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DETERMINATION OF THE MAGNITUDE AND ENANTIOSELECTIVITY OF LIGAND BINDING TO RAT AND RABBIT SERUM ALBUMINS USING IMMOBILIZED-PROTEIN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY STATIONARY PHASES

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Abstract—Rat, rabbit and human serum albumins were immobilized on an HPLC stationary phase, and the resulting phases were tested for their abilities to determine the extent and enantioselectivity of ligand binding to the respective albumins. A series of achiral and chiral compounds were chromatographed on the phases including benzodiazepinones, non-steroidal anti-inflammatory drugs, amino acids, warfarin and leucovorin. The chromatographic retentions of the benzodiazepinones and one series of non-steroidal anti-inflammatory agents were compared with protein binding data from ultrafiltration studies. The observed correlation factors (r) were consistently 0.999, indicating that the albumin phases can be used to determine the magnitude of binding to the respective proteins. The enantioselectivity was also investigated, and the results indicate that the stationary phases can be used to determine relative enantioselectivities and intraspecies differences in this stereoselectivity. For example, when *R*- and *S*-warfarin were studied, *R*-warfarin was retained to a greater extent than *S*-warfarin by the rabbit serum albumin-stationary phase, whereas the opposite enantioselectivity was found for the rat and human albumins. Binding interaction studies were also conducted on the rabbit and rat albumin stationary phases by sequentially adding increasing concentrations of octanoic acid to the chromatographic mobile phase. The octanoic acid reduced the retention of a series of non-steroidal anti-inflammatory agents, and the results of the experiments suggest that the interaction takes place at two or more sites on the albumin molecule and by anti-cooperative allosteric interactions and competitive displacement. The results of this study demonstrate that the immobilized serum albumin columns can be used to quantitate and probe ligand binding interactions.

The reversible attachment to serum proteins often plays a significant role in pharmacokinetics and pharmacodynamics, and a clear understanding of this process is fundamental in the development and rational use of many therapeutic agents. Since serum albumin (SA‖) is a major component of plasma proteins, the mechanism and degree of drug–SA binding have been studied extensively. The initial studies of this process described it as non-specific, comparing the mechanism to adsorbance by charcoal. However, it quickly became clear that the binding to SA exhibits structural and physicochemical selectivity.

The three-dimensional structural selectivity, i.e. the ability of SA to differentially bind the stereoisomers of a compound, has been demonstrated for SAs from the human (HSA), rat (RtSA), rabbit (RbSA) and a variety of other species [1]. The degree and direction of the stereoselective drug binding process are not necessarily constant between species [1]; for example, HSA binds *S*-phenprocoumon to a greater extent than *R*-phenprocoumon, whereas RtSA and RbSA show the opposite enantioselectivity [2]. A similar qualitative difference exists in the binding of *R*- and *S*-warfarin, where HSA and RtSA bind *S*-warfarin to a greater extent than *R*-warfarin and RbSA has the opposite enantioselectivity [1, 3]. Since animal models are often used in protein binding, pharmacokinetic and pharmacodynamic studies and the conclusions derived from these studies extrapolated to humans, it is important to identify any intraspecies differences in the extent and enantioselectivity of the protein binding process.

Numerous methods have been described for the determination of drug–protein binding. Most involve equilibration of the drug with a solution of the target protein followed by the separation of free drug from bound using equilibrium dialysis, ultrafiltration, or ultracentrifugation. When the enantioselectivity of the binding is under investigation, these methods

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‖ Abbreviations: SA, serum albumin; HSA, human serum albumin; RtSA, rat serum albumin; RbSA, rabbit serum albumin; SP, stationary phase; QSRR, quantitative structure–retention relationship; BDZ, benzodiazepinone; NSAIDs, non-steroidal anti-inflammatory drugs; and 2-APA NSAIDs, 2-aryl-propionic acid non-steroidal anti-inflammatory drugs.

suffer from a number of disadvantages including the need for relatively large quantities of the separate enantiomers or the availability of a suitable enantioselective assay for the determination of the free concentration of the stereoisomers.

These problems can be avoided by immobilizing the protein of interest on a high performance liquid chromatographic (HPLC) support and chromatographing the target compounds on the resulting stationary phase (SP). Studies with a human serum albumin-based stationary phase (HSA-SP) have demonstrated that the chromatographic retention of a compound, expressed as capacity factor, k', is a direct reflection of the degree of binding to HSA [4, 5] and that the stereochemical resolutions achieved on this column reflect the stereoselectivity of the free protein [6–8]. Additional studies have been carried out on the HSA-SP by addition of compounds that act as competitive and non-competitive displacers to the mobile phase and evaluation of the changes in the chromatographic retentions of marker ligands using chemometric and quantitative structure–retention relationship (QSRR) analysis. The results of these investigations have shown that the enantioselective resolution of chiral benzodiazepinones (BDZs) is the result of two separate binding sites: a high-affinity site to which the M-conformers of the BDZs bind and a low-affinity site that binds both the M- and P-conformers of the drug [7, 9]. The QSRR studies were also used to develop a molecular description of the binding process [9].

This manuscript reports the extension of this approach to drug binding on RtSA and RbSA. Both proteins were immobilized on an HPLC stationary phase to produce a RtSA-SP and a RbSA-SP, and a series of chiral and non-chiral compounds were chromatographed on these phases. The results of these studies indicate that the RtSA-SP and the RbSA-SP can be used to determine the degree and enantioselectivity for each of these proteins as well as compare these properties between the two species and humans. Also reported are the initial investigations with the RtSA-SP and RbSA-SP of the effect of increasing concentrations of octanoic acid on chromatographic retention and enantioselectivity for a series of 2-aryl-propionic acid non-steroidal anti-inflammatory drugs (2-APA NSAIDs). The results indicate that octanoic acid displaces the 2-APA NSAIDs and that this binding interaction occurs at two or more sites and by two mechanisms, an anti-cooperative allosteric interaction and competitive displacement.

MATERIALS AND METHODS

Compounds used in the study (and the suppliers). BDZs: (**1**) (*R,S*)-lormetazepam (Sigma Chemical Co., St. Louis, MO); (**2**) (*R,S*)-lorazepam (Sigma); (**3**) (*R,S*)-temazepam (Sigma); (**4**) clorazepate (Sigma); (**5**) (*R,S*)-oxazepam (Sigma); (**6**) (*R,S*)-oxazolam (Sigma); (**7**) alprazolam (Upjohn Co., Kalamazoo, MI); and (**8**) (*R,S*)-4-hydroxy-alprazolam (Upjohn). NSAIDs: (**9**) phenylbutazone (Sigma); (**10**) oxyphenbutazone (Sigma); (**11**) piroxicam (Sigma); (**12**) sulfinpirazone (Sigma); (**13**) sulindac (Sigma); (**14**) diclofenac (Sigma); (**15**) diflunisal (Sigma); (**16**) flufenamic (Sigma); (**17**) mefenamic (Sigma); (**18**) niflumic (Sigma); (**19**) (*R,S*)-fenoprofen (Sigma); (**20**) (*R,S*)-ketoprofen (Sigma); (**21**) (*R,S*)-suprofen (Sigma); (**22**) (*R,S*)-ibuprofen (Upjohn); (**23**) (*S*)-ibufprofen (Upjohn); (**24**) (*R,S*)-flurbiprofen (Upjohn); and (**25**) (*S*)-flurbiprofen (Upjohn). Amino acids: (**26**) (D,L)-tryptophan (Sigma); (**27**) (L)-tryptophan (Sigma); and (**28**) *N*-benzoyl-(D,L)-phenylalanine (Sigma). Coumarins: (**29**) (*R,S*)-warfarin (Sigma); and (**30**) (*S*)-warfarin (DuPont Merck Pharmaceutical Co., Wilmington, DE). Anticancer drug: (**31**) (6*R*,6*S*)-leucovorin (Sigma).

Materials. Human, rat and rabbit albumins, fraction V powders, were purchased from the Sigma Chemical Co. Octanoic acid was obtained from the Aldrich Chemical Co. (Milwaukee, WI). All the other chemicals were of analytical grade and were obtained from the usual suppliers.

Preparation of immobilized SA stationary phases. The immobilization of RtSA, RbSA and HSA was carried out in semi-preparative size columns (100 mm × 10 mm i.d.) packed with diol-bonded silica (7 μm, 300 Å) following a previously described method [6]. The diol-bonded stationary phase was initally washed by pumping 100 mL of acetone through the column at a flow rate of 1 mL/min (this flow rate was used throughout the synthesis) followed by 100 mL of anhydrous dioxane. A solution of 1,1-carbonyl diimidazole (CDI) in anhydrous dioxane [30 g in 500 mL] was then pumped through the column, and the reaction between the CDI and the diol-bonded stationary phase was followed spectrophotometrically by monitoring the decrease in absorbance at 254 nm. The activation was completed in 6 hr and the stationary phase was washed with another 100 mL of anhydrous dioxane.

RtSA, RbSA and HSA were dissolved in 500 mL potassium phosphate buffer [1 mM, pH 7] to yield concentrations of 8, 8 and 10 g/L, respectively. The solutions were circulated over the activated stationary phases and the immobilization was followed by monitoring the UV absorbance at 236.5 nm. At the end of 4 hr, the columns were flushed sequentially with 100 mL of: potassium phosphate buffer [50 mM, pH 7] (BUFFER); sodium chloride [50 mM in water]: BUFFER, 50:50 (v/v); glycine [100mM in water]; BUFFER; sterile water.

The amount of protein immobilized on the silica support was calculated by measuring the decrease in absorbance at 236.5 nm of sequential 10-mL fractions collected throughout the synthesis. The stationary phases synthesized in this project contained the following mg protein/g silica support: RtSA-SP, 90 mg; RbSA-SP, 107 mg; HSA-SP, 95 mg. After completion of the synthesis, the 100 mm × 10 mm columns were unpacked and the stationary phases were repacked in 100 mm × 4.6 mm i.d. stainless steel columns that were used in this study.

High performance liquid chromatographic experiments. Chromatography was carried out isocratically using a modular HPLC system, which consisted of: a Spectra-system P1000 pump (Spectra-Physics, San Jose, CA), a Reodyne 7125 injection valve equipped with a 20-μL loop, a 783 programmable absorbance

detector (ABI Analytical, Ramsey, NJ) and DataJet integrator (Spectra-Physics). The mobile phases employed were based on sodium dihydrogen phosphate-disodium hydrogen phosphate [100 mM, pH 7.0], with or without 6% (v/v) 1-propanol, flow rate 0.8 mL/min. A 20-μL sample of a 100 μM solution of each compound was injected onto the HPLC system and detection of the solutes was by UV absorbance. The chromatographic parameters measured were: (1) capacity factor (k') which is defined as $(t_r - t_0)/t_0$, where t_r is the retention time of interest and t_0 is the retention time of an unretained solute; in this study 1-propanol was used to determine t_0; (2) enantioselectivity factor (α) which is the ratio of the k' of the second eluted enantiomer divided by the k' of the first eluted enantiomer.

Ultrafiltration experiments. The ultrafiltration was carried out as previously described using a disposable Centrefree Micropartition System (Amicon, Beverly, MA) [4]. Briefly, 50 μL of a 10-mM solution in 1-propanol of each compound was incubated for 2 hr at room temperature in 1 mL of a buffer solution [100 mM, pH 7.4] containing albumin [4 g, 100 mL]. After centrifugation, the concentrations of the compounds in the ultrafiltrates were quantitated photometrically, except for the experiments involving the BDZs, compounds **1–8**, and rat albumin. In these cases, an interfering absorbance was detected in the ultrafiltrate and the concentrations of the BDZs were determined chromatographically using the HPLC system described above. The enantioselectivity of the binding of compounds **22** and **24** was determined by ultrafiltration studies utilizing the separate enantiomers.

Analysis of results. The chromatographic and ultrafiltration data were compared using a previously derived approach [4, 10] in which the chromatographic data are expressed as $k'/(k' + 1)$ and are correlated with the degree of protein binding expressed as percent bound, $\{(C_{TD} - C_{FD})/C_{TD}\} \times 100$, where C_{TD} and C_{FD} are the total and free drug concentrations, respectively.

RESULTS AND DISCUSSION

Comparison of chromatographic retention on an immobilized SA-SP and extent of binding to the free albumin. A series of BDZ, compounds **1–8**, were chromatographed on stationary phases composed of immobilized RtSA-SP and RbSA-SP using a phosphate buffer mobile phase. The k' values for each compound was determined and compared with the corresponding albumin binding data obtained by ultrafiltration studies. The correlations between the results of the chromatographic and ultrafiltration experiments for both RtSA and RbSA were excellent with $r = 0.999$ (Table 1).

When the mobile phase was composed of only phosphate buffer, the elution times of some of the test compounds were too long for convenient analysis. This problem was overcome by the addition of 6% 1-propanol to the mobile phase, which dramatically reduced the retention of most of the solutes; for example, the retention of compound **9** on the RtSA-SP was reduced from $k' = 36$ (50.8 min) to $k' = 11.8$ (20 min).

To determine the effect on the correlation between chromatographic retention and protein binding caused by the addition of 1-propanol to the mobile phase, the experiments involving the BDZs were repeated with 1-propanol (6%, v/v) in the phosphate buffer. The results of this comparison are presented in Table 1 and indicate that the addition of 1-propanol did not alter the correlation significantly.

The retention of a solute on immobilized-protein SPs is a combination of specific binding interactions with the protein and non-specific interactions with the SP; the latter interactions include binding to unreacted diol and silanol moieties on the original diol-bonded SP. The addition of relatively small amounts of 1-propanol appears to have little effect on the solute–protein interactions perhaps because the alkyl chain of the alcohol is too short to effectively compete for hydrophobic binding sites or the hydrogen bonding interactions with the protein are similar to those of the aqueous medium. However, the addition of the alcoholic modifier will mask the silanol and hydroxyl moieties on the SP reducing the non-specific interactions and will also change the polarity of the mobile phase, thereby increasing the solubility of the solute in this phase. These effects would lead to a reduction in the chromatographic retention without altering the solute–protein interactions.

A series of achiral NSAIDs, compounds **9–18**, was also chromatographed on an immobilized HSA-SP, on the RtSA-SP and on the RbSA-SP. In these experiments, 6% 1-propanol was added to the phosphate buffer mobile phase to elute the compounds. The k' value for each compound was determined and compared with the corresponding albumin binding data from ultrafiltration studies. The correlations between the results of the chromatographic and ultrafiltration experiments for HSA, RtSA and RbSA were excellent with $r = 0.999$, and are presented in Table 1 and Fig. 1.

The results of these studies indicate that the RtSA-SP and RbSA-SP can be used to determine the extent of protein binding to the respective albumins. This is consistent with previous results obtained on the HSA-SP with a series of BDZs and a series of coumarin derivatives [4]. The data from this study and previous work also demonstrate that once the correlation between ultrafiltration studies and chromatographic retention has been established for a particular albumin stationary phase, relative protein binding studies for a wide variety of compounds can be carried out rapidly using standard chromatographic techniques. However, the chromatographic retention of a compound on one immobilized-SA phase, expressed as either k' or $k'/(k' + 1)$, cannot be used to predict protein binding to a different SA, since there are differences in both the amount of protein immobilized on each stationary phase and in the properties of the respective SAs.

Comparison of interspecies enantioselectivity in serum albumin binding. Proteins are chiral polymers with three-dimensional structures containing chiral ravines and pockets that are involved in ligand binding. These stereochemical environments are the source of the enantioselectivity of the protein and are ultimately a function of the primary amino acid

Table 1. Correlation coefficients between chromatographic retention expressed as $k'/(k'+1)$ and degree of protein binding determined from ultrafiltration studies for a series of BDZs and achiral NSAIDs

% 1-Propanol in mobile phase	Correlation coefficients					
	Rat		Rabbit		Human	
	BDZs	NSAIDs	BDZs	NSAIDs	BDZs	NSAIDs
0	0.999	NE‡	0.999	NE	0.999§	NE
6	0.996	0.999	0.997	0.999	ND‖	0.999

* (1) (*R,S*)-lormetazepam; (2) (*R,S*)-lorazepam; (3) (*R,S*)-temazepam; (4) clorazepate; (5) (*R,S*)-oxazepam; (6) (*R,S*)-oxazolam; (7) alprazolam; (8) (*R,S*)-4-hydroxy-alprazolam.

† (9) Phenylbutazone; (10) oxyphenbutazone; (11) piroxicam; (12) sulfinpirazone; (13) sulindac; (14) diclofenac; (15) diflunisal; (16) flufenamic; (17) mefenamic; (18) niflumic.

‡ Compounds not eluted from the column.

§ From Ref. 4.

‖ Not determined.

sequence. Thus, subtle interspecies differences in the primary structure of albumins can be reflected in changes in both the extent and direction of enantioselective binding. These differences have been observed in this study for both of the purported binding sites on the albumin molecule.

The comparative enantioselectivities of the binding of chiral drug substances to site II of RtSA, RbSA and HSA were investigated using BDZs (compounds **1–4**), 2-aryl-propionic acid NSAIDs (2-APA NSAIDs, compounds **19–22**, **24**) and D,L-tryptophan (**26**) which are thought to bind at this site. The results of these studies are presented in Table 2 as degree of enantioselectivity (α) and, where possible, Table 2 also indicates the relative enantioselectivity as expressed by the elution order of the separate enantiomers. Representative chromatograms are presented in Fig. 2.

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-SP than the other SPs except for compound **1** where the α values were 1.70 and 1.65 on the RtSA-SP and HSA-SP, respectively, and 2.10 on the RbSA-SP. Since the BDZs used in this study rapidly racemize in aqueous solutions [11], it was not possible to determine the relative enantioselectivities. However, previous studies on the HSA-SP, which utilized oxazepam hemisuccinate (OXH), a BDZ that does not racemize in aqueous solutions, have demonstrated that *S*-OXH binds to a greater extent to HSA than *R*-OXH [7]; ultrafiltration studies confirmed this relative enantioselectivity. Quantitative structure–retention analysis of BDZ retention on a HSA-SP has also indicated that the observed enantioselectivity is the result of two separate binding sites: a high-affinity site to which the M-conformer of the BDZs binds and a low-affinity site that can bind both the M- and P-conformers of the compound [9].

When the chiral 2-APA NSAIDs were 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-SP. The relative enantioselectivities were investigated by chromatographing unequal mixtures of the enantiomers. On all of the SPs, 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-SPs resulted in the stereochemical separation of these enantiomers. In this case, the degree of enantioselectivity displayed by the RtSA-SP was about 3.5-fold higher than the other two proteins while the relative enantioselectivity was the same for all three SAs (L-tryptophan bound to a greater extent than D-tryptophan). The relative enantioselectivity is consistent with previously reported findings [1, 3].

Other differences in the enantioselectivities of the three SAs were observed with *N*-benzoyl-phenylalanine and leucovorin. In the case of *N*-benzoyl-phenylalanine, enantioselective separations were observed on the RtSA-SP ($\alpha = 1.47$) and HSA-SP ($\alpha = 2.13$) but not on the RbSA-SP. Leucovorin is a diastereomeric compound which is epimeric at the 6-carbon of the tetrahydropteridine ring, while the chirality of the (*S*)-glutamic acid moiety is fixed in both isomers. The stereoselective separation of the active (6*S,S*)-isomer from the inactive (6*R,S*)-form was observed on the HSA-SP but not on the RtSA-SP and RbSA-SP.

When the enantioselective binding of *R*- and *S*-warfarin to site I of the respective SAs was investigated, the magnitudes of the observed α values were indistinguishable. However, on the RtSA-SP and HSA-SP, *R*-warfarin eluted before *S*-warfarin while the opposite elution order was observed on the RbSA-SP. These enantioselectivities are consistent with previously reported data from warfarin protein binding studies using HSA, RtSA

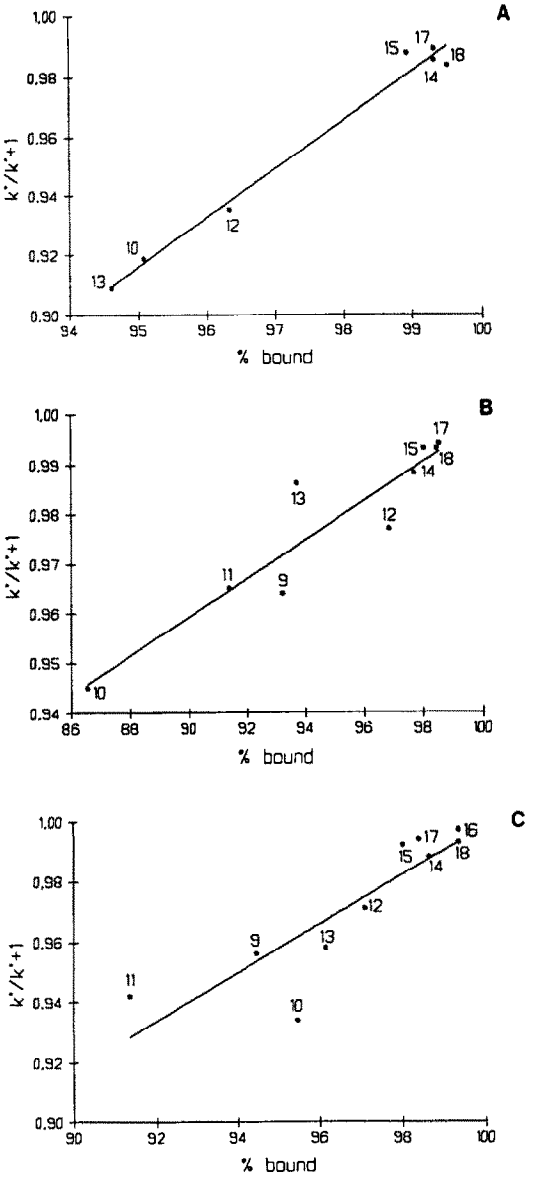

Fig. 1. Relationship between protein binding, expressed as percent bound, and chromatographic retention, expressed as $k'/(k'+1)$, for a series of achiral non-steroidal anti-inflammatory drugs, compounds **9–18**. Key: (A) HSA, (B) RtSA, and (C) RbSA; and (**9**) phenylbutazone; (**10**) oxyphenbutazone; (**11**) piroxicam; (**12** sulfinpirazone; (**13**) sulindac; (**14**) diclofenac; (**15**) diflunisal; (**16**) flufenamic; (**17**) mefenamic; and (**18**) niflumic.

and RbSA [12]. 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.

Effect of octanoic acid concentration on the chromatographic properties of RtSA-SP and RbSA-SP. The effect of medium chain fatty acids on the binding of 2-APA NSAIDs to RbSA and RtSA was investigated using the RtSA-SP and RbSA-SP. In these experiments, compounds **19–22** and **24** were chromatographed on the two SPs using a mobile phase composed of phosphate buffer and 6% 1-propanol. Increasing concentrations of octanoic acid were then added sequentially to the mobile phase, and the effect on the respective k' values was determined. As has been observed previously for this class of NSAIDs on the HSA-SP [5], the addition of octanoic acid to the mobile phase reduced the k' values of all the test drugs. The results are presented in Table 3 and illustrated using compound **22** in Fig. 3.

Previous studies on the HSA-SP have shown that the addition of octanoic acid to the mobile phase reduced the retention of compounds **20** and **21** through a combination of an anti-cooperative allosteric interaction and competitive displacement [5, 8]. The results of the present study are consistent with this model. The large average decreases in k' resulting from the addition of a relatively small concentration of octanoic acid [0.63 mM] to the mobile phase, 80% on the RtSA-SP and 74% decrease on the RbSA-SP, is indicative of an anti-cooperative allosteric interaction. This is supported by the observation that the compounds with the highest binding affinities, i.e. k's, experienced the greatest amount of displacement by the acid. If the displacement were simply competitive, then the inverse would have been observed and the lesser bound solutes would have experienced the greater displacement.

When the enantioselectivities of the binding interactions are considered, the addition of octanoic acid reduced the enantioselectivity on both the RtSA-SP and RbSA-SP. While the direction of the effect was the same on both phases, the magnitude differed indicating that there is at least a quantitative difference in the binding affinities.

Under the initial chromatographic conditions with no octanoic acid in the mobile phase, all of the test solutes, compounds **19–22** and **24**, were stereoselectively resolved on the RbSA-SP, whereas only compounds **19**, **22** and **24** were separated on the RtSA-SP. The addition of 0.10 mM octanoic acid destroyed the enantiomeric separation of compound **19** on the RbSA-SP, and the resolution for compounds **20** and **21** was lost when the octanoic acid concentration reached 0.31 mM. In contrast, the enantioselective separation of compound **19** on the RtSA-SP was not lost until the addition of 0.63 mM octanoic acid.

The effect of octanoic acid concentration on the enantioselective binding of ibuprofen (IBU, compound **22**) also illustrates the similarities and differences between the two SAs. On the RtSA-SP, the addition of 0.63 mM octanoic acid to the mobile phase reduced the k' values of *S*-IBU and *R*-IBU by 72 and 82%, respectively, and the observed enantioselectivity (α) was reduced by 31%, from 2.42 to 1.67. An almost 10-fold increase in the octanoic acid concentration (6.00 mM) decreased the k' values for *S*-IBU and *R*-IBU by 50 and 56%, respectively, but without an equivalent decrease in α which fell by 13% to 1.45.

On the RbSA-SP, the retention of *R*-IBU was too great to measure when there was no octanoic acid

Table 2. Enantioselectivity (α) and elution order of solutes chromatographed on RtSA-, RbSA- and HSA-SPs

Compounds	Enantioselectivity (elution order)		
	RtSA-SP	RbSA-SP	HSA-SP
(1) Lormetazepam	1.70[a]	2.10[a]	1.65[a]
(2) Lorazepam	1.05[a]	2.60[a]	1.36[a]
(3) Temazepam	1.25[a]	1.60[a]	2.46[a]
(4) Clorazepate	1.72[a]	2.10[a]	2.60[a]
(19) Fenoprofen	1.24[b]	1.22[b]	1.51[b]
(20) Ketoprofen	1.00[b]	1.32[b]	1.24[b]
(21) Suprofen	1.00[b]	1.85[b]	3.96[b]
(22) Ibuprofen	2.41[b] (*S,R*)	2.18[c] (*S,R*)	1.60[b] (*S,R*)
(24) Flurbiprofen	1.43[d] (*S,R*)	1.75[c] (*S,R*)	2.09[b] (*S,R*)
(26) Tryptophan	7.40[a] (D,L)	2.14[a] (D,L)	2.20[a] (D,L)
(28) *N*-Benzoyl-phenylalanine	1.47[b]	1.00[b]	2.13[b]
(29) Warfarin	1.47[b] (*R,S*)	1.57[b] (*S,R*)	2.56[b] (*R,S*)
(31) Leucovorin	1.00[b]	1.00[b]	2.33[b]

Chromatographic conditions: (a) 100% phosphate buffer (100 mM, pH 7), (b) phosphate buffer (100 mM, pH 7):1-propanol (94:6, v/v), (c) 1 mM octanoic acid in mobile phase composed of phosphate buffer (100 mM, pH 7):1-propanol (94:6, v/v), (d) 0.3125 mM octanoic acid in mobile phase composed of phosphate buffer (100 mM, pH 7):1-propanol (94:6, v/v).

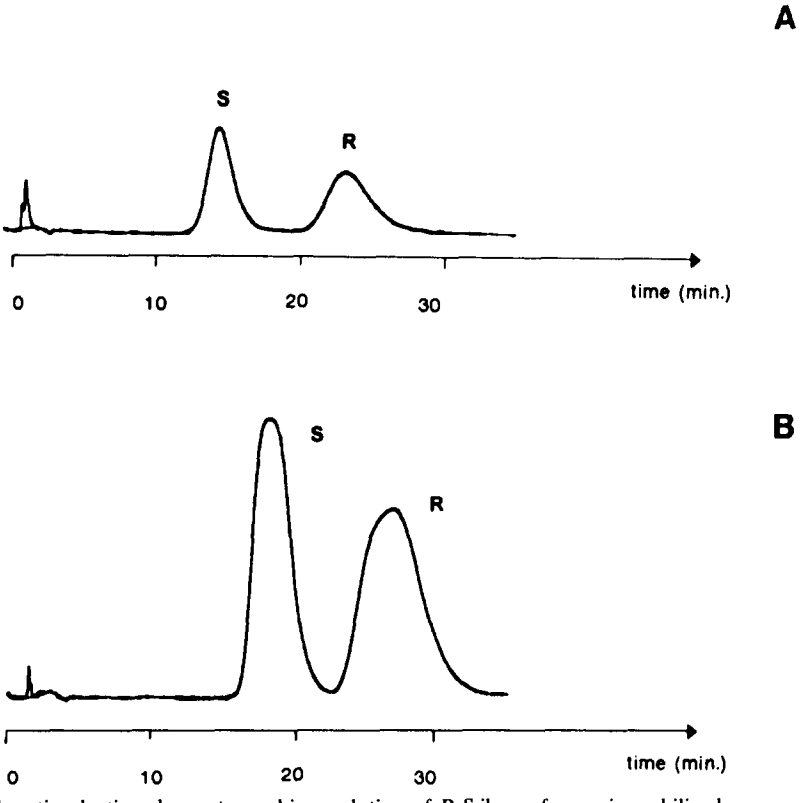

Fig. 2. Enantioselective chromatographic resolution of *R,S*-ibuprofen on immobilized-serum albumin stationary phases. Key: (A) chromatographic trace from the RtSA-SP; (B) chromatographic trace from the RbSA-SP; (S) the peak corresponding to *S*-ibuprofen; and (R) the peak corresponding to *R*-ibuprofen. See text for chromatographic conditions.

Table 3. Effect of octanoic acid concentration in the mobile phase on the chromatographic retention, expressed as capacity factor (k'), of a series of chiral 2-aryl-propionic acid non-steroidal anti-inflammatory drugs on the RtSA-SP and RbSA-SP

		Concentration of octanoic acid (mM)								
		0.00	0.10	0.31	0.63	1.25	2.50	4.00	5.00	6.00
Compounds		Chromatographic retention (k') on the Rt-SA-SP								
(19) Fenoprofen*		82.24	31.61	15.00	12.70	8.68	8.58	8.33	6.62	5.66
		101.65	36.78	15.80						
(20) Ketoprofen		31.70	14.82	5.58	4.82	4.50	4.14	3.33	2.76	2.35
(21) Suprofen		21.29	11.44	5.80	4.78	3.55	3.48	3.44	2.80	2.40
(22) Ibuprofen	*S*	31.80	26.63	10.30	8.90	7.75	7.66	6.81	5.71	4.50
	R	76.75	54.96	18.20	14.86	11.51	11.30	10.57	8.39	6.54
(24) Flurbiprofen	*R*	NE†	146.28	53.56	37.62	22.60	22.57	21.74	16.45	13.76
	S	NE	NE	76.83	52.37	29.98	29.51	27.66	20.58	17.01
		Chromatographic retention (k') on the RbSA-SP								
(19) Fenoprofen*		49.00	26.24	17.03	13.97	10.54	10.40	10.40	9.95	9.35
		59.66								
(20) Ketoprofen		13.46	7.50	5.72	4.79	3.90	3.80	3.77	3.38	3.29
		17.83	8.85							
(21) Suprofen		24.26	13.99	7.16	5.87	4.31	4.26	4.30	3.98	3.72
		44.94	10.14							
(22) Ibuprofen	*R*	47.47	20.81	13.71	11.13	8.48	8.00	7.88	8.05	7.80
	S	NE	45.30	23.40	16.95	11.20	9.85	9.59		
(24) Flurbiprofen	*R*	NE	96.01	48.74	38.76	23.90	22.87	22.85	22.19	20.62
	S	NE	168.56	73.84	53.33	29.89	24.80	24.79		

The octanoic acid was dissolved in a mobile phase which consisted of sodium phosphate [100 mM, pH 7.0] modified with 1-propanol (6%, v/v); see text for other experimental conditions.

* Enantiomeric elution order was not determined.

† Not eluted.

in the mobile phase. With 0.10 mM octanoic acid, the observed α was 2.18 which dropped to 1.53 when the octanoic acid concentration was 0.63 mM and was lost at 4.00 mM octanoic acid. The same pattern was observed for compound **24**, where on the RtSA-SP the enantioselectivity was reduced but still evident ($\alpha = 1.24$) with 6.00 mM octanoic acid, while on the RbSA-SP the enantioselectivity was lost after the addition of 4.00 mM octanoic acid.

These results can be explained using a single binding site model where both an anti-cooperative allosteric interaction and competitive displacement occur. Another possible description of the data is a two (or more) binding site model where one site(s) is affected by the anti-cooperative allosteric interaction while the other(s) is affected by competitive displacement. In this model, the affinity at the site(s) affected by the allosteric interaction is responsible for the majority of the observed binding and at least some of the enantioselectivity. The site(s) affected by the competitive displacement interactions also contributes to the magnitude and enantioselectivity of the ligand–SA interactions, but for compounds **19–21**, the site(s) appears to be of secondary importance. Compounds **22** and **24** either have increased affinities for the competitive displacement site(s) or bind at additional enantioselective sites.

The results from a competitive displacement study on the HSA-SP support the multiple binding site model [13]. In this study, *R*-IBU and *S*-IBU were used as the mobile phase modifiers and a series of benzodiazepines were used as the ligands. The displacement patterns produced by the addition of the IBU enantiomers indicated that the test ligands bound to at least three sites that were identified as $IBU_{R,S}$, IBU_R and IBU_S, where the designations were made according to the enantioselectivity of the respective sites.

In conclusion, these studies have illustrated that the use of HPLC stationary phases based on immobilized albumins of different species can be used to determine the magnitude and enantioselectivity of ligand binding to the respective proteins and to probe intraspecies differences in this binding. This chromatographic approach combined with chiral solutes can also provide information on binding behaviour, which is not readily available from studies involving only standard protein binding studies or binding displacement experiments. The effect of octanoic acid on the binding of the 2-APA NSAIDs observed in these preliminary studies illustrates this point. The interaction is clearly more complicated than simple competitive displacement from a single site. A complete analysis of the 2-APA NSAID–octanoic acid interaction is currently underway and will be reported elsewhere.

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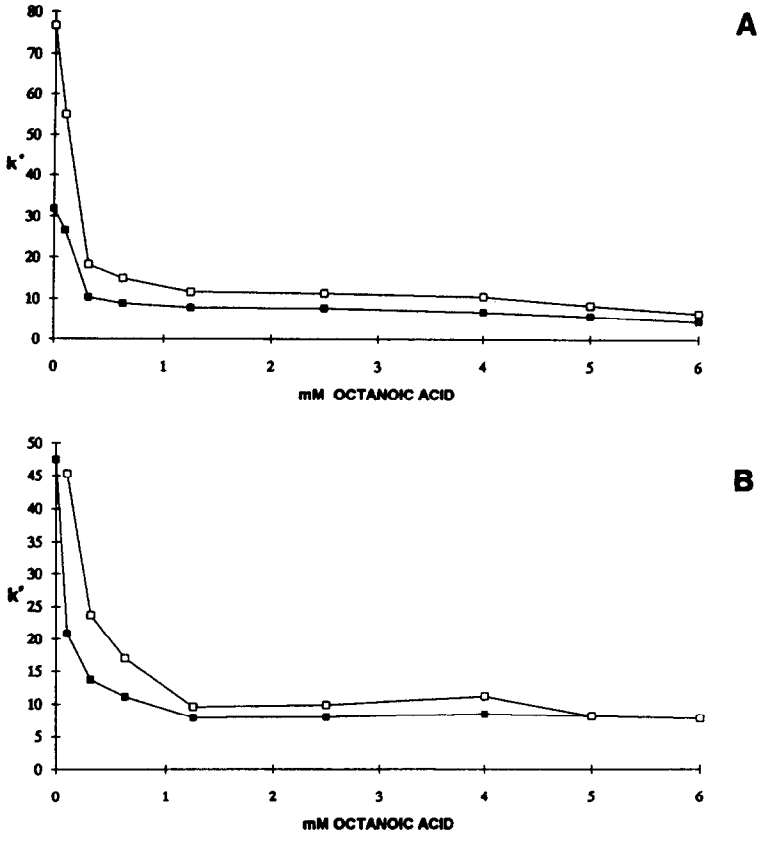

Fig. 3. Effect of the sequential addition to the chromatographic mobile phase of increasing concentrations of octanoic acid on the chromatographic retention, k', of *R*-ibuprofen and *S*-ibuprofen. Key: (A) RtSA-SP; (B) RbSA-SP; (□) the k' of *R*-ibuprofen; and (■) the k' of *S*-ibuprofen. See text for chromatographic conditions.

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