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Allosteric and competitive displacement of drugs from human serum albumin by octanoic acid, as revealed by high-performance liquid affinity chromatography, on a human serum albumin-based stationary phase

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ABSTRACT

A chiral stationary phase for high-performance liquid chromatography, based upon immobilized human serum albumin (HSA), was used to investigate the effect of octanoic acid on the simultaneous binding of a series of drugs to albumin. Octanoic acid was found to bind with high affinity to a primary binding site, which in turn induced an allosteric change in the region of drug binding Site II, resulting in the displacement of compounds binding there. Approximately 80% of the binding of suprofen and ketoprofen to HSA was accounted for by binding at Site II. Octanoic acid was found to also bind to a secondary site on HSA, with much lower affinity. This secondary site appeared to be the warfarin–azapropazone binding area (drug binding Site I), as both warfarin and phenylbutazone were displaced in a competitive manner by high levels of octanoic acid. The enantioselective binding to HSA exhibited by warfarin, suprofen and ketoprofen was found to be due to differential binding of the enantiomers at Site I; the primary binding site for suprofen and ketoprofen was not enantioselective.

INTRODUCTION

Most drugs undergo some degree of reversible binding to plasma proteins, a process which may often have significant effects on the overall activity profile of the compounds. The major plasma protein in this respect is human serum albumin (HSA), which has been the subject of an enormous amount of research. It is believed that the binding of drugs to HSA occurs at a small number of loci, with two major sites appearing to account for the bulk of drug binding. This is experimentally supported by the fact that ligands binding to one or other of these sites are able to displace most of the drugs which bind to HSA. These two sites have become known for the ligands supposed to bind there; at one site warfarin is the prototypical ligand (this is also termed Site I), while at the other, indoles and benzodiazepines appear to bind (Site II) [1–5]. A small number of drugs appear to bind to neither of these sites, and so several other, minor, binding sites have been proposed [4,5].

While a site-oriented approach to drug binding may successfully describe the majority of experimental findings, a large number of observations are poorly explained using this perspective. For instance, in displacement studies, the drugs war-

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farin, phenylbutazone and azapropazone behave exactly as if they competitively bind to the same site. However, when a single tryptophan residue, thought to be located within binding Site I, was chemically modified, the binding of warfarin alone was clearly decreased, with no effect being seen on the binding of phenylbutazone and azapropazone [6]. It is also difficult to reconcile the observation that the (*S*)-enantiomer of warfarin is able to induce an allosteric enhancement in the binding affinity of (*S*)-lorazepam methyl ether, while the (*R*)-enantiomer is not [7], with site-oriented binding, as the two enantiomers appear to bind at the same site and in the same orientation. Apparently anomalous observations such as these have been explained by proposing that binding occurs not at particular, small receptor-like sites, but over larger, less localized areas on the protein surface [8,9].

Conventional techniques for the study of protein binding are poorly adapted to the precise examination of interactions between two or more cobinding solutes. A preferable approach is that of affinity chromatography, in which the protein is immobilized on a suitable support, the ligand is injected onto the column, and competing agents are added to the mobile phase (*e.g.* ref. 10). In this way, small changes in binding affinity, which are reflected in chromatographic retention times, may be precisely monitored. However, the method suffers from the disadvantage that it is comparatively slow, and the method of detection in which fractions of column eluent are collected for subsequent analysis is cumbersome.

Recently, this group reported a chiral stationary phase (CSP) for use in high-performance liquid chromatography (HPLC), based upon immobilized human serum albumin (the HSA-CSP). The protein is immobilized within a commercially available diol column, which is first activated with 1,1-carbonyldiimidazole. The protein is immobilized to the silica surface through a urethane linkage, a process which does not significantly affect the drug binding properties [11,12] or the conformational mobility [13] of the protein. The HSA-CSP therefore offers what may be termed a "high-performance liquid affinity chromatographic" [14] approach to the examination of interactions between solutes binding to HSA. We here report the use of the HSA-CSP in the study of the effect of octanoic acid on the binding of a range of solutes to HSA, as a model system to illustrate the utility of this approach.

EXPERIMENTAL

Chemicals

Racemic warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin], phenylbutazone, tolbutamide, racemic ketoprofen and racemic suprofen were obtained from Sigma (St. Louis, MO, USA). Racemic oxazepam hemiscuccinate was prepared by acylation of oxazepam with succinic anhydride in the presence of pyridine, following a previously reported procedure [15]. Octanoic acid was obained from Aldrich (Milwaukee, WI, USA).

Chromatography

Chromatography was carried out using a modular HPLC system, which consisted of a Spectroflow 400 pump, a 480 injector module equipped with a 20-μl loop, a 783 programmable absorbance detector (ABI Analytical, Ramsey, NJ, USA) and a DataJet integrator (Spectra-Physics, San Jose, CA, USA). The column temperature was maintained at 25 ± 0.1°C, using a CH-30 temperature-regulating jacket (FIAtron Laboratory Systems, Oconomowoc, WI, USA).

The HSA-CSP was prepared as previously described [11], by Shandon Scientific (Runcorn, UK), using a 15 cm × 4.6 mm I.D. diol column.

Chromatographic conditions

The mobile phases employed in these studies were based on sodium dihydrogenphosphate–disodium hydrogenphosphate (50 m*M*, pH 6.90) modified with 10% (v/v) propan-1-ol. The flow-rate was 0.8 ml min^{-1} throughout. When octanoic acid was added to the mobile phase, the required volume of acid was first dissolved in the alcohol fraction, before addition of the buffer. All mobile phases were filtered (0.45 μm) and degassed by ultrasonication immediately prior to

use. Detection of the solutes was by their UV absorbance, at the relevant λ_{max}. The amount injected of each solute was 0.5 μg for the racemic compounds or 0.25 μg for the non-chiral solutes.

Effect of octanoic acid on the chromatographic capacity factor, k'

The chromatographic capacity factor (k') is defined as $(t_R - t_0)/t_0$, where t_R is the retention time of the solute of interest and t_0 is that of an unretained solute (water). The k' values of a range of solutes, some enantiomeric, on the HSA-CSP were determined in the phosphate buffer–propan-1-ol eluent describe above. Octanoic acid was then added to the mobile phase, and the resulting solution passed through the column until equilibrium was reached. The attainment of equilibrium was followed by monitoring the absorption of the column eluent at 220 nm. After saturation of the column, the octanoic acid could be seen to elute as a sharp front, followed by a plateau. The detector baseline was re-zeroed and allowed to stabilize. The solutes were then re-injected, and their new k' values were calculated. This process was repeated over a wide range of octanoic acid concentrations.

Determination of amount of immobilized protein

The number of moles of immobilised HSA participating in the chromatographic process (m_L) was determined by frontal elution of L-tryptophan on the HSA-CSP [16]. Solutions of L-tryptophan, in the mobile phase described above, ranging in concentration ([A]) between 1 and 100 μM, were sequentially introduced at the head of the column. This was achieved using a six-port valve to switch between pumps delivering either L-tryptophan solution or unmodified mobile phase. After a period of time inversely proportional to the concentration of the solution, a breakthrough profile was observed at the detector, and the relevant breakthrough volume [$(V - V_0)$, where V is the breakthrough volume and V_0 is the system void volume] was determined. L-Tryptophan was chosen as a probe of m_L as it is generally assumed to bind to a single site on HSA, at low ligand/protein ratios [17–19], therefore facilitating the calculation of the amount of immobilized HSA. A plot of the reciprocal of $\{(V - V_0)\,[A]\}$ against the reciprocal of [A] produced a straight line, the y-axis intercept of which, in the case of L-tryptophan, was equal to the reciprocal of the number of moles of immobilized protein, and the x-intercept of which was equivalent to the negative reciprocal of the dissociation constant [16].

Competition at a single site

The use of zonal elution in the determination of equilibrium constants by affinity chromatography is well described [20,21]. In this technique, a known concentration of a competing agent (I), which in the present instance is octanoic acid, is continuously applied to a column containing an immobilized ligand (L), while injections of a solute (A) are made. In the case where I and A compete at a single site on L, and A binds to no other loci on the matrix, reaction eqns. 1 and 2 and eqns. 3 and 4 may be used to represent the processes taking place in the column:

$$A + L \overset{K_3}{\leftrightharpoons} AL \tag{1}$$

$$I + L \overset{K_2}{\leftrightharpoons} IL \tag{2}$$

$$K_3 = \frac{\{AL\}}{[A]\,\{L\}} \tag{3}$$

$$K_2 = \frac{\{IL\}}{[I]\,\{L\}} \tag{4}$$

In eqns. 3 and 4, K_2 and K_3 represent the association constants for the binding of I and A to L, respectively. The terms [A] and [I] represent the mobile phase concentrations of A and I, while {AL} and {IL} represent the surface concentrations of their respective complexes with the immobilized ligand (HSA). Assuming that only a small amount of A is injected onto the column (*i.e.* $\{AL\} << \{IL\}$), and expression can be de-

rived to describe how the retention of A changes as [I] is varied [22,23]:

$$k'_A = \frac{K_3 m_L}{V_m (1 + K_2 [I])} \quad (5)$$

In eqn. 5, k'_A is the capacity factor of solute A, V_m is the void volume of the column, m_L represents the number of moles of HSA binding sites within the column, and [I] is the concentration of competing agent applied to the column. By taking the reciprocal of both sides of eqn. 5, the following equation is obtained:

$$\frac{1}{k'_A} = \frac{V_m K_2 [I]}{K_3 m_L} + \frac{V_m}{K_3 m_L} \quad (6)$$

Therefore, in the case of simple, single-site competition, eqn. 6 predicts that a plot of $1/k'_A$ against [I] will yield a linear relationship, the slope of the line being $V_m K_2 / K_3 m_L$ and the intercept being $V_m / K_3 m_L$. By calculating the ratio of the slope to the intercept for such a plot, the value of K_2 can be directly obtained.

Competition at a single site, with solute binding at secondary site(s), unaffected by competing agent

Eqns. 1–6 describe the case in which a solute and competing agent undergo simple, single-site competition for binding sites on the column. A slightly more complex situation occurs when the solute interacts with additional sites (specific and/or non-specific), which are unaffected by the addition of the competitor. In this case, the following processes will occur on the column in addition to those described in eqns 1–4 above:

$$\begin{array}{lcl} & K_{N1} & \\ A + L_{N1} & \leftrightharpoons & AL_{N1} \\ & . & \\ & . & \\ & . & \\ & K_{Nn} & \\ A + L_{Nn} & \leftrightharpoons & AL_{Nn} \end{array} \quad (7)$$

where $L_{N1}...L_{Nn}$ are the secondary binding sites for A, at which I does not bind, and $K_{N1}...K_{Nn}$ are their corresponding association constants. Based on an analogous treatment to that used to obtain eqn. 5, the following expression can be derived relating the retention of A to [I]:

$$k'_A = \frac{K_3 m_L}{V_m} \cdot \frac{1}{(K_2 [I] + 1)} + \frac{(K_{N1} m_{LN1} + ...K_{Nn} m_{LNn})}{V_m} \quad (8)$$

Note that the final term of this equation is independent of competitor concentration, and therefore may be reduced to a single term X, which is obtained by iterative testing. By substituting the term X into eqn. 8, and with rearrangement, the following relationship is obtained:

$$\frac{1}{(k'_A - X)} = \frac{V_m K_2 [I]}{K_3 m_L} + \frac{V_m}{K_3 m_L} \quad (9)$$

If this model applies, then a plot of $1/(k'_A - X)$ against [I] will be linear, with slope and intercept having the same meaning as in eqn. 6. The slope divided by the intercept will give K_2, which is the affinity constant for the binding of the competing agent to the site in question.

An equation analogous in form to eqn. 9 has been derived by Hermansson and Eriksson [24], to successfully describe the chromatographic interrelationship between weakly anionic solutes and dimethyloctylamine on a CSP based upon α_1-acid glycoprotein.

RESULTS

Determination of amount of immobilized protein

A plot of the reciprocal of $\{(V - V_0)\ [A]\}$ against $1/[A]$ was linear ($r^2 = 0.9999$). The y-intercept was $3.92_5 \pm 0.4 \cdot 10^5$, and the slope was 32.2 ± 0.6. From this, m_L (1/intercept) = $2.5_{48} \pm 0.1_4 \cdot 10^{-6}$ mol, or approximately 169 mg HSA. The association constant of L-tryptophan (intercept/slope) was $1.2_{17} \cdot 10^4\ M^{-1}$.

TABLE I

INFLUENCE OF OCTANOIC ACID (0–8 m*M*) ON THE CAPACITY FACTORS OF SOME TEST SOLUTES, ON THE HSA-CSP

A and B signify the first- and second-eluting enantiomers of ketoprofen and suprofen, the absolute enantiomeric elution orders of which are not known.

Compound	Capacity factor						
	0 m*M*	0.25 m*M*	0.5 m*M*	1.0 m*M*	2.5 m*M*	4. 0 m*M*	8.0 m*M*
(*R*)-Warfarin	35.7	29.4	27.8	23.8	18.5	16.1	13.9
(*S*)-Warfarin	52.6	37.0	34.5	29.4	20.4	17.0	13.9
Phenylbutazone	49.0	42.4	38.0	32.2	24.1	21.6	17.1
Tolbutamide	8.5	5.5	4.7	4.3	3.3	3.0	2.5
(*R*)-Oxazepam hemisuccinate	10.8	10.5	10.1	9.8	8.6	7.8	7.6
(*S*)-Oxazepam hemisuccinate	44.6	14.5	13.2	11.7	9.5	8.1	7.6
Ketoprofen A	62.0	15.1	12.9	10.8	7.9	7.0	5.7
Ketoprofen B	83.1	17.4	14.8	12.3	8.5	7.0	5.7
Suprofen A	66.9	13.9	11.7	10.7	8.1	6.5	5.6
Suprofen B	149.0	25.0	20.1	14.9	10.1	7.6	6.1

Chromatographic studies

The addition of octanoic acid to the mobile phase of the HSA-CSP had a concentration-dependent effect on the k' of the test solutes examined (Table I). Plotting of the chromatographic data in Table I, according to eqn. 6, did not reveal a linear relationship between the reciprocal of k' and concentration of acid for any of the compounds examined (Fig. 1), indicating that a simple competitive model does not apply. However, when the model for competition at a single site, with further binding of the solute at other

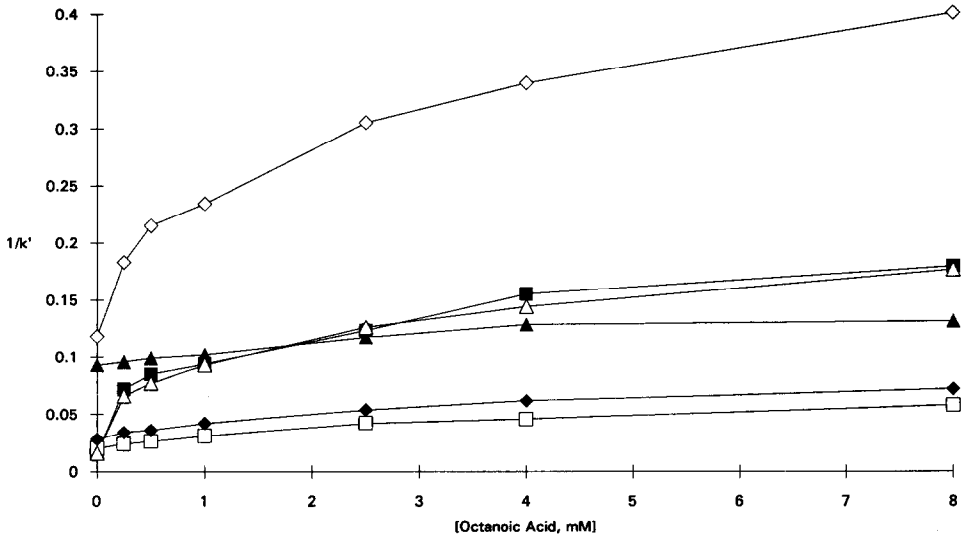

Fig. 1. Selected data, plotted according to the relationship for competitive displacement of solutes by octanoic acid, from a single site. ■ = Suprofen A; □ = phenylbutazone; ◆ = (*R*)-warfarin; ◇ = tolbutamide; ▲ = (*R*)-oxazepam hemisuccinate; △ = ketoprofen A. (A indicates the first-eluting enantiomer, where the absolute configuration, or optical rotation, is not known).

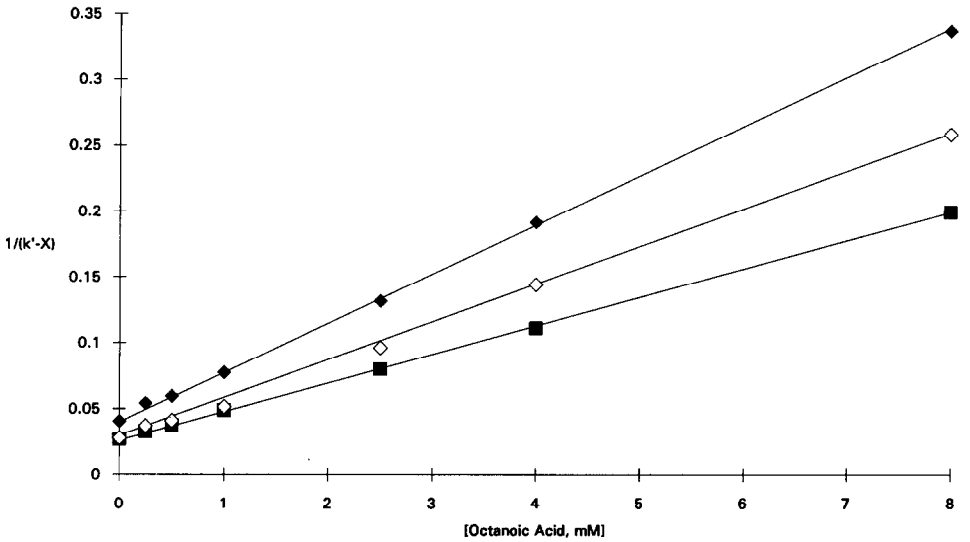

Fig. 2. Influence of octanoic acid on the retention of (*R*)-warfarin (◆), (*S*)-warfarin (◇) and phenylbutazone (■), plotted according to a model describing competition at a single site, with further binding of the solute at other site(s), which are unaffected by octanoic acid. X is the contribution to k' from such unaffected sites.

sites not affected by octanoic acid (eqn. 9), was applied, linear relationships for several compounds were obtained.

The binding behavior of both enantiomers of warfarin and phenylbutazone could be well described by the latter model (Fig. 2). In these three cases, correlation coefficients of 0.999, or greater, were obtained, with small standard deviation of slope and intercept, indicating good agreement with the proposed mechanism of displacement (Table II). The constant K_2 pertains to the binding affinity of octanoic acid, at the site from which it displaces the solute. For phenylbutazone and the enantiomers of warfarin, K_2 varied between 670 and 1150 M^{-1}.

The k' data for tolbutamide, when treated according to eqn. 9, gave a plot which appears to divide into two linear regions (Fig. 3). The pa-

TABLE II

PARAMETERS OBTAINED FROM CHROMATOGRAPHIC DATA IN TABLE I, WHEN FITTED ACCORDING TO EQN. 9

X is the residual k', resulting from binding at sites unaffected by octanoic acid. Other parameters are described in the text. V_m = 0.00175 l, $m_L = 2.5_{48} \pm 0.1_4 \cdot 10^{-6}$ mol.

Compound	X	Intercept (V_m/K_3m_L)	S.D. intercept	K_3 ($\times 10^{-4}$ M^{-1})	S.D. K_3 ($\times 10^{-4}$ M^{-1})
(*R*)-Warfarin	10.9	0.042_2	0.001_1	1.70_0	0.04_5
(*S*)-Warfarin	10.0	0.025_4	0.001_4	2.87_8	0.2_2
Phenylbutazone	11.6	0.028_2	0.002_2	2.5_0	0.21_9
Tolbutamide	2.0	0.25_1	0.01_2	0.28_1	0.02_1
Suprofen A	4.3	0.076_6	0.014_2	0.90_3	0.2_2
Suprofen B	4.4	0.027_3	0.005_3	2.65_8	0.5_2
Ketoprofen A	4.2	0.077_2	0.004_4	0.91_4	0.07_7
Ketoprofen B	4.1	0.054_0	0.004_4	1.3_1	0.1_2

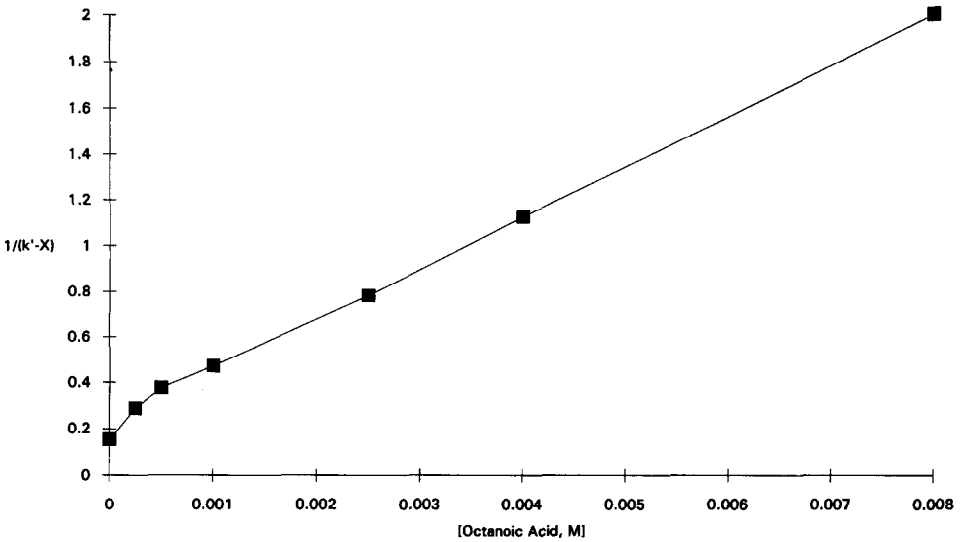

Fig. 3. Influence of octanoic acid on the retention of tolbutamide on the HSA-CSP, plotted according to a model describing competition at a single site, with further binding of the solute at other site(s), which are unaffected by octanoic acid. X is the contribution to k' from binding of tolbutamide to sites unaffected by the acid.

rameter X, for tolbutamide, is 2.0, indicating that binding to sites at which octanoic acid does not bind, accounts for approximately 25% of the total binding to HSA.

The 2-arylpropionic acid non-steroidal agents (NSAIDs), ketoprofen and suprofen, were relatively well described by eqn. 9, after a sharp decrease in affinity upon initial addition of octanoic acid (Figs. 4 and 5). For these compounds, the binding to sites not affected by the acid accounts for only 3–7% of the total. The initial decline in the k' values of the NSAIDs upon addition of

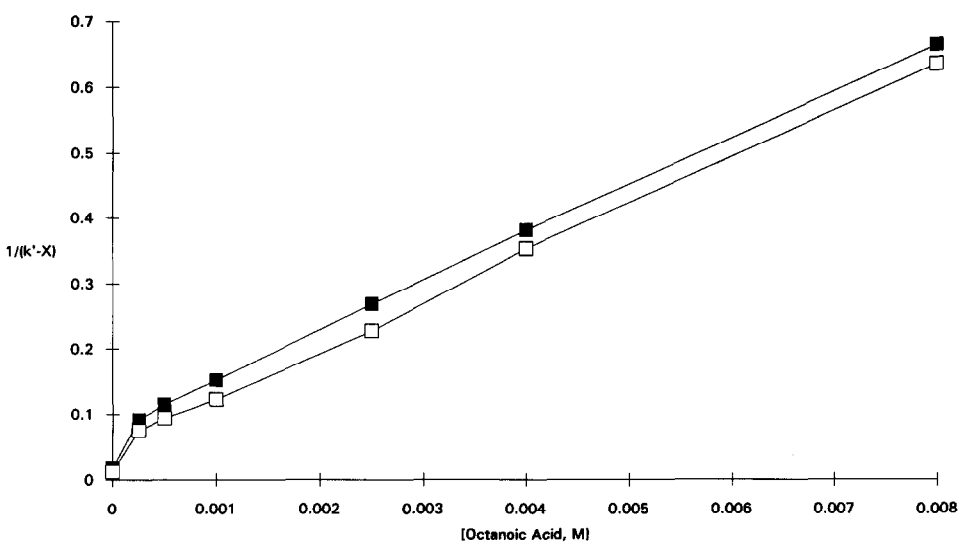

Fig. 4. Influence of octanoic acid on the retention of the first eluting (■) and second-eluting (□) enantiomers of ketoprofen on the HSA-CSP, plotted according to a model describing competition at a single site, with further binding of the solute at other site(s), which are unaffected by octanoic acid. X is the contribution to k' from binding to sites unaffected by octanoic acid.

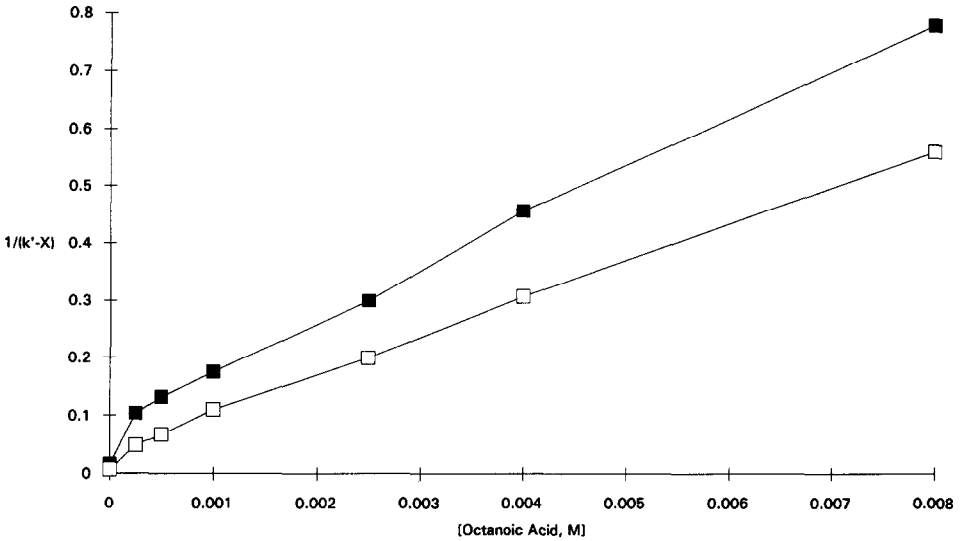

Fig. 5. Influence of octanoic acid on the retention of the first-eluting (■) and second-eluting (□) enantiomers of suprofen on the HSA-CSP, plotted according to a model describing competition at a single site, with further binding of the solute at other site(s), which are unaffected by octanoic acid. X is the contribution to k' from binding of suprofen enantiomers to sites unaffected by the acid.

octanoic acid represents a decrease of approximately 75–80%. This occurs with only a very small effect on the enantioselectivity factor of these compounds, indicating that the site from which they are initially displaced by octanoic acid is not that which accounts for the enantioselectivity observed in their binding to HSA.

The binding behavior of the enantiomers of oxazepam hemisuccinate (OXH) to the HSA-CSP was very poorly described by the present models, with both enantiomers giving different profiles. In a fashion that appeared similar to the behavior of the NSAIDs, initial addition of octanoic acid caused a large (approximately 70%) decrease in the k' of (*S*)-OXH. The (*R*)-enantiomer of OXH did not show this type of behavior.

DISCUSSION

The immobilization of HSA by the method used to produce the columns employed in this study does not significantly affect the binding properties, or the conformational mobility, of the protein. This has been demonstrated by the ability of the HSA-CSP to reproduce displacement phenomena [11,12] and allosteric interactions [13], initially reported using other techniques. It would therefore appear that the HSA-CSP provides a valid method for the study of the interactions between solutes and HSA.

Eqn. 6, which describes simple, single-site competition, was unsuccessful in fitting the actual chromatographic data obtained for the present solutes and octanoic acid, indicating that the processes involved were somewhat more complex. However, eqn. 9, which includes a term for solute binding at sites which are unaffected by the competitor, was able to very well describe the behavior of certain solutes. For the enantiomers of warfarin and for phenylbutazone, very good correlations were obtained between a factor containing the capacity factor and the concentration of acid added to the mobile phase. From the magnitude of factor X in eqn. 9 (Table II), approximately 70–80% of the total binding observed for these compounds arises from attachment to a site at which octanoic acid also binds. For warfarin, this site is enantioselective, as addition of sufficient acid results in the loss of chiral recognition. This site is probably that described as the warfarin–azapropazone binding area [8], which is also known as drug binding Site I. The linear relation-

ships obtained for phenylbutazone and the enantiomers of warfarin, indicate that K_3, the binding constant of the solutes at the site from which they are displaced by octanoic acid, does not change with the addition of acid. This implies that the displacement of warfarin and phenylbutazone from Site I, by octanoic acid, is mediated by a simple competitive mechanism. This is contrary to the conclusions of Rippie [25] on the displacement of warfarin and phenylbutazone from HSA by medium- to long-chain fatty acids (C_{12}–C_{18}). For these acids, the mechanism of displacement appears to be allosteric in nature, however, shorter-chain acids, such as octanoic acid, may have their own, independent binding sites [26].

The magnitude of the binding affinity (K_2) of octanoic acid at the site from which it displaces phenylbutazone and the enantiomers of warfarin (approximately $1 \cdot 10^3\ M^{-1}$) is in good agreement with that previously determined for the binding of the acid to its secondary binding site [27]. It therefore appears that drug binding Site I is the secondary binding site of octanoic acid.

The displacement of the other solutes examined in this study by octanoic acid appears to be more complex, and the chromatographic results are therefore not adequately described by the model outlined above. Tolbutamide, ketoprofen and suprofen displayed bilinear plots, when treated according to eqn. 9. These compounds have been proposed to bind to both Site I and Site II [5], which may account for this observation.

The presence of small quantities (<0.25 mM) of octanoic acid in the mobile phase resulted in large (approximately 80%) decreases in the capacity factors of the enantiomers of the two NSAIDs examined. Despite these large decreases in retention, there were no concurrent reductions in enantioselectivity. This would indicate that the site from which the NSAIDs were displaced by initial addition of octanoic acid, the site which accounts for the majority of their binding to HSA, is not enantioselective. As the concentration of acid was increased, its effect on the retention of the NSAIDs was relatively well described by eqn. 9, indicating competitive displacement of the solutes from a second site. This secondary displacement did result in an eventual loss of chiral recognition, indicating the involvement of an enantioselective site. Examination of the contribution of the parameter X to the k' values of the NSAIDs on the HSA-CSP (Table II) shows that for these compounds there is very little (3–7%) binding at sites which are unaffected by the presence of octanoic acid.

The nature of the displacement of the NSAIDs by low concentrations of octanoic acid is not immediately apparent. However, we have previously determined [28], that the addition of a constant concentration of octanoic acid to the mobile phase of the HSA-CSP affected the binding of a series of NSAIDs in a manner proportional to their initial binding affinities, *i.e.* those compounds with the highest binding affinities experienced the greatest amount of displacement by the acid. If the displacement were simply competitive in nature, then the inverse of this situation would have been observed, *i.e.* the lesser bound members of the series would have experienced the greater displacement. The initial, large displacement of suprofen and ketoprofen by octanoic acid is therefore probably allosterically mediated. The primary binding site for the ibuprofen-like NSAIDs has been said to be Site II [4,5]. Binding of octanoic acid to its primary binding site therefore induces a conformational change in the micro-environment of drug binding Site II, causing the displacement of compounds binding there.

The linear regions of the plots obtained for suprofen and ketoprofen represent the simple competitive displacement of the NSAID enantiomers by octanoic acid, and therefore values of K_2 may be extracted. Using only the linear portion of the plots for suprofen and ketoprofen, values of K_2 of between 1200, for suprofen A, and 3500, for suprofen B, are obtained, with the enantiomers of ketoprofen falling between these extremes. These values are consistent with that determined above for the binding affinity of octanoic acid in its displacement of phenylbutazone and the enantiomers of warfarin (*i.e.* at binding Site I). Sjöholm *et al.* [4,5] have suggested that the minor binding site for NSAIDs is the warfarin site, and

that tolbutamide binds approximately equally to both major sites. This would certainly account for the behavior of suprofen, ketoprofen and tolbutamide observed on the HSA-CSP.

The influence of various alkanoic acids on the chromatography of a range of solutes, including ketoprofen, on a CSP based upon bovine serum albumin (BSA), has been examined by Allenmark and Andersson [29,30]. The results of these authors are consistent with those reported here, retention and enantioselectivity factors for the NSAIDs being reduced by addition of the alkanoic acid. They also found [29] that the displacement of the NSAIDs could not be explained by simple competition at a single site.

The binding behavior of the enantiomers of OXH was poorly described by eqns. 6 and 9. Earlier studies employing the HSA-CSP have shown that the enantiomers of OXH do not compete for the same binding site(s) [12]. The (*S*)-enantiomer of OXH is strongly displaced by substrates which bind to Site II, such as the enantiomers of ibuprofen, whereas the binding of *R*)-OXH is unaffected. Only (*S*)-OXH, but not (*R*)-OXH, displayed a sharp decrease in affinity (corresponding to approximately 70%) with the addition of 0.25 m*M* acid, in a manner similar to that observed for the NSAIDs. This would be consistent with (*S*)-OXH binding to Site II on HSA. However, at least two other types of behavior may be discerned at higher acid concentrations. We are currently examining alternative strategies to explain the observations obtained for OXH.

Further evidence for the validity of the models described in this paper comes from the fact that the ratios of the affinity constants (K_3), obtained for the enantiomers of warfarin, ketoprofen and suprofen, were proportional to the chromatographic enantioselectivity factors (α) observed on the HSA-CSP (Fig. 6). Oxazepam hemisuccinate, which was poorly described by the present models, again proved to be anomalous, when viewed from this perspective.

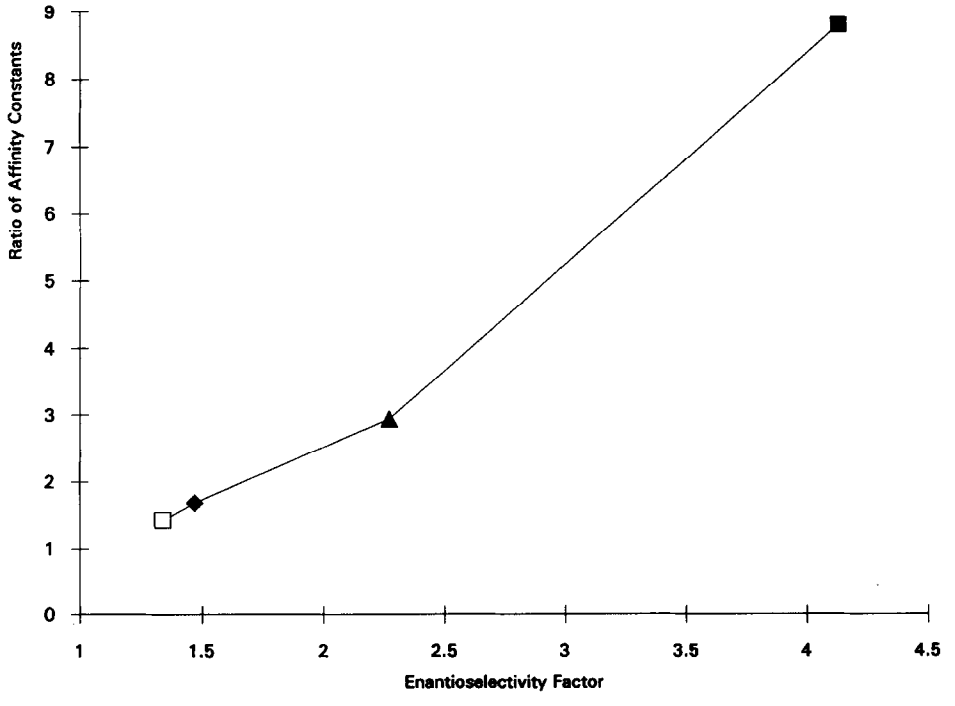

Fig. 6. Relationship between enatioselectivity factor (α), as determined on the HSA-CSP under the conditions described in the text, and the ratio of the binding affinities of the enantiomers (K_3, determined from the plot of $1/(k' - X)$ *versus* concentration of octanoic acid). □ = Ketoprofen; ▲ = suprofen; ◆ = warfarin; ■ = oxazepam hemisuccinate.

CONCLUSION

Protein-based stationary phases for HPLC present a new and useful approach to the study of the interaction between compounds co-binding to the immobilized protein. The present study, using a stationary phase based on HSA, shows that this approach, when combined with the application of appropriate mathematical treatments, can provide subtle information on the interaction between binding sites on the protein molecule.

The results of the present study indicate that binding of octanoic acid at its primary site causes an allosteric change in binding Site II, which results in the displacement of suprofen and ketoprofen, and possibly (*S*)-oxazepam hemisuccinate and tolbutamide. At higher concentration levels, the acid appears to bind directly at Site I, the warfarin–azapropazone binding area, from which it is able to displace target solutes in a competitive fashion.

The binding of suprofen and ketoprofen to their primary binding site, Site II, is not enantioselective; the difference in the binding of the enantiomers of these solutes to HSA arises from differential attachment at Site I.

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