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PLASMA PROTEIN BINDING AND INTERACTION STUDIES WITH DIFLUNISAL, A NEW SALICYLATE ANALGESIC

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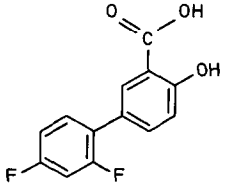
Abstract—The binding of diflunisal to human serum albumin and normal human plasma has been studied by equilibrium dialysis at 37°, pH 4. The plasma protein binding data were analysed according to a Scatchard model with two independent classes of binding sites. The number of binding sites and the corresponding association constants have been estimated by nonlinear least-squares regression analysis: $N_1 = 2.1$, $K_1 = 5.28 \times 10^5\ M^{-1}$, $N_2 = 7.7$ and $K_2 = 0.17 \times 10^5\ M^{-1}$. At a diflunisal plasma concentration of 50 μg/ml on average 99.83 per cent of the drug was bound to plasma proteins. The *in vitro* plasma protein binding of diflunisal was impaired by salicylic acid and phenprocoumon, while diflunisal itself was displaced from its primary binding sites in plasma by salicylic acid and bilirubin. Tolbutamide had no effect on the binding of diflunisal to plasma proteins.

Plasma protein binding is an important determinant of drug disposition in the body [1, 2]. The amount of drug bound in plasma is dependent on the protein concentration, the number of binding sites on the protein, the affinity of each binding site for the drug, and the total drug concentration [3]. Alterations in any one of these factors will change the fraction of drug in the free form. A decrease in protein concentration or binding capacity and displacement of a bound drug by other drugs, drug metabolites or endogenous substances will increase the free drug concentration. This increase may enhance the effects of the drug and alter its distribution and elimination characteristics [4, 5].

Diflusinal, a new derivative of salicylic acid ($pK_a = 3.30$) (Fig. 1), has been shown to possess



DIFLUNISAL

Fig. 1. Chemical structure of diflunisal [5-(2′, 4′-difluorophenyl)salicylic acid].

potent analgesic, anti-inflammatory and uricosuric properties both in animals and in man [6–8]. Preliminary pharmacokinetic experiments revealed a plasma protein binding of approximately 99 per cent in man, but detailed information is not available [9, 10].

This report describes the binding characteristics of diflunisal both to pure human serum albumin and to plasma of healthy volunteers. The influence of salicylic acid, tolbutamide and bilirubin on the *in vitro* binding characteristics of diflunisal was evaluated. The displacing effect of diflunisal on the *in vitro* binding of salicylic acid and phenprocoumon to plasma was also investigated.

MATERIALS AND METHODS

Materials. Human serum albumin (HSA), essentially fatty acid free, was purchased from Sigma Company, St. Louis, MO. HSA-solutions were prepared in isotonic phosphate buffer, pH 7.4, assuming a molecular weight for albumin of 65,100.

Blood samples, obtained from healthy volunteers, were collected on citrate and plasma was immediately separated by centrifugation. All plasma samples were directly used for the *in vitro* binding experiments. Unlabelled and labelled diflunisal (^{14}C, 16 μCi/mg) was kindly donated by the MSD Research Laboratories, Rahway. The radiochemical purity of the labelled diflunisal was checked by thin-layer chromatography: of the total radioactivity applied on the plate, about 98.5 per cent was located in one spot. ^{14}C-Salicylic acid (21 μCi/mg) was purchased from the Radiochemical Center, Amersham, England and ^{3}H-phenprocoumon (73 μCi/mg) was a gift from Hoffman-Laroche, Basle, Switzerland. Pure salicylic acid (Aldrich, Beerse, Belgium), tolbutamide (Boehringer GmbH, Mannheim, Germany) and bilirubin (Serva Feinbiochemica, Heidelberg, Germany) were used in the *in vitro* displacement experiments.

Measurement of binding. Plasma protein binding was determined by equilibrium dialysis (E.D.) at 37° against isotonic phosphate buffer, pH 7.4, in a Dianorm Equilibrium Dialyzer (Diachema A.G., Zürich, Switzerland) using Spectrapor 2 membranes

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(Spectrum Medical Industries, Los Angeles, CA). Determinations were made at diflunisal concentrations of 5–1000 μg/ml, salicylic acid concentrations of 10–1500 μg/ml and phenprocoumon concentrations of 0.5–250 μg/ml. Duplicate determinations using 1 ml of plasma (or HSA-solution) and buffer were performed. The time used for equilibration was 4 hr, as longer equilibration periods did not alter the degree of binding. After equilibrium was achieved, 0.75 ml sample aliquots from both sides of the dialysis cells were mixed with 10 ml of Instasolve (Packard Instrument Company, IL). Radioactivity was determined with a Packard Tricarb 3255 Liquid Scintillation Spectrometer using the method of external standardization. Per cent binding was calculated as follows:

$$\%B = \frac{C_p - C_b}{C_p} \cdot 100 ,$$

with C_p being drug concentration in plasma and C_b being drug concentration in buffer.

The extent of plasma protein binding was also determined by centrifugal ultrafiltration (U.F.) using seamless visking dialysis tubing (Medicell International, London, England) as previously described by Jusko [11].

Determination of binding parameters. The analysis of the protein binding data obtained with plasma was based upon the assumption that binding occurred exclusively to albumin. The concentration of albumin was determined by an immunological technique [12].

Binding data were interpreted by computer-fitting, assuming two classes of independent binding sites (Scatchard-model) [13] with the number of binding sites N_1 and N_2, and the association constants K_1 and K_2. The curve-fitting procedure, using the nonlinear least-squares program NONLIN [14], was based upon the following equation:

$$C_t = [D] + [PD]$$

$$C_t = [D] + \frac{N_1K_1[D][A]}{1+K_1[D]} + \frac{N_2K_2[D][A]}{1+K_2[D]} ,$$

where: C_t = unbound plasma concentration of diflunisal;
$[D]$ = unbound plasma concentration of diflunisal;
$[PD]$ = bound plasma concentration of diflunisal;
$[A]$ = plasma albumin concentration.

An IBM 370/158-MVS digital computer was used. The binding data obtained from the *in vitro* displacement studies were plotted in a double reciprocal way [15]. This approach was used to determine the nature of the displacement for only the low, clinically relevant drug concentration data (primary binding sites). Linear regressions were performed to determine the best line through the experimental points.

In the text and tables, results are presented as mean ± S.E.M. Where appropriate, statistical analysis was performed using Student's *t*-test. A P-value of 0.05 or less was considered to be statistically significant.

RESULTS

Methodological aspects. Binding data of diflunisal obtained by equilibrium dialysis and centrifugal ultrafiltration did not differ significantly (Table 1). However, the Dianorm Equilibrium Dialyzer System was used throughout this study, since only small amounts of plasma (1 ml) are needed to perform binding studies with this procedure. Drug binding to membranes or cells was found to be less than 3 per cent.

The effect of buffer composition and temperature on the plasma protein binding of diflunisal was investigated (Table 1). No change in binding characteristics of diflunisal could be observed when the isotonic phosphate buffer, pH 7.4, in the equilibrium technique was replaced by Krebs–Henseleit bicarbonate buffer, pH 7.4. Decreasing the temperature from 37° to 24° slightly but significantly increased the binding of diflunisal to plasma from 99.83 ± 0.01 per cent to 99.89 ± 0.01 per cent.

Binding of diflunisal to pure HSA and normal plasma. Figure 2 shows the Scatchard curves for the binding of ^{14}C-diflunisal to pure HSA and normal plasma samples. In both cases, the heterogeneity of the binding sites is expressed by the non-linearity of the Scatchard plot. The mean binding parameters resulting from the analysis of these data are summarized in Table 1. The number of primary binding sites for diflunisal on the albumin molecule as derived from binding studies to normal plasma was, on average, 2.1 ± 0.1. K_1, the association constant for this class of binding sites, was 5.28 ± 0.26 × 10^5 M^{-1}. The secondary, less important class had, on average, 7.7 ± 0.3 diflunisal binding sites with an association constant K_2 of 0.17 ± 0.02 × 10^5 M^{-1}. The binding of diflunisal was even more pronounced when studied in a pure HSA-buffer solution (Table 1). K_1 for the diflunisal–HSA interaction was in this case approx-

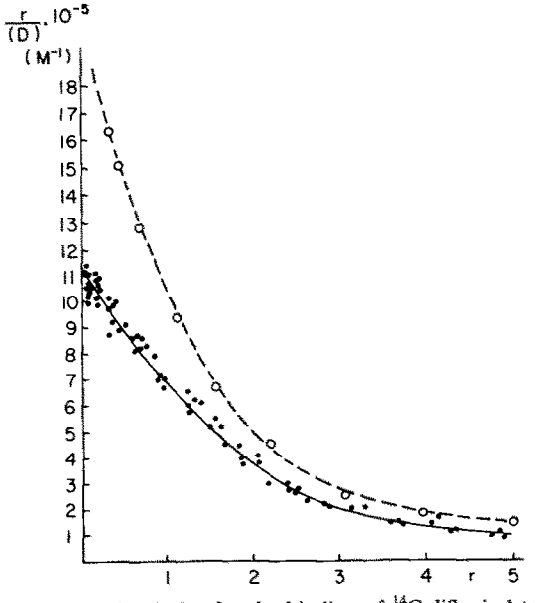

Fig. 2. Scatchard plot for the binding of ^{14}C-diflunisal to pure HSA (○— — —○) and to normal human plasma (anticoagulant: citrate) (●——●). All measurements were made by E.D. vs isotonic phosphate buffer (pH 7.4) at 37°. Each point represents the mean value of at least two experiments. r = number of moles of diflunisal bound per mole of albumin, (D) = molar concentration of free diflunisal.

Table 1. Binding characteristics of difunisal to citrated normal plasma and pure HSA*

Method	Binding to	N_1	K_1 (M^{-1}) × 10^{-5}	N_2	K_2 (M^{-1}) × 10^{-5}	%B	%U
E.D., 37° phosph. buffer	normal plasma ($n = 7$)	2.1 ± 0.1	5.28 ± 0.26	7.7 ± 0.3	0.17 ± 0.02	99.83 ± 0.01	0.17 ± 0.01
E.D., 37° K.-H buffer	normal plasma ($n = 4$)	2.0 ± 0.1	5.38 ± 0.45	8.1 ± 0.4	0.15 ± 0.01	99.84 ± 0.01	0.17 ± 0.01
E.D., 24° phosph. buffer	normal plasma ($n = 4$)	1.6† ± 0.1	9.21† ± 0.24	7.3 ± 0.4	0.27† ± 0.01	99.89† ± 0.01	0.11† ± 0.01
U.F., 37°	normal plasma ($n = 3$)	2.2 ± 0.1	4.18† ± 0.23	7.6 ± 0.4	0.15 ± 0.01	99.82 ± 0.01	0.18 ± 0.01
E.D., 37° phosph. buffer	pure HSA	1.8	11.1	5.0	0.4	99.90	0.10

* All binding parameters were obtained by fitting the binding data to the formula described under Methods. However, the binding parameters for the diflunisal–HSA interaction were determined graphically from the Scatchard plot.

† Value significantly different ($P < 0.05$) from the control value obtained by E.D., 37°, phosph. buffer.

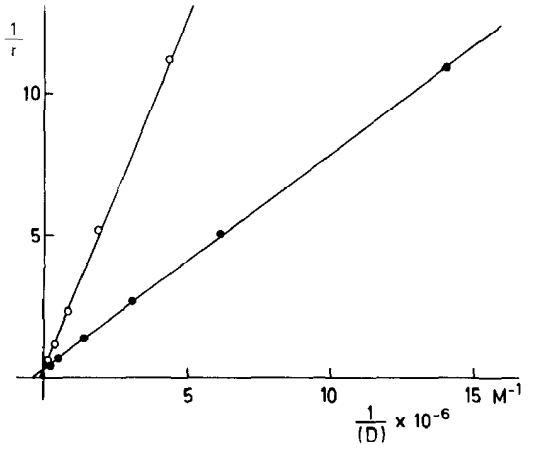

Fig. 3. Double reciprocal plot illustrating the effect of salicylic acid on the primary binding sites of diflunisal to normal plasma. For further explanation, see Fig. 2. ●——●: control (0 μg salicylic acid/ml plasma), ○— — —○: 250 μg salicylic acid/ml plasma.

imately 11.0 × 10^5 M^{-1} and the number of primary binding sites was 1.8. These data indicate that the binding capacity of pure HSA for diflunisal is about twice as high as the binding capacity of normal plasma for the drug.

Per cent unbound drug at a total diflunisal concentration of 50 μg/ml was, on average, 0.17 ± 0.01 per cent.

Effect of salicylic acid, tolbutamide and bilirubin on the in vitro *plasma binding of diflunisal.* The displacing effect of two highly plasma bound drugs (salicylic acid, tolbutamide) and one endogenous substance (bilirubin) on the binding of diflunisal to its primary binding sites in plasma was studied. In the presence of 250 μg salicylic acid per ml plasma, the binding of diflunisal was substantially reduced (Fig. 3).

Adding tolbutamide (50, 100 and 150 μg/ml) to normal plasma, however, did not influence the plasma protein binding of diflunisal. Bilirubin was added to normal plasma so that concentrations of approximately 0.03, 0.06 and 0.12 mg/ml were

Table 2. Displacing effect of salicylic acid, tolbutamide and bilirubin on the *in vitro* plasma protein binding of diflunisal (50 μg/ml plasma)

Displacing agent	Concentration of displacing agent (μg/ml)	Unbound diflunisal (%)
Salicylic acid	0	0.15
	250	0.53 ± 0.04 ($n = 3$)
Tolbutamide	0	0.19
	50	0.20
	100	0.19
	150	0.17
Bilirubin	~ 10	0.16
	~ 30	0.20
	~ 60	0.22
	~ 120	0.27

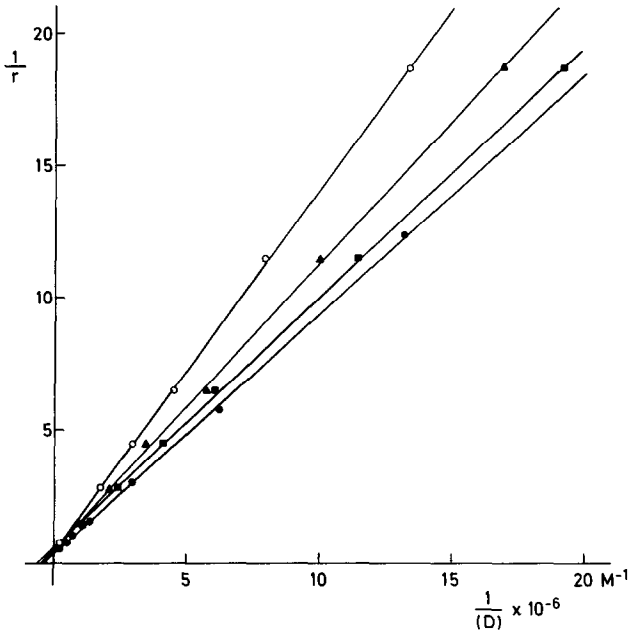

Fig. 4. Double reciprocal plot illustrating the effect of bilirubin on the primary binding sites of diflunisal to normal human plasma. For further explanation, see Fig. 2. ●——●: 0.01 mg bilirubin/ml plasma; ■——■: 0.03 mg bilirubin/ml plasma; ▲——▲: 0.06 mg bilirubin/ml plasma; ○——○: 0.12 mg bilirubin/ml plasma.

achieved. Plasma protein binding data for diflunisal obtained with these plasma samples indicate that bilirubin slightly inhibits the binding of this drug (Fig. 4). Table 2 shows the displacing effect of salicylic acid, tolbutamide and bilirubin on the binding of diflunisal at a diflunisal plasma concentration of 50 μg/ml.

Effect of diflunisal on the in vitro *plasma protein binding of salicylic acid and phenprocoumon.* The displacing effect of diflunisal (75 and 150 μg/ml) on the plasma protein binding of ^{14}C-salicylic acid and ^{3}H-phenprocoumon was investigated. Both concentrations of diflunisal exert an inhibiting effect on the primary binding parameters of salicylic acid and phenprocoumon, as is demonstrated by the double reciprocal plots in Figs. 5A and B. Table 3 shows per cent unbound salicylic acid and phenprocoumon at the therapeutic plasma concentrations of, respectively, 200 and 1 μg/ml without diflunisal, and in the presence of 75 and 150 μg diflunisal per ml plasma. These data show that the unbound fraction of salicylic acid and phenprocoumon is significantly increased by 75 μg and even more so by 150 μg diflunisal per ml plasma. Phenylbutazone, at concentrations of 75 and 150 μg/ml, increased the percentage of unbound phenprocoumon from a control value of 0.6 to 1.3 and 2.9%, respectively (Table 3).

* F. W. Gribnau, personal communication.

DISCUSSION

There have been very few reports of studies on the pharmacokinetics and plasma protein binding of diflunisal, a new acidic analgesic compound. Previous papers, dealing with the disposition of diflunisal in man, reported a plasma protein binding of about 98–99 per cent [9, 10]. Gribnau found a plasma protein binding of 99.6 and 99.4 per cent at diflunisal plasma concentrations of 15 and 50 μg/ml, respectively.* Results of the present investigation confirm this high plasma protein binding and show that the

Table 3. Displacing effect of diflunisal and phenylbutazone on the *in vitro* plasma protein binding of salicylic acid (100 μg/ml plasma) and phenprocoumon (1 μg/ml plasma)

Displacing agent	Concentration of displacing agent (μg/ml)	Unbound salicylic acid (%)	Unbound phenprocoumon (%)
Diflunisal	0	11.64	0.7
	75	14.18	0.9
	150	17.44	1.2
Phenylbutazone	0		0.6
	75		1.3
	150		2.9

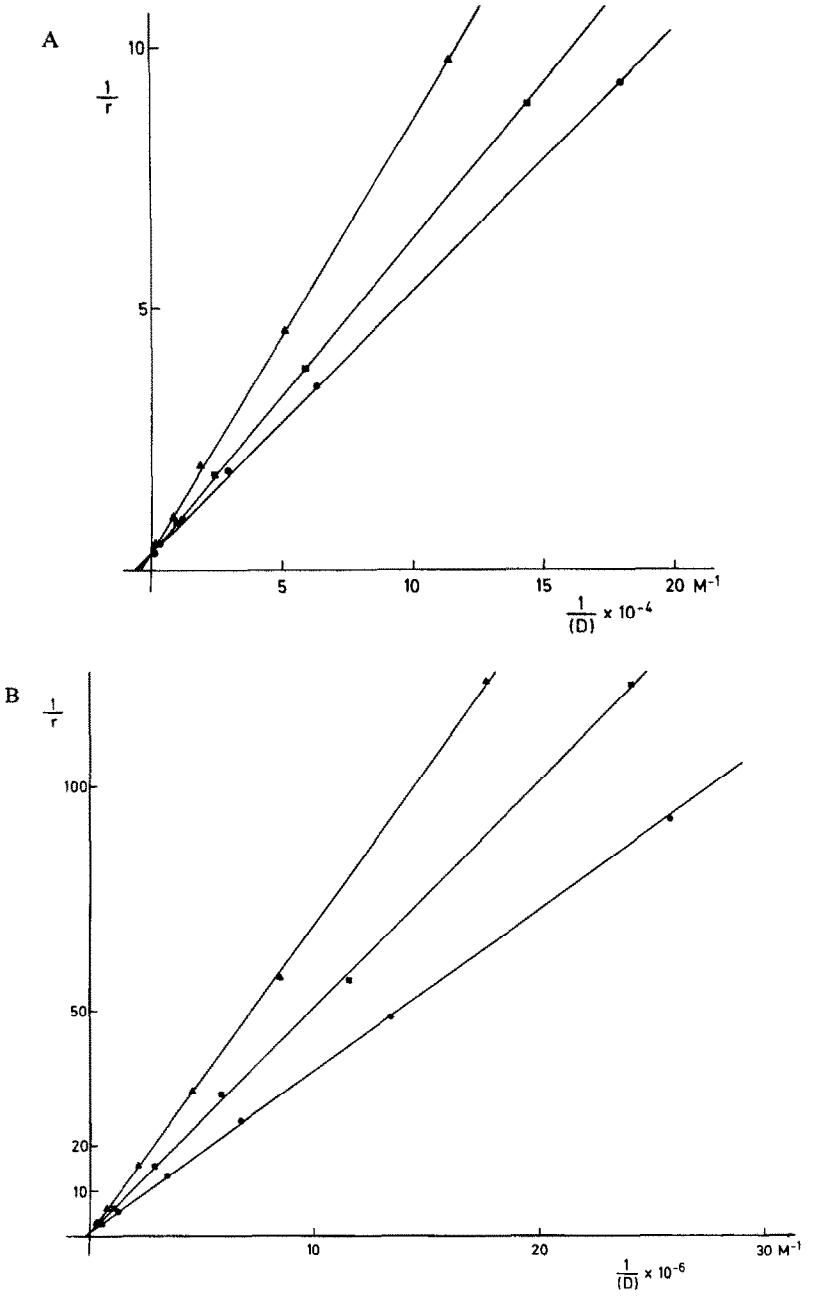

Fig. 5. Double reciprocal plot illustrating the displacing effect of diflunisal on the primary binding sites of ^{14}C-salicylic acid (A) and ^{3}H-phenprocoumon (B) to normal human plasma. For further explanation, see Fig. 2. ●——●: control (0 μg diflunisal/ml plasma); ■——■: 75 μg diflunisal/ml plasma; ▲——▲: 150 μg diflunisal/ml plasma.

binding data can be analysed according to a Scatchard model with two different classes of independent binding sites. The values of the binding parameters obtained in normal plasma by equilibrium dialysis at 37° are $N_1 = 2.1$, $K_1 = 5.28 \times 10^5$ M^{-1}, $N_2 = 7.7$ and $K_2 = 0.17 \times 10^5$ M^{-1}. Unbound diflunisal at a plasma concentration of 50 μg/ml is, on average, 0.17 ± 0.01%. A similar extensive plasma protein binding has been observed with other anti-inflammatory analgesics (phenylbutazone, naproxen, benoxaprofen) and with oral anticoagulants (dicoumarol, warfarin, phenprocoumon) [2, 16–19]. As a result, a small change in binding may appreciably affect the distribution and elimination characteristics of this drug.

The primary affinity constant (K_1) for the binding of diflunisal to pure HSA in buffer is about twice as high as the K_1-value obtained with normal plasma

(Table 1). This finding suggests that albumin is the only binding protein of importance in human plasma. The difference in diflunisal binding observed between plasma and HSA may be due to endogenous compounds present in plasma which inhibit diflunisal binding to the albumin molecule. Similar discrepancies between plasma and pure albumin binding have previously been described for salicylic acid and warfarin [20].

The clinical use of a new agent which is extensively bound to plasma protein requires consideration of possible interactions with the protein binding of other drugs. The *in vitro* interaction studies described were performed at drug concentrations within the therapeutic range for both the displacing agent and displaced drug. The plasma protein binding of diflunisal has been shown to be reduced in the presence of salicylic acid and vice versa. This is to be expected as both compounds are structurally related (Fig. 1). Tolbutamide, a hypoglycemic agent highly bound to plasma protein and especially to albumin [21, 22], does not affect the binding of diflunisal, suggesting that the primary binding sites on albumin for both compounds are distinct. Hyperbilirubinemia causes a slight reduction in the plasma protein binding of diflunisal. We also studied the effect of diflunisal on the plasma protein binding of the anticoagulant drug phenprocoumon. At diflunisal concentrations of 75 and 150 μg/ml, the percentage unbound phenprocoumon is increased from 0.7% control value to 0.9 and 0.12%, respectively, at a therapeutic phenprocoumon concentration of 1μg per ml plasma. However, phenylbutazone which is known to interact *in vivo* with oral anticoagulant therapy because of competition for a common binding site on albumin [23], displaced phenprocoumon *in vitro* to a much greater extent under identical experimental conditions (Table 3). These *in vitro* displacement data indicate that diflunisal may possibly interact *in vivo* with the oral anticoagulant agent phenprocoumon when simultaneously used. Furthermore, according to the concept of specific albumin binding sites [24–26], drugs such as phenprocoumon, warfarin, acenocoumarin, phenylbutazone, sulfinpyrazone are all bound to the same, specific site on the albumin molecule. Therefore, interactions with diflunisal and the above mentioned drugs may be anticipated since we found that diflunisal and one of the above listed compounds (phenprocoumon) share a specific albumin binding site. In addition, warfarin is known to be displaced by bilirubin [27] and therefore the observed interaction between bilirubin and diflunisal fits well with this specific site concept. Since tolbutamide does not interfere with the primary binding site of diflunisal, these two drugs must be primarily bound to different sites on the albumin molecule.

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