Intraindividual Relationships between Serum Protein Binding of Drugs in Normal Human Subjects, Patients with Impaired Renal Function, and Rats

AVRAHAM YACOBI and GERHARD LEVY ˣ

Abstract □ The serum protein binding of phenytoin, salicylic acid, sulfisoxazole, and warfarin was determined in normal human adults, in patients with impaired renal function (kidney donor and recipient), and in adult male Sprague–Dawley rats. The free fraction values for salicylate and sulfisoxazole were significantly correlated in all three groups. The other correlations were statistically significant in only one or two of these groups. There was a statistically significant negative correlation between albumin concentration and the free fraction values of salicylic acid and sulfisoxazole (but not of phenytoin and only under special circumstances with warfarin) in normal human subjects and of phenytoin, salicylic acid, and sulfisoxazole (but not warfarin) in rats. No such correlation was observed for any of the drugs in patients with impaired renal function. These observations show that no single weakly acidic drug can serve as an index for quantitatively determining the effect of disease or species differences on the serum protein binding of other weakly acidic drugs.

Keyphrases □ Protein binding, serum—phenytoin, salicylic acid, sulfisoxazole, and warfarin compared, normal and renally impaired humans, rats □ Binding, serum protein—phenytoin, salicylic acid, sulfisoxazole, and warfarin compared, normal and renally impaired humans, rats □ Phenytoin—serum protein binding compared to salicylic acid, sulfisoxazole, and warfarin, normal and renally impaired humans, rats □ Salicylic acid—serum protein binding compared to phenytoin, sulfisoxazole, and warfarin, normal and renally impaired humans, rats □ Sulfisoxazole—serum protein binding compared to phenytoin, salicylic acid, and warfarin, normal and renally impaired humans, rats □ Warfarin—serum protein binding compared to phenytoin, salicylic acid, and sulfisoxazole, normal and renally impaired humans, rats

The serum protein binding of drugs is affected by age and disease and differs among species (1–4). There are pronounced interindividual differences in the protein binding of some drugs even among relatively homogeneous groups of healthy humans (5) or animals (6). Since serum protein binding can significantly affect the pharmacokinetic characteristics of drugs (7, 8), considerable effort has been directed to the study of different variables that affect the binding of drugs to serum protein. It would be convenient if one or two acidic, neutral, and basic drugs could serve as representative index compounds in such studies. Therefore, the intrasubject relationships between the serum protein binding of several weakly acidic drugs (phenytoin, salicylic acid, sulfisoxazole, and warfarin) in normal human adults, patients with impaired renal function, and normal adult rats were studied.

EXPERIMENTAL

A single venous blood sample was obtained from 39 normal adults[1], consisting of 19 males (age 31.6 ± 8.5 years, range 21–54) and 20 females (age 27.1 ± 7.8 years, range 18–52). Periodic venous blood samples were obtained from a 9-year-old white male recipient of a renal transplant and from the donor, his 29-year-old mother. Blood sampling began before the transplant, while the recipient was bilaterally anephric, and continued for almost 3 months, during which the recipient experienced a rejection episode which was overcome in about 18 days. Further details of this case have been published elsewhere (9). Single blood samples were obtained from adult male Sprague–Dawley rats, 300–460 g, maintained on a standard diet[2].

Serum was separated, and phenytoin (15 μg/ml, including ^{14}C-phenytoin), salicylic acid (300 μg/ml), sulfisoxazole (100 μg/ml), and ^{14}C-warfarin (0.8 μg/ml) were added to different portions of each serum sample or to the buffer phase of the dialysis system. The serum samples were dialyzed to equilibrium against an equal volume of pH 7.4 phosphate buffer at 37°. Details of the dialysis procedures and analytical methods were described elsewhere (5, 9, 10).

The binding data are expressed as free fraction values, *i.e.*, the ratio of concentrations of unbound to total (free and bound) drug. Total serum protein concentration was determined by the method of Gornall *et al.* (11), and the fraction of albumin was measured with a commercial[3] electrophoresis system. Crystalline human and rat albumins were used as standards.

Table I—Relationship between Serum Free Fraction (*f*) Values of Various Drugs in Normal Human Adults

Drug Pair	*f* Range[a]	*r*	*n*	*p*
Phenytoin–salicylic acid	0.111–0.151 (I) 0.181–0.296 (II)	0.122	30	>0.6
Phenytoin–sulfisoxazole	0.111–0.151 (I) 0.0819–0.125 (III)	0.398	22	>0.1
Salicylic acid–sulfisoxazole	0.182–0.296 (II) 0.0819–0.125 (III)	0.736	22	<0.001
Warfarin–phenytoin	0.00503–0.0186 (IV) 0.111–0.155 (I)	0.032	39	>0.8
Warfarin–salicylic acid	0.00503–0.0172 (IV) 0.181–0.296 (II)	−0.292	30	>0.1
Warfarin–sulfisoxazole	0.00503–0.0172 (IV) 0.0819–0.125 (III)	0.007	22	>0.9

[a] Ranges of *f* are listed for phenytoin (I), salicylic acid (II), sulfisoxazole (III), and warfarin (IV) for the stated number of subjects (*n*).

RESULTS

The results of all protein binding correlation studies are summarized in Tables I–III. These tables include the results of previously published

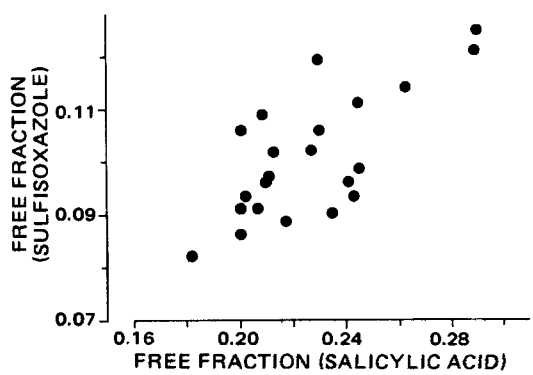

Figure 1—*Relationship between free fraction values of salicylic acid and sulfisoxazole in serum of 22 normal human adults;* r = *0.736,* p < *0.001.*

[1] Thirty-seven were Caucasian, one was Oriental, and one was of Peruvian ancestry. Most of the subjects were faculty, graduate students, and research personnel.

[2] Charles River Formula 4RF.

[3] Gelman Sepratek.

Table II—Relationship between Serum Free Fraction (f) Values of Various Drugs in Patients with Impaired Renal Function[a]

Drug Pair	f Range	r	n	p
Phenytoin–salicylic acid	0.143–0.233 (I) 0.192–0.374 (II)	0.867	17	<0.001
Phenytoin–sulfisoxazole	0.143–0.233 (I) 0.080–0.261 (III)	0.789	17	<0.001
Salicylic acid–sulfisoxazole	0.190–0.374 (II) 0.075–0.261 (III)	0.822	24	<0.001
Warfarin–phenytoin	0.00686–0.0162 (IV) 0.143–0.233 (I)	0.768	8	<0.05
Warfarin–salicylic acid	0.00686–0.0162 (IV) 0.190–0.374 (II)	0.837	13	<0.001
Warfarin–sulfisoxazole	0.00686–0.0162 (IV) 0.075–0.261 (III)	0.798	13	<0.005

[a] Designations as in Table I, except that n indicates the number of blood samples obtained from a kidney donor and recipient over a period of time.

Table III—Relationship between Free Fraction (f) Values of Various Drugs in Normal Adult Rats[a]

Drug Pair	f Range	r	n	p
Phenytoin–salicylic acid	0.179–0.280 (I) 0.390–0.690 (II)	0.866	18	<0.001
Phenytoin–sulfisoxazole	0.179–0.280 (I) 0.0685–0.196 (III)	0.785	18	<0.001
Salicylic acid–sulfisoxazole	0.390–0.690 (II) 0.0685–0.196 (III)	0.817	20	<0.001
Warfarin–phenytoin	0.00405–0.0296 (IV) 0.179–0.280 (I)	0.079	18	>0.7
Warfarin–salicylic acid	0.00405–0.0386 (IV) 0.390–0.690 (II)	0.323	20	>0.1
Warfarin–sulfisoxazole	0.00405–0.0386 (IV) 0.0685–0.196 (III)	0.289	20	>0.2

[a] Designations as in Table I.

investigations of protein binding correlations of warfarin–phenytoin in normal human adults (5) and of salicylic acid–sulfisoxazole in patients with impaired renal function (9) and in rats (12). The additional protein binding data reported here were obtained from the same serum samples, except that not all binding determinations could be done on all samples because of the limited volume of serum. The results of the determinations of the relationship between serum albumin concentration and the free fraction values of the different drugs are summarized in Tables IV–VI.

Studies in Normal Human Subjects—There was a statistically significant correlation between the free fraction values of salicylic acid and sulfisoxazole (Fig. 1) but not between any of the other combinations. The relatively widest range of serum free fraction values was found for warfarin (approximately 3.5-fold); the other three drugs had a considerably smaller intersubject variation (approximately 1.5-fold). There was a

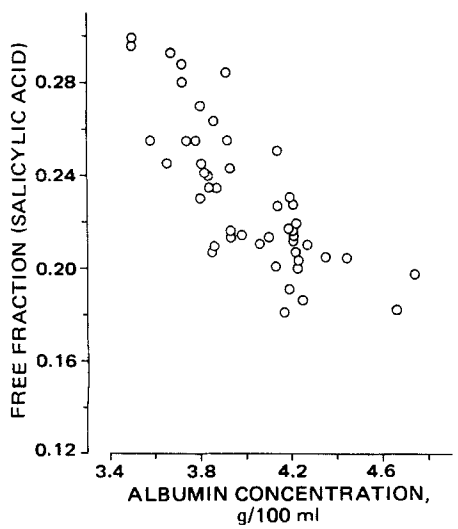

Figure 2—*Relationship between free fraction values of salicylic acid and albumin concentration in serum of 48 normal human adults; r = −0.772, p < 0.001. Eighteen subjects were from a previous study.*

Table IV—Relationship between Albumin Concentration and Free Fraction (f) Values of Various Drugs in Serum of Normal Human Adults

Drug	Range of f and A[a]	r	n	p
Phenytoin	0.111–0.155 3.50–4.72	−0.188	39	>0.2
Salicylic acid	0.181–0.299 3.50–4.74	−0.772	48	<0.001
Sulfisoxazole	0.0819–0.125 3.50–4.66	−0.699	22	<0.001
Warfarin	0.00503–0.0172 3.50–4.66	(See *Discussion*)		

[a] Range of f is on upper line; range of albumin concentration (A) is on lower line.

Table V—Relationship between Albumin Concentration and Free Fraction (f) Values of Various Drugs in Serum of Patients with Impaired Renal Function

Drug	Range of f and A[a]	r	n	p
Phenytoin	0.143–0.233 3.55–5.00	−0.259	17	>0.3
Salicylic acid	0.190–0.374 3.55–5.25	−0.126	24	>0.5
Sulfisoxazole	0.075–0.261 3.55–5.25	0.034	24	>0.8
Warfarin	0.00686–0.0162 3.95–5.12	−0.093	13	>0.7

[a] Range of f is on upper line; range of albumin concentration (A) is on lower line.

statistically significant negative correlation between the serum albumin concentration and the serum free fraction values of salicylic acid (Fig. 2) and sulfisoxazole. No such correlation was found for phenytoin and warfarin.

Studies on Patients with Impaired Renal Function—Figure 3 shows the change in free fraction values of phenytoin in the donor and recipient of a renal transplant over ~3 months. The serum free fraction in the donor increased shortly after removal of the kidney and eventually returned to normal. The free fraction in the serum of the recipient was substantially elevated while he was anephric, decreased to near normal shortly after the transplant, increased again during the rejection episode, and then decreased to normal some time after the rejection episode had been overcome. Similar patterns were observed with respect to the serum protein binding of salicylic acid and sulfisoxazole (9) and warfarin.

Statistically highly significant correlations were found between the free fraction values of all combinations of the four drugs (Table II). Since there was no significant difference between donor and recipient with respect to the correlation slopes (Figs. 4 and 5 and Ref. 9), the data were combined for the statistical analysis. There was no apparent correlation between the serum albumin concentration and the free fraction values of any of the drugs (Table V).

Studies on Normal Adult Rats—Highly statistically significant correlations between serum free fraction values were found for the drug pairs phenytoin–salicylic acid, phenytoin–sulfisoxazole, and salicylic acid–sulfisoxazole, while no significant correlation was apparent for warfarin–phenytoin, warfarin–salicylic acid, or warfarin–sulfisoxazole (Table III). The free fraction values for phenytoin, salicylic acid, and sulfisoxazole showed a significant negative correlation with the albumin concentration in serum; no such correlation was found with respect to warfarin (Table VI).

Table VI—Relationship between Albumin Concentration and Free Fraction (f) Values of Various Drugs in Serum of Normal Adult Rats

Drug	Range of f and A[a]	r	n	p
Phenytoin	0.179–0.280 3.00–3.79	−0.646	18	<0.005
Salicylic acid	0.390–0.690 3.00–3.79	−0.565	20	<0.01
Sulfisoxazole	0.0685–0.196 3.00–3.79	−0.543	20	<0.02
Warfarin	0.00405–0.0386 3.00–3.79	−0.0277	20	>0.9

[a] Range of f is on upper line; range of albumin concentration (A) is on lower line.

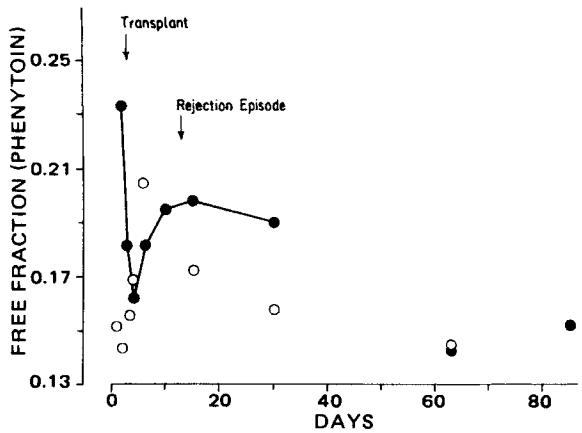

Figure 3—*Free fraction of phenytoin in serum of donor (○) and recipient (●) of a kidney transplant as a function of time.*

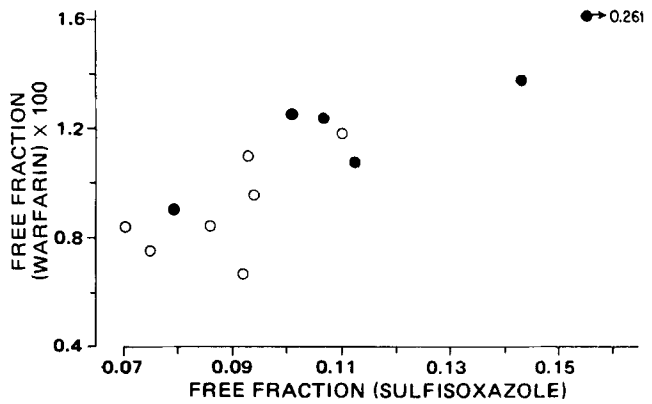

Figure 5—*Relationship between the serum free fraction values of sulfisoxazole and warfarin in serum samples from a kidney donor (○) and recipient (●) obtained over ~3 months;* r = *0.798,* p < *0.005.*

DISCUSSION

There are several possible approaches to the study of correlations between the serum protein binding of different drugs under various conditions. A relatively fundamental approach is the determination of the classic binding parameters of the drugs in solutions of pure albumin. This investigation focused more directly on the clinically relevant aspects of the problem by use of undiluted serum and widely used drugs at concentrations in the therapeutic range.

In comparing the results obtained with serum from normal humans, normal rats, and humans with impaired renal function, it should be recognized that data from the latter group essentially reflect intra- rather than interindividual differences because only two patients were studied and serum was obtained repeatedly during periods of changing renal function. It was considered desirable to use this study design rather than to make one-point-in-time determinations of protein binding in serum from a larger group of patients since it is important to determine, from a clinical pharmacokinetic point of view, the time course of events in patients with impaired renal function.

The serum free fraction values of all drugs studied in this investigation varied appreciably between subjects in the normal humans and rats. The most pronounced variation was found with respect to warfarin, particularly in rats. Such variation may be due to differences in albumin concentration or structure or be caused by endogenous inhibitors of protein binding (13) that may differ among subjects in concentration or kind. Albumin concentration is an important determinant of the serum protein binding of salicylic acid and sulfisoxazole in normal humans and rats and of phenytoin in rats (Table II). A statistically significant negative correlation was demonstrated between the albumin concentration and the warfarin free fraction in the serum of fasted normal humans (14) but not of subjects whose food intake was not restricted or controlled (5), as in this study (Table IV). Apparently, variations in the concentration of fatty acids or other food-derived substances obscure the effect of interindividual differences in the serum albumin concentration on the serum protein binding of warfarin. The very pronounced interindividual variation in the serum protein binding of warfarin in rats may be due to genetic influences, since the free fraction values show a trimodal frequency distribution in these animals (6) but are unimodal in humans (5).

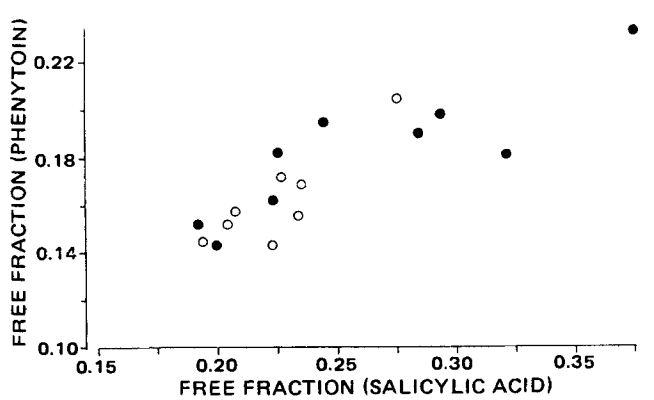

Figure 4—*Relationship between the free fraction values of phenytoin and salicylic acid in serum samples from a kidney donor (○) and recipient (●) obtained over ~3 months;* r = *0.867,* p < *0.001.*

The only statistically significant correlation of free fraction values in all three groups was found for salicylic acid–sulfisoxazole. Data suggesting competitive inhibition between salicylate and a sulfonamide for binding sites on albumin were reported previously (15). All possible pairs of drugs showed statistically significant correlations of free fraction values in the patients with impaired renal function. There is indirect evidence that this pathological condition is associated with pronounced elevation of endogenous inhibitors of serum protein binding (13), and this variable apparently affects all drugs used in this investigation. It also apparently obscures the effect of variations in albumin concentration, since there was no statistically significant relationship between free fraction values for any of the drugs and the serum albumin concentration in the serum of the two patients. However, if these relationships are examined separately for donor and recipient, a statistically significant negative correlation is found between the albumin concentration and the free fraction of salicylate in the serum of the donor ($r = -0.718$, $p < 0.01$).

The observations on the change in the free fraction values of phenytoin and warfarin after renal transplantation are in agreement with results obtained by Odar-Cederlöf (16), but this investigator studied the patients for a shorter period, did not encounter a rejection episode, and did not examine the serum binding characteristics of kidney donors.

There can be several reasons for the lack of correlation between the serum free fraction values of different drugs. They may have different binding sites, their association constant with albumin may differ significantly so that an endogenous inhibitor may effectively displace one but not the other drug, and the molar concentration ratio of drug to albumin in the therapeutic drug concentration range may differ appreciably. The latter two variables can influence the sensitivity of the free fraction value to changes in albumin concentration. These same factors, as well as qualitative and quantitative differences in albumin and/or endogenous inhibitors, may be responsible for species differences and differences between healthy subjects and patients with renal or other diseases (7). Consequently, it appears that no single drug can serve as an index compound for a group of other drugs. The investigation described in this report demonstrates this fact with respect to a group of weakly acidic drugs.

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Isolation and Identification of Morphine 3- and 6-Glucuronides, Morphine 3,6-Diglucuronide, Morphine 3-Ethereal Sulfate, Normorphine, and Normorphine 6-Glucuronide as Morphine Metabolites in Humans

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Abstract □ Morphine metabolites were isolated with column chromatography on a resin and neutral aluminum oxide and TLC from the urine of morphine-dependent subjects maintained on morphine sulfate at a dose of 240 mg/day. These metabolites were characterized as morphine 3-glucuronide, morphine 6-glucuronide, morphine 3,6-diglucuronide, morphine 3-ethereal sulfate, normorphine, normorphine 6-glucuronide, and, possibly, normorphine 3-glucuronide by free phenol and glucuronide tests, enzymatic hydrolysis, GLC, TLC, UV spectroscopy, and GLC–mass spectrometry.

Keyphrases □ Morphine metabolites, various—column chromatographic and TLC isolation from human urine □ Chromatography, column—isolation of various morphine metabolites from human urine □ TLC—isolation of various morphine metabolites from human urine □ Narcotic analgesics—various morphine metabolites, column chromatographic and TLC isolation from human urine

Studies with animals and humans have shown metabolism of morphine by the following pathways: (*a*) conjugation to give morphine 3-glucuronide, morphine 6-glucuronide, and morphine 3-ethereal sulfate (1–8); (*b*) *N*-demethylation to yield normorphine, which was then conjugated (9–15); (*c*) *O*-methylation to form codeine (16, 17); and (*d*) oxidation to form dihydromorphinone (18). Recently, a minor metabolite, tentatively identified as morphine 2,3-quinone, was reported in the urine of rats given morphine and after incubation of morphine with rat brain homogenates (19). Also, one study was unable to confirm the conversion of morphine to codeine (20).

Only morphine 3-glucuronide has been isolated as a morphine metabolite in humans (2), and a small amount of morphine 6-glucuronide was detected by TLC (5). The present paper reports the isolation and identification of morphine 3- and 6-glucuronides, morphine 3,6-diglucuronide, morphine 3-ethereal sulfate, normorphine, normorphine 6-glucuronide, and, possibly, normorphine 3-glucuronide from the urine of morphine-dependent human subjects.

EXPERIMENTAL

Materials and Subjects—The morphine sulfate USP used was a commercial product; normorphine hydrochloride was used as received[1]. Pseudomorphine and morphine *N*-oxide were synthesized according to the procedures of Fulton (21) and Freund and Speyer (22), respectively. Morphine 3-glucuronide, codeine 6-glucuronide, and morphine 3-ethereal sulfate were isolated from the urine of dogs and cats given morphine and codeine, respectively (3, 9, 23).

Four adult postaddict males[2] were judged to be in good health from recent history and physical and laboratory examinations, including tests of hematological, renal, hepatic, and cardiac functions. The subjects had an average age of 33 years (range of 27–41) and an average weight of 66 kg (range of 61–71). All subjects were given morphine sulfate injections in gradually increasing doses from 10 to 60 mg sc four times daily. Urine was collected from subjects during maintenance on morphine sulfate at 60 mg after several months and stored in a refrigerator.

Extraction of Morphine and Its Metabolites—Morphine, normorphine, and other possible metabolites were extracted from samples according to the procedure described previously (14). Samples were adjusted to about pH 10, buffered at pH 10.4 with 2 ml of 40% phosphate buffer (37% K_2HPO_4 and 3% K_3PO_4), salted, and extracted with 1,2-dichloroethane (glass distilled) containing 30% (v/v) 2-propanol.

The organic phase was shaken with 1 *N* HCl; then the acidic aqueous phase was separated and adjusted to pH 10.4, buffered, salted, and extracted again with the organic solvent. The residue obtained upon evaporation of the organic phase to dryness was used for TLC and GLC identification of morphine metabolites. For GLC identification, a derivative was prepared using 25% trimethylsilylimidazole in pyridine[3].

TLC—TLC was performed with either 250-μm silica gel plates with a preadsorbent area[4] or instant TLC sheets impregnated with silica gel[5].

[1] Courtesy of Dr. E. L. May, National Institutes of Health.
[2] Federal prisoner volunteers, incarcerated at the National Institute on Drug Abuse Research Center. Informed consent was obtained in writing in the presence of a witness.
[3] Pierce Chemical Co., Rockford, Ill.
[4] Quantum Industries, Fairfield, N.J.
[5] Gelman Instrument Co., Ann Arbor, Mich.