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Stereoselective drug distribution and anticoagulant potency of the enantiomers of phenprocoumon in rats

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The elimination, distribution and anticoagulant activity of *S*(−)-, *R*(+)-, and *R,S*(±)-phenprocoumon were determined in male Wistar-Lewis rats after intravenous injection of a single dose of 0·6 mg kg^{-1}. From the plasma concentrations which elicited the same anticoagulant effect, *S*(−)-phenprocoumon was 4 to 5 times more potent than *R*(+)-phenprocoumon. The potency of the racemate was between those of the enantiomers. The mean biologic half-life of the *S*(−)-enantiomer was shorter (12·5 h) than that of *R*(+)-phenprocoumon (17·8 h). No differences were observed in the apparent volume of distribution. However, the mean liver : plasma concentration ratio was higher for the *S*(−)-(6·9) than for the *R*(+)-enantiomer (5·2). At a total concentration of 16·8 μg ml^{-1} the percentage of unbound drug in rat serum was significantly higher for the *S*(−)- (1·13%) than that for the *R*(+)-enantiomer (0·76%). Values obtained for the racemate were always between those of the enantiomers. It is concluded that stereoselective differences in the distribution between plasma and liver, and consequently in the rate of elimination are most likely due to stereoselective differences in serum protein binding. The greater anticoagulant potency of the *S*(−)- over the *R*(+)-enantiomer, cannot be explained primarily by the observed pharmacokinetic differences.

The pharmacokinetics and pharmacodynamics of the enantiomers of warfarin have been studied in man (Hewick & McEwen, 1973; Breckenridge, Orme & others, 1974; Levy, O'Reilly & Wingard, 1974; O'Reilly, 1974) and rat (Eble, West & Link, 1966; Breckenridge & Orme, 1972; Hewick, 1972; Yacobi & Levy, 1974). Intriguing species differences in the elimination kinetics of the enantiomers were found. In man *S*(−)-warfarin is eliminated faster than *R*(+)-warfarin. The opposite is true in rats. A stereospecific metabolism was thought to be responsible for these differences in the elimination rate in man (Hewick & McEwen, 1973; Breckenridge & others, 1974; Lewis, Trager & others, 1974; O'Reilly, 1974) and rat (Breckenridge & Orme, 1972†). Stereoselective differences in drug distribution were recently observed in man for the enantiomers of the structurally related anticoagulant drug phenprocoumon (Jähnchen, Meinertz & others, 1976). The apparent volume of distribution was smaller and the plasma clearance slower for the *S*(−)- than for the *R*(+)-enantiomer. These differences in the pharmacokinetics have been related to marked stereoselective differences in the binding of the enantiomers to human serum albumin.

Detailed information on the pharmacokinetics of the enantiomers of phenprocoumon in rats is lacking. There is a report that after administration of the ^{14}C-labelled enantiomers the urinary excretion of total activity is more rapid for the *R*(+)- than that for the *S*(−)-enantiomer (Goding & West, 1969). Also, like warfarin, *S*(−)-phenprocoumon was found to be more potent in rats than its *R*(+)-enantiomer (West & Link, 1965). We have examined the anticoagulant effects of the enantiomers and the racemate in rats to compare the findings with those in man and to see if species differences exist between man and rat in stereoselective drug distribution.

MATERIALS AND METHODS

Materials

Racemic phenprocoumon and its enantiomers were gifts from Hoffmann-La Roche, Basel, Switzerland. $[\alpha]_D$ *S*(−)-drug −113·8° (3·8% in methanol) *R*(+)-drug +114° (3·4% in methanol)*. The racemate was a 1 : 1 mixture.

Male Wistar Lewis rats (inbred strain, Charles River, Wilmington, Mass., USA), initially 300 to 360 g, had free access to food (Altromin, Lage, Germany) and water.

Animal experiments

Three randomly selected groups each of 5 rats were used. In three consecutive experiments a single dose

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† A report definitively showing a stereoselective metabolism of wafarin by rat liver microsomes appeared recently (Pohl, L. R. Nelson, S. O. & others, 1976, *Biochem. Pharmac.*, **25**, 2153–2162).

* Kindly performed by Hoffmann-La Roche.

of each form of phenprocoumon was administered to each group. The time between these experiments was about 6 weeks. The drugs were administered in the following order: group 1: *R*;*S*;*R*,*S*, group 2: *R*,*S*;*R*;*S*, group 3: *S*;*R*,*S*;*R* for the 1st, 2nd and 3rd experiments respectively. One animal of group 1 died between the first and second experiment. Collection of several blood samples from rats by puncture of the tail artery and measurement of prothrombin times in plasma samples of 10 μl were according to Wingard & Levy (1973). Briefly, the drugs were injected in the proximal part of a tail vein and thereafter 9 to 11 blood samples 0·45 ml 6% were taken in 60–84 h, diluted with 0·05 ml sodium oxalate, centrifuged and the plasma frozen at −20°.

Determination of phenprocoumon

Racemic phenprocoumon and the enantiomers were determined by a modification of the fluorimetric assay of Jähnchen & others (1976).

Determination in plasma: plasma samples of 0·1 ml were adjusted to pH 3·1 by the addition of 0·5 ml citric acid phosphate buffer (1·5 M, pH 3·1). The drugs were extracted into 2·5 ml of n-heptane (reagent grade), shaken and centrifuged, 2·0 ml of the heptane phase was then removed and back-extracted into 0·5 ml of 0·5 N NaOH. The fluorescence was measured at 390 nm using a excitation wavelength of 313 nm in a Perkin-Elmer Fluorimeter (Model 204) equipped with microcuvettes. 0·5 N NaOH served as reference solution. A quinidine standard (0·125 μg ml^{-1} 0·1 N H_2SO_4) and a standard curve of phenprocoumon in distilled water and rat plasma was run through each set of determinations. Plasma blanks so obtained were about 0·15 μg ml^{-1} apparent phenprocoumon. There was no difference in fluorescence intensity between the enantiomers. The recovery of racemate and enantiomers from plasma was complete (>95%).

Determination in the liver: rats were killed under ether anaesthesia by withdrawal of all blood from the abdominal aorta. The livers were removed, blotted under slight pressure and homogenized with an Ultra-Turrax (Janke & Kunkel KG, Staufen i. Br., Germany) in ice cold 0·9% sodium chloride solution (1:4 w/v). The homogenate was stored at −20° until assayed. To 4 ml of liver homogenate were added 4 ml of citric acid phosphate buffer (1·5 M, pH 3·1) and 10 ml ethylene dichloride. This was shaken for 30 min and centrifuged. 8 ml of the organic layer were removed and the drug was back-extracted into 3 ml of 0·5 N NaOH. A 2·5 ml portion of the aqueous layer was acidified by adding 1 ml of 3 N HCl and then extracted with 5 ml of n-heptane. 4 ml of the heptane phase were back-extracted into 3 ml of 0·5 N NaOH. The fluorescence was measured according to Jähnchen & others, 1976). There was no difference in the recovery of the three forms of drug. A mean value of 84·6 (1·3%) (mean with s.d., n = 14) was obtained in the 2 to 4 μg g^{-1} concentration range. Thin-layer chromatographic studies of [^{3}H]phenprocoumon in rats revealed, that the fluorimetric method described can be considered as specific for unchanged drug. Metabolites present in the plasma, liver, bile and urine of treated rats were separated from the parent drug by extraction with n-heptane (Buffleb & Jähnchen, unpublished observations).

Serum protein binding

Blood was collected from 5 rats by puncture of the abdominal aorta under ether anaesthesia. The blood was permitted to clot and subsequently centrifuged for 15 min at 1000 *g*. The serum obtained from all animals was removed, mixed and divided into three parts of 7 ml. To each part of serum one of the forms of phenprocoumon (*R*,*S*; *S*; or *R*) was added, yielding in a final drug concentration of 16·8 μg ml^{-1}. The pH of the serum was measured and when necessary, adjusted to pH 7·4 with 0·1 N HCl. Portions of 2 ml were dialysed for 24 h at 25° against 2 ml of phosphate buffer (0·15 M, pH 7·4) using Cellophane dialysis membranes (Union Carbide Corp., Chicago, Ill.). The same experiment was repeated on the following day using serum from 5 more rats. Drug concentrations were determined fluorimetrically after extraction with n-heptane.

Pharmacokinetic analysis

The apparent first order elimination rate constant for phenprocoumon (k_{app}) was obtained from the slope of a log plasma concentration *vs* time plot determined by the method of least squares. The apparent volume of distribution of phenprocoumon (V_d) was calculated by dividing the dose by the extrapolated plasma concentration at time zero (C_p^o). The plasma clearance (Cl_p) was obtained by multiplying the first order elimination rate constant by the apparent volume of distribution.

The pharmacokinetic approach of Levy (1970) was used to calculate an intrinsic elimination rate constant (k) for racemic phenprocoumon and the enantiomers according to the equation: $k = k_{app}/F$, where F is the fraction of drug in the liver.

F was calculated as described by Jähnchen & Levy (1974) for dicumarol: F = $A°_L$/dose where $A°_L$, the initial amount of drug in liver,

$$= (W_L \cdot C_p^o \cdot C_L/C_P)/1000$$

W_L = weight of the livers in g, C_p^o = extrapolated zero time plasma concentration, C_L/C_p = liver/plasma concentration ratio of phenprocoumon.

The synthesis rate of prothrombin complex activity (R_{syn}) was calculated according to Nagashima, O'Reilly & Levy (1969) using the prothrombin complex activity (PCA) values during the recovery phase after a single dose of phenprocoumon. Since the plasma concentration could only be measured with accuracy up to 42 h after drug administration (compare Fig. 3) it was necessary for the construction of the R_{syn} *vs* log plasma concentration plot to extrapolate plasma concentrations after 42 h from the half-life slopes. The degradation rate constant of PCA (k_d) in Wistar Lewis rats was measured in a separate study by blocking the synthesis of PCA with 15 mg kg^{-1} warfarin (Martini & Jähnchen, unpublished results). A k_d-value of 3·2 (0·2) (mean with s.d., n = 10) was obtained.

RESULTS

Anticoagulant activity

The time course of the anticoagulant effect obtained after administration of a single dose (0·6 mg kg^{-1}) of the three forms of phenprocoumon is shown in Fig. 1. For the *R*(+)-enantiomer, the mean of the maximum depression of prothrombin complex activity was 15·2 (2·5%) (mean with s.d., n = 10) and occurred at about 12 h; the mean activity returned to a value of 80% of normal at about 48 h. For the *S*(−)-enantiomer, the maximum depression of activity was lower than 1·5% of normal and occurred between 24 and 30 h; the mean activity returned to 80% of normal at about 70 h. The mean activity of the racemate was always between that of the enantiomers.

The plasma concentration-response relation of the enantiomers and racemate is shown in Fig. 2.

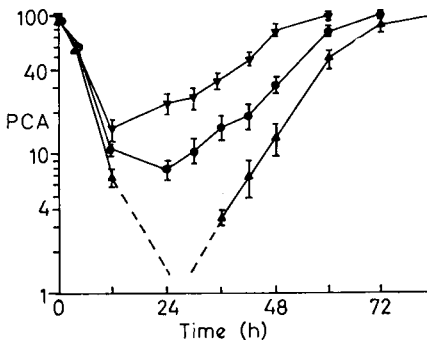

FIG. 1. Time course of prothrombin complex activity (PCA) (% of normal) in the plasma after intravenous administration of 0·6 mg kg^{-1} *R*(+)-phenprocoumon (▼), *R,S*(±)-phenprocoumon (●) and *S*(− -phenprocoumon (▲) to rats. Mean with s.d., 9 to 10 animals which have been repeatedly tested (see material and methods for experimental design). Prothrombin times measured in diluted plasma 24 and 30 h after administration of *S*(−)-phenprocoumon were longer than 15 min (lower than 1·5% PCA).

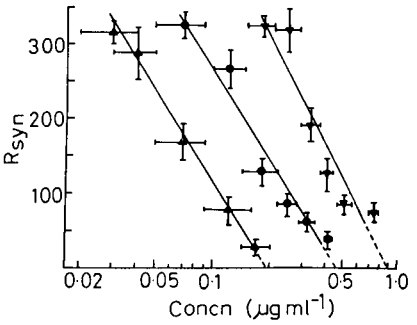

FIG. 2. Relation between the synthesis rate of prothrombin complex activity (R_{syn}) (% day^{-1}) and plasma concentration (μg ml^{-1}) of phenprocoumon in rats. (▼) *R*(+)-phenprocoumon; (●), *R,S*(±)-phenprocoumon; (▲), *S*(−)-phenprocoumon. Mean with s.d., 9 to 10 animals which have been repeatedly tested (see methods for experimental design).

For each of the three forms the synthesis rate of PCA (R_{syn}) appeared to be linearly related to the logarithm of the plasma concentration. There was an essentially parallel shift to lower plasma concentrations for the *S*- and to higher plasma concentrations for the *R*-enantiomer, compared with the racemate. The extrapolated hypothetical plasma concentration, which led to inhibition of prothrombin complex synthesis (C_{max}) was 0·19 μg ml^{-1} for the *S*(−)-enantiomer, 0·46 μg ml^{-1} for the racemate and 0·90 μg ml^{-1} for the *R*(+)-enantiomer.

Elimination kinetics

After intravenous injection of the three forms of phenprocoumon, concentrations in the plasma of rats declined monoexponentially with time (Fig. 3). There was a distinct difference in the rate of elimination between the three forms. The following plasma half-life values were obtained (mean with s.d.): 12·5 (1·2) h (n = 9) for *S*(−)-, 17·8 (1·8) h (n = 10) for *R*(+)-enantiomers and 15·0 (1·7) h (n = 10)

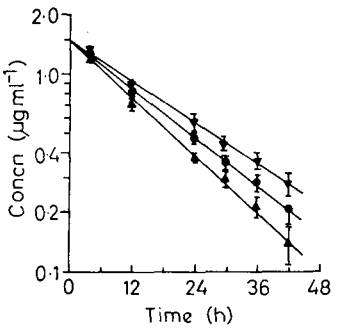

FIG. 3. Plasma concentration ($\mu g\ ml^{-1}$) of *R*(+)-phenprocoumon (▼), *R,S*(±)-phenprocoumon (●) and *S*(−)-phenprocoumon (▲) as a function of time after administration of a single dose (0·6 mg kg^{-1}, i.v.). Mean with s.d., 9 to 10 animals which have been repeatedly tested (see materials and methods for experimental design).

for the racemate. The difference between *S*(−) and *R*(+)-phenprocoumon was statistically significant ($P < 0{\cdot}001$, Student's *t*-test).

There was no difference in the apparent volume of distribution between the three forms. Values obtained were 403 (24 ml) kg^{-1} [*S*(−)-], 406 (20 ml) kg^{-1} [*R*(+)-] and 411 (27 ml) kg^{-1} (racemate). The plasma clearance of the *S*(−)-enantiomer, 22·5 (1·1 ml) $h^{-1}\ kg^{-1}$, was faster than that for the *R*(+)-enantiomer, 15·9 (1·0 ml) $h^{-1}\ kg^{-1}$. This difference was statistically significant ($P < 0{\cdot}001$). The plasma clearance of the racemate, 19·1 (0·9 ml) $h^{-1}\ kg^{-1}$ was between that of the enantiomers.

Distribution between plasma and liver

After a single intravenous dose of the three forms of drug the animals were killed in the third experiment at a time when the concentration in plasma was calculated (calculations based on the pharmacokinetic parameters obtained in the first and second experiment) to be about 0·40 $\mu g\ ml^{-1}$. There was an excellent agreement between the calculated and experimentally obtained values (Table 1).

The concentrations of all three forms of the drug in liver were appreciably higher than in plasma (Table 1). For the *S*(−)-enantiomer the concentration was 1·3 times higher than that of *R*(+)-enantiomer, resulting in a liver : plasma concentration ratio of 6·9 and 5·2 for *S*(−)- and *R*(+)-enantiomers, respectively. The liver concentration and the liver : plasma concentration ratio of racemate was between that of the enantiomers.

The time course of the concentration of racemate in the plasma and in the liver of rats was measured simultaneously in a separate experiment (Fig. 4). At all times (from 3 to 40 h after i.v. injection) there was an essentially parallel decline of drug concentrations in the plasma and in the liver with a liver : plasma concentration ratio of 5·7 (0·3) mean with s.d., n = 7).

Table 1. *Liver : plasma distribution ratio and serum protein binding of* R,S(±)*-phenprocoumon,* S(−)*-phenprocoumon and* R(+)*-phenprocoumon in rats.*[a]

	R,S(±)	*S*(−)	*R*(+)	*t*-test[d]
Time[b] (h)	28·5	24	34	
Plasma concn ($\mu g\ ml^{-1}$)	0·42 (0·02) (n = 4)	0·41 (0·04) (n = 5)	0·42 (0·02) (n = 5)	NS
Liver concn ($\mu g\ g^{-1}$)	2·43 (0·07) (n = 4)	2·84 (0·07) (n = 5)	2·16 (0·05) (n = 5)	$P < 0{\cdot}001$
Liver : plasma concn ratio	5·8 (0·25) (n = 4)	6·9 (0·5) (n = 5)	5·2 (0·2) (n = 5)	$P < 0{\cdot}001$
Free concn in serum[c] (% of total concn)	0·97 (0·05) (n = 6)	1·13 (0·06) (n = 6)	0·76 (0·07) (n = 6)	$P < 0{\cdot}001$

[a] Values are means (s.d.), n = number of animals or experiments.
[b] Time after injection of a single dose (0·6 mg kg^{-1}) at which the animals were killed.
[c] Determined in pooled serum of 10 rats at a total concentration of 16·8 $\mu g\ ml^{-1}$.
[d] Differences between *S*(−)- and *R*(+)-phenprocoumon.

Serum protein binding

When the serum protein binding of the three forms of drug was measured by equilibrium dialysis using a total concentration of 16·8 $\mu g\ ml^{-1}$, the free concentration of the *S*(−)- was about 1·5 times higher than that of *R*(+)-enantiomer with the racemate values falling between (Table 1).

DISCUSSION

The pharmacokinetics and pharmacodynamics of the enantiomers of phenprocoumon were studied in

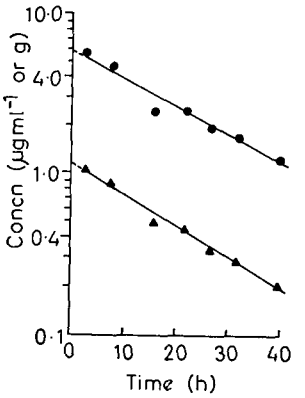

FIG. 4. Time course of racemic phenprocoumon in plasma (▲) and liver (●) of rats after intravenous injection of 0·6 mg kg^{-1}. Values for plasma and liver concentration were obtained at the same time from each of 7 animals killed at various times after drug.

inbred Wistar-Lewis rats because of the small intersubject differences which appeared with respect to the measured parameters. Such homogeneity of the animal material under investigation was also confirmed for warfarin (Martini & Jähnchen, unpublished observation). This is in contrast to results obtained with dicumarol and warfarin in outbred Sprague-Dawley rats, where large intersubject differences exist in the elimination, distribution and intrinsic anticoagulant activity (Jähnchen & Levy, 1974; Yacobi, Wingard & Levy, 1974; Yacobi, Lai & Levy, 1975). When Wistar-Lewis rats were used, highly reproducible results were obtained and this permits conclusive studies in a limited number of animals.

The enantiomers of phenprocoumon differ widely in their anticoagulant activities (Fig. 1). From the total plasma concentration (bound and free) required to produce a defined intensity of anticoagulant effect, the *S*-enantiomer is 4 to 5 times more potent than the *R*-enantiomer (Fig. 2). This potency ratio is higher than that observed in man, where a ratio of about 2 was established (Jähnchen & others, 1976). Studies in rats on the anticoagulant activity of the enantiomers of warfarin revealed a mean ratio between 2 and 3 (Breckenridge & Orme, 1972; Yacobi & Levy, 1974).

In male Wistar-Lewis rats the biologic half-life of *R*-phenprocoumon is about 1·4 times as long as that of *S*-phenprocoumon. Since phenprocoumon is eliminated in rats mainly by biotransformation (Haddock, Trager & Pohl, 1975) and is also highly bound to plasma proteins (Table 1), stereoselective differences in either rate of metabolism or drug distribution (or both) could account for the slower elimination of *R*-phenprocoumon. To resolve this question, drug distribution between liver and plasma was measured for all three forms (Table 1). It is evident, that the liver concentration of *S*- is significantly higher than that of *R*-, resulting in a 1·3 times higher liver : plasma concentration ratio for *S*-. Equilibrium dialysis studies with rat serum of untreated animals revealed that the free concentration of *S*- is 1·5 times higher than that of *R*-phenprocoumon. If such differences in the protein binding also occur *in vivo* (because of the detection limit of the analytical method it was not possible to measure the protein binding in native serum samples of treated animals) the differences in the liver : plasma ratio of the enantiomers can be explained by the different degree of binding of the enantiomers to serum proteins. From these results it appears, that the differences in the biologic half-life of the enantiomers can be related to differences in the distribution of the drugs between plasma and liver. This was explored further by applying the pharmacokinetic relation proposed by Levy (Nagashima, Levy & Sarcione, 1968; Levy & Nagashima, 1969; Levy, 1970; Jähnchen & Levy, 1974), where the apparent first-order elimination rate constant (k_{app}, which is equal to 0·693/biologic half-life) is a function of the fraction of the dose in the liver (F) and of the intrinsic elimination rate constant (k). While the k_{app} value reflects both metabolism and distribution, the k value may be looked upon as a measure of the activity of the metabolizing enzyme system(s). Table 2 shows that the differences observed in the k_{app} values of the enantiomers are mainly due to F, while the k values differ only slightly. However, these differences in the liver : plasma concentration ratio cannot be recognized by measuring the apparent volume of distribution. Similar V_d-values were obtained for the enantiomers and racemate.

Table 2. *Apparent elimination rate constant* (k_{app}) *and intrinsic elimination rate constant* (*k*) *for* R,S(±)-*phenprocoumon*, S(−)-*phenprocoumon and* R(+)-*phenprocoumon in rats.*[a]

	R,S(±)	*S*(−)	*R*(+)	*t*-test[d]
Fraction of dose in the liver (F)	0·345 (0·022) (n = 4)	0·399 (0·041) (n = 5)	0·295 (0·021) (n = 5)	$P < 0.005$
kapp[b] (h^{-1})	0·0464 (0·0051) (n = 10)	0·0556 (0·0054) (n = 9)	0·0392 (0·0039) n(= 10)	$P < 0.001$
k[c] (h^{-1})	0·134 (0·010) (n = 4)	0·139 (0·014) (n = 5)	0·133 (0·006) (n = 5)	NS

[a] Values are mean (s.d.).
[b] Obtained from the first and second experiment.
[c] k = *kapp*/F.
[d] Differences between *S*(−) and *R*(+)-phenprocoumon.

These studies have several interesting implications. First, there are intriguing species differences in the distribution of the enantiomers of the drug between rat and man, which can be related to stereoselective differences in plasma protein binding. Equilibrium dialysis studies with human serum albumin revealed that *S*- is bound to a higher degree than *R*-phenprocoumon (Jähnchen & others, 1976). The opposite is true in rat serum (Table 1). There is evidence that optical enantiomers can bind to plasma proteins to a different degree (Karush, 1952; Nazareth, Sokoloski & others, 1974; Sellers & Koch-Weser, 1975). There is also evidence that the relative proportion of the binding strength of enantiomers may differ from one species to another (Müller & Wollert, 1974, 1975). However, a complete inversion of the

binding strength in two species has not been described to our knowledge. In keeping with these protein binding studies is the observation that the plasma clearance of *S*-phenprocoumon is slower in man (Jähnchen & others, 1976; Hewick & Shepherd, 1976) but faster in rats than *R*-phenprocoumon.

A second interesting aspect appears by comparing the pharmacokinetics in rats of the enantiomers of the structurally related anticoagulant warfarin. The biologic half-life of *S*-(−)-warfarin is 1·4 to 1·8 times longer (Breckenridge & Orme, 1972; Hewick, 1972; Yacobi & Levy, 1974) and the binding to rat serum is higher (the mean ratio of the free fraction values *R*(+)-: *S*(−)- in the serum of 11 adult male Sprague Dawley rats being about 1·6; Yacobi & Levy, 1977) when compared to *R*(+)-warfarin. As in our study, the apparent volume of distribution is similar for the enantiomers of warfarin (Breckenridge & Orme, 1972; Yacobi & Levy, 1974). The opposite results are demonstrated for the enantiomers of phenprocoumon with respect to the elimination kinetics and plasma protein binding. Stereoselective differences in drug distribution between plasma and liver as shown here for phenprocoumon have not so far been detected for warfarin.

The anticoagulant potency, however, is in the same direction for the warfarin and phenprocoumon enantiomers in rats and man. Quantitative differences in the potency ratio between the enantiomers of both drugs are described above, are based on total drug in the plasma. They could be minimized, however, when related to free drug concentration in the plasma. This provides further evidence that the differences in the pharmacokinetics are not responsible for the profound differences in the anticoagulant effect.

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