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Stereoselective binding of 2,3-substituted 3-hydroxypropionic acids on an immobilised human serum albumin chiral stationary phase: stereochemical characterisation and quantitative structure–retention relationship study

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Abstract

The binding characteristics of a series of 2,3-substituted 3-hydroxypropionic acids, with anti-inflammatory properties, bearing two chiral centres, were studied by HPLC upon HSA (human serum albumin)-based stationary phase. The compounds were analysed in their stereoisomeric erythro and threo forms and the chromatographic conditions for enantioseparation of the erythro and threo forms were studied on human serum albumin stationary phase. The enantiomer elution order was determined by injection of the enriched samples or by carrying out the CD spectra of each enantiomeric fraction. The absolute configuration of the single enantiomers was assigned on the basis of their CD spectra. A QSRR study was performed by subjecting the chromatographic data of the compounds to multiparameter regression analysis against various molecular descriptors to have insight into the chiral recognition mechanism. The lipophilicity appeared to be the most important parameter in determining the affinity to the protein, the compounds' capacity factors being linearly correlated to the experimental RP-HPLC partition coefficients ($\log k'_w$). The enantioselectivity factors (α) related to the enantiomers of the erythro and threo forms were studied taking into consideration both the physico-chemical parameters and the conformational behaviour of the compounds.

Keywords: Enantiomer separation; Chiral stationary phases, LC; Quantitative structure–retention relationship; 3-Hydroxypropionic acid; Human serum albumin

1. Introduction

Studies on drug–human serum albumin binding are important in pharmacology and pharmacokinetics, because drug–protein binding affects the pharmacological activities and side effects of the drugs as well as the drug distribution and elimination.

Affinity chromatography using human serum albumin immobilised on a stationary phase (HSA-

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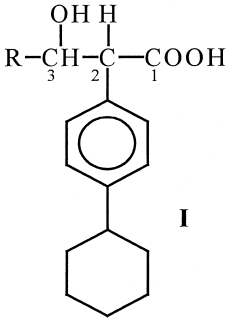
CSP) is a widely used method to study drug binding phenomena [1–3]. Because the binding of a drug to HSA may have considerable influence on its biological activity, quantitative structure–retention relationships (QSRRs) derived from retention data on the HSA-CSP can be of direct pharmacokinetic and pharmacodynamic relevance. In fact, the structural requirements essential for modulating affinity to the main sites of HSA and the mechanism of chiral discrimination on these sites are still the object of intense study to rationalise chiral drug–HSA binding and to predict enantioseparation.

The object of the present work was to further define the mechanism for enantiodiscrimination on the HSA-CSP, through the analysis of a series of anti-inflammatory arylpropionic acids **I**, which contain two chiral centres in their molecular skeleton (Fig. 1). To this aim, the stereochemistry of the single enantiomers was characterised by enantioselective HPLC analysis on HSA, Chiracel OJ stationary phases and by CD studies.

As QSRR studies have been reported for the chromatography of a series of chiral drugs binding to HSA [4–9], the relationship between the molecular descriptors of the solutes and the observed chromatographic retention and enantioselectivities was investigated. In order to obtain useful experimental parameters as molecular descriptors for hydrophobicity and enantioselectivity, reversed-phase HPLC $\log k'_w$ and the preferred conformations of the molecules were determined.

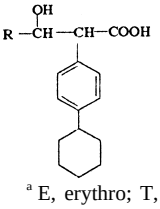


I

Fig. 1. General molecular structure of the compounds used in the study (for R see Table 1).

Table 1
Structures, capacity factors (k') and enantioselectivity (α) of racemates obtained on HSA column, mobile phase: 1-propanol:(pH 7.0, 0.1 *M*) potassium phosphate buffer (10:90, v/v) containing 5 m*M* octanoic acid; flow-rate 1 ml/min

Structures[a]	*R*	$k'(1)$	α
1E	CH_3	2.14	1.08
1T	CH_3	5.90	1.41
2E	Ph	8.23	1.65
2T	Ph	21.72	1.42
3E	$PhCH_3$(p)	12.56	1.54
3T	$PhCH_3$(p)	31.41	1.45
4E	PhCl(p)	27.14	1.18
4T	PhCl(p)	71.23	1.08
5E	PhBr(p)	37.51	1.17
5T	PhBr(p)	96.89	1



[a] E, erythro; T, threo.

2. Materials and methods

2.1. Materials

The studied arylpropionic acids (Table 1) were synthesised as previously reported [18].

HPLC-grade methanol, 1-propanol, 2-propanol, hexane (Promochem, Germany) were used to prepare the mobile phases. Water was doubly distilled. To prepare the pH 7.0 phosphate buffer solution potassium dihydrogenphosphate and dipotassium hydrogen phosphate trihydrate of analysis quality (Carlo Erba, Italy) were used. Octanoic acid (99%) was purchased from Aldrich Italia (Milan, Italy).

The buffer solutions were filtered through a 0.45-μm membrane filter and degassed before their use for HPLC.

2.2. Apparatus

The solvent delivery system was a quaternary HP 1050 Ti series pump, equipped with a Reodyne Model 7125 injector with a 20-μl sample loop. The

eluents were monitored by a Multiwavelength HP 1050 Detector connected to a computer station (HP Chemstations, Vectra VT). For routine analyses the detector wavelength was set at λ=220 nm and λ=275 nm.

A Jasco PU-980 solvent delivery system, connected to a Jasco MD 910 multiwavelength detector and a computer station, with a Reodyne model 7125 injector and a 20-μl loop was also used.

Circular dichroism (CD) measurements were carried out by a Jasco J-810 Spectropolarimeter (Jasco, Tokyo, Japan), using either methanol or phosphate buffer (0.1 *M*, pH 7.0) solutions (0.5–3 m*M*), with 1 cm pathlength, at room temperature.

2.3. Determination of the lipophilic properties (log k'_w) by RP-HPLC

The stationary phase was an Alltima C_{18} 5 μm (150×4.6 mm I.D.) column. The flow-rate was 1 ml/min.

The mobile phase composition ranged from 40 to 80% (v/v) methanol with 0.02 *M* phosphate buffer at pH 7.0.

The dead volume of the system was measured as the first distortion of the baseline after injection of pure water. The stock solutions of tested compounds (1 mg/ml in methanol) were diluted with water to the final injected concentration (50 μg/ml). A 20-μl injection was made in duplicate. According to their chromatographic behaviour, the retention times of the solutes were determined at four different methanol–phosphate buffer mixtures, ranging from 40 to 80% (v/v) methanol. At each mobile phase composition, the capacity factor k' was calculated according to $k'=(t_r-t_0)/t_0$, where t_r and t_0 were the retention times of analyte and the non-retained compound, respectively. The log k'_w values (log k' at 100% aqueous mobile phase) were obtained from the *y*-intercepts of plots of log k' versus percent of methanol in the mobile phase (Table 2). Correlation studies were performed using a statistical program (Graph Pad Prism).

2.4. Enantioselective chromatography

The chromatographic conditions for enantioseparation of the erythro and threo forms were studied on 7 μm HSA Shandon stationary phase (150×4.6 mm I.D.) at ambient temperature (Table 1).

Table 2
Dependence of the C_{18} capacity factors on the mobile phase composition: $\log k' = \log k'_w + a\ c$[a]

No.	Log k'_w	*a*	*r*
1E	3.456±0.011	−0.0496±0.0002	0.99997
1T	4.347±0.046	−0.0584±0.0009	0.99975
2E	4.677±0.032	−0.0631±0.0005	0.99983
2T	5.335±0.014	−0.0691±0.0002	0.99998
3E	5.130±0.009	−0.0677±0.0001	0.99999
3T	5.849±0.011	−0.0741±0.0002	0.99999
4E	5.593±0.016	−0.0724±0.0001	0.99998
4T	6.202±0.035	−0.0776±0.0005	0.99994
5E	5.660±0.029	−0.0718±0.0004	0.99995
5T	6.180±0.147	−0.0755±0.0024	0.99895

[a] *a*, slope; *r*, correlation coefficient; *c*, percent methanol; $\log k'_w = y$ intercept.

The mobile phase composition was 1-propanol:(pH 7.0, 0.1 *M*) potassium phosphate buffer (10:90, v/v) containing 5 m*M* octanoic acid. The flow-rate was 1 ml/min. The stock solutions of tested compounds (1 mg/ml in 1-propanol) were diluted either with buffer or with 2-propanol to the final injected concentration (50 μg ml^{-1}).

The values of enantiomeric excess (e.e.% = $A_2 - A_1/A_2 + A_1$) were calculated from the chromatographic areas of the enantiomeric peaks. For the enantiomers of compounds **1E, 1T, 2T, 3E, 3T, 4T** a HSA column was used following the above-reported conditions. The values of e.e.% of enantiomers of **4E, 5E** were determined using Chiracel OJ (250×4.6 mm I.D.; Daicel Chemical Industries), using hexane:2-propanol:glacial acetic acid (90:10:0.2, v/v) as eluent at 0.8 ml/min, with UV detection at 220–275 nm.

For compound **5T** the values of the e.e.% of the available enantiomers were not determined, because no enantioseparation was possible on the two chiral columns (HSA and OJ).

The elution order of the single enantiomer on HSA was performed by injecting erythro and threo racemate enriched with one pure enantiomer (Table 5). For compound **2E** the single enantiomers were not available and a preparative separation was performed on HSA column.

2.5. Molecular modeling and QSRR analysis

Three-dimensional models of all the compounds were created by means of the MacroModel Ver. 5.5 molecular modeling program [10]. In order to find a reliable conformation for each molecule, Monte Carlo/stochastic dynamics simulations [11] were run using the OPLS force field [12] and the GB/SA solvation model for water [13]. An equilibration time of 5 ps at 1000 K was set, then the stochastic dynamics simulation was run at 1000 K for 100 ps with a time step of 1 fs. Two hundred conformations were stored for each molecule, then the average was energy minimised and taken as representative of that molecule.

Classical hydrophobic ($\log P$), electronic (σ, F) and steric (MR, L) parameters were either calculated ($\log P$, MR) or retrieved (σ, F, L) by the C-QSAR program Ver. 1.87 [14]. It has to be noted that the theoretical method used to calculate the $\log P$ and MR values does not differentiate between threo and erythro isomers, so that the same value is given to each pair of isomers.

From the molecular models obtained as described above, some parameters related to the conformation were calculated. Solvent-accessible surface, molecular volume and the distance between the centroids of the farthest cycle in the 2-substituent (the cyclohexyl ring) and of the R group (Distance in Table 3) were calculated with MacroModel. In order to achieve a more accurate description of the electronic properties of the compounds than that provided by the substituent constants, LCAO_MO theoretical indices (E_{HOMO}, E_{LUMO}, q_{C2}, q_{C3}, q_{CR}) were computed. They were obtained by means of the semi-empirical quantum-mechanical method AM1 [15] implemented in the SYBYL molecular modeling package [16].

Regression analysis was performed with the C-QSAR program, while the multivariate analysis and the calculation of the q^2 statistics were run by means of the PLS method [17] implemented in SYBYL.

3. Results

3.1. Determination of the lipophilic properties (log k'_w) by RP-HPLC

The studied chiral compounds (Fig. 1 and Table 1) are propionic acid derivatives, bearing two chiral centres. They were synthesised as potential anti-inflammatory drugs and were obtained in their stereoisomeric erythro and threo forms, whose structure and configurational assignments were determined by ^{1}H NMR studies and previously reported [18].

As the $\log P$ theoretical values could not differentiate the diverse lipophilicity of erythro and threo stereoisomers, the lipophilic properties of the series of compounds under study were determined by RP-HPLC. In fact, by means of HPLC, hydrophobicity measures can be determined, which better describe

Table 3
Enantioretention indices and physico–chemical and geometric parameters of the compounds studied[a]

No.	Log k'_1	Log k'_2	Log k'_w	Confor	Distance (Å)
1E	0.330	0.380	3.456	Folded	7.7
1T	0.771	0.941	4.347	Linear	9.7
2E	0.915	1.152	4.677	Linear	10.8
2T	1.337	1.496	5.335	Folded	7.5
3E	1.099	1.298	5.130	Linear	10.8
3T	1.497	1.661	5.849	Folded	7.3
4E	1.434	1.506	5.593	Linear	10.9
4T	1.853	1.885	6.202	Folded	7.3
5E	1.574	1.645	5.660	Linear	10.8
5T	1.986	1.986	6.180	Folded	7.3

[a] k'_1, k'_2, capacity factors of the first and second eluting enantiomer on HSA column; k'_w, capacity factor at 100% of aqueous mobile phase on RP-18 column; Confor, conformation; Distance, the distance between the centroids of the farthest cycle in the 2-substituent (the cyclohexyl ring) and of the R group.

the hydrophobic interactions of solutes with hydrophobic binding sites on proteins [19].

The capacity factors ($\log k'$) were measured at different concentrations (40–80%) of methanol in the mobile phase. The value corresponding to 0% of methanol ($\log k'_w$) was then obtained by extrapolation of the linear graph $\log k'$ versus volume fraction of methanol (Table 2). This parameter ($\log k'_w$) was retained as a valuable index of lipophilicity [20] and was used in the QSRR study.

All the threo diastereoisomers showed higher $\log k'_w$ than the corresponding erythro ones (Tables 2 and 3), accordingly to the behaviour displayed on the HSA column (Table 1), and demonstrating the higher chromatographic lipophilicity of threo with respect to the other isomer.

3.2. Retention on HSA

The enantiomeric separation of each erythro and threo racemate was performed on the HSA column by using 5 m*M* octanoic acid as additive in a mobile phase consisting of 1-propanol:(pH 7.0, 0.1 *M*) potassium phosphate buffer (10:90, v/v). The capacity factor k'_1 (first eluting enantiomer) and enantioselectivity (α) factor for each racemate are reported in Table 1.

The addition of the modifier (octanoic acid) was required to decrease the retention times on HSA, as reported for the highly bound non-steroidal anti-inflammatory drugs [21–23]. All the threo diastereoisomers showed higher k'_1 than the corresponding erythro ones (Table 1), similar to the behaviour displayed on the reversed-phase system (C_{18}) (Table 2).

Moreover, for each compound, the threo enantiomers were completely resolved from the erythro ones (Fig. 2). Therefore, the four stereoisomers were completely separated with the exception of compound **5T**, for which enantioselectivity was not achieved (Table 1).

The retention parameters obtained on the HSA stationary phase were used for the QSRR analysis. The quantitative relationships between the chromatographic retention indices determined in the HSA column ($\log k'_1$, $\log k'_2$, Table 3) and the physicochemical descriptors were studied by means of both regression analysis and PLS methods. However, after

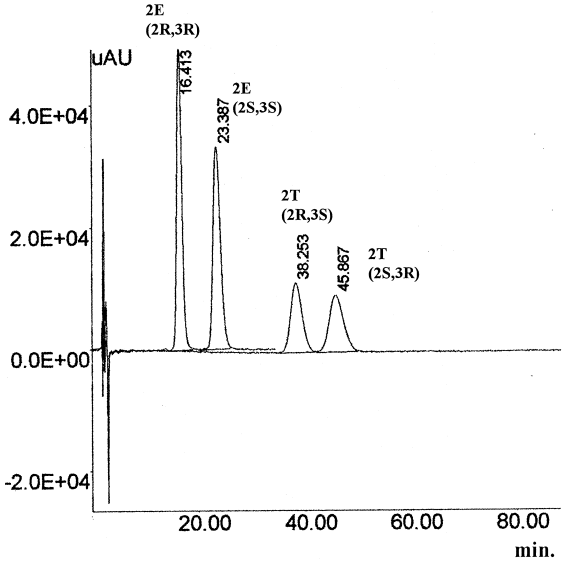


Fig. 2. Overlaid enantioselective chromatographic separations of *rac*-2E and *rac*-2T on HSA-CSP. Chromatographic conditions: mobile phase, 1-propanol:(pH 7.0, 0.1 *M*) potassium phosphate buffer (10:90, v/v) containing 5 m*M* octanoic acid; flow-rate, 1 ml/min, UV detection at 220 nm.

consideration of all the available descriptors (lipophilic, electronic, and steric, see Section 2), only the lipophilic properties of the compounds proved to be correlated with the variation of the HSA retention indices within the series. For compounds **1–5**, the following highly significant relationships correlating the variation of the first and the second eluted enantiomer capacity factors to the C_{18} retention index taken as a measure of lipophilicity were obtained:

$$\log k'_1 = 0.574(\pm 0.084) \log k'_w - 1.731(\pm 0.446)$$
$$n = 10 \quad r^2 = 0.969 \quad \mathrm{SD} = 0.095$$
$$q^2 = 0.944 \quad F_{1,8} = 249.31 \qquad (1)$$

$$\log k'_2 = 0.545(\pm 0.054) \log k'_w - 1.463(\pm 0.285)$$
$$n = 10 \quad r^2 = 0.986 \quad \mathrm{SD} = 0.061$$
$$q^2 = 0.976 \quad F_{1,8} = 547.53 \qquad (2)$$

In the above equations, the numbers in parentheses represent the 95% confidence interval, n is the number of compounds from which the equation was

calculated, r^2 is the conventional squared correlation coefficient, SD is the standard deviation of the equation, q^2 is the cross-validated correlation coefficient, and F is the value of the F-test.

Considering the very favourable statistics of both Eqs. (1) and (2), and considering also that no other descriptor was found to significantly improve the correlation, one can conclude that the lipophilicity as expressed by the chromatographic index log k'_w is the physicochemical factor influencing mostly the affinity for the albumin stationary phase for both the most and the least retained enantiomer of compounds **1–5**.

3.3. Stereochemical characterisation

The diastereomers of compounds **1–5** were characterised for their stereochemistry, determining the e.e. by enantioselective chromatography and the absolute configuration of the prevailing enantiomer by CD study. The relative configuration, erythro and threo, of compounds **1–5** was previously determined by ^{1}H-NMR studies [18].

The absolute configuration of the single diastereomers was determined by studying their circular dichroism (CD) spectra. In particular the benzene sector rule proposed by Smith [24] was applied in order to assign the absolute configuration to carbon-2 and to carbon-3 of the erythro and threo diastereomers of compounds **1-5**.

This sector rule is reported to be reliable in determining the absolute configuration of the chiral centre in α position to the aromatic group, on the basis of the sign of the CD band at about 260 nm, allied to ^1L_b electronic transition of the benzene chromophore. Only this chiral centre is considered to give a significant contribution to the lowest energy CD band. However a reliable application of this sector rule deserves some conformational restrictions. In particular the benzylic hydrogen should eclipse the edge of the benzene ring.

Thus, the most representative conformations for each compound was first determined by running Monte Carlo/stochastic dynamics simulations, using the OPLS force field and the GB/SA solvation model for water. The hydrogen atom at the chiral centre was eclipsed or almost eclipsed for all the compounds, in agreement with the literature data on chiral benzylic derivatives [25–28]. The application of the benzene sector rule [24] to compounds **1–5**, suggests a positive contribution to the CD at 260 nm for an (*S*) absolute configuration, both at carbon-2 and carbon-3.

This assignment is based on the assumption of a different contribution to the CD signal for each substituent at carbon-2 and carbon-3. In particular the carboxylate group and the main chain substituent are expected to give a larger contribution at carbon-2 and carbon-3, respectively.

On this premise a (2*S*,3*S*) absolute configuration can be assigned to the erythro compounds showing a positive CD band at 260 nm, in phosphate buffer solution (Table 4), except for compounds 1, where a methyl group replaces a benzene one in position three. This structural change determines a formal inversion of the absolute configuration at carbon-3. Consequently a (2*S*,3*R*) configuration is assigned to the erythro diastereoisomer of **1**, showing a positive CD band at 260 nm (Tables 4 and 6). It is worth noting that the benzene sector rule is applicable only to carbon-2 in the case of compounds **1E** and **1T**.

Table 4
Stereochemical data

Compound	CD (^1L_b band)		Absolute configuration[b]
	λ (nm)[a]	$\Delta\varepsilon$[a]	
1 E (+)	263	−0.048	2*R*,3*S*
1 T(+)	263	−0.033	2*R*,3*R*
rac-2 E[c]	265	−	2*R*,3*R*
	265	+	2*S*,3*S*
2 T (−)	273	+0.074	2*S*,3*R*
	257	−0.366	
3 E (−)	271	+0.043	2*S*,3*S*
3 T (−)	273	+0.092	2*S*,3*R*
	255	−0.197	
4 E (−)	270	+0.054	2*S*,3*S*
4 T (+)	273	−0.076	2*R*,3*S*
	252	+0.094	
5 E (−)	271	+0.038	2*S*,3*S*
5 T (−)	274	+0.074	2*S*,3*R*
	250	−0.080	

[a] Phosphate buffer solution; the $\Delta\varepsilon$ values were corrected for 100% e.e., with the exception of compound 5T(−).

[b] Determined by applying the benzene sector rule [24].

[c] CD analysis carried out on the enantiomeric fractions obtained by HPLC resolution on HSA-CSP.

As far as the threo compounds are concerned, the assignment of the absolute configuration by CD is not obvious. An opposite sign is expected for the contribution to the CD at 260 nm allied to carbon-2 and to carbon-3. CD investigation using methanol or phosphate buffer as the solvent suggests that the sign of the CD band at about 260 nm mainly reflects the absolute configuration at carbon-3, in the case of threo diastereomers.

The CD spectra between 280 and 240 nm of compounds (−)-**2T**, (−)-**3T,** (−)-**4T** in phosphate buffer are bisegnate, with a low intensity CD band in the lower energy part of the spectra, and a much higher intensity CD signal in the shorter wavelength region (Fig. 3, Table 4). Only a negative CD band was observed instead, when the CD spectra were carried out in methanol (Fig2). In agreement with the literature data [24,29], the change of the sign of the CD band by changing the solvent can be rationalised on the basis of the difference in the contribution to the CD of the different substituents on the chiral centre.

In particular, as far as compounds (−)-**2T** and (−)-**3T** in methanol solution are concerned, the substituted carbon-3 group should give a higher contribution to the CD with respect to the carboxyl group. The situation should be reversed in buffer solution. Here the carboxylate group prevails as contribution to the CD.

Thus, on the basis of the obtained CD data and by applying the sector rule, an (*S*) absolute configuration can be tentatively assigned to carbon-2 of the threo diastereomers showing a bisegnate (positive/negative) CD band at about 260 nm in phosphate buffer (Table 4). Furthermore the strong negative contribution to the CD band at 260 nm, either for methanol or buffer solution, suggests an (*R*) absolute configuration for carbon-3.

The fully determined stereochemistry of erythro and threo **1–5** compounds is reported in Table 4.

3.4. Enantioselectivity and elution order on the HSA-based HPLC column

The α values of enantioselectivity for compounds **1–5** were higher in the erythro series (except for the **1E**,**1T** pair), the halogen-substituted compounds showing the highest retention but the lowest enantioselectivity (Table 1).

The elution order of compounds **1–5**, erythro and threo, has been determined by injecting, on the HSA-based CSP, the single enantiomers or enriched enantiomer mixtures previously characterised for their e.e. and their absolute configuration (Table 5).

This approach was not possible for compound **2E**, because it was available only as a racemate. Thus the preparative enantioseparation was performed on the HSA-CSP, and the CD spectra were carried out on the two enantiomeric fractions. The less retained enantiomer showed a negative CD band at 260 nm and then a (2*R*,3*R*) absolute configuration could be assigned, still by applying the benzene sector rule. As a consequence the absolute configuration (2*S*,3*S*) was assigned to the more retained enantiomer, showing a positive CD band at 260 nm.

This procedure was performed also in the case of the sample (−)-**4T**, in order to confirm the elution order determined by injection of the enriched enantiomer fraction. The uncertainty in the determination of the elution order was due to the low e.e. of the injected sample and to the low enantioselectivity observed in the chromatographic HPLC analysis of **4T** (Table 1).

4. Discussion

Chromatographic retention and selectivity in an enantioselective system is the summation of both non-specific and enantioselective interactions with the stationary phase. This study utilized reversed-phase conditions and it is not surprising that quantitative structure–retention relationship analysis identified hydrophobicity as the key retention index. The relative importance of this parameter is reflected in the resolutions of the threo and erythro isomers obtained on the HSA column.

Threo and erythro isomers are diastereomers and are chemically distinct entities. The separation of these compounds reflects non-specific interactions between the solutes and the stationary phase. In this study, the erythro isomers eluted before their corresponding threo isomers with an average separation factor of 2.62 (range 2.50–2.76) (Fig. 2, Table 1). The erythro/threo elution order is consistent with the

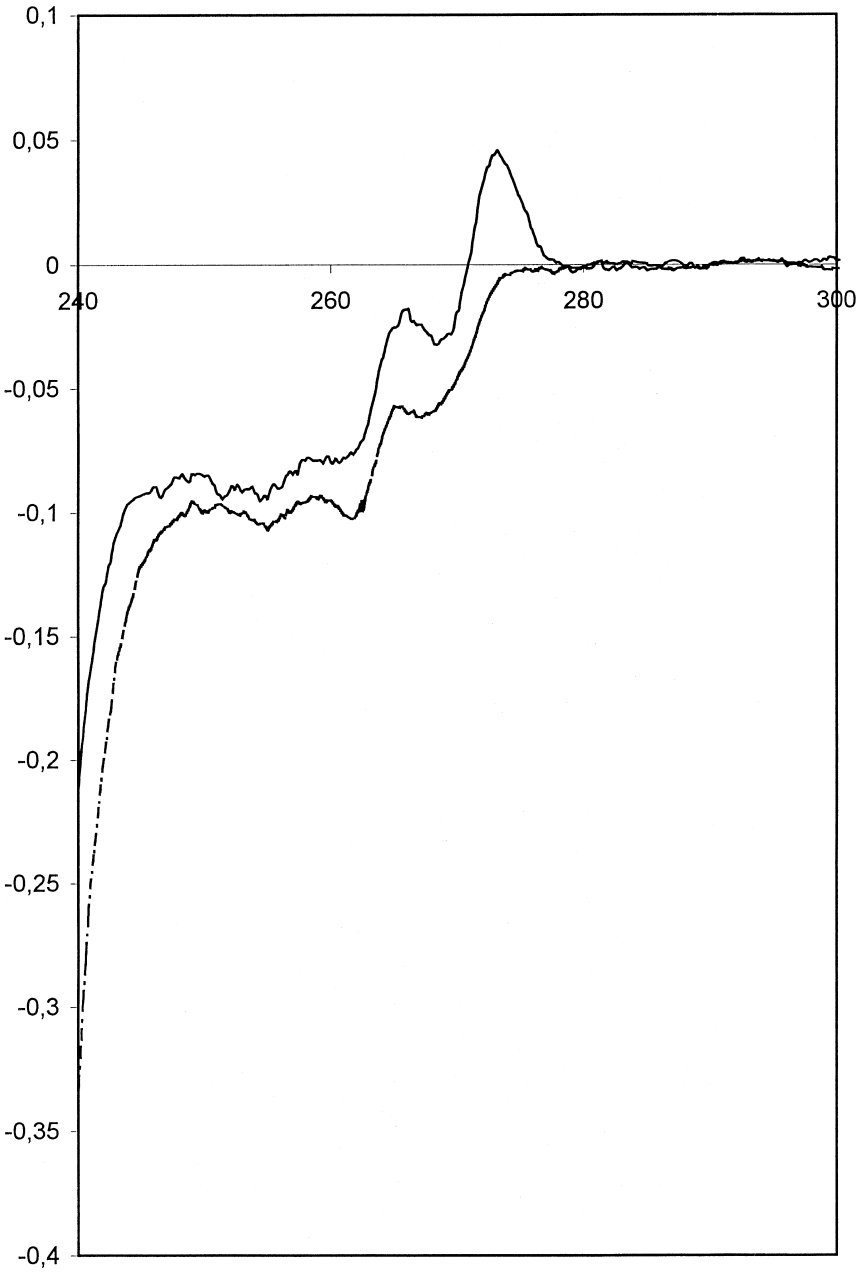


Fig. 3. CD spectra of (2*S*,3*R*)-3T in (pH 7.0, 0.1 *M*) potassium phosphate buffer (full line) and in methanol (dotted line) solutions.

calculated chromatographic lipophilicities, where $\log k'_w$ of each erythro isomer was less than $\log k'_w$ of the corresponding threo isomer (Tables 2 and 3).

Unlike diastereomers, enantiomers have the same physicochemical properties and, therefore, differences in hydrophobicity cannot be the source of the observed enantioselective separations. Other factors must play a role in generating the enantioselectivity in this chromatographic system. The data from this study provides some insight into this question.

Table 5
Elution order on HSA-CSP

Compound (Rac.)	Elution order (first/second)	Absolute configuration[b]
1 E	+/−	2*R*,3*S*
1 T	+/−	2*R*,3*R*
2 E[a]		2*R*,3*R*
2 T	+/−	2*R*,3*S*
3 E	+/−	2*R*,3*R*
3 T	+/−	2*R*,3*S*
4 E	+/−	2*R*,3*R*
4 T	+/−	2*R*,3*S*
5 E	+/−	2*R*,3*R*
5 T	n.d.	n.d.

[a] The elution order was obtained on the basis of the CD spectra of the enantiomeric fraction as obtained by resolution on HSA-CSP.
[b] Less retained enantiomer.

In Table 1, the observed enantioselective separations (expressed as the enantioselectivity factor α) are presented for all of the matched erythro **E** and threo **T** isomers. For compounds **2–5**, the α values achieved for the *E* enantiomers, i.e., the (2*S*,3*S*) and (2*R*,3*R*) isomers, were greater than the α values achieved with the corresponding **T** enantiomers, i.e., (2*R*,3*S*) and (2*S*,3*R*) isomers. However, the situation was reversed for compound **1** where the enantioselective separation of the **1T** enantiomers was greater than the separation achieved for the **1E** enantiomers, $\alpha = 1.41$ and 1.08, respectively.

The change of the relative absolute configuration at carbon-2 and carbon-3 cannot be the source of the $T > E$ enantioselectivity. In fact, the change of the absolute configuration at carbon-3 for **1** with respect to compounds **2–5** is only formal. This change is due to the methyl group in **1** replacing the benzene group at carbon-3 for **2–5** (Tables 1 and 6).

Therefore, the data in Table 1 should be analyzed as the enantioselectivities achieved with linear molecules as compared to those achieved with molecules with folded conformations, not as *E* vs. *T*.

The average enantioselectivity achieved with the five molecules with a linear conformation was $\alpha =$ 1.39 (range, 1.17–1.65; five of five separations). With the five molecules with a folded conformation, the observed enantioselectivity was $\alpha = 1.21$ (range, 1.0–1.45; four of five separations). The free energy differences ($\Delta\Delta G$) reflected by the observed enantioselectivities can be calculated using the relationship $\Delta\Delta G = RT \ln \alpha$ [30]. Using this approach the average $\Delta\Delta G$ associated with the enantioselective resolution of compounds with a linear conformation is 190 cal (range 93–297 cal, $n = 5$). The average $\Delta\Delta G$ associated with the enantioselective resolution of compounds with a folded conformation is 130 cal (range 46–220 cal, $n = 4$).

The source of this difference may lie in the structure of the binding site on the HSA molecule at which the enantiospecific retention occurs. Arylpropionic acids have been shown to bind at Site II or the 'indole–benzodiazepine binding site' by a variety of displacement and chromatographic techniques [21,31,32]. Semiquantitative structure–activity studies of the binding of chiral 2-arylpropionic acid non-steroidal anti-inflammatory agents to Site II on HSA have been reported by Wanwimolruk et al. [33]. Their results indicate that the binding site is a linear hydrophobic cleft, about 16 Å long and about 8 Å wide with a cationic group located near the surface at one end of the cleft. This model has been supported by chromatographic studies of the quantitative structure–retention relationships for a series of benzodiazepines on an immobilized HSA stationary phase [5].

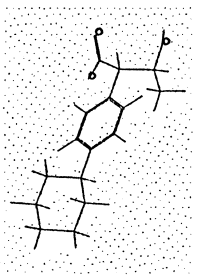
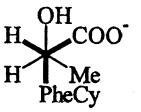
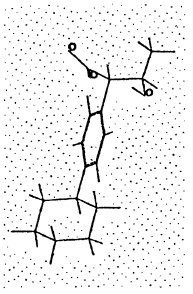
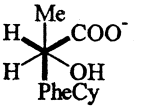
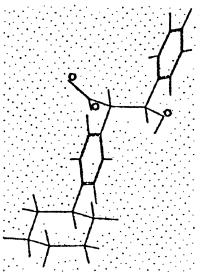
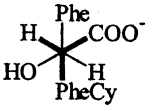
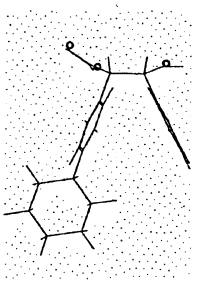
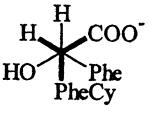
The properties of the Site II binding area suggest the following two-step interaction mechanism:

Step one: the solutes interact with Site II through an electrostatic interaction between the anionic carboxylate moiety on the solute and the cationic groups on the edge of the cleft.

Step two: the hydrophobic portions of the solute molecules enter into the hydrophobic cleft of Site II forming stabilized diastereomeric solute–protein complexes.

The interaction between the carboxylate moiety and the stationary phase tethers the solute relative to the hydrophobic cleft and this attachment is key to the enantioselective process. This is illustrated by the fact that the enantioselectivity was lost when compounds **1E** and **1T** were chromatographed as their methyl ester derivatives. However, while this interaction forms the solute–Site II complex, it cannot be the source of the observed enantioselectivity. Electrostatic interactions are long-range interactions (2 Å or greater) and the solute need not come into full contact with the chirality of the protein.

Table 6
Low energy conformation of some representative compounds studied

no.	Stereochemistry	Preferred conformation	Newman projection
1E	Erythro (2R,3S)		
1T	Threo (2R,3R)		
2E	Erythro (2R,3R)		
2T	Threo (2R,3S)		

The source of the enantiomeric discrimination is the process through which the solute–Site II complex is completed and stabilized. This process involves conformational adjustments of the solute and protein to allow for the insertion of the hydrophobic portion of the solute into the hydrophobic cavity at Site II, and the actual insertion of the solute into this cavity. The former process requires the expenditure of energy while the latter releases energy and is the overall driving force of the process.

The necessity for an initial conformational adjustment of the solute is supported by the differences between the magnitude of the enantioselective separations of the molecules in the linear and folded conformers. It can be assumed that the molecules with folded conformations will expend more energy assuming conformations capable of interacting with the hydrophobic cavity than those molecules whose preferred conformations are linear. The more energy that is required to create the diastereomeric solute–Site II complexes, the lower the expressed energetic differences between these complexes and the smaller the observed enantioselectivity. In this case, the average $\Delta\Delta G$ for the folded conformations is 60 cal lower than the average $\Delta\Delta G$ for the linear conformations producing a decrease in the average observed enantioselectivity from $\alpha = 1.39$ to $\alpha = 1.21$.

While the conformation adjustments appear to affect the magnitude of the enantioselectivity, the actual source of the enantio-differentiation appears to be steric interactions between the solute and the chirality of Site II that occur during the insertion of the solute into this cavity. This is suggested by the enantiomeric elution order. In both the erythro and threo series, the least retained enantiomers had an absolute configuration of *R* at carbon-2, the carbon adjacent to the carboxyl moiety (Fig. 1, Table 5). In the electrostatic tethering→conformational adjustment→hydrophobic insertion model, once the molecule has been tethered, the conformational adjustment initially and predominately brings the chirality at carbon-2 into the position to interact with the chirality of the hydrophobic cleft, and it is this interaction which occurs as the solute enters into the hydrophobic cavity, that drives the overall enantioselectivity.

5. Conclusions

The possibility of a predominantly conformationally driven chiral recognition mechanism demonstrates one of the problems associated with use of quantitative structure retention relationships to describe enantioselective separations. There is no question that the equations developed in this study can be used to predict enantioselective separations within this series of compounds. However, the non-specific hydrophobic interactions are so great that they mask the subtle enantioselective interactions making mechanistic determinations difficult. For example, the average diastereomeric separation factor for the erythro and threo compounds is 2.62, representing an energy difference ($\Delta\Delta G$) of 571 cal between the solute–stationary phase complexes of the two diastereomers. This is three times greater than the average $\Delta\Delta G$ calculated for the enantioselective separations of the linear molecules.

In this study, the inability of the chemometric approach to identify a molecular descriptor responsible for the enantioselective separations is both a function of the number of solutes ($n = 10$) and the small energetic differences. While a dramatic increase in the size of the experimental cohort is one avenue for further experimentation, perhaps a better experimental approach would be to apply fast-kinetic techniques to this system. The latter possibility is currently under investigation.

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