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The pharmacokinetics and pharmacodynamics of single dose (R)- and (S)-warfarin administered separately and together: relationship to *VKORC1* genotype

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- The contribution of (S)-warfarin to the clinical effect of *rac*-warfarin is well understood. The extent to which (R)-warfarin contributes to the clinical effect of *rac*-warfarin is unclear.

WHAT THIS STUDY ADDS

- Using unequivocally pure (R)- and (S)-warfarin we have demonstrated that (R)-warfarin contributes to the hypoprothrombinaemic effect of single large doses of warfarin.
- The extent of the interaction is dependent on *VKORC1* genotype.

AIMS

1) To determine the pharmacokinetics and pharmacodynamics of (R)- and (S)-warfarin given alone and in combination and 2) to determine whether the relative potency of (R)- and (S)-warfarin is dependent on *VKORC1* genotype.

METHODS

A three way crossover study was conducted in which 17 healthy male subjects stratified by *VKORC1* 1173 C>T genotype and all *CYP2C9* 1*/1* received (R)-warfarin 80 mg, (S)-warfarin 12.5 mg and *rac*-warfarin sodium 25 mg. Plasma (R)- and (S)-warfarin unbound and total concentrations and prothrombin time were determined at multiple time points to 168 h.

RESULTS

Pharmacokinetic parameters for (R)- and (S)-warfarin were similar to the literature. (R)-warfarin 80 mg alone resulted in a mean AUC_{PT} (0,168 h) of 3550 s h (95% CI 3220, 3880). *Rac*-warfarin sodium 25 mg containing (S)-warfarin 11.7 mg produced a greater effect on AUC_{PT} (0,168 h) than (S)-warfarin 12.5 mg (mean difference 250 s.h, 95% CI 110, 380, $P < 0.002$) given alone. In a mixed effects model the ratio of response between (R)- and (S)-warfarin ($AUC_{PT((R)\text{-warfarin})}$: $AUC_{PT((S)\text{-warfarin})}$) was 1.21 fold higher (95% CI 1.05, 1.41, $P < 0.02$) in subjects of *VKORC1* TT genotype compared with the CC genotype.

CONCLUSIONS

(R)-warfarin has a clear PD effect and contributes to the hypoprothrombinaemic effect of *rac*-warfarin. *VKORC1* genotype is a covariate of the relative R/S potency relationship. Prediction of drug interactions with warfarin needs to consider effects on (R)-warfarin PK and *VKORC1* genotype.

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Introduction

Rac-warfarin has been the main drug used worldwide for the prophylaxis of thromboembolic disease. The clinical use of warfarin remains problematic due to the 10-fold range in dosage requirements between individuals [1–5], narrow therapeutic index, complex clinical pharmacology, multiple reported drug interactions and the potentially severe consequences of both under-anticoagulation and over-anticoagulation.

Multiple patient factors are known to influence the response to warfarin including age, weight, gender, smoking status, vitamin K intake and mutations in coagulation factors [1, 6]. Polymorphisms in both the *CYP2C9* and the *VKORC1* genes have been shown to have a large contribution to the interindividual variation in warfarin dose [3–5, 7]. Despite accounting for all of these genetic and non-genetic factors, only 60% of the variability in warfarin dose requirements in Caucasians can be explained, suggesting other factors need to be considered.

One of the major barriers to the wider clinical use of warfarin remains the real and perceived risk of drug–drug interactions. In a systematic review by Holbrook *et al*. [8], over 120 drugs had been reported to be implicated in clinically significant interactions with warfarin. Anthony *et al*. [9] assessed three drug information compendia and the warfarin sodium (Coumadin™) USA product label for information regarding substances that interact with warfarin and found 648 entries from the four sources, with only 50 being common to all. Given that the majority of interactions with warfarin are of a pharmacokinetic basis, it should be possible to use a mechanism-based approach to predict the frequency and intensity of a particular drug interacting when used with warfarin. However the majority of interactions have been detected through non-systematic approaches such as sporadic case reporting [8].

The pharmacokinetics and pharmacodynamics of (R)- and (S)-warfarin differ. Both enantiomers are fully bioavailable [6] and hence it is variability in clearance which determines variability in exposure. The clearance of (S)-warfarin is almost exclusively (>85%) by CYP2C9 which catalyzes the 6- and 7- hydroxylation reactions [10], with (S)-7-hydroxywarfarin being the predominant metabolite [11]. The clearance of (R)-warfarin is more complicated, with the formation of 6-, 7-, 8- and 10-hydroxylated metabolites and reduction to an alcohol all contributing [12]. CYP1A2 is the principal enzyme catalyzing 6-hydroxylation (71%) but is minor for 7- and 8-hydroxylation [10]. CYP2C19 is the principal enzyme catalyzing 8-hydroxylation but is minor for 6- and 7-hydroxylation and CYP3A4 is exclusively responsible for 10-hydroxylation [10].

Several decades ago O'Reilly *et al*. [13] administered single doses of 1.5 mg kg^{-1} of separate (R)- and (S)-warfarin to 10 healthy volunteers. They found that (R)-warfarin had a lower apparent total clearance than (S)-warfarin such that the area under the plasma concentration–time curve (AUC) of (R)-warfarin was 1.9-fold greater that the AUC of (S)-warfarin. However for the same dose, (S)-warfarin showed a 1.8-fold greater hypoprothrombinaemic effect than (R)-warfarin as measured by the area under the prothrombin–time curve (AUC_{PT}). This confirmed that both enantiomers had activity, but based on the relative AUC of the enantiomers and the hypothrombinaemic response, the estimated eudismic potency ratio of (R)-warfarin was approximately 3.4 times less than that of (S)-warfarin. Breckenridge and colleagues [14] titrated warfarin to a target INR (International Normalized Ratio, the standard reporting of prothrombin time) in healthy subjects with (R)-warfarin alone and (S)-warfarin alone using a steady-state crossover design and found an *in vivo* dose potency difference in humans of 1.6. This suggested that the dose potency of R : S warfarin was approximately 1.6:1 whereas the eudismic potency was approximately 4:1. These findings showed that although (S)-warfarin is the dominant species for action, the contribution of (R)-warfarin may be important. In addition these studies did not report on unbound plasma concentrations.

At variance with these findings, later studies in which the *in vivo* contribution of (R)-warfarin was sought from PK/PD [15] or population analyses [16] concluded that there was no significant contribution from (R)-warfarin and that the clinically significant biological effect was attributable to (S)-warfarin. Chan *et al*. [15] hypothesized that contamination of the allegedly pure (R)-warfarin by (S)-warfarin was a flaw in the previous studies [13, 14], despite the amount of contamination (8%) being insufficient to explain the results. These more recent studies have led to the widely held view that differences in the metabolism of (R)-warfarin, or drug interactions affecting (R)-warfarin metabolism, are unlikely to be clinically significant. However, of the long list of interactions with warfarin, many of the drugs are known to inhibit enzymes only responsible for the metabolism of (R)-warfarin. Hence it is plausible that these clinically significant interactions are mediated through the inhibition of (R)-warfarin metabolism.

The pivotal studies establishing the dose and concentration potency of warfarin enantiomers were completed several decades ago, prior to our current understanding of pharmacogenomics and ability to genotype, in small groups of subjects who were likely to be relatively homogeneous. On the basis of these studies, the potential significance of variability in (R)-warfarin metabolism both genetic and drug-related has been largely ignored or dismissed. This is despite numerous drugs (for example erythromycin, cimetidine, esomeprazole, fluvoxamine, quinolones, rosuvastatin [17]) being reported as potentially interacting with warfarin in the product information or case reports, where an interaction mediated via alteration in (R)-warfarin pharmacokinetics is more likely than alteration in (S)-warfarin pharmacokinetics. In addition, while it is clearly established that genotype of *VKORC1* determines

pharmacodynamic response to *rac*-warfarin, it is not known if there is an interaction between *VKORC1* genotype and relative R : S potency. This would not necessarily be anticipated as the most clinically important *VKORC1* genotypes result from the polymorphisms −1639G>A and 1173C>T occurring in the gene promoter and intron 1 of the gene respectively. These polymorphisms result in altered gene expression and are not known to affect the final protein structure [18–20]. There is strong linkage disequilibrium between these two polymorphisms [21].

These discrepant results may be partly attributable to heterogeneity in the clinical population with regard to the relative potencies of the enantiomers and heterogeneity in metabolic pathways. We aimed to examine the pharmacokinetics and pharmacodynamics of *rac*-warfarin and its R- and S-enantiomers, given individually, in subjects of different *VKORC1* genotypes.

Methods

Participants

The intention was to recruit 18 healthy male participants between the ages of 18 and 45 years. All participants were genotyped for *CYP2C9* *1, *2 and *3 and only *CYP2C9* *1/*1 genotype subjects were included. By design there were to be equal distributions of homozygous wild-type, heterozygous and homozygous-variant for *VKORC1* genotype (1173 C>T). Participants were healthy with normal ECG and calculated creatinine clearance by the Cockcroft–Gault method >80 ml min^{-1}. Exclusion criteria were current illicit drug use, history of gastrointestinal bleeding, any regular medication use, ingestion of aspirin within 7 days of a study period or NSAIDs within five half-lives of any treatment period, alcohol consumption >two standard drinks a day on average and or more than four standard drinks in 1 day, current smoking, engaged in occupation or recreation which is at high risk of physical injury or trauma during the study period, BP>140/90 mmHg, abnormal fundoscopic examination, abnormal urinalysis, abnormal INR/APTT or platelet count at screening, history of bleeding disorders, clotting disorders or multiple miscarriage in first degree relatives or exclusively vegetarian diet.

Warfarin

To obtain pure (R)- and (S)-warfarin ($C_{19}H_{16}O_4$, MW 308.33), *rac*-warfarin was synthesized using the method of Bush & Trager [22]. The racemic mixture was resolved by the method of West *et al*. [23] and checked for purity by melting point, optical rotation and h.p.l.c. [24] by the Chemistry Department of the University of Adelaide, Australia. (R)- and (S)-warfarin purity was confirmed as >99% pure with no evidence of cross enantiomer contamination. Pure (R)- and (S)-warfarin were prepared as a 50 ml suspension for dosing using the method of Sharley *et al*. [25]. For dosing *rac*-warfarin 25 mg of commercially available warfarin sodium (Coumadin™, Sigma Company Ltd, Clayton, Australia, $C_{19}H_{15}NaO_4$, MW 330.3) tablets (5 × 5 mg) were crushed and prepared as a 50 ml suspension for administration.

Clinical study procedures

The study protocol was reviewed and approved by the Royal Adelaide Hospital Research Ethics Committee, Adelaide, Australia. Written informed consent for participation was obtained from all participants before enrolment in the study. Study participants were asked not to make significant changes in their diet for the duration of the study. We performed an open label, three way crossover study where participants on three separate occasions received 25 mg *rac*-warfarin sodium, 12.5 mg (S)-warfarin and 80 mg (R)-warfarin. A fixed dose design was chosen as the key comparisons were within subject. Each drug dose was at least 21 days apart. For each period, participants attended the study centre after an overnight fast from food for 10 h and water for 4 h. Subjects were then dosed with the warfarin suspension followed by a 2 h fast. Blood samples ($n = 15 \times 10$ ml) were collected over 168 h for warfarin assay and prothrombin time at 0, 0.5, 1, 2, 4, 6, 8, 12, 24, 28, 72, 96, 120, 144 and 168 h. Subjects were directly questioned with regard to the occurrence of adverse events at these time points and at study closure. For the initial eight subjects enrolled in the protocol, dosing order was randomized. This was converted to open-label, fixed dose order (*rac*-warfarin 25 mg, (S)-warfarin 12.5 mg, (R)-warfarin 80 mg) when trace (R)-warfarin was detected in plasma following (R)-warfarin 80 mg despite at least a 21 day wash out period.

Analysis

Venous blood samples for warfarin assay were centrifuged and plasma stored at −80°C. Total (R)- and (S)-warfarin concentrations were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS). The full details of the assay are reported elsewhere [26]. Briefly, 50 µl plasma was acidified with 5% formic acid and extracted by liquid–liquid extraction with methyl tertbutyl ether (MTBE) using d_6-warfarin (Cerilliant, Round Rock, TX, USA) as internal standard. The supernatant was evaporated and reconstituted in 100 µl 40% methanol. A 5 µl aliquot was injected onto a chiral 150 × 2.1 mm Chirobiotic V column (Astec, Supelco, USA) and eluted isocratically with 40% methanol/0.03% acetic acid. MS/MS detection was performed using negative mode electrospray ionization, monitoring the ion transitions *m/z* 307/161 for warfarin and 313/161 for d_6-warfarin, on an AB SCIEX 4000 Q TRAP mass spectrometer (AB Sciex, Foster City, Ca, USA). The assay was validated according to FDA guidelines [27]. Standard curves of 20–2000 µg l^{-1} of (R)- and (S)-warfarin were made by quadratic regression, weighted 1/X and $r^2 \geq 0.995$. Inter- and intra-day precision and inaccuracy were determined for the lower limit of quantification (LLOQ) of 20 µg l^{-1} and for

quality controls (QCs) at 50, 500 and 2000 μg l^{-1} ($n = 6$ at each level, each run). Interday coefficient of variation (CV) was <11% and bias was <13% ($n = 6$). Intraday CV was <4% and bias <8% ($n = 42$). Samples >2000 μg l^{-1} were diluted with blank plasma before analysis and samples <20 μg l^{-1} were considered below the limit of quantification. Both (R)- and (S)-warfarin concentrations were determined in all plasma samples regardless of dosing group in order to assess for contamination or evidence of chiral inversion.

Unbound warfarin concentrations were measured in triplicate on the 1 h samples by ultrafiltration [26]. In short, 500 μl plasma was ultrafiltered for 15 min at 32°C and 2000 *g* using Centrifree 30 K ultrafiltration devices (Millipore, Bedford, MA, USA). There was no evidence of non-specific binding to the devices. Ultrafiltrate samples (100 μl) were acidified with 5% formic acid and extracted by liquid–liquid extraction with MTBE using d_6-warfarin as internal standard. The supernatant was evaporated and reconstituted in 100 μl 40% methanol before injection of 10 μl onto the chiral Chirobioitc V column and detected by MS/MS as for total concentrations. Standard curves were made in spiked ultrafiltrate by quadratic regression, weighted 1/X with $r^2 \geq 0.998$. The analytical range was 1–20 μg l^{-1}. LLOQ was 1 μg l^{-1} and QCs were 2.5, 10 and 20 μg l^{-1}. CV was <10% and bias was <9% ($n = 6$ at LLOQ and each QC level). Samples >20 μg l^{-1} were diluted with blank ultrafiltrate before LC/MS analysis. Outliers from triplicate analysis were excluded based on deviation from mean >20%.

Genomic DNA was extracted using a QIAamp DNA mini kit (Qiagen, Australia). The *CYP2C9* variants *2 and *3 were classified by detection of 430C>T (R144C, rs1799853) and 1075A>C (I359L, rs1057910), respectively, using Taqman Drug Metabolism Genotyping Assays and Taqman universal PCR master mix with Amperase UNG (Applied Biosystems, Australia) as per manufacturer's instructions. The *VKORC1* 1173C>T variant (C6484T, rs 9934438) was detected using a previously published real-time PCR method with minor modifications [28]. Control samples for all assays were confirmed by direct DNA sequencing.

Prothrombin time (s) was determined by STAR Evolution Coagulation Analyzer, (Stago, Doncaster, Australia) (SA Pathology). The within run precision of this automated testing is a CV of <3%.

Pharmacokinetics

The pharmacokinetic parameters AUC(0,∞), t_{max}, C_{max}, $t_{1/2}$ for (R)- and (S)-warfarin were calculated by noncompartmental analysis using PKSolver [29] in Microsoft Excel. Apparent total oral clearance was calculated as dose/AUC(0,∞) for R- and S-warfarin. For *rac*-warfarin the dose of each enantiomer of warfarin administered was considered to be 11.67 mg after accounting for the molecular weight of the salt. AUC(0,∞) for the unbound fraction ($AUC(0,\infty_{unbound})$) for (R)- and (S)-warfarin was approximated by multiplying the fraction unbound (f_u) at 1 h by AUC(0,∞). Unbound clearance (CL_u) was subsequently estimated using $AUC(0,\infty_{unbound})$.

Pharmacodynamic response

The pharmacodynamic response was calculated as the AUC of prothrombin time from 0 to 168 h (AUC_{PT}) calculated using the linear trapezoidal rule. The response was also assessed as the AUC of change in prothrombin time after subtraction of PT at time 0 (AUC_{PTADJ}). In addition the ratio of response to (R)-warfarin 80 mg was compared with (S)-warfarin 12.5 mg with regard to *VKORC1* genotype using a mixed model approach.

Statistical analysis

Measurements from the same participants were compared by paired *t*-tests for normally distributed data. Comparisons between participants were made by unpaired *t*-tests or one way ANOVA. For the pharmacodynamic response repeated measures ANOVA was used to compare the AUC_{PT} response. The data were analyzed using GraphPad Instat version 3.00 for Windows (GraphPad Software, San Diego, California USA, http://www.graphpad.com). $P < 0.05$ was considered significant. A mixed model approach accounting for treatment period, sequence and genotype was used to examine the interaction between *VKORC1* genotype and the relative potency of (R)- and (S)-warfarin (SAS 9.2, SAS Institute Inc., Cary, North Carolina USA) as reflected in $AUC_{PT((R)\text{-warfarin})} : AUC_{PT((S)\text{-warfarin})}$. Data are expressed as ratios with 95% confidence intervals.

Results

Seventeen male participants of mean age 26 (range 17–41) years and mean weight 76 (range 55–105) kg were recruited into the study of whom six participants were *VKORC1* CC (homozygous wild-type), six participants *VKORC1* CT (heterozygotes) and five were *VKORC1* TT (homozygous variant). Baseline demographic characteristics are summarized in Table 1. Subjects with CT genotype were older than those recruited with CC or TT genotype ($P < 0.02$). The weight of subjects with TT genotype was less

Table 1

Baseline demographic characteristics of participants stratified by *VKORC1* genotype (1173C>T)

VKORC1 genotype	*n*	Age (years) (range)	Weight (kg) (range)
CC	6	23 (17–26)	82 (74–91)
CT	6	33 (23–41)*	81 (56–105)
TT	5	23 (19–27)	61 (55–70)*

*$P < 0.02$.

than those recruited with the CC or CT genotype ($P < 0.02$). Adverse events reported during the trial were all minor and were not attributed to study drug. There were no serious adverse events and no instances of bleeding. There was no difference in tolerability of study drug on the three dosing occasions.

Pharmacokinetics

There was no evidence of contamination or chiral inversion in the plasma sample analysis. The pharmacokinetic parameters from the three dose groups are summarized in Table 2. The exposure to (S)-warfarin 12.5 mg administered alone was greater than the exposure to (S)-warfarin 11.7 mg administered as *rac*-warfarin. The ratio of total $AUC_{(S)\text{-warfarin alone}}$: total $AUC_{(S)\text{-warfarin racemic}}$ was 1.12 (95% CI 1.01, 1.24). When approximating unbound $AUC_{(S)\text{-warfarin alone}}$: unbound $AUC_{(S)\text{-warfarin racemic}}$ the ratio was similar at 1.14 (95% CI 0.96, 1.32).

Total (S)-warfarin clearance was higher than total (R)-warfarin clearance (difference 131 ml h^{-1}, 95% CI 91.0, 172, $P < 0.0001$). Similarly unbound (S)-warfarin clearance was higher than unbound (R)-warfarin clearance (difference 25 l h^{-1}, 95% CI 16, 33, $P < 0.0001$). The mean difference between unbound (S)-warfarin clearance administered alone or as racemate was not significantly different (0.1 l h^{-1}, 95% CI –6.0, 2.6). Similarly, the mean difference in unbound (R)-warfarin clearance when administered alone or as a racemate was not significantly different (0.5 l h^{-1}, 95% CI –3.4, 4.4).

Pharmacokinetics by genotype for the pure (R)-warfarin and (S)-warfarin are summarized in Tables 3A and 3B. There was no significant difference in apparent oral clearance of total or unbound (R)-warfarin or (S)-warfarin between *VKORC1* genotypes ($P = 0.48$ total (R)-warfarin, $P = 0.82$ total (S)-warfarin, $P = 0.88$ unbound (R)-warfarin, $P = 0.11$ unbound (S)-warfarin).

Trace (R)-warfarin concentrations were detected in four of the first eight subjects during the pure (S)-warfarin dosing period. These concentrations were all <0.06 µg ml^{-1} compared with C_{max} (R)-warfarin 80 mg 7.69 µg ml^{-1} and

Table 2

Pharmacokinetic parameters of (R)-warfarin and (S)-warfarin administered alone and in racemic combination ($n = 17$). Note effective dose of racemic (R)- and (S)-warfarin 11.7 mg due to administration as a salt. Data are mean (95% CI) or (range)

	C_{max} (95% CI) (µg ml^{-1})	t_{max} (range) (h)	AUC(0,∞) (95% CI) (µg ml^{-1} h)	$t_{1/2}$ (95% CI) (h)	CL/*F* (95% CI) (ml h^{-1})	% unbound warfarin (95% CI)	AUC(0,∞ unbound) (95% CI) (µg ml^{-1} h)	$CL_{unbound}/F$ (95% CI) (l h^{-1})
(R)-warfarin 80 mg alone	7.69 (6.78, 8.59)	2.4 (0.5–12)	504 (442, 569)	51.1 (45.9, 56.4)	168 (146, 189)	0.98 (0.86, 1.1)	4.8 (4.2, 5.5)	18 (15, 21)
(R)-warfarin 11.7 mg racemic	1.34 (1.17, 1.50)	0.9 (0.5 –2)	74.4 (64.9, 84.0)	49.3 (44.8, 53.9)	166 (145, 186)	0.99 (0.88, 1.1)	0.73 (0.62, 0.84)	17 (15, 20)
(S)-warfarin 12.5 mg alone	1.36 (1.20, 1.53)	0.8 (0.5–2)	44.5 (38.5, 50.6)	33.1 (29.7, 36.5)	299 (259, 340)	0.72 (0.65, 0.79)	0.32 (0.27, 0.37)	43 (36, 50)
(S)-warfarin 11.7 mg racemic	1.37 (1.20, 1.53)	0.9 (0.5–2)	40.1 (35.3, 44.8)	34.1 (30.3, 38.0)	306 (270, 343)	0.72 (0.66, 0.78)	0.29 (0.25, 0.32)	43 (38, 48)

Table 3A

Pharmacokinetics of (R)-warfarin given as a single 80 mg oral dose according to *VKORC1* genotype ($n = 17$). Data are mean (95% CI) or mean (range)

(R)-warfarin 80 mg alone	C_{max} (95% CI) (µg ml^{-1})	t_{max} (range) (h)	AUC (95% CI) (µg ml^{-1} h)	$t_{1/2}$ (95% CI) (h)	CL/*F* (95% CI) (ml h^{-1})	AUC(0,∞ unbound) (95% CI) (µg ml^{-1} h)	$CL_{unbound}/F$ (95% CI) (l h^{-1})
CC ($n = 6$)	7.97 (6.09, 9.85)	2.1 (0.5–8)	462 (354, 570)	53.0 (45, 61)	181 (134, 229)	4.4 (4.0, 4.9)	18 (16, 20)
CT ($n = 6$)	6.82 (5.17, 8.47)	3.5 (0.5–1)	542 (442, 643)	56.1 (50, 63)	151 (124, 179)	5.0 (3.8, 6.2)	17 (12, 21)
TT ($n = 5$)	8.39 (6.06. 10.7)	1.6 (0.5–6)	509 (295, 723)	42.9 (27, 59)	171 (103, 155)	5.1 (2.6, 7.6)	19 (8, 30)

Table 3B

Pharmacokinetics of (S)-warfarin given as a single 12.5 mg oral dose according to *VKORC1* genotype ($n = 17$). Data are mean (95% CI) or (range)

(S)-warfarin 12.5 mg alone	C_{max} (95% CI) (µg ml^{-1})	t_{max} (range) (h)	AUC (95% CI) (µg ml^{-1} h)	$t_{1/2}$ (95% CI) (h)	CL/*F* (95% CI) (ml h^{-1})	AUC(0,∞ unbound) (95% CI) (µg ml^{-1} h)	$CL_{unbound}/F$ (95% CI) (l h^{-1})
CC ($n = 6$)	1.25 (0.86, 1.63)	0.75 (0.5–2)	43.9 (28.3, 59.4)	34.8 (28.4, 41.2)	312 (202, 423)	0.32 (0.23, 0.40)	42 (31, 53)
CT ($n = 6$)	1.35 (1.13, 1.57)	1 (0.5–2)	43.3 (32.1, 54.6)	30.6 (26.7, 34.4)	301 (234, 369)	0.27 (0.18, 0.36)	51 (35, 67)
TT ($n = 5$)	1.51 (1.01, 2.02)	0.6 (0.5–1)	46.8 (32.9, 60.7)	34.2 (22.2, 46.1)	281 (193, 368)	0.39 (0.27, 0.51)	34 (23, 45)

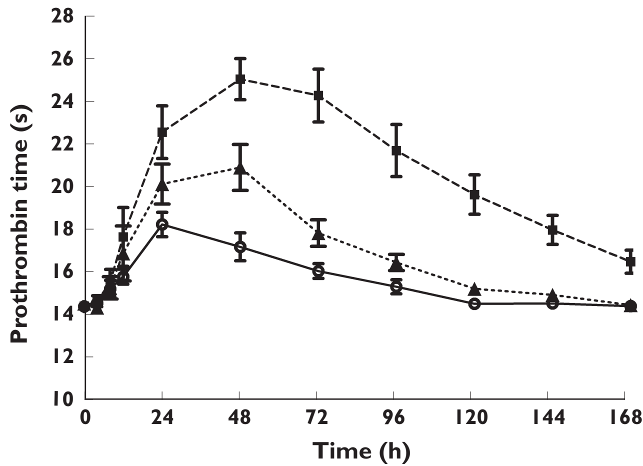

Figure 1

Mean (±SEM) prothrombin time (PT) response in healthy participants (*n* = 17) administered single dose (R)-warfarin 80 mg, (S)-warfarin 12.5 mg and *rac*-warfarin 25 mg. ■, (R)-warfarin 80 mg; ⊖, (S)-warfarin 12.5 mg; ▲, *rac*-warfarin 25 mg

Table 4

Prothromin time (PT) response by dose occasion for (R)-warfarin 80 mg, (S)-warfarin 12.5 mg and *rac*-warfarin 25 mg in 17 healthy subjects. Note effective dose of racemic (R)- and (S)-warfarin 11.7 mg due to administration as a salt

Dose	AUC_{PT} (s h) (range)	AUC_{PTADJ} (s h) (range)
R-warfarin	3550 ± 638 (2500–5350)	1120 ± 602 (198–2800)
S-warfarin	2650 ± 178 (2400–3080)	233 ± 154 (24–579)
Racemic warfarin	2900 ± 382 (2430–4020)	458 ± 369 (16–1570)

Data are mean ± SD (range). AUC_{PT} is area under the PT–time curve from time 0 to 168 h. AUC_{PTADJ} is area under the PT–time curve after adjustment for baseline PT from time 0 to 168 h.

C_{max} (S)-warfarin 12.5 mg 1.36 μg ml^{-1}. In subsequent subjects following the change to fixed dosing these residual concentrations of (R)-warfarin were no longer detected with administration of pure (S)-warfarin 12.5 mg.

Warfarin pharmacodynamics

All PTs had returned to the normal range (12–16 s) prior to beginning the next dosing period and there was no significant difference between baseline PT between dosing periods (P = 0.70). All three treatments produced a clear response–time profile (Figure 1, Table 4). The AUC_{PT} of *rac*-warfarin sodium 25 mg was 2900 s h (95% CI 2700, 3100) compared with the AUC_{PT} of (S)-warfarin 12.5 mg of 2650 s h (95% CI 2560, 2740), resulting in a mean difference of 250 s h (95% CI 110, 380, $P < 0.002$).

The pharmacodynamic response to (R)-warfarin 80 mg was greater than the response to either *rac*-warfarin (difference in AUC_{PT} 649 s h, 95% CI 502, 795, $P < 0.0001$) or

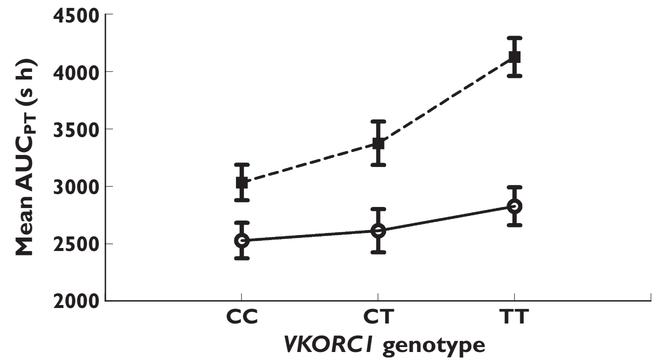

Figure 2

Mean (±SEM) AUC_{PT} response in participants (n = 17) administered single dose (R)-warfarin 80 mg and (S)-warfarin 12.5 mg by *VKORC1* (1173C>T) genotype. ■, (R)-warfarin 80 mg; ⊖, (S)-warfarin 12.5 mg

(S)-warfarin (difference in AUC_{PT} 895 s h, 95% CI 623, 1170, $P < 0.0001$). After subtracting for PT at time 0, *rac*-warfarin 25 mg demonstrated a 2-fold greater pharmacodynamic response over 168 h than (S)-warfarin 12.5 mg alone (mean difference in AUC_{PTADJ} 225 s h, 95% CI 78.0, 225, $P < 0.01$).

Mean response to (R)-warfarin 80 mg and (S)-warfarin 12.5 mg by *VKORC1* genotype is shown in Figure 2. Analysis of the interaction between *VKORC1* genotype and the relative potency of (R)- and (S)-warfarin on PT ($AUC_{PT((R)\text{-warfarin})} : AUC_{PT((S)\text{-warfarin})}$) was significant overall. Compared with the CC genotype, the ratio for TT genotype was 1.21-fold higher (95% CI 1.05, 1.41, $P < 0.02$). The ratio for the CT genotype was intermediate between those of CC and TT being 1.07-fold (95% CI 0.97, 1.18) higher than CC but 1.13-fold (95% CI 0.97, 1.32) lower than TT but these differences were not statistically significant (P = 0.11). There was no significant difference in mean response by sequence (P = 0.56) or period (P = 0.38).

Discussion

This is the first human study in which unequivocally pure (R)-warfarin demonstrated a therapeutic effect both alone and when administered with (S)-warfarin, replicating the findings of O'Reilly [13] and Breckenridge *et al*. [14] from three decades ago where the purity of warfarin was questioned.

The 80 mg dose of (R)-warfarin was intentionally chosen given doubt had previously been raised with regard to its potency [15, 16] and we wished to maximize our chances of achieving a pharmacodynamic response. (S)-warfarin 12.5 mg was given alone in order to ascertain the pharmacodynamic contribution of (R)-warfarin when *rac*-warfarin 25 mg was administered. The AUC_{PT} of *rac*-warfarin sodium 25 mg was greater than the AUC_{PT} of (S)-warfarin 12.5 mg confirming the contribution of (R)-

warfarin to the pharmacodynamic response of *rac*-warfarin. The greatest pharmacodynamic response was to (R)-warfarin 80 mg, attributable to the large dose.

Our findings are at variance with previous population pharmacokinetic studies such as those of Hamberg *et al*. [16] which have failed to detect concentrations of (R)-warfarin as significant covariates of the PT response. This discrepancy is likely to be due to the different methodology. We have used a carefully controlled crossover experiment in which (R)-warfarin was administered at two different doses which enabled a clear estimate of its effect. The analysis of Hamberg *et al*. came from populations where patients on potentially interacting concomitant medications were excluded, reducing the sensitivity to detect the effects of elevated concentrations of (R)-warfarin due to metabolic inhibitory concomitant medication. It is noted to be difficult in population analysis to detect the effect of a covariate in the presence of a related covariate with much larger effect. In the study of Kerbusch *et al*. [30], in which population PK/PD was used to estimate the *in vivo* potency of a metabolite, a situation analogous to that in the current study, it was noted that precision was highly dependent on a specific dataset in which the parent to metabolite ratios were remarkably different from the other studies, enabling signal detection. Hence the inability to detect (R)-warfarin as a significant covariate in population analysis should not be taken as evidence against the clear demonstration of effect in this study.

A potential criticism of the applicability of our study to the clinical use of warfarin is that it used single dose data rather than steady-state. However, well-accepted PK/PD models of warfarin [31] have incorporated single dose pharmacokinetics. Additionally in the population PK/PD study of Hamberg *et al*. [16], slightly more than half of the data came from single dose administration which was combined with data following multiple dose administration, confirming that both types of data are compatible and informative. Furthermore the agreement between single dose and multiple dose approaches with regard to the relative *in vivo* potency of (R)- and (S)-warfarin by O'Reilly [13] and Breckenridge *et al*. [14], respectively, suggests that our single dose design is appropriate to investigate relative clinical potencies.

A fixed dose design was selected as opposed to a body weight adjusted regimen as our principal comparison with regard to the relatively potency of (R)- and (S)-warfarin were within subject comparisons. Although the intention was to dose 25 mg *rac*-warfarin and 12.5 mg (S)-warfarin in order to achieve identical exposure to (S)-warfarin, different formulations of warfarin were used by necessity as we had limited quantities of pure (R)- and (S)-warfarin. The effective dose of (R)-warfarin and (S)-warfarin administered with racemic dosing from the commercial tablet was 11.7 mg. This resulted in approximately 10% less exposure to (S)-warfarin as determined by total AUC when it was administered in the racemate compared with the administration of 12.5 mg pure (S)-warfarin which is entirely accounted for by the use of warfarin sodium for racemic dosing. Some subjects showed delayed t_{max} when administered (R)-warfarin 80 mg, however drug exposure was dose proportional as reflected by the identical apparent oral clearance for both (R)-warfarin and (S)-warfarin when given alone or together. Despite the use of the two formulations, the pharmacokinetics of the enantiomers when given alone or together in this study were consistent with those reported in the literature [31]. Additionally, the apparent oral clearance estimates for each enantiomer were almost identical from each formulation, suggesting they were bioequivalent.

Pure (R)-warfarin administered alone showed a robust pharmacodynamic response. In addition, (R)-warfarin made a clear contribution to the activity of the racemate as exhibited by greater pharmacodynamic response to the racemate compared with the (S)-warfarin component given alone. The hypoprothrombinaemic effect of (R)-warfarin in *rac*-warfarin may in fact be underestimated in this study, given the exposure to (S)-warfarin in the racemic dosing was less than with (S)-warfarin alone and yet a clearly greater pharmacodynamic response resulted. This is contrary to the conventional wisdom that (R)-warfarin makes little contribution to the clinical effect of *rac*-warfarin.

For both enantiomers, *VKORC1* genotype was a determinant of response, with participants of TT genotype being both more sensitive to (S)-warfarin and relatively more sensitive to (R)-warfarin. Given that the effect size was modest and the sample size was small this needs to be interpreted with caution, but is potentially important as it suggests that patients with TT genotype might show disproportionately more anticoagulant response following co-administration of an inhibitor of (R)-warfarin metabolism. A mechanism by which *VKORC1* genotype alters the eudismic potency of the warfarin enantiomers is not readily apparent as the 1173 C>T mutation is in a non-coding part of the gene and hence is not expected to result in structural modification of the protein product, although may affect protein stability. Alternatively the mutation may be a marker for other genetic changes which affect the drug–protein interaction.

VKORC1 genotype had no apparent impact on either pharmacokinetic parameters including clearance, despite previous speculation that there may be an interaction between *VKORC1* genotype and clearance of warfarin enantiomers [32]. It has recently been demonstrated that polymorphisms in *CYP2C19* and *CYP3A4* do impact on (R)-warfarin clearance but we did not measure these in this study [33]. However any mutation reducing (R)-warfarin clearance without affecting (S)-warfarin clearance will increase the R : S dose potency ratio in those individuals.

Given the large number of drug interactions with warfarin, many of which are not readily explained by alterations in (S)-warfarin clearance (CYP2C9), alterations in

vitamin K production or pharmacodynamic factors this supports the contention that these interactions may be mediated through changes in (R)-warfarin clearance and thus enhanced pharmacodynamic effect. There is evidence to suggest that (R)-warfarin drug interactions are clinically significant, although there has been inconsistency when this has been formally examined. Given that typical warfarin interaction studies have small sample sizes, often 12 participants, this might be an explanation for case reports of clinically significant interactions occurring when prospective studies in volunteers have not shown a significant overall effect. For example, there are case reports of patients stable on warfarin who developed clinical overanticoagulation with co-administration of cimetidine [34], an inhibitor of CYP1A2 and CYP3A4. This was studied by O'Reilly [35] who examined the anticoagulant effect of 1.5 mg kg^{-1} *rac*-warfarin in combination with 1200 mg cimetidine and found it augmented both the hypoprothrombinaemic effects and blood concentrations of (R)-warfarin, a clinically significant result. Later, Toon *et al*. [36] found that in 12 healthy male volunteers, 800 mg cimetidine caused an approximately 20% reduction in the clearance of (R)-warfarin with no effect on (S)-warfarin and no effect on INR. Toon *et al*. concluded that this interaction would not have clinical consequences. The relative contribution of (R)-warfarin to drug–drug interactions may be dependent on *VKORC1* genotype and hence drug–drug interaction studies where (R)-warfarin is a possible suspect may be falsely reassuring unless *VKORC1* genotype has been taken into account.

This study has established the basics of the PK/PD relationship of the individual enantiomers of warfarin in man. From these data it will be possible, using PK/PD modelling combined with *in vitro* drug metabolism data to simulate the likely frequency and extent of pharmacokinetic drug–drug interaction with warfarin in a population. Another important finding from this study is that in addition to its known effect on warfarin dose requirements, *VKORC1* genotype may be a determinant of the relative contribution of (R)-warfarin to the clinical effect of warfarin and thus to drug–drug interactions. Hence such modelling should include *VKORC1* genotype as a potential covariate, in addition to genotypes for relevant drug metabolizing polymorphisms. (R)-warfarin has anticoagulant activity and cannot be dismissed as having no relevance.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare this work has been funded internally by the Discipline of Pharmacology, University of Adelaide. No support from any external organization has been received for the submitted work. There are no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years. There are no other relationships or activities that could appear to have influenced the submitted work.

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