UTILITY OF A NOVEL SELF-EMULSIFYING SYSTEM FOR A LIPOPHILIC DRUG WITH LOW ORAL BIOAVAILABILITY

by

CHOY WAI PENG

Thesis submitted in fulfilment of the requirements

for the degree of

Master of Science

May 2006

To Father in Heaven,

my beloved husband, Victor Joseph and my

dearest parents, Choy Min You and Teh Yuet Ying

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my deepest thanks and appreciation to my supervisor, Professor Dr. Yuen Kah Hay for his wisdom, inspiration, guidance and support. I am most honoured that he has given me the chance to pursue my MSc. under his supervision, which turns out to be moments of constant enlightenment that I will cherish always. He has provided me a very constructive and conducive working environment in which, I have the opportunity to know many wonderful people that has enriched my life.

I am equally indebted to Dr. Ng Bee Hong, who has spent most of her time and effort in helping me not only in this study but also in many other ways. Her invaluable advice is truly appreciated and well treasured. My heartfelt gratitude goes to my special friend, Ms Lim Ai Beoy, who never fails to be around whenever I need a shoulder and a helping hand. Also, I am obliged to Dr. Wong Jia Woei for her invaluable contributions in the study.

My sincere appreciation is extended to all my good friends and colleagues, namely, Sheau Chin, Dr. Irene Yap, Dr. Sharon Ling, Dr. Cheah Phaik Yeong, Yin Wai, Yit Hong, Saadiah, Hooi Ling, Ching Khee, Enrico, Dr. Nurzalina Khan, Shaun, Sandy, Pay Kim, Sin Yee, Siew Siew, Gamal, Mahmood, Samer, Jiyauddin, Mani, Sabiha, Kamarul, Erena, Bee Yean, Phaik Chin, Mei Mei, Jolene, Su Yee and May Thing for their constructive comments, advice, support and above all, friendship.

I would like to express my acknowledgement to Mr. Wan Teow Seng, Mr. Toh Weng Tuck, En. Norshimi, Mr. Somasundram, En. Azlan, En. Jaafar, Mr. Mahadevan, En. Zaidi, En Hamid, En. Ibrahim, En. Rahim, En. Yusuf, En. Adnan, Mr. Tan Seow Peng,

Mr. Chuah Lian Siah, Puan Yong Mee Nyok, En. Fizal and Puan Zaidah for their assistance.

I would like to thank Hovid Bhd. for their generous support and contribution in this study. My appreciation also goes to the Dean, Deputy Dean for Postgraduate Studies and the staff of the School of Pharmaceutical Sciences, USM for their support and assistance.

I am immensely indebted to my parents, brother and sister for their continuous support and love and lastly, my deepest gratitude goes to my husband, Mr. Victor Joseph for his boundless love, patience, encouragement and most of all for believing in me.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE

Abbreviation	Full description
ACN	Acetonitrile
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AR	Analytical grade
AUC	Area under the plasma concentration-time curve
AUC_{0-144h}	Area under the plasma concentration from time 0 to 144 hours after dosing
AUC_{0-t}	Area under the plasma concentration from time 0 to the last sampling time, t
$AUC_{t-\infty}$	Area under the plasma concentration from time t to infinity
$AUC_{0-\infty}$	Area under the plasma concentration from time 0 to infinity
C_{max}	Peak plasma concentration
C_{tl}	Plasma concentration at the last sampling point
C.V.	Coefficient of variation
CYP3A4	Cytochrome P450 subfamily 3A4
CYP450	Cytochrome P450
D(3,2)	Equivalent surface area mean diameter
D(4,3)	Equivalent volume mean diameter
D50	Volume median diameter
ECD	Electrochemical detector
GC	Gas chromatography
HIV	Human immunodeficiency virus
HLB	Hydrophile-lipophile balance
HPLC	High performance liquid chromatography
IPA	2-Propanol
k_e	Elimination rate constant
LOD	Limit of detection

LOQ	Limit of quantification
Log P	Log octanol/water partition coefficient
N.D.	Not detectable
o/w	Oil in water
PCS	Photon Correlation Spectroscopy
PEG	Polyethylene glycol
P-gp	P-glycoprotein
r	Correlation coefficient
S.D.	Standard deviation
SE	Self-emulsifying
SEDDS	Self-emulsifying drug delivery system
S.E.M.	Standard error of mean
SME	Self-microemulsifying
SMEDDS	Self-microemulsifying drug delivery system
SPE	Solid phase extraction
THF	Tetrahydrofuran
TLC	Thin layer chromatography
T_{max}	Time to reach peak plasma concentration
UV	Ultra violet
Vis	Visible
v/v	Volume over volume
w/w	Weight over weight
ZAve	Z average diameter

LIST OF APPENDICES

PENGGUNAAN SISTEM PENGEMULSIAN KENDIRI NOVEL BAGI SATU DRUG LIPOFILIK BERBIOKEPEROLEHAN RENDAH

ABSTRAK

Kajian ini dijalankan untuk membangunkan satu sediaan sistem pengemulsian kendiri (SES) untuk meningkatkan penyerapan sistemik drug yang berbiokeperolehan rendah dengan menggunakan ubiquinone sebagai drug model. Satu kaedah kromatografi cecair prestasi tinggi yang mudah, dan mempunyai spesifisiti, ketepatan dan kejituan yang diperlukan telah dibangunkan untuk menentukan kepekatan ubiquinone dalam plasma tikus dan manusia.

Beberapa aspek yang membawa kepada perumusan SES yang sesuai telah dikaji. Ini termasuk, penentuan kelarutan ubiquinone dalam lemak (log $P_{oktanol/akueus}$), penilaian beberapa jenis larutan pengangkut yang berpotensi dan kepekatan agen aktif permukaan, kecekapan untuk mengemulsi secara kendiri, penilaian saiz titisan yang terbentuk dari pelbagai gabungan larutan pengangkut dan agen permukaan dan akhir sekali, penilaian muatan drug dan kestabilan kimia ubiquinone dalam sediaan-sediaan pengemulsian kendiri (SE). Daripada kajian-kajian ini, sediaan SE optima yang mengandungi campuran asid α-linolenik dan Cremophor® EL pada nisbah 9:1 w/w serta dimuatkan dengan 60 mg/g ubiquinone didapati mempunyai prestasi *in vitro* yang paling memuaskan.

Dalam satu kajian *in vivo* yang melibatkan penggunaan tikus Sprague-Dawley, biokeperolehan oral ubiquinone yang diperolehi daripada sediaan SE optima didapati setanding dengan yang dihasilkan oleh sediaan pengemulsian mikro kendiri (SME) ubiquinone, Q-Gel® Forte yang boleh diperolehi secara komersial. Satu lagi kajian *in vivo* telah dijalankan menggunakan sukarelawan manusia yang sihat, di mana prestasi

in vivo sediaan SE yang optima dibandingkan dengan Q-Gel® Forte serta satu sediaan ubiquinone dalam bentuk ampaian berlemak, Bio-Quinone yang juga boleh didapati di pasaran. Tahap biokeperolehan ubiquinone yang dicapai oleh sediaan SE optima didapati meningkat (walaupun tidak mempunyai perbezaan yang signifikan secara statistik) jika dibandingkan dengan Q-Gel® Forte, manakala kedua-dua sediaan ini menghasilkan tahap biokeperolehan ubiquinone yang lebih tinggi secara signifikan ($p<0.05$) jika dibandingkan dengan sediaan ampaian berlemak, Bio-Quinone.

Sebagai kesimpulan, sediaan SE optima yang terdiri daripada campuran asid α-linolenik dan Cremophor® EL pada nisbah 9:1 w/w, dapat mengemulsi secara kendiri dengan memuaskan dan juga dapat meningkatkan biokeperolehan oral drug lipofilik seperti ubiquinone.

UTILITY OF A NOVEL SELF-EMULSIFYING SYSTEM FOR A LIPOPHILIC DRUG WITH LOW ORAL BIOAVAILABILITY

ABSTRACT

The present study was conducted to formulate a self-emulsifying system (SES) for enhancing the systemic absorption of poorly bioavailable lipophilic drugs, using ubiquinone as the model drug. A simple high performance liquid chromatographic method with the required specificity, precision and accuracy was first developed for quantification of ubiquinone in rat and human plasma.

Several aspects leading to the formulation of a suitable SES for ubiquinone were first studied. They included the determination of the ubiquinone lipid solubility (log $P_{octanol/aqueous}$), evaluation of various potential oily vehicles, type and concentrations of surfactants, the self-emulsification efficiency and emulsion droplet sizes of various surfactant and vehicle combinations and finally the drug loading and chemical stability of ubiquinone in the self-emulsifying (SE) formulations. From these studies, an optimised SE formulation comprising a 9:1 w/w mixture of α-linolenic acid and Cremophor® EL loaded with 60 mg/g of ubiquinone was found to have the most satisfactory *in vitro* performance.

An *in vivo* study conducted using Sprague-Dawley rats revealed that the oral bioavailability of the optimised SE formulation was comparable to that of a commercially available self-microemulsifying (SME) ubiquinone formulation, Q-Gel® Forte. Another *in vivo* study was also carried out using healthy human volunteers, comparing the optimised SE formulation to Q-Gel® Forte as well as another commercially available oily suspension of ubiquinone, Bio-Quinone. It was found that

the extent of bioavailability of the optimised SE formulation was slightly higher (although not statistically significant) than that of Q-Gel® Forte and both were significantly ($p<0.05$) higher than the oily drug suspension, Bio-Quinone.

In conclusion, the optimised SE formulation comprising a 9:1 w/w mixture of α-linolenic acid and Cremophor® EL could self-emulsify satisfactorily and also enhance the oral bioavailability of lipophilic drugs such as ubiquinone.

CHAPTER 1 INTRODUCTION

1.1 GENERAL PRINCIPLES OF ORAL BIOAVAILABILITY

Due to ease and convenience, the oral route remains by far the most common and preferred mode of drug administration (Gudzinowicz and Gudzinowicz, 1984; Shargel and Yu, 1999). However, not all drugs are well absorbed orally and their bioavailability has always been a major concern during development of an oral dosage form. Bioavailability refers to the rate and extent (amount) in which a therapeutically active drug reaches the systemic circulation after its administration (Shargel and Yu, 1999). Upon oral administration, a drug must be released from its dosage form to be dissolved in the gastrointestinal fluids. The dissolved form will then have to pass through the gastrointestinal barrier in order to gain access into the systemic circulation. A vast majority of orally administered drugs is absorbed from the mesenteric-branched arterioles and channelled through the hepatic portal vein leading to the liver prior to reaching the systemic circulation. On the other hand, absorption of certain lipophilic drugs can also occur via the intestinal lymphatic system, bypassing the hepatic-portal vein and therefore, the liver. Hence, in order to formulate an oral dosage form with satisfactory drug bioavailability, it is essential to understand the physicochemical, physiological and formulation factors that can affect the systemic absorption of a drug. These factors will be elaborated in the following sections.

1.1.1 PHYSICOCHEMICAL FACTORS

The physicochemical properties of a drug that can influence its passage into solution and transfer across membranes include lipid solubility, chemical stability, particle size and complexation potential (Ashford, 2002).

Prior to absorption, a drug has to be present in the dissolved form. Factors that can affect the dissolution rate are particle size, wettability, solubility and the physical form of the drug (either in salt or free form as well as crystalline or amorphous form) (Ashford, 2002).

An increase in the total surface area of drug particles in contact with the gastrointestinal fluids results in an increase in the drug dissolution rate. Provided that each particle of drug is intimately wetted by the gastrointestinal fluids, the effective surface area exhibited by the drug is directly related to the particle size of the drug. For a similar mass of drugs, the smaller the particle, the bigger will be the effective surface area (Ashford, 2002). Small particles with greater surface area dissolve more rapidly than larger particles even though both have the same intrinsic solubility. Nevertheless, particle size appears to have less influence on the absorption of drugs with high aqueous solubility, but it may have pronounced effect on the absorption of drugs with low aqueous solubility (Kwan *et al.*, 1986).

As the pH value varies from acidic in the stomach to slightly alkaline in the small intestine, the solubility of a drug differs as it travels along the gastrointestinal tract. Theoretically, solubility of an acidic drug gradually increases as it advances towards the small intestine whereas a basic drug has the highest solubility in the stomach. However, in reality, the large intestinal surface area compared to that of the stomach as well as the relatively longer residence time of the drug in the intestine than in the stomach, facilitate primary drug absorption from the small intestine regardless of its acidic or basic nature.

Certain drugs have poor solubility in the gastrointestinal fluids and their solubility can be increased with the appropriate soluble salt formation. For instance, the solubility of the free acid form of naproxen, a non-steroidal anti-inflammatory drug, is lower than that of the salt form, sodium naproxen (Sevelius *et al.*, 1980).

Polymorphism occurs when an organic compound has the capability of existing in more than one crystal form (Carstensen, 2002). Polymorphs may exhibit significantly different physical properties such as solubility, melting point and density. Metastable crystals, which possess higher energy, are less stable and hence, will be converted to the stable form eventually. Thus, the more stable forms are preferable for use in the final dosage form. Meanwhile, less energy is required to dissolve the amorphous form of a drug compared to the crystalline form, indicating that the amorphous form is more soluble than the crystalline form.

Lipid solubility of a drug has an impact on the permeation rate of the drug through the gastrointestinal barrier. Lipid soluble and non-ionisable drugs with high partition coefficients are capable of penetrating a variety of lipidic boundaries of the body cells and tissues (Gudzinowicz and Gudzinowicz, 1984). The lipidic characteristic of a drug depends on the presence of hydrophobic non-polar groups in the molecule. As the number of non-polar groups such as an alkyl group increases, the lipidic characteristic becomes more pronounced resulting in enhanced lipid solubility.

Drugs with low chemical stability are subjected to hydrolysis and enzymatic degradation in the gastrointestinal fluids. Hence, the amount for absorption is decreased, leading to reduced bioavailability as exemplified by erythromycin

base, which is rapidly destroyed by gastric acid. In order to protect erythromycin from degradation, its dosage form is enteric coated. Alternatively, it can be administered as a prodrug, namely, erythromycin stearate (Posti and Salonen, 1983).

Complexation of a drug may occur in the gastrointestinal fluids. The complexing agent may be a substance naturally present in the gastrointestinal tract, from a dietary source or an excipient in the dosage form. The solubility of the complexed form of a drug may be different from that of its original form. Therefore, complexes with better solubility are able to enhance bioavailability while those of poorer solubility will reduce drug bioavailability (Ashford, 2002).

1.1.2 PHYSIOLOGICAL FACTORS

Biological membranes, being the major structure in cells, function not only to enclose the cell contents but also act as selective barriers to the passage of molecules as a result of their lipidic and semipermeable nature. Two important pathways are involved in the transportation of drugs across the gastrointestinal barriers into the systemic circulation, namely the transcellular and paracellular pathways. Transcellular pathway refers to the transportation of drug molecules across the cell whereas paracellular pathway involves the movement of drug molecules through the gaps or tight junctions between the cells.

Transcellular absorption is further divided into passive diffusion, carrier-mediated transport (active transport and facilitated diffusion) and endocytosis. Passive diffusion is a process that involves the movement of drugs across a concentration gradient between the gastrointestinal tract and the systemic circulation with no assistance from external energy (Notari, 1987; Shargel and Yu, 1999). It is the preferred route of transport for relatively small molecules

and thus many drugs are transported via passive diffusion. On the other hand, certain compounds and many nutrients are absorbed transcellularly by a carrier-mediated transport mechanism of which there are two main types, active transport and facilitated diffusion. Active transport involves the delivery of a drug across the cell membrane against the concentration gradient with the assistance of a carrier. In contrast, facilitated diffusion requires a concentration gradient as the driving force because, unlike active transport, this mechanism cannot transport drug against the concentration gradient. Transportation of substance by this mechanism is at a much faster rate than would be anticipated for a normal diffusion process based on the molecular size and the polarity of the molecule. Endocytosis can be described as the engulfment of macromolecules by the cell membrane and subsequent transportation of the entire molecule into the cell (Ashford, 2002).

Present in the membranes of the enterocytes lining the gastrointestinal tract are certain counter transport efflux proteins, particularly P-glycoprotein (P-gp). These counter transporters can limit drug bioavailability by transporting the absorbed drugs back into the gastrointestinal lumen. In addition, the enterocytes also contain drug-metabolising enzymes, especially CYP3A4, which belongs to a subfamily of cytochrome P450. Being co-localised in the enterocytes of gastrointestinal tract, P-gp and CYP 3A4 are capable of working synergistically, as both have overlapping substrate activity (Watkins, 1997).

Other physiological factors that can affect oral bioavailability include gastric emptying rate, gastrointestinal motility as well as food status and food – drug interactions.

1.1.3 FORMULATION FACTORS

Various dosage forms have been used to administer drugs orally and their bioavailabilities differ from one another. Drug administered orally as a solution is absorbed faster than that in the form of a tablet or capsule. On the other hand, sustained-release dosage forms are devised to release their drug contents over an extended period of time, thereby providing a slow and sustained rate of absorption compared to conventional immediate-release dosage forms (Shargel and Yu, 1999).

Additionally, excipients used in the formulation of pharmaceutical dosage forms may also affect the bioavailability of a drug. For instance, magnesium stearate, a lubricant, is lipophilic and has water repellent characteristic. Its hydrophobic nature hinders liquid penetration into the tablet or capsule formulation and hence can impede the disintegration and dissolution of the drug in the gastrointestinal fluids. Surfactants, on the other hand, are able to increase the wettability of certain lipophilic drugs resulting in an increase in the rate of disintegration and dissolution, leading to improved oral bioavailability (Shargel and Yu, 1999).

1.2 LIPIDS AS DRUG DELIVERY CARRIERS

The ability of lipids to solubilise lipophilic drugs within the matrices of dosage forms often results in improved drug bioavailability, due to increased aqueous solubility and dissolution rate of drugs in the gastrointestinal fluids (Wasan, 2001). According to Humberstone and Charman (1997), lipid formulations can reduce the inherent limitations of slow and incomplete dissolution of poorly water-soluble drugs through the formation of solubilised phases from which absorption may occur.

Lipids offer versatility in oral drug administration as they can be formulated as solutions, suspensions, emulsions, self-emulsifying systems and

microemulsions. The availability of unit dosage forms such as soft or sealed hard gelatin capsules not only makes lipid formulations feasible but also practical and aesthetically acceptable to consumers.

1.3 LIPID SOLUTION AND SUSPENSION

The increased bioavailability of poorly soluble drugs with co-administration of food has prompted the investigation of using digestive lipids to dissolve or suspend this class of drugs for enhancing bioavailability. It has been shown that the bioavailability of corn oil suspensions of griseofulvin (Carrigan and Bates, 1973) and phenytoin (Chakrabakti and Belpaire, 1978) was 200% and 150% compared to that of an aqueous suspension of the respective drugs. In a study carried out by Abrams *et al.* (1978) comparing the bioavailability of a lipid-based solution of a lipophilic steroid derivative and an aqueous suspension, it was found that the bioavailability of the lipid-based solution was three-fold higher than that of the aqueous suspension. Besides that, lipid based formulations of a lipophilic anti-malarial amine (Stella *et al.*, 1978), cinnarizine (Tokumura *et al.*, 1987) and progesterone (Hargrove *et* al., 1989) also showed improved bioavailability compared to their respective capsule, tablet and powdered formulations.

1.4 SELF-EMULSIFYING DRUG DELIVERY SYSTEM (SEDDS)

1.4.1 INTRODUCTION

Pouton (1985 and 1997) defined a self-emulsifying drug delivery system (SEDDS) as an isotropic mixture of oil and surfactant (sometimes with added cosurfactant) that forms emulsion upon aqueous dilution with little or no energy input. It represents an efficient vehicle for the *in vivo* administration of emulsions (Constatinides, 1995). The mixture, either in the form of solution or suspension, can be encapsulated in soft or hard gelatin capsules. After oral

administration, the content of the capsule will be liberated from the capsule shell. Upon contact with the gastrointestinal fluids, the self-emulsifying formulation spreads readily in the gastrointestinal tract and self-emulsification will be initiated by the gentle agitation provided by digestive motility of the stomach and the small intestine (Shah *et al.*, 1994). In a recent review by Craig *et al.* (2000), the formulation of SEDDS is relatively simple in theory, as it only requires the incorporation of the drug into a suitable oil-surfactant mixture. A comprehensive review on SEDDS has also been published by Gursoy and Benita (2004).

1.4.2 FORMULATION

While self-emulsification has been shown to be dependent on the nature of oil/surfactant mixture, surfactant concentration and oil/surfactant ratio as well as temperature at which self-emulsification occurs (Pouton, 1985; Wakerly *et al.*, 1986; Wakerly *et al.*, 1987), the oily vehicle used is also an important determinant for a successful formulation of SEDDS. Besides solubilising lipophilic drugs and facilitating self-emulsification, the oily vehicle can also promote transport of lipophilic drugs via the lymphatic system, hence helping to increase oral bioavailability (Charman and Stella, 1991; Lindmark *et al.*, 1995; Gershanik and Benita, 2000; Holm *et al.*, 2002). Several long- and medium-chain glycerides have been used as vehicles or model systems according to a review by Constatinides (1995). It has been suggested that medium-chain triglycerides are more preferable compared to long-chain triglycerides in the formulation of SEDDS due to their better solvent capacity as well as ease of self-emulsification. To date, except for the data reported by Mulye (2000) and Ng (2005), no other data on SEDDS using fatty acids as the oily vehicles has been published. Fatty acids have been used by Kajita *et al.* (2000) for the preparation of an emulsion to improve mucosal absorption of vancomycin

hydrochloride in rats. Oleic acid, a monounsaturated fatty acid has been used in the formulation of a nonself-emulsifying product of testosterone undecanoate, Andriol® (Organon, NJ, USA).

As surfactant molecules contain both polar and non-polar groups, they tend to adsorb at the interfaces between the oil and aqueous phases with the hydrophilic group located in the aqueous phase while the lipophilic group in the oil phase (Salager, 2000). Non-ionic surfactants are often preferred in pharmaceutical formulations, especially oral preparations as these compounds are generally less toxic and safer than the ionic surfactants. Moreover, being non-ionic in nature, the activity of these surfactants is less influenced by changes in pH and ionic strength (Constatinides and Scalart, 1995). An important property of these surfactants concerning the bioavailability of drugs is that they can cause moderate reversible changes in the permeability of the intestinal wall (Wakerly *et al.*, 1986). The hydrophile-lipophile balance (HLB) of surfactants has been used in their selection for formulating SEDDS (Eccleston, 1992; Atwood, 1994). Surfactants with HLB values ranging from 3 to 8 are usually used for formulating water-in-oil emulsions whereas those of HLB values in the range of 8 to18 are applied in oil-in-water emulsions (Osborne *et al.*, 1988; Constatinides and Scalart, 1995).

Apart from surfactants, cosolvents such as ethanol, propylene glycol, polyethylene glycols and glycerol are occasionally incorporated into a SEDDS in order to increase the solvent capacity. Vonderscher and Meinzer (1994) have substantiated the addition of cosolvents to produce very fine dispersion under gentle agitations (Constantinides, 1995; Constantinides and Scalart, 1995) in their formulation of a SE preparation of cyclosporine A.

1.4.3 MECHANISM OF SELF-EMULSIFICATION

According to Constantinides (1995), spontaneous emulsification of SEDDS occurs because the free energy required for emulsification is low and could be either positive or negative. As the oil/surfactant mixture is diluted with water, a liquid crystalline phase is formed between the oil/surfactant and water phases. The effective swelling of this liquid crystalline phase allows spontaneous formation of an interface between the oil droplets and the water (Groves and Galindez, 1976; Wakerly *et al.*, 1986). The other possible mechanism proposed by these researchers was the rapid infiltration of water into the liquid crystalline phase resulting from gentle agitation instigates interface disruption and droplet formation. The presence of liquid crystalline phase surrounding the oil droplets gives rise to a stable self-emulsified system that prevents coalescence of the oil droplets.

Several studies to determine the involvement of liquid crystalline phase in the emulsification process have been carried out by Wakerly *et al.* (1986), Wakerly *et al.* (1987), Pouton *et al.* (1987), Craig *et al.* (1993) and Craig *et al.* (1995). According to Craig *et al.* (1993), the formation of the liquid crystalline phase was assumed to be largely dependent on the ratio of oil to surfactant and to water, which in turn accounted for the high specificity of the proportions of oil and surfactant required for self-emulsification as suggested by Pouton (1985). In addition, the drug in the formulation may alter the emulsion characteristics, probably by interacting with the liquid crystalline phase (Craig *et al.*, 1993). The complex relationship between the formation of liquid crystalline and emulsification was further substantiated by Craig *et al.* (1995) through examining a series of Imwitor 742 (a mixture of mono- and diglycerides of capric and caprylic acids) and Tween 80 systems using particle size analysis and low frequency dielectric spectroscopy.

1.4.4 DRUG ABSORPTION FROM SEDDS

Craig *et al.* (2000) has identified two differences in the drug uptake between a SEDDS and an ingested oily liquid. First, in SEDDS, oil is presented to the gastrointestinal tract in fine droplets, thus increasing the surface area for absorption instead of a single continuous phase. Second, the presence of emulsifying agents in the SEDDS facilitates emulsification, leading to better absorption. Charman *et al.* (1992) carried out a formulation and biopharmaceutic study on a lipophilic anti-viral compound, WIN 54954, in which the bioavailability of this compound in SEDDS was compared with that of a PEG 600 formulation. Although no significant difference was observed between the mean bioavailability of the two formulations, greater reproducibility was obtained with the SEDDS. Shah *et al.* (1994) also reported that the bioavailability of Ro-150778, a highly lipophilic naphthalene derivative, obtained with a SEDDS was four-fold and twenty-fold higher than that of a PEG 400 solution and a standard tablet preparation of the compound respectively. The improved bioavailability of drugs administered in the form of a SEDDS compared to a normal oily solution was further detailed by Humberstone and Charman (1997). More recently, Ja and Young (2000) showed that the oral bioavailability of indomethacin (a poorly water-soluble drug) in rats was increased by 57% when administered in the form of SEDDS compared to that of a methylcellulose suspension.

1.5 SELF-MICROEMULSIFYING DRUG DELIVERY SYSTEM

Self-microemulsifying drug delivery system (SMEDDS) is a SEDDS which forms transparent microemulsion with droplet sizes of less than 50 nm. Improved oral bioavailability as a result of the formation of nanosize droplets was demonstrated by the improved version of cyclosporine A formulation, Sandimmune Neoral compared to the older formulation, Sandimmune (Holt *et*

al., 1994). The former, which could produce a microemulsion, showed 1.3 to 1.5 times higher in the extent of systemic absorption compared to the latter, which could only yield a crude emulsion. Often, cosurfactants and/or cosolvents are utilised instead of a single surfactant system in order to obtain emulsions with very fine droplet sizes. Moreover, the amount of surfactant used is normally higher than that used in the formulation of a normal SEDDS (Karim *et al.*, 1994; Vondercher and Meinzer, 1994; Constantinides, 1995; Meinzer *et al.*, 1995).

1.6 UBIQUINONE

1.6.1 INTRODUCTION

Ubiquinone is also known as coenzyme Q_{10}, coenzyme Q(50), ubidecarenone and mitoquinone. It was first isolated from the mitochondria of beef heart by Crane *et al.* (1957) in USA. In 1958, Dr. Karl Folkers and his co-workers elucidated the molecular structure of the compound to be 2,3 dimethoxy-5-methyl-6-decaprenyl benzoquinone (Sinatra, 2005) as shown in Figure 1.1, with a molecular weight of 863.37. It has a melting point of 48°C. At room temperature, ubiquinone appears as a yellow to orange crystalline powder. It is practically insoluble in water, soluble in acetone and very slightly soluble in ethanol. It gradually decomposes and darkens upon exposure to light (British Pharmacopeia, 2003).

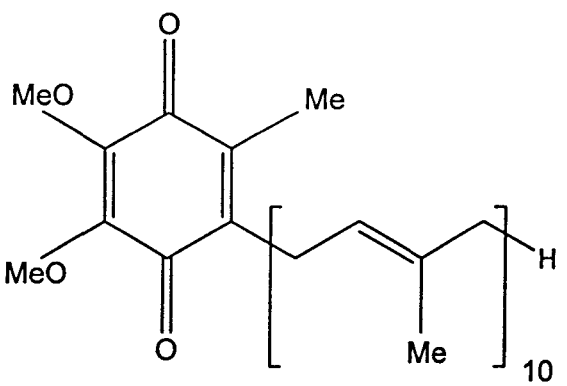


Fig. 1.1 : Structural formula of ubiquinone

Ubiquinone is basically a vitamin or vitamin-like substance found naturally in every cell of the body as it is synthesised in all the tissues in the body. Relatively high concentrations of ubiquinone are found in the heart, liver, kidney and pancreas (Linn *et al.*, 1959). Although ubiquinone is also present in a wide variety of food, daily dietary intake of approximately 2-5 mg is insufficient to produce any substantial clinical benefits (Sinatra, 2005).

1.6.2 THERAPEUTIC BENEFITS OF UBIQUINONE

About 40 to 50% of ubiquinone is concentrated in the mitochondria of a cell. It is the coenzyme for at least three mitochondrial enzymes, which are crucial in the production of adenosine triphosphate (ATP), required for cellular functions, as well as the enzymes in other parts of the cell (Langsjoen, 1994). Besides being involved in the production of ATP, ubiquinone also functions as a potent antioxidant and a membrane stabiliser. Due to its pivotal role in ATP production and antioxidant activity, ubiquinone thus exhibits many therapeutic benefits.

The findings by Folkers *et al.* (1970), Littarru *et al.* (1972a and 1972b), Folkers *et al.* (1985) and Frustaci *et al.* (1985) that heart tissues are generally deficient in ubiquinone when patients suffer from heart diseases, have prompted extensive investigations to evaluate the benefits of ubiquinone in treating heart diseases. There were at least nine placebo-controlled studies documented, all confirming the effectiveness and safety of ubiquinone in the treatment of heart diseases. Besides that, there were eight international symposia being held from 1976 to 1993 uncovering the biomedical and clinical aspects of ubiquinone via more than 300 presentations by scientists and physicians from 18 countries (Langsjoen, 1994). The majority of these studies related to the treatment of heart diseases showed that ubiquinone significantly improved heart muscle function with no obvious adverse effects.

Apart form treating heart diseases, studies on the benefits of ubiquinone as a supplement to reduce hypertension have also been carried out. Yamagami *et al.* (1976) reported a reduction in systolic and diastolic pressure in selected patients upon repleting their ubiquinone deficiency. Greenberg and Frishman (1990) who conducted a double blind placebo-controlled study involving 20 hypertensive patients with low serum ubiquinone concentrations, found a significant reduction in systolic and diastolic pressure in patients receiving 33.3 mg of ubiquinone 3 times per day while no improvement was observed in the placebo group. In an open-label study conducted by Langsjoen *et al.* (1994), average daily dose of 225 mg ubiquinone for six months in combination with standard antihypertensive drug therapy, enabled 51% of the participants to discontinue one to three of their antihypertensive medications. In another randomised, double blind, placebo-controlled study carried out by Burke *et al.* (2001), 55% of participants with systolic hypertension, but normal diastolic pressure who received 60 mg of ubiquinone per day responded positively after a twelve-week period compared to the placebo group.

The involvement of ubiquinone in oxidative/reductive reactions has rendered it a potent antioxidant (Ozawa, 1985; Ernster and Forsmark-Andree, 1993) by inhibiting lipid and protein peroxidation as well as scavenging free radicals. The reduced form gives up electron readily to neutralise oxidants/radicals. Being a powerful antioxidant, ubiquinone has the potential to be an anti-cancer and immune-stimulating agent. Studies carried out by Folkers *et al.* (1988) and Lockwood *et al.* (1995) showed that there was primary evidence that ubiquinone might help in improving the immune system of HIV/AIDS patients and inhibit or reduce the growth of breast cancer cells. In recent years, it has also been widely used in skin care products to combat aging despite the paucity of scientific evidence.

In addition, therapeutic benefits of ubiquinone for combating diseases such as dental disease (Wilkinson *et al.*, 1977), chronic renal failure (Singh *et al.,* 2000), Huntington disease (The Huntington Study Group, 2001), Parkinson disease (Shults *et al.*, 2002) and migraines (Rozen *et al.,* 2002) are still being extensively investigated.

1.6.3 ABSORPTION OF UBIQUINONE

As mentioned under Section 1.6.1, ubiquinone exists in crystalline powder at room temperature. Although it is highly lipophilic, it has limited solubility in lipids. Moreover, the compound has relatively high molecular weight. These factors have contributed to the poor and erratic absorption of ubiquinone upon oral administration (Miles *et al.*, 2002; Joshi *et al.*, 2003). Being a lipophilic substance, the absorption of ubiquinone mimics similar pathway to that of fat-soluble drugs, which involves emulsification with bile salts in the intestines, followed by the formation of micelles prior to absorption. Upon absorption from the gastrointestinal tract, ubiquinone will be taken up by the chylomicrons. According to Greenberg and Frishman (1990), a major portion of the exogenous ubiquinone is deposited in the liver and packaged into VLDL lipoprotein.

Various methods of increasing the oral bioavailability of ubiquinone have been documented as well as patented. Oily suspension of ubiquinone was found to yield better absorption compared to the powdered form (Weis *et al.*, 1994). Formulation of ubiquinone into self-emulsifying preparations has been shown to significantly improve the oral bioavailability of ubiquinone compared to the oily suspensions and powdered preparations (Chopra *et al.*, 1998; Wahlqvist *et al.*, 1998; Miles *et al.*, 2002; Molyneux *et al.*, 2004)

1.7 SCOPE OF THE STUDY

To date, the most successful commercially available ubiquinone formulation, with enhanced oral bioavailability, is a self-microemulsifying (SME) formulation comprising almost 90% of surfactants according to the patented formula (Goldman, 2000). High amounts of surfactants were required to obtain small droplet sizes. However, the high amounts of surfactants present in SME formulations might cause permeability changes in the intestinal walls and thus might not be recommended or safe for long-term oral consumption. There is also the issue of safety/toxicity in using high amounts of surfactants.

Therefore the present study was conducted to investigate the feasibility of using minimal amounts of a single surfactant system in the formulation of a SEDDS for enhancing the oral absorption of ubiquinone. The study was carried out in stages with the following objectives:

1. to develop a high performance liquid chromatographic method for quantification of ubiquinone in plasma matrices;

2. to formulate a SEDDS comprising a suitable lipidic vehicle and a single surfactant with efficient self-emulsifying properties;

3. to evaluate the *in vitro* performance of SEDDS based on droplet size analysis, physical stability of the resultant emulsion products and chemical stability of ubiquinone in the optimised SE formulation;

4. to evaluate the *in vivo* performance of an optimised SE formulation of ubiquinone in adult male Sprague-Dawley rats;

5. to evaluate the *in vivo* performance of an optimised SE formulation of ubiquinone in healthy human volunteers.

CHAPTER 2 DEVELOPMENT OF A SIMPLE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR ANALYSIS OF UBIQUINONE IN RAT AND HUMAN PLASMA

2.1 INTRODUCTION

Various analytical methods using high performance liquid chromatography (HPLC) have been reported for quantifying ubiquinone and ubiquinol in plasma, erythrocytes, urine and animal tissues. Ubiquinol, the reduced form of ubiquinone, is the most common form found *in vivo* (Kontush *et al.*, 1997), but it can be easily oxidised to ubiquinone not only at room temperature but also at -20°C (Edlund, 1988; Eaton *et al.*, 2000). Despite its poor stability, it is sometimes necessary to determine the ubiquinol levels, since its ratio to the total ubiquinone can be used to determine ubiquinone deficiency related diseases (Kontush *et al.,* 1997; Menke *et al.*, 2000). However, quantification of total ubiquinone in plasma or serum is deemed sufficient for determining the rate and extent of ubiquinone absorption in bioavailability studies, noting that the majority of bioavailability studies on ubiquinone reported in the literature were based on quantification of total ubiquinone.

Karpinska *et al.* (1998) described an ultra-violet (UV) spectrophotometric method for the quantification of plasma ubiquinone, which was claimed to be simpler and faster than methods based on HPLC. The method utilised derivatisation spectrophotometry, in which the approach was based on a mathematical transformation of the spectral curve obtained. However, spectrophotometric methods are generally deemed not specific. Moreover, no internal standard could be utilised in the double extraction procedure during sample clean-up because such detection method is unable to distinguish the

compound of interest from the internal standard, which customarily has a similar UV absorbance to that of the compound of interest.

Various HPLC methods using electrochemical detection (ECD) have also been reported in the literature. Lang and Packer (1987) reported such a method with sensitivity of approximately 40 ng/ml, being comparable to that achieved using HPLC-UV detection (Kommuru *et al.*, 1998; Mosca *et al.*, 2002), but no mention was made regarding its precision and accuracy. Another method reported by Edlund (1988) could produce clean chromatograms and simultaneously quantify ubiquinol, ubiquinone and vitamin E. However, the method was not validated for precision and accuracy. Grossi *et al.* (1992) also reported a method that could achieve excellent sensitivity of 5 ng/ml by coupling solid-phase extraction (SPE) and on-line column sample clean-up using column switching system with ECD. Besides being tedious and laborious to perform, methods employing ECD in general, often encounter problems of baseline stability.

Quantification of plasma ubiquinone using HPLC methods with UV detection has also been reported. Okamoto *et al.* (1985) reported a HPLC-UV method, which required double sample clean-up involving liquid-liquid extraction followed by thin-layer chromatography (TLC). However, its limit of detection was only 100 ng/ml, being less sensitive than other HPLC-UV methods. Also, the accuracy, precision and recovery of the method were not reported. Although an HPLC-UV method by Kommuru *et al.* (1998) could obtain a detection limit of 50 ng/ml, the analytical procedure was lengthy and tedious requiring triple extraction of the samples. Moreover, the analysis run time for each sample was long, taking up to 30 minutes per sample.

More recently, Mosca *et al.* (2002) reported a HPLC-UV method, which has a good sample clean-up recovery of 97.3%. The method also has a high sensitivity of 32 ng/ml, with good precision and accuracy where the coefficient of variation and error values were less than 11%. A simple direct deproteinisation of the sample was used as the clean-up procedure. The setback of this method is that a relatively large volume of 200 µl of the deproteinised plasma supernatant was required to be injected into the HPLC column. This might lead to rapid clogging of the HPLC column resulting in shortening of the column life span. Also the relatively long run time of 13.5 minutes might be impractical, especially when a large number of samples need to be analysed, such as those obtained from bioavailability studies.

Therefore, the aim of this part of the study was to develop a simple, rapid, economical and yet specific as well as sufficiently sensitive HPLC method using UV detection for the determination of ubiquinone in rat and human plasma. According to Edlund (1988), ubiquinone is negligibly found in rat plasma but present at significant levels in human plasma. Hence, method validation was performed for both human and rat plasma due to the differences in their matrices.

2.2 MATERIALS

Hexane, 2-propanol (IPA), acetonitrile (ACN) and tetrahydrofuran (THF) of either HPLC or analytical reagent (AR) grade were purchased from Mallinckrodt (Paris, KY, USA), while methanol of AR grade was acquired from Merck (Darmstadt, Germany). Ubiquinone reference standard was obtained from European Pharmacopeia, Strasbourg, France.

2.3 METHODS

2.3.1 INSTRUMENTATION

The HPLC system comprised a Jasco PU-980 pump (Jasco, Tokyo, Japan), a Gilson 151 UV/Vis detector (Gilson, Villiers-Le-Bel, France), a Rheodyne 7125 sample injector fitted with a 50 μl sample loop (Rheodyne, CA, USA) and a Hitachi D-2500 Chromato-integrator (Hitachi, Tokyo, Japan). A Zorbax XDB C18 (Palo Alto, CA, USA) column (250 x 4.6 mm i.d., 5 μm), fitted with a refillable guard column (Upchurch Scientific, Oak Harbour, WA, USA), was used for the chromatographic separation. The mobile phase consisting of IPA, ACN and distilled water at a ratio of 76 : 20 : 4 v/v, was delivered at a flow rate of 1.2 ml/min. The detection wavelength was set at 275 nm with a sensitivity range of 0.001 a.u.f.s.

2.3.2 SAMPLE PREPARATION

An aliquot of 100 μl of plasma (obtained from either rat or human) was accurately pipetted into a microcentrifuge tube (Eppendorf, Hamburg, Germany) and deproteinised using 200 μl of THF. The mixture was mixed thoroughly for 1 minute on a vortex-mixer (Thermolyne, Iowa, USA) before being centrifuged (Minispin® Plus Eppendorf, Hamburg, Germany) at 12 800 g for 10 minutes. Finally, 50 μl of the supernatant obtained was directly injected onto the HPLC system.

2.3.3 STANDARD SOLUTIONS AND CALIBRATION CURVES

A stock solution of ubiquinone was prepared in IPA at a concentration of 10 mg/ml. The stock solution was then diluted using mobile phase to yield a working solution of 400 μg/ml of ubiquinone. Calibration curves of ubiquinone in plasma at 10000.0, 5000.0, 2500.0, 1250.0, 625.0, 312.5, 156.3 and 78.1 ng/ml

were constructed by first spiking 10 ml of pooled blank plasma with 250 µl aliquot of ubiquinone working solution (to give a plasma concentration of 10 000 ng/ml), followed by serial dilution with blank plasma to obtain the subsequent plasma concentrations. These samples were also used for the recovery, precision and accuracy studies of the assay method.

Further serial dilutions of the working solution (400 µg/ml) with IPA were carried out to obtain concentrations of 100.0, 50.0, 25.0, 12.5, 6.3, 3.1, 1.6 and 0.8 µg/ml. These were then used to prepare drug solutions for recovery evaluation, by accurately spiking 10 µl of each of these samples into an appropriate amount of deproteinised blank plasma (prepared according to the deproteinisation procedure as described in section 2.3.2) to obtain similar concentrations as the plasma samples. The solutions were then vortex-mixed for 10 seconds and centrifuged at 12 800 g for 5 minutes prior to analysis.

2.3.4 RECOVERY, PRECISION AND ACCURACY

Plasma samples were quantified using peak height. For human plasma, corrections were made for the interference from endogenous ubiquinone by subtracting the peak height from the blank plasma used in the preparation of the calibration curves. Recovery, within- and between-day precision and accuracy of the method were determined at concentrations of 78.1, 156.3, 625.0, 1250.0, 2500.0, 5000.0 and 10000.0 ng/ml, respectively (using the plasma samples prepared in section 2.3.3 above). For within-day accuracy and precision, 6 replicates of each concentration were determined in a single day. For between-day evaluation, analysis was carried out on one sample of each concentration daily over 6 days with a calibration curve constructed on each day of analysis. The accuracy was reported as percentage of the measured

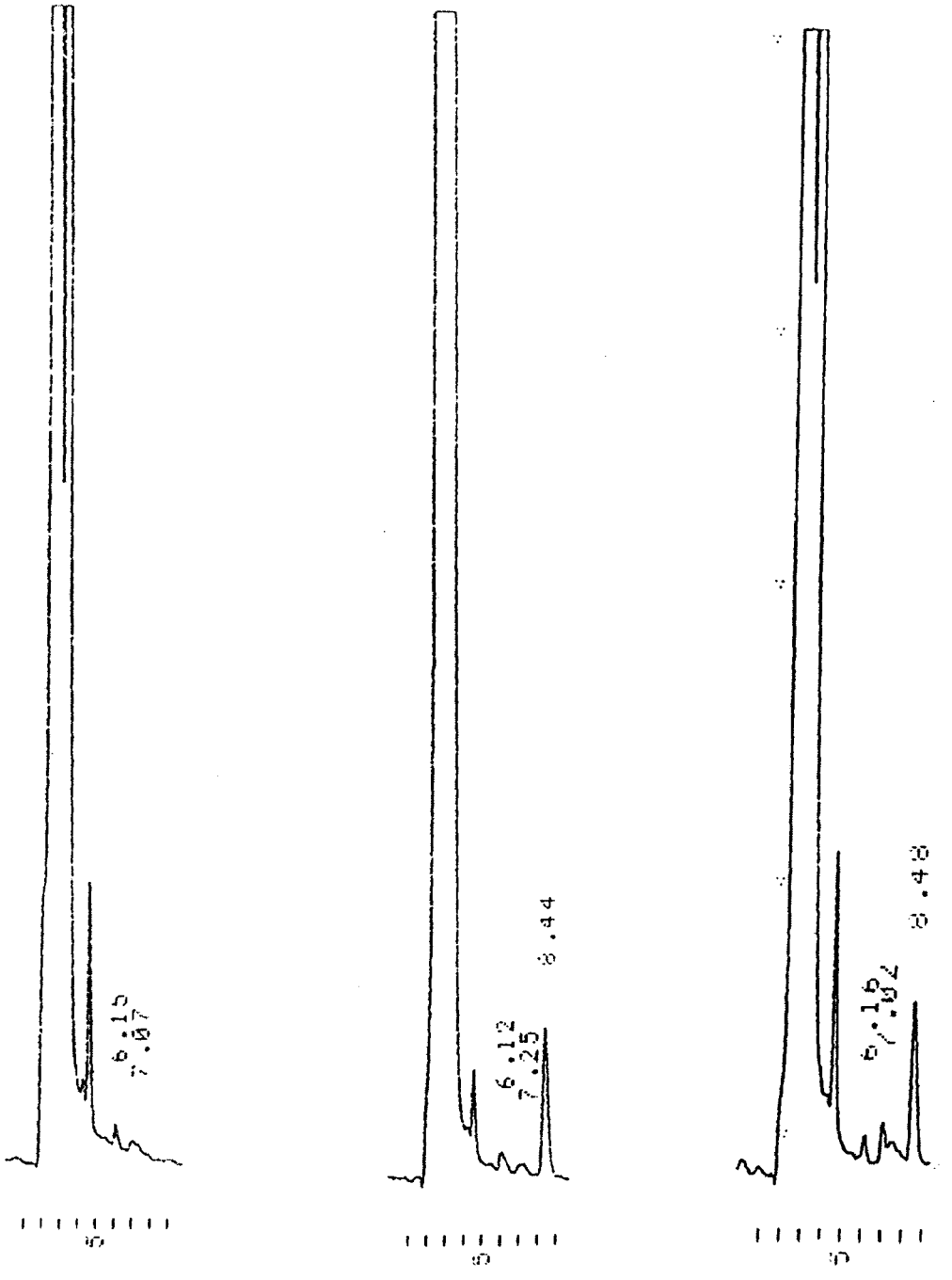
concentration over that of the theoretical spiked value, whereas the precision was denoted using the coefficient variation of the 6 replicates. The absolute recovery (n=6) of the sample preparation method was estimated by comparing the peak height obtained from the plasma samples with that obtained from similar concentrations of the drug spiked in deproteinised blank plasma (prepared according to procedure described in section 2.3.3).

2.4 RESULTS

Chromatograms obtained with blank rat plasma and rat plasma spiked with 1250.0 ng/ml of ubiquinone are shown in Figures 2.1 (a) and (b), while Figure 2.1 (c) illustrates a chromatogram of rat plasma containing 1133.3 ng/ml of ubiquinone, obtained 36 hours after oral administration of 400 mg/kg of ubiquinone in a self-emulsifying (SE) formulation. The chromatogram of the blank rat plasma was clean and devoid of interference from other endogenous compounds at the retention time of ubiquinone.

Figures 2.2 (a) and 2.2 (b) show the chromatograms obtained with blank human plasma and human plasma spiked with 1250.0 ng/ml of ubiquinone, while the chromatogram of human plasma sample containing 1745.6 ng/ml of ubiquinone, obtained 6 hours after oral administration of 180 mg of ubiquinone SE formulation is illustrated in Figure 2.2 (c). Unlike the rat plasma samples, endogenous plasma ubiquinone was found to be present at 0.6 to 1.0 μg/ml in the blank human plasma. Therefore, as mentioned earlier, corrections were made to accommodate the interference from endogenous ubiquinone in the present analysis.

Calibration curves (n=6), obtained by plotting the peak height of ubiquinone in rat plasma versus the plasma concentration of ubiquinone, were linear over the



(a) (b) (c)

igure 2.1. Chromatograms for the analysis of ubiquinone in (a) blank rat plasma, (b) lank rat plasma spiked with 1250.0 ng/ml ubiquinone and (c) rat plasma containing 133.3 ng/ml of ubiquinone, obtained 36 hours after oral administration of 400 mg/kg of ɔiquinone in SE formulation (y-axis, attenuation 6; x-axis, chart speed 2.5 mm/min). etention time of ubiquinone ≈ 8.4 mins

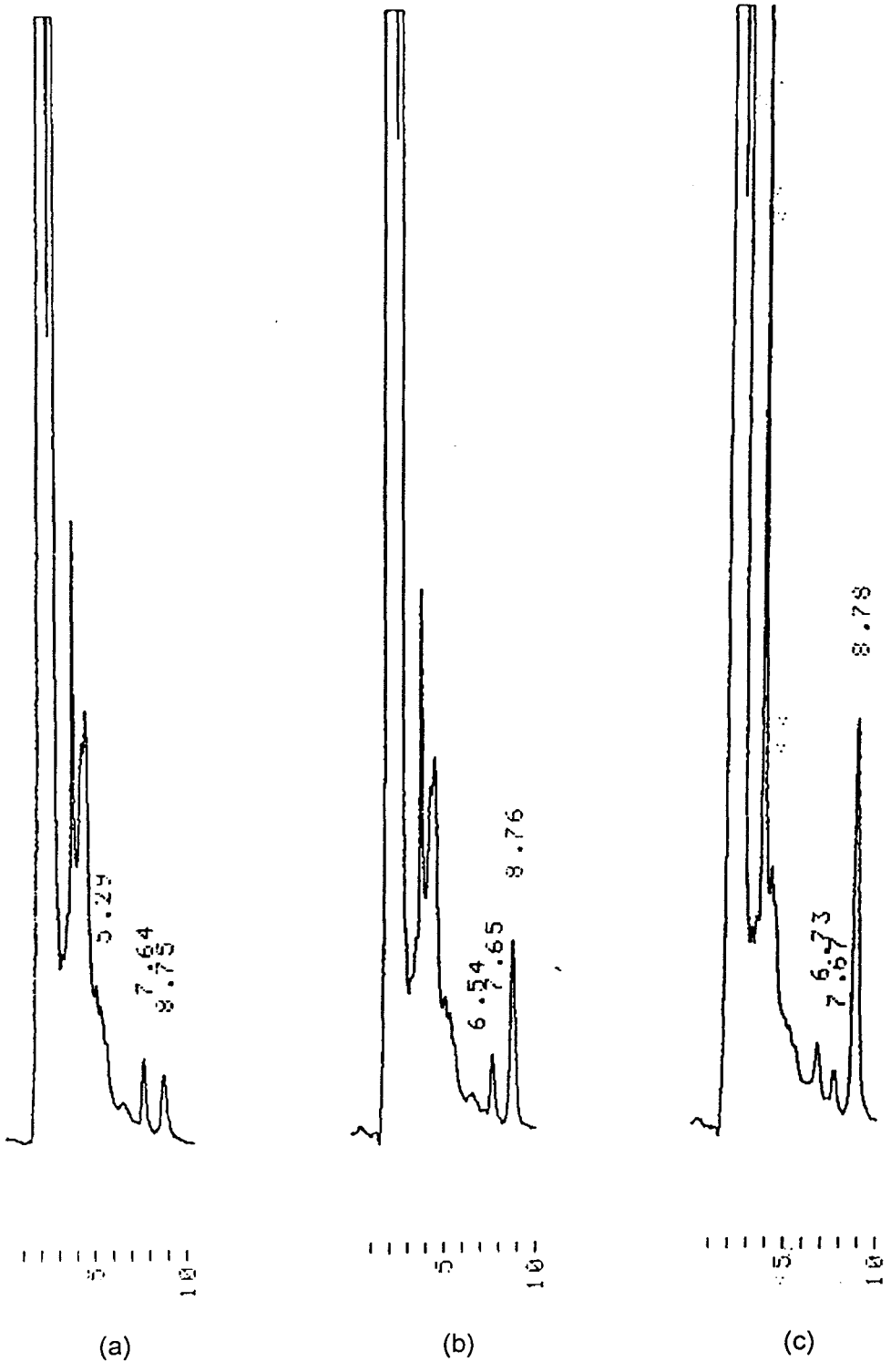


Figure 2.2. Chromatograms for the analysis of ubiquinone in (a) blank human plasma, (b) blank human plasma spiked with 1250.0 ng/ml ubiquinone and (c) human plasma containing 1745.6 ng/ml of ubiquinone, obtained 6 hours after oral administration of 180 mg of ubiquinone SES formulation (y-axis, attenuation 6; x-axis, chart speed 2.5 mm/min). Retention time of ubiquinone ≈ 8.7 mins