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INTRAVASCULAR FACTORS AFFECTING DIAZEPAM BINDING TO HUMAN SERUM ALBUMIN

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Abstract—The binding of a benzodiazepine derivative (diazepam) to human serum albumin was studied by equilibrium dialysis. The influence on this interaction of various endogenous substances (fatty acids, uric acid and bilirubin) and the metabolites of diazepam (desmethyldiazepam and oxazepam) were investigated. Binding studies were analyzed by fitting a model which utilizes the two independently measured quantities, the free drug concentration and the total amount of drug, to the experimental data points with a least squares method. Oleic acid decreases the binding of diazepam at all molar ratios of fatty acid:albumin. In contrast, palmitic acid at ratios of < 1:1 enhanced diazepam binding. Both uric acid and bilirubin had negligible effects on diazepam binding. The metabolites were bound to a less extent than diazepam (diazepam > desmethyldiazepam > oxazepam), and both metabolites reduced diazepam binding to albumin. *In vivo*, the absolute ratio of specific fatty acids to each other is probably as important a source of variations as the quantitative changes in total fatty acids or metabolites.

Albumin is the protein accounting for the majority of drug binding in human serum [1]. Drugs typically bind to only a limited number of protein binding sites on the albumin molecule, such that interactions can arise between drugs through either competitive or allosteric mechanisms [2]. As a consequence of such an interaction, the free concentration of the displaced drug increases, transiently enhancing the drug effect and increasing total drug clearance.

Albumin carries a complement of endogenous substances. For example, long-chain, saturated free fatty acids (FFA) bind to three classes of sites on albumin with association constants 100 to 1000 times larger than those reported for other organic ligands [3]. Unconjugated bilirubin binds avidly to albumin at two classes of binding sites [4]. The binding of endogenous substances can result in interactions with albumin-bound drugs. Studies by Rudman *et al.* [5] demonstrated inhibitory effects of both palmitic and oleic acids on the binding of anionic ligands to albumin, only when the molar ratio of FFA/albumin was 3.5 or greater. The two hypolipidemic drugs, chlorophenoxyisobutyrate (CPIB) and halofenate, are displaced from albumin by palmitic and oleic acids (4.0 moles/mole of albumin) [6]. FFA in excess of 3.0 moles/mole of albumin diminishes warfarin binding to albumin [7, 8]. Lauric acid, in a molar ratio of 4.7 displaces the benzodiazepine, diazepam, from albumin [9].

Diazepam is extensively bound to human albumin [10, 11]. Since the mode of elimination of diazepam is restrictive [12], the free drug concentration becomes important in determining drug clearance. Therefore, any factor that increases the free fraction of the drug could lead to alterations in total drug concentration, drug body clearance and possibly pharmacological effect [13]. The following studies were undertaken to determine the factors that modify free diazepam concentrations and possibly account for inter-individual variations in plasma protein binding *in vivo* [14]. The effects of different concentrations of two common plasma fatty acids, palmitic and oleic acids, and of bilirubin and uric acid on the binding of diazepam to defatted human serum albumin were determined. In addition, since the concentrations of the major metabolite of diazepam, desmethyldiazepam, can accumulate upon chronic administration to five times the level of its parent compound [15], the possible displacement of diazepam by desmethyldiazepam and oxazepam was investigated.

MATERIALS AND METHODS

Chemicals. Non-radiolabeled diazepam, [^{14}C]diazepam (specific activity of 26 μCi/mg), and *N*-desmethyldiazepam were donated by Hoffman-La Roche Limited, Vandreuil, Quebec. The radiochemical purity of [^{14}C]diazepam was verified by thin-layer chromatography on silica gel plates (E. Merck, Darmstadt, Germany) and found to be greater than 98 per cent. Oxazepam was obtained from Wyeth Limited, Toronto, Ontario. Essentially fatty acid free human serum albumin (FAF-HSA) (Sigma Chemical Co., St. Louis, MO, less than 0.005% fatty acids) was used without further purification. Palmitic acid, oleic acid, bilirubin and uric acid (Sigma Chemical Co.) were approximately 99% pure.

Preparation of solutions. Stock albumin solutions were prepared by dissolving crystallized human serum albumin in 0.067 M phosphate buffer at pH 7.4 to a concentration of approximately 3.1×10^{-5} M.

Palmitic acid was dissolved in absolute ethanol to a concentration of 5 mg/ml, and appropriate amounts were pipetted into test tubes. The ethanol was evaporated under nitrogen until a thin film of the fatty acid remained around the wall of the test tube. Fifty ml of FAF-HSA were then added and the solution was incubated overnight at 4°. Oleic acid was added directly in microliter quantities to test tubes and stored overnight at 4° with 50 ml of FAF-HSA.

Stock solutions of uric acid (6 mg/ml) and bilirubin (0.75 mg/ml) were prepared in 0.1 M sodium hydroxide. Appropriate microliter quantities were pipetted into test tubes and refrigerated overnight with 30 ml of FAF-HSA. *N*-desmethyldiazepam and oxazepam were dissolved in absolute ethanol to concentrations of 2.6 mg/ml and 2.75 mg/ml respectively. One hundred aliquots of each stock were mixed with 30 ml of FAF-HSA and stored overnight at 4°.

Equilibrium dialysis. The binding of diazepam to defatted albumin was measured by a modification of a previously reported equilibrium dialysis procedure using 0.067 M phosphate buffer (pH 7.4) at 37° [16]. Equilibrium was established after 6 hr of dialysis. Triplicates of each of eight determinations were performed over a diazepam concentration range of 0.878 to 8.780×10^{-5} M. Binding of drug to the glass wall of the test tube was negligible, although binding to the membrane was accounted for in the analysis of the binding data. Diazepam concentrations were measured post-dialysis with a Beckman DB-GT Grating Spectrophotometer, using an extinction coefficient of 2.8×10^4 mole^{-1} cm^{-1} at 240 nm [17]. For experiments conducted in the presence of desmethyldiazepam and oxazepam, the free diazepam concentration was determined by [^{14}C]diazepam, in which 2 per cent of the total drug present at all determinations constituted radiolabeled drug. Albumin concentrations were measured spectrophotometrically at 280 nm using $E^{1\%}_{1\,cm} = 5.3$ and a molecular weight of 69,000 [18].

Data analysis. A mathematical model incorporating the only two measured quantities, namely, the independent variable, D_t (the total amount of drug within the system), and the dependent variable, $[D_f]$ (the free drug concentration), was developed to determine the binding constants of diazepam to FAF-HSA. The equation

$$D_t = \left[\frac{n_1 k_1 [D_f]}{1 + k_2[D_f]} + \frac{n_2 k_2 [D_f]}{1 + k_2[D_f]}\right] P + (M[D_f] + c) + V[D_f]$$

assumes both primary and secondary sites for diazepam binding, where n_1, k_1, n_2 and k_2 are the binding constants and P is the total number of moles of albumin. The amount of drug bound to the dialysis membrane was determined to be linearly related to $[D_f]$ within the free drug concentration range and, therefore was expressed as $M[D_f] + c$, where M and c are the slope and intercept of the best-fitting straight line after regression analysis. The amount of drug in the water phase of the system was calculated from the free drug concentration by multiplying by volume V.

The binding results were analyzed by fitting the model to the experimental data points by minimization of least squares differences between predicted and observed values, using the computer program NLIN [19, 20]. The initial estimates of n_1 and n_2 were arbitrarily assigned to be 1.0 and 5.0 respectively. Starting values of k_1 and k_2 were derived from graphical extrapolations to the ordinate axis from Scatchard plots of the experimental data. The program calculates the final estimate, standard error and confidence interval for each parameter. Student's t-test was applied to the binding parameters, n_1 and k_1, of all experiments with those of diazepam binding to FAF-HSA alone. Statistics were not performed on the parameters of the secondary class since the standard errors were large and insufficient experimental data had been collected to allow meaningful interpretation. Statistics on the products n_1k_1 and n_2k_2 were omitted because of the inability to define the distributions of these terms and, therefore, derive satisfactory standard errors. "Best-fitting" curves were determined by substitution of the final estimates of each parameter into the mass-action equation of a reversible reaction, assuming two classes of binding sites.

RESULTS

The best-fitting lines to the experimental data points for the binding of diazepam to defatted albumin in the presence of varying concentrations of palmitic and oleic acid are shown in Fig. 1. The calculated numbers of binding sites and association constants of diazepam in the presence of palmitic and oleic acid are summarized in Table 1. The effects of both fatty acids were examined by increasing the concentrations from 0 to 4 moles of FFA per mole of albumin. Diazepam was displaced from its binding sites on albumin at all concentrations of oleic acid. Only 2 or more moles of palmitic acid reduced the degree of diazepam binding to albumin.

On the number of primary binding sites (n_1), primary association constants (k_1) and the product (n_1k_1), palmitic and oleic acids exerted different influences at low and high concentrations (Fig. 2). At low concentrations (0.5 and 1 mole FFA per mole of albumin), palmitic acid enhanced binding of diazepam to albumin due to an increase in the primary association constant. The same concentrations of oleic acid resulted in diminished values. High concentrations (2 and 4 moles FFA per mole of albumin) of both fatty acids caused decreased diazepam binding association constants. Reduced values for n_1 were observed at low concentrations of palmitic acid, while high concentrations resulted in values similar to those observed for diazepam binding to defatted albumin alone. A decrease in the value of the number of primary binding sites occurred at all concentrations of oleic acid.

The best-fitting curves for diazepam, desmethyldiazepam and oxazepam binding to defatted albumin are shown in Fig. 3 (left panel). Desmethyldiazepam and oxazepam bind to a lesser extent than diazepam, at all free drug concentrations. The final estimates of the primary association constants (k_1) for desmethyldiazepam. Only oxazepam showed reduced values of n_1 (Table 2). Radiolabeled diazepam was displaced in binding studies conducted in the presence of desmethyldiazepam and oxazepam (1.03×10^{-5} M) (Fig. 3, right panel). The association constants for diazepam in both instances are reduced significantly from diazepam binding to defatted albumin alone. Values of n_1 in both cases also varied from diazepam binding alone (Table 2). Binding parameters for cold and radiolabeled diazepam binding to defatted albumin did not differ, verifying the reproducibility of the experimental method.

Neither uric acid (up to 3.57×10^{-5}M) nor bilirubin (up to 1.28×10^{-5} M) altered the binding of diazepam to defatted albumin.

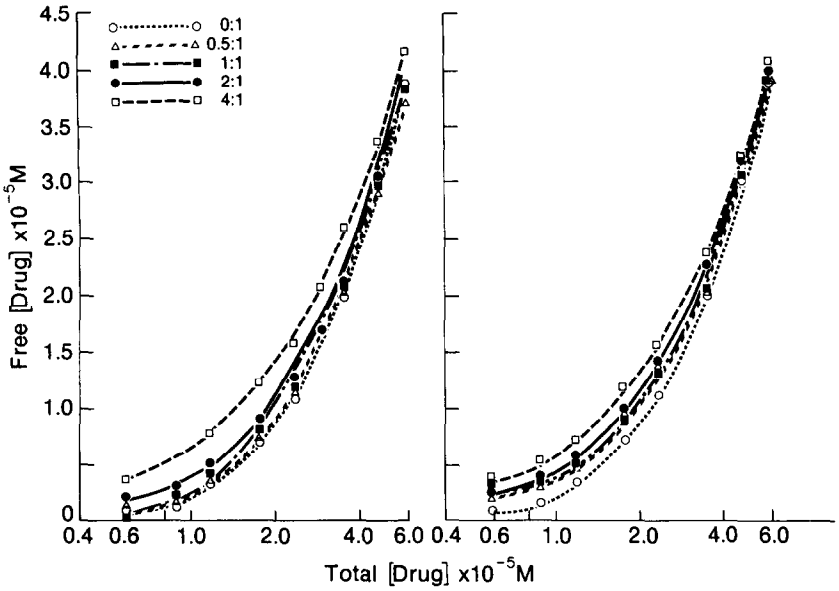

Fig. 1. Free versus total drug concentrations for the binding of diazepam to defatted human serum albumin in the presence of different concentrations of palmitic (left panel) and oleic acid (right panel). Each point represents the average of three experimental determinations. Studies were conducted in 0.067 M phosphate buffer (pH 7.4) at 37°.

DISCUSSION

The data analysis methodology applied in these studies was developed to circumvent a variety of problems inherent in more commonly used methods [1]. In the plot of r (moles of drug bound per mole of albumin) against log $[D_f]$, the value of r is dependent on the measurement of $[D_f]$. Consequently, any error in the measurement of $[D_f]$ is incorporated in r. In the plot according to Scatchard [21], the ordinate $r/[D_f]$ and the abscissa r are both calculated from the measurement of $[D_f]$. By applying a model using at least two independently measured variables, D_t and $[D_f]$, interdependence of variables and their errors is eliminated. Throughout the range of drug concentrations used, the errors in the measurements of free drug concentrations (coefficient of variation 1.0 to 3.7 per cent) and total drug (coefficient of variation 0.2 to 2.2 per cent) remained constant, and no weighting was necessary. The estimates of n_1 and k_1 obtained by this method are in general agreement with the results of other investigators, but are less likely to have been biased by the commonly applied data analysis techniques used in their method of derivation.

The association constant (k_a) for diazepam binding to human serum albumin has been reported previously: from gel filtration studies, a total binding constant (nk_a) of $4.92 \times 10^5 M^{-1}$ [22]; using ultrafiltration technique, $3.9 \times 10^5 M^{-1}$ [9]; and using circular dichroism, $1.8 \times 10^5 M^{-1}$ [23]. Since the possibility of a second class of binding sites was included in our curve fitting procedure, the value of the primary binding constant is

Table 1. Effects of fatty acids on the binding parameters of diazepam with fatty acid free human serum albumin (FAF-HSA)*

Fatty acid	Ratio	n_1	k_1 ($M^{-1} \times 10^5$)	n_1k_1 ($M^{-1} \times 10^5$)	n_2	k_2 ($M^{-1} \times 10^3$)	n_2k_2 ($M^{-1} \times 10^3$)	Σn_ik_i ($M^{-1} \times 10^5$)
FAF-HSA	0.1	0.91	9.47	8.62	5.30	1.90	10.07	8.72
Palmitic acid	0.5:1	0.72†	16.46†	11.85	2.42	8.17	19.77	12.05
	1:1	0.71†	18.43†	13.09	4.03	4.08	16.44	18.59
	2:1	1.06‡	1.26†	1.34	8.24	0.86	7.09	1.41
	4:1	1.05§	0.01†	0.01	5.76	4.50	25.92	0.27
Oleic acid	0.5:1	0.69†	3.79†	2.62	8.99	1.65	14.83	2.77
	1:1	0.76†	2.42	1.84	4.64	3.23	14.99	1.99
	2:1	0.47†	4.84†	2.27	5.14	1.34	6.89	2.34
	4:1	0.49†	0.67†	0.33	7.44	2.94	21.87	0.55

* Ratio = moles FFA/mole albumin; n_1 = number of binding sites in primary class; k_1 = association constant of primary binding sites; n_2 = number of binding sites in secondary class; and k_2 = association constant of secondary binding sites. [Albumin] = 3.1×10^{-5} M. All comparisons are to diazepam binding with FAF-HSA alone.

† $P < 0.05$.

‡ $P < 0.001$

§ Not significant.

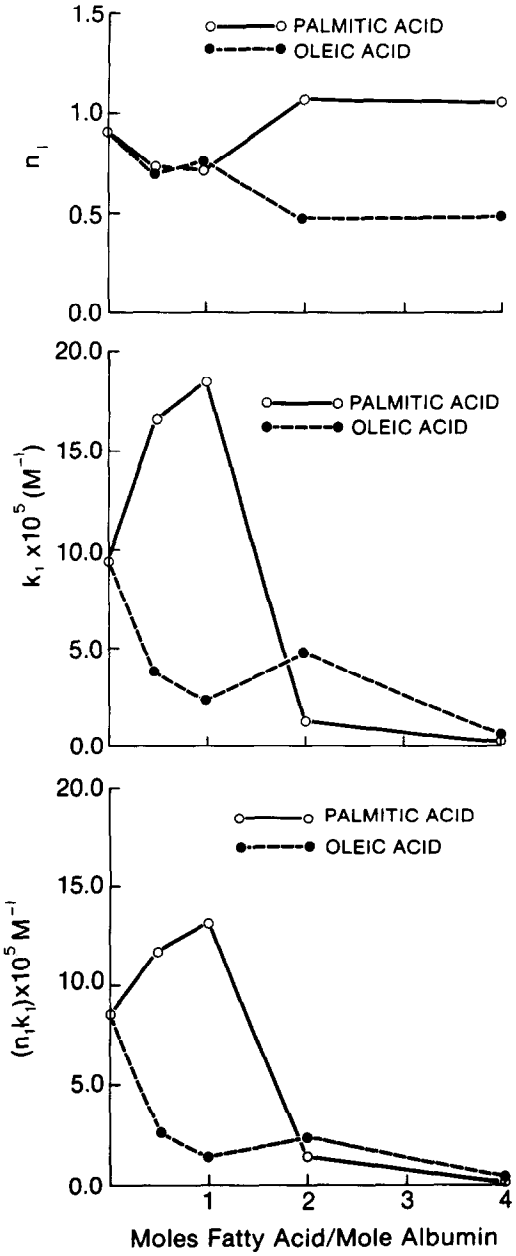

Fig. 2. Number of primary binding sites (n_1), primary binding association constants (k_1) and the product n_1k_1 for diazepam in the presence of varying molar ratios of palmitic and oleic acid.

somewhat different. Corresponding estimates from our study are $n_1 = 0.91$, $k_1 = 9.47 \times 10^5 M^{-1}$ and $n_1k_1 = 8.62 \times 10^5 M^{-1}$. Some differences among reports in the literature may reflect differences in FFA content of human serum albumin.

Our studies indicate that a secondary class of binding sites may exist with a capacity for 5 molecules of diazepam, with a lower association constant of the order of $10^4 M^{-1}$. Insufficient experimental data and interference by competition with membrane binding of comparable magnitude make difficult the assessment of whether the secondary binding is specific or not.

The relative binding of diazepam and its major metabolites to defatted human serum albumin is similar to their binding to whole human plasma or serum, i.e. diazepam 98.3 and 96.8 per cent and desmethyldiazepam 96.3 and 96.6 per cent [12, 24]. Of all three compounds, oxazepam is reported to have the lowest bound fraction of 95.7 per cent [24], reflected in a reduction in binding affinity compared to diazepam or desmethyldiazepam [22, 23]. Our studies show that oxazepam and desmethyldiazepam, present in drug:albumin ratios of 1:1, caused significant displacement of diazepam from FAF-HSA. Variations in values of n_1 for diazepam in these interaction studies may have been due to the metabolite binding molecular conformational changes on defatted albumin molecules, which are known to be thermodynamically unstable [25]. Since the albumin concentration *in vivo* is approximately 20-fold greater than that used in our studies, displacement by drug metabolites with lesser affinity that the parent drug is likely to be only a few per cent of the free diazepam concentration and, therefore, of dubious clinical relevance. Certainly bilirubin and uric acid will not be important at clinically occurring concentrations.

Other studies have shown that the fatty acid, lauric acid, displaces diazepam from defatted albumin [9]. However, these investigators used one of the least commonly occurring fatty acids at molar ratios in excess of that occurring physiologically. Our experiments used two fatty acids, palmitic and oleic acids, that predominate in the human, at concentrations of 0–4 moles per mole of albumin.

Increasing the palmitic acid concentration from 0 to 1 mole per mole of albumin enhances diazepam binding by increasing the affinity of albumin for diazepam. Recent experiments showed that low concentrations of fatty acids (0–3 moles per mole of albumin) did not displace the drug warfarin from albumin but rather increased its binding to albumin [7, 8]. Since fatty acids are known to stabilize the albumin molecule, FFA concentrations of 2–3 moles per mole of albumin place the molecule in a more favourable thermodynamic state than totally defatted albumin [25]. Stabilization of the albumin tertiary structure presumably caused an allosteric change in the distant warfarin binding site in such a manner as to enhance drug binding. A similar phenomenon presumably occurs for palmitic acid effects on diazepam binding.

In contrast, concentrations of oleic acid below 1 mole per mole of albumin cause immediate interference with diazepam binding. Considering these observations, it appears likely that the higher binding affinity of oleic acid ($k_a = 1.1 \times 10^8$ M^{-1}) over palmitic acid ($K_a = 6.0 \times 10^7$ M^{-1}) [3] results in a higher affinity binding of the former fatty acid to the primary fatty acid binding site, leading to a larger change of either the whole albumin structure or the conformation of the distant benzodiazepine binding site than would be possible for palmitic acid. The findings of McMenamy [26] on the comparison of the pH-binding profiles of defatted and non-defatted albumin strongly support the placement of the primary fatty acid binding site at or near the indole ligand binding site, also known to be specific for the benzodiazepine derivatives [27]. Close proximity of the two binding sites would suggest that, depending on the affinity of the fatty acid for the

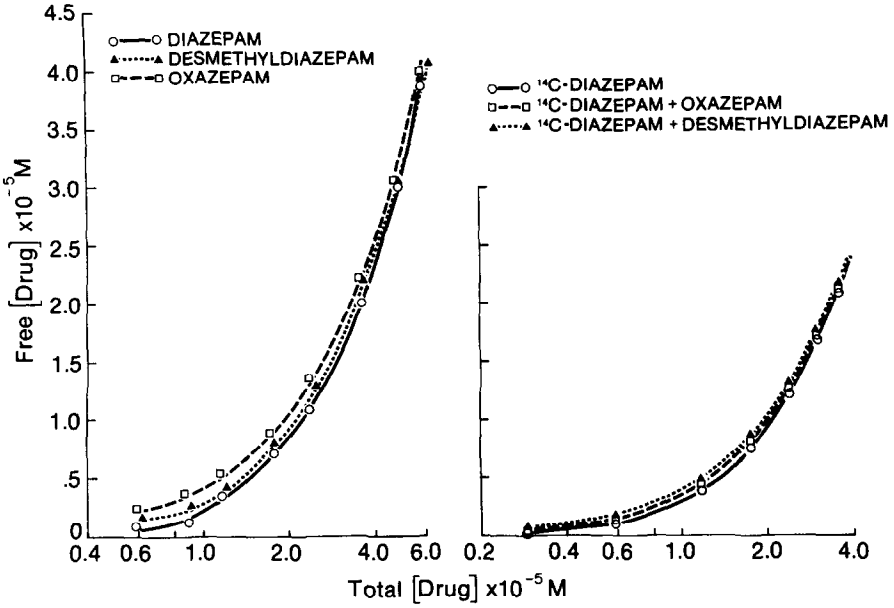

Fig. 3. Free versus total drug concentrations for the binding of diazepam, desmethyldiazepam and oxazepam to defatted human serum albumin (left panel) and the influence of desmethyldiazepam and oxazepam on [^{14}C]diazepam binding (right panel). Each point represents the average of three experimental determinations. Studies were conducted in 0.067 M phosphate buffer (pH 7.4) at 37°.

primary binding site, profound conformational changes would be easily induced in the local environment of the benzodiazepine binding site. The recent suggestion of Sjodin [28], that the displacement of bound diazepam by oleic acid at molar ratios even below 1 occurs primarily through an allosteric mechanism, is supported by the present studies.

Highly significant inter-individual variations in fraction free diazepam exist *in vivo* [14]. The results from the fatty acid studies suggest that differences in the ratio of oleic to palmitic acid (or other fatty acids) among individuals could account for such differences in diazepam binding *in vivo*.

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Table 2. Binding parameters of diazepam, desmethyldiazepam and oxazepam with FAF-HSA and the effects of metabolites on [^{14}C]diazepam binding*

	n_1	k_1 ($M^{-1} \times 10^5$)	n_1k_1 ($M^{-1} \times 10^5$)	n_2	k_2 ($M^{-1} \times 10^5$)	n_2k_2 ($M^{-1} \times 10^3$)	Σn_ik_i ($M^{-1} \times 10^5$)
Diazepam + FAF-HSA	0.91	9.47	8.62	5.30	1.90	10.07	8.72
Desmethyldiazepam + FAF-HSA	0.88†	4.38‡	3.85	4.55	4.39	19.97	4.05
Oxazepam + FAF-HSA	0.74‡	6.48§	4.80	6.30	1.39	8.76	4.81
[^{14}C]Diazepam	0.90	9.56	8.51	7.14	2.76	19.71	9.76
[^{14}C]Diazepam + desmethyldiazepam‖	0.70‡	4.74‡	3.32	4.21	6.26	26.35	3.49
[^{14}C]Diazepam + oxazepam‖	1.06‡	3.07‡	3.25	2.74	4.65	12.74	3.38

* See Table 1 for abbreviations n_1, k_1, n_2 and k_2. All comparisons are to diazepam binding parameters as appropriate for non-labeled and radiolabeled drug. [Albumin] = 3.1×10^{-5} M.

† Not significant.

‡ $P < 0.001$.

§ $P < 0.01$.

‖ [Desmethyldiazepam] and [oxazepam] = 1.03×10^{-5} M.

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