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PROTEIN BINDING OF OXAZEPAM AND ITS GLUCURONIDE CONJUGATES TO HUMAN ALBUMIN

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Abstract—The binding of oxazepam and its glucuronide conjugates to human serum albumin (HSA), as well as the binding interactions of the drug and its metabolites, were examined by equilibrium dialysis and kinetic probe studies. Oxazepam and its $S(+)$ glucuronide are bound to the HSA molecule with affinity constants of $3.5 \times 10^5\ M^{-1}$ and $5.5 \times 10^4\ M^{-1}$, respectively, which were independent of protein concentration over a range of 0.1 to 5.0 g/dl. The $R(-)$ glucuronide bound weakly to albumin, with the binding parameter, $N{\cdot}K$, increasing at lower albumin concentrations. Pre-acetylation of fatty acid free-HSA resulted in decreased binding of all three compounds, probably by altering the conformation of the binding sites. Kinetic probe studies with *p*-nitrophenyl acetate indicate that oxazepam and its $S(+)$ glucuronide shared a common binding site on HSA, but that the $R(-)$ glucuronide bound at another site. Oxazepam binding was unaffected by the presence of its glucuronide conjugates but was inhibited by fatty acids. The percentage of oxazepam bound to plasma proteins in patients with renal impairment (94%) was lower than in normal volunteers (97%). This lower binding can neither be attributed to lower albumin concentrations because of the large binding capacity of the protein and linearity of $N{\cdot}K$ nor to displacement by elevated concentrations of glucuronide conjugates, but it may be ascribed partly to increased plasma fatty acids.

Alterations in the protein binding of drugs caused by endogenous substances, other drugs, or metabolites of the drug can have important clinical and pharmacokinetic implications. The effects of endogenous substances such as fatty acids and bilirubin on drug plasma protein binding have been studied extensively, while less attention has been paid to displacement effects by drug metabolites. It has been shown that oxazepam and desmethyldiazepam can compete with and displace the parent compound, diazepam, from its binding site on human albumin [1]. High concentrations of the monodealkylated metabolite of disopyramide significantly lower the binding of the parent drug [2]. Acetylsulfapyridine, a metabolite of sulfapyridine, binds to plasma proteins more avidly than the parent compound [3].

Oxazepam binds with high affinity to a single binding site on the human serum albumin molecule known as the indole and benzodiazepine binding site [4–6] or binding site II [6, 7]. Most benzodiazepines, tryptophan, and many other compounds are also bound at this site [4–7].

Oxazepam is metabolized almost exclusively by glucuronide conjugation [8], producing concentrations of the metabolite in plasma similar to those of oxazepam [9]. Preliminary experiments have shown that both the dextrorotatory, $S(+)$, and levorotatory $R(-)$, isomers of oxazepam glucuronide [10] are bound to human serum albumin. Thus, the potential for a plasma protein binding interaction between oxazepam and its conjugates exists. It has been suggested that the decreased oxazepam binding seen in patients with renal dysfunction [11–13] may be due to competition by elevated plasma concentrations of the glucuronide [12]. The purpose of the present investigation was to characterize the binding of oxazepam and its $S(+)$ and $R(-)$ glucuronides to human albumin and to examine any potential binding interaction between the three compounds.

MATERIALS AND METHODS

Albumin. Human serum albumin (HSA) and essentially fatty acid free human serum albumin (FAF-HSA) were obtained from the Sigma Chemical Co. (St. Louis, MO). Pre-acetylation of FAF-HSA was accomplished by reacting equimolar concentrations of *p*-nitrophenyl acetate (NphOAc) (Aldrich Chemical Co. Inc., Milwaukee, WS) with FAF-HSA dissolved in 0.02 M triethanolamine/HCl (Sigma) buffer of pH 8 [14, 15]. Acetylated human serum albumin (Ac-HSA) was separated from the *p*-nitrophenylate ion by gel filtration on a column of Sephadex G-25 equilibrated with 0.067 M Na_2HPO_4 buffer, pH 7.4. The Ac-HSA was further purified by dialysis against phosphate buffer at 2° for 16 hr. Various concentrations of FAF-HSA and Ac-HSA were made by diluting the protein solution with appropriate volumes of phosphate buffer, pH 7.4

Human plasma was prepared from blood collected in heparinized vacutainers (Becton-Dickinson, Rutherford, NJ) from normal volunteers. Each plasma sample was screened for the presence of

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benzodiazepines or other interferences by gas–liquid chromatography [9]. Plasma was also obtained from a clinical study of normal volunteers and patients with renal dysfunction at 4 hr after the administration of 30 mg of oxazepam (Serax, Wyeth, 15-mg tablets).

Protein binding studies. Protein binding measurements were done by equilibrium dialysis [16] using the Dianorm Equilibrium Dialyzer (1 ml cells, Markson Science Inc., Delmar, CA) and Spectrapor II (Spectrum Medical Industries, New York, NY) membrane with a molecular weight cutoff of 12,000–14,000. Albumin solutions (1 ml), with drug added, were dialyzed against 0.067 M sodium phosphate buffer (1 ml), pH 7.4, for 4 hr at 37°. Preliminary experiments ascertained that 4 hr was sufficient for equilibration of the unbound oxazepam and glucuronides.

Due to the limited water solubility of oxazepam, the protein binding of oxazepam and its $S(+)$ and $R(-)$ glucuronides (provided by Cesario Tio of Wyeth Laboratories) to FAF-HSA and Ac-HSA was characterized at various protein concentrations while maintaining drug and metabolite concentrations constant.

Oxazepam binding was also measured in normal volunteers and in patients with renal dysfunction in samples obtained at 4 hr after the oral administration of 30 mg of oxazepam. Studies examining binding interactions between oxazepam and its metabolites were done in pooled human plasma. The binding of each compound was examined separately, as well as in the presence of one or both of the other compounds. To assess the effects of fatty acids, binding was examined in FAF-HSA, HSA and human plasma. The fatty acid content of these albumin solutions was determined by the method of Duncombe [17].

Drug and metabolite measurements. Oxazepam concentrations were determined by a modification of the gas–liquid chromatographic method of Knowles and Ruelius [9]. Concentrations of the $S(+)$ and $R(-)$ glucuronides were determined by high performance liquid chromatography. After the addition of an internal standard, lorazepam $R(+)$ glucuronide (provided by Cesario Tio of Wyeth), samples were extracted with the aid of Sep-Pak C18 cartridges (Waters Associates, Inc., Milford, MA) pretreated with 20 ml methanol and 20 ml water. The glucuronides were eluted from the Sep-Pak with 5 ml methanol (HPLC grade, Fisher Scientific Co., Fairlawn, NJ) after two 5-ml water rinses. The methanol was evaporated at 45° under a stream of nitrogen, and the samples were reconstituted with 0.2 ml of distilled water. Samples were then washed three times with 1 ml of diethyl ether (AR grade, Mallinckrodt, Paris, KY), warmed to 45° for 5 min and filtered through 0.45 μm Swinnex-HA filters (Millipore Corp., Bedford, MA) by centrifugation for 2 min at 750 *g*. The extracts were analyzed with the Analyst series 7800 liquid chromatograph (Laboratory Data Control, Riviera Beach, FL) equipped with a Rheodyne model 7120 injector. The glucuronides were resolved on a 30 cm × 4.6 mm i.d. Chromegabond C18, 10 μm particle size column (E.S. Industries, Marlton, NJ) with a 3 cm MPLC guard column (Brownlee Labs, Santa Clara, CA) using 17% acetonitrile in 0.1 M ammonium acetate adjusted to pH 5.0 with acetic acid as a mobile phase at a flow rate of 2.2 ml/min. A detection wavelength of 254 nm was used.

Probe studies with NphOAc. The rapid reaction of HSA with NphOAc was used to monitor the binding of oxazepam and its glucuronides to a specific binding site on the HSA molecule [14, 15]. Naproxen, which has been shown to bind to this specific site [15], was used as a control. The drugs were dissolved in 6.5×10^{-5} M FAF-HSA in 0.02 M triethanolamine/HCl buffer, pH 8, to yield ligand to albumin molar ratios ranging from 0.25 to 10. Ten microliters of $0.61 \cdot 10^{-5}$ M NphOAc was rapidly added to 2 ml of drug–albumin solution that had been preincubated at 25° for 1 min in a double beam spectrophotometer (Beckman Instruments, Fullerton, CA). The rate of the reaction between FAF-HSA and NphOAc was monitored by the formation of *p*-nitrophenylate ion recorded at 400 nm as percent transmission versus time with triethanolamine/HCl buffer used as the reference solution.

Data analysis. Two methods were employed to generate the protein binding parameters for the binding of the three compounds to FAF-HSA and Ac-HSA. The fraction of bound drug (F_B) can be described as a function of protein concentration (P) and free drug concentration (D_F) by the relationship:

$$F_B = \frac{1}{1 + \frac{D_F}{N \cdot P} + \frac{1}{N \cdot K \cdot P}} \quad (1)$$

where N is the number of binding sites and K is the association constant.

Least-squares estimates [18] of N and K were obtained by using equation 1 with protein and free drug concentrations serving as the independent variables (Method I). At low drug concentrations (i.e. $K^{-1} >> D_F$), the middle term in the denominator of equation 1 becomes negligible and the equation can be simplified to:

$$\frac{F_B}{1-F_B} = N \cdot K \cdot P \quad (2)$$

where the left-hand side of equation 2 is the ratio of bound to free drug (B/F). The slope of a plot of the binding ratio versus protein concentration will be equal to $N \cdot K$ providing this parameter is independent of the protein concentration (Method II).

When a drug is displaced from its binding site by a competing ligand that interacts at the same binding site, the fractional binding of the drug can be described by the relationship

$$F_B = \frac{1}{1 + \frac{D_F}{N \cdot P} + \frac{1}{N \cdot K \cdot P} + \frac{K_c \cdot C_F}{N \cdot K \cdot P}} \quad (3)$$

where C_F is the concentration of the competitor and K_c is its binding association constant. Equations 1 and 3 were used to predict the effects of competition between the three compounds. Association constants (K and K_c) used were those found for the binding of oxazepam and the $S(+)$ and $R(-)$ glucuronides to FAF-HSA.

The results of the NphOAc probe study were

examined by plotting the ratio of first-order rate constants for the reaction between NphOAc and FAF-HSA in the presence and absence of ligand (k/k_o) versus the molar ratio of ligand and HSA concentrations [15]. Oxazepam and its glucuronides were examined in relation to naproxen which has been shown to bind primarily at the NphOAc reaction site [15].

RESULTS

Binding studies. The percentage binding of oxazepam is presented as a function of HSA concentration in Fig. 1A. The extent of oxazepam binding varied moderately (95 to 99.5%) over a 10-fold range of HSA concentrations (0.38 to 3.96 g/dl). However, at lower HSA concentrations (< 0.1 g/dl) the binding of oxazepam decreased sharply with protein concentration. This pattern was seen with both albumin solutions; however, oxazepam binding to FAF-HSA was slightly greater than its binding to Ac-HSA. Nonlinear least-squares regression (Eq. 1) of the data resulted in good fittings only after the omission of data obtained at albumin concentrations of 0.1 g/dl or lower. The estimates of the protein binding parameters obtained for both albumins are presented in Table 1. The fractional binding of oxazepam at albumin concentrations less than 0.1 g/dl was lower than expected, suggesting a corresponding decrease in $N \cdot K$.

Figure 1B shows the relationship between the oxazepam binding ratio (B/F) and albumin concentration. Linear binding was found at albumin concentrations greater than 0.1 g/dl for FAF-HSA ($r = 0.884$) and Ac-HSA ($r = 0.988$). This also suggests that $N \cdot K$ was constant at albumin concentrations greater than 0.1 g/dl but changed at lower concentrations. The estimates of the protein binding parameters obtained by Method I agree with those obtained by Method II (Table 1). FAF-HSA exhibited a slightly higher affinity for oxazepam than did Ac-HSA.

The binding profile for the $S(+)$ glucuronide of oxazepam in relation to albumin concentration is presented in Fig. 2A. The fractional binding of the $S(+)$ glucuronide was highly dependent on albumin concentration, and protein binding to Ac-HSA was markedly lower (10–25%) than binding to FAF-HSA. The best nonlinear least-squares regression fittings (Eq. 1) were obtained after omission of binding data obtained at albumin concentrations of 0.1 g/dl and lower. The binding parameters generated are presented in Table 1. As with oxazepam (Fig. 1A), lower than expected binding was seen at lower albumin concentrations.

The relationship between the $S(+)$ glucuronide binding ratio and albumin concentration (> 0.1 g/dl) was linear for binding to both FAF-HSA ($r = 0.985$) and Ac-HSA (0.993) as shown in Fig. 2B. The $N \cdot K$ parameter was constant at albumin concentrations greater than 0.1 g/dl. As shown in Table 1, there is good agreement between the binding parameters generated by Methods I and II. FAF-HSA bound the $S(+)$ glucuronide with an affinity constant approximately three times greater than that of Ac-HSA.

Figure 3A illustrates the relationship between the percentage binding of the $R(-)$ glucuronide of oxazepam and albumin concentration. This glucuronide was bound to a lesser extent than were oxazepam and its $S(+)$ glucuronide. The fractional binding decreased sharply with albumin concentration except at lower concentrations where the binding decreased only slightly. The relationship between the binding ratio and albumin concentration (Fig. 3B) for the $R(-)$ glucuronide was not linear;

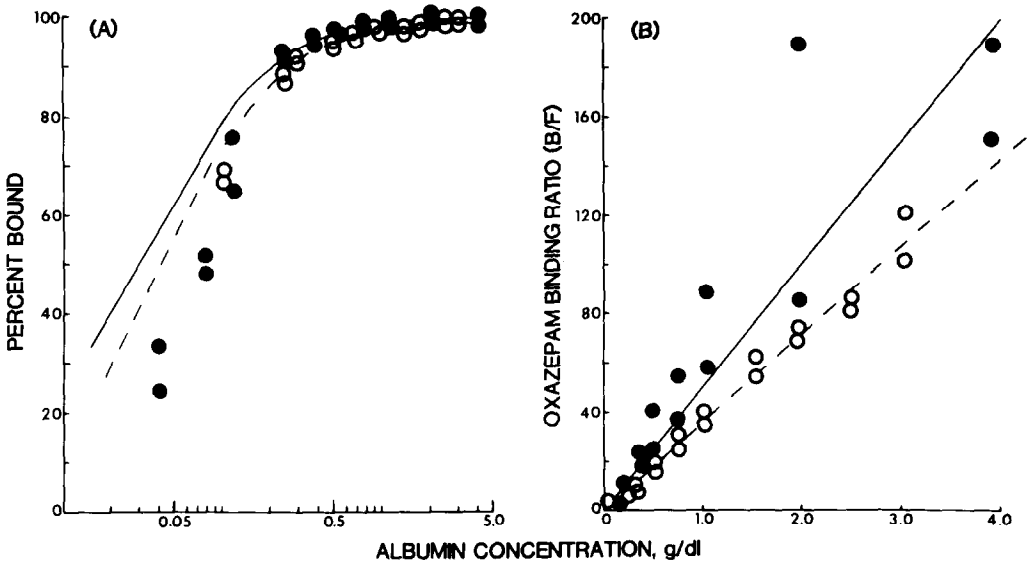

Fig. 1. Oxazepam binding. (A) Percent bound as a function of FAF-HSA (●) and Ac-HSA (○) concentrations. The lines represent the nonlinear least-squares regression fittings for oxazepam binding to FAF-HSA (−) and Ac-HSA (– – –) using Equation 1. (B) Relationship between the binding ratio (bound/free) of oxazepam and albumin concentration. Regression lines (Equation 2) for the data obtained with FAF-HSA ($r = 0.884$) and Ac-HSA ($r = 0.988$) are shown.

Table 1. Protein binding parameters for oxazepam and its *S*(+) glucuronide obtained by two data fitting methods*

Compound and protein	Method II	Method I	
	$N \cdot K$ (M^{-1})	N	K (M^{-1})
Oxazepam			
FAF-HSA	3.46×10^5	0.99	3.79×10^5
Ac-HSA	2.47×10^5	1.00	2.50×10^5
S(+) Glucuronide			
FAF-HSA	4.93×10^4	0.99	5.87×10^4
Ac-HSA	1.90×10^4	1.00	1.96×10^4

* Method I used Equation 1. Method II used Equation 2.

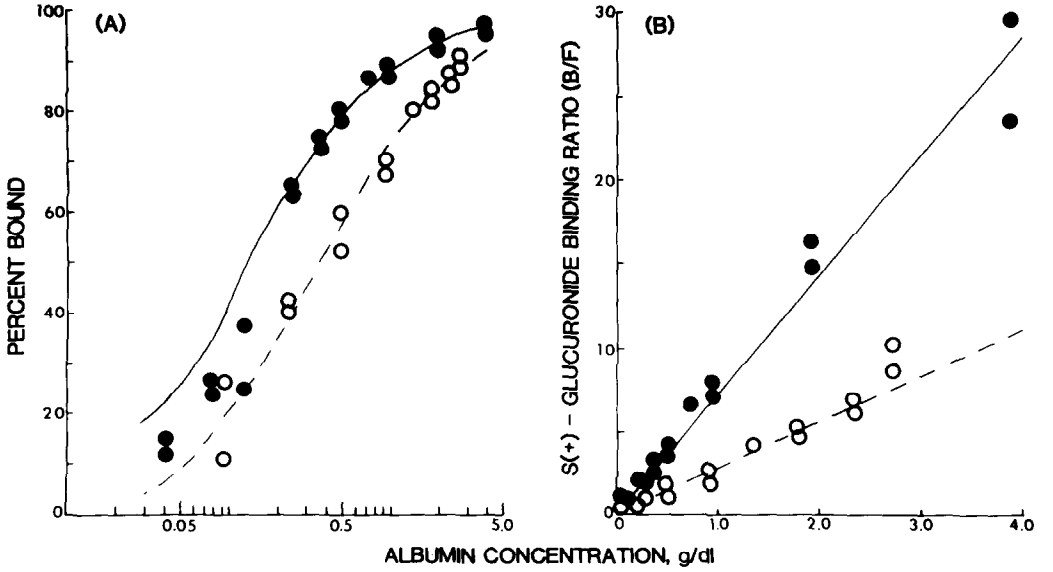

Fig. 2. *S*(+) Oxazepam glucuronide binding. (A) Percent bound as a function of FAF-HSA and Ac-HSA concentrations. (B) Relationship between the binding ratio for the *S*(+) glucuronide and albumin concentration ($r = 0.985$ for FAF-HSA and $r = 0.993$ for Ac-HSA). Symbols and lines are defined as in the legend to Fig 1.

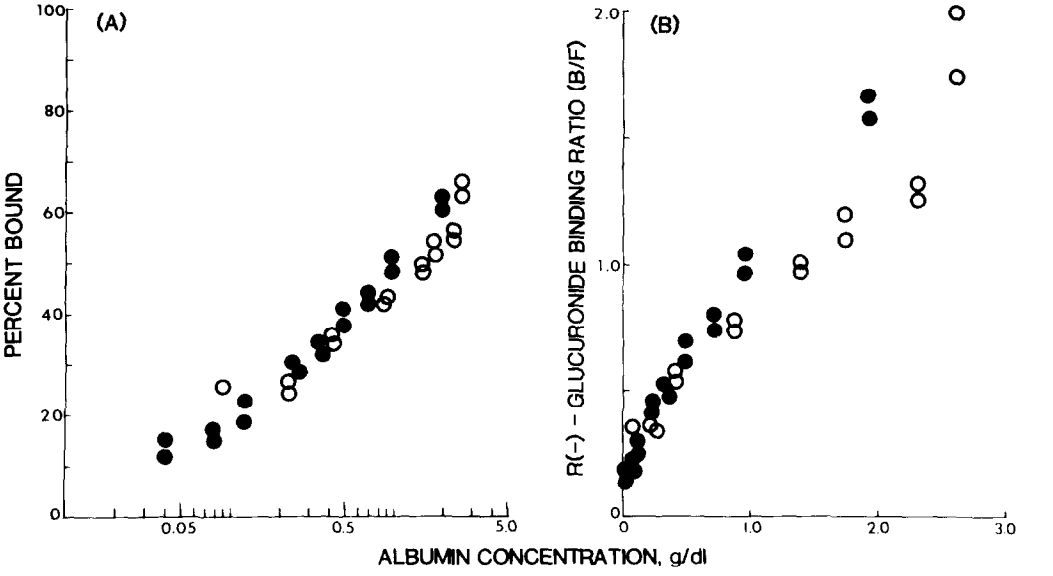

Fig. 3. *R*(−) Oxazepam glucuronide binding. (A) Percent bound as a function of FAF-HSA and Ac-HSA concentrations. (B) Relationship between the binding ratio for the *R*(−) glucuronide and albumin concentration. Symbols are defined as in the legend to Fig. 1.

therefore $N \cdot K$ could not be generated by Method II. Efforts to generate binding parameters by Method I also failed.

Plasma binding. The values for protein binding of the three compounds in FAF-HSA (3.0 g/dl) and human plasma (total protein = 5.4 g/dl, albumin = 3.0 g/dl) were compared. Oxazepam was 99.2% bound to FAF-HSA and 96.6% bound to human plasma. The *R*(−) glucuronide was bound to the same extent (55%) in both protein solutions. The binding of the *S*(+) glucuronide was also examined in non-defatted HSA (3.0 g/dl). Binding was 95.3% to FAF-HSA, 87.3% to human plasma, and 56.2% to non-defatted HSA. Fatty acid concentrations were 1400–1500 μM in non-defatted HSA, 200–300 μM in human plasma, and negligible in FAF-HSA.

Plasma was obtained 4 hr after the administration of 30 mg of oxazepam from eight normal volunteers and six patients with renal impairment. Oxazepam concentrations in normal volunteers averaged 534 ± 232 ng/ml and binding was 97.3 ± 0.74%. Patients with renal impairment exhibited oxazepam concentrations of 214 ± 115 ng/ml and the drug was 94.1 ± 2.4% bound. The fraction of oxazepam bound did not correlate with either total protein or albumin concentration in plasma and was independent of oxazepam concentration over a range of 225 to 1150 ng/ml.

Binding interaction studies. Protein binding of oxazepam and its *S*(+) and *R*(−) glucuronides was determined individually and in mixture of the compounds. A summary of the results is presented in Table 2. The predicted values of percent bound are slightly higher than those measured due to the use of association constants determined with FAF-HSA rather than plasma albumin. Oxazepam protein binding was not altered by high concentrations of either the *S*(+) glucuronide or a mixture of both glucuronides. This is consistent with the anticipated results. As predicted, the *S*(+) glucuronide was not displaced by oxazepam or the *R*(−) glucuronide. The *R*(−) glucuronide also was not displaced by oxazepam or the *S*(+) glucuronide; however, at high concentrations of both glucuronides a small decrease in the binding at the *R*(−) glucuronide was predicted.

Probe studies with NphOAc. The results of the probe study with NphOAc are presented in Fig. 4. Decreases in the rate constants are seen with naproxen, oxazepam, and the *S*(+) glucuronide. Naproxen and oxazepam almost completely inhibited the reaction between NphOAc and FAF-HSA at drug to albumin ratios of 1. The *S*(+) glucuronide, at the same molar concentration as FAF-HSA, also inhibited the reaction to a large extent. The *R*(−) glucuronide, however, had no effect on the reaction.

Table 2. Measured and predicted* effects of the binding interactions between oxazepam and its *S*(+) and *R*(−) glucuronides†

Ligand	Ligand concn (ng/ml)	Displacer	Displacer concn (ng/ml)	% Bound without displacer		% Bound with displacer	
				Predicted	Measured	Predicted	Measured
Oxazepam	310 ± 47‡	*S*(+) and *R*(−)§	1460	99.4	97.0 ± 0.2‡	99.4	97.7 ± 0.4‡
Oxazepam	1074 ± 65	*S*(+)	950	99.4	96.5 ± 0.1	99.4	97.8 ± 0.5
S(+) Glucuronide	380 ± 14	Oxazepam	250	96.2	87.8 ± 1.8	96.1	86.8 ± 0.9
S(+) Glucuronide	4674 ± 140	*R*(−)	1925	95.9	83.9 ± 1.1	95.9	82.9 ± 1.0
R(−) Glucuronide	819 ± 36	Oxazepam	250	75.1	75.0 ± 3.4	74.9	74.0 ± 1.9
R(−) Glucuronide	205 ± 8	*S*(−)	570	75.1	64.8 ± 2.4	74.9	66.7 ± 3.5
R(−) Glucuronide	1872 ± 74	*S*(+)	4300	75.0	68.7 ± 1.2	73.6	70.0 ± 3.0

* Predicted by the use of Equations 1 or 3.
† Experiments were done using pooled human plasma with an albumin concentration of 3.0 g/dl (4.4×10^{-4} M). The molecular weights of oxazepam and its glucuronides are 286.74 and 462.88 respectively.
‡ Mean ± S.D. of two to four experiments.
§ K_c used was the average of K_c values for the *S*(+) and *R*(−) glucuronides.

DISCUSSION

Binding of oxazepam and its S(+) glucuronide. The protein binding of oxazepam cannot be completely characterized at normal physiologic albumin concentrations as saturation of albumin binding sites cannot be attained owing to the low aqueous solubility of the drug and the large binding capacity of the protein. Protein binding of the benzodiazepines

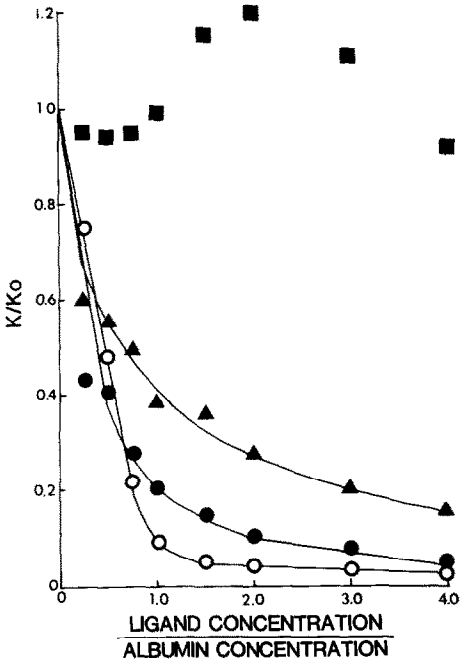

Fig. 4. Inhibition of the rapid reaction between NphOAc and HSA by (●) oxazepam, (▲) the $S(+)$ glucuronide, (■) the $R(-)$ glucuronide and (○) naproxen, presented as the ratio of the first-order rate constants in the presence (k) and absence (k_0) of ligand versus the molar ratio of ligand to HSA at 6.5×10^{-5} M. The reaction was conducted at 25° with 0.61×10^{-5} M NphOAc.

has been characterized at lower than physiologic albumin concentrations [4, 5]. However, protein binding parameters (i.e. $N \cdot K$) of some drugs can vary with protein concentration [19, 20], and extrapolation to physiologic albumin concentrations in such cases would be inappropriate. Varying the protein concentration allows its effect on $N \cdot K$ to be assessed with constant values of the latter allowing predictions of binding at higher protein concentrations.

The binding affinities of oxazepam and its $S(+)$ glucuronide for FAF-HSA and Ac-HSA were constant at albumin concentrations greater than 0.1 g/dl (Figs. 1 and 2); however, at lower concentrations less than expected binding suggests a decrease in $N \cdot K$. A similar phenomenon has been found for the binding of cortisol, corticosterone, and testosterone to α_1-acid glycoprotein [21]. Either contaminants present in small quantities in commercial albumin preparations or conformational changes in the albumin molecule at very low protein concentrations may cause this change. Thus, data obtained at very low albumin concentrations were not used in the estimation of the binding parameters.

Oxazepam was bound by FAF-HSA with the highest affinity ($3.5 \times 10^5\ M^{-1}$) of the three compounds. There was little change in the fractional binding of oxazepam over a wide range of albumin concentrations (Fig. 1A) as a result of the high binding affinity for this drug. Its binding is also linear at therapeutic drug concentrations [22]. The affinity constant for the $S(+)$ glucuronide ($5 \times 10^4\ M^{-1}$) was approximately one-tenth that for oxazepam, so that decreases in the albumin concentration resulted in proportional decreases in the fractional binding of the $S(+)$ glucuronide (Fig. 2A).

The rapid acetylation reaction between NphOAc and albumin [14] and the binding of ligands to the indole and benzodiazepine binding site on the HSA molecule are both associated with highly reactive tyrosine residues [5]. Indeed, *l*-tryptophan and chlordiazepoxide inhibit the rapid reaction between NphOAc and albumin [15], indicating that the reaction and binding sites are the same. Oxazepam bound with high affinity exclusively to this site as shown by the complete inhibition of this fast reaction at a drug to albumin ratio of 1. The computer fitting (Eq. 1) of the oxazepam binding data (Fig. 1A) also yielded an N of 1. These findings are in agreement with other reports that show oxazepam binds with high affinity to a single binding site [4, 7]. The $S(+)$ glucuronide, in a molar ratio of 1 with albumin, inhibited the reaction to a great extent but not completely. This could be due to either a second class of lower affinity binding sites or lower binding affinity for the $S(+)$ glucuronide. Computer fitting of $S(+)$ glucuronide data generated an N value of 1, suggesting the latter.

Binding of the $R(-)$ *glucuronide of oxazepam.* The binding of the $R(-)$ glucuronide was much different than that of oxazepam or the $S(+)$ glucuronide in that it was either not bound or was only very weakly bound to the indole and benzodiazepine binding site. This stereospecific binding is not unexpected. *l*-Tryptophan is bound by albumin with high affinity primarily to one site, whereas the *d*-isomer is bound with approximately one-hundredth of this affinity to different sites [23]. *d*-Oxazepam hemisuccinate is bound to albumin with an affinity about forty times greater than *l*-oxazepam hemisuccinate [24]. The $R(-)$ glucuronide of oxazepam was bound to albumin with an affinity at most one-tenth that of the $S(+)$ glucuronide.

Also, in contrast to oxazepam and the $S(+)$ glucuronide, the protein binding of the $R(-)$ glucuronide was highly dependent on albumin concentration. Scatchard analysis of the data resulted in a plot with a positive slope, whereas typical Scatchard plots exhibit negative slopes. It has been suggested that Scatchard plots with positive slopes are caused by decreases in $N \cdot K$ which occur as albumin concentrations increase [20]. This phenomenon has been shown to occur with the binding of numerous drugs [19, 20]. Contaminants in commercial albumin preparations such as *N*-acetyl-L-tryptophan and other indoles [25] and fatty acids can inhibit the binding of drugs. Increasing the albumin concentration will also result in increasing the contaminant concentration, thereby causing decreases in $N \cdot K$. Fatty acids can be ruled out as the changes in $N \cdot K$ were also seen with FAF-HSA, and indoles would affect the binding of oxazepam and the $S(+)$ glucuronide more than the $R(-)$ glucuronide. However, inhibition by other unknown contaminants cannot be excluded. Another possible explanation for the changes seen in the binding parameters is an interaction between the protein molecules. The dimer form of HSA binds digitoxin with a lower affinity than the monomer form [26]. At lower protein concentrations there is

a decrease in the percentage of albumin dimers, hence the affinity can increase. Although $N \cdot K$ for the $R(-)$ glucuronide was variable with protein concentration and could not be determined precisely, it was approximately $10^3\ M^{-1}$.

Binding interactions. Pre-acetylation of FAF-HSA with NphOAc resulted in decreased binding of all three compounds. This was expected for oxazepam and the $S(+)$ glucuronide as HSA is acetylated by NphOAc at a tyrosine residue that is associated with benzodiazepine binding. Computer fittings of the binding data obtained with Ac-HSA suggest that the association constant was decreased by acetylation while the number of binding sites remained constant (Table 1). Acetylation, therefore, probably causes a conformational change at or near this binding site. Modification of this tyrosine residue by other means has also resulted in a decrease in the binding affinities without altering the number of binding sites for *l*-tryptophan and diazepam [27]. However, simple occlusion of the binding sites cannot be excluded. The binding of the $R(-)$ glucuronide was also affected by pre-acetylation with NphOAc, probably by an allosteric mechanism.

As oxazepam and its $S(+)$ glucuronide are bound at the same site on the human serum albumin molecule, the possibility for a competitive interaction between the parent drug and its metabolite exists. No interaction was found, however, as the large binding capacity of albumin results in an abundance of benzodiazepine binding sites. Even at very low albumin concentrations, oxazepam would not be displaced by the glucuronide due to the 10-fold greater binding affinity of the parent drug compared to the metabolite. The $R(-)$ glucuronide was bound at different sites and therefore did not compete with oxazepam.

The percentage of oxazepam bound to plasma proteins was found to be lower in patients with renal dysfunction (94%) than in normal volunteers (97%). This 2-fold increase in the free fraction in patients with renal impairment has been observed previously [11–13]. The decreased binding was not due to lower total protein or albumin concentrations [12]. The binding of oxazepam was found to be linear over therapeutic concentrations [22], and differences in oxazepam concentrations between the two groups thus would not explain the differences in binding.

High concentrations of metabolites have been shown to decrease the binding of some drugs [1, 2]. In patients with impaired renal function, concentrations of the conjugated metabolites of oxazepam can exceed concentrations of the parent compound by as much as 50-fold [13]. Oxazepam binding was not altered by concentrations of metabolites normally observed and simulations predict that, even at glucuronide concentrations fifty times greater than those of oxazepam, no decrease in binding would be seen. Therefore, increased concentrations of metabolites or changes in protein concentrations are not the cause of the doubling of the oxazepam free fraction seen in patients with renal impairment. The accumulation of plasma fatty acids or other endogenous compounds that may inhibit oxazepam binding remains a possible cause for the decrease in oxazepam binding. Indeed, fatty acids inhibit the binding of oxazepam, its $S(+)$ glucuronide, and diazepam [1, 28, 29].

In summary, oxazepam and its $S(+)$ glucuronide were bound by the same site on the HSA molecule; however, there was no competitive binding interaction between the two compounds. The $R(-)$ glucuronide did not appear to be bound by this site. Pre-acetylation of HSA resulted in a decrease in the binding of all three compounds, probably by an allosteric mechanism. Patients with renal dysfunction exhibited a 2-fold increase in oxazepam free fraction, but this decreased binding cannot be attributed to either lower albumin concentrations or displacement by accumulated metabolites.

REFERENCES

1. G. B. Wong and E. M. Sellers, *Biochem. Pharmac.* **28**, 3265 (1979).
2. P. H. Hinderling, J. Bres and E. R. Garrett, *J. pharm. Sci.* **63**, 1684 (1974).
3. C. Fischer and U. Klotz, *J. Chromat.* **162**, 237 (1979).
4. W. Müller and U. Wollert, *Naunyn-Schmiedeberg's Archs Pharmac.* **280**, 229 (1973).
5. W. E. Müller and U. Wollert, *Naunyn-Schmiedeberg's Archs Pharmac.* **288**, 17 (1975).
6. K. J. Fehske, W. E. Müller and U. Wollert, *Biochem. Pharmac.* **30**, 687 (1981).
7. T. Sjodin, N. Roosdorp and I. Sjöholm, *Biochem. Pharmac.* **25**, 2131 (1976).
8. S. F. Sisenwine, C. O. Tio, S. R. Shrader and H. W. Ruelius, *Arzneimittel-Forsch.* **22**, 682 (1972).
9. J. A. Knowles and H. W. Ruelius, *Arzneimittel-Forsch.* **22**, 687 (1972).
10. H. W. Ruelius, C. O. Tio, J. A. Knowles, S. L. McHugh, R. T. Schillings and S. F. Sisenwine, *Drug Metab. Dispos.* **7**, 40 (1979).
11. T. G. Murray, S. T. Chiang, H. H. Koepke and B. R. Walker, *Clin. Pharmac. Ther.* **30**, 805 (1981).
12. U. Busch, M. Molzahn, G. Bozler and F. W. Koss, *Arzneimittel-Forsch.* **31**, 1507 (1981).
13. I. Odar-Cederlöf, J. Vessman, G. Alván and F. Sjöqvist, *Acta pharmac. tox.* **40** (Suppl.), 52 (1977).
14. G. E. Means and M. L. Bender, *Biochemistry* **14**, 4989 (1975).
15. N. P. Sollene and G. E. Means, *Molec. Pharmac.* **15**, 754 (1979).
16. C. A. Homon, E. R. Fluck, F. W. Janssen and H. W. Ruelius, *Agents Actions* **12**, 211 (1982).
17. W. G. Duncombe, *Clinica chim. Acta* **9**, 122 (1964).
18. C. M. Metzler, G. K. Elfring and A. J. McEwen, *Biometrics* **30**, 562 (1974).
19. J. Judis, *J. pharm. Sci.* **69**, 71 (1980).
20. C. J. Bowmer and W. E. Lindup, *J. pharm. Sci.* **67**, 1193 (1978).
21. J. Kerkay and U. Westphal, *Biochim. biophys. Acta* **170**, 324 (1968).
22. R. F. Johnson, S. Schenker, R. K. Roberts, P. V. Desmond and G. R. Wilkinson, *J. pharm. Sci.* **68**, 1320 (1979).
23. R. H. McMenamy and J. L. Oncley, *J. biol. Chem.* **233**, 1436 (1958).
24. W. E. Müller and U. Wollert, *Molec. Pharmac.* **11**, 52 (1975).
25. G. L. K. Bargren and J. I. Routh, *Clin. Biochem.* **7**, 290 (1974).
26. A. Brock, *Acta pharmac. tox.* **38**, 497 (1976).
27. K. J. Fehske, W. E. Müller and U. Wollert, *Biochim. biophys. Acta* **577**, 346 (1979).
28. E. Tsutsumi, T. Inaba, W. A. Mahon and W. Kalow, *Biochem. Pharmac.* **24**, 1361 (1975).
29. T. Sjödin, *Biochem. Pharmac.* **26**, 2157 (1977).