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Improved enantioselective method for the determination of the enantiomers of reboxetine in plasma by solid-phase extraction, chiral derivatization, and column-switching high-performance liquid chromatography with fluorescence detection

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Abstract

A rapid enantioselective method is described for the quantitation of the reboxetine (*R*,*R*)- and (*S*,*S*)-enantiomers in plasma utilizing solid-phase extraction, derivatization, normal-phase high-performance liquid chromatography, and fluorescence detection. Plasma samples (0.1 ml) with added internal standard were applied to activated solid-phase extraction discs containing a nonpolar/strong cation mixed-phase, washed, eluted, evaporated to dryness, and derivatized for 5 min with (+)-1-(9-fluorenyl)ethyl chloroformate. After termination of the derivatization reaction, the samples were analyzed by isocratic normal-phase HPLC using a silica column and ethanol–heptane (1:124, v/v) as mobile phase. The derivatized reboxetine peak was column-switched onto cyano and Chiralcel OD-H columns in series using ethanol–heptane (1:49, v/v) as mobile phase to resolve the diastereomeric derivatives of the enantiomers and separate interferences. The column effluent was monitored with fluorescence detection at 260/315 nm. The range of quantitation of each enantiomer was 2–2000 ng/ml. One sample was injected every 18 min.

Keywords: Enantiomer separation; Column switching; Reboxetine

1. Introduction

Reboxetine (Fig. 1) is a noradrenaline reuptake inhibitor used for the treatment of depressive illness [1]. Since the drug, containing two chiral centers, is marketed as a racemic mixture of (*R*,*R*)- and (*S*,*S*)-enantiomers, it was necessary to monitor plasma concentrations of the individual enantiomers in pre-clinical safety studies [2]. The enantiomers have been resolved by reversed-phase chromatography following derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate [(+)-FLEC] [3]. Al-

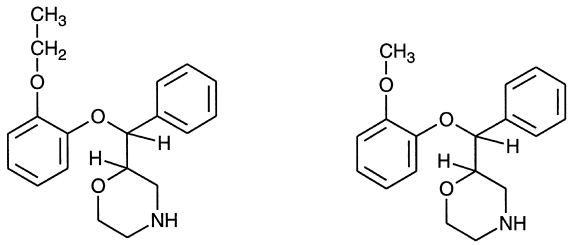

Fig. 1. Chemical structures of reboxetine and PNU-154176 (internal standard). The salt moieties are not shown.

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though the assay was quite sensitive (1 ng/ml limit of quantitation using 1 ml of plasma), the separation required 90 min and multiple liquid–liquid extraction steps. It also lacked an internal standard.

The improved procedure described in this report utilizes a single solid-phase extraction (SPE) step, derivatization with (+)-FLEC, and normal-phase chromatography using a column-switching system containing a silica column, a cyano column and a Chiralcel OD-H column. It also incorporates the use of an analog of reboxetine as internal standard.

2. Experimental

2.1. Chemicals and reagents

Reboxetine {*rac*-2-[(2-ethoxyphenoxy)phenylmethyl]morpholine}, and its enantiomers, (+)-(*S*,*S*)-reboxetine and (−)-(*R*,*R*)-reboxetine, were prepared at Pharmacia and Upjohn as methanesulfonate salts (formula mass 409.51). All concentrations are reported in free base equivalents (formula mass 313.40). The internal standard, PNU-154176A (Fig. 1), also prepared at Pharmacia and Upjohn, was a hydrochloride salt (formula masses of salt and free base were 335.83 and 299.37, respectively). The derivatizing agent, (+)-FLEC, was received from Aldrich (Milwaukee, WI, USA) as an 18 m*M* solution in acetone, which was diluted with acetone to 0.05 m*M* for use in this assay. Blank dog plasma was from Buckshire (Perkasie, PA, USA) and contained EDTA as anticoagulant.

Mobile phases A and B were ethanol–heptane mixtures, (1:124, v/v) and (1:49, v/v), respectively. An acetonitrile–ammonium acetate (30:70, v/v) buffer was prepared from acetonitrile and 0.01 *M* ammonium acetate. A sodium phosphate–triethylamine (TEA) buffer, pH 7.0, contained 0.05 *M* NaH_2PO_4 and 1% (v/v) TEA, with the pH adjusted to 7.0 with sodium hydroxide.

2.2. Solid-phase extraction instrumentation

SPEC PLUS 3ML MP1 columns with 15 mg of sorbent were from Ansys (Irvine, CA, USA) and contained a nonpolar/strong cation mixed phase in a disc format. The extraction procedure was performed using a Visiprep vacuum manifold (Supelco, Bellefonte, PA, USA). An evaporator (TurboVap LV, Zymark) was used to dry the collected eluates and to terminate the derivatization reaction.

2.3. Chromatographic instrumentation

The chromatographic system is described in Fig. 2. The system operated at ambient temperature (22–24°C). All samples were injected using a full loop injection volume of 100 μl, followed by a needle wash with 100 μl of ethanol–heptane (10:90, v/v). One sample was injected every 18 min. The samples in the autosampler were also maintained at ambient temperature (22–24°C). The autosampler was equipped with an extra valve that was programmed to column-switch the drug peak from 6.5–8 min after each injection. Data acquisition was initiated at 6.5 min after sample injection; data were collected from both fluorescence detectors using a dual channel data acquisition system (PE-Nelson 900 Series interface, Norwalk, CT, USA, connected to a Concurrent

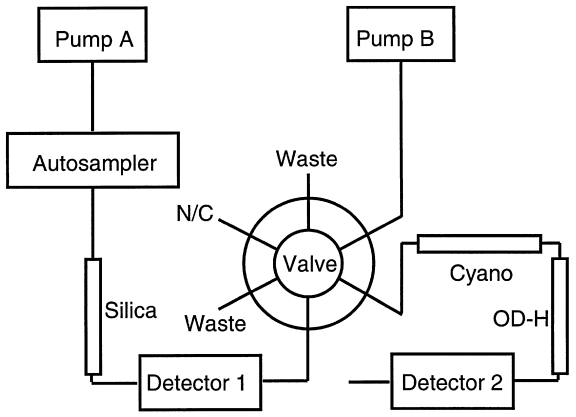

Fig. 2. Diagram of the column-switching system. N/C=Not connected. Pump A (1.0 ml/min): Beckman 127S (Fullerton, CA, USA). Pump B (1.5 ml/min): Beckman 114M. Autosampler (100 μl loop) with column-switching valve: Beckman 507e. Silica column: Spherisorb 250×4.6 mm, 3 μm (Metachem, Redando Beach, CA, USA). Cyano column: Zorbax CN, 250×4.6 mm, 5 μm (MacMod, Chadds Ford, PA, USA). OD-H column: Diacel Chiralcel OD-H, 150×4.6 mm, 5 μm (Chiral Technologies, Exton, PA, USA). Detectors 1 and 2 (260 nm excitation, 315 nm emission): Waters 470 with 5 μl and 16 μl flowcells, respectively (Milford, MA, USA).

Computer Corp. Nighthawk 5800 series computer, Fort Lauderdale, FL, USA).

2.4. Standard solutions and quality controls

A stock standard solution was prepared with racemic reboxetine methanesulfonate in the acetonitrile–ammonium acetate buffer at a concentration of 261 μg/ml, or 200 μg/ml of the free base (100 μg/ml free base of each enantiomer). Hereafter, all concentrations are reported in free base equivalents. The stock solution was serially diluted with acetonitrile–ammonium acetate buffer to prepare 12 working standard solutions with enantiomer concentrations ranging from 2–10 000 ng/ml. Stock and working standard solutions were stored at 2–5°C.

Quality control stock solutions were similarly prepared from the individual enantiomers at concentrations of 50.0 μg/ml. Each was diluted in acetonitrile–ammonium acetate buffer to prepare a 4000 ng/ml control stock. Preparation of quality control samples in dog plasma is detailed in Table 1. Some of the controls were prepared with unequal concentrations of the two enantiomers so that the chromatographic peaks could be definitively assigned. The quality control stock solutions were stored at 2–5°C. The quality control plasma samples were stored at ≤−10°C.

The internal standard stock solution containing PNU-154176A was prepared in acetonitrile–ammonium acetate buffer at a concentration of 100 μg/ml free base equivalents. The stock solution was diluted with acetonitrile–ammonium acetate buffer to prepare a working internal standard solution at a concentration of 5000 ng/ml. Both the stock and working internal standard solutions were stored at 2–5°C.

2.5. Sample preparation

Plasma unknowns or quality control samples (100 μl) were transferred to 75×12 mm disposable glass test tubes containing 100 μl of internal standard solution and 100 μl of acetonitrile–ammonium acetate buffer. Standard samples were prepared by adding 100 μl of the appropriate standard solution to a 75×12 mm disposable glass test tube containing 100 μl of internal standard and 100 μl of blank dog plasma. Standards, controls and unknowns were diluted with 1 ml of sodium phosphate–TEA buffer and vortexed to mix. SPE columns were activated by sequential washings with 0.3 ml of acetone–acetonitrile–TEA (50:50:0.2, v/v/v) and 1 ml of sodium phosphate–TEA buffer. The prepared samples were applied to the activated SPE columns and allowed to drain to the top of the sorbent bed by gravity. Full vacuum was then applied to remove the final bed volume. Next, the SPE columns were washed with one column volume of water (approximately 3.75 ml), drained under maximum vacuum, washed with 0.5 ml of 1 *M* acetic acid, washed with 0.5 ml of methanol, dried under maximum vacuum for 1 min, washed with 0.3 ml heptane, and dried under maximum vacuum for 3 min. The SPE columns were eluted by gravity with 0.3 ml of acetone–acetonitrile–TEA (50:50:0.2, v/v/v) into clean 75×12 mm disposable glass test tubes. Positive pressure was used to remove the final bed volume. The samples were transferred to an evaporator and evaporated to dryness at room temperature for approximately 10

Table 1
Preparation of quality control samples

Control name	Preparation method[a]	Concentration (*S,S*)-/(*R,R*)-reboxetine (ng/ml)
C1	Dilute 25 μl of the 4000 ng/ml (*S,S*)-reboxetine solution and 250 μl of the 4000 ng/ml (*R,R*)-reboxetine solution to 10 ml	10/100
C2	Dilute 250 μl of the 4000 ng/ml (*S,S*)-reboxetine solution and 25 μl of the 4000 ng/ml (*R,R*)-reboxetine solution to 10 ml	100/10
C3	Dilute 200 μl of the 50 μg/ml (*S,S*)-reboxetine solution and 200 μl of the 50 μg/ml (*R,R*)-reboxetine solution to 10 ml	1000/1000

[a] Diluent is pooled blank dog plasma with EDTA anticoagulant.

min under nitrogen flow. To derivatize the samples, 50 μl of 0.05 m*M* (+)-FLEC in acetone was added to the sample tubes, which were then placed in a heated block (Vortex-Evaporator, Searle, Fort Lee, NJ, USA) where they were incubated at 30°C for 5 min. To terminate the derivatization reaction, the samples were transferred back to the evaporator and evaporated to dryness under nitrogen flow at room temperature for approximately 5 min. The dried eluate was reconstituted in 0.3 ml of heptane, vortex mixed, transferred to autosampler vials, capped, and placed in the high-performance liquid chromatography (HPLC) autosampler tray. Chromatographic conditions are outlined in Section 2.3.

2.6. Quantitative determination

Separate calibration curves were generated using the peak height ratios of the derivatized (*R,R*)- or (*S,S*)-reboxetine enantiomer peak from the second detector relative to the derivatized internal standard peak from the first detector. Regression analysis was utilized to fit the calibration data, which was weighted by 1/concentration, to a straight line with intercept. The concentrations of the enantiomers in the unknown samples were obtained by back-calculation from the calibration curves, and are reported as ng/ml in free base equivalents.

3. Results and discussion

Since an assay for reboxetine enantiomers in plasma based on derivatization with (+)-FLEC has already been reported [3], the challenge was to develop a method faster than the previously reported 90 min separation which could be routinely applied to a large number of samples. The original method also lacked an internal standard, which we wanted to include so that the assay would be more robust.

Although reversed-phase separations of diastereomers formed by derivatization with (+)-FLEC have been successfully reported for a number of compounds [4–7], our method development efforts indicated that reversed-phase conditions yielded poor selectivity for separation of the two reboxetine diastereomer peaks. We then tried normal-phase conditions, which sometimes improve the separation of isomers, but also found the selectivity to be relatively poor on silica or cyano columns. Finally, we applied a cellulose tris-3,5-dimethylphenyl carbamate chiral column (Chiralcel OD-H) and found it to have very good selectivity for the two diastereomers. However, (+)-FLEC is a relatively non-specific derivatizing agent, and elimination of interferences proved to be a major problem. We applied column-switching to solve this problem. A silica column yielded an excellent separation of derivatized reboxetine from derivatized internal standard, and we then column-switched the derivatized reboxetine peak onto the Chiralcel OD-H column to separate the diastereomers. Still, some matrix and/or (+)-FLEC components interfered with the analysis, so we added a cyano column in series with the Chiralcel OD-H column to shift the drug peaks away from the interferences. We also were able to decrease the amount of (+)-FLEC used in the assay nearly 30-fold [from 0.2 ml of 0.36 m*M* (+)-FLEC in the original assay [3] to 0.05 ml of 0.05 m*M* (+)-FLEC] because the derivatization takes place in a nonaqueous environment. In the final procedure reported here, we nevertheless observed small background peaks at the retention times of the derivatized reboxetine enantiomers; these limited the lower limit of quantitation of the assay to about 2 ng/ml for each enantiomer.

3.1. Specificity

Derivatized reboxetine and derivatized internal standard were well resolved on a silica column with retention times of approximately 1 and 3 min, respectively (Fig. 3), relative to the initiation of data acquisition (6.5 min after injection). The background was generally low at the internal standard position, but a late eluting peak sometimes eluted close to the internal standard. Therefore, use of a high concentration of internal standard is important. After column-switching of the reboxetine peak (0–1.5 min after initiation of data acquisition), the derivatives of the (*R,R*)- and (*S,S*)-enantiomers (detector 2) were also well resolved ($k_2=3.80$, $\alpha=1.07$, $R_S=1.25$), with retention times of approximately 14 and 15 min, respectively, relative to initiation of data acquisition. Blank dog plasma samples from several sources exhibited small endogenous peaks at positions corre-

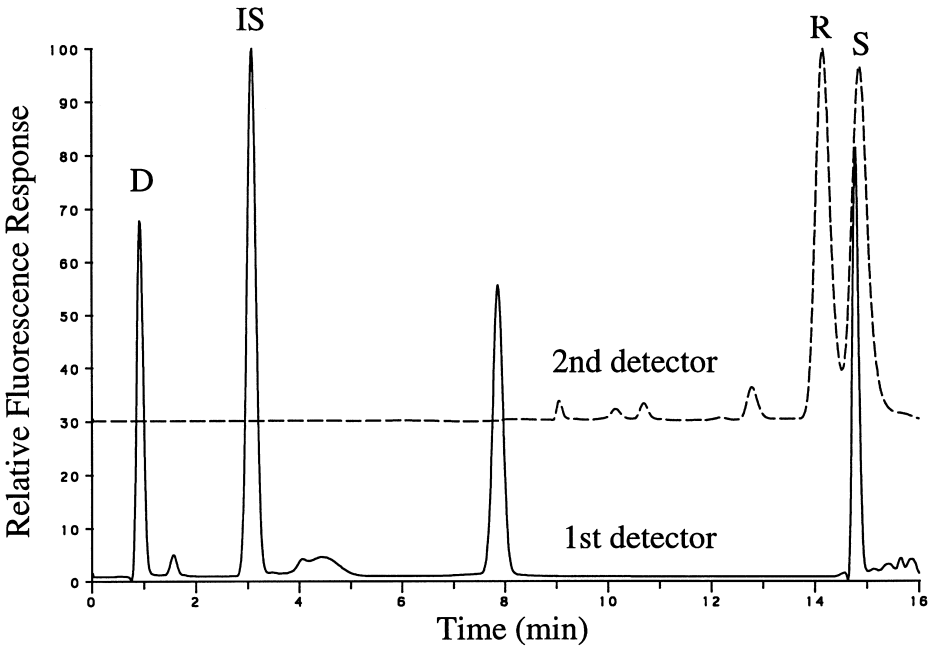

Fig. 3. Chromatograms of an extract of a dog plasma calibration standard containing 2000 ng/ml reboxetine. Lower chromatogram: derivatized drug and internal standard peaks after separation on the first column (silica). Upper chromatogram: derivatized enantiomer drug peaks after column-switching and separation on the second (cyano) and third (Chiralcel OD-H) columns. D=Reboxetine; I.S.=internal standard; R=(*R*,*R*)-reboxetine; S=(*S*,*S*)-reboxetine.

sponding to the enantiomers (Figs. 4 and 5); however, it appeared that impurities in the (+)-FLEC reagent were primarily responsible for these peaks.

3.2. Extraction efficiency

Absolute extraction recovery was investigated by analysis of extracted samples versus directly derivatized samples. Samples containing 10 μl of 10 μg/ml reboxetine in acetone were either directly derivatized or mixed with plasma and extracted and derivatized according to the usual procedure. The absolute recovery was 75±13% (n=4).

3.3. Linearity

Linearity of the assay was determined by generation of standard curves from peak height ratios of (*R*,*R*)- and (*S*,*S*)-reboxetine to internal standard in spiked plasma samples. The data were weighted by 1/concentration and fitted to a straight line with intercept using linear regression analysis. Summaries of calibration curve data from both enantiomers in dog plasma are shown in Table 2.

3.3.1. (R,R)-Reboxetine

Standard curves were found to be linear on three separate days between the range of 2–2000 ng/ml (*R*,*R*)-reboxetine using 10 standard levels (Table 2). Mean accuracies at 2 and 2000 ng/ml were both 100%. The precision (relative standard deviation, R.S.D.) was 10% and 1.6% at 2 and 2000 ng/ml, respectively. The average correlation coefficient (r) over three days was 0.9999±0.0001 (mean±S.D.). Average values of the slope and intercept were 6.15±0.21 (mean±S.D.) and 12.2±0.6, respectively. Chromatograms of low level standards are shown in Fig. 4.

3.3.2. (S,S)-Reboxetine

Standard curves were found to be linear on three separate days between the range of 2–2000 ng/ml (*S*,*S*)-reboxetine using 10 standard levels (Table 2). Mean accuracy and precision at 2 and 2000 ng/ml

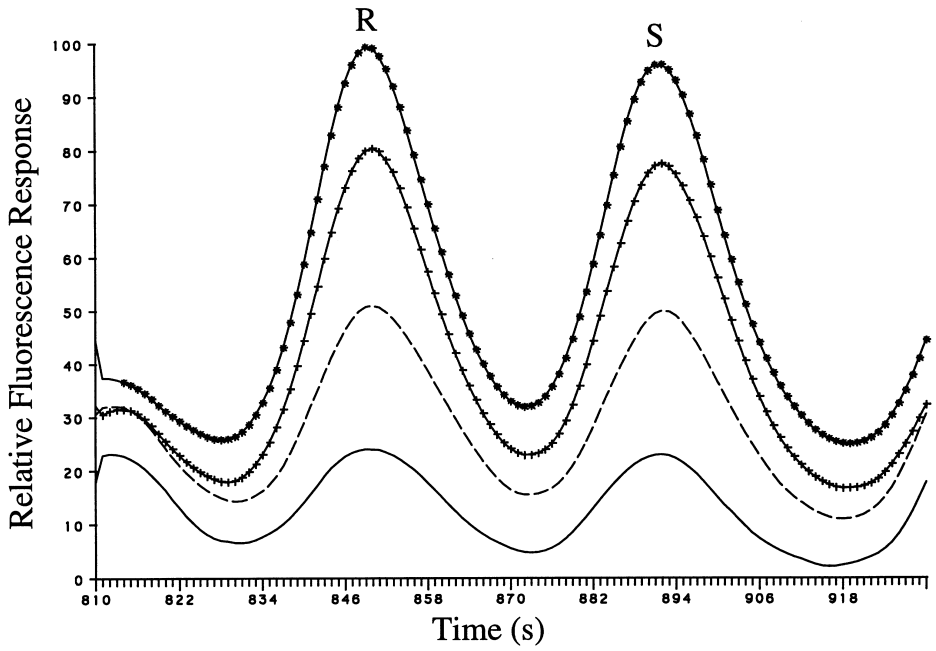

Fig. 4. Expanded chromatograms (2nd detector) of extracts of a dog plasma blank and low standards. From top to bottom: dog plasma standards containing 10, 5, 2 and 0 ng/ml of each enantiomer of reboxetine. R=(*R*,*R*)-Reboxetine; S=(*S*,*S*)-reboxetine.

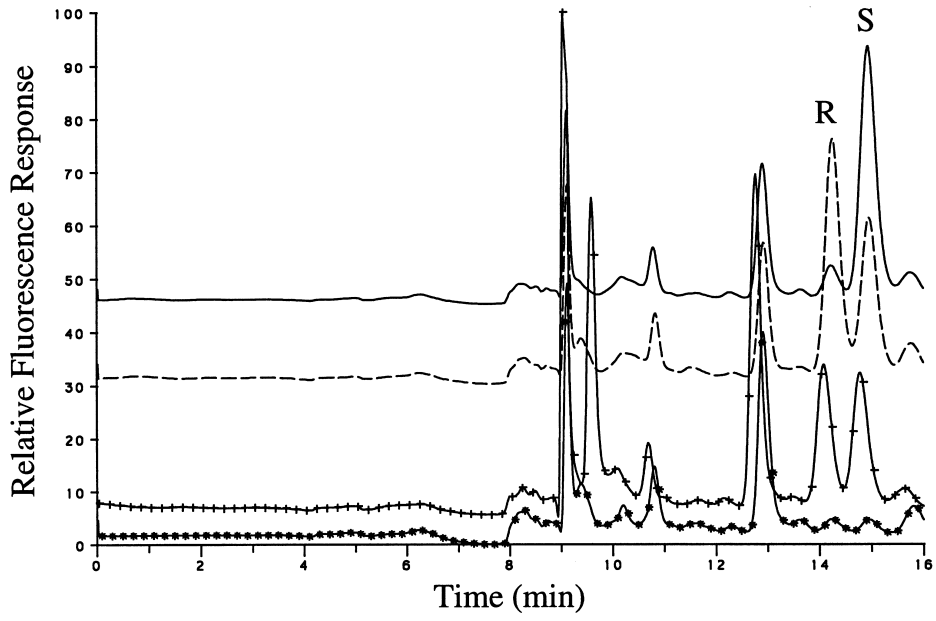

Fig. 5. Chromatograms (2nd detector) of extracts of dog plasma. From top to bottom: a dog plasma quality control sample containing 10 ng/ml (*R*,*R*)-reboxetine and 100 ng/ml (*S*,*S*)-reboxetine, a plasma sample drawn at 32 h after dosing from a dog administered a single 60 mg/kg oral dose of reboxetine, a dog plasma standard containing 20 ng/ml of each enantiomer, and a dog plasma blank. R=(*R*,*R*)-Reboxetine; S=(*S*,*S*)-reboxetine.

Table 2
Calibration curve data for reboxetine enantiomers in dog plasma (mean of three assay runs; highest standard and two lowest standards assayed in duplicate)

Theoretical concentration	(*R*,*R*)-Reboxetine			(*S*,*S*)-Reboxetine		
(ng/ml)	*n*	Accuracy (%)	R.S.D. (%)	*n*	Accuracy (%)	R.S.D. (%)
2	6	100	10	6	99	11
5	6	102	3.0	6	102	4.0
10	3	100	2.8	3	101	2.0
20	3	99	1.0	3	99	2.1
50	3	98	2.1	3	99	2.3
100	3	98	1.5	3	99	0.7
200	2	100	2.9	2	100	2.8
500	3	101	2.7	3	101	3.0
1000	3	101	0.6	3	101	0.8
2000	6	100	1.6	6	100	1.5

were 99±11% and 100±1.5%, respectively. The average correlation coefficient (r) over three days was 0.9999±0.0001 (mean±S.D.). Average values of the slope and intercept were 5.64±0.19 (mean±S.D.) and 12.4±0.8, respectively. Chromatograms of low level standards are shown in Fig. 4.

3.4. Limits of detection and quantitation

The limits of detection (LODs) and quantitation (LOQs) were determined by measuring background noise in plasma samples and calculating the concentrations that corresponded to signal-to-noise (S/N) ratios of 3 (LOD) and 10 (LOQ). The determinations of LODs and LOQs were accomplished utilizing the average noise as calculated from 11 plasma samples over two assay runs. The calculated LODs for both enantiomers corresponded to a concentration of approximately 0.3 ng/ml. The LOQ was found to be equivalent to a concentration of approximately 1 ng/ml for both enantiomers. Due to the small endogenous peaks observed in blank plasma at positions corresponding to the derivatives of the enantiomers (Section 3.1), it was necessary to raise the LOQ of the assay to 2 ng/ml (*R*,*R*)- and (*S*,*S*)-reboxetine in order to obtain reproducible results with acceptable accuracy and precision.

3.5. Precision and accuracy

Accuracy and precision were evaluated using quality control samples prepared at three concentration combinations of (*R*,*R*)- and (*S*,*S*)-reboxetine (Table 1). Intra-day determinations were evaluated by assaying quality controls in triplicate at each level. Inter-day evaluations were made from the data collected over three days of intra-day precision and accuracy trials. Accuracy was determined by calculating the mean recovery at each level, while precision was assessed from the R.S.D. of the mean concentration. A summary of quality control data is provided in Table 3. A chromatogram of a control sample is shown in Fig. 5.

3.5.1. (R,R)-Reboxetine

Quality control samples were assayed at concentrations of 10, 100 and 1000 ng/ml (*R*,*R*)-reboxetine (Table 3). Intra-day mean accuracy ranged from 91.0–106% with precision of 0.6–6.0%. The mean inter-day accuracy ranged from 94–104% with mean precision of 3–4%. Overall accuracy and precision for (*R*,*R*)-reboxetine control samples was 98±5%.

3.5.2. (S,S)-Reboxetine

Quality control samples were assayed at concentrations of 10, 100 and 1000 ng/ml (*S*,*S*)-reboxetine (Table 3). Intra-day mean accuracy ranged from 89.8–117% with precision of 0.6–5%. The mean inter-day accuracy ranged from 92.8–110% with mean precision of 2.3–6%. Overall accuracy and precision for (*S*,*S*)-reboxetine control samples was 102±8%.

Table 3
Intra- and inter-day accuracy and precision data for dog plasma samples fortified with reboxetine enantiomers

Day	Theoretical concentration (ng/ml)	(*R*,*R*)-Reboxetine			(*S*,*S*)-Reboxetine		
		n	Accuracy (%)	R.S.D. (%)	*n*	Accuracy (%)	R.S.D. (%)
1	10	3	91.0	2.1	3	106	4.0
	100	3	101	4.0	3	99.1	1.1
	1000	3	93.1	0.7	3	89.8	0.6
2	10	3	95.8	2.4	3	117	5.0
	100	3	105.2	0.7	3	102.3	0.8
	1000	3	99.3	1.0	3	95.6	0.9
3	10	3	96.0	6.0	3	109.1	2.6
	100	3	106	3.0	3	103.7	1.5
	1000	3	97.5	0.6	3	93.0	0.7
Inter-day	10	9	94.0	4.0	9	110	6.0
	100	9	104	3.0	9	101.7	2.3
	1000	9	96.6	3.0	9	92.8	2.8
Overall	–	27	98	5.0	27	102	8.0

3.6. Stability

3.6.1. Standard solutions and internal standard solutions

Stability of racemic reboxetine in water has been proven for at least three months at 4°C [3]. The stabilities of working standard solutions have been proven for at least six months when stored at ≤−10°C. The internal standard working solution has demonstrated stability for at least four months at 2–5°C.

3.6.2. Drug in matrix

Reboxetine was stable for one week in plasma at room temperature and at least four months at ≤−10°C.

3.6.3. Effect of freeze–thaw cycles

Quality control samples in dog plasma, stored at ≤−10°C, were subjected to three freeze–thaw cycles. No significant concentration changes were observed.

3.6.4. (+)-FLEC derivatization solution

The 0.05 m*M* (+)-FLEC solution, prepared in acetone, has been proven stable for at least one week when stored at 2–5°C.

3.6.5. Prepared samples

Prepared samples were stable at ambient temperature in the autosampler for at least three days.

3.7. Ruggedness

The three analytical columns are very durable in this assay. No substantial retention time shifts, peak shape degradation, or increase in column back-pressure were observed over the course of approximately 2000 injections of plasma extracts.

The conditions used for derivatization [(+)-FLEC concentration, temperature, and reaction time] are well above the minimum required for complete derivatization.

3.8. Application

The reported method has been used for the determination of reboxetine enantiomers in dog, rat, rabbit and mouse plasma samples in several toxicokinetic, bioavailability and protein binding studies. A chromatogram from a drug-treated dog is

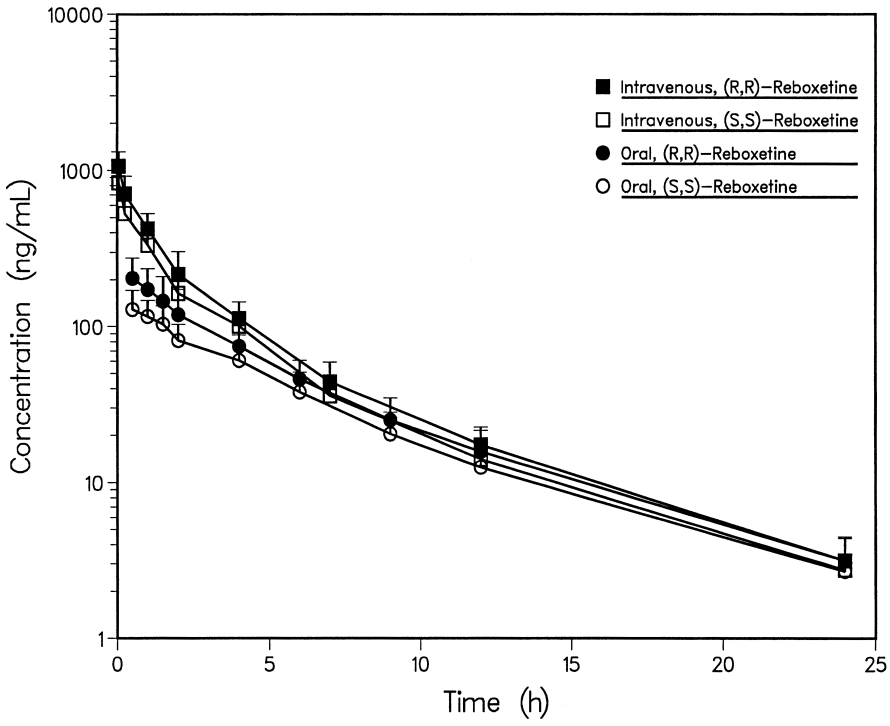

Fig. 6. Semi-logarithmic mean plasma concentration–time profile of reboxetine enantiomers in dogs ($n=6$) following single oral and intravenous administration of a solution formulation at a dose of 3 mg/kg. The error bars represent one standard deviation.

shown in Fig. 5. Fig. 6 illustrates the pharmacokinetics of the enantiomers in dogs ($n=6$) following single 3 mg/kg oral and intravenous doses of an aqueous solution of reboxetine. Reboxetine was rapidly absorbed after oral administration. The bioavailabilities of the (*R*,*R*)- and (*S*,*S*)-enantiomers were essentially identical (48±7% and 46±7%, respectively), as were the half-lives (4.5±0.9 h and 4.5±1.1 h, respectively), but the overall exposure to the (*R*,*R*)-enantiomer was about 30% greater than that of the (*S*,*S*)-enantiomer, based on plasma area-under-the-curve, following both oral and intravenous administration. This enantioselectivity can be observed in a chromatogram from a drug-treated dog (Fig. 5).

4. Conclusions

Although derivatization of enantiomers with (+)-FLEC yields diastereomers, we found that a chiral column separated the diastereomers better than achiral reversed-phase or normal-phase columns. A rapid SPE scheme using extraction disc technology was developed with an analog of reboxetine as internal standard. Using a column-switching system, the assay was rapid (total run time of 18 min) and sensitive (range of quantitation was 2–2000 ng/ml for each enantiomer in dog plasma, using 0.1 ml plasma samples). The calibration curves were linear with correlation coefficients averaging 0.9999 for each enantiomer in three assay runs. The recoveries of quality control plasma samples at three concentrations averaged 98±5% for (*R*,*R*)-reboxetine and 102±8% for (*S*,*S*)-reboxetine. Absolute extraction recoveries averaged 75±13%.

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