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Short communication

Automated determination of reboxetine by high-performance liquid chromatography with column-switching and ultraviolet detection

Sebastian Härtter, Harald Weigmann, Christoph Hiemke*

Department of Psychiatry, University of Mainz, Untere Zahlbacher Strasse 8, D-55131 Mainz, Germany

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Abstract

A fully automated method including column-switching and isocratic high-performance liquid chromatography (HPLC) was developed for quantitative analysis of the new antidepressant reboxetine, a noradrenaline reuptake inhibitor. After serum injection into the HPLC system and on-line sample clean-up on a silica C_8 (10×4.0 mm I.D.) clean-up column with an eluent consisting of 2.5% acetonitrile in deionized water, the chromatographic separation was performed on an analytical column (Lichrospher CN; 250×4.6 mm I.D.) with an eluent of acetonitrile–aqueous potassium phosphate buffer (0.008 *M*, pH 6.4) (50:50). The UV detector was set at 273 or 226 nm. The limit of quantification was about 15 ng/ml at 273 nm and about 4 ng/ml at 226 nm. The day-to-day relative standard deviation ranged between 2.7 and 6.7% with recovery rates ≥90%. Linear regression analyses revealed correlation coefficients >0.998. The method can be applied to therapeutic drug monitoring of reboxetine as well as pharmacokinetic studies.

Keywords: Reboxetine

1. Introduction

Reboxetine (Fig. 1) is a recently introduced antidepressant which exerts its therapeutic effects by selective blockade of the noradrenaline (NA) reuptake [1]. It is a rationally developed new antidepressant acting on a distinct target structure similar to the well known selective serotonin reuptake inhibitors (SSRIs), e.g., fluoxetine or sertraline [2]. Some of the tri- or tetracyclic antidepressants like nortriptyline, desipramine or maprotiline also potentially and preferentially block the NA reuptake [3]. However, they all give rise to relevant toxic side effects due to their widespread affinity to central-nervous receptors like muscarinic- or adrenergic receptors [4]. The concomitant therapeutic drug monitoring (TDM) of tricyclic antidepresants like desipramine

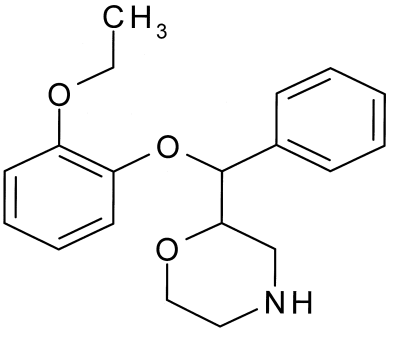

Fig. 1. Chemical structure of reboxetine {(*RS*)-2-[(*RS*)-α-(2-ethoxyphenoxy)benzyl] morpholine}.

*Corresponding author. Fax: +49-6131-176-690.
E-mail address: hiemke@mail.uni-mainz.de (C. Hiemke)

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or nortriptyline is strictly recommended primarily to avoid toxic side effects but secondly to improve the therapeutic response [5,6]. The value of TDM for the newer antidepressants like reboxetine is unclear, so far, since they all have almost no receptor blocking properties and are therefore suggested to be free of any serious side effects [7]. As for all kinds of psychotropic drugs, however, compliance is a major confounding factor of psychopharmacological therapy [8] and the most reliable check of compliance is by TDM of the administered drug in blood. This holds even more true for drugs without measurable and visible side effects like reboxetine. In addition, some recent studies on the correlation between serum concentrations and response of SSRIs like fluvoxamine, sertraline and paroxetine gave evidence for concentration ranges with a higher incidence of response [9–11]. This might be also applicable to reboxetine. Although an enantiomereselective method including derivatization and fluorescence detection has been published recently [12] a method suitable for routine TDM of reboxetine has not been published, so far. The aim of this study was to establish a fully automated high-performance liquid chromatography (HPLC) method, that enables the quantitative analysis of reboxetine for pharmacokinetic and TDM purposes and studies on possible correlations between reboxetine serum concentrations and therapeutic response.

2. Experimental

2.1. Chemicals

Reboxetine methansulfonate was kindly supplied by Pharmacia & Upjohn (Erlangen, Germany). Acetonitrile (HPLC grade), orthophosphoric acid and dipotassium hydrogenphosphate trihydrate (analytical-reagent grade) were obtained from Merck (Darmstadt, Germany). Water was deionized and filtered through a Milli-Q water processing system (Millipore, Eschborn, Germany).

2.2. Standards

Stock solutions for either quality control (QC) samples or calibration (C) samples were prepared by dissolving 10 mg of substance in 10 ml methanol each. They were diluted with deionized water and mixed with drug-free plasma from healthy volunteers to obtain calibration standards of free bases at five different concentrations, 15.00, 38.00, 77.00, 115.0, 153.0 ng/ml reboxetine (calculated as free base). QC samples were prepared at four different concentrations, 30.00, 60.00, 120.0 and 180.0 ng/ml. All standards could be stored in the dark at −20°C for several months without measurable decomposition.

2.3. Plasma or serum samples

Human plasma or serum samples were obtained from healthy non treated volunteers or from patients treated with reboxetine. Patients' blood for preparation of serum was collected in the morning immediately before the first daily dose.

Serum samples could be stored frozen at −20°C for several months without measurable decomposition. When stored for 24 h at room temperature no change in concentrations of analytes could be observed.

2.4. Instrumentation

The HPLC system consisted of a SIL-10 A autosampler, an electric six-port switching valve (FCV-12 AH) coupled to the autosampler and two HPLC pumps (LC-10 AS). One HPLC pump was used for loading plasma or serum onto the clean-up column and subsequent washing. The second pump was used to pump the analytical mobile phase through the analytical column. A variable-wavelength ultraviolet (UV) detector, type SPD-10A was used to monitor absorption at two different wavelengths (226 and 273 nm). All HPLC components were purchased from Shimadzu (Kyoto, Japan). Data acquisition and integration was performed by means of the Shimadzu Class LC 10 software (Version SPA 1993).

The analytical column (250×4.6 mm I.D.) was packed with Lichrospher CN (5 μm particle size) by MZ-Analysentechnik (Mainz, Germany). The clean-up column (10×4.6 mm) was filled with 20 μm

particles of C_8 bonded silica material (MZ-Analysentechnik).

2.5. Chromatographic procedure

Sample clean-up and chromatographic separation were performed at room temperature.

0–3 min: After recentrifugation of serum (10 000 *g* for 5 min), 100 μl of the supernatant were injected onto the clean-up column. Proteins and other interfering compounds were washed to waste by using deionized water containing 2.5% (v/v) acetonitrile at a flow-rate of 1.5 ml/min.

3–8 min: After the electric six-port valve had been switched at 3 min, the analytical run was started. The analytes to be determined were eluted onto the analytical column (back flush) and separated by the analytical mobile phase (second HPLC pump) of acetonitrile–K_2HPO_4 (0.008 *M*), adjusted to pH 6.4 by H_3PO_4 (50:50) at a flow-rate of 1.5 ml/min.

8–15 min: Five min after the start of the analytical run (at 8 min) the switching valve was reset.

The clean-up column was replaced after injection of 75 to 100 serum or plasma samples.

2.6. Interferences

To control for possible interferences with drugs that may be used in combination with reboxetine the suggested interfering compounds were prepared in blank plasma as described for QC and C samples of reboxetine. Concentrations were used according to reported therapeutic serum concentrations of each drug, e.g., haloperidol was tested at 50 ng/ml, diazepam at 500 ng/ml, carbamazepine at 10 μg/ml.

2.7. Calculations

The peak heights obtained from spiked serum or plasma, containing known amounts of drugs, were subjected to linear regression analysis for the calculation of correlation coefficients, slopes and intercepts. Drug concentrations in samples containing unknown amounts of drug were calculated on the basis of the computed regression lines.

2.8. Precision and accuracy

Precision and accuracy were evaluated by nine replicate analyses of QC samples on three different days to evaluate inter-assay variability. Precision was calculated as relative standard deviation (RSD), while accuracy was determined from the difference between nominal and determined concentrations. Recovery was analyzed by comparing the peak-heights of a serum QC sample after column-switching with an aqueous sample of the same amount injected directly onto the analytical column.

3. Results and discussion

Column-switching techniques are most useful for automated and rapid analysis of drugs in complex matrices [13–15]. In addition to a conventional isocratic HPLC system, only a pre-column for sample clean-up, a second HPLC pump and a six-port switching valve are needed. The procedure can be easily automated and urgent samples can be analyzed within 1 h.

We injected human serum directly into the system. Separation of reboxetine was conducted by HPLC within less than 15 min on a CN column (Fig. 2A–C).

In the described method we used only 2.5% acetonitrile solution as clean-up eluent, since at higher concentrations of acetonitrile, the recovery of reboxetine decreased. Nevertheless, the clean-up column did not have to be changed more frequently than with a clean-up eluent containing higher acetonitrile concentrations.

Inter-assay variabilities (precision) according to the analysis of QC samples ranged between 3 and 12% (Table 1). The inaccuracies were between 0.5 and −9.2% (Table 1). Recoveries were always higher than 90%. A precision below 15% was considered acceptable for therapeutic drug monitoring, since it was in the range recommended for

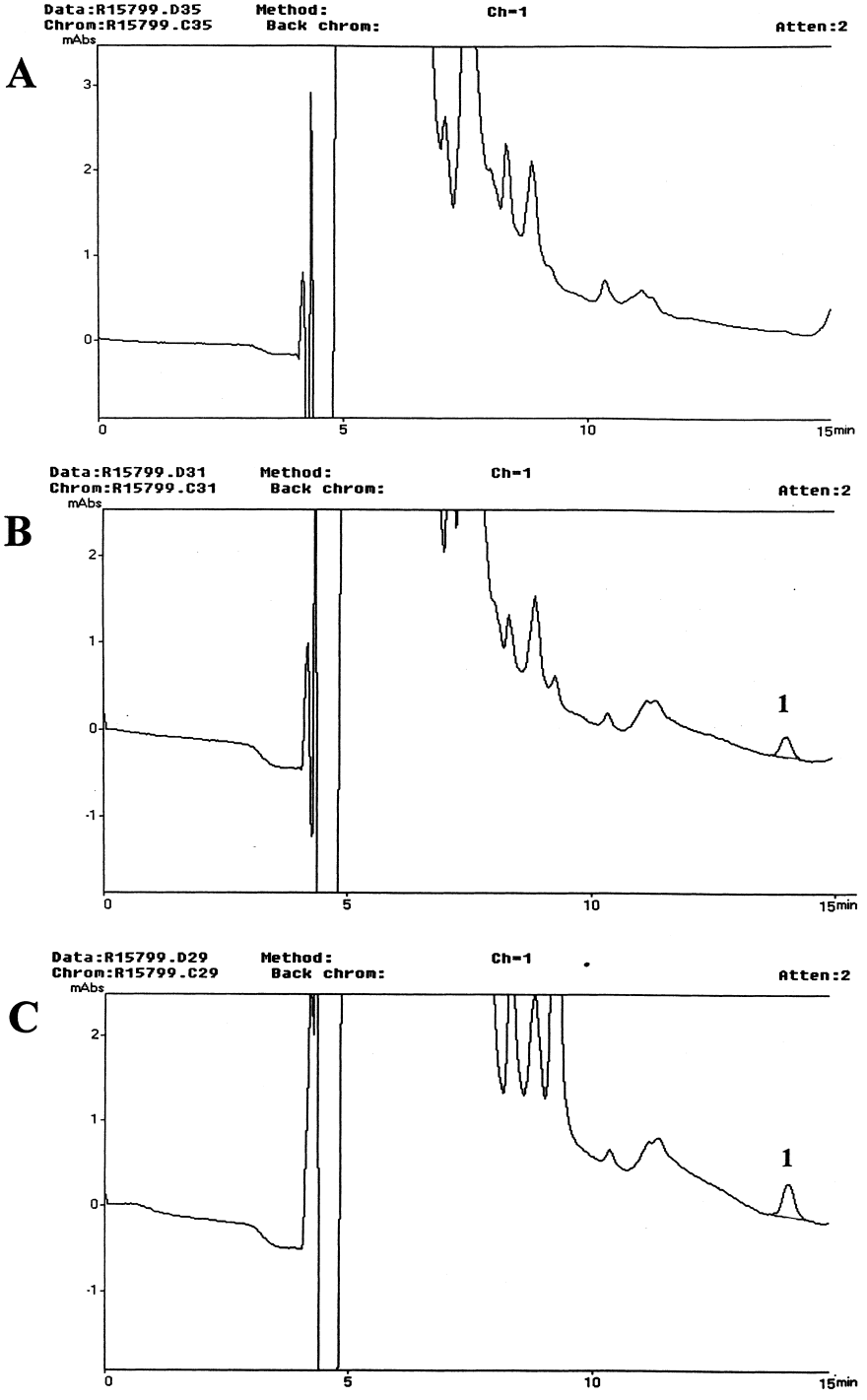

Fig. 2. Representative chromatograms of (A), a drug-free serum; (B), a drug-free serum supplemented with 38.0 ng/ml reboxetine (1); and (C) a chromatogram obtained after analysis of authentic human serum of a subject after chronic administration of 2×2 mg reboxetine/day (1=reboxetine=51 ng/ml).

Table 1
Precision and accuracy of the reboxetine assay according to the analysis of quality control samples on different days[a]

Analyte	Concentration added (ng/ml)	Mean (ng/ml)	Precision (%)	Inaccuracy (%)	N
Reboxetine, 226 nm	5.70	5.72	8.92	0.26	4
	22.50	22.32	12.50	−0.82	4
Reboxetine, 273 nm	30.00	30.15	6.66	0.51	9
	60.00	55.75	2.72	−7.09	9
	120.00	109.78	4.30	−8.52	9
	180.00	163.36	3.59	−9.24	9

[a] For the determination of concentrations below 15 ng/ml at 226 nm only two concentrations were tested.

determination of antiepileptics according to legal requirements [16,17].

The sensitivity of the assay could be easily increased by detection of the analytes at 226 nm. Even at the lowest quantifiable concentration of only 3.8 ng/ml reboxetine (free base) a precision of 6.4% and an inaccuracy of −4.4% were found (Table 2). The main disadvantage of the detection at the lower wavelength was the higher susceptibility for interfering compounds either from matrix constituents or from coadministered drugs. Detection at 273 nm was thus regarded as more reliable for the routine TDM of reboxetine.

The method revealed linearity between 3.8 and 22.95 ng/ml with a correlation coefficient (R)≥0.98 and between 15 and 153 ng/ml with R≥0.998.

Testing standard solutions containing other psychotropic drugs that may be applied in combination with reboxetine, interferences could be observed with the neuroleptics amisulpiride, olanzapine and risperidone (Table 3). This should be taken into account when these drugs are combined with reboxetine.

No remarkable degradation of reboxetine in plasma was seen after storage at −20°C for several weeks. However, we had not experience with long-term storage or samples thawed and frozen again more than two times. Therefore, we can not fully exclude degradation of reboxetine in plasma samples after storage over years or after multiple extreme temperature changes.

The method described here offers the possibility to analyse samples automatically. Batchwise analysis, which is usual for routine procedures that include off-line pre-extractions, is therefore not necessary for our procedure.

In conclusion, the described method has sufficient accuracy and precision and may be applied not only for therapeutic drug monitoring of reboxetine, but also to pharmacokinetic studies.

Table 2
Precision and accuracy of the reboxetine assay according to the analysis of calibration samples on different days[a]

Analyte	Concentration added (ng/ml)	Mean (ng/ml)	Precision (%)	Inaccuracy (%)	N
Reboxetine, 226 nm	3.80	3.63	6.36	−4.38	9
	7.65	8.13	10.44	6.32	9
	11.48	11.71	7.07	1.96	9
	22.95	22.33	8.67	−2.69	9
Reboxetine, 273 nm	15.00	15.50	10.52	3.34	9
	38.00	37.72	4.35	−0.72	9
	77.00	77.08	3.02	0.10	9
	115.00	114.26	1.91	−0.64	9
	153.00	153.55	1.13	0.36	9

[a] For the determination of concentrations below 15 ng/ml at 226 nm only four concentrations were used for regression analysis.

Table 3
List of tested interferences and retention time differences to reboxetine

Drug	Retention time (min)±reboxetine
Alprazolam	n.d.
Amisulpiride	+0.09
Carbamazepine	n.d.
Clozapine	+1.51
Diazepam	n.d.
Fluoxetine	+11.98
Fluvoxamine	+3.64
Haloperidol	n.d.
Lorazepam	+9.31
Melperon	+7.91
N-Desmethylclozapine	−1.28
Nefazodone	−2.86
Olanzapine	−0.54
Oxazepam	n.d.
Paroxetine	+8.07
Reboxetine	0
Risperidone	−0.34
Sertraline	+12.88
Sulpiride	−2.298
Temazepam	n.d.
Venlafaxine	+3.69
Zolpidem	n.d.

n.d.=No detectable peak from 0 to 30 min.

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