SOLID LIPID NANOPARTICLES AS DRUG CARRIERS FOR ATOVAQUONE

NORATIQAH BINTI MOHTAR

UNIVERSITI SAINS MALAYSIA

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SOLID LIPID NANOPARTICLES AS DRUG CARRIERS FOR ATOVAQUONE

by

NORATIQAH BINTI MOHTAR

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LIST OF ABBREVIATION & SYMBOLS

µl	=	Microlitre
°C	=	Degree centigrade
ACN	=	Acetonitrile
ANOVA	=	Analysis of Variance
ATQ	=	Atovaquone
ATQ-SLNs	=	Atovaquone-Solid Lipid Nanoparticles
ATQ-PD	=	Atovaquone-Pure Drug
AUC	=	Area Under Curve
C_{max}	=	Maximum plasma concentration
DSC	=	Differential Scanning Calorimetry
EE	=	Entrapment Efficiency
HPLC	=	High Performance Liquid Chromatography
Hr	=	Hour
IS	=	Internal Standard
K_e	=	Elimination rate constant
LLE	=	Liquid-Liquid Extraction
LOD	=	Limit of Detection
LOQ	=	Limit of Quantification
mg	=	Milligram
min	=	Minute
ml	=	Millilitre
N	=	Theoretical plate number
ng	=	Nanogram
ng/ml	=	Nanogram per millilitre
nm	=	Nanometer
PPT	=	Protein Precipitation Technique
QC	=	Quality Control
RE	=	Relative Error
RH	=	Relative Humidity
rpm	=	Rotation per minute
RSD	=	Relative Standard Deviation
SGF	=	Simulated Gastric Fluid

SIF	=	Simulated Intestinal Fluid
SLNs	=	Solid Lipid Nanoparticles
$t_{1/2}$	=	Half life
T_{max}	=	Time taken to reach maximum plasma concentration
USFDA	=	United States Food and Drug Administration
USP	=	United States Pharmacopoeia
UV	=	Ultraviolet
v/v	=	Weight per weight
w/v	=	Weight per volume
WHO	=	World Health Organization

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NANOPARTIKEL LIPID PEPEJAL SEBAGAI PEMBAWA DRUG UNTUK ATOVAQUONE

ABSTRAK

Nanopartikel lipid pepejal untuk atovaquone (ATQ-SLNs) telah disediakan menggunakan kaedah penyeragaman ricih tinggi dengan tripalmitin, trilaurin dan Compritol 888 ATO sebagai matriks lipid dan lesitin soya terhidrogen, Tween 80 dan poloxamer 188 sebagai surfaktan. Pengoptimuman formulasi telah dijalankan menggunakan 6 set kajian bentuk faktorial 2^4 berdasarkan empat pembolehubah tidak bersandar iaitu kitar penyeragaman, kepekatan lipid, kepekatan surfaktan utama, dan kepekatan surfaktan bersama. Pembolehubah bersandar adalah saiz partikel dan indeks polisebaran. Kesan keempat-empat pembolehubah tidak bersandar terhadap kedua-dua respon telah dianalisa menggunakan ANOVA dan plot permukaan respon. Akhirnya, formulasi terbaik yang menunjukkan efisiensi pemerangkapan tertinggi telah dipilih daripada setiap sistem lipid iaitu TPT 8 and TLT 16 untuk sistem tripalmitin dan trilaurin, masing-masing. Compritol 888 ATO pula telah dikecualikan untuk kajian seterusnya. Proses pengeringan dengan menggunakan trehalose sebagai agen pelindung krio telah dijalankan untuk memperbaiki kestabilan kedua-dua formulasi. Inkorporasi trehalose semasa proses penyeragaman menunjukkan penambahbaikan dalam saiz partikel dan efisiensi pemerangkapan apabila dibandingkan dengan inkorporasi selepas penyeragaman dalam kedua-dua formulasi. TLT 16 DH telah dipilih sebagai formulasi terakhir dengan diameter purata sebanyak 84.63 ± 1.51nm sebelum dan 217.9 ± 8.42nm selepas pengeringan, dan efisiensi pemerangkapan sebanyak 93.57 ± 3.07% sebelum dan 66.04 ± 1.74% selepas proses pengeringan. TLT 16 DH menunjukkan penambahbaikan dalam

kelarutan ATQ dalam kedua-dua medium bendalir simulasi gastrik (SGF) dan bendalir simulasi usus (SIF) dalam kajian in vitro beserta ciri-ciri pelepasan pantas di mana pelepasan sebanyak 100% telah dicapai dalam masa 5min. Kajian kestabilan menunjukkan TLT 16 DH adalah stabil dalam keadaan penyimpanan 4^{o}C selama 6 bulan. Di samping itu, dua kaedah HPLC-UV telah dibangunkan dan divalidasi secara berasingan untuk kuantifikasi ATQ dalam medium pelepasan dan plasma arnab. Kajian farmakokinetik dalam arnab mendedahkan penambahbaikan C_{max} dan $AUC_{0\text{-}24h}$ sebanyak 4.61-kali dan 4.55-kali, masing-masing, dalam ATQ-SLNs apabila dibandingkan dengan drug bebas. $t_{1/2}$ ATQ-SLNs juga diturunkan sebanyak 3.19-kali apabila dibandingkan dengan drug bebas tanpa formulasi. Kesimpulannya, satu formulasi SLNs untuk ATQ telah disediakan dengan jayanya dan boleh menjadi alternatif yang menjanjikan penyerapan yang lebih baik dan penurunan dos terapeutik untuk ATQ.

SOLID LIPID NANOPARTICLES AS DRUG CARRIERS FOR ATOVAQUONE

ABSTRACT

Solid lipid nanoparticles of atovaquone (ATQ-SLNs) were prepared by high shear homogenization method using tripalmitin, trilaurin, and Compritol 888 ATO as the lipid matrix and hydrogenated soy lecithin, Tween 80 and, poloxamer 188 as the surfactants. Optimization of the formulations was conducted using 6 sets of 2^4 factorial design study based on four independent variables which were homogenizing cycle, concentration of lipid, concentration of main surfactant, and concentration of co-surfactant. The dependent variables were particle size and polydistribution index. The effect of the four independent variables towards the responses was analyzed using ANOVA and response surface plots. Finally, the best formulations with the highest entrapment efficiency were chosen from each lipid system which were TPT 8 and TLT 16 for tripalmitin and trilaurin system, respectively, while Compritol 888 ATO was excluded for further study. Lyophilization process by using trehalose as the cryoprotectant was done to improve stability of both formulations. Incorporation of trehalose during homogenization process showed an improvement in the particle size and entrapment efficiency when compared to the incorporation after homogenization in both formulations. TLT 16 DH was selected as the final formulation with an average diameter of 84.63 ± 1.51nm before and 217.9 ± 8.42nm after lyophilization, and entrapment efficiency of 93.57 ± 3.07% before and 66.04 ± 1.74% after lyophilization process. TLT 16 DH showed an improvement in the solubility of ATQ in both simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) medium in the in vitro study with a fast release property where 100%

release was achieved within 5min. A stability study showed that TLT 16 DH was stable in 4^{o}C storage condition for 6 months. In addition, two HPLC-UV methods were developed and validated separately for the quantification of ATQ in the dissolution medium and rabbit's plasma. The in vivo pharmacokinetic study in rabbits revealed an improvement in the C_{max} and $AUC_{0\text{-}24h}$ by 4.61-fold and 4.55-fold, respectively, in ATQ-SLNs when compared to the free drug. The $t_{1/2}$ of ATQ-SLNs was also reduced by 3.19-fold when compared to the non-formulated free drug. In conclusion, an SLNs formulation of ATQ was successfully prepared and can be a promising alternative for better absorption and therapeutic dose reduction for ATQ.

CHAPTER 1

INTRODUCTION

1.1 Malaria

1.1.1 What is malaria?

Malaria is a vector-borne disease which spread without boundaries worldwide. This endemic disease affects all parts of the world especially in lower income countries including Africa, Asia, Latin America, the Middle East and Europe with most cases and deaths in sub-Saharan Africa. Triggering factors such as wet weather, flood or the movements of mass population driven by conflict add up the risk of spreading the disease. According to World Health Organization (WHO), malaria has recorded 247 million cases in 2006 and causing mortality of around 880,000; and consequently increased the health costs for the prevention and treatment of malaria for up to 40% of the public health expenditures in some of the heavy-burden countries (WHO, 2009). In Malaysia, the implementation of Malaria Eradication Programme in 1967 had successfully reduced the malarial cases from 150,000 cases in 1967 to 5,000 cases over the year of 2003 to 2005 (MOHM, 2006).

Malaria is caused by Plasmodium parasite which is transferred to human by Anopheles mosquitoes. Five Plasmodium species have been known to cause malaria in human which are P. falciparum, P. vivax, P. ovale, P. malariae, and P. knowlesi (Greenwood et al., 2008). Of all species, P. falciparum has a distinguished ability to cause majority of deaths from malaria. It can bind to endothelium during blood stage of the infection and sequester organs including brain.

Invasion of Plasmodium parasites in human body involves the life cycle of the parasites itself (Fig. 1.1). The cycle starts when the sporozoites are injected into the blood vessels in the skin during the vector's blood meal on human. Then the sporozoites will further undergo exoerythrocytic and intraerythrocytic cycle (Greenwood et al., 2008, Jones and Good, 2006). The liver stage infection will be initiated when the sporozoites migrate to liver cells (hepatocytes). This stage is also known as exoerythrocytic phase which produces merozoites within 1 week. The infected hepatocytes will rupture and cause the release of merozoites in aggregated form known as merosomes which will then evade antibodies and invade erythrocyte. These intraerythrocytic or asexual forms of the parasite are susceptible to immune responses and have been related to both protection and disease. The disease might be prevented by antibodies that block binding of falciparum-infected eryhthrocytes or may cause presentation of diverse sequelae affecting different organ systems. Finally, a mosquito will ingest the circulated sexual stages (gametocytes) to complete the cycle. Apart from migrating through blood vessels, some sporozoites will enter the lymphatic system and penetrate lymph vascular endothelial cells in lymph nodes (Amino et al., 2006). This lymph node form does not contribute to the life cycle as most of them are degraded but some may be important in modulating immune response.

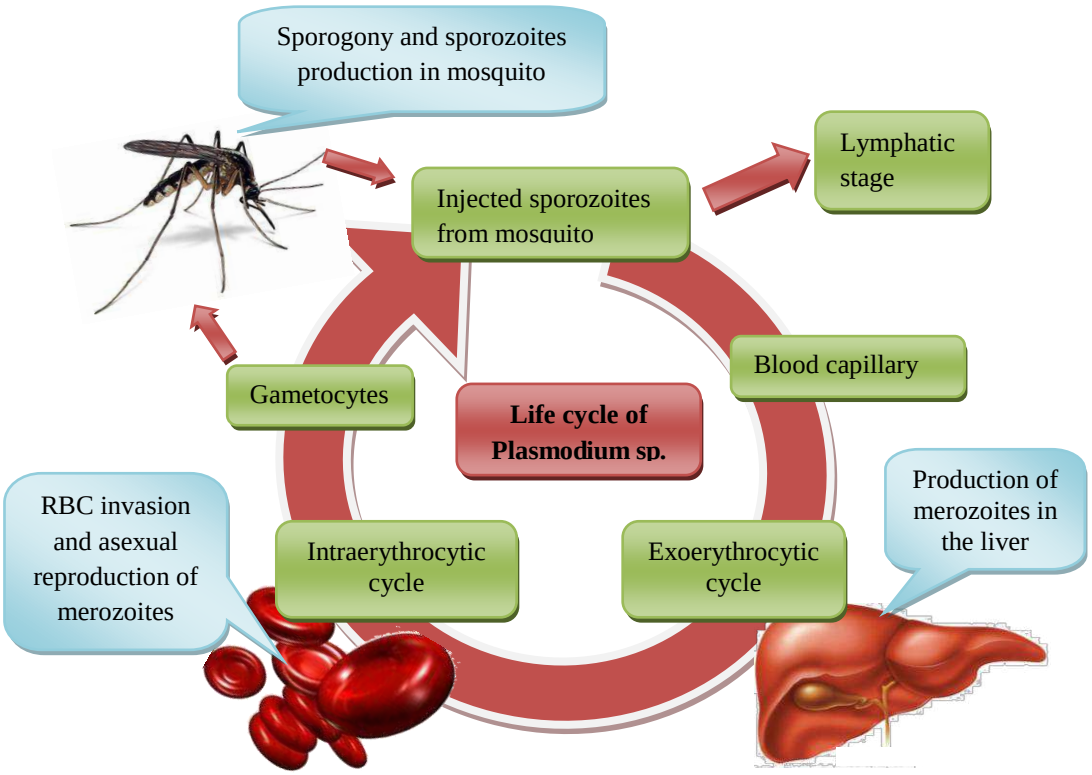

Fig 1.1: Life cycle of Plasmodium sp. in malaria infection

1.1.2 Drug therapy in malaria

Many tools and method have been implemented for the prevention and treatment of malaria worldwide. The World Health Assembly in 2005 has set a coverage target of 80% or more for their four key interventions which includes pharmacological and non-pharmacological approaches, namely insecticide-treated nets (ITNs), appropriate antimalarial drugs for probable of confirmed malarial cases, indoor residual spraying (IRS), and intermittent preventive treatment in pregnancy (WHO, 2009). As non-pharmacological approaches stress more on preventive measures, pharmacological approach remains crucial for both prophylaxis and treatment means. Each antimalarial drugs acts on different processes or metabolic pathways in different subcellular organelles (Table 1.1).

Table 1.1: Targets for antimalarial chemotherapy (Fidock et al., 2004).

Target location	Pathway/mechanism	Existing therapies
Cytosol	Folate metabolism	Pyrimethamine, proguanil, sulphadoxine, dapsone
	Unknown	Artemisinin
Parasite membrane	Membrane transport	Quinolines
Food vacuole	Haem polymerization	Chloroquine
	Free radical generation	Artemisinin
Mitochondrion	Electron transport	Atovaquone
Apicoplast	Protein synthesis	Tetracycline, clindamycin
	DNA synthesis	Quinolones
	Transcription	Rifampin

Chloroquine which acts at hemozoin will be concentrated inside the acidic food vacuole and further bind to β-hematin. This will cause build up of toxic free haem. On the other hand, antibiotics including tetracycline and clindamycin act inside the apicoplast to inhibit protein translation. The inhibition will cause the death of the progeny (delayed-death phenotype). Meanwhile, antifolates such as pyrimethamine and proguanil interrupt with de novo biosynthesis of folate while atovaquone inhibit the electron transport in the mitochondrion.

Combination therapy of antimalarial is recommended since the emergence of resistance strains towards monotherapy in all classes of antimalarials including artemisinin derivatives (Dhanawat et al., 2009, WHO, 2010). Combinations of drugs with different mechanisms of action can prevent or delay the onset of resistance by increasing the efficacy. This will lead to shorter duration of treatment that will further increase the compliance and reduce the risk of resistant parasites (Kremsner and Krishna, 2004). Thus, WHO suggested drug combination for all types of malaria

infections which can be divided into artemisinin-based combination therapy (ACT) and non-artemisinin based combination therapy (WHO, 2010).

1.2 Atovaquone

1.2.1 Physicochemical properties

Atovaquone (566C80) is a yellow crystalline solid which belongs to the class of naphthalenes with chemical name of trans-2-[4-(4-chlorophenyl) cyclohexyl]-3-hydroxy-1, 4-naphthalenedione (GlaxoSmithKline, 2008). It is a structural analogue of protozoan ubiquinone (Fig 1.1), a mitochondrial protein involved in electron transport or known as coenzyme Q (Baggish and Hill, 2002). The molecular formula for atovaquone is $C_{22}H_{19}ClO_3$ with a molecular weight of 366.84. It has a melting point of 216-219°C. A hydroxyl group in the molecule acts as a weak acid with a calculated pKa ≈ 5.0 (Lindegårdh and Bergqvist, 2000)

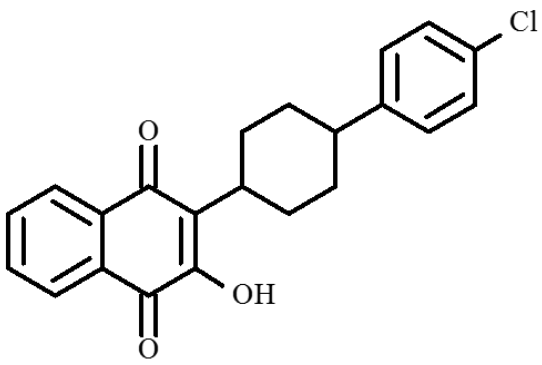

Fig 1.2: Chemical structure of atovaquone

According to United States Pharmacopeia (USP 28, 2005), atovaquone is practically insoluble in water (Table 1.2). Its hydrophobicity renders its solubility in many organic solvents including tetrahydrofuran and chloroform.

Table 1.2: Atovaquone solubility (USP 28, 2005)

Solubility	Solvent
Freely soluble	N-methyl-2-pyrrolidone, tetrahydrofuran (THF)
Soluble	Chloroform
Sparingly soluble	Acetone, di-n-butyl adipate, dimethylsufoxide (DMSO), polyethylene glycol (PEG) 400
Slightly soluble	Alcohol, 1,3-butanediol, ethyl acetate, glycerine, octanol, PEG 200
Very slightly soluble	0.1N NaOH
Insoluble	Water

1.2.2 Pharmacology of atovaquone

Atovaquone acts in the mitochondrion by inhibiting the electron transport in such organelles. It has a broad-spectrum antiparasitic activity towards causing organisms of pneumocystis pneumonia (Hughes et al., 1993, Hughes et al., 1998), toxoplasmosis (Djurković-Djaković et al., 2002) and malaria (Mulenga et al., 1999). In Plasmodium species, atovaquone inhibits the binding of coenzyme Q-complex III at Q_o cytochrome domain (Baggish and Hill, 2002). The inhibition will further cause collapse of the mitochondrial membrane potential. As several metabolic enzymes depend on this mitochondrial transport chain involving ubiquinone, atovaquone will cause indirect inhibition of these enzymes. Consequently, the synthesis of nucleic acid and adenosine triphosphate (ATP) will be blocked and causing the parasite death (GlaxoSmithKline, 2008).

Atovaquone is effective as a single antimalarial agent. However, its single usage have been associated with recurrence, reduction of susceptibility during following

treatment (Baggish and Hill, 2002), and rapid resistance due to gene mutation (Dhanawat et al., 2009). Therefore, atovaquone has been formulated in synergistic combination with proguanil (Canfield et al., 1995), an antifolate biguanide drug which inhibits plasmodial dihydrofolate reductase enzyme (DHFR). The combination has been favoured because of the high efficacy and tolerability compared to other antimalarial agents such as chloroquine and pyrimethamine/sulphamethoxazole (Mustafa and Agrawal, 2008). Their activity covers multi-drug resistance P. falciparum and P. ovale which did not response to other first line agents.

1.2.3 Pharmacokinetic of atovaquone

Despite of its potent antiparasitic activity, atovaquone's usage is hindered by its poor bioavailability. The bioavailability of ATQ depends on formulation and diet. The suspension formulation (Mepron®) increases the bioavailability by approximately 2-fold compared to the tablet formulation (Malarone®) which is from 23 ± 11% to 47 ± 15% (GlaxoSmithKline, 2008). Administration of atovaquone with food enhances its solubility in gastrointestinal tract hence improves the absorption (Schmidt and Dalhoff, 2002).

The volume of distribution at steady state (Vd_{ss}) is 0.60 ± 0.17 L/kg and it is highly bound to plasma proteins (99.9%) over the concentration range of 1 to 90 mcg/ml (GlaxoSmithKline, 2008). It has a long plasma half life of 2 to 3 days which is thought to be due to enterohepatic cycling. More than 94% of the administered dose was excreted unchanged in feces over 21 days with less than 0.6% excretion in the urine (Sweetman, 2005).

1.3 Solid Lipid Nanoparticles (SLNs)

Solid lipid nanoparticles (SLNs) are particles prepared from solid lipid or blends of solid lipid by replacing oil from o/w emulsion with a mean photon correlation spectroscopy (PCS) diameter of ~50-1000nm (Müller et al., 2000). For the past decade, studies have been done and promising results were shown for administration of SLNs by oral, parenteral, topical, rectal and ophthalmic route (Üner and Yener, 2007). It is getting an increasing attention because of its ability to increase lipophilic drug bioavailability with some data for controlled-release delivery (Li et al., 2009, Kumar et al., 2007). Moreover, it avoids toxicity problems by using physiological lipids and surfactants which are generally recognized as safe (GRAS) (Wissing et al., 2004) including lipids such as triglycerides and surfactants such as soy lecithin and poloxamer 188. Solvent avoidance is possible by using the production method of high pressure homogenization which also enables a simple and cost-efficient large scale production. This SLNs system is also capable of protecting incorporated labile drugs inside the solid matrix from degradation in storage and physiological conditions.

1.3.1 General compositions

1.3.1 (a) Lipid

Lipids can be defined as biological material which occurring naturally or derivation of those occurring naturally. It is water insoluble but soluble in organic solvents such as alcohol. The degree of fatty acid chain unsaturation, chain length, and homogeneity determines the physical form of the lipids in room temperature (Cannon and Long, 2008). As derived from the name, lipids which are solid at room temperature were used in the production of SLNs. Among the commonly used lipids

are monoglycerides such as glyceryl monostearate (Varia et al., 2008, Hou et al., 2003), triglycerides such as tripalmitin (Venkateswarlu and Manjunath, 2004, Kumar et al., 2007), and mixture of mono-, di-, and triglycerides such as Compritol 888 ATO (Kuo and Chen, 2009, zur Muhlen et al., 1998).

1.3.1 (b) Surfactant

Surfactants are important in preparing a formulation containing aqueous and lipid phase. Non-ionic surfactants with low hydrophile-lipophile balance (HLB) values (1-9) are more lipophilic and are commonly used in preparing water-in-oil emulsion whereas surfactants with high HLB value (>10) are more hydrophilic in nature and are useful in facilitating the formation of oil-in-water emulsion (Cannon and Long, 2008). Since SLNs involves in the initial formation of oil-in-water emulsion, non-ionic surfactants with high HLB values are used such as Poloxamers (Kumar et al., 2007, Venkateswarlu and Manjunath, 2004), and polysorbates (Hou et al., 2003, Kuo and Chen, 2009). Apart from that, natural emulsifiers such as soy lecithin and bile acids (Bunjes et al., 2001) were also being used in combination with the non-ionic emulsifiers.

1.3.2 Methods of production

Various techniques have been explored to produce SLNs including high shear homogenization, high pressure homogenization, microemulsion method, solvent emulsification method, solvent injection/nanoprecipitation method and membrane contractor method (Date et al., 2007). However, only four methods will be discussed as they are the most widely used method in the production of the SLNs.

1.3.2 (a) High shear homogenization (HSH) and ultrasonication

HSH and ultrasonication are the initially used technique in producing SLNs. HSH was being favoured due to its widespread availability, feasibility and the ease of handling. This method is usually combined with ultrasonication to further reduce the particle size (Abdelbary and Fahmy, 2009, Hou et al., 2003). However, as HPH is normally used to produce microemulsion, the quality of the nanodispersion will be compromised with the presence of microparticles. Furthermore, an extra caution should be taken with the use of ultrasonication as it can cause metal contamination from the probe into the preparation.

1.3.2 (b) High pressure homogenization (HPH)

HPH has been formerly used in formulating nanoemulsions for parenteral nutrition. Therefore, scaling up of the production from laboratory to industrial scale is feasible. Production of nanoemulsion involves high pressure (100-200bar) which pushes the liquid through a narrow gap and further causes acceleration of the fluid in a very short distance with high velocities (Mäder, 2006). Therefore, SLNs dispersions with narrow size distribution and higher particle content are possible without the presence of solvents (Üner and Yener, 2007). There are two general approaches in high pressure homogenization technique; namely hot homogenization and cold homogenization.

Hot homogenization involves melting of the lipid matrix at 5-10°C above the melting point. Simultaneously, an aqueous phase containing surfactant is heated at the same temperature and mix with the lipid melt to form preemulsion. The formed preemulsion is further homogenized in HPH at optimized pressure and cycles (Yang

et al., 1999, Varia et al., 2008) as further increment of the parameters will only cause an increment in kinetic energy of particle and thus causing the particle size to increase due to particle coalescence (Mäder, 2006). Finally, cooling down the preparation at or below room temperature will cause crystallization of the lipid to form SLNs.

In cold homogenization technique, avoidance of high temperature offers an advantage for thermolabile drugs. This method is also suitable for hydrophilic drug as it avoids the distribution of the drug into the aqueous phase which can occurs during hot homogenization and it can also bypass the complexity of crystallization step which sometimes lead to modification of the lipid structure or formation of supercooled melts (Mehnert and Mäder, 2001). Initially, the drug is solubilised in the lipid melt. The subsequent step involves rapid cooling of the melts to cause homogenous distribution of drug within lipid matrix. The cooled preparation is milled into microparticle sizes (50-100µm) which is then further suspended in chilled emulsifier solution. Finally, the presuspension is subjected to high pressure homogenization. In this technique, the production temperature has to be controlled to be at or below room temperature to ensure the solid state of the lipid (zur Muhlen et al., 1998).

1.3.2 (c) Microemulsion method

Microemulsion method involves the addition of an aqueous phase containing surfactant into the dispersion of drug in lipid melt under mild stirring. Both phases are maintained to be above the melting temperature of the lipid. Then, an aliquot of the microemulsion was diluted in a cold aqueous medium (2-3°C) under mild stirring

(Kuo and Chen, 2009). The speed of mixing is important so that the small size of the particles is due to precipitation and not mechanically induced by stirring process (Müller et al., 2000).

1.3.2 (d) Solvent emulsification

Solvent emulsification or solvent evaporation method has been commonly used in the production of polymeric microparticle and nanoparticles. In this method, water immiscible organic solvent such as chloroform and dicloromethane is used to dissolve the solid lipid prior to emulsification with aqueous phase which contains emulsifier. Subsequently, the oil droplets in the emulsion are formed into nanoparticles dispersion in the aqueous phase during the evaporation of solvent (Li et al., 2009, Luo et al., 2006). However, mean particle size depends on the concentration of lipid as high lipid concentration increases the viscosity of preparation. High viscosity will reduce the efficacy of homogenization thus increase the particle size (Mäder, 2006).

1.4 Literature review

Nanoparticulate drug delivery in medicine can be viewed as nanometer scale system ranging from 10-1000nm (De Jong and Borm, 2008). It is widely explored to improve drug targeting and delivery to ensure optimum therapeutic effects while reducing drug toxicity. Thus, it can lead to greater safety and biocompatibility and faster development of new safe medicines. SLNs is a relatively new nanoparticulate system when compared to other systems such as liposomes and polymeric nanoparticles. This drug carrier is particles made of solid lipids with a mean diameter of ~50-1000nm.

SLNs offers a prominent advantage over other nanoparticulate systems as it uses physiological lipids and surfactants which are widely being used in pharmaceutical formulations and generally recognized as safe (GRAS). In addition, the possibility of solvent avoidance by using high pressure homogenization can helps to avoid biotoxicity problem of carriers in human (Müller et al., 2000, Wissing et al., 2004). SLNs involve the formation of relatively rigid core consisting of lipids which are solid at room temperature. This solid carrier provides protection for the drug from gastric and intestinal degradation after oral administration (Üner and Yener, 2007). In one study, incorporation of quercetin in solid lipid nanoparticle has shown controlled-released of the drug in the in vitro study for more than 48 hours and an extension of the half-life when given intraduodenally to the rats (Li et al., 2009). The same finding was also reported by Kumar et. al (2007) when nitrendipine was formulated into SLNs. Both studies suggest a slow release of the drugs from the solid matrix over time which can ensure prolong circulation of the drug in the systemic circulation. Furthermore, the protection by the lipid can also help to improve stability of the incorporated drug (Faraji and Wipf, 2009).

Oral bioavailability of certain drugs can be improved using this delivery system as it has the possibility for uptake and transport through intestinal mucosa into blood and lymphatic circulation. An efficient uptake in intestine involves lymphatic uptake of particles with size between 20-500nm (Yuan et al., 2007). Lymphatic uptake of the SLNs can help bypassing the hepatic metabolism especially for the drug with high first-pass metabolism. Although the lymphatic uptake has been associated with small size of the particles, the advantage of SLNs in this case is the lipid (i.e. triglycerides) content in the formulation which may also influence the activation of this

transportation although chain length of the fatty acid may affect the uptake (Porter and Charman, 2001). This unique property of the SLNs has attracted researchers to explore the system for targeted-delivery of drug, particularly in cancer chemotherapy. A superior bioavailability of methotrexate, an anticancer drug, via the lymphatic uptake when formulated in the SLNs has been shown when compared to the plain solution of the drug. The increase in the bioavailability will offer a possibility of the effective dose reduction while reducing the dose-dependent toxicity (Paliwal et al., 2009). Moreover, the low distribution of the SLNs formulation into certain organs may be an advantage to reduce organ-related toxicity of idarubicin (Zara et al., 2001). Apart from this, surfactant content in the preparation will also help to improve the bioavailability of drug by increasing the membrane permeability and promotes bioadhesion of the drug in the intestinal membrane (Manjunath and Vankateswarlu, 2005).

To date, a wide range of research has been conducted by manipulating the SLNs system to be administered via several routes including oral, parenteral, topical, rectal, and ophthalmic (Üner and Yener, 2007). Therefore, a wide range of drugs can be potentially incorporated into the SLNs as a potential delivery system. Among the drugs, absorption of biopharmaceutics classification system (BCS) class II drugs may be improved using this delivery system. This class of drug exhibit a low aqueous solubility due to the hydrophobic nature but high membrane permeability. Thus dissolution of the drug in the intestinal medium is the rate-limiting step of the absorption. An antimalarial drug, atovaquone, falls into this category with poor bioavailability and exhibit a dependency of its bioavailability with food administration. As a compound with poor solubility in water, any modification which

can improve solubility and dissolution rate in the luminal tract will enhance the absorption (Fleisher et al., 1999). Therefore, co-administration of the drug with food has been shown to increase the bioavailability of ATQ in both tablet and suspension form (Rolan et al., 1994, Dixon et al., 1996). The presence of food may increase the solubility of ATQ in the intestinal lumen and stimulate the biliary secretion which is important in the digestion and absorption of fats in the small intestines, including the lipophilic drug. However, the extent of absorption was affected by the fat content of the meal where a high fat meal showed further increase in the absorption when compared to the low fat meal (Rolan et al., 1994). This variation may necessitate a proper monitoring of the patient's meal if an optimum absorption is to be achieved. Moreover, administration of the drug alone in fasting condition may cause sub-therapeutic plasma concentration and affect the therapy. Thus, a formulation which can diminish the food-dependent effect such as SLNs may be an attractive solution to this problem.

1.5 Statement of the problem

Formulation of ATQ in the conventional tablet form showed a low bioavailability which led to the administration of the drug in a large dose. The absorption of the drug was highly dependent on the food intake of the patient even in the micro-suspension formulation. This will cause variation in the pharmacokinetic profiles between patients and complicate the treatment. Therefore, incorporation of ATQ into the SLNs formulation may be a promising solution to increase the bioavailability of ATQ and further reduce the administration dose.

1.6 Objectives of present study

The development of ATQ-SLNs was carried out in various stages with following objectives;

i. To optimize ATQ-SLNs formulations using different lipids and surfactants in 2^4 factorial design study.

ii. To improve the stability of the selected SLNs by lyophilization of the formulations using trehalose as a cryoprotectant.

iii. To develop and validate two HPLC-UV methods for the quantification of ATQ in the release medium for the in vitro release study and for the quantification of ATQ in rabbit plasma in the in vivo pharmacokinetic study.

iv. To evaluate the in vivo pharmacokinetic parameters of ATQ-SLNs after oral administration in comparison to ATQ-free drug using rabbits.

CHAPTER 2

DEVELOPMENT AND VALIDATION OF HPLC-UV METHOD FOR THE DETERMINATION OF ATQ IN THE DISSOLUTION MEDIA

2.1 Introduction

Analysis of active pharmaceutical ingredients (API) in formulations is very crucial in the development of pharmaceutical dosage form. Other than determining the API's content, analysis also need to be done to evaluate the release of the drug in the in vitro release study. Therefore, a method of analysis has to be developed to meet the purposes.

UV spectrophotometer is one of the analytical methods which are widely being used for the detection of API because of its simplicity and fast analysis. Even though ATQ can be detected by UV; the method is not specific as other excipients can interrupt the signal of the analyte at the maximum wavelength. Therefore, visible light range can be used since ATQ is a coloured substance and other excipients are less likely to interfere the signal. One method using visible light detection was reported for the determination of ATQ in Malarone® tablet containing ATQ and proguanil using flow-through dissolution apparatus (Butler and Bateman, 1998). Nonetheless, visible light has its limitation of low sensitivity compared to UV light.

HPLC method is a more preferable analytical tool because of its high sensitivity, specificity and reproducibility. Detection of ATQ using HPLC has been conducted by a group of researchers to quantify the loading and encapsulation of ATQ in liposome (Cauchetier et al., 1999) and nanocapsules (Cauchetier et al., 2003). Thus,

a sensitive and specific method of HPLC-UV was developed and validated for the determination of ATQ for the quantification of ATQ in release medium during in vitro release study.

2.2 Materials

Atovaquone was purchased from Hallochem Pharmaceutical Co. Ltd. (Chongqing, China) and acetonitrile (HPLC grade) was purchased from J.T. Baker (Phillipsburg, USA). Ammonium acetate was bought from Bendosen Laboratory Chemicals (UK) and glacial acetic acid was bought from QRëC (Selangor, Malaysia).

2.3 Methods

2.3.1 Instrumentation

Analysis was performed using a Shimadzu liquid chromatographic system (Kyoto, Japan) with CBM-20A system controller, LC-20AD solvent delivery pump, SPD-20A UV-VIS detector, SIL-20A autosampler, and CTO-10ASvp oven system. Data acquisition and analysis was performed using Shimadzu LCsolution software (Kyoto, Japan).

2.3.2 Chromatographic condition

Chromatographic separation was performed at 45°C using a C18 column (Phenomenex, 150 x 4.60 mm i.d, 5 µm particle size) fitted with a universal guard cartridge (Thermoscientific, 4 x 4.6 mm i.d.). Samples of 20µl were injected into the HPLC system and the detection wavelength was set at 254nm. The mobile phase contained a mixture of 20mM ammonium acetate buffer (adjusted to pH3 with glacial acetic acid) and acetonitrile (ACN) in the ratio of 15:85 (v/v). System flow

rate was maintained at 1.0ml/min. The mobile phase was filtered under vacuum through 0.45µm nylon membrane filter (Whatman, England) and degassed for 10 min prior to use.

2.3.3 Preparation of standard and quality control solutions

A primary standard stock solution of atovaquone (400µg/ml) was prepared in methanol-dimethylformamide (99:1 v/v). The stock solution was further diluted with mobile phase to obtain a working standard solution of 40µg/ml. Solutions for calibration were prepared by diluting working standard solution with mobile phase to give concentration in the range of 40 to 4000ng/ml. Three concentrations for quality control (QC) were prepared at low, medium, and high concentration of 120, 1500, and 3500ng/ml, respectively. These solutions were stored at 4°C prior to use.

2.3.4 Method validation

2.3.4 (a) System suitability

System suitability test was performed to ensure well performance of the chromatographic system. The involved parameters were capacity factor (k'), tailing factor (T), theoretical plate number (N) and precision/injection repeatability. This test was conducted using atovaquone solution at a concentration of 1500ng/ml in five injection replicates.

Capacity factor (k') is used to measure relative elution time of the analyte compare to void volume using following equation

$$k' = (t_R - t_m)/t_m \qquad \text{(Eq. 2.1)}$$

where t_R is the retention time of the analyte and t_m is the retention time of the non-retained component. Commonly, the value of k' is more than 2 (USFDA, 1994).

Tailing factor (T) is an important parameter to measure symmetry of the peak. Quantitation will be affected as increasing in peak tailing reduces the accuracy. It is calculated using following equation

$$T = W_{0.05}/2f \qquad \text{(Eq. 2.2)}$$

where $W_{0.05}$ is the width of the peak at 5% height and f is the distance between maximum and the leading edge of the peak. T value of ≤ 2 is recommended by USFDA (USFDA, 1994).

Theoretical plate number (N) is a parameter of column efficiency which is the amount of peaks located per unit run-time of the chromatogram using the following equation

$$N = 16\,(t/W) \qquad \text{(Eq. 2.3)}$$

where t is the retention time of the analyte and W is the width of peak measured by extrapolating the relative straight line to the baseline. Generally, theoretical plate number should be more than 2000 although it depends on elution times.

Injection precision is expressed as relative standard deviation (RSD) which indicates the performance of the chromatography at the sample analyzing time. The recommended RSD is $\leq 1\%$ for $n \geq 5$ (USFDA, 1994).

2.3.4 (b) Specificity

Specificity of the method should portray the absence of interference from other substances at the retention time of the analyte and the analyte is well-resolved from other peaks. For the evaluation, blank-SLN was prepared containing all excipients except ATQ. The blank-SLNs was treated the same as ATQ-SLNs in the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) to be analyzed in six replicates.

2.3.4 (c) Linearity

The linearity of the method was evaluated using five calibration curves prepared on five consecutive days in the concentration range of 40 to 4000 ng/ml of ATQ. The curves were plotted using peak area of ATQ against corresponding concentration using linear regression analysis.

2.3.4 (d) Limit of detection and limit of quantification

Limit of detection (LOD) determination was carried out by injecting samples at subsequent low concentration of ATQ. The limit was determined at a signal to noise ratio of 3:1. Limit of quantification (LOQ) was defined as the lowest concentration of analyte in the calibration curve that can be determined with acceptable precision and accuracy under the stated experimental condition.

2.3.4 (e) Precision and accuracy

Precision is defined as percentage of relative standard deviation (%RSD) of the calculated concentration while accuracy is expressed as relative percentage error (%RE) using following equations

$$\text{Precision (\%RSD)} = \frac{\text{Standard deviation}}{\text{Mean value}} \times 100\% \quad \text{(Eq. 2.4)}$$

$$\text{Accuracy (\%RE)} = \frac{(\text{Calculated concentration} - C_{std})}{C_{std}} \times 100\% \quad \text{(Eq 2.5)}$$

where C_{std} is the nominal concentration of the standard solution in ng/ml.

Accuracy and precision for each point in the calibration curve were analyzed from the previously prepared five curves. For inter-day and intra-day precision and accuracy, QC samples at low, medium, and high concentration of 120, 1500 and 3500ng/ml were injected in five replicates. For intra-day variation, the replicates were analyzed on the same day while for inter-day variation; five replicates of three QC levels were analyzed on three consecutive days.

2.3.4 (f) Robustness

Robustness is the ability of the method to remain unaffected by deliberate variations in method parameters. The robustness was determined by analysis of samples with same concentration under several conditions including small change in pH (± 0.2), percentage of acetonitrile in mobile phase (± 1%), flow rate of the mobile phase (± 0.1ml/min) and detector wavelength (± 2nm).

2.3.4 (g) Solution stability

Stability study was performed using LQC, MQC and HQC samples to evaluate the stability of ATQ in the mobile phase under different conditions, namely, bench top stability (6 hr at room temperature), post-preparative stability (24 hr in the autosampler), and short term stability (14 days at 4°C).

2.3.5 Statistical analysis

The results in robustness test were analyzed for the difference using one-way analysis of variance (ANOVA), using SPSS version 16.0 software. The difference was statistically significant when $p < 0.05$. For a significant difference, a post hoc Tukey's HSD (honestly significant difference) test was conducted.

2.4 Results and Discussion

2.4.1 Method development and optimization

The chromatographic conditions were adjusted in order to provide a good performance of the assay. Selection of the detection wavelength is imperative to achieve maximum absorbance. Thus, a UV spectrophotometer scan was conducted in the range of 200 to 450nm (Figure 2.1) and the maximum absorbance was detected at 254nm. Therefore, 254nm was set as the detection wavelength for the study.

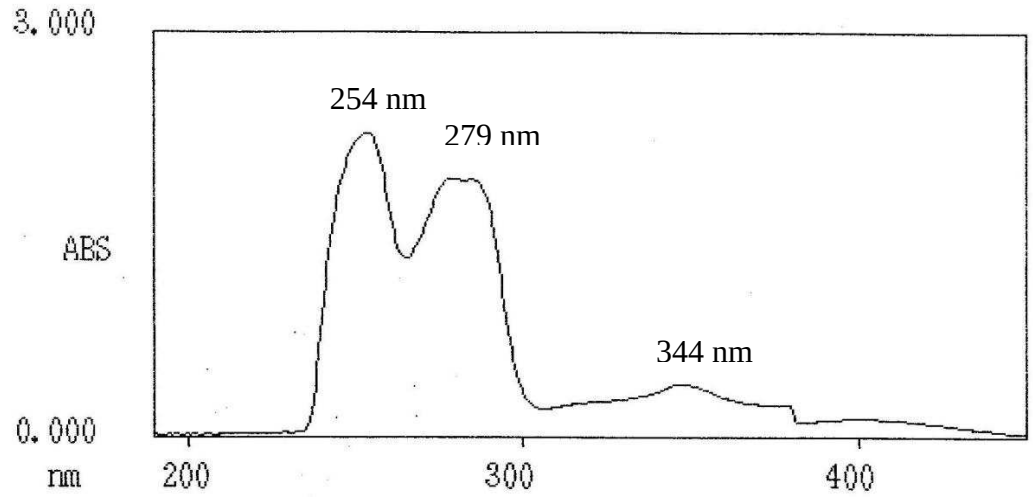

Fig. 2.1: UV absorption spectrum of ATQ from 200 to 450nm.

As for the mobile phase, several compositions of buffer to organic solvent (ACN) were tested at ratios of 10:90, 15:85, and 20:80 (v/v). Increasing the percentage of ACN increased hydrophobicity of the mobile phase. Therefore, elution of highly

hydrophobic compound such as ATQ became faster. A composition of 15:85 was chosen as it gave sufficient retention time (~5min) and a peak with high area and good shape.

pH of ammonium acetate buffer was varied from pH 5 to pH 3. As the pH decreased, retention time of ATQ was shortened and the peak area was increased. Tailing factor were also improved indicating symmetricity of the peak. For a weak acid substance such as ATQ, pH above the pKa value will cause the acidic analyte to carry a negative charge and behaves as an extremely polar molecule. Thus, delaying the elution of analyte in hydrophobic mobile phase. Since pKa of ATQ is ≈5.0 (Lindegårdh and Bergqvist, 2000), pH lower than pH 5 will cause elution of ATQ to be faster. Therefore, pH 3 was chosen as it gave sufficient retention time, highest peak area and good peak shape.

At first the temperature of the column was set at 25°C. However, it did not give a stable baseline. Thus, a higher temperature of 45°C was used instead as it gave stable baseline, faster elution of ATQ with a better peak shape. Finally, the mobile phase containing a mixture of 20mM ammonium acetate buffer (pH 3) and ACN (15:85, v/v) was chosen for the analysis at a flow rate 1.0ml/min and column temperature of 45°C. The wavelength of detection was set at 254nm.