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Research Paper

Effect of rutin on warfarin anticoagulation and pharmacokinetics of warfarin enantiomers in rats

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Abstract

Objectives The effects of the flavonoid rutin on the anticoagulant activity of oral warfarin and the protein binding and pharmacokinetics of its enantiomers were investigated in rats.
Methods A single dose of racemic warfarin, 1.5 mg/kg, was administered orally to rats either alone or on day 5 of an 8-day oral regimen of rutin, 1 g/kg daily.
Results Rutin reduced the anticoagulant effect of racemic warfarin, evident as a 31% reduction in the area under the prothrombin complex activty–time curve ($P < 0.05$).
Key findings Rutin had no apparent effect on pre-treatment baseline blood coagulation. It enhanced the in-vitro serum protein binding of S- and R-warfarin (reflected by 40% and 26% reductions in unbound fraction, respectively), and thus restricted distribution by 33 and 21%, respectively. Treatment with rutin significantly decreased the elimination half-life of S-warfarin by 37% as a result of the 69% increase in unbound clearance of the S-enantiomer. This effect was attributed to a significant 77% increase in the unbound formation clearance of the overall oxidative and reductive metabolites, and an increase in the unbound renal clearance of the more potent S-enantiomer of warfarin.
Conclusions Concurrent rutin administration is likely to reduce the anticoagulant effect of racemic warfarin, reflecting a significant decrease in the elimination half-life of the more potent S-enantiomer.
Keywords pharmacodynamics; pharmacokinetics; stereoselective drug interactions; rutin; warfarin

Introduction

Warfarin is a widely prescribed oral anticoagulant. It is available as a racemic mixture consisting of equal proportions of R- and S-warfarin. The S-enantiomer of warfarin is much more potent than the R-enantiomer.[1,2] Warfarin is extensively metabolised, involving highly stereospecific pathways catalysed by the cytochrome P450 (CYP) system.[3,4] Some properties of warfarin, such as narrow therapeutic index, high protein binding and CYP-dependent metabolism, render it prone to many drug interactions.[5–9] Interacting drugs can affect the metabolism of warfarin in a stereochemical manner, resulting in markedly different effects on the two entantiomers and modifying the anticoagulant response.[7,10–15]

Rutin is a flavonoid glycoside abundant in the plant kingdom and is widely used in complementary and alternative medicines.[16] Rutin derivatives (e.g. oxerutin and troxerutin) are used to treat various cardiovascular conditions.[17–19] Reports have shown their usefulness in treating abnormal fragility of the capillaries and as a vasoprotectant.[20–23] Rutin is also reported to relieve venous insufficiency of the lower limbs and capillary impairment.[24,25] Rutin and its derivatives have been used with warfarin in cardiovascular patients.[26,27] A clinical study has shown that patients with severe non-reconstructable chronic critical leg ischaemia benefited from initial therapy with intravenous rutin combined with long-term oral warfarin treatment.[26]

Quercetin is the aglycone of rutin and meets the structural requirements for a strong antioxidant.[28,29] Both quercetin and rutin form integral parts of various nutritional supplements and herbal preparations.[30–32] A semi-quantitative food frequency questionnaire in 1990 showed an estimated average intake of flavonoids in the USA of 20–22 mg per day; of the flavonols and flavones studied, quercetin contributed 73% in women and 76% in men.[33]

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Following oral administration, rutin is metabolised to the aglycone quercetin, which is then conjugated, in both rats[34] and humans.[16,34,35] Rutin is absorbed more slowly than quercetin in both rats and humans. Rutin is likely to be hydrolysed by the digestive microflora and converted to quercetin before being absorbed.[16,35] Quercetin binds strongly to human serum albumin (99.1–99.4 ± 0.5%) *in vitro*[36] and inhibits CYP3A4 in human liver microsomes,[37] the enzyme responsible for the metabolism of the R-enantiomer of warfarin, and also modulates P-glycoprotein, a drug efflux transporter.[38] Therefore, rutin and quercetin could directly or indirectly influence some of the metabolic pathways and thus affect the pharmacological actions of warfarin. However, literature data on the effects of rutin and quercetin on the pharmacokinetics and metabolism of warfarin enantiomers are still lacking. The present study was therefore undertaken to assess the interaction by quantification of the pharmacological response produced by warfarin, measurement of pharmacokinetic parameters associated with the R- and S-enantiomers of warfarin, and determination of their respective metabolic fates with and without the concurrent administration of rutin in rats. In-vitro serum protein binding studies were carried out to explore the effects of quercetin on the binding of warfarin enantiomers, as rutin is detected mainly as its aglycone quercetin in the body following oral administration.[16,34,35]

Materials and Methods

Chemicals and reagents

Racemic warfarin sodium salt, chlorowarfarin, barbital, carbobenzyloxy-L-proline, dicyclohexylcarbodiimide, rutin hydrate, quercetin and carboxy methyl cellulose (CM-Cellulose) were obtained from Sigma Chemical Co. (St Louis, MO, US). The warfarin alcohols – 4′-, 6-, and 7-hydroxywarfarin – were synthesised according to previously reported methods with some modifications.[39,40] Analar-grade trisodium citrate dihydrate and sodium chloride were purchased from Merck Co. (Schuchardt, Germany). The solvents used included acetonitrile, *n*-butylamine, chloroform, diethyl ether, ethyl acetate, *n*-hexane and methanol and were either of analytical grade for extraction or HPLC grade (Labscan Limited Co., Dublin, Ireland).

The Neoplastine CI Plus 5 kit was purchased from Diagnostica Stago (Taverny, France). Protein assay kits were purchased from Bio-Rad Laboratories (Hercules, CA, US). Tween 80 was provided by the Pharmaceutical Department, Health Ministry of Singapore. Milli-Q water was obtained using the MilliporeContinental Water Systems (Burlington, MA, US).

Animals

Male Sprague-Dawley rats (250–300 g) were purchased from the Laboratory Animal Center, Singapore. Animal experiments were approved by the National University of Singapore Institutional Animal Care and Use Committee (IACUC Protocol no. 756/05).

Animals were housed separately in metabolic cages and kept in a 12 h light–dark cycle for a minimum of 3 days before being used in experiments. Food and water were available *ad libitum*.

Effect of quercetin on serum protein binding of warfarin enantiomers *in vitro*

Serum pooled from eight randomly selected male Sprague-Dawley rats was spiked with racemic warfarin, 0.050 and 0.100 μmol/ml. To 1 ml aliquots of this serum were added 10 μl quercetin (dissolved in methanol) to yield final concentrations of 0 and 0.050 μmol/ml. Serum protein binding of warfarin enantiomers was determined by equilibrium dialysis at 37°C for 4 h, using a Spectrum equilibrium dialyser (Spectrum Laboratories Inc., Rancho Dominguez, CA, US). Samples obtained from the buffer and serum sides were analysed by HPLC. The total protein concentration in the serum before and after dialysis was determined using the Bio-Rad protein assay. The observed unbound fraction was corrected for volume shift during dialysis to calculate the true unbound fraction, using the equation established by Chan *et al.*[41] Protein leakage into the buffer compartment was checked using the Lowry protein assay.[42]

In-vivo studies

Two groups of male Sprague-Dawley rats (eight per group), in a parallel design, received either rutin (1 g in 1% CM-cellulose/kg per day) or an equal volume (5 ml/kg) of 1% CM-cellulose (as the control), by oral intubation, once a day for 8 consecutive days. During this time animals had access to food and water *ad libitum* except when fasted overnight before administration of warfarin and for 4 h afterwards. 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, either alone or with rutin. Blood samples were taken before administration of warfarin and at 1, 3, 6, 12, 24, 48, 72 and 96 h afterwards. Urine samples were collected beforehand, and over the 0–24, 24–48, 48–72, 72–96 and 96–120 h intervals after warfarin administration. About 400 μl blood obtained without anticoagulation was centrifuged (10 min at 3000*g*) to separate the serum. Serum samples obtained were then stored at −20°C before HPLC analysis.

For measurement of the subsequent anticoagulant effect, 50 μl of each blood sample was used for preparation of diluted plasma according to the established procedure.[43]

Assessment of anticoagulation

The degree of anticoagulation (hypoprothrombinaemic response) was assessed by means of the prothrombin time (PT), determined by the one-stage PT test on the diluted plasma sample, using a fully automated coagulation analyser (Sysmex automated blood coagulation analyser, CA 530, Sysmex Corporation, Kobe, Japan). The clotting time was recorded in seconds. The baseline Pt (PT_0) was determined from the plasma samples collected before drug (rutin and warfarin) administration. PT (in seconds) obtained was then transformed to the prothrombin complex activity (PCA) (in percent of normal activity). The parameters used to assess the degree of blood coagulability were the minimum prothrombin complex activity (PCA_{min}), a measure of maximum warfarin anticoagulation, time to achieve PCA_{min} ($t_{PCA,min}$) and the area under the PCA–time curve (AUC_{PCA}), a measure of the overall warfarin anticoagulation effect, calculated using the linear trapezoid method.[43]

Chemical analysis

Concentrations of warfarin enantiomers in serum samples, and the enantiomers of the various reductive and oxidative warfarin metabolites, including S,S-, S,R-, R,S- and R,R-warfarin alcohols, S- and R-4′-hydroxywarfarin, S- and R-6-hydroxywarfarin, and S- and R-7-hydroxywarfarin, in urine samples were determined using the previously reported stereospecific HPLC assay[44] with some modifications.[7] The limits of detection and quantitation of the assay for the analytes in serum and urine and the standard calibration ranges are shown in Table 1. All values for accuracy and intra- and inter-day variability values were within acceptable limits. Linearity was reflected by the excellent coefficient of determination ($r^2 > 0.99$).

Pharmacokinetic analysis

The serum drug concentration–time curves were analysed using WinNonlin Professional software (Version 5.0.1, Pharsight Corporation, Cary, NC, US), an iterative curve-fitting program based on non-linear regression analysis. The serum concentration–time profiles for the S- and R-enantiomers of warfarin following its oral administration were adequately described using the one-compartment open model with first-order absorption and elimination. The administered dose (1.5 mg/kg) was divided by 2 to obtain the equivalent enantiomeric dose.[45] The rate of warfarin absorption was assumed to be non-stereoselective and complete absorption was assumed.[7]

The peak serum concentration (C_{max}), the time of its occurrence (t_{max}), the absorption rate constant (k_a), elimination half-life ($t_{1/2}$), apparent volume of distribution (V_d) and the serum clearance (CL) were estimated from the non-linear regression analysis. The total area under the serum concentration–time curve from time zero to the last quantifiable time point ($AUC_{0–t}$) and from time zero to infinity ($AUC_{0–\infty}$) was estimated using the linear trapezoidal method. The fractions of the administered enantiomeric dose eliminated as the parent enantiomer (f_e) and its metabolites (f_m) were estimated from their respective cumulative amounts (expressed in warfarin equivalents) in urine. The unbound concentration of each warfarin enantiomer was calculated by multiplying the total plasma concentration by the respective mean value of f_u. The corresponding unbound clearance (CL_u) and unbound volume of distribution (V_u) were calculated from $CL_u = CL/f_u$ and $V_u = V_d/f_u$, respectively. The unbound renal clearance (CLu_R) of each warfarin enantiomer and the unbound formation clearance (CLu_f) of its metabolites were calculated using the equations $CLu_R = f_e\ CL_u$ and $CLu_f = f_m\ CL_u$, respectively.[46,47]

Statistical analysis

Data analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, US). Sample data were expressed as means ± SD. Comparisons of means of related samples were made using the Student's paired-samples *t*-test; comparisons of means of two independent groups were made using Student's independent-samples *t*-test. A *P* value of less than 0.05 was considered statistically significant.

Results

Effect of quercetin on serum protein binding of warfarin

The effect of quercetin on the protein binding of warfarin enantiomers in pooled rat serum was tested using relatively high concentrations of unlabelled racemic warfarin

Table 1 Parameters associated with the chemical analysis of the warfarin enantiomers in serum and urine and associated metabolites in urine

Species	Limit of detection[a] and quantitation[b] (ng/ml)	Mean absolute error[c] (*n* = 6) (%)	Variability of lowest concentration on standard curve (*n* = 6) (ng/ml; CV%)	Variability of highest concentration on standard curve (*n* = 6) (ng/ml; CV%)	r^2 over six-point calibration curve
Serum					
S-Warfarin	6.17, 15.4	5.00	15.4, 6.2[d], 3.4[e]	247, 13[d], 5.0[e]	0.9966
R-Warfarin	6.17, 15.4	3.80	15.4, 6.6, 4.1	247, 8.0, 8.1	0.9964
Urine					
S-Warfarin	12.3, 30.8	4.50	30.8, 4.2, 7.4	493, 5.9, 2.0	0.9980
R-Warfarin	12.3, 30.8	4.16	30.8, 3.2, 7.8	493, 5.6, 1.8	0.9919
S,R-Warfarin alcohol	6.49, 81.1	5.28	81.1, 10, 6.8	1297, 3.7, 6.7	0.9971
S,S-Warfarin alcohol	6.49, 81.1	5.96	81.1, 6.5, 1.0	1297, 7.1, 5.0	0.9950
R,S-Warfarin alcohol	3.24, 81.1	1.20	81.1, 6.8, 3.5	1297, 3.8, 3.8	0.9967
R,R-Warfarin alcohol	6.49, 81.1	7.64	81.1, 9.1, 6.0	1297, 1.5, 2.7	0.9921
S-6-Hydroxywarfarin	13.0, 81.1	3.48	81.1, 15, 12	1297, 3.0, 12	0.9934
S-7-Hydroxywarfarin	35.7, 81.1	6.64	81.1, 15, 6.3	1297, 4.4, 10	0.9980
R-6-Hydroxywarfarin	22.7, 81.1	9.90	81.1, 14, 9.4	1297, 3.1, 7.9	0.9921
R-7-Hydroxywarfarin	45.4, 81.1	8.95	81.1, 16, 12	1297, 9.6, 7.9	0.9916
S-4′-Hydroxywarfarin	29.2, 201	6.30	201, 11, 8.0	3243, 6.3, 16	0.9945
S-4′-Hydroxywarfarin	35.7, 201	9.80	201, 13, 5.1	3243, 5.2, 4.4	0.9975

[a]Limit of detection, defined as the analyte concentration with a signal-to-noise ratio of 3; [b]limit of quantitation, determined as the lowest calibration concentration at which the intra- and inter-day coefficient of variation (CV) was no greater than 20%; [c]accuracy, defined as absolute percentage of difference between the analysed and the spiked concentration over that of the spiked value; [d]intraday coefficient of variation; [e]interday coefficient of variation.

Table 2 Effect of quercetin on in-vitro serum protein binding of individual warfarin enantiomers

	Percent unbound			
	S-warfarin		R-warfarin	
	25 μM	50 μM	25 μM	50 μM
Control	0.61 ± 0.13 (6)	0.64 ± 0.15 (6)	0.94 ± 0.21 (6)[†]	0.96 ± 0.27 (6)[†]
Total	0.63 ± 0.15 (12)		0.95 ± 0.21 (12)[†]	
Quercetin (50 μM)	0.36 ± 0.12 (6)**	0.41 ± 0.13 (6)**	0.73 ± 0.16 (6)*,[†]	0.67 ± 0.18 (6)*,[†]
Total	0.38 ± 0.12 (12)**		0.70 ± 0.15 (12)*,[+]	

Values are percent unbound, mean ± SD; figures in parentheses indicate the number of determinations. *$P < 0.05$, **$P < 0.01$ vs control group. [†]$P > 0.01$ vs S-warfarin.

(50-100 μM), which permitted direct determination of the respective unbound fraction values by chiral HPLC. The unbound fraction of individual warfarin enantiomers in serum appeared to be concentration-independent, as similar fraction values were found at 25 and 50 μM. The unbound fraction of R-warfarin was significantly higher than that of S-warfarin ($P < 0.01$) (Table 2). The average enantiomeric ratio (R/S) of the unbound fraction for warfarin alone was 1.5, whereas in the presence of quercetin the R/S ratio was slightly increased to 1.8. Quercetin reduced the unbound fraction of S-warfarin by 40% ($P < 0.01$) and of R-warfarin by 26% ($P < 0.05$).

Effect of rutin on the anticoagulant activity of warfarin

Comparison of the PCA time course between the control (warfarin alone) and rutin groups is shown in Figure 1. As expected, the PCA declined following administration of racemic warfarin. The hypoprothrombinaemic response to warfarin was maximal at 24 h in all rats. The mean PCA value for the rutin group was significantly increased at 72 h compared with the control group ($P < 0.01$). As shown in Table 3, the baseline PT was not affected by rutin pretreatment. On the other hand, co-administration of rutin with warfarin significantly increased the mean AUC_{PCA} by 31% ($P < 0.05$).

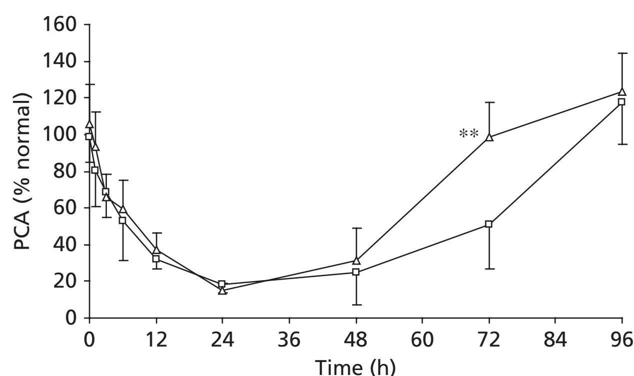

Figure 1 Time courses of changes in prothrombin complex activity (PCA) following oral administration of racemic warfarin (1.5 mg/kg) to rats alone (control; □) and during rutin treatment (△). Values are mean ± SD (n = 8). **$P < 0.01$ vs control.

Table 3 Estimated pharmacodynamic parameters following oral administration of racemic warfarin (1.5 mg/kg) to rats, alone (control) and on day 5 of an 8 day regimen of rutin (1 g/kg daily)

Parameter	Control	Rutin
PT_0 (s)	43.9 ± 8.5	41.5 ± 11.3
PCA_{min} (% normal)	14.4 ± 2.8	13.7 ± 3.0
$t_{PCA,min}$ (h)	24 ± 0	24 ± 0
AUC_{PCA} (% normal·h)	4431 ± 1272	5819 ± 768*

Values are mean ± SD (n = 8). PT_0, baseline prothrombin time; PCA_{min}, minimum prothrombin complex activity; $t_{PCA,min}$, time to achieve PCA_{min}; AUC_{PCA}, area under the PCA–time curve from time 0 to last sampling time point. *$P < 0.05$ vs control group.

Pharmacokinetic parameters

Warfarin was undetectable in the predose samples in all rats, confirming that rutin does not interfere with the warfarin assay. Figure 2 shows the concentration–time profiles of total and unbound serum S- and R-warfarin in rats receiving warfarin alone and with concurrent rutin treatment. The serum concentrations of warfarin enantiomers appeared to peak at 4–5 h and to decline monoexponentially; R-warfarin was eliminated more rapidly than S-warfarin. Rutin significantly increased the total, but not the unbound, peak serum concentrations of both S- and R-warfarin, by 71 and 60%, respectively. Total and unbound serum concentrations of S- and R- warfarin tended to be somewhat lower from 24 h to 96 h in the presence of rutin. Significant decreases in the serum concentrations of S- and R-warfarin between the control and rutin treatment groups were observed at several time points (Figure 2).

The estimated pharmacokinetic parameters of warfarin enantiomers in rats after receiving warfarin alone or with concomitant administration of rutin are presented in Table 4. With regard to absorption, there was a trend towards higher k_a and C_{max} and shorter t_{max} for both the R- and S-enantiomers of warfarin, although the difference between the control and rutin treatment groups was only significant for C_{max} ($P < 0.05$). With respect to disposition, co-administration of rutin significantly reduced the V_d of S- and R-warfarin, by 33% and 21%, respectively (both $P < 0.05$). Rutin reduced the elimination $t_{1/2}$ of S-enantiomer by 37% ($P < 0.01$) but had a marginal effect on that of the R-enantiomer; the CL of both S- and R-warfarin was reduced. On the other hand, rutin significantly increased the

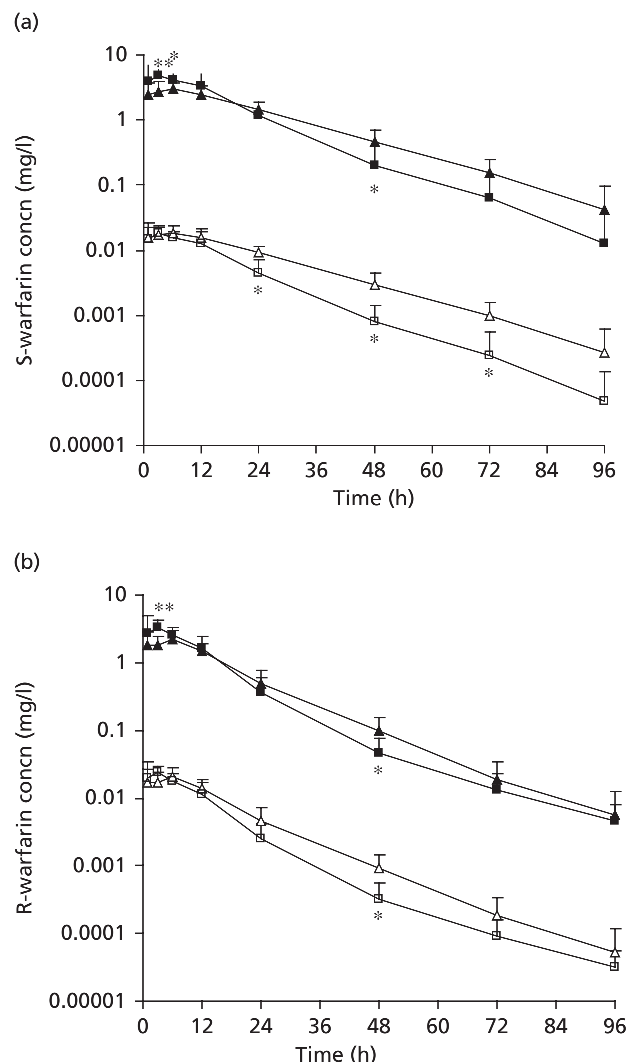

Figure 2 Time courses of serum concentrations of total and unbound (a) S- and (b) R-enantiomers of warfarin following oral administration of racemic warfarin (1.5 mg/kg) to rats alone (control) and on day 5 of 8 days' treatment with rutin. ▲, Total drug, control group; ■, total drug, rutin group; △, unbound drug, control group; □, unbound drug, rutin group. Data are mean ± SD (n = 8). *$P < 0.05$, **$P < 0.01$ vs control.

CL_u of the S-enantiomer by 69%, but not that of the R-enantiomer (6% increase). There was no obvious change in the V_u of either S- or R-warfarin in the presence of rutin.

Urinary excretion data

Urinary excretion data for warfarin enantiomers and metabolites (both oxidative and reductive) are summarized in Table 5. In the control group, the total urinary recovery of the enantiomers of warfarin and its metabolites (expressed as warfarin equivalents) was 66% of the racemic dose administered (67% for S-warfarin and 64% for R-warfarin). With respect to excretion of the unchanged parent enantiomers, recovery was higher with R-warfarin than S-warfarin. The recovery of 6- and 7-hydroxylated metabolites and reductive metabolites was greater with R-warfarin than S-enantiomer, whereas recovery of the 4′-hydroxylated metabolite of R-warfarin was only 40% of that of S-warfarin. With respect to the total oxidative products, the recovery of the R-form products was less than that of S-form products. Moreover, the metabolism of warfarin via reduction was found to be of minor importance when compared with oxidative pathways, accounting for less than 7% of the recovered dose.

Co-administration of rutin appeared to have marginal effects on the urinary recovery of the warfarin enantiomers and their reductive and oxidative metabolites, except that of R-7-hydroxywarfarin, which was decreased significantly by 41%. Overall, total recovery of the S- and R-enantiomeric dose was decreased by 10% and 31%, respectively, in the presence of rutin.

Combined serum and urinary excretion data

Combined information relating to cumulative drug and metabolite excretion and the total area under the unbound drug concentration–time curve enables detailed examination of the effect of rutin on the unbound clearance associated with the renal excretion of unchanged warfarin enantiomers and the formation of the respective metabolites. Table 6 summarises estimates of the CLu_R values of S- and R-warfarin and the CLu_f values of their respective metabolites. Rutin appeared to significantly increase the CLu_R of the S- and R-enantiomers (by 77% and 36%, respectively), the CLu_f of most reductive metabolites (72%, 106% and 73% for S,S-, S,R- and R,S-warfarin alcohol, respectively) and of most of the hydroxylated metabolites of S-warfarin – by 48% and 53% for 7- and 4′-hydroxywarfarin, respectively. In contrast, there were insignificant decreases in the CLu_f values for all of the hydroxylated metabolites of R-warfarin, ranging from 4 to 19%. With respect to the overall oxidative products, the CLu_f of the total S-form metabolites was increased by 77% in the presence of rutin ($P < 0.05$), whereas that of the total R-form metabolites was not significantly affected.

Discussion

Rutin is widely used in complementary and alternative medicines and numerous health supplements, as a constituent as well as in pure form.[35] Apart from the cardiovascular benefits, rutin has also been shown to lower plasma lipids and hepatic cholesterol levels.[48–50] Rutin has been reported to counteract the activity of the oral anticoagulant dicoumarol (a coumarin derivative) in rats by reducing PT.[51] As rutin combined with warfarin is reported to be beneficial in limb salvage and survival in patients with severe critical leg ischaemia,[26] co-administration of the two has clinical promise. However, warfarin has a low therapeutic index and is known to interact with various drugs and herbs; thus, potential drug interactions need to be scrutinised carefully. The present study demonstrates some evidence of interaction between rutin and warfarin in rats. The hypoprothrombinaemic response to warfarin is diminished in the rats treated concurrently with rutin (Figure 1, Table 3). The baseline PT was not affected by rutin pretreatment (Table 3), suggesting that rutin has no effect on blood coagulation *per se*.

Table 4 Estimated pharmacokinetic parameters for the R- and S-enantiomers of warfarin after oral administration of racemic drug (1.5 mg/kg) to rats in the absence (control) and presence of rutin

Parameter	S-warfarin		R-warfarin	
	Control	Rutin	Control	Rutin
C_{max} (mg/l)	3.11 ± 0.74	5.33 ± 1.48**	2.37 ± 0.71	3.79 ± 1.26*
t_{max} (h)	4.6 ± 2.0	3.9 ± 1.9	4.7 ± 2.3	3.5 ± 1.7
$AUC_{0-\infty}$ (mg/l·h)	94.0 ± 19.9	93.1 ± 38.6	46.1 ± 7.49	48.4 ± 15.5
k_a (h^{-1})	0.64 ± 0.63	0.86 ± 0.6	0.70 ± 0.55	0.94 ± 0.79
$t_{1/2}$ (h)	14.7 ± 3.8	9.3 ± 2.8**	10.1 ± 1.5	9.3 ± 1.6
V_d (l/kg)	0.18 ± 0.04	0.12 ± 0.03*	0.24 ± 0.05	0.19 ± 0.03*
CL (ml/h per kg)	8.3 ± 1.8	9.2 ± 3.4	16.7 ± 2.7	16.8 ± 4.9
V_u (l/kg)	29.0 ± 6.3	31.0 ± 13.4	25.5 ± 6.3	27.1 ± 9.4
CL_u (ml/h per kg)	1406 ± 355	2372 ± 826**	1949 ± 421	2066 ± 986

Values are mean ± SD (n = 8). C_{max}, peak serum concentration; t_{max}, time of C_{max}; $AUC_{0-\infty}$, total area under the serum concentration–time curve from time 0 to infinity; k_a, first-order absorption rate constant; $t_{1/2}$, elimination half-life; V_d, apparent volume of distribution; CL, total serum clearance; V_u, unbound volume of distribution; CL_u, unbound serum clearance. *$P < 0.05$, **$P < 0.01$ vs control group.

Table 5 Urinary recovery of unchanged S- and R-enantiomers of warfarin and metabolites in the absence (control) and presence of rutin

Species[a]	S-warfarin		R-warfarin	
	Control	Rutin	Control	Rutin
Warfarin	1.0 ± 0.6	1.1 ± 0.7	5.4 ± 6.0	4.4 ± 9.5
S-Warfarin alcohol[b]	2.3 ± 1.2	2.5 ± 1.9	3.3 ± 2.5	4.2 ± 1.1
R-Warfarin alcohol[c]	0.60 ± 0.84	0.76 ± 4.4	1.1 ± 0.4	0.85 ± 0.74
6-Hydroxywarfarin	11.6 ± 3.9	7.2 ± 4.6	13.0 ± 6.5	7.8 ± 5.6
7-Hydroxywarfarin	22.3 ± 14.3	20.3 ± 8.1	29.8 ± 5.3	17.7 ± 5.3***
4′-Hydroxywarfarin	29.4 ± 15.3	27.6 ± 18.9	11.9 ± 10.2	9.2 ± 14.7
Total hydroxyl[d]	63.3 ± 20.9	55.1 ± 25.4	54.7 ± 16.5	34.7 ± 29.7
Total dose recovered[e]	67.3 ± 22.5	60.5 ± 23.4	64.4 ± 21.5	44.2 ± 38.3

Values are means ± SD percentage of the administered dose recovered from urine (n = 8). [a]Expressed in warfarin equivalents; [b]metabolite formed by S-reduction; [c]metabolite formed by R-reduction; [d]total of all hydroxylated (oxidative) metabolites of warfarin; [e]total of warfarin and all the reductive and oxidative metabolites. ***$P < 0.001$ vs control group.

Table 6 Estimated unbound renal clearance of S- and R-warfarin and unbound formation clearance of metabolites in the absence (control) and presence of rutin

Species	S-warfarin		R-warfarin	
	Control	Rutin	Control	Rutin
Unbound renal clearance (ml/h per kg)	14.1 ± 3.1	25.0 ± 9.3**	99.3 ± 15.6	134.6 ± 31.3*
Unbound formation clearance (ml/h per kg)				
S-Warfarin alcohol	32.5 ± 7.0	55.8 ± 20.5**	60.4 ± 12.2	104.7 ± 26.9**
R-Warfarin alcohol	8.3 ± 1.8	17.1 ± 6.3**	20.2 ± 4.1	21.4 ± 6.1
6-Hydroxywarfarin	160.5 ± 34.7	163.2 ± 60.0	240.8 ± 48.9	196.4 ± 55.2
7-Hydroxywarfarin	308.5 ± 66.7	457.0 ± 152.7*	552.0 ± 121.1	445.1 ± 132.8
4′-Hydroxywarfarin	406.7 ± 88.3	621.3 ± 227.6*	220.4 ± 47.8	230.1 ± 67.2
Total hydroxy metabolites[a]	700.0 ± 151.8	1240 ± 680*	1013 ± 220	871.6 ± 255.2

Values are means ± SD (n = 8). [a]Unbound formation clearance of total observable hydroxylated metabolites of warfarin enantiomers. *$P < 0.05$, **$P < 0.01$ vs control group.

Changes in f_u and V_d values in the presence of rutin (or its aglycone quercetin) (Tables 2 and 4) suggest that the flavonoid has an effect on the serum protein binding of the warfarin enantiomers and their distribution. The protein binding of quercetin is extensive – at least 99% in plasma.[52] The concentration of quercetin used in the present protein binding study was 0.050 mM. A previous study had reported that the average peak plasma concentration of quercetin is 0.0013 mM in rats receiving gastric intubation of rutin (50 μmol/kg or 0.0305 g/kg).[34] Thus, a peak serum quercetin concentration of at least 0.043 mM was expected in the present in-vivo study, as the rats were given a higher dose of rutin (1 g/kg per day for

8 consecutive days). An increase in serum protein binding of S- and R-warfarin could account for the corresponding significant decrease in V_d and significant increase in C_{max} in the rutin-treated rats (Table 4, Figure 2).

Up-to-date information on the effect of quercetin on warfarin protein binding is lacking in the literature. It is thus unclear how quercetin augments the binding of warfarin to serum protein, since both warfarin and quercetin bind strongly to the subdomain IIA (having a large hydrophobic cavity) of albumin as the primary binding site[52,53] In-vitro studies have shown that quercetin is displaced non-competitively only by the presence of a large excess (1000 μM) of warfarin.[54] Nevertheless, the current binding data suggest that quercetin has a positive allosteric effect on the interaction of warfarin enantiomers with human serum albumin.

Higher k_a and C_{max} and shorter t_{max} values found in the rutin group indicate that the flavonoid may augment the absorption of warfarin enantiomers (Table 4). Rutin appears to significantly affect the elimination kinetics of the S-enantiomer, but not the R-enantiomer, since only the elimination $t_{1/2}$ of S-warfarin was substantially shortened as a result of the significant increase in the CL_u of S-warfarin (Table 4).

Close scrutiny of the urinary excretion data together with the unbound serum data reveals the extent to which the excretion and metabolism of warfarin enantiomers are affected by rutin. The baseline results of the urinary excretion study (Table 5) are consistent with those reported previously,[55] indicating that both enantiomers of warfarin are predominantly metabolised by oxidation, as well as being partly metabolised by reduction and partly excreted as unchanged drug. Concurrent rutin administration increases the renal excretion of S- and R-warfarin, the formation of most of the reductive metabolites of both enantiomers and that of most of the oxidative metabolites of S-enantiomer, but decreases excretion of most of the oxidative metabolites of the R-enantiomer to varying degrees (Table 6). The net effect is that the unbound clearance of the more potent S-warfarin is significantly increased in the presence of rutin, while that of the less potent R-warfarin remains unchanged.

An in-vitro study has shown that quercetin has an inhibitory effect on both tolbutamide 4-methylhydroxylation and testosterone 6β-hydroxylation in human liver microsomes, used as index metabolic reactions for CYP2C9 and CYP3A4, respectively.[56] The former isozyme is also known to be responsible for the oxidative metabolism of S-warfarin, mainly 6 and 7-hydroxylation, while the latter is responsible mainly for the 7-hydroxylation of R-warfarin.[8] The present study demonstrates that rutin marginally inhibits the formation of R-7-hydroxywarfarin, but has little effect on the formation of S-6-hydroxywarfarin and significantly increases formation of S-7-hydroxywarfarinin *in vivo* (Table 6). In-vitro studies using specific CYP isozymes may help to elucidate the metabolic interaction between warfarin and rutin.

Conclusions

Data obtained in the present animal study suggest that concurrent rutin administration is likely to reduce the anticoagulant effect of racemic warfarin, which is mainly attributed to a significant decrease in the elimination half-life of S-warfarin as a result of the substantially increased unbound clearance of the more potent S-enantiomer, with a little change in its unbound volume of distribution. Further investigation to verify this interaction in the clinic is warranted.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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