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Automated determination of pirlindole enantiomers in plasma by on-line coupling of a pre-column packed with restricted access material to a chiral liquid chromatographic column

P. Chiap [a], A. Ceccato [a], R. Gora [a,1], Ph. Hubert [a], J. Géczy [b], J. Crommen [a,*]

[a] *Department of Analytical Pharmaceutical Chemistry, Institute of Pharmacy, University of Liège, CHU, B36, B-4000 Liège 1, Belgium*

[b] *Therabel Research, Rue E. Van Ophem 110, B-1180, Brussels, Belgium*

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Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday.

Abstract

A fully automated liquid chromatographic method has been developed for the determination of the enantiomers of pirlindole, an antidepressant drug, in human plasma. The method is based on the use of a pre-column packed with restricted access material (RAM) (LiChrospher ADS RP-4) for sample clean-up coupled to a column containing a cellulose tris-(3,5-dimethylphenylcarbamate) based chiral stationary phase (Chiralcel OD-R) for the separation and quantitative analysis of pirlindole enantiomers. A 50-μl plasma volume was injected directly onto the pre-column using a mixture of phosphate buffer (pH 5.0) and methanol (97:3; v/v) as washing liquid. By rotation of a switching valve, the analytes were then eluted in the back-flush mode with the LC mobile phase. A complete separation of pirlindole enantiomers was obtained in 22 min on the Chiralcel OD-R column, using a mobile phase made of a mixture of phosphate buffer (pH 5.0) containing 50 mM sodium perchlorate and acetonitrile (65:35; v/v). The flow-rate was 0.6 ml/min and the analytes were detected fluorometrically using 295 and 340 nm as excitation and emission wavelengths, respectively. The method was then validated and was found to be linear in the 2.5–200 ng/ml range. The limit of detection was lower than 1 ng/ml. Repeatability and intermediate precision at a concentration of 50 ng/ml were about 1.5 and 3.5%, respectively.

Keywords: Pirlindole enantiomers; Chiral liquid chromatography; Plasma; Sample preparation; Column-switching; Restricted access material

* Corresponding author. Tel.: +32-4-366-4345; fax: +32-4-366-4347.
E-mail address: jcrommen@ulg.ac.be (J. Crommen).
[1] Present address: Department of Analytical Chemistry, Faculty of Natural Sciences, Comenius University, Mlynská Dolina, SK-842 15 Brastislava, Slovak Republic.

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1. Introduction

Pirlindole is a chiral tetracyclic compound (Fig. 1) characterised as a potential antidepressant drug used so far as a racemate [1–7]. Its main mechanism of action consists of selective and reversible inhibition of monoamine oxidase [2,4]. It also inhibits the neuronal reuptake of noradrenaline and 5-hydroxytryptamine, but it has no effect on the dopaminergic and cholinergic systems [2].

As shown in Fig. 1, pirlindole presents one asymmetric centre, giving two optical isomers. The enantiomers of chiral drugs can exhibit differences in the processes of absorption, distribution, metabolism and excretion [8]. Their pharmacological and toxic effects may also be different [8]. Therefore, it is necessary to determine the pharmacokinetic parameters of both enantiomers.

Nowadays, the majority of enantiomeric separations in bioanalysis are performed by liquid chromatography (LC). They are based either on the formation of diastereomeric derivatives after derivatisation of the analyte with an optically pure reagent (indirect approach) or more frequently on the use of chiral stationary phases (CSPs) (direct approach) [9]. Over the past decade, a wide variety of CSPs have been developed for the enantioseparation of chiral substances. Cellulose- or amylose-based CSPs were found to be particularly effective for the direct separation and quantitation of drug enantiomers, either in the normal-phase or in the reversed-phase (RP) mode [10].

Nevertheless, the direct analysis of protein rich fluids, such as plasma, by LC is generally impossible. A sample pre-treatment prior to analysis is usually needed to remove macromolecular sample components, e.g. proteins, in order to protect the LC column and to maintain the performance of the analytical system. The other aims of sample preparation are the removal of interfering low molecular weight compounds and the analyte enrichment.

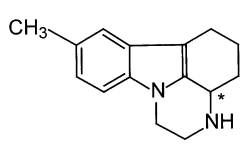

Fig. 1. Structure of pirlindole. The chiral centre is marked by an asterisk (*).

Since, the number of samples to be analysed in pharmacokinetic or clinical studies, is rather large, an automated method for sample pre-treatment has been developed. Among the different automated sample preparation techniques, the use of a pre-column packed with restricted access material (RAM) coupled to the LC column in a column-switching system constitutes an useful approach for the on-line analysis of drugs in plasma. A family of restricted access sorbents, namely alkyl diol silica (ADS), belonging to the group of internal surface reversed phase (ISRP) supports, has been developed by Boos et al. [11,12]. These restricted access supports packed in small pre-columns have been applied successively for the clean-up of biological samples in column switching systems [13–22]. This sample preparation technique has also been used for the determination of drug enantiomers in plasma [18,19,21].

A schematic representation of the topochemistry of the ADS sorbent is shown in Fig. 2. The access restriction is obtained by use of silica particles with an appropriate pore diameter (6 nm). This kind of RAM is characterised by a molecular weight cut-off of about 15 kDa. The physical diffusion barrier prevents the access of macromolecules, such as proteins, to the internal surface, on which either a butyryl (C_4), capryloyl (C_8) or stearoyl (C_{18}) moiety is bonded. Consequently, only low molecular mass compounds, such as drugs, have a free access to the internal surface and are retained mainly by hydrophobic interactions. Moreover, the adsorption and denaturation of proteins are prevented by hydrophilic and electroneutral diol groups bound to the external surface of the particles.

The aim of this paper is to develop and validate a fully automated procedure combining a pre-column packed with RAM to a chiral LC column for the determination of enantiomers of pirlindole in plasma.

A LC method for the determination of pirlindole in plasma and urine was reported, but it was non-stereoselective [23]. Moreover, the enantiomeric separation of pirlindole by LC has been

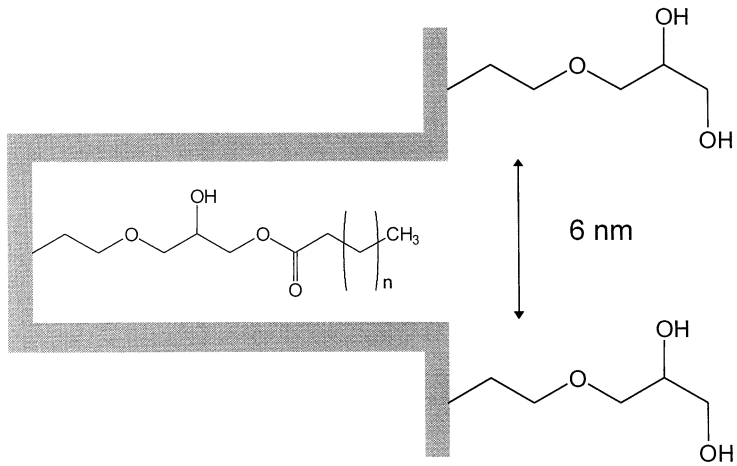

Fig. 2. Schematic representation of the topochemistry of the ADS sorbent [11,13]. $n = 1$: butyryl (C_4-ADS), $n = 5$: capryloyl (C_8-ADS), $n = 15$: stearoyl (C_{18}-ADS).

proposed, but only for the control of the enantiomeric purity of single enantiomers [24,25]. Consequently, no stereoselective method has been made available until now for the determination of the enantiomers of pirlindole in plasma and for the evaluation of their pharmacokinetics.

2. Experimental

2.1. Chemical and reagents

Racemic pirlindole hydrochloride was supplied by Therabel Research S.A./N.V. (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 [26]. The identification of each enantiomer was performed by polarimetry and their absolute configuration was determined by crystallographic experiments. Sodium dihydrogenphosphate dihydrate, sodium hydroxide, sodium perchlorate were purchased from Merck (Darmstadt, Germany) and were of analytical grade. Methanol and far UV acetonitrile, both of LC grade, were obtained from Fisher Scientific (Leicestershire, UK). The water used in all experiments was purified by means of a Milli-Q system (Millipore Corporation, Bedford, MA, USA).

The pre-column containing LiChrospher RP-4 ADS (particle size: 25 μm) was supplied by Merck. The analytical and guard columns were pre-packed with cellulose tris-(3,5-dimethylphenylcarbamate) coated on silica (particle size: 10 μm) (Chiralcel OD-R) from Daicel (Tokyo, Japan) and with LiChrospher 100 Diol (particle size: 5 μm) from Merck, respectively.

2.2. Apparatus

A schematic representation of the column-switching system was shown elsewhere [18,19].

The analytical system was composed of a model 422 LC pump from Kontron Instruments (Schlieren, Switzerland) (pump 1) and the following units from Merck-Hitachi, a model L-6200 A pump (pump 2), a model AS-2000 A autosampler equipped with a 100 μl injection loop, a model L-4250 UV-Vis detector and a model F-1050 fluorescence detector.

The LiChroCART pre-column (25 × 4 mm, i.d.) packed with LiChrospher RP-4 ADS was fitted to a Valco model VICI AG six-port switching valve (Valco Europe, Schenkon, Switzerland).

The separation was performed on a Chiralcel OD-R column (250 × 4.6 mm, i.d.) from Daicel

preceded by a LiChroCART guard column (4 × 4 mm, i.d.) from Merck. They were thermostated at 25 ± 0.1 °C in a model L-5025 programmable column oven (Merck).

The different modules were connected through an interface (D-6000, Merck) with an IBM compatible computer (PC-AT; CPU type Pentium) for the control of the analytical system and data collection. The model 422 pump from Kontron (pump 1) was controlled manually.

2.3. Chromatographic conditions

The chromatographic separation was performed in the isocratic mode. The mobile phase consisted of a mixture of 50-mM phosphate buffer containing sodium perchlorate (50 mM), adjusted to pH 5.0 with a 10% solution of sodium hydroxide, and acetonitrile (65:35, v/v). Prior to use, the mobile phase was degassed for 15 min in an ultrasonic bath. The chromatographic separation was performed at 25 °C using a constant flow-rate of 0.6 ml/min. The two analytes were monitored fluorometrically using 295 and 340 nm as excitation and emission wavelengths, respectively.

2.4. Standard solutions

A stock solution of pirlindole was prepared by dissolving 50 mg of racemic pirlindole in 50 ml of methanol. This solution was stored in a refrigerator at 4 °C when not in use.

2.4.1. Solutions used for method development

During method development, an intermediate solution of pirlindole was prepared by diluting the stock solution with water to obtain a concentration of 10 μg/ml. This diluted solution was stored in a refrigerator at 4 °C and was found to remain stable for at least 1 week. It was then diluted with water or plasma to reach a final concentration of about 500 ng/ml for each enantiomer. The latter solutions were prepared daily.

2.4.2. Solutions used for method validation

Two intermediate solutions of pirlindole were prepared by diluting a stock solution of pirlindole with water to obtain concentrations of 10 and 1 μg/ml, respectively. These diluted solutions were stored in a refrigerator at 4 °C and were prepared daily. They were used to spike plasma samples (2.0 ml) in order to obtain concentrations of 2.5, 5, 10, 25, 50, 100 and 200 ng/ml for each enantiomer. Aqueous standard solutions at four concentration levels (2.5, 5, 50 and 200 ng/ml of each enantiomer) were also prepared for the determination of the absolute recovery.

Stock solutions of S-(+)-pirlindole and R-(−)-pirlindole were prepared by dissolving 5 mg of these compounds in 10 ml of water. Each solution was then diluted with water to obtain an intermediate solution (concentration: 10 μg/ml), which was used to spike plasma samples (2.0 ml) in order to reach a final concentration of 200 ng/ml.

2.5. Automated sample preparation

After thawing the plasma sample at ambient temperature, the only manual operations were the centrifugation of the plasma sample at 3900 × *g* for 15 min and the introduction of an aliquot (0.5-ml) into a vial (0.85-ml) located in the appropriate rack of the autosampler. All other sample handling operations were then executed automatically.

The automatic sequence was performed in the following way:

- *Sample application and fractionation* (flow-rate: 0.6 ml/min): 50 μl of plasma sample were injected onto the ADS column with a mobile phase consisting of a mixture of 50 mM phosphate buffer (pH 5.0) and methanol (97:3; v/v) delivered by pump 1. The pre-column was then washed with this solution for 8 min. Meanwhile, the chiral column was re-equilibrated with the LC mobile phase delivered by pump 2 at a flow-rate of 0.6 ml/min.
- *Transfer* (flow-rate: 0.6 ml/min): by rotation of the switching valve, the analytes were then eluted in the back-flush mode with the LC mobile phase and transferred to the analytical column.
- *Reconditioning* (flow-rate: 0.6 ml/min): 4 min later, the switching valve was returned to its initial position allowing the ADS pre-column

to be re-equilibrated with the washing liquid for 10 min. Simultaneously, the chromatographic separation was started.

The total analysis time of a sample was 30 min (8 min for sample clean-up and 22 min for the chromatographic separation). However, the handling of the next sample was started during the chromatographic analysis of the previous one.

3. Results and discussion

The general strategy proposed for the development of automated bioanalytical methods based on the on-line coupling of a pre-column packed with ADS to LC can be summarised as follows:

- Selection of an appropriate detection mode and suitable LC conditions.
- Development of the sample preparation method:
  - selection of a suitable ADS support;
  - selection of the washing liquid (composition, flow-rate);
  - determination of the elution profiles of the analyte(s) and the biological matrix;
  - determination of the period of time needed for analyte transfer;
  - optimisation of the times for the rotation of the switching valve.
- Method validation.

3.1. Selection of the detection mode and suitable chromatographic conditions

The detection mode was selected according the properties of the analyte and the sensibility and selectivity required. Since, pirlindole presents native fluorescence properties, it was monitored fluorometrically at 295 and 340 nm, as excitation and emission wavelengths, respectively, owing to the higher sensitivity and selectivity provided by this detection mode.

The LC system was previously optimised for the enantiomeric separation of pirlindole. Indeed, an optimal enantioseparation of this compound had been obtained using tris-(3,5-dimethylphenylcarbamate) (Chiralcel OD-R) as CSP. This kind of CSP presents a good stability and allows the use of a relatively high percentage of organic modifier in the mobile phase, which is particularly useful for obtaining a peak compression at the top of the column during the transfer step of the analytes from the pre-column to the LC column.

3.2. Development of the automated sample preparation method

In order to develop the sample clean-up method, the first step consisted of determining the retention capability of the ADS support for pirlindole and to select the most appropriate washing liquid. Three ADS sorbents (RP-4, RP-8 and RP-18) packed in small pre-columns (25×4 mm, i.d.) were tested.

The elution profiles of pirlindole with a washing liquid, consisting of a mixture of 50 mM phosphate buffer (pH 5.0) and methanol were determined by monitoring the fluorescence absorbance of an aqueous solution of pirlindole injected on the pre-column connected directly to the fluorescence detector. The percentage of methanol in the washing liquid was varied from 0 to 15%.

As expected, the retention of pirlindole on the ADS support increased with the alkyl chain length. Since, pirlindole was retained sufficiently on the less hydrophobic phase (RP-4 ADS), this support was finally selected in order to minimise the adsorption and enrichment of potentially interfering plasma components and consequently to enhance method selectivity. The optimal content of methanol in the washing liquid was found to be 3% (v:v). The addition of this small amount of methanol was sufficient to achieve the release of pirlindole from the binding sites of the plasma proteins. Under these conditions, the capacity factor of pirlindole was 90 and the breakthrough time (T_A), corresponding to the beginning of elution of pirlindole from the pre-column, was 28 min when using a flow-rate of 0.6 ml/min for the washing liquid.

In order to determine the first time for the rotation of the switching valve, it was also necessary to examine the elution profile of the biological matrix. Indeed, the transfer step could only take place after the complete elution of the

plasma matrix from the pre-column. Fifty microliters of a blank plasma sample were injected onto the pre-column connected to an UV detector set at 280 nm. The flow-rate of the washing liquid was obviously the same as that used for the determination of the elution profile of pirlindole. The UV absorbance was monitored and the fractionation step was considered complete when the detector signal reached the baseline.

With a washing liquid consisting of a mixture of phosphate buffer (pH 5.0) and methanol (97/3; v:v), the time for a complete elimination of proteins (T_M) was 3 min. Since, T_A was higher than 10 min, it was preferable to settle the time for the rotation of the switching valve at 8 min ($T_M + 5$ min) [27]. During this period of time, the plasma matrix was directly flushed into waste, whilst the analyte was extracted and enriched on the internal surface of the ADS sorbent.

The third step consisted of determining the period of time needed to transfer pirlindole quantitatively from the pre-column to the analytical column. The determination of this time was performed with the fluorescence detector directly connected to the switching valve. Due to the strong eluting strength of the LC mobile phase consisting of a mixture of 50 mM phosphate buffer (pH 5.0) containing sodium perchlorate (50 mM) and acetonitrile (65:35; v/v), pirlindole was desorbed from the ADS pre-column in about 3 min. The flow-rate of the LC mobile phase was set at 0.6 ml/min. Since, the content of the organic modifier in the mobile phase used for transfer and separation was much higher than in the washing liquid, peak compression could be expected at the top of the analytical column.

Finally, a time period of 4 min was selected for the elution of the analyte from the pre-column in the back-flush mode and its quantitative transfer to the chiral column in a narrow elution band. Twelve minutes after sample application, the switching valve was returned to its initial position, allowing the ADS pre-column to be re-equilibrated with the washing liquid. The next sample application was carried out 10 min later and a new cycle could be started.

3.3. Method validation

Since, the developed procedure was used to evaluate the pharmacokinetics of pirlindole enantiomers, its validation was needed in order to ensure the reliability of the results within well defined limits and hence the confidence that could be placed on the results generated in these studies.

3.3.1. Stability of pirlindole in plasma samples

The response factors of pirlindole enantiomers in a spiked plasma sample at a concentration of 5 ng/ml, freshly prepared and stored for 24 h at ambient temperature were compared. Six independent determinations ($n = 6$) were carried out.

Student's test was applied to assess whether there was any influence of the storing time at room temperature on the stability of pirlindole enantiomers in plasma. Since, the t values calculated for S-(+)-pirlindole ($t = 1.31$) and for R-(−)-pirlindole ($t = 2.19$) were lower than the critical value shown in a t table with $(n_1 + n_2 - 2)$ degrees of freedom at the 5% significance level ($t_{(0.05;10)} = 2.23$), n_1 et n_2 representing the number of replicates at T_0 and T_{24}, the compounds were stable for at least 24 h in plasma samples.

3.3.2. Method selectivity towards endogenous components of plasma

The absence of interfering endogenous components of plasma at the retention times of pirlindole enantiomers was demonstrated in Fig. 3, which shows typical chromatograms obtained by analysis of a plasma spiked with racemic pirlindole at a concentration of 5 ng/ml and a blank plasma sample obtained from six different sources of the same matrix.

The order of elution of pirlindole enantiomers was determined by analysing separately solutions of each enantiomer and is comparable with that mentioned in literature [24,25]. The capacity factors of S-(+)-pirlindole and for R-(−)-pirlindole were 1.05 and 1.88, respectively.

3.3.3. Extraction efficiency

The extraction efficiency was calculated at four concentration levels (2.5, 5, 50 and 200 ng/ml) covering the entire calibration range by compar-

ing peak areas for each enantiomer with those found after direct injection of an aqueous standard solution at the same concentrations using the same autosampler equipped with the same loop of 100 μl.

As shown in Table 1, the mean absolute recovery was about 94% for each enantiomer and the extraction efficiency was relatively constant over the entire range considering the relative standard deviations (R.S.D.) obtained for each enantiomer (2.0 and 2.1%; $n = 4$).

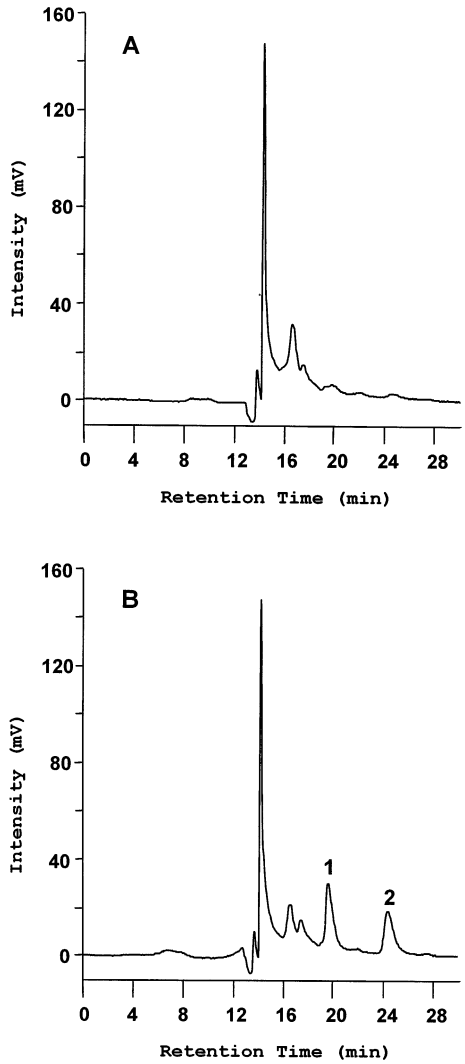

Fig. 3. Typical chromatograms obtained after on-line coupling of a pre-column packed with restricted access material to a chiral chromatographic column, (A) chromatogram of a blank plasma sample, (B) chromatogram of a plasma sample spiked with pirlindole (concentration 2.5 ng/ml of each enantiomer). Operating conditions given in Section 2. Peaks: (1) S-(+)-pirlindole; (2) R-(−)-pirlindole.

Table 1
Method validation

Validation criterion	S-(+)-pirlindole	R-(−)-pirlindole
Absolute recovery (mean ± S.D.; $n = 4$)	94.0 ± 2.0%	93.8 ± 2.1%
Response function ($n = 3$; $k = 7$)		
Concentration range	2.5–200 ng/ml $y = 191\,602x - 62\,419$ ($r^2 = 0.9998$)	2.5–200 ng/ml $y = 187\,929x - 65\,199$ ($r^2 = 0.9995$)
F for the slope	$F_1 = 113\,561$	$F_1 = 41\,029$
F-test for fitting	$F_2 = 2.80$	$F_2 = 2.89$
LOD	0.6 ng/ml	0.7 ng/ml
LOQ	2.0 ng/ml	2.5 ng/ml
Repeatability ($n = 6$; 1 day)		
2.5 ng/ml	5.0	5.2
5.0 ng/ml	3.1	5.6
50 ng/ml	1.0	1.6
200 ng/ml	0.9	0.8
Intermediate precision ($n = 18$; 3 days)		
2.5 ng/ml	13.3	6.3
5.0 ng/ml	12.6	6.0
50 ng/ml	2.5	4.3
200 ng/ml	1.7	2.0
Overall accuracy ($n = 18$)		
t-test for the slope	1.05	0.92
t-test for the origin	0.30	0.70

3.3.4. Analysis of the response function

In order to determine the response function, a calibration curve for each enantiomer was constructed in the range comprised between 2.5 and 200 ng/ml by selecting seven concentration levels ($k = 7$). Three independent samples ($n = 3$) were analysed at each concentration level. The peak areas for each enantiomer were considered as analytical responses. A simple linear regression model was selected.

The equations of the regression lines with their coefficients of determination (r^2) are presented in Table 1. Moreover, an analysis of variance (ANOVA) was carried out on calibration curves in order to confirm the linearity (F_1) and to verify the adequacy of the regression model (F_2). As can be seen in Table 1, the F_1 calculated was much greater than the F value of the table with (1, $N-2$) degrees of freedom at the 5% significance level ($F_{(0.95;\ 1.19)} = 4.38$). Consequently, linearity was assessed. The adequacy of the model was also confirmed by the lack of fit test, the F_2 value obtained from this test being lower than the critical F value of the table with ($k-2$, $N-k$) degrees of freedom at the 5% significance level ($F_{(0.95;\ 5.14)} = 2.96$).

3.3.5. Precision

The precision of the bioanalytical procedure was determined by computing the R.S.D. for repeatability and time-different intermediate precision from the analytical responses obtained for validation samples prepared at four concentration levels (2.5, 5, 50 and 200 ng/ml). These validation samples, corresponding to quality control samples, were analysed six times for 3 consecutive days. The R.S.D. values presented in Table 1 did not exceed 15% [28] and illustrated the relatively good precision of the developed procedure.

3.3.6. Accuracy

In order to determine accuracy, validation samples were prepared at three concentration levels (2.5, 50 and 200 ng/ml) ($k = 3$) covering the entire range considered and were analysed six times ($n = 6$). The concentrations of these validation samples were then estimated.

The overall accuracy of the procedure was assessed by fitting regression lines on the estimated concentration (y) as a function of the introduced concentration (x). A linear regression model based on the least squares method was applied. The following equations were obtained:

S-(+)-pirlindole:

$$y = 0.998x + 0.0793 \quad (r^2 = 0.9999)$$

R-(−)-pirlindole:

$$y = 0.996x + 0.310 \quad (r^2 = 0.9998)$$

The procedure developed for the determination of pirlindole enantiomers was considered accurate, since, the slopes and the origins of the regression lines were not significantly different from unity and zero, respectively, at the 5% significance level. Indeed, by applying Student's test, the t values calculated were lower than the critical value found in a t table with ($N-2$) degrees at the considered significance level ($t_{(0.05,\ 16)} = 2.12$), as can be seen in Table 1.

3.3.7. Detectability

The limits of detection (LOD) and quantitation (LOQ) were determined as analyte concentrations giving rise to signal-to-noise ratios of 3 and 10, respectively. The LODs for S-(+)-pirlindole and for R-(−)-pirlindole were evaluated at 0.6 and 0.7 ng/ml, respectively, while the LOQs for S-(+)-pirlindole and for R-(−)-pirlindole were found to be 2.0 and 2.5 ng/ml, respectively. Finally, the LOQs for both enantiomers were considered to be equal to 2.5 ng/ml, since, precision and accuracy were also assessed at this concentration level.

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