Journal of Chromatography A, 666 (1994) 221–234
Elsevier Science B.V., Amsterdam

CHROM. 25 640

Enantioselective high-performance liquid affinity chromatography as a probe of ligand–biopolymer interactions: an overview of a different use for high-performance liquid chromatographic chiral stationary phases

Irving W. Wainer

Department of Oncology, McGill University, Montreal, Quebec (Canada)

ABSTRACT

HPLC chiral stationary phases (CSPs) have become routine analytical and preparative tools. These phases have also been the subject of extensive studies concerned with the chiral recognition mechanisms responsible for the observed enantioselectivity. The mechanistic studies have provided the possibility to stretch the use of CSPs beyond separation science and into the study of ligand–biopolymer interactions. The essential observation in this strategy is that in an enantioselective chromatographic process, the non-specific interactions between the enantiomeric solutes, stationary phase and mobile phase can be cancelled out and the intermolecular interactions between the enantiomeric solutes and chiral selector isolated and studied. When the chiral selector is a biopolymer such as a protein or enzyme, this technique can be used to probe the qualitative and quantitative aspects of the ligand–biopolymer interactions. Examples using immobilized serum albumin- and α-chymotrypsin-based CSPs are presented.

CONTENTS

0021-9673/94/$26.00
SSDI 0021-9673(93)E1083-C

1. INTRODUCTION

Chiral stationary phases (CSPs) are typically regarded only as implements for use in analytical and preparative separations of enantiomeric compounds. While these phases have been extremely successful chromatographic tools, this view ignores another important property of CSPs, their ability to probe intermolecular interactions. This latter application is based upon the role that CSPs play in the chromatographic process and indeed, the chromatographic process itself.

The retention and enantioselective separation of a chiral compound on a CSP is the sum total of multiple interactions between enantiomeric solutes, stationary phase and mobile phase. It is these intimately connected solute–mobile phase and solute–CSP processes which create the potential to use these phases as biochemical probes. The key is the recognition that the observed chromatographic retention is the result of two interrelated but separate processes: (1) non-enantioselective interactions between the enantiomeric solutes, stationary phase and mobile phase which are identical for each enantiomer of the solute; (2) enantioselective interactions between the enantiomeric solutes and the chiral selector which differ for each enantiomer if enantioselectivity is observed.

The essential observation is that in an enantioselective chromatographic process, the non-specific interactions between the enantiomeric solutes, stationary phase and mobile phase can be cancelled out because the physicochemical properties of enantiomers are equivalent. Thus the intermolecular interactions between the enantiomorphs of the solute and chiral selector can be isolated and studied.

This observation in and of itself is not novel. Since the introduction by Pirkle *et al.* [1] of the (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine CSP, chiral recognition mechanisms have been extensively studied using methods as diverse as computational chemistry [2,3], quantitative structure–retention relationships (QSRR) [4], NMR [5] and "common chemical sense". These studies have revealed a subtle process in which a free energy difference ($\Delta\Delta G$) of 410 cal/mol can produce an enantioselectivity factor (α) of 2.00 [6]. In addition, the observed enantioselectivity can be the result of a single chiral recognition mechanism or the sum of two or more complementary or antagonistic processes [7].

The efforts involved in the studies of chiral recognition mechanisms have been directed primarily at improving analytical methods and designing new CSPs. The rapid evolution of commercially available CSPs is the direct consequence of the knowledge acquired from these studies. While these endeavours are important activities, they are not the only purpose for studying chiral recognition. Another area where the enantioselective chromatographic process can be utilized is the investigation of ligand–biopolymer interactions.

This can be accomplished by combining the attributes of affinity chromatography with those of biopolymer-based CSPs and by using some of the basic experimental and computational methods employed in the study of chiral recognition mechanisms.

2. AFFINITY CHROMATOGRAPHY USING BIOPOLYMER-BASED HPLC CSPs

The use of biopolymers, in particular proteins, enzymes and antibodies, as chromatographic stationary phases is an integral part of affinity chromatography. Although this technique is primarily perceived as method to isolate and purify biologically active compounds, it can also be used to assess ligand–protein, substrate–enzyme and protein–protein interactions [8,9]. The evolution of silica-based supports for affinity chromatography has resulted in the development of high-performance liquid affinity chromatography (HPLAC) which has also been used to measure equilibrium or kinetic constants of ligand–protein and protein–protein interactions [9]; for example, the rate constants for the association of sugar derivatives with concanavalin A, a saccharide-binding protein [10].

HPLAC can also be used to investigate competitive protein binding interactions [10,11]. When immobilized concanavalin A was the stationary phase and *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-α-D-glucopyranoside

and *p*-nitrophenyl-α-D-glacatopyranoside were the solutes, the addition of α-methyl-D-mannoside or α-methyl-D-glucoside to the mobile phase resulted in a decrease in the chromatographic retention, k', of the solutes. A plot of $1/k'$ *versus* modifier concentration, *i.e.* α-methyl-D-mannoside or α-methyl-D-glucoside, yielded straight lines indicating that the modifiers and the solutes were competing for the same binding site on the protein.

Another series of HPLAC phases have been developed for a conceptually different but related area of work. These are the biopolymer-based CSPs which were developed for enantioselective analytical and preparative separations of chiral molecules. The chiral selectors on these CSPs include derivatized cellulose and amylose, cyclodextrins, proteins and enzymes.

The protein- and enzyme-based CSPs utilize the inherent enantioselectivity of these biopolymers and are closely related to HPLAC phases. This relationship is illustrated by an early study of the effect of mobile phase additives on k' and enantioselectivity (α) of warfarin (WAR) enantiomers on a bovine serum albumin-based CSP (BSA-CSP) [12,13]. One of the modifiers studied was trichloroacetic acid (TCA) which was chosen based on previous work which demonstrated that TCA displaced WAR from human serum albumin (HSA) [14]. When TCA was consecutively added to the mobile phase, up to a concentration of 5 m*M*, there was approximately a 50% reduction in the k' values of both (*R*)-WAR and (*S*)-WAR, while α was reduced by only 3%, from 1.20 to 1.16. A plot of $1/k'$ *versus* TCA concentration yielded straight lines for both (*R*)- and (*S*)-WAR, Fig. 1. This indicates that (*R*)- and (*S*)-WAR bind at the same site on the BSA molecule as TCA does.

A series of *in vitro* protein binding studies were performed to determine whether the decreases in k' produced by TCA reflected similar decreases in the total BSA protein binding of the WAR enantiomers. When log k' was plotted against percent of the drug bound to BSA, straight lines were obtained for both (*R*)- and (*S*)-WAR with correlation coefficients (R^2) of 0.991 and 0.999, respectively. The slope of the line from (*S*)-WAR was significantly greater than

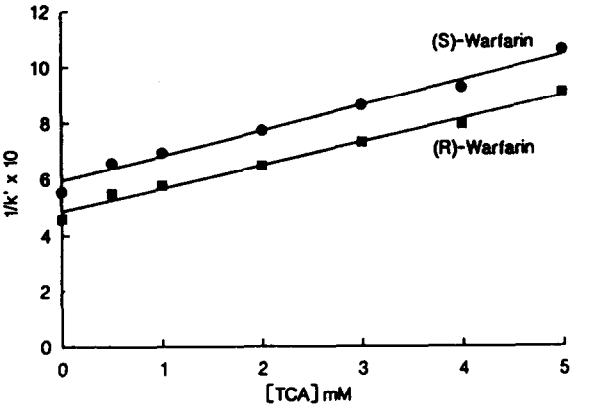

Fig. 1. The effect of trichloroacetic acid (TCA) on the retention of warfarin enantiomers [(*R*)-WAR and (*S*)-WAR] on a bovine serum albumin-based stationary phase, where ● = (*S*)-WAR, ■ = (*R*)-WAR. Adapted from ref. 12.

that of the line from (*R*)-WAR reflecting the more than two-fold difference between the affinity constants of the enantiomers, $570 \cdot 10^{-3}$ and $250 \cdot 10^{-3}$, respectively [15].

3. USING ENANTIOSELECTIVE HPLAC TO PROBE LIGAND–BIOPOLYMER INTERACTIONS

The development of efficient and reproducible biopolymer-based HPLC stationary phases and the demonstration that chromatographic retention and stereochemical selectivity on these phases reflect the properties of the non-immobilized biopolymer, has led us to propose the following experimental strategy. This strategy incorporates the observations that in an enantioselective chromatographic process, the non-specific interactions between the enantiomeric solutes, stationary phase and mobile phase can be cancelled out because the physicochemical properties of enantiomers are equivalent. The intermolecular interactions between the enantiomers of the solute and chiral selector can be isolated and studied by using one enantiomer as the internal control for the other. This approach is an extension of and an improvement upon "Analytical Affinity Chromatography" described by Chaiken [8] as: "a means to use matrix–mobile interactant systems to study mechanisms of biomolecular interactions and therein to attain an understanding of such interactions which are

often not easily achieved by solution methods alone".

In addition to the central strategy and immobilized biopolymer-based HPLC stationary phases, this method employs two experimental techniques —displacement chromatography and the development of QSRR. These procedures are described below.

3.1. High-performance displacement chromatography

High-performance displacement chromatography, which has been previously described for affinity-based chromatographic systems [8,10,11], is based upon interactions between two compounds which result from their mutual binding to a common site on the stationary phase. This technique can be used to determine the magnitude of the interactions and the site or sites on the protein at which they occur. The experimental technique is straightforward and has been previously described in the following terms [16]: (1) one of compounds, the "solute", is injected onto the biopolymer-based stationary phase and its retention, k', measured; (2) the other compound, the "displacer", is added to the mobile phase and its concentration is systematically increased during a series of experiments; (3) the effect of the "displacer" concentrations on the k' of the "solute" is then measured; (4) either compound can be used as the "displacer" or the "solute". When the "solute" is chromatographed using a mobile phase which does not contain the "displacer", k' is directly proportional to its binding affinity for the immobilized biopolymer. When the "displacer" is added to the mobile phase, the magnitude and direction of the resulting changes in k' can be used to determine the binding site of the ligand and to identify non-competitive or allosteric interactions.

3.2. Quantitative structure–retention relationships (QSRRs)

QSRRs are the result of the application of the methodology used for quantitative structure–biological activity relationships (QSAR) to the analysis of chromatographic data [17]. Two types of data are needed for QSRR studies: (1) a set of quantitatively comparable retention data for a sufficiently large set of solutes; (2) a set of molecular structural descriptors which reflect the physicochemical and structural properties of the solutes. The experimental approach requires that the chromatographic conditions, *i.e.* temperature, mobile phase and stationary phase, remain constant while a series of related solutes are chromatographed.

Through the use of multiparameter regression analysis or factor analysis, the logarithms of the observed k' values and the molecular structural descriptors can be mutually related. If statistically significant and physically meaningful QSRRs are obtained, they can be used to predict k' for a new solute; identify the most informative structural descriptors; gain insight into the molecular recognition mechanisms operating in a given chromatographic system; evaluate complex physicochemical properties of solutes; and even predict relative biological activities within a set of solute xenobiotics. When the chromatographic phase is a biopolymer, QSRRs can be used to investigate the strength of solute–biopolymer interactions as well as the mechanism of this interaction.

4. APPLYING ENANTIOSELECTIVE HPLAC

In order to give the reader an idea of the utility of this approach, an overview of some of the initial results using this strategy are presented below.

4.1. Determination of protein binding sites using a HSA-CSP

HSA is a globular, hydrophobic protein which has been shown to enantioselectively bind small enantiomeric molecules [18]. There are two commonly accepted drug-binding sites on HSA: the warfarin-azapropazone-binding area (Site I) and the indole and benzodiazepine binding site (Site II). An additional one (digitoxin [19]) to three sites (digitoxin, bilirubin and fatty acid [20]) have also been proposed.

Both Site I and Site II are enantioselective in

their binding interactions [15]. The enantioselectivity of the warfarin-azapropazone-binding area has been investigated by a number of laboratories, and, as stated above, (*S*)-WAR is more highly bound to HSA than (*R*)-WAR [14]. The indole and benzodiazepine binding site has also been the subject of a number of investigations and appears to be more enantioselective than the warfarin-azapropazone-binding area. For example, the ratio of the affinity constants of (+)-oxazepam hemisuccinate/(−)-oxazepam hemisuccinate is 49.5 [15].

The different binding sites on HSA for 140 compounds have been studied using competitive binding experiments [19]. This approach used HSA entrapped in spherical, macroporous polyacrylamide microparticles and radioactively labelled markers. However, the method was cumbersome and time consuming and could not determine the enantioselectivity of the binding interactions. In addition, when a compound displaced more than one marker, this technique could not clearly delineate between primary and secondary binding sites nor could it identify allosteric interactions. These issues can be readily addressed using enantioselective HPLAC.

When (*R*,*S*)-ibuprofen (*R*,*S*-IBU) was chromatographed on the HSA-CSP, the enantiomers were resolved with an $\alpha = 3.4$. When (*R*)-IBU and (*S*)-IBU were added independently to the mobile phase as part of a competitive displacement chromatographic study, there was a clear interaction between the two enantiomers, Table 1 [7]. The addition of (*R*)-IBU decreased the k' of (*S*)-IBU and the same effect was observed when (*S*)-IBU was the displacer, although the magnitude of the effect differed —100 μM (*R*)-IBU reduced the k' of (*S*)-IBU by 51% while 100 μM (*S*)-IBU reduced the k' of (*R*)-IBU by 44%. The difference in the magnitude of the effect is consistent with previous studies which determined that (*R*)-IBU had a higher binding affinity than (*S*)-IBU [15]. The results of this study indicate that at least part of the binding of (*R*)- and (*S*)-IBU to HSA occurs at the same site on the protein and involves the same mechanism.

A different result was obtained with the enantiomers of the benzodiazepine (BDZ) derivative oxazepam hemisuccinate (OXH) [21]. It has been generally assumed that the enantiomers of a chiral BDZ bind at the same site on HSA. However, the results of the competition studies carried out on the HSA-CSP, Table 1, do not support this hypothesis. The addition of up to 0.050 mM of (*S*)-OXH to the mobile phase had relatively little effect on the k' of (*R*)-OXH and, conversely, the addition of up to 0.020 mM of (*R*)-OXH had only a slight effect on the k' of (*S*)-OXH. These results indicate that (*R*)-OXH and (*S*)-OXH do not bind at the same site on the HSA molecule.

Further proof of different binding sites for (*R*)- and (*S*)-OXH was provided by competitive

TABLE 1

CHANGES IN THE CHROMATOGRAPHIC RETENTION OF THE ENANTIOMERS OF IBUPROFEN, (*R*)- AND (*S*)-IBU, AND OXAZEPAM HEMISUCCINATE ENANTIOMERS, (*R*)- AND (*S*)-OXH, AS A RESULT OF THE ADDITION OF THE OPPOSITE ENANTIOMER TO THE MOBILE PHASE

Data obtained from refs. 7 and 21.

Solute/competitor	Competitor concentration (mM)	k'
(*S*)-IBU/(*R*)-IBU	0.000	21.37
	0.005	18.27
	0.010	16.33
	0.050	11.69
	0.100	10.47
(*R*)-IBU/(*S*)-IBU	0.000	73.41
	0.020	66.84
	0.040	61.68
	0.100	41.17
	0.200	31.83
(*R*)-OXH/(*S*)-OXH	0.000	8.36
	0.005	8.31
	0.010	8.26
	0.020	8.17
	0.050	8.00
(*S*)-OXH/(*R*)-OXH	0.000	22.86
	0.005	22.80
	0.010	22.69
	0.020	22.20
	0.050	nd[a]

[a] Not determined.

displacement studies using the IBU enantiomers [21]. When (*R*)- and (*S*)-IBU were added to the mobile phase, the *k'* of (*S*)-OXH was reduced while the *k'* of (*R*)-OXH remained constant, Fig. 2. Since IBU is known to bind at Site II [15], these studies confirm that only (*S*)-OXH is bound to the BDZ binding site.

4.2. Determination of interspecies differences in protein binding using albumin-based CSPs

In addition to determining ligand binding to HSA, enantioselective HPLAC techniques can also be used to probe interspecies differences in protein binding. This chromatographic approach combined with chiral solutes can determine the magnitude and enantioselectivity of ligand binding to the respective proteins and also provide information on binding behaviour which is not readily available from standard protein binding studies. This is illustrated by the results obtained on CSPs made from three different serum albumins —rat (RtSA-CSP), rabbit (RbSA-CSP) and human (HSA-CSP) [22].

A series of achiral and chiral compounds were chromatographed on the CSPs including BDZs, non-steroidal anti-inflammatory drugs (NSAIDs), amino acids, WAR and leucovorin. The observed enantioselectivities, and where

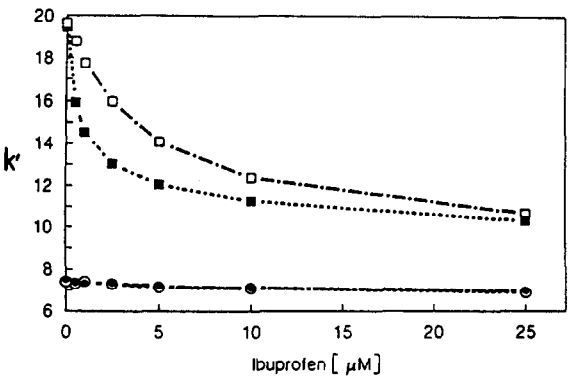

Fig. 2. The effect on the *k'* values of the enantiomers of oxazepam hemisuccinate [(*R*)-OXH; (*S*)-OXH of the addition of (*R*)- and (*S*)-ibuprofen to the mobile phase, where ■ = *k'* of (*S*)-OXH [(*R*)-ibuprofen in the mobile phase]; □ = *k'* of (*S*)-OXH [(*S*)-ibuprofen]; ● = *k'* of (*R*)-OXH [(*R*)-ibuprofen]; ○ = *k'* of (*R*)-OXH [(*S*)-ibuprofen]. Adapted from ref. 21.

possible elution orders, for the chiral solutes are presented in Table 2 [22].

All of the chiral BDZs were stereochemically resolved on each of the albumin stationary phases indicating that the binding process for these compounds was enantioselective. The observed α values were smaller on the RtSA-CSP than the other CSPs except for compound 1 where the α values were 1.70 and 1.65 on the RtSA-CSP and HSA-CSP, respectively and 2.10 on the RbSA-CSP. Since the BDZs used in this study rapidly racemize in aqueous solutions [23], it was not possible to determine the relative enantioselectivities.

When the chiral NSAIDs where chromatographed, a different pattern was observed. There was no significant difference in the degree of enantioselectivity for compounds 19, 22 and 24 on the three albumins while for compounds 20 and 21, no enantioselectivity was observed on the RtSA-CSP. The relative enantioselectivities were investigated by chromatographing unequal mixtures of the enantiomers. On all of the CSPs, the (*S*)-enantiomers eluted before the (*R*)-enantiomers indicating a greater affinity for the (*R*)-form. The relative enantioselectivities for compounds 22 and 24 and the lack of enantioselectivity in the binding of compounds 20 and 21 to RtSA were confirmed by ultrafiltration studies.

The chromatography of D- and L-tryptophan on the SA-CSPs resulted in the stereochemical separation of these enantiomers. In this case, the degree of enantioselectivity displayed by the RtSA-CSP was about 3.5-fold higher than the other two proteins while the relative enantioselectivity, L-tryptophan bound to a greater extent than D-tryptophan, was the same for all three SAs. The relative enantioselectivity is consistent with previously reported findings [15,24].

Other differences in the stereochemical selectivities of the three SAs were observed with N-benzoylphenylalanine and leucovorin. In the case of N-benzoylphenylalanine, enantioselective separations were observed on the RtSA-CSP ($\alpha = 1.47$) and HSA-CSP ($\alpha = 2.13$) but not on the RbSA-CSP. Leucovorin is a diastereomeric compound and the separation of the active (6*S*,*S*)-isomer from the inactive (6*R*,*S*)-form was

TABLE 2

ENANTIOSELECTIVITY (α) AND ELUTION ORDER OF SOLUTES CHROMATOGRAPHED ON HPLC CSPs BASED UPON RAT RtSA-CSP), RABBIT (RbSA-CSP) AND HUMAN (HSA-CSP) SERUM ALBUMINS

Data obtained from ref. 22.

Compound	Enantioselectivity (elution order)		
	RtSA-CSP	RbSA-CSP	HSA-CSP
1 Lormetazepam	1.70	2.10	1.65
2 Lorazepam	1.05	2.60	1.36
3 Temazepam	1.25	1.60	2.46
4 Clorazepate	1.72	2.10	2.60
19 Fenoprofen	1.24	1.22	1.51
20 Ketoprofen	1.0	1.32	1.24
21 Suprofen	1.0	1.85	3.96
22 Ibuprofen	2.41(*S*,*R*)	2.18(*S*,*R*)	1.60(*S*,*R*)
24 Flurbiprofen	1.43(*S*,*R*)	1.75(*S*,*R*)	2.09(*S*,*R*)
26 Tryptophan	7.40(D,L)	2.14(D,L)	2.20(D,L)
28 N-benzoylphenylalanine	1.47	1.0	2.13
29 Warfarin	1.47(*R*,*S*)	1.57(*S*,*R*)	2.56(*R*,*S*)
31 Leucovorin	1.0	1.0	2.33

observed on the HSA-CSP but not on the RtSA-CSP and RbSA-CSP.

When the enantioselective binding of (*R*)- and (*S*)-WAR to site I of the respective SAs was investigated, the magnitudes of the observed α values on the RbSA and RtSA were indistinguishable. However, on the RtSA-CSP and HSA-CSP, (*R*)-WAR eluted before (*S*)-WAR while the opposite elution order was observed on the RbSA-CSP. These enantioselectivities are consistent with previously reported data from WAR protein binding studies using HSA, RtSA and RbSA [15,24]. The results indicate that the stereochemical environment of site I in the RbSA, and therefore, the binding properties, are dramatically different from those found in the RtSA and HSA.

4.3. Determination of protein binding interactions using a HSA-CSP

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 altered. The resulting allosteric interactions can produce an increase in binding affinity (a cooperative interaction) or a decrease (an anti-cooperative interaction). These interactions are difficult to detect using standard protein binding techniques, but can be readily identified using enantioselective HPLAC.

4.3.1. Cooperative interactions

One cooperative interaction which has been identified on HSA occurs between (*S*)-WAR which binds at Site I and the enantiomers of lorazepam (LO) which bind at Site II [25]. This interaction was investigated using the HSA-CSP, (*S*)-WAR and (*R*)- and (*S*)-lorazepam hemisuccinate (LOH) [26]. When a (*S*)-WAR concentration of 10 μM was added to the mobile phase, there was no increase in the k' of (*R*)-LOH and a 72% increase in the k' of (*S*)-LOH resulting in a 76% increase in enantioselectivity, from $\alpha = 1.40$ to $\alpha = 2.47$, Fig. 3. This change in enantioselectivity effect clearly demonstrates a cooperative interaction between (*S*)-WAR and (*S*)-LOH.

Cooperative interactions have also been demonstrated between chiral BDZs [temazepam (TZ) and oxazepam (OX)] and digoxin [27]. In this study, as in the study utilizing (*S*)-WAR, the addition to the mobile phase of 100 μM digoxin

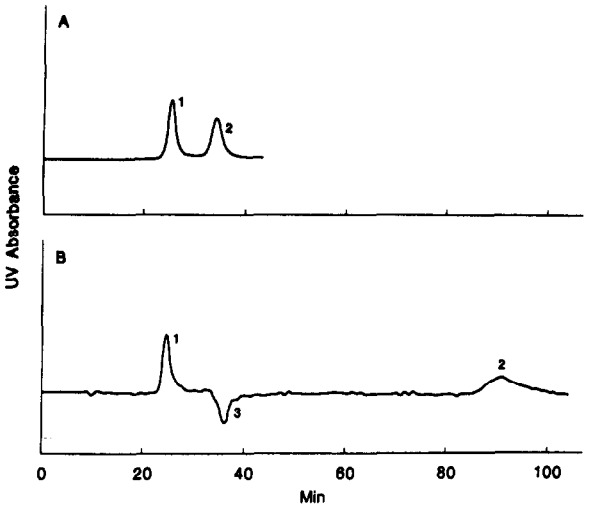

Fig. 3. The chromatographic profile following the injection of (*R*,*S*)-lorazepam hemisuccinate (LOH) onto the HSA-CSP; (A) without (*S*)-warfarin in the mobile phase; (B) with 40 μM (*S*)-warfarin in the mobile phase. Key: 1 = (*R*)-LOH; 2 = (*S*)-LOH; 3 = system peak corresponding to (*S*)-warfarin; adapted from ref. 26.

had no effect on the first eluted enantiomers of TZ and OX, nor was there an effect on the corresponding enantiomer of LO, Table 3. However, the presence of digoxin in the mobile phase produced a significant increase in the retention of the second eluted enantiomers of TZ and OX, but had only a slight effect on the corresponding enantiomer of LO.

The difference in the observed effect of digoxin on k' and α for TZ and OX *versus* LO is opposite of the effect observed when WAR is the mobile phase modifier. At 10 μM, WAR had no effect on the k' or α of TZ or OX [26,27] while when the WAR concentration reached 150 μM, the k' and α values decreased [27]. When LO was the solute, the addition of WAR (10 μM [26] and 50–150 μM [27]) produced an increase in the k' of the second eluted enantiomer producing an increase in α. This data illustrates the unique data which can be rapidly obtained using biopolymer-based CSPs.

4.3.2. *Anti-cooperative interactions*

Anti-cooperative interactions are difficult to detect since they can often be confused with displacement by direct competition. However, these interactions can be recognized using enantioselective HPLAC techniques. This is illustrated by the effect of octanoic acid on the retention on the HSA-CSP of a series of NSAIDs [28,29].

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' values of the solutes; for example, the addition of 4 mM octanoic acid to the mobile phase resulted in an 83% reduction in the k' of (*S*)-IBU [28,29]. The 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' values were the result of competitive displacement, 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, in this case, the displace-

TABLE 3

THE EFFECT OF DIGOXIN ON THE RETENTION AND ENANTIOSELECTIVITY ON THE ENANTIOMERS OF THREE CHIRAL BENZODIAZEPINES ON THE HSA-CSP

Data obtained from ref. 27.

Benzodiazepine	Without digoxin			With digoxin (0.1 mM)		
	k_1'	k_2'	α	k_1'	k_2'	α
Temazepam	2.7	19.3	7.2	2.7	32.0	11.8
Oxazepam	3.7	12.0	3.2	3.7	15.3	4.1
Lorazepam	5.0	7.0	1.4	5.3	8.0	1.5

ment of NSAIDs by octanoic acid, the mechanism appears to be anti-cooperative.

This conclusion was confirmed by graphical plotting of the effect of different concentrations of octanoic acid on k' [29]. The relationship between k' and octanoic acid concentrations can be expressed by the following equation [29]:

$$\frac{1}{(k'-X)} = \frac{V_M K_2[D]}{K_3 m_L} + \frac{V_M}{K_3 m_L} \quad (1)$$

where V_M = void volume of the column; K_2 and K_3 = equilibrium constants for the binding of the displacer and test solute, respectively; m_L = moles of the test solute bound to the HSA stationary phase; [D] = concentration of the displacer in the mobile phase; X = residual k' resulting from binding at sites unaffected by the displacer.

If both the test solute and the displacer bind at only one site on the HSA, then $X = 0$, and a plot of $1/k'$ *vs.* [D] will be linear. This was not observed for ketoprofen, Fig. 4, where the initial addition of 0.1 m*M* octanoic acid to the mobile phase produced an 75% decrease in k' and a discontinuity in the curve described by eqn. 1. It is important to note that the HSA-CSP is equilibrated with the octanoic acid containing mobile phase before the injection of the test solutes. Thus, even low concentrations of the acid will saturate the binding sites of the protein and induce the conformational changes which result in the decreased binding affinity.

The discontinuity in the curve appears to be indicative of a non-cooperative interaction. In addition, the displacement occurs with only a very small effect on the observed enantioselectivity indicating that the site from which the ketoprofen enantiomers were initially displaced was not the site which accounts for the enantioselective binding to HSA.

4.4. Determination of binding affinity constants

Eqn. 1 predicts that a plot of $1/(k' - X)$ *versus* [D] for a system with competition at a single site should give a linear relationship with a slope of $(V_M K_2/K_3 m_L)$ and an intercept of $(V_M/K_3 m_L)$. The value of K_2, the binding affinity constant for the displacer, can be determined directly by calculating the ratio of the slope to intercept for this plot.

When (*R*)-IBU was used as a displacer for a series of BDZs, the calculated K_2 for (*R*)-IBU was $1.4 \cdot 10^6\ M^{-1}$ [30]. The binding affinities of the individual enantiomers of IBU for HSA have not been reported, although the binding affinity of the racemate was determined by equilibrium

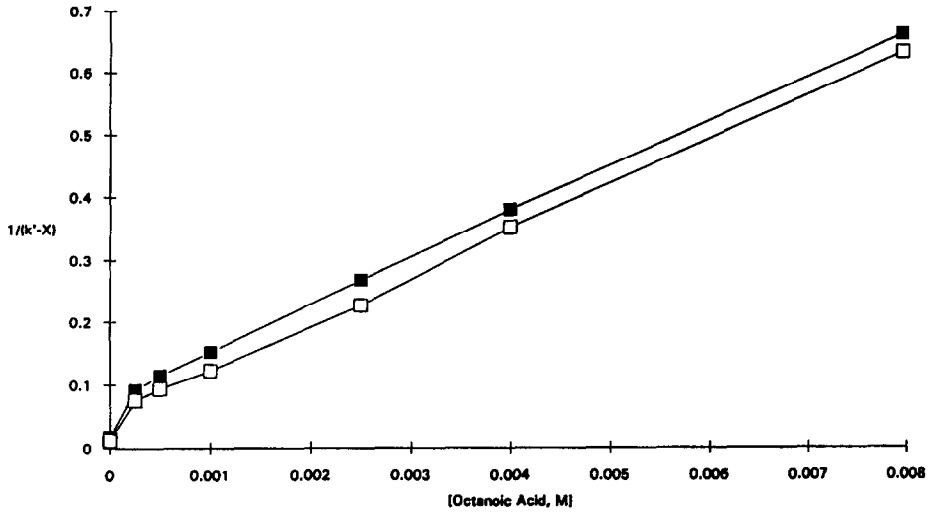

Fig. 4. The effect of octanoic acid on the retention of ketoprofen on the HSA-CSP; where ■ = first eluted enantiomer of ketoprofen, □ = second eluted enantiomer of ketoprofen; adapted from ref. 29.

dialysis to be $2 \cdot 10^6\ M^{-1}$ [31]. In the same study, the calculated K_2 for diazepam at the BDZ binding site was $7.2 \cdot 10^5\ M^{-1}$ [30] which is in agreement with the previously reported value of $4.7 \cdot 10^5\ M^{-1}$ [32].

4.5. Using molecular biochromatography for the computational prediction of retention and enantioselectivity on the HSA-CSP

QSRRs were derived for the chromatography of a series of 1,4-benzodiazepines (BDZs) on the HSA-CSP [4,33]. Molecular modelling of the solutes allowed the determination of various structural descriptors which included: P_{SM}, a submolecular polarity parameter; f_y and f_x, hydrophobicity of the substituent at position 7 in the fused benzene ring and at position 2′ of the phenyl system, respectively; $C(3)$, excess positive charge on carbon C(3) of the 1,4-diazepine system; W, width of the molecule; β_{CCN} angle formed by atoms C(2)-C(3)-N(4) of the diazepine ring. Using these descriptors, the retention for the first eluting peak of a chiral benzodiazepine could be described using eqn. 2 [33]:

$$\log k'_1 = -1.7497 + 0.3895(\pm 0.0751)\log f_y - 1.8392(\pm 0.5020)C(3) - 0.1609(\pm 0.0485)W + 0.0354(\pm 0.0150) \times \beta_{CCN} + 0.1736(\pm 0.0939)f_x \quad (2)$$

$$n = 21 \quad R = 0.8926 \quad F = 10.5 \quad p < 2 \cdot 10^{-4}$$

Combining P_{SM} with the retention parameter of the first eluting enantiomer permitted precise prediction of the retention of the second eluting enantiomer using eqn. 3 [33].

$$\log k'_2 = -0.1049 + 1.0739(\pm 0.0616)\log k'_1 + 0.5458(\pm 0.0318)P_{SM} \quad (3)$$

$$n = 16 \quad R = 0.9884 \quad F = 276 \quad p < 10^{-6}$$

Highly statistically significant regression equations were also derived which described both achiral and enantioselective retentions in terms of non-empirical molecular descriptors. The calculated enantioselectivity (α_{clcd}) correlated well with the experimentally observed values (α_{detd}): $\alpha_{detd} = 0.0994 + 0.8710\alpha_{clcd}$; $n = 16$, $R = 0.9974$ [33].

This approach could also be used to elucidate the structural and stereochemical aspects of the BDZ binding to HSA [33]. The results of these studies are presented in Fig. 5I and 5II.

4.6. Immobilized enzyme CSPs

Immobilized enzyme CSPs, IME-CSPs, have been synthesized through the covalent immobilization on HPLC supports of α-chymotrypsin (ACHT) [34,35], trypsin (TRYP) [36] and cellulase [37]. ACHT and TRYP phases have also been constructed by trapping the enzymes in inter-phases on an immobilized artificial membrane (IAM) HPLC support [38]. In some cases, the immobilized enzymes retained their activity and mirrored the properties of the free enzymes [38,39]. The active IME-CSPs could be used to

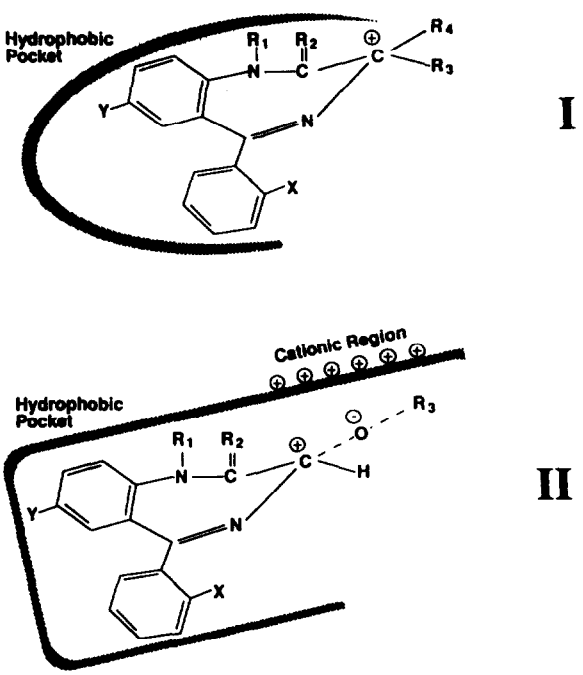

Fig. 5. Model for the structural requirements of the two postulated types of benzodiazepine binding to human serum albumin; where I = non-enantiospecific binding for benzodiazepines in the P conformation; II = enantiospecific binding for benzodiazepines in the M conformation; for experimental details see ref. 30.

rapidly screen for both enzyme substrates as well as determine the kinetic properties of the respective enzymes [38,39]. In addition, the IME-CSPs could also be used to identify and investigate the properties of enzyme inhibitors including a qualitative assessment of the type of enzyme inhibition, *i.e.* competitive, non-competitive, etc., and a quantitative evaluation of inhibition constants (K_I) [38,40]. The IME-SPs were stable and could be used for multiple studies. Enzyme-based stationary phases containing lipase and microsomes have been recently reported [41,42].

4.7. Identification of binding sites on α-chymotrypsin using an ACHT-CSP

An active form of the covalently immobilized ACHT-CSP was capable of resolving a number of enantiomeric compounds including D,L-tryptophan amide and N-benzoyl-D,L-leucine [34,39]. The enantioselective resolution of D,L-tryptophan amide on this CSP was a function of the activity of the enzyme which hydrolyzed L-tryptophan amide but not the D-enantiomer. Thus, the observed chromatographic resolution was actually the separation of L-tryptophan and D-tryptophan amide. However, neither the L- or D- forms of N-benzoylleucine are substrates for ACHT and the observed chiral separation of this compound was based only on differential binding to the protein.

Since it is assumed that substrates and pseudo-substrates bind at the active site of an enzyme, the observed enantioselectivity of the ACHT-CSP should be the result of interactions at a single site, *i.e.* the active site of the ACHT. One method to determine whether this is indeed the case is to block the active site of the enzyme. This can be accomplished using N-tosyl-L-phenylalanine chloromethyl ketone (TPCK). If chiral recognition took place solely at the active site of the enzyme, the inactivation should result in a loss of stereoselectivity.

When the ACHT-CSP was treated with TPCK, the immobilized enzyme was deactivated. The column was no longer able to hydrolyze L-tryptophanamide and the chiral resolutions based on enzymatic activity were lost [39]. In addition, the TPCK-inactivated ACHT-CSP was also unable to stereochemically resolve N-benzoyl-D,L-leucine and the chiral resolutions based upon solute structure were also lost [39]. These results support a single site chiral recognition process.

However, the results obtained from the chromatography of racemic amino acid esters on the active and TPCK-inactive forms of the ACHT-CSP are not consistent with this chiral recognition process [39]. The results from the chromatography of three esters are presented in Table 4. Alanine is not a substrate of ACHT and the chiral resolution is due to the differential binding of the two enantiomers. L-Tyrosine methyl ester and L-tryptophan ethyl ester are substrates and are hydrolyzed by ACHT while the D-forms are not.

If a single site chiral recognition process is operating, then TPCK inactivation should result in the loss of stereoselectivity as was observed for D,L-tryptophanamide and N-benzoyl-D,L-leucine. However, the observed enantioselectivities for the esters actually increased on the inactive form of the ACHT-CSP, Table 4.

These results suggest that solute binding sites on the ACHT molecule exist outside of the active site of the enzyme and that the binding of amino acid esters at these sites is stereoselective. Therefore, the observed chiral resolution of the ester solutes on the ACHT-CSP must be the result of interactions at multiple sites on the ACHT molecule.

This conclusion was supported by the results obtained during the study of the chromatography of aspartame stereoisomers on the ACHT-CSP [43]. Aspartame, Nα-aspartyl-phenylalanine 1-methyl ester (APME), is a dipeptide which exists as 4 stereoisomers: LL-APME, DD-APME, DL-APME and LD-APME. The LL-/DD- and DL-/LD- are enantiomeric pairs and the two pairs of enantiomers are related to each other as diastereomers. The enantiomeric and diastereomeric pairs can be resolved on the ACHT-CSP.

The effect of the molarity of the phosphate buffer in the mobile phase on retention and stereoselectivity of APME stereoisomers on the ACHT-CSP was investigated and the results are presented in Fig. 6. It is of interest to note that the molarity of the phosphate buffer had a

TABLE 4

ENANTIOSELECTIVITY AND CHROMATOGRAPHIC RETENTION OF AMINO ACID ESTERS ON ACTIVE AND TPCK-DEACTIVATED *A*-CHYMOTRYPSIN-BASED HPLC CHIRAL STATIONARY PHASES (ACHT-CSP)

Data obtained from ref. 40.

Solute	ACHT-CSP (active)		ACHT-CSP (TPCK)	
	k_1' [a]	α [b]	k_1' [a]	α [b]
L-Alanine benzyl ester	4.19	1.74	1.22	4.06
D-Alanine benzyl ester	7.28		4.95	
L-Tyrosine methyl ester	0.41	1.0	0.38	1.98
D-Tyrosine methyl ester	0.42		0.72	
L-Tryptophan ethyl ester	1.21	1.26	0.98	1.46
D-Tryptophan ethyl ester	1.53		1.43	

[a] k' of the first eluted enantiomer.
[b] $\alpha = k_{\text{second eluted enantiomer}}/k'_{\text{first eluted enantiomer}}$.

greater and opposite effect on the APME stereoisomers containing L-phenylalanine than those containing D-phenylalanine.

When the molarity of the phosphate buffer was raised from 0.050 to 0.500 *M*, the k' for DL-APME dropped from 1.10 to 0.32 and the k' for LL-APME fell from 1.29 to 0.33. Over the same concentration range, the k' values for LD- and DD-APME rose from 0.17 to 0.20 (LD-) and 0.18 to 0.22 (DD). The observed α values also decreased —for LD/DL from 6.67 (0.050 *M*) to 1.60 (0.500 *M*) and for DD/LL from 7.33 (0.050 *M*) to 1.54 (0.500 *M*).

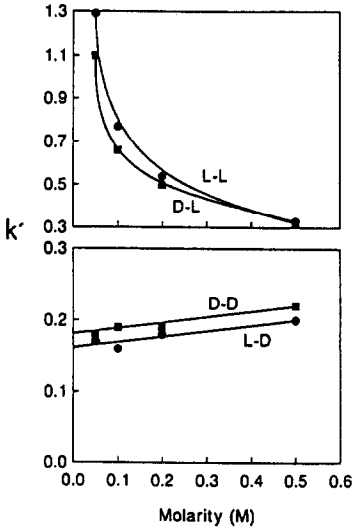

Fig. 6. The effect of phosphate buffer concentration on the retention of aspartame (APME) stereoisomers, where L-L = LL-APME; D-L = DL-APME; D-D = DD-APME; L-D = LD-APME. Adapted from ref. 40.

These results suggest the existence of two separate binding sites, the L-phenylalanine (L-Phe) and D-phenyl-alanine (D-Phe) sites. The decrease in k' for the DL- and LL-isomers indicates that the phosphate ion either competes for binding at the L-Phe site or that the increase in the ionic strength of the mobile phase decreases the affinity of the site for these isomers. If LD- and DD-APME were bound at the same site, their k' values should be affected in the same manner by the phosphate buffer concentration. Since the observed k' values of the LD- and DD-isomers increases, it appears that the phosphate ion does not compete at the D-Phe site and that a mobile phase with a high ionic strength increases the affinity of LD- and DD-APME for this site.

Standard techniques in enzymology can be used to measure global substrate/enzyme affinities, but, if multiple binding sites are present, these methods cannot easily separate the binding

process into its component parts. The possible existence of multiple binding sites and/or mechanisms is easier to probe with an enzyme-based stationary phase and chiral solutes because even subtle changes in the binding are reflected in changes in the observed enantioselectivities.

5. A LOOK TO THE FUTURE

The results of these studies demonstrate that the serum albumin-based stationary phases described in this review[a] can be used to study all aspects of ligand–protein binding including interspecies differences. The determination of chromatographic retention will yield qualitative and quantitative measures of the extent of protein binding when used in conjunction with a series of standards. If an enantioselective separation is observed, it will be a direct reflection of the magnitude and direction of the enantioselectivity involved in the binding of the ligand to the target protein. In addition, the use of displacement chromatography and QSRR analysis provide quantitative measures of drug–drug binding interactions and binding affinities as well as the molecular parameters describing the binding mechanisms.

The former application, detection of drug–drug binding interactions, is of particular interest in the pharmaceutical and clinical areas. When enantioselective HPLAC and displacement chromatography are combined, clinically relevant interactions can be rapidly identified. For example, patients undergoing treatment for arthritis with NSAIDs are often taking a variety of other drugs at the same time. The effect on the retention and enantioselectivity of the NSAID of adding these drugs to the mobile phase will be a direct indication of possible *in vivo* interactions. In this manner, a large number of potential interactions can be screened and the number of necessary clinical studies reduced.

The enzyme-based phases can be used in much the same manner. The injection of a potential substrate onto the phase will yield kinetic parameters and metabolic patterns. One can envision an on-line system coupled to a mass spectrometer which will yield direct information on the structure of the enzymatically produced compounds. When known or suspected enzyme inhibitors are added to the mobile phase, the type and mechanism of the inhibition can be readily identified. In this manner new drugs and potential drug interactions at the metabolic level can be identified.

The biopolymer-based CSPs do not have to be limited to serum proteins and enzymes. For example, one could envision stationary phases based upon receptors which would be used to rapidly screen new drug candidates. One possibility is a stationary phase based upon the calcium channel. This receptor has been shown to be enantioselective in its binding with (*S*)-verapamil bound to a greater extent than (*R*)-verapamil. Using the strategy outlined in this presentation, (*R*)-verapamil can be used as the internal control for the non-specific chromatographic interactions, while (*S*)-verapamil is the probe of the ligand–receptor interactions. The effect of adding a test compound to the mobile phase on the enantioselective separation of verapamil will yield a direct measurement of the relative affinity of the test compound for the receptor. If a related series of compounds are studied, a QSRR analysis can be undertaken leading to the design of new and more effective ligands.

Thus, the results presented in this manuscript can be viewed not as the conclusion of a body of work, but rather the start of new areas of research. In this manner, the lessons learned in enantioselective analytical and preparative chromatography can be used to solve new problems.

[a] At the present time there are at least two commercially available HSA-based CSPs. The phase used in the studies described in this paper was prepared by Shandon Scientific PLC, Runcorn, Cheshire, UK.

REFERENCES

1 W.H. Pirkle, J.M. Finn, J.L. Schriner and B.C. Hamper, *J. Am. Chem. Soc.*, 103 (1981) 3964.
2 K.B. Lipkowitz, D.A. Dementer, R. Zegarra, R. Larter, T. Darden, *J. Am. Chem. Soc.*, 110 (1988) 3446.
3 D.W. Armstrong, T.J. Ward, R.D. Armstrong and T.E. Beesley, *Science*, 232 (1986) 1132.

4 R. Kaliszan, T.A.G. Noctor and I.W. Wainer, *Chromatographia*, 33 (1992) 546.
5 W.H. Pirkle and T.C. Pochapsky, *J. Am. Chem. Soc.*, 109 (1987) 5975.
6 S.G. Allenmark, *Chromatographic Enantioseparations: Methods and Applications*, Ellis Horwood, Chichester, 1988, pp. 70–71.
7 I.W. Wainer, T.A.G. Noctor, E. Domenici and P. Jadaud, in S. Ahuja (Editor), *Chiral Separations by Liquid Chromatography*, American Chemical Society, Washington, DC, 1990, pp. 141–154.
8 I.M. Chaiken, *J. Chromatogr.*, 376 (1986) 11.
9 A. Jaulmes and C. Vidal-Madjar, *Adv. Chromatogr.*, 28 (1989) 1–64.
10 A.J. Muller and P.W. Carr, *J. Chromatogr.*, 184 (1984) 33.
11 D.J. Anderson, J.S. Anhalt and R.R. Walters, *J. Chromatogr.*, 359 (1986) 369.
12 I.W. Wainer and Y.-Q. Chu, *J. Chromatogr.*, 455 (1988) 316.
13 I.W. Wainer, in D. Stevenson and I.D. Wilson (Editors), *Recent Advances in Chiral Separations*, Plenum Press, New York, 1990, pp. 15–23.
14 E.M. Sellers and J. Koch-Weser, *Ann. N.Y. Acad. Sci.*, 179 (1971) 213.
15 T.A.G. Noctor, in I.W. Wainer (Editor), *Drug Stereochemistry: Analytical Methods and Pharmacology*, Marcel Dekker, New York, 2nd ed., 1993, pp. 337–364.
16 I.W. Wainer, R. Kaliszan and T.A.G. Noctor, *J. Pharm. Pharmacol.*, 45 (Suppl. 1) (1993) 367.
17 R. Kaliszan, *Quantitative Structure–Chromatographic Retention Relationships*, Wiley, NY, 1987.
18 G. Sudlow, D.J. Birkett and D.N. Wade, *Mol. Pharmacol.*, 11 (1975) 824.
19 I. Sjöholm, B. Ekman, A. Kober, I. Lunjstedt-Pahlman, B. Seiving and T. Sjödin, *Mol. Pharmacol.*, 16 (1979) 767.
20 K.J. Fehske, W.E. Müller and U. Wollert, *Biochem. Pharmacol.*, 30 (1981) 687.
21 E. Domenici, C. Bertucci, P. Salvadori, S. Motellier and I.W. Wainer, *Chirality*, 2 (1990) 263.
22 G. Massolini, A.F. Aubry and I.W. Wainer, *Biochem. Pharmacol.*, 46 (1993) 1285.
23 M. Stromar, V. Sunjic, T. Kovac, L. Klasinc and F. Kajfez, *Croatica Chem. Acta*, 46 (1974) 265.
24 C. Lagercrantz, T. Larsson and I. Denfors, *Comp. Biochem. Physiol.*, 69C (1981) 375.
25 I. Fitos, Z. Tegyey, M. Simonyi, I. Sjoholm, T. Larsson and C. Lagercrantz, *Biochem. Pharmacol.*, 35 (1986) 263.
26 E. Domenici, C. Bertucci, P. Salvadori and I.W. Wainer, *J. Pharm. Sci.*, 80 (1991) 164.
27 E. Chosson, S. Uzan, F. Gimenez, I.W. Wainer and R. Farinotti, *Chirality*, 5 (1993) 71.
28 T.A.G. Noctor, G. Félix and I.W. Wainer, *Chromatographia*, 31 (1991) 55.
29 T.A.G. Noctor, D.S. Hage and I.W. Wainer, *J. Chromatogr.*, 577 (1992) 305.
30 T.A.G. Noctor, C.D. Pham, R. Kaliszan and I.W. Wainer, *Mol. Pharmacol.*, 42 (1992) 506.
31 J.B. Whitlam, M.J. Crooks, K.F. Brown and P.V. Pederson, *Biochem. Pharmacol.*, 28 (1979) 675.
32 U. Kragh-Hansen, *Biochem. J.*, 209 (1983) 135.
33 R. Kaliszan, T.A.G. Nocotor and I.W. Wainer, *Mol. Pharmacol.*, 42 (1992) 512.
34 I.W. Wainer, P. Jadaud, G.R. Schonbaum, S.V. Kakodokar and M.P. Henry, *Chromatographia*, 25 (1988) 903.
35 I. Marle, A. Karlsson and C. Pettersson, *J. Chromatogr.*, 604 (1992) 185.
36 S. Thelohan, P. Jadaud and I.W. Wainer, *Chromatographia*, 28 (1989) 551.
37 I. Marle, P. Erlandsson, L. Hansson, R. Isaksson, C. Pettersson and G. Pettersson, *J. Chromatogr.*, 586 (1991) 233.
38 W.-K. Chui and I.W. Wainer, *Anal. Biochem.*, 201 (1992) 237.
39 P. Jadaud, S. Thelohan, G.R. Schonbaum and I.W. Wainer, *Chirality*, 1 (1989) 38.
40 T. Alebic-Kolbah and I.W. Wainer, *J. Chromatogr. A*, 653 (1993) 122.
41 T. Alebic-Kolbah and I.W. Wainer, *J. Chromatogr.*, 646 (1993) 289.
42 X.-M. Zhang and I.W. Wainer, *Tetrahedron Lett.*, 34 (1993) 4731.
43 P. Jadaud and I.W. Wainer, *Chirality*, 2 (1990) 31.