Comparative Pharmacokinetics of Coumarin Anticoagulants XLIV: Dose-Dependent Pharmacokinetics of Warfarin in Rats

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Abstract □ The purpose of this investigation was to determine the effect of dose on warfarin pharmacokinetics in rats. First, in a crossover experiment, rats received ^{14}C-warfarin, 0.2 mg/kg iv, 12 hr after an injection of either nonradioactive warfarin (0.5 mg/kg) or saline solution. Warfarin concentrations in plasma declined triexponentially as a function of time. Pharmacokinetic analysis revealed that pretreatment with warfarin significantly decreased the apparent volume of distribution, total plasma clearance, and intrinsic plasma clearance of the drug. In the second part of the investigation, rats received single intravenous warfarin injections in the order of 0.1–1.0–0.1 or 1.0–0.1–1.0 mg/kg at 2-week intervals. The apparent volume of distribution, total plasma clearance, and intrinsic plasma clearance of the 1.0-mg/kg warfarin dose were appreciably lower than those of the 0.1-mg/kg dose. The decrease in the apparent volume of distribution of warfarin with increasing dose is consistent with the previously observed concentration dependence in hepatic uptake of the drug.

Keyphrases □ Coumarin anticoagulants—warfarin, dose-dependent pharmacokinetics, rats □ Warfarin—dose-dependent pharmacokinetics, rats □ Pharmacokinetics—warfarin, dose dependence, rats

Hepatic uptake of warfarin in rats, as reflected by the ratio of total warfarin concentration in the liver to the free warfarin concentration in serum during the postdistributive (β) phase, is highly dose or concentration dependent. A determination of warfarin concentrations in serum and liver of rats 6 hr after injection of either 0.1 or 1.0 mg/kg showed that concentrations in serum increased much more than proportionally and that concentrations in the liver increased much less than proportionally with dose (1). Therefore, the liver–serum concentration ratio decreased as the dose was increased from 0.1 to 1.0 mg/kg.

Since warfarin in the liver can account for a large fraction of the total drug in the body of rats (2), dose-dependent hepatic uptake may result in a dose-dependent change in the apparent volume of distribution of the drug. This possibility, as well as the possibility of other dose-dependent changes in warfarin pharmacokinetics in rats, was explored in this investigation. The nonlinear uptake or binding of warfarin by the liver in a concentration range in which serum protein binding of the drug is almost independent of concentration provides an opportunity to study a system that has been the subject of considerable theoretical interest and exploration (3, 4).

EXPERIMENTAL

The investigation was carried out in two parts. First, the pharmacokinetics of a small ^{14}C-warfarin dose were determined in a crossover study on rats that had been given either saline solution or a larger dose of nonradioactive warfarin 12 hr earlier. Second, in a triple crossover study, rats received either a high, then a low, and then again a high dose of warfarin or a low, then a high, and then again a low dose of the drug.

In the first part of the study, 10 male Sprague–Dawley rats, 360–505 g, were used. Rats 1a–4a were obtained from a supplier[1] who was unable to provide additional animals at that time; therefore, Rats 6a–10a were obtained from a different source[2]. A two-piece cannula of silicone rubber–polyethylene was implanted in the right jugular vein, under light ether anesthesia (5, 6), 2 days before the start of the experiment. The rats were placed in individual plastic metabolism cages with food[3] and water freely available.

Five rats received an intravenous injection of 0.9% sodium chloride solution while the other five animals received an intravenous injection of nonradioactive racemic warfarin, 0.5 mg/kg, through the cannula at 8 pm. The injection volume was ~2 ml/rat. Twelve hours later, all animals received an intravenous injection of racemic ^{14}C-warfarin[4] (158 μCi/mg), ~3.5 μCi/rat, together with enough nonradioactive racemic warfarin to constitute a total dose of 0.2 mg/kg. Mainstream blood samples (0.15–0.20 ml) were obtained at 5, 15, 30, 45, 60, 80, 100, 120, 150, and 200 min and then at less frequent intervals for ~50 hr by a technique described previously (6), except that saline solution rather than heparinized saline solution was used to displace blood from the external portion of the cannula.

The blood was transferred to heparinized capillary tubes[5], which were

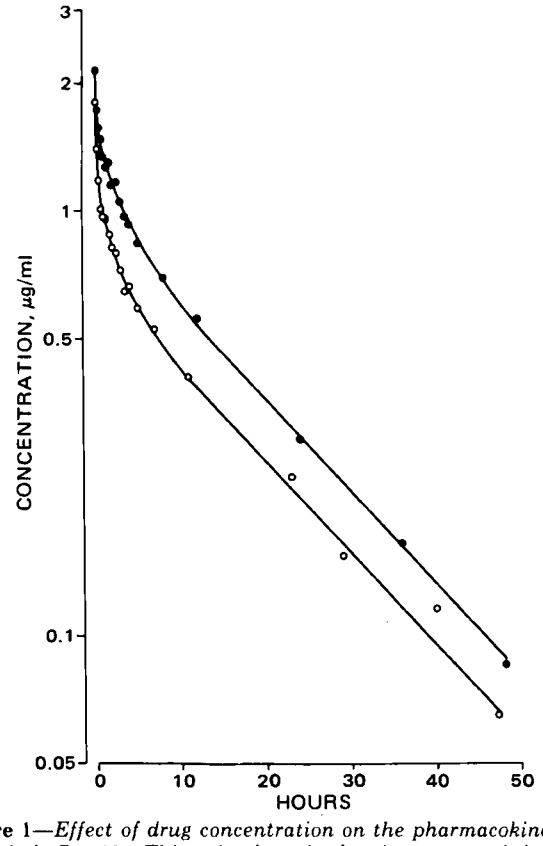

Figure 1—*Effect of drug concentration on the pharmacokinetics of warfarin in Rat 10a. This animal received an intravenous injection of ^{14}C-warfarin 12 hr after injection of saline solution (control experiment, open circles) and, 1 week later, the same dose of ^{14}C-warfarin 12 hr after intravenous injection of nonradioactive warfarin, 0.5 mg/kg (solid circles). The data points show the time course of ^{14}C-warfarin concentrations in serum. The lines were fitted to the data by computer.*

[1] Blue Spruce Farms, Altamont, N.Y.

[2] Holtzman Co., Madison, Wis.

[3] Charles River RMH 1000, Agway Inc., Syracuse, N.Y.

[4] Amersham Corp., Arlington Heights, Ill.

[5] Caraway, Sherwood Medical Industries, St. Louis, Mo.

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Table I—Effect of Drug Concentration on the Pharmacokinetics of Warfarin in Rats: Directly Determined Pharmacokinetic Constants and Serum Protein Binding [a]

Rat	Pre-treatment[b]	Body Weight, g	Constants of the Triexponential Equation[c]						Serum Free Fraction × 100[d]		Serum Protein Concentration, g/100 ml
			P	A	B	π	α	$\beta \times 100$	0.5 μg/ml	5 μg/ml	
1a	−	503	1.23	0.384	1.03	4.47	0.400	4.28	2.39	2.96	6.2
	+	505	2.36	0.127	1.39	9.75	0.867	4.72			
2a	−	487	0.956	0.150	1.02	3.10	0.519	3.47	1.20	1.42	7.1
	+	480	1.24	0.558	1.22	5.59	0.260	3.89			
3a	−	490	1.22	0.369	1.20	4.40	0.563	4.67	1.13	1.35	6.8
	+	482	1.38	0.512	0.995	5.61	0.572	3.77			
4a	−	486	1.74	0.771	0.896	5.11	0.400	2.00	1.02	1.22	6.2
	+	456	1.61	0.608	1.32	4.02	0.145	2.44			
5a	−	388	1.96	0.187	1.43	5.83	0.505	1.11	1.51	1.71	5.8
	+	373	2.03	0.316	1.29	3.63	0.441	1.19			
6a	−	360	1.42	0.402	0.805	6.71	0.622	4.08	—[e]	—[e]	—[e]
	+	361	0.460	0.483	0.876	5.46	0.786	2.54			
7a	−	418	1.19	0.337	0.900	5.01	0.216	4.86	2.81	3.18	7.1
	+	409	1.06	0.331	0.957	3.36	0.284	5.09			
8a	−	434	1.06	0.540	0.602	4.06	0.238	5.25	3.32	3.66	6.5
	+	431	1.03	0.270	1.07	3.52	0.372	6.87			
9a	−	462	1.08	0.300	0.902	3.54	0.247	5.38	2.50	2.98	7.6
	+	441	0.766	0.602	0.971	3.74	0.355	5.26			
10a	−	458	0.963	0.485	0.679	4.53	0.398	4.88	2.66	2.80	6.0
	+	485	0.760	0.599	0.919	3.23	0.276	4.83			
Mean	−	449	1.28	0.393	0.946	4.68	0.411	4.00	2.06 ± 0.80	2.36 ± 0.88[f]	6.6 ± 0.6
SD		46	0.33	0.178	0.242	1.06	0.142	1.42			
Mean	+	442	1.27	0.441	1.10	4.79	0.436	4.06			
SD		50	0.59	0.168	0.19	1.99	0.236	1.66			
Difference between + and −		NS	NS	NS	NS	NS	NS	NS			
Without Rat 5a											
Mean	−	455	1.21	0.415	0.893	4.55	0.400	4.32	2.13 ± 0.88	2.45 ± 0.96[f]	6.7 ± 0.6
SD		45	0.25	0.173	0.183	1.04	0.147	1.05			
Mean	+	450	1.19	0.454	1.08	4.92	0.435	4.38			
SD		45	0.56	0.172	0.185	2.06	0.250	1.40			
Difference between + and −		NS	NS	NS	$p < 0.05$	NS	NS	NS			

[a] The rats received an intravenous injection of ^{14}C-warfarin, 0.2 mg/kg 12 hr after an intravenous injection of either saline solution or nonradioactive warfarin, 0.5 mg/kg, in crossover fashion 1 week apart. [b] The − equals saline injection, and the + equals nonradioactive warfarin. Even-numbered rats received saline injection in the first experiment. [c] The P, A, and B values are in micrograms per milliliter based on a 0.2-mg/kg dose; π, α, and β values are in hours^{-1}. These constants describe the time course of warfarin concentrations: $C_t = Pe^{-\pi t} + Ae^{-\alpha t} + Be^{-\beta t}$, where C_t is the total drug concentration at time t. [d] Serum was collected 5 days after the end of the second experiment, and portions were spiked with ^{14}C-warfarin to yield total concentrations of about 0.5 and 5 μg/ml. [e] Rat 6 died 2 days after the second experiment, before serum for protein binding studies could be collected. [f] Significantly different from free fraction value at a total concentration of 0.5 μg/ml ($p < 0.001$ by paired t test).

centrifuged and cut at the erythrocyte–plasma interface after determination of the hematocrit. The plasma was removed, and warfarin was extracted, separated from its metabolites by TLC, and assayed by scintillation spectrometry as described previously (7). An aliquot of the injection solution was assayed in the same manner, and the plasma warfarin concentrations were normalized for small (9.5 ± 5.9%) interindividual variations from the designated dose. Crossover experiments were carried out 7 days later; animals that previously received saline pretreatment were pretreated with nonradioactive warfarin and *vice versa*. Cannula patency was maintained by replacing the saline solution in the cannula every day.

Five days after the end of the second experiment, blood was removed from the aorta of all animals under light ether anesthesia, and serum was separated. Portions of the serum were used to determine the free fraction of warfarin by equilibrium dialysis (2, 8) after addition of racemic ^{14}C-warfarin, ~0.5 and 5 μg/ml, respectively. The dialyses were done in duplicate. Total protein concentrations in serum were determined by the method of Gornall *et al.* (9) with rat albumin as the standard.

The second part of the study was carried out on adult male Sprague–Dawley rats[1] weighing 350–485 g at the beginning of the first experiment. They were cannulated and placed in metabolism cages as described in an earlier paragraph. The animals received intravenous injections of ^{14}C-warfarin, ~3.5 μCi, together with sufficient nonradioactive racemic warfarin to constitute a total dose of either 0.1 or 1.0 mg/kg. Two rats received the low, high, and low doses in that order, with a 14-day interval between each dose. Two other animals received the doses in the order high, low, and high dose.

Two additional rats received the high dose and then the low dose but expired due to apparent infection before the third (high) dose could be administered. These were the first two animals studied, and the infection problem was not encountered in the subsequently studied animals, apparently because the nonsterile saline solution previously used to fill the cannulas was replaced by sterile saline solution.

Blood sampling and analyses were carried out as in the first part of the investigation. Serum for protein binding determinations was obtained 14 days after the end of the third experiment.

The plasma warfarin concentration data obtained in the first and second parts of the investigation were fitted to a triexponential equation ($C_t = Pe^{-\pi t} + Ae^{-\alpha t} + Be^{-\beta t}$, where C_t is the drug concentration at time t) by a nonlinear least-squares regression procedure (10). Convergence was defined as a relative change in the residual sum of squares less than 10^{-4}. Data in all functions were weighted numerically equal. The apparent volume of the hypothetical central compartment was determined by dividing the injected dose by $P + A + B$. The area under the concentration–time curve (AUC) was calculated by the trapezoidal method to the last experimentally determined concentration, to which the area Ci/β was added, where Ci is the computer-estimated value of the last experimentally determined concentration. This AUC value was used to calculate total clearance (dose/AUC) and V_{area} (dose/$AUC \cdot \beta$). The AUC also was determined from the constants of the triexponential equation as the sum of P/π, A/α, and B/β. Intrinsic clearance was calculated by dividing the total clearance by the serum free fraction (f), using the average of the f values obtained at the 0.5- and 5-μg of warfarin/ml concentrations.

Statistical analyses were done by Student's t test (paired, two tailed).

RESULTS

Results of the first part of the investigation, in which 10 rats received an intravenous injection of ^{14}C-warfarin 12 hr after an injection of saline solution or of nonradioactive warfarin in a crossover experiment, are summarized in Tables I and II. Figure 1 shows the time course of ^{14}C-warfarin plasma concentrations in a typical animal; Fig. 2 shows the re-

Table II—Effect of Drug Concentration on the Pharmacokinetics of Warfarin in Rats: Derived Pharmacokinetic Constants [a]

Rat	Pre-treatment[b]	V_c, ml/kg	V_{area}, ml/kg	Total Clearance[c], ml/hr	Intrinsic Clearance[d], ml/hr	*AUC* by Trapezoidal Method, μg hr/ml	P/π, μg hr/ml	A/α, μg hr/ml	B/β, μg hr/ml	*AUC* from Constants[e], μg hr/ml
1a	−	75.8	177	3.81	142	26.4	0.275	0.960	24.1	25.3
	+	51.5	138	3.28	122	30.8	0.242	0.146	29.4	29.8
2a	−	93.9	190	3.21	245	30.3	0.308	0.289	29.4	30.0
	+	66.2	150	2.80	214	34.3	0.222	2.15	31.4	33.7
3a	−	71.7	157	3.60	290	27.2	0.277	0.655	25.7	26.6
	+	69.2	188	3.42	276	28.2	0.246	0.895	26.4	27.5
4a	−	58.7	214	2.07	185	46.9	0.341	1.93	44.8	47.1
	+	56.5	139	1.55	138	58.9	0.400	4.19	54.1	58.7
5a	−	55.9	140	0.602	37.4	129	0.336	0.370	129	130
	+	54.9	153	0.678	42.1	110	0.559	0.717	108	109
6a	−	76.0	236	3.46	—[f]	20.8	0.212	0.646	19.7	20.6
	+	11.0	220	2.02	—[f]	35.7	0.0842	0.615	34.5	35.2
7a	−	82.3	205	4.16	139	20.1	0.238	1.56	18.5	20.3
	+	85.1	189	3.93	131	20.8	0.315	1.17	18.8	20.3
8a	−	90.9	270	6.16	177	14.1	0.261	2.27	11.5	14.0
	+	84.4	171	5.07	145	17.0	0.293	0.726	15.6	16.6
9a	−	87.7	200	4.97	181	18.6	0.305	1.21	16.8	18.3
	+	85.5	168	3.89	142	22.7	0.205	1.70	18.5	20.4
10a	−	94.0	262	5.87	215	15.6	0.213	1.22	13.9	15.3
	+	87.7	189	4.43	162	21.9	0.235	2.17	19.0	21.4
Mean	−	78.7	205	3.79	179	34.9	0.277	1.11	33.3	34.8
SD		13.7	42	1.67	72	34.4	0.046	0.66	34.9	34.8
Mean	+	75.1	171	3.11	152	38.0	0.280	1.45	35.6	37.3
SD		18.5	26	1.36	64	27.9	0.127	1.17	27.8	27.9
Difference between + and −		NS	$p < 0.05$	$p < 0.01$	$p < 0.01$	NS[g]	NS	NS	NS	NS
Without Rat No. 5										
Mean	−	81.2	212	4.15	197	24.4	0.270	1.19	22.7	24.2
SD		11.8	38	1.31	51	10.0	0.042	0.64	10.1	10.1
Mean	+	77.3	172	3.37	166	30.0	0.249	1.53	27.5	29.3
SD		18.2	27	1.12	53	12.6	0.085	1.22	12.0	12.8
Difference between + and −		NS	$p < 0.02$	$p < 0.01$	$p < 0.01$	$p < 0.01$	NS	NS	NS	$p < 0.02$

[a] Based on P, A, B, π, α, and β values in Table I. [b] See footnote *b* in Table I. [c] Dose divided by *AUC* determined by the trapezoidal method. [d] Total clearance divided by the serum free fraction value obtained by averaging the two free fraction values for each animal listed in Table I. [e] Sum of P/π, A/α, and B/β. [f] Cannot be calculated due to lack of free fraction values. [g] $p < 0.01$ upon statistical analysis of $1/AUC$ values.

sults obtained in Rat 5a, which exhibited very much lower total and intrinsic clearances of warfarin than the rest of the group. The unusual animal is also noteworthy for its very long (≃ 60 hr) warfarin half-life (which showed excellent reproducibility in the crossover study) and its low serum protein concentration, suggestive of impaired liver function.

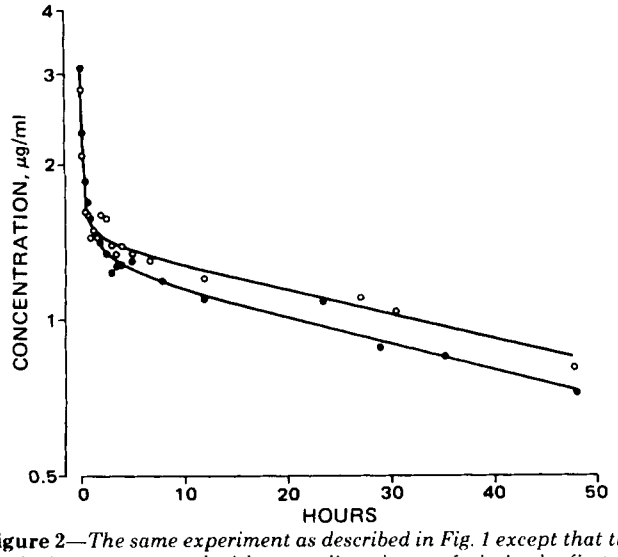

Figure 2—*The same experiment as described in Fig. 1 except that this rat (5a) was pretreated with nonradioactive warfarin in the first experiment and with saline solution in the second experiment. This rat had a much lower total clearance of warfarin and a lower serum protein concentration than the other nine animals, suggesting impaired liver function. The very long and reproducible half-life (60 hr compared to an average of 16 hr in the other rats) should be noted.*

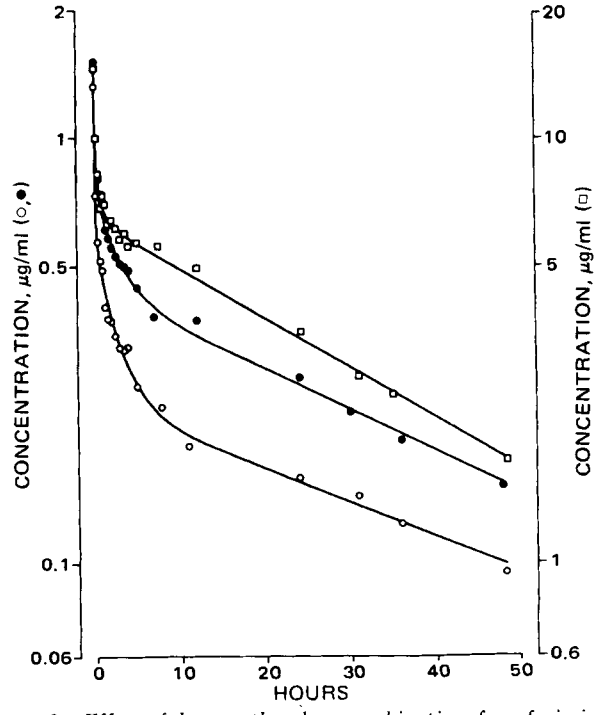

Figure 3—*Effect of dose on the pharmacokinetics of warfarin in Rat 1. This animal received intravenous injections of 0.1, 1.0, and 0.1 mg of ^{14}C-warfarin/kg at 2-week intervals. Key: ○, first dose (0.1 mg/kg); □, second dose (1.0 mg/kg); and ●, third dose (0.1 mg/kg). The data points show the time course of drug concentrations in plasma. The lines were fitted to the data by computer.*

Table III—Effect of Dose on the Pharmacokinetics of Warfarin in Rats: Directly Determined Pharmacokinetic Constants and Serum Protein Binding

Rat	Week	Dose, mg/kg	Body Weight, g	Constants of Triexponential Equation[a]						Serum Free Fraction × 100[b]	
				P	A	B	π	α	$\beta \times 100$	0.5 μg/ml	5 μg/ml
1	1	0.1	353	1.22	0.323	0.237	6.60	0.371	1.76	1.51	1.54
	3	1.0	374	10.8	2.19	6.48	6.66	1.18	2.68		
	5	0.1	393	1.11	0.341	0.446	5.41	0.409	2.18		
2	1	0.1	485	0.644	0.185	0.239	5.34	0.740	4.88	2.23	2.38
	3	1.0	452	4.27	1.10	5.99	4.71	0.471	5.87		
	5	0.1	509	0.634	0.154	0.356	4.26	0.556	4.76		
3	1	1.0	406	9.95	2.09	6.18	5.12	0.798	1.72	1.10	1.25
	3	0.1	421	1.12	0.221	0.453	4.05	0.422	2.41		
	5	1.0	442	8.99	3.85	6.97	10.3	1.51	1.57		
4	1	1.0	352	8.20	1.26	7.17	3.44	1.34	1.79	0.513	0.589
	3	0.1	380	1.01	0.316	0.525	3.94	0.567	2.22		
	5	1.0	401	9.15	1.64	7.45	5.90	0.791	1.69		
5[c]	1	1.0	457	7.60	3.21	5.36	6.84	0.685	2.51	—	—
	3	0.1	449	1.12	0.355	0.367	5.80	0.732	2.35		
	5	1.0	—	—	—	—	—	—	—		
6[c]	1	1.0	360	7.76	2.89	6.13	5.86	0.802	3.05	—	—
	3	0.1	391	1.01	0.407	0.425	5.87	0.495	2.81		
	5	1.0	—	—	—	—	—	—	—		

[a] The P, A, and B values are in micrograms per milliliter; the π, α, and β values are in hours^{-1}. These constants describe the time course of warfarin concentrations in serum; $C_t = Pe^{-\pi t} + Ae^{-\alpha t} + Be^{-\beta t}$, where C_t is the total drug concentration at time t. [b] Serum was collected 2 weeks after the end of the third experiment, and portions were spiked with ^{14}C-warfarin to yield total concentrations of about 0.5 and 5 μg/ml. [c] These animals died before the third experiment and before serum for free fraction determination could be obtained.

Therefore, the average results for the group and the statistical analyses in Tables I and II are presented not only for all 10 rats but also for the group without Rat 5a.

The time course of warfarin concentrations in plasma of all animals was best described by a triexponential equation, consistent with previous observations (11). The constants of that equation for each animal are listed in Table I. Pretreatment with 0.5 mg of warfarin/kg 12 hr earlier (a period somewhat shorter than the average biological half-life of the drug in this group of rats) had no apparent effect on the constants that characterize the so-called distribution phase of drug concentrations in plasma, *i.e.*, P, A, π, and α. There was also no apparent effect on β or, therefore, on the biological half-life of the drug. On the other hand, B was significantly increased by pretreatment with warfarin. The free fraction of warfarin in serum was almost constant over a 10-fold concentration range encompassing the maximum concentration of radioactive and nonradioactive drug in warfarin-pretreated animals and concentrations representing ≤20% of the initial warfarin concentration in these animals when they were pretreated with saline solution (Table I).

The apparent volume of the hypothetical central compartment (V_c) and the partial areas of the distribution phase, P/π and A/α, were not affected by warfarin pretreatment. However, V_{area} and total clearance were decreased significantly by that pretreatment (Table II). The intrinsic clearance of the drug decreased also. The excellent agreement of the AUC values determined by the trapezoidal method with those determined by summing the P/π, A/α, and B/β values reflects the good fit of the experimental data to the triexponential equation.

Consistent with theoretical considerations and previous observations (12), there was an essentially linear relationship between total clearance and serum free fraction of warfarin for the nine rats (*i.e.*, excluding Rat 5a), with a correlation coefficient of 0.87 ($p < 0.01$) for clearance values obtained in the control (saline pretreatment) experiments.

The second part of the study was a difficult three-way crossover experiment designed to confirm the effect of dose on V_{area} and total clearance and to examine the possible effect of dose on the distribution phase over a wider (10-fold) dose range. This experiment required measurements on each animal over 6 weeks and could be completed in only four rats during the time available. Figure 3 shows results obtained in one of two rats that received a 0.1-mg/kg dose first, a 1-mg/kg dose 2 weeks later, and another 0.1-mg/kg dose after 2 more weeks and that were sacrificed 2 weeks after the third dose to obtain serum for protein binding determinations. Figure 4 shows results obtained in one of two animals that received first the high dose, then the low dose, and then again the high dose of warfarin.

The results of the balanced three-way crossover experiment on four rats as well as results obtained from two rats that expired before the third dose are summarized in Tables III and IV. No averaging or statistical analyses were done because only two rats completed any one dosing sequence. It should be noted that P, A, and B will increase proportionally with dose if the pharmacokinetics are linear. In fact, P and A increased less than proportionally with only one exception (Rat 3, 5th week). On the other hand, B increased more than proportionally in all experiments (Table III). There were no apparently dose-dependent trends in π, α, and β. Serum free fraction values at warfarin concentrations of 0.5 and 5 μg/ml did not differ appreciably in any one animal (Table III).

The partial areas P/π and A/α did not increase proportionally with dose while B/β increased more than proportionally with dose (Table IV). There was no remarkable effect of dose on V_c, there occurred a consistent decrease of V_{area} at the higher dose, and there were consistent decreases of the total and intrinsic clearances at the higher dose. In all four rats receiving the three-dose sequence, V_c and V_{area} of the third dose were lower than those of the first (and equal) dose (Table IV).

DISCUSSION

The results of this investigation demonstrate a dose dependence in the pharmacokinetics of warfarin: V_{area}, intrinsic clearance, and, therefore, total clearance decreased with increasing dose in the dose range studied. The decrease in V_{area} is probably due, at least in part, to the previously demonstrated decreased uptake of warfarin by the liver with increasing dose (1) since the liver accounts for a major fraction of the total warfarin in the body following administration of a relatively small dose (2). Such a decrease in V_{area} is quite unusual; much more common is an increase in V_{area} with increasing dose due to decreased plasma protein binding of a drug at higher concentrations. However, O'Reilly *et al.* (13) reported a decrease in the apparent volume of distribution of dicumarol with increasing dose over the 150–600-mg dose range in adult humans. On the other hand, they did not observe such dose dependence with warfarin over the 50–200-mg dose range in the two humans studied (13). A limited study in this laboratory, carried out more than 10 years earlier when ^{14}C-warfarin was not available, revealed no apparent difference in the volume of distribution of warfarin in rats given injections of 4 and 12.5 mg/kg (14). Interestingly, the apparent volume of distribution of these very large doses (110–130 ml/kg) was slightly less than that of the 1-mg/kg dose in the present study.

Theoretical considerations suggest that, all else being equal, a reduction in liver uptake should cause an increase in β and, therefore, a decrease in the biological half-life of warfarin (15). However, the same considerations indicate that β will decrease if the intrinsic clearance is decreased. Thus, the lack of appreciable dose dependence of β in the present study may be rationalized by the opposing effects of decreased warfarin binding or uptake by the liver and decreased intrinsic clearance at the higher dose. It is possible that hepatic warfarin uptake may be due largely to specific binding of the drug to warfarin-metabolizing enzymes and that the decreased hepatic uptake and intrinsic clearance of warfarin at higher doses may be reflections of the same dose-dependent phenomenon.

Adequate blood volumes for serum protein binding studies were obtained only at the end of the crossover experiments to prevent premature exsanguination of the animals. It has been demonstrated previously in

Table IV—Effect of Dose on the Pharmacokinetics of Warfarin in Rats: Derived Pharmacokinetic Constants [a,b]

Rat	Week	Dose, mg/kg	V_c, ml/kg	V_{area}, ml/kg	Total Clearance, ml/hr	Intrinsic Clearance, ml/hr	*AUC* by Trapezoidal Method, μg hr/ml	P/π, μg hr/ml	A/α, μg hr/ml	B/β, μg hr/ml	*AUC* from Constants, μg hr/ml
1	1	0.1	56.1	390	2.43	159	14.5	0.185	0.871	13.5	14.6
	3	1.0	51.3	150	1.50	98.0	250	1.62	1.86	242	245
	5	0.1	52.7	211	1.81	118	21.7	0.205	0.834	20.5	21.5
2	1	0.1	93.4	365	8.64	374	5.61	0.121	0.250	4.90	5.27
	3	1.0	87.6	159	4.22	183	107	0.907	2.34	102	105
	5	0.1	87.6	263	6.37	276	7.99	0.149	0.277	7.48	7.91
3	1	1.0	54.9	159	1.11	61.7	365	1.94	2.62	359	364
	3	0.1	55.8	211	2.14	119	19.7	0.277	0.524	18.8	19.6
	5	1.0	50.5	144	1.00	55.6	442	0.873	2.55	444	447
4	1	1.0	60.2	139	0.873	158	403	2.38	0.940	401	404
	3	0.1	53.9	184	1.55	281	24.5	0.256	0.557	23.6	24.4
	5	1.0	54.9	134	0.905	164	443	1.55	2.07	441	445
5	1	1.0	61.7	181	2.07	—[c]	221	1.11	4.69	214	220
	3	0.1	54.3	261	2.74		16.4	0.193	0.485	15.6	16.3
	5	1.0	—	—	—		—	—	—	—	—
6	1	1.0	59.4	157	1.72	—[c]	209	1.32	3.60	201	206
	3	0.1	54.5	219	2.41		16.2	0.172	0.822	15.1	16.1
	5	1.0	—	—	—		—	—	—	—	—

[a] Based on P, A, B, π, α, and β values in Table III. [b] See footnotes *c*, *d*, and *e* in Table II for additional information. [c] Cannot be calculated due to lack of free fraction values.

this laboratory that serum free fraction values of warfarin in rats remain constant for at least 9 days (16). In another study, in which rats received a large single dose of warfarin, then daily smaller doses for 13 days, and then again a large dose, serum free fraction values were determined before and after this sequence (the time interval being ~35 days) and decreased by 18% on the average (17). We have no direct evidence that the free fraction values remained constant over 6 weeks of the present study. Therefore, there is some uncertainty concerning the *absolute* intrinsic clearance values in this study but not concerning the fact that intrinsic clearance did decrease with increasing dose (since the experiments were carried out in crossover fashion).

The three-way crossover experiments confirmed the results of the first part of the investigation in that they also demonstrated a decrease of V_{area} and intrinsic clearance with increasing dose. In addition, the three-way crossover experiments revealed a *relative* decrease in P and A and, therefore, in P/π and A/α with increasing dose. This result is probably due to an increased serum free fraction at the initial concentrations produced by the 1-mg/kg dose. It has been calculated that the free fraction of warfarin remains essentially constant at plasma warfarin concentrations up to 6 μg/ml and that it increases by 25 and >200% at concentrations of 96 and 282 μg/ml, respectively (18). Initial plasma warfarin concentrations following the 0.1- and 1-mg/kg doses in the present study were ~2 and 17 μg/ml, a difference large enough to expect some change in the free fraction. On the other hand, the first part of this study, in which V and A were not affected by pretreatment with warfarin, involved less than a twofold difference in initial concentration (considering the sum of the concentrations of ^{14}C-warfarin and nonradioactive drug) and these initial concentrations were well below 6 μg/ml.

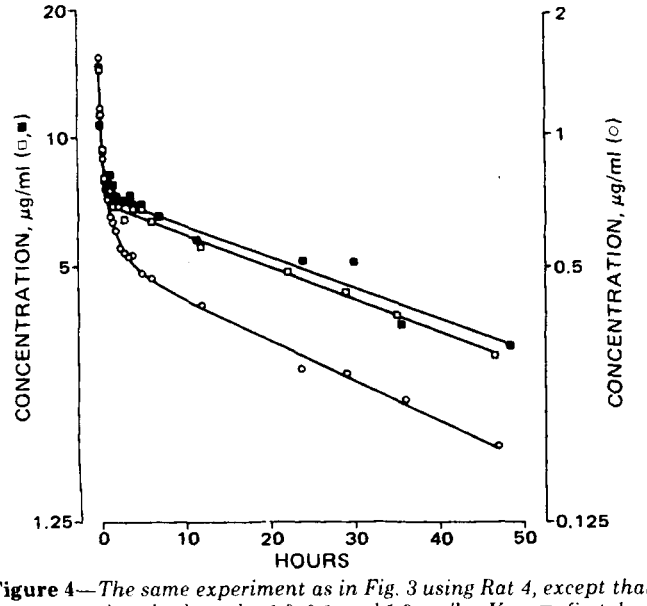

Figure 4—*The same experiment as in Fig. 3 using Rat 4, except that doses were given in the order 1.0, 0.1, and 1.0 mg/kg. Key:* □, *first dose (1.0 mg/kg);* ○, *second dose (0.1 mg/kg); and* ■, *third dose (1.0 mg/kg).*

The suggestion that the decrease in the P and A values after the 1-mg/kg dose (as compared to the corresponding values after the 0.1-mg/kg dose) is due to decreased plasma protein binding of warfarin finds support from results of a previous investigation (11). Studies of warfarin pharmacokinetics were carried out on 14 rats with widely different serum free fraction values (from 0.3×10^{-2} to 2.9×10^{-2}); all animals received a single 0.51-mg/kg injection. There was a statistically significant negative correlation between serum free fraction and P ($r = -0.873$, $p < 0.001$; the negative sign of the correlation coefficient was inadvertently omitted in the cited report).

In summary, warfarin pharmacokinetics in rats are dose dependent; the apparent volume of distribution of the drug, the intrinsic clearance, and the total clearance decrease with increasing dose in the 0.1–1-mg/kg dose range. At least one of these phenomena, *i.e.*, the decrease in the apparent volume of distribution, is probably due largely to decreased hepatic uptake of the drug. In addition, the apparent distribution phase of warfarin concentrations in plasma following intravenous injection of 1 mg/kg is less pronounced than that following a 0.1-mg/kg dose, probably due to decreased protein binding of the drug at the high initial plasma concentrations produced by the larger dose.

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Comparative Pharmacokinetics of Coumarin Anticoagulants XLV: Pharmacokinetic and Pharmacodynamic Studies of Acute Interaction between Warfarin and Phenylbutazone in Rats

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Abstract □ A comprehensive investigation of the effect of phenylbutazone on warfarin pharmacokinetics and anticoagulant activity was carried out in rats to identify and quantify various aspects of the interaction between these drugs. Adult male rats received intravenous racemic warfarin alone and together with phenylbutazone in a crossover experiment. Prothrombin complex activity and the plasma concentrations of phenylbutazone and of free and total (free plus protein-bound) warfarin were determined repeatedly for up to 60 hr. The total plasma clearance, the apparent volume of distribution, and the disposition rate constant (β) of warfarin were significantly increased and the intrinsic plasma warfarin clearance was significantly decreased during phenylbutazone administration. Phenylbutazone decreased the serum protein binding of warfarin both *in vitro* and *in vivo*, but the *in vivo* effect was much more pronounced, apparently due to the displacing effect of phenylbutazone metabolite(s). Phenylbutazone alone had no apparent effect on prothrombin complex activity *in vitro* but caused a modest, yet statistically significant, anticoagulant effect *in vivo*. The anticoagulant effect–plasma warfarin concentration curves for total and free warfarin were shifted to a considerably lower concentration range during phenylbutazone treatment. Thus, the interaction between phenylbutazone and warfarin involves at least three processes: an inhibition of warfarin biotransformation (decreased intrinsic clearance); displacement of warfarin from plasma protein binding sites (increased free fraction); and apparent potentiation of the anticoagulant action produced by a given plasma warfarin concentration. The latter may have been caused, at least in part, by a direct anticoagulant effect of phenylbutazone and/or its metabolite(s). The net effect of decreased protein binding and decreased intrinsic clearance was an increase in the total plasma warfarin clearance. The results of this investigation demonstrate that drug interactions can be complex and multifactorial.

Keyphrases □ Warfarin—interaction with phenylbutazone, pharmacokinetics, *in vitro* and *in vivo*, rats, prothrombin complex activity □ Phenylbutazone—interaction with warfarin, pharmacokinetics, *in vitro* and *in vivo*, rats, prothrombin complex activity □ Anticoagulants—warfarin, interaction with phenylbutazone, pharmacokinetics, *in vitro* and *in vivo*, rats, prothrombin complex activity

There is a tendency to attribute most drug interactions to only one of several theoretically possible mechanisms. This approach is probably unrealistic and reflects the limited scope and the relative lack of pharmacokinetic quantitation characteristic of most drug interaction studies. For example, one drug may displace another from plasma protein binding sites and cause the displaced drug to be eliminated more rapidly. Unless the increased clearance of the displaced drug is *quantitatively* consistent with the increase of its free fraction in plasma, additional interaction mechanisms must be sought. These additional mechanisms may operate in the same direction (*i.e.*, increased clearance) or in the opposite direction from that attributable to reduced plasma protein binding. Moreover, pharmacokinetic as well as pharmacodynamic interactions may occur so that an assessment of the therapeutic implications of the interacting system requires consideration of the net effect resulting from both types of interactions. These potential complexities suggest that comprehensive model drug interaction studies are needed for the development of more effective strategies for exploring potential or suspected interactions involving new drugs.

The interaction between the anticoagulant warfarin and the anti-inflammatory agent phenylbutazone is phenomenologically well established, and its potentially disastrous clinical consequences are generally appreciated (1–3). The interaction between these two drugs has been studied in humans (4–8) and in dogs (9). However, the recent development of clearance concepts (10, 11) and the availability of an animal model exhibiting wide interindividual differences in the plasma protein binding of warfarin under normal physiological conditions (12) have provided a more rational basis and a promising means for better exploration of the interaction.

The investigation described here consisted of a rigorous quantitative determination of the phenylbutazone effect on the elimination kinetics and anticoagulant action of warfarin in a crossover study on rats specially selected for wide interindividual differences in serum protein binding of warfarin and, therefore, in warfarin clearance. The phenylbutazone effect on serum protein binding of warfarin was determined *in vitro* and *in vivo*. The relationship between anticoagulant effect and warfarin concentration in plasma was determined for free and total (free plus bound) drug. The phenylbutazone effect *per se* on the coagulation process was examined *in vitro* and *in vivo*.

It is believed that the results of these studies will demonstrate the potential complexity and multifaceted characteristics of drug interactions, will illustrate the practical limitations of many drug interaction studies in humans,

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