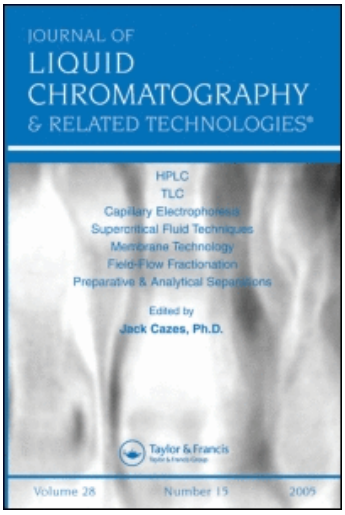

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The Use of Displacement Chromatography to Alter Retention and Enantioselectivity on a Human Serum Albumin-Based HPLC Chiral Stationary Phase: A Mini-Review

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THE USE OF DISPLACEMENT CHROMATOGRAPHY TO ALTER RETENTION AND ENANTIOSELECTIVITY ON A HUMAN SERUM ALBUMIN-BASED HPLC CHIRAL STATIONARY PHASE: A MINI-REVIEW

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ABSTRACT

The chromatographic properties of protein-based HPLC chiral stationary phases (CSPs) have been manipulated using a variety of neutral and charged mobile phase modifiers. These compounds have usually been alkyl alcohols and amines, acetonitrile and octanoic acid. This mini-review discusses another approach to the control of chromatographic retention (k') and enantioselectivity (α) on a human serum albumin based-CSP, HSA-CSP, which utilizes the binding characteristics of the protein. The addition to the mobile phase of compounds known to bind to HSA such as warfarin, ibuprofen and tryptophan altered k' and α for a variety of solutes. In some instances, direct competition reduced these variables, but the opposite result was also obtained due to allosteric interactions. These interactions, their chromatographic applications and pharmacological implications are examined in this presentation.

INTRODUCTION

The enantiomeric composition of pharmacologically active chiral compounds has become a key question in the development and use of therapeutic

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agents. This concern is a recognition of the fact that enantiomers of bioactive compounds often exhibit very different therapeutic and toxic effects as well as different pharmacokinetic, pharmacodynamic and metabolic fates [1]. The increased consciousness of the impact of stereochemistry on biological activity has been due to a great extent to the development of specific chromatographic techniques for the direct analytical and preparative separation of enantiomers. The most successful of these approaches has been high performance liquid chromatography (HPLC) utilizing chiral stationary phases (HPLC-CSPs) [2-4].

The chromatographic utility of the HPLC-CSPs has resulted in the development of over 55 commercially available forms which can be divided into 5 classes (I - V) based on the chiral recognition process operating on the CSP [2,5,6]. As far as drugs and related compounds are concerned, the most useful phases have been the class V CSPs which are based upon immobilized proteins [2-4]. Since proteins are chiral polymers, the class V CSPs are successful because they exploit the natural enantioselectivity of these biopolymers. At the present time, the class V CSPs include phases derived from α_1-acid glycoprotein (AGP) [7], bovine serum albumin (BSA) [8], human serum albumin (HSA) [9] and ovomucoid (OVM) [10].

A key factor in the ability of a protein to discriminate between enantiomorphs is its tertiary structure which creates the hydrophobic pockets and ravines often necessary for the formation of the diastereomeric complexes. However, the tertiary structure is not fixed. Proteins are flexible and their three-dimensional structures can be dramatically altered by irreversible or reversible processes. Covalent immobilization on a silica support is an irreversible process which can modify a protein by restricting its mobility and freezing it into "unnatural" conformations, i.e. a tertiary structure which the native protein will not normally assume. Reversible conformational changes can occur when the protein's environment is altered; these environments include the buffer in which a native protein is dissolved or a mobile phase used with an immobilized protein CSP.

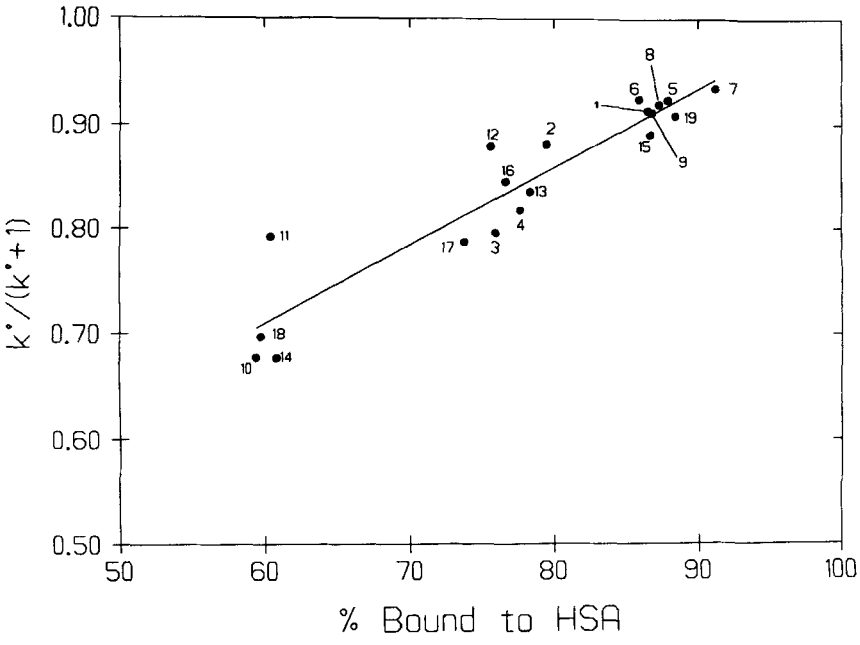

Figure 1. Relationship between the retention of a series of benzodiazepines on the HSA-CSP and their extent of binding to free HSA; for experimental details see Reference 15.

The effect that the immobilization of AGP, OVM and BSA has on their respective tertiary structures is unknown, we have determined that the immobilization of HSA onto silica does not significantly alter its ligand binding properties [11-15], nor its ability to alter its conformation in response to the binding of various ligands [12,13]. It is therefore possible to use the HSA-CSP to rapidly investigate the extent of binding to HSA and the enantioselectivity of this process [15]. This property of the HSA-CSP is illustrated by a comparison of the k's of 19 1,4-benzodiazepines on the HSA-CSP with the percentage of binding of each compound to free HSA, Figure 1 [15]. A correlation factor of 0.999 was obtained illustrating that the retention of a solute on the HSA-CSP is

clearly related to its binding affinity for the free protein and that this stationary phase can be used as a rapid probe of drug-protein binding.

THE USE OF MOBILE PHASE MODIFIERS

Since the tertiary structure of a protein can be significantly altered by changing the constitution of the solvent in which it is bathed, it is no surprise that the chromatographic properties of immobilized protein CSPs are highly dependent on the composition of the mobile phase. Retention (k') and enantioselectivity (α) are particularly sensitive to the addition of uncharged or charged modifiers to the mobile phase; pH, ionic strength and other factors, such as temperature also exert a significant influence on the chromatographic results. These effects are based, in part, on the fact that the retention of solutes on class V CSPs is largely due to hydrophobic and electrostatic interactions between the analytes and the immobilized biopolymer [16,17]. These interactions can be disrupted by addition of an organic solvent and by binding of a charged molecule to the protein.

Uncharged Mobile Phase Modifiers

The impact of uncharged modifiers on the chromatographic properties of a class V CSP are illustrated by the effect of adding 2-propanol or acetonitrile to the mobile phase used with the AGP-CSP. The enantiomers of the anti-arrhythmic drug verapamil were easily resolved on the AGP-CSP when an eluent containing acetonitrile was used. However, when an approximately iso-eluotropic amount of 2-propanol was used as modifier, no enantioseparation was observed [18]. The opposite results were obtained for methylphenobarbital where addition of 0.6 M 2-propanol produced an enantioselective separation (α = 1.25) while no stereoselectivity was observed with equimolar acetonitrile [18]. In addition, the enantiomers of warfarin were found to undergo a reversal in elution order on changing modifier from 2-propanol to acetonitrile [19]. These observations are reflective of the fundamental influence of uncharged organic modifiers on the conformation of the immobilized protein.

The effects of alcoholic modifiers and pH on the chromatographic properties of an OVM-CSP have also been investigated using acidic, basic and neutral solutes [20]. A series of primary, secondary and tertiary alcohols and pH's ranging from 3.5 to 6.0 were used in this study. Both the shape and the hydrophobicity of the alcoholic modifier affected k' and α with an increase in the hydrophobicity producing a decrease in k's and α's. However, this was not the case when *tert*-butanol was the modifier, suggesting that the size of the alkyl moiety attached to the carbinol carbon also contributes to the chromatographic results. The results of this study indicate that on the OVM-CSP, retention, enantioselectivity and chromatographic efficiency are a function of hydrophobic and coulombic interactions between the solute and the immobilized protein and that these parameters are influenced by the tertiary structure of the protein. This presents the possibility to dynamically alter the properties of the OVM-CSP by manipulating the composition of the mobile phase.

Charged Mobile Phase Modifiers

In addition to neutral organic compounds, charged molecules can be used as mobile phase modifiers, often with quite dramatic results. This approach has been extensively investigated for the AGP-CSP [3,21]. One of the major charged modifiers is N,N-dimethyloctylamine, DMOA, and addition of DMOA to the mobile phase reduces the k' and α for most basic compounds while having an opposite effect on acidic compounds [21]. In the latter instance, the addition of 10 μM DMOA increased the observed α for naproxen from 1.2 to 4.6 and for 2-phenoxypropionic acid from 1.03 to 2.3 [21]. Other charged modifiers such as tetrabutylammonium bromide and octanoic acid have also been utilized [22,23].

USING COMPETITIVE BINDING INTERACTIONS TO ALTER CHROMATOGRAPHIC RESULTS: A DIFFERENT APPROACH TO MOBILE PHASE MODIFIERS

HSA is the main plasma component responsible for the binding of drugs and it has been extensively studied and characterized. At the present time, it is

thought that ligand binding to HSA takes place at a small number of well defined drug binding sites [24]. The exact number of these sites remains the subject of some debate, but there is agreement on the existence of two major binding sites; the warfarin-azapropazone, and the indole-benzodiazepine sites (also known as Site I and Site II, respectively) [24]. A small number of compounds, such as digitoxin and tamoxifen, appear not to bind to either of these sites, necessitating the postulation of further, minor binding sites [24].

While the existence of distinct binding sites would seem to dictate a situation where only compounds which bind at the same site can interact, this is not the situation. Compounds which bind at different sites can affect each other by inducing conformational changes in the protein, i.e. allosteric interactions. Honoré [25], recently reviewed the potential interactions between ligands which simultaneously bind to a protein. The various possibilities are: 1) the ligands bind independently of each other and there is no interaction (**independent** binding); 2) binding of one ligand facilitates the binding of the other (**cooperative** binding); 3) binding of one of the ligands induces an allosteric change which decreases the ability of the second compound to bind (**anti-cooperative** binding); 4) two ligands bind competitively (**non-cooperative** binding).

Since chromatographic retention and enantioselectivity on an HSA-CSP reflects the binding affinity of the solute for the protein [15], the co-binding of another ligand should also affect k' and α. Thus, another class of mobile phase modifiers are compounds known to bind to the protein-based CSP. The affect of this class of modifiers on k' and α are discussed below.

Displacement Effects (Non-Cooperative Binding)

When a compound added to the mobile phase binds to the same site on the protein as a solute injected onto the column, competition exists for that binding site, resulting in a decrease in k' for the injected solute. For instance, the results of studies on free HSA suggest that the drugs ibuprofen and oxazepam hemi-succinate (OXH) compete for binding at the same site (the indole-benzodiazepine

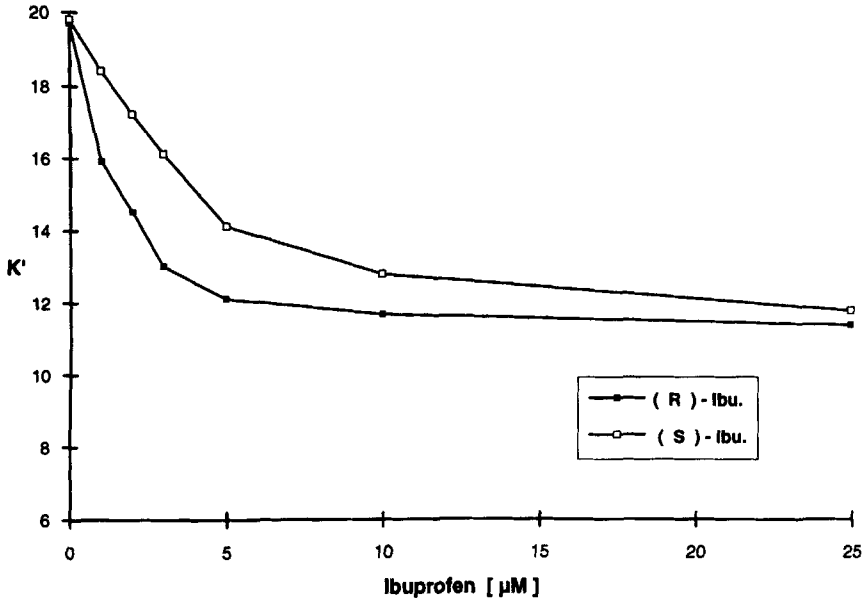

Figure 2. The effect on the k' S-oxazepam hemisuccinate (OXH) of the addition of R- and S-ibuprofen (IBU) to the mobile phase. Where ■ = k' S-OXH [R-IBU added to the mobile phase]; □ = k' S-OXH [S-IBU added to the mobile phase]; for experimental details see Reference 11.

binding site) [26]. This competition was reflected by a decrease in the k' of (S)-OXH when either enantiomer of ibuprofen was added to the mobile phase, Figure 2, [11]. The retention of (R)-OXH, on the other hand, was unaffected by ibuprofen in the mobile phase. This indicates that the chiral discrimination of the enantiomers of OXH by HSA results from the binding of the individual isomers at different sites on the protein. Only (S)-OXH appears to bind at the same site as (R) and (S)-ibuprofen. Figure 2 also demonstrates that the effect of (R)-ibuprofen on the retention of (S)-OXH is greater than that of its enantiomer; reflecting the higher affinity of (R)-Ibuprofen for HSA [26].

A similar, although much smaller, effect on the retention of the enantiomers of OXH was seen when the enantiomers of tryptophan (Trp) were

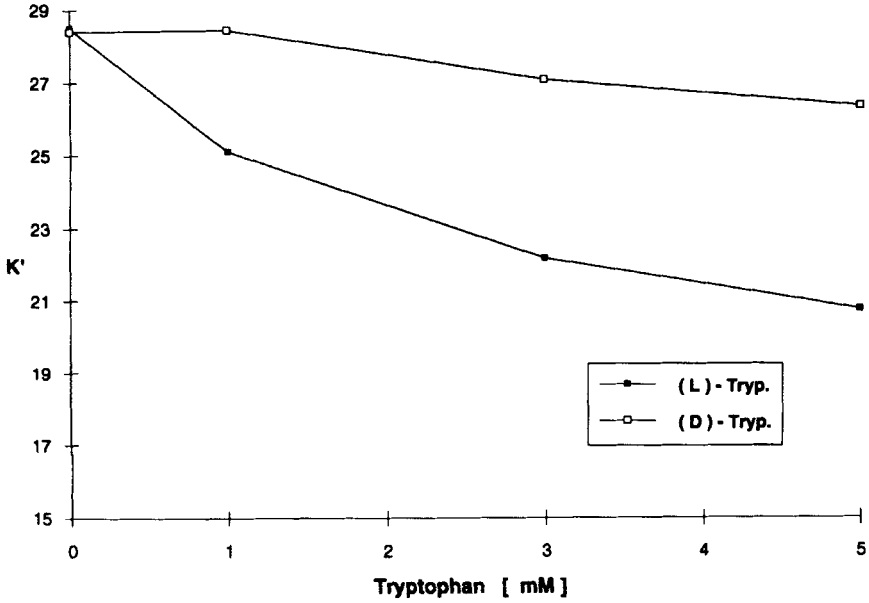

Figure 3. The effect on the k' S-oxazepam hemisuccinate (OXH) of the addition of L and D-tryptophan (TRP) to the mobile phase. Where ■ = k' S-OXH [L-TRP added to the mobile phase]; □ = k' S-OXH [D-TRP added to the mobile phase]; for experimental details see Reference 11.

individually added to the mobile phase, Figure 3, [11]. In this case, much higher amounts of specific modifier (mM, as opposed to the μM amounts used with ibuprofen) were required to effect displacement of (S)-OXH, and the effect of L-Trp was significantly greater than that of the D-enantiomer. This observation is consistent with the fact that the binding affinity of (S)-OXH for native HSA is considerably greater than that of L-Trp, which in turn is much larger than that of the D-form.

The displacement of one enantiomer, injected onto the column, by its opposite antipode added to the mobile phase, can be indicative of the mechanism by which HSA is able to express enantioselectivity towards a particular solute.

TABLE 1. The Chromatographic Interaction of Ibuprofen Enantiomers on a HSA-CSP. Where the competitor is the opposite enantiomer to that injected. See Reference 27 for the chromatographic conditions.

ENANTIOMER INJECTED	CONCENTRATION OF COMPETITOR (μM)	K'
R-Ibuprofen	0.0	73.41
	2.0	66.84
	4.0	61.86
	6.0	56.26
	8.0	45.27
	10.0	41.17
S-Ibuprofen	0.0	21.37
	1.0	16.33
	2.0	14.58
	4.0	12.72
	6.0	11.54
	8.0	10.79
	10.0	10.26

For instance, the addition of (R)-ibuprofen to the chromatographic mobile phase effects a significant reduction in the k' of the (S)-enantiomer injected onto the column, Table 1 [27]. Similarly, the (S)-enantiomer of ibuprofen, when used as a mobile phase additive, affects the retention of the (R)-form, Table 1, although higher levels are required to effect similar reductions in k', due to the lower affinity of this isomer for HSA [26]. These results indicate that both enantiomers of ibuprofen bind to a large degree to the same site on HSA, and that this site is the likely source of the enantioselectivity observed in the binding of the drug to the native protein.

Allosteric Enhancement of Enantioselectivity (Cooperative Binding)

Many compounds which bind to HSA cause a reversible change in the protein's conformation. In certain cases, this conformational change may affect a remote binding site in such a way that its ability to bind particular ligands is significantly enhanced. Occasionally, the ability of the affected site

to distinguish between enantiomers binding there is augmented, leading to an increase in enantioselectivity. Such "allosteric" effects have been characterized for native HSA, and are retained in the HSA-CSP [12,13].

Domenici *et al.* recently reported the effect on the k's and α of (R)- and (S)-lorazepam hemisuccinate (LOH) when the enantiomers of warfarin are used as mobile phase additives [12]. When (R)-warfarin was added to the mobile phase there was no effect on the retention of either enantiomer of LOH. However, when (S)-warfarin was used as the mobile phase modifier, there was a stereoselective effect on retention - the k' of (R)-LOH remained constant while the k' of (S)-LOH dramatically increased. The addition of 10 μM of (S)-warfarin to the mobile phase resulted in a 72% increase in the k' of (S)-LOH and a 76% increase in enantioselectivity, from α = 1.40 to α = 2.47, Figure 4.

When lorazepam was the solute, a similar although smaller (4%), increase in the k' of the second eluting enantiomer of lorazepam was observed. This effect resulted in an increase in α from 1.10 to 1.14 which significantly improved a marginal enantioseparation. In this case, the cooperative binding interaction was of practical benefit, whereas in the case of LOH it was not needed since the natural enantioselectivity of the HSA-CSP was adequate for analytical purposes.

Allosteric Decrease of Enantioselectivity (Anti-Cooperative Binding)

Allosteric interactions between binding sites need not always result in cooperativity. In many cases, the changes induced in the microenvironment of the affected site will result in a decreased ability to bind certain ligands. This form of "anti-cooperative" binding interactions is illustrated by the effect of fatty acids on the chromatography of a series of nonsteroidal antiinflammatory drugs related to ibuprofen, NSAIDs [13,27]. When the NSAIDs were chromatographed on the HSA-CSP, the addition of octanoic acid to the mobile phase resulted in a significant reduction in the k's of the solutes [13,27]. The

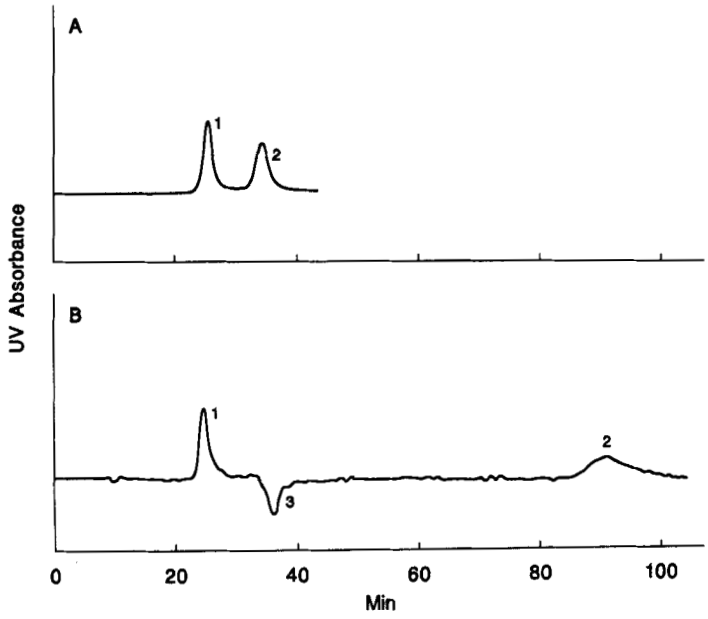

Figure 4. The chromatographic profile following the injection of (R,S)-lorazepam hemisuccinate (LOH) onto the HSA-CSP; where A: without (S)-warfarin in the mobile phase; B: with 40 μM (S)-warfarin in the mobile phase. Key: 1 = (R)-LOH; 2 = (S)-LOH; 3 = system peak corresponding to (S)-warfarin; for experimental deatails see Reference 12.

magnitudes of the observed reductions were proportional to the initial binding affinities of the NSAIDs, i.e. the stronger the affinity of the drug for HSA, the larger the decrease in k'. If the decreases in k's were the result of competitive displacement of the NSAIDs by octanoic acid, an inverse relationship between affinity and k' would have been observed, i.e. the more weakly bound solutes would have been more greatly affected. Thus, the displacement of NSAIDs by octanoic acid was anti-cooperative.

This conclusion was confirmed by graphical plotting of the effect of different concentrations of octanoic acid on k' [13]. If a plot of the inverse of the capacity factor, or the inverse of the capacity factor from which a constant

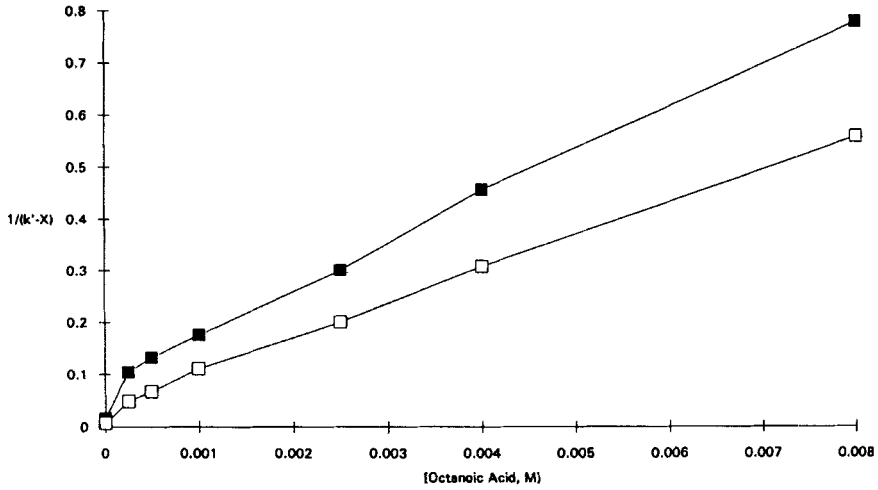

Figure 5. The effect of octanoic acid on the retention of suprofen on the HSA-CSP; where ■ = first eluted enantiomer of suprofen, □ = second eluted enantiomer of suprofen; for experimental details see Reference 13.

has been subtracted, against the concentration of the mobile phase additive is linear, then the binding affinity of the solute throughout the concentration range studied is constant, and therefore, by inference, the mechanism of displacement could not be allosteric. However, as demonstrated by the plot for the enantiomers of the NSAID suprofen, Figure 5, there is an initial discontinuity in the plot indicating that an allosteric interaction has occurred.

Anti-cooperative interactions can be used to improve chromatography on the HSA-CSP. For example, medium chain fatty acids (C_8 - C_{10}) can be added to the mobile phase to reduce retention and improve chromatographic performance. This is illustrated by the development of an analytical method for the enantiomers of ibuprofen on the HSA-CSP [27]. Due to the high affinity of ibuprofen for HSA, the k's of its enantiomers on the HSA-CSP were extremely high (total analysis time > 3 h), even at the upper levels of organic modifier permissible on this phase (15 -20%, v/v). However, addition

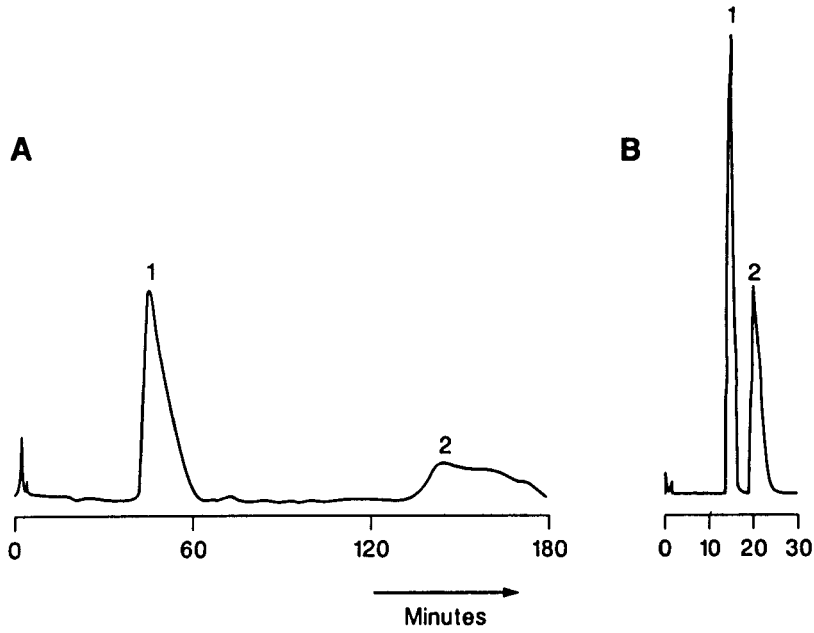

Figure 6. Enantioselective resolution of ibuprofen where 1 = (S)-ibuporfen and 2 = (R)-ibuprofen. A: without octanoic in the mobile phase; B: with 4 mM octanoic acid added to the mobile phase; for experimental details see Reference 27.

of a small quantity (4 mM) of octanoic acid to the mobile phase resulted in a large reduction in the k' of both enantiomers, to give an analysis time of just over 20 min, while maintaining adequate enantioselectivity, Figure 6 [27].

Independent Binding

Another possibility arising from the addition of specific modifiers to the chromatographic mobile phase is that the additive and the injected solute bind to different sites, and no energetic interaction occurs between them. Clearly, this situation is of no value in the improvement of chromatography for analytical purposes. However, even the fact that a mobile phase additive has

TABLE 2. The Effect on the Chromatographic Retention (k') of Oxazepam Hemisuccinate Enantiomers, (R)-OXH and (S)-OXH, as a Result of the Addition of the Opposite Enantiomer (designated as the Competitor) to the Mobile Phase. See Reference 11 for experimental details.

ENANTIOMER INJECTED	COMPETITOR CONCENTRATION (Mm)	k'
(R)-OXH	0.000	8.36
	0.005	8.31
	0.010	8.26
	0.020	8.17
	0.050	8.00
(S)-OXH	0.000	22.86
	0.005	22.80
	0.010	22.69
	0.020	22.20
	0.050	nd[a]

[a] Not determined due to large background noise in the chromatogram and poor peak efficiency.

no effect on the retention of a test solute may prove significant, given the useful predictive nature of the HSA-CSP for the behavior of the native protein.

When one enantiomer of OXH is added to the mobile phase, it has no effect on the retention of the other isomer, Table 2 [11]. It has been proposed that the high enantioselectivity observed in the binding of benzodiazepines, including OXH, results from binding of the enantiomers to the same site, with different affinities. However, the results of the competition studies shown in Table 2 do not support this hypothesis.

The presence of one enantiomer in the mobile phase, even at reasonably high concentrations, does not significantly affect the k' of the other isomer. This would imply that the large degree of enantioselectivity observed in the binding of OXH to HSA results from the separate attachment of the individual enantiomers to discrete sites. This assumption is borne out by protein binding studies, Table 3 [11], and by the fact that neither enantiomer

TABLE 3. The Binding Affinities of the Enantiomers of Oxazepam Hemisuccinate, (R)-OXH AND (S)-OXH, Alone and in Presence of the Opposite Enantiomer (designated as the Competitor) as Determined by Ultrafiltration Studies. See Reference 11 for experimental details.

COMPOUND	WITHOUT COMPETITOR	WITH COMPETITOR
(R)-OXH	$n_1k_1 = 9.5 \times 10^3$	$n_1k_1 = 8.0 \times 10^3$
(S)-OXH	$n_1k_1 = 2.9$ x0 10^5 (n_1 0.69)	$n_1k_1 = 2.8 \times 10^5$ ($n_1 = 0.73$)
(S)-OXH	$n_2k_2 = 8.0 \times 10^3$	$n_2k_2 = 8.5 \times 10_3$

of ibuprofen is able to cause a displacement of (R)-OXH, even though they were both able to displace the much more highly bound (S)-enantiomer at the same concentrations [11]. Studies on other chiral benzodiazepines have shown that this behavior holds true for the class as a whole [14].

The Effect of Mixed Modifiers

While it appears that the effects observed using the HSA-CSP are generally reflective of properties of the native protein, some care should be exercised in translating observations made in one system directly to the other. For instance, it is generally accepted, based on the results of studies carried out on native HSA, that L-tryptophan and the benzodiazepines share a binding site on HSA. In the chromatographic situation, when L-Trp (2 mM) was added to a mobile phase of buffer containing 10% (v/v) 1-propanol, a 20% decrease in the k' of (S)-OXH was observed; which is consistent with the anticipated competitive displacement. However, when the organic component of the mobile phase was changed to 10% (v/v) acetonitrile, the addition of 2 mM L-Trp produced a 15% increase in the k' of (S)-OXH. Clearly, the presence of acetonitrile induced a change in the tertiary structure of the immobilized protein, causing an inversion of the "natural" binding behavior.

Somewhat analogous differences in the effects of acetonitrile and alcoholic modifiers have been encountered with the AGP-CSP, as pointed out

above [18]. In the case of the AGP-CSP, it was postulated that certain organic solvents were able to induce changes in the secondary structure of the immobilized protein, inducing regions of helicity, and as a consequence radically altering its overall conformation, and hence enantioselectivity [18]. It seems highly likely that similar effects are operative on the HSA-CSP.

CONCLUSION

The use of CSPs based upon immobilized proteins provides a means of unparalleled versatility for the resolution of drug enantiomers. In the case of the HSA-CSP, behavioral properties of the native protein can be used to gain insight into the processes involved in retention and enantioseparation, and thereby to assist the development of chromatographic methods. In turn, data collected using the HSA-CSP, which benefit from chromatographic precision and reproducibility, can be used to further explore the nature of binding sites on the protein, and the interrelationships that exist between them.

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