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ELIMINATION OF PEAK DEFORMATION IN THE LIQUID CHROMATOGRAPHIC SEPARATION OF A STRONGLY PROTEIN-BOUND DRUG FROM DIRECTLY INJECTED BLOOD PLASMA SAMPLES

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SUMMARY

Direct injection of blood plasma samples into reversed-phase columns resulted in skewed chromatographic peaks for the drug naproxen. The skew is shown to be due to strong binding of naproxen to albumin present in the blood plasma. Methods to eliminate the peak skew have been investigated. They include changes of the composition of the eluent and of the sample solution in order to decrease the degree of binding of the drug to albumin. The methods studied were dilution, addition of displacers, change of pH and change of methanol concentration. Calculations based on known binding constants indicate that the degree of peak skew was directly influenced by the degree of protein binding of the drug in the sample solution.

INTRODUCTION

Reversed-phase liquid chromatography is potentially useful for direct on-line determinations of drugs and metabolites in biological samples. Its application to untreated blood plasma requires that the influence of the plasma matrix on the procedure be understood and controlled. Matrix components can influence both the chromatographic resolution of the drug and the physical condition of the column. When only a small sample volume is needed, the determination can be performed in an isocratic system[1,2], but a pre-column venting procedure may be advantageous in order to improve the separation from early eluted matrix components[1].

Pre-columns can be used not only as guard columns for the separation column[2] but also for the purpose of purification of the sample by using a step gradient[3,4] or, in the case of large sample volumes, for a trace enrichment procedure[4–8].

In a previous study[2] we have examined the chromatographic conditions that will enable the determination of drugs by direct injection of untreated blood plasma samples into liquid chromatographic columns. In the present paper we have included higher concentrations of an organic solvent in the eluent in order to be able to regulate the retention within wider limits. However, this chromatographic system gave a deformation of the chromatographic peak of the drug naproxen. As this drug is

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known to be bound to plasma albumin[9,10], we suspected that the peak deformation was due to the presence of a drug–albumin complex in the injected sample. Several authors[3,4,6] have suspected that such complexes could lead to errors in the determination of the total concentration of a drug in plasma, but as the complex results from a chemical equilibrium, the position of the latter can be shifted to yield a decreased degree of complexation. We have, therefore, studied methods for improving the chromatographic performance by dissociating the drug–albumin complex. The experimental factors studied were dilution, pH, methanol concentration and addition of displacers. The principles given might be used in other cases where an injected sample contains a complex of the substance to be chromatographed.

EXPERIMENTAL

Chemicals

Naproxen, (+)-2-(6-methoxy-2-naphthyl)propionic acid, and flurbiprofen, 2-fluoro-α-methyl-1,1′-biphenyl-4-acetic acid, was of pharmacopoeial grade and albumin was human serum albumin, Fraction V, obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade.

LiChrosorb RP-2 and RP-8 were obtained from E. Merck (Darmstadt, F.R.G.) and had particle diameters of 6 μm (RP-2) and 5 μm (RP-8). Partisil 5-C_8 was obtained from Whatman (Clifton, NJ, U.S.A.). It had a particle diameter of 5 μm.

Equipment

The pump was an LDC solvent delivery system 711-47 (Milton-Roy Minipump with pulse dampener; LDC, Riviera Beach, FL, U.S.A.). The injector was a Rheodyne syringe loading injector 7120 (Rheodyne, Berkeley, CA, U.S.A.) with a 10-μl loop. The detector used was an LDC UV III SpectroMonitor or a Model 440 UV Monitor (Waters Assoc., Milford, MA, U.S.A.). The separation columns were LiChroma tubes (316 stainless steel, Handy and Harman), 100 × 4.6 mm I.D., equipped with modified Swagelok connectors and Altex 2-μm stainless-steel frits (Altex, Berkeley, CA, U.S.A.). The pre-columns, 5 × 3.2 mm I.D., were constructed similar to the separation columns after modification of the fittings. Altex 2-μm stainless-steel frits were placed in both ends. The pre-column was connected to the separation column by a piece of 1/16 in. I.D. tubing via a Swagelok zero dead-volume union. Millipore filters (Millipore, Bedford, MA, U.S.A.), 0.45-μm filter, type HA and a Millipore stainless-steel filter holder (13 mm) were used.

Procedures

Spiking of plasma with naproxen was done by addition of 100 μl of a solution of a known concentration of naproxen in buffer (pH 7) to 1000 μl of carefully centrifuged plasma, followed by filtration through a 0.45-μm filter. After addition of an equal volume of buffer (pH 7) or water the resulting solution was injected into the chromatograph. Albumin solutions were made up in buffer (pH 7). Standards (st) were prepared like the plasma samples, except that a buffer was used instead of plasma (omitting centrifugation and filtration).

Asymmetry factors (asf) were measured by drawing a perpendicular from the

vertex, formed by the two peak tangent lines, to the baseline. The rear part of the peak baseline divided by the front part gives the asf. Plate numbers, N, were calculated from the basewidth, cut off on the baseline by the two peak tangent lines, and the retention time measured at the peak maximum.

All experiments were performed at 25.0°C. The buffers had an ionic strength of 0.1. The separation columns were slurry-packed with a high-pressure pump. The pre-columns, which contained the same support material as the separation columns, were also slurry-packed but by suction. The slurry for the pre-columns was made up in methanol–dichloromethane (1:1). The pre-columns were used to protect the separation columns and were routinely exchanged after about ten plasma injections.

RESULTS AND DISCUSSION

Sample pretreatment

In a previous study[2] the plasma sample was only centrifuged and then injected into the chromatograph, and this occasionally resulted in a sudden increase of the column back-pressure after a certain number of injections. In the present investigation, all plasma samples were centrifuged, filtered and diluted (1:1) in buffer. This gave a clear solution, which upon injection produced a much lower increase of the back-pressure, namely less than 0.6 bars per injection.

Chromatographic phase systems

In a previous study[2] emphasis was laid on the use of eluents free from organic solvents in order to prevent precipitation of plasma proteins. It was noticed then that no pronounced precipitation occurred until the methanol content exceeded 50%. We have now studied the performance of phase systems containing methanol for the separation of naproxen from plasma. This will enable a more efficient regulation of the retention, since the exploitation of pH for this purpose is restricted[2].

Table I summarizes the phase systems tested. Two concentration level of methanol were used: 30 and 50%. Even at 50% methanol the performance with respect

TABLE I

PHASE SYSTEMS AND PEAK SYMMETRY

No.	*Support*	*Eluent*	*Sample: 0.03–0.2 mM naproxen dissolved in*	*Symmetry, asf*
1	LiChrosorb RP-2	Phosphate buffer (pH 7) methanol (7:3)	Buffer	1.1
			Plasma*	0.5
			0.3 m*M* albumin	0.5
2	LiChrosorb RP-2	Acetate buffer (pH 4.8) methanol (1:1)	Buffer	1.3
			Plasma*	1.3
3	LiChrosorb RP-8	Phosphate buffer (pH 6) methanol (1:1)	Buffer	1.5
			Plasma*	1.5
4	Partisil 5-C_8	Phosphate buffer (pH 6) methanol (1:1)	Buffer	1.1
			Plasma*	1.1

* Blood plasma–buffer (1:1).

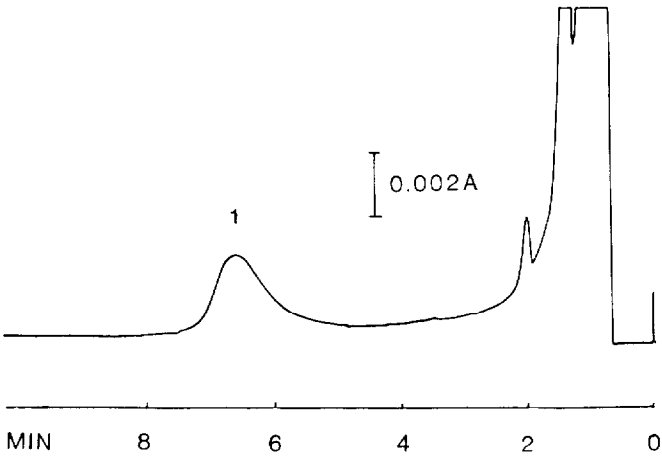

Fig. 1. Chromatogram of plasma containing naproxen (1). Sample (10 μl): blood plasma buffer (1:1), $6.6 \cdot 10^{-5}$ *M* naproxen. Phase system 1. Flow-rate: 1.1 ml/min. Detection 261 nm.

to the back-pressure was acceptable, but a continuous decrease in the column efficiency was observed. This might be caused by a slight precipitation of the proteins. The retention volume was constant, however.

Peak deformation

There was a marked difference in peak symmetry between the two methanol concentrations used. As noted in Table I, the peaks of naproxen (in blood plasma and albumin solution), obtained with 30% methanol were skewed, with a shoulder occurring on the front edge of the peak. One example of such a chromatogram is shown in Fig. 1. With 50% methanol in the eluent, the peak was symmetrical.

It is obvious that the peak skew is caused by the presence of the plasma matrix, since a naproxen sample dissolved in buffer gave a symmetrical peak. Since the shoulder occurs on the front edge, and the retention time measured at the peak was appreciably shorter than for the naproxen sample dissolved in buffer, it is possible that the effect is caused by the presence of a complexing agent in the plasma and that the complex formed is less strongly retained than the free naproxen. It is probable that this agent is identical to plasma albumin, since it is well known that naproxen is strongly bound to albumin[10]. This is supported by the fact that a sample of naproxen, dissolved in an albumin solution, also gave a skewed peak (Table I). In the following, we report tests that are aimed at confirming this hypothesis and methods by which the disturbing effect can be eliminated.

Retention of albumin

If albumin influences the retention of naproxen, the retention of albumin itself will be of importance for the effect. The retention volume of albumin in phase system 1 was lower than that for an unretained substance (KNO_3), and the porosity, defined as the retention volume divided by the volume of the column tube, was 0.45 for albumin and 0.61 for KNO_3. This shows that albumin, due to its size, is to a great extent excluded from the pores of the stationary phase and, therefore, it will migrate

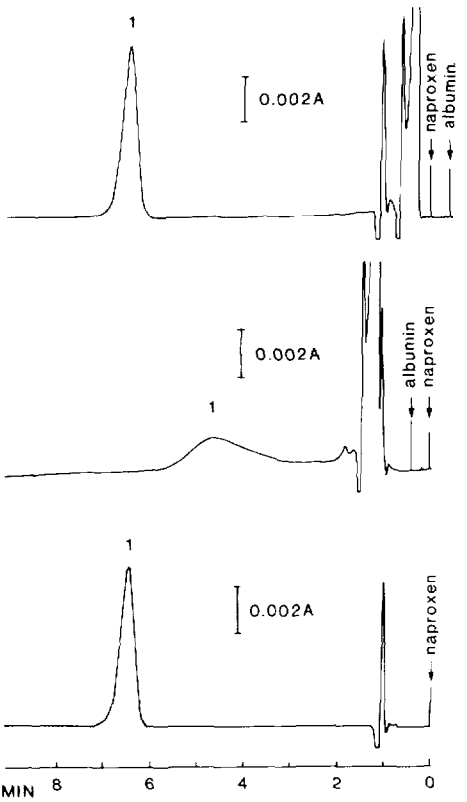

Fig. 2. Effect of albumin on peak symmetry of naproxen (1). Samples (10 μl): $6.6 \cdot 10^{-5}$ M naproxen and $6.0 \cdot 10^{-4}$ M albumin (in buffers). Phase system 1.

through the column even faster than unretained substances. The complex between naproxen and albumin should also be excluded and have a very low retention.

Influence of albumin on the chromatography of naproxen

Based on the knowledge of the retention of albumin, some simple experiments were designed that would show if albumin affects the retention of naproxen. Isolated samples of naproxen and albumin were injected immediately after each other. If the naproxen injection precedes the albumin injection the peak shape of naproxen should be influenced, since the faster migrating zone of albumin will pass over the slower migrating zone of naproxen. If injected in the reverse order, no effect on the naproxen peak would be expected since albumin should migrate ahead of naproxen and their zones will never meet.

Fig. 2 shows the results. It supports the hypothesis that complex formation between albumin and naproxen has caused the skewed peak in Fig. 1. Also, since the retention is lower when albumin is injected after naproxen, it indicates that the complex is less strongly retained than naproxen, as was predicted. Similar results were obtained when the albumin sample was exchanged for a plasma sample, and this seems to confirm that plasma albumin has caused the peak skew.

Elimination of peak skew

The peak skew can be eliminated by decreasing the degree of binding between the drug and albumin. This can be done in four different ways:

(1) by dilution

(2) by adding a displacer that will displace the drug on albumin

(3) by adding a component that changes the chemical structure of albumin so that it cannot bind the drug:

(a) change of the primary structure

(b) change of the secondary and tertiary structure

(4) by adding a component which changes the chemical structure of the drug so that it cannot bind to albumin.

Practically, methods 2–4 can be applied in two ways: (a) in the eluent, or (b) in the sample solution. Case (b) will be preferred since it is simpler to perform and gives more freedom to choose the composition of the eluent in order to regulate the retention. The effect of the added component must be so pronounced that it remains as long as the drug and albumin are present in the same zone in the column. This is due to the fact that the peak skew effect of albumin only occurs when albumin is present together with the drug. The dilution, which occurs in the column, counteracts the effect of the additive but, on the other hand, the separation of the zones of albumin and drug, which occurs due to their different retentions, puts less demands on the additive.

Dilution

The required degree of dilution will depend on the binding strength as well as on the total concentration of the drug and the protein. Assume the following complexation equilibrium between a drug, X, and a binding site, A_1, on the protein:

$$X + A_1 \rightleftharpoons XA_1$$

The corresponding equilibrium constant is then defined by:

$$K_{XA(1)} = \frac{[XA_1]}{[X][A_1]} \tag{1}$$

The brackets indicate the molar concentration in the solution. Assume that 1 mol of protein contains n_1 moles of binding sites A_1. Then the total molar concentration of binding sites will be defined by

$$n_1 C_P = [A_1] + [XA_1] = [A_1](1 + K_{XA(1)}[X]) \tag{2}$$

where C_P is the total molar concentration of the protein. Combination of eqns. 1 and 2 results in

$$\frac{[XA_1]}{C_P} = \frac{n_1 K_{XA(1)}[X]}{1 + K_{XA(1)}[X]} \tag{3}$$

which expresses the number of moles of drug bound per mole of protein and has

often been used in the evaluation of the values of n_1 and $K_{XA(1)}$, *e.g.*, by the Scatchard plot[10].

If the protein contains a second type of binding site, characterized by the binding constant $K_{XA(2)}$, a second term has to be added to eqn. 3. The ratio of the amounts of bound to free drug (binding ratio) can then be expressed by the equation

$$\frac{C_{XA}}{[X]} = C_P \left(\frac{n_1 K_{XA(1)}}{1 + K_{XA(1)}[X]} + \frac{n_2 K_{XA(2)}}{1 + K_{XA(2)}[X]} \right) \quad (4)$$

where C_{XA} represents the total molar concentration of drug bound to sites A_1 and A_2.

In order to decrease the risk of obtaining a skewed chromatographic peak when a plasma sample is injected, one should decrease the binding ratio in the sample solution. This can be done by a dilution, which decreases the protein concentration. At relatively low concentrations of the unbound drug, when $K_{XA}[X] \ll 1$, the binding ratio will decrease linearly with the protein concentration. At higher concentrations of the drug the binding ratio will decrease less efficiently by dilution, because [X] will decrease to a lesser degree than C_P owing to the release of X from the complex.

To illustrate the influence of albumin on peak deformation some experiments with dilution were performed with samples containing the drug, dissolved in $5.5 \cdot 10^{-4}$ *M* albumin solution, which corresponds to the normal plasma level of $5 \cdot 10^{-4}$ *M*[11]. The results are summarized in Table II.

The plate number and the asymmetry factor were taken as measures of the peak deformation and are expressed in relation to a standard. When the albumin sample was diluted 1:50, the efficiency and symmetry were restored. Very similar results were obtained with plasma samples containing the same initial concentration of naproxen.

Calculation of binding ratio and degree of binding. To illustrate further the effect of protein binding on the chromatographic behaviour the resulting peak skew was

TABLE II

EFFECT OF DILUTION ON THE BINDING RATIO, BINDING DEGREE, PEAK EFFICIENCY AND SYMMETRY OF NAPROXEN

Phase system: No. 1 (Table I). Sample: $C_X = 4.7 \cdot 10^{-4}$ *M*, $C_P = 5.5 \cdot 10^{-4}$ *M*; X = naproxen; P = albumin. Injected volume: 10 µl.

Dilution of sample	N/N_{st}	asf/asf_{st}	*Calculated binding ratio** $C_{XA}/[X]$	*Calculated degree of binding (%)** $(C_{XA}/C_X) \times 100$
0	0.41–0.55	0.60–0.70	138	99
1:10	0.74–0.80	0.84–0.94	18	95
1:50	0.98–1.07	0.87–1.04	5	84

* $K_{XA(1)} = 1.8 \cdot 10^6\ M^{-1}$; $n_1 = 1.0$ (ref. 10); $\frac{C_{XA}}{C_X} = \frac{C_{XA}}{[X]\left(1 + \frac{C_{XA}}{[X]}\right)}$.

correlated with the binding ratio of the drug in the injected albumin solution (Table II). The binding ratio can be calculated from eqn. 4 if the number of binding sites, the stability constants, the total concentration of albumin and the total concentration of the drug are known.

The concentration of bound and unbound drug are interrelated by

$$C_X = C_{XA} + [X] \tag{5}$$

where C_X is the total concentration of the drug. When binding to only one type of site has to be taken into account, the concentration of unbound drug, [X], can be calculated from a second-order equation after combination of eqns. 3 and 5. With two types of sites, a third-order equation must be used.

Table II shows the calculated binding ratios, as well as the binding degree, C_{XA}/C_X, for a sample containing albumin at the normal level which was diluted to different degrees. At a dilution of 1:50, where the peak skew was eliminated, the binding ratio was 5, corresponding to a binding degree of 84%.

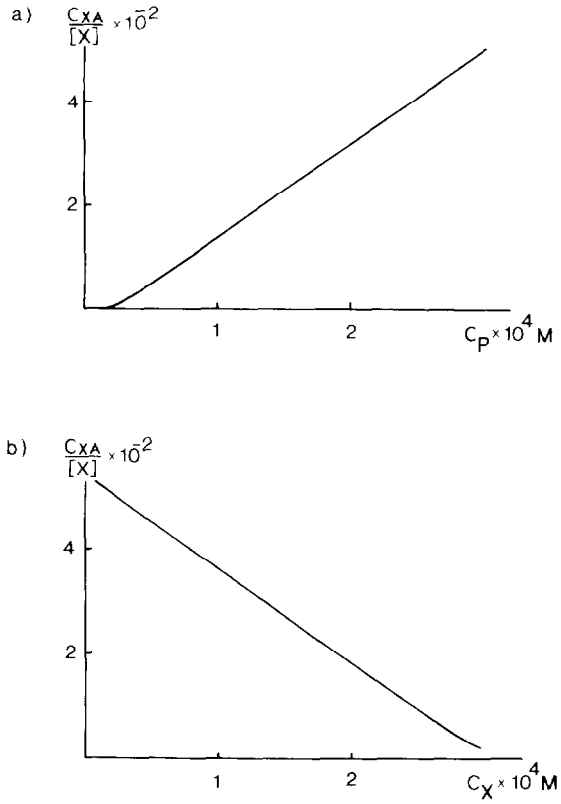

Fig. 3. Calculated binding ratios for the binding of naproxen to albumin at constant naproxen concentration ($C_X = 2.2 \cdot 10^{-5}$ *M*) and varying albumin concentration (a) and at constant albumin concentration ($C_P = 3.0 \cdot 10^{-4}$ *M*) and varying naproxen concentration (b).

The calculation was based on binding only to one type of site even though a second type of site exists[10,12]. Calculations based on binding constants for bovine serum albumin[12] showed that the binding to the second type of site was negligible at the low total concentration of the drug used in this study.

The binding ratio is dependent on the total concentration of the protein and on the total concentration of the drug. The latter point is important to realize, since it means that the binding ratio may be different for different concentration levels of the drug. Fig. 3 shows the calculated binding ratios for naproxen as a function of the total concentrations of naproxen and albumin when they were varied one at a time.

For constant C_X the binding ratio increases linearly above a certain value of C_P. For constant C_P the binding ratio assumes a constant maximal value when C_X is sufficiently small (linear binding isotherm) and decreases asymptotically to zero when C_X increases. For example, in experiments in which an excess concentration of naproxen was used no peak deformation was observed.

Fig. 4 shows the peak efficiency of naproxen in relation to a standard, plotted as a function of the calculated binding degree in the injected albumin solution. The different binding degrees were obtained by three different methods: dilution, change of the total concentration of the drug and change of the total concentration of albumin. For each method, the same binding degree seems to give similar chromatographic results. This shows the importance of the binding degree in the sample for the resulting chromatographic performance.

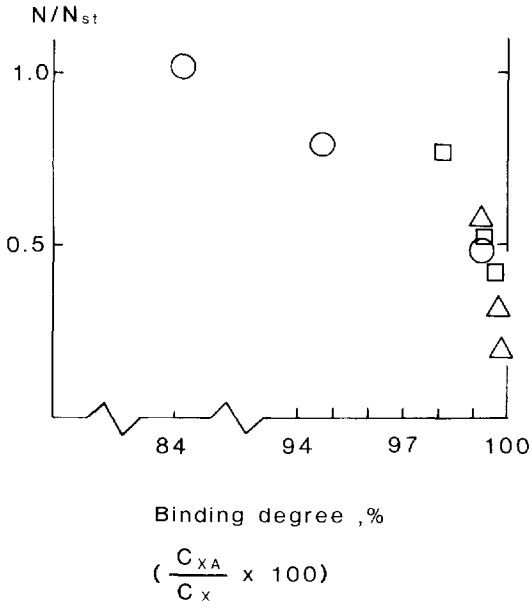

Fig. 4. Effect on the plate number of naproxen of its degree of binding to albumin in the injected sample. Comparison of three different methods for changing the binding degree. Phase system 1 (average of triplicates). ○, Dilution, results from Table II; □, change of albumin concentration, concentration range according to Fig. 3a, $C_X = 2.16 \cdot 10^{-5}$ M, $C_P = (0.5\ 1.5) \cdot 10^{-4}$ M; △, change of naproxen concentration, concentration range according to Fig. 3b, $C_P = 3.01 \cdot 10^{-4}$ M, $C_X = (0.2\ 2.4) \cdot 10^{-4}$ M.

Addition of displacer

The displacer, Y, should bind to the same type of sites as the drug. Assume that the drug only binds to one type of site. Then eqn. 2 takes the form

$$n_1 C_P = [A_1] + [XA_1] + [YA_1] \quad (6)$$

and the binding ratio of X will now be expressed by:

$$\frac{C_{XA}}{[X]} = C_P \left(\frac{n_1 K_{XA(1)}}{1 + K_{XA(1)}[X] + K_{YA(1)}[Y]} \right) \quad (7)$$

Obviously, an increasing concentration and stability constant of the displacer will decrease the binding ratio.

Displacers have been reported in the literature on the protein binding of drugs. For our study we chose flurbiprofen, for which a binding constant has been determined and which binds to the same site on albumin as naproxen[10]. Other possible displacers may be found among the alkyl sulphates, since they have also been shown to bind to albumin[13]. We have tested dodecyl sulphate and octyl sulphate.

Table III shows that a slight improvement in peak symmetry was obtained with the alkyl sulphates, whereas flurbiprofen had an almost complete effect (but still, the peak of naproxen shows a slight tendency to fronting, as illustrated in Fig. 5). The beneficial influence of octyl sulphate may be the reason why we did not observe any skewed peaks in a previous study[2], since in those experiments the eluent contained octyl sulphate.

The concentration of the displacer should be chosen so that the degree of

TABLE III

ELIMINATION OF PEAK SKEW BY ADDITION OF DISPLACERS

Phase system: No. 1 (Table I). Sample: naproxen (X) dissolved in blood plasma–buffer (1:1), $3.0 \cdot 10^{-4}$ *M* albumin solution or buffer. Sample volume: 10 µl.

$C_X \cdot 10^4$ (*M*)	*Displacer (Y)*	$C_Y \cdot 10^4$ (*M*)	*Calculated binding degree (%)**	N/N_{st}	asf/asf_{st}
Albumin samples					
2.0	–	–	99.4	0.4	0.5
2.0	Octyl sulphate	2.5	–	0.6	0.7
2.0	Flurbiprofen	1.2	92	0.4	0.6
2.0	Flurbiprofen	2.4	56	0.6	0.8
2.0	Flurbiprofen	4.7	18	0.8	0.8
0.66	Flurbiprofen	16	8	0.9	1.0
Blood plasma samples					
0.66	–	–		0.2	0.5
0.54	Octyl sulphate	20		0.7	0.8
0.54	Dodecyl sulphate	15		0.4	0.6
0.66	Flurbiprofen	16		0.9	0.9

* $K_{XA(1)} = 1.8 \cdot 10^6\ M^{-1}$; $n_1 = 1.0$; $K_{YA(1)} = 5.0 \cdot 10^6\ M^{-1}$ (ref. 10).

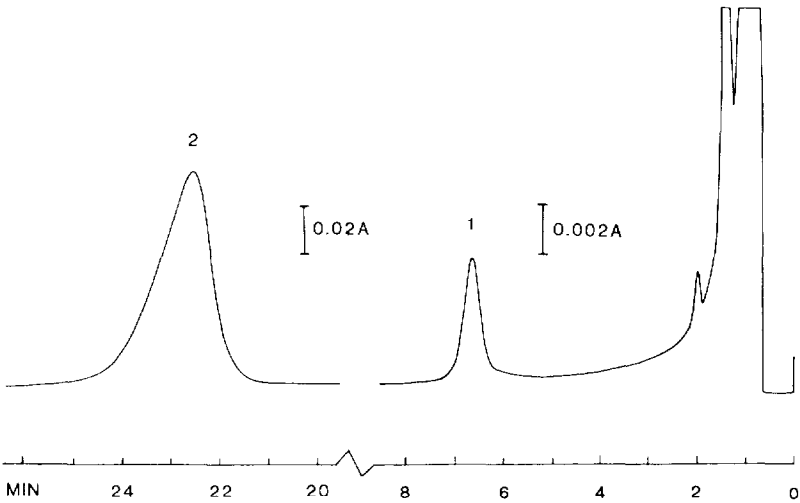

Fig. 5. Use of displacer, flurbiprofen (2), to eliminate the peak skew of naproxen (1). Sample (10 μl): blood plasma buffer (1:1), $6.6 \cdot 10^{-5}$ *M* naproxen and $1.6 \cdot 10^{-3}$ *M* flurbiprofen. Phase system 1. Flow-rate: 1.1 ml/min. Detection 261 nm.

binding of the drug is reduced to a sufficient level. Table III gives the binding ratio at different concentrations of flurbiprofen for a sample of naproxen, dissolved in an albumin solution. The degree of binding was calculated from known constants for human serum albumin by solving a form of eqn. 7 for known total concentrations of albumin, flurbiprofen and naproxen. Obviously the degree of binding must be well below 10% to give peaks of good efficiency and symmetry. This figure will not be generally valid and is much lower than that obtained at dilution, since the effect of the displacer will be dependent on its retention in the chromatographic system. The more strongly retained it is, the faster it will separate from the zone of albumin, and its displacing effect will disappear. The ideal displacer should have the same retention as albumin so that the two migrate in the same zone.

Change of structure of albumin and naproxen

These methods must be based on a detailed knowledge of the type of binding forces between naproxen and albumin. Such information is lacking, but some guidelines might be gleaned from the binding of other weak organic acids to albumin. Sulphonamides seem to be bound predominantly in the anionic form and preferably by electrostatic attraction to positively charged amino groups in albumin, but the uncharged part of the molecule is probably also taking part in the binding[14]. This indicates that the binding of naproxen might be influenced by a change of pH. It has been shown there is no change of the binding of naproxen between pH 5 and 9 (ref. 12), which can be expected, since the acid dissociation constant is probably around 4 and the charges of the amino groups on albumin do not change. At a pH value of 10, a decrease of the binding of sulphonamides due to changes of the albumin conformation was observed[14].

It may be assumed that adjustment of the pH to extremely acidic and basic values can decrease the binding of naproxen, both due to a breakdown of the albumin

TABLE IV

ELIMINATION OF PEAK SKEW BY ADDITIVES IN THE SAMPLE SOLUTION

Sample: naproxen (X) dissolved in blood plasma buffer (1:1), $3.0 \cdot 10^{-4}$ *M* albumin solution or buffer. Sample volume: 10 µl.

Phase system (Table I)	*Methanol in eluent (%)*	*Sample solution*	$C_X \cdot 10^4$ *(M)*	*Additive*	N/N_{st}	asf/asf_{st}
1	30	Plasma	0.66	–	0.2	0.5
			0.36	0.25 *M* H_3PO_4	1.0	1.0
			0.59	0.25 *M* NaOH	1.0	1.0
		Albumin	2.0	–	0.4	0.5
			2.0	0.25 *M* H_3PO_4	1.0	1.0
			2.0	0.25 *M* NaOH	1.0	1.0
2	50	Plasma	0.41	–	0.8	1.0
			0.41	0.25 *M* H_3PO_4	1.0	1.0
3	50	Plasma	0.35	–	0.8	1.0
			0.35	0.25 *M* H_3PO_4	1.0	1.0
4	50	Plasma	0.29	–	0.7	1.0
			0.29	0.25 *M* NaOH	0.7	1.0
			0.29	0.25 *M* H_3PO_4	1.0	1.0

structure and, at an acidic pH, due to the transformation of naproxen to the uncharged form.

A further possibility to decrease the binding of the drug might be to destroy the primary structure of the protein, *e.g.*, by enzymatic hydrolysis[6].

Table IV shows the effect of additives. The skewed peaks in phase system 1 are eliminated both by acidic and basic pH. The phase systems with 50% methanol in the eluent do not give skewed peaks, but the efficiency is somewhat decreased in relation to a standard ($N/N_{st} = 0.8$). However, the efficiency was fully restored by addition of phosphoric acid to the plasma samples.

The better performance of the phases systems with 50% methanol in the eluent might be explained by the denaturing effect of methanol on proteins, which can change the conformation of the protein. A further possibility is that the stability constants decrease due to, *e.g.*, a change of the solvation of the drug in the eluent.

CONCLUSIONS

Among the different methods for decreasing the binding degree of a drug the dilution method is the simplest, but it can only be used if the drug has so high a concentration in plasma that it can be diluted and still give a suitable detector response. This limits the applicability of the method, since most drugs are present at very low concentrations.

The use of displacers is somewhat limited if they give a detector response, as illustrated in Fig. 5 by the peak eluted late.

The best method is to use, if possible, an eluent having a composition that gives a low binding degree. If necessary, it can be combined with the use of additives in the sample solution.

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