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ACKNOWLEDGMENTS AND ADDRESSES

Received March 29, 1976, from the *Department of Pharmaceutical Chemistry, College of Pharmacy, Rutgers—the State University, New Brunswick, NJ 08903.*

Accepted for publication June 9, 1976.

The authors express their appreciation to Carole A. Bailey for technical assistance and to Dr. Thomas Medwick for helpful discussions.

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Comparative Pharmacokinetics of Coumarin Anticoagulants XXI: Effect of Plasma Protein Binding on Distribution Kinetics of Warfarin in Rats

AVRAHAM YACOBI and GERHARD LEVY [x]

Abstract □ The purpose of this investigation was to determine the effect of plasma protein binding on the pharmacokinetic parameters for warfarin that are used conventionally to describe its distribution kinetics on the basis of the time course of plasma warfarin concentrations. Following rapid intravenous injection, warfarin concentrations in the plasma of 14 selected adult male rats declined triexponentially, with the terminal exponential phase starting at about 5 hr. The free fraction, f, of warfarin in the serum of individual animals ranged from 0.303×10^{-2} to 2.89×10^{-2}. The parameters of the equation $C_t = Pe^{-\pi t} + Ae^{-\alpha t} + Be^{-\beta t}$ for plasma concentration C_t at time t were obtained from the experimental data by nonlinear least-squares computer fitting and varied markedly between animals. Strong and highly statistically significant positive correlations with f were obtained for P, B, and β, but no significant correlation was found for A, π, and α. Rate constants and apparent volumes for a three-compartment open mammillary model with elimination from the central compartment were calculated. No apparent correlation was found between f and the intercompartment distribution rate constants. However, strong positive correlations between f and the elimination rate constant, the volume of the central compartment, and the volume of distribution, V_{area}, were observed. There also was a strong linear correlation between f and total clearance. Excellent replication of the experimental data was obtained when the experiments were repeated in some animals after 2 weeks. A detailed analysis of practical pharmacokinetic problems associated with and revealed by such repeated experiments is presented.

Keyphrases □ Warfarin—distribution kinetics, effect of plasma protein binding, rats □ Distribution—pharmacokinetic parameters, effect of plasma protein binding, rats □ Pharmacokinetics—warfarin distribution, effect of plasma protein binding, rats □ Binding, plasma protein—effect on warfarin distribution kinetics, rats □ Protein binding, plasma—effect on warfarin distribution kinetics □ Anticoagulants—warfarin, distribution kinetics, effect of plasma protein binding, rats

Plasma protein binding is a major determinant of the elimination kinetics of warfarin in rats (1) and humans (2). Consistent with theoretical considerations (3), the total clearance, TC, of warfarin by the body was found to be proportional to the free fraction, f, of warfarin in plasma or serum. Wide intersubject differences in f and, consequently, in the TC of warfarin have been observed.

The purpose of this investigation was to determine the relationship between f and the pharmacokinetic constants conventionally used to describe the kinetics of warfarin distribution on the basis of the time course of drug concentrations in plasma after rapid intravenous injection. A detailed analysis of certain practical problems of pharmacokinetic data interpretation was also undertaken.

EXPERIMENTAL

Single 3-ml blood samples were obtained from 63 adult male Sprague–Dawley rats[1] for determination of f, and 14 animals with widely differing f values were selected for further study. Three weeks later, with

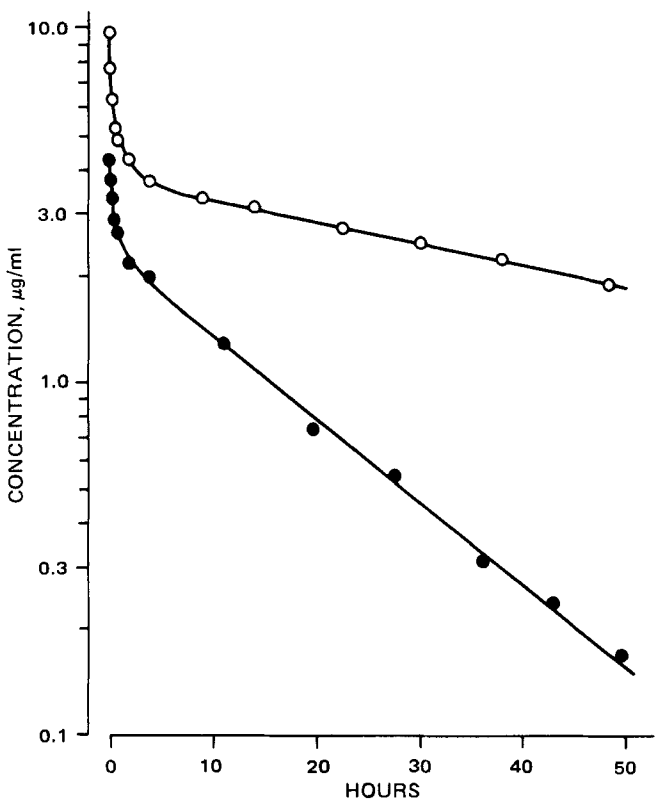

Figure 1—*Warfarin concentration in the plasma of two rats as a function of time after intravenous injection of* ^{14}*C-warfarin, 0.51 mg/kg. Key:* ●, *Rat 1; and* ○, *Rat 14. The curves were fitted to the data by a nonlinear least-squares computer program.*

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

Table I—Warfarin Kinetics in Rats

Parameter	Mean	Range
P, μg/ml[a]	4.67	1.28–8.78
A, μg/ml[a]	1.76	0.359–3.59
B, μg/ml[a]	3.08	2.36–4.10
π, hr^{-1}	6.98	2.34–19.0
α, hr^{-1}	1.03	0.348–1.83
β, hr^{-1}	0.0310	0.0117–0.0580

[a]For a 0.51-mg/kg dose.

Table II—Warfarin Kinetics in Rats

Constant	Mean	Range
k_{10}, hr^{-1}	0.0851	0.0422–0.141
k_{12}, hr^{-1}	3.11	0.134–8.73
k_{21}, hr^{-1}	4.21	1.73–9.18
k_{13}, hr^{-1}	0.640	0.0897–1.97
k_{31}, hr^{-1}	0.552	0.248–1.13
V_c, ml/kg	59.6	31.7–96.9
V_{area}, ml/kg	165	123–209
$V_{intercept}$, ml/kg	170	124–220
TC, ml/(hr × kg)	5.59	1.74–11.4

the animals then weighing 380–490 g, a two-piece cannula of silicone rubber–polyethylene was implanted in the right jugular vein under light ether anesthesia (4, 5). One or 2 days after cannulation, the rats were placed in individual metabolism cages with food[2] and water freely available.

Racemic warfarin, 0.51 mg/kg including about 5 μCi of ^{14}C-warfarin[3]/kg, was injected rapidly through the cannula; 0.25-ml blood samples were obtained at 5, 10, 20, 40, 60, and 120 min and then at less frequent intervals for about 50 hr by a technique described previously (5). Plasma was separated, and warfarin was extracted, purified by TLC, and assayed as previously described (6). At the end of the experiment, a large volume of blood was obtained for the determination of the free fraction of warfarin in serum by equilibrium dialysis (1, 7).

The warfarin concentrations in plasma as a function of time after injection were fitted to a multiexponential equation by nonlinear least-squares regression (8). Convergence was defined as a relative change in the residual sum of squares $<10^{-4}$. Data in all functions were weighted numerically equal. The parameter estimates of the multiexponential equation were used to calculate rate constants for a multicompartment open model (9). Simulations were carried out with a digital analog simulator program (10).

RESULTS

Figure 1 shows the time course of plasma warfarin concentrations in two rats, one a relatively rapid eliminator and the other a relatively slow

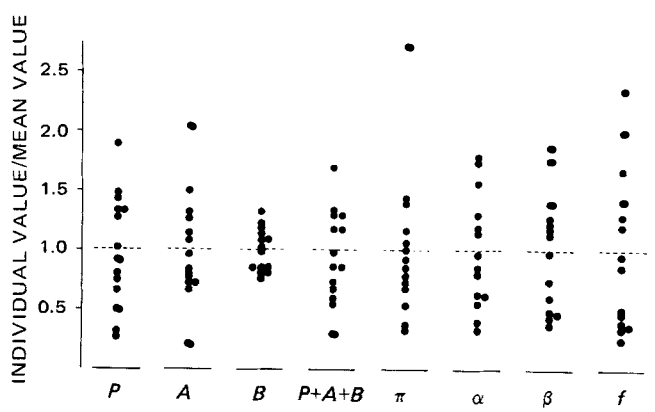

Figure 2—*Intersubject distribution of values for the parameters of the triexponential equation that describes the time course of plasma warfarin concentrations and of values for the free fraction of warfarin in the serum of 14 rats.*

[2] Charles River formula 4RF.
[3] Amersham/Searle Corp., Arlington Heights, Ill.

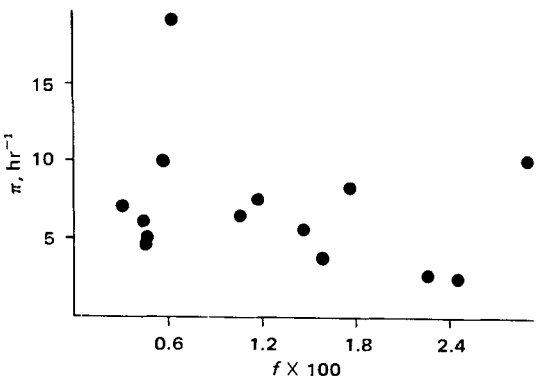

Figure 3—*Relationship between the serum free fraction, f, of warfarin and the parameter π, which characterizes the slope of the first exponential phase of the triexponential decline of warfarin concentrations in the plasma of 14 rats after intravenous injection.*

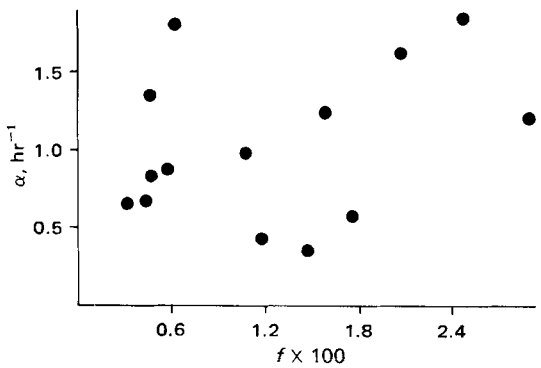

Figure 4—*Relationship between the serum free fraction, f, of warfarin and the parameter α, which characterizes the slope of the second exponential phase of the triexponential decline of warfarin concentrations in the plasma of 14 rats after intravenous injection.*

eliminator of the drug. Experimental data of similar quality were obtained for the other 12 animals. A triexponential equation of the type $C_t = Pe^{-\pi t} + Ae^{-\alpha t} + Be^{-\beta t}$ was required to describe the plasma warfarin concentrations, C_t, as a function of time, t. Substantially fewer than 20 iterations were required in all cases to obtain convergence; the correlation coefficient was ≥0.997 for each set of data.

The mean values and ranges for the parameters of the triexponential equation fitted to the individual data from 14 animals are listed in Table I. As is evident from the values of these constants and from Fig. 1, the terminal exponential phase of plasma concentrations began about 5 hr after injection. Rate constants for a linear three-compartment mam-

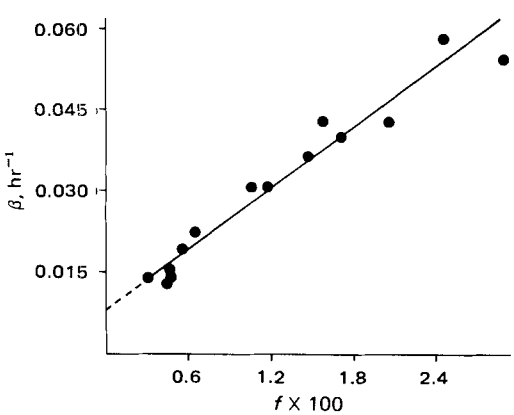

Figure 5—*Relationship between the serum free fraction, f, of warfarin and the parameter β, which characterizes the slope of the terminal exponential phase of the triexponential decline of warfarin concentrations in the plasma of 14 rats after intravenous injection. Correlation coefficient = 0.962, p < 0.001.*

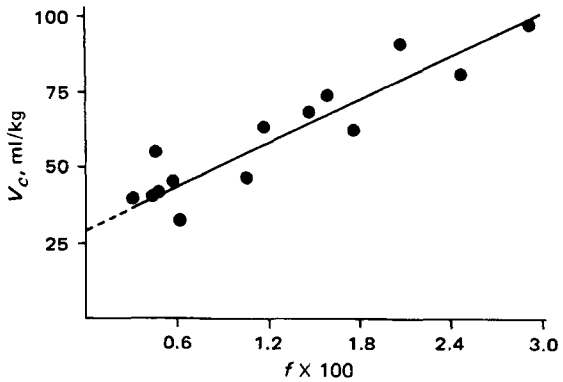

Figure 6—*Relationship between the serum free fraction, f, of warfarin and the volume of the hypothetical central compartment,* V_c *[which is dose* $(P + A + B)^{-1}$*] for warfarin in 14 rats. Correlation coefficient = 0.921,* $p < 0.001$.

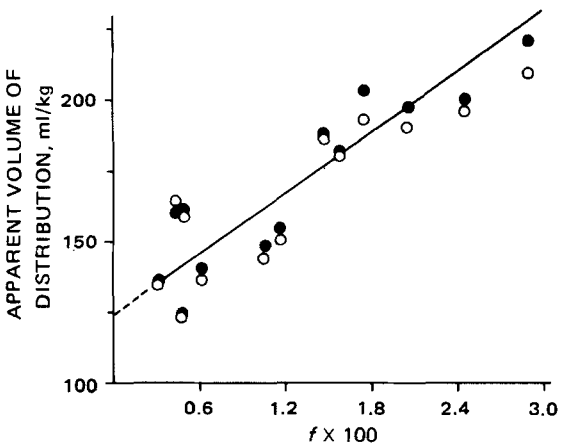

Figure 7—*Relationship between the serum free fraction, f, of warfarin and the apparent volume of distribution of the drug in 14 rats. Key:* ●, *based on the terminal exponential curve [one-compartment model, (dose)* B^{-1}*]; and* ○, *based on the triexponential curve [three-compartment model,* $V_{area} = (dose)\,(P/\pi + A/\alpha + B/\beta)^{-1}\,\beta^{-1}$*]. Regression line fitted to* ●, *correlation coefficient = 0.911,* $p < 0.001$.

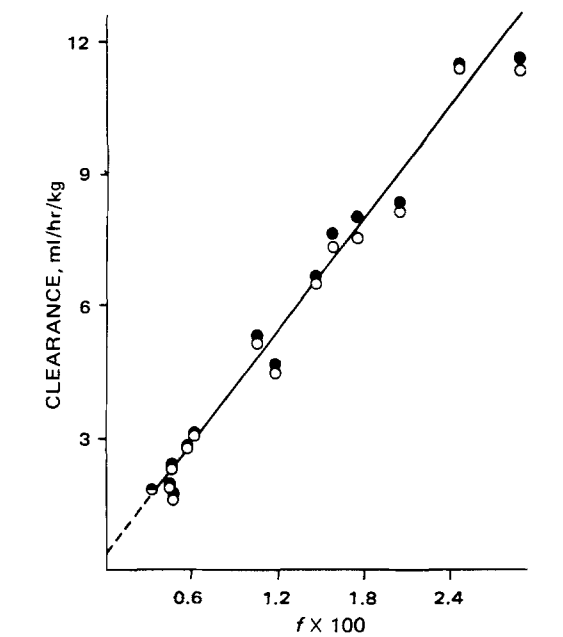

Figure 8—*Relationship between the serum free fraction, f, of warfarin and the total body clearance of warfarin in 14 rats. Key:* ●, *based on one-compartment model [(dose)* $B^{-1}\beta$*]; and* ○, *based on three-compartment model (*$V_{area}\beta$*). Regression line fitted to* ●, *correlation coefficient = 0.988,* $p < 0.001$.

Table III—Correlation between Pharmacokinetic Parameters for Warfarin in Rats and the Free Fraction of Warfarin in Serum

Parameter	Correlation Coefficient	Statistical Significance[a]
P	0.873	+
A	0.384	–
B	0.816	+
π	0.234	–
α	0.310	–
β	0.962	+
k_{10}	0.810	+
k_{12}	0.287	–
k_{21}	0.350	–
k_{13}	0.066	–
k_{31}	0.453	–
V_c	0.921	+
V_{area}	0.871	+
$V_{intercept}$	0.911	+
TC	0.988	+

[a] + = statistically significant ($p < 0.001$), and – = not statistically significant ($p > 0.1$).

millary open model, with elimination arbitrarily defined as occurring from the central compartment (Compartment 1), were obtained for each set of experimental data and are listed in Table II.

Table II also contains the values for the apparent volume of the central compartment, V_c, the apparent volume of distribution based on the area under the triexponential curve, V_{area}, the apparent volume of distribution estimated from the dose divided by B, $V_{intercept}$, and the total body clearance, $TC = V_{area}\,\beta$ or $\simeq V_{intercept}\beta$. The average ± SD of the individual ratios of $V_{intercept}/V_{area}$ was 1.03 ± 0.051, indicating only a very small contribution of the initial distribution phase to the total area under the concentration–time curve.

Figure 2 shows the distribution of values for the triexponential equation (to be referred to as the direct pharmacokinetic parameters) and for f, the serum free fraction, which ranged from 0.303×10^{-2} to 2.89×10^{-2}. Since the 14 rats were selected from 63 animals to obtain a wide range of f values, the distribution patterns in Fig. 2 are not characteristic of a normal population.

The direct pharmacokinetic parameters π and α were apparently not affected by changes in f (Figs. 3 and 4), but the β values showed a strong positive correlation with f (Fig. 5). The volumes V_c, V_{area}, and $V_{intercept}$ were also strongly and positively correlated with f (Figs. 6 and 7). Linear

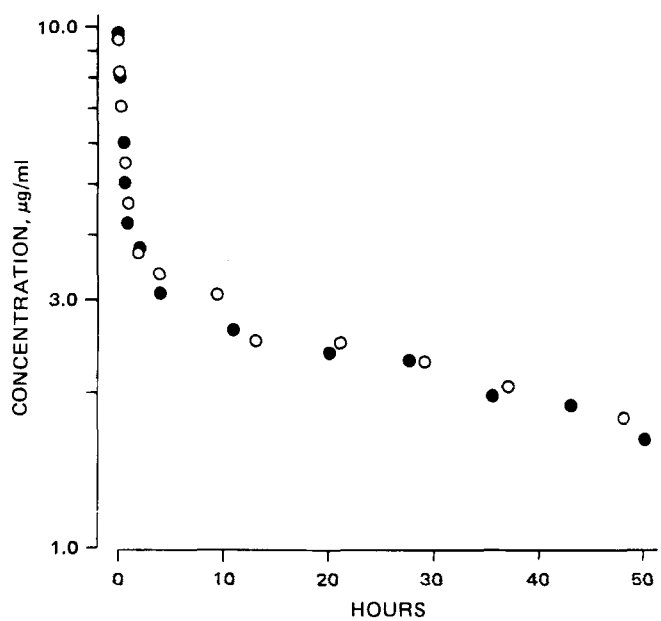

Figure 9—*Warfarin concentrations in the plasma of Rat 13 as a function of time after intravenous injection of* ^{14}C*-warfarin, 0.51 mg/kg, on two separate occasions. Key:* ●, *first experiment; and* ○, *2 weeks later.*

Table IV—Warfarin Kinetics in Rat 3 in Two Separate Experiments

Direct Parameter	First Experiment	Second Experiment[a]	Derived Constant	First Experiment	Second Experiment[a]
P, μg/ml[b]	1.38 ± 13.5[c]	5.17 ± 0.65	k_{10}, hr^{-1}	0.0894	0.160
A, μg/ml[b]	1.60 ± 13.7	0.766 ± 0.131	k_{12}, hr^{-1}	0.320	6.23
B, μg/ml[b]	2.60 ± 0.19	2.40 ± 0.12	k_{21}, hr^{-1}	2.36	4.09
π, hr^{-1}	2.87 ± 12.2	10.5 ± 1.50	k_{13}, hr^{-1}	0.711	0.208
α, hr^{-1}	1.36 ± 4.63	0.407 ± 0.153	k_{31}, hr^{-1}	0.790	0.316
$\beta \times 100$ hr^{-1}	4.28 ± 0.32	4.84 ± 0.20	V_c, ml/kg	91.4	61.2
			V_{area}, ml/kg	191	203
			TC, ml/(hr × kg)	8.17	9.82

[a] Two weeks after first experiment. [b] For a 0.51-mg/kg dose. [c] Standard deviation.

Table V—Warfarin Kinetics in Rat 8 in Two Separate Experiments

Direct Parameter	First Experiment	Second Experiment[a]	Derived Constant	First Experiment	Second Experiment[a]
P, μg/ml[b]	6.27 ± 0.45[c]	3.56 ± 0.37	k_{10}, hr^{-1}	0.113	0.0924
A, μg/ml[b]	1.27 ± 0.48	2.27 ± 0.32	k_{12}, hr^{-1}	2.95	2.12
B, μg/ml[b]	3.49 ± 0.09	3.23 ± 0.081	k_{21}, hr^{-1}	3.00	3.97
π, hr^{-1}	6.43 ± 0.97	6.32 ± 0.63	k_{13}, hr^{-1}	0.717	0.476
α, hr^{-1}	1.00 ± 0.46	0.752 ± 0.154	k_{31}, hr^{-1}	0.697	0.444
$\beta \times 100$ hr^{-1}	3.68 ± 0.10	3.42 ± 0.11	V_c, ml/kg	46.2	56.4
			V_{area}, ml/kg	142	152
			TC, ml/(hr × kg)	5.24	5.21

[a] Two weeks after first experiment. [b] For a 0.51-mg/kg dose. [c] Standard deviation.

regression lines in Figs. 5–7 do not necessarily imply an exactly linear relationship between the variables. There was a strong ($r = -0.913$) and highly statistically significant ($p < 0.001$) negative correlation between the plasma warfarin concentration at 5 min and f. This finding constitutes an independent and direct verification of the positive correlation between f and the computer-derived values of V_c. As demonstrated previously (1–3), there was a direct linear relationship between f and the total clearance of warfarin by the body (Fig. 8).

A summary of the relationship between f and the various direct and derived pharmacokinetic parameters for warfarin is presented in Table III. Of the derived constants, the elimination constant k_{10} was strongly correlated with f while the intercompartment distribution rate constants k_{12}, k_{21}, k_{13}, and k_{31} were not.

The warfarin experiment was repeated in three animals 2 weeks after the first experiment. Excellent replication of experimental data was obtained, as shown in Figs. 9 and 10 for one of the best and the "worst" case. Nevertheless, nonlinear least-squares regression analyses yielded substantial numerical intrasubject differences in P, A, π, and α (Tables IV–VI). On the other hand, there was very little intrasubject variation in B and β, and these values had small standard deviations. The same pattern obtained with respect to the derived pharmacokinetic constants and the volume terms; there was good replication of k_{10}, V_c, and V_{area} but not of the other constants (Tables IV–VI).

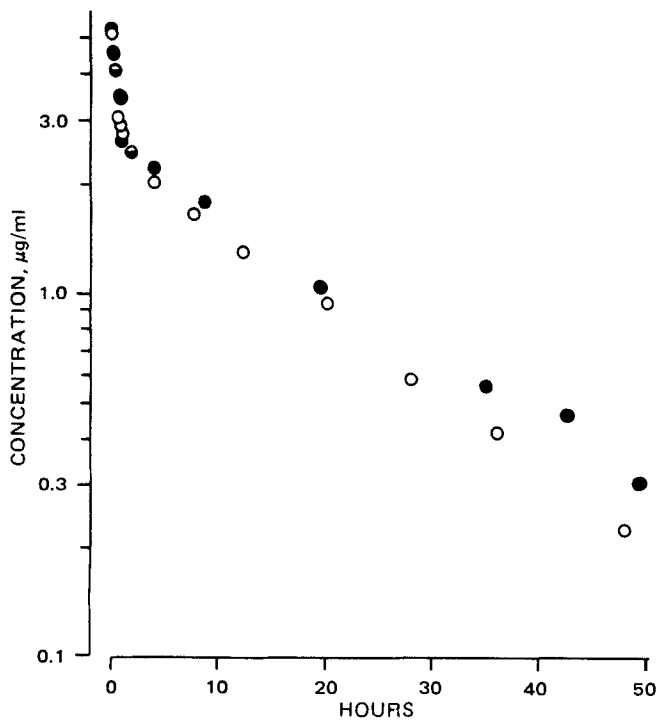

Figure 10—*Warfarin concentrations in the plasma of Rat 3 as a function of time after intravenous injection of ^{14}C-warfarin, 0.51 mg/kg, on two separate occasions. Key: ●, first experiment; and ○, 2 weeks later.*

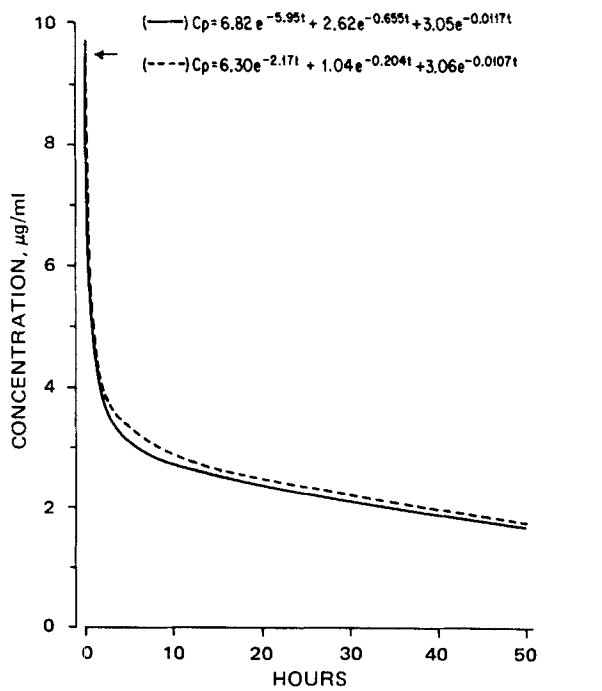

Figure 11—*Curves fitted to the data in Fig. 9 by nonlinear least-squares computer program. Key: ——, first experiment; and - - - -, second experiment.*

Table VI—Warfarin Kinetics in Rat 13 in Two Separate Experiments

Direct Parameter	First Experiment	Second Experiment[a]	Derived Constant	First Experiment	Second Experiment[a]
P, μg/ml[b]	6.82 ± 0.61[c]	6.30 ± 0.44	k_{10}, hr^{-1}	0.0472	0.0352
A, μg/ml[b]	2.62 ± 0.46	1.04 ± 0.38	k_{12}, hr^{-1}	2.67	1.16
B, μg/ml[b]	3.05 ± 0.13	3.06 ± 0.34	k_{21}, hr^{-1}	2.88	0.897
π, hr^{-1}	5.95 ± 1.09	2.17 ± 0.33	k_{13}, hr^{-1}	0.679	0.137
α, hr^{-1}	0.655 ± 0.178	0.204 ± 0.193	k_{31}, hr^{-1}	0.336	0.149
$\beta \times 100$ hr^{-1}	1.17 ± 0.13	1.07 ± 0.31	V_C, ml/kg	40.6	49.0
			V_{area}, ml/kg	164	162
			TC, ml/(hr × kg)	1.92	1.73

[a] Two weeks after first experiment. [b] For a 0.51-mg/kg dose. [c] Standard deviation.

Table VII—Effect of Initial Estimates on Computer-Derived Parameters for Triexponential Equation, Rat 14

Parameter	Initial Estimate	Computer Output[a]				
		N.C.	−30%*	+30%*	−30%* +30%+	+30%* −30%+
P	5.00	6.74	6.74*	6.74*	6.74*	6.74*
A	3.00	2.32	2.33*	2.32*	2.32+	2.32+
B	3.80	3.77	3.77	3.77	3.77	3.77
π	5.00	7.08	7.08*	7.06*	7.07*	7.07*
α	0.693	0.652	0.652*	0.650*	0.651+	0.651+
$\beta \times 100$	1.40	1.38	1.38	1.38	1.38	1.38

[a] The computer outputs when the initial estimates were not changed (N.C.) and when P, A, π, and α were increased or decreased by 30% as specified by the superscripts in the column headings.

To examine the effect of the initial estimates on the computer-calculated direct pharmacokinetic parameters, the initial estimates for P, A, π, and α were increased or decreased in different combinations by 30%[4]. For 12 of the 14 rats, this change had no effect (<5%) on the calculated values. Tables VII and VIII show the results of this exercise for one of the best and for the worst case, respectively.

Despite the numerically pronounced intrasubject differences in several direct pharmacokinetic parameters for warfarin (Tables IV–VI), the plasma concentration–time curves generated by the triexponential equations for replicate experiments in any one animal were very similar (Figs. 11 and 12). However, the curves obtained from the derived constants for the time course of warfarin in the two hypothetical peripheral compartments of the three-compartment model differed greatly between experiments in any one animal (Figs. 13 and 14).

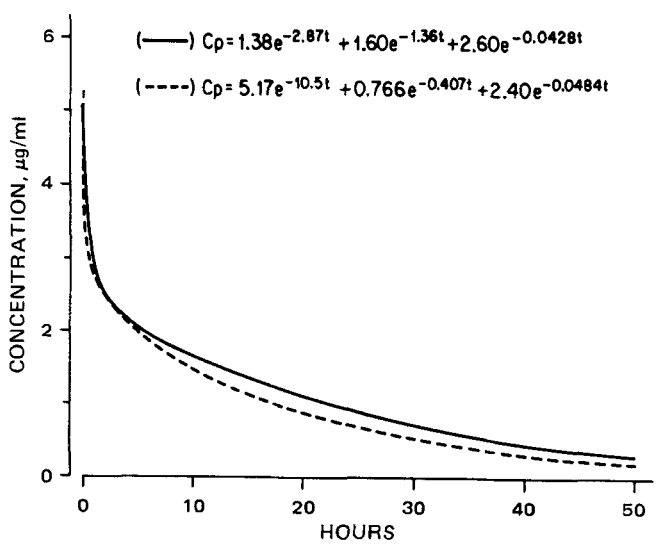

Figure 12—*Curve fitted to the data in Fig. 10 by nonlinear least-squares computer program. Key: ——, first experiment; and - - - -, second experiment.*

[4] The initial estimates for B and β were so close to the computer outputs in all cases that there was no need to vary these estimates.

DISCUSSION

The experiments were carried out under relatively ideal conditions for pharmacokinetic analysis: a large number of blood samples were obtained through an indwelling cannula; a sensitive, specific, and precise assay method was used; the experiments were done twice in some animals; and the concentration data were sufficiently "clean" so that computer calculations were not affected by substantial variations in the initial estimates of the parameters.

As expected on the basis of earlier investigations (1–3), plasma protein

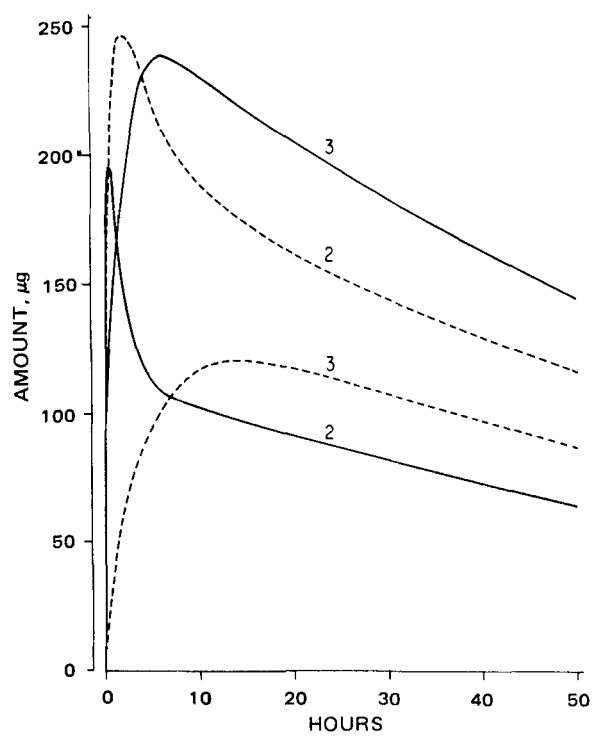

Figure 13—*Inferred time course of warfarin in the hypothetical shallow and deep peripheral compartments, based on the triexponential curves shown in Fig. 11. Key: ——, first experiment; - - - -, second experiment; 2, Compartment 2; and 3, Compartment 3.*

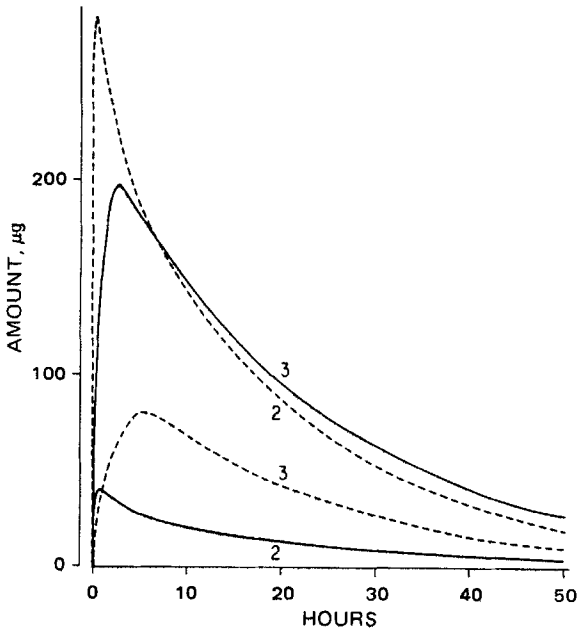

Figure 14—*Inferred time course of warfarin in the hypothetical shallow and deep peripheral compartments, based on the triexponential curves shown in Fig. 12. Key: ——, first experiment; - - - -, second experiment; 2, Compartment 2; and 3, Compartment 3.*

binding had a pronounced effect on the total clearance of warfarin. Our previous practice of using $V_{intercept}$ rather than V_{area} values to estimate TC[5] was entirely acceptable for warfarin, since the differences were on the order of 3 ± 5%.

Protein binding of warfarin in plasma had no apparent effect on the directly determined pharmacokinetic parameters π and α and on the derived rate constants k_{12}, k_{21}, k_{13}, and k_{31}, which are conventionally used to describe the distribution kinetics of a drug on the basis of plasma concentration data. This conclusion must be viewed in the context that these parameters are also the ones with the greatest uncertainty in their calculated values (as reflected by their relative coefficients of variation and by the results of replicate experiments).

Of interest are the strong correlations of P and V_c [*i.e.*, $D/(P + A + B)$] with f (Table III). If V_c is the volume of a "real" initial homogeneous compartment, then differences in plasma protein binding should not affect its value. The increase of V_c with f suggests that V_c is a hybrid affected by some aspects of drug distribution and that it does not reflect the volume of a distinct, homogeneous, well-defined, and instantaneously accessible (by intravenous injection) physiological space under the experimental conditions. However, V_c is essentially equal to the plasma volume of 35 ml/kg in Sprague–Dawley rats (11) when f is very small (Fig. 6).

From a theoretical assessment of problems associated with the analysis of pharmacokinetic models, Westlake (12) concluded that: "When a pharmacokinetic model is fitted to blood levels of a drug, the estimates of the pharmacokinetic parameters are likely to be subject to considerable error. These errors are probably unimportant as long as the model is used only to predict blood levels. However, when the parameters are used to predict other features of the system (*e.g.*, tissue drug levels), considerable errors in prediction may result." The results of our replicate studies, carried out under ideal conditions for good replication, afford the best opportunity to examine this problem experimentally.

[5] This practice is necessitated by the requirement of nonheparinized blood for most of these studies, since it is usually desired to determine not only drug concentrations but also the intensity of the anticoagulant effect. Blood samples must then be collected by serial arterial punctures (rather than by cannula) and at more frequent time intervals at later times, thereby limiting the number and frequency of early samples.

Table VIII—Effect of Initial Estimates on Computer-Derived Parameters for Triexponential Equation, Rat 3

Parameter	Initial Estimate	Computer Output[a] N.C.	−30%*	+30%*	−30%* +30+	+30%* −30%+
P	1.20	1.38	1.36*	1.78*	1.50*	0.888*
A	2.00	1.60	1.39*	1.19*	1.51+	2.35+
B	2.59	2.60	2.59	2.57	2.61	2.67
π	3.50	2.87	2.42*	2.37*	2.15*	5.14*
α	1.40	1.36	1.01*	1.32*	1.93+	1.97+
$\beta \times 100$	4.26	4.28	4.27	4.23	4.29	4.40

[a] The computer outputs when the initial estimates were not changed (N.C.) and when P, A, π, and α were increased or decreased by 30% as specified by the superscripts in the column headings.

Pharmacokinetic analysis of the data demonstrated that Westlake's conclusions are fully justified. The tool of pharmacokinetics is powerful and effective when focused on directly measurable concentrations or effects but becomes a potentially misleading and meaningless mathematical manipulation when used to infer the events or conditions that are thought (often mistakenly) to occur in the "black box" portion of the body and that cannot be measured directly.

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ACKNOWLEDGMENTS AND ADDRESSES

Received March 10, 1976, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214.*

Accepted for publication June 14, 1976.

Supported in part by Grant GM 20852 from the National Institute of General Medical Sciences, National Institutes of Health.

Previous paper in this series: A. Yacobi, J. A. Udall, and G. Levy, *Clin. Pharmacol. Ther.*, **20**, 300 (1976).

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