ELSEVIER

Journal of Chromatography A, 947 (2002) 247–254

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Simultaneous determination of reboxetine and *O*-desethylreboxetine enantiomers using enantioselective reversed-phase high-performance liquid chromatography

Daniel Öhman[a,*], Björn Norlander[a], Curt Peterson[a], Finn Bengtsson[a,b]

[a]*Department of Medicine and Care, Division of Clinical Pharmacology, University Hospital, SE-581 85 Linköping, Sweden*
[b]*Department of Neuroscience and Locomotion, Division of Psychiatry, University Hospital, SE-581 85 Linköping, Sweden*

Received 2 February 2001; received in revised form 18 December 2001; accepted 28 December 2001

Abstract

Current knowledge of stereoselective pharmacokinetics and different potencies of drug enantiomers requires the performance of stereoselective analysis during therapeutic drug monitoring in clinical practice. However, in the case of the new antidepressant drug reboxetine, no effort has been made so far to find a such a suitable system. Therefore, as a step towards developing an enantioselective bioanalytical method for reboxetine and the *O*-desethylreboxetine metabolite, three stereoselective chromatographic approaches have been investigated. Several chiral columns were tested, among them Chiral-AGP, ChiraGrom 2 and Chiral-CBH, which were able to simultaneously separate the two compounds into enantiomers in total running times of 28, 18 and 12 min, respectively.

Keywords: Enantiomer separation; Reboxetine; *O*-Desethylreboxetine

1. Introduction

The chiral compound reboxetine (RBX) [1] was introduced as a clinical antidepressant drug [2] in 1999. In contrast to most other available antidepressant drugs, it acts relatively selectively as a neural noradrenaline reuptake inhibitor (NARI) in the brain [3]. Chemically, RBX shares important structural elements with other commonly used antidepressant drugs, such as fluoxetine, which has a chiral centre structurally similar to one of the chiral centres of RBX [4] (Fig. 1). Still, the primary pharmacodynamic actions differ clearly between the drugs, fluoxetine belonging to the important clinical group of selective serotonin reuptake inhibitors (SSRI). Accordingly, the enantioselective features of fluox-

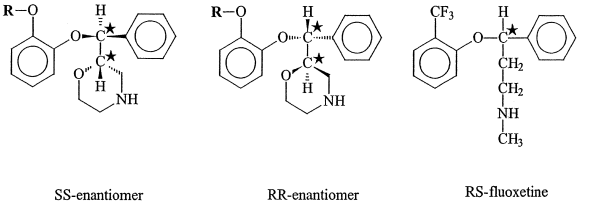

Fig. 1. The basic chemical structure of the RBX and *O*-RBX enantiomers (when R=CH_2CH_3, it is the RBX molecule and when R=H, it is the *O*-RBX molecule) and the structure of fluoxetine, ★ indicating the chiral centres of the molecule.

*Corresponding author. Tel.: +46-13-221-794; fax: +46-13-104-195.

E-mail address: daniel.ohman@imv.liu.se (D. Öhman).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00012-2

etine and its main metabolite have also been scrutinised kinetically [5]. There are, in fact, two chiral centres in the RBX molecule, but due to a stereospecific manufacturing process [6], the drug is prescribed to patients only as the enantiomers (−)-(*R*,*R*)-RBX and (+)-(*S*,*S*)-RBX (Fig. 1). Further unravelling of the kinetic features of the enantiomers of RBX and some of its main metabolites is therefore highly desirable.

All modern psychoactive drugs (including RBX) undergo extensive metabolism in the human body. In the case of racemic drugs, the metabolism is often more or less stereoselective and predicts for a large inter-individual variability in the concentration outcome of the enantiomers. Moreover, for RBX, the plasma concentration of the *R*,*R*-enantiomer has generally been found to be twice the concentration of the *S*,*S*-enantiomer after administration of a single oral dose of racemic RBX to healthy volunteers [7,8]. Importantly, the two enantiomers of RBX show a difference in NARI potency in rat hypothalamic synaptosomes, leading to the conclusion that the *S*,*S*-enantiomer is likely to be to be over 20 times more potent (IC_{50} 3.6 n*M*) than its *R*,*R*-stereomer (IC_{50} 85 n*M*) [9].

At least four routes for metabolic transformation have been identified in the human metabolism of RBX. These include 2-*O*-dealkylation to yield *O*-desethyl-RBX (*O*-RBX) (Fig. 1) [10]. There is no known inversion of the chiral centres of the molecule in this metabolic step.

Racemic RBX in plasma has been analysed by high-performance liquid chromatography (HPLC) and UV detection after liquid–liquid extraction with diethylether [11] and, in another study, after on-line solid-phase extraction [12]. Metabolites of RBX have previously been analysed in pre-clinical studies with [^{14}C]RBX and radio-HPLC detection [1] and, more recently, a bioanalytical method for simultaneous analysis of RBX and a metabolite in a clinical setting has been reported from our group [13].

Enantioselective analysis of RBX has previously been performed using chiral derivatisation with (+)-1-(9-fluorenyl)ethyl chloroformate [8]. The method proved reliable but the selectivity for the diastereomers was poor and therefore an unacceptably long chromatographic time (90 min) for clinical routine use was used here to achieve separation. With an improved version of the method [14], using normal-phase chromatography and column switching, it was possible to analyse the two diastereomers in 16 min. By using normal-phase chromatography combined with MS–MS detection, Fleishaker et al. [15] performed a direct, fast analysis of the RBX enantiomers on a Chiralcel OD-H analytical column.

In a routine clinical laboratory setting, none of the above mentioned methodologies is optimal.

This relates to (i) the lack of a simultaneous determination of a suitable metabolite which is very useful for tracing deviant drug metabolism and/or non-compliance in treatment failure [16], (ii) the use of a complicated derivatisation step, and (iii) the use of volatile, hydrophobic organic solvents. Retention times can be difficult to keep stable on normal phase-based systems in an everyday running application and, furthermore, with regards to the working environment, the aim should be to avoid hazardous solvents in routine applications.

The aim of this study was therefore to find a chromatographic system in which the enantiomers of both RBX and *O*-RBX could be separated. In addition, the chromatography was to be compatible with a routine laboratory setting, i.e. easy to handle, without the use of unpolar, volatile solvents as well as being considered cost-effective for the public health care system to use. Therefore, an effort was made to find a column, based on reversed-phase HPLC, that simultaneously separated the enantiomers of both RBX and *O*-RBX without derivatisation to diastereomers.

2. Experimental

2.1. Chemicals

Racemic reboxetine (RBX), the pure enantiomers (*RR*-RBX and *SS*-RBX) and racemic *O*-desethylreboxetine (*O*-RBX) were kindly supplied by Pharmacia and Upjohn (Kalamazoo, MI, USA), RBX, *RR*-RBX and *SS*-RBX as methanesulphonate salts (MW 409.5, analytical grade) and *O*-RBX as free base (MW 285.4, research grade). All concentrations are given as free base equivalents.

HPLC-grade acetonitrile and methanol were obtained from LabScan Analytical Sciences (Dublin,

Ireland), 2-propanol, sodium phosphate, EDTA (Triplex® III), sodium acetate and acetic acid from Merck (Darmstadt, Germany) and potassium phosphate from Fluka (Buchs, Switzerland). Water was obtained from a Milli-Q station (Millipore, Stockholm, Sweden). Human serum for preparing spiked samples was delivered from the blood centre at the Department of Transfusion Medicine, Linköping University Hospital (Linköping, Sweden).

2.2. Instrumentation

The HPLC system consisted of an Alliance 2690 with a column heater and a 2487 dual λ UV detector (Waters, Milford, USA).

Several chiral columns were tested during method development; some had chiral recognition for *O*-RBX or RBX (data not shown), but only three chiral analytical columns showed satisfactory chiral recognition simultaneously for *O*-RBX and RBX. They were (i) a 100×4 mm cellobiohydrolase (Chiral-CBH) column with a particle size of 5 μm, (ii) a 100×4 mm α_1-acid-glycoprotein (Chiral-AGP) column with a particle size of 5 μm, both of these columns being purchased from ChromTech (Stockholm, Sweden), and (iii) a 50×2 mm ChiraGrom-2 (CG-2) column with 8-μm particles from Grom Analytic+HPLC (Herrenberg, Germany). The chromatograms were recorded and processed using the Millennium 32 chromatography data system from Waters (Milford, USA).

2.3. Stock solutions, standards and quality controls

Stock solutions of racemic RBX and *O*-RBX, 100 and 6 μ*M*, respectively, and stock solutions of *RR*-RBX and *SS*-RBX with a concentration of 500 μ*M* were prepared in Milli-Q water and stored in dark glass bottles below 8 °C. From the stock solutions, retention time check solutions were prepared in the adequate mobile phase, *RR*-RBX and *SS*-RBX to a concentration of 200 n*M* and racemic RBX and *O*-RBX to 200 and 100 n*M*, respectively.

The human serum was spiked to a concentration of 400 n*M* *RR*-RBX, 700 n*M* *SS*-RBX and 400 n*M* racemic *O*-RBX.

For determination of linearity, the limit of quantification (LOQ) and the limit of detection (LOD), calibration points were prepared in MQ-water for direct injection in the concentration range 10 to 100 n*M* for the RBX and *O*-RBX racemates. LOD was set to the point where the peak/noise ratio did go below 3. LOQ was set to the point where the coefficient of variance from five repeated injections exceeded 15%.

2.4. Chromatographic conditions

The flow rate was set to 0.9 ml/min for the Chiral-AGP and Chiral-CBH columns. The injection volume was 25 μl, which gave the amounts of 5 pmol of RBX and 2.5 pmol of *O*-RBX in the injection of the racemates and 5 pmol of each enantiomer in the injection of the pure enantiomers. The smaller ChiraGrom 2 column had a flow rate of 0.22 ml/min. The injection volume was 10 μl and consequently 40% of the amounts in the Chiral-AGP and CBH applications was injected.

The mobile phase compositions after optimisation are given under the particular column in the Results and discussion section. The temperature was set to 25 °C for all columns and the detection was performed with UV at 210 nm. RBX has a more selective absorbance maximum at 273 nm, but the molecular absorbance is only about one tenth of the absorbance at 210 nm. Linearity, LOD and LOQ were determined at 210 nm. Solid phase extraction of human serum was performed with 1 ml of serum using the methodology described in Ref. [13]. The dried sample extract was resolved in 100 μl of the adequate mobile phase and injected as described above.

3. Results and discussion

3.1. The Chiral-AGP column

In many previous applications [17–21], immobilised α_1-acid glycoprotein has been used for the separation of enantiomers. For example, the fluoxetine enantiomers have also been separated on the Chiral-AGP column [22] and interestingly, as mentioned before, fluoxetine has a chiral centre similar to one of the chiral centres of the RBX molecule (Fig. 1). Furthermore, in protein binding studies conducted

on RBX, it has been shown that RBX is extensively bound to proteins, mainly to α_1-acid glycoprotein (AGP) [11] and, as a working hypothesis, we anticipated that the substances would interact in some way with the Chiral-AGP packing material.

The protein matrix of the Chiral-AGP column consists of a single peptide chain, 183 amino acids long, with an isoelectric point of 2.7 [23]. By using the pH range of 2–7 it is possible to change the conformation of the (at least) two chiral recognition sites [24] in the protein and obtain chiral separation for both anionic, non-protolytic and cationic compounds.

In the present application, the parent compound, RBX, showed excellent chiral recognition over the pH range 3–7 and was easily separated within 5 min using a 10 m*M* sodium acetate buffer of pH 5 with the addition of 3–15% of an uncharged organic modifier. The retention time (t_R) for both RBX peaks increased with increasing pH and decreased with increasing modifier concentration. (Methanol, ethanol, propanol, 2-propanol, acetonitrile and propionitrile were tested; data not shown.) However, the enantiomers of the metabolite showed no tendency to be separated with these mobile phases based on a sodium acetate buffer. Nevertheless by using a phosphate buffer instead, separation could be achieved also for *O*-RBX on the Chiral-AGP column, but only when the organic modifier did not contain an hydroxy group. This finding indicates that hydrogen bonding to the phenol of the metabolite is essential for chiral selectivity. Still, RBX, having an ethoxy group in that position, did indeed show a good chiral recognition also with alcohol modifiers. One explanation for this could be that RBX and *O*-RBX may utilise different binding sites and that their retention mechanisms therefore cannot readily be compared. It should be recalled that Enquist and Hermansson have characterised at least two binding sites, a high- and a low-affinity site for the chiral drug terodiline in the AGP protein [24]. As a consequence, all hydroxylic modifiers (i.e. alcohols) were excluded and the optimisation preceded testing only propionitrile and acetonitrile.

The ionic strength of the mobile phase buffer was also evaluated. An increase in the resolution of the *O*-RBX enantiomers was obtained with increased buffer concentration, indicating an ion-pair mechanism with one of the phosphate anions for the chiral recognition. This observation also indicates the utilisation of different binding sites in the AGP protein as only a minor effect on the RBX retention time compared to *O*-RBX, although both RBX and *O*-RBX have almost identical cationic properties. A baseline separation of the enantiomers of the metabolite was obtained with a 200 m*M* phosphate buffer at pH 6.1. Such high concentration of a phosphate buffer is however not recommended in routine clinical laboratory applications, neither by the manu-

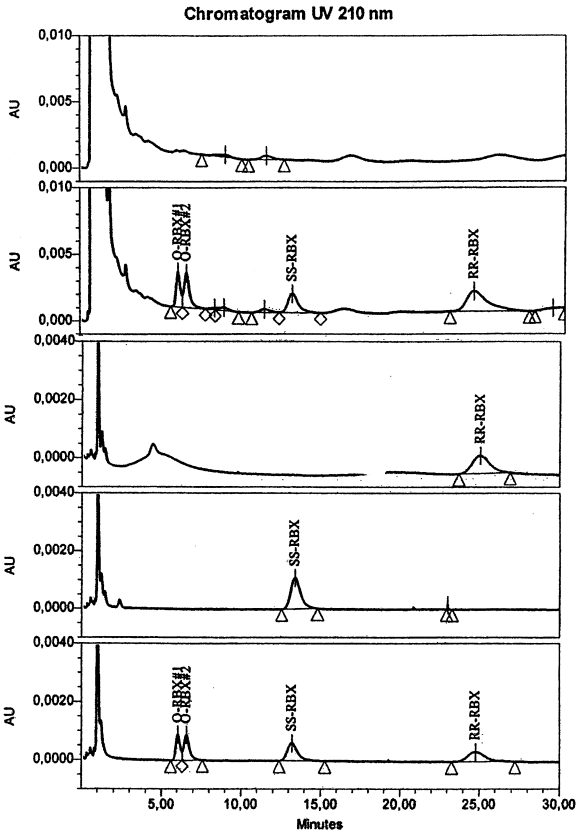

Fig. 2. Chromatograms obtained on the AGP column (4×100 mm, 5-μm particles). Mobile phase composition: 12% (vol.) acetonitrile in 25 m*M* phosphate buffer, pH 6.0. From the top, the chromatograms of extracted blank serum, extracted spiked plasma (400 n*M* *O*-RBX racemate, 400 n*M* *RR*-RBX and 700 n*M* *SS*-RBX), the pure *RR*-RBX and *SS*-RBX enantiomers (5 pmol of each enantiomer injected). At the bottom, a chromatogram after simultaneous injection of the RBX and *O*-RBX racemates (5 and 2.5 pmol injected, respectively). The enantiomers of *O*-RBX are designated #1 and #2 based only on retention order.

facturers of the column nor for good care of the other chromatographic equipment used.

An acceptable separation, within a reasonable chromatographic time for the last eluting peak, was obtained with a mobile phase composition of 12% (vol.) acetonitrile in a 25 m*M* phosphate buffer at pH 6.0 (Fig. 2, Table 1).

The chromatography proved to be robust with good linearity for both the RBX and the *O*-RBX enantiomers (Table 1). LOD was below 1 n*M* for the *O*-RBX enantiomers and the *SS*-RBX enantiomer and the LOQ was ~5 n*M*. The late eluting *RR*-RBX enantiomer had slightly higher LOD and LOQ at 3 and 12.5 n*M*, respectively (data not shown).

3.2. The Chiral-CBH column

The glycoprotein cellulase, CBH 1, originates from fungi and bacteria in nature. The protein has a molecular mass of 64 kDa and an isoelectric point of 3.9 [25]. The chiral recognition site is a 4×7×40 Å-long tunnel in the core of the protein. The tunnel contains acidic amino acids such as aspartic acid and other amino acids such as tryptophan, tyrosine and serine [26]. Accordingly, this means that ion exchange, hydrogen bonding and hydrophobic interactions might be involved in the retention mechanisms. The protein has been immobilised on silica for the separation of chiral acidic and basic drugs [27]. The

Table 1
Chromatographic performances of the three columns tested

Column	Peak name	Average calibrator equation, 5–50 n*M* (n=5)	R^2	t_R (n=25)	N (n=5)	α (n=5)	*Rs* (n=5)
ChiraGrom-2	*O*-RBX #1	$y = 14.88x - 11.74$	0.9995	4.11	781		
						1.26	1.55
	O-RBX #2	$y = 12.66x - 16.57$	0.9992	5.15	734		
						1.64	3.33
	RR-RBX	$y = 7.63x + 2.07$	0.9988	8.43	808		
						1.67	3.86
	SS-RBX	$y = 4.75x - 3.74$	0.9965	13.92	1052		
Chiral-AGP	*O*-RBX #1	$y = 9.42x + 0.97$	0.9996	6.07	3153		
						1.09	1.02
	O-RBX #2	$y = 9.50x - 1.43$	0.9999	6.59	2002		
						2.09	7.91
	SS-RBX	$y = 4.82x - 1.26$	0.9997	13.21	2361		
						1.91	7.53
	RR-RBX	$y = 2.64x - 0.12$	0.9967	24.79	2486		
Chiral-CBH	*RR*-RBX	$y = 12.25x + 9.17$	0.9995	3.49	1263		
						1.25	1.81
	SS-RBX	$y = 13.91x - 1.83$	0.9996	4.23	1244		
						1.33	2.27
	O-RBX #1	$y = 6.95x - 6.15$	0.9971	5.48	1275		
						1.74	4.6
	O-RBX #2	$y = 5.39x - 6.96$	0.9968	9.35	1302		

Raw data are taken from the chromatograms obtained after simultaneous injection of the *O*-RBX and RBX racemates (Figs. 2–4). The equation for the calibrator curve was obtained by taking the average peak height (n=5) at each calibrator level and correlating it to the concentration.

The selectivity (α) is calculated as $\alpha = k'_2/k'_1 = (t_{R2} - t_{R0})/(t_{R1} - t_{R0})$. The resolution (*Rs*) is calculated from the formula $Rs = (t_{R2} - t_{R1})/0.5 \times (w_1 + w_2)$. The plate count ($N$) is calculated using the formula $N = 16 \times (t_R/w)^2$. t_R and w denote peak retention time and peak width, respectively. t_0 was determined by injection of pure mobile phase, setting the time of the first baseline disturbance to t_0. N, α and *Rs* were calculated as means of five repeated injections of calibrator level 3 (25 n*M*). t_R was calculated as a average of 25 repeated injections in the concentration range 5–50 nM.

The enantiomers of *O*-RBX are designated #1 and #2, respectively, based only on retention order.

commercially available ''Chiral-CBH'' column has been characterised regarding the fundamental chromatographic properties by Hermansson and Grahn [28].

Numerous applications have been presented for this column [19,28–31], and it seems that the best chiral recognition is achieved for phenol amines. The selectivity increases with increasing numbers of hydroxyls in the aromatic ring [28] and it decreases with the number of carbon atoms between the amino group and the hydroxyl [27].

In this application, it can be assumed that the *O*-RBX metabolite, satisfying most of the criteria for good chiral recognition in the binding site (i.e. having an aromatic hydroxy group and a cationic amine), would yield a good separation. However, the parent compound has no hydrogen-donating hydroxyl group (but still the possibility of electron donation and hydrogen-acceptance in the hydrogen-binding area of the chiral recognition site) and therefore one would assume a less pronounced enantioselectivity.

The mobile phase composition of the Chiral-CBH column was optimised by using a non-hydroxylic organic modifier, acetonitrile and a hydroxylic organic modifier, 2-propanol. The pH of the 25 m*M* phosphate buffer was also optimised. It proved that an increase in pH from 6.1 to 6.7 did increase the selectivity (α) and the resolution (*Rs*) for both chiral couples and, to some extent, also between RBX and *O*-RBX. An increase in the concentration of acetonitrile and 2-propanol decreased the retention times for all peaks. However, 2-propanol had approximately three times the impact on the retention times of RBX as compared to acetonitrile. The impact on the *O*-RBX enantiomers was more similar for the two modifiers. Also, the enantioselectivity was affected more by 2-propanol than by acetonitrile, especially the selectivity for the metabolite. When analysing hydroxylic compounds the hydrogen-bonding properties of 2-propanol makes it a strong competitor in the hydrogen-bonding area in the CBH core, stronger than a modifier without this hydrogen-donating possibility, i.e. acetonitrile. The influence of 2-propanol is therefore more pronounced when the compounds analysed have both hydrogen-donating and hydrogen-accepting properties. This is why the *O*-RBX enantioselectivity is more affected than the RBX enantioselectivity.

With a mobile phase containing 6% acetonitrile and 9% 2-propanol (vol.) and 50 μ*M* EDTA in a 10 m*M* phosphate buffer at pH 6.1, RBX was well separated within 5 min. The metabolite had, as expected, a better chiral recognition than RBX, but also a longer retention time (Fig. 3, Table 1).

The chromatography proved to be robust with good linearity for both the RBX and the *O*-RBX enantiomers (Table 1). LOD was ~1 n*M* for both the RBX and the *O*-RBX enantiomers and the LOQ was below 5 n*M* for *RR*-RBX, *SS*-RBX and *O*-RBX ≠2.

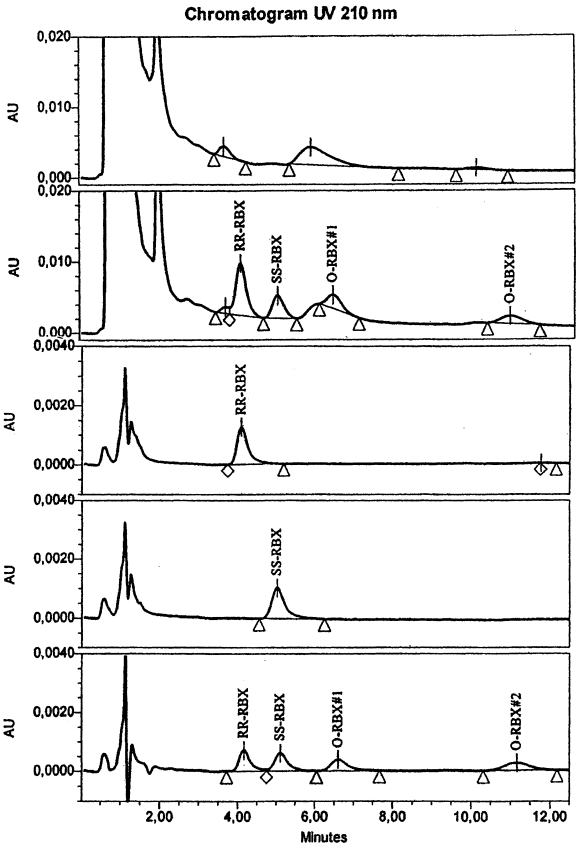

Fig. 3. Chromatograms obtained on the CBH column (4×100 mm, 5-μm particles). Mobile phase composition: 6% acetonitrile and 9% 2-propanol (vol.) and 50 μ*M* EDTA in 10 m*M* phosphate buffer, pH 6.1. From the top, chromatograms of extracted blank serum, extracted spiked plasma (400 n*M* *O*-RBX racemate, 400 n*M* *RR*-RBX and 700 n*M* *SS*-RBX), the pure *RR*-RBX and *SS*-RBX enantiomers (5 pmol of each enantiomer injected). At the bottom, the chromatogram after simultaneous injection of the RBX and *O*-RBX racemates (5 and 2.5 pmol injected, respectively). The enantiomers of *O*-RBX are designated ≠1 and ≠2 based only on retention order.

The first eluting *O*-RBX enantiomer had however a slightly higher LOQ between 5 and 12.5 n*M* (data not shown).

3.3. The CG-2 column

To the best of our knowledge, the retention mechanisms and the capabilities of the CG-2 column have not been published. The column matrix is not revealed by the manufacturers and the only additional information available is that the column contains different chiral polymeric layers bound to silica and that the phase is a built up of ''polar and unpolar phases for reversed- and normal-phase modes tailor-made for the separation of enantiomeric pharmaceuticals, agrochemicals, flavourings, chiral drug monitoring, etc.'' [32].

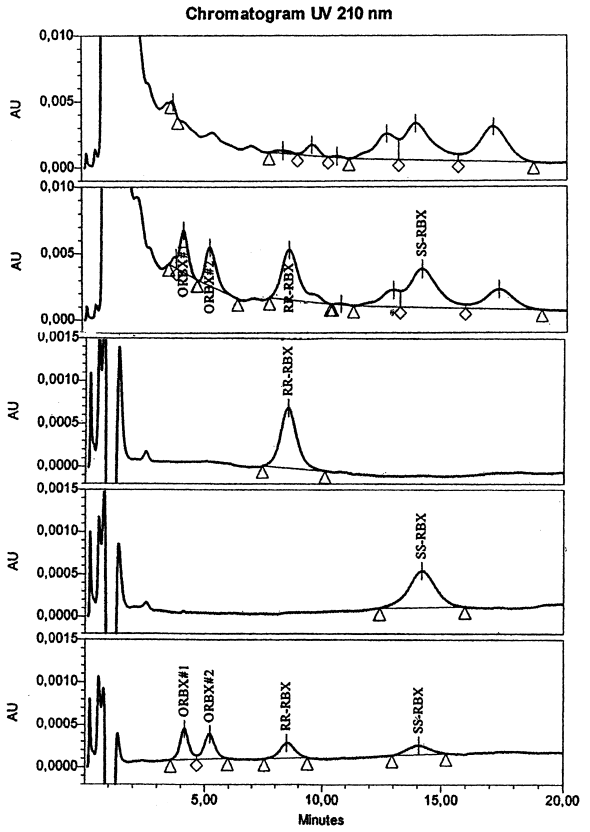

Fig. 4. Chromatograms obtained on the ChiraGrom 2 column (2×50 mm, 8-μm particles). Mobile phase composition: 27% acetonitrile (vol.) in 0.1 *M* phosphate buffer, pH 7.8. From the top, the chromatograms of extracted blank serum, extracted spiked plasma (400 n*M* *O*-RBX racemate, 400 n*M* *RR*-RBX and 700 n*M* *SS*-RBX), the pure *RR*-RBX and *SS*-RBX enantiomers (2 pmol of each enantiomer injected). At the bottom, simultaneous injection of the RBX and *O*-RBX racemates (2 and 1 pmol injected, respectively). The enantiomers of *O*-RBX are designated ≠1 and ≠2 based only on retention order.

For the analytical chemist it is questionable if it is compatible with good laboratory practice (GLP) or good manufacturing practice (GMP) to use such products in routine applications in a fully accredited laboratory.

There are, however, many examples of chiral applications in the application guide and in our application, the column is performing well for the chiral separation of RBX and *O*-RBX. The best separation was achieved with a mobile phase composition of 27% acetonitrile (vol.) in 0.1 *M* phosphate buffer, pH 7.8 (Fig. 4). The plate count is rather low for all peaks although the injected volume has been kept relatively low. This is perhaps explained by the large particle size (8 μm) and the short column (50 mm). The plate count (N), the retention times (t_R), α and Rs were evaluated and are presented in Table 1.

The chromatography proved to be robust with good linearity for both the RBX and the *O*-RBX enantiomers (Table 1). LOD was ~2 n*M* for the *O*-RBX enantiomers and slightly higher for the RBX enantiomers (~4 n*M*). The LOQ was below 5 n*M* for the *O*-RBX enantiomers, between 5 and 12.5 for the *RR*-RBX enantiomer and 12.5 for the *SS*-RBX enantiomer (data not shown).

4. Conclusion

It has been demonstrated that the chiral drug RBX and its metabolite *O*-RBX can be readily separated into pure enantiomers using three different chiral stationary phases, Chiral-AGP, Chiral-CBH and ChiraGrom-2 in the reversed-phase mode. No time-consuming derivatisation into diastereomers was necessary. The equipment used is easy to handle and can probably be found in most routine application laboratories. The three chiral columns found all have their advantages and disadvantages and which column is preferable is related to application. As shown in Figs. 2–4 endogenous interferences are present in all chromatograms obtained after injection of extracted serum samples. Therefore, if (or when) the

methodology is developed further to include concentration determinations in biological samples, new precautions will have to be taken and therefore it is unwise to exclude any column at this stage.

The AGP column has the best chiral separation for RBX and the overall best plate count. In an LC–MS application where phosphate buffers should of necessity be avoided, it is probably difficult to separate the enantiomers of the metabolite. The column in and of itself, however, does not exclude simultaneous determination of the *O*-RBX enantiomers and therefore this column would be preferable in an application using UV detection.

The Chiral-CBH column has the best chiral recognition for the metabolite. The sensitivity might be affected though if the parent compound is to be separated on the same chromatogram since the metabolite appears late in the chromatogram and has a relatively low plate count.

The ChiraGrom 2 column has advantages over the Chiral-CBH and Chiral-AGP columns.

Compared to the Chiral-AGP, the *Rs* for the metabolite is better and the total chromatographic run can be conducted in 18 instead of 28 min. Compared to the Chiral-CBH, the metabolite and the parent compound elute in reversed order, which increases the sensitivity to the metabolite. However, to be able to follow the guidelines for a fully accredited method development that applies to a laboratory working in accordance with GLP and GMP, this column must be rejected until the stationary phase is revealed and the retention mechanisms have been properly investigated.

References

[1] G. Cocchiara, R. Battaglia, P. Pevarello, M. Strolin Benedetti, Eur. J. Drug Metab. Pharmacokinet. 16 (1991) 231.
[2] D. Baldwin, C. Hawely, Int. J. Psychiatry Clin. Pract. 2 (1998) 195.
[3] E.H. Wong, M.S. Sonders, S.G. Amara, P.M. Tinholt, M.F. Piercey, W.P. Hoffmann, D.K. Hyslop, S. Franklin, R.D. Porsolt, A. Bonsignori, N. Carfagna, R.A. McArthur, Biol. Psychiatry 47 (2000) 818.
[4] P. Dostert, M.S. Benedetti, I. Poggesi, Eur. Neuropsychopharmacol. 7 (Suppl. 1) (1997) S23.
[5] R.F. Bergstrom, L. Lemberger, N.A. Farid, R.L. Wolen, Br. J. Psychiatry (Suppl.) 3 (1988) 47.
[6] P. Melloni, A. Della Torre, E. Lazzari, G. Mazzini, M. Meroni, Tetrahedron 41 (1985) 1393.
[7] S. Caccia, Clin. Pharmacokinet. 34 (1998) 281.
[8] E. Frigerio, E. Pianezzola, M. Strolin Benedetti, J. Chromatogr. A 660 (1994) 351.
[9] M. Strolin Benedetti, E. Frigerio, P. Tocchetti, G. Brianceschi, M.G. Castelli, C. Pellizzoni, P. Dostert, Chirality 7 (1995) 285.
[10] L.C. Wienkers, C. Allievi, M.J. Hauer, M.A. Wynalda, Drug Metab. Dispos. 27 (1999) 1334.
[11] D.M. Edwards, C. Pellizzoni, H.P. Breuel, A. Berardi, M.G. Castelli, E. Frigerio, I. Poggesi, M. Rocchetti, A. Dubini, M. Strolin Benedetti, Biopharm. Drug Dispos. 16 (1995) 443.
[12] S. Hartter, H. Weigmann, C. Hiemke, J. Chromatogr. B 740 (2000) 135.
[13] D. Öhman, B. Norlander, C. Peterson, F. Bengtsson, Ther. Drug Monit. 23 (2001) 17.
[14] R.R. Walters, S.C. Buist, J. Chromatogr. A 828 (1998) 167.
[15] J.C. Fleishaker, M. Mucci, C. Pellizzoni, I. Poggesi, Biopharm. Drug Dispos. 20 (1999) 53.
[16] F. Bengtsson, in: F. Bengtsson (Ed.), Overbridging the Concept of Experimental Pharmacology To Clinical Practice in Rational Drug Development. Focus On Modern Clinical Psychopharmacology, ADIS International, Chester, UK, 1996, p. 24.
[17] A. Ceccato, J. Hubert, J. Crommen, J. Chromatogr. A 760 (1997) 193.
[18] I. Abraham, J.P. Fawcett, J. Kennedy, A. Kumar, R. Ledger, J. Chromatogr. B 703 (1997) 203.
[19] A. Karlsson, A. Aspegren, J. Chromatogr. A 866 (2000) 15.
[20] J. Luksa, D. Josic, M. Kremser, Z. Kopitar, S. Milutinovic, J. Chromatogr. B 703 (1997) 185.
[21] J. Hermansson, A. Grahn, J. Chromatogr. A 694 (1995) 57.
[22] ChromTech, Separation of Chiral Compounds On Chiral-AGP, Chiral-CBH, Chiral-HSA, ChromTech, Hägersten, 1998.
[23] P.G. Board, I.M. Jones, A.K. Bentley, Gene 44 (1986) 127.
[24] M. Enquist, J. Hermansson, J. Chromatogr. 519 (1990) 285.
[25] R. Bhikhabhai, G. Johansson, G. Pettersson, J. Appl. Biochem. 6 (1984) 336.
[26] C. Divne, J. Stahlberg, T. Reinikainen, L. Ruohonen, G. Pettersson, J.K. Knowles, T.T. Teeri, T.A. Jones, Science 265 (1994) 524.
[27] I. Marle, P. Erlandsson, L. Hansson, R. Isaksson, C. Pettersson, G. Pettersson, J. Chromatogr. 586 (1991) 233.
[28] J. Hermansson, A. Grahn, J. Chromatogr. A 687 (1994) 45.
[29] H.Y. Aboul-Enein, V. Serignese, Biomed. Chromatogr. 11 (1997) 47.
[30] I. Marle, S. Jönsson, R. Isaksson, C. Pettersson, J. Chromatogr. 648 (1993) 333.
[31] D.J. Mayo, F.P. Tomasella, J.E. Noroski, J. Pharm. Biomed. Anal. 14 (1996) 457.
[32] GromAnalytik+HPLC GmbH, Column choice for chiral separations, in: Application Guide, 1997, pp. 1–16.