



# A highly sensitive HPLC method with automated on-line sample pre-treatment and fluorescence detection for determination of reboxetine in human plasma

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## ABSTRACT

A fully automated, rapid and highly sensitive HPLC method with automated sample pre-treatment by column-switching system and fluorescence detection has been developed for the trace quantitative determination of the new antidepressant reboxetine (RBX) in human plasma. A simple pre-column derivatization procedure with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) reagent was employed. Paroxetine (PXT) was used as an internal standard. Plasma samples containing both RBX and PXT, after filtration, were derivatized by heating with NBD-F in borate buffer of pH 8 at 70 °C for 30 min. The derivatized plasma samples were injected into the HPLC system where an on-line sample clean up was achieved on the pre-treatment column (Co-sense Shim-pack MAYI-ODS) with a washing mobile phase (acetonitrile:2% acetic acid; 40:60, v/v) at a flow rate of 5 mL min<sup>-1</sup> for 1 min. After an automated on-line column switching to the analytical Hypersil phenyl 120A column (250 mm × 4.6 mm, 5 μm), the separation of the derivatized RBX and PXT was performed using a mobile phase consisting of sodium acetate buffer (pH 3.5):tetrahydrofuran:acetonitrile (55:35:10, v/v/v) at a flow rate of 2.0 mL min<sup>-1</sup>. The eluted derivatives were monitored by a fluorescence detector set at an excitation wavelength of 470 nm and an emission wavelength of 530 nm. Under the optimum chromatographic conditions, a linear relationship with good correlation coefficient ( $r = 0.9995$ ,  $n = 5$ ) was found between the peak area ratio of RBX to PXT and RBX concentration in the range of 2–500 ng mL<sup>-1</sup>, with limits of detection and quantification of 0.5 and 1.7 ng mL<sup>-1</sup>, respectively. The intra- and inter-day precisions were satisfactory; the relative standard deviations were 2.25 and 3.01% for the intra- and inter-day precisions, respectively. The accuracy of the method proved as the mean recovery values were 100.11 ± 2.24% and 100.99 ± 2.98% for the intra- and inter-day assay runs, respectively. The proposed method involved simple and minimum sample preparation procedure and short run-time (<12 min) and therefore it can be applied to the routine therapeutic monitoring and pharmacokinetic studies of RBX.

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## 1. Introduction

Reboxetine (RBX); (RS)-2-[(RS)-α-(2-ethoxyphenoxy) benzyl-morpholine methane sulphonate, was first introduced in 1997 as the first drug of the new selective noradrenalin reuptake inhibitor (SNRI) antidepressant class [1]. It has similar therapeutic potency to tricyclic antidepressants [2,3]. The drug does not affect dopamine or serotonin reuptake, and it has low affinity for adrenergic, cholinergic, histaminergic, dopaminergic and serotonergic receptors [4]. RBX provides an alternative rationale for patients resistant to the conventional antidepressants therapy with an advantage of significantly improved adverse effects profile [5,6]. In contrast to certain serotonergic drugs, there is no evidence of any withdrawal syndrome upon sudden discontinuation or tapering of RBX ther-

apy [7]. RBX is also prescribed in the treatment of narcolepsy [8], panic disorders [9], and Parkinson's disease [10]. RBX is rapidly and extensively absorbed following oral administration. The maximum plasma concentration is achieved within 2–2.5 h after a 4 mg oral dose; and it is highly bound (~98%) to plasma proteins mainly α1-acid glycoprotein [11]. It is eliminated mainly via urine with a relatively short elimination half-life (~13 h), therefore it is given twice daily [12].

Most psychiatric patients are usually subjected to simultaneous poly-pharmacotherapy to achieve successful control of the symptoms. Therefore, careful monitoring and subsequent optimization of dosing regimen of potent drugs, such as RBX, in the plasma of the patients are extremely necessary. Because of the small doses of RBX, its concentrations in plasma samples are expected to be quite low [11]. For these reasons, a sensitive, selective, and accessible method is required for quantitative determination of RBX in plasma. Considerable efforts have been made to provide competent analytical techniques for the determination of RBX in plasma.

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These techniques include two related gas chromatography with mass detector [13,14], and several HPLC methods with UV or fluorescence [15–21], mass [22,23] and tandem mass [24] detectors. All these methods involved lengthy liquid–liquid or solid-phase extraction for RBX from the plasma samples prior to their analysis. Indeed, these pre-treatment procedures are cumbersome and time-consuming. They will also lead to large amount of errors in the recovery of RBX. Accordingly, these methods do not meet the needs of pharmacokinetic studies, which require an accurate and rapid feedback for the analytical information of pre-clinical and clinical specimens. Moreover, the cost and complexity of the instrumentation of HPLC–MS limited its applications in clinical laboratories.

The present work describes, for the first time, the development of a new automated highly sensitive and accurate HPLC method with fluorescence detection for the determination of RBX in plasma. The method involved a pre-column derivatization with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F), and an automated highly efficient on-line pre-treatment procedure by using a bio-sample analysis column-switching system (co-sense for BA) equipped with a Shim-pack MAY1-ODS bio-sample pre-treatment column. The method was successfully applied to the determination of RBX in spiked human plasma samples.

## 2. Experimental

### 2.1. Chemicals and materials

Reboxetine (RBX) and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Paroxetine (PXT) was obtained from SmithKline Beecham Pharmaceuticals (Brentford, England). Human plasma samples were collected from normal healthy volunteers at King Khaled University Hospital (Riyadh, Saudi Arabia), and they were kept frozen at  $-20^{\circ}\text{C}$  until analysis. Acetonitrile, tetrahydrofuran and all the other solvents were of HPLC grade (Merck, Darmstadt, Germany). All other materials were of analytical grade.

### 2.2. Preparation of stock solutions

#### 2.2.1. Reboxetine (RBX) and paroxetine (PXT) standard solutions

An accurately weighed amount (10 mg) of each of RBX and PXT was quantitatively transferred into a 10-mL volumetric flask, dissolved in distilled water, and completed to volume with the same solvent to produce stock solutions of  $1\text{ mg mL}^{-1}$ . The stock solutions were further diluted with water to obtain working standard solutions containing  $2000\text{ ng mL}^{-1}$  and  $400\text{ ng mL}^{-1}$  for RBX and PXT, respectively.

#### 2.2.2. 7-Fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) derivatizing reagent

An accurately weighed amount (5 mg) of NBD-F was transferred into a 10-mL volumetric flask, dissolved in 10 mL acetonitrile, completed to volume with the same solvent to produce a stock solution of 0.05% (w/v). The solution was freshly prepared and protected from light during use.

#### 2.2.3. Borate buffer solution

Weighed amounts of boric acid (0.62 g) and potassium chloride (0.75 g) were dissolved in 100 mL distilled water. A volume of 4.0 mL of  $0.2\text{ mol L}^{-1}$  NaOH and 40 mL of ethanol were added and the mixture was diluted to 200 mL with distilled water. The pH of the solution was adjusted to  $8 \pm 0.1$  by a calibrated pH-meter (Microprocessor pH meter BT-500, Boeco, Germany).

### 2.3. Chromatographic system

HPLC apparatus consisted of a Shimadzu system (Shimadzu Corporation, Kyoto, Japan) equipped with two solvent delivery systems (LC-20AD VP) with FCV-12AH high-pressure flow channel changeover valve, DGU-20AS on-line degasser, SIL-20A auto-sampler, co-sense BA LC system, CTO-20A column oven, SPD-20A UV–vis detector, SPD-M20A photodiode-array detector, RF-10A XL fluorescence detector, and CBM-20A system controller. The pre-treatment column was a Shim-pack MAY1-ODS (10 mm length  $\times$  4.6 mm i.d., 12 nm pore diameter, and  $50\text{ }\mu\text{m}$  particle diameter). The chromatographic separations were performed on an analytical column Hypersil 120A (250 mm length  $\times$  4.6 mm i.d.,  $5\text{ }\mu\text{m}$  particle diameter) manufactured by Phenomenex (USA). The column temperature was kept constant at  $25 \pm 2^{\circ}\text{C}$ . Separations were performed in isocratic mode. The pre-treatment mobile phase consisted of acetonitrile:2% acetic acid (40:60, v/v) pumped for 1 min at a flow rate of  $5\text{ mL min}^{-1}$ . The analytical mobile phase used for the separation consisted of  $10\text{ mmol L}^{-1}$  sodium acetate buffer (pH 3.5):tetrahydrofuran:acetonitrile (55:35:10, v/v/v) pumped at flow rate of  $2.0\text{ mL min}^{-1}$ . The mobile phases were filtered by a Millipore vacuum filtration system equipped with a  $0.45\text{ }\mu\text{m}$  pore size filter, degassed by ultrasonication, and further by passing through the DGU-20AS on-line degasser. The samples ( $50\text{ }\mu\text{L}$  each) were injected by the aid of the auto-sampler. The fluorescence detector was set at 470 nm as an excitation wavelength and 530 nm as an emission wavelength. The system control and data acquisition were performed by Shimadzu “LC solution” software (Shimadzu Corporation, Kyoto, Japan). The ratio of peak area of RBX to that of the internal standard (paroxetine; PXT) was used for the quantitation.

### 2.4. Sample preparation, derivatization and quantitation

The stock solutions of RBX and PXT were brought to room temperature, and working stock solutions of RBX ( $2000\text{ ng mL}^{-1}$ ) and PXT ( $400\text{ ng mL}^{-1}$ ) were prepared. The calibration standard samples were prepared by spiking blank human plasma with RBX to yield concentrations of 0, 2, 5, 10, 20, 50, 100, and  $500\text{ ng mL}^{-1}$ . The spiked plasma sample was filtered through a  $0.45\text{ }\mu\text{m}$  pore size Millipore filter or mixed with an equal volume of acetonitrile, vortexed for 30 s, and centrifuged for 20 min at 13,000 rpm by Biofuge Pico centrifuge (Heraeus Instruments, Germany). A  $500\text{ }\mu\text{L}$  of the filtrate (or the supernatant solution) was transferred into a screw-capped reaction tube using a micropipette followed by  $200\text{ }\mu\text{L}$  of the working solutions of the internal standard (PXT),  $1000\text{ }\mu\text{L}$  of the borate buffer solution, and  $200\text{ }\mu\text{L}$  of the NBD-F solution. The tube was capped, swirled, and left to stand in a thermostatically controlled water-bath (MLW type, Memmert GmbH, Co. Schwa Bach, Germany) at  $70^{\circ}\text{C}$  for 30 min. After cooling the tube rapidly to room temperature, a volume of  $100\text{ }\mu\text{L}$  of HCl was added, mixed, and  $50\text{ }\mu\text{L}$  of the resulting solution was injected into the HPLC system with the aid of the auto-sampler. The calibration curve was constructed by plotting the peak area ratio of RBX to PXT versus the concentration of RBX. The data were analyzed by least square regression and the intercept, slope, and correlation coefficient were calculated. The linear regression equation was used for calculating the concentrations of RBX in the spiked plasma samples based on their peak area ratios.

## 3. Results and discussion

### 3.1. Design and strategy for the method development

Reboxetine molecule contains weak chromophoric activity, and does not have a native fluorescence, therefore the determination

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