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Capillary electrophoretic and high-performance liquid chromatographic studies of the enantioselective separation of α_1-adrenoreceptor antagonists

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Abstract

Capillary electrophoresis (CE) and chiral stationary phase (CSP) HPLC methods were investigated for the determination of enantiomeric purity of α_1-adrenoreceptor antagonists related to WB 4101. In the CE study, the enantioseparation of the analytes was performed by studying the effect of different types of cyclodextrin in the buffer, namely heptakis (2,6-di-O-methyl)-β-cyclodextrin (DMCD), hydroxypropyl-β-cyclodextrin (HPCD) and β-cyclodextrin (β-CD). HPCD was found to be the most effective chiral selector in the enantioseparation of all the compounds, with high resolution values. A HPLC method, using immobilised serum protein columns, human serum albumin (HSA) and α_1-acid glycoprotein (AGP), was also investigated. Two benzodioxane racemates were well resolved on a mixed type (50% HSA and 50% AGP) column, with enantioselective binding on AGP column. © 1998 Elsevier Science B.V.

Keywords: Chiral stationary phases, LC; Enantiomer separation; Immobilised proteins; Benzodioxanes

1. Introduction

Several antagonists have been shown to discriminate among α_1-adrenoreceptor subtypes so far identified, namely, α_{1A}, α_{1B} and α_{1D} [1–3]. Benzodioxane bearing compounds related to WB 4101 {2-[[[2-(2,6-dimethoxyphenoxy)ethyl]amino]methyl] - 1,4 - benzodioxane}; (±**I**) (Fig. 1) represent a valuable tool for the characterization of α_1-adrenoreceptor subtypes [4]. Since they have been shown to posses significant stereoselectivity, it is of paramount importance to make available simple methods for the determination of enantiomeric purity.

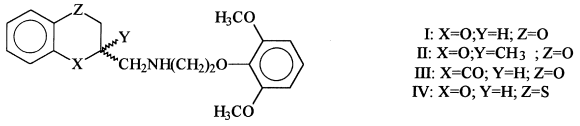

Fig. 1. Structures of α_1-adrenoreceptor antagonists.

A reversed-phase HPLC method for the separation and determination of the optical purity of WB 4101 (**I**) was previously reported [5], in which (±)**I** was converted into the amide of N-tosyl-(*S*)-proline.

This method was not found suitable for the other structurally related compounds, therefore both CE (capillary electrophoresis) and HPLC methods using chiral stationary phases (CSPs) based on immobilized proteins, were investigated as suitable ap-

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proaches for the resolution of the enantiomers of compounds **I**–**IV**.

CE is in fact a merging technique for quick and efficient separations of chiral compounds, and it has been used for the resolution of chiral drugs using chiral selectors as additives in the buffer [6,7].

A comparative CE study of different cyclodextrin derivatives is reported here for the enantioseparation of compounds **I**–**IV**; a validated method is also proposed for the determination of the enantiomeric purity of compound **IV**.

An alternative HPLC study was performed in order to check the binding properties of these compounds to the plasma proteins [8] α_1-acid glycoprotein (AGP) and human serum albumin (HSA) and to test the applicability of a mixed column (50% AGP and 50% HSA) to a broad range CSP for the enantioseparation of drugs [12].

2. Experimental

2.1. Chemicals and reagents

Purified water from a TKA ROS 300 system was used to prepare buffers and standard solutions. Phosphoric acid (85%) was from Carlo Erba (Milan, Italy), triethanolamine, heptakis (2,6-di-O-methyl)-β-cyclodextrin (DMCD), hydroxypropyl-β-cyclodextrin (HPCD) were purchased from Aldrich (Milan, Italy) and β-cyclodextrin (β-CD) was from Janssen (Geel, Belgium).

The benzodioxane related compounds WB 4101 (**I**), WS (**II**), WB4CO (**III**), and WBS (**IV**) were from the medicinal chemistry laboratory of the Department (Professor C. Melchiorre).

2.2. Apparatus

All CE measurements were carried out on a ^{3D}CE system (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode-array detector; the signals were recorded and processed with an HP Vectra 486/100 XM2 computer.

A fused-silica capillary of 48.5 cm (40 cm to the detector cell)×50 μm I.D. from HP was used. The experiments were performed at 15°C and constant voltage (+25 kV); the samples were introduced hydrodynamically for 10 s (injection pressure 5 kPa) and the analytes were monitored by UV detection at 220 nm.

All the compounds were analysed using a SpectraSystem P 1000 HPLC pump. The detector was a Spectra 100 fixed at 220 nm. The chromatograms were registered on a Data Jet (Thermo Separation Products San Jose, CA, USA) integrator. A 20-μl sample loop for the Rheodyne valve was used.

2.3. CE procedure

All carrier electrolytes were filtered through 0.45 μm Millex-HV filter units (Millipore, Milford, MA, USA). The capillary was rinsed (3 min) between runs with the separation electrolyte consisted of 0.1 *M* phosphoric acid adjusted to pH 3.00 with triethanolamine and containing β-CD or its derivatives.

The sample solutions were prepared in a diluted buffer solution (1:10) without cyclodextrin at a concentration of 50 μg/ml of racemic compound and stored at ambient temperature.

2.4. HPLC analysis

All the compounds were first dissolved in methanol, and then diluted with 0.1 *M* phosphate buffer (pH 7.4) to concentration ranging from 15 to 50 μ*M*.

The CSPs were: 10 μm HSA column, AGP column and a mixed column (50% AGP and 50% HSA) (150×4.6 mm I.D.). The three columns were prepared according to reference [12]. The amount of protein immobilised on each of the analytical column was as follows: HSA CSP: 85 mg HSA per gram silica; AGP CSP: 105 mg AGP per gram of silica; AGP–HSA column: 80 mg HSA per gram of silica and 100 mg AGP per gram of silica.

The mobile phase compositions were 1-propanol–0.1 *M* potassium phosphate buffer, pH 7.4 (10:90, v/v) (no. 1) and 5:95 v/v (no. 2) for all the compounds. The buffer was prepared by mixing solutions of 0.1 *M* K_2HPO_4 and KH_2PO_4 to get the desired pH. All mobile phases were filtered with a 0.45-μm micropore filter. The column was washed with 1–propanol–water (5:95, v/v) between experiments.

The chromatographic separations were performed

at 37°C with a column heater, at a flow-rate of 1 ml min^{-1}.

3. Results and discussion

3.1. CE analysis

Optimal parameters (CD type and concentration, buffer pH etc.) for the chiral separation of the considered analytes were studied. A reversal of the electroosmotic flow was chosen using phosphate–triethanolamine buffer (pH 3) because under these conditions a resolution enhancement for the basic racemates was reported [9]. Resolutions were estimated using USP definition based on peak width at half-height.

3.1.1. Effect of neutral cyclodextrins

In order to study the best conditions for the enantioresolutions of the examined racemates (**I**–**IV**), the effect of CD concentration was evaluated

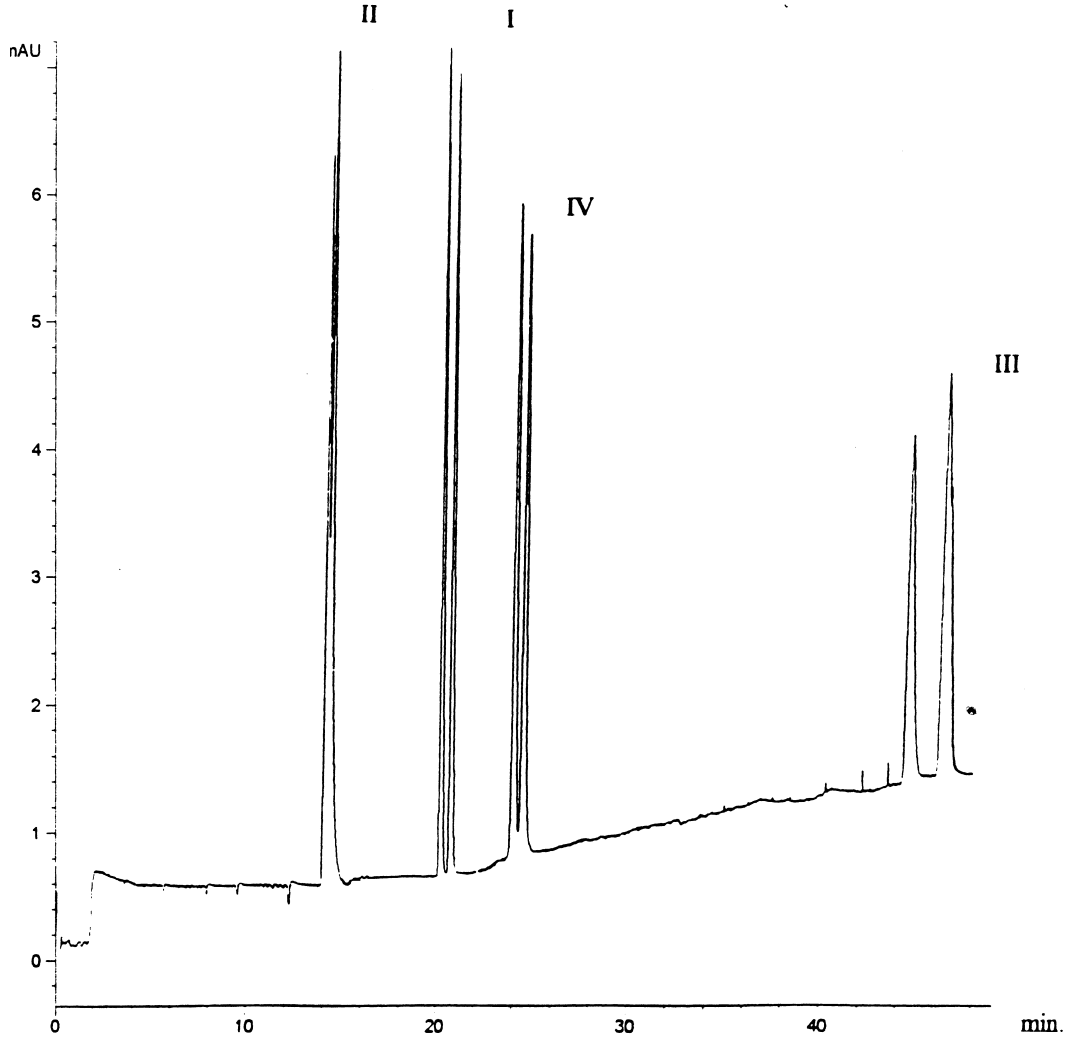

Fig. 2. Separation of enantiomers of compounds (**I**–**IV**) by CZE with β-CD. Solutes as in Fig. 1. Conditions: 7.5 m*M* β-CD in 100 m*M* phosphoric acid adjusted to pH 3.0 with triethanolamine; separation tube, 48.5 cm (effective length 40 cm)×50 μm I.D.; applied voltage, +25 kV; detection 220 nm; temperature, 15°C; injection time of the sample solution, 10 s.

since the degree of separation between the enantiomers is reported to be a function of the chiral selector concentration [10].

β-CD was used at concentrations lower than 15 m*M*, due to its poor water solubility; under these conditions compound **III** was fully resolved (R_s=4). The other racemates were also resolved but with only partial resolution. The CE separations of the four tested compounds with native β-CD as the chiral selector are presented in Fig. 2. This system seems to be very selective, allowing the separation between the different analytes, and also the partial or complete enantioresolution for each compound.

When DMCD was used as chiral selector, long migration times were observed; for example compound **II** migrated over 60 min at 7.5 m*M* and 10 m*M* concentration of DMCD. Moreover, the enantioresolution was lost in the range 20–40 m*M* (concentration of DMCD) but with higher concentration of DMCD the separation increased again (R_s=0.8 at 60 m*M* and R_s=1.0 at 80 m*M*). This

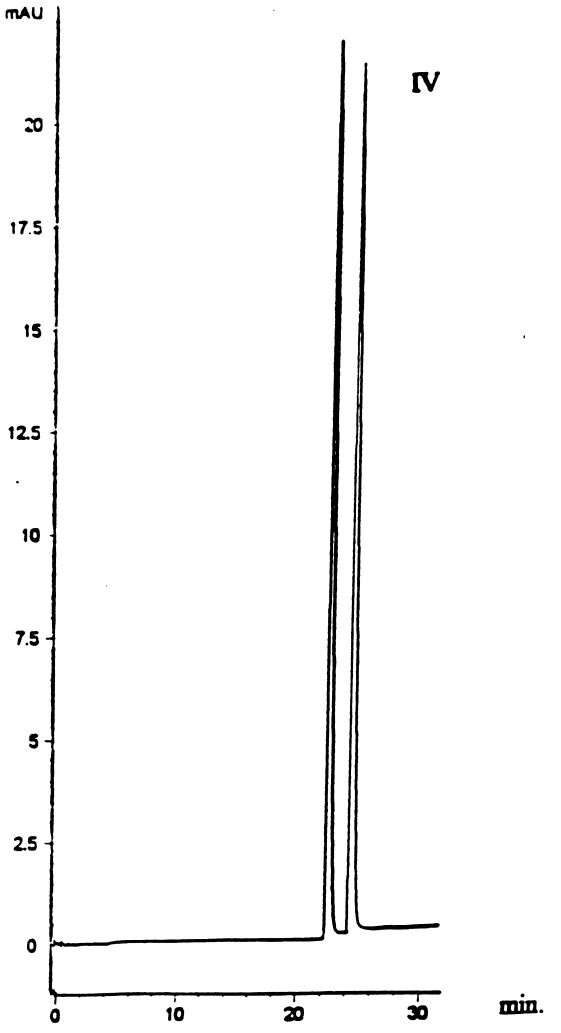

Fig. 3. Enantioseparation of compound **IV** by CZE with HPCD. Conditions: 20 m*M* HP-CD in 100 m*M* phosphoric acid adjusted to pH 3.0 with triethanolamine; other conditions as described in Fig. 2.

Table 1
Resolution values (n=3) for benzodioxane enantiomers (**I**–**IV**) separated by capillary electrophoresis with neutral cyclodextrin

Concentration	CD	**I**	**III**	**IV**	**II**
1 m*M*	β-CD	[a]	0.7	[a]	[a]
	DMCD	1.0	1.9	3.1	0.8
	HPCD	1.0	0.5	0.8	1.5
5 m*M*	β-CD	2.0	1.8	1.0	0.8
	DMCD	1.6	2.5	3.3	2.5
	HPCD	5.0	1.0	2.9	4.3
7.5 m*M*	β-CD	2.3	3.2	1.7	0.9
	DMCD	1.7	3.1	2.7	[b]
	HPCD	6.3	1.3	3.6	5
10 m*M*	β-CD	2.8	4.0	1.7	0.9
	DMCD	1.8	3.3	2.3	[b]
	HPCD	6.1	1.1	3.5	4.7
12.5 m*M*	β-CD	2.8	2.8	1.4	1
	DCMD	[b]	[b]	[b]	1
	HPCD	6.8	1.4	3.5	5
15 m*M*	β-CD	2.8	2.1	1.4	[b]
	DCMD	[b]	[b]	[b]	1
	HPCD	7.1	1.5	3.4	5
20 m*M*	DCMD	[b]	[b]	[b]	[a]
	HPCD	7.5	1.7	3.2	5
30 m*M*	DMCD	[b]	[b]	[b]	[a]
	HPCD	7.1	2.0	3.1	5
40 m*M*	DMCD	[b]	[b]	[b]	[a]
	HPCD	5.4	2.0	2.3	5.3
50 m*M*	DMCD	[b]	[b]	[b]	[a]
	HPCD	5.4	1.6	2.3	[a]
60 m*M*	DMCD	[b]	[b]	[b]	0.8
	HPCD	4.6	1.6	2.0	[b]

Resolution at the baseline was obtained at R_s=1.5
[a] No resolution.
[b] Migration time over 60 min.

phenomenon might be due to a reversed elution order.

A typical electrophoretic enantioresolution of compound **IV** obtained with HPCD (20 m*M*) is presented in Fig. 3. This chiral selector was found to be the most effective as very good resolution values were obtained for all the drugs with a relatively short migration times (Table 1). The good resolutions obtained under these conditions were applied for testing the chiral purity of the enantiomers of compound **IV**. This was of great interest because the two enantiomers display significant enantioselectivity toward α_1-adrenoreceptor subtypes [11].

*3.1.2. Determination of chiral purity of (±**IV**)*

The ability of the method to determine the purity of the enantiomers of compound **IV** was initially evaluated by direct analysis of each enantiomer and was confirmed by the 'addition standard method'. In the latter approach increasing amounts of an enantiomer of known purity was added to a known concentration of the other enantiomer, in order to verify the linearity of the response to the detector. A 7.5 m*M* concentration of HPCD was chosen as the best compromise between a good enantioresolution (R_s = 3.4) and short migration time (Table 1).

The ability of the method to detect minor contamination of one enantiomer by the other was investigated using relative (+)/(−) and (−)/(+) ratios of 1.0/99.0 to 5.0/95.0. To the pure (+) isomer (150 μg/ml), 1.5–7.5 μg/ml of (−) isomer were added to give ratios of 99.0/1.0 to 95.0/5.0. The ratios (*Y*) of peak corrected area (area/migration time) of impurity to analyte were calculated at each calibration point. The linear regression analysis obtained plotting the ratios (*Y*) versus impurity concentration (*C*) showed excellent correlation coefficients: r^2=0.9998 for the (−)-enantiomer and r^2=0.9996 for the (+)-enantiomer.

The method was applied to experimental samples of enantiomer (+) and (−). By the interpolation of the standard addition plot, the initial contamination of (−)-enantiomer and (+)-enantiomer was found to be 95.2/4.8 and 95.7/4.3, respectively.

The relative standard deviations (R.S.D.s, n=5) for peak area measurements were evaluated at 0.5%, 1% and 2% spike level for each enantiomer; the R.S.D.s were 1.47%, 1.39% and 0.6% for (−)-enantiomer and 1.8%, 1.6% and 0.8% for (+)-enantiomer, respectively.

The detection (LOD) and quantification (LOQ) limits for both the enantiomers, expressed as the signal-to-noise ratios of 3 and 10 respectively, were evaluated by the progressive dilution of an enantiomer solution (about 95% purity). LOD was found to be 0.2 μg/ml and LOQ 0.6 μg/ml, corresponding respectively to 0.13% and 0.4%.

3.2. Chromatography

Three types of stationary phases packed with: (a) 100% AGP, (b) 100% HSA, (c) a mixed 50% AGP and 50% HSA were tested. The mixed column with both the immobilised serum proteins, was already found to have an extended range of application for acidic and basic drugs [12], showing a good ver-

Table 2
Capacity factors (k'_1) and enantioselectivity (α) of racemate (**I**–**IV**) obtained on the three types of protein stationary phases

Compound	HSA–AGP column				AGP column				HSA column			
	Mobile phase 1		Mobile phase 2		Mobile phase 1		Mobile phase 2		Mobile phase 1		Mobile phase 2	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α	k'_1	α	k'_1	α
I	5.3	1	17.8	1.22	7.5	1	21.1	1.21	5.2	1	12.4	1
II	4.4	1.57	Nd	Nd	9.0	1.74	Nd	Nd	5.8	1	16.8	1
III	0.7	1	1.4	1	0.6	1	1.5	1	0.7	1	1.4	1
IV	12.1	1	49.5	1.02	26.1	1	Nd	Nd	9.9	1	26.6	1

Nd=not done.
Mobile phase 1: 1-propanol–0.1 *M* potassium phosphate buffer, pH 7.4 (10:90, v/v); mobile phase 2: 1-propanol–0.1 *M* potassium phosphate buffer, pH 7.4 (5:95, v/v).

satility. It is well recognised that HSA preferably binds acidic compounds [13,14], while AGP is more favourable for cationic compounds [15], so the two together can complement their performance.

The separation of the reported series of chiral compounds was studied on the HSA, the AGP and the AGP–HSA CSPs with two mobile phase with different percentage of *n*-propanol (5% and 10%). The separations were not systematically optimised but the retention and enantioselectivity of test compounds were compared on the three columns under the same conditions.

The chromatographic retention was expressed as a capacity factor (k') and the enantioselectivity factor (α) was calculated as the ratio of the k' values of the second eluting enantiomer to the first eluting enantiomer.

The capacity factor of the first eluted enantiomer and the enantioselectivity on all the three CSPs for the four compounds are summarised in Table 2.

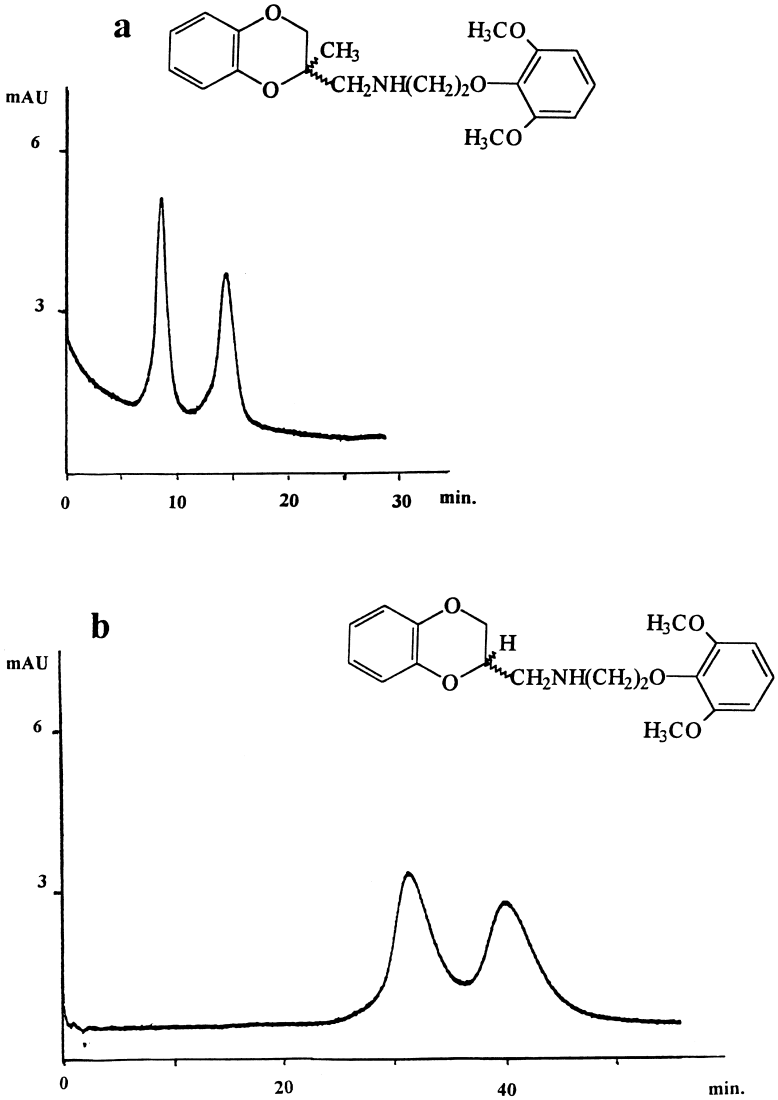

Fig. 4. HPLC enantioseparation of benzodioxane derivatives (**I**, R_s=1.13; **II**, R_s=1.66) on a mixed 50% AGP and 50% HSA CSP; mobile phase: 1-propanol– (pH 7.4, 0.1 *M*) potassium phosphate buffer (10:90, v/v) (a) and (5:95, v/v) (b).

Due to their basicity, the compounds were separated on the AGP column. This confirms the stereoselective binding of protonated drugs on AGP at physiological pH. Compounds **I** and **II**, bearing two oxygen on the etherocycle, were resolved on the AGP (α=1.21; 1.74) and the mixed protein columns (α=1.22; 1.57) (Fig. 4), while the sulphur derivative **IV** showed excessive affinity on AGP, without enantioseparation on both the HSA and AGP columns. On the mixed phase it showed less retention than AGP. On the contrary the presence of the carbonyl group in compound **III** conferred low affinity and non-reproducible chiral separation on all the three CSPs. No enantioselectivity for all the compounds was observed on the HSA column.

As expected [12], the capacity factors on the mixed phase were found to be the average of the capacity factors on the two single protein columns, while the enantioselectivity was maintained as on the AGP column, being enantioselective only for the binding on this protein.

The HPLC method was therefore found useful for the determination of the enantiomeric excess of compounds **I** and **II**, whereas the CE method was found to be general applicable.

4. Conclusion

The enantioseparation of benzodioxane related compounds was performed by CE and HPLC. CE allowed high enantioresolution to be achieved for each compounds in the series. By using the proposed method, the enantiomeric purity, whose determination is critically important for the biological activity, can be easily evaluated. For this application the choice of the suitable CD derivative proved to be a critical factor in improving enantioseparation.

HPLC study with different types of immobilised protein stationary phases afforded information about the binding properties of the examined compounds to the serum proteins. Their affinity was found to be higher on AGP than on HSA, confirming the transport role for basic drugs of this protein, with enantioselective binding for benzodioxane derivatives.

From the point of view of the analytical performances, AGP and the mixed type stationary phases had the same capacity for enantiodiscrimination, the latter showing advantages in terms of shorter retention times.

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