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STUDIES ON A KETONE REDUCTASE IN HUMAN AND RAT LIVER AND KIDNEY SOLUBLE FRACTION USING WARFARIN AS A SUBSTRATE

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Abstract—The enantiomers of warfarin were reduced to warfarin alcohols on incubation with human and rat liver and kidney cytosol. The metabolism was NADPH-dependent with NADH being only one tenth as effective at supporting the reduction. R-warfarin was reduced at a greater rate than S-warfarin by each of the tissues examined. At a concentration of 2·08 mM the rate of S-warfarin reduction ranged from 5 to 50% of the rate occurring with R-warfarin in the same tissue fraction. R-warfarin (1·3 mM) was mainly reduced to warfarin alcohol 1 (RS) (77%) the remainder being warfarin alcohol 2 (RR). S-warfarin formed slightly more warfarin alcohol 2 (SS) (57%) than warfarin alcohol 1 (SR). The apparent K_m values for the reduction of R-warfarin by human and rat liver and kidney cytosol ranged from 0·54 to 1·55 mM. The human liver reductase, even 3·5–8·5 hr after death, was significantly more active (2 times) than the rat liver reductase. Stability studies using rat liver indicated that about half of the reductase activity activity may be lost during the first 6 hr after death. This would suggest that human liver had more than four times the warfarin reductase activity of rat liver.

The molecule of the oral anticoagulant warfarin contains an asymmetric centre and therefore the drug can exist in two optically active forms, namely R-(+)-warfarin and its mirror image isomer S-(−)-warfarin. The warfarin as used clinically and rodenticidally is a racemic (equimolar) mixture of these two enantiomers.

In patients maintained on racemic warfarin, major plasma metabolites include warfarin alcohols which are present at about one third of the plasma warfarin concentration[1]. These alcohols are formed by reduction of the ketone group in the warfarin molecule and represent about 18% and 7% of urinary metabolites of racemic warfarin in man and the rat respectively[2, 3]. When R-warfarin alone is administered the proportion of warfarin alcohols in human urine rises to 37%[2].

Reduction of the warfarin ketone group to produce warfarin alcohols creates a second asymmetric centre and two diastereoisomeric alcohols, warfarin alcohol 1 and warfarin alcohol 2, may be formed. Each diastereoisomer exists in two enantiomeric forms, namely SR and RS for alcohol 1, and SS and RR for alcohol 2[4]. About five times more warfarin alcohol is found in the plasma of man after a single dose of R-warfarin than after an identical dose of S-warfarin. After R-warfarin administration the alcohol found in the plasma is almost entirely RS-warfarin alcohol and after S-warfarin it is mainly SS-warfarin alcohol[2, 1].

Ikeda *et al.*[5] reported the formation of an unidentified non-phenolic metabolite (metabolite X) of racemic warfarin in the presence of rat heptaic soluble fraction. Metabolite X was produced at about 20% of the rate at which warfarin was hydroxylated by the rat hepatic microsomol fraction. The present study was undertaken to see if metabolite X formation was due to ketonic reduction of warfarin by enzymes in the cytosol. In addition we wished to ascertain if stereoselectivity by such enzymes could be responsible for the higher plasma and urinary levels of warfarin alcohols seen after the administration of R-warfarin compared with S-warfarin[1, 2].

MATERIALS AND METHODS

Thin layer chromatography. Silica gel thin layer chromatography plates were used with the following solvent systems: (A) ethylene dichloride–acetone (9:1)[6], (B) cyclohexane–ethyl formate–formic acid (100:200:1)[6], (C) ether–acetic acid (99:1)[7], (D) ether–chloroform–acetone (60:30:1)[7], (E) ether–benzene–acetone–acetic acid (60:50:10:1)[7], (F) ether–ethyl acetate–acetic acid (80:30:1)[7], (G) ethylene dichloride–acetone (7:3)[6].

Preparation of warfarin enantiomers and warfarin alcohols. The warfarin enantiomers were prepared from racemic warfarin according to the method of West *et al.*[8]. The rotation of R-warfarin was $[\alpha]_D^{20} + 144{\cdot}4°$ (cl, 0·5 N NaOH) and the optical purity was 98·4%. The rotation of S-warfarin was $[\alpha]_D^{20} - 140{\cdot}6°$ (cl, 0·5 N NaOH) and the optical purity was 97·5%.

The warfarin alcohols 1 and 2, used as standards for thin layer chromatography and spectrophotofluorimetry, were prepared from racemic warfarin by reduction with sodium borohydride[9] and the diastereoisomers were then separated by column chromatography, essentially by the method of Chan, Lewis and Trager[4]. The alcohols were dissolved in a minimal amount of acetone and added to a column of silica gel (MFC, 100–200 mesh, Hopkin and Williams, Essex) suspended in cyclohexane–ethyl formate–formic acid (300:200:1). The column was fitted to an automatic

fraction collector and then eluted with the above solvent system. The fractions were analysed by silica gel thin layer chromatography using solvent system B and pooled appropriately. The solvent was evaporated *in vacuo* and the fraction recrystallized from acetone.

Animals and tissue preparation. Liver and kidney (both cortex and medulla) tissue was obtained from 200-g female Wistar rats directly after cervical dislocation or from human cadavers 3·5–8·5 hr after death. To determine whether phenobarbitone pretreatment induced the enzyme system under investigation a group of rats were given phenobarbitone sodium intraperitoneally, 75 mg/kg per day for 3 days, the enzyme assay being performed on the fourth day. The drug-treated group were compared with a group of saline-treated controls. Each group consisted of 4 rats.

After removal, tissue samples were cooled to 0–4° and kept at this temperature during subsequent manipulations. The samples were homogenised with 3 vol KCl (1·15%) containing 5 mM phosphate buffer, pH 7·4, using a Measuring and Scientific Equipment Ltd homogeniser (14,000 rev/min for 1 min) and centrifuged at 9000*g* for 20 min in an International Equipment Co. Centrifuge. The supernatant obtained was then centrifuged at 160,000*g* for 1 hr in a Beckman L2-65B ultracentrifuge to obtain the soluble fraction as supernatant. The protein content of the soluble fraction was estimated by the method of Lowry *et al.*[10], using human serum albumin as a standard.

Incubation details. Incubations were carried out at 37° for 50 min in a shaking incubator under an atmosphere of air. The basic incubation mixture comprised liver or kidney soluble fraction (containing 15 or 7·5 mg of protein respectively) with the following (mM); phosphate buffer (pH 7·4), 100; racemic or R-warfarin, 1·3; glucose 6-phosphate (G 6-P), 7; $MgSO_4$, 5; $NADP^+$, 1·4; in a total volume of 2·5 ml.

In some experiments the basic mixture was changed by including ATP (0·57 mM) and nicotinamide (34·3 mM) or by replacing $NADP^+$ and G 6-P with NADH (5 mM) or NADPH (5 mM). In particular, the reduced coenzyme, NADPH, was used for the reductase assays in the pH optimisation and phenobarbitone pretreatment experiments to eliminate any effects due to differences in the NADPH-generating system (G 6-P dehydrogenase activity).

For the determination of the pH optimum a series of 0·5 M phosphate buffer solutions were prepared with pH values, 5·0, 5·5, 6·0, 6·5, 7·0, 7·5, 8·0 and added to a series of incubation mixtures according to the standard procedure. The pH of each mixture was measured at 37° with a pH meter after incubation. When boiled cytosol was required for incubation this was prepared by heating soluble fraction plus buffer in a boiling water bath for 30 min and cooling to room temperature prior to making other additions. The effect of substrate concentration and enzyme stereoselectivity was studied by determining the amount of alcohols formed from R- and S-warfarin at concentrations ranging from 0·13 to 2·08 mM.

Assay of warfarin alcohols. After incubation the reaction was stopped by transferring 2 ml of the reaction mixture to a tube containing 1 ml of 3N hydrochloric acid. Then the mixture was extracted with 2 ml ethylene dichloride and assayed for warfarin alcohols 1 and 2 by the method of Lewis, Ilnicki and Carlstrom[11] as modified by Hewick and McEwen[1]. This involved silica gel thin layer chromatography of the concentrated ethylene dichloride extract firstly using solvent system A to separate unchanged warfarin from the warfarin alcohols and then re-running in solvent system B to separate warfarin alcohols 1 and 2. These were then quantified spectrophotofluorimetrically. The values obtained for alcohol 1 and alcohol 2 were usually summated to give 'total warfarin alcohols'.

Enzyme kinetic studies. The data obtained from experiments in which the warfarin concentration was varied were plotted according to the method of Hofstee[12], namely rate of alcohol formation (V) versus V/S, where S is the corresponding warfarin concentration. In each experiment a regression line was fitted to the data points by the method of least squares so that the Michaelis constant (K_m) could be calculated from the slope ($-K_m$) of the line and the maximal rate of warfarin alcohol formation (V_{max}) could be directly obtained from the intercept of the line with the ordinate.

Enzyme stability studies. To determine the stability of reductase activity in frozen cytosol, rat liver soluble fraction from 4 rats was assayed for reductase activity immediately after preparation and again after freezing for 24 hr at −20°.

To obtain an indication of the *post mortem* stability of the reductase enzyme system, three rats were killed, the carcasses placed in polythene bags in a water bath at 37° and liver samples taken from them after 0, 1·5, 3, 5 and 7 hr. During this time the temperature of the water bath was allowed to fall by 1°/hr to simulate the *post mortem* cooling of the human body. Samples obtained at each time were pooled, the soluble fraction was prepared and immediately assayed for warfarin reductase activity and G 6-P dehydrogenase activity. The latter was measured using a Perkin–Elmer 402 u.v./visible double-beam spectrophotometer by following the increase in optical density at 340 nm (temperature 23°) of the following system (mM); phosphate buffer (pH 7·4), 100; G 6-P, 7; $MgSO_4$, 5; $NADP^+$, 1·5; hepatic soluble fraction containing 10 mg of protein; in a total volume of 2·5 ml. The reference cell consisted of the above system without G 6-P.

RESULTS

Incubation of racemic warfarin and rat hepatic soluble fraction with the cofactors ATP, G 6-P, $NADP^+$, NAD^+ and nicotinamide as used by Ikeda *et al.*[5] and subsequent thin layer chromatography in systems A and B indicated the formation of two metabolites. One metabolite appeared to be formed in greater quantity than the other as indicated by its more pronounced fluorescence on the chromatogram. This metabolite (I) possessed the same fluorescence properties (excitation and emission maxima at 345 nm and 405 nm respectively), the same chromatographic mobility (Table 1) and the same absorption spectra (extinction maximum at

Table 1. Silica gel thin layer chromatography of warfarin and its reduced metabolites

Compound	R_f values and solvent systems*						
	A	B	C	D	E	F	G
Metabolite (I)	0·07	0·71	0·73	0·04	0·45	0·64	0·08
Alcohol 1	0·12	0·71	0·76	0·05	0·48	0·61	0·07
Alcohol 2		0·63	0·61		0·36	0·49	
Warfarin	0·51	0·70	0·75	0·42	0·53	0·70	0·44

* Solvent systems are as described under Materials and Methods.

309 nm in NaOH, 276 and 286 nm in acid) as warfarin alcohol 1. The other metabolite, which was formed in small amounts only, appeared (on the basis of chromatographic mobility) to be alcohol 2.

Preliminary experiments showed that R-warfarin was reduced faster than S-warfarin so the former enantiomer was used for the experiments aimed at characterising the reductase enzyme. These experiments, using rat hepatic soluble fraction and measuring 'total alcohols' (alcohol 1 plus alcohol 2) showed that warfarin reduction was mediated by an NADPH-dependent enzyme with a pH optimum of 7·4. The cofactors NAD^+, ATP and nicotinamide were not required and NADH (5 mM) appeared only one-tenth as effective as NADPH (5 mM) at supporting the reduction (Table 2). No warfarin alcohols were produced on incubation with boiled hepatic cytosol or when NADPH or components of its generating system had been omitted from the incubation mixture (Table 2). Subsequent experiments revealed that warfarin reduction was also mediated by similar NADPH-dependent enzymes in the soluble fraction of human liver and kidney, and rat kidney.

Phenobarbitone pretreatment of rats caused a slight (1·4-fold) but significant ($P < 0·01$) increase in hepatic warfarin reductase activity. The rate of R-warfarin reduction rose from $1·44 \pm 0·04$ to $2·00 \pm 0·10$ nmoles/mg protein per 50 min (mean ±S.E.M.).

R-warfarin was reduced at a faster rate than S-warfarin both by human and rat liver and kidney soluble fraction (Table 3). In none of the tissues used was S-warfarin reduction detectable below a concentration of 0·52 mM. Above this S-warfarin concentration the amount of reduction increased; at 2·08 mM the rate of S-warfarin reduction varied with tissue source but ranged from 5 to 50% of the rate occurring with R-warfarin in the same tissue fraction (Table 3). Figure 1 shows typical results obtained over the complete concentration range of 0·13–2·08 mM using human kidney soluble fraction. With this concentration range it was not possible to obtain K_m values for S-warfarin and attempts to

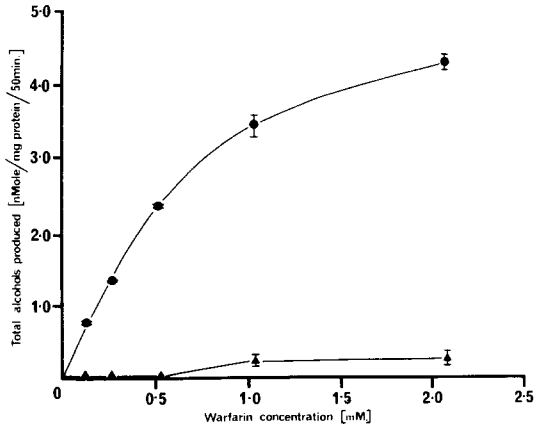

Fig. 1. Stereoselectivity of human kidney warfarin reductase. Total warfarin alcohols produced from R-warfarin (●) or S-warfarin (▲) at concentrations of 0·13–2·08 mM. Each point is the mean ±S.D. of duplicate determinations on cytosol prepared from a single tissue sample.

Table 2. Cofactor requirements for R-warfarin reduction in rat hepatic soluble fraction*

Cofactor added to incubation mixture†	Warfarin alcohols formed‡
$NADP^+$, G 6-P, NAD^+, ATP, nicotinamide	2·2
$NADP^+$, G 6-P, NAD^+	2·2
$NADP^+$, G 6-P	2·8
G 6-P	<0·1§
$NADP^+$, G 6-P‖	<0·1
NADH	0·2
NADPH	2·0
None	<0·1

* The pool of soluble fraction used for each incubation was prepared from 3 rat livers.

† Concentrations of cofactors used and other incubation conditions are as described under Materials and Methods.

‡ Total warfarin alcohols, i.e. alcohol 1 and alcohol 2 (n moles/mg protein per 50 min).

‖ Cytosol boiled before incubation with these cofactors.

§ Limit of detection.

Table 3. Effect of warfarin concentration on the stereoselectivity of warfarin reductase in human and rat hepatic and renal soluble fraction

Source of soluble fraction*	Enantiomer of warfarin	Rate of reduction† at a warfarin concentration of	
		0·52 mM	2·08 mM
Rat liver	R	0·44	0·96
	S	<0·1‡	0·44
Human liver	R	0·75	1·61
	S	<0·1	0·82
Rat kidney	R	3·45	5·83
	S	<0·1	1·32
Human kidney	R	2·34	4·31
	S	<0·1	0·24

* Rat soluble fraction was freshly prepared from 3 animals. Human cytosol was prepared from single tissue samples obtained 3·5–8·5 hr after death.

† Production of total warfarin alcohols, i.e. alcohol 1 and alcohol 2 (nmoles/mg protein per 50 min).

‡ Limit of detection.

use concentrations over 2·08 mM were unsuccessful since overloading of the chromatographic plate resulted.

When R-warfarin (1·3 mM) was reduced in the presence of rat hepatic enzyme 77% of the total warfarin alcohols formed was warfarin alcohol 1, the remainder being warfarin alcohol 2. When S-warfarin was reduced however, slightly more warfarin alcohol 2 was formed (57% of the total alcohols) than warfarin alcohol 1.

Figure 2 shows typical data obtained for R-warfarin reduction using rat liver and kidney soluble fraction plotted according to the method of Hofstee[12]. The regression lines have similar slopes indicating similar K_m values and the difference in reductase activity is indicated by the intercepts of the line with the ordinate (V_{max} values). The apparent K_m values for the reduction of R-warfarin for all the tissue fractions used were similar, ranging from 0·54 to 1·55 mM (Table 4). For the reduction of R-warfarin to warfarin alcohols the rat kidney reductase was 3–6 times more active than the rat liver enzyme (Fig. 2, Table 3, Table 4, Table 5). The human liver reductase, even 3·5–8·5 hr after death, was significantly more active than the rat liver reductase (Table 5, also Tables 3 and 4). The human kidney reductase of the two tissue samples examined was almost as active as the rat kidney enzyme (Table 5).

When the rat cadavers were cooled 1°/hr for 7 hr after death the reductase activity of the liver cytosol rose slightly in the first 1·5 hr and then

Table 4. The maximal reaction velocities (V_{max} values) and Michaelis constants (K_m values) of R-warfarin reductase in the liver and kidney of man and the rat

Source of soluble fraction*	$V^{\dagger}_{max}$	K_m (mM)
Rat liver	1·34	0·54
Human liver	2·88	1·55
Rat kidney	8·63	0·90
Human kidney	6·52	0·98

* Rat soluble fraction was freshly prepared from 3 animals. Human cytosol was prepared from single tissue samples obtained 3·5–8·5 hr after death.

† Production of total warfarin alcohols, i.e. alcohol 1 + alcohol 2 (nmoles/mg protein per 50 min) calculated from an R-warfarin concentration range of 0·13–2·08 mM.

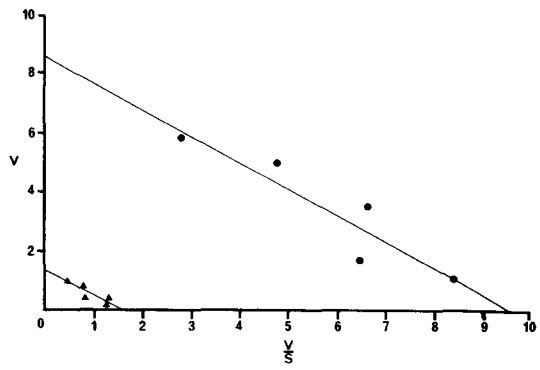

Fig. 2. R-warfarin reduction by rat liver (▲) and kidney (●) reductase plotted according to the method of Hofstee[11]. Total warfarin alcohols produced (V) are plotted versus V/S, where S is the corresponding R-warfarin concentration (0·13–2·08 mM).

Table 5. Mean rates of reduction of R-warfarin (1·3 mM) by human and rat liver and kidney soluble fraction

Source of soluble fraction*	Warfarin alcohols formed†	N
Rat liver	1·49 (± 0·23)	10
Human liver	3·17‡ (± 0·90)	4
Rat kidney	4·76 (± 0·29)	5
Human kidney	3·85 (± 0·19)	2

* In each rat experiment soluble fraction was freshly prepared from a pooled group of at least 3 rats. Human soluble fraction was prepared from single tissue samples obtained 3·5–8·5 hr after death. N indicates the number of experiments for each tissue.

† Total warfarin alcohols, i.e. alcohol 1 + alcohol 2 (nmoles/mg protein per 50 min). Values are expressed as means ±S.E.M.

‡ Significantly greater than rat liver reductase activity ($P < 0·05$).

Table 6. Post mortem survival of R-warfarin reductase activity in rat liver soluble fraction*

Time (hr)	Warfarin alcohols produced†
0	2·58 (±0·02)
1·5	3·14 (±0·06)
3·0	2·05 (±0·02)
5·0	1·81 (±0·02)
7·0	1·05 (±0·05)

* Soluble fraction prepared from a pool of the livers of 3 rats at each time interval.

† Total warfarin alcohols assayed in duplicate, i.e. alcohol 1 + alcohol 2 (nmoles/mg protein per 50 min).

steadily declined until at 7 hr it was 41% of the zero time level (Table 6). The G 6-P dehydrogenase activity of the soluble fraction surprisingly showed an apparent increase during this time so that it was 1·5–2·0 times the zero time level during the last 4 hr of the experiment.

Rat liver reductase activity fell significantly ($P < 0.001$) when the soluble fraction was stored at −20° for 24 hr. The rate of R-warfarin reduction fell from 2·44 ± 0·06 to 1·55 ± 0·05 nmoles/mg protein per 50 min (mean ±S.E.M.).

DISCUSSION

There appears to be some correlation between the stereoselectivity of warfarin reduction by man *in vivo* and by human tissue *in vitro*. Of the two enantiomers, R-warfarin is reduced more rapidly *in vitro* by human kidney and liver cytosol. This may explain why more warfarin alcohol is found in human urine and plasma after R- than after S-warfarin. In addition our studies *in vitro* using human (and rat) tissues show that R-warfarin is reduced mainly to alcohol 1 (RS) while S-warfarin forms a mixture containing slightly more alcohol 2 (SS) than alcohol 1 (SR). This may explain the presence of RS-warfarin alcohol in the plasma of man after R-warfarin and the smaller amounts of SS-warfarin alcohol after S-warfarin administration.

In the tissue samples investigated human liver and kidney cytosol reduced R-warfarin (1·3 mM) at about 2·1 and 2·6 times the rate of rat liver soluble fraction respectively (Table 5). All of the rat tissue studies were carried out on fresh tissues prepared immediately after death. The human tissues however, were obtained 3·5–8·5 hr after death with a mean delay of about 6 hr. Rat liver reductase activity declined when the rat carcasses were allowed to cool slowly from body temperature after death. After 6 hr the residual activity was about half the original activity determined immediately after death. This loss of activity was not due to a decline in the activity of the NADPH generating system. If human liver warfarin reductase activity declined at the same rate as this then the determinations on our *post mortem* human tissue samples could underestimate the activity in fresh human tissue by more than 50%. This would indicate that human liver had more than four times the warfarin reductase activity of rat liver and may explain the occurrence of greater amounts of warfarin alcohols in the plasma of man after warfarin administration than in the plasma of rats which have been similarly treated.*

The cofactor dependence of warfarin reduction makes it unlikely that alcohol dehydrogenase, which is also found in the soluble fraction, is responsible since the latter is NADH dependent and not NADPH dependent. A number of NADPH-dependent ketone reductases have been reported to occur in liver and kidney cytosol[13–15]. Culp and McMahon[13] described the partial purification of an aromatic aldehyde–ketone reductase from rabbit kidney cortex which also occurred in rabbit liver. This enzyme reduced a wide range of aldehyde and ketone substrates. Using acetophenone they found that the reduction formed an asymmetric centre which was chiefly of the S configuration. A similar phenomenon was observed in the present study in that the reduction appeared to favour the formation of the S configuration.

The warfarin reductases from the two species and two tissues investigated had similar apparent K_m values and displayed a similar stereoselectivity. It is possible that warfarin reduction is mediated by identical enzymes occurring in liver and kidney cytosol and that they are similar to the rabbit aldehyde–ketone reductase reported by Culp and McMahon[13].

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