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DICLOFENAC BINDING TO ALBUMIN AND LIPOPROTEINS IN HUMAN SERUM

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Abstract—The binding of diclofenac to human serum albumin (HSA) and to lipoproteins was studied *in vitro* by equilibrium dialysis. Binding to HSA is characterized by two classes of sites with one site each ($K_1 = 5 \times 10^5\ M^{-1}$ and $K_2 = 0.6 \times 10^5\ M^{-1}$). The binding to lipoproteins was shown to be saturable with a larger number of binding sites and low association constants. The evidence of two specific binding sites on HSA was confirmed by circular dichroism data. In addition, an identification of those sites was performed by displacement of fluorescent probes. The data show that the high affinity site ($K_1 = 5 \times 10^5\ M^{-1}$) is likely to be shared by benzodiazepines while the second one ($K_2 = 0.6 \times 10^5\ M^{-1}$) is common with the warfarin site.

Diclofenac (Fig. 1) is a non-steroidal antiinflammatory drug (NSAID). As most NSAID diclofenac is a weak acid ($pK_a = 4$) which is highly bound to plasma proteins [1]. Most of NSAID have been shown to be mainly bound to one of the two selective binding sites on human serum albumin [2]. There is now good evidence for the existence of binding sites on albumin with structural selectivity for a large variety of drugs, organic ligands and some endogenous substances [3]. The two distinct binding sites for anionic drugs have specific fluorescent markers, warfarin for site I and dansylsarcosine for site II [4].

Since diclofenac has an original chemical structure (see Fig. 1), we thought it would be interesting to characterize the plasma protein binding of this drug. The present study examines the interactions of diclofenac with albumin and lipoproteins. We also attempted to characterize and to identify the human serum albumin binding sites of diclofenac by using circular dichroism and fluorescence measurements.

MATERIALS AND METHODS

Proteins and serum. Human serum albumin (HSA, Sigma A 1887) was dissolved at a 10 μM concentration in phosphate buffer (pH 7.40, 0.066 M, $\mu = 0.176$). The HSA solution contained 0.04 mole of free fatty acid (FFA), expressed as palmitic acid, per mole of HSA. Plasma lipoproteins were isolated by sequential ultracentrifugal flotation of plasma at increasing density as previously described [5]. The isolated lipoproteins were dialysed before use against phosphate buffer containing M/15 NaCl. Lipoprotein concentration was calculated by summing chemical composition data obtained by standard methods: protein [6], cholesterol [7], phospholipid [8] and triacylglycerol [9]. Lipoproteins were adjusted to 2 g/l in the equilibrium dialysis experiments.

The serum binding of diclofenac was determined in a serum pool from healthy subjects. It had the following characteristics: total proteins 70 g/l, HSA 600 μM, alpha-1-acid glycoprotein (AAG) 14 μM and FFA 420 μM, expressed as palmitic acid concentration. FFA levels were measured by a gas chromatographic method that was previously described [10].

Drugs. ^{14}C-diclofenac (6.14 mCi/mmole, Ciba Geigy) was 98.5% pure, checked by thin layer chromatography: toluene–formic acid–heptane (80/12/8, v/v).

Unlabelled diclofenac was dissolved in ethanol and phosphate buffer. The percentage of ethanol in the final solution never exceeded 1%. We studied serum interactions between diclofenac and other drugs. These were used at therapeutic concentration levels: warfarin (6.5 μM), acenocoumarol (5.6 μM) and salicylic acid (1.4 mM). Serum diclofenac concentration was fixed to 6 μM.

Equilibrium dialysis. Diclofenac protein binding was studied by equilibrium dialysis as previously described in detail [10]. The experiments were carried out at 37°, pH 7.4, for 3.5 hr under a constant stirring at 20 rev/min (Dianorm apparatus). A preliminary experiment showed that equilibrium was attained at that time. There was no significant binding of diclofenac to the dialysis membrane (Visking). At the end of each experiment, concentrations in both compartments were measured by liquid scintillation counting (Ready-Solv^{MP}, Beckman).

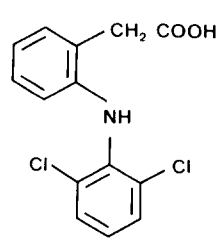

Fig. 1. Diclofenac chemical structure.

Fluorescent probe studies. Fluorescent measurements were made at room temperature in an Aminco SPF 500 spectrofluorometer. Diclofenac and fluorescent probes were added into the cell as microlitre volume of concentrated stock solution. Dansylsarcosine was obtained from Sigma Chemical Company and warfarin from Merrel. The wavelengths of the exciting light were 315 and 350 nm and fluorescence was measured at 375 and 475 nm for warfarin and dansylsarcosine respectively.

Circular dichroism (CD) measurements. The spectra were recorded at room temperature using a Jobin-Yvon Mark III dichrograph equipped with a Nicolet 1171 signal averager. Rectangular cell with a path length of 1 cm was used. The absorbance was kept below 1.4. CD is expressed in terms of the molar dichroic absorbance, $\Delta\varepsilon$, based on the HSA concentration. The spectropolarimetric titration was performed at 290 nm. The ligand was added as microlitre volumes of concentrated stock solution in the cell containing the HSA solution.

Estimation of binding parameters. All binding parameters were estimated by fitting the experimental data to a theoretical relationship for drug–protein binding derived from the mass-action law:

$$\frac{B}{R} = \sum \frac{n_i K_i F}{1 + K_i F} \qquad (1)$$

where B and F are the bound- and free-drug concentrations respectively, n and K the number of binding sites and the association constant, and R the total protein concentration. The data were analysed by an iterative non-linear regression program using a Gauss–Newton algorithm on a Tektronix 4051 [11]. All errors are expressed as standard deviations.

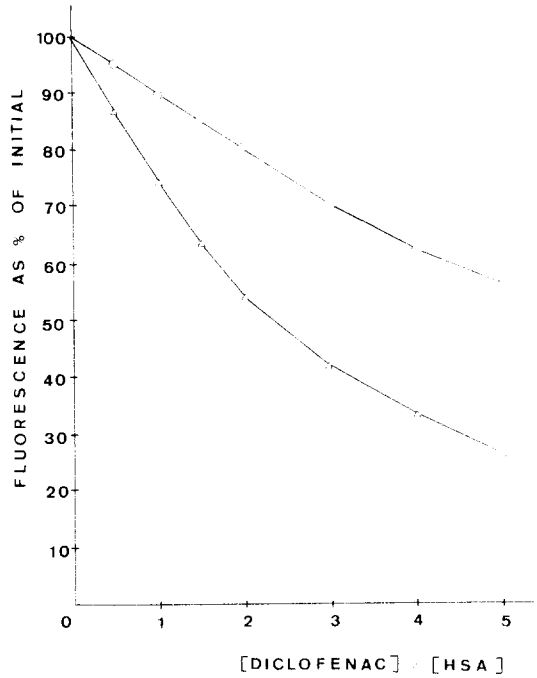

Fig. 3. Effects of diclofenac on the fluorescence of warfarin (○) and dansylsarcosine (△). HSA was used at a 10 μM concentration. Molar concentration ratios of fluorescent probes to HSA were 1/1. Fluorescence is expressed as a percentage of that before addition of diclofenac. The binding constants of the fluorescent probes as calculated by Sudlow *et al.* [3] are $n_1 = 0.9$, $K = 0.25 \times 10^6\,M^{-1}$ for warfarin and $n_1 = 1$, $K_1 = 0.167 \times 10^6\,M^{-1}$ for dansylsarcosine.

RESULTS

Characteristics of the binding to HSA

Two successive saturable processes occurred when diclofenac was bound to HSA as shown by the Scatchard plot inserted in Fig. 2. The data were fitted to equation (1) which yielded the following estimates: $n_1 = 0.54 \pm 0.20$, $K_1 = 1.15 \pm 0.52 \times 10^6 M^{-1}$ ($n_1K_1 = 6.20 \pm 5.0 \times 10^5 M^{-1}$) and $n_2 = 1.63 \pm 0.13$, $K_2 = 0.072 \pm 0.034 \times 10^6 M^{-1}$ ($n_2K_2 = 1.17 \pm 0.64 \times 10^5 M^{-1}$). The model was modified by fixing n_1 and n_2 as 1 in order to allow more precise comparisons with published data. The computed total binding constants (n_1K_1 and n_2K_2) were not significantly different from the previous model. The

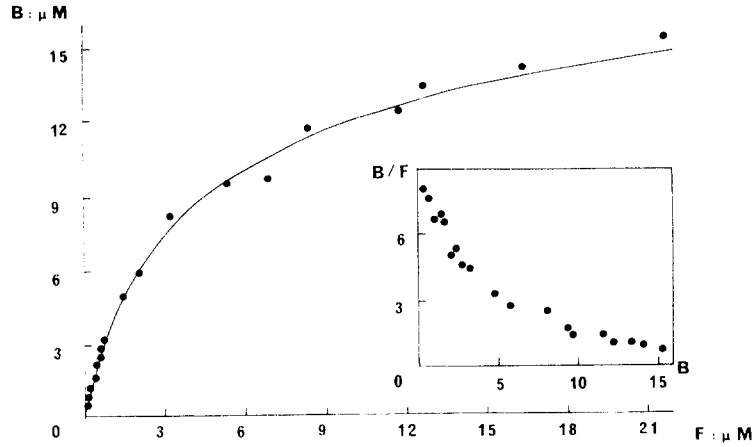

Fig. 2. Binding of diclofenac to HSA. B and F denote the bound and free concentrations of diclofenac. Each point is the mean of three determinations. Inset: Scatchard plot showing two successive binding processes; correlation: 0.998. The HSA concentration was 10 μM in phosphate buffer at pH 7.4. The equilibrium dialysis was performed at 37° during 3.5 hr.

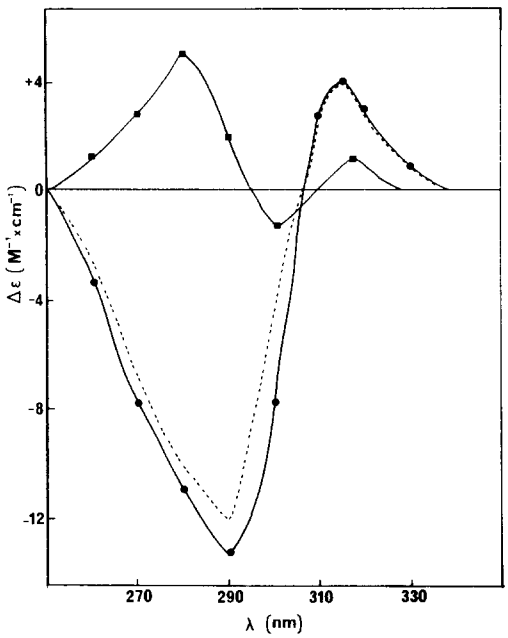

Fig. 4. Different CD spectra of diclofenac-HSA, at ligand to HSA molar ratios of 1/1 (●), 2/1 (---) and 4/1 (■), after subtraction of the HSA spectrum. Experimental conditions: HSA 15 μM in phosphate buffer, pH 7.4, cell path-length 1 cm, scale setting 5×10^{-6} mm^{-1}, room temperature.

relevant association constants for each binding site were $K_1 = 5.0 \pm 0.4 \times 10^5\,M^{-1}$ and $K_2 = 0.60 \pm 0.05 \times 10^5\,M^{-1}$.

Fluorescence studies

The binding sites of diclofenac were identified by fluorescence measurements. Figure 3 shows the effects of diclofenac on the fluorescence of warfarin (marker for the azapropazone/warfarin binding site) and dansylsarcosine (marker for the benzodiazepine binding site). The drug caused a net decrease in the HSA-bound dansylsarcosine fluorescence and a weaker effect in the HSA-bound warfarin fluorescence.

Circular dichroism measurements

The different CD spectra of the complexes between diclofenac at two different concentrations and HSA are shown in Fig. 4. In both cases, large amplitude CD bands were generated on diclofenac binding to HSA. At the lowest drug to HSA molar ratio (1/1), when diclofenac was mainly bound to its primary site, a strong negative band, centred at 290 nm and a weaker positive band at 315 nm were induced. At higher diclofenac to HSA molar ratio (2/1), the binding of diclofenac to its secondary site involved a net decrease in the amplitude of the band at 290 nm and only a slight reduction in the size of the band at 315 nm. When the diclofenac concentration was increased to a four-fold molar excess (saturation of the secondary binding site), positive bands at about 280 and 315 nm and a small negative band at 300 nm were generated, resulting from the larger contribution of diclofenac interaction with its secondary binding site. As seen in Fig. 5, the induced Cotton effect at 290 nm depends on the diclofenac concentration. As expected, negative Cotton effects were initially obtained but when the first site was saturated, the positive Cotton effect from the secondary site will neutralize the negative one and will dominate completely.

To locate the diclofenac binding sites by circular dichroism, two drugs, azapropazone and flurbiprofen were used as probes. Evidently, azapropazone did not affect significantly the CD spectrum obtained from the binding of diclofenac to HSA as equimolar amount relative to HSA, whereas flurbiprofen

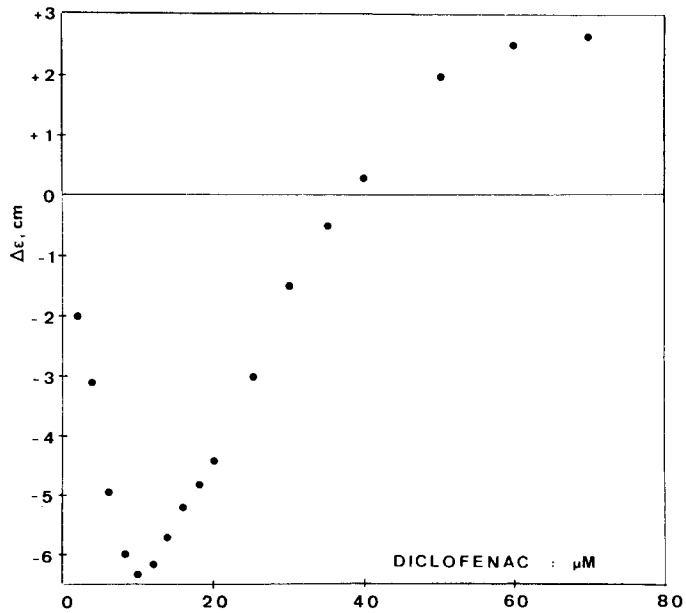

Fig. 5. Spectropolarimetric titration of HSA with diclofenac at 290 nm. HSA concentration: 10 μM in phosphate buffer pH 7.4, cell path length: 1 cm, scale setting: 2×10^6 mm^{-1}.

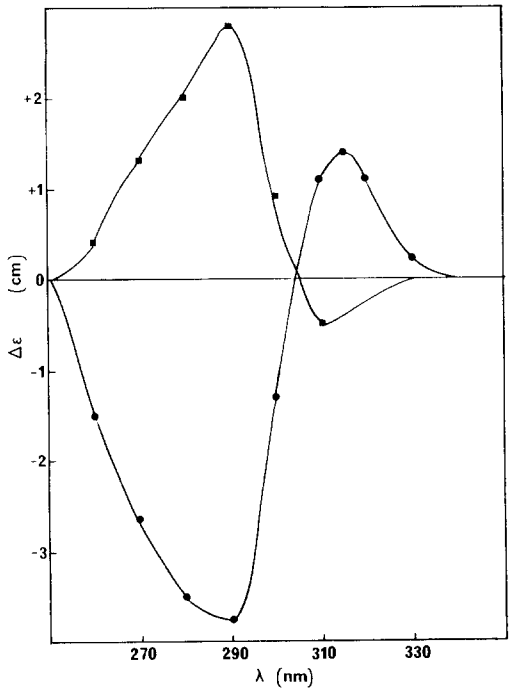

Fig. 6. The CD difference spectra obtained with diclofenac-HSA in the presence of the following binding sites probes: flurbiprofen (■) or azapropazone (●) after subtraction of the corresponding probe-HSA spectrum. The molar ratios of diclofenac-probe-HSA were 1/1/1. The experimental conditions were the same as in Fig. 4.

involved a dramatic change in both signs and amplitudes of the CD bands (Fig. 6). Comparing Fig. 6 to Fig. 4, it is obvious that the strong negative band induced by diclofenac at equimolar amount is blocked by flurbiprofen and the strong positive band at 290 nm in Fig. 6 originates from the interaction of diclofenac with its second site. Consequently, one may assume that diclofenac shares its primary binding site with flurbiprofen. Correspondingly, Fig. 6 shows that azapropazone does not affect the primary binding site of diclofenac, since only the CD bands originating from the primary site are induced at equimolar concentration of diclofenac.

Characteristics of the binding to lipoproteins

Diclofenac was dialysed over a wide range of concentrations (3–1500 μM). The binding of diclofenac to these lipoproteins followed a saturable process (Fig. 7). The binding parameters are presented in Table 1. The effect of pH on diclofenac binding to lipoproteins is summarized in Table 2. As the pH increased from 6.0 to 7.4, a decrease in the bound fraction of diclofenac was observed, corresponding to a decrease in the unionized fraction of diclofenac.

Serum binding

In the therapeutic concentration range (from 1.5 to 6 μM), diclofenac was highly bound to serum (containing 600 μM HSA), with a per cent of binding of 99.5% which remained constant over this range. Similarly, diclofenac was extensively bound to 600 μM HSA, with a per cent of binding more than 99%. There was no significant binding to AAG and globulins: the percentage bound to these proteins did not exceed 10%.

Influence of diclofenac on the binding of acidic drugs

The results are summarized in Table 3. Obviously diclofenac did not modify the *in vitro* serum binding of these drugs.

DISCUSSION

Diclofenac is characterized by a high association constant to HSA, as most anionic drugs. By contrast, the binding of diclofenac to lipoproteins is an uncommon feature of acidic ligands. Diclofenac binding to HSA follows two saturable processes. The numerical calculation of the binding constants yielded a satisfactory fit with a two single site program. The binding

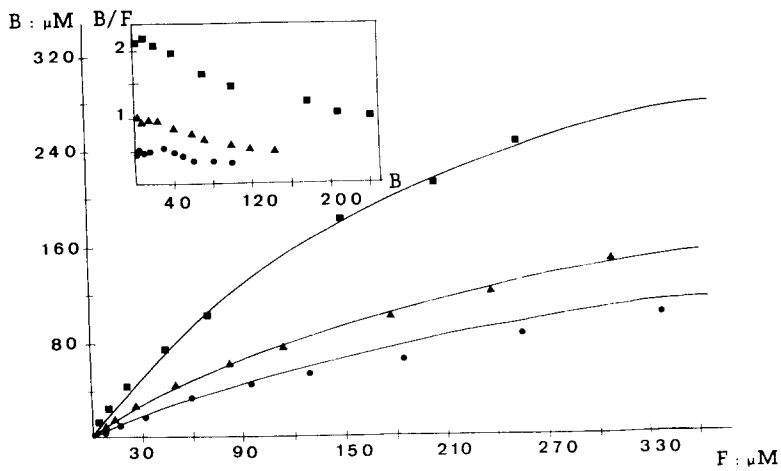

Fig. 7. Binding of diclofenac to VLDL (▲), LDL (●) and HDL (■). *B* and *F* denote the bound and free concentrations of diclofenac. Each point is the mean of three determinations. The lipoproteins concentrations were 2 g/l in phosphate buffer, pH 7.4. The equilibrium dialysis was performed at 37° for 3.5 hr.

Table 1. Binding parameters for diclofenac–lipoproteins interactions

	Percentages of binding	n	K (M^{-1})	n/(lipoprotein total-lipid volume) (ml^{-1})
HDL	60–48	42 ± 2	3970 ± 330	500 ± 24
LDL	31–22	390 ± 30	2270 ± 360	154 ± 17
VLDL	48–31	2840 ± 180	3020 ± 310	149 ± 9

The following molecular weights were used for calculations, VLDL: 19.6×10^6, LDL: 2.3×10^6, HDL: 0.3×10^6. The lipoproteins total-lipid volume was obtained by subtracting the volume occupied by protein (molecular weight × per cent protein × partial specific volume of protein) from the total lipoprotein volume. Values for lipoprotein characteristics were obtained from Ref. 12. Partial specific volume of apolipoprotein was 0.738 ml/g [13]. Errors are expressed as standard deviations.

constant for the primary binding site is high ($0.5 \times 10^6\,M^{-1}$), in the range of values reported for other NSAID while that for the second site is ten times lower. Wagner and Sulc [14] also found two classes of binding sites: one with a high affinity and low capacity ($K_1 = 1.03 \times 10^5\,M^{-1}$, $n_1 = 3.6$) and the other with a low affinity and high capacity ($K_2 = 3.05 \times 10^3\,M^{-1}$, $n_2 = 12$). The reasons for the discrepancies relevant to the parameters are twofold. First, these authors have used normal HSA while our investigations were performed with HSA free of fatty acids. Second, the two sets of binding parameters were calculated with different HSA concentrations. Many workers have found that drug protein binding parameters paradoxically depend upon the HSA concentrations used [15, 16]. The CD spectra of the complexes between HSA and diclofenac at molar ratios of 1/1 and 4/1 were quite distinct and the spectropolarimetric titration showed that the induced Cotton effect at 290 nm change sign when the diclofenac to HSA molar ratio increased over 1/1. Similar findings were reported by Rosen [17] and by Ekman *et al.* [18] for the binding of oxyphenbutazone and of indomethacin to HSA. It was concluded that the drugs successively bound to two specific binding sites, the change in the asymetric conformation around the ligand chromophore leading to different Cotton effects.

Our fluorescent measurements could indicate that diclofenac binds with a high affinity to site II (benzodiazepine site) and with a weaker affinity to site I (warfarin site). Diclofenac gave rise to a strong negative Cotton effect around 290 nm when bound to its primary site on HSA, while binding to the secondary site induced a positive Cotton effect in this wavelength region. Azapropazone and flurbiprofen are bound to just their primary site, the HSA site I and site II respectively, when present in equimolar amount to HSA, and their association constants are high, in the range of $10^6\,M^{-1}$ [2]. In this study, we have shown that flurbiprofen blocks the formation of the specific Cotton effects from the interaction of diclofenac with its primary binding site on HSA. This conclusion agrees with the structural features defined by Sudlow *et al.* [4] which allow ligand binding to one of these specific sites:

drugs which bind to the HSA site II (benzodiazepine site) are aromatic carboxylic acids, largely ionized at physiological pH. The configuration of these molecules is generally extended and the negative charge is specifically located at one end of the molecule, away from the non polar region, and,

drugs which bind to site I (azapropazone/warfarin binding site) are more bulky heterocyclic molecules with the negative charge more centered in the molecule.

Since the basic paper of Vallner [19], only cationic drugs were thought to be capable of binding to lipoproteins. In our experiments, we found a significant binding of diclofenac to the three classes of lipoproteins that follows a saturable process. For the lipoproteins, there is a decrease in the binding of diclofenac as pH increases, i.e. as the fraction of unionized diclofenac, free in buffer water, decreases. We suggest that the decrease of the binding is related to the displacement of the ionization equilibrium favouring the water soluble form (ionized) of diclofenac. Consequently, diclofenac is strongly expected to interact with these plasma proteins mainly by hydrophobic bonds. For VLDL and LDL, the number of binding sites are related to the lipoprotein total lipid volume, despite the wide differences in lipid composition of these two lipoproteins. Thus, the interaction of diclofenac with VLDL and LDL resembles a liposolubilization of the drug into the hydrophobic core of these lipoproteins. For HDL, the ratio of the number of binding sites to the lipoprotein total lipid volume is higher than for the two other lipoproteins (see Table 1). This enhanced binding may reflect additional diclofenac interaction with the HDL apolipoproteins or surface monolayer of phospholipids [20]. Another experimental work is warranted to confirm this hypothesis. The large number of binding sites observed on these lipoproteins should be viewed as non-specific sites. This

Table 2. Effects of pH on diclofenac binding to lipoproteins.

pH	Per cent unionized	Diclofenac bound fraction to: VLDL	LDL	HDL
6.0	1.00	0.50	0.45	0.74
6.8	0.16	0.43	0.30	0.68
7.4	0.04	0.39	0.27	0.65

Lipoprotein concentrations were each 2 g/l in phosphate buffer, pH 7.4. Diclofenac concentration was 30 μM.

Table 3. Effects of diclofenac on the serum binding of warfarin, acenocoumarol and salicylic acid: (mean ± standard deviation, $n = 5$).

Drug	Binding percentage to serum	Binding percentage to serum loaded with unlabelled diclofenac (6 μM/l)
^{14}C-Warfarin	97.18 ± 0.08	97.18 ± 0.04
^{14}C-Acenocoumarol	98.64 ± 0.23	98.70 ± 0.10
^{14}C-Salicylic acid	86.64 ± 0.71	86.12 ± 0.40

Therapeutic levels of drugs are used in all cases, diclofenac: 6 μM, warfarin: 6.5 μM, acenocoumarol: 5.6 μM and salicyclic acid: 1.4 μM. Diclofenac did not modify the binding of these drugs. The Mann and Whitney test did not show any significant difference between the three series of experiments, i.e. with and without diclofenac.

"binding" reflect a partitioning phenomenon of the drug between the aqueous phase of the medium and the lipid core of lipoproteins [20].

With a simulation program described elsewhere [21], we estimated the distribution of diclofenac on each different serum proteins. The binding per cent values in the therapeutic range of concentration are shown in Table 4. These estimations prove that in the total serum, HSA is the main binding protein. The distribution of a drug between the different serum proteins depends on the respective nK products and on the respective concentrations of each protein in the total serum.

Regarding the clinical standpoint, diclofenac, as many other acidic drugs, is extensively bound to serum proteins. In the therapeutic range of concentration, the binding percentage remain constant, because therapeutic concentrations are largely infrasaturating concentrations with respect to HSA. We studied interaction between diclofenac and warfarin, acenocoumarol and salicylic acid at therapeutic concentrations. These three drugs can commonly be associated with diclofenac in routine clinical practice. The negative results of these *in vitro* experiments are easily explained by the infrasaturating concentrations that were used for the four drugs.

Table 4. Distribution of diclofenac among the different serum binding proteins

Proteins concentrations in serum	Estimated percent of binding
HSA: 600 μM	
First binding site	93.9
Second binding site	4.1
HDL: 23 μM	1.1
LDL: 1.5 μM	0.3
VLDL: 0.06 μM	0.15
(Free fraction)	(0.45)

Using the estimated binding parameters for each protein, the distribution of the bound diclofenac in plasma was calculated as previously described in detail in Ref. 21.

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REFERENCES

1. W. Riess, H. Stierlin, P. Gegen, J. W. Faigle, A. Gérardin, J. Moppert, A. Sallmann, K. Schmid, A. Schweizer, M. Sulc, W. Theobald and J. Wagner, *Scand. J. Rheumatol. Suppl.* **22**, 17 (1978).
2. A. Kober and I. Sjöholm, *Molec. Pharmac.* **18**, 421 (1980).
3. K. J. Fehske, W. E. Müller and U. Wollert, *Biochem. Pharmac.* **30**, 687 (1981).
4. G. Sudlow, D. J. Birkett and D. N. Wade, *Molec. Pharmac.* **11**, 824 (1975).
5. S. Glasson, R. Zini and J. P. Tillement, *Biochem. Pharmac.* **31**, 831 (1982).
6. O. H. Lowry, N. J. Rosebrough, N. T. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
7. G. R. Bartlett, *J. biol. Chem.* **234**, 466 (1959).
8. C. C. Allain, L. S. Poon, C. S. G. Chan, W. Richmond and P. C. Fu, *Clin Chem.* **20**, 470 (1974).
9. G. Bucolo and H. David, *Clin. Chem.* **19**, 476 (1973).
10. R. Zini, J. Barré, P. D'Athis and J. P. Tillement, *Biochem. Pharmac.* **32**, 2909 (1983).
11. J. P. Tillement, R. Zini, P. D'Athis and G. Vassent, *Eur. J. clin. Pharmac.* **7**, 307 (1974).
12. B. W. Shen, A. M. Scanu and F. J. Kézdy, *Proc. natn. Acad. Sci. U.S.A.* **7**, 837 (1977).
13. J. Gwynne, H. B. Brewer and H. Edelhock, *J. biol. Chem.* **250**, 2269 (1975).
14. J. Wagner and M. Sulc, *Akt. Rheumatol.* **4**, 153 (1979).
15. C. J. Bowmer and W. E. Lindup, *Biochem. Pharmac.* **27**, 937 (1978).
16. D. Shen and M. Gibaldi, *J. Pharm. Sci.* **63**, 1698 (1974).
17. A. Rosen, *Biochem. Pharmac.* **19**, 2075 (1970).
18. B. Ekman, T. Sjödin and I. Sjöholm, *Biochem. Pharmac.* **29**, 1759 (1980).
19. J. J. Vallner and L. Chen, *J. Pharm. Sci.* **66**, 420 (1977).
20. H. P. Shu and A. V. Nichols, *Biochim. biophys. Acta* **665**, 376 (1981).
21. E. Albengres, S. Urien, J. F. Pognat and J. P. Tillement, *Pharmacology* **28**, 139 (1984).