Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 133–139

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Sensitive quantitation of reboxetine enantiomers in rat plasma and brain, using an optimised reverse phase chiral LC–MS/MS method

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ARTICLE INFO

Article history:
Received 11 August 2008
Received in revised form 3 October 2008
Accepted 10 October 2008
Available online 5 November 2008

Keywords:
S,S-reboxetine
R,R-reboxetine
Enantiomers
Racemate
Electrospray ionisation

ABSTRACT

A sensitive liquid chromatography–mass spectrometry (LC–MS) method has been developed for stereoselective determination of reboxetine in rat plasma and brain homogenate (LLOQ, 50 pg/ml). The method optimised ionisation efficiency with an electro-ionspray source, by adjusting the composite flow conditions (rate, pH, organic content) from column eluent and post-column organic modifier. LC conditions utilized a chiral AGP column (5 μm) with 12.5 mM ammonium carbonate buffer adjusted with formic acid (pH 6.7) and included a wash step (0.05% acetic acid in water) to maintain assay robustness and chromatographic performance. The total method cycle time was 23 min. Imprecision (R.S.D.) was below 10% and inaccuracy (% error) below 7% for both enantiomers in plasma and brain homogenate, over a 2000-fold dynamic range (0.05–100 ng/ml). An automated liquid–liquid extraction technique was used (borate buffer, pH 10/*tert*-butyl methyl ether) and the matrix type used produced no difference in the assay performance. The method was successfully applied to determine the pharmacokinetic profiles of S,S- and R,R-reboxetine in rats, following subcutaneous administration of racemate drug.

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1. Introduction

Reboxetine is a selective noradrenaline reuptake inhibitor (SNRI) that was introduced as a clinical antidepressant in 1999 [1]. In recent years it has been used as a pharmacological tool in both experimental neuropharmacology studies [2,3] and in vitro receptor binding investigations [4,5]. Its structure contains two chiral centres (Fig. 1) with four possible stereoisomers but as a consequence of the manufacturing process employed, racemate drug comprises only two enantiomers, S,S- and R,R-reboxetine [6]. The marketed compound is administered therapeutically in this racemic form; although original in vitro work established the primary SNRI pharmacology of S,S-reboxetine at rat hypothalamic synaptosomes, is considerably more potent than its antipode [7]. More recent clinical developments with the drug have sought to explore the benefits of using the single S,S-enantiomer in lower back and neuropathic pain syndromes [8,9]. In addition, the pharmacokinetic characterisation of orally dosed racemate in humans shows plasma concentrations of R,R-reboxetine can exceed those of S,S-reboxetine by up to a factor of 2 but the latter is thought to be responsible for much of the effect [10,11]. Therefore, historically it has been necessary to monitor the kinetics and dynamics of individual enantiomers to understand the concentration effect relationships more clearly. Equally, with future clinical applications of single enantiomer drug it can be considered important to contrast results against previous racemate data and establish clear evidence of the benefits.

Reboxetine has been used as a marker to study receptor occupancy levels in specific brain regions of the central nervous system due to its selective binding to the noradrenaline receptor family over related neurotransmitter receptors such as serotonin [4]. Furthermore, its high binding affinity can be utilized ex vivo as a competitor ligand, to measure the central nervous system (CNS) binding of novel agents administered in vivo [12]. At Pfizer Research and Development laboratories, the need arose to administer reboxetine racemate to rats and measure the plasma and brain concentrations of individual enantiomers to a sensitive level for pharmacokinetic characterisation over an extended period. This paper describes the analytical methodology that was put in place to assist in this research.

Several analytical methods to separate reboxetine enantiomers and quantify drug levels have previously been published [6,10,13–16], using chiral derivatisation, cellulose- and amylose-based stationary phases and most recently chiral α_1 acid glycoprotein [AGP]-based stationary phases which exploit the high binding affinity of reboxetine to this plasma component [17]. The older chiral separation techniques utilized non-volatile mobile phase buffers incompatible with the mass spectrometric detection

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doi:10.1016/j.jpba.2008.10.033

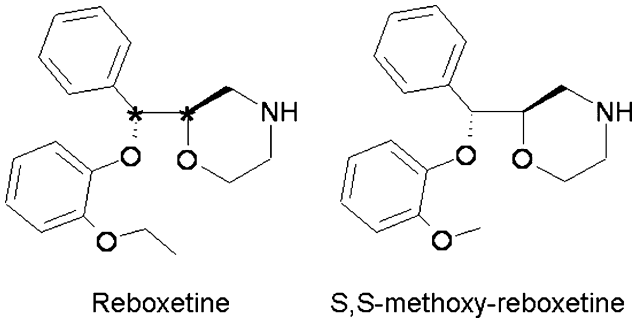

Fig. 1. The structure of reboxetine (*chiral centre) and internal standard S,S-methoxy-reboxetine.

which is the predominant technique employed for pharmaceutical bioanalysis today. One method used mass spectrometry to quantify both reboxetine enantiomers [15]; however the limit of detection achieved was approximately 20 ng/ml, insufficient for our investigation. The intention therefore in developing an alternate method was to utilize and optimise mass spectrometric detection operated with an electrospray ionisation (ESI) source, anticipated to offer the best chance of improving sensitivity [18].

2. Experimental

2.1. Chemicals and reagents

Racemic reboxetine (RBX) and pure enantiomer S,S-RBX methanesulphonate salts (m.w. 409.5) and internal standard (ISTD) S,S-methoxy-RBX hydrochloride salt (m.w. 335.8) were supplied by Pfizer Global R&D, Sandwich laboratories, Kent, UK (analytical grade). All concentrations expressed are given as free base equivalents. Analytical reagents were HPLC grade or better (Sigma–Aldrich Ltd, Dorset, UK) and water was obtained from a Milli-Q station. These were used without further purification. Control plasma was obtained from Charles River, UK and control brain tissue in-house.

2.2. Instrumentation

The HPLC system used was an ARIA TX2 platform (Thermofisher, Hemel Hempstead, UK) comprised of a Hewlett Packard 1100 series binary pump that enabled isocratic flow of mobile phase or column washing media and a further 1100 series quaternary pump (operated using a single channel and connected through a T-piece) that enabled a secondary flow of organic solvent to join, prior to entering the mass spectrometer. A post-column flow splitting setup and bypass switching valve were also positioned prior to the secondary flow which enabled the total volume and composition of eluant entering the mass spectrometer (MS) to be controlled (Fig. 2). An API 4000^{TM} Triple Quadrupole MS (Applied Biosystems/MDS Sciex, Canada) was utilized to monitor the analytes.

2.3. Stock solutions, standards and quality controls

Stock solutions of S,S-RBX, racemic-RBX and ISTD were prepared in DMSO at 1 mg/ml and stored in the fridge at 4 °C and thawed before use. Two separate racemate stock solutions were prepared; one for the preparation of calibration curve standards and the second for the preparation of quality control (QC) samples. Working solutions containing RBX racemate at 2, 0.2, 0.02 and 0.002 μg/ml in methanol–water (50:50, v/v) were obtained by serial dilution. S,S-RBX was used to confirm column retention times and equimolar concentrations of both S,S & R,R enantiomers were present in the racemate solution for quantitative purposes.

2.4. Sample extraction procedure and chromatographic conditions

Sample clean-up was achieved using an automated liquid–liquid extraction technique (Hamilton Microlab Star, Bonaduz, Switzerland). 100 μl sample matrix and 10 μl of 0.5 μg/ml ISTD solution were mixed with 300 μl borate buffer (pH 10; 1 M) and extracted into 1 ml *tert*-butyl methyl ether solvent. The mixture was centrifuged at 4 °C (3000 × *g*) for 10 min and 0.85 ml of the supernatant was removed. This process was repeated a second time to maximise extraction recovery and the extract was evaporated dryness under a stream of nitrogen at 40 °C. Plasma samples were defrosted and extracted the same day prior to analysis. Brain homogenate samples were provided diluted (1:14, brain:water, w/v), defrosted and vortexed before use. Blank control matrix was prepared with a Polytron PT3000 homogenisation machine (Kinematica, Newark, US) and treated in the same manner as the samples. Extracted sample residues were reconstituted in 125 μl of mobile phase A and 70 μl was injected onto the system.

Chromatographic separation was achieved using a Chrom Tech Chiral-AGP column (150 mm × 4.0 mm, 5 μm; Cheshire, UK) with an inline frit (2 μm) thermostated to a generic analytical temperature of 38 °C with a Hotsleeve column heater. Mobile phase A was a mixture of water and acetonitrile (85:15, v/v) buffered with 12.5 mM ammonium carbonate and adjusted to pH 6.7 with formic acid solution. It was pumped isocratically at 1.2 ml/min for 15 min. Mobile phase B was 0.05% acetic acid (pH 4.0) and utilized to wash the column for 6 min after each separation; a further 2 min re-equilibration of mobile phase A was then applied. The post-column flow of organic modifier was acetonitrile–isopropanol (50:50, v/v) with 0.15% formic acid (pH 3.8) pumped at 0.4 ml/min during the 15 min MS acquisition period. All flow during the washing and re-equilibration steps was directed to waste and the total cycle time for each sample was 23 min.

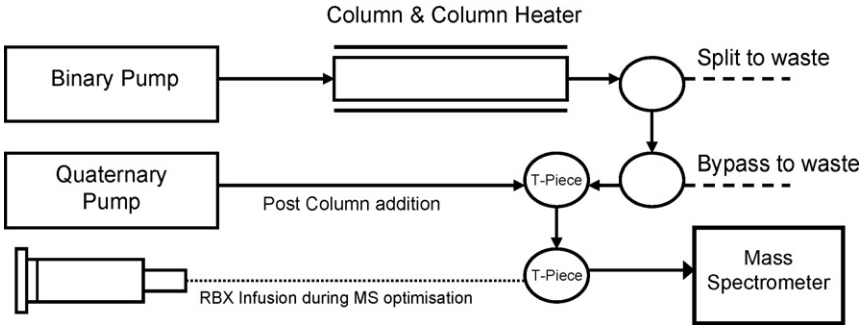

Fig. 2. Schematic of the LC-setup.

2.5. Mass spectrometry: optimised conditions

The API4000™ MS was operated in positive ion mode with a TurboIonspray interface (ESI) heated to 700 °C and performed using multiple reaction monitoring (MRM scan, dwell time 600 ms) operated with unit Q1 resolution and low Q3 resolution. Detection conditions applied for the RBX ions (m/z 314 > 175.8) and S,S-methoxy-RBX ions (m/z 300 > 175.8) were a collision energy 17 eV, ionspray voltage 4500 kV, declustering potential 61 V with nitrogen as the collision gas and curtain gas (set at 6 and 20 units, respectively).

The final composite flow rate entering the ion source was 1.3 ml/min, consisting of 0.9 ml/min column eluate (split 3:1) and 0.4 ml organic modifier (see above). MS parameters were optimised by altering the post-column flow conditions (organic flow rate, composition and pH) with 10 ng/ml RBX solution infused at 15 μl/min, through a T-piece (Fig. 2).

2.6. Data analysis, assay characterisation and stability

Data was collected and integrated using Analyst v1.4.1 software (Applied Biosystems/MDS Sciex, Canada) and stored in Watson 7.2 database (Thermofisher, Hemel Hempstead, UK). Eleven calibration standards were prepared at 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 ng/ml and the calibration curve plotted the peak area ratio (drug/ISTD) versus the concentration in ng/ml, using a least squares linear regression model, weighted $1/y^2$. Imprecision and inaccuracy were assessed by performing replicate analysis of QC's at four levels (LLOQ, 0.5, 5 and 50 ng/ml, $n = 6$). The procedure was repeated on three different days for the plasma matrix ($n = 3$) to determine inter-day accuracy and precision values and the same method was applied to assay brain homogenate in a single batch ($n = 1$). Reported parameters used the back calculated concentrations of standards and QCs against the nominal spiked concentration.

The stability of both RBX enantiomers in rat plasma and brain homogenate was evaluated by comparing low and high QC concentrations (0.25 and 200 ng/ml, $n = 3$) prepared on the day of analysis versus samples stored at −20 °C for 24 h, and long-term storage of 3 months undergoing three freeze/thaw cycles ($n = 3$). The conditions adequately covered the storage conditions of the study samples analysed. Equivalent stability checks were also prepared for single enantiomer S,S-RBX (10 ng/ml, $n = 3$, measuring also the R,R-form) to assess whether enantiomeric conversion occurred with either the racemate drug dosed to animals, during storage or in preparation of the assay standards.

3. Results and discussion

3.1. Chromatographic performance

The principal aims during the method development were to achieve a rapid enantiomeric separation, commensurate with a reverse phase HPLC setup (resolution factor ≥1.3 within 15 min) and maintain peak shape over multiple injections. Initial assessment of chirobiotic- (teicoplanin and vancomycin) and cellulose (Diacel OJ)-based stationary phases showed poor selectivity for chiral recognition of RBX enantiomers. Öhman et al. [15] demonstrated a reverse phase method using the chiral-AGP stationary phase (dynamic range 20–200 ng/ml), however some limitations

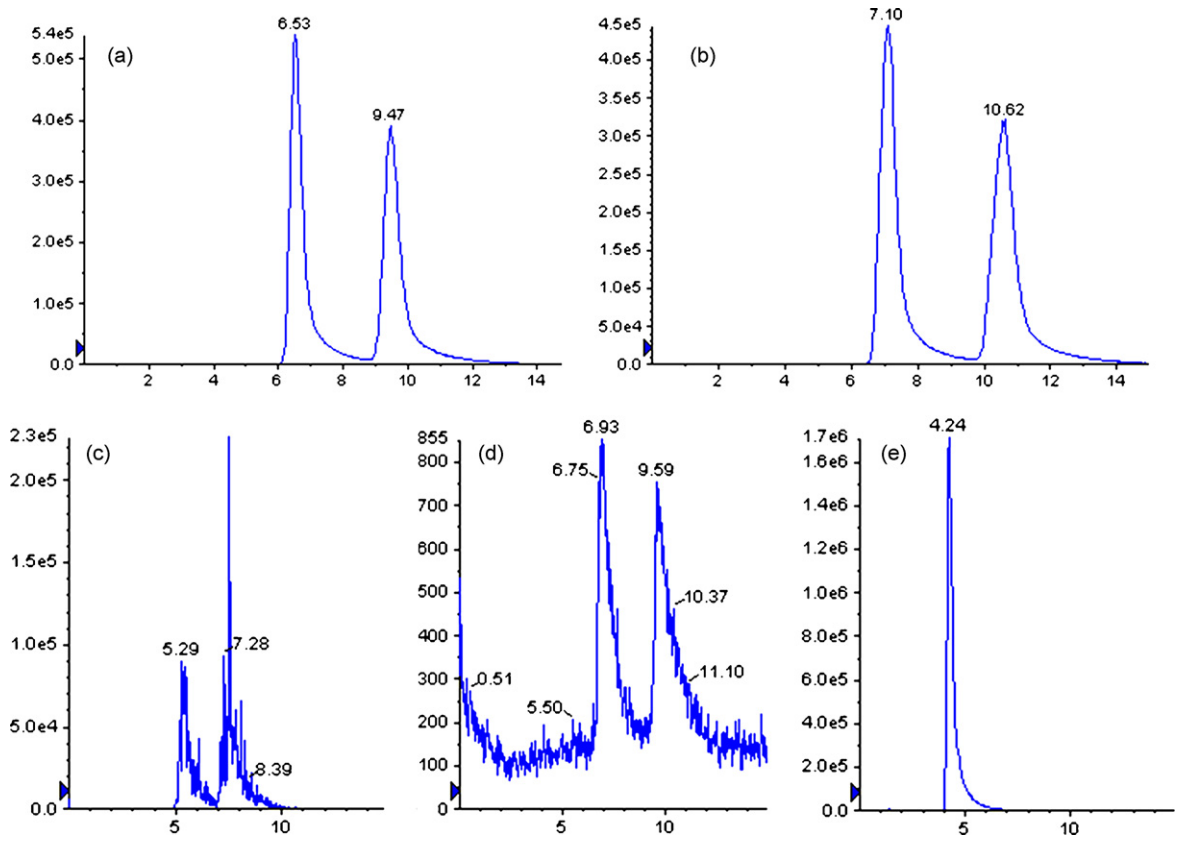

Fig. 3. MRM scans for RBX enantiomers (m/z 314 > 175.8) and ISTD S,S-methoxy-reboxetine (m/z 300 > 175.8) eluted in the order ISTD, S,S-RBX, R,R-RBX; obtained from (a) rat plasma 50 ng/ml sample injection no. 12, (b) rat plasma 50 ng/ml sample injection no. 40, (c) 50 ng/ml equivalent injection performed without post-column modifying agent, (d) rat plasma 0.05 ng/ml LLOQ and (e) ISTD 50 ng/ml. For chromatographic conditions see text.

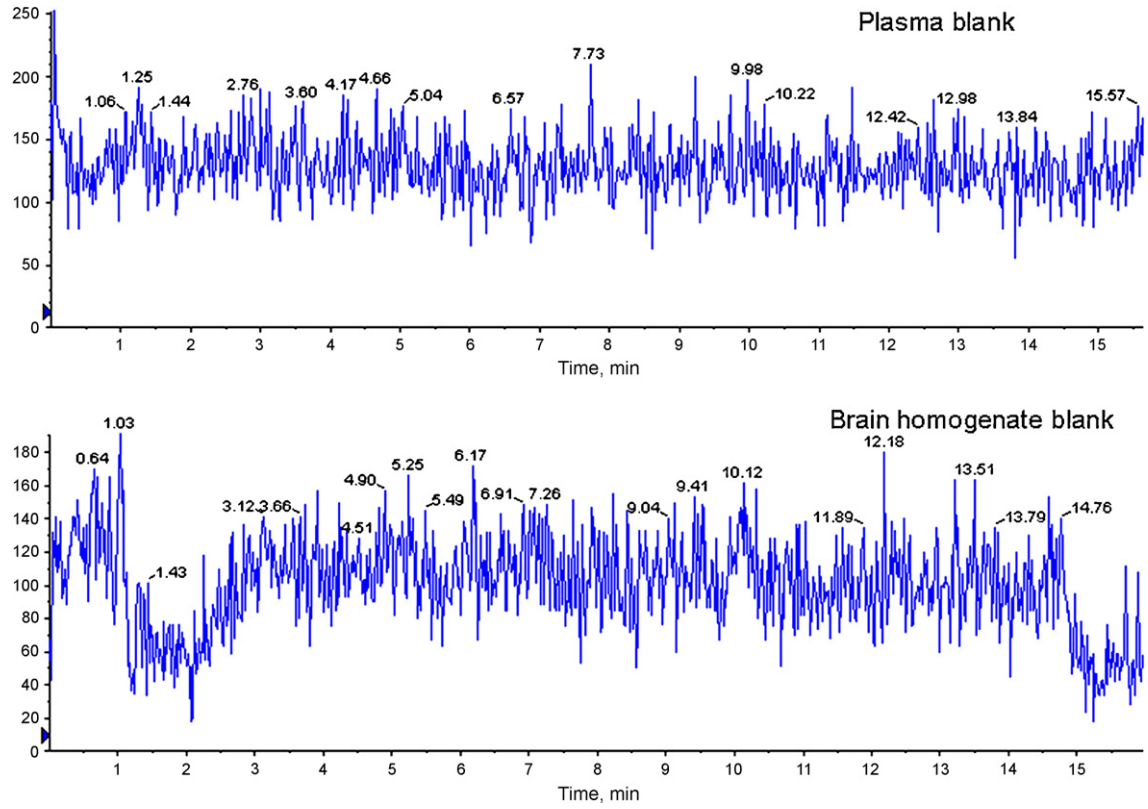

Fig. 4. Representative chromatogram of blank rat plasma and brain homogenate.

of this assay were a gradual lowering of peak intensity and robustness over the course of a batch of 20–40 samples and no internal standard was present to monitor systematic variability within the assay. Following the evaluation of different mobile phase buffers we determined ammonium carbonate (buffering range pH 6–9) produced the smoothest peak shape and largest MS response for both enantiomers and internal standard, which were eluted in the order S,S-methoxy-RBX (R_T 4.2–4.7 min), S,S-RBX (R_T 6.5–7.5 min) and R,R-RBX (R_T 9.5–11 min). A small increase in mobile phase pH (for example pH 6.6–6.8) significantly increased the column retention and separation power (resolution $\gg$2.0), but band broadening became the limiting factor as it produced a smaller signal to noise ratio. Therefore, the optimal mobile phase pH occurred at pH 6.7 where both peak resolution and sharpness were suitable for a sensitive assay. This is shown in Fig. 3(a) and (b) where through the course of an analytical batch, the slight variations in pH contributed to changes in signal to noise (12% decrease in response versus an increase in resolution from 1.5 to 1.8). Due to the sensitivity of chromatography pH, it was necessary to adjust the mobile phase pH on the day of use. However, the overall relative standard deviation (R.S.D.) of retention times for both enantiomers in any single analytical run performed never exceeded 5%.

The isocratic mobile phase chosen for the chiral separation of reboxetine was not sufficient to elute all other matrix components recovered in the sample extraction procedure, apparent through the gradual loss of chromatographic performance (peak shape, retention time) after approximately 15–20 injections [15]. Therefore, an extra column washing step was incorporated in the HPLC method using 0.05% acetic acid in water over 6 min to remove residual matrix material and this enabled >80 sequential injections to be performed in a single batch without any alteration in chromatography. Several analytical runs were performed altogether using the same column, totalling >250 injections. Acetic acid has been used as an additive in chiral-AGP HPLC separations previously [19,20] but in this assay incorporating acetic acid to adjust eluting mobile phase pH (pH 6.7) was not as effective as using a separate aqueous wash (pH 4) to desorb matrix components from the α1-acid glycoprotein stationary phase.

3.2. Assay selectivity, extraction recovery and sample stability

The analysis of blank matrices from plasma and brain homogenate showed no evidence of interfering endogenous components at the retention times of the two enantiomers (Fig. 4) and ISTD (not shown). Both extraction recovery and matrix suppression of the MS response were assessed by comparing neat injected drug solution to three batches of plasma and one batch of brain homogenate, spiked pre- and post the sample extraction procedure (n = 3 injections, 0.5 and 50 ng/ml). Mean recovery of RBX ranged from 82 ± 6 to 92 ± 5% in plasma and 98 ± 5% in brain homogenate. Blank extract (both plasma and brain homogenate) spiked with an equivalent drug concentration provided a signal response 87–107% of the maximum response possible with neat injection of RBX in mobile phase alone (R.S.D. of 7%), indicating matrix effects (signal suppression) was minimal. Similarly, the recovery of ISTD (S,S-methoxy-RBX) was 85% and matrix suppression was equivalent with a mean reponse 96 ± 13% of the maximum.

Following stability measurements for samples stored at −20 °C for 24 h and for 3 months undergoing three freeze/thaw cycles, it was determined no statistical decrease in nominal concentration appeared by comparison with the reference values, for either matrix used. Mean recoveries for both S,S- and R,R-RBX ranged from 85 to 106% and were within the variability of the assay. The results corroborate previous stability investigation during chiral analysis of RBX [10] where spiked serum samples stored in plastic tubes at −20 °C were found stable for 5 months.

A second stability feature that needed addressing when using racemic RBX to dose the animals and also prepare the assay standards, was ensuring no enantiomeric conversion occurred between S,S- and R,R-RBX forms, either in solution or during the sample extraction process. Unfortunately our laboratories were unable to source any R,R-RBX; however, equivalent storage conditions as described above with the stability samples were applied to single enantiomer S,S-RBX diluted in solution and spiked into plasma and brain homogenate (10 ng/ml, $n = 3$). No enantiomeric conversion was detected after both short-term (24 h) and long-term (3 month) storage. Moreover, the ratio of S,S- to R,R-RBX concentrations measured in the stability samples spiked with racemate, did not alter significantly from a value of 1.

3.3. Optimising MS response

Through the course of the method development process, it became apparent a suitable mobile phase for the α_1-acid-AGP column would contain a low percentage of organic solvent and have a neutral pH (~7), not optimally suited to electrospray ionisation with positive MS polarity. Water has a high solvation energy making it more difficult to desolvate than organic solvents like acetonitrile [21]. Therefore, optimisation of the MS response was achieved by post-column addition of organic modifier to mobile phase entering the ion source, and the effects were measured during the tuning process by altering the organic flow and simultaneously infusing RBX at a low flow of 15 μl/min (Figs. 2 and 5).

Sensitivity increased 2-fold when increasing volumes of organic solvent were added, peaking at a flow rate of 0.4 ml/min before dropping off at higher flows. The momentary drops in response occurred when the pump flow rate was adjusted. Similar mobile phase composition experiments with morphine [22], which is a polar neutral analyte, have shown a higher organic content during ESI does increase sensitivity and this is related to the increased volatility of the mobile phase [21]. RBX in contrast, is a relatively lipophilic and basic analyte ($\log D$ $pH_{7.4}$ of 1.7 and pK_a of the morpholine moiety 8.3) so the addition of acid would also be expected to increase the tendency for ionisation in positive ion mode with more available protons. The actual improvement measured was a mean response increase of 47% when 0.1% formic acid was included (Fig. 5); however, subsequent increases in acid content did not significantly increase the response further. The final composite flow conditions used were 0.9 ml/min eluting mobile phase plus 0.4 ml/min organic modifier containing 0.15% formic acid and resulted in an adjustment of the organic content from 15 to 41% and change in pH from 6.7 to 4.7. The change improved sensitivity approximately 4–5-fold from the basal level of organic flow applied (0.1 ml/min) but most importantly, at least some organic modifier was necessary to maintain a consistent response in the MS because the complete absence of organic modifier running at high aqueous flow into the ion source (≥0.9 ml/min) produced an extremely varied analyte response and made peak integration difficult. This is shown in Fig. 3(c) compared with optimised conditions for (a), (b) and (d). Sciex API4000 turbo-V ionisation sources are normally capable of vaporising flows of 1–2 ml/min provided sufficient organic content is present. In this experiment, including the modifier meant only a small portion of column flow (25%) needed to be directed to waste and thus MS detection could be maximised. Directing all column flow (1.2 ml/min) to the MS generated peaks similar to that shown in Fig. 3(c), which needed to be offset with a much larger volume of organic modifier, undesirable for ensuring complete vaporisation or cleanliness and maintenance of the MS.

3.4. Accuracy, precision and lower limit of quantitation (LLOQ)

The assay was linear over a 2000-fold dynamic range (0.05–100 ng/ml) in both plasma and brain homogenate and the quantitation accuracy and precision for both enantiomers was shown to be equivalent. Mean calibration line parameters found were as follows: $Y = 0.0202X + 0.000105$ ($r^2 = >0.993$), where Y equals the internal ratio (analyte/ISTD) of the peak area response and X equals the concentration spiked. Overall, the imprecision and inaccuracy for the assays performed were within ±15% for both intra- and inter-day analysis, shown by the QC results in Table 1, comparable with previously reported methods [6,10,15]. The data was therefore acceptable for discovery bioanalysis conducted in non-GLP regulated conditions and offered a sensitivity improvement ≥20-fold. Inter-day inaccuracy (% error) for each of the four QC levels ranged from 1 to 6% and additionally inter-day inaccuracy (% error) for each of the 11 calibration standards ranged from 0.4 to 10% (not shown).

The matrices used in the method (rat plasma or brain) did not affect the assay performance. The LLOQ of the assay was chosen by the peak to noise ratio present in the low calibration range (greater than three times background noise, Fig. 3d). In all chromatograms the R,R-RBX peak was marginally smaller than S,S-enantiomer but were clearly definable against the background noise and enabled accurate quantitation. No measurable peaks were identified in

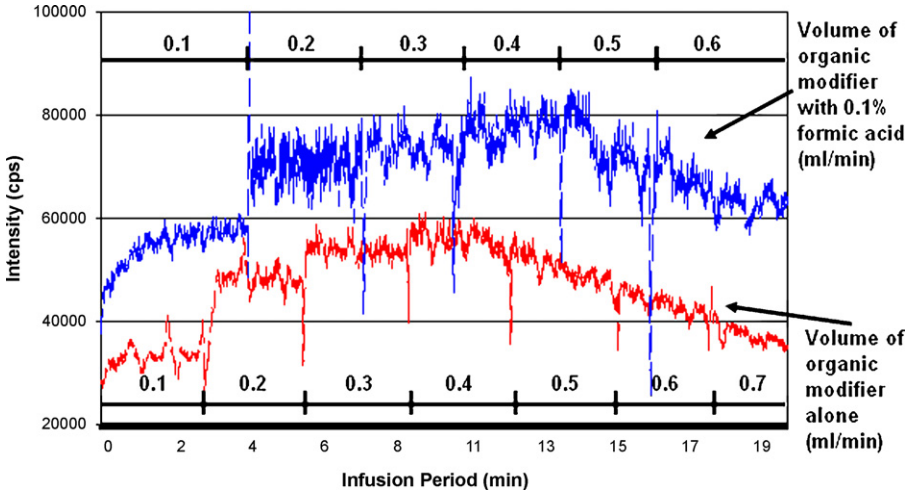

Fig. 5. Change in MS response when organic composition of mobile phase is increased (10 ng/ml reboxetine infusion).

Table 1
Imprecision and inaccuracy data in rat plasma and brain homogenate for S,S- and R,R-reboxetine.

Batch number	Spiked concentration (ng/ml)	Measured concentration S,S-reboxetine ($n=6$)		Imprecision (% R.S.D.)	Inaccuracy (% error)	Measured concentration R,R-reboxetine ($n=6$)		Imprecision (% R.S.D.)	Inaccuracy (% error)
		Mean (ng/ml)	(±S.D.)			Mean (ng/ml)	(±S.D.)		
1 (plasma)	0.05	0.0470	0.00171	3.6	−6.4	0.0496	0.00361	7.3	−0.8
	0.5	0.472	0.0264	5.6	−5.9	0.466	0.0375	8.0	−7.3
	5	4.90	0.243	5.0	−2.0	4.98	0.339	6.8	−0.4
	50	47.9	1.380	2.9	−4.4	47.4	1.66	3.5	−5.5
2 (plasma)	0.05	0.0457	0.00370	8.1	−9.4	0.0488	0.00538	11.0	−2.5
	0.5	0.475	0.0426	9.0	−5.3	0.450	0.0398	8.8	−11.1
	5	5.02	0.342	6.8	0.4	4.76	0.363	7.6	−5.0
	50	46.8	1.89	4.0	−6.8	45	3.82	8.5	−11.1
3 (plasma)	0.05	0.0559	0.00235	4.2	10.6	0.054	0.00725	13.4	7.4
	0.5	0.510	0.0301	5.9	2.0	0.509	0.0360	7.1	1.8
	5	4.92	0.109	2.2	−1.6	4.88	0.221	4.5	−2.5
	50	48.3	1.62	3.4	−3.5	50.4	1.86	3.7	0.8
4 (brain)	0.05	0.0547	0.00202	3.7	8.6	0.0521	0.00382	7.3	4.0
	0.5	0.499	0.0161	3.2	−0.2	0.497	0.0114	2.3	−0.6
	5	4.76	0.129	2.7	−5.0	4.71	0.128	2.7	−6.2
	50	46.6	0.599	1.3	−7.3	45.7	1.38	3.0	−9.4
Overall	0.05	0.0508	0.00245	4.9	0.8	0.0511	0.00502	9.8	2.2
	0.5	0.489	0.0288	5.9	−2.4	0.481	0.0312	6.6	−4.1
	5	4.90	0.206	4.2	−2.1	4.83	0.263	5.4	−3.5
	50	47.4	1.37	2.9	−5.5	47.1	2.18	4.7	−6.1

blank samples following injection of the top calibration standard and therefore carryover did not compromise the LLOQ or dynamic range of the assay.

3.5. Pharmacokinetic samples in the rat

The pharmacokinetic analyses in rat were performed to support ex vivo receptor occupancy investigations (the concentration–time effect relationship) of RBX dosed as a racemate to individual animals. Semi-logarithmic plots of the mean (±S.D.) RBX plasma and brain homogenate concentration–time profiles are illustrated in Fig. 6, showing separate enantiomer profiles.

The concentration results demonstrate a small change in the S,S/R,R-enantiomeric ratio occurred in rat plasma and no change within brain tissue following subcutaneous (SC) administration up to 16 h post-dose (approximate ratios, 0.8 [plasma], 1.0 [brain]). Also observable was the measured whole brain concentrations (ng/g, adjusted for the dilution factor in homogenisation) were on average 3–4-fold higher than plasma samples. This possibly occurs as a consequence of more extensive distribution and protein binding of RBX in this compartment. The findings are markedly different to the stereoselective pharmacokinetics demonstrated previously in rat and humans following oral administration of racemate [7,15] where enantiomeric ratios were in the range of 0.2–0.8 (higher R,R-RBX concentrations) and S,S-RBX was reported to exhibit the most potent pharmacological activity. However the findings described here are supported by similar in vivo investigations where RBX enantiomers and racemate were administered subcutaneously, stating no difference in biological effect could be seen [2]. One possible explanation for this disparity is the significant influence of enantiospecific absorption and first pass metabolism characteristics following oral reboxetine administration, versus the more simple distribution and elimination characteristics associated with direct subcutaneous dosing. The results in Fig. 6 show the rapid elimination of RBX from rat plasma and brain compartments concur with its high in vivo clearance [1]. The sensitivity of the assay enabled brain concentrations (the particular compartment of interest in this study) to be monitored out to 16 h despite the significant dilutions samples were subject to and showed the relative levels of S,S- and R,R-RBX did not change during this period.

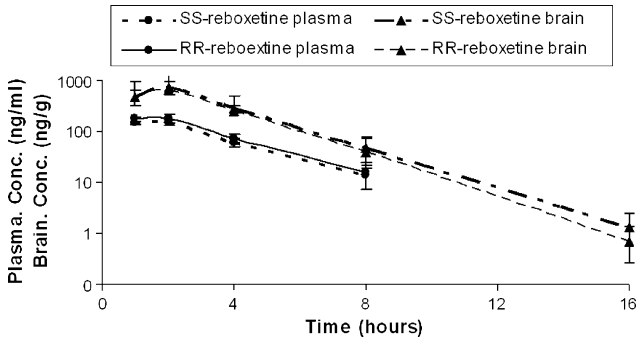

Fig. 6. Mean (±S.D.) plasma and brain homogenate concentrations vs. time for S,S-RBX (- - -) and R,R-RBX (—) following subcutaneous administration of racemate drug (10 mg/kg) in rat ($n=4$ per time point).

4. Conclusion

Mass spectrometry can offer a significant advantage in both analyte detection sensitivity and specificity over other detector systems and nowadays is being used increasingly in the drug discovery environment to monitor stereospecific drug kinetics. In the present study, a highly sensitive quantitative method for the analysis of RBX enantiomers in rat plasma and brain homogenate is described, that used a rapid chiral reverse phase separation, optimised for ESI-MS with post-column addition of organic modifier to enhance ionisation efficiency. The method shows significantly improved sensitivity and quantitation limits (LLOQ 0.05 ng/ml) over previously described methods and good analytical robustness. A suitable method characterisation was performed for pharmaceutical analysis and enabled the extended quantitation of pharmacokinetic plasma and brain samples in rat following SC dose administration. The sample clean-up steps were automated and the cycle time for analysis relatively short; therefore the method could be applied in

a clinical setting (given appropriate validation with human matrices) where a sensitive characterisation of stereoselective reboxetine kinetics is required.

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