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PROTEIN BINDING OF DIAZEPAM AND DIGITOXIN IN UREMIC AND NORMAL SERUM

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Abstract—The protein binding of diazepam and digitoxin in serum from uremic patients has been studied by equilibrium dialysis and compared to that in normal serum. Comparisons have also been made with isolated human serum albumin (HSA) from uremic and normal individuals. Diazepam and digitoxin are bound to different sites on HSA. Their binding was impaired in the serum from the patients when compared to that in the normal serum owing to decreased affinity constants for the binding to the primary sites on albumin. In the uremic serum the number of binding sites for diazepam is increased compared with the number in normal serum and HSA. For digitoxin the number of binding sites is larger both in the normal and the patient serum than that obtained with HSA. The fact that apparently an increased number of binding sites is made use of, is probably due to the presence of substances which inhibit the binding to the primary sites. The binding of the drugs was improved after charcoal-treatment of the uremic albumin at pH 3.0.

The decreased protein binding of many drugs in uremic patients is well established [1–4]. The decreased binding is primarily unrelated to the low serum albumin concentrations often seen in these patients, but is essentially due to the presence of endogenous substances which inhibit the binding of the drugs to albumin [1, 2]. Also, in normal serum the binding of drugs is to some extent inhibited by endogenous substances though the effects are less pronounced than in uremia [1].

Also in liver diseases inhibiting substances are frequently present in blood, resulting in decreased drug-binding capacity. However, it has recently been shown that the decreased binding is not a phenomenon experienced with all protein-bound drugs, but apparently a differentiated effect on specific binding sites of the serum albumin [5]. Thus, the binding of diazepam is decreased as compared with that in normal serum, while the binding of warfarin is unaffected in liver cirrhosis. These results suggest that there are specific inhibitors present in sera from patients with liver cirrhosis and that diseases might influence the binding of various drugs in different ways.

Albumin is the principal binding protein for many drugs in serum, and at least three different specific drug-binding sites are recognized on the molecule [6–8]. It has been shown that warfarin, diazepam and digitoxin, three drugs which are highly protein-bound, bind to different sites on the albumin molecule. It has also been shown that the binding of warfarin in uremic serum is inhibited, owing to a decreased affinity constant, K_{app}, of the drug–albumin complex. The decreased binding of diazepam has been established by ultracentrifugation [9] and qualitatively by circular dichroism [1]. The purpose of the present investigation was to study in more detail the influence of uremia on the binding of diazepam and digitoxin, representing drugs that are bound to other sites on albumin than warfarin.

MATERIALS AND METHODS

Patient material. Ten patients with uremia took part in the study after informed consent. Clinical data and drug treatment are given in Table 1. The patients were treated at the Renal Unit, Dept. of Internal Medicine, Karolinska Hospital, Stockholm, Sweden. As far as possible such patients were selected who were not treated with drugs known to act as inhibitors of drug protein binding. Some of them were treated with digitoxin. However, the therapeutic serum concentration is only 10–50 nmole/l and will thus not interfere in the *in vitro* studies.

Serum. Serum samples were collected and stored frozen at −20°. After analysis, the sera were pooled to give two serum pools; from one pool albumin was separated, while the other was used for the serum binding studies. Serum was also collected from 10 drug-free healthy volunteers, to give a serum pool for reference purposes.

Human serum albumin. HSA was prepared from normal and uremic serum as described earlier [1]. Some of the albumin was treated with activated charcoal at pH 3.0 according to Chen [10].

Drugs. [^{14}C]Diazepam (59.4 mCi/m-mole) was synthesized and analyzed as described earlier [5]. [^{3}H]Digitoxin (7.4 Ci/m-mole) was purchased from the Radiochemical Centre, Amersham, and purified as

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Table 1. Clinical data of the uremic patients

Patient	Age	Sex	Diagnosis	Serum values			Medication (grams daily)
				Creatinine μ moles/l	Urea m-moles/l	Albumin g/l	
UJ	47	F	Dysplastic kidneys	1280	29	31	Sodium bicarbonate, 2.0; allopurinol, 0.3; aluminium hydroxide; sodium polystyrene sulfonate, 15.0.
GL	69	F	Chronic nephritis	630	25	37	Digoxin, 0.0001; hydralazine, 0.075; bendroflumethiazide, 0.010; spironolactone, 0.075; alprenolol, 0.150.
KL	61	M	Prostatic cancer Pyelonephritis	1640	73	42	Hydralazine, 0.030; furosemide, 0.120; sodium bicarbonate, 3.0; sodium polystyrene sulfonate, 30.0.
NB	65	M	Chronic nephritis Heart failure	675	15	32	Digoxin, 0.0001; furosemide, 0.240; sodium bicarbonate, 3.0; calcium lactate; aluminium hydroxide.
PZ	66	M	Chronic glomerulonephritis	940	33	39	Digitoxin, 0.0001; furosemide, 0.080; sodium bicarbonate, 6.0; calcium lactate; aluminium hydroxide; calcium polystyrene sulfonate, 45.0.
KB*	62	M	Polycystic kidneys	1020	25	40	Digoxin, 0.00013; furosemide, 0.240; calcium lactate, 2.0; aluminium hydroxide; vitamins.
SS	59	F	Polycystic kidneys	1080	18	45	Digitoxin, 0.0001; furosemide, 1.0; spironolactone, 0.075; sodium bicarbonate, 2.0; calcium lactate; aluminium hydroxide; sodium polystyrene sulfonate, 30.
HB	55	M	Hypertension nephrosclerosis	840	28	43	Digitoxin, 0.0003; furosemide, 0.120; hydralazine, 0.100.
DW	30	M	Chronic glomerulonephritis	1840	47	45	Sodium bicarbonate, 4.0; aluminium hydroxide.
KG*	58	F	Polycystic kidneys	1020	27	43	Digitoxin, 0.0001; furosemide, 0.080; aluminium hydroxide; allopurinol, 0.200; ferrous sulphate, 0.300; vitamins.

* Patients in chronic intermittent hemodialysis.

described [6]. The corresponding unlabelled drugs were added to the isotope solutions to achieve suitable drug concentrations. Unlabelled drugs were obtained as gifts from the manufacturers.

Equilibrium dialysis. Serum protein binding was determined at 37° as described earlier [1]. The time used for equilibration was 5 hr for diazepam and 10 hr for digitoxin. The drugs were dissolved in ethanol and suitable amounts were dispensed into glass tubes. The ethanol was then evaporated and serum was added. Controls showed that the drugs redissolved completely in the serum. Serum albumin concentration was determined by immunochemical quantitation according to Mancini *et al.* [11] using M-Partigen® plates (Hoechst Behringwerke AG). The determinations were made at the end of the dialysis.

Gelfiltration. Serum was fractionated by gelfiltration on a Sephadex G-200 column (2.5 × 100 cm) in an isotonic 0.02 M sodium phosphate buffer, pH 7.4. The protein content of the fractions was analyzed by polyacrylamide-gel electrophoresis [12], at pH 8.3. The gels were stained by Coomassie Brilliant Blue [13]. Selected fractions were pooled and concentrated in a Diaflo® apparatus.

Mathematical analysis. The serum protein binding data were analyzed in the same way as described earlier [1]. K_{app}, the apparent association constant, obtained with the different samples, was determined from the Scatchard equation [14],

$$\frac{r}{[D]} = n \cdot K_{app} - r \cdot K_{app} \qquad (1)$$

In this equation r = moles of bound drug/mole of albumin, [D] = the concentration of unbound drug and n = the number of sites on albumin.

The possible effects of inhibitors present in the samples can be studied in diluted samples, as earlier advanced [1], by the following equation:

$$K_{app} = K_a^* - K_{app} \cdot C_{se} \cdot I_0 \cdot K_i \qquad (2)$$

C_{se} is the fractional concentration of the serum in the different diluted samples and I_0 the initial unbound concentration of the inhibitor in the serum. When K_{app} is plotted against $K_{app} \cdot C_{se}$, a straight line is obtained when the binding is competitively inhibited, and K_a^*, characterizing the particular drug–protein equilibrium, can be obtained from the intercept on the y-axis. The slope of the line $(-I_0 \cdot K_i)$ will be characteristic for the particular inhibitor–serum system. Linear regression analysis was used to characterize linear relationships. The values on the x-axis were then used as the independent and the values on the y-axis as the dependent variables.

RESULTS

Equilibrium dialysis studies. The percentual serum protein binding at the lowest drug concentrations used are given in Table 2, where the values refer to the binding in undiluted serum. The binding of both diazepam and digitoxin was decreased in the uremic serum compared to the situation in normal serum. The Scatchard plots for both diazepam and digitoxin (Figs. 1–3) show that the decreased binding of the drugs is primarily due to a decreased binding affinity.

The Scatchard plots obtained in the binding of diazepam with HSA and normal serum, respectively, shown in Fig. 1, give intercepts on the r-axis close to $r = 1$.

Table 2. Serum protein binding in per cent of total drug concentration

Serum	Diazepam 0.01 mM	Digitoxin 0.005 mM	Serum Albumin mg/ml
Normal	98.8	95.7	39.6
Uremic	95.3	93.4	33.3

These results indicate that diazepam (under the conditions used) has one primary binding site on HSA, and also that albumin is the primary protein responsible for the binding in normal serum. In uremic serum, the binding affinity is markedly decreased as is seen from the lower intercepts on the r/D axis and the lower K_{app} values obtained. Moreover, the intercept on the r-axis is increased.

Figure 2 shows the Scatchard plots for the binding of digitoxin in normal serum and to isolated HSA, and Fig. 3 shows the binding in uremic serum. With isolated charcoal-treated albumin, the n-value was close to 1 (Fig. 2), while both in normal and uremic undiluted serum the n-values were significantly higher. The binding affinity was decreased in the uremic serum when compared to that in normal serum. The values obtained from the plots are summarized in Table 3. Values obtained with albumin isolated from uremic serum are

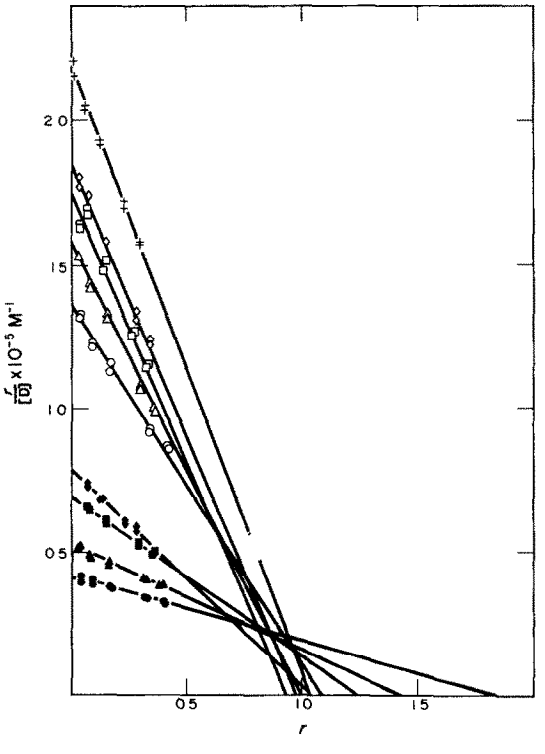

Fig. 1. Scatchard plots of the binding of diazepam to normal serum (○——○ undiluted, △——△ diluted 1 + 1, □——□ diluted 1 + 4, ◇——◇ diluted 1 + 9), uremic serum (●——● undiluted, ▲——▲ diluted 1 + 1, ■——■ diluted 1 + 4, ◆——◆ diluted 1 + 9) and charcoal treated HSA (+——+) studied by equilibrium dialysis. The sera were diluted with an isotonic phosphate buffer, pH 7.4.

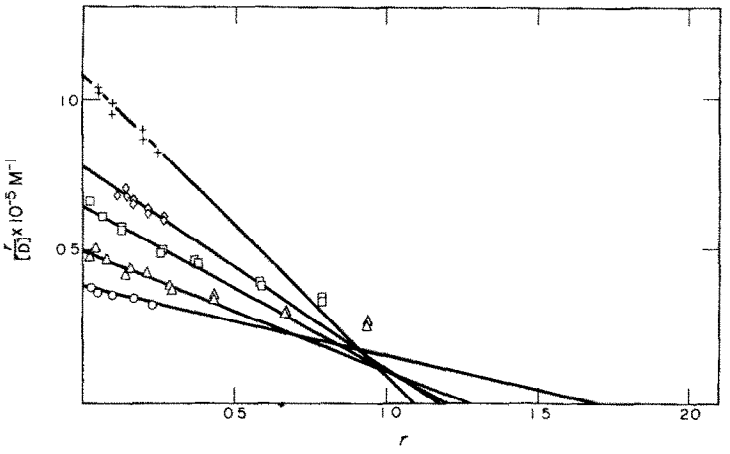

Fig. 2. Scatchard plots of the binding of digitoxin to normal serum (○——○ undiluted, △——△ diluted 1 + 1, □——□ diluted 1 + 4, ◇——◇ diluted 1 + 9) and to charcoal-treated HSA (+——+) studied by equilibrium dialysis. The sera were diluted with an isotonic phosphate buffer, pH 7.4.

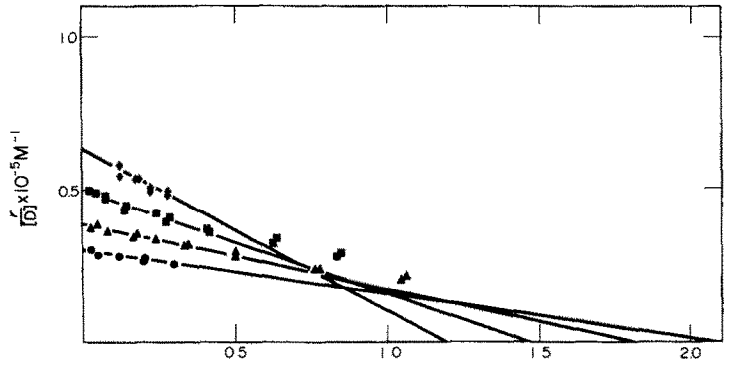

Fig. 3. Scatchard plots of the binding of digitoxin to uremic serum (●——● undiluted, ▲——▲ diluted 1 + 1, ■——■ diluted 1 + 4, ◇——◇ diluted 1 + 9) studied by equilibrium dialysis. The sera were diluted with an isotonic phosphate buffer, pH 7.4.

also included in Table 3. Compared to normal HSA the binding to the albumin from the uremic patients was decreased but was improved on charcoal treatment at pH 3.0.

The binding of the two drugs was also studied at various dilutions in buffer of uremic and normal sera (Figs. 1–3). The effects of the Donnan equilibrium are small and will not significantly influence the results [1]. The intercept on the r/D axis ($n \times K_{app}$) increased with increasing dilution of serum for the two drugs in both normal and uremic serum.

With diazepam the r-intercept was close to 2 in undiluted uremic serum, but decreased with dilution (Fig. 1). Since n was not constant, a closer investigation of the mechanisms responsible for the inhibition according to the procedure described under "Methods"

Table 3. Protein binding data obtained from Scatchard plots

	Diazepam			Digitoxin		
	$n \times K_{app}$ $M^{-1} \times 10^{-5}$	n	K_{app} $M^{-1} \times 10^{-5}$	$n \times K_{app}$ $M^{-1} \times 10^{-4}$	n	K_{app} $M^{-1} \times 10^{-4}$
Normal serum pool	1.4	1.1	1.3	3.8	1.7	2.2
Uremic serum pool	0.4	1.8	0.2	3.0	2.1	1.5
Charcoal-treated HSA from normal serum	2.2	1.0	2.1	10.8	1.1	10.0
Untreated HSA from uremic serum	1.0	0.8	1.4	6.9	1.4	5.1
Charcoal-treated HSA from uremic serum	1.5	0.8	1.8	8.0	0.9	8.5

by plotting K_{app} vs $K_{app} \times C_{se}$ was not possible. Such an analysis could however be performed with normal serum as shown in Fig. 4. The experimental points fall on a straight line with slope $K_i \times I_0$ (equation 2). The intercept on the y-axis yields K_a^*, which coincided with the values obtained with charcoal-treated HSA.

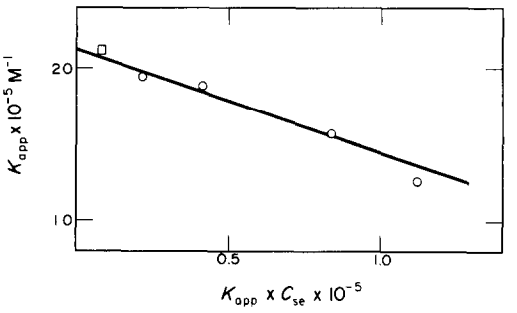

Fig. 4. Graph of K_{app} vs $K_{app} \cdot C_{se}$ for diazepam in normal serum (○——○). K_a value for the binding of diazepam to charcoal-treated HSA (.).

The number of binding sites involved in the binding of digitoxin cannot apparently be limited to the primary site on HSA. In normal undiluted serum the r-intercept was close to 2, and in uremic serum it was even higher. In a search for proteins other than albumin capable of binding digitoxin in serum, the components of normal serum were fractionated on Sephadex G-200. As is well documented in other studies, such a gel filtration separates the serum proteins into three fractions. The first and third fractions showed significant binding of digitoxin in equilibrium dialysis. The third fraction containing *i.a.* albumin bound digitoxin with an affinity constant of 1×10^5 M^1 (Table 3). The first fraction contains large proteins e.g. β-lipoproteins. After concentration of this fraction to 1.5 mg protein/ml (about half the normal serum concentration) the binding of digitoxin was 20 per cent. In this solution no traces of albumin were found by quantitative immunochemical analysis and polyacrylamide-gel electrophoresis. A determination of the binding affinity using a Rosenthal plot [15] gave the K_{app} value 1×10^5 M^{-1} for the binding of digitoxin.

DISCUSSION

Uremia has in several studies been shown to have a profound effect on the protein binding of several drugs. In specific cases, the increased concentration of endogenous substances, which can be removed by charcoal treatment of the serum albumin [1, 2], inhibits the binding by competitive and/or allosteric mechanisms as exemplified with salicylic acid and warfarin [1]. At least three specific drug binding sites can be identified on HSA [6, 8] and it has recently been shown that diazepam, digitoxin, and warfarin are suitable markers for these sites. They all show impaired binding in uremia [1, 2, 16, 17]. So far, no detailed quantitative studies have been undertaken with diazepam and digitoxin.

The present work has unequivocally shown that the impaired binding of diazepam and digitoxin in uremia is due to a decreased binding affinity to HSA, established by a decreased K_{app}. Also in normal serum, the binding is hampered due to the presence of endogenous substances when compared to the binding to purified, charcoal-treated HSA. However, the mechanisms responsible for the decreased binding in uremia are more complex than those earlier proposed for salicylic acid and warfarin. Thus, a decreased affinity constant is accompanied by a change of the number of binding sites involved in the binding of both diazepam and digitoxin.

At low concentrations (used in the present work), diazepam is exclusively bound to only one site on HSA [18, 19] and only this site seems to be effective in normal serum. The same situation is valid for digitoxin with respect to the binding to HSA [20], while also other proteins seem to play a role in normal serum, since n-values between 1.2 and 1.7 are found in different dilutions of the normal serum. Both with diazepam and digitoxin the n-value increases in uremic serum. Thus, other sites on HSA or on other proteins are made use of when the primary binding sites are inhibited. Unfortunately, this means that the earlier advanced procedure for studying the inhibition mechanism is not applicable with these drugs in uremia. In any case, it has been shown by dilution of the sera and by treatment of the albumin with activated charcoal that the inhibition is reversible.

At higher concentrations of diazepam, binding to secondary sites on HSA can be detected [18] and it is thus feasible to speculate that these may be effective in uremia when the primary site is inhibited. The effect of uremia on the binding of digitoxin is smaller than in the case of diazepam, which might explain the conflicting results presented by other authors [2, 16, 17, 21, 22]. The n-value is significantly increased in uremic serum and other binding sites are involved to an increasing extent when the binding to HSA is inhibited. The high molecular weight fractions of the serum proteins were isolated by gelfiltration, and shown to have high affinity for digitoxin. The binding constant was about 1×10^5 M^{-1} and the binding capacity of this protein fraction was high enough to give a binding degree of about 20 per cent at the conditions described. Our findings agree with those of Brock [23], who identified a lipoprotein fraction with digitoxin-binding properties.

The present study shows that the binding of both diazepam and digitoxin to their primary binding sites on HSA is inhibited by endogenous substances in uremia. Both diazepam and digitoxin are eliminated mainly by hepatic metabolism and are typical exponents of the group of low clearance drugs, i.e., their total blood clearance represents a minor fraction of the liver blood flow, which is approximately 1.5 l./min [24]. For diazepam, total blood clearance averages 0.076 l./min [25] and for digitoxin a plasma clearance of 0.003 l./min has been reported [26]. For such drugs it can be theoretically predicted that the total plasma clearance will increase in direct proportion to the free drug fraction in plasma [24, 27]. Furthermore it has been shown that for drugs which, in similarity to digitoxin and diazepam, are extensively tissue-localized, the apparent volume of distribution (V) will increase in direct proportion to the fraction of free drug in plasma [24]. Since plasma clearance is the product of $V \cdot \beta$, where β is the overall elimination rate constant, it can be concluded that the increased free fraction

observed for diazepam and digitoxin in uremic plasma would theoretically lead to increased volume of distribution and increased plasma clearance, but essentially constant biological half-life [$t_{1/2} = 0.693/\beta$].This would only be true, however, provided that intrinsic clearance of free drug in the liver and tissue binding are constant and not affected by the diseased state. Since data are lacking on these points the only recommendation that can be made is that free rather than total plasma levels of diazepam and digitoxin should be measured in uremic patients in attempts to relate plasma levels and drug effects.

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