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# THE USE OF THE CACO-2 CELL CULTURE MODEL TO INVESTIGATE LIPID VEHICLE EFFECTS ON LIPID METABOLISM AND DRUG PERMEATION

Being a thesis submitted for the degree of

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by

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This thesis is submitted by the undersigned to the University of Dublin, Trinity College, for examination for the degree of Doctor of Philosophy. It has not been submitted as an exercise for a degree at any other University. I myself carried out all of the experimental work described, except where duly acknowledged. This manuscript was completely written by me with the aid of editorial advice from Prof. Caitriona M. O'Driscoll and Dr Marianne B. Ashford.

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Sueballuck F., Ashford M.B. and O'Driscoll C.M. The effects of Plurenic block copolymers of lipid absorption and metabolism in the Caco-2 model. Paster Presentation - AstraZeneca Stude Presentations Day Macelestickid Manchester UK. January 2009.

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Solemens (AAPS), Sair Lake City, Ut, October, 2003.

#### Publications, presentations and abstracts

#### **Publications**

Seeballuck F., Ashford M.B. and O'Driscoll C.M. (2003). The effects of Pluronic® block copolymers and Cremophor® EL on intestinal lipoprotein processing and the potential link with P-glycoprotein in Caco-2 cells. *Pharmaceutical Research* **20**, 1085-1092.

#### Presentations and abstracts

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Seeballuck F., Ashford M.B. and O'Driscoll C.M. Factors affecting lipoprotein secretion in the Caco-2 model. *Poster Presentation* - Annual Trinity College Dublin/ Queens University Belfast Joint Research Seminar, Dublin, March, 2001.

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Seeballuck F., Ashford M.B. and O'Driscoll C.M. Comparative effects of polysorbate 80 and 60 on lipid metabolism and lipoprotein secretion in the Caco-2 cell model. *Poster Presentation* - Annual Trinity College Dublin/ Queens University Belfast Joint Research Seminar, Belfast, March, 2002.

Seeballuck F., Ashford M.B. and O'Driscoll C.M. Cremophor EL – a reversible inhibitor of triglyceride rich lipoprotein secretion. *Poster Presentation* - Annual Trinity College Dublin/ Queens University Belfast Joint Research Seminar, Dublin, March 2003.

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#### Summary

The aim of this thesis was to assess the usefulness of the Caco-2 model for screening lipid vehicle excipient effects on intestinal lipoprotein production and secretion, with emphasis on chylomicron secretion. In addition, the potential of the model to identify lymphotropic formulations and to predict lymphatic drug transport, by monitoring drug association with chylomicrons, was investigated.

The Caco-2 model, previously established in the laboratory for studying passive drug absorption was optimised for detection of lipid metabolism, specifically the production and secretion of triglyceride rich lipoproteins (TRL - chylomicrons and VLDL). It was found that cells of a higher passage number (68-84 versus 30-50) were required to produce significant levels of TRL. Cell response to various fatty acid stimuli was consistent with reported *in vivo* data. Oleic acid proved to be a potent stimulator of chylomicron secretion.

A series of non-ionic pharmaceutical surfactants were screened for their effects on TRL secretion in the Caco-2 model. It was found that Pluronic block copolymers, Cremophor EL and TPGS all inhibited TRL secretion in a dose dependent manner. Potency of surfactants as inhibitors was found to be HLB dependent, with the more lipophilic Pluronic L81 (HLB 1-7) producing selective and significant inhibition of chylomicron secretion at 0.003%w/v, and the more hydrophilic Pluronic F68 (HLB >24) only demonstrating inhibition from 4% onwards. Results showed that the reduced TRL secretion was not attributable to decreased cellular production of triglyceride or apo B, and was not associated with cell toxicity. A close correlation between surfactant inhibitory effects on TRL secretion and P-gp activity was demonstrated for Cremophor EL and Pluronic L81. As all of the tested surfactants contained a polyoxyethylene moiety, the effects of PEG 400 (an ethylene oxide polymer and a recognised P-gp inhibitor) on TRL secretion were investigated. This was found to inhibit chylomicron secretion, but with low potency (40% decrease in chylomicron secretion at 5%w/v PEG 400).

In contrast to these findings, a further non-ionic surfactant, Polysorbate 80, an oleate ester of polyoxyethylene sorbitan, was found to promote TRL secretion in Caco-2 cells. Secreted chylomicron response to Polysorbate 80 was dose dependent, reaching a maximum at 0.5% w/v. Further data indicated that this response was due to the oleate component – present both in free form and liberated from the ester by pancreatic or cell mediated enzyme hydrolysis.

Excipient data were used to assist in selecting excipients for a lymphotropic microemulsion formulation. In addition, the TRL stimulating effects of a number of long chain oils were investigated in Caco-2 cells. Oils were found to promote TRL secretion only following simulated

intestinal digestion. Pre-digested olive oil was found to have the highest potential to promote chylomicron secretion.

Surfactants were evaluated for their potential to inhibit intestinal lipolysis, of the investigated compounds, only Pluronic P85 was found to substantially inhibit pancreatic lipase activity under simulated intestinal conditions.

A potentially lymphotropic, oleate rich microemulsion preconcentrate vehicle was formulated with olive oil, Peceol, Cremophor EL and polysorbate 80. The vehicle was found to produce a stable microemulsion on dilution in simulated gastro-intestinal fluids. In the non-digested state, this vehicle substantially enhanced TRL secretion when administered to Caco-2 cells. It was also found to enhance the aqueous solubility and cellular permeability of halofantrine, a highly lipophilic drug, as compared to simple olive oil or Miglyol 812 emulsions. However, the microemulsion formulation failed to promote chylomicron-associated drug transport. When the oleate rich microemulsion was administered to cells in a pre-digested state there was a substantial decrease in its ability to promote chylomicron secretion. It also failed to promote halofantrine transport following digestion. In contrast, when a simple olive oil emulsion was presented to cells in a pre-digested form, its ability to promote chylomicron secretion and to increase halofantrine permeability was considerably enhanced as compared to a non-digested emulsion, but again, halofantrine failed to associate with secreted chylomicrons. Of the investigated vehicles, only a pre-digested polysorbate 80 solution was found to promote chylomicron-associated drug transport.

The Caco-2 model proved to be useful for predicting intestinal response to lipid vehicles and lipid vehicle excipients, as observed findings correlated well with reported *in vivo* data. However, the lack of TRL associated drug transport in the presence of olive oil and an oleate rich microemulsion vehicle indicates some limitations with the model in terms of predicting lymphatic drug transport.

#### TABLE OF CONTENTS

Title			i
Declar	ation		ii
Publica	ations, p	presentations and abstracts	iii
Acknov	vledgem	nents	iv
Summa	ry		v
Table o	of conte	nts	vii
Abbrev	iations		xiv
Origin	and sco	рре	xvi
СНАР	TER 1:	GENERAL INTRODUCTION	
1.1	Dige	estion and absorption of lipids	2
	1.1.1	Gastric Lipase	2
	1.1.2	Pancreatic Lipase	4
	1.1.3	In vitro models of triglyceride digestion	6
	1.1.4	Phospholipid and cholesterol ester digestion	7
	1.1.5	Intestinal milieu during lipolysis	7
		1.1.5.1 Lipolysis mechanism	7
		1.1.5.2 Role of calcium	8
		1.1.5.3 Uptake of triglyceride digestion products	10
		1.1.5.4 Uptake of phospholipid and cholesterol	11
1.2	Intes	stinal lipoprotein assembly	12
	1.2.1	Intracellular trafficking of fatty acids	12
	1.2.2	Re-esterification of lipids	13
	1.2.3	Lipoprotein composition and structure	14
	1.2.4	Overview of assembly path for chylomicrons	18
	1.2.5	Two step theory of lipoprotein assembly	18
	1.2.6	Alternative routes for intestinal VLDL and chylomicron assembly	19
	1.2.7	Importance of apoB for TRL secretion	22
	1.2.8	Regulation of apoB synthesis and secretion	22
	1.2.9	MTP and apoB secretion	23
1.3		d vehicles and drug absorption	24
	1.3.1	Increased drug solubility	25
	1.3.2	Gastric emptying	27
	1.3.3	Membrane permeability	27
	1.3.4	Aqueous boundary layer	33

	1	1.3.5	Lymphat	tic drug uptake	33
1.	4	Lymp	hatic dru	g uptake	35
	1	1.4.1	Anatomy	and physiology of the intestinal lymphatics	35
	1	1.4.2	Required	drug characteristics for lymphatic uptake	36
	1	1.4.3	Lipid ve	hicle effects on lymphatic drug transport	37
			1.4.3.1	Fatty acid chain length in administered lipid	39
			1.4.3.2	Degree of fatty acid saturation in administered lipid	39
			1.4.3.3	Digestibility of co-administered lipid	40
			1.4.3.4	Lipid structure	41
	1	1.4.4	Classes o	of lipid vehicles	41
			1.4.4.1	Self-emulsification and microemulsions	46
			1.4.4.2	Microemulsions	43
			1.4.4.3	$Formulation\ considerations\ for\ self-emulsifying\ lipid\ vehicles$	44
			1.4.4.4	Self-emulsifying and self-microemulsifying vehicle effects on d	rug
				bioavailability	46
1.	5	Asses	sment too	ols for lymphatic transport	50
		1.5.1	Whole a	nimal models	50
			1.5.1.1	Anaesthetised rat	50
			1.5.1.2	Conscious rat model	50
			1.5.1.3	Genetic mouse models	51
			1.5.1.4	Dog model	51
		1.5.2	In vitro	Caco-2 cell model	52
			1.5.2.1	Barrier properties of Caco-2 cells	52
			1.5.2.2	Suitability of Caco-2 cells as a model for lipid metabolism	53
			1.5.2.3	In vitro-In vivo correlation	54
			1.5.2.4	Factors affecting Caco-2 lipoprotein response	54
			1.5.2.5	Differences between lipid metabolism in Caco-2 model and	
				human intestine	56
1.	.6	Aims			59
CHA	PT	TER 2:	MATERI	IALS AND METHODS	
2.	.1	Mate	rials		61
2.	.2	Instru	imentatio	n	66
2.	.3	Meth	ods		68
	2	2.3.1	General	cell culture	68
		2.3.2	Lipid me	etabolism studies	71

	2.3.3	vandatic	on of unracentifugation technique	, 0
	2.3.4	Colorime	etric MTT toxicity assay for cell viability	77
	2.3.5	Quantific	cation of apolipoprotein B using an ELISA technique	77
	2.3.6	Measure	ment of efflux activity	80
	2.3.7	Fatty aci	d uptake	81
	2.3.8	CMC de	termination	81
	2.3.9	Determin	nation of free fatty acid content and sensitivity to digestion of	
		polysorb	ate surfactants	82
	2.3.10	Halofant	rine solubility study	83
	2.3.11	HPLC as	ssay for halofantrine	84
	2.3.12	In vitro l	lipolysis studies	90
	2.3.13	Microen	nulsion formulation and characterisation	92
	2.3.14	Adminis	stration of lipid vehicles to Caco-2 monolayers	93
	2.3.15	Gas chro	omatography	95
	2.3.16	Data ana	alysis	96
RESU	LTS AN	ND DISC	USSION	
CHAI	PTER 3	3: OPTI	MISATION OF CACO-2 MODEL FOR STUDYING	LIPII
MET/	BOLIS	M		
3.1		duction		98
3.2		and objection	ctives	99
3.3			ctives	100
0.0	3.3.1		f filter type on monolayer formation and lipid metabolism	100
	3.3.2		size analysis of TRL	106
	3.3.3		f passage number on lipid metabolism	107
	3.3.4		icibility of TRL secretion over the investigated passage range	112
	3.3.5		erisation and validation of optimised model	114
	3.3.3	3.3.5.1	Monolayer characterisation	114
		3.3.5.2	Concentration dependence of TRL response to oleic acid stim	
		3.3.3.2	concernation dependence of TIW response to office deld still	119
		3.3.5.3	Comparison of 'fed-' and 'fasted-state' vehicle effects on lipid	
		3.3.3.3	metabolism in Caco-2 monolayers	122
		2 2 5 1		
		3.3.5.4	Lipid composition of secreted lipoprotein fractions  Differential response to fathy acids of varying about length are	125
		3.3.5.5	Differential response to fatty acids of varying chain length an	
		2256	saturation	127
		3.3.5.6	Inhibition of TRL secretion by verapamil	132

3.4	Key	manigs		134
3.5	Discu	ission		134
СНАР	TER 4	: INHIBI	TORY EFFECTS OF PHARMACEUTICAL EXCIPIENT	S ON
LIPOP	ROTEI	N PROCE	ESSING IN CACO-2 MONOLAYERS	
4.1	Intro	duction		142
4.2		and object	tives	143
4.3	Resu			147
	4.3.1	CMC det	termination	147
	4.3.2	Excipien	t toxicity	147
	4.3.3	Excipien	t effects on lipid metabolism	150
		4.3.3.1	Effects of Pluronic block copolymers on lipoprotein secretion	150
		4.3.3.2	Effects of Pluronic L81 on fatty acid uptake	151
		4.3.3.3	Effects of Pluronic block copolymers on intracellular accumul	ation o
			lipid and apoB	152
		4.3.3.4	Effects of Cremophor EL, TPGS and PEG 400 on lipoprotein	
			secretion	158
		4.3.3.5	Effects of Cremophor EL on fatty acid uptake	159
		4.3.3.6	Effects of Cremophor EL, TPGS and PEG 400 on intracellular	•
			accumulation of lipid and apoB	159
	4.3.4		of Pluronic L81 and Cremophor EL on drug efflux	165
	4.3.5		ral application of Pluronic L81 and Cremophor EL	170
	4.3.6		FATP supplementation on TRL secretion in the presence of Plus	
			and Cremophor EL	172
	4.3.7		y of cells from the inhibitory effects of excipients	174
			TRL secretion recovery following wash-out	174
		4.3.7.2	TRL secretion recovery following wash-out and administration	
		C 1:	'fed-state' vehicle	176
4.4		findings		178
4.5	Disc	ussion		178

CHAPTER FIVE: EFFECTS OF POLYSORBATE SURFACTANTS ON LIPOPROTEIN

PROCESSING IN THE CACO-2 CELL MODEL

Introduction

Aims and objectives

5.1

5.2

184

185

.3	Resul	lts		186
5.3.1 Effects of polysorbate surfactants on cell viability and monolayer inte				ty
				186
	5.3.2	Polysorb	pate surfactant effects on lipid metabolism	187
		5.3.2.1	Vehicles	187
		5.3.2.2	Effects of polysorbate surfactants on lipoprotein secretion in	
			'fed-state' cells	187
		5.3.2.3	Effects of polysorbate surfactants on fatty uptake in Caco-2 cell	ls
				188
		5.3.2.4	Effects of polysorbate surfactants on intracellular accumulatio	n of
			lipids and apoB in 'fed-state' cells	188
		5.3.2.5	Effects of polysorbate 80 on lipoprotein secretion in 'fasted-sta	ite'
			cells	193
		5.3.2.6	Effects of polysorbate 80 on intracellular accumulation of trigl	ycerid
			and apoB in 'fasted-state' cells	196
	5.3.3	Compara	ative effects of polysorbate surfactants and free fatty acids on lip	id
		metaboli	ism in Caco-2 cells.	198
		5.3.3.1	Comparison of the effects of polysorbate 80 versus polysorbate	60 on
			the TRL secretion response in 'fasted-state' Caco-2 cells	198
		5.3.3.2	Effects of polysorbate surfactants versus free fatty acids on	
			basolateral secretion of triglyceride and apoB in Caco-2 cells	199
		5.3.3.3	Effects of polysorbate surfactants versus free fatty acids on	
			intracellular accumulation of triglyceride and apoB	199
		5.3.3.4	Colorimetric triglyceride assay analysis of cellular and basola	teral
			samples	203
	5.3.4	Dual lab	pelling of lipids	205
		5.3.4.1	Dual label composition of secreted and cellular lipids	205
		5.3.4.2	Density gradient ultracentrifugation analysis of basolateral sa	mples
				206
	5.3.5	Digestio	on and fatty acid composition of polysorbate surfactants	208
		5.3.5.1	Fatty acid composition	208
		5.3.5.2	Free fatty acid content of polysorbate solutions	208
		5.3.5.3	Sensitivity of polysorbate 80 to digestion	209
		5.3.5.4	Importance of polysorbate digestion for the TRL response	210
	5.3.6	Effect o	f polysorbate 80 on Cremophor EL mediated inhibition of chylor	nicron
		secretion	n	212
	537	Effects (	of nolysorbate surfactants on drug efflux	213

5.4	Key	findings		216
5.5	Disc	ussion		216
CHAP'	TER :	SIX: FOR	MULATION AND CHARACTERISATION OF LIPIE	-BASED
VEHIC	CLES			
6.1	Intro	oduction		221
6.2		s and object	ctives	222
6.3	Resi			224
	6.3.1		n of vehicle oil phase	224
		6.3.1.1	Lipid vehicle toxicity	227
		6.3.1.2	Effects of oil vehicles (digested and non-digested) on lipopro	otein
			secretion	228
		6.3.1.3	Effects of oil vehicles (digested and non-digested) on intrace	llular
			accumulation of triglyceride and apo B	229
	6.3.2	Solubilit	y of halofantrine in investigated oils	233
	6.3.3	Influence	e of vehicle excipients on lipid digestion	234
		6.3.3.1	Titrimetric analysis of lipolysis	235
		6.3.3.2	Lipolysis of $[^{14}C]$ triolein	240
	6.3.4	Microen	nulsion formulation and characterisation	243
		6.3.4.1	Effect of Peceol concentration on microemulsion formation	245
		6.3.4.2	Effect of olive oil concentration on microemulsion formation	245
		6.3.4.3	Effect of Cremophor EL: polysorbate 80 ratio on microemul	sion
			formation	245
		6.3.4.4	Effect of cell culture medium on microemulsion formation	246
		6.3.4.5	Effect of halofantrine on microemulsion droplet size	246
	6.3.5	Effect of	f co-administered lipid vehicles on the aqueous solubility of	
		halofant	rine	251
		6.3.5.1	Halofantrine solubility in vehicle preconcentrates	251
		6.3.5.2	Halofantrine solubility following vehicle dilution in gastroin	itestinal
			fluids	251
6.4	Key	findings		255
6.5	Dis	cussion		255
CHAF	PTER	SEVEN: E	EFFECTS OF LIPID VEHICLES ON THE CELLULAR	UPTAKE
AND	PERM	EABILITY	OF HALOFANTRINE IN THE CACO-2 MODEL	
7.1	Intr	oduction		261

	7.2	Aims	and objectives	261			
	7.3	Resul	lts	263			
		7.3.1	Vehicle preparation	263			
		7.3.2	Lipid vehicle (non-digested and digested) toxicity	264			
		7.3.3	Effect of in vitro lipolysis on free fatty acid content of lipid vehicles	268			
		7.3.4	Vehicle effects on lipid metabolism in Caco-2 cells	269			
			7.3.4.1 Secreted lipoprotein profile	269			
			7.3.4.2 Secreted lipid profile	270			
			7.3.4.3 Intracellular lipid and apoB	270			
		7.3.5	Effects of lipid based vehicles on halofantrine uptake and permeability is	in the			
			Caco-2 model	276			
		7.3.6	Lipid vehicle effects on density gradient distribution of halofantrine in				
			basolateral samples	281			
		7.3.7	Quantitative analysis of halofantrine in density gradient fractions	285			
		7.3.8	Correlation between halofantrine and chylomicron transport in Caco-2				
			monolayers	285			
		7.3.9	Effect of halofantrine on the bi-directional transport of cyclosporin A	290			
	7.4	Key	findings	291			
	7.5	Disci	ussion	291			
C.	HAP	TER E	IGHT: GENERAL DISCUSSION AND FUTURE DIRECTIONS				
	8.1	Back	ground	300			
	8.2	Aims		301			
	8.3	Inhib	oition of TRL secretion by lipid vehicle excipients	301			
	8.4	Inter	Interrelationship between P-gp and lipid metabolism				
	8.5	Polys	sorbate surfactants	307			
	8.6	Drug	solubilisation and lymphatic uptake	309			
	8.7	Caco	-2 cells as a model for predicting lymphatic drug transport	313			
	8.8	Key	findings of this thesis	315			
	8.9	Futu	re directions	316			
A	PPE	NDICE.	S	317			
R	EFEF	RENCE	S	334			

#### **Abbreviations**

A area of membrane (cm<sup>2</sup>)

ABC ATP binding cassette (proteins)

ABL aqueous boundary layer

ANOVA analysis of variance ApoB apolipoprotein B

Apobec-1 apolipoprotein B mRNA editing enzyme

BSA bovine serum albumin

°C degrees celsius

Co initial concentration

CsA Cyclosporin A
CE cholesterol ester
CM chylomicrons

CMC critical micellar concentration

DMEM Dulbecco's modified Eagle's medium

DPM disintegrations per minute

dq/dt steady state flux

EDTA ethylene diamine tetraacetic acid

ELISA enzyme linked immunosorbent assay

EO ethylene oxide

ER endoplasmic reticulum

I-FABP intestinal fatty acid binding protein

L-FABP liver fatty acid binding protein

FABP<sub>pm</sub> plasma membrane fatty acid binding protein

FBS foetal bovine serum

FFA free fatty acid

g gram(s)

GC gas chromatography

HBSS Hanks balanced salt solution
HDL high density lipoprotein (s)

Hepes {N-(2-hydroxyethyl) piperazine-N-(2-ethanesulphonicacid)}, sodium salt

HLB hydrophilic lipophilic balance

HGL human gastric lipase

HPL human pancreatic lipase

HPLC high performance liquid chromatography

IS internal standard

 $\lambda$  wavelength

l litre(s)

LCT long chain triglyceride

LDL low density lipoprotein (s)

Log P log partition coefficient octanol-water

μg microgram(s)
 μl microlitre(s)
 μM micromolar
 μmole(s) micromole(s)

mg milligram(s)
ml millilitre(s)
mM millimolar

mmole(s) millimole(s)

MCT medium chain triglyceride
MDR multiple drug resistant

M-me Miglyol 812 based microemulsion MRP multiple drug resistance protein

MTP microsomal triglyceride transfer protein

MW molecular weight ng nanogram(s)

nmole(s) nanomole(s)

NEFA non-esterified fatty acid

 $\Omega$  oleic acid ohms

O-me olive oil based microemulsion

Papp apparent permeability coefficient (cm/sec)

PC polycarbonate

PET polyethylene terephthalate

P-gp P-glycoprotein
PL phospholipid
P80 polysorbate 80
P60 polysorbate 60
PO propylene oxide

RER rough endoplasmic reticulum

Rpm revolutions per minute

s second

SA stearic acid

SD standard deviation

SDS PAGE sodium lauryl sulfate polyacrylamide gel electrophoresis

SER smooth endoplasmic reticulum

T time

TBME tertiary butyl methyl ether

TBU tributyrin unit

TLC thin layer chromatography

TG triglyceride

TPGS tocopheryl polyethylene glycol 1000 succinate

TRL triglyceride rich lipoprotein (s)

UV ultraviolet

VLDL very low density lipoprotein (s)

Wt weight

#### Origin and scope

The employment of combinatorial chemistry and genetic targeting in drug design has resulted in a large number of highly lipophilic drugs reaching early product development. These compounds typically have high binding affinity for target receptors *in vivo* and, as a result, are highly lipophilic. Such compounds have poor biopharmaceutical properties – they are typified by low aqueous solubility and high membrane permeability and as such, fall into the Class II of the biopharmaceutical classification system. Many bioavailability enhancing efforts to date for such compounds have been aimed at maximising drug dissolution. Although these compounds typically have good membrane permeability properties, the intestinal mucosa presents a number of additional physical and biochemical barriers to lipophilic drug absorption including: an aqueous boundary layer across which lipophilic drugs must pass before reaching the lipid bilayer, the presence of counter-transport proteins such as P-glycoprotein (P-gp) and MRP-1 and enterocyte based metabolic enzymes such as cytochrome P450 3A. All of these features act to limit intestinal drug absorption.

Digestible lipid vehicles are increasingly being recognised as a means of overcoming absorption barriers to poorly water soluble drugs. Mechanisms of absorption enhancement for such vehicles include: increased drug solubility in aqueous gastrointestinal medium - both via direct solubilisation in dispersed vehicle and via promotion of lipid digestion, facilitation of drug transport through the aqueous boundary layer, inhibition of cell associated enzymes and counter-transport proteins and stimulation of lymphatic drug uptake. Lymphatic uptake of highly lipophilic drugs occurs by drug association with the triglyceride core of intestinal chylomicrons. Lymphatic drug uptake carries the advantages of circumventing hepatic first pass metabolism and providing localised drug delivery to the lymph. Currently, there are a few commercially successful and clinically useful examples of lipid vehicle formulations, namely, Neoral (cyclosporin A), Norvir (ritonavir) and Fortavase (saquinavir). However, developments and widespread acceptance in this field have been slow. This reticence to employ lipid based vehicles is largely due to the lack of understanding of how lipid vehicles act to enhance drug absorption, particularly with regard to lymphatic drug transport. To date, research in this area has largely been conducted in whole animal models, typically in a lymph cannulated rat model, using techniques that are highly skilled and labour intensive. The lack of an in vitro model capable of high throughput screening for lipid vehicle effects on intestinal lipid metabolism and lymphatic drug transport has hindered progress in this area. The work described in this thesis focuses on the development of an in vitro Caco-2 model for rapidly assessing the interacting effects of lipid vehicle excipients on lipid metabolism, specifically on chylomicron secretion, and for investigating the potential of lipid vehicles to promote chylomicron-associated drug transport, thereby predicting lymphatic drug transport. An *in vitro* tool for screening intestinal response to lipid vehicles will accelerate research in this field and further our understanding of how lipid vehicles can benefit oral drug delivery.

## Chapter One

# **General Introduction**

#### 1 General Introduction

#### 1.1 Digestion and absorption of lipids

Prior to investigating the effects of lipid vehicles on intestinal drug bioavailability it is important to have a fundamental understanding of the processes that such vehicles are subject to on entry to the gastrointestinal tract. Intestinal luminal digestion can significantly alter the properties of lipids and lipid vehicles and so must be considered.

The majority of dietary lipids are long chain triglycerides (>90%), with the remainder largely comprising phospholipids or cholesterol (Thompson *et al.*, 1989; Tso, 1994). Long chain triglycerides are large, hydrophobic molecules that tend to form lipid globules when added to aqueous medium (Small, 1968). Although the less hydrophobic medium chain triglycerides may be absorbed intact (Chow *et al.*, 1989), a digestion step is required to present consumed long chain triglycerides in an easily absorbable form to enterocytes – i.e. as component monoglycerides and free fatty acids (Mattson and Volpenhein, 1964; Tso, 1994). This step is accomplished in the human gastrointestinal tract by lipases. Two such lipases have been identified as playing a major role in lipid digestion: gastric lipase and pancreatic lipase (Tso, 1994).

#### 1.1.1 Gastric Lipase

Digestion is generally initiated in the stomach by human gastric lipase (HGL), a 50kDa, acid stable glycoprotein, which operates optimally at pH 4-6 (Miled *et al.*, 2000). Gastric lipase is active at an oil-water interface, such as in a crude emulsion, and hydrolyses ester bonds to liberate free fatty acid (Gargouri *et al.*, 1989; Carriere *et al.*, 1991; Cartwright and Higgins, 1992). This enzyme is active on *sn*-1 and *sn*-3 esters of triglyceride, and shows stereopreference for the *sn*-3 bond (Rogalska *et al.*, 1990; Carriere *et al.*, 1997). Hydrolysis at the *sn*-2 position has been reported *in vitro* by one group (Tiruppathi and Balasubramanian, 1982), but this may have been a result of acyl migration within the molecule (Rogalska *et al.*, 1990). The typical end products of gastric lipolysis are diglycerides (50-70% as *sn*-1,2 diacylglyceride) and free fatty acids (Armand *et al.*, 1996; Carriere *et al.*, 1997).

Gastric lipase activity may be dependent on fatty acid chain length. There are, however, conflicting reports on this subject. One *in vitro* study, by Tiruppathi and Balasubramanian (1982), found that HGL was >3.5 times more active against tributyrin (C4:0) than triolein (C18:1) at pH 5.3, whereas, Gargouri *et al.* (1986b) reported comparable rates for *in vitro* HGL digestion of short and long chain triglycerides. Differences in experimental conditions may

explain these discrepancies. Tiruppathi and Balasubramanian (1982) included 5mM sodium taurocholate in their assay mix, whilst Gargouri et al. (1986b) used bile free buffer. Sodium taurocholate has been reported to selectively stimulate gastric lipolysis of short chain fatty acid esters (Tiruppathi and Balasubramanian, 1982; Gargouri et al., 1986b), and so may be responsible for the different observations.

The overall contribution of the gastric step of lipolysis to gastrointestinal fat digestion is very small, estimated as approximately 10% (Armand et al., 1994; Carriere et al., 1997). It has been postulated that this low level of lipolysis may be due to negative influences imparted by the inhospitable gastric milieu during digestion, rather than being due to the relative inactivity of HGL as an enzyme (Gargouri et al., 1986b). Most notably, gastric lipase is inhibited by protonated long chain fatty acids (Gargouri et al., 1986b; Kaminsky et al., 1988; Juhel et al., 2000), which accumulate at the oil-water interface during lipolysis and displace lipase from the oil surface (Gargouri et al., 1986b).

The gastric phase of digestion serves to prepare fat for subsequent and more extensive intestinal digestion. The mechanical churning action of the stomach (generated by antral contractions) in combination with the presence of protein and amphiphilic lipid digestion products generates a fine lipid emulsion. The median diameter of emulsified lipid droplets within the stomach is <30µm within one hour of fat ingestion (Armand *et al.*, 1994). This provides a large interfacial area for intestinal digestion. In addition, the end products of gastric lipolysis may stimulate secretion and activation of pancreatic lipase (Gargouri *et al.*, 1986b; Bernbäck *et al.*, 1989; Döppenschmitt *et al.*, 1998).

Recent findings have suggested an additional role for HGL in intestinal digestion. *In vivo* studies conducted by Carriere *et al.* (1993) have shown that a considerable amount of HGL is deposited in the jejunum along with chyme and that it remains active within the post-prandial intestinal milieu. It has been estimated that HGL may account for 13% of effective duodenal digestion. As indicated above, the optimum pH for gastric lipase is 4-6, which is easily achievable within the upper intestine in the post-prandial state (Dressman *et al.*, 1990; Carriere *et al.*, 2000). It should be noted that as digestion progresses and gastric emptying increases, the pH of the intestinal luminal contents may decrease to a level that is more suited to the activity of gastric versus pancreatic lipase activity. This may explain why certain individuals who are deficient in pancreatic lipase, as in cystic fibrosis, are still able to absorb approximately 50% of ingested lipid (Ross, 1954).

#### 1.1.2 Pancreatic lipase

The key enzyme responsible for in vivo lipolysis of ingested lipids is human pancreatic lipase This 50kDa, colipase dependent, glycoprotein is secreted into the duodenum by pancreatic acinar cells. As with gastric lipase, this hydrolase acts specifically at an oil-water interface (Brockerhoff, 1969) and demonstrates stereo-preference for sn-1 and sn-3 ester bonds (Carriere et al., 1997). In contrast to gastric lipase, there are no reports of HPL activity at the sn-2 position, and the principal products of digestion are sn-2 monoacylglycerol and free fatty Pancreatic lipase demonstrates 2-step hydrolysis kinetics, generating initially 1,2acids. diacylglycerol followed by 2-monoacylglycerol. Studies on triolein digestion indicate that the second hydrolysis step (hydrolysis of 1,2-dioleoylglycerol to 2-monooleoylglycerol) occurs 2.8times faster than the first (hydrolysis of the trioleoylglycerol to 1,2-dioleoylglycerol) (Lykidis et al., 1995). Pancreatic lipase may promote complete hydrolysis in some cases, liberating glycerol and free fatty acids. This occurs as a result of steric rearrangement, with the remaining fatty acid on 2-monoacylglyceride shifting to a free sn-1 or sn-3 position. In addition, 2monoglyceride may undergo non-enzymatic chemical hydrolysis under alkaline conditions to liberate glycerol and free fatty acid (Embleton and Pouton, 1997).

Pancreatic lipase possesses two topologically distinct sites – a large N terminal domain, which is responsible for its catalytic activity, and a C-terminal domain, which interacts with a crucial coenzyme, colipase (Winkler et al., 1990). In aqueous solution the enzyme is in a dormant or 'closed' state. In this conformation, the active lipolytic site, which comprises a catalytic triad of Ser 152, Asp 176 and His 263, is covered by a surface loop or 'lid' (residues 237-261) stabilised by disulphide bridges. In close association with this 'lid' there are two small side chains, which also act to hinder access to the active site. Introduction of an oil phase creates an interface. When HPL binds to the oil-water interface, a conformational rearrangement occurs, which exposes the active site (Borgström and Erlanson, 1973; Winkler et al., 1990; Brzozowski et al., 1991; van Tilbeurgh et al., 1993; Lykidis et al., 1995) allowing lipolysis to commence. Once in the 'open' form, the affinity of the colipase-HPL complex for the interface is increased because the lid structure and side chains close to the active site contain hydrophobic residues, which are reoriented towards the lipid phase (Miled et al., 2000). In vitro work has shown that in the absence of mediators, HPL activity at the interface is short lived. This occurs because, in common with other enzymes, HPL is susceptible to unfolding and irreversible inactivation at an oil-water interface and secondly because the amphiphilic fatty acid products of digestion will collect at the oil-water interface, displacing HPL from the interface and so inhibiting activity (Brockerhoff, 1971; Chapus et al., 1975; van Tilbeurgh et al., 1992). These problems are overcome in the gastrointestinal tract by two critical mediators - colipase and bile.

#### Colipase and pancreatic lipase

Colipase is a 10kDa protein that acts to anchor HPL to the oil phase in the presence of interfacially located amphiphilic compounds. Colipase is secreted from pancreatic acinar cells as a pro-form, pro-colipase, which is converted to colipase in the intestine by cleavage of five amino acids (Larsson and Erlanson-Albertsson, 1981; Armstrong and Carey, 1982; van Tilbeurgh *et al.*, 1992). Colipase contains three finger-shaped projections, each containing hydrophobic residues, which anchor to the lipid surface. Attached to these lipophilic 'fingers' is a binding site for lipase. The C-terminal domain of HPL binds to this site in a 1:1 ratio via H-bonding (Borgstrom, 1975; Brzozowski *et al.*, 1991; van Tilbeurgh *et al.*, 1992). HPL is brought close to the interface by this interaction and is activated. This complex is securely bound to the interface and unlike the free HPL is not easily displaced by bile salts or lipid digestion products (van Tilbeurgh *et al.*, 1992). For example, Borgström and Erlanson (1973) showed that although bile salts, when added at concentrations exceeding the critical micelle concentration (CMC), produced inhibition of HPL-catalysed lipolysis of tributyrin, this effect was reversed by addition of colipase to the system.

#### Bile salts and pancreatic lipase

Bile salts are a family of water-soluble, amphiphilic hydrocarbon molecules. Structurally, they consist of a steroid nucleus, which has a hydrophobic convex side ( $\beta$  side) and a more hydrophilic concave side ( $\alpha$  side) that contains hydroxyl groups (at C-3, 7 and/ or 12) and an aliphatic side chain extending from the C17 position. The aliphatic side chain may be conjugated at C24 by a strongly ionic taurine or glycine group, which contributes a strong hydrophilic moiety to the molecule. Because of their amphiphilic nature, bile salts have surfactant like properties and so will form micelles at concentrations above the CMC (Armstrong and Carey, 1982).

Bile salts fulfil a multitude of roles in lipid digestion. With regard to enzyme stability, bile salts are important for preventing unfolding and inactivation of HPL at the inhospitable oil-water interface and also for increasing the structural stability of the active/ open HPL-colipase complex (Brockerhoff, 1971; Momsen and Brockman, 1976). In addition to this stabilising action, preliminary small angle neutron scattering studies have indicated that mixed bile salt micelles may bind to the colipase-HPL complex and act to maintain the complex in its active/ open form in the absence of a distinct lipid phase (Pignol *et al.*, 2000). Bile salts further promote HPL by lowering its optimum pH for activity from pH 8-9 to pH 6-7 (Borgstrom, 1954), which is much closer to that obtained in the post-prandial intestinal lumen (Dressman *et al.*, 1990; Carriere *et al.*, 2000). Furthermore, surface active bile salts are important for creating an environment that favours solubilisation of the end products of digestion and clearance of

these potentially inhibitory products from the oil-water interface, both via formation of a water soluble mixed micellar phase (Gargouri et al., 1983; Hernell et al., 1990) and by lowering the effective pKa of long chain fatty acids to facilitate their ionisation and hence increase their aqueous solubility (Hofmann, 1968; Porter, 1999).

#### Calcium and pancreatic lipase

Although calcium has long been recognised as an essential element for HPL activity, its precise role in the lipolytic process has not yet been fully elucidated. It has been implicated both in maintaining thermal stability of HPL (Vonk and Western, 1984) and in protecting HPL from inactivation by the inhibitory end products of long chain triglyceride lipolysis (Benzonana and Desnuelle, 1968).

#### 1.1.3 In vitro models of triglyceride digestion

The majority of studies on pancreatic lipase-mediated lipid digestion have been conducted in vitro using a titrimetric pH stat technique (Benzonana and Desnuelle, 1968; Gargouri et al., 1983; Sek et al., 2001; Zangenberg et al., 2001; Miled et al., 2002). In this method, pancreatic lipase is added to a lipid substrate dispersed in a simulated intestinal buffer. As digestion progresses, free fatty acid is liberated and is titrated against NaOH solution. The substrate pH is kept constant as neutralisation occurs and the volume of NaOH required to maintain the pH is directly equated to the amount of free fatty acid released. Care must be taken in comparing data from this model as results can vary depending on digestion buffer composition, pancreatic lipase/ colipase concentration and the nature of the substrate (Benzonana and Desnuelle, 1968; Borgström and Erlanson, 1973; Gargouri et al., 1986a). There is a requirement for fatty acids to be ionised before they can be titrated, hence, this model is not well suited to detecting liberated long chain fatty acids (approximately pKa 8) under simulated post-prandial duodenal conditions (pH 5-7) (Hamosh et al., 1975; Dressman et al., 1990; Carriere et al., 2000). It is possible to increase assay detection of long chain fatty acids by employing conditions that favour ionisation, for example, by raising the experimental pH to 7.5-8.5 (Sek et al., 2001) or by increasing the calcium content of buffer (Chatterton et al., 1991). Recently, a high performance thin layer chromatography (HPTLC) technique has been described that overcomes titrimetric detection problems (Sek et al., 2001). This method quantifies lipolytic products directly via chromatographic analysis. Samples are collected from a digestion mix at intervals and lipids are solvent-extracted before being separated via high performance thin layer chromatography (HPTLC). Separated lipid products are detected by charring and quantified using densitometry. This is a more accurate and descriptive method than the pH stat, but is also more costly and time consuming and is not well suited to producing rate data.

#### 1.1.4 Phospholipid and cholesterol ester digestion

Luminal phospholipid is largely sourced from secreted bile and is present predominantly as phosphatidyl choline (lecithin) (Shen *et al.*, 2001). Phosphatidyl choline is a polar lipid, containing a hydrophilic phosphocholine head group attached to two relatively hydrophobic fatty acid moieties. This amphiphilic structure confers surfactant like properties, which allow phosphatidylcholine to lower interfacial tension and to form mixed micelles in association with amphiphilic bile salts (Naylor *et al.*, 1995). Phospholipase  $A_2$  is responsible for intestinal digestion of phosphatidyl choline. This enzyme is primarily secreted by the pancreas, but may also be derived from the intestinal mucosa, where it is concentrated in the brush border. As with pancreatic lipase, bile salt is required for enzyme activity. Phospholipase  $A_2$  acts to hydrolyse the sn-2 ester position of phosphatidyl choline, liberating free fatty acid and lysophosphatidylcholine, which are taken up by cells (Shen *et al.*, 2001).

The majority of dietary cholesterol is present as free cholesterol, which can be taken up intact by cells. Approximately 10-15% is present as a sterol ester, which requires hydrolytic degradation prior to absorption. Cholesterol ester hydrolysis is performed by cholesterol esterase, which is secreted by the pancreas. Enzyme activity is enhanced by the presence of bile salts and it has a broad specificity, with activity against triglyceride, cholesterol esters and phosphoglycerides. Cholesterol ester hydrolysis liberates free fatty acid and free cholesterol (Shen *et al.*, 2001).

#### 1.1.5 Intestinal milieu during lipolysis

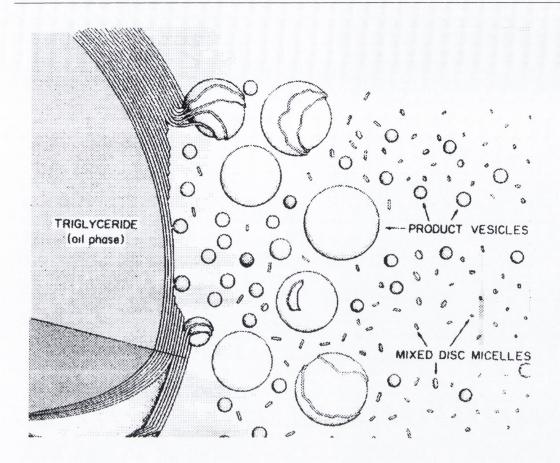
#### 1.1.5.1 Lipolysis mechanism

During lipid digestion, biliary lipids adsorb, with the pancreatic lipase/ colipase complex, to the surface of dispersed lipid droplets. Lipolysis proceeds at the surface, resulting in shrinkage of the droplet core and accumulation of fatty acids and monoglycerides at the interface. These amphiphilic products 'bud off' from the core to form multilamellar crystalline bilayers (Rigler et al., 1986; Hernell et al., 1990). Intestinal lipid digestion products promote a cholecystokinin mediated contraction of the gall bladder, which results in the expulsion of bile into the intestine. The luminal concentration of bile salt is increased from an average of 3-6mM in the fasted state, to 12-15mM during lipid digestion (Hernell et al., 1990; Armand et al., 1996; Lindahl et al., 1997). At concentrations exceeding the CMC, bile salts form micelles, which solubilise lipid products to create a mixed micellar phase (Figure 1.1). Evidence for this process comes from a combination of ultracentrifugation, light microscopy and freeze fracture analysis of aspirated and simulated duodenal contents. A key light microscopy study conducted by Patton and Carey

(1979) revealed at least three phases associated with *in vitro* digestion of long chain triglyceride (triolein) (in the presence of 13mM taurodeoxycholate and 8mM calcium), these were: (a) an oil phase, mostly composed of undigested triglyceride with smaller amounts of mono- and diglycerides, (b) a precipitated calcium soap phase, comprising calcium salts of ionised fatty acids and (c) a viscous isotropic phase containing monoglycerides and ionised fatty acids. The authors have postulated that this isotropic phase consists of a liquid crystalline phase in equilibrium with a mixed micellar phase of bile salt, monoglyceride and free fatty acid. Similar observations have been made via freeze fracture analysis of an *in vitro* digestion process (Rigler *et al.*, 1986). Additionally, ultracentrifugal analysis of isolated post-prandial human duodenal contents has confirmed that this 'isotropic' phase contains unilamellar liquid crystalline vesicles dispersed in a mixed micellar phase. It is thought that these unilamellar vesicles are the primary dispersed product of fat digestion formed at the oil droplet interface. As fatty acids are absorbed from the intestinal lumen, lipid vesicles will intercalate into the mixed micellar phase. The isotropic phase has been shown to contain bile salt, fatty acid, monoglyceride, phospholipid and cholesterol (Holt *et al.*, 1986; Hernell *et al.*, 1990).

#### 1.1.5.2 Role of calcium

Calcium plays an important role in keeping the oil-water interface free of liberated fatty acids, before micelle solubilisation takes effect. Light microscopy studies have shown that calcium reacts with liberated fatty acids (one calcium ion with two fatty acid molecules) (Zangenberg et al., 2001) to form insoluble calcium salts. These fatty acid salts accumulate in a crystalline phase (visible under light microscopy) close to the droplet surface, to produce a crystalline 'shell' of digestion products. This 'shell' exerts mechanical pressure on large, undigested droplets, causing them to erupt and form smaller droplets with increased surface area (Patton and Carey, 1979; Patton et al., 1985). The crystalline product is forced away from the droplet interface and can be isolated as an insoluble pellet via ultracentrifugation. As lipolysis progresses, monoglycerides accumulate and inhibit further calcium salt formation, thus preventing excessive generation of insoluble fatty acid calcium salts (Patton et al., 1985).



**Figure 1.1:** Schematic diagram of the accumulation of lipid digestion products at the oil droplet surface and their dispersion into a mixed micellar phase as per Rigler *et al.* (1986). The Biliary lipids, together with pancreatic lipase/colipase complex, adsorb to the surface of crude emulsion oil droplets. Lipolysis of triglycerides and diglycerides at the oil-water interface results in shrinkage of the oil core and accumulation of lipolytic products - monoglycerides and fatty acids- in addition to phospholipids and bile salts at the interface. These products dissociate to form multilamellar liquid crystalline bilayers and are solubilised by more bile salt to produce unilamellar vesicles and a mixed micellar phase.

#### 1.1.5.3 Uptake of triglyceride digestion products

Lipid digestion products (free fatty acids, monoglycerides, phospholipid and cholesterol) are absorbed at the apical surface of enterocytes from the digested mixed mixed mixellar phase (Johnston and Borgstrom, 1964; Mattson and Volpenhein, 1964). Before lipid digestion products reach the enterocytic membrane from the bulk luminal contents they must first cross the aqueous boundary layer (ABL) or unstirred water layer (UWL). This is a poorly stirred layer of water, mucus and glycocalyx, which is associated with the intestinal mucosal surface (Westergaard and Dietschy, 1974). Whilst there is some debate over the thickness of this bound aqueous layer, with estimates ranging from 40-600 µm (Westergaard and Dietschy, 1974; Anderson et al., 1988; Levitt et al., 1990), it is widely acknowledged as a significant barrier to lipid absorption (Westergaard and Dietschy, 1976). Micellar solubilisation considerably enhances fatty acid solubility in the intestinal lumen (up to 1000-fold) (Hofmann, 1970; Hermosa et al., 1996) and also facilitates transport of lipid digestion products across the ABL (Simmonds, 1972), thus presenting a high concentration of lipid digestion products for absorption at the enterocytic Studies in the Caco-2 model have highlighted the importance of bile salts in membrane. intestinal fatty acid uptake. For example, Walgren et al. (1998) reported a >15 fold enhancement in rate of oleic acid uptake from a mixed micellar fatty acid/ bile salt vehicle as compared to a standard BSA complex. Levin et al. (1992) reported similar findings, with a 10fold enhancement of fatty acid uptake from mixed micellar bile salt solution versus BSA complex in the first 15 minutes of exposure. Bile salt micelles are not absorbed intact, hence, lipid digestion products must dissociate from the mixed micellar phase prior to cellular uptake (Westergaard and Dietschy, 1976). It is thought that the low pH associated with the brush border 'microclimate' promotes dissociation of fatty acids from the micellar phase, thus enhancing fatty acid uptake (Shiau and Levine, 1980).

Short chain fatty acids are absorbed across enterocytes via passive diffusion, as has been demonstrated in the Caco-2 model for octanoic acid (Trotter et al., 1996). However, cellular uptake of long chain fatty acids is a more complex issue. Currently there is much debate over whether translocation of long chain fatty acids across the cell membrane involves active or passive transport processes. Based on studies using unilamellar phospholipid vesicles, Kamp and Hamilton (1992) proposed a mechanism for rapid and passive transcellular movement of fatty acids. This process involves fast flip-flop movement of fatty acids across lipid bilayers. Long chain fatty acids bind to the outer leaflet of the membrane. The majority of fatty acids are presented from the lumen to the mucosa in an ionised form. The acidic microclimate associated with the mucosal surface ensures that fatty acids penetrate into the phospholipid membrane in un-ionised form. Once within the lipid bilayer, 50% of long chain fatty acid are present in un-

ionised form. Un-ionised fatty acids diffuse across the membrane rapidly and spontaneously to the inner membrane leaflet, where they become ionised again. Transfer of the un-ionised fatty acids across the membrane is sufficiently rapid ( $t_{1/2}$  <2 seconds) to make additional active transport processes unnecessary. An earlier study by Bröring *et al.* (1989) drew similar conclusions, when it was found that the observed passive rate of translocation of oleic acid across cell membranes of isolated erythrocytes was sufficiently rapid to meet absorption requirements ( $t_{1/2}$  of <15 seconds).

Additional evidence has also pointed to the involvement of an active uptake protein (designated plasma membrane fatty acid binding protein, FABP<sub>pm</sub>) in the fatty acid uptake process. For example, Stremmel *et al.* (1985) found that the binding of [14C] oleic acid to rat microvillus membrane was saturable and sensitive to competitive inhibition by unlabelled fatty acid. Immunofluorescence studies by this group (with rabbit antibody against FABP isolated from rat liver cell membrane) have demonstrated the presence of a FABP in association with the brush border region of jejunal cells. Furthermore, when rat jejunal cells were preincubated with a specific antibody against hepatic FABP they were shown to have impaired long chain fatty acid uptake (Stremmel, 1988). These findings are supported by data from the Caco-2 model (Trotter *et al.*, 1996), in which a saturable long chain fatty acid uptake mechanism has been identified. In Caco-2 cells fatty acid uptake was found to be sensitive to competitive inhibition by medium and long chain fatty acids, as well as by monoglycerides, implying involvement of a common uptake protein for both products. Reported Caco-2 data implicate the involvement of both passive and active transport in long fatty acid uptake, with passive absorption predominating in the post-prandial phase when luminal lipid content is high (Trotter *et al.*, 1996).

#### 1.1.5.4 Uptake of phospholipid and cholesterol

Cells absorb lysophosphatidylcholine passively from the luminal mixed micellar phase. Absorbed lysophosphatidylcholine may be reacetylated to form phosphatidyl choline for incorporation into lipoproteins or cell membranes and the remainder is hydrolysed to glycerol-3-phosphorylcholine, which is transported via portal blood to the liver (Shen *et al.*, 2001).

Cholesterol uptake can occur passively, but may also involve an active protein uptake mechanism. Exogenous cholesterol is handled differently from endogenous cholesterol and is preferentially esterified and transported out of cells in association with triglyceride rich lipoproteins into the lymph. The rate of cholesterol esterification regulates its entry into the lymph. Esterification is largely mediated by acyl-CoA cholesterol acyltransferase (ACAT), which is concentrated in small intestine enterocytes (Tso, 1994; Shen *et al.*, 2001).

#### 1.2 Intestinal lipoprotein assembly

#### 1.2.1 Intracellular trafficking of fatty acids

Once fatty acids are absorbed into enterocytes they can either diffuse passively through cells for transport into portal blood supply or they can be reassembled into lipids and incorporated into secreted lipoproteins. Fatty acid chain length determines the intracellular route that will be taken. In general, the more hydrophilic short chain fatty acids (carbon chain length <12-14), which account for approximately 10% of dietary lipid, are destined for passive diffusion into portal blood capillaries, and more lipophilic, long chain fatty acids for re-esterification to triglycerides and subsequent incorporation into lipoproteins (Kiyasu *et al.*, 1952). More recent evidence has indicated that a significant amount of long chain fatty acid can be transported directly via the portal blood, for example, levels of up to 58% of linoleic acid and 28% of stearic acid have been observed in portal blood. The rate of perfusion through the intestinal lumen influences the proportion of fatty acid entering the lymphatic pathway, with higher rates resulting in increased lymphatic and reduced portal venous transport (McDonald *et al.*, 1980).

Lipid synthesis occurs in the smooth endoplasmic reticulum (SER). Hence, long chain fatty acids must be transported from the absorptive site, through the viscous, aqueous cytosol, to the SER (Tso, 1994). The cytosol presents a highly viscous aqueous barrier to the movement of unionised fatty acids. Intracellular trafficking is thought to be mediated by a water soluble transport fatty acid binding protein (McArthur et al., 1999). Two such cytoplasmic transport proteins have been identified in the human intestine, and are named after the tissue from which they were first isolated - intestinal fatty acid binding protein (I-FABP) and liver fatty acid binding protein (L-FABP) (Ockner and Manning, 1974; Gordon et al., 1983; Alpers et al., 1984). Both I-FABP and L-FABP are found in high concentrations in the upper intestine, but I-FABP predominates. Both proteins preferentially bind long chain polyunsaturated fatty acids, with a FABP: fatty acid ratio of 1:1 for I-FABP and 1:2 for L-FABP (Richieri et al., 1994). Given the high binding affinity of these proteins for long chain fatty acids and their concentration within enterocytes, they present an effective sink for absorbed fatty acids. Evidence indicates that I-FABP acquires absorbed fatty acids from plasma membranes via an active collisional process, whilst L-FABP receives them by passive diffusion, suggesting that I-FABP may be much more efficient at transporting absorbed fatty acids from the plasma membrane to the SER (Storch and Thumser, 2000; Thumser and Storch, 2000).

#### 1.2.2 Re-esterification of lipids

Absorbed fatty acids are re-esterified to triglyceride in the smooth endoplasmic reticulum (SER) (Bell *et al.*, 1981). The monoacylglycerol pathway is the predominant pathway for triglyceride synthesis and supplies approximately 80% of the chylomicron triglyceride load (Mansbach and Parthasarathy, 1982; Tso, 1994). A second pathway for intestinal triglyceride synthesis exists, the glycerol-3-phosphate (phosphatidic acid/  $\alpha$ -glycerophosphate) pathway, but is considerably less active then the monoacylglycerol pathway during the absorptive phase (Mansbach and Parthasarathy, 1982).

#### a) Monoacylglycerol Pathway Acyl-CoA Acyl-CoA -R2 Monoacylglycerol Diacylglycerol Acyltransferase Acyltransferase -OH OH Diglyceride Glycerol-3-Phosphate Pathway b) Acyl-CoA OH Acyl-CoA OH Glycerophosphate Phosphatidate Diacylglycerol Phosphohydrolase Acyltran sfera se Acyltransferase Glycerophosphate Phosphatidic Acid Diglyceride Triglyceride Glycerophospholipid Synthetase

Figure 1.2: Panel a: monoacylglycerol pathway for triglyceride synthesis, using exogenous monoacylglycerol and activated free fatty acids as substrates. Panel b: glycerol-3-phosphate ( $\alpha$ -glycerophosphate) pathway for triglyceride synthesis, using endogenous glycerol-3-phosphate and activated free fatty acids as substrates. FA, R1, R2 and R3 denote fatty acids. Figure taken from Tso, 1994.

The monoacylglycerol pathway is rapid and unidirectional, and involves the direct acylation of absorbed monoglyceride by activated fatty acids. This occurs in two steps, mediated sequentially by monoacylglycerol acyltransferase and diacylglycerol acyltransferase (Tso, 1994). This mechanism is summarised in Figure 1.2a. The secondary glycerol-3-phosphate pathway predominates in the fasted state when monoglyceride supply is restricted. This pathway synthesises triglyceride from glycerol-3-phosphate, derived from endogenous sources, and absorbed fatty acids (Mansbach and Parthasarathy, 1982). The glycerol-3-phosphate pathway is summarised in Figure 1.2b. Triglyceride synthesis by this route is slow and the resulting triglyceride may efflux back into the intestinal lumen. Diglyceride intermediates from the  $\alpha$ -glycerophosphate pathway may be used for *de novo* synthesis of phosphatidyl choline via the Kennedy pathway (Tso, 1994).

#### 1.2.3 Lipoprotein composition and structure

The intestine secretes two classes of triglyceride rich lipoproteins (TRL) - chylomicrons and very low density lipoproteins (VLDL). Chylomicrons are assembled during lipid absorption, predominantly from triglyceride derived from the monoacylglycerol pathway (Green and Glickman, 1981; Mansbach and Parthasarathy, 1982; Tso, 1994). Chylomicrons are large macromolecules, comprising a neutral lipid core (almost all triglyceride, with a small amount of cholesterol ester) surrounded and stabilised by an amphiphilic monolayer of phospholipid. Cholesterol is also present and partitions either into the core or within the phospholipid membrane (Figure 1.3). Specialised proteins, called apolipoproteins, are associated with the surface monolayer and act to regulate transport, uptake and utilisation of lipoproteins. With the exception of apoB, which acts as an integral membrane protein, all apolipoproteins can dissociate from one lipoprotein complex and transfer to another (Glickman et al., 1986; Knott et al., 1986; Chatterton et al., 1991). The triglyceride loading dictates the size of secreted chylomicrons and is dependent on the dietary intake (Hayashi et al., 1990). Chylomicron diameters can range from 75-6000nm, but tend to be 100-200nm on average (Bisgaier and Glickman, 1983; Anderson et al., 1989; Hayashi et al., 1990). Chylomicrons are responsible for transporting exogenous lipids to the body - their large diameter means that they are selectively transported via the lymphatics. Lymphatically transported chylomicrons enter the systemic circulation via the thoracic duct at the junction of the left internal jugular vein and the left subclavian vein (Sabesin and Frase, 1977; O'Driscoll, 1992). Once in the blood, chylomicrons deliver their triglyceride load to cells and are converted into cholesterol ester rich chylomicron remnants. These are taken up by liver cells and used either to generate hepatic VLDL (Hussain et al., 1996) or as a source of cholesterol for bile acid synthesis (Bravo et al., 1996).

VLDL have a smaller diameter and triglyceride loading than chylomicrons. Their assembly and secretion predominates in the 'fasted-state' and they are involved in supplying the body with triglyceride synthesised largely from endogenous sources (Ockner et al., 1969a; Ockner et al., 1969b). Their smaller size means that they may be transported via the lymph or the portal blood supply in the intestine. Although the systemic processing pathway of hepatic VLDL has been well characterised, there is little data available specifically on intestinal VLDL behaviour. Data from Ockner et al. (1969a) show that intestinal VLDL contain the apoB 100 isoform, in common with hepatic VLDL, and hence should follow a similar processing route. Hepatic VLDL undergo conversion to intermediate density lipoproteins (IDL) and low density lipoproteins (LDL) as the triglyceride load is delivered systemically. The resulting LDL are taken up by the liver or by macrophages. In contrast, Tso et al. (1984) have suggested that intestinal VLDL and chylomicrons share a common apolipoprotein composition. If this is the case, systemic processing of intestinal VLDL is likely to follow the chylomicron route.

Evidence indicates that the intestine secretes one further lipoprotein class – high density lipoproteins (HDL). These are thought to be secreted in a nascent form, as cholesterol ester deficient discoidal HDL particles containing apolipoprotein A-I and phospholipid (Green and Glickman, 1978). Additional surface apolipoproteins, cholesterol ester and phospholipids are gained systemically by exchange with tissues and TRL (Shepherd, 1994).

Because of the difficulties associated with isolating human lymph, the majority of data on human lipoprotein composition are typically sourced from plasma samples. General characteristics of human plasma lipoproteins are summarised in Table 1.1.

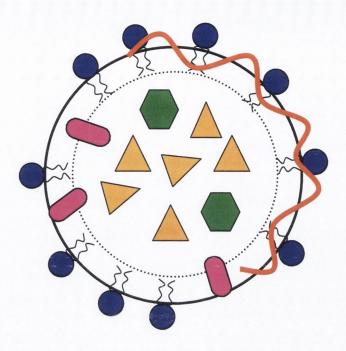


Figure 1.3: Basic structure of a chylomicron as modified from Wasan and Cassidy (1998). Chylomicrons comprise a neutral lipid core of triglyceride ( $\triangle$ ) and cholesterol ester ( $\blacksquare$ ) surrounded by a surface monolayer of amphiphilic phospholipids ( $\frac{\blacksquare}{8}$ ) in which apolipoproteins, including apolipoprotein B ( $\sim$ ) are embedded. A small amount of cholesterol ( $\blacksquare$ ) is present and partitions between the core and the phospholipid monolayer.

Table 1.1: General characteristics associated with human plasma lipoproteins

	Chylomicrons	VLDL	IDL	LDL	HDL
Density (g/ml)	< 0.95	0.95-1.006	1.006-1.019	1.019-1.063	1.063-1.21
Svedberg flotation (Sf)	>400	20-400		0-20	
Diameter (nm)	75-120 a	3-8	2.5-3.5	1.8-2.5	<1.2
Protein (% dry wt)	1-2	8	19	22	30
TG (% dry wt)	86	55	23	6	4
PL (% dry wt)	7	18	20	22	30
CE (% dry wt)	5	19	38	50	19
Ratio TG:PL	12.3	3.05	1.15	0.27	0.13
Major apolipoproteins	B48, A-I, A-II,	$B100^{b}$	B-100,	B100	A-I, A-II, A-IV
	A-IV,				
Minor apolipoproteins	C, E (largely	C, E	C, E		C1, C2, C3, E
	acquired via plasma				
	exchange)				

Modified from (Wasan and Cassidy, 1998) with additions from Forte and Nichols (1972), Shepherd (1994) and Tso (1994).

a: Lymphatic chylomicrons will contain a higher triglyceride load and have a larger diameter as compared to plasma lipoproteins because lipid exchanges occur during passage to the blood.

b: Plasma VLDL are largely derived from hepatic lipoprotein assembly, hence the reported apolipoprotein composition may differ from that of intestinally derived VLDL

# 1.2.4 Overview of Assembly Path for Chylomicrons

The general pathway followed during lipoprotein assembly has been elucidated by electron microscopic ultrastructural and immunolocalisation studies (Sabesin and Frase, 1977; Christensen et al., 1983). Triglyceride is re-assembled in the smooth endoplasmic reticulum (SER). Following fat feeding, large triglyceride lipid droplets accumulate in the channels of the endoplasmic reticulum (ER). ApoB is synthesised in the rough endoplasmic reticulum (RER). ApoB moves towards the SER and at the RER/ SER junction it associates with lipid. Large chylomicron sized lipoproteins are formed (60-350nm) and accumulate within the golgi apparatus. The golgi become distended and enlarged by their lipid load. Lipoprotein secretion occurs via exocytosis, whereby golgi vesicles fuse with the lateral plasmalemma, releasing chylomicrons into the intercellular space. The process is complete within 25 minutes.

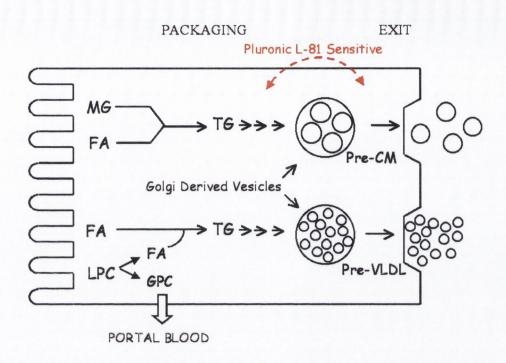
### 1.2.5 Two Step Theory of Lipoprotein Assembly

The interactions of apoB 100 with lipid have been widely studied and are well characterised. Similar interactions may be predicted for apoB 48 as a result of its structural identity. ApoB 100 is a large 524kDa glycoprotein composed of 4536 residues (Knott *et al.*, 1986; Yang *et al.*, 1986). Numerous hydrophobic regions (mostly comprising amphipathic α helical segments) span the length of the protein, and facilitate tight binding to the lipidic lipoprotein core (Knott *et al.*, 1986; Chatterton *et al.*, 1991). As the nascent, flexible protein is synthesised it associates with the inner leaflet of the RER and will only enter the lumen once it adopts the correct conformation. An initial lipidation step occurs during translocation. The growing peptide chain must associate with a certain amount of structural phospholipid and triglyceride to produce the correct conformation. In the absence of this lipidation step, the hydrophobic regions in the peptide chain will associate with eachother, resulting in misfolding (Gordon and Jamil, 2000). As the protein wraps itself around a small core of lipid, anchoring itself via hydrogen bonding, the elongated apoB molecule takes the form of a lipid deficient 'primordial' or 'nascent' lipoprotein. ApoB 48 forms HDL sized primordial lipoproteins and apoB 100, with its longer, more lipid dependent structure, forms LDL sized particles (Borén *et al.*, 1994).

As these lipid deficient primordial lipoproteins travel through the lumen of the endoplasmic reticulum (ER) towards the golgi, further lipidation or 'core expansion' occurs, resulting in fully lipidated primordial TRL. Core expansion may occur either by a continuous mechanism whereby lipid is transferred sequentially to the particle core, or via fusion of lipid deficient primordial lipoproteins with preformed lipid droplets. The lipid droplet fusion theory is supported by the ultrastructural studies of Sabesin and Frase (1977) and Christensen *et al.* (1983)

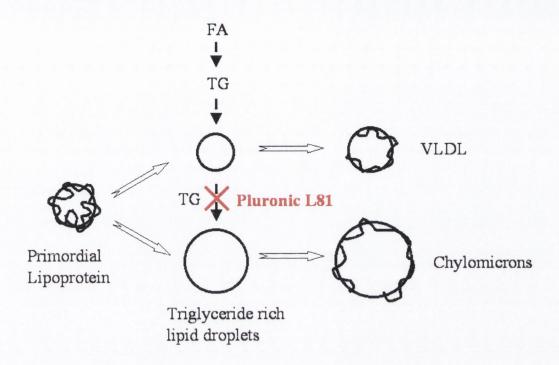
# 1.2.6 Alternative routes for intestinal VLDL and chylomicron assembly

Current data point to the existence of two separate routes for the assembly of VLDL and chylomicrons in the intestine. This concept was first introduced by Ockner and colleagues (Ockner et al., 1969a; Ockner et al., 1969b) following the observation of selective stimulation of VLDL or chylomicron secretion in response to different fatty acid stimuli. Results by this group found that duodenal infusion of stearic acid in the rat produced selective secretion of VLDL, whilst infusion of oleic or linoleic acid promoted secretion of chylomicrons. Extensive studies by Tso and colleagues have contributed much to the elucidation of this assembly path. Using a rat model, this group found that addition of Pluronic L81 to a triolein infusion significantly impaired lymphatic triglyceride output (Tso et al., 1980) and that this was due to reduced output of chylomicron sized lipoproteins (Tso et al., 1981). It was further shown that VLDL secretion, selectively promoted by phospholipid infusion, was unaffected by Pluronic L81 (Tso et al., 1984), thus demonstrating selective inhibition of chylomicron, but not VLDL, output. This led the authors to propose the model shown in Figure 1.4. In this model, the VLDL pathway preferentially obtains its triglyceride from the glycerol-3-phosphate pathway, which uses phophatidyl choline and fatty acid as substrates. In contrast, the chylomicron pathway obtains its triglyceride preferentially from the monoacylglycerol pathway, which uses degradation products of triglyceride (long chain unsaturated monoglycerides and free fatty acids) as its substrates. It is suggested that the chylomicron, but not the VLDL, synthetic pathway is susceptible to Pluronic L81 inhibition. Following the observation of large lipidic droplets in the intestinal ER of Pluronic L81 treated rats, it has been proposed that inhibition of chylomicron secretion occurs only at the final stage of assembly or secretion. This model has been supported by observations of Mahley et al. (1971), who showed that golgi vesicles tend to contain single, distinct size classes of TRL rather than a mixture. The presence of separate assembly paths for TRL would explain the preferential increase in chylomicron secretion observed following fat-feeding (Hayashi et al., 1990).



**Figure 1.4:** Schematic diagram for divergent pathways of chylomicron and VLDL assembly in the intestine as per Tso *et al.* (1984). MG = monoglyceride, FA = fatty acid, LPC = lysophosphatidylcholine, GPC = glycerophosphorylcholine.

Studies in the Caco-2 model by Luchoomun and Hussain (1999) support a slightly modified, but still divergent, assembly path. Studies by this group were conducted in cells using a dual-labelling technique to distinguish between pre-formed and newly synthesised lipids. Findings showed that although VLDL and chylomicrons both derived their phospholipid load predominantly from an intracellular, pre-formed pool, the triglyceride loading differed. Chylomicrons were found to carry an excess of newly synthesised versus pre-formed triglyceride, whilst VLDL carried equal amounts of each. These observations resulted in the sequential lipidation model. Lipid deficient 'primordial' lipoproteins are assembled as a first step to TRL assembly. These undergo fusion with preformed lipid droplets to produce either VLDL or chylomicrons, depending on the size of the lipid droplets and the extent of lipidation. It has been postulated that Pluronic L81 preferentially inhibits the formation of larger lipid droplets, thereby, selectively inhibiting formation of large chylomicrons (illustrated in Figure 1.5). This differs from the model proposed by Tso *et al.* (1984) in that the initial stages of the assembly path are shared by both TRL and effects are on lipid droplet formation as opposed to the lipid-protein interaction.



**Figure 1.5:** Schematic diagram for divergent pathways of chylomicron and VLDL assembly in the intestine as per Hussain (2000). FA = fatty acid, TG = triglyceride.

## 1.2.7 Importance of apoB for TRL secretion

ApoB is essential for the assembly and secretion of TRL. Studies conducted in transgenic mice have shown that in the absence of intestinal apoB, fat feeding results in the accumulation of large chylomicron sized lipid droplets within the intestinal cells and a deficiency of triglyceride secretion (Hamilton *et al.*, 1998). In these studies, lipid droplets were found to accumulate in the enterocytic ER and failed to transfer to the golgi.

# 1.2.8 Regulation of apoB synthesis and secretion

In humans, apoB exists in two isoforms - apoB 100 and apoB 48 (Glickman et al., 1986). Both forms are products of a single gene (Chen et al., 1987) and are synthesised in the RER. ApoB 100 is one of the largest known proteins (550kDa, 4536 residues) (Knott et al., 1986; Yang et al., 1986) and apoB 48 (264kDa, 2153 residues) is a truncated form comprising the initial 48% of the NH<sub>2</sub>-terminal of apoB 100. The deciding step in protein synthesis occurs via a posttranscriptional change in mRNA (Chen et al., 1987). This additional modification of mRNA is brought about by an organ-specific enzyme-dependent deamidation of cytidine at position 6666 of the nuclear transcript (Powell et al., 1987; Lau et al., 1991; Innearity et al., 1996) that introduces a UAA coded stop codon instead of a CAA coded glutamine at amino acid position 2153 and hence truncates the protein. The apobec-1 gene is involved in producing the editing enzyme involved in this process (Hirano et al., 1996). In humans, expression of the apoB 48 isoform only occurs in the intestine and apoB 100 is present in the liver (Glickman et al., 1986; Teng et al., 1990). Although chylomicrons are only secreted by the intestine in humans (Glickman et al., 1986) and only in association with apoB 48 (Kane et al., 1980), the apoB 48 isoform is not an absolute requirement for chylomicron assembly. Recent studies in apobec-1 knockout mice, which exclusively express the apoB 100 isoform in the intestine, have demonstrated that triglyceride transport may be effectively mediated by apoB 100 in the intestine (Ng et al., 2000). Further studies have shown that although apoB 48 is more efficient than apoB 100 at facilitating the early stages of chylomicron assembly, this impairment is overcome in the fat-fed state when later stages of assembly are rate limiting (Kendrick et al., 2001).

ApoB 100 secretion is controlled, in the liver, by intracellular degradation, which is in turn regulated by lipid availability. As apoB is translocated from the inner leaflet of the RER to the lumen, lipid must be added to the nascent peptide to stabilise the structure. When there is a deficiency of lipid the peptide undergoes misfolding and is subsequently tagged by ubiquitin for cytosolic degradation via proteasomal enzymes (Fisher *et al.*, 1997). It is not yet known

whether a similar mechanism regulates apoB secretion in the intestine, but the absence of any detectable levels of apoB ubiquitinylation in the intestine suggest that another mechanism may be involved in regulating secretion (Liao and Chan, 2000).

There are conflicting reports on the effects of fatty acid exposure on intestinal apoB secretion. A widely accepted view is that the administration of fatty acid does not alter the level of apoB secreted, but causes redistribution of the secreted lipoprotein profile, with fatty acid stimulation causing a shift towards larger and more triglyceride rich VLDL and chylomicrons. This theory is supported by data generated from *in vitro* and *in vivo* models (Hughes *et al.*, 1988; Hayashi *et al.*, 1990). Alternatively, some authors have shown an increase in the level of apoB secretion following fatty acid stimulation (Luchoomun and Hussain, 1999). This difference in opinion may be model related and requires further investigation.

There is convincing evidence for the involvement of calcium in the regulation of apoB secretion in intestinal cells. Calcium supplementation has been shown to increase the secretion of apoB by Caco-2 cells and to upregulate apoB mRNA editing (Hughes *et al.*, 1988; Chen *et al.*, 2000).

#### 1.2.9 MTP and apoB secretion

Lipidation of apoB is mediated, at least in the initial stages, by microsomal triglyceride transfer protein (MTP). This is a water-soluble heterodimer comprising a 58 kDa protein disulfide isomerase, and a large 97 kDa protein that possesses lipid transfer activity. It is located in the lumen of the ER and is found in both the intestine (mostly in the proximal jejunum) and the liver (Wetterau and Zilversmit, 1985; Wetterau et al., 1997). MTP is critically important for assembly of TRL. This is illustrated by the autosomal inherited disease, abetalipoproteinaemia, in which there is an absence of intestinal MTP as a result of gene defects (Wetterau et al., 1992; Sharp et al., 1993). Patients with this condition have extremely low circulating levels of apoB and related lipoproteins (chylomicrons, VLDL and LDL) and as a result, develop severe fat and fat-soluble vitamin deficiency (Aguie et al., 1995). This indicates a role for MTP in the very initial stages of lipoprotein assembly.

Studies involving the Hep G2 cell line indicate that there is a direct physical interaction between apoB 100 and MTP during lipoprotein assembly (Wu et al., 1996). This supports the role of MTP in early stage translocation and transport of apoB across the ER membrane. Wu et al. (1996) reported a 3-5 fold increase in MTP-apoB binding in the presence of long chain fatty acids. This observation is consistent with the ability of oleic acid to protect apoB from intracellular degradation by facilitating its translocation across the ER membrane. MTP may be

more critical for ensuring the intracellular stability of the larger apoB 100 molecule than for apoB 48. A study by Nicodeme *et al* (1999) found that low concentrations of a selective MTP inhibitor resulted in complete intracellular degradation of apoB 100. However, a number of smaller apoB molecules (apoB 51 and smaller) were still secreted, albeit in a lipid deficient form, and were only affected by higher concentrations of MTP inhibitor (10-fold higher concentration). This may be related to the size of the protein – longer protein structures contain more hydrophobic regions with high structural lipid dependence and so have greater capacity for misfolding.

There is some debate over the role of MTP in the process of core expansion. Data from the McA RH 7777 liver cell line have shown that administration of the selective MTP inhibitor, BMS-192951, following the initial formation of HDL sized lipoproteins, produced no effect on core expansion. MTP activity was inhibited by up to 75%, but this did not affect the size of the secreted lipoproteins (Gordon *et al.*, 1996). In contrast, addition of a selective MTP inhibitor, BMS-200150, to Caco-2 cells immediately after fatty acid administration (prior to initial formation of lipid deficient lipoproteins) produced a decrease in the secretion of both apoB and triglyceride, indicating a decrease in the size and the number of secreted lipoproteins (van Greevenbroek *et al.*, 1998). These differences may be model dependent and this step requires further clarification.

# 1.3 Lipid vehicles and drug absorption

Lipids and lipid vehicles have been shown to increase the bioavailability of co-administered lipophilic drugs. Such vehicles typically comprise a digestible lipid with (in the case of more complex self-emulsifying formulations) a blend of surfactants, co-surfactants and co-solvents. A number of mechanisms have been proposed for lipid vehicle enhancement of systemic drug absorption and are summarised below (Aungst, 1993):

- Increased aqueous solubilty in gastrointestinal fluid
- Reduced gastric movement
- Increased passage across the aqueous boundary layer
- Enhanced membrane permeation
- Inhibition of apically polarised drug efflux activity
- Inhibition of enterocytic drug metabolism
- Promotion of lymphatic uptake

## 1.3.1 Increased drug solubility

Fat digestion results in the formation of a mixed micellar phase containing high concentrations of bile salts, amphiphilic lipid digestion products and phospholipids. This creates an environment that is favourable to the solubilisation and wetting of lipophilic drugs (Hernell et al., 1990). Co-administration of a digestible oil can therefore increase aqueous drug solubility by taking advantage of the natural lipolysis process (MacGregor et al., 1997). For example, bile salt solutions have been shown to enhance aqueous solubility of lipophilic drugs in a concentration dependent manner. Increased rate and extent of drug dissolution occurs through a combination of wetting (Luner and Kamp, 2001), which predominates at sub-micellar concentrations, and micellar solubilisation (Bates et al., 1966; Bakatselou et al., 1991; MacGregor et al., 1997). Bates et al. (1966) demonstrated an increase in the aqueous equilibrium solubility of two hydrophobic compounds, griseofulvin and hexestrol, with concentrations of taurocholic acid in excess of the CMC, attributable to micelle solubilisation. Addition of 40mM taurocholic acid to the test system was found to increase the aqueous solubility by 148-fold for hexestrol and 5.6-fold for griseofulvin. Similarly, Bakatselou et al. (1991) reported a 2-fold increase in the aqueous solubility of hydrocortisone and a 20-fold increase in that of danazol in the presence of 30mM taurocholic acid, which was again attributable to micelle solubilisation. The bile salt effect on drug solubility appears to be dependent on drug properties – with drugs of higher log P being more susceptible to bile saltmediated solubilisation (Mithani et al., 1996). Bile salts have also been shown to increase the initial rate of dissolution through a combination of wetting and solubilisation effects. Baketselou et al (1991) reported a bile salt concentration dependent increase in the initial rate of dissolution for a number of steroids, including hydrocortisone, triamcinolone, dexamethasone In each case, a concentration of 15mM sodium taurocholate increased the dissolution rate by 50-100 fold versus simple 0.1N NaCl solution.

Incorporation of phospholipids into the bile salt solution may further enhance solubilisation capacity. For example Naylor *et al.* (1995) found that a mixed micellar bile salt: lecithin system (15mM:3.75mM) produced a 5-fold higher increase in the aqueous saturation solubility of danazol (log P 4.53) as compared to a simple 15mM bile salt solution. The initial rate of dissolution was also increased by the incorporation of lecithin by a factor of approximately 5.5. Addition of lipolytic products such as long chain and medium chain fatty acid may impact on drug solubilisation. MacGregor *et al.* (1997) reported a >3- fold increase in progesterone solubility in a 15mM bile salt solution containing 0.5% long chain or medium chain lipolytic products versus a simple 15mM bile salt solution. Additionally, Kossena *et al.* (2003) found that incorporation of long and medium chain fatty acids and monoglycerides into bile salt buffer

considerably enhanced the aqueous solubility of lipophilic drugs grisofulvin, danazol and halofantrine relative to bile salt buffer alone. The effects of digested lipid vehicles on aqueous drug solubility are dependent both on drug properties and on the nature of the administered lipid. Long chain triglyceride digestion products have a higher capacity for increasing the aqueous solubility of highly lipophilic drugs in bile salt/ phospholipid solutions (Kossena *et al.*, 2003). However, under conditions of incomplete triglyceride digestion, drug may remain within the undigested oil phase and fail to partition into the aqueous/ mixed micellar phase. This may limit aqueous dissolution/ solubilisation and hence impair drug bioavailability (Kaukonen *et al.*, 2002).

Increased aqueous drug solubility in the intestinal lumen will result in enhanced bioavailability if drug solubilisation is the rate limiting step to drug absorption, as typified by Type II drugs of the Biopharmaceutics Classification Scheme (BCS) shown in Table 1.2 (Amidon *et al.*, 1995).

Table 1.2: Biopharmaceutics Classification Scheme as described by Amidon et al. (1995)

Type I	Type II		
High aqueous solubility	Low aqueous solubility		
High membrane permeability	High membrane permeability		
Type III	Type IV		
High aqueous solubility	Low aqueous solubility		
Low membrane permeability	Low membrane permeability		

Co-administration of surfactant agents with drug vehicles may increase drug solubility by providing additional wetting and solubilisation effects or by improving drug and oil dispersion (Goodhart and Martin, 1962; Humberstone and Charman, 1997; Meaney and O'Driscoll, 2000). Certain oil-surfactant combinations can produced microemulsion or micellar systems on dilution, hence presenting incorporated drug in a suitably disperse form to eliminate the need for further lipid digestion (Pouton, 2000).

When formulating vehicles that contain digestible lipids, it is important to consider the effects that incorporated surfactants may have on lipid digestion. For example, an *in vitro* lipolysis study conducted by Solomon *et al.*, (1996) found that a series of nonyl phenol ethoxylates inhibited pancreatic lipase mediated digestion of medium chain triglyceride in a HLB dependent manner. Inhibition was observed for hydrophilic surfactants, with HLB>12. This is a particular concern because many oil-in-water emulsifiers fall in this HLB range. However, a similar *in vitro* study by Gargouri *et al.* (1983) found that a number of hydrophilic non-ionic surfactants

including polysorbate 80, polysorbate 20 and Triton X, produced no inhibition of pancreatic lipase under simulated *in vivo* conditions (bile salt and colipase present).

### 1.3.2 Gastric emptying

Small amounts of digestible oil may increase gastric emptying (by stimulating gastric motility), whilst large volumes delay it. This can have important consequences for drug absorption (Aungst, 1993). A study on intestinal absorption of a model lipophilic compound, SL-512, revealed a direct relationship between gastric emptying rate and the appearance of drug in plasma (Yamahira et al., 1978). Prolonged gastrointestinal residence is favourable for slowly dissolving drugs or drugs that undergo saturation kinetics at the site of absorption because the drug is released slowly into the intestine from the stomach and so is presented at its window of absorption in a gradual fashion - allowing more time for absorption (Aungst, 1993). Correlation between phenytoin bioavailability and residence time in stomach has been shown, with total bioavailability (measured as AUC) increasing with delayed gastric motility (Shinkuma et al., 1985a; Shinkuma et al., 1985b). The effects of lipids on gastric emptying may, however, complicate absorption kinetics when lymphatic drug uptake is applicable. Charman and Stella (1986a) postulated that delayed gastric emptying may decrease the relative concentration of drug per unit volume of co-administered lipid delivered to the intestinal lumen. This would impair the lymphotropic potential of the lipid vehicle and result in decreased lymphatic drug uptake.

### 1.3.3 Membrane permeability

Where membrane permeability is the rate limiting step to drug absorption, as with Type III or IV drugs of the BCS, bioavailability can be increased by lipid vehicle effects on membrane integrity. Although the lipid bilayer membrane may not present a significant barrier to the majority of lipophilic drugs (mostly Type II of the BCS), the effects of lipid vehicle components on physical membrane integrity can be substantial and so will be discussed here. Lipolytic degradation products, bile salts and surfactant molecules have all been shown to disrupt membrane integrity, either by altering cell membrane fluidity or by opening tight junctions between adjacent cells. Surfactants have been shown to alter membrane fluidity by solubilising out lipid components from the membranes, or by penetrating into the lipid bi-layer (Evrard et al., 2001; Dimitrijevic et al., 2000; Regev et al., 1999). Additionally, a number of lipid digestion products may alter the permeability of tight- junctions, thus increasing paracellular drug transport (Meaney and O'Driscoll, 2000; Anderberg et al., 1992). In particular, medium chain fatty acids are well recognised as paracellular permeation enhancers. Fatty acids of carbon chain length 8-12 have been shown to enhance Caco-2 monolayer permeability to

hydrophilic paracellular markers, such as mannitol (Lindmark et al., 1995; Anderberg et al., 1993), fluorescein (Lindmark et al., 1998) and fluoroscein isothiocyanate dextran (Hayashi et al., 1999). Promotion of paracellular transport is associated with reversible dilatation of tight junctions and is concentration dependent (Anderberg et al., 1993; Lindmark et al., 1995). Available data indicate that the opening of tight junctions by medium chain fatty acids may be associated with activation of the intracellular phospholipase C pathway. This produces an increase in intracellular calcium content, which in turn stimulates calmodulin-dependent contraction of actin fibres, resulting in opening of tight junctions (Lindmark et al., 1995). In contrast, long chain fatty acids tend to increase membrane permeability by penetrating into the lipid bilayer and disrupting its structure, hence altering membrane fluidity (Muranishi, 1990). Bile salt solutions and mixed bile salt: fatty acid micellar systems have been shown to increase paracellular membrane permeability, by opening tight junctions, and also to increase transcellular permeability, by altering membrane fluidity (Aungst, 2000; Meaney and O'Driscoll, 2000). The paracellular effect has been shown in the rat model by Lane et al (1994), following the observation of increased intestinal permeability to a range of paracellular permeants, including mannitol, PEG 400 and PEG 600, following co-administration in sodium cholate or mixed sodium cholate: linoleic acid micelles. More recent studies by Meaney and O'Driscoll (1999 and 2000) have also shown this response in the Caco-2 model. The authors of this work demonstrated an increase in Caco-2 monolayer membrane permeability both to hydrophilic paracellular markers (mannitol, PEG 900 and PEG 4000) and to a lipophilic transcellular marker (dexamethasone) in the presence of mixed sodium taurocholate: linoleic acid micelles.

#### Inhibition of drug efflux and pre-absorptive metabolism

Although permeability across the physical enterocytic cell membrane barrier tends not to be rate limiting to lipophilic compounds, the presence of a number of counter-transport proteins and phase I metabolic enzymes constitutes a significant biochemical barrier to drug absorption (Wacher *et al.*, 1996). The key protein involved in drug efflux in the intestine is P-glycoprotein (P-gp) or multiple drug resistance (MDR) protein. P-gp is a member of the extensive ATP binding cassette (ABC) superfamily of transport proteins. These proteins act as energy dependent pumps to transport a wide variety of compounds across membranes against a concentration gradient (Leslie *et al.*, 2001). P-gp is a 170kDa protein, encoded by the *ABCA* gene, and it acts to transport a broad range of xenobiotics out of cells. It is expressed in a number of tissues, including the liver, kidney, blood brain barrier and the small intestine (Hidalgo and Jibin, 1996). Intestinal P-gp is concentrated in the villus tip, where it acts to limit drug bioavailability by transporting drugs back into the intestinal lumen. P-gp is composed of two transmembrane domains, each containing six membrane spanning α-helices and two

cytosolic ATP binding domains (Linton et al., 2003). Substrates for P-gp are broad ranging and daunorubicin), alkaloids (reserpine, anthracyclines (doxorubicin, vinblastine), taxoids (taxol), peptides (cyclosporin A), steroid hormones (dexamethasone, hydrocortisone, aldosterone) and dye molecules (rhodamine R123) (Ford and Hait, 1990; Hunter and Hirst, 1997; Seelig, 1998). In general, substrates tend to be weakly basic lipophilic or amphiphilic compounds. An extensive study by Seelig (1998) identified the presence of two electron donor groups with a spacial separation of either 2.5±0.3Å or 4.6±0.6 Å as being essential for compound interaction with P-gp. As many substrates are lipophilic in nature, it has been proposed that protein binding/ interaction sites are located in the transmembrane region (Higgins and Gottesman, 1992; Ferté, 2000). Evidence points to the existence of multiple binding sites on the protein (Garrigos et al., 1997; Pascaud et al., 1998; Buxbaum, 1999). A number of mechanisms of drug transport have been proposed for P-gp. mechanism is that of a flippase action as described by Higgins and Gottesman (1992). By this process, drugs partition into the lipid bilayer of the cell membrane and bind to the transmembrane region of P-gp within the inner leaflet of the membrane. Drug binding induces an energy (ATP) dependent conformational change in the protein, such that drugs are transferred ('flipped') either to the outer leaflet of the membrane or directly into the extracellular aqueous phase.

In addition to efflux proteins, intestinal cells contain enzymes of the Cytochrome P450 (CYP450) family, which act to catalyse the oxidation of a wide range of xenobiotics (Hasler et al., 1999). Cytochrome P450 3A (CYP3A) is the predominant form found in the intestine and metabolises approximately 50% of all drugs that are substrates for the CYP450 family in humans. CYP3A and P-gp tend to be co-localised in tissues, including the intestinal villous tip, and demonstrate a broad overlap of substrate and inhibitor specificity (Table 1.3) (Wacher et al., 1995; Wacher et al., 1996; Kim et al., 1999; Benet et al., 1999; Cummins et al., 2002). It is therefore thought that these proteins act synergistically to form a concerted barrier to drug absorption. Synergy results because P-gp presents drugs to the CYP3A protein in a gradual and cyclical fashion, allowing for effective metabolism without enzyme saturation. In addition, oxidised metabolites of CYP3A substrates that might otherwise act to inhibit CYP3A, may also be transported from the inner leaflet of the cell membrane by P-gp (Benet and Cummins, 2001).

A number of typical lipid vehicle excipients have demonstrated considerable inhibitory activities against counter-transport proteins, thus increasing substrate drug bioavailability (Wagner *et al.*, 2001). The surfactant class of excipients is of particular importance in this regard. Cremophor EL, a commonly used pharmaceutical non-ionic surfactant, is a potent inhibitor of P-gp. Woodcock *et al.* (1990) first demonstrated this activity in the R100 cell line

(a multidrug resistant phenotype of the CCRF-CEM bone marrow cell line). Co-administration of Cremophor EL was found to make drug resistant cells sensitive to the cytotoxic effects of two chemotherapeutic agents, daunorubicin and vinblastine. Further to this report, a wider range of surface active agents have been evaluated for their inhibitory effects on efflux activity in R100 cells. Cremophor EL, polysorbate 80 and Solutolol have all been identified as P-gp inhibitors in this cell line (Woodcock et al., 1992). Studies in the Caco-2 model have shown that Cremophor EL and polysorbate 80 inhibit the efflux of Acf(N-Mef)<sub>2</sub>NH<sub>2</sub>, a model substrate peptide, in a concentration dependent manner (Nerurkar et al., 1996). Inhibition was observed at concentrations below and above surfactant CMC values, thus implying that inhibitory effects are associated both with surfactant monomers and additionally, with micelles. observations have been made for Pluronic block copolymer surfactants in the Caco-2 cell line (Batrakova et al., 1998; Weintraub et al., 1997). D-α-tocopherol polyethylene glycol 100 succinate (TPGS), a vitamin E derived surfactant has been shown to significantly increase the oral bioavailability (measured as AUC) of cyclosporin, a recognised P-gp substrate, in humans. Beneficial effects were attributed to combined inhibition of intestinal drug efflux and increased gastrointestinal drug solubility (Chang et al., 1996). In vitro-in vivo correlation of drug efflux has been demonstrated for TPGS, as it has also been shown to inhibit the efflux of P-gp substrates (amprenavir, Rhodamine 123, vinblastine and paclitaxel) in the Caco-2 model (Yu et al., 1999; Dintaman and Silverman, 1999).

It is currently thought that non-ionic surfactants inhibit P-gp by altering membrane fluidity. Pgp is located at the apical surface of cells and adopts a very specific conformation within the lipid bilayer membrane (Linton et al., 2003). Non-ionic surfactants can integrate within cell membranes, altering membrane fluidity and producing conformational changes in membrane proteins, thus modifying activity. Additionally, surfactant micelles may solubilise cell membrane components, thus producing changes in protein structure and function (Nerurkar et al., 1996; Rege et al., 2002). A recent study by Regev et al. (1999) showed that specific membrane fluidising agents such as ether and chloroform produced generalised inhibition of Pgp activity, as demonstrated by increased flip-flop of daunorubicin across cell membranes. A number of tested non-ionic surfactants including polysorbate 80 and Cremophor EL demonstrated similar inhibition characteristics to the anaesthetic agents, showing an absence of classic specific enzyme inhibition kinetics and implicating membrane fluidisation as the principle mechanism of action. Furthermore, inhibition associated with these surfactants was found to increase at concentrations approaching the CMC (Nerurkar et al., 1996), at which concentration membrane fluidisation also becomes significant (Regev et al., 1999). A further mechanism of action has been proposed for the Pluronic block copolymers (Batrakova et al., 2000). Work by Batrakova and colleagues has shown that Pluronic block copolymers have the

ability to interfere with energy dependent processes in the cell by lowering intracellular ATP concentrations. The drop in cellular ATP levels has been shown to correlate closely with drug efflux activity in a range of cells (Batrakova *et al.*, 2001; Kabanov *et al.*, 2003). This mechanism may be applicable to a wider range of surfactants.

Lipid digestion products have been shown to interfere with P-gp activity. For example Lo and Huang (2000) have shown that sodium caprate, a medium chain fatty acid, significantly impairs the active efflux of the amphiphilic anthracycline, epirubucin, in Caco-2 cells. Inhibitory potential of sodium caprate was similar to that of verapamil, a recognised and potent inhibitor of P-gp. Bile salt and mixed bile salt micelles at physiologically relevant concentrations have also been shown to interfere with drug efflux (Lo and Huang, 2000; Ingels *et al.*, 2002).

Although there are no published findings on direct lipid vehicle effects on CYP3A, the close association between CYP3A and P-gp in terms of substrate specificity and possible functional synergy, means that inhibition of P-gp will impact on CYP3A activity. For example, increased cellular concentration of a P-gp substrate may result in saturation of CYP3A, resulting in decreased efficacy of metabolism.

Table 1.3: Substrates for and Inhibitors of CYP3A and P-gp, taken from Wacher et al. (1995)

CYP3A substrate	P-gp <sup>a</sup>	CYP3A substrate	P-gp <sup>a</sup>
Antiarrhythmics		Hormones	
Amiodarone	I	Dexamethasone	S
Lidocaine	I	Estradiol	Sc
Quinidine	I	Hydrocortisone	S, I
Antifungals		Progesterone	I
Itraconazole	I	Testosterone	I
Ketoconazole	I	Immunosuppressants	
Calcium-channel blockers		Cyclosporin A	S, I
Diltiazem	S, I	FK506	S, I
Felodipine	I	Rapamycin	S
Nicardipine	S, I	Other	
Nitrendipine	I	Digitoxin	$S^d$
Nifedipine	I	Erythromycin	I
Verapamil	S, I	RU 486	I
Chemotherapeutic agents		Tamoxifen	I
Etoposide	S	Terfenadine	I
Morpholino doxorubicin	$S^b$		
Paclitaxel	S		
Vinblastine	S		
Vincristine	S		
Vindesine	S		

a S, substrate; I, inhibitor

### Potential association between efflux proteins and lipid metabolism

Field et al. (1995) have proposed a potential role for ABC proteins in the assembly of TRL. A preliminary study by this group found that when a number of drugs with recognised P-gp inhibitory activity, including verapamil, triufluoperazine and progesterone, were administered with an oleic acid vehicle to Caco-2 cells, they produced a decrease in secretion of apoB and triglyceride. These compounds also significantly inhibited the transfer of plasma membrane cholesterol to the ER. Additionally, Pluronic L81, which is recognised as an inhibitor of chylomicron secretion, has been shown to impair drug efflux in MDR cells. A further association between ABC transport proteins and lipid metabolism has recently been highlighted in the studies of Berge et al. (2000). Findings by this group found that mutations in the genes encoding two members (ABCG5 and ABCG8) of the ABC protein family were associated with patients suffering from sitosterolemia, an autosomal recessive condition in which excessive intestinal absorption and impaired biliary output of sterols is observed (Berge et al., 2000). In addition, ABC-1 transporter proteins have been shown to play a central role in cholesterol

b Doxorubicin, a precursor of morpholino doxorubicin, is a P-gp substrate

c 17-β estradiol glucuronide, a phase II metabolite of estradiol, is a P-gp substrate

d Digoxin, a metabolite of digitoxin, is a P-gp substrate

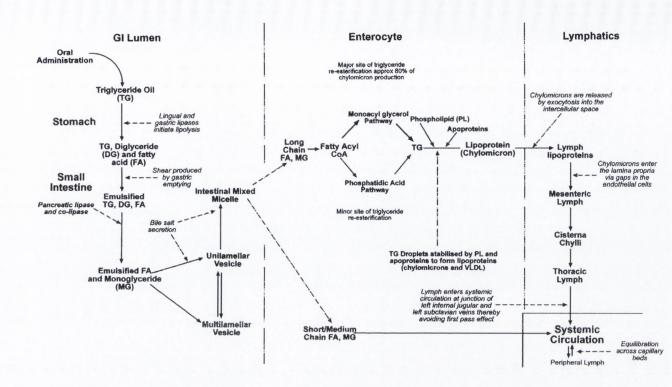
homeostasis by regulating the amount of cholesterol absorbed from the diet and also, may be involved in intracellular trafficking of phospholipid (McNeish et al., 2000).

### 1.3.4 Aqueous boundary layer

The aqueous boundary layer (ABL) has been discussed as a barrier to cellular absorption of lipid digestion products (Section 1.1.5.3). This layer of water, mucus and glycocalyx also poses a considerable physical barrier the passage of lipophilic drugs (Westergaard and Dietschy, 1974; Karlsson and Artursson, 1991; Naruhashi et al., 2003). For example, Karlsson and Artursson (1991) found that the permeability of testosterone across Caco-2 monolayers was increased 2.8fold when the aqueous boundary layer thickness was reduced from 1544µm to 128µm via mechanical agitation. Amidon et al (1982) made similar findings for the passage of capric acid across a synthetic lipid-like membrane in an earlier study, with a 2-fold increase in drug passage as membrane agitation was increased from 60 to 300 rpm. The mixed micellar phase produced during lipid digestion serves to facilitate passage of solubilised lipophilic drugs across the ABL in a similar manner to that described for lipid digestion products (Section 1.1.5.3). Micellar concentrations of synthetic surfactants or microemulsion-style lipid vehicles may also facilitate drug passage across the ABL (O'Driscoll et al., 1991b; Aungst, 1993; O'Reilly et al., 1994). For example, a cell-free study conducted by Amidon et al, using a synthetic lipid-like membrane, found that progesterone transport across a bound water layer was increased 2-3 fold following incorporation of drug into Polysorbate 80 micelles.

## 1.3.5 Lymphatic drug uptake

In addition to the direct effects of digestible lipids on drug solubilisation and cellular uptake, lipids may also enhance drug bioavailability by promoting lymphatic transport of lipophilic drugs. The majority of intestinally absorbed drugs diffuse through enterocytes and into the portal blood supply. Highly lipophilic drugs, however, may associate with newly synthesised triglyceride that is being assembled into large chylomicrons. Once incorporated into chylomicrons, lipophilic drugs gain direct access to the lymphatics (Counsell and Pohland, 1982). Evidence has shown that lymphatic drug uptake occurs as a result of drug association with the triglyceride core of lymphatic TRL (Pocock and Vost, 1974; Sieber, 1976; McIntosh *et al.*, 1999). The route by which digestible lipids gain access to the lymphatics is summarised in Figure 1.6. Co-administered lipophilic drugs are expected to enter the enterocytes in association with the intestinal mixed micellar phase and to gain access to the lymphatics in association with the triglyceride pool of the chylomicron, hence, following a similar route to the long chain fatty acids. This subject forms a core part of the underlying theory to this thesis, and hence will be covered in detail in the following section.



**Figure 1.6:** Schematic diagram describing the sequential steps in the digestion of lipids and subsequent absorption via the portal blood and intestinal lymphatics (Porter and Charman, 1997).

# 1.4 Lymphatic drug uptake

## 1.4.1 Anatomy and physiology of the intestinal lymphatics

The lymphatic system comprises a network of drainage vessels spread throughout the body. The lymphatics trace the path of the blood circulatory system and function to return plasma fluid and protein that have leaked into the interstitial space back into the blood system. In this manner, it acts to maintain the body's water balance. Initial or capillary lymphatic vessels are suited to this function as they have uniquely 'leaky' walls composed of a single layer of nonfenestrated endothelial cells with a non-continuous or absent basal membrane. (O'Driscoll, 1992; Swartz, 2001). At the level of the intestine, the lymph vessels have an important additional role in the transport of absorbed lipids from the enterocyte to the blood system, as their permeable nature permits selective absorption of the triglyceride rich chylomicrons, which are too large to permeate blood capillaries. Each of the gut villi is drained by a central lacteal, which drains fluid and absorbed lipids via the lymphatic capillaries to the mesenteric lymph duct. Lymph from the mesenteric duct (along with lymph from the hepatic and lumbar regions) drains into the cisternae chyli and from there, into the thoracic lymph duct. The thoracic lymph duct ascends upwards and empties into venous blood at the junction of the left internal jugular vein and the left subclavian vein (O'Driscoll, 1992; Porter, 1997). Thus, mesenteric lymph carries products from the intestine and into the systemic circulation, in a unidirectional manner, without passing through the liver. In contrast, products transported via blood pass into portal veins and undergo first pass metabolism in the liver before reaching systemic circulation.

Blood flow at the level of intestine is approximately 500 times faster than that of the lymph. This means that there is a natural movement of small, hydrophilic molecules into the portal blood and little opportunity for directing such molecules into mesenteric lymph (Pocock and Vost, 1974). Specific lymphatic delivery is possible when molecules are restricted from portal blood transport because of size, charge or hydrophobicity (Porter, 1997). For example, the preferential passage of chylomicrons into mesenteric lymph versus portal blood is dictated by size. Structurally, endothelial cells of blood vessels form a tight barrier featuring intercellular junctions and a basal lamina. Intercellular spaces preclude transport of compounds >10,000Da. In contrast, as mentioned above, initial or capillary lymphatic vessels have 'leaky' walls composed of a single layer of non-fenestrated endothelial cells with a non-continuous or absent basal membrane (O'Driscoll, 1992; Swartz, 2001). This allows for selective lymphatic passage of chylomicrons (diameter typically >100nm).

# 1.4.2 Required drug characteristics for lymphatic uptake

In order for a drug to undergo extensive lymphatic transport, it is necessary for it to have sufficient affinity for lymphatic triglyceride to overcome the higher mass transfer of fluid flow through the portal blood. It has been calculated that the mass transfer ratio between mesenteric lymph and portal blood is 1:50,000. This is based on the fact that portal blood flow is 500-times faster than that of the mesenteric lymph, and the lipid content of lymph, with which drugs associate, only accounts for 1% of the lymph volume. Based on this mass transfer difference, it has been calculated that a log P >5, and a triglyceride solubility of >50mg/ml are required for appreciable lymphatic transport. The importance of triglyceride solubility is illustrated by the superior lymphatic transport (13-fold difference) of DDT (log P 6.19, solubility 97.5mg/ml peanut oil) versus hexachlorobenzene (log P 6.53, solubility 7.5mg/ml peanut oil) when administered in an oleic acid dispersion to lymph cannulated rats (Charman and Stella, 1986b). These characteristics are favourable to lymphatic uptake, but do not guarantee it. For example, penclomidine, an experimental cytotoxic agent with a log P of 5.83 and a triglyceride solubility of 175mg/ml, demonstrated poor lymphatic uptake in a rat model when administered in soybean oil emulsion (<3% of administered dose as compared to 15% of DDT under similar conditions) (Myers and Stella, 1992). Drug delivery via mesenteric lymph provides a number of potential benefits to drug bioavailability. Firstly, as mesenteric lymph carries products in a unidirectional manner, without passing through the liver, hepatic first pass metabolism is avoided, thus increasing systemic levels of susceptible drugs (Porter and Charman, 2001). Secondly, the lymphatics act as a primary route for dissemination of tumour metastases and may also harbour a significant proportion of the HIV viral burden in infected individuals (Pantaleo et al., 1993; Tamalet et al., 1994) - site specific delivery of drugs to the lymphatics is therefore advantageous in these conditions (Porter and Charman, 2001).

#### Halofantrine as a model drug for lymphatic drug transport

Halofantrine is a highly lipophilic anti-malarial compound. Its log P is estimated at >8.5 and it has a reported triglyceride solubility of >50mg/ml (in arachis oil), thereby fitting the desired characteristics for lymphatic transport outlined by Charman and Stella (1986b). Clinically, halofantrine is useful in the treatment of drug resistant falciparum malaria and is well tolerated. However, the clinical use of halofantrine is restricted by its poor and variable oral bioavailability. Halofantrine is available as a tablet form and is administered three times daily as 500mg doses (Bryson and Goa, 2002). Oral bioavailability demonstrates wide inter- and intra-subject variability (Bryson and Goa, 2002) and has been estimated as <9% in beagle dogs (Humberstone *et al.*, 1996). Clinical treatment failure has been associated with sub-therapeutic concentrations of drug in plasma resulting from poor oral absorption. Therefore, halofantrine

requires a more effective delivery vehicle for ensuring adequate bioavailability. bioavailability of halofantrine was found to be increased significantly (approximately 3-fold in humans and 12-fold in beagle dogs) by co-administration with a fatty meal, thereby showing that co-administered lipids are beneficial to drug uptake (Humberstone et al., 1996; Bryson and Goa, 2002). The low aqueous solubility of halofantrine has been identified as a major contributing factor to its low oral bioavailability. Published findings have indicated that although increased drug solubilisation in the post-prandial intestinal milieu may be important for lipid vehicle-enhanced drug bioavailability (Humberstone et al., 1996), lymphatic drug uptake is also likely to play a key role. Up to 20% of administered halofantrine base was found to be transported via mesenteric lymph in a triple lymph cannulated rat model when administered with long chain oil (Porter et al., 1996b) and up to 54% of an administered halofantrine dose was subject to lymphatic transport in a post-prandial dog model (Khoo et al., 2001). Furthermore, a close correlation between lymphatic triglyceride output and halofantrine transport has been demonstrated in a rat model (Holm et al., 2001). Halofantrine has been shown to preferentially associate with the apolar lipid fraction of lipoproteins (McIntosh et al., 1999), thus implying that halofantrine is transported in association with the lipid core of lymphatic lipoproteins. Hence, halofantrine is an ideal model compound for studying chylomicron associated drug transport.

## 1.4.3 Lipid vehicle effects on lymphatic drug transport

Lipids can significantly enhance the intestinal lymphatic uptake of a co-administered lipophilic drug. However, the nature and characteristics of the selected lipid is critical for the outcome. As a general rule, a digestible oil rich in long chain unsaturated fatty acids will tend to favour lymphatic drug uptake by promoting lymphatic triglyceride output, whilst short chain or non-digestible oils will not. Desirable characteristics of the administered oil are discussed in further detail in the following sections and a summary of reported simple lipid vehicle (solutions or suspensions of drug in oil) effects on lymphatic drug uptake is shown in Table 1.4:

Table 1.4: Effects of co-administered oil on lymphatic drug uptake

Drug	Vehicle	Model	Lymphatic uptake	Reference
DDT	Sunflower oil	Thoracic lymph cannulated rat	55% transported within 4 hours via the lymph in association with chylomicrons	(Pocock and Vost, 1974)
o, p-DDT	Corn oil	Corn oil Thoracic lymph cannulated rat 48% transported via the lymphatics as compared to 24% from an ethanolic solution		(Sieber, 1976)
Halofantrine	Triolein, trilinolein, trilinolenin	Mesenteric lymph cannulated rat - conscious	11.1% transported in the lymph with trilinolein. Lymphatic transport in trilinolein> trilinolein> triolein	(Holm et al., 2001)
DDT	Soybean	Mesenteric lymph cannulated rat	19.37% transported in the lymph over 11 hours	(O'Driscoll et al., 1991a)
Probucol	Arachis oil, liquid paraffin	Thoracic lymph cannulated rat	$7.05\mu g/hr$ transported in the lymph with arachis oils versus $0.17\mu g/hr$ with liquid paraffin or $0.32\mu g/hr$ from an aqueous suspension.	(Palin et al., 1982)
DDT	Soybean oil	Mesenteric lymph cannulated rat	15% transported in the lymph	(Myers and Stella, 1992)
Halofantrine	Peanut oil, captex, tributyrin	Mesenteric lymph cannulated rat - conscious	15.8% dose transported in the lymph with peanut oil versus 0.34% from aqueous suspension. Lymphatic transport in peanut oil>>captex>tributyrin	(Caliph et al., 2000)
Mepitiostane	Triolein, Sesame oil	Thoracic lymph cannulated rat	41.2% of dose transported in the lymph in the presence of sesame oil and 45% with triolein as compared to 7.5% from an aqueous suspension.	(Ichihashi et al., 1992)

### 1.4.3.1 Fatty acid chain length in administered lipid

The preferential transport of fatty acids into portal blood or intestinal lymph is highly dependent on their chain length. The majority of fatty acids with carbon chain length <12-14 typically diffuse unaltered into portal blood, whilst more lipophilic long chain fatty acids may be incorporated into TRL and transported via the lymph (Kiyasu et al., 1952). In keeping with the general trend, Caliph et al. (2000) showed that lymphatic triglyceride output in a cannulated rat model was increased with C18 rich peanut oil, and followed a decreasing trend with decreasing fatty acid chain length of: peanut oil (C18)> Captex (medium chain, C8-C10)> tributyrin (short chain, C4). In this study, lymphatic transport of a co-administered lipophilic drug, halofantrine, followed a similar trend, such that lymphatic drug transport was closely related to triglyceride output. Palin and co-workers made a similar observation for lipid vehicle effects on lymphatic uptake of two lipophilic compounds, DDT and probucol. Their studies found that lymphatic drug uptake was substantially improved when drugs were co-administered with peanut oil and that a medium chain oil, Miglyol 812 and a non-digestible oil, liquid paraffin, failed to produce any such enhancements (Palin et al., 1982; Palin and Wilson, 1984). As peanut oil was the only administered oil with potential to promote TRL secretion, results were attributed to this response. In vivo human findings are consistent with these results. A study on the effects of administered lipid vehicles on the oral absorption of vitamin D3, a fat-soluble vitamin that is almost exclusively absorbed via the lymphatic route, found that oral bioavailability was substantially enhanced by administration in peanut oil as compared to medium chain Miglyol 812 (3-fold enhancement) (Holmberg et al., 1990).

### 1.4.3.2 Degree of fatty acid saturation in administered lipid

The degree of fatty acid saturation has a significant effect on the ability of lipid vehicles to promote lymphatic triglyceride output. In general, the size and triglyceride loading of secreted lipoproteins tend to be larger with unsaturated, as compared to saturated, fatty acids of equivalent chain length. Studies by Feldman *et al.* (1983) in the rat, using electron microscopy, showed that lipid vehicles containing increasing proportions of saturated (palmitic acid, tripalmitin): unsaturated (C18:1 rich safflower oil) fatty acids produced a progressive decrease in the size of intestinal lymph chylomicrons and an increase in size of secreted VLDL and HDL. Bergstedt *et al.* (1990) also noted an increase in secreted chylomicron size following administration of unsaturated versus saturated triglycerides to a rat. Lipid absorption was also higher with the unsaturated fatty acid. These findings are further supported by *in vitro* Caco-2 data. Studies by van Greevenbroek *et al.* (1996 and 2000) in Caco-2 cells found that whilst unsaturated fatty acids such as oleic acid (C18:1) and linoleic acid (C18:2) stimulated secretion of TRL, administration of saturated fatty acids, such as palmitic acid (16:0) and stearic acid

(18:0), promoted secretion of lipoproteins of equivalent density to LDL. Although the effect of unsaturated versus saturated oils on TRL secretion is well established, there is some disagreement over the relative enhancements of lymphatic triglyceride output with mono-, diand tri-unsaturated oils. For example, Cheema *et al.* (1987) demonstrated that polyunsaturated fatty acids stimulated lymphatic triglyceride output with a rank order of: linoleic (C18:1) >linolenic (C18:2) >oleic (C18:1) acid. In contrast, Holm *et al.* (2001) found a rank order of: linoleic >oleic> linolenic acid and van Greevenbroek *et al.* (1996) reported a trend of oleic>linoleic>stearic acid. As with fatty acid chain length, the effects of fatty acid saturation on lymphatic drug transport closely follow the lymphatic triglyceride output response. For example, Holm *et al.* (2001) found a linear relationship between cumulative transport of triglyceride and halofantrine in the lymph over 12 hours in the presence of oleic, linoleic and linolenic acid (r<sup>2</sup>>0.96).

### 1.4.3.3 Digestibility of lipid

The importance of lipid digestion in enhancing drug solubilisation in the gastrointestinal tract has been discussed above. Lipid digestion is also highly relevant to promoting lymphatic drug uptake. Long chain triglycerides can only be absorbed by cells and processed into triglycerides following luminal digestion into fatty acids and monoglycerides (Mattson and Volpenhein, For example, Porter et al. (1996a) found that when a oleate rich lipids were 1964). administered intra-duodenally to anaesthetised rats in a 'pre-digested' form - i.e. as a monoglyceride/free fatty acid micellar solution - lymphatic triglyceride response was considerably enhanced (in terms of the rate of onset and overall secretion) versus an administered peanut oil emulsion. Furthermore, when vehicles were administered orally to conscious rats (allowing for an additional gastric mixing and digestion step) the lymphatic triglyceride response to both vehicles was similar. Studies conducted by Palin and co-workers in the rat model found that lymphatic uptake of lipophilic drugs, DDT and probucol, was considerably lower following co-administration with a non-digestible mineral oil, liquid paraffin, than with a digestible lipid, peanut oil (Palin et al., 1982; Palin and Wilson, 1984). Levels of drug uptake with liquid paraffin were similar to those obtained from an aqueous suspension. It was hypothesised that association of lipophilic drugs with a non-digestible, nonabsorbable oil may result in the drug passing through the intestine and out of the body with the oil phase.

### 1.4.3.4 Lipid structure

The fatty acid composition of triglycerides and the positioning of fatty acid esters on the glyceride backbone can impact on TRL stimulating potential. Structured triglycerides are gaining increasing interest in clinical nutrition. They are synthesised with a specific fatty acid composition and specific fatty acid ester configuration on the glycerol backbone. triglycerides can incorporate medium chain fatty acids, that are easily hydrolysed and absorbed in the gastrointestinal tract with essential long chain fatty acids such as linoleic acid, which are less easily absorbed, to provide nutrition to individuals with lipid malabsorption conditions (Tso et al., 1999; Kishi et al., 2002). The significant effects that structured triglycerides can have on lymphatic drug absorption have been demonstrated by Holm et al. (2002). In this study, the lymphatic transport of halofantrine in a mesenteric lymph cannulated rat model was found to be similar following co-administration with either a long chain oil (sunflower oil) or a structured long-medium-long (L-M-L) chain triglyceride. In one set of tested rats, lymphatic uptake of halofantrine was also found to be similar in the presence of a medium-long-medium chain triglyceride (M-L-M). The fact that medium chain fatty acids can be incorporated into structured triglycerides without loss of lymphotropic potential means that benefits associated with medium chain fatty acids, such as superior solubilising properties, may be gained, thus providing higher drug concentrations for absorption.

## 1.4.4 Classes of lipid vehicles

The simplest form of lipid vehicle comprises a dispersion of drug in a suitable digestible oil. As described above, such vehicles can produce significant enhancements in lipophilic drug bioavailability via a number of mechanisms, including increased gastrointestinal solubilisation and lymphatic drug uptake. There are, however, a number of problems associated with employing such crude formulations. Firstly, the lipid solvent capacity for incorporated drug may be quite small, and hence will limit the administrable dose. Secondly, a triglyceride or mixed glyceride vehicle will require preliminary intestinal digestion before its benefits to drug absorption can be gained. This means that the lipid vehicle effects are subject to inter- and intra-patient variability and may be slow in onset. It is possible to overcome such issues and to further improve drug bioavailability by incorporation of additional excipients such as surfactants and co-solvents, which act to increase initial vehicle dispersion in gastrointestinal fluid (Humberstone and Charman, 1997). Lipid vehicles are typically administered in a concentrated (preconcentrate) form consisting of oil, surfactant and co-solvent components, and are diluted *in vivo* to generate oil-in-water dispersions. Lipid vehicle preconcentrates can be classified according to their ease of dispersion, digestibility and their oil-in-water droplet size

following aqueous dilution. Pouton (2000) devised a suitable classification scheme, which is summarised in Table 1.5.

**Table 1.5:** Classification of lipid based formulations and their typical properties as described by Pouton (2000).

Increasing hydrophilic content —						
Characteristics	$Type\ I$	Type II	Type IIIa	Type IIIb		
Typical composition (%) triglycerides or mixed glycerides	100	40-80	40-80	<30		
Surfactants (%)	_	20-60 (HLB <12)	20-40 (HLB >11)	20-50 (HLB >11)		
Hydrophilic co-solvents (%)	_	-	0-40	20-50		
Particle size of dispersion (nm)	Coarse	100-250	100-250	50-100		
Significance of digestibility	Crucial requirement	Not crucial, but likely to occur	Not crucial, but maybe inhibited	Not required and not likely to occur		

According to this classification system, Type I formulations consist of triglycerides or blends of mono-, di- and triglycerides that exhibit poor oil-in-water dispersion initially and that require extensive lipolytic degradation in order to facilitate emulsification in gastrointestinal fluid and solubilisation of any incorporated drug. Type II systems represent self-emulsifying drug delivery systems (SEDDS), which are isotropic mixtures of oil and surfactant (typically nonionic surfactants because of their low oral toxicity). These vehicles disperse easily on contact with aqueous medium to form a crude emulsion, but require lipolytic degradation to produce a fine emulsion with increased droplet surface area. Type III lipid systems contain blends of glycerides with surfactants and additional co-solvents or hydrophilic co-surfactants to increase rate of vehicle dispersion and drug solubilisation in the gastrointestinal tract. formulations are similar to Type II formulations, but may disperse more readily in aqueous medium. Type IIIb formulations have a high surfactant/ co-surfactant/ co-solvent: oil ratio and hence disperse spontaneously in water to produce a very fine oil-in water emulsion (typically droplet size 100nm), generally referred to as a microemulsion. These systems are sometimes referred to as 'self-microemulsifying drug delivery systems' (SMEDDS). Resulting type IIIb microemulsions are optically clear and thermodynamically stable as distinct from SEDDS. which show a tendency for phase separation when left to stand.

#### 1.4.4.1 Self-emulsification and microemulsions

Self-emulsifying drug delivery vehicles offer a means of overcoming some of the issues associated with simple oil dispersions. Firstly, the presence of a blend of oil phase components and surfactant compounds means that vehicle solvent capacity for lipophilic drug may be increased. Additionally, the vehicles tend to be reasonably viscous and so can be formulated as liquid gel capsules, providing a unit dose form that ensures increased accuracy of dosage and increased patient compliance. Self-emulsifying formulations generally comprise an oil phase (typically a digestible long chain oil or mixed glycerides) and a surfactant (generally non-ionic surfactants because of their good oral acceptability) (Gershanik and Benita, 2000; Constantinides, 1995). Surfactants are selected to give an appropriate hydrophile-lipophile balance (HLB) for producing an oil-in-water dispersion, typically, HLB>11. Surfactants act to lower surface tension at the oil-water interface, thus increasing ease of oil dispersion in gastrointestinal fluid. Self-emulsification is thought to occur when the entropy change that favours dispersion is greater than the energy required for the increase in surface energy associated with the increased surface area. This is summarised in equation 1.1:

$$\Delta G_f = \gamma \Delta A - T \Delta S$$

## Equation 1.1 (as per Lawrence and Rees, 2000)

Where:  $\Delta G_f$  is the free energy of formation,  $\gamma$  is the surface tension of the oil-water interface,  $\Delta A$  is the change in interfacial area,  $\Delta S$  is the dispersion entropy and T is the temperature.

Emulsification with minimal agitation is possible when  $\Delta G_f$  is extremely low. This can be achieved by minimising  $\gamma$ , for example with addition of high concentrations of surfactant. This is the principle associated with SEDDS formulation. Spontaneous emulsification becomes possible when  $\Delta G_f$  is negative. This can be achieved by minimising  $\gamma$  and increasing  $\Delta S$ , for example, by addition of high concentrations of surfactants that undergo dynamic micellemonomer surfactant exchanges. As the formation of oil-in-water dispersions is spontaneous, there is no tendency for phase separation on standing, thus the resulting system is stable. This is the principle associated with SMEDDS formulations (Lawrence and Rees, 2000).

#### 1.4.4.2 Microemulsions

A microemulsion may be defined as a system of water, oil and amphiphile which is forms a single optically isotropic and thermodynamically stable liquid solution (Danielsson and Lindman, 1981). This is in contra-distinction to a classic emulsion, which is kinetically

unstable and typically cloudy in appearance (Gershanik and Benita, 2000). The optical clarity associated with a microemulsion is a result of the fine dispersed droplet size, which is typically <100nm (Ritschel, 1991). These systems generally contain complex microstructural phases as a result of the high surfactant concentration (Gershanik and Benita, 2000). Drugs are solubilised in the microemulsion and presented at the site of absorption in a finely dispersed, almost colloidal form, such that the vehicles have a lower dependence on intestinal digestion for facilitating drug absorption. In terms of drug dispersion, these are clearly superior vehicles, but their low oil content may pose an issue in relation to solvent capacity for highly lipophilic drugs.

## 1.4.4.3 Formulation considerations for self-emulsifying lipid vehicles

Self-emulsifying formulations, as indicated above, contain a mixture of oil, surfactants and, optionally, co-solvents. The primary consideration in oral formulation design is excipient toxicity. For this reason, digestible, food grade oils and non-ionic surfactants comprise the bulk of formulation excipients (Lawrence and Rees, 2000). In terms of oral acceptability, unmodified edible oils are the preferred oil phase. However, limitations with regard to solvent capacity for certain drugs and poor emulsification properties often restrict the use of such oils. Hydrolysed oils and modified oils, such as polyglycolysed glycerides, have been employed to overcome these issues (Shah *et al.*, 1994; Constantinides, 1995; Gershanik and Benita, 2000; Kawakami *et al.*, 2002). With regard to fatty acid chain length, long chain oils are favoured in lymphotropic vehicles because they promote TRL secretion, however, medium chain oils may be beneficial for drug solubilisation because they are more easily emulsified in aqueous medium (Gershanik and Benita, 2000).

Non-ionic surfactants are the surfactants of choice for oral formulations because of their low oral toxicity (Swenson and Curatolo, 1992). Certain non-ionic surfactants are classed as food grade or have GRAS (generally recognised as safe) status. In order to promote oil-in-water emulsification, surfactants typically require a combined HLB value of 8-18, with the specific value being dependent on the oil phase and presence of additional excipients (Constantinides, 1995). Of the available surfactants, polysorbates are particularly useful because of their availability in a wide range of HLB values (Gershanik and Benita, 2000) and their good oral acceptability (polysorbates 60, 80 and 65 are all GRAS listed and polysorbates 20, 40, 60 and 80 are accepted as food additives in Europe and are on the FDA Inactive Ingredients Guide) (Lawrence, 2003). Other popular surfactants for lipid vehicle formulations include polyoxyethylene castor oil derivatives (the Cremophor series) and ethoxylated polyglycolysed glycerides (Gershanik and Benita, 2000). Recently, TPGS has been investigated as a surfactant

for oral lipid vehicle formulations. This compound was originally used as a water-soluble vitamin E supplement for use in lipid malabsorption conditions, but its effective solubilising properties and absorption enhancing properties have since been noted (Hong-Wei Wu and Hopkins, 1999). Co-solvents, when added, act to further solubilise drug and surfactant within the concentrated vehicle and aid drug solubilisation on dilution. Ethanol, polyethylene glycol and propylene glycol are most commonly used in this respect (Gershanik and Benita, 2000). However, high concentrations of co-solvent tend to be problematic when formulating vehicles as liquid capsules as they can leech into soft capsule shells, resulting in drug precipitation and alteration of vehicle composition. For this reason, co-solvent free vehicles incorporating a blend of surfactants with oil phase are favoured (Constantinides, 1995).

#### HLB

An important consideration in selection of a non-ionic surfactant in lipid vehicle design, as mentioned above, is its HLB (hydrophile-lipophile balance) number. This number is a dimensionless value assigned to a surfactant to describe its relative oil-water solubility and associated ability to emulsify or solubilise given ingredients. The system was originally devised by William C. Griffin, who was working for the Atlas Powder Company (which later became part of ICI) in 1949 to simplify surfactant selection for emulsion formulation. Complex experimental methods were originally used to measure HLB values based on solubility and dispersability measurements, but simple equations were later devised by Griffin to allow calculation of HLB values for non-ionic surfactants based on the relative proportions of oxyethylene (hydrophilic) and hydrocarbon (lipophilic) groups in the surfactant molecule or based on saponification and acid values. The HLB numbers scale surfactants, typically in a range of 0-20, based on relative hydrophilic/lipophilic balance. Surfactants with HLB values of ≤ 6 have relatively high oil solubility and hence favour formation and stabilisation of water-inoil emulsions. Surfactants with HLB values of 8-18 are more hydrophilic and typically act as oil-in-water emulsifiers and the most hydrophilic of this range (HLB 15-18) are good solubilisers. This scaling allows for a reduction in trial and error associated with initial surfactant selection to meet requirements and has been widely adopted within a number of markets including pharmaceutical and chemical coating industries (Shinoda and Friberg, 1986; Becher, 2001).

This classic system assigns HLB values in a fairly systematic manner, but it does not take into consideration the effects of temperature, surfactant concentration, rheological properties of dispersing medium, ionic strength of the aqueous phase or oil phase properties, all of which impact on emulsion formation, It also does not account for or explain the fact that mixtures of surfactants often display superior emulsifying properties to single surfactant systems. Attempts

have been made to account for such variables as temperature and environment by calculating HLB values from phase inversion temperature (the temperature at which an oil-in-water emulsion switches to a water-in-oil emulsion, or vice versa) or dielectric constants. Attempts have also been made to make more accurate structural determinations of HLB based on atomic structure (e.g. Davies and Lin methods) (Becher, 2001).

Although the assignment of HLB values lack exactness, they are still a useful guiding system for preliminary excipient selection in emulsions (especially where an oil has been given a 'required HLB' value for emulsion formation) and they are a good way of estimating oil or water solubility.

Self-emulsifying and self-microemulsifying vehicle effects on drug bioavailability 1.4.4.4 SEDDS formulations have been investigated widely in recent literature. They offer considerable advantages in terms of drug solubilisation as compared to Type I formulations and have been shown to significantly enhance bioavailability of co-administered drugs. Some key examples of formulation-based bioavailability enhancements are summarised in Table 1.6. For example, Hauss et al. (1998) found that administration of ontazolast in a SEDDS formulation comprising a Peccol oil phase with Gelucire 44/14 as surfactant produced a 14-fold increase in oral bioavailability and a 6-fold increase in lymphatic drug transport. These values were similar to data obtained with a crude soybean oil emulsion, but the t<sub>max</sub> was significantly lower. Improved drug bioavailability and quicker onset of action with the SEDDS formulation were attributed to increased lymphatic drug uptake, increased rate of drug solubilisation and possible vehicle effects on gastrointestinal transit time or drug metabolism. Charman et al. (1992) also reported a significant improvement in the reproducibility of the plasma profile of an investigational lipophilic compound, WIN 54954, following administration in a Neobee M5 medium chain oil/ Tagat TO surfactant SEDDS formulation versus a PEG dispersion.

The ease of dispersion of the SEDDS formulations is important for enhancing oral drug bioavailability. A study by Tarr and Yalkowsky (1989) showed that dispersed emulsion droplet size had a significant effect on the absorption of cyclosporin A. Using an *in situ* perfused rat intestine model, it was found that apparent permeability of cyclosporin A from a test emulsion prepared by homogenisation of Sandimmune® in water (nominal droplet size 2µm), was approximately twice that of an emulsion prepared by stirring (4µm droplet diameter). Similarly, Shah *et al.* (1994) demonstrated an inverse relationship between dispersed droplet size and release rate of a model lipophilic drug, RO 15-0778, from a series of SEDDS formulations.

Enhanced drug release or absorption profiles were attributed, in each case, to an increase in drug solubility facilitated by the dispersed state.

Enhanced drug solubilisation as a direct result of microemulsion formulation is exemplified by the first commercially successful microemulsion preconcentrate, Sandimmun Neoral®. This formulation produces a finely dispersed microemulsion on contact with gastrointestinal fluids, with an average droplet size of 39nm. In contrast, the crude emulsion formed by the original Sandimmune® self-emulsifying formulation produced an average droplet size of 864nm (Gao et al., 1998). The newer, self-microemulsifying formulation has been shown to produce a superior pharmacokinetic drug profile in humans, with significantly reduced t<sub>max</sub> and inter-subject variability. Additionally, the newer formulation is less sensitive to food effects. Bioavailability enhancement observed for the newer self-microemulsifying formulation versus the original self-emulsifying formulation has been attributed to a more rapid dissolution step. Drug solubilisation occurs on mixing with aqueous gastrointestinal fluids and the drug is presented in an absorbable form along the full length of the intestine, without relying on an initial vehicle digestion step (Kovarik et al., 1994). Further examples of commercially successful lipid formulations are given in Table 1.7.

Table 1.6: Effect of SEDDS and SMEDDS on bioavailability of poorly water-soluble drugs

Formulation	Drug	Oil phase	Surfactants	Response	Reference
SEDDS	WIN 54954	Neobee (medium chain triglyceride)	Tagat TO	√tmax (2-fold) and √ intersubject variability versus PEG solution	(Charman <i>et al.</i> , 1992)
SEDDS	RO 15-0778	Peanut oil/ Neobee	Labrafac CM10, polysorbate 80	↑Cmax (7-fold) ↑ bioavailability (11-fold) relative to capsule	(Shah et al., 1994)
SEDDS	Ontazolast	Peceol (mono and di- glycerides of oleic acid)	Gelucire 44/14	↑Cmax (27-fold) ↑ bioavailability (14-fold) relative to aqueous suspension	(Hauss et al., 1998)
SEDDS	Co enzyme Q <sub>10</sub>	Captex 200	Labrafac CM10, Lauroglycol	↑Cmax (2.2-fold) ↑ AUC (2.2-fold) relative to powder formulation	(Kommuru <i>et al.</i> , 2001)
SMEDDS	Cyclosporin A	Captex 355	Cremophor EL, Transcutol,	†absolute bioavailability - 3.3-fold increase versus Sandimmune® and 1.25-fold increase versus Neoral®.	(Gao et al., 1998)
SMEDDS	Halofantrine	Soybean, Maisine 35-1 (mixed glycosides of linoleic acid)	Cremophor EL	↑oral bioavailability (8-fold) relative to tablet	(Khoo et al., 1998)
SMEDDS	Cyclosporin A	Hydrolysed corn oil	*Polyoxyl 40 hydrogenated castor oil, DL-α-tocopherol, propylene glycol, ethanol	↓tmax (1.5-fold) and ↓ intersubject and intrasubject variability versus emulsion preconcentrate	(Kovarik <i>et al.</i> , 1994)

<sup>\*</sup> Neoral ® formulation as per Physicians' Desk Reference, 53rd edn. (1999).

Table 1.7: Commercial lipid-based formulations exhibiting enhanced bioavailability (Roman, 1999)

Product	Formulation Type	Strength (capsules)	Dose	Excipients	Bioavailability Enhancement
Neoral® (cyclosporin)	Liquid filled gelatin capsules	25 and 100mg	Up to 1g/day	Mono-di-triglycerides, polyoxyl 40 hydrogenated castor oil, DL-α-tocopherol and propylene glycol	20-50% compared to Sandimmune®
Norvir® (ritonavir)	Semi-solid filled hard gelatin capsule	100mg	600mg twice daily	Caprylic/ capric triglycerides, polyoxyl 35 castor oil, citric acid, ethanol, polyglycolysed glycerides, polysorbate 80 and propylene glycol	Similar to an oral solution
Fortovase® (saquinavir)	Liquid filled soft gelatin capsule	200mg	1200mg three times daily	Medium chain mono and diglycerides, povidone and DL-α-tocopherol	AUC increased 3.3 fold compared to Invirase®
Agenerase® (amprenavir)	Liquid filled soft gelatin capsule	150mg	1200mg twice daily	TPGS, polyethylene glycol 400 and propylene glycol	Conventional oral formulation gave no detectable blood levels

# 1.5 Assessment tools for lymphatic transport

#### 1.5.1 Whole animal models

#### 1.5.1.1 Anaesthetised rat

To date, the lymph cannulated anaesthetised rat has been the most widely used model for investigating intestinal lymphatic drug absorption. This model has been employed in this capacity for over 30 years and has been used to generate the bulk of available data on lymphatic drug transport. The rat is a robust model, which is cost-effective and readily available. A triple cannulated model has been described for lymphatic transport studies and is most commonly used now (Fukui et al., 1989; Porter et al., 1996a). The surgery involves cannulation of the mesenteric lymph duct, the jugular vein and the duodenum. The animal is anaesthetised for the duration of the experiment. Test vehicles are infused into the duodenum to simulate 'oral' delivery. The mesenteric lymph is sampled to determine the amount of drug absorbed via intestinal lymph and the jugular vein is sampled for systemic blood levels. This provides a good estimate of the contribution of lymphatic uptake to oral bioavailability.

Limitations with this model are related both to the experimental conditions and to interspecies differences. Firstly, anaesthesia eliminates the possibility of administering a test vehicle orally, thus removing the gastric pre-mixing step. This may have a significant impact on bioavailability from poorly dispersed formulations (Porter et al., 1996a). Anaesthesia also significantly reduces the rate of lymph flow, which may influence absorption by reducing the available lymph volume for drug uptake. Furthermore, the rate of absorption of poorly water soluble, lipophilic compounds, which are the most likely candidates for lymphatic transport, tends to be slow. This necessitates a prolonged experimental period in order to investigate the full pharmacokinetic profile, resulting in a lengthy period of anaesthesia, which is associated with a high mortality rate (Edwards et al., 2001). Interspecies differences may also complicate interpretation of data from the rat model. In humans, bile secretion is stimulated by the presence of luminal fat, however, in the rat, bile flow is continuous. This means that it is not possible to simulate 'fed' and 'fasted' states in the rat model, as found in vivo in humans. In addition, the small size of the animal precludes the administration of equivalent human dose volumes (Edwards et al., 2001).

#### 1.5.1.2 Conscious rat model

A conscious rat model has been developed to overcome the issues associated with anaesthesia. Porter *et al.* (1996b) have described an unrestrained, cannulated, conscious rat model. Surgery involves cannulation of the mesenteric lymph vessel for collection of intestinal lymph and

cannulation of the carotid artery for sampling systemic blood levels. The duodenum is cannulated to facilitate continuous rehydration of the animals. In some cases, the jugular vein may be cannulated to allow *iv* dose administration for estimating absolute drug bioavailability. Carotid, jugular and duodenal cannulae are tunnelled under the skin and exteriorised at the back of the neck. The mesenteric lymph cannula is exteriorised at the abdomen. Rats are allowed to recover following surgery and are tethered throughout experiments to allow free mobility. Rats are fitted with a jacket to hold collection bottles for lymph.

This model confers two key advantages over the anaesthetised model described above. Firstly, as anaesthesia is not maintained throughout experiments, longer sampling times are permitted with decreased risk of mortality. Secondly, oral dosing is possible, thus introducing a gastric mixing and pre-digestion step, which is particularly relevant for assessing bioavailability from poorly dispersed lipid vehicles (Porter *et al.*, 1996b). Issues associated with interspecies difference are still a problem with the conscious rat model.

#### 1.5.1.3 Genetic mouse models

Recently, a series of genetically modified mouse models have been employed for elucidating metabolic pathways. By blocking the expression of a single enzyme or protein, it is possible to accurately assess the importance of each step in a biochemical pathway and to remove some of the complexity associated with a standard *in vivo* system. In the context of lipid metabolism, a number of these models have been developed for investigating different steps in lipid processing. Elements that have been targeted include mdr-2 (multiple drug resistance protein 2) and apobec (apo B mRNA editing enzyme) (Ng *et al.*, 2000; Oude Elferink *et al.*, 1997). Because of the cost involved in developing genetically modified models, studies are restricted to mice. Again, in terms of lipid vehicle delivery the same problems arise as seen with the standard rat model. Mice are very small, and the surgery required for investigating lymphatic drug absorption requires much skill. The small size of these animals restricts the sample volume, thus necessitating the use of highly sensitive detection methods for drug concentration. Mechanistically, genetically modified models offer an important tool for understanding lipid processing, but in terms of absolute drug bioavailability studies, they are limited.

#### 1.5.1.4 Dog model

Recently, Khoo *et al.* (2001) described a robust, lymph cannulated dog model for evaluating the lymphatic transport of orally administered drugs. The use of such a large animal model overcomes the limitations associated with using rodent species in terms of lymph flow and dose volume. Lymph flow in the dog, in common with the human, is dependent on administration of

lipid, with lymph flow being stimulated by the ingestion of lipids. This permits a more accurate simulation of 'fed' and 'fasted' state response. Administration of dose volumes equivalent to those used in humans is also possible and drug metabolism may reflect the *in vivo* human situation more closely. In the described triple cannulated, conscious greyhound model, the thoracic lymph duct is cannulated for determining lymphatic drug transport and the jugular and carotid vessels are cannulated for rehydration and for blood sampling respectively. The dogs are allowed to recover following surgery and as anaesthesia is not maintained, lengthy sampling periods are feasible.

In terms of assessing results, the key disadvantage with this model is that lymph is collected from the thoracic rather than the mesenteric lymph duct. Thoracic lymph may provide an overestimate of intestinal lymphatic drug transport as it contains lymph that has drained from peripheral lymph vessels in addition to that associated with the intestine. This model is not suitable for routine screening because of a combination of ethical issues and practical limitations, which include high cost, a requirement for large animal facilities and considerable surgical skill.

#### 1.5.2 In vitro Caco-2 cell model

All of the above described animal models for lipid metabolism studies are poorly suited to rapid screening processes and all carry ethical issues with regard to vivisection. For these reasons, an *in vitro* model capable of predicting lipid vehicle effects on drug absorption, and specifically on lymphatic drug uptake, is highly desirable. To date, no such model has been identified, but the Caco-2 intestinal cell line has been used widely for elucidating pathways of intestinal lipid metabolism. It is therefore conceivable that this model may offer a means of studying the interaction between lipid metabolism drug absorption. An *in vitro* system also offers more control over experimental conditions and the possibility of studying processes in isolation.

# 1.5.2.1 Barrier properties of Caco-2 cells

The Caco-2 cell model has long been established in the pharmaceutical field as an *in vitro* intestinal model for evaluating drug transport (Hilgers *et al.*, 1990; Artursson, 1990; Artursson and Karlsson, 1991). This model has high predictive potential, demonstrating good *in vitro* - in *vivo* correlation in terms of passive and active drug transport (Dias and Yatscoff, 1996; Pade and Stavchansky, 1998; Saha and Kou, 2000).

# 1.5.2.2 Suitability of Caco-2 cells as a model for lipid metabolism

Caco-2 cells have also been widely employed in medical and biochemical fields as an in vitro model for investigating lipid metabolism. The value of this model in such studies was first reported by Hughes et al. (1987) and since then it has been used to accumulate a considerable body of information on the various intracellular stages of lipoprotein synthesis, transport and Prior to this, understanding of human intestinal lipid metabolism was severely impaired by a lack of suitable investigative models. Caco-2 cells have many features in common with the enterocyte, but as with any in vitro model, there are differences, which can limit predictive potential (Levy et al., 1995). In terms of lipid processing, the main function of the enterocyte is to absorb the end products of lipid digestion (largely fatty acids and monoglycerides) and deliver them to the body in a usable form. The enterocyte achieves this in vivo, by synthesising triglycerides and phospholipids from absorbed products and packaging them, along with specific functional proteins, into large triglyceride rich lipoprotein particles, which are then secreted into the intercellular space (Tso, 1994). The Caco-2 model has been shown to adequately perform this function. Hughes et al. (1987) first demonstrated lipoprotein secretion by Caco-2 cells grown on plastic supports. This original study monitored secreted apolipoprotein distribution into lipoprotein density fractions. It was found, under basal conditions, that Caco-2 cells secreted lipoproteins equivalent to VLDL, LDL and HDL density, with HDL predominating. Supplementation of cells with oleic acid altered the profile, resulting in preferential secretion of VLDL (Hughes et al., 1988). Shortly after, Traber et al. (1987) characterised lipoprotein secretion by Caco-2 monolayers grown to confluence (6-10 days postseeding) on semi-permeable filters. Cells were shown to demonstrate polarity of lipoprotein secretion, with fatty acids being preferentially absorbed on the apical side and secreted into basolateral medium. Experiments monitoring incorporation of [35S] methionine and [14C] oleic acid into lipoprotein fractions showed that, in the presence of oleic acid (~0.1µM), secreted lipoproteins were principally of LDL density. The apolipoprotein composition of secreted lipoproteins was found to be very similar to that found in vivo, with the key difference being the nature of the secreted apoB. In the adult intestine, apoB is present specifically as apoB 48. In contrast, Caco-2 cells secrete a mixture of apoB 100 and apoB 48. This apolipoprotein pattern is similar to that found in foetal intestine (Teng et al., 1990). Luchoomun and Hussain (1999) later confirmed the secretion of chylomicrons by fully differentiated Caco-2 monolayers in response to oleic acid stimulation.

#### 1.5.2.3 In vitro-In vivo correlation

Reported findings in the Caco-2 model provide good evidence of effective correlation with in vivo data, lending support to the validity of these cells as a model for the human intestinal The key components recognised as essential for lipoprotein secretion have been identified in this model including: intestinal fatty acid binding protein (FABP) (Levin et al., 1992), microsomal triglyceride transfer protein (MTP) (Mathur et al., 1997), cholesterol acyl transferase (ACAT) (Field et al., 1988), and a full range of apolipoproteins (Traber et al., 1987; Dashti et al., 1990). Caco-2 cells have been shown to respond to fatty acid stimulation in a similar way to the in vivo situation. Firstly, their increased TRL output on exposure to administered oleic acid (Field et al., 1988; Dashti et al., 1990; van Greevenbroek et al., 1996), with chylomicron secretion only occurring at higher fatty acid concentrations (Luchoomun and Hussain, 1999), closely reflects the *in vivo* fed/ fasted response, where chylomicron secretion is 'switched on' by fatty acid absorption (Ockner et al., 1969a; Ockner et al., 1969b). Furthermore, Caco-2 cells have been shown to respond to different fatty acid classes in a similar way to a whole animal rat model. For example, supplementation of Caco-2 cells with long chain mono- or di- unsaturated fatty acids such as oleic or linoleic acid stimulates TRL secretion, whereas exposure to saturated fatty acids such as palmitic and stearic acid, promotes secretion of smaller, LDL sized lipoproteins. This trend is consistent with findings in the rat model (Holm et al., 2001; Porsgaard and Hoy, 2000; Caliph et al., 2000). The lipoproteins secreted by Caco-2 cells share many characteristics with those found in human plasma. With regard to chylomicrons, the lipid composition and calculated diameter is very close to the human data (Luchoomun and Hussain, 1999). The key difference between the Caco-2 and human intestinal TRL is in the apolipoprotein composition. Human intestinal chylomicrons and VLDL carry the apoB 48 isoform exclusively, whilst Caco-2 TRL carry a combination of apoB 100 and apoB 48, depending on level of differentiation (Traber et al., 1987).

# 1.5.2.4 Factors affecting Caco-2 lipoprotein response

#### Fatty acid supplementation

As indicated above, the administration of oleic acid to Caco-2 cells produces an increase in the secretion of triglyceride. It has further been noted that oleic acid also stimulates the intracellular synthesis of triglyceride (Field *et al.*, 1988; Dashti *et al.*, 1990; van Greevenbroek *et al.*, 1996; Luchoomun and Hussain, 1999). In addition to the triglyceride response, oleic acid has been shown to promote the secretion, and redistribution to TRL fractions, of apoB (Traber *et al.*, 1987; Moberly *et al.*, 1990). Following the additional observation that oleic acid has no

effect on the intracellular concentrations of apoB or of apoB mRNA, it has been postulated that oleic acid may regulate apoB secretion in a post-transcriptional manner (Moberly et al., 1990).

Extensive studies on the effects of fatty acid chain length and degree of fatty acid saturation on lipoprotein metabolism have been conducted (Field et al., 1988; van Greevenbroek et al., 1996; van Greevenbroek et al., 1997). van Greevenbroek et al. (1996) showed that oleic acid (C18:1) and linoleic acid (C18:2) stimulated secretion of TRL, whereas saturated fatty acids, such as palmitic (C16:0) and stearic acid (C18:0), stimulated secretion of LDL sized lipoproteins. The monounsaturated C18:1 produced 1.5-fold higher secretion levels of lipid than the polyunsaturated linoleic acid (C18: 2). Field et al. (1988) also noted a similar trend of increased TRL and triglyceride secretion on administration of unsaturated versus saturated long chain fatty acid, again with C18:1 being the most potent stimulator of TRL secretion. Similar findings have been reported for hepatic cells (Dokko et al., 1998).

Further studies by van Greevenbroek *et al.* (1998) have considered the effects of geometrical configuration (cis versus trans) and double bond position on C18:1 fatty acid stimulation of TRL. It was found that the double bond position was a pertinent factor in lipoprotein secretion. Oleic acid (c-18:1 $\Delta$ 9) and elaidic acid (t-18:1 $\Delta$ 9) both produced significantly higher levels of triglyceride (approximately 2.5-3.5 fold difference) and apoB (approximately 1.5 fold difference) secretion than the related vaccenic acid (c-/t- 18:1 $\Delta$ 11). The geometrical configuration was found to be less important, but did have a small effect on triglyceride secretion, with (t-18:1 $\Delta$ 9) stimulating secretion of 1.3 times more triglyceride.

#### Degree of cellular differentiation

The degree of cellular differentiation can influence lipid metabolism in the Caco-2 model. For example, the expressed apoB profile and the TRL secretion capacity are dependent on the state of differentiation. Cells grown on impermeable plastic surfaces demonstrate a deficiency of secreted apoB (Hughes et al., 1987). In comparison, cells grown to confluence (6-9 days) on semi-permeable filters have been shown to secrete apoB, predominantly of the apoB 100 isoform in addition to a small, but detectable, amount of apoB 48 (Traber et al., 1987). Studies using more differentiated cell monolayers have reported equivalent secretion levels of apoB 48 and apoB 100 (Moberly et al., 1990; Wagner et al., 1992; Luchoomun and Hussain, 1999). These results are thought to be similar to those observed in developing foetal intestine, where the expression level of apoB 48 increases with maturity (Teng et al., 1990). Expression of apoB

48 is associated with an additional post-transcriptional step in apoB mRNA editing, hence it is associated with more highly developed tissue (Traber et al., 1987).

There is further evidence in current literature to support a relationship between lipoprotein secretion and Caco-2 differentiation. An extensive study by Mehran *et al.* (1997) has shown that lipid secretion in the Caco-2 model is highly sensitive to the level of cell differentiation. Triglyceride secretion in Caco-2 cells was found to be dependent on time in culture. Minimal levels of triglyceride secretion were observed before cells reached confluence at day 9 post-seeding, and maximum levels were achieved after 20 days in culture (at which stage cells were fully differentiated with maximum sucrase activity and fully developed microvilli) and maintained throughout the remaining test period of 30 days. Triglyceride output corresponded to an increase in VLDL secretion with time in culture. Further support for these findings comes from analysis of the time dependence of apolipoprotein secretion. It has been shown that apoB secretion in cells grown on plates only occurs after 11 days in culture and that a maximum level is not achieved until day 15 (Dashti *et al.*, 1990). The expression of Microsomal triglyceride transfer protein (MTP), a critical protein in the assembly of lipoproteins, has been shown to rely heavily on the stage of differentiation. A parallel between differentiation-dependent expression of MTP, apoB and of lipoprotein secretion has been reported (Mathur *et al.* 1997).

Luchoomun and Hussain (1999) were the first group to demonstrate chylomicron secretion in the Caco-2 model. They attributed their unique observation of chylomicron secretion in a fully differentiated Caco-2 model to the time allowed post-seeding for monolayer differentiation (21 days). However, as this was the first group to employ a centrifugation technique capable of distinguishing between chylomicrons and VLDL, it is possible that this observation may be partly due to analytical technique.

#### 1.5.2.5 Differences between lipid metabolism in Caco-2 model and human intestine

The main limitation identified with the Caco-2 model is its relatively poor lipoprotein secretory capacity. Caco-2 cells demonstrate high ability to absorb administered fatty acids and to synthesise triglyceride, but the subsequent secretion of triglyceride is restricted (Levy *et al.* 1995). The quoted literature values for triglyceride secretion efficiency are variable because of the variety of culture and experimental techniques used, but they are consistently low. Taking a few examples: Ranheim *et al.* (1992) reported ~10% secretion of synthesised triglyceride over a 5-hour period for Caco-2 monolayers supplemented with 0.6mM oleic acid, Dashti *et al.* (1990) reported ~11.5% secretion of synthesised triglyceride after a 15-19 hour assay period with

0.8mM oleic acid, Mehran et al. (1997) reported up to 6% secretion for fully differentiated monolayers after 2 hour incubation with 0.8mM oleic acid and van Greevenbroek et al. (1996) reported~15% secretion after 24 hours with 0.5mM oleic acid. A comparative study of lipid metabolism in Caco-2 cells and human foetal colon explants found that Caco-2 cells had a considerably higher capacity for synthesising triglyceride from administered oleic acid (28-fold difference), but that the export was limited, such that Caco-2 cells only secreted ~10% of synthesised triglyceride versus the almost 100% secretion by foetal colon tissue (Levy et al. 1999). The relative inefficiency of lipid transport in Caco-2 cells may be due to a number of factors. Mehran et al. (1997) suggested that the low activity of the 2-monoacylglycerol pathway in Caco-2 cells or a lack of microsomal triglyceride transfer protein could explain this observation. It is also possible that the relative apo B 48/ 100 distribution in Caco-2 cells is contributory.

In vivo, in the absorptive or 'fed' state, the enterocyte synthesises triglyceride from absorbed monoglycerides and fatty acids via the monoacylglycerol pathway. During the 'fasted' state, when luminal fat is not present, the phosphatidic acid pathway, which utilises glycerophosphate and fatty acid as substrates, takes over (Mansbach and Parthasarathy, 1982). In contrast to the in vivo scenario, the phosphatidic acid pathway is the principle route of triglyceride synthesis in Caco-2 cells both in the 'fed' and the 'fasted' state. Evidence for this comes from a comparative metabolic study of rat jejunum and Caco-2 monolayers (Trotter and Storch, 1993). It was found, in this study, that activity of monoacylglycerol acyltransferase, a key and exclusive enzyme of the monoacylglycerol pathway, was extremely low in Caco-2 cells and present at <7% of the amount found in rat jejunum. In contrast, levels of glycerol-3-phosphate acyltransferase, a key component of the phosphatidic acid pathway were present at comparable levels in both tissues. As chylomicron triglyceride is preferentially sourced from the monoacylglycerol pathway in vivo, the predominance of the phosphatidic acid pathway in Caco-2 cells may be a limitation to TRL output.

MTP, the enzyme involved in preliminary, structural lipidation of the growing apoB molecule, is another likely candidate for limiting lipid transport in Caco-2 cells. MTP has been identified and characterised in the Caco-2 model (Mathur *et al.* 1997). Its activity has been measured, but no direct comparison with human intestinal MTP has been made. Available data does, however, indicate that MTP activity in the Caco-2 model, a colon derived cell line, is relatively low. A comparative study of MTP activity in human foetal jejunum and human foetal colon quoted values of activity (expressed as % of triglyceride transferred from donor to acceptor vesicles per

μg protein in 1 hour) as approximately 15% and 1% respectively (Levy et al. 2001). This indicates that colon derived tissue has an inherently low ability to secrete apoB containing lipoproteins as compared to the jejunum. Indeed, this study also found that triglyceride output of colonic explants was only approximately 20% of the jejunal output and that apoB production was only 30-40% of the jejunal level (Levy et al. 2001). A study on MTP activity in Caco-2 cells using a similar transfer experiment reported values of approximately 0.055% transfer per μg protein per hour (Mathur et al. 1997). This is strong evidence for MTP expression being the rate-limiting step to TRL output in Caco-2 cells.

Finally, the nature of the apoB protein secreted by Caco-2 cells may also be a causative factor in the low TRL secretion efficiency. As indicated earlier, chylomicron assembly and secretion in the upper intestine in humans is reliant on the production of apoB 48. In humans, apoB48 is predominantly expressed in the intestine and apo B100 in the liver. In contrast, Caco-2 cells express a combination of both forms of apoB, the relative distribution relying on the state of differentiation (Mehran *et al.* 1997). Although chylomicrons secreted by the human intestine exclusively carry the apoB 48 isoform, it has been shown in rats that apoB 100 can form chylomicrons under appropriate fatty acid stimulation (Ng *et al.*, 2000). Further work in an *apobec-1* knockout mouse has shown that the initial chylomicron assembly step is much slower with apoB 100 as compared to apoB 48 (Kendrick *et al.* 2001). This problem is overcome by increasing the lipid load, but may still be a contributing factor to the low secretion efficiency of Caco-2 cells. In the presence of low MTP levels there is documented evidence to suggest that apoB 100 is more susceptible to intracellular degradation than apoB 48 (Nicodeme *et al.* 1999), again this may be a contributory factor.

# 1.6 Aims

- The Caco-2 model will be investigated as a surrogate for in vivo animal studies for predicting the effects of lipid vehicles and lipid vehicle excipients on lipoprotein secretion. Emphasis in these studies will be placed on lymphatically transported lipoproteins, specifically chylomicrons, to evaluate the potential of the Caco-2 model in lymphatic drug transport studies.
- A range of pharmaceutical surfactants (typical lipid vehicle components), including Pluronic block copolymers, Cremophor EL, TPGS and polysorbate 80, will be investigated, using the Caco-2 model, for their effects on triglyceride rich lipoprotein secretion. Studies will focus on non-ionic surfactants because of their low oral toxicity.
- A number of long chain oils will be investigated for their potential to promote triglyceride rich lipoprotein secretion, as a means of selecting a suitable oil phase for incorporation into a lymphotropic lipid based vehicle.
- A lipid based microemulsion formulation will be developed to simultaneously enhance the aqueous solubility of an incorporated lipophilic drug and to promote chylomicron secretion. Such a vehicle will maximise the potential for lymphatic drug transport. Vehicle formulation will be systematic and will take into consideration findings for excipient effects on lipid metabolism and lipolysis.
- The effects of a range of lipid based vehicles of varying complexity on secreted lipoprotein profile in the Caco-2 model will be compared and correlated with their effects on the cellular uptake and permeation of a model lipophilic drug.
- The usefulness of the Caco-2 model for predicting lipid vehicle effects on intestinal absorption of a lipophilic drug will be assessed.

# **Chapter Two**

**Materials and Methods** 

# 2 Materials and methods

# 2.1 Materials

#### Cell culture

- Dulbecco's modified Eagle's medium (DMEM) (1X), without sodium pyruvate, with 4500mg/ml glucose, with pyridoxine HCl, Gibco, 41965-039
- Gentamicin (10mg/ml), Gibco, 15710-049
- L-glutamine (200mM) (100X), Gibco, 15710-031
- Foetal bovine serum (FBS), Gibco, 10270-106
- Trypsin-EDTA in HBSS (1X) liquid, Gibco, 25300-054
- Sodium pyruvate MEM (100mM), Gibco, 11360-039
- Phosphate buffered saline, pH 7.4 (10X) solution, without calcium chloride, without magnesium chloride, Gibco, 70011-036
- Tryptan blue solution (0.4%), Sigma, T-8154
- Dimethylsulfoxide (DMSO), Sigma, D-8779
- 6-Well plates, Nunc, 1-50229A
- 12-Well plates, Costar®, 3512
- Transwell<sup>TM</sup> filters (polycarbonate), 0.4µm pore, 4.71cm<sup>2</sup>, Costar®, 3412
- Transwell<sup>TM</sup> filters (polycarbonate), 3μm pore, 4.71cm<sup>2</sup>, Costar®, 3414
- Transwell<sup>TM</sup> filters (polycarbonate), 0.4μm pore, 1.14cm<sup>2</sup>, Costar®, 3402
- Cell culture filters (polyethylene terephthalate), 1µm pore, 4.2cm², Falcon®, 3102
- Sterile acrodisc syringe filters, 0.2μm pore, Gelman, 4192
- Supor 200 membrane filters, 13mm, 0.2μm pore, Gelman, 60298
- Plastic filter holders, 13mm, Gelman, 4317
- Mycoplasma detection kit, Roche Diagnostics, 1 296 744
- Sterican 21 gauge needles, Braun
- Rubber cell scrapers, Sarstedt

All plastics for tissue culture were obtained from Gibco or Costar®.

All solutions used in cell work were sterilised either by filtration through a 0.2µm pore filter or by autoclaving.

# Toxicity testing

- MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue), Sigma, M-5655
- Sodium dodecyl sulfate, Sigma, L-6026
- Hydrochloric acid (1.023N), Aldrich, 31, 894-9
- Tissue culture treated plates (96-well, sterile), Falcon®, 3872

# Permeability/ transport experiments

- DL-[4-³H] Propranolol hydrochloride (1mCi/ml, 25Ci/mmol), Amersham Pharmacia Biotech, TRK 495
- D-[1-14C] Mannitol (200µCi/ml, 59µCi/mmol), Amersham Pharmacia Biotech, CFA 238
- [³H] Taxol (1mCi/ml, 5.0Ci/mmol), Moravek Biochemicals, MT552
- D-[1-3H (N)] Mannitol (1mCi/ml, 19.7Ci/mmol), New England Nuclear, NET101
- [G-<sup>3</sup>H] Vinblastine sulphate (250μCi/ml, 12.5Ci/mmol), Amersham Pharmacia Biotech, TRK 507
- [6, 7-3H (N)] Dexamethasone (1mCi/ml, 35Ci/mmol), New England Nuclear, NET467
- [mebmt-β-³H] Cyclosporin A (1mCi/ml, 9Ci/mmol), Amersham Pharmacia Biotech, TRK 904
- Fluorescein, sodium salt, Sigma, F-6377
- HBSS (Hank's buffered saline solution) (10X) liquid, Gibco, 14065-049
- D (+) Glucose (dextrose corn sugar), Sigma, G-6152
- Hepes (N-[2-hydroxyethyl] piperazine-N'-[2-ethane sulfonic acid]), sodium salt, Sigma, H-3784
- Verapamil, Sigma, V-4629
- Cyclosporin A, donated by ACI, Tipperary, Ireland.
- Ultima gold scintillation fluid, Packard Bioscience B. V., 6013329
- Disodium hydrogen phosphate Dodecahydrate, Merck, 1.06579.0500
- Sodium dihydrogen phosphate dihydrate, Merck, 1.06345.1000

#### Lipid studies

- [9,10 (n)-3H] Oleic acid (5mCi/1ml, 10Ci/mmol), Amersham Pharmacia Biotech, TRK 140
- [1-14C] Oleic acid (100μCi/ml, 60mCi/mmol), Amersham Pharmacia Biotech, CFA 243
- Albumin, bovine, essentially fatty acid free, Sigma, A-6003
- Triglyceride assay kit, Bio-Merieux, PAP 150
- Oleic acid, Sigma, O-1008
- Butyric acid, Aldrich, B10,350-0
- Capric acid, Sigma, C-1875
- Linoleic acid, Sigma, L-1376
- Palmitic Acid, Sigma, P-5585
- Stearic acid, Sigma, S-4751
- NaOH, 1.0 N, Sigma Diagnostics, 930-65
- Ethanol, Merck, UN 1170
- ATP (Adenoside-5'-triphosphate), cell culture tested, Sigma, A-6419
- Dodecylamine, Sigma, D-3639
- Sodium carbonate, Sigma, S-7795
- Phenylmethylsulfonyl fluoride (PMSF), Sigma, P-7626
- Sodium azide (NaN<sub>3</sub>), Sigma, S-8032
- Ethylenediaminetetraacetic Acid (EDTA), disodium salt, Sigma, E-1644
- ε-Amino-n-caproic acid (EACA), Sigma, A-7824
- Butylated hydroxytoluene (BHT), Sigma, B-1378
- Triton X, Sigma, T-9284
- Borex<sup>TM</sup> Disposable borosilicate glass culture tubes (12 x 75mm and 16 x 100mm), Sparks Laboratory Supplies

## Protein assay

- Bio-Rad DC protein assay kit, Bio-Rad Laboratories, 500-0112
- Albumin, bovine (minimum 98%), Sigma, A-7030
- Polystyrene (96-well) plates, Nunc, 2-69620A

# Ultracentrifugation

- Potassium bromide, Sigma, P-9881
- Polyallomer centrifuge tubes, Beckman, 331372

# Apolipoprotein B ELISA

Visking dialysis tubing (pore size approximately 2.4nm), Scientific Instrument Centre Ltd

- Nunc-immuno maxisorp (96-well) Plates, 96-well, Nunc 4-42404A
- Marvel milk powder
- 1D1 Monoclonal antibody, University of Ottowa Heart Institute
- Anti-apolipoprotein B (anti-human apoB antiserum isolated from sheep), Boehringer
   Mannheim, 726 494
- Alkaline phosphatase-conjugated rabbit affinity purified antibody to sheep IgG (whole molecule), Cappel, 56991
- Sigma Fast<sup>TM</sup> p-nitrophenyl phosphate tablet set, Sigma, N-2770
- Polyoxyethylenesorbitan monolaurate (Tween 20), Sigma, P-1379
- Trizma base, Sigma, T-6066
- Sodium carbonate, Sigma, S-7795
- Aprotinin, Sigma, A-1153

#### **HPLC**

- Tert-butyl methyl ether, spectrophotometric grade, Aldrich, 29,586-8
- Lauryl sulfate, sodium salt, Sigma, L-3771
- Acetic acid, glacial, Sigma, A-6283
- C8 Column (5μm, 4.6mm x 25cm), Beckman, 235332
- C8 Guard Column (4mm x 3mm), Phenomenex, AJO-4290.

# SDS PAGE

- High molecular weight markers, prestained, Bio-Rad, 161-0309
- Protogel, 30%w/v acrylamide: 0.8%w/v bisacrylamide stock solution (37.5:1), National Diagnostics
- TEMED (N,N,N',N'-tetramethylethylenediamine), Sigma, T-9281
- Ammonium persulfate, Sigma, A-3678
- Coomassie brilliant blue R-250, Bio-Rad, 161-0400
- 2-Mercaptoethanol, Sigma, M-3178
- Bromophenol blue, sodium salt, Sigma, B-8074
- Glycerol, Sigma, G-5516
- Lauryl sulfate, sodium salt, Sigma, L-3771
- Trizma (tris[hydroxymethyl]aminomethane) base, Sigma, T-6791
- Trizma hydrochloride, Sigma, T-3253

#### Microemulsion vehicles and lipid vehicle excipients

Olive oil BP, Pharmacy Brand

- Arachis/ peanut oil, Sigma, P-2144
- Corn oil, Sigma, C-8267
- Miglyol 812, donated by Sasol, Witten, Germany
- Soybean oil, Sigma, S-7381
- Cremophor EL (polyoxyl 35 castor oil), donated by BASF, Ludwigshafen am Rhein,
   Germany
- Cremophor RH40, donated by BASF, Ludwigshafen am Rhein, Germany
- Pluronic L81, donated by BASF, Ludwigshafen am Rhein, Germany
- Pluronic P85, donated by BASF, Parsipanny, NJ
- Pluronic (Lutrol) F68, donated by BASF, Parsipanny, NJ
- TPGS, vitamin E TPGS NF grade, donated by Eastman Chemical Co, TN
- Polyoxyethylenesorbitan monooleate (Tween 80), Sigma, P-8074
- Polyoxyethylenesorbitan monostearate (Tween 60), Sigma, P-1629
- Peceol (glycerol monooleate EP), donated by Gattefossé, Saint-Priest, France
- Labrafil M 1944 CS, donated by Gattefossé, Saint-Priest, France
- Captex 355, donated by Abitec Corp, Janesville, Wi
- Aquasil<sup>TM</sup> siliconizing fluid, Pierce, 42799

#### Lipolysis

- Pancreatin (from porcine pancreas, equivalent to 8X USP), Sigma, P-7545
- Pancreatin (from porcine pancreas, equivalent to 4X USP), cell culture tested, Sigma, P-3292
- Bile salt extract, Sigma, B-8631
- Taurocholic acid, sodium salt, Sigma, T-4009
- Lecithin (Lipoid E phosphatidyl choline, egg lecithin), Lipoid GmbH, 105016-1
- Halofantrine, obtained from SmithKline Beecham Pharmaceuticals courtesy of W. N. Charman, Monash University, Australia
- Halofantrine HCl, obtained from SmithKline Beecham Pharmaceuticals courtesy of W. N. Charman, Monash University, Australia
- Internal standard (IS) 2,4-Dichloro-6-trifluoromethyl-9- {1-[2-(dibutylamino)ethyl]}- phenanthrenemethanol, obtained from SmithKline Beecham Pharmaceuticals courtesy of W. N. Charman, Monash University, Australia.
- Acetic acid, 50% Solution, HPLC Grade, Fluka, 45754
- Acetonitrile, HPLC Grade, Riedel-de Haën, 34851
- Tert-butyl methyl ether (TMBE), Aldrich, 29-5868
- Trizma maleate, Sigma, T-3128

- Tributyrin (C4:0, 1,2,3-tributyryl glycerol), Sigma, T-8626
- Calcium chloride, CaCl<sub>2</sub>, dihydrate, Sigma, C-5080,
- Triolein (C18:1, [cis]-9), Sigma, T-7140
- Glycerol tri [1-<sup>14</sup>C] oleate (100μCi/ml, 59mCi/mmol), Amersham Pharmacia Biotech, CFA
   258
- Sucrose, Sigma, S-0389
- Dithiothreitol, Sigma, D-9779

# Thin layer chromatography (TLC)

- Whatman chromatography paper (grade 1 Chr), Whatman, 3031-681
- Silica 60 A LK6D 19 lane TLC plate, Whatman, 4865-821
- Corn oil BP, Sigma, C-8267
- Chloroform, HPLC Grade, Lab-Scan
- Methanol, HPLC Grade, Lab-Scan
- Acetic acid, glacial, Sigma, A-6283
- Diethyl ether, HPLC Grade, Sharlau
- Petroleum ether 40/60, Lab-Scan
- Iodine, Sigma, I-3380
- Cholesteryl oleate, Sigma, C-9253
- Cholesterol, Sigma, C-8667
- L-α Phosphatidylcholine (L-α-lecithin, from frozen egg lecithin), Sigma, P-7318

# Gas chromatography

- Boron trifluoride-methanol complex, Aldrich, 13,482-1
- Fatty acid methyl ester standards, Sigma, 1891-1AMP, Sigma 189-13 and Sigma, P-9667
- 25M BP 21 Polar aluminium silica column, Scientific Glass Engineering Ltd, Milton Keynes, England

#### 2.2 Instrumentation

- Autoclave, Priorclave
- Autosizer Lo-C, Malvern
- Autoanalyser, Technicon RA-XT<sup>TM</sup> System, Bayer Diagnostics
- Gel scanner: white/ UV transilluminator, with hood and Gelcam, UVP, with Gelworks ID and Grab-IT software to image gels and calculate band density
- Gilson transfer pipettes, P20, P200, P1000
- Grid haemocytometer, Hawksley

- HPLC, Waters 610 LC Module 1 Plus pump and injector with a Waters 486 UV detector (257 nm), with Millenium software
- Hydrometers (1.3-1.4g/ml and 1.0-1.1g/ml), T&A, UK
- Inverted light microscope, Wilovert
- Liquid scintillation counter, Packard Tri-Carb 2100 series
- Microcentrifuge, Sigma 113
- Microplate reader, Bio-Tek, EL 311 with KC Jr software
- Mini-Protean II gel electrophoresis apparatus, Bio-Rad
- N-Evap, Organomation
- Nuaire class II Type A/B3 laminar air flow hood
- Nuaire water-jacketed infrared automatic CO<sub>2</sub> incubator
- Orion pH meter with electrode.
- Oven, Carbolyte
- pH Stat 718 autotitrator, Metrohm
- Portable pipet-aid, Drummond
- Sigma 203 bench top centrifuge
- Ultrasonic disrupter sonifier II, with probe tip, Branson
- Shimadzu GC-14A series gas chromatograph fitted with a Shimadzu C-R6A Integrator
- Solvent filter apparatus, Millipore
- Spectrophotometer, Spectronic Genesys 5
- Temperature controlled, shaking water bath, Precision
- Tensiometer, Lauda TD-1
- Ultracentrifuge, Beckman L8-55 with SW41 rotor
- Ultracentrifuge (Bench top), Beckman, TL-100 with TLA 100.1 rotor
- Victor fluorescent plate reader (excitation/emission filters 495/535nm), Wallac
- Voltmeter/ ohmmeter fitted with chopstick electrodes, World Precision Instruments
- Water bath with immersion thermostat, Techne
- Peristaltic pump, Watson Marlow
- Whirlimixer, Fisons
- Water purifier and deioniser, Purite Lab Water RO100HP

# 2.3 Methods

#### 2.3.1 General cell culture

#### Cell line

Caco-2 cells (passage 30), a human colonic adenocarcinoma cell line, were obtained from the European Collection of Animal Cell Cultures (ECACC), UK. Cells were tested for mycoplasma at regular intervals using a mycoplasma detection kit, (Roche, Cat. No.1 296 744). Cells tested as mycoplasma negative.

#### Maintenance/ culture medium

Cells were cultured using Dulbecco's modified Eagle's medium (DMEM) (1X solution, Gibco) supplemented with 10%v/v foetal bovine serum (FBS), 2mM L-glutamine, 100µg/ml gentamicin and 1mM sodium pyruvate. Details of preparation are given in Appendix I.

All nutrient media were prepared aseptically in a laminar flow hood and stored at 4°C. Prepared media were tested for batch sterility by incubating a 5ml volume under standard culture conditions in an incubator at 37°C with an atmosphere of 5% CO<sub>2</sub> and 90% relative humidity for 72 hours and visually checking for signs of microbial contamination after a week. Media were used within 3 weeks of preparation.

#### Maintenance procedure

Cells were cultured routinely in 75cm<sup>2</sup> tissue culture flasks in culture medium and grown in an incubator at 37°C with a controlled atmosphere of 5% C0<sub>2</sub> and 90% relative humidity. Medium (10 ml per flask) was changed on Monday, Wednesday and Friday of each week to maintain a pH of 7.2-7.4. Cells were checked for confluence by light microscopy and sub-cultured when 70-90% confluent (every 5-7 days).

# Sub-culturing procedure

Cells were sub-cultured as follows: cells were rinsed with 1X PBS, pH 7.4, solution (10ml per flask) and then incubated with 5ml trypsin-EDTA (0.25%w/v trypsin and 0.02%w/v EDTA) for 5-10 minutes at 37°C until detached from flask. 5ml of culture medium was added and the resulting cell suspension was centrifuged at 1000 rpm for 10 minutes to precipitate cells. The supernatant, containing trypsin, was pipetted off and the cell pellet re-suspended in 1ml of fresh culture medium. Cell number and viability were determined using the tryptan blue exclusion test and a grid haemocytometer. 20µl of tryptan blue was added to 20 µl of cell suspension and mixed gently. 20µl of this mixture was transferred to a haemocytometer, which was then overlaid with a cover slip and viewed under an inverted light microscope. The viable cells in

the central grid were counted (dead cells were stained blue, live cells were clear) and cell concentration (cells/ml) determined using Equation 2.1:

Number of cells/ml = Number of cells in 25 grids on haemocytometer  $\times 2 \times 10^4$ 

# Equation 2.1

Cells were sub-cultured into flasks at an approximate seeding density of  $5 \times 10^4$  cells/ flask by transferring the appropriate suspension volume to a new  $75 \text{cm}^2$  flask and adding 10ml of culture medium.

#### Preparation of cell monolayers

Cells were seeded onto semi-permeable filters and grown for 19-23 days to allow differentiation (full phenotypic expression). Cells were seeded onto filter supports (apical side) from a resuspended cell pellet at a density of 63,000 cells/cm² (Hidalgo *et al.*, 1989). Unless otherwise indicated, cells were seeded onto Costar® Transwell<sup>TM</sup> polycarbonate filters, 0.4µm pore (either of 24mm diameter, 4.71cm² surface area or 12mm diameter, 1.14cm² surface area as indicated)

#### Cell storage and recovery procedure

At 70-90% confluence, cells were detached from flasks as outlined above and the pellet resuspended in fresh culture medium. Cell concentration was determined and suspension diluted to a final concentration of 6 x 10<sup>6</sup>cells/ml. Cell suspension was mixed with an equal volume of freezing medium (10%v/v DMSO in FBS) and transferred to a cryo-vial (Costar®) (1.5ml per 2ml vial). Vials were snap-frozen in dry ice and stored in either a -80°C freezer for short term storage, or liquid nitrogen for long-term storage. The initial freezing step was conducted rapidly to avoid prolonged contact of cells with the cryopreservative, DMSO, at room temperature, where it is cytotoxic.

For recovery, the contents of a single cryo-vial were rapidly thawed at 37°C and diluted to 10 ml with fresh culture media. The resulting cell suspension was centrifuged at 1000 rpm for 10 minutes to produce a cell pellet. The supernatant, containing the cryopreservative, DMSO, was pipetted off and discarded and the cell pellet re-suspended in fresh media. Cells were transferred to a single flask and cultured under standard conditions. Cells were passaged twice after recovery before use in experiments.

# Transepithelial electrical resistance measurements

Transepithelial electrical resistance (TEER) measurements were taken before and after each experiment as an indicator of monolayer integrity. TEER measurements were made with a World Precision Instruments EVOM fitted with chop stick electrodes. In each case, measurements were taken at the same position on the insert filter to minimise variability. TEER readings taken with chopstick electrodes gave a resistance ( $\Omega$ ) reading. Resistance measurements were multiplied by monolayer surface area to express the monolayer resistance ( $\Omega$ cm<sup>2</sup>).

# Measurement of monolayer permeability characteristics

Mannitol was used as a paracellular marker and propranolol as a transcellular marker to determine monolayer permeability characteristics over the passage range employed. Marker permeability was measured at regular intervals to check monolayer quality. Experiments were performed using either 12mm or 24mm diameter polycarbonate filters (0.4μm pore). Apical-basolateral transport of markers was measured over a 2.5 hour period. [¹⁴C] mannitol (0.25μCi/ml) and [³H] propranolol (0.5μCi/ml) were added in transport buffer (TB) (composition give in Appendix II). Transport was initiated by addition of markers to the apical (donor) chamber and basolateral samples were taken at 15-30 minute intervals. Sink conditions were maintained by transferring filter inserts to fresh wells containing TB. Basolateral samples were analysed for mannitol/ propranolol content via liquid scintillation counting (dual [³H]/ [¹⁴C] count). Where excipient effects on marker permeability were investigated, excipients were added to the donor solution (containing markers) at the appropriate test concentration prior to administering solutions to cells. Cells were maintained at 37°C in the incubator throughout the experiment.

As an additional means of characterising monolayers, permeability values for fluorescein (a paracellular marker) and [ $^3$ H] dexamethasone (transcellular marker) were also determined in the high passage cells. Transport experiments were conducted as described above. Fluorescein was added at a concentration of  $20\mu g/ml$  and dexamethasone at  $3.5\mu Ci/ml$  to the apical cell surface. Basolateral samples were collected, in each case, at 30 minute intervals over 2.5 hours. Dexamethasone was assayed using liquid scintillation counting and fluorescein using a Victor fluorescent spectrometer ( $\lambda_{ex} = 495 nm$ ,  $\lambda_{em} = 535 nm$ ).

Apparent permeability coefficients were calculated for each marker using Equation 2.2:

 $Papp = Q/Co \times A$ 

#### Equation 2.2

where

Q = Steady state flux (total amount of drug transported per second), calculated from the slope of a plot of the cumulative amount of drug transported (DPM) against time (minutes). Where there was a lag phase or where more than 10% of drug was transported across monolayers – only the linear portion of plot was used for determination of steady state flux. With the exception of basolateral-apical flux for vinblastine, taxol and cyclosporin A, no more than 5% of stock was transported across monolayers. In all cases, slope was determined from a minimum of four data points.

Co = initial stock concentration (amount of drug, DPM, per cm<sup>3</sup>)

A = surface area of monolayer (cm<sup>2</sup>)

Papp = permeability constant, as cm/sec

#### Protein assay

Protein content of cell monolayers was determined according to the Lowry method using a Bio-Rad DC protein assay kit. Lysed cell suspensions were assayed for protein using this assay and unknown sample protein content was determined from a calibration curve using BSA as a standard. Absorbance, at 650nm, was plotted against BSA concentration and the equation of the line (y = mx + c) was fitted by regression analysis with Microsoft® Excel 2000. A representative calibration curve is shown in Appendix III.

#### 2.3.2 Lipid metabolism studies

# Lipid vehicles

'Fed' simulated vehicle comprised 0.5mM oleic acid bound to 0.125mM bovine serum albumin (BSA) and was prepared as previously described by Field *et al.* (1988) and van Greevenbroek *et al.* (1996) using the following method:

Vehicles were prepared from a 50mM ethanolic oleic acid stock solution in ethanol. The necessary aliquot of oleic acid solution was dried under nitrogen with [<sup>14</sup>C] oleic acid (2μl/ml, equivalent to 0.2μCi/ml), this label being a widely used marker of lipoprotein metabolism in published cell culture literature (Traber *et al.*, 1987; van Greevenbroek *et al.*, 1996; Abumrad *et al.*, 1998). The sodium salt was prepared by neutralisation with ethanolic sodium hydroxide

(100mM) solution. After drying under nitrogen, the fatty acid salt was reconstituted in hot water (>80°C). BSA in serum-free culture medium was added at half the final volume to produce a 2X solution containing 1mM oleic acid with 0.25mM BSA. This solution was incubated for 2 hours at 37°C to allow equilibration. A clear solution was formed. This was then diluted to full volume with serum-free culture medium (DMEM supplemented with 1mM sodium pyruvate, 2mM L-glutamine and 100μg/ml gentamicin) or solutions of excipients in serum-free culture medium to final working concentration. The final vehicle was sterilised by filtration through a 0.2μm pore filter.

'Control'/ 'fasted-state' vehicle comprised 0.125mM BSA and was prepared as described for 'fed-state' vehicle. Again, control was labelled with trace amount of [14C] oleic acid.

BSA was included in the above vehicles a carrier for fatty acids. BSA contains 7 key binding sites for fatty acid and acts to increase fatty acid solubility (aqueous solubility for oleic acid has been estimated at <1μM) and to reduce its cellular toxicity (Spector et al., 1971; Vorum et al., 1992). In vivo, human serum albumin is used as a carrier for fatty acids in the plasma to deliver them in usable form to various tissues. Although the uptake process has not yet been fully elucidated, it is thought that fatty acid dissociation from the albumin complex may not be necessary prior to cellular absorption (Hamilton, 1998). It has been suggested that an albumin binding site may facilitate fatty acid uptake in hepatic cells (Weisiger et al., 1981). In the intestine fatty acids are typically delivered as part of a mixed micellar phase. However, attempts in our laboratory to produce a mixed oleic acid-bile salt micellar solution required concentrations upwards of 10mM sodium taurocholate to solubilise 0.5mM oleic acid. At this concentration, cell toxicity was observed. As BSA has been widely employed in Caco-2 and Hep G2 literature for delivering fatty acids to cells in a non-toxic form, it was selected as a carrier in these studies. The administered ratio of BSA to fatty acid has been reported to impact significantly on fatty acid uptake capacity, but a ratio of 4:1 has been shown to be optimal for the Caco-2 model. Given the poor aqueous solubility of oleic acid in buffer solutions, it is likely that >99% of oleic acid is bound to BSA in our model.

# Standard lipid experiments

Fully differentiated monolayers (19-21 days old) grown on 24mm diameter filters were washed thrice with serum-free culture medium and then incubated (1.5ml in apical chamber, 3ml in basolateral chamber) with serum-free culture medium for 3 hours. TEER measurements were taken. Medium was removed and the test or control vehicle (labelled with trace [14C] oleic acid) added to the apical chamber (1.5ml). 3ml serum-free culture medium was placed in the basolateral chamber. Monolayers were incubated for 20 hours under standard conditions (37°C,

5% CO<sub>2</sub>, 90% relative humidity). This long incubation time was required to allow sufficient accumulation of secreted lipoproteins for accurate quantification and ultracentrifugation analysis (Mehran *et al.*, 1997). After incubation, TEER values were again read to check monolayer integrity.

# Preparation of basolateral and cell samples for lipid, apoB and lipoprotein analyses

Basolateral contents were collected and centrifuged immediately at 5000 rpm for 10 minutes to remove cellular debris and the supernatant collected for further analysis. EDTA (25μl of a 100mM aqueous solution), NaN<sub>3</sub> (sodium azide) (25μl of a 100mM aqueous solution) and PMSF (10μl of a 2mM ethanolic solution) were added as preservatives. Samples were stored at 4°C for up to 3 days until further analysis. Apical contents were collected and a 100μl aliquot analysed by liquid scintillation counting to determine end apical concentration of label (unabsorbed label). For intra-cellular determination monolayers were washed twice in 3%w/v BSA in phosphate buffered saline (pH 7.4) (PBS) (to remove bound fatty acid) and then twice in PBS alone. Cells were scraped into ice cold PBS using a rubber cell scraper and lysed by repeated passage through a 21 gauge needle and addition of Triton X (to 0.5%w/v). Preservatives were added, as above, and the volume was made to 2ml with PBS, pH 7.4. Cell lysates were stored at 4°C for up to 3 days before further analysis. One aliquot was removed for lipid analysis and the remainder used for protein determination (Lowry method, using a Bio-Rad assay kit).

#### Lipid extraction

Lipid analysis was conducted on cell lysate and basolateral samples. Lipids were extracted in chloroform: methanol (2:1) according to the method of Folch *et al.* (1957). An extraction mixture of chloroform: methanol, 2:1 with 0.01%v/v BHT (as anti-oxidant) was prepared. 5ml of extraction mixture was added to 0.5ml of basolateral sample or 200µl of cell lysate in a 16x100mm borosilicate glass tube. The mixture was vortexed for one minute. The tubes were centrifuged at 4000 rpm for 10 minutes to separate organic and aqueous phases. The bottom organic layer (containing lipids) was removed with a glass pasteur pipette and transferred to a 12 x 75mm borosilicate glass tube. 2ml of chloroform was added to the aqueous phase to remove any residual lipid. This mixture was vortexed for 1 minute and centrifuged for a further 10 minutes at 4000 rpm. The organic phase was again removed and added to the first isolate. The pooled organic sample was dried under nitrogen in an N-evap evaporator. Dried samples were stored at -20°C until further analysis.

Separation of lipids (triglyceride, phospholipid and cholesterol ester) from dried extracts

The various lipid classes were separated by thin layer chromatography (TLC) as described (Gibney and Connolly, 1988). Silica 60 LKD 19 lane TLC plates (Whatman) were washed in chloroform, dried and then activated by heating in an oven at 100°C for 1 hour. Plates were allowed to cool. Lipid extracts were reconstituted in 40µl of chloroform with vortexing. Reconstituted extracts were spotted onto individual lanes of a TLC plate (Whatman LK6D 19 lane silica gel plate) in 5µl aliquots, drying between each addition. A further 30µl of chloroform was added to the extract residue and transferred, again in 5µl aliquots, to the loading band to ensure full sample recovery. Basolateral and cellular lipid extracts isolated from a single Transwell<sup>TM</sup> filter were spotted onto the plate in neighbouring lanes. Triglyceride, phospholipid and cholesterol ester standards were also spotted onto individual lanes of each plate. Standards were prepared as follows and stored under nitrogen at -80°C for up to 2 months:

Triglyceride: 10µl/ml solution of pure corn oil (Sigma) in chloroform (15µl spotted onto plate). Phospholipid: 2mg/ml solution of phosphatidyl choline (Sigma) in chloroform (20µl spotted onto plate).

Cholesterol Ester: 3mg/ml solution of cholesterol oleate (Sigma) in chloroform (15µl spotted onto plate).

TLC plates were allowed to dry at room temperature and then partially developed in methanol by allowing the solvent to run just beyond the loading band, as recommended by the manufacturer. This allowed the phospholipids to wash onto the running strip of the lane. Plates were air-dried and then developed fully in a TLC tank containing 80ml of developing solvent petroleum ether: diethyl ether: glacial acetic acid, 80:20:1. The TLC tank was lined with Whatman filter paper and equilibrated with developing solvent for 1 hour prior to use. After developing for 40 minutes the plates were air-dried and then transferred to a glass tank containing iodine vapour (generated from iodine granules). Lipid spots were stained brown by the iodine. Various lipid classes were identified by comparison with standards. Cellular lipids were visualised directly by this technique, but basolateral samples were not always visible (lipid content being too low). The exact location of lipid spots for individual basolateral samples was identified by comparison with the neighbouring cellular extract (isolated from same Transwell<sup>TM</sup> filter). This accounted for any minor differences in retention value of lipid classes caused by administration of different vehicles. Lipid spots were marked with pencil and then scraped into scintillation vials. 4ml of scintillation fluid was added and the vial vortexed for

1 minute to solubilise lipid. Content of radiolabelled lipid was determined by liquid scintillation counting.

Because of the possibility of cell loss during the scraping process, all cell lipid data were corrected for cell protein content. Secreted lipid and lipoprotein data were expressed per filter (as cells were seeded under uniform conditions and from a single batch of cells for each comparison).

# Separation of lipoprotein classes by ultracentrifugation of basolateral samples

Lipoproteins were isolated from basolateral samples according to flotation density using density gradient ultracentrifugation (Karpe *et al.*, 1996; Luchoomun and Hussain, 1999). Briefly, 2ml of basolateral medium was adjusted to a density of 1.1g/ml with solid KBr. The sample was overlaid with a gradient of KBr density solutions comprising 3ml x 1.063g/ml, 3ml x 1.019g/ml and 2ml x 1.006g/ml. To minimise mixing between density junctions, salt solutions were allowed to gravity feed down the centrifuge tube wall via a 22 gauge needle attached to a 1ml syringe. Density solutions were prepared from stock salt solutions of density 1.35g/ml (3M [357g/l] KBr/ 2.6M [152g/l] NaCl) and density 1.005g/ml (0.15M NaCl [8.77g/l]), each preserved with 0.05%w/v EDTA, according to Equation 2.3:

$$A * 1.005 + B * 1.35 = (A + B) * X$$

#### Equation 2.3

Where: A=volume of 1.005g/ml solution required

B=volume 1.35g/ml solution required

X = required density

Solutions of 1.063g/ml (equivalent to LDL density), 1.019g/ml (equivalent to IDL density) and 1.006g/ml (equivalent to VLDL density) were prepared and pH adjusted to 7.4 with conc. HCl. Solution density was measured at room temperature using a hydrometer and where necessary, minor adjustments were made using stock salt solutions to give required densities.

Ultracentrifugation of samples was carried out in an SW41 Beckman rotor at 39,000 rpm. Lipoprotein fractions from basolateral samples were not visible within the gradient and hence were isolated based on location in the gradient. After 3.5 hours, chylomicron fraction (gradient fraction 1) was aspirated from the top ml of the gradient via a pasteur pipette with narrowed tip

and this volume was replenished with 1ml 1.006g/ml solution. Following a further 18 hours ultracentrifugation at 39,000 rpm, the gradient was aspirated off in 1ml fractions, VLDL were taken as the top 1ml of the gradient (fraction 2), LDL were taken as fractions 5-8 (density 1.019-1.006g/ml) and HDL (density 1.1-1.063g/ml) as fractions 9-12. Lipoprotein content was quantified by measuring the amount of radiolabelled oleic acid incorporated into each fraction by liquid scintillation counting.

# 2.3.3 Validation of ultracentrifugation technique

# Measurement of solution density within the resulting gradient

PBS, pH 7.4 was substituted for cell media in the above technique in order to characterise the final gradient density distribution. Following ultracentrifugation, the mass of each 1ml aliquot was determined (in 250µl volumes) on a microbalance, and density calculated as g/l. The resulting density gradient profile is shown in Appendix IV.

#### Analysis of human plasma by ultracentrifugation

Human plasma was used to validate the ultracentrifugation technique employed. Blood (20ml) was collected from a human subject into tubes containing EDTA (final concentration 4mM) 2 hours post-feeding. Plasma was separated by centrifugation at 4000 rpm for 15 minutes at 4°C. Plasma was stored on ice and used on the day of collection.

3ml plasma was adjusted to a density of 1.1g/ml with solid KBr. Samples were transferred to a Beckman polyallomer centrifuge tube and overlaid with density gradient solutions. Ultracentrifugation was performed as described for basolateral samples at 39,000 rpm. After 3.5 hours, chylomicrons (fraction 1) were isolated in the top 1ml and the volume replenished. After a further 18 hours, the resulting gradient was aspirated off and collected into 1.5ml eppendorf tubes in 1ml aliquots (fraction 2-12). VLDL were located at the top of the gradient (density <1.006g/ml), LDL were located at the 1.019g/ml - 1.063g/ml density interface and HDL at the 1.063g/ml -sample interface. In human plasma samples, chylomicrons and VLDL were each visible as a milky white layer, LDL and HDL were visible as orange-yellow bands and lipoprotein deficient plasma remained at the bottom of the tube as a red layer. Each gradient fraction was analysed for protein, triglyceride and cholesterol content. Protein analysis was conducted using a Bio-Rad assay kit and triglyceride and cholesterol content was determined using a Technicon RA-XT autoanalyser. The relative triglyceride/ protein/ cholesterol composition of each fraction indicates lipoprotein composition and hence was used to confirm location of each lipoprotein class throughout the gradient (Appendix V).

# 2.3.4 Colorimetric MTT (tetrazolium) toxicity assay for cell viability

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a tetrazolium salt that is cleaved by mitochondrial dehydrogenase in living cells to give a dark blue product (Mossman, 1983; Tada et al., 1986). Cell viability, indicated by dehydrogenase activity, can therefore be assessed by measuring the ability of cells to convert MTT to its coloured byproduct. An MTT assay for cell viability was performed, with modifications, according to the method described by Saarinen-Savolainen et al. (1998). Initially a calibration curve was produced to select the optimum cell concentration for the assay. Caco-2 cells were plated in the concentration range of 5-70 x 10<sup>3</sup> cells/ 100µl media/ well in 96-well Falcon® tissue culture treated plates. Plates were incubated under standard tissue culture conditions for 48 hours. Medium was removed and replaced with serum-free DMEM (100µl/well) and 25µl MTT (5mg/ml PBS) was added to each well. Plates were incubated for 2 hours at 37°C. The blue formazan reaction product of MTT was solubilised by addition of 100µl extraction buffer (10%w/w SDS in 0.1N HCl) and further incubation at 37°C. Absorbance was measured at 570nm. The calibration curve was linear up to 50,000 cells/well and a concentration of 35,000 cells was selected for subsequent assays based on absorbance value. Linear regression analysis revealed an r<sup>2</sup> value of 0.991 for the region between 5 x 10<sup>3</sup> and 35 x 10<sup>3</sup> cells. A representative plot is shown in Appendix VI.

The assay was modified for determination of vehicle toxicity to account for the long exposure time used in lipid experiments (20 hours). Caco-2 cells were seeded at a density of 35,000 cells/well onto 96-well tissue culture treated plates as described above and grown until confluent for 2-3 days in full culture medium. Culture medium was removed and 100µl of test vehicle added and incubated for 20 hours under standard culture conditions. Wells were washed thrice with serum-free DMEM and then incubated for 2 hours with serum-free DMEM (100µl/well) and 25µl MTT solution (5mg/ml PBS) as described above. Assay was continued as described above for the calibration curve from this point. For determination of excipient IC<sub>50</sub>, i.e. the concentration at which 50% cell viability was observed, a series of dilutions of test compounds were added to cells and IC<sub>50</sub> determined from a plot of % intracellular dehydrogenase activity against excipient concentration.

# 2.3.5 Quantification of apolipoprotein B using an ELISA technique

#### ELISA standard

Human LDL was used as a standard for the apoB ELISA as LDL protein comprises >95% apoB (Karpe and Hamsten, 1994). Blood (20ml) was collected from a human subject following an

overnight fast, and plasma isolated as described above (Section 2.3.3). LDL was separated from fasted plasma by density gradient ultracentrifugation (identified as yellow band in 1.019-1.063g/ml region of the gradient) and then dialysed extensively against PBS, pH 7.4 to remove KBr. For dialysis LDL was transferred to a length of Visking tubing, sealed at both ends with clips. The tubing was then transferred to 1000ml of stirred PBS, pH 7.4 containing 0.02%w/v EDTA and 0.02%w/v NaN<sub>3</sub> (sodium azide). Dialysis was conducted at 4°C over a 48-72 hour period, with at least 2 changes of buffer. LDL was then transferred to eppendorf tubes and aprotinin and PMSF were added as preservatives. The protein content of LDL was measured using a Bio-Rad protein assay, this is a measure of the ApoB content. SDS PAGE was used to confirm protein purity. LDL was stored for up to 3 months at 4°C for use as an ELISA standard. Fresh standards were prepared at 3 month intervals.

# SDS PAGE analysis of LDL standard

LDL was delipidated as described by Karpe *et al.* (1996) prior to SDS PAGE analysis. A volume of LDL equivalent to 1mg protein (as determined by Bio-Rad assay) was pipetted slowly, with mixing, into 4ml methanol contained in a 12ml glass conical centrifuge tube. 4ml ice-cold diethyl ether was added and the sample vortexed to mix. The mixture was centrifuged at 4000 rpm for 30 minutes, at 4°C, at which stage solvent was removed by aspiration. The protein, visible as a white haze in the bottom of the tube, was resuspended in diethyl ether, vortexed and centrifuged again. Solvent was removed and the protein pellet dried under nitrogen. The pellet was dissolved in 1ml of 1X SDS PAGE sample buffer (composition given in Appendix V), transferred to an eppendorf tube and denatured by heating at 80°C for 10 minutes. Condensation on the inside of the eppendorf was recovered by centrifuging sample at 10,000 rpm for 2 minutes at room temperature. Sample was aliquotted and stored at -20°C before further analysis.

SDS PAGE was conducted on a 6% SDS polyacrylamide running gel, with a 4.8% stacking gel, according to the method of Laemmli (Laemmli, 1970). Details of gel preparation and buffer composition are outlined in Appendix VII. Gels were prepared on the day of use and transferred to a Bio-Rad Mini-Protean II electrophoresis chamber filled with running buffer. Samples (10-20µl) were pipetted into sample wells on the gel. Standard molecular weight markers were added to a blank well and purified human plasma LDL, donated by Muriel Caslake, Lipid Research Department, Glasgow Royal Infirmary, was employed as a standard for apoB 100. Electrophoresis was conducted for approximately 40 minutes at 150V until the sample dye front reached the bottom of the gel. The protein was stained with coomassie blue stain (composition given in Appendix VII) over 40 minutes and excess stain removed overnight by soaking in destain solution (composition given in Appendix VII). Gel imaging was

conducted using a UVP transilluminator with Gelcam fixed focal length polarised camera with Grab-IT and Gelworks 1D advanced software. LDL was considered pure if a single apoB band was visible near the origin and any secondary bands were less than 10% of the main band intensity. Purity was confirmed by determining the band intensity for a series of samples of protein content 1µg-10µg/well and plotting protein content against band intensity. A correlation coefficient of 0.95 or higher confirmed protein purity (Karpe *et al.*, 1996). A representative gel is shown in Appendix VIII.

# Apolipoprotein B ELISA

The apoB content of basolateral samples and cell lysates was determined using an indirect sandwich ELISA as described (Bakillah et al., 1997):

- 96-well Nunc Maxisorp plate was coated overnight at 4°C with monoclonal antibody 1D1 (1:1000 in PBS, pH 7.4, 100μl/well)
- Wells were washed thrice with PBS-Tween 20 (0.05%v/v), pH 7.4
- Wells were blocked at room temperature for 2 hours with 'Marvel' milk protein 3%w/v in PBS, pH 7.4 (200µl/well)
- Wells were incubated for 2 hours at 37°C with human LDL standard (0.5-10ng LDL protein per well, in 100µl PBS, pH 7.4, equivalent to 5-100ng/ml)
- Wash step was repeated
- Wells were incubated for 1 hour at 37°C with sheep anti-human ApoB polyclonal antibody (1/1000 in PBS, pH 7.4, 100µl/well)
- Wash step was repeated
- Wells were incubated for 1 hour at 37°C with alkaline phosphatase linked rabbit anti-sheep
   IgG (1/2000 in PBS, pH 7.4, 100μl/well)
- Wells were washed twice with PBS-Tween (0.05%v/v), pH 7.4 and then twice with 0.2M tris base/ HCl buffer, pH 9.8-10
- P-nitrophenol phosphate (1mg/ml) in tris-HCl buffer, pH 9.8-10 (from Sigma Fast<sup>™</sup> tablet set) was added to wells (100µl/well) and incubated at room temperature for 10-20 minutes (or until absorbance for 100ng/ml standard reached 0.8)
- Absorbance was measured at 405nm to quantify apoB content

A calibration curve of absorbance against standard concentration was prepared. The calibration curve was linear up to a concentration of 150 ng/ml. Unknown sample concentrations were determined against the calibration curve. Regression analysis was conducted using Microsoft Excel® 2000 to confirm linearity of the calibration curve. In each case, the correlation coefficient ( $r^2$ ) was found to be >0.95. A representative curve is shown in Appendix IX.

# 2.3.6 Measurement of efflux activity

Cyclosporin A (CsA) was routinely used as a model substrate drug for efflux studies. CsA is a substrate for P-gp (Augustijns *et al.*, 1993; Hunter and Hirst, 1997) and to a much lower extent, for MRP (Holló *et al.*, 1996). Affinity for P-gp is 4-fold higher than for MRP (Holló *et al.*, 1996), so that CsA efflux can be taken to reflect P-gp activity.

Efflux activity was calculated by comparing apical-basolateral (influx) and basolateral-apical (efflux) transport of CsA, as described by Augustijns *et al.* (1993). All transport experiments were carried out on monolayers grown on 12mm diameter polycarbonate filters (0.4μm pore) using a transport buffer (TB) comprising Hanks buffered saline solution (HBSS) supplemented with 10mM Hepes and 25mM glucose at pH 7.4.

Stock (100 $\mu$ M) solution of CsA in DMSO was prepared. This was diluted 1 in 200 in TB to produce a test vehicle containing a final concentration of 0.5%v/v DMSO and 0.5 $\mu$ M CsA. 0.2 $\mu$ Ci/ml of [ $^{3}$ H] CsA was added as a trace label. [ $^{14}$ C] mannitol (0.15 $\mu$ Ci/ml) was added as a marker for membrane integrity.

Caco-2 monolayers were washed thrice with TB and allowed to equilibrate for 30-60 minutes. TEER measurements were taken and test vehicle added to the donor compartment to initiate experiments. Blank TB was added to the acceptor compartment. To investigate the effect of surfactants on CsA influx, various dilutions of excipient were added to the test vehicle in the apical compartment and basolateral samples were taken at 30 minute intervals over 2.5 hours, moving filters to a new basolateral well each time to maintain sink conditions. For efflux experiments, various dilutions of surfactant were added to the apical compartment with blank TB and test vehicle was added to the basolateral well. At 30 minute intervals over 2.5 hours, the entire apical volume was removed for sampling and the volume replaced with fresh TB containing the appropriate concentration of surfactant. Cells were maintained at 37°C in the incubator throughout the experiment, with the exception of sample collection points. Samples were collected as described (Augustijns et al., 1993; Hosoya et al., 1996), 20µl of 0.1M cold CsA solution in DMSO was added to basolateral samples and incubated for 1 hour before harvesting basolateral samples to ensure full recovery of label. Samples, with pipette tips, were transferred to scintillation vials. Ultima Gold (5ml) scintillation fluid was added and samples analysed by liquid scintillation counting.

Papp values were calculated for both apical-basolateral and basolateral-apical transport using Equation 2.2.

Taxol and vinblastine were used as additional substrates for P-gp to characterise Caco-2 monolayer efflux activity. Bi-directional transport studies were conducted on [ $^3$ H] taxol (0.5 $\mu$ Ci/ml, equivalent to 0.1 $\mu$ M) and [ $^3$ H] vinblastine (1.25 $\mu$ Ci/ml, equivalent to 0.1 $\mu$ M) as described for CsA to determine efflux activity.

# 2.3.7 Fatty acid uptake

Fatty acid uptake was monitored as described by Trotter and Storch (1991). Caco-2 cells were seeded onto 12mm diameter Transwell<sup>TM</sup> filters as described in Section 2.3.1 and grown for 19-21 days until fully differentiated. Oleic acid vehicles were prepared as described in Section 2.3.2, using HBSS TB as diluent. Final fatty acid concentration was 0.5mM oleic acid, complexed to 0.125mM BSA. Vehicles were labelled with [<sup>3</sup>H] oleic acid (20μCi/ml) and test excipients included at concentrations shown to inhibit TRL secretion as determined by lipid metabolism studies. Monolayers were rinsed in transport buffer three times and incubated for 1 hour at 37°C to equilibrate. Uptake was determined at 37°C. Fatty acid vehicles were added to cells for either 5 minutes or 1 hour to monitor initial uptake rate. After the specified incubation time, uptake medium was aspirated off with a pasteur pipette and filters were immediately dipped into ice-cold PBS, pH 7.4, containing BSA (0.5%w/v). Filters were then rinsed twice more with ice-cold BSA (0.5%w/v) solution to ensure complete removal of extracellular/ adsorbed fatty acid and then three times with ice-cold PBS. Cells were scraped into 0.05%w/v Triton X solution (1ml), and lysed by repeated passage through a 21 gauge needle. Cell associated activity was determined by liquid scintillation counting (0.5ml aliquot) and protein content determined using the Bio-Rad DC assay kit (Section 2.3.2).

#### 2.3.8 CMC determination

Surfactant CMC values were determined using a Du Nuoy ring (platinum ring, 4cm diameter) tensiometer (Lauda, TD-1) under conditions simulating those used throughout the cell work. Serial dilutions of excipient solutions were prepared in 'fed-state' media (0.5mM oleic acid: 0.125mM BSA in serum-free culture medium) or transport buffer and surface tension measurements taken at 37°C. Temperature was maintained at 37°C using a water jacketed vessel. Surface tension was calculated from the force required to pull the platinum ring away from the solution surface. The CMC value was determined from a plot of surface tension against concentration, and was taken as the point where no further decrease in surface tension occurred with increasing surfactant concentration.

# 2.3.9 Determination of free fatty acid content and sensitivity to digestion of polysorbate surfactants

# Free fatty acid quantification

Solutions of polysorbate 80 (0.25%w/v) were prepared in serum-free DMEM. Free fatty acid content was determined using a colorimetric non-esterified free fatty acid (NEFA) test kit (Randox). Background absorbance was measured for blank serum-free DMEM. All determinations were made in triplicate.

# In vitro digestion of polysorbate 80

In order to assess the sensitivity of polysorbate 80 to intestinal digestion, solutions prepared in serum-free DMEM (0.25%w/v) were subject to in vitro digestion by either (a) cell homogenate, (b) apical medium from cultured monolayers or (c) pancreatin. In the case of (a), Caco-2 homogenate was obtained from fully differentiated cell monolayers (passage 68-84) grown on onto 24mm polycarbonate Transwell<sup>TM</sup> filters (0.4µm pore) and for 19-22 days. homogenate was prepared according to the method of Spalinger et al. (1998) as follows. Monolayers were rinsed with culture medium and scraped with a cell scraper into 0.5ml of 0.25M sucrose containing 3mM EDTA and 1mM dithiothreitol and homogenised by sonication (Branson sonifier, with probe, 2 min, 10s intervals). Five monolayers were pooled and protein content determined using the Bio-Rad DC protein assay. Pooled homogenate was stored at -80°C for up to one week before use. For polysorbate digestion experiments, homogenate equivalent to 1.6mg protein (approximating to 1 monolayer and contained in 521µl homogenate volume) was added to serum-free DMEM to a final volume of 1.5ml. Polysorbate 80 was added to a concentration of 0.25%w/v and the resulting solutions were incubated over 20 hours at 37°C (to simulate typical lipid experiment conditions). For (b), serum-free medium was incubated with Caco-2 monolayers for 20 hours and then harvested using a pasteur pipette. Polysorbate 80 (0.25%w/v) was added to the harvested apical medium and incubated for 20 hours at 37°C. For (c), solutions of polysorbate 80 were prepared in serum-free DMEM containing 5mM sodium taurocholate. Pancreatin (8X USP, 0.04mg/100ml) was added and the solution incubated for 2 hours at 37°C, with agitation of 150 rpm in a shaking water bath to simulate intestinal luminal digestion. In each system, free fatty acid content was measured using the NEFA test kit, on systems pre- and post-digestion. Controls were prepared in each case in the absence of polysorbate 80 and processed in the same manner as test systems to determine background absorbance.

# 2.3.10 Halofantrine solubility study

# Solubility of halofantrine in lipid vehicles (as preconcentrates)

Glass tubes (10ml nominal volume) were siliconized with Aquasil<sup>TM</sup>. Excess Halofantrine (100mg) was weighed into the siliconized glass tubes. Samples (1g) of each lipid vehicle (olive oil, Miglyol 812, olive oil microemulsion, Miglyol 812 microemulsion and polysorbate 80) were added (each in triplicate) and tubes were vortexed to mix. Tubes were screw-capped and agitated in a shaking water bath at 150 rpm at 25°C (to simulate room temperature) for 96 hours. At 24-hour intervals, tubes were centrifuged at 5000 rpm on a bench top centrifuge (Sigma 203) for 30 minutes to precipitate undissolved drug. A 50mg aliquot of supernatant was aspirated off with a pasteur pipette and weighed into a clean siliconized glass tube. 5ml of chloroform: methanol (2:1) was added and tubes were vortexed to dissolve the oily vehicles. A 100µl aliquot was removed and diluted 1 in 50 in acetonitrile, with vortexing, in another siliconized glass tube. The resulting solution was assayed directly via HPLC for halofantrine (Section 2.3.11). Equilibrium solubility was reached when consecutive concentration values differed by less than 5%. In all cases, this was reached within 72 hours.

# Aqueous solubility of halofantrine from diluted lipid vehicles

In order to assess the maximum intestinal aqueous drug concentrations achievable from drug saturated lipid vehicles, aqueous halofantrine solubility was determined following dilution of halofantrine-saturated vehicles in simulated gastrointestinal media. Vehicle preconcentrates were first saturated with halofantrine at 25°C by agitating in a shaking water bath for 72 hours as described above, and undissolved drug was sedimented out by centrifugation. Aliquots (40µg) of each saturated system were subsequently weighed, in triplicate, into 10ml siliconized glass tubes with screw cap lids. Vehicles were then diluted 1 in 200 (to simulate predicted dilution in vivo, for a 1g vehicle being diluted in 200ml fluid) in either 0.1N HCl, bile salt: phospholipid buffer (10:2.5mM) or bile salt: phospholipid (10:2.5mM) buffer with pancreatin (8X USP, 4mg/ml) to simulate gastric, pre-digestive intestinal, and digestive intestinal fluids respectively. Tubes were agitated at 150 rpm at 37°C for 2 hours. Aliquots of 3ml were subsequently transferred to polycarbonate centrifuge tubes (Beckman, 349622) and centrifuged at 300,000g for 30 minutes, on a Beckman TL-100 centrifuge, to sediment any undissolved drug and to separate oil (emulsified/ undigested oil) and aqueous phases (Sek et al., 2001). Where an undigested oil phase was present, this was aspirated off using a pasteur pipette prior to Aliquots of clear aqueous phase were then collected via a pasteur pipette and sampling. transferred to siliconized glass tubes. 500µl aliquots of the aqueous phase were collected with a pipette, transferred to siliconized glass tubes and diluted 1 in 4 with acetonitrile with vortexing. Samples were then assayed for halofantrine content via HPLC (Section 2.3.11). Depending on

the gastrointestinal medium, the aqueous phase comprised bile salt solution, mixed micellar phase, surfactant/oil microemulsion, polysorbate 80 solution, or monoglyceride/ diglyceride/ fatty acid oil components. The oil phase was only seen where crude emulsions were dispersed in gastrointestinal medium, and predominantly contained undigested di- and triglycerides.

# 2.3.11 HPLC assay for halofantrine

# HPLC assay technique

A HPLC assay was established in the lab for halofantrine according to the method of Humberstone *et al.* (1995). The HPLC system consisted of a Waters 610 LC Module 1 Plus pump and injector with a Waters 486 UV detector (257 nm), a Luna C8 bonded phase column (5µm particle size, 25 x 4.6 mm internal diameter, Beckman) and a C8 guard column (Phenomenex). The mobile phase consisted of 0.2%w/v SDS and 0.2%v/v glacial acetic acid in acetonitrile: water (75:25), the flow rate was 1.5 ml/min. Run time was 12 minutes for halofantrine samples that contained no IS, and 14 minutes where IS was included. Assay temperature was maintained at 25°C using a column heater. Column was equilibrated for at least 3 hours before conducting analyses.

#### HPLC standards

Stock solutions of halofantrine HCl were prepared in acetonitrile equivalent to  $100\mu g/ml$  halofantrine base and stored in siliconized glass volumetric flasks at  $-20^{\circ}C$  for up to 6 weeks. Standard solutions were subsequently prepared from stocks on a daily basis. Stock solution of internal standard was prepared in acetonitrile at a concentration of  $100\mu g/ml$  and stored at  $-20^{\circ}C$  for up to 6 weeks.

#### Sample preparation and calibration for solubility samples

#### Sample preparation

Concentrated lipid vehicles saturated with halofantrine were subject to a 2-step dilution step prior to HPLC quantification – samples were initially diluted (50mg per 5ml) in chloroform: methanol (2:1) and subsequently 1 in 50 in acetonitrile. Lipid vehicles diluted in simulated gastrointestinal media (1 in 200) were diluted 1 in 4 in acetonitrile prior to injecting onto the HPLC column.

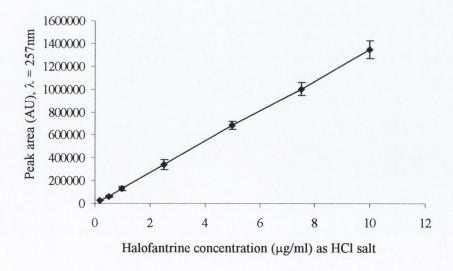
#### Calibration curve for unextracted samples

Standards were prepared daily by dilution of halofantrine HCl stock in acetonitrile to give a final concentration range equivalent to 0.05-10µg/ml halofantrine. Standard

curves were prepared by plotting standard peak area against halofantrine concentration. Unknown sample concentrations were determined from the equation of the line: y=mx +c, as calculated using linear regression of the standard curve (using Microsoft® Excel). A correlation coefficient was calculated using correlation statistics of the regression line to confirm linearity. A representative calibration curve is shown in Figure 2.1.

#### Assay validation

Intra-assay accuracy and precision were determined by replicate analyses (n=5) of standard halofantrine solutions at five different concentrations (0.5, 1, 2.5, 5 and 10μg/ml) in the expected sample concentration range. Accuracy was expressed as the percentage ratio of the observed to the theoretical concentration of halofantrine and precision as the relative standard deviation (RSD) of replicate analyses for each concentration. Inter-assay (inter-day) accuracy and precision were determined on five separate days using standards of the same concentration. Results are shown in Table 2.1. Intra-assay accuracy, as determined by replicate analyses of standards (n=5) on a single day, was 98.3-103.4% at all concentrations (0.5-10μg/ml). The variability was less than 2.4% (RSD) for the given concentration range. Inter-assay accuracy (measured over 5 days) was found to be 101.6-103.5% for all concentrations and variability was less than 4.5% (RSD). The limit of detection (LOD), taken as a signal to noise ratio of 3:1 (as compared to background) was found to be 0.03μg/ml and the limit of quantification (LOQ), taken as a signal to noise ratio of 10:1, was found to be 0.1μg/ml.



**Figure 2.1:** Representative HPLC standard calibration curve for halofantrine prepared using halofantrine HCl solutions in acetonitrile. Values represent the mean  $(n=4) \pm SD$ . The equation of the line was fitted using linear regression, with Microsoft® Excel 2000: y=134609x-783.99,  $r^2=0.999$ .

**Table 2.1:** Inter- and intra-assay accuracy (% relative to theoretical value) and precision (% RSD) of the halofantrine HPLC assay technique. Inter-assay validation was conducted on spiked samples over 5 days and intra-assay validation was conducted on 5 sets of spiked samples on a single day.

	Intra-assay		Inter-assay	
Basolateral Concentration (μg/ml)	Accuracy (%± SD)	Precision (RSD%)	Accuracy (%± SD)	Precision (RSD%)
0.5	$98.3 \pm 2.6$	2.4	$102.2 \pm 2.6$	2.6
1	$104.3 \pm 1.7$	1.6	$103.0 \pm 2.0$	2.0
2.5	$102.6 \pm 0.8$	0.7	$102.5 \pm 2.0$	2.0
5	$104.4 \pm 1.5$	1.4	$103.5 \pm 2.7$	2.6
10	$103.4 \pm 1.0$	0.9	101.6 ± 4.6	4.5

# Peak Interference

To ensure that lipid vehicle components did not interfere with peak detection, blank lipid vehicles were diluted as described for sample preparation and were analysed via HPLC to obtain a chromatogram. None of the vehicles- either in digested or undigested state were found to produce peaks in the halofantrine region (retention time  $8.80 \pm 0.12$  minutes).

# Sample preparation and calibration for Caco-2 samples

#### Halofantrine extraction technique

An extraction technique was required for stock vehicles applied to cells and for cellular and basolateral samples collected from Caco-2 cells to remove protein content and also as a means of concentrating samples to improve detection. Samples and standards were extracted using the same procedure. Extraction was performed as described by (Humberstone *et al.*, 1995). 1ml acetonitrile was added to samples and tubes were vortexed for 2 minutes to precipitate protein. 8ml of tert-butyl-methyl ether (TBME) was added and tubes were capped and vortexed vigorously for a further 2 minutes. Tubes were centrifuged at 4000 rpm for 10 minutes at room temperature to separate aqueous and organic layers. 8ml of the upper TBME layer was transferred using a glass pasteur pipette to another, clean polypropylene tube containing 100µl of 0.005N HCl in acetonitrile (to prevent degradation of halofantrine). Samples were

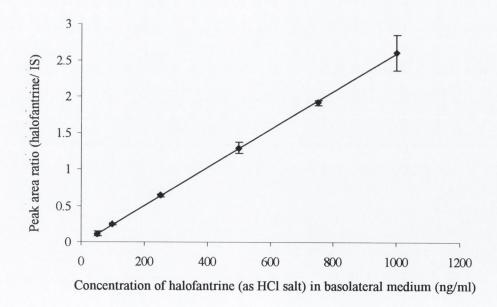
evaporated to dryness under nitrogen at 40°C on an N-Evap evaporator. Samples were reconstituted in 100µl of acetonitrile (500µl in the case of stock vehicles) with vigorous vortexing over 2 minutes and assayed via HPLC.

# Spiked standards for extracted calibration curves

100μl aliquots of diluted halofantrine HCl stock solution were added to 1000μl culture medium containing 20%v/v delipidated serum in 12ml polypropylene centrifuge tubes to give a final concentration equivalent to 50, 100, 250, 500, 750, 1000 and 2500ng halofantrine base/1.1ml media. Standards were then spiked with 100μl of internal standard (IS) solution (2000ng/ml acetonitrile, prepared daily from stock solution) and mixed with vortexing. Spiked standards were extracted prior to HPLC analysis.

#### Calibration curve

Standard curves were prepared by plotting standard peak area ratio (ratio of halofantrine peak area: IS peak area) against halofantrine concentration. Unknown sample concentrations were determined from the equation of the line: y = mx + c, as calculated using linear regression of the standard curve. A correlation coefficient was calculated using correlation statistics of the regression line to confirm linearity. A representative calibration curve is shown in Figure 2.2.



**Figure 2.2:** Representative standard calibration curve for halofantrine assay, expressed as peak area ratio (halofantrine/ internal standard) against theoretical concentration. Values represent the mean (n=4)  $\pm$  SD. Curve fitted by linear regression analysis (y = 0.0026x-0.015,  $r^2$ = 0.9998)

# Sample preparation for extraction

Basolateral or gradient fraction samples were thawed at room temperature and 1ml aliquots transferred to 12ml polypropylene tubes. Cellular samples and halofantrine stock vehicles were thawed at room temperature and 100µl aliquots were added to 900µl of culture medium (containing 20% delipidated serum) in polypropylene tubes. 100µl acetonitrile was added to samples to compensate for the 100µl of acetonitrile in the stock solutions added to spiked standards. All standards/ samples were then spiked with 100µl of IS (2000ng/ml) and mixed by vortexing.

# Assay validation

Intra-assay accuracy and precision were determined by replicate analyses of spiked standards (prepared in cell culture medium containing 20% delipidated serum) containing 50, 100, 250, 500 and 1000ng/ml halofantrine, with IS added. Spiked standards were subject to the same extraction process as the calibration standards and samples. For intra-assay values, replicate analyses (n=5) were performed on a single day. Accuracy was expressed as the percentage ratio of the observed to the expected concentration. Precision was expressed as the relative standard deviation (RSD) of replicate analyses for each concentration. Inter-assay (inter-day) accuracy and precision were determined on five separate days. Results for intra- and inter-assay validation are shown in Table 2.2. Intra-assay accuracy, as determined by replicate analyses of standards (n=5) on the one day, was 98.5-103.2% at all concentrations. The variability was less than 6.5% (RSD) or concentrations of 100ng/ml-1000ng/ml. At the lowest investigated concentration of 50ng/ml, the precision was 14.6% (RSD). Inter-assay accuracy (measured over 5 days) was found to be 101.5-105.1% for all concentrations and variability was less than 10% (RSD). The limit of detection (signal to noise ratio of 3:1) was taken as 20ng/ml and limit of quantification as 50ng/ml (signal-noise ratio of 10:1). The recoveries of halofantrine and IS were determined by comparison of the peak areas of halofantrine and IS recovered from extracted standards with the peak areas obtained from injection of unextracted standards. Recovery of halofantrine was >80% for all tested concentrations (Table 2.2), and IS recovery was on average  $82.6\pm11.2\%$ .

# Peak interference

In order to ensure that extracted matrices did not interfere with detection of halofantrine or IS, blank and spiked media were extracted and injected and chromatogram traces compared for signs of peak interference. Extracted basolateral medium, gradient fractions and cell lysate (diluted 1 in 5 in DMEM) showed no interfering peaks in the

halofantrine or IS region (retention times of  $9.11 \pm 0.15$  and  $12.03 \pm 0.23$  minutes respectively).

**Table 2.2:** Inter- and intra-assay accuracy (% relative to theoretical value) and precision (% RSD) of the extracted halofantrine HPLC assay technique. Inter-assay validation was conducted on spiked samples over 5 days and intra-assay validation was conducted on 5 sets of spiked samples on a single day.

Halofantrine concentration (ng/ml)	Accuracy (%± SD)	Precision (RSD%)	Recovery (%± SD)
Intra-assay (n=8)			
50	$103.2 \pm 10.6$	14.6	$89.5 \pm 6.2$
100	$101.7 \pm 6.7$	6.5	$84.5\pm19$
250	$101.4 \pm 12.2$	4.7	$80.5 \pm 13.7$
500	$98.5 \pm 4.4$	2.4	$82.0 \pm 11.2$
1000	$99.6 \pm 2.7$	0.3	$82.3 \pm 21.0$
Inter-assay (n=5)			
50	$103.9 \pm 9.4$	9.1	$91.9 \pm 18.6$
100	$105.1 \pm 9.9$	9.5	$84.4 \pm 16.7$
250	$101.9 \pm 7.0$	6.8	$80.1 \pm 8.7$
500	$101.5\pm4.4$	4.4	$80.0\pm8.7$
1000	$100.6\pm6.6$	6.6	$83.1 \pm 8.2$

# Effect of media matrix on extraction efficiency

Throughout the study, samples were extracted from a number of different matrices – basolateral medium and density gradient fractions. As inadequate blank volumes of each of these media were available for the routine preparation of standard concentration curves, cell culture medium containing 20% delipidated serum (equivalent to basolateral medium) was used on a regular basis for preparation of standard calibration curves. In order to verify that calibration curves prepared by extracting standards from culture medium were representative of standard calibration curves extracted from other media, a single experiment was conducted to compare the extraction efficiency from each medium. Standard curves were prepared from culture media (with 20% delipidated serum) and gradient fractions (obtained by centrifuging blank density gradient layered over control cell culture medium containing 20% delipidated serum) equivalent

to TRL, LDL and HDL. The concentration of halofantrine present in a single set of samples was calculated against each calibration curve and the resulting concentration values compared to identify any significant differences. Results are shown in Table 2.3. At a 95% level of significance, no significant differences were found between sample concentrations calculated using calibration curves generated from standards extracted from basolateral medium (cell culture medium with 20% delipidated serum) and standards extracted from different gradient fractions.

**Table 2.3:** Comparison of calculated sample concentrations using calibration curve standards extracted from different density gradient fractions and from basolateral medium (cell culture medium with 20% delipidated serum). Values represent the mean (SD) (n=3).

			antrine concentrati		••••••
	sta	andard calibration	curves extracted i	from different med	dia
Theoretical concentration (ng/ml)	Basolateral medium	1.006g/ml	1.019g/ml	1.063g/ml	Lipoprotein deficient medium
50	51.6 (4.4)	53.9 (1.4)	57.4 (5.5)	53.4 (4.7)	48.2 (1.9)
100	101.7 (6.7)	102.8 (2.6)	109.2 (10.5)	101.8 (9.0)	91.9 (3.7)
250	253.6 (30.5)	251.2 (6.4)	267.1 (25.7)	248.8 (22.0)	224.6 (9.0)
500	513.4 (63.1)	493.2 (12.5)	524.3 (25.4)	488.3 (43.2)	440.9 (17.6)
1000	996.3 (27.3)	1009.1 (25.7)	1072.8 (51.5)	999.3 (88.5)	902.1 (36.1)
p-value		0.987	0.7129	1.000	0.574

# 2.3.12 In vitro lipolysis studies

# pH stat technique

In vitro titrimetric analysis was conducted to determine the rate and extent of triglyceride hydrolysis by pancreatic lipase. Porcine pancreatic extract (pancreatin) was used as a source of pancreatic lipase and of co-lipase. Lipolysis was conducted based on the technique described by Sek et al. (2001) using a Metrohm pH stat 718 autotitrator. The assay buffer comprised 10mM tris -maleate buffer, pH 7.2, with 150mM NaCl, 2mM CaCl<sub>2</sub> and 1% w/v bile extract. For each assay, 0.2g of test oil was crudely emulsified in 10ml assay buffer by stirring for 10 minutes at 37°C. The pH was equilibrated to 7.2 by addition of NaOH or HCl as required. The reaction was initiated by addition of 0.2g pancreatin (equivalent to 10,000 TBU, determined as described in Appendix XI) and was allowed to proceed at 37°C for 2400 seconds. The pH stat

autotitrator maintained the pH at 7.2 and liberated free fatty acid was neutralised by addition of 0.1N NaOH solution. Readings were taken at 80 second intervals for the cumulative volume of NaOH added to maintain pH. The amount of NaOH (in µmoles) added to the reaction vessel to neutralise liberated fatty acid was used to quantify the extent of triglyceride digestion, as one mole of NaOH neutralises 1 mole of liberated fatty acid. Lipolysis of medium chain oils was most easily detected under the experimental conditions; hence, Miglyol 812 was used as a model oil for most of this work. Where excipient effects on lipid digestion were investigated, the required amount of each excipient was added to oil at the beginning of the experiment, prior to emulsification.

# [14C] triolein substrate technique

Glycerol tri-[14C]-oleate ([14C] triolein) was used as a substrate for lipolysis induced by pancreatin and by Caco-2 homogenate. Caco-2 homogenate was prepared as described in Section 2.3.8. Lipolytic activity was determined on an assay system that consisted of 14mM triolein (unlabelled) with 118µCi/ml (2mM) [14C] triolein (61mCi/mmol), 10mM tris-NaCl (pH 8.6) +/- 10mM sodium taurocholate based on that used by (Spalinger et al., 1998). 5%v/v FBS was added to aid lipid emulsification. A stable emulsion was formed by sonicating the mixture (2 minutes sonification, Bransen sonifier). 25µl of this incubation substrate was mixed with 10µl test surfactant solution by vortexing. 15µl of diluted cell homogenate (containing 30µg cell protein) or of pancreatin solution (containing 0.5µg pancreatin) was added to initiate lipolysis. Final reaction mixture contained 7mM triolein and 59µCi/ml (1mM) [14C] triolein +/-5mM sodium taurocholate +/- 0.1%w/v test surfactant. The reaction tubes (glass 12 x 75mm tubes) were incubated at 37°C for 2 hours in a shaking water bath. The reaction was stopped by the addition of the fatty acid extracting solvent. Free fatty acids were extracted using the method of Belfrage and Vaughan (1969) - the reaction was stopped by the vigorous addition of 1.6ml of ice-cold methanol: chloroform: hexane mixture (1.41: 1.25: 1), followed by 0.5ml of ice-cold potassium carbonate buffer, pH 10 (potassium carbonate-potassium borate-potassium hydroxide buffer, pH 10, 0.05 M). Test tubes were vortexed vigorously for a few seconds and then centrifuged at 5000 rpm for 10 minutes to separate phases. The upper phase volume was 1.1ml following this procedure. A 0.5ml aliquot of this was transferred to a 6ml polyethylene scintillation vial and 3ml Ultima Gold scintillation fluid was added. Scintillation vials were vortexed and then radioactive free fatty acid content was quantified on a liquid scintillation counter.

# 2.3.13 Microemulsion formulation and characterisation

# Preconcentrate formulation

Surfactant components were weighed and mixed, on a heated magnetic stirrer, in 10ml glass push cap vials at 40-50°C. The oil phase was added and stirred in until a homogenous system formed. The resulting microemulsion 'preconcentrate' was allowed to equilibrate for 24 hours before further analysis. Preconcentrates were stored at ambient temperature for one month to allow detection of formulation separation.

# Characterisation of microemulsions

## Microemulsion formation

Preconcentrate samples were diluted 1 in 200 in 0.1N HCl, or simulated intestinal fluids – low bile salt buffer (tris-maleate buffer, pH 6.8, containing 3mM sodium taurocholate: 0.75mM lecithin) or high bile salt buffer (tris-maleate buffer, pH 6.8, containing 15mM sodium taurocholate: 3.75mM lecithin) and agitated at 37°C and 150 rpm in a shaking water bath to produce oil-in-water dispersions.

# Assessment of system clarity

System appearance was noted initially by visual assessment and clarity classified as clear, translucent, slightly hazy or cloudy. Sample turbidity was measured as a more objective measure of system clarity to support this initial evaluation. Turbidity was determined from sample absorbance taken at 400nm by a conversion formula given in equation 2.4:

$$\tau = (2.303*absorbance)/l$$

Equation 2.4 (Fletcher and Morris, 1995)

Where  $\tau$  = turbidity and l=pathlength of cuvette (1cm)

Any systems with good clarity (clear, translucent or slightly hazy) were taken to represent microemulsion formulations, and were further subjected to particle size analysis by PCS using a Malvern Lo-C particle sizer.

# Photon correlation spectroscopy (PCS)

Particle size analysis was conducted using photon correlation spectroscopy (PCS). A Malvern autosizer Lo-C apparatus with a 64 channel 7032 correlator and argon ion external laser providing 75-100mW at 488nm was employed. Formulation preconcentrates were diluted 1 in

200 in buffers that had been filtered twice through a 0.2 µm pore filter. Dilutions were carried out in dust-free 20ml Sterilin vials. Formulations were agitated at 37°C and 150 rpm in a shaking water bath to ensure efficient dispersion. Resulting microemulsions were transferred to a quartz cuvette (scattering cell, Suprasil) and allowed to equilibrate for 15 minutes at 25°C in the temperature controlled sample unit of the autosizer. Measurements of light scatter were taken at a fixed angle of 90° with a 1cm pathlength and detected on a photon detection system, which transmitted the signal to the .64 channel 7032 correlator. PCS is a dynamic light scattering technique that measures changes in light scatter patterns resulting from Brownian motion of particles. Brownian motion of particles (generally in the nm range) occurs when particles are bombarded by solvent molecules, and is dependent on particle size, with smaller particles exhibiting more rapid movement. Measurements of laser light scatter due to Brownian motion of dispersed oil droplets, were averaged over 10 sub-runs and were converted using cumulants analysis (via a Malvern application program, LoC, version PCS: v126) to a Zave (nm) reading, which was used as an estimate of droplet diameter. Each measurement was made in triplicate. A latex bead standard (Nanosphere<sup>TM</sup> size standard, 220nm, Duke Scientific Corp) was analysed routinely to ensure that readings were accurate.

# 2.3.14 Administration of lipid vehicles to Caco-2 monolayers

# Vehicle preparation

# Halofantrine loading of lipid vehicles

Test vehicles for administration to cells included: olive oil (long chain triglyceride, LCT), Miglyol 812 (medium chain triglyceride, MCT), polysorbate 80, LCT based microemulsion (ome) and MCT based microemulsion (m-me). Where halofantrine was added, vehicles (contained in 10ml siliconized glass tubes) were saturated with halofantrine base by agitation in a shaking water bath at 150 rpm over 72 hours, and at 25°C. Tubes were centrifuged at 5000 rpm for 10 minutes to precipitate undissolved drug. The halofantrine-saturated supernatant was then processed as described in the following section.

### Lipid vehicle dilution or digestion in simulated intestinal media

Simulated intestinal buffer comprised 10mM tris buffer, pH 6.8, with 100mM sodium chloride, 1mM CaCl<sub>2</sub>, 10mM sodium taurocholate and 2.5mM lecithin. Simulated digestion buffer was the same, with the addition of pancreatin (cell culture grade, 4X USP, Sigma) 4μg/ml. The vehicle digestion technique was modified from that used by Garrett *et al.* (2000). Lipid vehicles were diluted 1 in 50 in simulated intestinal media and mixed by agitating at 150 rpm for 2 hours at 37°C to simulate intestinal conditions. Subsequently, mixtures were labelled with 0.2μCi/ml

[14C] oleic acid by incubating for 30 minutes at 37°C with the dried, sodium salt of labelled fatty acid and then diluted 1 in 4 with culture medium containing 20% v/v delipidated serum. Any trypsin present in the pancreatin was deactivated by the addition of delipidated serum to the diluted systems. Prepared media were sterile filtered through a 0.2µm filter in the laminar flow hood and warmed to 37°C prior to administering to cells.

# Administration of halofantrine loaded vehicle to cell monolayers

# Vehicle administration

Halofantrine loaded vehicles were administered to cells (1.5ml per monolayer) as per standard lipid experiments. Filters were transferred to clean 6-well plates containing 2ml culture medium with 20% v/v delipidated serum (to minimise drug adherence to plastic) as the basolateral medium. Monolayers were incubated overnight (20 hours) with vehicles at 37°C, 5% CO<sub>2</sub>. TEER values were taken prior to and following vehicle exposure to monitor monolayer integrity throughout the experiment. Following the incubation period, the apical medium was aspirated off and saved for determination of unabsorbed halofantrine content. Basolateral medium was pooled from 2 monolayers for each determination to increase sensitivity of detection.

# Sample processing

Basolateral medium was pooled from 2 monolayers and transferred to siliconized glass tubes. Tubes were centrifuged at 5000 rpm for 10 minutes to sediment cell debris. The supernatant was transferred to a clean, siliconized glass tube. Preservatives were added as per Section 2.3.2. Cell lysates were collected and processed as per Section 2.3.2.

# Gradient ultracentrifugation

For each pooled basolateral sample, 3ml was transferred to a polyallomer centrifuge tube and 1ml was reserved for determination of total basolateral halofantrine content. 0.51g of KBr was added to centrifuge tubes and allowed to dissolve at room temperature to increase sample density to approximately 1.1g/ml. Samples were swirled gently until thoroughly mixed and overlaid with a density gradient as described in Section 2.3.2. Samples were centrifuged at 39,000 rpm. After 3.5 hours the chylomicron fraction was removed in the top ml and replenished with 1.006g/ml solution. After a further 18 hours, the gradient was aspirated off in 1 ml fractions which were transferred, using siliconized glass pasteur pipettes to eppendorf vials. Samples were stored at -20°C until further analysis via HPLC for halofantrine content. All determinations were carried out in triplicate. Preliminary centrifugal analysis of spiked

halofantrine samples, in serum-free medium, showed that recovery of drug from the gradient was only approximately 50%. Addition of delipidated serum increased recovery to 80-85%.

Cell lysate, basolateral medium, gradient fractions and stock solutions were all analysed for halofantrine content using HPLC as per Section 2.3.11.

# 2.3.15 Gas chromatography

# Transmethylation and extraction

Fatty acids were transmethylated and extracted from polysorbate using the method described by Hamilton *et al.* (1992). This was conducted as follows: Polysorbate sample (250mg) was weighed into a 50ml round-bottomed flask. 4ml NaOH solution (2%w/v in methanol) was added and the reaction mixture heated under reflux for 5-10 minutes until clear. 5ml BF<sub>3</sub>-methanol complex solution was added through the condenser and the mixture was refluxed for 2 min. 3ml hexane was added through the condenser and the mixture boiled for a further minute and allowed to cool. Approximately 10ml of saturated NaCl solution was added to the flask and the mixture swirled gently. Further NaCl solution was added to fill the flask and allow phase separation. 1ml of the upper hexane layer was removed and residual water removed with anhydrous sodium sulphate. The hexane extract was dried under nitrogen and stored at −80°C until further analysis. Samples were reconstituted in 500μl hexane prior to injection onto the gas chromatography column.

# Gas chromatography conditions

Fatty acid methyl esters extracted from polysorbate solutions were identified by gas liquid chromatography with a Shimadzu GC-14A series gas chromatograph fitted with a Shimadzu C-R6A integrator (Mason Technologies, Dublin, Ireland). A 25M BP 21 polar aluminium silica column (Scientific Glass Engineering Ltd, Milton Keynes, England) was fitted. The split ratio was set at 1/20 and the analytical conditions were as follows:

Make-up gas: Oxygen-free nitrogen 0.5Kg/cm<sup>3</sup>

Column gas: Oxygen free nitrogen 1.0Kg/cm<sup>3</sup>

Hydrogen 0.6Kg/cm<sup>3</sup>
Dry air 0.5Kg/cm<sup>3</sup>

Temperature programme: Column initial temperature 120°C

8°C/min for 10 min to 200°C Hold at 200°C for 12 min 4°C/min for 5 min to 220°C Hold at 220°C for 12 min

Injection temperature 250°C Detection temperature 260°C

The fatty acid methyl esters were identified with a flame ionisation detector and identified by their retention times against a fatty acid methyl ester standard mix (Sigma). Fatty acid composition (%) was calculated using peak areas. Samples were injected by Anne Mullins, Department of Clinical Medicine, Trinity College, Dublin, who also optimised the gas chromatography conditions based on the method of Roche (1995).

# 2.3.16 Data analysis

Data analysis was conducted using Minitab<sup>TM</sup> 13.2 software. Where multiple samples were compared against a control, an analysis of variance (ANOVA) was conducted and significance relative to control calculated using Dunnett's multiple comparison test. Where multiple samples were compared against each other, an ANOVA was conducted and significance calculated using Tukey's multiple comparison test. A paired student t-test was used for two-sample comparisons. A p-value of <0.05 was considered significant and a p-value of <0.01 was highly significant.

# **Chapter Three**

# Optimisation of Caco-2 Model for Studying Lipid Metabolism

# 3 Optimisation of Caco-2 model for studying lipid metabolism

# 3.1 Introduction

Currently, there is a growing interest in the use of lipid-based formulations for improving the oral bioavailability of poorly water-soluble drugs. This has largely been sparked by the employment of combinatorial chemistry and genomics in drug discovery, resulting in the generation of a large number of highly active, lipophilic lead compounds with serious bioavailability issues (Charman, 1998; Venkatesh and Lipper, 2000). application of lipid formulation design to improving oral delivery of cyclosporin A (Neoral®), ritonavir (norvir®) and saquinavir (Fortovase®) has further prompted this interest (Porter and Charman, 2001). A number of mechanisms of bioavailability enhancement have been proposed for such vehicles. These include increased drug solubility in the intestinal lumen, alteration of intestinal barrier properties and promotion of lymphatic drug uptake (Counsell and Pohland, 1982; Pouton, 2000; Porter and Charman, 2001; O'Driscoll, 2002). Although there are in vitro models available for studying drug solubility and intestinal barrier properties (van de Waterbeemd, 1998; Braun et al., 2000; Kretz and Probst, 2001), lymphatic drug uptake studies, to date, have been limited to whole animal models (Edwards et al., 2001), predominantly rat studies. Whole animal models are poorly suited to the level of high throughput screening required to meet the demands of new drug discovery programs because of limitations imparted by ethical issues, cost and high labour and skill requirements (Rodrigues, 1997; Venkatesh and Lipper, 2000). For this reason, an in vitro cell culture model capable of predicting the in vivo lymphatic transport response to lipid drug formulations is highly desirable (Venkatesh and Lipper, 2000; Kretz and Probst, 2001).

Caco-2 cells have been used widely in biochemical and medical fields for elucidating the complex mechanisms associated with lipid metabolism (Hughes *et al.*, 1987; Trotter and Storch, 1991; Levy *et al.*, 1995; Mathur *et al.*, 1997; van Greevenbroek *et al.*, 1998). Caco-2 cells have many features in common with human duodenal enterocytes, making them ideal for such studies. Cultured cells possess essential pathways for synthesis of triglycerides, phospholipids, cholesterol esters and apolipoproteins and for assembly of lipoproteins (Hughes *et al.*, 1987; Trotter and Storch, 1993; Levy *et al.*, 1995; Mathur *et al.*, 1997). Additionally, when grown as monolayers, cells demonstrate polarity, absorbing fatty acids at the apical/ mucosal surface and secreting assembled lipoproteins at the basolateral/ serosal side as seen *in vivo* (Traber *et al.*, 1987). When grown to maturity and allowed to differentiate fully, Caco-2 cells secrete the full range of human lipoproteins – chylomicrons, VLDL, LDL and HDL (Levy *et al.*, 1999; Luchoomun and Hussain, 1999; van Greevenbroek *et al.*, 2000). Furthermore, the Caco-2

model is well established in the pharmaceutical field for studying barrier properties of the human intestine (Hilgers *et al.*, 1990). *In vitro-in vivo* correlations with regard to simple passive drug absorption have been proven (Artursson, 1990; Arturrson and Karlsson, 1991; Lennernäs *et al.*, 1996; Yamashita *et al.*, 1997; Yamashita *et al.*, 2000) and key intestinal transport proteins involved in active drug uptake and efflux (Hunter *et al.*, 1993; Hidalgo and Jibin, 1996; Hirohashi *et al.*, 2000; Nakamura *et al.*, 2002), as well as proteins involved in intracellular drug metabolism (Kim *et al.*,1999; Engman *et al.*, 2001), have been identified. Taking these factors into consideration, the Caco-2 model presents a potential alternative to whole animal models for studying interacting effects of lipid vehicles on lipophilic drug absorption, including lymphatic drug uptake. Initial studies have shown that the Caco-2 model can be used to study the inhibitory effects of Pluronic L81 on lipoprotein secretion (Luchoomun and Hussain, 1999), and that resulting data is qualitatively similar to that obtained with a rat intestinal model (Tso *et al.*, 1984).

# 3.2 Aims and objectives

Key studies published to date on lipid metabolism in the Caco-2 model employ a wide range of different experimental and culture conditions. Taking into consideration differences in analytical techniques and expression of results, this makes it difficult to compare findings. Prior to commencing further studies on lipid metabolism, it was necessary for us to establish a Caco-2 model capable of producing easily detectable and reproducible levels of triglyceride rich lipoprotein secretion. Initial studies employing the culture conditions reported by Luchooman and Hussain (1999), with Caco-2 cells of medium passage number grown on 3μm pore polycarbonate filters, produced minimal lipoprotein secretion levels. Hence studies were undertaken to optimise culture conditions taking into consideration conditions used for existing permeability studies in our laboratory and those employed for lipid studies by van Greevenbroek et al (1995) (passage 69-92 cells, 0.45μm pore polycarbonate filters), Trotter and Storch (1991) (passage 20-42 cells, 0.4μm pore polycarbonate filters) and Levin *et al* (1992) (passage 37-38 cells, 3μm pore polycarbonate filters).

Further aims of this chapter were to investigate the suitability of this *in vitro* model for predicting *in vivo* lipid vehicle effects on lipid metabolism and to characterise the model in terms of its barrier properties to a variety of paracellular and transcellular markers and P-gp substrates.

# 3.3 Results

# 3.3.1 Effect of filter type on monolayer formation and lipid metabolism

The Caco-2 model was established previously in the laboratory for studying passive drug absorption. In these studies, cells were grown on 0.4µm pore polycarbonate filters. As the chylomicron diameter has been reported to range from 75-600nm (Bisgaier and Glickman, 1983), filters with larger pore sizes were investigated to ascertain whether they would facilitate higher TRL secretion levels than the 0.4µm pore filter. Cells were seeded onto filters using standard culture conditions described in Section 2.3.1. The three filters used were:

- Costar Transwell<sup>TM</sup> polycarbonate (PC) filters, 0.4μm pore, 24mm diameter, 4.71cm<sup>2</sup> surface area.
- Costar Transwell<sup>TM</sup> polycarbonate (PC) filters, 3µm pore, 24mm diameter, 4.71cm<sup>2</sup> surface area
- Falcon® cell culture polyethylene terphthalate (PET) filters, 1μm pore, 23.1mm diameter, 4.2cm² surface area.

The 0.4µm and 3µm pore filters were made of polycarbonate, whilst the 1µm pore filter was made from PET. The reason for using different filter materials was that of commercial product availability. Polycarbonate was the material of choice, partly because it was well established in the laboratory for Caco-2 monolayer permeability studies and also because, it is the most widely used filter material in published literature (Artursson, 1990; Yamashita *et al.*, 1997; Braun *et al.*, 2000; Yamashita *et al.*, 2000), demonstrating well characterised cell growth. However, as there was no 1µm pore polycarbonate filter available, PET was used as an alternative. In each case, cells were seeded at a density of 63,000 cells/ cm² as per Section 2.3.1. Fully differentiated cells of passage 30-50 were used for this preliminary study as this was the standard passage range used in the laboratory for permeability experiments. The barrier properties of cell monolayers grown on each filter were characterised by measuring transepithelial electrical resistance (TEER) and by determining apparent permeability values for paracellular (mannitol) and transcellular (propranolol) permeants (Section 2.3.1).

With regard to lipid studies, cells were administered either 'fed-' or 'fasted-state' control vehicles and the intracellular levels of triglyceride and secreted TRL were compared for the three filters using techniques outlined in Section 2.3.2. Cell lysate, and additionally, secreted lipid and lipoprotein data, were corrected for monolayer protein content in this set of experiments to account for differences in available monolayer growth surface area (4.71cm² for polycarbonate and 4.2cm² for PET filters).

# Monolayer resistance and permeability to markers

Table 3.1 summarises the findings for monolayer permeability to transcellular (propranolol) and paracellular (mannitol) markers in addition to TEER values. As can be seen, there were no significant differences between apparent permeability coefficients or monolayer resistance for cells grown on polycarbonate filters with different pore size. However, cell monolayers grown on the 1μm pore PET filter demonstrated slightly higher monolayer resistance (approximately 18% higher than the 0.4μm pore polycarbonate filter, p<0.05) and reduced permeability to administered mannitol (approximately 45% lower than the 0.4μm pore filter, p<0.05). These data suggest that the 1μm pore PET filter produced monolayers with slightly different barrier properties to the polycarbonate filters.

**Table 3.1:** Monolayer resistance (TEER) values and permeability coefficients for mannitol (paracellular marker) and propranolol (transcellular marker) across Caco-2 monolayers grown on different filters. Values represent the mean (n=4) (SD).

	0.4μm, PC	1μm, PET	3μm, PC
Mannitol (Papp, cm/s x10 <sup>6</sup> )	2.28 (0.15)	1.28 (0.32)	2.08 (0.27)
Propranolol (Papp, cm/s x10 <sup>6</sup> )	16.46 (1.58)	14.50 (1.16)	14.1 (1.56)
TEER value ( $\Omega cm^2$ )	992.6(12.45)	1171.6 (42.3)	943.2 (63.7)

# Basolateral secretion of TRL, lipids and apoB

Data for TRL secretion (Figure 3.1a), as obtained via density gradient ultracentrifugation, show that the highest levels of chylomicron and VLDL secretion, in response to 'fed-state' (0.5mM oleic acid) vehicle were obtained for cells grown on the 0.4µm pore filter. Levels were lower for cells grown on 1µm pore filters, but the difference was not statistically significant. Secreted TRL levels obtained for cells grown on 3µm pore filters were significantly lower (p<0.05) than those with the smaller pore (0.4 and 1µm pore) filters. Data for total secreted triglyceride in 'fed-state' cells, as determined via TLC analysis, (Figure 3.1b) followed a similar trend, confirming a reduction in TRL secretion with the 3µm and 1µm pore filters. Secreted phospholipid (Figure 3.2a) and apoB (Figure 3.2b) levels also followed a similar decreasing trend with increasing pore size. As phospholipid is a surface lipid and triglyceride a core lipid, the relative triglyceride: phospholipid ratio gives an indication of lipoprotein size (Miller and

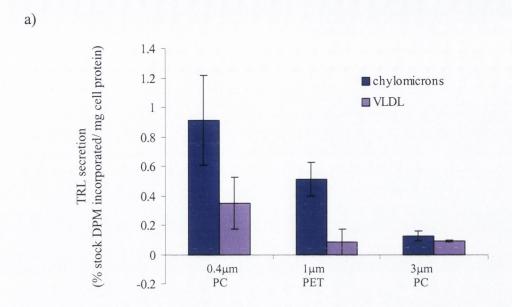
Small, 1983; Wasan and Cassidy, 1998). The amount of secreted apoB is used as a measure of the number of secreted lipoproteins because there is one surface apoB present on each secreted chylomicron or VLDL complex (Phillips *et al.*,1997) and the secreted triglyceride: apoB ratio gives an additional indication of lipoprotein triglyceride loading. Looking at the triglyceride: phospholipid and triglyceride: apoB ratios as shown in Table 3.2 and the secreted apoB levels in Figure 3.2b, it is apparent that filter pore size had a more pronounced effect on the number, as compared to the size, of secreted lipoproteins. For all filters, control 'fasted-state' levels of triglyceride secretion were similar and significantly lower than 'fed-state' data. A consideration of pore size alone suggests that larger pore filters should allow higher secretion levels of TRL. As this was not found to be the case in this study, it is likely that other factors are influencing the cell monolayer response.

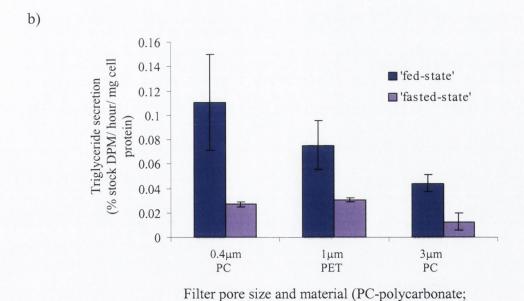
**Table 3.2:** Summary of secreted triglyceride: phospholipid and triglyceride: apoB ratios in response to 'fed-state' vehicle for Caco-2 monolayers grown on different filters

	0.4µm	1µm	3µm
TG: PL (DPM/DPM)	2.7	1.6	2.3
TG: apoB (DPM/µg apoB)	0.22	0.17	0.18

# Intracellular accumulation of lipids and apoB

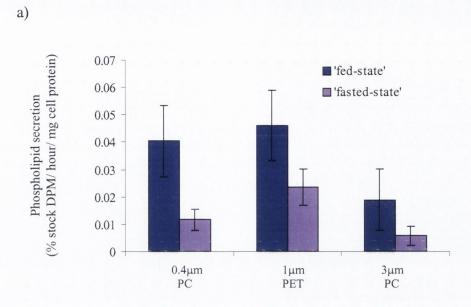
In contrast to basolateral data, intracellular triglyceride levels in response to 'fed-state vehicle (Figure 3.3a) were found to be similar for cells grown on each of the tested filters. Similarly, intracellular content of phospholipid (Figure 3.3b) and apoB (Figure 3.4) was largely unaffected by filter type in the 'fed-state'. The only significant difference resulting from filter type was for intracellular apoB content for cells grown on the 3µm pore filter in response to 'fasted-state' vehicle, where observed intracellular levels were approximately 40% lower than with the 0.4µm pore filter. Looking at the 'fed-' versus 'fasted-state' levels, differences for intracellular lipid content were only seen for triglyceride (2-3.5 fold higher in response to 'fed-state'), and not for phospholipid or apoB.





**Figure 3.1:** Effects of pore size and filter type on the basolateral secretion of TRL, as determined by density gradient ultracentrifugation, in 'fed-state' cells (panel a) and on basolateral secretion of triglyceride in 'fed-' and 'fasted-state' cells (panel b). Values represent the mean  $\pm$  SD (n=4).

PET- polyethylene terephthalate)



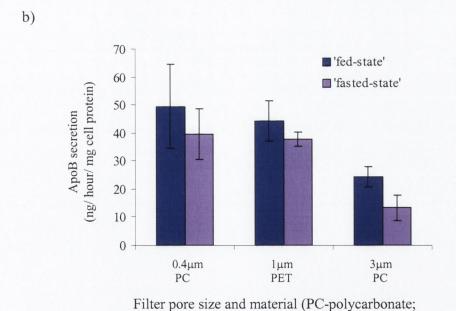
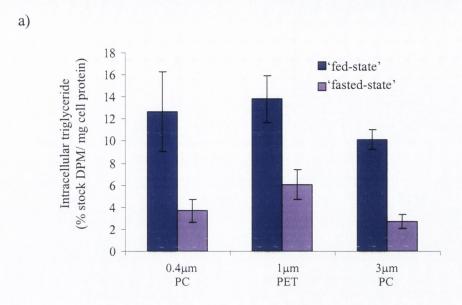
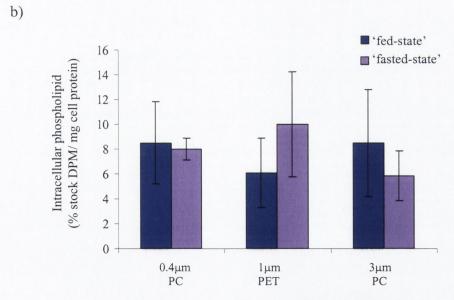


Figure 3.2 Effects of pore size and filter type on the basolateral secretion of phospholipid (panel a) and apoB (panel b) in 'fed-' and 'fasted-state' cells. Values represent the mean  $\pm$  SD (n=4).

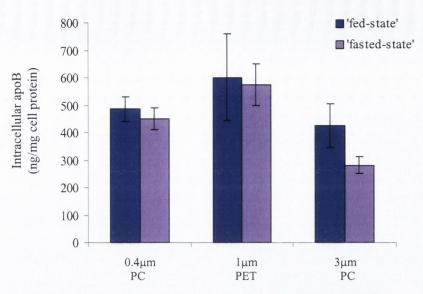
PET- polyethylene terephthalate)





Filter pore size and material (PC-polycarbonate; PET- polyethylene terephthalate)

Figure 3.3: Effects of pore size and filter type on the intracellular accumulation of triglyceride (panel a) and phospholipid (panel b) in 'fed-' and 'fasted-state' cells. Values represent the mean  $\pm$  SD (n=4).



Filter pore size and material (PC-polycarbonate; PET- polyethylene terephthalate)

Figure 3.4: Effects of pore size and filter type on the intracellular accumulation of apoB in 'fed-' and 'fasted-state' cells. Values represent the mean  $\pm$  SD (n=4).

# 3.3.2 Particle size analysis of TRL

The above data have shown that cells grown on polycarbonate filters of 0.4μm pore permit passage of secreted chylomicrons and that larger pore filters fail to offer any advantages in terms of chylomicron output. This suggests that the majority of secreted chylomicrons are smaller than 0.4μm in diameter. In order to estimate what proportion of secreted chylomicrons are likely to pass through 0.4μm filter pores, chylomicrons isolated from rat mesenteric lymph were subject to particle size analysis. Size measurements were made using photon correlation spectroscopy (PCS) as detailed in Section 2.3.12. Mesenteric rat lymph was supplied by Emma Lawless, School of Pharmacy, Trinity College, Dublin. Lymph was collected from rats that had been administered a long chain triglyceride microemulsion formulation (20%w/w olive oil, 20% w/w Peceol, 40%w/w Cremophor EL and 20%w/w polysorbate 80) seven hours previously using the method described by Griffin (2001). Lymph was subject to density gradient ultracentrifugation to isolate chylomicrons as described in Section 2.3.3. The milky chylomicron layer was isolated and diluted 1 in 20 in filtered saline (0.2μm pore filter).

Samples were sized on a Malvern Lo-C particle sizer. The average chylomicron diameter was found to be 126nm, with maximum of 564nm (Table 3.3) (particle size distribution trace given in Appendix XII). These values are consistent with reported average values of 100-200nm for chylomicron diameter (Haberbosch *et al.*, 1982; Hayashi *et al.*, 1990) and maximum range quoted as 600nm (Bisgaier and Glickman, 1983). Using intensity values (presented in Appendix XII), the proportion of chylomicrons with diameter of 400nm or greater was calculated. >99% of secreted chylomicrons were less than 400nm in diameter. This indicates that a filter with a nominal pore size of 0.4µm will permit free passage of the majority of secreted chylomicrons.

**Table 3.3:** Size analysis of mesenteric lymphatic chylomicron fraction (density <1.006g/ml). Lymph was collected from rats administered an olive oil microemulsion vehicle.

	Zave (nm) ± SD (n=6)	Polydispersity	Minimum (nm)	Maximum (nm)	% > 400nm
Diameter size	1260 + 10	0.240	25.9	563.9	0.0
distribution	$126.0 \pm 1.0$	0.240	23.9	303.9	0.9

# 3.3.3 Effect of passage number on lipid metabolism

Once it was established that polycarbonate filters of 0.4µm pore were suitable for growing cell monolayers for lipid studies, the effects of cell passage number on lipid metabolism were investigated. This aspect was explored because a wide range of Caco-2 passage numbers (passages 20-92) are employed in published lipid studies (van Greevenbroek *et al.*, 1996; Levy *et al.*, 1999; Moberly *et al.*, 1990; Gedde-Dahl *et al.*, 1999) and because passage number has been shown to significantly impact on cellular metabolism (Walter and Kissel, 1995; Hosoya *et al.*, 1996; Hongshi *et al.*, 1997). Prior to commencing lipid metabolism studies, the effects of passage number on membrane barrier properties were established.

# Monolayer resistance and permeability to markers

As shown in Table 3.4, Caco-2 passage number over the investigated ranges did not significantly alter cell monolayer permeability to the transcellular marker, propranolol or the paracellular marker, mannitol, nor did it affect monolayer TEER values.

**Table 3.4:** Monolayer resistance (TEER) values and permeability coefficients for mannitol (paracellular marker) and propranolol (transcellular marker) across Caco-2 monolayers of different passage number. Values represent the mean (SD).

	Passage 30-50	Passage 68-84
Mannitol (Papp, cm/s x10 <sup>6</sup> )	2.56 (0.39) (n=8)	2.36 (0.95) (n=12)
Propranolol (Papp, cm/s x10 <sup>6</sup> )	17.03 (1.72) (n=8)	16.3 (1.58) (n=8)
TEER value ( $\Omega cm^2$ )	986.5 (31.4) (n=8)	965.7 (109.6) (n=12)

A cut-off of passage 84 for the higher passage cells was selected based on the observation, during preliminary studies, of excessive cell growth on filters beyond passage 88. Cells were seen at this passage to 'overgrow' on filters and onto the plastic filter mount. This was accompanied by increase in variability of TEER values. Cell overgrowth is a problem associated with high passage cells, and may be due to preferential selection of fast growing clones (Hongshi *et al.*, 1997).

# Basolateral secretion of TRL, lipids and apoB

Although passage number had no significant effects on monolayer barrier properties, it did impact on the lipoprotein output from cells. Following exposure to 'fed-state' vehicle, the stimulated chylomicron secretion level was 2.5-fold higher (p<0.01) from cells of passage 68-84 as compared to cells of lower passage (Figure 3.5). VLDL secretion was also elevated somewhat, but the difference was not statistically significant. The increase in TRL secretion was accompanied by a corresponding increase (2-fold, p<0.05) in basolateral triglyceride secretion (Figure 3.6a). Cells of higher passage number also demonstrated elevated levels of apoB secretion (1.6-fold, p<0.01) relative to lower passage number cells (Figure 3.6c), suggesting an increase in the number of secreted lipoproteins. Secreted phospholipid levels were not substantially altered (Figure 3.6b). 'Fasted-state' cells at the two passage ranges demonstrated similar triglyceride (Figure 3.6a) and phospholipid (Figure 3.6b) secretion levels. In contrast, apoB secretion levels in 'fasted-state' cells were elevated with the higher passage cells (Figure 3.6c).

# Intracellular accumulation of lipids and apoB

Under 'fed-state' conditions, intracellular triglyceride levels were approximately 1.4-fold higher in passage 68-84 cells versus low passage cells, but the difference was not significant (Figure 3.7a). Phospholipid levels were similar for both passage ranges (Figure 3.7b). The intracellular concentration of apoB was slightly elevated in passage 68-84 cells (1.3-fold) as compared to low passage cells, but the difference was not significant (Figure 3.7c). When looking at the 'fasted-state' response (Figures 3.7a-c), higher passage cells showed increases in intracellular accumulation of triglyceride (p<0.01) and phospholipid (p<0.05) versus low passage cells, but no change in intracellular apoB.

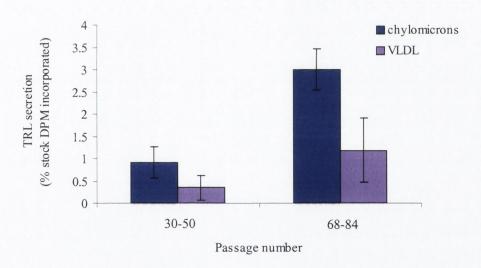
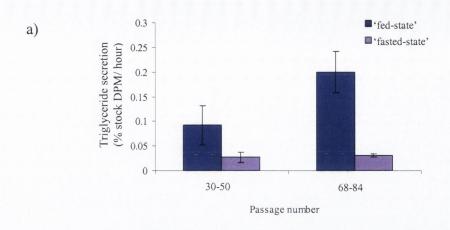
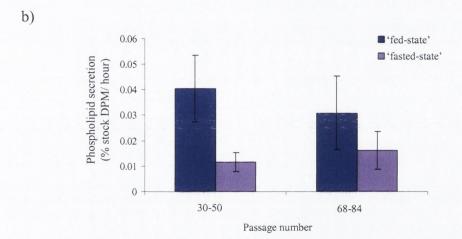


Figure 3.5: Effects of passage number on the basolateral secretion of TRL in 'fed-state' cells. Results are expressed as % of administered [ $^{14}$ C] oleic acid stock incorporated into density gradient fractions. Values represent the mean  $\pm$  SD (n=4).





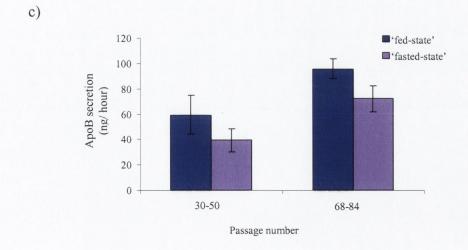
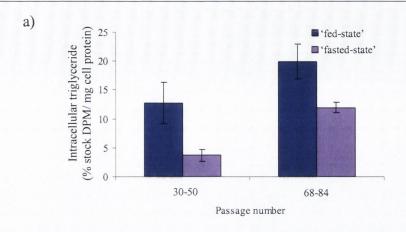
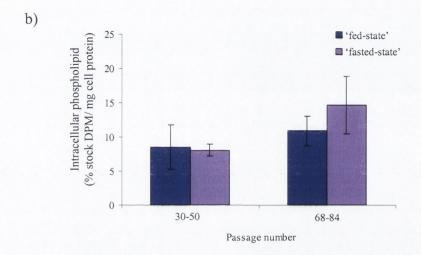


Figure 3.6: Effects of passage number on the basolateral secretion of triglyceride (panel a), phospholipid (panel b) and apoB (panel c) in 'fed-' and 'fasted-state' cells. Values represent the mean  $\pm$  SD (n=4).





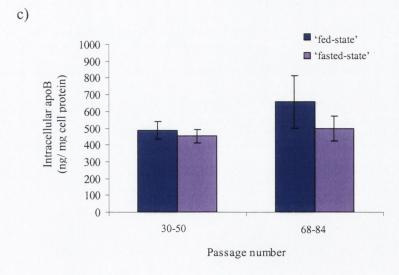


Figure 3.7: Effect of passage number on the intracellular accumulation of triglyceride (panel a), phospholipid (panel b) and apoB (panel c) in 'fed-' and 'fasted-state' cells. Values represent the mean  $\pm$  SD (n=4).

# 3.3.4 Reproducibility of TRL secretion over the investigated passage range

In order to verify reproducibility of data from the Caco-2 model, it was necessary to characterise the lipid production and secretion potential of the selected model over the full cell passage range employed throughout this study. This was achieved by including a 'fed-state' control with each batch of lipid experiments and collectively analysing the resulting intracellular and basolateral lipid levels over the investigated passage range (68-84).

Levels of intracellular and secreted triglyceride and apoB for 'fed-' stimulated Caco-2 cells were monitored at intervals over the investigated passage range. As can be seen from data presented in Tables 3.5 and 4.6, there were no significant changes or trends in either the intracellular accumulation or the basolateral secretion of triglyceride and apoB (key TRL components).

Table 3.5: Basolateral secretion of triglyceride and apoB by 'fed-' stimulated Caco-2 cells of passage 68-84, 0.4µm pore, polycarbonate filters. Values are expressed as the mean (SD) (n=3-4).

	Passage							
	68	72	74	76	78	80	82	84
Triglyceride (%	0.17	0.22	0.18	0.22	0.18	0.19	0.26	0.25
stock DPM/ hr)	(0.02)	(0.04)	(0.03)	(0.02)	(0.02)	(0.02)	(0.04)	(0.08)
ApoB	132.60	137.80	118.32	129.41	154.32	125.89	111.02	160.2
(ng/hr)	(5.21)	(10.44)	(15.62)	(17.14)	(8.38)	(33.00)	(5.00)	(5.52)

Table 3.6: Intracellular accumulation of triglyceride and apoB by 'fed-' stimulated Caco-2 cells of passage 68-84, 0.4µm pore, polycarbonate filters. Values are expressed as the mean (SD) (n=3-4).

	Passage							
	68	72	74	76	78	80	82	84
Triglyceride (%	21.3	18.1	21.5	22.0	18.9	18.9	22.03	20.5
stock DPM/mg	(2.7)	(1.9)	(5.0)	(2.4)	(0.3)	(5.4)	(1.8)	(5.8)
protein)								
ApoB	1073	1390	1268	1322	1186.4	971.8	787.1	865.1
(ng/mg protein)	(108)	(322)	(282)	(240)	(132.7)	(295.7)	(151.3)	(37.1)

Secreted chylomicron levels were also monitored over the passage range, as these are the key lipoproteins of interest in lymphatic lipid transport. These are illustrated in a control chart (Figure 3.8.), 95% confidence interval regions are indicated (dotted lines). Again, there were no significant differences in chylomicron secretion, and no clearly defined trends over the passage range.

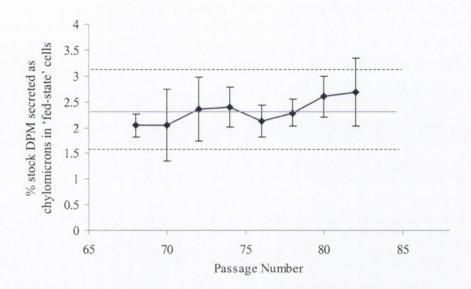


Figure 3.8: Control chart for chylomicron secretion in Caco-2 cells of passage 68-84,  $0.4\mu m$  pore polycarbonate filters. Chylomicrons were isolated from basolateral medium following a 20 hour incubation period using density gradient ultracentrifugation (Section 2.3.2). 95% confidence intervals (dotted lines) and mean (solid, central line) are shown. Values represent the mean  $\pm$  SD (n=4).

# 3.3.5 Characterisation and validation of optimised model

The above data indicate that Caco-2 monolayers of passage 68-84 cells, grown on 0.4µm pore polycarbonate filters are optimal for TRL secretion studies. These experimental conditions were therefore selected for further studies. Prior to initiating lipid vehicle studies, the model was characterised and validated.

Monolayers were characterised in terms of barrier properties (passive permeability to hydrophilic and hydrophobic markers), active drug efflux and lipid processing capacity as a validation step prior to commencing further studies. Substantial inter-laboratory variation has been reported for the Caco-2 cell model, and as a result, it has been advised that permeability to para- and transepithelial markers and P-glycoprotein (P-gp) substrates be assessed under standard experimental conditions to facilitate comparison with published data and to ensure consistency of data (Wunderli-Allenspach, 2001). Mannitol and fluorescein were chosen as hydrophobic markers for paracellular transport and propranolol and dexamethasone as hydrophobic permeants for transcellular passage. Bi-directional transport studies were conducted on cyclosporin A, vinblastine and taxol in order to characterise active drug efflux properties. Sensitivity of cyclosporin A efflux to inhibition by verapamil was also investigated as a further validation step for the cell model to confirm P-gp involvement.

Validation of lipid metabolism in the Caco-2 model was performed by a series of experiments to determine:

- concentration dependence of TRL response to oleic acid exposure
- differential response to 'fed-' versus 'fasted- state' vehicles
- lipid composition of secreted lipoproteins
- differential response to fatty acids of varying chain length/ saturation
- sensitivity of lipoprotein secretion to inhibition by verapamil

# 3.3.5.1 Monolayer Characterisation

# Monolayer permeability to transcellular and paracellular markers

Caco-2 monolayer integrity was confirmed using paracellular permeants, mannitol and fluorescein. Transmembrane permeability was measured using propranolol (log P 2.53) (Yee, 1997) and dexamethasone (log P 1.82) (Yee, 1997). Transport experiments were conducted as described in Section 2.3.1. Results are presented in Table 3.7. Permeability values for paracellular markers were similar to literature data for fully differentiated monolayers, confirming tight junction formation in monolayers. Permeability coefficients for transcellular markers were again found to be similar to published findings (Table 3.7).

Monolayer permeability to mannitol and propranolol was measured at intervals over the experimental passage range to investigate reproducibility of monolayer formation. Data presented in Figure 3.9 show no significant differences or trends between Papp values obtained over the passage range, thus confirming consistency of growth.

Table 3.7: Average permeability values for paracellular (mannitol and fluorescein) and transcellular (propranolol and dexamethasone) markers across Caco-2 monolayers (passage 68-84 cells, 0.4μm pore, polycarbonate filters). Values represent the mean (SD) (n=4).

Marker	Experimental findings	Literature Data
Marker	Papp (cm/sec x 10 <sup>6</sup> )	Papp (cm/sec x10 <sup>6</sup> )
Mannitol (n=12)	2.36 (0.95)	0.14-5.23 a,b,c,d
Fluorescein (n=4)	2.31 (0.79)	$0.098-5.24^{d}$
Propranolol (n=8)	16.30 (1.58)	2.16-34.1 a,b,c,e
Dexamethasone (n=4)	13.37 (0.56)	1.12-23.4 b,f

a (Pade and Stavchansky, 1998)

b (Yee, 1997)

c (Gres et al., 1998)

d (Walter and Kissel, 1995)

e (Cogburn *et al.*, 1991)

f (Yamashita et al., 2000)

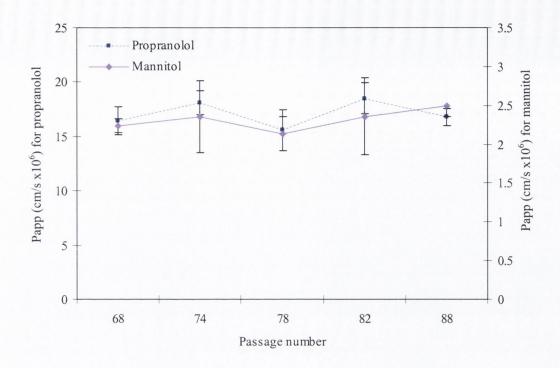


Figure 3.9: Apparent permeability values for mannitol (paracellular marker) and propranolol (transcellular marker) across Caco-2 monolayers measured at intervals over the passage 68-84 range. Values represent the mean  $(n=3-4) \pm SD$ .

# Active drug efflux in monolayers

Active drug efflux was evaluated in Caco-2 monolayers for three P-gp substrates – cyclosporin A (CsA) (a neutral hydrophobic cyclic peptide), vinblastine (a vinca alkaloid) and taxol (a taxoid). Bi-directional transport studies were conducted as described in Section 2.3.5. Apparent permeability coefficient (Papp) values (bi-directional) for each drug are listed in Table 3.8. As illustrated by the Papp ratios (Papp basolateral-apical/ Papp apical-basolateral), net efflux (basolateral-apical movement) was observed for each of these markers, with rank order of efflux sensitivity being vinblastine >taxol> cyclosporin A (Table 3.8). The Papp ratios obtained for each substrate are consistent with reported literature values (Table 3.8).

**Table 3.8:** Bi-directional transport of P-glycoprotein substrates across Caco-2 monolayers (passage 68-84). Values represent the mean (SD) (n=4).

Commonad	Down A D	Down D. A	Down notice	Literature Papp
Compound	Papp A-B	Papp B-A	Papp ratio	ratio
Cyclosporin A (n=18)	1.38 (0.962)	14.41 (6.9)	10.44	10 <sup>a</sup>
Vinblastine (n=6)	0.315 (0.051)	24.25 (1.27)	77.99	42.5 <sup>b</sup>
Taxol (n=4)	1.11 (0.107)	51.77 (4.59)	46.64	38.5 <sup>b</sup>

a obtained in Caco-2 cells (Augustijns *et al.*, 1993)

Published data have demonstrated the ability of verapamil to inhibit P-gp drug efflux in Caco-2 cells (Hunter *et al.*, 1993). As a further validation step, the effect of verapamil on CsA efflux was investigated in the established model to confirm transport sensitivity to this compound.

Inhibition of CsA efflux was measured by comparing apical-basolateral and basolateral-apical transport of CsA across Caco-2 monolayers in the presence and absence of verapamil. Verapamil was added to both apical and basolateral media and the bi-directional transport experiment conducted as described in Section 2.3.5. Monolayer resistance and intracellular dehydrogenase activity were measured following exposure of cells to verapamil to monitor cell viability as per Sections 2.3.1 and 2.3.4.

# Assessment of verapamil toxicity to Caco-2 cells

It was important to determine cell viability in the presence of verapamil in order to confirm that observed effects on drug transport were not a result of cell or monolayer damage. Data presented in Table 3.9 for monolayer resistance (presented as TEER values) and cell viability (as measured using an MTT assay) show that Caco-2 cell monolayers remained fully viable and intact in the presence of 50μM verapamil following a 2.5 hour exposure. However, at the higher concentration of 100μM, verapamil produced a 76% reduction in monolayer integrity, and a 58% decrease in cell viability. For this reason, further experiments were conducted using 50μM verapamil only.

b obtained in MDR-MDCK cells (Ward et al., 1999)

Table 3.9: Caco-2 cell viability and monolayer integrity in the presence of verapamil. Values represent the mean (SD), n=3-5. Viability was measured using an MTT assay and monolayer integrity was measured routinely using TEER values following a 2.5 hour exposure. Values are expressed as % relative to pre-exposure level (TEER) or % relative to control cells incubated without verapamil (MTT); \*\*p<0.01 relative to starting value or positive control respectively.

Concentration of verapamil (µM)	TEER values (% relative to initial value, SD)	MTT viability (% cell viability, SD)
50	93.9 (8.4), n=4	98.62 (8.96), n=5
100	23.80 (3.52)**, n=3	42.35 (6.54)**, n=5

# Effects of verapamil on cyclosporin A flux

Addition of verapamil ( $50\mu M$ ) to transport buffer significantly (p<0.01) inhibited the efflux (basolateral-apical movement) of CsA. In the presence of verapamil, the Papp ratio for CsA was effectively 1, with no difference between apical-basolateral and basolateral-apical movement of CsA. The apical-basolateral transport of CsA was not affected by verapamil addition under the experimental conditions (Figure 3.10).

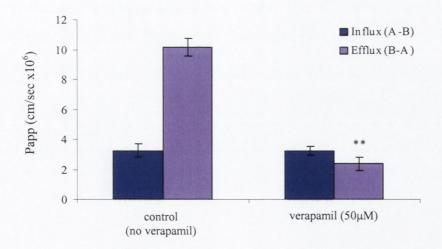


Figure 3.10: Effects of verapamil on the permeability of CsA in both the apical-basolateral and the basolateral-apical direction. Results are expressed as the mean (n=4)  $\pm$  SD. Statistical significance of excipient effects relative to the control (CsA flux in the absence of verapamil) is shown: \*\*p<0.01.

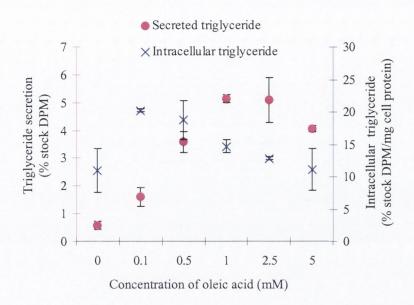
# 3.3.5.2 Concentration dependence of TRL response to oleic acid stimulus

Cells were supplemented with increasing concentrations of oleic acid to establish a concentration-response relationship with the ultimate aim of selecting an appropriate 'fed-state' oleic acid concentration for further studies. Oleic acid vehicles were prepared by increasing the molar strength of oleic acid stocks and adding them to BSA solutions, as per Section 2.3.2, in each case, maintaining a 4:1 molar ratio of oleic acid to BSA. All vehicles were labelled with trace [<sup>14</sup>C] oleic acid (0.2µCi/ml). Vehicles were incubated with Caco-2 monolayers for 20 hours. Cell lysates and basolateral samples were collected for lipid and lipoprotein analysis as described in Section 2.3.2.

Increasing concentrations of oleic acid produced a corresponding rise in the secretion of triglyceride (Figure 3.11a) and apoB (3.11b) relative to 'fasted-state' (0mM oleic acid), reaching a plateau at 1mM oleic acid. The increase in secretion for 1mM oleic acid versus 'fasted-state' was more pronounced for triglyceride (8.87-fold, p<0.01) than for apoB (2.36-fold, p<0.01), indicating an increase in both the number and the triglyceride enrichment of secreted lipoproteins. This was further shown by the secreted TRL data obtained from density gradient analysis (Figure 3.12). Concentrations of up to 1mM oleic acid produced a concentration dependent increase in chylomicron secretion. Thereafter, a plateau was reached. At 1mM oleic acid, chylomicron secretion was 180-fold higher than the control 'fasted-state' level (p<0.01). VLDL secretion showed an increasing trend as far as 0.5mM and thereafter showed a decline. VLDL secretion at 0.5mM oleic acid was 10-fold higher than the 'fasted-state' level (Figure 3.12).

In contrast to secreted lipid data, intracellular triglyceride was raised relative to 'fasted-state' by administration of 0.1mM oleic acid (2-fold, p<0.05), but thereafter followed a decreasing trend with increasing administered oleic acid concentration, returning to the initial 'fasted-state' level at 2.5mM oleic acid (Figure 3.11a). Oleic acid had no significant effects on intracellular apoB content, although there appeared to be a slightly increasing trend (Figure 3.11b). As oleic acid had a more pronounced effect on secreted triglyceride levels than on intracellular triglyceride content, the secretion efficiency of triglyceride was seen to improve with higher administered oleic acid doses (Figure 3.13).

a)



b)

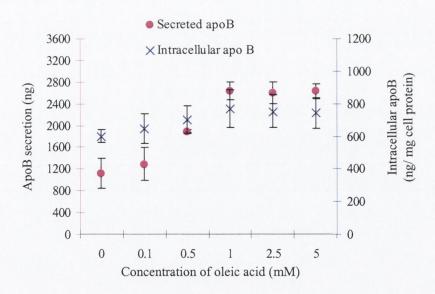


Figure 3.11: Effect of oleic acid concentration on intracellular and secreted levels of triglyceride (panel a) and apoB (panel b) in Caco-2 cells. Values represent the mean  $\pm$  SD (n=3-5).

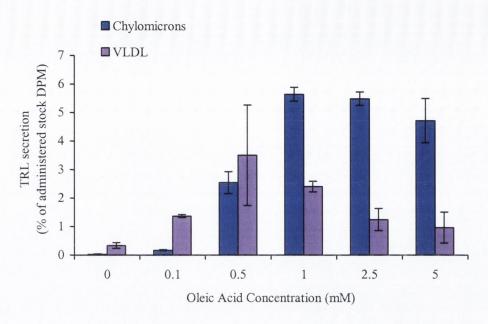
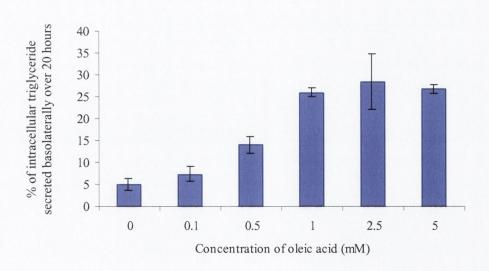


Figure 3.12: Effect of oleic acid concentration on TRL secretion response in Caco-2 cells. Values represent the mean  $\pm$  SD (n=3-5).



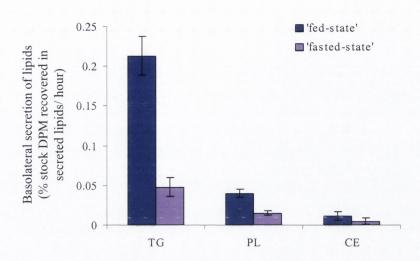
**Figure 3.13:** Effect of oleic acid concentration on triglyceride secretion efficiency (measured as the proportion of total cellular triglyceride secreted basolaterally over  $\frac{1}{2}$ 0 hours) in Caco-2 cells. Values represent the mean  $\pm$  SD (n=3-5).

# 3.3.5.3 Comparison of 'fed-' and 'fasted-state' vehicle effects on lipid metabolism in Caco-2 monolayers

Cells of passage 68-84 were cultured as described in Section 2.3.1 onto 24mm, 0.4µm pore polycarbonate filters and grown for 19-22 days. 'Fed-' (0.5mM oleic acid: 0.125mM BSA) or 'fasted-state' (0.125mM BSA) vehicles were applied to rinsed cell monolayers (n=4) and incubated over night as per Section 2.3.2. Cell lysate and secreted basolateral samples were collected following a 20-hour incubation and analysed for lipid and apoB content as per Section 2.3.2.

Administration of 'fed-state' vehicle to Caco-2 monolayers stimulated an increase in the secretion of triglyceride and phospholipid as compared to 'fasted-state' (Figure 3.14a). Triglyceride secretion was increased by >4-fold (p<0.01) and phospholipid secretion by approximately 2-fold (p<0.01). 'Fed-state' vehicle did not alter the secretion of cholesterol ester. Intracellular levels of triglyceride were also increased significantly by the administration of 'fed-state' vehicle (1.8-fold increase, p<0.01), but to a lesser extent than secreted levels (Figure 3.14b). Intracellular phospholipid and cholesterol ester levels remained unchanged. In addition, 'fed-state' vehicle increased the cellular secretion of apoB by 1.8-fold (p<0.05) relative to 'fasted-state', without altering intracellular levels (Figure 3.15 a,b). These results indicate a selective increase in the number and triglyceride loading of lipoproteins relative to 'fasted-state', with some stimulation of triglyceride production. The secreted lipoprotein profile (Figure 3.16) further illustrates the selective effects of 'fed-state' on TRL secretion. Chylomicron and VLDL secretion were substantially enhanced (50- and 37-fold respectively) by administration of 'fed-' versus 'fasted-state' vehicles.





# b)

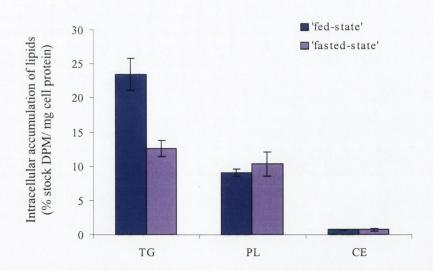


Figure 3.14: Comparative effects of 'fed-' and 'fasted-state' vehicles on secreted (panel a) and intracellular (panel b) lipid (triglyceride, phospholipid and cholesterol ester) levels. Values represent the mean  $\pm$  SD (n=4).

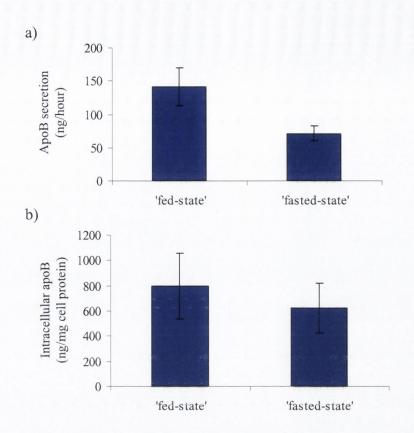


Figure 3.15: Comparative effects of 'fed-' and 'fasted-state' vehicles on secreted (panel a) and intracellular (panel b) apoB levels in Caco-2 cells. Values represent the mean  $\pm$  SD (n=4).

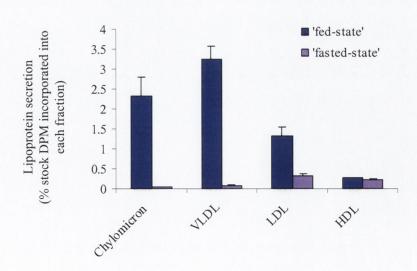


Figure 3.16: Comparative effects of 'fed-' and 'fasted-state' vehicles on the secreted lipoprotein profile for Caco-2 cells. Values represent the mean  $\pm$  SD (n=4).

#### 3.3.5.4 Lipid composition of secreted lipoprotein fractions

Cell monolayers (n=3) were administered 'fed-' or 'fasted-state' vehicles with a high [\$^{14}\$C] label content (0.8µCi/ml, four-fold higher than in routine lipid studies) and incubated for 20 hours. Basolateral samples were subject to density gradient ultracentrifugation to isolate each lipoprotein class (Section 2.3.2.). Lipids were extracted from each of the isolated lipoprotein fractions using the Folch extraction method, (Section 2.3.2) and subsequently analysed for lipid composition using TLC and liquid scintillation counting.

The lipid composition of secreted lipoproteins (isolated according to flotation density) was determined, as per Section 2.3.2, following administration of 'fed-state' vehicle. Data are presented in Table 3.10 and expressed as % total basolateral label incorporated into each of the lipid classes (triglyceride, phospholipid, cholesterol ester). Remaining label was either present as free fatty acid or was lost during processing. Chylomicron and VLDL fractions were composed almost entirely of triglyceride. As the lipoprotein density increased (from chylomicrons through to HDL), there was a decreasing trend in the ratio of triglyceride: phospholipid and also for triglyceride: cholesterol ester (Figure 3.17).

On comparing the lipid composition of the secreted Caco-2 lipoproteins with human lipoproteins of similar flotation density (as summarised in Table 3.11) it can be seen that the chylomicron composition is very similar, with >85% as triglyceride versus other lipids. Additionally, the trend towards increasing cholesterol/ phospholipid content and decreasing triglyceride with higher density lipoproteins is consistent with the human profile. With regard to more specific lipid composition, there were notable differences in LDL and HDL lipid composition for experimental Caco-2 findings as compared to reported human data. Caco-2 derived LDL had a substantially higher triglyceride (10.8-fold) and lower phospholipid (3-fold) and cholesterol (2-fold) content versus the human counterpart. HDL also contained considerably more triglyceride (5-fold difference) and cholesterol (2-fold difference) than the human form, and demonstrated lower (7.5-fold) phospholipid content.

**Table 3.10:** Lipid composition of secreted TRL fractions ('fed-state'), % of secreted stock DPM recovered in each lipid fraction. Values represent the mean  $\pm$  SD (n=3).

Lipoprotein fraction	Triglyceride	Phospholipid	Cholesterol ester
Chylomicron	84.1 (0.2)	4.8 (1.2)	9.0 (0.6)
VLDL	78.6 (6.5)	4.8 (0.1)	9.8 (5.0)
LDL	64.8 (15.7)	7.1 (4.3)	23.4 (10.1)
HDL	20.9 (8.6)	4.0 (3.7)	40.2 (12.1)

**Table 3.11:** Lipid and protein composition of human lipoproteins (% composition) as described by Wasan and Cassidy (1998)

Lipoprotein fraction	Triglyceride	Phospholipid	Cholesterol	Protein
Chylomicron	86	7	5	1-2
VLDL	55	18	19	8
LDL	6	22	50	22
HDL	4	30	19	47

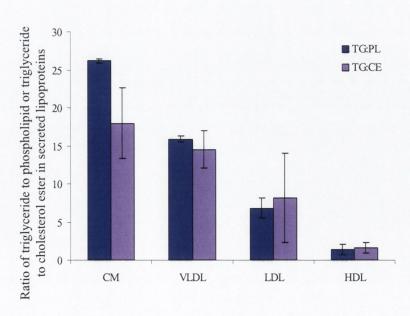


Figure 3.17: Ratio of triglyceride to phospholipid and triglyceride to cholesterol ester in secreted lipoproteins from 'fed' stimulated Caco-2 monolayers. Values represent the mean  $\pm$  SD (n=3).

#### 3.3.5.5 Differential Response to fatty acids of varying chain length and saturation

Fatty acid chain length and saturation is important for the *in vivo* lipoprotein response. A number of studies have recorded the effects of saturated and unsaturated fatty acids of different chain length on plasma and lymph triglyceride levels (Ockner et al., 1969; Bergstedt et al., 1990; van Greevenbroek et al., 1996; Caliph et al., 2000). To further validate the use of the Caco-2 cell model described here for predicting the *in vivo* response, the effects of a number of fatty acids on intracellular and secreted lipid levels were assessed and compared to reported in vivo findings. In addition to the monounsaturated oleic acid (C18:1), a number of saturated fatty acids of increasing chain length were investigated - butyric acid (C4:0), capric acid (C10:0), palmitic acid (C16:0) and stearic acid (C18:0). Fatty acid vehicles were prepared in a similar manner to the 'fed-state' vehicle described in Section 2.3.2, supplementing the appropriate fatty acid stock solution for oleic acid. All vehicles were labelled with trace [14C] oleic acid, as it has previously been shown that this can be used as a general marker for different fatty acid vehicles without altering the differential cell response (van Greevenbroek et al., 1996). Vehicles were incubated for 20 hours with fully differentiated Caco-2 monolayers (passage 68-84 cells seeded on 24mm diameter polycarbonate filters, 0.4µm pore). Samples of cell lysate and basolateral medium were processed as per Section 2.3.2 to quantify triglyceride and apoB content as well as to characterise the secreted lipoprotein profile.

Toxicity of the investigated fatty acids to Caco-2 cells was measured using an MTT assay for intracellular dehydrogenase activity (Section 2.3.4) over a range of fatty acid concentrations (0.5-5mM) and monolayer integrity was confirmed using TEER values (Section 2.3.1) following a 20-hour application of fatty acid vehicles to Caco-2 monolayers.

None of the investigated fatty acids significantly altered cell viability or monolayer integrity when added at a concentration of 0.5mM. At the higher concentration of 5mM, oleic acid and stearic acid reduced cell viability by approximately 20% (p<0.05), whilst other fatty acids had no effect. At the highest investigated concentration of 10mM, all of the fatty acids had a significantly negative effect on cell viability (Table 3.12).

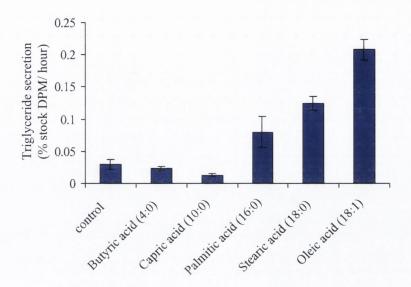
Fatty acid class had a significant impact on the lipoprotein secretion response. In the case of the saturated fatty acids, triglyceride secretion was seen to increase proportionally with fatty acid chain length. The mono-unsaturated oleic acid promoted the highest triglyceride secretion level (1.6-fold higher than with the equivalent saturated fatty acid, stearic acid, p<0.01). Butyric and capric acid did not promote the secretion of triglyceride above background control levels. The ability of fatty acids to stimulate triglyceride secretion followed a decreasing trend of: oleic>

stearic (p<0.01)> palmitic (p<0.05) > butyric (p<0.01) = capric (Figure 3.18a). ApoB secretion followed a similar trend, but the difference between responses was not as pronounced as for triglyceride levels (Figure 3.18b), indicating an increase in both the number and triglyceride enrichment of secreted lipoproteins. This was confirmed by the secreted lipoprotein response. Oleic showed a selective enhancement in chylomicron and VLDL secretion and stearic acid promoted secretion primarily of VLDL and LDL. Palmitic acid produced a predominance of LDL, whilst butyric and capric acid failed to produce any changes in lipoprotein secretion above baseline (Figure 3.19). Of further note is the increasing trend of triglyceride secretion response with increasing fatty acid chain length (Figure 3.20). With regard to intracellular lipids, palmitic acid, oleic acid and stearic acid promoted slightly higher triglyceride levels than the shorter chain fatty acids, however differences between triglyceride levels were not statistically significant (Figure 3.21a). Intracellular apoB levels were similar for each of the investigated fatty acids (Figure 3.21b).

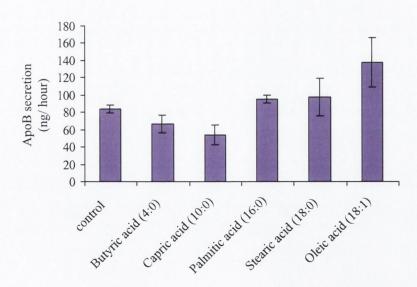
Table 3.12: Fatty acid effects on Caco-2 viability (measured using an MTT assay) and monolayer integrity (measured as TEER). Values represent the mean (n=5 for MTT and n=3 for TEER measurements) (SD). Statistical significance relative to positive control (cells incubated without excipients) (MTT assay) or starting value (TEER measurements) is shown: \*\*p<0.01; \*p<0.05 relative to control.

Fatty acid	% Integrity (as per TEER) at 0.5mM	% Viability (as per MTT assay) at 0.5mM	% Viability (as per MTT assay) at 5mM	% Viability (as per MTT assay) at 10mM
Oleic acid (C18:1)	96.5 (2.3)	92.7 (6.3)	78.2 (8.5)*	70.3 (6.6)**
Stearic acid (C18:0)	102.1 (5.6)	95.2 (2.3)	79.9 (6.6)*	43.5 (2.1)**
Palmitic acid(C16:0)	93.3 (4.5)	104.8 (4.4)	91.1 (2.5)	74.4 (3.8)**
Capric acid (C10:0)	97.2 (5.1)	102.9 (5.0)	87.2 (6.6)	81.2 (3.1)*
Butyric acid (C4:0)	92.5 (3.6)	94.7 (5.4)	111.7 (8.9)	72.2 (2.9)**

a)



b)



**Figure 3.18:** Effects of fatty acid chain length on basolateral secretion of triglyceride (panel a) and apoB (panel b) in Caco-2 monolayers. Fatty acids were administered to cells at a concentration of 0.5mM. Values are expressed as the mean  $\pm$  SD (n=5 for oleic acid and 3 for remaining fatty acids).

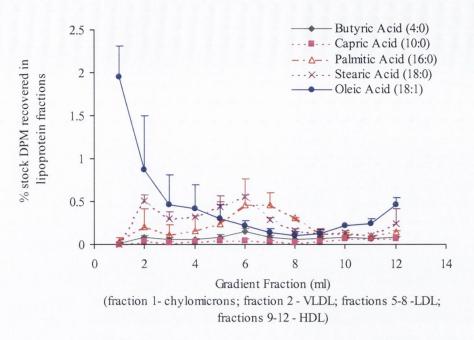


Figure 3.19: Secreted lipoprotein profile response to fatty acids of different chain length. Fatty acids were administered to cells at a concentration of 0.5mM. Data are expressed as the mean  $\pm$  SD (n=5 for oleic acid and 3 for remaining fatty acids).

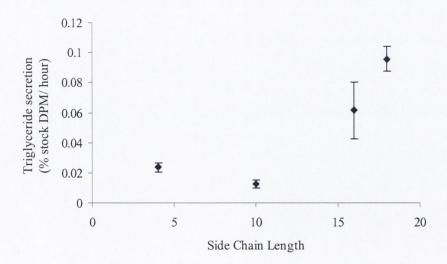
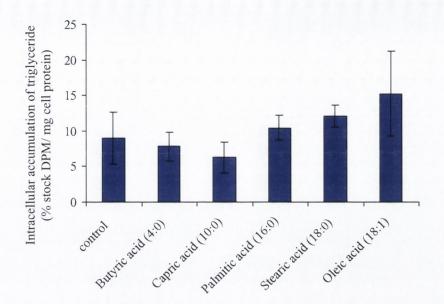


Figure 3.20: Correlation between saturated fatty acid chain length and triglyceride secretion. Fatty acids were administered to cells at a concentration of 0.5mM. Results are expressed as the mean  $\pm$  SD (n=5 for oleic acid and 3 for remaining fatty acids).

a)



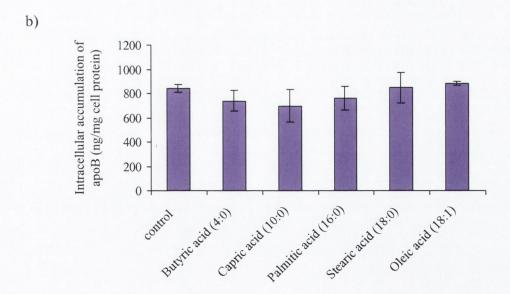


Figure 3.21: Effects of fatty acid chain length on intracellular accumulation of triglyceride (panel a) and apoB (panel b) in Caco-2 monolayers. Fatty acids were administered to cells at a concentration of 0.5mM. Values are expressed as the mean  $\pm$  SD (n=5 for oleic acid and 3 for remaining fatty acids).

#### 3.3.5.6 Inhibition of TRL secretion by verapamil

For the TRL study, cells were administered a 'fed-state' vehicle to the apical compartment and serum-free DMEM was placed in the basolateral chamber. Verapamil was added to the apical and the basolateral volume to a final concentration of 50µM as described by Field *et al.* (1995). The TRL secretion response in the presence of verapamil was compared against a positive 'fed-state' control.

Incubation of cells with  $50\mu M$  verapamil over 20 hours did not alter cell viability as measured via an MTT assay ( $92.0 \pm 5.8$ ) Similarly, overnight incubation with  $50\mu M$  verapamil did not significantly reduce monolayer resistance (TEER values  $96.3 \pm 5.3\%$  relative to starting value).

When verapamil ( $50\mu M$ ) was added to basolateral and apical medium in 'fed' stimulated Caco-2 cell monolayers, chylomicron and VLDL secretion levels were reduced by 50 and 35% respectively (p<0.01) (Figure 3.22). Verapamil at this concentration had no significant effects on the intracellular content of apoB, triglyceride or phospholipid (Figure 3.23).

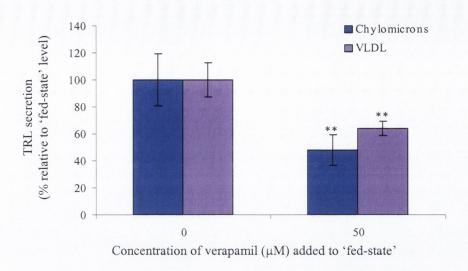


Figure 3.22: Effect of verapamil ( $50\mu M$ ) on the secretion of TRL in Caco-2 monolayers. Results are expressed as the mean  $\pm$  SD (n=4). Statistical significance of excipient effects relative to the 'fed-state' control is shown: \*\*p<0.01.

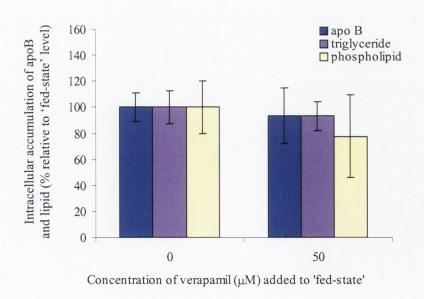


Figure 3.23: Effect of verapamil ( $50\mu M$ ) on the intracellular accumulation of apoB, triglyceride and phospholipid in 'fed' stimulated Caco-2 monolayers Results are expressed as the mean  $\pm$  SD (n=4).

#### 3.4 Key findings

Polycarbonate filters of 0.4µm pore were identified as being suitable for lipid metabolism studies as they facilitated formation of Caco-2 monolayers with good, predictable barrier properties and TRL secretion capacity. This pore size should allow>99% of generated chylomicrons to pass into basolateral medium.

Under the selected conditions for lipid metabolism studies, cells demonstrated reproducible lipoprotein secretion and metabolism profiles. Secreted lipoproteins were similar in composition to human plasma lipoproteins.

Cells were found to respond to oleic acid stimulus in a concentration dependent manner, showing an increase in triglyceride secretion capacity with increasing oleic acid concentration. A concentration of 0.5mM oleic acid was identified as being a suitable 'fed-state' vehicle for subsequent lipid metabolism studies as it produced a significant, yet sub-optimal, TRL secretion response.

Caco-2 cell response to a range of fatty acid vehicles was comparable to that previously reported for *in vivo* rat and *in vitro* Caco-2 studies- with high levels of chylomicron secretion in response to monounsaturated long chain fatty acid (oleic acid) and preferential secretion of LDL in response to saturated long chain fatty acid (stearic acid).

Barrier properties of cell monolayers, in terms of resistance to paracellular and transcellular permeants and P-gp efflux potential, were comparable to those cited in published literature. P-gp efflux and chylomicron secretion were both found to be sensitive to inhibition by verapamil.

#### 2.5 Discussion

Prior to commencing studies on lipid metabolism in Caco-2 cell monolayers, some preliminary optimisation work was conducted to ensure high levels of triglyceride and TRL secretion in this model for ease of detection. Cell monolayers were used when fully differentiated (day 19-22 post-seeding) as lipoprotein secretion has been shown to be maximal at this time (Mehran *et al.*, 1997). Initially, the filter on which cells were grown was a concern. The lab had previously established a Caco-2 model for studying monolayer permeability using the Costar Transwell<sup>TM</sup> system, with a 0.4μm pore, polycarbonate semi-permeable filter (Meaney, 1997). Cells of passage 30-50 were used routinely in these studies. However, triglyceride rich chylomicrons,

the principle triglyceride carrying lipoproteins in lymph, are quoted in literature as having an average diameter of 100-200 nm, with a broad range from 75-600nm (Haberbosch *et al.*, 1982; Bisgaier and Glickman, 1983; Hayashi *et al.*, 1990; Wasan and Cassidy, 1998), hence there was some concern over the ability of larger lipoproteins to pass through the filter easily. For this reason, a number of different filter pore sizes were evaluated for cell growth and lipoprotein secretion - 0.4µm, 1µm, 3µm pore sizes. As a result of commercial availability, the 0.4µm and 3µm pore filters employed were made of polycarbonate, and came from the Costar Transwell<sup>TM</sup> range, whilst the 1µm pore filter was made of polyethylene terephthalate (PET), and came from the Falcon® cell culture insert range.

#### Effect of filter type on monolayer formation and lipid metabolism

The pore size in the polycarbonate filters did not markedly affect Caco-2 monolayer resistance or permeability to passively absorbed markers (paracellular and transcellular), and hence did not appear to impact on physical barrier properties. However, cell monolayers grown on the 1µm pore PET filter had slightly, but significantly, higher TEER values and also showed a decrease in mannitol permeability as compared to the polycarbonate filters, indicating altered membrane properties.

The filter pore size had a significant impact on TRL secretion in response to 'fed-state' vehicle (0.5mM), with cells grown on the  $0.4\mu\text{m}$  pore filter demonstrating highest TRL levels, and the  $3\mu\text{m}$  pore filter producing considerably lower secretion levels than either the  $0.4\mu\text{m}$  or  $1\mu\text{m}$  pore filters.

Higher secretion levels of triglyceride would have been expected for the 1µm pore filter versus the 0.4µm pore filter, taking TRL diameter into consideration, but this was not observed. The reason for poor lipid metabolism in the cell monolayers grown on the 3µm pore filters may be due to migration of cells through the large pores, as demonstrated by Tucker *et al.* (1992). In the study by Tucker *et al.* (1992), cell migration through large pores was shown to produce poorly differentiated cell layers on the underside of the filter and to block pores. Cell migration into pores would explain the low TRL secretion levels. Morphology related changes in metabolism may explain the low cellular levels of triglyceride observed in the current study. As a reasonably small filter pore diameter was selected for this work, it was necessary to estimate what proportion of large chylomicrons were likely to be filtered out by the limited pore size. As an estimate, rat mesenteric lymph chylomicrons were sized by PCS and the average diameter (expressed as a Zave value) found to be approximately 126nm, with <1% of lymph

chylomicrons being > 400nm. These data indicate that the majority of chylomicrons should pass unrestricted through a  $0.4\mu m$  filter.

The lower TRL secretion levels observed for the 1µm pore filters may have been a result of filter support material. PET filters have been shown to encourage multiple cell layer formation (Rothen-Rutishauser *et al.*, 2000) and to impede cell differentiation (Behrans and Kissel, 2003). Altered morphology has been shown to result in loss of monolayer polarity (Braun *et al.*, 2000) and reduced permeability to paracellular permeants (Behrans and Kissel, 2003). Such reports are consistent with the experimental findings presented here, where monolayers grown on PET filters exhibited an increased monolayer resistance and reduced permeability to propranolol (20% decrease) and mannitol (44% decrease) relative to the 0.4µm pore polycarbonate filter. Altered morphology may interfere with TRL production. As the 1µm pore filter did not offer any clear advantage over the 0.4µm pore filter in terms of lipoprotein secretion, and as PET has been associated with multilayer formation, it was decided to continue employing the well-established 0.4µm pore polycarbonate filters for lipid studies.

#### Effect of passage number on monolayer formation and lipid metabolism

Cell passage number had a significant impact on secreted TRL levels. Presented data found that cells of higher passage (68-84) secreted considerably higher levels of chylomicrons and VLDL than cells of lower passage (30-50) in response to 'fed-state' vehicle. Secreted TRL levels at passage 68-84 were consistent with those obtained by van Greevenbroek et al. (1996) using cells of a similarly high passage range. These data highlight the importance of cell passage in lipid metabolism. The impact of passage number on Caco-2 metabolism has been discussed previously in relation to expression of intestinal transport proteins. For example, a study by Anderle et al. (1998) found that passaging of Caco-2 cells resulted in a decrease in expression of P-gp if cells were passaged after becoming confluent, and an increase in P-gp expression if passaged when 70-90% confluent. Such reports are consistent with our findings of increased TRL secretion with higher passage, as cells were passaged when approximately 70% confluent. In contrast, Hongshi et al. (1997) found that excessive passaging of cells (at 90% confluence) resulted in a reduction in the functional expression of brush border enzymes and transport As this observation was also associated with a reduction in morphological heterogeneity of the cell population, it was concluded that the passaging process selected fastgrowing, less metabolically active, Caco-2 sub-clones. Such contradictory findings may be a result of inter-laboratory variability, which impacts significantly on Caco-2 behaviour (Walter and Kissel, 1995; Braun et al., 2000), or as a result of the rather aggressive passaging process

adopted by Hongshi *et al* (1997) (cells trypsinised from passage 18 to 108) (Hongshi *et al.*, 1997).

#### Monolayer Characterisation

Permeability characteristics of the cell model were determined to ensure that barrier properties were in line with reported literature values for fully differentiated cell layers. Mannitol and fluorescein were used as paracellular markers to ensure tight junction formation and verapamil and dexamethasone as transcellular permeants. It must be noted that there is considerable interlaboratory variability in reported values for permeability and for active drug transport (Walter and Kissel, 1995; Braun *et al.*, 2000). As it stands, a number of variables have been implicated in the significant inter-laboratory differences observed. These include cell culture medium, cell passage number, cell source, sub-clone selection, filter support and age of cultured monolayers (Braun *et al.*, 2000; Wunderli-Allenspach, 2001). As a result, the experimental permeability values can only be compared against target ranges as opposed to specific values. In the case of trans and paracellular markers, monolayer permeability values were consistent with published data ranges for fully differentiated, intact Caco-2 monolayers (Table 3.7). As good correlation has been demonstrated between published Caco-2 and *in vivo* human intestinal permeability data (Lennernäs *et al.*, 1996; Lennernäs, 1997), these findings indicate that cell monolayers provide an effective model for the intestinal mucosal barrier.

With regard to drug efflux, the Papp ratios of drug substrates (taxol, vinblastine and cyclosporin A) obtained here indicate that drug efflux is operational and consistent with reported findings (Augustijns *et al.*, 1993; Ward *et al.*, 1999). The inhibitory effects of verapamil, a well-established P-gp inhibitor, on cyclosporin A efflux confirm the involvement of P-gp in cyclosporin A transport, and again are consistent with published data trends (Hunter *et al.*, 1993; Garrigos *et al.*, 1997). These findings confirm the formation of an intact and active model for predicting barrier properties of intestinal epithelium.

#### Validation of Caco-2 cells as a model for lipid metabolism

Further to this initial characterisation of barrier properties, the usefulness of the established Caco-2 model for predicting *in vivo* lipid response was examined and validated. Prior to commencing further studies, the concentration of oleic acid used in simulated 'fed-state' vehicle was optimised based on a dose-response relationship. Work presented up to this point used a 0.5mM oleic acid vehicle to simulate 'fed-state' based on previous reported studies, which employed concentrations of 0.5-1mM oleic acid (Field *et al.*, 1988; Dashti *et al.*, 1990; van Greevenbroek *et al.*, 1996; Luchoomun and Hussain, 1999). Administration of oleic acid at increasing concentrations to Caco-2 monolayers produced a concentration dependent increase in

chylomicron and triglyceride secretion relative to 'fasted-state' levels, which was significant (p<0.05) upwards from 0.1mM oleic acid. Intracellular triglyceride levels, showed an initial rise relative to control 'fasted-state', but remained constant from 1mM oleic acid onwards. This progressive increase in triglyceride secretion combined with relatively constant cellular triglyceride levels resulted in an increase in triglyceride secretion efficiency (amount of secreted versus cellular lipid). Effects on triglyceride secretion were more pronounced than on apoB or phospholipid secretion, indicating selective increase in the triglyceride loading of secreted The selective promotion of chylomicron secretion without a corresponding lipoproteins. accumulation of cellular triglyceride stores has previously been reported in vivo (Mansbach and Parthasarathy, 1982; Mansbach and Arnold, 1986) and is consistent with the steady state model for triglyceride delivery to the lymphatics proposed by Mansbach and Arnold (1986). Furthermore, the transfer of assembled lipoproteins from the endoplasmic reticulum (ER) to golgi has been identified as the rate limiting step in chylomicron secretion and as a potential site for up-regulation during feeding (Kumar and Mansbach, 1997; Kumar and Mansbach, 1999). The plateau in TRL secretion response to oleic acid dose was not associated with cell toxicity, but may reflect saturation of the lipid pathway in the cell. Published data have indicated a maximum lymphatic triglyceride secretion rate in a lymph cannulated rat model (Tso et al., 1982), and hence, confirm the presence of saturable steps in the pathway.

As a concentration of 0.5mM oleic acid produced an intermediate level of TRL secretion, whilst maintaining cell viability, it was deemed appropriate for further studies of negative or positive vehicle effects on TRL secretion.

#### In vitro-in vivo correlation

The differential Caco-2 cell response to 'fed-' or 'fasted-state' vehicles was investigated in more detail. The observation of promoted TRL secretion (accompanied by an increase in triglyceride, phospholipid, apoB output) and of increased intracellular triglyceride content following administration of a simulated 'fed-state' vehicle (0.5mM oleic acid) versus 'fasted-state' vehicle is consistent with published Caco-2 findings (Field *et al.*, 1988; Dashti *et al.*, 1990; van Greevenbroek *et al.*, 1996). A similar response has also been noted *in vivo* in the mesenteric lymph cannulated rat model (Hayashi *et al.*, 1990).

Looking more specifically at the observed lipid composition of the secreted lipoproteins, there are striking similarities with reported Caco-2 data (Dashti *et al.*, 1990; Mehran *et al.*, 1997), verifying that the established Caco-2 model in this work is capable of reproducing published findings. The lipid composition of secreted TRL from Caco-2 cells corresponds well with that of human lipoproteins of similar flotation density (as summarised in Table 3.9). The trend for

increasing cholesterol/ phospholipid content and decreasing triglyceride with higher density lipoproteins is also consistent with human profile. However, the Caco-2 model data tends to over-estimate the relative triglyceride content of higher density fractions, specifically, LDL and HDL. This may be a result of model differences. Caco-2 cells are isolated colon carcinoma cells and so may exhibit different lipoprotein profiles from small intestinal cells. Additionally, the human lipoproteins are isolated from plasma as opposed to lymph, and so may have lost triglyceride through tissue interactions. Differences may also arise as an artefact of the experimental technique employed — lipids from Caco-2 cells are quantified based on incorporation of a radiolabelled oleic acid tracer, which is preferentially incorporated into newly synthesised triglyceride in the cell (Mansbach and Parthasarathy, 1982), whereas, phospholipids and cholesterol may be derived from endogenous sources (Shen *et al.*, 2001), and hence, may not incorporated into newly absorbed fatty acid.

Once the 'fed' versus 'fasted' state vehicle responses had been determined, the ability of the cell model to distinguish between differential effects of a number of fatty acids on lipoprotein secretion was investigated. Short chain fatty acids, butyric acid (C4:0) and capric acid (C:10), produced no stimulation of lipoprotein secretion. In contrast, the long chain saturated fatty acids, palmitic (C16:0) and stearic (C18:0) acid stimulated secretion of LDL, and also, in the case of stearic acid, VLDL. Of the investigated fatty acids, only oleic acid (C18:1), a long chain monounsaturated fatty acid, promoted secretion of chylomicrons. These data are consistent with findings by van Greevenbroek et al. (1996) who found that fatty acids stimulated TRL secretion with a rank order of: oleic acid > linoleic acid > stearic acid> palmitic acid. Such findings are also in keeping with reported in vivo data. For example, Caliph et al. (2000) showed that lymphatic triglyceride output in a lymph cannulated rat model was increased with C18 rich peanut oil, and followed a decreasing trend with fatty acid chain length of: peanut oil > Captex (medium chain, C<sub>8</sub>-C<sub>10</sub>)> tributyrin (short chain, C<sub>4</sub>). Additionally, it has been shown, in the rat, that although long chain fatty acids are incorporated into lymphatic triglycerides, short chain fatty acids (typically <C12), are passively absorbed across intestinal cells and pass directly into the portal blood (Kiyasu et al., 1952). This is consistent with the absence of lipoprotein secretion following administration of butyric and capric acid to Caco-2 cells. The observation of significant chylomicron secretion with oleic, but not stearic acid, in this work is also supported by animal data. For example, electron microscopy studies of rat lymph by Feldman et al. (1983) showed that an increase in the proportion of saturated (palmitic acid, tripalmitin) versus unsaturated (C18:1 rich safflower oil) fatty acids in a lipid vehicle produced a progressive decrease in the size of intestinal lymph chylomicrons. Similarly, Kalogeris and Story, (1992) found that administration of a corn oil emulsion (rich in long chain unsaturated

fatty acids) produced considerably larger chylomicrons in rat lymph than administration of butter oil (high in saturated fatty acids).

#### Sensitivity of lipoprotein response to inhibition by verapamil

As the negative and positive influences of lipid vehicles on lipoprotein secretion are to be evaluated in this work, it is important to demonstrate that the model employed is sensitive to lipoprotein modulating compounds. The positive response to oleic acid supplementation has already been demonstrated. In order to ascertain sensitivity to inhibitors, the effects of verapamil on TRL secretion in 'fed' stimulated cells was evaluated. Verapamil, was selected because, in addition to its inhibitory effects on P-gp activity, it has been shown by Field et al. (1995) to effectively impair the basolateral secretion of triglyceride and TRL. Verapamil, at a concentration of 50µM produced 75% reduction in chylomicron secretion and 60% decrease in chylomicron secretion, without altering intracellular accumulation of triglyceride, phospholipid or apoB. In a similar model, Field et al. (1995) found significant inhibition of triglyceride and apoB secretion (95 and 75% respectively), accompanied by a small reduction in cellular triglyceride (18%), but not in cellular apoB following administration of 50µM verapamil with 0.25mM oleic acid. Hence our model followed a similar response, albeit moderately less sensitive, to that described by Field and co-workers. Differences in sensitivity may be a result of the different administered oleic acid doses (Field and co-workers administered 50% lower dose).

#### Efficiency and reproducibility of TRL secretion

The overall secretion efficiency of the investigated Caco-2 model was quite low, with only approximately 13% of cellular triglyceride being secreted basolaterally over 20 hours in 'fed-state' cells. Such secretion levels are consistent with published cell data (Dashti *et al.*, 1990) and are considerably lower than predicted efficiency of intestinal mucosa (Levy *et al.*, 1999; Mansbach and Parthasarathy, 1982). This low lipid transport efficiency means that although the model may be useful for making qualitative predictions of the *in vivo* response, the ability to make quantitative comparisons may be limited (Levy *et al.*, 1995).

As a final validation step, the reproducibility of lipid production and lipoprotein secretion was examined in the optimised Caco-2 model (high passage cells grown on 0.4µm pore polycarbonate filters). At a significance level of 0.05, values for intracellular lipid and apoB accumulation and secreted TRL were not found to differ over the investigated passage range. Caco-2 cells of passage 68-84 were found to produce superior levels of TRL secretion to cells of passage 30-50.

### **Chapter Four**

Inhibitory Effects of Pharmaceutical Excipients on Lipoprotein Processing in Caco-2 Monolayers

# 4 Inhibitory effects of pharmaceutical excipients on lipoprotein processing in Caco-2 monolayers

#### 4.1 Introduction

Lipid based formulations are being increasingly recognised as a means of improving the oral bioavailability of lipophilic drugs (Charman, 1998). Microemulsion and self-emulsifying formulations are of particular interest because of their ability to form fine oil-in-water dispersions on mixing with gastrointestinal fluids, thus solubilising high concentrations of lipophilic drugs and providing a higher effective drug concentration and interfacial area for absorption (Constantinides, 1995; Lawrence and Rees, 2000; Kawakami et al., 2002). Such formulations tend to be multicomponent, comprising an oil phase in combination with a blend of surfactants, co-surfactants and co-solvents (Constantinides, 1995; Gershanik and Benita, 2000; Lawrence and Rees, 2000). To date, vehicle formulation design has been largely empirical, with no clear guidelines on excipient selection or formulation composition. Consideration tends to be given to producing stable, welldispersed formulations with high drug solubility (Khoo et al., 1998; Kawakami et al., 2002). However, recent findings have demonstrated significant bio-modulating effects associated with many of the pharmaceutically acceptable excipients incorporated into such formulations, in particular with non-ionic surfactants. For example, Pluronic L81, a lipophilic member of the commonly employed Pluronic block copolymer surfactant family, has been shown to inhibit lymphatic triglyceride and chylomicron output in the rat model (Tso et al., 1981). This may have serious implications for the lymphatic uptake of lipophilic drugs which may be mediated by drug association with the chylomicron triglyceride pool (Counsell and Pohland, 1982; O'Driscoll, 2002). Similar inhibitory effects of Pluronic L81 on chylomicron output have also been shown in the Caco-2 model (Luchoomun and Hussain, 1999). In addition, a number of polyoxyethylated nonionic surfactants, including Cremophor EL, Triton X, Polysorbate 80, TPGS and Pluronic block copolymers have been shown to interfere with intestinal membrane fluidity and/ or to inhibit efflux activity of P-glycoprotein (P-gp) and associated anti-transport proteins (Woodcock et al., 1992; Nerurkar et al., 1996; Batrakova et al., 1999; Regev et al., 1999; Batrakova et al., 2001b; Rege et al., 2002). Such effects may significantly alter membrane barrier properties, hence modifying drug bioavailability (Regev et al., 1999; Rege et al., 2002).

Observed inhibition of P-gp by pharmaceutical excipients is of additional interest because reported findings, using verapamil as an inhibitor, have suggested an association between drug-mediated inhibition of TRL secretion and P-gp activity (Field *et al.*, 1995). As pharmacologically active drugs can simultaneously modulate both processes, it is conceivable that other agents, with demonstrated P-gp inhibitory activity, including pharmaceutical excipients, may also interfere with lymphatic drug uptake. As many lipophilic drugs are substrates for P-gp (Seelig, 1998) and are

also likely to be candidates for lipid mediated lymphatic uptake (Charman and Stella, 1986), the interacting and confounding effects of co-administered vehicle excipients on lipophilic drug bioavailability need to be considered at a preformulation stage.

#### 4.2 Aims and objectives

The previous chapter demonstrated the ability of Caco-2 cells to secrete TRL in a similar manner to the *in vivo* intestine. The Caco-2 TRL secretion response was found to be sensitive to modulation by various fatty acid vehicles. The current chapter aims to investigate the effects of a number of pharmaceutical non-ionic surfactants on lipoprotein synthesis and secretion using this model.

The dual effects of selected excipients on P-gp related drug efflux and on lipoprotein secretion will be evaluated to test the hypothesis of an association between these two processes.

As all of the investigated excipients are non-ionic surfactants comprising a hydrophilic polyoxyethylene side chain esterified to a hydrophobic moiety, the effects of PEG 400 (polyoxyethylene) on lipoprotein metabolism will also be evaluated to determine the importance of the hydrophilic side chain for excipient activity. PEG 400 has the added advantage, in this study, as being a proven inhibitor of P-gp, and so will further test the hypothesised relationship between P-gp and lipoprotein secretion.

#### Chemical structures of excipients

The chemical structures of the investigated pharmaceutical excipients are shown here. Additional characteristics are summarised in Table 4.2.

#### Pluronic block copolymers

HO 
$$\left\{CH_2CH_2O\right\}_X$$
  $\left\{CH_2CHO\right\}_Y$   $\left\{CH_3CH_2O\right\}_X$  EO

**Figure 4.1:** General structure of Pluronic block copolymers [( $\alpha$ -Hydro- $\omega$ -hydroxypoly(oxyethylene)poly(oxypropylene)poly(oxyethylene)] block copolymers, taken from Kabanov *et al.* (2002).

These consist of segments of ethylene oxide (EO) and propylene oxide (PO) arranged in an EO<sub>x</sub>-PO<sub>y</sub>-EO<sub>x</sub> arrangement, which results in an amphiphilic molecule whose molecular size and hydrophilic-lipophilic balance (HLB) depends on the number of hydrophilic EO and hydrophobic PO units in each segment. The specific EO and PO content for block copolymers used in this work is given in Table 4.1.

**Table 4.1:** Average number of EO and PO units in Pluronic block copolymers as calculated from molecular weight values by Batrakova *et al* (1999)

Copolymer	Average number of	Average number of	Average Mwt
	EO units (x)	PO units (y)	
L81	6.25	42.67	2500
P85	52.27	39.66	4500
F68	152.73	28.97	8800

#### PEG 400

$$HO \longrightarrow C \longrightarrow (CH_2 - O - CH_2)_m \longrightarrow C \longrightarrow OH$$

**Figure 4.2**: Structure of polyethylene glycol (PEG) 400, m=8.7 (average number of ethylene oxide units per molecule), taken from Price (2003).

Polyethylene glycol (PEG) is a water-soluble polymer of ethylene oxide and water. The number of repeating units (as shown in Figure 4.2) in a specific PEG molecule dictates its physical properties and its molecular weight. PEG 400 has an average of 8.7 repeating units and so is a liquid. It may be used as a solvent, co-solvent, suspending agent or emulsion stabiliser in oral lipid vehicles (Price, 2003).

#### **TPGS**

**Figure 4.3:** Structure of TPGS, Vitamin E-TPGS, n= 22.3 (average number of ethylene oxide units in per molecule of PEG 1000), taken from Wu and Hopkins (1999).

TPGS (d-alpha tocopheryl polyethylene glycol 1000 succinate) is a water-soluble derivative of vitamin E. The presence of a hydrophilic polyethylene glycol chain attached to a lipophilic tocopherol acid succinate group confers amphiphilic properties, making TPGS a useful solubilising and absorption enhancing agent (Wu and Hopkins, 1999).

#### Cremophor EL

**Figure 4.4:** Structure of glycerol polyethylene glycol ricinoleate (main constituent of Cremophor EL).

Cremophor EL (Polyoxyl 35 castor oil/ glycerol polyethylene glycol ricinoleate) is produced by reacting castor oil with ethylene oxide, and as such, does not have a single molecular component. It is largely comprised of glycerol esters of ricinoleic acid ethoxylated with 30-50 molecules of ethylene oxide (Figure 4.4) (Gunstone, 1992) and small amounts of macrogol ricinoleate with corresponding free glycols. Other components include polyethylene glycols, glycerol ethoxylates and residual castor oil. Cremophor EL composition is approximately 83% hydrophobic and 17% hydrophilic (Yu, 2003).

#### 4.3 Results

#### 4.3.1 CMC determination

In order to characterise surfactants, critical micelle concentration (CMC) measurements were made, as per Section 2.3.8 in 'fed-state' media or HBSS buffer at 37°C to simulate the experimental conditions employed throughout the cell work. Results are shown in Table 4.2 and representative plots are shown in Appendix XIII. A trend towards lower CMC values with increasing surfactant hydrophobicity was observed, with lowest values being obtained for Pluronic L81 and highest values for Pluronic F68. Values are consistent with published findings for aqueous surfactant solutions (Batrakova *et al.*, 1999).

#### 4.3.2 Excipient Toxicity

Initially, an IC<sub>50</sub> value (concentration of surfactant required to produce 50% inhibition of cellular dehydrogenase activity) was determined for each compound, using an MTT assay (as described in Section 2.3.4), in order to set a maximum test dose for lipid experiments. Looking initially at the Pluronic block copolymer findings, summarised in Table 4.2, it can be seen that the highly lipophilic Pluronic L81 exhibited the highest toxicity, with an IC<sub>50</sub> value of 0.02%w/v, which was 15-fold lower than that obtained for Pluronic P85 and >700-fold lower than that of Pluronic F68. The rank order for block copolymer toxicity was L81> P85> F68. This corresponds with an increase in copolymer hydrophilicity and hence may reflect the ability of surfactants to permeate the cell membrane. TPGS was found to be highly cytotoxic, with an IC<sub>50</sub> value of 0.05%w/v and Cremophor EL was similar to Pluronic P85, with an IC<sub>50</sub> value of approximately 0.5%w/v. These values were used to select excipient concentrations for further lipid studies.

**Table 4.2**: Summary of excipient characteristics – values are shown for excipient HLB, CMC, IC<sub>50</sub> and average molecular weight

••••••	HLB	CMC <sup>a</sup> (%w/v)	CMC <sup>b</sup> (%w/v)	IC <sub>50</sub> <sup>c</sup> (%w/v)	Average Mwt
	(literature)				
Pluronic L81	1-7 <sup>d</sup>	0.005	0.005	0.02	2500 <sup>d</sup>
Pluronic P85	12-18 <sup>d</sup>	0.03	N.D.	0.30	4500 <sup>d</sup>
Pluronic F68	>24 <sup>d</sup>	0.30	N.D.	14.30	$8800^{\rm d}$
Cremophor EL	12-14 <sup>d</sup>	0.01	0.02	0.53	2515 <sup>d</sup>
TPGS	15-19 <sup>d</sup>	0.01	N.D.	0.05	1513 <sup>d</sup>
PEG 400	N.A.	N.A.	N.A.	7.60	285-315 <sup>e</sup>

N.D. - not determined

N.A. - not applicable

- a. Determined in 'fed-state' media, at 37°C using a Du Nuoy tensiometer (Section 2.3.8)
- b. Determined in HBSS, at 37°C using a Du Nuoy tensiometer (Section 2.3.8)
- c. Determined via an MTT assay for intracellular dehydrogenase activity (Section 2.3.4)
- d. Technical bulletin supplied by manufacturer (BASF Corp)
- e. Handbook of Pharmaceutical Excipients (Price, 2003)

During the course of lipid experiments, TEER values were taken (as per Section 2.3.1) to monitor cell monolayer integrity following exposure to investigated systems. Dehydrogenase activity was additionally measured for the excipient concentrations employed in this work using an MTT assay (Section 2.3.4). Cell monolayers remained intact and cells viable for the majority of investigated excipient concentrations. In the case of Pluronic L81, monolayer resistance was found to decrease significantly at a test concentration of 0.012%w/v (27% decrease, p<0.01). This was accompanied by a 45% reduction in cellular dehydrogenase activity (p<0.01) (Table 4.3a). Pluronic F68 showed no significant drop in viability or monolayer integrity over the concentration range and Pluronic P85 showed only a reduction in TEER values (23% decrease, p<0.05) at the upper concentration of 0.06%w/v (p<0.05.). Cremophor EL produced significant cell toxicity at concentrations of 0.6 and 1%w/v, with substantial reductions in both monolayer integrity and dehydrogenase activity (45% decrease in dehydrogenase activity, p<0.01 and 20% decrease in monolayer resistance, p<0.05, at 0.06%w/v) (Table 4.3b). Cell viability and monolayer integrity with TPGS were maintained at up to 0.02%w/v and were substantially reduced at 0.2%w/v (76% decrease in dehydrogenase activity, p<0.01 and 60% decrease in monolayer resistance, p<0.01) (Table 4.3b). Cell viability and monolayer integrity were maintained at 5%w/v PEG 400 (Table 4.3b).

**Table 4.3:** The effects of Pluronic block copolymers (a) and Cremophor EL, TPGS and PEG 400 (b) on cell viability (measured using an MTT assay for cell dehydrogenase activity) and monolayer integrity (measured as TEER). Values represent the mean (SD) (n=5 for MTT and n=3-6 for TEER measurements). Statistical significance relative to positive control (MTT assay) or starting value (TEER measurements) is shown: \*\*p<0.01, \*p<0.05.

a)

b)

Test compound and concentration (%w/v)		MTT viability (% relative to control)	TEER measurements (% relative to start value)	
Pluronic L81				
	0.0008	102.7 (2.0)	106.9 (12.0)	
	0.003	107.7 (9.9)	105.9 (13.1)	
	0.006	104.5 (2.3)	97.4 (13.0)	
	0.012	73.1 (2.2)**	55.8 (15.2)**	
Pluronic P85				
	0.003	97.7 (7.6)	101.2 (13.6)	
	0.03	99.0 (2.0)	94.9 (5.0)	
	0.045	92.3 (5.1)	88.4 (7.9)	
	0.06	80.7 (8.6)*	89.7 (11.8)	
Pluronic F68				
	0.1	119.6 (15.6)	103.4 (8.5)	
	1	99.1 (2.9)	106.6 (3.5)	
	4	94.9 (2.3)	107.8 (6.1)	
	8	90.8 (9.0)	96.0 (6.1)	

Test compound and concentration (%w/v)		MTT viability (% relative to control)	TEER measurements (% relative to start value)	
Cremophor EL				
	0.01	109.9 (23.4)	90.3 (9.6)	
	0.025	94.8 (5.3)	100.1 (4.2)	
	0.1	109.2 (18.2)	96.9 (0.6)	
	0.25	93.6 (10.3)	92.8 (3.2)	
	0.6	55.2 (6.0)**	79.6 (4.2)*	
	1	14.9 (1.0)**	43.1 (9.0)**	
TPGS				
	0.0002	107.7 (7.6)	98.7 (2.3)	
	0.002	109.5 (6.7)	102.3 (4.6)	
	0.02	107.3 (4.1)	95.6 (3.5)	
	0. 2	23.3 (1.1)**	38.2 (6.8)**	
PEG 400	5	92.0 (8.4)	88.6 (10.1)	

#### 4.3.3 Excipient effects on lipid metabolism

The effects of a range of pharmaceutical excipients on lipid metabolism were investigated in the Caco-2 model. Excipients were co-administered with 'fed-state' vehicle to evaluate their effects on 'fed' stimulated cells. 'Fed state' vehicle was prepared in serum-free culture medium at a 2X concentration as described in Section 2.3.2. This vehicle was subsequently diluted 1 in 2 with either serum-free culture medium (for control systems) or in solutions of test excipient (prepared in serum-free medium) to produce vehicles containing 0.5mM oleic acid, 0.125mM BSA and the relevant concentration of excipient. Vehicles were labelled with trace [ $^{14}$ C] oleic acid ( $^{0.2}\mu$ Ci/ml). 'Fasted-state' vehicle, comprising 0.125mM BSA and labelled with trace [ $^{14}$ C] oleic acid, was prepared as in Section 2.3.2.

Test vehicles were administered to fully differentiated monolayers and samples were analysed as described in Section 2.3.2. Test vehicle data were compared with and expressed relative to 'fed-state' control. Analyses were conducted to determine lipid and apoB content of cell lysates and basolateral samples and to characterise the secreted lipoprotein density profile, as per Section 2.3.2.

#### 4.3.3.1 Effects of Pluronic block copolymers on lipoprotein secretion

In the absence of any additional excipients, administration of 'fed-state' vehicle to fully differentiated Caco-2 cells specifically promoted the secretion of TRL (chylomicrons and VLDL) (Section 3.3.5.3). Co-administration of Pluronic L81 with 'fed-state' produced a concentration dependent decrease of chylomicron (Figure 4.5a) and, to a lesser extent, of VLDL (Figure 4.5b) secretion as compared to a 'fed-state' control. Chylomicron secretion was significantly impaired upwards from 0.006%w/v Pluronic L81, with approximately 60% reduction at 0.006%w/v (p<0.05) and >90% reduction at 0.012%w/v (p<0.01) (Figure 4.5a). Inhibition of VLDL secretion was only observed at 0.012%w/v (Figure 4.5b), but as cytotoxicity was also observed at this concentration (Table 4.3a), the results may be attributable, in part to cell condition.

Pluronic P85 was much less potent as an inhibitor of TRL secretion, with decreased chylomicron secretion occurring from 0.03%w/v (p<0.05). As observed with Pluronic L81, chylomicron secretion was more sensitive to the inhibitory effects of Pluronic P85 than VLDL secretion. For example, selective inhibition of chylomicron secretion occurred at 0.03%w/v (Figure 4.5a). Reduced VLDL secretion was observed at 0.045% and 0.06%w/v Pluronic P85 (Figure 4.5b), and was not associated with any loss in cell viability or monolayer integrity (Table 4.3a). Pluronic F68 produced no inhibition of TRL secretion at concentrations of up to 1%w/v (Figures 4.5a,b), but did impair both chylomicron and VLDL secretion at 8%w/v (p<0.05), with selective inhibition of chylomicrons at 4%w/v (Figure 4.5a,b).

LDL secretion was partially reduced in the presence of the highest investigated concentrations of Pluronic L81 (55% reduction at 0.012%w/v, p<0.01) and Pluronic P85 (45% reduction at 0.06%w/v, p<0.05), but not Pluronic F68 (Figure 4.5c). HDL secretion was unaffected by Pluronic block copolymers at tested concentrations (Figures 4.5d).

Values for total basolateral secretion of lipids and apoB support that obtained using density gradient ultracentrifugation. The level of triglyceride secretion (Figure 4.6a) in the presence of increasing concentrations of Pluronic L81 followed a similar trend to that of chylomicron secretion (Figure 4.5a) with a concentration dependent reduction upwards from 0.006%w/v (p<0.01). Pluronic P85 presented a similar profile, with significant concentration dependent decreases in triglyceride secretion upwards from 0.03%w/v (Figure 4.6b). Pluronic F68, the least potent inhibitor, produced decreases in triglyceride secretion (40%, p<0.05 and 45%, p<0.01 respectively) at the higher concentrations of 4% and 8% (w/v) (Figure 4.6c). In general, secreted phospholipid data followed a similar trend to that obtained for triglyceride.

In all cases, apoB secretion was less sensitive than triglyceride secretion to the inhibitory effects of applied excipients (Figures 4.6a-c). This resulted in a general trend towards a reduction in triglyceride: apoB ratio. Again indicating preferential inhibition of TRL.

#### 4.3.3.2 Effects of Pluronic L81 on fatty acid uptake

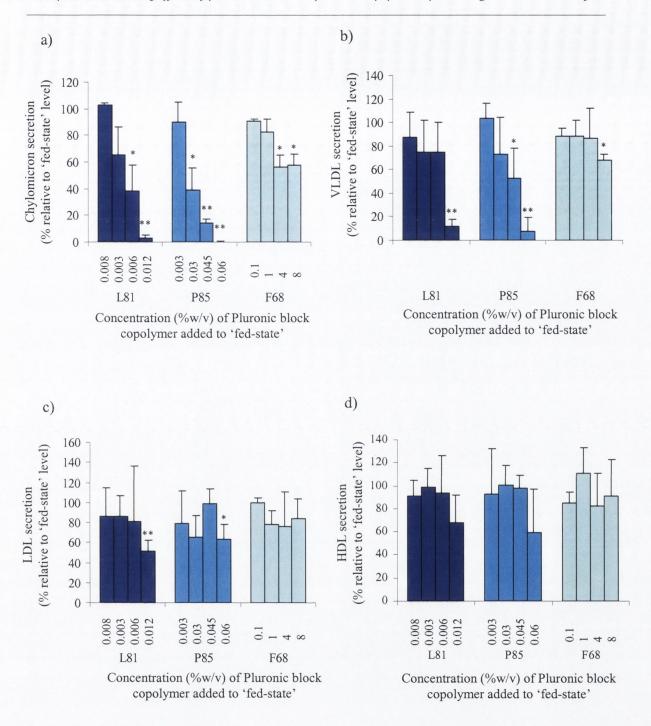
Lipoprotein metabolism consists of a number of steps, commencing with fatty acid uptake. Although fatty acids are passively absorbed by Caco-2 cells, it has also been proposed that active uptake, mediated by a membrane fatty acid binding protein, may be a contributing factor (Stremmel, 1988; Trotter *et al.*, 1996). Surfactant agents interfere with membrane fluidity (Regev *et al.*, 1999), and hence have the potential to interfere with transporter-mediated and passive absorption of fatty acids. To investigate if the observed inhibitory effects of Pluronic L81 on lipoprotein secretion were mediated by inhibition of this initial step, the effects of Pluronic L81on fatty acid uptake were assessed.

The initial rate of fatty acid uptake was monitored as described in Section 2.3.7. Experiments were initiated by administration of [³H] oleic acid to cell monolayers. Uptake was measured by quantifying fatty acid content of cells after 5 minutes and 1 hour exposure. Pluronic L81 (0.006%w/v) was added to the fatty acid uptake vehicle for the test system. Fatty acid uptake was also estimated following 20-hour exposure as part of the lipid metabolism studies (Section 2.3.2) by measuring total intracellular and secreted [¹⁴C] oleic acid content in aliquots of cell lysate and basolateral medium.

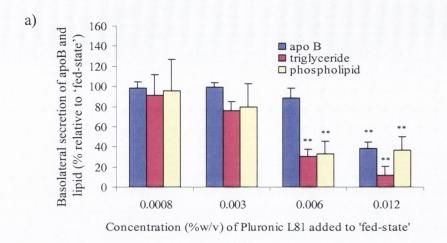
Looking at the data presented in Figure 4.7, it can be seen that Pluronic L81 had no statistically significant effects on fatty acid uptake at any of the time intervals tested.

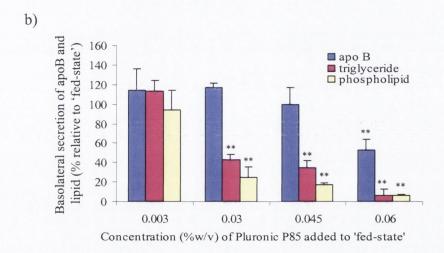
## 4.3.3.3 Effects of Pluronic block copolymers on intracellular accumulation of lipid and apoB

Intracellular levels of lipid and apoB were not significantly affected over the range of investigated concentrations for any of the investigated Pluronic block copolymers (Figures 4.8a-c), even when limited cell viability was observed (i.e. 0.012%w/v Pluronic L81). This constant level of triglyceride production in the cell, in combination with a concentration dependent decrease in triglyceride secretion, means that there is an accompanying decrease in the ratio of secreted to intracellular triglyceride over the evaluated range. This was the same for all surfactants investigated, albeit over different concentration ranges. This reduction in secretion efficiency is demonstrated in Figure 4.9 for Pluronic L81.



**Figure 4.5:** The effects of Pluronic block copolymer addition on the secretion of lipoproteins in 'fed' stimulated Caco-2 cell monolayers. Data are shown for chylomicron (panel a), VLDL (panel b), LDL (panel c) and HDL (panel d) secretion. Values represent the mean  $\pm$  SD (n=3-6). Statistical significance relative to 'fed-state' control is shown: \*p<0.05, \*\*p<0.01.





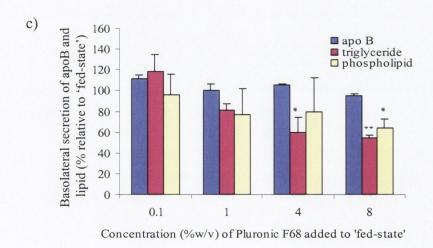


Figure 4.6: Effects of Pluronic block copolymer addition on the basolateral secretion of apoB, triglyceride and phospholipid in 'fed' stimulated Caco-2 cell monolayers. Data are shown for Pluronic L81 (panel a), Pluronic P85 (panel b) and Pluronic F68 (panel c). Values represent the mean  $\pm$  SD (n=3-6). Statistical significance relative to 'fed-state' control is shown: \*p<0.05, \*\*p<0.01.

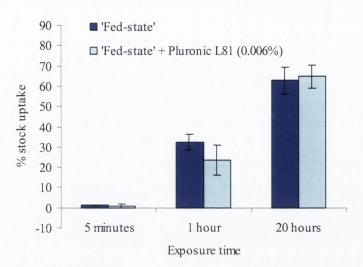
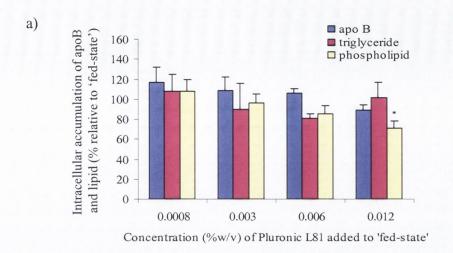
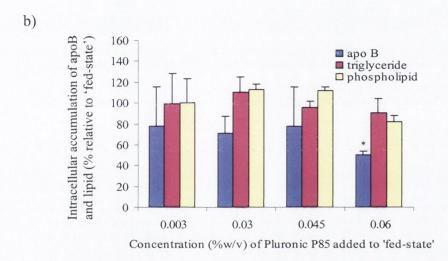


Figure 4.7: Effect of Pluronic L81 (0.006% w/v) on fatty acid uptake in 'fed' stimulated Caco-2 cells. 'Fed-state' control (no excipients) was applied for comparison. Values represent the mean  $\pm$  SD (n=4).





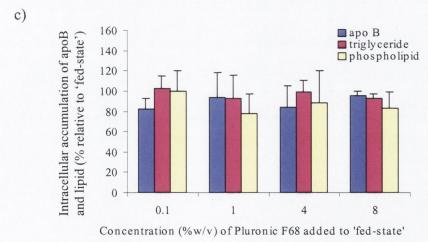


Figure 4.8: Effects of Pluronic block copolymer addition on the intracellular accumulation of apoB, triglyceride and phospholipid in 'fed' stimulated Caco-2 cell monolayers. Data are shown for Pluronic L81 (panel a), Pluronic P85 (panel b) and Pluronic F68 (panel c). Values represent the mean  $\pm$  SD (n=3-6). Statistical significance relative to 'fed-state' control is shown: \*p<0.05.

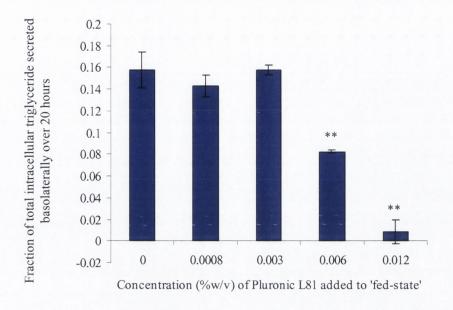


Figure 4.9: Effect of Pluronic L81 addition on the secretion efficiency of triglyceride in Caco-2 monolayers expressed as the total amount of triglyceride secreted over 20 hours as a proportion of the total amount of intracellular triglyceride accumulated over the same period. Values represent the mean  $\pm$  SD (n=3-6), \*\*p<0.01.

#### 4.3.3.4 Effects of Cremophor EL, TPGS and PEG 400 on lipoprotein secretion

As observed with Pluronic surfactants, co-administration of Cremophor EL or TPGS with 'fed-state' produced a concentration dependent decrease in the secretion of TRL, with more pronounced effects on chylomicron, as opposed to VLDL, secretion. Cremophor EL produced significant inhibition of chylomicron secretion from 0.06%w/v (p<0.01) onwards, with complete blockade at 0.25%w/v (Figure 4.10a). Inhibition of VLDL secretion was observed at 0.25%w/v (Figure 4.10b) and was not associated with cell toxicity (Table 4.3b). TPGS presented a similar profile in terms of chylomicron secretion, with 60% inhibition of chylomicron secretion being seen at 0.02%w/v (p<0.01) and complete blockade at 0.2%w/v (Figure 4.10a). In contrast to the other investigated surfactants, inhibition of VLDL secretion was particularly pronounced with TPGS and commenced at the same concentration as the chylomicron response (0.02%w/v) (Figure 4.10b). Cell toxicity was also quite pronounced in the presence of TPGS, with an 80% reduction in viability at 0.2%w/v (Table 4.3b). Effects of Cremophor EL and TPGS on secretion of LDL and HDL secretion were negligible (Figures 4.10c,d), with reduced LDL secretion only being observed at 0.2%w/v TPGS, and which was most likely a result of cytotoxicity.

As all of the investigated excipients contain a polyoxyethylene component, the effects of PEG 400, a commonly used co-solvent on TRL secretion were investigated. PEG 400 has exhibited P-gp inhibitory activity (Johnson *et al.*, 2002), and as such, is of particular interest. PEG 400 was applied to cells at a single concentration of 5%w/v. Chylomicron secretion was reduced by approximately 45% (Figure 4.10a), but secretion levels of VLDL, LDL and HDL remained unchanged at this concentration (Figures 4.10b-d). Cell viability was not affected by this concentration of PEG 400 (Table 4.3b).

Once again, secreted triglyceride and apoB levels were used to support data obtained from density gradient separation. As seen with Pluronic block copolymer data, addition of Cremophor EL to 'fed-state' vehicle produced a concentration dependent decrease in the secreted triglyceride to apoB ratio (Figure 4.11a), which is consistent with the observed reduction in chylomicron secretion. Significant decreases in triglyceride secretion were noted for concentrations of 0.06%w/v Cremophor EL and above. Data for TPGS differed in that there was a corresponding decrease in both apoB and triglyceride secretion, such that the ratio of triglyceride to apoB remained relatively constant (Figure 4.11b). Significant inhibition of both triglyceride and apoB secretion was seen at 0.02%w/v, which would support the observed decrease in secretion of both chylomicron and VLDL density fractions at this concentration. With regard to PEG 400, inhibition of triglyceride secretion at 5%w/v was substantial (>60%, p<0.01), whilst apoB secretion remained constant (Figure 4.11c).

#### 4.3.3.5 Effects of Cremophor EL on fatty acid uptake

The effects of Cremophor EL (0.1%w/v) on fatty acid uptake were assessed as described above for Pluronic L81 (Section 4.3.3.2). Data presented in Figure 4.12 show that, in common with Pluronic L81, Cremophor EL did not impair fatty acid uptake by Caco-2 cells at either the initial stages of application (5 minutes and 1 hour) or following a 20-hour incubation. The negative effects of Cremophor EL on lipoprotein output were therefore not a result of impaired fatty acid uptake by cells.

## 4.3.3.6 Effects of Cremophor EL, TPGS and PEG 400 on intracellular accumulation of lipid and apoB

With the exception of TPGS at 0.2%w/v, there were no appreciable changes in the intracellular levels of apoB or triglyceride over the investigated concentration range for Cremophor EL, TPGS or PEG 400 (Figures 4.13a-c). The reduced intracellular triglyceride content with TPGS at 0.2%w/v was associated with significant reduction in cell viability, and so may be a result of cell condition. As with the Pluronic surfactants, the constant level of intracellular triglyceride paired with reduced secretion levels, resulted in a decrease in secretion efficiency. This is demonstrated more clearly for Cremophor EL in Figure 4.14.

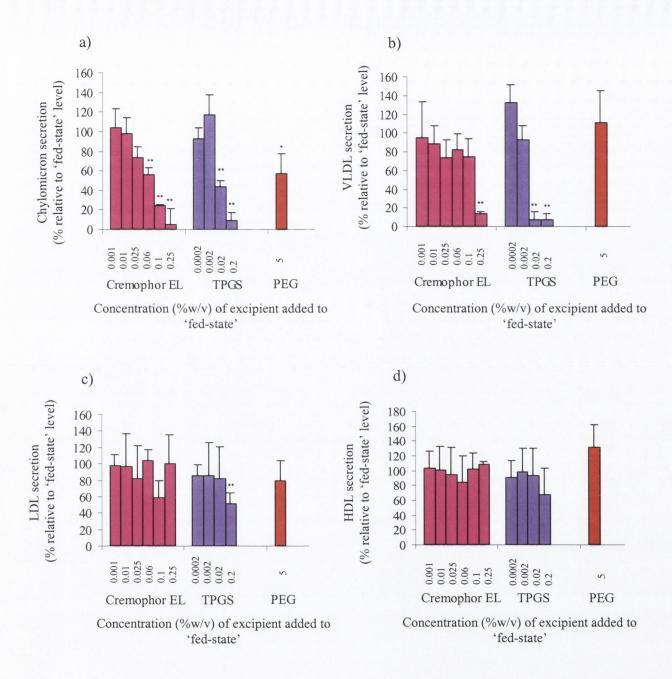


Figure 4.10: Effects of excipient addition on the basolateral secretion of lipoproteins in 'fed' stimulated Caco-2 cell monolayers. Data are shown for chylomicron (panel a), VLDL (panel b), LDL (panel c) and HDL (panel d) secretion. Values represent the mean  $\pm$  SD (n=3-6). Statistical significance relative to 'fed-state' control is shown: \*p<0.05, \*\*p<0.01.

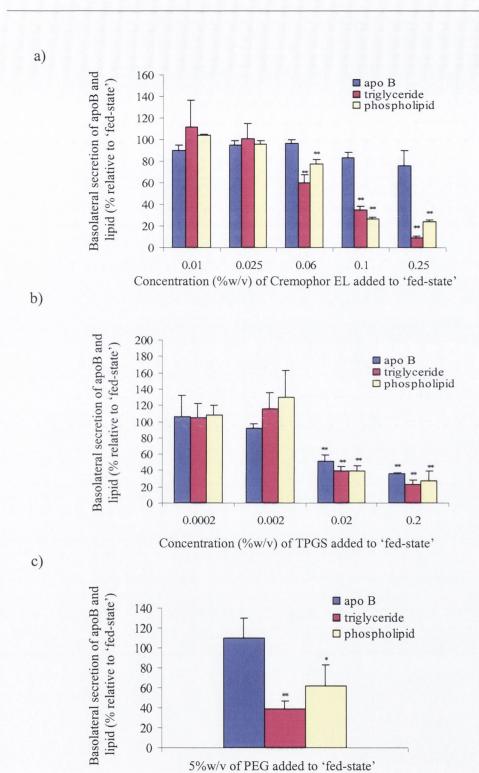


Figure 4.11: Effects of excipient addition on the basolateral secretion of apoB, triglyceride and phospholipid in 'fed' stimulated Caco-2 cell monolayers. Data are shown for Cremophor EL (panel a), TPGS (panel b) and PEG 400 (panel c). Values represent the mean  $\pm$  SD (n=3-6). Statistical significance relative to 'fed-state' control is shown: \*p<0.05, \*\*p<0.01.

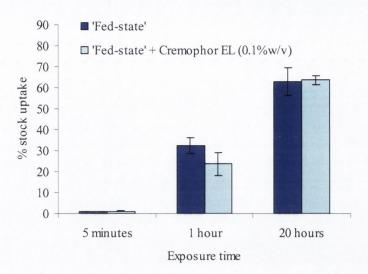
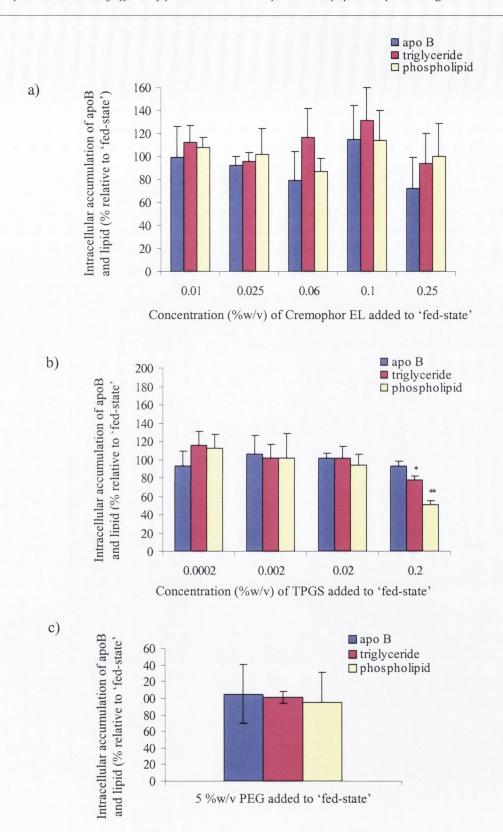


Figure 4.12: Effect of Cremophor EL (0.1% w/v) on fatty acid uptake in 'fed' stimulated Caco-2 cells. 'Fed-state' control (no excipients) was applied for comparison. Values represent the mean  $\pm$  SD (n=4).



**Figure 4.13:** Effects of excipient addition on the intracellular accumulation of apoB, triglyceride and phospholipid in 'fed' stimulated Caco-2 cell monolayers. Data are shown for Cremophor EL (panel a), TPGS (panel b) and PEG 400 (panel c). Values represent the mean  $\pm$  S.D. (n=3-6). Statistical significance relative to 'fed-state' control is shown: \*p<0.05, \*\*p<0.01.

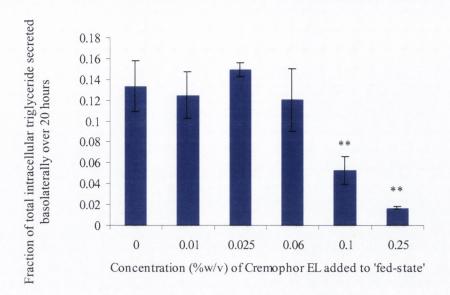


Figure 4.14: Effect of Cremophor EL addition on the secretion efficiency of triglyceride in Caco-2 monolayers expressed as the total amount of triglyceride secreted over 20 hours as a proportion of the total amount of intracellular triglyceride accumulated over the same period. Values represent the mean  $\pm$  SD (n=3-6), \*\*p<0.01.

#### 4.3.4 Effects of Pluronic L81 and Cremophor EL on drug efflux

One of the aims of this work is to investigate further the potential involvement of ABC transporter family proteins in lipoprotein metabolism as first hypothesised by Field *et al.* (1995). Work by this group found that verapamil, trifluoperazine and progesterone, all well recognised inhibitors of P-gp, also inhibited secretion of triglyceride and apoB in Caco-2 cells. Work presented above (Section 4.3.3) has shown the negative effects that non-ionic surfactants, including Cremophor EL and Pluronic L81, have on TRL secretion. To investigate a possible link between inhibition of P-gp and TRL secretion for such excipients, the effects of Cremophor EL and Pluronic L81 on P-gp activity, at concentrations corresponding to those used in the lipid studies, were investigated. P-gp activity was assessed, as described in Section 2.3.5 using bi-directional transport studies with cyclosporin A (CsA) as a substrate. Experiments were conducted on mature Caco-2 cell monolayers grown on 12mm diameter Transwell<sup>TM</sup> filters with 0.4µm pores. Excipients were added to the apical medium for studies of drug influx (apical-basolateral drug transport) or drug efflux (basolateral-apical drug transport). Data obtained for drug efflux were plotted against results obtained in Section 4.3.3 for excipient effects on chylomicron secretion to investigate any correlation.

With increasing concentrations of Pluronic L81, P-gp mediated efflux (basolateral to apical movement) of CsA was found to decrease in parallel with inhibition of chylomicron secretion (Figure 4.15a). Below the CMC, at 0.003%w/v, the inhibition of efflux was significant (p<0.05), a decrease in chylomicron secretion was also observed, however this was not significant due to higher variability. At concentrations of 0.006%w/v and above, Pluronic L81 produced significant inhibition of both chylomicron secretion and efflux activity (p<0.05).

Cremophor EL data followed a similar trend towards decreasing chylomicron secretion and CsA efflux with increasing concentrations. Inhibition was initiated at higher concentrations than with Pluronic L81. At 0.025%w/v, Cremophor EL produced significant inhibition of CsA efflux (p<0.05), without a corresponding decrease in chylomicron secretion (Figure 4.15b). However, when the concentration was increased further (>0.025%w/v) Cremophor EL induced parallel inhibition of both P-gp activity and chylomicron secretion. CsA influx (apical to basolateral movement) initially increased with increasing concentrations of both surfactants, due to reduced drug efflux. However, at concentrations approaching the surfactant CMC (0.005%w/v for Pluronic L81 and 0.2%w/v for Cremophor EL), the influx of CsA decreased again. This effect was particularly evident with Pluronic L81 (Figure 4.15a), and maybe due to a reduction in free CsA concentration following micelle formation and solubilisation.

Correlation plots of chylomicron secretion against drug efflux (Papp, B-A) or net drug efflux (1-[Papp  $_{A-B}$ /Papp  $_{B-A}$ ]) are shown for Pluronic L81 and Cremophor EL (Figures 4.16 a,b). Both plots show a good relationship between drug efflux and chylomicron secretion in the presence of increasing concentrations of surfactants, with correlation being particularly high for Pluronic L81 ( $r^2$ =0.9565).

[14C] Mannitol was included in each transport experiment as a paracellular marker for monolayer integrity. In contrast to CsA data, mannitol did not undergo net efflux across monolayers, thus confirming that it is not a substrate for P-gp (Figure 4.17). Administration of Cremophor EL and Pluronic L81 to cells had no significant effects on mannitol transport in either the apical-basolateral or the basolateral-apical directions within the investigated concentration range, verifying that observed results for CsA were not due to effects on membrane permeability. At concentrations where cell monolayer toxicity was observed (as indicated by cellular dehydrogenase activity and TEER values presented in Table 4.3) – i.e. at 0.25 and 1%w/v Cremophor EL and at 0.012%w/v Pluronic L81, mannitol permeability was increased (up to 1.5-fold for Cremophor EL at 1%w/v and up to 1.4-fold for Pluronic L81) relative to the control (mannitol transport in the absence of added excipients), but the differences in values were not statistically significant.

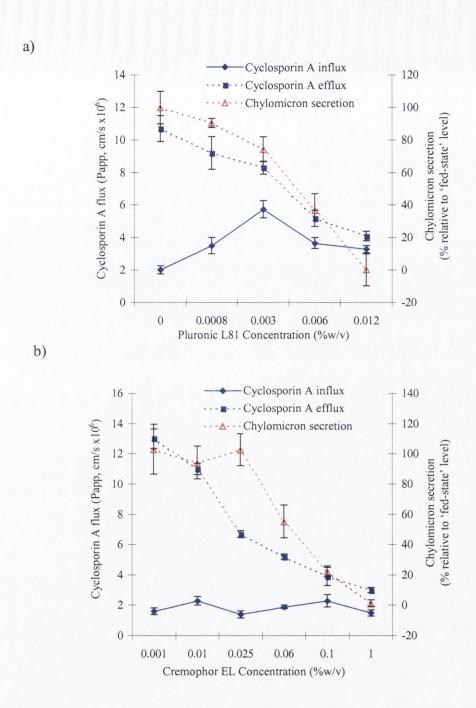
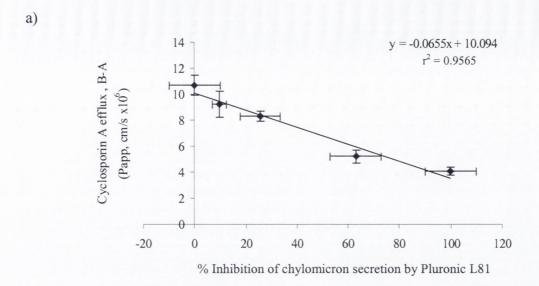
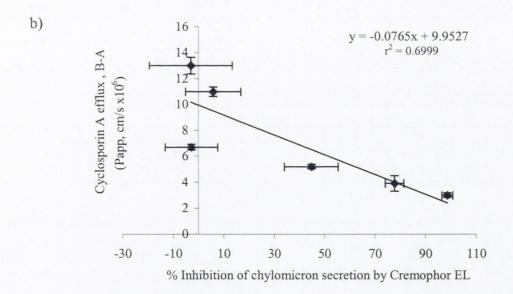


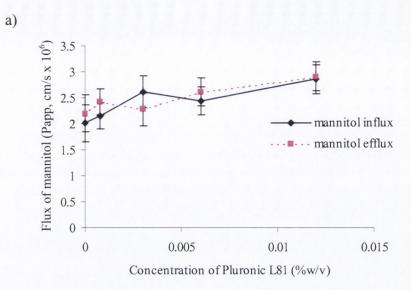
Figure 4.15: Comparison of the inhibitory effects of various concentrations of Pluronic L81 (panel a) and Cremophor EL (panel b) on cyclosporin A flux (apical-basolateral transport and basolateral to apical transport) and chylomicron secretion. Values represent the mean  $\pm$ SD (n=3-4).

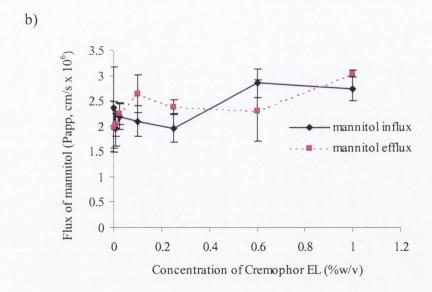
Please note – the shown lines are only added to help distinguish between the three series of data shown in each graph – the x-axes are not to scale, hence, the reader needs to take these factors into consideration when interpreting data.





**Figure 4.16:** Correlation between inhibition of chylomicron secretion and efflux of cyclosporin A in the presence of various concentrations of Pluronic L81 (panel a) and Cremophor EL (panel b). Values represent the mean  $\pm$  SD (n=3-4).



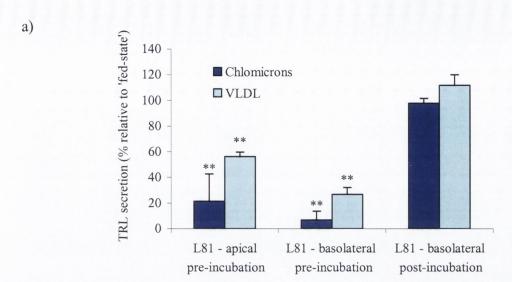


**Figure 4.17:** Mannitol flux (apical-basolateral transport and basolateral to apical transport) across Caco-2 monolayers in the presence of various concentrations of Pluronic L81 (panel a) and Cremophor EL (panel b). Values represent the mean ±SD (n=3-4).

#### 4.3.5 Basolateral application of Pluronic L81 and Cremophor EL

In the above lipid metabolism studies, excipients were all added to the apical surface of cell monolayers. An additional experiment was conducted to see if the site of excipient exposure was important for its effects. As above, 'fed-state' vehicle was applied to the apical surface of Caco-2 cell monolayers, but this time, excipients were added to the basolateral well with the receiving serum-free culture medium. Cell monolayers were incubated for 20 hours and samples processed as for standard lipid experiments, as described in Section 2.3.2. Results were again compared to a 'fed-state' control with no excipient added. There was a concern that surfactants added to the basolateral medium may interfere with flotation density of lipoproteins and hence interfere with lipoprotein separation during density gradient ultracentrifugation. To ensure that this was not an issue, a 'fed-state' control vehicle was applied to cell monolayers in the absence of any excipients and the basolateral media collected. Basolateral media from 6 monolayers were pooled and divided into six aliquots. Three aliquots were spiked with test excipient and the remaining three reserved as controls. Samples were subject to density gradient ultracentrifugation and the results compared.

When Pluronic L81 (0.006%w/v) and Cremophor EL (0.1%w/v) were applied to the basolateral surface of 'fed' stimulated Caco-2 monolayers (presented in the receiving basolateral volume), inhibition of TRL secretion was observed. Inhibition was more pronounced than that observed following apical application of excipients with 'fed-state' vehicle (Figures 4.18 a,b). Looking at the data for 'fed-state' basolateral samples spiked with excipients (Figures 4.18 a,b - 'post-incubation' data) it is clear that the test surfactants had no significant effects on the distribution of TRL within the density gradient during ultracentrifugation



Site of Pluronic L81 (0.006%w/v) application

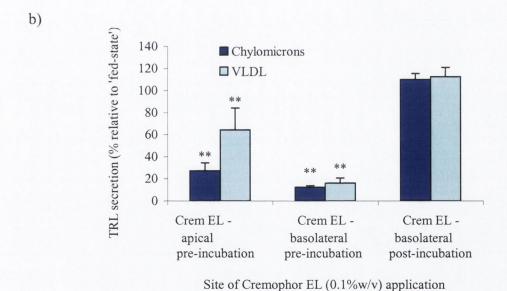


Figure 4.18: Effects of basolateral administration of Pluronic L81 (0.006%w/v) (panel a) and Cremophor EL (0.1%w/v) (panel b) on TRL secretion in 'fed' stimulated cells. Data are shown for excipients added to cells during exposure to 'fed-state' vehicle (preincubation) and excipients added to media collected from 'fed-state' cells (post-incubation). Values represent the mean  $\pm$  SD (n=4). Significance relative to 'fed-state' control is shown: \*\*p<0.01.

# 4.3.6 Effect of ATP supplementation on TRL secretion in the presence of Pluronic L81 and Cremophor EL

It has been proposed that the inhibitory effects of Pluronic P85 against P-gp efflux activity are mediated, at least in part, by an intracellular ATP depletion mechanism. This theory was put forward by Batrakova *et al.* (2001a) following the observation of a parallel decrease in intracellular ATP levels and inhibition of P-gp efflux in the BBMEC (bovine brain microvessel endothelial cell) cell line in the presence of Pluronic P85. As ABC transporter proteins are highly energy dependent, an effect on ATP levels would have a negative impact on active drug efflux. In order to establish whether this ATP depleting mechanism was applicable to TRL secretion in Caco-2 cells, supplementary ATP was added to inhibited systems, where cells were exposed to 'fed-state' vehicles with Pluronic L81 (0.006%w/v) or Cremophor EL (0.1%w/v), to see if it could reverse the observed inhibitory effects. Methodology was based on that used by Batrakova *et al.* (2001b) to study the effects of ATP supplementation on P-gp efflux in BBMEC cells. Caco-2 monolayers were incubated either with standard vehicles ('fed-state' control or 'fed-state' containing excipients, prepared as in Section 2.3.2) or standard vehicles supplemented with ATP (200μM) and dodecylamine (20μM) (as a cell permeabilising agent to assist uptake of ATP).

Co-administration of 50 or 100µM ATP with vehicles did not reduce the inhibitory effects of Pluronic L81 or Cremophor EL on TRL secretion (Figure 4.19 a). Additionally, co-administration of ATP with 'fed-state' vehicle did not alter secreted TRL levels as compared to levels obtained with 'fed-state' control (with no additional ATP). Additional ATP did not alter the intracellular levels of triglyceride or apoB in any of the investigated systems (Figure 4.19 b).

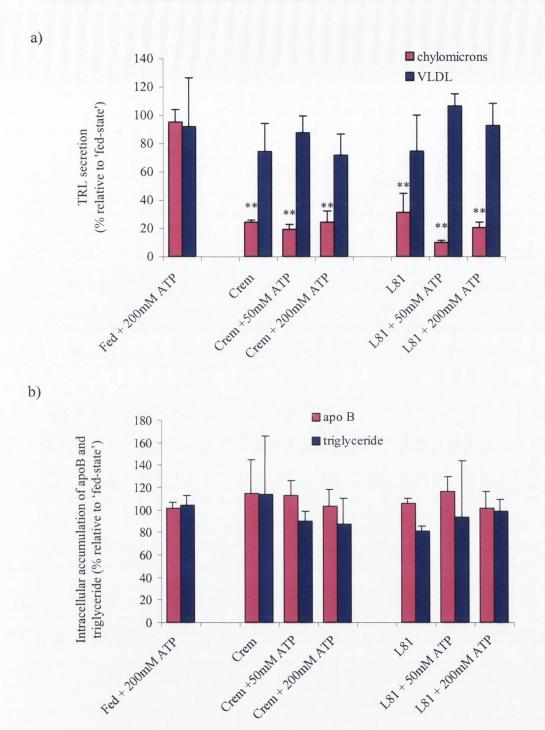


Figure 4.19: Effects of supplementary ATP on the response of 'fed' stimulated Caco-2 cells to Pluronic L81 and Cremophor EL. Results are shown for TRL secretion (panel a) and intracellular accumulation of triglyceride and apoB (panel b). Values represent the mean  $\pm$  SD (n=4). Statistical significance relative to 'fed-state' control is shown: \*p<0.05, \*\*p<0.01.

#### 4.3.7 Recovery of cells from the inhibitory effects of excipients

#### 4.3.7.1 TRL secretion recovery following wash-out

Tso et al. (1982) showed, in a lymph cannulated rat model, that the inhibitory effects of Pluronic L81 on TRL secretion (in the presence of an oleate rich vehicle) were acute and that excipient withdrawal resulted in a sudden burst release of triglyceride into lymph, taken by the authors to represent the maximum intestinal triglyceride output rate. The Caco-2 response to excipient withdrawal under 'fed' stimulated, conditions was examined to see if it was similar to the rat data. 'Fed-state' vehicles with or without Pluronic L81 (0.006%w/v) were applied to equilibrated monolayers as per Section 2.3.2 and basolateral samples assayed for triglyceride content every 2.5 hours for 10 hours and then at the 24-hour time point. After this time, cells were rinsed three times with serum-free medium and incubated with plain serum-free medium for a further 20 hours. Again, samples were taken at 2.5 hour intervals for 10 hours and then at the 24-hour time point to monitor triglyceride levels post-exposure.

Co-administration of Pluronic L81 with 'fed-state' vehicle produced a significant decrease in secreted triglyceride level as compared to a 'fed-state' control. Following a washout step and addition of fresh serum-free DMEM, triglyceride secretion in cells previously administered 'fed-state' control vehicle showed a sudden drop in triglyceride output (Figure 4.20). Cells previously exposed to a combined 'fed-state'/ Pluronic L81 vehicle showed a small, but short-lived increase in triglyceride levels following the wash-out step, which was particularly marked, and significant (p<0.05) at the 29-hour time point (5 hours post-wash-out). Following wash-out, the amount of triglyceride released by cells previously exposed to Pluronic L81 did not equal the difference between triglyceride secretion in 'fed-state' control and Pluronic L81 treated cells during the first 24 hour exposure, nor did it equal the rate of output seen during exposure to 'fed-state' control vehicle (Figure 4.20). This indicates that, unlike in the rat model employed by Tso *et al.* (1982), withdrawal of Pluronic L81 did not result in rapid secretion of a massive intracellular loading of triglyceride.

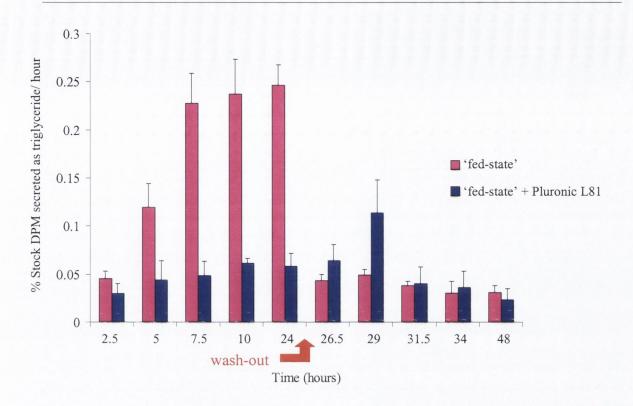


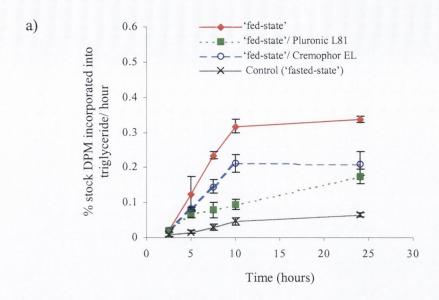
Figure 4.20: Effect of co-administered Pluronic L81 (0.006% w/v) on the rate of triglyceride secretion (expressed as the % stock DPM incorporated into triglyceride per hour) over a 24-hour period in the presence of 'fed-state' vehicle in Caco-2 monolayers and on the triglyceride output of cells following vehicle withdrawal (initiated by a wash-out step). Values represent the mean  $\pm$  SD (n=4).

#### 4.3.7.2 TRL secretion recovery following wash-out and administration of 'fed-state' vehicle

A dual labelling technique was devised to investigate whether cell response to a 'fed-state' vehicle was affected by prior exposure to inhibitory surfactants (Pluronic L81 and Cremophor EL). Monolayers were first incubated with [\frac{14}{C}] oleic acid labelled 'fed-state' vehicle or 'fed-state' vehicle plus excipient. Basolateral samples were taken at 2.5-hour intervals for the first 10 hours (each time moving the monolayer to a fresh sample well containing 3ml serum-free medium) and then at the 24-hour time point. After 24 hours the vehicle was removed and monolayers washed thrice in serum-free culture medium. A recovery 'fed-state' vehicle was then administered. This vehicle was labelled with [\frac{3}{H}] oleic acid as a fatty acid probe in order to distinguish between lipids synthesised from pre- and post-recovery fatty acid vehicles. Basolateral samples were again taken at 2.5-hour intervals over 10 hours and then at the 24-hour time point.

Administration of 'fed-state' control vehicle to Caco-2 cells produced a triglyceride secretion profile with a 2.5-hour lag period, and a maximum rate of triglyceride secretion at approximately 10 hours (Figure 4.21a). In the presence of Pluronic L81 or Cremophor EL, a reduced rate of secretion was observed, with significant differences (p<0.05) occurring from 5 hours onwards for Pluronic L81 and from 7.5 hours onwards for Cremophor EL (Figure 4.21a). Inhibition was more pronounced in the presence of Pluronic L81 as compared to Cremophor EL. Cells administered a 'fasted-state' control BSA vehicle (labelled with trace amounts of [\frac{14}{C}] oleic acid) demonstrated minimal triglyceride secretion.

Following the wash-out step and administration of a [³H] labelled 'fed-state' vehicle, cells previously treated with Pluronic L81 or Cremophor EL, recovered to give secretion profiles similar to 'fed-state' control (not previously treated with excipient). Cells administered a 'fasted-state' control BSA vehicle (with trace amounts of [³H] oleic acid only) again demonstrated minimal triglyceride secretion (Figure 4.21b). The plateau reached by cells given a recovery 'fed-state' vehicle (i.e. cells previously exposed to 'fed-state' plus or minus excipient) tended to be slightly, but not significantly, higher than that reached during the initial [¹⁴C] 'fed-state' exposure (Figure 4.21b).



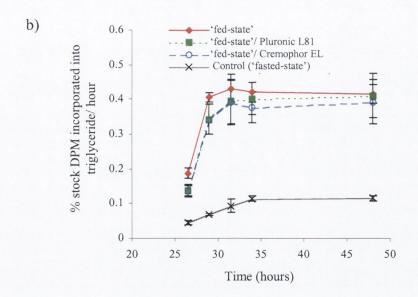


Figure 4.21: Effect of co-administered Pluronic L81 (0.006% w/v) and Cremophor EL (0.1% w/v) on the rate of triglyceride secretion (expressed as the % of stock [ $^{14}\text{C}$ ] oleic acid incorporated into triglyceride per hour) over a 24-hour period in 'fed' stimulated Caco-2 monolayers (panel a) and on the rate of triglyceride secretion (expressed as the % of stock [ $^{3}\text{H}$ ] oleic acid incorporated into triglyceride per hour) following wash-out step at 24 hours and administration of recovery [ $^{3}\text{H}$ ] oleic acid labelled 'fed-state' vehicle (panel b). Values represent the mean  $\pm$  SD (n=4).

#### 4.4 Key findings

Pluronic block copolymers inhibited lipoprotein secretion in Caco-2 cells in a concentration dependent and reversible manner. Inhibition was selective for chylomicrons at low excipient concentrations. Inhibitory potential and cytotoxicity was highest for more lipophilic Pluronic block copolymer members.

Cremophor EL and TPGS, two further non-ionic, polyoxyethylated surfactants, also inhibited TRL secretion in Caco-2 cells.

Surfactants produced inhibition of TRL output without altering fatty acid uptake or cellular triglyceride and apo B levels, indicating inhibition at the assembly/ secretion steps of the lipoprotein pathway.

Cremophor EL and Pluronic L81 produced a corresponding decrease in P-gp activity with their inhibitory effects on TRL secretion, thus implying an association between the two actions.

The increase in inhibitory potential with increasing surfactant lipophilicity suggests that cellular uptake is critical for effects on TRL secretion.

The tested surfactants, in addition to having reported P-gp inhibitory effects, all contained a polyoxyethylene moiety. For this reason, PEG 400 (a polymer of ethylene glycol with reported P-gp inhibitory activity) was added to cells. PEG 400 was found to produce moderate inhibition of TRL secretion when added at a relatively high concentration. This implies that the polyoxyethylene moiety is important for effects on TRL secretion.

The similarity between reported *in vivo* rat response to Pluronic L81 and that of the Caco-2 model presented here in terms of selective inhibition of chylomicron secretion, rapid recovery from inhibition and absence of effects on intracellular triglyceride synthesis further highlights the potential that Caco-2 cells have to predict *in vivo* response.

#### 4.5 Discussion

Published findings have demonstrated the inhibitory effects of Pluronic L81 on intestinal TRL output *in vivo* in the rat (Tso *et al.*, 1981; Pidlich *et al.*, 1996) and *in vitro* in the Caco-2 model (Luchoomun and Hussain, 1999). As an extension of these observations, the effects of a range of Pluronic block copolymers and of other, structurally diverse, non-ionic surfactants on lipid metabolism in the Caco-2 model were explored.

All of the investigated Pluronic block copolymers were found to inhibit TRL secretion in the 'fedstimulated' Caco-2 model to some extent. In each case, inhibition was concentration dependent and was selective for chylomicron secretion at lower administered excipient concentrations. Observations of selectivity and concentration dependence of the inhibitory effects of copolymers on TRL secretion are in agreement with published findings. For example, both Luchoomun and Hussain (1999) and Tso and Gollamudi (1984) found that lower concentrations of Pluronic L81 inhibited secretion of chylomicrons in preference to VLDL. Such findings are also consistent with the existence of two separate assembly pathways for chylomicrons as proposed by Tso et al. (1984) and Hussain (2000). There is some discrepancy in the reported sensitivities of the TRL secretion response to Pluronic L81. Tso et al. (1981) found that a concentration of 0.017%w/v was required to induce inhibition of intestinal triglyceride output, whereas the data presented here found inhibition at 0.006%w/v. This difference may be a result of model variables. Tso and colleagues used a lymph fistulated rat model and infused test vehicles through the duodenum whereas data here were generated using an isolated Caco-2 (colon carcinoma) cell model. Luchooman (1999) reported inhibition of chylomicron secretion by Pluronic L81 in the Caco-2 model at much lower concentrations (1-8µg/ml) than observed in the results presented here. These differences may have been a result of experimental conditions. Luchoomun and Hussain (1999) used Caco-2 cell monolayers grown on 3µm pore filters, which may have altered monolayer differentiation (Tucker et al., 1992). Indeed, their reported oleic acid-stimulated apoB secretion level (10.6ng/filter/hour) was approximately 8-fold lower than that observed throughout this work. This may account for sensitivity differences.

The potency of Pluronic block copolymers as inhibitors of TRL secretion was dependent on excipient HLB. The most lipophilic copolymer, Pluronic L81 proved to be the most potent inhibitor, producing inhibition of chylomicron secretion at concentrations as low as 0.006%w/v (p<0.05). Inhibition of TRL secretion occurred with the other tested Pluronic block copolymers, but required higher excipient concentrations (approximately 5-fold higher for Pluronic P85 and >600-fold higher for Pluronic F68). The cytotoxicity of administered Pluronic block copolymers was found to follow a similar HLB dependent trend, with more lipophilic compounds proving to be the most toxic. Work by Batrakova *et al.* (1999) shows that lipophilic block copolymers are more effective at inhibiting P-gp activity in MDR cancer cells than the more hydrophilic group members. This HLB dependence of Pluronic block copolymer activity may reflect the ability of excipients to enter cells and to interact with intracellular targets. Evidence from *in vitro* and *in vivo* models has shown that Pluronic block copolymers are taken up intracellularly (Kabanov *et al.*, 1992; Bergstedt *et al.*, 1991). *In vitro* studies, using a series of fluorescently labelled Pluronic block copolymers, have also shown that uptake is dependent on compound structure and HLB – with hydrophobic

copolymers containing intermediate length propylene oxide blocks (30-60 units), such as Pluronic L81 and Pluronic P85 being most easily transported into and within BBMEC cells (Kabanov *et al.*, 2002) and hydrophilic block copolymers showing limited or no uptake. Pluronic L81, with its high propylene oxide content (43 units) and HLB of 1-7, has a high potential for intracellular transport.

Cremophor EL and TPGS, two structurally different non-ionic surfactants, were also found to inhibit TRL secretion in a concentration dependent manner. The potency of these compounds as inhibitors was similar to that of Pluronic P85, with which they also share similar HLB values. Such findings are important in that they demonstrate that these inhibitory effects extend beyond the Pluronic block copolymers to a broader range of surfactant agents.

All of the tested non-ionic surfactants were found to inhibit TRL secretion, without affecting fatty acid uptake or intracellular synthesis of lipid components (triglyceride or phospholipid) or apoB. Additionally, inhibition was seen when surfactants were applied to either the apical or basolateral surface, implicating an intracellular site of action. As inhibition was more pronounced following basolateral application, it is likely that an intracellular site located towards the basolateral surface may be involved. These data are consistent with findings by Tso and colleagues in the lymph fistulated rat model (Tso et al., 1980; Tso et al., 1981). This group found that duodenal perfusion of Pluronic L81 with triolein blocked lymphatic chylomicron output without affecting the level of intracellular lipid re-esterification. Triglyceride was found to accumulate in the rat intestinal mucosa in tandem with the reduced secretion, resulting in the formation of large lipid droplets within the endoplasmic reticulum (Tso et al., 1981). Such findings indicate a block in intracellular transfer of developing lipoproteins.

As all of the above investigated surfactants comprised a hydrophilic polyoxyethylene side chain attached to a lipophilic moiety (polypropylene oxide in the case of the copolymers, ricinoleic acid for castor oil and vitamin E for TPGS) it was decided to investigate the specific effects of an isolated polyoxyethylene chain on lipid metabolism. For this reason, PEG 400 was administered to cells. PEG 400 was shown to inhibit TRL secretion in 'fed-state' cells when added at a concentration of 5%w/v. The potency of inhibition was considerably lower than with the hydrophobic surfactants and was comparable to that obtained with the hydrophilic Pluronic F68 compound, which again may reflect an inability of this hydrophilic excipient to permeate into cells and reach intracellular targets. These data do, however, indicate that the polyoxyethylene grouping on tested excipients may be important for inhibiting TRL secretion, but that the additional hydrophobic moiety is necessary for increasing potency, most likely by facilitating cellular uptake.

Following withdrawal of inhibitory surfactants, Pluronic L81 and Cremophor EL, the Caco-2 triglyceride secretion response to 'fed-state' vehicle was found to recover rapidly to normal, uninhibited 'fed-state' levels. Such findings are consistent with data from the lymph cannulated rat model (Tso et al., 1981; Tso et al., 1982). However, unlike the rat model, withdrawal did not result in a burst release of accumulated intracellular triglyceride, possibly indicating further model differences.

Results for Cremophor EL and Pluronic L81 clearly demonstrate dual inhibition of both P-gp efflux activity and chylomicron secretion over similar excipient concentration range. Good correlation was found between effects on both these processes. These data are consistent with findings by Field *et al.* (1995) in the Caco-2 model, where pharmacologically active drugs, such as verapamil and trifluoperazine, which are recognised inhibitors of P-gp were shown to be effective at inhibiting TRL secretion. Inhibition of P-gp by Pluronic L81 and Cremophor EL has previously been reported using cell models (Woodcock *et al.*, 1990; Batrakova *et al.*, 1999).

The common effects of polyoxyethylated surfactants on P-gp activity have long been recognised (Woodcock *et al.*, 1992; Hugger *et al.*, 2002). One theory for this effect is that of a general membrane fluidization mechanism brought about by these amphiphilic compounds (Dudeja *et al.*, 1995; Batrakova *et al.*, 2001a). Many commonly used non-ionic surfactants demonstrate the ability to alter membrane fluidity by penetrating into the lipid bilayer or solubilising out membrane lipids (Dimitrijevic *et al.*, 2000). As P-gp is located in the apical membrane of enterocytes and as it has transmembrane domains and intermembrane binding sites, it may be highly sensitive to lipid bilayer conformation and open to manipulation by compounds that interfere with membrane integrity (Beisson *et al.*, 2001). The relationship between membrane fluidity and P-gp function has been shown to be quite high for a number of surfactants (Woodcock *et al.*, 1992; Dudeja *et al.*, 1995). More specific and direct effects of surfactants on transport protein activity have also been suggested. Evidence for direct protein interactions comes from the observation of inhibited drug efflux in the presence of submicellar concentrations of Pluronic block copolymers (Batrakova *et al.*, 1998) (micelle formation being important for membrane fluidisation) and inhibition of drug substrate binding to P-gp by low concentrations of Triton X (Dimitrijevic *et al.*, 2000).

Intracellular energy depletion by Pluronic block copolymers has been implicated in inhibition of drug efflux activity in a number of multiple drug resistant (MDR) cell lines. The ability of Pluronic block copolymers to deplete intracellular ATP levels has been closely correlated with their ability to inhibit drug efflux (Batrakova *et al.*, 2001b). Furthermore, supplementation of inhibited cells with exogenous ATP has been shown to abolish copolymer induced effects and to restore function of drug efflux. Pluronic block copolymers and other polyoxyethylated surfactants have been

shown to act as potassium ionophores and hence interrupt the electron transport chain in cell mitochondria (Brierley et al., 1972; Rapoport et al., 2000; van Zutphen et al., 1972). Such activity may uncouple oxidative phosphorylation and impair production of ATP (Kabanov et al., 2003). This may present a possible mechanism of ATP depletion by these reagents.

The applicability of ATP depletion to inhibited TRL secretion in Caco-2 cells was tested in the present study by administering exogenous ATP to Pluronic L81 and Cremophor EL inhibited 'fed-state' Caco-2 cells. Unlike the reported P-gp results, exogenous ATP did not restore the TRL secretion response in cells exposed to Pluronic L81 or Cremophor EL. This may reflect alternative or additional mechanisms of action for the inhibitory effects of surfactant on TRL secretion, such as by direct alkalinisation of intracellular transport vesicles or interaction with binding sites on ABC proteins. Alternatively, it may reflect poor absorption of ATP by Caco-2 cells. In the published studies Kbv, LLC-MDR1 and BBMEC cells were exposed to ATP in the presence of dodecylamine, a permeabilising agent (Batrakova *et al.*, 2000; Batrakova *et al.*, 2001b), these cell lines represent oropharyngeal epithelium, kidney epithelium and blood brain barrier respectively and as such may have different permeability properties to the Caco-2 colon model. In the presented work, ATP was added to both apical and basolateral surfaces of cells at the highest dose that did not produce cell toxicity. The added concentration could therefore not be increased further. There was no available data on supplementation of Caco-2 cells with ATP, hence it was not possible to assess how well exogenous ATP is taken up intracellularly.

In summary, data presented here indicate that a wide range of polyoxyethylated non-ionic surfactants have the potential to inhibit intestinal TRL secretion in a concentration dependent manner. As many of the tested excipients produced inhibition of TRL secretion at pharmaceutically relevant concentrations, and as there is a close correlation between P-gp activity and TRL secretion, the confounding bio-modulating effects of such excipients must be considered during formulation design. For example, given a highly lipophilic drug that is both a substrate for P-gp driven efflux, and also a candidate for lymphatic drug uptake, the benefits to oral bioavailability of inhibiting drug efflux may be counteracted by the loss of lymphatic drug uptake in response to decreased lymphatic triglyceride output.

### **Chapter Five**

Effects of Polysorbate Surfactants on Lipoprotein Processing in the Caco-2 Cell Model

# 5 Effects of Polysorbate Surfactants on Lipoprotein Processing in the Caco-2 Cell Model

#### 5.1 Introduction

Data presented in Chapter Three show, in concordance with published literature (Luchoomun and Hussain, 1999; Dashti *et al.*, 1990), that long chain fatty acids promote secretion of lipoproteins in Caco-2 cells and that oleic acid, in particular, is highly effective at promoting TRL secretion. In contrast, findings from Chapter Four show that a number of non-ionic surfactants, namely Pluronic block copolymers, Cremophor EL and TPGS, inhibit TRL secretion in the Caco-2 model. The investigated surfactants all share the common features of a polyoxyethylene side chain attached to a hydrophobic moiety. Surfactant effects on TRL secretion were found to be dependent on the surfactant lipophilicity, with surfactants of HLB 12-18 producing significant inhibition at concentrations of 0.01-0.03%w/v.

Polysorbate (Tween) surfactants are available in a wide range of HLB values and many are classed as food grade or have GRAS (generally recognised as safe) status, and as such are frequently included in oral drug delivery vehicles. Additionally, polysorbate 80 is widely employed as a wetting or solubilising agent for *in vivo* bioavailability and *in vitro* permeability studies (Nishigaki *et al.*, 1976; Li and Zhao, 2002; Takahashi *et al.*, 2002; Porter *et al.*, 1996) to facilitate dispersion or solubilisation of poorly-water soluble drugs. For these reasons, it is imperative to have a thorough understanding of the potential bio-modulating effects of this excipient family.

Structurally, polysorbates are a series of fatty acid esters of sorbitol and its anhydrides copolymerised with ethylene oxide (Lawrence, 2003), as illustrated in Figure 5.1. They differ from one another in the length of the polyoxyethylene chain and also in the nature of the associated fatty acid. In terms of lipid metabolism studies, this class of surfactant essentially combines the properties of a non-ionic, polyoxyethylated surfactant with those of an esterified fatty acid. Polysorbate 80 is of particular interest here because it incorporates oleic acid, which promotes TRL secretion, and because it fits the criteria for potent TRL inhibitory effects as determined in Chapter Four (HLB of 15 and the presence of a polyoxyethylene side chain).

A close association between inhibition of energy dependent drug efflux and TRL secretion response in Caco-2 cells by common excipients was outlined in Chapter Four. As polysorbate 80 is a recognised inhibitor of P-gp (Nerurkar *et al.*, 1996; Woodcock *et al.*, 1992), evaluation of its dual effects on TRL secretion and P-gp activity will further elucidate this hypothesised relationship.

#### 5.2 Aims and objectives

The objectives of the current study were to evaluate the effects of polysorbate 80 on lipid metabolism, particularly on TRL secretion, and to compare effects with those of polysorbate 60. The sensitivity of polysorbate 80 to intestinal digestion was also investigated *in vitro* to establish whether or not the fatty acid component is likely to be liberated *in vivo*. In addition, the effects of Polysorbates 80 and 60 on drug efflux were investigated to further explore the relationship between drug efflux and TRL secretion.

Figure 5.1: Chemical structures of polysorbates 80 and 60 as per Lawrence (2003)

#### 5.3 Results

#### 5.3.1 Effects of polysorbate surfactants on cell viability and monolayer integrity

The cytotoxicity of polysorbate surfactants was determined by measuring cell dehydrogenase activity using an MTT assay following excipient exposure as described (Section 2.3.4). Additionally, TEER measurements were taken (as per Section 2.3.1) during lipid experiments to monitor the effects of excipient exposure on monolayer integrity. Results are shown in Table 5.1.

**Table 5.1:** The effects of Polysorbate 80 and Polysorbate 60 on Caco-2 cell viability (measured using an MTT assay for cell dehydrogenase activity) and monolayer integrity (measured as TEER). Values represent the mean (n=5 for MTT and n=3-6 for TEER measurements) (SD). Statistical significance relative to positive control (cells incubated without excipients) (MTT assay) or starting value (TEER measurements) is shown: \*\*p<0.01, \*p<0.05.

Concentration (%w/v)	Polysorbate 80		Polysorbate 60	
	MTT	TEER	MTT	TEER
	(% viability)	(% relative to start)	(% viability)	(% relative to start)
0.0005	107.1 (10.0)	103.9 (2.4)	101.9 (7.3)	102.3 (4.5)
0.005	103.9 (8.2)	104.4 (7.8)	95.6 (7.8)	99.7 (1.2)
0.05	108.2 (8.5)	99.3 (12.4)	95.5 (11.7)	103.6 (4.4)
0.15	109.6 (5.4)	96.4 (3.9)	105.6 (7.3)	ND
0.25	100.3 (5.3)	98.6 (3.6)	91.2 (6.0)	91.2 (6.0)
0.5	105.4 (8.9)	101.4 (3.4)	50.3 (6.7) **	76.1 (5.4)*
1	54.1 (12.1) **	88.6 (6.1)	17.6 (0.43) **	ND

ND - not determined

In the presence of polysorbate 80, cell viability remained close to 100% at concentrations up to 0.5%w/v, but was reduced (45%, p<0.01) at 1%w/v. Polysorbate 60 produced a significant reduction in intracellular dehydrogenase activity at 0.5%w/v (p<0.01), with full viability being maintained at up to 0.25%w/v. Cell monolayer resistance measurements presented similar findings, with monolayer integrity being compromised at 1%w/v polysorbate 80 (12% decrease, but not statistically significant) and 0.5%w/v polysorbate 60 (25% decrease, p<0.05). Toxicity values for the polysorbate surfactants are comparable to those achieved for Cremophor EL, a non-ionic surfactant of similar HLB, in Section 4.3.2.

#### 5.3.2 Polysorbate surfactant effects on lipid metabolism

#### 5.3.2.1 Vehicles

Vehicles were formulated as described in Section 2.3.2, with polysorbate solutions (in serum-free DMEM) being added to concentrated 'fed-' or 'fasted-state' vehicles before diluting to the correct volume and concentration. Polysorbate surfactants were added over the concentration range of 0.0005-1%w/v. For standard lipid metabolism studies, vehicles were labelled with trace [<sup>14</sup>C] oleic acid (0.2μCi/ml). Caco-2 cells were incubated with vehicles over 20 hours, after which time basolateral samples and cell lysates were analysed for lipid and apoB content as per Section 2.3.2. Basolateral samples were also subject to density gradient ultracentrifugation (Section 2.3.2) to isolate lipoprotein classes according to flotation density.

#### 5.3.2.2 Effects of polysorbate surfactants on lipoprotein secretion in 'fed-state' cells

In contrast to findings for non-ionic surfactants presented in Chapter Four, administration of polysorbate 80 to 'fed-' stimulated cells resulted in a concentration dependent increase in chylomicron secretion relative to 'fed-state' level (Figure 5.2a). The increase in chylomicron secretion was significant at and above a concentration of 0.15%w/v. Maximum chylomicron secretion was reached at 0.5%w/v polysorbate 80, with a 3.5 fold increase in chylomicron secretion relative to 'fed-state'. VLDL secretion was not altered from 'fed-state' level by co-administration of polysorbate 80. At 1%w/v polysorbate 80, chylomicron secretion showed a decrease relative to that seen at 0.5%w/v, which may have been a result of cell toxicity, as indicated by MTT data (Table 5.1). Supplementation of 'fed' stimulated cells with polysorbate 60, however, produced no change in the TRL secretion levels (Figure 5.2b) over the investigated concentration range (0.0005-0.5%w/v).

Looking at the data for polysorbate 80 effects on total secreted apoB and lipids (Figure 5.3a), it can be seen that the secreted triglyceride levels followed a concentration dependent increase in the presence of polysorbate 80. The increase in triglyceride secretion was significant from 0.25%w/v onwards (p<0.01) (Figure 5.3a) and was consistent with findings for chylomicron secretion obtained from density gradient ultracentrifugation (Figure 5.2a). Although secreted triglyceride levels were seen to increase with polysorbate 80 content, the apoB and phospholipid secretion levels remained relatively constant. This resulted in an elevation in the secreted triglyceride to apoB and secreted triglyceride: phospholipid ratios, thereby indicating triglyceride enrichment of secreted lipoproteins. Phospholipid secretion did show a slight increase relative to control 'fed-state' level in response to polysorbate 80, but the difference was not significant. In contrast to polysorbate 80 findings and in agreement with density gradient

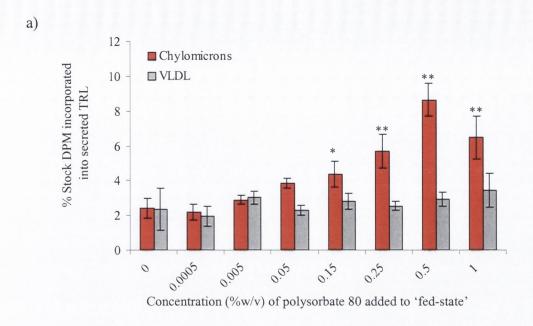
ultracentrifugation data, Polysorbate 60 supplementation had no significant effects on secreted apoB, triglyceride or phospholipid levels relative to 'fed-state' (Figure 5.3b).

#### 5.3.2.3 Effects of polysorbate surfactants on fatty uptake in Caco-2 cells

It has been suggested that rapid and efficient uptake of administered fatty acid is necessary for the assembly and secretion of large chylomicrons (Luchoomun and Hussain, 1999). An experiment was therefore conducted to see if the enhanced TRL secretion observed in Caco-2 cells in response to polysorbate 80 was partly due to an increase in the rate of uptake of fatty acid at the apical surface. Polysorbate 80 and polysorbate 60 were added to [14C] labelled 'fed-state' vehicle and applied to Caco-2 monolayers. Initial rate of fatty acid uptake was monitored by assaying cellular radiolabel content at intervals of 5 minutes and 1 hour, as per Section 2.3.8. Additionally, Fatty acid uptake over a 20-hour period was determined by measuring the total [14C] oleic acid content of cells and basolateral medium. Findings are presented in Figure 5.4. The data show that polysorbate 80 had no significant effects on fatty acid uptake at any of the time intervals tested relative to the control 'fed-state' vehicle or the 'fed-state' vehicle supplemented with polysorbate 60. Hence, fatty acid uptake was not an important factor in the observed TRL response to polysorbate 80.

## 5.3.2.4 Effects of polysorbate surfactants on intracellular accumulation of lipids and apoB in 'fed-state' cells

Co-administration of polysorbate 80 or polysorbate 60 with 'fed-state' vehicle did not alter the levels of intracellular triglyceride, phospholipid or apoB over the investigated excipient concentration range (Figure 5.5a,b). In the case of polysorbate 80, this means that the observed increase in triglyceride secretion output (Figure 5.3a) was not associated with any corresponding change in intracellular triglyceride production. Therefore, the secreted: cellular ratio (secretion efficiency) of triglyceride was increased proportionately with polysorbate 80 dose (Figure 5.6).



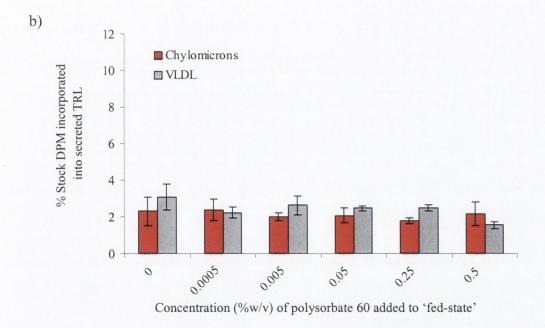
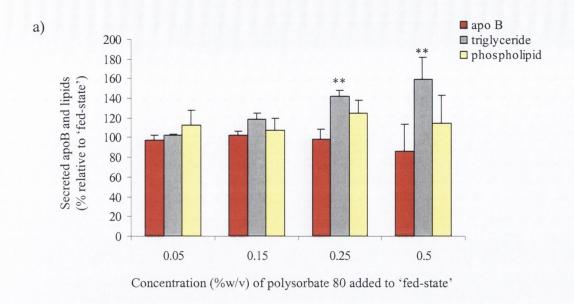


Figure 5.2: Effects of increasing concentrations of polysorbate 80 (panel a) and polysorbate 60 (panel b) on TRL secretion in 'fed' stimulated cells. Results are expressed as % of administered [ $^{14}$ C] oleic acid stock incorporated into density gradient fractions over 20 hours. Values represent the mean  $\pm$  SD (n=3-5). Statistical significance of excipient effects relative to 'fed-state' control is shown: \*\*p<0.01, \*p<0.05.



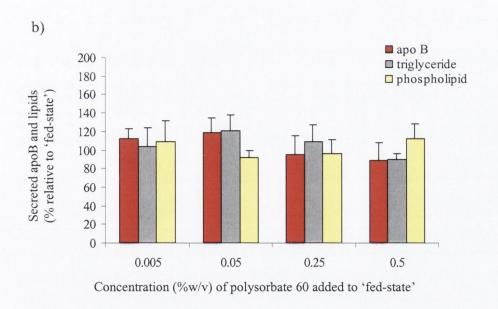


Figure 5.3: Effects of increasing concentrations of polysorbate 80 (panel a) and polysorbate 60 (panel b) on triglyceride, phospholipid and apoB secretion in 'fed' stimulated cells. Results are expressed as secretion levels relative (%) to 'fed-state' level. Values represent the mean  $\pm$  SD (n=3-5). Statistical significance of excipient effects relative to 'fed-state' control is shown: \*\*p<0.01.

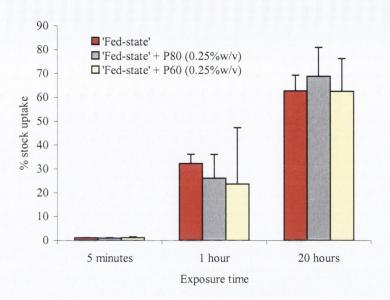
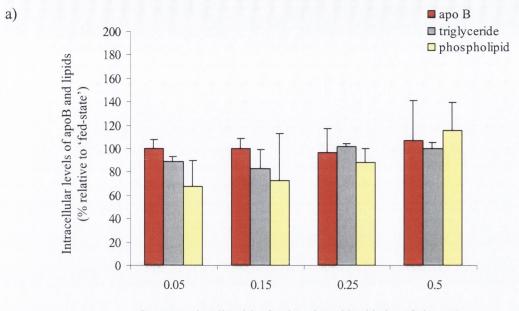
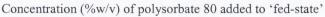
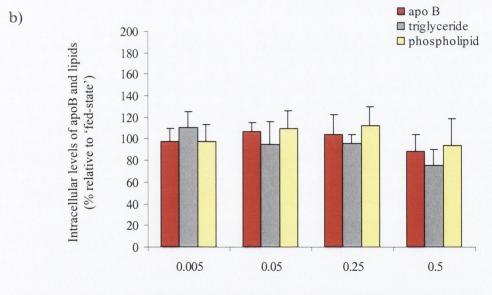


Figure 5.4: Effects of polysorbate 80 and polysorbate 60 on fatty acid uptake in 'fed' stimulated Caco-2 cells. 'Fed-state' control was applied for comparison. Values represent the mean  $\pm$  SD (n=4).







Concentration (%w/v) of polysorbate 60 added to 'fed-state'

**Figure 5.5:** Effects of increasing concentrations of polysorbate 80 (panel a) and polysorbate 60 (panel b) on intracellular triglyceride, phospholipid and apoB levels in 'fed' stimulated cells. Values represent the mean  $\pm$  SD (n=3-5).

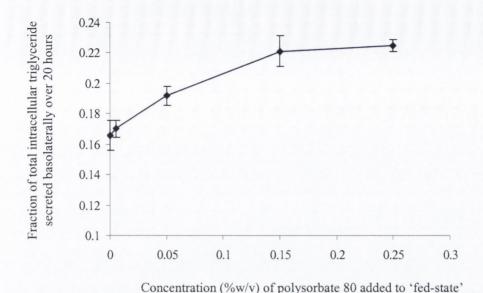


Figure 5.6: Effect of polysorbate 80 addition on the secretion efficiency of triglyceride in Caco-2 monolayers, expressed as the total amount of triglyceride secreted over 20 hours as a proportion of the total amount of intracellular triglyceride accumulated over the same period. Values represent the mean  $\pm$  SD (n=3-5).

#### 5.3.2.5 Effects of polysorbate 80 on lipoprotein secretion in 'fasted-state' cells

The above data have shown that polysorbate 80 promotes TRL secretion in Caco-2 cells when co-administered with a 'fed-state' vehicle comprising 0.5mM oleic acid: 0.125mM BSA. The effect of polysorbate 80 supplementation on 'fasted' cells was subsequently investigated to see if this response occurred in the absence of additional administered free fatty acid. This time, polysorbate 80 was administered to Caco-2 monolayers with a 'fasted-state' vehicle of 0.125mM BSA labelled with trace [14C] oleic acid, as per section 2.3.2. The concentration dependence of the response was assessed by co-administering 0.15-0.5%w/v polysorbate 80. As with 'fed-state' studies, the secreted chylomicron and VLDL levels were initially measured via density gradient ultracentrifugation (Section 2.3.2). In common with 'fed-state' data, polysorbate 80 supplementation of 'fasted' cells produced a concentration dependent increase in chylomicron secretion, with statistical significance relative to the 'fasted-state' control from the lowest tested concentration (0.15%w/v) upwards. In contrast to 'fed-state' findings, VLDL secretion was also seen to increase relative to the control, with statistical significance at 0.15%w/v. VLDL secretion reached a maximum (20-fold increase relative to 'fasted-state' control with a dose of 0.25%w/v polysorbate 80 (Figure 5.7), whilst, chylomicron secretion continued to follow a dose-response curve, reaching a 30-fold increase (p<0.01) relative to 'fasted-state' control at the highest tested concentration of polysorbate 80 (0.5%w/v).

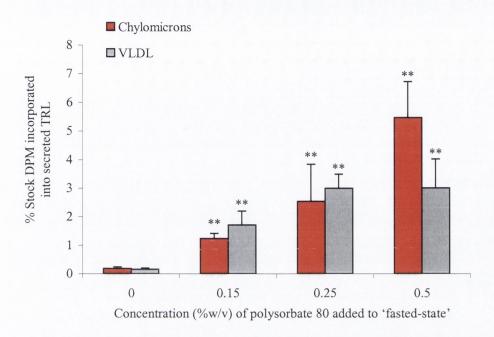
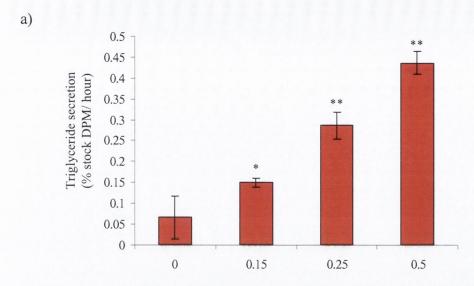
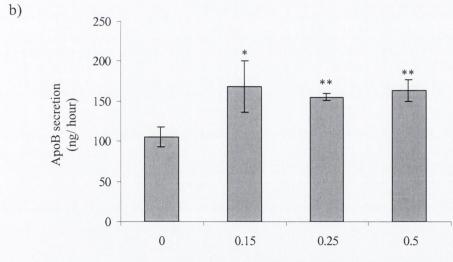


Figure 5.7: Effects of increasing concentrations of polysorbate 80 on TRL secretion in 'fasted-state' cells. Results are expressed as % of administered [14C] oleic acid stock incorporated into density gradient fractions over 20 hours. Values represent the mean  $\pm$  SD (n=3-5). Statistical significance of excipient effects relative to 'fasted-state' control is shown: \*\*p<0.01.

In support of density gradient ultracentrifugation data (Figure 5.7), triglyceride secretion levels (as determined by TLC analysis) demonstrated a concentration dependent increase over the investigated polysorbate 80 dose range. The elevated triglyceride secretion response was significant (p<0.05) versus 'fasted-state' at 0.15%w/v polysorbate 80, and highly significant (p<0.01) thereafter (Figure 5.8a). ApoB secretion (as determined using an ELISA) showed an initial increase (1.5-fold increase, p<0.05) relative to 'fasted-state' at 0.15%w/v polysorbate 80, with no further increase at higher concentrations (Figure 5.8b). Taking into consideration the combined triglyceride and apoB responses, these data indicate a rise in the secreted triglyceride: apoB ratio, which is consistent with triglyceride enrichment of secreted lipoproteins.



Concentration (%w/v) of polysorbate 80 added to 'fasted-state'

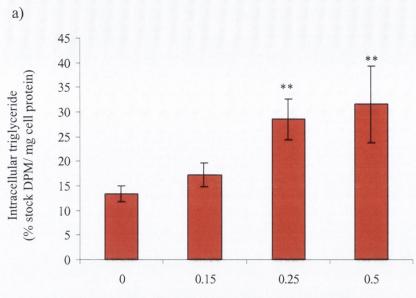


Concentration (%w/v) of polysorbate 80 added to 'fasted-state'

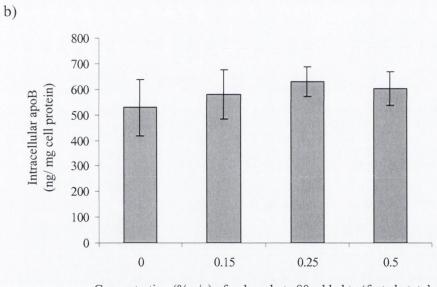
Figure 5.8: Effects of increasing concentrations of polysorbate 80 on triglyceride (panel a) and apoB (panel b) secretion in 'fasted-state' cells. Values represent the mean  $\pm$  SD (n=3-5). Statistical significance of excipient effects relative to 'fasted-state' control is shown: \*p<0.05, \*\*p<0.01.

# 5.3.2.6 Effects of polysorbate 80 on intracellular accumulation of triglyceride and apoB in 'fasted-state' cells

In contrast to 'fed-state' findings, supplementation of 'fasted-state' cells with polysorbate 80 produced a concentration dependent increase in intracellular triglyceride levels (Figure 5.9a), however, the response was less pronounced than that observed for triglyceride secretion. The rise in intracellular triglyceride content was significant (p<0.01) relative to 'fasted-state' at 0.25%w/v polysorbate 80. Intracellular triglyceride content reached a maximum at 0.25%w/v polysorbate 80. This is in contradistinction to secreted triglyceride levels, where a further 1.4-fold increase (p<0.05) was observed at 0.5%w/v polysorbate 80 versus 0.25%w/v. Following supplementation with 0.5%w/v polysorbate 80, intracellular triglyceride levels were approximately 2.3-fold higher than in the 'fasted-state' control. This is compared to a 6.6-fold increase in triglyceride secretion versus control at the same concentration, thus indicating a selective rise in triglyceride secretion efficiency. Intracellular apoB levels showed no statistically significant change over the investigated concentration range (Figure 5.9b).



Concentration (%w/v) of polysorbate 80 added to 'fasted-state'



Concentration (%w/v) of polysorbate 80 added to 'fasted-state'

**Figure 5.9:** Effects of increasing concentrations of polysorbate 80 on intracellular triglyceride (panel a) and apoB (panel b) accumulation in 'fasted-state' cells. Values represent the mean  $\pm$  SD (n=3-5). Statistical significance of excipient effects relative to 'fasted-state' control is shown: \*p<0.05, \*\*p<0.01.

### 5.3.3 Comparative effects of polysorbate surfactants and free fatty acids on lipid metabolism in Caco-2 cells.

The above findings for polysorbate 80-mediated enhancement of TRL secretion in 'fasted-state' Caco-2 cells are similar to those observed for oleic acid supplementation in Section 3.3.5.2. As polysorbate 80 is an ester of oleic acid, it is likely that the observed effects were due to the component fatty acid. The absence of any TRL-promoting effects by polysorbate 60, a stearic acid ester, is consistent with this theory. A number of techniques were subsequently employed to determine whether or not this was the case. As a preliminary step, the effects of polysorbate 80 and polysorbate 60 on the secreted lipoprotein profile of 'fasted-state' Caco-2 cells were compared. These two surfactants differ only in their fatty acid moiety - polysorbate 80 being an ester of oleic acid and polysorbate 60 an ester of stearic acid. Findings in Section 3.3.5.4 showed that cells responded differently to stearic acid and oleic acid - secreting VLDL and LDL in response to the former and VLDL and chylomicrons with the latter. It was hypothesised that polysorbate surfactants would produce secreted lipoprotein profiles resembling those of their free fatty acid counterparts if the fatty acid component were responsible for the effects on TRL For direct comparison, the effects of free oleic acid and stearic acid on lipid output. metabolism were also determined. A fatty acid concentration of 0.5mM was selected for these studies in keeping with standard 'fed-state' vehicle and fatty acid studies presented in Section 3.3.5.4.

# 5.3.3.1 Comparison of the effects of polysorbate 80 versus polysorbate 60 on the TRL secretion response in 'fasted-state' Caco-2 cells

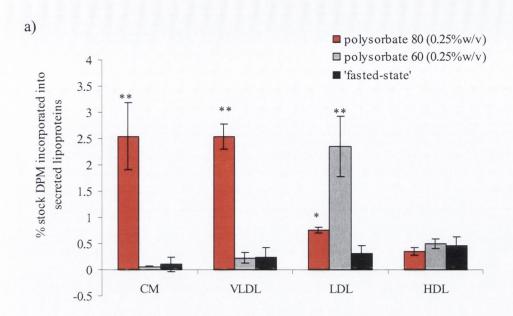
When polysorbate 80 (0.25%w/v) was added to 'fasted-state' vehicle, it produced a marked and selective increase in TRL secretion. Chylomicron and VLDL output were each increased by approximately 20-fold, relative to 'fasted-state' control (p<0.01) (Figure 5.10a). There was also a small, but significant increase in secretion of lipoproteins of flotation density equivalent to LDL (approximately 2-fold, p<0.05). In contrast, supplementation of 'fasted-state' cells with polysorbate 60 (0.25%w/v) resulted in a selective increase in LDL secretion (7-fold increase, p<0.01) (Figure 5.10a). Taking into consideration the data obtained for free fatty acid supplementation of 'fasted-state' cells (Figure 5.10b), it can be seen that the secreted lipoprotein responses obtained for polysorbate 80 and polysorbate 60 closely mirror the trends observed for oleic acid (0.5mM) and stearic acid (0.5mM) respectively. From these findings it can be inferred that the differential effects of the investigated polysorbates on lipoprotein secretion are most likely mediated by their component fatty acids.

### 5.3.3.2 Effects of polysorbate surfactants versus free fatty acids on basolateral secretion of triglyceride and apoB in Caco-2 cells

In further support of data obtained using density gradient ultracentrifugation, the effects of polysorbate surfactants and their component fatty acids on triglyceride and apoB secretion in 'fasted-state' cells were compared. Findings are summarised in Figure 5.11. Of the administered vehicles, only Polysorbate 80 and its fatty acid, oleic acid significantly stimulated secretion of triglyceride. Polysorbate 80 (0.25%w/v) and oleic acid (0.5mM) both produced a >4-fold increase in triglyceride secretion versus 'fasted-state' control (Figure 5.11a). ApoB secretion levels also showed an increase in the presence of these vehicles (Figure 5.11b), with 2.1- and 2.6-fold increases corresponding to polysorbate 80 and oleic acid respectively (p<0.01). Polysorbate 60 also promoted secretion of apoB, but to a lower extent (1.7-fold increase, p<0.05). Stearic acid produced a small increase in apoB secretion relative to 'fasted-state' control, but the difference was not statistically significant.

# 5.3.3.3 Effects of polysorbate surfactants versus free fatty acids on intracellular accumulation of triglyceride and apoB

Data for accumulated intracellular triglyceride and apoB levels in the presence of polysorbate 80, polysorbate 60 and their component fatty acids are shown in Figure 5.12. Only polysorbate 80 and oleic acid were found to increase intracellular triglyceride levels versus the 'fasted-state' control, with an approximately 2-fold increase with polysorbate 80 (0.25%w/v) and 1.6-fold increase with oleic acid (0.5mM) (p<0.01) (Figure 5.12a). This complements the secreted TRL data and again highlights the similarity between the effects of polysorbate 80 and free oleic acid on Caco-2 lipid metabolism.



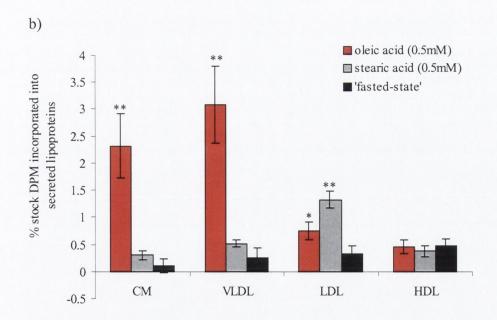
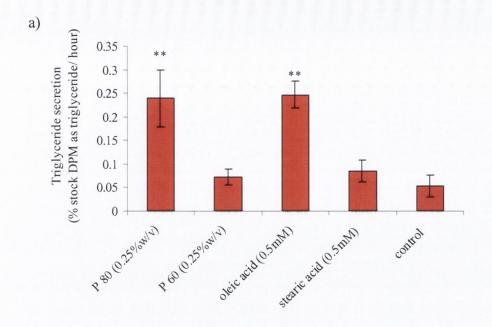


Figure 5.10: Effects of polysorbate 80 (0.25% w/v) and polysorbate 60 (0.25% w/v) (panel a) and of oleic acid (0.5mM) and stearic acid (0.5mM) (panel b) on lipoprotein secretion in 'fasted-state' cells. Results are expressed as % of administered [14C] oleic acid stock incorporated into density gradient fractions over 20 hours. Values represent the mean  $\pm$  SD (n=3-5). Statistical significance of vehicle effects relative to 'fasted-state' control is shown: \*\*p<0.01, \*p<0.05.



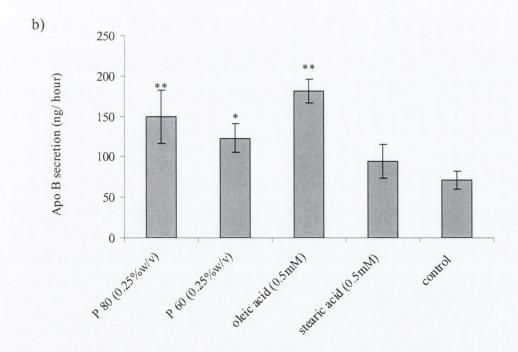
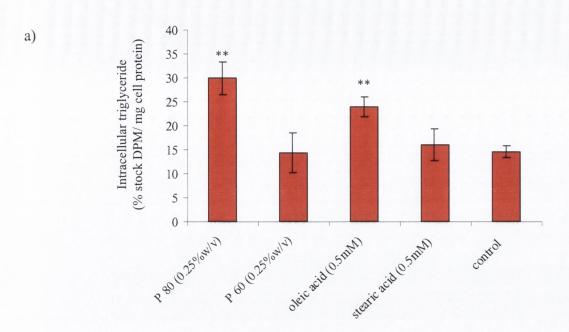


Figure 5.11: Effects of polysorbate 80 (0.25% w/v), polysorbate 60 (0.25% w/v), oleic acid (0.5mM) and stearic acid (0.5mM) on triglyceride (panel a) and apoB (panel b) secretion in 'fasted-state' cells. Values represent the mean of 3-5 monolayers  $\pm$  SD. Statistical significance of vehicle effects relative to 'fasted-state' control is shown: \*\*p<0.01, \*p<0.05.



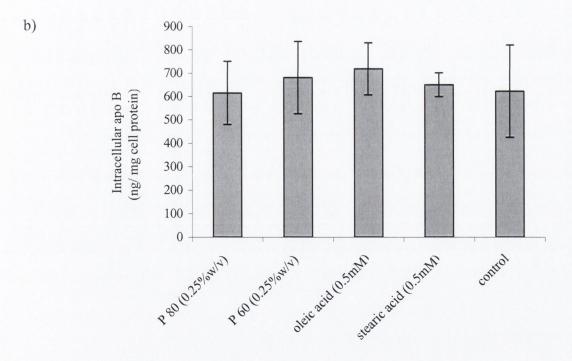
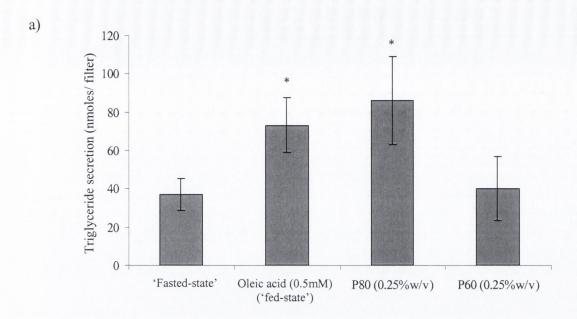


Figure 5.12: Effects of polysorbate 80 (0.25%w/v), polysorbate 60 (0.25%w/v), oleic acid (0.5mM), and stearic acid (0.5mM) on intracellular triglyceride (panel a) and apoB (panel b) levels in 'fasted-state' cells. Values represent the mean  $\pm$  SD (n=3-5). Statistical significance of vehicle effects relative to 'fasted-state' control is shown: \*\*p<0.01.

#### 5.3.3.4 Colorimetric triglyceride assay analysis of cellular and basolateral samples

Results presented thus far for triglyceride and associated TRL secretion have been obtained by following fatty acid radiolabel ([14C] oleic acid) incorporation into lipids. triglyceride assay kit based on glycerol detection (Bio Merieux PAP 150 triglyceride assay kit) Test vehicles - 0.25%w/v was employed to confirm the triglyceride radiolabel data. polysorbate 80, 0.25%w/v polysorbate 60 and 0.5mM oleic acid, each added to 'fasted-state' medium, were prepared according to Section 2.3.2. Caco-2 cell monolayers were incubated with test vehicles or 'fasted-state' control over 20 hours and cell lysate and basolateral samples collected as described (Section 2.3.3). In order to increase assay detection, samples were pooled from two Caco-2 monolayers for each determination. Cell lysates and basolateral medium were extracted (each in 1ml aliquots) as described in Section 2.3.2. Extracts for basolateral or cell lysate were pooled and dried under nitrogen. Extracts were reconstituted in isopropanol (40 µl), vortexed for 2 minutes and subsequently assayed directly using the BioMerieux test kit. This assay monitors triglyceride levels according to glyceride content and so is a good complement to the radiolabelled fatty acid data. Data obtained using the colorimetric assay kit are summarised in Figure 5.13. Supplementation of 'fasted' cells with polysorbate 80 (0.25%w/v) or oleic acid (0.5mM) ('fed-state') produced significant increases in triglyceride secretion compared to the 'fasted-state' control (p<0.05) (Figure 5.13a). contrast, supplementation with polysorbate 60 produced no significant change in triglyceride secretion (Figure 5.13a). These data are similar to the trends observed for triglyceride secretion using [14C] oleic acid as a lipid marker (Figure 5.11a). Intracellular levels of triglyceride were increased following administration of polysorbate 80 (>2-fold, p<0.05) and oleic acid (not statistically significant due to high variability) (Figure 5.13b), whilst administration of polysorbate 60 failed to bring about any alteration in intracellular triglyceride levels. Again, these findings are consistent with radiolabel data (Figure 5.12a), thus verifying the validity of radiolabel data obtained in this study.



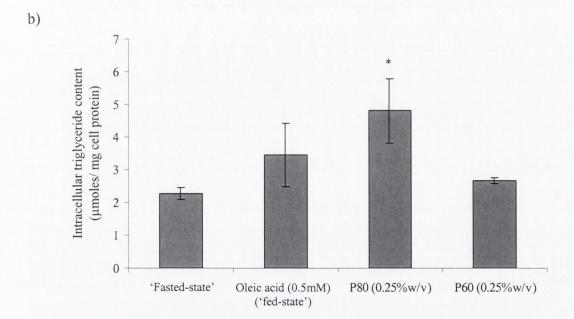


Figure 5.13: Measurement of secreted (panel a) and intracellular (panel b) triglyceride using a colorimetric assay kit following incubation of Caco-2 cells with polysorbate 80 (0.25% w/v), polysorbate 60 (0.25% w/v), 0.5mM oleic acid ('fed-state') or 'fasted-state' control. Values represent the mean  $\pm$  SD (n=4, each comprising samples pooled from 2 filters). Statistical significance relative to the 'fasted-state' control is shown: \*p <0.05.

#### 5.3.4 Dual labelling of lipids

Findings presented in this chapter demonstrate that the fatty acid component of polysorbate 80 is responsible for its promotive effects on TRL secretion. It is most likely that polysorbate 80 acts as a source of oleic acid for triglyceride synthesis. Alternatively, it is possible that polysorbate 80, as an intact ester, may stimulate cells to mobilise endogenous fatty acid stores for triglyceride synthesis, possibly via interaction with a membrane receptor. In order to distinguish between these alternative scenarios, a dual-labelling technique was devised. In this method, cell lipids were pre-labelled over 20 hours with [3H] oleic acid (0.2μCi/ml, added as 'fasted-state', 0.125mM BSA, vehicle) to label intracellular lipids. Cells were rinsed, and [14C] oleic acid (0.2µCi/ml) labelled test vehicles were applied. The test vehicles administered to cells were 'fed-state' (0.5mM oleic acid) or polysorbate 80 (0.5%w/v). Polysorbate 80 was used in this study at 0.5%w/v, the concentration shown to produce a maximum TRL secretion response (Section 5.3.2), to ensure maximum sensitivity of detection. Following a further 20hour incubation, cell lysates and secreted basolateral samples were collected for analysis as described in Section 2.3.2. The relative amounts of [3H] and [14C] oleic acid incorporated into secreted lipids (triglyceride, phospholipid and cholesterol ester) and into secreted lipoprotein fractions (isolated via density gradient ultracentrifugation) were determined. The amount of [3H] oleic acid represented incorporation of endogenous cellular lipids into secreted lipids and the amount of [14C] oleic acid represented newly administered, exogenous fatty acid incorporation. If polysorbate 80 is used as a source of oleic acid, then the relative incorporation of [3H] (endogenous) and [14C] (exogenous) fatty acid into secreted lipids should match that of the free oleic acid vehicle.

#### 5.3.4.1 Dual label composition of secreted and cellular lipids

The dual [³H]/ [¹⁴C] label composition of secreted lipid classes (triglyceride, phospholipid and cholesterol ester) following administration of polysorbate 80 (0.25%w/v) to Caco-2 cells closely mirrored that obtained with a 'fed-state', oleic acid vehicle (Table 5.2). In each case, there was a tendency towards higher [¹⁴C] versus [³H] label incorporation, implying a preference for secretion of lipids synthesised *de novo* from apically absorbed fatty acid (p<0.05). The dual label composition of intracellular lipid classes was also similar for both vehicles (Table 5.3). This suggests that both vehicles act as a source of oleic acid for lipid synthesis and promote secretion of endogenous lipids to a similar extent in Caco-2 cells.

#### 5.3.4.2 Density gradient ultracentrifugation analysis of basolateral samples

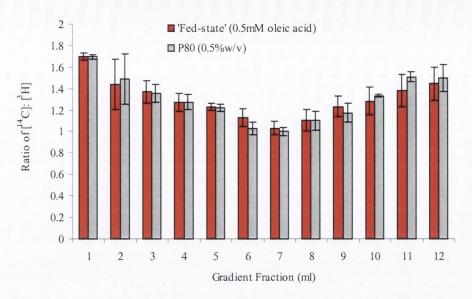
Basolateral samples were subject to density gradient ultracentrifugation (as per Section 2.3.2) and the relative distribution of [³H] and [¹⁴C] into each 1ml fraction of the gradient determined to characterise the secreted lipoprotein profile. The first plot – Figure 5.14 – shows the ratio of [¹⁴C]: [³H] recovered in each fraction, with values >1 indicating higher relative [¹⁴C] content. Lipoproteins from each fraction of the gradient had a similar dual label composition in response to both vehicles, as illustrated by the close parallel between distribution curves. Figure 5.15 compares the relative amounts of [³H] and [¹⁴C] oleic acid recovered in TRL fractions. Both oleic acid and polysorbate 80 enhanced secretion of TRL versus control 'fasted-state', and in each case, the relative [³H]: [¹⁴C] label distribution was similar. As seen with secreted lipid data, there was a tendency towards a higher [¹⁴C] oleic acid content of secreted TRL, demonstrating preferential incorporation of newly absorbed fatty acid into TRL associated lipids. These data reinforce the similarity between free fatty acid and polysorbate 80 effects on lipid metabolism, and imply that polysorbate 80 is used as a source of oleic acid for lipid synthesis in Caco-2 cells.

Table 5.2: Dual [ ${}^{3}H$ ]/ [ ${}^{14}C$ ] label composition of secreted lipids following supplementation of Caco-2 cells with either 'fed-state' or polysorbate 80 (0.5%w/v) vehicles. Results represent the mean (n=4) (SD).

	Relative (%) dual label composition of secreted lipids		
	Triglyceride	Phospholipid	Cholesterol Ester
'Fed-state [3H] label	41.51 (3.91)	24.11 (2.40)	37.89 (6.18)
'Fed-state' [14C] label	58.49 (5.01)	75.89 (12.50)	62.11 (6.61)
Polysorbate 80 [ <sup>3</sup> H] label	44.70 (2.54)	24.48 (1.40)	39.13 (7.32)
Polysorbate 80 [14C] label	55.30 (2.16)	75.52 (10.37)	60.87 (8.97)

Table 5.3: Dual [ $^{3}$ H]/ [ $^{14}$ C] label composition of intracellular lipids following supplementation of Caco-2 cells with either 'fed-state' or polysorbate 80 (0.5%w/v) vehicles. Results represent the mean (n=4) (SD).

	Relative (%) dual label composition of secreted lipids		
	Triglyceride	Phospholipid	Cholesterol Ester
'Fed-state' [3H] label	39.62 (3.9)	30.46 (3.2)	54.94 (2.36)
'Fed-state' [14C] label	60.38 (3.13)	69.54 (12.5)	45.06 (6.18)
Polysorbate 80 [ <sup>3</sup> H] label	45.62 (2.54)	32.48 (5.2)	41.33 (5.19)
Polysorbate 80 [14C] label	54.38 (2.17)	67.52 (1.4)	58.67 (7.32)



**Figure 5.14:** Relative ratio of [ $^{14}$ C]: [ $^{3}$ H] oleic acid in each 1ml fraction of the density gradient following ultracentrifugation of basolateral samples from Caco-2 cells supplemented with 'fed-state' or polysorbate 80 (0.5% w/v) vehicles. Fraction 1= chylomicrons; fraction 2 = VLDL; fractions 5-8 = LDL; fractions 9-12, HDL. Values represent the mean (n=4)  $\pm$  SD.

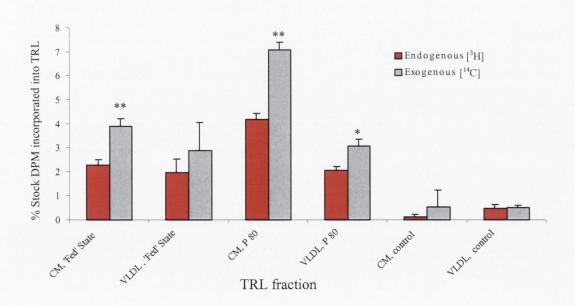


Figure 5.15: Dual [ ${}^{3}$ H]/ [ ${}^{14}$ C] label composition of secreted chylomicrons and VLDL following supplementation of cells with 'fed-state', polysorbate 80 or control 'fasted-state' vehicles. Values represent the mean  $\pm$  SD (n=4). Statistical significance of differences between [ ${}^{3}$ H] and [ ${}^{14}$ C] label incorporation is shown: \*\*p<0.01, \*p<0.05.

#### 5.3.5 Digestion and fatty acid composition of polysorbate surfactants

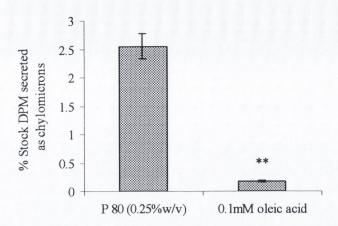
Oleic acid is present in polysorbate surfactants either as free fatty acid contaminant (from manufacture or ester hydrolysis on poor storage) or as an ester of polyoxyethylene sorbitan. Further experiments were conducted to establish whether free fatty acid in the supplied polysorbate 80 stock was sufficient to produce the level of TRL secretion observed above, or whether further liberation of fatty acid from the ester was necessary.

#### 5.3.5.1 Fatty acid composition

The fatty acid composition of polysorbate 80 stock was characterised using gas chromatography, as outlined in Section 2.3.15 in order to determine the proportion of fatty acid present as long chain unsaturated fatty acid. Using gas chromatography, the fatty acid composition of polysorbate 80 was confirmed to be 75.6±1.6 (wt)% oleic acid (with approximately 7.5 (wt)% as linoleic acid and the remainder as palmitic, palmitoleic and stearic acid) and that of polysorbate 60 to be 52.0±0.6 (wt)% stearic acid (with the remainder being largely comprised of palmitic acid). This is in accordance with product specifications as given by the supplier (Sigma-Aldrich, Dublin, Ireland). As both oleic acid and linoleic acid have been shown to be potent stimulators of TRL secretion, then approximately 83% of the polysorbate 80 fatty acid content has potential to promote TRL output.

#### 5.3.5.2 Free fatty acid content of polysorbate solutions

A colorimetric assay kit for non-esterified fatty acid (NEFA test kit, Randox) was employed to calculate the amount of free fatty acid in polysorbate 80 solutions. The total free fatty acid content of a 0.25%w/v polysorbate 80 solution (prepared in serum-free medium) was found to be 0.10 ±0.02mM, which equates to approximately 0.083%w/v oleic acid/ linoleic acid. To establish whether this free fatty acid content was sufficient to produce TRL secretion, cells were administered 'fasted-state' vehicles supplemented with either 0.25%w/v polysorbate 80 or 0.1mM oleic acid. Chylomicron secretion was compared for these vehicles. As shown in Figure 5.16, polysorbate 80 (0.25%w/v) produced a significantly (approximately 20-fold, p<0.01) higher chylomicron output level than the corresponding free fatty acid vehicle (0.1mM oleic acid). This confirms that the free fatty acid content of polysorbate 80 is not sufficient for the excipient effects on TRL secretion.



**Figure 5.16:** Comparative effects of polysorbate 80 (0.25% w/v) and oleic acid (0.1mM) on chylomicron secretion in Caco-2 cells. Values represent the mean  $\pm$  SD (n=4). Significant difference relative to polysorbate 80 (0.25% w/v) solution is shown: \*\*p<0.01.

#### 5.3.5.3 Sensitivity of polysorbate 80 to digestion

A number of experiments were conducted subsequently to investigate the sensitivity of the polysorbate fatty acid ester to hydrolytic degradation by Caco-2 cells (to determine likelihood of hydrolysis during lipid experiments) and by intestinal luminal digestion. These are outlined in detail in Section 2.3.9.

Incubation of polysorbate 80 solution with porcine pancreatin under simulated intestinal conditions over 2 hours resulted in liberation of free fatty acid (13.4-fold increase, p<0.01), thus demonstrating sensitivity to intestinal digestion. Similarly, incubation of polysorbate 80 (0.25%w/v) with Caco-2 homogenate over 20 hours produced a 14.5-fold increase in the level of free fatty acid in the test solution (p<0.01) (Table 5.4). Incubation of polysorbate 80 (0.25%w/v) over 20 hours with apical medium harvested from Caco-2 monolayers (following 20-hour exposure of medium to cells) produced a smaller, 5.2-fold increase (p<0.01), resulting in a final concentration of approximately 0.52mM free fatty acid. [In the latter experiment, apical medium harvested from cells was expected to contain enzymes released from mucosal side of cells, which could contribute to luminal digestion]. These findings indicate that Caco-2 cells have the capacity to digest the fatty acid ester, possibly due to cell-associated esterase or lipase enzymes, intracellularly and in media isolated from cell monolayers. The sensitivity of polysorbate 60 to luminal digestion was also investigated to see if ester hydrolysis was The free fatty acid content of 0.25%w/v solution of applicable to other polysorbates.

polysorbate 60 pre- and post- incubation (2 hours) with pancreatin was found to be  $0.15\pm0.04$ mM and  $1.22\pm0.07$ mM respectively, thus demonstrating sensitivity to digestion.

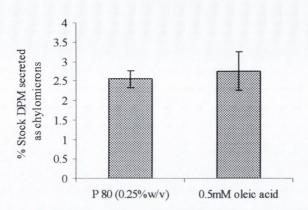
**Table 5.4:** Free fatty acid content of polysorbate 80 solution (0.25% w/v) prior to and following in vitro digestion. Values represent the mean (SD) (n=3).

Incubation conditions for polysorbate 80 (0.25%w/v)	Free fatty acid concentration* (mM) (SD)	
Solution in DMEM - pre-incubation	0.10 (0.02)	
Incubation in blank DMEM (20 hours)	0.12 (0.05)	
Incubation with pancreatin (2 hours)	1.34 (0.12)	
Incubation (20 hours) in apical medium (harvested from cell monolayers following a 20-hour incubation)	0.52 (0.09)	
Incubation (20 hours) with cell homogenate	1.45 (0.30)	

<sup>\*</sup> values were corrected for background readings of each test system incubated without polysorbate 80.

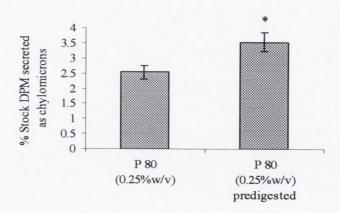
### 5.3.5.4 Importance of polysorbate digestion for the TRL response

Under the standard lipid experimental conditions, polysorbate vehicles are incubated with the apical surface of Caco-2 cell monolayers for 20 hours. Data presented in Table 5.4 shows that these conditions are likely to result in liberation of oleic acid/ linoleic to a final concentration of 0.52mM free fatty acid, which approximates to 0.42mM oleic acid/ linoleic acid. comparative oleic acid-polysorbate 80 experiment was therefore conducted to determine whether a concentration of 0.5mM fatty acid was sufficient for polysorbate 80 effects on TRL secretion. Chylomicron secretion was again used as an index for TRL output. Administration of polysorbate 80 (0.25%w/v) to Caco-2 cells in the 'fasted-state' produced a level of chylomicron secretion (2.54±0.25% of administered stock DPM) similar to that achieved with 0.5mM oleic acid (2.26±0.49 % of administered stock DPM) (Figure 5.17). The difference in mean values was not statistically significant. This indicates that apical metabolism of polysorbate 80 may release sufficient free fatty acid for the observed TRL response. Although differences were not significant, there was a slightly higher TRL response from the polysorbatesupplemented cells. Given that the free oleic acid concentration added for comparison was also somewhat higher than the theoretical content of the apically-digested polysorbate solution, it is possible that additional degradation of polysorbate 80 within the cell may contribute to the TRL secretion response.



**Figure 5.17:** Comparative effects of polysorbate 80 (0.25% w/v) and oleic acid (0.5mM) on chylomicron secretion in Caco-2 cells. Values represent the mean  $\pm$  SD (n=4).

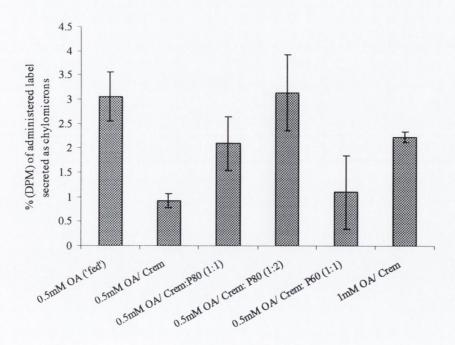
The chylomicron secretion response to polysorbate 80 (0.25%w/v) was enhanced (1.4-fold increase, p<0.05) when the solution was presented to cells following a preliminary simulated intestinal digestion step (Figure 5.18). This was most likely a result of increased fatty acid liberation and shows that luminal digestion will promote utilisation, by intestinal cells, of polysorbate 80 as a fatty acid source



**Figure 5.18:** Effect of pre-digested polysorbate 80 (0.25% w/v) solution on chylomicron secretion in Caco-2 cells. Values represent the mean  $\pm$  SD (n=4). Significant difference relative to polysorbate 80 (0.25% w/v) solution (undigested) is shown: \*p<0.05.

### 5.3.6 Effect of polysorbate 80 on Cremophor EL mediated inhibition of chylomicron secretion

Cremophor EL (0.1%w/v) was previously shown to inhibit TRL secretion in 'fed-state' Caco-2 cells (Chapter Four). To investigate if this inhibitory response could be partly reversed or ameliorated by the promotional effects of polysorbate 80, blends of polysorbate 80: Cremophor EL (1:1 and 2:1, wt:wt) were administered to 'fed' stimulated Caco-2 cells. When polysorbate 80 was administered with Cremophor EL at a 1:1 ratio, a significant improvement in chylomicron secretion was observed relative to a Cremophor EL (0.1%w/v) control, resulting in a 60% reduction in Cremophor EL mediated inhibition (p<0.05) (Figure 5.19). administration of polysorbate 80 at a 2:1 ratio with Cremophor EL resulted in complete reversal of the inhibitory effects of Cremophor EL. Substitution of polysorbate 60 for polysorbate 80 in the blend failed to reverse Cremophor mediated inhibition of TRL secretion (Figure 5.19). These data indicate that additional oleic acid, supplied by polysorbate 80, acts to overcome the inhibitory effects of Cremophor EL on lipid metabolism. To confirm that the oleic acid component was responsible for this reversal, the effects of free oleic acid supplementation of Cremophor EL-inhibited cells were investigated. Supplementation of cells with an additional 0.5mM oleic acid (final concentration of 1mM oleic acid in the vehicle) also acted to ameliorate the observed inhibition of TRL secretion (approximately 60% reduction in inhibition, p<0.01).



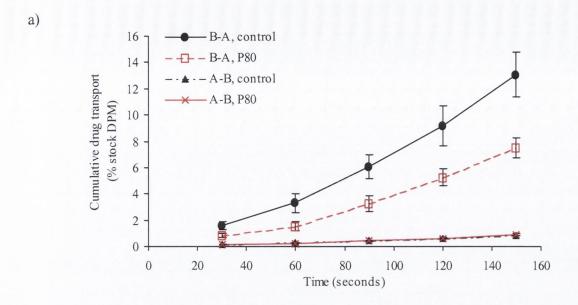
**Figure 5.19:** Effects of polysorbate 80, polysorbate 60 and additional oleic acid on 'inhibited' chylomicron secretion in 'fed-state' cells exposed to Cremophor EL (0.1%w/v). Values represent the mean  $(n=4) \pm SD$ .

#### 5.3.7 Effects of polysorbate surfactants on drug efflux

The previous chapter outlined a putative relationship between inhibition of active drug efflux and of TRL secretion by non-ionic surfactants (Section 4.3.4). All of the tested excipients that were shown to inhibit TRL secretion have also been reported to inhibit drug efflux. Additionally, a strong, correlation was shown between concentration dependent inhibition of TRL secretion and P-gp activity for two surfactants – Pluronic L81 and Cremophor EL.

Data presented here have shown that, in contrast to other tested surfactants, polysorbate 60 has no significant effect on lipid export, and polysorbate 80 promotes TRL secretion. In spite of this, reports in published literature suggest that polysorbate 80 is an effective inhibitor of P-gp related drug efflux (Woodcock *et al.*, 1992; Nerurkar *et al.*, 1996). In order to confirm this action in the Caco-2 model employed in this project, the effects of polysorbate 80 and polysorbate 60 on cyclosporin A (CsA) efflux were investigated using bi-directional transport experiments as described in Section 2.3.6.

Polysorbate 80 (0.25%w/v) was found to inhibit the basolateral-apical transport of CsA as compared to a control (containing no excipient) (Figure 5.20a), resulting in a significant decrease in the basolateral-apical permeability coefficient (approximately 50% decrease, p<0.05) (Table 5.5). Polysorbate 60 (0.25%w/v) produced a slight decrease in the efflux of CsA, but this difference was not significant (Figure 5.20b, and Table 5.5). Mannitol transport was monitored concurrently to verify monolayer integrity and was not affected by polysorbate surfactants (Table 5.6).



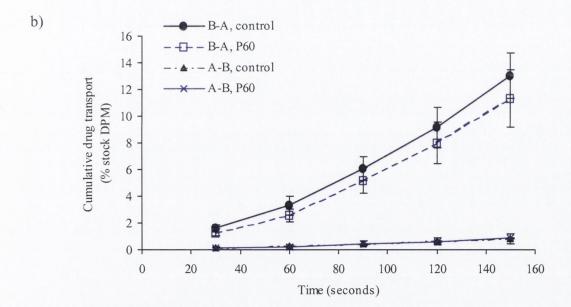


Figure 5.20: Effect of polysorbate 80 (0.25% w/v) (panel a) and polysorbate 60 (0.25% w/v) (panel b) on the transport of cyclosporin A across Caco-2 monolayers in the apical-basolateral and the basolateral-apical directions. Values are expressed as the mean  $\pm$  SD (n=4).

**Table 5.5:** Summary table of permeability coefficients for cyclosporin A across Caco-2 monolayers in the presence of polysorbate surfactants. Values represent the mean  $\pm$  SD (n=4). The Papp ratio (Papp<sub>B-A</sub>/ Papp<sub>A-B</sub>) is also shown.

	Papp*, A-B	Papp*, B-A	Papp ratio
Control	1.33 (0.15)	15.98 (2.90)	12.02
Polysorbate 80 (0.25%w/v)	1.67 (0.71)	9.45 (0.87)	5.67
Polysorbate 60 (0.25%w/v)	1.44 (0.59)	14.22 (2.87)	9.85

<sup>\*</sup>Papp, cm/sec x 10<sup>6</sup>

**Table 5.6:** Summary table of permeability coefficients for mannitol across Caco-2 monolayers in the presence of polysorbate surfactants. Values represent the mean  $\pm$  SD (n=4). The Papp ratio (Papp<sub>B-A</sub>/ Papp<sub>A-B</sub>) is also shown.

	Papp*, A-B	Papp*, B-A	Papp ratio
Control	1.82 (0.18)	1.98 (0.16)	1.24
Polysorbate 80 (0.25%w/v)	1.72 (0.05)	1.93 (0.12)	1.28
Polysorbate 60 (0.25%w/v)	1.66 (0.04)	1.99 (0.13)	1.38

<sup>\*</sup>Papp, cm/sec x 10<sup>6</sup>

#### 5.4 Key findings

Addition of polysorbate 80 (an ester of oleic acid) to 'fed' stimulated cells increased the basolateral secretion of chylomicrons in a concentration dependent manner (initiated at 0.15%w/v, maximum effect at 0.5%w/v).

Addition of polysorbate 80 to 'fasted-state' cells stimulated the secretion of both chylomicrons and VLDL. Effects were concentration dependent, and accompanied by an increase in cellular triglyceride content.

In all cases, effects of polysorbate 80 on TRL output were more pronounced than on cellular triglyceride, resulting in an increase in triglyceride secretion capacity.

Addition of polysorbate 60, an ester of stearic acid, to 'fasted-' or 'fed-state' cells failed to stimulate TRL output, but stimulated LDL secretion in 'fasted-state' cells.

Effects of polysorbate 80 and polysorbate 60 on the lipoprotein secretion profile in Caco-2 cells reflected the responses observed to their component fatty acids in Chapter 3. Thus, indicating that the polysorbate fatty acid moiety is responsible for observed effects.

The fatty acid ester of polysorbate 80 was shown to be susceptible to hydrolysis by Caco-2 homogenate, pancreatic extract and apical cell medium.

Ester hydrolysis within apical medium, and possibly within cell monolayers/ brush border, was found to be important for the effects of polysorbate 80 on lipoprotein secretion in the Caco-2 cell model. This indicates that hydrolysis is required to make incorporated fatty acid available for cellular uptake and metabolism.

Polysorbate 80 was able to reverse the inhibitory effects of Cremophor EL on TRL output in Caco-2 cells.

#### 5.5 Discussion

#### Effects of polysorbate surfactants on lipoproteins secretion in the Caco-2 model

Polysorbate 80, an oleic acid ester of polyoxyethylene sorbitan, was found to produce a concentration dependent increase in TRL secretion in Caco-2 cells. This response was observed when polysorbate 80 was added either to 'fed-state' or 'fasted-state' cells. These observations

are consistent with *in vivo* data previously obtained in our laboratory using a mesenteric lymph cannulated rat model. Data from the rat model showed a concentration dependent increase in lymphatic triglyceride output when polysorbate 80 was administered via intraduodenal infusion at concentrations of 4 and 10%w/v (Lawless *et al.*, 2003). The Caco-2 model showed a TRL secretion response at lower concentrations, with significant effects at 0.15%w/v. Intracellular triglyceride levels in 'fed-state' cells were not affected by polysorbate 80 supplementation, but did show a slight increase in 'fasted-state' cells. The observed selective increase in TRL secretion, without a corresponding increase in cellular lipid or apoB accumulation is similar to the concentration dependent response observed following administration of free oleic acid in Chapter Three. In contrast to polysorbate 80 data, supplementation of 'fed' stimulated cells with polysorbate 60 produced no significant changes in the secreted lipoprotein profile.

#### Comparison of polysorbate versus free fatty acid effects on lipoprotein secretion

The effects of polysorbate 80 and polysorbate 60 on lipoprotein secretion in the Caco-2 model were markedly different. As these two surfactants only differ in the fatty acid component, as shown in Figure 5.1, it is likely that the difference in response is due to the fatty acid moiety. Polysorbate 80 is an oleic acid monoester of polyoxyethylene sorbitan and polysorbate 60 is a stearic acid monoester of polyoxyethylene sorbitan. Oleic acid, a long chain monounsaturated fatty acid, has been shown to promote secretion of chylomicrons and increase lymphatic triglyceride output (Bergstedt *et al.*, 1990; van Greevenbroek *et al.*, 1996), whereas stearic acid, a saturated long chain fatty acid, has been shown to produce low levels of triglyceride output in the rat (Bergstedt *et al.*, 1990) and to stimulate secretion of LDL-sized lipoproteins in the Caco-2 model (van Greevenbroek *et al.*, 1996). It is therefore likely that the promotional effects of polysorbate 80 on TRL secretion are due to its oleic acid moiety. This is further confirmed by the similarity between the secreted lipoprotein profiles obtained with oleic acid versus polysorbate 80 (predominantly chylomicrons and VLDL) and stearic acid versus polysorbate 60 (predominantly LDL).

#### Dual labelling of cellular and secreted lipids

The concentration dependence of the TRL response to polysorbate 80 suggests that the surfactant may be used as a source of oleic acid for triglyceride synthesis. However, there is also a possibility that polysorbate 80 acts as a stimulus or signal for triglyceride production in cells, rather than as a substrate. A dual labelling technique was used to disentangle these mechanisms. Data showed a striking similarity between the effects of oleic acid and polysorbate 80 on labelled fatty acid incorporation into lipoproteins in response to oleic acid and polysorbate 80, with exogenous label being preferentially incorporated into secreted lipoproteins and associated triglyceride. This indicates that polysorbate 80 is being used, in a

similar manner to free oleic acid, as a substrate for lipid synthesis in a similar manner to oleic acid. The observation of preferential incorporation of exogenous versus endogenous fatty acid into secreted TRL is consistent with the existence of a dedicated processing path for shuttling absorbed fatty acids into lymph directed lipoproteins as proposed by Mansbach and Parthasarathy (1982). The absence of a corresponding increase in cellular triglyceride with the elevated TRL output is compatible with the steady state model of triglyceride secretion proposed by Mansbach and Arnold (1986).

#### Sensitivity of polysorbate 80 to digestion

Assuming that polysorbate 80 acts as a source of free oleic acid for triglyceride synthesis, further work was conducted to determine whether the TRL promoting effects were due to unesterified oleic acid present in the surfactant blend (as supplied) or as a result of hydrolysis of the surfactant ester bond in vivo to release additional free oleic acid. The sensitivity of polysorbate 80 to intestinal hydrolysis was assessed by measuring the free fatty acid content of polysorbate 80 solutions prior to and following exposure to pancreatic lipase (as pancreatin). Results showed a significant increase in the free fatty acid content of polysorbate 80 solution following exposure to pancreatic extract, thus demonstrating that polysorbate 80 is sensitive to intestinal digestion at the ester bond. Following a 2-hour incubation with pancreatin under simulated physiological conditions approximately 1.8 µmoles of fatty acid were liberated into the 1.5ml test volume, producing a final concentration of 1.3mM, which is sufficient to promote significant TRL secretion in Caco-2 cells. Susceptibility of polysorbate surfactants to enzyme hydrolysis by a number of mammalian and bacterial lipases and esterases has previously been reported (Saito et al., 1983; Plou et al., 1998). A study by Plou et al. (1998) using a turbidometric assay for enzyme hydrolysis, found that polysorbate 80 acted as an intermediate substrate for both esterases and lipases. The sensitivity of polysorbate 80 to hydrolysis by porcine pancreatic lipase was considerably lower than that of standard substrate oils such as triolein (10-fold difference) or olive oil (150-fold difference). The reason for lower observed sensitivity is likely to be a result of the higher aqueous solubility of polysorbate 80. Lipases are active at oil-water interfaces because the act of binding induces a conformational rearrangement, which exposes the active lipolytic site (Borgström and Erlanson, 1973; van Tilbeurgh et al., 1993; Egloff et al., 1995; Brzozowski et al., 1991). Thus a dispersed oil phase is a more suitable substrate than a micellar polysorbate system. Plou et al. (1998) found that polysorbate 80 was more sensitive (6.5-fold difference) to hydrolysis by porcine liver esterase than by porcine pancreatic lipase. Esterases are present in pancreatin and are active in aqueous solution on dissolved substrates and therefore may have contributed to the observed response.

Endogenous lipase activity, as determined by triolein hydrolysis, has been reported in Caco-2 cells and has been shown to be located primarily in the cytosol and the brush border (Spalinger et al., 1998). This would imply that Caco-2 cells possess the inherent ability to digest lipids and also, potentially, polysorbate surfactants. In order to determine whether or not polysorbate 80 was sensitive to hydrolysis by Caco-2 cells, the free fatty acid content of polysorbate 80 solutions was measured prior to and following incubation with Caco-2 homogenate and apical cell medium. A 20 hour incubation period was selected to reflect lipid experiment conditions. Incubation of polysorbate 80 solutions with Caco-2 homogenate or apical medium harvested from individual Caco-2 monolayers produced a significant increase in free fatty acid concentration. This confirms that Caco-2 cells are capable of digesting polysorbate 80 to liberate free fatty acid. The homogenate was 2.8-times more active than apical medium at hydrolysing polysorbate 80. This was most likely due to a higher concentration of active enzymes within the homogenate as compared to apical medium.

Intestinal mucosal lipase and esterase activity has been identified in the rat (Fernandez-Lopez *et al.*, 1976; Tipton *et al.*, 1989; Rao and Mansbach, 1993; Mahan *et al.*, 2001), and intestinal mucosal esterase activity has been found in the human (Negrel *et al.*, 1977). Thus, it is possible that polysorbate 80 hydrolysis may be mediated by a combination of esterase and lipase activity in both the intestinal lumen and within the mucosa. Hydrolytic enzyme activity in apical medium may be due to secretion of brush border enzymes into medium, or may result from the release of intracellular enzymes via cell lysis.

#### Quantitative comparison of polysorbate 80 versus oleic acid effects on TRL secretion

The Caco-2 TRL secretion level in response to polysorbate 80 solution (0.25%w/v) was found to be equivalent to that produced by 0.5mM oleic acid. This is close to the amount of free fatty acid found in polysorbate 80 samples following overnight incubation in apical medium and is 5-times higher than the free fatty acid content of undigested solution. This implies that the free fatty acid content of polysorbate 80, as supplied, is insufficient to produce the TRL response observed in Caco-2 cells, but exposure to hydrolytic enzymes (esterases or lipases) in apical medium may liberate sufficient fatty acid for this effect. The sensitivity of polysorbate 80 to digestion by pancreatic extract also means that esterified fatty acid in polysorbate will be liberated during intestinal digestion and made available for lipid synthesis.

#### Effects of polysorbate surfactants on P-gp activity

Chapter Four outlined a potential association between the inhibitory effects of non-ionic surfactants on TRL secretion and P-gp activity. However, although polysorbate 80 was found to promote TRL secretion in the Caco-2 model, it was found, in common with published

literature (Woodcock et al., 1992; Nerurkar et al., 1996; Lo, 2003), to inhibit transport protein mediated efflux of cyclosporin A. Differences in polysorbate 80 effects on TRL secretion and P-gp may be due to the presence of oleic acid in the compound, which overcomes any inhibitory effects that the surfactant may have on TRL secretion. Alternatively, cross sensitivity of P-gp activity and TRL secretion to surfactant inhibition may not extend to all polyoxyethylated surfactants.

Polysorbate 60 was found to have no significant inhibitory effects against either P-gp or TRL secretion. However, according to Chapter Four, the presence of a polyoxyethylene grouping and the HLB value of 15 would suggest that this surfactant should have similar TRL inhibitory effects to TPGS, Cremophor EL and Pluronic P85. Polysorbate 60 contains stearic acid, which does not promote high levels of TRL secretion, and hence is unlikely to overcome any inhibitory effects on TRL export that may be associated with polyoxyethylene grouping. This suggests that the criteria for TRL inhibitory activity outlined in Chapter Four may be oversimplistic and that further structural considerations are applicable. Looking at the specific polysorbate structure, the conformation of the polyoxyethylene blocks may be an important factor because the component polyoxyethylene groupings are relatively short and are distributed around the sorbitol ring as four separate ester groups, whereas the polyoxyethylene blocks on the other investigated surfactants are linear.

Differences between the effects of polysorbate 80 and polysorbate 60 on P-gp efflux were unexpected and may reflect different binding affinities resulting from the slight difference in fatty acid saturation or alkyl chain flexibility (oleic acid being monounsaturated and stearic acid being saturated). Polysorbate 60 has not been reported as a P-gp inhibitor in published literature to date.

In summary, polysorbate 80, because of its oleic acid content and sensitivity to intestinal digestion, is an ideal excipient for incorporation into lymphotropic vehicles. As a primary surfactant, polysorbate 80 has the potential to promote lymphatic triglyceride output and as a co-surfactant, it can act to counteract the inhibitory effects of co-administered surfactants on TRL secretion. Its ability to inhibit drug efflux makes polysorbate additionally attractive for incorporation into lymphotropic vehicles, as many lipophilic drug candidates are substrates for P-gp – thus, the combination of inhibited efflux and facilitation of lymphatic transport may result in considerable bioavailability enhancements.

### **Chapter Six**

Formulation and Characterisation of Lipid-Based Vehicles

### 6 Formulation and Characterisation of Lipid-Based Vehicles

#### 6.1 Introduction

There is growing interest in the use of lipid-based vehicles for increasing the bioavailability of co-administered lipophilic drugs. Complex self-emulsifying and microemulsion lipid vehicles are of particular interest in this regard because of their ability to rapidly disperse in the gastrointestinal tract and because of their potential to increase oral bioavailability via a range of mechanisms including increased aqueous drug solubility, altered membrane permeability and modulation of intestinal transport proteins (Shah *et al.*, 1994; Constantinides, 1995; Lawrence and Rees, 2000). Commercial development and design of such formulations to date has, however, been hindered by a lack of suitable guidelines on excipient selection (Charman, 1998). In order to rationally select excipients for such formulations, it is important to have a thorough understanding of the interacting (bioactive and physicochemical) effects excipients may have on lipophilic drug absorption in the gastrointestinal tract. Work presented in Chapters Four and Five has described the use of the Caco-2 model for predicting possible bioactive effects of lipid vehicle excipients on TRL secretion and on intestinal anti-transport proteins. Such data are important for facilitating appropriate excipient selection.

The ability of the vehicle to solubilise an adequate dose of an incorporated lipophilic drug and to maintain it in solution following dilution in gastrointestinal fluids is a further consideration in formulation design. Oil phase selection is critical, and must ensure high drug solubility and, where possible, promotion of lymphatic triglyceride transport. Surfactants are required to ensure rapid vehicle dispersion and enhanced drug solubilisation. Sensitivity of the formulation to intestinal digestion is also critical, as the intestinal milieu post-lipid digestion is rich in bile salts, phospholipids and amphiphilic monoglycerides and fatty acids, which are particularly favourable to solubilisation of lipophilic drugs (Naylor *et al.*, 1995; Mithani *et al.*, 1996).

Halofantrine was selected as a model lipophilic compound to address the effects of lipid vehicles on drug solubilisation. Halofantrine was selected for this work because of its high lipophilicity (log P >8.5) and its reported long chain triglyceride solubility of >50mg/ml, which make it an ideal candidate for lymphatic transport. Halofantrine (Figure 6.1) is an antimalarial 9-phenanthrene-methanol derivative with a melting point of 85°C. It is practically insoluble in water, with an estimated aqueous solubility of <20ng/ml (Lim and Go, 2000). As a tertiary amine, the pKa of halofantrine is predicted to be >10, however, its apparent pKa in simulated 'fed-state' media has been measured as 6.92 using UV spectrophotometric studies (Khoo *et al.*, 2002) and 7.5 using potentiometry experiments (Taillardat-Bertschinger *et al.*, 2003). UV spectrophotometric studies on aqueous buffer solutions (incorporating N-methyl-2-pyrrolidine

as cosolvent) have estimated the aqueous pKa of halofantrine as 5.58 (Khoo *et al.*, 2002), however, more recent potentiometry studies in cosolvent-free systems have measured its pKa as 8-9 (Taillardat-Bertschinger *et al.*, 2003). Molecular modelling calculations to determine low-energy structural orientations of halofantrine suggest that its lower than predicted pKa value could be due to folding of the side chain, facilitating intermolecular hydrogen bonding between the tertiary amine and the benzylic hydroxyl group or the phenanthrene ring, thereby reducing the electron density and associated basicity of the nitrogen atom. The hydrochloride salt of halofantrine has a reported aqueous solubility of 1.3µg/ml and a long chain triglyceride solubility of 0.44mg/ml and is therefore unlikely to undergo lymphatic transport (Khoo *et al.*, 2002).

**Figure 6.1:** Chemical structures of halofantrine base, MW 500.43 (panel a) and Internal standard (IS) (2,4-dichloro-6-trifluoromethyl-9-{1-[2-(dibutylamino)ethyl]}-phenanthrenemethanol, HCl), MW 536.89 (panel b).

#### 6.2 Aims and objectives

The objective of the current study was to develop a microemulsion formulation with high theoretical lymphotropic potential and high capacity for solubilisation of a model lipophilic drug, halofantrine. Initial oil phase selection was made by assessing oil phase potential to enhance chylomicron secretion in the Caco-2 model and ability to solubilise a high dose of halofantrine base. Surface active excipients were chosen to minimise negative effects on chylomicron formation (using data from Chapters Four and Five), and to ensure fine dispersion

of oil phase and incorporated drug on dilution in gastrointestinal fluids. Furthermore, consideration was given to the digestibility of the formulated vehicle to avail of the additional solubilisation properties imparted by lipid digestion

#### 6.3 Results

#### 6.3.1 Selection of vehicle oil phase

Findings presented in Section 3.3.5 and in published literature (Palin and Wilson, 1984; Cheema et al., 1987; van Greevenbroek et al., 1996; Caliph et al., 2000) have shown that an oil phase rich in oleic acid or other long chain, unsaturated fatty acids is most favourable for maximising lymphatic triglyceride output. The relative TRL promoting effects of a number of such oils were compared using the Caco-2 model.

Test oils were selected based on their fatty acid composition. Oils high in oleic and linoleic acid were of particular interest because of their reported effects on TRL secretion (Cheema *et al.*, 1987; Holm *et al.*, 2001). The selected oils included olive oil, arachis oil and corn oil as representative long chain unsaturated oils. Miglyol 812, a medium chain oil, was also investigated for comparative purposes. Fatty acid compositions of investigated long chain oils and of Miglyol 812 are summarised in Table 6.1 and given in detail in Appendix XIV.

Table 6.1: Fatty acid composition (%) and relative density values of investigated oils

Oil	Description and fatty acid composition	Density (g/ml)
Olive <sup>a</sup>	Mixed glycerides of: oleic (55-83%), palmitic (3.5-21%),	0.909-0.915
	linoleic (3.5-21%) and stearic (0.5-5%) acids	(25°C)
Corn <sup>a</sup>	Triglycerides of: linoleic (58.9%), oleic (25.8%), palmitic	0.915-0.918
	(11%) and stearic (1.7%) acids	(25°C)
Arachis <sup>a</sup>	Mixed glycerides of: oleic (56%), linoleic (26%), palmitic	0.915
	(8.3%), stearic (3.1%) and arachidic (2.4%) acids.	(25°C)
Miglyol	Triglycerides of caprylic (50-65%) and capric acids (30-	0.94-0.95
812 <sup>b</sup>	45%)	(20°C)

a: Handbook of Pharmaceutical Excipients (2003)

b: Product information from manufacturer, Sasol, Witten, Germany

Oils were administered to Caco-2 monolayers either as simple emulsions or as pre-digested emulsions to predict the *in vivo* lipid metabolism response. Oil emulsions were stabilised with sodium taurocholate (final concentration of 2.5mM) and vehicle digestion was performed using pancreatin as per Section 2.3.14. In all cases, oils were presented to cells with a final dilution of 1 in 200 in sample buffer: cell medium (1:3) as described in Section 2.3.14.

The viability of cells in the presence of the digestion mix was a particular concern. The lipase used to digest vehicles was added as pancreatin, a pancreatic extract. This extract, in addition to containing pancreatic lipase and colipase, contains a number of other pancreatic enzymes including the protease, trypsin, which is toxic to cells. High concentrations of protein deactivate this enzyme via saturation, and hence, delipidated serum was added to counteract the enzyme. Viability in the presence of digestion buffer diluted in serum-containing medium was determined using an MTT assay for dehydrogenase activity (Section 2.3.4) and monolayer integrity was confirmed by monitoring TEER values (Section 2.3.1), and by measuring monolayer permeability to mannitol (paracellular marker) and propranolol (transcellular marker) (Section 2.3.1), following a 20-hour exposure to digestion buffer. Results were compared to those obtained following incubation of cells with digestion buffer diluted in serum-free culture medium to illustrate the importance of serum.

In the absence of serum, the diluted digestion buffer produced a 70% decrease (p<0.01) in cell dehydrogenase activity. In contrast, dehydrogenase activity was maintained in full when serum was added to the diluting cell culture medium (Table 6.2).

In agreement with MTT findings, supplementation of cells with digestion buffer diluted in serum-free medium produced a significant increase in mannitol permeability (>4-fold, p<0.01) (Figure 6.2a) and a corresponding decrease in monolayer resistance (Table 6.2), which was not observed when serum was added to the medium. Digestion buffer had no statistically significant effects on propranolol permeability (Figure 6.2b).

Table 6.2: MTT and TEER values for Caco-2 cells following 20-hour exposure to digestion buffer (with and without serum). MTT values are expressed relative to a positive control (cells incubated with serum-free medium). TEER values are expressed relative to pre-exposure levels. Values are expressed as the mean (n=3 or 5) (SD). Significance is indicated relative to control or starting value: \*\*p<0.01

	MTT values (n=5) (% relative to control)	TEER values (n=3) (% relative to starting value)
Digestion buffer (without serum)	29.45 (13.51)**	44.3 (12.4)**
Digestion buffer (with serum)	96.8 (11.34)	94.3 (6.2)

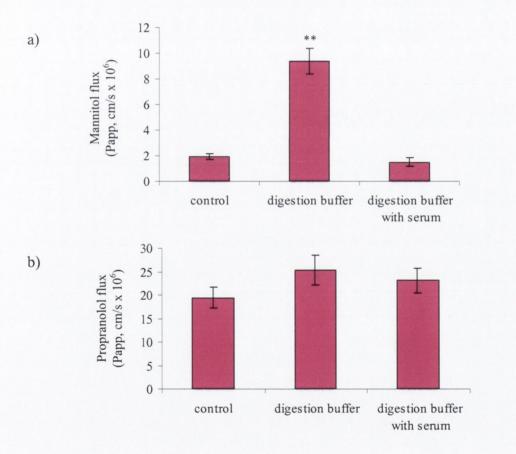


Figure 6.2: Caco-2 Monolayer permeability to mannitol (panel a) and propranolol (panel b) following 20-hour incubation with control buffer or digestion buffer diluted 1 in 4 in serum-free culture medium or culture medium supplemented with 20%v/v delipidated serum. Results represent the average (n=3)  $\pm$  SD. Statistically significant differences from the control level are indicated: \*\*p<0.01.

#### 6.3.1.1 Lipid vehicle toxicity

Lipid vehicle toxicity to cultured cells was a major concern. Cytotoxicity was evaluated by monitoring cell viability and cell monolayer integrity following exposure to test vehicles (non-digested and digested). Cell viability was assessed using an MTT assay for intracellular dehydrogenase activity (Section 2.3.4) and cell monolayer integrity was determined using TEER measurements (Section 2.3.1). The MTT assay used a positive control of cells exposed to serum free culture medium for reference. TEER measurements used the monolayer resistance value taken before vehicle exposure as a control.

Cytotoxicity data for the non-digested oils (Table 6.3) showed no statistically significant changes in monolayer integrity or intracellular dehydrogenase activity following a 20-hour exposure of cells to emulsified oils.

**Table 6.3:** Effects of oil emulsions on cell viability (measured using an MTT assay for cell dehydrogenase activity) and monolayer integrity (measured as TEER). Values represent the mean (n=4 or 5) (SD). Cells were exposed to lipid vehicles for 20 hours prior to taking measurements. Statistical significance relative to positive control (MTT assay) or pre-exposure value (TEER measurements) is shown: \*\*p<0.01.

***************************************	Cell viability (n=5)	TEER values (n=4)
	(% relative to control)	(% relative to start)
Control buffer <sup>a</sup>	95.2 (3.1)	93.7 (4.8)
Olive	92.7 (4.9)	88.9 (7.1)
Miglyol 812	94.0 (11.5)	94.7 (5.6)
Arachis	100.8 (3.9)	95.8 (4.3)
Corn	98.0 (4.1)	95.2 (3.8)

a: control buffer comprised 10mM Tris buffer, pH 6.8, containing 100mM sodium chloride, 1mM CaCl<sub>2</sub>, 10mM sodium taurocholate and 2.5mM lecithin diluted 1 in 4 in serum free culture medium.

Looking at the digested oil data (Table 6.4), a significant decrease in cell monolayer resistance (35% drop, p<0.01) is evident for cells exposed to the digested Miglyol 812 vehicle. A decrease in dehydrogenase activity relative to control was also noted for this vehicle, but the difference was not statistically significant. Cell viability and monolayer integrity remained unchanged in the presence of digested long chain oils under the experimental conditions employed (Table 6.4).

**Table 6.4:** Effects of digested oil emulsions on cell viability (measured using an MTT assay for cell dehydrogenase activity) and monolayer integrity (measured as TEER). Values represent the mean (n=4 or 5) (SD). Cells were exposed to lipid vehicles for 20 hours prior to taking measurements. Statistical significance relative to positive control (MTT assay) or pre-exposure value (TEER measurements) is shown: \*\*p<0.01.

	Cell viability (n=5)	TEER values (n=4)
	(% relative to control)	(% relative to start)
Control buffer <sup>b</sup>	96.8 (11.34)	94.3 (6.2)
Olive	91.4 (4.2)	89.2 (2.6)
Miglyol 812	84.0 (12.7)	65.4 (16)**
Arachis	93.8 (5.6)	90.3 (6.1)
Corn	94.1 (8.0)	92.2 (7.4)

b: control buffer comprised 10mM Tris buffer, pH 6.8, containing 100mM sodium chloride, 1mM  $CaCl_2$ , 10mM sodium taurocholate and 2.5mM lecithin with 4 $\mu$ g/ml pancreatin diluted 1 in 4 in serum free culture medium.

#### 6.3.1.2 Effects of oil vehicles (digested and non-digested) on lipoprotein secretion

Supplementation of Caco-2 cells with long chain oil emulsions produced a shift towards higher secreted TRL levels relative to control buffer, but the response was only statistically significant (p<0.05) for VLDL secretion in the presence of arachis oil. LDL secretion was elevated by the Miglyol 812 and arachis oil emulsions, but again, the difference was only statistically significant for arachis oil (Figure 6.2a).

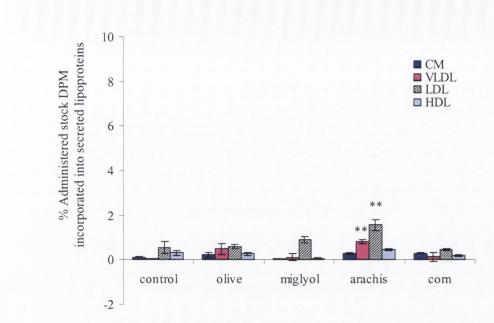
The digested bile salt buffer produced higher secreted lipoprotein levels in Caco-2 cells than the non-digested buffer (significant, p<0.05, for chylomicrons and VLDL). This was most likely a result of free fatty acid being liberated from lecithin, a constituent of the bile salt buffer, by phospholipase A<sub>2</sub>, which is a present in pancreatin. Pre-digestion of long chain oil vehicles resulted in a substantial increase in the secreted chylomicron response in Caco-2 cells. The difference was highest for olive oil (30-fold increase in chylomicron secretion with digested versus non-digested olive oil, p<0.01) as compared to arachis oil (10-fold increase, p<0.01) or corn oil (12-fold increase, p<0.01) (Figure 6.3a,b). For each of the digested long chain oils, chylomicron secretion was substantially higher (p<0.01) than that observed in the presence of control digestion buffer. Although digested long chain oils tended to promote higher secretion levels of VLDL than the non-digested oils, the levels were not statistically different from those obtained with the control digestion buffer (Figure 6.3b). The only difference noted in LDL or HDL levels for digested versus non-digested long chain oils was a decrease (p<0.05) in LDL secretion with digested arachis oil, possibly resulting from a shift towards higher TRL

production (Figure 6.3a,b). The digested Miglyol 812 vehicle failed to stimulate lipoprotein secretion in Caco-2 cells (Figure 6.3b), and produced significantly lower (p<0.01) levels of LDL output than the non-digested vehicle.

Trends observed for the secreted lipoprotein distribution profiles (as obtained via density gradient ultracentrifugation) were reflected by the secreted triglyceride and apo B data. Supplementation of Caco-2 cells with long chain oil emulsions (non-digested) produced a small increase in triglyceride secretion, which was statistically significant (p<0.05) for arachis and corn oils (Figure 6.4a). Miglyol 812 (medium chain oil) had no effect on the secreted triglyceride response. In contrast, administration of either long or medium chain oils resulted in a significant increase (p<0.05) in apo B secretion relative to the bile salt buffer (Figure 6.4b). Vehicle digestion resulted in a substantial increase in the secreted triglyceride response to all administered long chain oils, especially olive oil (Figure 6.4a). The digested olive oil vehicle produced a >5-fold increase (p<0.01) in triglyceride secretion versus the non-digested vehicle, as compared to a <2-fold increase (p<0.01) for arachis and corn oils (Figure 6.4a). Miglyol 812 did not elevate triglyceride secretion when presented in either the non-digested or digested state. Digestion of long chain oils had no effect on the apo B secretion levels relative to non-digested emulsions, hence indicating selective triglyceride enrichment of secreted lipoproteins, as shown by the density gradient ultracentrifugation findings. Digestion of Miglyol 812 resulted in a significant drop (p<0.01) in secreted apo B (Figure 6.4b), which is consistent with a decrease in The observed increase in apo B (p<0.05) and triglyceride (not statistically LDL output. significant) secretion with the digested versus non-digested control buffer is consistent with stimulation of lipoprotein secretion by lecithin-derived fatty acid, and supports density gradient findings.

# 6.3.1.3 Effects of oil vehicles (digested and non-digested) on intracellular accumulation of triglyceride and apo B

Supplementation of Caco-2 cells with long chain oils tended to raise intracellular triglyceride levels, but differences versus the control buffer were not statistically significant (Figure 6.5a). Digestion of long chain oils produced no change in the intracellular content of triglyceride relative to the non-digested oil vehicles, in spite of the observed increase in triglyceride output. In contrast to the secreted triglyceride findings, the digested Miglyol vehicle resulted in a substantial (3.5-fold, p<0.01) elevation in intracellular triglyceride relative to the non-digested vehicle response (Figure 6.5a). Intracellular apo B content was not affected by oil vehicles (long or medium chain) administered in either the non-digested or digested states (Figure 6.5b).



a)

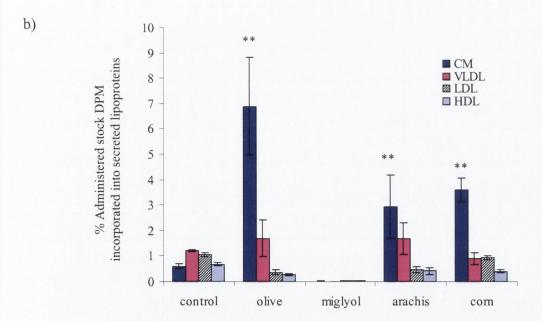
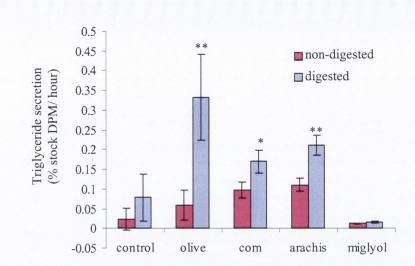


Figure 6.3: Effects of non-digested (panel a) and digested (panel b) oil emulsion vehicles on the secreted lipoprotein profile. Results are expressed as the mean (n=4)  $\pm$  SD. Statistically significant differences between data obtained with oil vehicles and with control buffer are shown: \*\*p<0.01.



a)

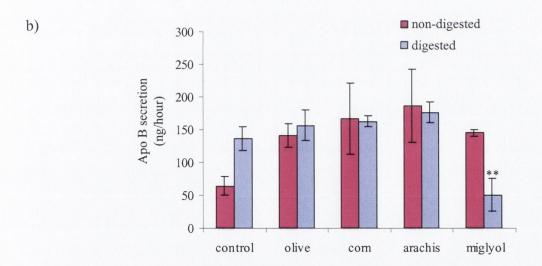
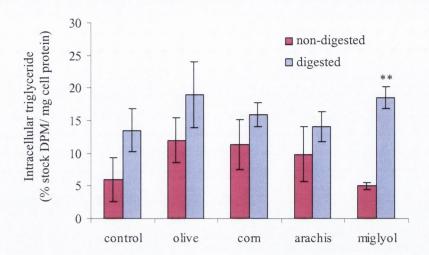


Figure 6.4: Effects of non-digested and digested oil emulsion vehicles on the basolateral secretion of triglyceride (panel a) and apo B (panel b) in Caco-2 cells. Results are expressed as the mean (n=4)  $\pm$  SD. Statistically significant differences between digested and non-digested systems are indicated: \*p<0.05, \*\*p<0.01.

a)



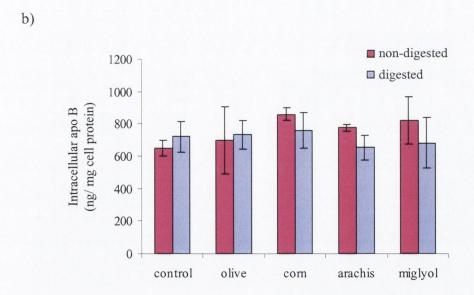


Figure 6.5: Effects of non-digested and digested oil emulsion vehicles on the intracellular accumulation of triglyceride (panel a) and apo B (panel b) in Caco-2 cells. Results are expressed as the mean  $\pm$  SD (n=4). Statistically significant differences between digested and non-digested systems are indicated: \*\*p<0.01.

#### 6.3.2 Solubility of halofantrine in investigated oils

In addition to having good TRL promoting potential, it is essential for the selected oil phase to have high solubilising capacity for the incorporated drug, in this case, halofantrine. As a result, the solubility of halofantrine was tested in each of the investigated oil phases prior to continuing formulation studies.

Solubility studies were conducted at room temperature (25°C), as per Section 2.3.10. Samples were initially taken at 24-hour intervals in order to establish and confirm equilibrium solubility. Equilibrium solubility was reached when differences between consecutive samples were no longer statistically significant (p>0.05). Equilibrium solubility was attained within 72 hours for all tested vehicles. Halofantrine content was assayed using HPLC (Section 2.3.11). Prior to injecting samples onto the column, oil vehicles were subject to a 2-step dilution process – first, they were diluted (50mg/5ml) in chloroform: methanol (2:1) and subsequently, 1 in 100 (v/v) in acetonitrile.

Data are presented in Table 6.5. Solubility in long chain oils was found to be 40-48mg halofantrine/g oil. Solubility was significantly higher (p<0.01) in the medium chain oil, Miglyol 812, (68.3mg/g oil) and significantly lower in Peceol, a monoglyceride of oleic acid (29mg/g Peceol).

To confirm recovery of halofantrine during the 2-step dilution process, samples of olive oil and an olive oil based microemulsion were spiked with halofantrine (30mg/g and 20mg/g vehicle respectively). Spiked samples were diluted as described for unknown samples and analysed via HPLC. Recovery was determined by comparing theoretical and experimentally determined values. Recovery was found to be >99% for each system, hence, the dilution process did not adversely affect drug recovery.

Table 6.5 Equilibrium solubility of halofantrine in long and medium chain oils. Values are expressed as mg halofantrine/g oil. Oil solubilities were determined at room temperature  $(25^{\circ}C)$ . Data represent the mean (n=3) (SD).

Oil phase	Equilibrium (72 hour) solubility (mg/g oil), 25	
olive	40.2 (1.9)	
corn	47.8 (1.5)	
arachis	42.9 (4.8)	
Miglyol 812	68.3 (8.0)	
Peceol	29.1 (1.8)	

#### 6.3.3 Influence of vehicle excipients on lipid digestion

Data presented in Figures 6.2 and 6.3 illustrate the importance of triglyceride digestion in facilitating an intestinal TRL secretion response. Non-ionic surfactants have been shown in some reported in vitro studies to impair pancreatic lipase activity (Gargouri et al., 1983; MacGregor et al., 1997; Patel et al., 1999). It was therefore critical to establish the effects of investigated excipients on lipolysis under simulated in vivo conditions. Initial studies were conducted via titrimetric assay using a pH stat autotitrator (Metrohm Titrino 740) as described in Section 2.3.12. The lipolysis buffer comprised a Tris-Maleate buffer, pH 7.2, with 1%w/v bile extract, which is equivalent to approximately 10mM mixed bile salts (Zangenberg et al., 2001) and within expected post-prandial human duodenal limits (Hernell et al., 1990; Dressman et al., 1998). This technique measures lipolytic activity by monitoring the amount of NaOH required to neutralise liberated fatty acids. There are limitations associated with this analytical method in that the neutralisation reaction requires fatty acids to be present in the ionised state under the given experimental conditions. This is not a problem for short and medium chain fatty acids (pKa<6), but can limit detection of long chain fatty acids, which are reported to have pKa values close to 8 (Shankland, 1970). The mixed micellar phase present in the post-prandial intestine does tend to lower the effective pKa of fatty acids (Benzonana and Desnuelle, 1968; Hernell et al., 1990), but not enough to ensure complete ionisation of long chain fatty acids. As a result, the lipolysis of short chain and medium chain oils is much more sensitive to detection by titration than that of long chain oils as previously demonstrated by Sek et al. (2001) and as shown in Figure 6.6. Because of this, excipient effects on lipid digestion were assessed using the model medium chain triglyceride, Miglyol 812 to ensure good detection sensitivity. The effects of bile salt on lipolysis were also investigated because of discrepancies in reported findings from experimental systems incorporating different amounts of bile.

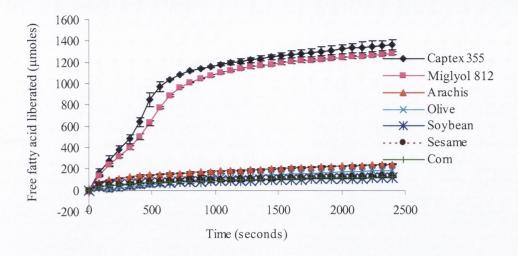
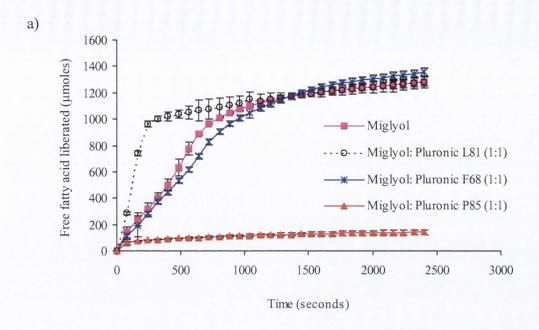


Figure 6.6: Lipolysis of mixed glycerides of long or medium chain fatty acids. Data were obtained by emulsifying oils (0.25g) in 10ml standard digestion buffer (10mM Tris maleate, pH 7.2, 1%w/v bile extract, 2mM CaCl<sub>2</sub>), 37%C and titrating against 0.1M NaOH. Plots represent the mean of 3 determinations  $\pm$  SD. Fatty acid compositions of investigated oils are given in Appendix XIV.

#### 6.3.3.1 Titrimetric analysis of lipolysis

Addition of Pluronic P85 to the Miglyol 812 emulsion (at either a 1:1 or a 1:2 surfactant: oil ratio, on a weight basis), in the presence of bile, produced a substantial drop in both the rate and the extent of *in vitro* lipolysis (Figure 6.7a,b). Inhibitory effects of Pluronic P85 were still maintained when the Pluronic P85 concentration was reduced by a factor of five (Figure 6.7b). In contrast, addition of Pluronic L81 to the standard digestion mix, at a 1:1 oil: surfactant ratio, resulted in an increase in the initial rate, but not the extent, of lipolysis (Figure 6.7a). When the Pluronic L81 concentration was halved, this effect was not seen (Figure 6.7b). Addition of Pluronic F68 to the digestion mix at a 1:1 or 2:1 oil: surfactant ratio produced no change in lipolysis (Figure 6.7a,b).

In the absence of bile, Pluronic surfactants all produced significant inhibition of lipolysis when added at an oil: surfactant ratio of 1:1. When the excipient concentration was halved, the inhibitory effects of Pluronic L81 on Miglyol 812 digestion were ameliorated, but the negative effects of Pluronic P85 and F68 persisted (Figure 6.8a,b).



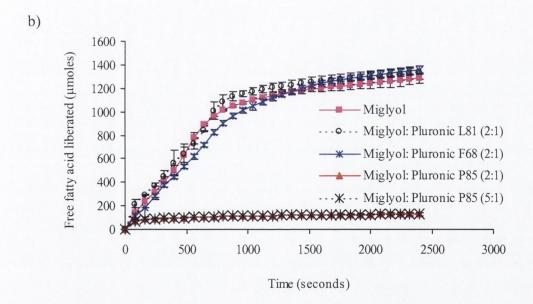
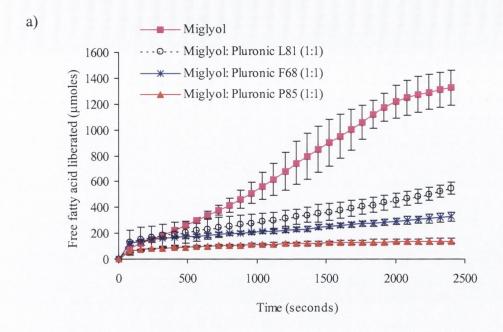
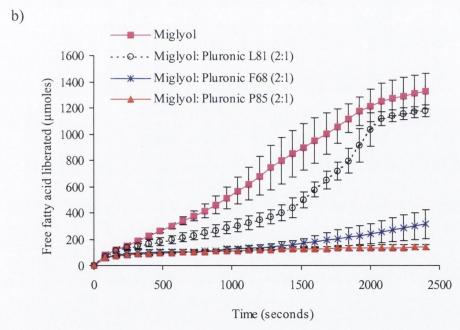


Figure 6.7: Effects of Pluronic block copolymers on the lipolysis of Miglyol 812 in standard buffer (10mM Tris maleate, pH 7.2, 1%w/v bile extract, 2mM CaCl<sub>2</sub>). Plots represent the mean of 3 determinations ± SD. Panel a- Miglyol 812: Pluronic, 1:1. Panel b- Miglyol 812: Pluronic, 2:1 or 5:1.





**Figure 6.8:** Effects of Pluronic block copolymers on the lipolysis of Miglyol 812 in bile-free buffer (10mM Tris maleate, pH 7.2, 2mM CaCl<sub>2</sub> buffer). Plots represent the mean of 3 determinations  $\pm$  SD. Panel a- Miglyol 812: Pluronic, 1:1. Panel b- Miglyol 812: Pluronic, 2:1.

When Cremophor EL and polysorbate 80 were added to the standard Miglyol 812 digestion mix at an oil: surfactant ratio of 1:1, there was a small decrease in the rate of lipolysis over the initial 25 minutes, however, by the end of the 40 minute investigative period, the overall extent of lipolysis was not affected (Figure 6.9).

In the absence of bile salt, Cremophor EL and polysorbate 80 were both found to effectively impair lipolysis of Miglyol 812 in the test system (Figure 6.10a). This inhibitory effect was attenuated somewhat when the excipient concentrations were halved, but the extent of hydrolysis was still less than 50% of the Miglyol 812 control level (Figure 6.10b).

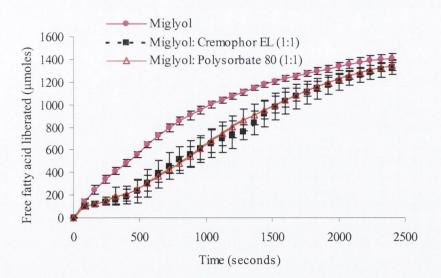
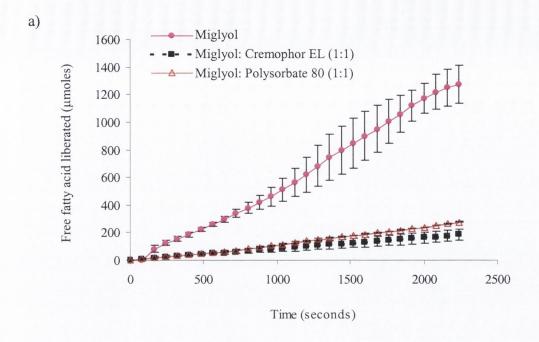


Figure 6.9: Effects of polysorbate 80 and Cremophor EL (at a Miglyol 812: surfactant ratio of 1:1) on the digestion of Miglyol 812 in standard buffer (10mM Tris maleate, pH 7.2, 1% w/v bile extract, 2mM CaCl<sub>2</sub>). Plots represent the mean of 3 determinations  $\pm$  SD.



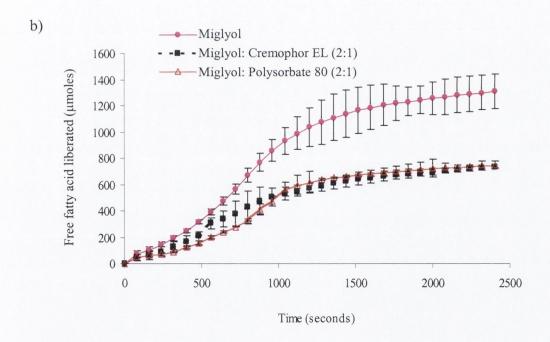


Figure 6.10: Effects of polysorbate 80 and Cremophor EL on the digestion of Miglyol 812 in bile-free buffer. Plots represent the mean of 3 determinations  $\pm$  SD.

Panel a- Miglyol 812: surfactant, 1:1. Panel b- Miglyol 812: surfactant, 2:1.

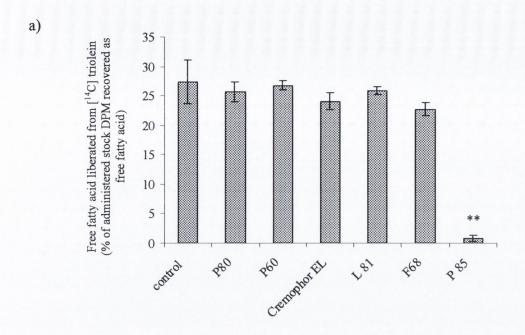
# 6.3.3.2 Lipolysis of [14C] triolein

Radiolabelled triolein (glycerol tri [14C] oleate) was used as a substrate for lipolysis in order to directly assess the effects of added excipients on long chain triglyceride digestion and to substantiate data obtained using the pH stat technique. The radiolabel assay was conducted as described in Section 2.3.12. Briefly, [14C] triolein was incubated with test excipients over a 2-hour period under simulated intestinal conditions. Free fatty acid ([14C] oleic acid) was extracted from the assay mix and quantified via liquid scintillation counting to give a value for total fatty acid release over 2 hours. No rate data are available using this technique, but detection sensitivity is considerably enhanced. As a result of the higher assay sensitivity, it was possible to investigate Caco-2 homogenate-mediated triglyceride digestion in addition to pancreatic lipase mediated data. Surfactants investigated here included: Cremophor EL, polysorbate 80, polysorbate 60 and Pluronic block copolymers P85, F68 and L81. Surfactants were added at a concentration of 0.1%w/v to simulate expected intestinal luminal concentrations.

In the presence of bile salt, Pluronic P85 (0.1%w/v) was found to significantly impair pancreatic lipase (5µg/ml pancreatin)-mediated digestion of triolein (Figure 6.11a). None of the other investigated excipients inhibited lipolysis under these experimental conditions.

In the absence of bile salts, all of the investigated surfactants resulted in a significant reduction in the extent of lipid digestion by pancreatin (Figure 6.11b). There was a 4-fold reduction in lipolysis of triolein in the presence of both Pluronic L81 and Pluronic F68, and a >12-fold decrease (p<0.01) with all other tested surfactants.

Triolein was sensitive to lipolytic degradation by Caco-2 homogenate, but activity associated with 1.6mg cell protein/ml was considerably lower (24-fold, p<0.01) than that observed with 5μg/ml of pancreatin. Digestion of triolein by Caco-2 homogenate (in standard bile salt buffer) was significantly impaired in the presence of: polysorbate 80, polysorbate 60, Cremophor EL and Pluronic P85. In each case, the reduction was no greater than 50% (Figure 6.12).



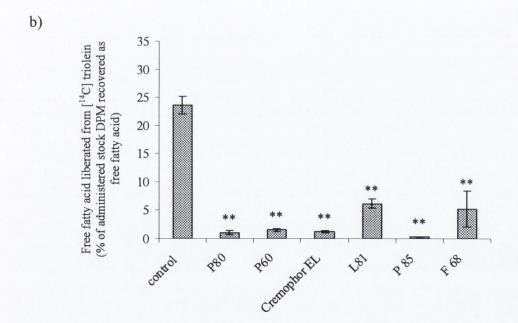
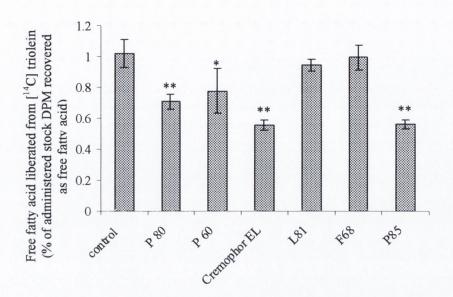


Figure 6.11: Effect of excipient addition (0.1%w/v) on digestion of glycerol tri-[ $^{14}$ C] oleate by pancreatin in the presence (panel a) or absence (panel b) of bile salt (10mM sodium taurocholate). Vehicles (50µl final volume) were incubated with 5µg/ml pancreatin for 2 hours at 37°C in a shaking water bath (100 rpm). Free fatty acid was subsequently extracted using the method of Belfrage and Vaughan (1969). Plots represent the mean (n=4)  $\pm$  SD.



**Figure 6.12:** Effect of excipient addition (0.1% w/v) on digestion of glycerol tri-[14C] oleate digestion by Caco-2 homogenate in the presence of bile salt (10mM sodium taurocholate). Vehicles (50µl final volume) were incubated with homogenate equivalent to 1.6mg/ml protein for 2 hours at 37°C in a shaking water bath (100 rpm). Free fatty acid was subsequently extracted using the method of Belfrage and Vaughan (1969). Plots represent the mean  $(n=4) \pm SD$ .

#### 6.3.4 Microemulsion Formulation and Characterisation

Data presented above (Figures 6.3 and 6.4) indicate that under physiological conditions, olive oil displays clear potential, in terms of promoting lymphatic triglyceride transport, versus other investigated long chain oils. For this reason, olive oil was selected as the oil component of a lymphotropic microemulsion vehicle. Surfactant selection for the formulation was based initially on data presented in Chapters Four and Five, with the aim of avoiding surfactantinduced inhibition of TRL secretion. Preliminary formulation work therefore focussed on polysorbate 80, Pluronic F68 and polysorbate 60 because negative influences on TRL secretion were either minimal or absent for these compounds. All formulations were prepared as a preconcentrate and subsequently diluted 1 in 200 in either 0.1N HCl (simulated gastric medium), or in bile salt/ lecithin solutions (low bile salt (BS): 3mM sodium taurocholate: 0.75mM lecithin solution as simulated 'fasted-state' intestinal fluid or high BS: 15mM sodium taurocholate: 3.75mM lecithin solution as simulated 'fed-state' intestinal fluid). Preliminary formulations incorporating olive oil with these surfactants failed to produce clear, stable microemulsions on aqueous dilution, as determined by visual assessment. selection was therefore expanded to include Cremophor EL, Pluronic P85 and TPGS in combination with polysorbate 80. Polysorbate 80 was added to ameliorate surfactant induced TRL inhibition because of its demonstrated promotive effects on chylomicron output (Chapter Five). Preliminary work found that a Cremophor EL: polysorbate 80 blend was most successful for generating microemulsions. It was further found that addition of Peccol (glycerol monooleate) to the oil phase helped to reduce microemulsion droplet size. This also had the added benefit of increasing the long chain unsaturated fatty acid loading of vehicles. The formulation was subsequently optimised by sequentially altering the relative amounts of each component to ensure maximum olive oil content and a small dispersed droplet size. Preconcentrate formulations were prepared as per Section 2.3.13.

A microemulsion can be described as a system of water, oil and amphiphile, which forms a single optically isotropic and thermodynamically stable liquid (Danielsson and Lindman, 1981). Hence, initial evaluation of diluted (1 in 200) vehicles was conducted by visual assessment of microemulsion clarity. In order to qualify this initial assessment, two further techniques were employed to characterise the oil-in-water dispersion - turbidity measurements and PCS size analysis (as per Section 2.3.13). Turbidity was used as a means of quantifying system clarity in a non-subjective manner. Throughout this work it was generally found that turbidity values of <1 corresponded to visually clear or slightly hazy, blue-tinged, systems which were consistent with microemulsion formation. Values greater than this were associated with cloudy systems or systems with phase separation, and therefore represented crude emulsions. Where systems

displayed good clarity, it was possible to employ PCS size analysis to obtain a more accurate estimate of microemulsion droplet size. A close correlation ( $r^2$ =0.822) was found between turbidity measurements and PCS size measurements throughout this work for droplet sizes of up to 150nm, as illustrated in Figure 6.13, thus confirming the relevance of clarity as a means of classifying microemulsions. A similar correlation between microemulsion droplet size and turbidity has previously been reported (Fletcher and Morris, 1995). Microemulsions typically have a droplet size of less than 100nm (Cortesi and Nastruzzi, 1999), which corresponds approximately to turbidity of <0.6.

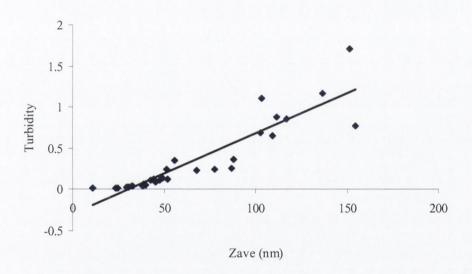


Figure 6.13: Correlation between formulation turbidity (as determined from absorbance measurements at  $\lambda$ =400nm) and Zave (nm, as determined via PCS analysis) for a range of lipid-based formulations. Linear regression analysis was conducted using Microsoft® Excel 2000. The fitted line equation was found to be: y=0.0097x-0.2942, r²=0.822.

#### 6.3.4.1 Effect of Peceol concentration on microemulsion formation

Preconcentrate formulation optimisation work was initiated with a fixed olive oil concentration of 20%w/w, and a surfactant ratio of Cremophor EL: polysorbate 80 (1:1). Peccol content was incrementally increased from 0-50%w/w. Results are shown in Figure 6.14 for turbidity and PCS data. The resultant microemulsion droplet size was found, in all media, to follow a decreasing trend, reaching a minimum at 20%w/w Peccol. Beyond this concentration, droplet size was found to rise with increasing Peccol content. Dispersed droplet size tended to be lower in the bile salt containing buffers versus acid solution. Smallest droplet size (40nm) obtained was for the formulation containing 20%w/w Peccol diluted in either low BS or high BS buffers.

#### 6.3.4.2 Effect of olive oil concentration on microemulsion formation

The effect of olive oil content on microemulsion formation was examined in a system containing 20%w/w Peccol and a preliminary surfactant ratio of Cremophor EL: polysorbate 80 (1:1). Findings for microemulsion turbidity and PCS data are shown in Figure 6.15. At concentrations of <20%w/w olive oil, the nature of the diluent was important, with the high BS buffer producing far superior microemulsions to either 0.1N HCl or low BS buffers. For microemulsions prepared in high BS buffer or HCl, an olive oil content of 20-25% was optimal, producing preparations with dispersed droplet size <70nm in all media. However, on standing for a week at ambient temperature, the product with 25%w/w oil showed phase separation, and hence was unstable. Further studies were conducted with 20%w/w olive oil and 20%w/w Peccol.

#### 6.3.4.3 Effect of Cremophor EL: polysorbate 80 ratio on microemulsion formation

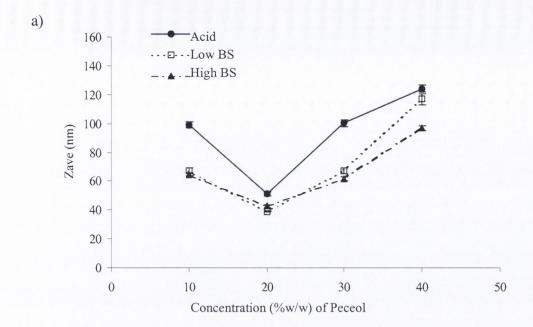
The ratio of polysorbate 80: Cremophor EL was varied to establish whether or not it was possible to elevate the polysorbate 80 content without adversely affecting microemulsion formation. Using a vehicle containing 20%w/w olive oil and 20%w/w Peceol, the polysorbate 80: Cremophor EL ratio was varied from 1:0 to 0:1 in increments. Data presented in Figure 6.16 show that a higher content of Cremophor EL was desirable for microemulsion formation, but that microemulsion characteristics were maintained at a ratio of polysorbate 80: Cremophor EL of 2:1. When diluted in acid, this polysorbate 80: Cremophor EL ratio produced a good microemulsion, with dispersed droplet size estimated at 53.4nm via PCS when diluted in 0.1N HCl. Dilution in bile salt buffers, representative of intestinal conditions, produced a significant (p<0.05) reduction in dispersed droplet size, resulting in Zave values of 42 and 32nm in low BS and high BS buffers respectively. As the higher polysorbate 80 ratio was beneficial to promoting TRL output in Caco-2 cells (as shown in Chapter 5), an investigational vehicle containing a polysorbate 80: Cremophor EL ratio of 2:1 was selected for further studies.

#### 6.3.4.4 Effect of cell culture medium on microemulsion formation

Subsequent studies will examine the effects of formulated lipid vehicles on lipid metabolism and drug transport in the Caco-2 model. When administering vehicles to cells, it is necessary to dilute them in cell culture medium (DMEM) containing delipidated serum. For this reason, the effects of culture medium on dispersed droplet size were evaluated. For this purpose, 0.1N HCl and bile salt solutions were prepared in culture medium instead of water. The culture medium-based buffers were used to dilute the developed microemulsion preconcentrate formulation, as described above, and particle size analysis conducted via PCS. Results were compared to those obtained using standard buffers. Findings, as presented in Table 6.6, showed that culture medium did not significantly alter the obtained Zave values and hence, was unlikely to impact significantly on microemulsion formation.

#### 6.3.4.5 Effect of halofantrine on microemulsion droplet size

On dilution in 0.1N HCl solution, incorporation of halofantrine (saturation concentration) into the microemulsion preconcentrate resulted in a small (7%, p<0.05) decrease in average dispersed droplet diameter versus the halofantrine-free system. However, on dilution in low BS buffer, halofantrine had no effect on dispersed droplet size (Table 6.7), indicating that it is unlikely to influence vehicle dispersion in the intestine.



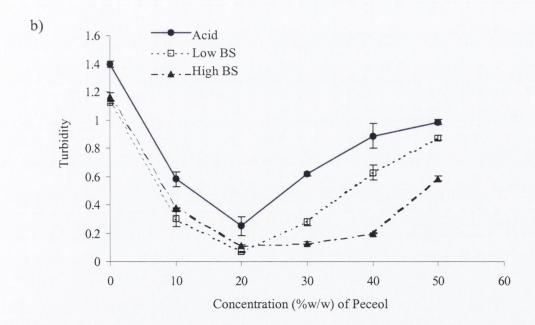
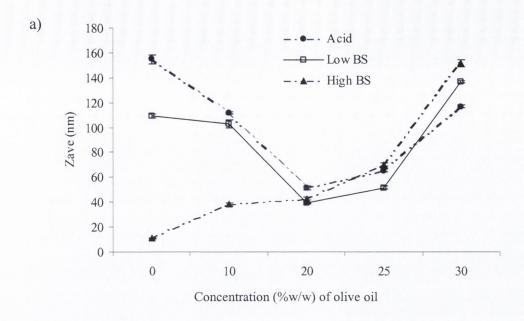


Figure 6.14: Effects of Peceol concentration on microemulsion droplet size as determined via PCS size analysis (panel a) and turbidity measurements as determined from absorbance measurements at  $\lambda$ =400nm (panel b) in a formulation comprising olive oil (20%w/w) and a surfactant blend of Cremophor EL: polysorbate 80 (1:1). Results are expressed as the mean (n=3)  $\pm$  SD.



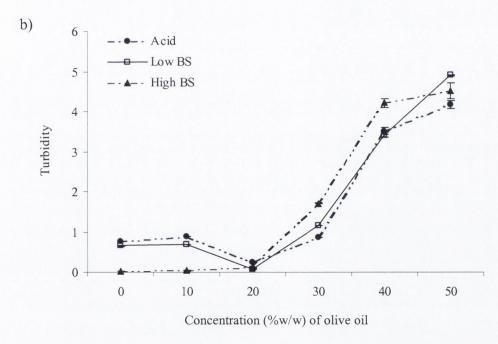
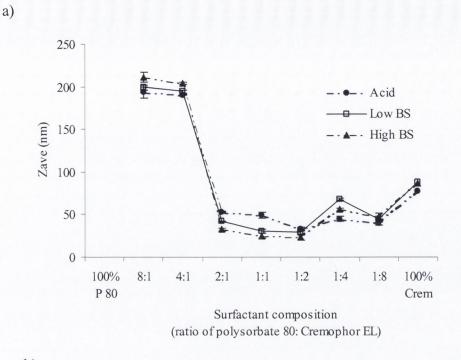


Figure 6.15: Effects of olive oil concentration on microemulsion droplet size as determined via PCS size analysis (panel a) and turbidity measurements as determined from absorbance measurements at  $\lambda$ =400nm (panel b) in a formulation comprising Peceol (20%w/w) and a surfactant blend of Cremophor EL: polysorbate 80 (1:1). Results are expressed as the mean (n=3)  $\pm$  SD.



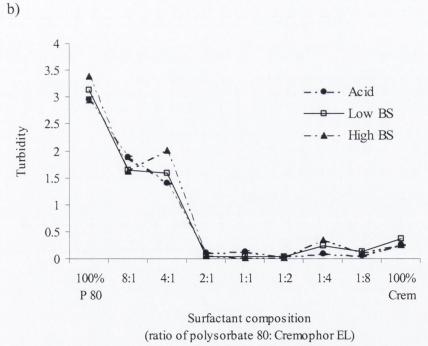


Figure 6.16: Effects of surfactant ratio on microemulsion droplet size as determined via PCS size analysis (panel a) and turbidity measurements (panel b) in a formulation comprising 20% w/w Peceol, 20%w/w olive oil and 60%w/w surfactant blend (polysorbate 80: Cremophor EL with varying ratio). Results are expressed as the mean (n=3) measurements  $\pm$  SD.

**Table 6.6:** Effect of buffer diluent (water versus cell culture medium) on dispersed microemulsion droplet size as measured via PCS (Zave, nm). The microemulsion preconcentrate formulation comprising olive oil (20%w/w), Peceol (20%w/w) and polysorbate 80: Cremophor EL (2:1) (60%w/w) was diluted 1 in 200 in each test buffer and incubated over 2 hours at 37°C, with agitation. Values represent the mean (n=3) (SD).

Solvent	0.1N HCl	Low BS buffer
Water	53.4 (1.3)	39.0 (0.4)
DMEM	56.7 (2.6)	40.0 (1.3)

**Table 6.7:** Effect of halofantrine on dispersed microemulsion droplet size as measured via PCS (Zave, nm). The microemulsion preconcentrate formulation comprising olive oil (20% w/w), Peceol (20% w/w) and polysorbate 80: Cremophor EL (2:1) (60% w/w) was saturated with halofantrine and subsequently diluted 1 in 200 in test buffer and incubated over 2 hours at 37°C, with agitation. Values represent the mean (n=3) (SD).

Diluent	Control (no halofantrine)	Halofantrine
0.1N HCl	53.4 (0.3)	49.5 (0.4)
Low BS buffer	39.0 (0.4)	38.8 (1.1)

#### 6.3.5 Effect of co-administered lipid vehicles on the aqueous solubility of halofantrine

#### 6.3.5.1 Halofantrine solubility in vehicle preconcentrates

The solubility of halofantrine in various oil phases was discussed above. However, pharmaceutically useful lipid vehicles tend to have a high surfactant content to ensure vehicle dispersion in gastrointestinal fluids. It is therefore important to measure drug solubility in the final vehicle to estimate deliverable dose. Drug solubility was measured as described in Section 2.3.10 at 25°C, and equilibrium solubility was taken at the 72-hour time point. Solubility was determined in the above described olive oil based microemulsion preconcentrate (20%w/w olive oil, 20%w/w Peceol and 60%w/w polysorbate 80: Cremophor EL, 2:1). For comparative purposes, a microemulsion preconcentrate incorporating Miglyol 812 was also evaluated. This microemulsion substituted Miglyol 812 for the oil phase (30%w/w) and contained a Cremophor EL: polysorbate 60 (2:1) surfactant blend. This microemulsion formed a good dispersion on mixing with acid or bile salt buffers (Zave <40nm in all cases, as measured using PCS), and contained no long chain unsaturated fatty acids. Solubility was also evaluated in polysorbate 80, because of the potential this excipient has for forming an oleate rich micellar system in aqueous media. Microemulsion preconcentrate compositions are summarised in Table 6.8 and drug solubility data in Table 6.9. Data for halofantrine solubility in microemulsion vehicles was compared to solubility data obtained in the constituent oils. Solubility data for these oils is reproduced in Table 6.8 to facilitate comparisons. Halofantrine solubility was found to be significantly higher (p<0.05) in the simple oil vehicles, as compared to more complex microemulsion formulations or polysorbate 80. Furthermore, solubility was higher (p<0.01) in Miglyol 812, a medium chain triglyceride, as compared to olive oil, a long chain oil (Table 6.10).

#### 6.3.5.2 Halofantrine solubility following vehicle dilution in gastrointestinal fluids

Additionally, the effects of co-administered lipid vehicles on the aqueous solubility of halofantrine in the gastrointestinal tract were evaluated *in vitro*. Vehicles were saturated with halofantrine and allowed to reach equilibrium solubility by agitating in a shaking water bath at 25°C for 72 hours as per section 2.3.13. Vehicles were subsequently diluted (1 in 200, 37°C, 2 hours), as described in Section 2.3.13, in each of three buffers - 0.1N HCl to simulate gastric fluid, 3mM sodium taurocholate: 0.75mM lecithin in Tris buffer, pH 7 to simulate fasted intestinal fluid and 3mM sodium taurocholate: 0.75mM lecithin in Tris buffer, pH 7 with added pancreatin (1000TBU/ml) to simulate initiation of intestinal digestion. For each test system, the aqueous phase was separated from the resulting mixture by ultracentrifugation (described in Section 2.3.13) at 300,000g for 30 minutes, diluted 1 in 10 or 1 in 20 in acetonitrile and assayed directly using HPLC for halofantrine content.

Following dilution in 0.1N HCl, the microemulsion formulations offered considerable advantages over the less complex formulations in terms of the obtained aqueous halofantrine concentration. Aqueous solubilisation levels for halofantrine were at least 15-fold (p<0.01) higher with the microemulsion formulations as compared to the corresponding oil emulsions and 1.4-1.6-fold higher than the polysorbate 80 solution (p<0.05) (Table 6.10). The administered halofantrine dose was dependent on the saturation solubility of drug in the preconcentrate vehicle, so data were dose corrected to determine the % of the administered halofantrine dose recovered in aqueous phase following dilution. It was found, upon dilution in 0.1N HCl, that the micellar polysorbate 80 solution and the Miglyol based microemulsion both facilitated complete solubilisation of their contained dose (Table 6.11). Drug solubility from these vehicles remained at approximately 100% upon dilution in bile salt and digestive buffers. The olive oil microemulsion facilitated solubilisation of 68-78% of its incorporated halofantrine dose in the test buffers. Although solubilisation appeared to be higher in bile salt and digested buffers versus the acid solution, differences were not statistically significant.

In contrast, the amount of drug solubilised from the crude oil dispersion was highly dependent on the nature of the diluent. In acid solution, the amount of halofantrine recovered in aqueous solution from the crude oil dispersions (olive oil or Miglyol 812) accounted for <3% of administered dose. This was, however, increased to 13-17% (p<0.01), when vehicles were diluted in bile salt buffer. Following simulated intestinal digestion of oil emulsions, the amount of drug recovered in aqueous solution was further increased to approximately 30% (p<0.01) of administered dose. These data demonstrate the importance of vehicle digestion for crude oils.

**Table 6.8:** Composition of microemulsion preconcentrate formulations employed in solubility studies

Olive oil microemulsion	Composition (%w/v)	Miglyol 812 microemulsion	Composition (%w/v)
Olive oil	20	Miglyol 812	30
Peceol	20	Polysorbate 60	20
Cremophor EL	20	Cremophor EL	50
Polysorbate 80	40		

**Table 6.9:** Solubility of halofantrine base in lipid-based vehicle preconcentrates. Equilibrium solubility was taken at 72 hours. Values are expressed as mg halofantrine/g oil. Measurements were taken at room temperature ( $25^{\circ}$ C). Data represent the mean (n=3) (SD).

Vehicles	Equilibrium solubility (mg/g), at 25°C in vehicle preconcentrates
Olive oil	40.2 (1.9)
Miglyol 812	68.3 (8.0)
Olive oil microemulsion	31.9 (0.8)
Miglyol 812 microemulsion	25.2 (3.6)
Polysorbate 80	14.9 (1.5)

**Table 6.10:** Aqueous solubility ( $\mu g/ml$ ) of halofantrine in gastrointestinal buffers following 1 in 200 dilution of halofantrine-saturated oils and lipid vehicle preconcentrates. [Vehicles were agitated at 150 rpm for 2 hours at 37°C, and centrifuged at 300,000g for 30 minutes to separate aqueous and oil phases and to precipitate undissolved drug/insoluble digestion products. Aqueous phase was assayed for drug content]. Values represent the mean (SD) (n=3).

Vehicle	Halofantrine solubility (µg/ml) in different media			
	0.1N HCl	Low BS	Low BS/ pancreating (Digestion buffer)	
Olive oil	5.9 (0.6)	35.2 (2.2)	66.7 (0.8)	
Miglyol 812	6.2 (1.9)	44.4 (4.1)	94.7 (22.4)	
Olive oil M/E	108.4 (16.3)	115.4 (17.7)	125.3 (21.5)	
Miglyol 812 M/E	121.0 (7.3)	138.9 (25.9)	106.4 (4.5)	
Polysorbate 80	74.3 (6.3)	82.0 (3.9)	72.9 (11.2)	

**Table 6.11:** % of halofantrine dose recovered in aqueous phase of gastrointestinal buffers following 1 in 200 dilution of halofantrine-saturated oils and lipid vehicle preconcentrates. [Vehicles were agitated at 150 rpm for 2 hours at 37°C, and centrifuged at 300,000g for 30 minutes to separate aqueous and oil phases and to precipitate undissolved drug/ insoluble digestion products. Aqueous phase was assayed for drug content]. Values represent the mean (SD) (n=3).

Vehicle	% Halofantrine dose recovered in aqueous phase			
	0.1N HCl	Low BS	Low BS/ pancreatin (Digestion buffer)	
Olive oil	2.9 (1.0)	17.5 (1.5)	33.3 (1.0)	
Miglyol 812	1.8 (2.4)	13.0 (8.7)	27.7 (11.1)	
Olive oil M/E	68.0 (16.5)	72.3 (8.8)	78.6 (10.7)	
Miglyol 812 M/E	96.0 (6.5)	110.2 (13.2)	84.4 (3.6)	
Polysorbate 80	99.7 (8.7)	110.1 (5.2)	97.8 (5.9)	

# 6.4 Key findings

A microemulsion formulation was designed to increase the aqueous solubility, and to potentially promote lymphatic uptake, of a lipophilic drug, halofantrine, with the ultimate aim of enhancing its oral bioavailability. Microemulsion formulation was designed using guided excipient selection.

The formulation oil phase was selected based on its ability to stimulate TRL secretion in Caco-2 cells. Of the tested oils, olive oil produced the highest secreted levels of TRL when administered in a pre-digested state. TRL promotive effects were attributable to liberated long chain unsaturated fatty acids.

Administered long chain triglycerides need to be digested prior to absorption. Free fatty acids, monoglycerides, diglycerides and glycerol, liberated from the digestion process, contribute to the formation of a mixed micellar phase, which is important for solubilisation of poorly soluble compounds. Surfactant effects on lipolysis were therefore a major concern and hence were investigated. Of the tested surfactants, only Pluronic P85 proved to be a potent inhibitor of pancreatic lipase in simulated gastrointestinal buffer, whilst Cremophor EL, polysorbate 80, Pluronic L81, Pluronic F68 and TPGS demonstrated little or no inhibitory effects.

Excipients were selected to facilitate oil dispersion and drug solubilisation in simulated gastrointestinal media without adversely affecting lipolysis or TRL output. A formulation containing 20%w/w olive oil, 20%w/w Peceol and 60%w/w polysorbate 80: Cremophor EL (2:1) produced a fine microemulsion (droplet size<40nm) when dispersed in gastrointestinal media

The aqueous solubility of halofantrine was considerable enhanced when co-administered in this microemulsion formulation and was independent of lipid digestion. In contrast, aqueous drug solubilisation from a crude oil dispersion was highly dependent on a pre-digestion step.

#### 6.5 Discussion

#### Comparative effects of non-digested and digested oils on lipid metabolism

In this chapter, a microemulsion formulation with potential to promote lymphatic uptake and aqueous solubility of a model lipophilic drug, halofantrine, was designed. An oil phase was selected based on lymphotropic potential. This was assessed by measuring TRL secretion levels in the Caco-2 model following supplementation with various long chain oils. Data showed that when long chain oils were presented to cells in a crude emulsion (in bile salt buffer) form, they

did not produce any substantial enhancements in TRL synthesis or secretion as compared to a control buffer or a medium chain oil emulsion. However, when long chain oils were subject to a preliminary digestion step, their TRL stimulating effects were significantly increased. Of the investigated long chain oils (olive oil, corn oil and arachis oil), the digested olive oil preparation demonstrated the highest potential for promoting TRL, and specifically chylomicron, secretion. In contrast, the medium chain oil, Miglyol 812, failed to promote TRL secretion when presented in either digested or non-digested states (Caliph et al., 2000). The long chain oils investigated in this work were largely composed of oleic acid, linoleic acid or linolenic acid whereas Miglyol 812 contained capric acid and caprylic acid and hence, the observed responses were likely to be related to fatty acid content of oils. These data are consistent with in vitro findings presented in Chapter Three for the impact of fatty acid chain length on lipoprotein secretion in the Caco-2 model. Data from Chapter Three showed that oleic acid, a monounsaturated long chain fatty acid was substantially more efficient at promoting chylomicron secretion than shorter chain or saturated fatty acids. This is further supported by in vivo data from the cannulated rat lymph model where long chain unsaturated fatty acids (oleic acid, linoleic and linolenic acid) were all shown to be far more efficient at promoting lymphatic triglyceride output than short-medium chain fatty acids (capric acid, caproic acid and butyric acid) (Porter et al., 1996; Caliph et al., 2000; Holm et al., 2001).

The oils used in this work took the form of di- and triglycerides of fatty acids, which are not readily absorbed by intestinal cells. Such oils need to be hydrolysed by lipase to monoglycerides and free fatty acids before they can be taken up by enterocytes (Mattson and Volpenhein, 1964). In vivo this is achieved by pancreatic lipase, but as Caco-2 cells have been shown to possess a small amount of brush border or cytosol associated lipase (Spalinger et al., 1998) investigated lipids were expected to impact on secreted lipoprotein profile to some extent. When lipids were administered to cells as non-digested emulsions, the secreted lipoprotein response was close to background, suggesting that cell associated lipase activity was not sufficient for facilitating uptake of the administered oils. In contrast, pre-digested long chain oils produced a significant increase in secretion of TRL, in particular, of chylomicrons, versus non-digested vehicles, with a rank order secretion of olive oil> corn oil> arachis oil. Predigestion caused the release of free fatty acid from the glyceride backbone, and thus increased the available amount of free fatty acid for cellular uptake and incorporation into secreted triglyceride. The comparative effects of the long chain oils on TRL secretion may be explained by differences in fatty acid composition. Olive oil contains up to 83% oleic acid, which has been shown to be effective at promoting TRL secretion in Caco-2 cells, whereas the arachis and corn oils both contain a higher amount of linoleic acid, which has been shown to have less pronounced effects than oleic acid on TRL secretion in the Caco-2 model (van Greevenbroek et al., 1996).

#### Excipient effects on lipid digestion

As the TRL stimulating effects of long chain oils proved to be highly reliant on lipid digestion, the effects of additional lipid vehicle excipients on intestinal lipolysis were evaluated. Studies were conducted in vitro to simulate intestinal conditions. Using a titrimetric assay to monitor free fatty acid release from the medium chain oil Miglyol 812 in simulated intestinal buffer, it was found that, Pluronic P85, a Pluronic block copolymer surfactant, was a potent inhibitor of pancreatic lipase. When Pluronic P85 was added to Miglyol 812 in a surfactant: oil ratio of 1:1, 1:2 or 1:5 (typical of lipid vehicle composition) lipolysis was completely blocked. In contrast, Pluronic L81 increased the initial rate of lipolysis when added at a 1:1 ratio. Addition of further non-ionic surfactants, Cremophor EL, polysorbate 80 and Pluronic F68, to the digestion mix, did not alter the rate or extent of lipolysis. Pluronic block copolymers, being composed of polymerised blocks of ethylene oxide (EO) and propylene oxide (PO), are all structurally similar and differ only in the chain length (molecular weight) and relative EO/PO ratio. Hence, differences in Pluronic block copolymer effects on intestinal lipolysis may be attributed to the relative EO/ PO composition. Pluronic F68 contains a high proportion of EO and hence is very hydrophilic in nature (HLB >24); Pluronic L81 contains a large proportion of PO, and is hydrophobic (HLB 1-7) whilst Pluronic P85 possesses intermediate lipophilicity (HLB 12-18). Taking lipophilicity into consideration, it is feasible that the intermediate HLB value of Pluronic P85 allows it to interact effectively at the oil-water interface of dispersed emulsion droplets, hence interfering with pancreatic lipase binding. Pancreatic lipase is only active at an oil-water interface because the act of binding induces a conformational change, which exposes an active triad of amino acids (Ser 152, Asp 176 and His 263) (Brzozowski et al., 1991; van Tilbeurgh et al., 1993) responsible for lipolysis. It has been shown that high concentrations of non-ionic surfactants inhibit pancreatic lipase activity, most likely by interference of the binding mechanism (Borgström and Erlanson, 1973; Solomon et al., 1996; MacGregor et al., 1997). Solomon et al. (1996) showed, for a series of nonylphenol ethoxylates, that surfactants of intermediate HLB (13-17) were most effective at inhibiting pancreatic lipase. It was postulated that inhibition resulted from surfactants docking to the oil-water interface and forming a hydrophilic oxyethylene mantle, which prevents lipase from binding.

The HLB dependence of lipase inhibition by nonylphenol ethoxylates (Solomon *et al.*, 1996) was attributed to affinity of the surfactant for the oil-water interface and also to the length of the EO chain, with chain length of >8 being required to produce an effective EO barrier at the interface. Pluronic P85 has an EO chain length of 52, and so should form a thick oxyethylene

layer around the oil droplet surface, thus preventing binding of the lipase-colipase complex. The failure of Pluronic F68 to inhibit lipolysis under physiological conditions may be a result of its high aqueous solubility, which gives it affinity for the aqueous versus oil phase. The lack of inhibitory effects for Pluronic L81 under physiological conditions is likely to be due to a combination of the short EO chain length of the compound (6 units), which gives it poor ability to form a substantial oxyethylene mantle, and its high lipophilicity (HLB 1-7) which gives it affinity for the bulk lipid phase. The initial acceleration of lipolysis observed at the higher concentration of this compound may result from enhanced lipid droplet dispersion, with a resulting increase in exposed interfacial surface area for lipase-colipase complex binding.

#### Bile salt effect on lipolysis

Bile salts have been shown to protect lipase from surfactant-induced inhibition. This effect may be due to bile salts solubilising surfactant molecules into a mixed micellar phase or by accumulating at the oil-water interface and preventing other surfactants from binding to the interface (Borgström and Erlanson, 1973). The colipase-lipase complex is sufficiently lipophilic to penetrate the bile salt shield and to gain access to the oil surface for binding (Brockerhoff, 1971; Borgström and Erlanson, 1973). As Pluronic P85 was found to inhibit lipolysis both in the presence and absence of bile salts, it is likely that this surfactant has a higher affinity for the lipid-water interface than the bile salt. Cremophor EL and polysorbate 80, two surfactants with equivalent HLB values to Pluronic P85, failed to impair lipolysis in simulated intestinal buffer. This infers that the correct surfactant lipophilicity is not sufficient to ensure inhibition of lipase binding. It may be that the EO/PO chain lengths or the structural conformation of Pluronic P85 are particularly favourable to interfacial binding. In the absence of bile salts, more generalised inhibition of pancreatic lipase by surfactants was observed. This is consistent with published findings and may be a result of surfactants having greater access to the oil-water interface to block pancreatic lipase binding, or may be due to surfactant-induced denaturation of the enzyme (Benzonana and Desnuelle, 1968; Borgström and Erlanson, 1973)

#### [14C] Triolein assay for lipolysis

The titrimetric method described above used a medium chain oil substrate for reasons of detection sensitivity, however, digestion of long chain oils is of greater concern with regard to promotion of lymphatic triglyceride turnover. Long chain fatty acids are less water-soluble than medium chain fatty acids and so will behave differently in the intestinal milieu, and thus may influence lipase activity in alternative ways to medium chain fatty acids. In order to directly monitor lipolysis of a long chain oil, a different technique was employed. This time, glycerol tri [<sup>14</sup>C] oleate was used as a lipase substrate. The amount of [<sup>14</sup>C] oleic acid liberated in the

lipolysis medium was quantified using liquid scintillation counting and used as a measure of lipase activity. Trends obtained using this method for surfactant effects on lipolysis were found to be similar to those obtained using the pH stat technique. Marked inhibition of pancreatic lipase activity in the presence of bile salts was only observed with Pluronic P85. More widespread inhibition by surfactants was noted when bile salts were excluded from test medium. Taken together, these data indicate that surfactant-mediated inhibition of intestinal digestion may not be a major problem for all non-ionic surfactants. However, the observation of potent inhibition by Pluronic P85 means that there is still a need to screen for possible excipient-lipase interactions prior to formulating a lymphotropic vehicle.

In the work presented here, lipolysis mediated by Caco-2 homogenate was more sensitive to surfactant inhibition than that mediated by pancreatin. As lipolysis was 20-fold higher in the pancreatin system versus homogenate, this disparity may result from differences in enzyme activity.

#### Solubilisation of halofantrine

High drug solubility in the vehicle oil phase is advantageous because it allows for increased drug loading and offers the potential for reduced dosage volume and elevated intra-luminal drug levels. Solubility of halofantrine in a number of oils was therefore determined to assess any relative advantages associated with test oils with regard to drug loading. Work presented here found that the medium chain oil, Miglyol 812, had 50% higher solvent capacity (p<0.05) (60mg/g oil versus 40mg/g oil) for halofantrine than the investigated long chain oils. Halofantrine solubility was similar in all of the tested long chain oils. Microemulsion preconcentrate vehicles demonstrated lower solubility for halofantrine (25-30mg/g vehicle) than the constituent oils, most likely as a result of higher drug solubility in lipidic triglycerides versus amphiphilic surfactants. These data are consistent with findings by Khoo et al. (1998). On dilution in simulated gastric buffer (0.1N HCl) or simulated intestinal buffer (3mM sodium taurocholate: 0.75mM lecithin), highest aqueous drug solubility was observed following coadministration with microemulsion vehicles or the polysorbate 80 micellar solution. Drug solubility values were 2-3-fold higher with such vehicles than with the crude oil emulsions. When vehicles were presented to cells in a pre-digested state, aqueous drug solubility was considerably enhanced for the oil vehicles (2-fold enhancement versus crude emulsion in bile salt buffer p<0.01), but was still higher for the more disperse formulations (microemulsions and polysorbate 80 micelles). Increased drug solubilisation with digested lipids has previously been reported and is due to the creation of a highly solubilising mixed micellar phase in the intestinal lumen comprising bile salts, phospholipids and amphiphilic end products of lipid digestion (Naylor et al., 1995; Nielsen et al., 2001). In general, the Miglyol 812 based formulations tended to facilitate higher levels of drug solubilisation than corresponding olive oil based formulations, however, the long chain oil was favoured for formulation development because of its promotive effects on TRL secretion.

#### Lipid vehicle dispersion

When considering microemulsion vehicle formulations in terms of ease of vehicle dispersion, it was found that Cremophor EL was most effective at producing a fine oil-in-water dispersion for the long chain, olive oil, on dilution in simulated intestinal buffer. Polysorbate 80 was added to reduce the dispersed oil droplet size, and to ameliorate inhibitory effects of Cremophor EL on TRL secretion. Peceol, a monoglyceride of oleic acid, was added to increase the oleate content of the oil phase and also acted to reduce the dispersed droplet size. A final formulation of 20%w/w olive oil, 20%w/w Peccol and 60%w/w polysorbate 80: Cremophor EL (2:1) was The inclusion of high concentrations of Cremophor EL in microemulsion developed. formulations has been reported to impede the initial rate of vehicle dispersion in aqueous media (Khoo et al., 1998). However, as the work presented here examined the effects of excipients on vehicle dispersion following dilution in gastrointestinal buffers under simulated physiological conditions i.e. using a 2 hour mixing interval, this was not an issue. Considering that reported gastrointestinal transit times are in the order of 3-4 hours (Carriere et al., 1993; Basit et al., 2001), a delay of 5-15 minutes in vehicle dispersion is unlikely to pose a bioavailability problem in vivo.

# **Chapter Seven**

Effects of Lipid Vehicles on the Cellular Uptake and Permeability of Halofantrine in the Caco-2

Model

# 7 Effects of Lipid Vehicles on the Cellular Uptake and Permeability of Halofantrine in the Caco-2 Model

#### 7.1 Introduction

Lymphatic drug transport may be an important route for increasing the oral bioavailability of highly lipophilic drug compounds (Porter and Charman, 1997). Lymphatic drug transport is thought to occur by partitioning of drugs into the triglyceride rich core of secreted chylomicrons. It is therefore possible to target this route by co-administering drugs with lipid vehicles that promote TRL secretion. Findings presented in this thesis have shown that oils, such as olive oil, that are rich in long chain unsaturated fatty acids are effective at promoting TRL output in the Caco-2 model when presented in a pre-digested state. Similarly, the oleate rich surfactant, polysorbate 80, is also capable of promoting TRL secretion. Using these data, and an understanding gained in Chapter Four of the possible negative effects that excipients can have on TRL secretion, an oleate rich microemulsion formulation was systematically designed in Chapter Six with the dual aim of maximising drug solubility in aqueous medium (by producing a fine oil-in-water dispersion) and of promoting triglyceride associated drug transport.

Halofantrine was selected as a model drug to monitor chylomicron associated drug transport in the Caco-2 model because it fits the criteria established by Charman and Stella (1986) for lymphatic uptake (high triglyceride solubility, >50mg/ml, and log P value, >5) and because extensive lymphatic transport (up to 50% of administered dose) has been demonstrated *in vivo* (Khoo *et al.*, 2001).

As lymphatic drug transport occurs largely by association of drugs with chylomicrons, the potential lymphotropic effects of lipid vehicles can theoretically be assessed in the Caco-2 model by monitoring chylomicron output and association of co-administered drugs with secreted chylomicrons.

# 7.2 Aims and objectives

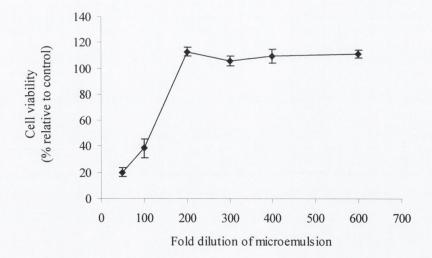
The aim of this work was to evaluate and compare the simultaneous effects of a range of lipid based vehicles on lipoprotein secretion and halofantrine permeability in the Caco-2 model as a means of identifying important vehicle requirements for effective lymphatic drug transport. Three oleate rich vehicles with different dispersion characteristics were investigated – a crude olive oil emulsion, a polysorbate 80 micellar system and the olive oil based microemulsion (o-me) developed in Chapter Six. For comparison, two vehicles with poor TRL promoting effects

were included in the study – a crude Miglyol 812 emulsion and a Miglyol 812 based microemulsion (m-me), as detailed in Chapter Six.

#### 7.3 Results

## 7.3.1 Vehicle preparation

For non-digested systems, vehicles were diluted 1 in 50 in bile salt solution (10mM sodium taurocholate with 2.5mM lecithin), agitated at 150 rpm for 2 hours at 37°C to simulate mixing in gastrointestinal fluid, and subsequently diluted 1 in 4 in medium containing 20%v/v delipidated serum and trace [14C] oleic acid, as described in Section 2.3.14. This last dilution step in cell media was used to produce a final vehicle dilution of 1 in 200 and also to provide sufficient nutrients for an overnight incubation with Caco-2 cells. A final 1 in 200 dilution of preconcentrate vehicles was used in all studies, partly to simulate predicted dilution of a 1g lipid dose in the intestine and also because attempts to use more concentrated lipid vehicles resulted in decreased cell viability (Figure 7.1).



**Figure 7.1:** Effect of microemulsion dilution factor on intracellular dehydrogenase activity in Caco-2 cells as determined using an MTT assay. O-me was diluted in serum-free cell culture medium and administered to cells over a 20-hour period for determination of microemulsion toxicity. Values represent the mean  $\pm$ SD (n=5).

Lipid vehicles were subject to a simulated intestinal digestion step in this study to determine the influence of digested versus non-digested vehicle effects on halofantrine transport across

Caco-2 monolayers. Vehicles were prepared as outlined above, with the addition of pancreatin (cell culture tested) to the initial bile salt buffer (4mg/100ml). Although pancreatin has protease activity (due to trypsin), the inclusion of delipidated serum in the diluting medium prevented any cytotoxic effects on Caco-2 cells. The viability of cells in the presence of digestion medium was confirmed in Chapter Six (Section 6.3.1).

# 7.3.2 Lipid vehicle (non-digested and digested) toxicity

# Vehicle effects on cell viability

Potential toxic effects of investigated vehicles (in non-digested and digested states) were screened initially using an MTT assay for intracellular dehydrogenase activity (Section 2.3.4).

Results for non-digested vehicle effects on Caco-2 cell viability are summarised in Table 7.1. None of the vehicles had a significant impact on cell viability as determined using an MTT assay. Digested lipid vehicles (diluted in serum-containing medium) did not produce any substantial changes in cell viability (Table 7.2) relative to control. Cellular dehydrogenase activity decreased by approximately 15% following exposure to the Miglyol 812 and m-me vehicles, but the difference was not statistically significantly from the control (cells exposed to serum-free culture medium over the same period).

# Vehicle effects on monolayer integrity

In addition to cell viability, the effects of lipid vehicles (non-digested and digested) on monolayer integrity were confirmed by monitoring monolayer resistance (as determined via TEER measurements, Section 2.3.1), and by determining permeability to mannitol and propranolol following a 20-hour exposure to test lipid vehicles (Section 2.3.1). Permeability values were compared to those obtained using a standard HBSS transport buffer (positive control). In agreement with MTT assay findings, monolayer integrity, as determined via TEER measurements (Table 7.1) and permeability values (Figure 7.2a.b), was maintained in full in the presence of non-digested lipid vehicles. Of the digested lipid vehicles, the only vehicle that produced a significant reduction in monolayer resistance was the digested Miglyol 812 emulsion (35% decrease in monolayer resistance, p<0.01) (Table 7.2). Monolayer permeability data supported these findings. Monolayer permeability to mannitol was only increased by pre-exposure of cells to the digested Miglyol 812 emulsion (2.2-fold increase, p<0.01) or the digested m-me (1.8-fold increase, p<0.01) (Figure 7.2a). Propranolol transport was not affected by pre-exposure to any of the tested lipid based vehicles (either in digested or non-digested state) (Figure 7.2b).

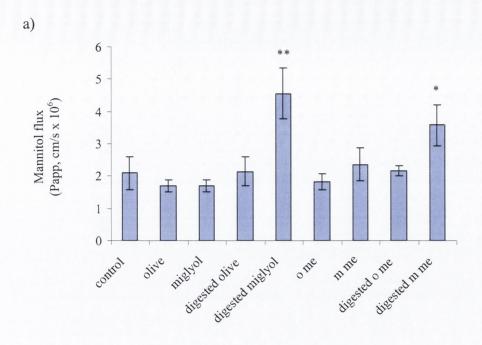
In order to ascertain whether the effects of the digested Miglyol 812 emulsion on paracellular transport of mannitol were reversible or not, cells were allowed to recover from Miglyol 812 exposure for 20 hours in the presence of cell culture medium before measuring marker transport. Results are shown in Figure 7.3. Mannitol flux returned to control levels following this recovery period. This was accompanied by an increase in monolayer resistance (returning to 96.3% of pre-exposure level).

Table 7.1: MTT and TEER values for Caco-2 cells following exposure to non-digested lipid vehicles. Cells were exposed to lipid vehicles for 20 hours prior to taking measurements. MTT values are expressed relative to a positive control (cells incubated with serum-free medium) and TEER values are expressed relative to pre-exposure levels. Results are expressed as the mean (n=4 or 5) (SD). O-me = olive oil based microemulsion, M-me = miglyol based microemulsion.

	MTT values $(n=5)$	TEER values $(n=4)$
	(% relative to control)	(% relative to starting value)
Olive	92.7 (4.9)	88.9 (7.1)
O-me	98.8 (3.9)	94.0 (8.7)
Miglyol 812	94.0 (11.5)	94.7 (5.6)
M-me	98.3 (12.9)	90.8 (11.1)
Polysorbate 80	100.3 (5.3)	98.6 (3.6)

Table 7.2: MTT and TEER values for Caco-2 cells following exposure to digested lipid vehicles. Cells were exposed to lipid vehicles for 20 hours prior to taking measurements. MTT values are expressed relative to a positive control (cells incubated with serum-free medium). TEER values are expressed relative to pre-exposure values. Results are expressed as the mean (n=4 or 5) (SD). O-me = olive oil based microemulsion, M-me = miglyol based microemulsion.

	MTT Values (n=5)	TEER Values (n=4)
	(% relative to control)	(% relative to start)
Olive	91.4 (4.2)	89.2 (2.6)
O-me	92.6 (9.6)	94.7 (12.9)
Miglyol 812	84.0 (12.7)	65.4 (16)**
M-me	87.3 (12.9)	93.1 (7.3)
Polysorbate 80	92.4 (6.7)	92.6 (6.7)



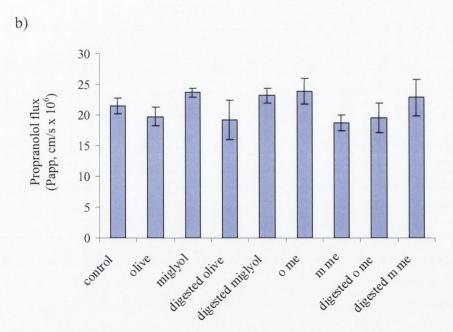


Figure 7.2: Effects of lipid vehicles on Caco-2 monolayer permeability to mannitol (paracellular marker) (panel a) and to propranolol (transcellular marker) (panel b). Cells were pre-incubated with lipid vehicles for 20 hours prior to measuring monolayer permeability. Permeability was determined by measuring cumulative marker transport over 2 hours. Results are expressed as the mean  $(n=4) \pm SD$ . Significant differences from the control level are indicated: \*\*p<0.01, \*p<0.05. O-me = olive oil based microemulsion, m-me = miglyol based microemulsion.

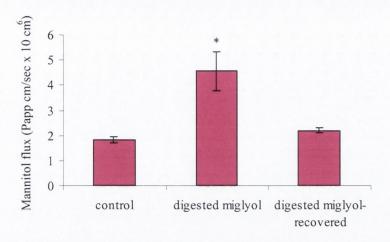


Figure 7.3: Caco-2 monolayer permeability to mannitol (paracellular marker) following 20-hour exposure to (a) control of culture medium containing 20% dilipidated serum, (b) digested Miglyol 812 emulsion vehicle or (c) digested Miglyol 812 vehicle, followed by an additional 20-hour exposure to cell culture medium, to allow recovery from Miglyol 812 effects. Results represent the average of 4 monolayers  $\pm$  S.D. Statistically significant differences from control levels are indicated: \*\*p<0.01.

### 7.3.3 Effect of *in vitro* lipolysis on free fatty acid content of lipid vehicles

Sensitivity of lipid vehicles to lipolysis by pancreatin was determined in a similar manner to that described for polysorbate surfactants (Section 2.3.9). Free fatty acid content of diluted lipid vehicles (digested and non-digested) was determined using a Randox NEFA test kit. Results are shown in Figure 7.4. As can be seen, the rank order of sensitivity to *in vitro* digestion was: Miglyol 812 emulsion> m-me>olive oil emulsion> o-me = polysorbate 80. In all cases, lipolysis produced a substantial (minimum of 6.7-fold, with o-me and maximum of 80-fold, with Miglyol 812) and highly statistically significant (p<0.01) increase in free fatty acid content.

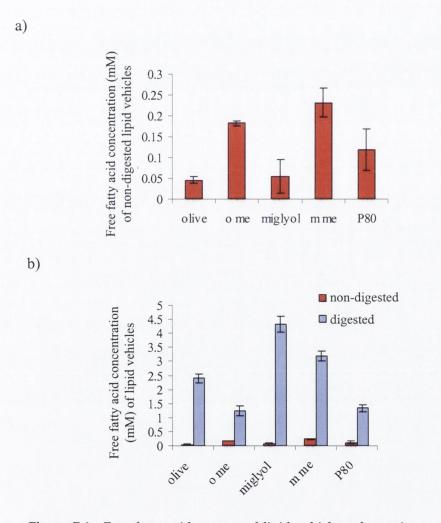


Figure 7.4: Free fatty acid content of lipid vehicles taken prior to and following *in vitro* lipolysis. Panel (a) shows results for non-digested vehicles panel (b) shows combined data for non-digested and digested vehicles. Determinations were made with a colorimetric non-esterified fatty acid (NEFA) test kit. Data represent the mean  $(n=3) \pm SD$ .

# 7.3.4 Vehicle effects on lipid metabolism in Caco-2 cells

Vehicle effects on lipid metabolism were investigated by supplementing Caco-2 monolayers with [¹⁴C] labelled lipid vehicles. Vehicles were prepared as above, by diluting systems in culture medium containing 20%v/v delipidated serum and labelling with [¹⁴C] oleic acid (final activity of 0.2μCi/ml). Lipid vehicles were presented in non-digested and digested states to assess the importance of intestinal lipolysis on the cellular response. Intracellular and secreted levels of triglyceride and apoB were quantified and the secreted lipoprotein profile characterised as per Section 2.3.2.

# 7.3.4.1 Secreted lipoprotein profile

The secreted lipoprotein profile for Caco-2 cells was highly sensitive to lipid vehicle effects. Supplementation of cells with the o-me and polysorbate 80 vehicles elicited a selective increase in secreted chylomicron and VLDL levels in Caco-2 cells (Figure 7.5). Following an in vitro pre-digestion step, the chylomicron stimulating effects of olive oil were considerably enhanced (40-fold, p<0.01) (Figure 7.5a), as previously observed in Chapter Six (Figure 6.3). In vitro digestion also enhanced the chylomicron stimulating potential of polysorbate 80, but the response was less profound (1.5-fold increase relative to non-digested level, p<0.05). In contrast, the effects of the o-me on chylomicron secretion were decreased somewhat by the predigestion step, but the difference was not significant. In the non-digested state, the rank order of vehicles, in terms of potential to promote chylomicron secretion, was: polysorbate 80> o-me>> (P<0.01) olive oil emulsion = m-me>Miglyol 812 emulsion=control. Following digestion, the rank order switched to: olive oil emulsion> (p<0.05) polysorbate 80 >> (p<0.01) o-me > (p<0.01) control > (p<0.01) Miglyol 812 emulsion = m-me. In the digested state, olive oil produced 1.4-fold higher chylomicron secretion levels (p<0.01) than digested polysorbate 80 solution and 7.5-fold higher secretion levels than the control digestion buffer (p<0.01) (Figure 7.5a). The VLDL secretion response showed a similar trend following supplementation with lipid vehicles, but effects were less pronounced than on chylomicron secretion. Again, highest levels of VLDL secretion were obtained with the digested olive oil emulsion (Figure 7.5b).

Lipid vehicle effects on LDL secretion were less pronounced than on TRL secretion. In the non-digested state, the m-me was the only vehicle found to produce a significant increase in LDL secretion relative to the control buffer (2.1-fold, p<0.01). Polysorbate 80 and Miglyol 812 emulsion vehicles also increased LDL secretion (each by approximately 1.4-fold) relative to control buffer, but the difference was not statistically significant (Figure 7.6a). *In vitro* digestion of the Miglyol 812 containing formulations resulted in a significant decrease in LDL promoting effects, whereas pre-digestion of polysorbate 80 resulted in an increase. Following

lipid vehicle digestion, polysorbate 80 was the only vehicle that significantly enhanced LDL secretion above control digestion buffer levels (Figure 7.6a). Secreted HDL levels were not affected by lipid vehicle supplementation (Figure 7.6b).

### 7.3.4.2 Secreted lipid profile

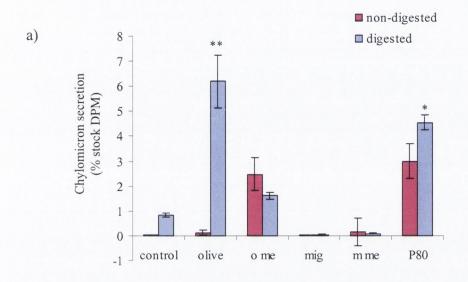
Secreted triglyceride and apoB data complement the above density gradient ultracentrifugation findings. Of the non-digested vehicles, only the o-me and polysorbate 80 vehicles produced a substantial increase in triglyceride secretion as compared to the control buffer (Figure 7.7a). The secretion level was elevated 5.7-fold (p<0.01) by polysorbate 80 and 3.9-fold (p<0.01) by the o-me as compared to control buffer. In vitro digestion of the olive oil and polysorbate 80 micellar vehicles produced an increase in their ability to stimulate triglyceride secretion. In the case of olive oil, triglyceride secretion was increased 5.6-fold (p<0.01) and for polysorbate 80 it was increased 1.3-fold (p<0.01). The control buffer vehicle also showed an increase in triglyceride secretion post-digestion, but the difference was not statistically significant (Figure 7.7a). The remaining digested vehicles displayed no significant changes in triglyceride secretion relative to the non-digested state. Secreted apoB data for non-digested vehicles showed an increase (p<0.05) relative to control for all of the non-digested vehicles (Figure 7.7b). As seen in Chapter Six, apoB and triglyceride secretion were higher with the digested versus non-digested buffer, most likely as a result of free fatty acid being liberated from lecithin in the buffer. Digestion of the lipid vehicles did not further enhance the secreted apoB level relative to either digested control buffer or to non-digested lipid vehicles, but in the case of Miglyol 812, a significant decrease in apoB secretion relative to the non-digested state was observed (Figure 7.7b). Secreted phospholipid levels were largely unaffected by supplementation of cells with lipid vehicles versus control buffer (Figure 7.9a). Following digestion, long chain lipid vehicles produced similar levels of phospholipid output to their nondigested counterparts. Digestion of the Miglyol 812 emulsion and m-me vehicles resulted in a significant decrease in the secreted phospholipid response, which is consistent with a reduction in lipoprotein output.

#### 7.3.4.3 Intracellular lipid and apoB

Supplementation of Caco-2 cells with non-digested microemulsion vehicles, polysorbate 80 solution and olive oil emulsion promoted an increase in intracellular triglyceride, however, this increase was only statistically significant (p<0.05) for the o-me and polysorbate 80 vehicles (Figure 7.8a). The effects of polysorbate 80 and o-me vehicles on intracellular triglyceride accumulation were less pronounced than on the secreted triglyceride response, with a 4-fold and 2.5-fold increase in intracellular triglyceride for o-me and polysorbate 80 vehicles respectively. When vehicles were subject to *in vitro* digestion, the Miglyol 812 emulsion and m-me both

produced a significant increase in the intracellular triglyceride level relative to corresponding non-digested vehicles, with 3.6-fold and 1.8-fold increases respectively (Figure 7.8a).

Intracellular levels of apoB (Figure 7.8b) and phospholipid (Figure 7.9b) were not affected by supplementation of cells with either non-digested or digested lipid vehicles.



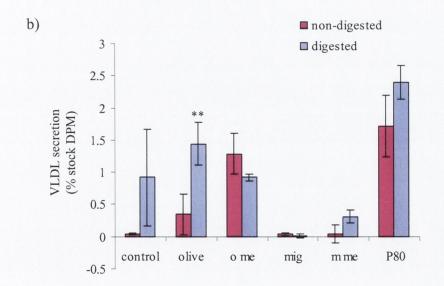
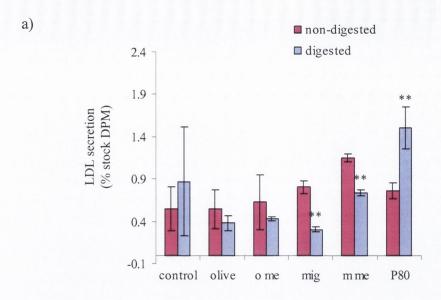
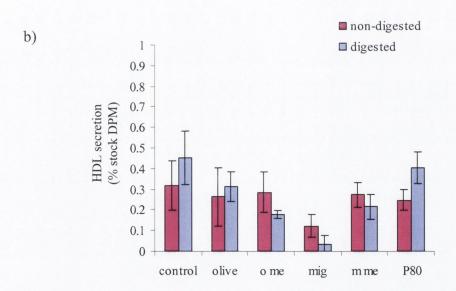
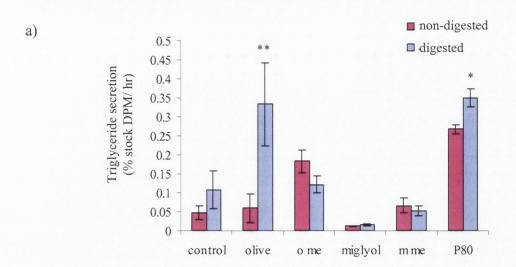


Figure 7.5: Effects of non-digested lipid vehicles on basolateral secretion of chylomicrons (panel a) and VLDL (panel b) in Caco-2 monolayers. Results are expressed as the mean (n=4)  $\pm$  SD. Statistically significant differences between results obtained with non-digested versus digested vehicles are shown: \*\*p<0.01.





**Figure 7.6:** Effects of lipid vehicles on basolateral secretion of LDL (panel a) and HDL (panel b) in Caco-2 monolayers. Results are expressed as the mean  $(n=4) \pm SD$ . Statistically significant differences between results obtained with non-digested versus digested vehicles are shown: \*\*p<0.01.



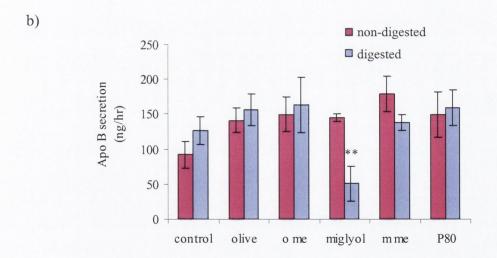
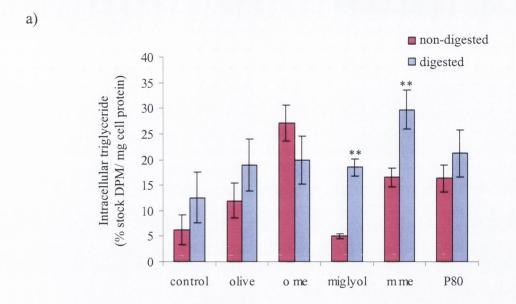


Figure 7.7: Effects of lipid vehicles on basolateral secretion of triglyceride (panel a) and apoB (panel b) in Caco-2 monolayers. Values represent the mean (n=4)  $\pm$  SD. Statistically significant differences between results obtained with digested versus non-digested vehicles are shown: \*\*p<0.01.



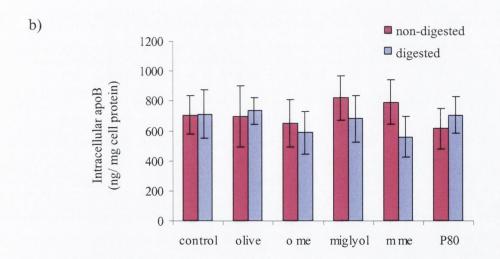
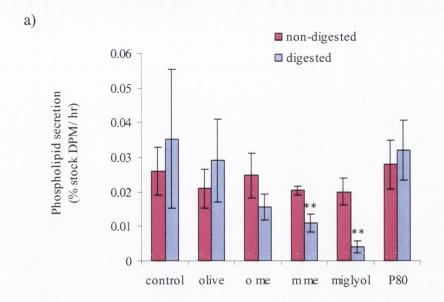
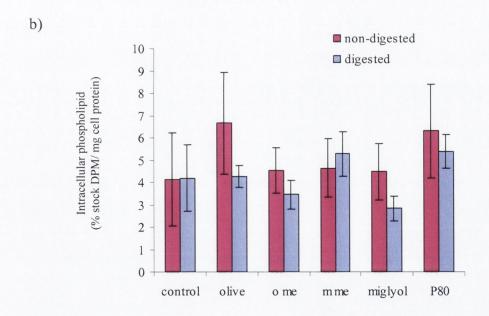


Figure 7.8: Effects of lipid vehicles on intracellular accumulation of triglyceride (panel a) and apoB (panel b) in Caco-2 monolayers. Values represent the mean (n=4)  $\pm$  SD. Statistically significant differences obtained with non-digested versus digested vehicles are shown: \*\*p<0.01.





**Figure 7.9**: Effects of lipid vehicles on the basolateral secretion (panel a) and intracellular accumulation (panel b) of phospholipid in Caco-2 monolayers. Values represent the mean  $(n=4) \pm SD$ . Statistically significant differences obtained with non-digested versus digested vehicles are shown: \*\*p<0.01.

# 7.3.5 Effects of lipid based vehicles on halofantrine uptake and permeability in the Caco-2 model

A key aim of this study is to assess the effects that lipid based vehicles have on the intestinal absorption of lipophilic drugs and the potential of the Caco-2 model to predict lymphatic drug uptake. Halofantrine was selected as model lipophilic drug in these studies because of its established *in vivo* absorption profile. For this work, lipid vehicles were saturated with halofantrine (as described in Section 2.3.14) prior to administering to Caco-2 cells. Caco-2 cells were incubated with vehicles for 20 hours as in standard lipid studies to ensure high levels of lipoprotein secretion. Following this interval, cell lysates and basolateral samples were collected. For each determination, samples were pooled from 2 monolayers as per Section 2.3.14 to increase detection. Basolateral samples (1ml) and cell lysate (0.5ml) were subject to extraction and the resulting extracts analysed for halofantrine content as described (Section 2.3.11). This gave data for halofantrine uptake and secretion. Basolateral samples (3ml) were also subject to density gradient ultracentrifugation to isolate lipoprotein fractions, and each fraction was then analysed for halofantrine content, following extraction. This gave data for halofantrine distribution between lipoprotein fractions.

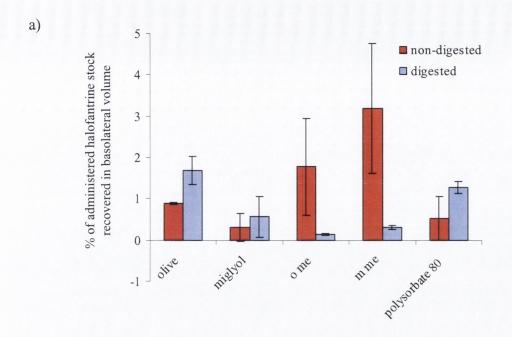
The effects of co-administered lipid vehicles on cellular uptake and basolateral transport of halofantrine are summarised in Figure 7.10. Looking initially at the results for basolateral recovery of halofantrine following co-administration with non-digested lipid vehicles, it is clear that the microemulsion formulations confer the highest advantages in terms of drug absorption, resulting in passage of up to 3.2 or 1.9% of administered stock with the m-me and o-me respectively. The lipid emulsions and the polysorbate 80 vehicle facilitated lower levels of halofantrine permeation, with approximately 0.5% and less, of administered stock being recovered in the basolateral well. When vehicles were subject to a pre-digestion step, the trend for halofantrine passage was altered. Of the digested vehicles, the olive oil emulsion and polysorbate 80 vehicles produced the highest levels of halofantrine permeability, with 1.7 and 1.2% of administered stock being recovered in basolateral medium respectively. comparing non-digested versus digested vehicles, it was found that halofantrine transport was significantly enhanced for the olive oil emulsion (p<0.01) and significantly decreased for both microemulsion vehicles (p<0.01)(Figure 7.10a) following pre-digestion. Additionally, although both of the non-digested microemulsion vehicles produced higher levels of halofantrine transport than the digested olive oil emulsion, the differences in results were not statistically significant because of the high variability associated with the microemulsion data.

Lipid vehicle effects on intracellular accumulation of halofantrine closely resembled the observed basolateral transport response, again with the microemulsion vehicles producing highest levels of drug passage in the non-digested state, and olive oil and polysorbate vehicles producing highest levels in the digested state (Figure 7.10b).

These data are all corrected for the administered stock concentration. Administered stock concentrations differed according to aqueous drug solubility following dilution of the preconcentrate vehicles, and are summarised in Table 7.3. As noted in Chapter Six, digestion of the crude emulsions facilitated higher aqueous solubility of halofantrine as a result of mixed micelle formation. An examination of the actual amount of halofantrine in basolateral samples, without any dose correction, may reflect better the *in vivo* situation. These data are shown in Figure 7.11. Data followed a similar trend, but the beneficial effects of the non-digested microemulsion vehicles were even more profound, with, for example, a 15-fold difference between basolateral levels with the Miglyol 812-based microemulsion versus polysorbate 80 solution (Figure 7.11a), as compared to a 6-fold difference for the equivalent dose corrected values (Figure 7.10a).

Table 7.3: Concentration of halofantrine in administered lipid vehicles (diluted 1 in 200 from preconcentrate in bile salt/ culture medium as per Section 2.3.14 and sterile filtered).

Vehicle	Non-digested (µg/ml)	Digested (μg/ml)
Olive emulsion	10.4	31.9
Miglyol 812 emulsion	53.8	82.5
O-me	66.3	46.9
M-me	86.3	63.8
Polysorbate 80	21.6	18.8



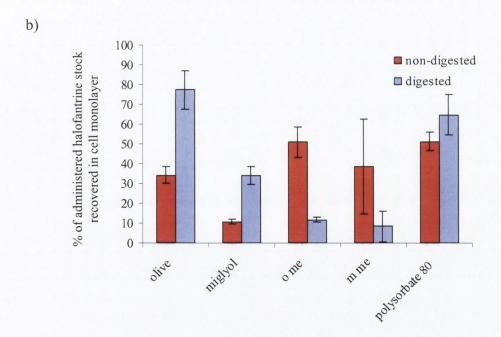


Figure 7.10: Percentage of halofantrine (corrected for stock concentration) recovered in basolateral samples (panel a) and cell lysate (panel b) following 20 hour incubation with halofantrine saturated lipid vehicles. Results are expressed as the mean  $(n=3) \pm SD$ .

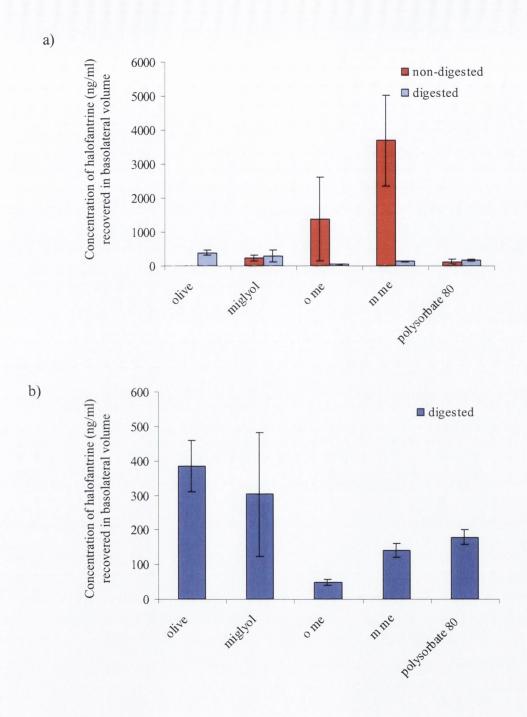


Figure 7.11: Amount of halofantrine (ng/ml) recovered in basolateral samples following 20 hour incubation with halofantrine saturated lipid vehicles. Results are shown for digested versus non-digested vehicles (panel a) and for non-digested vehicles alone (panel b). Results are expressed as the mean  $(n=3) \pm SD$ .

### Mass Balance of halofantrine

Mass balance analysis showed that for most vehicles, halofantrine recovery in samples was >80% (Table 7.5). In each case, the loss in mass balance may have been due to drug adhering to cell monolayers or to cell filter assemblies (filter, filter walls or plastic wells). Any drug bound to the outer surface of the cell monolayer is lost during the rinsing step prior to cell lysis and so is not assayed. The Miglyol 812 emulsion proved to be an exception, with only 46-54% recovery. The additional loss in drug recovery with this particular vehicle may have been due to drug precipitating from the digested medium chain mixed micellar phase as the lipid digestion products were taken up by cells. As aqueous drug solubility was highest with the digested Miglyol 812 vehicle, it is likely that there was a high reliance on the presence of amphiphilic digestion products to maintain drug in a solubilised form. This would result in the system being more susceptible to precipitation than the other tested vehicles. Medium chain triglycerides and their digestion products are rapidly absorbed by cells, if absorption of the lipid products is more rapid than drug uptake, precipitation may occur. Precipitation may result in additional drug adhering to cell monolayer surfaces or plastic-ware, thus causing an additional drop in drug recovery.

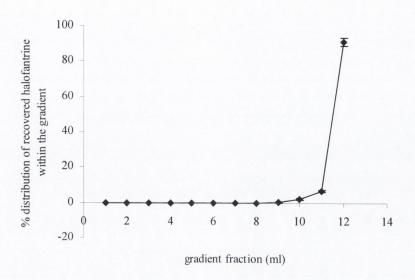
**Table 7.5:** Total recovery of halofantrine in cellular, basolateral and apical samples as a % of administered stock. Data represent the mean  $(n=3) \pm SD$ .

Vehicle	Digested vehicle data	Non-digested vehicle data	
Olive oil emulsion	$80.5 \pm 5.1$	$106.3 \pm 8.1$	
Miglyol 812 emulsion	$46.8 \pm 7.1$	$54.1 \pm 8.5$	
O-me	$93.6 \pm 11.9$	$85.4 \pm 1.3$	
M-me	$89.3 \pm 8.6$	$80.3 \pm 2.8$	
Polysorbate 80	$87.1 \pm 6.6$	$84.7 \pm 10.1$	

# 7.3.6 Lipid vehicle effects on density gradient distribution of halofantrine in basolateral samples

Basolateral samples were subject to density gradient analysis as per Section 2.3.14, and the amount of drug associated with each lipoprotein density fraction was quantified. To facilitate comparisons between vehicles, data were expressed as distribution (%) of recovered halofantrine within the density gradient. Prior to commencing work, the natural flotation density of halofantrine was established by spiking culture medium (containing 20%v/v dilipidated serum) with 200ng/ml halofantrine (added as ethanolic stock, giving a final concentration of 2%v/v ethanol) and subjecting this halofantrine solution to density gradient ultracentrifugation as per 2.3.14. Halofantrine was recovered in the final 2 fractions of the gradient, corresponding to HDL and lipoprotein deficient medium (LPDM), following ultracentrifugation of the spiked samples (Figure 7.12).

When halofantrine was co-administered with the olive or Miglyol 812 emulsions, drug recovered in basolateral medium was shown to be associated with the final two fractions of the gradient (HDL and lipoprotein deficient medium, LPDM). As this corresponds with the natural flotation density of halofantrine, it is likely that halofantrine recovered in the terminal fractions was present in aqueous solution and not associated with secreted lipoproteins. The pattern of halofantrine distribution within the gradient was the same regardless of whether oil emulsion vehicles were digested or not (Figures 7.13a and 7.13b). Administration of halofantrine in nondigested microemulsion vehicles (olive-oil based or Miglyol 812-based) caused halofantrine to associate largely with the HDL/ LPDM fraction of the density gradient fraction, but there was also some association with LDL fractions (15-20%) and a small amount with the VLDL fraction (10-20%) (Figures 7.13c and 7.14a). On administering halofantrine with the non-digested polysorbate 80 micellar solution, halofantrine was found to associate primarily with the HDL (approximately 60%) fraction of the gradient and to a lesser extent with LDL (10%), VLDL (20%) and chylomicrons (10%). Pre-digestion of the polysorbate 80 solution caused an increase in the association of basolateral halofantrine with TRL fractions of the gradient, such that 30% of the recovered drug was found in the chylomicron fraction and 30% in the VLDL fraction, with the remainder being split between LDL and HDL fractions (Figure 7.14b). The increase in association of drug with chylomicrons post-digestion was significant compared to the nondigested state (p<0.05).



**Figure 7.12:** Distribution of halofantrine within blank density gradient. Results represent the mean  $\pm$  S.D (n=3).

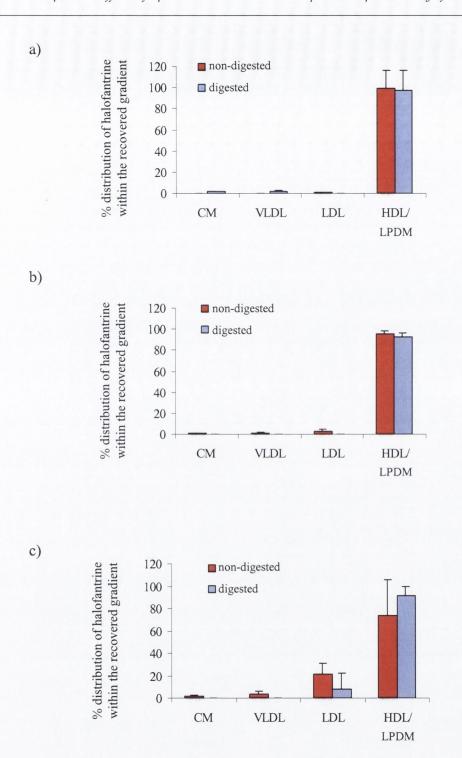
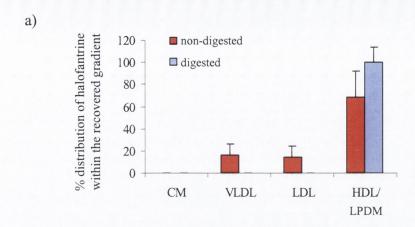


Figure 7.13: Effects of olive oil emulsion (panel a), Miglyol 812 emulsion (panel b) and ome (panel c) vehicles on density gradient distribution of halofantrine recovered in basolateral samples. Data are presented for non-digested and digested vehicles. Values represent the mean  $(n=3) \pm SD$ .



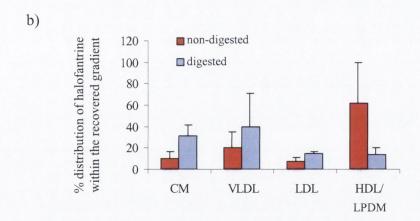


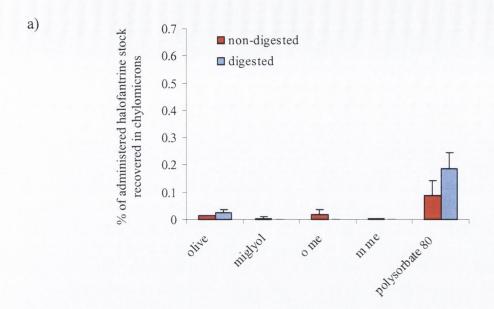
Figure 7.14: Effects of m-me (panel a) and polysorbate 80 (panel b) vehicles on density gradient distribution of halofantrine recovered in basolateral samples. Data are presented for non-digested and digested vehicles. Values represent the mean  $(n=3) \pm SD$ .

# 7.3.7 Quantitative analysis of halofantrine in density gradient fractions

The above data described the relative distribution of halofantrine within the density gradient, expressed as a % of total amount of halofantrine recovered within the total gradient. In order to extend the comparative analyses of the vehicles, the amount of halofantrine (as a % of administered stock) associated with each of the density gradient fractions was compared across the investigated vehicle range. Looking initially at the chylomicron data it can be seen that polysorbate 80 was the only vehicle to promote association of halofantrine with chylomicron fractions of the gradient (Figure 7.15a). In contrast, the highest degree of halofantrine association with the VLDL fraction was obtained for the m-me, followed by the polysorbate 80 solution (Figure 7.15b), with an approximately 2-fold difference between the two responses. Both microemulsion formulations equally promoted the association of halofantrine with LDL to a much greater extent than any of the other lipid vehicles (Figure 7.16a). Highest levels of halofantrine overall were recovered in the HDL/ LPDM fraction of the gradient, implying that drug is present mostly in 'free' form. Recovered levels in HDL/ LPDM fraction were particularly high in the presence of the non-digested microemulsion formulations and the digested olive oil emulsion (Figure 7.16b).

# 7.3.8 Correlation between halofantrine and chylomicron transport in Caco-2 monolayers

A correlation plot of halofantrine transport into basolateral medium and accompanying chylomicron secretion for Caco-2 cells administered halofantrine-saturated lipid vehicles is shown in Figure 7.17a. Data showed no clear relationship between variables as evidenced by the correlation coefficient ( $r^2$ ) of 0.066 (Figure 7.17a). Looking at a plot of the amount of halofantrine recovered in the chylomicron fraction against chylomicron secretion, a stronger correlation is seen, with  $r^2 = 0.4173$ , but this is still very low (Figure 7.17b). As lipid digestion can have a significant effect on halofantrine transport, the data for digested versus non-digested vehicles were examined in isolation. A strong correlation was found between halofantrine recovery in the chylomicron fraction and chylomicron secretion for the non-digested lipid vehicle data ( $r^2$ =0.9462) (Figure 7.18b), which was superior to that obtained for the digested vehicles ( $r^2$ =0.4427) (Figure 7.18a).



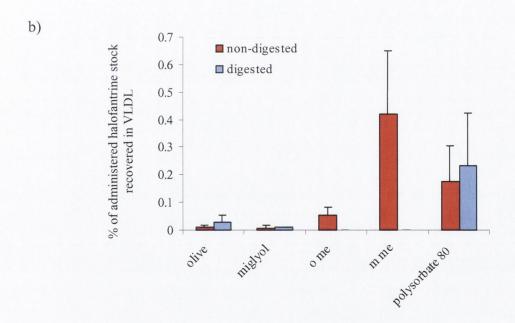
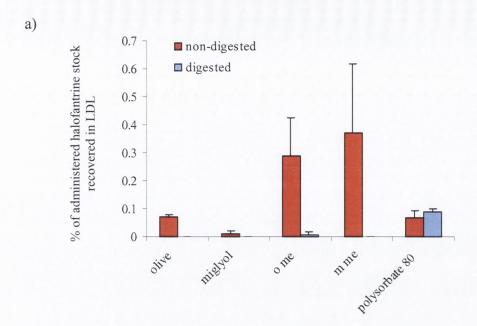


Figure 7.15: Effects of lipid vehicles on the association of administered halofantrine with chylomicron (panel a) and VLDL (panel b) fractions of the density gradient. Data are presented for non-digested and digested vehicles. Values represent the mean  $(n=3) \pm SD$ .



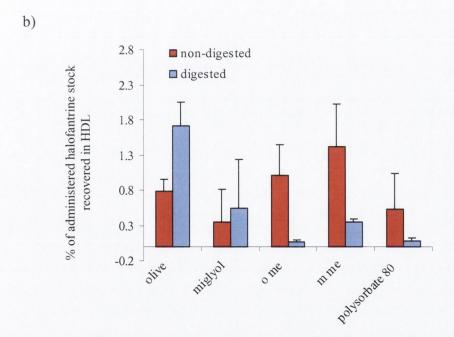
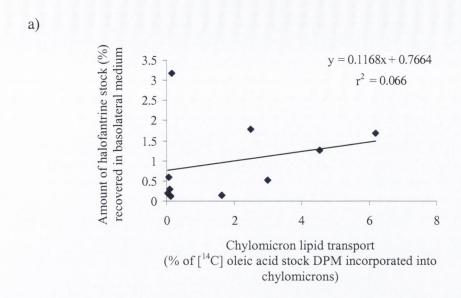


Figure 7.16: Effects of lipid vehicles on the association of administered halofantrine with LDL (panel a) and HDL (panel b) fractions of the density gradient. Data are presented for non-digested and digested vehicles. Values represent the mean (n=3)  $\pm$  SD.



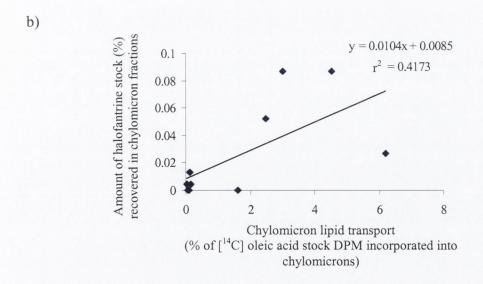
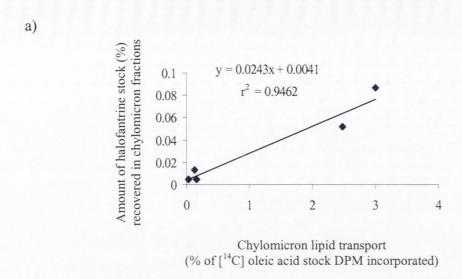


Figure 7.17: Correlation between halofantrine transport into basolateral medium and chylomicron lipid output (panel a) and halofantrine association with chylomicrons and chylomicron secretion (panel b) following supplementation of Caco-2 cells with non-digested and digested halofantrine-saturated lipid vehicles.



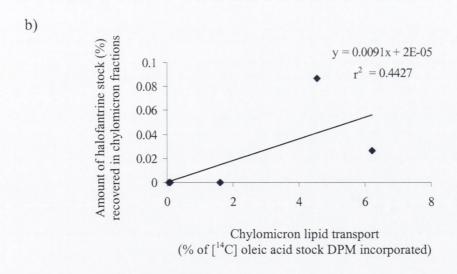


Figure 7.18: Correlation between halofantrine association with chylomicrons and chylomicron lipid transport following supplementation of Caco-2 cells with non-digested (panel a) and digested (panel b) halofantrine-saturated lipid vehicles.

# 7.3.9 Effect of halofantrine on the bi-directional transport of cyclosporin A

Halofantrine is a lipophilic compound with an alkaline side chain, and as such, fits required criteria for a P-gp substrate (Seelig, 1998). Sensitivity of halofantrine to anti-transport processes was checked initially using standard transport studies based on those described in Section 2.3.1. For these preliminary studies, halofantrine was administered as a 200μM aqueous solution and samples were taken at 30-60 minute time intervals from receiving medium. Using this technique, it was not possible to detect halofantrine in the collected samples with the established HPLC assay due to limitations in assay sensitivity. As a compromise, the influence of halofantrine (200μM) on the bi-directional transport of cyclosporin A ([³H] labelled), a recognised P-gp substrate, was evaluated to indicate potential for binding to P-gp. Data presented in Figure 7.19 show that inclusion of halofantrine in the transport medium resulted in a small, but significant decrease (25%) in cyclosporin A efflux (basolateral-apical transport) relative to control, without any statistically significant change in the apical-basolateral transport.

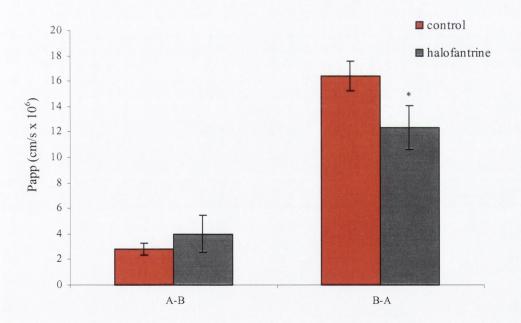


Figure 7.19: Effect of halofantrine ( $200\mu M$ ) on the bi-directional transport of cyclosporin A across Caco-2 monolayers. Values represent the mean  $\pm$  SD (n=3).

# 7.4 Key findings

A number of lipid vehicles were compared for their effects on TRL secretion in Caco-2 cells. When administered in a non-digested state, vehicles stimulated TRL secretion according to the rank order (highest-lowest) of: polysorbate 80> olive oil-based microemulsion>>olive oil emulsion = Miglyol 812-based microemulsion> Miglyol 812 emulsion = control. When vehicles were administered to cells in a pre-digested state, the rank order switched to: olive oil emulsion> polysorbate 80> >> olive oil-based microemulsion> control> Miglyol 812 emulsion = Miglyol 812-based microemulsion.

When halofantrine-saturated vehicles were administered to Caco-2 cells in a non-digested state, highest levels of halofantrine transport across Caco-2 monolayers were observed for the microemulsion vehicles. Cellular uptake of halofantrine followed a similar trend to transmembrane transport.

Following vehicle digestion, the promotive effects of administered microemulsion vehicles on transmembrane transport of halofantrine were considerably and significantly reduced.

When halofantrine-saturated vehicles were administered to Caco-2 cells in a pre-digested state, the highest levels of halofantrine transport across Caco-2 monolayers were observed for the olive oil emulsion and the polysorbate 80 vehicles. Again, cellular uptake of halofantrine followed a similar trend to transmembrane transport.

Of the administered vehicles, only the polysorbate 80 solution (in a non-digested or digested state) was capable of promoting association of halofantrine with secreted TRL, even though highest levels of TRL secretion were observed for the digested olive oil emulsion.

Although there was no clear correlation between TRL output and drug association with TRL fractions for the digested lipid vehicles, there was a good correlation between these variables for the non-digested vehicles ( $r^2=0.942$ ).

#### 7.5 Discussion

In the previous chapter, the systematic development of a lipid based microemulsion preconcentrate formulation for enhancing lymphatic drug uptake was described. The selected olive oil-based formulation produced a finely dispersed microemulsion on dilution in gastric or intestinal medium and contained a high concentration of oleic acid as glyceride esters (in olive

oil or Peccol), free fatty acid or as polysorbitan esters (in polysorbate 80). Additionally, the formulation components were either food grade edible oils, approved for oral delivery or had GRAS (generally recognised a safe) status.

Following on from this work the effects of the developed microemulsion vehicle on the absorption of halofantrine, a model lipophilic drug, in Caco-2 monolayers were assessed. Results were compared with those obtained using a number of different lipid vehicles to determine whether any specific advantages were conferred by this olive oil-based microemulsion (o-me). The comparison formulations were: a medium chain oil (Miglyol 812) emulsion, a long chain oil (olive oil) emulsion, a Miglyol 812-based microemulsion (m-me), the developed olive oil-based microemulsion (o-me from Chapter Six) and a polysorbate 80 micellar solution.

#### Vehicle Toxicity

Vehicles administered in the non-digested state did not adversely affect Caco-2 cell viability or compromise monolayer integrity under the given experimental conditions. However, digested vehicles containing medium chain oils tended to decrease cell monolayer integrity. As discussed in the previous chapter, this was most likely a result of liberated medium chain fatty acids (capric acid and caprylic acid), which have been shown to increase intestinal membrane permeability by dilating tight junctions via an action on phospholipase C (Anderberg *et al.*, 1993; Lindmark *et al.*, 1995; Lindmark *et al.*, 1998). Data presented here showed that the effects on monolayer permeability were reversible following vehicle withdrawal.

#### Non-digested lipid vehicle effects on lipid metabolism

Lipid vehicles promoted an increase in secreted levels of apoB relative to control (buffer supplemented) cells, and with the exception of the Miglyol 812 emulsion, all tended to promote an increase in intra-cellular triglyceride accumulation with a rank order effect of: o-me > polysorbate 80 = m-me > olive emulsion > Miglyol 812 emulsion = control. The o-me and polysorbate 80 vehicles also significantly enhanced the secretion of triglyceride versus control (p<0.01) levels and produced an increase in the secreted triglyceride: apoB ratio, which was associated with a selective increase in TRL (chylomicron and VLDL) output. The effects of polysorbate 80 on TRL secretion were somewhat higher than those of o-me. The m-me was the only other non-digested vehicle to significantly impact on the secreted lipoprotein profile, eliciting a selective increase in LDL secretion relative to the control (p<0.01). As the o-me and polysorbate 80 vehicles, but not the olive oil emulsion, produced selective stimulation of TRL secretion, it is likely that the polysorbate 80 and Peceol components of the microemulsion formulation contributed to the TRL response. Peceol is a monoglyceride of oleic acid and

hence is readily transported across the Caco-2 lipid membrane (Ho and Storch, 2001) and polysorbate 80 has been shown in this project to act as a source of free oleic acid for synthesis of secreted triglyceride (present both as free fatty acid contaminant in the surfactant or liberated by ester hydrolysis) (Chapter 5). The olive oil emulsion contains significantly less free fatty acid than the polysorbate 80 solution (0.05mM versus 0.1mM) and the lack of a TRL stimulating response suggests that it may also be less sensitive to mucosal digestion than polysorbate 80. This suggests that cell-associated esterase as opposed to lipase may play a more important role in Caco-2 mediated digestion of polysorbate 80.

#### Digested lipid vehicle effects on lipid metabolism

When pre-digested vehicles were applied to Caco-2 monolayers the secreted lipoprotein response was significantly altered. This was most profound for the olive oil emulsion, with triglyceride, chylomicron and VLDL secretion being increased 6-, 40- and 4- fold respectively (p<0.01). The TRL secretion response to polysorbate 80 was also increased by the pre-digestion step, but to a lower extent, with only a 1.3-fold increase in triglyceride secretion (p<0.05) relative to non-digested results. In contrast, the secreted triglyceride and accompanying TRL response to o-me was reduced following digestion by approximately 30%, but this difference was not statistically significant.

The increased TRL secretion response to the digested polysorbate 80 and olive oil vehicles can be attributed to the increase in vehicle free fatty acid content (predominantly as oleic acid) following lipolysis. Olive oil is a natural substrate for pancreatic lipase (Tietz et al., 1989; Armand et al., 1994), and polysorbate 80 has also been shown to have limited susceptibility to this enzyme (Plou et al., 1998). All of the investigated lipid vehicles were found to be sensitive to intestinal lipolysis (Figure 7.10), as evidenced by a significant rise in diluted vehicle free fatty acid content. Lipase activity against the olive oil and Miglyol 812 emulsions was particularly high, with 50- and 80- fold increases in fatty acid concentration respectively as compared to 11-fold for polysorbate 80 Mansbach and Nevin (1998). It is interesting to note that although the oleate rich o-me formulation was also sensitive to pancreatic lipase activity, experiencing a 6.7-fold increase in vehicle free fatty acid content on digestion, the TRL stimulating potential of this vehicle was reduced by lipolysis. As this was not accompanied by any reduction in cell viability or monolayer permeability, the findings could not be attributed to cell condition. One possible explanation for these findings is that of increased affinity of liberated fatty acid for the dispersed phase following digestion. Lipolysis generates free fatty acids and monoglycerides from dispersed di- and triglycerides (Armand et al., 1994; Embleton and Pouton, 1997), and may also hydrolyse ester bonds of non-ionic surfactants such as polysorbate 80 (Plou et al., 1998) and Cremophor EL (Howes et al., 1998), liberating free fatty

acids, glycerides and ethylene oxide groupings. On mixing with bile salts in the simulated intestinal buffer, such products create a highly solubilising mixed-micellar phase (Carey and Small, 1970; Hernell *et al.*, 1990). Before fatty acids can be absorbed by enterocytes, it is necessary for solubilised free fatty acids to dissociate from the micellar phase at the brush border (Shiau and Levine, 1980). It is possible that the affinity of fatty acid for the mixed micellar phase following microemulsion digestion is increased, such that additional liberated fatty acid is not available in free form for absorption. Alternatively, Cremophor EL, which has already been shown to impair TRL secretion (Chapter 4) may have additional inhibitory effects on TRL secretion when presented in a digested form, possibly through increased cellular uptake.

Of further note is the observation of increased intracellular triglyceride levels in the presence of the digested Miglyol 812 and m-me vehicles versus findings for non-digested medium chain vehicles. This was accompanied by a selective reduction in the secreted LDL response. Cytotoxicity data for these digested vehicles revealed a decrease in monolayer integrity, most likely due to medium chain fatty acid effects on tight junctions. The increase in cellular triglyceride may be attributed to the massive increase in free fatty acid content of digested vehicles (80-fold for the Miglyol 812 emulsion and 13-fold for the m-me respectively), which is available for cellular absorption. The decrease in LDL secretion observed for digested medium chain vehicles, in combination with the reduced monolayer integrity, may reflect impaired cellular metabolism in this case. Intracellular dehydrogenase activity was not altered, but as the lipoprotein assembly path involves a number of complex steps, including vesicular transport and secretion of assembled lipoproteins (Sabesin and Frase, 1977; Mansbach and Nevin, 1998; van Greevenbroek and de Bruin, 1998), it is possible that one or more of these steps were impaired.

# Non-digested lipid vehicle effects on the uptake and cellular passage of halofantrine

Looking initially at dose corrected data for non-digested lipid vehicles, it was found that highest degree of cellular uptake (as a % of administered stock) of halofantrine was obtained with the polysorbate 80 and o-me vehicles. For all, but the Miglyol 812 emulsion, > 30% of administered stock was recovered in the cell lysate. The Miglyol 812 emulsion produced intracellular halofantrine levels of approximately 10% of administered stock content. The amount of halofantrine transported into the basolateral chamber was also vehicle dependent. A similar trend to cellular data was followed, but the differences were more pronounced. The two microemulsion formulations facilitated highest transport levels of halofantrine. Although transport was 30% higher with the m-me as compared to the o-me, the difference was not statistically significant because of high variability. The rank order of halofantrine transport with non-digested lipid vehicles was: m-me > o-me > olive emulsion > polysorbate 80 > Miglyol 812 emulsion. These data represent dose corrected values. If the uncorrected (absolute) values for

halofantrine transport are considered, the relative enhancements offered by the microemulsion vehicles are magnified because of their superior drug solubilisation properties. Using uncorrected values, the m-me and o-me formulations facilitated transport of 180-fold and 70-fold more halofantrine respectively than the olive oil emulsion, as compared to 3.5-fold and 2-fold with the dose corrected data. As the microemulsion formulations both enhanced drug transport to a similar extent and to a much greater degree than any of the other administered vehicles, it can be assumed that either the microemulsion excipients or the dispersed state of the microemulsion is responsible for the observed effect. As the results were similar for both the medium chain and long chain microemulsion formulations, it is unlikely that lipid pathways are a major contributing factor to drug passage in the Caco-2 model for these vehicles. Both microemulsion formulations contained a large amount of the non-ionic surfactants, polysorbate 80 and/ or Cremophor EL.

As discussed and illustrated in Chapters 4 and 5, both Cremophor EL and polysorbate 80 have been shown to demonstrate significant inhibitory activity against P-gp mediated drug efflux. There are no published reports on halofantrine sensitivity to P-gp, but available data does indicate that it is a substrate for Pgh 1, a homologue of P-gp that is found in *Plasmodium falciparum*, and which has been associated with drug resistance (Reed *et al.*, 2000). Additionally, results presented here indicate that halofantrine has some ability to impair P-gp-mediated efflux of cyclosporin A, a P-gp substrate, in the Caco-2 model, suggesting indirectly, that it may also act as a substrate for this protein. However, because of limitations in assay sensitivity, it was not possible to directly study the passage of halofantrine across the Caco-2 monolayer from an aqueous solution in a standard transport experiment because of limitations in assay sensitivity.

With the exception of the polysorbate 80 micellar solution, none of the non-digested formulations promoted incorporation of halofantrine into the chylomicron or VLDL fractions, in most cases, halofantrine was associated with LDL or HDL/ LPDM fractions. Microemulsions promoted incorporation of halofantrine into LDL, with 10% of gradient halofantrine recovered in LDL for the long chain microemulsion and 20% for the medium chain microemulsion. LDL association may have occurred as a result of drug-protein binding. The polysorbate 80 micellar solution resulted in 10% of basolateral drug being incorporated into chylomicrons, 20% into VLDL, 8% into LDL and 62% into the HDL/ LPDM fraction. As halofantrine falls naturally into the most dense fractions of the gradient, it is likely that the presence of halofantrine in the TRL fractions is associated with secreted lipoproteins. However, as chylomicrons only accounted for 10% of basolateral halofantrine transport in the Caco-2 model employed here, lymphatic drug transport is not predicted to be extensive.

# Digested lipid vehicle effects on the uptake and cellular passage of halofantrine

When lipid vehicles were subject to a pre-digestion step their effects on halofantrine uptake and transmembrane transport were significantly altered. Cellular uptake was found to be increased for the two emulsion vehicles and for the polysorbate 80 solution, but the difference was only statistically significant for the olive oil and Miglyol 812 emulsions. In contrast, cellular uptake of halofantrine from the two microemulsion vehicles was substantially reduced following digestion, even taking administered dose adjustments into consideration. Lipid vehicle digestion produced an increase in the transmembrane transport of halofantrine from the olive oil emulsion (1.9-fold, p<0.01) and a significant decrease in transport from the microemulsion vehicles (10-fold for m-me and 12.5-fold for o-me). Both the polysorbate 80 solution and Miglyol 812 emulsion showed an increase in transmembrane transport of halofantrine following vehicle digestion, but the differences were not statistically significant versus non-digested data because of sample variability. Following digestion, the rank order of halofantrine transport was: olive oil emulsion> polysorbate 80> Miglyol 812 emulsion> m-me=o-me. Where data were not dose corrected, values followed the same trend, but the differences were even more pronounced for the effects of digestion on the olive oil emulsion and microemulsion vehicles.

Taking these findings into consideration, it is clear, that although the investigated microemulsion vehicles confer significant advantages in terms of intestinal halofantrine absorption in the non-digested state, these advantages are removed once vehicles are subject to intestinal luminal digestion. The reason for this may again be related to an increase in drug affinity for the mixed micellar phase produced following lipid vehicle digestion, resulting in a decrease in the 'free' fraction of drug available for absorption. Similar findings have been previously reported for bile salt effects on drug absorption (Charman *et al.*, 1997). Gradient data did show some involvement of LDL and VLDL in drug transport for non-digested microemulsion vehicles, which was eliminated post-digestion. However, as basolateral transport was decreased by 90% overall, whilst LDL and VLDL transport only accounted for 30% of drug transport, then it is likely that decreased drug-lipoprotein association only plays a small role in the observed reduction in drug transport. Chylomicron association was not a significant factor for the o-me vehicle in the Caco-2 model employed here, and hence, lymphatic drug transport is not predicted to be a contributing factor.

Data for the Miglyol 812 emulsion showed very low levels of cellular uptake and transmembrane transport of halofantrine, with <0.5% of administered stock being recovered in the basolateral volume. Digestion of the Miglyol 812 vehicle resulted in a significant increase in the cellular uptake of halofantrine and a small increase in basolateral transport. As the digested Miglyol 812 vehicle also reduced membrane integrity, this increase in uptake may be a

result of increased membrane permeability. As transported halofantrine was found in association with the HDL/ LPDM fraction of the gradient, in which 'free' halofantrine naturally falls, it is likely that drug passed through cells via passive diffusion and did not associate with secreted lipoproteins.

The influence of the olive oil emulsion on drug absorption in Caco-2 cells is of particular interest. The olive oil emulsion, in a non-digested state promoted uptake of approximately 30% of administered halofantrine and basolateral transport of 0.8% of administered stock. Following vehicle digestion, the amount of halofantrine taken into cells was increased to 70% of administered stock and the basolaterally-transported level was increased to 1.5% (p<0.01). The triglyceride output of cells was increased in tandem with the increase in halofantrine absorption, hence, the impact of lipoprotein secretion on drug transport should be considered. However, from the density gradient data, it can be seen that the basolaterally-recovered halofantrine was associated with the HDL/LPDM fraction of the gradient, and hence TRL mediated transport did not occur. Drug absorption is therefore likely to have occurred via passive diffusion. The digested olive oil vehicle may additionally have increased drug absorption by altering membrane fluidity. Long chain free fatty acids have been shown to insert into the lipid bilayer of cells and to disrupt the structure, thereby increasing membrane fluidity and 'leakiness' (Muranishi, 1990). Monolayer TEER values were not significantly altered in this work, but it is still possible for membrane fluidity to be altered sufficiently to permit increased drug absorption, without increasing paracellular permeability or altering transpoithelial transport of other, easily absorbed, transport markers such as propranolol. Alternatively, the possibility that halofantrine passes through cells in association with TRL and then dissociates during the incubation period must also be considered.

The absence of any TRL associated transport of halofantrine in the Caco-2 model in the presence of olive oil is not consistent with *in vivo* findings. Porter *et al.* (1996a), Caliph *et al.* (2000) and Holm *et al.* (2001) all found a significant increase in lymphatic uptake of halofantrine in the rat model following co-administration with lipid vehicles containing long chain unsaturated fatty acids. Most notably Porter *et al.* (1996b) found that up to 20% of halofantrine was transported in the lymph of a conscious, lymph cannulated rat model when administered in a peanut oil dispersion. Caliph *et al.* (2000) and Holm *et al.* (2001) also reported a clear relationship between lymphatic triglyceride output and lymphatic transport of halofantrine. Such findings are not supported by the Caco-2 data presented here. The beneficial effects of oleate and other long chain unsaturated fatty acid rich oils on the lymphatic transport of a wide range of lipophilic drugs have also been widely reported for *in vivo* rat studies (Nishigaki *et al.*, 1976; Palin and Wilson, 1984; Charman and Stella, 1986; Myers and Stella,

1992; Ichihashi *et al.*, 1992), thus demonstrating that this is a highly relevant mechanism of action *in vivo* for lipid vehicle effects on drug uptake.

Of the investigated systems, polysorbate 80 was the only vehicle that appeared to promote drug passage via TRL association. In the non-digested state, polysorbate 80 promoted uptake of approximately 50% of administered halofantrine stock, which was increased to 60% in the digested state. Basolateral transport of halofantrine was increased from 0.5% to 1% of administered stock by vehicle digestion. This was not a statistically significant difference, and the level of halofantrine transport was still only 60% of the digested olive oil vehicle, but what is of note here is that 30% of the basolaterally-transported dose was associated with TRL when administered with the non-digested solution and this was increased to 70% (30% with chylomicrons and 40% with VLDL) when administered with a digested solution. This implies that polysorbate 80 is capable of enhancing lymphatic drug uptake and may therefore confer more significant advantages in terms of lymphatic drug targeting than the developed microemulsion. The differences observed between digested olive oil and polysorbate 80 effects on TRL association of halofantrine may be related to the different dispersed states of the vehicles.

Micellar solutions of polysorbate 80 have previously been shown to promote lymphatic uptake of lipophilic drugs *in vivo* (Nishigaki *et al.*, 1976; Ichihashi *et al.*, 1992; Porter *et al.*, 1996a; Porter *et al.*, 1996b). These effects have largely been attributed to drug dispersion, but as polysorbate 80 has the potential to promote lymphatic triglyceride output, the relevance of lymphatic mediated transport should also be considered.

# **Chapter Eight**

**General Discussion** 

#### 8 General Discussion

# 8.1 Background

There is a growing trend towards the employment of combinatorial chemistry and genomics in drug discovery programs. These techniques are aimed at rapidly identifying highly potent, site directed compounds. Such compounds tend to have low aqueous solubility, high lipophilicity and high molecular weights, and therefore face serious oral bioavailability issues, which can hinder product development (Venkatesh and Lipper, 2000). Formulation scientists are therefore moving towards the use of novel or non-conventional formulation strategies to overcome the formidable barriers to oral drug absorption faced by lipophilic drugs.

Lipid-based vehicles have been shown to increase the oral bioavailability of co-administered lipophilic compounds. In spite of this, there has been a general reluctance, until recently, to advance such formulations to the market. This reticence is largely due to an absence of clear guidelines on formulation design and a paucity of information regarding vehicle effects *in vivo*. However, in light of the increasing trend towards highly potent, lipophilic drug candidates and the clinical and commercial successes of several lipid-based formulations incorporating problem drugs - namely cyclosporine (Neoral®), ritonavir (Norvir®) and saquinavir (Fortovase®) – there has been a renewed interest in this research field.

Lipid-based vehicles provide a means of increasing the oral bioavailability of lipophilic drugs via a number of mechanisms including: increased aqueous solubility in the gastrointestinal tract, enhanced transport across the unstirred water layer, inhibition of enterocyte-based metabolism and counter-transport processes and promotion of lymphatic drug uptake via association with intestinal TRL. Lymphatic uptake is of particular interest because, in addition to facilitating absorption of highly lipophilic drugs, this route offers a means of avoiding hepatic first pass metabolism and of targeting drugs specifically to lymphatic sites. Recent findings from a conscious dog model suggest that up to 50% of a delivered lipophilic drug (halofantrine in this example) dose may be transported via the lymphatics, when co-administered with food, demonstrating the significance of this route of transport (Khoo *et al.*, 2001).

To date, lymphatic drug uptake has been studied on a small scale in whole animal models, largely in lymph cannulated rats. However, given the demand for high throughput screening required by modern drug discovery programs, an *in vitro* model capable of predicting the *in vivo* lymphatic response is required. The Caco-2 model has been shown to secrete a similar range of lipoproteins to the intestine. As lymphatic drug transport occurs largely by drug association with the triglyceride core of large, triglyceride rich chylomicrons, lymphatic transport potential

can theoretically be estimated by monitoring the extent of chylomicron or triglyceride secretion in isolated cells and examining drug-lipoprotein partitioning behaviour.

#### 8.2 Aims

This project aims to investigate the effects of a number of lipid vehicle excipients on lipoprotein secretion in the Caco-2 model, and to further delineate associated mechanisms of action. These findings will facilitate a more rational approach to excipient selection for lymphotropic vehicle formulations. Additionally, the work examines the ability of the Caco-2 model to simulate *in vivo* responses to lipid vehicles and to predict lymphatic drug association.

# 8.3 Inhibition of TRL secretion by lipid vehicle excipients

Non-ionic surfactants and co-solvents are frequently incorporated into lipid-based vehicles at high concentrations to facilitate rapid and efficient solubilisation and dispersion of the vehicle and its drug load on contact with aqueous gastrointestinal fluid. Evidence in published literature, however, has pointed to potential bio-modulating effects of non-ionic surfactants on lipid metabolism. Extensive studies by Tso and colleagues, have shown that Pluronic L81, a lipophilic block copolymer surfactant, has potent and acute inhibitory actions on chylomicron secretion (Tso *et al.*, 1981; Tso and Gollamudi, 1984). Work, conducted by this group, using a mesenteric lymph cannulated rat, has shown that concentrations as low as 0.17mg/ml Pluronic L81 can produce complete block of lymphatic chylomicron output. As lymphatic drug transport is proportional to triglyceride output, this can have serious implications for the bioavailability of lipophilic drugs. Given the widespread use of non-ionic surfactants in formulation design and the potency of Pluronic L81 as an inhibitor, it was decided to investigate the effects of a range of commonly used pharmaceutical surfactants on lipoprotein secretion in our established Caco-2 model.

A number of tested surfactants - TPGS, Cremophor EL and Pluronics L81, P85 and F68 - were found to inhibit TRL secretion, with selectivity for chylomicron secretion at lower concentrations (Chapter Four). The rank order for inhibitory potency against chylomicron secretion was: Pluronic L81>> Pluronic P85  $\approx$  TPGS > Cremophor EL >> Pluronic F68. This followed a similar trend to the surfactant HLB, as summarised in Figure 8.1. A good correlation was found between surfactant inhibitory potential and HLB across the full surfactant range (correlation coefficient=0.722) and this was more pronounced for the Pluronic block copolymer class as shown in the inset (correlation coefficient = 0.888).

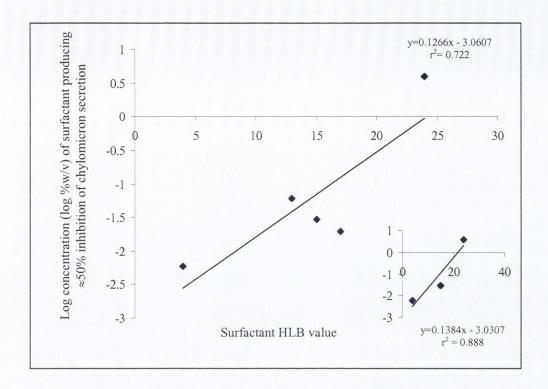


Figure 8.1: Correlation between surfactant HLB and inhibition of chylomicron secretion (expressed as the log concentration of surfactant producing approximately 50% inhibition of chylomicron secretion),  $r^2 = 0.722$ . The inset shows the same data, for Pluronic block copolymers in isolation,  $r^2 = 0.888$ .

Although the negative effects of Pluronic L81 on TRL secretion are well established, the mechanisms associated with inhibition have not yet been elucidated. The data presented here show that inhibition is common to other classes of non-ionic surfactant and that it is related to surfactant HLB. Work by Batrakova and colleagues found a similar HLB dependence of Pluronic block copolymer mediated inhibition of P-gp efflux. Lipophilic block copolymers with HLB <20 and an intermediate propylene oxide chain length (30-60 units) were found to be most potent at inhibiting P-gp (Kabanov *et al.*, 2003). As these structural features were also associated with an increase in the cellular uptake of block copolymers, it has been postulated that the HLB dependence of P-gp inhibition may be related to ease of compound transport within the cell and access to intracellular metabolic targets. Pluronic F-68 is too hydrophilic to be readily absorbed by cells, whilst Pluronic L81 and Pluronic P85 fit criteria for high uptake and demonstrate significant inhibition of P-gp at low concentrations (<0.005%w/v) (Batrakova *et al.*, 1999). It is feasible that the observed HLB dependence of TRL inhibition also reflects the ability of surfactants to gain access to intracellular targets.

Data presented in Chapter Four found that chylomicron and VLDL output were severely inhibited at appreciably low concentrations of surfactant. The intracellular levels of triglyceride and apoB, key components of TRL were, however, largely unaffected, suggesting that inhibition occurs subsequent to lipid and apoB synthesis. These findings are consistent with observations in the rat model by Tso and colleagues. Findings by this group showed that intra-duodenal administration of Pluronic L81 with an oleate rich vehicle caused a block in chylomicron secretion and a corresponding accumulation of large lipidic droplets within the enterocytic endoplasmic reticulum (ER) (Tso et al., 1981). Lipid droplets were up to 10-times larger than standard chylomicrons. On withdrawal of the vehicle, chylomicron secretion was found to recover rapidly, giving a burst release of chylomicron-sized lipoproteins. The typical lipoprotein assembly route, as delineated by Sabesin and Frase (1977), commences in the ER. Absorbed fatty acids are re-assembled into triglycerides within the smooth endoplasmic reticulum (SER) and apoB is synthesised within the rough endoplasmic reticulum (RER). ApoB associates with lipid droplets in the SER/ RER junction, forming primordial lipoproteins. These are then transported to the golgi, where they are packed into vesicles and transported into the intercellular space before passing into lymph. Little is known about the changes that occur en route from ER to golgi or how the process is regulated, but the fact that Pluronic L81 causes lipids to accumulate in the ER implies that Pluronic L81 either interferes with the association of apoB with the lipid mass or it prevents transport of nascent lipoproteins to the golgi.

The effects of Pluronic L81 on lipid metabolism in the rat model are similar to the characteristics of chylomicron retention disease in humans. This inherited condition is associated with fat malabsorption, hypocholesterolemia and an accumulation of lipid with enterocytic ER, resulting from an inability to secrete apoB containing lipoproteins. In chylomicron retention disease, fasting plasma triglyceride levels and hepatic VLDL output are normal (Levy et al., 1987). A study by Raabe et al. (1998), using a transgenic mouse model expressing hepatic, but not intestinal apoB, produced similar symptoms, with an absence of chylomicron secretion and a massive accumulation of cytosolic fat droplets in the absorptive enterocytes. It is conceivable that apoB is required for transporting nascent lipoproteins from the ER to the golgi and that the effects of Pluronic L81 may be due to inhibition of the lipid-apoB complexation process.

A recent *in vitro* study by Morita *et al.* (2003) has shown that Pluronic L81 alters the conformation of apoB 100 adopted on the surface of LDL and causes lipid droplets and lipoproteins to aggregate. The failure of more hydrophilic Pluronic L64 to produce similar effects was attributed to a specific HLB requirement for observed responses. The authors postulated that Pluronic L81 achieved its multiple effects by destabilising lipid and lipoprotein

particle surfaces, thereby interfering with the lipid-apoB interaction, which is essential for maintaining correct apoB conformation, and causing lipid droplets to coalesce. This mechanism is consistent with our findings of selective inhibition of TRL secretion without corresponding changes in intracellular triglyceride and apoB synthesis and is also consistent with observations by Tso *et al* (1981) of excessively large lipid droplet accumulation in the enterocytic ER of rats administered a combined lipid- Pluronic L81 vehicle.

An energy dependent transfer route has been proposed for the transport of primordial/ nascent lipoproteins from the ER to the golgi and has been shown to be rate limiting to TRL secretion (Kumar and Mansbach, 1997; Mansbach and Nevin, 1998). *In vitro* studies by Kumar and Mansbach (1999) have isolated a vesicle that vectorially transfers lipid from ER to golgi. The isolated vesicle is approximately 200nm in diameter, and appears to carry a single lipid mass, suggesting that one chylomicron is transported per vesicle. The authors proposed that vesicle mediated transfer may act to protect triglyceride from lipolysis by intracellular lipases. As this route is energy dependent, any interference of energy utilisation in the cell will affect this transfer step. This is another possible site of regulation by surfactants and will be discussed in further detail below. Inhibition of vesicle transfer from ER to golgi would also be consistent with our findings of selective inhibition of lipoprotein secretion.

Collectively, the observation of significant impairment of TRL secretion with low concentrations of pharmaceutically useful surfactants has serious implications for lymphatic drug transport. The effects of lipid vehicle excipients on lymphatic triglyceride output, therefore, need to be taken into consideration in early stages of lymphotropic vehicle formulation.

# 8.4 Interrelationship between P-gp and lipid metabolism

It is a widely accepted view that inhibition of P-gp by non-ionic surfactants is mediated by a surface active effect on plasma membranes. Surfactants increase membrane fluidity either by insertion into the lipid bilayer or by solubilising out membrane lipids (Dimitrijevic *et al.*, 2000; Evrard *et al.*, 2001). The increase in membrane fluidity may alter the conformational arrangement of the membrane bound P-gp protein and hence interfere with its activity. Regev *et al.* (1999) showed that membrane fluidisation by anaesthetics abolished P-gp ATPase activity in a similar manner to surfactants, and Batrakova and colleagues demonstrated a close relationship between Pluronic block copolymer effects on membrane fluidity and corresponding P-gp inhibition (Batrakova *et al.*, 2001b; Batrakova *et al.*, 2003; Kabanov *et al.*, 2003).

Data presented in Chapter Four indicate that P-gp inhibition by surfactants may be a more complex process. Our findings showed that a range of non-ionic surfactants were able to inhibit TRL output in the Caco-2 model. All of these excipients had been previously reported to inhibit P-gp activity. Further investigation demonstrated a close correlation between the concentration dependence of surfactant mediated P-gp inhibition and inhibition of chylomicron secretion by Cremophor EL and Pluronic L81. Both processes were found to be impaired at 0.06%w/v Cremophor EL and 0.006% Pluronic L81, thus implying a common regulatory mechanism between the two processes.

Work presented by Field *et al.* (1995) found that a range of pharmacologically active drugs, including verapamil, trifluoperazine and progesterone, with recognised P-gp inhibitory effects, acted to impair lipid and apoB output and cholesterol trafficking in the Caco-2 cell model, thereby suggesting that a common regulatory mechanism exists to modulate P-gp activity and lipid metabolism. Field *et al.* (1995) showed that in addition to P-gp modulating drugs, nigericin, a potassium ionophore that produces alkalinisation of intracellular vesicles, also caused a profound inhibition of cholesterol transport and apoB secretion. It has been postulated that P-gp or related ATP dependent transport proteins may be involved in maintaining the acidity of transport vesicles and that inhibition of these proteins results in impaired lipoprotein output.

As discussed above, an energy dependent transfer vesicle has been implicated in transporting primordial chylomicrons from the ER to the golgi and is likely to be the rate limited step to TRL secretion (Mansbach and Nevin, 1998; Kumar and Mansbach, 1999). Alkalinisation of this transport vesicle by the mechanism proposed by Field *et al.* (1995) may explain the effects of surfactants and other P-gp modulating drugs on TRL secretion.

Recent findings by Batrakova and colleagues have shown that Pluronic P85 depletes intracellular levels of ATP in MDR cells and inhibits ATPase activity (Batrakova *et al.*, 2000). A correlation between inhibitory effects of Pluronic P85 on P-gp and ATP depletion has been demonstrated and it has been found that supplementation of inhibited cells with exogenous ATP has the effect of reversing the P-gp inhibitory response (Batrakova *et al.*, 2001a). Data by this group also showed that the ATP depleting mechanism was closely related to block copolymer HLB and PO chain length, such that lipophilic block copolymers with intermediate PO chain length chain length proved to be most efficient at depleting ATP (Batrakova *et al.*, 2003). These Pluronic structural features were also important for P-gp inhibitory potential, intracellular movement and surfactant effects on membrane fluidity.

Brierley et al. (1972) and van Zutphen et al. (1972) have shown that non-ionic polyoxyethylated surfactants, including Triton X, nonylphenol ethoxylates and polysorbate 20 can act as ionophores, increasing lipid bilayer permeability to monovalent cations, particularly to potassium. The ability of polyoxyethylated surfactants to act as ionophores is closely related to their ability to complex potassium and also to their lipid solubility. It is therefore thought that the polyoxyethylene chain forms a complex with potassium or other monovalent cations, and that the resulting lipid soluble cation-surfactant complex passes through the lipid bilayer, thereby disrupting membrane resistance. This has been shown in isolated phospholipid bilayer membranes and also in mitochondria, where potassium uptake was found to increase in the presence of surfactants, resulting in mitochondrial swelling. Ionophore activity was found to be concentration dependent, with more rapid onset at higher surfactant concentrations. Potassium ionophore activity can impact directly on transport vesicles by interfering with the ion gradient that maintains vesicle acidity or may result in energy depletion by altering the electron transport chain in mitochondrial membranes. Brierley et al. (1972) and Rapoport et al. (2000) have demonstrated that non-ionic surfactants can inhibit electron transport in mitochondria, resulting in decreased respiration, phosphorylation and ATPase activity. Hence, potassium ionophore activity may be responsible for multiple effects of surfactants on energy dependent processes in the cell.

All of the surfactants found to inhibit TRL secretion in Chapter Four contained a polyoxyethylene grouping in their structure. Administration of an isolated polyoxyethylene group (as PEG 400) produced inhibition of TRL secretion, but the potency was low and similar to that obtained with the most hydrophilic block copolymer, Pluronic F68, again, possibly reflecting poor membrane permeation. The requirement of a polyoxyethylene grouping in surfactants and the increase in potency with more lipophilic compounds is consistent with an intracellular ionophore mechanism of inhibition.

In addition, the polyoxyethylene chain may be important for direct inhibition of P-gp by certain surfactants. Work by Loo and Clarke (1998) has shown that nonylphenol ethoxylates bind to specific sites on the P-gp complex and that the polyoxyethylene chain is critical in the protein-surfactant interaction, as the isolated nonylphenol molecule does not exhibit similar binding. The authors hypothesised that electron donating groups on the polyoxyethylene chain may be important for the binding mechanism (Loo and Clarke, 1998). Due to the cross-over of surfactant actions on TRL secretion and P-gp activity, the polyoxyethylene group may also be important for excipient interaction with other members of the ABC transporter family that are involved in lipid metabolism and hence it is feasible that surfactants have a direct inhibitory effect on ATP dependent proteins.

Energy dependent proteins of the ABC transporter protein family have recently been implicated in lipid metabolism. Berge *et al.* (2000) reported that mutations in the genes encoding two members (ABCG5 and ABCG8) of the ABC protein family may be responsible for the excessive intestinal absorption and impaired biliary output of sterols observed in the autosomal recessive condition sitosterolemia (Berge *et al.*, 2000). Additionally, ABC-1 transporter proteins have been shown to play a central role in cholesterol homeostasis by regulating the amount of cholesterol absorbed from the diet and may also be involved in intracellular trafficking of phospholipid (McNeish *et al.*, 2000). Furthermore, liver X receptors (LXRs) and farnesoid X receptors (FXRs), which are nuclear hormone receptors activated by oxysterols and bile acids respectively, have been shown to play a role in lipid homeostasis by regulating the expression of genes involved in lipid metabolism (Repa *et al.*, 2000; Edwards *et al.*, 2002; Joseph and Tontonoz, 2003). These nuclear receptors have been shown to regulate expression of ABCG5, ABCG8, ABAC1 and fatty acid synthase (Edwards *et al.*, 2002). Hence, these receptors may present another possible target for excipient mediated modulation of TRL secretion.

#### 8.5 Polysorbate surfactants

In apparent contradiction to findings from Chapter Four, polysorbate 80, a non-ionic polyoxyethylated surfactant of intermediate HLB, was found to selectively stimulate the secretion of chylomicrons in a concentration dependent manner (Chapter Five). The TRL stimulating effects of polysorbate 80 were most likely attributable to the oleic acid moiety, which was liberated from the polysorbitan oleate ester following digestion by Caco-2 brush border enzymes or pancreatic lipase. Reports from in vivo rat studies have demonstrated that simple micellar solutions of lipophilic drugs can promote selective lymphatic drug transport. For example, Nishigaki et al. (1976) reported a 2-fold enhancement in lymphatic transport of retinyl palmitate following administration in an aqueous polysorbate solution as compared to a long chain triglyceride emulsion and Ichihashi et al. (1992) found that lymphatic transport of mepitiostane, following administration in an aqueous polysorbate 80 solution, was comparable to that obtained with a sesame oil or triolein emulsion. Possible explanations for apparent 'lipid-free' micellar solution effects on lymphatic drug transport include enhanced drug transport as a function of increased aqueous solubilisation, enhanced drug absorption via surfactant effects on membrane permeability partitioning of drugs into lymphatic lipoproteins synthesised from endogenous lipids or partitioning of drugs into lymphatic lipoproteins synthesised from lipolytic degradation products of surfactants (such as oleic acid from polysorbate 80). Our findings show that concentrations as low as 0.15%w/v polysorbate 80 can produce significant increases in triglyceride and chylomicron secretion in the Caco-2 model.

and that 0.25%w/v polysorbate 80 can produce secretion levels equivalent to 0.5mM oleic acid. Given that many of the *in vivo* rat studies have used high concentrations of polysorbate 80 (up to 10%) it is likely that increased lymphatic triglyceride output resulting from polysorbate 80 digestion contributes to lymphatic drug transport.

When halofantrine was administered to Caco-2 cells in a pre-digested olive oil vehicle, the resulting association of drug with secreted chylomicrons was 7-fold lower than when halofantrine was administered in a pre-digested polysorbate 80 solution (Chapter Seven). Triglyceride secretion levels were similar for both vehicles, but specific chylomicron lipid secretion was higher with the olive oil vehicle by approximately 40%. This suggests that factors other than chylomicron triglyceride load influence drug partitioning into the lymph, such as the dispersed state of the drug or effects on the membrane barrier (biochemical and physical).

Assuming that the oleic acid content of polysorbate 80 is sufficient to overwhelm any inhibitory potential that the remaining structure may have on lipid metabolism, inhibition of TRL secretion by polysorbate 60 would still be expected, as its saturated fatty acid component, stearic acid, unlike monounsaturated oleic acid, does not tend to promote chylomicron secretion. However, findings in Chapter Five showed that this was not the case, thus indicating that an intermediate HLB and the presence of a polyoxyethylene chain is not sufficient to ensure TRL inhibition by surfactants and that further structural features must be considered. Unlike the other investigated polyoxyethylated surfactants, the hydrophilic polyoxyethylene chains on polysorbates are distributed around the sorbitol ring as four short chains rather than being present in a single, long spiralling chain. The four chains sum to a total of 20 ethylene oxide subunits, such that each chain contains, on average, 5 units. As discussed above, chain length is critical for the efficacy of surfactants as potassium ionophores, as the polyoxyethylene chain needs to surround the cation. Studies by Brierley et al. (1972) showed that potassium ionophore activity was reasonably low in polysorbate 80 and absent in polysorbate 60. As polysorbate 20, a structurally similar compound based on the lauric acid (C12:0), was found to have potent ionophore activity, the length or flexibility of the fatty acid moiety esterified to one of the four polyoxyethylene side chains may also influence the binding mechanism. An absence of ionophore activity for polysorbate 60 would explain the lack of TRL and P-gp inhibitory effects noted in our studies. The presence of low ionophore activity for polysorbate 80 would explain why P-gp inhibition was observed in Chapter Five and the presence of oleic acid would account for the absence of any observed TRL inhibition.

It is also feasible that the conformation of the polyoxyethylene side chain is important for direct binding interactions with ABC proteins, and may therefore influence the ability of surfactants to interact with specific binding sites/ protein receptors.

Given the dual effects of polysorbate 80 on TRL secretion and on P-gp efflux, this excipient offers much promise in the formulation of lymphotrophic vehicles for lipophilic drugs. Bioavailability enhancements for lipophilic drug candidates may be considerable as a result of the combined reduction in luminally directed drug efflux and increased the lymphatic drug transport.

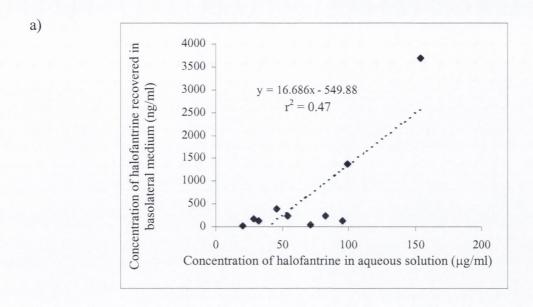
#### 8.6 Drug solubilisation and lymphatic uptake

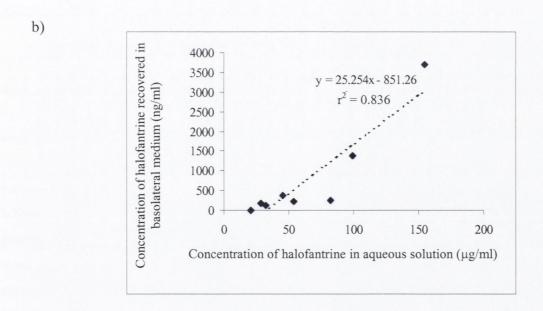
The post-prandial luminal contents are ideal for solubilising high concentrations of lipophilic drugs. This is largely facilitated by the presence of bile salts, phospholipids and amphiphilic end products of lipid digestion, which create a mixed micellar phase into which drugs can partition. The luminal contents form fairly complex phases that can be separated into a mixed micellar phase, a crystalline phase and lipid phase. The mixed micellar phase facilitates lipophilic drug absorption by transporting drug molecules across the aqueous boundary layer and by presenting drugs in a molecularly dispersed form at the enterocyte membrane.

The impact of post-prandial luminal contents on the aqueous solubility of lipophilic drugs, has been widely investigated. High concentrations of bile salts have been found to significantly enhance the aqueous solubility of lipophilic drugs. For example, Naylor et al. (1995) showed that the aqueous solubility of danazol, a highly lipophilic steroid (log P 4.53), was increased 11fold in the presence of 15mM sodium taurocholate versus bile free buffer and 40-fold in the presence of 15mM sodium taurocholate: 3.75mM lecithin. Furthermore, Humberstone et al. (1996) reported a 200-fold increase in the aqueous solubility of halofantrine with 15mM sodium taurocholate versus bile-free solution and >1700-fold increase with 15mM sodium taurocholate: 3.75mM lecithin. The solubility profile can be further modified by incorporation of lipolytic degradation products into the mixture. For example Kossena et al. (2003) found that addition of long or medium chain monoglycerides and free fatty acid (simulating end products of triglyceride digestion) to a bile salt/lecithin micellar solution produced significant enhancements in the aqueous solubility of three lipophilic drugs – griseofulvin (log P 2.18), danazol (log P 4.53) and halofantrine (log P >8). The most lipophilic of the three, halofantrine, experienced greatest solubility enhancements with the long chain digestion products (approximately 2-fold higher levels than with medium chain digestion products and >10-fold higher than the blank bile salt/ lecithin solution), as a result of its affinity for the lipid depots in the mixed micellar phase.

The effects of a range of lipidic vehicles on the aqueous solubility of halofantrine in the presence and the absence of simulated intestinal digestion were investigated in this project. In the absence of digestion, it was found that the two microemulsion vehicles and the polysorbate 80 micellar solution produced highest levels of aqueous drug solubility. Aqueous solubilisation in the presence of these vehicles was independent of the diluting buffer composition. In contrast, aqueous solubility from the oil emulsions was highly sensitive to the diluting medium, demonstrating substantial enhancements with the addition of bile salt/ lecithin and further enhancements following lipid digestion. These data show the importance of the dispersed state on lipophilic drug solubility, with fine micellar, microemulsion or mixed micellar systems being most effective at promoting aqueous solubility. These findings are consistent with observations by Tarr and Yalkowsky (1989) of increased drug solubility with decreasing dispersed lipid droplet size. Kaukonen et al. (2002) found that halofantrine solubility was higher in the presence of long chain versus medium chain lipid digestion products (2-fold difference). In contrast, our results showed that the digested Miglyol 812 vehicle (medium chain triglyceride) produced higher aqueous concentrations of halofantrine than the digested olive oil vehicle (long chain triglyceride) (approximately 1.5-fold higher). However, Kaukonen et al. (2002) specifically added final stage lipid digestion products to bile salt buffer for their solubility studies whereas ours relied on digestion of administered triglycerides to generate a mixed micellar phase. As triglyceride digestion was more complete for the medium chain versus long chain oil (liberating 1.8-fold more free fatty acid) in our studies, then the amount of monoglyceride and free fatty acid available for mixed micelle solubilisation is expected to be higher, hence accounting for higher drug solubilisation.

The oral bioavailability of halofantrine from tablet form is significantly enhanced when administered in the post-prandial versus fasted state. Humberstone *et al.* (1996) attributed this to an increase in drug absorption resulting from substantial enhancements in luminal drug solubility with the presence of bile constituents in the post-prandial intestinal milieu. In order to establish whether increased drug solubility was sufficient to explain patterns observed for halofantrine permeability across Caco-2 monolayers in the present study, data for absolute solubility versus basolateral drug concentration were plotted (Figure 8.2a). The correlation between these parameters was poor ( $r^2$ =0.47), indicating that factors other than solubility and dispersed lipid droplet size are involved in dictating halofantrine permeability across Caco-2 monolayers. However, if the digested microemulsion data, which gave particularly low levels of drug transport, are removed from the plot, the correlation is much better ( $r^2$ =0.84) (Figure 8.2b), but still indicates involvement of other parameters in dictating drug permeation.





**Figure 8.2:** Correlation between the aqueous solubility of halofantrine in simulated intestinal buffer and permeability across Caco-2 monolayers following co-administration with lipid vehicles (digested and non-digested). Panel a: data for the full range of investigated lipid vehicles. Panel b: data for all, but the digested microemulsion vehicles.

Lymphatic drug uptake has been shown to contribute to the oral bioavailability of a range of lipophilic drugs, including halofantrine. A study by Khoo et al. (2001) showed that up to 50% of an orally administered halofantrine dose could be transported in the lymph of a fed dog (beagle) model. Previous studies in the lymph cannulated rat model have reported up to 20% lymphatic drug transport (Porter et al., 1996). Given the poor oral bioavailability of halofantrine, lymphatic transport presents an important route of absorption. In the current study, attempts were made with the Caco-2 model to predict lymphatic transport of halofantrine by measuring the association of halofantrine with secreted chylomicrons. Previous studies in the rat model have shown that long chain triglycerides are very efficient at promoting lymphatic halofantrine transport (Caliph et al., 2000; Holm et al., 2001), however, less than 2% of basolaterally recovered halofantrine was associated with chylomicrons following administration with a digested olive oil vehicle. This accounted for only 0.03% of the total administered halofantrine dose. The highest levels of chylomicron drug association were obtained with the digested polysorbate 80 vehicle, but transport still only accounted for approximately 30% of basolaterally recovered halofantrine and less than 0.2% of the administered dose. These findings indicate a discrepancy between the Caco-2 model and in vivo findings.

In Chapter Six, an oleate rich microemulsion formulation was developed to promote lymphatic drug transport. However, findings from Chapter Seven show that this formulation did not successfully promote association of halofantrine with secreted chylomicrons. Solubility data showed that both medium chain and a long chain oil based microemulsions produced considerable enhancements in the extent and the consistency of halofantrine solubility in various gastrointestinal fluids. However, the effects of vehicles on halofantrine uptake did not reflect the solubility. When administered in a non-digested state, microemulsion vehicle produced higher levels of halofantrine permeability across Caco-2 monolayers than any of the other investigated vehicles. Transport was not related to drug association with TRL, nor did it correlate with triglyceride secretion. Permeability may have been mediated by a combination of increased drug solubility, facilitated transport across the aqueous boundary layer and alterations in membrane permeability. Additionally, data presented in Chapter Seven indicate that halofantrine may be sensitive to P-gp efflux, hence inhibition of P-gp activity by the microemulsion vehicle may have contributed to the observed halofantrine transport. It is not entirely clear why digestion of microemulsion vehicles resulted in a decrease in halofantrine permeability without any changes in drug solubility or triglyceride transport. It may be that drug affinity for the digested microemulsion system is increased to a level that restricts the amount of dissociated or 'free' drug available for cellular uptake. These findings clearly highlight the importance of considering luminal processing of lipid vehicles when trying to predict effects on drug absorption.

#### 8.7 Caco-2 cells as a model for predicting lymphatic drug transport

In terms of the lipid metabolism response to administered fatty acids and lipid vehicles, the Caco-2 model appears to be a good surrogate model for intact intestine. Data presented in Chapter Three demonstrated the similarity between the Caco-2 response to fatty acids of different chain length and saturation and reported in vivo findings. Further studies in our lab (Lawless et al., 2003a) have shown a close correlation between triglyceride secretion in the Caco-2 model and lymphatic triglyceride output in a cannulated rat model in response to a range of lipid vehicles. The similarity between the Caco-2 and in vivo response is, however, qualitative at best. In terms of absolute triglyceride secretion the Caco-2 model demonstrates substantially lower secretion levels than the intestine. The triglyceride secretion level in 'fedstate' cells was found to be 73nmoles (approximately 65µg) over a 20-hour period per filter. In comparison, reported mesenteric lymph triglyceride secretion levels for the lymph cannulated rat model (following administration of oleate rich lipid vehicles) are in the order of 30-140mg over 12 hours (Caliph et al, 2000; Griffin, 2001; Holm et al, 2001). This 800-3000 fold difference in triglyceride output is considerable. Whilst the use of a radiolabelled fatty acid precursor may facilitate reasonably accurate predictions of lipid metabolism responses in the Caco-2 model, the low level of TRL output is unlikely to be sufficient to provide a good lipid depot for drug incorporation. The model therefore may prove to be a powerful tool for screening lipid vehicle effects on the lipoprotein secretion response, but is unlikely to play a major role in predicting lymphatic drug transport potential. The low efficiency of triglyceride secretion in the Caco-2 model was probably a major contributing factor to the poor TRL-drug association observed for halofantrine in Chapter Seven.

Caco-2 cells are a human colon carcinoma derived cell line. The main function of the colon *in vivo* is to absorb water from luminal contents. As such, the colon is not suited to absorbing and processing large amount of lipid products. Findings from current literature and from the work presented here have shown that Caco-2 cells possess basic lipid processing pathways and the ability to secrete lipoproteins, but not to the same level of efficiency as the duodenum. Key differences therefore exist between the Caco-2 model and the small intestine, which may explain the limited secretion capacity of the cell model. Firstly, the most active triglyceride synthesis route in the Caco-2 model is that of the phosphatidic acid pathway (Trotter and Storch, 1993), which uses glycerol-3-phosphate and fatty acid as precursors. In the duodenum, this is only a minor route of triglyceride synthesis that predominates during fasted state lipid turnover. During lipid digestion, the monoacylglycerol pathway functions in the intestine to supply triglyceride for chylomicron assembly (Mansbach and Parthasarathy, 1982) and it is

thought that an increase in the amount of triglyceride being assembled via this route is responsible for the observed increase in triglyceride export following feeding (Mansbach and Arnold, 1986; Mansbach and Nevin, 1998). The absence of an active monoacylglycerol pathway may redirect absorbed lipids into the cellular pool rather than the secreted triglyceride pool.

In addition, activity of MTP, a key protein involved in initial complexation of lipids with apoB, has been shown to be approximately 15-fold lower in human foetal colon tissue than in foetal jejunum (Levy et al., 2001). This may limit available apoB for transporting triglyceride out of the cell. The nature of the apoB secreted by Caco-2 cells may also be a causative factor in the low TRL secretion efficiency. Chylomicron assembly and secretion in the upper intestine in humans is reliant on the production of apoB 48. In humans, apoB 48 is predominantly expressed in the intestine and apoB 100 in the liver, where it is associated with VLDL. In contrast, Caco-2 cells express a combination of both forms of apoB, the relative distribution relying on the state of differentiation (Mehran et al., 1997). Under normal conditions, the human intestine will secrete both VLDL and chylomicron sized TRL, depending on lipid supply, whilst the liver will only secrete VLDL. Hence, the apoB 48 form appears to be critical for chylomicron formation. Recent evidence has shown that apoB 100 can be incorporated into chylomicrons in the absence of apoB 48, but that the initial chylomicron assembly step tends to be much slower (Kendrick et al., 2001). This problem is overcome by increasing the lipid load, but may still be a contributing factor to the low secretion efficiency of Caco-2 cells. In the presence of low MTP levels there is documented evidence to suggest that apoB 100 is more susceptible to intracellular degradation than apoB 48, again, this may be a contributory factor (Nicodeme et al., 1999).

#### 8.8 Key findings of this thesis

- A number of polyoxyethylated non-ionic surfactants were found to selectively inhibit triglyceride rich lipoprotein secretion in the Caco-2 model. Inhibition was found to depend on the HLB of the surfactant, with more lipophilic compounds being most potent inhibitors. The polyoxyethylene side was identified as an important feature for inhibition.
- A close association between inhibitory effects of surfactants on triglyceride rich lipoprotein secretion and on P-glycoprotein mediated drug efflux was found, suggesting a common regulatory link between the processes.
- Polysorbate 80, in spite of being a polyoxyethylated non-ionic surfactant of intermediate HLB, was found to promote chylomicron secretion in Caco-2 cells. The effects of polysorbate 80 on chylomicron secretion were due to its oleic acid moiety, which was liberated from the polysorbitan oleate ester via Caco-2 based lipase/ esterase or pancreatin catalysed hydrolysis.
- Microemulsion formulations produced a substantial enhancement in the aqueous solubility of halofantrine, a highly lipophilic anti-malarial compound. On co-administration in a non-digested state, microemulsion formulations produced significant enhancements in drug permeability across Caco-2 cells as compared to simple lipid emulsions. However, when administered in a pre-digested state, the permeability enhancing effects were lost.
- Halofantrine association with secreted chylomicrons in the Caco-2 model was considerably lower than reported for *in vivo* rat and dog models and may be a result of the low lipoprotein secretion capacity of the Caco-2 model versus duodenal enterocytes.

#### 8.9 Future Directions

The data collected here have shown that the Caco-2 model offers promise as a screening tool for predicting trends in the *in vivo* lymphatic lipid response to various lipid vehicles and lipid vehicle excipients. However, the model has a low triglyceride and TRL secretion capacity as compared to published findings for the human intestine, which may limit direct quantitative estimation of the *in vivo* response and prediction of lipid vehicle effects on drug association with lymphatic TRL. Efforts should be made to either boost the triglyceride secretion efficiency of the existing cell model or to select an intestinal cell culture model that more closely mimics the triglyceride secretion efficiency of the upper intestine. As a result of the possible involvement of ABC proteins in lipid metabolism, the effects of P-gp inducing agents, such as vinblastine and doxorubicin, on TRL output should be investigated. Caco-2 sub-clones should be screened for triglyceride secretion to see if they offer any advantages over the standard Caco-2 model and the possibility of transfecting Caco-2 cells with apobec (to increase apoB 48 expression) or MTP (to increase lipidation of apoB) should be considered. The use of human intestinal explants, which have good triglyceride secretion capacity, should be considered to support initial Caco-2 data.

The investigated long chain triglyceride rich microemulsion in these studies failed to promote the association of halofantrine with secreted TRL under the experimental conditions. Furthermore, the secreted TRL response to this vehicle was considerably lowered when this vehicle was presented to cells in a digested as compared to undigested state. Further work needs to be conducted to formulate a long chain fatty acid rich microemulsion vehicle that promotes TRL secretion in cells when presented in either a digested or non-digested state. The long chain triglyceride oil phase may need to be changed to a more easily dispersed mixed glyceride or a structured MLM triglyceride to decrease the required concentration of surfactant for vehicle dispersion. More extensive formulation work should be conducted with feedback from *in vitro* cell studies.

# Appendices

# Appendix I

#### Preparation of 500ml whole, maintenance, culture medium

Dulbecco's modified Eagle's medium (DMEM, 1X) was obtained from Gibco in sterile, 500ml volumes. All modifications were made aseptically, in a laminar flow hood. A volume of 65ml was removed from the sealed 500ml bottle and the following additions made:

50ml FCS 5ml Gentamicin, 10mg/ml 5ml Sodium pyruvate, 100mM 5ml L-glutamine, 200mM

### Appendix II

#### Preparation of 1000ml transport buffer (TB)

Transport studies were carried out in HBSS (Hank's buffered saline solution) containing 25mM glucose and 10mM Hepes during the course of this work. This was prepared as follows:

50ml HBSS (10X) solution, Gibco

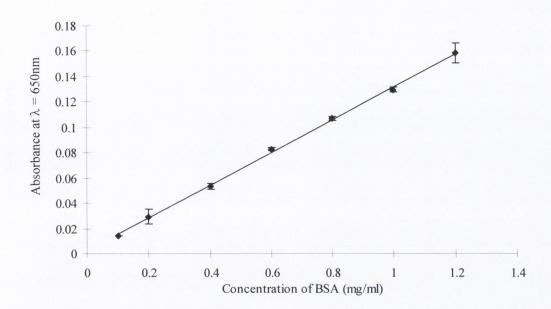
3.7g Glucose

2.6g Hepes

Sterile water to volume

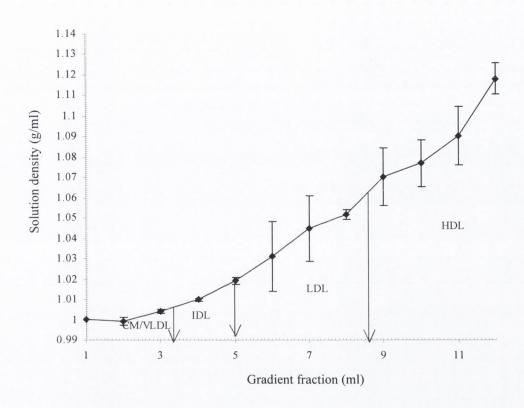
The appropriate quantities of ingredients were initially dissolved in approximately 500ml of water, and the pH adjusted to 7.35 with NaOH solution. Solution was subsequently made to 1000ml with sterile water and aseptically filtered through a  $0.2\mu m$  filter in a laminar flow hood.

# Appendix III



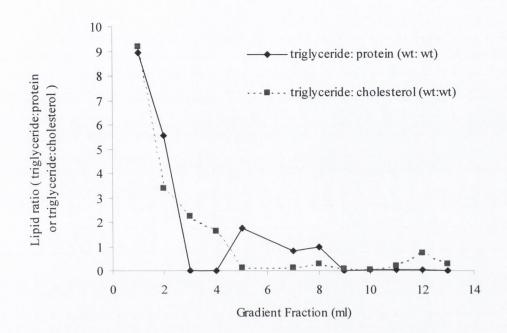
Calibration curve for BioRad protein assay plotted as standard absorbance (read at  $\lambda$ =650nm) against protein concentration (protein present as BSA, Sigma, A-7030). Data are averaged from six individual calibration curves obtained on different days. The equation of the line was calculated using linear regression analysis with Microsoft® Excel 2000 and was found to be: y = 0.129x + 0.0027, with correlation coefficient (y = 0.9989). Calibration curves typically had an y = 0.9989.

### Appendix IV



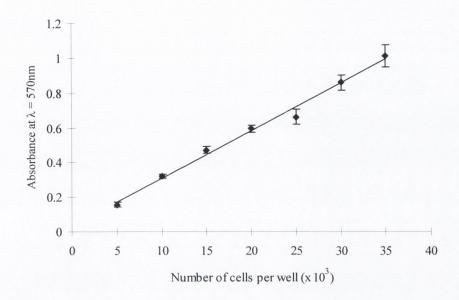
Solution density distribution following ultracentrifugation of a blank density gradient (Section 2.3.3). Phosphate buffered saline (3ml), adjusted to 1.1g/ml with solid KBr was used as the test solution. This was overlaid with KBr density solutions in the following sequence: 3ml x 1.063g/ml, 3ml x 1.019g/ml and 2ml x 1.006g/ml. This density gradient was centrifuged at 39000 rpm for 3.5 hours and the top 1ml fraction collected (fraction 1). This was replenished with 1ml 1.006g/ml solution and the gradient centrifuged for a further 18 hours. The resulting centrifuged gradient was aspirated off in 1ml fractions (Fractions 2-12). 250µl of each fraction was weighed on a microbalance to determine fraction density. Predicted location of each lipoprotein class within the gradient based on flotation density: chylomicrons<1.006g/ml; VLDL, 0.95g/ml-1.006g/ml; IDL, 1.006-1.019g/ml; LDL, 1.019-1.063g/ml; HDL, 1.063-1.21g/ml.

# Appendix V



Distribution of lipids throughout the density gradient following ultracentrifugation of plasma (Section 2.3.3). Triglyceride content of each fraction was determined using a BioRad colorimetric assay kit and the cholesterol and triglyceride content were determined using a Technicon RA-XT<sup>TM</sup> autoanalyser. The ratios of triglyceride: protein and triglyceride: cholesterol are plotted. The lipid ratio is consistent with the presence of chylomicrons and VLDL in the first fractions (1.006g/ml density, chylomicrons collected after 3.5 hours centrifugation at 39,000 rpm and VLDL collected following a further 18 hours centrifugation), LDL in fractions 5-8 and HDL in fractions 9-12, as per Table 1.1.

# Appendix VI



Calibration curve for preliminary selection of Caco-2 seeding density in the MTT assay (Section 2.3.4). Final cell absorbance (at  $\lambda$ =570nm) is plotted against cell concentration (number of cells per well, in 100µl) (n=8). The equation of the line was fitted using linear regression in Microsoft® Excel 2000. The equation was found to be: y = 0.0273x + 0.036, with correlation coefficient of  $r^2$ =0.991.

#### Appendix VII

#### Laemmli Buffer System for SDS PAGE

Lower gel buffer (100ml):		olution (pH 8.8) (final 1.5M) olution (final 0.4%w/v)		75ml 4ml 21ml
Upper gel buffer (100ml):		olution (pH 6.8) (final 0.5M) solution (final 0.4%w/v)		50ml 4ml 46ml
Sample buffer (5X, 10ml):	50%v/v glycer 2-mercaptoetha	pH 6.8) (final 60mM) ol solution (final 25%v/v) anol (final 14.4mM) ohenol blue solution (final 0.1%v	w/v)	0.6ml 5ml 0.5ml 1ml 0.9ml
Running Buffer (1000ml):	Tris (final 25m Glycine (final SDS (final 0.1 Water	192mM)	to 1000	3g 14.4g 1g 0ml
Coomassie Blue Staining Solut	tion (1000ml):	Coomassie blue R-250 Methanol Glacial acetic acid Water	1g 450ml 100ml 450ml	
Destaining Solution (1000ml):		Methanol Glacial acetic acid Water	100ml 100ml 800ml	

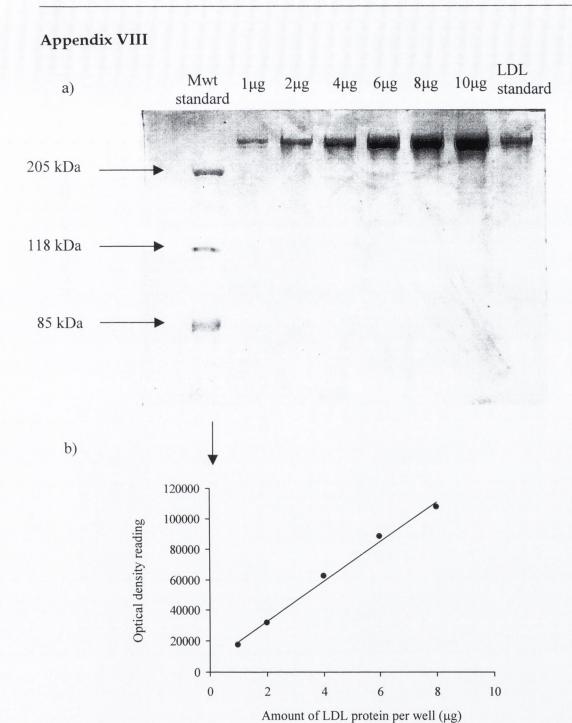
6% running gel and 4.8% stacking gel were prepared as follows:

6% running gel: 2ml lower gel buffer, 4.4ml water, 1.6ml acrylamide (30%), 12.5μl TEMED and 35μl ammonium persulfate (10%w/v in water).

4.8% stacking gel: 1.25ml upper gel buffer, 2.95ml water, 0.8ml acrylamide (30%),  $12.5\mu$ l TEMED and  $35\mu$ l ammonium persulfate (10%w/v in water).

#### Preparation of 6% running gel with 4.8% stacking gel

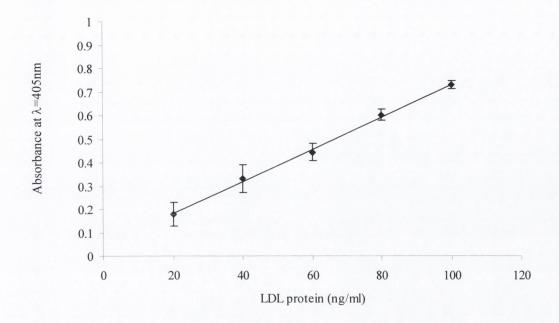
In each case, water, buffer and acrylamide were pipetted into a glass vial and mixed gently. TEMED and ammonium persulfate were added simultaneously and mixed in by swirling to initiate polymerisation. 4ml gel mix was then pipetted into a BioRad mini-protean II gel mould, taking care to avoid air bubbles. Isopropanol was sprayed gently over the surface to maintain a flat surface and the gel was allowed to set for 30-60 minutes. Once set, the gel surface was rinsed with water to remove isopropanol. Stacking gel was prepared in a similar manner and pipetted onto the running gel, a gel comb was inserted into the surface to form sample wells and the gel was allowed to set (30 minutes). The comb was then removed carefully and the gel surface rinsed with water.



Panel a: SDS PAGE gel with LDL protein (Section 2.3.5). Protein was added to wells in concentration range of 1-10  $\mu$ g/well. BioRad high molecular weight standards were added to the same gel (shown on far left), The band to the far right of the gel represents an LDL standard obtained from the Lipid Research Group, Glasgow Royal Infirmiary.

Panel b: Calibration curve for LDL (predominantly apoB 100) standard plotted as gel band density against protein content. The equation of the line was fitted using linear regression analysis and was found to be: y=13095x+6337.9,  $r^2=0.9924$ .

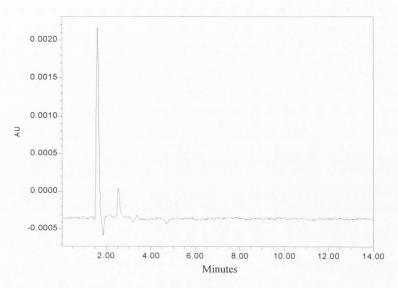
# Appendix IX



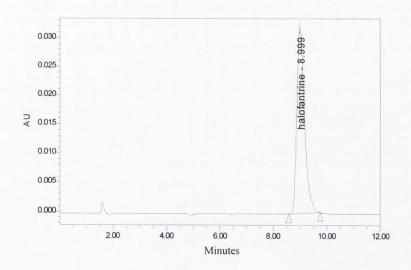
Calibration curve for apoB ELISA (Section 2.3.5) plotted as standard absorbance (read at  $\lambda$ =405nm) against LDL protein concentration. Data are plotted from six individual calibration curves obtained on different days. The equation of the line was calculated using linear regression analysis with Microsoft® Excel 2000 and was found to be: y = 0.0068x + 0.0485, with  $r^2 = 0.9976$ . Calibration curves typically had an  $r^2$  value >0.9.

# Appendix X (a)

Representative chromatograms for halofantrine assay as per section 2.3.10

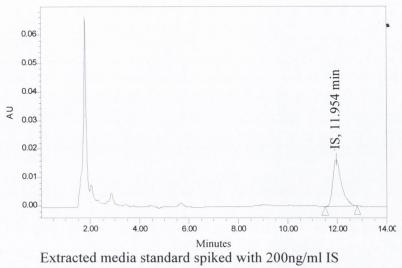


Acetonitrile injection

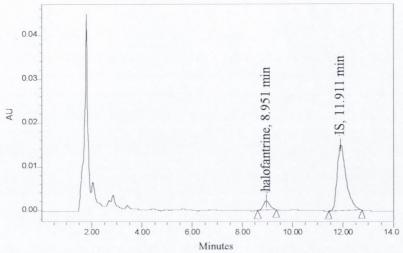


5μg/ml halofantrine standard (unextracted)

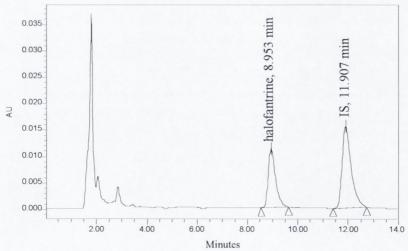
# Appendix X (b)



# Appendix X (c)

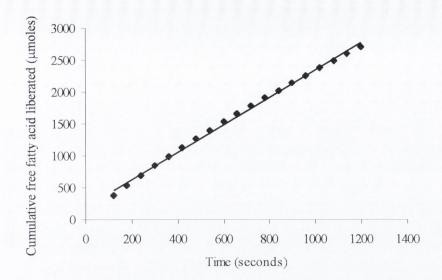


Extracted media standard spiked with 50 ng/ml halofantrine and 200 ng/ml IS



Extracted media standard spiked with 250ng/ml halofantrine and 200ng/ml IS  $\,$ 

#### Appendix XI

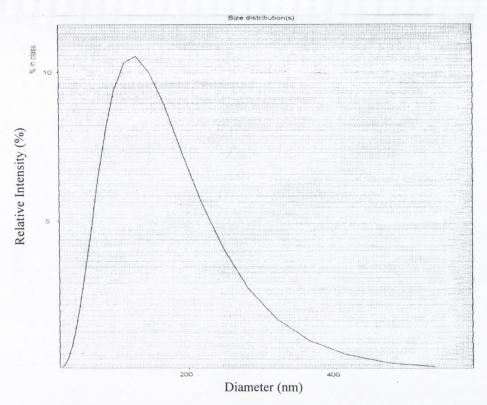


Determination of tributyrin units (TBU) in pancreatin for Section 2.3.12:

2ml of tributyrin was emulsified in 8ml of assay buffer (10mM Tris-maleate, pH 7.2, with 150mM NaCl, 2mM CaCl<sub>2</sub> and 1%w/v bile extract) by stirring at 37°C over 10 minutes. Pancreatin (2.6mg) was added to the oil emulsion to initiate lipolysis. System pH was maintained at 7.2 in a pH stat autotitrator by neutralisation of liberated free fatty acid with 0.5N NaOH solution. Lipolysis was allowed to continue for 1800 seconds and cumulative volume readings (for NaOH addition) were taken at 60 second intervals. The amount of NaOH ( $\mu$ moles) added to the system was directly proportional to the amount of fatty acid liberated in the digestion process. The amount of free fatty acid liberated was plotted against time and the steady state rate of lipolysis (taken from the linear portion of the lipolysis curve, 120-1200 seconds) was used to determine pancreatin activity. The equation of the line, as fitted using linear regression, was: y = 2.134x + 204.76,  $r^2 = 0.9972$ . The TBU (tributyrin unit) value was used as a measure of activity. 1 TBU is the amount of enzyme that liberates 1 $\mu$ mole of free fatty acid from tributyrin:

- 2.6mg of pancreatin liberates 2.134µmoles of free fatty acid per second
- This is equivalent to 123.5 μmoles per minute
- 1 TBU is the amount of enzyme that liberates 1 \mu mole of free fatty acid
- 0.0203mg pancreatin liberates 1µmole of free fatty acid
- Therefore, there are 49.25 TBU per mg of pancreatin

# Appendix XII

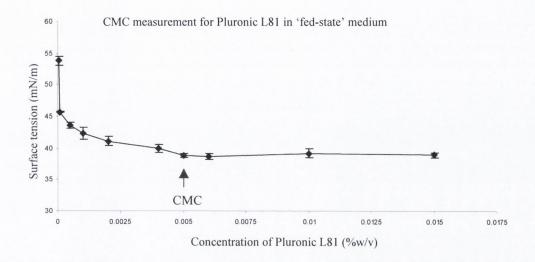


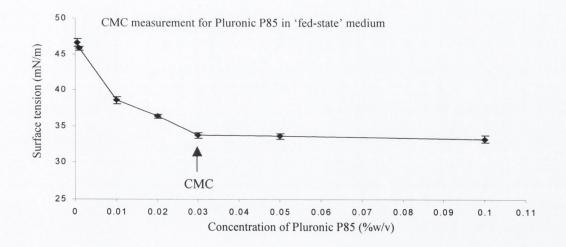
Particle size distribution of rat mesenteric lymph chylomicrons analysed using PCS as per section 3.3.2.

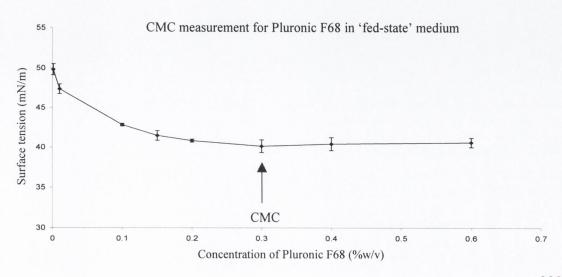
Size (nm)	intensity	volume
25.90	0.10	2.60
29.70	0.20	3.50
33.90	0.40	4.80
38.80	0.70	6.10
44.30	1.30	7.30
50.70	2.10	8.10
57.90	3.30	8.50
66.20	4.80	8.40
75.70	6.40	7.80
86.60	8.10	6.80
99.00	9.40	5.60
113.10	10.30	4.50
129.30	10.50	3.40
147.90	10.00	2.60
169.00	8.90	2.00
193.20	7.30	1.60
220.90	5.70	1.40
252.60	4.10	1.60
288.70	2.70	3.90
330.10	1.70	5.60
377.10	1.00	2.90
431.40	0.50	0.40
493.20	0.30	0.30

### Appendix XIII

Representative plots of surface tension (mN/m) (as measured by the Lauda Tensiometer in section 4.3.1) versus surfactant concentration for determination of CMC in fed state medium. Plots are shown for Pluronic L81 and Pluronic P85.







# Appendix XIV

**Table 1:** Fatty acid (%) composition of investigated long chain oils as per the Handbook of Pharmaceutical Excipients (4th Edition, 2003).

Fatty acid	Olive	Corn	Arachis	Soybean	Sesame
14:0 (myristic)	≤0.5				
16:0 (palmitic)	7.5-20	11	8.3	9-13	9.1
16:1 (palmitoleic)	0.3-0.5				
17:1 (hepatodecenoic)	≤0.3				
18:0 (stearic)	0.5-5	1.7	3.1	3-6	4.3
18:1 (oleic acid)	55-83	25.8	56	17-26	45.4
18:2 (linoleic)	3.5-21	58.9	26	50-57	40.4
18:3 (linolenic)	≤0.9	1.1		5-10	
20:0 (arachidic)	≤0.6		2.4		0.8
20:1 (eicosaenoic)	≤0.4				
22:0 (behenic)	≤0.2		3.1		
24:0 (lignoceric)	≤0.1		1.1		

Table 2: Fatty acid (%) composition of investigated medium chain oils

Fatty acid	Miglyol 812 <sup>a</sup>	Captex 355 <sup>b</sup> 20-50 50-80	
10:0 (capric)	30-45		
C8:0 (caprylic)	50-65		
C6:0 (caproic)	<2	<2	
C12:0 (lauric)	<2	<3	
C14:0 (myristic)	<1	<1	

a Product information from Sasol, Witten, Germany.

b Product information from Abitec Corp, Janesville, Wi.

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