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The Disposition of The Enantiomers of Warfarin Following Chronic Administration to Rats: Relationship to Anticoagulant Response

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Abstract—A model of chronic anticoagulation has been used to investigate the whole liver and subcellular disposition of the individual enantiomers of warfarin in the Wistar rat in relation to anticoagulant response. Consistent pharmacodynamic responses were achieved by dosing daily with *R*-warfarin (0·4, or 0·8 mg kg^{-1} day^{-1} i.p.) or *S*-warfarin (0·1 mg kg^{-1} day^{-1} i.p.). After the administration of increasing doses of *R*-warfarin, prothrombin times were dose-dependent (16·3 ± 0·5 s, 0·1 mg kg^{-1} day^{-1}; 21·6 ± 1·7 s, 0·4 mg kg^{-1} day^{-1}; 55·1 ± 9·0 s, 0·8 mg kg^{-1} day^{-1}; results all measured 24 h after the final dose). Increasing doses of *R*-warfarin also produced increases in plasma, whole liver, and cytosolic concentrations of warfarin. However, there were no significant differences between the microsomal concentrations of *R*-warfarin in the three groups. The dose of *S*-warfarin required to produce a consistent and significant increase in the prothrombin time was four-fold lower than the dose of *R*-warfarin required to cause a similar effect. Plasma concentrations of *S*-warfarin were not significantly different from those seen after 0·4 mg kg^{-1} day^{-1} *R*-warfarin. Whole liver and cytosolic concentrations of the *S*-enantiomer were lower than those observed after a dose of 0·4 mg kg^{-1} day^{-1} *R*-warfarin. However, consistent with microsomal concentrations following increasing doses of *R*-warfarin, there was no significant difference between microsomal concentrations of *R*(+)- and *S*(−)-warfarin. Thus, in contrast to plasma, whole liver or liver cytosol, microsomal concentrations of warfarin do not appear to be related to either dose or pharmacological response. Our results appear consistent with the possibility of there being a saturable binding site in the microsomal fraction of the liver, with a capacity of approximately 4·0 $\mu g\ g^{-1}$ protein.

Four-hydroxycoumarin anticoagulants inhibit the postribosomal γ-carboxylation of vitamin K_1-dependent clotting factors II, VII, IX, and X, by inhibition of the microsomal enzymes vitamin K_1 epoxide reductase, and vitamin K_1 quinone reductase (Bell & Matschiner 1972; Whitlon et al 1978). Warfarin, which is the most common anticoagulant used in clinical practice, exists as two distinct stereoisomers: *R*(+)- and *S*(−)-warfarin. In both man and rat, the *S*-enantiomer is a more effective inhibitor of clotting factor synthesis than *R*-warfarin, yet the half-lives of the two enantiomers differ significantly between species (Breckenridge et al 1973; Breckenridge & Orme 1972; O'Reilly 1974; Wingard et al 1978). For example, in rats, *S*-warfarin has a longer half-life and is a more effective anticoagulant, but in man, although *S*-warfarin has the shorter half life it is a more potent anticoagulant than the *R*-enantiomer. Moreover, Fasco & Principe (1982) showed that when either *R*- or *S*-warfarin were administered to rats in-vivo at doses which produced comparable plasma warfarin concentrations, the *S*-enantiomer was a more potent inhibitor of the microsomal vitamin K_1 epoxide cycle than the *R*-enantiomer, but when warfarin was added directly to microsomal preparations in-vitro, there was no difference between the inhibitory activities of the two enantiomers. Thus, the differences in potency between the enantiomers cannot entirely be explained by different rates of metabolism, and one explanation for the apparent disparity between the available data is that the hepatic disposition of warfarin may be stereoselective.

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In order to investigate this possibility, we have now examined the disposition of the two enantiomers of warfarin under conditions in which the pharmacodynamic actions were constant and comparable. We have established a model that was based on the daily administration of either *R*- or *S*-warfarin in doses which produced consistent levels of anticoagulation in the Wistar rat. Using this model it was possible to compare the pharmacodynamic effects of warfarin (measured by changes in prothrombin time; PT), with the concomitant plasma concentrations of the drug. Furthermore, it was also possible to measure, and thereby compare, whole liver and the hepatic subcellular distribution of warfarin with the measured pharmacodynamic responses.

Materials and Methods

Male Wistar rats (200–250 g), bred in the departmental animal house, had free access to both food (Labshore R 14) and water for the duration of the experiment. Drugs used were *R*(+)- and *S*(−)-warfarin, which were gifts from Ward Blenkinsop, Widnes. All reagents were obtained from BDH (Poole, UK), except rabbit brain thromboplastin, which was purchased from Manchester Comparative Reagents (Manchester, UK). Solvents (HPLC-grade) were obtained from Fisons (Loughborough, UK).

Production of steady-state anticoagulation

Groups of rats (n = 3 in each experiment) were injected with

either *R*(+)- or *S*(−)-warfarin for at least 7 days. The doses of *R*-warfarin were 2·0, 1·0, 0·8, 0·4 and 0·2 mg kg^{-1} day^{-1} i.p. The doses of *S*-warfarin used were 0·4, 0·2 and 0·1 mg kg^{-1} day^{-1} i.p. Before the first dose of warfarin, and up to 10 days of dosing, rats were lightly anaesthetized with diethylether for the collection of blood from the tail artery. Blood (1·0 mL) was collected into 10% w/v trisodium citrate. This volume of blood was adequate for the measurement of prothrombin time and for the determination of plasma warfarin concentrations. Warfarin was extracted from plasma (100 μL) into methyl-tertiary-butyl ether (200 μL) after precipitation of the protein with hydrochloric acid (6M); concentrations were then measured by normal-phase high performance liquid chromatography (HPLC-NP). The standard curves derived from this assay were gradient = 1·17, intercept = 0·086, r = 0·98.

Intrahepatic disposition of R- *or* S-*warfarin during steady-state anticoagulation*

Rats were injected with either *R*-warfarin (0·1, 0·4, 0·8 mg kg^{-1} day^{-1} i.p.; n = 6 for each group), or *S*-warfarin (0·1 mg kg^{-1} day^{-1} i.p.; n = 6). These doses produced steady state levels of anticoagulation (see Results). Four days after the rats had reached steady state, and 20–24 h after the last dose of warfarin, blood (10 mL) was withdrawn by cardiac puncture during ether anaesthesia. The livers were then perfused in-situ with ice-cold phosphate buffer (1/15 M; pH 7·4) and homogenized. Liver microsomes, and liver cytosolic fractions were prepared by the method described by Maggs et al (1983) and were frozen (−20°C) until use.

Analysis of whole liver, cytosol and microsomal fractions was performed using HPLC-NP (flow rate 2·2 mL min^{-1} through a Spherisorb 5-nitrile column (25 cm × 4·5 mm i.d.) and Partisil 10 Si precolumn). Mobile phase used for plasma and cytosolic assays was 90% hexane: 5% propan-2-ol: 5% dichloromethane. For whole liver and microsomal assays, mobile phase composition was 85%: 10%: 5%. An aliquot of 1% glacial acetic acid was added to all mobile phases to suppress ionization of the hydroxyl moiety of warfarin and thereby prevent peak tailing. Detection of warfarin was by ultraviolet absorbance at 313 nm. For the extraction of warfarin from whole liver homogenate 1 mL homogenate was extracted into methyl-tertiary-butyl ether (2 mL), after precipitation of protein (6M HCl). Extraction was facilitated by tumbling for 20 min before centrifugation. A 50 μL aliquot of the supernatant was then injected directly onto the HPLC. Conditions for the extraction of warfarin from liver cytosol and liver microsomes were identical, except that after centrifugation, the supernatant was evaporated to dryness under vacuum, and the residue redissolved in 200 μL methyl-t-butyl ether. A 50 μL aliquot of this solution was then analysed. Concentrations of warfarin were derived from peak-height ratios using the internal standard acenocoumarol. Concentrations of warfarin in cytosol and microsomes are expressed per mg of cytosolic or microsomal protein; protein concentrations were determined by the method of Lowry et al (1951). Standard curves derived for whole liver were gradient = 1·71, intercept = 0·00, r = 0·99, for liver cytosol were gradient = 1·27, intercept = 0·09, r = 0·99, and those from liver microsomes were gradient = 1·19, intercept = 0·03, r = 0·99.

Results

Production of steady-state anticoagulation

Resting prothrombin times were 18·4 + 0·18 s (PCA 100%). Daily dosing with *R*-warfarin produced marked increases in prothrombin times (74·6 ± 14·1s, 2 mg kg^{-1} day^{-1}; 179·3 ± 21·1s, 1 mg kg^{-1} day^{-1}, on day 3 and day 10, respectively), which corresponded to a PCA of less than 5% of normal, indicating almost complete inhibition of clotting factor synthesis. The marked anticoagulant effect in these animals was accompanied by significant ($P < 0·05$) increases in plasma concentrations of warfarin (2·78 ± 0·5 μg mL^{-1}, 2·0 mg kg^{-1} day^{-1}; 2·53 ± 0·2 μg mL^{-1}, 1 mg kg^{-1} day^{-1}). No significant anticoagulant effect was observed over the 10 day period with *R*-warfarin 0·2 mg kg^{-1} day^{-1} despite being detected in the plasma of these animals (0·22 ± 0·08 μg mL^{-1}).

Three days of dosing with the *S*-enantiomer (0·4 mg kg^{-1} day^{-1}) produced a large increase in prothrombin time (141·2 ± 34·5 s; PCA < 5% of normal). Half this dose caused a smaller change (prothrombin time 39·6 ± 4·8 s; PCA 19·0 ± 3·4% of normal) over the first 5 days, after which there was a profound increase in prothrombin time (146·5 ± 11·5 s; PCA < 5%). These changes in PCA were accompanied by corresponding increases in plasma warfarin concentrations (1·01 ± 0·11 μg mL^{-1}, 0·4 mg kg^{-1} day^{-1}; 0·33 ± 0·2 μg mL^{-1}, 0·2 mg kg^{-1} day^{-1}).

Ongoing and consistent anticoagulation was achieved at doses of 0·4 and 0·8 mg kg^{-1} day^{-1} of the *R*-enantiomer. A dose of 0·4 mg kg^{-1} day^{-1} produced an increase in prothrombin time (21·2 ± 2·1 s), and a fall in PCA (44·3 ± 6·4%; Fig. 1). The change in prothrombin time and PCA was maintained throughout the experiment, and was accompanied by stable plasma warfarin concentrations (Fig. 1). PCA and plasma warfarin concentrations were more variable following administration of the higher dose of *R*-warfarin. However, the responses remained consistent, and did not result in the ultimate development of the marked anticoagulant response seen at greater doses.

The dose of *S*-warfarin required to produce significant and stable anticoagulation was 0·1 mg kg^{-1} day^{-1} (Fig. 1; PT 21·7 ± 2·2 s; PCA 35·5 ± 2·8%) This was lower than that required to produce consistent anticoagulation with the *R*-enantiomer. We occasionally found that dosing with *S*-warfarin revealed animals that were extremely sensitive to the effects of *S*-warfarin, and as a consequence of its administration developed an abrupt and profound hypoprothrombinaemia, irrespective of the dose used. Animals that developed this marked response were not used in the remaining studies.

The intrahepatic disposition of R(+)- *and* S(−)-*warfarin during steady-state anticoagulation*

These results were produced in a separate group of animals to those described above. Increasing doses of *R*-warfarin produced significant ($P < 0·05$) increases in the plasma concentrations of the enantiomer (Table 1). Likewise, there were significant ($P < 0·05$) increases in both liver homogenate and liver cytosolic concentrations of warfarin (Table 1). However, although the concentration of *R*-warfarin in liver microsomes was slightly lower after 0·1 mg kg^{-1} day^{-1} than

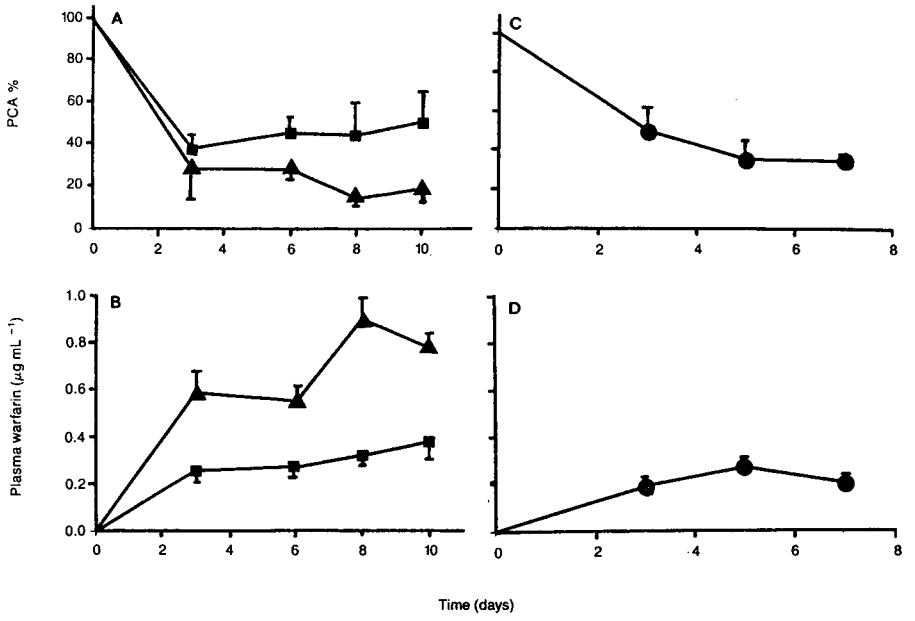

FIG. 1. Prothrombin complex activity (PCA; %) and plasma warfarin concentrations ($\mu g\ mL^{-1}$) against days of dosing for *R*(+)-warfarin (A,B) and *S*(−)-warfarin (C,D) after 0·8 (▲) and 0·4 (■) mg kg^{-1} day^{-1} *R*(+)-warfarin and 0·1 (●) mg kg^{-1} day^{-1} *S*(−)-warfarin.

Table 1. Concentrations of *R*(+)- and *S*(−)-warfarin in plasma, whole liver homogenate, liver cytosol, and liver microsomes (n = 6) as measured by HPLC following seven daily administrations of the enantiomers. Warfarin concentrations are compared with prothrombin times.

Dose	[dose] (mg kg^{-1} day^{-1})	[plasma] ($\mu g\ mL^{-1}$)	[whole liver] (μg g liver^{-1}	[cytosol] ($\mu g\ g^{-1}$*)	[microsomal] ($\mu g\ g^{-1}$*)	PT (s)
R-Warfarin	0·1	0·08±0·00	0·8±0·1	0·5±0·0	2·8±0·8	16·3±0·5
R-Warfarin	0·4	0·3±0·04	1·3±0·04	2·7±0·24	3·9±0·5	21·6±1·7
R-Warfarin	0·8	0·6±0·05	2·3±0·2	5·5±0·48	4·0±0·5	55·1±9·0
S-Warfarin	0·1	0·2±0·02	0·9±0·1	0·9±0·24	3·9±0·33	19·7±0·6

* Protein

after the higher doses of warfarin, there were no significant differences between the microsomal concentrations of *R*-warfarin in the 3 groups.

Administration of *S*-warfarin (0·1 mg kg^{-1} day^{-1}) resulted in plasma levels of warfarin not significantly different from those seen after a dose of 0·4 mg kg^{-1} day^{-1} *R*-warfarin. Concentrations of *S*-warfarin in whole liver homogenate and cytosol were significantly lower than after *R*-warfarin ($P < 0·05$). In contrast to these data, concentrations of *S*-warfarin in liver microsomes were not different from those seen after the three doses of *R*-warfarin.

Discussion

Coumarin anticoagulants are used clinically for either primary or secondary treatment of thromboembolic and vascular disease. They are prescribed for long-term use in doses below those required to produce complete inhibition of clotting factor synthesis. Studies in animals, however, frequently concentrate on the effects of administration of large single doses of coumarins on their site(s) of action, vitamin K_1-quinone reductase and epoxide reductase. In order to be able to relate pharmacodynamics and pharmacokinetics to the outcome of continued administration of warfarin, we have established a model of anticoagulation in rats in which prolonged clotting times can be maintained over a period of several days.

Warfarin has a single asymmetric centre, and exists as two separate enantiomers *R*(+)- and *S*(−)-warfarin; it was possible to establish a consistent anticoagulant response with each enantiomer of warfarin using the present model. The data derived from our initial studies are expressed both as prothrombin times and as PCA in order for direct effects of warfarin on clotting factor activity to be compared. Whilst the doses of *R*- and *S*-warfarin which produced stable and comparable levels of anticoagulation caused small increases in prothrombin time (2-4 s), this represented a fall in PCA to levels 50% of normal. Thus, small changes in prothrombin times represented quite marked changes in clotting factor synthesis.

The present studies showed that a controlled anticoagulant response was only achieved, during chronic administration, within a narrow dosing range (0·4-0·8 mg kg^{-1} day^{-1}) with the *R*-enantiomer, and at a single dose of the *S*-enantiomer. These observations are in agreement with the narrow therapeutic index of warfarin, and known difficulties in the establishment and maintenance of steady state warfarin in patients (Holford 1986). In agreement with previous

in-vivo studies (Eble et al 1966; Breckenridge & Orme 1972), we observed no significant difference between the consistent pharmacodynamic response achieved with *S*-warfarin compared with a four fold higher dose of the *R*-enantiomer. However, this relationship was not maintained when the dose of *S*-warfarin was doubled, since it was not possible to maintain consistent levels of anticoagulation with the greater dose (above). Since we could only establish controlled anticoagulation at one dose of the *S*-enantiomer, it would appear that the dose response curve for the action of this isomer was much steeper than that for *R*-warfarin, which may, in part, be due to differences in the mechanism of action of the enantiomers on vitamin K_1-epoxide reductase (Thijssen et al 1988).

Vitamin K_1 2,3-epoxide reductase is a microsomal enzyme, and since warfarin appears to bind covalently to the active site of the enzyme (Thijssen & Baars 1987), it could be predicted that the pharmacodynamic response to warfarin, as measured by prothrombin time, reflected the concentration of the drug at its site of action. In this study we found that consistent plasma, whole liver and liver cytosol concentrations of the enantiomers correlated well with prothrombin time, but that there was no correlation between microsomal concentrations of the enantiomers and pharmacodynamic response. Moreover, the microsomal concentrations of *R*- or *S*-warfarin were not different in any of the groups. Thus, while the microsomal concentration of *R*-warfarin was independent of dose, there was a progressive increase in anticoagulant effect. One explanation for this finding is that the microsomal concentrations measured in these experiments reflected not only binding to vitamin K-epoxide reductase itself, as suggested by Thijssen & Baars (1987), but to a secondary site within the microsomal fraction of the liver. The in-vivo stereoselective release of *R*(+)- or *S*(−)-warfarin from such a binding site may explain the apparent disparity between in-vitro and in-vivo inhibition of vitamin K_1 epoxide reductase by the enantiomers of warfarin (Fasco & Principe 1982). Alternatively, the level of inhibition of clotting factor synthesis could be related to cytosolic concentrations of warfarin given that we have found that both cytosolic concentrations and pharmacodynamic responses were dose dependent.

In conclusion, the relationship between the pharmacological response to, and the intra-hepatic disposition of warfarin has been determined after chronic administration of the individual enantiomers of the drug. It would appear that there is saturable binding of warfarin to the endoplasmic reticulum. However, this binding appears neither to be stereoselective nor directly related to inhibition of clotting factor synthesis. These data are consistent with the hypothesis that the endoplasmic reticulum contains more than one binding site for warfarin, only one of which is associated with clotting factor synthesis.

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