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Disposition of warfarin enantiomers and metabolites in patients during multiple dosing with *rac*-warfarin

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1 The disposition of warfarin enantiomers and metabolites has been studied in 36 patients receiving chronic *rac*-warfarin therapy, titrated to approximately the same anticoagulant response.

2 A stereoselective h.p.l.c. assay was employed to determine the concentrations of (R)- and (S)-warfarin, (R,S)-warfarin alcohol and (S)-7-hydroxywarfarin in plasma and 24 h urine samples. The concentrations of (R)-7-hydroxywarfarin, (S,S)-warfarin alcohol and (R)-6- and (S)-6-hydroxywarfarin were also determined in urine samples. The fractions unbound of warfarin enantiomers were determined using equilibrium dialysis.

3 Wide variability was observed in daily dose requirements (mean 6.1 mg; range: 2.5–12 mg), in plasma concentrations of (S)-warfarin (0.48 mg l^{-1}; 0.11–1.02 mg l^{-1}), (R)-warfarin (0.87 mg l^{-1}; 0.29–1.82 mg l^{-1}), (R,S)-warfarin alcohol (0.31 mg l^{-1}; 0.02–0.72 mg l^{-1}) and (S)-7-hydroxywarfarin (0.25 mg l^{-1}; 0.07–0.37 mg l^{-1}) and the percentage unbound of (S)-warfarin (0.53%; 0.29%–0.82%) and (R)-warfarin (0.54%; 0.26%–0.96%).

4 The mean plasma clearances of warfarin enantiomers were 7.5 l day^{-1} per 70 kg (2.5–22.1) for (S)-warfarin and 3.6 l day^{-1} per 70 kg (1.6–8.8) for (R)-warfarin. There was a significant correlation between the estimated formation clearance of (S)-7-hydroxywarfarin and the clearance of (S)-warfarin, which accounted for much of the variability in the latter.

5 The renal clearance of (S)-7-hydroxywarfarin (mean 5.6 l day^{-1} per 70 kg; range 0.8–18.8) and (R,S)-warfarin alcohol (2.8 l day^{-1} per 70 kg; 0.1–16.7) was variable across the study group.

Keywords warfarin enantiomers warfarin metabolites pharmacokinetics variability

Introduction

Patients taking warfarin differ widely in the daily dose required to achieve the same degree of anticoagulation [1,2,3]. While attempts have been made to account for this variability [4] interpretation is complicated because warfarin is used clinically as a racemic mixture. Furthermore, (S)-warfarin is approximately five times more potent as an anticoagulant [5] and is metabolised more rapidly [6,7] than its optical antipode. In the past investigations have been made into the pharmacokinetics of warfarin in patients at steady-state [3,4,8]. However, much of the pharmacokinetic data for warfarin have been obtained using non-stereoselective assays. Two recent studies have examined the disposition of warfarin enantiomers at steady-state in healthy subjects [9] and anticoagulated patients [10]. Preliminary reports from these studies indicate that the serum concentrations of (R)-warfarin exceed those of the (S)-enantiomer at steady-state. The concentrations of the major warfarin metabolites in patients receiving

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chronic warfarin therapy have not previously been reported. Banfield *et al.* [10] have highlighted the need to study unbound concentrations of the pharmacologically relevant warfarin enantiomer in the analysis of warfarin pharmacokinetic data.

The present study was conducted with the aim of describing the disposition of warfarin enantiomers and its major metabolites in patients undergoing chronic warfarin therapy. The protein binding of warfarin enantiomers and the urinary metabolic profile in patients has also been investigated.

Methods

This study had the approval of the North Staffordshire Health District Ethics Committee.

Patients

Thirty-six outpatients from the Medical Anticoagulant Clinic at North Staffordshire Hospital Centre who were receiving *rac*-warfarin as part of their anticoagulant therapy and had demonstrated stable anticoagulation over some weeks were recruited into the study. Each patient had previously been titrated to a prothrombin index in the range 30% to 50%. The patient group consisted of 17 females and 19 males. The mean age of the patients was 52.2 years (range; 20–75) and their mean weight was 75.8 kg (range; 49–106). No patient was taking or had taken any drug known to affect the pharmacokinetics or pharmacodynamics of warfarin. Biochemical and haematological tests prior to patient enrolment confirmed that none showed evidence of renal or hepatic insufficiency. Twenty-two patients were taking concomitant medications. These included digoxin, sedatives (e.g. nitrazepam) and diuretics (e.g. bumetanide). Five patients were cigarette smokers (4–30 per day) and 13 patients consumed alcohol regularly (10–100 g ethanol per week).

Dose administration and sample collection

The mean duration of warfarin therapy for patients in the study group was 3 months (range; 1–5 months). Each patient had been stabilised on the daily dose used in the study for at least 1 month prior to sample collection. Plasma concentrations of warfarin and metabolites obtained from all patients were assumed to be at steady-state. A single blood sample was taken on the day of the clinic visit (approximately 12 h after dose administration). Plasma was harvested to allow the measurement of warfarin and metabolite concentrations. A portion of plasma was retained for protein binding analysis and for the analysis of total protein and albumin concentrations. Patients were asked to collect all urine over the 24 h dose interval. Total urine volume was measured and a portion was retained for drug and metabolite analysis. Plasma and urine samples were frozen at –20°C until the time of analysis. At the same time a blood sample was collected for prothrombin time determination.

Sample analysis

Plasma and urine were analysed using a stereoselective h.p.l.c. assay for warfarin and metabolite enantiomers [12,13]. Total protein and human serum albumin concentrations were determined using the biuret [14] and bromocresol green [15] methods, respectively. Prothrombin time (in seconds) for each test sample (sample PT) was measured using Quick's one-stage prothrombin test and was converted to prothrombin index (% P.I.) by dividing the test result by the control prothrombin time (control PT) using the following equation,

$$\%\ \text{P.I.} = \frac{\text{control PT}}{\text{sample PT}} \cdot 100$$

Determination of unbound fraction

The unbound fraction (f*u*) of each warfarin enantiomer was determined using equilibrium dialysis at 37° C for 4 h after the addition of 15 μg *rac*-warfarin to 1 ml of patient plasma [11]. Volume shift during equilibrium dialysis was monitored by measuring the concentration of total protein. Where appropriate the unbound fraction was corrected for volume shift [16]. A control protein binding experiment was conducted to examine the contribution of the variability of the method to observed variability in f*u*. Twenty 1 ml aliquots of drug-free plasma were spiked with 15 mg l^{-1} *rac*-warfarin and subjected to the same equilibrium dialysis protocol as patient plasma samples.

Pharmacokinetic analysis

Total clearance (CL) of warfarin enantiomers from plasma was estimated using the following equation, assuming that *rac*-warfarin bioavailability [17] and compliance was complete,

$$\text{CL} = \frac{D}{2\tau \cdot C_{ss,av}} \qquad (2)$$

where D is the dose of *rac*-warfarin given daily (τ = 24 h) and $C_{ss,av}$ is the average steady-state plasma concentration of each warfarin enantiomer. The corresponding unbound clearance was determined using Equation 3,

$$\text{CLu} = \frac{\text{CL}}{\text{fu}} \qquad (3)$$

The formation clearance of warfarin metabolites (CL_f) was estimated, assuming that all metabolite formed undergoes urinary excretion, using the following equation

$$CL_f = \frac{Ae(\text{m})/\tau}{C_{ss,av}} \qquad (4)$$

where $Ae(m)$ is the total amount of metabolite excreted in the urine (expressed as warfarin equivalents) over the dose interval (τ) of 24 h. Renal clearance of warfarin metabolite, $CL(m)_R$, was estimated using the following equation,

$$CL(m)_R = \frac{Ae(m)/\tau}{C(m)_{ss}} \quad (5)$$

where $C(m)_{ss}$ is the steady-state plasma concentration of metabolite.

Statistical analysis

Results are expressed as mean data and standard deviation (s.d.) or coefficient of variation where shown. The Statistical Package for the Social Sciences, was used for the bivariate correlation analysis. A two-tailed test of significance was employed at the 0.05 significance level. Where appropriate the 95% confidence interval was also calculated.

Results

Patients were taking a wide range of daily warfarin doses to achieve the same degree of anticoagulation. The mean (± s.d.) dose for the group was 6.1 ± 2.3 mg, but the daily dose ranged from as low as 2.5 mg up to 12 mg. The distribution of doses appeared to be unimodal indicating that the study group did not contain a distinct group of patients who were warfarin resistant. The mean (± s.d.) steady-state plasma concentration of (S)-warfarin was 0.48 ± 0.25 mg l^{-1} (range; 0.11–1.02) and of (R)-warfarin was 0.87 ± 0.35 mg l^{-1} (range; 0.29–1.82). The total and unbound steady-state plasma concentrations of warfarin enantiomers are shown in Figure 1. The enantiomeric ratio (S/R) of warfarin plasma concentrations displayed marked variability, ranging from 0.21 to 1.28 (mean; 0.55 ± 0.22). The mean plasma clearance of (S)-warfarin was 7.5 ± 4.4 l day^{-1} per 70 kg (range; 2.5–22.1) and for the (R)-enantiomer it was 3.6 ± 1.4 l day^{-1} per 70 kg (range; 1.6–8.8). The mean percentage unbound of (S)-warfarin was 0.53 ± 0.13% (range; 0.29–0.82) and for (R)-warfarin it was 0.54 ± 0.14% (range; 0.26–0.96). In the control protein binding experiment the percentage unbound varied from 0.45 to 0.61% (mean; 0.51%, %CV 9.7) for (S)-warfarin and from 0.49 to 0.69 (mean; 0.59%, %CV 10.2) for (R)-warfarin. The calculated unbound clearance (CLu) was 1440 l day^{-1} per 70 kg (range; 533–4014) for (S)-warfarin and for (R)-warfarin it was 695 l day^{-1} per 70 kg (range; 349–1671).

The mean (± s.d.) steady-state plasma concentration of (R,S)-warfarin alcohol was 0.31 ± 0.18 mg l^{-1} (range; 0.02–0.72) and for (S)-7-hydroxywarfarin it was 0.25 ± 0.09 mg l^{-1} (0–0.37). Table 1 shows the daily urinary excretion rate and formation clearance values for the enantiomers of the major warfarin

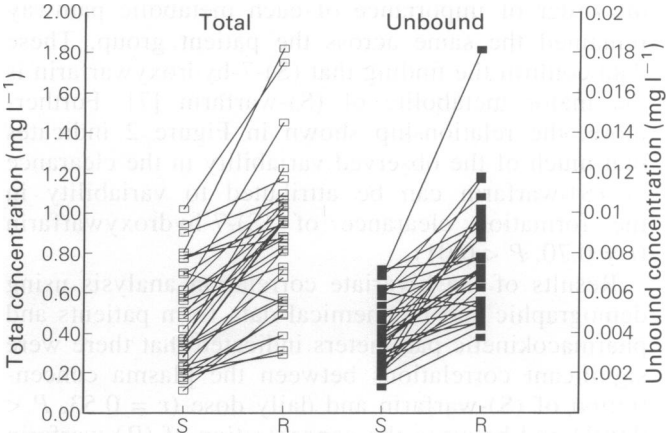

Figure 1 Steady-state plasma concentrations (mg l^{-1}) of total and unbound (S)-warfarin and (R)-warfarin in 36 patients. Individual observations are joined by solid lines.

Table 1 Urinary excretion rate and estimated formation clearance values for warfarin metabolites in 36 patients during steady-state dosing. Mean ± s.d. with range shown in bracket below

Metabolite	*UER (mg day^{-1})*	*$CL(m)_R$ (l day^{-1} per 70 kg)*	*CL_f (l day^{-1})*
(S)-7-hydroxywarfarin	1.38 ± 0.85 (0.24–3.80)	5.64 ± 4.34 (0.83–18.78)	3.48 ± 2.03 (0.29–9.46)
(R)-7-hydroxywarfarin	0.11 ± 0.07 (0.03–0.25)	ND	0.13 ± 0.06 (0.04–0.25)
(S)-6-hydroxywarfarin	0.32 ± 0.21 (0.04–1.15)	ND	0.79 ± 0.48 (0.05–2.01)
(R)-6-hydroxywarfarin	0.47 ± 0.35 (0.04–1.65)	ND	0.57 ± 0.41 (0.02–1.64)
(R,S)-warfarin alcohol	0.49 ± 0.32 (0.05–1.50)	2.77 ± 3.80 (0.10–16.70)	0.64 ± 0.44 (0.03–2.08)
(S,S)-warfarin alcohol	0.22 ± 0.10 (0.10–0.52)	ND	0.54 ± 0.31 (0.17–1.50)

UER, urinary excretion rate; $CL(m)_R$, renal plasma clearance of metabolite; CL_f, formation clearance of metabolite; ND, not determined.

metabolite enantiomers. Marked variability was observed in all these parameters. (S)-7-Hydroxywarfarin and (R,S)-warfarin alcohol were the only two metabolites that could be measured accurately in plasma to allow estimation of renal clearance; the plasma concentrations of other metabolites were below the limit of determination of the assay. The renal clearances of (S)-7-hydroxywarfarin (mean 5.6 l day^{-1} per 70 kg; range 0.8–18.8) and (R,S)-warfarin alcohol (2.8 l day^{-1} per 70 kg; 0.1–16.7) showed wide inter-subject variability. Using the CL_f data shown in Table 1 it is possible to calculate the fraction of warfarin metabolised by each metabolic pathway (*fm*), as CL_f/CL, where CL is the total clearance of the respective parent enantiomer. The calculated *fm* (%CV) values, shown in decreasing order of importance, were 0.45 (44) for (S)-7-hydroxywarfarin, 0.18 (71) for (R,S)-warfarin alcohol, 0.15 (66) for (R)-6-hydroxywarfarin, 0.10 (52) for (S)-6-hydroxywarfarin, 0.08 (43) for (S,S)-warfarin alcohol and 0.03 (42) for (R)-7-hydroxywarfarin. Although there was notable inter-subject variability in *fm* values for each metabolite the order of importance of each metabolic pathway remained the same across the patient group. These data confirm the finding that (S)-7-hydroxywarfarin is the major metabolite of (S)-warfarin [7]. Furthermore, the relationship shown in Figure 2 indicates that much of the observed variability in the clearance of (S)-warfarin can be attributed to variability in the formation clearance of (S)-7-hydroxywarfarin ($r = 0.70$, $P < 0.01$).

Results of the bivariate correlation analysis using demographic and biochemical data from patients and pharmacokinetic parameters indicated that there were significant correlations between the plasma concentration of (S)-warfarin and daily dose ($r = 0.53$, $P < 0.001$) and between the concentration of (R)-warfarin and dose ($r = 0.61$, $P < 0.001$). Even stronger correlations were detected between the unbound plasma concentrations of (S)-warfarin and daily dose ($r = 0.64$, $P < 0.001$) and also (R)-warfarin and dose ($r = 0.64$, $P < 0.001$). Weak but significant correlations were also demonstrated between the unbound fractions of both enantiomers and albumin ((S)-warfarin $r = -0.38$, $P = 0.029$; (R)-warfarin $r = -0.39$, $P = 0.023$) but not with total protein concentration in plasma. There was a significant correlation between the unbound fractions of (S)- and (R)-warfarin ($r = 0.92$, $P < 0.001$).

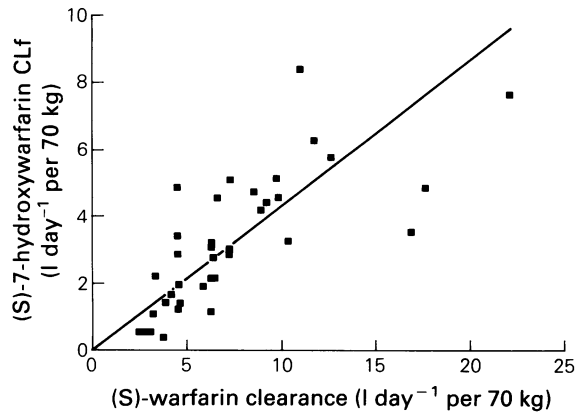

Figure 2 Relationship between the estimated formation clearance of (S)-7-hydroxywarfarin and the plasma clearance of (S)-warfarin. The solid regression line is shown (slope = 0.43, $r = 0.70$, $P < 0.01$).

Older patients in the study group (above 50 years) tended to have a lower dose requirement of warfarin (5.7 mg; 95% C.I. 4.5–6.9 mg) compared with the younger patients (less than 50 years) (6.7 mg; 95% C.I. 5.9–7.5 mg). However, these differences did not reach statistical significance. There were no apparent age related differences in plasma concentration, unbound fraction or clearance of either warfarin or metabolite enantiomers. A comparison of the 95% confidence intervals for the dose requirements (4.7–6.6 mg *vs* 5.6–8.2 mg) and steady-state concentration of (S)-warfarin (0.31–0.51 mg l^{-1} *vs* 0.45–0.71 mg l^{-1}) indicated that there was no difference in these parameters for patients receiving and not receiving concomitant medications, respectively. The influence of gender and regular alcohol intake on the study parameters was also examined but the results were unremarkable. A comparison of the pharmacokinetic parameters between smoking and non-smoking patients was not possible because too few patients ($n = 5$) were smokers.

Discussion

Sampling protocol simulations

A possible limitation of the present investigation is the estimation of steady-state pharmacokinetic data for warfarin and metabolite enantiomers using a single plasma sample obtained at approximately 12 h after dose administration. Previous studies examining the pharmacokinetics of *rac*-warfarin [4,18] and warfarin enantiomers [10,19–21] at steady-state have used a single point determination. To understand the implication of this sampling protocol a simulation study was conducted to estimate the plasma concentration profile of *rac*-warfarin and individual enantiomers over a 24 h time period at steady-state. Plasma concentrations were simulated following an oral dose using a one-compartment disposition model with the assumption of first order absorption, a model that has been widely used to describe warfarin pharmacokinetics [11,22]. Population pharmacokinetic estimates for *rac*-warfarin [22] and individual subject estimates of enantiomer half-lives [11] were employed in the simulations. The results indicated that a plasma sample taken at 12 ± 2 h after drug administration would provide concentration within 1 to 6% of the average plasma drug concentration observed over that dosage interval. This finding is in agreement with data presented by Holford [2]. The concentration-time profile during a dosage interval at steady-state shows a less than 2-fold concentration range because both warfarin enantiomers have relatively long half-lives (25 h for (S)-warfarin and 37 h

for (R)-warfarin [11,19]) when compared with the dosing interval (24 h). The elimination of warfarin metabolites is formation-rate limited [6] so the concentration of metabolite is virtually at steady-state with respect to parent drug. Therefore, the concentration of metabolite at 12 h after dose administration can be viewed as a good approximation of the average plasma metabolite concentration at steady-state.

Warfarin dose and enantiomer concentrations

The distribution of doses of *rac*-warfarin required to achieve therapeutic anticoagulation observed in the present study is in agreement with data presented by others [3,4,23]. Given that patients in the study group were taking a range of doses that varied 6-fold it is not surprising that a range of plasma enantiomer concentrations were observed (Figure 1). The steady-state plasma concentrations of (R)- and (S)-warfarin were in good agreement with steady-state serum concentrations in 10 patients reported by McAleer *et al.* [10], (R)-warfarin 0.61 ± 0.29 mg l^{-1} and (S)-warfarin 0.49 ± 0.23 mg l^{-1}, and in 5 patients reported by Chu & Wainer [24], (R)-warfarin 1.32 ± 0.52 mg l^{-1} and (S)-warfarin 0.26 ± 0.14 mg l^{-1}.

When plasma concentrations from the present study were corrected for dose a wide inter-patient variability remained (data not shown) indicating that dose alone is not the major factor contributing to the observed variability in warfarin enantiomer concentrations. Warfarin is rapidly and completely absorbed from the gastrointestinal tract [17] suggesting that incomplete bioavailability is an unlikely source of variability [25]. While every effort was made to ensure that patients were compliant with the prescribed warfarin dose regimen, it was not possible to exclude patient non-compliance as a potential source of variability.

Unbound fraction of warfarin enantiomers in patients

The protein binding of warfarin is known to be independent of concentration over a wide concentration range (up to 25 mg l^{-1}) [11]. In the present study the addition of 15 μg of *rac*-warfarin to each sample prior to protein binding determination should not alter the experimentally determined value of f*u*. The possibility that the observed inter-patient variability in unbound fraction may arise due to variability in the method was addressed using a control protein binding experiment. The small difference in f*u* observed in the control experiment (less than 1.4-fold) indicates that the wide differences observed in the 36 patients studied (2.8-fold for (S)-warfarin and 3.7-fold for (R)-warfarin) are due mainly to inter-individual differences in protein binding. The correlation observed between albumin concentration and the unbound fraction of each warfarin enantiomer and lack of correlation with total plasma protein concentrations are consistent with the observation that albumin is the major binding protein for warfarin in plasma [26]. This result is in contrast to the findings of Yacobi *et al.* [8] who reported that differences in the unbound fraction of *rac*-warfarin were unrelated to albumin concentrations in the patient population studied. However, the study of Yacobi *et al.* [8] assessed patient unbound serum concentrations after spiking samples with [^{14}C]-warfarin and subsequent equilibrium dialysis for 20 h. No check of radiochemical purity was reported. The significant correlation observed between the f*u* of (S)- and (R)-warfarin indicates that differences in albumin concentation could, in part, account for the variability in f*u*. However, the possibility that concomitantly administered drugs may alter protein binding cannot be discounted. Although no patient was taking any drug that is known to affect warfarin binding, the fraction unbound for both (R)- and (S)-warfarin did tend to be higher in the sub-group of patients taking concomitant medications.

Clearance of warfarin and metabolites

The observation that the mean enantiomeric ratio (S/R) is less than unity (0.55 ± 0.22) confirms the known stereoselective difference in the clearance of warfarin enantiomers [2,7,11,19,27]. The absolute values for the clearance and unbound clearance of warfarin enantiomers obtained in the present study would be affected by patient non-compliance, but not values of metabolite formation clearance or metabolite renal clearance. However, the mean estimates of clearance for warfarin enantiomers obtained in the present study are in good agreement with data presented by others following a single dose [2,7,11,19, 27] and during steady-state dosing [9,10]. Mungall *et al.* [22] identified patient weight as an important population covariate governing the observed variability in warfarin clearance. In the present study we have presented clearance values corrected to a weight of 70 kg to gain a better indication of the true variability in clearance across the study group.

The metabolism of (S)-warfarin to the inactive metabolite, (S)-7-hydroxywarfarin, is responsible for the termination of warfarin's anticoagulant activity [7]. In the present study there was wide variability in CL*u* for (S)-warfarin (533–4014 l day^{-1} per 70 kg). This variability is, in part, due to the variability in the metabolic activity of the enzyme system.

The calculation of formation clearance for each metabolite is dependent on the assumption of total urinary excretion of these compounds. This assumption appears reasonable considering that (R,S)- and (S,S)-warfarin alcohols are largely excreted unchanged when administered to man [28]. Similarly, conjugates of the 6- and 7-hydroxylated metabolites have not been found in the urine [11]. However, a small fraction of the hydroxylated metabolites is thought to undergo biliary excretion [7]. The absolute value of f*m* could be in error if patients were non-compliant, however the relative values for different metabolites would remain the same. The values of f*m* determined for each metabolite in patients at steady-state are in good agreement with data presented by Banfield *et al.* [11] for f*m* values determined in healthy subject following a single 1.5 mg kg^{-1} *rac*-warfarin dose. The sum of f*m* values should add to one, as shown by Toon *et al.* [27], but in the present

analysis this is not the case. This may be due to clearance of warfarin via metabolic pathways not examined in the present study [7,29] or to patient non-compliance.

Several reports have indicated that age influences warfarin dose requirements [4,22,23,29]. The results of the present study confirm this trend between patient age and dose. A population pharmacokinetic study by Mungall *et al.* [22] using data from 163 patients (age range; 18–77 years) demonstrated that the clearance of *rac*-warfarin decreases by approximately 1% with each year of age. Similarly, Shepherd *et al.* [30] found a 14% decrease in *rac*-warfarin clearance between the young and more elderly in a group of 26 subjects (range; 20–94 years). Bradbury *et al.* [9] compared the steady-state serum concentrations and clearances of (R)- and (S)-warfarin in a group of six young (31 ± 12 years) and eight elderly (80 ± 2 years) healthy volunteers. There was no difference in the serum concentrations of enantiomers or the clearance of (S)-warfarin between young and elderly subjects, but the clearance of (R)-warfarin was lower in the older subjects [9]. Our findings suggest that, although warfarin clearance is variable, there was no trend associated with patient age. The observation that older patients had a lower dose requirement and the lack of alteration in the steady-state pharmacokinetic parameters of warfarin is consistent with previous reports that elderly patients have a greater intrinsic sensitivity to anti-coagulant effects of warfarin [30,31]. Bradbury *et al.* [9] also reported that the ratio of *rac*-warfarin to warfarin alcohol tended to be lower in elderly subjects (1.32 ± 0.63) when compared with younger (2.51 ± 1.24) subjects. Results from the present study indicated no difference in the ratio of *rac*-warfarin to warfarin alcohol between elderly and young patients (overall mean 6.99 ± 7.61).

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