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Enantiomeric separation of pirlindole by liquid chromatography using different types of chiral stationary phases

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Abstract

The enantioseparation of pirlindole by liquid chromatography (LC) was investigated using three different chiral stationary phases (CSPs) containing either cellulose tris-(3,5-dimethylphenylcarbamate) (Chiralcel OD-R), ovomucoid (OVM) or β-cyclodextrin (β-CD). The effects of the mobile phase pH on retention, enantioselectivity and resolution were studied. Methanol and acetonitrile were tested as organic modifiers while the influence of the addition to the mobile phase of sodium alkanesulfonates or sodium perchlorate was also investigated. Sodium perchlorate was only used on the Chiralcel OD-R column while sodium alkanesulfonates were tested as mobile phase additives on the three kinds of CSPs. The enantioseparation of pirlindole could be obtained on all CSPs tested, the best results with respect to chiral resolution being achieved on the Chiralcel OD-R and the OVM columns. The use of sodium octanesulfonate (NaOS) was found to improve the enantioseparation of pirlindole on the OVM column while enantioselectivity was considerably enhanced by addition of sodium perchlorate on the Chiralcel OD-R column.

Keywords: Pirlindole; Chiral stationary phases; β-Cyclodextrin; Ovomucoid; Cellulose tris-(3,5-dimethylphenylcarbamate); Ionic modifiers

1. Introduction

Pirlindole [2,3,3a,4,5,6-hexahydro-8-methyl-1H-pyrazino (3,2,1-j,k) carbazole hydrochloride] is a chiral tetracyclic compound, characterised as an antidepressant drug (cfr. Fig. 1). This substance has been used so far as a racemate [1–8]. Clearly, however, the enantiomers of chiral drugs can present differences in pharmacological activity and efficacy.

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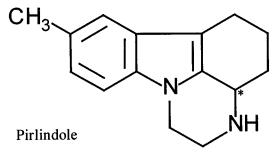

Fig. 1. Structure of pirlindole. The chiral centre is marked by an asterisk.

It is therefore important to develop enantioselective analytical methods for pharmacokinetic and metabolism studies and for quality control. Nowadays, many enantiomeric separations are performed by liquid chromatography (LC), using either derivatisation of the analyte with an optically pure reagent and subsequent separation on an achiral stationary phase (indirect approach) or a chiral stationary phase (CSP) which makes it possible to resolve directly the enantiomers (direct approach) [9].

Each type of CSP can be classified according to the nature of the chiral selector. Different groups of chiral selectors can be distinguished, small molecules involving charge transfer complexation ($\pi-\pi$ bonding interactions), crown-ethers, cyclodextrins (CDs) or derivatised CDs, polysaccharides and polysaccharide derivatives, proteins and macrocyclic antibiotics have been used successfully in CSPs for the enantioseparation of various kinds of compounds [9–12].

In the present study, three different CSPs are evaluated with respect to their enantioselectivity towards pirlindole. They consist in an ovomucoid (OVM) stationary phase (Ultron ES-OVM column), a β-cyclodextrin (β-CD) bonded phase (Chiradex) and a cellulose tris-(3,5-dimethylphenyl)carbamate coated on silica (Chiralcel OD-R).

These three CSPs can be used in the reversed phase mode and have been reported to be suitable for the chiral separation of a wide range of substances [13–38].

The aim of this study is to determine the most suitable LC conditions for the enantiomeric separation of pirlindole. The influence of the mobile phase pH, the nature and concentration of neutral modifiers and the addition of ionic modifiers, such as sodium alkanesulfonates or sodium perchlorate on the enantiomeric resolution of pirlindole has been investigated for the three CSPs examined.

2. Experimental

2.1. Chemical and reagents

Racemic pirlindole hydrochloride was supplied by Therabel Research (Brussels, Belgium), *S*-(+)-pirlindole and *R*-(−)-pirlindole were synthesised by derivatisation of racemic pirlindole with *R*-phenethylisocyanate, separation of the corresponding diastereoisomers by preparative LC, hydrolysis and recrystallisation [39]. Each enantiomer was identified by polarimetry and their absolute configuration was determined by crystallographic experiments.

Sodium monohydrogen phosphate, sodium dihydrogen phosphate, sodium hydroxide, sodium bromide, potassium bromide, sodium perchlorate

Table 1
Influence of pH and acetonitrile concentration on retention and separation of pirlindole enantiomers on Chiralcel OD-R[a,b]

		20%	25%	30%	35%	40%	45%	50%
pH 5.0	k'_S	4.8	2.7	1.6	1.0	0.71	0.56	0.47
	α	1.58	1.46	1.38	1.31	1.34	1.24	1.22
	Rs	2.6	2.2	1.6	1.2	1.0	<0.7	<0.7
pH 6.0	k'_S	–	–	6.2	4.0	2.9	2.2	1.9
	α	–	–	1.20	1.18	1.17	1.16	1.14
	Rs	–	–	1.5	1.3	1.1	0.9	0.7

[a] Stationary phase, Chiralcel OD-R; mobile phase, 50 mM phosphate buffer containing acetonitrile.
[b] k'_S, retention factor of *S*-(+)-pirlindole (first eluting enantiomer); α, selectivity; *Rs*, resolution; (–), not determined.

Table 2
Effect of ionic modifiers on retention and enantioresolution for pirlindole on Chiralcel OD-R[a,b]

		10^{-2} M	2.10^{-2} M	5.10^{-2} M	0.1 M	0.2 M	0.5 M
Sodium hexane-sulfonate	k'_S	0.54	0.61	0.65	0.69	–	–
	α	1.45	1.50	1.61	1.75	–	–
	Rs	1.4	1.7	2.1	2.7	–	–
Sodium perchlorate	k'_S	0.77	0.86	1.1	1.2	1.4	1.6
	α	1.51	1.60	1.73	1.84	1.92	2.05
	Rs	1.7	2.2	3.0	3.7	4.4	5.3
Sodium bromide	k'_S	0.54	0.52	0.49	0.48	0.46	–
	α	1.34	1.37	1.42	1.50	1.55	–
	Rs	1.1	1.2	1.6	2.0	3.1	–
Potassium bromide	k'_S	0.52	0.52	0.48	0.51	0.45	–
	α	1.36	1.37	1.41	1.44	1.49	–
	Rs	1.2	1.2	1.3	1.4	1.4	–

[a] Stationary phase, Chiralcel OD-R; mobile phase, 50 mM phosphate buffer (pH 5.0)-acetonitrile (60:40, v/v).
[b] k'_S, retention factor of *S*-(+)-pirlindole (first eluting enantiomer); α, selectivity; *Rs*, resolution (–), not determined.

and phosphoric acid were of p.a. quality from Merck (Darmstadt, Germany). Butane-, hexane- and octane-sulfonic acids (sodium salts) were obtained from Sigma (St Louis, MO, USA). Acetonitrile and methanol were of HPLC grade from Fischer Scientific (Loughborough, UK). The water used in all experiments was of Milli-Q quality from Millipore (Bedford, MA, USA).

The Ultron ES-OVM analytical column (150 × 4.6 mm i.d.) was packed with a CSP containing OVM chemically bonded to aminopropylsilica (particle size, 5 μm) from Shinwa (Kyoto, Japan). The Chiradex column from Merck (250 × 4 mm i.d.) was filled with β-CD covalently bonded to silica gel (5 μm). The third CSP used in this study was a Chiralcel OD-R column (250 × 4.6 mm i.d.) packed with cellulose tris-(3,5-dimethylphenylcarbamate) coated on silica (10 μm) from Daicel Chemical Industries (Tokyo, Japan). These three CSPs were preceded by a LiChroCart guard column prepacked with Lichrospher 100 DIOL (5 μm) from Merck.

Table 3
Influence of the mobile phase pH on retention and enantioseparation for pirlindole on Ultron ES-OVM[a,b]

	3.0	4.0	4.5	5.0	5.5	6.0
k'_S	0.30	1.1	1.8	3.3	7.2	12.3
α	1	1.10	1.15	1.20	1.19	1.15
Rs	–	<0.7	1.3	1.9	2.1	1.7

[a] Stationary phase, Ultron ES-OVM; mobile phases, 50 mM phosphate buffer-methanol (85:15, v/v) Sample, (±)-pirlindole 20 μg ml^{-1}.
[b] k'_S, retention factor of *S*-(+)-pirlindole (first eluting enantiomer); α, selectivity; *Rs*, resolution, (–), not determined.

2.2. Apparatus

The LC equipment consisted of a model L-6200 A pump, a model AS-2000 A autosampler equipped with a 100 μl loop, a L-5025 programmable column oven and a L-4250 UV-VIS detector, all from Merck-Hitachi (Merck).

The data were collected on a Compaq DeskPro 5100 (Houston, Tx, USA) computer and the results were printed on a HP Deskjet 500 (Hewlett-Packard, Palo-Alto, USA). The whole chromatographic system was controlled by the same computer, using a Merck-Hitachi D-7000 HPLC Manager software.

The pH of mobile phase buffers was adjusted by means of a model Delta 345 pH meter from Mettler (Greifensee, Switzerland). The guard column placed before each chiral column was a LiChroCart (4 × 4 mm i.d.) from Merck.

Table 4
Influence of the addition of an uncharged modifier on retention and enantioseparation for pirlindole on Ultron ES-OVM[a,b]

		0%	5%	10%	15%	20%	25%
Acetonitrile	k'_R	18.6	6.9	2.0	1.1	0.66	0.43
	k'_S	19.6	8.6	2.2	1.1	0.66	0.43
	α	1.05	1.25	1.13	1	1	1
	Rs	<0.7	2.8	1.2	–	–	–
Methanol	k'_S	19.6	–	5.3	3.3	2.2	1.7
	k'_R	18.6	–	6.5	3.9	2.4	1.8
	α	1.05	–	1.24	1.18	1.13	1.10
	Rs	<0.7	–	2.3	1.8	1.1	0.7

[a] Stationary phase, Ultron ES-OVM; mobile phase, 50 mM phosphate buffer (pH 5.0) containing acetonitrile or methanol.
[b] k'_S, retention factor of *S*-(+)-pirlindole; k'_R, retention factor of *R*-(−)-pirlindole; α, selectivity; *Rs*, resolution; (–), not determined.

2.3. Chromatographic systems

The mobile phases consisted of mixtures of 50 mM phosphate buffer and an organic modifier (methanol or acetonitrile), to which an alkanesulfonate or sodium perchlorate was possibly added. The aqueous fraction of the mobile phases was adjusted to the pH with a 10% sodium hydroxide solution if necessary.

Before use, all mobile phases were filtered through a 0.22 μm filter and degassed for 15 min in a Sonicor SC-100-22TH ultrasonic bath (Copiague, NY, USA). The flow rate was 0.5 ml min^{-1} when the Chiralcel OD-R column was used and 0.9 ml min^{-1} for the Ultron ES-OVM and the Chiradex CSPs. UV detection was performed at 220 nm in all experiments.

2.4. Standard solutions

For the studies of the influence of the mobile phase pH and the addition of neutral and ionic modifiers, a stock solution was prepared by dissolving 50 mg of racemic pirlindole in 50 ml of methanol. This solution was then diluted with water to obtain a final concentration of 10 μg ml^{-1} for each enantiomer.

The stock solution of *S*-(+)-pirlindole was prepared by dissolving 5 mg of this enantiomer in 10 ml of water. This solution was then diluted with water to obtain a final concentration of 10 μg ml^{-1}. The solution of *R*-(−)-pirlindole was prepared in the same way.

3. Results and discussion

3.1. Chiralcel OD-R

3.1.1. Influence of mobile phase pH and acetonitrile

The influence of the pH of the mobile phase on the retention and the enantioseparation of pirlindole enantiomers is illustrated in Table 1. A decrease in pH from 6 to 5 seems to have a favourable influence on enantioselectivity. At acetonitrile concentrations higher than 30%, however, lower chiral resolution values were obtained at pH 5, due to the insufficient retention of pirlindole enantiomers under these conditions. No useful results were found at pH < 5, because of too low retention and pH values > 6 were avoided in order to preserve the lifetime of the CSP.

As can be seen in Table 1, the enantiomers of pirlindole could be completely resolved at both pH (5 and 6) with an acetonitrile concentration of 30%. A maximum chiral resolution value of 2.60 was obtained with a mobile phase consisting of a pH 5 buffer containing 20% (v/v) of acetonitrile.

3.1.2. Influence of ionic modifiers

Four ionic modifiers were tested in the mobile phase with this CSP: sodium hexanesulfonate (NaHS), frequently used in ion-pair LC, sodium perchlorate, which was previously reported to be very useful for chiral separations on Chiralcel OD-R [27–31,36,37], sodium bromide and potassium bromide.

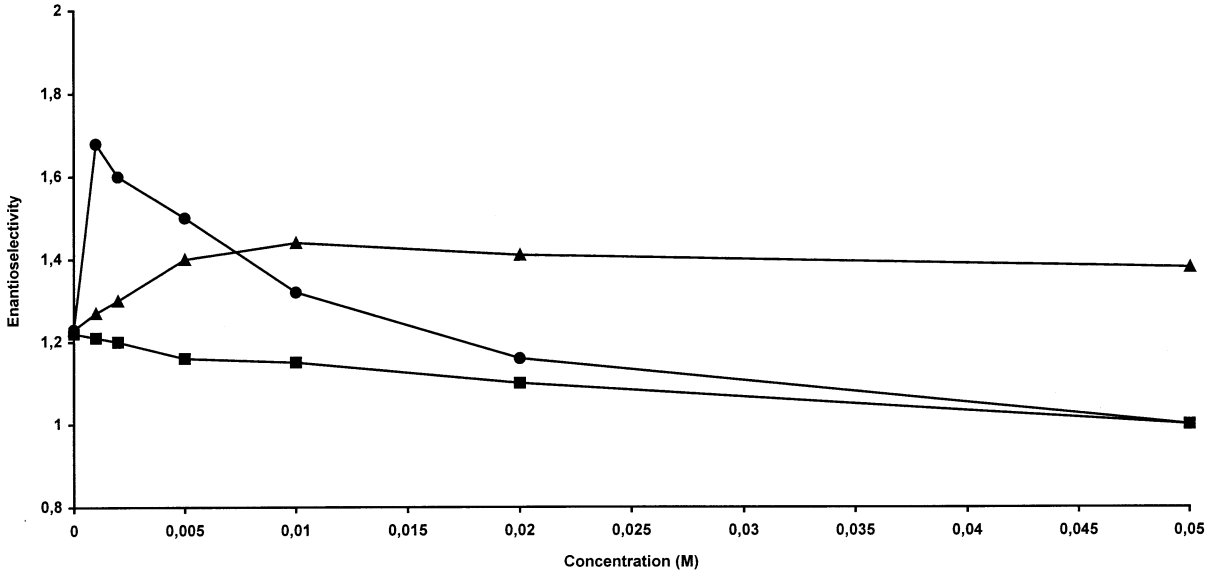

Fig. 2. Influence of the nature and concentration of alkanesulfonate on enantioselectivity for pirlindole on the Ultron ES-OVM. Stationary phase, Ultron ES-OVM; mobile phase, 50 mM phosphate buffer (pH 5.0)-acetonitrile (95:5, v/v); sample, (±)-pirlindole, 20 μg ml^{-1}. (■), NaBS (▲), NaHS (●), NaOS.

Pirlindole (pKa = 7.7) was essentially present in cationic form in the LC mobile phases used in this study (pH 5.0 buffer, acetonitrile, 60:40, v:v).

The effect of these ionic modifiers on retention and enantioseparation is shown in Table 2. With increasing concentration of NaHS and sodium perchlorate in the mobile phase, the retention of pirlindole enantiomers was found to increase while k' values were almost unaffected by the addition of sodium or potassium bromide.

Table 5
Influence of the pH of mobile phase on retention and enantioseparation for pirlindole on Chiradex[a,b]

pH	3.0	4.0	5.0	6.0	7.0	7.5
k'_R	1.91	2.62	4.54	8.9	16.17	18.26
α	1.11	1.13	1.14	1.14	1.14	1.15
Rs	0.95	1.36	1.58	1.9	1.73	1.75

[a] Stationary phase, Chiradex; mobile phase, 50 mM phosphate buffer-acetonitrile (85:15, v/v).

[b] k'_R: retention factor of R-(−)-pirlindole (first eluting peak); α, selectivity; *Rs*, resolution; (–), non determined values.

On the contrary, an increase in enantioselectivity and enantioresolution was obtained with increasing concentration of each ionic modifier.

It should be noted that the best enantioseparation of pirlindole was obtained with sodium perchlorate at 0.5 M concentration, with α and *Rs* values of 2.05, and 5.3, respectively. The hexanesulfonate concentration range necessary to obtain an increase in retention and a complete enantioseparation of pirlindole was unusually high (0.02–0.1 M).

It is interesting to note that significantly lower resolution values were obtained with KBr than with NaBr.

Results obtained with perchlorate and hexanesulfonate can probably be explained by an ion-pairing effects [27]. They confirm the very important role of the counter-ion in the optimisation of enantiomeric separations of basic compounds on this kind of CSP in the reversed phase mode [27]. Results obtained with sodium and potassium bromide seem to indicate that the nature of the co-ion has also an influence on enantioselectivity, probably due to a competition effect [27].

Table 6
Influence of the addition of an uncharged modifier on retention and enantioseparation for pirlindole on Chiradex[a,b]

		5%	10%	15%	20%	25%	30%	35%	40%	45%
Methanol	k'_R	–	–	8.9	7.6	6.5	5.4	4.2	3.2	2.5
	α	–	–	1.15	1.15	1.15	1.15	1.15	1.14	1.14
	Rs	–	–	1.9	1.9	1.8	1.8	1.7	1.6	1.5
Acetonitrile	k'_R	7.0	3.9	2.2	1.2	0.75	0.57	0.45	0.31	0.29
	α	1.14	1.14	1.14	1.13	1.13	1.12	1.11	–	1.00
	Rs	2.0	1.8	1.6	1.2	0.9	–	–	–	–

[a] Stationary phase, Chiradex; mobile phase, 50 mM phosphate buffer (pH 6.0) containing methanol or acetonitrile.
[b] k'_R, retention factor of R-(−)-pirlindole (first eluting peak); α, selectivity; *Rs*, resolution; (–), not determined.

3.2. Ultron ES-OVM

It is worth noting that according to Haginaka et al. [40], the chiral recognition ability of this stationary phase seems to be related to an ovoglycoprotein present as an impurity in the OVM bonded to silica.

3.2.1. Influence of the mobile phase pH

The influence of the mobile phase pH on the enantioseparation of pirlindole was investigated in the range from 3 to 7, using mobile phases consisting of mixtures of phosphate buffer and methanol (85:15, v/v).

As can be seen from Table 3, the retention factors of pirlindole enantiomers increased with increasing pH. When the mobile phase consisted of pH 7.0 buffer and methanol, the retention times of pirlindole enantiomers were higher than 75 min and the α and *Rs* values were not determined. This increase in retention, particularly pronounced between pH 6 and 7, can be attributed not only to the decrease in the protonation of the amino group of pirlindole but also to conformational changes of the protein on the stationary phase [13,14].

The effect of the mobile phase pH on the separation of pirlindole enantiomers is also shown in Table 3. With methanol as modifier, maximum enantioselectivity and resolution values were obtained at intermediate pH (5.0–5.5). These results can be again related to conformational changes in the immobilised protein occurring in that pH range [13,14].

3.2.2. Influence of uncharged modifier

Acetonitrile and methanol were tested as uncharged modifiers on this CSP. Their influence on retention and enantioseparation can be seen in Table 4. As could be expected in reversed phase systems, an increase in methanol or acetonitrile concentration resulted in a decrease in retention and enantioselectivity. However, it should be noted that a small amount of organic solvent seems to be necessary for an adequate enantioseparation of pirlindole on this CSP, the highest chiral resolution values being obtained with 5% of acetonitrile and 10% of methanol. It is interesting to note that the elution order of pirlindole enantiomers was reversed by addition of methanol to the mobile phase.

Similar observations about reversals of elution order caused by changes of uncharged modifiers on the OVM CSP were previously reported for propranolol and related substances [15,16]. These results suggest the existence of different kinds of binding sites, chiral and non-chiral, on this CSP.

3.2.3. Influence of ionic modifiers

Sodium-butanesulfonate (NaBS), NaHS, and NaOS were tested as anionic modifiers on Ultron ES-OVM. They were added to mobile phases made of 50 mM phosphate buffer (pH 5.0) and acetonitrile (95:5, v/v).

In opposition to the observations made on Chiralcel OD-R, an increase in the concentration of each alkanesulfonate caused the retention of pirlindole enantiomers to decrease, which does not correspond to the usual behaviour observed for an ion-pairing system.

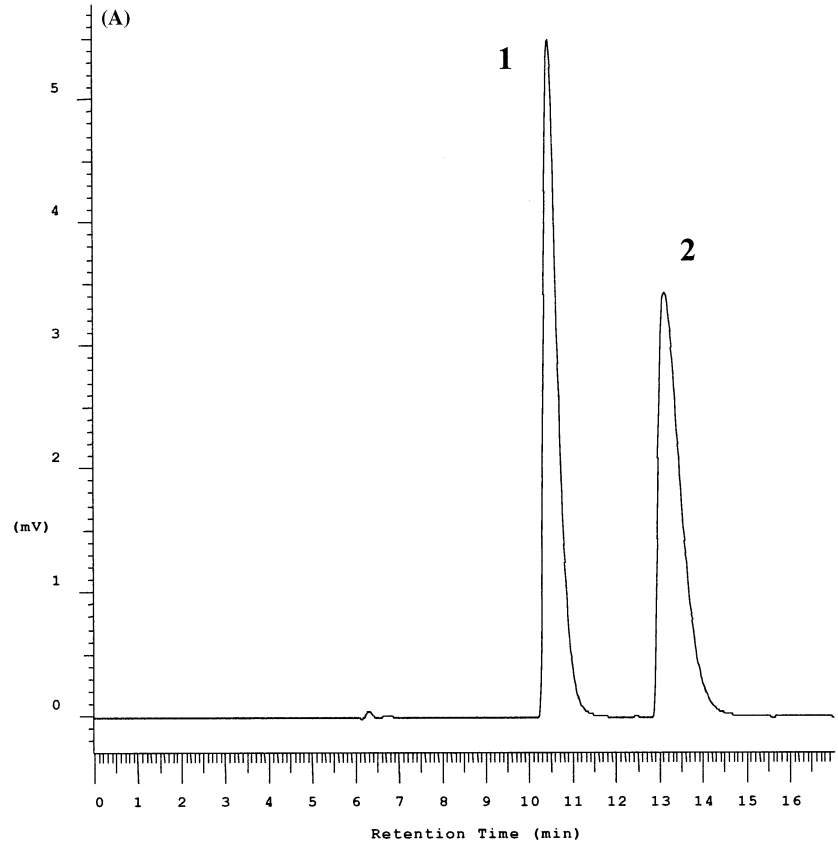

Fig. 3. Enantiomeric separation of pirlindole on the three CSPs. (A) Chiralcel OD-R-0.05 M $NaClO_4$ in pH 5.0 phosphate-acetonitrile (60:40, v/v); (B) Ultron ES-OVM-10^{-3} M NaOS in pH 5.0 phosphate buffer-acetonitrile (95:5, v/v); (C) Chiradex-pH 6.0 phosphate buffer-methanol (65:35, v/v). UV detection, 220 nm. Temperature, 27°C. Sample, (±)-pirlindole 20 μg ml. 1. *S*-(+)-Pirlindole; 2. *R*-(−)-Pirlindole.

The influence of the nature and concentration of the alkanesulfonate on enantioselectivity and enantioresolution was also investigated. Fig. 2 clearly shows that the effect of the alkanesulfonate on the enantioseparation of pirlindole using this CSP, strongly depends on the length of the alkyl chain. In the lower concentration range (≤ 5 mM), the addition of NaHS led to a significant improvement in the enantioseparation of pirlindole while at higher NaHS concentrations, enantioselectivity remained fairly constant.

When low concentrations of NaOS (≤ 5 mM) were added to the LC mobile phase, enantioselectivity for pirlindole was considerably increased. Nevertheless, in contrast with hexanesulfonate, enantioselectivity strongly decreased at higher NaOS concentrations. The effects of the alkanesulfonates on chiral resolution are similar to those observed for enantioselectivity.

The addition of NaOS to the LC mobile phase using Ultron ES-OVM as CSP was previously reported by Miwa et al. [35]. They also noted a decrease in retention for basic and acidic compounds by addition of NaOS but they could not observe any improvement in enantioseparation. Clearly, however, in the case of pirlindole, low concentrations of NaOS or NaHS can significantly improve chiral resolution. This favourable effect could be explained by interactions between the alkanesulfonate and the protein, leading to conformational changes of the protein in the vicinity of the chiral sites.

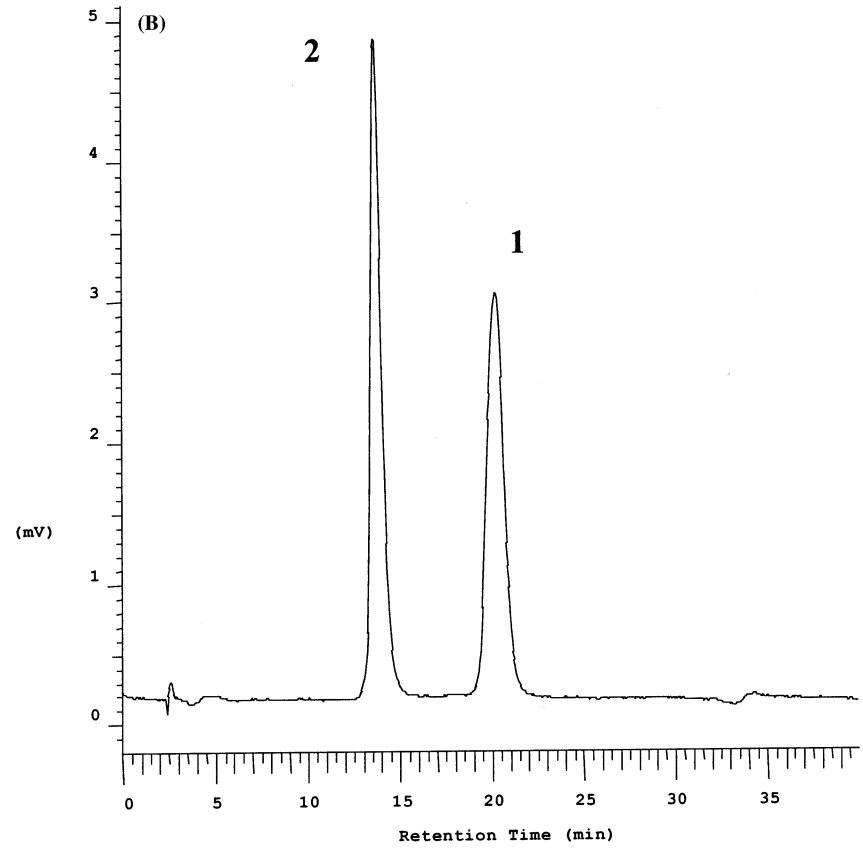

Fig. 3. (*Continued*)

3.3. Chiradex

The chiral recognition mechanism of CDs usually results from the formation of inclusion complexes between the hydrophobic moiety of the analyte and the relatively non-polar interior of the CD cavity [23].

3.3.1. Influence of the mobile phase pH

The influence of the pH of the mobile phase, consisting of a mixture of 50 mM phosphate buffer and acetonitrile (85:15, v/v) was investigated in the range from 3 to 7.5 (cf. Table 5). The increase in pH caused the retention of both enantiomers of pirlindole to increase. Changes in the mobile phase pH had very little influence on enantioselectivity while an increase in pH was found to have a favourable effect on chiral resolution, at least up to pH 6, at which a maximum *Rs* value (1.9) was observed.

3.3.2. Influence of uncharged modifier

Table 6 shows the influence of the concentration of methanol and acetonitrile on retention, enantioselectivity and resolution for pirlindole enantiomers. As could expected in a reversed phase system, an increase in the organic modifier concentration led to a decrease in retention and enantioresolution of the enantiomers. However, the decrease in enantioselectivity was less pronounced, especially with methanol, the α values being almost constant in the whole concentration range with this sorbent. Therefore, methanol should be preferably used to regulate retention on this kind of CSP [23,26].

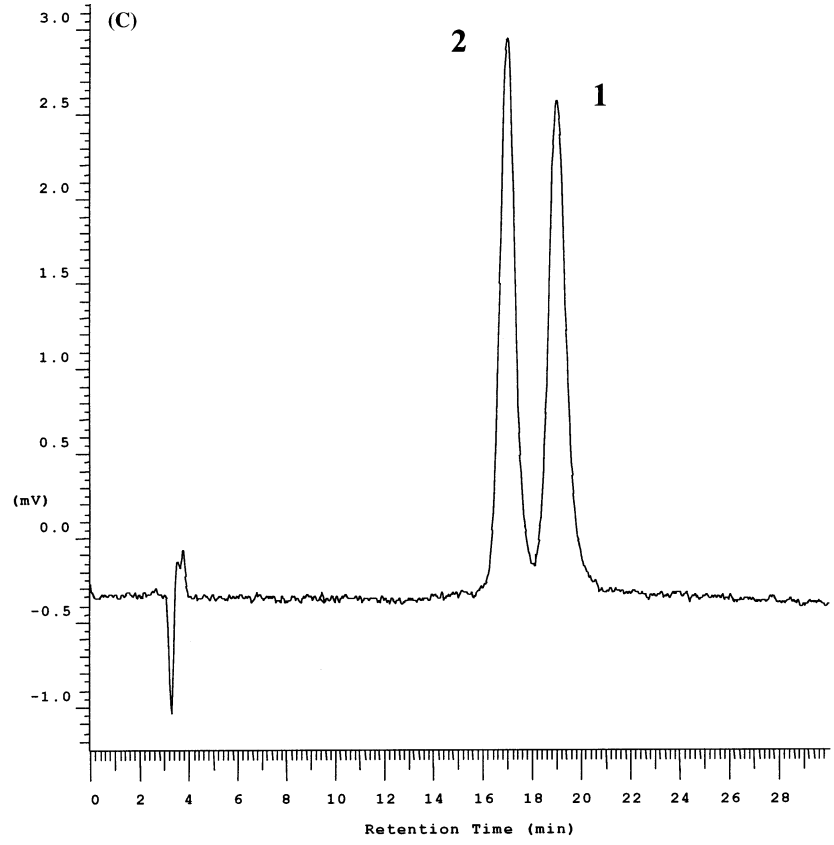

Fig. 3. (*Continued*)

3.3.3. Influence of ionic modifier and other mobile phase additives

NaBS, NaHS and NaOS were added to mobile phases consisting of pH 5.0 phosphate buffer and acetonitrile (90:10, v/v) in order to investigate possible effects on the enantioseparation of pirlindole on Chiradex. The addition of these compounds did not give rise to any improvement of the enantioseparation of pirlindole. In the presence of NaOS, enantiomeric resolution was almost completely lost.

4. Conclusion

The enantiomeric separation of pirlindole could be achieved on the three CSPs tested (cf. Fig. 3). The highest resolution values were obtained on the Ultron ES-OVM and Chiralcel OD-R columns.

The effect of alkanesulfonates was found to be of prime importance for the separation of pirlindole enantiomers on Ultron ES-OVM. A particularly favourable effect on chiral resolution was observed with octanesulfonate at 1 mM concentration. Moreover, the nature of the non-ionic modifier had a strong influence on the elution order of *R*-(−)- and *S*-(+)-pirlindole. With Chiralcel OD-R as CSP, the sodium perchlorate concentration in the mobile phase was confirmed to be a key parameter in the optimisation of the enantioseparation of pirlindole, besides the mobile phase pH and the acetonitrile concentration. On Chiradex, the addition of alkanesulfonates had a detrimental effect on the enantioseparation of pirlindole.

In order to perform the determination of enantiomeric purity or the pharmacokinetic studies of pirlindole, the Chiralcel OD-R column, combined

with a mobile phase consisting of a mixture of pH 5.0 phosphate buffer containing $NaClO_4$ (0.05 M) and acetonitrile (60:40, v/v) was found to be the most adequate LC system, due to the high enantioresolution obtained, the good stability of this kind of CSP and the high content of organic modifier in the mobile phase. The latter is particularly interesting in bioanalytical applications where sample enrichment at the top of the analytical column is generally needed. Using this chiral LC method, it was possible to measure concentrations lower than 0.1% of one enantiomer of pirlindole in its antipode [41].

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