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Short communication

Enantioseparation of the antidepressant reboxetine

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ABSTRACT

The enantioseparation of reboxetine by HPLC was investigated using chiral stationary phases (CSPs) containing cellulose Tris(3,5-dimethylphenyl)carbamate on silica gel (Chiralcel OD column) as the chiral selector. Reversed phase HPLC was the technique of choice for the analytical enantioseparation of reboxetine, while the chiral semipreparative separation was obtained with the same CSP, but in normal phase conditions. The effects of the mobile phase pH and composition on analytical retention, enantioselectivity and resolution were investigated. The best performance was obtained using a mobile phase composed of 0.5 M sodium perchlorate at pH 6 and acetonitrile in the 60/40 (v/v) ratio. The semipreparative separation has allowed obtaining pure enantiomers, but required the preparation of reboxetine free base. Different *n*-hexane/alcohol mixtures were tested as mobile phases, varying both the nature of the alcohol and its percentage in the mobile phase. Different *n*-hexane/alcohol mixtures were tested as mobile phase and the best results were obtained by using a mobile phase composed of *n*-hexane and 2-propanol (80:20, v/v).

1. Introduction

Depression is a chronic, debilitating mental disease affecting more than 10% of the general population [1–5]. Traditional tricyclic antidepressants (TCAs) increase the synaptic availability of norepinephrine (NE) [6,7]. However, conventional tricyclic antidepressants have a relatively high affinity for a broad range of receptors, including adrenergic, muscarinic and histaminergic receptors [8]. This low selectivity causes a wide range of adverse side effects that contribute to low compliance among patients. The introduction of the selective serotonin reuptake inhibitors (SSRIs), which have a more favourable side effect profile, shifted the emphasis toward a potential crucial role of serotonergic dysfunction in affective disorders. Although the reduction in side effects associated with SSRIs has contributed to their popularity, many patients remain treatment resistant. A continuing effort to identify more efficacious antidepressant compounds has led to the emergence of the selective NE reuptake inhibitors (NRIs) [9].

The first NRI commercially available for major depression was Reboxetine, 2-[α-(2-ethoxyphenoxy)phenylmethyl]-morpholine (RBX, Fig. 1) [10]. Unlike conventional tricyclic antidepressants, RBX has only minimal sedative and cardiovascular liabilities, probably due to increased pharmacological specificity [8]. Compared to SSRIs, it has demonstrated to cause less sexual dysfunctions and gastrointestinal side effects [8].

RBX represents a valuable therapeutic tool to investigate the role of NE in depression and in antidepressant therapy and provides a rational alternative for patients resistant to conventional antidepressant therapy.

RBX possesses two chiral centres, and is marketed as the mesilate of the racemic mixture of the (+)-(2*S*,3*S*)- and (−)-(2*R*,3*R*)-enantiomers [11]. *In vitro* and *in vivo* receptor binding models suggest that the *S*,*S*-enantiomer is the most potent NRI, although its plasma concentrations are about two times lower than those of the *R*,*R*-enantiomer after administration of the racemate [12,13]. However, this is evidently not the result of stereoselective metabolism, since both enantiomers are oxidised to approximately the same degree by the cytochrome isoform CYP3A4 in *in vitro* studies [14–16]. This is supported by the fact that the enantiomers show similar elimination half-lives, regardless of the route of administration. RBX may be an example of a racemic drug with one clearly active enantiomer, where accurate definition of the dose–effect relationship has been obscured by enantioselective disposition.

This hypothesis highlights the necessity to develop reliable methods for the separation and analysis of RBX enantiomers. Several papers are present in the literature, which analyse RBX, mainly

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by means of high-performance liquid chromatography (HPLC) with UV detection, or with fluorimetric detection after derivatisation, or by means of capillary electrophoresis [17–25]. A few of these methods have been subsequently used for pharmacokinetic studies, in rat and dog plasma. Ficarra et al. described a method for the direct separation of RBX enantiomers in normal phase mode on different cellulose- and amylose-based CSPs [20]. Fleishaker et al. performed a direct, fast analysis of RBX enantiomers on a Chiralcel OD-H analytical column [13]. Ohman et al. described the use of three different CSPs (Chiral-AGP, Chiral-CBH and ChiraGrom-2) in reversed phase mode [18,19] for the same purpose.

However, none of these papers report a method for the determination of RBX enantiomers in formulations. The aim of this study was to develop a reliable analytical method which could be used for the enantioselective quality control of RBX enantiomers in commercially available pharmaceutical formulations (such as Edronax® and Davedax® tablets).

2. Experimental

2.1. Chemicals

RBX racemic powder (for its chemical structure see Fig. 1) and Edronax® tablets were kindly provided by Pharmacia & Upjohn Italia S.p.A. (Milan, Italy). Phosphoric acid, sodium hydroxide and methanol analytical grade were from Carlo Erba (Milan, Italy). HPLC-grade 2-propanol, *n*-hexane and acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA); 1,3,5-tri-*tert*-butylbenzene and sodium perchlorate, analytical grade, were from Fluka (Buchs, Switzerland). Ultrapure water (18.2 MΩ cm) was obtained by means of a Millipore (Milford, MA, USA) Milli-Q apparatus.

2.2. Apparatus and chromatographic conditions

The chromatographic apparatus consisted of a Jasco (Tokyo, Japan) PU-980 Intelligent Pump and a Jasco J-710 spectropolarimeter detector or a Jasco 875-UV spectrophotometric detector. A Rheodyne 7000 valve installed post-column allowed for the trapping of peak fractions for the acquisition of circular dichroism or UV spectra with the on-line installed spectropolarimeter detector. Chromatograms were recorded via a Jasco J-700 or HSM Hitachi system software.

The analytical enantioseparation of RBX was carried out isocratically at room temperature on a Chiralcel OD-R column (cellulose Tris(3,5-dimethylphenylcarbamate); 250 mm × 4.6 mm i.d.; 10 μm particle diameter). The mobile phase consisted of 0.5 M $NaClO_4$ and acetonitrile (60:40, v/v), at a flow rate of 0.5 mL/min. Pure enantiomers of RBX were obtained by semipreparative, normal phase HPLC on a Chiralcel OD column (cellulose Tris(3,5-dimethylphenylcarbamate); 250 mm × 10 mm i.d.; 10 μm particle diameter) with a mobile phase consisting of *n*-hexane and 2-propanol (80:20, v/v), at a flow rate of 2 mL/min.

Fractions of (+)- and (−)-RBX were collected and the mobile phase was evaporated with a stream of nitrogen at room temperature.

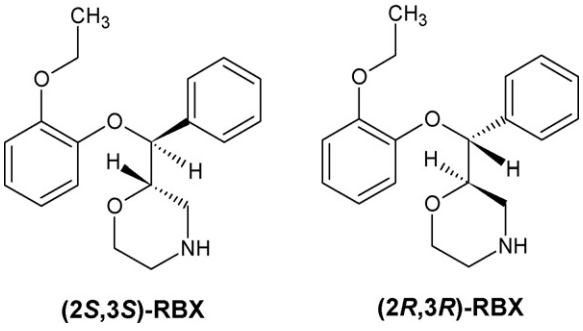

Fig. 1. Chemical structures of RBX enantiomers.

2.3. Solutions

Racemic RBX stock solutions for analytical enantioseparation were prepared by dissolving 26.12 mg of RBX methanesulfonate (MW 409.5) in 20 mL of water, in order to obtain a final concentration of RBX, calculated as the free base (MW 3134), equal to 1 mg/mL. Standard solutions of 10 μg/mL were obtained from stock solutions by dilution with 0.5 M $NaClO_4$. Stock solutions of RBX free base for direct phase HPLC were prepared by dissolving 200 mg of RBX methanesulfonate in 20 mL of NaOH 1 M, then extracting with 20 mL of ethyl acetate. The organic extract was dried under vacuum, then 80 mg of RBX free base redissolved in 1 mL of 2-propanol and directly injected into the HPLC system.

2.4. Chromatographic parameters

The resolution factor (R_s) was calculated by the formula

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2}$$

where t_1 and t_2 are the enantiomer retention times (in s), while w_1 and w_2 are the enantiomer peak widths (in s) at the baseline.

The retention factor (k') was calculated as $k' = (t_R - t_0)/t_0$, where t_R is the retention time of each enantiomer and t_0 is the dead time of the column; the latter was determined by injection of 1,3,5-tri-*tert*-butylbenzene.

The separation factor (α) was calculated as $\alpha = k'_2/k'_1$.

2.5. Analysis of standard solutions

Standard solutions were analysed by RP-HPLC under the reported working conditions. The analyte peak areas were plotted against the analyte concentrations and 10-point calibration curves were obtained for each single enantiomer by means of the least square method. Each concentration was injected in triplicate. Precision assays were carried out at three different concentrations, corresponding to the lower limit, middle point and upper limit of the calibration curve. The solutions were prepared and injected six times at each concentration level to obtain the relative standard deviation (R.S.D.%) of both peak areas and retention times.

2.6. Sample preparation from commercial formulations

Edronax® tablets were extracted according to an already implemented method [24], and analysed. Each tablet declaredly contains 4 mg of RBX, calculated as the free base. Ten tablets were weighed, then thoroughly ground and mixed, to obtain a fine, uniform powder. An aliquot corresponding to the amount of powder, which contains 10 mg of RBX free base, was weighed and transferred into a test tube. To the powder, 10 mL of a pH 2.5, 50 mM phosphate buffer were added and the resulting mixture was sonicated for 10 min, then centrifuged at 1400 × *g*. The supernatant is a clear solution containing RBX at a nominal concentration of 1 mg/mL, expressed as the free base. This solution was then diluted to a nominal concentration of 10 μg/mL with water and injected into the HPLC apparatus.

2.7. Method validation

The formulation samples were treated as described above; linearity was assessed by injecting solutions at 10 different concentrations in triplicate. Sample solutions at three different concentrations (corresponding to the lower limit, middle point and upper limit of the calibration curve) were prepared and injected six times during the same day (to evaluate repeatability) and over six different days (to evaluate intermediate precision). The mean amount of drug found of label claim was also calculated. According to the United States Pharmacopoeia guidelines [29], the limit of quantitation (LOQ) and limit of detection (LOD) were obtained as the analyte concentrations which gave rise to peaks whose heights were 10 and 3 times the baseline noise, respectively.

Accuracy was assessed by means of recovery assays. Known amounts of the analyte were added to the formulations in order to obtain final spikings (after suitable dilution) corresponding to a low, a middle and an high concentration of the calibration curve. After sample pre-treatment, the resulting solution was analysed and the recovery of the added analyte was calculated. This procedure was repeated six times for each concentration in order to obtain mean recovery and R.S.D.% values.

3. Results and discussion

A good strategy to obtain the enantioseparation of basic drugs such as RBX is the use of HPLC with chiral stationary phases (CSPs). The chiral selectors used in preparing CSPs include polysaccharide derivatives, cyclodextrin derivatives, macrocyclic antibiotics, proteins, ligand exchange complex, crown ethers, imprinted polymers and low-molecular-mass selectors such as Pirkle-type compounds [26]. Among the various chiral columns available, those based on cellulose derivatives are among the most commonly used [27].

In our previous study, we have developed an electrophoretic method to separate the enantiomers of RBX [25]. Since the pure enantiomers of the drug could not be obtained, we purified a limited amount of the individual enantiomers by HPLC using a cellulose Tris(3,5-dimethylphenyl)carbamate CSP on silica gel (Chiralcel OD column). The enantiomers were then used as standard compounds for capillary electrophoresis characterisation. The good separation obtained with a Chiralcel OD column has prompted us to use the same CSP for this HPLC study. This kind of stationary phase can be used both in normal phase mode (Chiralcel OD column) and in reversed phase mode (Chiralcel OD-R column) [27,28].

Reversed phase HPLC was the technique of choice for the analytical separation, due to the ionic properties of RBX, which is sold as the methanesulfonate. The effect of mobile phase composition on the separation of RBX enantiomers was evaluated by studying the pH and the acetonitrile and perchlorate contents.

3.1. Optimisation of the HPLC separation

The Chiralcel OD-R stationary phase is usually employed with aqueous mobile phases. It has been previously reported that sodium perchlorate is an ionic modifier, which can notably improve chiral resolution on Chiralcel OD-R sorbents [30–33]. For this reason, the effect of sodium perchlorate on retention and enantioseparation was tested. The results are shown in Table 1.

Enantioselectivity, enantioseparation and retention times were almost unaffected when increasing the sodium perchlorate concentration in the mobile phase from 0 to 0.3 M. On the contrary, an increase in enantioselectivity and enantioseparation was obtained when increasing the concentration of the ionic modifier from 0.3 to 0.5 M. Both parameters decreased sharply at higher concentrations of perchlorate. The best separation of RBX was obtained using sodium perchlorate 0.5 M, with α and R_s values of 1.39 and 3.38, respectively. The results obtained with perchlorate can probably be explained by ion pairing effects [30]. They confirm the very important role of the counter ion in the enantioseparation of basic compound on CSP cellulose Tris(3,5-dimethylphenylcarbamate) in the reversed phase mode [34].

Table 1
Influence of the concentration of sodium perchlorate on retention and enantioseparation of RBX on Chiralcel OD-R[a]

$[HClO_4]$ (M)	k'_1	k'_2	α	R_s
0.00	1.75	2.40	1.37	1.85
0.20	1.97	2.73	1.39	1.73
0.30	2.01	2.78	1.39	1.79
0.50	2.89	4.01	1.39	3.38
1.00	2.10	2.39	1.14	1.20

[a] Mobile phase: sodium perchlorate/acetonitrile (60/40, v/v).

The influence of the pH of the mobile phase on retention times, enantioseparation and enantioselectivity is shown in Table 2. An increase in the pH of the mobile phase from 5.0 to 6.0 seems to have a favourable influence on enantioselectivity. No useful enantioresolution was found at pH <5.0, due to insufficient retention of RBX enantiomers; pH >6.0 was avoided to preserve the lifetime of the CSP. As can be seen in Table 2, the enantiomers of RBX are completely resolved at pH 6.0.

The influence of acetonitrile percentages on the enantioseparation was investigated (Table 3). As it could be expected in reversed phase systems, an increase in acetonitrile percentage resulted in a decrease in retention times. The highest value of resolution of RBX enantiomer peaks was obtained by using a mobile phase composed of water: acetonitrile, 60:40 (v/v), with α and R_s values of 1.39 and 3.38, respectively.

The effect of column temperature on the RBX enantioseparation was studied (between 20 and 40 °C). The results obtained indicated that, as it could be expected with a Chiralcel OD-R column, an increase in temperature resulted in decreased capacity factors. The separation factor and resolution slightly increase with an increase in temperature, but the differences are not very marked and any temperature in this range could be suitable for analysis. The working temperature of 25 °C was chosen because it is nearest to normal room temperatures, thus easily maintained.

The enantioseparation of a 10 μg/mL standard solution of racemic RBX methanesulfonate is reported in Fig. 2. This chro-

Table 2
Influence of the pH on retention and enantioseparation of RBX on Chiralcel OD-R[a]

pH	k'_1	k'_2	α	R_s
3.0	1.49	1.60	1.07	0.40
4.0	1.51	1.61	1.07	0.55
5.0	1.25	1.34	1.07	0.35
6.0	2.61	3.85	1.48	3.21

[a] Mobile phase: 0.5 M sodium perchlorate/acetonitrile (60/40, v/v).

Table 3
Influence of the organic modifier acetonitrile on retention and enantioseparation of RBX on Chiralcel OD-R[a]

% Acetonitrile	k'_1	k'_2	α	R_s
60	0.48	0.65	1.36	1.11
50	0.83	1.09	1.32	1.47
40	2.89	4.01	1.39	3.38
30	4.88	6.10	1.25	2.41

[a] Mobile phase: pH 6.0, 0.5 M sodium perchlorate/acetonitrile.

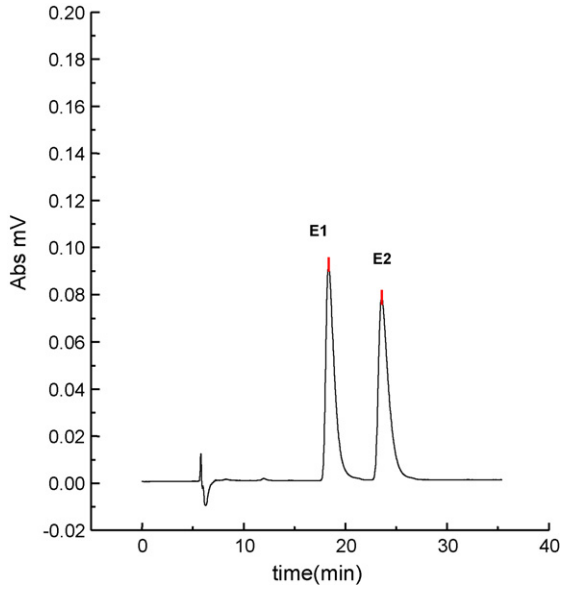

Fig. 2. Chromatogram of a RBX methanesulfonate standard solution (10 μg/mL, 20-μL loop) on a Chiralcel OD-R column, obtained using a mobile phase composed of 0.5 M sodium perchlorate (pH 6.0)/acetonitrile (60/40, v/v), at the temperature of 25 °C, at a flow rate of 0.5 mL/min. E1 and E2 correspond to the first and second eluted peak, respectively.

matogram was obtained using a mobile phase composed of pH 6.0, 0.5 M sodium perchlorate and acetonitrile (60/40, v/v), at the temperature of 25 °C and a flow rate of 0.5 mL/min. The two enantiomer peaks are very well separated (R_S = 3.2) and there are no other significant peaks.

3.2. Semipreparative HPLC

Since pure RBX enantiomers could not be obtained, a semipreparative HPLC method was developed to separate and purify them.

The chiral semipreparative separation of RBX was carried out using the same CSP used for analytical separation, but in normal phase conditions.

The use of a normal phase system requires that RBX is in its free base form, in order to prepare standard solutions in alcohols such as ethanol or 2-propanol, which are suitable for injection in these systems.

Different mobile phases were tested, varying the nature of the alcohol and its percentage in the mobile phase. The mobile phases tested were *n*-hexane/2-propanol and *n*-hexane/ethanol mixtures in different ratios. It was verified from the start that the enantioseparation was unaffected by the addition of small amounts of diethylamine or trifluoroacetic acid, as suggested by common column use procedures.

The best results in terms of resolution and analysis time were obtained with a mobile phase composed of *n*-hexane/2-propanol (80:20, v/v) at a flow rate of 2 mL/min. The elution of the enantiomers was monitored with an UV detector set at 226 nm.

With this system, it was possible to inject 40 mg of racemic RBX in a single chromatographic run (Fig. 3), obtaining each single enantiomer as a different eluate fraction. The fractions were collected and dried at reduced pressure and at room temperature.

An amount of about 70 mg of each enantiomer (enantiomer 1, E1 and enantiomer 2, E2) was obtained, with an enantiomeric excess (e.e.) greater than 99.5%, evaluating by injecting each fraction in

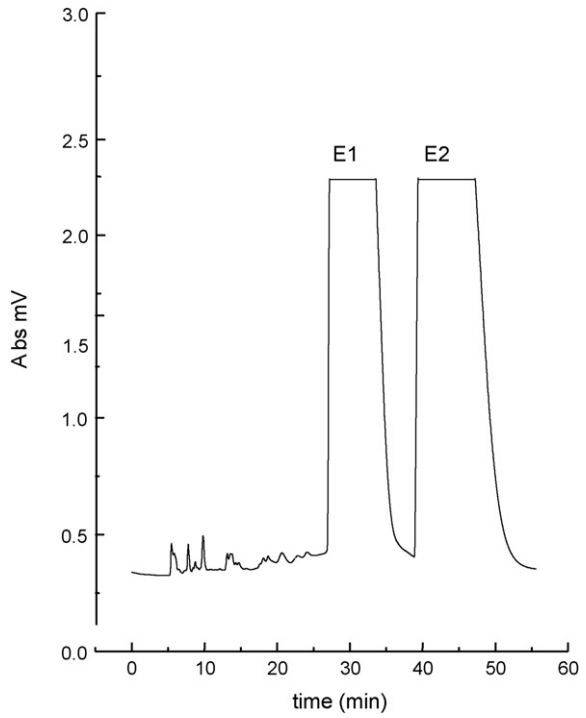

Fig. 3. Semipreparative resolution of 40 mg of racemic RBX on a Chiralcel OD column (250 mm × 10 mm). Mobile phase, *n*-hexane/2-propanol (80/20, v/v); flow rate, 2 mL/min.

the same chromatographic system. The enantiomeric excess was calculated by the standard definition:

$$\text{e.e.} = \frac{\text{major enantiomer} - \text{minor enantiomer}}{\text{major enantiomer} + \text{minor enantiomer}} \times 100$$

The HPLC system was connected online with a circular dichroism detector to verify the elution order of the two separated chromatographic fractions to be used as reference standards in reversed phase mode. A 7000 Reodhyne valve post-column permits to isolate the cell of the spectropolarimeter detector for the acquisition of circular dichroism (CD) and UV spectra. A spectrum of the first eluted peak is compared with a spectrum corresponding to the second eluted peak in Fig. 4. As can be observed, the UV absorbance

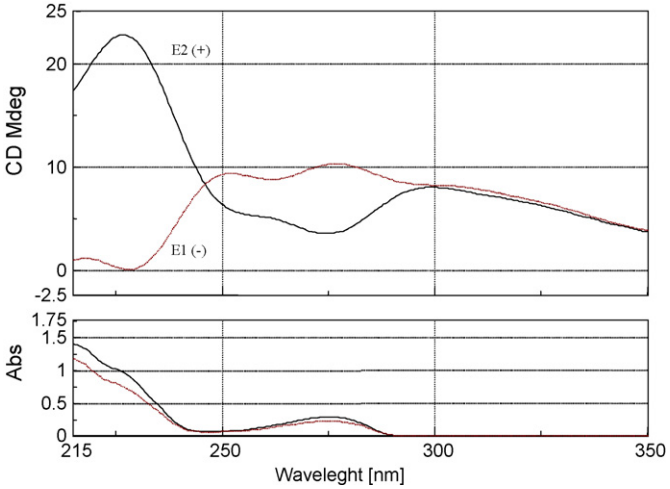

Fig. 4. Circular dichroism (above) and UV (below) spectra of RBX enantiomers recorded online in a mobile phase composed of *n*-hexane/2-propanol (80/20, v/v).

Table 4
Linearity parameters and values assessed on samples extracted from commercial tablets

Analyte	Linearity range (μg/mL)	Linearity equation, $y = a + bx$[a]		r_c	LOQ (μg/mL)	LOD (μg/mL)
		$a \pm$ S.D.	$b \pm$ S.D.			
E1	0.5–100.0	−120.3 ± 58.20	326.5 ± 1.416	0.9998	0.5	0.2
E2	0.5–100.0	−118.4 ± 59.67	323.9 ± 1.452	0.9998	0.5	0.2

[a] y is the enantiomer area, expressed as mV s; x is the enantiomer concentration, expressed as μg/mL.

Table 5
Validation parameters assessed on samples extracted from Edronax® tablets

Analyte	Nominal concentration (μg/mL)	Amount found of label claim (%)	Peak area repeatability (R.S.D.%)	Peak area intermediate precision (R.S.D.%)	Retention time intermediate precision (R.S.D.%)
E1	0.5	98	2.0	2.1	0.6
	50.0	101	1.3	1.6	0.4
	100.0	100	0.8	1.5	0.4
E2	0.5	98	2.2	2.4	0.4
	50.0	101	2.1	2.5	0.6
	100.0	99	1.6	2.0	0.1

of the two enantiomers are identical and the Cotton effects are opposite.

The results of the single enantiomer injections show that the first and the second eluted enantiomer on Chiralcel OD-R corresponded to E1 (−) and E2 (+), respectively, indicating with (+) and (−) the signal of circular dichroism.

3.3. Analysis of standard solutions

The analytical method thus developed was applied to standard solutions of RBX. Good linearity ($r_c > 0.9996$) was found over the 0.5–100 μg/mL concentration range (racemic concentration 0.25–50.0 μg/mL for each enantiomer). The limit of quantitation (LOQ) was 0.5 μg/mL (racemic concentration), while the limit of detection (LOD) was 0.2 μg/mL (racemic concentration). Precision assays at three different levels (0.5, 50 and 100 μg/mL) also gave good results, with R.S.D. (relative standard deviation) values always lower than 2% for both enantiomers peak areas and retention times.

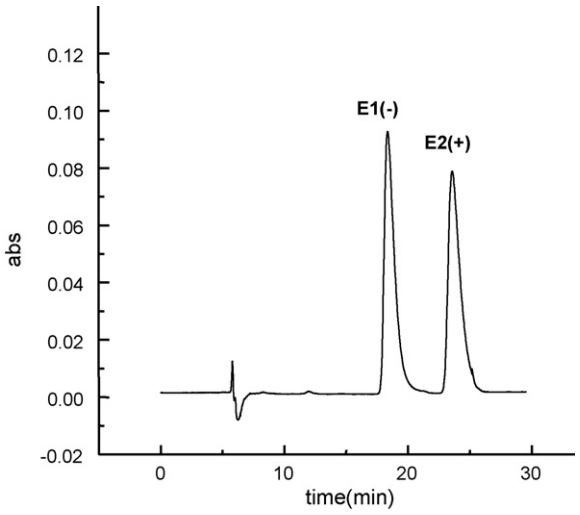

Fig. 5. Chromatogram of a solution of Edronax® (nominal concentration, 10 μg/mL; 20-μL loop) on a Chiralcel OD-R column, obtained using a mobile phase composed of 0.5 M sodium perchlorate (pH 6.0)/acetonitrile (60/40, v/v), at the temperature of 25 °C, at a flow rate of 0.5 mL/min. E1 and E2 correspond to the first and second eluted peak, respectively.

3.4. Application of the method to formulations

A previously developed extraction method used methanol to extract RBX from Edronax® tablets [25]. Since RBX is present in tablets as the methanesulfonate salt, in this study water was used instead of methanol to extract the active principle. Preliminary assays allowed to ascertain that RBX could be quantitatively extracted from the tablets of Edronax® with this simple water treatment. Subsequently, the extract was diluted with a sodium perchlorate aqueous solution (0.5 M, pH 6.0) and analysed by means of the developed reversed phase analytical method. The chromatogram corresponding to an extract from Edronax® tablets is shown in Fig. 5. As it can be seen, no interference from the matrix is present and the two enantiomers are completely separated. The chromatograms of samples from Edronax® tablets are superimposable to those obtained from standards.

3.5. Method validation

The method was validated in terms of linearity, precision and accuracy. Good linearity was found on extracts from tablets for both enantiomers ($r_c > 0.9998$) in the 0.5–100 μg/mL range (racemic concentration). The LOD was found to be 0.2 μg/mL and the LOQ 0.5 μg/mL for each enantiomer. Linearity parameters can be found in Table 4. Precision assays carried out at three different nominal concentrations (0.5, 50 and 100 μg/mL, racemic) gave good results, with R.S.D. values always lower than 2.8% (enantiomer areas) and 0.7% (migration times). The amounts of drug found of label claim were in the 99–102% range. Thus, the formulations analysed are well within the limits given by the United States Pharmacopoeia for tablets, which are 93–107% [29]. The complete results of the assays on tablets are reported in Table 5. Method accuracy was evaluated

Table 6
Recovery values for accuracy assays carried out on Edronax® tablets

Analyte	Concentration added (μg/mL)	Recovery (%)	Repeatability (R.S.D.%)
E1	0.5	102	2.0
	50.0	101	1.5
	100.0	100	1.5
E2	0.5	101	1.9
	50.0	99	1.3
	100.0	102	1.6

by means of recovery assays at three different concentration levels. The results of the recovery assays are reported in Table 6; they are very satisfactory, with mean recovery values ranging from 99% to 102%.

The proposed enantioselective HPLC method is based on a cellulose OD CSP and has been validated in terms of linearity, precision and accuracy and has been successfully applied to the quality control of RBX in commercial formulations. Although the Chiralcel OD-R column has been used for the resolution of other chiral compounds, to the best of our knowledge it has never been applied to the enantioseparation of RBX. Finally, the isolation of the optical isomers of RBX was obtained using a semipreparative column packed with the same cellulose OD CSP. This latter method could prove useful for scale-up studies aimed at producing large amounts of pure enantiomers for different purposes: for example, pharmacokinetic and pharmacological studies, or the preparation of enantiopure formulations.

4. Conclusion

The HPLC method described herein achieves the enantioseparation of RBX and has been applied to the quantitative determination of its enantiomers in commercial formulations. The method uses a Chiralcel OD-R column as the chiral selector. The method is quite sensitive (LOQ = 0.5 μg/mL of racemic RBX), is quite rapid (simple extraction procedure, chromatographic run shorter than 30 min) and feasible; with respect to normal phase methods, it is also inexpensive and uses lower amounts of toxic organic solvents. Furthermore, precision and accuracy are satisfactory (R.S.D. < 2.8%, mean recovery 102%). Thus, the method seems to be suitable for the quality control of RBX enantiomers in commercial formulations. Moreover, a semipreparative separation of enantiomers was developed, which has provided pure RBX enantiomers. Hence, it was possible to assign a unequivocal identity to their chromatographic peaks. Since it seems that RBX enantiomers differ in their pharmacological properties, the semipreparative enantioseparation could be scaled up to conduct enantioselective pharmacokinetic studies, as well as to develop formulations containing an excess of one enantiomer, in order to obtain better therapeutic results.

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