Comparative Pharmacokinetics of Coumarin Anticoagulants XLIII: Concentration-Dependent Hepatic Uptake of Warfarin in Rats

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Abstract □ The purpose of this study was to determine if hepatic warfarin uptake, which has a major quantitative effect on warfarin distribution in rats, is concentration dependent. Adult male rats received either 0.1 or 1.0 mg of racemic warfarin/kg iv and were killed 6 hr later. With increasing dose, the concentrations of free and total (free plus protein-bound) serum warfarin increased much more than proportionally, and the total warfarin concentration in the liver increased much less than proportionally. The liver to serum total warfarin concentration ratios 6 hr after injection of the 0.1- and 1.0-mg/kg doses were 11.3 ± 1.7 and 0.814 ± 0.222, respectively (mean ± *SD*, $n = 6$, $p < 0.001$). The ratio of the total drug concentration in the liver to the free drug concentration in serum (mean ± *SD*) was 866 ± 105 in animals that received the 0.1-mg/kg dose and 111 ± 42 in animals that received the 1.0-mg/kg dose ($p < 0.001$). It is concluded that hepatic warfarin uptake decreases with increasing drug concentration and that this may cause the apparent volume of distribution of warfarin to decrease with increasing dose in rats.

Keyphrases □ Coumarin anticoagulants—warfarin, hepatic uptake, concentration dependence, rats □ Warfarin—hepatic uptake, concentration dependence, rats □ Anticoagulants—warfarin, hepatic uptake, concentration dependence, rats □ Pharmacokinetics—anticoagulants, warfarin, hepatic uptake, rats

The pharmacokinetics of warfarin in rats provide a classical example of the effect of serum protein binding on the elimination of a drug with a hepatic clearance much lower than, and therefore independent of, the hepatic blood perfusion rate (1). Very large interindividual differences in total warfarin clearance by rats are associated with, and can be related to, corresponding differences in the serum free fraction of the drug (2). However, theoretical considerations suggest that the pharmacokinetic components of total clearance, *i.e.*, the apparent volume of distribution and β, are affected by changes in tissue binding of the drug, even though tissue binding should have no effect on the total clearance *per se* (3, 4).

Despite being bound extensively to serum proteins ($\simeq$ 99% in the pharmacologically realistic drug concentration range), warfarin concentrates in rat liver. In a previous study in this series, an average liver to serum concentration ratio of 2.5 was found in rats injected with a 0.6-mg/kg dose of warfarin and sacrificed when the serum warfarin concentration had declined to ~0.4 μg/ml (2). The rat livers contained nearly one-half the amount of warfarin in the bodies at the time of sacrifice. Whether caused by saturable binding to hepatic tissues or by a saturable transport process, this pronounced warfarin uptake by the liver could be drug concentration dependent. Such concentration dependence may have important effects on warfarin pharmacokinetics and was explored in this investigation.

EXPERIMENTAL

Twelve adult male Sprague–Dawley rats, 284–323 g, with unrestricted access to food and water received an intravenous injection of racemic ^{14}C-warfarin[1] (158 μCi/mg), about 3.5 μCi, together with sufficient nonradioactive warfarin to constitute a total dose of 0.1 or 1.0 mg/kg. Six rats received the low dose and six received the high dose. The drug was injected rapidly into the femoral vein. Six hours later, the animals were anesthetized with ether and exsanguinated from the aorta by syringe.

Serum was separated from the blood, and warfarin was extracted and separated from its metabolites by TLC to allow quantitation by scintillation spectrometry (5). The liver was excised, and the large left lobe was removed, weighed, and homogenized with two volumes of ice-cold 0.9% sodium chloride by means of a polytef pestle tissue grinder (30-ml chamber volume) at 2000 rpm for 5 min. Warfarin in the liver homogenate was extracted, separated from its metabolites, and assayed as described previously (2). Recovery of added warfarin from the liver homogenate was 88 ± 7% (mean ± *SD*, $n = 8$) and was concentration independent in the range of 0.2–22 μg/g wet weight of liver.

The free fraction of warfarin in serum was determined by equilibrium dialysis at 37° (2), without drug addition to either the buffer or the serum phase. The free warfarin fraction in the liver was calculated as described previously (6), based on the assumptions that the free warfarin concentrations in serum and in liver water are identical and that the liver contains 70% water.

Statistical significance of differences was determined by the Student t test (unpaired, two tailed).

RESULTS

The serum and liver drug concentrations were determined 6 hr after intravenous injection when serum warfarin concentrations were in the terminal exponential (β) phase (7). Injection of a 0.1-mg/kg dose of warfarin resulted in average total (free and bound) drug concentrations of 0.102 μg/ml in serum and 1.14 μg/g wet weight of liver (Table I). Upon injection of a 1.0-mg/kg dose, the average total concentrations were 4.65 μg/ml in serum and 3.58 μg/g of liver (Table II). Thus, a 10-fold increase in the warfarin dose produced a 46-fold increase in the total serum warfarin concentration but only a threefold increase of the concentration in the liver. Therefore, the average liver to serum concentration ratio of total warfarin decreased from 11.3 at the lower dose to only 0.814 at the higher dose.

The concentrations of free warfarin in serum (mean ± *SD*, $n = 6$) were 0.00133 ± 0.00016 and 0.0352 ± 0.0105 μg/ml in rats that received 0.1 and 1.0 mg/kg of warfarin, respectively, representing a 26-fold increase. The difference between the dose-normalized concentrations (*i.e.*, concentration/dose) of free drug is highly significant ($p < 0.001$).

Liver to serum warfarin concentration ratios at an average serum concentration intermediate between those observed in the present study were available from a previous investigation (2). However, the experimental conditions of the previous study were somewhat different in that the animals were older (body weight 400–450 g), the warfarin dose was 0.6 mg/kg, and the animals were killed when the total serum warfarin concentration had decreased to ~0.4 μg/ml. Therefore, the animals were killed after a period of time equal to about three times the biological half-life of the drug. The ratios of total liver drug concentration to total serum drug concentration (C_L^t/C_S^t) and total liver drug concentration to free serum drug concentration (C_L^t/C_S^f) obtained in the previous investigation and in this study are listed in Table III. Both ratios decreased strikingly with increasing serum warfarin concentration.

DISCUSSION

Direct *in vitro* equilibrium dialysis studies showed that rat liver ho-

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

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Table I—Free and Total Warfarin Concentrations in Rat Serum and Liver 6 hr after a 0.1-mg/kg iv Dose

Rat	Body Weight, g	Total Concentration, μg/ml or μg/g		Liver to Serum Total Concentration Ratio	Free Fraction × 100	
		Serum	Liver		Serum	Liver[a]
1 A	284	0.0985	1.13	11.5	1.21	0.0737
2 A	298	0.0981	1.10	11.2	1.53	0.0955
3 A	287	0.114	1.19	10.4	1.32	0.0888
4 A	295	0.110	1.04	9.45	1.13	0.0835
5 A	300	0.113	1.20	10.6	1.22	0.0805
6 A	298	0.0807	1.16	14.4	1.41	0.0688
Mean	293	0.102	1.14	11.3	1.30	0.0818
SD	7	0.010	0.06	1.7	0.15	0.0098

[a] Estimated by the method described in Ref. 6.

Table II—Free and Total Warfarin Concentrations in Rat Serum and Liver 6 hr after a 1.0-mg/kg iv Dose

Rat	Body Weight, g	Total Concentration, μg/ml or μg/g		Liver to Serum Total Concentration Ratio	Free Fraction × 100	
		Serum	Liver		Serum	Liver[a]
1 B	316	3.52	3.63	1.03	1.19	0.808
2 B	300	3.43	3.33	0.971	1.08	0.782
3 B	322	5.34	3.42	0.640	0.569	0.622
4 B	293	5.34	3.78	0.708	0.755	0.746
5 B	310	4.04	4.11	1.02	1.12	0.770
6 B	323	6.21	3.18	0.512	0.264	0.361
Mean	311	4.65[b]	3.58[c]	0.814[d]	0.830[e]	0.682[d]
SD	12	1.14	0.34	0.222	0.366	0.170

[a] Estimated by the method described in Ref. 6. [b] Significantly higher than the dose-normalized concentration 6 hr after a 0.1-mg/kg dose ($p < 0.001$). [c] Significantly lower than the dose-normalized concentration 6 hr after a 0.1-mg/kg dose ($p < 0.001$). [d] Significantly different from result obtained after injection of 0.1 mg/kg ($p < 0.001$). [e] Significantly different from result obtained after injection of 0.1 mg/kg ($p < 0.002$).

Table III—Relationship between Serum Concentration and Liver to Serum Concentration Ratio of Warfarin in Rats

Total Concentration, μg/ml or μg/g		Free Fraction in Serum × 100	Liver to Serum Total Concentration Ratio	Liver Total to Serum Free Concentration Ratio	Data Source
Serum	Liver				
0.102 ± 0.010[a]	1.14 ± 0.06	1.30 ± 0.15	11.3 ± 1.7	866 ± 105	This study; 0.1-mg/kg dose
0.397 ± 0.101	0.948 ± 0.156	0.956 (0.227–2.02)[b]	2.50 ± 0.59	451 ± 368	Reference 2
4.65 ± 1.14	3.58 ± 0.34	0.830 ± 0.366	0.814 ± 0.222[c]	111 ± 42[c]	This study; 1.0-mg/kg dose

[a] Mean ± *SD*. [b] Range (values not normally distributed). [c] Significantly different from the ratio obtained after the 0.1-mg/kg dose ($p < 0.001$).

mogenate binds warfarin (2). The results of this investigation demonstrate that *in vivo* warfarin uptake by the rat liver is drug concentration dependent, as reflected by a striking decrease in the liver to serum warfarin concentration ratio with increasing drug concentration (Table III). The more than proportional increase in the free and total serum warfarin concentrations with increasing dose (Tables I and II) may reflect a decrease in the apparent distribution volume, but this result can also be due to a dose-dependent change in the intrinsic drug clearance. Studies are now in progress to clarify this problem.

The free fraction of warfarin in serum of rats is concentration independent over a wide concentration range but exhibits considerable interanimal variation (2). The free warfarin fractions in serum and liver in any one rat are strongly correlated (2, 6). Consequently, the interindividual variation of the liver to serum warfarin concentration ratio at a given drug concentration is much smaller than the interindividual variation of free fraction values (6). The ratio of the total warfarin concentration in the liver to the free serum warfarin concentration during the β-phase is an unambiguous index of the hepatic warfarin uptake[2]. The fact that this ratio is very high (866 at 6 hr after a 0.1-mg/kg dose) and decreases markedly with increasing concentration suggests that warfarin is bound to saturable hepatic tissues and/or transported from plasma to the liver by a saturable process.

Kekki *et al.* (9) attempted to determine warfarin concentrations in rat plasma and liver as a function of time after intravenous injection of 1, 2, 8, and 40 mg/kg, using a spectrophotometric assay. They reported that assay difficulties prevented the determination of warfarin concentrations in the liver after the 1- and 2-mg/kg doses. The liver to plasma total concentration ratio 6 hr after injection of 8 mg/kg was ~0.2, and the plasma warfarin concentration was 38.4 ± 4.8 μg/ml (mean ± *SE*). These data are not inconsistent with those summarized in Table III. The liver to plasma total concentration ratio 6 hr after injection of 40 mg/kg was actually somewhat *higher* than that produced by the 8-mg/kg dose, apparently because of the threefold increase in the free fraction of warfarin in plasma at these very high and pharmacologically unrealistic concentrations.

Consideration of the results of this investigation together with those of Kekki *et al.* (9) indicates that a concentration-dependent warfarin uptake by the liver causes the liver to plasma warfarin concentration ratio to decrease with increasing concentration over the pharmacologically realistic concentration range (*i.e.*, in the range where synthesis of vitamin K-dependent clotting factors is inhibited or blocked) and that concentration-dependent plasma protein binding may reverse this trend at higher concentrations. Since the liver accounts for a major fraction of the total warfarin in the body of a rat following administration of a pharmacologically realistic dose (2), concentration-dependent hepatic drug uptake apparently may result in nonlinear (dose-dependent) warfarin pharmacokinetics in intact animals and particularly in a decrease in the apparent volume of distribution of the drug with increasing dose in the pharmacologically realistic dose range. Studies are now in progress to examine that possibility.

[2] The ratio of drug concentrations in the liver and in serum from blood obtained from the aorta is not necessarily identical to the ratio of drug concentrations in the liver and in serum from the hepatic venous outflow. With warfarin, a drug whose hepatic clearance is very much lower than the hepatic blood flow, the two ratios can be expected to be virtually identical on the basis of the criteria established by Chen and Gross (8).

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COMMUNICATIONS

Pharmacokinetics of *cis*-Dichlorodiammine Platinum(II) in Rats Using an External Loop–Eigenfunction Expansion Technique

Keyphrases □ Pharmacokinetics—organometallic antineoplastic agents, external loop blood sampling techniques, eigenfunction expansion analysis, Fourier transform analysis □ External loop technique—blood collection, pharmacokinetics, eigenfunction expansion analysis □ Antineoplastic agents—organometallic, pharmacokinetics, external loop technique, eigenfunction expansion analysis

To the Editor:

An external loop technique for collecting many closely spaced blood concentration–time data points, early in an experiment when the concentration changes rapidly, was developed recently for compounds labeled with γ-emitting isotopes (1, 2). The large amount of accurate data collected at such frequent intervals allows the use of the more objective methods of Fourier transform or eigenfunction expansion analysis for the determination of pharmacokinetic parameters.

With conventional data collection methods, determination of the optimum number of exponentials necessary to describe adequately the time course of the drug is difficult. With a limited number of data points collected over a relatively short period (4–6 hr), a maximum of three exponentials has been suggested (3). For studies conducted over several days, it is possible to resolve more exponentials (4).

The external loop method permits continuous monitoring of radiopharmaceuticals and provides several advantages over traditional blood sampling techniques:

1. There is no sampling time error.
2. The number of samples is ~500 *versus* 40 since there is no blood volume loss to limit the sampling.
3. The sample volume does not change. Such a change affects both the compartment size and the loss of activity.
4. The continuous cumulative count of the analyzer gives an accurate average activity over the time interval.
5. The time interval may be varied or kept constant with a lower limit of less than a millisecond. This flexibility allows the collection of different data sets, thus enhancing numerical analysis by gaining more information at a crucial time.

The large amount of data collected permits the use of Fourier transform analysis to resolve the number of exponentials. Fourier transform analysis was used previously to analyze multicomponent exponential decay curves (5–8). By this method, it is possible to transform the raw data and obtain a plot of the transformed data *versus* the rate constants (λ). The major contributing rate constants can be determined as major peaks in this curve. Provencher (9) recently described a digital computer program for data analysis (9) that is described by a multiexponential equation using the Fourier transform method proposed by Gardner *et al.* (5, 6). Subsequently, an alternative method using an eigenfunction procedure was proposed (10).

cis-Dichlorodiammine platinum(II) (I) represents the first of a series of organometallic antineoplastic agents to be marketed. This compound is used clinically, particularly in the treatment of metastatic testicular carcinoma. Pharmacokinetic parameters for the platinum moiety from I were determined in the rat using traditional blood sampling techniques (4, 11) and an external loop method (2). As an initial step in further studies using I and related agents, we investigated the kinetics of I using an external loop and analyzed the data with an eigenfunction procedure[1].

Male Sprague–Dawley rats, 300–350 g, were prepared as previously described (1). Hexachloroplatinic acid containing platinum-195m[2] was used as the starting material

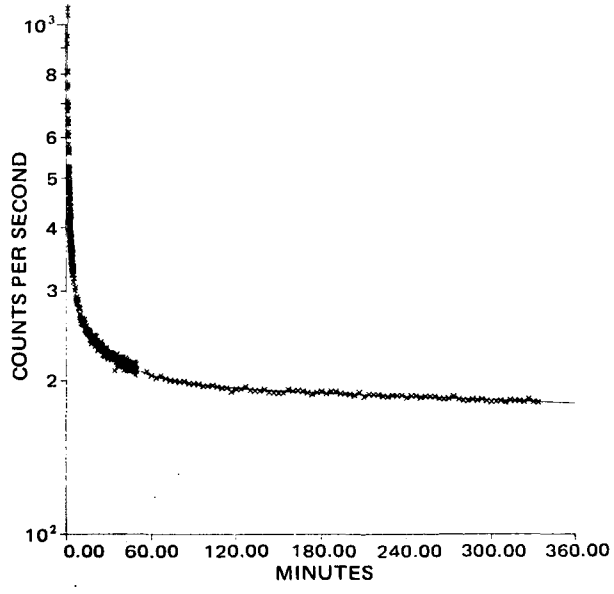

Figure 1—*Plot of counts per second* (×) versus *time measured in an external blood loop following intravenous administration of 1.1 mg of* cis*-dichlorodiammine platinum(II)/kg to Rat 1. The solid line was calculated using the equation that best fit the data.*

[1] A digitial computer program for performing the analysis was kindly provided by S. W. Provencher, Max-Planck-Institut fur Biophysikalische Chemie, D-3400 Gottingen-Nikolausberg, Federal Republic of Germany.
[2] Oak Ridge National Laboratory, Oak Ridge, Tenn.

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