Effect of Herbal Medicines on the Pharmacokinetics and Pharmacodynamics of Warfarin in Healthy Subjects

By

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Preface

The work described in this thesis was carried out by the author in the Faculty of Pharmacy, The University of Sydney, under the supervision of Dr Andrew J McLachlan and Dr Colin C Duke. This thesis has not been submitted for a degree at any other university. Full acknowledgement has been made where the work of others has been cited or used. A list of publications is included in support of this thesis.

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Publications in Support of this Thesis

- Jiang X, Williams KM, Liauw W, Ammit AJ, Roufogalis BD, Duke CC, Day RO, McLachlan AJ, Effect of St John's wort and ginseng on the pharmacokinetics and pharmacodynamics of warfarin in healthy subjects, *British Journal of Clinical Pharmacology* 2004;57:592-9.

- Jiang X, Williams KM, Liauw W, Ammit AJ, Roufogalis BD, Duke CC, Day RO, McLachlan AJ, Effect of ginkgo and ginger on the pharmacokinetics and pharmacodynamics of warfarin in healthy subjects, *British Journal of Clinical Pharmacology* (Under revision).

Papers Presented at Scientific Meeting

- Jiang X, Williams KM, Liauw W, Ammit AJ, Roufogalis BD, Duke CC, Day RO, McLachlan AJ, Pharmacokinetic-pharmacodynamic modelling to investigate herb-drug interactions with warfarin. 8th World Congress on Clinical Pharmacology & Therapeutics (2004). Poster

- Jiang X, Williams KM, Liauw W, Ammit AJ, Roufogalis BD, Duke CC, Day RO, McLachlan AJ, Effect of ginkgo and ginger on the pharmacokinetics and pharmacodynamics of warfarin in healthy subjects. Australasian Pharmaceutical Science Association **(APSA)** Annual Conference, Sydney (2003). Podium.

- Jiang X**,** Williams KM, Liauw W, Ammit AJ, Roufogalis BD, Duke CC, Day RO, McLachlan AJ, Effect of St John's wort and ginseng on the pharmacokinetics and pharmacodynamics of warfarin in healthy subjects. American Association of Pharmaceutical Scientist **(AAPS)** Annual Conference and Exposition – Salt Lake City, 26-30, October, 2003. Poster.

- Jiang X**,** Williams KM, Liauw W, Ammit AJ, Roufogalis BD, Duke CC, Day RO, McLachlan AJ, Effect of St John's wort and ginseng on the pharmacokinetics and pharmacodynamics of warfarin in healthy subjects (Preliminary Study). Australasian Pharmaceutical Science Association **(APSA)** Annual Conference – Melbourne (2002). Poster.

- Jiang X**,** Williams KM, Roufogalis BD, Duke CC, Day RO, McLachlan AJ, Effect of St John's wort and ginseng on the pharmacokinetics and pharmacodynamics of warfarin: results of a pilot study. The University of Sydney, The College of Health Sciences **(CoHS)** Research Conference – Leura (2002). Miniposter/Podium.

Abbreviations

ADP	Adenosine disphosphate
ANOVA	Analysis of variance
APTT	Activated partial prothrombin time
ATP	Adenosine triphosphate
AUC	Area under the plasma drug concentration-time curve
BP	British Pharmacopoeia
cAMP	Adenosine 3',5'-cyclic phosphate
cGMP	cyclic guanosine monophosphate
CI	Confidence interval
C_{max}	Maximum drug concentration observed in plasma
C_{min}	Minimum drug concentration observed in plasma
C_R	R-warfarin concentration
C_S	S-warfarin concentration
$C_{50,R}$	Concentration of R-warfarin that produces 50% inhibition of PCA
$C_{50,S}$	Concentration of S-warfarin that produces 50% inhibition of PCA
CL	Total clearance of drug from plasma
CLu	Clearance of unbound drug
CNS	Central nervous system
CV	Coefficient of variation
CYP	Cytochrome P450
DA	Dopamine
cDNA	Complementary deoxyribonucleic acid
E_{max}	Maximum effect
Em	Emission
Ex	Excitation
F	Bioavailability of drug or herbal constituent
FDA	Food and Drug Administration
fe	Fraction of drug or herbal constituent excreted unchanged in urine
fu	Fraction of unbound drug concentration in plasma
GC	Gas chromatography
GCP	Good Clinical Practice
GMP	Good Manufacturing Practice
GS	Ginseng
h	Hour
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IC_{50}	The concentration that produces 50% inhibition of PCA
INR	International normalised ratio of prothrombin time
I.S.	Internal standard
k	Elimination rate constant
ka	Absorption rate constant
k_d	Degradation rate constant of the prothrombin complex
LCMSMS	Liquid chromatography mass spectrometry/mass spectrometry
LOQ	Limit of quantitation
mRNA	Messenger ribonucleic acid

min	Minute
MW	Molecule weight
NA	Noradrenaline
NSAIDs	Non steroidal anti-inflammatory drugs
QC	Quality control
PAF	Platelet activating factor
PCA	Prothrombin complex activity
PK/PD	Pharmacokinetics/Pharmacodynamics
PT	Prothrombin time
PXR	Pregnane X receptor
r^2	Coefficient of determination
R_{deg}	Degradation rate of prothrombin complex
R_{syn}	Synthesis rate of prothrombin complex
SD	Standard deviation
sec	Second
SJW	St John's wort
$t_{1/2}$	Half-life
t_d	Time between the start of absorption of the drug and the initiation of anticoagulant
TGA	Therapeutic Goods Administration
TXA_2	Thromboxane A_2
TLC	Thin-layer Chromatography
t_{max}	Time at which the highest drug concentration occurs
UV	Ultraviolet
USP	United States Pharmacopoeia
UER	Urinary excretion rate
V	Volume of distribution
WF	Warfarin
γ	Slope factor in concentration-response relationship

Abstract

Herbal medicines are widely used in our community. A survey of Australian consumers indicated that 60% had used complementary and/or alternative medicines in the past year with the majority not informing their doctor that they were using herbal medicines. Little is known about the potentially serious consequences of interactions between herbal and conventional medicines. Warfarin has an important role in treating people with heart disease, yet it has a narrow therapeutic range, is highly bound to plasma proteins, and is metabolised by cytochrome P450. This creates the potential for life-threatening interactions with other drugs and foods leading to excessive bleeding. Hence, warfarin is one of the most frequently investigated drugs for interaction studies. Early clinical reports suggest that there exists the potential for an interaction between warfarin and four herbal medicines: St John's wort, ginseng, ginkgo and ginger. However, these herb-drug combinations have never been conclusively studied.

The two clinical studies conducted as part of this research had an identical study design. Twenty-four healthy male subjects were recruited into the two separate studies. This was an open label, three-way crossover randomised study in twelve healthy male subjects, who received a single 25 mg dose of warfarin alone or after 14 days pre-treatment with St John's wort, or 7 days pre-treatment with ginseng. Dosing with St John's wort or ginseng was continued for 7 days after administration of the warfarin dose in study I or who received a single 25 mg dose of warfarin alone or

after 7 days pre-treatment with recommended doses of ginkgo or ginger from single ingredient products of known quality. Dosing with ginkgo or ginger was continued for 7 days after administration of the warfarin dose in study II. Platelet aggregation, international normalised ratio (INR) of prothrombin time, warfarin enantiomer protein binding, warfarin enantiomer concentrations in plasma and S-7-hydroxywarfarin concentration in urine were measured in both studies. Statistical comparisons were made using ANOVA and 95% confidence interval (CI) for mean value and 90% CI for geometric mean ratio value are reported.

In study I, the mean (95% CI) apparent clearance of S-warfarin after warfarin alone or with St John's wort or ginseng were, respectively, 198 (174 – 223) ml/h, 269 (241 – 297) ml/h and 220 (201 – 238) ml/h. The respective apparent clearances of R-warfarin were 110 (94 – 126) ml/h, 142 (123 – 161) ml/h and 119 (106 – 131) ml/h. The mean ratio of apparent clearance for S-warfarin was 1.29 (1.16-1.46) and for R-warfarin was 1.23 (1.11-1.37) when St John's wort was co-administered. The mean ratio of $AUC_{0\text{-}168}$ of INR was 0.79 (0.70 - 0.95) when St John's wort was co-administered. The urinary excretion ratio of S-7-hydroxywarfarin after administration of warfarin alone was 0.04 (0.03 – 0.06) mg/h and there was no significant difference following treatment with either St John's wort 0.03 (0.02 – 0.04) mg/h or ginseng 0.03 (0.02 – 0.04) mg/h. The ratio of geometric means for S-7-hydroxywarfarin UER was 0.82 (0.61-1.12) for St John's wort, and 0.68 (0.50-0.91) for ginseng. St John's wort and ginseng did not affect the apparent volumes of distribution or protein binding of warfarin enantiomers.

In study II, the mean (95% CI) apparent clearance of S-warfarin after warfarin alone, with ginkgo or ginger were 189 (167 – 210) ml/h, 200 (173 – 227) ml/h and 201 (171 – 231) ml/h, respectively. The respective apparent clearances of R-warfarin were 127 (106 – 149) ml/h, 126 (111 – 141) ml/h and 131 (106 – 156) ml/h. The mean ratio of apparent clearance for S-warfarin was 1.05 (0.98 -1.12) and for R-warfarin was 1.00 (0.93 -1.08) when co-administered with ginkgo. The mean ratio of $AUC_{0\text{-}168}$ of INR was 0.93 (0.81 -1.05) when co-administered with ginkgo. The mean ratio of apparent clearance for S-warfarin was 1.05 (0.97 -1.13) and for R-warfarin was 1.02 (0.95 -1.10) when co-administered with ginger. The mean ratio of $AUC_{0\text{-}168}$ of INR was 1.01 (0.93 -1.15) when co-administered with ginger. The urinary excretion ratio (UER) of S-7-hydroxywarfarin after administration of warfarin alone was 0.04 (0.03 – 0.05) mg/h and there was no significant difference following treatment with either ginkgo 0.04 (0.03 – 0.04) mg/h or ginger 0.03 (0.02 – 0.04) mg/h. The ratio of geometric means for S-7-hydroxywarfarin UER was 1.07 (0.69-1.67) for ginkgo, and 1.00 (0.64-1.56) for ginger. Ginkgo and ginger did not affect the apparent volumes of distribution or protein binding of either S-warfarin or R-warfarin.

In conclusion, St John's wort significantly induced the apparent clearance of both S-warfarin and R-warfarin, which in turn resulted in a significant reduction in the pharmacological effect of *rac*-warfarin. Ginseng, ginkgo and ginger at recommended doses affect neither clotting status, nor the pharmacokinetics or pharmacodynamics of either S-warfarin or R-warfarin in healthy subjects.

Chapter 1 Introduction

1.1 Overview

Herbal medicines are widely used in our community [1-3]. A survey of Australian consumers indicated that 60% had used complementary and/or alternative medicines in the past year with the majority not informing their doctor that they were using herbal medicines [4]. Little is known about the potentially serious consequences of herbal medicines and conventional medicine interactions. Furthermore, herbal products are not marketed with proof of efficacy or safety as is normally required for conventional medicines by the Therapeutical Goods Administration (TGA) or Food and Drug Administration (FDA). In the literature, most herb-drug interactions are based on case reports or suspected interactions [5]. Fugh-Berman reported that of one hundred and eight cases of suspected interactions identified in a review article assessing the reliability of reported herb- drug interactions, seventy-four (68.5%) did not contain sufficient information to evaluate the likelihood of an interaction [6]. Additionally, there is only variable evidence supporting herb-drug interactions, much of which includes conflicting *in vitro* and *in vivo* data [7]. There have been a number of case reports of suspected interactions and *in vitro* data indicating the possible effects of herbal medicines on blood coagulation and clot formation [8]. However there has been no systematic investigation of the potential interactions between

herbal medicines and the anticoagulant drug warfarin. This presents a challenge for health care practitioners.

Warfarin has an important role in treating people with heart diseases, yet it has a narrow therapeutic range, exhibits stereoselective metabolism, is approximately 99% bound to plasma proteins, and is metabolised by cytochrome P450 (CYP). This creates the potential for life-threatening interactions with other drugs and foods leading to excessive bleeding or therapeutic failure. Hence, warfarin is one of the most frequently investigated drugs for interaction studies [9]. Early clinical reports suggest that there exists the potential for an interaction between warfarin and four herbal medicines: St John's wort, ginseng, ginkgo and ginger. However, these herb-drug combinations have never been conclusively studied. Warfarin is widely used in the community where people have free access to herbal and alternative medicines [4]. Hence, there is considerable potential for clinically significant drug interactions. The systematic study of potential interactions between warfarin and herbal medicines proposed in this research will lead to safer dosing guidelines for the use of warfarin in combination with these supposedly "safe" herbal medicines. Excessive warfarin response can be life threatening or cause excessive bleeding leading to serious consequences. The identification and avoidance of significant drug interactions with warfarin can reduce patient problems caused by bleeding and reduce the length of hospital stays and decrease the need for frequent blood monitoring. Warfarin is a substrate for the drug metabolising enzyme CYP2C9, so the present study will also provide a valuable insight into possible interactions with other medicines that are substrates for CYP2C9.

The aims of the studies reported in this thesis were

- To investigate the herb-drug interactions between four commonly used herbal medicines (St John's wort, ginseng, ginkgo, and ginger) and the anticoagulant drug warfarin
- To identify the drug interaction mechanisms
- To investigate St John's wort, ginseng, ginkgo, and ginger's effect on clotting status, respectively

The findings of these studies can be used to infer whether there are potentially serious herb-drug interactions between these four herbal medicines and other conventional medicines in addition to warfarin.

1.2 Physicochemical Properties of Warfarin

Warfarin, $C_{19}H_{16}O_4$, ((RS)-2-oxo-3- (3-oxo-1-phenylbutyl)-2H-chromen-4-olate) is a colourless crystalline material with a MW of 308.3 and melting point between 159^{o}C to 160^{o}C. Warfarin can exhibit natural fluorescence with excitation and emission wavelengths of 290-342 nm and 385 nm, respectively. It is practically insoluble in water, moderately soluble in ethanol and readily soluble in acetone. On the other hand, warfarin sodium, $C_{19}H_{15}NaO_4$ (MW: 330.32), (sodium (RS)-2-oxo-3-(3-oxo-1-phenylbutyl)-2H-chromen-4-olate) is a white hygroscopic powder which is very soluble in water and in alcohols, soluble in acetone, very slightly soluble in ether and in methylene chloride. Warfarin has an apparent dissociation constant (pKa) of 5.0 (at 20^{o}C) and a partition coefficient (log P) (octanol/pH 8.0) of 0.0 [10].

Warfarin is a chiral molecule. The asymmetric carbon at position 9 of warfarin gives rise to two enantiomers, namely R-(+)-warfarin (R-warfarin) and its mirror image isomer S-(-)-warfarin (S-warfarin) (Figure 1-1). The main monohydroxylated metabolites of warfarin, such as 4'-, 6-, 7- and 8-hydroxywarfarin, also contain a single asymmetric centre (Table 1-1) [11].

Figure 1-1. Three dimensional structures of warfarin enantiomers.

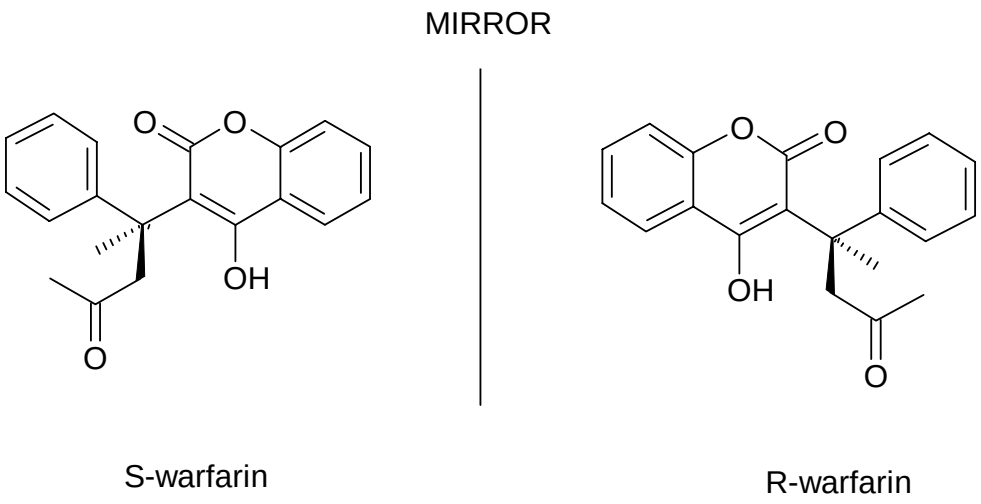
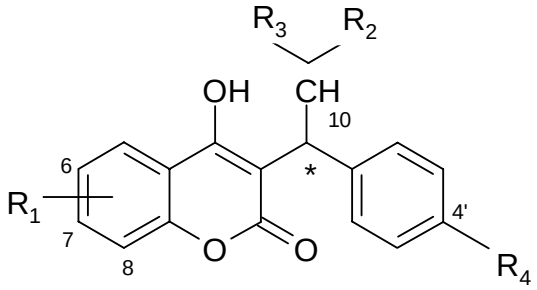
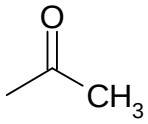
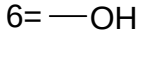
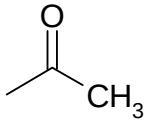
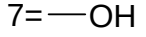
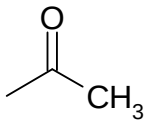
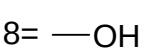
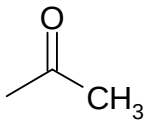
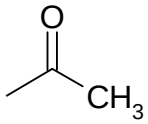
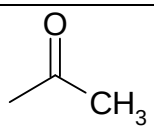
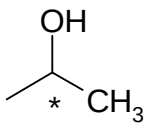


Table 1-1. Structures of warfarin and its main metabolites. Adapted from [12]**.**



Compound	Structure			
	R1	**R2**	**R3**	**R4**
Warfarin	H		H	H
6-Hydroxywarfarin			H	H
7-Hydroxywarfarin			H	H
8-Hydroxywarfarin			H	H
4'-Hydroxywarfarin	H		H	OH
10-Hydroxywarfarin	H		OHa	H
Warfarin alcohol	H		H	H

Note: *: Asymmetric centre; a: The second asymmetric centre generated at C-10 position.

1.3 Clinical Use of Warfarin

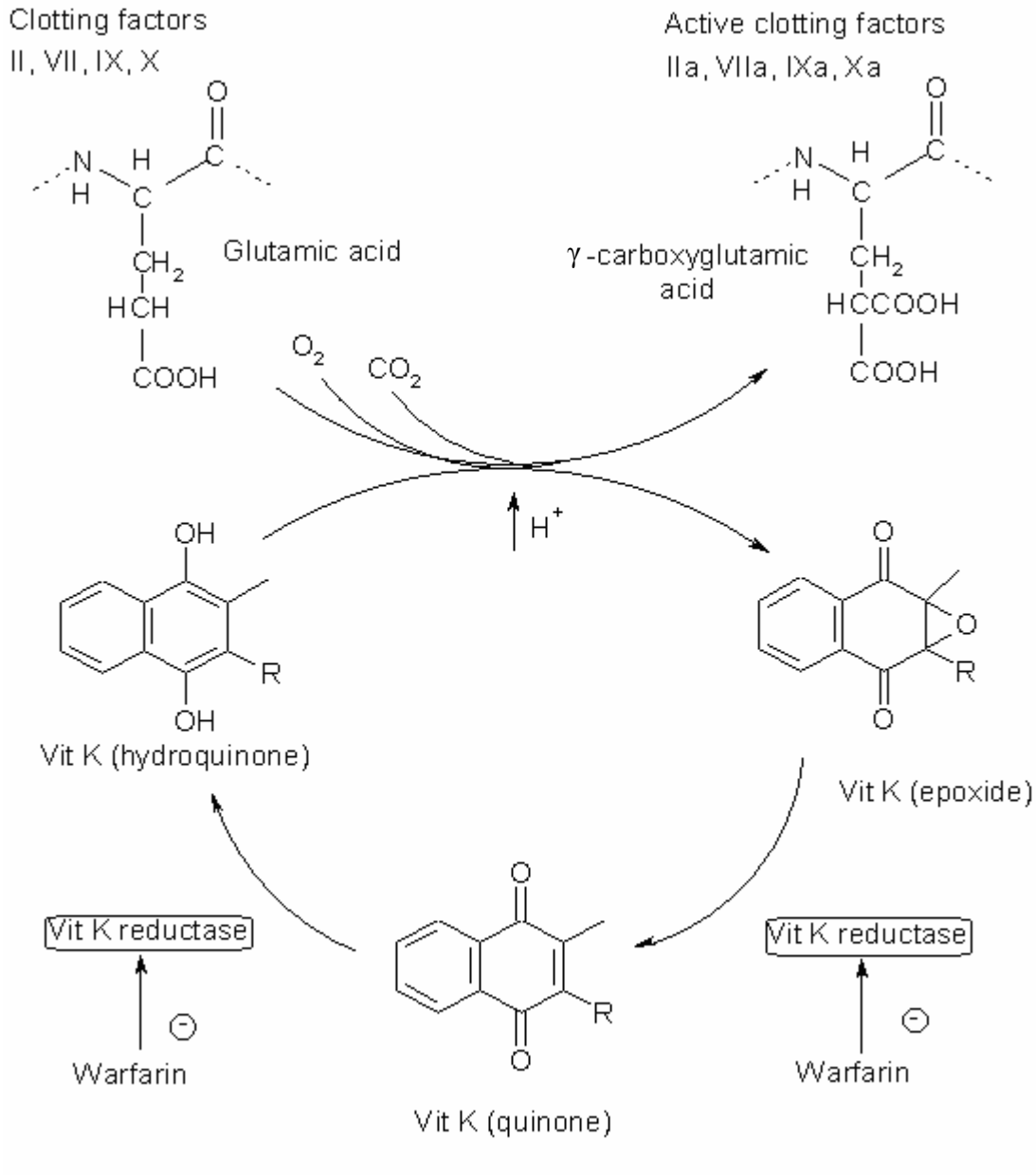
In the clinical setting, warfarin is administered for the prevention and treatment of venous thromboses and pulmonary embolism [13]. It is also used for the prophylaxis and treatment of thromboembolic complications associated with atrial fibrillation, diabetes mellitus and hypertension associated with an otherwise normal heart. It is additionally used as an adjunct in the treatment of coronary occlusion. Warfarin does not affect established thrombi or reverse tissue ischaemia but is instead used to prevent clot growth and secondary complications [14]. The following ranges of International normalized ratio (INR) of prothrombin time are recommended by the British Society of Haematology. 2.0 to 2.5: prophylaxis of deep vein thrombosis including high risk surgery; 2.0 to 3.0: treatment of deep vein thrombosis, pulmonary embolism and atrial fibrillation; 3.0 to 4.5: recurrent deep vein thrombosis and pulmonary embolism; arterial disease including myocardial infarction, arterial grafts; cardiac prosthetic valves and grafts.

1.4 Pharmacodynamics of Warfarin

1.4.1 Mechanism of Action

Warfarin is a vitamin K dependent factor anticoagulant. It acts by inhibiting the synthesis of vitamin K dependent clotting factors (Factors II, VII, IX and X and clotting protein C and S) [15] (Figure 1-2).

Figure 1-2. The probable mechanism of action of vitamin K and the site of action of oral anticoagulants. Adapted from [16]**.**



Reduced vitamin K (the hydroquinone) acts as a cofactor in the conversion of glutamic acid (Glu) to γ-carboxyglutamic acid (Gla). During this reaction, the reduced form of the vitamin K is converted to the epoxide, which in turn is reduced to the quinone and then the hydroquinone. Warfarin has similar structural characteristics to vitamin K, the inhibition of vitamin K reductase is competitive and only exerts its actions *in vivo* without affecting clotting if added to blood *in vitro* [17].

Warfarin does not alter the degradation rate of clotting factors already in circulation; it only affects the synthesis rate of clotting factors [18]. Therefore, the onset of anticoagulation induced by warfarin is delayed. The anticoagulation effect of warfarin takes about 8 hours to become apparent as a result of the time taken for degradation of carboxylated factors [18]. The onset of action of warfarin depends on the elimination half-lives of the relevant factors. Factor VII, with half-life of 6 hours, is affected first, then Factors IX, X and II with half-lives of 24, 40 and 60 hours, respectively [19, 20]. The two enantiomers of warfarin display different anticoagulant potency in humans. S-warfarin is considerably more potent than R-warfarin in terms of the anticoagulant effect [14].

1.4.2 Pharmacodynamic Models Used to Describe Warfarin Response

Various models have been reported to describe the relationship between warfarin concentration (C) and the inhibitory effect of warfarin on the synthesis rate of vitamin K-dependent clotting factors. The simplest is the linear pharmacodynamic model [21]. The liner pharmacodynamic model is described in Equation 1-1.

$$f(C) = 1 - S \times C \qquad \text{Equation 1-1}$$

where f(C) is an appropriate pharmacodynamic model describing the relationship between warfarin concentration and its inhibiting effect on prothrombin complex activity (PCA) synthesis rate; S is a slope parameter reflecting the patient's sensitivity to warfarin.

A power function has also been employed to define the effect of warfarin on the inhibition of clotting factor synthesis (Equation 1-2). This modified version of the linear pharmacodynamic model successfully deals with high warfarin concentrations but fails with a zero concentration [22].

$$f(C) = 100 \times 1 - 1/(S \times C^n) \qquad \text{Equation 1-2}$$

in this model n is the power parameter.

Nagashima *et al* [23] proposed the first log-linear pharmacodynamic model to describe warfarin pharmacodynamics. This was the first comprehensive model, which described the *in vivo* effect of warfarin on the synthesis of clotting factors. The hypothetical minimum effective plasma concentration (C_{min}) is obtained by extrapolating the percent inhibition of clotting factor synthesis rate versus log concentration line to zero inhibition. In theory, the log-linear pharmacodynamic model will overpredict the true effect when synthesis rate is more than 80% inhibited

and underpredict the effect when synthesis rate is less than 20% inhibited. This model is described in Equation 1-3.

$$f(C) = 1 - M \times [\log (C) - \log(C_{min})] \qquad \text{Equation 1-3}$$

where M is the slope of the log warfarin concentration versus percentage inhibition of clotting factor synthesis rate profile.

The E_{max} pharmacodynamic model [24] has a more secure base in pharmacological theory, and was able to describe the entire range of warfarin concentrations and effects. However, this model assumes a fixed value for the gradient of the concentration-effect relationship. The inhibitory E_{max} pharmacodynamic model is described in Equation 1-4.

$$f(C) = 1 - 1/(IC_{50}+C) \qquad \text{Equation 1-4}$$

where IC_{50} is the warfarin concentration producing 50% inhibition of clotting factor synthesis rate.

Chan *et al.* [18] detailed a further elaboration of the E_{max} model, the sigmoid E_{max} pharmacodynamic model, which has been used to describe the simultaneous effect of the S- and R- warfarin enantiomers on the inhibition of clotting factor synthesis rate. This model is described in Equation 1-5.

$$f(C) = 1 - \frac{1}{1 + \left(\frac{C_S}{IC_{50S}}\right)^{\gamma} + \left(\frac{C_R}{IC_{50R}}\right)^{\gamma}}$$

Equation 1-5

where C_S and C_R are concentrations of S-warfarin and R-warfarin, IC_{50S} and IC_{50R} are the corresponding concentrations of the enantiomers which individually produce 50% inhibition of prothrombin complex synthesis, and γ is the steepness parameter.

As described in Section 1.4.1, warfarin acts by inhibiting the synthesis of vitamin K-dependent clotting factors. At any time, the PCA expressed as a percentage of maximum response in blood is the net effect of synthesis rate (R_{syn}) and degradation rate (R_{deg}) of the prothrombin complex (Equation 1-6 and 1-7) [18].

$$dPCA/dt = R_{syn} - R_{deg} \qquad \text{Equation 1-6}$$

$$dPCA/dt = R_{syn} - k_d \times PCA \qquad \text{Equation 1-7}$$

where k_d is the degradation or elimination rate constant of the prothrombin complex. The effect of an anticoagulant drug (eg. warfarin) on R_{syn} can be expressed in terms of its fractional effect of the pre-treatment (control) synthesis rate ($R_{syn,0}$):

$$dPCA/dt = R_{syn,0}\,[1-f(C)] - k_d \times PCA \qquad \text{Equation 1-8}$$

The unbound concentration of warfarin enantiomers are considered to be responsible for the anticoagulant response at the active site and can be included in the model. Combining the Equation 1-8 with Equation 1-5, the pharmacodynamic model is described in Equation 1-9.

$$\frac{dPCA}{dt} = k_d \left[\frac{100}{1 + \left(\frac{Cu(t)}{Cu_{50,S}}\right)^{\gamma_S} + \left(\frac{Cu(t)}{Cu_{50,R}}\right)^{\gamma_R}} - PCA \right]$$

Equation 1-9

where $Cu_{50,S}$ and $Cu_{50,R}$ are the unbound concentrations of S- and R-warfarin, respectively, required to produce a 50% inhibition of prothrombin complex activity. Cu(t) is the unbound concentration of each enantiomer at time t, and γ is a measure of the steepness of the concentration-response curve for each warfarin enantiomer. Since the anticoagulant effect of racemic warfarin is predominantly contributed by S-warfarin [18]. The Equation 1-9 can be simplified to Equation 1-10.

$$\frac{dPCA}{dt} = k_d \left[\frac{100}{1 + \left(\frac{Cu(t)}{Cu_{50,S}}\right)^{\gamma_S}} - PCA \right]$$

Equation 1-10

1.5 Pharmacokinetics of Warfarin

Warfarin is a racemic mixture of the R- and S-enantiomers. In studies that administered warfarin enantiomers separately, it was found that the S-enantiomer exhibits two to five times more anticoagulant activity than the R-enantiomer in humans but generally has a more rapid clearance [25, 26]. Furthermore Chan *et al* [18] found that the anticoagulant effect of warfarin is predominantly contributed by S-enantiomer when warfarin was administered as *rac*-warfarin.

1.5.1 Absorption

Warfarin is essentially completely absorbed after oral administration and has a systemic bioavailability of more than 90% in humans [18]. The peak concentration after oral dosing is generally achieved within 60–90 min. No identifiable enantiomer-specific differences in absorption have been reported while both the rate and extent of absorption are independent of the dose administered [20, 27].

1.5.2 Distribution

Warfarin enantiomers distribute into a relatively small apparent volume of distribution (V) of about 0.14 ± 0.03 L/kg (V_S); 0.15 ± 0.03 L/kg (V_R) in humans [27-29]. A distribution phase last 6 to 12 h in distinguishable after rapid intravenous or oral administration of an aqueous solution. Model-based estimates of volume of distribution were close agreement for each enantiomer [18]. Approximately 99.5% of the warfarin is bound to human plasma serum proteins, primarily albumin, where it is pharmacologically inactive and is protected from biotransformation and excretion, but the plasma protein bound drug is not permanently which is reversible [30]. No

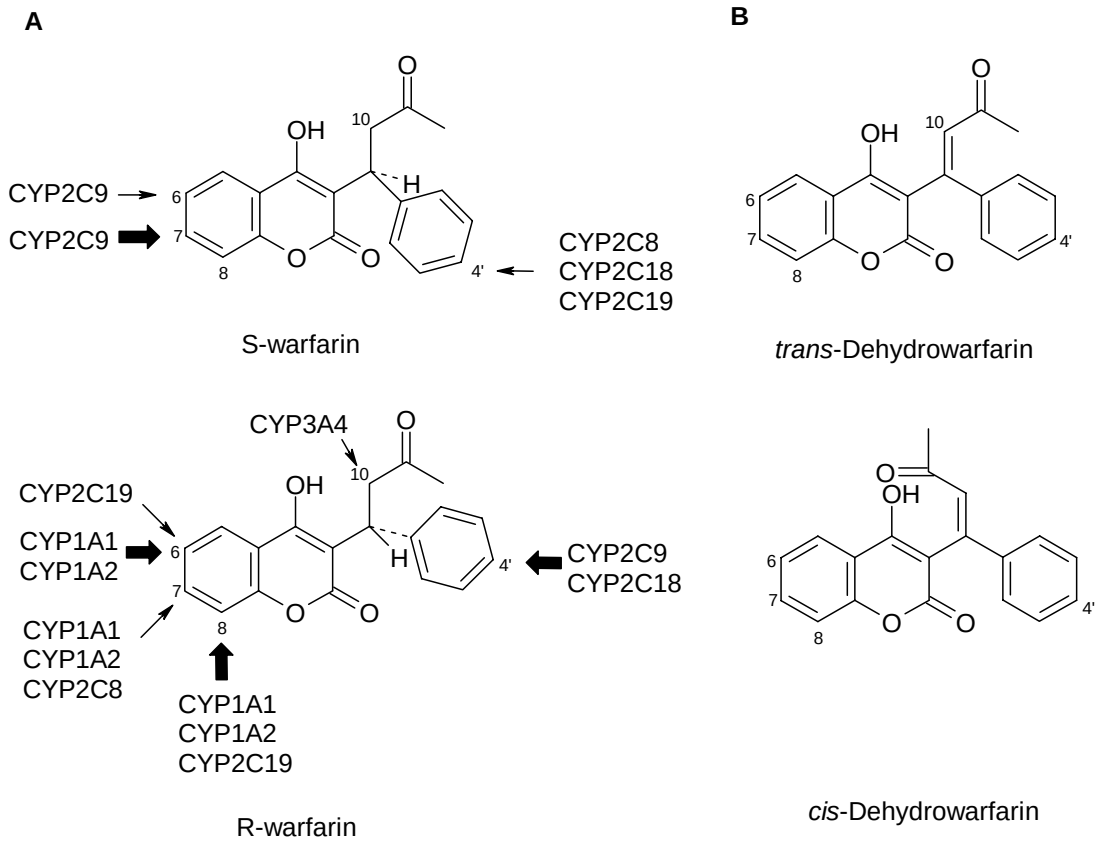
stereoselective differences in protein binding of warfarin enantiomers has been found (fu_S = 0.0051 ± 0.0005, fu_R = 0.0062 ± 0.0005, fu, fraction unbound drug concentration) [30, 31].

1.5.3 Metabolism

The elimination of warfarin is almost entirely accounted for by hepatic enzymatic metabolism (Figure 1-3). Warfarin is stereoselectively metabolised by hepatic microsomal enzymes (cytochrome P450) located in the hepatic smooth endoplasmic reticulum to inactive hydroxylated metabolites (predominant route) and also by reductases to reduced metabolites (warfarin alcohols).

Hepatic metabolism of warfarin is the major determinant of inter-subject variability in the warfarin dose-concentration-response relationship [20, 32]. Warfarin is metabolized in a complex manner involving: (1) keto reduction resulting in the formation of four diastereoisomeric metabolites designated as warfarin alcohols;

Figure 1-3. A: Sites of hydroxylation of S- and R-warfarin catalysed by human cytochrome P450 isozymes to yield the hydroxylated metabolites of warfarin. B: Structures of the dehydrowarfarin metabolites of warfarin. ➡ , major metabolic pathway; → , minor metabolic pathway. Adapted from [33]**.**



(2) oxidation to yield regioisomeric 4'-, 6-, 7-, 8- and 10-hydroxywarfarin; (3) dehydration of warfarin alcohol to the cyclic metabolite and (4) various conjugation reactions [34]. The warfarin alcohols have minimal anticoagulant activity. Table 1-1 shows the structures of warfarin and its main metabolites.

Based on *in vitro* studies, S-warfarin is metabolised predominantly to the inactive metabolite S-7-hydroxywarfarin by the cytochrome P450 2C9 (CYP2C9) [35-37] while R-warfarin is mainly metabolised to R,S-warfarin alcohol by liver cytosolic ketone reductases [38]. A small percentage of R-warfarin is converted to oxidative metabolites by several CYP450 enzymes including CYP1A2 [37], CYP3A4 [39] and CYP2C19 [40]. Very little is known about the Phase II metabolism of the warfarin metabolites in humans [33]. One *in vivo* study demonstrated that 10-hydroxywarfarin was fully conjugated, 8-hydroxywarfarin was 66% conjugated and the extent of 6-hydroxywarfarin conjugation varied between 66 and 100% in different patients by treating the urine with glucuronidase and sulfatase enzymes [33].

CYP2C9 is known to be polymorphic [33]. Two known allelic variants CYP2C9*2 and CYP2C9*3 differ from the wild-type CYP2C9*1. Both allelic variants display impaired hydroxylation of S-warfarin when expressed *in vitro* compared to wild type. The CYP2C9*3 variant is less than 5% as efficient as the wild-type activity while CYP2C9*2 shows about 12% of wild-type activity [41, 42]. Furthermore, some CYP2C9 polymorphisms are associated with increased risk of excessive bleeding for patients undergoing warfarin anticoagulation in the clinical setting [43, 44].

1.5.4 Excretion

Both reductive and oxidative metabolites of warfarin are excreted in urine and in bile while enterohepatic circulation has been observed for the parent drug [45]. The metabolites are principally excreted into the urine and, to a lesser extent, into the bile. Only 2-5% of the warfarin administered appears unchanged in human urine [46, 47]. Warfarin enantiomers have different elimination rate constants (k_S = 0.024 ± 0.009 1/h; k_R = 0.017 ± 0.003 1/h) [18]. Since the volumes of distribution of warfarin enantiomers are similar, the clearance of R-warfarin is generally half of S-warfarin unbound clearance (CLu_S = 675 ± 212 ml/h/kg; CLu_R = 399 ± 58 ml/h/kg) [18]. Hence, the half-life of R-warfarin ranges from 37 to 89 h while the half-life of S-warfarin ranges from 21 to 43 h [27].

1.5.5 Pharmacokinetic Modelling of Warfarin

Warfarin plasma concentration-time data after oral administration has been described by both one- and two-compartment pharmacokinetic models [46, 48]. Although the two compartment pharmacokinetic model may enhance the precision of data fitting, a simple model should be adequate for drug-drug interactions and combined pharmacokinetic-pharmacodynamic (PK/PD) studies [18]. The one compartment pharmacokinetic model is described in Equation 1-11 and 1-12.

$$C_S(t) = \left[\frac{F \cdot D}{V_S}\left[\frac{ka}{ka - k_S}\right]\left[e^{-k_S t} - e^{-kat}\right]\right]$$

Equation 1-11

$$C_R(t) = \left(\frac{F \cdot D}{2 \cdot V_R}\right)\left(\frac{ka}{ka - k_R}\right)\left(e^{-k_R t} - e^{-kat}\right)$$

Equation 1-12

where D is the dose of racemic warfarin administered (divided by 2 to obtain the equivalent dose of each enantiomer), F is the fraction of the dose absorbed, ka is the first order absorption rate constant, k_S and k_R are the respective elimination rate constants for individual warfarin enantiomers, and V_S and V_R are the respective volumes of distribution.

1.5.6 Warfarin Pharmacokinetic-Pharmacodynamic Modelling

Equations 1-11 and 1-12 have been combined with Equation 1-9 to generate a combined PK/PD model. An additional pharmacodynamic parameter was introduced to account for the observed delay in PCA change after warfarin administration. The parameter t_d represents the time between the onset of absorption of the drug and the initiation of anticoagulant response. Therefore t in the combined PK/PD model was replaced with a corrected time value t*, where $t^* = t - t_d$.

Since the anticoagulant response data are generally obtained as an INR, the response data can be transformed to PCA with the use of the functional relationship between INR and PCA (percentage of normal activity) described in the following equation:

$$\text{PCA}(\%\quad \text{normal}) = \frac{a}{\text{INR} - b}$$

Equation 1-13

The values of the constants a and b are determined by fitting this Equation 1-13 to PCA and INR data obtained using serial dilutions of pooled normal plasma (See Section 2.5).

1.6 Warfarin Drug Interaction Mechanisms

Drug interactions with warfarin can occur via three main mechanisms: 1) Pharmacodynamic interactions: modification of the pharmacological effect of warfarin without altering its concentration in the body; 2) Pharmacokinetic interactions: alteration of the concentration of warfarin reaching its site of action and 3) Physicochemical interactions. Clinically important drug interactions occur when either of the interacting drugs have a steep concentration-response curve and narrow safety margin such that a small change in plasma concentration leads to a substantial change in beneficial or adverse effect [16].

As a result of its narrow therapeutic range, high binding to plasma proteins and metabolism by cytochrome P450, warfarin is prone to life-threatening interactions. There have been more than 250 compounds, including clinically used drugs, herbal medicines and other xenobiotics, proven to or suspected of having an interaction with warfarin [49]. Understanding the mechanism of warfarin drug interactions provides an insight into the possible clinical significance of an interaction and can help elucidate strategies to avoid or minimise the impact in a given patient.

1.6.1 Pharmacodynamic Interactions

Drugs or herbal medicines can alter the pharmacodynamics of warfarin by their influence on vitamin K intake and absorption, the rate of synthesis and clearance of vitamin K-dependent clotting factors, by direct effects on blood coagulation or platelet function. Pharmacodynamic interactions can occur in many different ways and by various mechanisms. Their mechanisms are discussed in the following sections.

Drugs that Affect Vitamin K Intake, Synthesis and Elimination of Vitamin K and Clotting Factors

Warfarin competes with vitamin K and prevents the hepatic synthesis of various coagulation factors. There are two main sources of vitamin K in humans. Vitamin K_1 is of plant origin and is ingested [50], while vitamin K_2 is synthesized by bacteria in human intestine [51]. Drugs that reduce the systemic availability of vitamin K in the intestine should be considered to potentially alter the patient's the response to oral anticoagulants. For example, Karlson *et al* [52] reported that a single administration of 250 μg vitamin K_1, 250 g spinach, 250 g broccoli and 37.5 ml wine did not affect prothrombin time values in patients fed an ordinary diet and warfarin therapy. However, when vitamin K_1, broccoli and spinach were given daily for one week, the prothrombin time was significantly reduced and the warfarin dose needed to be adjusted. Furthermore, in a clinical trial study, Kim *et al* [53] reported that INR on day 2 following a dose of 10 mg warfarin was 1.18 ± 0.19, which differed significantly from baseline (INR = 1.00 ± 0.05) and warfarin (10 mg) orally with vitamin K (10 mg) (INR = 1.06 ± 0.07). INR at baseline was not significantly

different from warfarin with vitamin K in a randomized crossover fasted study in eleven (3 men, 8 women) healthy adults by investigating the effects of oral vitamin K on S- and R-warfarin. On the other hand, if vitamin K_2 production in the intestine is inhibited, for example, by administration of broad spectrum antibiotics [49] that kill intestinal microflora, the anticoagulant action of warfarin is increased.

Drugs that Affect Blood Coagulation and Platelet Aggregation

Drugs that have an independent effect on blood coagulation or platelet aggregation may affect bleeding by distinct mechanisms in patients receiving warfarin therapy. For example, halofenate [54] and paracetamol [55] have a pharmacodynamic interaction with warfarin by independently affecting the activity of circulating coagulation factors. Mercaptopurine [56] increases the activity of circulating of Factor II which may correspondingly decrease the pharmacological effect of warfarin. Aspirin and most NSAIDs [57] increase the risk of bleeding in patients receiving warfarin by inhibition of platelet thromboxane A2 biosynthesis. An added concern with NSAIDs is the risk of damage to the integrity of epithelial surfaces leading to major bleeding [58].

1.6.2 Pharmacokinetic Interactions

Pharmacokinetic mechanisms for drug interactions with warfarin comprise mainly of induction or inhibition of drug metabolising enzymes and to a lesser extent alteration of plasma protein binding. It is important to note that some drugs may interact by multiple mechanisms. All of the four major processes that determine the

pharmacokinetic behaviour of a drug: absorption, distribution, metabolism and excretion can be affected by co-administration of other drugs.

Drugs that Affect Warfarin Absorption

Gastrointestinal absorption is slowed by drugs that inhibit gastric emptying or is accelerated by drugs, which hasten gastric emptying. Alternatively, one drug may interact with another drug in the gut in such a way as to inhibit absorption of warfarin. Co-administration of cholestyramine [59], a bile acid binding resin used to treat hypercholesterolemia, binds to warfarin in the gastrointestinal tract to prevent its absorption if administered simultaneously. Despite a number of possible interactions influencing warfarin absorption, the clinical significance of the interaction is limited because generally warfarin is very rapidly absorbed and also has a delay in the onset of its pharmacological response.

Drugs that Affect Warfarin Distribution

Although one drug may alter the distribution of another, such interactions are seldom clinically important [60]. Warfarin is very highly bound to plasma protein (fu_S = 0.0051 ± 0.0005, fu_R = 0.0062 ± 0.0005) especially to albumin [18] and as a consequence, small changes in protein binding will therefore lead to correspondingly large changes in circulating unbound drug. Displacement of a drug from binding sites in plasma or tissues transiently increases the unbound concentration of warfarin. However, warfarin has a low hepatic extraction ratio so an increase in fraction unbound leads to an increased hepatic clearance; a new steady state results in which

total drug concentration in plasma is reduced but the unbound drug concentration is similar with that before. In this case, it should be appreciated that the target therapeutic concentration range (with respect to total drug) will be altered by co-administration of a displacing drug. For example, phenylbutazone is capable of competing with warfarin for these plasma protein binding sites and it has been shown to increase the unbound fraction of S-warfarin from 0.0062 ± 0.0005 to 0.0111 ± 0.0011 and from 0.0052 ± 0.0005 to 0.0114 ± 0.0015 for R-warfarin [18]. In other words, the unbound concentration of warfarin almost doubled in over a short period of time before returning to pre-treatment levels. Furthermore, the protein binding affinity can be stereoselective. For example, sulfinpyrazone [61] can displace S-warfarin from its protein binding site to a greater extent than R-warfarin.

Drugs that Affect Warfarin Metabolism

Drug interactions can occur via inhibition or induction of drug metabolism with the risk of toxicity or reduced drug activity. These cases will be discussed in the following sections.

Enzyme Induction

Many clinically important drug interactions result from enzyme induction, particularly for cytochrome P450s. A number of drugs and xenobiotics cause enzyme induction and thereby decrease the pharmacological activity of a range of other drugs [62, 63]. For example, the antibiotic rifampicin [64], given for 3 days, reduces the effectiveness of warfarin as an anticoagulant. In humans, members of the CYP3A subfamily of P450 enzymes are particularly relevant to drug metabolism because of

their broad substrate specificity and their abundance in the liver and intestine. CYP3A4 alone is involved in the metabolism of greater than 50% of prescription drugs [62] while approximately 16% of clinically used drugs are metabolized by CYP2C9 [65]. Furthermore, it has become increasingly evident that the pregnane X receptor (PXR) as well as other nuclear receptors mediate CYP3A and CYP2C9 [66-69]. Activation of the PXR and the subsequent induction of CYP3A, CYP2C9 and other drug metabolising enzymes and transporters by xenobiotics can result, in turn, in an accelerated metabolism of some medications.

Enzyme Inhibition

Many drugs cause enzyme inhibition, particularly of the cytochrome P450 system. This can lead to increased drug and metabolite concentrations leading to excessive drug effects at a given dose. Inhibition of drug metabolism can be either stereoselective or non-stereoselective. For example, phenylbutazone selectively inhibits S-warfarin metabolism and cimetidine selectively inhibits R-warfarin metabolism, whereas amiodarone is known to inhibit the metabolism of both isomers [36, 70].

Drugs that Have Effects on Warfarin Excretion

Warfarin mainly undergoes hepatic metabolism. After administration of warfarin to humans, only trace amounts of unchanged warfarin are recovered in the urine [71]. Metabolites of warfarin excreted in urine are inactive, so drug interactions with the renal excretion of warfarin metabolites are not likely to be clinically significant [27, 71, 72].

1.7 St. John's Wort

St John's wort, also called hypericum, consists of the whole plant or cut dried flowering tops of *Hypericum perforatum*, harvested during flowering time. This herb contains not less than 0.08% total hypericins usually expressed as the content of hypericin ($C_{30}H_{16}O_8$; MW 504.4), calculated with reference to the dried herb [73]. St John's wort is widely used in the community for the management of a range of conditions including depression. The constituents, pharmacology and drug interactions of St John's wort have been the subject of several review articles [8, 73-75].

1.7.1 Chemistry and Pharmacology

St John's wort has been shown to contain at least nine groups of compounds that may contribute to the herb's pharmacological effect; however, the pharmacology of the many constituents is not yet fully known (Table 1-2).

In a manner similar to conventional antidepressant pharmacology, it is reported that constituents of St John's wort may exert a significant affect on catecholamine neurotransmission via known pathways, including: 1) inhibition of neurotransmitter metabolism; 2) modulation of neurotransmitter receptor density and sensitivity; and 3) synaptic reuptake inhibition [76]. St John's wort extracts have been shown to inhibit the uptake of serotonin (5-HT) [77], noradrenaline (NA) and dopamine (DA) and it has also been shown to have a potent affinity for the adenosine, serotonin 5-HT_1, and benzodiazepine and γ-aminobutyric acid (GABA) receptors as well as weakly inhibiting monoamine oxidase [78].

Table 1-2. The main constituents of St John's wort and possible pharmacological activity [74-76]**.**

Constituent and percentage of composition	**Possible action**
Anthraquinone derivatives (naphthodianthrones): Hypericin and pseudohypericin (0.1 – 0.15%) and isohypericin; Protohypericin and protopseudohypericin (biosynthetic precursors of hypericin and pseudohypericin, respectively); cyclopseudohypericin	Affinity for σ-opioid receptors
Flavonoids Flavonols (2-5%) (eg. kaempferol, quercetin); Flavones (eg. luteolin); Glycosides (eg. hyperoside (0.5-2%), isoquercitrin (0.3%), quercitrin (0.3%), rutin (0.3-1.6%)); Biflavonoids including biapigenin (a flavone) and amentoflavone (a isomeric to biapigenin derivative); Catechins (flavonoids often associated with condensed tannins)	Inhibition of MAO-A Agonist at benzodiazepine and GABA receptor
Prenylated phloroglucinols Hyperforin (2.0 – 4.5%) Adhyperforin (0.2 – 1.9%) Oxygenated analogues of hyperforin	Affinity for GABA receptors Inhibition of uptake of 5-HT, norepinephrine (NE), DA
Tannins (8-9%) Proanthocyanidins (condensed type)	Unknown
Other phenols; Caffeic; Chlorogenic; p-coumaric; ferulic; ρ-hydroxybenzoic; Vanillic acids	Unknown
Volatile oils (0.05 – 0.9%) Methyl-2-octane (saturated hydrocarbon)	Unknown

It has been reported that interleukin-6 could be affected by St John's wort and that this might be related to the antidepressant activity of St John's wort. Hyperforin is the constituent most likely to contribute to the St John's wort antidepressant activity [79]. There have been no published reports suggesting that St John's wort could affect platelet aggregation or the coagulation system.

1.7.2 Pharmacokinetics of St John's Wort Constituents

Hyperforin

Biber *et al* [80] investigated the oral bioavailability of hyperforin from hypericum extracts in rats and humans. After oral administration of 300 mg/kg St John's wort extract (containing 5% hyperforin) to rats, the C_{max} of hyperforin of 370 ng/ml were reached after 3 h, and the estimated half-life and apparent clearance were 6 h and 70 ml/min/kg, respectively. In this study healthy volunteers received a film-coated tablet containing 300 mg St John's wort extract, which is equivalent to a dose of 14.8 mg hyperforin. LC/MS/MS was used to measure the hyperforin concentration in the plasma. The hyperforin $C_{max,}$ t_{max} and half-life were estimated to be 150 ng/ml, 3.5 h and 9 h, respectively. Hyperforin pharmacokinetics were found to be linear up to a dose of 600 mg of the St John's wort extract. Increasing the doses to 900 or 1200 mg of St John's wort extract resulted in lower that expected C_{max} and AUC. Multiple doses of St John's wort were also used in the study and no accumulation of hyperforin in plasma was observed and the steady-state plasma concentrations of hyperforin were 100 ng/ml after 3 x 300 mg/day of the St John's wort extract [80].

Hypericin and Pseudohypericin

The single- and multiple-dose pharmacokinetics of the naphthodianthrones hypericin and pseudohypericin derived from St. John's wort (*Hypericum perforatum*, LI 160, Lichtwer Pharma GmbH, Berlin) were studied in 12 healthy male subjects. After a single oral dose of 300, 900 or 1800 mg of dried St John's wort extract (equivalent to 250, 750, or 1500 µg hypericin and 526, 1578, or 3156 µg pseudohypericin), the median maximal plasma concentrations were 1.5, 4.1, and 14.2 ng/ml for hypericin and 2.7, 11.7, and 30.6 ng/ml for pseudohypericin, respectively [81]. The elimination half-lives of hypericin ranged from 24.8 to 26.5 h, and varied from 16.3 to 36.0 h for pseudohypericin. Ranging between 2.0 to 2.6 h, the median lag-time of absorption was longer for hypericin when compared with pseudohypericin (0.3 to 1.1 h). The area under the plasma drug concentration-time curve (AUC) showed a non-linear increase with increasing dose; this effect was statistically significant for hypericin. During long-term dosing (3 x 300 mg/day) steady state was reached after 4 days. Mean maximal plasma concentrations during the steady-state dosing regimen were 8.5 ng/ml for hypericin and 5.8 ng/ml for pseudohypericin, while mean trough concentrations were 5.3 ng/ml for hypericin and 3.7 ng/ml for pseudohypericin [81].

1.7.3 St John's Wort Drug Interactions

Numerous clinical trials have demonstrated that St John's wort is an antidepressant indicated in patients with mild to moderate depression [82-84]. Recently, St John's wort has been implicated in numerous herb-drug interactions [8]. In response to the suspected St John's wort drug interactions, the Food and Drug Administration (FDA) published a Public Health Advisory announcement [85], stating that warnings need

to be added to the St John's wort labelling. In Australia, the Therapeutic Goods Administration (TGA) highlighted potentially serious interactions between St John's wort preparations and some prescribed medicines including warfarin [86]. But the suspected interaction between St John's wort and warfarin has not been systemically investigated. Furthermore, conflicting results appear in the literature between *in vitro* and *in vivo* studies. St John's wort drug interactions are reviewed in detail in the following sections.

Alprazolam

The benzodiazepine alprazolam is a substrate of CYP3A4 [87]. Markowitz *et al* [88] suggested that pre-treatment with St. John's wort (300 mg 3 times daily for three days) taken at recommended doses for depression is unlikely to affect alprazolam activity in healthy volunteers. The long-term use of St John's wort can induce CYP3A4 as demonstrated in several studies [8]. The fact that no significant difference was found in this study [88] could be related to short term use of St John's wort.

Amitriptyline

Several cytochrome P450 enzymes are involved in the metabolism of the tricyclic antidepressant amitriptyline including CYP2D6, CYP2C19 and CYP3A4 [89]. Johne *et al* [90] reported that plasma concentrations of amitriptyline, nortriptyline (a metabolite of amitriptyline) and other hydroxylated metabolites decreased when

amitriptyline was co-administrated with St John's wort (900 mg daily for 14 days). Cumulative urinary excretion of amitriptyline and metabolites decreased to the same extent as plasma concentrations upon co-administration with St John's wort based on a clinical trial in twelve patients[89]. Induction of cytochrome P-450 enzymes or drug transporters (P-glycoprotein) by St. John's wort extract may contribute to this pharmacokinetic interaction.

Caffeine

Two drug interaction studies [91, 92] have been reported with St John's wort and caffeine using different dose regimens; 1) 300 mg, 3 times daily for 28 days, 2) a single 900 mg oral dose and 300 mg, 3 times daily for 14 days based on clinical trials. No significant effect was observed on the activity of CYP1A2 using a probe-drug cocktail including caffeine [91, 92]. Caffeine is a substrate of CYP1A2 [93] and it was concluded that St John's wort does not affect CYP1A2 activity. However the cocktail approach has several limitations. First, the substrates of CYPs are not directly relevant to a particular drug class. Second, it is important to use a validated cocktail where there is not drug-drug interaction in them. Furthermore, a cocktail approach is designed to study drug interaction mechanism in metabolism, which does not allow assessing pharmacokinetic mechanism (including absorption, distribution, metabolism and excretion) and pharmacodynamic mechanism.

Carbamazepine

The anticonvulsant carbamazepine is a predominantly metabolised by CYP3A4 and partly by CYP2C8. Burstein *et al* [94] reported that treatment of eight healthy volunteers with St John's wort (300 mg 3 times daily for 14 days) did not induce the clearance of carbamazepine. Carbamazepine is subject to autoinduction so the authors suggested that St John's wort might not be potent enough to alter an enzyme system that is already induced.

Chlorzoxazone and Debrisoquine

By using a probe-drug cocktail including chlorzoxazone and debrisoquine which are substrates for CYP2E1 [95] and CYP2D6 [96], respectively and measuring single-time point phenotypic metabolic 6-hydroxychlorzoxazone/chlorzoxazone serum and debrisoquine urinary recovery ratios, Gurley *et al* [91] reported that St John's wort (300 mg, 3 times daily for 28 days) did not significantly affect the activity of CYP2E1 and CYP2D6 suggesting that St John's wort does not affect the metabolic activity of CYP2E1 and CYP2D6.

Cyclosporin

Numerous case reports of drug interactions between the immunosuppressant cyclosporin and St John's wort have been identified including eight kidney transplant recipients, one heart transplant recipient and one liver transplant recipient [97-105]. In these cases cyclosporin blood concentrations were consistently documented to be subtherapeutic during co-administration with St John's wort. One patient developed

acute rejection possibly due to low cyclosporin concentrations [97]. Another patient developed acute graft rejection due to low cyclosporin concentrations, after stopping treatment with St John's wort, cyclosporin blood levels remained within the therapeutic range and liver function recovered completely [102]. Dresser *et al* [106] reported that multiple doses of St John's wort (300 mg 3 times daily for 12 days) increased the clearance of cyclosporin after an oral dose in 21 young healthy subjects. Furthermore, Bauer *et al* [107] reported that administration of a St John's wort extract (600 mg once daily for 14 days) to renal transplant recipients resulted in a rapid and significant reduction of blood cyclosporin concentrations. Cyclosporin is a substrate for the CYP3A4 isoenzyme and P-glycoprotein [108, 109], so the induction of both CYP3A4 and P-glycoprotein by constituents of St John's wort may act to reduce the blood concentration of cyclosporin to subtherapeutic levels. This can lead to clinically significant consequences such as the rejection of a transplanted organ.

Dextromethorphan

Several drug interaction studies have been performed using the cough suppressant dextromethorphan as a substrate for CYP2D6 [110] in clinical trials. Roby *et al* [111] reported that St John's wort (300 mg 3 times daily orally for 14 days) failed to elicit a statistically significant change in dextromethorphan – dextrorphan ratios in thirteen healthy volunteers. Markowitz *et al* [88] suggested that pre-treatment with St. John's wort (300 mg 3 times daily for 3 days) taken at recommended doses for depression is unlikely to effect CYP2D6 activity in seven healthy volunteers. Furthermore, Wang *et al* [92] reported that short-term (3×300 mg, a single oral dose) and long-term St

John's wort (300 mg 3 times daily for 12 days) had no significant effect on dextromethorphan pharmacokinetics (and by inference CYP2D6 activity) in twelve healthy subjects.

Digoxin

Digoxin, a cardiac inotropic agent, is a substrate for P-glycoprotein [112]. In one case report, Cheng *et al* [113] reported a St John's wort interaction with digoxin. Durr *et al* [114] provided evidence from a rat study where St John's wort extract was administered to rats over 14 days resulting in a 3.8-fold increase of intestinal P-glycoprotein expression and a 2.5-fold increase in hepatic CYP3A2 expression using western blot analyses. In the clinical arm of this study, the administration of St John's wort extract (300 mg × 3 times) to eight healthy male volunteers for 14 days resulted in an 18% decrease of digoxin exposure after a single digoxin dose (0.5 mg), a 1.4- and 1.5-fold increase in expression of duodenal P-glycoprotein and CYP3A4, respectively [114]. These researchers showed a 1.4-fold increase in the functional activity of hepatic CYP3A4 using the ^{14}C-erythromycin breath test. These results indicate that St John's wort induces intestinal P-glycoprotein in rats and humans, hepatic CYP3A2 in rats, and intestinal and hepatic CYP3A4 in humans. In a separate study, Johne *et al* [115] investigated the pharmacokinetics of digoxin in a single-blind, placebo-controlled parallel study. After the achievement of steady state for digoxin concentrations on day 5, 13 healthy volunteers received digoxin either with placebo or with St John's wort (900 mg/d) for another 10 days. No statistically significant change was observed after the first dose of St John's wort extract;

however, 10 days of treatment with St John's wort extract resulted in a significant decrease of digoxin AUC_{0-24} by 25%.

Fexofenadine

Several drug interaction studies have been carried out using fexofenadine as a typical substrate for the drug transporter P-glycoprotein [116] in clinical trials. Dresser *et al* [106] reported that St John's wort (300 mg, 3 times daily for 12 days) increased the clearance of fexofenadine after oral administration to 21 young healthy volunteers. In a study by Wang *et al* [117] fexofenadine (60 mg) was orally administered before a single dose of St John's wort (900 mg), and after 2 weeks of treatment with St John's wort (300 mg 3 times a day) to determine P-glycoprotein activity using a three-period, open-label, fixed-schedule study design. A single dose of St John's wort significantly increased the maximum plasma concentration of fexofenadine by 45% and significantly decreased the apparent clearance by 20%, with no change in half-life or renal clearance. Long-term administration of St John's wort did not cause a significant change in fexofenadine disposition relative to the control phase. Compared with the single-dose treatment phase, long-term St John's wort caused a significant 35% decrease in maximum plasma concentration and a significant 47% increase in fexofenadine apparent clearance [117]. These results therefore suggest that single dose of St John's wort is an inhibitor of P-glycoprotein activity but is an inducer of P-glycoprotein activity after long-term use (two weeks).

Indinavir and Nevirapine

Drug interactions between antiretroviral agents and St John's wort have been identified in several case reports. St John's wort was found to lower nevirapine, Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs) or indinavir concentrations in people with human immunodeficiency virus (HIV) who were co-administered St John's wort [118-121]. *In vivo* studies in humans have shown that nevirapine is extensively biotransformed via cytochrome P450 metabolism to several hydroxylated metabolites. *In vitro* studies with human liver microsomes suggested that oxidative metabolism of nevirapine is mediated primarily by isoenzymes from the CYP3A family, although other isoenzymes may have a secondary role [8, 122]. Furthermore, in a clinical trial in sixteen healthy volunteers, St John's wort significantly reduced the AUC of the HIV-1 protease inhibitor indinavir by a mean of 57% and decreased the extrapolated 8 h indinavir trough concentration by 81% [123].

Irinotecan

Mathijssen *et al* [124] reported the effect of St John's wort on the metabolism of irinotecan, a pro-drug of SN-38 and a known substrate for CYP3A4, in 5 cancer patients treated with intravenous irinotecan in both the presence and absence of St John's wort (900 mg daily, orally for 18 days) in an unblinded, randomized crossover study design. The plasma concentrations of the active metabolite SN-38 decreased by 42% following St John's wort treatment. The degree of myelosuppression was substantially worse in the absence of St John's wort.

Midazolam

Several drug interaction studies with St John's wort have been reported using the sedative hypnotic midazolam as a typical substrate for CYP3A4 [125, 126]. Dresser *et al* [106] reported that St John's wort (300 mg, 3 times daily for 12 days) increased the clearance of midazolam after oral and intravenous administration in 21 young healthy volunteers. Furthermore, Gurley *et al* [91] reported that St John's wort (300 mg, 3 times daily for 28 days) significantly induced the activity of CYP3A4 using a probe-drug cocktail including midazolam by measuring single-time point phenotypic metabolic ratios. Interestingly, Wang *et al* [92] reported that short-term administration of St John's wort (a single 3×300 mg oral dose) had no effect on CYP activities but longer-term St John's wort (300 mg, 3 times daily for 14 days) administration caused a significant increase in the apparent clearance of midazolam using both oral midazolam (to study intestinal wall and hepatic CYP3A) and intravenous midazolam (to investigate hepatic CYP3A) in 12 healthy subjects.

Pravastatin and Simvastatin

The effects of St John's wort on the pharmacokinetics of the lipid lowing drugs simvastatin and pravastatin were investigated by Sugimoto *et al* [127]. St John's wort capsule (300 mg, three times a day for 14 days) was taken by sixteen healthy male subjects (n = 8 in group 1 and n = 8 in group 2) in a double blind, crossover study. On day 14, a single oral dose of simvastatin (10 mg) and pravastatin (20 mg) was given to subjects in group 1 and group 2, respectively. Plasma concentrations of simvastatin and simvastatin hydroxyl acid, an active metabolite, were significantly

reduced by coadministration with St John's wort while plasma concentrations of pravastatin were not affected. Simvastatin is metabolized by CYP3A4 in the intestinal wall and liver. No significant differences were observed in the elimination half-life of simvastatin or pravastatin. The results of this study suggest that the interaction is caused by the enhancement of the CYP3A4-mediated first-pass metabolism of simvastatin in the small intestine and liver. The lack of an interaction with pravastatin is probably because the major metabolites of pravastatin are produced by chemical degradation in the stomach rather than by cytochrome P450-dependent metabolism in the liver and is mainly excreted into urine and bile [128].

Theophylline

The bronchodilator theophylline is a substrate for CYP1A2 [129]. Nebel *et al* [130] reported the potential metabolic induction of theophylline by coadministration of St John's wort based on a case report. In an *in vitro* study, Karyekar *et al* [131] reported that St John's wort increased the expression of CYP1A2 in LS180 cells in a concentration dependent manner. The induction was time-dependent, since enzyme levels returned to baseline within 4-8 hours after removal of St John's wort constituents. This induction may be responsible for the observation of reduced plasma theophylline concentrations during co-administration of St John's wort [8, 131]. But this finding is in contrast with the results of an *in vivo* study using caffeine as typical substrate for CYP1A2 [91, 92].

Tolbutamide

Tolbutamide is a substrate of CYP2C9 [132]. Wang *et al* [92] reported that short-term administration of St John's wort (3×300 mg, a single oral dose) and long-term St John's wort (300 mg 3 times daily for 12 days) administration in twelve healthy subjects did not significantly affect tolbutamide pharmacokinetics.

Warfarin

Potential St John's wort interactions with warfarin have been reported in the literature. There are a number of case reports suggesting that co-administration of St John's wort decreases the effects of warfarin [8]. The Medical Products Agency (MPA, Sweden) has received seven case reports of a reduced anticoagulant effect and decreased INR of warfarin associated with co-administration of St John's wort [75]. Similarly, over the period October 1992 to September 2000, the United Kingdom (UK) Committee on Safety of Medicines and the Medicines Control Agency received 35 reports of suspected interactions between St John's wort and conventional medicines of which four were related to potential interactions with warfarin [74], two reported an increase in INR and two cases reported a decrease in INR. However no systematic investigation of the clinical significance and possible mechanism of a St John's wort interaction with warfarin have been reported.

Oral contraceptives

Oral contraceptive steroids are metabolised by cytochrome P450 [8]. Two drug interactions with St John's wort have been identified based on case reports. Schwarz

et al [133] reported an unwanted pregnancy upon self-medication with St John's wort despite hormonal contraception while Ratz *et al* [134] reported a case of irregular bleeding upon co-administration of St John's wort and oral contraceptives. While these cases are suggestive of herb-drug interaction, two recent controlled clinical trials have provided more definitive information.

Hall *et al* [135] reported that coadministration with St John's wort was associated with a significant increase in the apparent clearance of norethindrone and a significant reduction in the half-life of ethinyloestradiol. The study was conducted in twelve healthy premenopausal women who had been using oral contraception for 3 months as a combination oral contraceptive pill containing ethinyloestradiol and norethindrone for 3 consecutive 28-day menstrual cycles. The participants were administered St John's wort (300 mg, 3 times a day) during the second and third cycles. The serum concentrations of ethinylestradiol (day 7), norethindrone (day 7), follicle-stimulating hormone (days 12-16), luteinizing hormone (days 12-16), progesterone (day 21), and intravenous and oral midazolam (CYP3A4) (days 22 and 23) were measured. The incidence of breakthrough bleeding was quantified during the first and third cycles. The apparent oral clearance of midazolam was significantly increased during St John's wort administration, but the systemic clearance of midazolam was unchanged. Serum concentrations of follicle-stimulating hormone, luteinizing hormone, and progesterone were not significantly altered by St John's wort treatment. Breakthrough bleeding occurred in 2 of 12 women in the control phase compared with 7 of 12 women in the St John's wort phase. Induction of ethinyloestradiol and norethindrone metabolism caused by St John's wort was

consistent with increased CYP3A activity, which was demonstrated by using midazolam as a probe for this drug metabolism enzyme. In another study, Pfrunder *et al* [136] investigated the interaction between an oral contraceptive and St John's wort using eighteen healthy females treated with a low-dose oral contraceptive (0.02 mg ethinyloestradiol, 0.150 mg desogestrel) alone or combined with St John's wort extract (300 mg, twice daily or three times daily). Ovarian activity was assessed by measuring follicle maturation and serum concentrations of oestradiol and progesterone. During co-administration of low-dose oral contraceptive and St John's wort, there was no evidence of ovulation and no significant change in follicle maturation or serum sex hormone concentrations. However, significantly more subjects reported intracyclic bleeding during combined treatment with St John's wort extract (300 mg, twice daily) (13/17) and coadministration with St John's wort extract (300 mg, three times daily) (15/17) than with oral contraceptives alone (6/17). The C_{max} and AUC_{0-24} of ethinyloestradiol were not changed during and study cycle, whereas the AUC_{0-24} and C_{max} of 3-ketodesogestrel decreased significantly during administration of both St John's wort extract regimens. These data strongly suggest the potential for a serious interaction between oral contraceptives and St John's wort.

Others Drugs

One *in vitro* study demonstrated that St. John's wort constituents (hyperforin and quercetin) significantly inhibited the metabolic activity of CYP1A2 (hyperforin IC_{50}: 3.87 μM; quercetin IC_{50}: 11.6 μM), CYP2C9 (hyperforin IC_{50}: 0.01 μM; quercetin IC_{50}: 3.14 μM), CYP2C19 (hyperforin IC_{50}: 0.02 μM; quercetin IC_{50}: 6.13 μM)

CYP2D6 (hyperforin IC_{50}: 12.03 μM; quercetin IC_{50}: 21.0 μM)and CYP3A4 (hyperforin IC_{50}: 4.2 μM; quercetin IC_{50}: 19.52 μM) by analysing the conversion of specific surrogate substrates measured fluorometrically in a 96-well plate format [137]. Roby *et al* [138] reported that treatment with St John's wort (300 mg 3 times a day for 14 days) resulted in significant increases in the urinary 6-beta-hydroxycortisol/cortisol (a phenotype indicator of CYP3A4) ratio using healthy volunteers. In an *in vitro* study, Obach *et al* [139] reported that crude extracts of St John's wort demonstrated inhibition of each of the five isoenzymes CYP2D6, CYP2C9, CYP3A4, CYP1A2 and CYP2C19. The three former enzymes were more sensitive than the two latter enzymes to inhibition by St John's wort crude extracts. The flavonoid compound I3, II8-biapigenin of St John's wort was shown to be a potent, competitive inhibitor of CYP3A4, CYP2C9 and CYP1A2 activities [139]. Hyperforin was a potent non-competitive inhibitor of CYP2D6 activity and competitive inhibitor of CYP2C9 and CYP3A4 activities. Hypericin also demonstrated potent inhibition of several CYP activities. Budzinski *et al* [140] reported that St. John's wort has demonstrated *in vitro* CYP3A4 inhibitory capability by means of a fluorometric microtitre plate assay. Furohyperforin, a mono-oxidized hyperforin isolated from St John's wort (MW 552, IC_{50} 4.49 μM) was reported to be less potent as an inhibitor compared to other hyperforin analogues (IC_{50} 0.072 – 0.2 μM). The hyperforin analogues with MW 588 (IC_{50} 0.072 μM) and MW 522 (IC_{50} 0.079 μM) were the most potent inhibitors of CYP3A4 [141] using a fluorometric method to measure the fluorescence of the substrate metabolite fluorescein. These *in vitro* data suggesting that St John's wort and its constituents are inhibitors of drug

metabolising enzymes is in striking contrast to *in vivo* data, which implicate long term St John's wort administration as an inducer of drug metabolism.

Enzyme Expression

Several *in vitro* studies have been performed to determine the effect of St John's wort on drug metabolising enzyme and transporter expression. In one *in vitro* study, Perloff *et al* [142] exposed LS-180 intestinal carcinoma cells to St John's wort extract for 3 days. P-glycoprotein expression was strongly induced after exposure to St John's wort extract in a concentration-dependent manner. In Caco-2 cell monolayers, St John's wort significantly induced P-glycoprotein expression at clinically relevant concentrations of its constituents. As previously discussed, the *in vivo* study by Durr *et al* [114] demonstrated that St John's wort extract induced hepatic CYP3A2 in rats, and intestinal and hepatic CYP3A4 in humans. In a clinical trial using healthy volunteers who were randomized to receive either St John's wort (600 mg three times daily) for 16 days (in 15 subjects) or placebo (in 7 subjects), Hennessy *et al* [143] reported that St John's wort extract increased expression and enhanced the drug efflux function of the multi drug transporters P-glycoprotein in peripheral blood lymphocytes.

In summary, long-term use of St John's wort extract in human subjects induces the activity of CYP3A4 and P-glycoprotein but the available evidence suggests that the herb has less of an effect on CYP1A2, 2E1, 2D6 and 2C9. Furthermore, conflicting results were found between *in vivo* and *in vitro* studies. The role of individual

constituents of St John's wort in herb-drug interactions has not been conclusively investigated *in vivo*.

1.8 Panax Ginseng

In this thesis the term ginseng, also called Korean ginseng, refers to the whole or cut dried root of *Panax ginseng*. Ginseng contains not less than 0.40% of combined ginsenosides Rg1 ($C_{42}H_{72}O_{14}$, $2H_2O$, = 837) and Rb1 ($C_{54}H_{92}O_{23}$, $3H_2O$, = 1163), calculated with reference to the dried herb [144]. Ginseng has been used in Traditional Chinese Medicines to enhance stamina and capacity to cope with fatigue and physical stress [145]. Numerous constituents, pharmacological activities and drug interactions of ginseng have been reported in the literature [146-148].

1.8.1 Chemistry and Pharmacology

The chemistry and pharmacology of ginseng constituents have been reviewed by Gillis *et al* [145] and Attele *et al* [147]. Ginseng typically contains panaxadiols including ginsenoside Rb1 (0.38%), Rb2 (0.13%), Rc (0.19%), Rd (0.04%), Rg3, Rh2 and Rh3, panaxatriols including ginsenoside Re (0.15%), Rf (0.09%), Rg1 (0.38%), Rg2 (0.02%) and Rh1, and oleanic acid including ginsenoside Ro. Most of the pharmacological actions of ginseng are attributed to the ginsenosides [147]. However, more than twenty ginsenosides have been isolated. Some major pharmacological effects are summarised in the following sections.

Ginseng has been claimed to have both stimulatory and inhibitory effects on the central nervous system (CNS) including memory, learning and behaviour [149] and may modulate neurotransmission. Ginsenosides Rb1 and Rg1 are thought to play a major role in the effects of ginseng on the CNS [150, 151]. Several ginsenosides show direct cytotoxic and growth inhibitory effects against tumour cells *in vitro* via different mechanisms [152, 153]. Other studies have shown ginseng to induce differentiation as well as to inhibit metastasis [154, 155] based on *in vitro* and animal studies. Furthermore, ginseng and individual ginsenosides have demonstrated effects on cardiovascular physiology by a mechanism involving nitric oxide [145].

Numerous *in vitro* and *in vivo* studies have been performed on the effect of ginseng constituents on platelet aggregation and coagulation. One *in vitro* study in humans by Park *et al* [156] reported that the non-saponin fraction from the roots of ginseng inhibited the aggregation of human platelets induced by thrombin in a dose-dependent manner by regulating the levels of cGMP and thromboxane A_2 (TXA_2). Teng *et al* [157] reported that panaxynol markedly inhibited the aggregation induced by collagen, arachidonic acid, adenosine diphosphate (ADP), ionophore A23187, platelet activating factor (PAF) and thrombin of washed platelets while ginsenosides had no significant effect on the aggregation. Even so, ginsenoside Ro (1 mg/ml) did inhibit the ATP release from platelets. Kimura *et al* [158] reported that among six saponins tested; only ginsenoside Rg1 inhibited adrenaline- and thrombin-induced platelet aggregation and serotonin release in a dose-dependent manner. Park *et al* [159] reported that the lipophilic fraction from ginseng increased cGMP directly and cAMP indirectly and thus inhibited thrombin- or collagen-induced platelet

aggregation in one *in vitro* rat study. In another *in vitro* study using rabbit platelet, Kuo *et al* [160] reported that panaxynol and ginsenosides Ro, Rg1, and Rg2 were found to be the main antiplatelet components. In addition, panaxynol inhibited the aggregation, release reaction, and thromboxane formation in rabbit platelets while ginsenosides Ro, Rg1, and Rg2 suppressed the release reaction only. In one *in vitro* study, Yun *et al* [161] reported using a platelet aggregation assay with human platelet rich plasma, Korean red ginseng significantly inhibited thrombin (IC_{50} >2 mg/ml), ADP (IC_{50}, 0.72 mg/ml), or collagen (IC_{50}, 0.32 mg/ml) induced platelet aggregation. Furthermore, in a study conducted in rats [159], both the prothrombin time and activated partial thromboplastin time (APTT) were prolonged *in vivo* by the action of the lipophilic fraction (25 mg) from ginseng on rat platelet aggregation induced by collagen or thrombin. Blood coagulation and cGMP levels were also significantly increased.

1.8.2 Pharmacokinetics of Ginseng Constituents

Few pharmacokinetic studies of the constituents of ginseng have been reported in human subjects. Cui *et al* [162] determined that approximately 1.2% of the oral dose of protopanaxatriol ginsenosides (3 mg) and smaller amounts of the protopanaxadiol ginsenosides not exceeding 0.2% of the administered dose (7 mg) were recovered in urine over five days after a single dose of ginseng to healthy subjects. However, Shibata *et al* [163] reported that neither the individual ginsenosides nor their metabolites could be identified except compound-K, which is the main intestinal bacterial metabolite of protopanaxadiol ginsenosides, was identified in human serum using a specific enzyme immunoassay 8 h after oral administration of ginseng. *Panax*

notoginseng is different with *Panax ginseng* but it also contains ginsenoside Rb1 and Rg1. Very low bioavailability of ginsenoside Rb1 and ginsenoside Rg1 were found by Xu *et al* [164] after *Panax notoginseng* was administered to rats and serum samples were measured using an established HPLC method to quantitate ginsenoside Rb1 and Rg1. The decline in the concentrations of Rb1 in serum was described by a two-compartment pharmacokinetic model. The distribution half-life was reported as 23.4 min and the elimination half-life was 18.0 h. Ginsenoside Rb1 was absorbed from the gastrointestinal tract and the absolute bioavailability after oral administration was estimated to be only 4.35%. The pharmacokinetics of Rg1 in rats also was described by a two-compartment model with distribution and elimination half-lives were 24.23 and 14.13 h, respectively. Ginsenoside Rg1 was absorbed in the gastrointestinal tract and the oral bioavailability was estimated to be 18.4% [164].

1.8.3 Ginseng Drug Interactions

Ginseng has been used for thousands years and was listed in the top five selling herbal products for 2002 [165]. It could be expected that co-administration of ginseng with other conventional medicines is likely to be very high. However, relatively little information regarding herb-drug interactions with ginseng can be found in the literature. The available data are summarised in the following section. This thesis will focus on ginseng (*Panax ginseng*) which is different to Siberian ginseng (*Eleuthroccus senticosus*) containing eleutherosides B and eleutherosides E [166] and *Panax notoginseng* containing ginsenoside Rb1 and Rg1 [164].

Caffeine, Chlorzoxazone, Debrisoquine and Midazolam

Gurley *et al* [91] reported that administration of ginseng had no significant effect on CYP3A4, CYP1A2, CYP2E1, and CYP2D6 activity in twelve healthy volunteers who were randomly assigned to receive either ginseng or other herbal medicines for 28 days with a 30-day washout period between each treatment phase. A cocktail of probe drugs including midazolam (CYP3A4), caffeine (CYP1A2), chlorzoxazone (CYP2E1), and debrisoquin (CYP2D6) were administered before ginseng (baseline) and after ginseng pre-treatment. To determine the relative metabolic activity of CYP3A4, CYP1A2, CYP2E1, and CYP2D6, metabolic ratios including 1-hydroxymidazolam/midazolam serum ratios (1-hour sample), paraxanthine/caffeine serum ratios (6-hour sample), 6-hydroxychlorzoxazone/chlorzoxazone serum ratios (2-hour sample), and debrisoquine urinary recovery ratios (8-hour collection) were used to compare with or without coadministration, respectively [91].

Warfarin

In a case report, a 47-year-old man had received anticoagulation therapy with warfarin since 1990 to prevent embolic events. The dosage of warfarin and INR had been stabilised for past nine months. The patient's INR was 3.1 four weeks before co-ingestion of ginseng. Two weeks after the patient started taking ginseng, his INR declined to 1.5. Ginseng was discontinued, and the INR returned to 3.3 in two weeks [167]. However, in an animal study, Zhu *et al* [168] reported that there was no significant impact of ginseng on the pharmacokinetics or pharmacodynamics of warfarin using either single or multiple doses administration in male rats. Despite these conflicting observations, the possible effects of ginseng's constituents on

platelet aggregation still provides the possibility of a pharmacodynamic interaction with warfarin in humans.

Other Drugs

Based on *in vitro* experiments that investigated the catalytic activity of c-DNA expressed cytochrome P450 isoforms, Henderson *et al* [169] reported that the ginsenosides including Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 and eleutherosides including B and E are not likely to inhibit the metabolism of coadministered medications in which the primary route of elimination is via cytochrome P450 including CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. Increasing concentrations of these ginsenosides and eleutherosides were incubated with a panel of recombinant human CYP isoforms and their effects on the conversion of specific surrogate substrates were measured fluorometrically in a 96-well plate format. In a clinical trial, Anderson *et al* [165] reported that administration of ginseng (100 mg standardized to 4% ginsenosides, two times a day for 14 days) did not significantly alter the urinary 6-β-hydroxy-cortisol/cortisol ratio, leading to the suggestion that ginseng does not affect CYP3A4. Kim *et al* [170] reported that the standardized saponin of red ginseng showed inhibitory effects on CYP450-associated monooxygenase activities in a dose-dependent manner. These *in vitro* studies found that p-nitrophenol hydroxylase activity, which has been shown to represent the CCl_4-activating CYP450 2E1 enzyme, was inhibited. Chang *et al* [171] reported on CYP1 catalytic activity as assessed by 7-ethoxyresorufin O-dealkylation. The ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1, either individually or as a mixture and at the levels reflecting those expected *in vivo* did not influence CYP1 activities. However,

at a higher ginsenoside concentration (50 mg/ml), Rb1, Rb2, Rc, Rd, and Rf inhibited the enzyme's activity.

In summary, no significant ginseng-drug interactions have been demonstrated in the literature by *in vivo* or *in vitro* studies in therapeutic range of concentrations of ginseng constituents. Furthermore, the poor bioavailability of ginseng constituents ginsenoside Rb1 and Rg1 following administration of ginseng casts doubt on potential interactions *in vivo* (See Section 1.8.2).

1.9 Ginkgo Biloba

Ginkgo is the dried leaf of *Ginkgo biloba*. It is generally collected in autumn when leaves are green and leaves are dried immediately. *Ginkgo biloba* contains not less than 0.40% of total amount of flavonol glycosides, calculated on the dry basis [172].

1.9.1 Chemistry and Pharmacology

The constituents of primary interest in ginkgo (*Ginkgo biloba* L.) leaf are terpenoid compounds including ginkgolides (ginkgolides A, B, C, J and M), bilobalide and phenolic compounds including flavonoids (flavones, biflavones, flavonols, tannins and associated glycosides) [173]. The standardised leaf extract is prepared by a multi step purification process to a 35 - 67:1 (average 50:1) ratio of dried leaves to final concentrated extract. The extract is standardised to contain 22-27% flavonol glycosides (determined as quercetin, kaemferol and isorhemnetin usually determined by HPLC and calculated as quercetin and kaemferol glycosides with molar masses of

756.7 and 740.7, respectively), 5%-7% terpene lactones consisting of approximately 2.8-3.4% ginkgolides A, B and C and approximately 2.6-3.2% bilobalide; and below 5 ppm ginkgolic acids. The ginkgo leaf extract of the EGb 761 is standardised to contain 24% flavonol glycosides and 6% terpene lactones [73].

Ginkgo has been claimed to have various pharmacological actions which have been reviewed in several articles [174-177]. Ginkgo has been reported to have effects on the cardiovascular and circulatory systems [178], peripheral vascular system [179], neurological, psychological and behavioural function [180], immune system [181], and the metabolic and nutritional systems [173].

The effects of ginkgo on thrombosis, hemostasis and embolism have also been reported [173]. These effects provide the suggestion of a potential pharmacodynamic interaction with warfarin. However, conflicting results have been observed in the literature regarding the effect of ginkgo on clotting status. Several *in vitro* studies have demonstrated that ginkgo extract or ginkgolides A, B and C can inhibit platelet PAF rather than ADP or arachidonic acid induced platelet aggregation [182-185]. Furthermore, in clinical trials, collagen-induced platelet aggregation was inhibited after an intravenous infusion of a ginkgo extract in 24 patients suffering from arteriosclerotic disorders [186]. In another study, ginkgo extract significantly reduced collagen but not PAF-mediated platelet aggregation in healthy volunteers and Type 2 diabetic subjects in a clinical trial in which 120 mg of standardised extract was ingested for 3 months [187]. Reports that ginkgo does not affect clotting status have also been reported. An *in vitro* study found that up to 0.2 mg/ml of ginkgo extract did

not inhibit ADP and collagen-induced aggregation of rat platelets [188]. In another prospective, double blind, randomised, placebo-controlled study in 32 young male healthy volunteers; ginkgo extract (EGb761) did not alter any haematological marker using three doses of ginkgo extracts 120, 240 and 480 mg/day for 14 days [189]. In this study, primary haemostasis was assessed by both the bleeding time measured by the 3-point Ivy-Neison technique and a direct quantitative measurement of blood loss according to the Bernal-Hoyos methods. The interaction between platelets and coagulation was assessed using the thrombin generation test in platelet rich plasma. Several methods were used to assess platelet function including (i) by platelet aggregation in citrated platelet rich plasma, induced by three different agonists: adenosine diphosphate (2.5 μM), thrombin receptor agonist peptide (25 μM) and collagen (1.25 μg/ml); (ii) by quantification of platelet membrane glycoproteins and (iii) by measurement of procoagulant activity assessed by annexin fixation on the platelet membrane [189].

1.9.2 Pharmacokinetics of Ginkgo Constituents

Ginkgolide A, B, C and bilobalide

Drago *et al* [190] reported that a dose of *Gingko biloba* extract (40 mg twice daily for 7 days) was accompanied by a significantly longer half-life of ginkgolide B ($t_{1/2}$ = 11.64 ± 5.2 h) than gingko biloba extract (a single 80 mg dose daily for 7 days) ($t_{1/2}$ = 4.31 ± 0.49 h), even though the latter caused a higher peak concentration of ginkgolide B. The t_{max} of ginkgolide B was 2.3 h after administration in two different dosage regimens for orally administered *Gingko biloba* extract using twelve healthy volunteers randomly assigned to different treatment groups with a 21 day washout

period. Fourtillan *et al* [191] reported that after oral administration of 120 mg EGb 761 containing ginkgolide A (1.44 mg), ginkgolide B (1.03 mg) and bilobalide (3.36 mg) to twelve healthy volunteers, the mean absolute bioavailability was 80%, 88% and 79% for ginkgolide A, B and bilobalide, respectively, while ginkgolide C was not bioavailable. A significant amount of the given dose was excreted unchanged in urine [191]: ginkgolide A (72.3%), ginkgolide B (41.4%) and bilobalide (31.2%) after oral administration. The $t_{1/2}$ was 4.5, 10.6 and 3.2 h for ginkgolide A, B and bilobalide, respectively. The volumes of distribution were 36.9 L for ginkgolide A and 53.6 L for ginkgolide B. There was no relevant influence of food on the pharmacokinetics of ginkgolide A, B and bilobalide (Table 1-3). The pharmacokinetics of these ginkgo constituents were found to be linear over the dose range of 80 to 240 mg of EGb 761. The clearances in elderly people were lower (7.1 vs. 10.1 L/h for ginkgolide A, 8.4 vs. 9.7 L/h for ginkgolide B, 20.1 vs. 52.2 L/h for bilobalide) when compared with young volunteers [191].

Table 1-3. Pharmacokinetic parameters of ginkgolide A, B and bilobalide after oral administration of 120 mg EGb 761 under fasted and fed conditions (Data extracted from [192]**).**

	C_{max} (ng/ml)	t_{max} (h)	AUC (ng.h/ml)	F (%)	fe(%)	$t_{1/2}$ (h)	CL (L/h)
Fasting							
Ginkgolide A	33.3 ± 9.1	1.1 ± 0.7	146.4 ± 21.5	0.8 ± 0.1	72.3 ± 12.3	4.5 ± 1.6	10.1 ± 1.6
Ginkgolide B	16.5 ± 5.0	1.2 ± 0.7	109.9 ± 20.6	0.9 ± 0.2	41.4 ± 12.9	10.6 ± 3.6	9.7 ± 2.2
Bilobalide	18.1 ± 8.8	1.2 ± 0.8	79.0 ± 39.0	0.8 ± 0.3	31.2 ± 10.1	3.2 ± 0.6	52.2 ± 26.2
After breakfast							
Ginkgolide A	21.1 ± 4.3	2.0 ± 0.5	159.4 ± 52.5	0.9 ± 0.2	58.4 ± 12.3	4.0 ± 0.6	9.7 ± 2.4
Ginkgolide B	11.5 ± 2.6	2.5 ± 0.6	112.3 ± 37.4	0.9 ± 0.2	40.0 ± 8.0	9.5 ± 3.2	9.9 ± 2.6
Bilobalide	11.8 ± 6.7	2.9 ± 1.4	77.0 ± 33.0	0.8 ± 0.3	14.2 ± 12.2	4.3 ± 2.1	50.0 ± 17.8

1.9.3 Ginkgo Drug Interactions

Ginkgo has been widely used in many countries with the claims that it assists peripheral circulation and improves memory and cognitive function [173]. A review of ginkgo-drug interactions is presented in the following section.

Caffeine, Chlorzoxazone, Debrisoquine and Midazolam

Gurley *et al* [91] found no significant effect on CYP3A4, CYP1A2, CYP2E1, and CYP2D6 metabolic activity in twelve healthy volunteers who were randomly assigned to receive either ginkgo or other herbal medicines for 28 days with a 30-day washout period between each treatment phase. In this study, a cocktail of probe drugs including midazolam (CYP3A4), caffeine (CYP1A2), chlorzoxazone (CYP2E1), and debrisoquine (CYP2D6) was administered before ginkgo and after pre-treatment with ginkgo to determine the relative metabolic actability of these enzymes. However, this study using the cocktail approach is not directly relevant to a particular drug class and pharmacodynamic interactions remain unknown.

Digoxin

An open-labeled, randomized, crossover trial was conducted in eight healthy human volunteers to investigate the potential interaction between ginkgo and digoxin. In the first phase of this study, volunteers were randomly assigned to receive ginkgo (80 mg, three times daily) for one week, then ingested a single dose of digoxin (0.5 mg) with ginkgo for another two days while other volunteers received digoxin alone. No

significant difference between treatments was observed with respect to digoxin pharmacokinetics [193].

Diltiazem

In vitro and *in vivo* studies in rats by Ohnishi *et al* [194] reported that the addition of ginkgo to small intestinal and hepatic microsomes inhibited the formation of N-demethyl diltiazem, an active metabolite of diltiazem produced by CYP3A, in a concentration-dependent manner. The pre-treatment significantly decreased the elimination rate constant (k) of diltiazem following intravenous administration of diltiazem. These results indicated that the concomitant use of *Ginkgo biloba* in rats increased the bioavailability of diltiazem by inhibiting both intestinal and hepatic metabolism, via inhibition of CYP3A [194]. However, data from animal may infer the potential interactions but are not possible to conclusively assess clinical significance in humans.

Ibuprofen

Meisel *et al* [195] described a case report of the fatal intracerebral mass bleeding associated with co-ingestion of *Ginkgo biloba* and the NSAID ibuprofen (600 mg daily) in a 71-year old patient who had been taking ginkgo (40 mg, twice daily) for at least 2 years and 6 months. The fatal intracerebral mass bleeding in this patient was suspected to be intensified due to the inhibition of the TXA_2–dependent platelet aggregation conferred by ibuprofen and ginkgo. However, uncontrolled observational study may overestimate the significance.

Nicardipine

An *in vivo* study in rats by Shinozuka *et al* [196] reported that the feeding of ginkgo extracts to rodents for 4 weeks significantly reduced the hypotensive effect of the calcium channel blocker nicardipine, which is reported to be metabolized by CYP3A2 in rats. But clinical significance remains unknown.

Warfarin

Engelsen *et al* [197] have conducted a randomised, double blind, placebo-controlled crossover clinical trial in twenty-four outpatients on stable, long-term warfarin treatment with two week wash out period. These researchers found that coadministration of ginkgo extract (100 mg daily for four weeks) did not influence the INR of warfarin. However, the effect of ginkgo on the pharmacokinetics of warfarin was not investigated in this study.

Other Drugs

Zou *et al* [137] reported that constituents of *Ginkgo biloba* (ginkgolic acids I and II rather than ginkgolide A, B and C), respectively, inhibited CYP1A2 (IC_{50} (μM): 4.81, 4.88), 2C9 (IC_{50} (μM): 2.41, 1.94), 2C19 (IC_{50} (μM): 4.22, 4.41), 2D6 (IC_{50} (μM): 10.42, 7.82) and 3A4 (IC_{50} (μM): 6.74, 6.25) metabolic activity of the cDNA human P450 isoforms during *in vitro* experiments to measure their effects on the conversion of specific substrates measured fluorometrically in a 96-well plate format. The constituent bilobalide was found to inhibit the metabolic activity of CYP2D6 (IC_{50} (μM): 11.23). However, isolated tissues or microsomes may not reflect *in vivo* response.

Enzyme Expression

Several *in vivo* studies in rats have suggested that ginkgo extract is capable of inducing drug metabolism. Shinozuka *et al* [196] reported that male rats were fed either a control diet or diet containing ginkgo for 4 weeks. The ginkgo diet markedly induced levels of CYP2B1/2, CYP3A1 and CYP3A2 mRNA in the rodent liver. But the levels of CYP1A1, CYP1A2, CYP2E1, CYP2C11 and CYP4A1 were unchanged. Furthermore, Umegaki *et al* [188] reported that in rats, the concentration of CYPs and activity of various CYP enzymes in the rodent liver were increased in a dose- and time-dependent manner. The induction of CYP2B enzyme by ginkgo extract was confirmed by Western blot analysis [188].

In summary, *in vitro* studies suggest ginkgo constituents inhibited enzyme activity of CYP3A whereas *in vivo* studies in animals suggest the herb is an inducer to CYP3A2, 2B1/2 and 3A1. Data from a number of studies also suggests that ginkgo does not affect CYP3A4, CYP1A2, CYP2E1, CYP2D6, CYP2C9, CYP1A1, CYP 2C11 and CYP4A1.

1.10 Ginger

Ginger consists of the peeled, dried, whole or cut rhizome of *Zingiber officinale*, either completely or from the wide flat surfaces only. Whole or cut, ginger contains not less than 15 ml/kg of essential oil, calculated with reference to the dried herb [198]. Constituents, pharmacological activity and potential drug interactions have been reported for ginger [199, 200].

1.10.1 Chemistry and Pharmacology

Ginger contains volatile oils (1-3%), and the main constituents of these oils are sesquiterpene hydrocarbons in which the most abundant are zingeberene (35%) and farnesene (10%). Pungent compounds are considered to be responsible for the biological effect of ginger [200]. The major constituents found among these are different types of gingerols (33%) which are a series of homologous compounds differentiated by the number of carbon atoms in their side-chain: 10, 12 and 14 carbon atoms give rise to [6]-, [8]- and [10]-gingerols, respectively. The [6]-gingerol constituent is the most common. With prolonged storage of ginger, large amounts of [6], [8]- and [10]-shogaols are also found which are the dehydrated form of the gingerols [200] .

Numerous pharmacological effects have been documented in the literature including gastrointestinal, cardiovascular, serotonin antagonistic effects [200]. The following section reviews the effect of ginger and its constituents on platelet aggregation and coagulation.

A series of synthetic gingerols and related phenylalkanol analogues were found to inhibit arachidonic acid-induced platelet serotonin release and aggregation based on an *in vitro* study using human platelets [201]. Furthermore, it was found in *in vitro* studies that a significant effect of ginger extract was to inhibit platelet aggregation. Srivastava *et al* [202-204] reported that ginger extract extracted using three organic solvents: n-hexane, chloroform and ethyl acetate reduced platelet thromboxane

formation from exogenous arachidonic acid and also inhibited platelet aggregation induced by arachidonic acid, epinephrine, ADP and collagen in a dose-dependent manner. Suekawa *et al* [205] reported that (6)-Shogaol, a pungent component of ginger, inhibited arachidonic acid - induced platelet aggregation in rabbits. In a clinical trial, 5 g of dry ginger in two divided doses with a fatty meal was reported to significantly inhibit the platelet aggregation induced by ADP and epinephrine in ten healthy male volunteers in whom platelet aggregation was enhanced by 100 g butter for 7 days while there was no significant alteration in platelet aggregation in the placebo control group (10 healthy male volunteers) [205]. In addition, using a single dose of 10 g powdered ginger, a significant reduction in platelet aggregation induced by ADP and epinephrine was observed in patients with coronary artery disease while no significant effect was found using a dose of 4 g daily for 3 months [206].

However, conflicting findings related to the effect of ginger constituents on platelet aggregation are found elsewhere in the literature. In animal studies, no significant effect on the coagulation parameters PT and APTT or on warfarin-induced changes in blood coagulation was found in rats using multiple 100 mg/kg doses of EV.EXT™33, which is a ginger extract of patented standardised ethanol extract of dry rhizomes of *Zingiber officinale* Roscoe [207]. Lumb *et al* [208] investigated the effects of 2 g dried ginger or placebo capsules on platelet function using eight healthy male volunteers in a randomised double blind study. Bleeding time, platelet count, thromboelastography and whole blood platelet aggregometry were measured before, 3 h, and 24 h after the capsules. There were no significant differences found between ginger and placebo [208].

1.10.2 Pharmacokinetics of Ginger Constituents

After bolus intravenous administration of a 3 mg/kg dose of ginger extract in rats, the pharmacokinetics of [6]-gingerol were described by a two-compartment open pharmacokinetic model. [6]-Gingerol was rapidly cleared from plasma with a terminal elimination half-life of 7.2 min and a total body clearance of 16.8 ml/min/kg. Serum protein binding of [6]-gingerol was reported to be 92.4% [209]. There are no data describing the pharmacokinetics of ginger constituents in humans.

1.10.3 Ginger Drug Interactions

Ginger is a spice which is used daily in our society and with claims for aiding travel sickness and nausea relief [73]. The possibility of herb-drug interactions with ginger is expected in our community. The following sections provide a literature review of the available evidence supporting ginger – drug interactions.

Paracetamol

In a randomised double-blind crossover trial, ginger (1 g) or placebo was coadministered with paracetamol to 16 healthy volunteers. Gastric emptying and the absorption of paracetamol was not affected by co-ingestion with ginger [210].

Warfarin

In studies conducted in rats, the effects of patented standardised ginger extract on blood coagulation were studied. It was found that ginger had no significant effect on warfarin-induced changes in blood coagulation, suggesting that this herb does not

interact with warfarin [207]. However, because of the possible effects of ginger's constituents on platelet aggregation, the possibility of a pharmacodynamic interaction with warfarin in humans cannot be excluded.

Enzyme Expression

Banerjee *et al* [211] investigated the influence of certain essential oils including ginger oil on carcinogen-metabolizing enzymes and acid-soluble sulfhydryls in mouse liver and found ginger oil (10 µl/day for 14 days by gavage) did not significantly affect CYP levels.

In summary, the available information suggests there is no conclusive evidence supporting pharmacokinetic drug interactions with ginger. However, ginger has been found to have a range of pharmacological effects including the inhibition of platelet aggregation based on several *in vitro* studies. The possible pharmacodynamic interaction with the anticoagulant drug warfarin remains to be clarified.

Chapter 2 General Methodology

2.1 Clinical Study

2.1.1 Overview of Warfarin Drug Interaction Clinical Trial Designs

The anticoagulant drug warfarin remains one of the most frequently investigated drugs in interaction studies with new chemical entities [9]. This section presents a review of the literature of warfarin drug interaction studies with a focus on methodological aspects of clinical trial design and in particular on the dose administration to healthy volunteers.

The source of data for this review was obtained from MEDLINE 1966 to 2003 including articles listed under the search terms “warfarin” and “interactions”. Studies were limited to clinical trials involving humans. Articles were reviewed to examine the study design, dose of warfarin and all cases of subject withdrawal from drug interaction studies involving warfarin related adverse events.

Table 2-1 summarises the outcome of the literature review. In this review there were 58 papers identified describing 50 studies conducted in healthy volunteers and eight studies conducted in both male and female patients. The use of a single 25 mg dose of warfarin was the most widely used dose in warfarin drug interaction clinical trials involving healthy subjects. There were only eight reports of volunteer withdrawal due to warfarin side effects from the 58 studies, which represented a total of 704 subjects.

Table 2-1. Summary of clinical study designs to assess warfarin-drug interactions.

Aspect of Study design		**Total**	**References**
Papers		58 papers	
Studies using healthy subjects		50 papers (male, 670 subjects; female, 34 subjects)	
Studies using patient subjects		8 papers (158 patients)	
Studies using multiple doses		26 papers (Dose adjusted according to the target INR)	
Studies using single dose : 31 papers	5 mg	1 paper	[212]
	7.5 mg	1 paper	[213]
	10 mg	1 paper	[214]
	20 mg	1 paper	[215]
	25 mg	16 papers	[216-231]
	30 mg	7 papers	[232-238]
	50 mg	1 paper	[239]
	0.36 mg/kg	1 paper	[240]
	0.75 mg/kg	1 paper	[241]
	1.5 mg/kg	1 paper	[18]
Number of volunteers withdrawn due to ADR to warfarin		8 subjects	

Note: In one paper the dose of warfarin administered was unknown.

ADR: Adverse drug reaction

Based on the data presented in this review, it can be concluded that it is safe and reasonable to study drug interactions of warfarin in healthy male volunteers taking a single oral dose of 25 mg of warfarin.

2.1.2 Subjects

The two clinical studies conducted as part of this research had an identical study design. Twenty-four healthy male subjects were recruited into the two separate studies (n=12 in each study). Subjects were non-smokers and selected based on the following subject inclusion and exclusion criteria. A *post-hoc* power calculation indicated that twelve subjects in a crossover study would provide an 80% chance of detecting a 20% difference in the $AUC_{0-\infty}$ of S-warfarin at the p=0.05 level of significance. All participants gave written informed consent before entering the study. The study was approved by both the St Vincent's Hospital Research Ethics Committee and Human Ethics Committee of the University of Sydney.

Subject Inclusion Criteria

Subjects who fulfilled the following criteria were eligible for inclusion into this study:

1. Subjects must be healthy male volunteers aged 18 to 50 years and within 15% of ideal body weight for height and build.
2. Subjects must be in good health based on medical history, physical examination, and clinical laboratory test results including haematological test with a full differential blood count and haemostasis investigation (platelet aggregation and INR) and serum concentrations of creatinine, albumin, total bilirubin and total protein.

3. The current or past medical conditions of subjects must NOT be likely to significantly affect their pharmacokinetic or pharmacodynamic response to warfarin.
4. Subjects must not have taken any medication for at least two weeks before commencing the study.
5. The subjects must be willing and able to comply with the "Information for Participants".

Subject Exclusion Criteria

The subjects who did not meet the subject inclusion criteria would not be eligible for inclusion into this study.

1. Subject requesting cessation of treatment.
2. Subjects with any current or past medical condition that might significantly affect their pharmacokinetic or pharmacodynamic response to warfarin were excluded from the study.
3. Subject experiencing an adverse event that deemed to be sufficiently severe. The adverse event must be recorded.
4. Subject that exhibits non-compliance with the protocol.
5. INR of subject is greater than 4.5 (at which time medical management might warrant vitamin K administration).

2.1.3 Warfarin and Herbal Medicine Products

Coumadin™ (Warfarin sodium, Boots Healthcare Australia Pty Ltd, North Ryde NSW, Australia) was used in these two clinical trials. The commercially available

preparations of St John's wort, ginseng, ginkgo and ginger used in these two studies were chosen according to an assessment of the quality of various brands and also conformity of the dose with that recommended in the Herbal Medicine-Expanded Commission E Monographs. The qualitative assessment of a range of herbal medicine products are described in Chapter 4. Proprietary products Bioglan® (St John's wort, each tablet containing standardised dry extract equivalent to 1 g *Hypericum perforatum* flowering herb top, 0.825 mg hypericin and 12.5 mg hyperforin; Batch 1331-2, Bioglan Ltd, Kirrawee NSW, Australia), Golden Glow (Korean ginseng, each capsule containing extract equivalent to 0.5 g *Panax ginseng* root and 8.93 mg ginsenosides as ginsenoside Rg1; Batch K0125, Queensland Biochemics Pty Ltd, Virginia Qld, Australia), Tavonin™ (*Ginkgo biloba*, each tablet containing the standardised dry extract, EGb761, equivalent to 2 g of *Ginkgo biloba* leaf, 9.6 mg of ginkgo flavonglycosides, 2.4 mg of ginkgolides and bilobalide, Batch 6250202, Epping NSW, Australia) and Blackmore's Travel Calm Ginger (*Zingiber officinale*, each tablet containing extract equivalent to 0.4 g of ginger rhizome powder, Batch 103863, Balgowlah NSW, Australia) were used in these two clinical trials.

2.1.4 Study Design and Dose Administration

Both study designs were based on a 3 × 3 Latin Square (Table 2-2). It was an open label, controlled three-treatment, three-period, three-sequence crossover study with at least 14-day washout period between period I, period II and period III of dosing. An equal number of subjects were randomly assigned to each of the three possible dosing sequences.

Table 2-2. 3 × 3 Latin Square study design.

Group	Period		
	I	II	III
1. (Subject 1 – 4)	A	B	C
2. (Subject 5 – 8)	B	C	A
3. (Subject 9 – 12)	C	A	B

Study I

A: A single 25 mg warfarin dose.

B: St John's Wort doses (one tablet, three times daily) for 13 days, then a single 25 mg warfarin dose co-administered with multiple St John's wort doses (one tablet, three times daily) for a further 7 days.

C: Ginseng doses (two capsules, three times daily) for 6 days, then a single 25 mg warfarin dose co-administered with multiple ginseng doses (two capsules, three times daily) for a further 7 days.

Study II

A: A single 25 mg warfarin dose.

B: Ginkgo doses (two tablets, three times daily) for 6 days, then a single 25 mg warfarin dose co-administered with multiple ginkgo doses (two tablets, three times daily) for a further 7 days.

C: Ginger doses (three tablets, three times daily) for 6 days, then a single 25 mg warfarin dose co-administered with multiple ginger doses (three tablets, three times daily) for a further 7 days.

2.1.5 Randomisation

Subject sequence numbers were allocated to the twelve subjects when they registered. Following which, subjects were assigned a random number generated using Microsoft Excel. In this manner, subjects were assigned to one of the three treatment groups (Table 2-3).

Table 2-3. Randomisation.

Subject Sequence No.	1	2	3	4	5	6	7	8	9	10	11	12
Subject Random No.	2	2	3	3	1	3	2	1	1	1	2	3
Subject Group No.	2	2	3	3	1	3	2	1	1	1	2	3

2.1.6 Sample Collection and Analysis

In both studies, blood samples (20 ml) were collected into both sodium citrate and EDTA tubes via an indwelling cannula or by venipuncture. Sampling times in relation to warfarin dosing were: -48, -24, 0, 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144 and 168 h. Whole blood was collected to measure platelet aggregation while plasma was harvested by centrifugation (at 1500 g for 10 min) to determine the INR. A portion of plasma was stored frozen until the time of drug concentration analysis. Urine (24 h) was collected before and after administration of warfarin dose for three days. The volume of urine was recorded and a portion was stored frozen for subsequent analysis.

2.2 HPLC Assay for Warfarin Enantiomers

The concentrations of S-warfarin and R-warfarin in plasma were determined using a modified version of the high performance liquid chromatography (HPLC) assay by Naidong *et al* [242] which employed a chiral HPLC column (Silica-bonded β-cyclodextrin, Cyclobond™, Astec, Alltech Associates Australia Pty Ltd, Baulkham Hills, NSW, Australia) with fluorescence detection. In brief, aliquots (0.5 ml) of plasma were spiked with the internal standard (I.S., naproxen) and sulfuric acid solution (0.35 ml, 0.5 M) and then extracted with dichloromethane: hexane (1:5, 4 ml) on a roller for 15 min. The organic phase was decanted after separation by centrifugation (at 1500 g for 10 min) and freezing the aqueous phase in dry ice. The organic layer was evaporated to dryness under nitrogen and reconstituted in acetonitrile (200 µl) and an aliquot (20 µl) was injected onto the column. The mobile phase comprised of acetonitrile: triethylamine: glacial acetic acid (100:0.2:0.3, v/v/v/v) with a flow rate of 1 ml/min. Measurements were made using a fluorescence detector (RF 535, excitation wavelength 310 nm, emission wavelength 400 nm, Shimadzu Scientific Instruments (Oceania) Pty Ltd, Rydalmere, NSW, Australia). Unknown concentration in plasma samples from the clinical trial were analysed in duplicate with five different concentrations of standard samples in plasma for standard curve and low, medium and high concentration of the quality control samples in plasma.

2.3 HPLC Assay for S-7-hydroxywarfarin

The concentrations of S-7-hydroxywarfarin in urine were determined using a modified version of the HPLC assay by Naidong *et al* [242] which employed a chiral HPLC column (Silica-bonded β-cyclodextrin, Cyclobond™, Astec, Alltech Associates Australia Pty Ltd, Baulkham Hills NSW, Australia) with fluorescence detection. In brief, aliquots (0.1 ml) of urine samples were spiked with internal standard (naproxen) and sulfuric acid solution (0.5 M, 0.1 ml) and then extracted with dichloromethane: hexane (1:2, 4 ml). After 30 min on a roller bed mixer, the organic phase was decanted after separation by centrifugation (at 1500 g for 10 min) and freezing the aqueous phase in dry ice. The organic layer was evaporated to dryness under nitrogen and reconstituted in acetonitrile (200 µl) and an aliquot (20 µl) injected onto the column. The mobile phase comprised of acetonitrile: methanol: triethylamine: glacial acetic acid (100:1.5:0.3:0.25, v/v/v/v) with a flow rate of 1 ml/min. Measurements were made using a fluorescence detector (Shimadzu RF 535, excitation wavelength 310 nm, emission wavelength 400 nm, Shimadzu Scientific Instruments (Oceania) Pty Ltd, Rydalmere, NSW, Australia). Unknown concentration urine samples from the clinical trial were analysed in duplicate with five different concentrations of standard samples in urine for standard curve and low, medium and high concentrations of the quality control samples in urine.

2.4 Plasma Protein Binding

The unbound fractions of S-warfarin and R-warfarin in plasma were assessed by ultrafiltration (Centrifree® YM-30, Millipore Australia Pty Ltd, North Ryde, NSW,

Australia). *Rac*-warfarin (10 μg) was added to 1 ml of pooled plasma obtained from each subject between the sampling times of 1-8, 12-72 and 96-168 h after warfarin dose. After centrifuging at 1800 g for 20 min at 37^{o}C, the ultrafiltrate was collected for drug concentration measurement. The concentration of each warfarin enantiomer in the ultrafiltrate and in plasma was measured by the validated HPLC method. In brief, plasma ultrafiltrate (0.2 ml) containing warfarin, 10 μl of I.S. solution (naproxen; 0.0341 mg/ml) and sulfuric acid (0.5 M, 140 μl) were added. After mixing, dichloromethane: hexane (1:5, 2 ml) was added as an extraction solvent. After roller bed mixing for 15 min, centrifuging at 1500 g for 10 min and freezing the aqueous phase in dry ice, the organic phase was separated and evaporated to dryness under nitrogen and the residue was reconstituted in 100 μl of the mobile phase. A 50 μl of the reconstituted residue was injected into the HPLC. The fraction unbound (fu) was calculated as the ratio of the concentration of each warfarin enantiomer in the ultrafiltrate to that in plasma. All of the samples were analysed in duplicate.

2.5 INR Measurement

Warfarin therapy can be monitored by measuring the prothrombin time (PT) or International Normalised Ratio (INR). A number of methods can be used to measure the effect of warfarin in individuals, including the measurements of vitamin K_1 and vitamin K_1 2,3 epoxide concentration [243], γ-carboxyglutamic acid concentration [244] and clotting factor concentrations [245]. Of the various methods available, the PT test or INR test is the most common method for monitoring the effect of oral anticoagulants [14]. The PT is responsive to depression of three of the four vitamin

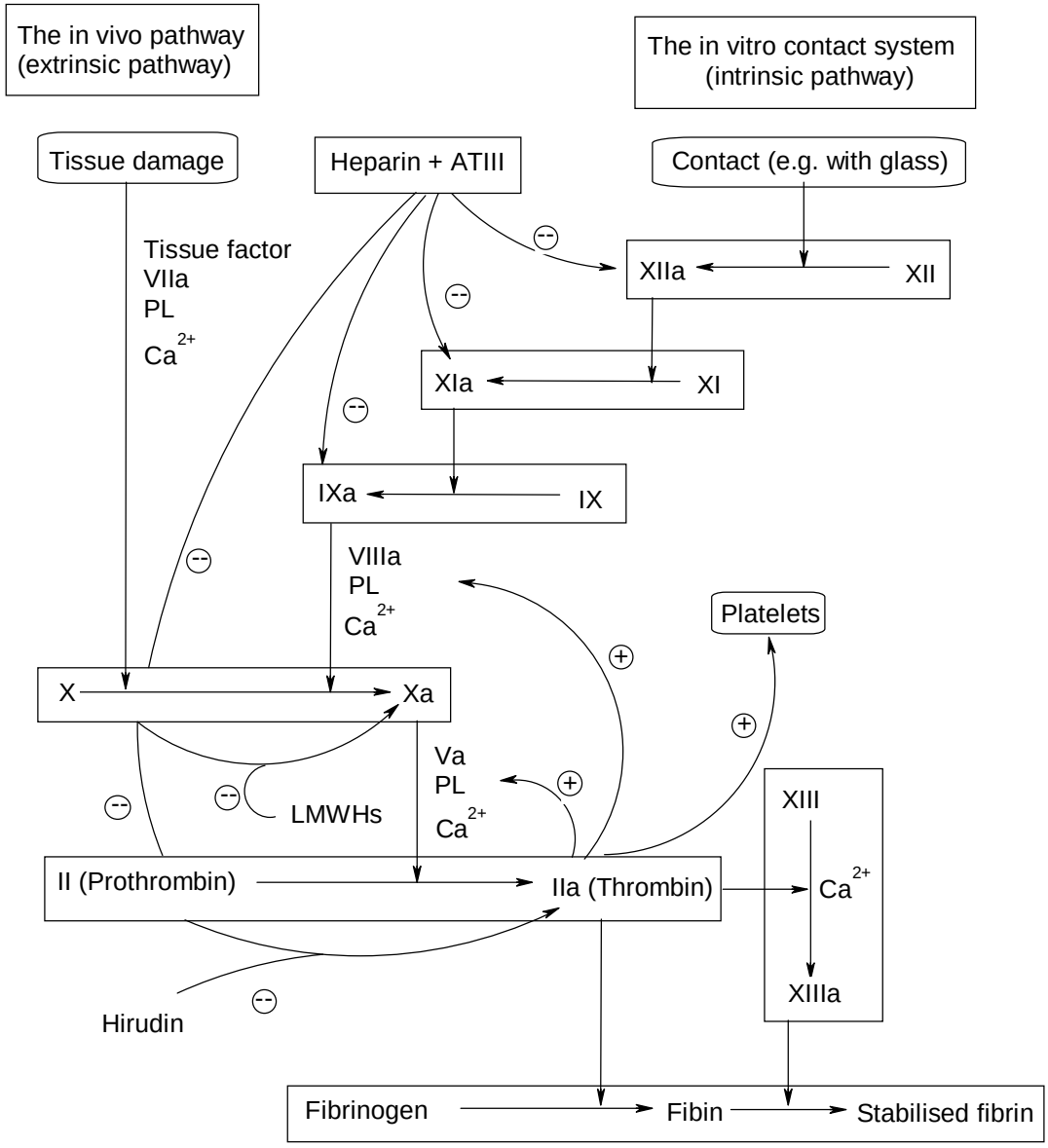
K-dependent clotting Factors (II, VII and X), which can be reduced in the presence of warfarin at a rate proportional to the respective half-lives of these factors (Figure 2-1). The principle of the method is described in Equation 2-1.

$$\text{Citrated plasma} + \text{Thromboplastin} + CaCl_2 \longrightarrow \text{Fibrin Clot} \quad \text{Equation 2-1}$$

Thromboplastin is a phospholipid-protein extract of tissue usually lung, brain or placenta, containing both the tissue factor and phospholipid necessary to promote the activation of Factor X by Factor VII. During induction of anticoagulant treatment, the PT primarily reflects the depression of Factor VII, since this factor has the shortest half-life (4-6 h) among the vitamin K-dependent factors. During maintenance therapy, this test is sensitive to depression of prothrombin (Factor II), Factor VII and Factor X [246].

Thromboplastins vary in their responsiveness to the anticoagulant effects of warfarin, depending on their source, phospholipid content and preparation [247]. The responsiveness of thromboplastins can be measured by assessing their International Sensitivity Index (ISI). Difference in thromboplastin responsiveness was the main reason for clinically important differences in oral anticoagulant dosing. In 1983, the INR system proposed by Kirkwood was adopted by the World Health Organization (WHO) which then made international PT standardisation possible [248]. The calculation of INR is described in Equation 2-2.

Figure 2-1. The coagulation cascade. Adapted from [16]**.**



Note: PL, negatively charged phospholipid supplied by activated platelets; ATIII, antithrombin III, LMWHs, low-molecular-weight heparins.

$$INR = (\text{patient PT}/\text{mean normal PT})^{ISI} \qquad \text{Equation 2-2}$$

The measurement of INR in the present study was conducted according to the instructions supplied by the manufacturer (Dade Behring, Australia) in the principles of the method. The coagulation process is triggered by incubation of plasma with the optimal amount of thromboplastin and calcium. The time to formation of a fibrin clot is then measured. Thromborel S (human thromboplastin/calcium reagent for one stage prothrombin time, Dade Behring, Diagnostics Pty Ltd, Lane Cove NSW, Australia) was reconstituted according to the labelled amount of distilled water and warmed to 37^{o}C before use (after reaching 37^{o}C the reagent must be incubated at this temperature for at least 30 minutes). Citrated plasma (50 µl) was pipetted into a Dade Behring cuvette pre-warmed to 37^{o}C and incubated for 1 min at 37^{o}C; 100 µl of Thromborel® S (warmed to 37^{o}C) was pipetted to the Dade Behring Coagulometer analyser. On the addition of the Thromborel® S reagent, the timer on the coagulometer (Dade Behring, Diagnostics Pty Ltd, Lane Cove NSW, Australia) was started and the PT was determined. INR is calculated using Equation 2-2, where International Sensitivity Index (ISI) is provided for the Thromborel® S reagent by the manufacturer. All of the samples were analysed in duplicate.

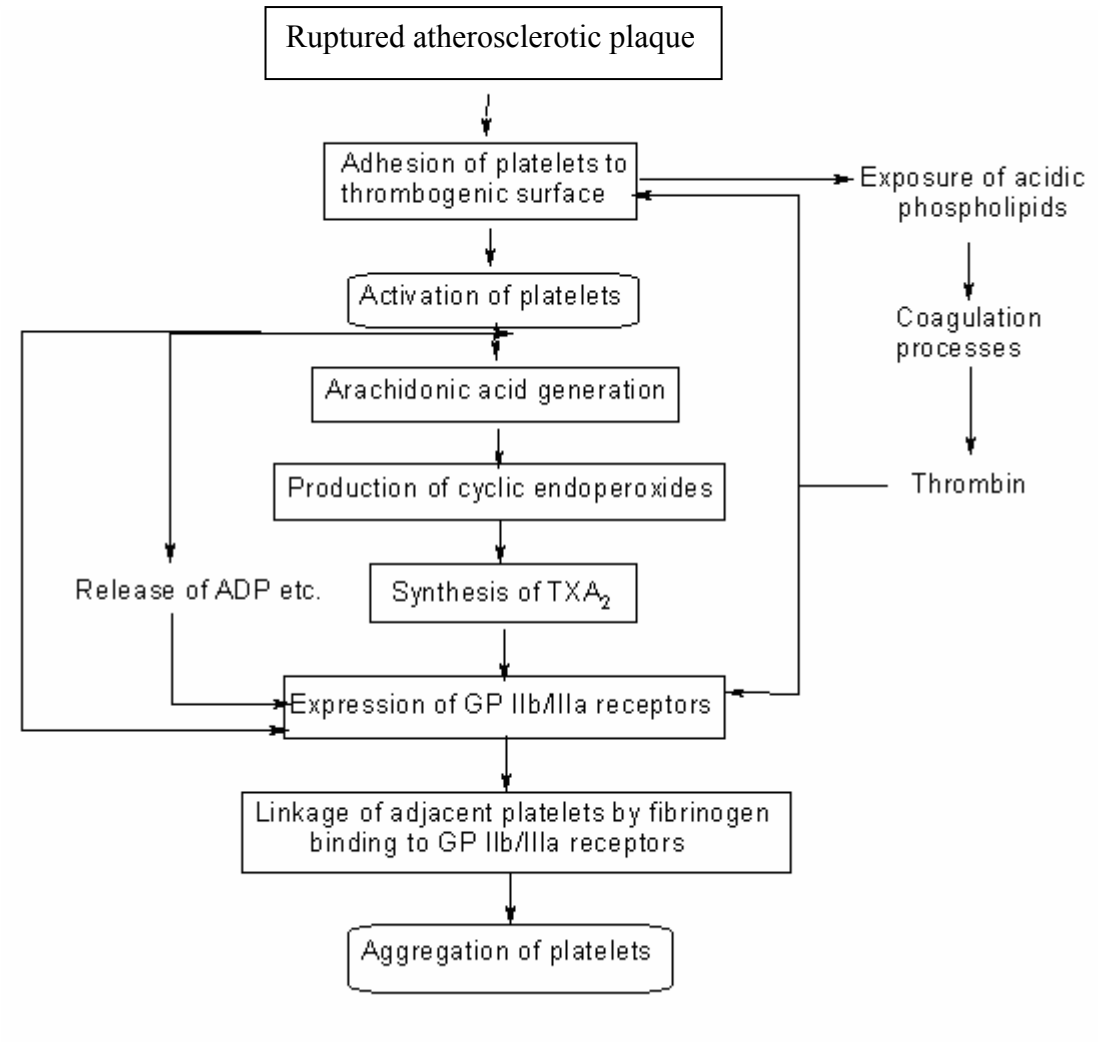
2.6 Platelet Aggregation Measurement

Numerous methods have been reported for measuring platelet function including photo-optical (turbidimetric) methods of platelet aggregometry, impedance methods

of platelet aggtegometry, viscometry, Clot retraction, PFA-100 and rapid platelet function assay. The advantages and disadvantages of these methods have been reviewed in several review articles [249-251]. Impedance platelet aggregation was introduced in 1980 by Cardinal and Flower [252] as an alternative to photo optical aggregometry that could also be applied to whole blood samples. The advantage of this method is that whole blood is more physiologic, a smaller quantity of blood is needed for testing, and it can be performed quickly and conveniently.

Platelet activation can be induced by a variety of chemical and mechanical methods via several distinct intracellular pathways [251] (Figure 2-2). Adenosine disphosphate (ADP) has been the agonist most commonly used for turbidimetric aggregation studies. The relevance of collagen as an agonist stems from the abundance of collagen in the subendothelium, which is exposed at the time of arterial injury. The use of collagen as an agonist is limited by variability between production lots that occur at the time of harvest. Arachidonic acid and TXA_2 are weak platelet agonists that are completely inhibited by aspirin. Thrombin is the most potent platelet agonist; however, thrombin-induced platelet aggregation has several limitations including lot-to-lot variability, storage requirements and the ability of thrombin to activate the coagulation cascade and thus interfere with the assay. Recently, a thrombin receptor agonist peptide (TRAP) has been used to replace thrombin to allow activation of platelets through the thrombin receptor without activating fibrinogen into fibrin [253].

Figure 2-2. Platelet activation cascade. Adapted from [16]**.**



Note: ADP, adenosine disphosphate; GP, glycoprotein; NO, nitric oxide; TXA_2, thromboxane A_2

Platelet aggregation was measured in these studies using a whole-blood aggregometer (Chrono-par®, Chrono-log Corporation, USA, supplied by Edward Keller Australia Pty Ltd, Hallam, Vic, Australia) according to the manufacturer's instructions. Briefly, pre-warmed whole blood (1 ml) diluted 1 in 2 (one part of blood to one part of saline) with normal saline was incubated at 37^{o}C for 2 min (stirring speed-1200 rpm). Platelet aggregation was induced by adding arachidonic acid (10 µl, 50 mM stock concentration, Chrono-par®, Chrono-log Corporation, USA, supplied by Edward Keller Australia Pty Ltd, Hallam, Vic, Australia). A change in impedance was recorded for 6 min after stimulation with arachidonic acid and reported as impedance aggregation (ohm). All of the samples were analysed in duplicate.

2.7 Data Analysis

2.7.1 Model Independent Pharmacokinetic and Pharmacodynamic Analysis

The pharmacokinetic parameters for warfarin enantiomers were estimated using two approaches; model independent and model dependent methods (See Section 2.7.2). The elimination rate constant (k) of warfarin enantiomers was obtained by linear regression analysis of the terminal log-linear portion of the warfarin enantiomer concentration – time curve. Elimination half-life ($t_{1/2}$) was calculated as ln2/k. The area under the plasma S-warfarin and R-warfarin concentration-time curves until the last concentration observation (AUC_{0-t}) were calculated using the trapezoidal rule. The AUC was extrapolated to infinity ($AUC_{0-\infty}$) using C_t/k where C_t is the last measured S-warfarin or R-warfarin concentration occurring at time "t". The highest S-warfarin and R-warfarin concentrations (C_{max}) and the time that these occurred

(t_{max}) were obtained by observation without interpolation. Apparent clearance (CL/F) and apparent volume of distribution (V/F) for the warfarin enantiomers were calculated as Dose/2/$AUC_{0-\infty}$ and CL/F/k, respectively. INR was reported as the area under the INR versus time curve (AUC_{0-168} of INR) and calculated by the trapezoidal method. Also the INR_{max} and t_{max} of INR were reported. Urinary excretion rate (UER) of S-7-hydroxywarfarin was calculated as the amount of metabolite eliminated (Ae,m) over the sample collection time interval (T).

2.7.2 Pharmacokinetic-Pharmacodynamic Modelling

Equation 2-3 and Equation 2-4 were combined with Equation 2-5 to produce a combined PK/PD model as described by Chan *et al* [18]: the enantiomer concentration-time data was described using the one-compartment Pharmacokinetic model with first order absorption (ka) and elimination (k). Such that;

$$C_R(t) = \frac{F \cdot D}{2 \cdot V_R} \cdot \frac{ka}{ka - k_R} \left(e^{-k_R (t - t_d)} - e^{-ka (t - t_d)} \right)$$

Equation 2-3

$$C_S(t) = \frac{F \cdot D}{2 \cdot V_S} \cdot \frac{ka}{ka - k_S} \left(e^{-k_S (t - t_d)} - e^{-ka (t - t_d)} \right)$$

Equation 2-4

where $C_R(t)$ and $C_S(t)$ are the concentrations R- and S-warfarin at time (t).

$$\frac{dPCA}{dt} = k_d \left[\frac{100}{\left(\frac{C_{u,S}}{EDu_{50,S}}\right)^{\gamma_S} + \left(\frac{C_{u,R}}{EDu_{50,R}}\right)^{\gamma_R} + 1} - PCA \right]$$

Equation 2-5

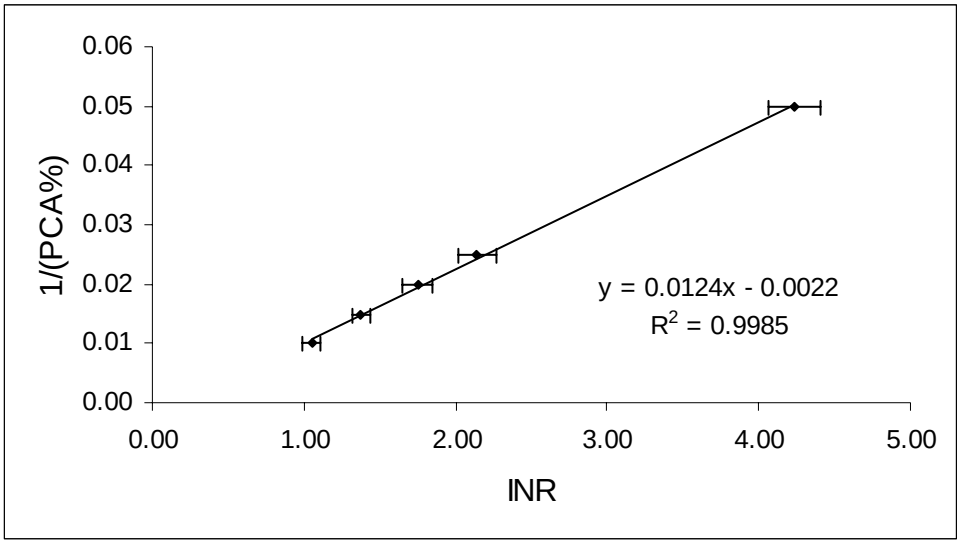
The terminology used in these equations is defined in Section1.4.2. An additional pharmacodynamic parameter was introduced to account for the observed delay in PCA change after warfarin administration. Anticoagulant response data was obtained as INR, so in order to fit these data using the proposed PK/PD model, response data was transformed to PCA with the use of the functional relationship between INR and PCA (percentage of normal activity) described in Equation 2-6.

$$PCA(\%\quad \text{normal}) = \frac{a}{INR - b}$$

Equation 2-6

in where a = 80.65 and b = 0.18. The values of the constants a and b were determined by fitting Equation 2-6 to PCA% and INR data (Figure 2-3) obtained using serial dilutions of pooled normal plasma. This relationship was substituted into the combined equation to allow direct application of INR data.

Figure 2-3. PCA%-INR standard curve (n=4).



Then the pharmacokinetic-pharmacodynamic modelling of warfarin enantiomer was analysed using Scientist® for Windows™ Version 2.0 (MicroMath, Inc., Salt Lake City, Utah 84121) to estimate PK/PD parameters.

2.7.3 Statistical Analysis

The PK/PD parameters were compared using analysis of variance (ANOVA) followed by the *post hoc* multiple comparisons with Dunnett using Stata® 5.0 (Stata Corporation, USA) and SPSS® 11.0 (SPSS Inc., USA) for sequence, period and treatment effects. The 95% confidence interval (CI) was used for the descriptive presentation of study parameters while the 90% CI of the ratio of logarithmically transformed parameters was used to compare control (warfarin alone) and treatment (warfarin with herbal medicine) phases. It was deemed that an interaction lacked clinical significance if the 90% CI values fell within the range 80%-125%. A p-value of less than 0.05 was considered significant.

Chapter 3 Validation of Analytical Methods

3.1 Calibration of HPLC Assay for Warfarin Enantiomers in Human Plasma and S-7-hydroxywarfarin in Urine

3.1.1 Introduction

An assay capable of quantifying warfarin enantiomers in human plasma and S-7-hydroxywarfarin in human urine is necessary for the present study. Several HPLC methods have been developed for the determination of warfarin enantiomers in human plasma in the literature. The HPLC methods can be classified into two categories: methods (Table 3-1) that employ derivatisation of warfarin to diastereoisomers followed by an achiral HPLC separation and methods (Table 3-2) that employ a chiral HPLC stationary phase column to directly separate enantiomers. The major issue of methods requiring derivatisation is the optical purity of the chiral derivatising agent and the reaction efficiency. Methods requiring derivatisation can also be time consuming and laborious hence most recent assays of warfarin enantiomers have focussed on using chiral stationary phase. There are several kinds of chiral stationary phases which are used in stereoselective HPLC that have been used to separate warfarin enantiomers, such as Chiralcel OC (Cellulose), Chiralcel OD (Cellulose), Achiral/chiral (C8/Chiral-AGP), Silica-bonded β-cyclodextrin and achiral/chiral (Bovine serum albumin/ Pinkerton) (Table 3-2).

Table 3-1. HPLC methods for using chiral derivatising agents for assay of warfarin enantiomers in human plasma.

Reference	Column type and derivatising agent	Resolution factor	Sensitivity	Accuracy	Precision	Detector
Banfield *et al* [254]	Silica; Carbobenzyloxy-L-proline	N/A	96-160 ng	N/A	N/A	UV: 313 nm
Banfield *et al* [255]	Silica and post-column aminolysis with n-post-column; Carbobenzyloxy-L-proline	N/A	50-100 ng	N/A	N/A	Fluorescence Ex: 313 nm Em: N/A
Carter *et al* [256]	Silica and hydrolysed post-column; (-)-HCA	N/A	5 – 10 ng/ml	105.9-108.6%	8.6-11%	Fluorescence Ex: 313 nm Em: 370 nm

N/A: Not available; HCA: hept-5-ene-2-carboxylic acid
Ex: Excitation; Em: Emission

Table 3-2. HPLC methods using chiral stationary phase for assay of warfarin enantiomers in human plasma.

Reference	Column type	Resolution factor	Sensitivity	Accuracy	Precision	Detector
Andersen *et al* [257]	Chiralcel OC (Cellulose)	>1.5	25 ng/ml	90%	R-: 3.6-9.5% S-: N/A	Fluorescence Ex: 315 nm Em: 370 nm
Takahashi *et al* [258]	Chiralcel OD (Cellulose)	5.6	R-: 20 ng/ml S-: 40 ng/ml	96.9-103	<9.6%	UV: 312 nm
McAleer *et al* [259]	Achiral/chiral (C8 / Chiral – AGP)	1.64	50 ng/ml	91.2-93.3%	N/A	Fluorescence Ex: 300 nm Em: 390 nm
Naidong *et al* [242]	Silica-bonded β-cyclodexrin	>1.5	12.5 ng/ml	N/A	S-: 12.1% R-: 9.7%	UV: 320 nm
Chu *et al* [260]	Achiral/chiral (Bovine serum albumin/Pinkerton)	1.05	N/A	N/A	N/A	N/A
Naidong *et al* [261]	Silica-bonded β-cyclodexrin	N/A	1 ng/ml	S-:<7.3%(bias) R-:5.8% (bias)	S-: <7.3% R-: <6.5%	MS/MS

N/A: Not available

The main disadvantage of such an approach is the expense and relatively short life of the column.

A modified version of the assay by Naidong *et al* [242] was employed in the present investigation of warfarin enantiomer concentrations in plasma and S-7-hydroxywarfarin in urine. This assay was chosen because it demonstrates excellent enantiomer resolution, high sensitivity and longer column stability compared with other assays.

3.1.2 Materials and Methods

Materials and Reagents

Racemic warfarin (98%, Lot 65H12131) was purchased from Sigma-Aldrich (Sigma-Aldrich Pty. Ltd., NSW, Australia), S-7-hydroxywarfarin (98%) was purchased from UFC Ltd (Manchester, England), naproxen (Lot B-6-JD-006) was obtained from Syntex laboratories, Inc. (Palo Alto, Calif., USA). All organic solvents were of analytical grade and purchased from Biolab (Mulgrave, Vic, Australia) including dichloromethane (Mallinkrodt, ChromAR®), hexane (Mallinkrodt, ChromAR®), acetonitrile (B&J Brand ®), triethylamine (AJAX Chemicals, UNILAB), acetic acid, glacial (AJAX Chemicals, UNIVAR) and sulfuric acid was purchased from (AJAX Chemicals, UNIVAR).

Instrumentation

The HPLC system consisted of an autosampler (Waters 715 Ultra WISP Sample processor), pump (Shimadzu, LC-10 AS), detector (Hitachi, F1000, Fluorescence Spectrophotometer, with the excitation wavelength was set at 310 nm and the emission wavelength was set 400 nm), the β-cyclodextrin analytical column (Cyclobond I 2000 column of 250×4.6 mm, Cat. No.20024, Ser. No. 13175, Astec, Whippany, NJ, USA, purchased from Alltech Associates Pty Ltd, Australia) and β-cyclodextrin guard column (Cyclobond I 2000 column of 20×4.0 mm, Cat. No. 21100, Ser. No. 000815, Astec, Whippany, NJ, USA, Alltech Associates Pty Ltd, Australia). The flow rate was 1.0 ml/min. The mobile phase was acetonitrile: triethylamine: glacial acetic acid (100:0.2:0.3, v/v/v) for the separation of the warfarin enantiomers in human plasma assay. Whereas, the mobile phase was acetonitrile: methanol: triethylamine: glacial acetic acid, (100:1.5:0.3:0.25, v/v/v) for S-7-hydroxywarfarin in the urine assay.

Data Management

The chromatograms were measured and data were captured using a Turbochrom data system (version 4, Perkin Elmer, purchased from Alltech Associates Pty Ltd, Australia).

3.1.3 Calibration of HPLC Assay for Warfarin Enantiomers in Human Plasma

Extraction Procedures

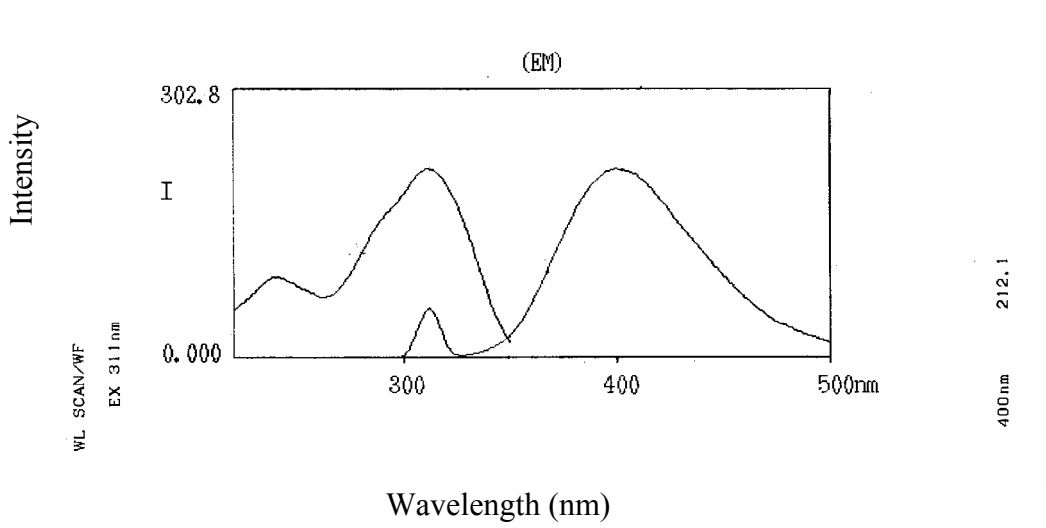
Aliquots (0.5 ml) of plasma were spiked with the internal standard (naproxen, 0.17 mg/ml) and sulfuric acid solution (0.35 ml, 0.5 M) and then extracted with dichloromethane: hexane (1:5, 4 ml). The organic phase was decanted after separation by centrifugation (at 1500 g for 10 min) and freezing the aqueous phase in dry ice. The organic layer was evaporated to dryness under nitrogen and reconstituted in acetonitrile (200 µl) and an aliquot (20 µl) injected onto the column.

Results

Warfarin Fluorescence Spectrum in Mobile Phase

The warfarin fluorescence spectrum was measured in mobile phase (acetonitrile: triethylamine: glacial acetic acid, (100:0.2:0.3, v/v/v)) using a fluorescence spectrophotometer (Hitachi, F-2000, Hitachi Ltd, Tokyo, Japan). The excitation wavelength was 311nm and the emission wavelength was 400 nm (Figure 3-1).

Figure 3-1. The fluorescence spectrum of warfarin in mobile phase.



Specificity

Figure 3-2 shows typical HPLC chromatograms of warfarin enantiomers. Twelve different batches of control plasma containing EDTA were used to investigate the specificity of the assay. No interfering peaks were observed in the different batches of drug free plasma samples at the retention time of warfarin enantiomers and internal standard (I.S.). The typical retention times were 5.7, 6.2 and 11.9 min for S-warfarin, R-warfarin and I.S., respectively. The analytical run time was 20 min per sample.

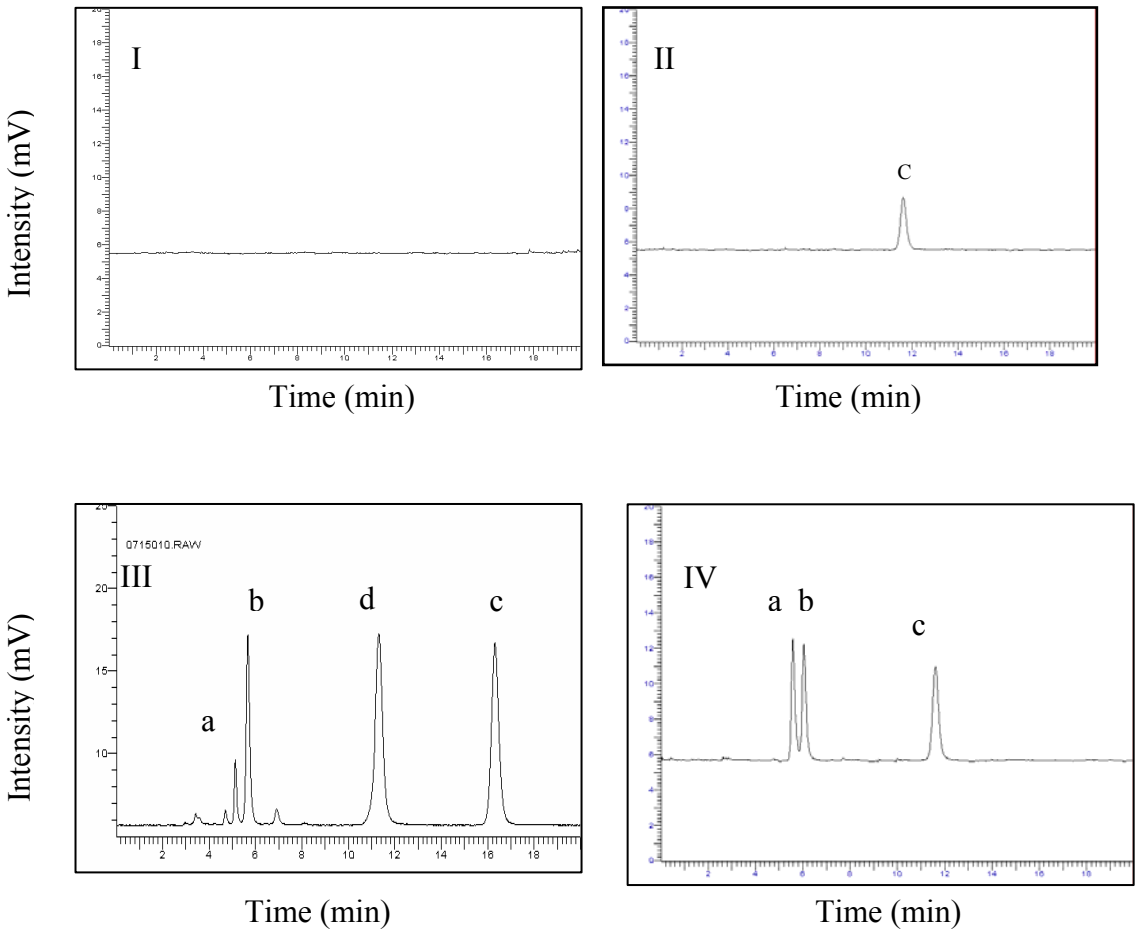


Figure 3-2. HPLC chromatogram of warfarin enantiomers.

I. Extracted blank plasma; II. Extracted blank plasma with I.S.; III. Extracted plasma sample from a subject at 144 h with I.S.; IV. Extracted blank plasma spiked with *rac*-warfarin and I.S.; a. S-warfarin; b. R-warfarin; c. I.S. (Naproxen); d. Unknown metabolite of warfarin

Calibration Curve

Limit of Quantitation (LOQ)

The LOQ for S-warfarin and R-warfarin is 36.9 ng/ml using 0.5 ml of plasma sample and injecting 20 μl of reconstituted residue. The typical peak height response at this concentration is five times greater than background interference in blank plasma samples at the retention time of the S-warfarin and R-warfarin (Figure 3-3). The peaks of the S-warfarin and R-warfarin were identifiable, discrete and reproducible. Table 3-3 shows the precision was less than 20% at 36.9 ng/ml.

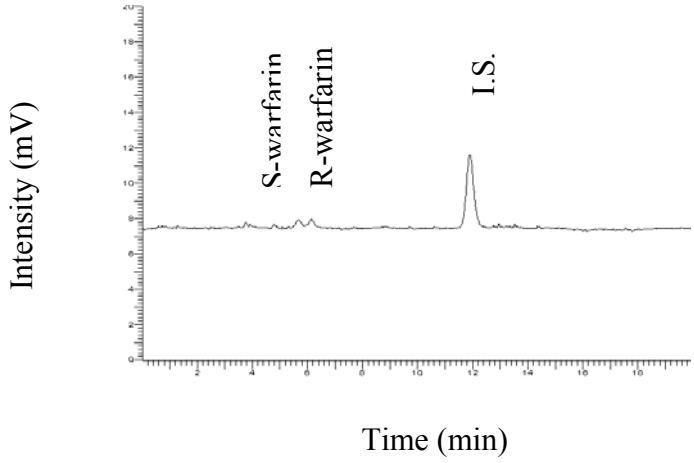


Figure 3-3. HPLC chromatogram of extracted low quality control plasma sample (36.9 ng/ml).

Linearity

The plasma concentration range of warfarin enantiomers expected in warfarin pharmacokinetic studies is 40 to 2500 ng/ml [216]. Eight different warfarin concentrations were used to establish the standard curve for each analytical run. Five

validation standard curves were run on five different days over three weeks during assay development.

Table 3-3. Standard curve statistics for spiked S- and R-warfarin in human plasma.

	Conc.	Ratio of warfarin (peak height) to I.S.							
	ng/ml	1	2	3	4	5	Mean	SD	CV%
S-warfarin	18.5	0.04	0.03	0.02	0.04	0.00	0.03	0.02	64.4
R-warfarin	18.5	0.03	0.03	0.03	0.00	0.00	0.02	0.02	91.3
S-warfarin	36.9	0.06	0.06	0.05	0.04	0.06	0.05	0.01	16.6
R-warfarin	36.9	0.06	0.06	0.06	0.05	0.06	0.06	0.01	7.7
S-warfarin	73.8	0.10	0.11	0.10	0.10	0.13	0.11	0.01	12.1
R-warfarin	73.8	0.10	0.11	0.09	0.09	0.09	0.10	0.01	9.3
S-warfarin	184.5	0.26	0.25	0.26	0.25	0.25	0.25	0.01	2.2
R-warfarin	184.5	0.28	0.25	0.23	0.23	0.24	0.25	0.02	8.4
S-warfarin	369.0	0.56	0.53	0.51	0.53	0.55	0.54	0.02	3.6
R-warfarin	369.0	0.54	0.53	0.48	0.52	0.52	0.52	0.02	4.4
S-warfarin	1107.0	1.61	1.58	1.63	1.61	1.63	1.61	0.02	1.3
R-warfarin	1107.0	1.58	1.53	1.55	1.56	1.60	1.56	0.03	1.7
S-warfarin	2214.0	3.12	2.96	3.15	3.30	3.30	3.17	0.14	4.5
R-warfarin	2214.0	3.01	2.95	3.09	3.26	3.16	3.09	0.12	3.9
S-warfarin	2952.0	3.93	4.28	4.20	4.37	4.58	4.27	0.24	5.6
R-warfarin	2952.0	3.89	4.19	4.10	4.20	4.44	4.16	0.20	4.8

Figure 3-4. Typical S-warfarin standard curve in plasma.

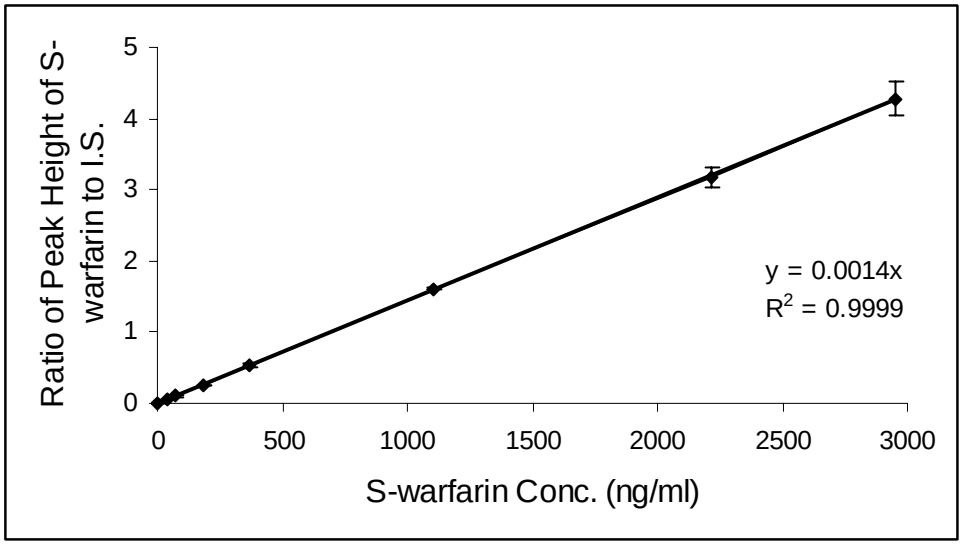


Figure 3-5. Typical R-warfarin standard curve in plasma.

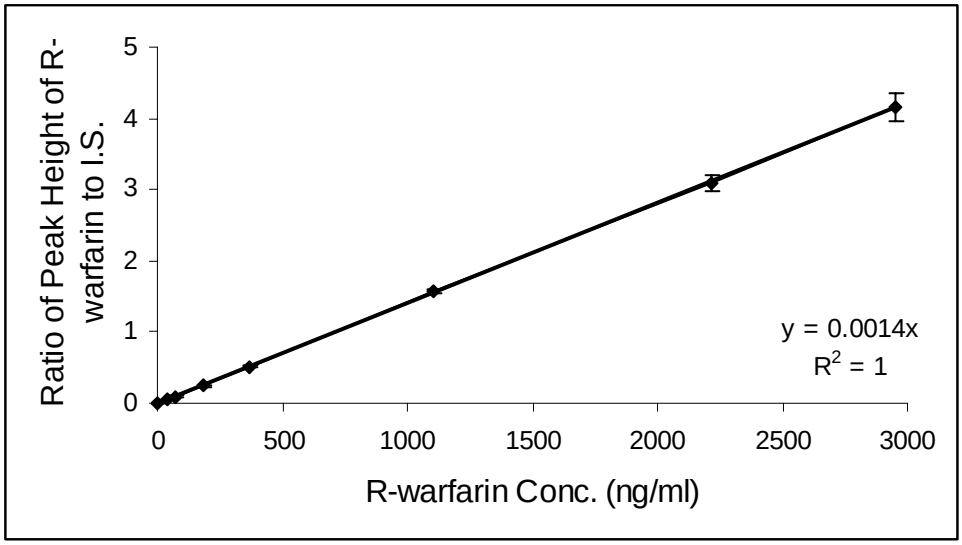


Table 3-3 shows that the precision at the LOQ is less than 20% and the deviation of standards other than LOQ in eight non-zero standards is less than 15%. The correlation coefficients (r^2) of S-warfarin and R-warfarin are greater than 0.995. Figure 3-4 and Figure 3-5 show that there are linear relationships in the plasma concentration range of 36.9 – 2952 ng/ml for both enantiomers of warfarin.

Precision and Accuracy

Precision and accuracy assessment was conducted at three different enantiomer concentrations of quality control samples in the range of expected concentrations with five determinations per concentration. Table 3-4 shows that the inter-day accuracy was within 15% of the actual value and precision did not exceed 15% CV at each concentration.

Recovery

The recovery experiment was performed by comparing the peak height of warfarin enantiomers of extracted plasma samples at three concentrations with unextracted standard solutions. Liquid-liquid extraction was used for sample clean up. Table 3-5 shows that the overall extraction recoveries were 90%, 91% and 87% for S-warfarin, R-warfarin and I.S., respectively. Also demonstrated are the precision, accuracy, reproducibility and efficiency.

Table 3-4. Precision and accuracy of S- and R-warfarin quality controls (n=5).

	S-Warfarin (ng/ml)			R-Warfarin (ng/ml)		
Nominal Conc.	65.4	981.0	2289.0	65.4	981.0	2289.0
Inter-day						
1	64.3	821.4	2007.1	69.2	892.3	2069.2
2	71.4	935.7	2335.7	71.4	907.1	2278.6
3	85.7	892.9	2064.3	78.6	878.6	1985.7
4	66.7	833.3	1873.3	64.3	871.4	1957.1
5	73.3	913.3	1960.0	80.0	873.3	1933.3
Mean	72.3	879.3	2048.1	72.7	884.6	2044.8
SD	8.3	50	175.3	6.6	15.0	140.4
CV%	11.5	5.7	8.6	9.0	1.7	6.9
RE%	110.5	89.6	89.5	111.2	90.2	89.3
Intra-day Run						
1	66.7	820.0	1913.3	53.3	786.7	1840.0
2	86.7	946.7	2166.7	73.3	953.3	2106.7
3	73.3	846.7	1873.3	66.7	820.0	1793.3
4	66.7	913.3	1880.0	53.3	860.0	1860.0
5	73.3	913.3	1960.0	80.0	873.3	1933.3
Mean	73.3	888.0	1958.7	65.3	858.7	1906.7
SD	8.2	52.6	121.2	12.0	63.0	122.7
CV%	11.2	5.9	6.2	18.3	7.3	6.4
RE%	112.2	90.5	85.6	99.9	87.5	83.3

SD: Standard deviation; CV: Coefficient of variation; RE%: Relative error or Accuracy; n: Number of replicates

Table 3-5. Recovery of S- and R-warfarin and internal standard in human plasma.

Nominal Conc.	S-Warfarin (ng/ml)			R-Warfarin (ng/ml)			Internal standard (mg/ml)
	104	552	2760	104	552	2760	0.012
Extracted peak height							
Mean (n=4)	645.9	2910.3	13954.1	644.7	2904.7	13649.0	484.2
SD	5.1	177.5	684.1	15.9	189.1	524.0	42.6
CV%	0.8	6.1	4.9	2.5	6.5	3.8	8.8
Unextracted peak height							
Mean (n=4)	698.1	3382.3	15320.6	693.6	3286.1	14876.9	555.4
SD	18.0	72.5	573.3	35.3	90.2	611.8	25.4
CV%	2.6	2.1	3.7	5.1	2.7	4.1	4.6
%Recovery	92.5	86.1	91.1	92.9	88.4	91.8	87.2
Mean Recovery%	90			91			87
SD	3.4			2.4			
CV%	3.8			2.6			

Recovery % = Extracted peak height/ Unextracted peak height × 100%

Stability

Table 3-6, Table 3-7 and Table 3-8 show that S-warfarin, R-warfarin and I.S. in human plasma QC samples stored at -4^{o}C were stable after 17 days and 5 freeze-thaw cycles. S-warfarin, R-warfarin and I.S. in this mobile phase were stable at the room temperature for 17 hours when stored in the autosampler. S-warfarin, R-warfarin and I.S. of stock standard solutions stored at 5^{o}C were stable over 15 days.

Table 3-6. Sample storage and freeze thaw stability of quality control samples in plasma.

		S-Warfarin (ng/ml)			R-Warfarin (ng/ml)		
Nominal		65.4	981.0	2289.0	65.4	981.0	2289.0
Freeze/Thaw Cycle	1	64.3	821.4	2007.1	69.2	892.3	2069.2
	2	71.4	935.7	2335.7	71.4	907.1	2278.6
	3	85.7	892.9	2064.3	78.6	878.6	1985.7
	4	66.7	833.3	1873.3	64.3	871.4	1957.1
	5	73.3	913.3	1960.0	80.0	873.3	1933.3
Observed mean		72.3	879.3	2048.1	72.7	884.6	2044.8
SD		8.3	50.0	175.3	6.6	15.0	140.4
CV%		11.5	5.7	8.6	9.0	1.7	6.9

Table 3-7. Autosampler stability of quality control samples in plasma.

		S-Warfarin (ng/ml)			R-Warfarin (ng/ml)			I.S. (mg/ml)
Nominal Conc.		65.4	981.0	2289.0	65.4	981.0	2289.0	0.2
Time (h)	0	71.4	914.3	2371.4	71.4	878.6	2335.71	3316.9*
	9	64.3	942.9	2357.1	64.3	928.6	2264.3	3482.5*
	17	71.4	950.0	2285.7	71.4	914.3	2228.6	3770.5*
Mean		69.1	935.7	2338.1	69.1	907.1	2276.2	3523.3*
SD		4.1	18.9	45.9	4.1	25.8	54.6	229.6
CV%		6.0	2.0	2.0	6.0	2.8	2.4	6.5

*Measured by comparing peak height

Table 3-8. Stock solution stability of quality control samples in plasma.

Time (Day)	S-Warfarin (ng/ml)			R-Warfarin (ng/ml)			I.S. (mg/ml)
Nominal Conc.	65.4	981.0	2289.0	65.4	981.0	2289.0	0.17
0	58.3	991.7	2400.0	58.3	958.3	2341.7	5329.8*
7	61.5	984.6	2323.1	61.5	961.5	2246.2	4854.1*
9	72.7	1163.6	2745.5	73.3	900.0	2309.1	5612.3*
14	61.5	815.4	2284.6	66.7	1000.0	2416.7	5199.5*
Mean	63.5	988.8	2438.3	65.0	955.0	2328.4	5248.9*
SD	6.3	142.2	210.3	6.5	41.3	71.0	314.6
CV%	9.9	14.4	8.6	10.1	4.3	3.1	6.0

*Measured by comparing peak height

3.1.4 Calibration of HPLC Assay for S-7-hydroxywarfarin in Human Urine

Extraction Procedures

Aliquots (0.1 ml) of urine samples were spiked with internal standard (naproxen, 0.17 mg/ml, 10 µl) and sulfuric acid solution (0.5 M, 0.1 ml) and then extracted with dichloromethane: hexane (1:2, 4 ml). After 15 min on a roller bed mixer, the organic phase was decanted after separation by centrifugation (at 1500 g for 10 min) and freezing the aqueous phase in dry ice. The organic layer was evaporated to dryness under nitrogen and reconstituted in acetonitrile (200 µl) and an aliquot (20 µl) injected onto the column.

Results

7-Hydroxywarfarin Fluorescence Spectrum in Mobile Phase

The 7-hydroxywarfarin fluorescence spectrum was measured in mobile phase (acetonitrile: methanol: triethylamine: glacial acetic acid, (100:1.5:0.3:0.25, v/v/v)) using a fluorescence spectrophotometer (Hitachi, F-2000, Hitachi Ltd, Tokyo, Japan). The excitation and emission wavelengths were 301 nm and 401 nm, respectively (Figure 3-6).

Figure 3-6. The fluorescence spectrum of 7-Hydroxywarfarin in mobile phase.

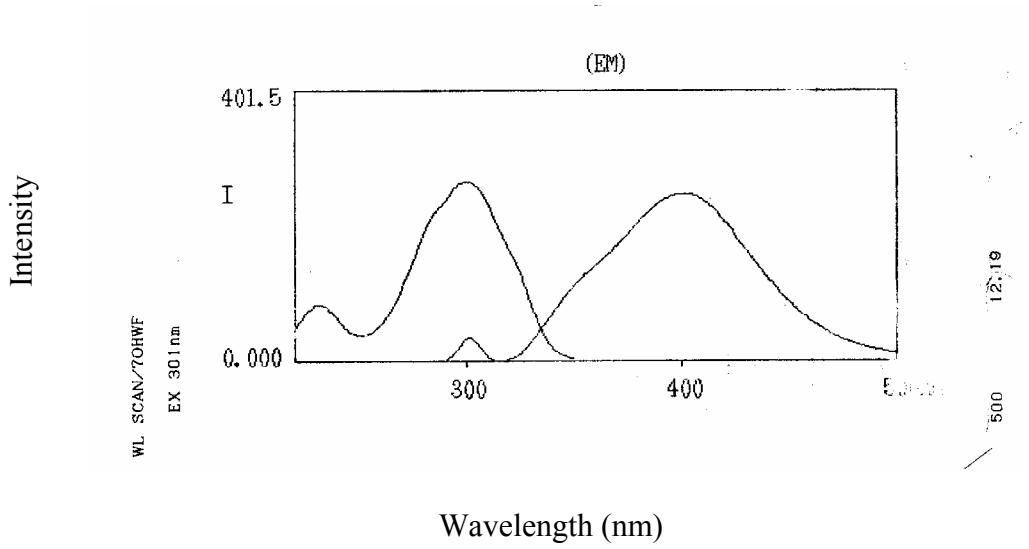


Figure 3-7 shows HPLC chromatograms of S-7-hydroxywarfarin in the mobile phase. Twelve different batches of control urine were used to investigate the specificity of the assay. No interfering peaks were found in the different batches of control urine samples at the retention time of S-7-hydroxywarfarin and I.S. The typical retention times were 12.8 and 15.6 min for S-7-hydroxywarfarin and I.S., respectively. The analytical run time was 20 min per sample.

Specificity

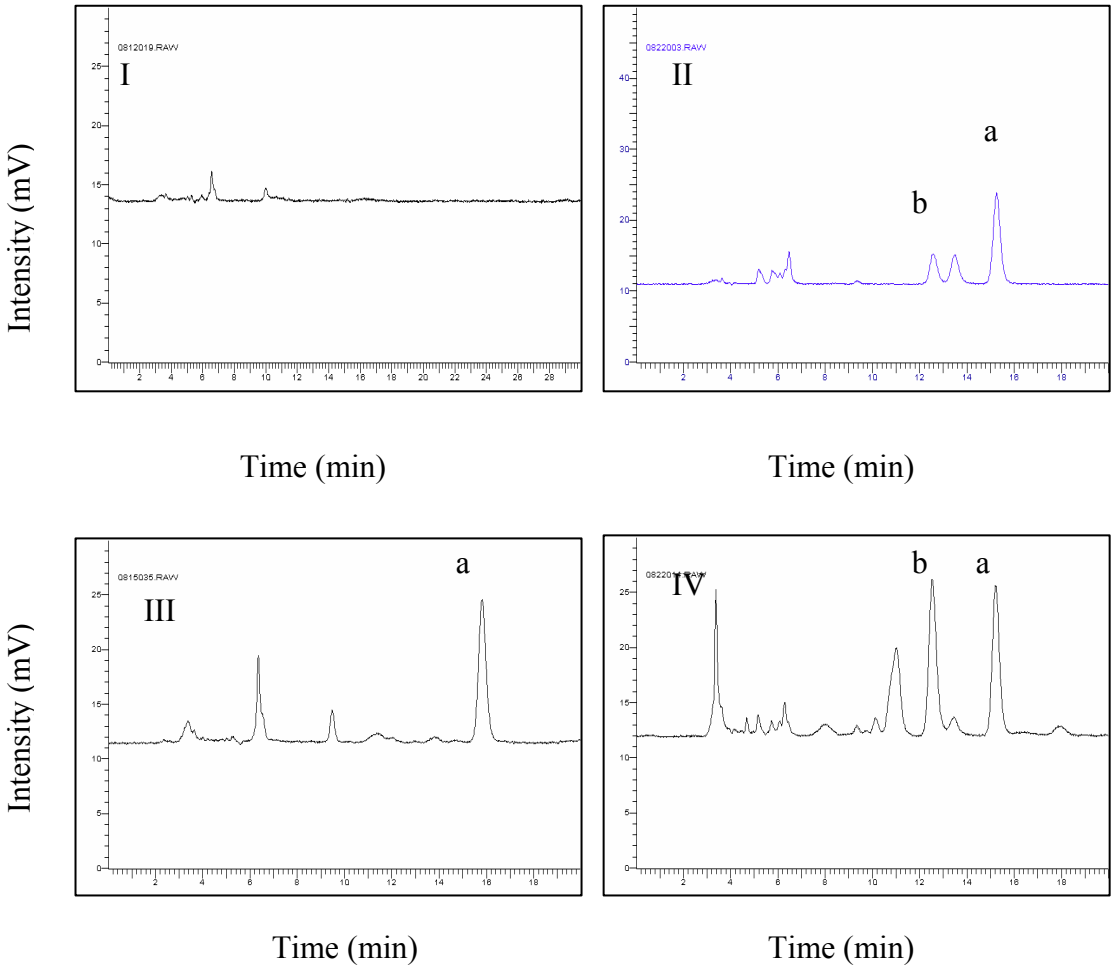


Figure 3-7. HPLC chromatogram of S-7-hydroxywarfarin.

I. Extracted blank urine; II. Extracted urine spiked S-7-hydroxywarfarin and I.S.; III. Extracted blank urine with I.S; IV. Extracted urine sample from a volunteer receiving 25 mg dose of warfarin with added I.S. a. I.S (Naproxen) b. S-7-hydroxywarfarin

Precision and Accuracy

Precision and accuracy assessment was conducted at three different 7-hydroxywarfarin concentrations of quality control (QC) samples in the range of expected concentrations with five determinations per sample. Table 3-9 shows that the inter-day accuracy were within 15% of the actual value while precision did not exceed 15% CV% at each concentration. Figure 3-8 shows that there is linear relationship in the urine concentration range of 82.5 – 1650 ng/ml for S-7-hydroxywarfarin.

Figure 3-8. S-7-hydroxywarfarin standard curve (n=4).

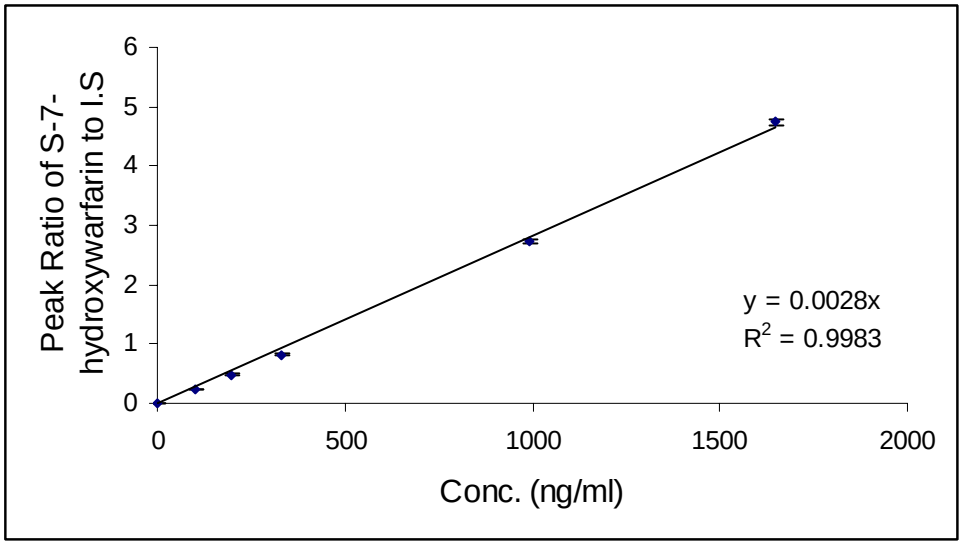


Table 3-9. Inter-day precision and accuracy of S-7-hydroxywarfarin quality control sample in urine (n=4).

	S-7-hydroxywarfarin (ng/ml)		
Nominal Conc.	165.0	330.0	660.0
Observed Mean	160.5	320.1	646.5
SD	16.6	15.8	14.3
CV%	10.3	4.9	2.2
RE%	97.2	97.0	98.0

RE%: Relative error or Accuracy; n: Number of replicates

Recovery

The recovery experiment was performed by comparing the peak height of S-7-hydroxywarfarin of extracted urine samples at three concentrations with unextracted standard solutions. Liquid-liquid extraction was used for sample clean up. Table 3-10 shows that the overall extraction recoveries were 83.0% and 83.3% for S-7-hydroxywarfarin and I.S., respectively. The data demonstrate the precision, accuracy, reproducibility and efficiency of the recovery of S-7-hydroxywarfarin from human urine.

Table 3-10. Recovery of S-7-hydroxywarfarin and internal standard (I.S.) in human urine.

	S-7-hydroxywarfarin (ng/ml)			I.S. (ng/ml)
Nominal Conc.	82.5	330.0	825.0	11100.0
Unextracted peak height				
Mean (n=3)	3483.3	13175.6	34419.6	15866.0
SD	213.2	686.4	1590.7	988.1
CV%	6.1	5.2	4.6	6.2
Extracted peak height				
Mean (n=3)	2810.9	11542.1	27748.4	13219.5
SD	389.3	260.0	2334.1	1026.5
CV%	13.8	2.3	8.4	7.8
Recovery%	80.7	87.6	80.6	83.3
Mean Recovery %	83.0			
SD	4.0			
CV%	4.8			

Recovery % = Extracted peak height/ Unextracted peak height × 100%

Discussion

Assay of Warfarin Enantiomers in Human Plasma

Twelve batches of control drug-free plasma (from St Vincent's Hospital, Darlinghurst, NSW, Australia) were used to investigate the specificity of the assay. No interference was observed in different control plasma samples at the retention time of warfarin enantiomers or the internal standard (naproxen). Fluorescence detection was chosen to measure the intensity of warfarin enantiomers in the HPLC column eluent rather than a UV detector because interference was observed at retention times of S-warfarin, R-warfarin and I.S. when a UV detector (320 nm) was used. This is modification to the assay published by Naidong *et al* [242].

Several extraction solvents have been used in an attempt to clean up the plasma sample, for example, diethyl ether, hexane, dichloromethane, chloroform, and a mixture of dichloromethane: hexane (1:5). Dichloromethane: hexane (1:5) was selected for use in this assay since the interference was smaller and the extraction recovery was higher for warfarin enantiomers compared with other solvents. This is another modification to the assay published by Naidong *et al* [242] in which ether was employed as an extraction solvent.

Sensitivity of this method was less (LOQ is 36.90 ng/ml) when compared with the assay published by Naidong *et al* [242] (LOQ is 12.5 ng/ml) [5]. However, 0.5 ml of plasma was used for this assay and reconstituted residue (20 μl) was used to inject into HPLC column, while plasma (1 ml) and reconstituted residue (50 μl) was used in the assay published by Naidong *et al* [242]), yet still covered the range of warfarin

enantiomer plasma concentrations expected in pharmacokinetic studies using the design outlined in this thesis. Hence this is a more practical method and has the added advantage of extending the working life of the column. If the sensitivity of this assay was found to be inadequate, it would be possible to improve sensitivity by increasing the plasma sample volume or by increasing the injection volume into HPLC column.

Assay of S-7-hydroxywarfarin in Human Urine

S-7-Hydroxywarfarin could not be detected in the human plasma but was quantifiable in urine samples. After modification of the assay published by Naidong *et al* [242], the present method can now be used to analyse S-7-hydroxywarfarin in human urine. Twelve batches of control urine were used to investigate the specificity of the assay. No interference was observed in different control samples at the retention time of S-hydroxywarfarin and I.S. (naproxen). Fluorescence detection was chosen to measure the intensity of S-7-hydroxywarfarin. Several extraction solvents have been used to try to clean up the urine sample including diethyl ether, hexane, dichloromethane, chloroform, a mixture of dichloromethane: hexane (1:5) and a mixture of dichloromethane: hexane (1:2). Dichloromethane: hexane (1:2) was selected for use in the present assay because the interference was smaller and the extraction recovery was higher for S-7-hydroxywarfarin. The limit of quantification of this method was 82.5 ng/ml.

3.2 Calibration of Warfarin Protein Binding

3.2.1 Introduction

The plasma protein binding of drugs has been shown to have significant effects on numerous aspects of clinical pharmacokinetics and pharmacodynamics. In many clinical situations, measurement of the total drug concentration does not provide the necessary information concerning the unbound fraction of drug in plasma, which is available for distribution, elimination, and pharmacodynamic action. Thus, accurate determination of unbound plasma drug concentrations is essential in the therapeutic monitoring of drugs [262]. Many methodologies are available for determining the extent of plasma protein binding of drugs including microdialysis, equilibrium dialysis, ultrafiltration, dynamic dialysis, ultracentrifugation, gel filtration, electrophoresis, and spectrophotometry and enzyme kinetic methods. In the clinical evaluation of drug therapy, equilibrium dialysis and ultrafiltration are the most routinely utilised methods. Both of these methods have been proven to be experimentally sound and to yield adequate protein binding data [256, 263].

Warfarin has high binding to plasma proteins (See Section 1.5.2). A small change in the fraction of unbound warfarin has the potential to significantly affect the anticoagulant activity in a transient manner [18]. Two main techniques for measuring unbound drug concentration, equilibrium dialysis and ultrafiltration using the addition of radiolabeled warfarin enantiomers or addition of racemic warfarin, have been reported to measure the concentration of unbound warfarin enantiomers [263, 264]. The present study examines the method of ultrafiltration with addition of racemic warfarin for the assessment of unbound fractions of warfarin enantiomers.

This approach relies on the assumption of concentration independent protein binding as established by Banfield *et al* [265].

3.2.2 Materials and Methods

Materials and Reagents

The materials and methods for section have been presented in Section 3.1.2.

Instrumentation

Ultrafiltration Tubes (Centrifree YM-30) were purchased from Amicon, Millipore Corporation (North Ryde, NSW, Australia). A fixed angle rotor centrifuge (Jouan CT422) was used.

Preparation of Ultrafiltrate

Warfarin standard buffer solution (1 ml, 10 μg/ml) was transferred to Centrifree® YM-30 tube. After centrifuging at 1800 g for 20 min at 37°C, 0.1 ml of the ultrafiltrate was transferred to a 10 ml volumetric flask and diluted to volume. The ultrafiltrate (0.1 μg/ml, in buffer) was then ready for analytical use.

Warfarin plasma standard (1 ml, 10 μg/ml) and different concentrations of warfarin in plasma for measuring linear range of warfarin unbound fraction were transferred to Centrifree® YM-30 tube. After centrifugation at 1800 g for 20 min at 37°C, the ultrafiltrate was ready for analytical use.

Extraction Procedures:

To 0.2 ml warfarin original buffer solution (0.1 μg/ml), warfarin buffer ultrafiltrate solution (0.1 μg/ml) and warfarin plasma ultrafiltrate (10 μg/ml), 10 μl of I.S. solution (0.0341 mg/ml, naproxen) and sulfuric acid (0.5 M, 140 μl) were added. After mixing, 2 ml of dichloromethane: hexane (1:5) was added to extract the compounds. After 15 min on a roller bed mixer and centrifugation at 1500 g for 10 min, the organic phase was separated and evaporated to dryness under nitrogen, followed by reconstitution in 100 μl of mobile phase. A 50 μl portion of the reconstituted residue was injected into the HPLC.

Data Treatment

The fraction unbound (fu) was calculated as the ratio of the concentration of each warfarin enantiomer in the ultrafiltrate to the corresponding concentration in plasma.

3.2.3 Results

Table 3-11 shows that after *rac*-warfarin was spiked into blank plasma the unbound fraction of S-warfarin and R-warfarin enantiomers were 0.0051 ± 0.0003 and 0.0057 ± 0.0002, respectively. The warfarin adsorbed by the ultrafiltrate device and membrane was less than 10% for S-warfarin and less than 5% for R-warfarin. Table 3-12 shows that warfarin protein bindings are independent of concentration in the range 8.6 – 13.8 μg/ml.

Table 3-11. Fraction of unbound warfarin and unadsorbed warfarin by ultrafiltration.

	S-warfarin		R-warfarin	
	Buffer	Plasma	Buffer	Plasma
Mean concentration before ultrafiltration (μg/ml)	5.0	5.00	5.0	5.00
Mean concentration after ultrafiltration (μg/ml)	4.6	0.03	4.9	0.03
Mean of unbound/unadsorbed fraction	0.917	0.005	0.977	0.006
SD	0.04	0.0003	0.03	0.0002
CV%	4.7	5.3	3.1	3.9
n	3	3	3	3

Note: Concentration of S-warfarin and R-warfarin is half of *rac*-warfarin.
n: Number of replicates

Table 3-12. Linear range of fraction of unbound warfarin (n=4).

Warfarin concentration in plasma (μg/ml)	S-warfarin fu	R-warfarin fu
8.6	0.005	0.005
10.4	0.005	0.006
12.1	0.005	0.005
13.8	0.004	0.004
Mean	0.005	0.005
SD	0.001	0.001
CV%	10.5	16.3

3.2.4 Discussion

The plasma protein binding of warfarin was independent over the range of 8.6 – 13.8 μg/ml in the present study. This is in agreement with the report of Chan *et al* [18] which demonstrated that plasma protein binding of warfarin was independent over the range of 0.275 to 10.55 μg/ml. Therefore, the addition of racemic warfarin to a plasma sample made at a concentration of 10 μg/ml before ultrafiltration is not expected to alter the original fraction of the unbound warfarin enantiomers in the sample. Furthermore, the ultrafiltrate warfarin enantiomers in plasma at this concentration can be measured by the presently established method. The results demonstrated by this method show good agreement with those previously reported by ultrafiltration and equilibrium dialysis methods using radiolabel warfarin enantiomers [265].

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Chapter 4 Variability in Constituents of Herbal Medicines

4.1 Introduction

Herbal medicines are included under the broader definition of complementary medicines. To be listed by the Therapeutic Goods Administration (TGA), herbal medicine products need to meet requirements for safety and quality, the latter requiring that they be manufactured under Good Manufacturing Practice (GMP) conditions. Most herbal medicines are listed. Listed herbal medicine products are not, however, individually assessed for efficacy but the sponsors are required to hold evidence to support efficacy claims for their product [266]. The World Health Organization (WHO) publishes guidance concerning the quality assurance of pharmaceuticals and the specifications for drug substances and dosage forms and provides advice on the validation of manufacturing processes and on the manufacture of both investigational products for clinical trials and herbal medicinal products. WHO also has a certification scheme to endorse the quality of pharmaceutical products moving in international commerce, and the assessment of herbal medicines and import procedures for pharmaceutical products [267].

However, there is relatively little information about the quality of herbal medicine products in the scientific literature. This partly stems from the complexity of these

medicines which often contain a complex mixture of phytochemicals. Several quality issues were found in the literature regarding the variability in the content of the constituents including *Hypericum perforatum* [268-270], *Ginkgo biloba* [271, 272] and their commercial extracts in herbal medicine products.

The aim of this chapter was to investigate the recommended dose regimen suggested by manufacturers and, using thin-layer chromatography (TLC) as a screening method, to assess the quality of different commercial herbal medicine products available in Australia. These included St John's wort (*Hypericum perforatum*), ginseng (*Panax ginseng*), ginkgo (*Ginkgo biloba*) and ginger (*Zingiber officinale*) that were to be used in the herb-drug interaction studies with warfarin presented later in this thesis.

4.2 Materials and Methods

4.2.1 Materials and Reagents

Commercially available herbal medicine products of St John's wort included

- Golden Glow St John's Wort (Batch H11092, Queensland Biochemics Pty Ltd, Virginia, Qld, Australia);
- Blackmores Hyperiforte™ (St John's Wort) (Batch 20183, Blackmores Ltd, Balgowlah, NSW, Australia);
- Nature's Own Hypericum (St John's Wort) (Batch 7890701, Bullivant's Natural Health Products Pty. Ltd, Virginia, Qld, Australia);
- Bioglan® Stress Relax with St. John's Wort (Batch 1331-2, Bioglan Ltd, Kirrawee, NSW, Australia);

- Greenridge Extra Strength Hypericum (St John's Wort), (Batch 514900B, Greenridge Botanicals (Australia) Pty. Ltd., Toowoomba, Qld., Australia).

Herbal medicine products containing ginseng included

- Golden Glow Korean Ginseng (Batch K01251, Queensland Biochemics Pty Ltd, Virginia, Qld, Australia);
- Blackmores Korean Ginseng (Batch 15698, Blackmores Ltd, Balgowlah, NSW, Australia);
- Nature's Own® Korean Ginseng (Batch 7852805, Bullivant's Natural Health Products Pty. Ltd, Virginia, Qld, Australia);
- VitaGlow GINZ!NG (Batch 12569, VitaGlow Pty Ltd, Balgowlah, NSW, Australia).

Ginkgo herbal medicine products evaluated included

- Blackmores Ginkgoforte™, (Batch 20897, Blackmores Ltd, Balgowlah, NSW 2093, Australia);
- Bioglan, (Batch 1268-2); Golden Glow Ginkgo Biloba, (Batch H11169, Queensland Biochemics Pty Ltd, Virginia, Qld, Australia);
- Nature's Own, Ginkgo Biloba (Batch 7859504, Bullivant's Natural Health Products Pty. Ltd, Virginia, Qld, Australia);
- BioGinkgo® (Batch 1268-2, Pharmanex Inc., Provo, UT 84601, USA);
- Flordis Tavonin™ Extract of Ginkgo biloba (Batch 6250202, made in Germany by Dr. Willmar Schwabe GmbH & Co. and are marketed in Australia by Flordis, Epping, NSW, Australia);
- Tebonin® forte (Batch 2860200, Dr. Willmar Schwabe Arzneimittel, Postfach 41 0925, 76209 Karlsruhe, Germany);

- Phytomedicine (Batch 41546A, Phytomedicine Pty Ltd, Dee Why, NSW, Australia);
- Herron Ginkgo Biloba (Batch 53261, Herron Pharmaceuticals Pty Ltd, Tennyson, Qld, Australia) and Medi Herb® Ginkgo biloba (Batch 112086, Mediherb Pty Ltd, Warwick, Qld, Australia).

Herbal medicine products that contained ginger included

- Blackmores Travel Calm Ginger (Batch 19782, Blackmores Ltd, Balgowlah, NSW, Australia); Golden Glow Ginger (Batch D10468, Queensland Biochemics Pty Ltd, Virginia Qld, Australia);
- Vcaps™ (Supplier not available);
- Zinaxin™ (Batch 904002, Ferrosan International Pty. Ltd., Gladesville, NSW, Australia).

Analytical standards of specific phyto-constituents including rutin, ginsenoside Rb1, ginsenoside Rg1, bilobalide, ginkgolide A, ginkgolide B and ginkgolide C were donated from the Phytolaboratory Services, Herbal Medicines Research and Education Centre (HMREC), Faculty of Pharmacy, The University of Sydney. Resorcinol was purchased from Sigma-Aldrich Pty. Ltd, (Sydney, Australia). The purity of standards was not less than 95%. All solvents and reagents were analytical grade or HPLC grade.

4.2.2 Instrumentation

The high performance thin layer chromatographic system consisted of plate of chromatography silica gel F254 (Merck 1.05554), TLC band loading and developing

device (Camag Linomat IV 1.16) and image device including Camag VideoScan Version 1.01, Camag HPTLC Vario System and Camag TLC Software"Cats" (Version 3.20) (CAMAG, Sonnenmattstr, 11, CH-4132 Muttenz, Switzerland).

4.2.3 Assessment of Recommended Dose Regimens of Different Brand Herbal Medicine Products

Dose regimens of different brands of herbal medicine products were evaluated and compared. Daily dosage equivalents of raw material and the main ingredients either known actives or markers were calculated and compared with those mentioned in the Commission E monographs for each herbal medicine [73].

4.2.4 Preparation of Reference Solutions

Reference solution of rutin (1 mg/ml), ginsenoside Rb1 (0.1 mg/ml) ginsenoside Rg1 (0.1 mg/ml), bilobalide (1 mg/ml), ginkgolide A (1 mg/ml), ginkgolide B (1 mg/ml) and ginkgolide C (1 mg/ml) and resorcinol (1 mg/ml) were prepared in methanol, and resorcinol (1 mg/ml) was prepared immediately before use.

4.2.5 Preparation of Test Solutions

Test Solutions of St John's Wort

Golden Glow, Blackmores, Nature's Own and Bioglan St John's wort tablets (10 tablets) were weighed and ground into powder using a mortar and pestle, respectively. According to the weight of each tablet and the labelled contents, Golden Glow (0.1974 g), Blackmores (0.2836 g), Nature's Own (0.1955 g), Bioglan (0.4555 g) and Greenridge (0.5 ml) St John's wort, equivalent to 0.5 g of dried *Hypericum*

perforatum were weighed, respectively. The powdered St John's wort tablets were stirred in 10 ml of methanol, sonicated for 15 min then centrifuged at 1500 g for 10 min. The supernatants of the methanolic extracts were used as test solutions and a 1 μl portion was spotted onto the TLC plate for each sample using the loading device.

Test Solutions of Ginseng

Blackmores ginseng (10 tablets) was weighed and ground into powder with a mortar and pestle. According to the weight of each tablet and the labelled contents, Blackmores (1.034 g), Golden Glow (1 capsule), Nature's Own (1 capsule) and VitaGlow (1 capsule) ginseng equivalent to 0.5 g of raw *Panax ginseng* were weighed, respectively. The powdered ginseng tablets or capsule contents were stirred in 5 ml of methanol, sonicated for 15 min and then centrifuged at 1500 g for 10 min. The methanolic supernatants were used as the test solutions and a 1 μl portion was spotted onto the TLC plate for each sample using the loading device.

Test Solutions of Ginkgo

Blackmores, Bioglan, Golden Glow, Nature's Own, BioGinkgo, Tavonin, Tebonin forte, Phytomedicine, Herron and Medi Herb ginkgo tablets (10 tablets) were weighed and ground, respectively as above. According to the weight of tablet and the labelled contents, Blackmores (2.2626 g), Bioglan (1.3198 g); Golden Glow (1.0403 g); Nature's Own (1.8008 g), BioGinkgo (0.3970 g), Tavonin (0.5543 g), Tebonin forte (0.5485 g), Phytomedicine (1.5945 g), Herron (1.5708 g) and Medi Herb (1.0010 g) ginkgo equivalent to 4 g of raw *Ginkgo biloba* were weighed, respectively.

The powdered ginkgo tablets were stirred in 5 ml of methanol, sonicated for 15 min and centrifuged at 1500 g for 10 min. The supernatants of methanolic extracts were used as the test solution and a 3 μl portion of test solution was spotted onto the TLC plate for each sample using the loading device.

Test Solutions of Ginger

Golden Glow, Blackmores and Vcaps™ ginger tablet (10 tablets) were weighed and ground with mortar and pestle, respectively. According to the weight of each tablet and the labelled contents, Golden Glow (0.8277 g), Blackmores (1.2930 g), Vcaps™ (0.3710 g) and 1 ml of Zinaxin™ (1 capsule was mixed in 6.6 ml of methanol) ginger equivalent to 1.0 g of raw *Zingiber officinale* were weighed, respectively. Methanol (5 ml) was added to each powdered tablet and methanol (4 ml) was added to 1 ml of Zinaxin™. Then the powdered ginger dose forms or capsule content of ginger extract were shaken for 15 min on a roller and centrifuged for 10 min at 1500 g. The supernatant of methanolic extracts were used as the test solution and a 10 μl portion was spotted onto the plate for each sample.

4.2.6 TLC of St John's Wort Preparations

TLC of St John's wort preparations was conducted according to the Hypericum Monograph in the British Pharmacopeia 2001 [273]. Briefly, extracts of products containing hypericum were examined using thin-layer chromatography on a TLC silica gel plate. Test and reference solutions (1 μl) were applied to the plate as 5 mm bands, spaced 4 mm apart using the Camag Linomat IV loading device. A path of 7

cm was developed using a mixture of 6 volumes of anhydrous formic acid, 9 volumes of water, and 90 volumes of ethyl acetate in the Camag HPTLC Vario System. The plate was allowed to dry at 100^{o}C to 105^{o}C for 10 min on the Camag heater. A 10 g/l solution of diphenylboric acid aminoethyl ester in methanol was then sprayed onto the plate and further treated with a 50 g/l solution of macrogol 400 in methanol. After 30 min, the plate was examined under ultraviolet (UV) light at 366 nm. The TLC image of the constituents of St John's wort dose forms were captured using the Camag VideoScan and Camag TLC Software"Cats".

4.2.7 TLC of Ginseng Preparations

TLC of ginseng preparations was conducted according to the Ginseng Monograph in the British Pharmacopeia 2001 [144]. Briefly, extracts of products containing ginseng were examined using a TLC silica gel plate. Test solution (1 μl) and reference solution (3 μl) were applied to the silica gel plate as 5 mm bands, spaced 4 mm apart using the Camag Linomat IV loading device. A path of 9 cm was developed using an unsaturated tank with the upper layer of a mixture of 2.5 ml of ethyl acetate, 5 ml of water and 10 ml of butanol, which had been allowed to separate for 10 min. The plate was allowed to dry in air and was then dipped in 10% methanolic sulfuric acid solution (20 ml of sulphuric acid, 10 ml of methanol and 170 ml of water) for 1 sec followed by heating at 120^{o}C for 2 min. The TLC image of the constituents of ginseng products were captured using the Camag VideoScan and Camag TLC Software"Cats" with VideoStore 2 under a UV source at 366 nm.

4.2.8 TLC of Ginger Preparations

TLC of ginger preparations was conducted according to the Ginger Monograph in the British Pharmacopeia 2001 [198]. Briefly, extracts of products containing *Zingiber officinale* were examined using TLC. Test solution (8 μl) and the resorcinol reference solution (1 μl) were applied to the silica gel plate as 5 mm bands, spaced 4 mm apart using the Camag Linomat IV loading device. The plate was developed over 10 cm in the unsaturated Camag HPTLC Vario System using a mixture of 40:60 (hexane: ether). The plate was allowed to dry in air. A 10 g/l solution of vanillin in sulfuric acid was sprayed onto the plate and examined in the daylight while heating at 100^{o}C to 105^{o}C for 10 min. The TLC image of the constituents of ginger dose forms was captured using the Camag VideoScan and Camag TLC Software"Cats".

4.2.9 TLC of Ginkgo Preparations

TLC of ginkgo preparations were conducted according to the Ginkgo Monograph in the Pharmacopeia of P. R. China, 2000 [172]. Briefly, extracts of products containing *Ginkgo biloba* were examined using TLC. Test and reference solutions (1 μl) were applied as 5 mm bands, spaced 5 mm apart using the Camag Linomat IV loading device to a plate pre-washed with methanol, dried then pre-treated with 4% sodium acetate for 20 min and then dried. The plate was developed over 9 cm using a mixture of 10 ml volume of toluene, 5 ml volume of ethyl acetate, 5 ml of acetone and 0.6 ml of methanol. The plate was allowed to dry and was then dipped in a mixture of 8 g of sodium acetate, 120 ml of ethanol, and 80 ml of water (4% sodium hydroxide) for 1 sec followed by heated at 160^{o}C for 30 min. The TLC image of the

constituents of ginkgo was captured with VideoStore 2 under a UV source at 366 nm using Camag VideoScan and Camag TLC Software"Cats".

4.3 Results

4.3.1 Comparison of Recommended Dose Regimens of Herbal Medicine Products

St John's Wort (Hypericum perforatum)

Hypericum perforatum, also named St John's wort, is an herbal medicine with claimed therapeutic effects on nervous tension and mild anxiety (See Section 1.7). There is clear evidence of its antidepressant actions (See Section 1.7). Daily dosage according to the Commission E monograph [73] for hypericum is 2-4 g per day of chopped or powdered herb for internal use, or 0.2-1.0 mg of total hypericin in other forms of preparation via alternative application. The daily dosage regimens recommended on the labels of different commercial herbal medicine products containing St John's wort as a single herbal ingredient are summarised in Table 4-1. Variation in the recommended dosage regimens and quality was found in different commercial products of St John's wort. For St John's wort, the daily dose recommended on Blackmores (raw: 5.4 g), Nature's Own (raw: 5.4 g) and Greenridge (raw: 6 g) product labels are higher than recommended according to the Commission E monographs for St John's wort (raw: 2-4 g). However, the recommended daily dose suggested on the Bioglan (raw: 3 g), Golden Glow (raw: 2-4 g) and Remotiv (raw: 2.73 g) product labels are consistent with the dose recommended by the Commission E monographs for hypericum.

Ginseng

Ginseng is an herbal medicine with claimed efficacy in "stress relief" (See Section 1.8). The daily recommended dosage regimens according to the Commission E monograph for *Panax ginseng* is 1-2 g of *Panax ginseng* root per day for up to three months [146]. The daily dosage regimen recommended on the labels of different commercial herbal medicine products containing ginseng as a single ingredient are summarised in Table 4-2. For ginseng, the daily doses given on the Blackmores (raw: 1g) and Nature's Own (raw: 1 g) product labels are consistent with the dose recommended by the Commission E.

Table 4-1. Daily dosage regimen of different commercial products of St John's wort .

(The recommended daily dosage regimen on Commission E monographs for hypericum: raw: 2-4 g)

Brand Name	**Adult dosage on product label and weight per tablet**	**Active ingredient per tablet or each 2 ml dose based on label claim**	**Daily dosage regimen equivalent to raw material and main ingredients**
Blackmores Hyperiforte™ (St John's Wort); AUST L 58665 Batch: 20183; Expiry date: 052004	Take one tablet three times daily with meals, or as professionally prescribed Weight per tablet: 1.0 ± 0.1 g	*Hypericum perforatum* (St John's Wort) extract equiv. to dry flowering herb top 1.8 g (standardised to contain hypericin derivatives 990 µg)	Raw material: 5.4 g Hypericin derivatives: 2.97 mg
Nature's Own; Hypericum; (St John's Wort) AUST L 66471 Batch: 7890701; Expiry date: 04 2004	Take one tablet three times a day. Or as advised by your health care professional. Weight per tablet: 0.7 ± 0.05 g	*Hypericum perforatum* (St John's Wort) concentrated extract equiv. to dry herb top flowers 1.8 g (standardised to contain hypericin 900 µg; hyperforin 15 mg)	Raw material: 5.4 g Hypericin: 2.7 mg Hyperforin: 45 mg
Bioglan® (St John's Wort) AUST L 61058 Batch: 1331-2 Expiry date: 052003	Take one tablet three times daily with meals, or as directed by a healthcare professional Weight per tablet: 0.9 ± 0.1 g	Standardised dry extract equivalent to: *Hypericum perforatum* (St John's Wort) herb top flowering approx. 1 g (equivalent to hypericin 825 µg; hyperforin 12.5 mg)	Raw material: 3 g Hypericin: 2.5 mg Hyperforin: 37.5 mg

Table 4-1. Continued.

Brand Name	Adult dosage on product label and weight per tablet	Active ingredient per tablet or each 2 ml dose based on label claim	Daily dosage regimen equivalent to raw material and main ingredients
Greenridge Hypericum (St John's Wort) AUST L 46088 Batch: 514900B Expiry date: 052004	Take 2 ml three times daily in a small quantity of water.	Each 2 ml contains *Hypericum perforatum* (St John's Wort) extract equiv. to 2 g dry herb	Raw material: 6 g
Golden Glow (St. John's Wort) AUST L 67606 Batch: H11092 Expiry date: 072004	Swallow 1 or 2 tablets daily or as directed by your health care professional. Weight per tablet: 0.8 ± 0.1g	*Hypericum perforatum* (St. John's Wort) herb approx. 2 g, Standardised equiv. Hypericin 903.2 µg – 1.129 mg	Raw material: 2-4 g Hypericin: 1-2 mg
Remotiv® Extract of Hypericum (St. John's Wort) AUST L 76830 Batch: 173111 Expiry date: 012004	Take one tablet morning and evening. Duration of therapy is recommended for four to six weeks. Weight per tablet: 0.6 ± 0.05 g	Extracts equivalent to dry: *Hypericum perforatum* herb 1.375 g standardised to 500 µg hypericin	Raw material: 2.75 g Hypericin: 1 mg

Table 4-2. Daily dosage regimen of different commercial products of ginseng.
(The recommended daily dosage regimen on Commission E monographs for *Panax ginseng* is 1-2 g)

Brand Name	**Adult dosage and weight per tablet or capsule**	**Active ingredient per tables or capsules**	**Daily dosage regiment equivalent to raw material and main ingredients**
Blackmores; Korean ginseng; AUST L 51987 Batch: 15698 Expiry date: 112003	Take two tablets daily with meals, or as professionally prescribed Weight per tablet: 1.0 ± 0.1 g	*Panax ginseng* (Korean ginseng) extract equivalent to dry root 500 mg (standardised to contain ginsenosides 9.3 mg)	Raw material: 1 g Ginsenosides: 18.6 mg
Nature's Own®; Korean ginseng; AUST L 66148 753/5 Batch: 7852805 Expiry date: 022003	Take two capsules daily with food. Weight per capsule: 1.4 ± 0.1 g	Each capsule contains: 500 mg of Korean ginseng	Raw material: 1 g
VitaGlow Korean ginseng; AUST L 51089 Batch: 12569 Expiry date: 032003	Take one to two capsules daily with food or as professionally prescribed. Weight per capsule: 0.6 ± 0.05g	Extract equiv. dry: *Panax ginseng* (Korean Ginseng) root approximately 500 mg (standardized equivalent ginsenosides (as ginsenoside Rg1) 8.93 mg)	Raw material: 0.5-1.0 g Ginsenosides: 9-18 mg
Golden Glow Korean Ginseng AUST L 59267 Batch: K01251 Expiry date: 102003	Swallow two capsules three times daily for periods of up to two months. Weight per capsules: 0.5 ± 0.05g	Each capsule contains extract equivalent to *Panax ginseng* (Korean Ginseng) root approximately 500 mg, standard equivalent to ginsenosides (as ginsenoside Rg1) 8.93 mg	Raw material: 3 g Ginsenosides: 53.58 mg

In contrast, the daily dose given on the VitaGlow (raw: 0.55-1.1 g) product label is lower than the dose recommended by the Commission E while the daily dose given on the Golden Glow (raw: 3g) product label is higher than the dose recommended by the Commission E.

Ginger

Ginger (*Zingiber officinale*) is an herbal medicine with claimed therapeutic benefits in travel sickness, morning sickness and indigestion (See Section 1.10). Recommended dosage according to the Commission E monograph for *Zingiber officinale* is dose of 2-4 g of cut rhizome or dried extract, or 0.25-1.0 g of powdered rhizome, three times daily [199]. Dosage regimens of ginger on the labels of different commercial product available in Australia are summarised in Table 4-3. For ginger, the recommended daily dose of the Golden Glow (raw: 2-4 g) product is consistent with dose recommended in the Commission E monograph, but daily dose raw 1-2 g given on the Vcaps™ product label is lower than the dose recommended in the Commission E. The dose recommended on the Blackmores product is only for short term use, so a valid comparison with the Commission E recommended dose is not appropriate.

Table 4-3. Daily dosage regimen of different commercial products of ginger.

(A recommended 2-4 g per day of cut rhizome or dried extract on the Commission E)

Brand Name	**Adult dosage and weight per tablet or capsule**	**Active ingredient per tables or capsules**	**Daily dosage regiment equivalent to raw material on labels**
Blackmores; Travel calm ginger AUST L 20201 Batch: 19782; Expiry date: 052004	Take 2 to 3 tablets half an hour before travelling, then 1 tablet every 2 hours. Weight per tablet: 0.5 ± 0.05 g	*Zingiber officinale* (Ginger) rhizome powder 400 mg	Raw material: 2.4 g
Golden Glow Ginger 1000 AUST L 68379 Batch: D10468 Expiry date: 042004	Swallow 1 to 2 tablets, two times per day Weight per tablet: 0.8 ± 0.07 g	Contains extract equivalent fresh *Zingiber officinale* (Ginger) rhizome. Approx. 1 g standardised equivalent [6]-Gingerol l 1 mg	Raw material: 2 to 4 g [6]-Gingerol: 2 to 4 mg
Zinaxin™ * Ferrosan International Batch: 904002	Weight per capsules: 0.7 ± 0.06g	*Zingiber officinale* extract equivalent to dry rhizome 6.6 g	

* Not available in Australia

Ginkgo

Ginkgo biloba leaf preparation is an herbal medicine with claimed therapeutic benefits in assisting blood circulation (See Section 1.9). The recommended daily dosage according to the Commission E monograph for *Ginkgo biloba* is 120-240 mg (raw material: 6 – 12 g) standardised dry extract in liquid or solid pharmaceutical dose form for oral administration, given in two or three divided daily doses [175]. Recommended daily dosage regimens on different commercial ginkgo product labels are summarised in Table 4-4. For ginkgo, the daily doses given on the Nature's Own (raw: 1.5-3 g) and Bioglan (raw: 2-6 g) product labels are lower than the dose recommended in the Commission E monograph for G*inkgo biloba*. The daily doses given on the Blackmores (raw: 6 g), BioGinkgo (raw: 6 g), Golden Glow (6 g), Tavonin 6-12 g), Phytomedicine (7.5 g), Herron (raw: 6 g) and Medi Herb (6 g) product labels are consistent with the dose recommended in the Commission E monograph for the herb.

Table 4-4. Daily dosage regimen of different commercial products of ginkgo.

(120-240 mg (raw material: 6 – 12 g) standardised dry extract was recommended on Commission E)

Brand name	**Adult dosage and weight per tablet or capsule**	**Active ingredient per tablet or capsule**	**Daily dosage regiment equivalent to raw material and main ingredients**
Blackmores Ginkgoforte™ AUST L 67101 Batch 20897 Expiry date: 072004	Take one tablet three times daily with meals. Weight per tablet: 1 g	*Ginkgo biloba* (Ginkgo) extract equivalent to dry leaf 2 g (standardised to contain ginkgo flavone glycosides 10.7 mg and ginkgolides and Bilobalide 2.7 mg).	Raw material: 6 g Ginkgo flavonglycosides: 32.1 mg Ginkgolides: 8.1 mg Bilobalide: 8.1 mg
Nature's Own; Ginkgo biloba; AUST L 28460 751/4 Batch: 7859504; Expiry date: 022004	Take 1 or 2 capsules twice daily. Or as advised by your health care professional. Weight per capsule: 0.34 g	Concentrated standardised *Ginkgo biloba* as extract equivalent to dry leaf 750 mg. Standardised to ginkgo flavone glycosides 3.6 mg (24%).	Raw material: 1.5-3 g Ginkgo flavone glycosides: 7.2-14.4 mg
Bioglan®; Super ginkgo 2000 AUST L 61461 Batch: 1268-2 Expiry date: 012005	Take 1-3 tablets daily with a meal. Weight per tablet: 0.66 g	Standardised extract dry conc. Equivalent dry *ginkgo biloba* leaf 2 g equivalent to ginkgo flavone glycosides 9.6 mg	Raw material: 2-6 g Ginkgo flavone glycosides: 9.6-28.8 mg

Table 4-4. Continued.

Brand name	Adult dosage and weight per tablet or capsule	Active ingredient per tablet or capsule	Daily dosage regiment equivalent to raw material and main ingredients
BioGinkgo® Extra Strength* Batch: 10801 Expiry date: 042003	Take 1 tablet twice daily with a meal. Do not chew tablet. Weight per tablet: 0.3 ± 0.03g	Leaf (50:1) extract standardized to scientifically- supported ratios of 27% ginkgo flavone glycosides and 7% terpene lactones	Raw material: 6 g Ginkgo (Ginkgo biloba) leaf extract (50:1): 120 mg
Golden Glow Ginkgo Biloba 2000; AUST L 63605 Batch: H11169 Expiry date: 072004	Swallow 1 tablet, 3 times daily. Weight per tablet: 0.5 ± 0.04 g	Extract equivalent dry *ginkgo biloba* leaf approximately 2 g standardized equivalent ginkgo flavone glycosides 10.7 mg, Standardized equivalent ginkgolides and Bilobalide 2.7	Raw material: 6 g Ginkgo flavonglycosides: 32.1 mg Ginkgolides: 8.1 mg Bilobalide: 8.1 mg
Tavonin™ (EGB 761) AUSTL 76759 Batch: 6250202 Expiry date: 012005	Take one to two tablets three times a day. Mean tablet weight: 0.27 ± 0.03 g	Each tablet contains extracts equivalent to dry *Ginkgo biloba* leaf 2 g standardised to: Ginkgo flavone glycosides 9.6 mg Ginkgolides and biobalide 2.4 mg	Raw material: 6-12 g Ginkgo flavonglycosides: 28.8-57.6 mg Ginkgolides: 7.2- 14.4mg Biobalide: 7.2-14.4 mg
Tebonin® forte (EGB 761) 40 mg* Batch: 2860200 Expiry date: 122004	One tablet three times daily before meals, Mean tablet weight: 0.27 ± 0.02 g	Each tablet contains extracts (40 mg) equivalent to dry *Ginkgo biloba* leaf 2 g (35 – 67:1).	Not available

* Not available in Australia

Table 4-4. Continued.

Brand name	Adult dosage and weight per tablet or capsule	Active ingredient per tablet or capsule	Daily dosage regiment equivalent to raw material and main ingredients
Phytomedicine ginkgo 2500 AUST L 75421 Batch: 41546A Expiry date: 062003	One tablet thrice daily before meals Table weight: 1.0 ± 0.1 g	*Ginkgo biloba* extract equiv. To dried leaf 2500 mg. Standardised to contain ginkgo flavone glycosides 13.35 mg	Raw material: 7.5 g Ginkgo flavone glycosides: 40.05 mg
Herron Ginkgo Biloba 2000 AUST L 76846 Batch: 53261 Expiry date: 072005	Adults: Take one tablet three times daily with food Table weight: 0.8 ± 0.05 g	Each tablet contains: Ginkgo (*Ginkgo biloba*) extract equivalent to dry leaf 2 g standardised to contain Ginkgo Flavonglycosides 10.7 mg, Ginkgolides and Bilobalide 2.7 mg,	Raw material: 6 g Ginkgo flavone glycosides: 32.1 mg Ginkgolides: 8.1 mg Bilobalide: 8.1 mg
Medi Herb® Phytosynergist™ Ginkgo biloba AUST L 76385 Batch: 112086 Expiry date: 052005	Take 1 tablet 3 times daily, Table weight: 0.5 ± 0.05g	Each tablet contains extract equiv. Dry: *Ginkgo biloba* leaf 2.0 g standard. to ginkgo flavone glycosides 10.7 mg standard to ginkgolides and bilobalide 2.7 mg	Raw material: 6 g Ginkgo flavone glycosides: 32.1 mg Ginkgolides: 8.1 mg Bilobalide: 8.1 mg

4.3.2 TLC of Herbal Medicines

Figure 4-1 shows TLC chromatograms of different commercial products of hypericum with a run time of 25 min. The chromatogram obtained with the reference solution shows a band in the lower third zones which is attributable to rutin and which shows yellow-orange fluorescence. The chromatogram obtained with the test solution shows reddish-orange fluorescent zones of rutin and hyperoside in the lower third, the band corresponding to pseudohypericin in the lower part of the upper third and above it, band corresponding to hypericin, both with red fluorescence. Other yellow or blue fluorescence zones are also visible. These results suggest that the Bioglan, Natures Own and Golden Glow products have a pattern and amount of constituents, which are consistent with comparable quality. In contrast, the Greenridge product appears to lack some constituents suggesting it is of lower quality. Based on the qualitative TLC results obtained from different commercial St John's wort products (Figure 4-1), the Bioglan product was selected for use in clinical trials to investigate drug interactions between warfarin and St John's wort that are described in this thesis.

TLC of St John's Wort (Hypericum)

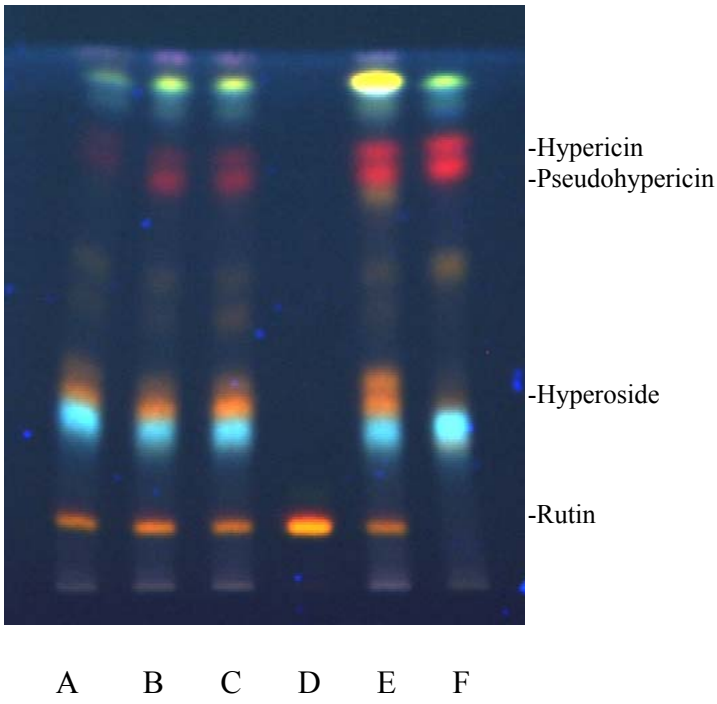

Figure 4-1. TLC of different commercial products of St John's wort and standard constituents.

A: Golden Glow; B: Blackmores Hyperiforte™; C: Nature's Own; D: Rutin (1 mg/ml); E: Bioglan® Stress Relax with St John's wort; F: Greenridge (The labelled standards have been identified based on their expected retention times in this TLC system according to the British Pharmacopoeia 2001).

Figure 4-2 shows the TLC chromatograms of different commercial products containing *Panax ginseng* over a run time of 35 min. From the TLC chromatograms of ginseng, it can be seen that the Golden Glow and Blackmores Korean ginseng products have well defined TLC finger prints while the pattern of constituents is less well defined for the Nature's Own and Vita Glow products. These qualitative data suggest that the Golden Glow and Blackmores products have comparable quality. In contrast, the quality of the Nature's Own and Vita Glow herbal medicine products are not as well established. Based on the TLC comparison of different commercial products of ginseng, the Golden Glow product was deemed to be of appropriate quality for use in the clinical studies.

Figure 4-3 and Figure 4-4 show TLC chromatograms of different commercial products of *Ginkgo biloba* with a run time of 20 min. It suggests that all products contain a similar range and amount of constituents. The *Ginkgo biloba* extract of EGb 761 is used in the Tavonin™ product. Several clinical trials using EGb 761 extract have already been performed with this ginkgo extract (as reviewed in Section 1.9), so the Tavonin™ product was selected for use in subsequent clinical trials to investigate drug interactions between warfarin and this herbal medicine.

Figure 4-5 shows TLC chromatograms of different commercial products of *Zingiber officinale*. The chromatogram obtained with the reference solution shows an intense red band (resorcinol) in the lower half of the TLC plate.

TLC of Ginseng

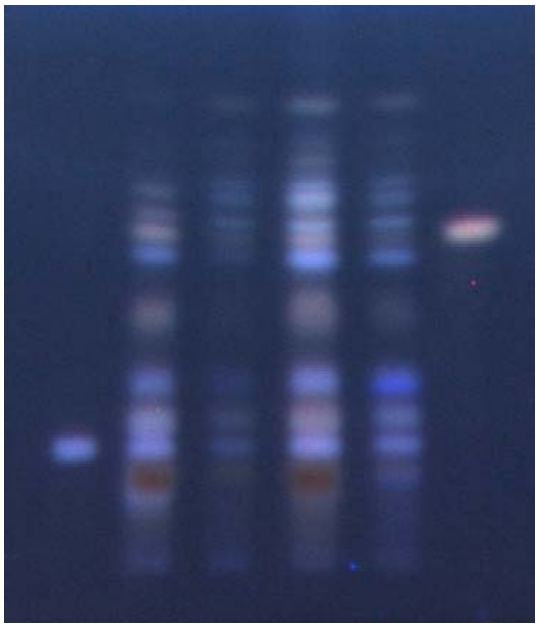

Figure 4-2. TLC of different commercial products of ginseng and standard constituents.

A: Ginsenoside Rb1 (0.1 mg/ml); B: Blackmores; C: Nature's Own; D: Golden Glow; E: Vita Glow; F: Ginsenoside Rg1 (0.1mg/ml).

TLC of Ginkgo

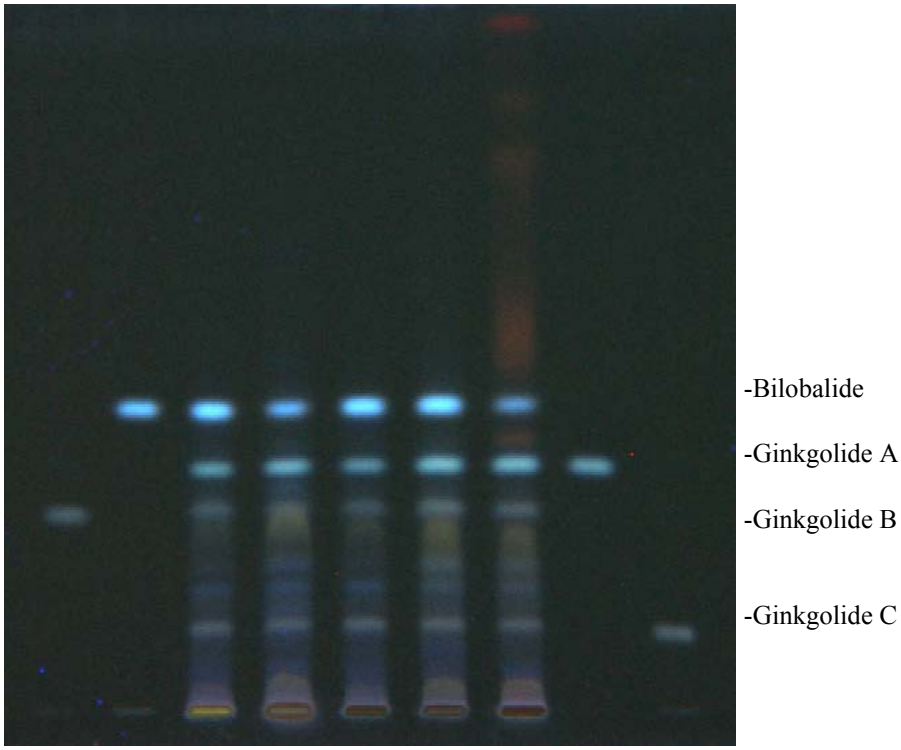

Figure 4-3. TLC of different commercial products of ginkgo and standard constituents.

A: Ginkgolide B (1 mg/ml); B: Bilobalide (1 mg/ml); C: Blackmores; D: Bioglan; E: Golden Glow; F: Nature's Own; G: BioGinkgo; H: Ginkgolide A (1 mg/ml); I: Ginkgolide C (1 mg/ml).

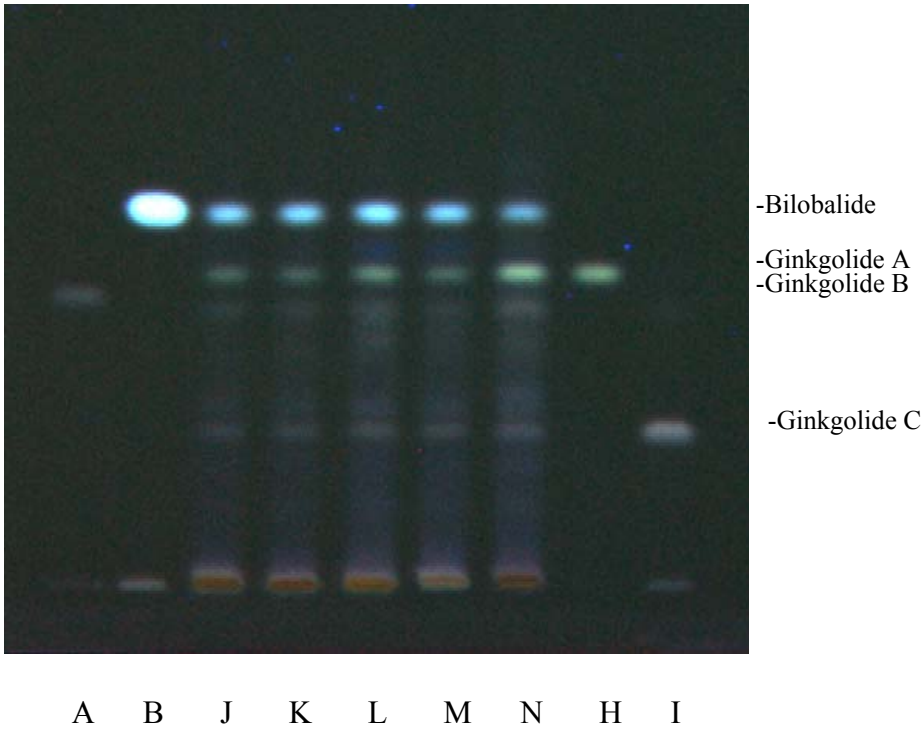

Figure 4-4. TLC of different commercial products of ginkgo and standard constituents.

A: Ginkgolide B (1 mg/ml); B: Bilobalide (1 mg/ml); H: Ginkgolide A (1 mg/ml); I: Ginkgolide C (1 mg/ml); J: Tavonin™; K: Tebonin®forte; L: Phytomedicine; M: Herron; N: Medi Herb.

TLC of Ginger

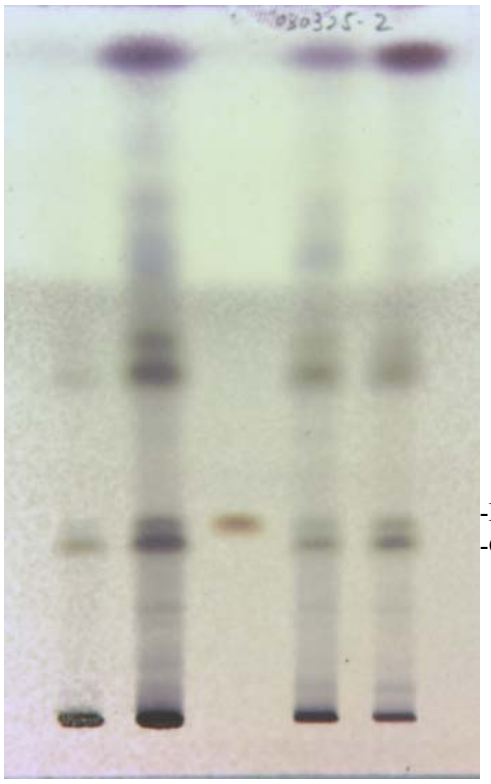

Figure 4-5. TLC of different commercial products of ginger and standard constituent.

A: Golden Glow; B: Blackmores, C Resorcinol (1 mg/ml), D: Vcaps™, E: Zinaxin™ (The standard of gingerol has been identified based on the expected retention time for this TLC system according to British Pharmacopoeia 2001).

The chromatogram obtained with the test solutions shows two intense violet bands (gingerols) below the resorcinol band. These data suggest that the Blackmores product has a well defined constituent finger-print and hence, this product was used in subsequent clinical trial to investigate drug interactions between warfarin and ginger.

4.4 Discussion

The daily dosage regimens of these four herbal medicines vary according to different commercial products. However, most are within the recommended dosage range suggested by the Commission E. The main ingredients were comparable in different commercial products of ginseng, ginger and ginkgo, but there were notable differences between different commercial products of St John's wort. The most likely explanation is that these differences between products arise from the different sources of herbal medicines and the different manufacturing processes used for these products including herb extraction, purification, drying and dosage formulation. TLC was used in this study as a method for quality screening of different brands of commercial herbal medicines. It should be noted that in this study pharmacopoeial methods have been used to investigate the quality of herbal medicines to facilitate product selection. The exact concentration of active or marker compounds has not been quantitated.

This qualitative study has highlighted some differences in the dose recommendations and pattern of different constituents between herbal medicine products. Similar

results regarding dosage issues of herbal medicines have also been noted in the literature. For example, Garrard *et al* [268] investigated the 10 most commonly purchased herbs based on their highest sales (as dollars) in 1998 including echinacea, St John's wort, ginkgo, garlic, saw palmetto, ginseng, goldenseal, aloe, Siberian ginseng and valerian. A large range in label ingredients and recommended daily dose were found in these 10 herbal medicine products. Among the 880 products investigated for these ten herbal medicines, 43% were consistent with product labels in ingredients and recommended daily dose, 20% in ingredients only, and 37% were either not consistent this recommended doses or the labeled information was insufficient to assess dosing guidelines.

In contrast to conventional medicines, the pharmaceutical properties of most herbal medicine products are poorly characterized. Since it is difficult to find the individual components, which are responsible for the observed or claimed pharmacological activities, in many cases the whole plant extract is considered to be the “active” part of the dose form. However, where the active components or extraction markers have been identified, these constituents should be used to establish a quality control monograph. Such a monograph can be used to ensure the quality of herbal medicine products from batch to batch, between different brands of commercial herbal medicine products involving the same herbal ingredients and to compare products with respect not only to content uniformity but also to their biopharmaceutical properties. With the increasing use of analytical methods such as HPLC, GC and TLC to investigate the quality control of herbal medicines, there is greater understanding of the complex nature of many herbal medicines.

Quality issues including content uniformity and stability are critical issues for herbal medicine products. Numerous quality studies have been done on products containing St John's wort but few studies have been conducted on ginseng, ginkgo and ginger. Bergonzi *et al* [269] investigated samples of *Hypericum perforatum*, including the dried extracts and four commercially available dried extracts, using HPLC to determine the relative concentration of the main constituents of this herb. The total flavonol content ranged from 4.58% to 15.90%; hypericins ranged from 0.05% to 0.11%; and hyperforins ranged from 1.37% to 20.80% in these products. For commercial dried extracts, their flavonol content varied from 10.64% to 15.01%, hypericins varied from 0.03% to 0.20%, and hyperforins varied from 1.18% to 6.54% [269]. Batch to batch reproducibility of St. John's wort herbal medicine products has been performed by Wurglics *et al* [274]. These researchers analyzed both hyperforin and hypericin content from five batches from each of eight manufacturers. Hyperforin concentrations were analyzed by HPLC, and total hypericin concentrations were determined by polarography, an electrochemical method. Widely differing amounts of hypericin and hyperforin were found in some products [274]. Bilia *et al* [275] evaluated thermal and photostability of a commercial dried extract and capsules of St. John's wort. In addition, capsules of different colors corresponding to different opacity and pigment contents were also evaluated as the primary packaging of the herbal medicine product. Tests in the secondary pack were performed using amber containers. HPLC was employed for determination of the stability of all the characteristic constituents including flavonols, hyperforins and hypericins. Results of the photostability test showed all the constituents to be photosensitive under the tested conditions. However, capsules of different opacity

and containing different pigments influenced the stability of the different classes of constituents. Amber containers, suggested as secondary packages, only partly influenced the photostability of the investigated constituents. Long-term thermal stability testing by measuring t_{90} of hyperforins and hypericins, respectively, showed the stable period was less than 4 months even if the antioxidants ascorbic and citric acids were added to the formulation.

Besides the quality issues mentioned above, issues related to phytoequivalence or bioequivalence of commercial herbal medicine remains an area of research. One study conducted by Westerhoff *et al* [276] investigated the dissolution of specific constituents of St John's wort products. The components of St John's wort including phloroglucines, naphthodianthrones and the flavonoids, which were known or suspected to play a role in its antidepressant activity, were measured in the dissolution study. The study demonstrated that products had notably different release profiles of selected constituents. Another conclusion was that bioequivalence was not likely to be demonstrated even though the label claims on these St John's wort products indicated that these products should be pharmaceutically equivalent [276].

Kressmann *et al* [271, 272] published two separate studies which aimed to identify the quality issues of ginkgo products and the influence on the bioavailability of ginkgo constituents. A variety of products on the USA market was studied including quantity of constituents and *in vitro* dissolution. Flavone glycosides ranged from 24% to 36%, terpene lactones from 4% to 11% and the ginkgolic acids from < 500

ppm to about 90000 ppm were found in these ginkgo products. Comparing the dissolution rates of terpene lactones and flavone glycosides within the single products, most of the products released more than 75% of the content of both components within 30 min. However, several products showed differences in dissolution rates. Furthermore, two different *Ginkgo biloba* brands were analysed for dissolution rates and relative bioavailability in terms of ginkgolide A, ginkgolide B and bilobalide. Dissolution rates at pH 1 and 4.5 were conducted according to the USP 23. The relative bioavailability was investigated after single oral administration of 120 mg *Ginkgo biloba* extract as tablets or capsules in twelve healthy male volunteers using an open-label, randomized crossover design. Significant difference in $AUC_{0-\infty}$, t_{max} and C_{max} in term of ginkgolide A, ginkgolide B and bilobalide were found in the test and reference products. The poor dissolution rate of the ginkgo product resulted in poor bioavailability. The results demonstrated that the two ginkgo products were not considered to be bioequivalent [271].

Only in the last few decades, reports of clinical trials started to appear in the literature that compare the constituents of different products using *in vivo* investigations [277]. Most reports have been based on observational trials and were rarely conducted according to the Good Clinical Practice (GCP). Recently, clinical studies with herbal medicines have started to be performed according to the GCP requirement by a few pharmaceutical companies. Compared with conventional medicines, the complex composition of herbal medicines makes it difficult to carry out pharmacodynamic and pharmacokinetics studies. It is not unreasonable that clinical development of herbal medicine products should be conducted according to

the general principles of conventional drug development. However, without rigorous phytoequivalence studies, the results of a clinical trial performed with a particular brand of herbal medicine product cannot be easily or reliably extrapolated to other brands of herbal medicine products.

Chapter 5 Effect of St John's Wort and Ginseng on the Pharmacokinetics and Pharmacodynamics of Warfarin in Healthy Subjects

5.1 Introduction

The anticoagulant warfarin has an important place in the management of cardiovascular disease in the community where people have free access to herbal medicines. The narrow therapeutic range of warfarin and its metabolism by cytochrome P450 (CYP) make it prone to potentially life-threatening interactions and result in warfarin being one of the most frequently investigated drugs with respect to drug interactions [9]. St John's wort (*Hypericum perforatum*) is a herbal medicine widely used in the community for the management of a range of conditions including depression. Numerous drug interactions with St John's wort have been documented based on case reports, and *in vitro* and *in vivo* studies [8, 74, 75]. There are a number of case reports suggesting that co-administration of St John's wort decreases the effects of warfarin [8]. The Medical Products Agency (MPA, Sweden) has received seven case reports of a reduced anticoagulant effect and decreased INR of warfarin associated with co-administration of St John's wort [75]. Similarly, over the period of October 1992 to September 2000, the UK Committee on Safety of Medicines and the Medicines Control Agency received 35 reports of suspected interactions between St John's wort and conventional medicines; of these four were related to potential

interactions with warfarin [74]; two cases reported an increase in INR and two cases reported a decrease in INR. Despite these observations, the possible interaction between warfarin and St John's wort has not been systemically investigated.

Similarly, ginseng (*Panax ginseng*) is also widely used in the community for a variety of indications, but few drug interaction studies have been undertaken. There is a published case report of decreased effect of warfarin in a patient receiving ginseng [167], but a study in rats found no effect of ginseng on the pharmacokinetics or pharmacodynamics of warfarin [168]. The conclusion from a recent systematic review was that patients who take warfarin with ginseng should regularly monitor their INR [148].

The aim of the present study was to investigate the possible drug interactions between warfarin and these two widely used herbal medicines in healthy subjects using single herbal ingredient products of known quality. The results from this chapter have been published in the British Journal of Clinical Pharmacology [278].

5.2 Materials and Methods

The materials and methods for this chapter have been presented in Chapter 2.

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5.3 Results

5.3.1 Subjects

Twelve healthy male subjects were recruited into and completed the study in three treatment groups. Subjects were aged between 20 to 39 years and within 15% of ideal body weight for height and build. Subjects came from a diverse ethnic mix (8 Caucasians, 4 Asians). All subjects were non-smokers and met the inclusion criteria outlined in Section 2.1.2. The demographic data for the subject cohort are described in Table 5-1.

5.3.2 Results of Statistical Analysis

ANOVA was performed on log transformed parameters including $AUC_{(0-\infty)}$ and C_{max} of S-warfarin and R-warfarin and AUC_{0-168} of INR. Sequence and period factors of $AUC_{(0-\infty)}$ of S-warfarin and R-warfarin and AUC_{0-168} of INR were not identified as being statistically significant while treatment was significantly different (Appendix 1-5). The lack of a period or sequence effect supports the observation that the trial design, especially the washout period, was adequate to exclude any carryover effect of St John's wort, ginseng and warfarin. This is especially important given that St John's wort has been shown to be an inducer of drug metabolising enzymes.

5.3.3 Pharmacokinetics of S-warfarin and R-warfarin

There was a significant difference in $AUC_{0-\infty}$, $t_{1/2}$ and CL/F for both S-warfarin and R-warfarin following pre-treatment with St John's wort. By contrast, there were no significant changes in these parameters following treatment with ginseng. Neither St

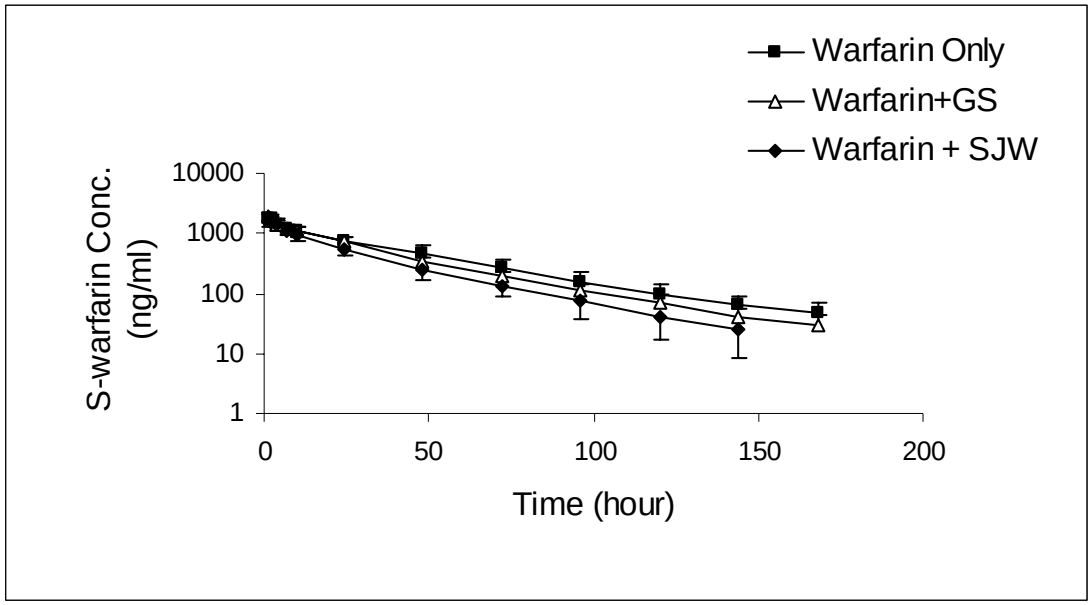
John's wort nor ginseng affected the apparent volume of distribution, C_{max} or t_{max} of the warfarin enantiomers (Figure 5-1 to 5-12, Table 5-2 and Table 5-3).

Table 5-1. Subject demographic data (n=12).

Subject	Age (years)	Weight (kg)	Height (cm)	Ethnicity
1	24	66	179	Caucasian
2	27	65	175	Caucasian
3	36	66.4	163	Asian
4	22	76	178	Asian
5	21	108.9	189.5	Caucasian
6	22	91	187.5	Caucasian
7	23	94	185	Caucasian
8	20	51	179	Caucasian
9	22	70	175	Caucasian
10	25	68	169.5	Asian
11	35	70	180	Caucasian
12	39	65	168	Asian
Mean ± SD	26.3 ± 6.6	74.3 ± 15.9	177.4 ± 7.9	

Figure 5-1. S-warfarin concentration-time profiles following administration of a single 25 mg oral dose of *rac*-warfarin either alone (warfarin only), or warfarin and ginseng (GS), or warfarin and St John's wort (SJW). Data are presented as Mean (± SD, n=12) with semi-log (A) and linear (B) plots. Individual subject data are shown in Appendix 11.

A:



B:

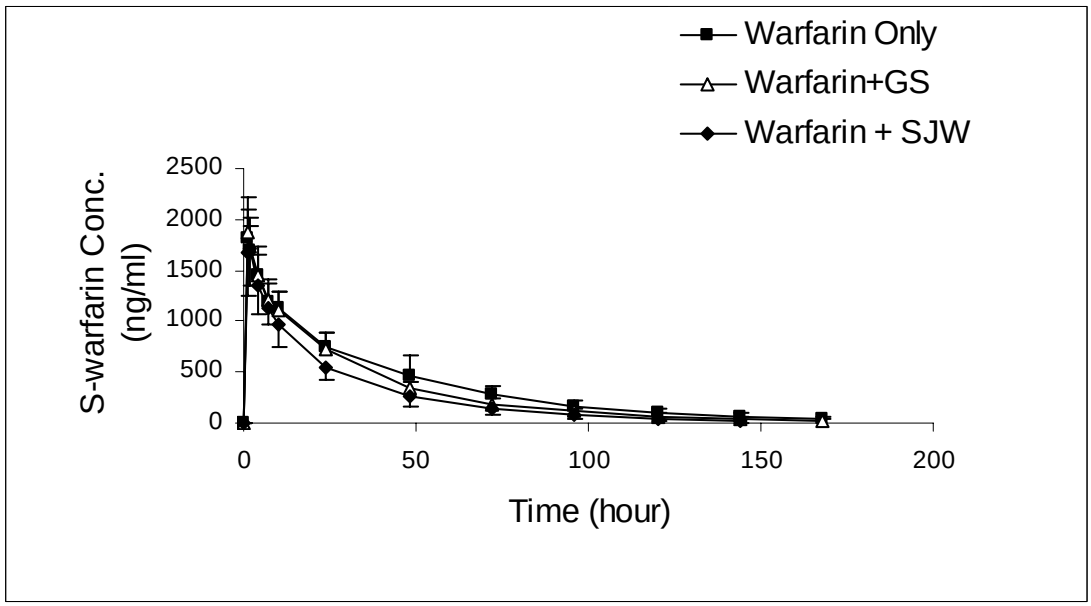
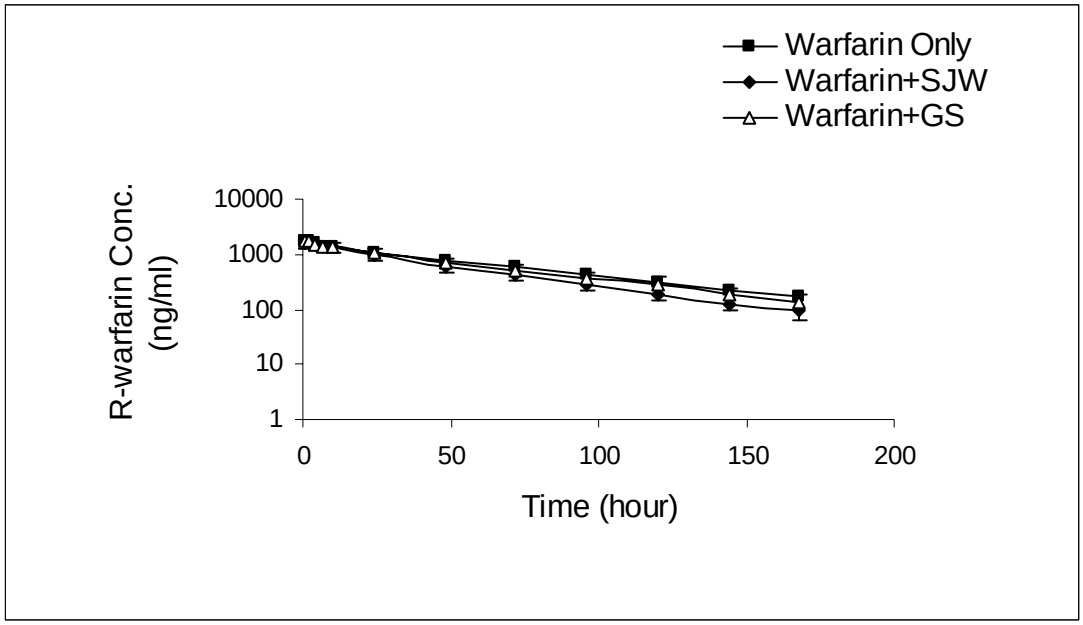


Figure 5-2. R-warfarin concentration-time profiles following administration of a single 25 mg oral dose of *rac*-warfarin either alone (warfarin only), or warfarin and ginseng (GS), or warfarin and St John's wort (SJW). Data are presented as mean (± SD, n=12) with semi-log (A) and linear (B) plots. Individual subject data are presented in Appendix 11.

A:



B:

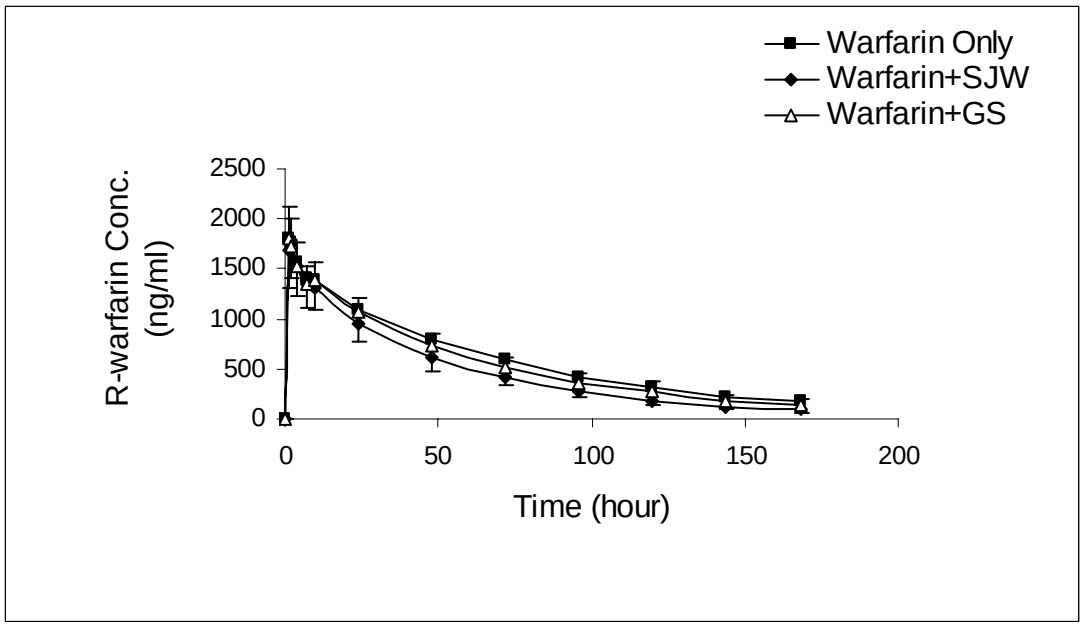


Table 5-2. Warfarin pharmacokinetic parameters following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either St John's wort (WF + SJW) or ginseng (WF + GS). Data are presented as mean ± SD (n=12). The 95% confidence interval is shown in brackets for each parameter. Individual subject data are presented in Appendices 13-19.

Treatment	WF alone	WF + SJW	WF + GS
fu			
S-warfarin	0.0034 ± 0.0011 (0.0027 - 0.0041)	0.0036 ± 0.0010 (0.0030 - 0.0043)	0.0039 ± 0.0016 (0.0029 - 0.0049)
R-warfarin	0.0048 ± 0.0006 (0.0044 - 0.0051)	0.0047 ± 0.0009 (0.0041 - 0.0052)	0.0046 ± 0.0008 (0.0041 - 0.0051)
$AUC_{0-\infty}$ (ng/ml×h)			
S-warfarin	65400 ± 13800 (56600 - 74100)	47700 ± 8300 (42400 - 53000)	57800 ± 7400 (53100 - 62500)
R-warfarin	120900 ± 32900 (99000 - 142700)	91100 ± 15400 (80800 - 101300)	108100 ± 18300 (95900 - 120200)
t_{max} (h)			
S-warfarin	1.29 ± 0.51 (0.97 - 1.62)	1.26 ± 0.46 (0.97 - 1.55)	1.30 ± 0.55 (0.95 - 1.65)
R-warfarin	1.34 ± 0.48 (1.02 – 1.66)	1.34 ± 0.48 (1.10 – 1.73)	1.30 ± 0.52 (0.95 – 1.65)
C_{max} (ng/ml)			
S-warfarin	1890 ± 260 (1700 - 2000)	1820 ± 340 (1600 - 2000)	1930 ± 310 (1700 - 2100)
R-warfarin	19200 ± 320 (1700 - 2100)	1840 ± 360 (1600 - 2100)	1890 ± 290 (1700 - 2000)
$t_{1/2}$ (h)			
S-warfarin	31.7 ± 4.5 (28.8 - 34.5)	25.1 ± 4.3 (22.4 - 27.9)	29.2 ± 5.2 (25.9 - 32.4)
R-warfarin	51.7 ± 9.6 (45.6 - 57.8)	40.3 ± 3.9 (38.0 - 42.7)	47.9 ± 7.8 (42.9 - 52.9)
CL/F (ml/h)			
S-warfarin	198 ± 38 (174 - 223)	270 ± 44 (241 - 297)	220 ± 29 (201 - 238)
R-warfarin	110 ± 25 (94 - 126)	142 ± 29 (123 - 161)	119 ± 20 (106 - 131)
V/F (ml/kg)			
S-warfarin	120 ± 30 (110 - 140)	130 ± 30 (110 - 150)	130 ± 30 (110 - 150)
R-warfarin	100 ± 20 (90 - 130)	100 ± 20 (100 - 130)	100 ± 20 (100 - 120)

Table 5-3. Mean ratios and 90% confidence intervals for log transformed S-warfarin and R-warfarin pharmacokinetic parameters comparing each herb treatment to the warfarin only arm as control (n=12).

Treatment	St John's wort (90% CI)	Ginseng (90% CI)
fu		
S-warfarin	1.12 (0.96 - 1.31)	1.10 (0.85 - 1.42)
R-warfarin	1.04 (0.95 - 1.14)	1.03 (0.94 - 1.13)
$AUC_{0-\infty}$		
S-warfarin	0.73 (0.65 - 0.83)*	0.89 (0.82 - 0.98)
R-warfarin	0.77 (0.67 - 0.87)*	0.91 (0.84 - 0.99)
t_{max}		
S-warfarin	1.13 (0.76 - 1.49)	1.20 (0.77 - 1.62)
R-warfarin	1.17 (0.80 - 1.54)	1.11 (0.78 - 1.44)
C_{max}		
S-warfarin	0.95 (0.86 - 1.05)	1.01 (0.90 - 1.12)
R-warfarin	0.96 (0.84 - 1.09)	0.98 (0.88 - 1.09)
$t_{1/2}$		
S-warfarin	0.79 (0.72 - 0.87)*	0.92 (0.85 - 0.99)
R-warfarin	0.79 (0.72 - 0.86)*	0.93 (0.88 - 0.99)
CL/F		
S-warfarin	1.29 (1.16 - 1.46)*	1.12 (1.03 - 1.22)
R-warfarin	1.23 (1.11 - 1.37)*	1.10 (1.01 - 1.20)
V/F		
S-warfarin	1.10 (0.97 - 1.24)	1.04 (0.94 - 1.14)
R-warfarin	1.06 (0.88 - 1.24)	1.03 (0.95 - 1.10)

ANOVA *$p<0.05$

Figure 5-3. S-warfarin apparent clearance (CL/F) following single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginseng (WF + GS) and warfarin + St John's wort (WF + SJW).

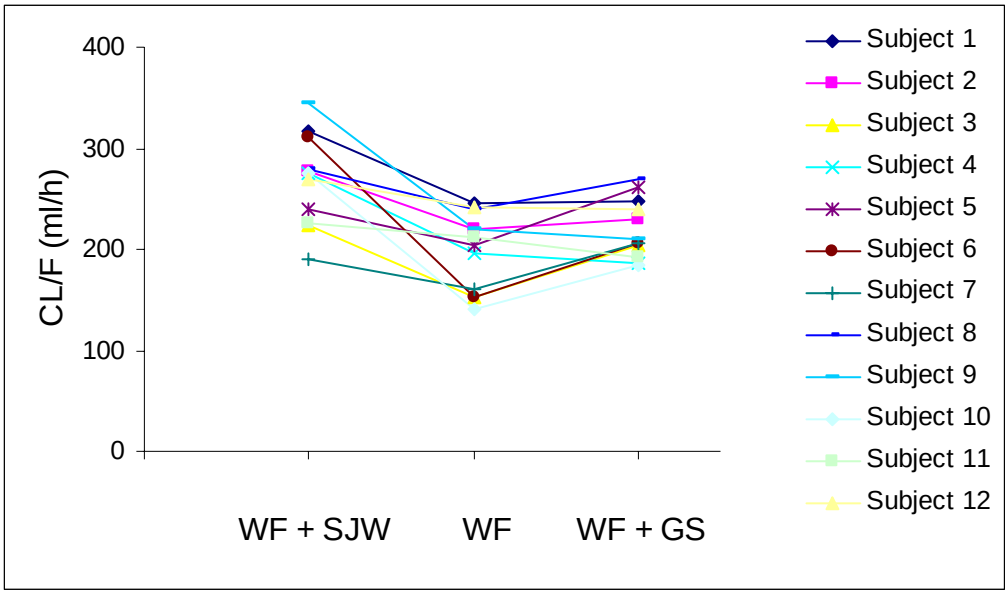


Figure 5-4. R-warfarin apparent clearance (CL/F) following single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginseng (WF + GS) and warfarin + St John's wort (WF + SJW).

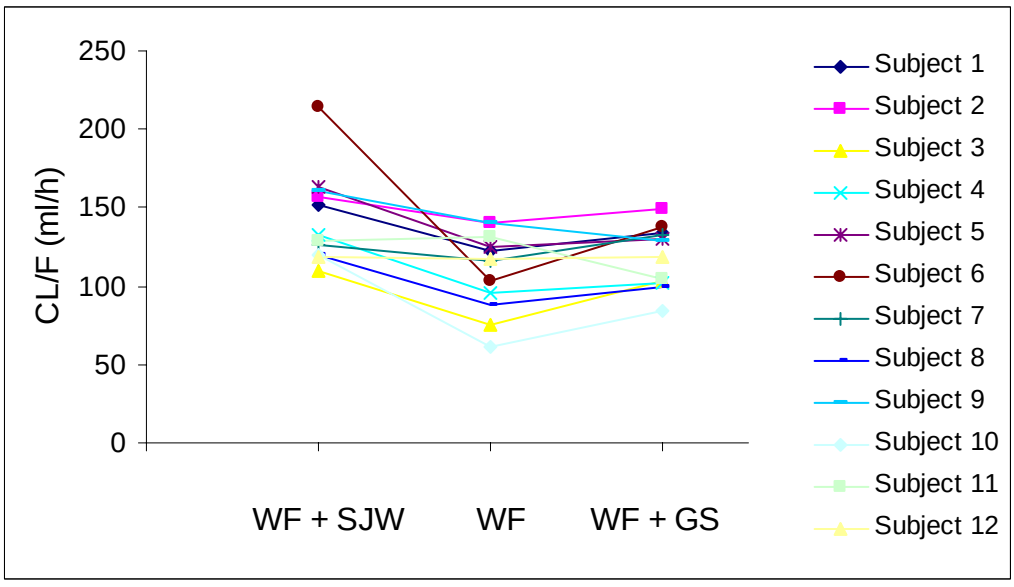


Figure 5-5. S-warfarin half-life ($t_{1/2}$) following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginseng (WF + GS) and warfarin + St John's wort (WF + SJW).

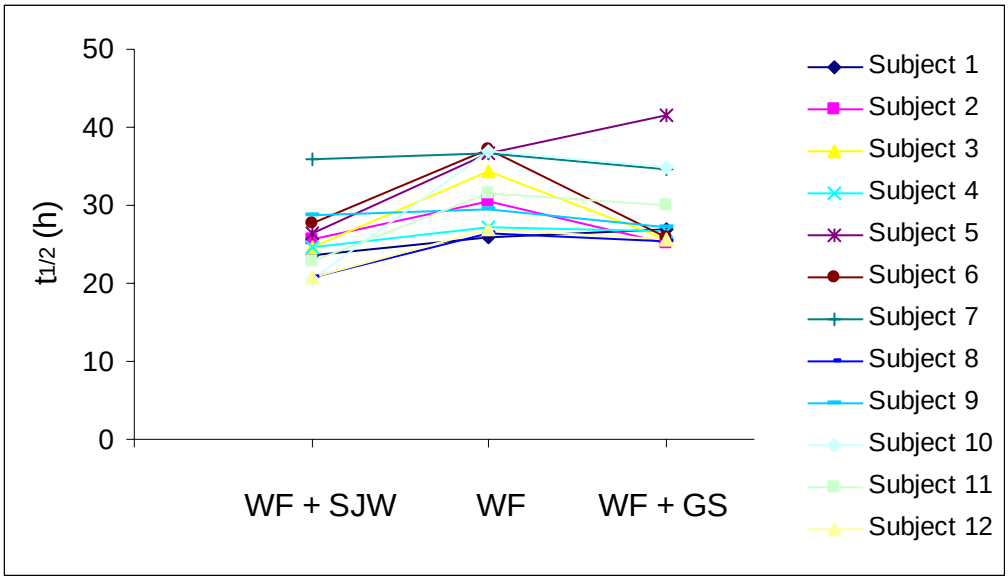


Figure 5-6. R-warfarin half-life ($t_{1/2}$) following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginseng (WF + GS) and warfarin + St John's wort (WF + SJW).

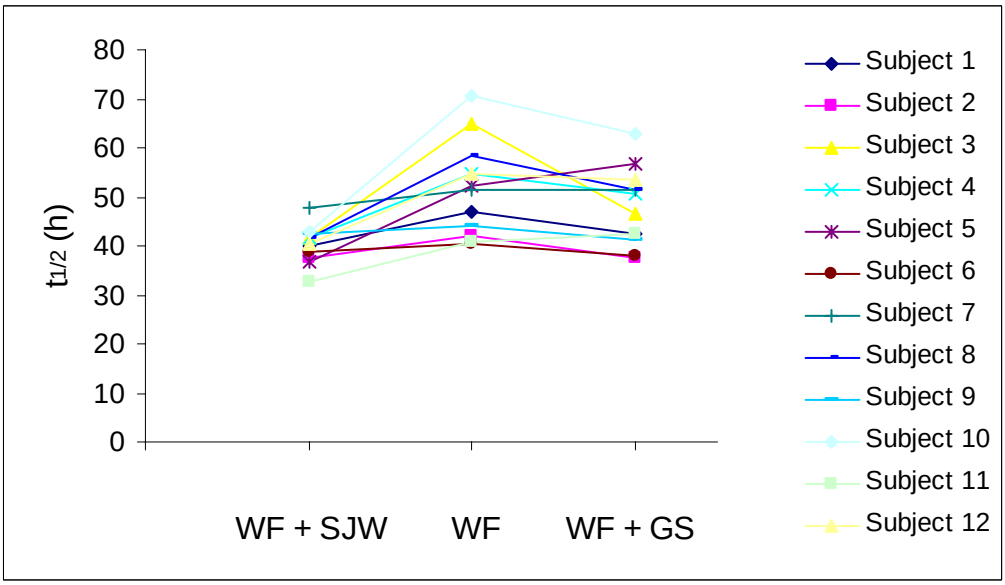


Figure 5-7. S-warfarin apparent volume of distribution (V/F) following single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginseng (WF + GS) and warfarin + St John's wort (WF + SJW).

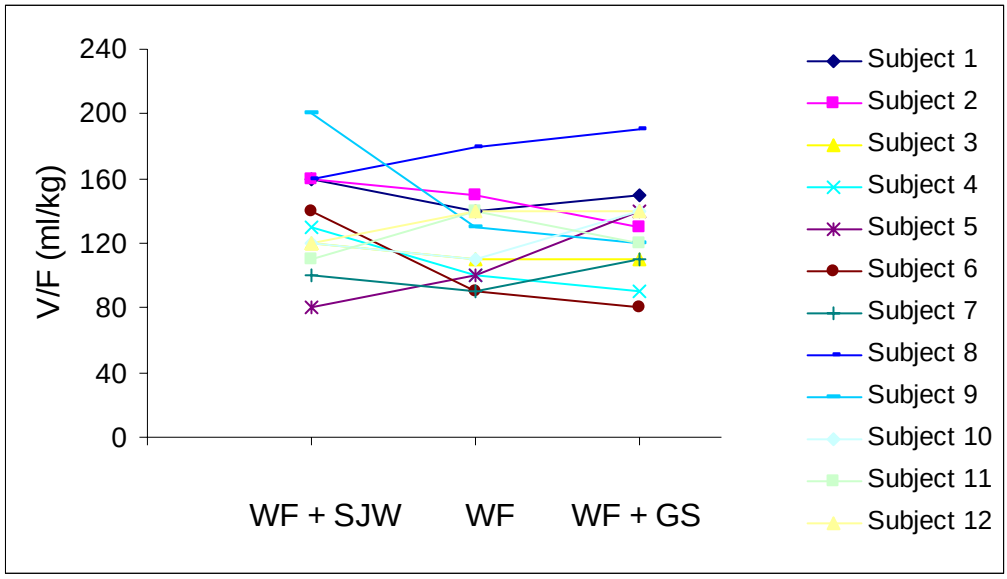


Figure 5-8. R-warfarin apparent volume of distribution (V/F) following single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginseng (WF + GS) and warfarin + St John's wort (WF + SJW).

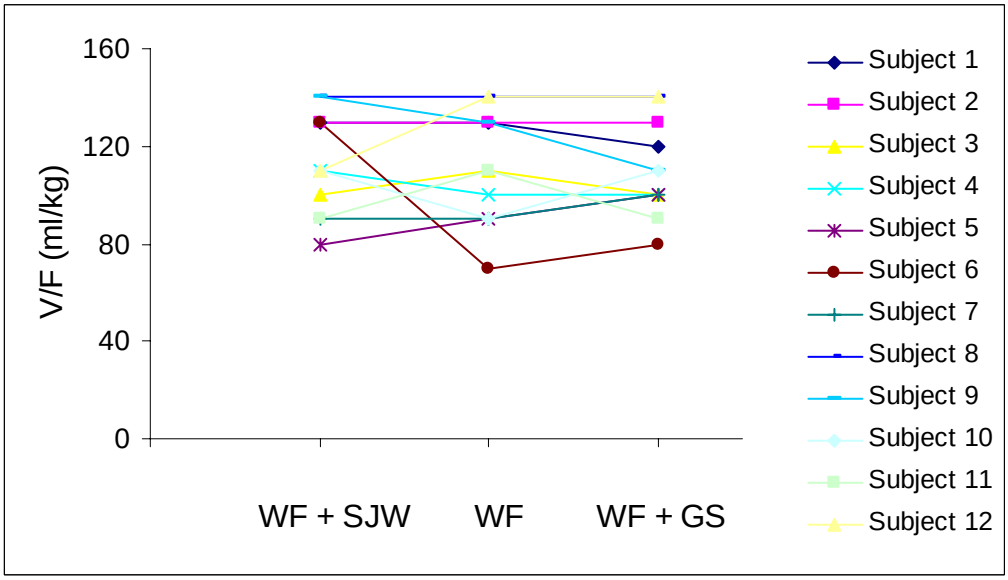


Figure 5-9. S-warfarin maximum plasma concentration (C_{max}) following single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginseng (WF + GS) and warfarin + St John's wort (WF + SJW).

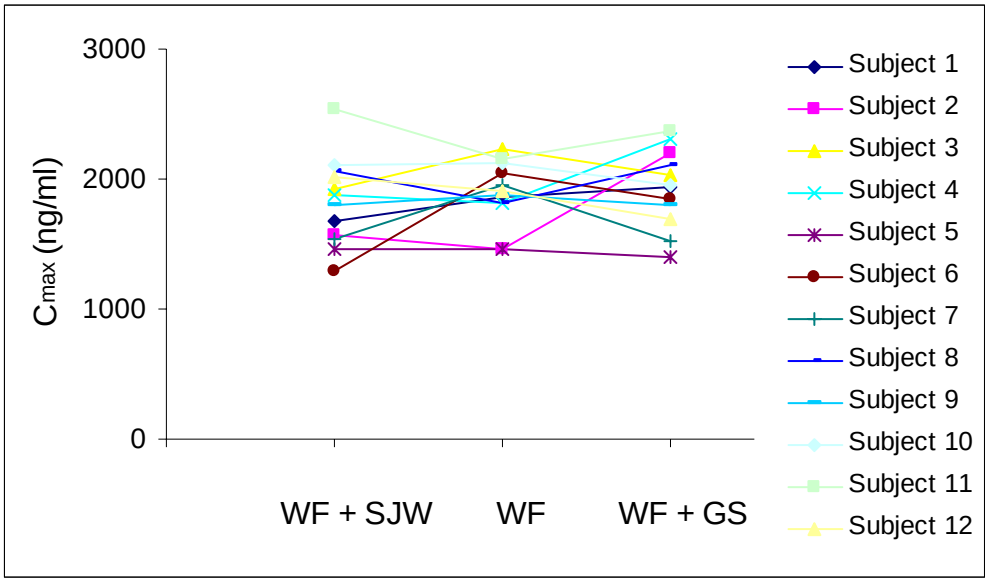


Figure 5-10. R-warfarin maximum plasma concentration (C_{max}) following single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginseng (WF + GS) and warfarin + St John's wort (WF + SJW).

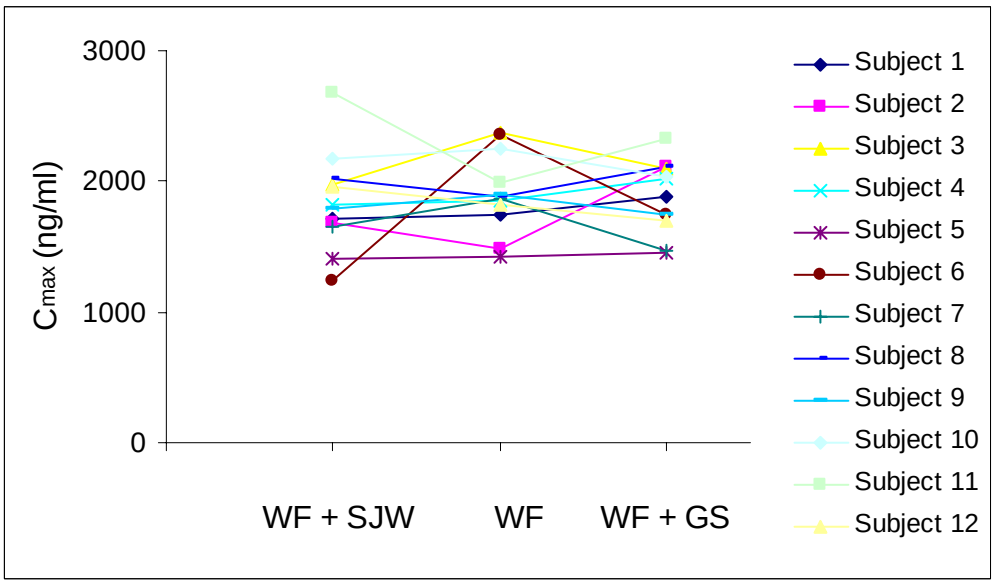


Figure 5-11. The time at which S-warfarin C_{max} occurs (t_{max}) following single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginseng (WF + GS) and warfarin + St John's wort (WF + SJW).

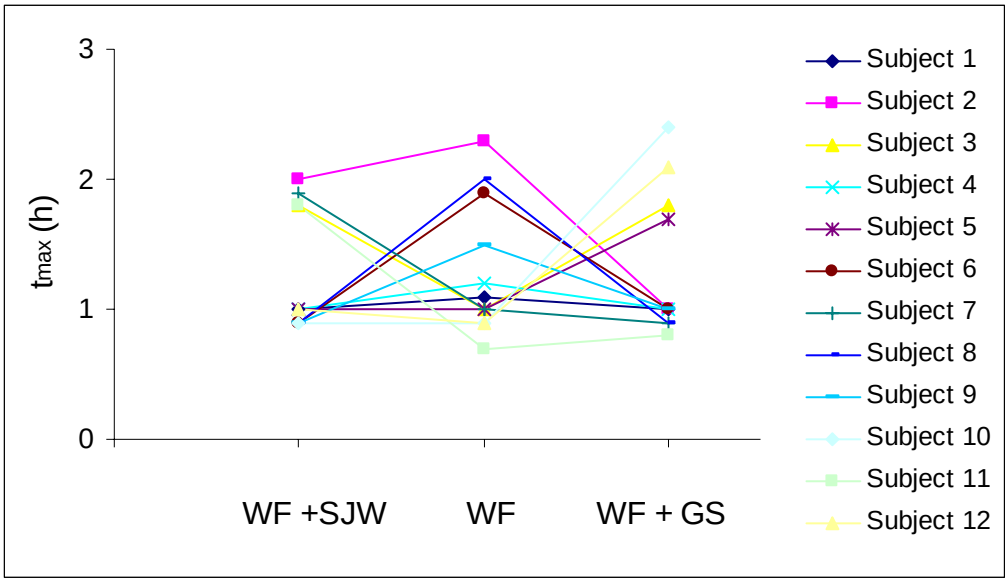
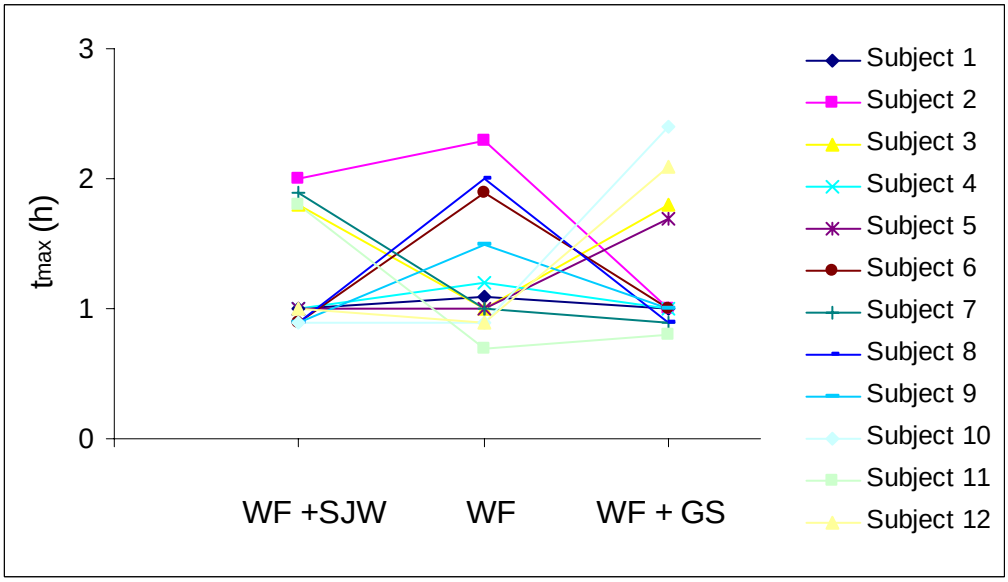


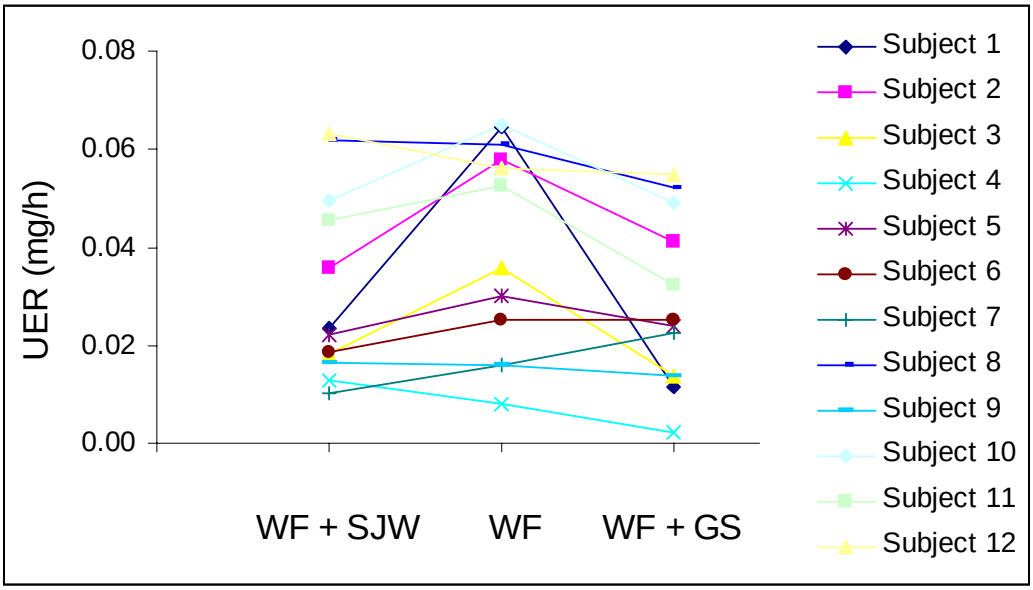
Figure 5-12. The time at which R-warfarin C_{max} occurs (t_{max}) following single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginseng (WF + GS) and warfarin + St John's wort (WF + SJW).



5.3.4 Urinary Excretion Rate (UER) of S-7-hydroxywarfarin

The urinary excretion rate of S-7-hydroxywarfarin after administration of warfarin alone (0.04 ± 0.02 mg/h) was not significantly different following treatment with St John's wort (0.03 ± 0.02 mg/h), but was reduced by treatment with ginseng (0.03 ± 0.02 mg/h) (Figure 5-13) as assessed by the ratio of geometric mean and 90% confidence intervals for the UER which was 0.82 (0.61-1.12) after pre-treatment with St John's wort and 0.68 (0.50-0.91) with ginseng.

Figure 5-13. Urinary excretion rate of S-7-hydroxywarfarin following a single oral 25 mg *rac*-warfarin alone (WF), and in combination with either St John's wort (WF + SJW) or ginseng (WF + GS).



5.3.5 Plasma Protein Binding of Warfarin

The fraction unbound of S-warfarin and R-warfarin were 0.0034 ± 0.0011 and 0.0048 ± 0.0006, respectively, following administration of warfarin alone, and 0.0036 ± 0.0010 and 0.0047 ± 0.0009 for warfarin following pre-treatment with St John's wort, was 0.0039 ± 0.0016 and 0.0046 ± 0.0008 following pre-treatment with ginseng, respectively. The protein binding of warfarin enantiomers did not change over the duration of the sample collection interval (Figure 5-14, 15).

Figure 5-14. Fraction of unbound S-warfarin following administration of a single oral 25 mg dose of *rac*-warfarin alone (WF), or in combination with either St John's wort (WF + SJW) or ginseng (WF + GS).

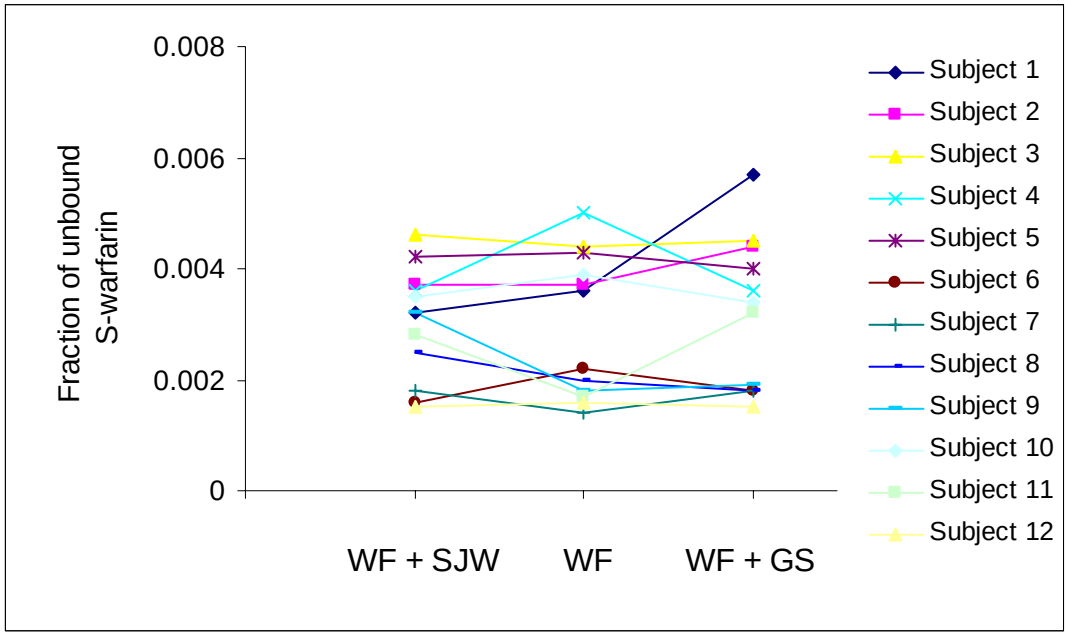
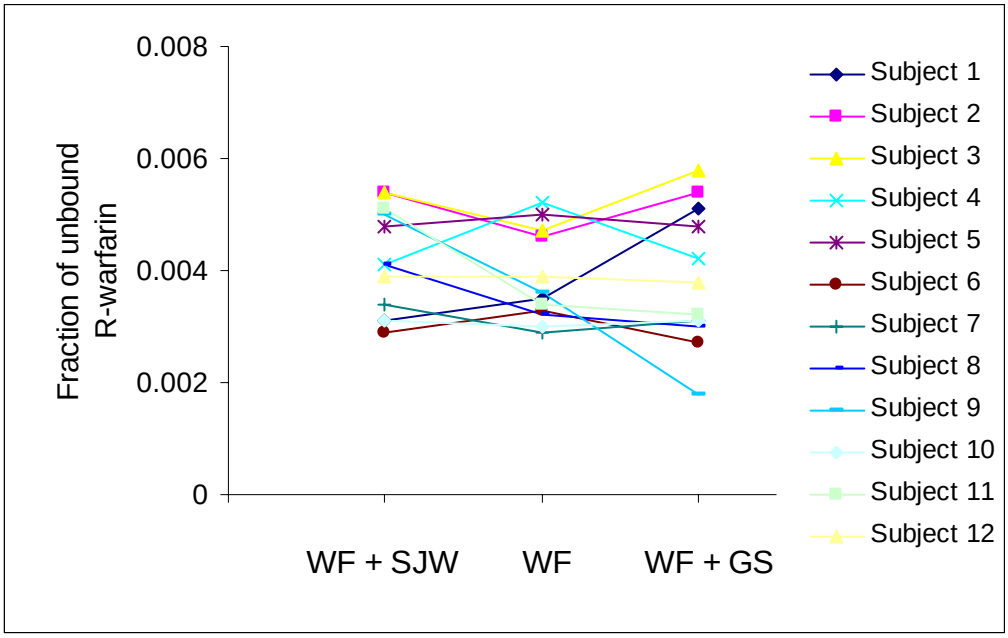


Figure 5-15. Fraction of unbound R-warfarin following administration of a single oral 25 mg dose of *rac*-warfarin alone (WF), in combination with either St John's wort (WF + SJW) or ginseng (WF + GS).



5.3.6 Pharmacodynamic Endpoints

After administration of a single 25 mg dose of warfarin, the INR for each subject increased to a peak at approximately 48 h after a delay of about 8-12 hours. INR values returned to normal after approximately 144 hours (Figure 5-16 and Table 5-4). The ratios of the $AUC_{0\text{-}168}$ of INR and the 90% confidence intervals were 0.79 (0.70-0.95) for treatment with St John's wort, and 1.01 (0.88-1.16) for ginseng (Table 5-5). There was a significant difference in warfarin pharmacodynamics between warfarin alone and co-administration with St John's wort. However, there was no significant difference in warfarin pharmacodynamics when warfarin was ingested following pre-treatment with ginseng. Neither St John's wort nor ginseng alone affected baseline INR or platelet aggregation (Table 5-5).

Figure 5-16. INR versus time profiles following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginseng (GS) and warfarin + St John's wort (WF + SJW) (mean ± SD, n=12).

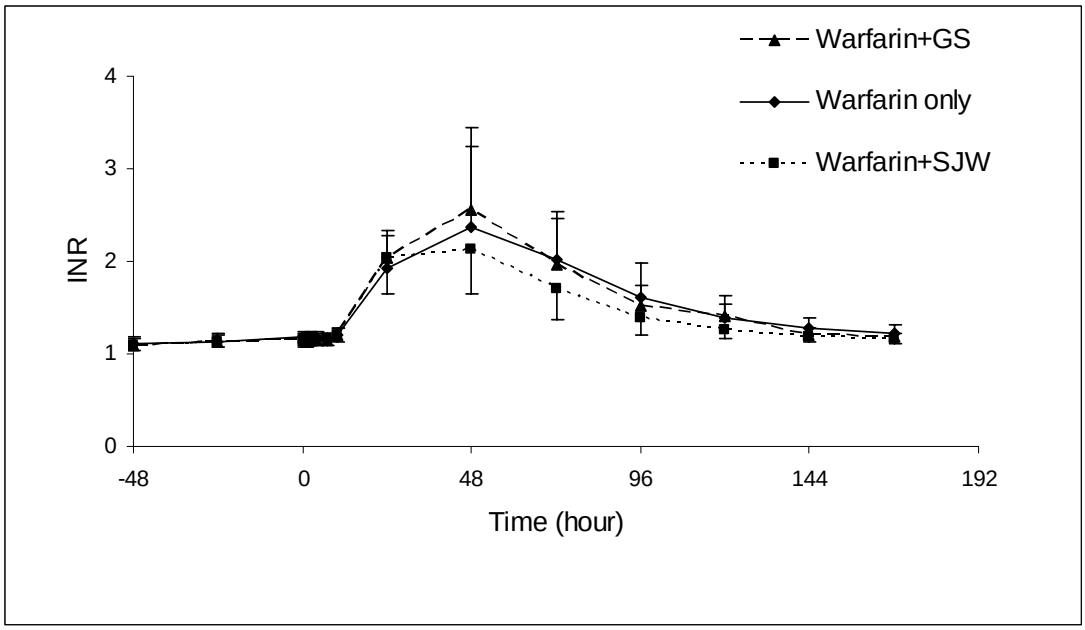


Figure 5-17. $AUC_{0\text{-}168}$ of INR following a single oral 25 mg *rac*-warfarin alone (WF), in combination with either St John's wort (WF + SJW) or ginseng (WF + GS).

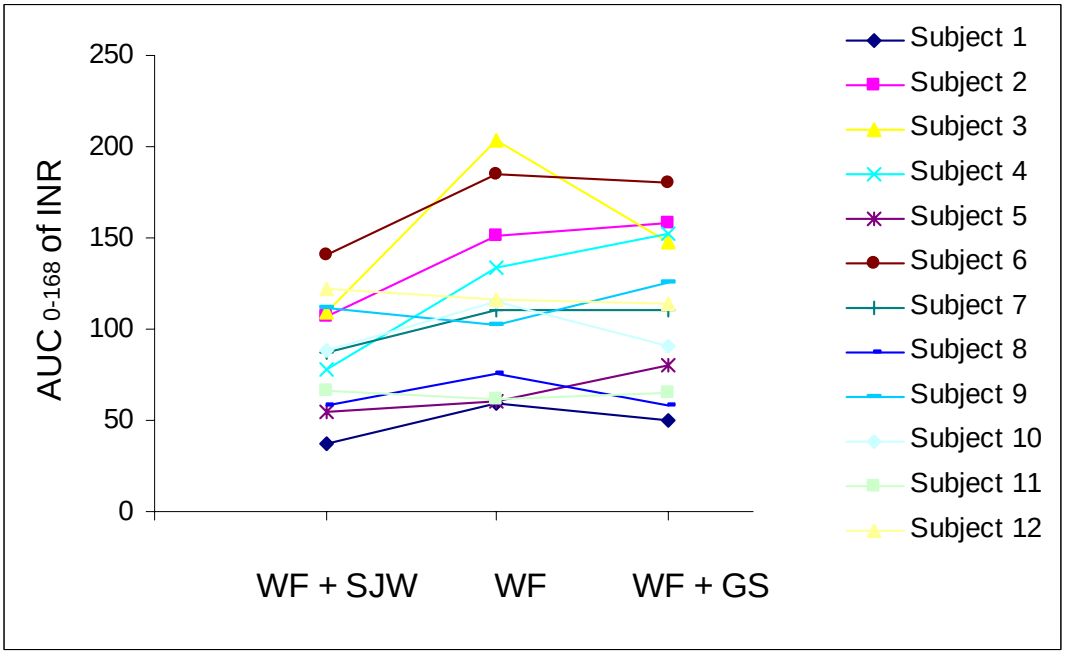


Table 5-4. Warfarin pharmacodynamic parameters following a single oral dose of 25 mg *rac*-warfarin alone (WF), and in combination with either St John's wort (WF + SJW) or ginseng (WF + GS). Data are presented as mean ± SD and 95% CI are shown in brackets (n=12). Individual subject data are presented in Appendices 20-24.

Treatment	WF alone	WF + SJW	WF + GS
$INR_{baseline}$*	1.14 ± 0.07 (1.08 - 1.20)	1.12 ± 0.06 (1.08 -1.20)	1.13 ± 0.05 (1.10 - 1.12)
AUC_{0-168} of INR	111.0 ± 49.3 (79.6 - 142.3)	88.3 ± 30.7 (68.8 - 107.8)	111.1 ± 43.1 (83.9 - 138.7)
t_{max} of INR (h)	45.5 ± 17.4 (34.5 – 56.6)	39.8 ± 12.7 (31.7 – 47.9)	41.2 ± 12.1 (33.6 – 48.9)
INR_{max}	2.4 ± 0.8 (2.0 – 2.9)	2.2 ± 0.5 (2.0 – 2.5)	2.6 ± 0.8 (2.1 – 3.1)
Baseline platelet aggregation (Ω)	7.7 ± 2.2 (5.6 - 9.1)	7.5 ± 1.1 (6.5 - 8.2)	7.1 ± 1.4 (6.0 - 7.8)

*$INR_{baseline}$: the INR after either 14 days pre-treatment with SJW or 7 days pre-treatment with ginseng prior to warfarin administration

Table 5-5. Mean ratios and 90% confidence intervals of the ratio of log-transformed warfarin pharmacodynamic parameters (n=12) after treatment with different herb medicines. (*p<0.05)

Treatment	St John's wort (90% CI)	Ginseng (90% CI)
$INR_{baseline}$	0.99 (0.96 - 1.01)	0.99 (0.97 - 1.02)
AUC_{0-168} of INR	0.79 (0.70 - 0.95)*	1.01 (0.88 - 1.16)
t_{max} of INR	0.89 (0.78 – 1.01)	0.93 (0.82 – 1.06)
INR_{max}	0.95 (0.84 – 1.08)	1.07 (0.94 – 1.22)
Baseline platelet aggregation	1.00 (0.88 - 1.14)	1.00 (0.85 - 1.06)

5.3.7 Pharmacokinetic and Pharmacodynamic Modelling

The warfarin enantiomer concentration – time data and the effect data (expressed as prothrombin complex activity, PCA) were best described by a one compartment pharmacokinetic model and a sigmoid E_{max} pharmacodynamic model, respectively. Mean data are presented in Figure 5-18 and individual fits to pharmacokinetic and pharmacodynamic data are presented in Appendix 11. The model dependent pharmacokinetic and pharmacodynamic parameters are presented in Table 5-6 and Figure 5-18 to 5-21**.** Their ratios between different herbal pre-treatments are presented in Table 5-7. It was not possible to obtain reliable model dependent estimates of the concentration of R-warfarin required to produce a 50% inhibition prothrombin complex activity ($C_{50,R}$). These results confirm the significant difference in $t_{1/2}$ and CL/F for both S-warfarin and R-warfarin following pre-treatment with St John's wort. Furthermore, these data indicate no significant changes observed in the pharmacodynamic parameters of S-warfarin in healthy male volunteers following treatment with St John's wort. Model dependent analyses confirm that ginseng did not affect either the pharmacokinetics or pharmacodynamics of warfarin. The pharmacokinetic parameters estimated based on the model-dependent methods and the pharmacokinetic parameters generated using the model independent approach (See Section 5.3.3) is in excellent agreement.

Figure 5-18. Prothrombin complex activity (PCA)-time profiles following administration of a single oral dose of 25 mg *rac*-warfarin either alone (WF), warfarin + ginseng (WF + GS) or warfarin + St John's wort (WF + SJW) (mean ± SD, n=12). Individual subject profiles are presented in Appendix 11.

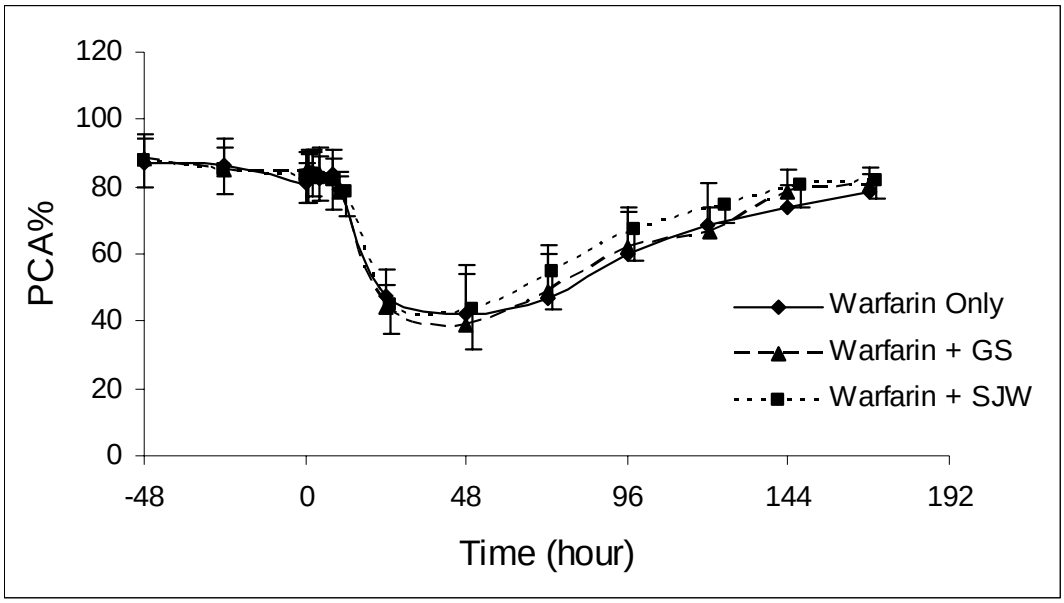


Figure 5-19. Model dependent estimates of the degradation rate constant of the prothrombin complex (k_d) following oral administration of a 25 mg *rac*-warfarin dose as warfarin only (WF), warfarin + St John's wort (WF + SJW) and warfarin + ginseng (WF + GS).

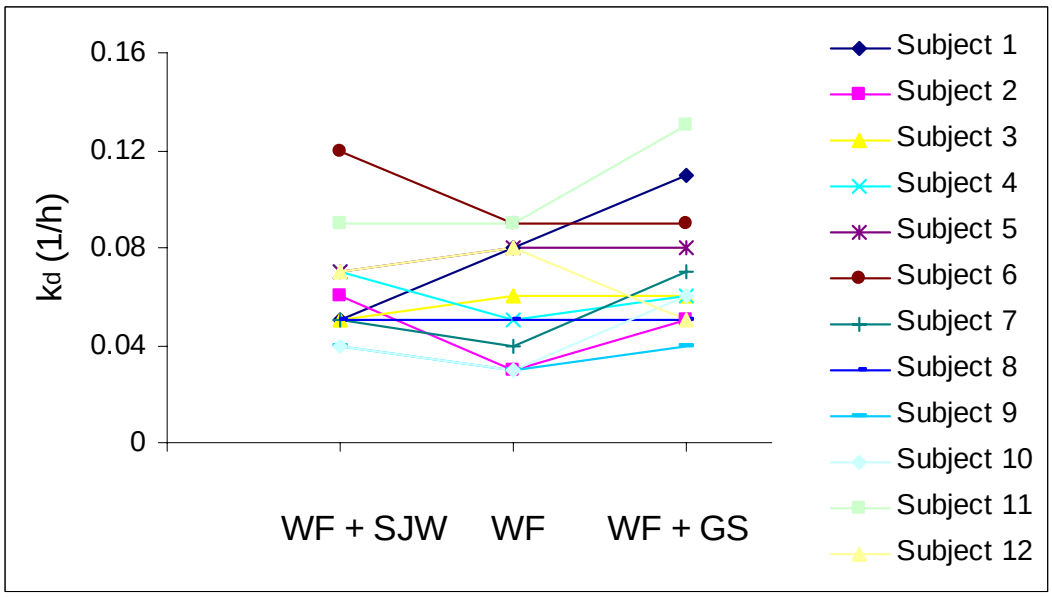


Figure 5-20. Model dependent estimates of concentration of S-warfarin required to produce a 50% inhibition of PCA ($C_{50,S}$) following oral administration of a 25 mg *rac*-warfarin dose as warfarin only (WF), warfarin + St John's wort (WF + SJW) and warfarin + ginseng (WF + GS).

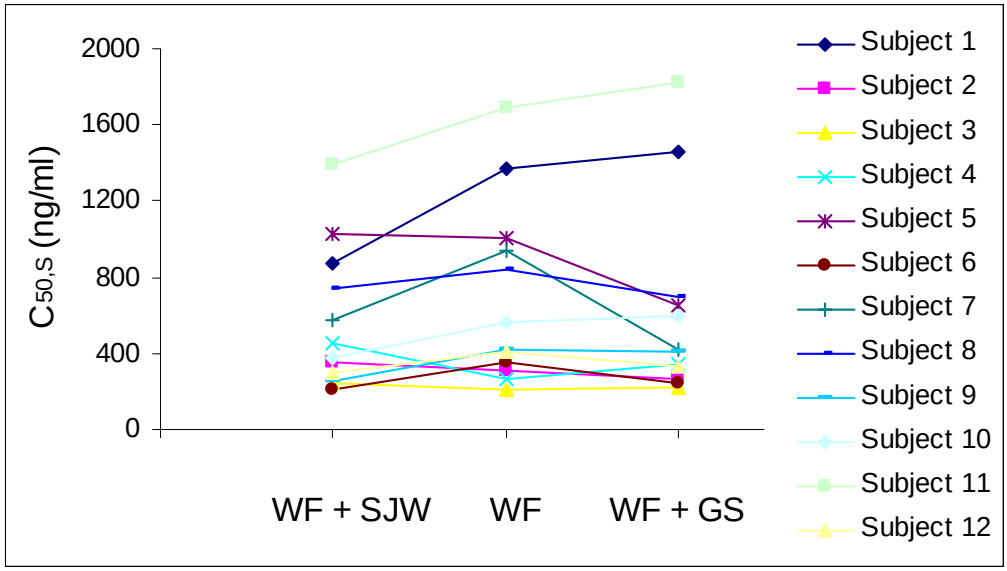


Figure 5-21. Model dependent estimates of the steepness of the concentration-response curve (γ) following oral administration of a 25 mg *rac*-warfarin dose as warfarin only (WF), warfarin + St John's wort (WF + SJW) and warfarin + ginseng (WF + GS).

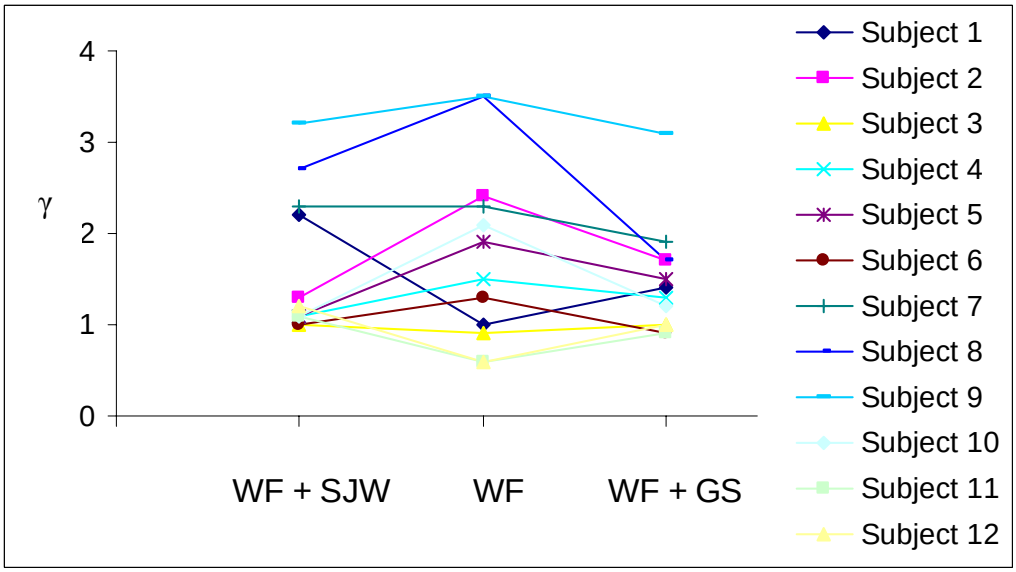


Table 5-6. Model dependent warfarin pharmacokinetic and pharmacodynamic parameters following a single oral dose of 25 mg *rac*-warfarin alone (WF) or in combination with either St John's wort (WF + SJW) or ginseng (WF + GS) (mean ± SD, 95% CI, n=12). Individual data are presented in Appendices 20-24.

Treatment	WF alone	WF + SJW	WF + GS
k_d (1/h)	0.06 ± 0.02 (0.04 – 0.07)	0.06 ± 0.02 (0.05 – 0.08)	0.07 ± 0.03 (0.05 – 0.09)
$C_{50,S}$ (ng/ml)	698 ± 477 (395 – 1000)	566 ± 371 (330 - 801)	622 ± 506 (300 – 943)
γ_S	1.8 ± 1.0 (1.2 – 2.4)	1.6 ± 0.8 (1.1 – 2.1)	1.5 ± 0.6 (1.1 – 1.9)
$t_{1/2}$ (h) S-warfarin R-warfarin	 30.9 ± 4.0 (28.4 – 33.5) 49.9 ± 8.8 (44.3 – 55.5)	 23.6 ± 4.4 (20.8 – 26.4) 38.8 ± 3.9 (36.3 – 41.3)	 27.6 ± 3.9 (25.1 – 30.1) 46.6 ± 6.5 (42.5 – 50.7)
CL/F (ml/h) S-warfarin R-warfarin	 202 ± 38 (178 – 226) 113 ± 25 (97 – 129)	 283 ± 50 (251 – 315) 147 ± 31 (127 – 166)	 223 ± 35 (201 – 245) 120 ± 19 (107 – 132)
V/F (ml/kg) S-warfarin R-warfarin	 120 ± 30 (100 – 140) 110 ± 20 (100 – 120)	 130 ± 30 (110 – 150) 110 ± 20 (100 – 120)	 120 ± 30 (100 – 140) 110 ± 20 (100 – 120)

Table 5-7. Mean ratios and 90% confidence intervals for selected log transformed pharmacokinetic and pharmacodynamic parameters between herb treatments compared to warfarin only (n=12).

Treatment	St John's wort (90% CI)	Ginseng (90% CI)
k_d	1.11 (1.03 – 1.18)	1.23 (1.14 – 1.32)
$C_{50,S}$	0.84 (0.78 – 0.90)	0.87 (0.81 – 0.94)
γ_S	0.96 (0.87– 1.05)	0.90 (0.82– 0.98)
$t_{1/2}$ S-warfarin R-warfarin	 0.76 (0.74 – 0.78)* 0.79 (0.76 – 0.81)*	 0.89 (0.87 – 0.92) 0.94 (0.91 – 0.97)
CL/F S-warfarin R-warfarin	 1.40 (1.34 – 1.47)* 1.31 (1.25 – 1.37)*	 1.13 (1.08 – 1.15) 1.07 (1.02 – 1.12)
V/F S-warfarin R-warfarin	 1.06 (1.00 – 1.12) 1.06 (0.97 – 1.06)	 0.98 (0.93 – 1.04) 1.00 (0.95 – 1.04)

*ANOVA, $p<0.05$

5.3.8 Adverse Events

Twelve subjects completed the study and no significant adverse events or bleeding episodes were observed during the study. Three subjects reported changes in sleeping habits (waking up early in the morning) during St John's wort treatment.

5.4 Discussion

This study investigated the effect of two commonly ingested herbal medicines on the pharmacokinetics and pharmacodynamics of warfarin and their independent effect on INR and platelet aggregation using a standard study design widely used in investigating warfarin–drug interactions. The major finding was that the co-administration of St John's wort at the Commission E recommended doses (1 tablet 3 times daily for 14 days) increased the apparent clearance of the warfarin enantiomers, leading to a subsequent reduction in the plasma concentrations of warfarin enantiomers and their pharmacodynamic effect.

In assessing potential herb-drug interactions, it is essential to use herbal medicines of known quality to provide the best chance of being able to rigorously detect significant effects. In this study, qualitative TLC and Pharmacopoeial methods were used to characterise the constituents of proprietary preparations of St John's wort and ginseng prior to the study (See Chapter 4). As some variability in the composition of different brands of commercial herbal medicines products of St John's wort and ginseng has been described, it was important to establish the quality of the herbal medicines under investigation. There remains debate about which constituents of St John's wort and ginseng [8, 74, 148] might be involved in potential herb-drug interactions, so the products employed in this study were not quantitatively assessed for the specific content of individual constituents but for their overall content and quality.

In this study, warfarin, a racemic mixture, was administered and the pharmacokinetics of the individual enantiomers was studied. It is known that S-warfarin exhibits a significantly greater anticoagulant activity when compared to the R-enantiomer when each enantiomer is administered separately [18]. Furthermore, there are stereoselective differences in the pharmacokinetics of warfarin enantiomers. S-warfarin has been reported to be two to five times more potent than R-warfarin in terms of the anticoagulant effect [26]. However, R-warfarin may actually be less potent than previously reported, as the source of R-warfarin used in the study to investigate its potency contained a significant amount of S-warfarin (7.5%;[25]) [26]. Chan *et al* [18] reported that the anticoagulant effect observed after the administration of racemic warfarin is almost entirely contributed by S-warfarin and the observation was confirmed in the PKPD modelling analysis in the present study. Elucidation of the pharmacokinetics of warfarin enantiomers thus allows greater insight into the mechanism of possible pharmacokinetic interactions with this drug. S-warfarin is metabolised to S-7-hydroxywarfarin by CYP2C9 [279] while R-warfarin is partly metabolised by CYP1A2 and CYP3A4 [18]. The simultaneous investigation of warfarin enantiomer pharmacokinetics thus provides insight into the possible effects of St John's wort and ginseng on these drug metabolism pathways.

In vitro studies have demonstrated that constituents of St John's wort inhibit the metabolic activity of CYP2C9, CYP2D6 and CYP3A4 [139, 140]. Paradoxically, St John's wort has also been reported to induce CYP1A2, CYP2E1 and CYP3A4 based on *in vivo* studies [91, 106, 114, 124, 127, 138, 280, 281]. This effect has been attributed to the activation of the human pregnane X receptor (PXR) by the St John's

wort constituent, hyperforin, which has been demonstrated both *in vitro* [282] and *in vivo* [283]. The present study employed a regimen of 14-day pre-treatment with St John's wort which was based on the study by Wang *et al* [92] who reported that following administration of St John's wort to healthy volunteers for 14 days, there was induction of CYP3A4 activity in the intestinal wall and liver. Interestingly, Wang *et al* [92] found no alteration in the metabolic activities of CYP2C9, CYP1A2, or CYP2D6. This conclusion was based on administration of a cocktail of substrates for specific cytochromes (these included tolbutamide, CYP2C9; caffeine, CYP1A2; dextromethorphan, CYP2D6; oral midazolam, intestinal wall and hepatic CYP3A; and intravenous midazolam, hepatic CYP3A). The studies reported in this thesis employed (at least) a 14-day washout period between treatment periods. As little is known about the offset of the induction of drug metabolising enzymes by St John's wort, this period was based on the half-lives of warfarin enantiomers [18] and the St John's wort constituents, hypericin and hyperforin [74]. The analysis of variance in this randomised three-period study demonstrated no period effect, suggesting the inductive effect of St John's wort on drug metabolising enzymes had dissipated in the 14 days between treatments.

In the present study, treatment with St John's wort significantly induced not only CYP1A2 and/or CYP3A4 as evidenced by the effects on R-warfarin, but also CYP2C9 as determined by the effects on the pharmacokinetics of the S-enantiomer. The induction of CYP2C9 reported in this thesis was in contrast to the observations of Wang *et al* [92] but confirms the suggestions raised by Henderson *et al* [8] in their review of St John's wort drug interactions and an *in vitro* study [67] that

demonstrated the human pregnane X receptor (hPXR) mediates induction of CYP2C9 by hyperforin (found in St. John's wort). Surprisingly, St John's wort did not significantly affect the UER of S-7-hydroxywarfarin (the main metabolite of S-warfarin) while it induced the clearance of S-warfarin. An increase in the UER would be expected due to induction of CYP2C9 by St John's wort. The mechanism of this effect is not clear and needs further study. Missed urine sample collection that was reported by a few volunteers during 24 hours urine collections, could have contributed to this observation and represents a disadvantage for using urine samples to investigate metabolite pharmacokinetics.

A few ginseng-drug interactions have been reported in the literature. In a case report, a 47-year-old man had received anticoagulation therapy with warfarin since 1990 to prevent embolic events. The dosage of warfarin and INR had been stabilised for the past nine months. The patient's INR was 3.1 four weeks before he started taking ginseng. Two weeks after the patient started taking ginseng, his INR declined to 1.5. Ginseng was discontinued, and the INR returned to 3.3 in two weeks [167]. However, no significant effect on drug metabolism was found in a clinical trial in humans wherein the activities of various cytochrome P450 isoenzymes were assessed using the phenotypic ratios of probe-drugs that included midazolam (CYP3A4), caffeine (CYP1A2), chlorzoxazone (CYP2E1) and debrisoquin (CYP2D6) [91]. Furthermore. A warfarin–ginseng interaction study in male Sprague-Dawley rats using both single (2 mg/kg) and multiples doses of warfarin (0.2 mg/kg daily x 6 days) found no significant effect of *Panax ginseng* on warfarin pharmacokinetics [168]. The authors also found the content of vitamin K was undetectable in the ginseng decoction [168].

Consistent with these latter reports, the present study confirmed that ginseng did not affect the pharmacokinetics or the pharmacodynamics of warfarin in human subjects. From these studies it is possible to confirm that *Panax ginseng* had no effect on the activity of CYP1A2, CYP3A4 or CYP2C9 in the healthy volunteers pre-treated with this herb.

Since approximately 99% of the warfarin in plasma is bound to plasma proteins, another possible drug interaction mechanism that may alter the hepatic clearance of warfarin enantiomers could be the effect of St John's wort or ginseng constituents on the protein binding of warfarin. No *in vitro* or *in vivo* studies were found in the literature regarding the ability of St John's wort or ginseng constituents to affect protein binding of warfarin. In this study, treatment with St John's wort and ginseng did not influence warfarin enantiomer protein binding or the distribution in the present study. It suggests that increased clearances of warfarin enantiomers are due to induction of CYP 2C9, CYP 3A4 and/or CYP1A2 by pre-treatment with St John's wort.

Several *in vitro* studies have demonstrated that constituents of ginseng may inhibit thrombin, collagen or arachidonic acid induced platelet aggregation using human platelet rich plasma [157, 159, 161]. Yun *et al* [161] evaluated the antithrombotic effects of Korean red ginseng using platelet aggregation and coagulation assays. In the platelet aggregation assay using human platelet rich plasma (PRP), extracts of Korean red ginseng were found to significantly inhibit thrombin and collagen-induced platelet aggregation. The IC_{50} values of Korean red ginseng were >2 mg/ml

for thrombin, 0.32 ± 0.01 mg/ml for collagen and 0.72 ± 0.25 mg/ml for ADP. In the coagulation assay, Korean red ginseng was found to significantly prolong APPT and PT as compared with control data. Park *et al* [159] studied the effect of dietary supplementation with 25 mg (0.0025% of the total diet) of a lipophilic fraction from *Panax ginseng* on rat platelet aggregation induced by collagen (100 μg/ml) or thrombin (0.1 units/ml), and on blood coagulation. The cGMP levels in collagen-stimulated platelets from rats fed 15% corn oil plus the lipophilic fraction of ginseng were significantly increased compared to rats only fed 15% corn oil. The levels of cAMP in thrombin-stimulated platelets in the ginseng fraction treated rats were decreased but were increased in collagen-stimulated platelets. Furthermore, the levels of both cGMP and cAMP were also increased by addition of the lipophilic fraction of ginseng to thrombin- and collagen-stimulated platelets *in vitro*. Both the prothrombin time and activated partial thromboplastin time were prolonged in citrated platelet-poor plasma in lipophilic fraction of ginseng group compared to corn oil only treated group. Teng *et al* [157] reported that panaxynol (0.1 mg/ml) isolated from the diethyl ether layer of *Panax ginseng* inhibited markedly the aggregation of washed platelets induced by collagen, arachidonic acid, ADP, PAF and thrombin while ginsenosides had no significant effect on the aggregation, however, ginsenoside Ro (1 mg/ml) inhibited the ATP release of platelets using human blood in *in vitro* study.

Contrary to these reports, pre-treatment with ginseng in healthy male subject did not significantly affect the baseline INR and platelet aggregation in the present study. This discrepancy between *in vitro* and *in vivo* effects could be related to metabolic biotransformation or poor bioavailability of ginseng constituents after oral dosing as

suggested by several bioavailability studies [163, 284]. Cui *et al* [162] determined that about 1.2% of the oral dose of protopanaxatriol ginsenosides (3 mg) and smaller amounts of the protopanaxadiol ginsenosides not exceeding 0.2% of the administered dose (7 mg) were recovered in five days in human urine after a single dose of ginseng. However, Shibata *et al* [163] reported that neither the individual ginsenosides nor their metabolites could be identified except compound-K which is the main intestinal bacterial metabolite of protopanaxadiol ginsenosides was identified in human serum using a specific enzyme immunoassay 8 h after oral administration of ginseng. *Panax notoginseng* is different with *Panax ginseng* but it contains ginsenoside Rb1 and Rg1 as well. Xu *et al* [164] found very low bioavailability of ginsenoside Rb1 (4.35%) and ginsenoside Rg1 (18.40%) after *Panax notoginseng* was administrated to rats. Serum samples were measured using an established HPLC method to quantitate ginsenoside Rb1 and Rg1.

In this study a combined PK/PD model was employed to characterise the warfarin concentration effect data obtained in the three phases of this clinical trial. This approach allows an assessment of the effect of these herbs on warfarin pharmacodynamic parameters. No lag time was found for drug absorption in the present study, but a characteristic of the pharmacological response of warfarin is the delay in the onset of action after oral administration of the drug [18]. In the combined PK/PD model this delay is represented by the parameter t_d. In the present study, it was not possible to estimate lag time of onset of anticoagulant (t_d) directly so it was fixed to the value of 8.0 h. Chan *et al* [18], who proposed the modelling approach used in this study, found that td ranged from 8.0 to 8.8 in their study of six

volunteers. The modelling approach could not provide reliable estimates of C_{50R} and γ_R probably because the anticoagulant effect of racemic warfarin is predominately determined by S-warfarin [26]. Co-administration with St John's wort or ginseng did not affect the protein binding of warfarin enantiomers so $C_{50,S}$ was reported rather than $Cu_{50,S}$. No significant difference was found in estimates of the pharmacodynamic parameters k_d, $C_{50,S}$ and γ_S obtained from data collected with and without pre-treatment with herbal medicines. The estimates of k_d, C_{50S} and γ_S from the present study are in close agreement with previously reported data [18].

In summary, this study found that St John's wort when administered to healthy male subjects in a single ingredient herbal product at recommended doses induced the metabolism of both S-warfarin and R-warfarin with a subsequent effect on INR. In another word, the PD relationship was not intrinsically altered by St John's wort. By contrast, *Panax ginseng* at recommended doses had no significant effect on warfarin metabolism or its pharmacological effect in healthy subjects. These findings suggest that there is a potential for significant herb-drug interactions with St John's wort for drugs that are the substrates for CYP2C9 and CYP3A4 and/or CYP1A2.

Chapter 6 Effect of Ginkgo and Ginger on the Pharmacokinetics and Pharmacodynamics of Warfarin in Healthy Subjects

6.1 Introduction

The anticoagulant warfarin has a narrow therapeutic index and displays high inter- and intra-subject variability in pharmacokinetics and pharmacodynamics. In the community, there is also widespread, often unreported, self-medication with a range of herbal medicines. The opportunity for potential life-threatening interactions between herbal medications and warfarin is therefore high [2]. Ginkgo (*Ginkgo biloba*) is one such herbal medicine. It is commonly used as EGb 761, a ginkgo extract, for promoting and maintaining mental alertness, concentration, focus and a wide range of other indications [173, 285]. In a review article published in the Cochrane Database, Birks *et al* [285] reviewed the effect of ginkgo on cognitive impairment and dementia in the literature. It is reported that cognition, activities of daily living, measures of mood and emotional function show benefit for ginkgo at doses less than 200 mg/day compared with placebo at less than 12 weeks.

Despite the utility of this herb and its extracts, relatively little is known about the potential for or clinical significance of ginkgo-drug interactions. A recent review article [2] identified one case report of an interaction between ginkgo and warfarin and four case reports of spontaneous bleeding associated with ginkgo use attributed

to possible effects of this herb on platelet function. *In vitro* studies indicate that constituents of *Ginkgo biloba* (ginkgolic acids I and II) inhibit drug metabolising enzymes including CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 [137]. In contrast, the feeding of ginkgo extracts to rats for 4 weeks reportedly reduced the hypotensive effect of nicardipine (which is metabolised by CYP3A2) and induced the expression of hepatic CYP2B1/2, CYP3A1 and CYP3A2 mRNA [196]. *In vitro*, animal and *in vivo* clinical studies have investigated the effect of ginkgo extracts on platelet aggregation and coagulation, but the results have been conflicting [183, 187-189].

Ginger (*Zingiber officinale*) is also widely used in the community for the prevention of motion sickness and a variety of other indications [200]. However few drug interaction studies using ginger have been undertaken. Several *in vitro* studies demonstrated that platelet aggregation was inhibited by ginger extracts [201-204, 286]. Furthermore, when patients received a single dose (10 g) of powdered ginger, platelet aggregation was found to be reduced [206]. These data suggest that a pharmacodynamic interaction with warfarin is possible.

The aim of the present study was to investigate the possible drug interactions between warfarin and these two widely used herbal medicines at recommended doses from herbal medicine products of known quality. The independent effects of ginger and ginkgo on clotting status also were investigated.

6.2 Materials and Methods

The materials and methods for this chapter have been presented in Chapter 2.

6.3 Results

6.3.1 Subject

Twelve healthy male subjects were recruited and completed this study in three treatment groups. Subjects were aged 22 to 31 years and within 15% of ideal body weight for height and build. Subjects came from 2 main ethnic groups (6 Caucasians, 6 Asians) (Table 6-1). All subjects were non-smokers and met the inclusion criteria as outlined in Section 2.1.2.

6.3.2 Results of Statistical Analysis

ANOVA was performed on the log-transformed parameters including $AUC_{0-\infty}$ and C_{max} of S-warfarin and R-warfarin and AUC_{0-168} of INR. Both sequence and period effects were found to be significant in the analysis but treatment was not. The statistical results of $AUC_{0-\infty}$ of S-warfarin, R-warfarin and AUC_{0-168} of INR are shown in Appendix 6 - 10. The reason for the sequence and period effects was unclear. Despite this, the results are acceptable based on *in vivo* bioequivalence guidance [287] because this was a single-dose study, which includes only healthy male subjects. Furthermore, an adequate washout period was used and no detectable warfarin concentration was measured prior to warfarin dose administration in each period.

6.3.3 Pharmacokinetics of S-warfarin and R-warfarin

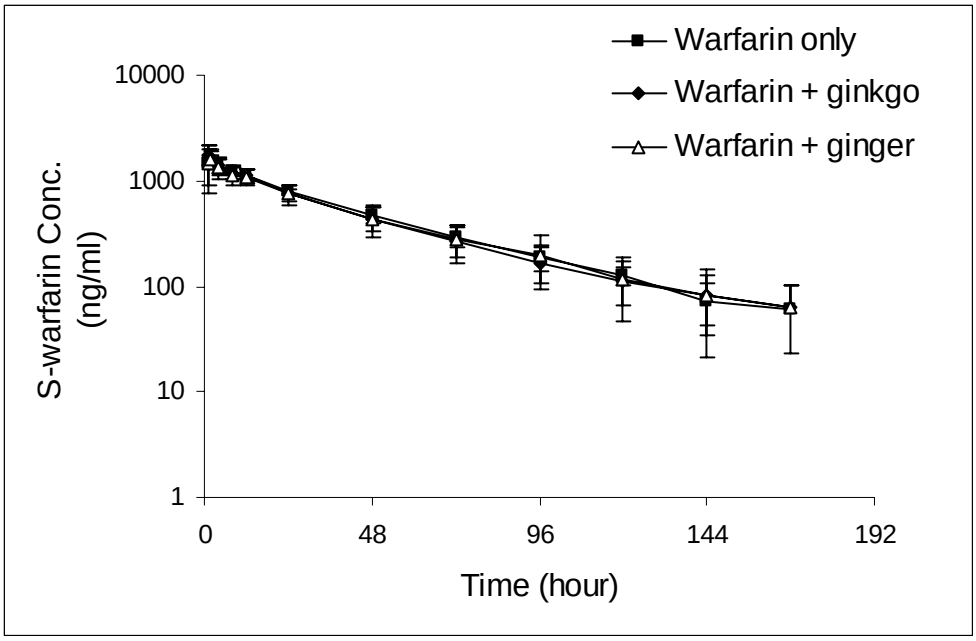
There were no significant changes observed in the pharmacokinetic parameters of S- or R-warfarin in healthy male volunteers following treatment with ginkgo or ginger (Table 6-2 and 6-3; Figure 6-1 to 6-12).

Table 6-1. Subject demographic data (n=12).

Subject	Age (years)	Weight (kg)	Height (cm)	Ethnicity
1	28	52.2	172.5	Asian
2	22	79.4	183.8	Caucasian
3	23	75.8	175.8	Asian
4	22	70.4	168.2	Asian
5	27	72.4	174.5	Asian
6	25	78	180.5	Caucasian
7	22	101	199.7	Caucasian
8	23	71.6	187.5	Caucasian
9	30	74.4	163	Asian
10	26	72.6	178	Caucasian
11	31	99.2	173	Asian
12	28	73.8	183	Caucasian
Mean ± SD	25.6 ± 3.2	76.7 ± 12.9	178.3 ± 9.6	

Figure 6-1. S-warfarin concentration-time profiles following administration of a single 25 mg oral dose of *rac*-warfarin either alone (warfarin only), or warfarin and ginkgo (WF + Ginkgo), or warfarin and ginger (WF + Ginger). Data are presented as mean (± SD, n=12) with semi-log (A) and linear (B) plots. Individual subject profiles are presented in Appendix 12.

A:



B:

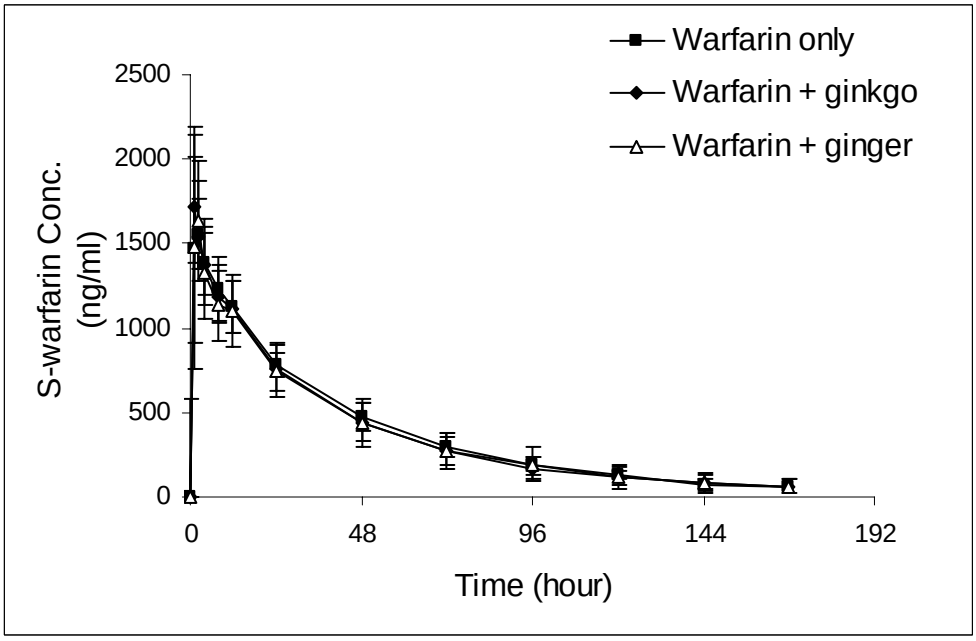
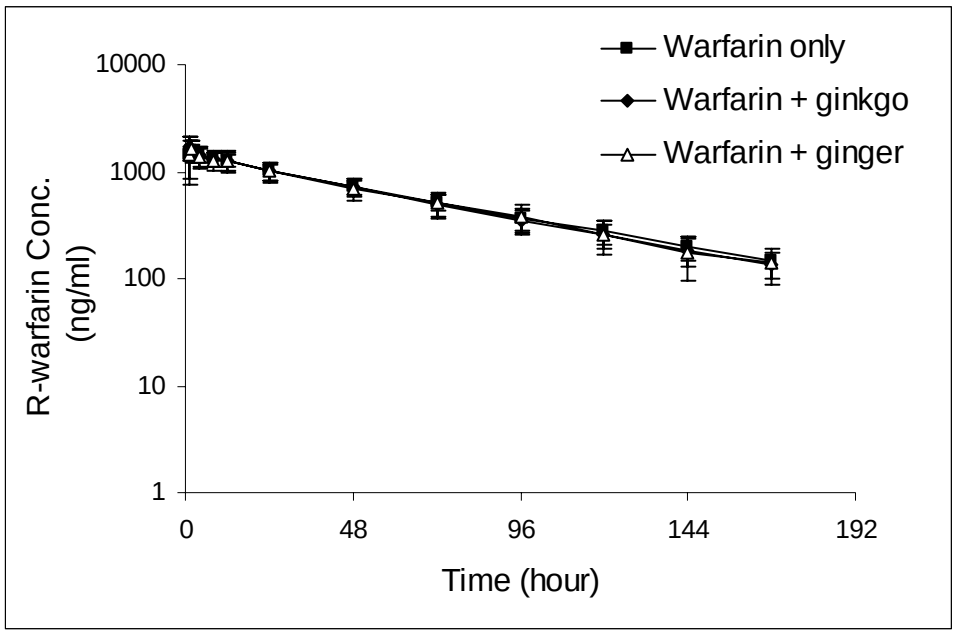


Figure 6-2. R-warfarin concentration-time profiles following administration of a single 25 mg oral dose of *rac*-warfarin either alone (warfarin only), or warfarin and ginkgo (WF + Ginkgo), or warfarin and ginger (WF + Ginger). Data are presented as mean (± SD, n=12) with semi-log (A) and linear (B) plots. Individual subject profiles are presented in Appendix 12.

A:



B:

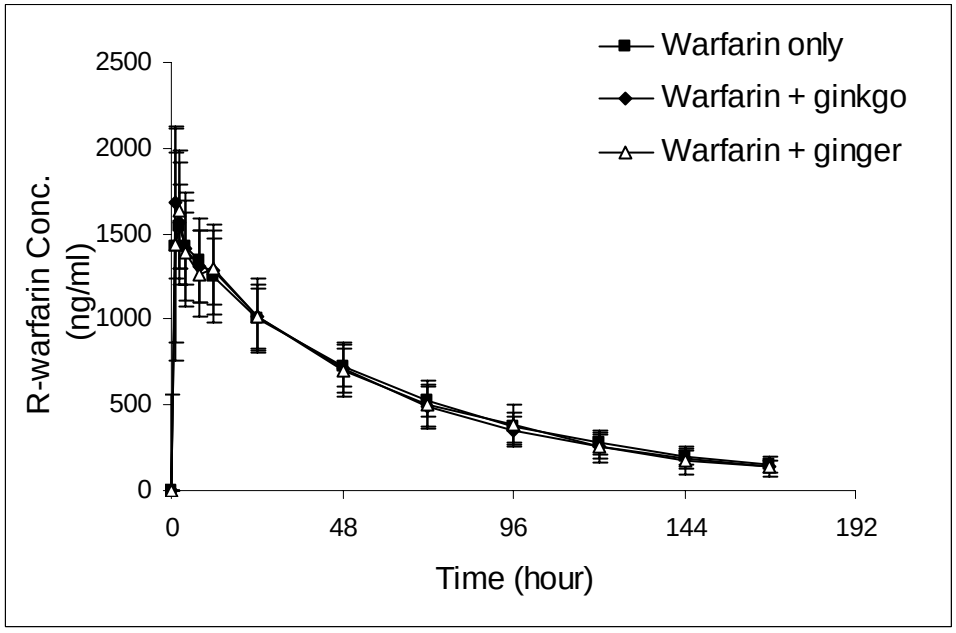


Table 6-2. Warfarin pharmacokinetic parameters following administration of a single oral dose of 25 mg *rac*-warfarin only (WF), or after pre-treatment with ginkgo (WF + ginkgo) or warfarin + ginger (WF + ginger) (mean ± SD, 95% CI, n=12). Individual subject data are presented in Appendices 25-31.

Treatment	Warfarin only	Warfarin + ginkgo	Warfarin + ginger
fu			
S-warfarin	0.0052 ± 0.0013 (0.0044 – 0.0060)	0.0049 ± 0.0012 (0.0042 – 0.0057)	0.0052 ± 0.0010 (0.0045 – 0.0058)
R-warfarin	0.0048 ± 0.0011 (0.0041 – 0.0054)	0.0047 ± 0.0009 (0.0041 – 0.0052)	0.0048 ± 0.0084 (0.0043 – 0.0054)
$AUC_{0-\infty}$ (ng/ml×h)			
S-warfarin	68000 ± 11400 (60800 – 75300)	65800 ± 16800 (55100 – 76500)	66000 ± 1900 (54000 – 78100)
R-warfarin	104000 ± 24700 (88400 – 120000)	102200 ± 18000 (90800 – 113600)	102600 ± 25500 (86400 – 118800)
t_{max} (h)			
S-warfarin	2.1 ± 1.1 (1.4 – 2.8)	1.4 ± 0.8 (0.9 – 1.9)	1.6 ± 0.8 (1.1 – 2.1)
R-warfarin	2.1 ± 1.1 (1.4 – 2.8)	1.6 ± 1.7 (0.6 – 2.7)	1.6 ± 0.8 (1.1 – 2.1)
C_{max} (ng/ml)			
S-warfarin	1700 ± 500 (1400 – 2000)	1800 ± 400 (1500 – 2000)	1700 ± 400 (1500 – 2000)
R-warfarin	1700 ± 500 (1400 – 2000)	1800 ± 400 (1500 – 2000)	1700 ± 400 (1500 – 1900)
$t_{1/2}$ (h)			
S-warfarin	35.8 ± 7.2 (31.1 – 40.3)	35.1 ± 6.7 (30.9 – 39.3)	35.7 ± 8.9 (30.0 – 41.3)
R-warfarin	50.3 ± 7.1 (45.8 – 54.9)	48.6 ± 6.1 (44.7 – 52.4)	47.7 ± 8.0 (42.6 – 52.8)
CL/F (ml/h)			
S-warfarin	189 ± 34 (167 – 210)	200 ± 43 (173 – 227)	201 ± 47 (171 – 231)
R-warfarin	127 ± 34 (106 – 149)	126 ± 24 (111 – 141)	131 ± 39 (106 – 156)
V/F (ml/kg)			
S-warfarin	120 ± 20 (110 – 140)	120 ± 10 (110 – 130)	120 ± 20 (110 – 130)
R-warfarin	120 ± 20 (100 – 130)	110 ± 10 (100 – 120)	110 ± 20 (100 – 120)

Table 6-3. Mean ratios and 90% confidence intervals for log transformed S-warfarin and R-warfarin pharmacokinetic parameters comparing each herb treatment to the warfarin only arm as control (n=12).

Treatment	Ginkgo	Ginger
fu		
S-warfarin	0.98 (0.90 – 1.02)	1.02 (0.89 – 1.15)
R-warfarin	0.99 (0.95 – 1.05)	1.03 (0.97 – 1.09)
$AUC_{0-\infty}$		
S-warfarin	0.97 (0.89 – 1.03)	0.95 (0.89 – 1.03)
R-warfarin	1.10 (0.92 – 1.07)	0.98 (0.91 – 1.06)
t_{max}		
S-warfarin	0.68 (0.63 – 0.73)	0.79 (0.73 – 0.85)
R-warfarin	0.72 (0.67 – 0.77)	0.79 (0.73 – 0.85)
C_{max}		
S-warfarin	1.04 (0.97 – 1.09)	1.01 (0.94 – 1.07)
R-warfarin	1.03 (0.97 – 1.10)	1.02 (0.95 – 1.07)
$t_{1/2}$		
S-warfarin	0.98 (0.93 – 1.04)	0.99 (0.94 – 1.04)
R-warfarin	0.97 (0.92 – 1.02)	0.94 (0.90 – 1.01)
CL/F		
S-warfarin	1.05 (0.98 – 1.12)	1.05 (0.97 – 1.13)
R-warfarin	1.00 (0.93 – 1.08)	1.02 (0.95 – 1.10)
V/F		
S-warfarin	1.03 (0.99 – 1.07)	1.03 (0.99 – 1.08)
R-warfarin	0.95 (0.95 – 1.01)	0.97 (0.93 – 1.00)

Figure 6-3. S-warfarin apparent clearance (CL/F) following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginger (WF + Ginger).

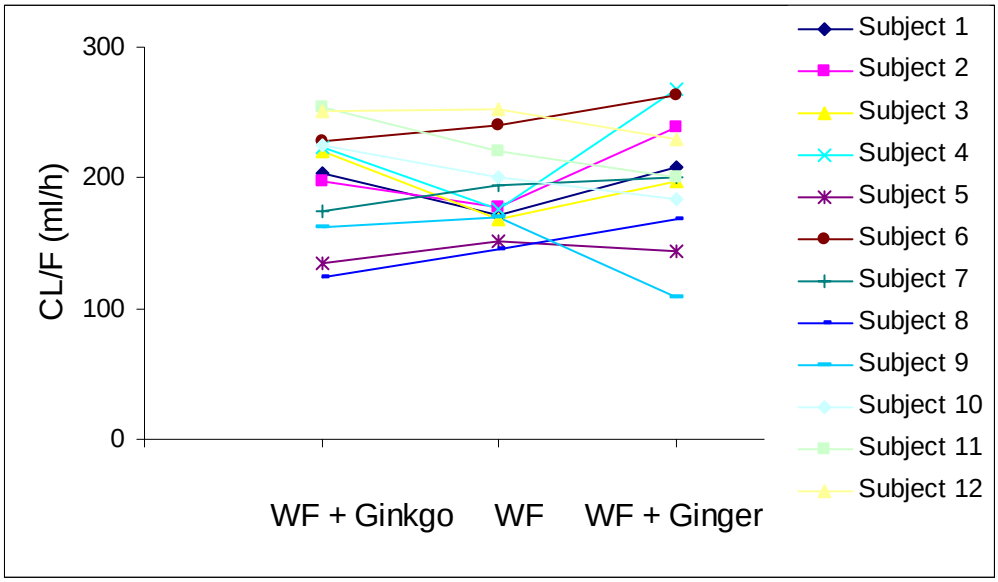


Figure 6-4. R-warfarin apparent clearance (CL/F) following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginger (WF + Ginger).

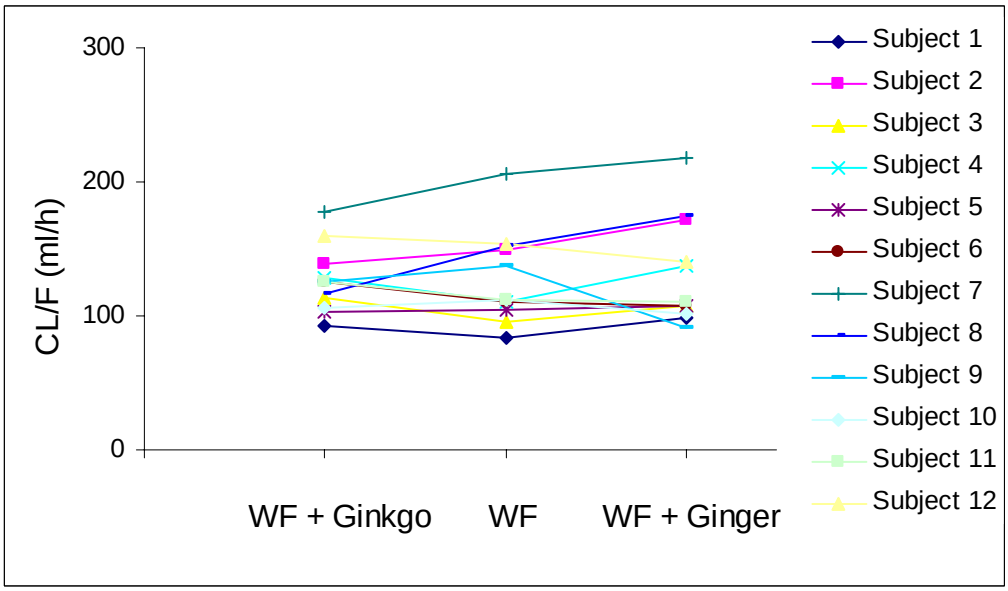


Figure 6-5. S-warfarin half-life ($t_{1/2}$) following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginger (WF + Ginger).

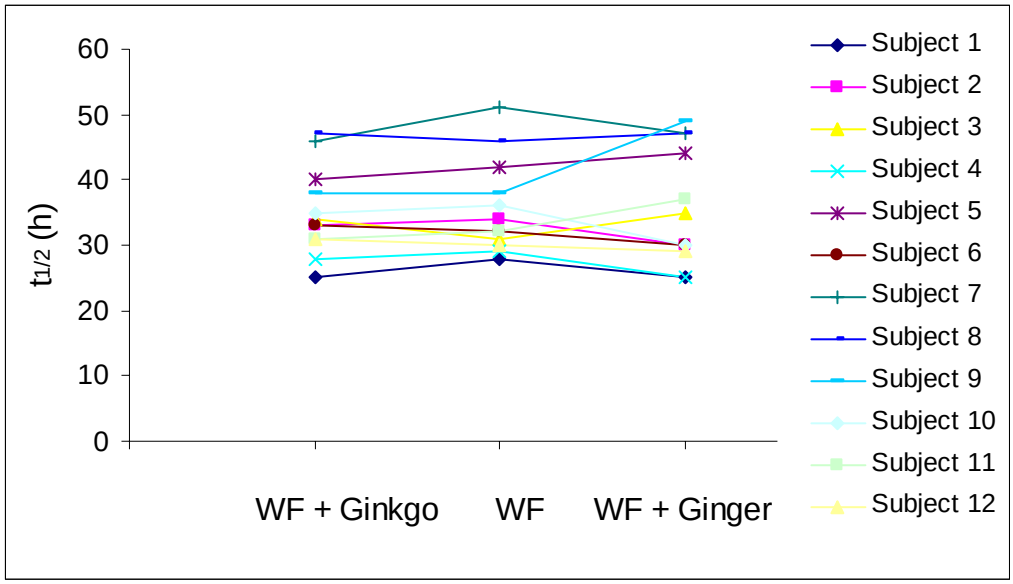


Figure 6-6. R-warfarin half-life ($t_{1/2}$) following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginger (WF + Ginger).

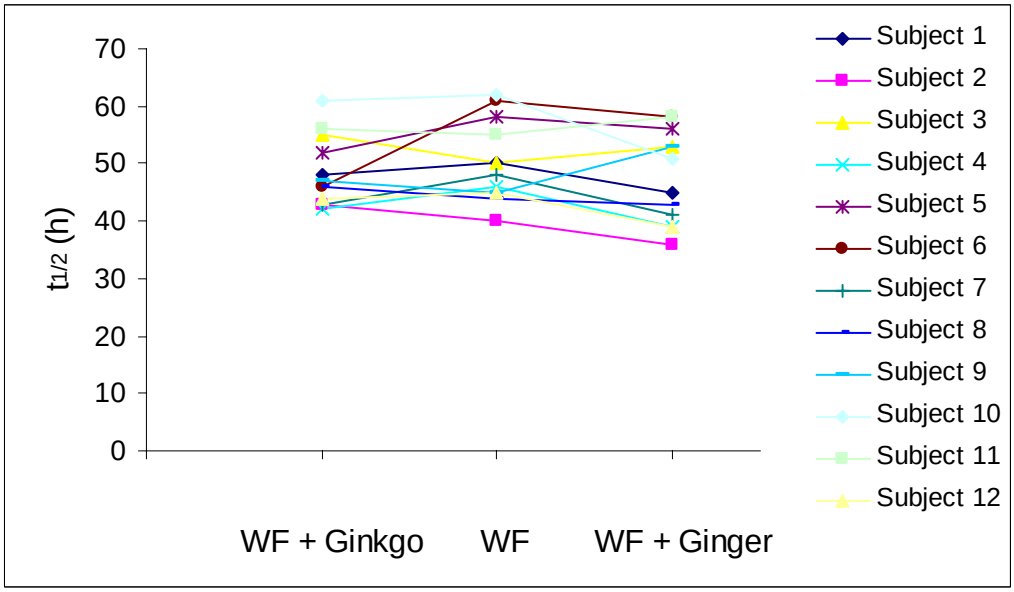


Figure 6-7. S-warfarin apparent volume of distribution (V/F) following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginger (WF + Ginger).

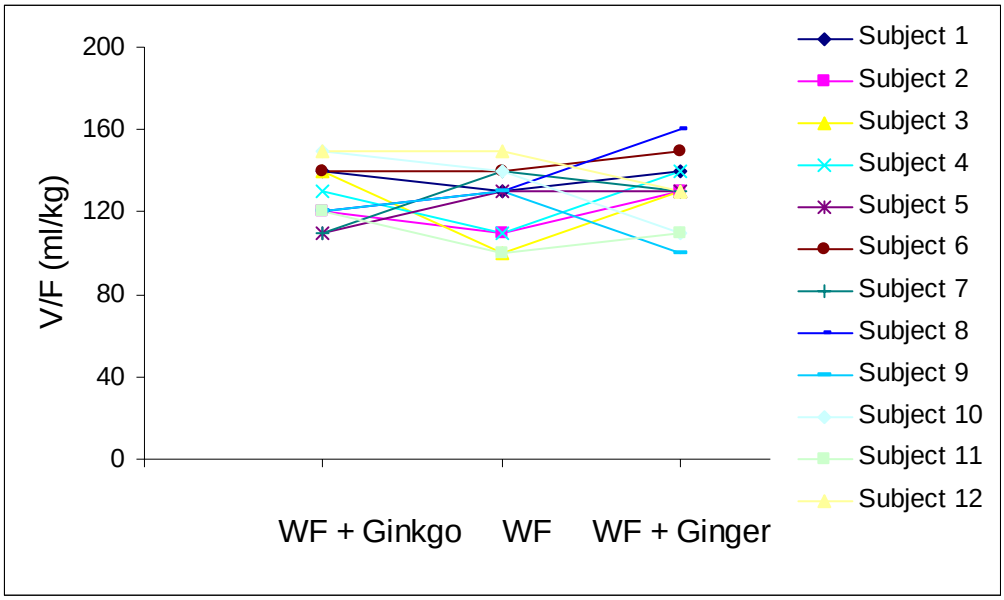


Figure 6-8. R-warfarin apparent volume of distribution (V/F) following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginger (WF + Ginger).

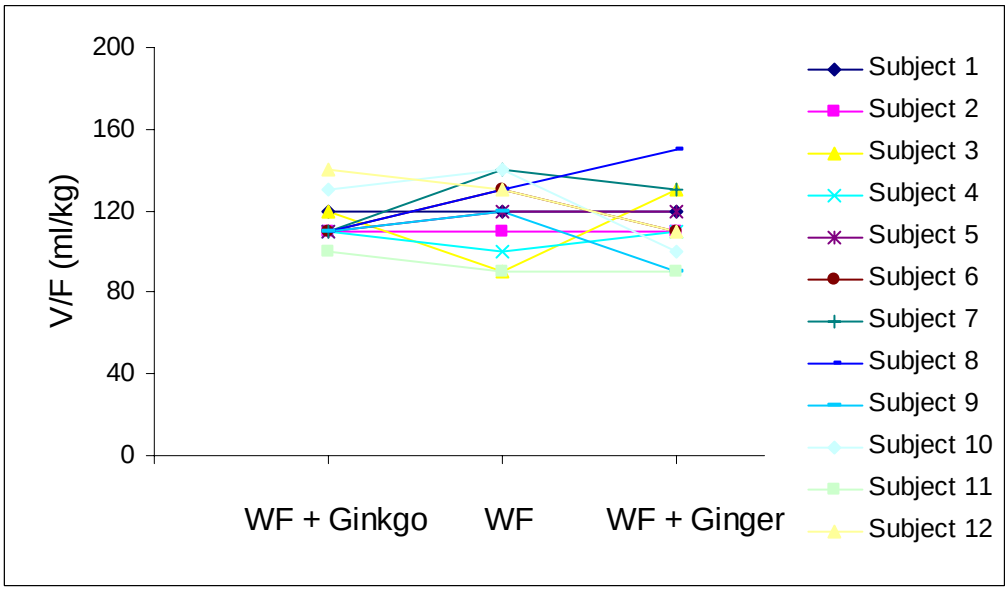


Figure 6-9. S-warfarin maximum plasma concentration (C_{max}) following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginger (WF + Ginger).

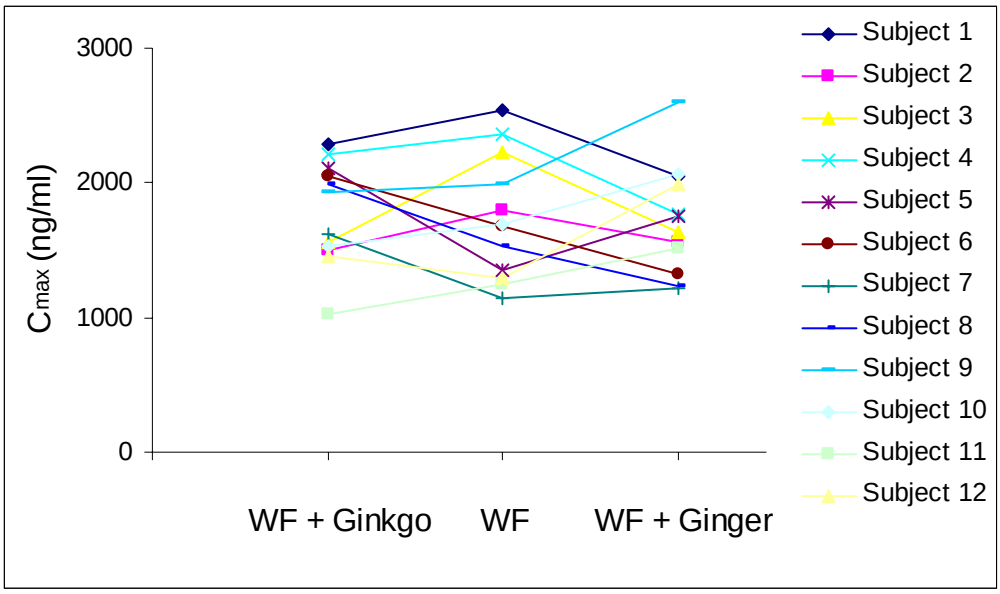


Figure 6-10. R-warfarin maximum plasma concentration (C_{max}) following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginger (WF + Ginger).

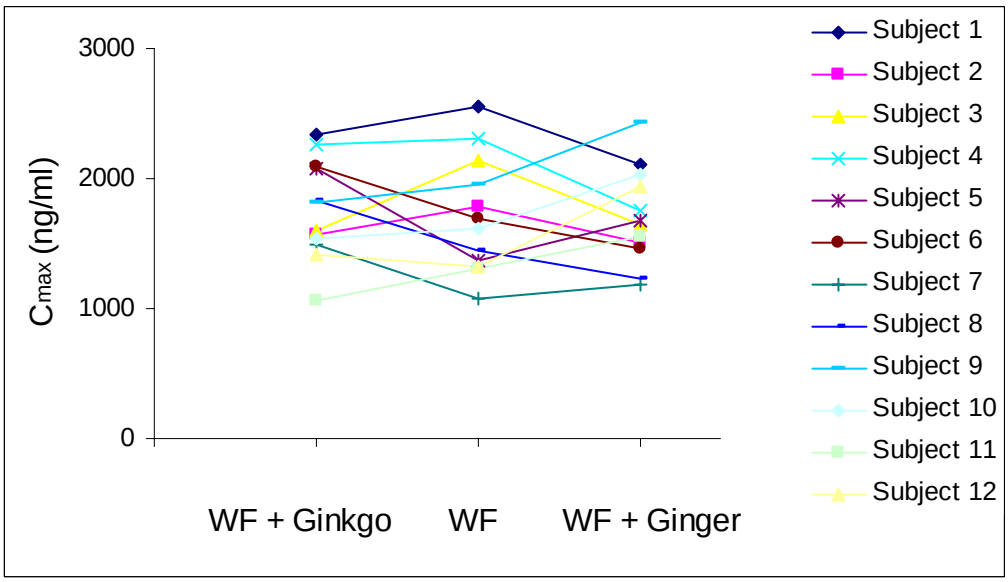


Figure 6-11. The time at which S-warfarin C_{max} occurs (t_{max}) following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginger (WF + Ginger).

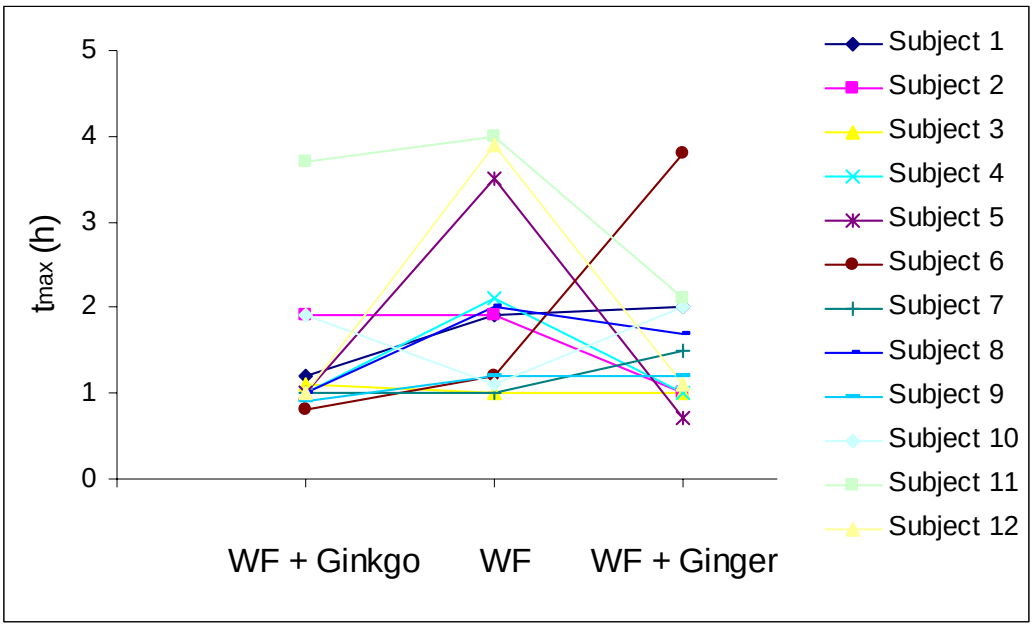
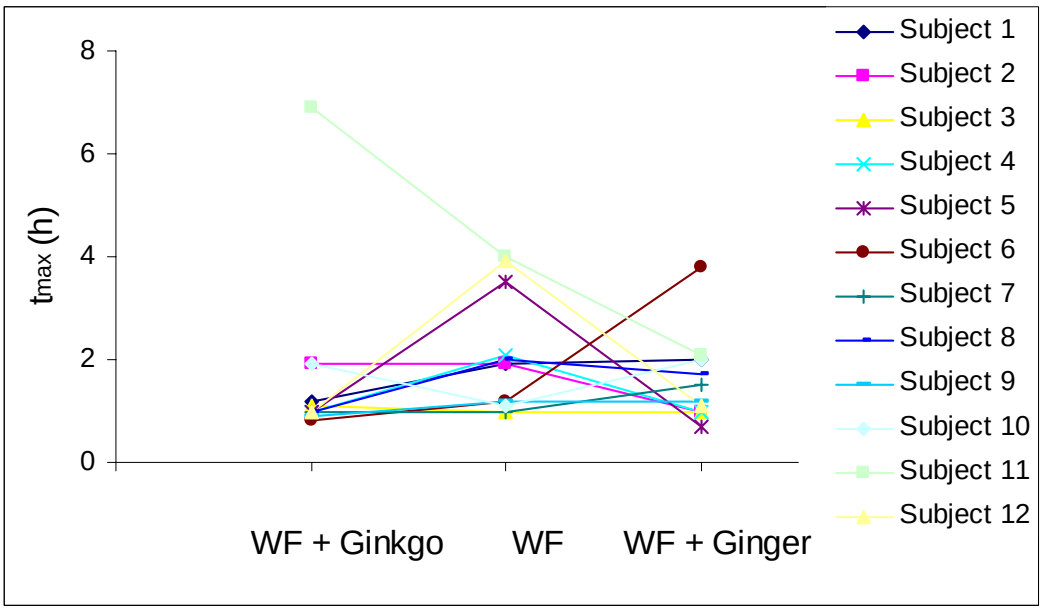


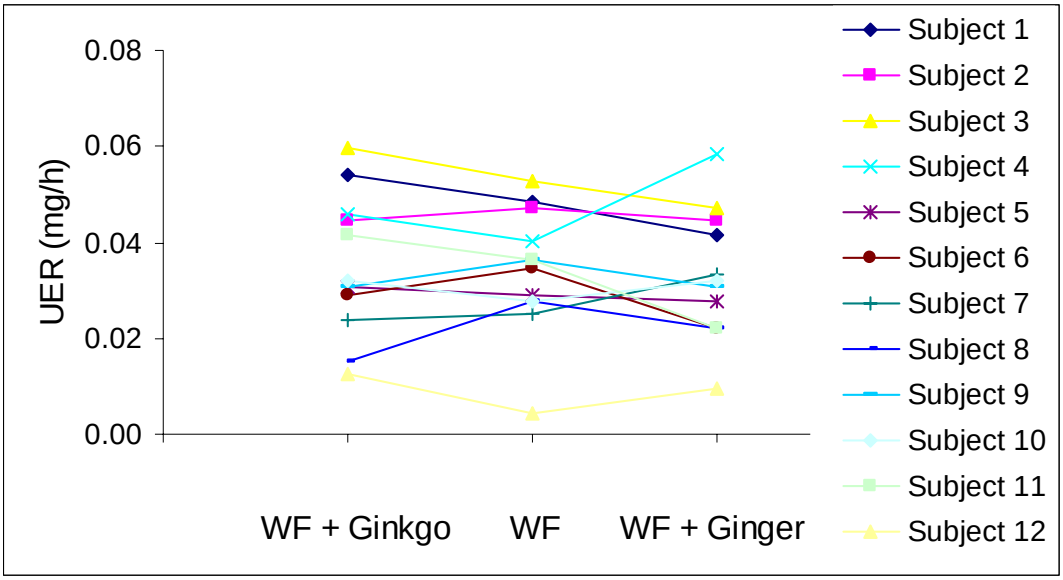
Figure 6-12. The time at which R-warfarin C_{max} occurs (t_{max}) following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginger (WF + Ginger).



6.3.4 Urinary Excretion Rate (UER) of S-7-hydroxywarfarin

UER of S-7-hydroxywarfarin after administration of warfarin alone was 0.04 ± 0.01 mg/h and there was no significant difference following treatment with either ginkgo (0.04 ± 0.01 mg/h) or ginger (0.03 ± 0.01 mg/h) (Figure 6-13). The ratio of geometric means (and 90% CI) for S-7-hydroxywarfarin UER was 1.07 (0.69-1.67) for ginkgo, and 1.00 (0.64-1.56) for ginger. These CIs of ratios were outside the predefined limit of 0.80 to 1.25 suggesting that a lack of difference cannot be concluded but appears unlikely based on the present data.

Figure 6-13. Urinary excretion rate of S-7-hydroxywarfarin following a single oral 25 mg *rac*-warfarin alone (WF), in combination with either ginkgo (WF + Ginkgo) or ginseng (WF + Ginger).



6.3.5 Plasma Protein Binding of Warfarin Enantiomers

The fraction of unbound S-warfarin and R-warfarin in plasma were 0.0052 ± 0.0013 and 0.0048 ± 0.0011, respectively, following administration of warfarin alone, and 0.0049 ± 0.0012 and 0.0047 ± 0.0008 for warfarin following administration with ginkgo. Similarly, following treatment with ginger, the fraction of unbound S-warfarin and R-warfarin in plasma were 0.0052 ± 0.0010 and 0.0048 ± 0.0008, respectively (Figure 6-14 and Figure 6-15). These differences were not significant. Furthermore, protein binding did not change over the time course of the study.

Figure 6-14. Fraction of unbound S-warfarin in plasma following administration of a single oral 25 mg dose of *rac*-warfarin alone (WF), in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger).

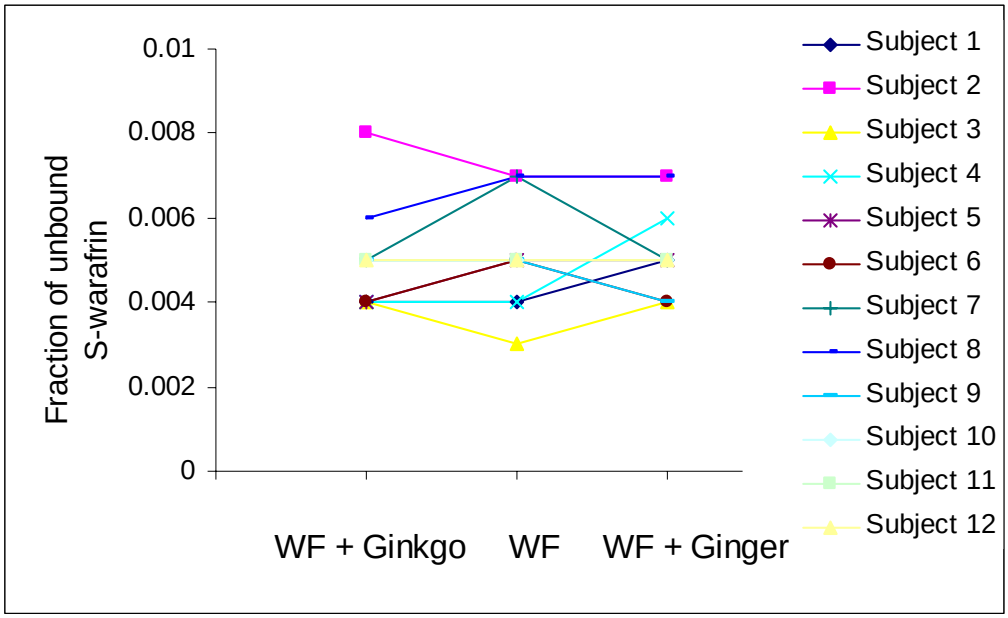
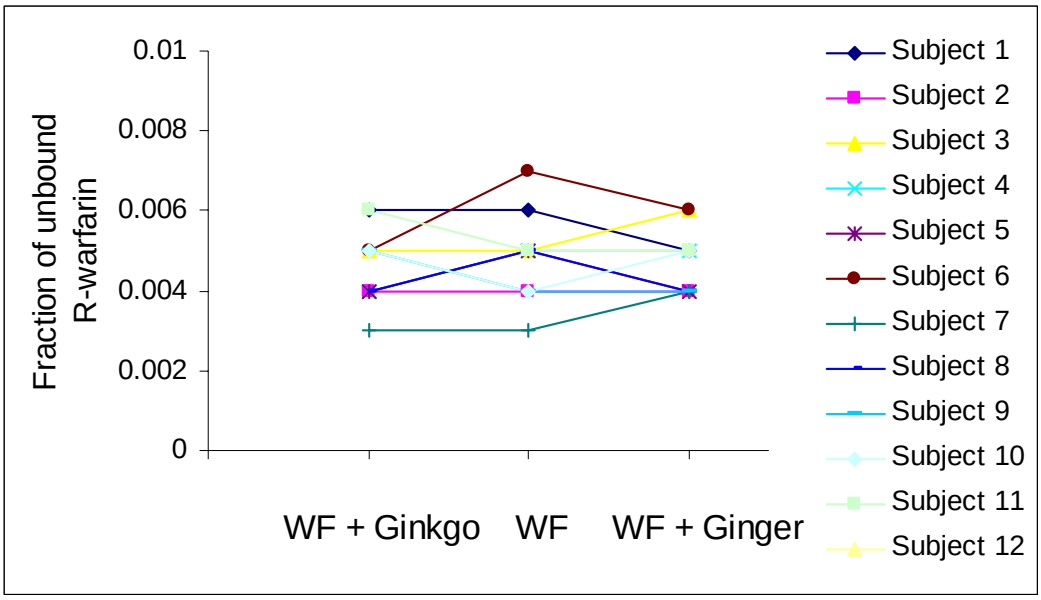


Figure 6-15. Fraction of unbound R-warfarin following administration of a single oral 25 mg dose of *rac*-warfarin alone (WF), in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger).



6.3.6 Pharmacodynamics

The mean ratios of the $AUC_{0\text{-}168}$ of INR (and 90% CI) were 0.93 (0.64-1.34) following treatment with ginkgo and 1.03 (0.72-1.49) for ginger (Table 6-4 and Table 6-5). While these confidence intervals included 1.0 suggesting no significant difference, the interval boundaries were not included in the 0.80 to 1.25 predefined limits. While a change in warfarin pharmacodynamics after ginkgo or ginger pre-treatment is unlikely, this cannot be concluded based on the present data. However, neither ginkgo nor ginger alone affected the baseline INR or platelet aggregation in response to arachidonic acid (Table 6-4, Table 6-5; Figure 6-16 and Figure 6-17).

Figure 6-16. INR-time profiles following a single oral 25 mg *rac*-warfarin dose with warfarin only, warfarin + ginkgo and warfarin + ginger (mean ± SD, n=12, warfarin dose administered on time 0).

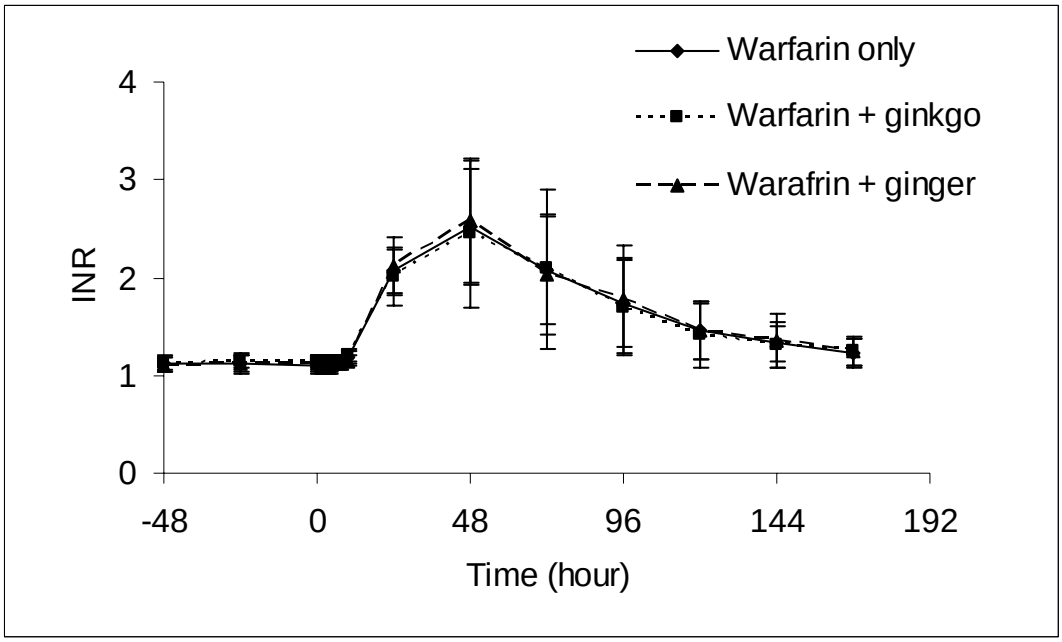


Figure 6-17. $AUC_{0\text{-}168}$ of INR following a single oral 25 mg *rac*-warfarin dose with warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginger (WF + Ginger).

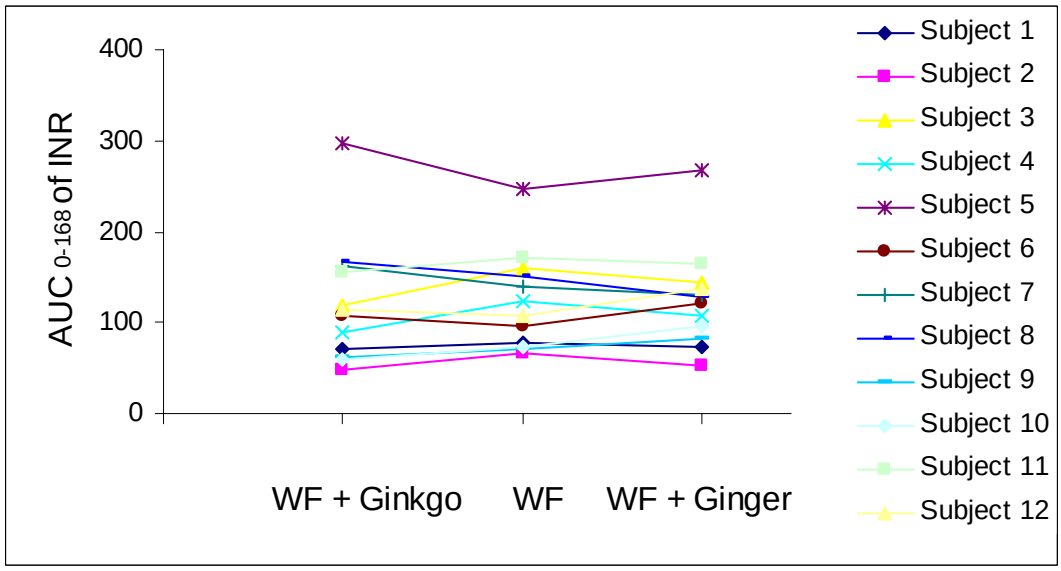


Table 6-4. Warfarin pharmacodynamic parameters following a single oral dose of 25 mg *rac*-warfarin alone (WF), in combination with either ginkgo (WF + ginkgo) or ginger (WF + ginger). Data are presented as mean ± SD and 95% CI are shown in brackets (n=12). Individual subject data are presented in Appendices 32-36.

Treatment	Warfarin alone	WF + ginkgo	WF + ginger
$INR_{baseline}$*	1.12 ± 0.08 (1.10 – 1.17)	1.14 ± 0.07 (1.10 – 1.18)	1.12 ± 0.07 (1.10 – 1.16)
t_{max} of INR (h)	45.7 ± 8.6 (40.2 – 51.1)	45.7 ± 16.6 (35 – 56.3)	43.3 ± 10.7 (36.6 – 50.1)
INR_{max}	2.6 ± 0.6 (2.2 – 2.9)	2.5 ± 0.8 (2.0 – 3.0)	2.6 ± 0.6 (2.3 – 3.0)
$AUC_{0\text{-}168}$ of INR	124 ± 54 (90 – 158)	121 ± 69 (77 – 165)	125 ± 55 (91 – 160)
Baseline platelet aggregation (Ω)	7.5 ± 1.5 (6.5 – 8.4)	8.4 ± 1.3 (7.6 – 9.2)	8.1 ± 0.9 (7.5 – 8.7)

* $INR_{baseline}$: the INR after 7 days pre-treatment with ginkgo or ginger prior to warfarin administration

Table 6-5. Mean ratios and 90% confidence intervals of the ratio of log-transformed warfarin pharmacodynamic parameters (n=12) after treatment with different herb medicines.

Treatment	Ginkgo (90% CI)	Ginger (90% CI)
$INR_{baseline}$	1.03 (1.00 – 1.05)	1.10 (0.98 – 1.02)
t_{max} of INR	0.96 (0.84 – 1.09)	0.94 (0.82 – 1.06)
INR_{max}	0.97 (0.85 – 1.09)	1.03 (0.91 – 1.17)
$AUC_{0\text{-}168}$ of INR	0.93 (0.81 – 1.05)	1.01 (0.93 – 1.15)
Baseline platelet aggregation (Ω)	1.14 (1.08 – 1.20)	1.11 (1.04 – 1.16)

6.3.7 Pharmacokinetic and Pharmacodynamic Modelling

As demonstrated in Chapter 5 the warfarin enantiomer concentration and effect-time data were best described by a one compartment pharmacokinetic model and the sigmoid E_{max} pharmacodynamic model. The results of the pharmacokinetic modelling confirmed that there were no significant changes observed in the pharmacokinetic parameters of S- or R-warfarin following treatment with ginkgo or ginger. The findings of the PKPD modelling confirmed the suggestion [18] that the anticoagulant effect of *rac*-warfarin is predominantly contributed by S-warfarin and the pharmacokinetic and pharmacodynamic modelling does not have the ability to reliably estimate $C_{50,R}$ and γ_R. Interestingly, the pharmacodynamic aspect of the modelling approach found that there was a significant difference in the degradation (or elimination) rate constant of the prothrombin complex (k_d) following a pre-treatment with ginger (Table 6-6, 6-7; Figure 6-18 to 6-21).

Figure 6-18. Prothrombin complex activity (PCA)-time profiles following administration of a single oral dose of 25 mg *rac*-warfarin alone (WF), warfarin + ginkgo (WF + ginkgo) or warfarin + ginger (WF + ginger) (mean ± SD, n=12). Individual subject profiles are presented in Appendix 12.

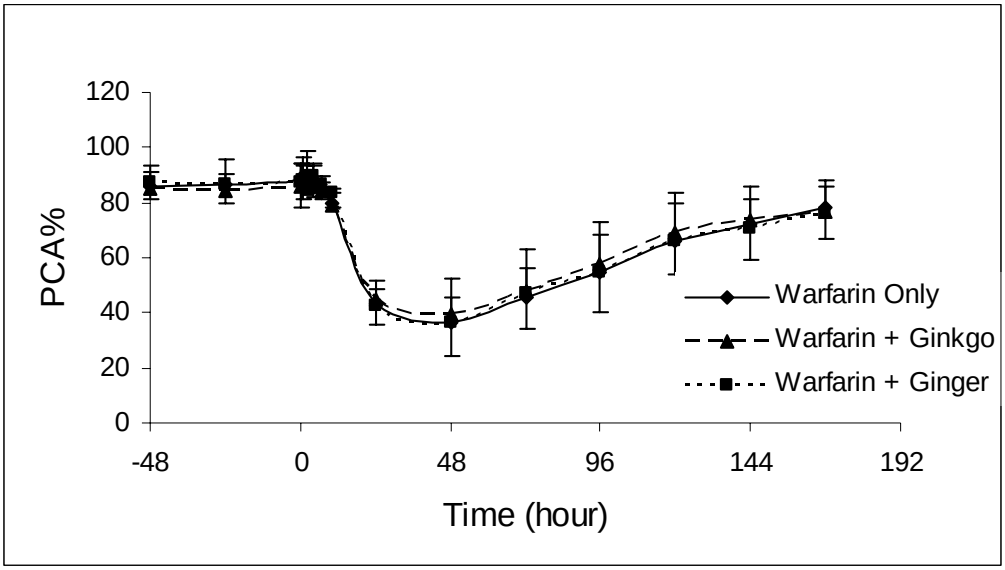


Figure 6-19. Model dependent estimates of the degradation rate constant of the prothrombin complex (k_d) following oral administration of a 25 mg *rac*-warfarin dose as warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginger (WF + Ginger).

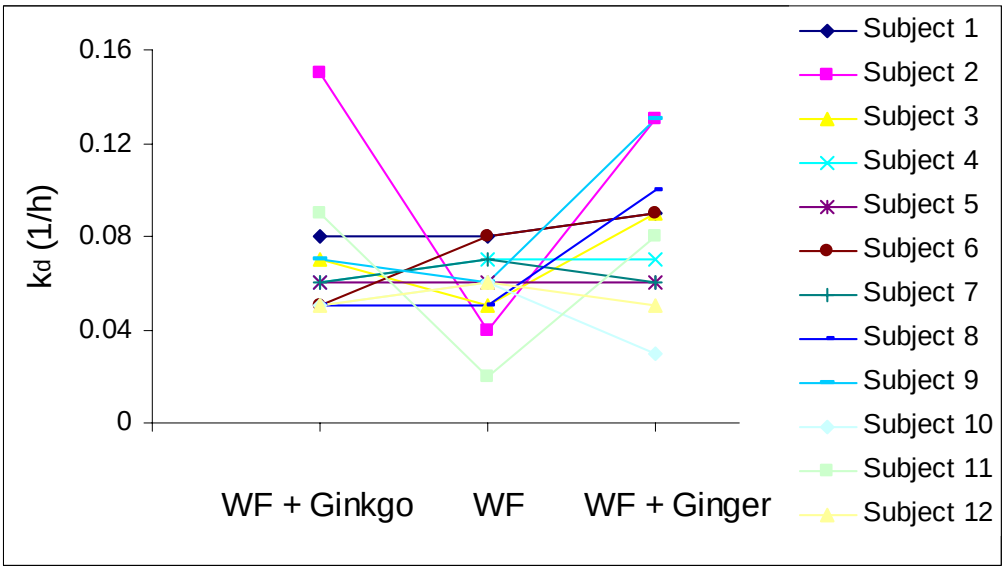


Figure 6-20. Model dependent estimates of concentration of S-warfarin required to produce a 50% inhibition of PCA ($C_{50,S}$) following oral administration of a 25 mg *rac*-warfarin dose as warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + Ginger (WF + Ginger).

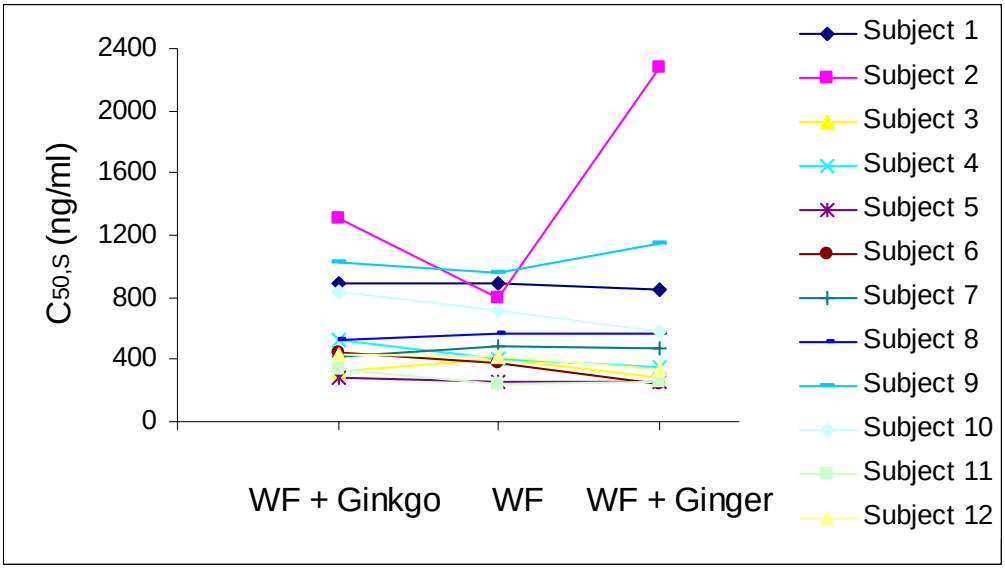


Figure 6-21. Model dependent estimates of the steepness of the concentration-response (γ) following oral administration of a 25 mg *rac*-warfarin dose as warfarin only (WF), warfarin + ginkgo (WF + Ginkgo) and warfarin + ginseng (WF + Ginger).

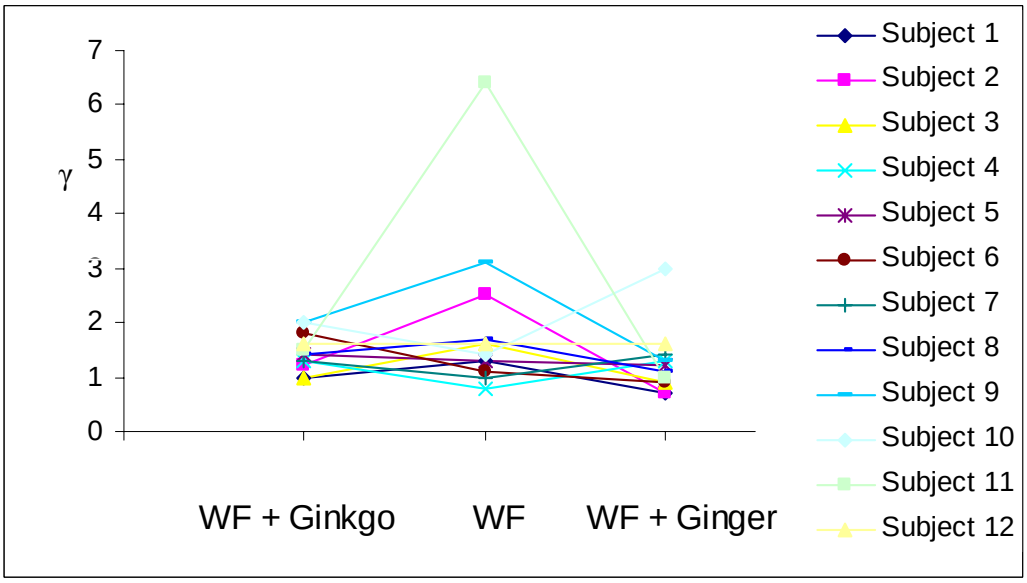


Table 6-6. Model dependent warfarin pharmacokinetic and pharmacodynamic parameters following a single oral dose of 25 mg *rac*-warfarin alone (WF) or in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger) (mean ± SD, 95% CI, n=12).

Treatment	WF alone	WF + Ginkgo	WF + Ginger
k_d (1/h)	0.06 ± 0.02 (0.05 – 0.07)	0.07 ± 0.03 (0.05 – 0.09)	0.08 ± 0.03 (0.06 – 0.10)
$C_{50,S}$ (ng/ml)	543 ± 241 (389 – 696)	611 ± 323 (406 - 817)	633 ± 586 (261 – 1005)
γ_S	2.0 ± 1.6 (1.0 – 3.0)	1.5 ± 0.3 (1.2 – 1.7)	1.8 ± 2.3 (0.3 – 3.2)
$t_{1/2}$ (h) S-warfarin R-warfarin	 35.1 ± 6.5 (31.0 – 39.3) 50.3 ± 11.2 (43.2 – 57.3)	 33.7 ± 6.4 (30.0 – 37.8) 48.1 ± 8.4 (42.8 – 53.5)	 35.6 ± 9.8 (29.4 – 41.8) 48.2 ± 8.7 (42.6 – 53.7)
CL/F (ml/h) S-warfarin R-warfarin	 194 ± 40 (168 – 219) 130 ± 33 (109 – 151)	 195 ± 42 (168 – 222) 124 ± 22 (110 – 139)	 199 ± 48 (169 – 229) 132 ± 41 (106 – 158)
V/F (ml/kg) S-warfarin R-warfarin	 130 ± 20 (120 – 140) 120 ± 20 (110 – 130)	 120 ± 10 (110 – 130) 110 ± 10 (100 – 120)	 130 ± 20 (110 – 140) 120 ± 20 (100 – 130)

Table 6-7. Mean ratios and 90% confidence intervals for selected log transformed pharmacokinetic and pharmacodynamic parameters between herb treatments compared to warfarin only (n=12).

Treatment	Ginkgo (90% CI)	Ginger (90% CI)
k_d	1.19 (1.06 – 1.33)	1.36 (1.21 – 1.52)*
$C_{50,S}$	1.10 (1.03 – 1.18)	0.98 (0.92 – 1.05)
γ_S	0.86 (0.75 – 0.97)	0.70 (0.61 – 0.79)*
$t_{1/2}$ S-warfarin R-warfarin	 0.96 (0.93 – 0.98) 0.97 (0.93 – 1.00)	 0.99 (0.97 – 1.02) 0.96 (0.93 – 1.00)
CL/F S-warfarin R-warfarin	 1.00 (0.97 – 1.03) 0.97 (0.94 – 1.01)	 1.02 (0.99 – 1.05) 1.01 (0.97 – 1.05)
V/F S-warfarin R-warfarin	 0.96 (0.93 – 0.99) 0.95 (0.93 – 0.97)	 1.00 (0.97 – 1.03) 0.97 (0.95 – 1.00)

ANOVA *$p<0.05$

6.3.8 Adverse Events

In the present study, twelve subjects completed the study. No significant adverse events or bleeding episodes were observed. One subject reported constipation during the first two days of ginkgo pre-treatment and mild diarrhoea during the first two days of ginger pre-treatment.

6.4 Discussion

This study investigated the effect of two commonly ingested herbal medicines on the pharmacokinetics and pharmacodynamics of warfarin enantiomers as well as the independent effects of these herbs on INR and platelet aggregation using a standard study design widely used in investigating warfarin–drug interactions. The major finding was that co-administration of recommended doses of ginkgo or ginger did not significantly affect pharmacokinetic and pharmacodynamic parameters of warfarin enantiomers after a single dose of the drug to healthy male subjects.

Both sequence and period effects were found to be significant in the ANOVA analysis of the log transformed parameters including $AUC_{0-\infty}$ of S- and R-warfarin and AUC_{0-168} of INR. The reason for the sequence and period effects was unclear. The results are considered acceptable according to sequence effect described in the *In Vivo* Bioequivalence Guidances (United States Pharmacopoeia) USP23 [287] because this was a single-dose study which included only healthy male subjects. An adequate washout period was used with no detectable warfarin concentrations prior to warfarin dose administration in each period and INR returned to baseline in each case.

Twelve healthy subjects were recruited in the present study. This sample size was determined by power calculation based on twelve subjects in a crossover study would provide an 80% chance of detecting a 20% difference in the $AUC_{0-\infty}$ of S-warfarin while forty-three subjects were needed to find the difference in the AUC_{0-168} of INR. Hence using twelve healthy subjects could not conclusively exclude a

pharmacodynamic interaction. However, such an interaction is unlikely because there was no significant change in the pharmacokinetics of S-warfarin and R-warfarin and there was no statistically difference in baseline INR following pre-treatment with ginkgo or ginger.

The *Ginkgo biloba* leaf extracts generally have a low incidence of side effects. It is reported in a review article by McKenna *et al* [173] that numerous clinical trials have demonstrated that EGb 761 is considered safe without causing any serious adverse effects. The reported side effects include gastrointestinal complaints, headache, sleep disturbances, dizziness, and allergic skin reactions. And there also are several case reports involving patients who experience haemorrhage. Ginger has been recognized by the US Food and Drug Administration (FDA) and is listed as a food additive that is "Generally Recognized as Safe" [200].

No batch to batch or brand to brand reproducibility studies of ginger preparations have been found in the literature. Kressmann *et al* [271, 272] published two separate articles to identify the quality issues of the ginkgo products and their influence on the bioavailability of ginkgo constituents (See Section 4.4). Herbal medicine products containing extracts of ginkgo or ginger were chosen for this study following a qualitative assessment of a range of commercially available single-ingredient herbal medicine products (See Chapter 4). Furthermore, the selected study formulations conformed to the dosage recommended in the Herbal Medicine-Expanded Commission E Monographs. There was good agreement in the composition of different commercial herbal medicine products containing ginkgo as a single herb ingredient. In contrast, notable variability was found in the composition of the

different brands of ginger. As stated in Chapter 5, this observation further reinforces the need to establish the quality of herbal medicines products used in clinical studies to evaluate their clinical efficacy or potential herb-drug interactions. The herbal medicine product chosen for the present study (Tavonin™) contained *Ginkgo biloba* refined extract, EGb 761 which has been investigated in numerous clinical trials and has been the subject of a systematic review [285]. This level of evidence, which supports the clinical use of this extract, is one of the reasons it was selected for investigation in this study.

Ginkgo extracts have been reported to inhibit the drug metabolising activity of CYP450. Zou *et al* [137] evaluated the effects of ginkgolide A, B, C, ginkgo acid I, II, bilobalide and isorhemnetin, purified compounds from *Ginkgo biloba,* on the catalytic activity of cDNA-expressed cytochrome P450 isoforms using *in vitro* experiments. Increasing concentrations of the compounds were incubated with a panel of recombinantly expressed human CYP isoforms and examined their effects on the conversion of specific substrates measured fluorometrically in a 96-well plate format. Ginkgolic acids I and II were found to be potent inhibitors of CYP1A2 (IC_{50} (μM): 4.81, 4.88), 2C9 (IC_{50} (μM): 2.41, 1.94), 2C19 (IC_{50} (μM): 4.22, 4.41), 2D6 (IC_{50} (μM): 10.42, 7.82) and 3A4 (IC_{50} (μM): 6.74, 6.25), respectively, while ginkgolide A, B, C, bilobalide and isorhemnetin did not inhibit these enzymes. Furthermore, Ohnishi *et al* [194] investigated the effects of *Ginkgo biloba* leaf extract, on the pharmacokinetics of diltiazem, a substrate for CYP 3A using both *in vitro* and *in vivo* studies in rats. A standardized method was used to prepare the *Ginkgo biloba* extract. The final quality of this extract was assured containing over 24% flavonoid glycosides and 6% terpene lactones and less than 1 ppm ginkgolic

acids, and the yield was about 2%. The simultaneous addition of this *Ginkgo biloba* extract to small intestine and liver microsomes inhibited the formation of N-demethyldiltiazem, an active metabolite of diltiazem generated by CYP3A, with an IC_{50} of about 50 and 182 μg/ml, respectively. Both the formation rate of N-demethyldiltiazem and the total amount of CYP in intestinal or hepatic microsomes decreased transiently and the area under the concentration-time curve and absolute bioavailability after oral administration of diltiazem (30 mg/kg) were significantly increased after a single oral pretreatment with *Ginkgo biloba* (20 mg/kg). The pretreatment with *Ginkgo biloba* extract (20 mg/kg) significantly decreased the elimination rate constant and increased the mean residence time after intravenous administration of diltiazem (3 mg/kg). Umegaki *et al* [188] reported that the addition of ginkgo extract resulted in a concentration dependent inhibition of various CYP enzymes, especially CYP2B based on *in vitro* studies using rats and human hepatic microsomes. Paradoxically, ginkgo extract has also been reported in the literature to induce cytochrome P450. Shinozuka *et al* [196] reported, based on studies in rats, that the levels of CYP2B1/2, CYP3A1 and CYP3A2 mRNA in the liver were significantly induced while CYP1A1, CYP1A2, CYP2E1, CYP2C11 and CYP4A1 remained unchanged and the hypotensive effect of nicardipine, metabolized by CYP3A2, was significantly reduced in rats after pre-treatment with 0.5% of ginkgo extract for four weeks. Umegaki *et al* [188] also reported that the concentration and activities of CYP enzymes, especially the CYP2B enzyme, were significantly increased on day 1 of feeding of a 0.5% ginkgo extract diet and after administration of ginkgo extract of 10 mg/kg body weight for 5 days by intragastric gavage in rats. However, these effects of ginkgo have not been observed in clinical trials in humans. Gurley *et al* [91] reported that a single-time point phenotypic metabolic ratios were

used to determine whether long-term administration of *Ginkgo biloba* and other herbal medicines affected CYP1A2, CYP2D6, CYP2E1 or CYP3A4 activity. Twelve healthy volunteers (6 males, 6 females) were randomly assigned to receive either *Ginkgo biloba* (60 mg, 4 times daily) or other herbal medicines for 28 days. For each subject, a 30-day washout period was used between each herbal medicine phase. Probe-drug cocktails of midazolam (CYP3A4), caffeine (CYP1A2), chlorzoxazone (CYP2E1) and debrisoquin (CYP2D6) were administered before herbal medicine treatment and at the end of herbal medicine treatment. Phenotypic trait measurements were determined for CYP3A4, CYP1A2, CYP2E1, and CYP2D6 by using 1-hydroxymidazolam/midazolam serum ratios (1-hour sample), paraxanthine/caffeine serum ratios (6-hour sample), 6-hydroxychlorzoxazone/chlorzoxazone serum ratios (2-hour sample), and debrisoquin urinary recovery ratios (8-hour collection), respectively. No significant effect on CYP activity was observed for *Ginkgo biloba*. The effect of ginkgo extract on the *in vivo* metabolic activity of CYP2C9 was not investigated by these researchers.

A recent clinical trial investigated the effect of ginkgo co-administration on warfarin pharmacodynamics using a randomized, double-blind placebo-controlled cross-over trial in patients using 100 mg of ginkgo extract daily over treatment periods of four weeks [197]. Twenty-four outpatients (fourteen women and ten men) were included who were receiving stable, long-term warfarin treatment. A two-week wash out period was employed. The INR was kept between 2.0 and 4.0 by appropriate adjustment of the warfarin dosage. The INR was stable during all treatment periods. The geometric mean dosage of warfarin did not change during the treatment periods

[197]. However, the effect of ginkgo on the pharmacokinetics of warfarin was not investigated in the study by Engelsen *et al* [197].

In the study reported in this thesis, recommended doses of a herbal medicine product containing EGb 761 (ginkgolic acids < 5 ppm) did not affect the clearance of warfarin enantiomers which suggests that this herb does not significantly influence CYP1A2, CYP3A4 or CYP2C9 metabolic activity at this dose. The conflicting observations about the possible effects of ginkgo constituents on cytochrome P450 in the literature could be caused by variability in constituents of ginkgo and different concentrations of ginkgo constituents used in the *in vivo* and *in vitro* studies reported in the literature. Therefore, the use of standardised methods to prepare herbal medicines and standardised methods to perform quality control is quite important to guarantee equivalent efficacy in different proprietary preparations.

Conflicting results have also been observed in the effect of ginkgo on clotting status in the literature. Several *in vitro* studies have demonstrated that ginkgo extract or ginkgolides A, B and C inhibit PAF but not ADP or arachidonic acid induced platelet aggregation [182-185]. Furthermore, in clinical trials, twenty-four patients suffering from arteriosclerotic disorders were divided into 2 groups. Twelve patients received sodium chloride (250 ml) with *Ginkgo biloba* extract (25 ml) while the other twelve patients were treated with sodium chloride (250 ml) only. The collagen induced platelet aggregation was determined before, immediately after infusion and on the following day. The platelet aggregation increased in both treatment groups after infusion. Platelet aggregation in the group treated with *Ginkgo biloba* extract after 1 day returned to normal while the aggregation remained increased in the sodium

chloride group [186]. Furthermore, ginkgo extract significantly reduced collagen but not PAF-mediated platelet aggregation in healthy volunteers (n=28) and Type 2 diabetic subjects (n=19) in which 120 mg of standardized ginkgo was ingested for 3 months [187].

However, several studies have concluded that ginkgo does not affect clotting status. In one study, up to 0.2 mg/ml of ginkgo extract did not inhibit ADP and collagen-induced platelet aggregation *in vitro* in rats [288]. In another prospective, double blind, randomised, placebo-controlled study in 32 young male healthy volunteers; ginkgo extract (EGb761) did not alter any haematological marker using three doses of ginkgo extracts 120, 240 and 480 mg/day for 14 days [189]. In this study, primary haemostasis was assessed by both the bleeding time measured by the 3-point Ivy-Neison technique and a direct quantitative measurement of blood loss according to the Bernal-Hoyos methods. The interaction between platelets and coagulation was assessed using the thrombin generation test in platelet rich plasma. Several methods were used to assess platelet function including (i) by platelet aggregation in citrated platelet rich plasma, induced by three different agonists: adenosine diphosphate (2.5 μM), thrombin receptor agonist peptide (25 μM) and collagen (1.25 μg/ml); (ii) by quantification of platelet membrane glycoproteins and (iii) by measurement of procoagulant activity assessed by annexin fixation on the platelet membrane [189]. In the present study, ginkgo did not significantly affect platelet aggregation induced by arachidonic acid and coagulation which is in agreement with these findings.

Few ginger-drug interactions have been reported in the literature. Banerjee *et al* [211] investigated the influence of certain essential oils including ginger oil, on

carcinogen-metabolizing enzymes and acid-soluble sulfhydryls in mouse liver and found ginger oil (10 μl/day for 14 days by gavage) did not significantly affect cytochrome P450 levels. This is consistent with the observations of a lack an effect of ginger on warfarin metabolism reported in this thesis.

Investigation of the effect of ginger on platelet aggregation or coagulation has been conducted by several researchers. A series of synthetic gingerols and related phenylalkanol analogues were found to inhibit arachidonic acid induced platelet serotonin release and aggregation based on an *in vitro* study of human platelets [201]. Furthermore, it was found in *in vitro* studies that a significant effect of ginger extract was to inhibit platelet aggregation. Srivastava *et al* [202-204] reported that organic ginger extract extracted in three organic solvents: n-hexane, chloroform and ethyl acetate reduced platelet thromboxane formation from exogenous arachidonic acid and also inhibited platelet aggregation induced by arachidonic acid, epinephrine, ADP and collagen in a dose-dependent manner. Suekawa *et al* [286] reported that (6)-Shogaol, a pungent component of ginger, inhibited arachidonic acid - induced platelet aggregation in rabbits platelet *in vitro*.

However, conflicting findings related to the effect of ginger constituents on platelet aggregation are found elsewhere in the literature. Lumb *et al* [208] reported the effects of 2 g dried ginger or placebo capsules on platelet function using eight healthy male volunteers in a randomised double blind study. Bleeding time, platelet count, thromboelastography and whole blood platelet aggregometry were measured before, 3 h, and 24 h after administration of ginger. There were no significant differences found between ginger and placebo [208]. In contrast, administration of 5

g of dry ginger in two divided doses with a fatty meal was reported to significantly inhibit the platelet aggregation induced by adenosine diphosphate and epinephrine in ten healthy male volunteers in whom platelet aggregation was enhanced by 100 g butter for 7 days while there was no significant alteration in platelet aggregation in the placebo control group (10 healthy male volunteers) [205]. In addition, using a single dose of 10 g powdered ginger, a significant reduction in platelet aggregation induced by ADP and epinephrine was observed in patients with coronary artery disease while no significant effect was found using a dose of 4 g daily for 3 months [206]. In animal studies, no significant effect on coagulation parameters PT and APTT or on warfarin-induced changes in blood coagulation was found in rats using multiple 100 mg/kg doses of EV.EXT™ 33, which is a ginger extract of patented standardised ethanol extract of dry rhizomes of *Zingiber officinale* Roscoe [207]. In the present study, no significant effect was found on platelet aggregation and coagulation in healthy human volunteers using a daily dose of 3.6 g of ginger for 5 days.

The conflicting results on clotting status in the literature and the present study could be attributed to a number of factors including the *in vivo* metabolic biotransformation of the ginger constituents after oral dosing of ginger, dose regimen or study population. There are no studies reported on the pharmacokinetics of ginger constituents in humans.

In this study a combined PK/PD model was employed to characterise the warfarin concentration-effect data obtained in the three phases of this clinical trial. This allows assessment of the effect of herbs on pharmacodynamic parameters.

Co-administration with ginger was found to significantly increase the degradation rate constant (k_d) of PCA and slop factor in concentration-response relationship (γ_S) while no significant difference was found in C_{50S} with and without these two herbal medicines. The clinical implications of the effect of ginger on the k_d of PCA and γ_S is unclear given that ginger did not significantly affect INR baseline and $AUC_{0\text{-}168}$ of INR. The mechanism by which ginger affects k_d or γ_S is not clear. C_{50S}, k_d and γ_S from the present study of control group are in agreement with previous reported [18].

In summary, this study found that neither ginkgo nor ginger when administered as single herbal ingredient medicines at recommended doses for a week affected the pharmacokinetics of either S-warfarin or R-warfarin in humans, nor did they affect coagulation status. These finding suggest that the coadministration of ginkgo or ginger at recommended doses is unlikely to affect warfarin response in healthy persons. The finding that ginger increased degradation rate constant deserves further investigation and the safety of these herbs in anticoagulant patients has yet to be established.

Chapter 7 General Discussion, Conclusions and Further Studies

7.1 General Discussion

These studies involved standard warfarin-drug interactions study design which is the most frequently used in the literature (See Section 2.1.1). A power calculation indicated that using 12 subjects to investigate warfarin drug interaction in a crossover study provides an 80% chance to detect a 20% difference in $AUC_{0-\infty}$ of S-warfarin. However, 43 subjects would be needed to show a similar difference in AUC_{0-168} of INR as pharmacodynamic parameter. A number of CI ranges of ratios for pharmacodynamic parameters were out side the predefined limit of 0.80 to 1.25 suggesting that a lack of significant difference cannot be concluded. While these two studies could not conclusively exclude a pharmacodynamic interaction such an interaction is unlikely because no significant change was observed in pharmacokinetics of S-warfarin while warfarin was coadministered with ginseng, ginkgo or ginger and also no statistically difference was found in baseline INR during pre-treatment with St John's wort, ginseng, ginkgo or ginger. For St John's wort, it induced the metabolism of warfarin and subsequently reduced the anticoagulant effect of warfarin.

C_{max} of S- and R-warfarin were not suitable to be used to test sequence and period effect, because the sampling time was not designed to find a difference in C_{max} of S- and R-warfarin.

The single 25 mg dose of warfarin used in the study was based on the literature review of warfarin drug interaction study design. It is the most frequently used in the literature (See Section 2.1.1). Warfarin has a narrow therapeutic range and serious side effects such as bleeding rarely happened after an in single dose of warfarin but are not uncommon during long-term use. The target therapeutic range of warfarin therapy INR (2-3) for treatment of venous thrombosis and pulmonary embolism (See Section 1.3) which is recommended by Therapeutic Guidelines: Cardiovascular (Australia) was reached after a single 25 mg dose of warfarin (Figure 5-17 and Figure 6-17). Furthermore, Figure 7-1 and Figure 7-2 show the concentration-effect relationship for warfarin derived from the PKPD modelling analysis and the PD parameter estimated. Over the S-warfarin concentration range (0 - 2 μg/ml) observed after a single 25 mg dose, the maximum inhibition of PCA is approximately 80%. This observation means that this study design was capable of detecting both increases and decreased in warfarin effect during drug interaction studies.

Figure 7-1. PCA-Concentration profiles following single oral 25 mg *rac*-warfarin dose with warfarin only, warfarin + St John's wort and warfarin + ginseng using mean data (n=12) derived from PD parameters presented in Table 5-6.

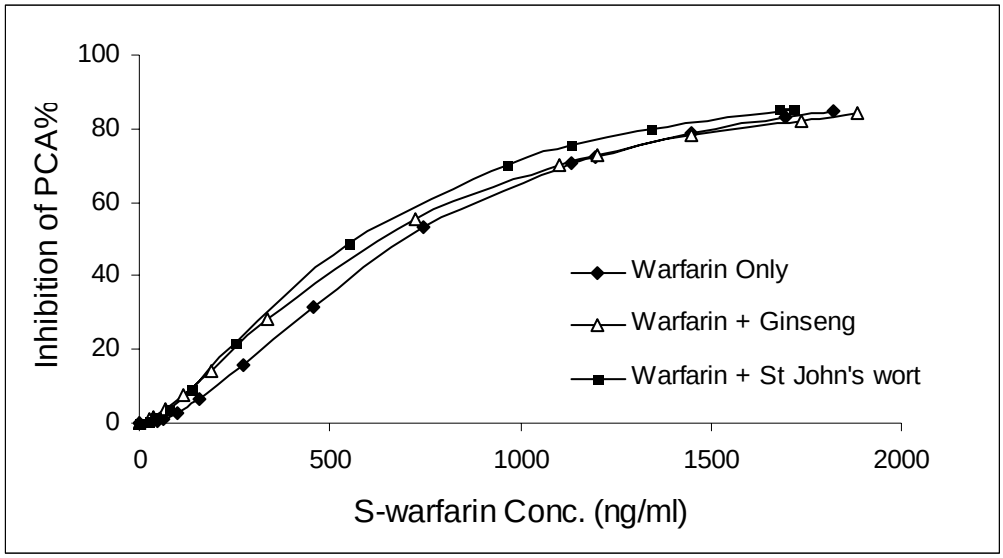
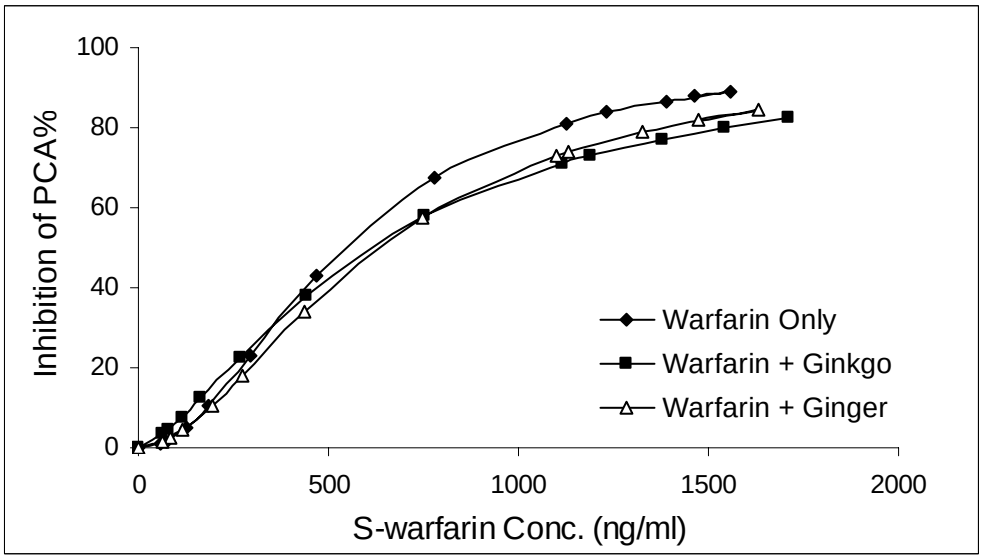


Figure 7-2. PCA-Concentration profiles following single oral 25 mg *rac*-warfarin dose with warfarin only, warfarin + ginkgo and warfarin + ginger using mean data (n=12) derived from PD parameters presented in Table 6-6.



7.2 General Conclusions

This thesis has investigated the effect of herbal medicines on the pharmacokinetics and pharmacodynamics of warfarin and their individual effect on coagulation and platelet aggregation in healthy subjects. This has been achieved using four of the most commonly used herbs St John's wort, ginseng, ginkgo and ginger. The results of this study revealed important information about the potential for herbs to influence human metabolism enzymes including CYP2C9, CYP1A2 and/or CYP3A4.

This chapter details the important findings of the work presented in this thesis. Key elements of the preceding chapters are discussed in a broader context and potential areas of further research are highlighted.

Chapter 4 describes the investigations into the quality of herbal medicine products. In this thesis commercially available herbal medicine products were selected as it would most likely reflect the type of products used by patients receiving warfarin. No significant variability was found between different commercial products of ginkgo. However, the variability between different commercial products of St John's wort, ginseng, and ginger was notable. TLC was used as a screening method to investigate the quality of herbal medicines because it is a simple, quick and productive method to perform batch tests investigating the qualitative differences between commercial products of herbal medicines and different constituents within such herbal medicines. Standardised quality control methods based on the British Pharmacopoeia 2001 and the People's Republic of China Pharmacopoeia 2000 were used in this thesis. The

use of standardised methods to prepare herbal medicines and to perform quality control is recommended. This is critical to ensure the quality of herbal medicines; to minimise the variability of constitutes in herbal medicines and to ensure efficacy of herbal medicines.

In Chapter 5, it has been demonstrated that pre-treatment with St John's wort can induce the metabolism of both S- and R- warfarin in humans by comparing pharmacokinetic and pharmacodynamic parameters with and without coadministration with St John's wort. S-warfarin is metabolised predominantly to S-7-hydroxywarfarin by CYP2C9 while R-warfarin is partly metabolised by CYP3A4 and CYP1A2. The study design and data analysis approach used in this thesis demonstrated that the drug interaction mechanism of St John's wort on warfarin is the result of induction of the CYP2C9, CYP3A4 and/or CYP1A2 rather than a reduction in the unbound fraction of warfarin leading to an increase in its clearance. Previous studies have not demonstrated an effect of St John's wort on CYP2C9. Potential drug interactions with St John's wort include other drugs that are substrates of CYP2C9, CYP3A4 and/or CYP1A2. Co-administration of St John's wort can reduce the efficacy of warfarin based on this pharmacokinetic interaction mechanism. This most certainly has implications for patients taking and the need to avoid or carefully monitor the use of St John's wort.

Studies in Chapter 5 and 6 of this thesis found that ginseng, ginkgo and ginger administered as single ingredient herbal medicines at the recommended dose had

little effect on the pharmacokinetics and pharmacodynamics of warfarin enantiomers. Furthermore, ginseng, ginkgo and ginger had little effect on substrates of CYP2C9, CYP3A4 and CYP1A2.

Administration of St John's wort, ginseng, ginkgo and ginger at recommended oral doses did not significantly affect coagulation and platelet aggregation in healthy subjects. Nevertheless, the safety of these herbs in patients receiving anticoagulants has yet to be established.

The lack of an interaction with ginseng ginger and ginkgo observed in this study does not allow one to conclude that such an interaction is not possible in patients. There is little information about the way that herbal medicines are used in our community but the suggestion is that people often take multiple combinations of herbs often at higher recommended dosed. In summary, these findings provide rigorous evidence to support the recommendation that close monitoring of INR should be undertaken in patients receiving this herb-drug combination. Further research is needed to clarify the implications of these findings for elderly patients (who are likely to receive warfarin) and in people using a range of herbal and complementary medicines often in a variety of doses.

7.3 Further Studies

The pharmacokinetic interactions between warfarin and these four herbal medicines have been clarified in this thesis. Understanding the mechanism of warfarin drug interactions provides an insight into the possible clinical significance of an interaction and can help elucidate strategies to avoid or minimise the impact in a given patient. Based on these findings in healthy male subjects, further studies should be conducted in patients receiving anticoagulants to obtain the most definitive data on the probability and magnitude of warfarin pharmacodynamic interactions with the four herbal medicines examined. Especially, the finding that ginger increased the degradation rate constant of the prothrombin complex deserves further study.

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Appendices

Appendix 1. ANOVA test of sequence, period and treatment effects on $logC_{max}$ of S-warfarin in study I investigating the effect of St John's wort and ginseng on warfarin.

Number of obs =	36	R-squared	= 0.2388		
Root MSE	= .066307	Adj R-squared	= 0.0813		
Source |	Partial SS	df	MS	F	Prob > F
Model |	0.040000003	6	0.006666667	1.52	0.2080
Treatment |	0.004999994	2	0.002499997	0.57	0.5725
Period |	3.1580e-15	2	1.5790e-15	0.00	1.0000
Sequence |	0.035000009	2	0.017500004	3.98	0.0297
Residual |	0.127500023	29	0.004396553		
Total |	0.167500026	35	0.004785715		

Appendix 2. ANOVA test of sequence, period and treatment effects on $logC_{max}$ of R-warfarin in study I investigating the effect of St John's wort and ginseng on warfarin.

Number of obs =	36	R-squared	= 0.2044		
Root MSE	= .071719	Adj R-squared	= 0.0398		
Source |	Partial SS	df	MS	F	Prob > F
Model |	0.03833334	6	0.00638889	1.24	0.3143
Treatment |	0.011666676	2	0.005833338	1.13	0.3356
Period |	1.2632e-14	2	6.3159e-15	0.00	1.0000
Sequence |	0.026666663	2	0.013333332	2.59	0.0921
Residual |	0.149166684	29	0.005143679		
Total |	0.187500024	35	0.005357144		

Appendix 3. ANOVA test of sequence, period and treatment effects on $logAUC_{0-\infty}$ of S-warfarin in study I investigating the effect of St John's wort and ginseng on warfarin.

Number of obs =	36	R-squared	= 0.4140		
Root MSE	= .073826	Adj R-squared	= 0.2928		
Source |	Partial SS	df	MS	F	Prob > F
Model |	0.111666994	6	0.018611166	3.41	0.0113
Treatment |	0.107222537	2	0.053611268	9.84*	0.0005
Period |	0.002222229	2	0.001111114	0.20	0.8167
Sequence |	0.002222229	2	0.001111114	0.20	0.8167
Residual |	0.158055956	29	0.005450205		
Total |	0.26972295	35	0.00770637		

*p<0.05

Appendix 4. ANOVA test of sequence, period and treatment effects on $logAUC_{0-\infty}$ of R-warfarin in study I investigating the effect of St John's wort and ginseng on warfarin.

Number of obs =	36	R-squared	= 0.2214		
Root MSE	= .100525	Adj R-squared	= 0.0603		
Source |	Partial SS	df	MS	F	Prob > F
Model |	0.083333206	6	0.013888868	1.37	0.2580
Treatment |	0.068888795	2	0.034444397	3.41	0.0468
Period |	0.010555517	2	0.005277758	0.52	0.5986
Sequence |	0.003888895	2	0.001944447	0.19	0.8260
Residual |	0.293055232	29	0.010105353		
Total |	0.376388439	35	0.010753955		

Appendix 5. ANOVA test of sequence, period and treatment effects on $logAUC_{0\text{-}168}$ of INR in study I investigating the effect of St John's wort and ginseng on warfarin.

Number of obs = 36 R-squared = 0.1574					
Root MSE = .184816 Adj R-squared = -0.0170					
Source	Partial SS	df	MS	F	Prob > F
Model	0.184999999	6	0.030833333	0.90	0.5065
Treatment	0.142222205	2	0.071111103	2.08	0.1429
Period	0.027222208	2	0.013611104	0.40	0.6750
Sequence	0.015555585	2	0.007777793	0.23	0.7978
Residual	0.990555452	29	0.034157085		
Total	1.17555545	35	0.033587299	.	

Appendix 6. ANOVA test of sequence, period and treatment effects on $logAUC_{0\text{-}\infty}$ of S-warfarin in Study II investigating the effect of ginkgo and ginger on warfarin.

Number of obs = 36 R-squared = 0.2745					
Root MSE = .097035 Adj R-squared = 0.1244					
Source	Partial SS	df	MS	F	Prob > F
Model	0.103333414	6	0.017222236	1.83	0.1281
Treatment	0.008888925	2	0.004444462	0.47	0.6284
Period	0.077222263	2	0.038611132	4.10	0.0270
Sequence	0.017222226	2	0.008611113	0.91	0.4119
Residual	0.27305601	29	0.009415724		
Total	0.376389424	35	0.010753984		

Appendix 7. ANOVA test of sequence, period and treatment effects on $logAUC_{0-\infty}$ of R-warfarin in study II investigating the effect of ginkgo and ginger on warfarin.

Number of obs = 36 R-squared = 0.1088					
Root MSE = .106269 Adj R-squared = -0.0755					
Source	Partial SS	df	MS	F	Prob > F
Model	0.039999924	6	0.006666654	0.59	0.7353
Treatment	0.001666663	2	0.000833332	0.07	0.9290
Period	0.011666644	2	0.005833322	0.52	0.6020
Sequence	0.026666616	2	0.013333308	1.18	0.3214
Residual	0.327499375	29	0.011293082		
Total	0.367499299	35	0.01049998		

Appendix 8. ANOVA test of sequence, period and treatment effects on $logC_{max,}$ of S-warfarin in study II investigating the effect of ginkgo and ginger on warfarin.

Number of obs = 36 R-squared = 0.5205					
Root MSE = .076314 Adj R-squared = 0.4213					
Source	Partial SS	df	MS	F	Prob > F
Model	0.183333441	6	0.030555573	5.25	0.0009
Treatment	0.003888895	2	0.001944447	0.33	0.7189
Period	0.117222246	2	0.058611123	10.06*	0.0005
Sequence	0.0622223	2	0.03111115	5.34*	0.0106
Residual	0.168888973	29	0.005823758		
Total	0.352222414	35	0.010063498		

*$p<0.05$

Appendix 9. ANOVA test of sequence, period and treatment effects on $logC_{max}$ of R-warfarin in study II investigating the effect of ginkgo and ginger on warfarin.

Number of obs =	36	R-squared	= 0.4961		
Root MSE	= .082988	Adj R-squared =	0.3919		
Source |	Partial SS	df	MS	F	Prob > F
Model |	0.196666749	6	0.032777791	4.76	0.0017
Treatment |	0.007222234	2	0.003611117	0.52	0.5975
Period |	0.115555547	2	0.057777774	8.39*	0.0013
Sequence |	0.073888968	2	0.036944484	5.36*	0.0104
Residual |	0.199722319	29	0.006886977		
Total |	0.396389068	35	0.011325402		

*p<0.05

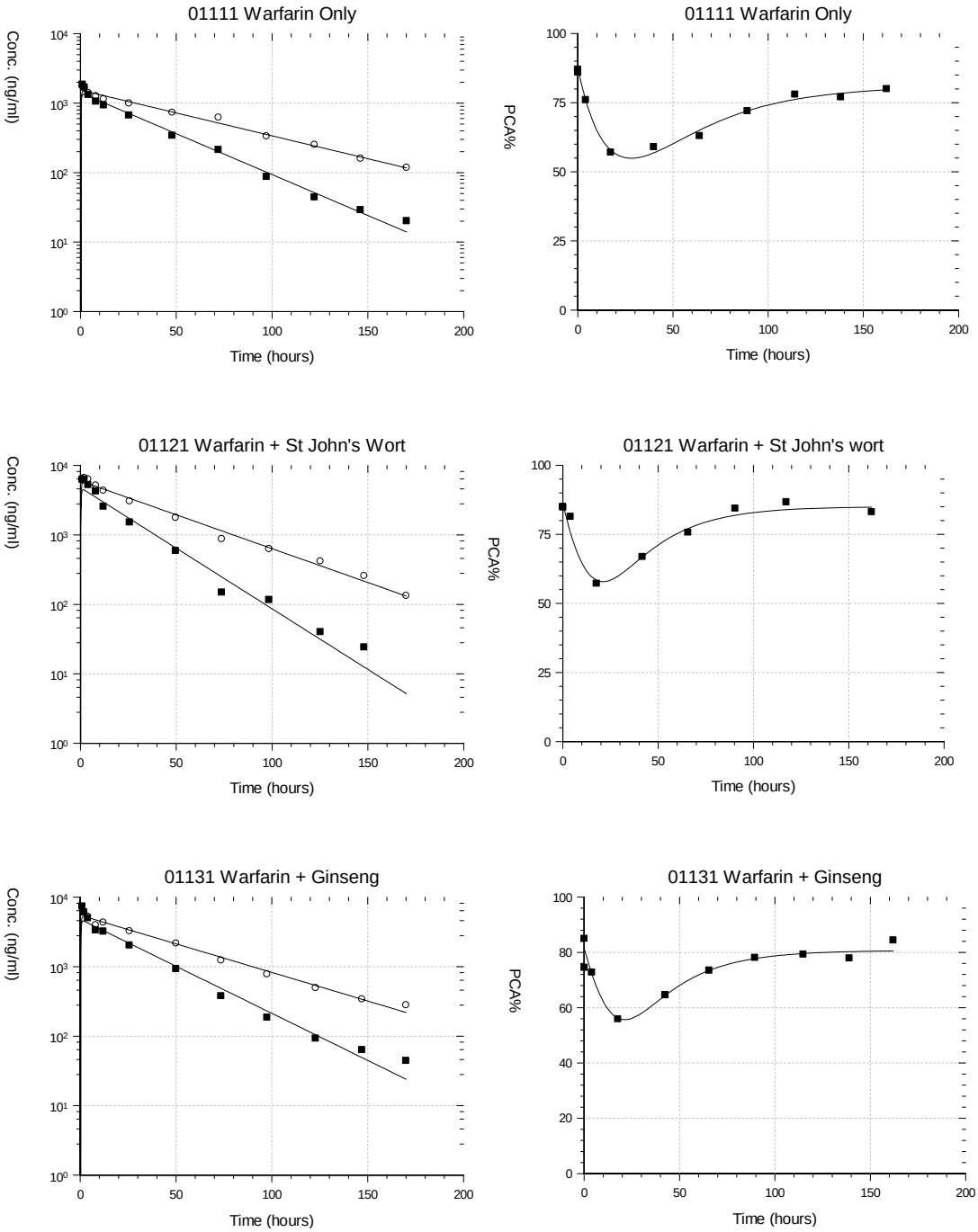
Appendix 10. ANOVA test of sequence, period and treatment effects on $logAUC_{0\text{-}168}$ of INR in study II investigating the effect of ginkgo and ginger on warfarin.

Number of obs =	36	R-squared	= 0.3296		
Root MSE	= .174005	Adj R-squared =	0.1909		
Source |	Partial SS	df	MS	F	Prob > F
Model |	0.431666714	6	0.071944452	2.38	0.0546
Treatment |	0.003888888	2	0.001944444	0.06	0.9379
Period |	0.037222233	2	0.018611117	0.61	0.5477
Sequence |	0.390555592	2	0.195277796	6.45*	0.0048
Residual |	0.878055759	29	0.030277785		
Total |	1.30972247	35	0.037420642		

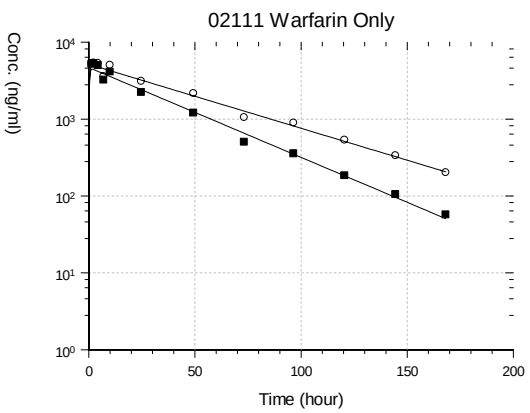
*p<0.05

Appendix 11. Study I plasma concentration – time profile for S-warfarin and R-warfarin and the PCA% -time (elapsing time – td) profile following a single oral dose of 25 mg *rac*-warfarin alone or with St John's Wort (warfarin + St John's Wort) or with ginseng (Warfarin + Ginseng). (■, observed data for S-warfarin; ○, observed data for R-warfarin; —, model predicted data).

Subject 1



Subject 2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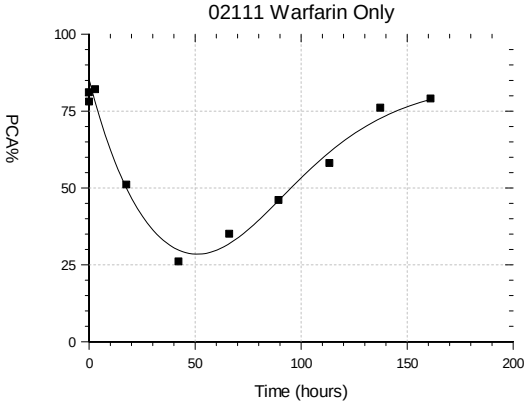
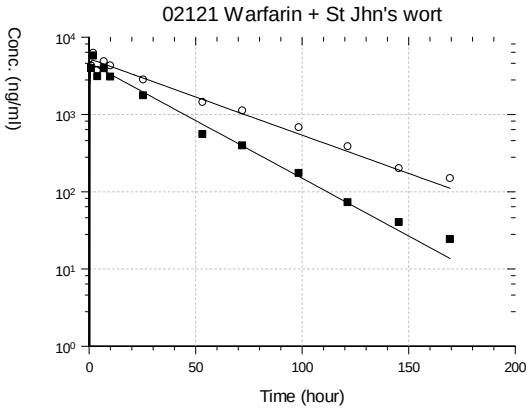
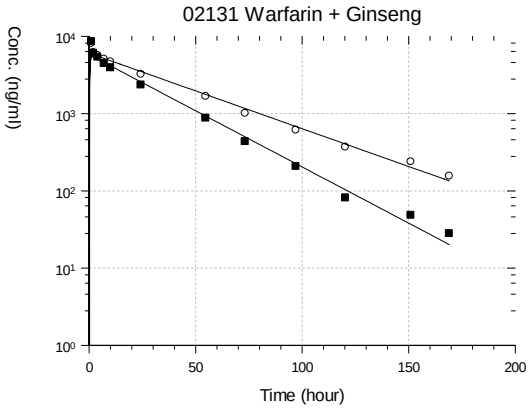
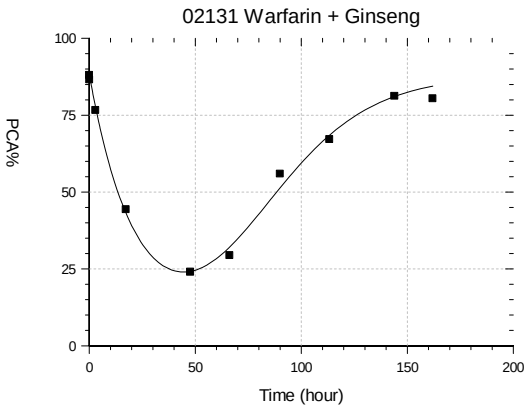
Conc. (ng/ml)
Time (hour)


02111 Warfarin Only
PCA%
Time (hours)

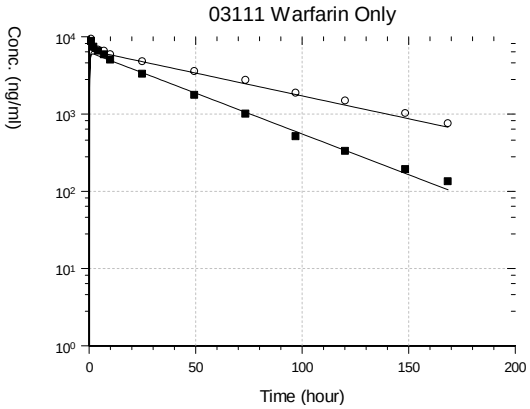

02121 Warfarin + St Jhn's wort
Conc. (ng/ml)
Time (hour)

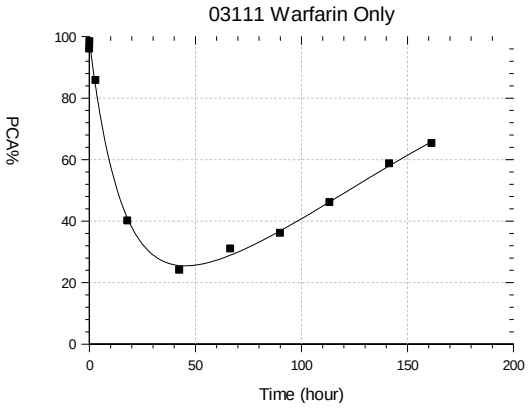

02121 Warfarin + St John's wort
PCA%
Time (hours)

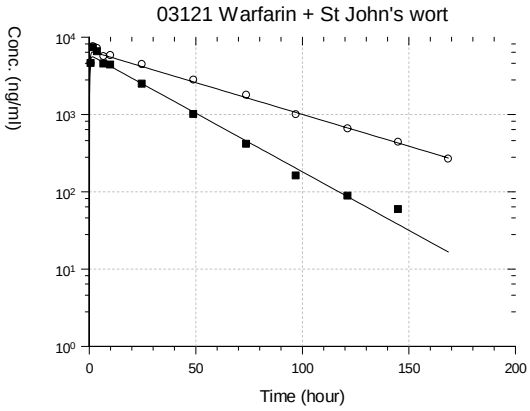

02131 Warfarin + Ginseng
Conc. (ng/ml)
Time (hour)

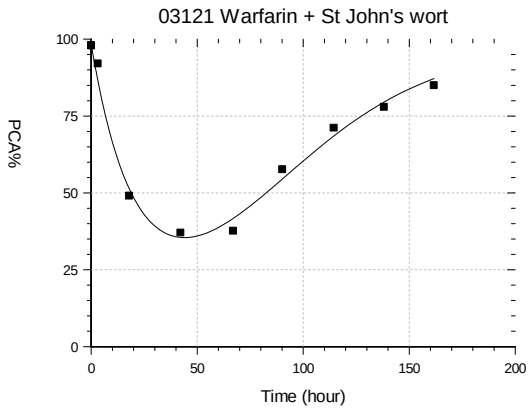

02131 Warfarin + Ginseng
PCA%
Time (hour)

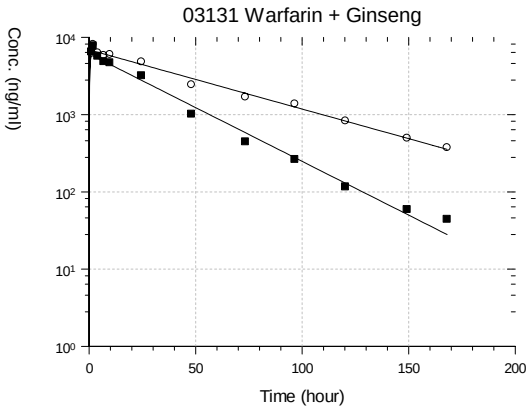
Subject 3

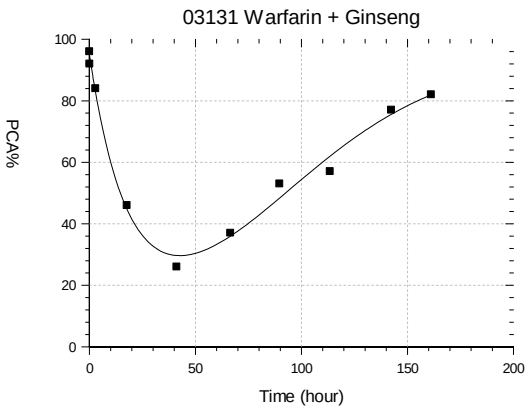

03111 Warfarin Only
Conc. (ng/ml)
Time (hour)


03111 Warfarin Only
PCA%
Time (hour)

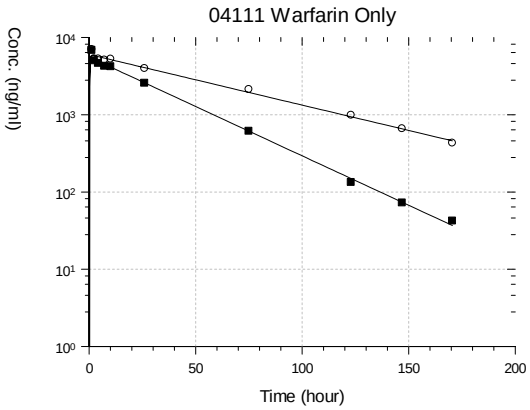

03121 Warfarin + St John's wort
Conc. (ng/ml)
Time (hour)

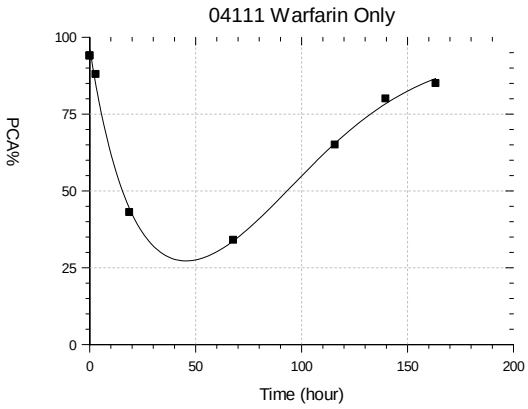

03121 Warfarin + St John's wort
PCA%
Time (hour)


03131 Warfarin + Ginseng
Conc. (ng/ml)
Time (hour)


03131 Warfarin + Ginseng
PCA%
Time (hour)

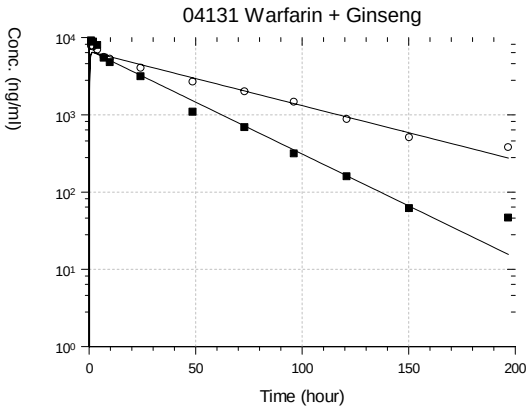
Subject 4

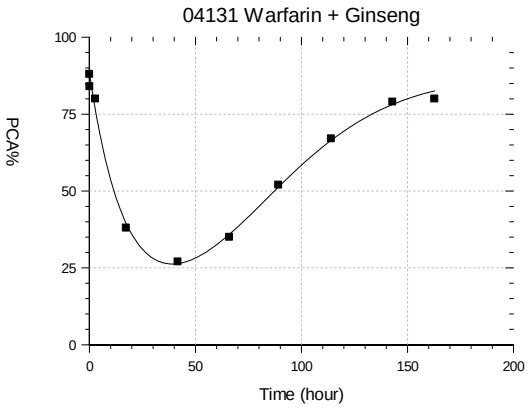

04111 Warfarin Only
Conc. (ng/ml)
Time (hour)


04111 Warfarin Only
PCA%
Time (hour)

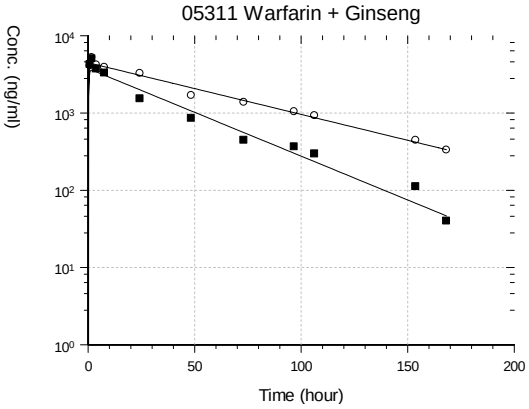

04121 Warfarin + St John's wort
Conc. (ng/ml)
Time (hour)

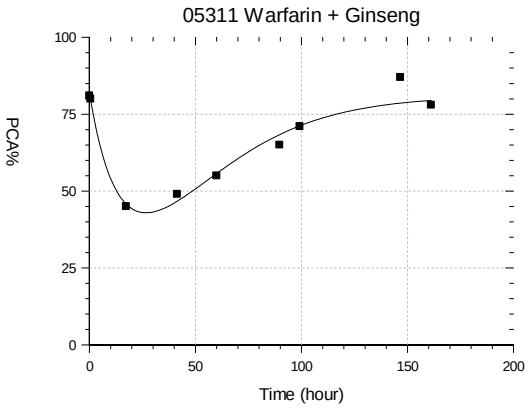

04121 Warfarin + St John's wort
PCA%
Time (hour)

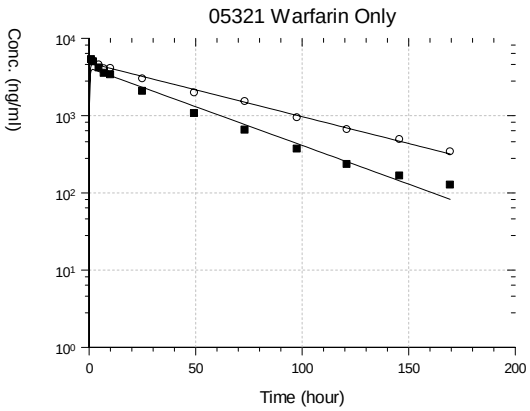

04131 Warfarin + Ginseng
Conc. (ng/ml)
Time (hour)

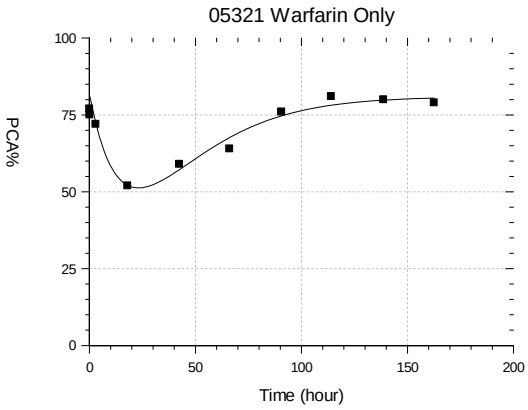

04131 Warfarin + Ginseng
PCA%
Time (hour)

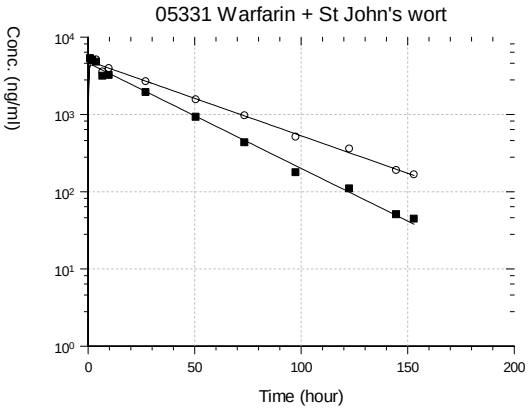
Subject 5

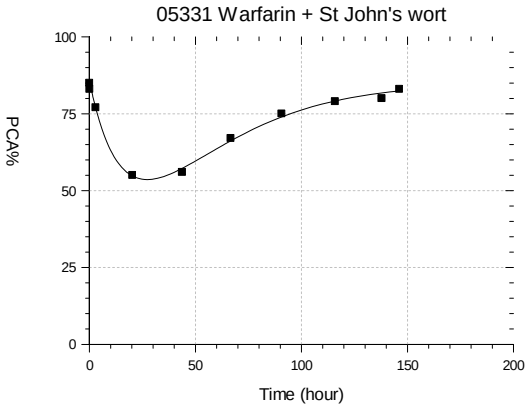

05311 Warfarin + Ginseng
Conc. (ng/ml)
Time (hour)


05311 Warfarin + Ginseng
PCA%
Time (hour)

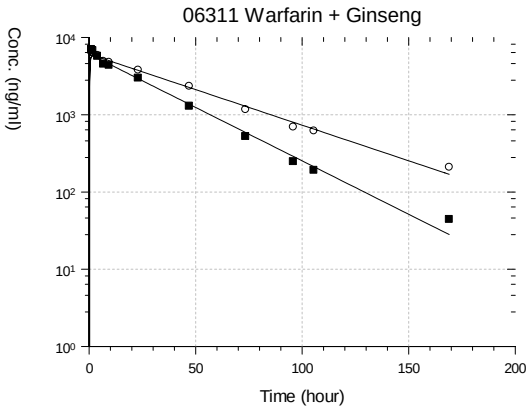

05321 Warfarin Only
Conc. (ng/ml)
Time (hour)

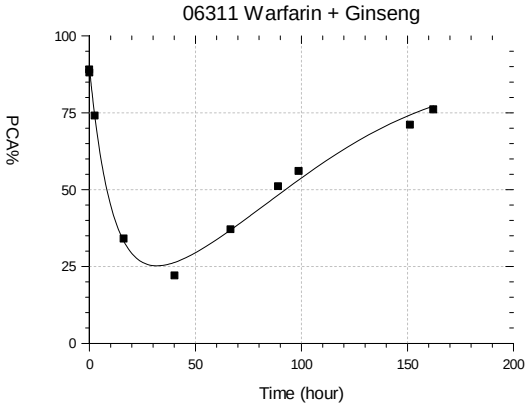

05321 Warfarin Only
PCA%
Time (hour)

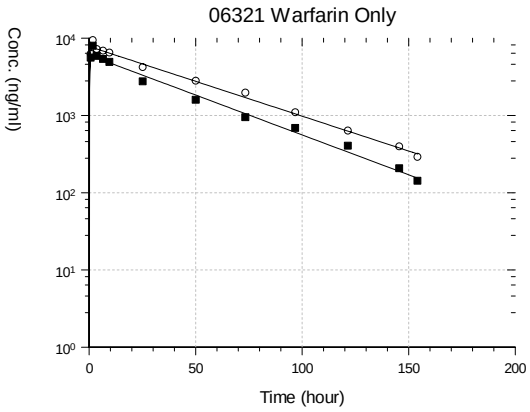

05331 Warfarin + St John's wort
Conc. (ng/ml)
Time (hour)

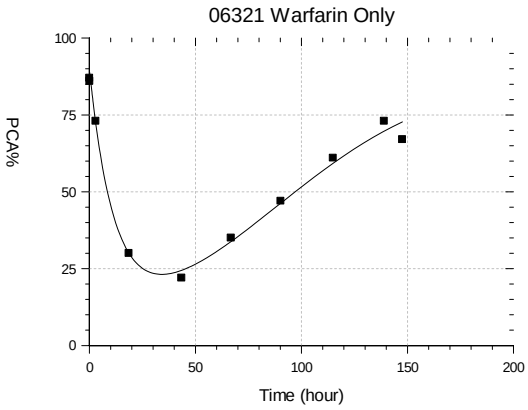

05331 Warfarin + St John's wort
PCA%
Time (hour)

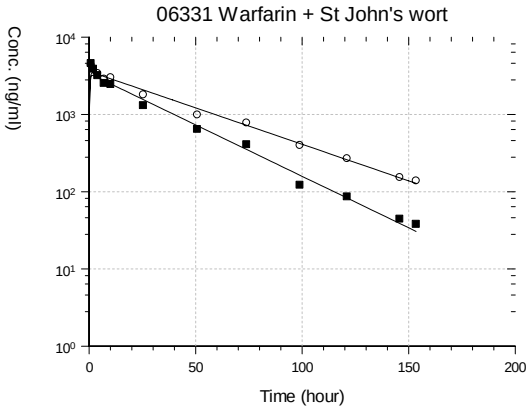
Subject 6

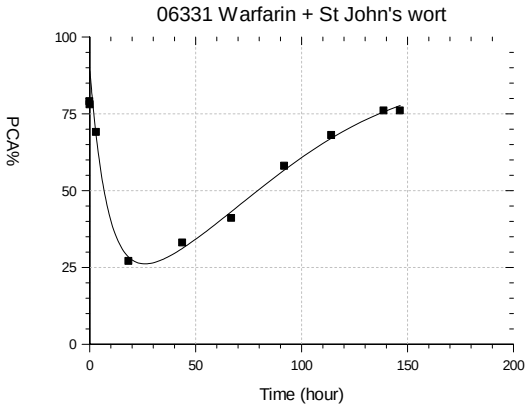

06311 Warfarin + Ginseng
Conc. (ng/ml)
Time (hour)


06311 Warfarin + Ginseng
PCA%
Time (hour)

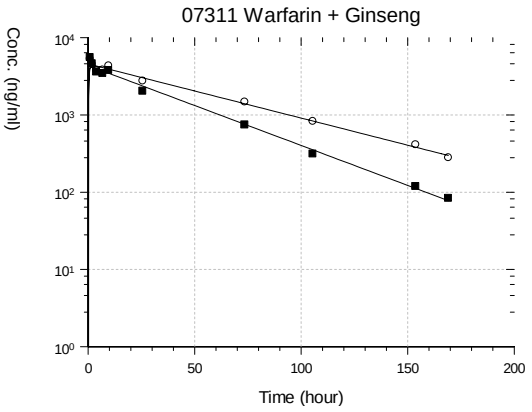

06321 Warfarin Only
Conc. (ng/ml)
Time (hour)

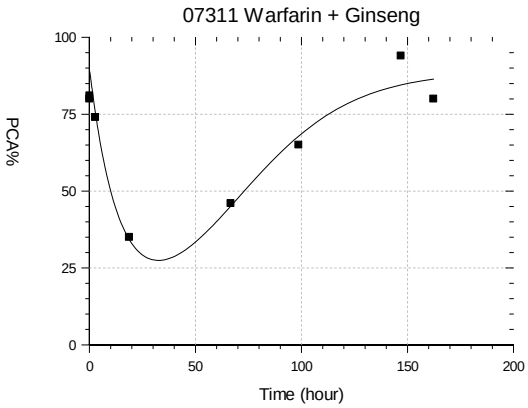

06321 Warfarin Only
PCA%
Time (hour)

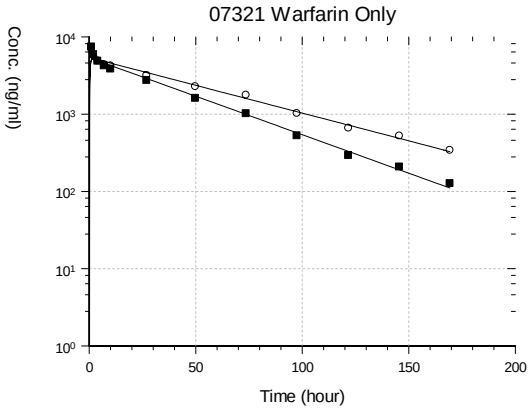

06331 Warfarin + St John's wort
Conc. (ng/ml)
Time (hour)

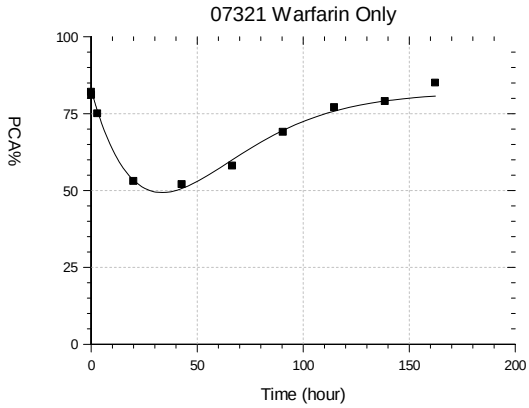

06331 Warfarin + St John's wort
PCA%
Time (hour)

Subject 7

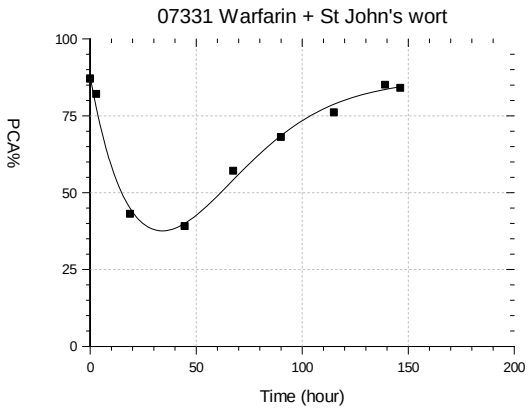

07311 Warfarin + Ginseng
Conc. (ng/ml)
Time (hour)


07311 Warfarin + Ginseng
PCA%
Time (hour)

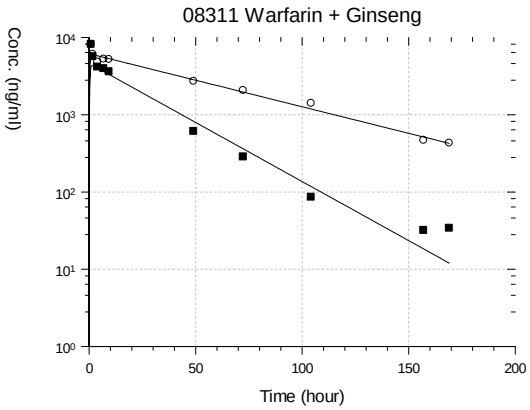

07321 Warfarin Only
Conc. (ng/ml)
Time (hour)

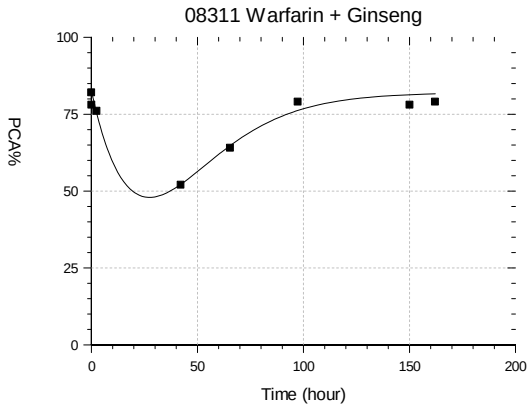

07321 Warfarin Only
PCA%
Time (hour)

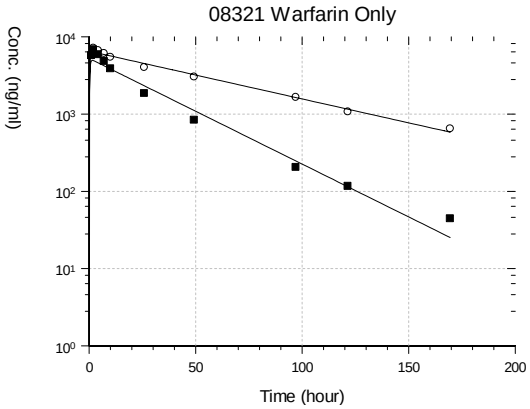

07331 Warfarin + St John's wort
Conc. (ng/ml)
Time (hour)

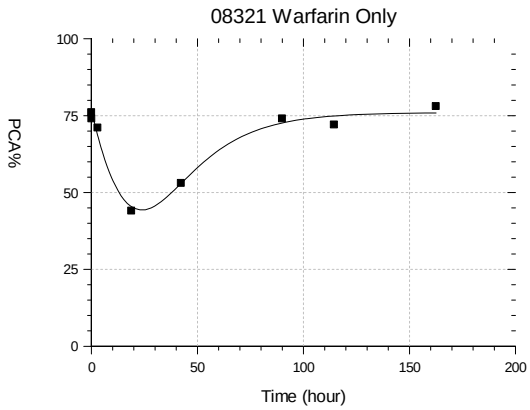

07331 Warfarin + St John's wort
PCA%
Time (hour)

Subject 8

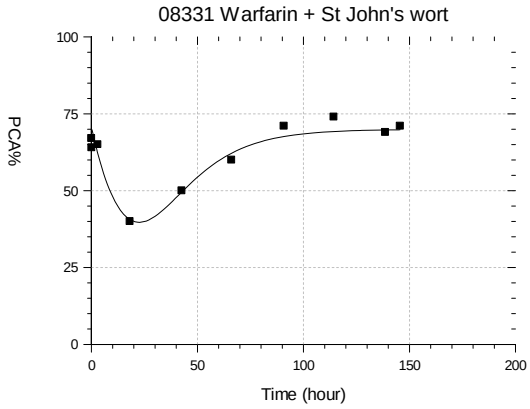

08311 Warfarin + Ginseng
Conc. (ng/ml)
Time (hour)


08311 Warfarin + Ginseng
PCA%
Time (hour)


08321 Warfarin Only
Conc. (ng/ml)
Time (hour)

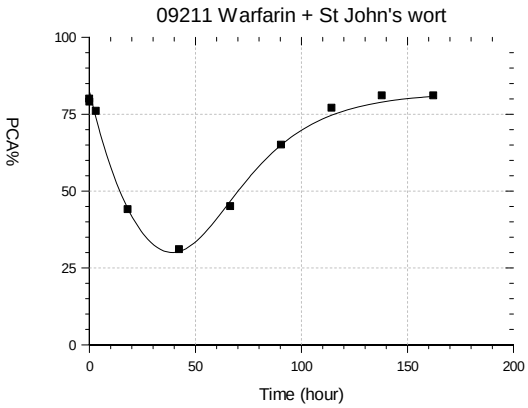

08321 Warfarin Only
PCA%
Time (hour)

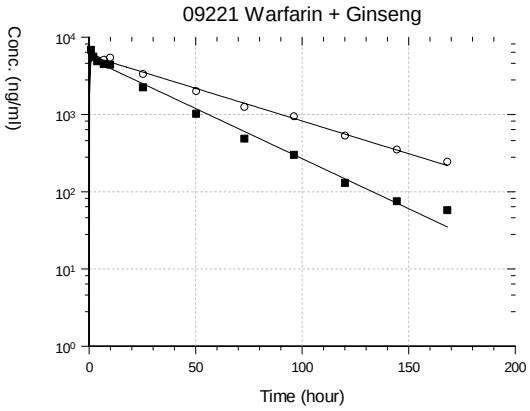

08331 Warfarin + St John's wort
Conc. (ng/ml)
Time (hour)

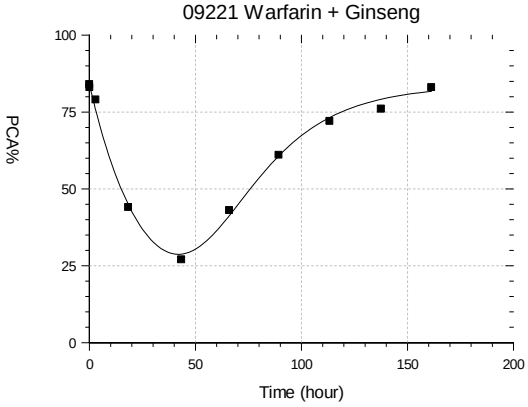

08331 Warfarin + St John's wort
PCA%
Time (hour)

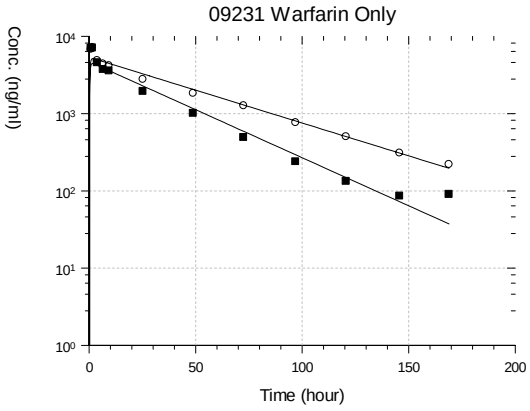
Subject 9

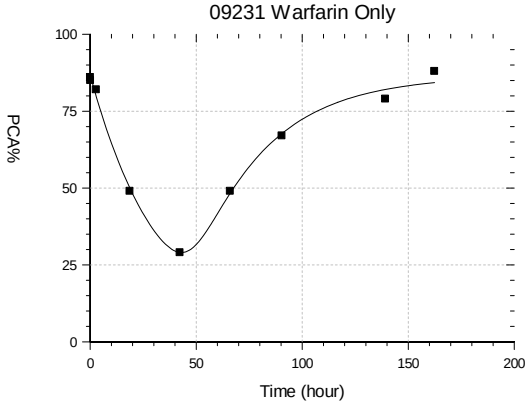

09211 Warfarin + St John's wort
Conc. (ng/ml)
Time (hour)


09211 Warfarin + St John's wort
PCA%
Time (hour)

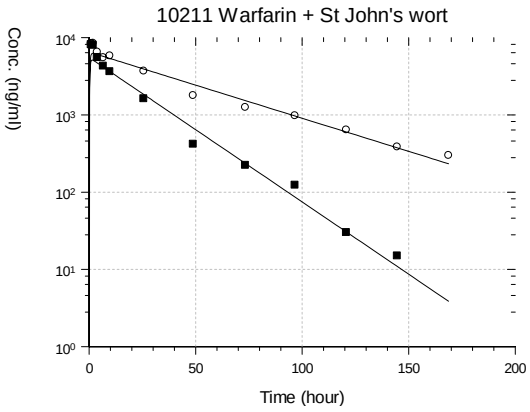

09221 Warfarin + Ginseng
Conc. (ng/ml)
Time (hour)

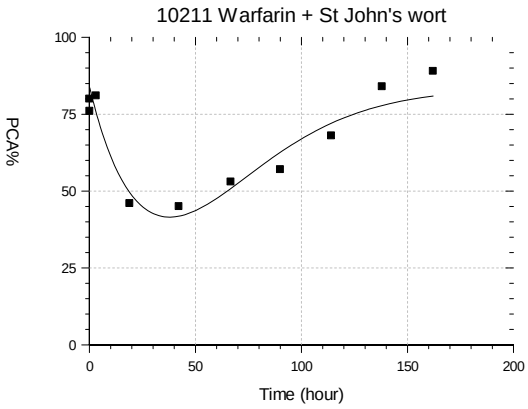

09221 Warfarin + Ginseng
PCA%
Time (hour)

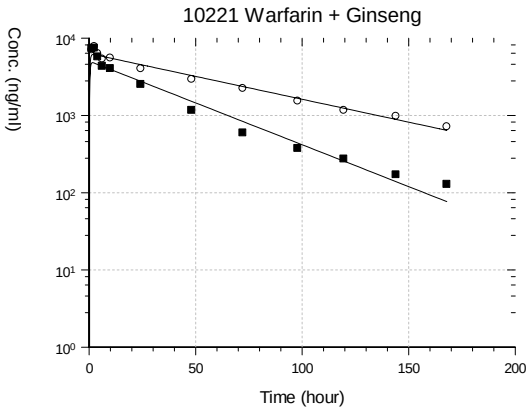

09231 Warfarin Only
Conc. (ng/ml)
Time (hour)

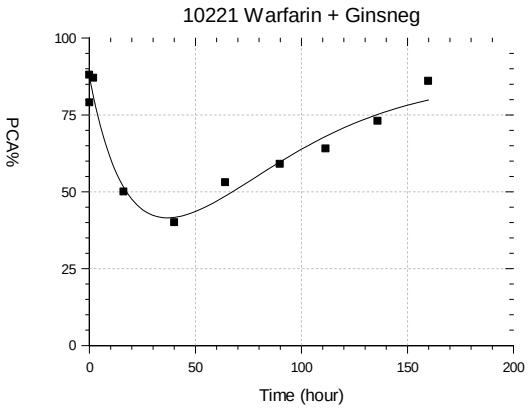

09231 Warfarin Only
PCA%
Time (hour)

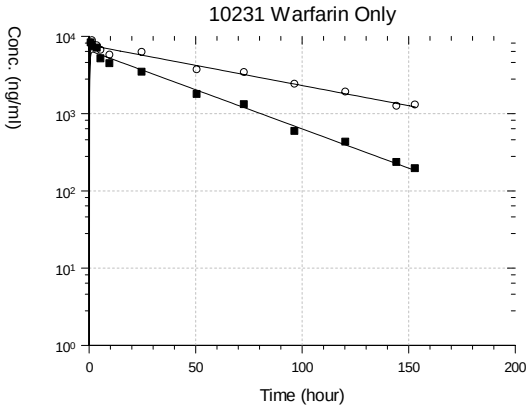
Subject 10

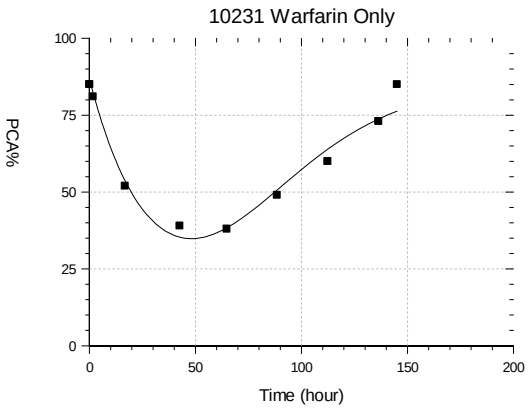

10211 Warfarin + St John's wort
Conc. (ng/ml)
Time (hour)


10211 Warfarin + St John's wort
PCA%
Time (hour)


10221 Warfarin + Ginseng
Conc. (ng/ml)
Time (hour)


10221 Warfarin + Ginsneg
PCA%
Time (hour)

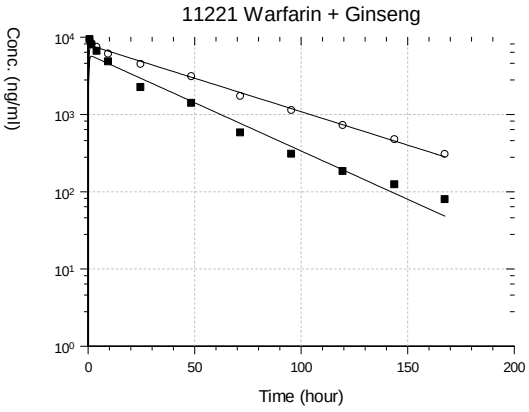

10231 Warfarin Only
Conc. (ng/ml)
Time (hour)

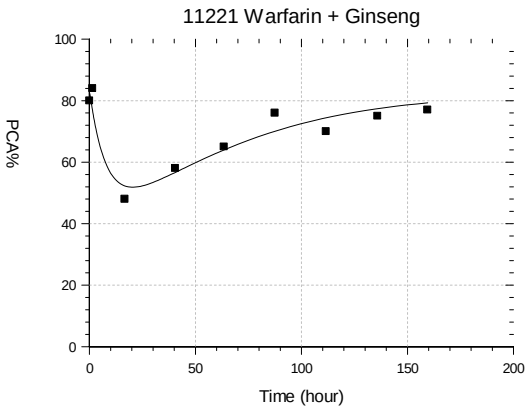

10231 Warfarin Only
PCA%
Time (hour)

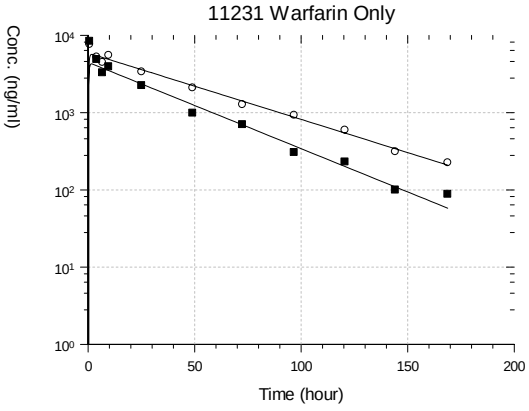
Subject 11

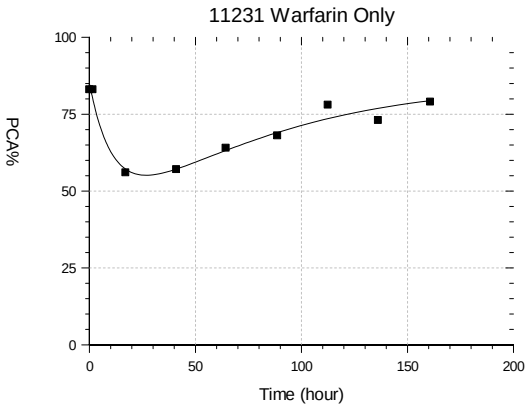

11211 Warfarin + St John's wort
Conc. (ng/ml)
Time (hour)


11211 Warfarin + St John's wort
PCA%
Time (hour)


11221 Warfarin + Ginseng
Conc. (ng/ml)
Time (hour)


11221 Warfarin + Ginseng
PCA%
Time (hour)

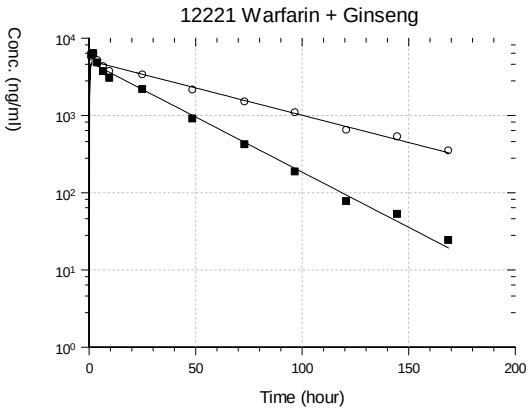

11231 Warfarin Only
Conc. (ng/ml)
Time (hour)

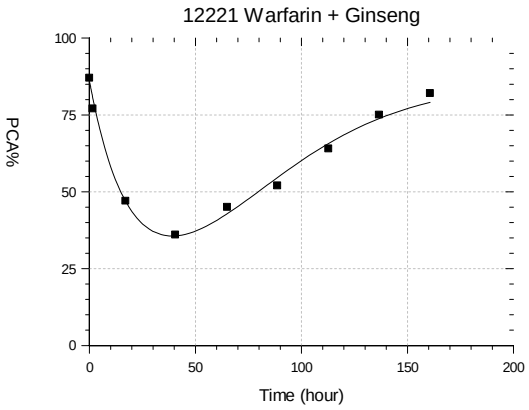

11231 Warfarin Only
PCA%
Time (hour)

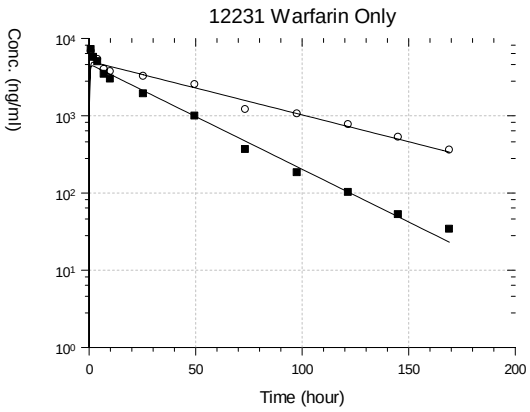
Subject 12

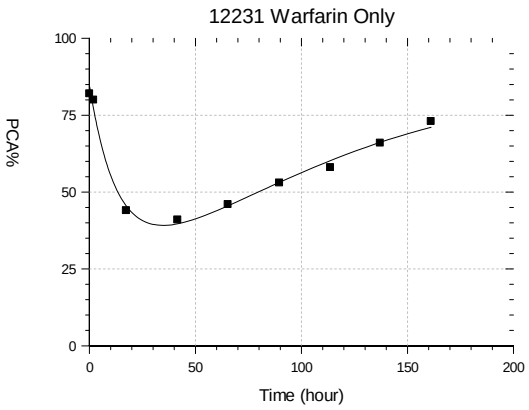

12211 Warfarin + St John's wort
Conc. (ng/ml)
Time (hour)


12211 Warfarin + St John's wort
PCA%
Time (hour)


12221 Warfarin + Ginseng
Conc. (ng/ml)
Time (hour)

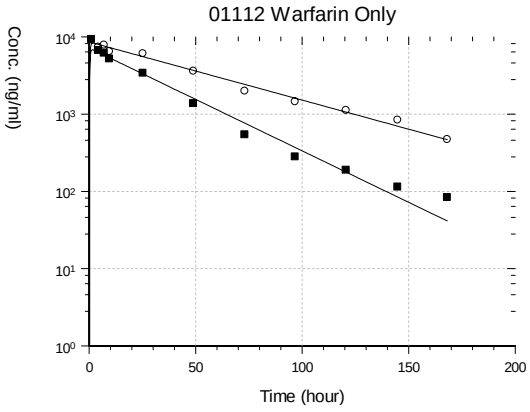

12221 Warfarin + Ginseng
PCA%
Time (hour)

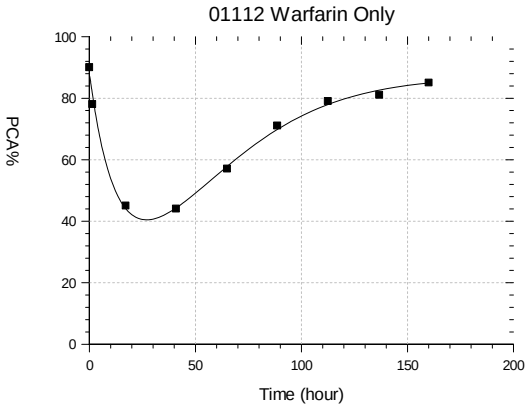

12231 Warfarin Only
Conc. (ng/ml)
Time (hour)

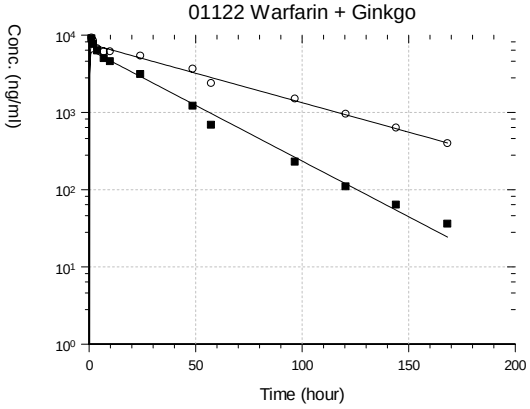

12231 Warfarin Only
PCA%
Time (hour)

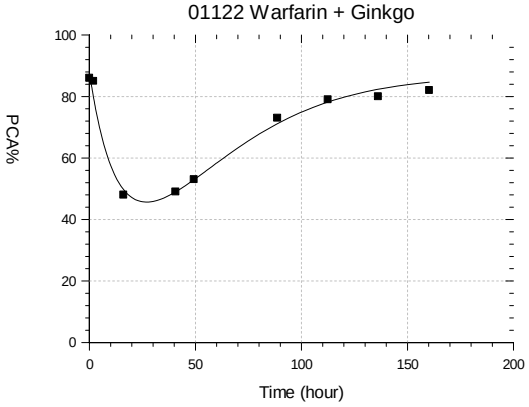
Appendix 12. Study II plasma concentration – time profile for S-warfarin and R-warfarin and the PCA% -time (elapsing time - td) profile following a single oral dose of 25 mg *rac*-warfarin alone or with ginkgo (Warfarin + Ginkgo) or with ginseng (Warfarin + Ginger) (■, observed data for S-warfarin; ○, observed data for R-warfarin; —, model predicted data).

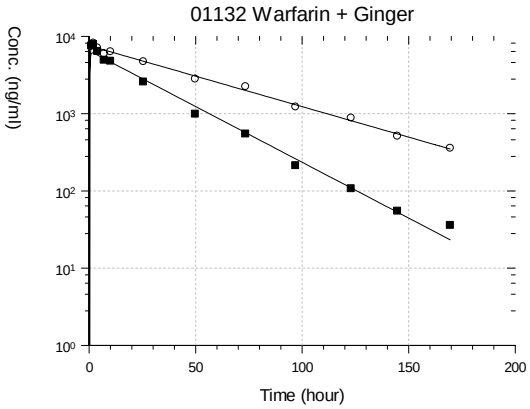
Subject 1

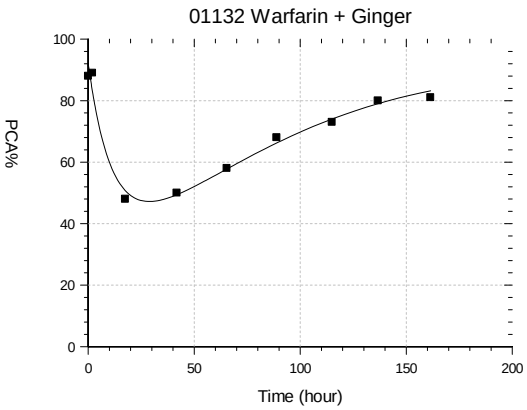












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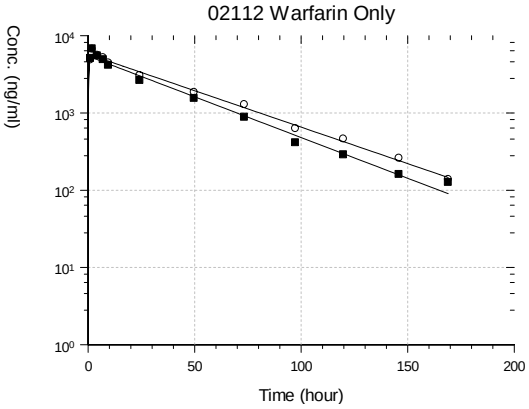
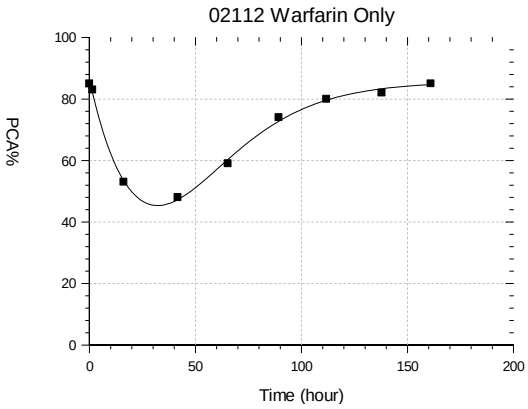
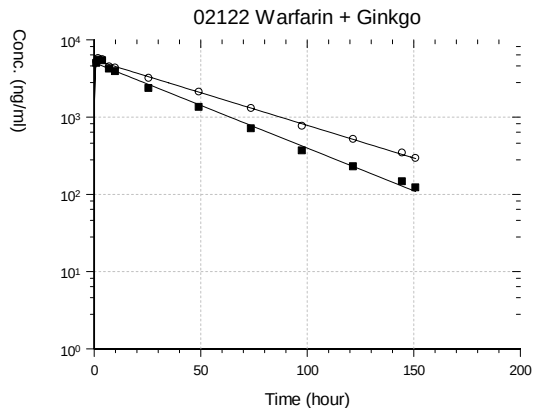
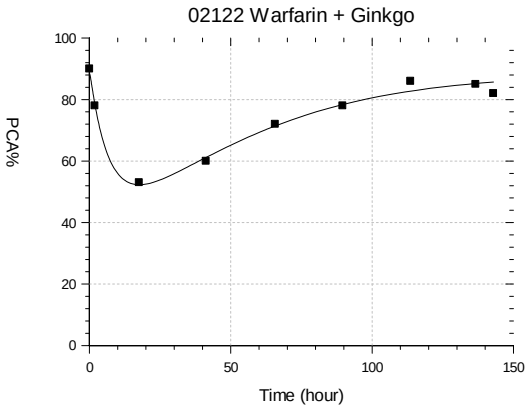
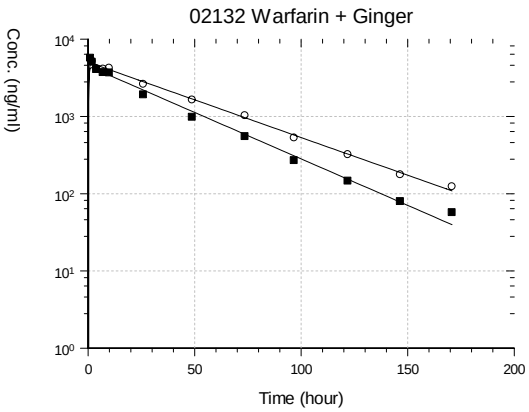
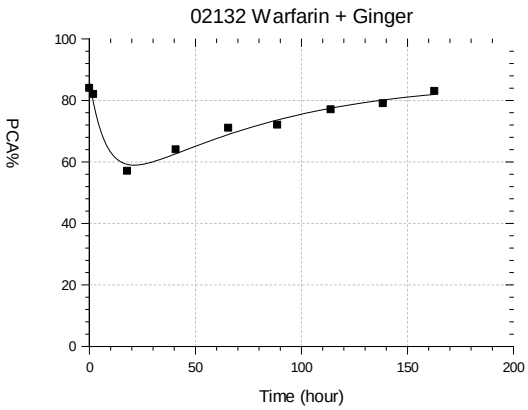

02112 Warfarin Only
Conc. (ng/ml)
Time (hour)


02112 Warfarin Only
PCA%
Time (hour)

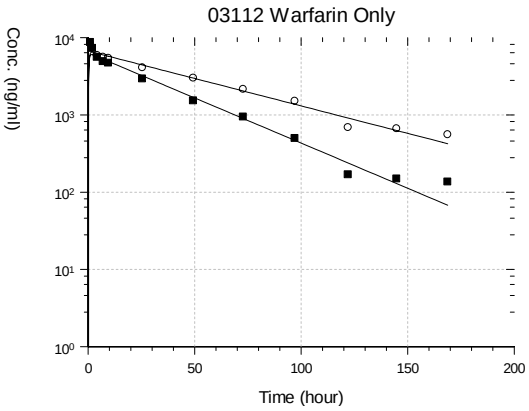

02122 Warfarin + Ginkgo
Conc. (ng/ml)
Time (hour)

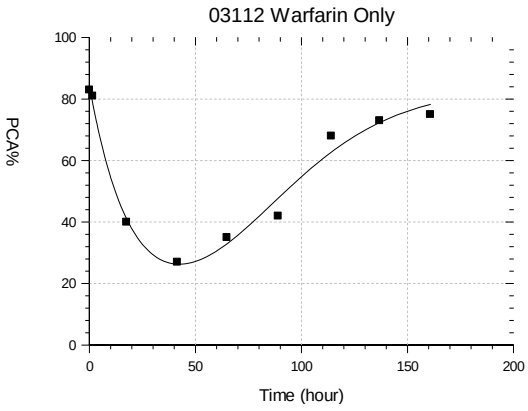

02122 Warfarin + Ginkgo
PCA%
Time (hour)

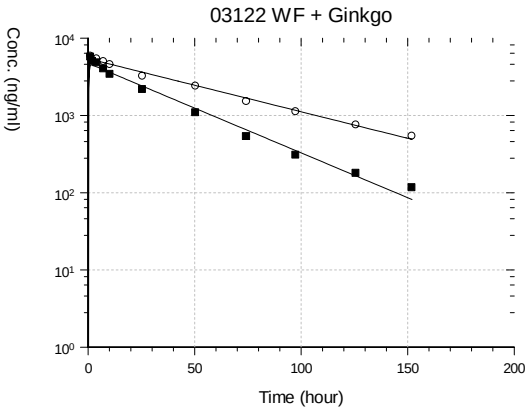

02132 Warfarin + Ginger
Conc. (ng/ml)
Time (hour)

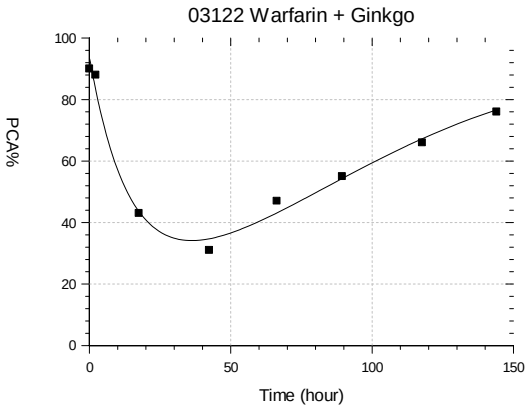

02132 Warfarin + Ginger
PCA%
Time (hour)

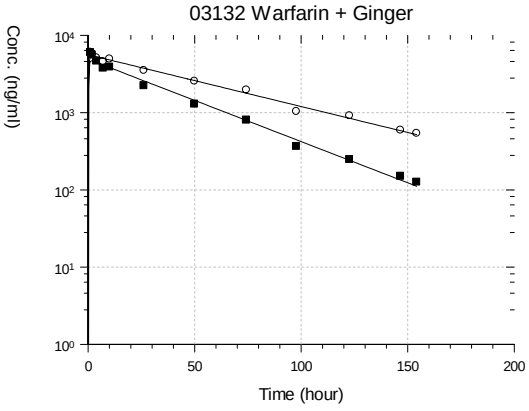
Subject 3

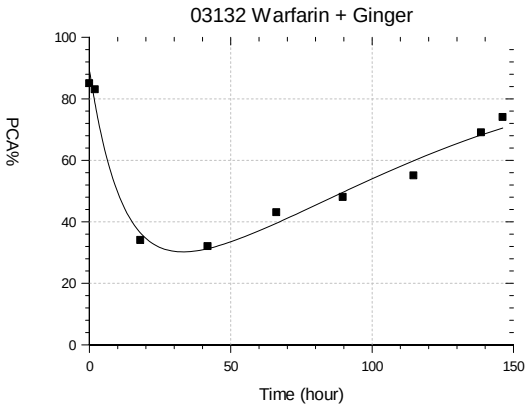

03112 Warfarin Only
Conc. (ng/ml)
Time (hour)


03112 Warfarin Only
PCA%
Time (hour)

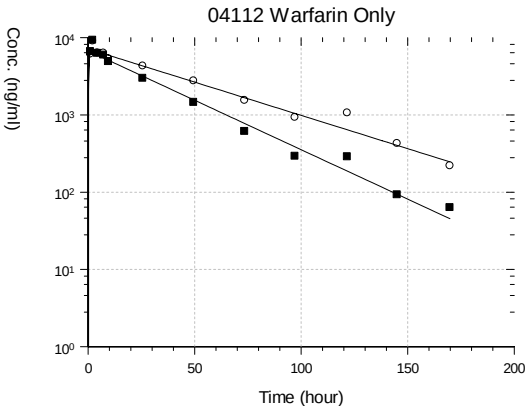

03122 WF + Ginkgo
Conc. (ng/ml)
Time (hour)

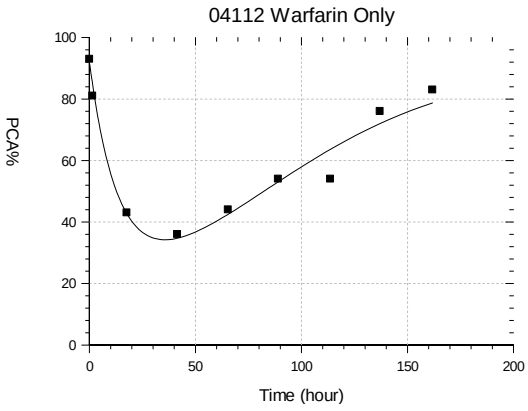

03122 Warfarin + Ginkgo
PCA%
Time (hour)

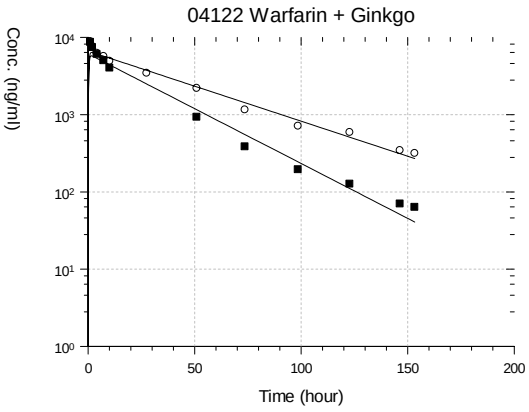

03132 Warfarin + Ginger
Conc. (ng/ml)
Time (hour)

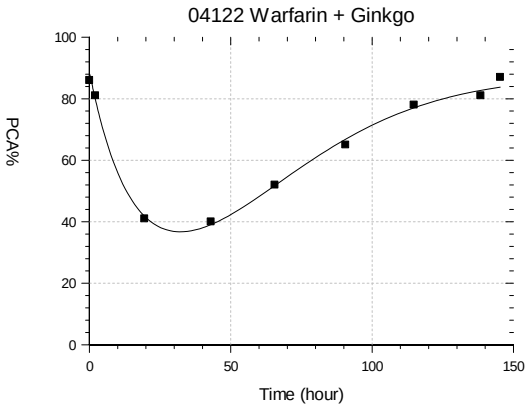

03132 Warfarin + Ginger
PCA%
Time (hour)

Subject 4


04112 Warfarin Only
Conc. (ng/ml)
Time (hour)


04112 Warfarin Only
PCA%
Time (hour)


04122 Warfarin + Ginkgo
Conc. (ng/ml)
Time (hour)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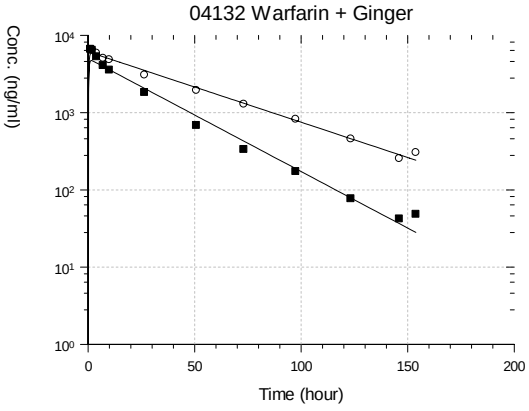
PCA%
Time (hour)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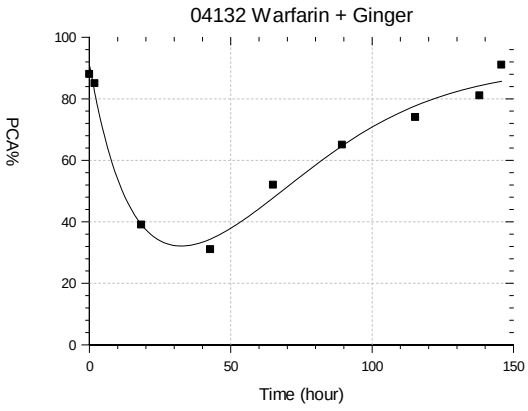
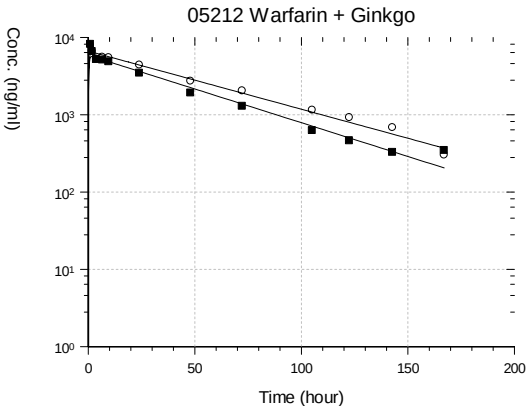
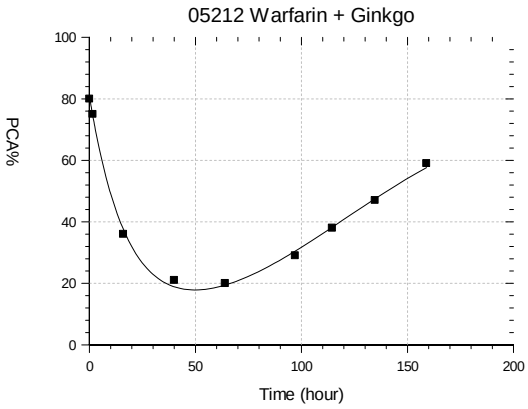
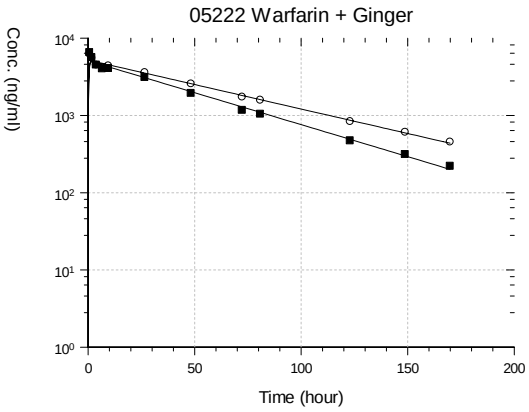
Conc. (ng/ml)
Time (hour)

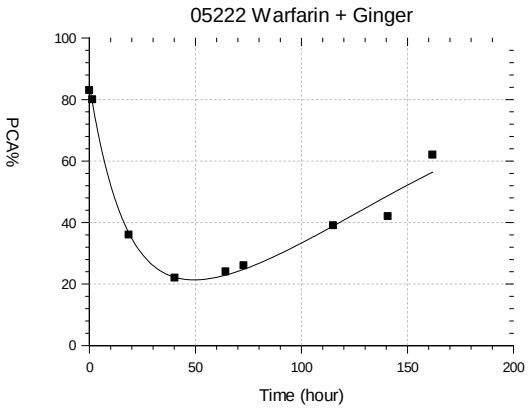

04132 Warfarin + Ginger
PCA%
Time (hour)

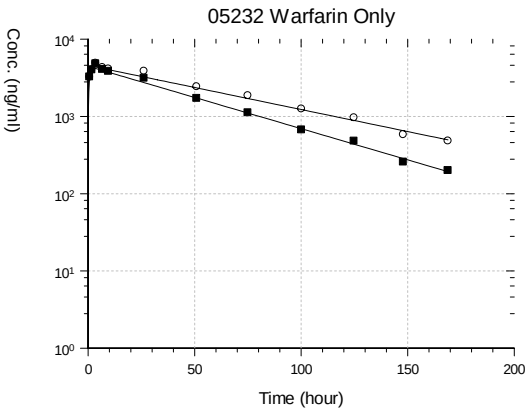
Subject 5

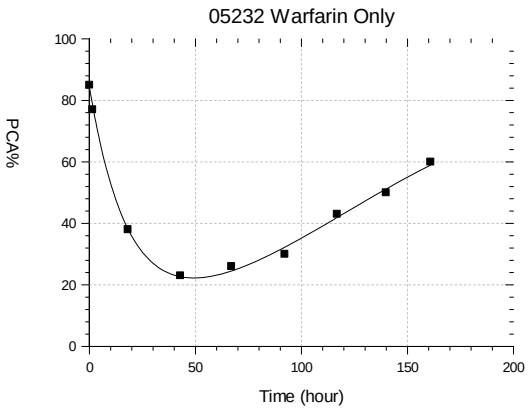

05212 Warfarin + Ginkgo
Conc. (ng/ml)
Time (hour)


05212 Warfarin + Ginkgo
PCA%
Time (hour)

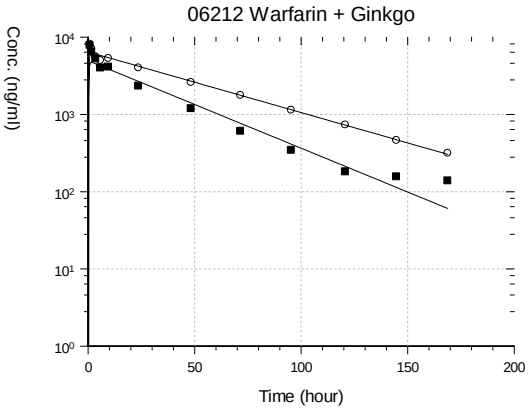

05222 Warfarin + Ginger
Conc. (ng/ml)
Time (hour)

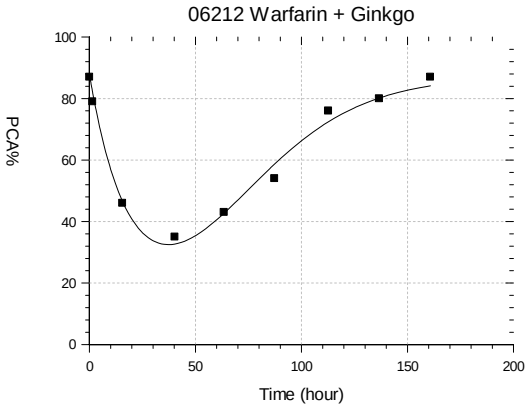

05222 Warfarin + Ginger
PCA%
Time (hour)

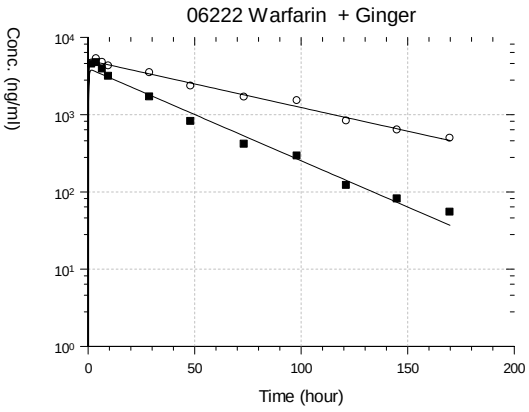

05232 Warfarin Only
Conc. (ng/ml)
Time (hour)

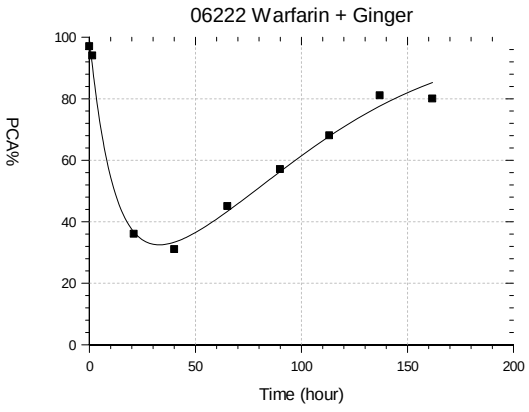

05232 Warfarin Only
PCA%
Time (hour)

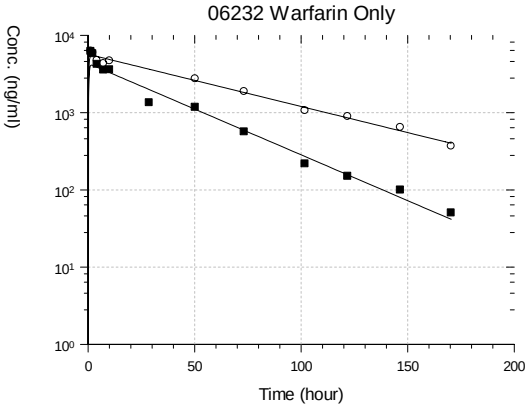
Subject 6

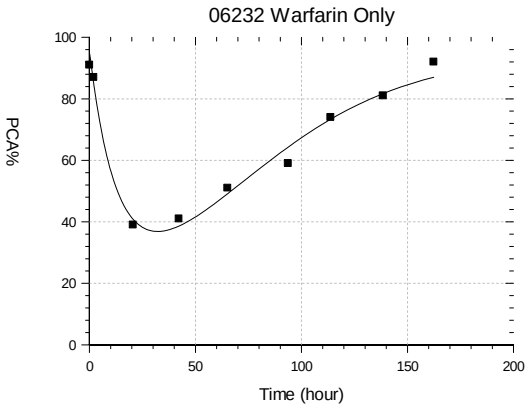

06212 Warfarin + Ginkgo
Conc. (ng/ml)
Time (hour)


06212 Warfarin + Ginkgo
PCA%
Time (hour)

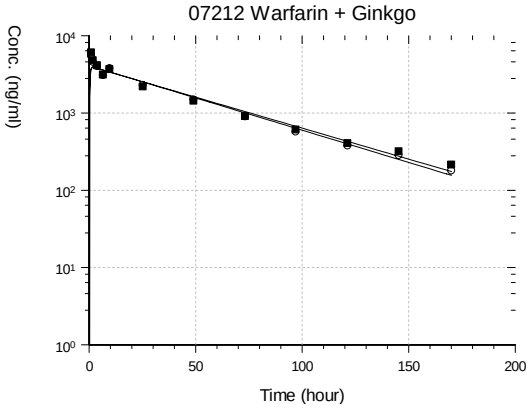

06222 Warfarin + Ginger
Conc. (ng/ml)
Time (hour)

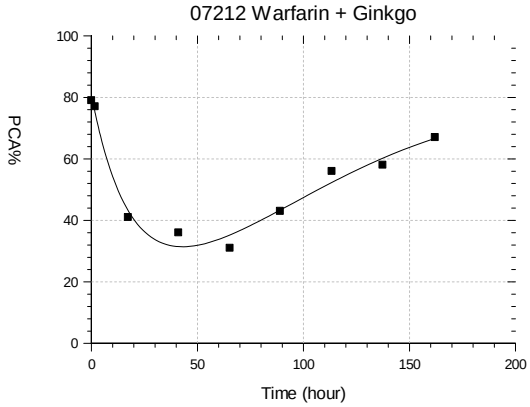

06222 Warfarin + Ginger
PCA%
Time (hour)

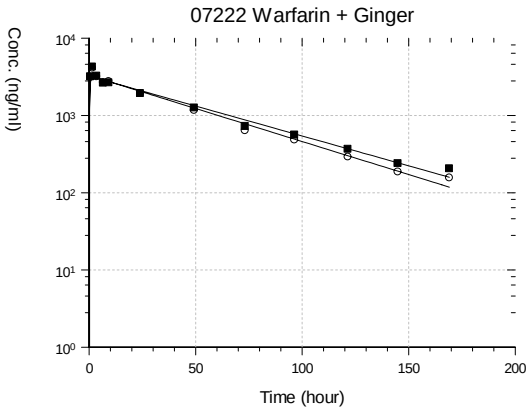

06232 Warfarin Only
Conc. (ng/ml)
Time (hour)

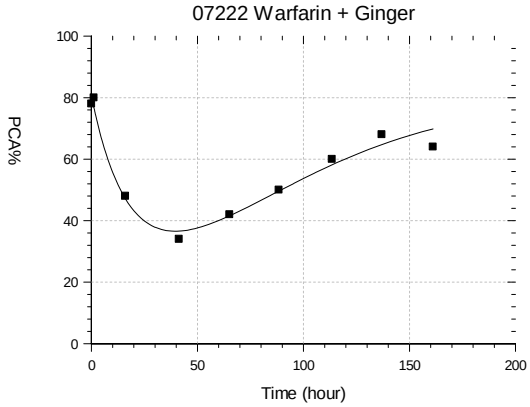

06232 Warfarin Only
PCA%
Time (hour)

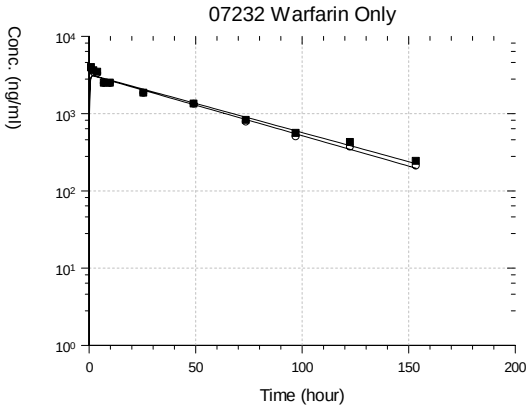
Subject 7


07212 Warfarin + Ginkgo
Conc. (ng/ml)
Time (hour)


07212 Warfarin + Ginkgo
PCA%
Time (hour)


07222 Warfarin + Ginger
Conc. (ng/ml)
Time (hour)


07222 Warfarin + Ginger
PCA%
Time (hour)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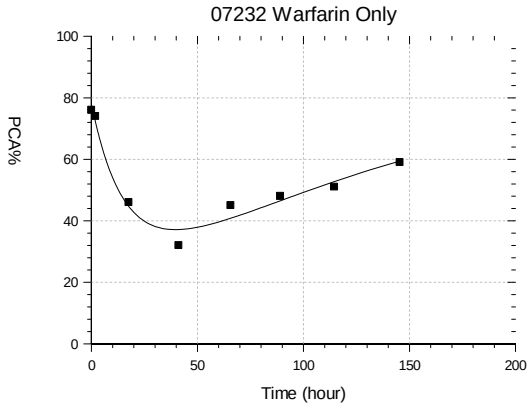
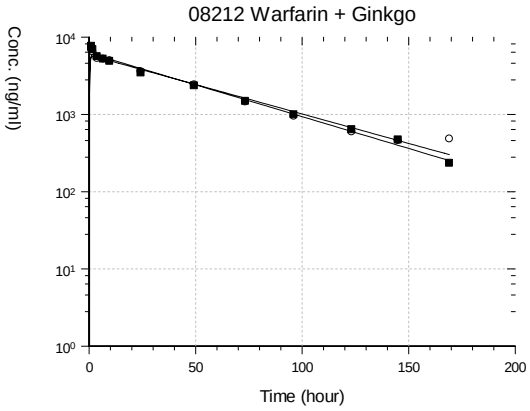
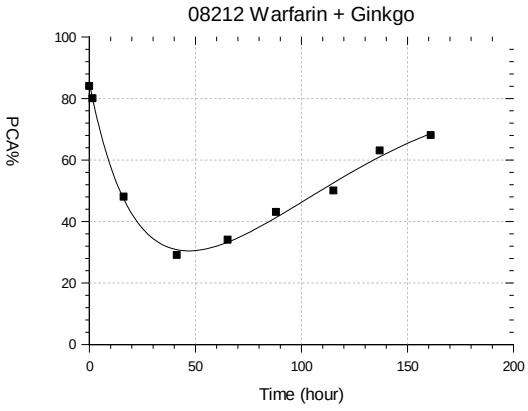
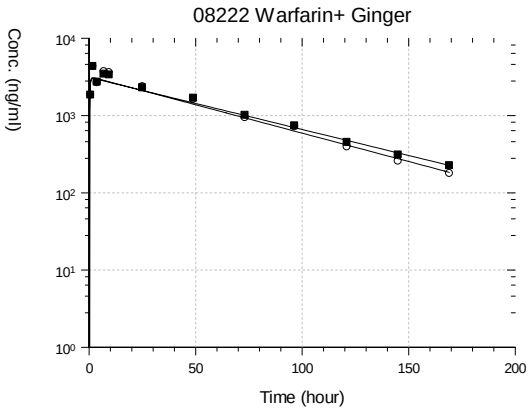
Conc. (ng/ml)
Time (hour)

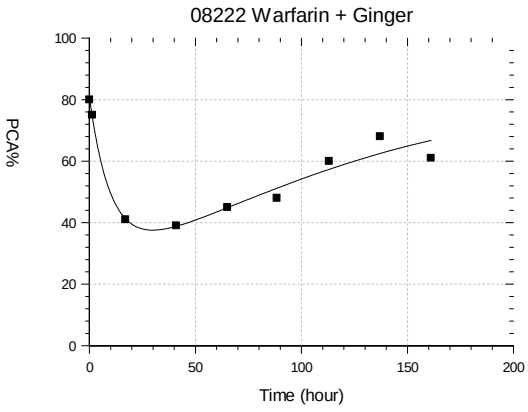

07232 Warfarin Only
PCA%
Time (hour)

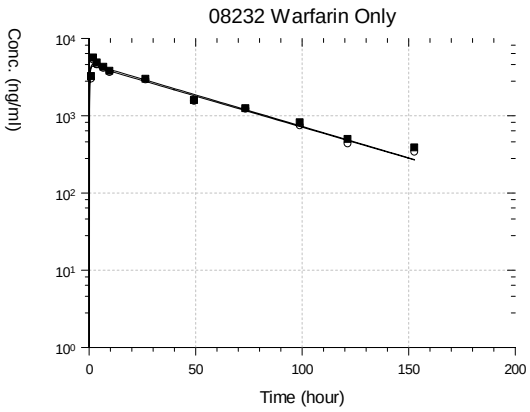
Subject 8

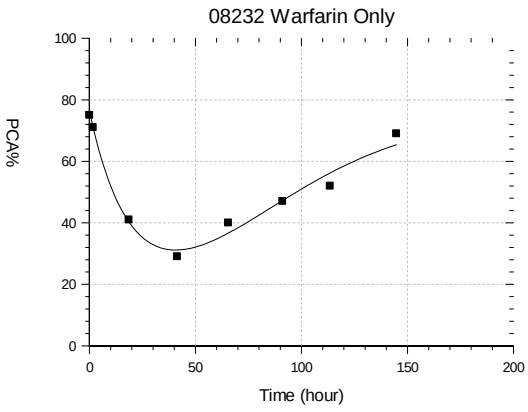

08212 Warfarin + Ginkgo
Conc. (ng/ml)
Time (hour)


08212 Warfarin + Ginkgo
PCA%
Time (hour)

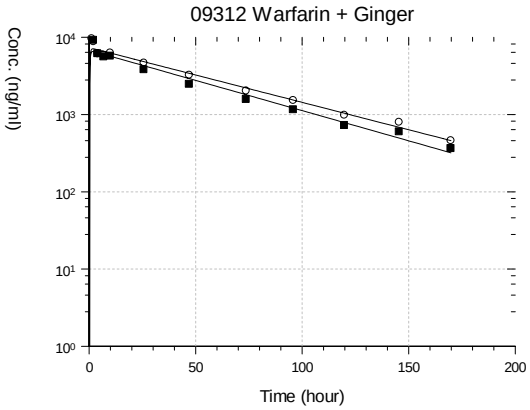

08222 Warfarin+ Ginger
Conc. (ng/ml)
Time (hour)

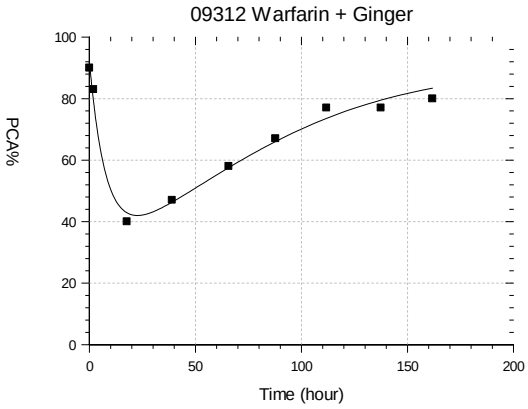

08222 Warfarin + Ginger
PCA%
Time (hour)

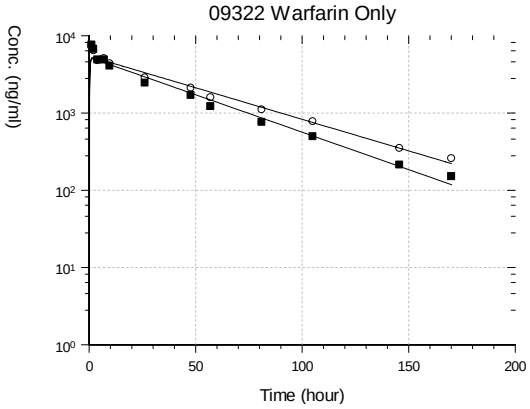

08232 Warfarin Only
Conc. (ng/ml)
Time (hour)

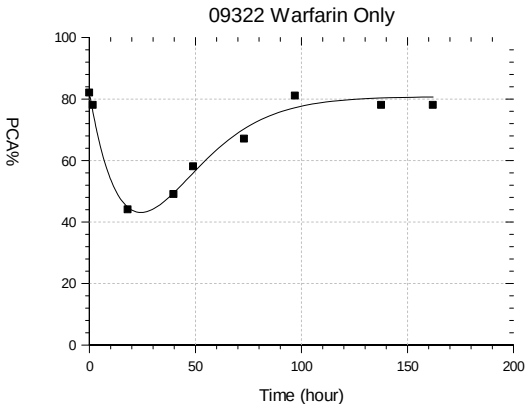

08232 Warfarin Only
PCA%
Time (hour)

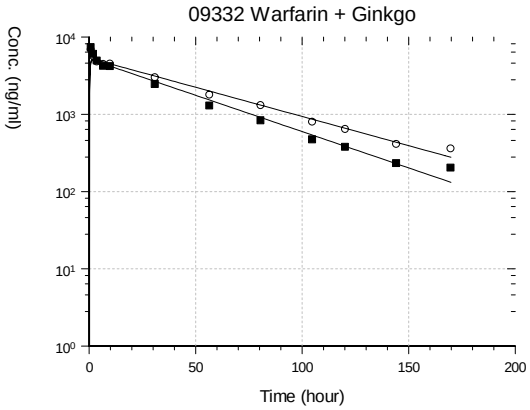
Subject 9

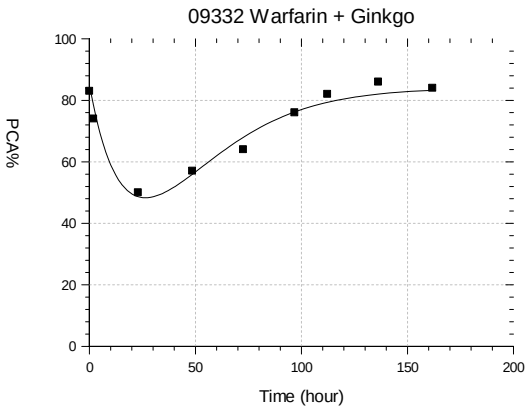

09312 Warfarin + Ginger
Conc. (ng/ml)
Time (hour)


09312 Warfarin + Ginger
PCA%
Time (hour)

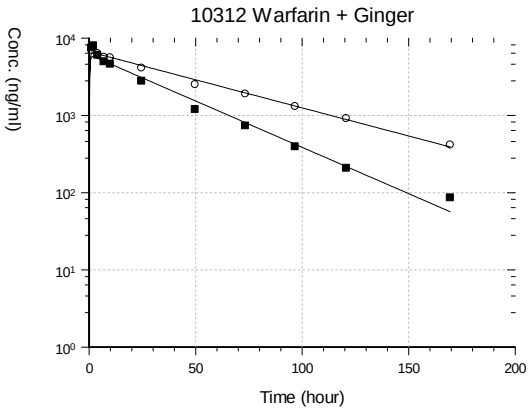

09322 Warfarin Only
Conc. (ng/ml)
Time (hour)

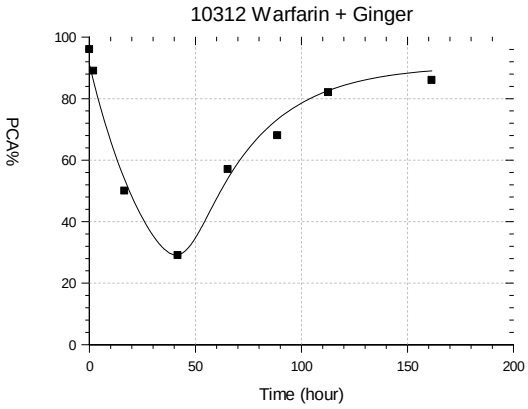

09322 Warfarin Only
PCA%
Time (hour)

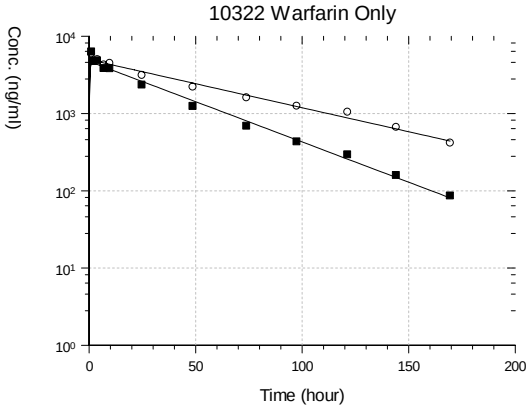

09332 Warfarin + Ginkgo
Conc. (ng/ml)
Time (hour)

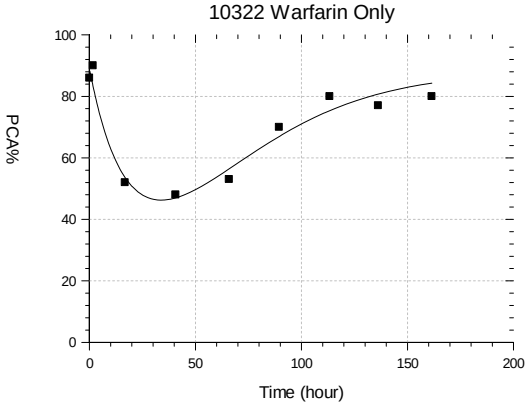

09332 Warfarin + Ginkgo
PCA%
Time (hour)

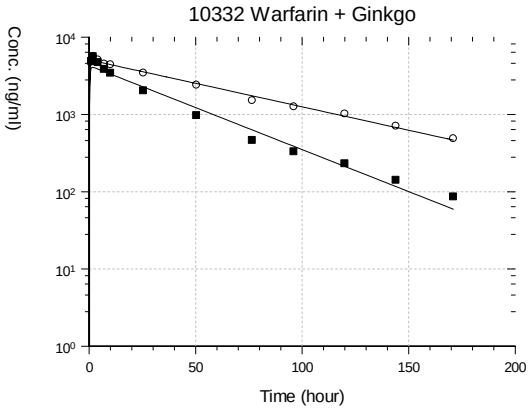
Subject 10

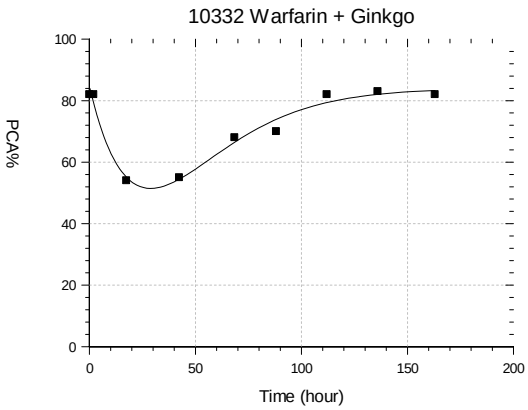

10312 Warfarin + Ginger
Conc. (ng/ml)
Time (hour)


10312 Warfarin + Ginger
PCA%
Time (hour)

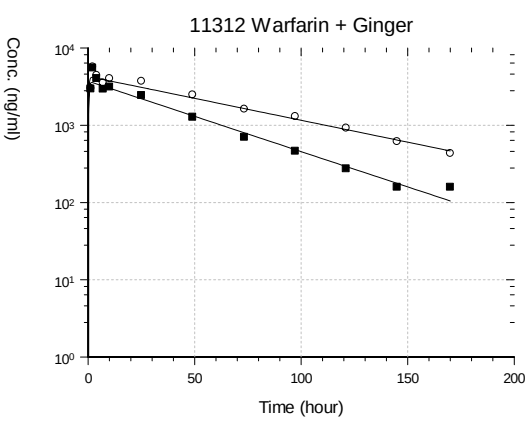

10322 Warfarin Only
Conc. (ng/ml)
Time (hour)

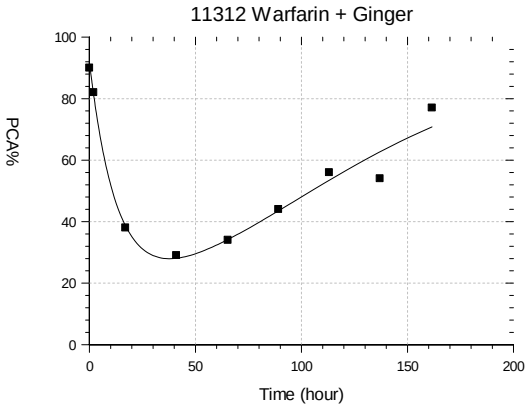

10322 Warfarin Only
PCA%
Time (hour)

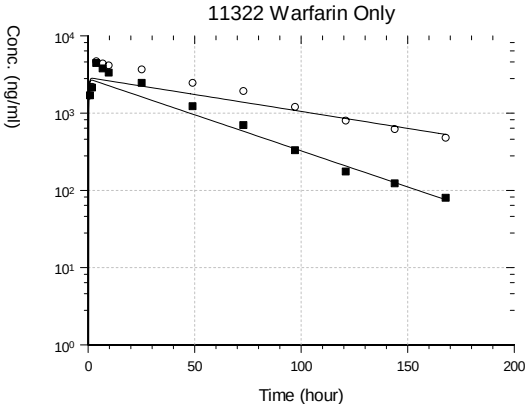

10332 Warfarin + Ginkgo
Conc. (ng/ml)
Time (hour)

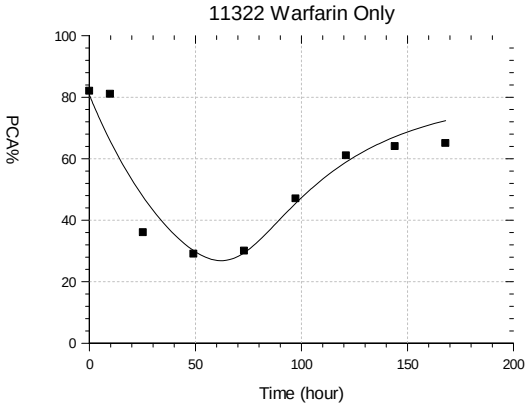

10332 Warfarin + Ginkgo
PCA%
Time (hour)

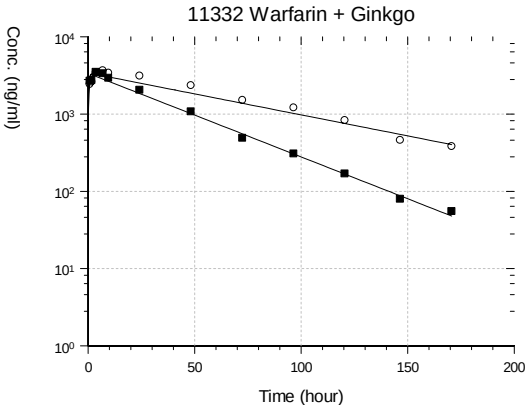
Subject 11

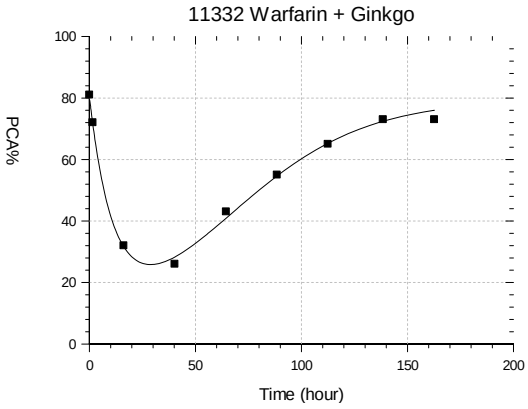

11312 Warfarin + Ginger
Conc. (ng/ml)
Time (hour)


11312 Warfarin + Ginger
PCA%
Time (hour)

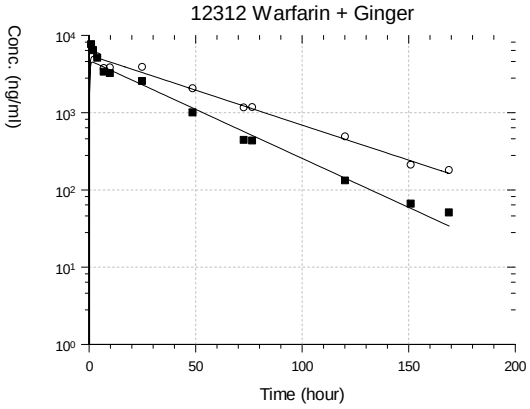

11322 Warfarin Only
Conc. (ng/ml)
Time (hour)

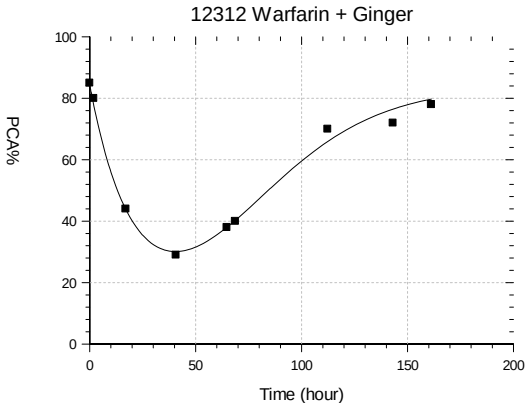

11322 Warfarin Only
PCA%
Time (hour)

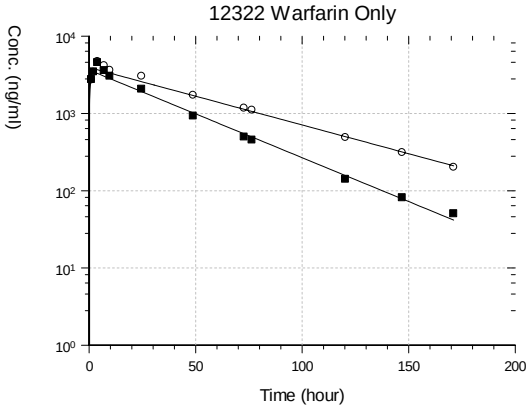

11332 Warfarin + Ginkgo
Conc. (ng/ml)
Time (hour)

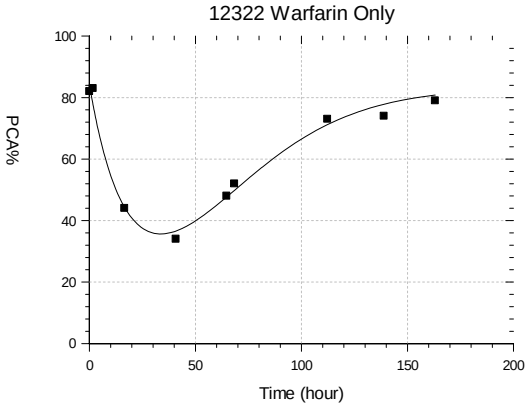

11332 Warfarin + Ginkgo
PCA%
Time (hour)

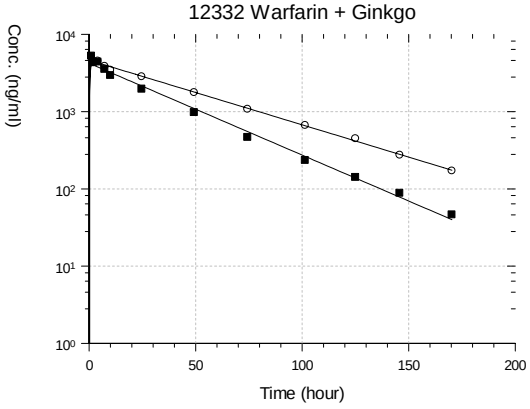
Subject 12

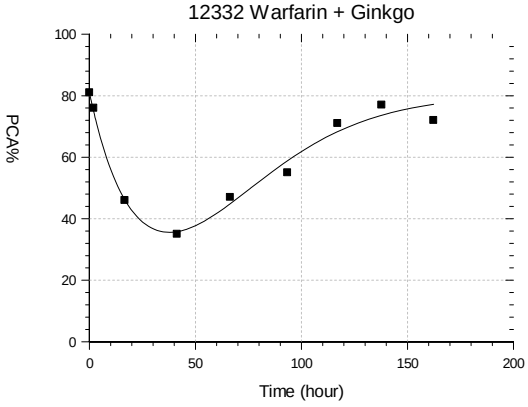

12312 Warfarin + Ginger
Conc. (ng/ml)
Time (hour)


12312 Warfarin + Ginger
PCA%
Time (hour)


12322 Warfarin Only
Conc. (ng/ml)
Time (hour)


12322 Warfarin Only
PCA%
Time (hour)


12332 Warfarin + Ginkgo
Conc. (ng/ml)
Time (hour)


12332 Warfarin + Ginkgo
PCA%
Time (hour)

Appendix 13. Individual fraction of unbound (fu) warfarin in study I following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either St John's wort (WF + SJW) or ginseng (WF + GS) (mean ± SD).

Subject No	WF alone		WF + SJW		WF + GS	
	fu of S-warfarin	fu of R-warfarin	fu of S-warfarin	fu of R-warfarin	fu of S-warfarin	fu of R-warfarin
1	0.004	0.004	0.003	0.003	0.005	0.004
2	0.004	0.005	0.004	0.005	0.005	0.005
3	0.004	0.005	0.005	0.005	0.005	0.006
4	0.005	0.005	0.004	0.004	0.004	0.004
5	0.004	0.005	0.004	0.005	0.004	0.005
6	0.003	0.004	0.004	0.004	0.002	0.004
7	0.002	0.004	0.002	0.005	0.003	0.004
8	0.003	0.005	0.003	0.005	0.002	0.004
9	0.003	0.005	0.004	0.006	0.003	0.005
10	0.005	0.004	0.005	0.004	0.005	0.004
11	0.002	0.005	0.004	0.004	0.007	0.004
12	0.002	0.006	0.002	0.006	0.002	0.006
Mean	0.003	0.005	0.004	0.005	0.004	0.005
SD	0.001	0.001	0.001	0.001	0.002	0.001

Appendix 14. Individual $AUC_{0-\infty}$ for warfarin in study I following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either St John's wort (WF + SJW) or ginseng (WF + GS) (mean ± SD).

Subject No	WF alone		WF + SJW		WF + GS	
	$AUC_{0-\infty}$ of S-warfarin (ng/ml×h)	$AUC_{0-\infty}$ of R-warfarin (ng/ml×h)	$AUC_{0-\infty}$ of S-warfarin (ng/ml×h)	$AUC_{0-\infty}$ of R-warfarin (ng/ml×h)	$AUC_{0-\infty}$ of S-warfarin (ng/ml×h)	$AUC_{0-\infty}$ of R-warfarin (ng/ml×h)
1	50900	101800	39500	82200	50300	93100
2	57100	88700	45100	80000	54500	83400
3	82500	165100	55900	113900	61400	121000
4	63500	129800	45300	93800	67300	121900
5	61400	100200	52200	76500	48000	96300
6	82500	120700	40200	58400	60007	90600
7	78000	107900	66000	99300	60500	94400
8	52400	142500	44800	104200	46300	126200
9	56700	89100	36200	77600	59700	96800
10	89200	202400	45400	104600	68000	148300
11	59300	95300	55400	97400	65000	119300
12	51400	107000	46400	104900	52000	105300
Mean	65400	120900	47700	91100	57800	108100
SD	13800	32900	8300	15400	7400	18300

Appendix 15. Individual t_{max} for warfarin in study I following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either St John's wort (WF + SJW) or ginseng (WF + GS) (mean ± SD).

Subject No	WF alone		WF + SJW		WF + GS	
	t_{max} of S-warfarin (h)	t_{max} of R-warfarin (h)	t_{max} of S-warfarin (h)	t_{max} of R-warfarin (h)	t_{max} of S-warfarin (h)	t_{max} of R-warfarin (h)
1	1.1	1.1	1.0	1.0	1.0	1.0
2	2.3	2.3	2.0	2.0	1.0	1.0
3	1.0	1.0	1.8	1.8	1.8	1.8
4	1.2	1.2	1.0	1.0	1.0	1.0
5	1.0	1.0	1.0	2.0	1.7	1.7
6	1.9	1.9	0.9	0.9	1.0	1.0
7	1.0	1.0	1.9	1.9	0.9	0.9
8	2.0	2.0	0.9	0.9	0.9	0.9
9	1.5	1.5	0.9	0.9	1.0	1.0
10	0.9	1.5	0.9	1.8	2.4	2.4
11	0.7	0.7	1.8	1.8	0.8	0.8
12	0.9	0.9	1.0	1.0	2.1	2.1
Mean	1.3	1.3	1.3	1.3	1.3	1.3
SD	0.5	0.5	0.5	0.5	0.6	0.5

Appendix 16. Individual C_{max} for warfarin in study I following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either St John's wort (WF + SJW) or ginseng (WF + GS) (mean ± SD).

Subject No	WF alone		WF + SJW		WF + GS	
	C_{max} of S-warfarin (ng/ml)	C_{max} of R-warfarin (ng/ml)	C_{max} of S-warfarin (ng/ml)	C_{max} of R-warfarin (ng/ml)	C_{max} of S-warfarin (ng/ml)	C_{max} of R-warfarin (ng/ml)
1	1860	1750	1680	1720	1940	1880
2	1450	1480	1560	1680	2200	2110
3	2230	2370	1920	1980	2030	2100
4	1810	1850	1870	1820	2310	2020
5	1460	1420	1460	1410	1410	1450
6	2050	2360	1290	1240	1850	1740
7	1950	1860	1540	1660	1520	1470
8	1810	1890	2050	2020	2110	2110
9	1880	1900	1810	1800	1800	1740
10	2130	2250	2100	2180	1950	2040
11	2160	2000	2530	2670	2370	2330
12	1900	1820	2010	1960	1700	1700
Mean	1890	1920	1820	1840	1930	1890
SD	260	320	340	360	310	290

Appendix 17. Individual $t_{1/2}$ for warfarin in study I following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either St John's wort (WF + SJW) or ginseng (WF + GS) (mean ± SD).

Subject No	WF alone		WF + SJW		WF + GS	
	$t_{1/2}$ of S-warfarin (h)	$t_{1/2}$ of R-warfarin (h)	$t_{1/2}$ of S-warfarin (h)	$t_{1/2}$ of R-warfarin (h)	$t_{1/2}$ of S-warfarin (h)	$t_{1/2}$ of R-warfarin (h)
1	26.0	47.1	23.6	39.8	27.0	42.5
2	30.5	42.0	25.6	37.7	25.2	37.7
3	34.3	64.8	24.6	41.7	25.6	46.5
4	27.3	54.6	24.5	41.8	26.7	50.6
5	36.7	52.1	26.5	36.9	41.5	56.8
6	37.3	40.3	27.6	38.7	26.0	37.9
7	36.7	51.3	35.9	47.8	34.5	51.3
8	26.3	58.2	20.8	41.5	25.5	51.3
9	29.5	43.9	28.8	42.5	27.3	41.3
10	36.9	70.7	20.4	43.0	35.0	63.0
11	31.5	40.8	22.7	32.5	30.0	42.5
12	26.9	54.6	20.7	40.3	25.7	53.3
Mean	31.7	51.7	25.1	40.3	29.2	47.9
SD	4.5	9.6	4.3	3.9	5.2	7.8

Appendix 18. Individual CL/F for warfarin in study I following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either St John's wort (WF + SJW) or ginseng (WF + GS) (mean ± SD).

Subject No	WF alone		WF + SJW		WF + GS	
	CL/F of S-warfarin (ml/h)	CL/F of R-warfarin (ml/h)	CL/F of S-warfarin (ml/h)	CL/F of R-warfarin (ml/h)	CL/F of S-warfarin (ml/h)	CL/F of R-warfarin (ml/h)
1	246	123	316	152	24	134
2	219	141	278	156	229	150
3	152	76	224	110	204	103
4	197	96	276	133	186	103
5	204	125	240	164	261	130
6	152	104	311	214	206	138
7	160	116	189	126	207	132
8	239	88	279	120	270	99
9	221	140	345	161	209	129
10	140	62	275	120	184	84
11	211	131	225	128	192	105
12	242	117	269	119	240	119
Mean	198	110	270	142	220	119
SD	38	25	44.0	29	29	20

Appendix 19. Individual V/F for warfarin in study I following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either St John's wort (WF + SJW) or ginseng (WF + GS) (mean ± SD).

Subject No	WF alone		WF + SJW		WF + GS	
	V/F of S-warfarin (L/kg)	V/F of R-warfarin (L/kg)	V/F of S-warfarin (L/kg)	V/F of R-warfarin (L/kg)	V/F of S-warfarin (L/kg)	V/F of R-warfarin (L/kg)
1	0.14	0.13	0.16	0.13	0.15	0.12
2	0.15	0.13	0.16	0.13	0.13	0.13
3	0.11	0.11	0.12	0.10	0.11	0.10
4	0.10	0.10	0.13	0.11	0.09	0.10
5	0.10	0.09	0.08	0.08	0.14	0.10
6	0.09	0.07	0.14	0.13	0.08	0.08
7	0.09	0.09	0.10	0.09	0.11	0.10
8	0.18	0.14	0.16	0.14	0.19	0.14
9	0.13	0.13	0.20	0.14	0.12	0.11
10	0.11	0.09	0.12	0.11	0.14	0.11
11	0.14	0.11	0.11	0.09	0.12	0.09
12	0.14	0.14	0.12	0.11	0.14	0.14
Mean	0.12	0.10	0.13	0.10	0.13	0.10
SD	0.03	0.02	0.03	0.02	0.03	0.02

Appendix 20. Individual $INR_{baseline}$ in study I during control and pre-treatment with St John's wort (SJW) or Ginseng (GS) (mean ± SD).

Subject No	Control	SJW	GS
1	1.15	1.14	1.16
2	1.12	1.08	1.08
3	1.04	1.05	1.08
4	1.02	1.03	1.05
5	1.25	1.14	1.18
6	1.16	1.13	1.14
7	1.17	1.08	1.16
8	1.27	1.20	1.19
9	1.14	1.14	1.10
10	1.10	1.15	1.07
11	1.14	1.21	1.21
12	1.09	1.10	1.13
Mean	1.14	1.12	1.13
SD	0.07	0.06	0.05

Appendix 21. Individual $AUC_{0\text{-}168}$ of INR for warfarin in study I following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either St John's wort (WF + SJW) or ginseng (WF + GS) (mean ± SD).

Subject No	WF	WF + SJW	WF + GS
1	58.8	37.8	49.9
2	151.1	107.0	158.7
3	203.0	109.8	147.9
4	133.2	78.0	152.2
5	60.2	54.5	79.8
6	184.6	140.9	180.7
7	110.0	85.8	112.3
8	75.9	58.6	58.1
9	102.7	111.3	126.1
10	114.9	88.3	90.3
11	62.1	66.2	65.6
12	116.4	121.5	113.9
Mean	111.0	88.3	111.1
SD	49.3	30.7	43.1

Appendix 22. Individual t_{max} of INR for warfarin in study I following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either St John's wort (WF + SJW) or ginseng (WF + GS) (mean ± SD).

Subject No	WF	WF + SJW	WF + GS
1	25.3	25.7	25.7
2	49.3	53.3	54.7
3	49.5	49.0	48.0
4	75.0	49.3	48.7
5	25.0	27.1	24.3
6	50.2	25.4	47.0
7	49.8	51.5	25.6
8	25.9	24.9	49.0
9	48.8	48.9	50.4
10	72.8	48.8	48.1
11	25.1	24.4	24.7
12	49.6	49.1	48.6
Mean	45.5	39.8	41.2
SD	17.4	12.7	12.1

Appendix 23. Individual INR_{max} for warfarin in study I following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either St John's wort (WF + SJW) or ginseng (WF + GS) (mean ± SD).

Subject No	WF	WF + SJW	WF + GS
1	1.6	1.6	1.6
2	3.2	2.6	3.5
3	3.5	2.3	3.3
4	2.6	2.2	3.2
5	1.7	1.6	2.0
6	3.8	3.1	3.9
7	1.7	2.2	2.5
8	2.0	2.0	1.7
9	3.0	2.8	3.2
10	2.3	2.0	2.0
11	1.6	1.9	1.9
12	2.2	2.7	2.4
Mean	2.4	2.2	2.6
SD	0.8	0.5	0.8

Appendix 24. Individual platelet aggregation for warfarin in study I during control, St John's wort (SJW) or ginseng (GS) pre-treatment (mean ± SD).

Subject No	Control	SJW	GS
1*	.	.	.
2	7.5	7.5	7.0
3	3.5	5.0	5.0
4	3.0	7.0	5.5
5	10.0	7.0	7.5
6	6.5	7.0	6.0
7	11.5	9.5	8.0
8	10.0	8.5	8.5
9	7.5	8.5	7.5
10	6.5	6.0	5.5
11	7.5	8.0	6.0
12	7.5	7.0	9.0
Mean	7.7	7.5	7.1
SD	2.2	1.1	1.4

* Not available

Appendix 25. Individual fraction of unbound (fu) warfarin in study II following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger) (mean ± SD).

Subject No	WF alone		WF + Ginkgo		WF + Ginger	
	fu of S-warfarin	fu of R-warfarin	fu of S-warfarin	fu of R-warfarin	fu of S-warfarin	fu of R-warfarin
1	0.004	0.004	0.004	0.005	0.005	0.006
2	0.007	0.006	0.008	0.006	0.007	0.005
3	0.003	0.004	0.004	0.004	0.004	0.004
4	0.004	0.005	0.004	0.005	0.006	0.006
5	0.005	0.005	0.004	0.004	0.005	0.005
6	0.005	0.005	0.004	0.004	0.004	0.004
7	0.007	0.007	0.005	0.005	0.005	0.006
8	0.007	0.003	0.006	0.003	0.007	0.004
9	0.005	0.005	0.005	0.004	0.004	0.004
10	0.005	0.004	0.005	0.005	0.005	0.004
11	0.005	0.004	0.005	0.005	0.005	0.005
12	0.005	0.005	0.005	0.006	0.005	0.005
Mean	0.005	0.005	0.005	0.005	0.005	0.005
SD	0.001	0.001	0.001	0.001	0.001	0.001

Appendix 26. Individual $AUC_{0-\infty}$ for warfarin in study II following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger) (mean ± SD).

Subject No	WF alone		WF + Ginkgo		WF + Ginger	
	$AUC_{0-\infty}$ of S-warfarin (ng/ml×h)	$AUC_{0-\infty}$ of R-warfarin (ng/ml×h)	$AUC_{0-\infty}$ of S-warfarin (ng/ml×h)	$AUC_{0-\infty}$ of R-warfarin (ng/ml×h)	$AUC_{0-\infty}$ of S-warfarin (ng/ml×h)	$AUC_{0-\infty}$ of R-warfarin (ng/ml×h)
1	72700	149300	61600	135300	60200	127800
2	70800	83700	63000	89900	52400	72800
3	74500	130300	56900	109900	63600	116800
4	71000	113700	55700	97000	46700	90800
5	82500	119300	93300	121300	86600	116000
6	52100	112700	54900	99500	47400	116700
7	64000	60700	72000	70600	62300	57300
8	86400	82300	101000	107600	74400	71600
9	73800	90900	76800	98900	114600	137100
10	62500	111900	55700	117700	67900	122700
11	56700	112100	49100	99600	62400	113100
12	49500	81800	49800	78700	54300	88400
Mean	68000	104000	65800	102200	66000	102600
SD	11400	24700	16800	18000	19000	25500

Appendix 27. Individual t_{max} for warfarin in study II following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger) (mean ± SD).

Subject No	WF alone		WF + Ginkgo		WF + Ginger	
	t_{max} of S-warfarin (h)	t_{max} of R-warfarin (h)	t_{max} of S-warfarin (h)	t_{max} of R-warfarin (h)	t_{max} of S-warfarin (h)	t_{max} of R-warfarin (h)
1	1.9	1.9	1.2	1.2	2.0	2.0
2	1.9	1.9	1.9	1.9	1.0	1.0
3	1.0	1.0	1.1	1.1	1.0	1.0
4	2.1	2.1	1.0	1.0	1.0	1.0
5	3.5	3.5	1.0	1.0	0.7	0.7
6	1.2	1.2	0.8	0.8	3.8	3.8
7	1.0	1.0	1.0	1.0	1.5	1.5
8	2.0	2.0	1.0	1.0	1.7	1.7
9	1.2	1.2	0.9	0.9	1.2	1.2
10	1.1	1.1	1.9	1.9	2.0	2.0
11	4.0	4.0	3.7	6.9	2.1	2.1
12	3.9	3.9	1.0	1.0	1.1	1.1
Mean	2.1	2.1	1.4	1.6	1.6	1.6
SD	1.1	1.1	0.8	1.7	0.8	0.8

Appendix 28. Individual C_{max} for warfarin in study II following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger) (mean ± SD).

Subject No	WF alone		WF + Ginkgo		WF + Ginger	
	C_{max} of S-warfarin (ng/ml)	C_{max} of R-warfarin (ng/ml)	C_{max} of S-warfarin (ng/ml)	C_{max} of R-warfarin (ng/ml)	C_{max} of S-warfarin (ng/ml)	C_{max} of R-warfarin (ng/ml)
1	2500	2600	2300	2300	2000	2100
2	1800	1800	1500	1600	1600	1500
3	2200	2100	1600	1600	1600	1600
4	2400	2300	2200	2300	1800	1800
5	1400	1400	2100	2100	1700	1700
6	1700	1700	2100	2100	1300	1500
7	1100	1100	1600	1500	1200	1200
8	1500	1500	2000	1800	1200	1200
9	2000	2000	1900	1800	2600	2400
10	1700	1600	1500	1500	2100	2000
11	1300	1300	1000	1100	1500	1600
12	1300	1300	1500	1400	2000	1900
Mean	1700	1700	1800	1800	1700	1700
SD	500	500	400	400	400	400

Appendix 29. Individual $t_{1/2}$ for warfarin in study II following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger) (mean ± SD).

Subject No	WF alone		WF + Ginkgo		WF + Ginger	
	$t_{1/2}$ of S-warfarin (h)	$t_{1/2}$ of R-warfarin (h)	$t_{1/2}$ of S-warfarin (h)	$t_{1/2}$ of R-warfarin (h)	$t_{1/2}$ of S-warfarin (h)	$t_{1/2}$ of R-warfarin (h)
1	28.0	50.0	25.0	48.0	25.0	45.0
2	34.0	40.0	33.0	43.0	30.0	36.0
3	31.0	50.0	34.0	55.0	35.0	53.0
4	29.0	46.0	28.0	42.0	25.0	39.0
5	42.0	58.0	40.0	52.0	44.0	56.0
6	32.0	61.0	33.0	46.0	30.0	58.0
7	51.0	48.0	46.0	43.0	47.0	41.0
8	46.0	44.0	47.0	46.0	47.0	43.0
9	38.0	45.0	38.0	47.0	49.0	53.0
10	36.0	62.0	35.0	61.0	30.0	51.0
11	32.0	55.0	31.0	56.0	37.0	58.0
12	30.0	45.0	31.0	44.0	29.0	39.0
Mean	35.8	50.3	35.1	48.6	35.7	47.7
SD	7.2	7.1	6.7	6.1	8.9	8.0

Appendix 30. Individual CL/F for warfarin in study II following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger) (mean ± SD).

Subject No	WF alone		WF + Ginkgo		WF + Ginger	
	CL/F of S-warfarin (ml/h)	CL/F of R-warfarin (ml/h)	CL/F of S-warfarin (ml/h)	CL/F of R-warfarin (ml/h)	CL/F of S-warfarin (ml/h)	CL/F of R-warfarin (ml/h)
1	172	84	203	92	208	98
2	177	149	198	139	239	172
3	168	96	220	114	197	107
4	176	110	224	129	268	138
5	151	105	134	103	144	108
6	240	111	228	126	264	107
7	195	206	174	177	201	218
8	145	152	124	116	168	175
9	170	138	163	126	109	91
10	200	112	225	106	184	102
11	220	112	254	125	200	111
12	253	153	251	159	230	141
Mean	188	127	200	126	201	131
SD	33	34	43	24	47	39

Appendix 31. Individual V/F for warfarin in study II following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger) (mean ± SD).

Subject No	WF alone		WF + Ginkgo		WF + Ginger	
	V/F of S-warfarin (L/kg)	V/F of R-warfarin (L/kg)	V/F of S-warfarin (L/kg)	V/F of R-warfarin (L/kg)	V/F of S-warfarin (L/kg)	V/F of R-warfarin (L/kg)
1	0.13	0.12	0.14	0.12	0.14	0.12
2	0.11	0.11	0.12	0.11	0.13	0.11
3	0.10	0.09	0.14	0.12	0.13	0.13
4	0.11	0.10	0.13	0.11	0.14	0.11
5	0.13	0.12	0.11	0.11	0.13	0.12
6	0.14	0.13	0.14	0.11	0.15	0.11
7	0.14	0.14	0.11	0.11	0.13	0.13
8	0.13	0.13	0.12	0.11	0.16	0.15
9	0.13	0.12	0.12	0.11	0.10	0.09
10	0.14	0.14	0.15	0.13	0.11	0.10
11	0.10	0.09	0.12	0.10	0.11	0.09
12	0.15	0.13	0.15	0.14	0.13	0.11
Mean	0.12	0.12	0.12	0.11	0.12	0.11
SD	0.02	0.02	0.01	0.01	0.02	0.02

Appendix 32. Individual $INR_{baseline}$ for warfarin in study II during control, ginkgo or ginger pre-treatment (mean ± SD).

Subject No	Control	Ginkgo	Ginger
1	1.1	1.1	1.1
2	1.0	1.1	1.1
3	1.1	1.2	1.1
4	1.1	1.1	1.0
5	1.1	1.2	1.2
6	1.0	1.0	1.0
7	1.2	1.2	1.2
8	1.3	1.2	1.2
9	1.1	1.1	1.1
10	1.1	1.1	1.1
11	1.2	1.2	1.1
12	1.1	1.2	1.2
Mean	1.1	1.1	1.1
SD	0.1	0.1	0.1

Appendix 33. Individual t_{max} of INR for warfarin in study II following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger) (mean ± SD).

Subject No	WF alone	WF + Ginkgo	WF + Ginger
1	48.9	24.1	25.5
2	49.7	25.6	25.9
3	49.4	50.4	49.9
4	49.5	51.0	50.8
5	50.9	72.2	48.3
6	28.6	48.3	48.1
7	49.2	73.3	49.3
8	49.4	49.3	48.9
9	26.2	31.0	25.7
10	48.7	25.5	49.7
11	49.0	48.3	49.0
12	48.8	49.3	48.7
Mean	45.7	45.7	43.3
SD	8.6	16.6	10.7

Appendix 34. Individual INR_{max} for warfarin in study II following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger) (mean ± SD).

Subject No	WF alone	WF + Ginkgo	WF + Ginger
1	2.04	1.86	1.86
2	1.87	1.70	1.59
3	3.19	2.80	2.70
4	2.45	2.18	2.80
5	3.65	4.27	3.80
6	2.27	2.47	2.82
7	2.73	2.79	2.54
8	2.96	3.01	2.27
9	2.02	1.79	2.21
10	1.85	1.67	2.99
11	2.99	3.33	2.95
12	2.53	2.47	2.99
Mean	2.60	2.50	2.60
SD	0.60	0.80	0.60

Appendix 35. Individual AUC_{0-168} of INR for warfarin in study II following administration a single oral 25 mg *rac*-warfarin (WF) alone, in combination with either ginkgo (WF + Ginkgo) or ginger (WF + Ginger) (mean ± SD).

Subject No	WF alone	WF + Ginkgo	WF + Ginger
1	78.0	70.0	74.0
2	67.0	47.0	52.0
3	161.0	119.0	143.0
4	123.0	90.0	107.0
5	248.0	297.0	267.0
6	97.0	108.0	121.0
7	139.0	163.0	130.0
8	151.0	167.0	129.0
9	70.0	61.0	83.0
10	74.0	59.0	97.0
11	172.0	155.0	165.0
12	108.0	114.0	137.0
Mean	124.0	121.0	125.0
SD	54.0	69.0	55.0

Appendix 36. Individual baseline platelet aggregation for warfarin in study II during control, ginkgo or ginger pre-treatment (mean ± SD).

Subject No	Control	Ginkgo	Ginger
1	7.5	8.0	7.0
2	5.5	7.5	8.0
3	5.5	8.5	8.0
4	6.0	9.0	7.5
5	7.5	9.0	7.5
6	7.5	8.0	9.0
7	7.5	10.5	9.5
8	7.5	8.0	6.5
9	7.0	7.5	9.5
10	11.0	11.0	8.0
11	8.5	6.5	8.5
12	8.5	7.5	8.0
Mean	7.5	8.4	8.1
SD	1.5	1.3	0.9