Journal of Pharmacokinetics and Biopharmaceutics, Vol. 3, No. 4, 1975

Rapid Equilibration of Warfarin Between Rat Tissue and Plasma

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Received Oct. 21, 1974—Final Mar. 11, 1975

Plasma and tissue concentrations of warfarin in the rat were measured as a function of time following a 10 mg/kg intravenous dose. The mathematical interpretation of the experimental results suggested that the data could be explained in terms of a two-compartment open model. Following equilibration, which occurred within a few minutes after injection, individual tissue levels and plasma levels of warfarin were found to be always directly proportional.

KEY WORDS: warfarin; linear pharmacokinetics; tissue binding; plasma protein binding; tissue distribution.

INTRODUCTION

The purpose of this study was to derive a pharmacokinetic model which would describe the relationship between tissue and plasma concentrations of warfarin in rats, as a function of time, following an intravenous dose of sodium warfarin, 10 mg/kg.

Forty male Sprague-Dawley rats with an average weight of 300 g were used in the study of eight time intervals: 1, 5, 15, 30, 60, 120, 180, and 240 min. Five animals were used for each time interval, four serving as recipients and the fifth as a blank.

Supported by Training Grant GM 1367–10 from the National Institute of General Medical Sciences, U.S. Public Health Service, Bethesda, Maryland 20014.

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EXPERIMENTAL

Animal Treatment

The rats were starved the night before the experiment and during the experiment; water only was allowed *ad libitum*. The rats were weighed in the morning prior to the experiment, and 3 mg of sodium warfarin was administered intravenously by tail vein, based on their average weight. The rats were not anesthetized during the injection.

At the end of each time interval, the corresponding rats were anesthetized with ether U.S.P. An abdominal incision was made, and an immediate blood sample was taken from the bifurcation of the inferior vena cava. The needle was then detached and the sample transferred slowly to prevent hemolysis against the inside of a 17- by 100-mm plastic vial containing 0.2 ml of sodium citrate, 25% w/v. The plasma was separated by centrifugation and transferred by pipette into new plastic vials, capped, and frozen at −4°C. The rats were then decapitated, and the following were organs excised, transferred to plastic vials, capped, and frozen at −4°C: brain, heart, lung, spleen, and liver.

Tissue homogenates were prepared and then refrozen until assayed. Each homogenate run represented the same organ for a given time interval. For example, all five hearts excised at 1 min after injection were first thawed, blotted, and weighed, and then exactly 0.7 g of tissue from each heart was weighed into a separate, tared 15-ml Broeck glass tissue grinder and homogenized with 7 ml of phosphate buffer, *p*H 7.25 (0.1 M). The homogenate was then transferred to glass tubes, stoppered, and frozen at −4°C until time of assay.

Assay Methodology

The amount of warfarin in plasma and tissues at various time intervals was determined by the modified O'Reilly method with a few modifications (1). The additional changes involved the extraction procedure, elimination of separatory funnels, and use of pyrex glass wool for filtering purposes. The final method is given in detail below.

Blank rat plasma and tissue samples freshly frozen at −4°C, followed by thawing, homogenizing with warfarin as a spike, and refreezing at the end of 4 months, and finally thawing again at intervals over the next 5 months, yielded excellent Beer's law plots. O'Reilly also reported that results of warfarin determinations on plasma, urine, and stool stored in the frozen state for several months, even when the specimens were repeatedly thawed and frozen, were not significantly different from those obtained on fresh samples (2). It was also determined that human plasma, O-positive and not

over 24 hr old, kept frozen for 2 weeks, gave the same blank value in the assay as when fresh. However, blank values were found to increase when the same plasma that had been kept refrigerated at 4°C was used.

The results of calibration studies on warfarin-spiked human plasma, rat plasma, and rat tissues are presented in Table I. Human plasma, 4-ml samples, was spiked from 0.13 to 12 μg/ml; 2-ml samples were spiked from 0.15 to 12 μg/ml. Rat plasma, 2-ml samples, was spiked from 0.25 to 7.5 μg/ml; rat tissues were spiked from 2 to 30 μg/g.

Table I. Warfarin Tissue and Plasma Assay Calibration Data: Slopes of Least-Squares Lines Forced Through the Origin for Plots of Absorbance Against Amount of Warfarin in μg/ml of Final NaOH Solution

Shaking time (min)	Sample	Slope[a]
20	Human plasma, 4 ml	0.263
20	Human plasma, 4 ml	0.314
20	Human plasma, 4 ml	0.289
20	Human plasma, 4 ml	0.282
20	Human plasma, 4 ml	0.282
10	Human plasma, 4 ml	0.312
10	Human plasma, 4 ml	0.299
10	Human plasma, 4 ml	0.299
10	Human plasma, 4 ml	0.329
10	Human plasma, 4 ml	0.380
20	Human plasma, 2 ml	0.311
20	Human plasma, 2 ml	0.274
20	Human plasma, 2 ml	0.265
10	Human plasma, 2 ml	0.359
10	Human plasma, 2 ml	0.336
10	Rat plasma, 2 ml	0.317
10	Rat plasma, 2 ml	0.338
20	Rat brain homogenate, 4 ml	0.321
20	Rat brain homogenate, 4 ml	0.289
20	Rat heart homogenate, 4 ml	0.298
20	Rat heart homogenate, 4 ml	0.320
20	Rat lung homogenate, 4 ml	0.348
20	Rat lung homogenate, 4 ml	0.327
20	Rat spleen homogenate, 4 ml	0.303
20	Rat spleen homogenate, 4 ml	0.328
20	Rat liver homogenate, 4 ml	0.336
20	Rat liver homogenate, 4 ml	0.334

[a]Average slope = 0.313, which is absorbance units/μg warfarin/ml final NaOH (2.5 N). (Student's *t* test gave no significant difference between slopes of 10-min vs. 20-min shaking periods nor between slopes of 4 ml plasma, 2 ml plasma, and 4 ml tissue homogenates.)

The assay procedure for the determination of warfarin in rat (or human) plasma and tissue was as follows:

1. Twenty milliliters of 1,2-ethylenedichloride (EDC), analytical grade, was pipetted into 50-ml round-bottom centrifuge tubes to receive teflon-lined screw caps for the given number of samples and blanks.
2. Samples were added as follows:
   a. Human plasma: Four milliliters was layered onto EDC surface, and warfarin spike (μl) was added and gently distributed into plasma without mixing layers, followed by addition of 3 ml HCl (1 N).
   b. Rat plasma: Two milliliters was layered and spiked as above, followed by 5 ml HCl (0.6 N).
   c. Rat tissue: Four milliliters of a uniformly dispersed 10% tissue homogenate, using the tissue grinder if previously prepared and frozen, in phosphate buffer, *p*H 7.25 (0.1 M), was layered as above onto EDC surface, and warfarin spike (μl) was added, followed by 3 ml HCl (1 N).
3. Centrifuge tubes were capped and agitated horizontally in a Kahn (Ebberback) shaker: 10 min for plasma extraction, 20 min for tissue homogenates. Too much shaking resulted in emulsification and high blank values; inadequate shaking time resulted in incomplete transfer of warfarin into the organic phase (2).
4. Tubes were then centrifuged at 500*g*, 4°C, for 4 min; the top aqueous layer was discarded; 5 ml of the same phosphate buffer was added to remove interfering substances extracted by the relatively polar EDC (2); tubes were capped and agitated horizontally for 10 min and again centrifuged as above.
5. The aqueous buffer was discarded; exactly 15 ml of the organic layer was transferred to a new set of 50-ml round-bottom centrifuge tubes, followed by exactly 5 ml of NaOH (2.5 N) and horizontal agitation for 5 min. The organic layer was reduced by 10 ml with a pipette, and the balance including the 5-ml aqueous layer was filtered through pyrex glass wool into 15-ml vacutainers and centrifuged at 500g, 8 min, at room temperature.
6. The ultraviolet absorbance of the warfarin solution was read within 2 hr using a Gilford model 2400 spectrophotometer and 7.5-cm quartz cuvettes. The instrument was nulled against the blank for each sample reading taken at 360 and 308 nm. The net absorbance was then calculated by taking the difference between meter readings at 308 and 360 nm. (The cuvettes were cleaned in 50% nitric acid for 24 hr following each assay series of 16 samples; absolute ethyl

alcohol was used to thoroughly dry the sample cuvette after cleaning between each sample reading.)

7. The inherent absorbance of the two cuvettes, following the nitric acid soak, was determined by use of distilled water to be 0.005 and 0.009. The difference between the two cuvettes was considered negligible, and therefore a correction factor was not included in the calculation of the net absorbance for each sample. The 7.5-cm cuvettes were tagged so that one was used only for the sample, the other for the blank.
8. Warfarin concentration in samples of plasma or tissue was calculated as follows based on a 7.5-cm pathlength:
   a. Warfarin concentration in plasma (μg/ml) = (net $A \times 5 \times 20$)/ $0.313 \times$ ml of plasma $\times 15$).
   b. Warfarin concentration in tissue (μg/g) = (net $A \times 5 \times 20 \times 1$)/ $(0.313 \times 4 \times 15 \times 0.1)$ = net $A/0.0188$.

Estimation of Free (Unbound) Warfarin Plasma Concentrations

The equation of Wosilait (3), shown as equation 1, was used for estimation of free (unbound) warfarin tissue concentrations. The constants are those for human serum albumin, but our values of 0.9–1.0% free, calculated with the equation, are very similar to the 0.2–1.5% free reported by Levy and Yacobi (4) for the rat:

$$C_B = \frac{K_1 n_1 P C_F}{1 + K_1 C_F} + \frac{K_2 n_2 P C_F}{1 + K_2 C_F} \tag{1}$$

where C_B is the concentration of protein-bound warfarin in nmoles/ml, C_F is the concentration of free (unbound) warfarin in nmoles/ml, K_1 and K_2 are the association constants in liters/mole, n_1 and n_2 are the binding capacities, and P is the concentration of serum albumin in nmoles/ml. Division of the top and bottom of the right-hand sides by K_1 and K_2, respectively, and substitution of Wosilait's values of $K_1 = 0.089$ ml/nmole (corresponding to 8.94×10^4 liters/mole), $K_2 = 0.0067$ ml/nmole (corresponding to 6.7×10^3 liters/mole), $n_1 = 2$, $n_2 = 4$, and $P = 580$ nmole/ml gave

$$C_B = \frac{1160 C_F}{11.24 + C_F} + \frac{2320 C_F}{149.25 + C_F} \tag{2}$$

If the total plasma concentration is represented by C_p, then C_p is given by

$$C_p = C_F + \frac{1160C_F}{11.24 + C_F} + \frac{2320C_F}{149.25 + C_F} \tag{3}$$

Let

$$y = C_F + \frac{1160C_F}{11.24 + C_F} + \frac{2320C_F}{149.25 + C_F} - C_p \tag{4}$$

A Hewlett–Packard electronic calculator program was written for the latter equation to solve for C_F iteratively from a given value of C_p. Trial values of C_F were entered until $y \simeq 0$. The free drug concentrations calculated by this method agreed very well with those given by Wosilait in his article. The free warfarin concentrations in rat plasma observed in this study, calculated by the above method, are shown in Table II below the total warfarin concentrations. The free drug concentrations varied from 1.0% (when $C_p = 80.1$ μg/ml) to 0.90% (when $C_p = 37.6$ μg/ml) in our rat studies. Concentrations were interconverted using C_F (nmoles/ml) = 3.24 C_F (μg/ml).

RESULTS

Table II lists the observed average total plasma and tissue concentrations and the calculated free warfarin plasma concentrations.

Table III lists the ratios of the average tissue concentration to the average total plasma concentration. For each tissue, the ratio remained essentially constant from 0.0833 hr (5 min) to 4 hr with reasonable small coefficients of variation. From the data of Anderson (5), similar ratios were calculated and these are shown in Table IV. The liver/plasma and brain/plasma ratios calculated from Anderson's data agree very well with those calculated data obtained in our study. We observed higher average lung/plasma and heart/plasma ratios than Anderson and a lower spleen/plasma ratio than Anderson. However, Anderson's data were much more variable than the data in our study, as indicated by the coefficients of variation. Since the dose used by Anderson was only 2 mg/kg, and the dose used in our studies was 10 mg/kg, and similar ratios were observed in the two studies, it appeared to be unnecessary to perform additional tissue studies. The dose range of interest was 0–10 mg/kg, and it appeared unlikely that there was a "dose dependency" in this range.

The diffusible form of warfarin is the free (unbound) form in plasma water, hence tissue concentrations should really be related to the free (unbound) concentrations. The ratios of tissue concentration to free warfarin

Table II. Average Observed Total and Calculated Free Warfarin Plasma Concentration (μg/ml) and Observed Average Tissue Concentrations[a] (μg/g) Following Rapid Intravenous Injection of 10 mg Sodium Warfarin (9.33 mg Warfarin) per Kilogram of Body Weight into the Tail Vein of Male Sprague-Dawley Rats

	Average concentration at indicated time							
Tissue	1 (min) 0.0166 (hr)	5 (min) 0.0833 (hr)	15 (min) 0.25 (hr)	30 (min) 0.5 (hr)	60 (min) 1.0 (hr)	120 (min) 2.0 (hr)	180 (min) 3.0 (hr)	240 (min) 4.0 (hr)
Total plasma (μg/ml)	80.1	66.6	56.6	51.4	49.5	47.3	39.9	37.6
Free plasma (μg/ml)	0.80	0.64	0.54	0.48	0.46	0.44	0.36	0.34
Liver (μg/g)	—	286	255	222	209	204	199	189
Lung (μg/g)	254	177	151	145	134	136	109	101
Heart (μg/g)	269	158	140	113	106	108	93.2	93.2
Spleen (μg/g)	132.5	78.6	52.6	46.4	53.5	45.9	57.1	37.7
Brain (μg/g)	62.8	33.1	17.6	34.4	24.4	10.2	8.15	—

[a]Tissue concentrations reported are uncorrected for the warfarin present in blood in the tissue since such corrections amount to only 1–2% of the total tissue concentration and were considered to be within assay error.

Table III. Ratios of Average Tissue Concentration (μg/g) to Average Total Plasma Concentration (μg/ml) Observed Following a 10 mg/kg Dose of Sodium Warfarin Intravenously in the Rat

	$\frac{\text{Average tissue concentration } (\mu g/g)}{\text{Average total plasma concentration } (\mu g/ml)}$ at indicated time									
Ratio	0.0166 (hr)	0.0833 (hr)	0.25 (hr)	0.5 (hr)	1 (hr)	2 (hr)	3 (hr)	4 (hr)	Average	CV[a] (%)
Liver/plasma	—	4.29	4.51	4.53	4.21	4.30	4.98	5.03	4.52	7.6
Lung/plasma	3.17	2.66	2.67	2.81	2.70	2.86	2.74	2.69	2.73	2.8
Heart/plasma	3.36	2.38	2.48	2.19	2.13	2.28	2.33	2.48	2.32	5.8
Spleen/plasma	1.65	1.18	0.93	0.90	1.08	0.97	1.43	1.00	1.07	17.3
Brain/plasma	0.78	0.50	0.31	0.67	0.49	0.22	0.20	—	0.40	4.6

[a]CV = coefficient of variation in percent = (standard deviation/average) × 100. Values were calculated omitting the 1-min (0.0166-hr) ratios.

Table IV. Ratios of Average Tissue Concentration (μg/g) to Average Total Plasma Concentration (μg/ml) Calculated from Data of Anderson (5) Following a 2 mg/kg Dose of Sodium Warfarin Intravenously in the Rat

	$\frac{\text{Average tissue concentration } (\mu g/g)}{\text{Average total plasma concentration } (\mu g/ml)}$ at indicated time							
Ratio	1 (hr)	2 (hr)	4 (hr)	6 (hr)	24 (hr)	48 (hr)	Average	CV (%)
Liver/plasma	6.74	6.54	2.94	2.5	2.7	6.3	4.62	45.4
Lung/plasma	1.5	0.64	0.45	1.1	0.72	1.7	1.0	49.3
Heart/plasma	1.1	0.48	0.80	0.82	0.85	3.1	1.2	80.2
Spleen/plasma	2.5	1.5	1.7	1.4	1.4	4.2	2.1	52.0
Brain/plasma	1.0	0.15	0.20	0.26	0.46	0.43	0.42	74.7

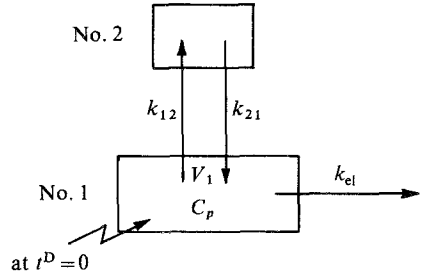
concentrations are given in Table V. For each tissue, from 0.0833 to 4 hr, the ratio remained essentially constant with reasonably small coefficients of variation. However, as indicated before, in the concentration range studied, free (unbound) warfarin ranged from only 0.90 to 1.0% of total plasma concentrations. This is well within analytical error; hence, in the case of warfarin in the rat, use of total plasma concentrations is satisfactory for the model shown in Scheme II.

Fitting of Total Warfarin Plasma Concentration–Time Data

The total plasma concentrations listed in Table II were "stripped" on semilogarithmic graph paper, using an electronic calculator to calculate the least-squares lines. The data were tentatively fitted to the equation

$$\hat{C}_p = 27.83e^{-8.66t} + 54.60e^{-0.0942t} \tag{5}$$

On the basis of these results, the classical interpretation is that the data obey the two-compartment open model with rapid intravenous injection:



Scheme I

Equations appropriate to this model are given below:

$$\hat{C}_p = \frac{C_p^0}{(\alpha - \beta)}[(k_{21} - \beta)e^{-\beta t} - (k_{21} - \alpha)e^{-\alpha t}] \tag{6}$$

$$\alpha = \tfrac{1}{2}[(k_{12} + k_{21} + k_{el}) + \sqrt{(k_{12} + k_{21} + k_{el})^2 - 4k_{21}k_{el}}] \tag{7}$$

$$\beta = \tfrac{1}{2}[(k_{12} + k_{21} + k_{el}) - \sqrt{(k_{12} + k_{21} + k_{el})^2 - 4k_{21}k_{el}}] \tag{8}$$

Equation 6 may also be written as equation 9:

$$\hat{C}_p = Ae^{-\alpha t} + Be^{-\beta t} \tag{9}$$

Table V. Ratios of Average Tissue Concentration (μg/g) to Calculated Average Free Warfarin Concentration in Plasma (μg/ml) Observed Following a 10 mg/kg Dose of Sodium Warfarin Intravenously in the Sprague-Dawley Male Rat

	Average tissue concentration (μg/g) / Calculated average free warfarin concentration in plasma (μg/ml) at indicated time								
Ratio	0.0833 (hr)	0.25 (hr)	0.5 (hr)	1 (hr)	2 (hr)	3 (hr)	4 (hr)	Average	CV (%)
Liver/plasma	446	473	463	453	462	552	557	487	9.7
Lung/plasma	227	280	301	291	308	304	297	294	4.0
Heart/plasma	248	260	234	229	245	259	274	250	6.3
Spleen/plasma	123	97.4	96.7	116	104	159	111	115	18.7
Brain/plasma	52	33	72	53	23	23	—	43	4.6

where

$$A = D(k_{21} - \alpha)/V_1(\beta - \alpha) \tag{10}$$

$$B = D(k_{21} - \beta)/V_1(\alpha - \beta) \tag{11}$$

The model parameters were estimated from the equations

$$V_1 = D/C_p^0 = D/(A + B) \tag{12}$$

$$k_{21} = (A\beta + B\alpha)/(A + B) \tag{13}$$

$$k_{el} = \alpha\beta/k_{21} \tag{14}$$

$$k_{12} = \alpha + \beta - k_{21} - k_{el} \tag{15}$$

Using the values $A = 27.83$, $\alpha = 8.66$, $B = 54.60$, and $\beta = 0.0942$ (by comparison of equations 5 and 9) and equations 13 through 15, the preliminary estimates $k_{21} = 5.77$, $k_{el} = 0.141$, and $k_{12} = 2.85\ \text{hr}^{-1}$ were obtained.

The plasma concentration–time data and these preliminary estimates were then used as input data, and the data were fitted to equations 6 through 8 using the program NONLIN and the IBM 360/67 digital computer. The results of the fitting are shown in Table VI. The fit was excellent. Substitution of the estimated parameters into equation 6 gave equation 16. Hence equation 16 is an improvement over equation 5 since the sum of squared deviations is lower for equation 16 than for equation 5.

$$\hat{C}_p = 29.97e^{-10.27t} + 54.73e^{-0.0953t} \tag{16}$$

Table VI. Results of Nonlinear Least-Squares Fitting of Average Total Plasma Concentration Data

Estimated parameter	Least-squares estimate	Standard deviation
$k_{12}(\text{hr}^{-1})$	3.45	0.846
$k_{21}(\text{hr}^{-1})$	6.67	1.39
$k_{el}(\text{hr}^{-1})$	0.147	0.0148
$C_1^0(\mu\text{g/ml})$	84.7	2.68

Derived parameter	Parameter value
$\alpha\ (\text{hr}^{-1})$	10.27
$\beta\ (\text{hr}^{-1})$	0.0953
V_1 (liters/kg)	0.110

Measures of fit	Value
Sum of squared deviations	7.74
Coefficient of determination	1.00
Correlation coefficient for $\hat{C}_p$ on C_p	0.997

Fitting of Tissue Concentration Data

Results presented in Table III suggested the plot shown in Fig. 1. In Fig. 1, the tissue concentration is plotted against the total plasma concentration. For each tissue, the data from 5 to 240 min appeared to be linear. Extrapolation of the lines and forcing the lines through the origin appeared to be justified on the basis of the data in Tables III and IV. The data obtained at 1 min after injection were lower than the "line values" since mixing had probably not been completed.

The ratios of average tissue concentration to total plasma concentration given in Table III are plotted in Fig. 2. For lung, heart, spleen, and possibly brain, the ratios appear to be randomly distributed about the average ("line values") drawn in the figure. However, for liver there appear to be definite trends in the data.

On the basis of the above results, the tissue concentration data were fitted as follows.

Approach No. 1: The total plasma concentrations and the lung, heart, spleen, and brain tissue concentrations were simultaneously fitted to equations, 7, 8, and 17 through 21 using the data from 0.0833 to 4 hr, the program NONLIN, and the IBM 360/67 digital computer.

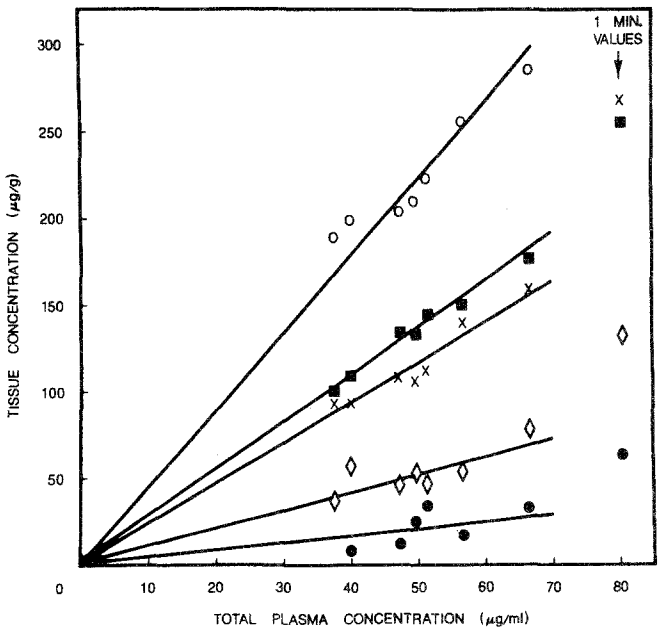


Fig. 1. Warfarin tissue concentrations relative to total plasma concentration. Symbols: ○, liver, slope 4.44; ■, lung, slope 2.73; ×, heart, slope 2.33; ◇, spleen, slope 1.03; ●, brain, slope 0.41.

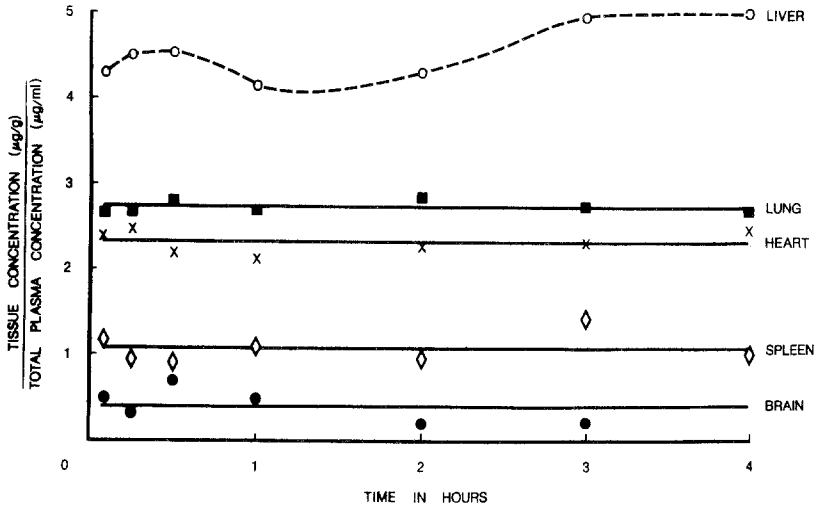


Fig. 2. Ratios of average tissue concentrations to total plasma concentration given in Table III.

$$\text{Plasma: } C_p = \frac{C_p^0}{\alpha - \beta}[(k_{21} - \beta)e^{-\beta t} - (k_{21} - \alpha)e^{-\alpha t}] \tag{17}$$

$$\text{Lung: } T_1 = B_1 \cdot C_p \tag{18}$$

$$\text{Heart: } T_2 = B_2 \cdot C_p \tag{19}$$

$$\text{Spleen: } T_3 = B_3 \cdot C_p \tag{20}$$

$$\text{Brain: } T_4 = B_4 \cdot C_p \tag{21}$$

The preliminary estimates of B_1 through B_4 were the slopes of the lines drawn in Fig. 1. The preliminary estimates of k_{12}, k_{21}, and k_{el} used were the least-squares estimates shown in Table VI. The results of the simultaneous fitting are shown in Table VII and Fig. 3. The fits were excellent. The overall coefficient of determination was 0.995 and the overall Corr value was 0.991. The standard deviations of the B_1 through B_4 values were very small relative to the size of the estimates. Both the univariate and *s*-plane 95% confidence intervals of the B_1 through B_4 values are perhaps the smallest reported to date for biological data. For example, the univariate 95% confidence interval for B_1 (lung) was 2.41–3.05 and the *s*-plane 95% confidence interval was 2.08–3.38. The percent deviations of the model-predicted concentrations from the observed concentrations are shown in Table VIII. The deviations appeared to be reasonably random. Figure 3 is a Cartesian coordinate plot showing the results of the simultaneous fitting of the plasma

Table VII. Results of Simultaneous Fitting of Tissue and Total Plasma Concentrations of Warfarin in the Rat Following Rapid Intravenous Injection of a 10 mg/kg Dose of Sodium Warfarin Equivalent to 9.33 mg Warfarin Acid per Kilogram

Data	Estimated parameter	Least-squares estimate	Standard deviation	Measures of fit r^2	Measures of fit Corr
Plasma	k_{12} (hr^{-1})	2.25	1.32	0.999	0.992
	k_{21} (hr^{-1})	4.65	1.89		
	k_{el} (hr^{-1})	0.117	0.0267		
	C_1^0 (μg/ml)	79.1	8.44		
Lung	B_1	2.73	0.151	0.998	0.970
Heart	B_2	2.33	0.131	0.998	0.968
Spleen	B_3	1.07	0.0758	0.983	0.802
Brain	B_4	0.423	0.0584	0.880	0.704

Derived parameters

α (hr^{-1})	6.94
β (hr^{-1})	0.0786
V_1 (liters/kg)	0.118

Other measures of fit	Value
Sum of squared observations	2.7×10^5
Sum of squared deviations	1.26×10^3
Overall coefficient of determination	0.995
Overall correlation coefficient for predicted *vs.* observed	0.991

concentration data and the tissue concentrations for lung, heart, spleen, and brain. The lines drawn through the points are based on equations 17 through 21 and the least-squares estimates of the parameters shown in Table VII. The theoretical line drawn through the liver concentration data in Fig. 3 is discussed subsequently.

Approach No. 2: The tissue concentrations of lung, heart, spleen, and brain were simultaneously fitted to equations 18 through 21 and C_p was given by equation 17. The program NONLIN and the IBM 360/67 digital computer were used as formerly. The initial estimates used for B_1 through B_4 were the slopes of the line shown in Fig. 1. The results of this fitting are given in Table IX. The least-squares estimates of the proportionality constants, B_1 through B_4, obtained by Approach No. 1 and Approach No. 2 are identical, as can be seen by comparing the estimates in Tables VII and IX. The simultaneous fit by Approach No. 2 was also excellent as seen by the measures of fit and low values of the standard deviations and 95% confidence intervals of the parameters shown in Table IX.

Fitting of Liver Concentration Data

Figure 2 indicates that unlike for the other tissues measured the liver concentration/plasma concentration ratio does not appear to be constant.

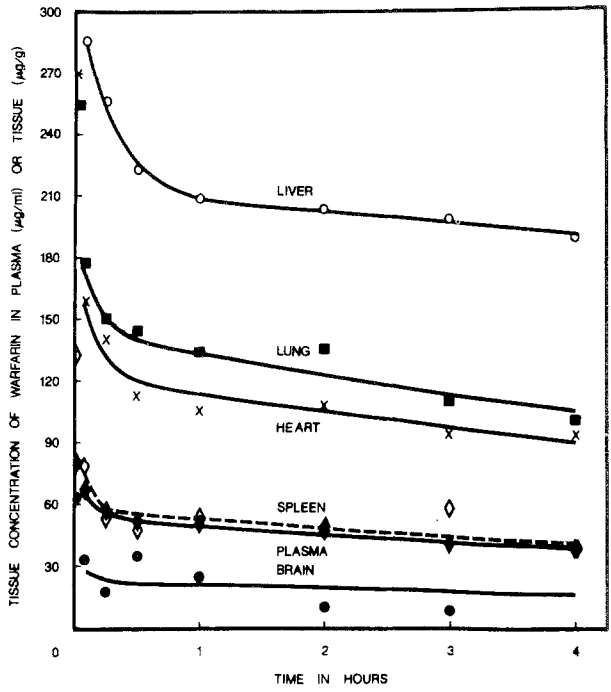


Fig. 3. Line through liver concentrations is based on equation (21): $\hat{L} = 102e^{-3.66t} + 212e^{-0.0257t}$. Lines through other tissue and plasma concentrations are based on simultaneous computer fitting (Table VII), resulting in the following equations: lung concentration = $2.73C_p$, heart concentration = $2.33C_p$, spleen concentration = $1.07C_p$; brain concentration = $0.423C_p$; $C_p = 52.73\,e^{-0.0786t} + 26.34\,e^{-6.94t}$, where C_p is the total plasma concentration.

The liver concentration data were separately fitted to a double exponential equation using the program NONLIN and the IBM 360/67 digital computer. The least-squares equation obtained is given as equation 22:

$$\hat{L} = 102e^{-3.66t} + 212e^{-0.0257t} \tag{22}$$

where $\hat{L}$ is the estimated liver concentration in μg/g and t is time in hours. The fit was excellent with $r^2 = 1.000$, Corr = 0.997, $\Sigma L^2 = 3.56 \times 10^5$, and $\Sigma(\hat{L} - L)^2 = 40.2$. The line drawn through the liver concentration points in Fig. 3 is based on equation 12.

The α value of 3.66 in equation 21 is quite different from the α value of 10.27 obtained in fitting the total plasma concentration data (Table VI) or the α value of 6.94 obtained in simultaneous fitting of the plasma concentrations and tissue concentrations observed in lung, heart, spleen, and brain

Table VIII. Percent Deviations of Model-Predicted Concentrations ($\hat{C}_p$ or $\hat{T}$) from Observed Concentrations (C_p or T)

	Percent deviation[a] at indicated time						
Concentration	0.0833 (hr)	0.25 (hr)	0.50 (hr)	1 (hr)	2 (hr)	3 (hr)	4 (hr)
Plasma	0.85	−0.43	0.24	−1.5	−4.7	4.4	2.4
Lung	3.2	1.5	−2.8	−0.65	−9.4	3.8	3.8
Heart	−1.5	−6.4	6.6	7.6	−2.7	4.1	−3.8
Spleen	−9.0	14.1	18.3	−2.9	4.6	−22.3	8.8
Brain	−14.1	35.5	−36.6	−15.4	87.0	116.0	—

[a]For plasma, percent deviation = $[(\hat{C}_p - C_p)/C_p] \times 100$. For tissue, percent deviation = $[(\hat{T} - T)/T] \times 100$.

(Table VII). Similarly the β value (0.0257) in equation 21 is quite different from the β value of 0.0953 obtained in fitting the total plasma concentration data (Table VI) or the β value of 0.0786 obtained in the simultaneous fitting (Table VII). These results and mass balance considerations strongly suggest that metabolites were being measured in liver as well as unchanged drug.

DISCUSSION

The data and the analyses suggest that the model for warfarin in the rat may be written as in Scheme II:

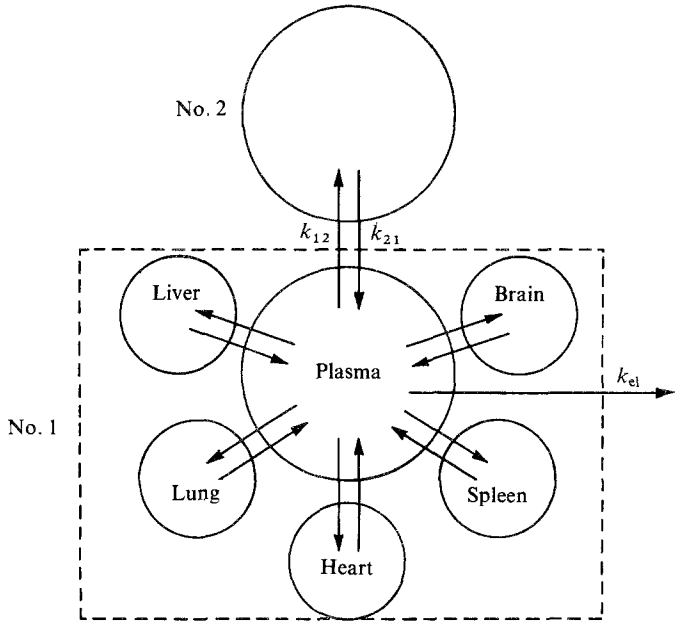
The reversible arrows between liver and plasma, lung and plasma, etc., suggest that equilibration has been achieved within a few minutes after injection into the plasma and that individual tissue levels and plasma levels within the dashed line are always directly proportional (i.e., binding is linear). The lack of direct proportionality between observed liver concentrations and plasma concentrations is attributed to measurement of metabolites along with unchanged drug when the liver tissue was analyzed, but it is assumed that proportionality would have existed if only unchanged drug had been measured directly. The portion enclosed by the dashed line in Scheme II is conceived of as "compartment No. 1" of the two-compartment open model. There may also be tissues other than those measured in this compartment. The compartment represented as the solid line circle at the top of the scheme is conceived of as "compartment No. 2" of the two-compartment open model. The first-order rate constants, k_{12} and k_{21}, are distribution rate constants for free (unbound) warfarin in plasma water and other body fluids. The "other fluids" are most likely what is termed "interstitial lymph," which averages 12% of body weight and 20% of body water (6). Other tissues are probably part of compartment 2, also.

Table IX. Results of Simultaneous Fitting of Tissue Concentrations of Warfarin in the Lung, Heart, Spleen, and Brain of the Rat Following Rapid Intravenous Injection of a 10 mg/kg Dose of Sodium Warfarin Equivalent to 9.33 mg Warfarin Acid per Kilogram[a]

	Proportionality constant (B_i)						
		Least-squares	Standard	95% confidence intervals		Measures of fit	
Tissue	Parameter	estimate	deviation	Univariate	*s*-Plane	r^2	Corr
Lung	B_1	2.73	0.0564	2.61–2.84	2.54–2.92	0.999	0.978
Heart	B_2	2.33	0.0564	2.21–2.44	2.14–2.52	0.996	0.949
Spleen	B_3	1.07	0.0564	0.949–1.18	0.876–1.25	0.982	0.793
Brain	B_4	0.424	0.0587	0.302–0.545	0.227–0.621	0.885	0.747
					Overall	0.995	0.990

[a] $\Sigma \text{obs}^2 = 2.55 \times 10^5$; $\Sigma \text{dev}^2 = 1.31 \times 10^3$.

Wosilait (3) stated: "Extensive binding of a drug by serum albumin is thought to reduce both the rate of distribution of the drug and its elimination from the body" and cited B. B. Brodie as a reference. Warfarin was 99.0–99.1 % bound to plasma albumin of the rat in these studies, but it was shown that within 1 min after intravenous injection the major fraction of the dose was bound to tissues. From 5 to 240 min following injection, it was shown that the major fraction of the dose could be accounted for in just five tissues.



Scheme II

It may be assumed that the drug which is tissue bound is at least as unavailable for elimination as is the drug which is bound to albumin. Hence these data strongly suggest that binding of warfarin to serum albumin does not reduce the rate of distribution to any appreciable degree.

Wosilait (3) stated: "The warfarin in the [rat] plasma was not metabolized to any great extent." This supports the assumption made that the warfarin measured in plasma in this study was unchanged warfarin. The assay used was the same as that used by Welling *et al.* (1), who showed that it gave the same results as obtained by a thin-layer chromatographic assay, when 93 human plasma samples containing warfarin were assayed by both methods. Yacobi *et al.* (7) have recently shown the same for the rat.

It should be noted that the treatment employed, in which tissue concentrations have been related to total plasma concentrations, was feasible only with warfarin since the fraction free in plasma (from 0.009 to 0.01 in the range of plasma concentrations studied) was essentially constant. If free drug concentrations had been used, the B_1 through B_4 values in Tables VII and IX would have been about 100 times greater.

It should also be noted that for the model shown as Scheme II it is implicitly assumed that the diastereoisomers are bound to tissues and plasma proteins and metabolized at the same rates.

Commonly it is assumed that compartment No. 1 of the two-compartment model is the "plasma compartment" and compartment No. 2 is the "tissue compartment." The results of this study strongly suggest that such a description is inadequate. In the case of warfarin in the rat, it may well be that the biphasic nature of the plasma concentration curve is principally caused by distribution of warfarin between "fluid" compartments.

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