AN INVESTIGATION OF THE FACTORS WHICH IMPACT ON THE ABSORPTION AND METABOLISM OF HALOFANTRINE

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'...let us run with perseverance the race marked out for us.'

Hebrews 12:1

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ABSTRACT

Halofantrine (Hf) is a highly lipophilic (log P ~ 8.5) antimalarial effective in the treatment of multidrug-resistant *Plasmodium falciparum* malaria. However, the clinical utility of Hf is limited by poor and highly variable absorption following oral administration of the commercially available Hf hydrochloride (Hf.HCl) tablet.

Co-administration of Hf.HCl with food has been shown to increase the oral bioavailability of Hf and historically was considered a practical means for increasing the absorption of Hf. However, this practice is now contraindicated as excessively high and poorly controlled Hf plasma concentrations may result post-prandially and have been associated with an increased risk of adverse cardiac effects.

The previously described utility of food to increase the absorption of Hf stimulated an investigation of the potential for lipidic formulations to improve the oral delivery of Hf. In these studies, a number of lipid-based self-emulsifying formulations were developed and afforded a 6- to 8-fold improvement in absolute oral bioavailability of Hf in fasted beagle dogs relative to the Hf.HCl tablet. The increase in bioavailability was likely to be a result of delivering Hf in a solubilized and rapidly dispersed manner.

Whilst the previous food studies showed that the absorption of Hf was markedly increased post-prandially, in the same studies, the proportion of Hf metabolized to its equipotent active metabolite, *N*-desbutylhalofantrine (Hfm), was significantly reduced. Consequently, mechanisms by which the post-prandial metabolism of Hf may be reduced were also explored in this thesis. A study evaluating the effect of ketoconazole, a specific inhibitor of CYP3A4, on the relative plasma profiles of Hf and Hfm after oral administration of Hf base to beagles indicated that the *in vivo* conversion of Hf to Hfm was likely mediated via CYP3A4. Administration of Hf with ketoconazole to fasted beagles also yielded similar plasma Hfm/Hf AUC ratios to those observed post-prandially. These data suggested that the decreased post-prandial metabolism of Hf

could be due to the inhibition of presystemic CYP3A4 metabolism by components present in food, or avoidance of pre-systemic hepatic metabolism via the post-prandial recruitment of intestinal lymphatic transport.

To address the potential issue of Hf lymphatic transport, a conscious dog model that allows simultaneous study of intestinal lymphatic and non-lymphatic drug transport and aspects of enterocyte-based metabolism was developed. After oral administration of 100 mg Hf base, the proportion of the dose transported by the intestinal lymphatics was 1.3% and 54% after fasted and post-prandial administration, respectively. It therefore appears that the previously observed decrease in the post-prandial metabolism of Hf was largely a consequence of significant post-prandial intestinal lymphatic transport of Hf (and therefore avoidance of hepatic first-pass metabolism).

Hf.HCl was not considered to be a likely candidate for lymphatic transport as its solubility in triglyceride lipid is low. However, after post-prandial administration of Hf.HCl (107 mg), the extent of lymphatic transport of Hf was surprisingly high (47% of administered dose). Subsequent physicochemical studies showed that the apparent pKa of Hf varied between 5.06 to 6.92, and is considerably lower than that anticipated for a tertiary amine. The extensive lymphatic transport observed was therefore most likely due to the conversion of solubilized Hf.HCl to the free base. In addition to using indicators such as log P and triglyceride solubility to predict lymphatic transport of potential drug candidates, possible conversion to the more lipid soluble unionized form should also be considered especially for salts of poorly water soluble acids and bases.

In this thesis, the potential utility of dispersed lipid-based formulations for oral delivery of lipophilic drugs was discussed. The intestinal lymphatics have been shown to be an important absorption pathway for highly lipophilic drugs and a new dog model was developed which will assist in the identification of key factors that impact on the bioavailability, lymphatic transport and metabolic profiles of highly lipophilic drugs.

STATEMENT OF ORIGINALITY

The Registrar
Victorian College of Pharmacy
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I hereby certify that the work presented in this thesis has not been submitted by myself or any other person for the award of a degree at Monash University or any other institution. To the best of my knowledge, this thesis does not contain material that has been published previously by another person except where due reference has been made.

Shui-Mei Khoo
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PUBLICATIONS

1. S. M. Khoo, A. J. Humberstone, C. J. H. Porter, G. A. Edwards and W. N. Charman. Formulation design and bioavailability assessment of lipidic self-emulsifying formulations of halofantrine. *Int. J. Pharm.* **167:**155-164 (1998).

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Abbreviations

ACN	acetonitrile
AR	analytical reagent
AUC	area under the plasma concentration versus time curve
BP	British Pharmacopoeia
CL	clearance
C_{max}	maximum plasma concentration
CMC	critical micellar concentration
CV	coefficient of variation
CYP	cytochrome P450
d	density
D	distribution coefficient
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
$ElogP_{oct}$	estimate of log P_{oct} by RP HPLC
FA	fatty acid
FTIR	Fourier transform infrared spectroscopy
h	hour
HCl	hydrochloride
HDL	high density lipoprotein
Hf	Halofantrine
Hfm	*N*-desbutylhalofantrine
HIV	human immunodeficiency virus
HLB	hydrophile-lipophile balance
HPLC	high performance liquid chromatography
i.d.	internal diameter

IS	internal standard
IV	intravenous
k'	capacity factor
KBr	potassium bromide
KC	ketoconazole
Kcps	kilo counts per seconds
LCT	long-chain triglyceride
LDL	low density lipoprotein
log P	logarithm of the octanol-water partition coefficient
LPDL	lipoprotein deficient lymph
MCT	medium-chain triglyceride
MG	monoglyceride
MQL	minimum quantifiable limit
NaTC	sodium taurocholate
nm	nanometre
NMP	*N*-methyl-2-pyrrolidone
o.d.	outer diameter
PCS	photon correlation spectroscopy
PEG	polyethylene glycol
P-gp	P-glycoprotein
pKa_m	apparent micellar ionisation constant
pKa_s	apparent spectrophotometric ionisation constant
RP-HPLC	reverse phase high performance liquid chromatography
rpm	revolutions per minute
SBO	soybean oil
SD	standard deviation

SE	standard error
SEDDS	self-emulsifying drug delivery system
SMEDDS	self-microemulsifying drug delivery system
$T_{1/2}$	terminal elimination half-life
TBME	*tert*-butyl methyl ether
TG	triglyceride
T_{max}	time at which maximum plasma concentration was recorded
USP	United States Pharmacopeia
UV	ultraviolet
UWL	unstirred water layer
VLDL	very low density lipoprotein
w/w	weight in weight

CHAPTER 1

Introduction

1.1 STATEMENT OF THE PROBLEM

Since its discovery and development, halofantrine (Hf), which is commercially available as the hydrochloride salt (Hf.HCl) has been an important alternative drug used for the treatment of multidrug-resistant *Plasmodium falciparum* infection. When administered in multiple doses, Hf provided effective treatment against falciparum malaria. However, a major factor limiting the clinical utility of Hf.HCl is its extremely low aqueous solubility, which has resulted in poor and erratic drug absorption and consequently, low and highly variable plasma concentration-time profiles. For Hf, persistent sub-therapeutic plasma concentrations have been responsible for treatment failures and may also facilitate the development of drug resistance.

The co-administration of Hf.HCl with a fatty meal has been reported to increase the rate and extent of drug absorption considerably. This has been attributed to improved drug solubilization and dissolution in the post-prandial intestinal milieu. Whilst food may improve the oral bioavailability of Hf and reduce inter-subject variability in Hf plasma concentrations, the practicality of this is questionable as acutely ill malaria patients are unlikely to consume a large fatty meal. Furthermore, excessively high Hf plasma concentrations have been associated with severe adverse cardiac effects, especially in patients with pre-existing cardiopathy.

Interestingly, whilst food markedly increased the oral bioavailability of Hf (3-fold in humans and 12-fold in beagles), a significantly lower proportion of Hf was metabolized to its major metabolite, *N*-desbutylhalofantrine (Hfm). The mean post-prandial plasma Hfm/Hf AUC ratios decreased 2.4-fold in humans and 6.8-fold in beagles. This could be of therapeutic importance as Hfm is equipotent to Hf against *Plasmodium falciparum* infection and has been reported to be largely devoid of the adverse cardiac effects associated with Hf treatment.

Possible factors contributing to the decreased metabolism of Hf in the fed state include recruitment of intestinal lymphatic drug transport and/or reduced presystemic (hepatic and enterocyte-based) metabolism, although further studies are required to clarify the role of these processes in the absorption of Hf. In addition to decreasing first-pass metabolism and systemic clearance of Hf, increased binding of Hf to lipoproteins in the post-prandial state could also influence the pharmacodynamic profile of Hf as the decreased free fraction of Hf has been shown to increase the IC_{50} of Hf.

With the rapid development of multidrug-resistant strains of malarial parasites, it would be beneficial to improve the oral delivery of Hf such that reproducible and optimal Hf blood concentrations can be provided for the safe and effective use of Hf in the treatment of malaria. Since lipid-based formulations have been successfully employed to improve the oral bioavailability of many poorly water soluble drugs by reducing the inherent limitations of slow and incomplete dissolution and presenting the drug in a solubilized manner, an aim of this thesis was to explore the potential utility of lipidic vehicles as a formulation strategy for improving the oral bioavailability of Hf.

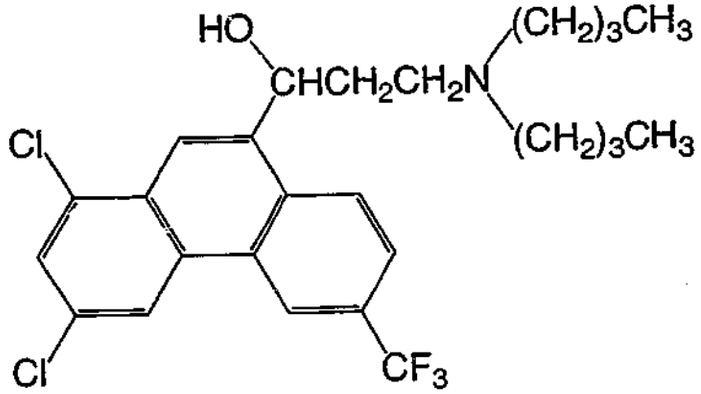
The focus of the research conducted in this thesis has also been to investigate some of the mechanistic aspects of the absorption and metabolism of Hf and to identify the basis by which food may decrease the metabolism of Hf. Whilst *in vitro* studies using human liver microsomes indicate that the conversion of Hf to Hfm is largely mediated via cytochrome P450 3A4, this has not been formally assessed *in vivo*. To date, the lymphatic transport of Hf in an animal model other than the rat has not been investigated. Due to differences in the luminal environment of the intestine in the rat, it is difficult to extrapolate lymphatic transport data from rats to higher species such as humans and dogs. Therefore, this necessitated the development a more appropriate animal model for the study of the intestinal lymphatic transport of Hf.

1.2 HALOFANTRINE

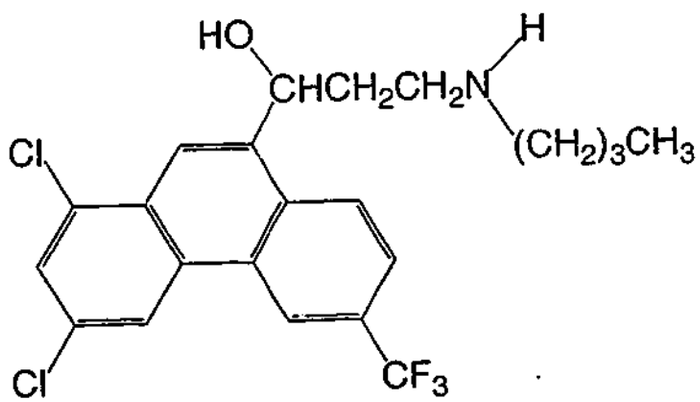
1.2.1 Background

Malaria is one of the major causes of morbidity and mortality in tropical and developing countries. This parasitic infection afflicts over half a billion people and kills approximately two million people each year. Most of the victims are children under the age of five living in sub-Saharan Africa. Of the four species of malaria parasites that infect humans, *Plasmodium falciparum* is the most prevalent. It is also responsible for the high fatality rate, and has developed resistance to the majority of the currently available antimalarial drugs.[1-5] Mefloquine, a fluorinated quinoline methanol, had been the drug of choice for the treatment of uncomplicated chloroquine-resistant falciparum malaria, however, its efficacy has declined rapidly in the past few years and mefloquine resistant parasites have been reported.[6] Due to the continual emergence and spread of resistance to antimalarial drugs, there is an urgent need for new and structurally different antimalarial agents.

In the 1960s, when it became clear that that the efficacy of chloroquine was diminishing, there was a renewed effort to identify new antimalarial agents to control the spread of drug resistant malaria parasites. The Walter Reed Army Institute of Research (WRAIR, of the US Army) undertook an extensive screening program. After testing over 300,000 compounds, halofantrine (Hf, Figure 1.1) emerged as one of the most promising compounds. Hf is a 9-phenanthrenemethanol derivative belonging to a class of arylaminoalcohols, which had been identified as potential antimalarial agents as early as World War II. Following a joint effort between WRAIR and SmithKline French Laboratories, Hf was developed and became commercially available in 1988 as tablets or a suspension of the hydrochloride salt (Hf.HCl, Halfan®).[7-9]



Halofantrine



N-Desbutylhalofantrine

Figure 1.1 Chemical structures of halofantrine and the major metabolite, *N*-desbutylhalofantrine.

1.2.2 Antimalarial activity and clinical efficacy of halofantrine

Hf is a blood schizontocide with selective inhibitory activity against mature parasites (trophozoites and schizonts).[10] It has no activity against gametocytes or the pre-erythrocytic hepatic stages of the parasite life cycle.[11] The mechanism of action of Hf remains poorly understood although it is believed to be similar in some respects to quinine and chloroquine. It has been suggested that Hf may form a complex with ferriprotoporphyrin IX in infected red blood cells and the resulting complex then damages the cell membrane, causing lysis and death of the parasite.[12] However, there has been conflicting data in this area as other investigations suggest that Hf may inhibit a proton pump at the parasite-erythrocyte interface and may also affect the mitochondria and haematozoin vesicles of the parasite.[11]

The antimalarial activity of Hf was initially assessed *in vivo* using various rodent and primate malaria models. *In vitro* studies were subsequently developed and confirmed that Hf was highly potent against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*. The IC_{50} (drug concentration corresponding to 50% inhibition of parasitic uptake of the radiolabelled marker) for Hf was 2.5 and 3.2 ng/mL against the two strains, respectively, compared to 6.7 and 7.8 ng/mL for mefloquine.[13] However, there has been some evidence indicating possible cross-resistance between the two agents.[14] The *in vitro* efficacy of Hf has also been verified through the use of clinical isolates of *P. falciparum* cases from Asia and Africa.[15,16] Hf is administered as a racemic mixture, where both the (+) and (-) isomers have been reported to be equally active.[17] The major metabolite of Hf, a *N*-desbutyl derivative (*N*-desbutylhalofantrine, Hfm, Figure 1.1), has demonstrated equivalent *in vitro* antimalarial activity to that of Hf.[18]

Hf has been widely prescribed for the treatment of uncomplicated, multidrug-resistant *Plasmodium falciparum* malaria. A review of early clinical studies indicated

that administration of a single or two-dose regimen of Hf resulted in low cure rates and high rates of recrudescence, particularly in non-immune patients and children.[19] However, subsequent dose-finding studies established that a dosage regime of three 500 mg (or 8 mg/kg for children weighing less than 40 kg) Hf.HCl doses taken 6 hourly was well-tolerated and provided the most effective treatment against *P. falciparum* infections. In 1990, Horton *et al.*[20] reviewed the use of the 3-dose regimen in 1315 patients (from 37 studies) infected with *P. falciparum* and found that parasitaemia cleared within 7 days in all but 8 patients (0.6%) and during the observation period of 28 days, recrudescence occurred in 77 patients (6%), mainly in children under the age of 2 years or non-immune patients. As a result, a second course of treatment, 7 days later was recommended to prevent further episodes of parasitaemia.

More recent clinical studies have reported varying efficacy of Hf in the treatment of *P. falciparum* infections. In general, the 3-dose regimen of Hf resulted in cure rates of 83 to 100%. However, in certain regions of Thailand, the cure rates decreased markedly to less than 30% over a 5 year period. A study in Eastern Thailand compared the efficacy of standard dosage regimens of Hf and mefloquine. After Hf treatment, a cure rate of 28% was reported and a correspondingly low cure rate of 33% was observed after mefloquine treatment. These results indicated cross-resistance of mefloquine-resistant *P. falciparum* to Hf and questioned the efficacy of Hf in mefloquine-resistant infections.[21,22] Others have also reported reduced sensitivity of *P. falciparum* to Hf and it was suggested that the decreased sensitivity to Hf was a result of drug pressure[23] and to cross-resistance with mefloquine.[24,25] Therefore, it appears that development of resistance of *P. falciparum* to Hf is inevitable.

Incomplete and variable drug absorption has been cited as the major cause of treatment failure with Hf. In an attempt to maximize Hf bioavailability and antimalarial efficacy, a regimen comprising an initial loading dose (three 500 mg doses every 4

hours on day 1) followed by daily maintenance doses of 500 mg each day for 6 days has been proposed. A study in infected Thai soldiers found this regimen was well-tolerated and produced good clinical response. The efficacy between this Hf regimen and the standard 7 day quinine/tetracycline therapy was not significantly different and cure rates were 92% and 85%, respectively.[26] However, the slow elimination of Hf may induce parasite resistance when used in endemic areas. Administration of the same total dose (4.5 g) over 3 days also afforded good cure rates but this was associated with electrocardiographic (ECG) abnormalities.[27,28]

1.2.3 Adverse effects of halofantrine

Hf is generally well-tolerated and has good patient acceptability. The majority of adverse effects experienced by patients were mild and transient and did not require specific treatment. The most commonly reported adverse effects were abdominal pain, pruritus, nausea, vomiting, diarrhoea, headache and rash.[20] Other reported side effects include phototoxicity[29] and intravascular haemolysis.[30,31] Although animal studies indicate that Hf is not mutagenic or teratogenic, it is embryotoxic and also affects the survival of rat pups exposed to milk from female rats dosed with Hf. Therefore, the use of Hf is contraindicated in pregnant and breast-feeding women.[29]

ECG abnormalities have been observed in patients receiving Hf therapy. Like quinine and quinidine, Hf appeared to consistently prolong the QT interval, which can be fatal in patients with a pre-existing cardiopathy.[27,28,32-35] The QT interval is the duration between the beginning of the QRS complex and the end of the T wave in an electrocardiogram and represents the time between the onset of ventricular depolarization and complete ventricular repolarization. The QT interval varies with heart rate and is usually corrected for heart rate to give the QTc interval. Clinically,

prolongation of QTc has been associated with serious ventricular arrhythmias (e.g. torsade de pointes) and cardiovascular related fatalities.[36]

Significant QTc prolongation (defined as an increase of > 25% from baseline values) was more likely to occur when Hf was given as a re-treatment following mefloquine failure than as a primary treatment (although mefloquine did not cause cardiac effects) or when high doses of Hf were administered.[28] Lengthening of the QTc interval also appeared to be directly correlated with Hf plasma levels but not with the metabolite, Hfm.[32,34,37] Maximum changes in QTc intervals coincided with the time when Hf plasma concentrations peaked and as Hf concentrations decreased, QTc intervals returned to baseline. These studies suggest that dosage regimens leading to high maximal concentrations of Hf should be avoided and additional studies determining the conditions and any drug interactions that may inhibit the metabolism of Hf will be required.

The cardiotoxicity of Hf has been reported to be stereoselective.[38-40] *In vitro* studies indicate that both racemic Hf and (+)-Hf are more cardiotoxic than the (-) enantiomer. Therefore, administration of (-)-Hf alone could decrease the cardiotoxic potential of Hf.

It has been proposed that Hfm could be a safer alternative antimalarial drug to Hf, as *in vitro* studies have indicated that Hfm has minimal cardiotoxicity[39] and it has equipotent antimalarial activity to Hf.[18] However, more recently, *in vivo* studies have shown that after intravenous administration of Hfm to anaesthetized rabbits, Hfm did cause QTc prolongation, and there was a significant positive correlation between plasma Hfm concentrations and QTc interval.[41]

1.2.4 Pharmacokinetic properties of halofantrine

The pharmacokinetic properties of Hf and its major metabolite, *N*-desbutylhalofantrine (Hfm), in healthy human volunteers and in patients infected with *P. falciparum* have been extensively reviewed by Bryson and Goa[8] and by Karbwang and Na Bangchang.[9]

An important feature of Hf pharmacokinetic studies is the poor and highly erratic absorption of Hf after oral administration of the tablet formulation, which results in large intra- and inter-subject variability in the plasma concentration-time profiles of Hf.[42-45] This has been attributed to the extremely low aqueous solubility (< 0.01%) of Hf.HCl.[46] The highly variable and often sub-therapeutic Hf and Hfm plasma concentrations are of concern as this has been associated with treatment failures and may accelerate the emergence of resistance to Hf.

1.2.4.1 Effect of dose, ethnic group and disease state

Dose-proportionality studies have indicated that the relationship between dose and area under the plasma concentration-time curve (AUC) is only linear over the 250 to 500 mg range. Increasing the dose above 500 mg resulted in disproportionately small increases in AUC and C_{max} (maximum plasma drug concentrations).[45] In an attempt to achieve therapeutic plasma concentrations, multiple dosage regimens have been suggested. The pharmacokinetics of Hf and Hfm after administration of a single 500 mg dose or three 500 mg doses at 6 hourly intervals have been reported.[47] As shown in Table 1.1, there was wide variability in the C_{max} and AUC values for both Hf and Hfm at both doses. Interestingly, increasing the dose of Hf 3-fold resulted in 11- and 9-fold increases in Hf C_{max} and AUC values, respectively. The authors suggested that the marked increase in Hf C_{max} and AUC values was possibly a function of enhanced absorption of Hf in the presence of food which was eaten by the subjects 2 h before the

second and third doses. In this study, the half-life ($T_{1/2}$) of Hfm was considerably longer than that of Hf.

The pharmacokinetic parameters of Hf and Hfm have been reported to be different between ethnic groups, and between malaria patients and healthy volunteers (Table 1.1). Karbwang *et al.*[48] found that after administration of three doses of 500 mg Hf at 6 hourly intervals, the mean Hf and Hfm C_{max} values in Thai volunteers were considerably lower than those reported for Caucasian volunteers (1163 ng/mL compared to 3205 ng/mL), although the T_{max} (time at which C_{max} was recorded) and $T_{1/2}$ values were similar in both groups.[47] It was suggested that this was a function of reduced absorption due to the lower fat content in the Thai diet.

Wide variability in the pharmacokinetics of Hf and Hfm have also been reported after administration of the standard dosage regimen of 3 x 500 mg 6 hourly to Thai and Melanesian patients with uncomplicated falciparum malaria.[43,44] Although the C_{max} and T_{max} values of Hf and Hfm in Thai patients were similar to healthy Thai volunteers, the apparent $T_{1/2}$ of Hf was significantly longer in the malaria patients. In contrast, the apparent $T_{1/2}$ of Hfm was shorter in malaria patients. These findings suggest that the elimination of Hf may be different in malaria patients compared to healthy volunteers. The longer $T_{1/2}$ of Hf in malaria patients is not desirable as any persistent sub-therapeutic concentrations of Hf may facilitate the induction of drug resistance.

Table 1.1 Mean (and range or ± SD) pharmacokinetic parameters of halofantrine and *N*-desbutylhalofantrine after oral administration of Hf.HCl tablets to healthy volunteers or patients with uncomplicated malaria

Ref. no.	Population (n)	Dose	Halofantrine				*N*-Desbutylhalofantrine			
			C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	AUC (μg.h/mL)	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2}$ (h)	AUC (μg.h/mL)
Healthy volunteers										
47	Caucasian (10)	500 mg	296 (180-656)	6 (6-8)	23 (9-42)	5.6 (2.9-16)	123 (58-247)	12 (6-32)	75 (23-156)	123 (59-250)
		3 x 500 mg	3205 (1557-4929)	15 (9-17)	38 (14-80)	49 (25-69)	3763 (1828-5785)	48 (32-56)	103 (62-218)	412 (364-482)
48	Thai (8)	3 x 500 mg	1163 (777-3006)	18 (16-22)	43 ± 2	82 ± 57	288 (74-904)	36 (18-72)	252 ± 86	58 ± 30
45	Caucasian (6)	250 mg (Fst)	184 ± 115	6 ± 1.3	81 ± 19	3.9 ± 2.6	79 ± 30	16 ± 6.5		8.8 ± 3.5
		250 mg (Fed)	1218 ± 464	3.3 ± 1.5	80 ± 10	11.3 ± 3.5				10.7 ± 3.2
Patients with uncomplicated malaria										
43	Thai (12)	3 x 500 mg	1192 ± 410	16 ± 2	113 ± 31	61 ± 24	397 ± 160	55 ± 26	118 ± 38	48 ± 22
44	Melanesian (6)	3 x 500 mg	896 ± 370	15 ± 5	91 ± 27	43 ± 11	491 ± 213	56 ± 25	79 ± 23	75 ± 40

Fst - dose administered to fasting volunteers.

Fed - dose administered with a fatty meal.

1.2.4.2 Effect of food

Milton *et al.*[45] demonstrated that the rate and extent of Hf absorption is substantially enhanced when the drug is administered with a fatty meal. After administration of a 250 mg Hf.HCl tablet to fed volunteers, mean C_{max} and AUC values increased 7- and 3-fold, respectively, compared to fasted volunteers (Table 1.1). It was suggested that the increase in Hf AUC values was due to an increase in absorption rather than a change in clearance or volume of distribution in the fed state. The AUC of Hfm was also increased in the fed state but not to the same extent as for Hf. Mean plasma Hfm/Hf AUC ratios after fasted and fed administration were 2.25 and 0.95, respectively.

In a more recent study, the absolute oral bioavailability of Hf.HCl in fasted and fed dogs was reported.[49] The mean absolute oral bioavailability of Hf.HCl after administration in the fasted state was 8.6%, which increased approximately 12-fold when administered post-prandially. A 20-fold increase in Hf C_{max} was also observed when Hf.HCl was administered post-prandially compared with the fasted state. These data, together with the determination of Hf as a low extraction ratio drug indicated that the low fasted bioavailability of Hf.HCl is due to poor absorption rather than an inherent metabolic limitation. The authors also suggested that the improved absorption of Hf.HCl in the fed state was most likely due to increased solubilization and dissolution of the drug in the presence of bile constituents and lipid digestion products within the post-prandial intestinal lumen. Whilst administration of food during treatment may increase oral bioavailability, this is not practical as most patients would have difficulty eating large fatty meals during an acute illness. Furthermore, excessively high and poorly controlled plasma Hf concentrations have been associated with cardiac toxicity in patients with a pre-existing cardiopathy.[35]

An interesting feature arising from this dog study was the lack of effect of food on the plasma AUC of Hfm. In spite of a 12-fold increase in mean post-prandial Hf AUC values, there was only a 2-fold increase in the mean post-prandial Hfm AUC values. This was reflected in the mean plasma Hfm/Hf AUC ratios, which decreased 6.8-fold from 0.54 in the fasted state to 0.08 in the fed state. Altered splanchnic blood flow or saturated metabolism have been proposed as possible explanations for the reduced first-pass and/or systemic clearance of Hf. However, Hf is a low extraction drug,[49] and changes in splanchnic blood flow would not be expected to affect the clearance of low extraction drugs.[50] Furthermore, saturation of hepatic *N*-dealkylation of Hf due to increased portal blood absorption of Hf is also unlikely at the dose studied.[49,51]

A mechanism whereby the proportion of drug subjected to first-pass metabolism could be reduced, in spite of an overall increase in drug absorption is the recruitment of the intestinal lymphatics as an alternate absorption pathway. Since lymphatically transported drugs gain direct access to the systemic circulation and avoid hepatic first-pass clearance,[52] this may explain why the plasma AUC .values for Hfm did not directly reflect the increase in post-prandial Hf absorption. The lymphatic transport of Hf has been reported in the rat model (see section 1.2.5). Although only 5% of the dose of Hf.HCl administered orally to fasted rats was collected in the lymph, this could represent a major proportion of the total dose absorbed.[53]

Hf is highly lipophilic and has been shown to have high affinity for plasma lipoproteins.[54-56] Increased association of Hf with plasma lipoproteins and the increased levels of plasma lipoproteins present after the ingestion of a fatty meal could decrease both first-pass and systemic clearance of Hf by decreasing the unbound fraction of the drug in plasma. A crossover study examining the pharmacokinetics of Hf after intravenous administration to fed or fasted dogs demonstrated a significant increase in

drug binding to post-prandial plasma lipoproteins. The reduction in unbound fraction of drug in plasma resulted in decreases in Hf clearance (15%) and volume of distribution (21%) and a corresponding increase in plasma Hf AUC (19%) in the fed state compared to the fasted state.[55] This study also indicated that decreased clearance and volume of distribution of Hf were contributing factors to the greater than 100% absolute oral bioavailability of Hf reported in the earlier fed dog study.[49] In addition to affecting the pharmacokinetics of Hf, food can potentially influence its pharmacodynamic profile. For example, a decrease in the free fraction of drug resulting from sequestration by triglyceride-rich lipoproteins has been shown to result in a marked increase in the *in vitro* IC_{50} values of Hf.[57]

Another possible reason for the different Hfm/Hf AUC ratios is that the liver may not be the only site of Hf elimination. It has been suggested that intestinal metabolism could significantly contribute to the first-pass clearance of poorly available drugs such as cyclosporine and midazolam.[58-60] These compounds are substrates for the cytochrome P450 3A4 (CYP3A4) enzymes which are expressed in the liver and intestinal epithelium. Recent *in vitro* studies using human liver microsomes indicated that conversion of Hf to Hfm is largely mediated via CYP3A4,[61,62] therefore, Hf may also be subjected to first-pass intestinal metabolism. Although the relative contributions of prehepatic (enterocyte-based) and hepatic metabolism of Hf to Hfm are unknown, the inability to detect plasma levels of Hfm after intravenous administration of Hf[49,63] suggests that prehepatic conversion of Hf to Hfm may be significant. The significantly decreased plasma Hfm/Hf AUC ratio after administration with food could be due to the inhibition of CYP3A4 by one or more specific food components or by saturation of metabolism due to higher concentrations of Hf present in the enterocyte after enhanced absorption. Alternatively, since Hf has also been reported to associate with chylomicrons and VLDL in mesenteric lymph,[53] it is possible that after the ingestion of

dietary lipids, Hf may be sequestered into the lipid processing pathways within the enterocyte, thereby avoiding metabolism by CYP3A4 enzymes.

1.2.4.3 Metabolism of halofantrine

It has been proposed that Hf is eliminated entirely by metabolism since unchanged Hf has not been detected in urine of human subjects after administration of Hf.HCl tablets.[45] However, significant recovery of drug in faeces has been reported, suggesting that a major fraction of the administered dose was not absorbed. Some biliary excretion and enterohepatic recirculation of the absorbed drug may also occur.[9]

N-Desbutylhalofantrine has been identified as the major metabolite of Hf in both dogs[29] and humans.[45] As mentioned previously, *in vitro* studies using human liver microsomes indicate that CYP3A4 is largely responsible for the metabolism of Hf and ketoconazole, a well-characterized inhibitor of CYP3A4,[64] has been shown to be a potent inhibitor of Hf metabolism.[61,62,65] Hfm has equivalent *in vitro* antimalarial activity against *P. falciparum* as Hf[18] and may contribute to antimalarial activity after administration of Hf. Consequently, Hfm plasma concentrations have been determined in the majority of pharmacokinetic studies. The $T_{1/2}$ of Hfm is generally much longer than the parent compound (Table 1.1), which is undesirable since the long plasma residence time may induce the rapid emergence of parasite resistance. The subsequent fate of Hfm is unknown, as less than 0.01% of an oral dose of Hf is detected in the urine as Hfm,[45] and negligible amounts of Hfm have been detected in the bile of an isolated perfused rat liver.[66]

Two other metabolites of Hf have been identified in dog plasma.[67] Both of these metabolites were formed by modification of the dibutylaminopropyl side chain of Hf, however, it is not known if these metabolites are formed from Hf or Hfm or in what proportions.

1.2.4.4 Enantiomers of halofantrine

Hf possesses an asymmetric carbon atom and is only available as a racemic mixture. Both the (+)-and (-)-enantiomers of Hf and Hfm have been shown to have equivalent *in vitro* activities against *P. falciparum*.[17,68,69]

After oral administration of racemic Hf.HCl, differences in the pharmacokinetics of the enantiomers of Hf and Hfm have been reported.[70-74] The plasma concentrations of (+)-Hf tended to be higher than (-)-Hf as (-)-Hf appeared to be cleared more rapidly than (+)-Hf.[70-72] An *in vitro* study (using liver homogenates) reported that the metabolism of Hf was stereoselective with the (-)-enantiomer preferentially metabolizing to Hfm.[75] Therefore, it appears that the pharmacokinetic differences between Hf enantiomers may be a function of stereoselective metabolism. More recently, the binding of Hf to plasma lipoprotein fractions has also been shown to be stereoselective, with the (+)-Hf preferentially associating with lipoprotein-rich fractions of plasma, and (-)-Hf was primarily associated with lipoprotein-deficient fractions.[73] It has been reported that the pharmacokinetics of Hfm enantiomers parallel those of Hf.[74] After intravenous and oral administration of (±)-Hfm to rats, plasma concentrations of (+)-Hfm exceeded those of (-)-Hfm.

1.2.5 Intestinal lymphatic transport of halofantrine

Considering the high partition coefficient of Hf (calculated log P of 8.5),[46] high miscibility of amorphous Hf free base with a triglyceride lipid (> 200 mg/mL)[76] and the documented food effects for Hf.HCl,[45,49] it was postulated that intestinal lymphatic transport may be a significant contributor to oral bioavailability of Hf. Accordingly, the intestinal lymphatic transport of Hf has been investigated in the rat model.[53,76,77]

Lymphatic transport studies conducted in anaesthetized and conscious rat models demonstrated that up to 20% of the administered dose could be recovered in

intestinal lymph when the highly lipid soluble free base form of Hf was administered in an appropriate lipid vehicle. The majority of Hf transported in lymph was also associated with the chylomicron fraction.[53,76,77] Interestingly, after administration of the poorly lipid soluble Hf.HCl with or without lipid, approximately 5% of the dose was lymphatically transported. The large differences in lymph transport between the two forms of Hf could largely be attributed to the greater solubility of Hf base in triglyceride lipid compared to Hf.HCl (solubility in peanut oil ~ 1 mg/mL), thereby facilitating greater association of Hf base with the products of lipid digestion and subsequently interaction with the intestinally derived chylomicrons.

Results from the above rat studies indicate that when orally administered with a suitable lipid source, a significant proportion of Hf dose can be transported via the intestinal lymphatics, primarily in association with chylomicrons. However, due to differences in the characteristics of the luminal environment of the rat intestine, it is difficult to predict the likely role of lymphatic transport in higher species such as humans or dogs.

The following sections provide some background into the areas that are relevant to the research conducted in this thesis. These include a brief overview of the factors influencing oral drug bioavailability, the role of lipids in the oral delivery of poorly water soluble drugs, aspects of lipid digestion and intestinal lymphatic drug transport.

1.3 FACTORS INFLUENCING THE ORAL BIOAVAILABILITY OF DRUGS

Oral drug absorption is a complex process involving many different steps. Identification of the causes of incomplete oral bioavailability has been the subject of extensive research as this has important clinical implications. For example, poor and variable drug absorption could lead to a poor clinical response or treatment failure and in some cases, the potential development of drug resistance. This section provides a

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brief overview of some of the factors which may affect oral bioavailability of drugs. For more detail, reviews on this subject should be consulted.[78-84]

1.3.1 Physicochemical characteristics of the drug

Drug dissolution and permeability across the intestinal membrane are two important steps governing oral drug absorption and are largely dependent on physicochemical properties such as aqueous solubility, lipophilicity (often expressed as the logarithm of the octanol-water partition coefficient, log P), molecular size and the potential of the drug to ionize (pKa). In order to be absorbed, drugs must be hydrophilic enough to be soluble in the gastrointestinal lumen, yet sufficiently lipophilic to cross the intestinal membrane. Whilst parameters such as log P, molecular weight and pKa are likely to dictate the membrane permeability of the compound,[85,86] they may also influence the aqueous solubility and consequently the dissolution rate. Physical (e.g. particle size, crystal structure, polymorphism) and physiological factors (e.g. pH, presence of bile salts) may also influence drug dissolution.[87,88]

Recently, computational strategies have been developed to assist medicinal chemists in the design and rapid selection of potential drug candidates that are orally active. These include Lipinski's Rule of Five,[80] the use of theoretical estimates of octanol/water partition coefficients (cLogP)[85] and approaches based on determination of the dynamic polar surface properties of drug molecules (related to molecular hydrogen bonding capacity).[89-91] Molsurf, which uses an electronic wave-function to calculate the relevant physicochemical descriptors for the prediction of membrane permeability,[92] and VolSurf which correlates three dimensional molecular structures with physicochemical and pharmacokinetic properties of lead compounds[93] are more recent computational tools. Whilst these tools vary in complexity, they are also proving to be

useful for the prediction of oral bioavailability at an early stage of drug discovery and development.

1.3.2 Stability within the gastrointestinal tract

Degradation within the gastrointestinal lumen may be due to chemical instability at acidic pH in the stomach or enzymatic degradation by luminal or brush border digestive enzymes, or luminal micro-organisms.[94,95] Degradation in the acidic environment of the intestinal lumen may be prevented by the use of enteric coating.

1.3.3 Physiological barriers to oral bioavailability

1.3.3.1 Intestinal epithelium

The intestinal epithelium represents the major physical barrier to absorption of orally administered drugs, and it restricts the free movement of drug molecules from the intestinal lumen to the bloodstream. This complex barrier is composed of a single layer of columnar epithelial cells, primarily enterocytes and goblet cells, joined at their apical surfaces by tight junctions.[83,96,97] In addition to the intestinal epithelium, the mucus layer may also provide a barrier to the absorption of certain molecules such as the tertiary amines chlorpromazine, promethazine, and imipramine.[98,99]

Transport of drug molecules across the intestinal epithelium may occur by either paracellular or transcellular processes and can take place along the entire length of the intestine. Paracellular transport involves passive diffusion of molecules through the tight junctions and into the aqueous environment between epithelial cells.[100] Absorption via this pathway is typically considered a minor absorption pathway due to the presence of tight junctions, which only allow the passage of small hydrophilic molecules with molecular radii of less than 11 Å.[101]

Transcellular transport involves the movement of a molecule across the apical cell membrane, through the cytoplasm of the cell and across the basolateral membrane by passive diffusion, or by a carrier- or receptor-mediated process. Since passive transcellular transport requires partitioning of drugs across both the apical and basolateral membranes it only occurs maximally for relatively small hydrophobic compounds.[84] However, specific carriers such as those for nutrients, vitamins and bile salts may actively transport certain classes of hydrophilic drugs across the intestinal epithelium.[102-104] Compounds absorbed via the transcellular route may also be recognized as substrates for secretory efflux systems such as P-glycoprotein and/or enterocytic intracellular metabolism by cytochrome P450 isozymes, and the implications of this is discussed in more detail in the following section.

1.3.3.2 P-glycoprotein efflux and enterocyte-based drug metabolism

It is now widely recognized that the intestinal multidrug efflux pump, P-glycoprotein, and the major phase I drug metabolizing enzymes, cytochrome P450 3A (CYP3A), present at the villus tip enterocytes of the small intestine, the primary site of drug absorption, act as biochemical barriers to limit the systemic availability of orally administered drugs.[58,105,106]

P-glycoprotein (P-gp) is a product of the multidrug resistance gene MDR1 and has been characterized as the ATP-dependent multidrug transporter responsible for the efflux of chemotherapeutic agents from resistant cancer cells. Over-expression of P-gp is one of the major causes of multidrug resistance in human cancers as this efflux transporter ultimately results in lower intracellular drug concentrations and therefore a reduction in the cytotoxic activity of anticancer drugs.[105] The expression of this 170 kDa transmembrane protein is not limited to tumor cells. P-gp is also widely expressed in a variety of normal human tissues such as the adrenal glands, lungs, apical surface of

epithelial cells in the liver (bile canaliculi), kidney (proximal tubule), bladder, pancreas, small and large intestine, and endothelial cells of blood capillaries in the brain.[82,102] The specific location of P-gp at these tissues indicates that P-gp functions to facilitate the excretion and/or minimize the absorption of xenobiotics and other potentially 'toxic' compounds. Since P-gp is expressed at high levels on the luminal surface of mature columnar epithelial cells of the small intestine and colon, it acts as a barrier to intestinal drug absorption by transporting drugs back into the intestinal lumen, as they are absorbed across the intestinal mucosa.[107,108] Expression of P-gp between subjects has been reported to be highly variable, and may therefore be responsible for some of the intersubject variability commonly seen in drug absorption profiles.[105] Substrates for P-gp cover a broad range of chemical structures with diverse therapeutic indications such as cancer chemotherapeutic agents, immunosuppressants, steroids, calcium channel blockers, HIV protease inhibitors and certain peptidomimetic drugs.[102,109,110]

In addition to P-gp, a number of other secretory transporters have been characterized in the intestine including the family of multidrug resistance-associated proteins (e.g. MRP2, MRP3), the breast cancer resistance protein (BCRP), organic cation transporters and members of the organic anion polypeptide family. Like P-gp, these efflux transporters may also be responsible for the cellular extrusion of many clinically important drugs.[102,111-113]

The contribution of intestinal cytochrome P450 enzymes (CYPs) to the overall metabolism of candidate drugs has historically been considered relatively minor compared to hepatic metabolism. However, this view has recently been re-examined following the identification of enzymes of the CYP3A subfamily at high levels in the mature villus tip enterocytes of the small intestine. CYP3A are the predominant phase I drug metabolizing enzymes found in humans, and its role in drug metabolism will be discussed in greater detail in chapter 3.[82,114-116]

CYP3A and P-gp have been reported to have a striking overlap in substrate specificity.[117] Furthermore, the shared location and spatial relationship of P-gp (traversing the plasma membrane) and CYP3A (inside the cell on the endoplasmic reticulum) in the small intestinal enterocytes suggest that the two processes may act in a complementary and synergistic manner to reduce the bioavailability of their substrates after oral administration. After being taken up by the enterocytes, some of the substrate drug molecules are metabolized, and those that escape metabolic conversion may be extruded from the cells into the lumen by P-gp. Drug molecules in the lumen may be subjected to the same cycle, resulting in repeated exposure of drug to metabolic enzymes, possibly at less than saturated concentrations, thereby further reducing bioavailability. The inhibition of either CYP3A4 or P-gp or both is expected to improve the bioavailability of many products and decrease the variability in systemic drug concentrations.[105] Recently, it has been reported that both CYP3A4 drug metabolism and P-gp drug efflux can be coordinately regulated by the steroid and xenobiotic receptor (SXR).[118] Therefore, the activity of SXR could also manipulated to control drug metabolism and drug efflux.

1.3.4 Effect of food on oral drug bioavailability

Historically, food has been regarded as a barrier to drug absorption. However, in addition to the physical and chemical interactions that may occur between drugs and specific food components, the ingestion of food also triggers a number of physiological events, some of which may assist the absorption of, in particular, poorly water soluble drugs. Changes such as alteration in the secretion of gastric acid, bile and pancreatic fluids; modified gastric and intestinal motility patterns; and altered hepatic/splanchnic blood and lymph flow may all have a significant impact on drug absorption.[119-122]

More recently, studies have shown that specific components in food can increase the oral bioavailability of certain drugs. For example, flavanoids such as naringin and quercetin and several furanocoumarins including 6',7'-dihydroxybergamottin present in grapefruit juice have been identified as potent inhibitors of CYP3A4 *in vitro*.[123-125] The inhibition of intestinal rather than hepatic CYP3A4 has been suggested since grapefruit juice has no effect on drug disposition after intravenous administration.[123,126,127] Therefore, the co-administration of grapefruit juice with drugs that are subject to metabolism by CYP3A4 may result in enhanced oral bioavailability.[128,129] Furthermore, components in grapefruit juice[130,131] and orange juice[132] have also been reported to inhibit P-gp. However, another recent study suggests that grapefruit juice significantly activates P-gp-mediated efflux of drugs that are substrates of P-gp, potentially counteracting the CYP3A-inhibitory effects of grapefruit juice.[133]

Food has been shown to significantly improve the oral bioavailability of many poorly water soluble, lipophilic drugs. The enhanced absorption has generally been ascribed to the lipid component of food.[122] Ingested lipids can stimulate a series of lipid digestion processes, which lead to a wide range of effects. For example, the presence of lipid in the duodenum stimulates the secretion of biliary and pancreatic fluids, which could potentially increase the rate of dissolution and/or solubility of certain compounds via enhanced wetting or by promoting drug solubilization within bile salt micelles thereby improving bioavailability.[134,135] Bile salts and lipid digestion products have also been reported to alter the intrinsic permeability of the intestinal membrane leading to increased absorption. Therefore, it appears that lipids could have a beneficial role in drug absorption.

1.4 THE ROLE OF LIPIDS IN THE ORAL DELIVERY OF POORLY WATER SOLUBLE DRUGS

The use of natural and synthetic lipids as a potential formulation strategy for improving the oral bioavailability of poorly water soluble, highly lipophilic drug candidates has generated much interest. Lipids can reduce the inherent limitations of slow and incomplete dissolution, and facilitate the formation of solubilized phases from which drug absorption may occur. The attainment of a solubilized phase will not necessarily arise directly from the administered lipid, but more frequently will result from the intraluminal processing (digestion and dispersion) to which lipids are subjected prior to absorption.[136] The co-administration of lipids with drugs can also impact on their absorption path. Whilst most orally administered drugs gain access to the systemic circulation via the portal vein, some highly lipophilic drugs are transported to the systemic circulation via the intestinal lymphatics (thereby avoiding presystemic hepatic metabolism).[52] In addition to delaying gastric transit and altering membrane permeability, certain lipids may effect biochemical changes in the enterocyte such as attenuation of the activity of cytochrome P-450 enzymes and drug efflux processes mediated via P-gp, leading to an increase in the permeability of drug across the absorptive barrier.[137]

The following sections briefly describe the physiological processing which are involved in the digestion and absorption of dietary lipids, as an understanding of these processes is crucial for the rational design of lipid formulations. Recent examples of the utility of lipid-based formulations to increase oral bioavailability will also be presented.

1.4.1 Lipids and the gastrointestinal tract

1.4.1.1 Digestion and absorption of lipids

The digestion and absorption of lipids has been extensively reviewed in the nutritional and physiology literature.[138-141] Briefly, lipid digestion involves three main sequential steps: (i) dispersion of fat globules into a coarse emulsion of high surface area, (ii) enzymatic hydrolysis of the fatty acid glyceryl esters at the oil/water interface and (iii) dispersion of the digestion products into an absorbable form.[139] Digestion of dietary or formulation derived lipids, which are predominantly in the form of triglyceride (TG) begins in the stomach where lingual and gastric lipases secreted by the salivary gland and gastric mucosa, respectively, initiate the hydrolysis of TG to the corresponding diglyceride (DG) and fatty acid (FA). Liberation of these amphiphilic lipid digestion products, in combination with the shear produced by antral contraction and gastric emptying, facilitates the formation of a crude emulsion that empties into the duodenum.[141]

The presence of lipid in the duodenum stimulates the secretion of bile salts and biliary lipids from the gall bladder and pancreatic fluids from the pancreas. Biliary lipids such as phospholipid and cholesterol ester adsorb to the surface of the crude emulsion, to stabilize and further reduce droplet size. Subsequently, the process of lipid digestion is completed under the action of pancreatic lipase and colipase to quantitatively produce one molecule of 2-monoglyceride (MG) and two FA molecules for each TG molecule.[138,141,142] Since FA and MG are effective emulsifying agents, and because FA promotes binding of lipase/co-lipase complex to the emulsion surface,[143,144] the overall process of lipolysis is self-promoting.

Early studies by Hofmann and Borgstrom[145,146] proposed that the *in vivo* products of lipid digestion partitioned between an oily phase and a solubilized bile salt mixed micellar phase. However, more recent studies on aspirates of human post-

prandial intestinal fluid identified the presence of unilamellar vesicles in equilibrium with bile salt mixed micelles.[147,148] Current understanding suggests that as lipolysis proceeds, digestion products 'pinch off' from the surface of the TG/DG emulsion to form large liquid crystalline structures, which in the presence of sufficient bile salt and phospholipid concentrations then form multilamellar and unilamellar vesicles. The efficient solubilization of lipid digestion products by bile salt micelles is pivotal to the trafficking of the lipid digestion products to the surface of the enterocyte.

The brush border membrane of enterocytes is separated from the bulk aqueous phase of intestinal contents by a poorly mixed unstirred water layer (UWL) that constitutes a major barrier to the absorption of poorly water soluble lipid digestion products and drug candidates. However, micellar solubilization which enhances the luminal solubility (up to 1000-fold) of the lipid digestion products, also facilitates their passage across the UWL close to the absorptive surface and provides a concentration gradient for the absorption of lipids (and presumably drugs solubilized in this phase).[141,142]

The specific mechanisms underpinning the absorption of lipid digestion products have not been completely elucidated, however, it is believed that bile salt micelles are not absorbed intact and that the various products of lipid digestion must dissociate from the intestinal mixed micellar phase prior to absorption into the enterocyte.[149,150] Dissociation of lipid from the mixed micellar phase may be stimulated by a microclimate of lower pH associated with the intestinal absorptive site.[151-153] In addition to passive diffusion, there is now evidence to suggest that specific membrane bound carrier proteins may facilitate the transport of lipid digestion products across the apical membrane of the enterocyte. For example, a microvillus membrane fatty acid binding protein and a fatty acid transporter have been identified and suggested to be responsible for the uptake of FA and other lipid substrates into the enterocyte.[154-156]

1.4.1.2 Impact of lipids on gastrointestinal transit profile

The products of lipid digestion delay gastric emptying to enable optimal digestion and absorption of lipids.[157] Delayed gastrointestinal motility could potentially enhance the absorption of poorly water soluble drugs by increasing the time available for dissolution. It has been suggested that the formulation of drugs with lipidic excipients such as a long-chain FA (oleic acid) could also activate the ileal brake, a feedback mechanism regulating movement in the small intestine, to prolong intestinal residence time and potentially increase drug bioavailability.[158,159] However, in comparison to the amount of lipid ingested in a meal, small pharmaceutically relevant volumes of lipid may not have a significant impact on transit profiles.

1.4.1.3 Impact of lipids on drug solubilization

Bile is released from the gall bladder in response to the intestinal presence of lipid digestion products. In addition to solubilizing lipid digestion products, bile may also improve the bioavailability of co-administered poorly water soluble drugs by enhancing their rate of dissolution and/or solubilization. An increase in the rate of dissolution may occur via a decrease in the interfacial energy barrier between solid drug and the dissolution medium (enhanced wetting), leading to an effective increase in surface area, or through an increase in solubility via micellar solubilization. In general, enhanced wetting predominates at bile salt concentrations below the critical micelle concentration (cmc), whereas enhanced solubility is dominant at concentrations above the cmc.[134,135,160,161]

As mentioned previously, the UWL presents a barrier to the absorption of poorly water soluble drugs. However, the solubilization of poorly water soluble drugs within bile salt micelles facilitates diffusion through the aqueous layer thereby enhancing absorption. In contrast, it is also important to be cognisant of the possibility that drug

solubilization may lead to a decrease in absorption due to a reduction in the intermicellar 'free' fraction of drug or in some cases the formation of insoluble, poorly absorbed drug-bile salt complexes.[122]

1.4.1.4 Impact of lipids on intestinal permeability

It is well known that bile salts, FA and MG may alter the intrinsic permeability of the intestinal membrane leading to increased absorption via paracellular or transcellular routes.[94,162] When considering these 'naturally occurring permeability enhancers', it is apparent that the intestinal mucosa is frequently damaged during normal digestive and absorptive processes, however mechanisms have evolved for rapid repair.

The use of various lipids (e.g. medium-chain glycerides and FA) and surfactants as permeability enhancers to improve drug absorption has received considerable attention.[83,86,94] However, tissue irritation and toxicity resulting from the use of such agents has raised safety concerns. In an attempt to reduce mucosal damage caused by permeability enhancers, a more recent approach to enhancing drug absorption has been the development of enhancers (e.g. sodium caprate) that act in a transient and reversible manner to regulate tight junctions and promote paracellular transport.[83,96,163]

1.4.1.5 Impact of lipidic excipients on the barrier function of P-gp and CYP3A

Recently, it has become apparent that lipidic formulation excipients could affect the barrier properties of P-gp and CYP3A. Commonly used pharmaceutical surfactants such as polyoxyethylene sorbitan fatty acid esters (e.g. Tween 80) and polyethoxylated castor oil (e.g. Cremophor EL) have been shown to inhibit P-gp in cell culture systems at concentrations below their cmc.[113,164] Vitamin E TPGS (D-α-tocopheryl polyethylene glycol 1000 succinate), a water soluble derivative of Vitamin E which acts to solubilize

lipophilic compounds has been reported to improve the oral bioavailability of cyclosporine.[165] This enhancement was originally attributed to increased solubility, improved permeability and/or reduced intestinal metabolism, however, recent studies have indicated that this lipidic excipient is also an inhibitor of P-gp mediated drug efflux.[109,166] Therefore, enhanced oral bioavailability of drugs (which are substrates of P-gp) co-administered with these surfactants may, in part, be due to inhibition of P-gp present in the enterocytes.

The potential for formulation excipients to inhibit CYP3A activity has also been reported. In a study by Mountfield *et al.*,[167] the majority of the excipients investigated inhibited enzyme activity, however, the high IC_{50} values (mM range) recorded suggest that the inhibition of CYP3A is unlikely to be significant clinically. Generally, greater inhibition of CYP3A activity was observed with amphiphilic ingredients such as mixed micellar solutions, the surfactants Tween 20 and Tween 80, and oleic acid. In addition, a recent patent has described the potential for lipids/essential oils to inhibit both CYP3A based metabolic processes and P-gp mediated anti-transport processes.[168]

Excipients in lipid dose forms could potentially enhance drug absorption by affecting P-gp mediated drug efflux and/or CYP3A mediated enterocyte metabolism. However, effective use of such excipients as modulators of CYP3A and P-gp will require further *in vivo* studies to determine the clinical and biopharmaceutic relevance of such interactions and the ramifications of other drug/drug and drug/excipient interactions.

1.4.2 Lipid-based formulations

A preference for solid dosage forms has prevailed over many years in the pharmaceutical industry, however, there is currently increasing interest in the development of lipid-based formulations for oral delivery of poorly water soluble

compounds. Reasons for this include the successful marketing of lipid-based products containing cyclosporine (Neoral®), the HIV protease inhibitors saquinavir (Fortovase®) and amprenavir (Agenerase®) and the lipid soluble vitamins; a trend towards the discovery and development of increasingly hydrophobic and potent new chemical entities, and the resolution of many of the previously problematic technology transfer, stability and regulatory issues associated with lipid-based delivery systems.[137,169]

The design of lipid-based formulations remains semi-empirical and poses a significant challenge to the pharmaceutical scientist. Progress has been impeded by the lack of predictive *in vitro* and *in vivo* testing methodologies which enable the determination of the fate of the drug and lipidic components during luminal digestion and processing. Whilst an understanding of the processes involved in the digestion of dietary lipids may provide a useful basis for the rational design of lipid-based dose forms, the large differences in volume and composition of pharmaceutical lipids compared to dietary lipids in a meal must be considered when attempting to evaluate the role of digestion of lipid vehicles on drug absorption.[136]

Lipid-based dose forms utilized for the oral delivery of hydrophilic and lipophilic drugs generally consist of a blend of different classes of excipients such as long- or medium-chain triglycerides, long- or medium-chain mixed mono- and diglycerides, individual or mixed surfactants (non-ionic, hydrophilic or lipophilic) and various hydrophilic co-solvents.[137,170] Depending on their composition, lipid formulations may exhibit a broad range of characteristics (e.g. varying degree of self-emulsification) which has made the systematic comparison and description of such formulations difficult. However, a classification system of different lipid formulations has recently been proposed to facilitate the comparison of published data.[171,172] The

typical properties and selected examples of each type of formulation in the classification system are presented below.

1.4.2.1 Lipid delivery systems: lipid solutions, SEDDS and SMEDDS

Three main types of lipid formulations have been proposed by Pouton[171,172] and the typical properties of each system are described in Table 1.2. The three main criteria by which lipid formulations can be distinguished include (i) does the formulation self-emulsify or remain poorly dispersed in water, (ii) when the formulation makes contact with water are some of the components lost by dissolving in the aqueous phase and (iii) is the dispersed formulation digestible by lipases.[171]

Type I systems are formulations which comprise drug dissolved in digestible lipids, such as triglycerides. Due to the low solvent capacity of triglycerides and the small volume required for each unit dose, these simple systems are often limited to the delivery of highly lipophilic compounds (log P > 4) and potent drugs. For less hydrophobic drugs, the solvent capacity can be improved by blending triglycerides with mixed monoglycerides and diglycerides. The digestibility of the chosen oil is important since bioavailability from these oil solutions is dependent on the lipolysis of the oily vehicles and the formation of digestion products which facilitate the solubilization of drug within the colloidal phases. Non-digestible lipids (such as mineral oil, liquid paraffin) that essentially remain unabsorbed in the intestinal lumen may limit or reduce drug absorption.[172] With these formulations, it is also important to establish that the drug remains in solution during their passage through the gut. This may be investigated using *in vitro* lipid digestion models followed by phase separation of the digestion products to enable the determination of drug in each phase.[173,174]

Table 1.2 Typical properties of Type I, II, IIIA and IIIB lipid formulations. Reproduced with permission from ref. 172.

	Increasing hydrophilic content →			
	Type I	**Type II**	**Type IIIA**	**Type IIIB**
Typical composition (%)				
TG or mixed glycerides	100	40-80	40-80	< 20
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 aqueous dilution	Limited importance	Solvent capacity unaffected	Some loss of solvent capacity	Significant phase changes and potential loss of solvent capacity
Significance of digestibility	Crucial	Not crucial but likely to occur	Not crucial but may be inhibited	Not required, and not likely to occur

TG, triglyceride; HLB, hydrophile-lipophile balance.

Although there are many earlier examples of drugs formulated in oily solutions,[52,136,175] with the exception of fat soluble vitamins, there are few recent examples. Formulation of cinnarizine, a weak base with poor aqueous solubility, as a solution in a long-chain fatty acid (oleic acid) resulted in a 4-fold increase in oral bioavailability in dogs compared with a standard tablet formulation.[176] Similarly, Abrams *et al.*[177] also found that in rats, the oral bioavailability of a lipophilic steroid

derivative from a sesame oil solution was 3-fold higher than from an aqueous suspension of solid drug.

Myers and Stella[178] investigated the effect of digestible and non-digestible lipid vehicles on the oral bioavailability of penclomedine, a highly lipophilic cytotoxic agent. Following intraduodenal administration to rats, the rank order of penclomedine bioavailability from the different lipid vehicles could be rationalized in terms of their digestibility: medium-chain triglyceride (MCT, rapid digestion) > long-chain triglyceride (LCT, slower and incomplete digestion) > non-digestible mineral oil > short-chain triglyceride (rapid digestion but dissipation of digestion products caused the drug to precipitate) > aqueous suspension.

The distinguishing features of Type II systems, also known as self-emulsifying drug delivery systems (SEDDS) are (i) efficient self-emulsification, and (ii) absence of water soluble components. SEDDS are isotropic mixtures of oil and surfactant, which form fine oil-in-water emulsions when introduced into aqueous phases under conditions of gentle agitation. Hydrophobic drugs can be dissolved in SEDDS and encapsulated into soft gelatin capsules to produce convenient unit dose forms. When the formulation is released into the gut lumen it disperses to form a fine emulsion enabling the drug to remain in solution, thereby avoiding the rate-limiting dissolution step. An additional advantage of SEDDS is the large interfacial area provided for partitioning of drug between oil and water, which could lead to improvements in oral bioavailability.[136,179]

SEDDS are best formulated with medium-chain triglycerides and/or mixed mono- and diglycerides, and non-ionic surfactants with HLB of 10-12 such as polysorbate 85. Non-ionic surfactants are preferred as they are generally recognized to be less toxic than the ionic surface active agents.[162] In addition to promoting emulsification, the surfactant may improve the solvent capacity of the formulation, enhance intestinal permeability and could potentially inhibit P-gp and/or CYP3A

thereby enhancing drug absorption.[164,167] SEDDS may vary in terms of their digestibility, but if a very fine dispersion is produced, bioavailability may be enhanced independently of digestion.[172,180]

Studies of binary mixtures of the surfactant, Tagat TO (polyoxyethylene-25-glyceryl trioleate), and a MCT (Miglyol) by Wakerly *et al.*[181,182] showed that surfactant concentrations of at least 25% were required for efficient self-emulsification. At surfactant concentrations of 30-50%, emulsification occurred rapidly and emulsions with a particle size of 100-250 nm could be produced. However, increasing the surfactant concentration beyond 60% resulted in the formation of viscous liquid crystalline gels at the oil-water interface, which retarded self-emulsification.

Charman *et al.*[183] utilized a MCT (Neobee M5) and a non-ionic surfactant (Tagat TO) to formulate a SEDDS for the delivery of an investigational lipophilic compound, WIN 54954. A study in fasted beagles showed that the absolute bioavailability of WIN 54954 from either the SEDDS or a PEG 600 solution was not significantly different, however, the SEDDS did improve the reproducibility of the plasma profiles in terms of C_{max} and T_{max}. The more consistent absorption of WIN 54954 from the SEDDS was mostly likely a function of the solubilizing and dispersing properties of the formulation.

SEDDS containing a range of polyglycolyzed glycerides (PGG) with varying fatty acid and polyethylene glycol (PEG) chain lengths and either a MCT (Neobee M5) or a LCT (peanut oil) were developed by Shah *et al.*[184] for the delivery of a lipophilic naphthalene derivative, Ro 15-0778. The authors found that the fatty acid chain length and degree of saturation, as well as the molecular weight and concentration of the PEG in the emulsifier influenced the particle size and the polarity of the droplets in the emulsion, and this consequently affected the release rate of the drug. Emulsifiers with HLB values of approximately 10 produced the best results. *In vivo* studies in dogs showed that the bioavailability of Ro 15-0778 from the SEDDS formulation was 4-fold

higher than a PEG 400 control solution and 20-fold greater than a standard tablet formulation. The reduced bioavailability from the PEG 400 solution was likely to be due to precipitation of the drug within the intestinal lumen.

Type III formulations generally produce very fine dispersions (< 100 nm) that are optically clear and thermodynamically stable. These systems have been referred to as 'self-microemulsifying' systems (SMEDDS) and typically comprise triglyceride, mixed glycerides, hydrophilic surfactants (HLB > 12) and/or hydrophilic co-solvents (such as ethanol, propylene glycol, polyethylene glycol, glycerol, glycofurol, transcutol) to improve the solvent capacity of the formulation for drugs with intermediate log P (between 2 and 4).[172,179,185]

The introduction of more hydrophilic components which result in a different mechanism of emulsification, distinguishes Type III from Type II systems. During dispersion, the hydrophilic co-solvent in Type III formulations tends to diffuse away from the oil into the aqueous phase resulting in 'diffusion and stranding' which may be the driving force for emulsification. While mixing with excess water, the bulk of the hydrophilic surfactants may also form a micellar solution. The fate of the drug and lipid components after mixing with water could be solubilization in a swollen micellar solution, dispersion as a fine emulsion, or isolation and precipitation. Sub-classification into Type IIIA and Type IIIB has been suggested to address the differences that may emerge as the oil content of the formulation is reduced. Being the most hydrophilic type of lipid formulation, Type IIIB formulations also present the highest risk of drug precipitation, but if the drug can be stabilized in solution, the finest dispersion may result. Given the low glyceride content, the biological activity of Type IIIB systems is independent of lipolysis but is dependent on the formation of a fine dispersion on dilution.[171]

An example of a Type III system is the lipid-based formulation of cyclosporine, Sandimmune Neoral®. This microemulsion pre-concentrate which consists of drug, surfactant, hydrophilic co-solvent and lipids produces a finely dispersed oil-in-water microemulsion when diluted in an aqueous phase. The new formulation afforded an approximately 2-fold increase in the oral bioavailability of cyclosporine compared with the original commercial formulation (Sandimmune® comprising drug, corn oil, ethanol and polyglycolyzed LCT) which produces a crude oil-in-water emulsion.[186] Inter- and intra-subject variability in cyclosporine pharmacokinetics also decreased after administration of the Sandimmune Neoral®.[187] Further studies indicate that the absorption of cyclosporine from the microemulsion formulation was largely independent of the ingestion of food.[188] This is likely to be due to greater dispersion of the new formulation on dilution in an aqueous phase, which provides increased efficiency and consistency for absorption. In contrast, absorption from the crude emulsion appears to be dependent on the *in vivo* digestion and dispersion of the administered lipid as the oral bioavailability of cyclosporine increased 37% when administered post-prandially compared to fasted administration.

Self-emulsifying lipid formulations that form positively charged emulsion droplets upon dilution have recently been shown to undergo enhanced electrostatic interactions with the negatively charged apical surface of Caco-2 cell monolayer and the mucosal surface of everted rat intestine. Such formulations resulted in enhanced oral bioavailability of progesterone in young female rats and higher blood levels of cyclosporine in perfused rats in comparison to the corresponding negatively charged emulsions.[189-191] More recent studies suggest that at high concentrations, positively charged emulsions affect the barrier properties of Caco-2 cell monolayers and reduce

cell viability, however, the cytotoxic effects are not likely to occur at physiological conditions, where the dilution in aqueous phase is greater.[192]

1.4.2.2 Solid dispersions

Solid dispersions were first introduced by Sekiguchi and Obi[193] in 1961 as a unique approach to reduce particle size and increase dissolution and absorption rates of poorly water soluble drugs. They are defined as a dispersion of one or more active ingredient in an inert, hydrophilic carrier or matrix at solid state prepared by the melting (fusion), solvent, or melting-solvent method.[194] The drug may be molecularly dispersed in the carrier in the form of a solid solution, be in the amorphous form or it can be dispersed as fine particles. On exposure to aqueous media, the carrier dissolves rapidly, releasing drug as very fine, dispersed colloidal particles of submicron size. The greatly enhanced surface area affords higher dissolution rates and is expected to increase the bioavailability of poorly water soluble drugs.[195,196]

Whilst solid dispersions offer many advantages as a delivery system, a major factor limiting the wider commercial application of this delivery system is the difficulty in ensuring physical stability over time. The presence of drug in a high energy, metastable or amorphous form can be problematic as there is a tendency for the drug to revert to the thermodynamically more stable crystalline form and this can affect the dissolution rate of the drug and ultimately result in diminished absorption. Gris-PEG® by Novartis (griseofulvin in polyethylene glycol) and Cesamet® by Lilly (nabilone in povidone) are two older commercially available solid dispersion products. Norvir® (by Abbott) was a more recently available solid dispersion comprising ritonavir (a poorly water soluble HIV protease inhibitor) and a mixture of surface active carriers such as Gelucire 50/13, polysorbate 80 and polyoxyl 35 castor oil. Formulation as a solid

dispersion enhanced the oral bioavailability of ritonavir. However, this product was later withdrawn from the market, due to the crystallization of ritonavir in the formulation, which consequently resulted in the product failing dissolution specifications.[169,196] Complexity in the method of preparation and scale up manufacturing processes, and difficulty in obtaining reproducible physicochemical properties were other limiting factors.[196]

Numerous carriers are currently available for the preparation of solid dispersions, of which the water soluble high molecular weight polyethylene glycols (PEG) and polyvinylpyrrolidones (PVP) are most commonly used.[195,197] The trend towards the discovery of poorly water soluble drug candidates has led to the development of amphiphilic lipid-based carriers with solubilizing properties. In comparison to the traditional matrices, these surface active carriers offer the added advantage of improving wetting characteristics and facilitating the dispersion and solubilization of the liberated drug, thereby enabling complete dissolution of poorly water soluble drugs and bioavailability enhancement. An example of such carrier is Gelucire 44/14, which is a mixture of glycerides and fatty acid esters of PEG 1500. The suffixes 44 and 14 refer to its melting point and HLB value, respectively.[198] Another lipid-based surface active carrier is a water soluble derivative of Vitamin E, Vitamin E TPGS (D-α-tocopheryl polyethylene glycol 1000 succinate), which has a HLB value of 13 and relatively low melting point of 38°C.[199] The low melting temperatures of these carriers enable direct filling of the molten drug/matrix into a gelatin capsule. These carriers spontaneously solubilize lipophilic drugs upon contact with an aqueous medium to form either a fine emulsion or microemulsion and hence, facilitate drug absorption.[196] Similarly, Polysorbate 80 has been incorporated into solid PEG dispersions to enhance dissolution and bioavailability by improving wetting and solubilization of the drug.[200]

The solubilizing properties of Gelucire 44/14 have been shown to contribute significantly to the enhancement of the dissolution and/or oral bioavailability of REV 5901 (a poorly water soluble lipoxygenase inhibitor),[201,202] UC-781 (a non-nucleoside reverse transcriptase inhibitor),[203] DMP 323 (a poorly soluble HIV protease inhibitor)[204] and ubidecarenone (coenzyme Q_{10}).[205]

1.5 INTESTINAL LYMPHATIC DRUG TRANSPORT

Following oral administration, drugs can gain access to the systemic circulation via two separate pathways, the portal blood or the intestinal lymphatics. Due to the high capacity of portal blood to transport both water soluble and poorly water soluble compounds, the majority of orally administered drugs are absorbed via this pathway. However, a disadvantage associated with absorption via the portal route is the potential for hepatic first-pass metabolism.

Although the primary role of the intestinal lymphatics is the absorption and transport of dietary lipids and lipid soluble vitamins to the systemic circulation, it also represents an alternate absorption pathway for highly lipophilic drugs and xenobiotics. Due to the unique anatomy and physiology of the lymphatic system, promotion of drug delivery via the intestinal lymphatics has certain advantages over the portal route. For drugs that undergo significant first-pass metabolism, absorption via the intestinal lymphatics will bypass the liver and result in avoidance of hepatic first-pass metabolism, thereby improving oral bioavailability. There is also the potential for targeted delivery of chemotherapeutic agents to regions of the lymphatic system that may be poorly perfused by the systemic circulation for the treatment of certain cancers and immunological diseases. Since the process of intestinal lymphatic drug transport often continues over longer periods than drug absorption via the portal route, it may also be possible to modulate the rate of drug delivery to the systemic circulation.[206]

In the following sections, the intracellular processing of lipids after absorption into the enterocyte and the characteristics of the intestinal lymphatics will be described. Factors influencing lymphatic drug absorption and approaches to promote lymphatic drug transport, namely by prodrug and lipid formulation will also be discussed.

1.5.1 Intracellular lipid processing

After absorption into the enterocyte, the fatty acid chain length of lipid digestion products dictates its transport. Generally, short- and medium-chain lipids (carbon chain length < 12), which account for approximately 10% of dietary lipid, are primarily transported by the portal blood.[207] In contrast, long-chain lipids (> 12 carbons) migrate from the absorptive site to the endoplasmic reticulum where re-esterification and assembly into intestinal lipoprotein carriers occur prior to secretion into mesenteric lymph (Figure 1.2). Realistically, this dependence of the route of lipid transport on chain length of the fatty acid is not always 'clear cut' as there are reports of lymphatic transport of medium-chain fatty acids and portal blood absorption of long-chain fatty acids.[208,209]

To date, the mechanism by which absorbed lipids (and potentially drug molecules) migrate from the absorptive site to the endoplasmic reticulum where FA are re-esterified to TG is unknown, however, it has been suggested that cytosolic fatty acid binding proteins (FABP) play an important role in the intracellular transport of absorbed FA.[210] Since FABP preferentially binds to long-chain FA and not medium-chain FA, this may explain why medium-chain FA are mainly reabsorbed through the portal system without being re-esterified.[142]

The resynthesis of FA to TG by the enterocyte may occur by one of two pathways (Figure 1.2). The major route for re-esterification is the monoacylglycerol pathway. This involves the direct acylation of absorbed exogenous 2-monoacylglycerol

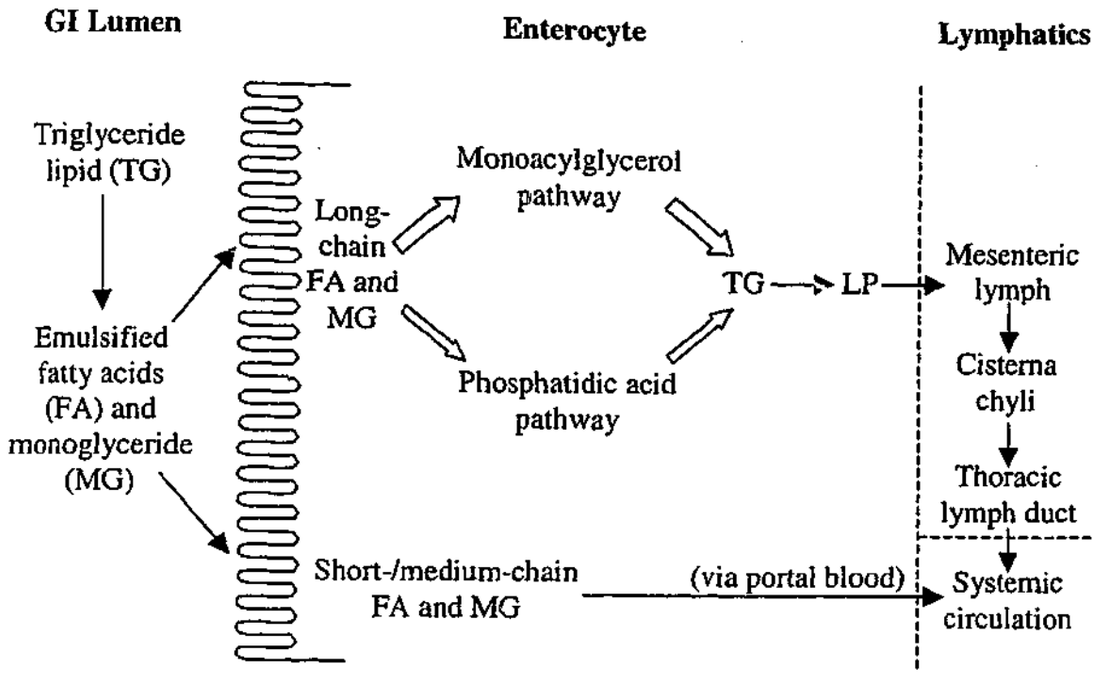

Figure 1.2 Schematic diagram of the intracellular processing of lipid digestion products following absorption into the enterocyte. Long-chain fatty acids (FA) and monoglycerides (MG) are re-esterified to form triglycerides (TG) via the monoacylglycerol or phosphatidic acid pathways and assembled into intestinal lipoproteins (LP), primarily chylomicrons. Chylomicrons are secreted into the mesenteric lymph and drain via the thoracic lymph duct into the systemic circulation. Short- and medium-chain lipids are absorbed directly into the portal blood. Adapted from ref. 206.

with suitably activated fatty acids to form the corresponding diglyceride, which is then sequentially acylated to form TG. During lipid absorption, this is the more important pathway for the formation of TG as it has the highest capacity, is the most rapid and energy efficient, and accounts for approximately 80% of chylomicron triglyceride synthesized.[138,211] The minor and slower pathway for TG resynthesis is the phosphatidic acid pathway (also known as the glycerol-3-phosphate or α-glycerophosphate pathway). It involves the sequential acylation of endogenous glycerol-3-phosphate with three molecules of activated fatty acid in a complex series of biochemical steps. In the fasted state, when the supply of 2-monoacyglycerol is lacking, this pathway becomes the major pathway for the formation of TG and the substrates used in TG synthesis are derived from glucose metabolism and endogenous fatty acids.[211] Whilst both esterification pathways utilize acyl CoA as the fatty acid donor and form 1,2-diglyceride as an intermediate, the two pathways function independently of each other.[142]

Following resynthesis, TG formed in the endoplasmic reticulum is processed through a number of intracellular organelles (namely the rough endoplasmic reticulum and Golgi apparatus) to become the main core lipid of intestinal lipoproteins, primarily chylomicrons. The sequential addition of polar phospholipids, specific apolipoproteins and unesterified cholesterol stabilize the hydrophobic core of chylomicrons in the aqueous environment of lymph. Chylomicrons then fuse with the basolateral membrane of the intestinal cell and are released into the lamina propria. Due to their size, chylomicrons are preferentially absorbed by the open capillaries of the mesenteric lymphatics and transported to the general circulation via the thoracic duct.[141]

1.5.2 Characteristics of the intestinal lymphatics

Whilst the gastrointestinal tract is richly supplied with lymphatic vessels, the anatomy and function of the lymphatics in the upper and lower intestinal regions are

somewhat different. The lymphatics of the small intestine originate as single, closed vessels (known as lacteals) located in the centre of each intestinal villus (Figure 1.3). The lacteals are approximately 20 μm in diameter and lie within the interstitium, approximately 50 μm below the epithelial cells. In the large intestine, the lymphatic vessels are fewer in number and smaller in size and consist of a well-developed network of lymph vessels that originate deep in the mucosa, 300-400 μm from the epithelial surface. Since the colon is devoid of villi, there are no lacteals in the colon.[212,213]

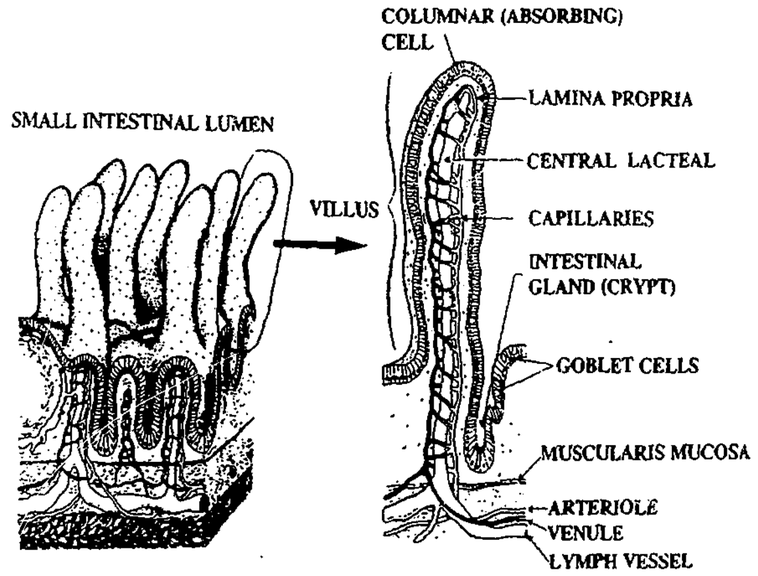

Figure 1.3 Schematic diagram of the small intestine illustrating the projection of villi into the intestinal lumen (left) and the anatomic features of a single villus (right). Reproduced with permission from ref. 78.

Lymphatic vessels from both the small and large intestines originate as a plexus of lymphatic capillaries in the mucosa and submucosa which eventually join to form larger mesenteric collecting lymph vessels. Mesenteric lymph runs into the cisterna chyli, which also collects hepatic and lumbar lymph. From the cisterna chyli, lymph is drained by the thoracic duct directly into the systemic circulation at the junction of the left subclavian and internal jugular veins without passing through the liver. Therefore, drugs absorbed via the intestinal lymphatics avoid hepatic first-pass metabolism.

The rate of fluid flow through the intestinal lymphatics is approximately 500-fold lower than portal blood.[214] Therefore, the opportunity for selective lymphatic transport of low molecular weight, water soluble drugs is minimal. However, the lymph capillaries, which comprise a single layer of endothelial cells and fragmented basement membrane are significantly more permeable than blood capillaries enabling the lymphatic transport of high molecular weight molecules or colloids.

The content of intestinal lymph varies between fed and fasted states. In the fasting state, lymph appears transparent and the composition is similar to that of other extracellular fluids in that it contains water, plasma proteins, enzymes, electrolytes, and lipids, although the concentrations of each of these components are different. Lymph collected after fat ingestion has a milky appearance which is primarily due to the presence of chylomicrons, the largest lipid carrying lipoprotein in lymph. Other lipoproteins present in lymph after fat ingestion include very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). These differ in size and lipid and apolipoprotein composition.[212,213]

Chylomicrons formed by the enterocyte in response to lipid ingestion are primarily responsible for the transport of exogenous lipid. The size (50-500 nm in diameter), rate of formation and composition of the chylomicrons vary with the composition and quantity of ingested lipid, although there is usually a component of

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endogenous lipid.[215] The spherical chylomicrons typically comprise 86-92% TG, 6-8% phospholipid, 2-4% sterols (free and esterified) and about 1-2% protein. They are composed of a nonpolar hydrophobic core containing TG, cholesterol esters and unesterified cholesterol, which is surrounded by an exterior surface coating of polar phospholipid, specific apolipoproteins, unesterified cholesterol, and some unsaturated TG. The surface coating of phospholipid and protein (covering up to 80% and 20% of chylomicron surface, respectively) stabilizes the hydrophobic core of the chylomicron in the aqueous environment of lymph.[142,216]

Unlike chylomicrons, VLDL only have a minor role in the transport of exogenous lipids and are usually formed by the enterocyte during fasting conditions for the lymphatic transport of lipids derived from endogenous intestinal and biliary sources.[217] VLDL are much smaller (20 to 50 nm) than chylomicrons and the triglyceride composition of the VLDL is only approximately 50%. Therefore, VLDL are likely to be less efficient in the transport of dietary-based lipid (and possibly lipophilic drugs) than the chylomicrons. It has been suggested that VLDL are synthesized by mechanisms independent of chylomicron synthesis and in different cellular locations. Studies by Tso and co-workers[218,219] have shown that chylomicron synthesis can be selectively inhibited by the surfactant, Pluronic L-81, whereas the pathway of VLDL synthesis is insensitive to this surfactant. HDL are also synthesized by the small intestine, but do not have a significant role in lymphatic drug transport.

1.5.3 Factors governing the intestinal lymphatic transport of lipophilic drugs

The intestinal lymphatics have been shown to contribute to the absorption of a number of highly lipophilic drugs such as cyclosporine,[220] probucol,[221] mepitiostane,[222] naftifine,[223] penclomedine,[224] halofantrine,[76] ontazolast,[225] CI-976,[226] MK-386,[227]

lipophilic vitamins and vitamin derivatives, xenobiotics including DDT and associated analogs,[228] benzo(a)pyrene,[229] and numerous lipophilic prodrugs.[230]

Although the mechanism(s) by which lipophilic drugs gain access to intestinal lymph is not fully understood, the majority of lymphatically transported lipophilic compounds are either solubilized within, or associated with the triglyceride core of chylomicrons. Small quantities of transported drug may also be associated with the VLDL fraction of lymph.[52] Therefore, promotion of lymphatic drug transport is likely to require the co-administration of an appropriate lipid source to stimulate lipoprotein synthesis by the enterocyte. Whilst post-prandial administration readily provides the lipid source required to support chylomicron formation, lipid-based formulations may also promote intestinal lymphatic drug transport by stimulating the turnover of lipoproteins through the enterocyte. The utility of lipid-based formulations to enhance lymphatic drug transport will be discussed in greater detail in section 1.5.5.2.

Charman and Stella[231] have established an apparent relationship between the physicochemical properties of an administered drug and the extent of intestinal lymphatic drug transport. They suggested that candidate drug molecules should have a log octanol/water partition coefficient (log P) of at least 5 and significant triglyceride solubility (> 50 mg/mL) before intestinal lymphatic transport is likely to become a significant contributor to oral bioavailability. However, these criteria are based on the assumption that the drug is completely absorbed and metabolically stable in the intestinal lumen and within the enterocyte.

The requirement for a high log P value takes into consideration the differences in the flow rate between portal blood and intestinal lymph (approximately 500-fold) and the maximum lipid content of lymph (1-2%). However, since the transport of lipophilic drugs in intestinal lymph is associated with the chylomicron fraction of intestinal lymph, it is the chylomicron flux and not the actual volumetric flow of intestinal lymph

that determines lymphatic transport. Therefore, for a drug to be equally transported by the portal blood and the lipid fraction of the intestinal lymph, it would require a partition coefficient of at least 50,000:1 in favour of chylomicron lipid, which translates to a log P of 4.7.

Appreciable solubility of a drug in a lipid such as a LCT is another important criterion for lymphatic drug transport as this reflects the solubility of the drug in the lipid core of the chylomicron. This has been illustrated by a study examining the cumulative lymphatic transport of two highly lipophilic compounds, hexachlorobenzene (HCB, log P 6.53) and DDT (log P 6.19) in an anaesthetized rat model. After intraduodenal administration, the lymphatic transport of DDT (solubility in peanut oil 13-fold higher than HCB) was substantially greater (33.5% dose) than the lymphatic transport of HCB (2.3% dose).[231]

Whilst high lipid solubility and log P are necessary for significant intestinal lymphatic transport, they are not the sole prerequisites for ensuring the intestinal lymphatic transport of a compound. The extent of protein and red blood cell binding are additional factors that need to be considered when attempting to predict lymphatic drug transport. Penclomedine is a highly lipophilic cytotoxic agent with log P of 5.5 and solubility of 175 mg/mL in triolein. After intraduodenal administration as either a soybean oil or triolein emulsion, only 3% of the absorbed dose was transported in lymph. The lower than expected lymphatic transport of penclomedine has been attributed to the high affinity of the drug for red blood cells and/or plasma proteins which increases the partitioning of drug towards the portal blood.[224]

1.5.4 Animal models for the assessment of intestinal lymphatic drug transport

A number of animal models have been described and reviewed recently[232,233] for estimating intestinal lymphatic drug transport. Whilst most studies are conducted in the

laboratory rat, limitations associated with this model have made the extrapolation of lymph transport data to species other than the rat difficult. For example, bile flow in the rat is continuous and independent of food intake whereas in the higher species, food/lipid is required to stimulate the numerous events that lead to the digestion and absorption of lipids. While the constant stream of bile in the fasted rat may be sufficient to process a small quantity of formulation lipid, a mass of lipid larger than that commonly used in oral formulations may be required to stimulate bile production and the related digestive events in higher species. Therefore, results obtained in the fasted rat may not be mirrored in higher species. Furthermore, the attainment of representative fed and fasted states and the evaluation of dose forms clinically relevant to humans are also not possible in the rat. Whilst these limitations may be addressed by utilizing a larger animal model such as the dog or pig, this is often limited by considerable logistical and economic constraints.[233]

The dog has been described as a lymphatic delivery model where the creation of a thoracic duct fistula enables the continuous collection of thoracic duct lymph.[234] Unlike the rat, dogs are capable of ingesting prototype human dose forms and have pre- and post-prandial intestinal environments that are similar to humans. In addition to establishing the contribution of lymphatic transport to oral bioavailability, this model could also be employed to assist in rational design of lipid-based formulations.

An anaesthetized pig has been employed as a lymphatic transport model.[235] The model enabled the simultaneous sampling of mesenteric lymph, hepatic portal blood and systemic blood for the determination of the effect of novel formulations on the absorption pathways of propranolol after intraduodenal administration. Although the study described approaches for periodic sampling of mesenteric lymph (and not its complete collection), it may be possible to modify the technique to collect all mesenteric lymph, thereby enabling drug transport to be quantitatively assessed.

Methodologies and protocols employed for the study of lymphatic drug transport often differ between research groups. The most common differences include the site of cannulation and lymph fistulation,[236] the extent of hydration and fasting/fed state of the animal after lymph duct cannulation,[237,238] whether the experiment is performed in a conscious or anaesthetized animal and the site of drug/lipid administration.[53] Therefore, to enable the comparison of data between studies it is important to standardize procedures. In addition to estimating lymphatic drug transport, sampling of portal blood absorption would enable the extent of lymphatic transport relative to absorption via the portal blood and the overall bioavailability of the drug to be estimated.

1.5.5 Approaches for enhancing intestinal lymphatic drug transport

Intestinal lymphatic drug transport may be enhanced by either changing the physicochemical properties of the drug via a prodrug approach (e.g. increase drug lipophilicity) or by employing lipid formulation strategies.

1.5.5.1 Prodrug approach

Two major prodrug approaches have been explored for increasing the overall lipophilicity of compounds and thereby increasing lymphatic drug transport. The first and simplest approach involves derivatization of compounds via simple ester or ether linkages. The second and chemically more challenging approach is the design of 'functional' prodrugs that mimic key components of the lipid digestion and re-esterification pathway, thereby facilitating the incorporation of prodrug molecules into the lipid transport pathway. Since the promotion of intestinal lymphatic transport using lipophilic prodrugs is not the primary focus of the thesis, this will not be discussed. Recent reviews have addressed the topic of lymph directing prodrugs in detail.[230,239,240]

1.5.5.2 Lipid formulation approach

Lipid formulations have been employed as an alternate approach to enhance the lymphatic transport of poorly water soluble, highly lipophilic drugs.[52,206] Lipid vehicles may facilitate lymphatic drug transport by stimulating lipoprotein synthesis within the enterocyte and providing an absorption sink into which lipophilic drugs can partition. There is currently a vast selection of pharmaceutical lipid vehicles, and the choice of co-administered lipid is important as this determines the extent of intestinal lymphatic drug transport. Lipids may be classified by the (i) lipid chain length, (ii) lipid class, (iii) degree of lipid saturation, and (iv) physical state of the administered lipid.[206,241,242]

1.5.5.2.1 Effect of lipid chain length

A number of studies have evaluated the impact of fatty acid chain length of a lipid vehicle on intestinal lymphatic drug transport.[221,243-246] Generally lipid formulations based on long-chain lipids resulted in greater enhancement of lymphatic drug transport than medium-chain lipids. These studies suggest that lipids with fatty acid chain length of less than 12 carbons do not promote the formation of chylomicrons and are sufficiently water soluble to be absorbed via the portal blood whereas longer chain fatty acids, once absorbed and re-esterified to triglyceride will promote chylomicron formation and consequently lymphatic drug transport.

The effect of lipidic vehicles with different fatty acid chain lengths on the intestinal lymphatic transport and absolute oral bioavailability of halofantrine (Hf, log P ~ 8.5) has recently been reported by Caliph *et al.*[77] The lymphatic transport of Hf in conscious, lymph cannulated rats was highly dependent on the fatty acid chain length of the co-administered triglyceride lipid, and increased with increasing chain length (lipid free vehicle, 0.34% of dose < tributyrin, short-chain (C_4) triglyceride, 2.22 % < Captex 355, medium-chain (C_{8-10}) triglyceride, 5.5% < peanut oil, long-chain (C_{18}) triglyceride,

15.8%). There was also a strong positive correlation between C_{18} triglyceride lipid transport and Hf transport in intestinal lymph suggesting that drug was transported in association with resynthesized exogenous long-chain lipids present in the core of lymph lipoproteins. Lymphatic transport also accounted for 70% of the total systemic availability of Hf in animals dosed with the long-chain triglyceride vehicle, whereas, after administration of the medium- or short-chain triglyceride or lipid free formulations, the majority of the drug was absorbed via the portal route.

1.5.5.2.2 Effect of lipid class

There have been a number of reports on the effect of lipid class (triglyceride or free fatty acid) on the rate and extent of intestinal lymphatic drug transport.[53,245-248] Whilst fatty acids can be absorbed without luminal modification, triglycerides are required to be hydrolyzed in the intestinal lumen prior to absorption and therefore, a longer lag time in lymphatic drug transport is expected with the triglyceride vehicle.

Charman *et al.*[247] reported that the intraduodenal administration of DDT (2 mg in lipid volume of 200 μL) to anaesthetized rats in either a long-chain fatty acid (oleic acid) or an oleic acid-monoolein vehicle, which represents the luminal digestion products of triglyceride resulted in significantly faster and greater extent of intestinal lymphatic transport of DDT than the long-chain triglyceride (LCT) vehicle (peanut oil). The longer lag time associated with administration of the triglyceride vehicle likely reflected the requirement for pre-absorptive hydrolysis to the corresponding fatty acids and monoglyceride, whereas the more rapid transport of DDT after administration in either oleic acid or the oleic acid-monoolein vehicles was consistent with more rapid resynthesis of triglyceride and chylomicron transport.

In contrast, Noguchi and co-workers[245] found that the rate and extent of lymphatic transport of testosterone undecanoate in unanaesthetized rats was greatest and

not significantly different when administered orally in either a long-chain fatty acid (oleic acid) or a LCT vehicle (peanut or safflower oil). In comparison, mixtures of oleic acid and monoolein (1:1 or 2:1) appeared to decrease the lymphatic transport of testosterone undecanoate relative to the triglyceride vehicle.

Although the necessary pre-absorptive hydrolysis of triglyceride has been proposed as a potential limiting step in the lymphatic transport of lipophilic compounds, the above examples show conflicting data, which may in part be due to the different study designs. The effects of lipid class appear to be less pronounced in the conscious rat model.

1.5.5.2.3 Effect of saturated and unsaturated fatty acids

The absorption and lymphatic transport of saturated and unsaturated fatty acids has been the subject of considerable interest due to their effects on health. Generally, the absorption and transport of saturated lipids is less efficient than that for unsaturated lipids, although this is also dependent on the chain length and extent of unsaturation.[249-251]

It has been reported that monounsaturated lipids generally enhance intestinal lymphatic drug transport to greater extents than polyunsaturated lipids.[245] Recently, the absorption of two carotenoids, lycopene and astaxanthin, from an emulsion containing either corn oil (polyunsaturated triglyceride) or olive oil (monounsaturated triglyceride) showed that both carotenoids were significantly better absorbed from the olive oil than from the corn oil emulsions.[252] The mechanism responsible for the decreased absorption with corn oil is unknown.

The effect of three different unsaturated triglycerides on the intestinal lymphatic transport of halofantrine in conscious rats was examined by Holm *et al.*[253] The rank order for the lymphatic transport of halofantrine was trilinolein ($C_{18:2}$) > trilinolenin

($C_{18:3}$) > triolein ($C_{18:1}$) but there was no statistically significant difference. A similar rank order of steady state chylomicron transport was observed by Cheema *et al.*[251] after administration of the corresponding fatty acids. These studies suggest that lipid vehicles containing linoleic acid could be more effective in the delivery of drugs to the lymphatic system. In contrast, Porsgaard and Hoy[254] have shown that the extent of lymphatic transport of α-tocopherol was not dependent on the degree of unsaturation of the co-administered lipids.

Based on the reported trends in the lymphatic transport of lipids of varying degrees of unsaturation, it is difficult to predict the effect that the different lipids would have on the lymphatic transport of a lipophilic drug and warrants further investigation.

1.5.5.2.4 Potential utility of structured triglycerides

Structured triglycerides (STG) are novel lipids incorporating both medium- and long-chain fatty acids on the same glycerol backbone. These lipids are chemically distinct and the effects of various STG on the absorption of fatty acids and cholesterol have been investigated by numerous groups.[255-260] Significant recovery of medium-chain fatty acids in lymph has been reported and generally, medium-chain fatty acids located in the 2-position of the STG were more efficiently absorbed via the lymphatics than those in the 1- and 3-positions. It was proposed that the medium-chain fatty acids in lymph could have been absorbed as 2-monoglycerides and were subsequently utilized in triglyceride resynthesis for lymphatic transport. Similarly, the lymphatic absorption of long-chain fatty acids was faster and greater when present in the 2-position than when present in the 1- and 3-positions. This may be due to the rapid and more complete hydrolysis of medium-chain fatty acids located in the 1- and 3-positions of the STG.

At present, the utility of STG in drug delivery has not been fully explored, however, a recent study by Holm *et al.*[261] suggests that these novel lipids could

potentially be efficient vehicles for promoting drug absorption via the lymphatic and portal routes.

1.5.5.2.5 Effect of lipid vehicle dispersion

Many lipid formulations with physical characteristics representative of the final stages of lipid digestion (i.e. mixed micellar systems containing 'digested' lipids such as fatty acid and monoglycerides) appear to promote the lymphatic transport of lipophilic compounds, which include a lipid regulator drug (CI-976),[226] mepitiostane,[248] cyclosporine,[262] *d*-α-tocopherol acetate,[263] retinol and retinyl palmitate.[264]

Intestinal lymphatic transport of the free base form of halofantrine (Hf), after intraduodenal infusion as either a lipid solution, a polysorbate 80 stabilized emulsion, or a polysorbate 80 mixed micellar system has been evaluated in an anaesthetized rat model.[76] The different lipid vehicles were chosen to represent the progressive physical states of lipid digestion and the lipid component of each formulation was 50 μL of a 2:1 molar ratio of oleic acid:glycerol monooleate. Up to 20% of the administered dose was recovered in intestinal lymph, suggesting that the intestinal lymphatics were a significant absorption path for Hf when administered in an appropriate lipid vehicle. The rank order effect of the vehicles for the promotion of lymphatic transport was micellar (17.7%) > emulsion (11.8%) > lipid solution (10.6%), supporting the contention that formulation of lipids as increasingly dispersed systems may enhance lymphatic transport.

However, in a subsequent study[53] where Hf base was dosed orally as a lipid solution or micellar system to conscious rats, the effect of various lipid vehicles on the promotion of lymphatic transport was no longer evident. In both cases, the extent of lymphatic transport of Hf was maximal (approximately 20% of administered dose). The lower extent of lymphatic transport of Hf from the lipid solution formulation in the

anaesthetized model may be due to the side effects of anaesthesia where reduced blood and bile flow may result in insufficient solubilization of the nondispersed formulations prior to the drug and lipid reaching the absorptive site. The decreased lymphatic transport of Hf from the nondispersed formulations in the anaesthetized rat model may also reflect a decrease in the ease and extent of processing of the intraduodenally administered lipid solutions compared with the crude emulsions that form *in situ* after gastric emptying in the conscious model. Therefore, preduodenal processing of lipid formulations, as well as the attainment of a micellar state in the duodenum, is likely to facilitate the uptake of lipid digestion products required for lymphatic drug transport.

Hauss and co-workers[225] recently evaluated the impact of formulation variables on the intestinal lymphatic transport of ontazolast, a lipophilic (log P 4.0), poorly water soluble anti-inflammatory compound intended for oral administration in humans. Ontazolast was formulated as an aqueous suspension, and in four different lipid vehicles: a 20% soybean emulsion, a solution in Peceol and as two self-emulsifying drug delivery system (SEDDS) for oral administration to conscious rats. The SEDDS incorporated the lipidic excipients Gelucire 44/14, which forms a very fine emulsion when in contact with the gastrointestinal fluids *in vivo*, and Peceol, a readily dispersible and absorbable solubilizing agent which provides a triglyceridogenic substrate for efficient chylomicron synthesis and subsequent lymphatic drug transport. Either equal parts of Gelucire 44/14 and Peceol or 8 parts Gelucire 44/14 to 2 parts Peceol were used. When compared to the aqueous suspension, the total amount of lymphatically transported ontazolast over a 24 h post-dosing period was approximately 20- to 25-fold greater for the lipid-based SEDDS, and 50-fold greater for the soybean oil emulsion. In this study, the lymphatic drug transport appeared to vary directly with the amount of concurrent lymphatic triglyceride transport.

The lymphatic uptake of MK-386, a specific sterol 5α-reductase inhibitor, from aqueous and lipid formulations has been investigated by Kwei *et al.*[227] The high lipophilicity and structural similarity of the compound to cholesterol suggest that MK-386 could be a candidate for intestinal lymphatic transport. After administration of a 5 mg/kg dose in either an aqueous suspension, a mono-diglyceride/polysorbate 80 (MDG/PS80) (1/1) vehicle or a soybean oil (SBO) solution to conscious rats, the total mass of MK-386 collected in lymph was in the order of aqueous suspension (4.8% of administered dose) > MDG/PS80 (0.45%) > SBO (0.10%). Low portal plasma concentrations and preferential partitioning of drug into the lymphatics suggest that the intestinal lymphatics were the major route of uptake of MK-386. The significant lymphatic uptake of MK-386 in aqueous suspension in the absence of exogenously administered lipid was surprising. This could be due to drug partitioning into intestinal lipoproteins (primarily VLDL) secreted by the enterocyte during the absorption of endogenous lipids. The rate of appearance of drug in lymph was slower for the lipid formulations, presumably due to delayed gastric emptying, and the slower digestion and absorption of lipids into the intestinal lymphatics. In this study, the sampling time was only for 6 h, during which, the lymphatic transport of MK-386 may not have peaked or reached completion. Consequently, this may explain the lower lymphatic transport of drug in the lipid formulations compared with the aqueous suspension.

1.5.5.2.6 Effect of lipid volume and requirement for co-administered lipid

It has been reported that majority of highly lipophilic compounds which are lymphatically transported typically associate with the triglyceride lipid core of chylomicrons.[52] This has led to the commonly held view that lipids are a prerequisite for optimal intestinal lymphatic drug transport as the administration of an appropriate lipid source would initiate the cascade of events leading to the synthesis of lipoproteins

(primarily chylomicrons and VLDL) by the enterocyte, and thus, providing an absorption sink into which lipophilic drugs can partition. However, the volume of lipid required to be administered to support chylomicron formation remains unclear and the effect of pharmaceutically relevant volumes of formulated lipids on lipoprotein synthesis in animal models other than the rat has also not been extensively studied.

In rat studies, the volume of lipid administered often varies between 0.05 and 1 mL. On a bodyweight conversion basis, 50 μL is equivalent to approximately 10 mL for a 70 kg human. The effect of different lipid dose volumes on the lymphatic transport of DDT[247] and benzo(a)pyrene[229] has been studied in the rat. Increasing the volume of lipid from 50 to 200 μL did not affect the extent of lymphatic transport of DDT, although the lag time for appearance of DDT in the lymph was decreased with the lower volume of administered lipid. Similarly, varying the mass of lipid vehicle by ten-fold (50 or 500 μmoles of olive oil) did not significantly affect the lymphatic transport of benzo(a)pyrene.

Whilst formulation lipids have been successfully utilized to enhance lymphatic drug transport, a number of studies have reported appreciable lymphatic drug transport in the absence of co-administered lipid. The lymphatic transport of MK-386 was significantly higher after administration in an aqueous suspension (4.8% of administered dose) than in a soybean oil emulsion (0.10%),[227] an enhancement of up to 10-fold in the intestinal lymphatic transport of cyclosporine was observed after administration of simple micellar solutions compared with mixed micellar systems and lipid solutions,[262] and the extent of lymphatic transport of mepitiostane (MP) was similar when administered in either a 10% polysorbate 80 micellar solution (40.9% of dose) or a sesame oil solution (41.2%) and a surprisingly high extent of lymphatic transport of MP was also observed after administration as an aqueous suspension (7.5%

of dose).[248] Interestingly, the co-administration of halofantrine hydrochloride (Hf.HCl) with exogenous lipid did not enhance the extent of lymphatic transport of Hf.HCl (4.6% dose) compared with administration as a simple 1% Tween 80 dispersion (6.1% dose), although there were significant differences in the distribution of drug between the various lymph lipoprotein fractions. The proportion of Hf recovered from the chylomicron fraction of lymph was significantly higher when administered with exogenous lipid (82 vs. 68%) and there was a corresponding increase in the proportion of Hf transported in the VLDL fraction when administered in the dispersion compared with the exogenous lipid formulation (26 vs. 12%).[53]

The above examples suggest that under certain circumstances, the co-administration of lipids may not be a pre-requisite for appreciable lymphatic drug transport. In the absence of exogenous lipid, drugs may gain access to mesenteric lymph in association with intestinal lipoproteins (predominantly VLDL) secreted by the enterocyte during the absorption of endogenous lipids. However, the limited capacity of endogenous lipid turnover via the VLDL pathway (in the fasted state) may limit the intestinal lymphatic transport of drugs utilizing this absorption pathway. The relatively high concentrations of surfactants used in some of the lipid-free solubilized formulations may also affect membrane integrity and enterocyte functionality, although this has not been addressed in detail.

In summary, absorption via the intestinal lymphatics offer the potential advantage of increased bioavailability due to a decrease in hepatic first-pass metabolism, the specific targeting of drugs to regions within the lymphatic system, and the possibility of controlling the rate of drug entry into the systemic circulation. Candidates for intestinal lymphatic transport are generally highly lipophilic and the compounds typically associate with the lipid core of chylomicrons in lymph. The synthesis of prodrugs to increase drug lipophilicity and development of lipidic

formulations are approaches which can be taken to enhance lymphatic drug transport. As many new drug candidates are proving to be poorly water soluble and highly lipophilic, drug delivery to the intestinal lymphatics could become an increasingly important area of research. A better understanding of the mechanistic aspects of lymphatic transport may aid in the design of delivery systems that promote lymphatic drug transport.

1.6 SUMMARY AND PLAN OF THESIS

In this chapter, the use of halofantrine in the treatment of multidrug-resistant malaria and its pharmacokinetic properties; factors affecting the oral bioavailability of drugs; the role of lipids in the oral delivery of poorly water soluble drugs; aspects of lipid digestion and absorption; and intestinal lymphatic drug transport have been discussed. This provides background into the studies that were undertaken in this thesis. The utility of lipidic dose forms as a formulation strategy to promote the oral absorption of Hf and the involvement of CYP3A4 in the *in vivo* metabolism of Hf are described in chapters 2 and 3, respectively. The intestinal lymphatic transport of Hf and Hfm after fasted and post-prandial administration of Hf base, fed administration of Hf.HCl and Hfm.HCl are assessed in chapters 4, 5 and 6, respectively. Finally, chapter 7 concludes the thesis and makes recommendations for future work.

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CHAPTER 2

LIPID-BASED SELF-EMULSIFYING FORMULATIONS FOR THE ORAL DELIVERY OF HALOFANTRINE

2.1 INTRODUCTION

Halofantrine (Hf) is a clinically important drug used for the treatment of uncomplicated, multidrug-resistant *Plasmodium falciparum* malaria.[1,2] Hf is orally active and well-tolerated, however, a major limitation associated with its clinical utility is the highly variable and often low plasma concentrations following oral administration of the commercially available tablet formulation (Halfan®) containing 250 mg Hf hydrochloride (Hf.HCl).[3,4] The poor intestinal absorption of Hf.HCl is presumably due to its extremely low aqueous solubility (< 2 μg/mL).[5] Sub-therapeutic Hf plasma concentrations are a major concern as this can result in treatment failures and predispose development of drug resistance.

In an attempt to enhance the intestinal absorption of Hf.HCl, a micronized formulation was developed to increase the surface area available for drug dissolution.[6] This improved the bioavailability of Hf.HCl by approximately 2-fold, however, the absorption of Hf remained highly variable. It is likely that the clinical usage of the micronized formulation would probably be subjected to the same limitations as the original unmicronized tablet formulation.

The absorption of Hf.HCl can be substantially enhanced when co-administered with a fatty meal.[5,7] The improved absorption of Hf.HCl when administered with food was most likely due to increased drug solubilization and dissolution in the presence of bile constituents and lipid digestion products in the post-prandial intestine.[5] However, the practice of co-administering food with Hf.HCl to improve oral bioavailability is now contraindicated as excessively high and poorly controlled Hf plasma concentrations have been associated with prolongation of the QTc interval of the ECG and an increased risk of cardiac arrhythmias.[8,9]

The enhanced oral bioavailability observed after the co-administration of many poorly water soluble drugs with food is often attributed to the lipid component of ingested food. Lipids can reduce the inherent limitations of slow and incomplete dissolution and facilitate the formation of solubilized phases from which absorption may occur.[10,11] Furthermore, lipid-based formulations have been successfully employed to improve the oral bioavailability of many poorly water soluble drugs.[11,12] Therefore, the potential for lipidic formulations to provide the means for improved and consistent oral delivery of Hf was investigated in this chapter.

In recent years, the use of self-emulsifying drug delivery systems (SEDDS) and self-microemulsifying drug delivery systems (SMEDDS) to improve the oral bioavailability of lipophilic drugs has received much attention.[13-21] SEDDS and SMEDDS are isotropic mixtures of oil(s), surfactant(s) and drug which form fine oil-in-water emulsions or microemulsions, respectively, when exposed to aqueous media under conditions of gentle agitation.[15] Whilst SEDDS typically produce droplets with particle size between 100 and 300 nm, SMEDDS form optically clear and thermodynamically stable dispersions with droplet size of less than 100 nm (see section 1.4.2.1.). The spontaneous formation of an emulsion in the gastrointestinal tract is advantageous as this presents the drug in a solubilized and highly dispersed form, and the small droplet size provides a large interfacial surface area for drug absorption.[22] Therefore, for lipophilic drugs that display dissolution rate-limited absorption, SEDDS and SMEDDS may offer an improvement in both the rate and extent of absorption and yield more reproducible plasma concentration profiles. Furthermore, these mixtures may be formulated into soft or hard gelatin capsules to produce precise and convenient unit dose forms.

2.2 OBJECTIVES

The primary aim of this study was to explore the potential for lipid-based formulations to improve the absolute oral bioavailability of Hf. Lipid-based SEDDS and SMEDDS were developed for the oral delivery of Hf as these delivery systems have the advantage of presenting Hf in a solubilized form, thereby avoiding the rate-limiting dissolution step. To maximize drug loading in a unit dose lipid formulation, Hf free base was utilized as its solubility in triglyceride lipidic solvents was considerably higher than Hf.HCl. The solubility of Hf.HCl in peanut oil was ~ 1 mg/mL whereas the amorphous form of Hf free base was miscible at concentrations in excess of 200 mg/mL.[23]

Prototype SEDDS and SMEDDS comprising a triglyceride, a mono-/diglyceride, a non-ionic surfactant, a hydrophilic phase and Hf in varying proportions were prepared. The efficacy of self-emulsification of the formulations was assessed visually and by particle size analysis. Three optimized formulations were chosen for bioavailability assessment in fasted beagle dogs. These included a medium-chain triglyceride (MCT) SMEDDS, a MCT SEDDS and a long-chain triglyceride (LCT) SMEDDS. The study was specifically designed to enable the assessment of the effect of glyceride chain length (MCT SMEDDS compared to LCT SMEDDS) and the degree of dispersion or particle size (SEDDS compared to SMEDDS) on the bioavailability of Hf.

2.3 MATERIALS AND METHODS

2.3.1 Materials

Crystalline Hf free base (SmithKline Beecham Pharmaceuticals, Mysore, India), Captex 355 and Capmul MCM (Abitec Corporation, Janesville, WI, USA), soybean oil B.P. (R.P. Scherer, Victoria, Australia), Maisine 35-1 (Gattefossé s.a., Saint-Priest, France), Cremophor EL (BASF, Ludwigshafen, Germany), and absolute ethanol (CSR,

NSW, Australia) were used as received. Air-filled oblong soft gelatin capsules (1 mL capacity) were supplied by R.P. Scherer (Victoria, Australia). Dilute hydrochloric acid (HCl, 0.1 M) was prepared by diluting concentrated HCl (36% w/w, Ajax Chemicals, NSW, Australia) in water. Intralipid® 10% (Kabi Pharmacia, Sweden), *N,N*-dimethylformamide (AR grade, Ajax Chemicals, NSW, Australia) and triacetin (Henkel, Dusseldorf, Germany) were used to prepare the intravenous formulation. For the plasma assay, *N*-desbutylhalofantrine HCl (major metabolite of Hf, Hfm) and the internal standard (2,4-dichloro-6-trifluoro-methyl-9-{1-[2-(dibutyl-amino)ethyl]}-phenanthrene-methanol HCl) were obtained from SmithKline Beecham Pharmaceuticals (King of Prussia, PA, USA). Acetonitrile (Mallinckrodt, Paris, KY, USA) and *tert*-butyl methyl ether (Fluka, Switzerland) were of HPLC grade. Analytical grade glacial acetic acid and sodium dodecyl sulphate were from BDH Laboratory Supplies (Poole, UK). Water was obtained from a Milli-Q (Millipore, Milford, MA, USA) water purification system.

2.3.2 Solubility determinations

The solubility of crystalline Hf base in each component of the lipid-based formulation and blank formulations was determined. An excess of Hf base (approximately 100 mg) was placed in screw-capped conical glass tubes with 1 g of vehicle. The tubes were shaken at 30°C in a thermostated water bath for 72 h and then centrifuged at 1200 *g* for 10 min to separate undissolved drug. A sample (20-30 mg) from the supernatant was accurately weighed into 20 mL volumetric flasks, diluted with acetonitrile and quantified by HPLC (section 2.3.6.2.4). Sampling was repeated at 24 h intervals until sequential solubility values varied by less than 5%, indicating equilibrium solubility. Reported values are averages of duplicate samples, with individual values varying by less than 5%.

2.3.3 Preparation of SEDDS and SMEDDS formulations

Approximately 5 to 8 g of each prototype formulation was prepared by firstly weighing Hf base into 12 mL teflon-lined screw-capped conical glass tubes, followed by addition of various proportions of glycerides and surfactants. The components were mixed by gentle vortexing, and placed in a 50°C water bath, until all drug had dissolved. Once cooled to ambient temperature, absolute ethanol was added, and the mixture mixed again to ensure uniformity. The formulation was then equilibrated at ambient temperature for at least 48 hours, and examined for signs of turbidity or phase separation prior to self-emulsification and particle size studies.

2.3.4 Assessment of the efficiency of self-emulsification

A standard USP dissolution apparatus (Erweka, Germany) was used to assess the efficiency of self-emulsification. Prototype formulations (1 mL) were added dropwise to 200 mL of medium (either 0.1 M HCl or purified water at 37°C in a 1 L round bottomed glass vessel). Gentle agitation was provided by a standard stainless steel dissolution paddle mounted immediately below the meniscus of the aqueous medium and rotating at 60 rpm. The temperature of the medium was held at 37°C by placing the vessel in a thermostated water bath. The efficiency of self-emulsification was assessed visually and/or by particle sizing after 10 min of stirring.

2.3.4.1 Visual assessment of self-emulsification

The self-emulsification efficiency of each prototype formulation was initially assessed visually according to the rate of emulsification and the final appearance of the emulsion. The following grading system was employed for the visual assessment of the *in vitro* performance of the formulations: *A* denotes a rapidly forming (< 1 min)

microemulsion which was optically clear or transparent with slightly bluish appearance; *B* denotes a rapidly forming, slightly less clear emulsion which has a bluish white appearance; *C* denotes a bright white emulsion (similar in appearance to milk) that formed within 2 min; *D* denotes a dull, greyish white emulsion with a slightly oily appearance that was slow to emulsify (> 2 min); and *E* denotes a formulation which exhibited either poor or minimal emulsification with large oil droplets floating on the surface of the medium. The dispersability of the formulation, and the formation of intermediate structures upon addition to the aqueous medium were also noted.

2.3.4.2 Particle size determination

The particle size distribution of the emulsions formed by the SEDDS/SMEDDS formulations was determined by photon correlation spectroscopy (PCS), which involves the measurement of light scattering due to the Brownian motion of the colloidal particles.[24] A Malvern Zetasizer 3000 photon correlation spectrometer (Malvern Instruments, Malvern, UK) was used to analyse the samples as the instrument measures particle diameters between 1 to 5000 nm, which encompasses the expected diameters of the emulsion droplets. Light scattering was monitored at a 90° angle and the sample compartment was thermostated at 25°C. Certified monodisperse polystyrene microspheres (Nanosphere®, Duke Scientific Corporation, Palo Alto, CA, USA) with a diameter of 220 ± 6 nm were used as a standard to ensure the performance of the instrument. Approximately 3 mL of emulsion (dispersed phase) was placed in a polystyrene sample cuvette and either measured directly, or diluted with an appropriate medium (0.1 M HCl or purified water which had been filtered through a 0.22 μm filter) to produce the recommended scattering intensity of 50 to 200 Kcps. Measurements were performed in triplicate. The data was processed by standard analysis, a high resolution

method which employs non-negative least squares fitting, and reported as mean particle size and standard deviation. Formulations producing finely dispersed microemulsions (< 100 nm) were classified as SMEDDS, whilst those producing less finely dispersed emulsions (100-300 nm) were labelled as SEDDS.

2.3.5 Preparation of Hf formulations for oral bioavailability assessment

2.3.5.1 Preparation of the oral lipid formulations

On the day prior to the study, each of the three optimized oral lipid formulations (1 g) was accurately filled into air-filled soft gelatin capsules using a 1 mL syringe with an 18 G hypodermic needle, after which the resulting perforation was sealed with molten gelatin. The composition of the optimized lipid formulations are shown in Table 2.1, each capsule was filled to contain 50 mg Hf base.

The short term chemical stability of Hf in the lipid formulations at 30°C was assessed by analysing the formulations immediately, one, two and four weeks after preparation. Approximately 20-30 μg of the formulation was accurately weighed into a 25 mL volumetric flask and made up to volume with acetonitrile (ACN). This solution was then sonicated in a sonic water bath (Bransonic 220, Bransonic, Shelton, CN, USA) for 20 min until a clear solution was obtained before analysis by HPLC (section 2.3.6.2.4).

Table 2.1 Composition of the optimized lipid-based self-emulsifying formulations selected for bioavailability assessment in fasted beagles (the dose of Hf base administered per unit formulation was 50 mg)

Components	MCT SEDDS (% w/w)	MCT SMEDDS (% w/w)	LCT SMEDDS (% w/w)
Halofantrine base	5	5	5
Captex 355	46.7	33.3	--
Capmul MCM	23.3	16.7	--
Soybean oil	--	--	29
Maisine 35-1	--	--	29
Cremophor EL	15	35	30
Absolute ethanol	10	10	7

2.3.5.2 *Preparation of the intravenous formulation*

A suitable formulation for the intravenous (IV) administration of Hf was required for the determination of the absolute bioavailability of Hf in beagle dogs. To date, a commercial Hf formulation suitable for IV administration has not been available. However, Humberstone *et al.*[5] successfully incorporated Hf base into a commercial IV lipid emulsion (Intralipid® containing 10% soybean oil) by adapting the method of El-Sayed and Repta.[25] The IV lipid emulsion was well-tolerated by the dogs and the pharmacokinetics of Hf in beagle dogs after IV administration of this lipid emulsion have been shown to be essentially no different to the pharmacokinetics reported after the IV administration of a developmental co-solvent formulation comprising Hf.HCl (50 mg/mL) in dimethylacetamide/polyethylene glycol (40/60 v/v).[5,26] Although the co-solvent formulation was reported to be well-tolerated in dogs, mild to moderate adverse reactions at the infusion site were experienced by human patients.[27] Consequently, the IV lipid emulsion formulation prepared in this study was used exclusively for the IV administration of Hf in the studies reported in this thesis and the method for incorporation of Hf base into Intralipid® is described below.

Hf base (250 mg) was weighed into a calibrated polypropylene centrifuge tube and melted in a 100°C oven to the amorphous form. *N,N*-Dimethylformamide was added to a volume of 0.5 mL and the mixture sonicated in a sonic water bath for 20 min to form a uniformly mixed solution. Triacetin (0.5 mL) was then added and the contents mixed by vortexing for 2 min. The resulting solution (400 μL) was added to 20 mL of Intralipid® 10%, which was placed in a 50 mL polypropylene beaker immersed in an ice bath. The Hf solution was added slowly as 100 μL aliquots while the emulsion was sonicated using a Sonicator® XL 2020 Ultrasonic Liquid Processor (Misonix Incorp., Farmingdale, NY, USA) fitted with a tapped horn probe with end diameter of 12.7 mm

and running at 275-330 Watts (50%-60% of maximum output power) with an output frequency of 20 kHz. The emulsion was sonicated for 1 min, then allowed to cool for 2 min to avoid excessive heating of the emulsion. This procedure was repeated four times after the addition of each 100 μL aliquot and the emulsion was sonicated for two additional 1 min periods after all 400 μL of the Hf solution had been added. The emulsion was sterilized by passing through a 0.22 μm Millex-GV filter (Millipore, Bedford, MA, USA) under aseptic conditions. The final drug concentration of the resulting emulsion was expected to be approximately 5 mg/mL but was verified by HPLC prior to dosing. The physical and chemical stability of the IV emulsion was assessed before and after administration using PCS (to determine particle size distribution) and HPLC, respectively. Prior to HPLC analysis (section 2.3.6.2.4), 100 μL of emulsion was dissolved in 25 mL of ACN.

2.3.6 Absolute oral bioavailability study

2.3.6.1 Study design

The absolute oral bioavailability study was approved and performed in accordance with the guidelines of the Melbourne University Animal Ethics Committee. A randomized, four-treatment, four way crossover study with a washout period of 7 days between each treatment was conducted in four male beagles (0.5 to 1.0 year old, 15 to 18 kg). The dogs were fasted for 24 hours prior to dosage administration and fed after the 10 h post-dosing sample. Water was available *ad libitum* throughout the study period. The individual treatments were an IV formulation (2 mg/kg) administered as a 5 min infusion and the three optimized lipidic oral formulations (Table 2.1), each containing 50 mg Hf base as a unit dose and administered with 50 mL of water.

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Blood samples (2.5 mL) were obtained either from the cephalic vein via an indwelling catheter or by individual venipunctures at predose (-10 min), 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 24, 32, 48 and 72 h following oral dosing, and at predose (-10 min), 0 (end of infusion), 15 and 30 min, 1, 1.5, 2, 3, 4, 6, 8, 10, 24, 32, 48 and 72 h after IV administration. Individual tubes containing dipotassium EDTA were used to store the collected blood samples. Plasma was separated from whole blood by centrifugation and stored at -70°C prior to analysis. The plasma concentrations of Hf and the major metabolite, *N*-desbutylhalofantrine (Hfm), were determined using a validated HPLC assay (section 2.3.6.2).

2.3.6.2 Quantitation of Hf and Hfm in plasma

A simplified method involving a single liquid-liquid extraction procedure and liquid chromatography previously developed and validated in this laboratory by Humberstone *et al.*[28] was employed for the determination of the plasma concentrations of Hf and Hfm in plasma. The methods are described below.

2.3.6.2.1 Plasma extraction procedure

Plasma samples (0.5 mL) placed in 12 mL polypropylene centrifuge tubes were initially spiked with a 200 μL aliquot of internal standard (IS, 2 μg/mL), and mixed by brief vortexing. ACN (1 mL for samples or 0.8 mL for plasma standards) was then added, the sample vortexed at high speeds for 2 min to precipitate plasma proteins and centrifuged for 2 min at 500 *g* using a Beckman GS-6R refrigerated bench top centrifuge (Beckman Instruments, Palo Alto, CA, USA). To this, the extraction solvent, *tert*-butyl methyl ether (TBME, 8 mL), was added. The contents in the tubes were vortexed for 2 min and then centrifuged at 1200 *g* for 10 min. The upper organic phase (8 mL) was

carefully removed using glass pipettes and placed in new polypropylene tubes containing 100 μL of 0.005 M HCl (prepared in ACN). The contents were evaporated to dryness under a stream of high purity N_2 at 30°C using a N-EVAP 112 evaporator (Organomation, Berlin, MA, USA). The dried extracts were reconstituted with 200 μL ACN and 25 μL was injected onto the HPLC column.

2.3.6.2.2 Standards and calibration

Stock solutions of Hf, Hfm and the IS were prepared at a concentration of 100 μg/mL (concentrations were reported as mass of the free base form per mL) by dissolving the corresponding HCl salt in ACN. Using the stock solutions, standard solutions were prepared in ACN at concentrations in the range of 0.05 to 20 μg/mL for Hf and Hfm and 2 μg/mL for the IS. All stock and standard solutions were stored in glass at –20°C and the chemical stability verified by HPLC prior to usage. On each analysis day, six or seven standards covering the plasma concentration range of 20 to 8000 ng/mL were prepared in triplicate by spiking blank plasma (0.5 mL) with 200 μL of the appropriate standard solution. After following the extraction procedure described above, the standards were analysed at the start, middle and end of the HPLC run. Standard curves were obtained by plotting peak height ratios (Hf:IS or Hfm:IS) against concentration following extraction and analysis of spiked plasma samples as described above. Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by linear regression of the standard curve, and linearity confirmed by calculating the correlation statistics of the regression line. All concentrations were expressed as the free base equivalent.

2.3.6.2.3 Assay recovery, precision and accuracy

Recovery was calculated by comparing the peak heights recovered from spiked plasma samples with the peak heights of the injected standard solutions. Intra-assay precision and the accuracy of the assay were determined by replicate analyses (n = 5) of spiked plasma samples at three different concentrations (20, 200, 2000 ng/mL), and inter-assay precision determined by analysis of spiked plasma samples (n = 5) at these concentrations on three different days of analysis. The minimum quantifiable limit (MQL) was determined as the minimum concentration of Hf and Hfm at which the intra-assay coefficient of variation was less than 20%.

2.3.6.2.4 Chromatography

Chromatography was performed using a HPLC system consisting of a Waters 590 programmable pump, a Waters 712 WISP injector, a Waters 486 absorbance detector (Millipore, Milford, MA, USA) set at 257 nm, an Ultrasphere C_8 reversed-phase column (5 μm particle size, 25 cm x 4.6 mm i.d., Beckman Instruments, Fullerton, CA, USA) and a Brownlee® RP-8 guard column (Aquapore 7 μm, 3.2 x 15 mm, Applied Biosystems, San Jose, CA, USA). Data were analysed on a Shimadzu C-R6A integrator (Shimadzu Corp., Japan). The mobile phase consisted of 72:25 (v/v) ACN:water with 0.2% (w/v) sodium dodecyl sulphate and 0.2% (v/v) glacial acetic acid, and was pumped isocratically at a flow rate of 1.5 mL/min. A 25 μL volume of sample was injected onto the column and all samples were separated at room temperature.

2.3.6.3 Pharmacokinetic analysis

From inspection of the plasma concentration versus time profiles for Hf and Hfm, the maximum plasma concentration (C_{max}) and the corresponding time (T_{max}) were

noted. The area under the plasma concentration-time curve ($AUC^{0-\infty}$) was calculated using the linear trapezoidal rule to the last sampling time (72 h), followed by addition of the extrapolated area ($AUC^{72h-\infty}$) calculated by dividing the concentration at 72 h by the terminal elimination rate constant. Hf plasma clearance (CL) was calculated by dividing IV dose with the corresponding AUC. The absolute bioavailability of Hf was calculated as a percentage of the AUC of an oral dose to the AUC of an IV dose normalized to the equivalent oral dose. One way analysis of variance and Student-Newman-Keuls multiple comparison test were performed using a standard statistical package (SigmaStat, Jandel Scientific, CA, USA) to determine the significance of the differences observed between formulations. The data were considered statistically significant at $p < 0.05$.

2.4 RESULTS AND DISCUSSION

The potential for dispersed lipid-based formulations to improve the oral bioavailability of Hf was investigated in this study. SEDDS and SMEDDS were chosen for the delivery of Hf as these formulations have previously been reported to improve the rate and extent of absorption of poorly water soluble drugs by presenting the drug in a highly dispersed form. The formulation and assessment of prototype self-emulsifying Hf formulations are discussed below (sections 2.4.1 and 2.4.2). Hf base, which has greater solubility in triglyceride lipids compared with the commercially available hydrochloride salt (Hf.HCl) was used for the preparation of the lipidic formulations. Optimized formulations selected for bioavailability assessment included a MCT containing SEDDS and two SMEDDS formulations, one containing a MCT, and the other containing a LCT. The study was designed to enable the effect of lipid chain length and degree of dispersion on the absorption of Hf to be examined. The pharmacokinetic parameters of Hf and its desbutyl metabolite (Hfm) after oral

administration of the lipid formulations to fasted beagles are presented and discussed in section 2.4.3.

2.4.1 Formulation of the SEDDS and SMEDDS

Pharmaceutically acceptable excipients were used to formulate the self-dispersing lipid-based formulations. The formulations were isotropic mixtures of glycerides (mono-, di- and triglycerides), a non-ionic surfactant and a co-solvent. The formulations could be divided into two groups according to the fatty acid chain length of the lipids. Medium-chain glycerides employed in this study were Captex 355 (C_8/C_{10} triglyceride) and Capmul MCM (C_8/C_{10} mono-/diglyceride, HLB = 5.5-6.0), and the long-chain glycerides examined were soybean oil (C_{18} triglyceride comprising predominantly linoleic ($C_{18:2}$) and oleic ($C_{18:1}$) fatty acids) and Maisine 35-1 (C_{18} mono-/diglyceride with oleic and linoleic fatty acids, HLB = 4). When formulating the oil-in-water systems using either the medium- or long-chain glycerides, the mono-/diglyceride essentially served as a low HLB co-surfactant. For all formulations, a non-ionic surfactant with high HLB, Cremophor EL (polyoxyethylated castor oil, HLB = 13.5), and a co-solvent (ethanol) were incorporated to facilitate the formation of stable water-in-oil (micro)emulsions.

Drug loading per unit formulation was a critical design factor and was dependent on the solubility of Hf in the various formulation components and the maximal volume reasonably encapsulated in either a hard or soft gelatin capsule. For the medium-chain glyceride formulations based on Captex 355/Capmul MCM combinations, the solubility of Hf base could be enhanced by increasing the proportion of Captex 355 as the solubility of Hf base was almost 2-fold higher in Captex 355 than in Capmul MCM (Table 2.2). However, this strategy led to less efficient emulsification due to an overall

Table 2.2 Equilibrium solubility of crystalline halofantrine base (Hf) in various vehicles and formulations at 30°C

Vehicle or formulation	Solubility of Hf[a] (mg/g)
Captex 355	82.2
Capmul MCM	47.7
Soybean oil	46.7
Maisine 35-1	40.9
Cremophor EL/Captex 355 (1:1 w/w)	20.0
Absolute ethanol	31.7
MCT SEDDS *(Cremophor EL 15%, Ethanol 10%,* *Captex 355/Capmul MCM (2:1 w/w) 75%)*	74.3
MCT SMEDDS *(Cremophor EL 35%, Ethanol 10%,* *Captex 355/Capmul MCM (2:1 w/w) 55%)*	68.0
LCT SMEDDS *(Cremophor EL 30%, Ethanol 7%,* *Soybean oil/Maisine 35-1 (1:1 w/w) 63%)*	49.3

[a] Data represent the average of duplicate samples with individual values varying by less than 5%.

reduction in the Capmul content of the formulation. A 2:1 (w/w) ratio of Captex 355 to Capmul MCM afforded a good balance between drug loading and efficient emulsification. For the long-chain glycerides, the solubility of Hf base in soybean oil and Maisine 35-1 were approximately the same (Table 2.2), therefore, there was no benefit in increasing the triglyceride concentration. However, in absolute terms Hf base was less soluble in long-chain compared with medium-chain glycerides thereby limiting the unit dose of Hf base in long-chain lipid formulations. Various miscible co-solvents were investigated as a means of increasing Hf solubility. The addition of 10% (w/w) ethanol in the formulations increased Hf solubility by approximately 20% as well as increasing the rate of dispersion/emulsification. The solubility studies indicated that a drug loading of 5% (w/w) Hf base could be incorporated into both the long- and medium-chain formulations which had been selected for bioavailability assessment (Table 2.2).

Temperature cycling studies were performed (daily 30°C/4°C cycles for 7 days) on the three optimized formulations to ensure maintenance of a single phase system in terms of drug solubility and the mutual miscibility of all formulation components. Interestingly, in spite of exceeding the solubility of Hf (in all three formulations) at the lower temperature, Hf did not precipitate out of solution. However, the long term stability of the super-saturated solutions at the lower temperature is not known and was not investigated. The various components in the formulations also remained miscible throughout the temperature cycling study.

2.4.2 *In vitro* assessment of the SEDDS and SMEDDS

A number of methods have been proposed for the evaluation of self-emulsifying efficiency of the lipid formulations, these include visual assessment, estimating particle

size distribution, measurement of emulsion droplet polarity and surface tension, and the use of low frequency dielectric spectroscopy.[20,22,29-31] In this study, the efficiency of self-emulsification of a series of medium- and long-chain lipid formulations was evaluated by visual assessment and analysis of particle size distribution. Whilst visual assessment provides important information about the self-emulsification process and of the resulting dispersion system, measurement of emulsion droplet size provides a more accurate indication of the efficiency of self-emulsification. The self-emulsification studies were conducted in both an acidic medium (0.1 M HCl) and in purified water to simulate the range of conditions likely to be encountered in the gastrointestinal tract.

The visual assessment and particle size determination of the emulsions formed by the medium-chain lipid formulations are presented in Table 2.3. Pseudo-ternary phase diagrams were also constructed and are shown in Figure 2.1. Although the formulations consisted of five different components, there were only three variables and each was represented on a separate axis of the ternary diagram. As the ratio of Captex 355 to Capmul MCM and the drug concentration were kept constant at 2:1 and 5% (w/w), respectively, they were considered a single variable and the other two variables were the non-ionic surfactant (Cremophor EL) and the co-solvent, ethanol.

The emulsification characteristics differed slightly between the two different dissolution media, with better visual gradings and smaller particle size values resulting when the formulations were dispersed in the acidic medium. The nature of the emulsion formed in aqueous media also depended on the concentration of Cremophor EL in the formulation. When dispersed in aqueous media, increasing the concentration of Cremophor EL generally led to an improvement in the clarity of the emulsions formed upon dissolution and a corresponding decrease in particle size data (Table 2.3). For the

Table 2.3 Visual assessment and particle size analysis (mean ± SD, n = 3) of emulsions formed after dispersion of 1 g of medium-chain lipid formulations in 200 mL of either 0.1 M HCl or purified water (gentle agitation was provided by a dissolution paddle rotating at 60 rpm)

Composition[a] (% w/w)				Visual grading[b]		Mean particle size (nm)	
Capt:Capm 2:1 (w/w)	Crem EL	Ethanol	Hf base	0.1 M HCl	Water	0.1 M HCl	Water
80	10	5	5	C	C	145.3 ± 0.6	133.0 ± 0.6
75	15	5	5	B	B	77.2 ± 0.1	92.9 ± 0.8
70	20	5	5	A	B	54.3 ± 0.7	98.2 ± 0.9
65	25	5	5	A	B	42.3 ± 0.9	93.4 ± 1.9
60	30	5	5	A	A	36.8 ± 0.5	41.9 ± 0.7
55	35	5	5	A	A	32.0 ± 0.5	27.3 ± 0.7
50	40	5	5	A[c]	A[c]	27.6 ± 0.6	22.0 ± 1.4
75	10	10	5	C	C	136.7 ± 0.9	161.5 ± 1.4
70	15	10	5	B	C	71.4 ± 1.2	118.5 ± 4.7
65	20	10	5	B	D	58.7 ± 0.5	201.9 ± 2.7
60	25	10	5	B	D	46.7 ± 0.2	251.4 ± 1.2
55	30	10	5	A	C	36.3 ± 0.2	141.4 ± 2.0
50	35	10	5	A	A	31.3 ± 0.5	51.5 ± 0.7
45	40	10	5	A[c]	A[c]	27.2 ± 0.2	27.9 ± 0.5
70	10	15	5	C	C	116.5 ± 0.6	127.7 ± 0.1
65	15	15	5	B	C	69.0 ± 0.9	151.5 ± 0.3
60	20	15	5	B	D	79.7 ± 0.4	284.3 ± 3.9
55	25	15	5	C	D	365.9 ± 59.3	278.6 ± 3.0
50	30	15	5	B	D	163.0 ± 1.6	266.2 ± 1.3
45	35	15	5	A[c]	C[c]	93.3 ± 5.6	184.3 ± 0.8

[a] Capt, Captex 355; Capm, Capmul MCM; Crem EL, Cremophor EL.

[b] **A** = rapidly forming (< 1 min), clear or slightly bluish microemulsion; **B** = rapidly forming, slightly less clear, bluish white emulsion; **C** = bright white emulsion similar to milk, formed within 2 min; **D** = dull, greyish white emulsion with a slightly oily appearance, slow to emulsify (> 2 min).

[c] Transparent, gel-like structures formed prior to dispersing completely (within 1 min).

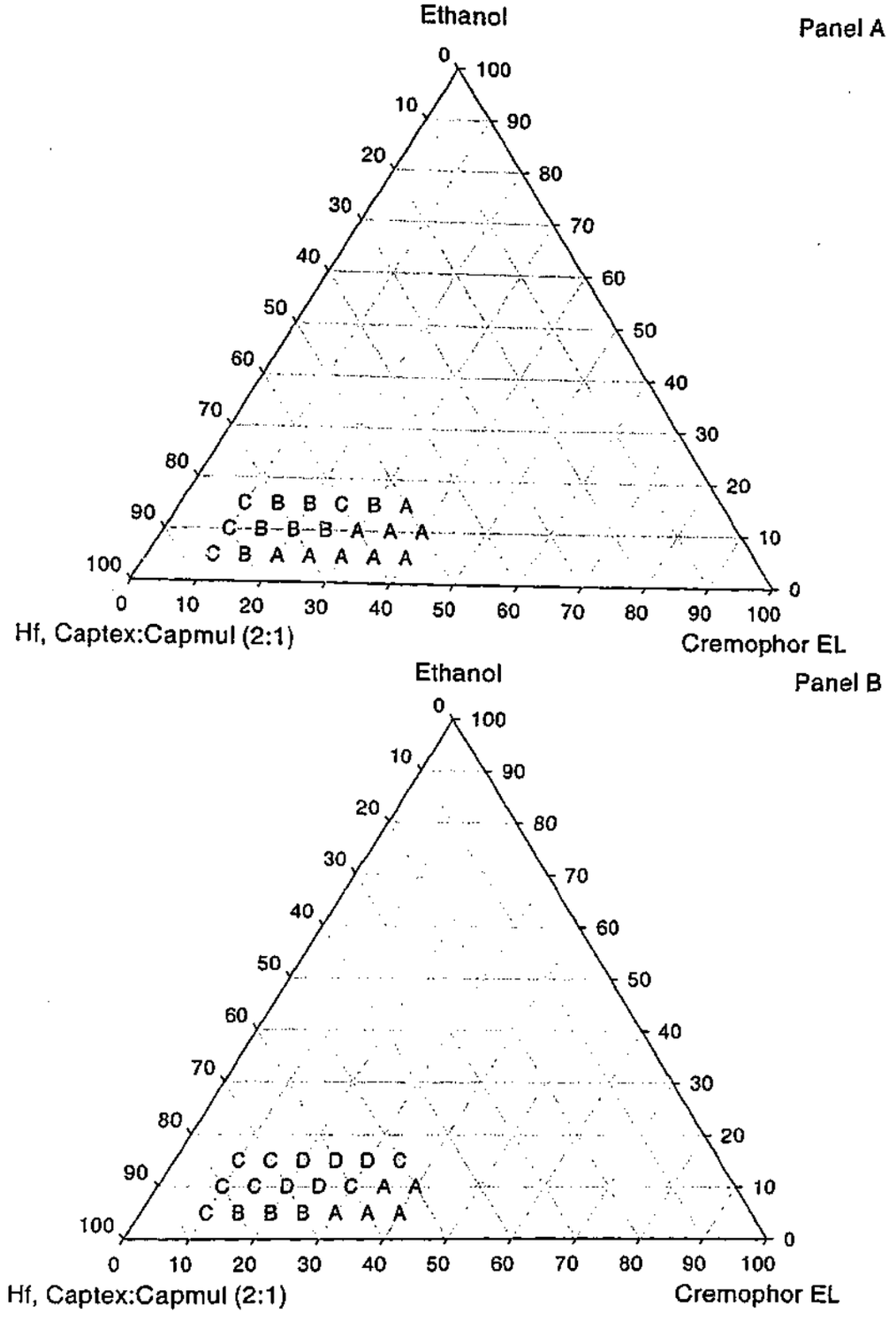

Figure 2.1 Pseudo-ternary phase diagrams of medium-chain lipid formulations containing Hf base (5% w/w) dispersed in either 0.1 M HCl (Panel A) or purified water (Panel B). Emulsions formed were assessed visually: **A** = rapidly forming (< 1 min), clear or slightly bluish microemulsion; **B** = rapidly forming, slightly less clear, bluish white emulsion; **C** = bright white emulsion similar to milk, formed within 2 min; **D** = dull, greyish white emulsion with slightly oily appearance, slow to emulsify (> 2 min).

medium-chain formulations, microemulsions formed when the concentration of Cremophor EL were above 30% (w/w). However, further increases in the concentration of Cremophor EL typically resulted in the formation of transparent, gel-like intermediate structures upon the addition of the formulation to aqueous media, and self-emulsification was often retarded, requiring at least an additional minute to fully disperse. The formation of viscous liquid crystalline phases at high surfactant concentrations has been reported by Pouton,[22] and is likely to be due to the high viscosity at the interface between droplet and water.

The mechanism of self-emulsification is poorly understood. However, Pouton[32] has proposed that for formulations containing a hydrophilic co-solvent, the mechanism of self-emulsification may involve diffusion of the co-solvent away from the bulk oil and into the aqueous phase, resulting in 'diffusion and stranding' which may be the driving force for emulsification. While mixing with excess water, the bulk of the hydrophilic surfactants may be transferred to the aqueous phase to form a micellar solution. The fate of the drug and lipid components after mixing with water could be solubilization in a swollen micellar solution, dispersion as a fine emulsion, or isolation and precipitation.

In comparison to the medium-chain glyceride formulations, the long-chain glyceride formulations tended to emulsify better in water than acidic media (Table 2.4), and they required higher concentrations of Cremophor EL (at least 25% w/w) to provide efficient emulsification. As observed with the medium-chain glyceride formulations, the use of Cremophor EL above 30% w/w resulted in the formation of transparent, gel-like structures upon the addition of the formulations to aqueous media. However, unlike the gel-like structures observed with the medium-chain glyceride formulations, these

Table 2.4 Visual assessment and particle size analysis (mean ± SD, n = 3) of emulsions formed after dispersion of 1 g of long-chain lipid formulations in 200 mL of either 0.1 M HCl or purified water (gentle agitation was provided by a dissolution paddle rotating at 60 rpm)

Composition[a] (% w/w)				Visual grading[b]		Mean particle size (nm)	
SBO:Mais 1:1 (w/w)	Crem EL	Ethanol	Hf base	0.1 M HCl	Water	0.1 M HCl	Water
80	10	5	5	E	E	n/m	n/m
75	15	5	5	E	E	n/m	n/m
70	20	5	5	E	E	n/m	n/m
65	25	5	5	E	C	n/m	229.7 ± 1.4
60	30	5	5	B	B	90.4 ± 0.7	78.7 ± 0.9
55	35	5	5	B[c]	A[c]	59.2 ± 0.4	38.1 ± 0.4
50	40	5	5	A[c]	A[c]	36.7 ± 0.6	31.9 ± 0.4
68	20	7	5	E	E	n/m	n/m
63	25	7	5	C	C	108.9 ± 0.7	123.7 ± 0.6
58	30	7	5	B	A	67.9 ± 0.8	49.6 ± 0.2
53	35	7	5	B[c]	B[c]	49.5 ± 0.7	42.6 ± 0.7
48	40	7	5	A[c]	A[c]	47.7 ± 0.6	54.9 ± 3.5
65	20	10	5	E	E	n/m	n/m
60	25	10	5	C	C	194.4 ± 0.3	161.5 ± 0.7
55	30	10	5	C	B	150.9 ± 1.8	76.5 ± 0.5
50	35	10	5	C[c]	B[c]	147.0 ± 3.4	87.8 ± 1.4

[a] SBO, soybean oil; Mais, Maisine 35-1; Crem EL, Cremophor EL.

[b] **A** = rapidly forming (< 1 min) clear or slightly bluish microemulsion; **B** = rapidly forming, slightly less clear bluish white emulsion; **C** = bright white emulsion similar to milk, formed within 2 min; **D** = dull, greyish white emulsion with a slightly oily appearance, slow to emulsify (> 2 min); **E** = poor or minimal emulsification with large oil droplets present on the surface.

[c] Transparent, gel-like intermediate structures formed prior to dispersing completely (requiring 5-15 min).

n/m Particle size measurements of the dispersion formed could not be accurately measured due to the presence of unemulsified oil.

structures required approximately 5 to 15 min to completely disperse and this was of concern in terms of affording efficient and rapid drug release *in vivo*. Therefore, to achieve efficient dispersion of the long-chain glyceride formulations, the concentration of Cremophor EL was limited to between 25 and 30% w/w.

The visual gradings and the corresponding mean particle size of a number of medium-chain glyceride and long-chain glyceride (micro)emulsions formed on dispersion are shown in Tables 2.3 and 2.4, respectively, with most particle size distributions being unimodal. In general, there was a good correlation between visual observations and particle size measurements. The SMEDDS formulations formed microemulsions (i.e. visual grading of *A*) where the particle size of the droplets were generally less than 50 nm, and the SEDDS usually formed emulsions which were graded *C* and these had a mean droplet size in the range of 100 to 200 nm. In some cases, it was not possible to measure the particle size of the emulsions formed. These emulsions were usually graded *D* or *E* and typically contained unemulsified oil which resulted in inaccurate particle size measurements. The data tabulated in Tables 2.3 and 2.4 indicate that increasing the concentration of Cremophor EL, while maintaining the ethanol concentration constant, usually resulted in improved emulsion clarity and a smaller particle size demonstrating the crucial role of Cremophor EL in determining the functionality of the resulting emulsion.

Based on the self-emulsification properties and particle size data, an optimized medium-chain SMEDDS, long-chain SMEDDS and medium-chain SEDDS (Table 2.1) were selected for *in vivo* evaluation in fasted beagle dogs. Both the SMEDDS formulations had similar emulsification properties and particle size range but contained different lipids to enable the effect of the lipid chain length on the bioavailability of Hf to be investigated. The medium-chain SEDDS, which had poorer self-emulsification

properties, was also included in the study to probe the importance of degree of dispersion on the absorption of Hf.

2.4.3 *In vivo* assessment of the lipid-based self-emulsifying formulations

2.4.3.1 Validation of plasma assay

Using the chromatography conditions described above (section 2.3.6.2.4), a run time of 12 min was obtained and the retention times for Hfm, Hf and IS were 5.4, 7.8 and 10.2 min, respectively. These conditions also afforded good separation of Hfm, Hf and IS peaks from underlying peaks associated with plasma. The minimum quantifiable limit was 20 ng/mL for both Hf and Hfm and standard curves were linear in the spiked plasma concentration range of 20 to 8000 ng/mL ($R^2 > 0.99$). Presented in Figure 2.2 is a representative calibration curve for Hf. The recovery, precision and accuracy data are shown in Table 2.5. The average recoveries of Hf and Hfm from spiked plasma samples were greater than 80% across the concentration range and the mean (± SD, n = 15) recovery of IS was 84.4 ± 2.9%. The assay method employed for the determination of plasma concentrations of Hf and Hfm in dog plasma was also precise and accurate as indicated by intra- and inter-assay coefficient of variation (CV) values of less than 15% across the concentration range and high accuracy where deviation from target concentrations were less than 10%.

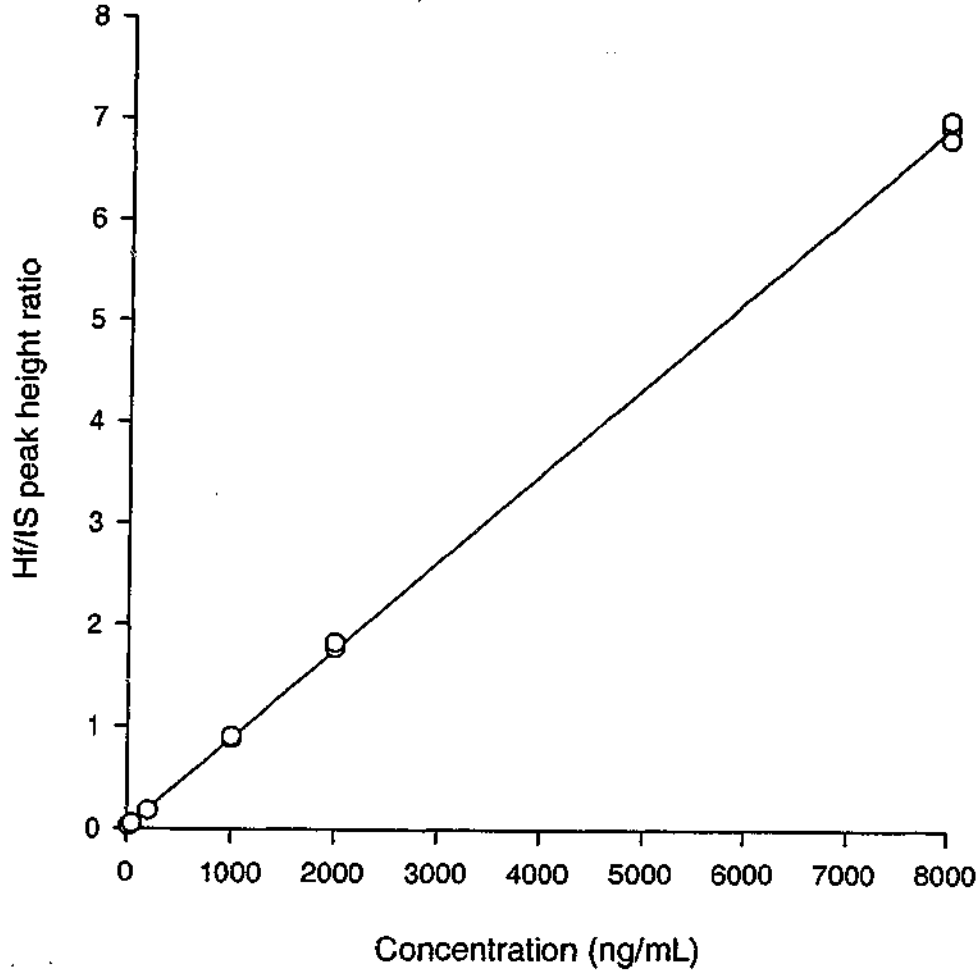

Figure 2.2 A representative calibration curve of halofantrine in spiked dog plasma. Symbols represent measured values (n = 3) and line was fitted by linear regression. The linear regression from these data was: Peak height ratio = $0.020 + 0.00087 \cdot$ Concentration, $R^2 = 0.999$.

Table 2.5 The recovery, intra- and inter-assay precision, and accuracy of the assay procedure for halofantrine (Hf) and *N*-desbutylhalofantrine (Hfm) from spiked plasma samples

	Concentration (ng/mL)	% Recovery (mean ± SD)	Precision (CV, %)		Accuracy (%)
			Intra-assay	Inter-assay	
Hf	20	104.5 ± 16.9	9.5	11.5	102.9
	200	101.5 ± 5.2	4.6	3.9	100.3
	2000	97.6 ± 6.6	1.8	1.5	98.9
Hfm	20	104.5 ± 23.4	13.8	9.99	96.8
	200	88.3 ± 10.1	12.4	8.6	91.9
	2000	81.2 ± 5.5	6.7	7.3	100.5

2.4.3.2 *Absolute oral bioavailability study*

After consideration of the temperature-dependent solubility of Hf base, the miscibility of individual components within the formulation, self-emulsification properties, and assessment of partial ternary phase diagrams and associated particle size data, optimized medium-chain and long-chain glyceride formulations were chosen for an absolute oral bioavailability study in fasted beagles. The chosen formulations are described in Table 2.1 and denoted as MCT SEDDS, MCT SMEDDS and LCT SMEDDS. For example, the MCT and LCT SMEDDS had similar *in vitro* emulsification properties and particle size profiles, but were formulated with lipids of different fatty acid chain lengths. In contrast, the MCT SEDDS and MCT SMEDDS formulations were based on the same lipid class but produced different particle size when evaluated *in vitro*.

Prior to commencing the absolute oral bioavailability study, the short term stability of Hf in the lipid formulations was assessed by HPLC as described in sections 2.3.5.1 and 2.3.6.2.4. No significant changes in the HPLC chromatograms were observed indicating that the formulations were stable at 30°C for at least four weeks after preparation. The area of the Hf peak (in the HPLC chromatograms) also accounted for greater than 98% of the total peak area.

Both the Intralipid® emulsion-based IV formulation and the oral self-emulsifying lipid formulations were well-tolerated by the dogs. The plasma concentration versus time profiles of Hf after IV administration of the Hf emulsion were highly reproducible, as shown in Figure 2.3. The mean (± SD, n = 4) plasma clearance was 301 ± 24 mL/h/kg, which was consistent with the value previously reported by Humberstone *et al.*[5] where the same IV emulsion was administered (at 1.6 mg/kg) to fasted beagle dogs.

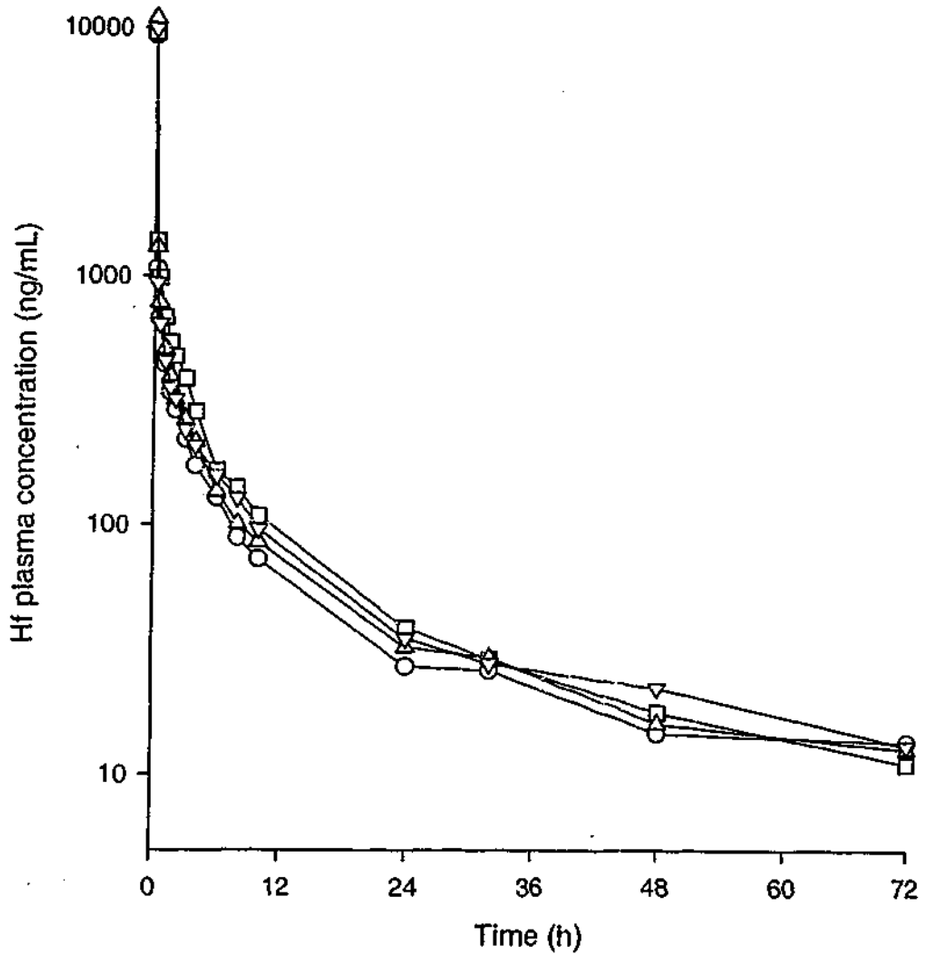

Figure 2.3 Individual halofantrine (Hf) plasma concentration versus time profiles after IV administration of an emulsion-based Hf formulation (2 mg/kg) to fasted male beagles (n = 4).

However, in comparison to the previous study[5] the apparent terminal elimination half-life ($T_{1/2}$) was slightly longer in this study at 34.8 ± 6.9 h.

The mean (± SD, n = 4) plasma concentration versus time profiles of Hf and Hfm following fasted oral administration of the three lipidic formulations are presented in Figure 2.4. The corresponding pharmacokinetic parameters for both Hf and Hfm are presented in Tables 2.6 and 2.7, respectively.

The C_{max} and AUC values of Hf following oral administration of the three lipid formulations (Table 2.6) were not significantly different ($p > 0.05$), however, the mean C_{max} and AUC values of the LCT SMEDDS tended to be higher than the medium-chain glyceride formulations. The degree of variability of C_{max} and AUC values was relatively high, and may be the result of one particular dog having consistently lower plasma profiles. The T_{max} values were statistically similar across the formulation groups, although the rate of absorption from the MCT SMEDDS formulation tended to be slightly lower than the other two oral formulations.

The mean absolute oral bioavailability of Hf free base for all three formulations ranged between 52 and 67%. When compared to previous data from this laboratory where the absolute oral bioavailability of the commercially available tablet formulation (containing 250 mg Hf.HCl) in fasted beagles was 8.6 ± 5.3%,[5] the current results represent an approximate six- to eight-fold improvement. The significant increase in the oral bioavailability of Hf is likely to be due to the ability of the lipid-based formulations to deliver Hf in a solubilized and highly dispersed manner, thereby overcoming the dissolution-rate limitations associated with the commercially available Hf.HCl tablet. The importance of solubilization on Hf absorption is further supported by a study conducted in fasted beagles where the absolute oral bioavailability of crystalline Hf

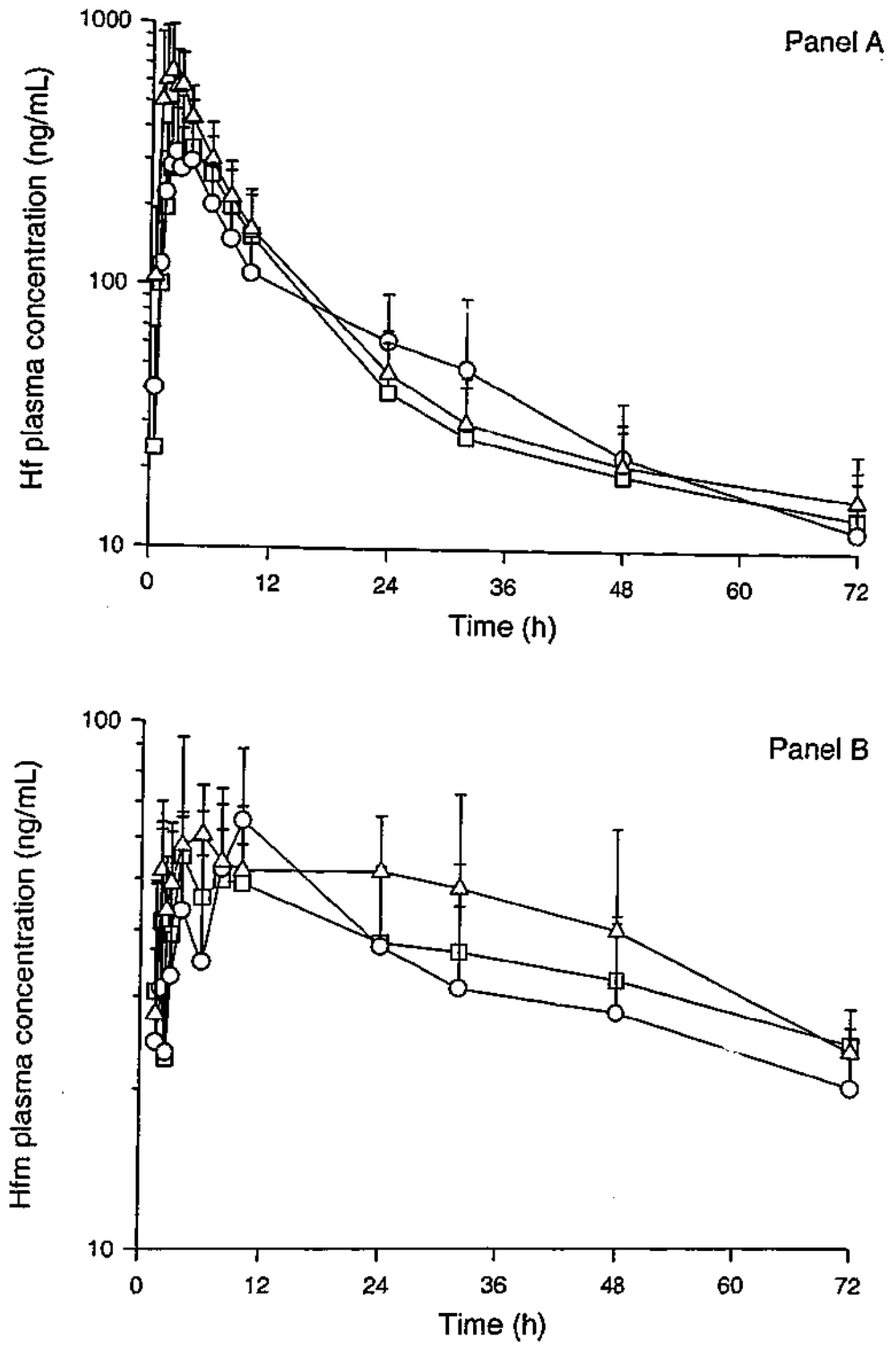

Figure 2.4 Mean (± SD, n = 4) plasma concentration versus time profiles of halofantrine (Hf, panel A) and *N*-desbutylhalofantrine (Hfm, panel B) following oral administration of MCT SEDDS (○), MCT SMEDDS (□) and LCT SMEDDS (Δ) formulations containing 50 mg Hf base to fasted beagles.

Table 2.6 Pharmacokinetic parameters (mean ± SD, n = 4) of halofantrine (Hf) following oral administration of a capsule containing 50 mg Hf base in either a MCT SEDDS, MCT SMEDDS or LCT SMEDDS to fasted beagles

Parameters	MCT SEDDS	MCT SMEDDS	LCT SMEDDS
C_{max} (ng/mL)	363 ± 156	374 ± 198	704 ± 308
T_{max} (h)	2.8 ± 0.8	4.2 ± 1.5	2.3 ± 0.5
$AUC^{0-\infty}$ (ng.h/mL)	5313 ± 1956	5426 ± 2481	6973 ± 2388
Absolute BA[a] (%)	51.6 ± 19.2	52.7 ± 24.0	67.3 ± 21.0

[a] Absolute oral bioavailability = $(AUC_{oral} / AUC_{IV}) \times (dose_{IV} / dose_{oral}) \times 100$, the dose normalized $AUC^{0-\infty}$ value of Hf after IV administration at a dose of 2 mg/kg was 10275 ± 929 ng.h/mL.

Table 2.7 Pharmacokinetic parameters (mean ± SD, n = 4) of *N*-desbutylhalofantrine (Hfm) following oral administration of a capsule containing 50 mg Hf base in either a MCT SEDDS, MCT SMEDDS or LCT SMEDDS; or after IV administration of a Hf formulation (2 mg/kg) to fasted beagles

Parameters	Oral Formulations			IV Hf
	MCT SEDDS	MCT SMEDDS	LCT SMEDDS	
C_{max} (ng/mL)	72.3 ± 16.8	67.9 ± 32.2	69.2 ± 11.5	53.3 ± 18.5
T_{max} (h)	6.5 ± 3.0	5.3 ± 2.2	3.5 ± 1.8	2.6 ± 1.1
AUC^{0-48h} (ng.h/mL)	1847 ± 494	1884 ± 731	2319 ± 448	1604 ± 559
Hfm/Hf AUC ratio[a]	0.46 ± 0.11	0.47 ± 0.17	0.44 ± 0.16	0.28 ± 0.07

[a] AUC^{0-48h} values for Hf and Hfm were used to calculate the Hfm/Hf AUC ratio.

base (233 mg) administered alone in a hard gelatin capsule was 2.9 ± 3.3% (unpublished data).

In addition to enhancing oral bioavailability by producing fine emulsion droplets, and thereby increasing surface area for improved drug dissolution, the surfactants and lipidic excipients present in the SEDDS and SMEDDS formulations may also increase drug absorption by other mechanisms. Solubilization of Hf may facilitate diffusion through the unstirred, aqueous layer, which acts as a barrier to hinder poorly water soluble drugs from reaching the absorption site.[10] Surfactants and medium-chain glycerides (e.g. sodium caprate) are also known to alter the intrinsic permeability of the intestinal membrane resulting in increased drug absorption via paracellular or transcellular routes.[33-38] More recently, commonly used surfactants such as Cremophor EL and Polysorbate 80 have been reported to enhance the intestinal absorption of drugs by inhibiting the P-glycoprotein efflux system, which functions to lower intracellular drug concentrations by active extrusion of drugs back into the intestinal lumen.[39-41] However, at present, it is not known if Hf is a substrate of P-glycoprotein.

Across the formulations studied, differences in particle size of the droplets in the emulsion formed did not significantly affect the rate or the extent of absorption of Hf, although this could be a function of the differences between the MCT SEDDS and MCT SMEDDS being insufficiently large to result in a difference in the absorption of Hf.

Whilst not statistically significant due to the small size of the study, there was a trend towards higher bioavailability from the LCT formulations compared with either of the MCT formulations. These results are consistent with the greater bioavailability of cyclosporine from LCT lipid vehicles compared to MCT.[42] The basis for this difference observed could be due to differences in intraluminal processing of MCT and LCT lipids where the release of bile salts and the formation of mixed micelles or different colloidal

phases associated with lipid digestion can influence drug absorption. However, it is not known to what extent such small volumes of lipid will induce these effects. Furthermore, as a result of the production of such fine dispersions, bioavailability enhancement is unlikely to be dependent on the digestion of the lipidic excipients.[13] Although significant intestinal lymphatic transport of Hf base has been reported after administration of LCT lipids in a rat model,[23,43] the ability of the small volume of LCT lipid employed in the current formulations to maximally stimulate intestinal lymphatic transport as a major contributor to the oral absorption of Hf is questionable. This contention is supported by the statistically similar plasma AUC values for Hfm across the three formulations. The consequence of significant intestinal lymphatic transport resulting from the LCT formulation would have been a reduction in hepatic first-pass metabolism (producing lower Hfm AUC values) which was not observed (Table 2.7).

There was no difference in the rate or extent of Hfm formation after oral administration of the lipid-based formulations, although the extent of Hfm formation tended to be lower (but not statistically significant, $p > 0.05$) after IV administration when normalized to the resulting Hf AUC values (i.e. Hfm/Hf AUC ratios). The lower Hfm/Hf AUC ratio observed after IV administration (Table 2.7) suggests that the liver may not be the only site of Hf metabolism. Recent *in vitro* studies have indicated that Hf is predominantly metabolized to Hfm by cytochrome P450 3A4 (CYP3A4).[44,45] As CYP3A4 is present in the liver and the intestinal epithelium,[46] and known to be a major determinant of oral bioavailability of important drugs such as cyclosporine[47] and midazolam,[48] non-hepatic metabolism of Hf via enterocyte-based CYP3A4 is consistent with the trend towards higher Hfm/Hf plasma AUC ratios observed after fasted oral administration compared to IV administration.

Previous Hf fed/fasted bioavailability data from this laboratory indicated that the extent of Hfm formation only increased 2-fold when Hf was administered post-prandially, in spite of a 12-fold increase in the extent of Hf absorption compared with fasted administration resulting in a low Hfm/Hf plasma AUC ratio of 0.08.[5] At this stage, it is not possible to rationalize the previously observed effect of food or currently described effect of the lipid-based formulations on the relative Hfm/Hf plasma AUC ratios. However, it does appear that enterocyte-based metabolism of Hf to Hfm may be modulated by food, and possibly lipidic formulation components. The basis for the differences in Hfm/Hf plasma AUC ratios may also include altered splanchnic blood flow, saturated metabolism of Hf, increased lymphatic transport of Hf and altered post-prandial plasma lipoprotein binding of Hf.

2.5 CONCLUSIONS

This study has identified a number of promising lipid-based self-emulsifying formulations for the oral delivery of Hf and potentially other lipophilic compounds. By using visual assessment and particle size analysis to evaluate the self-emulsification efficiency of the formulations, optimized SMEDDS and SEDDS formulations producing fine (micro)emulsions with mean droplet diameters of approximately 50 nm and between 100 to 200 nm, respectively, were selected for an absolute oral bioavailability study in fasted beagles. The lipid-based self-emulsifying formulations containing Hf free base afforded a six- to eight-fold improvement in absolute oral bioavailability compared to previous data of the standard Hf.HCl tablet formulation. The significant increase in oral bioavailability was likely to be a consequence of delivering the poorly water soluble drug in a solubilized and rapidly dispersed manner. Although not statistically significant, there was a trend towards higher bioavailability from the LCT formulation

compared with either of the MCT formulations. For drugs that undergo dissolution rate-limited absorption, significant bioavailability enhancement can be achieved through rational design of lipid-based formulations.

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CHAPTER 3

ASSESSMENT OF THE ROLE OF CYP3A4 IN THE *IN VIVO* CONVERSION OF HALOFANTRINE TO ITS EQUIPOTENT METABOLITE, *N*-DESBUTYLHALOFANTRINE

3.1 INTRODUCTION

After oral administration, *N*-desbutylhalofantrine (Hfm) has been identified as the major metabolite of halofantrine (Hf) (Figure 3.1).[1,2] This metabolite has been shown to have equivalent *in vitro* antimalarial activity to Hf against *Plasmodium falciparum*[3] and may therefore have therapeutic importance. Furthermore, Hfm appears to be largely devoid of the limiting cardiac side-effect profile observed after administration of Hf hydrochloride.[4,5] An interesting feature of Hfm plasma profiles is that although food increased the oral bioavailability of Hf 3-fold in humans[2] and 12-fold in beagles,[6] there was a significantly lower proportion of Hf metabolized to Hfm when administered post-prandially. For example, the mean Hfm/Hf plasma $AUC^{0-\infty}$ ratios after fasted and fed administration in humans were 2.25 and 0.95,[2] and Hfm/Hf plasma AUC^{0-72h} ratios after fasted and fed administration in beagles were 0.54 and 0.08.[6] The mechanism by which this decrease in post-prandial metabolism occurs is currently unknown.

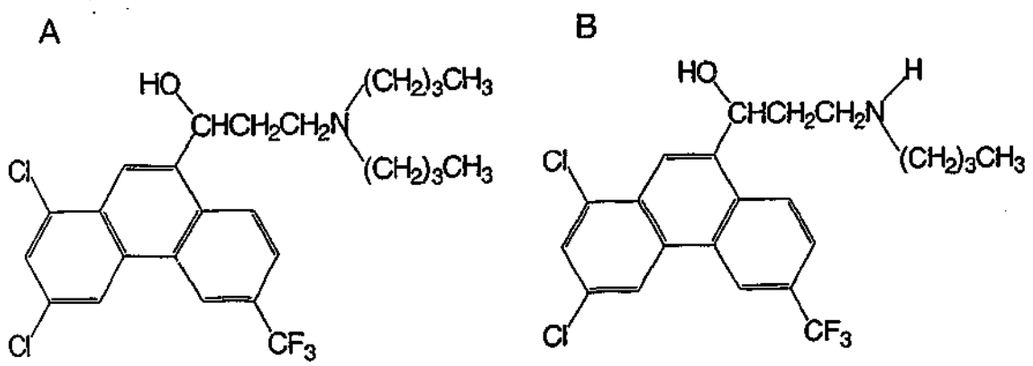

Figure 3.1 The chemical structures of (A) halofantrine and (B) the major metabolite, *N*-desbutylhalofantrine.

In vitro studies using human liver microsomes indicate that the conversion of Hf to Hfm is largely mediated via cytochrome P450 (CYP) 3A, a major subfamily of the CYP enzymes.[7,8] Although the two isoforms of CYP3A, CYP3A4 and CYP3A5, are both commonly expressed in the liver are involved in the metabolism of Hf, CYP3A4 is the major isoform responsible for Hf metabolism as the contribution of CYP3A5 to the formation of Hfm was less significant than CYP3A4. Other CYPs (e.g. CYP2D6 and CYP2C8) have also been reported to mediate the conversion of Hf to Hfm but their contribution is relatively minor in comparison to CYP3A4. *In vitro* studies have also shown that ketoconazole (KC), a well characterized inhibitor of CYP3A4,[9] non-competitively inhibited the formation of Hfm with an inhibition constant (K_i) of 0.05 μM.[8,10]

Greater than 50% of drugs in use today are reported to be substrates of CYP3A, the major phase I drug metabolising enzyme found in humans.[11,12] In addition to being present in the liver, CYP3A is also expressed at high levels in the villus tip of enterocytes in the small intestine.[13] It accounts for 30% and 70% of total CYP content in liver and intestine, respectively.[14,15] Among the CYP3A subfamily, CYP3A4 is the most abundant isoform and the most important enzyme in drug metabolism.[16] However, its expression is highly variable (10- to 100-fold variation in CYP3A4 levels) between individuals.[17] CYP3A5, another isoform of CYP3A is 83% homologous to CYP3A4 but is expressed at much lower levels than CYP3A4 and is present in about 25% to 30% of human livers, and in the small intestine of approximately 70% of adults.[18] The expression of a third isoform of CYP3A, CYP3A7 (previously found in fetal liver), has not been found in human duodenum.[19]

CYP3A4 has broad substrate specificity and is known to catalyse the metabolism of a large and growing number of structurally diverse and clinically important drugs via

metabolic processes such as hydroxylation and *N*-dealkylation reactions.[20,21] Many substrates of CYP3A4 display low and variable bioavailability after oral administration. This is not surprising considering the presence of CYP3A4 at high levels in the villus tip of enterocytes in the small intestinal epithelium where a large surface area is available for the enzyme to interact with absorbed drug. This together with the presence of CYP3A4 in the liver can result in extensive first-pass (presystemic) drug metabolism. Moreover, drug interactions involving enzyme induction or inhibition are common. CYP3A4 may be induced by a number of structurally unrelated compounds including dexamethasone, rifampicin (rifampin), phenobarbitone and phenytoin. Known inhibitors of CYP3A4 include azole antifungals (e.g. ketoconazole, itraconazole), macrolide antibacterials (e.g. erythromycin, troleandomycin), HIV protease inhibitors (e.g. saquinavir, ritonavir), and certain constituents in grapefruit juice.[21,22]

The role of CYP3A in intestinal (pre-hepatic) first-pass drug metabolism is rapidly emerging[16,23,24] and has recently been recognized to be a major determinant of oral bioavailability of several important drugs including cyclosporine, tacrolimus, midazolam and HIV protease inhibitors.[25-29] Such processes may also have a significant role in the pre-hepatic metabolism of Hf, and could potentially be responsible at least in part for the low and variable oral bioavailability of Hf. Although the relative contributions of pre-hepatic and hepatic metabolism of Hf to Hfm are unknown, the low and often undetectable plasma levels of Hfm observed after intravenous administration of Hf to beagles[6] and humans[30] suggests that pre-hepatic conversion of Hf to Hfm may be significant.

3.2 OBJECTIVES

To date, the involvement of CYP3A4 in the *in vivo* metabolism of Hf has not been formally assessed. Establishment of the *in vivo* role of CYP3A4 in mediating Hf metabolism is of clinical importance given that many inhibitors and/or inducers of CYP3A4 are widely used in clinical practice, and if administered in combination with Hf, they could affect the metabolism of Hf and result in potentially unfavourable clinical outcomes. For example, the co-administration of a drug which is an inducer of CYP3A4 could further decrease plasma concentrations of Hf and result in treatment failure. In contrast, inhibition of CYP3A4 and hence Hf metabolism could increase Hf plasma concentrations and result in a beneficial therapeutic outcome. However, excessively high Hf plasma concentrations could also increase the occurrence of adverse cardiac effects as significant prolongation in QTc interval of the electrocardiogram which leads to serious ventricular arrhythmias has been reported to be directly correlated with Hf plasma concentrations.[4,5] Similar adverse cardiac effects have been reported to occur following the concomitant administration of CYP3A4 substrates (e.g. non-sedating antihistamines and cisapride) with an inhibitor of CYP3A4 enzyme activity, this is presumably due to markedly elevated plasma drug concentrations.[31-33]

Accordingly, the current study was undertaken to confirm the putative role of CYP3A4 in the *N*-dealkylation of Hf to Hfm by evaluating the effect of co-administering ketoconazole, a potent inhibitor of CYP3A4, on the relative plasma profiles of Hf and Hfm after fasted oral administration to beagles.

3.3 METHODS

3.3.1 Preparation of solid dispersions for oral administration of Hf

The oral administration of Hf employed a solid dispersion of amorphous Hf base prepared in polyethylene glycol 6000 (PEG 6000) as this presents Hf in a finely dispersed form and afforded an approximate 5-fold increase in absolute oral bioavailability compared with the commercial Hf hydrochloride tablet.[34] The formulation also did not contain excipients that are likely to interact with CYP3A4.

Crystalline Hf base was firstly heated in a beaker at 90°C on a hotplate to produce the amorphous form prior to the addition of PEG 6000 (Ajax Chemicals, NSW, Australia). The fusion of drug and carrier was allowed to occur at 70-80°C. Once uniformly mixed, the molten mixture was filled directly into pre-cooled suppository moulds and placed in a refrigerator (5-8°C) for rapid solidification. Each solid dispersion was prepared to contain 75 mg Hf base and to have a fill weight of 700 mg.

Following preparation of the solid dispersion, the chemical stability of Hf was determined by HPLC (section 3.3.3) to ensure that Hf had not undergone chemical decomposition during the fusion process. Prior to HPLC analysis, a pre-weighed, whole unit dose was dissolved in 100 mL of acetonitrile and diluted to appropriate concentrations before injection onto the column. Hf remained stable during the fusion process as the area under the Hf peak accounted for greater than 99% of the total peak area and degradation peaks were not observed in the HPLC chromatograms. The mean (± SD, n = 5) content of Hf was 100.2 ± 1.85% of the nominal concentration of 75 mg.

The dispersion characteristics of the solid dispersions were also determined. Dispersion studies were conducted using a standard USP dissolution apparatus. The solid dispersions were placed in 500 mL of medium (either 0.1 M HCl or purified water maintained at 37°C) and gentle agitation was provided by rotating the dissolution paddle

at 100 rpm. Complete dissolution of the water soluble PEG 6000 matrix was achieved within 30 min in both media, liberating the drug as fine, dispersed particles.

3.3.2 *In vivo* study design

The study was approved and performed in accordance with the guidelines of the Melbourne University Animal Experimentation Ethics Committee, and was conducted in two phases (Figure 3.2) in four fasted male beagles (12-16 kg). Phase I was a randomized, crossover study with a 6-day washout period where the drug treatments were either an intravenous (IV) infusion of Hf (prepared as described in section 2.3.5.2) administered as a 2 mg/kg dose over 5 min, or an oral dose of 150 mg Hf base (two solid dispersions). Phase II commenced on study day 15 with daily administration of a single 200 mg ketoconazole (KC) tablet (Nizoral®, Janssen Cilag, NSW, Australia) to each beagle during feeding time in the afternoon, which continued until study day 25. The dose of KC administered corresponded to a daily dose of 12.5-16.7 mg/kg, which was expected to induce maximal inhibition of CYP3A4 in dogs.[35] On study day 22 (after 7 days of KC pre-treatment), an oral dose of 150 mg Hf was administered to each fasted dog. On this day, the KC dose was administered 2 h prior to Hf administration. Blood tests were taken prior to, during and after the ketoconazole treatment period. The results of these tests indicated normal hepatic function and liver enzyme activity.

Blood samples (2.5 mL) were collected either from the cephalic vein via an indwelling catheter (that was not the same as that used for IV administration of Hf) or by individual venipunctures at predose (-10 min), 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 24, 32, 48, 72 and 96 h after oral administration, and at predose (-10 min), 0 (end of infusion), 15 and 30 min, 1, 1.5, 2, 3, 4, 6, 8, 10, 24, 32, 48, 72 and 96 h after IV administration. Plasma was separated from whole blood by centrifugation and stored at

Phase I (without KC)

Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	Hf (oral/IV)							Hf (oral/IV)						

Phase II (with KC)

Day	15	16	17	18	19	20	21	22	23	24	25
	KC	KC	KC	KC	KC	KC	KC	KC Hf (oral)	KC	KC	KC

Figure 3.2 Schematic of the study design. Phase I was a randomized, crossover study where halofantrine (Hf) was administered either orally (150 mg) or by intravenous infusion (2 mg/kg over 5 min). In phase II, ketoconazole (KC, 200 mg) was administered daily and after 7 days of KC pre-treatment, oral Hf (150 mg) was administered.

-70°C prior to analysis. Hf and Hfm were extracted from plasma using the method previously described in section 2.3.6.2.1 and plasma concentrations were determined using a validated gradient HPLC assay (section 3.3.3).

3.3.3 Gradient chromatography

HPLC was performed using a Beckman System Gold® 126 Programmable Solvent Module, a Beckman System Gold® 168 Diode Array detector and a Beckman 507e autosampler (Beckman Instruments, Fullerton, CA, USA). An Ultrasphere C_8 reversed-phase column (5μm particle size, 25 cm x 4.6 mm i.d., Beckman Instruments, Fullerton, CA, USA) and a Brownlee® RP-8 guard column (Aquapore 7 μm, 3.2 x 15 mm, Applied Biosystems, San Jose, CA, USA) were used and both were maintained at 37°C. The mobile phase was pumped at a flow rate of 1 mL/min and consisted of a mixture of solvent A (10% acetonitrile:90% Milli-Q water containing 0.2% w/v sodium dodecyl sulphate and 0.2% v/v glacial acetic acid) and solvent B (90% acetonitrile:10% Milli-Q water containing 0.2% w/v sodium dodecyl sulphate and 0.2% v/v glacial acetic acid). Chromatography commenced with 80% solvent B, which was increased to 90% solvent B over 7 min, maintained at 90% solvent B for 4 min and then increased to 95% solvent B over 1 min. The gradient remained at 95% solvent B for 1.5 min before returning to 80% solvent B over 0.5 min. The column was then allowed to re-equilibrate with 80% solvent B for 1 min. The samples were maintained at 10°C in the autosampler prior to injection. A 25 μL sample was injected onto the column and detection was conducted by single wavelength monitoring at 258 nm. The data was processed using the Beckman Nouveau software.

Using the gradient chromatography conditions described above, Hfm, Hf and IS eluted at 6.2, 8.3 and 9.7 min, respectively (Figure 3.3). The minimum quantifiable

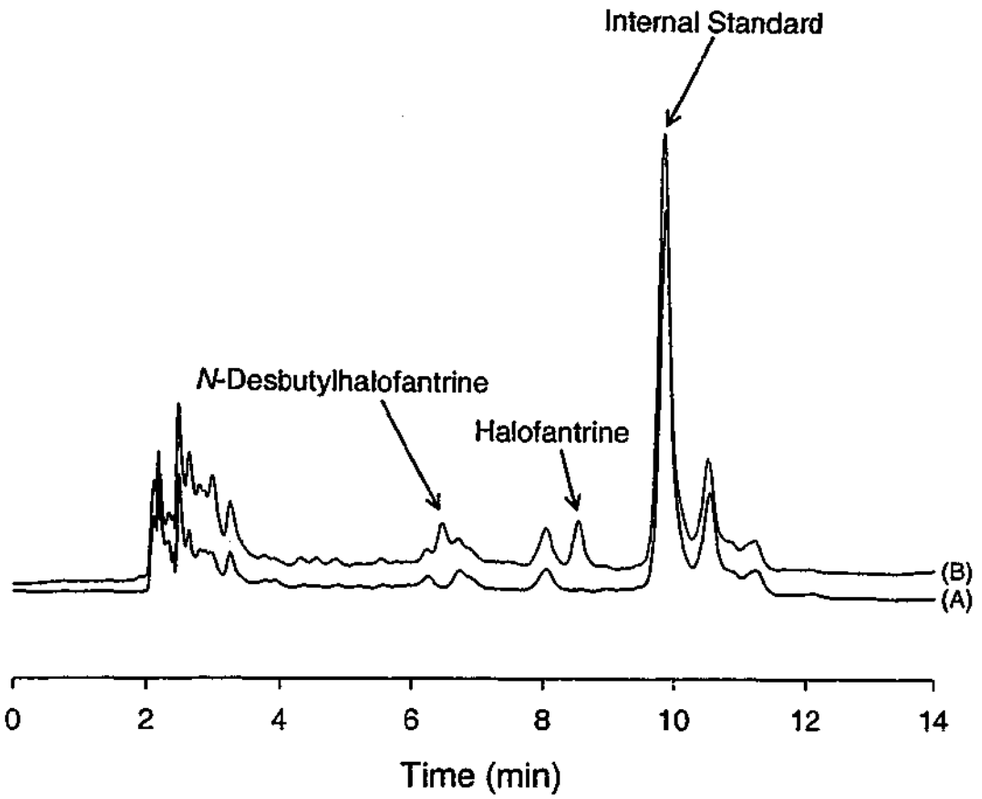

Figure 3.3 Representative HPLC chromatograms for (A) extracted blank plasma spiked with internal standard, and (B) extracted plasma sample spiked with *N*-desbutylhalofantrine and halofantrine (corresponding to 20 ng/mL), and internal standard.

limit (MQL), defined as the lowest level of quantification (with coefficient of variation of less than 15%) was 10 ng/mL for both Hfm and Hf. The standard curve was linear between spiked plasma concentration of 10 and 2000 ng/mL ($R^2 > 0.99$) for Hfm and between 10 and 8000 ng/mL ($R^2 > 0.99$) for Hf. Extraction efficiency was greater than 85%, and the intra- and inter-day coefficient of variation for Hf and Hfm were less than 15% across the concentration range. Accuracy deviated by ± 10% from nominal concentrations.

3.3.4 Pharmacokinetic data analysis

Peak plasma concentrations (C_{max}) and the time for their occurrence (T_{max}) were noted directly from the individual plasma concentration-time profiles for Hf and Hfm. The area under the plasma concentration-time curve ($AUC^{0-\infty}$) was calculated using the linear trapezoidal rule to the last sampling time (96 h), followed by addition of the extrapolated area calculated by dividing the concentration at 96 h by the terminal elimination rate constant (k_e). The k_e was determined from regression analysis of the terminal linear phase. In one instance (for a dog which received oral Hf without KC) the plasma samples after 10 h were contaminated and could not be accurately analysed. In this case, the plasma $AUC^{0-\infty}$ value was estimated using the trapezoidal method to the 10 h plasma concentration, and the extrapolated tail area was estimated using the individually determined elimination rate constant after IV administration. It was estimated that this approach may have over-estimated the particular plasma $AUC^{0-\infty}$ value by as much as 30%. However, any such over-estimation would then directly minimize the difference in the respective plasma Hf AUC values between the with- and without-KC treatments. Differences in parameters between the treatment groups were analysed by a paired *t*-test and were considered significant if $p < 0.05$.

Plasma clearance (CL_{IV}) after IV administration of Hf, and absolute oral bioavailability (F) were calculated using the following standard equations:

$$CL_{IV} = Dose_{IV} / AUC_{IV} \qquad \text{Equation 3.1}$$

$$F = (AUC_{oral} / AUC_{IV}) \times (Dose_{IV} / Dose_{oral}) \qquad \text{Equation 3.2}$$

Oral bioavailability of Hf may be affected by at least three factors: the fraction of the drug dose absorbed from the gut lumen into the gut epithelium (F_{abs}), the fraction of the absorbed dose that passes through the gut into the hepatic portal vein unmetabolized by the enzymatic processes in the gut membrane (F_G), and the fraction of the dose absorbed intact into the hepatic portal vein that is not lost to hepatic first-pass metabolism (F_H), as depicted in Figure 3.4. Therefore, the measured oral bioavailability (F) will be the product of these three terms:[25]

$$F = F_{abs} \cdot F_G \cdot F_H \qquad \text{Equation 3.3}$$

Gut and hepatic availability may be defined as one minus the extraction ratio (ER) at each site:

$$F = F_{abs} \cdot (1\text{-}ER_G) \cdot (1\text{-}ER_H) \qquad \text{Equation 3.4}$$

After intravenous administration, it is possible to estimate the hepatic extraction ratio, which is the ratio of the hepatic blood clearance to the hepatic blood flow (Q_H):

$$ER_H = CL_{IV} / Q_H \qquad \text{Equation 3.5}$$

The hepatic extraction ratio was calculated using a value of 1.8 L/h/kg for liver blood flow in dogs[36,37] and the previously measured Hf blood/plasma ratio of 0.82.[6] In these calculations, it is assumed that Hf is predominantly cleared by the liver after IV administration and that KC does not affect liver blood flow to any significant degree.

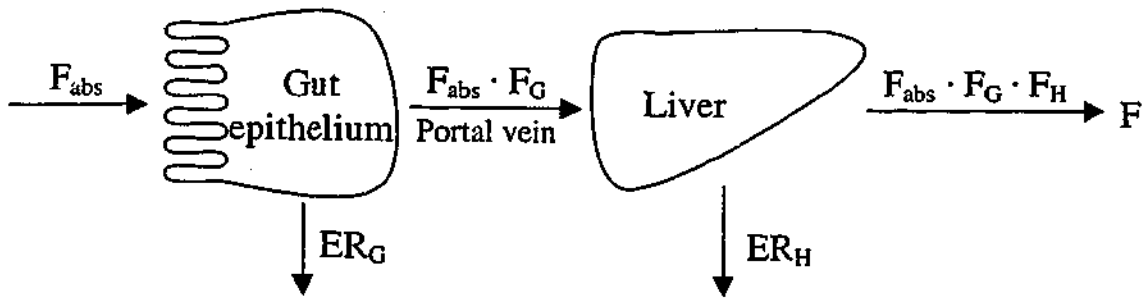

Figure 3.4 Schematic diagram depicting the effects of absorption, and gut and hepatic first-pass extraction on drug oral bioavailability (F). F_{abs}, fraction of drug dose absorbed from the gut lumen; ER_G, gut extraction ratio; F_G, fraction of the absorbed dose not metabolized by gut enzymes; ER_H, hepatic extraction ratio; F_H, hepatic first-pass availability. Adapted from ref. 25.

3.4 RESULTS AND DISCUSSION

The present study evaluated the effect of co-administered ketoconazole (KC), a highly potent and selective inhibitor of CYP3A4, on the relative plasma profiles of Hf and Hfm after fasted oral administration to beagles to assess the putative involvement of CYP3A4 in the *in vivo* metabolism of Hf. Considering that CYP3A4 is not only expressed abundantly in the liver but is also present at high levels in the villus tip of enterocytes in the jejunum, the primary site of drug absorption, CYP3A4 may have an important role in mediating the pre-systemic metabolism of Hf and could potentially influence the oral bioavailability of Hf.

KC has similar affinity for liver and intestinal microsomes and the mechanism of inhibition of CYP3A4 activity by KC has been demonstrated to be non-competitive.[38] Recent studies using a modified Caco-2 cell culture system reported that KC causes a persistent and slowly reversible inhibition of CYP3A4 activity. These results suggest that the inhibitory effects of KC on intestinal CYP3A4 are likely to persist beyond the residence time of KC in the intestinal lumen.[39]

The relative contributions of pre-hepatic and hepatic metabolism to the *N*-dealkylation of Hf to Hfm have not been formally studied. However, Hfm plasma concentrations between 50-100 ng/mL were observed after oral administration of Hf to beagles whereas Hfm plasma concentrations after intravenous administration of Hf were below the 10 ng/mL MQL of the assay.[6] This suggests that pre-systemic metabolism of Hf to Hfm may be significant after oral administration. The pharmacokinetics of Hf after the administration of three separate 1 h intravenous infusions of Hf (1 mg/kg, at 8 h intervals) in malaria patients has been described by Krishna *et al.*[30] A peak Hfm concentration of 27 ± 18.5 ng/mL (mean ± SD, n = 12) was observed 35 h after the first infusion of Hf, although greater than 90% of this peak value was observed 15 h after the

first infusion. The low concentrations precluded further pharmacokinetic analysis of Hfm and the authors suggested that since intravenous administration avoids first-pass metabolism of Hf, the fraction of Hf converted to Hfm after intravenous administration would be much lower than that observed after oral administration.

3.4.1 The effect of ketoconazole on the pharmacokinetics of Hf and Hfm

In Phase I of this study (without KC), intravenous administration of Hf to beagles produced low but detectable plasma concentrations of Hfm such that the calculated mean (± SD, n = 4) plasma Hfm/Hf $AUC^{0\text{-}72h}$ ratio was 0.19 ± 0.11. The presence of detectable plasma concentrations of Hfm after intravenous administration of Hf was unexpected based on previous data,[6] and may be the result of a higher Hf dose administered in this study. Nevertheless, Hfm plasma concentrations in the present study were generally low and variable.

Figure 3.5 presents the Hf and Hfm plasma concentration-time profiles observed after oral administration of Hf to fasted beagles with- and without-KC, and a summary of the corresponding pharmacokinetic data are shown in Table 3.1. The co-administration of Hf with KC produced non-significant changes in the measured plasma C_{max} and T_{max} values of Hf. However, the difference in the plasma Hf $AUC^{0\text{-}\infty}$ values between the with- and without-KC treatment groups was significantly different ($p < 0.05$). Co-administration of Hf with KC inhibited the formation of Hfm after fasted oral administration as plasma concentrations of Hfm were reduced from approximately 50-100 ng/mL (without KC) to below the 10 ng/mL MQL of the assay. The mean (± SD, n = 4) ratio of the plasma Hfm/Hf $AUC^{0\text{-}72h}$ values for fasted administration were 0.56 ± 0.18 (without KC) and less than 0.05 (with KC). These data suggest that the *in vivo*

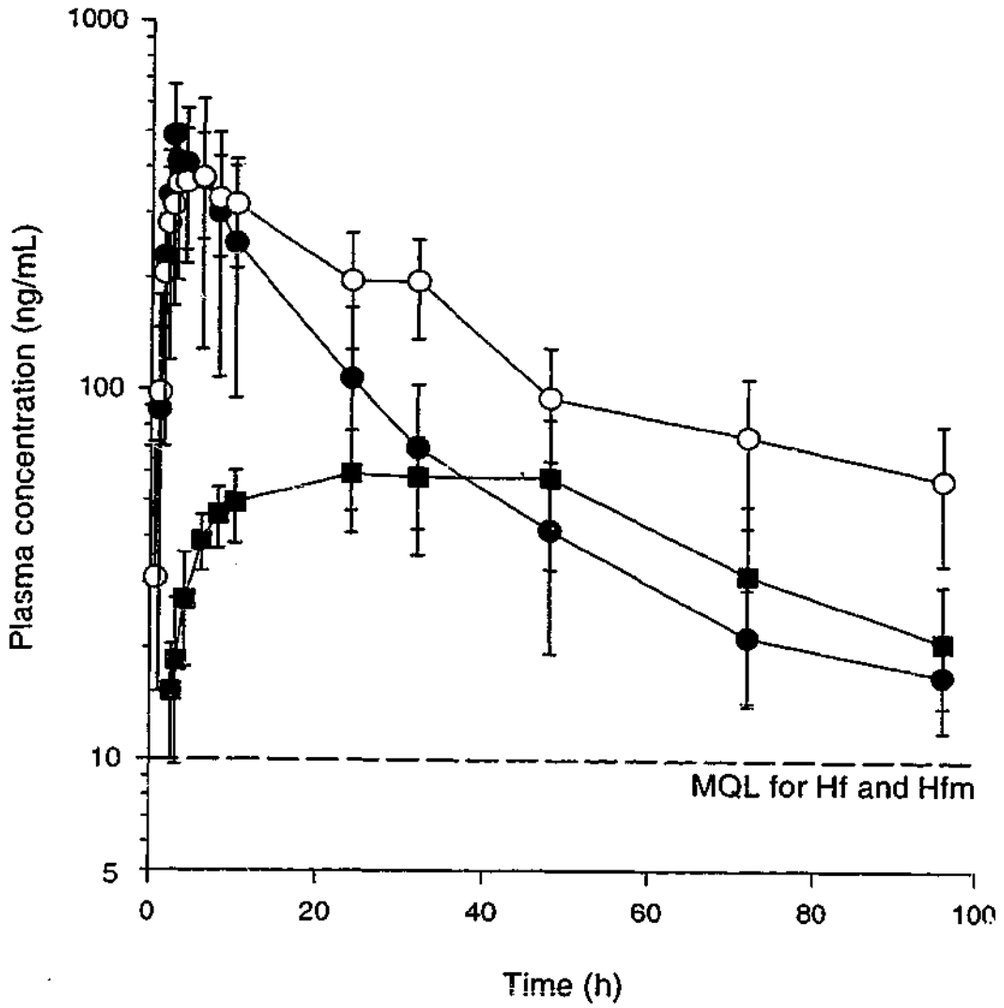

Figure 3.5 Mean (± SD, n = 4) plasma concentration-time profile of halofantrine (Hf, ● or ○) and the major metabolite, *N*-desbutylhalofantrine (Hfm, ■), after fasted oral administration of 150 mg Hf without ketoconazole (closed symbols) or after ketoconazole pre-treatment (open symbols). When Hf was co-administered with ketoconazole, the concentration of Hfm was below the 10 ng/mL MQL of the assay at all time points.

Table 3.1 Summary pharmacokinetic parameters (mean ± SD, n = 4) of halofantrine (Hf) and *N*-desbutylhalofantrine (Hfm) after oral administration of Hf (150 mg) to fasted beagles with or without co-administration of ketoconazole

	Parameters	Oral halofantrine (without ketoconazole)	Oral halofantrine (with ketoconazole)
Hf	C_{max} (ng/mL)	476 ± 144	419 ± 130
	T_{max} (h)	3.3 ± 1.9	4.1 ± 2.2
	$T_{1/2}$ (h)	26.9 ± 2.7	38.2 ± 3.5
	$AUC^{0\text{-}96h}$ (ng.h/mL)	7842 ± 3775	14822 ± 4233
	$AUC^{0\text{-}\infty}$ (ng.h/mL)	8454 ± 3853[a]	18280 ± 5593[a]
Hfm	C_{max} (ng/mL)	68.3 ± 21.3	None detected
	T_{max} (h)	34.4 ± 10.3	None detected
	$AUC^{0\text{-}96h}$ (ng.h/mL)	4581 ± 1419	None detected

[a] Significantly different ($p < 0.05$).

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	$AUC^{0\text{-}\infty}$ (ng.h/mL)	8454 ± 3853[a]	18280 ± 5593[a]
Hfm	C_{max} (ng/mL)	68.3 ± 21.3	None detected
	T_{max} (h)	34.4 ± 10.3	None detected
	$AUC^{0\text{-}96h}$ (ng.h/mL)	4581 ± 1419	None detected

[a] Significantly different ($p < 0.05$).

conversion of Hf to Hfm is likely to be mediated via CYP3A4, extending the previous *in vitro* identification of CYP3A4 involvement.[7,8] Furthermore, based on the localization of functional CYP3A4 in the small intestinal epithelium, it is likely that both pre-hepatic (enterocyte-based) and hepatic CYP3A4 contribute to the pre-systemic metabolism of Hf after oral administration.

The nucleotide and deduced amino acid sequence of dog CYP3A12 (the major isoform of CYP3A in dogs) has been reported to be 83% and 79% homologous, respectively, to human CYP3A4.[40] Dog intestinal tissue has also been shown to be functionally similar to human intestinal tissue with respect to testosterone-6β-hydroxylase activity (a marker for CYP3A4 activity) although the intrinsic enzyme activity was consistently lower than that observed with human intestinal tissue.[41] This is further supported by a more recent study employing liver microsomes.[42] *In vitro* studies have demonstrated that Hf may be converted to Hfm via CYP2D6,[7] however, the role of CYP2D6-mediated conversion of Hf in dogs is likely to be minor due to the low activity of CYP2D6 in dogs.[41]

The measured oral bioavailability of Hf (F_{meas}) and pharmacokinetic parameters estimating the impact of intestinal and hepatic metabolic processes on the oral bioavailability of Hf for individual dogs are presented in Table 3.2. Phase I of the study (without KC) confirmed that Hf is a low extraction drug. The mean (± SD, n = 4) hepatic extraction ratio (ER_H) and the corresponding F_H (fraction of absorbed dose not metabolized by the liver) of Hf were 0.162 ± 0.020 and 0.838 ± 0.020, respectively. These results suggest that the low oral bioavailability of Hf is unlikely to be attributed to extensive hepatic first-pass clearance.

In the present study, the ($F_{abs} \cdot F_G$) values, which represents the fraction of dose absorbed from the intestinal lumen (F_{abs}) multiplied by the fraction of absorbed dose not

Table 3.2 Pharmacokinetic parameters estimated from halofantrine (Hf) plasma profiles after oral (150 mg) and intravenous (2 mg/kg) Hf administration without ketoconazole pre-treatment (Phase I)

Dog no.	AUC_{IV} (ng.h/mL)	AUC_{oral} (ng.h/mL)	F_{meas}[a] (%)	F_H[b]	$F_{abs} \cdot F_G$[c]	CL_{IV} (mL/h/kg)
1	7867	6249	18.2	0.828	0.220	254
2	7444	8165	18.4	0.818	0.225	269
3	8487	5429	14.0	0.840	0.166	236
4	9973	13971	23.5	0.864	0.272	201
Mean	8843	8454	18.5	0.838	0.221	240
SD	1106	3853	3.89	0.020	0.043	29.3

[a] $F_{meas} = (AUC_{oral}/AUC_{IV}) \times (Dose_{IV}/Dose_{oral})$

[b] $F_H = 1 - CL_{IV}/Q_H$, calculated from intravenous parameters, assuming Hf is metabolized exclusively by the liver after intravenous dosing

[c] $F_{abs} \cdot F_G = F_{meas}/F_H$

Abbreviations: AUC, area under the concentration-time curve; F_{meas}, measured oral bioavailability; F_H, fraction of absorbed dose not metabolized by the liver; F_{abs}, fraction of oral dose absorbed; F_G, fraction of absorbed dose not metabolized by gut enzymes; CL, clearance.

metabolized by the enzymatic processes in the gut membrane (F_G), was considerably lower than F_H. The low ($F_{abs} \cdot F_G$) values could be attributed to factors such as poor and incomplete drug absorption in spite of delivering Hf as a PEG 6000 solid dispersion (to overcome rate-limited dissolution), extensive metabolism by CYP3A4 in the gut epithelium (resulting in high gut extraction ratio, ER_G) and/or P-glycoprotein (P-gp) mediated drug efflux. P-gp is a product of the multidrug resistance gene, MDR1, and functions as an energy-dependent drug efflux pump to lower intracellular drug concentrations.[43] In addition to being expressed in tumour cells, P-gp is also present at high levels on the apical surface of epithelial cells in the liver, small intestine and colon.[43] It has been suggested that P-gp and CYP3A4 may act synergistically to limit oral drug bioavailability due to the shared location and spatial relationship of P-gp and CYP3A4 within the absorptive enterocytes of the small intestine and the significant overlap in substrate specificity.[16,17,44,45] Although Hf has not been identified as a substrate of P-gp, it is possible that Hf may interact with the intestinal P-gp efflux pump as there has been a strong association between resistance to Hf and an amplification and overexpression of the multidrug resistant-like gene homologue, pfmdr1, in *Plasmodium falciparum* malarial parasites.[46-49] Further studies using for example, wild type and mdr1a (-/-) knockout mice, which do not express functional P-gp[50,51] would be required to establish the role of P-gp in limiting the oral bioavailability of Hf.

Co-administration of oral Hf with KC (phase II) increased the AUC_{oral} of Hf approximately 2-fold ($p < 0.05$) (Table 3.1). As IV Hf was not included in phase II of the study, the additional pharmacokinetic parameters could not be estimated. However, being a low extraction drug, the co-administration of KC with IV Hf is unlikely to effect significant changes in F_H, CL_{IV} and AUC_{IV}. Therefore, the significant increase in AUC_{oral} observed after concomitant oral administration of Hf with KC is likely to be

attributed to an increase in ($F_{abs} \cdot F_G$), where absorption processes and gut extraction may have been affected by KC.

Studies with tacrolimus, cyclosporine and K02, a novel vinylsulfone peptidomimetic cysteine protease inhibitor, have demonstrated that oral KC dosing had little effect on the pharmacokinetics of the respective drug after IV administration. However, after oral drug administration, significant increases in oral bioavailability were reported.[26,52,53] The greater impact of KC on oral rather than IV pharmacokinetics of the drug suggests that metabolic processes occurring in the gut and not the liver are a major determinant of the oral bioavailability of these drugs. As the above drugs are substrates for both CYP3A4 and P-gp, and since KC is an inhibitor of both CYP3A4 and P-gp efflux activity,[53,54] the substantial increase in oral drug bioavailability observed after concomitant administration of KC is likely to be due to inhibition of both intestinal CYP3A4 metabolism and P-gp drug efflux. Similarly, the significant increase in Hf AUC_{oral} observed after co-administration of Hf with KC may have been attributed to decreased intestinal first-pass effect of enterocyte-based CYP3A4 metabolism and potentially reduced P-gp drug efflux.

3.4.2 Comparison of plasma Hfm/Hf AUC ratios

Table 3.3 summarizes the published data from dogs and humans describing the conversion of Hf to Hfm after intravenous administration, fed and fasted oral administration, and the ketoconazole-related data from the current study. For both humans and beagles, the plasma Hfm/Hf AUC ratio after fasted oral administration of Hf was larger than all other treatments, and post-prandial administration resulted in 2.4-

Table 3.3 A comparison of mean (± SD) pharmacokinetic parameters of halofantrine (Hf), and plasma desbutylhalofantrine (Hfm)/Hf AUC ratios compiled from published studies conducted in beagles and humans, and data from the current study

Species	Parameters	Published data		
		Oral/Fasted	**Oral/Fed[a]**	**Intravenous**
Beagles (n = 3) (ref. 6)	C_{max} (ng/mL)	275 ± 156	5540 ± 2107	
	T_{max} (h)	2.6 ± 0.6	2.0 ± 1.0	
	$AUC^{0-\infty}$ (ng.h/mL)	4201 ± 2784	51496 ± 9379	47185 ± 7632[b]
	$T_{1/2}$ (h)	17.0 ± 2.2	20.7 ± 4.5	18.4 ± 2.9
	Hfm/Hf AUC^{0-72h} ratio	0.54	0.08	less than 0.05[c]
Humans (n = 6) (ref. 2, 30)	C_{max} (ng/mL)	184 ± 115	1218 ± 464	
	T_{max} (h)	6.0 ± 1.3	3.3 ± 1.5	
	$AUC^{0-\infty}$ (ng.h/mL)	3900 ± 2600	11300 ± 3500	
	$T_{1/2}$ (h)	81 ± 19	80 ± 10	7.5 ± 7.7
	Hfm/Hf $AUC^{0-\infty}$ ratio	2.25	0.95	less than 0.1[c]
		Current study		
		Oral/Fasted (without KC)	**Oral/Fasted (with KC)**	**Intravenous**
Beagles (n = 4)	C_{max} (ng/mL)	476 ± 144	419 ± 130	
	T_{max} (h)	3.3 ± 1.9	4.1 ± 2.2	
	$AUC^{0-\infty}$ (ng.h/mL)	8454 ± 3853[d]	18280 ± 5593[d]	44198 ± 10908[b]
	$T_{1/2}$ (h)	26.9 ± 2.7	38.2 ± 3.5	28.8 ± 4.2
	Hfm/Hf AUC^{0-72h} ratio	0.56	less than 0.05[c]	0.19 ± 0.11

[a] Dogs ingested 600 g of canned dog food (2.5% fat, 7.5% protein), and humans ingested sausages, scrambled egg, fried potato, one pint of milk (60 g of fat).

[b] The IV dose was 1.6 mg/kg in published study, and 2.0 mg/kg in current study, AUC values were dose normalized to oral dose.

[c] The maximal Hfm/Hf ratios were estimated by assuming that any non-detected plasma Hfm levels were at the MQL of the respective plasma assays.

[d] Significantly different ($p < 0.05$).

and 6.8-fold decreases in the plasma Hfm/Hf AUC ratio in humans and beagles, respectively (despite significant bile salt solubilization mediated increases in Hf absorption).[2,6] In the current study, the rank order of plasma Hfm/Hf AUC ratios was oral fasted > IV > oral fasted with co-administered ketoconazole.

An interesting feature arising from comparison of the current ketoconazole data with previous fed/fasted Hf data obtained from this laboratory was that the post-prandial administration of Hf produced a similar plasma Hfm/Hf AUC profile as did the co-administration of Hf with ketoconazole in the fasted state. A recent report described essential oils as having the ability to increase oral drug bioavailability through inhibition of enterocyte-based CYP3A metabolism and/or inhibition of P-glycoprotein efflux mechanisms.[55] It may be possible that similarly active lipidic components were present in the ingested food in the studies described in Table 3.3 which may have decreased the pre-systemic CYP3A4 conversion of Hf to Hfm. However, other mechanisms by which the pre-systemic metabolism of post-prandially administered Hf may be decreased include lipid-induced recruitment of intestinal lymphatic transport,[56] and the localization and trafficking of Hf through the enterocyte such that metabolism is avoided. As it may be possible that Hf is a substrate of the intestinal P-gp efflux pump, inhibition of P-gp mediated counter-transport activity and its consequential effect on drug recycling would lead to decreased exposure and susceptibility to intestinal metabolism.[11,17]

Whilst the primary role of the intestinal lymphatics is in the absorption and transport of dietary lipids and lipid soluble vitamins to the systemic circulation, it has also been recognized as an alternate absorption pathway for highly lipophilic drugs and xenobiotics. Due to the unique anatomy and physiology of the lymphatic system, drugs absorbed via the intestinal lymphatics will bypass the liver and result in avoidance of hepatic first-pass metabolism, thereby improving the oral bioavailability of drugs that

undergo significant first-pass metabolism. Lymphatically transported drugs are typically solubilized within, or associated with the triglyceride core of chylomicrons, which are formed in response to the oral ingestion of triglyceride lipids. Therefore, promotion of lymphatic drug transport would require the co-administration of an appropriate lipid source to stimulate lipoprotein synthesis by the enterocyte.[57,58]

Recent studies in rats indicate that when orally administered with a suitable lipid source, a significant proportion of Hf dose can be transported via the intestinal lymphatics, primarily in association with chylomicrons.[56,59,60] However, due to differences in the characteristics of the luminal environment of the rat intestine, it is difficult to predict the likely role of intestinal lymphatic transport in higher species such as humans or dogs. Therefore, in order to establish the relative contribution of intestinal lymphatic transport in the decreased pre-systemic metabolism of Hf (after post-prandial administration), a suitable animal model that enables the examination of intestinal lymphatic drug transport after oral ingestion of prototypic dose forms in representative fed and fasted states is required and this was addressed in chapter 4.

3.5 CONCLUSIONS

The results from this study indicate that the *in vivo* metabolism of Hf to Hfm was primarily mediated via CYP3A4 as the co-administration of Hf with ketoconazole, a potent inhibitor of CYP3A4, inhibited the formation of Hfm after fasted oral administration. Although the relative contributions of prehepatic (enterocyte-based) and hepatic CYP3A4 metabolism could not be determined in this study, inhibition of both processes and potentially P-gp drug efflux by ketoconazole may largely be responsible for the improved oral absorption of Hf. Whilst the oral bioavailability of Hf may be substantially enhanced by concomitant administration of Hf with a CYP3A4 inhibitor

such as ketoconazole, this would not be recommended in clinical practice as the increased Hf plasma concentrations could result in adverse cardiac effects such as life-threatening ventricular arrhythmias which are associated with prolongation in QTc interval of the electrocardiogram. Interestingly, the decreased plasma Hfm/Hf AUC ratio observed after fasted administration of Hf with KC was similar to the value previously observed after post-prandial administration. It was postulated that lipidic components present in food may inhibit enterocyte-based CYP3A4 metabolism and/or P-gp efflux mechanisms, however, further studies are required to determine the contribution of other processes such as recruitment of intestinal lymphatic transport, which may also decrease the presystemic metabolism of Hf. This has formed the basis for the studies conducted in chapters 4 and 5.

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CHAPTER 4

DEVELOPMENT OF A CONSCIOUS DOG MODEL FOR THE SIMULTANEOUS ASSESSMENT OF THE ABSORPTION, ENTEROCYTE-BASED METABOLISM AND INTESTINAL LYMPHATIC TRANSPORT OF HALOFANTRINE

4.1 INTRODUCTION

As previously described, the co-administration of halofantrine (Hf) with food markedly increased the oral bioavailability of Hf. However, the proportion of Hf metabolized to its equipotent active metabolite (*N*-desbutylhalofantrine, Hfm) was significantly reduced after post-prandial administration as the mean post-prandial plasma Hfm/Hf AUC ratios decreased 2.4-fold in humans[1] and 6.8-fold in beagles.[2] The basis by which food may decrease the metabolism of Hf has not been fully elucidated but may include the post-prandial recruitment of intestinal lymphatic transport of Hf thereby avoiding first-pass metabolism and/or inhibition of presystemic (hepatic and enterocyte-based) CYP3A4 metabolism by one or more specific component(s) present in food.

The intestinal lymphatics are a specialized absorption pathway by which dietary lipids and fat-soluble vitamins are transported to the systemic circulation. In recent years, the intestinal lymphatic system has increasingly been shown to be a significant contributor to the uptake and transport of highly lipophilic drugs to the systemic circulation after oral administration.[3-5] Drug transport via the intestinal lymphatics offers the advantage of enhanced oral bioavailability as intestinal lymph drains via the thoracic duct directly into the systemic circulation thereby avoiding hepatic first-pass metabolism. Although the mechanisms by which lipophilic drugs gain access to the intestinal lymphatics has not been fully established, the majority of lymphatically transported drugs typically associate with the triglyceride lipid core of chylomicrons, which are the primary lipoproteins formed by the enterocyte in response to lipid ingestion. Therefore, the co-administration of an appropriate lipid source (e.g. food or a pharmaceutical lipid vehicle) is generally required to stimulate lipoprotein synthesis and promote intestinal lymphatic drug transport.[6,7] Furthermore, it has been suggested that

candidate drugs require a log octanol/water partition co-efficient (log P) in excess of 5, and reasonable solubility in long-chain triglycerides (at least 50 mg/mL), before intestinal lymphatic transport is likely to become a significant contributor to oral bioavailability (as discussed in section 1.5.3).[8]

Hf was considered a likely candidate for intestinal lymphatic transport as the calculated log P was ~ 8.5,[9] and the free base form of Hf was highly soluble in a triglyceride lipid (> 50 mg/g in peanut oil).[10] Studies in the conscious rat indicated that after oral administration of Hf base in a lipid vehicle, approximately 20% of the 2 mg dose was recovered in intestinal lymph.[11] However, due to a number of limitations associated with the rat model, it is difficult to extrapolate lymphatic transport data from rats to higher species (such as humans and dogs). In the rat, bile flow is essentially continuous and independent of food intake, whereas in higher species, food or lipid is required to stimulate the numerous digestion-related events. This precludes the attainment of pre- and post-prandial intestinal states that are representative of the human situation. Moreover, the formulations administered to rats are often not relevant to higher species as the physically small size of the rat also does not allow the administration of full size dose forms used in humans.[12]

In chapter 3, it was demonstrated that the *in vivo* conversion of Hf to Hfm was largely mediated via CYP3A4, and interestingly, the administration of Hf to fasted beagles pre-treated with ketoconazole (an inhibitor of CYP3A4) yielded similar plasma Hfm/Hf AUC ratios as those observed post-prandially. It was postulated that active lipidic components present in ingested food may inhibit enterocyte-based CYP3A4 metabolism thereby decreasing the presystemic conversion of Hf to Hfm. Whilst it appears that specific components in food may decrease the presystemic CYP3A4 metabolism of Hf, the relative contribution of other processes such as recruitment of

intestinal lymphatic transport cannot be excluded. Therefore, the aims of this chapter were to develop a suitable animal model which would enable the role of intestinal lymphatic transport in the oral bioavailability of Hf following fasted and fed administration to be examined.

4.1.1 Large animal models for the study of intestinal lymphatic drug transport

The limitations associated with the rat model may be addressed by utilizing larger animal models such as the pig and dog for the study of intestinal lymphatic drug transport. Since these larger animal models are physiologically more representative of the human situation, they provide a more accurate assessment of the absorption pathways of lipophilic drugs after oral administration of the prototypical dose forms that would be used in higher species (e.g. human).

Based on the similarities in the gastrointestinal profiles between the pig and human,[13] an anaesthetized pig model which enables the simultaneous sampling of mesenteric lymph, hepatic portal blood and systemic blood has been described by White *et al.*[14] However, this model only allowed for periodic sampling (and not the continuous collection) of lymph, and hence, intestinal lymphatic drug transport could not be quantitatively assessed.

A number of methods have been described for the cannulation of the thoracic lymph duct of dogs thereby enabling the continuous collection of thoracic lymph. Witte *et al.*[15] and Briscoe[16] have described methods which involve the direct cannulation of the thoracic duct at the left external jugulo-subclavian venous juncture in the neck of the dogs. Although the authors reported high success rates, successful cannulation was reported to be difficult when multiple lymphatic channels converged to a short thoracic duct ampulla. Extreme care was also required while inserting the cannula to avoid

puncturing the thin duct wall. Rajpal and Kirkpatrick[17] developed a technique in which thoracic duct lymph was collected via a reservoir pouch, created by ligation of the external jugular vein in the neck of dogs, above and below the entry point of the thoracic duct into the vein. Insertion of a cannula into this isolated segment of the jugular vein then allowed for chronic collection of thoracic lymph. This method was reported to be more robust and less invasive. Models allowing the reinfusion of collected lymph into the venous circulation have also been described,[18,19] however, clotting of lymph inside the cannula in spite of a continuous infusion of anticoagulant have resulted in high failure rates. The models described above have predominantly been used to either study the immune system or aspects of lymph fluid and physiology in selected pathologic states.

4.2 OBJECTIVES

Unlike the rat, dogs have pre- and post-prandial intestinal environments that are more representative of the human situation,[13,20] and they are capable of ingesting dose forms that are clinically relevant (with respect to dose and size) to humans. Therefore, the aims of the present study were to develop a conscious dog model which enables the simultaneous examination of intestinal lymphatic and non-lymphatic (i.e. portal blood) drug absorption and the assessment of potential enterocyte-based drug metabolism. By examining the recovery of triglyceride lipid in thoracic duct lymph, the model could be internally validated. The model developed was utilized to establish the role of intestinal lymphatic transport in the oral bioavailability of Hf and to probe aspects of its metabolic profile following fasted and fed oral drug administration. As this model allowed the collection of thoracic duct lymph rather than mesenteric lymph and may result in an over-estimation of the extent of intestinal lymphatic drug transport, the lymphatic

transport of Hf after intravenous administration to post-prandial lymph cannulated dogs was studied to enable the potential redistribution of drug from the systemic circulation to peripheral lymph to be determined.

4.3 EXPERIMENTAL METHODS

4.3.1 Chemicals, reagents and Hf formulation

Hf base was supplied by SmithKline Beecham Pharmaceuticals (Mysore, India), and PEG 6000 (polyethylene glycol 6000) was from Ajax Chemicals (Australia). Acetylpromazine maleate (Delvet Pty. Ltd., Australia), propofol (Schering-Plough, Australia), cephazolin (Sigma Pharmaceuticals, Australia), carprofen (Pfizer, Australia), Iohexol (Nycomed, Australia) and polydioxinone sutures (Ethicon, USA) were used as received. Normal saline (0.9% sodium chloride) and Lactated Ringer's Solution (an isotonic solution containing 3.22 g/L sodium lactate, 6.0 g/L sodium chloride, 0.40 g/L potassium chloride and 0.27 g/L calcium chloride) were obtained from Baxter Healthcare (Australia). A solid dispersion of amorphous Hf base (100 mg) in PEG 6000 prepared by the fusion method (as previously described in section 3.3.1) was employed for the oral administration of Hf and the intravenous (IV) formulation containing Hf (5 mg/mL) solubilized in an IV lipid emulsion was prepared as described in section 2.3.5.2.

4.3.2 Surgical and related procedures

All surgical and experimental procedures were performed in accordance with the guidelines and approval of the Melbourne University Animal Experimentation Ethics Committee. Adult male greyhound dogs (28-35 kg) were employed for the study and their health status was verified by a veterinarian. Each dog was fed a small lipid meal

(corn oil) to facilitate identification and visualisation of the thoracic duct prior to surgical premedication with a subcutaneous injection of acetylpromazine maleate (0.5 mg/kg). Anaesthesia was then induced with an intravenous injection of propofol (3-6 mg/kg), and the dog intubated. Surgical anaesthesia was maintained by delivery of halothane (1.5%) and oxygen. Each dog also received intravenous infusion of Lactated Ringer's solution (300 mL/h) during surgery and a post-operative intravenous injection of the antibiotic cephazolin (20 mg/kg) and the analgesic carprofen (4 mg/kg).

4.3.2.1 Portal vein cannulation

The dog was placed in dorsal recumbency and the ventral abdomen was clipped and prepared for aseptic surgery. A 5 cm long ventral midline laparotomy was performed to allow a loop of jejunum to be exteriorized (Figure 4.1). A 133 mm (5.25 in) long, 16 Gauge cannula was then inserted into a jejunal vein to the level of the portal vein. The cannula was stabilized in both the portal and jejunal veins with a ligature of 3-0 silk and a minimal volume extension tube, filled with heparinized saline, was then attached to the cannula. The extension tube was externalized through a separate stab incision in the left flank 4 cm lateral to the midline and stabilized with sutures. The laparotomy wound was closed in anatomical layers with 3-0 polydioxinone sutures. The position of the cannula in the portal vein was confirmed by X-ray following the injection of a contrast dye (Iohexol) (Figure 4.2). The patency of the cannula was maintained by frequent flushing with a small volume of heparinized saline (1 U/mL).

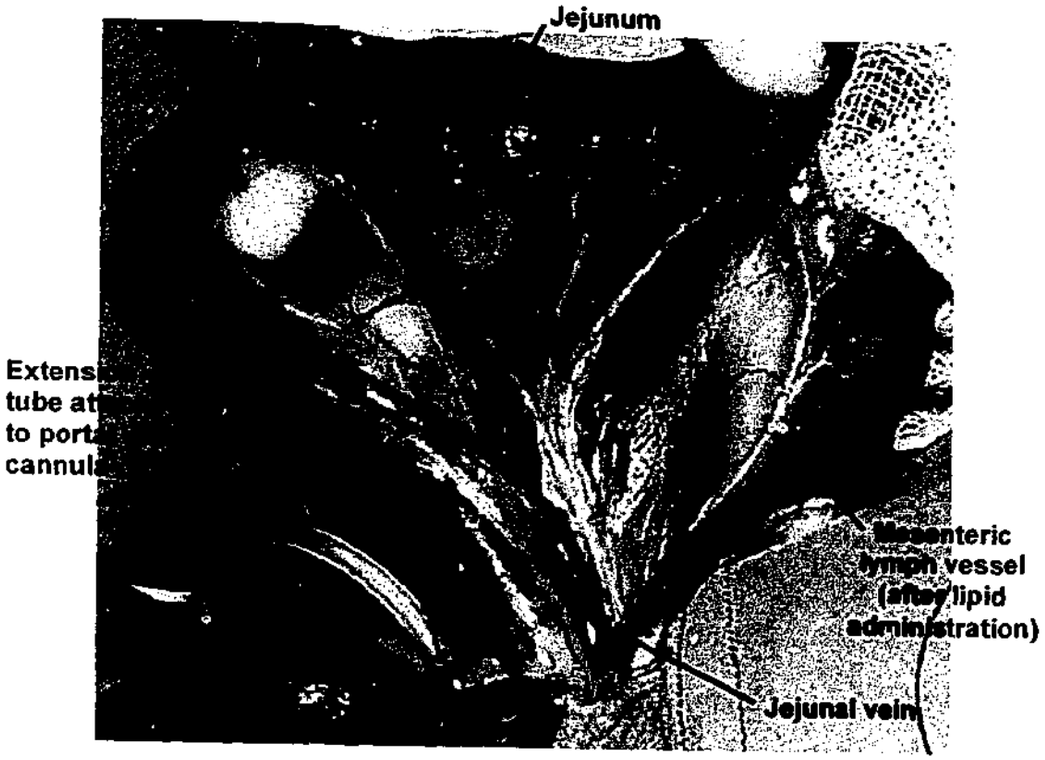

Figure 4.1 A loop of jejunum exteriorized for the insertion of a cannula to the portal vein (via a jejunal vein).

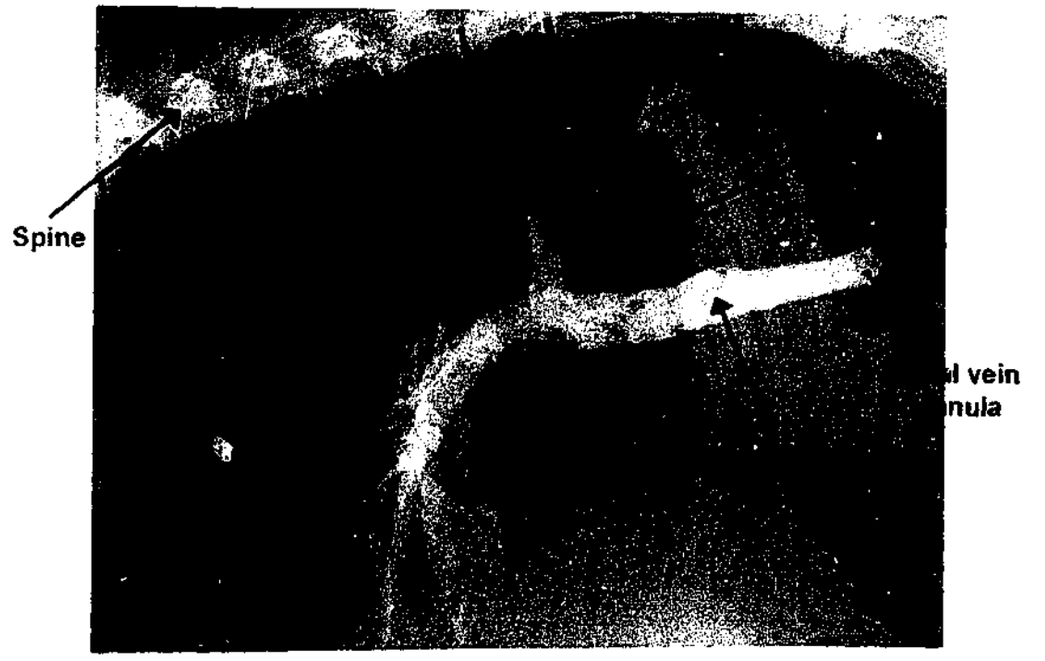

Figure 4.2 An X-ray showing the position of the inserted cannula in the portal vein after infusion of a contrast dye (Iohexol) via the portal vein cannula.

4.3.2.2 *Thoracic duct cannulation*

The thoracic lymph duct was cannulated by an adaptation of a previous method.[17] The dog was placed in right lateral recumbency with the front left leg pulled caudally to facilitate cannulation of the thoracic duct. The left lateral area of the neck and cranial thorax were clipped and prepared for aseptic surgery. A curved incision was made over the left external jugular vein and the vein was then dissected from the surrounding connective tissues to the axillary vein. Exposure was aided by retraction of the sternocephalicus muscle, and the ligation and division of the distal communicating branch of the cephalic vein and the descending branch of the omocervical artery. At the junction of the left internal and external jugular veins, the thoracic duct ampulla, which has a whitish appearance was identified by careful dissection (Figure 4.3). The thoracic duct was then ligated at the point of entrance into the external jugular vein (Figure 4.4), and all tributaries converging to the thoracic duct were identified and ligated to ensure the return of lymph through the main lymphatic canal. The thoracic duct was cannulated directly with PVC tubing (1.4 mm i.d.; 1.9 mm o.d., BioService, Australia) (Figure 4.5) which was brought through the skin via a separate stab incision and stabilized in place with 3-0 silk ligatures and cyanoacrylate adhesive. The surgical wound was closed in layers with 3-0 polydioxinone sutures. The externalized cannula was placed in a collection bottle held in place by a bag hung around the neck of the dog.

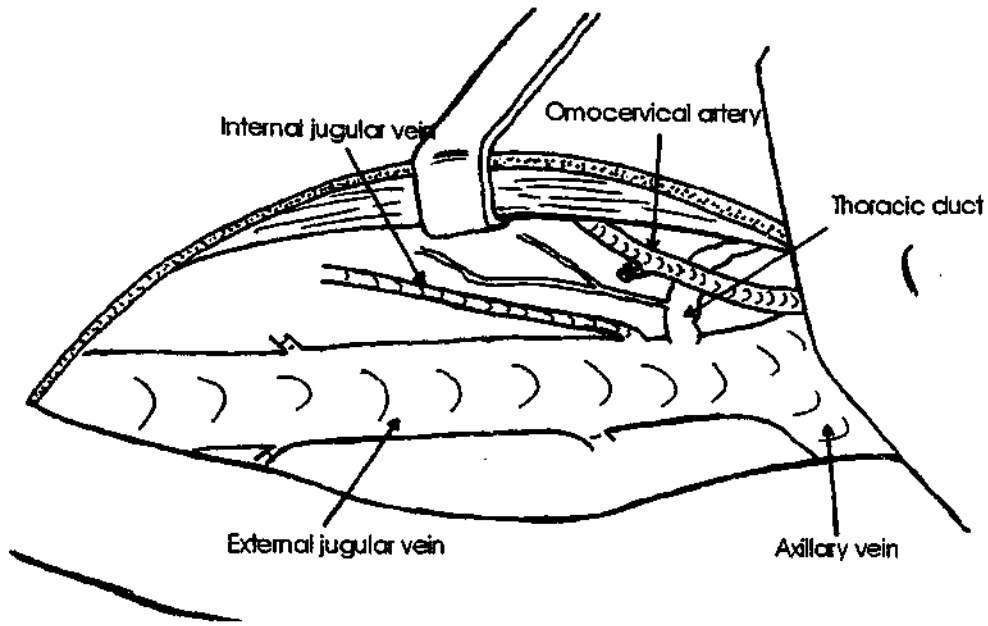

Figure 4.3 Schematic diagram of the left lateral neck area of a dog during surgery to insert a thoracic lymph duct cannula. Reproduced with permission from ref. 21.

duct

Figure 4.4 Swelling of the thoracic lymph duct following ligation at the point of its entry into the external jugular vein.

Figure 4.5 Lymph cannula (PVC tubing, 1.4 mm i.d.; 1.9 mm o.d.) inserted directly into the thoracic lymph duct.

4.3.2.3 Post-surgical recovery

Following surgery, the dogs were allowed to recover unrestrained in a closed run for a period of 12-16 h. In the initial recovery period, fluids were administered intravenously to ensure adequate hydration and to prevent hypoproteinaemia. The dogs were allowed to return to normal ambulatory movement before the commencement of a study. Immediately prior to the start of a study, a 20 Gauge intravenous catheter was inserted into the cephalic vein to enable serial blood sampling during the study. For dogs receiving an intravenous formulation of Hf, an additional catheter was inserted into the cephalic vein of the opposite leg for intravenous dosing (the catheter was not subsequently used for blood sampling). The catheters were kept patent by flushing with

heparinized saline. The dogs were encouraged to drink water throughout the study as long-term loss of lymph may result in dehydration, electrolyte imbalances and hypoproteinaemia. At the conclusion of the study, the dogs were sacrificed by an intravenous injection of pentobarbitone.

4.3.3 Experimental procedures

For studies in the fasted state, food was withheld from the dogs throughout both the recovery and study periods. To obtain a post-prandial state, the dogs were fed a standard can of commercial dog food (680 g) containing 5% crude fat and 7.5% protein 30-45 min prior to drug administration. In a parallel study design, either an intravenous formulation of Hf (2 mg/kg) infused over 10 min was administered to post-prandial dogs (n = 3) or 100 mg (0.2 mmol) Hf base prepared as a PEG 6000 solid dispersion was orally administered with 50 mL of water to fasted (n = 3) and fed (n = 4) dogs. To limit possible dehydration due to the continuous collection of thoracic lymph, 25 mL normal saline was administered hourly by IV bolus during the sampling period. Water was available *ad libitum* and the dogs were fed after collection of either the 10 or 12 h samples. Although the current study was limited to 24 h, study periods of greater than 48 h could readily be achieved provided the dogs were adequately hydrated.

Systemic and portal vein blood samples (2.5 mL) were obtained via indwelling cannulas and collected into individual tubes containing dipotassium EDTA. After intravenous administration, blood was collected at predose (-5 min), 0 (end of infusion), 10, 20, 30 and 45 min, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 and 24 h post-dosing, whereas blood samples were collected at predose (-5 min), 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10 and 24 h following fasted and post-prandial oral administration, with an additional

sample taken at 12 h after post-prandial administration. Plasma was separated by centrifugation, and then stored at -20°C prior to analysis.

After administration of Hf, lymph was collected continuously into 50 mL tubes containing 75 mg disodium EDTA (to prevent clot formation) for the 10-12 h post-dosing period. Individual lymph samples for each hourly collection period were combined and the mass of lymph collected per hour was determined gravimetrically. A 1 mL aliquot of lymph was then taken from each hourly sample, placed into individual 1.5 mL tubes and stored between 5-8°C prior to analysis (within 24 h).

4.3.4 Analytical procedures

4.3.4.1 Quantitation of Hf and Hfm in plasma and lymph

Plasma concentrations of Hf and the major metabolite, Hfm, were determined using the validated plasma extraction procedure and gradient HPLC assay described in sections 2.3.6.2.1 and 3.3.3, respectively. Briefly, the minimum quantifiable limit of the assay was 10 ng/mL for both Hf and Hfm and the assay was linear between 10 and 8000 ng/mL. The extraction efficiency was greater than 85%, and the intra- and inter-day coefficient of variation for Hf and Hfm were less than 15% across the concentration range (10 to 8000 ng/mL).

Analysis of Hf in lymph involved dilution of 100 µL of lymph to 10 mL with acetonitrile and then vortexing at high speeds for 2 min. The insoluble protein-based components were removed by centrifugation at 1200 *g* for 10 min, and the supernatant was analysed by HPLC (section 3.3.3). Using this procedure, recovery of spiked Hf from blank lymph was greater than 95%. As all lymph draining from the thoracic duct was collected, the mass of drug transported was calculated by multiplying the lymph drug concentration by the corresponding mass of lymph from each collection period.

4.3.4.2 *Analysis of triglyceride concentrations in lymph and plasma*

Triglyceride concentrations (expressed as mg equivalents of C_{18} triglyceride) in lymph and plasma were determined using an automated Roche Cobas Mira clinical chemistry analyzer (Roche, Basle, Switzerland). The analyzer employed a commercial enzyme-based colorimetric assay (Boehringer Mannheim, Germany) and was externally calibrated for each run. Total cholesterol and triglyceride concentrations in lymph lipoprotein fractions was also be determined using the Cobas Mira analyzer.

4.3.4.3 *Fractionation of lymph lipoproteins*

Thoracic duct lymph collected during the 1-2, 2-3 and 3-4 h post-dosing periods from post-prandial dogs administered oral Hf were fractionated by ultracentrifugation using a Beckman Optima® XL-100K ultracentrifuge (Beckman Instruments, Palo Alto, CA, USA) and a Beckman SW 60 Ti rotor (Beckman Instruments, Ireland) in a manner similar to that previously described.[22] Chylomicrons were firstly separated by layering lymph (2 mL) under a sodium chloride density solution (2 mL, d = 1.0063 g/mL) in a 4 mL ultracentrifuge tube (Seton Polyclear, 11 x 60 mm, Seton Scientific, Sunnyvale, CA, USA) using a semi-automated layering device. Following centrifugation at 44,100 rpm (15°C) for 1 h and 20 min, the bottom of the tube was pierced with a needle for removal of the remaining lymph (without chylomicrons) situated at the bottom of the tube, and leaving the chylomicron fraction which had floated to the top of the tube to form a thick, white, semi-solid layer. The chylomicrons were then collected and redispersed in the sodium chloride density solution left in the tube.

The remaining lymph lipoprotein fractions were separated in a single spin using a density gradient and ultracentrifugation at 58,000 rpm (15°C) for 19 h. Density gradient solutions were prepared by adding appropriate quantities of dried KBr to the

background salt density of lymph as previously described,[22,23] and the solution densities were confirmed using refractometry. The density gradient was formed by firstly layering 0.5 mL of d = 1.21 g/mL solution into a 4 mL ultracentrifuge tube, followed by 0.75 mL of d = 1.15 g/mL solution, 0.75 mL of d = 1.02 g/mL solution, 1 mL of lymph (without chylomicrons) and lastly 1 mL of d = 1.0063 g/mL density solution. After centrifugation, each tube was removed and placed on a tube piercer, and a high-density solution (Fluorinert, Sigma Chemicals, St Louis, MO, USA) was pumped into the bottom of the pierced tube at 24 mL/h. The solution collected from the top of the tube was passed through a UV detector set at 280 nm prior to automated collection of 20 x 200 µL aliquots. Individual aliquots were assigned as either very low density lipoproteins (VLDL), low density lipoproteins (LDL), high density lipoproteins (HDL), or lipoprotein deficient lymph (LPDL) on the basis of triglyceride and cholesterol concentrations and the UV absorbance at 280 nm (as an indication of protein content). The concentration of Hf in each lipoprotein fraction was determined using the extraction procedure and validated HPLC assay described in sections 2.3.6.2.1 and 3.3.3, respectively.

4.3.5 Data analysis

The area under the plasma concentration-time profiles (AUC^{0-t}) was calculated using the linear trapezoidal method up to the last measured concentration. The terminal elimination rate constant could not be accurately determined, as the sampling period of the study was less than one half-life of the parent drug. The peak plasma concentrations (C_{max}) and the time for the occurrence (T_{max}) were noted directly from individual profiles. Clearance (CL) was calculated as the ratio of intravenous dose/AUC^{0-t}, however this was only an estimate as the $AUC^{0-\infty}$ could not be accurately determined.

The proportion of dose absorbed via the non-lymphatic (portal blood) route was calculated from the ratio of the dose-normalized AUC values determined after oral and IV administration, respectively. Differences in pharmacokinetic parameters between the treatment groups (fasted versus post-prandial administration) were analysed by Student's t-test at a significance level of $\alpha = 0.05$. A non-parametric Sign test at the 1% level was employed to determine if the difference between the portal and systemic plasma Hfm concentrations was greater than zero over the 10 h post-dosing period.

4.4 RESULTS AND DISCUSSION

The rat model is often employed to investigate the intestinal lymphatic transport of lipophilic drugs and for examining the effects of prototype lipidic formulations on lymphatic drug transport and oral bioavailability. However, extrapolation of rat data to higher species is difficult, as bile flow in the rat is essentially continuous and independent of food intake. To address these limitations, a new triple-cannulated conscious dog model (Figure 4.6) was developed to allow the simultaneous collection of thoracic duct lymph, and sampling of portal and systemic blood, thereby enabling the determination of intestinal lymphatic transport, non-lymphatic absorption (portal route) and pre-systemic metabolism of drugs after oral administration of prototypic dose forms in representative fed and fasted intestinal states. This model has been utilized to study the lymphatic transport and putative enterocyte-based metabolism of Hf as previous data[2,10,11,24] have indicated that intestinal lymphatic transport may be a contributing process to the post-prandial oral bioavailability profile of Hf. The potential redistribution of Hf from the systemic circulation to lymph was also addressed.

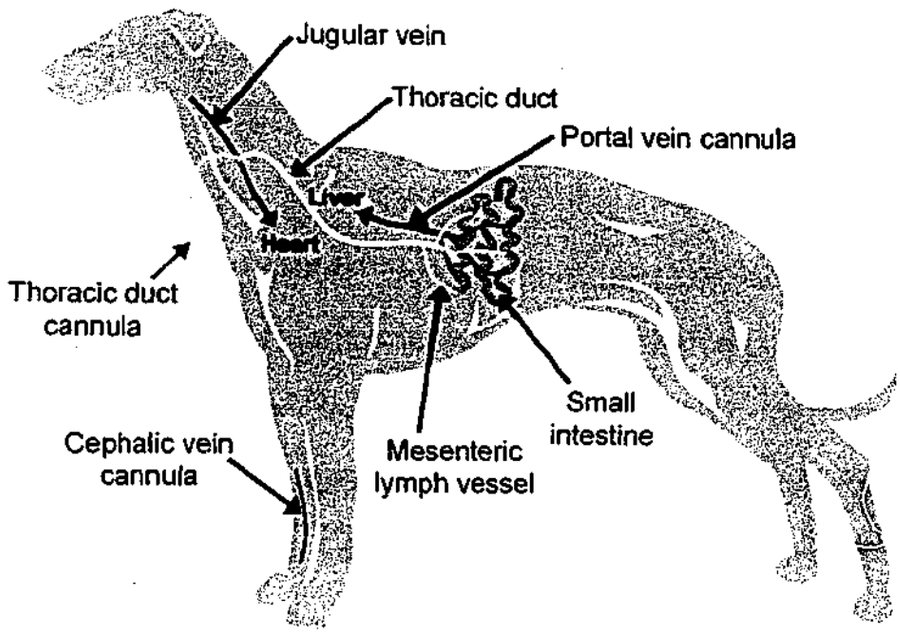

Figure 4.6 A schematic diagram of the triple-cannulated conscious dog model. The thoracic duct, portal and cephalic veins were cannulated to enable the simultaneous collection of thoracic duct lymph, portal and systemic blood, respectively.

4.4.1 Development of the triple-cannulated conscious dog model

The technique described by Rajpal and Kirkpatrick[17] for the creation of a thoracic duct fistula was initially employed for the collection of thoracic duct lymph in dogs. However, this technique was surgically difficult and challenging because of the required depth of surgical dissection into the thoracic inlet and variability in the point of entry of the thoracic duct into the external jugular vein. These factors often made it difficult to isolate the vein segment containing the thoracic duct ampulla without causing a pneumothorax or obstruction of the venous return at the caval bifurcation. After numerous unsuccessful experiences with this cumbersome and surgically-involved technique, a simpler and more reliable surgical method involving the direct cannulation of the thoracic lymph duct was developed for the continuous collection of lymph.

The modified method afforded a robust model with a success rate of greater than 80%. Failures have predominantly been due to the removal of the lymph cannula by the dogs during the recovery period. In comparison to the method of Rajpal and Kirkpatrick,[17] direct cannulation of the thoracic duct in the neck area required less surgical intervention and reduced the operating time to less than an hour, and dogs were often standing upright within an hour after surgery. The incidence of clot formation and subsequent cannula blockage was also reduced with this method. Whilst the anatomy of the lymphatic system in the upper neck region of dogs has been reported to be highly variable[25,26] the direct cannulation method provided flexibility and allowed ready accommodation of the inevitable anatomical variations, which was often not possible with the previously published method. Identification and visualisation of the thoracic duct was aided by the administration of a small lipid meal prior to surgery.

Long-chain dietary lipids are quantitatively transported to the systemic circulation by the intestinal lymphatics, therefore, the amount of triglyceride (TG)

collected in thoracic lymph should reflect the mass of ingested lipid. In order to confirm the integrity of the lymph duct cannulation, and to ensure the complete collection of thoracic lymph, the total mass of TG transported in thoracic duct lymph and the systemic plasma concentrations of TG were determined periodically. As the mass of triglyceride recovered in fasted dogs (representative of the basal levels of triglyceride production) was less than 5% of the amount of triglyceride typically recovered in fed dogs (Table 4.1), the triglyceride values reported for post-prandial dogs were not corrected for basal levels and represent the endogenous and exogenous sources of triglyceride.

Acceptance criteria for the post-prandial model included the recovery of greater than 85% (by mass) of the ingested TG lipid (present in food) in thoracic duct lymph, and correspondingly low systemic plasma TG concentrations. In the lymph cannulated dogs, plasma TG levels were similar in either fed or fasted animals and ranged between 0.3 and 0.6 mg/mL. This compares with levels in control (non-cannulated) animals of approximately 0.5-1 mg/mL under fasted conditions and 1-5 mg/mL in the post-prandial state. Therefore, the poor recovery of TG in post-prandial lymph and/or high systemic plasma TG concentrations in lymph cannulated animals (> 0.7-0.8 mg/mL) would indicate lymphatic leakage, or a failure to detect and ligate collateral lymph vessels, thereby allowing passage of lymph into the systemic circulation. These circumstances form the basis for excluding data from individual dogs. Using these criteria, the model was essentially internally validated and ensured the quality of the data from a limited number of dogs.

Lymph flow may be affected by extrinsic factors such as the dog's movements or its level of activity (i.e. resting or walking), degree of hydration and by the ingestion of food or lipid. As expected, the lymph flow rates were considerably higher in post-

prandial dogs compared with dogs in the fasted state (Figure 4.7). In the initial 3-4 h period after feeding, greater than 100 g of lymph was collected each hour, and the total mass of lymph collected over a 12 h period was 1047 ± 124 g (mean ± SD, n = 4). In contrast, the mean ± SD (n = 3) total mass of lymph collected from fasted dogs over a 10 h period was much lower at 279 ± 230 g.

The lymph TG output rates in fasted dogs and dogs fed a standard meal containing 34 g of lipid are shown in Figure 4.8. Typically, approximately 90-105% of the ingested TG was recovered in lymph over the 12 h post-dosing period. The visual appearance of lymph collected from fasted and fed dogs was also vastly different. Lymph from fasted dogs appeared clear and straw-coloured, whereas lymph collected from post-prandial dogs was milky white, indicating the presence of chylomicrons.

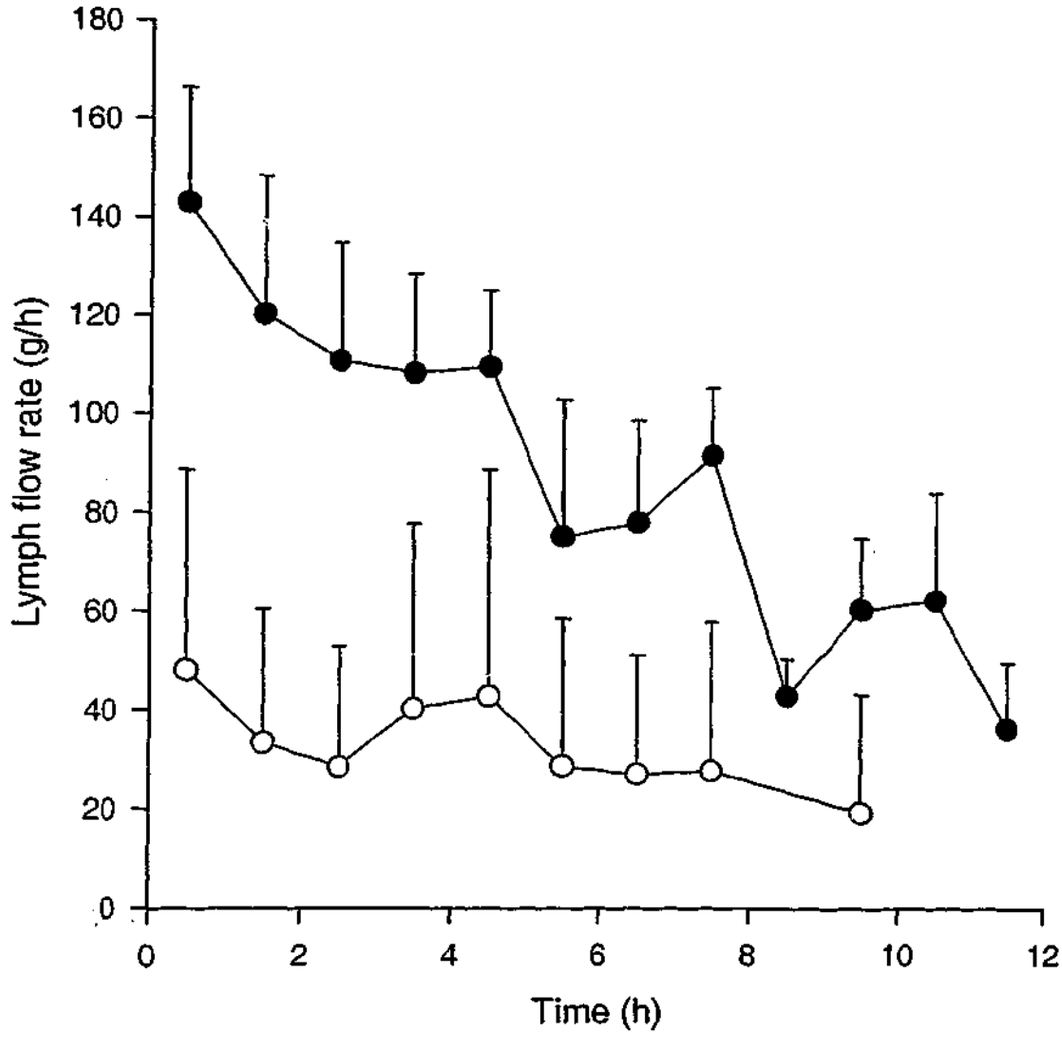

Figure 4.7 The mean (± SD) lymph flow rate in fasted (○, n = 3) greyhound dogs and in dogs fed a standard meal containing approximately 34 g lipid (●, n = 4).

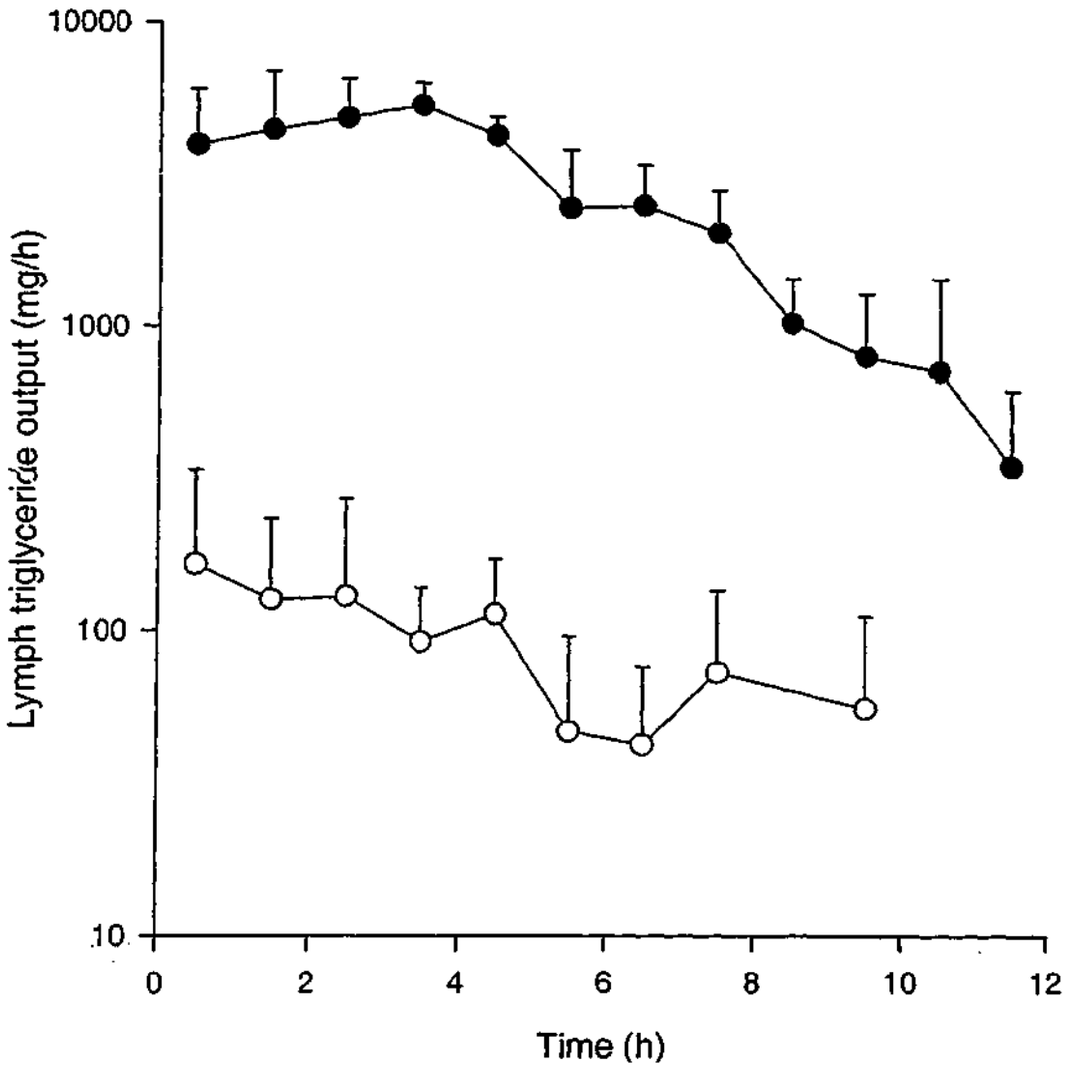

Figure 4.8 The mean (± SD) triglyceride output in fasted (○, n = 3) greyhound dogs and in dogs fed a standard meal containing approximately 34 g lipid (●, n = 4).

4.4.2 Potential redistribution of halofantrine from blood to lymph

In the current dog model, lymph was collected from the thoracic lymph duct rather than the mesenteric lymph duct due to reasons of practicality, integrity and the importance of having a conscious rather than an anaesthetized dog model. Moreover, cannulation of the mesenteric lymph duct is more surgically-involved and maintaining the cannula in position for the continuous collection of lymph would be difficult in a conscious and unrestrained dog.

However, the collection of thoracic lymph in rats has been reported to marginally overestimate the true extent of intestinal lymphatic drug transport as the thoracic duct not only collects mesenteric lymph which originates solely from the small intestine but also peripheral and hepatic lymph.[27] The potential for an orally administered drug absorbed via the portal blood to the general systemic circulation to equilibrate across capillary beds and gain access to peripheral lymph and/or the possible transfer of drug absorbed via the portal blood to hepatic lymph could contribute to an overestimation of the extent of intestinal lymphatic drug transport after oral administration (Figure 4.9). Therefore, the recovery of drug in thoracic duct lymph may not only reflect drug absorption via the intestinal lymphatics but also drug redistribution between the systemic circulation and peripheral and/or hepatic lymph. For example, Noguchi *et al.*[27] observed greater amounts of intravenously administered DDT in the thoracic lymph (3.6% of dose) of rats than the intestinal lymph (0.6% of dose). The possibility of drug redistribution between the general blood circulation and lymph has also been observed for cyclosporine,[28,29] *p*-aminosalicylic acid[30] and some lipophilic vitamins.[31] Nevertheless, considerations dictated that, in this study, lymph was collected from the thoracic duct.

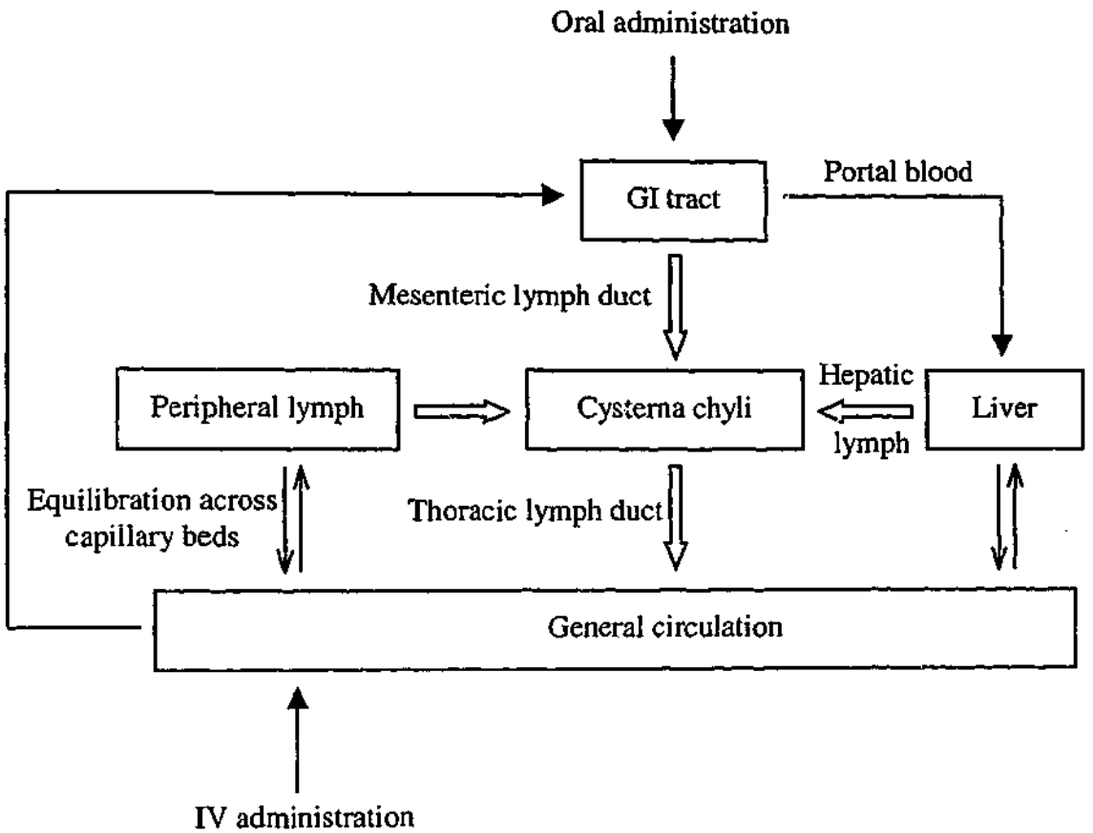

Figure 4.9 Schematic representation illustrating the potential for equilibration of drug between the general circulation and the peripheral and/or hepatic lymph. Adapted from ref. 22.

To address the issue of potential blood-lymph drug redistribution, the lymphatic transport of Hf following intravenous administration of an emulsion-based Hf formulation (2 mg/kg) to post-prandial thoracic lymph duct cannulated dogs was investigated. The study was conducted in post-prandial dogs to maximize the lipid load in lymph, thereby allowing maximal drug transfer to occur. After post-prandial intravenous administration of Hf, the redistribution of Hf from blood to lymph was extremely low, with only 1.45 ± 0.22% (mean ± SD, n = 3) of the dose recovered in thoracic duct lymph over the 12 h post-dosing period (Figure 4.10). This suggests that the recovery of drug in thoracic duct lymph after post-prandial oral administration of Hf is likely to be quantitatively the result of drug absorption via the intestinal lymphatics as drug equilibration across capillary beds is minimal for Hf.

4.4.3 Pharmacokinetic profile of halofantrine after intravenous administration

In addition to assessing potential drug redistribution from blood to lymph after intravenous administration of the emulsion-based Hf formulation to post-prandial lymph fistulated dogs, the pharmacokinetic profile of Hf was also determined to enable the proportion of drug absorbed into the systemic circulation via the portal blood after oral administration to be estimated. The mean (± SD, n = 3) Hf plasma concentration versus time profile after intravenous administration of Hf (2 mg/kg) is presented in Figure 4.11. The dose normalized Hf plasma $AUC^{0\text{-}24h}$ and Hf clearance were 6993 ± 330 ng.h/mL and 475 ± 12.4 mL/h/kg, respectively. Consistent with previous studies, the plasma concentration of the major metabolite, *N*-desbutylhalofantrine (Hfm), following intravenous administration was generally very low, near the minimum quantifiable limit of 10 ng/mL.

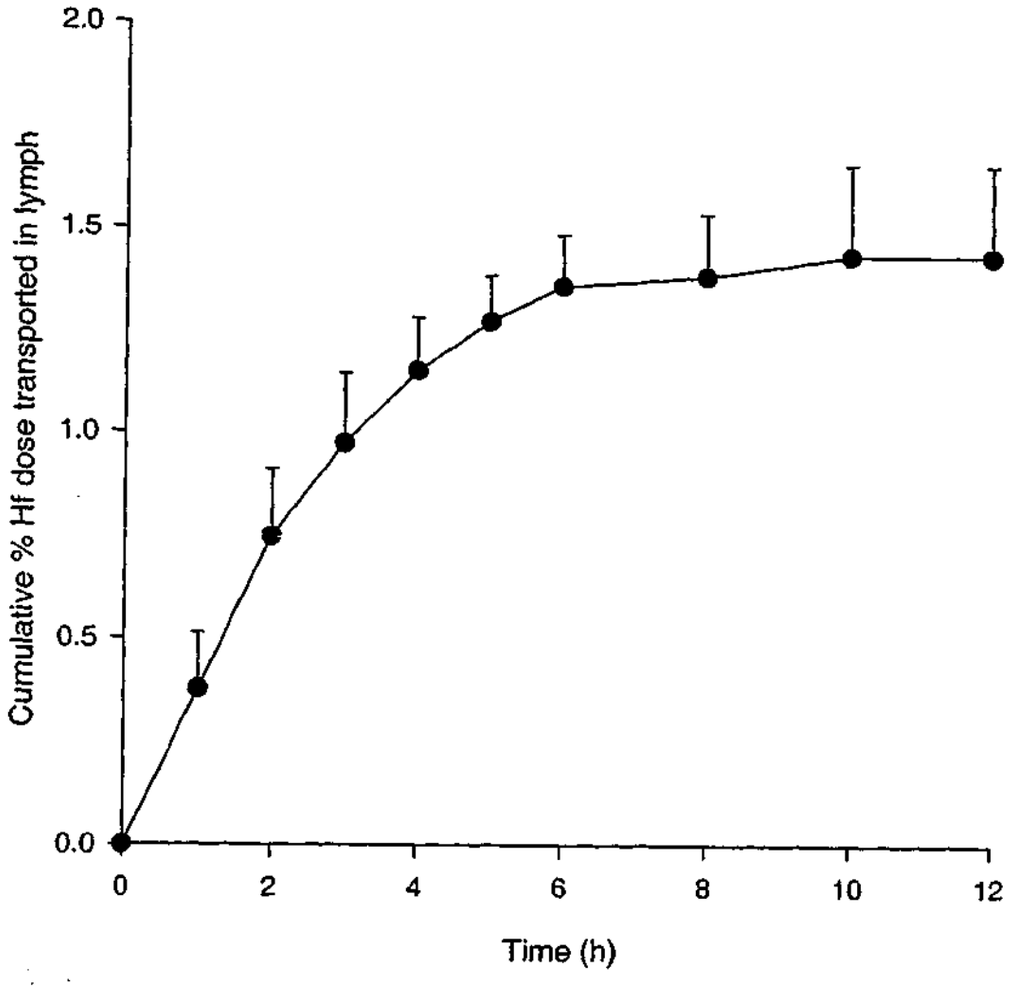

Figure 4.10 Cumulative lymphatic transport of halofantrine (Hf) (% dose, mean ± SD, n = 3) after intravenous administration of an emulsion-based formulation of Hf (2 mg/kg) to post-prandial thoracic lymph duct cannulated dogs.

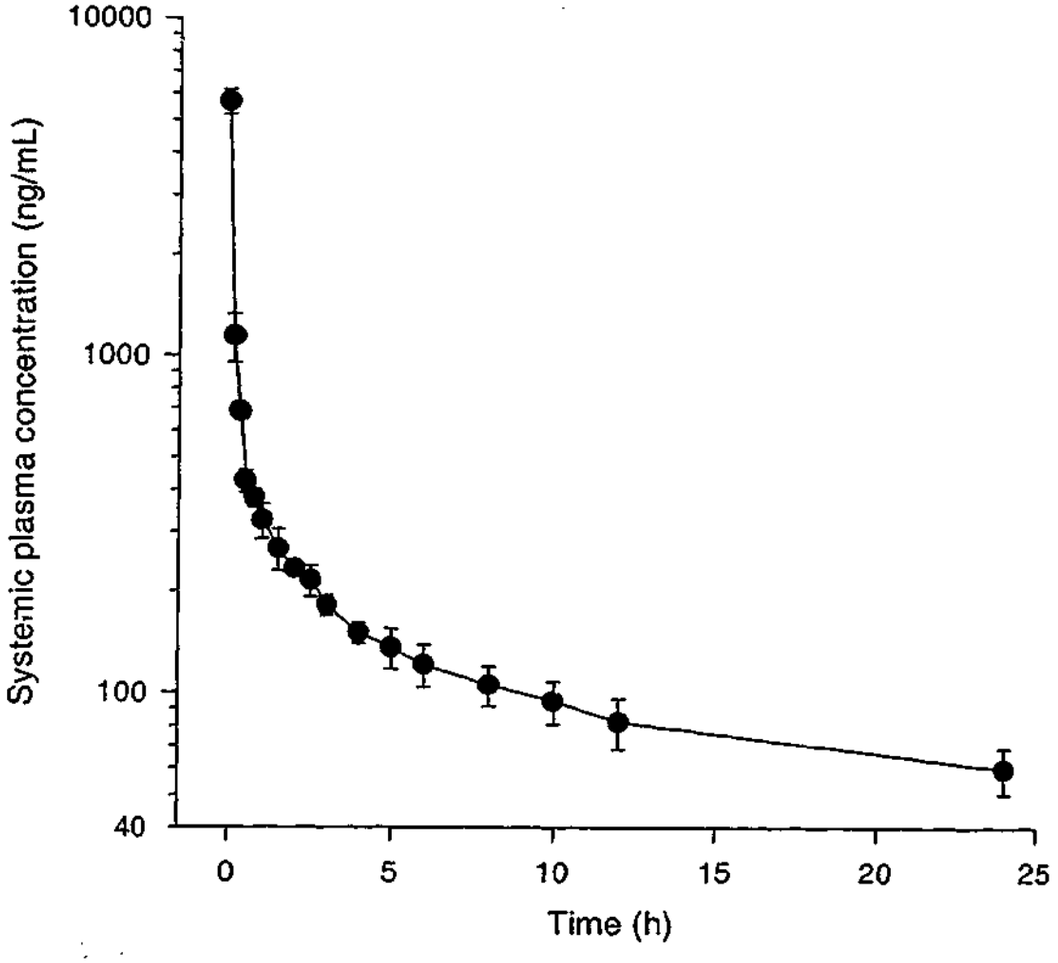

Figure 4.11 Systemic plasma concentration versus time profile of Hf (mean ± SD, n = 3) after intravenous administration of an emulsion-based Hf formulation (2 mg/kg) to post-prandial thoracic lymph duct cannulated dogs.

4.4.4 Intestinal lymphatic drug transport after oral administration of Hf base to fasted and post-prandial dogs

4.4.4.1 Cumulative lymphatic drug transport

The cumulative lymphatic transport of Hf after fasted or post-prandial administration of 100 mg Hf base in a non-lipid oral dose form is presented in Figure 4.12. The mean (± SD, n = 3) lymphatic transport of Hf in the fasted dogs was extremely low at 1.3 ± 0.7% of the administered dose. This is presumably due to the absence of an appropriate lipid source which is typically required to promote intestinal lymphatic drug transport. However, considering that the absolute oral bioavailability of Hf was only 8.6 ± 5.3% after fasted administration of 250 mg Hf.HCl, the contribution of intestinal lymphatic transport was quite significant.

After post-prandial oral administration, the mean (± SD, n = 4) cumulative lymphatic transport of Hf was 54 ± 8.2% of the administered dose. Greater than 90% of the post-prandial lymphatic transport of Hf occurred within 4 h of dosing (Table 4.1). In comparison with previous lymphatic transport studies in fasted conscious rats where approximately 10% of the orally administered dose of 2 mg Hf base in 50 μL lipid was transported via the intestinal lymphatics in the initial 4 h post-dosing period, the present data demonstrate that intestinal lymphatic drug transport in the dog was more extensive and rapid compared with the rat model. As the maximum oral bioavailability of Hf in dogs has previously been reported to be approximately 80%,[2] the current data indicate that intestinal lymphatic transport is likely to be the predominant absorption pathway for Hf after post-prandial administration.

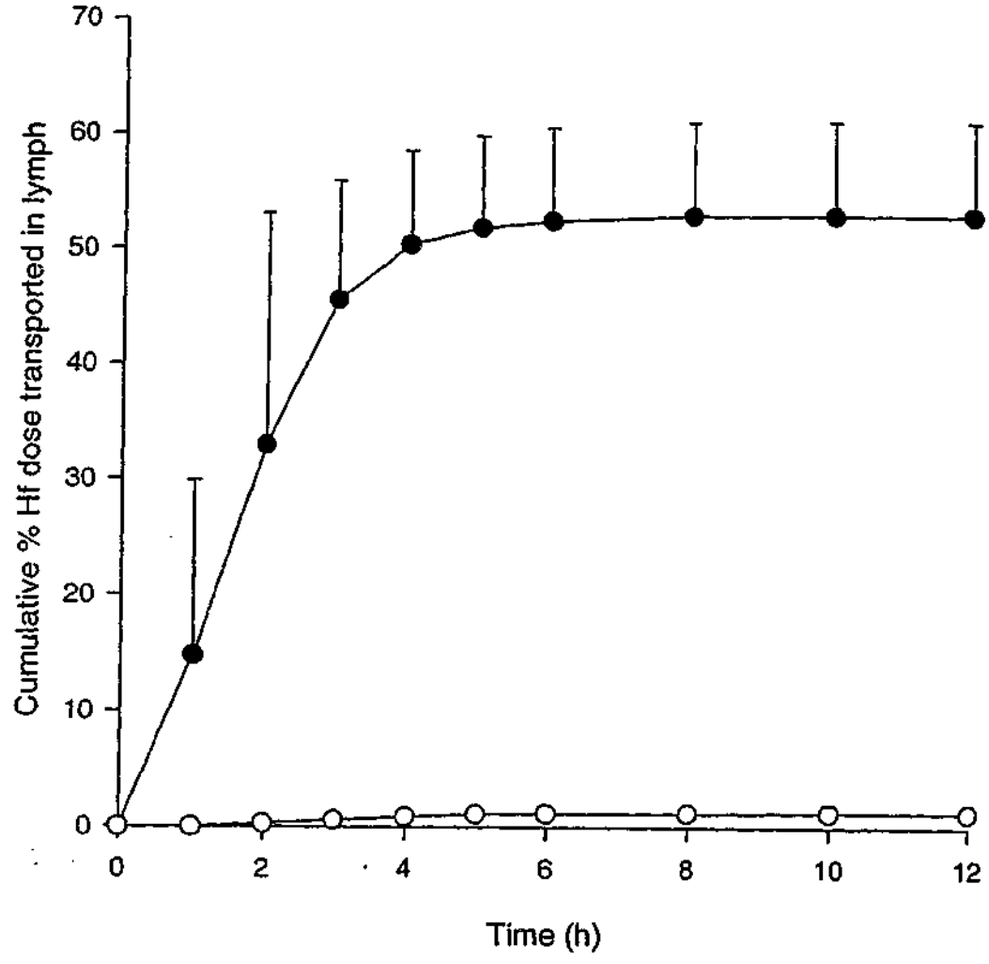

Figure 4.12 Cumulative lymphatic transport of halofantrine (Hf) (% dose, mean ± SD) in thoracic lymph duct cannulated dogs after fasted (○, n = 3) or post-prandial (●, n = 4) administration of 100 mg Hf base.

Table 4.1 Mean (± SD) cumulative lymphatic transport of halofantrine (Hf) and triglyceride (TG), and lymph flow, after fasted or post-prandial administration of 100 mg Hf base to thoracic lymph duct cannulated dogs

	Cumulative transport of Hf (% dose)			Cumulative mass of TG in lymph (g)			Cumulative lymph flow (g)		
Post-dosing Period	0-4 h	4-8 h	8-12 h[a]	0-4 h	4-8 h	8-12 h[a]	0-4 h	4-8 h	8-12 h[a]
Fasted (n = 3)	0.9 ± 0.3	1.2 ± 0.6	1.3 ± 0.7	0.3 ± 0.1	0.4 ± 0.2	0.5 ± 0.4	174 ± 97	246 ± 178	279 ± 230
Fed (n = 4)	50.7 ± 8.2	53.7 ± 8.2	54.0 ± 8.2	18.4 ± 6.7	30.6 ± 5.2	33.6 ± 3.5	483 ± 86	841 ± 87	1047 ± 124

[a] Final lymph collection period for fasted dogs was 8-10 h.

4.4.4.2 *Association of halofantrine with lymph lipoproteins*

Hf has been reported to bind significantly to human and beagle plasma lipoproteins and the extent of binding was markedly higher in post-prandial plasma compared with fasted plasma. Increased association of Hf with TG rich plasma lipoproteins has been implicated in the decreased post-prandial clearance of Hf. It has also been suggested that the mechanism of association between Hf and plasma lipoproteins is primarily via solubilization in the apolar lipid core (i.e. TG and cholesterol ester) of the lipoproteins.[23,32]

Highly lipophilic compounds transported by the intestinal lymphatics typically associate with the chylomicron fraction of lymph. In contrast, only small quantities of lymphatically transported drugs are likely to associate with the VLDL fraction.[7] Accordingly, the distribution of Hf between different lipoprotein fractions in post-prandial lymph was determined. The method employed to isolate the lipoprotein fractions afforded good separation as depicted in Figure 4.13 where a typical separation profile of post-prandial lymph monitored using TG and total cholesterol concentrations present in individual fractionation aliquots is shown. The percentage distribution of Hf between lipoprotein fractions in lymph collected from post-prandial dogs (n = 2) in the 1 to 4 h post-dosing period is presented in Table 4.2. In the present study, greater than 98% of lymphatically transported Hf was associated with the chylomicron fraction of lymph. The association of Hf with the chylomicron fraction was greater in this study compared to previous chylomicron-based lymphatic transport data obtained in fasted rats where ~ 80% of Hf was associated with the chylomicron fraction when Hf was co-administered with lipid.[11] This difference may be due to the administration of a higher lipid load (present in food) resulting in an increased formation of chylomicrons (the primary lipoprotein responsible for the transport of exogenous TG) in the post-prandial

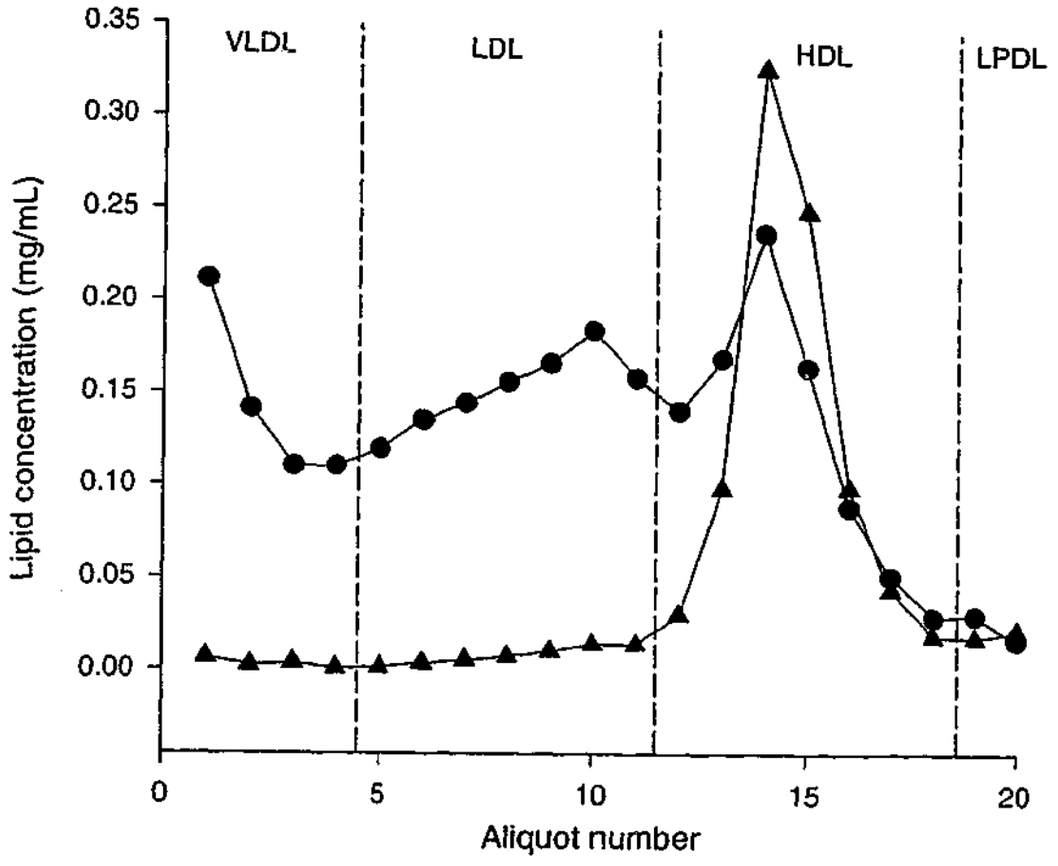

Figure 4.13 Representative profile of triglyceride (●) and total cholesterol (▲) concentrations as a function of fractionation aliquot obtained after density gradient centrifugation of post-prandial lymph (without chylomicrons). Aliquots 1-4 were combined for the very low density lipoprotein (VLDL) fraction, aliquots 5-11 for the low density lipoprotein (LDL) fraction, aliquots 12-18 for the high density lipoprotein (HDL) fraction and aliquots 19-20 were designated the lipoprotein deficient lymph fraction (LPDL).

Table 4.2 Percentage distribution of halofantrine between different lipoprotein fractions in thoracic duct lymph collected in the 1-4 h post-dosing period from two individual post-prandial dogs administered 100 mg Hf base

	Percentage distribution of halofantrine					
	Post-dosing period (Dog 1)			Post-dosing period (Dog 2)		
Lipoprotein fraction[a]	**1-2 h**	**2-3 h**	**3-4 h**	**1-2 h**	**2-3 h**	**3-4 h**
Chylomicron	98.98	99.32	98.85	99.26	98.82	98.66
VLDL	0.010	0.0	0.267	0.024	0.046	0.041
LDL	0.062	0.028	0.118	0.079	0.068	0.105
HDL	0.340	0.282	0.366	0.262	0.529	0.736
LPDL	0.611	0.374	0.403	0.375	0.538	0.461
% Recovery[b]	84.60	94.28	91.80	87.79	90.85	92.40

[a] VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LPDL, lipoprotein deficient lymph.

[b] Recovery was defined as the % mass of drug recovered after lymph fractionation divided by the mass of drug present in the original unfractionated lymph sample.

dogs. Not surprisingly, analysis of the lipid content in the lipoprotein fractions revealed that greater than 99% of the total TG content in lymph was also present in the chylomicron fraction. The amount of Hf present per unit mass of TG lipid in the chylomicrons ranged between 0.25 and 4.14 mg/g. These data indicate the importance of the formation and transport of chylomicrons for the intestinal lymphatic transport of Hf.

4.4.4.3 Lymphatic transport rates of halofantrine and triglyceride

The rates of post-prandial lymphatic transport of Hf and TG (comprising both endogenous and exogenous TG sources) are presented in Figure 4.14. The peak rate of Hf transport occurred during the 2-3 h post-dosing period and the majority of drug transport was complete within 3-4 h of administration (Figure 4.14). The maximal rates of TG transport occurred over a 5 h post-dosing period, after which the rate of TG transport gradually declined. The cumulative post-prandial TG transport in the 12 h period post-dosing was 33.6 ± 3.5 g (mean ± SD, n = 4) which accounted for approximately 100% of lipid present in the ingested food (Table 4.1). In fasted dogs, TG transport was much lower at 0.5 ± 0.4 g (mean ± SD, n = 3), reflecting basal transport levels arising from endogenously derived lipid.

When comparing the relative rates of Hf and TG transport after post-prandial administration (Figure 4.14), it is interesting to note that their transport appeared linked for the initial 2-3 h, after which the rate of Hf transport decreased in spite of substantial and continuing TG transport. This discontinuity, occurring approximately 3 h post-dosing, represents a decrease in Hf concentration per unit mass of TG. This observation has a number of important implications. Firstly, the mass of TG lipid required to support significant lymphatic transport of Hf during the 2-3 h post-dosing period was

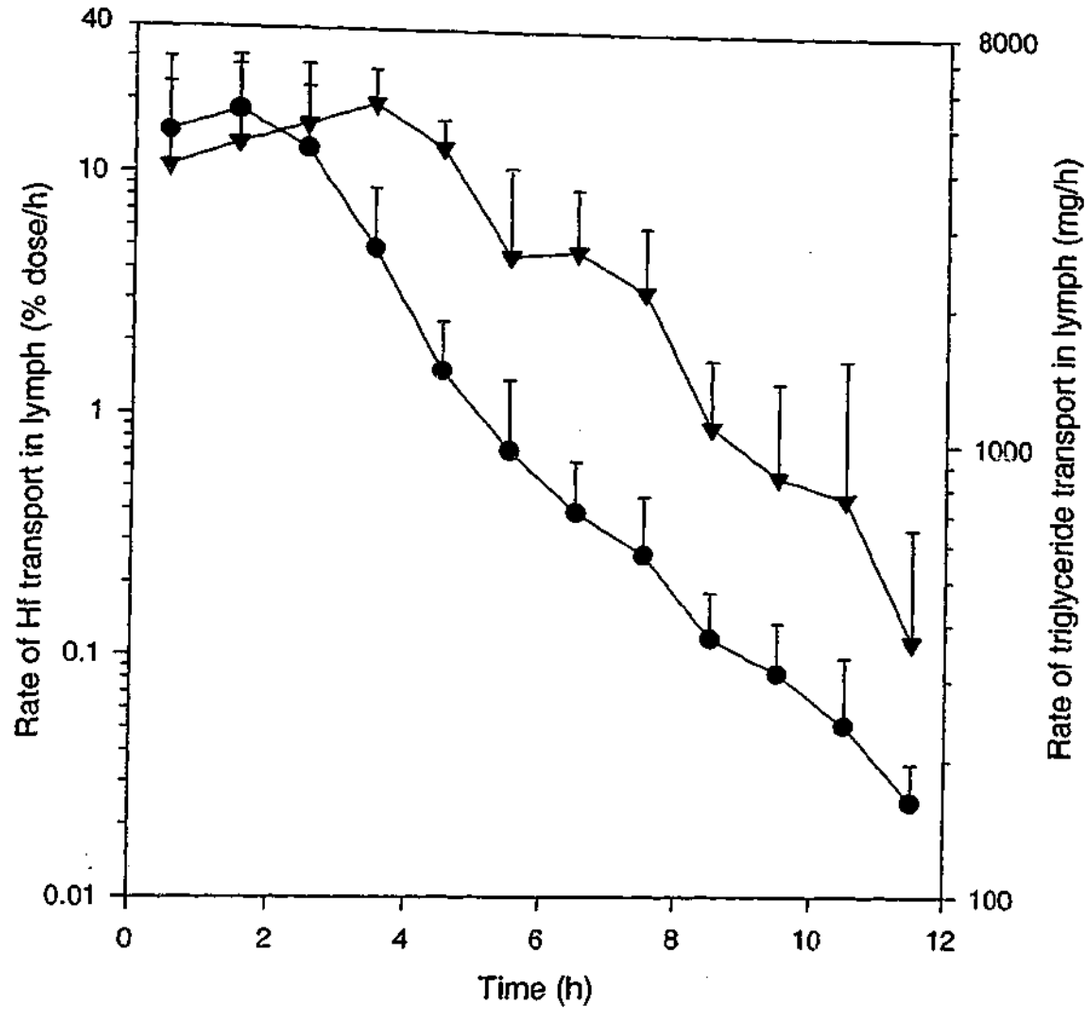

Figure 4.14 The mean (± SD, n = 4) rate of lymphatic transport of halofantrine (Hf) (●, expressed as % Hf dose transported per hour), and triglyceride (▼, expressed as mg triglyceride per hour) after post-prandial administration of 100 mg Hf base to thoracic lymph duct cannulated dogs.

approximately 30% of that present in the administered meal (and hence, quantities of TG much less than present in a "meal" can support lymphatic transport). Secondly, as lymphatic transport of Hf was rapid, and linked with TG transport, it may be possible to develop formulation and lipid selection strategies that further enhance transport (e.g. achieving a high Hf concentration in lipid digestion and enterocyte lipid processing domains). Thirdly, the discontinuity between Hf and TG transport requires a change in either processing, or kinetic factor(s), in order to produce the relative decrease in Hf per mass of TG lipid. It is likely that the discontinuity arises from a decrease in Hf concentration within the enterocyte lipid-processing microdomains, reflecting a decrease in the mass of Hf remaining to be absorbed (and hence, a decreased concentration within pre-absorptive lipid digestion phases) and the continual partitioning of Hf from lipid microdomains into the cytosol of the enterocyte, and eventual absorption via portal blood.

4.4.4.4 Effect of lymph flow on the lymphatic transport of halofantrine

The extent of rehydration in the animal may affect lymph flow and the rate of appearance of intestinal lymph lipoproteins after lipid administration. Tso *et al.*[33] have demonstrated that the lymph flow in hydrated rats is considerably faster than dehydrated rats and increased lymph flow resulted in a decrease in the time taken for chylomicrons to appear in lymph. However, despite the differences in the rate of formation and transport of lymph lipoproteins between hydrated and non-hydrated rats, the overall extent of lymph lipid transport was not dissimilar.

In this study, a similar schedule for administering fluids was adhered to at all times to avoid any possible variation in hydration. Figure 4.15 shows the post-prandial lymph flow rate and rate of Hf transport in lymph. The relationship between lymph flow

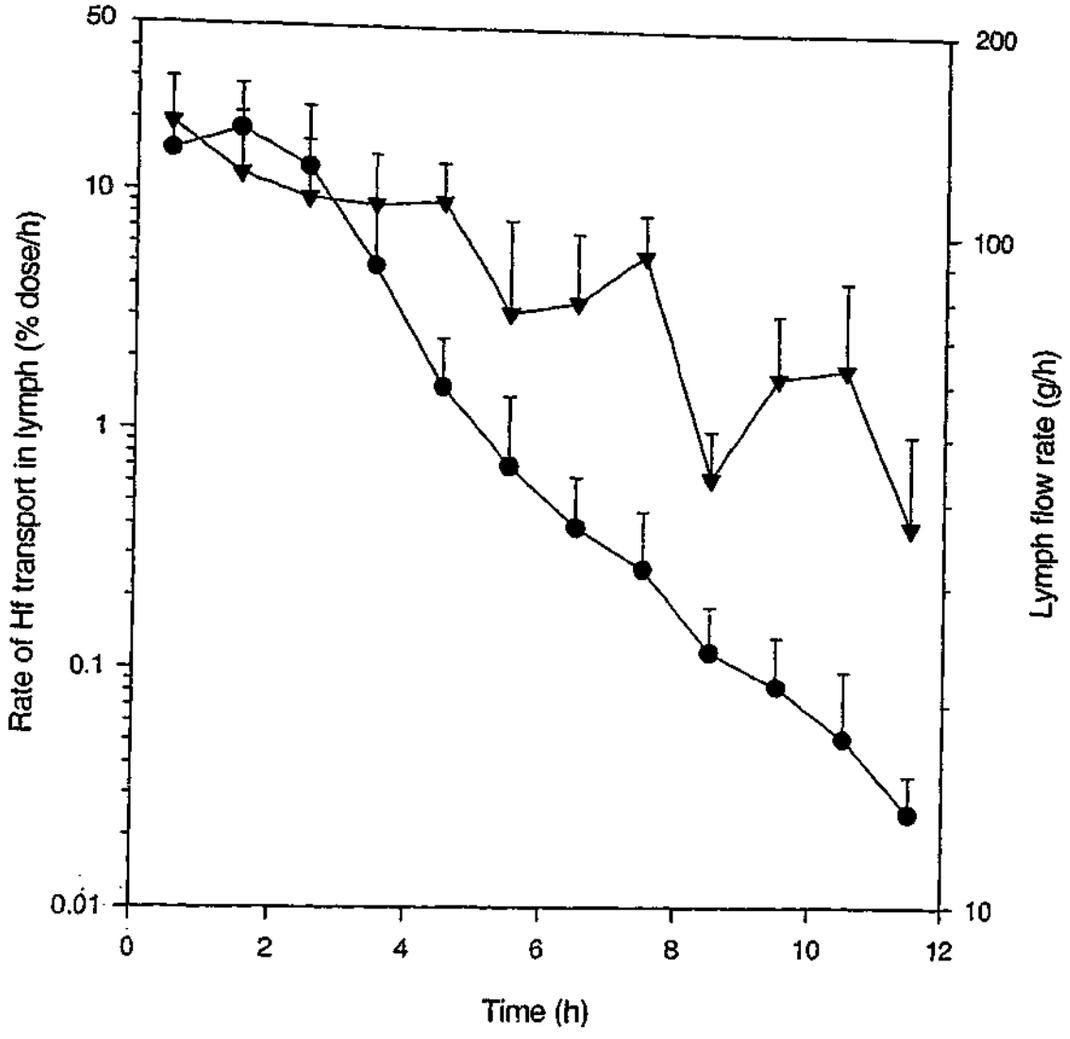

Figure 4.15 The mean (± SD, n = 4) rate of lymphatic transport of halofantrine (●, Hf) and lymph flow rate (▼) after post-prandial administration of 100 mg Hf base to thoracic lymph duct cannulated dogs.

and the transport of Hf in lymph does not appear to be as clear as the one between rate of triglyceride transport and lymph transport of Hf. The rate of lymphatic transport of Hf declined 2-3 h post-dosing in spite of maximal lymph flow rates occurring over the 5 h post-dosing period.

Presented in Figure 4.16 are the concentration of Hf in lymph and lymph flow rate after post-prandial administration of Hf base. It is apparent that the concentration of Hf in lymph did not correlate directly with the lymph flow rate. However, the peak Hf lymph concentrations observed 2-3 h post-dosing coincided with the time period where the rate of Hf transport in lymph was also maximal. In comparison to typical systemic Hf plasma concentrations of 0.1 to 1 μg/mL observed after oral administration, the maximal post-prandial Hf lymph concentrations were several orders of magnitude higher. The clinical implications of this are discussed in section 4.5.

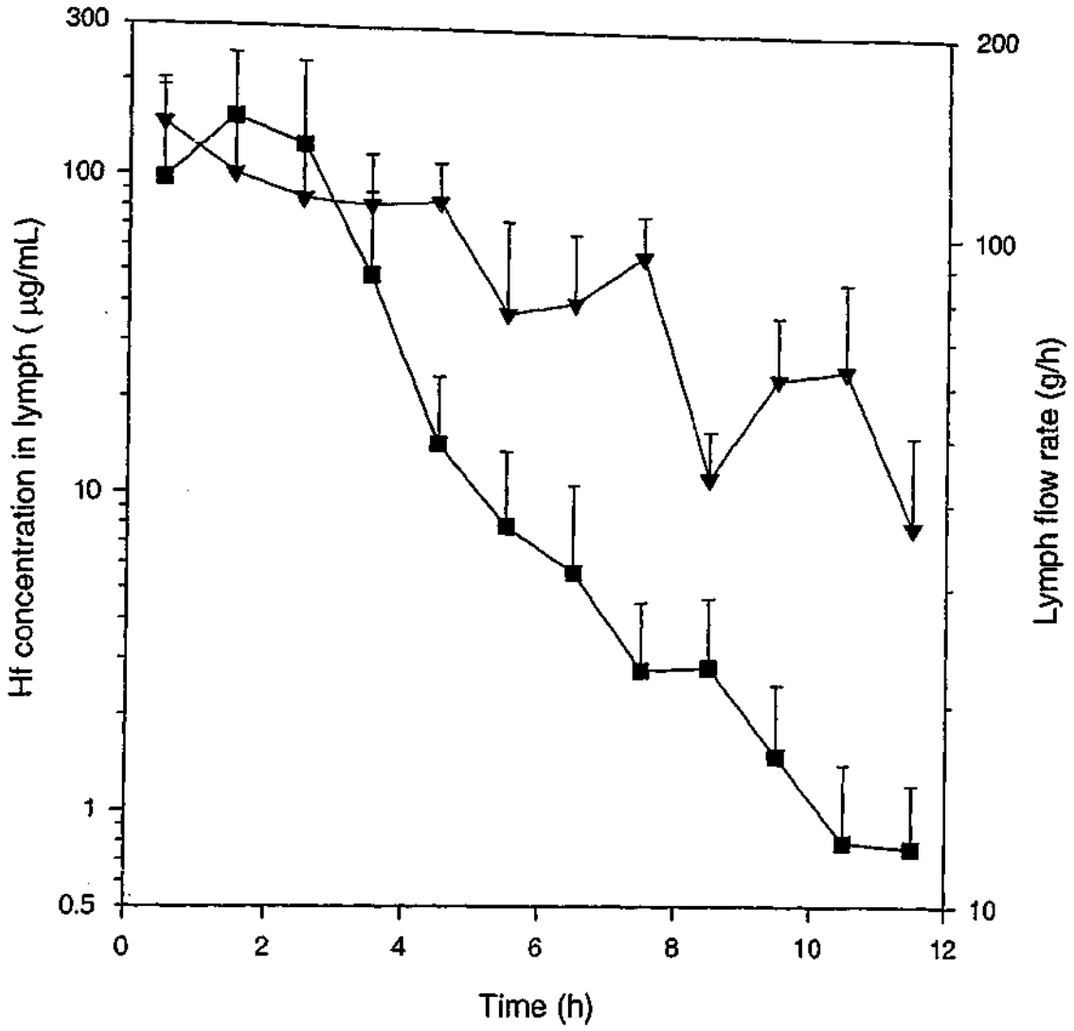

Figure 4.16 The mean (± SD, n = 4) concentration of halofantrine (Hf) in lymph (■) and lymph flow rate (▼) after post-prandial administration of 100 mg Hf base to thoracic lymph duct cannulated dogs.

4.4.5 Absorption of halofantrine via the portal route after oral administration

The portal and systemic plasma concentration versus time profiles of Hf and Hfm in the thoracic lymph duct cannulated dogs, and the relevant pharmacokinetic parameters, after fasted and post-prandial administration of 100 mg Hf base are presented in Figure 4.17 and Table 4.3, respectively. The systemic plasma T_{max} values for Hf were significantly different between fasted and post-prandial states, with the more rapid post-prandial T_{max} value likely reflecting enhanced dissolution and solubilization of Hf within the post-prandial intestinal milieu.[2] Additionally, the rapid post-prandial lymphatic transport of Hf would decrease its absorption time profile and mass remaining to be absorbed, leading to a lower T_{max} relative to the fasted state.

In lymph duct cannulated dogs, there was a clear trend towards higher portal and systemic plasma Hf AUC values after fasted compared with post-prandial administration, however, these differences were not statistically significant at the 5% level. This lack of significance is most likely a consequence of the limited size of the parallel study design (albeit, the study adequately assessed lymph transport as quantitation of TG transport which is essentially internally consistent for each dog), and the abbreviated blood sampling schedule (due to the 24 h study period) which prevented the adequate definition of the terminal plasma profiles of Hf and Hfm and the associated differences between fasted and post-prandial administration. The Hf AUC values also appeared to be more variable after fasted administration than after post-prandial administration, this may be a reflection of the more variable rate and extent of drug solubilization in the fasted state.

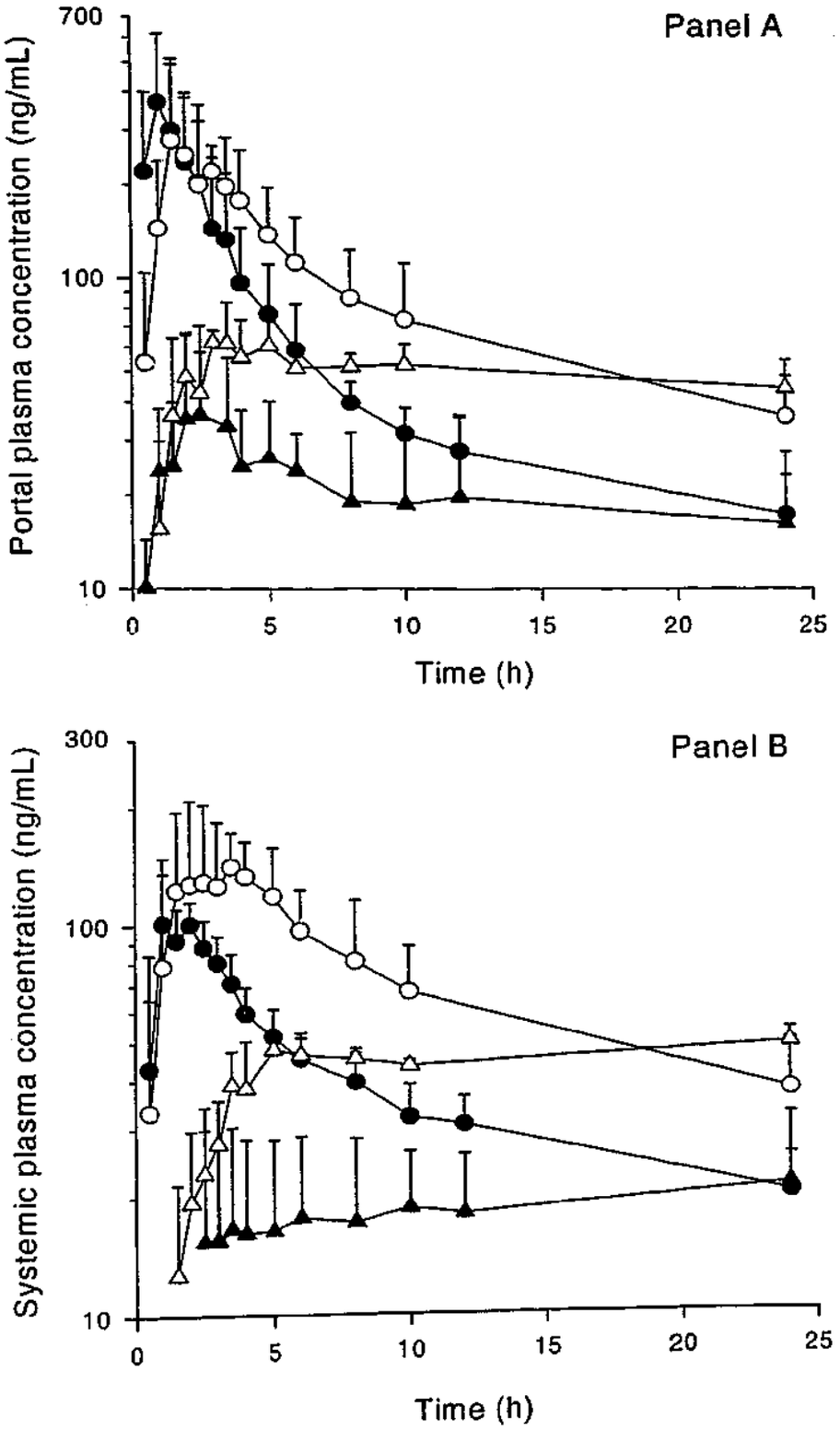

Figure 4.17 Portal plasma concentration (panel A) and systemic plasma concentration (panel B) versus time profiles of Hf (○, ●) and Hfm (△, ▲) after fasted (open symbols, n = 3) or post-prandial administration (closed symbols, n = 4) of 100 mg Hf base to thoracic lymph duct cannulated dogs. Data are presented as mean ± SD.

Table 4.3 Mean (± SD) portal and systemic plasma pharmacokinetic parameters of halofantrine (Hf) and *N*-desbutylhalofantrine (Hfm) after fasted (n = 3) or post-prandial (n = 4) administration of 100 mg Hf base to thoracic lymph duct cannulated dogs

	Pharmacokinetic parameter	Portal Plasma		Systemic Plasma	
		Fasted	Post-prandial	Fasted	Post-prandial
Hf	C_{max} (ng/mL)	364 ± 149	493 ± 197	154 ± 52.8	124 ± 2.7
	T_{max} (h)	2.2 ± 0.7	1.4 ± 0.7	3.4 ± 0.8	1.3 ± 0.5[a]
	$AUC^{0\text{-}24h}$ (ng.h/mL)	2697 ± 1735	1522 ± 481	2073 ± 1176	935 ± 112
Hfm	C_{max} (ng/mL)	74.0 ± 5.1	45.8 ± 25.9	52.0 ± 3.7	27.1 ± 11.2[a]
	T_{max} (h)	3.9 ± 1.0	3.0 ± 2.1	13.2 ± 10.3	11.8 ± 8.8
	$AUC^{0\text{-}24h}$ (ng.h/mL)	1183 ± 199	491 ± 304[a]	1003 ± 13.7	418 ± 201[a]
	Hfm/Hf AUC ratio	0.55 ± 0.26	0.33 ± 0.22	0.59 ± 0.28	0.45 ± 0.20

[a] Significantly different ($p < 0.05$) compared with the corresponding fasted value.

The total bioavailability of Hf in the lymph duct cannulated dogs is presented in Table 4.4. This is defined as the percentage dose recovered over 12 h in thoracic duct lymph plus the proportion of the dose absorbed via the portal blood into the systemic circulation (relative to an IV control). After post-prandial oral administration, the total availability of Hf was 67.1 ± 8.7% with intestinal lymphatic transport contributing to approximately 80% of the total dose absorbed. Administration in the fasted state resulted in significantly lower ($p < 0.05$) and more variable availability of Hf, and absorption of Hf was primarily via the portal blood (Table 4.4). Therefore, the higher fasted Hf plasma profiles observed in Figure 4.17 were likely to be due to a greater amount of absorbed drug leaving the enterocyte via the portal blood in the fasted state (as lymph transport only accounted for 1.3% of the dose). In contrast to the post-prandial situation where 54% of the dose was lymphatically transported, the amount of Hf available for absorption via the portal blood was substantially decreased, although the proportion of drug absorbed into the systemic circulation via the portal route was not significantly different after fasted or fed administration. Therefore, the current data suggest that intestinal lymphatic transport was the predominant absorption pathway after post-prandial administration of Hf base, whereas, after fasted administration, the majority of drug was absorbed non-lymphatically via the portal route.

Table 4.4 Total availability of halofantrine (Hf, mean % dose ± SD) estimated as the percentage dose appearing in thoracic duct lymph and percentage dose absorbed into the systemic circulation (via portal blood) after fasted or fed administration of 100 mg Hf base to thoracic lymph duct cannulated dogs

	Lymphatic transport[a]	**Systemic availability[b]**	**Total Hf availability (Lymph + Blood)**
Fasted (n = 3)	1.3 ± 0.7	30.2 ± 18.7	31.5 ± 18.3
Fed (n = 4)	54.0 ± 8.2[c]	13.1 ± 1.6	67.1 ± 8.7[c]

[a] Cumulative % dose of Hf recovered in thoracic duct lymph over the 10 h post-dosing period in fasted dogs and over the 12 h post-dosing period in fed dogs.

[b] The percentage dose of Hf absorbed directly into the systemic circulation via portal blood (i.e. non-lymphatic absorption) was based on the systemic plasma $AUC^{0\text{-}24h}$ relative to the $AUC^{0\text{-}24h}$ obtained after IV administration (section 4.4.3).

[c] Significantly different ($p < 0.05$) from fasted administration.

4.4.6 Enterocyte-based metabolism of halofantrine

Hf is metabolized to its major (and equipotent) metabolite, *N*-desbutylhalofantrine (Hfm), by *N*-dealkylation. *In vitro*[34,35] and *in vivo* (Chapter 3) studies indicate that CYP3A4 is largely responsible for this metabolic conversion, with the liver, and possibly the intestine, being important sites of metabolism. Propofol, a short-acting anaesthetic agent used to anaesthetize the dogs for surgery has been reported to inhibit CYP3A4 activity.[36,37] However, the *in vitro* human liver microsomal studies conclude that any possible inhibitory effects of propofol on CYP3A4 are unlikely to be significant at clinical doses, as 10-fold higher doses were required to produce a significant inhibitory effect. Furthermore, in comparison to selective inhibitors such as ketoconazole, propofol is at least a 100-fold less potent. In this study, a single and low dose of propofol was administered by intravenous injection to induce anaesthesia, and the dogs were allowed a recovery period of 12-16 h prior to Hf administration. Therefore, it is extremely unlikely that propofol would have had a significant effect on the CYP3A4 mediated metabolism of Hf.

In the present study, Hfm portal plasma concentrations were consistently higher than systemic Hfm plasma concentrations during the post-dosing absorption period (Figure 4.17). When a non-parametric Sign test was applied at a 1% confidence level, the differences in portal and systemic Hfm plasma concentrations were significantly greater than zero, consistent with likely enterocyte-based conversion of Hf to Hfm. However, further studies are required to confirm this tentative conclusion by excluding the possibilities of luminal formation of Hfm and/or reabsorption of biliary recycled Hfm, neither of which are expected.

An objective of the current study was to explore the possible factors responsible for the observed decrease in the extent of post-prandial metabolism of Hf.[1,2] The portal

and systemic plasma Hfm AUC^{0-24h} values in Table 4.3 were significantly higher in the fasted state compared with post-prandial administration, however, when the data were corrected for the differing amounts of Hf absorbed under fasted and post-prandial conditions (i.e. by calculating the corresponding Hfm/Hf AUC^{0-24h} values), the ratios were no longer statistically significant. Hence, although this preliminary study indicates that Hf is likely to be metabolized to Hfm during passage through the enterocyte, the degree of conversion was not significantly influenced by administration of Hf in either fasted or post-prandial states. Therefore, to reconcile the previously observed 2.4-fold and 6.8-fold post-prandial decrease in plasma Hfm/Hf AUC values in humans and dogs, respectively, it appears that post-prandial recruitment of intestinal lymphatic transport of Hf (which bypasses hepatic first-pass metabolism) is a likely key factor underlying the observed changes in metabolic profile of Hf.

4.5 CONCLUSIONS AND IMPLICATIONS

In summary, a new and robust triple-cannulated conscious dog model has been developed for the simultaneous assessment of absorption, enterocyte-based metabolism and lymphatic transport of candidate lipophilic drugs under realistic pre- and post-prandial conditions. The model enables the rapid identification of lipophilic drug candidates which may be substrates for intestinal lymphatic transport and may also assist in the development of lipid-based formulations to maximize oral drug bioavailability. Unlike the rat model, this model allowed the oral administration of dose forms that are clinically relevant to humans in representative fed and fasted states.

Using the dog model to study Hf absorption, lymphatic transport accounted for 1.3% and 54% of an administered 100 mg dose of Hf base after fasted and fed oral administration, respectively. Furthermore, after post-prandial administration, intestinal

lymphatic transport contributed to approximately 80% of the total dose absorbed. These data suggest that significant post-prandial lymphatic transport of Hf is an important factor contributing to the observed decrease in the post-prandial metabolism of Hf in dogs and humans.

From a clinical perspective, elevated post-prandial plasma Hf concentrations have been implicated in QTc interval prolongation in some patients.[38,39] In this study maximal post-prandial Hf lymph concentrations of 0.1-0.3 mg/mL were observed (Figure 4.16). When compared with "typical" systemic Hf plasma concentrations of ~ 0.1-1 μg/mL after oral administration, it is possible that post-prandial lymphatic transport of Hf may be a contributing factor to the observed effects on the QTc interval (as thoracic lymph empties into the systemic circulation at the junction of the jugular vein directly above the heart). However, further studies will be required to investigate this putative linkage.

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CHAPTER 5

A PHYSICOCHEMICAL BASIS FOR THE EXTENSIVE INTESTINAL LYMPHATIC TRANSPORT OF POORLY LIPID SOLUBLE, HALOFANTRINE HYDROCHLORIDE, AFTER POST-PRANDIAL ADMINISTRATION TO DOGS

5.1 INTRODUCTION

In chapter 4, a triple-cannulated conscious dog model which enabled the simultaneous examination of intestinal lymphatic and non-lymphatic (i.e. portal blood) absorption of candidate lipophilic drugs under realistic pre- and post-prandial conditions was developed. Using this dog model, intestinal lymphatic transport was shown to be a significant contributor to the oral bioavailability of Hf. After post-prandial administration of the highly lipid soluble free base form of halofantrine (Hf base), intestinal lymphatic transport accounted for 54% of the administered 100 mg dose compared to 1.3% following fasted administration. The extensive lymphatic transport of Hf was not surprising considering the high calculated log P (~ 8.5) and high solubility of Hf base in triglyceride lipids (~ 50 mg/mL), both of which are important parameters for significant intestinal lymphatic drug transport.[1]

Based on its low lipid solubility (~ 1 mg/mL), the commercially available hydrochloride salt of Hf (Hf.HCl) was not expected to be a likely candidate for intestinal lymphatic transport. However, previous studies in conscious rats have demonstrated that after the oral administration of 2 mg Hf.HCl with 50 μL of a lipid vehicle comprising oleic acid and glycerol monooleate (2:1 molar ratio), approximately 5% of the dose was lymphatically transported.[2] Although the extent of lymphatic drug transport following administration of Hf.HCl was significantly lower than that after the administration of a similar lipid vehicle containing Hf base (4.6% vs 20% of 2 mg dose, respectively), the lymphatic transport of Hf.HCl was surprisingly high considering the limited solubility of Hf.HCl in triglyceride lipid. A potential explanation for the higher than expected lymphatic transport of the poorly lipid soluble Hf.HCl is the partial conversion of solubilized Hf.HCl to the free base form of Hf. However, being a tertiary amine, the

pKa of Hf is expected to be greater than 10,[3] and at intestinal pH values, less than 0.1% of Hf is likely to be present as the free base.

Given that the earlier documented food effects were observed following oral administration of Hf.HCl,[4,5] the potential intestinal lymphatic transport of Hf after post-prandial administration of Hf.HCl was investigated in the present study using the triple-cannulated dog model described in the previous chapter. Interestingly, the cumulative lymphatic drug transport observed after post-prandial administration of Hf.HCl was unexpectedly high (47% of administered 107 mg dose) and not significantly different from the highly lipid soluble Hf base. In order to elucidate the mechanism by which extensive intestinal lymphatic drug transport may occur after post-prandial administration of the poorly lipid soluble Hf.HCl, additional studies investigating the physicochemical properties of Hf.HCl were conducted.

5.2 EXPERIMENTAL METHODS

5.2.1 Materials

Hf.HCl and Hf base were supplied by SmithKline Beecham Pharmaceuticals (King of Prussia, PA, USA and Mysore, India, respectively). For the formulation of a dry capsule containing Hf.HCl, Avicel® (microcrystalline cellulose) and Explotab® (sodium starch glycolate) were purchased from Asahi Chemical Industry (Osaka, Japan) and H. Bleakley Pty. Ltd. (NSW, Australia), respectively. Sodium taurocholate (NaTC) and *N*-methyl-2-pyrrolidone (99+%, HPLC grade) were purchased from Sigma Chemicals (St Louis, MO, USA). Purified egg yolk lecithin and super refined soybean oil (SBO) were donated by Pharmacia (Sweden) and Croda Surfactants (Victoria, Australia), respectively. All other chemicals were of analytical reagent grade and

solvents were of HPLC grade. Water was obtained from a Milli-Q (Millipore, Milford, MA, USA) water purification system.

5.2.2 Assessment of the absorption of Hf.HCl in post-prandial dogs

5.2.2.1 Surgical procedures

All surgical and experimental procedures were approved by the local Institutional Animal Experimentation Ethics Committee. Following the induction of surgical anaesthesia, the thoracic lymph duct and portal vein of healthy adult male greyhound dogs (28-35 kg) were cannulated as previously described in section 4.3.2. After surgery, the dogs were allowed to recover unrestrained in a run for a period of 12-16 h, and during this time, they returned to normal ambulatory movement. An intravenous catheter was inserted into the cephalic vein prior to drug administration to enable serial blood sampling during the study period.

5.2.2.2 Experimental procedure

The dogs (n = 3) were fed a standard can of commercial dog food (680 g) containing 5% crude fat approximately 30-45 min prior to the administration of an oral dose of 107 mg Hf.HCl (equivalent to 100 mg Hf base on a molar basis). The oral administration of Hf.HCl employed a dry capsule (size 0) containing Avicel® (160 mg) and Explotab® (160 mg). In addition to allowing the dogs free access to drinking water, 25 mL of 0.9% sodium chloride solution was administered by IV bolus injection at hourly intervals to limit possible dehydration due to continual collection of thoracic lymph. Administration of food was not allowed until after collection of the 12 h sample.

Systemic and portal vein blood samples (2.5 mL) were obtained via indwelling cannulas at predose (-5 min), 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12 and 24 h after

dosing. Blood samples were collected into individual tubes containing dipotassium EDTA, plasma was separated by centrifugation and then stored at –20°C prior to analysis.

Lymph was collected continuously into 50 mL tubes containing 75 mg disodium EDTA (to prevent clot formation) for the 12 h post-dosing period. Lymph samples collected each hour were combined and the total mass of lymph collected per hour was determined gravimetrically. The samples were stored between 5-8°C prior to analysis (within 24h).

5.2.2.3 Analytical procedures

Plasma concentrations of Hf and the major metabolite, *N*-desbutylhalofantrine (Hfm), were determined using the validated plasma extraction procedure and gradient HPLC assay described in sections 2.3.6.2.1 and 3.3.3, respectively. The analysis of Hf in lymph has previously been described in section 4.3.4.1. As all the lymph draining from the thoracic duct was collected, the absolute amount of drug transported lymphatically was calculated by multiplying the concentration of drug in lymph by the corresponding mass of lymph produced during each collection period.

Lymph and plasma triglyceride concentrations were measured using a clinical chemistry analyzer (Roche Cobras Mira, Basle, Switzerland) and a commercial enzyme-based colorimetric assay kit (Boehringer Mannheim, Germany). The lipoprotein fractions in lymph collected during the 1-2, 2-3 and 3-4 h post-dosing periods were separated by ultracentrifugation as previously described in section 4.3.4.3.

5.2.2.4 *Data analysis*

The area under the plasma concentration-time curve (AUC^{0-t}) was calculated by the linear trapezoidal rule from time zero to the last measured plasma concentration. The peak plasma concentration (C_{max}) and the time for this occurrence (T_{max}) were noted directly from individual profiles. The proportion of dose absorbed into the systemic circulation via the non-lymphatic route was calculated from the ratio of the dose-normalized AUC values determined after oral and IV (previously determined in chapter 4, section 4.4.3) administration, respectively. The terminal elimination rate constant could not be accurately determined, as the sampling period of the study was less than one half-life of the parent drug.

5.2.3 Physicochemical studies

5.2.3.1 *Determination of pKa by UV spectrophotometry*

The method employed was similar to that described by Albert and Serjeant.[3] However, due to the very low aqueous solubility of Hf.HCl, the addition of a co-solvent was required. *N*-Methyl-2-pyrrolidone (NMP) was chosen, as it has been reported to be an effective co-solvent for several poorly water soluble drugs[6,7] and in comparison with other solvents was the most efficient in maintaining solubility of Hf.HCl in aqueous solution. Buffer solutions (20 mM, ionic strength adjusted to 0.1 M with sodium chloride) of pH values ranging from 2 to 10 were prepared using either phosphate, acetate, TRIS or carbonate buffer salts and adjusted to the required pH using either 1 M sodium hydroxide or 1 M hydrochloric acid. For each sample, 0.1 mL of Hf.HCl stock solution (1 mg/mL in NMP) was added to 2.9 mL NMP and then diluted to 10 mL with the appropriate buffer solution to give a final drug concentration of 10 μg/mL. Duplicate samples were prepared and scanned between 230 and 400 nm at a rate of 600 nm/min

using a Cary 3 Bio UV-Visible spectrophotometer (Varian, Australia). The apparent pH value of each solution was determined using an Activon pH electrode (model AEP 341, Activon, Australia) connected to a Radiometer pH meter (model PHM 290, Radiometer, Denmark) which had been calibrated (± 0.02 pH) with appropriate pH standards at 35°C. This corresponded to the temperature of the solution upon mixing, and the temperature at which the UV measurements were performed.

The UV spectra were examined to determine the wavelength at which there was a maximum change in absorbance as the pH was varied. Absorbance values at this wavelength were plotted against pH and the data fitted to a sigmoidal equation using Sigmaplot for Windows version 4.00 (SPSS Inc., USA). The corresponding sigmoidal equation was:

$$y = y_0 + a / (1 + \exp(-(x-x_0)/b)) \qquad \text{Equation 5.1}$$

where y is the absorbance, y_0 is the minimum absorbance, x is the pH, x_0 is the pH value corresponding to the midpoint of the curve, a is the difference between maximum and minimum absorbance units and b is the weighted slope of the curve. From the derived equation, the pH value corresponding to the midpoint of the curve (x_0) was determined and this was taken to be the apparent spectrophotometric pKa of Hf, denoted pKa_s. To determine the effect of using NMP as a co-solvent in the buffer solutions on the pKa_s of Hf, experiments were repeated using varying concentrations of NMP (5-40% v/v) to enable extrapolation to 0% NMP.

5.2.3.2 Molecular modelling

Molecular modelling was performed with Chem3D Ultra version 4.0 (CambridgeSoft, Cambridge, MA, USA), run on an HP Vectra Pentium III

microcomputer. Molecular models were energy minimized with a modified version of Allinger's MM2 force field.[8] The modifications to the MM2 force field included a Parr-Pariser-Pople self-consistent field computation that improves the performance of sigma bond parameters attached to conjugated pi-electron systems, as in halofantrine. Force field models were minimized to either a final RMS gradient of < 0.001 kcal. mol^{-1}.$angstrom^{-1}$, or else to the condition of no change in the steric energy (± 0.001 $kcal.mol^{-1}$) for > 100 iterations.

5.2.3.3 Solubility determinations

The solubility of Hf.HCl and Hf base in soybean oil (SBO), and of Hf.HCl in buffer solutions (pH 2 to 9, 20 mM, ionic strength 0.1 M adjusted with NaCl) containing sodium taurocholate (NaTC) and lecithin in a 4:1 molar ratio were determined. To mimic fasted and post-prandial intestinal states, 3.75 and 15 mM NaTC were used, respectively.[9] Screw-capped glass centrifuge tubes (10 mL) containing excess drug and either 2 mL SBO or 5 mL of the respective NaTC:lecithin mixed micellar buffer solution were equilibrated with agitation at 37°C in a thermostated water bath and sampled periodically up to 72 h or until equilibrium solubility had been reached (i.e. when sequential sampling afforded solubility values which varied by less than 5%). After centrifugation at 1200 *g* for 20 min, samples (100 μL) were taken from the supernatant and diluted appropriately prior to analysis by HPLC. Measurements were performed in duplicate and the reported values are averages of the duplicate samples, with individual values differing by less than 5%.

5.2.3.4 *Partitioning studies*

The partitioning of Hf.HCl between an oil phase (SBO) and aqueous micellar solutions of varying pH values was investigated. Buffer solutions (acetate, phosphate or TRIS, pH 2 to 9, 20 mM, ionic strength 0.1 M adjusted with NaCl) containing either a combination of 15 mM sodium taurocholate and 3.75 mM lecithin or 42 mM sodium dodecyl sulphate, 30 mM cetrimide, or 0.042% (w/v) Tween 80 alone were prepared. Since the concentration of bile salt used to represent the post-prandial state (15 mM) is approximately 30-fold higher than the critical micelle concentration (CMC),[10] the concentration of the other surfactant systems used also corresponded to a 30-fold increase from their individual CMC values.[11-13] SBO (0.5 g) and the aqueous micellar solutions (10 mL) containing Hf.HCl at a concentration corresponding to 80% saturated solubility (at the individual pH values) were then placed in 12 mL centrifuge tubes and allowed to equilibrate on a roller at 37°C for 24 h. After equilibration, the two phases were separated by centrifugation at 37°C, and each phase was assayed for Hf by HPLC. The pH of the aqueous phase was also measured using a calibrated pH electrode and pH meter as mentioned in section 5.2.3.1. All measurements were performed in duplicate. The distribution coefficient (D) was calculated as the ratio of the concentration of Hf in the oil phase to that in the aqueous micellar phase. The D and pH values were then fitted to a sigmoidal equation (Equation 5.1) using Sigmaplot, and the mid-point of the sigmoidal curve was taken to be the apparent micellar pKa value, denoted pKa_m.

5.2.3.5 *Fourier transform infrared spectroscopy (FTIR)*

FTIR was employed to identify the species of drug (i.e. Hf base or Hf.HCl) present in the oil phase at the conclusion of the partitioning experiments. A sample of the oil phase was spread evenly onto the surface of an attenuated total reflectance crystal

(ATR crystal, Pike Laboratories, Madison, WI, USA) and 2000 scans from 4000 to 600 cm^{-1} were collected using a Bio-Rad Excalibur spectrometer (Bio-Rad Laboratories, Cambridge, MA, USA). The spectra were then corrected by subtraction of that for a blank soybean oil sample.

5.2.3.6 Self-association of halofantrine

The self-association of Hf in aqueous solutions of varying apparent pH values was investigated using UV spectrophotometry. Buffer solutions (apparent pH 6 to 10, 20 mM, ionic strength 0.1 M) containing 30% v/v NMP and Hf.HCl (2.5 to 30 μg/mL) were prepared and scanned between 230 to 400 nm. The absorbance at the maximum wavelength (λ_{max}) was recorded and plotted against Hf concentration, to enable the resultant curve to be examined. Molar absorptivity (ε) of Hf in the various solutions was determined using the Beer-Lambert law:

$$\varepsilon = A / C \cdot l \qquad \text{Equation 5.2}$$

where A is the absorbance at λ_{max}, C is the molar concentration of the sample and l is the length (in cm) of the path of the light beam through the sample cell.[14]

5.3 RESULTS AND DISCUSSION

The previous chapter demonstrated that after post-prandial administration of the highly lipid soluble Hf base, the intestinal lymphatics were the predominant absorption pathway for Hf. This is not surprising considering its high calculated log P (~ 8.5) and significant solubility in long-chain triglyceride lipids (~ 50 mg/mL). In contrast, the commercially available hydrochloride salt of Hf (Hf.HCl) was not considered a likely substrate for intestinal lymphatic transport as its solubility in long-chain triglyceride

lipids is low (~ 1 mg/mL). However, given that the earlier documented food effects were observed after oral administration of Hf.HCl,[4,5] the potential intestinal lymphatic drug transport following post-prandial administration of the poorly lipid soluble Hf.HCl was investigated in the present study.

5.3.1 Assessment of the absorption of Hf.HCl after post-prandial administration

5.3.1.1 Intestinal lymphatic drug transport

The cumulative lymphatic transport of Hf after post-prandial oral administration of 107 mg Hf.HCl (molar equivalent to 100 mg Hf base) to conscious, thoracic lymph duct cannulated dogs is presented in Figure 5.1 and Table 5.1. The mean (± SD, n = 3) lymphatic transport was 47.3 ± 4.3% of the administered dose. Considering the low lipid solubility of Hf.HCl, the extent of lymphatic drug transport after post-prandial administration of Hf.HCl was surprisingly high and not significantly different from the post-prandial lymphatic transport of the highly lipid soluble Hf base (54.0 ± 8.2% of 100 mg dose). However, a lag phase of 1-2 h in the lymphatic transport of Hf was observed in this study suggesting a minor yet consistent difference in the kinetics of the lymphatic transport profiles. After post-prandial administration of Hf.HCl, lymphatic drug transport was largely complete within 6-8 h compared to 4 h for Hf base (Figure 5.1 and Table 5.1). The intestinal lymphatic transport of Hf following fasted administration of Hf.HCl was not studied as this is expected to be low in the absence of food or a suitable lipid source with which to promote chylomicron synthesis.[15]

The cumulative transport of triglyceride (TG) in lymph was also determined to confirm the integrity of lymph duct cannulation and to ensure complete collection of thoracic lymph. After oral administration of Hf.HCl to post-prandial dogs, the mean (± SD, n = 3) cumulative TG transport (comprising endogenous and exogenous TG

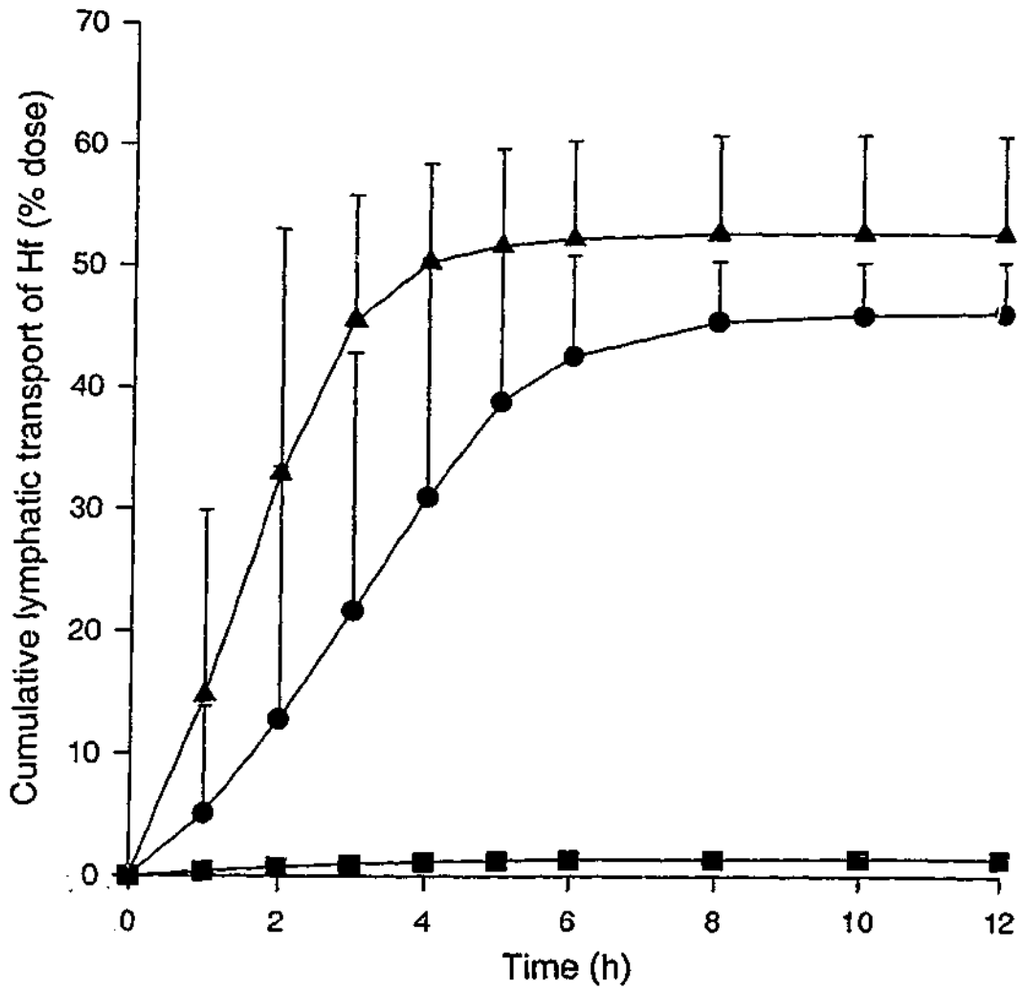

Figure 5.1 Cumulative lymphatic transport of halofantrine (Hf) (% dose, mean ± SD) in thoracic lymph duct cannulated dogs after post-prandial administration of 107 mg Hf.HCl (●, n = 3), 100 mg Hf base (▲, n = 4, from chapter 4) or after fasted administration of 100 mg Hf base (■, n = 3, from chapter 4).

Table 5.1 Mean (± SD) cumulative lymphatic transport of halofantrine (Hf) and triglyceride (TG), and lymph flow after post-prandial administration of either 100 mg Hf base or 107 mg Hf.HCl to thoracic lymph duct cannulated dogs

	Cumulative transport of Hf (% dose)			Cumulative mass of TG in lymph (g)			Cumulative lymph flow (g)		
Post-dosing period	0-4 h	4-8 h	8-12 h	0-4 h	4-8 h	8-12 h	0-4 h	4-8 h	8-12 h
Hf base[a] (n = 4)	50.7 ± 8.2	53.7 ± 8.2	54.0 ± 8.2	18.4 ± 6.7	30.6 ± 5.2	33.6 ± 3.5	483 ± 86	841 ± 87	1047 ± 124
Hf.HCl (n = 3)	31.3 ± 19.7	46.3 ± 5.0	47.3 ± 4.3	18.2 ± 4.4	30.5 ± 7.1	32.5 ± 6.7	391 ± 43	668 ± 98	844 ± 124

[a] Data from chapter 4.

sources) in the 12 h post-dosing period was 32.5 ± 6.7 g (Table 5.1), accounting for greater than 90% of the lipid present in the ingested food. The values observed in this study are consistent with those obtained in the previous chapter.

As mentioned previously, lipophilic compounds transported by the intestinal lymphatics typically associate with the chylomicron fraction of lymph.[15,16] Table 5.2 presents the percentage distribution of Hf between the lipoprotein fractions of lymph collected between 1 and 4 h post-dosing period. These data are consistent with those previously observed after the post-prandial administration of Hf base as shown in Table 4.2. Greater than 97% of the drug present in lymph was associated with the chylomicron fraction. Interestingly, during periods of maximal lymphatic drug transport, the amount of Hf present per mass of chylomicron triglyceride (fractionated from lymph) was as high as 2.5 mg/g, exceeding the previously reported TG solubility of Hf.HCl in peanut oil (~ 1 mg/mL).[17]

It was proposed that Hf.HCl could dissolve in food-related TG as a prelude to it appearing in chylomicron TG. However, this would be considered a minor contribution to lymph transport, as ingested TG is hydrolysed prior to absorption and then re-synthesized within the enterocyte prior to TG lymph transport. As a result of this processing, the expected concentration of Hf.HCl that might ultimately appear in chylomicron TG would only be a fraction of its saturated TG solubility (and an even smaller fraction of the observed drug concentration of 2.5 mg/mL in chylomicron TG).

Table 5.2 Percentage distribution of halofantrine (Hf) between different lipoprotein fractions in thoracic duct lymph collected in the 1-4 h post-dosing period from two individual post-prandial dogs administered 107 mg Hf.HCl

	Percentage distribution of halofantrine					
	Post-dosing period (Dog 1)			**Post-dosing period (Dog 2)**		
Lipoprotein fraction[a]	**1-2 h**	**2-3 h**	**3-4 h**	**1-2 h[b]**	**2-3 h**	**3-4 h**
Chylomicron	97.12	99.52	99.40	0	99.38	98.79
VLDL	0.240	0.045	0.056	0	0.021	0.486
LDL	0.098	0.058	0.044	0	0.069	0.166
HDL	1.022	0.144	0.260	0	0.242	0.264
LPDL	1.518	0.231	0.241	0	0.284	0.292
% Recovery[c]	85.80	95.31	84.37		90.24	83.90

[a] VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LPDL, lipoprotein deficient lymph.

[b] Negligible lymphatic drug transport occurred during this sampling period.

[c] Recovery was defined as the % mass of drug recovered after lymph fractionation divided by the mass of drug present in the original unfractionated lymph sample.

Depicted in Figure 5.2 is a possible explanation for the extensive lymphatic transport of Hf after post-prandial administration of Hf.HCl. This may include enhanced solubilization of Hf.HCl in the post-prandial intestinal milieu and/or partial conversion within the intestinal lumen to the highly lipid soluble free base thereby facilitating the association of Hf with products of lipid digestion and subsequent incorporation into lymph lipoproteins for lymphatic transport. However, Hf is a tertiary amine and is expected[3] to have a pKa of greater than 10. At physiological intestinal pH (~ 6-7),[18] less than 0.1% of Hf is likely to be present in the unionized form. Further studies elucidating the mechanism by which Hf may undergo extensive lymphatic transport after post-prandial administration of Hf.HCl were conducted, and will be discussed in the latter parts of this chapter.

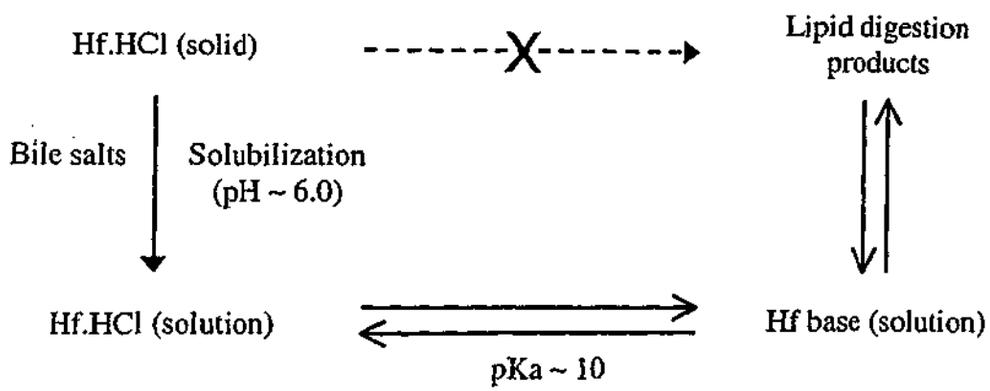

Figure 5.2 Schematic representation of the mechanism proposed for the extensive lymphatic transport of halofantrine (Hf) after post-prandial administration of Hf.HCl to dogs.

The rate of lymphatic transport of Hf and TG following post-prandial oral administration of Hf.HCl is presented in Figure 5.3. Maximal transport rates for both species occurred during the 3 to 5 h post-dosing period, after which the rate of transport gradually declined. Interestingly, the transport of Hf and TG appeared to be linked for almost the entire study period, which suggests that the co-administration of lipid is important for significant intestinal lymphatic drug transport, as the formation of lipoproteins by the enterocyte is dependent on the absorption of lipid digestion products.

Figure 5.4 demonstrates the rate of lymphatic transport of Hf after the post-prandial oral administration of either 107 mg Hf.HCl or 100 mg Hf base. It is apparent that the kinetics of the lymphatic transport of Hf after oral administration of the two different forms of Hf varied slightly in the initial post-dosing period. Following the administration of Hf base, peak Hf transport rates were observed in the first 2-3 h, after which the rate declined rapidly. In comparison, the initial rate of Hf transport was lower after the administration of Hf.HCl, but the transport of Hf remained at maximal rates for a longer period, up to 5 h post-dosing. The differences in the kinetics of lymphatic drug transport may reflect the different dissolution, solubilization and absorption rates of Hf after oral administration of the two different forms of Hf (free base or HCl salt). Unlike the rate of lymphatic transport of Hf, the rate of TG transport in lymph was similar after administration of either Hf.HCl or Hf base (Figure 5.5) and would therefore, be an unlikely contributor to the observed differences in the kinetics of Hf transport in lymph.

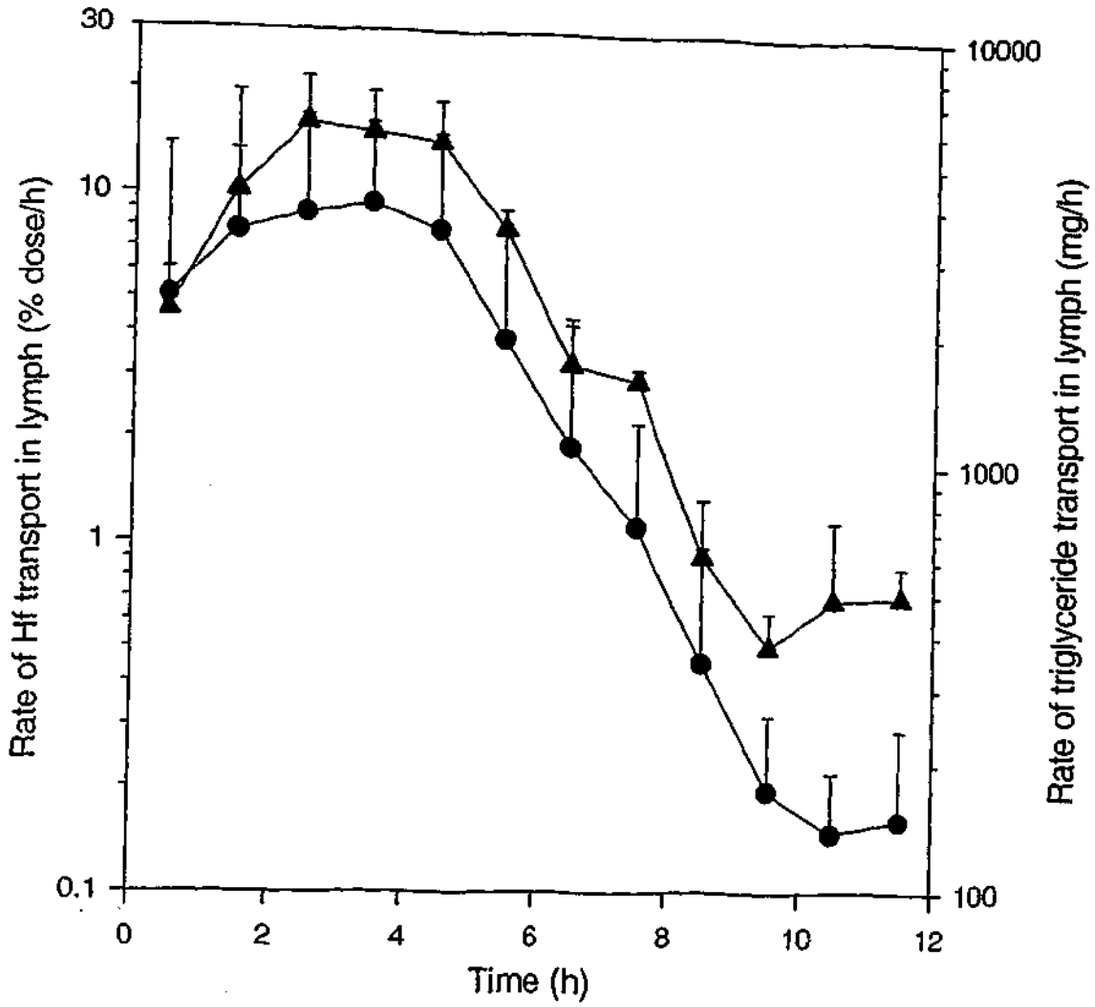

Figure 5.3 The mean (± SD, n = 3) rate of lymphatic transport of halofantrine (●, expressed as % Hf dose transported per hour) and triglyceride (▲, expressed as mg triglyceride per hour) after post-prandial administration of 107 mg Hf.HCl to thoracic lymph duct cannulated dogs.

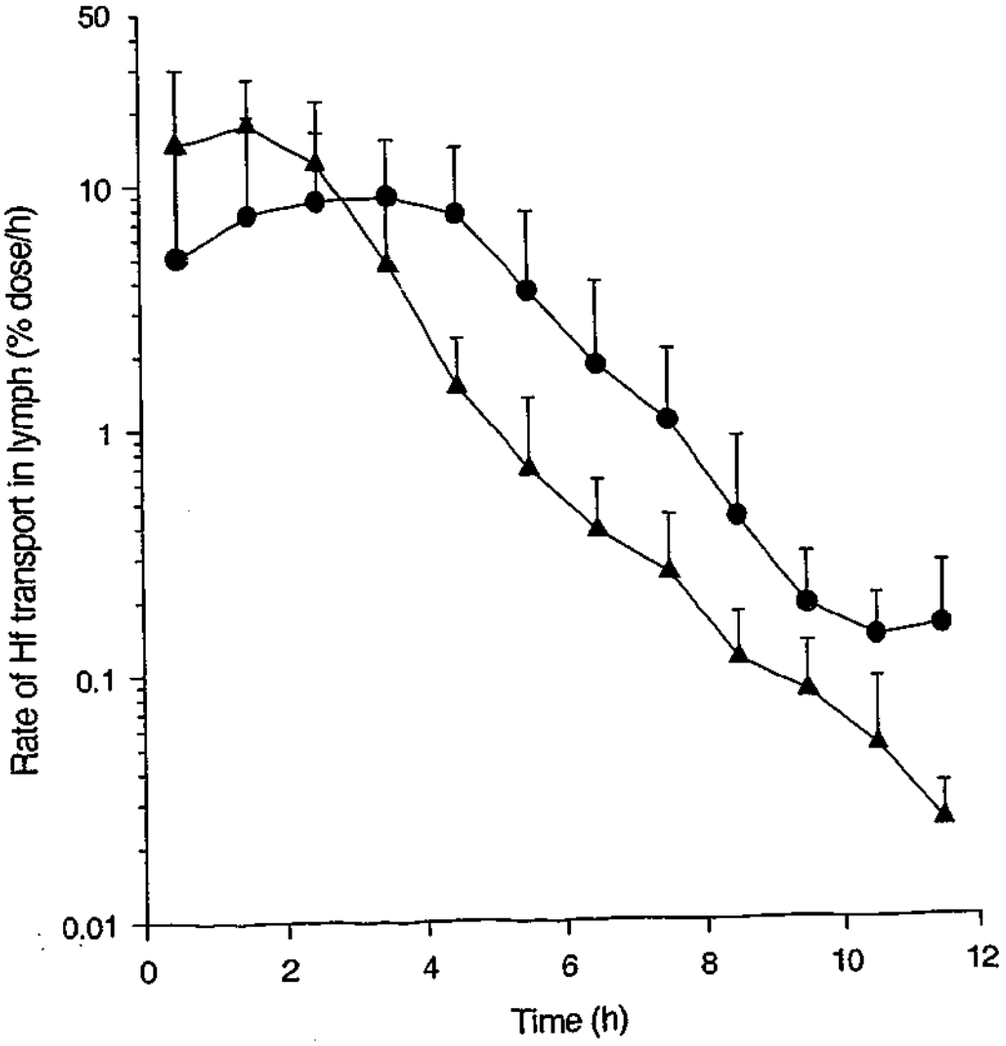

Figure 5.4 The mean (± SD) rate of lymphatic transport of halofantrine (Hf) after post-prandial administration of either 107 mg Hf.HCl (●, n = 3) or 100 mg Hf base (▲, n = 4, from chapter 4) to thoracic lymph duct cannulated dogs.

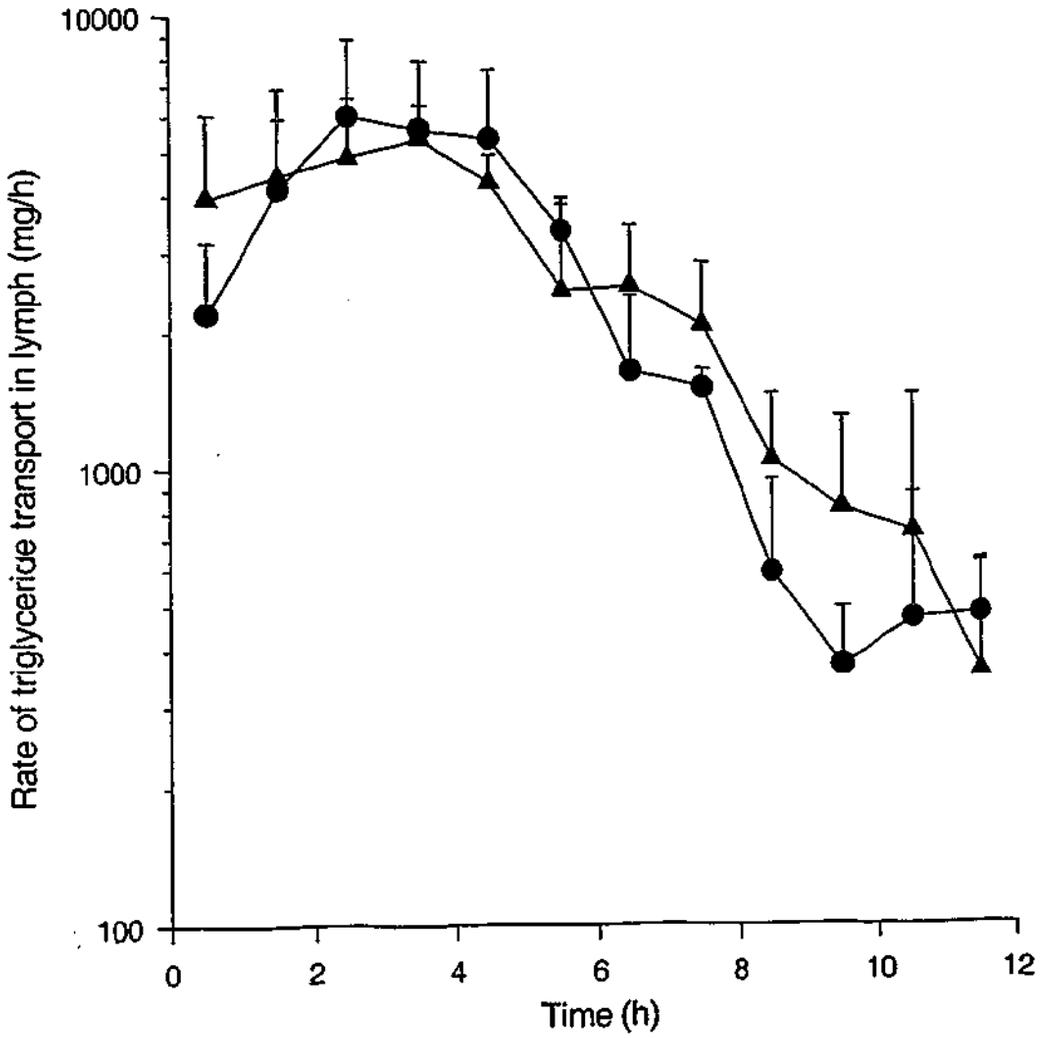

Figure 5.5 The mean (± SD) rate of triglyceride transport in lymph after post-prandial administration of either 107 mg Hf.HCl (●, n = 3) or 100 mg Hf base (▲, n = 4, from chapter 4) to thoracic lymph duct cannulated dogs.

5.3.1.2 *Absorption of Hf into the systemic circulation via the portal route*

The portal and systemic plasma concentration versus time profiles of Hf and the metabolite, *N*-desbutylhalofantrine (Hfm), following post-prandial oral administration of 107 mg Hf.HCl to thoracic lymph duct cannulated dogs are presented in Figure 5.6 and the corresponding pharmacokinetic parameters are tabulated in Table 5.3. These data represent drug absorption into the general systemic circulation via the portal (non-lymphatic) route only as the contribution of drug absorbed via the intestinal lymphatics has been removed. As expected, during the absorption phase the portal Hf plasma concentrations were higher than the systemic plasma concentrations, although the plasma concentrations were relatively variable following administration of the unformulated Hf.HCl. Similarly, the portal Hfm plasma concentrations were consistently higher than systemic Hfm plasma concentrations during the post-dosing absorption period (Figure 5.6), indicating likely enterocyte-based conversion of Hf to Hfm (as discussed in section 4.4.6).

The mean systemic plasma C_{max} and $AUC^{0\text{-}24h}$ values for Hf (Table 5.3) observed in the lymph fistulated dogs (where contribution to the systemic blood levels from the lymph has been removed) were considerably lower than the values previously reported in non-lymph fistulated, post-prandial beagles administered 250 mg Hf.HCl where the mean (± SD, n = 3) C_{max} and plasma $AUC^{0\text{-}\infty}$ values for Hf were 5540 ± 2107 ng/mL and 51496 ± 9379 ng.h/mL, respectively.[4] Although the differences in the dose of Hf.HCl administered (i.e. 107 mg versus 250 mg) and the different breeds of dogs used (greyhound versus beagles) do not allow a direct comparison of the two studies, these two factors alone are unlikely to account for the large (greater than 20-fold) differences in C_{max} and AUC values. Hence, the magnitude of the differences in C_{max} and AUC values observed between lymph fistulated and non-lymph fistulated dogs

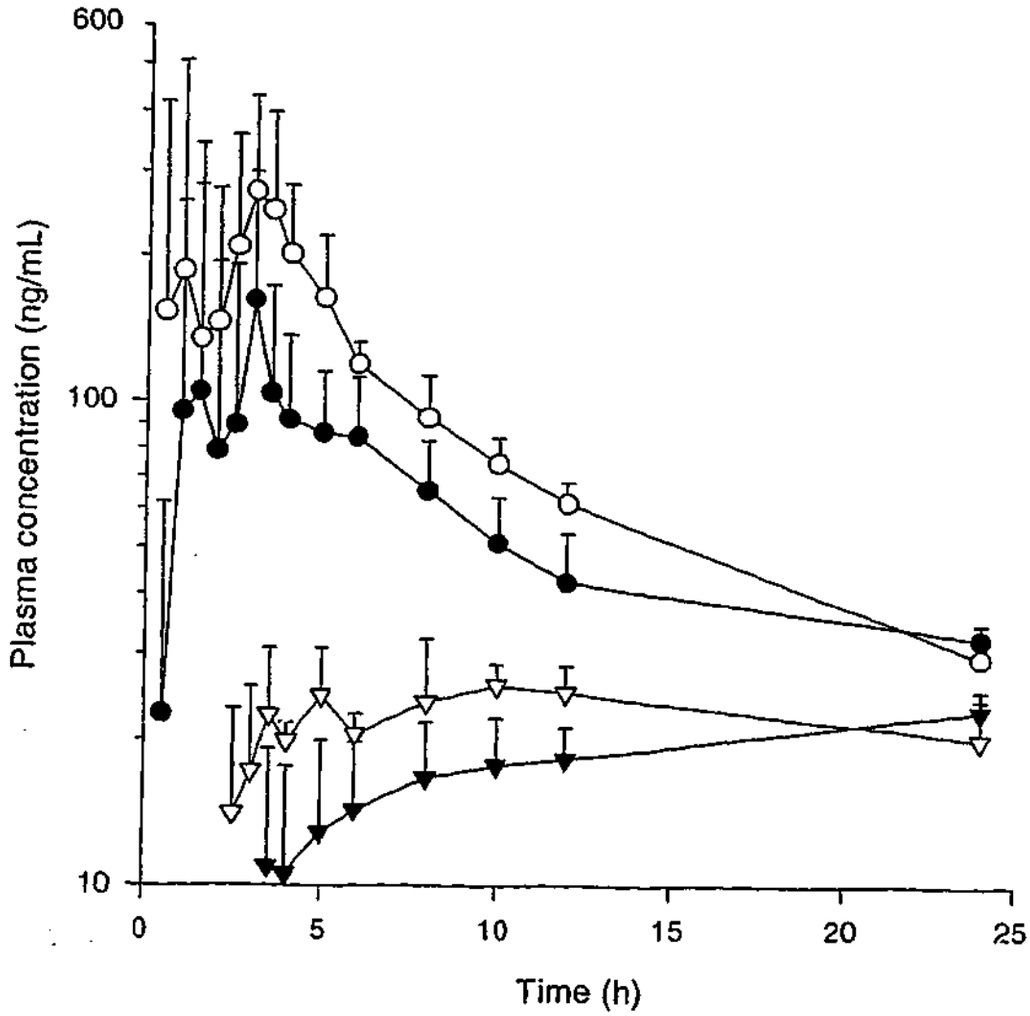

Figure 5.6 Portal (open symbols) and systemic (closed symbols) plasma concentration versus time profiles of halofantrine (●) and the major metabolite, *N*-desbutylhalofantrine (▼), after oral administration of 107 mg Hf.HCl to post-prandial thoracic lymph duct cannulated dogs. Data are presented as mean ± SD, n = 3.

Table 5.3 Portal and systemic plasma pharmacokinetic parameters (mean ± SD, n = 3) of halofantrine (Hf) and *N*-desbutylhalofantrine (Hfm) after post-prandial administration of 107 mg Hf.HCl to thoracic lymph duct cannulated dogs

	Pharmacokinetic parameter	Portal plasma	Systemic plasma
Hf	C_{max} (ng/mL)	432 ± 140	223 ± 139
	T_{max} (h)	2.7 ± 1.5	3.2 ± 1.8
	$AUC^{0\text{-}24h}$ (ng.h/mL)	2201 ± 178	1355 ± 581
Hfm	C_{max} (ng/mL)	29.2 ± 5.2	27.6 ± 7.9
	T_{max} (h)	7.8 ± 4.3	21.3 ± 4.6
	$AUC^{0\text{-}24h}$ (ng.h/mL)	492 ± 45	404 ± 68
	Hfm/Hf AUC ratio	0.22 ± 0.03	0.33 ± 0.12

suggests that intestinal lymphatic transport may be a significant contributor to oral bioavailability of Hf after post-prandial administration.

In the present study, estimates of the percentage dose of Hf absorbed via portal blood (relative to IV administration, previously determined in chapter 4) was 19.0 ± 8.1% (mean ± SD, n = 3). The total availability of Hf as a consequence of intestinal lymphatic transport and absorption via the portal route was 66.3 ± 12.4% (Table 5.4), with intestinal lymphatic transport contributing to approximately 70% of the total dose absorbed. Therefore, the current data suggest that the intestinal lymphatic transport was the predominant absorption pathway after post-prandial administration of Hf.HCl.

Table 5.4 Total availability of halofantrine (Hf) (mean % dose ± SD, n = 3) after post-prandial administration of 107 mg Hf.HCl to thoracic lymph duct cannulated dogs

	Lymphatic transport[a]	**Systemic availability[b]**	**Total Hf availability (Lymph + Blood)**
Hf.HCl (fed)	47.3 ± 4.3	19.0 ± 8.1	66.3 ± 12.4

[a] Cumulative % dose of Hf recovered in thoracic duct lymph over a 12 h post-dosing period.

[b] The percentage dose of Hf absorbed directly into the systemic circulation via portal blood (i.e. non-lymphatic absorption) was based on the systemic plasma $AUC^{0\text{-}24h}$ relative to the $AUC^{0\text{-}24h}$ obtained after IV administration. Dose-normalized $AUC^{0\text{-}24h}$ after IV administration of Hf was 6693 ± 330 ng.h/mL (section 4.4.3).

5.3.2 Physicochemical studies of Hf.HCl

The following sections discuss the results of the physicochemical studies conducted to elucidate the mechanism(s) by which the poorly lipid soluble Hf.HCl was extensively transported via the intestinal lymphatics after post-prandial administration to dogs.

5.3.2.1 pKa determination by UV spectrophotometry

An estimate of the apparent pKa of Hf was determined since this has not been reported previously. As a tertiary aliphatic amine, Hf would be predicted[3] to have a pKa of at least 10. UV spectrophotometry was employed to determine the pKa of Hf as it is too poorly soluble in aqueous solutions for potentiometric titration to be used. The addition of NMP (30% v/v) as a co-solvent enabled a suitable concentration of Hf (10 μg/mL) in buffer solutions to be obtained for the UV spectrophotometric studies. The ionized (apparent pH 4.35 and 6.23) and unionized (apparent pH 8.78) species of Hf exhibit different spectra (Figure 5.7A). However, below the apparent pH of 6.23, where Hf was predominantly in the ionized form, the UV spectra of the ionized species were identical as shown in the uppermost spectra in Figure 5.7A. At apparent pH values above 9, the UV spectra of Hf showed slight irregularities, which may be due to the instability of Hf at the high pH values and have therefore been omitted. A sharp and clear isosbestic point at ~ 270 nm indicated that Hf is likely to undergo a single ionization, presumably involving the tertiary amine. At 260 nm, the greatest difference between the absorbances of the ionized and unionized species was observed. When the absorbance at 260 nm was plotted as a function of pH (Figure 5.7B) and fitted to a sigmoidal equation, the apparent pKa_s of Hf was estimated to be 7.64 ± 0.02 (± SE of estimation).

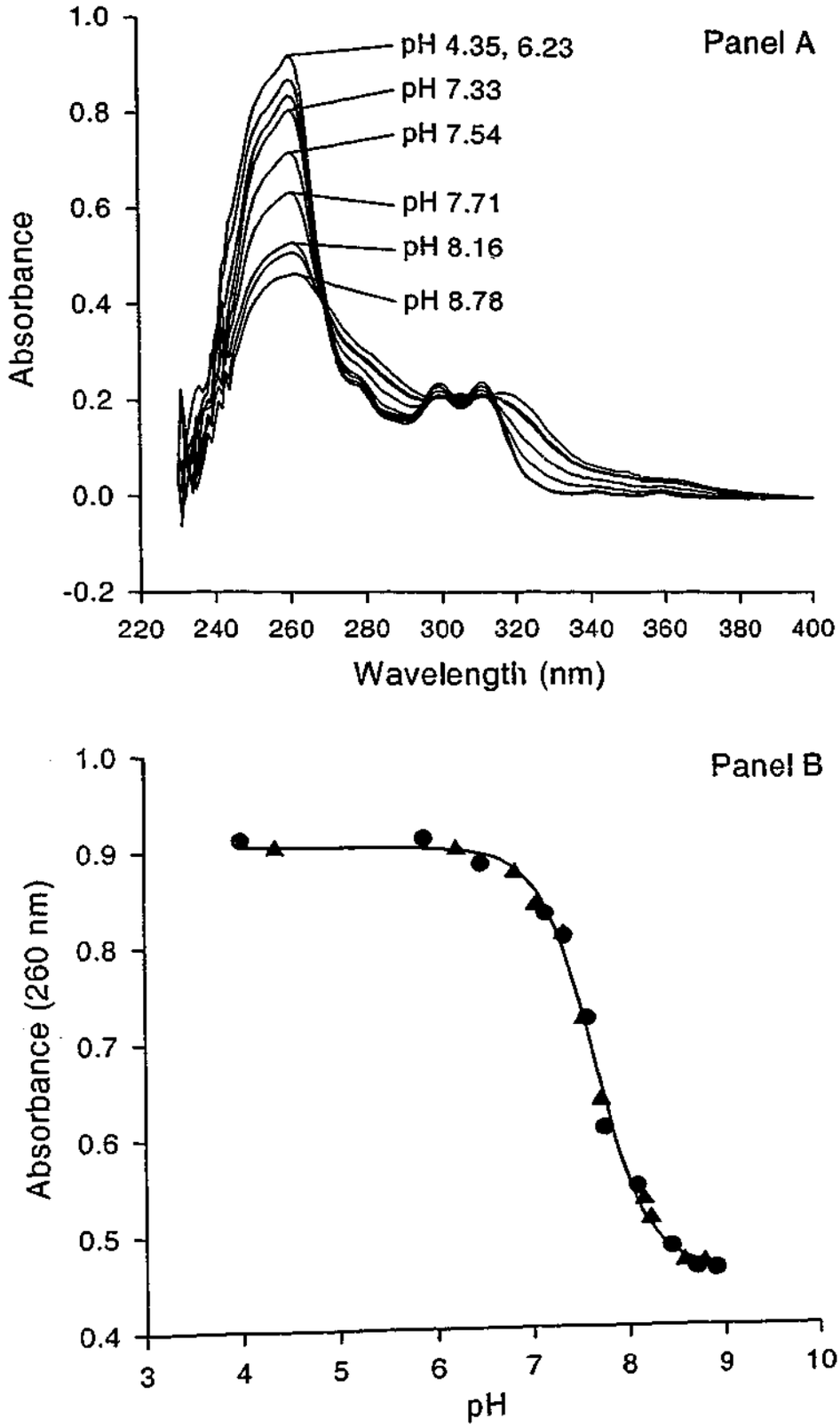

Figure 5.7 UV spectra of Hf (10 μg/mL in aqueous buffer solutions containing 30% v/v *N*-methyl-2-pyrrolidone) as a function of pH (panel A), and Absorbance (at 260 nm) versus pH profile of Hf (panel B). For panel A, some spectra have been omitted for clarity, and for panel B, symbols represent measured values (n = 2) and the line is a sigmoidal fitted curve.

Whilst initial pKa_s determinations employed 30% v/v NMP as a co-solvent, additional studies using varying amounts of NMP (5 to 40% v/v) were subsequently conducted to determine the effect of using NMP as a co-solvent in the buffer solutions on the pKa_s of Hf. A plot of mean pKa_s values (n = 2, individual values varying by < 0.05) against NMP concentration (Figure 5.8) revealed a positive linear relationship (R^2 = 0.995) with all the data points lying within the 95% confidence interval. Following extrapolation to 0% NMP, the pKa_s of Hf was estimated to be 5.58 ± 0.07 (± SE), which is ~ 5 log units lower than the expected value for a tertiary amine. These data suggest that at intestinal pH, a significant proportion of solubilized Hf.HCl is likely to convert to the highly lipid soluble free base form, which would facilitate the association of drug with lipid digestion products and promote integration into chylomicrons during lipoprotein synthesis and consequently increase the extent of lymphatic drug transport.

Molecular modelling calculations determining low energy structural orientations of Hf suggest that the low pKa value could be due to the folding of the side chain such that the tertiary amine is in close proximity to the benzylic hydroxyl group, enabling intramolecular hydrogen bonding to occur and thereby reducing the basicity of the nitrogen atom. Triethanolamine is another example of a tertiary amine having a lower than expected pKa of 7.87,[19] due to intramolecular hydrogen bonding between the hydroxyl groups affecting the electron density of the nitrogen atom, and resulting in triethanolamine being a weaker base. However, in comparison to Hf, the decrease in pKa value of triethanolamine is not as significant. There appears to be a contribution from another low energy orientation of Hf, in which the tertiary nitrogen is positioned in close proximity to the aromatic phenanthrene ring system. This conformation further reduces the electron density on the nitrogen, thus decreasing its basicity. Evidence

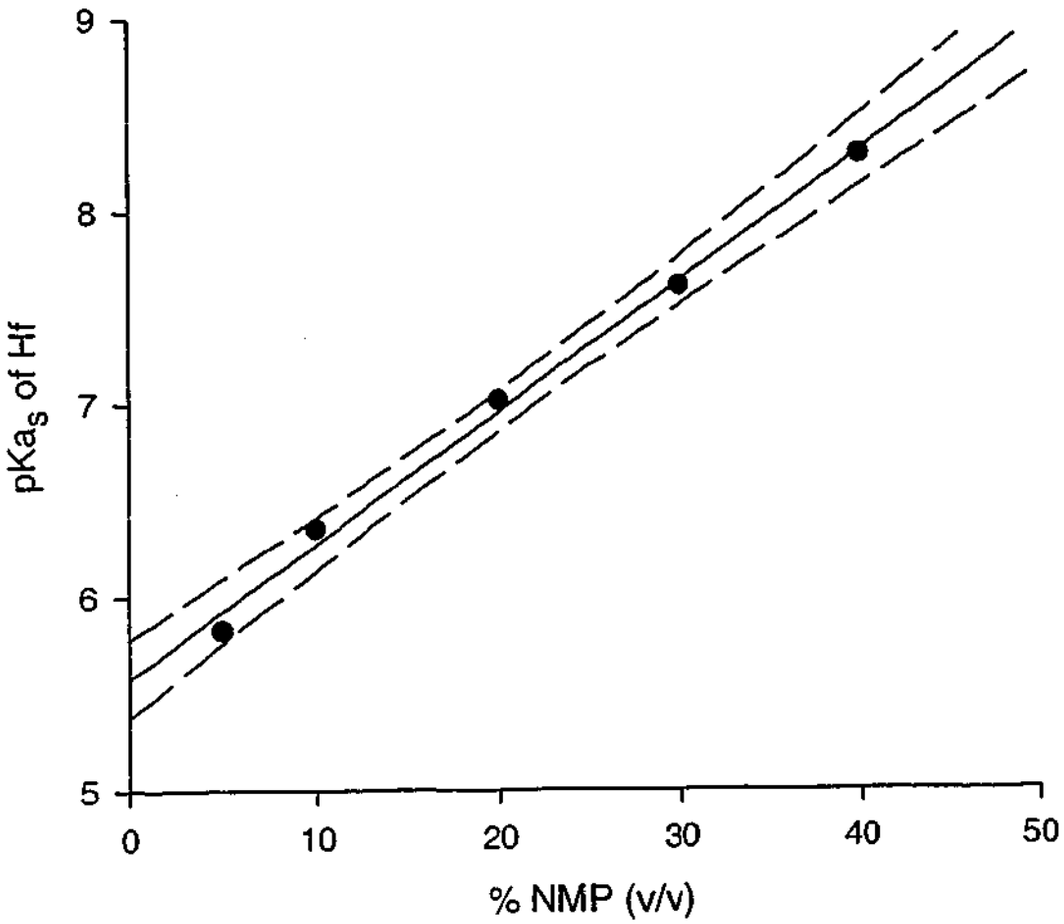

Figure 5.8 The effect of the use of NMP as a co-solvent (in buffer solutions) on the pKa_s of Hf (as determined by UV spectrophotometry). Symbols represent mean measured values (n = 2), the solid line was fitted by linear regression and the dotted lines represent the 95% confidence interval.

supporting this explanation is that the UV spectral changes in Hf as a function of pH have a number of points of similarity with those of aniline, a typical aromatic amine which is an extremely weak base (pKa = 4.60). For both Hf and aniline, increasing the pH of the buffer solution resulted in a slight shift of the UV spectrum to the right.[3,20]

5.3.2.2 Solubility and partitioning behaviour of halofantrine

Additional physicochemical studies were conducted to investigate the solubility and partitioning behaviour of Hf.HCl in representative fed state intestinal fluids. The effect of different micellar systems on the partitioning behaviour of Hf.HCl was also studied.

The equilibrium solubility of Hf.HCl in a long-chain TG, soybean oil (SBO), was 0.44 mg/mL. Not surprisingly, the solubility of Hf base in SBO was ~ 100-fold higher at 48.7 mg/mL. In accordance with a previous study,[4] the solubility of Hf.HCl increased markedly from 0.32 μg/mL in pH 6 buffer to 5.52 μg/mL in representative fasted state mixed micellar solution (3.75 mM NaTC:lecithin 4:1, pH 6) and even higher to 2311 μg/mL in representative fed state mixed micellar solution (15 mM NaTC:lecithin 4:1, pH 6). Solubilization of the drug in fed state mixed micellar solution occurred extremely rapidly, with maximum concentrations recorded within one hour. These results suggest that the bile constituents present in post-prandial intestinal milieu greatly enhanced drug solubilization, thereby increasing the amount of drug available for absorption.

The partitioning behaviour of Hf between a long-chain triglyceride (TG) lipid (SBO) and various aqueous micellar solutions was studied to determine the distribution coefficient, D. It was envisaged that the log D value obtained at physiological intestinal pH would indicate the affinity of solubilized Hf for the lipid digestion phases within the

intestinal lumen and once absorbed into the enterocyte, the likelihood of Hf being incorporated into the triglyceride core of chylomicrons during lipoprotein synthesis. Furthermore, since log D is dependent on the ionization state of Hf, and can therefore vary as a function of pH, an apparent micellar ionization constant, pKa_m, for Hf can also be determined when log D is studied as a function of pH in micellar solutions. Whilst the sodium taurocholate (NaTC):lecithin (15:3.75 mM) mixed micellar system was chosen to represent fed state intestinal fluids, three other surfactants were selected to probe the effect of ionic charge of the micellar species on the partitioning behaviour of Hf.

Figure 5.9 presents the pH-dependent partitioning profiles of Hf between SBO and the four different aqueous micellar systems. A sigmoidal relationship between log D and pH was observed for all systems, and these profiles also resemble the pH-dependent partitioning profiles previously reported for basic drugs.[21,22] At low pH values, where Hf was present as the ionized form and preferred to remain in the micellar phase, low log D values (-0.5 to 0) were observed. However, as the pH of the aqueous micellar phase was increased, the distribution of Hf between the two phases changed. At higher pH values, where Hf was more likely to exist as the unionized form, maximal partitioning of Hf into the oil phase occurred as indicated by increases in log D values of 2 to 3 log units. Interestingly, as the pH of the micellar solutions was further increased, the log D values declined. It is postulated that at high pH values, ionization of the benzylic hydroxyl group may have occurred and the increase in polarity of Hf resulted in decreased partitioning of Hf to the oil phase and consequently, lower log D values were observed. Alternatively, the decrease in log D values could have been due to an increased incidence of self-association of the unionized hydrophobic Hf molecules in the aqueous micellar phase, thereby hindering the partitioning of Hf into the oil phase. The

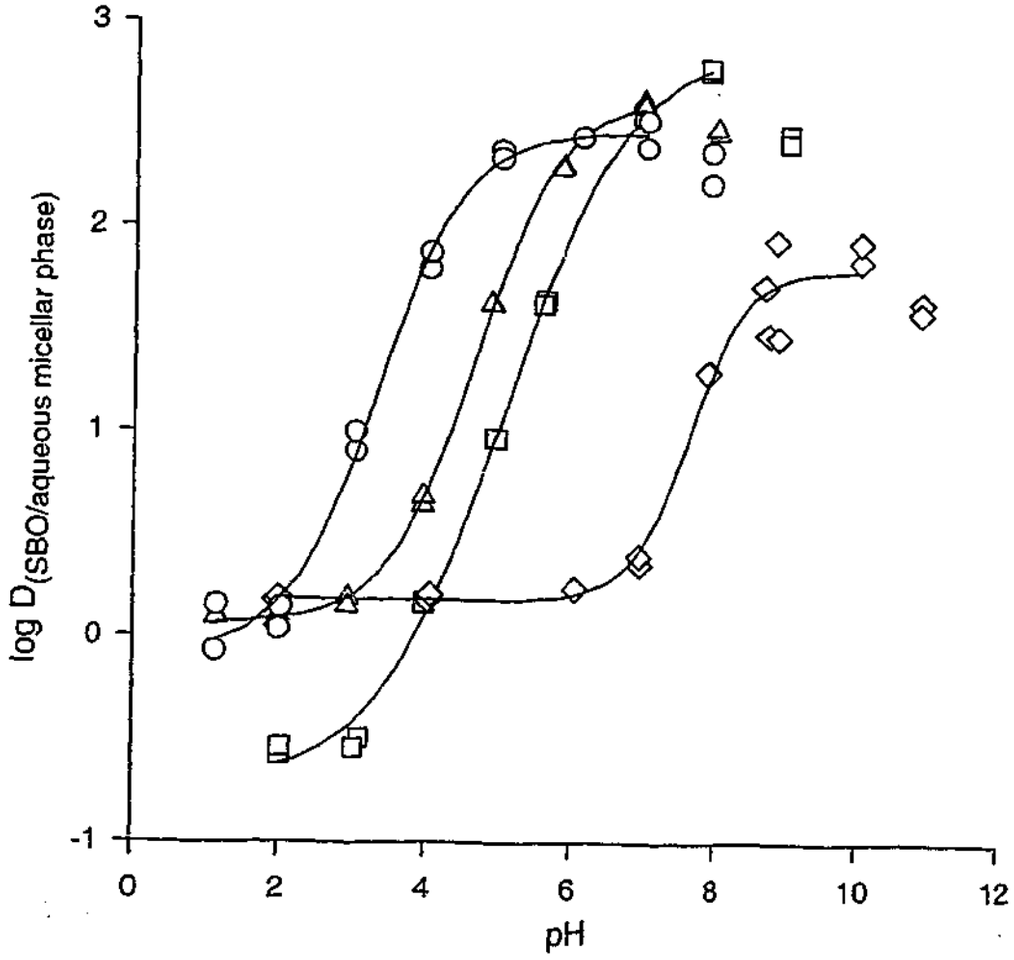

Figure 5.9 pH-dependent partitioning of Hf between soybean oil and different aqueous micellar solutions: 0.042% w/v Tween 80 (○), 30 mM cetrimide (Δ), 15:3.75 mM sodium taurocholate:lecithin (□) and 42 mM sodium dodecyl sulphate (◊). Symbols represent measured values (n = 2) and the lines represent sigmoidal fitted curves.

occurrence of self-association was further investigated using UV spectrophotometry and will be discussed in the following section.

Although the log D values at low and high pH values did vary slightly between the different micellar systems, the maximal log D values (2.0 to 2.8) in this study were considerably lower than the octanol/water log P of Hf, which was calculated to be 8.86 using the ACD Log P DB version 4.56 program (Advanced Chemistry Development, Inc., Ontario, Canada). The magnitude of this difference is likely to be due to the use of different partitioning phases in this study and since Hf is extremely poorly water soluble, this precludes an accurate determination of the octanol/water log P of Hf.

Apparent pKa_m values of Hf in each micellar system were determined by fitting the pH-dependent partitioning profiles (i.e. D versus pH) to a sigmoidal equation (Equation 5.1), although data points where the D values began to decline (i.e. at pH values above the maximum D values) were excluded. From the equation, the midpoint of the curve could be determined and this was taken to be the apparent pKa_m of Hf. The pKa_m values of Hf in the different micellar systems varied between 4.39 to 8.59 (Table 5.5). Whilst the anionic surfactant (sodium dodecyl sulphate, SDS) resulted in Hf having the highest pKa_m value, the non-ionic Tween 80 surfactant system afforded the lowest pKa_m value. The pKa_m value obtained for the sodium taurocholate:lecithin system (representative of the post-prandial intestinal state) was 6.92 ± 0.02, which is approximately 1.34 pH units higher than the value previously determined by UV spectrophotometry (pKa_s = 5.58). With a pKa_m value of 6.92, approximately 10% of solubilized Hf.HCl is expected to convert to the free base form of Hf at physiological intestinal pH (~ 6.0). As Hf base has a greater tendency to associate with lipid digestion products and be incorporated into chylomicrons for lymphatic transport, this may drive the equilibrium towards the continual conversion of solubilized Hf.HCl to Hf base.

Table 5.5 Apparent micellar pKa value (pKa_m) of Hf determined from pH-dependent partitioning studies using soybean oil and different aqueous micellar systems

Aqueous micellar system[a]	pKa_m (± SE)
Tween 80 (0.042% w/v)	4.39 ± 0.18
Cetrimide (30 mM)	5.98 ± 0.04
Sodium taurocholate:lecithin (15:3.75 mM)	6.92 ± 0.02
Sodium dodecyl sulphate (42 mM)	8.59 ± 0.31

[a] The concentration of the surfactants used were approximately 30-fold higher than the individual CMC values.

The basis for the different pKa_m values observed has not been elucidated. Further studies will be required to examine the manner in which Hf molecules are incorporated into the micelles, the solubilizing capacity of the surfactant and the size and aggregation number of the micelles. For example, a strong interaction between the anionic SDS micelles and the protonated form of Hf may prevent Hf from partitioning into the oil phase, hence the low log D values. It is only at higher pH values where the proportion of unionized Hf is increased, and the interaction with the micelles is weaker that partitioning of Hf into the oil phase occurs to a greater extent. Strong interactions between cationic drug species and anionic SDS micelles have been reported to increase pKa values.[23] In contrast, Tween 80 is a non-ionic surfactant which forms micelles with a hydrophobic core surrounded by a polyoxyethylene shell (pallisade layer).[24] Since the pallisade layer is extremely hydrated, it is unlikely to be a favourable environment for Hf, and may limit the extent of solubilization of Hf within the hydrophobic core. As a consequence, the solubilizing capacity of Tween 80 for unionized Hf is likely to be lower and partitioning to the oil phase is more likely to occur.

The conversion of Hf.HCl to Hf base was further supported by ATR-FTIR studies. Analysis of the oil phases at the conclusion of the partitioning studies afforded spectra that were similar to that of Hf base, as indicated by the absence of a characteristic vibration at 2600 cm^{-1} (evident on a spectrum of a sample of Hf.HCl dissolved in soybean oil) which corresponds to the salt of a tertiary amine (Figure 5.10).[25] These studies suggest that even though the partitioning studies commenced with Hf.HCl in the aqueous micellar phase, conversion to the more lipid soluble free base form resulted in the partitioning of Hf base into the oil phase and being the predominant species present in the oil phase. This would also explain the higher than expected concentrations of Hf observed in the oil phase (up to 22 mg/mL).

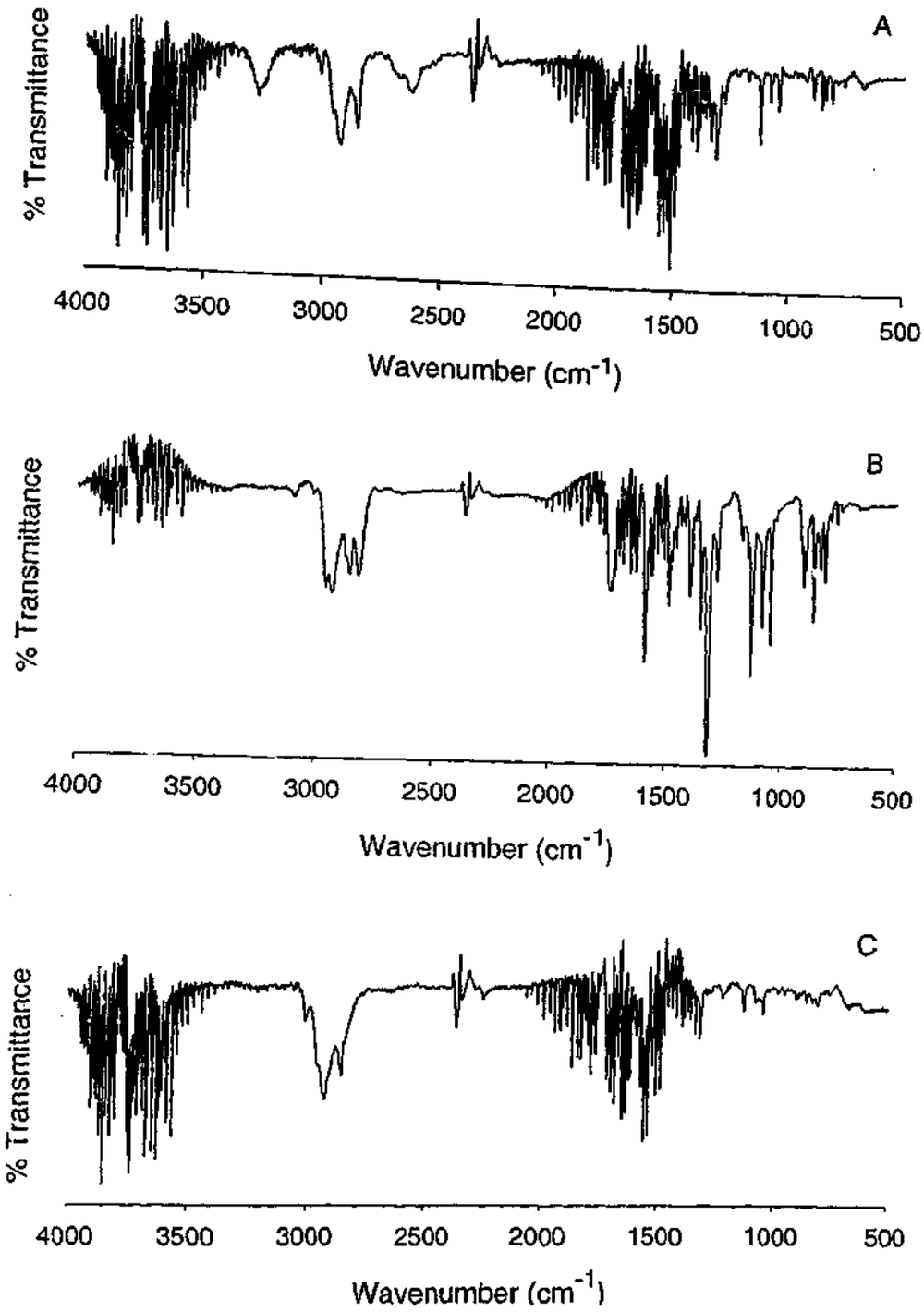

Figure 5.10 ATR-FTIR spectra of (A) Hf.HCl (0.5 mg/mL) and (B) Hf base (50 mg/mL) dissolved in soybean oil, and (C) a representative soybean oil sample at the conclusion of a partitioning study (containing 2 mg/mL Hf). Spectra were corrected by subtraction of a blank soybean oil sample.

5.3.2.3 *Self-association of halofantrine*

Self-association of Hf monomers has been postulated as a possible explanation for the decrease in log D observed at high pH values (Figure 5.9). Since deviations from the Beer-Lambert law with increasing drug concentration has been reported to be an indication of self-association for certain compounds,[26] UV spectrophotometry was employed to study the occurrence of self-association of Hf in aqueous solutions at several pH values.

Figure 5.11 presents the absorbance (at λ_{max}) versus concentration profiles of Hf as a function of pH. At pH 6.3, where Hf was predominantly in the ionized form, the Beer-Lambert law was obeyed, and the molar absorptivity (ε) was calculated to be approximately 49,000 M^{-1} cm^{-1}. In contrast, deviation from linearity at the higher Hf concentrations became apparent when the pH of the solutions was increased to 7.6, 8.0 and 9.2. At these pH values, a greater proportion of Hf was present in the hydrophobic unionized form, thereby increasing the likelihood of self-association of Hf monomers. Curve fitting of the data suggests that the curvature in the profiles was real and not due to random variability, and the data could be fitted more closely to a quadratic rather than a linear equation. The ε values calculated for the pH 7.2, 8.0 and 9.2 samples varied between 20,000 and 40,000 M^{-1} cm^{-1} indicating the presence of different species of Hf, which may include ionized and unionized Hf monomers as well as associated forms of Hf (dimers or larger complexes). Interestingly, at pH 10.6, deviation from linearity was no longer apparent and the data could be fitted by linear regression. At this pH, the ε values were approximately 31,000 M^{-1} cm^{-1}, suggesting that the Hf species present at pH 10.6 was different from that at pH 6.3. It is possible that the benzylic hydroxyl group may become ionized at this pH, and being more polar, Hf was less likely to self-

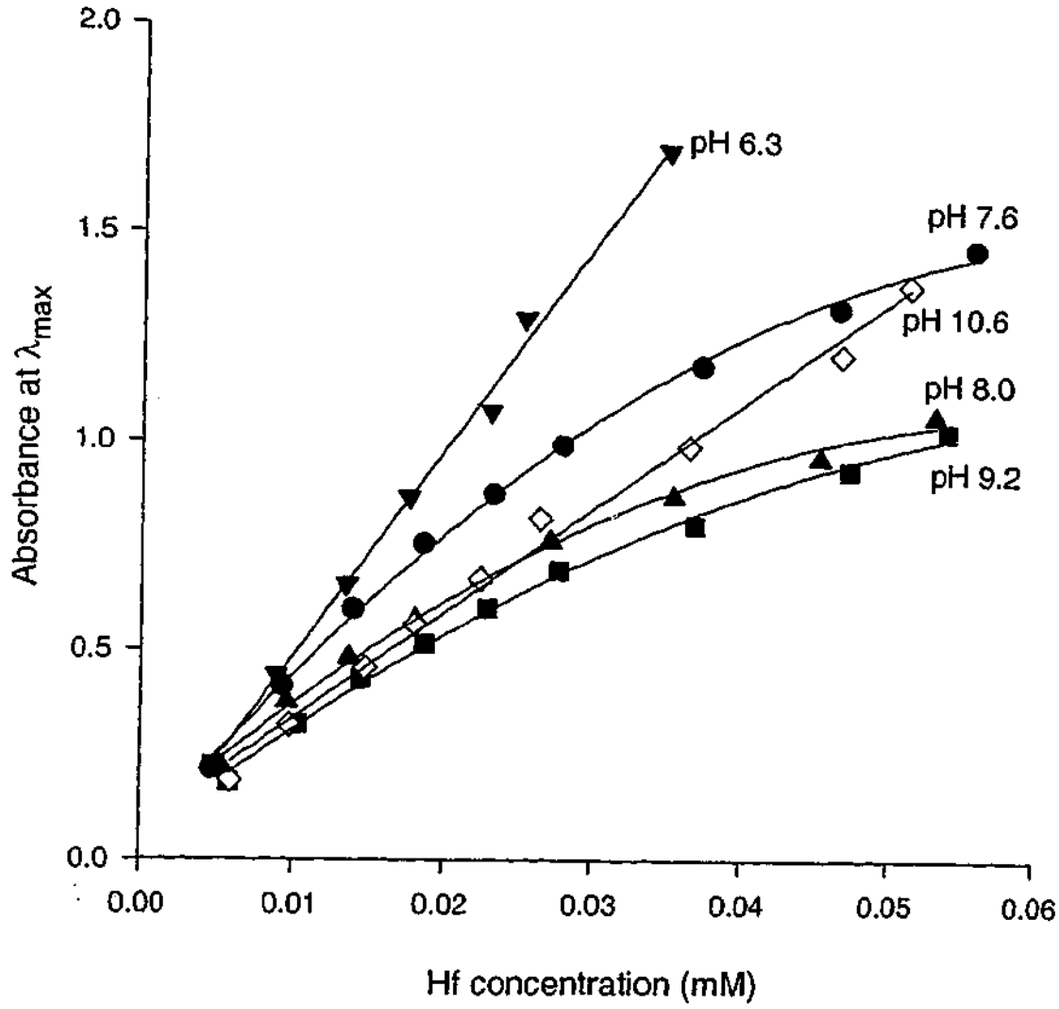

Figure 5.11 Absorbance (at λ_{max}) versus Hf concentration profiles as a function of pH: pH 6.3 (▼), pH 7.6 (●), pH 8.0 (▲), pH 9.2 (■) and pH 10.6 (◊). Symbols represent measured values and lines are fitted curves.

associate. Therefore, the decrease in log D at high pH values observed in the partitioning studies is likely to be due to greater partitioning of ionized Hf (as the anion) back into the aqueous micellar phase.

As expected, there is a greater tendency for hydrophobic unionized species of Hf to self-associate in aqueous solutions than the ionized species. The mechanism by which the Hf monomers may interact to form dimers or larger complexes is unknown but may include intermolecular hydrogen bonding or a stacking interaction of the planar (phenanthrene ring) region of the molecule, whereby each monomer can lie flat on top of another monomer or a stack of monomers. This stepwise self-association may continue indefinitely resulting in the formation of large complexes or multimers.[27] Further studies will be required to investigate this phenomenon and also to examine the rate and extent at which self-association occurs.

5.4 CONCLUSIONS AND IMPLICATIONS

Following post-prandial administration of Hf.HCl, intestinal lymphatic transport accounted for 47% of the administered dose whilst 19% of the dose was absorbed via the portal route. Based on the low triglyceride solubility of Hf.HCl, such extensive intestinal lymphatic drug transport was surprising. Physicochemical studies indicate that Hf.HCl was extensively solubilized when administered post-prandially, and the apparent pKa of Hf determined by UV spectrophotometry and partitioning studies in representative fed state mixed micellar solutions were 5.58 and 6.92, respectively. These results suggest that at intestinal pH, there will be some conversion of solubilized Hf.HCl to the highly lipid soluble free base form. Being more lipid soluble, the free base form of Hf is more likely to associate with lipid digestion products and be incorporated into chylomicrons during lipoprotein synthesis for subsequent intestinal lymphatic transport.

The present data suggest that intestinal lymphatic transport may be an important absorption pathway for highly lipophilic drugs. In addition to using indicators such as log P and TG solubility to predict the intestinal lymphatic transport of potential drug candidates, factors such as drug solubilization in post-prandial intestinal conditions followed by possible conversion to the more lipid soluble unionized form should also be considered especially for poorly lipid soluble weak acids and bases.

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CHAPTER 6

AN INVESTIGATION OF THE INTESTINAL LYMPHATIC TRANSPORT OF *N*-DESBUTYLHALOFANTRINE AFTER POST-PRANDIAL ORAL ADMINISTRATION

6.1 INTRODUCTION

The studies conducted in chapters 4 and 5 demonstrated that the post-prandial administration of either Hf base or Hf.HCl results in significant intestinal lymphatic drug transport (~ 50% of the administered dose). Furthermore, the maximal concentrations of Hf in thoracic lymph after post-prandial administration may be as high as 0.1-0.3 mg/mL, which is several orders of magnitude above the typical systemic plasma concentrations of 0.1-1 μg/mL. Considering that thoracic lymph enters the systemic circulation at the junction of the left jugular vein which then drains directly into the heart, it is possible that extensive post-prandial lymphatic transport of Hf and more importantly, the exposure of the heart to high concentrations of Hf may directly contribute to the onset of adverse cardiac effects previously reported with Hf therapy. This would also explain the lack of significant prolongation of the rate-corrected QT (QTc) interval of the electrocardiogram (ECG) after fasted Hf administration where intestinal lymphatic transport is minimal. However, further studies will be required to fully investigate the impact of high lymph drug concentrations on adverse cardiac effects.

It has been suggested that the major metabolite of Hf, *N*-desbutylhalofantrine (Hfm), could potentially be a safer alternative antimalarial drug to the parent compound. Hfm has equipotent antimalarial activity[1] and an *in vitro* study by Wesche *et al.*[2] indicated that Hfm had minimal effect on the QT interval and was unlikely to cause cardiotoxicity, thereby, supporting earlier clinical studies which suggested that Hfm had little or no effect on the QTc interval.[3,4] However, contrary to previous suggestions, a recent *in vivo* study using an anaesthetized rabbit model reported that the intravenous administration of Hfm resulted in a dose dependent prolongation of the QTc interval.[5] Moreover, the effects of Hfm on QTc prolongation were very similar to that of Hf.[6] At

present, the basis for the apparent differences in Hfm cardiotoxicity when assessed *in vivo* and *in vitro* has not been established.

6.2 OBJECTIVES

In light of the putative linkage between high lymph drug concentrations and the onset of adverse cardiac effects, the primary aim of this study was to examine the extent of intestinal lymphatic transport of Hfm after post-prandial oral administration as this may provide some indication of the likely cardiotoxicity profile of Hfm. However, prior to investigating the potential intestinal lymphatic transport of Hfm, the physicochemical properties of Hfm (i.e. log P_{oct} and solubility in triglyceride lipids) were determined to ascertain if Hfm is a likely candidate for intestinal lymphatic transport. The triple-cannulated dog model described in chapter 4 was utilized to verify the predictions made from the results of the physicochemical studies.

6.3 EXPERIMENTAL METHODS

6.3.1 Materials

N-Desbutylhalofantrine hydrochloride (Hfm.HCl) was supplied by SmithKline Beecham Pharmaceuticals (King of Prussia, PA, USA). Super refined soybean oil, Captex 355 and purified egg yolk lecithin were gifts from Croda Surfactants (Victoria, Australia), Abitec Corporation (Janesville, WI, USA) and Pharmacia (Sweden), respectively. Sodium taurocholate was purchased from Sigma Chemicals (St Louis, MO, USA). Avicel® (microcrystalline cellulose) and Explotab® (sodium starch glycolate) were obtained from Asahi Chemical Industry (Osaka, Japan) and H. Bleakley Pty. Ltd. (NSW, Australia), respectively, for the formulation of a dry fill capsule containing Hfm.HCl. All other chemicals were of analytical reagent grade and solvents

were of HPLC grade. Water was obtained from a Milli-Q (Millipore, Milford, MA, USA) water purification system.

6.3.2 Preparation of Hfm free base

Hfm free base was prepared by firstly dissolving Hfm.HCl (2.4 g) in a mixture of 95% ethanol (264 mL) and water (34 mL) at 35 to 40°C. To this, 1 M sodium hydroxide (6 mL) was added to alkalinize the solution. The resultant mixture was stirred for 10 min before the addition of water (55 mL) to precipitate Hfm free base out of solution. The crystalline drug was then filtered and washed with a 1:1 mixture of 95% ethanol/water and dried under vacuum in a desiccator containing P_2O_5. Hfm free base was identified and characterized by ^1H NMR spectroscopy, electrospray mass spectroscopy, infrared spectroscopy and differential scanning calorimetry.

6.3.3 Estimation of the log P_{oct} of Hfm by RP-HPLC

Reverse phase HPLC (RP-HPLC) was employed to estimate the lipophilicity of Hfm. The method utilized in this study was an adaptation of that described by Lombardo *et al.*[7] which was based on the observation that RP-HPLC retention data correlated well with the relative lipophilicity values determined by octanol-water partition experiments. By determining and converting retention data of a series of standards with known log P_{oct} values to a relative capacity factor (log k'_{water}) and plotting these values against the corresponding log P_{oct} values, an estimate of the log P_{oct} (ElogP_{oct}) of an unknown compound can be determined by simple regression.

6.3.3.1 HPLC conditions

All chromatographic runs were performed using a Waters Alliance® 2690 separation module, a Waters 996 photodiode array detector (Waters Corporation,

Milford, MA, USA) set at 208 and 254 nm and a SupelcoSil LC-ABZ column (5μm particle size, 120 Å, 4.6 mm i.d. x 5 cm, with an electrostatically coated silica stationary phase) maintained at 25°C. The mobile phase consisted of 20 mM MOPS (3-morpholinopropanesulfonic acid) buffer adjusted to pH 7.4 (with 1 M sodium hydroxide) and methanol in varying proportions (15%-80% v/v). Octanol-saturated water was used to prepare the buffer and 0.25% (v/v) of octanol was added to methanol. The solvent was pumped isocratically at a flow rate of 0.5 to 1.5 mL/min, depending on the lipophilicity range of the compound (Table 6.1). Samples were dissolved in 1:1 methanol/water to a concentration 50 μg/mL and a 5 μL volume of sample was injected onto the column.

Table 6.1 Recommended HPLC conditions for the determination of ElogP$_{oct}$ of compounds with a given range of log P$_{oct}$ value

Group	log P$_{oct}$ range	Flow rate (mL/min)	Solvent (% Methanol)
1	-0.5 to 1	0.5	15, 20, 25
2	1 to 3	1.0	40, 45, 50
3	3 to 5	1.5	60, 65, 70
4	> 5	1.5	70, 75, 80

6.3.3.2 *Calculation of capacity factor (k')*

The capacity factor (k') of a compound at a given concentration of solvent can be calculated using Equation 6.1:

$$k'_{\Phi} = (t_r - t_0) / t_0 \qquad \text{Equation 6.1}$$

where k'_{Φ} is the capacity factor of the compound at a given solvent concentration, t_r is the retention time and t_0 is the dead time (volume) of the column, which can be determined by injecting pure acetone.

For each standard and unknown compound, k' at a minimum of three different concentrations of mobile phase solvent was determined. By plotting log k' against solvent concentration and extrapolating the linear curve to the intercept of the Y-axis, the capacity factor in water (k'_{water}, or k' for 0% solvent) can be derived.

6.3.3.3 *Calibration curve*

For the calibration curve, six standard compounds with known (or predicted) log P_{oct} values were chosen and these included paracetamol, hydrocortisone acetate, estradiol, danazol, cinnarizine and halofantrine. The log k'_{water} values of these compounds were determined as described above and plotted against the corresponding log P_{oct} value. Linear regression of the data afforded the standard equation, $y = mx + c$, which was then used to estimate the log P_{oct} for unknown compounds. Each standard curve was accepted if the regression equation was within two standard deviation units of the mean. In all cases, the square of the correlation coefficient (R^2) was > 0.98. All regression analyses were performed using Sigmaplot for Window version 4.00 (SPSS, Inc., USA).

6.3.4 Solubility determinations

The equilibrium solubility of Hfm.HCl and Hfm free base in Captex 355 (medium-chain triglyceride) and soybean oil (long-chain triglyceride) at 30°C was determined as described previously in section 2.3.2. Further studies were also conducted (as described in section 5.2.3.3) to determine the solubility of Hfm.HCl and Hfm free base in sodium acetate buffer solutions (pH 5.5, 20 mM, ionic strength 0.1 M adjusted with sodium chloride) containing sodium taurocholate (NaTC) and lecithin in a 4:1 molar ratio at 37°C. The fasted and fed intestinal states were mimicked by using 5 and 15 mM NaTC, respectively. The samples were analysed by HPLC (section 3.3.3) and the reported values are the average of duplicate samples, with individual values varying by less than 5%.

6.3.5 Assessment of the intestinal lymphatic transport of Hfm

Healthy adult male greyhounds (28-35 kg, n = 4) were used to investigate the intestinal lymphatic transport of Hfm after post-prandial oral administration. The thoracic lymph duct and portal vein of the dogs were cannulated as previously described in section 4.3.2 and the dogs were allowed to recover unrestrained for a period of 12-16 h after surgery. An intravenous catheter was inserted into the cephalic vein prior to drug administration to enable serial blood sampling during the study period.

In order to obtain a post-prandial state, the dogs were fed a standard can of dog food (680 g) containing 5% crude fat approximately 30-45 min prior to drug administration. A 96 mg (0.2 mmol) dose of Hfm.HCl (equivalent to 100 mg Hf base on a molar basis) prepared as a simple dry fill with Avicel® (180 mg) and Explotab® (180 mg) in a size 0 hard gelatin capsule was administered orally with 50 mL of water. The dogs were allowed free access to drinking water and 25 mL of 0.9% sodium chloride

solution was administered by intravenous bolus injection at hourly intervals to limit dehydration due to continual collection of thoracic lymph.

Lymph was collected continuously into 50 mL tubes containing 75 mg disodium EDTA over a 12 h post-dosing period. Lymph samples collected each hour were combined and the total mass of lymph collected per hour was determined gravimetrically. The samples were stored between 5-8°C prior to analysis.

Systemic and portal vein blood samples (2.5 mL) were obtained via indwelling cannulas at predose (-5 min), 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12 and 24 h after dosing. Blood samples were collected into individual tubes containing dipotassium EDTA, plasma was separated by centrifugation and then stored at –20°C prior to analysis.

The method described in section 4.3.4.1 was employed to determine the concentration of Hfm in lymph. Recovery of Hfm from spiked blank lymph was greater than 90%. The absolute amount of Hfm collected in thoracic duct lymph over the 12 h post-dosing period was calculated by multiplying the concentration of drug in lymph by the corresponding mass of lymph produced during each collection period. Plasma concentrations of Hfm was determined using the validated plasma extraction procedure and gradient HPLC assay described in sections 2.3.6.2.1 and 3.3.3, respectively.

Lymph and plasma triglyceride concentrations were also measured using a clinical chemistry analyzer (Roche Cobras Mira, Basle, Switzerland) and a commercial enzyme-based colorimetric assay kit (Boehringer Mannheim, Germany).

6.4 RESULTS AND DISCUSSION

In the present study, the physicochemical properties of the equipotent metabolite of Hf, *N*-desbutylhalofantrine (Hfm) were investigated to determine the likelihood of Hfm as a candidate for intestinal lymphatic transport. The potential intestinal lymphatic transport of Hfm after post-prandial administration of Hfm.HCl was then verified using the conscious triple-cannulated dog model previously described in chapter 4. From a clinical perspective, the extent of intestinal lymphatic transport of Hfm after oral administration may have important implications as extensive lymphatic drug transport and exposure of the heart to high drug concentrations may contribute directly to the onset of adverse cardiac effects. This putative linkage has been observed with Hf.

6.4.1 Physicochemical properties of Hfm

Physicochemical parameters such as log octanol/water partition coefficient (log P_{oct}) and triglyceride solubility are useful indicators for predicting the potential intestinal lymphatic transport of orally administered drugs. It has been suggested that candidate drug molecules generally require a log P_{oct} in excess of 5, and triglyceride solubility of at least 50 mg/mL before intestinal lymphatic transport is likely to become a major contributor to oral bioavailability.[8] These estimations were based on the difference between portal blood and lymph flow (500:1), the lipid load in lymph, the relative affinity of the absorbed drug for the lipoidal and aqueous environments of the enterocyte, and the likely association of the drug with the triglyceride core of chylomicrons. In addition to using these two indicators to predict the intestinal lymphatic transport of potential drug candidates, the results from the previous chapter suggest that drug solubilization in representative fed state intestinal conditions and possible conversion to the more lipid soluble unionized form should also be considered especially for poorly water soluble acids and bases.

6.4.1.1 *Estimation of log P_{oct} of Hfm*

A RP-HPLC method was employed to estimate the log P_{oct} ($ElogP_{oct}$) of Hfm. The method was an adaptation of that described by Lombardo *et al.*,[7] and was based on the observation that RP-HPLC retention data correlated well with relative lipophilicity values determined by octanol-water partition experiments. RP-HPLC was used in preference to the classical shake-flask method as the poor aqueous solubility of Hfm and the limit of detection of current available instrumentation may reduce the accuracy of the latter method.

Table 6.2 presents the calculated capacity factor in water (log k'_{water}) and known log P_{oct} values for six standard compounds. These values were used to construct the calibration curve shown in Figure 6.1 and regression analysis of the data afforded equation 6.2:

$$\log P_{oct} = 1.528 \cdot \log k'_{water} - 1.079 \qquad (R^2 = 0.984) \qquad \text{Equation 6.2}$$

Using this equation, the individual $ElogP_{oct}$ value for each of the standard was also calculated and these are presented in Table 6.2. The $ElogP_{oct}$ values were very similar to the reported log P_{oct} values which had been obtained by the shake-flask method suggesting that the RP-HPLC method may be used to provide relatively accurate estimation of the log P_{oct} values of unknown compounds.

The calculated log k'_{water} value of Hfm was 5.59, therefore, using equation 6.2, the log P_{oct} of Hfm was estimated to be 7.46. The log P_{oct} of Hfm was also calculated using the ACD Log P DB version 4.56 program (Advanced Chemistry Development, Inc., Ontario, Canada) which afforded a value of 6.73. Despite the slight discrepancy between both methods, it appears that Hfm is a highly lipophilic compound and could potentially be a candidate for intestinal lymphatic transport.

Table 6.2 Capacity factor in water (log k'_{water}) and log P_{oct} values for six standard compounds

Standard compound	log k'_{water}	log P_{oct}[a]	ElogP$_{oct}$[b]	Ref. no.[c]
Paracetamol	1.14	0.51	0.66	9
Hydrocortisone acetate	1.83	2.19	1.72	10
Estradiol	3.28	4.01	3.93	11
Danazol	4.01	4.53	5.05	12
Cinnarizine	4.50	5.60	5.80	13
Halofantrine	6.29	8.86[d]	8.53	

[a] Log P_{oct} data obtained by the shake-flask method as reported in literature.
[b] Data obtained using Equation 6.2.
[c] References for the reported log P_{oct} data.
[d] Calculated value using the ACD Log P DB v. 4.56 program (Advanced Chemistry Development, Inc., Ontario, Canada).

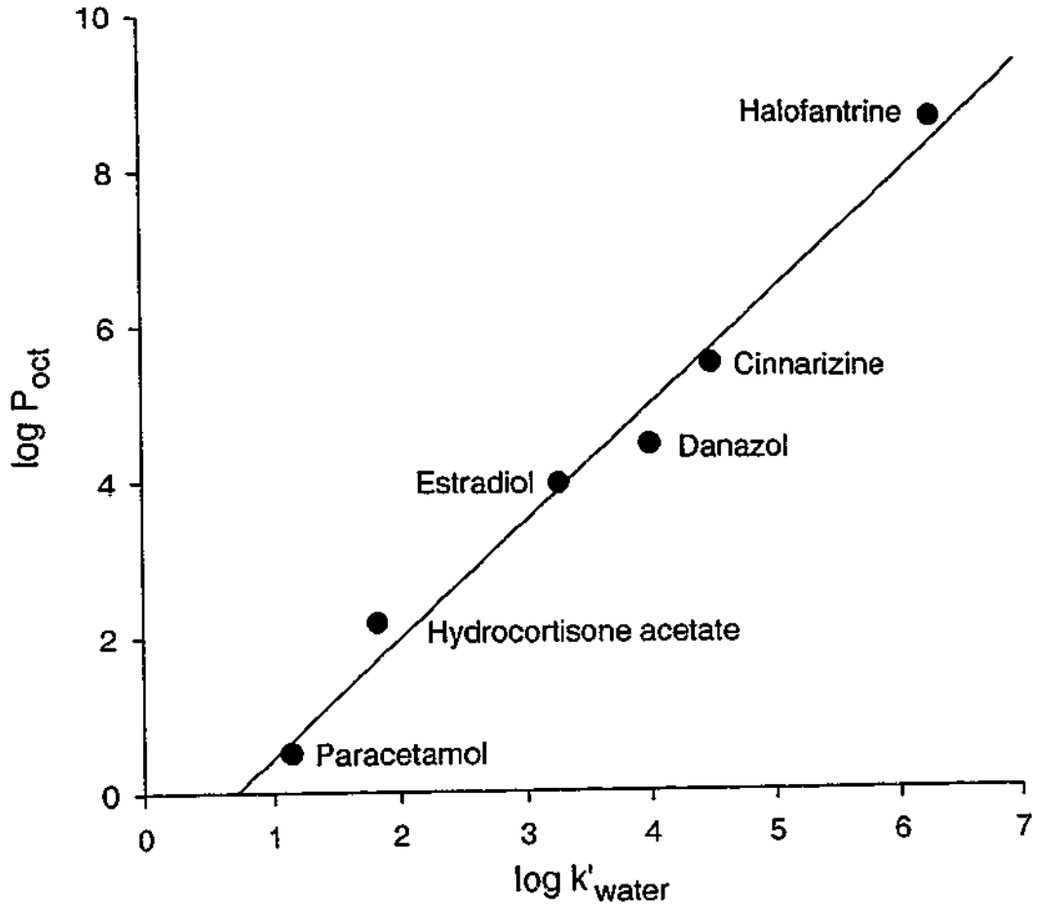

Figure 6.1 Relationship between log P_{oct} and log k'_{water} values for six standard compounds. The line was fitted by linear regression to afford equation 6.2: $\log P_{oct} = 1.528 \cdot \log k'_{water} - 1.079$, $R^2 = 0.984$.

6.4.1.2 *Solubility of Hfm*

In addition to high partition coefficient being a pre-requisite for intestinal lymphatic transport, lipid solubility is another important parameter to consider. Presented in Table 6.3 are the equilibrium solubility data of Hfm.HCl and Hfm base in Captex 355 (medium-chain triglyceride) and soybean oil (long-chain triglyceride). Considering that the solubility of both forms of Hfm in triglyceride lipids were considerably lower (at least 6-fold) than Hf base and also well below the nominal pre-requisite value of 50 mg/mL, the extent of intestinal lymphatic transport of Hfm is expected to be lower than Hf base. This assumption was based on a previous study with DDT and hexachlorobenzene (HCB),[8] which showed the importance of lipid solubility in determining the lymphatic transport of drugs with high partition coefficients. Although both compounds were regarded as highly lipophilic as evidenced by high log P_{oct} values (> 6), the 13-fold lower triglyceride solubility of HCB compared with DDT was reflected in a 14.6-fold decrease in extent of intestinal lymphatic transport.

Table 6.3 Equilibrium solubility of Hfm.HCl, Hfm base and Hf base in triglyceride lipid vehicles at 30°C

Lipid vehicle	Solubility (mg/g)		
	Hfm.HCl[a]	Hfm base[a]	Hf base[b]
Captex 355	< 0.05	16.74	82.2
Soybean oil	< 0.05	7.67	46.7

[a] Data represent the mean of duplicate samples, with individual values varying by less than 5%.

[b] Data had previously been obtained under similar conditions and reported in chapter 2 (Table 2.2).

Table 6.4 presents the solubility of Hfm.HCl and Hfm base in buffer and bile salt mixed micellar solutions representing fasted and fed intestinal states. The marked increase in solubility of both forms of Hfm in the representative fed state mixed micellar solution (15 mM NaTC:lecithin, pH 5.5) suggests that the bile constituents present in the post-prandial intestinal milieu is likely to greatly enhance drug solubilization, thereby increasing the amount of drug available for absorption.

Table 6.4 Solubility of Hfm.HCl and Hfm base in buffer, representative fasted state (3.75 mM NaTC:lecithin, 4:1) and fed state (15 mM NaTC:lecithin, 4:1) mixed micellar solutions at 37°C

Solution[a]	Solubility[b] (μg/mL)	
	Hfm.HCl	**Hfm base**
Buffer (pH 5.5)	7.05	12.95
3.75 mM NaTC:lecithin (4:1, pH 5.5)	11.85	12.30
15 mM NaTC:lecithin (4:1, pH 5.5)	2255	1408

[a] NaTC, sodium taurocholate.
[b] Data represent the mean of duplicate samples, with individual values varying by less than 5%.

6.4.2 Assessment of the absorption of Hfm after post-prandial administration

6.4.2.1 Intestinal lymphatic transport

Although Hfm has been shown to be a highly lipophilic compound ($ElogP_{oct}$ of 7.46), the limited solubility of Hfm in long-chain triglyceride lipid suggests that Hfm is unlikely to be a candidate for significant intestinal lymphatic transport after post-prandial oral administration. Nevertheless, the results of the physicochemical studies were verified by examining the extent of lymphatic transport of Hfm after post-prandial administration of 96 mg Hfm.HCl to thoracic lymph duct cannulated dogs. The study was conducted in post-prandial dogs as the administration of food readily provided the lipid source required to support lipoprotein synthesis (primarily chylomicrons and VLDL) by the enterocyte. Moreover, the solubility studies indicate that the solubilization of Hfm.HCl is likely to be enhanced in the post-prandial state.

The cumulative lymphatic transport of Hfm after post-prandial administration of Hfm.HCl to conscious thoracic lymph duct cannulated dogs is presented in Figure 6.2 and Table 6.5. The previously reported lymphatic transport data of Hf after post-prandial administration of an equivalent dose (on molar basis) of Hf base (100 mg) or Hf.HCl (107 mg) has also been included to enable the data to be compared. Unlike Hf, the intestinal lymphatics do not appear to be the primary absorption pathway for Hfm as the extent of lymphatic transport of Hfm was extremely low (less than 1% of administered dose) following post-prandial administration of Hfm.HCl. This was not surprising considering the poor triglyceride solubility of Hfm.HCl. Although Hf.HCl has also been shown to be poorly lipid soluble, the extensive lymphatic transport observed after post-prandial administration was largely due to the conversion of Hf.HCl to the more lipid soluble free base form. However, for Hfm, the triglyceride solubility of both forms of Hfm (free base and HCl salt) was poor and hence, may have precluded

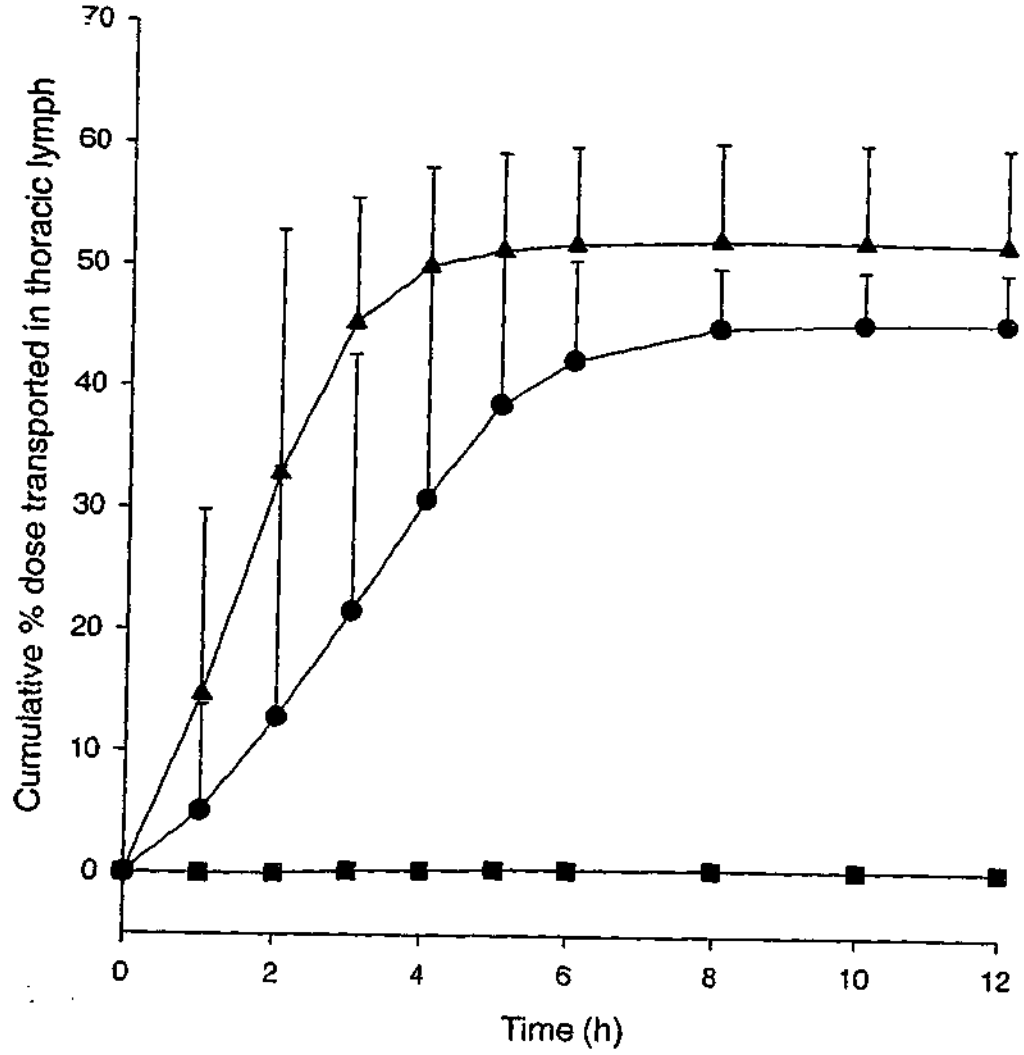

Figure 6.2 Cumulative lymphatic transport of Hfm and Hf (% dose, mean ± SD) in thoracic lymph duct cannulated dogs following post-prandial oral administration of either 96 mg Hfm.HCl (■, n = 4), 107 mg Hf.HCl (●, n = 3, from chapter 5) or 100 mg Hf base (▲, n = 4, from chapter 4).

Table 6.5 Mean (± SD) cumulative lymphatic transport of Hfm or Hf, maximum drug concentration in lymph, cumulative mass of triglyceride (TG) in lymph and cumulative lymph flow after post-prandial administration of Hfm.HCl, Hf.HCl and Hf base to thoracic lymph duct cannulated dogs

	Hfm.HCl (96 mg, n = 4)	**Hf.HCl**[a] (107 mg, n = 3)	**Hf base**[a] (100 mg, n = 4)
Cum. drug transport[b] (% dose)	0.6 ± 0.1	47.3 ± 4.3	54.0 ± 8.2
C_{max} in lymph[c] (μg/mL)	1.9 ± 0.7	226 ± 112	241 ± 74
Cum. mass of TG[b] (g)	31.8 ± 6.4	32.5 ± 6.7	33.6 ± 3.5
Cum. lymph flow[b] (g)	841 ± 110	844 ± 124	1047 ± 124

[a] Data for Hf.HCl and Hf base were obtained from chapters 5 and 4, respectively.
[b] Cumulative data were collected over the 12 h post-dosing period.
[c] Maximum drug concentration in lymph.

the significant lymphatic transport of Hfm. This study further demonstrates the importance of employing both partition coefficient and triglyceride solubility (of the unionized and ionized forms) of the candidate drug as indicators for predicting intestinal lymphatic drug transport. As a result of the large differences in the extent of lymphatic transport between Hfm and Hf, a vast difference in maximum drug concentrations in lymph was also observed (Table 6.5).

The cumulative mass of triglyceride transported in lymph and the cumulative lymph flow over the 12 h post-dosing period are shown in Table 6.5. The values obtained in this study were consistent with those reported in chapters 4 and 5. Recovery of greater than 90% (by mass) of the lipid present in the ingested food confirmed the integrity of the lymph duct cannulation and ensured the complete collection of thoracic lymph. Therefore, the low lymphatic transport of Hfm is unlikely to be due irregularities associated with the model.

6.4.2.2 Absorption of Hfm into the systemic circulation via the portal route

In addition to assessing the intestinal lymphatic transport of Hfm, its absorption into the systemic circulation via the portal route was also investigated. Figure 6.3 depicts the portal and systemic plasma concentration versus time profiles of Hfm, and the corresponding pharmacokinetic parameters are presented in Table 6.6. As expected, the portal plasma concentrations were higher than the systemic plasma concentrations during the post-dosing absorption phase and it appears that absorption was largely complete after 8 h. The systemic and portal plasma C_{max} values for Hfm were not significantly different ($p > 0.05$) from that after Hf administration (base or HCl), however, T_{max} for Hfm did appear to be later compared to Hf (base or HCl). In this study, the percentage dose of Hfm absorbed via the portal route could not be ascertained as the pharmacokinetics of Hfm after intravenous administration were not determined.

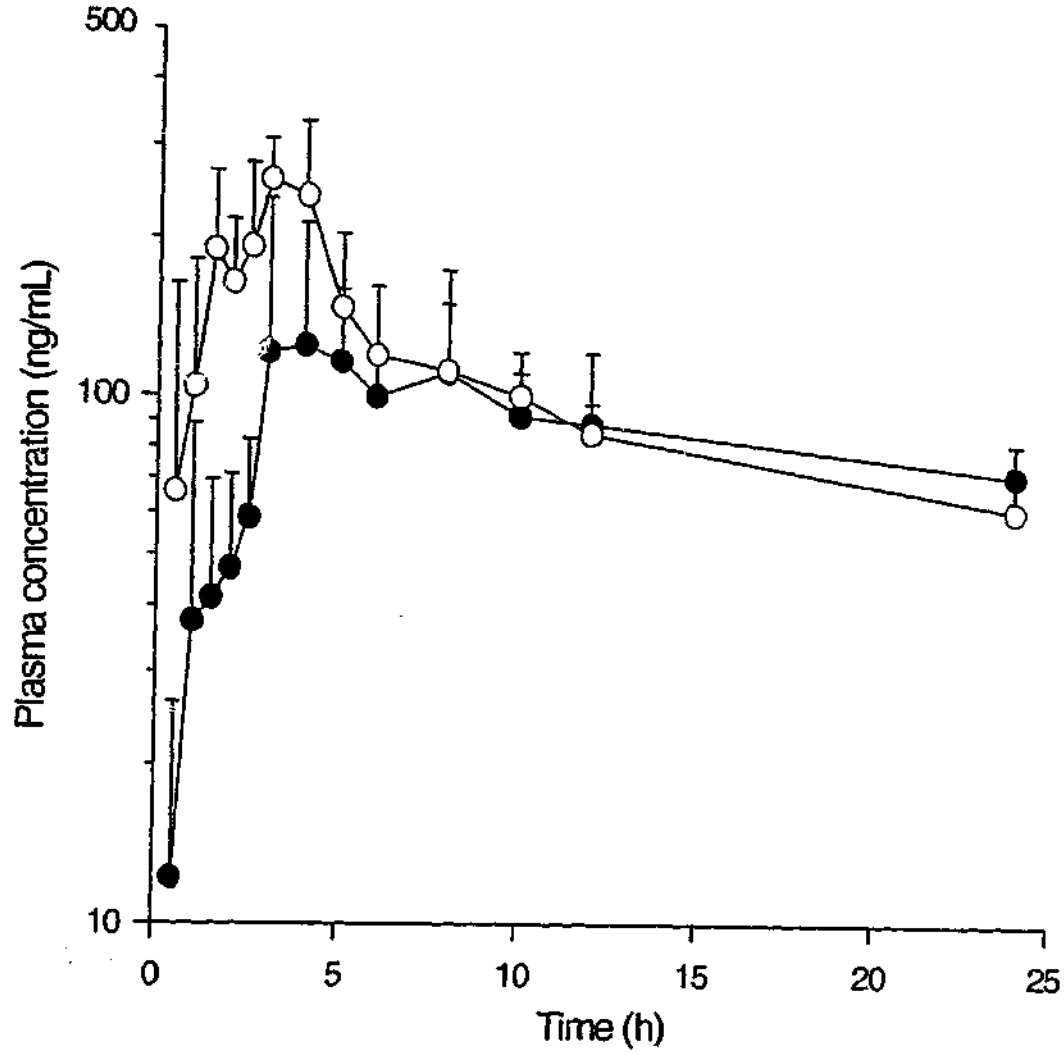

Figure 6.3 Portal (open symbols) and systemic (closed symbols) plasma concentration versus time profiles of Hfm after post-prandial oral administration of 96 mg Hfm.HCl to thoracic lymph duct cannulated dogs. Data are presented as mean ± SD, n = 4.

Table 6.6 Portal and systemic plasma pharmacokinetic parameters (mean ± SD, n = 4) of Hfm after post-prandial administration of 96 mg Hfm.HCl to thoracic lymph duct cannulated dogs

Parameter	Portal plasma	Systemic plasma
C_{max} (ng/mL)	302 ± 65	155 ± 99
T_{max} (h)	3.2 ± 0.6	6.6 ± 4.0
AUC^{0-24h} (ng.h/mL)	2649 ± 161	2116 ± 553

6.4.3 Clinical implications

Hfm has been reported to be largely devoid of the adverse cardiac effects that have limited the clinical utility of Hf in the treatment of multi-drug resistant malaria.[2-4] However, in contrast to previous suggestions, a recent study using an anaesthetized rabbit model has demonstrated that when administered intravenously, Hfm does lead to a dose dependent prolongation of the QTc interval.[5]

It has been suggested that the clinical cardiac consequences observed after Hf administration result, at least in part, from a contribution of extensive lymphatic drug transport and high post-prandial drug concentrations in lymph. Whilst the intrinsic effects of Hfm on repolarisation of the heart after intravenous administration has been shown to be similar to that of Hf, the minimal lymphatic transport of Hfm after post-prandial oral administration may protect the heart from exposure to high drug concentrations (Table 6.5) and potentially minimize cardiotoxicity. It is therefore possible that Hfm could be a safer alternative to Hf for the treatment of malaria.

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However, further studies will be required to investigate the adverse cardiac effects of Hfm and the role of lymphatic drug transport in the onset of adverse cardiac effects.

6.5 CONCLUSIONS

The intestinal lymphatic transport of Hfm after post-prandial administration of Hfm.HCl was investigated in conscious triple-cannulated dogs. Although Hfm has been shown to be a highly lipophilic compound (ElogP_{oct} of 7.46), less than 1% of the administered dose was lymphatically transported after post-prandial administration. This was presumably attributed to the poor solubility of both forms of Hfm (free base and HCl) in long-chain triglyceride lipid. The results of the present study are consistent with a previous report,[8] which has suggested that partition coefficient and solubility in a long-chain triglyceride lipid are both important parameters to consider when predicting intestinal lymphatic drug transport after oral administration.

It was proposed that extensive lymphatic drug transport and exposure of the heart to high drug concentrations may contribute directly to the onset of adverse cardiac effects. However, the limited extent of lymphatic transport of Hfm after post-prandial oral administration may protect the heart from exposure to high drug concentrations and potentially minimize cardiotoxicity even though Hfm has been reported to lead to a dose dependent prolongation of the QTc interval after intravenous administration.

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CHAPTER 7

SUMMARY AND RECOMMENDATIONS FOR FUTURE WORK

7.1 SUMMARY

In this thesis, a number of factors which may impact on the absorption and metabolism of Halofantrine (Hf), a highly lipophilic phenanthrenemethanol antimalarial were investigated. Although Hf is well-tolerated and orally active, the clinical utility of Hf in the treatment of malaria is limited by the poor and erratic absorption of Hf after oral administration of the commercially available Hf.HCl tablet (Halfan®). The resulting low and highly variable plasma concentrations have not only been associated with treatment failures but may potentially accelerate the emergence of resistance to Hf. The extent of Hf absorption increases markedly following post-prandial administration of Hf.HCl, and is at least in part a result of enhanced drug solubilization and dissolution within the post-prandial intestinal milieu.[1] However, this practice is now contraindicated due to the possibility of post-prandial administration leading to high and poorly controlled Hf plasma levels which have previously been associated with an increased incidence of adverse cardiac effects.

Lipids are a major constituent of ingested food which may effect physicochemical and physiological changes within the gastrointestinal tract that significantly influence the absorption and oral bioavailability of poorly water soluble and highly lipophilic drugs.[2] An initial objective of the work described in this thesis was therefore to examine the potential for lipid-based formulations to improve the oral delivery of Hf in a more controlled and reproducible manner than that observed previously after post-prandial administration. Through rational design and utilizing *in vitro* assessment methods developed during these studies, a number of promising multi-component formulations which produced fine (micro)emulsions on dispersion in the gastrointestinal tract were evaluated in fasted beagles. These lipid-based self-emulsifying formulations afforded a 6- to 8-fold improvement in absolute oral bioavailability relative to the commercial Hf.HCl tablet. Although not statistically

significant, there was a trend towards higher bioavailability after administration of the formulation containing long-chain lipid components compared with the equivalent formulation containing medium-chain lipid components. The significant increases in oral bioavailability seen after administration of these formulations were likely to be a consequence of delivering Hf in a solubilized and rapidly dispersed manner, thereby overcoming the dissolution rate-limitation to the absorption of Hf. These data illustrate the potential utility of dispersed lipid-based formulations for the improved oral delivery of Hf and also suggest the utility of similar formulations for other poorly water soluble drugs. Importantly, the magnitude of bioavailability enhancement (6- to 8-fold) that was achieved in these studies through rational design of lipid-based formulations is significant and represents a substantial improvement over the currently available formulation.

An interesting feature arising from the previous fed and fasted studies of Hf.HCl[1,3] was that although food markedly increased the oral bioavailability of Hf, the proportion of Hf metabolized to its equipotent active metabolite, *N*-desbutylhalofantrine (Hfm), was significantly reduced. Therefore, a second focus of this thesis was to elucidate the mechanism(s) by which post-prandial administration of Hf may lead to a decrease in its metabolism. Possible factors for this include inhibition of presystemic (enterocyte-based) metabolism by components present in food and/or post-prandial re. .uitment of intestinal lymphatic transport of Hf leading to avoidance of hepatic first-pass metabolism.

In the work described in this thesis, the co-administration of Hf with ketoconazole, a potent inhibitor of CYP3A4, inhibited the formation of Hfm and improved the oral bioavailability of Hf, suggesting that both systemic and presystemic metabolism of Hf to Hfm was primarily mediated via CYP3A4 *in vivo*. Furthermore, based on the localization of functional CYP3A4 in the intestinal epithelium and liver, it

is likely that both prehepatic (enterocyte-based) and hepatic CYP3A4 contribute to the presystemic metabolism of Hf after oral administration. Interestingly, the decreased plasma Hfm/Hf AUC ratios observed after fasted administration of Hf with ketoconazole were similar to the low values previously observed after post-prandial administration. These data suggested that the co-administration of Hf with food may result in the direct inhibition of presystemic CYP3A4-mediated metabolism of Hf by components present in food, or alternatively that the co-administration of food may stimulate intestinal lymphatic transport of Hf thereby leading to avoidance of hepatic presystemic metabolism.

Hf was considered a likely candidate for lymphatic transport as the calculated log P was ~ 8.5 and the free base form of Hf was highly soluble in long-chain triglyceride lipid. Earlier studies in conscious rats indicated that after oral administration of Hf base in a lipid vehicle, approximately 20% of a 2 mg dose was recoverable in intestinal lymph.[4] However, it is difficult to extrapolate rat transport data to higher species especially under fed and fasted conditions, as bile flow in the rat is essentially continuous and independent of food intake (unlike the situation in humans), therefore precluding the attainment of representative pre-and post-prandial intestinal states. Moreover, due to size constraints it is not possible to administer prototypical dose forms for higher species to small animals such as a rat.

The limitations of the rat model were addressed by the development of a robust triple-cannulated conscious dog model which enabled the simultaneous assessment of absorption, enterocyte-based metabolism and lymphatic transport of candidate lipophilic drugs under realistic pre- and post-prandial conditions. The physical size of the dog also allowed the oral administration of dose forms that are clinically relevant to humans. By monitoring the recovery of triglyceride lipid in thoracic duct lymph, the model was also essentially internally validated. This model will not only enable the rapid identification

of lipophilic drug candidates which may be substrates for intestinal lymphatic transport but may also facilitate identification of the key factors that impact on the bioavailability, lymphatic transport, and metabolic profiles of highly lipophilic drugs. Furthermore, it can assist in the development of lipid-based formulations to maximize oral drug bioavailability.

After post-prandial administration of 100 mg Hf base to thoracic lymph duct cannulated dogs, 54% of the administered dose was lymphatically transported, accounting for approximately 80% of the total dose absorbed. Not surprisingly, the lymphatic transport of Hf was much lower (1.3% of dose) after fasted administration, presumably due to the lack of an adequate lipid source to drive chylomicron formation. Whilst comparison of portal and systemic plasma Hfm concentrations suggested the occurrence of enterocyte-based conversion of Hf to Hfm, the proportion of Hf metabolized to Hfm was similar after fasted or post-prandial administration. Therefore, it appears that the previously observed decrease in post-prandial metabolism of Hf is largely a consequence of significant post-prandial intestinal lymphatic transport which effectively bypasses hepatic first-pass metabolism as opposed to the direct impact of a component of food on metabolism.

In contrast to Hf base, the commercially available Hf.HCl was not considered a likely candidate for intestinal lymphatic transport as its solubility in long-chain triglyceride lipids is low (< 1mg/mL). However, after administration of 107 mg of Hf.HCl to post-prandial dogs, lymphatic drug transport was surprisingly high at 47% of the administered dose. It was postulated that partial conversion of solubilized Hf.HCl to the highly lipid soluble free base form within the intestinal lumen might account for the extensive lymphatic transport. However, Hf is a tertiary amine with an estimated pKa of at least 10 and the fraction of Hf present as the free base is likely to be extremely low at intestinal pH values. The results of a number of physicochemical studies suggest that

Hf.HCl was extensively solubilized in the post-prandial intestinal milieu and surprisingly that the apparent pKa of Hf determined by UV spectrophotometry and partitioning studies employing soybean oil and simulated fed state mixed micellar solutions was 5.58 and 6.92, respectively. Molecular modelling calculations suggest that the low pKa values of Hf could be due to Hf adapting a low energy structural orientation which enables the basicity of the tertiary nitrogen to be reduced. It appears therefore that the extensive lymphatic drug transport observed after fed administration of Hf.HCl was likely to be due to the conversion of solubilized Hf.HCl to the free base prior to absorption.

In addition to indicators such as log P and triglyceride solubility, these data suggest that factors such as drug solubilization in representative fed state intestinal conditions, the potential impact of this solubilization on apparent pKa and the possible conversion to the more lipid soluble unionized form should also be considered when predicting the potential lymphatic transport of salts of poorly water soluble weak acids and bases.

The maximal concentrations of Hf in lymph after post-prandial administration of either Hf base or Hf.HCl were ~ 0.1-0.3 mg/mL, which is several orders of magnitude above the typical systemic plasma concentrations of 0.1-1 μg/mL. Excessively high Hf plasma concentrations (which typically occur after post-prandial administration) have been associated with significant prolongation of the QTc interval of the ECG in some patients.[5,6] Considering that thoracic lymph enters the systemic circulation at the junction of the left jugular vein directly above the heart, it is possible that exposure of the heart to high concentrations of Hf may directly contribute to the onset of adverse cardiac effects associated with Hf therapy. In light of this putative linkage, the lymphatic transport of Hfm, which has been reported to be largely devoid of adverse cardiac effects[7,8] was investigated. Although the estimated log P of Hfm is high (7.46),

the poor lipid solubility of both forms of Hfm (free base and HCl salt) when compared to Hf (lipid solubility of Hfm base in long-chain triglyceride was at least 6-fold lower than Hf base) precluded significant lymphatic transport of Hfm after post-prandial administration of Hfm.HCl (< 1% of dose). It is possible therefore, that the limited extent of lymphatic transport of Hfm may protect the heart from exposure to high drug concentrations and potentially minimize cardiotoxicity. The potential link between lymphatic transport of highly lipophilic drugs and QTc prolongation is an interesting outcome from this work and deserves further investigation (see later).

In summary, the data presented in this thesis indicate the potential utility of dispersed lipid-based formulations for the oral delivery of lipophilic drugs. A new dog model was developed to enable the rapid identification of lipophilic drug candidates that may be substrates for intestinal lymphatic transport. Using this model, the intestinal lymphatics have been shown to be a more important absorption route for highly lipophilic drugs (and in particular for the salts of highly lipophilic drugs) than previously assumed. This absorption pathway offers the advantage of avoidance of hepatic first-pass metabolism and opportunities for targeted delivery. The model may also assist in the development of lipid formulations to maximize oral drug bioavailability and facilitate the identification of key factors that impact on the bioavailability, lymphatic transport and metabolic profiles of highly lipophilic drugs.

7.2 RECOMMENDATIONS FOR FUTURE WORK

The following are suggestions for future work which may be conducted to further increase current knowledge of the factors which may impact on the absorption and metabolic processing of Hf and potentially other lipophilic drugs.

CYP3A4 and P-glycoprotein (P-gp) have been reported to have a significant overlap in substrate specificity and may act synergistically to limit oral drug

bioavailability.[9] Hf has yet to be identified as a substrate for intestinal P-gp, however, further studies using for example, wild type and P-gp knockout mice to establish the effect of P-gp on the oral bioavailability of Hf may be beneficial, especially in addressing potential drug/drug, drug/excipient and drug/food interactions.

Lymphatically transported drugs are typically solubilized within the triglyceride lipid core of chylomicrons, which are formed in response to orally ingested lipids.[10] Hence, for lymphatic transport to be significant, candidate drugs need to be administered in the presence of an adequate lipid source. Whilst post-prandial administration readily provides the lipid source required to support chylomicron formation, the effect of pharmaceutically relevant volumes of formulated lipids on chylomicron formation in animal models other than the rat has not been studied. The lymphatic transport data in the post-prandial dog suggests that the quantities of triglyceride required to support lymphatic transport may be much lower than that present in a meal. Therefore, studies examining the potential for lipid-based formulations (such as those developed in chapter 2) to stimulate chylomicron formation and consequently intestinal lymphatic drug transport after fasted oral administration to an appropriate animal model (such as the dog) may provide insight into formulation strategies which may be adopted to enhance lymphatic drug transport. Further studies will also be required to investigate the intracellular processes that occur prior to lymphatic drug transport as these remains poorly understood.

The putative linkage between high lymph drug concentrations and the onset of adverse cardiac effects such as prolongation of the electrocardiographic QTc interval and ventricular arrhythmias also requires further investigation. Assessment of the potential lymphatic transport of drugs (e.g. terfenadine, cisapride) known to induce such cardiac effects at elevated plasma drug concentrations may assist in the establishment of this linkage. Development of an appropriate model for investigating the cardiotoxicity

of Hfm will also be required to determine if Hfm is potentially a safer alternative antimalarial drug to the parent compound.

Assessment of the mass balance of lymphatic transport of Hf in lymph cannulated and non-lymph cannulated dogs may be warranted given that a recent study[11] in rats has indicated that where lymphatic transport was the primary mechanism of drug transport to the systemic circulation, the bioavailability of Hf assessed in non-lymph cannulated rats was lower than the extent of total availability measured in lymph cannulated rats. It has been suggested that these results may be attributed to the presystemic clearance of Hf within the lymphatics or an increase in systemic clearance of lymphatically transported Hf.

Modification of the triple-cannulated dog model such that the portal blood flow may be measured could be advantageous as this would not only enable an estimation of the fraction of dose absorbed from the gut into the portal circulation but also allow the relative contribution of hepatic and enterocyte-based metabolism to be evaluated. The modified model may also be utilized to assess the effects of various surfactants and lipidic excipients on enterocyte-based metabolism.

A recent study has reported the stereoselective binding of Hf enantiomers to lipoprotein fractions of fasted plasma.[12] The results indicated that the (+)-enantiomer is preferentially associated with the lipoprotein-rich fractions of plasma, whereas the (-)-enantiomer is primarily associated with lipoprotein deficient fractions. The stereoselective binding of Hf enantiomers to lymph lipoprotein fractions and the potential for selective lymphatic transport of Hf enantiomers after post-prandial administration has yet to be investigated. Given that the association of Hf with plasma lipoproteins has been shown to affect the pharmacokinetics and potentially the pharmacodynamics of the drug, it may be necessary to further investigate the binding of

Hf to lymph lipoproteins after oral administration and to determine the likely effect of post-prandial administration on lipoprotein binding.

Additional physicochemical studies investigating the interaction between Hf and various micellar systems and the impact of this on the partitioning behaviour of Hf may also be beneficial. The results of such studies would also be applicable to other ionizable compounds and would therefore assist in providing more definitive criteria for predicting the lymphatic transport of potential drug candidates.

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