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Plasma half-lives, plasma metabolites and anticoagulant efficacies of the enantiomers of warfarin in man*

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S-(−)-Warfarin was found to be a more potent anticoagulant than *R*-(+)-warfarin in man. However, *S*-warfarin was cleared more rapidly from the plasma; respective mean plasma half-lives (from four subjects) for *R* and *S*-warfarin were 45·4 and 33·0h. Unlike the assay of Lewis, Ilnicki & Carlstrom (1970), the assay of Corn & Berberich (1967) for measuring plasma warfarin gave spuriously long half-life values, particularly with *R*-warfarin. The apparent volumes of distribution of the enantiomers were not significantly different. A major plasma metabolite detected was warfarin $alcohol_1$, which was seen in much greater quantities after giving *R*-warfarin than after *S*-warfarin. The corresponding diastereoisomer, warfarin $alcohol_2$, was seen in trace amounts after *S*-warfarin only.

The oral anticoagulant warfarin (1-(4′-hydroxy-3′-coumarinyl)-1-phenyl-3-butanone), as available for clinical and rodenticidal use, is a racemic mixture comprising two enantiomers. In normal and warfarin-resistant rats *S*-(−)-warfarin is a more potent anticoagulant (Eble, West & Link, 1966; Breckenridge & Orme, 1972; Hewick, 1972) and is cleared less rapidly from the plasma than *R*-(+)-warfarin (Breckenridge & Orme, 1972; Hewick, 1972). We have examined the anticoagulant potencies, plasma half-lives and plasma metabolites of the warfarin enantiomers in man.

After administration of racemic warfarin in man Lewis & Trager (1971) found three plasma metabolites. They were 7-hydroxywarfarin and two diastereoisomeric alcohols, warfarin $alcohol_1$ and warfarin $alcohol_2$ respectively. The warfarin alcohols are formed by the reduction of the ketone group of warfarin, with the creation of a second asymmetric centre in the molecule. Each diastereoisomer exists in two enantiomeric forms, namely *RS* and *SR* for warfarin $alcohol_1$ and *RR* and *SS* for warfarin $alcohol_2$†.

We have attempted to quantitate these plasma metabolites as well as plasma warfarin, after administration of the warfarin enantiomers. We have also compared the respective assays of Lewis, Ilnicki & Carlstrom (1970) and Corn & Berberich (1967) for measuring plasma warfarin and determining plasma warfarin half-lives. The latter assay has been reported to be non-specific for warfarin in the presence of its metabolites and may give spuriously long plasma warfarin half-lives (Lewis & others, 1970).

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† These absolute configurations have been reassigned according to Chan, K., Lewis, R. & Trager, W. (1972), *J. mednl Chem.*, **15**, 1265–1270. They differ from the tentative absolute configurations originally assigned by Lewis & Trager (1971).

MATERIALS AND METHODS

Volunteers

These comprised four normal subjects (two male, A and B; two female C and D) and six male patients (E, F, G, H, I and J). The subjects A, B, C and D weighed 70, 67, 41 and 54 kg respectively. The four patients (E, F, G and H) to receive the warfarin enantiomers had been receiving proprietary racemic warfarin (Marevan, BDH) daily for the previous six months but were scheduled to complete anticoagulant therapy within a few weeks. Patients I and J had been receiving Marevan daily for about two weeks and had stable thrombotest activities within the range 5–8 %.

Drugs

Racemic warfarin was purchased from Ward Blenkinsop & Co. Ltd. Warfarin $alcohol_1$ and warfarin $alcohol_2$ were a gift from Ward Blenkinsop & Co. Ltd. The warfarin enantiomers were prepared according to the method of West, Preis & others (1961). The melting points of the *R*-(+)-warfarin and *S*-(−)-warfarin were 177–8° and 176–8° respectively. The rotation of the *R*-(+)-warfarin was $[\alpha]_D^{20} + 152{\cdot}7°$ (*c* 2, 0·5N sodium hydroxide). The rotation of the *S*-(−)-warfarin was $[\alpha]_D^{20} - 151{\cdot}6°$ (*c* 2, 0·5N sodium hydroxide).

Administration of drugs

Racemic warfarin and the enantiomers, as the free acids in a lactose base, were dispensed in the form of hard gelatin capsules.

In one experiment, the normal subjects A, B, C and D received 100 mg single doses of either *R* or *S*-warfarin with 50 mg vitamin K_1 (Konakion, Roche). In a separate experiment subjects A and B received *R* or *S*-warfarin at 15 and 30 mg dose levels without the administration of vitamin K_1. Several weeks were allowed to elapse between each dose of warfarin so that blood coagulation had returned to normal and no warfarin remained in the plasma before a further dose of warfarin was given. In patients E, F, G and H our preparations of racemic, *R* or *S*-warfarin, each for a week consecutively, were substituted in identical dose for the previously taken Marevan. All drugs were given orally.

Blood collection and measurement of anticoagulant effect

Blood (9 ml) obtained by venepuncture was mixed with 1 ml trisodium citrate (0·106M). The coagulability of blood was monitored by means of thrombotest determinations, performed as directed by the suppliers (BDH Chemicals Ltd.) on whole blood.

Determination of plasma warfarin and metabolites

To obtain the plasma the blood was centrifuged at 3000 *g* for 10 min.

Plasma warfarin was measured by the assays of Corn & Berberich (1967) and Lewis & others, (1970). In the former assay, acetone is added to the plasma to precipitate the protein and extract the warfarin, and the fluorescence of the acetone solution is measured before and after acidification. There is a linear relation between the concentration of warfarin in acetone and the loss of fluorescence after acidification.

The method of Lewis & others (1970), which should be specific for unchanged warfarin, involves extracting the acidified plasma with ethylene dichloride and subject-

ing the organic extract to thin-layer chromatography using ethylene dichloride–acetone (9:1, by volume) on silica gel to separate unchanged warfarin. The warfarin is then eluted with acetone and the warfarin fluorescence measured as in the Corn & Berberich assay (1967). To obtain reproducible results we found it necessary to add one drop of ammonia (0·88) to the warfarin-acetone eluate before spectrophotofluorometry. Possibly this was necessary to completely neutralize any hydrochloric acid carried over from the earlier extraction procedure.

The assay of Lewis & others, (1970) was slightly modified to enable the warfarin alcohols to be assayed. After chromatography of the plasma in the ethylene dichloride-acetone system, the upper part of the dried chromatogram associated with the warfarin loci was cut off, while the lower part was rechromatographed in the solvent system cyclohexane–ethyl formate–formic acid (50:100:0·5, by volume) (Lewis & Trager 1971) to separate warfarin $alcohol_1$ from warfarin $alcohol_2$. The warfarin alcohols were located, eluted and assayed similarly to warfarin (Lewis & others, 1970). A linear relation was observed between the concentration of warfarin alcohol in plasma and the net fluorescence of acetone eluates of warfarin alcohol chromatographic loci. This linear relation held true for plasma samples containing from 0·2 $\mu g\ ml^{-1}$ (about the minimum concentration of warfarin alcohol that could be assayed) to at least 10 $\mu g\ ml^{-1}$. Three plasma samples containing warfarin alcohol 0·5, 1·5 and 2·5 $\mu g\ ml^{-1}$ respectively were each assayed three times in duplicate. The mean coefficient of variation was 3·1 % (range 2·3 to 3·7 %). Where very small amounts of warfarin alcohol(s) appeared to be present and it was difficult to visibly separate the two diastereoisomers, a larger area of the plate associated with both alcohols was eluted and "total alcohols" was calculated using $alcohol_1$ as standard. Fluorescence was measured using an Aminco Bowman spectrophotofluorometer. For warfarin and the warfarin alcohols the respective excitation and emission wavelengths were 345 and 405 nm. The relative fluorescence intensity of warfarin $alcohol_1$ was about 1·5 times that of $alcohol_2$ and about twice that of warfarin.

On each plate to be chromatographed, a standard extract was applied that had been prepared from plasma containing warfarin (2 $\mu g\ ml^{-1}$), warfarin $alcohol_1$ (1 $\mu g\ ml^{-1}$) and warfarin $alcohol_2$ (1 $\mu g\ ml^{-1}$). In addition a "blank" extract from plasma containing no drug was applied.

Test for conjugated metabolites in plasma

Plasma samples (4 ml) at pH 3·8 (acetate buffer 0·15M) were incubated for 4h at 37° with 60 000 units of β-glucuronidase enzyme containing some sulphatase activity (Sigma Type II). Control incubations containing no enzyme were also performed. The incubated plasma samples were then extracted and the warfarin and warfarin alcohols determined essentially as indicated previously.

Calculation of half-lives and apparent volumes of distribution

A warfarin plasma half-life was determined from the slope of the regression line of the semilogarithmic plot of the plasma warfarin concentration versus time after warfarin administration. To mask any variability in rates of absorption, data points obtained earlier than 24h were excluded from calculation of the slope. The regression line was fitted to the data by the method of least squares. The apparent volume of distribution (Vd) was obtained by extrapolation of the regression line to the zero-time

warfarin concentration (Cp°). Vd (as percentage of body weight) is the dose of drug administered (mg) multiplied by 100 and divided by Cp° (mg $litre^{-1}$) and bodyweight (kg).

RESULTS

Table 1 shows that in normal human subjects (unlike in rats) *S*-warfarin has a shorter plasma half-life than *R*-warfarin. The difference in half-lives between the enantiomers is more marked when using the assay of Corn & Berberich (1967) than when using that of Lewis & others (1970). This is mainly due to the higher half-life values obtained for *R*-warfarin using the former assay. The apparent volumes of distribution of the enantiomers ranged from about 8 to 12% of body weight. There was no significant difference between the apparent volumes of distribution of the two enantiomers.

Under the conditions used, thin-layer chromatographic analysis of plasma samples revealed apparently only warfarin alcohols as metabolites. Incubation of the plasma samples with β-glucuronidase enzyme possessing some sulphatase activity did not increase the amount of free warfarin or warfarin alcohols, indicating the absence of glucuronide and probably sulphate conjugates.

Fig. 1 shows the plasma concentrations of warfarin and warfarin alcohols at various times after administration of *R* or *S*-warfarin to subject B. *R*-Warfarin administration was associated with only warfarin $alcohol_1$ in the plasma. The alcohol reached a maximum plasma concentration of about 2·6 μg ml^{-1} about 36h after R-warfarin administration. Four days after giving the drug the concentration of $alcohol_1$ was still about 1·8 μg $ml.^{-1}$ *S*-Warfarin administration was associated with much smaller amounts of warfarin $alcohol_1$ in the plasma. It was difficult to be sure, but traces of $alcohol_2$ may have been present, therefore "total alcohols" were estimated. The "total alcohols" reached a maximum plasma concentration of

Table 1. *Comparison of the plasma half-lives of the enantiomers of warfarin in volunteers using the assays of Lewis & others (1970) and Corn & Berberich (1967).* The subjects received 100 mg single doses of either *R* or *S*-warfarin with 50 mg vitamin K_1. The drugs were given orally. Plasma warfarin concentrations were determined daily for 5 or 6 days. The concentration measured on the first day was excluded from the half-life calculation.

Subject	Enantiomer	Apparent volume of distribution* (% body weight)	Half-life (h) as determined by the assay of Lewis & others (1970)	Half-life (h) as determined by the assay of Corn & Berberich (1967)
A	*R*	11·5	64·2	78·8
	S	10·4	51·6	51·9
B	*R*	11·5	34·9	48·0
	S	11·5	28·8	28·3
C	*R*	10·6	45·0	61·2
	S	12·25	26·0	29·6
D	*R*	7·9	37·5	43·9
	S	9·3	23·5	27·8

* Calculated from zero-time plasma warfarin concentrations determined from data using the assay of Lewis & others (1970).

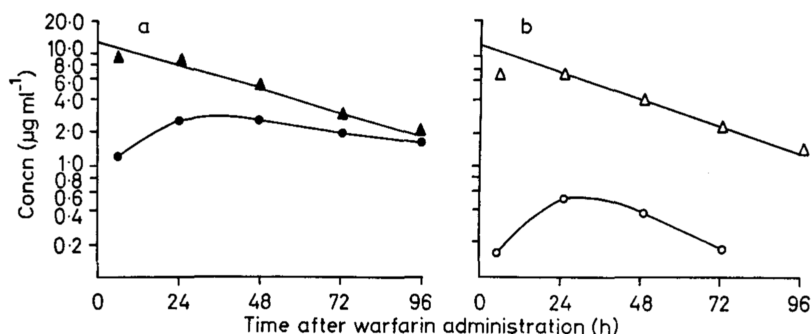

FIG. 1. The plasma concentrations of warfarin and warfarin alcohol(s) after administration of a) *R*-warfarin or b) *S*-warfarin to subject B. The subject received a 100 mg single dose of either *R* or *S*-warfarin with 50 mg vitamin K_1. The drugs were given orally. ▲, *R*-warfarin; △, *S*-warfarin; ●, warfarin alcohol$_1$; ○, "total alcohols" (may include alcohol$_2$ with alcohol$_1$). The plasma levels of warfarin were determined by the method of Lewis & others (1970).

about 0·5 μg ml^{-1} about 28h after *S*-warfarin administration. Four days after giving the drug no warfarin alcohols remained in the plasma.

Similar results to those obtained in subject B were obtained in the other 3 subjects. Table 2 gives the approximate times, for all four subjects, when the plasma levels of warfarin alcohols were highest and the corresponding alcohol concentrations at those times.

All subjects receiving a 100 mg dose of warfarin also received 50 mg of vitamin K_1. Taking vitamin K_1 reduced but did not eliminate the anticoagulant effect of the warfarin enantiomers. Table 3 gives the times of the maximum anticoagulant effect for the enantiomers and the corresponding Thrombotest percentages in the subjects receiving warfarin plus vitamin K_1. Under these conditions *S*-warfarin was the more potent enantiomer. We found that *S*-warfarin was also the more potent anticoagulant (using subjects A and B) when no vitamin K_1 was administered. This was so at both 15 mg and 30 mg dose levels (Table 4).

S-Warfarin was also found to be a more potent anticoagulant than *R*-warfarin in patients. Fig. 2 shows that in four patients the change from proprietary racemic

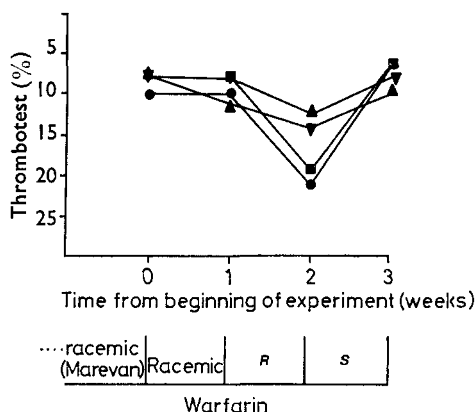

FIG. 2. The effect of the enantiomers of warfarin on anticoagulant control in 4 male patients. The daily doses of warfarin were E, 11 mg (▲); F, 3 mg (●); G, 4 mg (▼); H, 3 mg (■). At the beginning of the experimental period proprietary racemic warfarin (Marevan) was exchanged for racemic warfarin formulated identically to the enantiomers to be subsequently used.

Table 2. *Comparison of warfarin alcohol concentration in plasma after administration of warfarin enantiomers to volunteers.* The subjects received 100 mg single doses of either *R* or *S*-warfarin with 50 mg vitamin K_1. The drugs were given orally. The plasma samples were those used for the determination of the warfarin plasma half-lives shown in Table 1. The warfarin alcohol concentrations given are the means of duplicates that did not differ by more than 5%.

Subject	Enantiomer	Approximate time after warfarin administration when maximum alcohol concentration in plasma (h)	Maximum plasma concentration of warfarin alcohol(s) (μg ml^{-1})
A	*R*	48–72	1·53[a]
	S	28	0·55[b]
B	*R*	28–48	2·62[a]
	S	28	0·52[b]
C	*R*	48	2·22[a]
	S	28	0·34[b]
D	*R*	48–72	2·22[a]
	S	28	0·46[b]

[a] Warfarin $alcohol_1$
[b] "Total alcohols" (may contain $alcohol_1$ with $alcohol_2$)

warfarin to our preparation of racemic warfarin was not associated with any marked overall change in anticoagulant effect. However, administration of *R*-warfarin resulted in decreased anticoagulation (increased Thrombotest percentages) in all patients. Substitution of *S*-warfarin for *R*-warfarin was associated with an increase in anticoagulant effect in all patients. Parallel plasma warfarin determinations (assay of Lewis & others, 1970) indicated that plasma warfarin concentrations increased during *R*-warfarin administration but decreased with *S*-warfarin administration.

Table 3. *Relative anticoagulant efficacies of the warfarin enantiomers in volunteers receiving vitamin* K_1. The subjects received 100 mg single doses of either *R* or *S*-warfarin with 50 mg vitamin K_1. The drugs were given orally. The blood samples were centrifuged and the resultant plasma samples used for the determination of the warfarin plasma half-lives shown in Table 1. The Thrombotest values are means of duplicates. The two clotting times from which the duplicate Thrombotest values were determined never differed by more than 2 s.

Subject	Enantiomer	Time of maximum anticoagulant effect after warfarin administration (days)	Thrombotest values (%) at time of maximum anticoagulant effect
A	*R*	6	27·0
	S	5	13·5
B	*R*	3	70·0
	S	4	31·0
C	*R*	4	35
	S	4	12·5
D	*R*	4	60·0
	S	4	31·0

Table 4. *Relative anticoagulant efficacies of the warfarin enantiomers in volunteers not receiving vitamin* K_1.

Enantiomer	Oral dose (mg)	Thrombotest value (%) on third day after drug* in subject: A	B
R	15	80	100
R	30	35	26·5
S	15	46	27
S	20	12	14

* With the 15 and 30 mg doses of warfarin the maximum anticoagulant effect occurred on the third day after drug administration. The Thrombotest values are means of duplicates. The two clotting times from which the duplicate Thrombotest values were determined never differed by more than 2 s.

Plasma levels of warfarin alcohols (estimated as "total alcohols") tended to parallel plasma warfarin levels. The alcohols were present in amounts about one quarter those of the corresponding warfarin concentrations.

The way in which plasma levels of warfarin alcohols ("total alcohols") tend to parallel those of unchanged warfarin (over a 24h period) is shown in Fig. 3 The data are from patients I and J who, at the start of the 24h experimental period, had received their usual daily doses of racemic warfarin (Marevan).

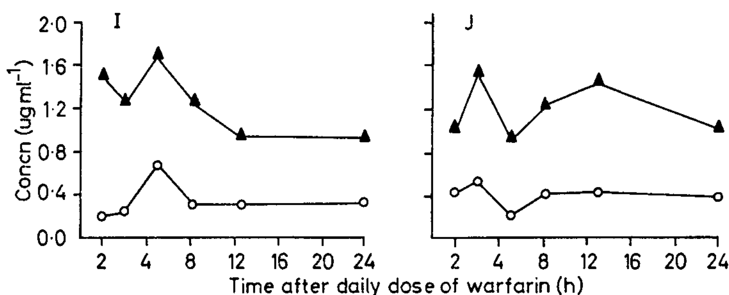

Fig. 3. The plasma concentrations of warfarin and warfarin alcohols in patients I and J over a 24 h period after each receiving their usual daily maintenance dose of racemic warfarin (Marevan). The daily maintenance doses for patients I and J were 4 and 2 mg respectively. The patients had been receiving these doses daily for at least two weeks prior to the experiment. ▲, Warfarin; ○, "total alcohols" (warfarin $alcohol_1$ and possibly warfarin $alcohol_2$). The warfarin was assayed by the method of Lewis & others, (1970).

DISCUSSION

In the rat, *S*-warfarin is 5–7 times more potent than *R*-warfarin as an anticoagulant (Eble & others, 1966; Breckenridge & Orme, 1972; Hewick, 1972). The present work shows that in man also, *S*-warfarin is more potent than *R*-warfarin, although the exact potency was not determined. Unlike in the rat, however, where *R*-warfarin is cleared from the plasma about twice as fast as *S*-warfarin (Breckenridge & Orme, 1972; Hewick, 1972), in man *S*-warfarin is cleared from the plasma about 40% faster than *R*-warfarin (based on the assay of Lewis & others, 1970). It is unlikely that the vitamin K_1, taken by our subjects would significantly affect the warfarin half-lives (O'Reilly, Aggeler & Leong, 1963). In man, as in the rat (Breckenridge & Orme,

1972; Hewick, 1972) the apparent volumes of distribution of the two enantiomers were not significantly different.

A major plasma metabolite was found to be warfarin $alcohol_1$. About five times more $alcohol_1$ was found with *R*-warfarin than with *S*-warfarin. It is almost certain that the presence of relatively large amounts of $alcohol_1$ in the plasma account for the markedly longer half-life values for *R*-warfarin determined by the assay of Corn & Berberich (1967) compared with those determined by the assay of Lewis & others, (1970). Therefore, in agreement with the latter authors, we find that the Corn & Berberich assay is not good for determining the plasma half-life of warfarin in man. On the other hand, studies in rats, giving 5 mg kg^{-1} doses of each enantiomer, revealed only traces of warfarin plasma metabolites and the two assays gave very similar half-life values for the respective enantiomers (D.S. Hewick unpublished observation).

Our studies indicate that, compared with warfarin $alcohol_1$, warfarin $alcohol_2$ and possibly 7-hydroxywarfarin are minor plasma metabolites. Since we had no 7-hydroxywarfarin available as a standard it is difficult for us to comment with certainty on the presence or absence of 7-hydroxywarfarin in the plasma.

Probably a factor contributing to the relatively high concentration of warfarin $alcohol_1$ in the plasma compared with other metabolites is its slow rate of clearance. In the one subject used, Lewis & Trager (1971) found that $alcohol_1$ had a plasma half-life (33h) that was similar to that of racemic warfarin (35h). Warfarin $alcohol_2$ (half-life 12h) was cleared from the plasma about three times faster than $alcohol_1$.

In the present study, the $alcohol_1$ produced after *R*-warfarin administration would have the *RS* configuration. The smaller amount of $alcohol_1$ and the trace of $alcohol_2$ after *S*-warfarin administration would have the *SR* and *SS* configurations respectively. Probably a major plasma metabolite of clinically-administered racemic warfarin therefore is warfarin $alcohol_1$ in the *RS* configuration.

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