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**THE EFFECT OF A FATTY ACID-BASED CARRIER ON THE
BIOAVAILABILITY OF EPIGALLOCATECHIN GALLATE**



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OPSOMMING

Agtergrond: Potensiële gesondheidsvoordele van groentee en groentee katesjien, epigallokatesjiengallaat (EGCG) in mense sluit in voorkoming van kanker, voorkoming van kardiovaskulêre siekte, voorkoming van diabetes mellitus, vermindering van die risiko van die ontwikkeling van hipertensie, voorkoming van vetsug, voorkoming van tandbederf, vermindering van die voorkoms van kognitiewe versteuring en vermindering van oksidatiewe stres. Verskeie studies het vasgestel dat EGCG 'n baie lae biobeskikbaarheid het en daar is dus 'n behoefte om maniere te vind om dit te verhoog. Die hipotese is gestel dat 'n vetsuurgebaseerde draer, Pheroid™, die biobeskikbaarheid van EGCG kan verhoog.

Doel: Die doel van die studie was om die effek van Pheroid™ op die biobeskikbaarheid van EGCG in gesonde vrywilligers te ondersoek.

Studie-ontwerp: 'n Ewekansige, dubbelblinde oorkruisontwerpstudie met 'n 3-dag inlooperperiode en 'n 3-dag uitwasperiode is uitgevoer. Proefpersone is voorsien van 'n flavonoïed/katesjienvry dieet. Twintig (20) proefpersone het 'n enkeldosis van 400 mg EGCG of 400 mg EGCG geïnkorporeer in Pheroid™ op twee afsonderlike geleenthede in ewekansige volgorde ingeneem, en onveranderd EGCG is in die bloed oor 8 ure op verskillende tydsintervalle gemeet.

Opset: Metaboliese Kliniek, Noordwes-Universiteit, Potchefstroom Kampus, Noordwes-Provinsie.

Resultate: Wanneer 'n nie-kompartementele farmakokinetiese analisemodel getoets is vir betekenisvolheid, het die areas onder die plasmakonsentrasiekurwes [$AUC_{(0-480 \text{ min})}$] vir die eerste periode 'n statisties betekenisvolle verskil tussen EGCG geïnkorporeer in Pheroid™ ($50744 \pm 26273 \text{ min} \cdot \text{ng/ml}$) en EGCG ($18106 \pm 13158 \text{ min} \cdot \text{ng/ml}$) getoon met 'n p-waarde van 0.005. Hierdie resultate moet egter versigtig geïnterpreteer word omdat slegs die eerste periode data geanaliseer is, wat dus die voordeel van 'n oorkruisontwerp verbeur. Maksimum konsentrasie vir EGCG plus Pheroid™ ($224 \pm 271 \text{ ng/ml}$) was hoër

as die van EGCG alleen (139 ± 117 ng/ml) en T_{\max} van EGCG plus Pheroid™ (200 ± 107 min) was korter as EGCG alleen (236 ± 65 min), wat suggereer dat daar verhoogde biobeskikbaarheid was, maar sonder statistiese betekenisvolheid. Soortgelyke resultate is verkry as data gegeneer van 'n een-kompartementele farmakokinetiese analisemodel geanaliseer is, wat bevestig dat Pheroid™ nie EGCG biobeskikbaarheid betekenisvol verhoog het nie.

Gevolgtrekking: 'n Dosis van 400 mg EGCG is veilig en word deur gesonde individue goed verdra. In hierdie studie is gevind dat Pheroid™ nie die biobeskikbaarheid van EGCG betekenisvol verhoog het nie.

ABSTRACT

Background: Potential health benefits of green tea and green tea catechin, epigallocatechin gallate (EGCG), in humans include prevention of cancer, prevention of cardiovascular disease, prevention of diabetes mellitus, reduction of the risk of hypertension development, prevention of obesity, prevention of dental caries, lowering of the prevalence of cognitive impairment and reduction in oxidative stress. Various studies have established that EGCG has very low bioavailability and thus there is a need to establish ways of increasing this. It is hypothesised that a fatty acid-based carrier, Pheroid™ could enhance the bioavailability of EGCG.

Aim: The aim the study is to investigate the effect of Pheroid™ on the bioavailability of EGCG in healthy volunteers.

Study design: A randomised, double-blinded crossover design study with a 3-day run-in period and a 3-day washout period was conducted. Subjects were provided with a low flavonoid/catechin free diet. Twenty (20) subjects ingested a single dose of 400 mg EGCG or 400 mg EGCG entrapped in Pheroid™ on two separate occasions in random order, and unchanged EGCG was measured in the blood over 8 hours at different time points.

Setting: Metabolic Clinic, North-West University, Potchefstroom Campus, North-West Province.

Results: When a non-compartmental pharmacokinetic analysis data was tested for significance, the areas under the plasma concentration curves [AUC_(0-480 min)] for the first period showed a statistically significant difference between EGCG incorporated in Pheroid™ (50744±26273 min*ng/ml) and EGCG (18106±13158 min*ng/ml) with a p value of 0.005. These results should however be treated with caution as only first period data was analysed, therefore, losing the advantages of a crossover design. Maximum concentration for EGCG plus Pheroid™ (224±271 ng/ml) was higher than that of EGCG alone (139±117 ng/ml) and T_{max} of EGCG plus Pheroid™ (200±107 min) was shorter

than the EGCG alone (236 ± 65 min), suggesting that there was an increased bioavailability, however with no statistical significance. Similar results were obtained when data generated from a one-compartmental pharmacokinetic analysis model was analysed, confirming that Pheroid™ did not increase EGCG bioavailability significantly.

Conclusion: A dosage of 400 mg EGCG is safe and well tolerated by healthy individuals. In this study, it was found that Pheroid™ did not increase bioavailability of EGCG significantly.

ABBREVIATIONS

3'-MeEC	3'- <i>O</i> -methyl(-)-epicatechin
4'-MeEGC	4'- <i>O</i> -methyl(-)-epigallocatechin
4''-MeEGCG	4''- <i>O</i> -methyl(-)-epigallocatechin gallate
4',4''-DiMeEGCG	4',4''-dimethyl(-)-epigallocatechin gallate
AAPH	2'-azobis(2-amidinopropane) hydrochloride
Ab	Total amount of drug in the body
ANOVA	Analysis of variance
AUC	Area under the curve
BHA	Butylated hydroxyanisole
BioMUST	<u>B</u> ioavailability of <u>M</u> icronutrients <u>U</u> sing <u>S</u> table <u>I</u> sotope <u>T</u> echnology
BHT	Butylated hydroxytoluene
BT	Black tea
C	(-)-Catechin or catechin
CG	Catechin gallate
CL	Clearance
CL/F	Total oral clearance
C _{max}	Maximum plasma concentration
COMT	Catechol- <i>O</i> -methyltransferase
C _p	Plasma concentration of drug
CVD	Cardiovascular diseases
DGT	Decaffeinated green tea
DHA	Docosahexaenoic acid
DM	Diabetes mellitus
EC	Epicatechin
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EPA	Eicosapentaenoic

F	Absolute bioavailability
FDA	Food and Drug Administration
FRAP	Ferric-reducing antioxidant power
GC	Gallocatechin
GCG	Gallocatechin gallate
GC-MS	Gas chromatography mass spectrometry
GI	Gastrointestinal
GT	Green tea
GTC	Green tea catechins
GTE	Green tea extract
GTS	Green tea supplement
HPLC	High performance liquid chromatography
HPLC-MSMS	High performance liquid chromatography-Mass Spectrometry/ Mass Spectrometry
IHD	Ischaemic heart disease
<i>i.v.</i>	Intravenous
K ₀₁	Rate of absorption
K ₁₀	Rate of elimination
LC-MSMS	Liquid chromatography-Mass Spectrometry/Mass Spectrometry
M4	(-)-5(3',4',5',-trihydroxyphenyl)- γ -valerolactone
M6	(-)-5(3',4'-dihydroxyphenyl)- γ -valerolactone
M6'	(-)-5(3',5'-dihydroxyphenyl)- γ -valerolactone
MRM	Multiple reaction monitoring
MW	Molecular weight
NaH ₂ PO ₄	Sodium dihydrogen orthophosphate
NCD	Non-communicable diseases
N	Nitrogen
ORAC	Oxygen radical absorbance capacity
O	Oxygen
<i>p.o.</i>	Per orally (oral administration)
PUFA	Polyunsaturated fatty acid

QC	quality control
R-PE	R-phycoerythrin
SAM	S-adenosylmethionine
$t_{1/2,abs}$	Absorption half life time
$t_{1/2,e}$	Elimination half-life time
TEAC	Trolox equivalent antioxidant capacity
T_{lag}	Lag time
T_{max}	Time taken to reach maximum plasma concentration
TRAP	Total radical-trapping antioxidant potential
V_d	Apparent (hypothetical) volume of distribution
V_d/F	Oral apparent volume of distribution
YLLS	Years of life lost

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1 CHAPTER 1: INTRODUCTION

1.1 BACKGROUND AND MOTIVATION

Globally, non-communicable diseases (NCD) such as cardiovascular diseases (CVD), cancer and diabetes mellitus are on the rise. In 2001, NCD accounted for almost 60% of the 56 million deaths annually and 47% of the global burden of disease (World Health Assembly, 2004). Projections of mortality viewed in terms of years of life lost (YLLS) due to premature death, show that in 1990 ischaemic heart disease (IHD) was the fourth leading cause of death and it is projected that in 2020 it will be the number one leading cause of death in both developing and developed countries (Murray & Lopez, 1996). Therefore, there is a need to explore ways of reducing and/or preventing NCD, in addition to modification of lifestyle-related risk factors such as an unhealthy diet, physical inactivity and tobacco use.

Catechins in green, black and oolong teas could play an important role in the prevention of NCDs (Liao *et al.*, 2001). Green tea (*Camellia sinensis* Theaceae) and green tea catechin, epigallocatechin gallate (EGCG), have been found to have potential health benefits in humans (Dulloo *et al.*, 1999; Leenen *et al.*, 2000; Pisters *et al.*, 2001; Hakim *et al.*, 2003; Yang *et al.*, 2004b; Nagao, *et al.*, 2005; Henning *et al.*, 2006; Hirasawa *et al.*, 2006; Kiruyama *et al.*, 2006; Widlansky *et al.*, 2007).

Epigallocatechin gallate is the most abundant catechin in green tea (Hidgon & Frei, 2003; Liao *et al.*, 2001) and is reported to have significant antioxidant and biological activities (Lu, *et al.*, 2003). After consumption, a substantial amount (77-92%) of epigallocatechin gallate in plasma is in the free form (unconjugated) (Chow *et al.*, 2003; Lee *et al.*, 2002). Other green tea catechins include catechin (C), epicatechin (EC), gallic acid (GA), epigallocatechin (EGC), epicatechin gallate (ECG) and gallic acid gallate (GCG) (Liao *et al.*, 2001), and occur highly conjugated with glucuronic acid and/or sulphate groups in plasma (Manach *et al.*, 2005).

Catechins are preventive antioxidants because they scavenge reactive oxygen species, terminate free radical chain reactions, and chelate transition metals involved in free radical generation (Pietta *et al.*, 2000). According to their one-electron reduction potentials, these catechins quench superoxide anion, peroxy, hydroxyl and alkoxy species producing more stable (less energetic) radicals (Pietta *et al.*, 2000). However, as reflected by a review published by Hidgon & Frei, (2003), the general antioxidative functions of tea catechins in plasma and other tissues following tea ingestion are not strong and sometimes are not significant. This may be attributed to the low bioavailability of tea catechins (Yang *et al.*, 2004a). Manach *et al.*, (2005) in a review assessing 97 bioavailability studies, concluded that gallates or galloylated tea catechins are one of the least absorbed polyphenols.

The health benefits of polyphenols amongst others depend on the amount consumed and on their bioavailability (Manach *et al.*, 2004). Catechins, especially gallated catechins, are very difficult to absorb into the systemic circulation from the gut and when absorbed are rapidly metabolised (Liao *et al.*, 2001). The bioavailability of green tea catechins depends on several factors, including catechin structure, purity, and dosage (Liao, *et al.*, 2001). These factors will be elaborated in chapter 2. Since bioavailability of tea catechins is low and their potential health benefits are high, there is a need to investigate whether bioavailability of catechin supplements can be improved on. One way of increasing bioavailability of green tea catechins could be the use of delivery systems. Different types of delivery systems exist; these include microsphere hydrogels, microparticles, emulsions/microemulsions, liposomes, micelles, nanoparticles and nanocrystals. Delivery systems are primarily used to modify compounds or drug molecules with low aqueous solubility.

The Pheroid™, a patented fatty acid-based carrier system has been shown to enhance the absorption of dermatological formulations (Goodfield *et al.*, 2003; Tzaneva *et al.*, 2003). This carrier system contains only components that have been recognised as pharmaceutically safe (Department of Health, 2004; FDA, 2005). The Pheroid™ is comprised of a mixture of six fatty acids; linoleic acid, oleic acid, hexadecanoic acid, stearic acid as well as trace amounts of docosahexaenoic acid (DHA) and eicosapentaenoic (EPA). It has been established that drugs carried in the Pheroid™

increase delivery of active compounds, decrease time to onset of action, reduce the minimal effective concentration, and increase therapeutic efficacy (North-West University, 2004).

The effects of Pheroid™ used as a carrier molecule of EGCG will be investigated in a randomized double-blinded cross-over study. This study will explore whether the Pheroid™ will have similar effects on orally administered EGCG, which potentially plays an important role in human health, in order to find out whether the bioavailability of EGCG will be increased or not. Hence, this study will look at the effect of the fatty acid-based carrier on the bioavailability of EGCG.

1.1.1 Aims, objectives and hypothesis

1.1.1.1 Aim

To investigate the effect of a fatty acid-based carrier on the bioavailability of EGCG in healthy volunteers.

1.1.1.2 Objective

- To estimate pharmacokinetic parameters after the control and intervention treatments and use these to determine whether Pheroid® can increase the bioavailability of EGCG.

1.1.1.3 Hypothesis

It is hypothesised that fatty acid-based carrier system will increase the bioavailability of EGCG.

1.2 OUTLINE OF THE MINI-DISSERTATION

Chapter 1 entails background information, aim, objectives and hypothesis of the study. Chapter 2 gives literature review of bioavailability of green tea catechins. Chapter 3 focuses on methods used to determine the effects of Pheroid™ on the bioavailability of epigallocatechin gallate. The findings of study will be reported in chapter 4, while discussion, conclusion and recommendations drawn from the results of the study will be in chapter 5. Bibliography will be cited at the end of all the five

(5) chapters outlined according to the guidelines of Quoting Sources of the North-West University (formerly known as the Potchefstroom University for Christian Higher Education) (van der Walt, 2004).

2 CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

This chapter will review the following: 1) bioavailability of drugs in general and the factors influencing this; 2) bioavailability of green tea catechins; 3) factors influencing oral administration of green tea; 4) catechin pharmacokinetics; and 5) delivery systems that may enhance bioavailability of drugs and/or other compounds, in particular, lipid-based delivery systems. A brief summary on the composition and mechanism of action of a patented fatty-acid carrier molecule, Pheroid™ which was used in the randomised clinical trial as a delivery system, will also be addressed.

Oral bioavailability, that is, absolute bioavailability of green tea catechins has been found to be very low in rodents, but has not been established in humans due to lack of pharmacokinetic studies following intravenous administration (Cai *et al.*, 2002). For a nutrient to be effective after consumption it needs to be bioavailable to the body. It is not enough only to know how much of a nutrient is present in a dietary supplement, but how much of that which is ingested is bioavailable (Srinivasan, 2001).

A higher bioavailability is commonly regarded as favourable, that is, often a food source with higher bioavailability or a nutritional supplement with a better delivery is promoted as superior (Solomons & Slavin, 2001). However, caution has to be taken as high bioavailability of nutrients may cause nutritional and/or other problems (Yang & Landau, 2000). Higher bioavailability may push the limits of safety. Scientific evidence is emerging that higher doses of epigallocatechin gallate, a green tea catechin, may be deleterious to health, at least *in vitro* and in animal models. Schmidt *et al.*, (2005) in an *in vitro* study concluded that high concentrations of green tea extract, containing high amounts of epigallocatechin gallate (EGCG), exerted acute toxicity in rat liver cells. Yun *et al.*, (2006) concluded that EGCG further impaired the beta-cell response to high glucose in diabetic rats and exacerbated the loss of islet cell mass and insulin-immunoreactivity in beta cells. Islam & Choi *et al.*, (2007), in a

dose response study found that lower dose of green tea (Green Tea Low, 0.5%) increases serum insulin concentration whereas higher dose (Green Tea High, 2.0%) increases serum fasting blood glucose in rats. This suggests that at lower doses EGCG can act as an antioxidant, but at higher doses it can act as a prooxidant. Safety studies done using TEAVIGO™, a high-concentration EGCG extract, indicate 1) that this catechin is not genotoxic (Isbrucker *et al.*, 2006a), 2) high dose of EGCG (2000 mg orally) was lethal to rats whereas no adverse effects were observed when a low dose of EGCG (500 mg/kg/day) was administered (Isbrucker *et al.*, 2006b), and 3) this same pattern was observed in a two-generation study in rats with the highest dose reducing growth rate of offspring and a slight increase in pup loss and the lowest dose was considered safe (Isbrucker *et al.*, 2006c). These studies further demonstrate that caution has to be taken not to give a high dose of EGCG as it may be deleterious to health, at least in animal models.

Bioavailability evaluation of nutritional supplements may be different from that of drugs; however, flavonoids bioavailability is measured using a similar approach as to that used for pharmacokinetic drug trials. For this reason, bioavailability of drugs and/or other compounds in general will be covered in the following section.

2.2 BIOAVAILABILITY

2.2.1 Definition of bioavailability

Bioavailability can be defined as the rate and extent (amount) to which a bioactive drug or compound reaches the systemic circulation and is available at the site of action (Shargel & Yu, 1999). Bioavailability is considered to be one of the most critical issues for any drug developed for extravascular administration, that is, oral, rectal, transdermal and subcutaneous (Kwon, 2001). It is usually expressed as a fraction or a percent. There are two types of bioavailability, these are, absolute bioavailability and relative (apparent) bioavailability. Shargel & Yu (1999) defines absolute bioavailability as the measure of availability of the active drug in the systemic circulation after non-intravenous or extravascular administration compared to that after intravenous (i.v.) administration; and relative bioavailability as the availability of the drug from a drug product as compared to a recognized standard.

The absolute bioavailability or oral bioavailability of a drug or compound is generally determined using a pharmacokinetic study to obtain a *plasma drug concentration versus time* plot for the drug or compound after both intravenous and extravascular administration (Anon, 2005). The absolute bioavailability is the dose-corrected area under the curve (AUC) extravascular divided by AUC intravenous:

$$\text{Absolute bioavailability (F)} = \frac{[\text{AUC/Dose}]_{\text{p.o.}}}{[\text{AUC/Dose}]_{\text{i.v.}}} \quad (3)$$

OR

$$F = \frac{\text{AUC}_{\text{p.o.}} * \text{Dose}_{\text{i.v.}}}{\text{AUC}_{\text{i.v.}} * \text{Dose}_{\text{p.o.}}}$$

where i.v. represents intravenous administration and p.o. oral administration (Shargel & Yu, 1999; Lambert *et al.*, 2003). A drug or compound given by the intravenous route has an absolute bioavailability of 1 (F=1), and those given by other routes usually have an absolute bioavailability of less than 1, that is, bioavailability of a drug or compound administered intravenously is 100% and via other routes is less (Shargel & Yu, 1999) due to a number of factors that will be discussed later in this section. When F is 1, it is known as unity.

For the relative bioavailability, it is difficult to ascertain a fraction of a dose systematically available from an oral drug product. The availability of a drug in the formulation is compared to the availability of a drug in a standard dosage formulation, usually a solution of the pure drug evaluated in a crossover study. The relative bioavailability of two drugs given at the same dosage level and by the same route of administration can be obtained using the following equation:

$$\text{Relative bioavailability} = \frac{[\text{AUC}]_{\text{A}}}{[\text{AUC}]_{\text{B}}} \quad (2a)$$

This fraction can be multiplied by 100 to obtain *percent* relative bioavailability (Shargel & Yu, 1999).

If different dosages are administered, then the dose-corrected AUC for drug product A is divided by AUC for drug product B:

$$\text{Relative bioavailability} = \frac{[\text{AUC}]_A / \text{Dose}_A}{[\text{AUC}]_B / \text{Dose}_B} \quad (2b)$$

where drug product B is the recognised reference standard (Shargel & Yu, 1999).

The bioavailability of a dietary component is determined by four processes, namely, absorption (A) from the small intestine or colon, metabolism (B) in the body, distribution (C) in the body, and excretion (D) from the body (**Figure 1**).

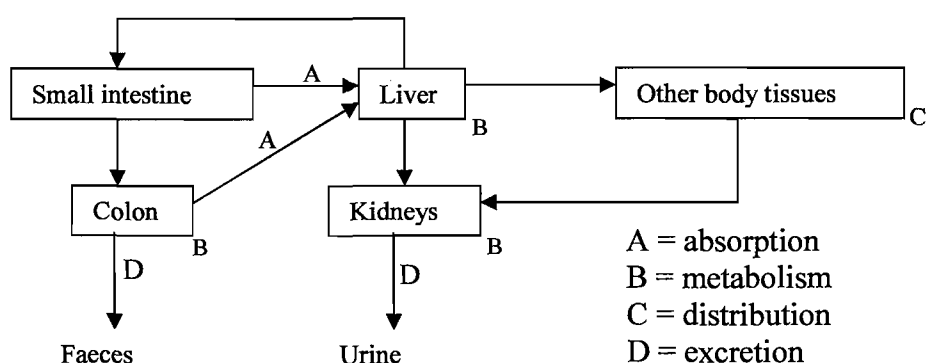


Figure 1: Schematic overview of the main bioavailability processes (Adapted from Olthof, 2001).

The schematic overview in **Figure 1** illustrates bioavailability processes which occur after an ingestion of a dietary component. Absorption takes place in the intestinal mucosa (small intestines and colon) and finally the dietary component is excreted into faeces. Metabolism, distribution and excretion processes determine the type of circulating metabolites, the sites that these metabolites can reach, and the period of time that they are available to target tissues (Olthof, 2001). Metabolism of a dietary component in humans mainly takes place in the colon and in the liver. However, kidneys also play an important role in metabolism because metabolites of the dietary component are finally removed from the body through urinary excretion (Olthof *et al.*, 2003).

2.2.2 Pharmacokinetic parameters used to determine bioavailability

The measurement of drug concentrations in blood, plasma or serum after drug administration is the most direct and objective way to determine drug or compound bioavailability (Shargel & Yu, 1999). Other methods include urinary drug excretion, acute pharmacodynamic effect, clinical observations and *in vitro* studies. The following are pharmacokinetic parameters that are used to determine bioavailability when the plasma drug concentration method is used.

2.2.2.1 Area under the curve

The area under the plasma drug concentration-time curve is used as a measure of the total amount of unaltered drug that reaches the systemic circulation. It is independent of the route of administration and processes of drug elimination as long as the elimination processes do not change and it is generally directly proportional to the dose given (Shargel & Yu, 1999). The units of AUC are concentration time (for example, hr* $\mu\text{g/ml}$, hr* $\mu\text{mol/ml}$, min* $\mu\text{g/ml}$, min*ng/ml, min* $\mu\text{mol/ml}$).

2.2.2.2 Peak plasma concentration

The peak plasma drug concentration (C_{max}) represents the maximum plasma drug concentration obtained after oral administration of a drug, and it provides a warning of possible toxic levels of a drug (Shargel & Yu, 1999). The units of C_{max} are concentration units (for example, $\mu\text{g/ml}$, $\mu\text{mol/ml}$, ng/ml, and nmol/ml)

2.2.2.3 Time of peak plasma concentration

The time of peak plasma concentration, T_{max} , corresponds to the time required to reach maximum drug concentration after drug administration (Shargel & Yu, 1999). The units of T_{max} are units of time (for example, hours, and minutes).

2.2.2.4 Elimination half-life

The elimination half-life ($t_{1/2,e}$) of a drug is the period of time over which its concentration in plasma decreases by half from a reference concentration at any given time (Kwon, 2001). There are several ways of estimating half-life of a drug from its plasma drug concentration-time curve, and these are:

- Visual inspection of the plasma drug concentration-time curve by eyeballing a time interval over which the concentration decreases by half from any reference time point
- Estimating $t_{1/2}$ between two drug concentrations (C_1 and C_2) at two different time points (t_1 and t_2):

$$t_{1/2} = \frac{(0.693)(t_2 - t_1)}{\ln(C_1/C_2)} \quad (3)$$

Units of elimination half life time are minutes or hours.

2.2.2.5 Volume of distribution

The volume of distribution for a drug or the apparent volume (V_d) in which the drug is dissolved, represents a volume that must be considered in estimating the amount of the drug in the body from the concentration of the drug found in the sampling compartment (Shargel & Yu, 1999). It is expressed as:

$$V_d = \frac{Ab}{C_p} \quad (4)$$

where Ab is the total amount of the drug in the body and C_p is the plasma concentration of the drug. The volume of distribution which is larger than the plasma compartment (3L) indicates that the drug is also present in tissues or fluids outside that compartment (Winter, 1994). Hypothetical volume of distribution is presented as V_d or V_F and its units are litres or millilitres.

2.2.2.6 Clearance

Clearance (CL) is the intrinsic ability of the body or its elimination organs (usually kidneys and liver) to remove a drug from the blood or plasma. It represents the theoretical volume of the blood or plasma, which is completely cleared of drug in a given period of time (Winter, 1994). Clearance is presented as CL or CL_F and its units are litres per minute or millimetres/minute.

2.2.3 Factors influencing oral bioavailability

There are factors which reduce the availability of a substance or compound prior to it entering systemic circulation. Such factors include physiological, physicochemical properties of drug molecules and dosage form factors.

2.2.3.1 Physiological factors

2.2.3.1.1 pH in the gastrointestinal tract

The pH in the gastrointestinal (GI) tract is one of the physiological factors which affect the gastrointestinal absorption of drugs. The pH of the stomach ranges from 1 to 3.5 in healthy people in a fasted state, then after ingestion of a meal the gastric juice is buffered to a less acidic pH, usually 3 to 7 which returns to the lower fasted-state values within 2-3 hours depending on the meal size (Ashford, 2002a). Intestinal pH values, from the duodenum to the ileum, ranges from 5 to 8 (Kwon, 2001). These higher pH values are due to neutralisation of the gastric acid with bicarbonate ions secreted by the pancreas into the small intestine. In the colon, the pH drops to about 6.5 due to break down of undigested carbohydrates into short-chain fatty acids by bacterial enzymes (Ashford, 2002a).

The intestinal pH may influence the chemical stability of the drug in the lumen, resulting in chemical degradation due to pH-dependent hydrolysis. This instability may result in a reduced bioavailability.

2.2.3.1.2 Influence of food in the gastrointestinal tract

The presence of food in the GI tract can influence bioavailability directly or indirectly via a number of mechanisms. This may be through the formation of insoluble complexes with food components; alteration of pH (as already mentioned); alteration of gastric emptying and GI motility (fats and fatty acids in the diet delays gastric emptying and thus delay the onset of action of certain drugs); stimulation of gastrointestinal secretions (can lead to degradation of drugs that are susceptible to enzymatic metabolism, hence reduction in bioavailability); competition between food components and drugs for specialised absorption mechanisms; increased viscosity of GI contents; and food induced changes in the blood flow (blood flow to the GI tract and liver increases shortly after a meal, increasing the rate at which drugs are presented to the liver) (Ashford, 2002a).

2.2.3.1.3 Intestinal microflora

Microflora residing in the GI tract can metabolise a variety of drugs, which can reduce the amount available for absorption (Kwon, 2001).

2.2.3.1.4 First-pass metabolism

Drugs administered orally undergo first pass or presystemic metabolism by the gut wall and/or hepatic enzymes, namely, cytochrome P450 enzymes, before reaching the systemic circulation (Washington *et al.*, 2001; Ashford, 2002a). This “first-pass effect” can substantially decrease the amount of an active drug reaching the systemic circulation, thus, reducing its bioavailability (Winter, 1994; Ashford, 2002a).

2.2.3.2 Physicochemical properties of drugs

2.2.3.2.1 Solubility

The gastrointestinal epithelia act as a lipid barrier on drugs which are absorbed by passive diffusion, and those that are lipid soluble will pass across the barrier more easily. As most drugs are weak electrolytes, the un-ionised form of weakly acidic or basic drugs (the lipid-soluble form) will pass across the gastrointestinal epithelia, whereas the gastrointestinal epithelia is impermeable to the ionised (poorly lipid-soluble) form of such drugs (Ashford, 2002b), thus, the un-ionised forms of drugs, if sufficiently lipophilic, are better absorbed as compared to their ionised counterparts.

2.2.3.2.2 Chemical structure

The number of hydrogen bonds within a molecule and molecular size can also influence the permeability of a drug across the gastrointestinal membrane, hence its overall bioavailability. This may be explained by Lipinski’s ‘rule of 5’ which states that poor absorption and permeation are more likely when two of the following parameters are out of range: a compound containing 5 or more hydrogen-bond donors (expressed as the sum of OH and NH groups); having a molecular weight (MW) of over 500; and 10 or more hydrogen-bond acceptors [expressed as the sum of nitrogens (N) and oxygens (O)] (Lipinski *et al.*, 1997). Too many hydrogen bonds within a molecule may hinder absorption. In general, not more than 10 hydrogen bond acceptors should be present if the molecule is to be absorbed well (Ashford, 2002b).

2.2.3.2.3 Dosage form

The type of dosage form (solution, suspension, solid) and its method of preparation or manufacture can influence its rate and/or extent of absorption from the GI tract. Bioavailability of a given drug tends to decrease in the following order of types of dosage forms: aqueous solutions > aqueous suspension > solid dosage forms (e.g. hard gelatine capsules or tablets) (Ashford, 2002b).

These are some of the factors that affect or influence bioavailability of drugs, and will help understand bioavailability of green tea catechins (GTC).

2.3 BIOAVAILABILITY OF GREEN TEA CATECHINS

Potential health benefits of green tea and green tea catechin, epigallocatechin gallate (EGCG), in humans include prevention of cancer (Pisters *et al.*, 2001; Henning *et al.*, 2006); prevention of cardiovascular disease (Widlansky *et al.*, 2007); prevention of diabetes mellitus (Kao *et al.*, 2006); reduction in the risk of hypertension development (Yang *et al.*, 2004); prevention of obesity (Dulloo *et al.*, 1999; Nagao, *et al.*, 2005; Kao *et al.*, 2006); prevention of dental caries (Hirasawa *et al.*, 2006); lowering prevalence of cognitive impairment (Kiruyama *et al.*, 2006); and reduction of oxidative stress (Leenen *et al.*, 2000; Hakim *et al.*, 2003).

It is important to consider the bioavailability of green tea catechins in order to get a comprehensive understanding of their biological properties, such as antioxidant activity and possible impact on human health. Green tea catechins have demonstrated that they have antioxidant properties *in vitro* and *in vivo*. Antioxidant activity is considered one of the primary functions of flavonoids such as green tea catechins, as they appear to be potent free radical scavengers. Free radicals have been found to cause oxidative damage. Oxidative damage is associated with and may contribute to the development of lifestyle diseases like cardiovascular diseases, stroke, type 2 diabetes mellitus and other age-related diseases. The antioxidant properties of green tea catechins are demonstrated in published research to impact on these diseases. The antioxidant activity of green tea catechins will be looked into later in this section.

Other biological properties of green tea catechins include anti-carcinogenic, anti-inflammatory and anti-microbial properties.

This review is limited to green tea catechins as there is enough published evidence on bioavailability of catechins in this type of tea. Focusing on green tea catechins will help compare the bioavailability of EGCG with other catechins in green tea. Catechins, especially gallated catechins, are very difficult to absorb into the systemic circulation from the gut, when absorbed, they are rapidly metabolised (Liao *et al.*, 2001). It is important to realise that the polyphenols that are most common in the diet are not necessarily the most active in the body, either because they have a low intrinsic activity or because they are poorly absorbed in the intestine, highly metabolised, or rapidly excreted largely within 24 hours (Liao *et al.*, 2001; Manach *et al.*, 2004). Direct evidence of the bioavailability of catechins has been obtained by measuring their concentrations in plasma and urine after ingestion of catechin-rich foods with known content, supplements of the pure catechin of interest or green tea extracts with a mixture of catechins (Scalbert & Williamson, 2000).

2.3.1 Distribution of catechins in foods

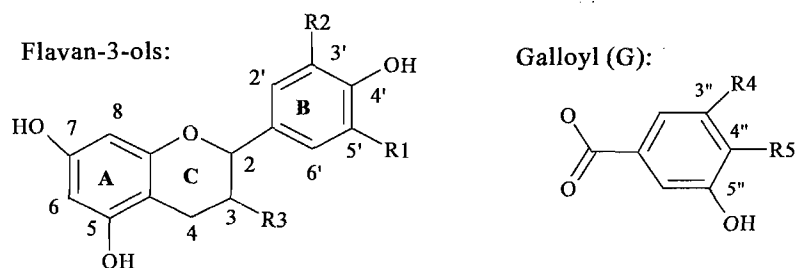
Catechins are most commonly found in most fruits [e.g. black grapes and their products (wine and juices), blackberries, cherries, apricots and some apple varieties]; some legumes (broad beans and faba beans); teas (black, green and oolong); chocolates (dark and milk chocolate) and cocoa (Arts *et al.*, 2000a). Catechin (C) and epicatechin (EC) are mainly found in fruits and chocolate (Arts *et al.*, 2000a). Epicatechin gallate (ECG), gallic catechin (GC), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) are found in some legumes, black grapes and tea, tea being the only reported beverage containing EGCG (Arts *et al.*, 2000b).

2.3.2 Chemical structure of green tea catechins

Flavan-3-ols or catechins are a subclass of flavonoids. Other subclasses of flavonoids are flavonols, flavones, isoflavones, flavanones and anthocyanidins. Flavanols are classified as C15 compounds and their derivatives possess two aromatic rings (A and B) connected by three carbon units (C-2, C-3 and C-4). The flavanol structure of

catechins (3, 3', 4', 5, 7-pentahydroxyflavan) contains two asymmetric carbon atoms at C-2 and C-3 (Liao *et al.*, 2001; Demeule *et al.*, 2002), as shown in **Figure 2**.

A) General structure



B) Tea catechins

Compounds	C-2	R ₁	R ₂	R ₃	R ₄	R ₅
1) Major catechins						
Epicatechin (EC)	R	H	OH	OH	---	---
Epigallocatechin (EGC)	R	OH	OH	OH	---	---
Epicatechin-3- <i>O</i> -gallate (ECG)	R	H	OH	G	OH	OH
Epigallocatechin-3- <i>O</i> -gallate (EGCG)	R	OH	OH	G	OH	OH
2) Epimers at the C-2 position						
Catechin (C)	S	H	OH	OH	---	---
Gallocatechin (GC)	S	OH	OH	OH	---	---
Catechin-3- <i>O</i> -gallate (CG)	S	H	OH	G	OH	OH
Gallocatechin-3- <i>O</i> -gallate (GCG)	S	OH	OH	G	OH	OH
3) Methylated derivatives						
Epigallocatechin-3- <i>O</i> -(3- <i>O</i> -methyl) gallate (EGCG3''Me)	R	OH	OH	G	OCH ₃	OH
Gallocatechin-3- <i>O</i> -(3- <i>O</i> -methyl)gallate (GCG3''Me)	S	OH	OH	G	OCH ₃	OH
Epigallocatechin-3- <i>O</i> -(4- <i>O</i> -methyl)gallate (EGCG4''Me)	R	OH	OH	G	OH	OCH ₃
Epicatechin-3- <i>O</i> -(3- <i>O</i> -methyl)gallate (EGCG3''Me)	R	H	OH	G	OCH ₃	OH

R: Rectus; S: Sinister

Figure 2: (A) General structures of catechins. (B) Major catechins, epimers at the C-2 position and methylated catechin derivatives found in tea. Adapted from Demeule *et al.*, (2002).

The major flavonoids found in green tea are flavan-3-ols, and they make up more than 75% of all the polyphenols found in green tea. The principal flavan-3-ols found in green tea include EGCG (the main constituent of green tea catechins), EC, EGC and ECG. Other flavan-3-ols include four epimers of EGCG, namely, catechin (C), GC, catechin gallate (CG) and gallocatechin gallate (GCG) and four catechin derivatives methylated at the 3'-*O*-position of the gallic acid (gallate) moiety (Demeule *et al.*, 2002) (**Figure 2**).

Figure 2 shows the differences in structures of the family of green tea polyphenols. For example, epicatechin has an *ortho*-dihydroxyl (catechol) group in the B ring at carbons 3' and 4' and a hydroxyl group at carbon 3 on the C-ring; EGC differs from EC in that it has a trihydroxyl group at carbons 3', 4' and 5' on the B ring; ECG differs from EC in that it has a galloyl or gallate moiety esterified at carbon 3 of the C ring; and EGCG has both a trihydroxyl group at carbons 3', 4' and 5' on the B ring and a gallate moiety esterified at carbon 3 of the C ring (Higdon & Frei, 2003). The presence of the hydroxyl groups and the gallate moiety in their chemical structure has been found to influence the antioxidant and/or scavenging activity of green tea catechins (Nanjo *et al.*, 1996).

2.3.3 Antioxidant activity

Radical scavenging activity is a measure of antioxidant capacity. Radical scavenging activity in plasma can be measured using various assays such as total radical-trapping antioxidant potential (TRAP), oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidant capacity (TEAC) and ferric-reducing antioxidant power (FRAP) to assess individual antioxidant status (Collins, 2005). In the TRAP assay, the rate of peroxidation induced by 2'-azobis(2-amidinopropane) hydrochloride (AAPH) is monitored through the loss of fluorescence of the protein R-phycoerythrin (R-PE); and the lag-phase is compared to that induced by Trolox (a water soluble analogue of vitamin E) in the same plasma sample. In the ORAC assay, the same principle as in the TRAP is applied, and it is based on the ability of a test substance to inhibit the oxidation of B-phycoerythrin by reactive oxygen species, relative to Trolox. The TEAC assay is based on the ability of molecules to scavenge the stable free radical of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) in comparison with Trolox. The FRAP assay is a colorimetric assay that measures the ability of plasma to reduce the intense blue ferric tripyridyltriazine complex to its ferrous form, thereby changing the absorbance (Rietveld & Wiseman, 2003).

The abovementioned assays may be used to test the antioxidant and/or scavenging activity of tea catechins. Gallated catechins (EGCG and GCG) have a stronger radical scavenging activity than non-gallated catechins (EGC, GC, EC, and C). The mechanism of action of flavonols as radical scavengers involves the transfer of an electron followed by deprotonation (**Figure 3**) to stabilise the radical species (Rice-Evans, 1996).

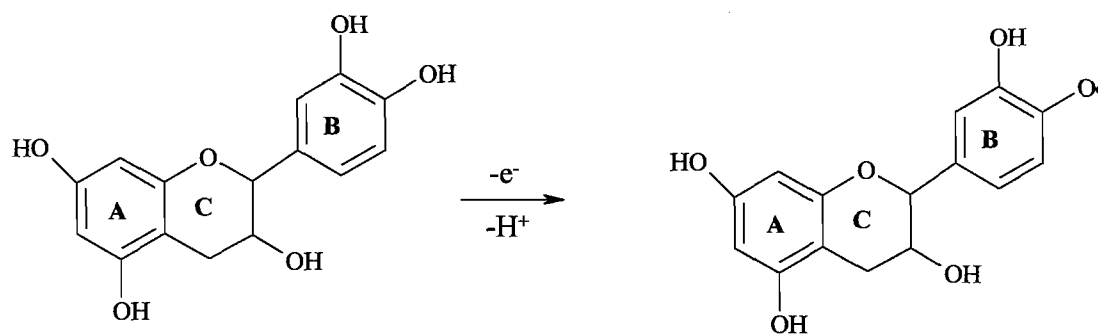


Figure 3: Formation of catechin phenoxyl radical by one electron oxidation [Adapted from Valcic *et al.*, (1999)]

The antioxidant and radical scavenging activities of catechins are directly related to the combination of aromatic rings and hydroxyl groups that make up their structure and these include *ortho*-3',4'-dihydroxyl group or 3',4',5'-trihydroxyl group in the B ring, a gallate moiety esterified at carbon 3 of the C ring, and the hydroxyl groups at carbon 5 and 7 positions of the A ring (Rice-Evans, 1996; Frei & Higdon, 2003). Nanjo *et al.*, (1999) have found that the presence of an *ortho*-3',4'-dihydroxyl group in the B ring and a gallate moiety at carbon 3 of the C ring may be important in scavenging the superoxide anion and the hydroxyl radicals respectively.

Epigallocatechin gallate has the highest antioxidant activity as compared to the other green tea catechins, that is, EGCG>EGC>ECG>EC (Nanjo *et al.*, 1996). Valcic *et al.*, (1999) have suggested that like other phenolic antioxidants, EGCG may act as a chain-breaking antioxidant which traps peroxy radicals and thus suppresses radical chain autoxidation. Kondo *et al.*, (1999) proposed the mechanisms of EGCG and ECG in radical oxidation where AAPH, which generates peroxy radicals by its action with oxygen, was used as an initiator of lipid peroxidation in the liposomal system and of radical oxidation in the aqueous solution (**Figure 4**).

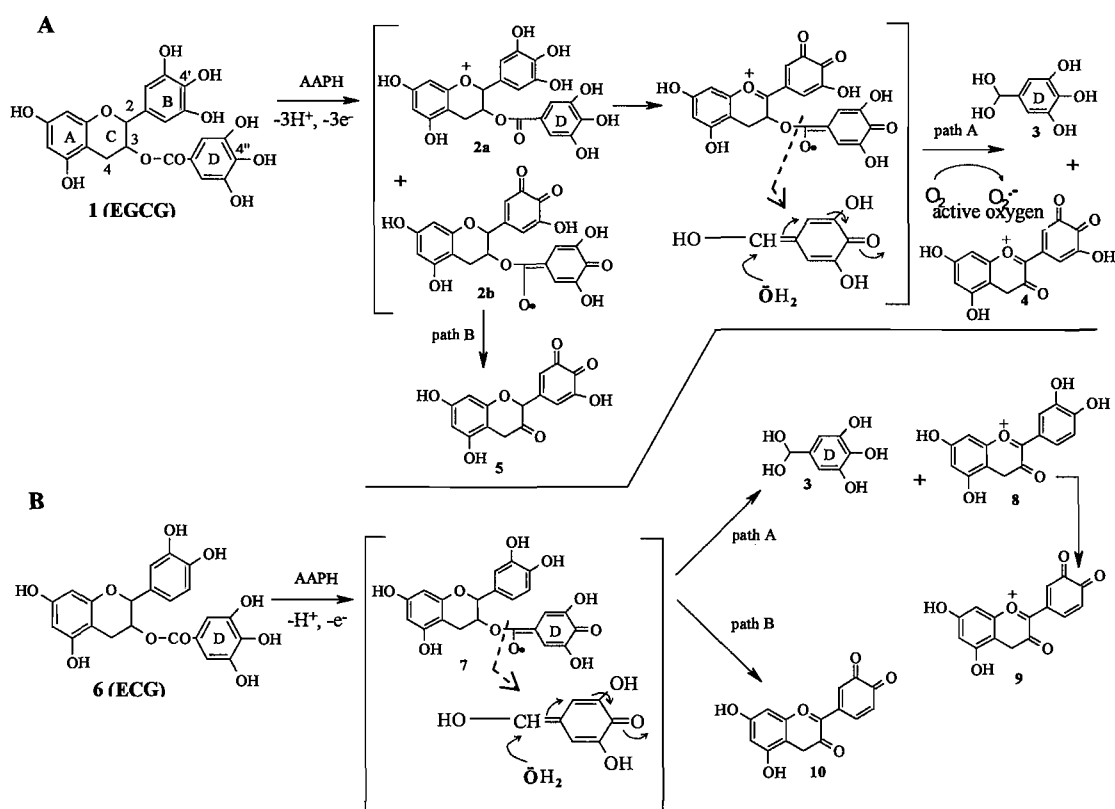


Figure 4: Proposed mechanisms of EGCG and ECG in radical oxidation [Adapted from Kondo *et al.*, (1999)]

Kondo *et al.*, (1999) indicated that EGCG can be converted to an anthocyanidin-like compound (compound 4, **Figure 4A**) followed by cleavage of the gallate moiety by oxidation, whereas ECG can be converted to an anthocyanin-like compound (compound 8, **Figure 4B**) after cleavage of the gallate moiety. A general structure of anthocyanidin is shown below for comparison (**Figure 5**) with the chemical structures of compounds 4 and 8.

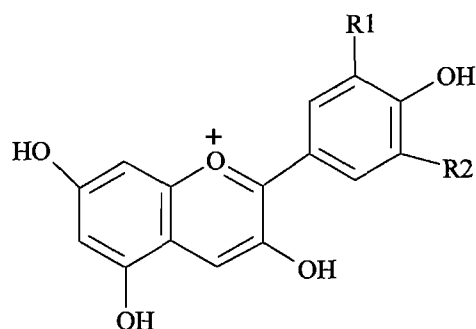


Figure 5: General structure of anthocyanidin

The antioxidant potential and their resulting potential bioactivity *in vivo* is dependent on the absorption, metabolism, distribution and excretion of these compounds within the body after ingestion, as well as the reducing properties of the resulting metabolites (Spencer, 2003). Microbial metabolites such as (-)-5(3',4',5'-trihydroxyphenyl)- γ -valerolactone (M4), (-)-5(3',4'-dihydroxyphenyl)- γ -valerolactone (M6) and (-)-5(3',5'-dihydroxyphenyl)- γ -valerolactone (M6') have been found to exert some antioxidant activity because of the di-/trihydroxyphenyl groups in their structure (Manach *et al.*, 2005). Therefore, green tea catechins and their metabolites exert antioxidant activity, with EGCG having the highest antioxidant capacity.

2.3.4 Scientific evidence on bioavailability of green tea catechins

Oral or absolute bioavailability of green tea catechins has been found to be very low in rodents, but it has not been established in humans because of lack of an intravenous formulation (Cai *et al.*, 2002). Thus, lack of pharmacokinetic studies after intravenous administration of green tea catechins in humans (Chow *et al.*, 2001). Chen *et al.*, (1997) found that oral bioavailability of green catechins in rats was at 1.6% (0.016). In another bioavailability study, Zhu *et al.*, (2000) assessed oral absorption and bioavailability of tea catechins in rats and found that absolute

bioavailability of EC, EGCG and ECG was 0.39 (39%), 0.14 (14%) and 0.06 (6%) respectively with a large apparent volume of distribution of 29.7, 38.5 and 63.0 litres/kg and an oral clearance of 0.046, 0.061 and 0.091 litres/min/kg. Since absolute bioavailability values range between 0 and 1, small values result in high oral clearance and a large oral apparent volume of distribution, which may be attributed to low oral bioavailability in humans (Cai *et al.*, 2002). In a study performed using mice, Lambert *et al.*, (2003) reported an absolute bioavailability of EGCG to be 26.5% (0.265), higher than that reported for rats. Furthermore, Xu *et al.*, (2004) determined the absolute bioavailability of tea epicatechins (EC, ECG, EGCG) and their epimers (GCG, GC, CG and C) to be 0.12 (12%) for EGCG, 0.31 (31%) for ECG, 0.08 (8%) for GCG and 0.23 (23%) for CG after oral administration of 4000 mg green tea extract (GTE)-epimer mixture/kg. These findings are consistent with the findings by Zhu *et al.*, (2000), who found an absolute bioavailability of tea catechins to range between 0.06 – 0.39 (6 – 39%). A recent bioavailability study on EGCG by Li *et al.*, (2007) reported an oral bioavailability of 4.95% in freely moving rats, further reflecting low bioavailability of this catechin.

Bioavailability studies have shown that maximum plasma concentrations in humans after oral administration of 90-150 mg of catechins in the form of green tea are in the order of 0.1-0.7 $\mu\text{mol/L}$ (Manach *et al.*, 2004) and are most often reached within 1-2 hrs after ingestion. Generally, catechins have a short half-life, with an elimination half-life period of 2-3 hours (Williamson & Manach, 2005). Manach *et al.*, (2005) performed a review on pharmacokinetic parameters of different groups of flavonoids in 97 bioavailability studies in humans after oral administration of a single dose of polyphenol provided as a pure compound, plant extract, or whole food/beverage. The results of the pharmacokinetic data for (epi)catechin, EGC, and EGCG are presented in **Table 1**. The pharmacokinetic data for all the studies were converted to correspond to a supply of 50 mg aglycone equivalent. Aglycone is a non-sugar compound remaining after replacement of the glycosyl group from a glycoside molecule with a hydrogen atom. Additionally, it is the aglycone form of a flavanoid that is utilised by the body. The results of the review by Manach *et al.*, (2005) are consistent with conclusions made by Manach *et al.*, (2004) and Williamson & Manach, (2005).

Table 1: Pharmacokinetic data for catechins

Catechin	T_{max} (hours)		C_{max} ($\mu\text{mol/L}$)		AUC ($\mu\text{mol h/L}$)		$t_{1/2,e}$ (hours)	
	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
(Epi)catechin	1.8 \pm 0.1	0.5-2.5	0.40 \pm 0.09	0.09-1.10	1.1 \pm 0.3	0.5-2.0	2.5 \pm 0.4	1.1-4.1
EGC	1.4 \pm 0.1	0.5-2.0	1.10 \pm 0.40	0.30-2.70	2.0 \pm 0.8	1.0-3.6	2.3 \pm 0.2	1.7-2.8
EGCG	2.3 \pm 0.2	1.6-3.2	0.12 \pm 0.03	0.03-0.38	0.5 \pm 0.1	0.2-0.9	3.5 \pm 0.3	2.5-5.1

AUC, area under the plasma concentration-time curve; C_{max} , maximum plasma concentration; EGC, epigallocatechin; EGCG, epigallocatechin gallate; (Epi)catechin, epicatechin and catechin; $t_{1/2,e}$, the elimination half life; SD, standard deviation; T_{max} , time to reach the plasma concentration.

Bioavailability studies have shown that relative bioavailability of green tea catechins is also relatively low. Several studies have estimated pharmacokinetics parameters of green tea catechins in humans following oral administration. Van Amelsvoort *et al.*, (2001) in a randomised crossover design study, orally administered 1.5 mmol ECG (664 mg), epigallocatechin (EGC) (459 mg) or EGCG (688 mg) to human subjects. They found that the mean C_{max} was 3.1, 5.0 and 1.3 $\mu\text{mol/L}$; the mean T_{max} 4, 1.4 and 2.9 hours; and the mean $t_{1/2,e}$ values 6.9, 1.7 and 3.9 hours for ECG, EGC and EGCG respectively. Lee *et al.*, (2002) estimated the pharmacokinetic parameters of EGCG, EGC and EC after administration of a single oral dose of green tea or decaffeinated green tea (20 mg tea solids/kg) or EGCG (2 mg/kg) to eight subjects. They reported a C_{max} of EGCG, EGC and EC at 77.9 ± 22.2 , 223.4 ± 35.2 and 124.03 ± 7.86 ng/ml respectively; AUC as 508.2 ± 227 , 945.4 ± 438.4 and 529.5 ± 244.4 ng.h.ml⁻¹ respectively; elimination half-lives of 3.4 ± 0.3 , 1.7 ± 0.4 and 2.0 ± 0.4 h respectively; and a T_{max} ranging between 1.3 to 1.6 hours. In another randomised study, Ullmann *et al.*, (2003) found that C_{max} of EGCG ranged between 130 and 3392 ng/ml (0.28 – 7.4 $\mu\text{mol/L}$) and T_{max} between 1.3-2.2 hours, and $t_{1/2,e}$ values were observed between 1.9 and 4.6 hours after an oral administration of 50 to 1600 mg pure EGCG were given. Chow *et al.*, (2001) in another study compared the pharmacokinetic variables of pure EGCG (200-800 mg) and Polyphenon E, a decaffeinated green tea catechin mixture given orally. They found that EGCG C_{max} ranged from 73.7 to 438.5 ng/ml (0.16-0.96 $\mu\text{mol/L}$), T_{max} from 109-241 min (1.8-4.0 hours), AUC from 22 to 167 min $\mu\text{g/ml}$, $t_{1/2}$ from 118 to 184 min (2-3.1 hours); and Polyphenon C_{max} ranged from 73 to 378 ng/ml (0.16-0.82 $\mu\text{mol/L}$), T_{max} from 145 to 249 min (2.4-4.2 hours), AUC from

22 to 167 min $\mu\text{g/ml}$, $t_{1/2,e}$ from 118 to 184 min (2-3.1 hours), depending on the dose. In the same study, they also measured clearance and apparent volume of distribution, which ranged from 6.0 to 18.0 litres/min and 1044 to 4774 litres, reflecting very low bioavailability of catechins in humans. The pharmacokinetic parameters of the pure EGCG were not significantly different from those in the catechin mixture.

Nagakawa *et al.*, (1997) in a study where healthy subjects orally consumed a green tea extract (GTE) containing 225, 375 and 525 mg EGCG and 7.5, 12.5, and 17.5 mg EGC respectively, detected 0.2-2.0% of ingested EGCG and 0.2-1.3% of ingested EGC dosages in plasma. Similarly, another study done in humans by Lee *et al.*, (2002), found that at the time point T_{max} , 0.16, 0.58 and 1.1% of the ingested doses of EGCG, EGC, and EC derived from 900-1700 mg [depending on each subject's body weight (20 mg/kg body weight)] green tea (GT) solids after oral administration were present in the circulating plasma. Furthermore, in the same pharmacokinetic study, when the same amount of decaffeinated green tea (DGT) was administered, the values in the blood were 0.13, 0.53, and 0.71% respectively; and when 90-170 mg (2 mg/kg body weight) pure EGCG was administered, only 0.1% of the ingested dose appeared in the blood at T_{max} , further demonstrating poor bioavailability of catechins in humans.

Low C_{max} and long T_{max} reflect a slow rate of absorption (Zhu *et al.*, 2000) of the catechins. In **Table 1** EGCG has lower C_{max} and longer T_{max} values than other catechins, suggesting that it has a slow rate of absorption, hence low bioavailability. Since half-lives of catechins in plasma are relatively short, this can contribute to less accumulation in blood with repeated intakes and therefore impacts negatively on their bioavailability. These results suggest that EGCG is less bioavailable than EGC and EC, which may partly be due to their chemical structural differences, which will be addressed in the section below.

2.4 FACTORS INFLUENCING ORAL ADMINISTRATION OF CATECHIN PHARMACOKINETICS

Oral bioavailability of various green tea catechins differs markedly, and it depends on several factors such as; biochemical, physiological, physicochemical and dosage

form. There are, however, no individual factors which independently influences the bioavailability of catechins, and bioavailability of these are usually dependent on interplay between a number of these factors. These factors will however be discussed independently.

2.4.1 Biochemical factors

2.4.1.1 Conjugation

Generally, flavonoids are present in plants conjugated to sugars, and it is these glycosides that are ingested in the diet and enter the GI tract (Spencer, 2003). Catechins in tea are an exception to this rule as they are present in the diet in the non-glycosylated form, as aglycones. Therefore, unlike other dietary flavonoids, there is no initial requirement for β -glucosidase action prior to absorption (Spencer, 2003).

Once absorbed, polyphenols are subjected to three main types of conjugation, namely, methylation, sulphation and glucuronidation (Manach *et al.*, 2005). Catechins, especially those without a gallate moiety are readily conjugated to glucuronides and sulphates (Yang & Landau, 2000). The relative importance of the three types of conjugation tends to vary according to the nature of the substrate and the dose ingested. Sulphation is generally a high-affinity, lower-capacity pathway than is glucuronidation, hence, when the ingested dose increases, a shift from sulphation toward glucuronidation occurs. Catechol-*O*-methyltransferase (COMT) catalyses the transfer of a methyl group from *s*-adenosylmethionine (SAM) to polyphenols having a catechol moiety such as catechins. For example, EC is methylated to form 3'-*O*-methyl(-)-epicatechin (3'-MeEC), EGC is methylated to form 4'-*O*-methyl(-)-epigallocatechin (4'-MeEGC), and EGCG 4',4''-dimethyl(-)-epigallocatechin gallate (4',4''-DiMeEGCG) as shown in **Figure 6**. *In vivo*, EGCG is first methylated to form 4''-*O*-methyl(-)-epigallocatechin gallate (4''-MeEGCG) and then further methylated to form 4',4''-DiMeEGCG (Meng *et al.*, 2002). In animals, phase II metabolism reactions are likely to compete with one another. The relative concentration of each enzyme and its activity for the tea polyphenols determine the metabolic profile *in vivo* (Lambert & Yang, 2003a).

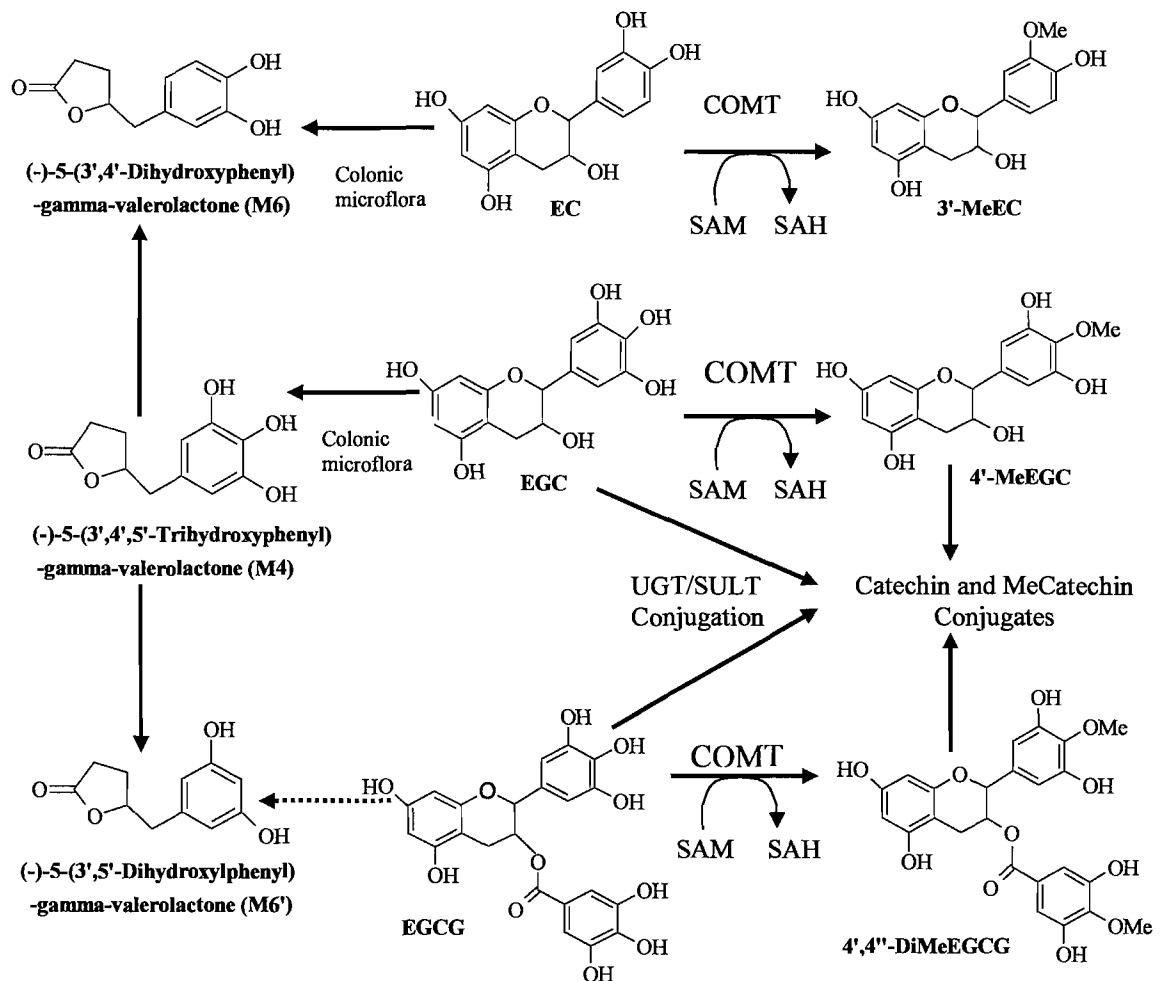


Figure 6: Metabolic fate of tea catechins (phase II metabolism reactions). 3'-MeEC, 3'-*O*-methyl(-)-epicatechin; 4'-MeEGC, 4'-*O*-methyl(-)-epigallocatechin; 4',4''-DiMeEGCG, 4',4''-dimethyl(-)-epigallocatechin gallate; COMT, catechol-*O*-methyltransferase; EC, epicatechin; EGC, epigallocatechin, EGCG, epigallocatechin gallate; SAM, s-adenosylmethionine; SAH, homocysteine; SULT, sulphotransferase; UGT, uridine 5'-diphosphoglucuronosyltransferase (Adapted from Lambert & Yang, 2003a; Lambert & Yang, 2003b).

After oral consumption, a substantial amount (77-92%) of EGCG in plasma is in the free form (unconjugated) (Lee *et al.*, 2002; Chow *et al.*, 2003). Other green tea catechins such as C, GC, GCG, EC, EGC and ECG occur highly conjugated with glucuronic acid and/or sulphate groups in plasma (Manach *et al.*, 2005). Major circulating catechin metabolites in human plasma are (-)-epicatechin-3'-*O*-glucuronide, 4'-*O*-methyl(-)-epicatechin-3'-*O*-glucuronide, 4'-*O*-methyl(-)-epicatechin-5- or 7-*O*-glucuronide, 3'-*O*-methyl(-)-epicatechin, (-)-epicatechin-7-*O*-glucuronide and 3'-*O*-methyl(-)-epicatechin-7-*O*-glucuronide (Natsume *et al.*, 2003). Most bioavailability studies only measure the unchanged catechins, ignoring their metabolites, which may lead to underestimation of bioavailability. Therefore, bioavailability studies on polyphenols should measure the total polyphenols in the blood after treatment (hydrolysis) with deconjugating enzymes (Scalbert & Williamson, 2000) such as β -glucuronidase and sulphatase before analysis.

2.4.1.2 Metabolism by the gut microflora

Polyphenols must be hydrolysed by intestinal enzymes or by the colonic microflora before absorption (Manach *et al.*, 2004). The extent of absorption of dietary polyphenols in the small intestine is relatively small, therefore, the majority of the ingested polyphenols reach the large intestine where they encounter colonic microflora (Scalbert & Williamson, 2000; Manach *et al.*, 2004). Extensive microflora in the colon also play a critical role in the metabolism of polyphenols (Williamson & Manach, 2005). This type of metabolism is known as ring-fission metabolism. After microbial enzyme-catalysed deconjugation of polyphenol conjugates that reach the colon, there are two possible routes, namely, absorption of the intact polyphenol through the colonic epithelium, and passage into the blood stream (as free or conjugated forms) or a breakdown of the original polyphenol structure into metabolites (Williamson & Manach, 2005). Microflora extensively metabolises the aglycones into various aromatic acids (Manach *et al.*, 2004). Two major tea catechin metabolites, M4 and M6 have been detected in human urine and blood (Li *et al.*, 2000; Lee *et al.*, 2002), which appeared to be formed by the intestinal flora in the human colon and then absorbed. In addition, Meng *et al.*, (2002) have also detected M6' in urine. These ring fission products are formed as a result of anaerobic fermentation of EGC, EC and ECG with human faecal microflora (**Figure 6**).

2.4.2 Physiological factors

2.4.2.1 pH in the gastrointestinal tract

Green tea catechins are stable in the stomach due to its low pH, when they proceed into the intestinal lumen where the pH is high, they are partly degraded before they are absorbed. EGCG has been observed to rapidly oxidize in authentic intestinal juice (pH 8.5) with the amount of EGCG decreased to 19.4% in only 5 minutes. A similar incubation in mouse plasma (pH 7.8) decreased to 60.7% (Yoshino *et al.*, 1999). In the same study, Yoshino *et al.*, (1999) observed that EGCG, ECG, EGC and EC were very stable under acidic conditions (pH 1.8-6.4), and when the pH was above 7.4, EGC and EGCG became unstable, whereas EC was only degraded under strong alkaline conditions (pH 11.2). Considering this, the high intestinal pH may lead to degradation of GTC, especially EGC and EGCG, and thus lower their bioavailability. Chemical degradation of catechins will further be discussed under the physicochemical factors section.

2.4.2.2 First-pass metabolism

First-pass effect may contribute to the low bioavailability of catechins by causing some losses through GI metabolism and/or extraction by the liver immediately after absorption (Zhu *et al.*, 2000). However, Cai *et al.*, (2002) in a study that assessed the contribution of pre-systemic hepatic extraction to the low bioavailability of green tea catechins in rats, found that first-pass hepatic elimination does not play any significant role in the pre-systemic loss of orally administered green tea catechins.

2.4.3 Physicochemical factors

2.4.3.1 Chemical structure

Understanding the structural factors that influence absorption and metabolism of catechins is essential. The chemical structure of catechins determines their rate and extent of intestinal absorption, and the nature of their metabolites circulating in the plasma (Scalbert & Williamson, 2000). Epigallocatechin gallate may be less bioavailable than other catechins such as EGC and EC partly due to chemical structural differences. This may be partly explained by Lipinski's 'rule of 5' which is based on the ability of a molecule to pass through transient pores formed in the

plasma membrane by the movement of the phospholipid acyl tails, and also by a molecule's ability to form hydrogen bonds (Lambert & Yang, 2003b). Epigallocatechin gallate has a MW of 458.4, which is relatively high, whereas EC and EGC have MW of 290.3 and 306.3 respectively. Its chemical structure has 8 OH groups whereas EC and EGC have 5 and 6 OH groups respectively. These differences are illustrated in **Figure 7**.

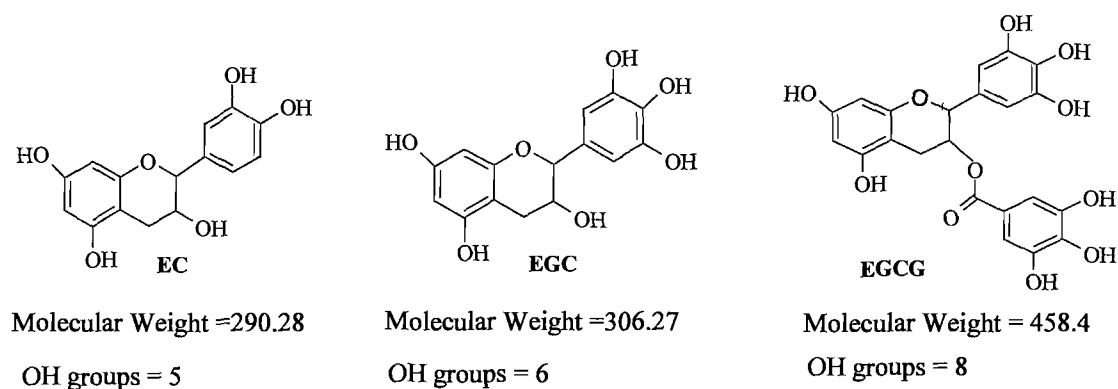


Figure 7: Structural differences in epicatechin, epigallocatechin and epigallocatechin gallate. EC, epicatechin; EGC, epigallocatechin; EGCG, epigallocatechin gallate, OH, hydroxyl group

In general, due to these structural differences, green tea catechins have low bioavailability, and that of EGCG is even lower, compared to EGC and EC.

2.4.3.2 Chemical stability

Contributing to the results of Yoshino *et al.*, (1999), Zhu *et al.*, (1997) additionally reported that the stability of green tea catechins between pH 4 and 8 is again pH-dependent (the lower the pH, the greater the stability). All of the GTC are stable in acidic solutions at a pH range of 1.8 to 6.4 except EC, which is found to be stable between pH 1.8 and 11.2. Epigallocatechin and EGCG are rapidly degraded at pH levels above 7.4, which is the pH of most body fluids. The pH value of the intestine and body fluids is neutral or slightly alkaline (Lam *et al.*, 2004). Since the pH of the intestinal tract ranges from 5 to 8, degradation of EGCG and EGC may occur in the intestinal lumen and may contribute to their systemic loss before absorption, leading to lower bioavailability. Stability of GTC in plasma (pH 7.8) may also contribute to their poor bioavailability, as they are pH-dependent. At high pH values, the basic

environment can easily attack the proton of the phenol group, leading to the generation of a phenoxide anion, which is highly reactive with electrophilic agents of the body such as free radicals, and also forms the semiquinone radical, which can further undergo dimerization or other reactions (Lam *et al.*, 2004). These catechins are further degraded to metabolites in other body fluids and organs after absorption. Other factors that affect chemical stability of GTC in aqueous solutions are oxygen concentration, temperature and ionic strength (Liao *et al.*, 2001).

2.4.3.3 Solubility

Zhu *et al.*, (2000) observed that catechin fractions are readily dissolved in water. However, under physiological conditions, catechins are compounds of phenolic nature and are weakly acidic, thus in principle, they should exist in the un-ionised form, and solubility of un-ionised molecules can be greatly reduced thereby hindering their absorption in the GI tract (Zhu *et al.*, 2000). Furthermore, galloyl (or gallated) catechins such as ECG, EGCG have the potential to bind to protein/membrane surfaces by hydrogen bonding through phenolic terminals, forming a catechin-protein complex which may hinder the absorption of these catechins by lowering their aqueous solubility (Chen *et al.*, 1997; Zhu *et al.*, 2000).

2.4.4 Dosage form

Bioavailability of catechins after oral administration may be affected by the dosage form, that is, whether taken as green tea catechins in solution or as hard gelatine capsules/tablets. Green tea catechins in solution are thought to be more bioavailable as compared to green tea catechins given as hard gelatine capsules or tablets.

Bioavailability studies have been done in specific food items rich in catechins and on green tea extracts that are either in a mixture or on one specific catechin in a pure form. Henning *et al.*, (2004) found that a green tea supplement (GTS) intervention resulted in a higher total plasma flavanol concentration ($0.66 \pm 0.05 \mu\text{mol/L}$) than did a black tea (BT) intervention ($0.47 \pm 0.04 \mu\text{mol/L}$), even though BT contained 28.5% more flavanols. This may have been due to the fact that BT has other polyphenols (theaflavins and thearubigins), which may interfere with catechin bioavailability as compared to GTS which contains only selected catechins.

The percentage of catechins in plasma that are sulphated or glucuronidated depends on the dose ingested (Williamson & Manach, 2005). It is assumed that bioavailability parameters, that is, C_{max} , T_{max} , AUC, $t_{1/2}$, and relative urinary excretion, increase linearly with the dose (Manach *et al.*, 2005). This has been demonstrated in humans for EGCG by Ullmann *et al.*, (2003) in a single ascending dose study.

The delivery of catechins to the circulation through oral administration is hindered by several factors, some of which have been discussed above. The gut appears to be a major factor limiting bioavailability of orally administered EGCG as it is extensively metabolised. EGCG also undergoes methylation, glucuronidation and sulphation in both rodents and humans (Lambert and Yang, 2003a). The potential health benefits of increased catechin intake justifies investigating the usefulness of technologies such as delivery systems to improve the bioavailability of catechins following oral consumption. Other delivery systems include transdermal formulations; whereby compounds are delivered across the skin into the systemic circulation. This delivery system bypasses poor absorption in the small intestines, extensive metabolism in the colon and liver, and other barriers that limit bioavailability of orally administered EGCG. Lambert *et al.*, (2006) transdermally delivered EGCG to mice and they found significantly longer $t_{1/2,e}$ and larger AUC (67- and 8-fold, respectively) indicating a higher bioavailability than when EGCG was orally administered in mice in their previous work (Lambert *et al.*, 2003).

2.5 DELIVERY SYSTEMS FOR THERAPEUTICS








Different types of delivery systems exist. Kostarelos (2003) has summarised the biological applications achieved by engineering the colloid and interface characteristics of the delivery systems such as pulmonary gene therapy, solid tumour chemotherapy, hepatocytes, endosomal escape, and blood circulation. According to Kostarelos (2003), the purpose of any delivery system is to control the pharmacological parameters (bioavailability, pharmacokinetics, biodistribution and pharmacodynamics) characteristic of the administered moieties. The therapeutic agents that are administered using a delivery system should exhibit different biological profiles compared to those obtained when administered alone. The author further explained that the optimal means for delivering therapeutic agents is either by

incorporation, encapsulation, adsorption or binding with a delivery system. Colloidal carriers have been used primarily to assist in the modification of drug molecules exhibiting low aqueous solubility, and these include emulsions, microemulsions, and micelles. In **Table 2**, the most commonly used colloid particle-based delivery systems are displayed with their typical average dimension.

2.5.1 Lipid-based drug delivery systems

Lipid-based drug delivery systems offer versatility for oral administration as they can be formulated as solutions, gels, suspensions, emulsions, self-emulsifying systems and microemulsions (Humberstone & Charman, 1997). They are ideally prepared as unit dose forms which could be filled into either a sealed hard or soft gelatine capsule (Pouton & Charman, 1997). For lipophilic, poorly water soluble drugs, oral bioavailability is commonly limited by solubility in the intestinal lumen, and a slow and incomplete dissolution from solid dose forms during transit down the GI tract (Kossena *et al.*, 2004). Even though the exact mechanism is not well defined, it has been established that lipid-based dose forms typically enhance oral bioavailability as the lipid solution reduces energy input required to overcome the crystal lattice energy of a solid dose form (Humberstone & Charman, 1997; Kossena *et al.*, 2004). Besides that, the digestion and dispersion of a lipidic vehicle results in the production of a dispersed lipidic microenvironment (micelles/vesicles/emulsion droplets) that persists during GI transit and provides a solubilisation sink for the drug, preventing precipitation. The solubility of drugs in the GI lumen is believed to be increased through the presence of lipids in the GI tract which stimulates an increase in the secretion of bile salts and endogenous biliary lipids including phospholipids and cholesterol. This leads to the formation of bile salts/phospholipids/cholesterol intestinal mixed micelles and an increase in the solubilisation capacity of the GI tract (Porter & Charman, 2001). Liposomes and micelles are examples of lipid-based delivery systems.

Table 2: Delivery system types, common delivery systems from each type and most widespread biomedical and pharmaceutical uses

Delivery system types and typical mean particle diameter	Representative systems of each type	Characteristic applications
 0.5 - 20 μm Microspheres hydrogels	alginate, gelatin, chitosan, PLGA microspheres synthetic, biodegradable, polymer hydrogels	sustained release of therapeutics scaffolds for cell delivery in tissue engineering
 0.2 - 5 μm Microparticles	polystyrene, microspheres	targeted delivery of therapeutics
 0.15 - 2 μm Emulsions, microemulsions	o/w, w/o/w, lipid, emulsions o/w microemulsions	controlled and targeted delivery of therapeutics
 30 - 1000 nm Liposomes	Phospholipids and polymer-based bilayer vesicles	targeted delivery of therapeutics
 3 - 80 nm Micelles	natural and synthetic surfactant	targeted delivery of therapeutics
 2 - 100 nm Nanoparticles	lipid, polymer, inorganic nanoparticles	targeted delivery of therapeutics <i>in vivo</i> navigation devices
 2 - 100 nm Nanocrystals	quantum dots	imaging agents

o/w, oil-in-water; w/o, water-in-oil; PLGA. Adapted from Kostanelos, (2003).

2.5.1.1 Liposomes

Liposomes are an example of lipid-based delivery system. They are small vesicles consisting of one or more concentric lipid bilayer, enclosing discrete aqueous spaces and are formed spontaneously when (phospho) lipids are suspended in aqueous media. The lipophilic parts of the molecules face inwards and the hydrophilic parts are exposed to the aqueous phase surrounding them (Laverman *et al.*, 1999). Liposomes vary in size, 30 – 1000nm (**Table 2**). Because of their structural versatility in terms of size, surface charge, composition, bilayer fluidity and because of their ability to encapsulate almost any drug, regardless of their solubility, it has been observed that liposomes could serve as carriers for drugs (Laverman *et al.*, 1999). Other delivery systems are discussed below.

2.5.1.2 Micelles

Another example of a lipid-based delivery system is a micelle. Micelles are natural and synthetic surfactants, spherical colloidal nanoparticles into which many amphiphilic molecules self assemble (Torchilin *et al.*, 2003). In water, hydrophobic fragments of amphiphilic molecules form the core of a micelle, which may then be used as a cargo space for poorly soluble pharmaceuticals (Lasic, 1992). Hydrophilic parts of the molecules form the micelle corona. A typical structure of a micelle and its mean particle diameter (3 – 80 nm) are shown in **Table 2**. Micelle encapsulation increases bioavailability of poorly soluble drugs, protects them from destruction in biological surroundings, and beneficially modifies their pharmacokinetics and biodistribution (Torchilin *et al.*, 2003).

2.5.1.3 Pheroid™ delivery system

Pheroid™ contains ethyl esters of the essential fatty acids, linoleic acid and linolenic acid, as well as oleic acid, emulsified in water saturated with nitrous oxide (Saunders *et al.*, 1999). The Pheroid™ comprises of a submicron emulsion/colloidal formulation capable of entrapping various drugs and delivering them with high efficiency. It has a particle size of about 300 nm – 2 µm. A brief description of Pheroid™ is given as it is not possible to give a detailed description due to proprietary information restriction. This system has been shown to enhance the absorption of dermatological formulations

by the skin (Saunders *et al.*, 1999, Tzaneva *et al.*, 2003; Goodfield *et al.*, 2004). It has been established that Pheroid™ increases the delivery of active compounds, decrease time to onset of action, reduce the minimal effective concentration, and increase therapeutic efficacy of the carried drug (North-West University, 2004). The pro-Pheroid have been designed to enable Pheroid™ to be formed once the product has been digested and/or exposed to body fluids, thereby increasing potential bioavailability and stability of drugs given through the oral route.

Pheroid™ was used in a randomised controlled trial as a carrier molecule for 94% pure crystalline EGCG (TEAVIGO™) in the present study. Epigallocatechin gallate alone or EGCG plus Pheroid™ were encapsulated and given to volunteers in a crossover design manner. It was hypothesised that this carrier molecule will enhance the bioavailability of EGCG.

2.6 SUMMARY

Oral bioavailability of green tea catechins is affected by a number of factors such as; pH of the GI tract, intestinal microflora, first-pass metabolism, biochemical processes in the GI tract, chemical structure, chemical stability, solubility and dosage forms. The main limitation on the determination of oral bioavailability of green tea catechins is the absence of intravenous formulations of green tea catechins for human use. Therefore, existing data on oral bioavailability is mainly from rodents, and the available data on humans is reported as pharmacokinetic parameters, and percentage of ingested green tea catechins detected in plasma after their oral administration. Areas under the plasma concentration-time curves for the different treatments given, which are the estimated pharmacokinetic parameters following oral administration of a drug or nutrient in human bioavailability studies, can be used to calculate the relative bioavailability fraction or percentage, but not oral bioavailability. The pharmacokinetic data has been used to establish that there is low bioavailability of green tea catechins in humans. From the existing evidence, EGCG seems to have a lower bioavailability compared to other green tea catechins.

It is vital for the bioavailability of green tea catechins to be enhanced as these compounds have been shown to have antioxidant and/or scavenging potential in human, animal and *in vitro* studies. Thus, they may play an important role in the prevention of diseases such as CVD, cancer, hypertension, DM, dental caries. The antioxidant potential is directly associated with the combination of aromatic rings and hydroxyl groups that make up their structure. There are existing delivery systems which have been formulated to help enhance bioavailability of drugs, and these may be extended to nutritional compounds such as green tea catechins. In this study, TEAVIGO™, a high-concentration EGCG extract was incorporated in the lipid-based delivery system, Pheroid™, and given to healthy volunteers in a crossover double blind controlled study. The methodology, results will be reported and discussed in the following chapters.

3 CHAPTER 3: METHODOLOGY

3.1 INTRODUCTION

A study on the effect of a fatty acid-based carrier (Pheroid™) on the bioavailability of epigallocatechin gallate (EGCG) was conducted in accordance with the ICH Harmonised Tripartite guidelines for Good Clinical Practice, (1997) and the guidelines set out in the World Medical Association Declaration of Helsinki, (1964).

The research protocol was approved by the Ethics Committee of the North-West University, Potchefstroom Campus. The protocol was not submitted to the Medicine Control Council (MCC) for approval because the components of the fatty acid based carrier are considered pharmaceutically safe by the Food and Drug Administration (USA) and the Public Health Authority of South Africa (South African MCC).

Prior to participation in the study, the purpose and nature of the study were fully explained both verbally and in writing to each volunteer before completing a written consent form (**Annexure A**). Volunteers were further provided with clinical trial information sheet explaining all the study procedures, what is expected from them and what to expect (**Annexure B**).

The research team maintained confidentiality of the clinical trial volunteers' records and the identities. All information obtained during the study was regarded as confidential. All relevant data collected during the study period was recorded in the study forms by authorised staff. All forms were produced and supplied by the North-West University. The exit assessment form (**Annexure C**) was completed and signed at the end of the study. The study staff took all reasonable measures to record data in accordance with the protocol. There were no deviations from the approved protocol. Data remains the property of the North-West University.

This chapter entails a section on materials and reagents used in the study, the experimental design, diet that the volunteers followed, sample collection, plasma analysis of EGCG using LC-MSMS, pharmacokinetic analysis and statistical analysis.

Twenty healthy volunteers (10 males and 10 females) were enrolled in a randomised, double-blind crossover study following set inclusion and exclusion criteria. Volunteers completed a medical history questionnaire for screening into the study (**Annexure D**). Volunteers ingested a single dose of 400 mg EGCG or 400 mg EGCG incorporated in Pheroid™ on separate occasions. Volunteers followed a low flavonoid/catechin free diet for three (3) days prior to the trial day, followed by an overnight stay and fast in the metabolic unit of the Nutrition Department, North-West University. During the trial day, blood samples were drawn over a period of 8 hours. One millilitre (1) ml of plasma was mixed with 100µl of ascorbate-EDTA solution and then immediately frozen at -80°C until EGCG analysis. Extractions were performed and 300 µl clear supernatant transferred to autosampler sample vials after which 15 µl of each sample was injected into the LC-MSMS. Plasma concentrations for all the time intervals (0 min, 30 min, 60 min, 90 min, 120 min, 180 min, 300 min and 480 min) were obtained for all the 20 volunteers. Plasma concentrations were modelled using the pharmacokinetic software WinNonlin version 5.0 (Pharsight Corp, Mountain View, California, USA). A one-compartmental pharmacokinetic analysis model was used and data from only 9 complete pairs were successfully modelled. Therefore, a noncompartmental pharmacokinetic analysis model was used to obtain pharmacokinetic data with a large sample size of 15 volunteers. Data from both the one-compartmental and the noncompartmental models underwent statistical analysis using a data analysis software system STATISTICA version 7.1 (Statsoft Inc., Tulsa, OK, USA).

3.2 METHODOLOGY

3.2.1 Materials and Reagents

TEAVIGO™ (94 % pure crystalline EGCG) provided by DSM Nutritional Products Ltd. EGCG alone and EGCG plus Pheroid™ (a fatty acid-based carrier) capsules were formulated by the School of Pharmacy, Department of Pharmaceutics, North-

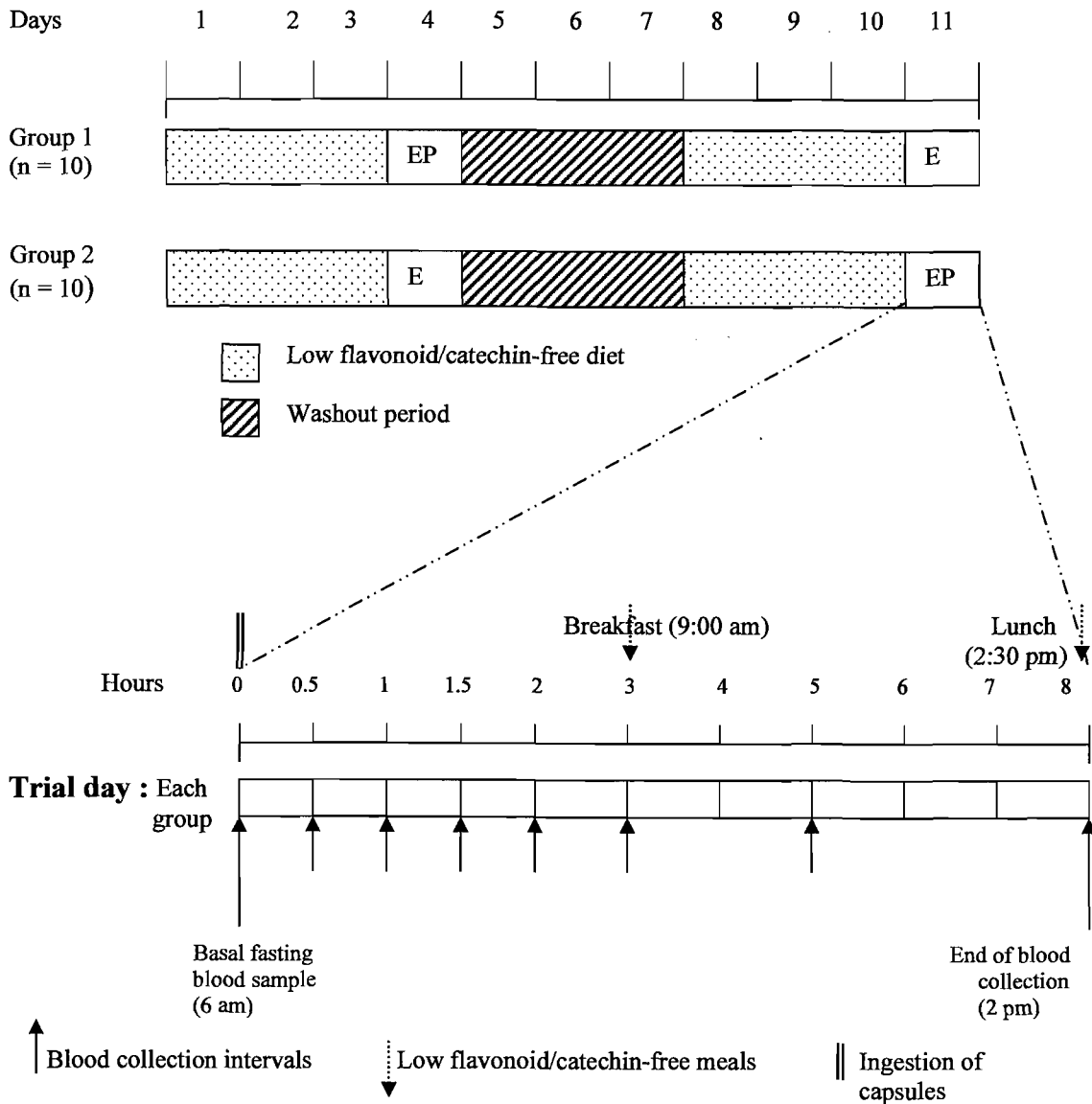
West University (Potchefstroom, South Africa) using TEAVIGO™ (**Annexure E**). All capsules contained 400 mg EGCG. The capsules were stored at room temperature. EGCG, β -D-glucuronidase / sulphatase (G7017) enzyme preparation from *Helix pommatia*, tyrosol, ethylenediaminetetraacetic acid (EDTA), and ascorbic acid were purchased from Sigma-Aldrich Chemical, Inc. (St Louis, MO, USA). High purity solvents (methanol, water, acetonitrile) were purchased from Honewell International Inc., Burdick & Jackson (Muskegon, MI, USA). Acetic acid and sodium hydroxide pellets were purchased from Saarchem (Gauteng, South Africa) and sodium dihydrogen orthophosphate anhydrous (NaH_2PO_4) was purchased from Saarchem-Holpro Analytic (Pty) Ltd (Krugersdorp, South Africa).

3.2.2 Experimental design

Twenty healthy post-graduate students of the North-West University, 10 men and 10 women aged 21 – 35 years, were recruited for the study. Potential volunteers were excluded if they reported chronic diseases e.g. cancer, cardiovascular disease, hypertension, diabetes mellitus, liver disease and/or renal disease, tuberculosis; acute infections such as common cold, flu, fever; pregnancy; lactation; alcohol intake averaging > 10 g (> 1 glass)/day for females and > 20 g (2 glasses)/day for males; smoking cessation for < 6 months and body mass index > 30 kg/m². The research protocol was approved by the Ethics Committee of the North-West University, Potchefstroom Campus (05M08) and all volunteers gave written informed consent.

A randomised, double-blinded crossover designed study with a 3-day run-in period and a 3-day washout period was conducted, during which volunteers were provided with a low flavonoid/catechin-free diet (**Annexure F**). Twenty (20) volunteers ingested a single dose of EGCG or EGCG incorporated in Pheroid™ on two separate occasions in random order, and unchanged EGCG was measured in the blood over a period of 8 hours. A computer random number generator was used to randomise volunteers. In the afternoons prior to the trial days (Day 3 and Day 10), volunteers were required to come to the metabolic unit at 5 p.m. for an overnight stay. Ten (10) of the volunteers ingested 400 mg EGCG, while the other ten had 400 mg EGCG incorporated in Pheroid, after an overnight fast. Each supplement was given as a capsule. A summary of the experimental design is shown in **Figure 8**. This design

indicates ingestion of capsules, collection of blood samples at different time points over 8 hours and consumption of breakfast and lunch done during the trial days on Day 4 and Day 11 for both groups 1 and 2 were done when either EGCG or EGCG incorporated in Pheroid™ were taken by the volunteers.



E, where 400 mg EGCG is given; EP, where 400 mg EGCG incorporated in Pheroid™ is given.

Day 1 Tuesday; Day 2 Wednesday; Day 3 Thursday; Day 4 Friday; Day 5 Saturday; Day 6 Sunday; Day 7 Monday; Day 8 Tuesday; Day 9 Wednesday; Day 10 Thursday; Day 11 Friday

Figure 8 Schematic design of the study

Volunteers were asked to refrain from taking vitamin and mineral supplements (including prescribed, over the counter, herbal and homeopathic therapies) during the

entire trial period. Volunteers kept records of medications taken during the trial (**Annexure G**). There were two side effects record charts, one was filled by the volunteers (**Annexure H**) and the other by the research nurses (**Annexure I**). The form filled in by the nurses was to be used to evaluate the safety and feasibility of EGCG and EGCG incorporated in Pheroid™.

On completion of the study an exit assessment form was filled by the investigator and a nurse (**Annexure C**).

3.2.3 Diet

Volunteers followed a low flavonoid/catechin-free diet for 3 days (three meals/day, breakfast, lunch and dinner) which was given from Day 1 to Day 3 followed by a trial period (Day 4) and a washout period (Day 5 to Day 7) as shown in **Figure 8**. The diet was planned by a Registered Dietitian who modified recipes to comply with the requirements of a low flavonoid, catechin-free diet. Energy content of the diet was not restricted in order to improve compliance. After the 3 day washout period on Day 8, volunteers followed a similar 3-day diet. The diet was prepared in the kitchen of the metabolic unit of the North-West University, Potchefstroom Campus. In addition, volunteers were given a list of foods low in flavonoids and free from catechins for guidance during controlled feeding and on the day of trial. The same list was used to plan the required diet and was drawn using the USDA database for the flavonoid content of selected foods (USDA Nutrient Laboratory Data, 2003). Volunteers were provided with a list of foods allowed and those that they were to avoid (**Annexure J**). They had to keep a food record chart of food eaten outside the study (**Annexure K**).

On Day 11, volunteers who, on Day 4, were given 400mg EGCG were given 400 mg EGCG incorporated in Pheroid™ and the other group 400 mg EGCG. During the trial days, volunteers were provided with a standardized breakfast (low flavonoid/catechin-free) approximately 3 hours after the consumption of EGCG or EGCG incorporated in Pheroid™. A standardized lunch whereby flavonoids and catechins were not restricted was given to all volunteers after the last blood sample collection after 8 hours.

3.2.4 Sample collection

Venous blood samples were collected from all volunteers by a qualified nursing sister using a Vascocan Braunnule with Ivasofix stylet cannula set. One blood sample (5 ml) was drawn after an overnight fast (0 minutes) and the others collected at 30, 60, 90, 120, 180, 300 and 480 minutes after ingestion of the food supplements. Volunteers were cannulated throughout the 0 – 480 min blood collection period. A blood sample record was kept for each volunteer (**Annexure L**). Blood samples were collected in heparinised tubes and immediately placed in ice. Plasma was prepared within 30 min after blood sampling by centrifugation at 2000 x g at 4°C for 10 minutes. One millilitre (1) ml of plasma was mixed with 100µl of ascorbate-EDTA solution (0.4 M NaH₂PO₄ buffer containing 20 % ascorbic acid – 0.1 % EDTA, pH 3.6) and then immediately frozen at -80°C until EGCG analysis (Lee *et al.*, 2000).

3.2.5 Plasma analysis of EGCG using LC-MSMS

3.2.5.1 Sample preparation

Sample preparation was done using an adapted method from Lee *et al.* (2000) and Henning *et al.*, (2006). To 200 µl of thawed plasma, 5 µl of ascorbate-EDTA buffer solution (10% ascorbic acid - 40 mM NaH₂PO₄ – 0.1% EDTA at pH 3.6), 20 µl of 50 mM NaH₂PO₄ buffer solution (pH 7.4) and 20 µl of a 10 µg/ml tyrosol solution (internal standard) made up in methanol containing 0.2% ascorbic acid-0.005% EDTA [50/50 (v/v)], was added. The mixture was vortexed followed by the addition of 15 µl β-D-glucuronidase (1500 U)/ sulphatase (113U) enzyme preparation from *Helix pommatia*. The mixture was vortexed again and incubated in a 37°C water bath for 45 min. The mixture was extracted twice with 2 ml ethyl acetate, by vortexing for 30 seconds and centrifugation for 3 minutes. Combined supernatants were mixed with 10 µl of 0.2% ascorbic acid and dried under nitrogen. The dried samples were reconstituted in 100 µl methanol and 200 µl of 15% aqueous acetonitrile. The reconstituted mixture was centrifuged at 14000 x g for 15 min to pellet any solid material. The clear supernatant was transferred to autosampler sample vials and 15 µl of each sample injected onto the LC-MSMS.

3.2.5.2 Analysis

All analyses were performed on an Agilent 1200 Series Liquid Chromatograph System (Agilent Technologies, Santa Clara, California, USA) interfaced to 6410 Triple-Quadrupole mass spectrometer. This system consisted of a binary pump, degasser, thermostated plate sampler and thermostated column compartment. Chromatographic separation was performed at 20°C using Zorbax Eclipse XDB-C8 2.1 x 150 mm, particle size 3.5 µm, column (Agilent Technologies, Santa Clara, California, USA). The Mobile Phase A consisted of water in 0.5% acetic acid and Mobile Phase B acetonitrile. The flow rate was 0.35 ml/min. The column was eluted at room temperature with a linear gradient of 85% Mobile Phase A from 0 to 5 minutes. The gradient was then progressively changed to 75%A from 5 to 6.2 minutes and back to 85%A at 6.2 minutes. This was done using a constant flow rate of 0.35 ml/min and maintained at 85%A from at 6.21 to 7 minutes at a flow rate of 0.5 ml/min.

The triple quadrupole mass spectrometer is equipped with an electrospray ionization source (ESI). Ionization was performed in the negative mode and nitrogen was used as drying and nebulizing gas. Nitrogen was delivered by a nitrogen generator model N2-14-K727. Drying gas flow and temperature were set to 10 L/min and 300°C respectively with a nebulizer gas pressure to 40 psi. The applied capillary voltage was 3840 V.

Mass spectrometric analysis was accomplished by means of multiple reaction monitoring (MRM), and parameters such as fragmentation and collision energy were optimized for maximum abundance of ion transitions for each compound. The LC-MSMS method was developed in the negative detection mode using two MRM transition at a dwell time of 100 ms. The MRM was used to monitor the transition of the deprotonated molecule with m/z 457 [M-H]⁻ to the product ions 125, 169 and 305 for EGCG analysis and m/z 137 to product ions 106 and 119 for tyrosol. Collision energies for EGCG product ion 125 was 30 V and for product ions 169 and 305, 15 V. Collision energies for the internal standard (tyrosol) product ions 106 and 119 were 25 V. Fragmentor voltage of 120 V was used for transitions. All LC-MSMS data were processed by the Agilent MassHunter Workstation software Version B.01.00.

Calibration curves for EGCG ($R^2 = 0.99550701$) were done using freshly spiked plasma at six different concentrations 10, 20, 40, 80, 160, 320 ng/ml. Each batch was analysed with two quality controls (QC) of 160 ng/ml and 80 ng/ml.

3.2.6 Pharmacokinetic analysis

Baseline corrections of plasma concentrations of unchanged EGCG for all the volunteers at individual level for both treatments were performed. Pharmacokinetic analyses were performed on each individual set of data using the pharmacokinetic software WinNonlin version 5.0 (Pharsight Corp, Mountain View, California, USA). The Gauss-Newton method with Levenberg and Hartley modification (Davies and Whiting, 1972) was used to estimate the primary pharmacokinetic parameters, hypothetical volume of distribution (V_d), rate of absorption (K_{01}), rate of elimination (K_{10}) and lag time (T_{lag}). The Pharmacokinetic Model 4, (1 compartment 1st order, lag time, 1st order elimination) was used to fit the observed plasma concentration levels. The model is described by the following equation:

$$C_{plasma,EGCG}(t) = \frac{D * K_{01}}{V_d * (K_{01} - K_{10})} * (e^{-K_{10}*t} - e^{K_{01}*t})$$

where $C_{plasma,EGCG}(t)$ is concentration in response to time (ng/ml), D is dose (mg); K_{01} is the rate of absorption (min); K_{10} the rate of elimination (min); V_d is the hypothetical volume of distribution (L) and t is the time (min).

Parameters that were estimated were areas under the plasma concentration-time curves (AUC) for treatment A and B, maximum plasma concentration (C_{max}), time to reach the plasma concentration (T_{max}), absorption half life time ($t_{1/2,abs}$), elimination half life time ($t_{1/2,e}$), lag time (T_{lag}), hypothetical volume of distribution (V_d) and clearance (CL). Relative bioavailability was calculated using the equation below:

$$\text{Relative bioavailability} = \frac{[AUC]_{EGCG \text{ incorporated in Pheroid}^{TM}}}{[AUC]_{EGCG}}$$

where $[AUC]_{EGCG}$ incorporated in Pheroid™ and $[AUC]_{EGCG}$ are the total areas under the plasma concentration–time curves following the administration of a single dose of EGCG incorporated in Pheroid™ and EGCG respectively.

Areas under the curves were also calculated by WinNonlin, Version 5.0 software (Pharsight Corp, Mountain View, California, USA) with a noncompartmental model. Areas under the plasma concentration-time curves, T_{max} , and C_{max} were calculated for the 15 volunteers, and the data analysed statistically.

3.2.7 Statistical analysis

Data from the one-compartmental ($n = 9$) and the noncompartmental ($n = 15$) pharmacokinetic models were analysed with a data analysis software system STATISTICA version 7.1 (Statsoft Inc., Tulsa, OK, USA). Initially, variables were tested for normality using the Shapiro Wilk's W test; data was not normally distributed and therefore was logarithmically transformed. Logarithms of AUC_{0-480} , C_{max} , T_{max} , $t_{1/2,abs}$, $t_{1/2,e}$, T_{lag} , V_d and CL (for the two sets of data) were tested for normality using normal probability plots which resulted in normally distributed values. There was no deviation from the assumption of homogeneity of variances using the Bartlett chi-square test. A two-way ANOVA, where volunteers were nested within sequence and volunteers as random effects, was used to test for the sequence effect. It was found that the sequence had an effect on $AUC_{0-480\ min}$, but not on C_{max} and T_{max} for the noncompartmental pharmacokinetic analysis model data. Therefore, $AUC_{0-480\ min}$ for EGCG incorporated in Pheroid™ and EGCG in phase 1 (first period) were compared using a two-sample t-test (correcting for possible unequal variances), and for C_{max} and T_{max} , a three-way ANOVA with main effects, that is, volunteer, treatments and phase) was used. A two-way ANOVA was also used in the one-compartmental pharmacokinetic analysis model data, there was sequence effect on $AUC_{0-480\ min}$, C_{max} , V_d and CL , and therefore, a two-sample t-test was used to test this data for statistical significance. For the remaining pharmacokinetic variables which did not have a sequence effect, T_{max} , $t_{1/2,abs}$, $t_{1/2,e}$ and T_{lag} , a three-way ANOVA with main effects (volunteer, treatments and phase) was used for all variables. Results were expressed as geometric means (95% CI) and/or mean \pm SD unless stated otherwise. A p-value of below 0.05 was considered significant.

4 CHAPTER 4: RESULTS

4.1 INTRODUCTION

In a randomized controlled cross over study, 20 volunteers were given 400 mg EGCG and 400mg EGCG incorporated in Pheroid™. After administration of EGCG and EGCG incorporated in Pheroid™ in random order, blood samples were collected at different time points over 8 hours. These blood samples were analysed with an LC-MSMS and plasma concentrations of unchanged EGCG were obtained. Baseline corrections of plasma concentrations for all volunteers at individual level for both EGCG and EGCG incorporated in Pheroid™ were performed. A pharmacokinetic software program, WinNonlin was used to get plasma concentration-time curves using baseline corrected plasma concentrations of unchanged EGCG from 0 to 480 min of administration of either EGCG or EGCG incorporated in Pheroid™ (**Annexure M**). The plasma concentration-time curves, 20 from EGCG and 20 from EGCG incorporated in Pheroid™ obtained were visually inspected. Ten of these (from five volunteers) were excluded as they had no clear elimination phase. The plasma concentration-time curves were fitted into a one-compartmental model and pharmacokinetic data calculated. Further, a noncompartmental model was used to calculate pharmacokinetic variables. Nine complete pairs of plasma concentration-time curves were successfully fitted into a one-compartmental model, and the pharmacokinetic data obtained were $AUC_{0-480 \text{ min}}$, C_{max} , T_{max} , $t_{1/2,e}$, $t_{1/2,abs}$, T_{lag} , V_d and CL . The noncompartmental pharmacokinetic analysis model processed data for all 15 volunteers, and variables selected were $AUC_{0-480 \text{ min}}$, C_{max} and T_{max} . Pharmacokinetic variables obtained from the two models underwent statistical tests. The results from these two models are presented in this chapter.

4.2 RESULTS

Twenty-one volunteers were recruited for the study, of which one withdrew due to other commitments. Therefore, 20 volunteers were enrolled into the study. All the 20

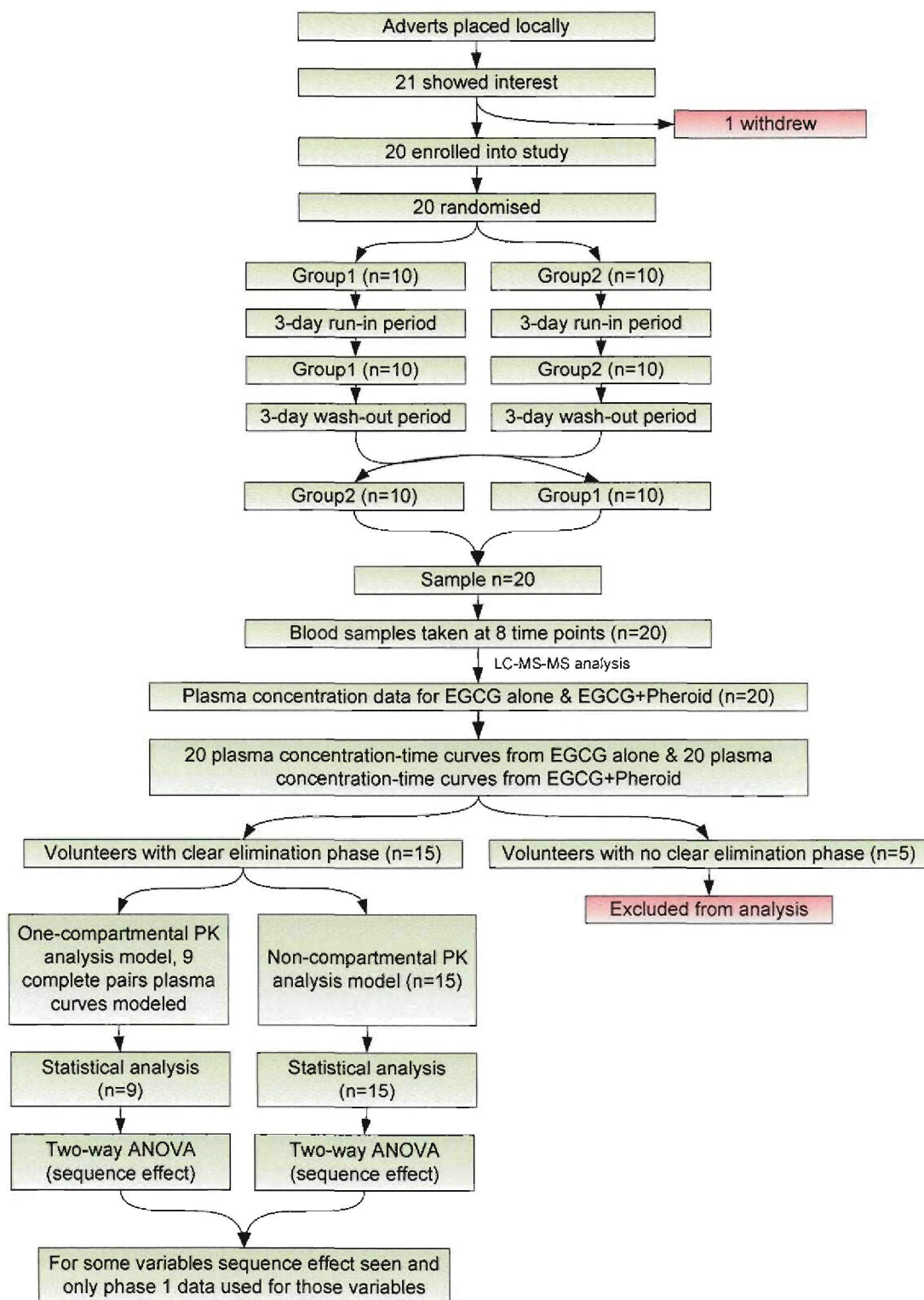


Figure 9: Schematic summary of crossover study and analyses where ANOVA, analysis of variance; EGCG, epigallocatechin gallate; LC-MSMS, liquid chromatography-mass spectrometry; PK, pharmacokinetic

volunteers (10 females and 10 males) completed the study. A schematic summary that depicts the research design and analyses performed is shown in **Figure 9**.

Data from five (5) volunteers was excluded from the pharmacokinetic and statistical analyses after visual inspection of the plasma concentration-time curves. Therefore, data from 15 volunteers was modelled either with a noncompartmental pharmacokinetic analysis model or a one-compartmental pharmacokinetic analysis model. The plasma concentration-time curves were excluded because they showed no clear elimination phase; these included 2 males and 3 females. One of these five volunteers experienced symptoms of hypoglycaemia during the trial. He reported that when he misses breakfast or any meal he experiences hypoglycaemic symptoms even though he does not have diabetes mellitus. This volunteer was given a snack (1 slice of bread with margarine and 1 glass of a caffeine-free fizzy drink) to alleviate the hypoglycaemic symptoms.

An example of a plasma curve that has no clear elimination phase is illustrated in **Figure 10** whereby the plasma concentration-time curve following administration of epigallocatechin gallate (EGCG) alone showed a continued rise in concentration.

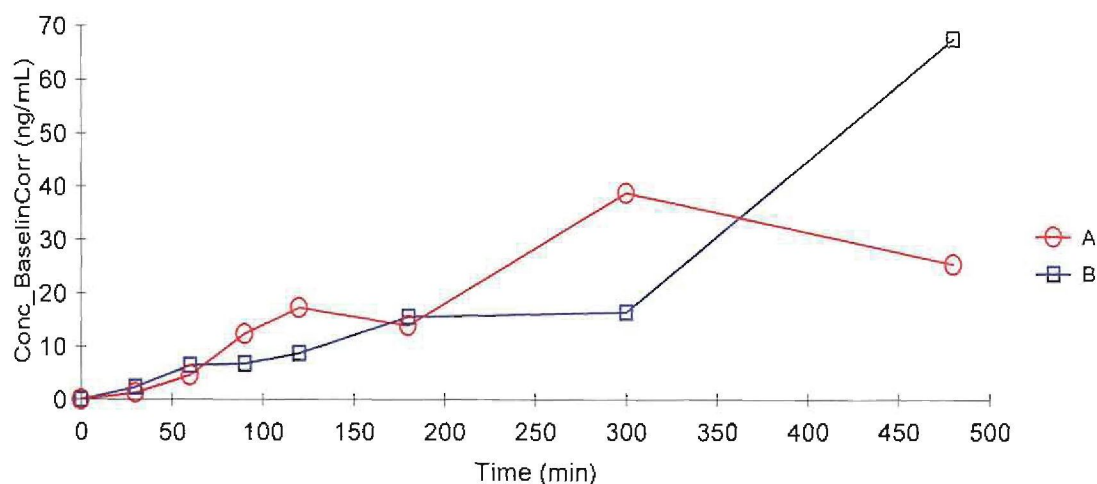


Figure 10: Plasma concentration-time curve for volunteer X

where A is EGCG incorporated in Pheroid™ and B is EGCG.

Table 3a and **Table 3b** show the descriptive characteristics of 20 volunteers (before exclusion of the 5 volunteers) and 15 volunteers (after exclusion of 5 volunteers) respectively.

Table 3a: Descriptive characteristics of volunteers who consumed 400 mg EGCG and 400 mg EGCG incorporated in Pheroid™ for all the 20 volunteers^{1,2}.

Characteristic	EGCG	EGCG incorporated in Pheroid™
Number of volunteers (n)	20	20
Males/females	10/10	10/10
Age (years)	23.55±0.67	23.55±0.67
Body mass index (kg/m²)	24.56±0.61	24.56±0.61

¹Data presented as means±SD

²EGCG, epigallocatechin gallate

Table 3b: Descriptive characteristics of volunteers who consumed 400 mg EGCG and 400 mg EGCG incorporated in Pheroid™ for 15 volunteers^{1,2}.

Characteristic	EGCG	EGCG incorporated in Pheroid™
Number of volunteers (n)	15	15
Males/females	8/7	8/7
Age (years)	23.73±3.33	23.73±3.33
Body mass index (kg/m²)	24.73±2.78	24.73±2.78

¹Data presented as means±SD

²EGCG, epigallocatechin gallate

Compliance to the intake of capsules was 100 % as there was close supervision and monitoring by the research team. All the 20 volunteers tolerated the dosages ingested. None of the 20 volunteers experienced negative side effects or adverse consequences following administration of either 400 mg EGCG or 400 mg EGCG incorporated in Pheroid™.

Mean baseline plasma concentrations of all the 20 volunteers was 5.15±0.350 ng/ml for EGCG incorporated in Pheroid™ and 5.10±0.192 ng/ml for EGCG (p=0.651), before baseline corrections of plasma concentrations were done at individual level.

This reflects that baseline plasma concentrations started at the same level before either EGCG incorporated in Pheroid™ or EGCG were given to volunteers. Mean baseline plasma concentrations for all the 20 subjects for the first period (4.99 ± 0.398 ng/ml) and for second period (5.16 ± 0.317) were also not statistically different from each other ($p=0.144$).

Observed plasma concentrations did not return to baseline values at time interval 480 min as seen in **Annexure M, Figure 10 and 11**.

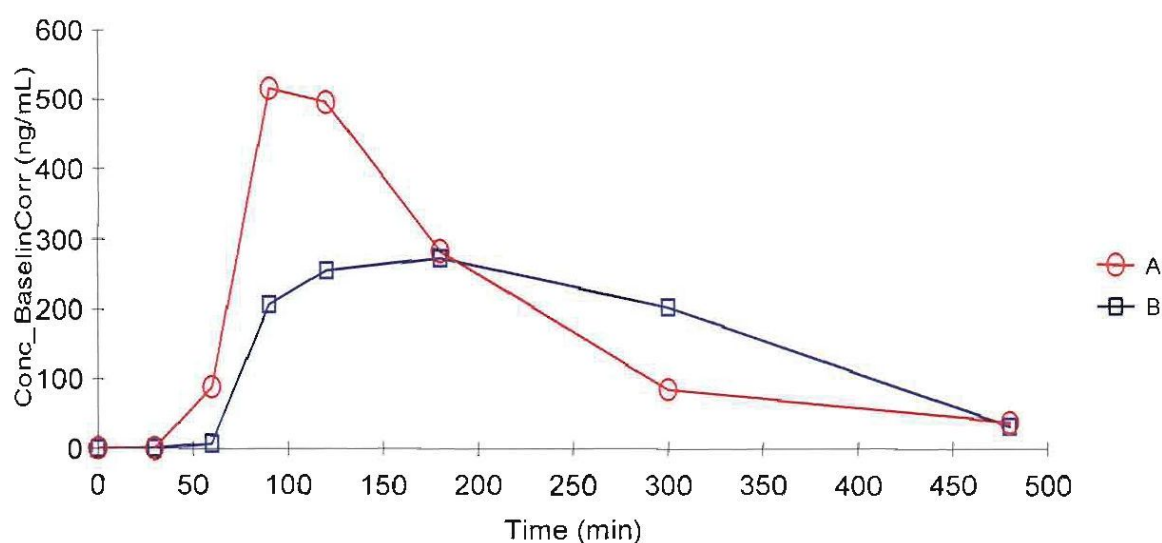


Figure 11: Plasma concentration-time curve for volunteer Y

where A is EGCG incorporated in Pheroid™ and B is EGCG

4.2.1 Noncompartmental pharmacokinetic model

The noncompartmental pharmacokinetic analysis model estimated areas under the plasma concentration-time curves ($AUC_{0 \rightarrow 480 \text{min}}$), the maximum plasma concentration (C_{max}) and the time taken to reach the maximum plasma concentration (T_{max}) data for 15 volunteers. Statistical analysis on this was subsequently performed. This data is presented in **Table 4**. A two-way ANOVA, where volunteers were nested within sequence and volunteers as random effects, was used to test for the sequence effect. It was found that the sequence had an effect on $AUC_{0 \rightarrow 480 \text{min}}$, but not on C_{max} and T_{max} for the noncompartmental pharmacokinetic analysis model data. Therefore, AUC for EGCG incorporated in Pheroid™ and EGCG in phase I were compared using a two-

sample t-test (correcting for possible unequal variances), and for C_{max} and T_{max} , a three-way ANOVA with main effects, that is, volunteer, treatments and phase) was used.

Table 4: Pharmacokinetic parameters obtained from a noncompartmental pharmacokinetic model

Parameter	EGCG			EGCG incorporated in Pheroid™			P-value
	Geometric mean (95% CI)	Mean±SD	N	Geometric mean (95% CI)	Mean±SD	N	
$AUC_{0 \rightarrow 480 \text{ min}}$, min*ng/ml [#]	14489 (7991-28220)	18106±13158	9	44797 (23172-78315)	50744±26273	6	0.005
T_{max} , min [‡]	227 (200-272)	236±65.0	15	167 (141-259)	200±107	15	0.130
C_{max} , ng/ml [‡]	96.2 (73.7-203)	139±117	15	133 (73.7-374)	224±271	15	0.150

where $AUC_{0-480 \text{ min}}$, area under the plasma concentration-time curve from time 0 to 480 min; CI, confidence interval; C_{max} , maximum plasma concentration; EGCG, epigallocatechin gallate; N, number of volunteers; SD, standard deviation; and T_{max} , time to reach the plasma concentration.

[#] Only data from first period was statistically analysed due to the sequence effect, a two-sample t-test was used.

[‡] For T_{max} and C_{max} , a three-way ANOVA test was used.

It seems the order in which EGCG and EGCG incorporated in Pheroid™ were given had an effect on $AUC_{0-480 \text{ min}}$ but not on C_{max} and T_{max} . A two-way ANOVA test showed a statistically significant sequence effect on $AUC_{0-480 \text{ min}}$ ($p=0.032$). Data for the second period was therefore ignored and statistical analysis confined to the first period alone as suggested by Sibbald & Roberts (1998).

Areas under the plasma concentration-time curves for the first period showed that there was a statistically significant difference between EGCG incorporated in Pheroid™ (50744±26273 min*ng/ml) and EGCG (18106±13158 min*ng/ml) with a p value of 0.005. Maximum concentration for EGCG incorporated in Pheroid™ (224±271 ng/ml) was higher than that of EGCG (139±117 ng/ml) and T_{max} of EGCG incorporated in Pheroid™ (200±107 min) was shorter than the EGCG (236±65 min).

These results suggest that there may be increased bioavailability, however, without significant changes to T_{max} and C_{max} .

Relative bioavailability calculated from the noncompartmental pharmacokinetic model data was found to be 1.72 ± 1.32 , a percent relative bioavailability of 72%.

4.2.2 One-compartmental pharmacokinetic model

Pharmacokinetic analysis performed on each individual set of data using a compartmental model yielded data from only 9 complete pairs. The 9 volunteers were 6 males and 3 females with mean age of 22.4 ± 1.9 years and mean body mass index of 25.6 ± 3.0 kg/m². These are reported in **Table 5**.

Table 5: Pharmacokinetic parameters obtained from a one-compartmental pharmacokinetic model

Parameter	EGCG			EGCG incorporated in Pheroid™			P-value
	Geometric mean (95% CI)	Mean±SD	N	Geometric mean (95% CI)	Mean±SD	N	
AUC ₀₋₄₈₀ , min*ng/ml [#]	19548 (9392-33258)	21325±9611	5	60988 (14071-120384)	67228±33406	4	0.013
T _{max} , min [‡]	180 (148-223)	185±49.0	9	132 (85.6-233)	159±95.7	9	0.326
C _{max} , ng/ml [#]	48.6 (25.4-79.7)	52.6±21.9	5	173 (-97.0-556)	229±205	4	0.052
T _{lag} , min [‡]	52.6 (34.4-104)	69.1±45.2	9	43.5 (24.6-78.7)	51.7±35.3	9	0.606
t _{½,abs} , min [‡]	37.3 (16.4-108)	62.2±59.6	9	29.5 (16.2-118)	67.1±66.3	9	0.703
t _{½,e} , min [‡]	158 (36.6-394)	215±233	9	154 (55.3-368)	212±204	9	0.969
V _d , L [#]	5571 (497-12838)	6667±4970	5	1304 (-18.9-3195)	1588±1010	4	0.024
CL, L/min [#]	20.5 (9.71-35.0)	22.3±10.2	5	6.56 (1.53-12.9)	7.23±3.58	4	0.013

where AUC₀₋₄₈₀, area under the plasma concentration-time curve; CL, clearance; C_{max}, maximum plasma concentration; EGCG, epigallocatechin gallate; t_{½,e}, the elimination half life; t_{½,abs} the absorption half life; T_{lag}, time to reach first appearance in plasma; T_{max}, time to reach the plasma maximum concentration and V_d, hypothetical volume of distribution.

[#] Only data from first period was statistically analysed due to the sequence effect, a two-way t-test was used.

[‡] For T_{max} and C_{max}, a three-way ANOVA test was used.

All the selected variables were tested for the sequence effect with a two-way ANOVA test. Area under the plasma concentration-time curve, C_{max}, hypothetical volume of

distribution and clearance had a sequence effect, therefore, a two-sample t-test was used to test for statistical significance. The remaining pharmacokinetic variables which did not have the sequence effect, T_{max} , T_{lag} , $t_{1/2,e}$ and $t_{1/2,abs}$, a three-way ANOVA test was done.

Areas under the plasma concentration-time curve from time 0 min to 480 min, maximum plasma concentration, hypothetical volume of distribution and clearance had a sequence effect; therefore only data for the first period was tested for statistical significance. All these variables were found to show statistical difference between EGCG incorporated in Pheroid™ and EGCG. AUC_{0-480} for EGCG incorporated in Pheroid™ (67228±33406 min*ng/ml) was higher than that of EGCG (21325±9611 min*ng/ml) with a p-value of 0.013. Maximum concentration of EGCG incorporated in Pheroid™ (229±205 min) was higher than that of EGCG (52.6±21.9) with a p-value of 0.052. Hypothetical volume of distribution was 1588±1010 litres for EGCG incorporated in Pheroid™ and 6667±4970 litres for EGCG (p=0.024); and clearance was 7.23±3.58 litres/min for EGCG incorporated in Pheroid™ and 22.3±10.2 litres/min for EGCG (p=0.013).

The time taken to reach maximum plasma concentration for EGCG incorporated in Pheroid™ (159±95.7 min) was slightly shorter than the EGCG (185±49.0 min). Elimination half lives for EGCG incorporated in Pheroid™ and EGCG were estimated to be 212±204 min and 215±233 respectively. Absorption half life for EGCG incorporated in Pheroid™ (67.1±66.3 min) was slightly higher than that of EGCG (62.2±59.6 min). The time to reach first appearance in the blood T_{lag} was 51.7±35.3 min for EGCG incorporated in Pheroid™ and for EGCG was 69.1±45.2 min. All variables which did not have a sequence effect showed no statistical difference between EGCG incorporated in Pheroid™ and EGCG.

4.3 SUMMARY

The pharmacokinetics in human volunteers were studied to determine whether a fatty acid delivery system Pheroid™ could increase the bioavailability of EGCG.

The results obtained from the variables that did not have a sequence effect from the noncompartmental and the one-compartmental pharmacokinetic analysis models suggest that Pheroid™ increases bioavailability of EGCG. However, this was not statistically significant for all variables. Significant differences between EGCG incorporated in Pheroid™ and EGCG were seen when AUC_{0-480} obtained from the noncompartmental model, and AUC_{0-480} , C_{max} , V_d and CL obtained from the one-compartmental model were treated differently from other variables due to sequence effect, but this should be treated with caution as the second period data was discarded, therefore losing the advantages of a crossover design.

A sequence effect was found in four out of eight pharmacokinetic variables chosen for the one-compartmental pharmacokinetic model variables, namely AUC_{0-480} , C_{max} , volume of distribution and clearance. A statistical significant difference between EGCG incorporated in Pheroid™ and EGCG was found to exist. These results should also be treated with caution as only first period data was tested for significance due to sequence effect.

5.1 INTRODUCTION

Following a randomised cross over designed study, plasma concentrations of unchanged EGCG were obtained from the LC-MSMS analysis. This data was processed in a pharmacokinetic software program, WinNonlin, which yielded the plasma concentration-time curves for all 20 volunteers for both EGCG and EGCG incorporated in Pheroid™. Individual plasma concentrations curves were then modelled using both the one-compartmental and the noncompartmental pharmacokinetic models. Data from these models was analysed statistically.

Pharmacokinetic variables calculated using a one-compartmental pharmacokinetic analysis model which had a sequence effect were $AUC_{0-480 \text{ min}}$, C_{max} , V_d and CL . It was found that for each of these variables there was a statistical difference between EGCG incorporated in Pheroid™ and EGCG. For the other variables which were calculated using this pharmacokinetic model which did not have any sequence effect, T_{max} , $t_{1/2,e}$, $t_{1/2,abs}$ and T_{lag} , it was found that there was no significant difference between EGCG incorporated in Pheroid™ and EGCG.

Data calculated using a noncompartmental model was found to have a sequence effect on AUC_{0-480} , but not on C_{max} and T_{max} . Using the first period AUC_{0-480} data, it was found that Pheroid™ significantly influenced the bioavailability of EGCG positively ($p = 0.005$) whereas data from C_{max} and T_{max} showed no significant difference between EGCG incorporated in Pheroid™ and EGCG.

This chapter will discuss these results, and draw conclusions and recommendations from the results obtained.

5.2 DISCUSSION

The study was designed to investigate the possibility of increasing bioavailability of EGCG using a fatty acid delivery system. This was because of the potential role of this flavonoid in the prevention of lifestyle diseases/conditions such as cardiovascular diseases, cancer, hypertension, obesity and diabetes mellitus in humans (Dulloo *et al.*, 1999; Pisters *et al.*, 2001; Yang *et al.*, 2004; Nagao *et al.*, 2005; Henning *et al.*, 2006; Kao *et al.*, 2006 and Widlansky *et al.*, 2007). Information on its bioavailability and disposition is therefore important for understanding the biological effects of green tea or green tea extracts.

Plasma concentrations before ingestion of EGCG incorporated in Pheroid™ and EGCG at baseline, that is, time zero (0) minutes was 5.15 ± 0.350 ng/ml and 5.10 ± 0.192 ng/ml respectively ($p=0.651$), even after an overnight fast. Mulder (2007) in a personal communication confirmed that they have found mean fasting EGCG concentrations of 10 nmol/L, an equivalent of approximately 5 ng/ml, in their pharmacokinetic studies. This may be due to the fact that very low levels of EGCG can occur in other foods that are not yet identified as sources of EGCG. Precautions were taken to exclude all the known sources of EGCG while providing the volunteers with a variety of foods low in flavonoids and free in catechin. Epigallocatechin gallate is the main catechin in green tea, and any other form of tea was not allowed in the study. Therefore the source of EGCG in the blood before administration of EGCG incorporated in Pheroid™ and EGCG is not known.

It was decided that both the noncompartmental and the one-compartmental pharmacokinetic analyses models be used to obtain data as the one-compartmental model successfully fitted only nine (9) complete pairs of plasma concentration-time curves, a very small sample with limited power. Even though the noncompartmental model can calculate only a limited number of pharmacokinetic variables as compared to the one-compartmental model, it successfully modelled data for 15 volunteers, providing more statistical power. Therefore, data obtained from both models was utilised.

Maximum plasma concentrations obtained from the one-compartmental pharmacokinetic model for EGCG incorporated in Pheroid™ was found to be 229 ± 205 ng/ml and that of EGCG was 52.6 ± 21.9 ng/ml ($p=0.052$). The time taken to reach maximum plasma concentration of EGCG incorporated in Pheroid was 159 ± 95.7 min (2.65 hours), slightly shorter than for EGCG which was found to be 185 ± 49.0 min (3.08 hours); with no statistical difference between EGCG incorporated in Pheroid and EGCG. Low C_{max} and long T_{max} show that there is a slow rate of absorption (Zhu *et al.*, 2000). A slow rate of absorption and therefore low bioavailability is indicated by a low C_{max} when EGCG alone was administered. Elimination half lives of EGCG incorporated in Pheroid™ and EGCG were estimated to be 212 ± 204 min (3.53 hours) and 215 ± 233 min (3.58 hours) respectively. This is similar to what is reported in the literature. Ullmann *et al.*, (2003) after administering 50 to 1600 mg pure EGCG orally obtained a T_{max} of 1.3 to 2.2 hours, C_{max} of 130 – 3392 ng/ml and elimination half life of 1.9 to 4.6 hours.

The results obtained from this study are also similar to those by Chow *et al.*, (2001) after oral ingestion of pure EGCG (200-800 mg). They found that C_{max} for EGCG ranged from 73.7 to 438.5 ng/ml (in this study 229 ± 205 ng/ml EGCG incorporated in Pheroid™ and 52.6 ± 21.9 ng/ml EGCG), T_{max} from 109-241 min (159 min EGCG incorporated in Pheroid™ and 185 min EGCG), AUC_{0-480} min from 22000 to 167000 min*ng/ml (67228 ± 33406 min*ng/ml EGCG incorporated in Pheroid™ and 21325 ± 9611 min*ng/ml EGCG). Elimination half life obtained by Chow *et al.*, (2001) was, however, lower than that detected in the current study for both EGCG incorporated in Pheroid™ and EGCG, at 118 to 184 min (212 ± 204 min EGCG incorporated in Pheroid™ and 215 ± 233 min EGCG). Pheroid™ did not change the elimination half life of EGCG significantly.

Area under the plasma concentration-time curve from time 0 min to 480 min calculated from a noncompartmental pharmacokinetic model for EGCG incorporated in Pheroid™ was 50744 ± 26273 min*ng/ml and EGCG was 18106 ± 13158 min*ng/ml. These results, though statistically significant ($p=0.005$), would have been stronger if we did not have sequence effect. Mean maximum plasma concentrations for EGCG

was 139 ± 117 ng/ml and for EGCG incorporated in Pheroid™ was 224 ± 271 ng/ml. The time to reach maximum plasma concentration for EGCG was 236 ± 65.0 min and for EGCG incorporated in Pheroid™ was 200 ± 107 min. Maximum plasma concentration and T_{max} in the current study are comparable with the values obtained by Chow *et al.*, (2001) after administration of 200 to 800 mg EGCG. The higher the C_{max} and the shorter the T_{max} , the more bioavailable is a compound, suggesting that Pheroid™ may have increased the bioavailability of EGCG, though not statistically significant.

The time to reach first appearance in plasma (T_{lag}) following oral administration of EGCG incorporated in Pheroid™ was not significantly different from that following oral administration of EGCG. EGCG first appeared in the plasma at 51.7 ± 35.3 min and 69.1 ± 45.2 min for EGCG incorporated in Pheroid™ and EGCG respectively. This could possibly be due to lack of power because we cannot exclude the possibility that there might be a significant difference if we had adequate power.

Apparent volume of distribution (V_d) was found to be 1588 ± 1010 litres and 6667 ± 4970 litres ($p=0.024$) for EGCG incorporated in Pheroid™ and EGCG respectively. Clearance (CL) was found to be 7.23 ± 3.58 litres/min and 22.3 ± 10.2 litres/min ($p=0.013$) for EGCG incorporated in Pheroid™ and EGCG respectively. These results are similar to those obtained by Chow *et al.*, 2001 who found that volume of distribution of EGCG ranged from 1044 to 4774 litres and likewise, clearance was 6.0 to 18.0 litres/min; reflecting very low bioavailability of EGCG in humans.

Relative bioavailability fraction estimated from the noncompartmental model was found to be 1.72 ± 1.32 , that is, percent relative bioavailability was about 72%. It was not possible to estimate absolute or oral bioavailability of EGCG as both forms of EGCG were only given orally and not intravenously. Absolute bioavailability of green tea catechins has not been established in humans due to lack of pharmacokinetic studies following intravenous administration (Cai *et al.*, 2002).

Results obtained from both the noncompartmental and the one-compartmental models suggest that EGCG has a low bioavailability, even when Pheroid™ was used. Though EGCG has been found to have some health benefits, vast evidence exist that EGCG is poorly bioavailable in humans (Nagakawa *et al.*, 1997; Chow *et al.*, 2001; Lee *et al.*, 2002; Ullmann *et al.*, 2003), the gut being the major limiting factor. This study has further confirmed that indeed EGCG administered orally has low bioavailability. Poor bioavailability may be due to poor absorption in the small intestines, and extensive metabolism in the colon and liver, only to mention a few. Therefore, other means of uptake which bypass the gut may be used to attempt to increase EGCG bioavailability, for example transdermal application of EGCG incorporated in Pheroid™. Lambert *et al.*, (2006) performed a study in mice and they concluded that EGCG transdermal gel was useful for delivering prolonged levels of EGCG to the plasma and tissues, and this may provide an alternative to oral administration of EGCG. Epigallocatechin gallate is delivered across the skin into the systemic circulation, thereby, bypassing poor absorption in the small intestines, extensive metabolism in the colon and liver, and other barriers that limit bioavailability of orally administered EGCG.

A potential mechanism explaining how Pheroid™ interacted with EGCG may be similar to that occurring for other lipid bilayers. Hashimoto *et al.*, (1999) concluded that the presence of gallic esters in the structure of EGCG is responsible for its high affinity to lipid bilayers and high amounts incorporated into the bilayer. In addition to the gallic esters in the structure of EGCG, the affinity of tea catechins for lipid bilayers is governed by the number of hydroxyl groups on the B-ring of the catechin and the stereochemical structure of the catechin (Kajiya *et al.*, 2002).

It is assumed that ingestion of a low flavonoid/catechin free breakfast after 3 hours of administering either 400 mg EGCG incorporated in Pheroid™ or 400 mg EGCG did not affect the absorption of EGCG negatively as the time taken for EGCG incorporated in Pheroid™ and EGCG to reach maximum concentration was found to be within 3 hours as reflected by the data calculated by a noncompartmental model (2.65 hours and 3.08 hours respectively). We therefore believe that a meal given 3 hours after the administration of EGCG did not influence the results. Food could have

reduced EGCG absorption if the volunteers had taken the two types of EGCG on full stomachs in a non-fasting state, as researchers like Ullmann (in an unpublished study) found that food reduced EGCG absorption in animal studies (Ullmann *et al.*, 2003). It was not practical to make volunteers to fast 8 hours post an overnight fast. Therefore, using the available literature from Manach *et al.*, (2005), who evaluated 97 bioavailability studies and concluded that the maximum time taken to reach maximum plasma concentration ranged between 1.6 to 3.2 hours, it was decided to give breakfast 3 hours after the administration of EGCG.

All reasonable precautions were taken to avoid conditions that could potentially hinder an increase in the bioavailability of EGCG or anything that has a possibility of giving false results. Volunteers were fed a low flavonoid/ catechin free diet, with all meals and snacks being provided; as the presence of other tea polyphenols in significant amounts may affect the absorption and disposition of EGCG (Yang *et al.*, 1998). Additionally, volunteers were given a list of foods to avoid and they were given guidance as to what to eat in case they felt hungry while out of campus. Further precautions were taken by designing a randomised controlled trial with a run-in period of 3 days and a washout period of 3 days. Blood samples were then drawn following a 3-day run-in period and an overnight fast before another administration of EGCG, followed by a 3-day washout period. Furthermore, study volunteers were fasted prior to EGCG administration, making conditions conducive for increased rate of EGCG absorption. Chow *et al.*, (2005) concluded that greater oral bioavailability of free EGCG could be achieved when capsules were taken on an empty stomach following an overnight fast. Human volunteers in their study tolerated the 400 and 800 mg well under fasting conditions, and only experienced nausea in the highest dosage of 1200 mg. It is possible that green tea catechins may be more stable in a fasted stomach than a fed stomach as they have been shown to be more stable under acidic conditions. After ingestion of a meal the gastric juice is buffered to a less acidic pH, which is known to adversely influence stability and hence bioavailability. The presence of food in the GI tract can influence bioavailability by a number of mechanisms. It may be through the formation of insoluble complexes with food components; alteration of pH, alteration of gastric emptying and GI motility (gastric emptying rate being delayed); stimulation of gastrointestinal secretions; competition

between food components and drugs for specialised absorption mechanisms; increased viscosity of GI contents; and food induced changes in the blood flow (Ashford, 2002a) as previously stated in the literature review.

Even though all reasonable precautions were taken, it is acknowledged that some variables obtained in both the noncompartmental and the one-compartmental models had a sequence effect which was a limitation in this study as it led to only the first period data of such variables to be statistically analysed, therefore losing the advantages of a cross over designed trial. Cross over designed trials efficiently eliminate the difference between volunteers, because each volunteer serves as his or her own control, it deals with the effects of trends or differences between periods and it can provide unbiased estimates for the differences between treatments (Senn, 2003; Reed, 2004). To avoid a carryover effect, volunteers had a washout period of 3 days, which was regarded adequate to clear the EGCG in the blood.

A carryover effect was not anticipated as generally catechins have short half-lives as compared to other polyphenols. Williamson & Manach (2005) established that catechins have an elimination half-life period of 2-3 hours. In addition, Yang *et al.*, (1999) has predicted that most catechins would be cleared from the body within 10 to 12 hours and would not accumulate in the body. In a personal communication with Mulder (2007), he further confirmed the above evidence and stated that due to relatively long plasma half-life of more than 4 hours, a washout period of more than 1 day is required, a washout period of 3 days (an equivalent of 18 half lives) was therefore more than enough to eliminate EGCG in the plasma. It is therefore likely that this was a sequence effect and not a carry over effect as first period baseline plasma concentrations were more or less at the same level as compared to the second period baseline plasma concentrations. For all the 20 volunteers, first period mean \pm SD baseline plasma concentration was 4.99 \pm 0.40 ng/ml and second period baseline plasma concentration was 5.16 \pm 0.32 ng/ml with no statistical difference between the two periods.

A sequence effect changes the effectiveness of the drug treatment produced by the order in which the drugs were administered, whereas a carryover effect occurs when

the effect of the drug given in the first period persists into the second period despite a washout period (Reed, 2004). The cause of a sequence effect is therefore not known. It may have occurred because there were only 10 rooms available in the metabolic unit where volunteers could be accommodated overnight and during the trial day, leading to the 20 volunteers who were enrolled being divided into two groups. Ten (10) volunteers per group instead of all the 20 volunteers, were given 400 mg EGCG incorporated in Pheroid™ and 400 mg EGCG in random order in one day. Following the administration of EGCG was a 3-day run-in and then 3-day washout period, followed by another administration of the different types of EGCG depending on what was given in the first period. Each group took two weeks to complete the study as indicated in the calendar for volunteers (**Annexure B** and **Figure 12** below). Group 1 was followed by group 2 which also underwent the same procedure. This design was therefore not the usual two-period, two-treatment design which is usually applied in crossover trials.

Date of Trial	Treatment A	Treatment B		
06/05/05	19, 21, 43, 77, 89	30, 31, 37, 55, 93	(Group 1)	} 1 st period
20/05/05	40, 54, 71, 81, 87	49, 67, 76, 80, 91	(Group 2)	
13/05/05	30, 31, 37, 55, 93	19, 21, 43, 77, 89	(Group 1)	} 2 nd period
27/05/05	49, 67, 76, 80, 91	40, 54, 71, 81, 87	(Group 2)	

Figure 12: Treatment allocation

where Treatment A is 400 mg EGCG incorporated in Pheroid™ and Treatment B is 400 mg EGCG

The other possible limitation of the study was that there was no pilot study before commencing the main study. A pilot study would have helped to establish the time to take the last sample (0-infinity), as for all the 20 volunteers' plasma concentration-time curves did not go back to baseline concentrations in a given period. Probably the 9th time point of blood collection at 24 hours could have been included. This was not possible because the volunteers were postgraduate students and we did not want to interfere with their academic work by keeping them in the metabolic unit for a longer period. Although a pilot study in this form is expensive and quite invasive, it would have helped to ensure that the investigators knew exactly how EGCG performed

under the test conditions, which could have aided in establishing more conclusive information on the ability of Pheroid™ to improve the bioavailability of EGCG.

5.3 CONCLUSION

From the results of the study obtained:

- It is safe for healthy individuals to ingest 400 mg EGCG orally in the presence or absence of Pheroid™. Volunteers tolerated the given dosage as no adverse effects were reported during the study following administration of EGCG.
- In general and taking all markers of bioavailability into account, Pheroid™ did not increase bioavailability of EGCG significantly. Both the one-compartmental and the noncompartmental pharmacokinetic results suggested that there was no significant difference between EGCG incorporated in Pheroid™ and EGCG. This is in exception for $AUC_{0 \rightarrow 480 \text{min}}$, (for both compartmental models), T_{max} , V_d and CL (for the one-compartmental model) where only data from the first period was statistically analysed following sequence effect.
- Data from the first period alone cannot be used to support the hypothesis as the advantages of a crossover design were lost due to sequence effect.
- It is tempting to conclude that there is a difference based on only $AUC_{0 \rightarrow 480 \text{min}}$ data. One has to remember that the totality of the information should be used to draw conclusions and when doing so, one cannot conclude that there is a significant difference in bioavailability. On the other hand, because of the limited power, it cannot be excluded that the possibility of drawing a false conclusion that there is no effect while in actual fact there is one. This is partly due to the sequence effect (and loss of data).

5.4 RECOMMENDATIONS

- Pilot study is a necessity. This could have helped to identify unclear or incomplete elimination phase before the study was commenced, and would

have determined the last time interval to draw a blood sample showing clear elimination. Therefore, for future bioavailability studies, pilot studies are strongly recommended.

- It may be prudent to recommend that the study be performed with a larger number of volunteers (power to be calculated based on these results).
- The cause of sequence effect in the areas under the plasma concentration curves of EGCG incorporated in Pheroid™ and EGCG when both the noncompartmental and the one-compartmental pharmacokinetic analysis models were used should be investigated further.
- The source or cause of the baseline fasting EGCG concentrations of 5.15 ± 0.350 ng/ml and 5.10 ± 0.192 ng/ml for EGCG incorporated in Pheroid™ and EGCG respectively, even after an overnight fast should be further investigated.
- EGCG metabolites should be monitored as they may have some health benefits.
- The primary role of EGCG could be in the gut, and some secondary effects may be responsible for probable health benefits, this would require some additional work, and scientific development.

Annexure A: CONSENT

Title of the project: The effect of the fatty acid based carrier on the bioavailability of epigallocatechin gallate.

I, the undersigned (full names) read/listened to the information on the project and I declare that I understand the information. I had the opportunity to discuss aspects of the project with the project leader and I declare that I participate in the project as a volunteer. I hereby give my consent to be a volunteer in this project.

I indemnify the University, also any employee or student of the University, of any liability against myself, which may rise during the course of the project.

I will not submit ant claims against the University regarding personal detrimental effects due to the project, due to negligence by the University, its employees or students, or any other volunteers.

(Signature of the volunteer)

Signed at On

Witnesses

1.
2.

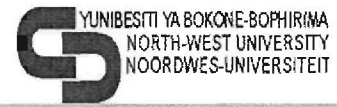
For experimenting with a married persons the following indemnity from the spouse is required.

I, (full names), the spouse of the volunteer in this project, hereby undertake not to submit any claims against the University regarding treatment in case of death or injuries of this person due to the project as described in this application, due to negligence of the University, its students or another volunteer, or in any other way.

Signature: Date:

Relationship:

Annexure B: INFORMATION FOR VOLUNTEERS



STUDY NAME: EGCG study

Information for volunteers

For more information contact the registered Nursing Sister,
Chrissie Lessing at the Metabolic Unit/Lipid Clinic, Potchefstroom Campus,
North-West University, Extension 2480
E-mail: VGEMCL@puk.ac.za



POTCHEFSTROOM CAMPUS



Annexure B continued

Background

There is scientific evidence which suggest that foods rich in antioxidants play an important role in the prevention of heart disease, cancer, skin damage, diabetes mellitus, just to mention a few. We know that less than 2% of the ingested antioxidant ends up in the blood. We aim to increase the absorption of this antioxidant from green tea by using a carrier molecule given as a capsule.





What is going to happen?

- Screening will be done to ensure that you meet the inclusion criteria.
- Your weight, height, temperature and blood pressure will be taken
- You are expected to complete a medical background questionnaire and give it to the Registered Nurse.
- You will be admitted in the Metabolic Unit of the nutrition Department of the north-West University on the 5th and 12th of May OR 19th and 26th of May 2005 (Thursdays) at 4 p.m. for an overnight stay.
- You will be provided with all meals in the metabolic Unit.
- Blood samples will be collected on the 6th and 13th of May 2005 OR 20th and 27th of May 2005 (Fridays), 8 samples per day and each sample will be 5 ml.
- After your second blood sample visit, on the 13th OR 27th of May 2005, you will be compensated with R500.

What is expected from you?

- You need to be committed and reliable for the duration of the study, the 3rd – 13th OR 17th - 27th of May 2005 (see calendar).
- To book in at the metabolic unit on your appointment date (5th and 12th May 2005 OR 19th and 26th of May 2005) at 4 p.m.
- To be willing to sleep over for the night and stay in the Metabolic Unit until the following day.
- To be willing to refrain from tea and coffee throughout the study period.
- To follow the planned menu on the specified times (3rd – 6th of May 2005 & 10th – 13th of May 2005 OR 17th – 20th of May 2005 & 24th – 27th 2005).
- To remember to record any food eaten that is not in the menu.
- To avoid taking vitamin and mineral supplements throughout the study period.
- To remember to complete the medical record chart.
- Not to smoke at all.
- If at all taking alcohol, females can have 1 glass and males less than 2 glasses of alcohol.
- To fast from 10 p.m. on the 5th and 12th of May OR 19th and 26th of May 2005 (Thursdays) and go to bed to rest.
- A cannula will be put in place on Friday morning to take blood samples and will stay in place until all 8 blood samples are taken. Thank you!

Annexure B continued

MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
2	3	4	5	6	7	8
	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
	3 DAY PRESCRIBED DIET			TRIAL DAY	WASHOUT PERIOD (HOME)	WASHOUT PERIOD (HOME)
	Breakfast	Breakfast	Breakfast	 Breakfast		
	Lunch	Lunch	Lunch	 Lunch		
	Dinner	Dinner	Dinner OVERNIGHT STAY	HOME		
MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY	SUNDAY
9	10	11	12	13	14	15
WASHOUT PERIOD (HOME)	DAY 7	DAY 8	DAY 8	DAY 10	DAY 11	DAY 12
	3 DAY PRESCRIBED DIET			TRIAL DAY		
	Breakfast	Breakfast	Breakfast	 Breakfast		
	Lunch	Lunch	Lunch	 Lunch		
	Dinner	Dinner	Dinner OVERNIGHT STAY	HOME		



Ingestion of a capsule



Blood sample collection

Annexure C : EXIT ASSESSMENT FORM

Protocol Number: NWU/ND/01/2005

Study Name: EGCG STUDY

1. PERSONS PRESENT AND DESIGNATION

M.C. LESSING
(SUSTER) GEREJISTREERDE
VERPLEEGKUNDIGE

2. TERMINATION OF STUDY

N/A YES NO

a) Study completed

b) Other

If other, specify _____

3. SUBJECT ASSESSMENT

a) Number of subjects planned: 20 Comment: _____

b) Number of subjects screened: 21 Comment: I dropped before commencing & study and was replaced.

c) Number of subjects enrolled: 20 Comment: _____

d) Number of subjects completed: 20 Comment: _____

e) Number of subjects discontinued: 0 Comment: _____

4. STUDY PROCEDURES

N/A YES NO

* Give details in comment section

EGM

a) Protocol / compliance to protocol

b) Informed consent completion

c) Have all severe adverse events (SAEs) been followed to completion

d) Have new SAEs occurred sine last visit

Annexure C continued

STUDY PROCEDURES Continued	N/A	YES	NO
e) Biological samples being correctly handled	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>
f) Any changes to trial team member	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>

5. STUDY SUPPLIES	N/A	YES	NO
a) Product supplies accountable	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>
b) Expiry date checked	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>
c) Removal of study supplies arranged	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
d) Have randomisation codes been opened	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
e) Randomisation codes removed	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>

6. STUDY ARCHIVING	N/A	YES	NO
a) Are study documents ready for archiving?	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>

7. COMMENTS

Section	Comment	Action	Date
4 c)	No SAEs reported		
4 d)	No SAEs reported		

Completed by: KGOMOTSO G. MORUISI Date: 27/05/05

Annexure D: MEDICAL HISTORY QUESTIONNAIRE

Protocol Number:
Study: EGCG Study

Date: _____ Interviewer: _____

VOLUNTEER INFORMATION

Name: _____ Volunteer Number:
Date of birth: _____ Gender: _____
Address: _____

Telephone: (h) _____ (w) _____ Cell: _____

ANTHROPOMETRY

Weight: _____ kg Height: _____ m BMI: _____ (kg/m²)

MEDICAL HISTORY:

Past Medical History:

Medical questions (please tick or circle in the box where necessary):

1a) Do you have any current illness(es)? Yes No
b) If yes, which one(s)? _____

2. Do you use any medication for above illness(es)? Yes No
3. What medication do you use? _____

4a) Do you have any allergies? Yes No
b) If yes, which one(s)? _____

5a) Do you drink tea or coffee? Yes No
b) If yes, how many cups/day? _____

6. Do you use any vitamin and/or mineral supplements? Yes No

7. Do you use any herbal products? Yes No

8. Do you use any food supplements? Yes No

9a) Do you take alcohol? Yes No
b) If yes, how many glasses do you take per week? _____

10a) Do you smoke? Yes No

b) If no, have you ever smoked? Yes No
c) If yes, when did you stop smoking? _____

Signature: _____

Annexure E: TEAVIGO PRODUCT INFORMATION

DSM Nutritional Products



Product Data Sheet

TEAVIGO™

Description

TEAVIGO™ is a highly purified extract from the leaves of green tea (*Camellia sinensis*) in the form of a fine, off-white to pale pink powder. It is composed of a minimum of 90% epigallocatechin gallate (EGCG), and has a melting point between approx. 210 °C and 215 °C.

Product identification

Product code: 50 0285 0

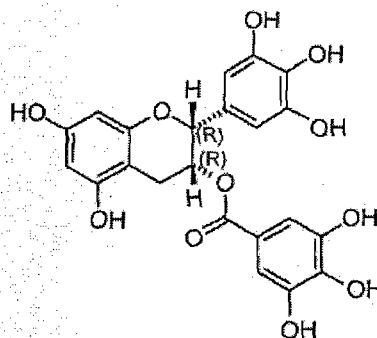
Chemical name: polyphenol (-)-epigallocatechin-3-gallate

Synonyms: epigallocatechin gallate (EGCG)

CAS No.: 989-51-5

INCI name: epigallocatechin gallate

Molecular mass: 458.4 g/mol



Specifications

Appearance: off-white to pale pink powder

Identity: corresponds

Loss on drying: max. 5%

Heavy metals: max. 10 ppm

Arsenic: max. 3 ppm

Lead: max. 5 ppm

Assay:

min. 94% EGCG (on dry material)

max. 0.1% caffeine

Microbiological purity: corresponds

Solubility

EGCG is fairly soluble in water, ethanol, methanol and acetone.

DSM Nutritional Products



Product Data Sheet

Stability and storage

EGCG may be stored for at least 24 months from the date of manufacture in the unopened original container and at a temperature below 25 °C. The 'best used before' date is printed on the label. Keep container tightly closed. Once opened, use contents quickly.

Uses

For capsules and tablets.

For fortification of foods and beverages.

For cosmetic oral hygiene products.

Safety

This product is safe for the intended use. Avoid ingestion, inhalation of dust or direct contact by applying suitable protective measures and personal hygiene.



For full safety information and necessary precautions, please refer to the respective DSM Material Safety Data Sheet.

Legal notice

The information given in this publication is based on our current knowledge and experience, and may be used at your discretion and risk. It does not relieve you from carrying out your own precautions and tests. We do not assume any liability in connection with your product or its use. You must comply with all applicable laws and regulations, and observe all third party rights.

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Human Nutrition and Health
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PO Box 3255
CH-4002 Basel
Switzerland

Tel.: +41 (0) 6168 73030
Fax: +41 (0) 6168 81592
Internet www.dsmnutritionalproducts.com

Safety Data Sheet		TEAVIGO	
1. Product and Company Identification			
Product name	TEAVIGO		
Product code	50 0285 0		
Company information	Enquiries:	Local representation:	
	DSM Nutritional Products AG	LV0000	
	Wurmisweg 576	LV1111	
	CH-4303 Kaiseraugst	LV2222	
	Switzerland	LV3333	
		LV4444	
	Phone +41-61/688 33 33	LV5555	
	Fax +41-61/688 33 30	LV6666	
		LV7777	
2. Composition/Information on ingredients			
Characterization	green tea extract with high content of epigallocatechin gallate (EGCG)		
Chemical name	- 3,4,5-Trihydroxybenzoic acid (2R,3R)-3,4-dihydro-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-2H-1-benzopyran-3-yl ester *1		
Synonyms	<ul style="list-style-type: none"> - Camellia sinensis ext. - Thea chinensis ext. - tea extract - EGCG extracted from green tea 		
CAS number	84650-60-2		
UN number	3077		
Ro number	Ro0267624-000		
Ingredient	Concentration	EU-Classification (pure ingredient)	
EGCG CAS: 989-51-5	> 90 %	 Xn, N R22, R36, R43, R51/53 S22, S24, S26, S36/37/39, S51 	
Polyphenols	~ 2 %		
Water			
*1 referring to:	EGCG		

TEAVIGO

3. Hazards identification	
Most important hazards	<ul style="list-style-type: none"> - Harmful if swallowed. - Irritating to eyes. - May cause sensitization by skin contact. - Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
4. First-aid measures	
Eye contact	<ul style="list-style-type: none"> - rinse immediately with tap water for 10 minutes - open eyelids forcibly - begin with medical treatment.
Skin contact	<ul style="list-style-type: none"> - remove immediately contaminated clothes, wash affected skin with water and soap - do not use any solvents
Inhalation	<ul style="list-style-type: none"> - remove the casualty to fresh air and keep him/her calm - in the event of symptoms get medical treatment
Note to physician	<ul style="list-style-type: none"> - treat symptomatically
5. Fire-fighting measures	
Suitable extinguishing media	<ul style="list-style-type: none"> - water spray jet, dry powder, foam, carbon dioxide
Specific hazards	<ul style="list-style-type: none"> - substance is hazardous for water: contain fire-fighting wastewater
Protection of fire-fighters	<ul style="list-style-type: none"> - precipitate gases/vapours/mists with water spray
6. Accidental release measures	
Environmental protection	<ul style="list-style-type: none"> - do not allow to enter drains or waterways
Methods for cleaning up	<ul style="list-style-type: none"> - collect solids (avoid dust formation) and hand over to waste removal
7. Handling and storage	
Handling	
Technical measures	<ul style="list-style-type: none"> - processing in closed systems, if possible superposed by inert gas (e.g. nitrogen) - local exhaust ventilation necessary - take precautionary measures against electrostatic charging
Suitable materials	<ul style="list-style-type: none"> - aluminium, enamel, glass, plastic
Storage	
Storage conditions	<ul style="list-style-type: none"> - cool and dry

Annexure E continued

TEAVIGO

Validity	- 24 months, < 25 °C, in the unopened original container, see "best use before" date stated on the label, after opening the container, the contents should be used within a short period
Packaging materials	- tightly closing; material: big bag (lined with polyethylene bag), fibredrums (lined with polyethylene bag), food-approved plastics
8. Exposure controls/Personal protection	
Engineering Measures	- see 7.
Monitoring	
Threshold value (Roche) air	- IOEL: 0.5 mg/m ³
Analytics	- sampling on glass fibre filter and gravimetric or chemical determination
Personal protective equipment	
Respiratory protection	- in case of open handling or accidental release: particle mask or respirator with independent air supply
Hand protection	- protective gloves (eg made of neoprene, nitrile or butyl rubber)
Eye protection	- tightly fitting safety glasses
Body protection	- disposable protective clothing including gloves, cap and overshoes
9. Physical and chemical properties	
Colour	off-white to pale pink
Form	crystalline powder partly with lumps
Density	1.59 g/cm ³ (19.4 °C; OECD No. 109)
Particle size	~ 70 % 125 to 250 µm (Fritsch Analysette 3 sieve analysis)
Surface tension	72.6 mN/m (20 °C) (OECD No. 115, "Surface Tension of Aqueous Solutions")
Solubility	~ 40'000 mg/l, water (19.5 °C, pH 4.9, OECD No. 105) ~ 10'000 mg/l, water (20 °C, pH 4)
Partition coefficient	log P _{ow} 1.1 (n-octanol/water 20 °C) pH 4.0 (Shake Flask Method, OECD No. 107) log P _{ow} < 0.3 (20 °C) pH 4.0 (HPLC Method, OECD No. 117)
pH value (19.5 °C)	~ 4.8 (saturated aqueous solution)

Annexure E continued

TEAVIGO



Hydrolysis	<p>$t_{1/2} = 50$ d (pH 4, 25 °C) $t_{1/2} = < 1$ d (pH 7, 25 °C) $t_{1/2} = < 1$ d (pH 9, 25 °C) (OECD No. 111)</p>
Melting temperature	> 200 °C (decomposes without clearly defined melting point; OECD No. 102)
Boiling temperature	> 200 °C (OECD No. 103) decomposes without clearly defined boiling point
Vapour pressure	< 0.000001 Pa (25 °C) (OECD No. 104)
Flammability	- not highly flammable (Flammability (solids) 92/69/EEC A.10.)
Note	- non-oxidizing (Expert Statement) - non-explosive (Expert Statement)
10. Stability and reactivity	
Stability	- stable under the conditions mentioned in chapter 7
Conditions to avoid	- temperatures above 100 °C
Note	- unstable in water, polymerisation with a half-life in the range of ca. 48 hours at room temperature
11. Toxicological information	
Acute toxicity	- LD ₅₀ 200 to 2'000 mg/kg (oral, rat) - LD ₅₀ > 2'000 mg/kg (dermal, rat)
Local effects	- skin: non-irritant (rabbit; OECD No. 404) - eye: irritant (rabbit)
Sensitization	- sensitizing (guinea pig); contact hypersensitivity (maximisation) test and open epicutaneous test - human experience: rare cases of asthma during handling of green tea extract reported
Subchronic toxicity	- NOEL 50 mg/kg/d (oral, dog; 13 weeks); capsules - NOEL 500 mg/kg/d (oral, rat; 13 weeks); feed admix - NOEL 45 mg/kg/d (oral, rat; 13 weeks); gavage
Mutagenicity	- not mutagenic (various in vivo and in vitro test systems); one Ames test and two micronucleus tests - mutagenic (in vitro test system) - no suspicion of mutagenicity in man
Reproduction toxicity	- not teratogenic, not embryotoxic (1000 mg/kg, limit dose; oral, rat)

TEAVIGO

12. Ecological information	
Ready biodegradability	- not readily biodegradable 17 %, 28 d (CO ₂ Evolution Test, Modified Sturm Test, OECD No. 301B)
Abiotic degradation	- unstable in water, probably polymerisation (aquatic ecotoxicity media) $t_{1/2} < 2$ h, - 20 °C
Ecotoxicity	- strongly toxic for fish (carp) LC ₅₀ (96 h) 7.5 mg/l (nominal concentration) NOEC (96 h) 5.6 mg/l (nominal concentration) LC ₁₀₀ (96 h) 10 mg/l (nominal concentration) (OECD No. 203) - moderately toxic for planktonic crustaceans (Daphnia magna) EC ₅₀ (48 h) 40 mg/l (nominal concentration) NOEC (48 h) 18 mg/l (nominal concentration) (OECD No. 202) - highly toxic for algae (Selenastrum capricornutum) EbC ₅₀ (72 h) 0.90 mg/l (nominal concentration) ErC ₅₀ (72 h) 3.4 mg/l (nominal concentration) NOErC (72 h) 0.46 mg/l (nominal concentration) (OECD No. 201) - no adverse influence on substrate biodegradation (activated sludge) concentration (28 d) 20.5 mg/l (OECD No. 301B, Modified Sturm Test) - barely toxic for microorganisms (activated sludge) NOEC (3 h) ≥ 100 mg/l EC ₂₀ (3 h) 171 mg/l EC ₈₀ (3 h) 520 mg/l (OECD No. 209)
Mobility	- high mobility (soil, sediment, activated sludge) K _{oc} = 7.7 (OECD No. 121)
Air pollution	- observe local/national regulations
13. Disposal considerations	
Waste from residues	- incinerate in qualified installation with flue gas scrubbing - observe local/national regulations regarding waste disposal

Annexure E continued

TEAVIGO

14. Transport information							
IATA	Class	UN/ID	PG		PI	Label	
	9	3077	III		911/911	9	
IMDG	Class	UN	PG	EmS	PI	Label	Mark
	9	3077	III	F-A S-F	P002/IBC08	9	
RID/ADR	Class	UN	PG	Haz.no	PI	Label	Classif.code
	9	3077	III	90	P002/IBC08	9	M7
Proper shipping name		ENVIRONMENTALLY HAZARDOUS SUBSTANCE, SOLID, N.O.S. Tea extract					
15. Regulatory information							
Classification and labelling according to EU directives							
		Xn		N			
R22	Harmful if swallowed.						
R36	Irritating to eyes.						
R43	May cause sensitization by skin contact.						
R51/53	Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.						
S22	Do not breathe dust.						
S24	Avoid contact with skin.						
S26	In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.						
S36/37/39	Wear suitable protective clothing, gloves and eye/face protection.						
S61	Avoid release to the environment. Refer to special instructions/Safety data sheets.						
Water hazard class (Germany)	2: hazardous for water (own classification according to directive VwVwS of 17.05.1999)						

TEAVIGO

16. Other information

Use	- for tablets and capsules - additive in processed food and beverages - additive for cosmetics
Safety-lab number	- BS-7196 - BS-6780
R phrases (chapter 2 ingredients)	
R22	Harmful if swallowed.
R36	Irritating to eyes.
R43	May cause sensitization by skin contact.
R51/53	Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
NFPA Rating	- Health: 2 Fire: 1 Reactivity: 1
Edition documentation	- changes from previous version in sections 4, 5, 9, 14, 15

Important Notice

DSM N.V., headquartered in Heerlen, The Netherlands, has acquired the vitamins, carotenoids, enzymes, food and feed ingredients, cosmetics ingredients and fine chemicals business (VFC Business) of the Roche group of companies, headquartered in Basel, Switzerland. Within the United States, DSM Nutritional Products, Inc. has purchased certain assets and assumed certain liabilities of the VFC Business formally conducted by Roche Vitamins Inc. Please note that corporate names, trade names, trade and service marks and domain names containing the word "Roche" and the "Roche" logo will continue to appear on our business documentation during our transition. We appreciate your understanding and cooperation as we complete our rebranding program. Should you have any questions, or if DSM can be of further assistance to you, please do not hesitate to contact your Account Manager or our Account Management Center at: +41-61/688 33 33.

Annexure F: COMPOSITION OF CONTROL DIET GIVEN TO VOLUNTEERS

	Food item	Day 1	Day 2	Day 3	Day 4
Breakfast	Dry cereal (Rice Krispies, Cornflakes, Weetabix, All bran flakes)	60g	60g	60g	60g
	Milk, full cream	250 ml	250 ml	250 ml	250 ml
	Sugar	10g	10g	10g	10g
	Eggs	2	2	2	2
	Grilled beef sausage	60g			
	Grilled bacon		60g		30g
	Gouda cheese			60g	
	Mayonnaise			1 tblsp	
	White bread	2 slices	2 slices	2 slices	2 slices
	Margarine (Canola)	14g	14g	14g	14g
	Fizzy drink (caffeine-free)	250 ml	250 ml	250 ml	250 ml
Lunch	Tasty mince	120g			
	Beef stir fry		200g		
	Chicken			120g	
	Grilled steak and sauce				150g
	Spaghetti	200g			
	Pitta bread		1		
	Pasta bake			200g	
	Oven roasted potatoes				200g
	Carrot and pineapple salad	60g			
	Greek salad				60g
	Fizzy drink (caffeine-free)	250 ml	250 ml	250 ml	250 ml
Supper	Chicken a la king	230g			
	Grilled pork chops		150g		
	Beef stroganoff			230g	
	Rice	200g		200g	
	Baked potato wedges		200g		
	Boiled mixed vegetables (corn, peas, carrots)	80g			
	Sweetened grilled pineapple		60g		
	Mixed vegetable bake		80g		
	Boiled peas			80g	
	Sweet and sour sauce		2 tblsp		
	Fizzy drink (caffeine-free)	250 ml	250 ml	250 ml	

Annexure H: REPORT OF ANY ADVERSE EFFECTS

Protocol Number:

Study Name: EGCG STUDY

VOLUNTEER INFORMATION

Name: _____

Volunteer Number:

Date of birth: _____

Gender: _____

Adverse effect	During Treatment A	During Treatment B	Day of adverse effect appearance

Annexure I: ADVERSE EVENTS REPORT FORM

Protocol Number:

Study Name: EGCG STUDY

VOLUNTEER INFORMATION

Name: _____

Volunteer Number:

Date of birth: _____

Gender: _____

1. Adverse Event experienced:

Date of onset: _____ Duration of adverse event: _____

Treatment code at which adverse event was experienced _____

Description of reaction (include abnormal tests/ lab data, vital signs, physical observations, etc).

2. Relevant medical history (allergies and other medical conditions)

3. Is this type of adverse event (AE) an unexpected occurrence?

Yes

No

4. How likely is this AE related to the food supplement given?

Definitely related

Probably not related / Unlikely

Probably related / Likely

Definitely not related

Possibly related / May be

Unknown

5. Adverse event severity

Not severe

Severe

Very severe

Annexure J: LIST OF FOODS

List of food that can be eaten

Cereals e.g. bread, rice, mealie meal, pasta

Scones, plain muffins

Potatoes

Fruits (only pineapple and banana)

Vegetables (only cabbage, mushrooms, carrots, cucumber, peas, sweet corn)

All types of meat, fish, milk

Plain yogurt, yoghurt with banana pieces

Eggs

Salted potato chips (Simba), salted pop corns

Sweets (not fruit based)

Note : Avoid mixed dishes outside the study as these may contain foods that need to be avoided

Foods that needs to be avoided

Green tea and green tea extracts

Tea, coffee, cocoa, milo

Chocolate and chocolate products

Wine

Other fruits (fresh or dried) and their fruit juices

Other vegetables except those in the list of foods that are allowed

Spices (except white and black peppers)

Fizzy drinks containing caffeine e.g. cola drinks

Soy sauce and other soy products

Vinegar

Please record everything you eat in outside the study in the provided forms

THANK YOU!

Annexure K: FOOD RECORD CHART

Protocol Number: _____

Study Name: EGCG STUDY

Name: _____

Volunteer Number:

Day: _____

Date: _____

Time	Place	Food eaten	Amount
Breakfast			
Mid-morning snack			
Lunch			
Mid-afternoon snack			
Supper			
Bed-time snack			

Annexure L: BLOOD SAMPLING RECORD

Protocol Number: _____

Study Name: EGCG STUDY

Name: _____

Subject Number:

Date: _____

Temperature: _____

Blood pressure: _____

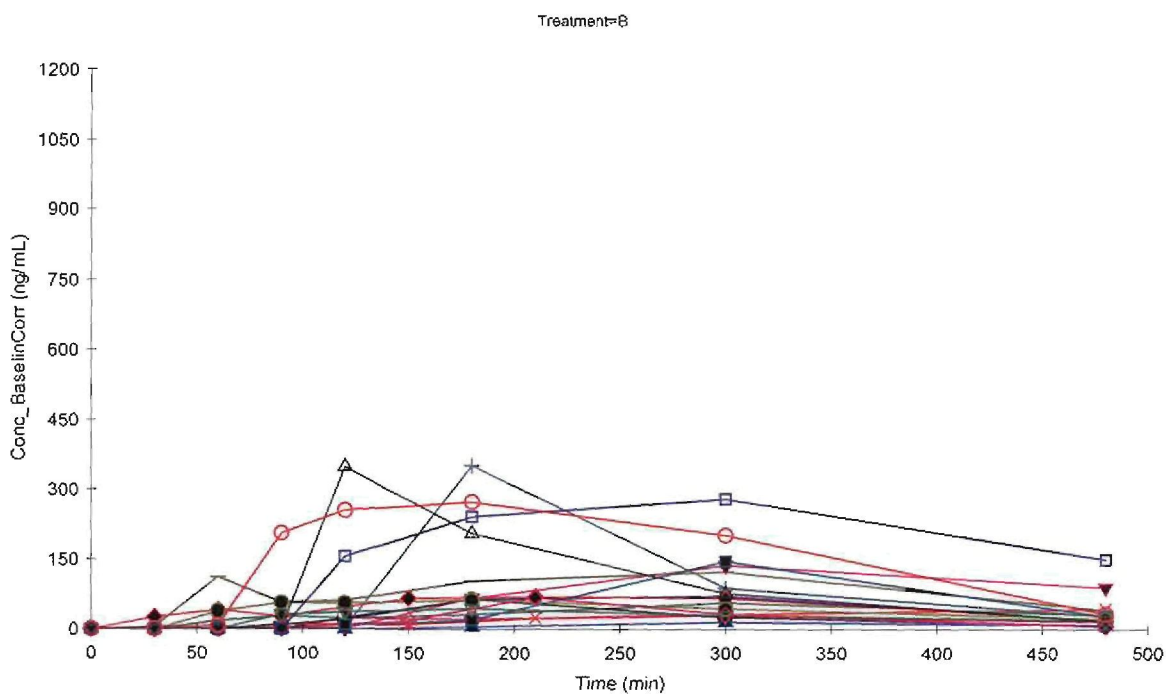
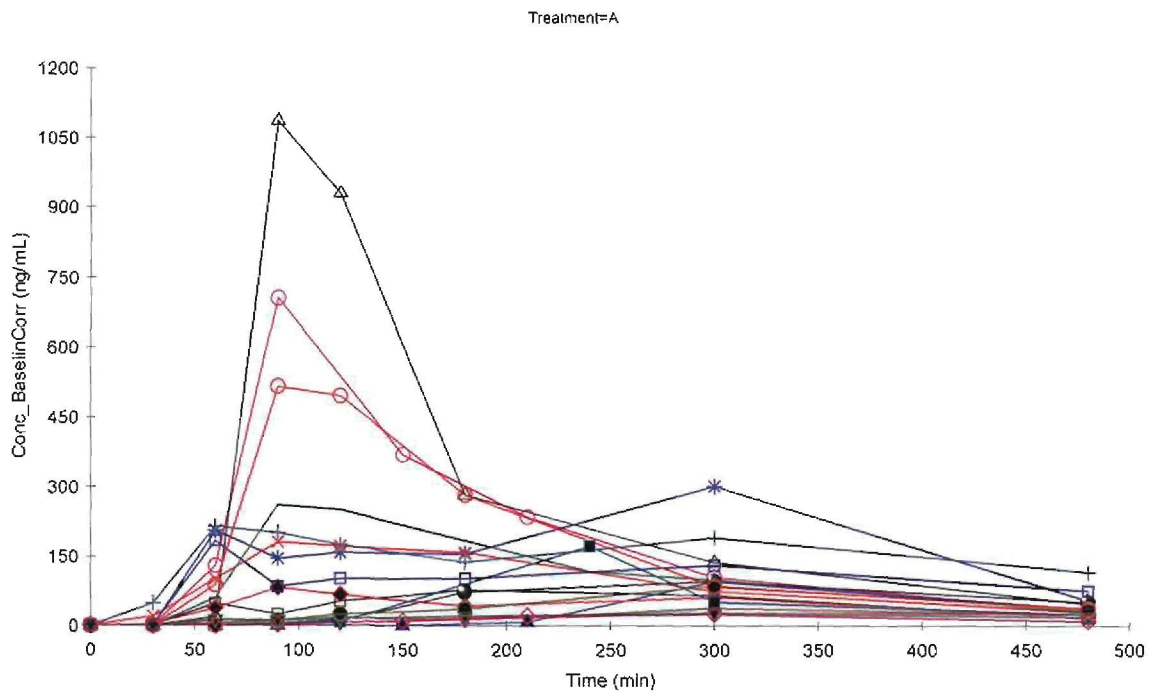
Blood samples:

Time intervals	Ideal time for withdrawal of blood sample	
T _{0min}	6:00 am	
T _{30 min}	6 :30 am	
T _{60 min}	7 :00 am	
T _{90 min}	7 :30 am	
T _{120 min}	8 :00 am	
T _{180 min}	9 :00 am	
T _{300 min}	11 :00 am	
T _{480 min}	2 pm	

Signature: _____

Date : _____

Annexure M: Plasma concentrations of all the 20 volunteers following EGCG incorporated Pheroid™ (Treatment A) and EGCG (Treatment B)



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