TOCOTRIENOLS IN SOLID DOSAGE FORM

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TOCOTRIENOLS IN SOLID DOSAGE FORM

by

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To my parents, family members and close friends

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TABLE OF CONTENTS

CHAPTER 4: VALIDATION OF AN ASSAY METHOD FOR TOCOMAX TABLETS

LIST OF TABLES

LIST OF FIGURES

LIST OF EQUATIONS

LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE

ACN	Acetonitrile
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the plasma concentration-time curve
$AUC_{0\text{-}12h}$	Area under the plasma concentration-time curve from time zero to the last sampling time, 12 hours after dosing
$AUC_{0\text{-}24h}$	Area under the plasma concentration-time curve from time zero to the last sampling time, 24 hours after dosing
C_{max}	Peak plasma concentration
CYP3A4	Cytochrome P450 subfamily 3A4
HPLC	High performance liquid chromatography
k_e	Elimination rate constant
MDR	Multidrug resistant
P-gp	P-glycoprotein
R	Correlation coefficient
R.S.D.	Relative standard deviation
S.D.	Standard deviation
SEDDS	Self-emulsifying drug delivery system
S.E.M.	Standard error of mean
SES	Self-emulsifying system
THF	Tetrahydrofuran
T_{max}	Time to reach peak plasma concentration

USM	Universiti Sains Malaysia
v/v	volume over volume
w/w	weight over weight
w/v	weight over volume

LIST OF APPENDICES

TOKOTRIENOL DALAM DOS PEPEJAL

ABSTRAK

Kajian biokeperolehan oral serbuk Tocomax® 20% yang mengandungi campuran beberapa jenis tokotrienol telah dijalankan dengan menggunakan tikus Sprague-Dawley. Kajian tersebut menunjukkan bahawa serbuk Tocomax® 20% mempunyai biokeperolehan yang separas dengan Tocovid® Suprabio (1.0, 1.0 dan 1.2 kali ganda untuk alpha, gamma dan delta tokotrienol masing-masing). Di samping itu, ia juga mempunyai biokeperolehan yang lebih tinggi daripada suspensi minyak Tocomin® 50% (1.9, 2.4 dan 2.9 kali ganda untuk alpha, gamma dan delta tokotrienol masing-masing).

Penggunaan serbuk Tocomax® 20% sebagai bahan aktif dalam formulasi tablet yang mengandungi tokotrienol menunjukkan bahawa tablet yang memuaskan dapat dihasilkan dengan menggunakan Neusilin UFL2 dan US2 sebagai diluents, Ac-Di-Sol sebagai disintegrant dan magnesium sebagai lubricant, dengan berat tablet sebanyak 500 mg setiap tablet. Tablet yang dihasilkan adalah sekata dari segi warna dan tidak mempunyai kecacatan seperti ‘capping’. Tablet tersebut juga mempunyai masa peleraian yang baik, iaitu 8 minit dan mempunyai berat yang seragam (kurang daripada 2%) Tablet dengan formulasi yang optimum juga berjaya dipindahkan ke skala yang lebih besar.

Suatu kaedah kromatografi cecair prestasi tinggi yang digunakan untuk menentukan kuantiti campuran tokotrienol dalam tablet disahkan berdasarkan garis panduan ICH. Kaedah asai itu dapat memuaskan criteria pengesahan dari segi 'linearity', kespesifikan, ketepatan dan kejituan. Kandungan tokotrienol dalam tablet yang dihasilkan adalah sebanyak 93.9, 92.6 dan 92.2% untuk alpha, gamma dan delta tokotrienol masing-masing. Eksperimen yang selanjutnya menunjukkan bahawa tablet yang diformulasikan hanya stabil apabila disimpan dalam suhu 4^0C dan bukannya apabila didedahkan kepada suhu 40^0C dan 70% RH (kelembapan relatif).

Seterusnya, satu kajian biokeperolehan yang melibatkan sukarelawan manusia dijalankan dengan menggunakan tablet Tocomax yang telah dirumuskan dan dibandingkan dengan suspensi minyak Tocomin®. Profil biokeperolehan yang didapati menunjukkan bahawa sukarelawan yang diberi tablet Tocomax adalah lebih tinggi daripada suspensi minyak Tocomin® (2.0, 1.7 and 1.9 kali ganda untuk alpha, gamma dan delta tocotrienol masing-masing.

TOCOTRIENOLS IN SOLID DOSAGE FORM

ABSTRACT

The bioavailability study of Tocomax® 20% powder containing mixed tocotrienols was carried out using Sprague-Dawley rats. The study showed that Tocomax® 20% powder had comparative oral bioavailability as the Tocovid® Suprabio (1.0, 1.0 and 1.2 times for alpha, gamma and delta tocotrienols respectively). In addition, it was also found to have better oral bioavailability as compared to Tocomin® 50% oily formulation (1.9, 2.4 and 2.9 times for alpha, gamma and delta tocotrienol respectively).

Subsequent usage of Tocomax® 20% powder as the active ingredient for the formulation of tablets containing tocotrienols showed that a satisfactory tablet could be obtained using Neusilin UFL2 and US2 as the diluent, Ac-Di-Sol as the disintegrant and magnesium stearate as the lubricant with the ideal tablet weight of 500 mg per tablet. The tablets were homogeneous in colour and with no physical defects like capping. The tablets also have good disintegration times of approximately 8 minutes and were uniform in weight (less than 2%). The tablets with the optimized formulation were also successfully up-scaled.

A simple high performance liquid chromatography method used to assay the mixed tocotrienols in the tablets was validated in accordance with the ICH guidelines. The

method was found to fulfill validation requirements such as linearity, specificity, recovery/ accuracy and precision. The assay of the freshly prepared tablet was 93.9, 92.6 and 92.2% for alpha, gamma and delta tocotrienols respectively. Further stability tests showed that the tablets were stable when kept at 4^0C but not at other storage conditions such as 40^0C and at 70% RH (relative humidity).

Finally, a bioavailability study involving healthy human volunteers was carried out using the formulated Tocomax tablets and compared against Tocomin® oily formulation. The profiles for volunteers given Tocomax tablets preparation obtained was consistently higher than that of the Tocomin oily formulation (2.0, 1.7 and 1.9 times for alpha, gamma and delta tocotrienol respectively) when administered as the tablet compared to the oily formulation.

CHAPTER 1: INTRODUCTION

1.1 ORAL BIOAVAILABILITY OF DRUGS

Oral bioavailability of drugs is a term which is misconstrued by many as oral drug absorption. It should be emphasized that while oral absorption involves the transit of drugs from the mucus membrane of the digestive tract into the blood stream, oral bioavailability, refers to the rate and amount of medication being moved from the digestive tract to the intended site of action in the body (Ashford, 2002a, Chiou, 2001).

1.2 FACTORS AFFECTING THE BIOAVAILABILITY OF AN ORAL DRUG

The oral route is the preferred mode of drug administration due to its ease and convenience to the patients (Ashford, 2002a). Oral administration also avoids the pain and risk of infection associated with parenteral administrations such as intravenous and intramuscular injections (Gavhane and Yadav, 2012). However, many factors can affect the bioavailability of drugs given orally. Thus, an understanding of these factors is crucial in the development of an effective oral medication (Muller et al., 2006). These factors can be classified into physiological, physicochemical and formulation factors.

1.2.1 PHYSIOLOGICAL FACTORS

Some of the main physiological factors which may affect the oral bioavailability include gastrointestinal pH, the unstirred water layer, gastrointestinal (GI) membrane, pre-systemic metabolism and physiological disorders of the GI tract.

1.2.1(a) Gastrointestinal pH

The pH of the gastrointestinal tract changes gradually from being acidic in the stomach region to being alkaline in the intestines and colon area (Fallingborg, 1999). The pH also differs between a fasting and a fed stomach (Ashford, 2002a). Since unionized drugs molecules penetrate the gastrointestinal membrane more effectively than ionized ones, a weakly acidic drug tend to have better absorption in the stomach with more drug molecules existing as unionized forms while weakly basic drugs tend to have better absorption in the intestine (Schanker et al., 1957). The presence of food however, increases the pH of the gastrointestinal tract (Arne, 1978) and prolongs the gastric emptying (Jackson et al., 2007, Dressman et al., 1990). The prolonged gastric emptying time increases the exposure time of the drug molecules to the acidic stomach pH, leading to degradation of pH sensitive drugs such as penicilins (Mizen et al, 1995, Simberkoff et al., 1970) and erythromycin (Cachet et al., 1989).

1.2.1 (b) The unstirred water layer

The unstirred water layer is the region sandwiched between the lumen and gastrointestinal membrane. It comprises mainly mucus and glycocalyx (Ashford, 2002a) and is approximately 500 μm thick (Atuma et al., 2000). While it offers protection towards the gastrointestinal tract (Strugala et al., 2003), the layer has also been shown to hinder the diffusion of certain drugs by binding the drugs to the mucus and hence prevents them from crossing the gastrointestinal membrane (Loftsson and Brewster, 2011; MacAdam, 1993).

1.2.1 (c) Gastrointestinal membrane

The gastrointestinal membrane, similar to a semi-permeable lipoidal sieve (Hogben et al., 1959), is a barrier which allows only the passing of small molecules and ions (example, water molecules) through a convoluted passage between the epithelial cells lining the gastrointestinal membrane. This is known as the paracellular pathway (Gumbiner, 1987). The other route, transcellular pathway, involves the movement of drug molecules across the epithelial cells. Transcellular pathway can be sub-divided into various mechanisms such as passive diffusion and carrier mediated transport (Ashford, 2002a). While the passive diffusion route functions by transporting small lipophilic molecules from a high concentration region to a low concentration region, the transportation of drugs with very hydrophilic molecules are restricted as they do not have sufficient lipid solubility to cross the gastrointestinal membrane (Adson et al., 1995). On the other hand, extremely hydrophobic drug molecules were unable to solubilize in the aqueous layer of the gastrointestinal lumen before penetrating the gastrointestinal membrane. Carrier mediated transport, is further branched into two types, namely, active transport and facilitated diffusion (transport). Active transportation involved the transportation of drug molecules against a concentration gradient through the gastrointestinal membrane and it requires energy, generated mainly from the hydrolysis of intracellular adenosine triphosphate (Komarova and Malik, 2010). In contrast, facilitated diffusion does not transfer drug molecules against a concentration gradient, and thus, does not require energy. The substrates are transported through facilitated diffusion down a concentration gradient at a rate based on the molecular size and polarity of the molecule itself (Ashford, 2002a).

1.2.1(d) Pre-systemic metabolism

Intestinal P-glycoprotein (P-g) and intestinal metabolizing enzyme (CYP3A4) are two widely studied physiological factors that can affect the bioavailability of drugs (Ashford, 2002a). While P-gp (encoded by the MDR gene) exerts its effect by expelling the drug out of the enterocytes (Juliano and Ling, 1976), CYP3A4 (a predominant Phase 1 drug metabolizing species found in humans), functions mainly by metabolizing the drug before any effective absorption could take place (Koudriakova et al., 1998; Shimada et al., 1994). Both are believed to work synergistically as the continuous process of expelling the drug into the lumen by the P-gp provided extra exposure for the CYP3A4 to metabolize the drug. P-gp also removes metabolites from the enterocytes of the gastrointestinal tract and expels them back into the lumen, some of which are substrates of CYP3A4 (Hochman et al., 2000; Meijerman et al., 2008; Watkins, 1997). However, like most enzymes, both P-gp and CYP3A4 are saturable and can be inhibited (Sandström et al., 2001) or induced (Lemahieu and Maes, 2007; Marzolini, 1994).

1.2.1(e) Physiological disorders

Aging individuals were shown to undergo physiological changes (Newton, 2004), potentially altering drug metabolism as well as its pre-systemic elimination process (Wilkinson, 1997). Wilkinson (1997) also noted that patients with liver diseases (usually the elderly), often display signs of increased drug bioavailability, especially drugs which exhibit high first pass metabolism effects. The impaired hepatic function reduces the extent of first pass metabolism, thus, increasing the oral bioavailability of the drug given. Other studies which discussed altered drug bioavailability due to

diseases such as Parkinson and diabetes include those carried out by Seeberger and Hauser (2007), Darwiche et al. (2001) and Hermann et al. (1998).

1.2.2 PHYSICOCHEMICAL FACTORS

Physicochemical factors refer to the properties of the drug molecule and these include solubility and permeability, stability, particle size and polymorphism of the drug (Hörter and Dressman, 2001).

1.2.2(a) Solubility and permeability

Drugs are classified into four classes under the Biopharmaceutics Drug Classification Scheme (BCS). While Class I drugs are highly soluble and permeable, the remaining classes of drugs faced either solubility, permeability or a combination of both problems (Martinez et al., 2002). Poor solubility of a drug will not only lead to undesirable drug precipitation (Dai, 2010), but will also produce drug solutions in nano-molar concentrations in the gastrointestinal tract. With only trace amounts of the soluble drug and even lesser amount of drug being able to permeate the intestinal barrier, the oral bioavailability of the drug administered is reduced and therefore, insufficient to induce a therapeutic effect on the patient (Bergstrom et al., 2004).

1.2.2(b) Stability of the drug

The stability of a drug is important so that minimal degradation occurs upon exposure to the harsh conditions of the gastrointestinal tract. A drug is said to be stable in the gastrointestinal tract if less than 5% of the drug is degraded after incubation in stimulated gastric fluids and stimulated intestinal fluids for 1 and 3 hours respectively (Asafu-Adjaye et al., 2007). To increase the stability of a drug,

numerous formulation techniques have been employed. An example would be the introduction of enteric coated tablets whereby the drug would be released from its enteric coat to the site of absorption upon contact with the alkaline pH (Fujii et al., 2011). Meanwhile, Petereit and Weisbrod (1999) utilized methacrylate copolymers as tablet coatings to make the drug more stable.

1.2.2(c) Particle size and polymorphism of the drug

Drugs with smaller particle size have larger surface volume, thus, enabling the drug to have maximum possible solubility before absorption takes place (Jinno et al., 2006). This concept has been utilized by formulators in efforts to increase the bioavailability of poorly water soluble drugs such as danazol (Liversidge and Cundy, 1995). The particle size of a drug can be reduced by various methods such as micronization, nanosuspension, sonocrystallisation and supercritical fluidation (Mohanachandran et al., 2010).

Polymorphism leads to the occurrence of the same drug having a series of different physicochemical properties (Raw et al., 2004). The difference is mainly due to the different crystal packing arrangements in the drug itself, thereby giving rise to different properties such as melting points, chemical reactivity, dissolution rates and therefore, different bioavailability (Ashford, 2002b and Rustichelli et al., 2000). Polymorphic forms such as crystallines are in general chemically more stable than their amorphous counterpart but are seldom favored due to difficulties in dissociation and dissolution (Choi et al., 2004; Snider et al., 2004). Hence, a meta-stable drug form is usually used in formulations (Kawakami, 2012; Singhal and Curatolo, 2004). The effects of different polymorphic drugs forms on bioavailability can be seen

through studies carried out by Kobayashi et al. (2001). It is also noted that polymorphic transformations between the different drug forms can be affected by environmental conditions such as humidity and moisture uptake (Cheng and Lin, 2008).

1.2.3 FORMULATION FACTORS

Formulation factors include the type of dosage forms and excipients used (Goole et al., 2010). The absorption of drugs in solid forms such as tablets is governed by the rate of disintegration followed by the rate of dissolution. Thus, drugs in liquid dosage forms such as suspensions and emulsions are more readily absorbed (Compton et al., 2006).

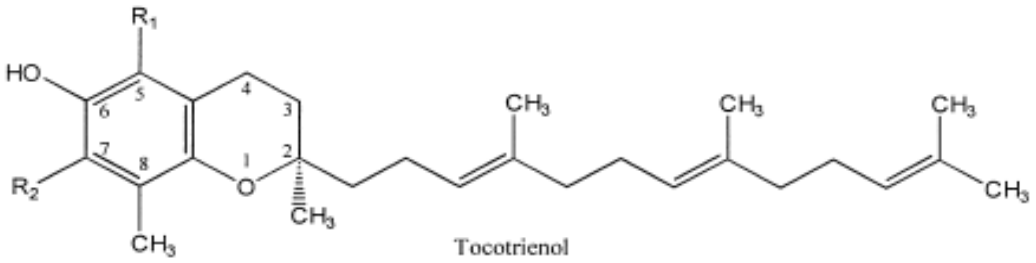
Pharmaceutical excipients constitute the bulk properties of tablets and contribute vastly towards the dosage, stability and bioavailability of an active ingredient (Pifferi and Restani, 2003; Schulze et al., 2005). Any incompatibility of tabletting excipients with the drug may cause undesirable interactions which may be determined through various methods such as differential scanning calorimetry or microscopic methods (Mura et al., 1998). Tablet lubricants such as magnesium stearate are commonly added to facilitate the smooth ejection of tablets from the tabletting machine. Excessive addition of magnesium stearate however, repels water and increases the disintegration time (Llusa et al., 2009). Another excipient, polyvinylpyrrolidone (PVP), is found to interact and form solid bridges with the actives upon storage at high moisture conditions, prolonging the disintegration time (Zelko et al., 2000).

Under formulation factor, the size and density of the granules are also found to influence the bioavailability of the drug as well (Hegedus and Pintye-Hodi, 2007). Heavier and denser granules are shown to be retained in the stomach region for a longer period of time whereas smaller size granules tend to empty quicker, giving rise to a difference in bioavailability though the formulation may consist of the same active drug and excipients (Newton, 2010). Other effects of formulation and process variables on the release of drugs include the method of manufacturing, for example; wet granulation or through direct compression (Kranz and Wagner, 2006). In their study, the authors found that formulation of the studied drug through wet granulation method produced tablets with faster release rate profiles as the method had produced tablets which were porous and were able to take up liquid rapidly.

1.3 TOCOTRIENOLS

1.3.1 INTRODUCTION

Tocotrienols are part of Vitamin E family, the other half of which is the highly popular tocopherols. Both tocotrienols and tocopherols have 4 isomeric members, namely the alpha, beta, gamma and delta (α, β, γ and δ). These isomers are easily identified by the number and position of the methyl groups on the chromanol head. While the tocopherols contain a chromanol head and a saturated phytyl tail, the tocotrienols on the other hand, possess a chromanol head with unsaturated isoprenoid side chain at positions 3', 7' and 11' (Figure 1.1).



Type of tocotrienol	R1	R2
α tocotrienol	CH_3	CH_3
β tocotrienol	CH_3	H
γ tocotrienol	H	CH_3
δ tocotrienol	H	H

Figure 1.1: Chemical structure of tocotrienols

1.3.2 PHARMACOKINETICS OF TOCOTRIENOLS

Several analytical methods have been developed to determine the pharmacokinetics of tocotrienols. These include the usage of high performance liquid chromatography (HPLC) with fluorescence detection (Grebenstein and Frank, 2012; Yap et al., 1999), HPLC with ultraviolet detection (Abuasal et al., 2011, Katsanidis and Addis, 1999) and LCMS/MS (Liang et al., 2013). Each method of detection has its own advantages and disadvantages. The optimal procedure is largely dependant on the matrix (plasma or animal tissues) containing the tocotrienols itself.

In general, the bioavailability of tocotrienols is poor and erratic. Nevertheless, studies carried out by Yap et al. (2003) showed that although the bioavailability of tocotrienols administered orally was low and incomplete, it was still better compared to other routes of administration such as intraperitoneal or intramuscular. The bioavailability of tocotrienols administered after an intake of oily food was also

observed to increase after healthy human volunteers were given a high fat meal just before administration of the drug.

1.3.3 POTENTIAL HEALTH BENEFITS

1.3.3(a) Antioxidant properties

Oxidative stress occurs whenever there is an imbalance between the production of reactive oxygen species and the corresponding protective detoxifying mechanisms in the body (Blokhina and Fagerstedt, 2010; Maccarrone et al., 2001). As a result, the deoxyribonucleic acid (DNA), bodily proteins and lipids may be damaged (Sies, 1991). Tocotrienols, which belong to the lipid soluble antioxidant group, demonstrate its antioxidative activities by neutralizing peroxyl and alkoxyl radicals produced during lipid peroxidation (Burton et al., 1983). Lipid peroxidation is detrimental to health because it modifies, inactivates and damages important cellular components (Yoshida et al., 2007). Peroxidation of low density lipoprotein (LDL)-lipids also initiates the pathogenesis of atherosclerosis. Due to LDL being oxidized, this modified particle is recognized by the scavenger cell receptor on macrophages. As a consequence, massive amounts of cholesterol are deposited into these cells and thus, foam cells are generated. Tocotrienols which are known to produce antioxidative effects are able to reduce foam cell formation by inhibiting LDL peroxidation (Steinberg et al., 1989).

1.3.3(b) Cholesterol lowering properties and prevention of cardiovascular diseases.

Tocotrienols as anti-cholesterol agents were first presented by Qureshi et al. (1986), whereby tocotrienol-rich fractions (TRF) extracted from barley was found to inhibit the biosynthesis of cholesterol when fed to rats and chickens. Tocotrienols inhibit the

synthesis of cholesterol by down-regulating HMG-CoA reductase enzyme (Elson and Qureshi, 1995; Minhajuddin et al., 2005) and by encouraging the degradation of this enzyme (Khor et al., 1995; Parker et al., 1993). Tocotrienols also reduce both apolipoprotein B and lipoprotein A, a component of VLDL (very low density lipoprotein) as high levels of apolipoprotein B increased the risk of premature coronary artery disease (Theriault et al., 1999).

Nevertheless, the property of tocotrienols as potential cholesterol lowering agent varies in different human studies. For example, tocotrienols given to human subjects did not alter the content of cholesterol in the serum (Tomeo et al. 1995). The same findings were published by Mensink et al. (1999); O'Byrne et al. (2000); Rasool et al. (2006, 2008). On the contrary, Ladeia et al. (2008) showed that the amount of serum lipid decreased statistically when a palm oil-rich diet was given to healthy young individuals.

1.3.3(c) Anti-cancer properties:

Tocotrienols were found to have anti-proliferative (Behery et al., 2012) and pro-apoptotic properties (Miyazawa et al., 2009) with the ability to reduce the incidence and recurrence of cancer (Schwenke, 2002; Sylvester and Shah, 2005). For example, women who consume less than 4 mg/ day of tocotrienols through food intake were at risk of developing cancer. This is because the natural female hormone, 17β-estradiol (E2), tends to undergo epoxidation under stressed conditions and thus, activating many of the common chemical carcinogens. Tocotrienols were found to reduce the activation of E2-eposide (Yu et al., 2005). Meanwhile, Srivastava and Gupta (2006) demonstrated that supplementation of tocotrienols induced cell cycle arrest and

apoptosis selectivity in human prostate cancer cells. Other studies of tocotrienols as promising anticancer drugs include research on breast cancer cells (Guthrie et al., 1997; Pierpaoli et al., 2010).

1.3.3(d) Diabetes and hypertension

The endothelium refers to the inner layer of blood vessels with the function to regulate vascular tone and to ensure the flow of blood in the vessels is not distrupted (Calles-Escandon and Cipolla, 2001). An impairment of the function of the endothelium (endothelial dysfunction), may result in the loss of physiological functions such as the ability to promote vasodilatation, fibrinolysis and anti-aggregation due to over production of reactive oxygen species and uncontrolled oxidative stress (Anderson, 2003). This will eventually lead to complications and diseases such as diabetes and hypertension (Balakumar et al., 2009). Experiments conducted by Muharis et al. (2010), showed that tocotrienols restored the endothelial functions in diabetic and hypertensive rats. The findings were also supported by Kuhad and Copra (2009) whereby tocotrienols and not tocopherols were shown to be more effective in preventing biochemical and molecular changes associated with diabetes in diabetic rats. Furthermore, hyperlipidaemic diabetic patients provided with dietary tocotrienols showed a reduction of L DL-C (Baliarsingh et al., 2005).

1.3.4 CURRENT DOSAGE FORM IN THE MARKET

Numerous tocotrienol preparations are available in the current market. Most of these are in oily liquid form, encapsulated by a softgel capsule shell. This type of formulation is popular since the tocotrienols raw materials are readily available in oily solution form.

1.4 DOSAGE FORMS

1.4.1 INTRODUCTION

Dosage forms can be defined as vehicles where the drugs are delivered specifically to the intended site of action in the body (Lachman et al., 1986) and some of these examples are listed out by Rouge et al. (1996). Various methods were employed to deliver these drugs to the specific site. Examples are floating (Whitehead et al., 1998), mucoadhesive (Cilurzo et al., 2010) and expendable gastroretentive (Klausner et al., 2003) dosage forms. Then, there are different types of dosage forms, formulated accordingly to their intended route of administration, for example, oral, topical, rectal, parenteral, vaginal, inhaled, ophtalmic and otic. They can also be categorized according to their respective physical forms, solid, semi-solid or liquid. While solid dosage medications include tablets, soft and hard gels, semi-solid preparations consist of topical preparations such as gels, ointments and cream based products. Liquid dosage forms are syrups and suspensions. Each of these dosage forms has its own advantages as well as disadvantages (Lachman et al., 1986).

1.4.2 ADVANTAGES OF SOLID DOSAGE FORMS

One of the more important advantages offered by solid dosage forms is safety and convenience towards the end users. This formulation benefits consumers with basic medical knowledge as it provides an accurate form of dosing. For example, it is more accurate to swallow a 500 mg dose tablet as compared to taking a liquid medication of which dosage is measured using spoons of different sizes.

Solid dosage forms are popular because of their excellent physical property and shelf life. Unlike liquids or syrups, formulations of solid drugs rarely contain preservatives

like parabens as microorganisms do not trive easily on solid matrix of tablets. From a manufacturer's point of view, it is more convenient to produce solid dosages as they are less bulky and less messy as liquids are not involved. The need for continuous stirring facilities is not required because these solids do not encounter separation problems, hence, lowering the overall manufacturing costs (Lachman et al., 1986; Stoltenberg et al., 2011).

1.5 TOCOMAX® 20% POWDER

Tocomax® 20% powder is a spray-dried powder, manufactured by Carotech® Bhd, Malaysia. It is named as such as it contains 20% (w/w) of mixed tocotrienols and alpha tocopherol, mainly extracted from the palm oil fruits, *Elaeis guineensis*. The remaining contents of the Tocomax® 20% powder include plant squalenes, phytosterols, Co-enzyme Q10 and mixed carotenoids. The product can be best described as an orange-coloured powder with a fine texture. Like most tocotrienols products, the powder is heat and light sensitive. Being extremely hygroscopic, the powder tends to absorb moisture from the environment and clump together, with the tendency to form a sticky and non free-flowing mass. Nevertheless, some of the advertised applications of Tocomax® 20% powder, include dietary nutritional supplements, functional ingredients or as vitamin premixes. This is true despite the fact that there is still a lack of information with no scientific journals being published regarding the bioavailability and tabletting capabilities of the Tocomax® 20% powder.

1.6 SUMMARY AND SCOPE OF STUDY

Although a number of spray-dried powder containing tocotrienols has emerged in the commercial market, most of these products have yet to be assessed for bioavailability. Subsequent issues on the tabletting and compressibility properties of these tocotrienols containing products were questionable as oil may leach out upon compression, giving the tablets an oily and unsightly mottled appearance (Takashima et al., 1999). Moreover, the tablets produced should be subjected to stringent *in vitro* and *in vivo* tests to provide more complete data regarding disintegration time, hardness, friability and stability.

The objectives of this project comprise the following:

(a) To determine the bioavailability of Tocomax® 20% powder using rats

(b) To formulate tablets using Tocomax® 20% powder as the active ingredient

(c) To validate a suitable assay for the formulation and to determine its stability in various storage conditions

(d) To determine the bioavailability of the said formulation using human subjects

CHAPTER 2: *IN VIVO* EVALUATION OF TOCOMAX® 20% POWDER VERSUS TWO DIFFERENT TOCOTRIENOL PREPARATIONS IN RATS

2.1 INTRODUCTION

The development of Tocomax® 20% powder (as described in Section 1.5) illustrates the application of the spray-drying technique for converting an oily drug into a solid. This technique has often been challenged with issues such as: (1) complexity of the process, (2) the expense of more resources such as time, effort and materials to fine tune the process and (3) producing a workable end product. Spray drying often generates unsuccessful results due to incorrect parameters, inadequate drying time which result in a sticky and moist mass and low yield due to thermal degradation of drug (Anwar et al., 2011; Freitas and Muller, 1997; Gharsallaoui, 2007). Thus, the successful commercialization of Tocomax® 20% powder in the local market has also proven that spray-drying an oily drug such as tocotrienols into a powder preparation is possible.

Despite the success of preparing the tocotrienols into a powder form, there is little documentation on the bioavailability of Tocomax® 20% powder. Therefore, the aim of this part of study was to investigate the systemic uptake of tocotrienols in the form of Tocomax® 20% powder in comparison with a normal oily preparation (Tocomin® 50%) and also a commercial self-emulsifying system of the tocotrienols with enhanced oral bioavailability (Tocovid® Suprabio). The study was carried out using Sprague-Dawley rats according to a 3-period, 3-sequence crossover study design.

2.2 MATERIALS

Tocomax® 20% powder was obtained from Carotech Bhd, Malaysia. Tocovid® Suprabio and Tocomin® 50% were both obtained from Hovid Bhd, Malaysia and the content of each type of tocotrienol isomer in the preparations are listed in Table 2.1.

Table 2.1 Contents of each type of tocotrienol isomers in Tocomax® 20% powder (% w/w), Tocovid® Suprabio (% w/v) and Tocomin® 50% (% w/v)

Tocotrienols preparations	Percentage of tocotrienol isomers (%)		
	alpha	gamma	delta
Tocomax® 20% powder (% w/w)	4.8	8.2	2.2
Tocovid® Suprabio (% w/v)	2.6	4.7	1.1
Tocomin® 50% oily formulation (% w/v)	12.3	20.6	5.3

Meanwhile, soya bean oil was also obtained from Hovid Bhd, Malaysia. All the other solvents used were either of analytical or HPLC grades and were purchased from Merck KGaA (Darmstadt, Germany).

2.3 METHODS

2.3.1 PREPARATION OF VARIOUS TOCOTRIENOL PREPARATIONS FOR ADMINISTRATION TO THE RATS

A total of 5 mg of mixed tocotrienols (alpha, gamma and delta) of each preparation was fed to each rat. The actual amount of each isomer for each preparation is shown in the table below (Table 2.2). Meanwhile, the method to prepare each type of tocotrienols preparations is stated in the following sections (2.3.1(a), 2.3.1(b) and 2.3.1(c)).

Table 2.2 The actual amount of tocotrienol isomer in each preparation

Tocotrienols preparations	Amount of tocotrienol isomers (mg)		
	alpha	gamma	delta
Tocomax® 20% powder (w/w)	1.6	2.7	0.7
Tocovid® Suprabio (w/v)	1.5	2.8	0.7
Tocomin® 50% oily formulation (w/v)	1.6	2.7	0.7

The values in Table 2.2 were obtained by multiplying the percentage of the respective isomers from Table 2.1 with the total amount of tocotrienols preparation stated in Section 2.3.1(a), (b) and (c).

2.3.1(a) Preparation of Tocomax® 20% powder

For each gram of Tocomax® 20% powder, there were 152.0 mg of mixed tocotrienols (alpha, gamma and delta tocotrienols). Therefore, to obtain 5.0 mg of mixed tocotrienols, 32.9 mg of Tocomax® 20% powder was weighed carefully into a 1 ml syringe. This preparation contained 1.6 mg of alpha tocotrienol, 2.7 mg of gamma tocotrienol and 0.7 mg of delta tocotrienol.

2.3.1(b) Preparation of Tocovid® Suprabio

Tocovid® Suprabio were in capsules form and to obtain its contents, the capsules were cut open carefully using a penknife. The contents were collected immediately into an amber cream jar and stirred for 15 minutes until a homogeneous mixture was obtained using a hot plate stirrer (502-P, PMC Industries, Inc, San Diego, USA). According to the product label, 600.0 mg (per capsule) of the Tocovid® Suprabio contains 50.0 mg of mixed tocotrienols. Therefore, in order to obtain 5.0 mg of mixed tocotrienols, 60.0 mg of the mixture was weighed carefully into a 1 ml syringe.

This 60.0 mg of Tocovid® Suprabio mixture contained 1.5 mg of alpha tocotrienol, 2.8 mg of gamma tocotrienol and 0.7 mg of delta tocotrienol.

2.3.1(c) Preparation of Tocomin® 50% oily formulation

According to the product label, 100.0 mg of Tocomin® 50% contains 38.2 mg of mixed tocotrienols Therefore, to obtain 5.0 mg of mixed tocotrienols, 13.1 mg of the Tocomin® 50% was required. However, this amount was considered too little to be weighed out and may give rise to weighing errors instead. Thus, 1310.0 mg (100 times higher than the amount required) of Tocomin® 50% was weighed into a beaker. 2690.0 mg of soya oil was then weighed into the same beaker and the mixture was then stirred for 15 minutes until a homogeneous suspension was obtained. The amount of soya oil was added as such in order to make up the final volume of 4000.0 mg. 40.0 mg of the prepared mixture was then weighed carefully into a 1 ml syringe, and this contained 1.6 mg of alpha tocotrienol, 2.7 mg of gamma tocotrienol and 0.7 mg of delta tocotrienol.

2.3.2 *IN VIVO* ABSORPTION STUDIES

The *in vivo* study protocol was reviewed and approved by the Ethics Committee on Animal Studies, Universiti Sains Malaysia. The study was conducted using 6 adult male Sprague Dawley rats, weighing 269.0 to 320 g, (mean = 313.0 g, S.D. = 15.0 g), according to a 3-period, 3-sequence crossover study design with a one week washout period between the phases. The rats were randomly divided into 3 groups of 2 rats each and were administered the tocotrienols preparations according to the sequence shown in Table 2.3.

Table 2.3 Sequence of administration of Tocomax® 20% powder, Tocovid® Suprabio and Tocomin® 50% oily formulation

Group	Sequence of administration		
(2 rats/ group)	Phase 1	Phase 2	Phase 3
1	Tocomax® 20% powder	Tocovid® Suprabio	Tocomin® 50% oily formulation
2	Tocovid® Suprabio	Tocomin® 50% oily formulation	Tocomax® 20% powder
3	Tocomin® 50% oily formulation	Tocomax® 20% powder	Tocovid® Suprabio

The rats were fasted for 12 hours prior to administration of the respective preparations via oral intubation. Food was also withheld the first 4 hours after dosing but the rats had free access to water throughout the study period. For all formulations, the dose of mixed tocotrienols administered was 5 mg per rat. Blood samples of approximately 0.3 ml were collected from the tail vein into heparinised microcentrifuged tubes at 0 (prior to dosing), 1, 2, 3, 4, 6, 8 and 12 hours post administration. The blood samples were then centrifuged for 20 minutes at 12800 g. Subsequently, 0.2 ml aliquot of plasma from each blood sample was then transferred into new microcentrifuge tube. All plasma samples were kept at -20^0C until analysis.

2.3.3 ANALYSIS OF ALPHA, GAMMA AND DELTA TOCOTRIENOLS

2.3.3(a) Instrumentation

Plasma levels of alpha, gamma and delta tocotrienols were determined using a high performance liquid chromatographic (HPLC) method developed and validated by Yap et al. (1999). The HPLC system comprised of a Waters 600E Multisolvent Delivery System (Maple Street Milford, USA), Waters 2475 Multi λ Fluorescence

Detector (Maple Street Milford, USA), a Waters 717 Plus Autosampler (Maple Street Milford, USA), fitted with a 50 µl sample loop (Rheodyne, California) and a data acquisition and analysis software, Waters Empower^{TM} 2 Data Software (Maple Street Milford, USA). A Genesis C_{18} (250 x 4.6 mm id, 4µ) (Grace Davison Discovery Sciences, Illinois, USA) analytical column, fitted with a refillable guard column (2mm x 2cm) (Upchurch Scientific, Oak Harbour, WA, USA), packed with Perisorb RP-18 (30 – 40 µm, pellicular, Upchurch Scientific, Oak Harbour, WA, USA), was used for the chromatographic separation. The mobile phase used was pure methanol. The system was operated at ambient room temperature (25^0C) with the detector operating at an excitation wavelength of 296 nm and emission wavelength of 330 nm while the sensitivity was set at 2000 EUFS. Analyses were run at a flow rate of 1.5 ml/minute and the samples were quantified using peak area.

2.3.3(b) Sample preparation

Prior to analysis, a 100 µl aliquot of sample was accurately measured into a microcentrifuge tube (Eppendorf, Hamburg, Germany) and was deproteinized by adding in 200 µl of acetonitrile: tetrahydrofuran (3:2, v/v). The mixture was then vortex-mixed for 2.5 minutes using a vortex mixer (Barnstead Thermolyne MaxiMix Vortex Mixer, USA) and then centrifuged (MiniSpin Plus, Eppendorf, Germany) at 12800 g for 20 minutes. Approximately 100 µl of the resulting supernatant was injected into the HPLC system.

2.3.4 DATA AND PHARMACOKINETIC ANALYSIS

Corrections were made for all measured values due to the presence of endogeneous peaks in the blank plasma itself. This was carried out by subtracting the tocotrienols concentration determined at 0 hour (prior dosing) from all the measured values obtained. The oral bioavailability of tocotrienols from three different preparations were compared using the pharmacokinetic parameters, area under the plasma concentration-time curve from time zero to the last sampling time, 12 hours after dosing ($AUC_{0\text{-}12h}$), peak plasma concentration (C_{max}) and time to reach peak plasma concentration (T_{max}). While C_{max} and T_{max} values were obtained directly from the plasma concentration values (Weiner, 1981), the $AUC_{0\text{-}12h}$ values were calculated using the trapezoidal formula. The other common parameter used for bioavailability studies, area under plasma concentration-time curve from time zero to infinity ($AUC_{0\text{-}\infty}$), was not taken into consideration as it would require the calculation of area under the plasma concentration-time curve from time t to infinity ($AUC_{t\text{-}\infty}$), which in turn, utilizes the relationship C_{tt}/k_e, whereby, C_{tt} is the plasma concentration at the last sampling point and k_e, the elimination rate constant. Since the amount of plasma samples that could be taken from the rats were rather limited (as compared to human subjects), the estimation of k_e values from the terminal slope of the plasma concentration-time curve is more difficult and may give rise to errors. This is especially true when plasma concentrations are low, where the last few concentrations were near the quantification limit of the assay method or below the detection limit. Also, it should be noted that for all 3 preparations, the C_{tt} values were very low and almost baseline values. Hence, $AUC_{t\text{-}12h}$ is deemed sufficient for comparing the bioavailability of the 3 preparations.

2.3.5 STATISTICAL ANALYSIS

The $AUC_{0\text{-}12h}$ and C_{max} values obtained were analyzed statistically using an analysis of variance (ANOVA) procedure appropriate for a three-way crossover study design that distinguishes effects due to group, subjects/groups, period and treatment (Wagner, 1975). These values were logarithmic transformed prior to statistical analysis. Pairwise comparisons of the three formulations were carried out using the Tukey's test when a statistically significant difference ($p < 0.05$) was detected using the ANOVA procedure. On the other hand, T_{max} values of the three preparations were also analyzed using the Friedman's Test. A statistically significant difference was considered at $p < 0.05$.

The 90% confidence intervals for the ratio of $AUC_{0\text{-}12h}$ and C_{max} values for all three test formulations were calculated using the two one-sided test procedures (Schuirmann, 1987).

2.4 RESULTS

The mean plasma alpha, gamma and delta tocotrienols concentrations versus time profiles obtained of the three different tocotrienols preparations are shown in Figures 2.1(a), 2.1(b) and 2.1(c) respectively. Since the doses of all 3 tocotrienol isoforms in all the 3 preparations were essentially similar (albeit a slight difference between the Tocomax® 20% powder and Tocovid® Suprabio in alpha and gamma tocotrienols), no adjustment or normalization of the doses was carried out. From the plots obtained, it can be seen that the Tocomax® 20% powder had the highest plasma levels for gamma and delta tocotrienols but showed comparable plasma profiles for the alpha tocotrienol isomer as compared to Tocovid® Suprabio. Meanwhile, Tocomin® 50% oily formulation showed the lowest plasma levels for all three different isomers of tocotrienols.

Tables 2.4(a), 2.4(b) and 2.4(c) show the individual numerical values of $AUC_{0\text{-}12h}$, C_{max} and T_{max} estimated from the plasma alpha, gamma and delta tocotrienols concentration versus time profiles of the three preparations respectively. There was a statistical significant difference among the logarithmic transformed $AUC_{0\text{-}12h}$ values ($p < 0.05$) of the three preparations for all three tocotrienol isomers. Further calculations using the Tukey's test showed that the significant difference lies between the $AUC_{0\text{-}12h}$ values of tocotrienols administered as Tocomax® 20% powder and Tocomin® 50% oily formulation as well as that between Tocovid® Suprabio and Tocomin® 50% oily formulation for all three alpha, gamma and delta tocotrienols. However, the Tukey's test also revealed that there were no significant differences for $AUC_{0\text{-}12h}$ values calculated between Tocomax® 20% powder and Tocovid® Suprabio.