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Simultaneous determination of pirlindole enantiomers and dehydropirlindole by chiral liquid chromatography

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Abstract

Liquid chromatography was employed for the determination of pirlindole enantiomers and its oxidation product dehydropirlindole (DHP). The direct separation of pirlindole enantiomers and DHP was achieved on a cellulose tris-(3,5-dimethylphenylcarbamate) chiral stationary phase (Chiralcel OD-R). Acetonitrile was used as the organic modifier and sodium perchlorate was used as an ionic additive in the mobile phase. The influence of acetonitrile and sodium perchlorate concentrations on enantioselectivity and achiral selectivity towards DHP was investigated in order to find suitable conditions for the determination of low amounts of each analyte. The mobile phase selected consisted of a mixture of acetonitrile and phosphate buffer (pH 5.0) containing sodium perchlorate (0.05 M) (35:65, v/v) and the UV detector was set at 220 nm. The method developed was validated and was found to be linear in the 0.1–5 μg ml^{-1} range ($r^2 = 0.999$ for the three compounds). Repeatability and the intermediate precision for the three analytes at a concentration of 0.1 μg ml^{-1} were about 3 and 4%, respectively. This concentration corresponds to the quantification of 0.1% for the minor enantiomer. Actual determinations of enantiomeric purity for single enantiomers of pirlindole were performed.

Keywords: Chiral liquid chromatography; Pirlindole enantiomers; Dehydropirlindole; Simultaneous determination

1. Introduction

The discovery of two distinct subtypes of mono-amine oxidase (MAO-A and MAO-B) and the development of reversible and selective inhibitors of MAO-A has led to a resurgence of interest in the use of MAO inhibitors for the treatment of depression.

Pirlindole (2,3,3a,4,5,6-hexahydro-8-methyl-1*H*-pyrazino (3,2,1-j,k) carbazole hydrochloride) is a tetracyclic compound that has been character-

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ized as a potential antidepressant drug in preclinical studies [1–3] and for which an interest has arisen due to its marked selectivity as a reversible inhibitor of MAO-A [4]. In clinical trials, the efficacy and safety of pirlindole has been demonstrated in comparison to reference standard drugs such as imipramine and amitryptilline [5].

More recently, the superiority of pirlindole was demonstrated versus placebo [6] and it was shown to be equivalent to moclobemide [7]. Another study has recently confirmed the potential interest of a reversible inhibitor of monoamine oxidase (RIMA) such as pirlindole for the treatment of major depression [8].

Pirlindole presents an asymmetric centre (Fig. 1) and therefore it has been decided to individually explore the pharmacology of its enantiomers.

Different analytical methods have been developed to perform the determination of pirlindole in pharmaceutical forms or to measure its pharmacokinetics in body fluids. These analytical methods are based on UV spectrophotometry [9–11], gas chromatography [12] or liquid chromatography [13,14]. Nevertheless, none of these analytical methods are non-stereoselective.

The enantiomeric separation of pirlindole was achieved in the reversed-phase mode on a cellulose tris-(3,5 dimethylphenylcarbamate) (Chiralcel OD-R) based chiral stationary phase (CSP). This chiral selector was previously used for the enantioselective separation of various drugs by LC using mixtures of aqueous buffer and organic modifier as mobile phases [15–26]. Nevertheless, it must be noted that the chiral separation of pirlindole could also be achieved on others CSPs such as an ovomucoid based stationary phase (Ultron ES-OVM, Shinwa) or a β-cyclodextrin bonded phase (Chiradex, Merck) [26].

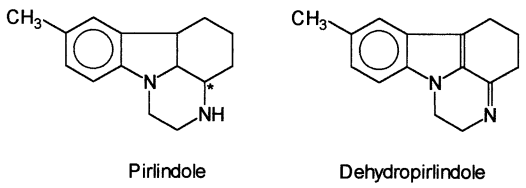

Fig. 1. Structures of pirlindole and DHP. The chiral centre is marked by an asterisk (*).

The aim of the method described in this paper is to achieve the enantiomeric purity determination of pirlindole and the simultaneous quantification of its main impurity, dehydropirlindole (DHP). DHP is the oxidation product of pirlindole but is not a chiral compound because of the loss of the hydrogen atom on the asymmetric carbon of pirlindole (Fig. 1).

The selection of the chiral LC conditions for the separation of (+)-(*S*)-pirlindole ((+)-P), (−)-(*R*)-pirlindole ((−)-P) and DHP has been discussed. The influence of sodium perchlorate and acetonitrile concentrations on the LC separation of these three compounds has been investigated. The analytical method developed has been validated and used to control the enantiomeric purity of single enantiomers of pirlindole obtained by synthesis [27] and to measure the DHP concentration present in these enantiomers.

2. Experimental

2.1. Chemical and reagents

Racemic pirlindole hydrochloride was supplied by Therabel (Brussels, Belgium), (+)-pirlindole and (−)-pirlindole were synthetised by derivatisation of racemic pirlindole with L-phenylalanine, separation of the diastereoisomers obtained by medium pressure liquid chromatography (MPLC), hydrolysis and recrystallization [27]. The structure of each enantiomer was confirmed by polarimetric measurement and their absolute configuration was determined by crystallographic experiments. The methyl ester of L-phenylalanine was obtained from Fluka (Buchs, Switzerland). L-Phenylalanine was from UCB (Brussels, Belgium). DHP hydrochloride was also synthesised at the department of Medicinal Chemistry of the University of Liège.

Sodium dihydrogenphosphate dihydrate, sodium perchlorate and sodium hydroxide were all of analytical grade from Merck (Darmstadt, Germany). Methanol and acetonitrile were of HPLC grade from Fischer (Loughborough, UK). The water used in all experiments was of Milli-Q quality (Millipore, Bedford, MA, USA).

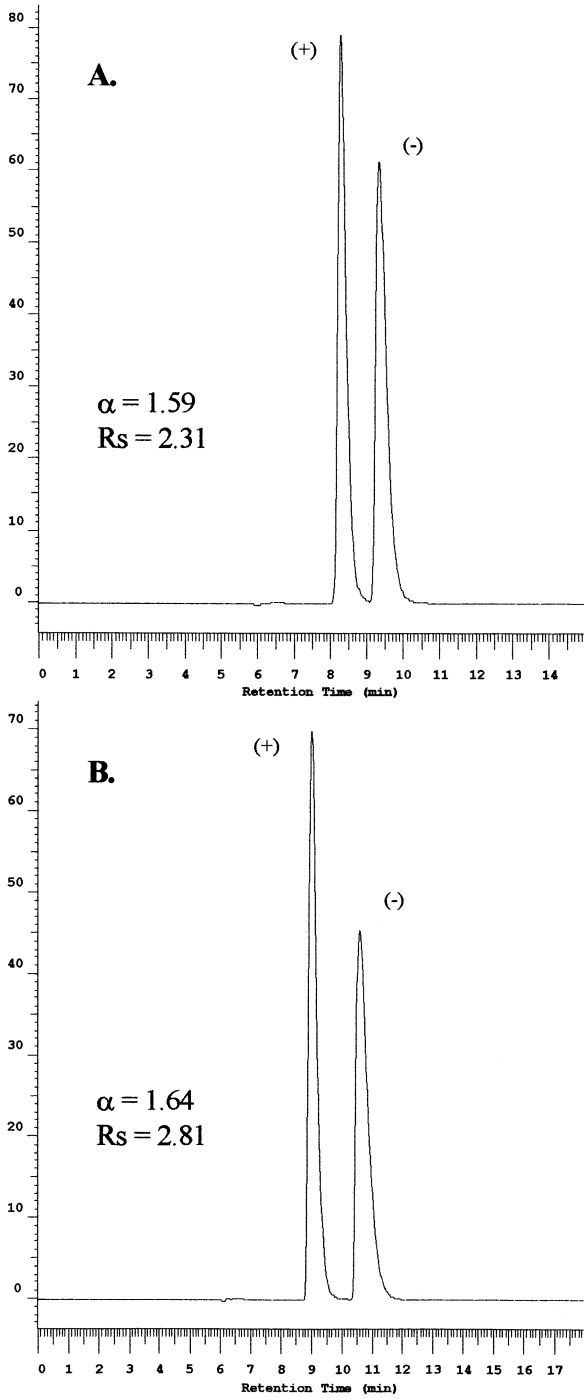

Fig. 2. Chiral separation of pirlindole. Stationary phase: Chiralcel OD-R; mobile phases: (A) phosphate buffer containing 50 mM $NaClO_4$ (pH 5.0)–acetonitrile (50:50); (B) phosphate buffer containing 50 mM $NaClO_4$ (pH 5.0)–acetonitrile (55:45). UV detection at 220 nm; flow rate, 0.5 ml min^{-1}; sample, (±)-pirlindole 100 μg ml^{-1}.

The chiral stationary phase was a Chiralcel OD-R column filled with cellulose tris-(3,5-dimethyphenylcarbamate) coated on silica (10 μm) from Daicel (Tokyo, Japan). The latter was preceded by a LiChroCart guard column prepacked with LiChrospher 100 DIOL (5 μm) from Merck.

2.2. Apparatus

The chromatographic system 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. The data were collected on an IBM compatible computer (PC-AT; CPU type Pentium) and the results were printed on a HP deskjet 500 (Hewlett-Packard, Palo Alto, CA, USA). The whole chromatographic system was controlled by the computer using the Merck-Hitachi D-7000 HPLC Manager.

2.3. Chromatographic technique

All chromatographic experiments were performed in the isocratic mode. For the determination of suitable condition for separating (+) and (−) enantiomers of pirlindole and DHP, the mobile phases consisted of mixtures of acetonitrile and of a 50 mM phosphate buffer containing sodium perchlorate, the pH of which was adjusted to 5.0 with a 10% solution of sodium hydroxide.

The mobile phase selected for the method validation and for the determination of the enantiomeric purity of the single enantiomers of pirlindole synthesised consisted of a mixture of a 50 mM phosphate buffer containing $NaClO_4$ (0.05 M), adjusted to pH 5.0 with a 10% solution of NaOH, and acetonitrile (65:35, v/v). Before use, all the mobile phases were degassed for 15 min in an ultrasonic bath. The flow-rate was 0.5 ml min^{-1} and the detection was performed at 220 nm.

2.4. Standard solutions

2.4.1. Solutions used for method development

Stock solutions of (+)-P, (−)-P, the methyl ester of L-phenylalanine, DHP and diastereoiso-

Table 1
Influence of sodium perchlorate on the separation of pirlindole enantiomers and DHP

	0 mM	20 mM	50 mM	100 mM	200 mM	500 mM
k' (+)-P	0.29	0.40	0.43	0.48	0.54	0.59
k' DHP	0.20	0.41	0.57	0.72	0.88	0.95
k' (−)-P	0.37	0.57	0.68	0.81	1.00	1.19
α (+/DHP)	1.49*	ND**	1.33	1.50	1.61	1.62
α (DHP/−)	1.89	1.82	1.19	1.12	1.14	1.24
α (±)	1.27	1.41	1.58	1.69	1.83	2.02
Rs (+/DHP)	1.60*	ND**	1.88	3.11	4.09	4.22
Rs (DHP/−)	2.65	1.82	1.22	0.96	1.25	2.23
Rs (±)	1.22	2.04	3.15	4.02	5.40	6.63

k', capacity ratios; α, selectivity; Rs, resolution, chromatographic conditions as specified in text.
* Selectivity between DHP and (+)-P; ** Not determined because of the simultaneous elution of (+)-P and DHP.

mers of (±)-pirlindole were prepared by dissolving 10 mg of each compound in 20 ml methanol. These solutions were then diluted 10 times with water to obtain a final concentration of 50 μg ml^{-1}. The solution of (±)-pirlindole was obtained by dissolving 20 mg of this analyte in 20 ml methanol and subsequent dilution (10 times) of the latter with water (100 μg ml^{-1}). The solution of L-phenylalanine was prepared by dissolving 10 mg L-phenylalanine in 20 ml water. This solution was then diluted 10 times with water (50 μg ml^{-1}).

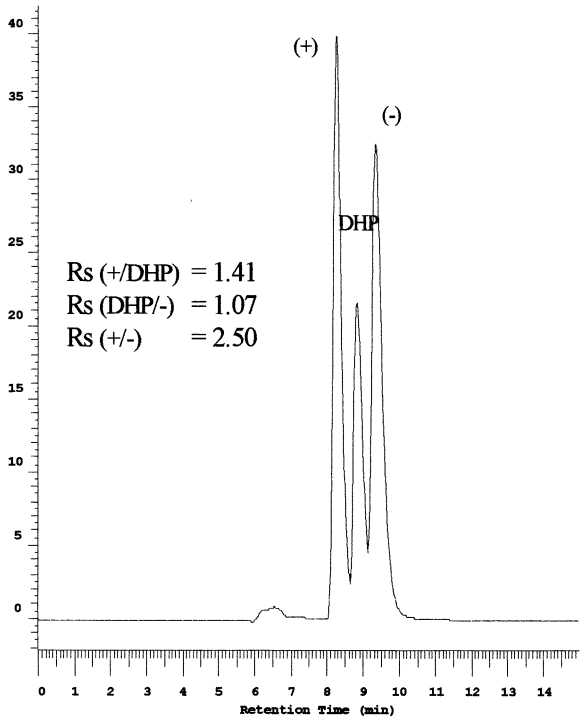

Fig. 3. Separation of DHP and pirlindole enantiomers on Chiralcel OD-R. Mobile phase: 50 mM phosphate buffer containing 50 mM $NaClO_4$ (pH 5.0)–acetonitrile (50:50) (see the text for the other chromatographic conditions). Sample, (±)-pirlindole 100 μg ml^{-1} and DHP 50 μg ml^{-1}.

2.4.2. Solutions used for validation

A mixed solution of racemic pirlindole and DHP was prepared by dissolving stock solutions of (±)-pirlindole and DHP with water to obtain a concentration of 5 μg ml^{-1} for (+)-P, (−)-P and DHP. The solutions were then prepared for calibration with final concentrations ranging from 0.1 to 5 μg ml^{-1} for each compound.

Table 2
Influence of acetonitrile on the separation of pirlindole enantiomers and DHP

	35%	38%	40%	45%	50%	55%
k' (+)-P	1.05	0.75	0.64	0.43	0.31	0.24
k' DHP	1.55	1.05	0.90	0.57	0.40	0.30
k' (−)-P	1.82	1.23	1.06	0.68	0.48	0.37
α (+/DHP)	1.48	1.40	1.40	1.33	1.30	1.25
α (DHP/−)	1.18	1.17	1.18	1.19	1.20	1.24
α (±)	1.75	1.64	1.64	1.58	1.56	1.54
Rs (+/DHP)	3.41	2.88	2.68	1.88	1.41	0.90
Rs (DHP/−)	1.65	1.37	1.35	1.22	1.07	0.99
Rs (±)	5.18	4.34	4.12	3.15	2.50	2.00

See first footnote for Table 1.

Table 3
Validation of the chiral LC method used the determination of enantiomeric purity of pirlindole

Validation criterion	(+)-P	DHP	(−)-P
Linearity ($n=6$, $k=3$) 0.1–5 μg ml^{-1}	$y=62223x-1882$	$y=27928x-864$	$y=61820x-1081$
r^2	0.9988	0.9993	0.9996
F-test for the slope	$F_1=13807$	$F_1=22028$	$F_1=46975$
F-test for the fit	$F_2=1.38$	$F_2=1.43$	$F_2=2.94$
LOD (μg ml^{-1})	0.012	0.019	0.018
LOQ (μg ml^{-1})	0.036	0.057	0.054
Repeatability ($n=6$, 1 day)			
0.1 μg ml^{-1} (%)	2.97	3.58	2.84
1.0 μg ml^{-1} (%)	1.88	1.32	2.24
5.0 μg ml^{-1} (%)	1.49	1.08	0.97
Intermediate precision ($n=6$, 3 days)			
0.1 μg ml^{-1} (%)	3.97	3.69	4.88
1.0 μg ml^{-1} (%)	2.10	2.47	2.72
5.0 μg ml^{-1} (%)	2.23	1.75	2.25
Overall accuracy ($n=18$)			
t-Test for the slope	1.66	1.02	1.91
t-Test for the origin	1.15	1.54	0.83

2.4.3. Sample solutions

The solutions of (+)-P or (−)-P used for the determination of enantiomeric purity were prepared by dissolving 10 mg of the corresponding enantiomer in a 10 ml volume of methanol and by diluting this solution 10 times with water in order to obtain a concentration of 100 μg ml^{-1}.

3. Results and discussion

3.1. Enantioseparation of pirlindole

As was reported elsewhere [26], the chiral separation of pirlindole can be achieved on different CSPs: the β-cyclodextrin bonded phase (Chiradex) or an ovomucoid based column (Ultron ES-OVM) can be used for the enantioseparation of this compound.

However, the chiral resolution for pirlindole obtained on Chiralcel OD-R was higher than for those achieved on the Chiradex and ES-OVM phases [26]. Moreover, the Chiralcel OD-R phase presents good stability and more predictable behaviour than the ES-OVM phase.

The main parameters to be taken into consideration for improving chiral separations on the Chiralcel OD-R column should be the pH, the organic modifier concentration and the sodium perchlorate concentration in the mobile phase [11,13,14,16–22]. Fig. 2 illustrates the enantioseparation of pirlindole and the effect of acetonitrile concentration on this chiral separation. By decreasing the acetonitrile content from 50 to 45%, enantioselectivity and enantioresolution were increased to 1.64 and 2.81, respectively. The order of elution of the enantiomers of pirlindole was determined by injecting separate solutions of each enantiomer.

3.2. Separation of pirlindole enantiomers from DHP

DHP is frequently present in pirlindole in low quantities. Moreover, it has been observed that DHP generally contaminates the single enantiomers of pirlindole prepared according to the method described by de Tullio et al. [27].

In the LC conditions described above for the chiral determination of pirlindole, DHP was found to elute in the same retention range as

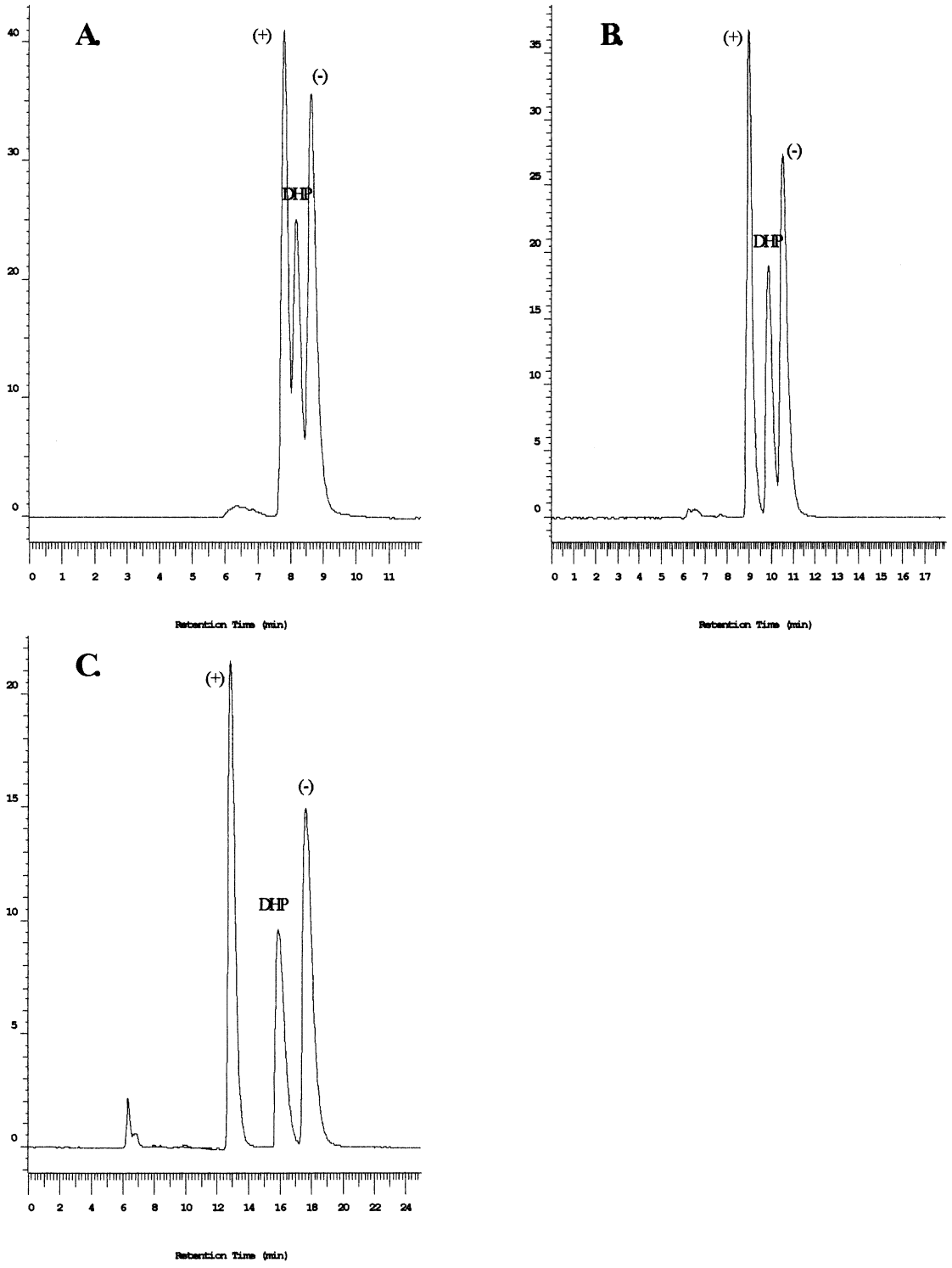

Fig. 4. Influence of acetonitrile concentration on the separation of DHP and pirlindole enantiomers. Stationary phase: Chiralcel OD-R; mobile phases: (A) buffer (pH 5.0)–acetonitrile (45:55); (B) buffer (pH 5.0)–acetonitrile (55:45); (C) buffer (pH 5.0)–acetonitrile (65:35) (see the text for the other chromatographic conditions). Sample, ($\pm$)-pirlindole 50 μg ml^{-1} and DHP 25 μg ml^{-1}.

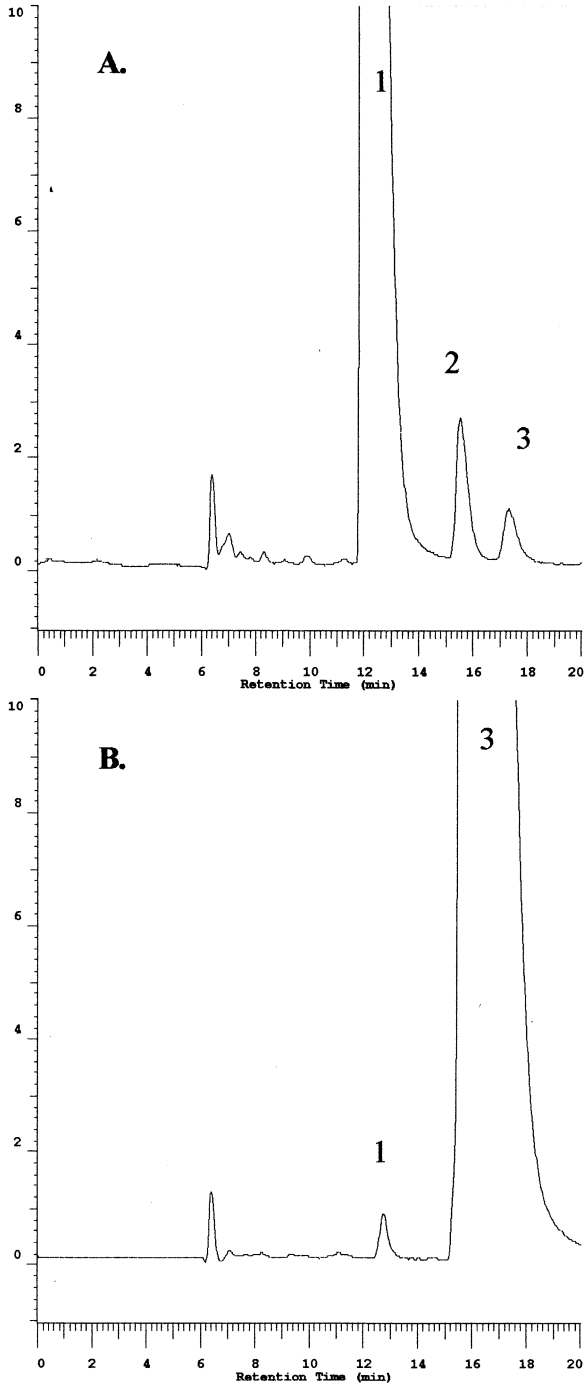

Fig. 5. Typical chromatograms of pirlindole enantiomers containing their stereoisomers. (A) 0.42% of (−) in (+)-enantiomer; (B) 0.26% of (+) in (−)-enantiomer (see the text for the chromatographic conditions). (1) (+)-P, (2) DHP, (3) (−)-P.

(+)-P and (−)-P (Fig. 3). Even if these three compounds were partly separated, the separation was not sufficient to quantify these analytes, particularly if one of them was present in low concentration.

3.2.1. Influence of sodium perchlorate concentration

The mobile phase used for the investigation of the effect of $NaClO_4$ concentration on the separation of the three analytes consisted of a mixture of acetonitrile and of a 50 mM phosphate buffer containing $NaClO_4$ and adjusted to pH 5.0 (45:55, v/v).

The influence of $NaClO_4$ concentration on the chiral separation of the basic compounds was previously reported. An increase in concentration improves enantioselectivity and enantioresolution, and causes the capacity ratios of both enantiomers to increase [11,13,14,16–22]. This is obviously also the case for pirlindole. The influence of $NaClO_4$ concentration on the separation of both enantiomers from DHP is illustrated in Table 1. When $NaClO_4$ was not present in the mobile phase, DHP eluted before (+)-P and was totally separated from (+)-P ($Rs = 1.60$). However, the enantioseparation of pirlindole was not complete. By increasing the $NaClO_4$ concentration, an inversion of the elution order between DHP and (+)-P was observed. Indeed, at a 20 mM concentration of $NaClO_4$, (+)-P and DHP eluted simultaneously and when the $NaClO_4$ was increased to 50 mM, the (+)-P eluted before DHP.

It is also interesting to note that the selectivity and the resolution between DHP and (−)-P decreased when the $NaClO_4$ concentration increased from 0 to 100 mM. However, when this concentration was increased to 200 or 500 mM, the selectivity and resolution values increased, leading to a better separation between these two compounds. The complete separation of DHP and (−)-P could be achieved when the 500 mM concentration of $NaClO_4$ was reached.

3.2.2. Influence of acetonitrile concentration

The mobile phase selected for studying the influence of acetonitrile concentration on separation of DHP from the (+) and (−) enantiomers of pirlindole consisted of a mixture of a 50 mM

phosphate buffer containing 50 mM $NaClO_4$ adjusted to pH 5.0 and acetonitrile.

The results obtained in this study are shown in Table 2. An increase in acetonitrile concentration caused the capacity factors of the three analytes to decrease. It also led to a decrease in selectivity and resolution between each analyte. The acetonitrile concentration was found to influence the retention of the three compounds. Fig. 4 shows the chromatograms obtained at three concentrations of acetonitrile. It clearly shows that a decrease in the organic modifier concentration causes the resolution and selectivity to increase. A 35% concentration of acetonitrile gives rise to a sufficient separation of DHP from (+)-P and (−)-P (Fig. 4C). The resolution between (+)-P and DHP and between DHP and (−)-P are 3.41 and 1.65, respectively. The enantioresolution under the same conditions was 5.18.

The results obtained show that a complete separation of the (+) and (−) enantiomers of pirlindole from DHP can be achieved by modifying either the sodium perchlorate concentration or the acetonitrile content in the mobile phase. Finally, the mobile phase selected consisted of a mixture of 35% acetonitrile and 65% 50 mM phosphate buffer containing 50 mM $NaClO_4$ adjusted to pH 5.0, in order to limit its lower concentration in salt.

3.3. Validation

3.3.1. Selectivity towards other compounds

The synthesis of single enantiomers of pirlindole consisted of the derivatisation of racemic pirlindole with L-phenylalanine methyl ester. The two diastereoisomers obtained were then separated by MPLC and hydrolysed to obtain the (+) and (−) enantiomers separately. The latter were purified by recrystallization [27].

The selectivity of the method developed was tested towards the compounds that could be present in the final products such as phenylalanine, the methyl ester of phenylalanine and the diastereoisomers. Phenylalanine is eluted in the solvent front, the methyl ester of phenylalanine ($k' = 0.11$) is eluted just after the solvent front and the diastereoisomers are eluted after 80 min. The capacity ratios of (+)-P, DHP and (−)-P are 1.05, 1.55 and 1.82, respectively. The technique developed is then selective for the determination of pirlindole enantiomers and DHP.

3.3.2. Linearity

The linear regression analysis for pirlindole enantiomers and DHP were made by plotting peak areas (y) versus analyte concentrations (x) in μg ml^{-1}. The following equations were obtained (range: 0.1–5 μg ml^{-1}):

(+)−P: $y = 62223x - 1882$ $r^2 = 0.9988$

DHP: $y = 27928x - 864$ $r^2 = 0.9993$

(−)−P: $y = 61820x - 1081$ $r^2 = 0.9996$

The determination coefficient (r^2) obtained for the regression lines of both enantiomers of pirlindole and DHP demonstrate the linearity of the relationship between the peak area and the analyte concentration. Moreover, an analysis of variance (ANOVA) was carried out on each calibration curve in order to confirm the linearity (F_1) and to test the quality of the fit (F_2) [28]. The linearity was assessed for the three compounds (Table 3).

3.3.3. Detectability

The limits of detection (LOD) and quantification (LOQ) were determined as the concentrations of analyte giving rise to signal-to-noise ratios of 3 and 10, respectively. The LODs and LOQs for (+)-P, DHP and (−)-P were found to be 36, 57 and 54 ng ml^{-1}, respectively.

3.3.4. Precision

The precision of the method developed was estimated by measuring the repeatability and reproducibility for the three analytes of interest at three concentrations levels, ranging from 0.1 to 5 μg ml^{-1}. The repeatability RSDs are 2.97, 3.58 and 2.84% for (+)-P, DHP and (−)-P, respectively, at a concentration of 0.1 μg ml^{-1}. The reproducibility RSDs at the same concentration are 3.97, 3.69 and 4.88%, for (+)-P, DHP and (−)-P, respectively.

3.3.5. Accuracy

The overall accuracy of the procedure was assessed by plotting the analyte amount found versus the amount really present in the solution at three concentrations levels ($n = 6$) ranging from 0.1 to 5 μg ml^{-1} ($r^2 = 0.9992$, 0.9994 and 0.9998 for (+)-P, DHP and (−)-P, respectively). *t*-Tests showed that the slopes of the regression lines were not significantly different from unity (the calculated values of t were 1.66, 1.02 and 1.91 for (+)-P, DHP and (−)-P, respectively) and that the intercepts were not significantly different from zero (the calculated values of t were 1.15, 1.54 and 0.83 for (+)-P, DHP and (−)-P, respectively). The critical value of t was 2.12 ($P = 0.05$).

The analytical method for the determination of pirlindole enantiomers and DHP can thus be considered as accurate within the overall concentration range investigated.

3.3.6. Application of the method

The method developed for the determination of concentrations of (+)-P, (−)-P and DHP in prepared single enantiomers has been applied successfully. Fig. 5 shows chromatograms of (+)-P containing 0.42% of the (−)-enantiomer and of (−)-P containing 0.26% of the (+)-enantiomer.

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