IN VIVO ASPECTS OF POTENTIAL STEREOSPECIFIC DRUG INTERACTIONS OF ORAL WARFARIN AND RUTIN IN RATS

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Dedicated to two of my most special friends who have made this possible for me,

Vishwa and Napsiah

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PRESENTATION:

1. Poster presentation at "2nd Asia-Pacific Conference and Exhibition on Anti-Ageing Medicine 2003", Singapore.
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SUMMARY

Polypharmacy is prevalent among most of the therapeutic regimens to treat patients. Approximately one third of adults in the United States take complementary and alternative medicines (CAM) (1). Interactions between these drugs may affect the pharmacological or adverse effects of each other and complicate the management of long-term drug therapies.

Numerous drug interactions are observed with oral anticoagulant warfarin. The effects of warfarin are highly sensitive to the co-administered drugs. Warfarin therapy is complicated by the fact that it has a narrow therapeutic index and its enantiomers vary in pharmacokinetic and pharmacodynamic properties. Flavonoids are known to affect the bioavailability of drugs through cytochrome P450 modulation (2, 3, 4, 5, 6). Rutin, a flavonoid glycoside, and its aglycone quercetin are abundant in nature, especially in fruits and vegetables. Rutin is also widely found as a constituent of multivitamin preparations and herbal remedies. The present study was designed to investigate the potential drug interactions between rutin and warfarin in rats.

In the single dose study, rats pretreated for four days with oral rutin (1 g in 1% CM-cellulose/kg) or an equal volume (5 ml/kg) of 1% CM-cellulose (as the control), were given a single dose of racemic warfarin (1.5 mg/kg) orally. With the rutin regimen continued, blood samples were collected at different intervals over 96 h. In the multiple dose study, rats pretreated for five days with oral warfarin (0.15 mg/kg/day) to attain steady state, were given rutin (1 g in 1% CM-cellulose/kg) or an equal volume (5 ml/kg) of 1% CM-cellulose (as the control) orally along with the daily

warfarin for another six days. Blood samples were collected at different intervals over 168 h. The S- and R- enantiomers of warfarin in serum were separated and analyzed by high performance liquid chromatography. Plasma prothrombin time was measured.

With the single dose of warfarin, hypoprothrombinaemia, as measured by reduced percentage of normal prothrombin complex activity, was observed in both rutin treated and control rats, but the recovery was found to be much faster in rutin treatment group compared to control animals. Of both S- and R- warfarin, the maximum serum concentration values, were increased, while the elimination half-life and apparent volume of distribution values, were significantly reduced with rutin treatment. There was an apparent increase in the rate of absorption and decrease in the time to reach peak serum concentration of both the enantiomers, though not statistically significant. With multiple doses of warfarin, rutin treatment resulted in higher percentage of normal prothrombin complex activity compared to control. Both rutin treated and control animals showed steady state serum levels of S- and R-warfarin with lower values of S- warfarin in the former group. Rutin treatment showed a trend to increase the steady state clearance, reduce the volume of distribution and elimination half-life, of S- warfarin.

These results indicate a potential interaction between rutin and warfarin. As rutin and quercetin are present in numerous diets of plant origin, precaution must be taken before starting warfarin therapy in subjects who are on a diet rich in these bioflavonoids.

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ABBREVIATIONS

AUC, total area under the serum concentration-time curve; C_{max}, maximum (peak) serum concentration; t_{max}, peak time; CL, total serum clearance; CM-Cellulose, carboxy methyl cellulose; CYP, cytochrome P-450; ka, absorption rate constant; PCA, prothrombin complex activity; PCA_{min}, minimum value of PCA; $t_{PCA,min}$, time to achieve PCA_{min}; PCA AUC_{0-96}, total area under the PCA–time curve from time zero to 96 h; PT, prothrombin time; PT_0, basal mean prothrombin time; $t_{1/2}$, elimination half-life; Vd, apparent volume of distribution.

CHAPTER 1 INTRODUCTION

Warfarin, a coumarin oral anticoagulant, is frequently used for the treatment and prevention of thromboembolic diseases. Racemate warfarin (mixture of R- and S-enantiomers) is normally used in clinical practice. S-warfarin is approximately five times more active than the R- isomer (7, 8) and is responsible for essentially all of the anticoagulant effects of the drug. It is also found to be more prone to drug interactions. Warfarin is highly bound to plasma protein (97.4-99%), especially albumin (8, 9). Given orally, warfarin is completely absorbed (10) and is metabolized by hepatic CYPs (11). S-Warfarin is metabolized by CYP2C9 to S-7-hydroxywarfarin in humans (11, 12), whereas R-warfarin is mainly oxidized to R-6-hydroxywarfarin, primarily by CYP1A2 and CYP3A4 (11, 12). Warfarin is a low extraction ratio drug (10).

Flavonoids, an important family of antioxidants, are ubiquitous in edible plants, fruits, foods and medicinal botanicals (13, 14, 15). Flavonoids are known to affect the bioavailability of drugs through cytochrome P-450 (CYP) (15) and/or P-glycoprotein modulation (2, 3, 4, 5, 16). In general they occur in food as glycosides (4, 14). Rutin is a flavonoid glycoside abundant in the plant kingdom (14). Rutin derivatives (e.g. oxerutin and troxerutin) are used to treat various cardiovascular conditions (17, 18, 19). Reports have shown its usefulness in treating abnormal fragility of the capillaries and as a vasoprotectant (20, 21, 22). Rutin is also reported to relieve venous insufficiency of the lower limbs and capillary impairment (18, 19). Rutin and its derivatives have been combined with oral anticoagulant such as warfarin in cardiovascular patients (23). Therefore, it could directly or indirectly influence some

of the metabolic pathways and thus affect the pharmacological actions of the co-administered drug.

Quercetin, the aglycone of rutin (24), meets the structural requirements for a strong antioxidant (24). Both quercetin and rutin form an integral part of various nutritional supplements and herbal preparations (25). Estimated average intake of quercetin in the USA is 20-22 mg per day (26). Consumption of rutin or quercetin diet resulted in the same conjugated metabolites in rat (14), pig (27) and human plasma (25, 28). Quercetin binds strongly to human serum albumin (HSA) (up to 99.1-99.4 ± 0.5 %) *in vitro* (24). When administered for a long time, quercetin accumulates in the blood and this could be attributed to its long elimination half-life (29). Quercetin has been shown to inhibit CYP3A4 enzymes in human microsomes *in vitro* (30), the enzyme responsible for the metabolism of R- enantiomer of warfarin. It also modulates P-glycoprotein, a plasma membrane transporter (13).

The present study was carried out to explore the pharmacokinetic and pharmacodynamic interactions of warfarin with rutin in rats, since warfarin is a commonly used oral anticoagulant (31) with wide variation in dose-response (32) and narrow therapeutic index and rutin is present in most of the foods, vegetables and dietary supplements. A retrospective clinical study has shown that patients with severe nonreconstructable chronic critical leg ischemia benefited from initial therapy with intravenous rutin combined with long-term oral warfarin treatment (23). To our knowledge, there have been no studies on the interactions between warfarin and rutin in rats. Thus, it was imperative to study the potential interactions between the two compounds.

1.1. LITERATURE REVIEW

Warfarin is one of the most frequently used anticoagulants for the treatment and prevention of thromboembolic diseases. It has the advantage over heparin as it can be administered orally in long-term therapy. With low costs, it turns out to be economical to patients if taken for years. Some of the indications for warfarin therapy are listed out in **Table 1.1**.

1.1.1. HISTORICAL PERSPECTIVE OF WARFARIN

Oral anticoagulants were discovered accidentally in the 1920s when livestock in North Dakota fed decomposed sweet clover developed bleeding disorder (33, 34). The animals were progressively unable to form clots and bled to death after 30 days of ingestion. The disorder was reversible when the clover was removed from the feed or when the animals were transfused with fresh blood from unaffected animals. Vitamin K administration also reversed the condition. In 1934, bishydroxycoumarin (dicoumarol) was isolated and identified as the cause of the disorder. Warfarin, a coumarin derivative, was synthesized in 1944 and used as a highly effective rat poison initially. In 1951, an army inductee survived a suicide attempt after ingesting 567mg of warfarin (34). The hemorrhage was reversed by administering vitamin K and blood transfusion. This led to the widespread use of warfarin in clinics as an effective anticoagulant with vitamin K as an antidote if bleeding complications arose.

Table 1.1 Indications for warfarin therapy

Treatment	Target INR/ treatment duration	Reference	Prophylactic therapy in patients	Target INR/ treatment duration	Reference
Deep venous thrombosis	2-3 / 3 months	61	Prosthetic heart valves	2-3.5-4.5 (3-12 months/ long-term)	128
Pulmonary embolism	2-3 / 3 months	61	Stents, filter devices, indwelling catheters in blood vessels	2.5-3.5/ lifelong	37
Arterial Thromboses	>3 / long-term	37			
Unstable angina	2-3	37	Atrial fibrillation	2-3 / long-term	129
Peripheral vascular disease	2-3	37	Myocardial infarction	2-3 / long-term	37
			Valvular heart diseases	2-3 / long-term	130
Certain renal diseases	-	37	Post-surgery or immobilization	1.6-2.3 / 4-6 weeks (low dose)	61
Small cell lung cancer	-	37			
Recurrent venous thrombo-embolism	2-2.85/ long-term	131	Antiphospholipid syndrome	3-4 / lifelong	34
			Antithrombin deficiency and Factor V Leiden mutation	3-6 months or long-term	34

1.1.2. CHEMISTRY

Warfarin [3-(α-acetonylbenzyl)-4-hydroxy coumarin] is a single-ring coumarin derivative (8) (**Fig. 1.1**). It is weakly acidic (pK_a 5.1) (35), insoluble in water and shows natural fluorescence with excitation at 290-342 nm and emission at 385 nm (36). The 4-hydroxycoumarin residue of warfarin with a nonpolar carbon substituent at the 3-position (asymmetrical carbon), is required for the pharmacodynamic properties (37). The 4-hydroxycoumarin ring binds to the reductase receptor. The side chain affects the disposition and metabolism of warfarin. Clinically warfarin is available as a racemic mixture of (R)- and (S)- enantiomers.

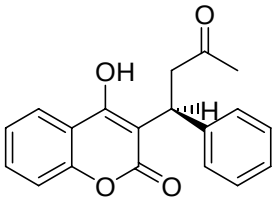
1.1.3. PHARMACODYNAMICS

1.1.3.1. Mechanism of action

Vitamin K is an important cofactor for the enzymatic pathway of blood coagulation. It is essential for the γ-carboxylation of glutamate residues on inactive forms of clotting factors to γ-carboxyglutamic acid (8, 34, 38, 39, 40, 41, 42, 43). γ-carboxylation permits these coagulation proteins to undergo a conformational change in the presence of calcium ions (43, 44). In the absence of γ-carboxylation clotting factors are unable to bind to calcium ions and phospholipid surfaces through calcium ion bridges (8, 34) and have reduced activity.

Warfarin acts as an anticoagulant by reducing the synthesis of the vitamin K-dependent clotting factors like factor II, VII, IX and X and thus decreases the risk of thrombosis (45, 46). The mechanism is not completely understood but involves

(S)-warfarin



(R)-warfarin

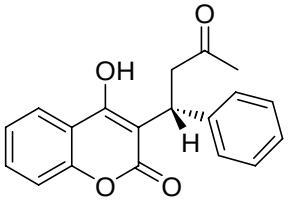


Figure 1.1 Warfarin chemical structure

inhibition of vitamin K reductase enzyme (**Fig. 1.2**), which is responsible for converting inactive vitamin K epoxide to active vitamin K (38, 39, 47). Thus warfarin blocks the regeneration of the active form of vitamin K and does so in a dose-dependent manner. The time to deplete vitamin K to a threshold level so as to affect the synthesis of clotting factors is responsible for the delay in onset of action after the warfarin dose (8). Vitamin K stores in liver are eventually depleted. The anticoagulant response to warfarin is unpredictable and requires a careful monitoring for potential interactions. Age, diet, illness, patient compliance, genetic factors, physical activity, concurrent drug therapy and other unknown factors can affect the response to warfarin.

1.1.4. PHARMACOKINETICS

Warfarin is a drug of choice for the pharmacokinetic modeling. It has a reliable onset and duration of action and good bioavailability. (S)-warfarin is approximately five times more potent (7, 8) and is metabolized more rapidly than the (R)- isomer in man (8, 48). The concentration of each isomer in plasma therefore varies within and among patients. (S)- isomer is responsible for essentially all of the anticoagulant response of the drug and is found to be more prone to drug interactions.

1.1.4.1. Absorption

Warfarin is absorbed rapidly and almost completely when administered orally (8). The bioavailability of racemic warfarin solutions is almost complete also when administered intramuscularly, rectally or intravenously (37). It is detectable in plasma

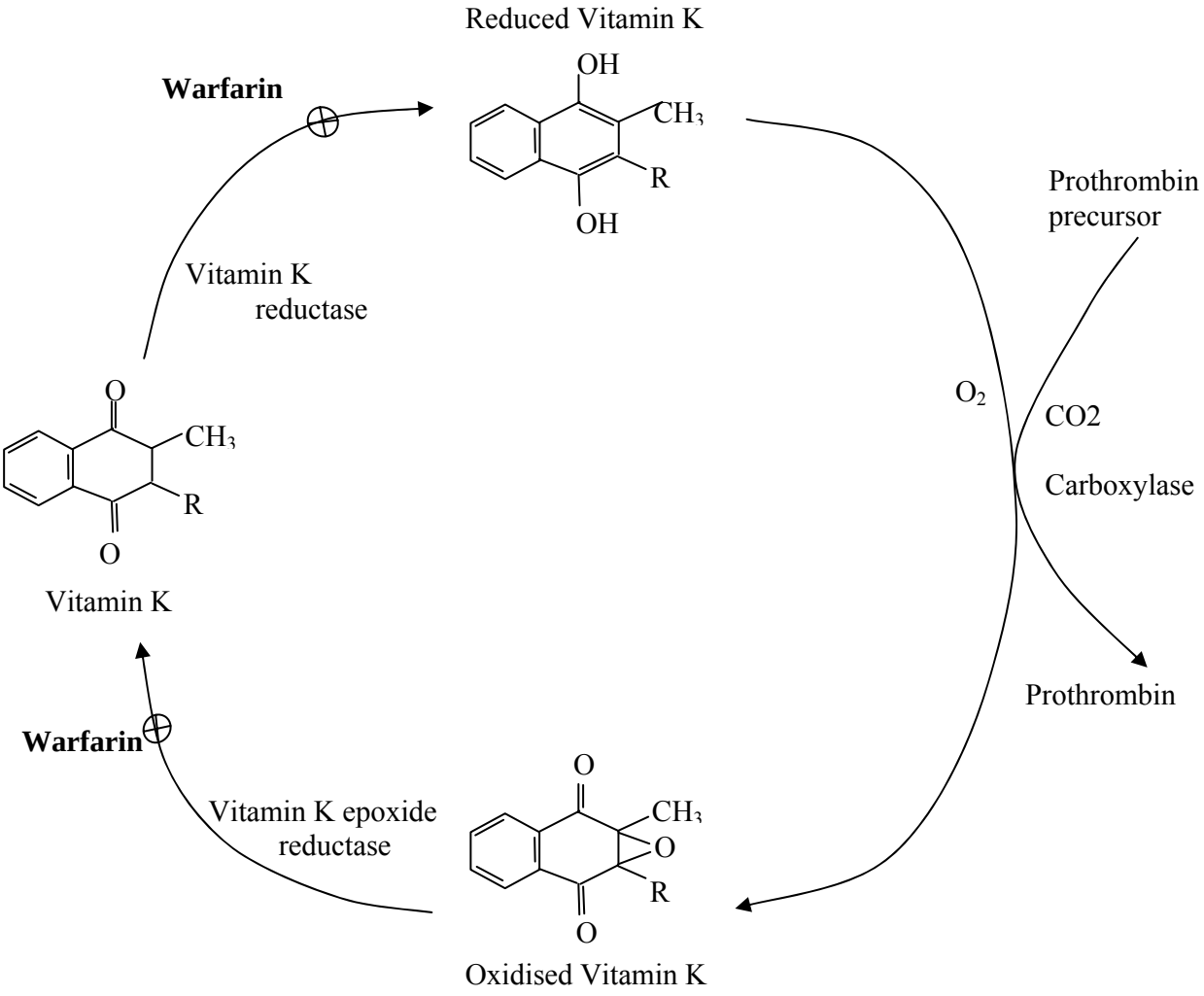


Figure 1.2 Mechanism of warfarin anticoagulation
Warfarin acts as an anticoagulant by reducing the synthesis of the vitamin K-dependent clotting factors. Its anticoagulant effects are achieved by interfering with cyclic interconversion of Vitamin K via inhibition of vitamin K reductase enzyme, which is responsible for converting inactive vitamin K epoxide to active vitamin K. Thus warfarin blocks the regeneration of the active form of vitamin K

within 1 hour of its oral absorption (37). Peak blood concentration is reached within 90 minutes (range between 2 to 8 hours) (37). The rate of absorption is delayed by the presence of food but the bioavailability is not affected. **Table 1.2** shows various pharmacokinetic parameters, estimated in rats after oral ingestion of single and multiple doses of warfarin (49).

1.1.4.2. Distribution

The volume of distribution of racemic warfarin in man is found to be 0.09-0.17 L/kg (8). Warfarin is highly bound (97.4-99.9 %) to plasma proteins, mainly albumin. The relative serum protein binding of warfarin is independent of concentration over a broad range (50). Bound warfarin lacks its activity and is protected from biotransformation and excretion (8). The protein binding affinity differs between the two enantiomers of warfarin. The S- isomer has a greater affinity to protein binding in man compared to the R- isomer. Some of the drug interactions of warfarin by protein binding displacement may involve only one of the two enantiomers. Thus, highly protein bound acidic drugs can displace warfarin stereospecifically from the binding sites (8). Such interactions may be clinically significant, but may go undetected if only the racemate warfarin concentration is measured. However displacement from protein binding sites may cause only transient increase in the concentration of unbound warfarin as the total body clearance is increased with more unbound drug available at the elimination sites (8).

Tissue binding of warfarin involves two classes of binding sites, one with high affinity and low capacity and the other with lower affinity and unlimited capacity (50)

Table 1.2 Pharmacokinetic parameters estimated in rats after oral ingestion of single and multiple doses of warfarin (49)

Parameters	Warfarin (oral)	
	Single dose (2 mg/kg)	Multiple doses (0.2 mg/kg/day, for 6 days)
C_{max} (µg/ml)	7.8 ± 0.5	9.4 ± 1.4
t_{max} (h)	2.6 ± 1.0	2.0 ± 0.0
AUC[a] (mg.h/ml)	118 ± 22	133 ± 10
$t_{1/2}$ (h)	14.3 ± 5.8	16.3 ± 3.0
Vd (ml/kg)	1483 ± 746	1419 ± 294
CL (ml/h)	17.5 ± 3.3	15.0 ±1.0

Values are mean ± S.D. (n = 6)
C_{max}, maximum plasma concentration; t_{max}, peak time; AUC, total area under plasma concentration-time curve; $t_{1/2,}$ elimination half life; Vd, apparent volume of distribution; CL, total plasma clearance; $PT_{0,}$ basal prothrombin time at time zero; $PT_{max,}$ maximal PT achieved; $T_{max,PT}$ time to PT_{max};
[a] $AUC_{0-\infty}$ and $AUC_{0-\tau}$ for single and multiple dose study, respectively.

Warfarin is extensively distributed to the liver and accumulates in microsomes to a large extent compared to other tissues (8) **(Table 1.3),** (50). Gathering evidence has shown the importance of hepatic uptake of warfarin as both plasma and tissue binding can affect the apparent volume of distribution and biological half-life of the drug (50). Hepatic uptake of warfarin is concentration dependent and is saturable with increasing concentration (50).

Body fat takes up warfarin by partitioning rather than by albumin binding (50). A substance with apparent unlimited capacity, other than albumin, could be responsible for warfarin binding in fat (50). Warfarin also crosses the placenta and is teratogenic, but active warfarin is not found in milk (37).

1.1.4.3. Metabolism and Excretion

Warfarin metabolism in humans is catalyzed mainly by cytochrome P450 isoenzymes in the liver (11). Also, the acetonyl side chain ketone moiety is reduced by ketone reductases (51). Metabolites include 4'-hydroxywarfarin, 6-hydroxy warfarin, 7-hydroxy warfarin, 8-hydroxy warfarin, 10-hydroxy warfarin, dehydrowarfarin and two pairs of diastereomeric warfarin alcohols (52). (S)-7-hydroxywarfarin is the predominant metabolite of (S)-warfarin in human and R-warfarin is mainly oxidized to (R)-6-hydroxy warfarin (12). The metabolites of warfarin do not appear to contribute to the activity of the drug. But metabolite estimation helps in understanding the source of variation and mechanism of interaction of drugs with warfarin. A better understanding of the warfarin drug interactions could be possible by either giving each enantiomer individually (a rare clinical practice) or using a stereo specific assay

Table 1.3 Apparent binding and tissue-serum partition constants for S-warfarin in rat tissues (50)

Tissue	$T_{max,}$ (μg/g)	K_d, (μg/ml)	R
Liver	0.666	-	0.251
Kidneys	0.161	-	0.195
Muscle	0.0405	-	0.0441
Fat	0.0174	8.46×10^{-4}	2.85

T_{max}, theoretical maximum capacity of the low capacity binding sites; K_d, dissociation- constant of S-warfarin-low capacity binding sites; R, ratio of the S-warfarin concentration in a tissue to that in serum.
T_{max} and R decreased in the order: liver > kidneys > muscle

to measure the plasma concentration of each isomer after the administration of the racemate drug (53).

Cytochrome P450 monooxygenase or CYP is the most important group of liver enzymes catalyzing phase- I metabolic biotransformation reactions (54). It has a broad range of substrate specificity. These heme-containing mixed-function oxidases catalyze the oxidation of a majority of chemical substances in the liver. Substrates of the human CYP isoenzymes are identified by assessing their metabolism directly with human CYP isoforms or by inhibiting their metabolism with antibodies or selective inhibitors. Hepatic P450 system consists of a number of isoforms.

CYP1 family is composed of at least two subfamilies, CYP1A and CYP1B. The well-characterized CYP1A subfamily consists of two members, CYP1A1 and CYP1A2. CYP1A2 is mainly confined to the liver. In humans it appears to be mainly responsible for the mutagenic activation of several heterocyclic amines. CYP2C subfamily is the largest of mammalian liver enzymes and has been studied most extensively (55). In humans, CYP2C8, CYP2C9, CYP2C18 and CYP2C19 have been expressed in liver, CYP2C9 being the most abundant. Human CYP2C enzymes metabolize a number of drugs, such as mephenytoin, warfarin, tolbutamide and phenytoin. (S)-warfarin 7-hydroxylation is almost exclusively catalyzed by CYP2C9 in humans (11). (R)- isomer 7-hydroxylation is carried out by several CYP enzymes, but primarily by CYP-1A2 and 3A4 (11).

CYP3A enzymes appear to be of great importance in man, major form being CYP3A4. It is the most versatile of the P450 superfamily, being involved in the

metabolism and activation of a number of structurally unrelated compounds (56). It is responsible for numerous drug interactions and is inducible by a number of structurally unrelated drugs and xenobiotics. Drugs metabolized by CYP3A4 include protease inhibitors, calcium channel blockers, benzodiazepines, estrogens, cyclosporins, cortisone etc (57). Significant amount of CYP3A4 is present in the gut wall epithelium and thus metabolizes many drugs before absorption (56).

Carbonyl reductases are a group of enzymes present widely in cytosolic and microsomal fractions of various tissues, but mainly in the liver. They reduce carbonyl groups of a number of drugs and endogenous substances and are NADPH- dependent. Ketone reduction results in the formation of alcohols. Microsomal ketone reductases differ from cytosolic ketone reductases in stereoselectivity. In in-vitro reduction of warfarin in the rat and in man, marked substrate selectivity is shown for (R)-enantiomer which is reduced mainly to (RS)- alcohol. The same stereoselectivity for product and substrate is shown in the in-vivo ketone reduction in man. In the rat, in-vivo reduction is selective for (S)- warfarin, reducing it to (SS)- alcohol (51).

Warfarin is excreted mainly as its metabolites by the kidneys in urine and stool. 15-20 % of the oral warfarin is excreted in the urine as the alcohols (11). The average warfarin plasma clearance rate is 0.045 $ml.min^{-1}.kg^{-1}$. The elimination half-life is between 25-60 hours (mean 40 hours) (37). Some of the pharmacokinetic parameters for warfarin elimination in different species are compiled in **Table 1.4**. The table can serve as a basis for comparing the pharmacokinetics of warfarin in different species. For example, the data shows that, unlike in man, S-warfarin is eliminated more slowly than R-warfarin in rats (58). Limitations of the data include, fewer number of animals

Table 1.4 Comparison of reported pharmacokinetic parameters of warfarin elimination in different animal species

Mean elimination half-life ($t_{1/2}$) (h) ±S.D				
Subject	S-Warfarin	R-Warfarin	Racemic warfarin*	Reference
Rat	13.85±4.33(10)	7.26±1.82(10)	11.56±3.73(10)	132
	22.4 [a] (11)	-	-	133
	23.6±4.6 (10)	17.5±2.2(10)	-	108
	-	-	7.07 [a] (13)	134
	-	-	10.4 [a] (2)	59
	19.5±3.4(10)	11.9±3.9(10)	-	104
Man	33±12.65(10)	58±15.81(10)	42±6.32(10)	7
	30.5±13.96(6)	41.2±4.16(6)	36.2±19.2(6)	135
	33±14.6(6)	43.4±8.4(6)	-	105
	37±35(12)	53±14(12)	-	136
	27.5±7.38(6)	36.8±14.23(6)	-	137
	36±13(6)	46±7(6)	-	138
	-	-	36.3±3.5(12)	139
	-	-	43±10(30)	59
	-	-	34 [a] (10)	134
Dog	-	-	22 [a] (2)	59
	-	-	18.4±4.6(4)	140
Monkey	-	-	14.4 [a] (4)	59
Rabbit	4.27±0.56(4)	6.39±0.36(4)	5.6±1.84(8)	141
	-	-	10.89±1.24(4)	142
Clearance (CL) (ml/min/kg body wt) ±S.D				
Subject	S-Warfarin	R-Warfarin	Racemate	Reference
Rat	0.08 [a] (11)	-	-	133
	-	-	0.36 [a] (13)	134

Man	0.0744±0.044	0.0357±0.014	-	142
	(36)	(36)		
	0.108±0.036(6)	0.077±0.017(6)	-	138
	-	-	0.0367 [a] (10)	134
	3.52±1.13 (6)	2.61±0.37 (6)	2.96±0.64(6)	135
Rabbit	2.82±0.54(4)	0.73±0.08(4)	1.33±0.23(8)	141
	-	-	3.15 [b] ±0.4(4)	142
	Plasma unbound fraction (f_u) ±S.D			
Subject	S-Warfarin	R-Warfarin	Racemate	Reference
Rat	0.00837±	0.0131±	0.0107±	48
	0.00487(11)	0.00836(11)	0.00652(11)	
	-	-	0.02 [a] (13)	134
	0.0059±	0.0090±	-	104
	0.0015(10)	0.0031(10)		
Man	0.0051±	0.0062±	-	105
	0.0005(6)	0.0005(6)		
	0.0058±	0.0059±	-	144
	0.0007(10)	0.0007(10)		
	0.00903±	0.0118±	0.0104±	48
	0.00319(31)	0.00401(31)	0.00347(31)	
	-	-	0.0078	142
			±0.0007(6)	
	-	-	0.08 [a] (10)	134
Dog	-	-	0.026	140
			±0.012(4)	

Number of animals or subjects in parenthesis; * Racemic warfarin, a mixture of (R)- and (S)- enantiomers, was estimated by UV spectrophotometric/fluorometric method; [a] S.D values not available; [b] CL is expressed as ml/min

studied in some cases, possible differences of strain and sex and variation in experimental conditions (59).

Physiologic pharmacokinetic models are useful in understanding the time course of drug concentrations in plasma as well as in main organs and tissues in animals (60). **Fig. 1.3** represents a physiologically based pharmacokinetic model for (S)-warfarin disposition in rats (60). This model requires estimation of actual drug concentration in various organs and tissues. Each of the assayed tissue was included as a separate compartment and the unanalyzed pooled into "rest of body" compartment. The model is based on the assumption that blood flow is solely responsible for the drug transport between compartments and the drug reaches equilibrium between tissue and blood instantaneously. Also it was assumed specifically for S-warfarin that elimination was solely through hepatic metabolism.

Physiologic pharmacokinetic models cannot be used in humans for obvious reasons, as data required are available only from animal studies. But useful alternatives of target-mediated pharmacokinetic models are available. Such models also allow characterization of data from the literature for racemic warfarin pharmacokinetics in man (60).

1.1.5. MEASUREMENT OF ANTICOAGULATION

Warfarin has a low therapeutic index. Careful monitoring of the patient is required to maintain the therapeutic window. The anticoagulant effect of warfarin is mainly monitored by the prothrombin time (PT) assay (34). PT reflects changes in three of the four vitamin K-dependent procoagulant clotting factors (factors II, VII and X)(61)

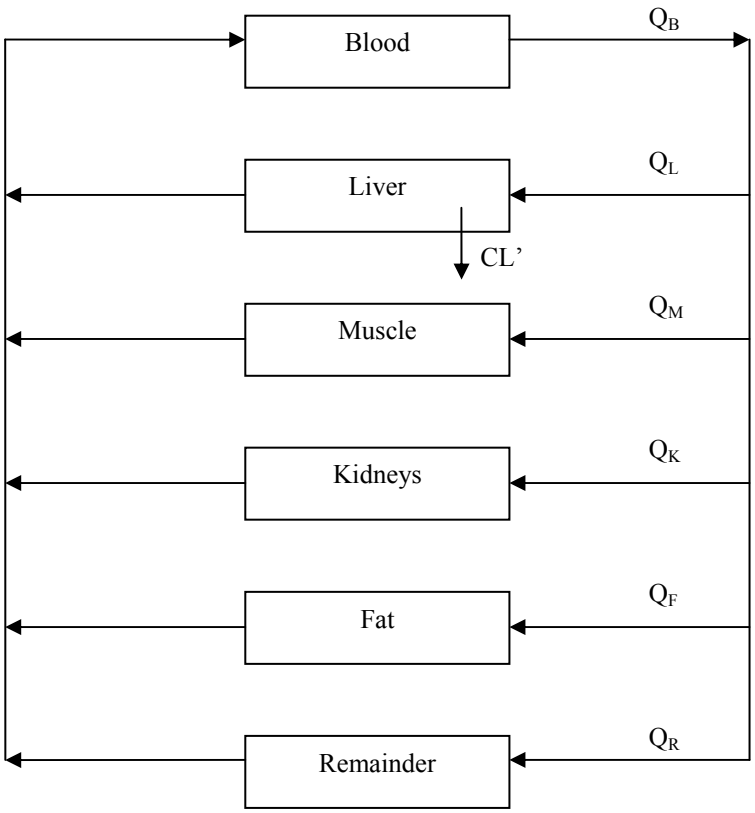


Figure 1.3 Physiologically based pharmacokinetic model for (S)-warfarin disposition in rats (60)
Blood flow rates in different tissues is represented by "Q"; CL', Intrinsic clearance.

Each of the assayed tissue was included as a separate compartment and the rest pooled as a remainder. The model is based on the assumption that blood flow is solely responsible for the drug transport between compartments and the drug reaches equilibrium between tissue and blood instantaneously. Also it was assumed specifically for S-warfarin that elimination was solely through hepatic metabolism.

It is most sensitive to the level of clotting factor VII during the first few days of therapy (61). PT is based on the time to form a clot after the addition of thromboplastin and calcium to the citrated plasma. The PT result may vary depending on the type of thromboplastin used in the reagent. The international normalized ratio (INR) may be used to standardize the results from different laboratories. A correction factor, international sensitivity index (ISI), assigned to each reagent is used to calculate INR from PT values. Ratio of patient PT to mean PT from normal people raised to the ISI power gives INR.

1.1.6. CHEMICAL ANALYSIS

Due to the differences in pharmacokinetic and pharmacological actions of enantiomers, chiral separation and assay in biological sample becomes an important consideration. High-performance liquid chromatography (HPLC) is one of the most commonly used analytical techniques for this purpose. Various non-stereospecific HPLC assays have been developed for warfarin in biological samples such as a reversed-phase HPLC with post-column alkalinization to increase fluorescence detection (62). However, chiral or achiral methods were used to selectively separate the enantiomers (63). Chiral separation techniques by HPLC can be carried out either by using chiral HPLC columns such as α_1-acid glycoprotein or β-cyclodextrin columns or by incorporating chiral reagents into HPLC mobile phase on achiral columns (64). Achiral procedures involve precolumn derivatization of enantiomers to diastereomeric esters using chiral derivatising agents and separation by normal-phase HPLC (65) or reversed-phase HPLC (66).

Chiral separation technique for warfarin enantiomers by normal phase HPLC was reported by Banfield, C. and Rowland M. 1984 (65) (**Fig. 1.4**). The assay method is able to successfully separate the enantiomers and detect fluorimetrically. It was found to be highly sensitive and specific.

1.1.7. WARFARIN DRUG INTERACTIONS

Drug interactions involving oral anticoagulants are broadly classified as Pharmacokinetic, Pharmacodynamic and Allergic or Idiopathic. Pharmacokinetic interactions involve changes in pharmacokinetic parameters such as clearance, half-life or volume of distribution. They are accompanied by changes in the amount of anticoagulants and are caused by alterations in protein binding, metabolism, absorption and/or excretion. Drugs may affect the pharmacodynamics of warfarin by increasing the metabolic clearance or inhibiting the synthesis of vitamin K-dependent coagulation factors, and/or by interfering with various pathways of hemostasis (61). Pharmacodynamic interactions can be synergistic or antagonistic and result in expected or nonpredictable activity. Generally no change in the circulating amounts of the anticoagulant is observed in these interactions. Idiopathic interactions are those with no known mechanism of interaction. One example is the interaction between ascorbic acid and oral anticoagulants (33).

Interactions affecting the pharmacokinetics of drugs are difficult to anticipate as they might result from various factors involving absorption, distribution, metabolism and excretion. Metabolism appears to be the major source of interaction in humans. Published reports show that numerous chemicals selectively inhibit the drug oxidation

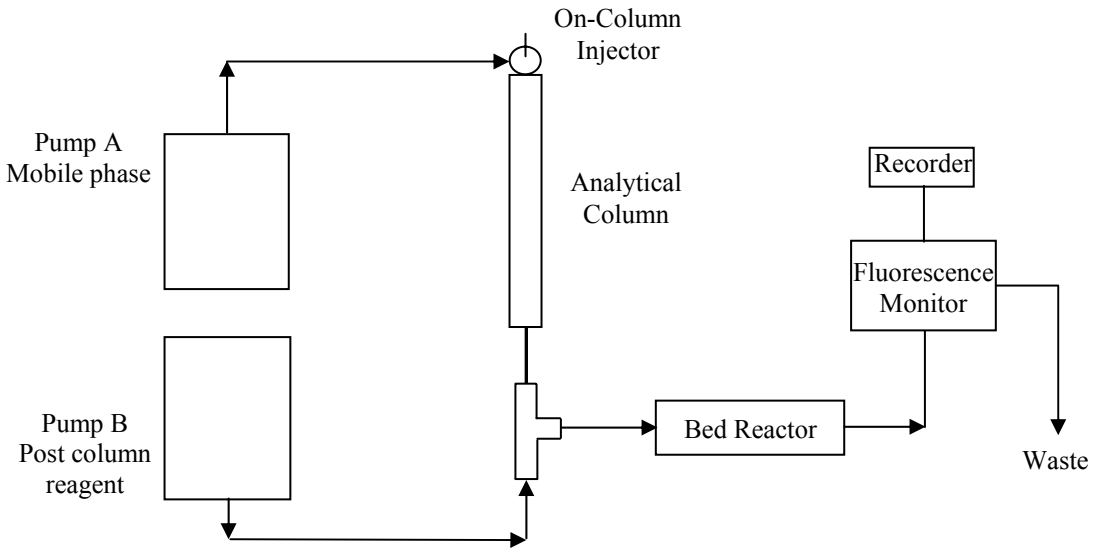


Figure 1.4 Schematic instrumental setup for chiral separation of warfarin enantiomers by HPLC (65)

reactions catalyzed by specific CYP isozymes. This is emerging to be important mechanism for understanding the nature of drug interactions (55). A drug which inhibits CYP2C9 to a considerable extent can have a major impact on the warfarin anticoagulant response because the clearance of (S) - warfarin is reduced. Thus Fluvastatin, which is a potent inhibitor of the CYP2C9 isoenzyme, affects clinical anticoagulation more than the other statins (67).

Routinely warfarin may be a part of multiple drug therapy regimens and drug interactions constitute a major problem of chronic polypharmacy. Pharmacokinetic interactions of warfarin are generally accompanied by changes in the warfarin circulating in blood. These interactions would lead to either loss of anticoagulation or increased risk of bleeding. Some of the interacting drugs as listed out in **Table 1.5** include amiodarone, propafenone, benzbromarone, barbiturates, fluconazole, cimetidine, metronidazole, trimethoprim-sulphamethoxazole combination, clonazepam, NSAIDs like phenylbutazone, bucolome. The list shows that most of the major categories of therapeutic agents can interact with warfarin.

In spite of negative pre-marketing drug interaction studies, post-marketing drug interactions have been reported for many drugs. Stereoselective inhibition of P450-2C9 leading to enhanced metabolism of (S)-warfarin has been demonstrated for miconazole, metronidazole, sulfinpyrazone, phenylbutazone, enoxacin, amiodarone, tricynafren, cimetidine, and omeprazole. Metabolically based drug interaction with fluconazole has been reported, with fluconazole inhibiting CYP-2C9 and 3A4. Bucolome is known to be a clinically potent inhibitor of CYP2C9 (68). Uricosuric drug benzbromarone inhibits CYP2C9-metabolism of (S)-warfarin. Fluvoxamine,

Table 1.5 Warfarin drug interactions (33, 34, 69) (The list is non-exhaustive)

Antibacterial:	**H_2-receptor antagonists:**	**Nonsteroidal anti-inflammatory drugs:**	**Miscellaneous:**
Chloramphenicol[a]	Cimetidine[a]	Indomethacin[a]	Allopurinol[a]
Ciprofloxaci[n]	Ranitidine[a]	Ketoprofen[a]	Thyroxine[a]
Cotrimoxazole[a]	**Analgesic/antipyretics:**	Piroxicam[a]	Dipyridamole[a]
Dicloxacillin[b]	Paracetamol-dextropropoxyphene[a]	Sulindac[a]	Ticlopidine[a]
Isoniazid[a]	**Anticonvulsants:**	Oxyphenbutazone[a]	Alcohol[c]
Enoxacin[a]	Carbamazepine[b]	Tolmentin[a]	Prednisone[c]
Erythromycin[a]	Phenobarbital[c]	Phenylbutazone[a]	Haloperidol[b]
Metronidazole[a]	Phenytoin[c]	Sulfinpyrazone[a]	Omeprazole[a]
Nalidixic acid[a]	**Sedative-Hypnotics:**	Aspirin[a]	Ascorbic acid[b]
Norfloxacin[a]	Secobarbital[b]	Rofecoxib[a]	Retinol[a]
Rifampicin[b]	Chloral hydrate[a]	Celecoxib[a]	Oral contraceptives[b]
Tetracyclins[a]	**Antihyperlipidaemics:**	**Diuretics:**	Sucralfate[b]
Nafcillin[b]	Lovastatin[a]	Ethacrynic acid[a]	Diazoxide[a]
Antifungal:	Fluvastatin[a]	Metolazone[a]	Sucralfate[b]
Fluconazole[a]	Clofibrate[a]	Spironolactone[b]	Danazol[a]
Miconazole[a]	Cholestyramine[c]	**Immuno-suppressants:**	Stanozalol[a]
Itraconazole[a]	Simvastatin[a]	Azathioprine[b]	Disulfiram[a]
Ketoconazole[a]	**Antineoplastic drugs:**	Cyclosporine[c]	Aluminium-hydroxide[b]
Griseofulvin[b]	Isofamide[a]	**Antimalarial:**	
Selective serotonin reuptake inhibitors:	Sulofenur[a]	Proguanil[a]	
Fluvoxamine[a]	Mitotane[a]	**Antiarrhythmics:**	
Fluoxetin[a]	Broxuridine[a]	Amiodarone[a]	
Paroxetin[a]	Mercaptopurine[b]	Propafenone[a]	
Sertraline[a]		Disopyramide[b]	
Citalopram[a]			

[a] Increase warfarin effect; [b] Decrease effect; [c] either increase or decrease effect

inhibitor of CYP-1A2, 3A4 and 2C9, is the most likely of SSRIs to interact with warfarin (69). Recently a possible interaction between warfarin and menthol cough drops has been reported (70). Though the mechanism of decrease in anticoagulation is not known, menthol is known to affect the CYP 450 metabolism.

1.1.7.1. Warfarin interactions with complementary and alternative medicines (CAM)

Recent reports show that the use of CAM is on the rise among patient populations throughout the world. Most of them use it for the treatment of chronic, incurable diseases including cancer and HIV/AIDS (71). With the surge in chronic diseases and the potential side effects of modern drugs, patients often explore various CAM (72). General public is of the opinion that "naturalness" is a guarantee for "harmlessness". About 50% of Americans use dietary supplements to treat various ailments and up to 20% use these products regularly (69). Along with the prescribed polypharmacy, patients take numerous over-the-counter medications, vitamins, herbs and foods. All these have the potential to interact. It is known that substances that inhibit cytochrome P450 isoforms have higher potential for drug interactions (73). But experimental data on herb-drug interactions are limited and scarce (74). Potential interactions of alternative medicine products with prescription medicines are all the more important in case of drugs with narrow therapeutic index (1). Therapeutic effects of such drugs add up due to interaction and the cumulative effect can be unpredictable. It may require a careful monitoring of the therapy.

Potential and documented interactions have also been reported between herbal

supplements and warfarin (**Table 1.6**) (1, 74, 75, 76). Numerous food and drug interactions of warfarin have been reported when compared to any other medication (1). Interactions with warfarin could be through various pathways. Patients who took warfarin along with traditional Chinese medicinal (TCM) herbs as food supplements have had warfarin adverse effects enhanced (77). Addition of prescription drugs to "herbal" products is ubiquitous with Chinese patent medicines (74). Quilinggao ("essence of tortoise shell") is one such popular Chinese herbal product that is reported to have caused bleeding when administered with warfarin (78). It contains various ingredients that interact with warfarin. Therefore estimating and managing warfarin interactions with herbal products are very important. Most of the available information on potential drug interactions between warfarin and herbal products is based on *in vitro* data, animal studies, or individual case reports. Definite mechanisms for such interactions are yet to be established (1).

New drug products are routinely examined *in vitro* and *in vivo* for the potential to cause drug interactions *via* inhibition of drug-metabolizing enzymes (73). But herbal preparations are not subject to such tests. Thus the potential exists that herbal preparation could cause drug interactions when administered with other medications. Herbal products that have been associated with published case reports of possible interactions with warfarin include danshen, devil's claw (harpagophytum procumbens), dong quai, green tea, ginkgo biloba, ginseng and papain. Dietary supplements like coenzyme Q10 and vitamin C, E and K have also been reported to adversely affect warfarin therapy (1). Dong quai, fenugreek, horse chestnut, red clove, sweet clover, sweet woodruff and chamomile contain coumarin like substances and have the potential to interact with warfarin (1). Furanocoumarins from ethyl acetate

Table 1.6 Warfarin interactions with herbs and herbal products (1, 69, 75, 76, 119) (Partial list)

	Interaction		Interaction
Ginseng St John's Wort Coenzyme Q_{10} Ascorbic acid Retinol Vitamin K Soy milk	Decreased INR	Green tea Broccoli Brussels sprouts cauliflower cabbage spinach	Decrease in warfarin effects
Papain Danshen Dong quai Anise Alfalfa Arnica Celery Chamomile Fenugreek Horse chestnut Licorice root Parsley Prickly ash Quassia Red clover Tonka beans	Increased INR	Garlic Ginkgo Clove Feverfew Ginger Onion Turmeric Meadowsweet Poplar Willow bark Angelica root Asafoetida Lovage root Borage seeds Bogbean Capsicum Devil's claw Vitamin E Cordyalis yanhuso Magnolia bark Pineapple Wintergreen leaf Kangen Karyu	Increased risk of bleeding

extract of grapefruit juice have decreased the activities of CYP-3A4, 1A2, 2C9, and 2D6 (79) and reported to inhibit 7-hydroxylation of coumarins in healthy volunteers (80). Excessive doses of *Kangen-karyu*, an anti-thrombotic TCM, prolonged the bleeding caused by warfarin by suppressing its metabolism (72). Thus benign use of alternative medicine among naïve subjects could lead to potentially fatal results.

1.1.8. FLAVONOIDS

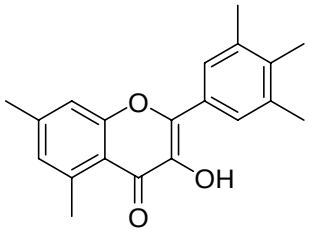
1.1.8.1. Introduction

Flavonoids are a diverse class of polyphenolic compounds found in plants (81). Flavonoids are divided into following groups: flavans, flavanones, isoflavanones, flavones, isoflavones, anthocyanidines, chalcones and flavonolignans (82). All flavonoids have the same basic structure, a three-ringed molecule with hydroxyl groups attached (C_6-C_3-$C_{6)}$ (**Fig. 1.5**). They occur in foods as a glycoside and have numerous biological activities in animals (81). The approximate daily intake of flavonoids from food in different countries is 3-70 mg with higher values in regions where intake of tea and vegetables is high. Their actions are related either to the chemical structure or to the enzyme activity.

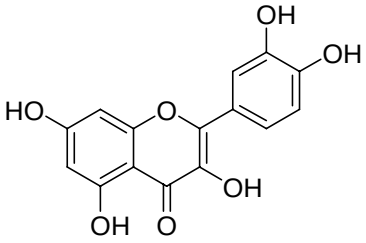
Flavonoids are known to affect the bioavailability of drugs by interacting with P-glycoprotein multiple drug transporters (16) or through cytochrome P450 modulation (2). In general, it is found that flavonoids containing hydroxyl group inhibit cytochrome P450 enzymes and flavonoids devoid of hydroxyl groups often stimulate the enzyme activities (82, 83). Flavonoids can stimulate or inhibit cytochrome P450 depending on the structure, amount and experimental conditions (4).

Figure 1.5 Chemical structures of flavonols, quercetin and rutin

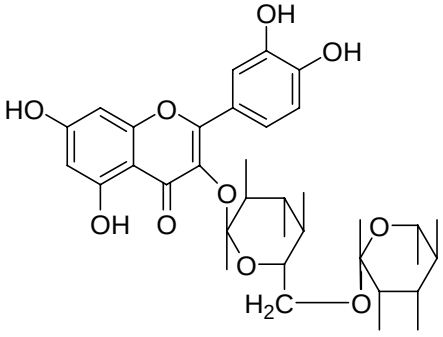
Flavonols



Quercetin



Rutin



1.1.8.2. Rutin and Quercetin

Rutin and its metabolite quercetin are the flavonoids widely distributed in the plant kingdom. Rutin was introduced as capillary stabilizer in 1949 (84). It can be prepared from Buckwheat or red beans (85). Rutin is soluble in hot ethyl alcohol (5%), precipitating slowly on cooling (20). It is one of the main flavonoids present in Hypericum species. **Table 1.7** summarizes some of the available information on rutin. The physiological functions of rutin include inhibition of capillary permeability and enhancement of capillary resistance, anti-inflammatory, antihydropic, aldose reductase inhibition and improvement in glaucoma by diabetes. Rutin is found to relax smooth muscles and lower hepatic and blood cholesterol in rats (86). Rutin derivatives (e.g. oxerutin and troxerutin) are used to treat various cardiovascular conditions (17, 18, 19). It is also reported to relieve venous insufficiency of the lower limbs and capillary impairment (18, 19). Rutin is known to be a strong scavenger of superoxide and hydroxyl radicals and as an antioxidant. It is used against lipid peroxidation, used as a coloring agent, nutrient, food additive (enzymatically decomposed rutin) (85) and in cosmetics.

Quercetin is found in citrus fruits, apples, onions, parsley, Gingko biloba, St John's wort, Echinacea purpurea, tea, red wine and other fruits and vegetables. It is the most abundant of the flavonoid molecules. It acts as an antioxidant. Quercetin is the aglycone of different flavonoids, including rutin (quercetin 3-O-glucose-rhamnose), quercetrin, isoquercetrin (quercetin 3-O-glucose). (Aglycone is the non-sugar compound remaining after replacement of the glycosyl group from a glycoside). It has higher activity when compared to these flavonoids. Thus both rutin and quercetin

Table 1.7 Drug information: Rutin (145)

Rutin	a flavonol glycoside of quercetin and rutinose
Description	Pale yellow substance, slightly soluble in water
Source	Found abundant in Buckwheat plant, black tea and apple peels
Molecular Formula	$C_{27}H_{30}O_{16}$
Molecular weight	610.53 daltons
Other names	Rutoside; quercetin-3-rutinoside; sophorin; 3, 3', 4', 5, 7- penta-hydroxyflavone-3-rutinoside; 3-rhamnosyl-glucosyl quercetin; 3-[[6-O-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-gluco pyranosyl] oxy]-2-(3, 4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one
Actions	Antioxidant, anti-inflammatory, anticarcinogenic, antithrombotic, cytoprotective and vasoprotective
Indications	Venous edema, varicose veins; to strengthen capillaries; as anti-inflammatory; against some toxins; anticancer; prevents oxidation of vitamin C
Adverse reactions	generally well tolerated; nausea, headache, mild tingling of the extremities
Interactions	Quinolone antibiotics, vitamin C, processed meat products
Dose	500 mg once/twice daily

present in Ginkgo biloba extract cause vasorelaxation, but quercetin has a stronger relaxation than rutin (87). Bioavailability of quercetin is affected by attaching or detaching a glucose molecule, but not by the position (88). It has effective anti-cancer, anti-ulcer, anti-allergic, anti-inflammatory, antiviral activities and gives cardiovascular protection and prevents diabetic cataracts. It is often used for allergic conditions such as asthma, hay fever, eczema, hives and also to treat gout, pancreatitis and prostatitis. Rutin and quercetin have low solubility in biological fluids (17, 89).

Quercetin strongly inhibits the activity of carbonyl reductases, the enzymes responsible for the biotransformation of various drugs and endogenous compounds, *in vitro* (90). Thus it can possibly affect the metabolism of drugs that are metabolized by carbonyl reduction such as quinines and warfarin. Furthermore, quercetin inhibits CYP3A4 enzymes in both human and rodent microsomes *in vitro*. CYP1A2 is most susceptible to quercetin inhibition (91). Effect of quercetin on CYP2C9 and CYP3A4 were recently studied in human liver microsomes (6). Ginseng quercetin inhibited both CYP2C9 and CYP3A4 significantly (6). Though *in vivo* data was not available, the results indicate that quercetin is capable of inhibiting the metabolic reactions mediated by CYP2C9 and CYP3A4 and thus have potential interactions with drugs metabolized by these enzymes. Quercetin is also reported to increase the expression of CYP1A1 (82).

Severe anticoagulation was reported in a patient who consumed a non-steroidal anti-inflammatory herbal drug with rutin as one of its constituents (92). The interaction could have been possibly due to contamination with coumadin, but the potential interaction with rutin has not been studied yet. Troxerutin, a rutin derivative, is known

to heal capillary endothelial defects and shown to be beneficial in combination with coumarin for treating chronic venous insufficiency (93). It was observed to protect liver from lipid peroxidation caused by coumarin (93). Rutin was reported not to have interacted with *in vitro* liver microsomal cytochrome P-450 dependent monooxygenases in a study. But many of the results of this study could not be extrapolated to *in vivo* studies (89). However, rutin could directly or indirectly modify the metabolic pathways of other active constituents, thus affecting the pharmacological actions of the interacting drug.

The absorption, metabolism, transportation and delivery of flavonoids to specific tissues may influence the effect of flavonoid-rich diet on health (94). There have been many contradictory reports on the mechanisms of absorption of flavonoids from the gastro-intestinal tract (25). Rutin is absorbed more slowly than quercetin in rats and humans (95). The former is likely to be hydrolyzed by the digestive microflora and converted to quercetin prior to being absorbed (14, 25, 95) though absorption of some of the intact flavonoid glycosides has been reported recently (96). Glucuronides and/or sulfates of quercetin and unconjugated quercetin aglycone, with no rutin, were detected when quercetin (8-50 mg dose) and rutin (16-100 mg dose) were administered orally to healthy volunteers (25) **(Table 1.8)**. With a single oral dose of 1 g/kg body weight, quercetin and rutin were detected in the body as aglycone or conjugates of quercetin and isorhamnetin up to three days post dose (97).

Pharmacokinetic variables of absorption and elimination of quercetin in human plasma after ingestion of various flavonoid foods are summarized in **Table 1.9** (88, 98, 99). **Table 1.10** shows the total area under plasma concentration-time curve and

Table 1.8 Pharmacokinetic parameters of oral quercetin and rutin in human plasma

Pharmacokinetic parameters	Quercetin			Rutin			
	8 mg[a]	20 mg[a]	50 mg[a]	16 mg[a]	40 mg[a]	160 mg[a]	200 mg[b]
C_{max}	41.4	66.1	86.1	23.5	47.6	89.9	320
t_{max}	1.9±1.2	2.7±1.9	4.9±2.1	6.5±1.8	7.4±2.2	7.5±2.2	6.98±2.9
AUC_{0-24}	527	882	1138	381	636	1017	2500
$t_{1/2}$	17.1±3.7	17.7±2.6	15.1±3.0	-	-	-	11.8±3.1
Protein binding [c]							
Plasma		99.1 ± 0.5			-		
HSA (40mg/ml)		99.4 ± 0.1			-		
K_1 [d]							
BSA		134 ± 6			10.7 ± 6.6		
HSA		50 ± 8			-		

[a] Values are mean (± S.D for t_{max} and $t_{1/2}$); (n = 16) (25)
[b] Values are mean (± S.D for t_{max} and $t_{1/2}$); (n = 12) (28)
[c] Values are mean (± S.D) fractions (%) of quercetin (1.5-15 μM) bound to plasma; (n=5) (117)
[d] Values are mean (± S.D) (n=4) (94)
C_{max}, maximum plasma concentration, (μg/l); t_{max}, peak time, (h); AUC_{0-24}, total area under plasma concentration-time curve from time zero to 24 h, (μg h/l); $t_{1/2,}$ elimination half life, (h); HSA, human serum albumin; K_1, Binding constant (10^3 M^{-1}); BSA, bovine serum albumin.

Table 1.9 Pharmacokinetic variables of absorption and elimination of quercetin from various foods/sources in human plasma (88, 98, 99)

Variable	Quercetin source (oral ingestion)					
	Quercetin-3-glucoside[a]	Quercetin-4-glucoside[a]	Onions	Apples	Rutinoside	Quercetin[a,b]
C_{max}	1526	1345	224±44	92±19	90±93	-
t_{max}	0.617	0.45	0.7±1.08	2.5±0.72	9.3±1.8	-
AUC_{0-36}	-	-	2330±849	1061±375	983±978	-
AUC_{0-72}	5775	5276	-	-	-	-
$t_{1/2}$	18.5	17.7	28±92	23±32	-	2.4
Amount in urine[c]	9.75	8.61	3.2±1.6	1.45±0.7	1.17±1.34	-
Vd	-	-	-	-	-	0.34
CL	-	-	-	-	-	34.26

Values are mean ± S.D (n = 9)
[a] S.D values not available
[b] Quercetin administered i.v. (100 mg)
[c] Amount of quercetin excreted in 24 h urine (µmol)
C_{max}, maximum plasma concentration, (µg/l); t_{max}, peak time, (h); AUC_{0-36}, total area under plasma concentration-time curve from time zero to 36 h, (µg h/l); AUC_{0-72}, total area under plasma concentration-time curve from time zero to 72 h, (µg h/l); $t_{1/2,}$ elimination half life, (h); V_d, apparent volume of distribution (l/kg); CL, total plasma clearance (l/h).

Table 1.10 Total area under plasma concentration-time curve (AUC_{0-24}) and mean (± S.E) 24 h urinary excretion of quercetin and methylated quercetin in rats administered quercetin and rutin (100)

Supplement	Metabolite	AUC_{0-24} (nmol/h)	Urinary excretion (nmol/24 h urine)
Quercetin	quercetin	4.13	32.4 ± 14.1
	methylated quercetin	1.91	50.7 ± 20.2
Rutin	quercetin	9.97	59.1 ± 10.3
	methylated quercetin	9.75	173.4 ± 40.2

mean 24 h urinary excretion of quercetin in rats administered quercetin and rutin (100). Further quercetin has been reported to accumulate in the blood when administered for a long time and is attributed to its long elimination half-life (98).

A recent report estimates that 15 million people in United States and 3.7 million in Australia may be at risk of drug-supplement interactions (75). Some of the available on-line resources for herbal product drug interaction are listed out in **Table 1.11.** Though confirmed drug-herb interactions are rare, adverse reactions remain under-reported most of the time. So the total figures could be much higher. If this is the case in the United States and Australia, then Singapore and most of the Asian societies might be at a greater risk as many of the traditional alternative medicines using herbs originated from these Asian countries and are still being used widely. The main purpose of this project is to study the pharmacokinetic and pharmacodynamic interactions of warfarin with rutin in rats, with particular emphasis on enantiomeric composition following administration of the racemic drug.

The present study was designed to carry out quantitative analysis of serum warfarin levels, with emphasis on individual enantiomers. The effect of rutin on the degree of anticoagulation was assessed by means of one-stage prothrombin time. Also rutin treatment study in the absence of warfarin was carried out to evaluate the effect of rutin alone on blood coagulation in rats.

Table 1.11 On-line resources for herbal product interaction with warfarin (69)

The natural Pharmacist	www.tnp.com
The Pharmacist's Letter	www.naturalpharmacist.com
National Institutes of Health	http://dietary-supplements.info.nih.gov
Special Nutritionals	http://vm.cfsan.fda.gov
Adverse event monitoring-	
MEDWATCH Reporting	www.fda.gov/medwatch

CHAPTER 2 OBJECTIVES

In this study, the potential drug interaction between rutin, a flavonoid glycoside and warfarin, the most widely prescribed oral anticoagulant, was investigated in rats. Warfarin drug interactions are clinically relevant due to its narrow therapeutic window. Since rutin and quercetin are present in numerous diets of plant origin, potential for drug interactions before and after starting warfarin therapy in subjects on rich diet of these bioflavonoids was evaluated in rats.

The study was designed to investigate the effects of both single (1.5 mg/kg) and multiple (0.15 mg/kg) oral doses of racemic warfarin in rats treated with and without rutin (1 g/kg). Quantitative analysis of serum warfarin levels, with emphasis on individual enantiomers, was carried out. The effect of rutin on the degree of anticoagulation was assessed by means of the one-stage prothrombin time.

Also, another study was designed separately to study the effect of rutin alone on blood coagulation.

CHAPTER 3 *IN VIVO* SINGLE DOSE STUDY

3.1. INTRODUCTION

Warfarin is the oral anticoagulant of choice in most of the countries (66). However about 250 drugs and a whole lot of nutritional supplements and herbal preparations are known to potentially interact with warfarin (75). Possibility of unearthing potential interactions in the future is immense. Rutin is present in many of the often-consumed foods, vegetables and medicinal botanicals. It is also widely used in complementary and alternative medicines. Thus potential drug interaction between rutin and warfarin needs to be evaluated.

In this part of the study, rats were treated with rutin orally for four days before administering a single dose of warfarin to possibly mimic a clinical case of introducing warfarin in a patient who has been on rutin either from diet or herbal remedies with rutin as one of the constituents.

3.2. OBJECTIVES

The experiment was designed to study the effect of rutin on pharmacokinetic and pharmacodynamic properties of warfarin administered as a single oral dose in rats. Concentrations of each of the warfarin enantiomers were quantitatively estimated in serum by HPLC assay and pharmacokinetic parameters calculated. Also the effect of rutin on prothrombin time was measured.

3.3. MATERIALS AND METHODS

3.3.1. Chemicals and reagents

Racemic warfarin, chlorowarfarin, and carbobenzyloxy-L-proline were obtained from Aldrich Chemical Company, Milwaukee, USA. 1, 3-dicyclohexylcarbodiimide was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Rutin hydrate from Sigma Chemical CO, St. Louis, MO, USA, and carboxy methyl cellulose (CM-Cellulose), sodium salt and quercetin dihydrate from Sigma-Aldrich CO, St. Louis, MO, USA were used for the study. Trisodium citrate dihydrate GR and sodium chloride GR were purchased from E. Merck (Darmstadt, Germany). The solvents n-hexane (GR, Merck KgaA, Darmstadt, Germany), butylamine (Merck-Schuchardt, Germany), methanol and acetonitrile (Mallinckrodt chrom AR®HPLC), ethyl acetate (HPLC) and diethyl ether (Analytical reagent AR) (both obtained from Lab Scan Analytical sciences, Bangkok) were used for the study. 10X PBS (Phosphate buffer saline containing 80 g/L NaCl, 2 g/L KCl, 14.4 g/L Na_2HPO_4, and 2.4 g/L KH_2PO_4; pH 7.4) was purchased from National University Medical Institutes (NUMI), Singapore.

The sodium citrate solution was prepared as an anticoagulant according to the reported method (101) based on Owren's theory of blood coagulation. (Sodium citrate solution – 231 ml of 34.2 mM Sodium citrate + 200 ml of Owren's buffer + 569 ml of 0.9% NaCl). Formulations of rutin were prepared just before use by weighing and suspending in 1% CM-cellulose. Neoplastine® CI Plus 5 kit (Cat. No. 00375) was obtained from Diagnostica Stago (France). The kit reagents consist of Reagent 1 (freeze-dried thromboplastine prepared from fresh rabbit cerebral tissues) and

Reagent 2 (aqueous solvent containing calcium with sodium azide as a preservative, 5-ml per vial). The ISI (International Sensitivity Index) values of Neoplastine, determined against a secondary standard of the rabbit brain thromboplastine, were 1.21 and 1.22 for Lot Nos. 022571 and 013334, respectively. The reagent contains a specific heparin inhibitor. Any prolongation of the prothrombin time is related to a real deficiency of the prothrombin complex activity.

Rat cubes, the standard diet for laboratory animals fortified with vitamins and minerals, were purchased from Glen Forest Stockfeeders, Australia. Milli-Q water from the Millipore® Continental Water Systems (Burlington, MA, USA) was used for all the experimental purposes.

3.3.2. Apparatus

The high performance liquid chromatography (HPLC) system for the assay of warfarin enantiomers consisted of a solvent delivery system (LC-10AT, Shimadzu), sample injector with standard sample loop (20 μl), a fluorescence detector (RF-10AXL, Shimadzu), a communication bus module (CBM101, Shimadzu), a computer and a workstation (Class-CR10, Shimadzu). The chromatographic conditions for isocratic normal phase HPLC were as follows: A stainless steel column (250 × 4 mm) packed with silica (MAXSIL 10 silica, 10 micron, Phenomenex, USA) maintained at 23°C column temperature, was used for the separation. Mobile phase was a mixture of ethyl acetate/n-hexane (22.5:77.5) at a flow rate of 0.8 ml/min. Bed reactor (stainless steel column 3 mm i.d., 25 cm) packed with glass beads (40 μm) was used for the post column aminolysis. Butylamine/methanol (1:1) was used as the post column reagent

at a flow rate of 0.4 ml/min. The effluent from the column was detected by excitation at 313 nm and emission at 400 nm using a fluorescence detector.

For the prothrombin time (PT) test, fully automated coagulation analyzer (Sysmex automated blood coagulation analyzer, CA 530, Sysmex Corporation, Kobe, Japan) was used. Siliconized capillary tubes for orbital bleeding (1.3 mm ID, 50 mm length, Fisher Sc. US) siliconized with dimethyl dichlorosilane, 1.5 ml capacity micro centrifuge tube- with 250 μl sodium citrate in it and Heparinized microhematocrit capillary tubes (1.1-1.2 mm ID) were used for blood sampling.

3.3.3. Animals and sampling method

Adult male Sprague-Dawley rats, each weighing 250-300 g were obtained from the Laboratory Animal Center, Singapore. The Institutional Animal Care and Use committee of laboratory animal centre, National University of Singapore, Singapore approved the animal experiments. Rats were housed in metabolic stainless steel cages individually in an air-conditioned room (23 ± 1°C). They were maintained on 12 h light/dark cycle for three days before the experiment. The animals had access to food (rat cubes) and water *ad libitum* during the experimental period, but fasted overnight before oral administration of warfarin and 4h after the administration.

3.3.3.1. Preparation of citrate buffer diluted plasma

Venous blood was obtained from the orbital sinus of rats by puncturing with siliconized capillary tube. First two drops of blood were discarded. Citrate-diluted

plasma was prepared as per the method established previously in our laboratory (102). Not less than 50 µl of blood was collected into micro centrifuge tube with 250 µl sodium citrate solution. Subsequently, hematocrit blood sample was obtained by introducing one end of the heparinized microhematocrit capillary tube into the orbital sinus of the eye. Both micro centrifuge tube & microhematocrit capillary tube were weighed on an analytical balance immediately. Length in mm of blood in the microhematocrit tube was recorded. Following equations were used to calculate the volume of blood collected into the micro centrifuge tube:

Gravity (mg/ml) = (W2-W1) × c/L

c - a coefficient determined by filling microhematocrit tube with a known volume of distilled water, measuring the length of the water column obtained & then calculating the ratio of the length to the volume.

Volume (µl) of blood received into the micro centrifuge tube with anticoagulant solution is given by, volume = (W2-W1)/gravity.

W- weight (in mg) of the micro centrifuge tube containing sodium citrate solution (250 µl)

After calculating the accurate volume of blood, extra sodium citrate solution was added to make up 1:6 dilution of the whole blood. The citrate-diluted blood was centrifuged for 10 min at 3000 rpm, plasma was separated and used for the One Stage Prothrombin Time test. Serum was prepared from about 400 µl of blood obtained without citrate. Concentration of the warfarin enantiomers in the serum was analyzed by normal phase HPLC method.

3.3.4. Chemical analysis

The stereospecific HPLC method established by Banfield, C. and Rowland, M. 1984 (65), was used for the chemical analysis. The S- and R- enantiomers of warfarin in the serum sample were separated by first converting them into diastereomeric esters with carbobenzyloxy L-proline and further separating on HPLC system. The effluent from the column was mixed with postcolumn n-butylamine for aminolysis to break the esters as warfarin loses its fluorescence due to the ester formed at 4-hydroxyl group. The resulting fluorescent enantiomers were detected on-column fluorimetrically.

3.3.5. Estimation of anticoagulation

Neoplastine reagent was prepared by reconstituting freeze-dried thromboplastine powder (Reagent 1) in 2.5 ml of the given aqueous solvent (Reagent 2) just before use. The test protocol of the machine was set to sample 100 µl of citrate-diluted plasma (102) and mix with 50 µl of the Neoplastine reagent automatically. The program of the machine allows the sampled plasma to be incubated for 180 s at 37°C before the addition of the reagent. The maximum reaction time was adjusted to 600 s. The clotting time in seconds (PT) as end point was read directly from the touch screen of the machine. The precision was tested by using blank pooled plasma at different dilutions within a range of 100-15% and was found to be acceptable (RSD 0.32-5.31%).

3.3.6. Animal study protocol

Two groups of male Sprague-Dawley rats (n = 8 per group), in a parallel design,

received rutin (1 g in 1% CM-cellulose/kg) and an equal volume (5 ml/kg) of 1% CM-cellulose (as the control), respectively, by oral intubation, once a day for eight consecutive days. On day 5, each rat received a single oral dose of racemic warfarin (1.5 mg/kg, dose volume = 5 ml/kg) by oral intubation. Blood samples were collected from each rat prior to, and 1, 3, 6, 12, 24, 48, 72 and 96 h after warfarin administration. About 400 µl of blood obtained without anticoagulation was centrifuged (10 min at 3000 × *g*) to separate serum. Serum samples obtained were then stored at −20°C before HPLC analysis. For the subsequent anticoagulant effect measurement, 50 µl of each blood sample was used for diluted plasma preparation according to the established procedure (102).

3.3.7. Data Analysis

3.3.7.1. Pharmacodynamic analysis

The PT (in seconds) obtained was converted to prothrombin complex activity (PCA) (as percentage of normal activity) using the calibration curve performed (103) as explained below. The pooled plasma from untreated rats was diluted to different dilutions ranging from 100 to 15 % (PCA), with 100 % being the undiluted plasma and the corresponding PT values were measured. The calibration curve was plotted using PT values (in seconds) on the y-axis & PCA % on the x-axis. The calibration equation thus obtained was used to calculate the PCA % corresponding to each of the PT value measured in the study. The minimum value of PCA (PCA_{min}), time to achieve PCA_{min} ($t_{PCA,min}$) and the total area under the PCA–time curve from time zero to 96 h after warfarin (PCA AUC_{0-96}), obtained using the linear trapezoidal rule, were used to assess the degree of blood coagulability (102). PCA_{min} corresponds to the

maximum anticoagulation achieved and the $t_{PCA,min}$ represents the time to reach maximum anticogulation.

3.3.7.2. Pharmacokinetic analysis

WinNonlin (version 1.1) iterative curve-fitting program based on nonlinear regression analysis (Scientific Consulting Inc., Lexington, KY, USA) was used to estimate various pharmacokinetic parameters (104). The time-profiles of the S- and R-enantiomers of warfarin following its oral administration to rats were adequately described using the one-compartment open model with first-order absorption and elimination. The administered dose of warfarin was divided by two to obtain the equivalent enantiomeric dose (105). The rate and extent of warfarin absorption were assumed to be non-stereoselective and complete, respectively (105, 106).

The peak serum concentration (C_{max}) for each enantiomer of warfarin and the corresponding time of its occurrence (t_{max}) were obtained by visual inspection of the serum concentration–time curves. The absorption rate constant (ka), elimination half-life ($t_{1/2}$), total serum clearance (CL) and apparent volume of distribution (V_d) were obtained from the nonlinear regression analysis. The total area under serum concentration-time curve from time zero to infinity ($AUC_{0-\infty}$) was calculated using linear trapezoidal rule.

3.3.7.3. Statistical analysis

The Statistical Package for the Social Sciences (SPSS 11.0 for windows, SPSS Inc., USA) was used for the data analysis. Data were expressed as mean ± standard

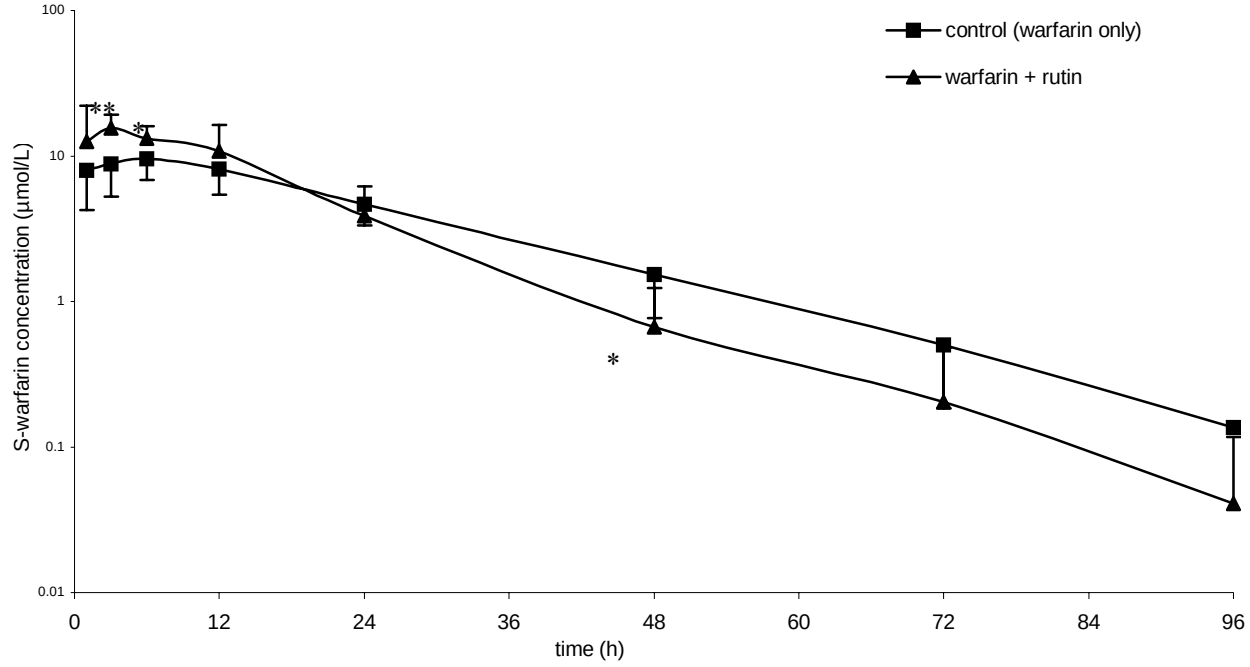
deviation (SD). Comparisons of means of related samples and from two independent groups were made using the Student's paired-samples and independent-samples *t*-tests, respectively. A *p*-value of less than 0.05 was considered as statistically significant.

3.4 RESULTS

3.4.1. Warfarin pharmacokinetics

The mean serum concentration-time profiles of the S- and R- enantiomers of warfarin after oral administration of a single dose of the racemic drug (1.5 mg/kg) in the presence and absence of rutin are shown in **Fig. 3.1**. Rutin treatment significantly increased both S- and R-warfarin serum levels initially compared to control group. Subsequently during the elimination phase both the enantiomer levels were lower with respect to that in control animals. The difference between the two groups in enantiomer levels was statistically significant at 3 ($p<0.01$), 6 ($p<0.05$) and 48 h ($p<0.05$) time-points in case of S-warfarin and 3 ($p<0.01$) and 48 h ($p<0.05$) for R-warfarin. The serum concentration profile of S- warfarin in control and rutin treated groups after WinNonlin curve fitting is shown in **Fig. 3.2 (a,b)** and that of R- warfarin are shown in **Fig. 3.3 (a,b).**

Estimates of pharmacokinetic parameters between the two groups are summarized in **Table 3.1**. With regard to those describing absorption kinetics, rutin treatment increased the S- and R- warfarin C_{max} values (by 71.3 and 59.7%, respectively; $p<0.01$ and $p< 0.05$) while tended to shorten the corresponding t_{max} values (by 27.3 and 27.7%, respectively). The respective ka values appeared to increase to a similar extent



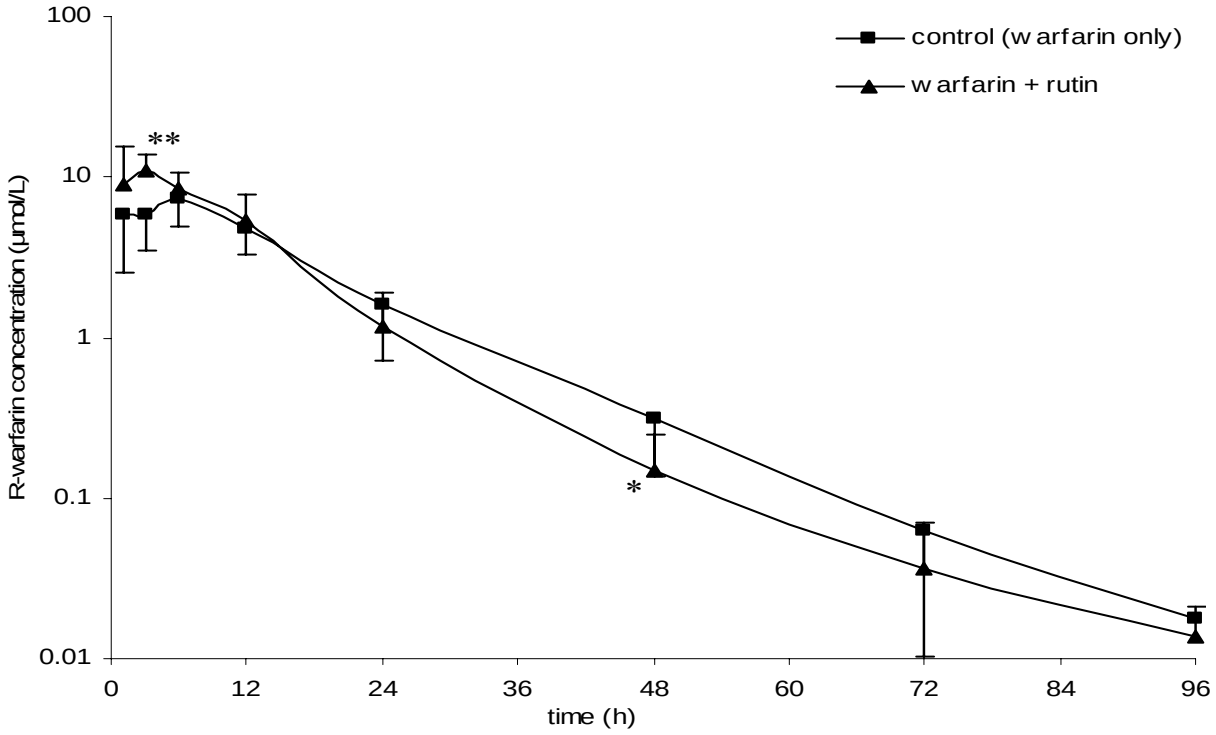
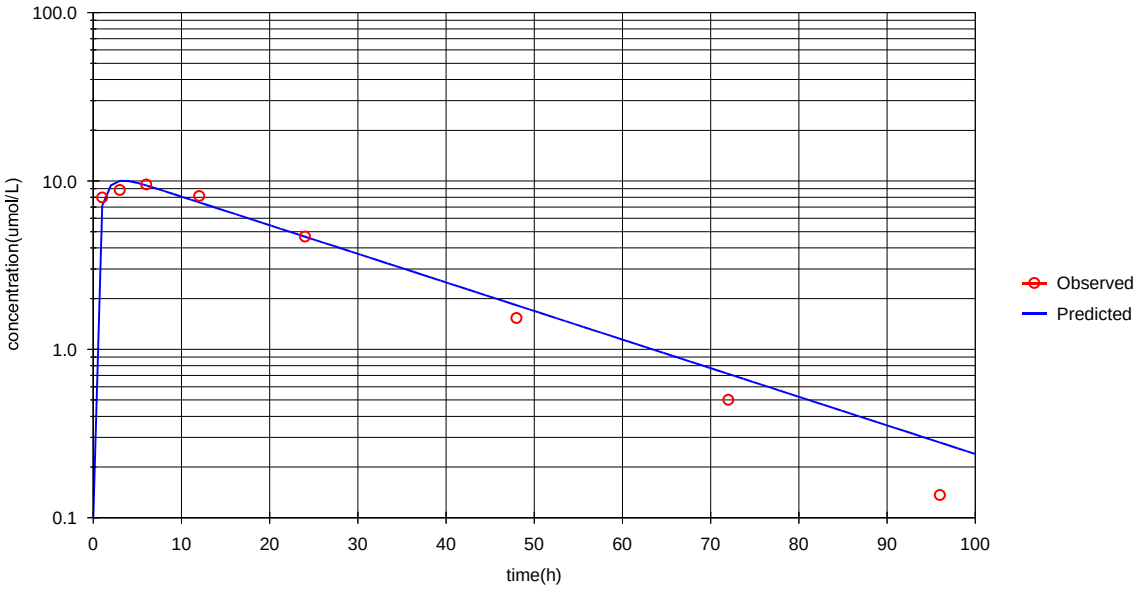


Figure 3.1 Time courses of total serum concentrations (mean ± S.D.) of S- and R-enantiomers of warfarin following a single oral administration of racemic dose (1.5 mg/kg) to rats alone (receiving 1% CM-cellulose, 5 ml/kg daily, as the control) and during 8-day treatment with rutin (1 g in 1% CM-cellulose, 5 ml/kg daily).

* $p < 0.05$ comparing the control group with rutin treatment group.
** $p < 0.01$ comparing the control group with rutin treatment group.

(a)



(b)

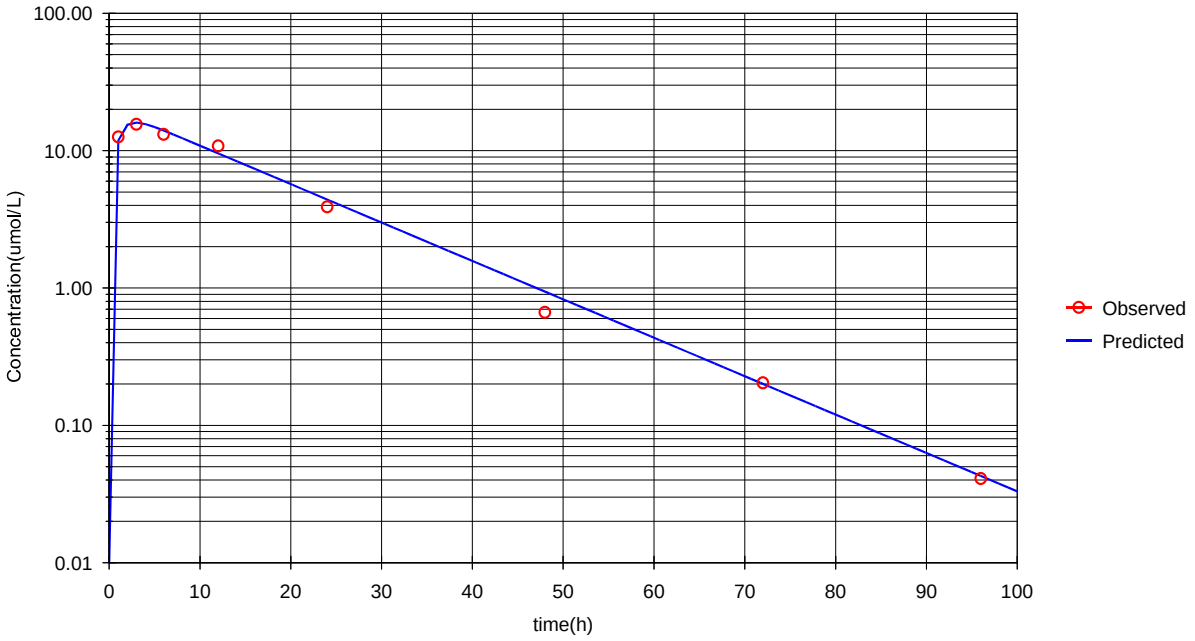
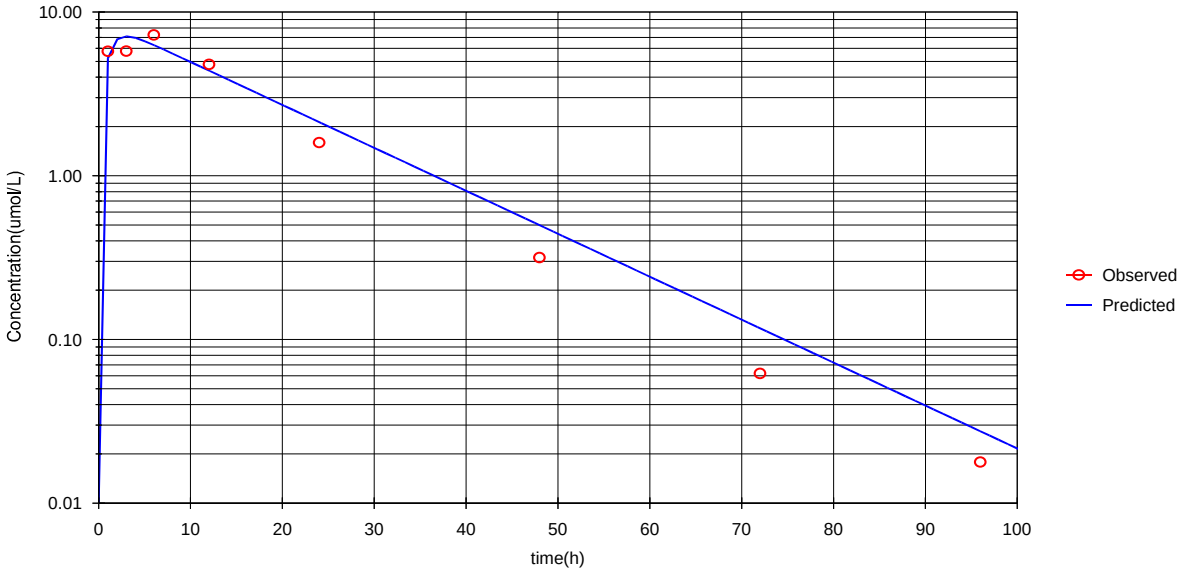


Figure 3.2 S- warfarin (mean) curve fitting for (a) control and (b) rutin treated rats in single dose study using WinNonlin. Control rats received 1% CM-cellulose, 5 ml/kg, daily and rutin group was given 1 g rutin in 1% CM-cellulose, 5 ml/kg daily for eight consecutive days. On day 5, each rat received a single oral dose of racemic warfarin (1.5 mg/kg, dose volume = 5 ml/kg). One compartment open model with no lag time, with 1st order absorption and 1st order elimination, and with user specified initial parameters and bounds was used to select the best fitting curve. The circular points indicate observed S- warfarin levels and the line connecting those shows predicted levels.

(a)



(b)

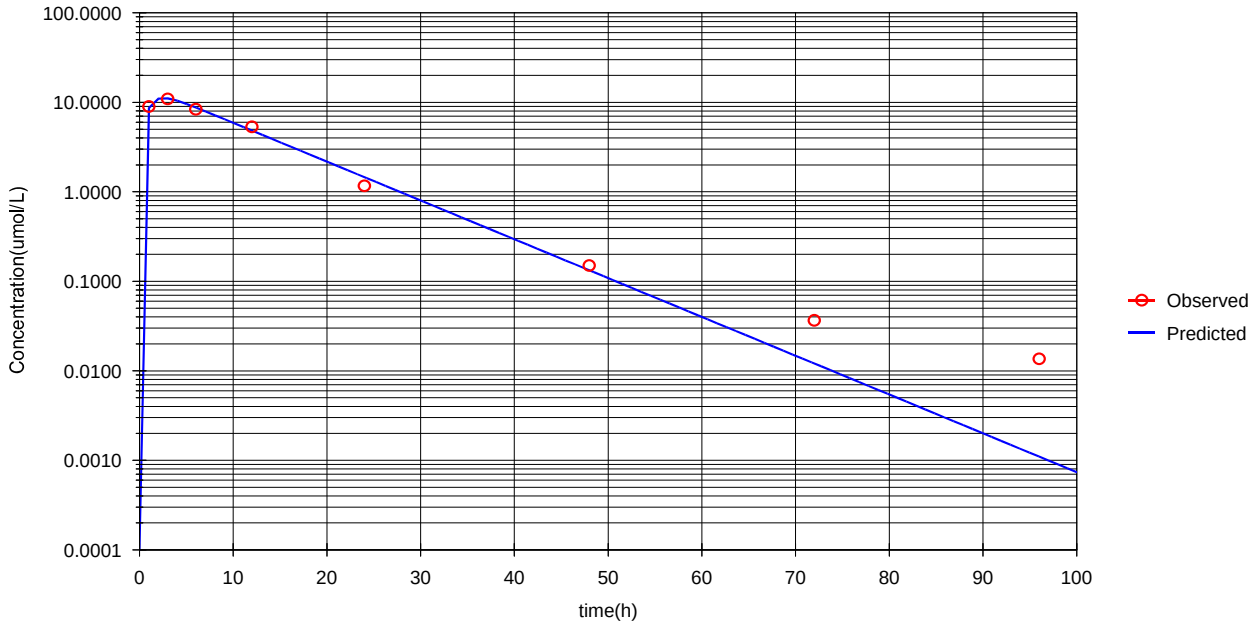


Figure 3.3 R- warfarin (mean) curve fitting for (a) control and (b) rutin treated rats in single dose study using WinNonlin. One compartment open model with no lag time, with 1st order absorption and 1st order elimination, and with user specified initial parameters and bounds was used to select the best fitting curve. The circular points indicate observed S- warfarin levels and the line connecting those shows predicted levels.

Table 3.1 Estimated pharmacokinetic parameters of (S)- and (R)- enantiomers of warfarin after a single oral administration of racemic drug (1.5 mg/kg) to rats in the absence and presence of rutin

Parameter	Control		Rutin	
	S-warfarin	R-warfarin	S-warfarin	R-warfarin
C_{max} (μmoles/L)	10.1 ± 2.4	7.7 ± 2.3 [a]	17.3 ± 4.8 [c]	12.3 ± 4.1 [d]
t_{max} (h)	5.30 ± 3.1	4.66 ± 2.2	3.85 ± 1.8	3.37 ± 1.4
$AUC_{0-\infty}$ (μmoles/L·h)	304.9 ± 64.5	149.4 ± 24.3 [a]	302.1 ± 125.2	157.0 ± 50.3
ka (h^{-1})	0.637 ± 0.63	0.698 ± 0.55	0.857 ± 0.60	0.938 ± 0.79
$t_{1/2}$ (h)	15.3 ± 2.1	10.1 ± 1.5 [a]	8.6 ± 2.6 [c]	6.0 ± 2.0 [c]
V_d (L/kg)	0.183 ± 0.044	0.242 ± 0.053 [b]	0.107 ± 0.028 [c]	0.137 ± 0.031 [c]
CL (ml/kg/h)	8.30 ± 1.8	16.67 ± 2.7 [a]	9.23 ± 3.4	16.83 ± 4.9

Values are mean ± S.D. (n = 8)
C_{max}, maximum serum concentration; t_{max}, peak time.; $AUC_{0-\infty}$, total area under serum concentration-time curve from time zero to infinity; ka, absorption rate constant; $t_{1/2}$, elimination half life; V_d, apparent volume of distribution; CL, total serum clearance.

[a] $p < 0.01$ comparing S- warfarin with R- warfarin in control group
[b] $p < 0.05$ comparing S- warfarin with R- warfarin in control group
[c] $p < 0.01$ comparing the control group with rutin treatment group
[d] $p < 0.05$ comparing the control group with rutin treatment group

(by 34.5 and 34.4%, respectively) in the rutin treatment group compared to the control group. With regard to parameters describing disposition kinetics, rutin treatment significantly reduced the V_d values (by 41.5 and 43.4%, respectively) and $t_{1/2}$ values (by 43.8 and 40.6%, respectively) of both S- and R- warfarin ($p < 0.01$). There was no significant difference in AUC and CL for either S- or R- warfarin between the control and rutin co-administration groups.

3.4.2. Effect of rutin treatment on warfarin anticoagulation

The basal mean prothrombin times (PT_0) between the control and rutin treatment groups were similar either with CM-cellulose (1%, 5 ml/kg daily) or rutin (1 g in 1% CM- cellulose, 5 ml/kg daily) treatment for four days. **Table 3.2** shows the pharmacodynamic parameters after acute dose of warfarin (1.5 mg/kg) in rutin treated and control rats. Rutin treatment appeared to reduce mean PCA_{min} (by 74.3%) and $t_{PCA,min}$ (by 30.8%, $p < 0.05$) compared to the control group. The mean PCA $AUC_{0\text{-}96h}$ value of rutin treatment group was significantly higher (by 31.3%, $p < 0.05$) than that of control rats. With the warfarin administration, the PT values increased in all the animals as shown in **Fig. 3.4**. Recovery from this hypoprothrombinaemia was faster with rutin treated animals as lower PT (and higher PCA) values were observed at both 48 h and 72 h ($p < 0.05$) post warfarin dose in the treatment group compared to that in the control rats.

3.5. DISCUSSION

Rutin, as a constituent as well as in pure form, is widely used in complementary and

Table 3.2 Estimated pharmacodynamic parameters (mean± S.D.) following a single oral administration of racemic warfarin (1.5 mg/kg) to rats alone (control) and during rutin treatment

Parameter	Control	Rutin
PT_0 (s)	43.9 ± 8.5	41.5 ± 11.3
PCA_{min} (% normal)	14.4 ± 2.8	3.7 ± 3.0
PT $AUC_{0\text{-}96h}$ (s·h)	19130.3 ± 4239.3	15676.7 ± 2497.2
PCA $AUC_{0\text{-}96h}$ (% normal·h)	4431.3 ± 1271.8	5818.9 ± 767.6 [a]
$t_{PCA,min}$ (h)	39.0 ± 12.4	27.0 ± 8.5 [a]

PT_0, basal prothrombin time at time zero; PCA_{min}, minimum prothrombin complex activity; PT $AUC_{0\text{-}96h}$, area under PT-time curve from time zero to last sampling point; PCA $AUC_{0\text{-}96h}$, area under PCA-time curve from time zero to last sampling point (96h); $t_{PCA,min}$, time to achieve PCA_{min}.

[a] $p < 0.05$ comparing the control group with rutin treatment group (n = 8)

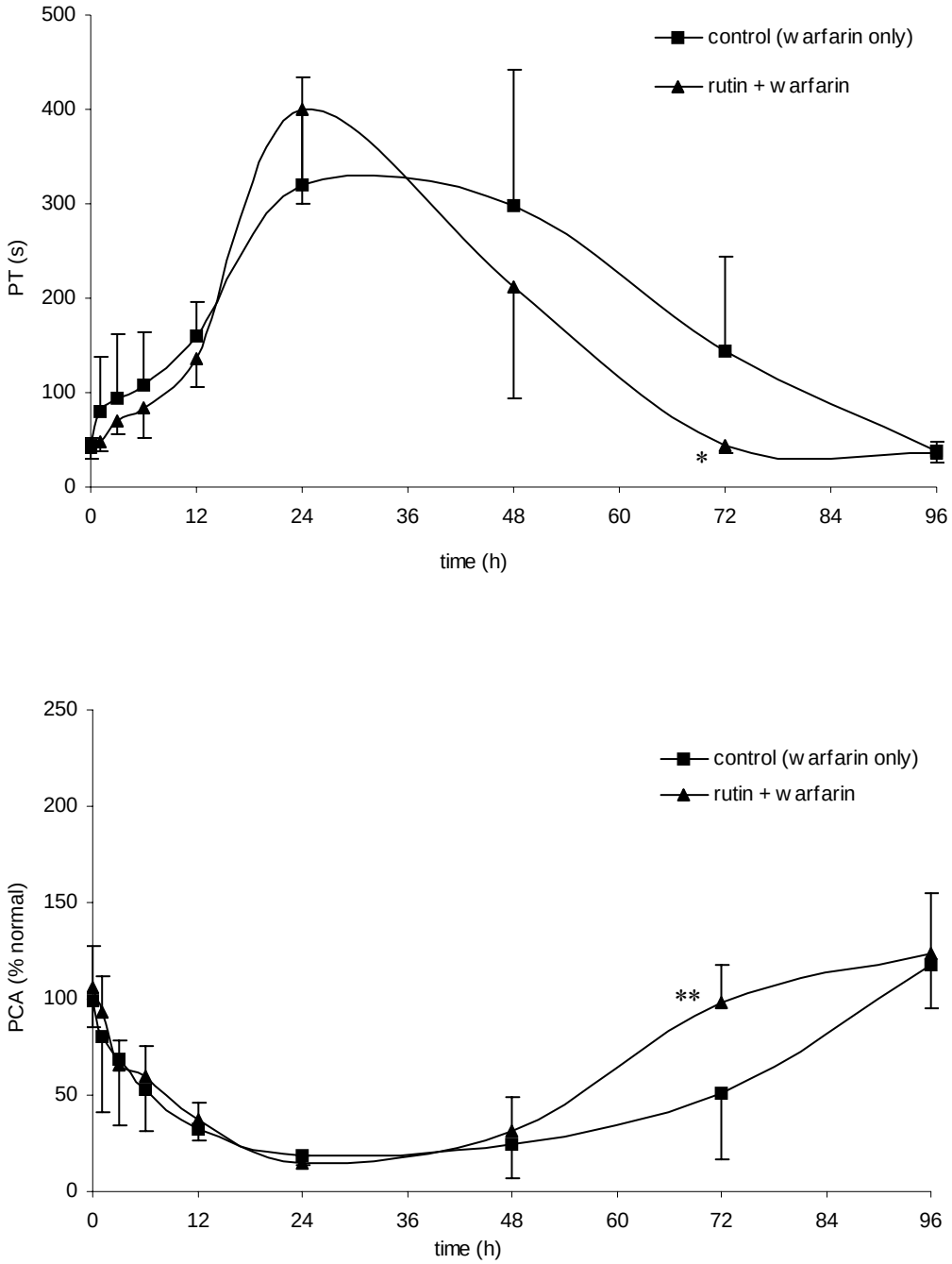


Figure 3.4 Changes in prothrombin time (PT in s) and prothrombin complex activity (PCA in percent of normal) with time following a single oral dose of racemic warfarin (1.5 mg/kg) to rats treated with and without rutin. Values are expressed as mean ± SD (n = 8).

* $p < 0.05$ comparing the control group with rutin treatment group. ** $p < 0.01$ comparing the control group with rutin treatment group.

alternative medicines and numerous health supplements. Apart from the cardiovascular benefits, rutin is also shown to lower plasma lipids and hepatic cholesterol levels (86). Rutin has been reported to counteract dicoumarol in rats (107). When rutin was coadministered orally with dicoumarol, PT was shorter compared to that with dicoumarol alone (107). As rutin combined with warfarin is reported to be beneficial in limb salvage and survival in patients with severe critical leg ischemia (23), coadministration of the two drugs clinically is a promising option. But warfarin with low therapeutic index and being known to interact with various drugs and herbs, such as Danshen (108), Ginseng (49), St. John's wort (109), potential drug interaction needs to be carefully considered. Our study has revealed some evidence of interaction between rutin and warfarin in rats.

Rats fed orally with rutin at a dose of 1 g/kg body weight for 22 days reportedly showed no toxic effects (97). Thus the rutin dose of 1 g/kg body weight, much higher than the normal dietary intake in man, was selected for our study to maximize the potential interactions, if any. Rats were treated with rutin orally for four days before administering a single dose of warfarin. This could possibly mimic a clinical case of starting warfarin in a patient who has been on a diet rich in rutin and also on dietary supplements and herbal remedies with rutin as one of the constituents. The rats were monitored closely throughout the study and all the animals were found to tolerate the treatment and the repeated blood sampling well.

In agreement with the initial assumption (105, 106), the present study showed no significant stereoselective difference in the rate of absorption of warfarin enantiomers in control animals receiving CM-cellulose only. The peak serum concentration (C_{max})

achieved with S-warfarin was significantly higher than that of R-warfarin. It is known that R-warfarin is eliminated faster in rats and slower in man compared to S-warfarin elimination kinetics (48). Consistent with literature reports (48, 110), significantly lower AUC, higher clearance and shorter elimination half-life for R-warfarin, in comparison to S-warfarin, were observed in control rats. Also, the V_d value of R-warfarin was larger than that of S-warfarin, due possibly to a higher binding affinity to albumin for the latter (8, 48). The t_{max} values of both the enantiomers were found to be slightly longer compared to those obtained previously in our laboratory in similar experiments (104, 111). This could be due to the use of CM-cellulose as a vehicle in the present study, though it needs to be verified further. It is also known that the presence of food can slow the rate of absorption of warfarin without affecting its bioavailability (8).

There was no significant difference in mean PT values between the rutin treatment and control groups prior to warfarin dosing (PT_0). Thus after four days of treatment, rutin by itself showed no effect on blood coagulation. (This was further supported by designing a study of effects of rutin on blood coagulation in the absence of warfarin. Rutin treatment alone showed no significant effect on blood coagulation compared to that of control). When a single dose of warfarin was administered to both the groups, warfarin caused hypoprothrombinaemia in all the animals, represented by increased PT (decreased PCA). The degree of hypoprothrombinaemic response was enhanced at 24 h only, but was reduced at all other time-points, with concurrent rutin administration. As a result, rutin treatment increased the total area under the PCA-time curve from 0 to 96 h post warfarin. This indicated an overall reduction in the anticoagulation response to warfarin. It was also interesting to note that the recovery

from warfarin anticoagulation was found to be much faster in the presence of rutin. The difference in PT (and PCA) between the two groups was especially significant at 72 h post warfarin dose.

Similarly, pharmacokinetic parameters of S- and R-warfarin showed a trend of interaction in the presence of rutin. There was an initial increase followed by lower levels of both the enantiomers in rutin treated rats. S-warfarin levels significantly differed between the two groups at 3, 6 and 48 h time-points and R-warfarin levels at 3 and 48 h after warfarin dose. The initial increase in serum levels of S-and R-warfarin is intriguing. Rapid absorption of a drug can lead to a higher peak concentration and shorter peak time. Our results showed an apparently higher ka and shorter peak time with rutin treatment, though not statistically significant. An increase in R-and S-warfarin levels would be expected, as quercetin, the aglycone of rutin, has been shown to inhibit *in vitro* human CYP3A4 and CYP2C9 (6), the hepatic microsomes responsible for the metabolism of R- and S-warfarin, respectively (11).

In vitro studies using specific CYP enzymes may be needed to shed more light on this interaction. Typically, the inhibitory constant, K_i, for the inhibitor drug is used to determine the inhibition efficiency (112). However, K_i value needs to be estimated very accurately to successfully predict from *in vitro* data, whether a drug inhibits CYP enzymes *in vivo* or not (112). If not, the prediction of *in vivo* drug interactions may not be very conclusive (112). As such it has been reported that *in vitro* and *in vivo* data on CYP isoform modulation could yield contradictory results (113, 114, 115, 116). Estimation of warfarin metabolites in urine is also important to understand the effect of rutin on warfarin. It can provide valuable insight into the metabolism and

clearance of warfarin and potential metabolic interactions with rutin, if any. .

Rutin appeared to have no effect on the total serum clearance values of R- and S-warfarin. However, the apparent volumes of distribution of the two enantiomers were significantly lower in case of the rutin group. Hence, shorter elimination half-life values of S- and R-warfarin were observed with rutin treated rats compared to that of control. This could have been responsible for the observed lower levels of S- warfarin and possibly the faster recovery of PT with rutin treatment. Quercetin and related flavonoids have been reported to bind (99.1-99.4%) almost exclusively and strongly to human serum albumin *in vitro* (24) as well as rodent albumin (14). Both warfarin and quercetin bind to the IIA domain of albumin (117, 118) and quercetin is shown to be displaced by large excess of warfarin, although not competitively (24, 94). Using fluorescence spectroscopy these researchers studied albumin binding of quercetin in the presence of large excess of warfarin but individual warfarin enantiomers were not quantitated. In our study, as total serum clearance was not altered much, rutin administered in large excess could have affected the serum and tissue protein binding of warfarin. Thus, further analysis of protein binding is needed to be carried out to understand the mechanisms of this interaction.

3.6. CONCLUSION

The single dose study reveals possible pharmacokinetic drug interactions between rutin and a single dose of warfarin. Rutin treatment increased the C_{max} and reduced the $t_{1/2}$ and V_d values of both S- and R- warfarin significantly and lowered the serum levels of both the enantiomers in the elimination phase compared to the control group.

Rutin treatment also resulted in a faster recovery from the hypoprothrombinaemia caused by warfarin. Thus, due to the risk of potential interactions between the two when co-administered, extra care must be taken to exclude high levels of rutin aglycone and conjugates in patient blood before starting warfarin therapy. In addition, if the patient has been stabilized on warfarin with concomitant use of rutin or quercetin, precaution should be taken to avoid sudden suspension of rutin as it may affect warfarin levels fatally. Therapeutic monitoring of warfarin is extremely important in this scenario and warfarin dose regimen needs to be adjusted accordingly.

CHAPTER 4 *IN VIVO* MULTIPLE DOSE STUDY

4.1. INTRODUCTION

Warfarin is used to treat various cardiovascular disorders and generally administered for a very long period. Warfarin drug interactions are clinically relevant since an interaction leading to increased action may cause hemorrhage and decreased action may cause reduced anticoagulation (115). With the initiation of warfarin therapy, notable and stable anticoagulation is achieved only after the existing vitamin K-dependent clotting factors are removed from the circulation by clearance (37, 61). Due to the long half-life of some of the coagulation factors, complete antithrombotic effect is not achieved for several days (37). But the PT may be prolonged sooner because of the rapid reduction of clotting factors with shorter half-life (37). Generally, 2 to 7 days are required to reach this steady state of anticoagulation depending on the dose used (61). However, in case of non emergency conditions, such as chronic stable atrial fibrillation, a maintenance dose of 4-5 mg/day may achieve steady-state anticoagulant effect in about 14 days (61). Thus with multiple dosing of warfarin and longer duration of treatment, introduction of an interacting constituent can have serious implications as warfarin has a very narrow therapeutic index.

It is known that although target anticoagulation (PT/INR) was maintained carefully in patients, a brief change in drug compliance or diet may precipitate fatal bleeding or clotting (115, 119). For example, when an herbal preparation with antiplatelet activity is introduced in a patient on chronic anticoagulation with stable PT/INR values, resulting interaction can lead to bleeding complications (119). Some of the clinical

reports of fatal interactions between long term multiple doses of warfarin and herbs are compiled in **Table 4.1** (119).

With a small therapeutic window of warfarin, such interactions are possible due to various reasons like, inherent anticoagulant/antiplatelet activities of the herbs/diet or effects on the pharmacokinetics of warfarin (119). Furthermore, due to its elimination half-life range of 25 to 60 hours (37), warfarin may take longer time before its plasma concentrations and anticoagulant effects come back to therapeutic levels. It is known that 3.5 to 5 half-lives are required to reach a steady state whenever there is a change (addition or deletion) in dose. So it is important to understand the mechanisms of potential interactions, not only with single dose, but also with multiple doses of warfarin and a longer treatment regimen.

Understanding and managing warfarin interactions with complementary and alternative medicines (CAM) is a challenge as relevant details such as active constituents, efficacy and adverse effects of most of these medicines are scarce. Herbal formulas such as garlic preparations may vary in the amount of their active constituents depending on the mode of preparation (120). Many of the multivitamins and herbal drugs can potentially affect the therapeutic benefits of warfarin (121) (**Table 1.6**). It was reported by Kurnik, D. et al 2003 (121) that 3 different patients on warfarin experienced sub- or supratherapeutic INR levels with initiation or cessation of a daily dose of multivitamin preparation containing vitamin K (121). As such the levels of these multivitamins in blood may be insignificant to affect but when given to patients on anticoagulation, vitamin K-containing multivitamins can inhibit the beneficial effects of warfarin (121).

Table 4.1 Clinical case reports of complications arising due to interactions between multiple warfarin dosing and herbs (119)

Patient age(years)/ gender	Herb used	dosage	complication
32/Female	Garlic	*	Prolonged post-operative bleeding
87/Male		2 g/day	Spontaneous epidural hematoma
72/Male		*	Postoperative bleeding
70/Male	Ginkgo Biloba	80 mg/day	Spontaneous hyphema
33/Female		120 mg/day	Spontaneous subdural hematoma
72/Female		150 mg/day	Spontaneous subdural hematoma
78/Female		*	Intracerebral hemorrhage
61/Male		160 mg/day	Subarachnoid hemorrhage
61/Male	Quiling gao	1 can/day [a]	Mucosal bleeding
25/Female	Herbal tea	*	menometrorrhagia
*	Devil's claw	*	Purpura
48/Female	Danshen	* [a]	Increased response to warfarin
66/Male		* [a]	Bleeding gastric carcinoma
62/Male		* [a]	Pleural hemorrhage
47/Male	Ginseng	3 capsules/day [a]	Reduced response to warfarin
44/Female		Face cream	Vaginal bleeding
72/Female		200 mg	Vaginal bleeding

* Not applicable

[a] Patient on chronic anticoagulation therapy with warfarin

In the next part of the study, rats were treated with daily doses of warfarin for five days before administering rutin. This was designed to possibly mimic a clinical case of a patient stabilized and responding well to a warfarin treatment regimen and taking subsequent rutin unknowingly along with warfarin.

As rutin is shown to have the potential to interact with single dose of warfarin, it was imperative to see if by itself rutin had any action on the blood coagulation in rats. Quercetin and rutin are strong anti-platelet and antithrombotic agents *in vivo* though *in vitro* potency is modest (122). Their antithrombotic property could be due to a combination of the anti-oxidant action and the ability to bind to platelet membranes (122). Thus in the next set of experiments rutin was evaluated alone, in the absence of warfarin, for its effect on blood coagulation, if any. Rats were treated with rutin orally for eight days and clotting time was measured at various time intervals over the treatment period.

4.2. OBJECTIVES

The objective was to study the effect of introduction of rutin on pharmacokinetic and pharmacodynamic properties of warfarin at steady state levels in rats. Concentrations of each of the warfarin enantiomers were quantitatively estimated in serum by HPLC assay and pharmacokinetic parameters calculated. Also the effect on warfarin anticoagulation was measured.

Further experiments were designed to study the effect of rutin alone on coagulability in rats in the absence of warfarin. The effect on coagulation was evaluated by

measuring prothrombin time and PCA (as percentage of normal prothrombin complex activity).

4.3. MATERIALS AND METHODS

4.3.1. Apparatus

The HPLC system for the assay of warfarin enantiomers was as follows: A solvent delivery system (LC-10AT, Shimadzu), sample injector with standard sample loop (20 μl), a fluorescence detector (RF-10AXL, Shimadzu), a communication bus module (CBM101, Shimadzu), a computer and a workstation (Class-CR10, Shimadzu). The chromatographic conditions for isocratic normal phase HPLC were as follows: A stainless steel column (250 × 4 mm) packed with silica (MAXSIL 10 silica, 10 micron, Phenomenex, USA) maintained at 23°C ambient temperature, was used for the separation. Mobile phase of a mixture of ethyl acetate/n-hexane (22.5:77.5) was run at a flow rate of 0.8 ml/min. Bed reactor (stainless steel column 3 mm i.d., 25 cm) packed with glass beads (40 μm) was used for the post column aminolysis. Butylamine/methanol (1:1) was pumped as the post column reagent at a flow rate of 0.4 ml/min. The effluent from the column was detected by excitation at 313 nm and emission at 400 nm using a fluorescence detector. For the prothrombin time (PT) test, fully automated coagulation analyzer (Sysmex automated blood coagulation analyzer, CA 530, Sysmex Corporation, Kobe, Japan) was used.

4.3.2. Animals and sampling method

Adult male Sprague-Dawley rats, each weighing 250-300 g were obtained from the Laboratory Animal Center, Singapore. The animal experiments were approved by the Institutional Animal Care and Use Committee of laboratory animal centre, National University of Singapore, Singapore. Rats were housed in metabolic stainless steel cages individually in an air-conditioned room (23 ± 1°C). They were maintained on 12 h light/dark cycle for three days before the experiment. The animals had access to food (rat cubes) and water *ad libitum* during the experimental period, but fasted overnight before oral administration of warfarin and 4h after the administration. Rat cubes, the standard diet for laboratory animals fortified with vitamins and minerals, purchased from Glen Forest Stockfeeders, Australia.

4.3.2.1. Preparation of citrate buffer diluted plasma

Venous blood was obtained from the orbital sinus of rats by puncturing with siliconized capillary tube. Citrate-diluted plasma was prepared as per the method established (102). The citrate-diluted blood was centrifuged for 10 min. at 3000 rpm, plasma was separated and used for the One Stage Prothrombin Time test. Serum was prepared from about 400 μl of blood obtained without citrate. Concentration of the warfarin enantiomers in the serum was analyzed by normal phase HPLC method.

4.3.3. Chemical analysis

The stereospecific HPLC method established by Banfield, C. and Rowland, M. 1984

(65), was used for the chemical analysis. The S- and R- enantiomers of warfarin in the serum sample were separated by first converting them into diastereomeric esters with carbobenzyloxy L-proline and further separating on HPLC system. The effluent from the column was mixed with postcolumn n-butylamine for aminolysis. The resulting fluorescent enantiomers were detected on-column fluorimetrically.

4.3.4. Estimation of anticoagulation

Neoplastine reagent was prepared by reconstituting freeze-dried thromboplastine powder (Reagent 1) in 2.5 ml of the given aqueous solvent (Reagent 2) just before use. The test protocol of the machine was set to sample 100 µl of citrate-diluted plasma (102) and mix with 50 µl of the Neoplastine reagent automatically. The maximum reaction time was adjusted to 600 s. The clotting time in seconds (PT) as end point was read directly from the machine. The precision was tested by using blank pooled plasma at different dilutions within a range of 100-15% and was found to be acceptable (RSD 0.32-5.31%).

4.3.5. Animal study protocol

4.3.5.1. Multiple dose study

Male Sprague-Dawley rats were divided randomly into control and treatment groups (n = 6 per group). For the first five days animals from both the groups received oral warfarin (0.15 mg/kg/day, dose volume = 5 ml/kg) daily to reach a steady state. On the sixth day, along with warfarin, control rats were also given CM-cellulose (1% w/v) (5 ml/kg) orally. In a parallel design, treatment group received rutin (1 g in 1 %

CM-cellulose/kg) at a dose of 5 ml/kg body weight apart from the warfarin dose. Daily dosing of warfarin and rutin/CM-cellulose, sodium salt was continued till 96 h post the first rutin dose. Blood samples were collected from each rat prior to, and 24, 48, 72, 96, 120, 144 and 168 h after the administration of the first dose of rutin. Plasma and serum samples were prepared (102) and separated by centrifugation (10 min at 3000 X g) and stored at –20°C until analysis.

4.3.5.2. Control rutin treatment study in the absence of warfarin

This treatment schedule was designed to study the effect of rutin alone on blood coagulation in rats. One group of male Sprague-Dawley rats (n = 6) was treated with rutin (1 g/kg/day, dose volume = 5 ml/kg body weight) for 5 days. The control rats (n = 6) received oral CM-cellulose (1% w/v) (5 ml/kg) for the same period. Blood samples were collected from each rat prior to, and 24, 48, 72, 96, 120, 144 and 168 h after the first dose of rutin. Plasma samples were prepared by appropriate dilutions of the blood with citrate buffer (102), centrifuged (10 min at 3000 X g), separated and stored at –20°C until before the analysis.

4.3.6. Data analysis

4.3.6.1. Pharmacodynamic analysis

The PT (in seconds) obtained was converted to prothrombin complex activity (PCA) (as percentage of normal activity) using the calibration curve performed (103). The minimum value of PCA (PCA_{min}) was used to assess the degree of blood coagulability (111). PCA_{min} corresponds to the maximum anticoagulation achieved.

4.3.6.2. Pharmacokinetic analysis

The time-profiles of the S- and R-enantiomers of warfarin following its oral administration to rats were evaluated. The administered dose of warfarin was divided by two to obtain the equivalent enantiomeric dose (105). The minimum serum concentration during steady state (C_{min}) for each enantiomer of warfarin was obtained from the serum concentration–time curve by taking the mean of all the trough concentration values during the steady state. WinNonlin (version 1.1) iterative curve-fitting program (Scientific Consulting Inc., Lexington, KY, USA) was used to estimate various pharmacokinetic parameters (104). The apparent volume of distribution (V_d) and absorption rate constant (k_a or k_{01}) obtained from the single dose study were used as a basis for curve fitting. Also the elimination rate constant (k or k_{10}) was calculated from the terminal phase of the serum concentration-time curve for each animal and the value was used as the initial parameter for curve fitting along with V_d and k_a. One compartment open model with no lag time, with 1^{st} order absorption and 1^{st} order elimination, and with user specified initial parameters and bounds was used to select the best fitting curve for each animal data and subsequent PK parameters. Elimination half life ($t_{1/2}$) was estimated from the k value. Steady state clearance value was estimated using the formula $CL_{ss} = (R_0/C_{min})$ where CL_{ss} is the clearance value associated with minimum serum concentration at steady state (C_{min}) and R_0 is the dosing rate. The apparent volume of distribution (V_{ss}) associated with minimum serum concentration at steady state is calculated using the equation $V_{ss} = CL_{ss}/k$.

4.3.6.3. Statistical analysis

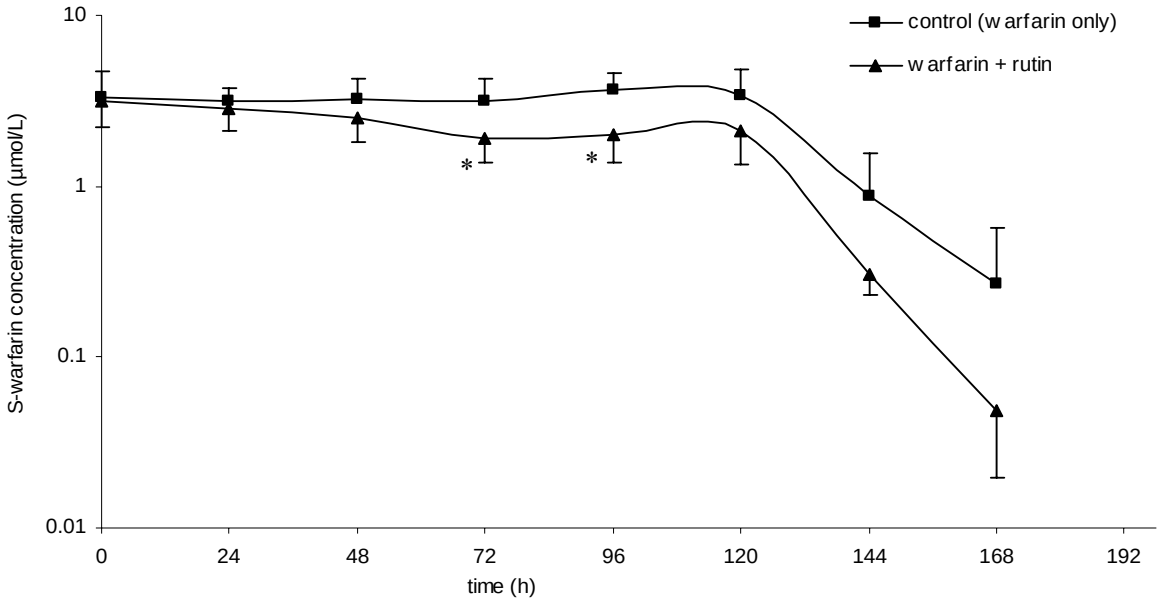
The Statistical Package for the Social Sciences (SPSS 11.0 for windows, SPSS Inc., USA) was used for the data analysis. Data were expressed as mean ± standard deviation (SD). Comparisons of means of related samples and from two independent groups were made using the Student's paired-samples and independent-samples *t*-tests, respectively. A *p*-value of less than 0.05 was taken as statistically significant.

4.4. RESULTS

4.4.1. Steady-state pharmacokinetics of warfarin

Fig. 4.1(a,b) illustrates the time course of mean (± SD) serum concentrations of S- and R- enantiomers of warfarin at steady state in the presence and absence of rutin. Warfarin treatment for the first five days in both groups resulted in similar serum levels of both the enantiomers. With rutin treatment, S- warfarin levels were significantly ($p < 0.05$) reduced at 72 and 96 h post first dose of rutin compared to that of control group. R- warfarin levels also declined in a similar pattern though the difference between the control and treated groups was not statistically significant. After the last dose of warfarin at 96 h, serum levels of both the enantiomers reduced by the first-order elimination kinetics. But S- warfarin was eliminated faster compared to R- warfarin. These results showed a similar trend to single dose study results with respect to the elimination phase. Steady state trough serum concentrations of S-warfarin in control and rutin treated groups after WinNonlin curve fitting are shown in **Fig. 4.2 (a,b)** and that of R- warfarin are shown in **Fig. 4.3 (a,b).** These WinNonlin graphs clearly show that with multiple dosing of warfarin, the estimated levels

(a)



(b)

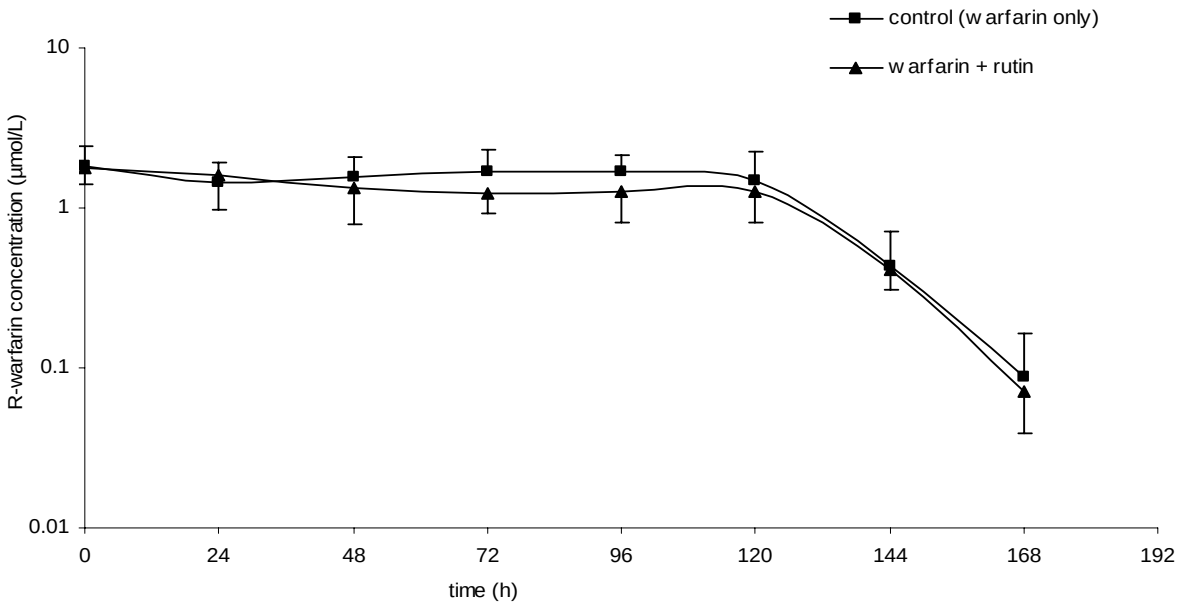
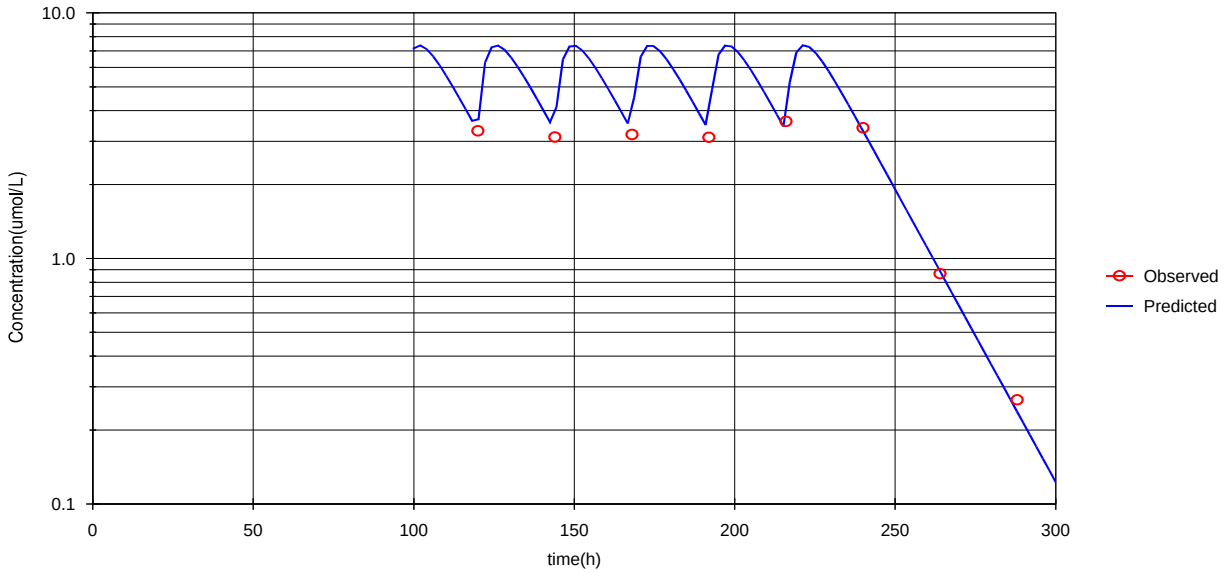


Figure 4.1 Time profiles of (a) S- and (b) R- warfarin concentrations (mean ± S.D.) in serum after multiple doses of warfarin (0.15 mg/kg body weight) in control and rutin treated rats (n = 6). Animals from both the groups received oral racemic warfarin daily, for initial five days (blood samples were not collected/analyzed during this period). From "0" h, along with warfarin, control rats were given daily CM-cellulose (1% w/v) (5 ml/kg) and treatment group received rutin (1 g in 1 % CM-cellulose/kg) (5 ml/kg body weight, orally for 96 h.
* $p<0.05$ comparing the control with rutin treatment group.

(a)



(b)

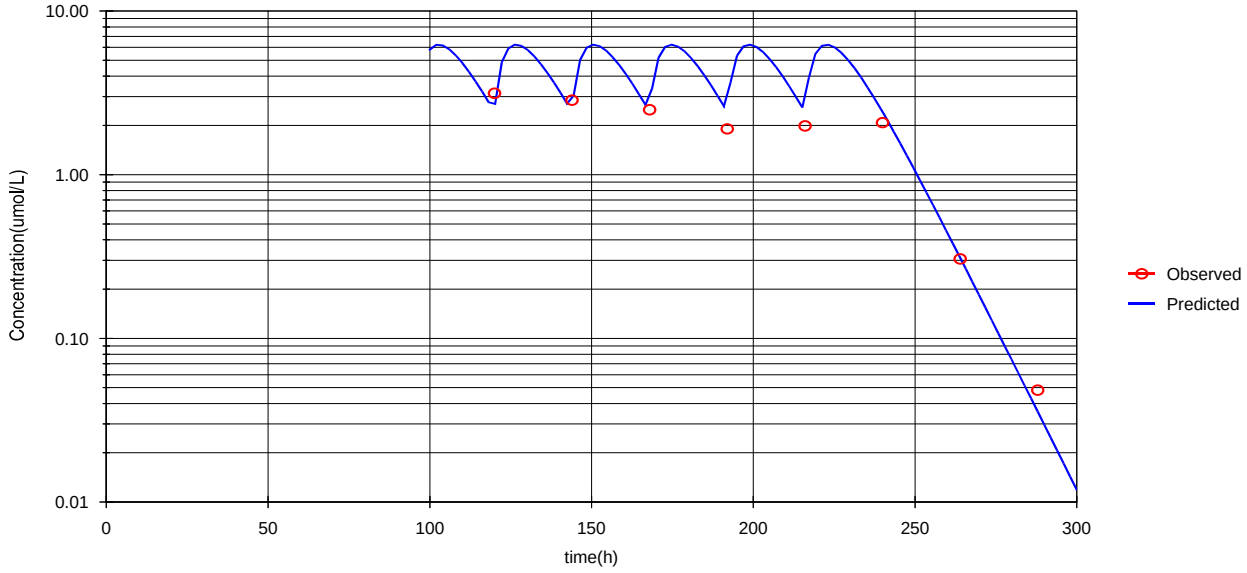
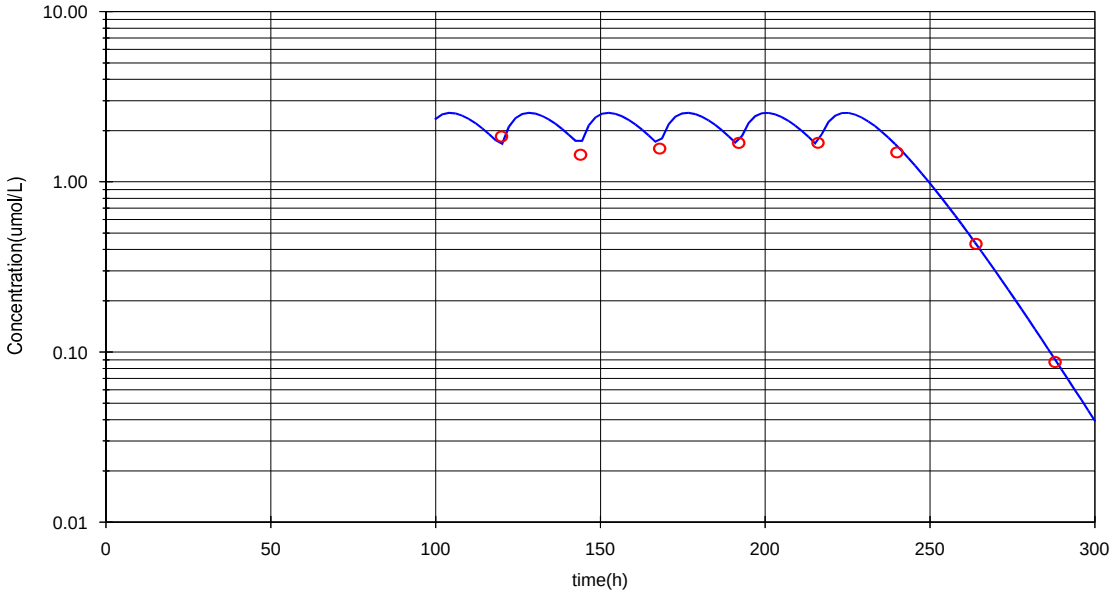


Figure 4.2 Mean S- warfarin curve fitting for (a) control and (b) rutin treated rats using WinNonlin. Animals from both the groups received oral racemic warfarin (0.15 mg/kg body weight) daily, for initial five days (blood samples were not collected/analyzed during this period). From 120 h post 1st dose of warfarin, along with warfarin, control rats were given daily CM-cellulose (1% w/v) (5 ml/kg) and treatment group received rutin (1 g in 1 % CM-cellulose/kg) (5 ml/kg body weight, orally for 96 h. One compartment open model with no lag time, with 1st order absorption and 1st order elimination, and with user specified initial parameters and bounds was used to select the best fitting curve. The circular points indicate observed S- warfarin levels and the line connecting those shows predicted levels.

(a)



(b)

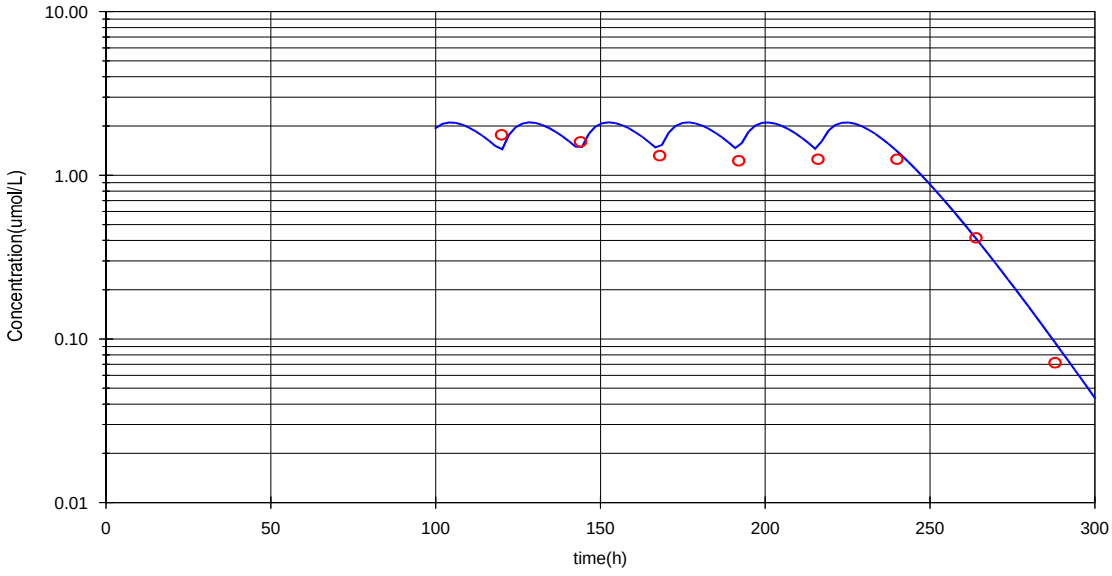


Figure 4.3 Mean R- warfarin curve fitting for (a) control and (b) rutin treated rats using WinNonlin. One compartment open model with no lag time, with 1st order absorption and 1st order elimination, and with user specified initial parameters and bounds was used to select the best fitting curve. The circular points indicate observed S- warfarin levels and the line connecting those shows predicted levels.

(marked as circular points in the graph) change from trough values to peaks between doses at steady state. Also the terminal elimination phase shows a linear pattern.

Pharmacokinetic profiles between the two groups for both the enantiomers of warfarin are compared in **Table 4.2**. Rutin treatment significantly reduced the minimum serum concentration value during steady state, C_{min}, (by 26.67 %) of S- warfarin ($p < 0.05$) compared to that of control group. R-warfarin minimum serum concentration levels during steady state reduced to a small extent (10.82 %) without any statistical significance. With rutin treatment, elimination half-life of S- warfarin reduced by 41 % but the difference was not statistically significant. However the elimination half-life of R- warfarin remained almost unchanged between control and rutin treated animals.

As the coefficient of variation of estimation of V_d and CL values from the WinNonlin curve fitting was very high, we based the CL_{ss} and V_{ss} estimation on minimum serum concentration value at steady state (C_{min}). CL_{ss} for S- warfarin increased with rutin treatment (by 31.1 %) compared to that of control group without statistical significance. However, CL_{ss} for R- warfarin was almost unchanged between control and rutin treated groups. Rutin treatment decreased the V_{ss} values of S– warfarin (by 36.11 %) but the decrease was not statistically significant. These results were similar to that of single dose study.

4.4.2. Effect of rutin treatment on warfarin anticoagulation at steady state

The time course of changes in PCA after multiple doses of warfarin with or without

Table 4.2 Pharmacokinetic parameters of control (treated with CM-cellulose only) and rutin treated (rutin in CM-cellulose) rats with respect to S- and R- warfarin after treatment with multiple doses of racemic warfarin (0.15 mg/kg body weight/day) orally.

Parameter	Control		Rutin treated	
	S- warfarin	R-warfarin	S- warfarin	R-warfarin
C_{min} (μmol/L)	3.30 ± 0.81	1.57 ± 0.52 [a]	2.42 ± 0.51 [c]	1.40 ± 0.38 [d]
Elimination $t_{1/2}$ (h)	13.35 ± 2.27	9.61 ± 2.32	7.87 ± 2.35	9.45 ± 0.87 [e]
V_{ss} (L/kg)[*]	0.072 ± 0.05	0.128 ± 0.07 [b]	0.046 ± 0.018	0.136 ± 0.031 [d]
CL_{ss} (ml/kg/h)[*]	3.31 ± 1.17	7.167 ± 2.63 [a]	4.34 ± 0.87	7.55 ± 1.48 [d]

Values are mean ± S.D. (n = 6)
C_{min}, minimum serum concentration during steady state; $t_{1/2,}$ elimination half life; V_{ss}, apparent volume of distribution associated with minimum serum concentration during steady state; CL_{ss}, total serum clearance associated with minimum serum concentration at steady state.

[*] based on the minimum serum concentration at steady state
[a] $p < 0.01$ comparing S- warfarin with R- warfarin in control group
[b] $p < 0.05$ comparing S- warfarin with R- warfarin in control group
[c] $p < 0.05$ comparing the control group with rutin treatment group
[d] $p < 0.01$ comparing S- warfarin with R- warfarin in rutin treatment group
[e] $p < 0.05$ comparing S- warfarin with R- warfarin in rutin treatment group

rutin treatment is represented by **Fig. 4.4 (a,b)**. The mean PCA values between the two treatment groups were similar before starting warfarin treatment and also before the 1st dose of rutin. With rutin treatment, the values appear to be higher from 72 h compared to control group levels. The difference was statistically significant at 72 h time point ($p < 0.01$). The average PCA_{min} values observed with rutin treatment group was 42.55 ± 11.95 and significantly higher ($p < 0.01$) when compared to that of control group (19.15 ± 5.96) (**Table 4.3**).

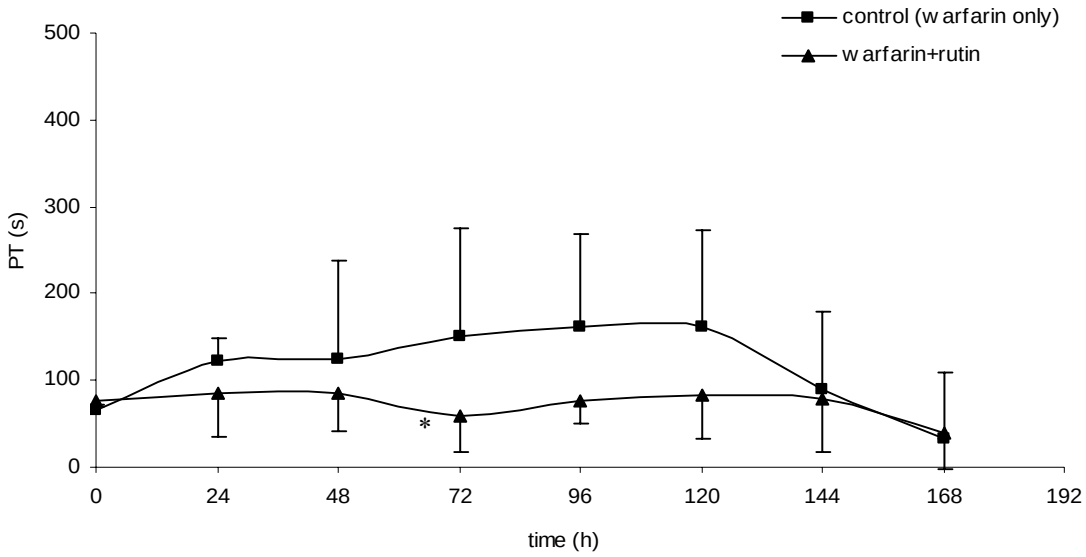
4.4.3. Effect of rutin treatment alone on blood coagulation

Effect of rutin treatment in the absence of warfarin on blood coagulation in rats is shown in **Fig. 4.5 (a,b).** The mean PCA values (as percentage of normal activity) were compared at different intervals over 168 h after the first dose of rutin. The time course of animals treated with rutin (1 g/kg/day) for 5 days showed no significant difference in mean PCA levels compared to the control rats that received only oral CM-cellulose for the same period. The average PT_0 (± SD) of rutin treated and control groups were 29.3 ± 2.3 and 34.8 ± 7.7, respectively.

4.5. DISCUSSION

Warfarin is used widely around the globe for the treatment and prevention of thromboembolic diseases. It is generally administered orally in long-term therapy and its low cost makes it more suitable for chronic dosing regimen. In spite of accumulating evidence over the years of its excellent therapeutic benefits, the usage is restricted by the inconvenience of constant therapeutic monitoring required and the

(a)



(b)

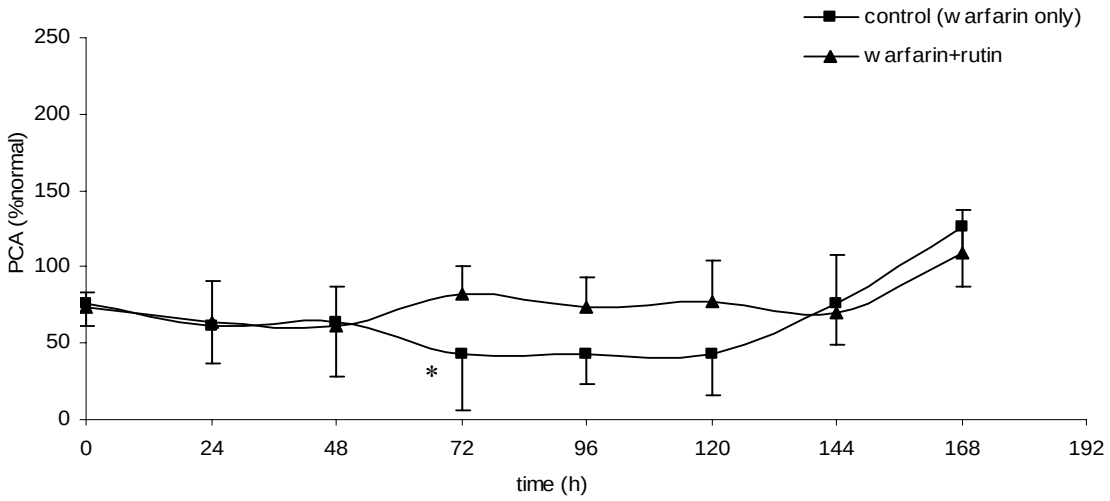


Figure 4.4 Changes in (a) prothrombin time (PT), (s) and (b) prothrombin complex activity (PCA), (percent of normal) with time following multiple doses of warfarin (0.15 mg/kg body weight) in control and rutin (1 g/kg body weight) treated rats. Animals from both the groups received oral racemic warfarin daily, for initial five days (blood samples were not collected/analyzed during this period). From "0" h, along with warfarin, control rats were given daily CM-cellulose (1% w/v) (5 ml/kg) and treatment group received rutin (1 g in 1 % CM-cellulose/kg) (5 ml/kg body weight, orally for 96 h.
Values are expressed as mean ± S.D (n = 6).

* $p < 0.01$ comparing the control group with rutin treatment group.

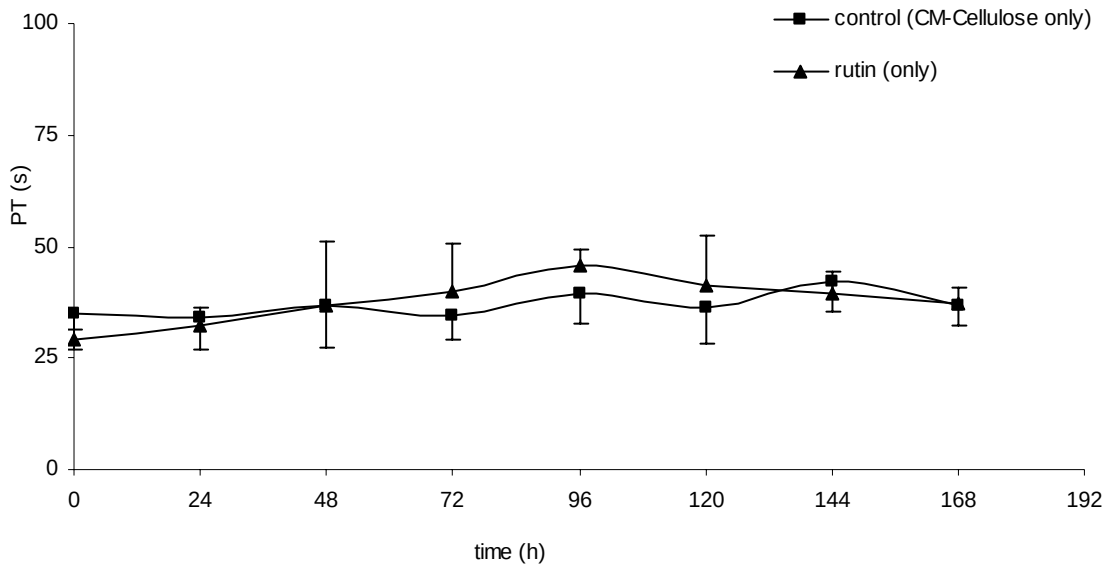
Table 4.3 Pharmacodynamic parameters of control (treated with CM-cellulose only) and rutin treated (rutin in CM-cellulose) rats after treatment with multiple doses of racemic warfarin (0.15 mg/kg body weight/day) orally.

Parameter	Control	Rutin treated
PT_0 (s)	36.68 ± 5.57	38.35 ± 4.42
PCA_{min} (% normal)	19.15 ± 5.96	42.55 ± 11.95 [a]

Note: values are mean ± S.D. (n = 6)
PT_0, basal prothrombin time at time zero; PCA_{min}, minimum prothrombin complex activity;

[a] $p < 0.01$ comparing the control group with rutin treatment group

(a)



(b)

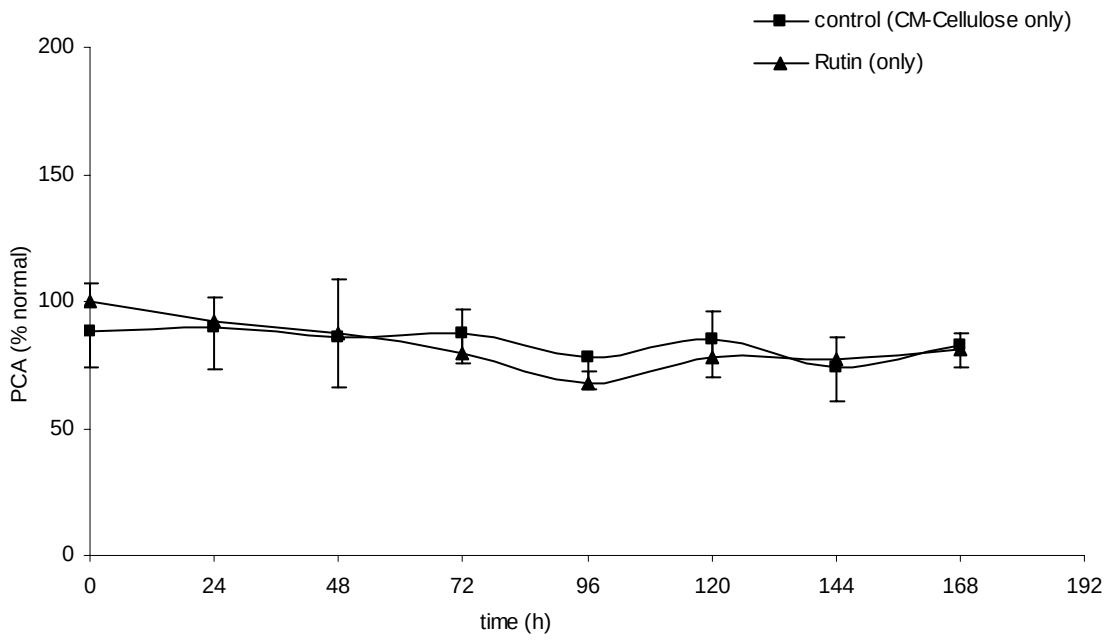


Figure 4.5 Changes in prothrombin time (a) (PT), (s) and (b) prothrombin complex activity (PCA), (percent of normal) with time in rats treated with and without (control) rutin (1 g/kg body weight), daily from 0 to 96 h. Values are expressed as mean ± S.D (n = 6). There was no statistically significant difference in both PT and PCA values between the control and rutin treatment groups.

potential interactions leading to fatal bleeding (8). The management of warfarin anticoagulation is especially difficult in the elderly and those with co-morbid conditions and those who need concomitant treatment with other drugs (8). Warfarin with its narrow therapeutic window, can lead to serious hemorrhagic conditions when an interacting drug is added to an ongoing, long term, multiple dosing regimen of warfarin (119). Thus it is important to evaluate the potential interactions of multiple doses of warfarin and a longer treatment regimen in addition to single dose-warfarin study.

Rutin is widely used in complementary and alternative medicines and numerous health supplements. Rutin has been reported to counteract dicoumarol in rats (107). When coadministered with warfarin, rutin is reported to be beneficial in patients with severe critical leg ischemia (23). Thus the two drugs together have a promising clinical application. But the potential for drug interaction needs to be carefully evaluated. Our study with single dose warfarin and rutin coadministration has shown some evidence of pharmacokinetic interactions between the two in rats. Hence *in vivo* multiple dose warfarin-rutin interaction study was designed.

Rats were treated with warfarin orally for five days before co-administering warfarin and rutin daily for subsequent five days. This could possibly mimic a clinical case of patient stabilized on warfarin therapy taking a rutin supplement or diet rich in rutin. Lower dose of warfarin (0.15 mg/kg/day) was selected to avoid fatalities in animals. The rats were monitored closely throughout the study and all the animals were found to tolerate the long treatment regimen and the repeated blood sampling well.

Consistent with literature reports that R-warfarin is eliminated faster in rats and slower in man compared to S-warfarin elimination kinetics (48, 110), higher clearance and shorter elimination half-life for R-warfarin, in comparison to S-warfarin, were observed in control rats. This was observed earlier in our single dose study as well. $t_{1/2}$ values of multiple dose study were similar to that of single dose study for both the enantiomers. It is known that the PK parameters do not vary much between single dose and multiple doses of the drug assuming that the absorption is very fast.

Pharmacokinetic parameters of S- and R-warfarin also showed a trend of interaction in the presence of rutin similar to single dose study. The serum levels of both the enantiomers in rutin treated rats were lower compared to that in control group. S-warfarin levels significantly differed between the two groups at 72 and 96 h time-points. R-warfarin levels also showed a decline with rutin treatment. The steady state serum clearance of S- warfarin tended to be higher in the rutin-treated group though the value showed no statistically significant difference when compared to the control group. There was an apparent lowering of steady state volume of distribution of S-warfarin compared to that of control group. Hence, shorter elimination half-life value of S- warfarin was observed with rutin treated rats compared to that of control. This in turn could have been responsible for the observed lower levels of S- warfarin and possibly the faster recovery of PT with rutin treatment, similar to single dose study. The steady state volume of distribution and clearance of R- warfarin showed no difference with rutin treatment and the elimination half-life value showed no change compared to control values. Elimination half-life of S- warfarin is shorter compared to that of R- Warfarin with rutin treatment though.

Quercetin was recently reported to inhibit both CYP2C9 and CYP3A4 significantly in human liver microsomes (6). This should correspond to increased serum levels of both S- and R- warfarin and reduced clearance value. But in our study of both single and multiple warfarin doses, rutin treatment appeared to reduce the levels of S-warfarin with apparent increase in clearance and shorter elimination half-life. Volume of distribution and clearance are two independent pharmacokinetic variables that can affect the elimination half-life of a drug ($t_{1/2}$ α V/CL). Depending on various physiological conditions like disease states, blood flow to the liver, intrinsic clearance, or protein binding, alteration of one or more of these factors can have a significant effect on the $t_{1/2}$ of the drug (123). When the clearance of the drug is slightly increased or unchanged, decrease in volume of distribution (possibly due to an increased plasma protein binding) can result in shorter elimination half-life. This could possibly explain the correlation between the increase in clearance and reduction in volume of distribution and the elimination half life of S- warfarin with rutin treatment in our study.

Warfarin is eliminated mainly by liver but it is a low hepatic extraction ratio drug (124). For low hepatic extraction ratio drugs, hepatic clearance ($CL_{h)}$ is dependent on unbound fraction of the drug (f_u) and intrinsic clearance (CL_{int}) of the liver ($CL_h = f_u.CL_{int}$). Increase in the intrinsic clearance of the liver can lead to increased hepatic clearance of warfarin. As the steady state drug concentration is inversely proportional to the clearance, the lowering of S-warfarin steady state concentration as seen in our study could possibly be due to a change in the intrinsic clearance or tissue unbound fraction. Moreover, various research works have shown that the data obtained from *in vitro* and *in vivo* work with respect to cytochrome P450 modulation do not always

correlate. Similar to the contradiction between our study and *in vitro* data on quercetin, the antiemetic drug Aprepitant (neurokinin-1 receptor antagonist) was reported to inhibit CYP3A4 when incubated with human liver microsomes *in vitro* (113), and in human subjects (*in vivo*) using midazolam as a probe substrate of CYP3A4 (114). But it was shown to have a modest inductive effect on both CYP3A4 and CYP2C9 *in vivo* using midazolam and tolbutamide as a probe substrate respectively (116). Aprepitant also induced S- warfarin metabolism in healthy subjects indicating an induction of CYP2C9 (115). Although due to various constraints the present study could not include interaction studies using specific hepatic microsomes, such *in vitro* studies in the future may help to understand this aspect of the interaction better.

Both warfarin and quercetin bind to the IIA domain of albumin (117, 118) and quercetin is shown to be displaced by large excess of warfarin, although not competitively (24, 94). In our study there was a trend to reduce the volume of distribution of S- Warfarin. At this point of the study, it is difficult to conclusively explain the observed interactions in various pharmacokinetic parameters. Further analysis of the warfarin metabolites in urine and the protein binding studies may help in understanding this potential interaction better. However the results so far clearly show the potential trend of interaction between rutin and warfarin both in single and multiple doses.

There was no significant difference in mean PT values between the rutin treatment and control groups prior to rutin dosing (PT_0). The results obtained with rutin treatment were similar to single dose study with respect to anticoagulation. The

degree of hypoprothrombinaemic response was reduced at all time-points with concurrent rutin administration compared to warfarin alone group. The higher levels of PCA and average PCA_{min} obtained indicated overall reduction in the anticoagulation response to warfarin. The difference in PCA between the two groups was especially significant at 72 h time point ($p < 0.01$). The lower serum levels of S-warfarin observed with rutin treatment compared to that in control rats provides an explanation for this effect of rutin on anticoagulation.

Rutin is reported to be well tolerated in rats when administered at a high dose for a long time (97). But there was no information on its effect on clotting time. Thus we further evaluated the effect of rutin on coagulation process in rats compared to that in control animals. The study was designed to determine the effects of rutin on blood coagulation in the absence of warfarin. The results indicated that rutin by itself had no effect on PT (PCA) and ruled out the possibility of rutin affecting coagulability directly. Rutin treatment alone showed no significant effect on blood coagulation compared to that of control. When administered with warfarin, the observed changes in PT (PCA) could not have been due to rutin acting on clotting mechanisms.

4.6. CONCLUSION

The results showed the potential interaction between rutin and multiple doses of warfarin. S- warfarin levels in serum were reduced and the PCA values increased with co-administration of rutin. PK parameters also varied between the control and rutin treated groups. The S- warfarin steady state clearance increased and volume of distribution and elimination half-life reduced with rutin treatment. The results were

similar to single dose study. The 2nd part of the study reveals that rutin by itself does not affect the blood coagulation and clotting process. Given at high doses, rutin treatment showed no significant difference compared to CM-cellulose treatment in PT and PCA values and the animals well tolerated the drug throughout the study period.

Thus the possible interactions should be carefully considered when a patient stable on warfarin treatment starts taking flavonoid (rutin) rich diet or health supplements. Studies have shown that many of the patients on warfarin therapy also self-medicate with alternative medicines (76). As warfarin has a narrow therapeutic window, the maintenance of coagulation within desirable range is critical and so is avoiding potential interacting substances.

CHAPTER 5 CONCLUSION

The potential drug interaction between rutin, a flavonoid glycoside and warfarin, the oral anticoagulant, was investigated in rats. The study was designed to estimate the effects of both single and multiple oral doses of racemic warfarin in rats treated with rutin. The results reveal a possible pharmacokinetic drug interaction between rutin and oral warfarin in both the studies.

Rutin treatment along with single dose of warfarin increased the C_{max} and reduced the $t_{1/2}$ and V_d values of both S- and R- warfarin significantly and lowered the serum levels of both the enantiomers in the elimination phase compared to the control group. Rutin treatment also increased the total area under the PCA-time curve from 0 to 96 h post warfarin indicating an overall reduction in the anticoagulation response to warfarin. Recovery from warfarin anticoagulation was found to be much faster in the presence of rutin.

With multiple doses of warfarin, rutin treatment resulted in lower serum levels of S-warfarin, higher PCA values and lower anticoagulation compared to control. Of S-warfarin, the clearance was increased, and the volume of distribution and elimination half-life reduced compared to corresponding control values. Also, rutin by itself did not affect the blood coagulation and clotting process.

Thus the potential interactions should be carefully evaluated when rutin and warfarin are coadministered. Extra care must be taken when a patient stable on warfarin treatment starts taking flavonoid (rutin) rich diet or health supplements or when high

levels of rutin aglycone and conjugates are present in patient blood before starting warfarin therapy. Furthermore, if the patient has been stabilized on warfarin with concomitant use of rutin or quercetin, precaution should be taken to avoid sudden suspension of rutin.

CHAPTER 6 POTENTIAL APPLICATIONS AND SUGGESTIONS FOR FUTURE WORK

6.1. POTENTIAL APPLICATIONS

Understanding the mechanism involved in a drug interaction can help to make better prediction of the potential interaction. Ability to quantitatively measure the interaction is crucial in this scenario. When the mechanism of the interaction is well understood and is both predictable and quantifiable, then we can weigh the benefits of such combinations of drugs more judiciously.

Warning both medical professionals and patients about the potential for interaction between complementary medicines and prescribed drugs may reduce the risk of some of the life threatening interactions. It is all the more important, considering the benign nature and prevalent use of herbal alternative medicines and for drugs with narrow therapeutic indices like warfarin. Although patients treated with warfarin are usually counseled for possible interactions with herbal medicines, noncompliance (use of herbal products or failure to follow the advice) can result in loss of anticoagulation and fatal bleeding. Also the composition of health food often differs among different brands and manufacturers. Thus proper evaluation and a detailed case history of all the drugs and alternative medicines taken should be carried out in patients with unstable anticoagulation state.

The results of the present study indicate a potential interaction between rutin and warfarin. As rutin and its aglycone – quercetin, are present in numerous diets of plant

origin, precaution must be taken when rutin and warfarin are coadministered. Interactions should be carefully considered when either rutin or warfarin treatment is initiated or suspended in a patient with high levels of either of them in blood. Therapeutic monitoring of warfarin is useful in this condition and warfarin dose should be adjusted accordingly.

6.2. SUGGESTIONS FOR FUTURE WORK

Although the present study indicates a potential interaction between warfarin and rutin, actual mechanisms of the interactions need to be evaluated further. Studies on the effect of rutin/quercetin on S- and R-warfarin metabolic profiles in urine, specific hepatic enzymes *in vitro*, and protein binding could give more insight into the potential interactions and the basis for underlying mechanisms.

Quercetin has been reported to bind strongly to human serum albumin *in vitro* (24) as well as rodent albumin (14). Both warfarin and quercetin bind to the IIA domain of albumin (117, 118) and quercetin is shown to be displaced by large excess of warfarin, although not competitively (24, 94). In the present study, as total serum clearance was not altered much, rutin administered in large excess could have affected the serum and tissue protein binding of warfarin. Analysis of protein and tissue binding should be carried out to understand the mechanisms of this interaction.

Quercetin is reported to inhibit the activity of carbonyl reductases *in vitro* (90), CYP1A2 (91) and both CYP2C9 and CYP3A4 in human liver microsomes (6). Quercetin is also reported to increase the expression of CYP1A1 (82). Thus it can

potentially affect the metabolism of warfarin. However, in our study S- warfarin levels were reduced with rutin treatment. Such discrepancies between *in vitro* and *in vivo* data have been reported in various other studies as well (113, 114, 115, 116, 125). Nonetheless, *in vitro* studies using specific CYP enzymes may shed more light on this interaction and the data needs to be carefully evaluated to scale upto clinical interactions.

Warfarin is extensively metabolized in the liver, with only 2-5 % of the dose present unchanged in urine in human and 5-9 % in rat urine (126, 127). As warfarin enantiomers and the metabolites are predominantly excreted by kidney, detailed estimation of warfarin metabolites in urine is useful to understand the extent to which warfarin metabolism and excretion are affected. It can provide valuable insight into the metabolism and clearance of warfarin enantiomers and potential metabolic interactions with rutin, if any. Modeling of the serum and urine data together with unbound concentrations of warfarin could present a better picture of the mechanisms of interaction.

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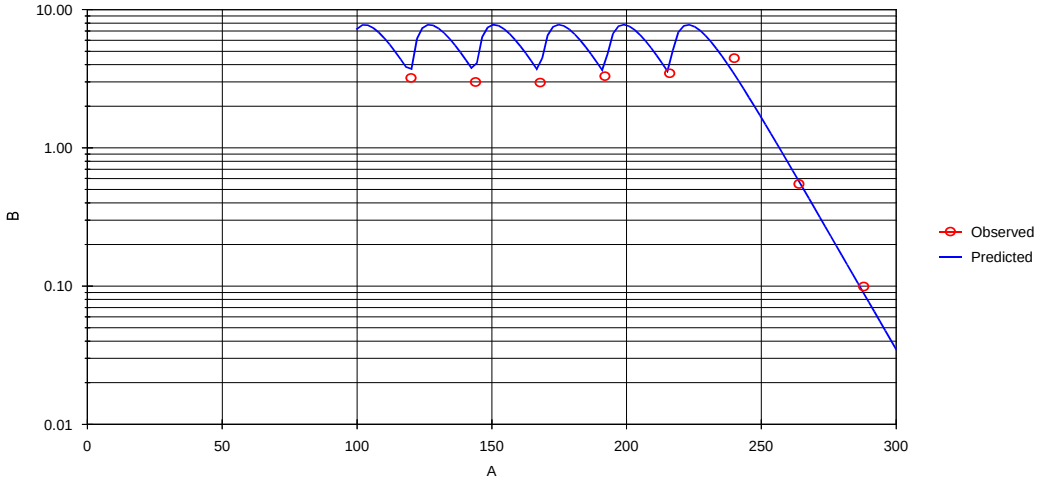
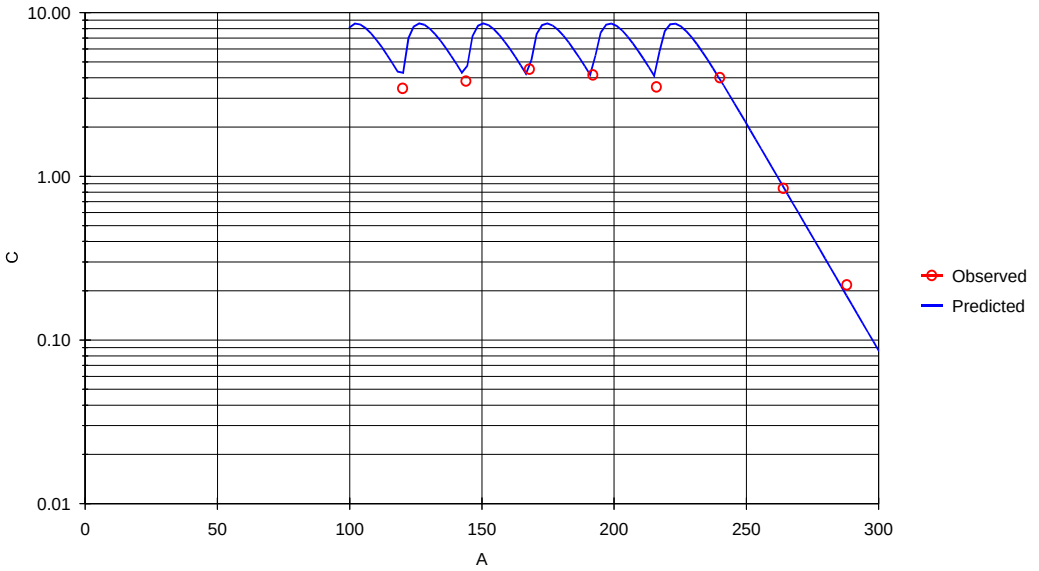
APPENDIX

4.1. S- warfarin curve fitting using WinNonlin for control rat #1.



S- warfarin curve fitting using WinNonlin for control rat #2



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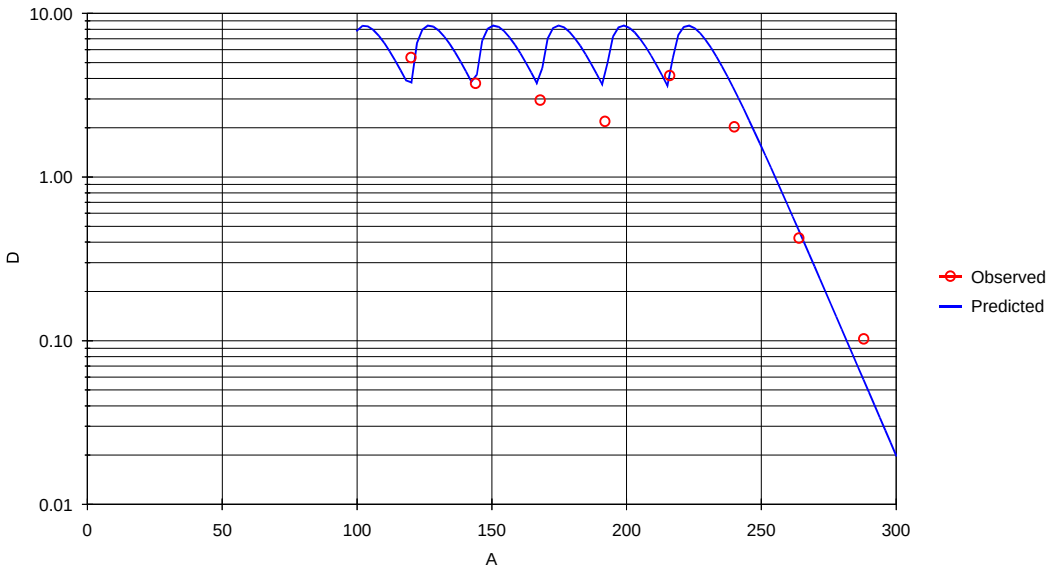

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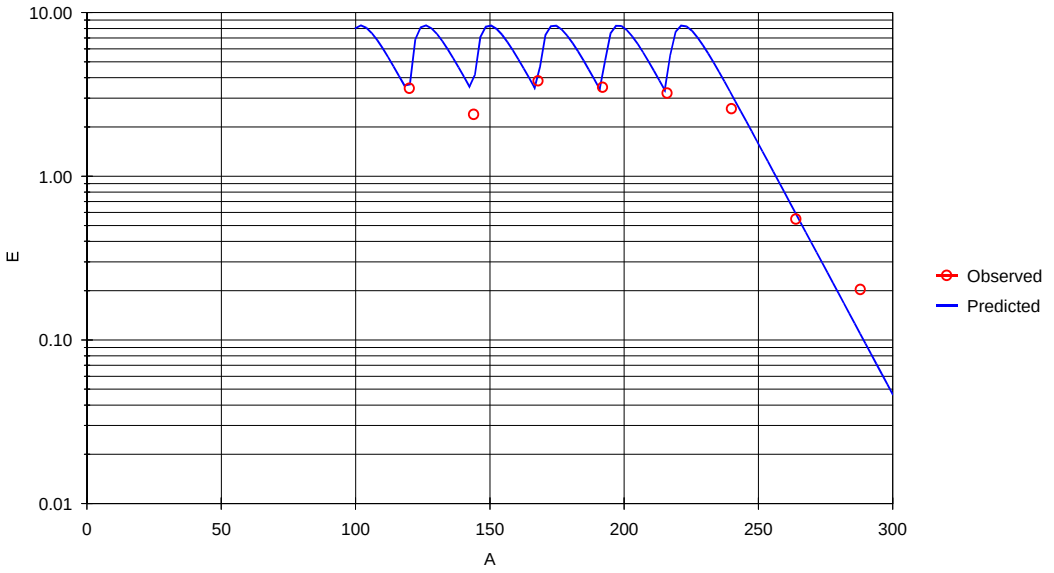

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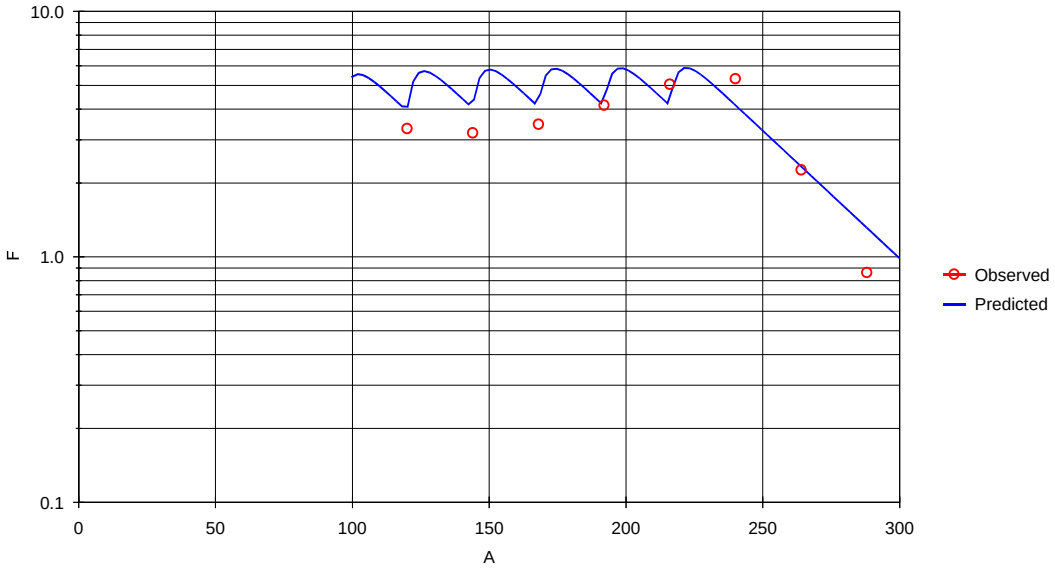
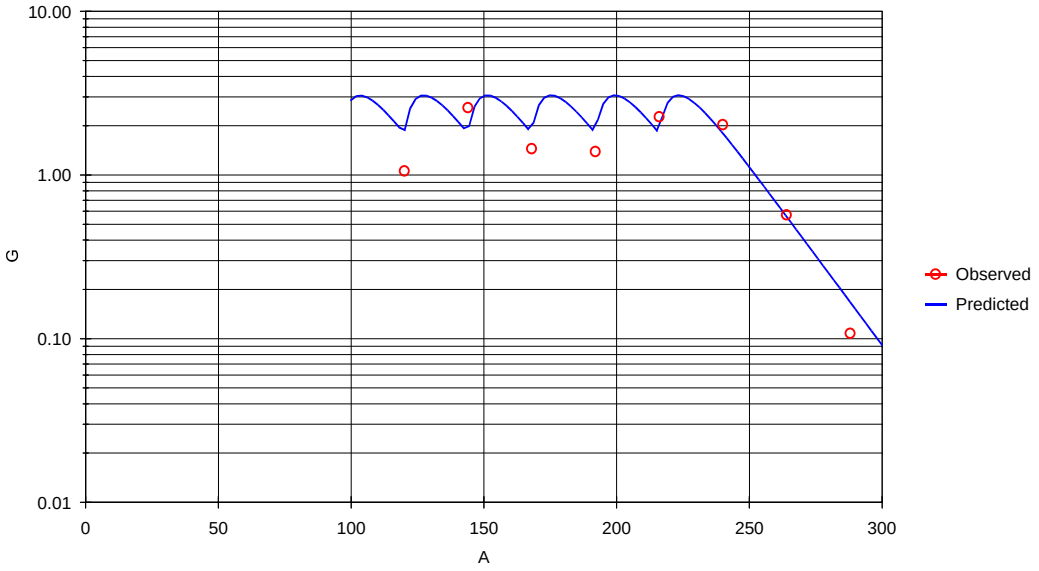
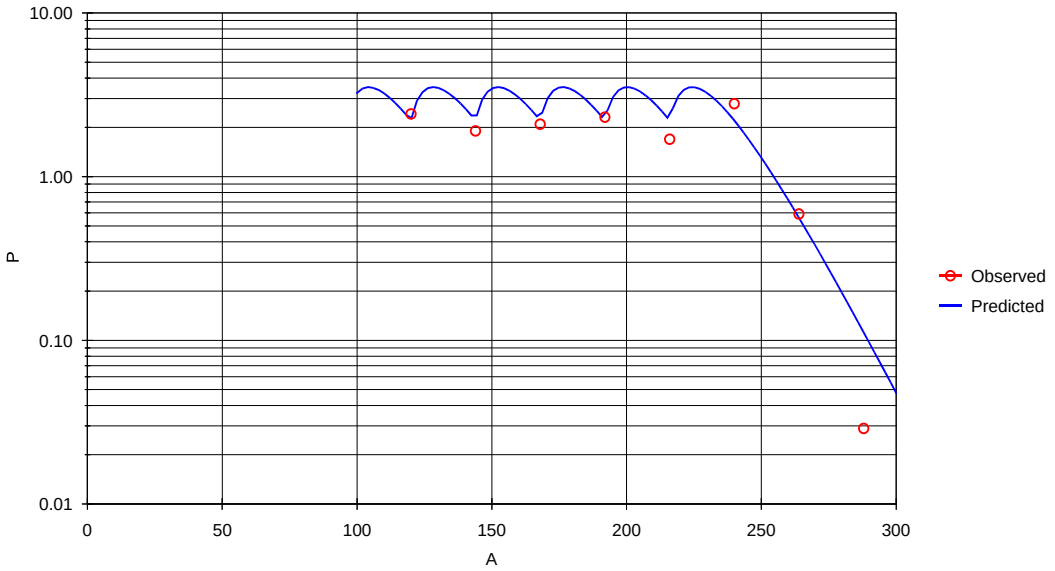


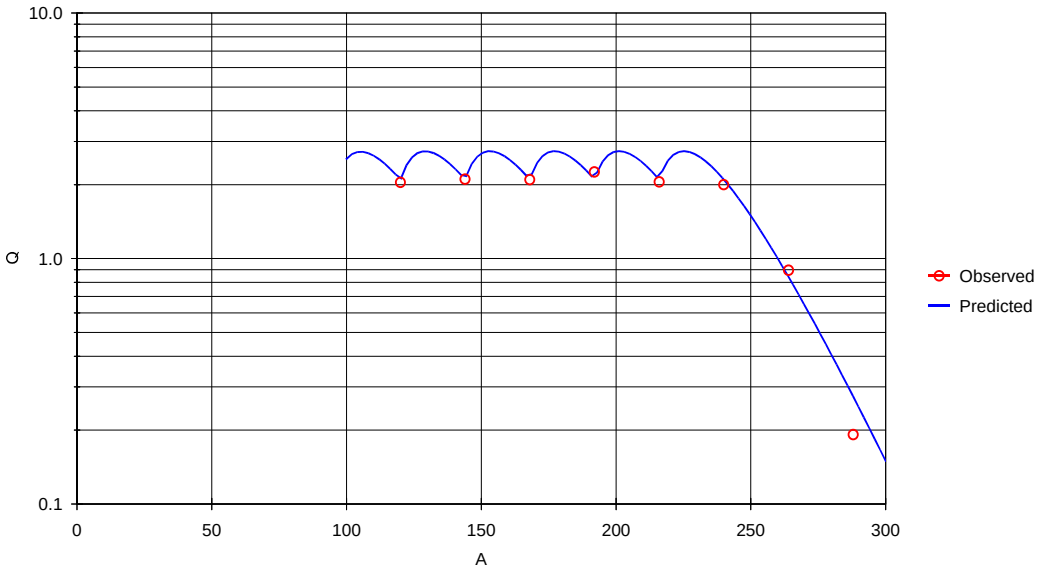
S- warfarin curve fitting using WinNonlin for control rat #6



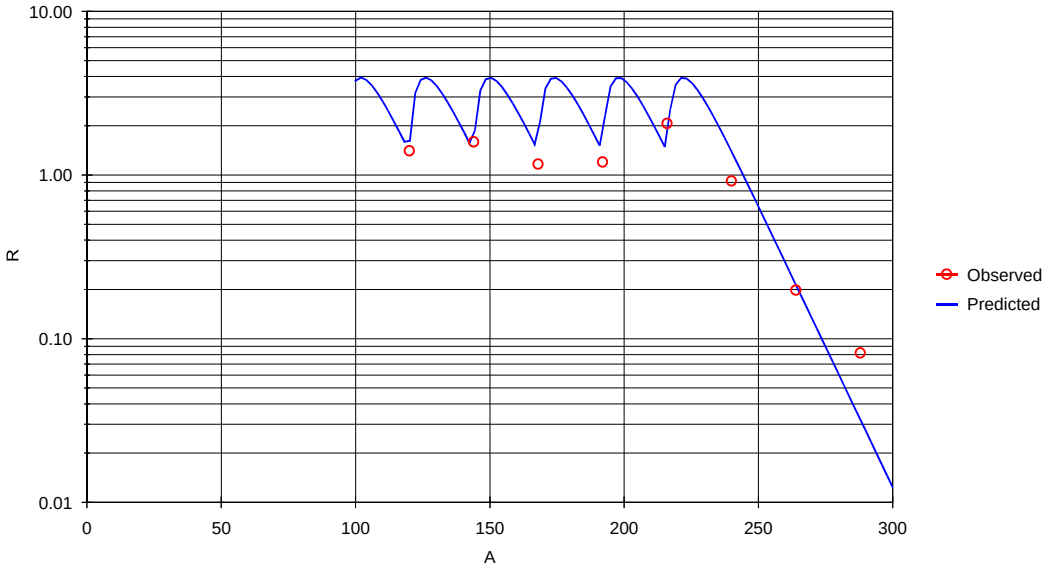
S- warfarin curve fitting using WinNonlin for rutin treated rat #1



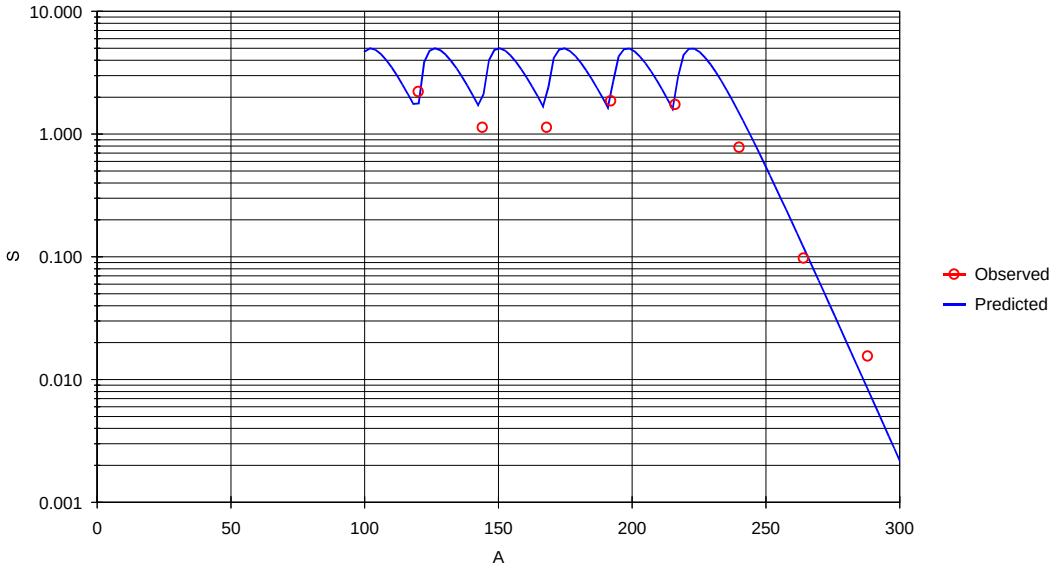
S- warfarin curve fitting using WinNonlin for rutin treated rat #2



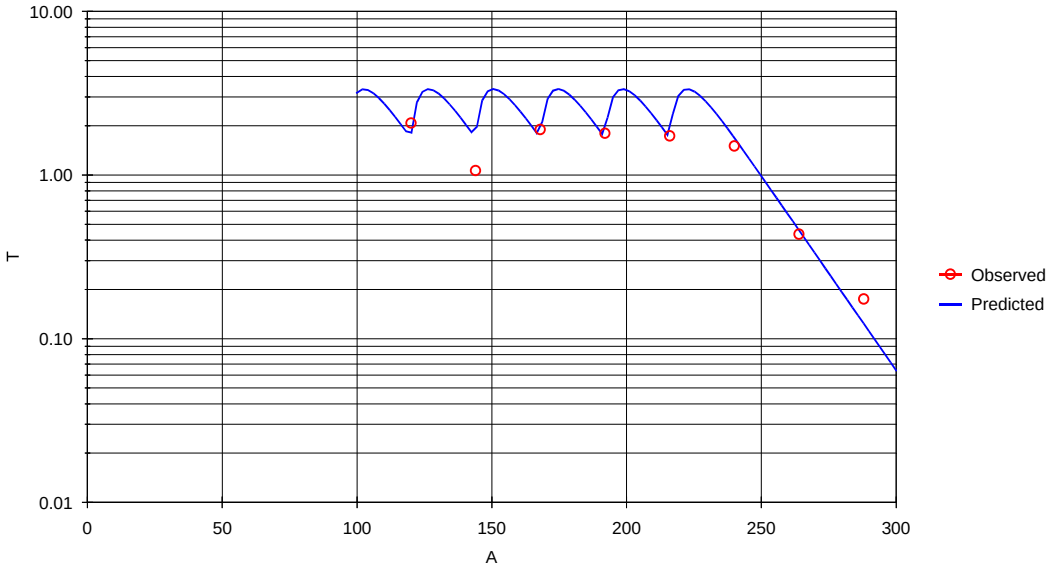
S- warfarin curve fitting using WinNonlin for rutin treated rat #3



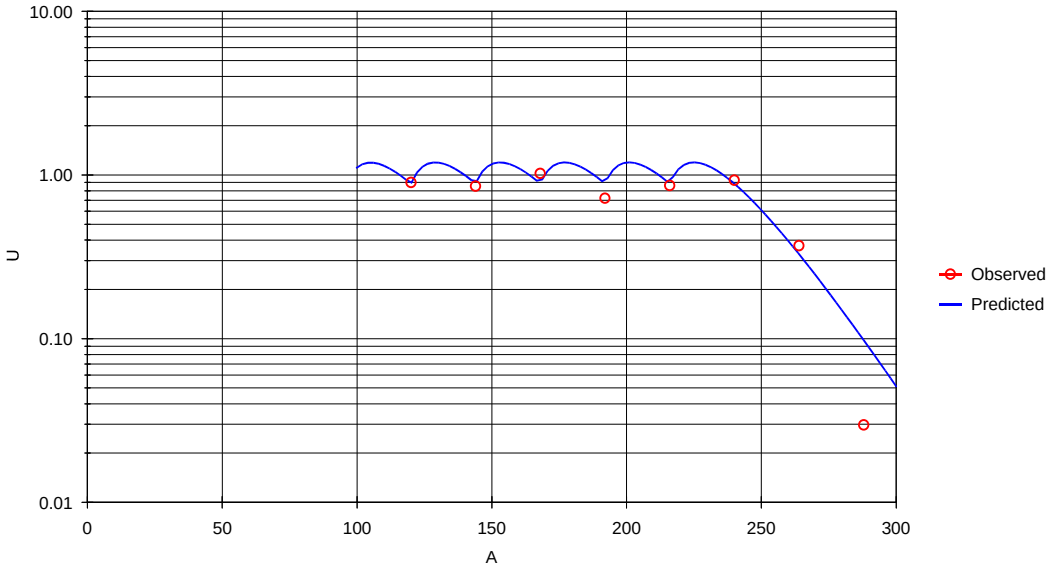
S- warfarin curve fitting using WinNonlin for rutin treated rat #4



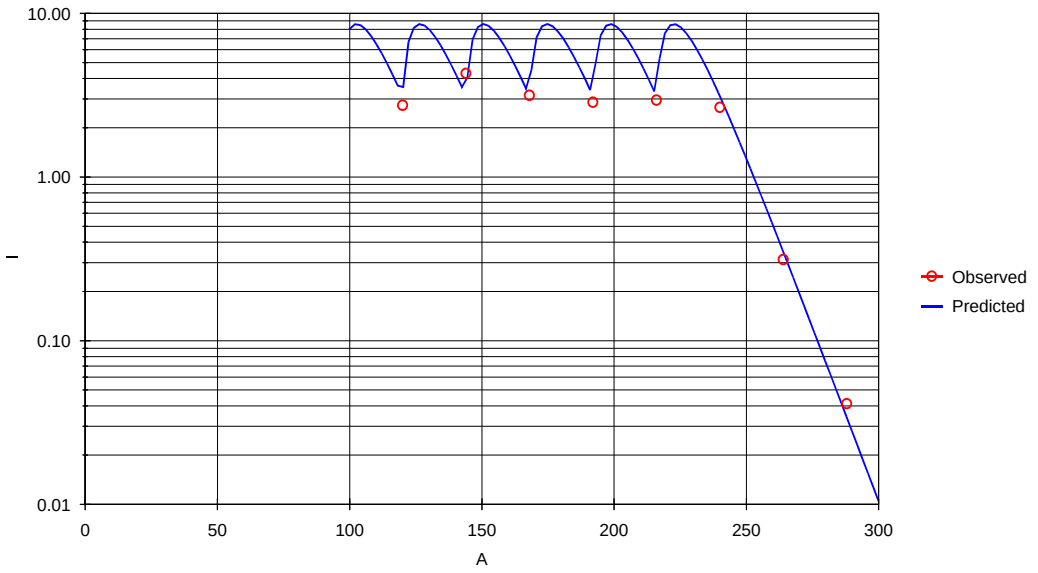
S- warfarin curve fitting using WinNonlin for rutin treated rat #5



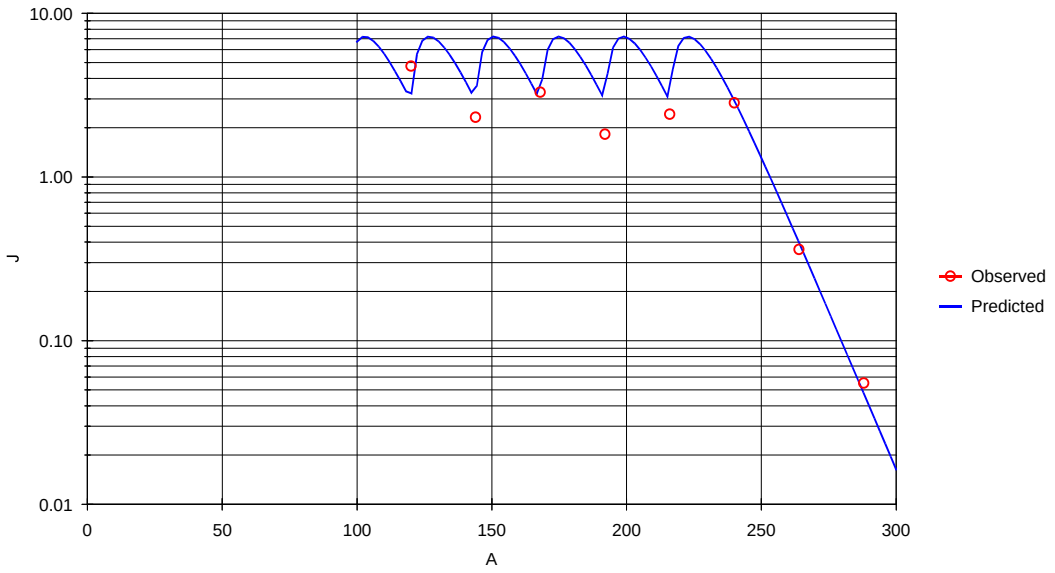
S- warfarin curve fitting using WinNonlin for rutin treated rat #6



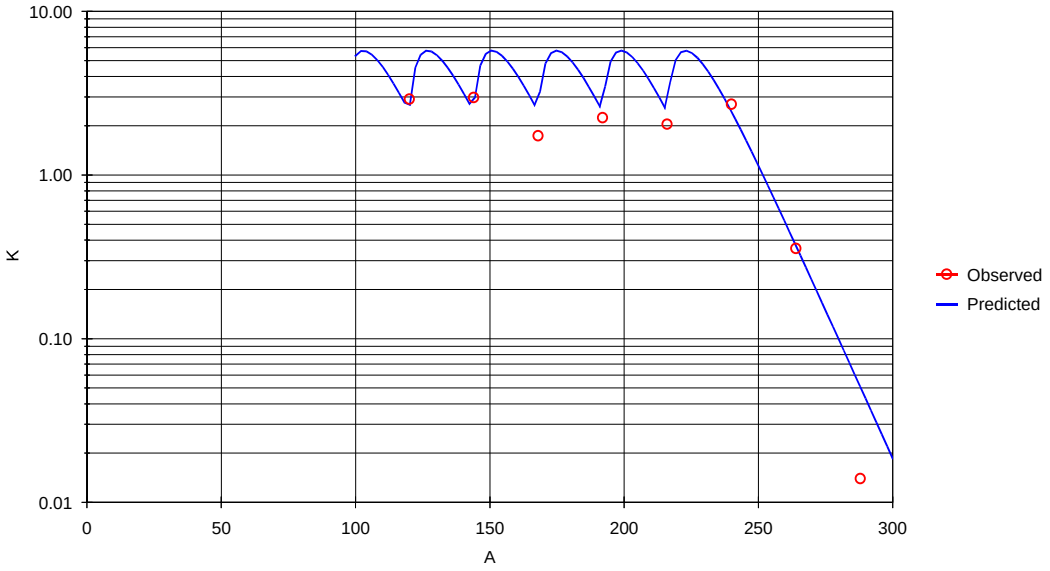
R- warfarin curve fitting using WinNonlin for control rat #1



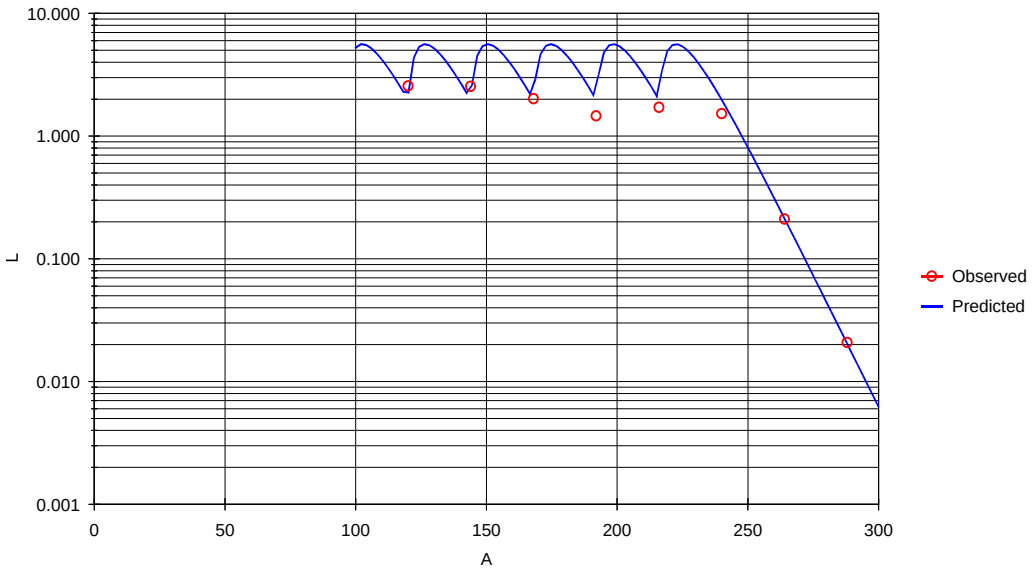
R- warfarin curve fitting using WinNonlin for control rat #2



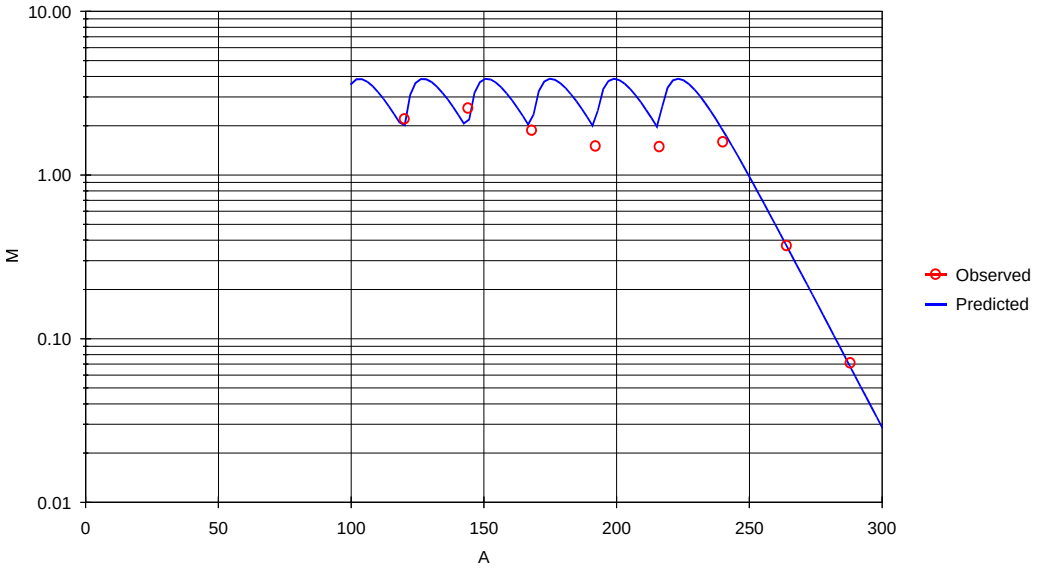
R- warfarin curve fitting using WinNonlin for control rat #3



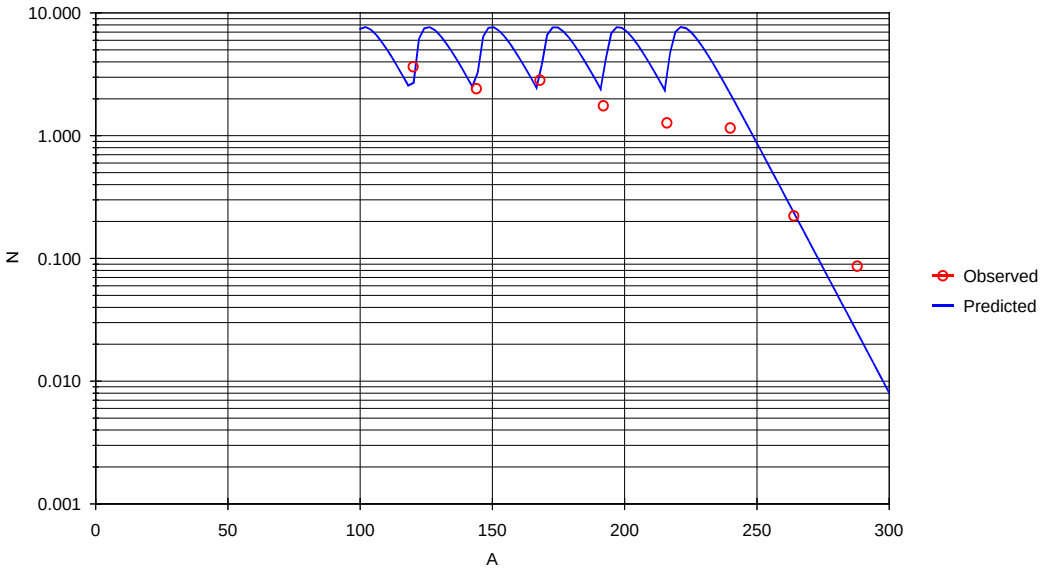
R- warfarin curve fitting using WinNonlin for control rat #4



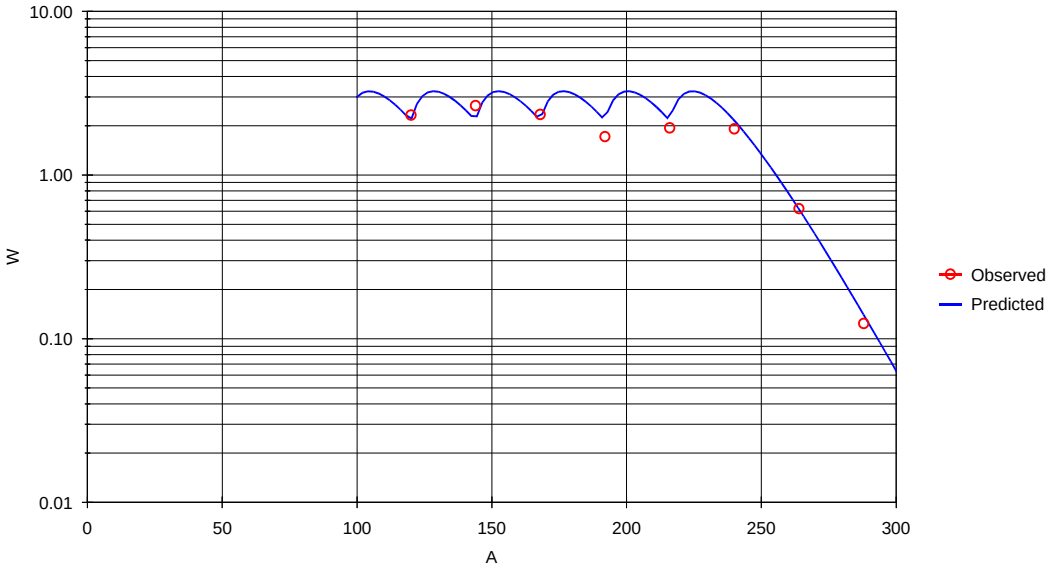
R- warfarin curve fitting using WinNonlin for control rat #5



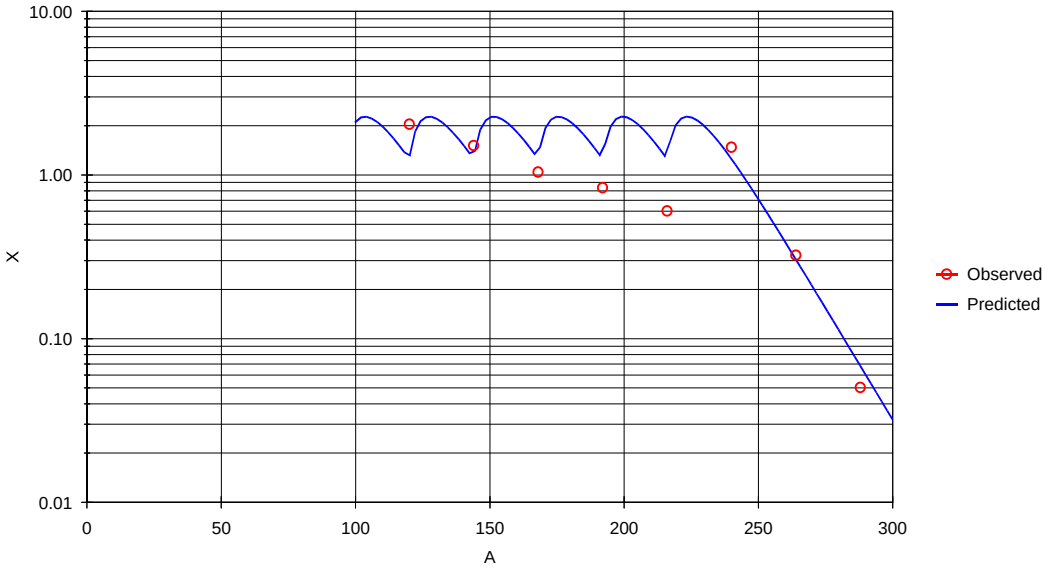
R- warfarin curve fitting using WinNonlin for control rat #6



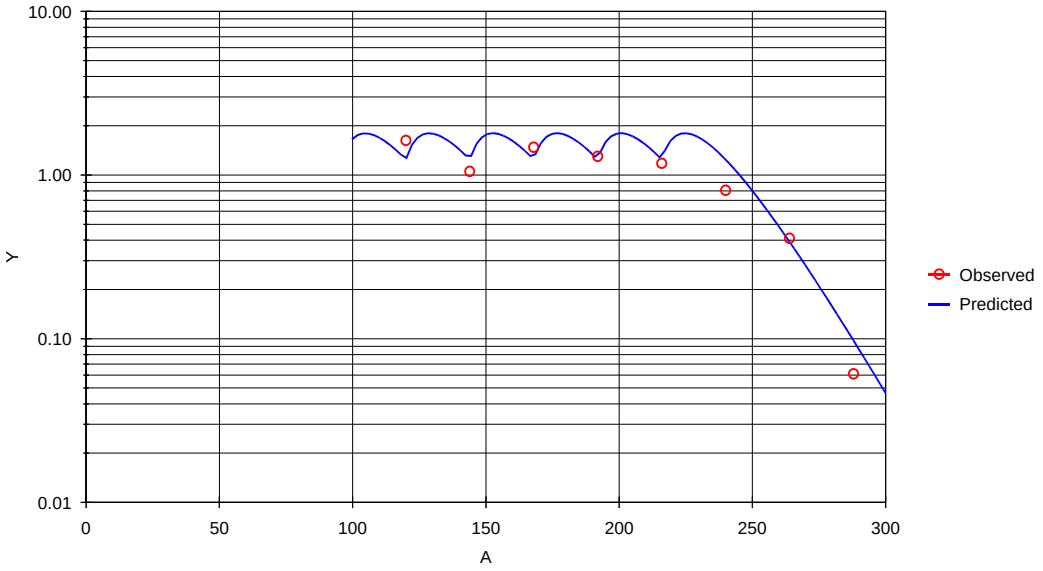
R- warfarin curve fitting using WinNonlin for rutin treated rat #1



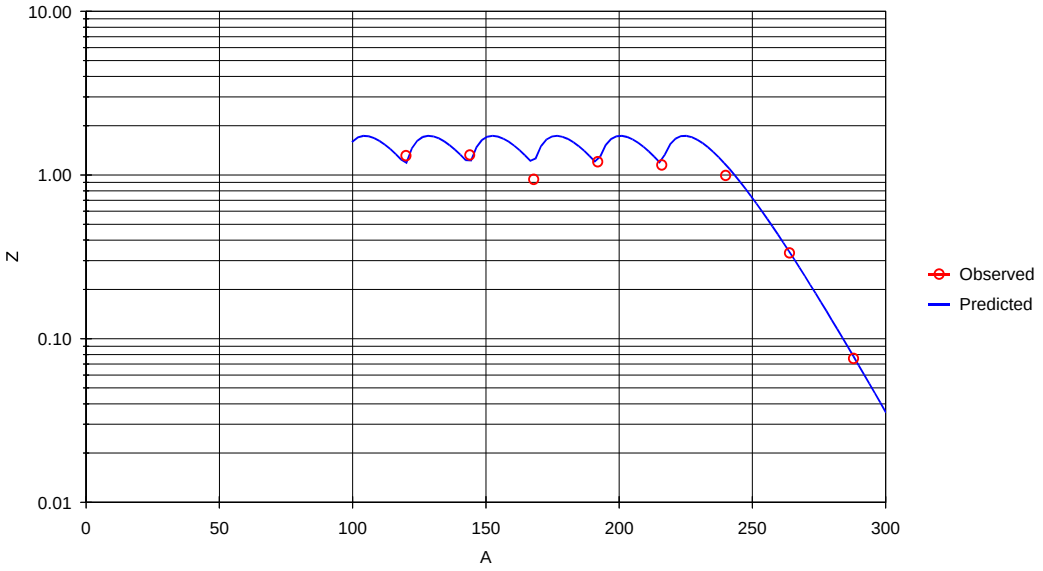
R- warfarin curve fitting using WinNonlin for rutin treated rat #2



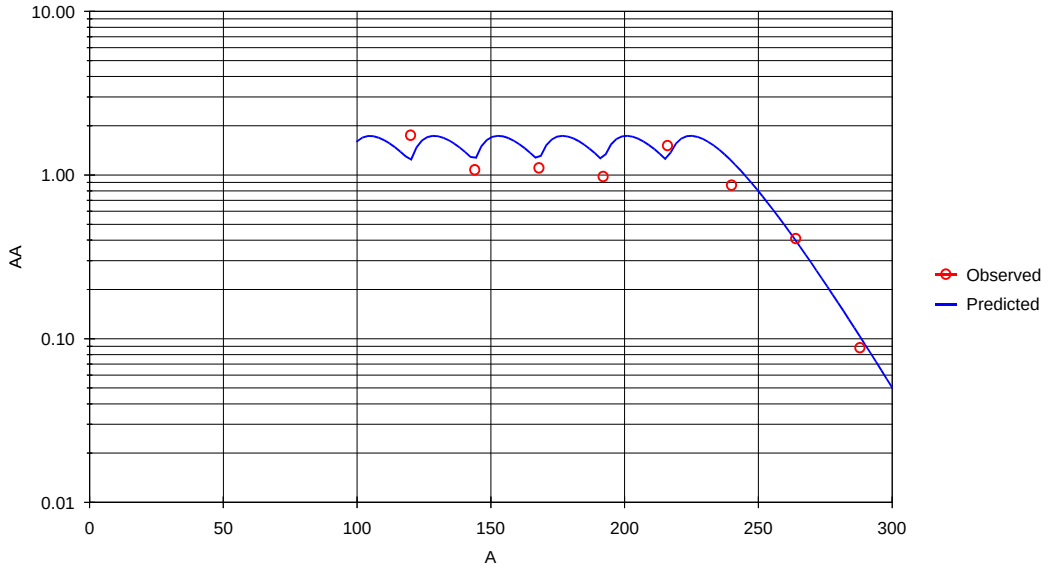
R- warfarin curve fitting using WinNonlin for rutin treated rat #3



R- warfarin curve fitting using WinNonlin for rutin treated rat #4



R- warfarin curve fitting using WinNonlin for rutin treated rat #5



R- warfarin curve fitting using WinNonlin for rutin treated rat #6