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In-vitro study on the competitive binding of diflunisal and uraemic toxins to serum albumin and human plasma using a potentiometric ion-probe technique

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Abstract

The competitive binding of diflunisal and three well-known uraemic toxins (3-indoxyl sulfate, indole-3-acetic acid and hippuric acid) to bovine serum albumin (BSA), human serum albumin (HSA) and human plasma was studied by direct potentiometry. The method used the potentiometric drug ion-probe technique with a home-made ion sensor (electrode) selective to the drug anion. The site-oriented Scatchard model was used to describe the binding of diflunisal to BSA, HSA and human plasma, while the general competitive binding model was used to calculate the binding parameters of the three uraemic toxins to BSA. Diflunisal binding parameters, number of binding sites, n_i and association constants for each class of binding site, K_i, were calculated in the absence and presence of uraemic toxins. Although diflunisal exhibits high binding affinity for site I of HSA and the three uraemic toxins bind primarily to site II, strong interaction was observed between the drug and the three toxins, which were found to affect the binding of diflunisal on its primary class of binding sites on both BSA and HSA molecules and on human plasma. These results are strong evidence that the decreased binding of diflunisal that occurs in uraemic plasma may not be solely attributed to the lower albumin concentration observed in many patients with renal failure. The uraemic toxins that accumulate in uraemic plasma may displace the drug from its specific binding sites on plasma proteins, resulting in increased free drug plasma concentration in uraemic patients.

Introduction

Drug protein binding is known to be an important factor in drug bioavailability, efficacy, transport and toxicity. Moreover, competitive binding phenomena resulting in displacement of drugs from plasma proteins have also been reported, particularly for drugs strongly bound to plasma proteins. Small changes in the percentage of binding of these drugs cause significant changes in their free plasma concentration, which is responsible for their pharmacological action. In the case of renal disease, defective drug binding occurs in plasma, especially for acidic drugs, even in the presence of a normal concentration of albumin, which is the main binding protein of this type of drug in blood (Lindup et al 1986). This situation can be caused by the accumulation of endogenous substances in plasma known as uraemic toxins, which are characterized by high binding affinity for the human serum albumin (HSA) molecule. Indoxyl sulfate, indole-3-acetic acid, hippuric acid and 3-carboxy-4-methyl-5-propyl-2-furanopropionic acid are reported to be the main uraemic toxins (Lindup et al 1985; Takamura et al 1997; Tsutsumi et al 1999). It has been shown that the presence of these toxins in plasma may enhance the pharmacological effect of several drugs by inhibiting their plasma protein binding, and altering their distribution and elimination characteristics (Bowmer & Lindup 1982; Lindup et al 1986; Mingrore et al 1997).

Diflunisal (5-(2,4-difluorophenyl)salicylic acid) is a salicylic acid derivative and well-known non-steroidal anti-inflammatory drug (NSAID) that is known to be more than 99% bound to normal human plasma (Verbeeck et al 1979; Lin 1989; Sideris et al 1994). Reduced plasma binding of diflunisal was observed in patients with chronic renal failure and attributed to decreased albumin synthesis and, consequently, to the lower plasma protein concentration found in many of these patients (Verbeeck & Schepper 1979). However, no work has been done on the influence of uraemic toxins on the protein binding of diflu-

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nisal and no data are available for the binding of diflunisal to plasma proteins in the presence of uraemic toxins.

Accordingly, the main objective and novelty of the present investigation was to study in-vitro the simultaneous binding of diflunisal and the three main uraemic toxins, 3-indoxyl sulfate (IS), indole-3-acetic acid (IA) and hippuric acid (HA), to bovine serum albumin (BSA), HSA and total human plasma under standardized conditions. For this purpose, a modification of the potentiometric ion-probe technique (Angelakou et al 1994) was applied for continuous monitoring of the free drug ion in the reaction mixture of protein–drug–toxin by direct potentiometry. The big advantage of ion selective electrode (ISE) direct potentiometry, when applied to protein binding studies, is its ability to measure directly the activity of the free drug anion in diluted and concentrated protein solutions and in undiluted biological specimens, thus overcoming the dilution step, which is the major drawback of spectroscopic techniques (Christopoulos & Diamandis 1990; Valsami et al 1991; Angelakou et al 1994, 1999; Sideris et al 1994).

Materials and Methods

Apparatus

The diflunisal electrode used was of the PVC type with an ion exchanger consisting of the diflunisal–tetraheptylammonium ion pair in 2-nitrophenyl-octyl ether (Sideris et al 1994). The reference electrode was of Ag/AgCl type. An Orion digital electrometer (readability ±0.1 mV) connected to a chart recorder was used for the potential measurements. All experiments were carried out in a double wall cell, thermostatted at 25°C, at pH 7.4. Additions of microvolumes to the monitored solution in the measurement cell were achieved accurately using Hamilton microsyringes and automatic pipettes for volumes over 100 μL.

Reagents

All solutions were prepared in deionized water. Diflunisal, IS, IA, HA and HSA (A-1887, ≥96%, fatty acid content ≈0.005%) were purchased from Sigma-Aldrich (St Louis, MO). Tetraheptylammonium bromide, 2-nitrophenyl-octyl ether and BSA (5476 fraction V, ≥96%) were from Fluka (Buchs, Switzerland). Other chemicals used were of analytical grade.

Phosphate buffer, 0.10 M, pH 7.4
This was prepared by dissolving sodium dihydrogen phosphate in water and adjusting the pH with an 18 M NaOH solution.

Diflunisal stock solution
This was 0.010 M with respect to diflunisal in phosphate buffer 0.1 M, pH 7.4.

BSA and HSA solutions
These solutions contained 22.5 g L^{-1} (3.36×10^{-4} M) of BSA or HSA in phosphate buffer 0.1 M, pH 7.4.

Plasma preparation
Blood was collected from nine healthy volunteers of either sex, aged between 25 and 35 years, who had not received any medication for at least 1 week before blood collection. Plasma was obtained by centrifugation at 3000 rpm for 10 min. Prepared plasma was divided into 5-mL volume glass tubes and stored at −40°C. The remaining total plasma protein concentration was determined by the Biuret reaction and was found to be 72.1 ± 6.2 g L^{-1}. In binding experiments plasma was used after 1:1 (v/v) dilution with phosphate buffer 0.1 M, pH 7.4.

Mixed diflunisal/protein and diflunisal/toxins solutions
These solutions contained diflunisal at a concentration of 0.010 M, protein at a concentration of 22.5 g L^{-1} or plasma 1:1 (v/v) diluted with phosphate buffer 0.1 M, pH 7.4 and/or the three uraemic toxins at various concentrations (45–300 μM) in phosphate buffer 0.1 M, pH 7.4.

Procedures

Calibration (response) curve of the ISE
The pair of electrodes was immersed in 5 mL of phosphate buffer, 0.10 M, pH 7.4, in a 25°C thermostatted cell. After stabilization of the potential (± 0.10 mV), small volumes of diflunisal stock solution (0.010 M) were added, covering a solution concentration range of 9.96×10^{-6} to 5.35×10^{-3} M. Potential values (mV) were recorded and measured after stabilization (±0.1 mV) following each addition. These values (E, mV) were plotted against the negative logarithm of diflunisal concentration ($-\log C_{diflunisal}$), according to the Nernst equation, to obtain the calibration (response) curve of the ISE, using linear least squares fitting. Volume correction after each diflunisal addition was performed. A calibration curve was always constructed before each binding experiment.

Binding experiments
Studies of the competitive binding of diflunisal and the three uraemic toxins to BSA, HSA and human plasma under standardized conditions of pH 7.4 and 25°C were performed using diflunisal ISE. All experiments were performed in triplicate or more.

Binding experiments in the absence of toxins
Five millilitres of BSA or HSA or plasma solution were titrated stepwise in the measurement cell by micro-volume additions of mixed protein–diflunisal solution or diflunisal stock solution in the case of plasma. The potential values were recorded to check stabilization after each addition (the total drug concentration range covered was 50–1800 μg mL^{-1} or 2.0×10^{-4} to 7.2×10^{-3} M). Binding experiments in the absence of toxins were performed in five replicates.

Binding experiments in the presence of toxins (displacement experiments)
Five millilitres of mixed protein/toxin(s) or plasma/toxins solution were titrated potentiometrically by micro-volume additions of mixed protein/diflunisal/toxin(s) or diflunisal/

toxins solution in the case of plasma (the total drug concentration range covered was 50–1500 μg mL^{-1} or 2.0×10^{-4} to 6.0×10^{-3} M). These experiments were performed in triplicate.

Data analysis

In all binding experiments the free diflunisal concentration (F) was calculated from the calibration curve of the electrode and the concentration of bound diflunisal (B) from the following equation:

$$B = T - F \quad (1)$$

where T is the total diflunisal molar concentration. In the binding experiments of diflunisal with BSA and HSA in the absence and presence of the uraemic toxins, the estimates of the binding parameters were obtained by non-linear least squares fitting of the Scatchard equation (equation 2) to the experimental data (Scatchard 1949):

$$r = \frac{B}{P_t} = \sum_{i=1}^{m} \frac{n_i \cdot K_i \cdot F}{1 + K_i \cdot F} \quad (2)$$

where r is the number of drug molecules bound to one protein molecule, while m, n_i and K_i denote the number of distinct classes of independent and non-interacting binding sites, the number of binding sites of the i^{th} class and the diflunisal association constant of the i^{th} class, respectively.

In the case of diflunisal binding to human plasma in the absence and presence of the uraemic toxins, the modified Scatchard model (equation 3) was fitted to the experimental data in order to obtain estimates of the binding parameters (Valsami et al 1991):

$$r = \frac{B}{P_t} \sum_{i=1}^{m} \frac{N_i \cdot K_i \cdot F}{1 + \left(\frac{K_i \cdot F}{1000 \cdot M} \right)} \quad (3)$$

where B and F are given in μg mL^{-1}, P_t is the total plasma protein content given in g L^{-1}, K_i is given in L mol^{-1}, N_i is the binding capacity of the i^{th} class of binding sites in mol g^{-1} and M is the molecular mass of the drug.

Statistical methods

Non-linear least-squares fitting of equation 2 or equation 3 to the experimental data was performed with the MINSQ non-linear least squares fitting program (MicroMath Scientific Software 1992). Both the F-statistic and MSC, a modified Akaike model selection criterion included in the MINSQ least-squares fitting program (the most appropriate model being that with the largest MSC), were used to evaluate differences between various models examined. The effect of uraemic toxins on the protein binding of diflunisal was examined statistically by paired *t*-test, since the normality test passed in all cases. The values of the estimated binding parameters of diflunisal in the presence of uraemic toxins were compared to the respective values in the absence of toxins.

Results

Effect of uraemic toxins on diflunisal ISE operation

Diflunisal ISE showed a near-Nernstian response (slope 56–60 mV dec^{-1}) in the diflunisal concentration range from 5×10^{-6} to 6×10^{-3} M. The limit of detection was 1×10^{-6} M and the operative life of the electrode was about 1 month. The electrode showed no drift in the presence of BSA, HSA or human plasma and was thus appropriate for the binding experiments performed.

In order to evaluate the effect of uraemic toxins on the diflunisal ISE operation, calibration curves were constructed in the presence of each toxin and the ISE characteristics, i.e. the slope and the intercept of the corresponding calibration curve, were calculated. The slope of the electrode was found to be 56.21 ± 0.45 mV dec^{-1} in the absence of toxins and 55.37 ± 0.41, 55.46 ± 0.45 and 56.29 ± 0.57 mV dec^{-1} in the presence of HA, IS and IA, respectively. In all cases, a paired *t*-test revealed statistically non-significant differences ($0.173 < P < 0.680$). The calculated y-intercept values were 12.59 ± 2.12 mV in the absence of toxins and 11.97 ± 1.6, 13.76 ± 1.46 and 12.1 ± 2.02 mV in the presence of HA, IS and IA, respectively. Again, a paired t-test revealed non-significant statistical differences ($0.102 < P < 0.555$).

Competitive binding of diflunisal and uraemic toxins to BSA

BSA was used as the model protein in our binding studies because of its structural similarity to HSA and its low cost. This similarity allows the extrapolation of the results from these experiments to diflunisal binding to HSA and human plasma.

The non-linear Scatchard plots (Figure 1) obtained for the binding of diflunisal to BSA, both in the absence and presence of the three uraemic toxins, revealed that the binding of diflunisal to this model protein involved more than one class of binding site, while data analysis according to the Scatchard

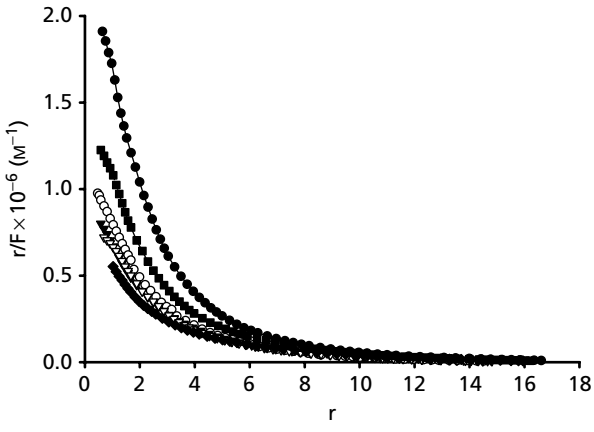

Figure 1 Representative Scatchard plots for the binding of diflunisal with BSA in the absence (●) of toxins and in the presence of HA (○, 300 μmol L^{-1}), IA (▼, 300 μmol L^{-1}), IS (∇, 300 μmol L^{-1}) and the three uraemic toxins at concentrations of 300 μmol L^{-1} (◆) or HA 357 μmol L^{-1}, IA 45 μmol L^{-1}, IS 152 μmol L^{-1} (■) at 25°C, pH 7.4.

Table 1 Estimated binding parameters[a]* for the binding of diflunisal to BSA and HSA in the absence and presence of the three uraemic toxins at 25°C, pH 7.4

Protein[b]	Competing agent	n_1	$K_1 \times 10^{-5}$ (M^{-1})	n_2	$K_2 \times 10^{-4}$ (M^{-1})	R^2
BSA	–	4.18 (0.42)	4.43 (0.34)	12.41 (0.63)	0.55 (0.04)	0.9995
BSA	HA[c]	3.51 (0.23)	3.07 (0.19) ($t_{exp} = 6.163 > t_{th}$, $P \leq 0.001$)	11.68 (0.42)	0.49 (0.04) ($t_{exp} = 3.098 > t_{th}$, $P = 0.021$)	0.9999
BSA	IA[c]	3.69 (0.32)	2.13 (0.11) ($t_{exp} = 10.860 > t_{th}$, $P \leq 0.001$)	11.39 (0.56)	0.44 (0.04) ($t_{exp} = 4.613 > t_{th}$, $P = 0.004$)	0.9999
BSA	IS[c]	3.72 (0.28)	2.09 (0.19) ($t_{exp} = 10.559 > t_{th}$, $P \leq 0.001$)	11.37 (0.35)	0.42 (0.03) ($t_{exp} = 5.148 > t_{th}$, $P = 0.002$)	0.9997
BSA	Three toxins[d]	3.53 (0.23)	1.72 (0.14) ($t_{exp} = 12.664 > t_{th}$, $P \leq 0.001$)	12.64 (0.58)	0.54 (0.05) ($t_{exp} = 0.610 < t_{th}$, $P = 0.564$)	0.9999
BSA	Three toxins[e]	3.74 (0.21)	3.35 (0.28) ($t_{exp} = 4.548 > t_{th}$, $P \leq 0.001$)	12.64 (0.48)	0.54 (0.05) ($t_{exp} = 0.487 < t_{th}$, $P = 0.644$)	0.9999
HSA	–	4.28 (0.34)	5.48 (0.34)	7.75 (0.26)	0.69 (0.06)	0.9995
HSA	Three toxins[d]	4.77 (0.43)	0.97 (0.06) ($t_{exp} = 18.662 > t_{th}$, $P \leq 0.001$)	8.99 (0.62)	0.22 (0.01) ($t_{exp} = 13.981 > t_{th}$, $P \leq 0.001$)	0.9998
HSA	Three toxins[e]	4.08 (0.21)	2.10 (0.18) ($t_{exp} = 13.485 > t_{th}$, $P \leq 0.001$)	7.49 (0.34)	0.48 (0.04) ($t_{exp} = 5.583 > t_{th}$, $P = 0.001$)	0.9997

[a]Standard deviations given in parentheses, calculated from three or more binding experiments, each one consisting of approximately 50 measurement points. [b]Protein concentration 22.5 g L^{-1}. [c]At concentration of 300 μmol L^{-1}. [d]At concentration of 300 μmol L^{-1} each. [e]At concentrations of 357, 45 and 152 μmol L^{-1} for HA, IA and IS, respectively. *Calculated t_{exp} values are given for paired *t*-test performed to examine differences between diflunisal binding constants (K_1, K_2) in the absence and presence of toxins (theoretical t value, $t_{th} = 2.447$ at $P = 0.05$, for 6 degrees of freedom).

model resulted in two classes of binding site in all cases. These results are in agreement with earlier reports on diflunisal binding to plasma proteins in the absence of uraemic toxins (Verbeeck et al 1979; Lin 1989; Sideris et al 1994). The estimated values of the binding parameters are presented in Table 1. In all cases the coefficient of variation of parameter estimation was less than 10%.

There is a significant influence of the uraemic toxins on the binding of diflunisal to BSA, which is reflected in the decreased values of parameter K_1 (Figure 1, Table 1). Statistically significant differences between the estimated K_1 values in the absence and presence of uraemic toxins were found when compared by paired *t*-test ($P \leq 0.001$, Table 1). These results indicate that uraemic toxins compete with diflunisal, mainly for its primary class of binding sites on the BSA molecule. The influence of toxins on drug-binding affinity for the secondary class of binding sites, although statistically significant ($0.002 \leq P \leq 0.021$), does not seem to affect the overall binding of diflunisal to BSA. However, this is an expected result, taking into account the almost two orders of magnitude higher binding affinity of the drug for its primary class of binding sites compared to that for its secondary class of binding sites, as reflected in the estimated K_1 and K_2 values (Table 1).

The results from the binding experiments of diflunisal to BSA in the presence of each toxin separately (Figure 1, Table 1) show that: (i) the greatest decrease in the value of K_1 (about 50%) is observed in the presence of IS and IA (300 μmol L^{-1}), (ii) the influence of HA (300 μmol L^{-1}) on the binding of diflunisal to BSA is less significant, probably due to the low binding affinity of this uraemic toxin for BSA, which is reported to be about 41% bound to plasma proteins (Lindup et al 1986) and (iii) in the presence of all three toxins, at concentrations of 300 μmol L^{-1}, the calculated K_1 value is about 40% of the initial value observed in the absence of toxins. This observation is less profound when the concentrations of the three uraemic toxins, HA, IA and IS, are 357, 45 and 152 μmol L^{-1}, respectively. In this case, the calculated K_1 value is greater than the values found in the presence of each toxin separately at a concentration of 300 μmol L^{-1} (Table 1), but significantly lower than the K_1 value derived in the absence of toxins. This result should probably be attributed to the lower concentrations of IS and IA used in these experiments in order to simulate the in-vivo conditions of the uraemic plasma (Mingrore et al 1997).

Calculation of the binding parameters of HA, IS and IA

The experimental data from the binding experiments of diflunisal to BSA in the presence of each uraemic toxin separately were used to calculate the protein binding parameters of the three uraemic toxins. In each binding experiment the free diflunisal concentration, F, calculated from the calibration curve of the electrode for each point of the binding titration experiment, was used for the determination of the toxin (inhibitor) binding parameters according to the general competitive binding model (Angelakou et al 1994). The model, based on the site-oriented Scatchard (1949) model, i.e. the classification of binding sites and equivalence between sites in each class, for the drug (denoted with subscript 1) and the uraemic toxin (denoted with subscript 2) is described by the following equations:

$$r_1 = \sum_{j=1}^{m} \frac{n_{1j} K_{1j} F_1}{1 + K_{1j} F_1 + K_{2j} F_2} \quad (4)$$

Table 2 Estimated binding parameters[a] for the three uraemic toxins to BSA, using the general competitive model[b]

Uraemic toxin	n_1	$K_1 \times 10^{-4}$ (M^{-1})	n_2	$K_2 \times 10^{-3}$ (M^{-1})	R^2
IS	5.0(0.5)	3.2(0.4)	6.8(0.3)	4.0(0.1)	0.9998
IA	1.2(0.2)	4.3(0.4)	2.9(0.1)	8.7(0.4)	0.9998
HA	9.2(2.0)	0.4(0.1)	23(0.9)	1.5(0.1)	0.9996

[a]Standard deviations given in parentheses, calculated from three binding experiments each one consisting of approximately 50 measurement points.
[b]Temperature 25°C, pH 7.4.

$$r_2 = \sum_{j=1}^{m} \frac{n_{2j}K_{2j}F_2}{1 + K_{1j}F_1 + K_{2j}F_2} \quad (5)$$

where, $r_i = B_i/P_t$ is the number of moles of the i^{th} ligand bound per mole of protein, B_i and F_i are the bound and free molar concentrations of the i^{th} ligand, respectively, P_t is the total protein molar concentration, n_{ij} is the number of binding sites for the j^{th} class, with respect to the i^{th} ligand, and K_{ij} is the binding constant for the association of the i^{th} ligand with the j^{th} class of binding sites (for i = 1, 2). F_2 is the free toxin concentration at each point of the binding experiment. The values of F_2 vary from a minimum (at the commencement of the binding experiment) to a theoretical maximum value, which is equal to the total toxin concentration, T_2 (when the toxin is totally displaced by the diflunisal).

Table 2 shows the results derived from the binding study of diflunisal with BSA in the presence of the three uraemic toxins, using the general competitive model. The binding parameters for each toxin were estimated by non-linear least-squares fitting of equations 4 and 5 to the experimental data using the MINSQ non-linear least squares fitting program (MicroMath Scientific Software 1992). The binding parameters of diflunisal (n_{1j}, K_{1j}) were entered in the model as predetermined constant values (Table 1), while T_2 was known and F_2 was treated as an implicit variable calculated either numerically or algebraically by a build-in root finder of the program for each iteration involving r_1 and F_1. In all cases the correlation coefficients of the fittings were extremely high (Table 2). Two classes of binding sites were determined for each uraemic toxin studied, in agreement with recent literature data, where two classes of binding sites were reported for the binding of IS to HSA (Sakai et al 2001). The binding affinity of the three uraemic toxins to BSA was found to increase in the following order: IA > IS > HA. However, if the total binding affinity ($n \times K$) is taken into account, IS appears to be more extensively bound to BSA than the other two uraemic toxins.

The results derived from the binding experiments of diflunisal with BSA in the absence and presence of the three uraemic toxins, HA, IS and IA, may serve as model studies for the interaction of this drug with plasma proteins, mainly with HSA, which is the major plasma binding protein for acidic drugs such as diflunisal.

Binding of diflunisal to HSA and human plasma in the absence and presence of uraemic toxins

Binding experiments of diflunisal with HSA and human plasma in both the absence and presence of all three uraemic toxins were performed based on the results obtained from the initial experiments with the model protein BSA. In experiments with HSA, the three uraemic toxins were used at concentrations of either 300 μmol L^{-1} or 357, 45 and 152 μmol L^{-1} for HA, IA and IS, respectively, while in experiments with human plasma, uraemic toxins were used only at concentrations of 357 μmol L^{-1} (HA), 45 μmol L^{-1} (IA) and 152 μmol L^{-1} (IS) in order to simulate the in-vivo conditions of uraemic plasma. The Scatchard plots obtained are shown in Figures 2 and 3 (for HSA and human plasma, respectively). Data analysis again revealed two classes of binding sites in all cases and the calculated binding parameters, derived from these experiments, are shown in Tables 1 and 3. In all cases the coefficient of variation of parameter estimation was less than 10%.

In the presence of the three uraemic toxins, the binding affinity of diflunisal to the HSA molecule and human plasma is decreased, as reflected in the decreased value of K_1 calculated in both cases (Tables 1 and 3). The differences between the estimated K_1 values in the absence and presence of uraemic toxins were examined by paired *t*-test and found to be statistically significant ($P \leq 0.001$, Tables 1 and 3). The estimated

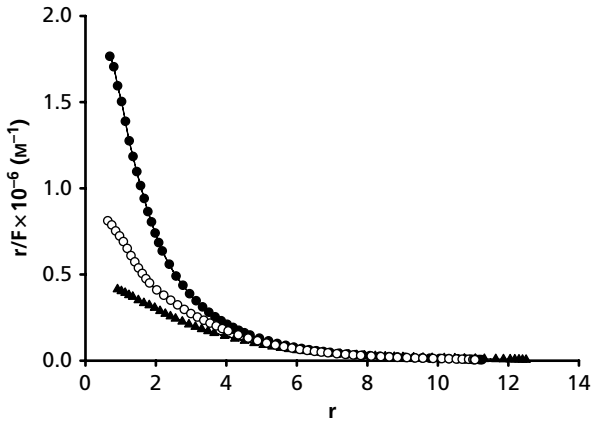

Figure 2 Representative Scatchard plots for the binding of diflunisal with HSA in the absence (●) of toxins and in the presence of the three uraemic toxins at concentrations of 300 μmol L^{-1} (▲) or HA 357 μmol L^{-1}, IA 45 μmol L^{-1}, IS 152 μmol L^{-1} (○) at 25°C, pH 7.4.

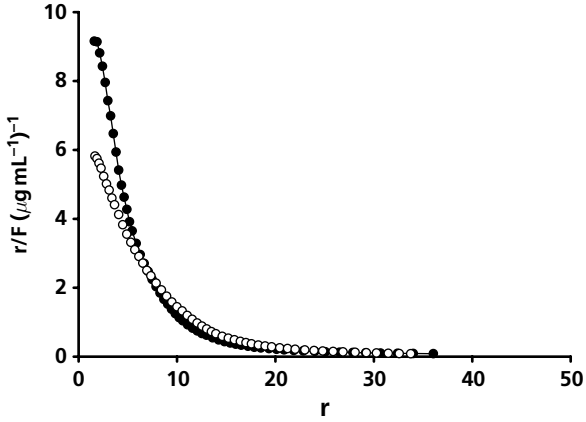

Figure 3 Representative Scatchard plots for the binding of diflunisal to human plasma diluted 1:1 (v/v) with phosphate buffer 0.10 M in the absence (●) and presence (○) of the three uraemic toxins at concentrations of HA 357 μmol L^{-1}, IA 45 μmol L^{-1}, IS 152 μmol L^{-1}, at 25°C, pH 7.4.

Table 3 Estimated binding parameters[a]* for the binding of diflunisal to human plasma[b] at 25°C in the absence and presence of the three uraemic toxins[c]

	Plasma	Plasma + three toxins
$n_1 \times 10^5$ (mol g^{-1})	3.60 (0.27)	4.30 (0.38)
$K_1 \times 10^{-5}$ (M^{-1})	2.65 (0.14)	1.41 (0.12)
		($t_{exp} = 11.024 > t_{th}$, $P \leq 0.001$)
$n_2 \times 10^5$ (mol g^{-1})	12.30 (0.82)	11.10 (0.38)
$K_2 \times 10^{-4}$ (M^{-1})	0.164 (0.03)	0.184 (0.03)
		($t_{exp} = -0.751 < t_{th}$, $P = 0.481$)
R^2	0.9994	0.9996

[a]Standard deviations given in parentheses, calculated from three binding experiments each one consisting of approximately 50 measurement points. [b]Plasma diluted 1:1 with phosphate buffer, 0.1 M, pH 7.4 (3.6 g dL^{-1}). [c]At concentrations of 357, 45 and 152 μmol L^{-1} for HA, IA and IS, respectively. *Calculated t_{exp} values are given for paired *t*-test performed to examine differences between diflunisal binding constants (K_1, K_2) in the absence and presence of toxins (theoretical t value, $t_{th} = 2.447$ at $P = 0.05$, for 6 degrees of freedom).

K_1 values for the binding of diflunsal to HSA and human plasma in the presence of uraemic toxins, at concentrations of 357, 45 and 152 μmol L^{-1}, for HA, IA and IS, respectively, is almost half of those in the absence of toxins (Tables 1 and 3). These results indicate that uraemic toxins greatly affect the binding of diflunisal, mainly to its primary class of binding sites on the HSA molecule. Regarding the influence of uraemic toxins on the binding affinity of the secondary class of the drug, the differences between the estimated values of K_2, examined by paired *t*-test, in the absence and presence of uraemic toxins, were also found to be statistically significant ($P \leq 0.001$). However, because of the low contribution of the secondary class of binding sites to the overall binding of diflunisal on HSA molecule, this influence can be considered to be of minor importance.

Comparing the decrease of K_1 value in the binding experiments with BSA to that with HSA, in the absence and presence of the three uraemic toxins at concentrations of 357, 45 and 152 μmol L^{-1} for HA, IA and IS, respectively (Table 1), the effect is more profound with HSA. This finding supports the idea that the three toxins (mainly the indole derivatives) have greater affinity to HSA than BSA. Although the general conformation of the binding sites on the molecules of BSA and HSA is similar, there is a partial differentiation on the aspect of selectivity influencing the binding affinity.

Discussion

Binding of the NSAID diflunisal to BSA, HSA and human plasma, in the absence and presence of the main uraemic toxins, HA, IS and IA, was studied. Since similar published data are lacking, the aim of the present study was to evaluate the effect of these endogenous substances, which accumulate in uraemic plasma, on diflunisal protein binding under standardized conditions (25°C, pH 7.4) over a wide range of total drug concentration (50–1500 μg mL^{-1}).

The binding affinity of diflunisal to BSA, HSA and human plasma was found to increase in the following order: HSA > BSA > plasma (Tables 1 and 3). It is noteworthy that the value of the binding constant for the primary class of binding sites, K_1, of diflunisal to human plasma is half of that for its binding to pure HSA (Tables 1 and 3). This finding confirms the belief that HSA is the main binding protein for diflunisal in human plasma. However, some differences may occur between the binding affinity of this drug to pure HSA and normal human plasma because of the presence of various endogenous substances in plasma, such as bilirubin and fatty acids, which may inhibit the binding of diflunisal to HSA (Mingrore et al 1997; Sakai et al 2001).

In the presence of the three uraemic toxins, the binding affinity of diflunisal to its primary class of binding sites on BSA, HSA molecule and human plasma is decreased, as reflected in the calculated K_1 values (Tables 1 and 3). Surprisingly, the reduction in the bound amount of diflunisal, caused by the presence of the three uraemic toxins, was only 1–4%, compared to that in the absence of toxins, while the respective increase in its free amount was 0.2–0.54% for a total diflunisal concentration ranging between 50 and 500 μg mL^{-1}. It should be noticed, however, that for drugs exhibiting extremely high protein binding, such as diflunisal, apparently minor changes in the bound fractions may induce important disturbances in their free (unbound) active fractions. Thus, for diflunisal, this small increase in its free fraction corresponds to an approximately 70–200% rise in the available active (free) drug concentration and this may have important therapeutic consequences.

It is also noteworthy that although diflunisal is primarily bound to site I of warfarin-azapropazone on the HSA molecule (Fehske et al 1982) and HA, IS and IA were recently found to bind with high affinity to site II of indole-benzodiazepine on the HSA molecule (Sakai et al 1995), there are

strong mutual interactions between these substances when simultaneous binding on albumin occurs. Site I and site II are located on subdomains IIA and IIIA of HSA, respectively (Fehske et al 1981), while recently site I was found to comprise at least three subsites, namely, Ia, Ib and Ic, corresponding to the high-affinity binding sites of warfarin, dansyl-L-asparagine (DNSA) and n-butyl-p-animobenzoate, respectively (Yamasaki et al 1996). Interactions between drugs and endogenous or exogenous substances that bind simultaneously on the HSA molecule are usually characterized either as competitive, when binding occurs on the same binding sites, or independent, when binding takes place on different sites on the HSA molecule (Brodersen et al 1977; Verbeeck et al 1979; Kragh-Hansen, 1981, 1988; Sakai et al 2001). However, the crystal structure of HSA shows that subdomains IIA and IIIA share a common interface (Min & Carter 1992) and, probably, indicates that ligands bound to subdomain IIIA affect conformational changes, as well as binding affinities, in subdomain IIA through heterotropic allosteric interactions at this interface. In accordance with this suggestion, Yamasaki et al (1999) found a competitive-like antagonism between the binding of DNSA, which is a site I probe, and ibuprofen and diazepam, which bind with high affinity to site II. In addition, Sakai et al (2001) observed mutual displacement between IS, which binds to site II, and the site I probe DNSA. In line with these observations, our results indicate that the presence of uraemic toxins (IS, IA and HA) in the plasma of patients with renal failure may increase the unbound diflunisal fraction because of the binding interactions taking place at the interface between site I and site II of the HSA molecule.

Conclusions

The main purpose of this study was to investigate, in vitro, the effect of three well-known uraemic toxins, HA, IS and IA, on the binding of diflunisal to BSA, HSA and human plasma. The potentiometric method used, in conjunction with the general competitive binding model, enabled the determination of binding parameters (n_i, K_i) not only for the interaction of diflunisal with plasma proteins in the absence and presence of the three uraemic toxins, but also for the interaction of each uraemic toxin with BSA. The results of the present study are strong evidence that uraemic toxins in plasma of patients with renal failure displace diflunisal from its specific binding sites on the albumin molecule, probably through interactions taking place at the interface between site I and site II of the HSA molecule. Hence, it may be reasonable that the decreased protein binding of diflunisal occurring in uraemic plasma may not be solely attributed to the lower albumin concentration observed in many patients with renal failure. Inhibition of diflunisal protein binding in the presence of uraemic toxins that accumulate in uraemic plasma, and the resulting increase of its free plasma concentration, should also be considered since defective drug protein binding is observed in the plasma of uraemic patients even in the presence of normal albumin concentration.

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