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Comparative Pharmacokinetics of Coumarin Anticoagulants XXIX: Elimination Kinetics and Anticoagulant Activity of (*S*)-(−)-Warfarin in Rats before and after Chronic Administration

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Abstract □ The kinetics of elimination and the anticoagulant effect of (*S*)-(−)-warfarin were determined in adult male rats before and after daily drug administration for 13 days. There was a small but statistically significant ($p < 0.05$) decrease in the body clearance of (*S*)-(−)-warfarin (from 4.84 to 4.37 ml/hr/kg) and an increase in the serum free fraction of racemic warfarin (added to serum *in vitro*) from 0.00850 to 0.0107 ($p < 0.05$). The concentration of (*S*)-(−)-warfarin in serum at which the synthesis rate of prothrombin complex activity is one-half of the prewarfarin rate increased from 0.532 to 0.655 μg/ml on the average ($p < 0.05$).

Keyphrases □ Warfarin—elimination kinetics and anticoagulant activity, effect of chronic administration, rats □ Elimination kinetics—warfarin, effect of chronic administration, rats □ Anticoagulants—warfarin, elimination kinetics and activity, effect of chronic administration, rats □ Coumarins—warfarin, elimination kinetics and anticoagulant activity, effect of chronic administration, rats

The coumarin anticoagulants act by inhibiting the synthesis of the vitamin K-dependent clotting factors II (prothrombin), VII, IX, and X. This inhibitory effect is accompanied by the formation of so-called abnormal prothrombin in humans (1–4), oxen and cows (5–8), and rats (9–12). In humans treated with a coumarin anticoagulant, abnormal prothrombin can be detected within 8–12 hr after drug administration and becomes the predominant form of prothrombin in plasma after 24–84 hr (3). The earlier investigations suggested that abnormal prothrombin has no coagulant activity; more recently, it has become apparent that there are several abnormal prothrombins and that some do have activity, but considerably less than that of normal prothrombin (4, 8). Apparently, abnormal prothrombin is a precursor of normal prothrombin and accumulates during treatment with coumarin anticoagulants, because these vitamin K antagonists interfere with the conversion of the precursor to its fully biologically active form (9, 11, 12).

The clinical implications of the accumulation of abnormal forms of prothrombin during chronic treatment with dicumarol or warfarin are not known. In view of the potential hazards of conducting such studies in humans, an investigation was carried out in rats to determine the relationship between the anticoagulant effect and the warfarin concentration in plasma before and after chronic drug administration. While the results may differ quantitatively from those in humans, it is considered likely that they will reflect in principle the events that may be encountered clinically.

EXPERIMENTAL

This investigation was carried out in five phases: (*a*) screening of rats for serum protein binding of warfarin, (*b*) administration of a single large dose of warfarin to rats whose serum free fraction of warfarin varied widely and determination of the time courses of drug concentration and anticoagulant activity in plasma, (*c*) daily administration of a maintenance dose of warfarin to these rats for 13 days, (*d*) administration of a second large dose of warfarin and determination of the time courses of drug concentration and anticoagulant activity in plasma, and (*e*) determination of serum protein binding of warfarin.

A 3-ml blood sample was taken from the tail artery of 26 adult male Sprague–Dawley rats, and the serum was separated. The serum was spiked with racemic ^{14}C-warfarin, about 1 μg/ml, and the free fraction was determined by equilibrium dialysis (13).

Based on the results of the screening study, 12 rats with widely differing serum free fraction values for warfarin were selected. Their body weights ranged from 350 to 440 g during all phases of the investigation. They received a 0.6-mg/kg iv injection of ^{3}H-(*S*)-(−)-warfarin (specific activity, 1.43 mCi/mg).

Blood samples (0.45 ml) were taken serially from the tail artery until prothrombin complex activity had returned to between 60 and 80% of the prewarfarin level. Plasma warfarin concentrations were determined by scintillation counting after extraction and TLC using a slight modification of a previously described method (14). To 0.2-ml samples of plasma was added 5 μl of unlabeled (*S*)-(−)-warfarin, 1 mg/ml, in acetone solution. The samples were then acidified and extracted with 2.5 ml of ethylene dichloride from which 2 ml was evaporated under nitrogen for chromatography (14). Recovery of ^{3}H-(*S*)-(−)-warfarin from spiked samples was 88.3 ± 2.4% (mean ± *SD*, $n = 16$) in the 0.013–6.33-μg/ml concentration range and was independent of concentration. Determinations of prothrombin complex activity and pharmacokinetic calculations were carried out as previously described (14).

After completion of the single-dose warfarin study, the rats received daily injections of ^{3}H-(*S*)-(−)-warfarin, 83–98 μg/kg ip, for 13 days to maintain prothrombin complex activity synthesis rate (R_{syn}) at about 30% of normal.

Two days after the last maintenance dose, the rats received another

Table I—Pharmacokinetic Constants of (*S*)-(−)-Warfarin before and after Chronic Drug Administration to Selected Rats

Constant	Range		Mean		Ratio[a], Before/After
	Before	After	Before	After	
Half-life, hr	10.4–46.7	10.3–47.8	22.4	24.1[b]	0.938 ± 0.114
Volume of distribution[c], ml/kg	88.2–145	86.7–145	117	113[b]	1.05 ± 0.089
Total clearance, ml/hr/kg	1.68–9.58	1.47–8.13	4.84	4.37[d]	1.14 ± 0.180
Serum free fraction of racemic warfarin, × 100	0.247–1.74	0.238–2.31	0.850	1.07[d]	0.819 ± 0.121

[a] Mean ± *SD*, $n = 11$. [b] Not statistically significantly different from the Before value. [c] The apparent volume of distribution after chronic administration was determined by dividing the intravenous dose by the extrapolated zero-time drug concentration minus the concentration immediately before injection. [d] Statistically significantly different ($p < 0.05$) from the Before value by paired t test.

0.6-mg/kg iv dose and serial blood samples were again obtained for the determination of warfarin concentration and prothrombin complex activity.

Ten days after the second large intravenous dose, the rats were sacrificed by removing blood from the aorta under ether anesthesia. Serum was separated and used for the determination of the serum free fraction values of racemic and (*S*)-(−)-warfarin.

The pharmacokinetic studies were preceded by an experiment to rule out artifacts due to possible tritium exchange. Two rats received a very large dose of (*S*)-(−)-warfarin (12 mg/kg iv) together with ^{3}H-(*S*)-(−)-warfarin, 0.6 mg/kg iv. Blood samples were obtained repeatedly over 29 hr and assayed by scintillation counting as previously described as well as by spectrophotometry (15). The ratio of warfarin concentrations determined by the two methods was 1.01 ± 0.05 (mean ± *SD*, $n = 7$) and was independent of time after injection.

RESULTS

The rats selected had serum free fraction values for racemic warfarin ranging from 0.00247 to 0.0174 (representing from 98.26 to 99.75% protein binding). Since one of the 12 rats died during the study, data from only 11 animals are reported. Figure 1 shows the time course of warfarin concentrations in the plasma of two animals, the most rapid and the slowest eliminator of the drug, before and after chronic warfarin administration. The results of all pharmacokinetic studies are summarized in Table I. As intended by selection of rats with widely varying serum warfarin free fraction values, the pharmacokinetic constants differed appreciably between animals. These constants changed only slightly after chronic drug administration, but the increases in total clearance and in serum free fraction were statistically significant.

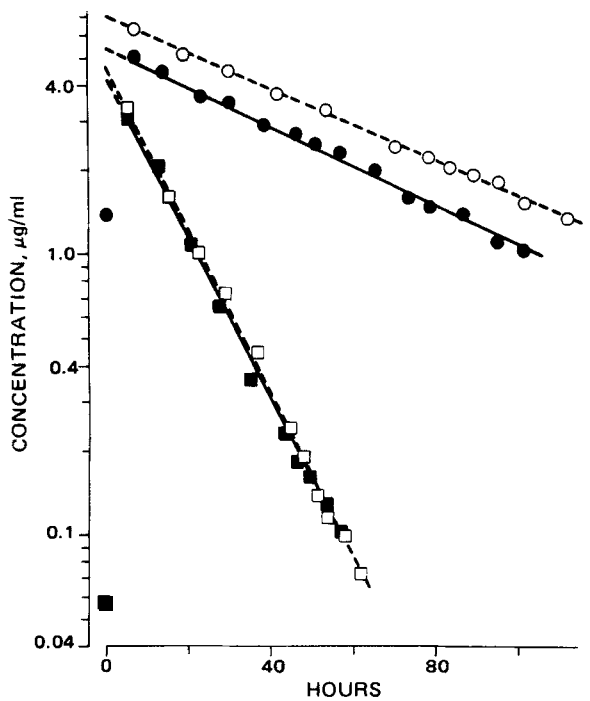

Figure 1—*Concentration of warfarin in serum of the rat with the highest clearance (squares) and the rat with the lowest clearance (circles) after intravenous injection of* (S)-(−)-*warfarin, 0.6 mg/kg, before (solid symbols) and after (open symbols) daily administration of the drug for 13 days. The symbols at zero time are the warfarin concentrations immediately before injection of the final dose.*

Strong and highly statistically significant ($p < 0.001$) correlations were observed between the pre- and postchronic administration values for the following pharmacokinetic constants in individual animals: serum free fraction of racemic warfarin ($r = 0.976$), biological half-life ($r = 0.957$), apparent volume of distribution ($r = 0.876$), and total clearance of (*S*)-(−)-warfarin ($r = 0.910$). The ratio of serum free fraction values, racemic/(*S*)-(−)-warfarin, was 1.28 ± 0.175 (mean ± *SD*) after chronic administration. Consistent with previously developed theory and experimental data on racemic warfarin (16), the total clearance of (*S*)-(−)-warfarin was proportional to the serum free fraction value of the drug in individual animals (Fig. 2).

The prothrombin complex activity of the animals before administration of the first warfarin dose was 105 ± 2% of the normal standard. Forty-eight hours after the last chronic (maintenance) dose, the prothrombin complex activity ranged from 30.5 to 91.7% (mean, 68.6%) of the standard. Animals with the highest total clearance of warfarin showed the most rapid recovery from the anticoagulant effect of the drug.

As in previous studies, the relationship between anticoagulant effect (inhibition of synthesis rate of prothrombin complex activity) and the logarithm of plasma warfarin concentration was essentially linear and could be characterized by a slope value (m) and a point. The latter is reported here as the concentration of (*S*)-(−)-warfarin at which the synthesis rate of prothrombin complex activity (R_{syn}) is one-half of normal (R^0_{syn}). The results obtained before and after chronic warfarin administration are presented in Table II. There was no significant change in slope, but there was a significant lateral shift of the effect–log concentration curve toward a higher concentration range, as reflected by the statistically significant increase in concentration at 0.5 R^0_{syn} after chronic warfarin treatment.

DISCUSSION

The two enantiomers of warfarin differ from one another with respect to elimination kinetics and anticoagulant potency (15). The ratio of plasma concentrations of the enantiomers changes, therefore, as a function of time (17). To circumvent possible interpretive problems in data analysis due to changing enantiomer concentration ratios, (*S*)-(−)-warfarin rather than the racemic mixture of (*S*)-(−)- and (*R*)-(+)-warfarin was used. As a further precaution, labeled drug was used not only for the single-dose experiments before and after chronic drug administration but also for the administration of maintenance doses. The use of unlabeled warfarin for maintenance dosing could introduce problems if a "deep" compartment exists for the drug that is not apparent upon administration of single doses.

Because of the pronounced interindividual differences in the serum protein binding of warfarin and of the consequent differences in the total clearance of the drug in rats (13), the serum free fraction of warfarin was determined in a relatively large group of rats and a smaller group with a relatively even and wide distribution of free fraction values was selected. It was possible, therefore, to examine the effect of chronic drug administration on rapid as well as on slow eliminators of warfarin.

There were only small and statistically not significant differences in the biological half-life and apparent volume of distribution of (*S*)-(−)-warfarin before and after chronic drug administration. However, the approximately 14% decrease in total clearance (based on individual clearance ratios) was statistically significant. Since the serum protein binding of racemic warfarin decreased by about 18% (based on individual free fraction ratios), the *intrinsic* clearance of warfarin (total clearance/free fraction) decreased even more than the total clearance. This conclusion is justified since there is a strong and linear correlation between the serum free fraction values of (*S*)-(−)- and racemic warfarin (18).

Essentially linear correlations were observed between the serum free fraction and total clearance values before and after chronic drug administration. These observations indicate that the changes in pharmacokinetic parameter values were of the same *relative* magnitude in

Table II—Relationship between Anticoagulant Effect and Serum Warfarin Concentration before and after Chronic Administration of (*S*)-(−)-Warfarin to Selected Rats

Rat	− Slope (− *m*)		Ratio of *m* Values, Before/After	Plasma Concentration at 0.5 R^0_{syn}, μg/ml		Ratio of Concentrations, Before/After
	Before	After		Before	After	
1	1.13	0.966	1.17	0.163	0.129	1.26
2	1.63	1.22	1.34	0.178	0.166	1.07
3	1.16	1.49	0.778	0.191	0.223	0.856
4	1.53	1.25	1.22	0.232	0.315	0.736
5	0.962	1.00	0.962	0.155	0.158	0.981
6	1.07	1.53	0.699	0.266	0.255	1.04
7	1.89	1.88	1.00	0.898	1.15	0.781
8	1.50	—[a]	—	0.529	—[a]	—
9	1.86	1.84	1.01	0.843	0.998	0.845
10	1.52	1.79	0.849	1.11	1.42	0.782
11	1.26	2.33	0.541	1.28	1.74	0.736
Mean[b]	1.40	1.53[c]	0.957	0.532	0.655[d]	0.909
SD			0.247			0.173

[a] This rat was given vitamin K to prevent bleeding. [b] All means without Rat 8. [c] Not statistically significantly different from the Before value. [d] Statistically significantly different ($p < 0.05$) from the Before value.

rapid and slow eliminators of warfarin. The reasons for these changes are not evident. They could be age dependent, but this possibility is unlikely since the time interval between experiments was well within the middle age phase of the animals. Another possibility, also entirely speculative, is that accumulation of drug metabolites caused product inhibition of warfarin biotransformation.

The anticoagulant effect–log plasma warfarin concentration curves were significantly shifted to the higher concentration range after chronic drug administration. The magnitude of this shift was even greater in terms of unbound drug concentration, since plasma protein binding of warfarin decreased somewhat after chronic drug administration. It would have been desirable to monitor steady-state drug concentrations and prothrombin complex activity during chronic administration, but this could not be done because the number of blood withdrawals from the tail artery of the rat is limited by scarification of the tail at the site of puncture.

The shift of the anticoagulant effect–log drug concentration curve toward higher concentrations could be due to changes in the normal rates of synthesis or degradation of vitamin K-dependent clotting factors or in the interaction between warfarin and its site of action. Accumulation of warfarin metabolities with anticoagulant activity would have had the opposite effect. On an entirely hypothetical basis, warfarin metabolites with no anticoagulant activity might, in fact, compete with warfarin at the latter's site of action and thereby act as inhibitors of the anticoagulant effect of warfarin. Finally, the observed effect can be a consequence of the accumulation, during warfarin treatment, of abnormal forms of prothrombin with some coagulant activity (4, 8). This possibility is most attractive, since it is based on experimental evidence rather than on inferences.

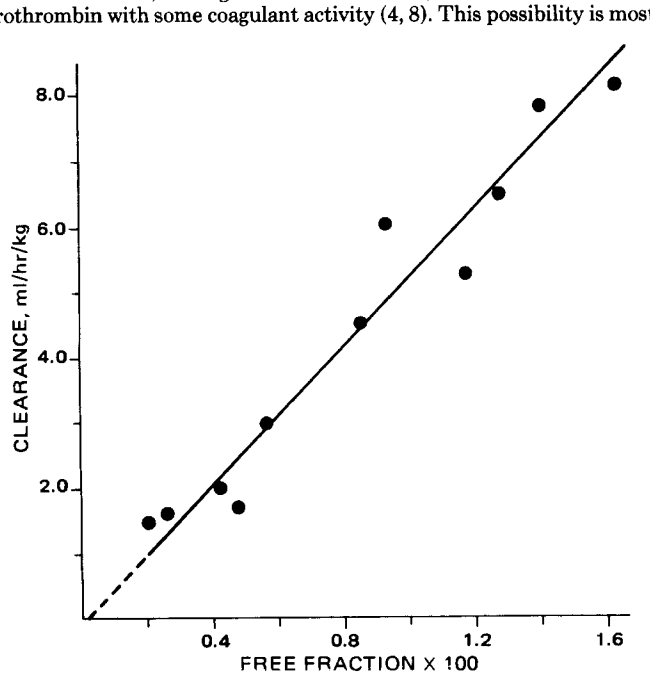

Figure 2—*Relationship between the total clearance and the serum free fraction of* (S)-(−)-*warfarin in 11 rats after chronic dosing* (r = *0.972*, p < *0.001*).

The changes in anticoagulant activity and total clearance of warfarin after chronic administration, as observed in this study, are relatively small and tend to cancel out. After chronic warfarin administration, higher drug concentrations are required to produce a given degree of anticoagulation, but the same dosing rate causes higher warfarin concentrations since drug clearance is decreased. The magnitude of these opposing effects in humans remains to be determined. It is possible that they occur in the initial "loading" or presteady-state phase of warfarin therapy and, therefore, may have little or no effect on the therapeutic management of patients receiving anticoagulant therapy.

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