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Determination of reboxetine in rat brain microdialysates and plasma samples using liquid chromatography coupled to fluorescence detection

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ABSTRACT

A liquid chromatographic method with fluorescence detection was developed and validated for the quantification of the antidepressant reboxetine (RBX), a selective noradrenalin reuptake inhibitor, in rat brain microdialysates. After modification of the method in terms of sample preparation and sensitivity, it was also validated for the quantification of RBX in rat plasma samples. To enable fluorescence detection, a pre-column derivatization step with 9-fluorenylmethyl chloroformate was included. Separations were performed on a reversed phase C_{18} column using gradient elution. The retention time for RBX was found to be 8.8 min. The assay of RBX in brain microdialysis samples showed a linear relationship in the calibration curve from 2 to 200 ng/mL, with a correlation coefficient ≥0.999. The limit of detection (LOD) and the lower limit of quantification (LLOQ) were 0.6 and 2.0 ng/mL respectively. The intra-day and the inter-day precision (RSD %) ranged between 1.5% and 11.7% with an average recovery of 101.2 ± 8.2% (mean ± SD, n = 40). For the analysis of plasma samples, the calibration curve was linear between 20 and 700 ng/mL with a correlation coefficient ≥0.999. LOD and LLOQ were 6 and 20 ng/mL respectively. The intra-day and the inter-day precision (RSD %) ranged between 1.7% and 11.5% with an average recovery of 98.5 ± 7.3% (mean ± SD, n = 40). We demonstrated the applicability of the method to determine the concentration–time profiles of RBX in brain and plasma following systemic administration.

1. Introduction

Depression is a chronic, debilitating mental disease affecting more than 10% of the general population [1]. Several different classes of antidepressants exist. They are classified according to their mechanism of action. Each antidepressant affects one or more of the neurotransmitters dopamine, noradrenalin and serotonin. Regardless of the mechanism of action, all antidepressant drugs demonstrate similar clinical efficacy, but they differ in terms of tolerability and side-effect profile [1–4].

Reboxetine (RBX; Fig. 1) or 2-[α-(2-ethoxyphenoxy)phenylmethyl]-morpholine is a potent and selective noradrenalin reuptake inhibitor (SNRI). RBX has a significantly improved adverse effect profile compared to the other classical antidepressant drugs [5,6]. RBX is a secondary amine with pK_a value of 8.3 ± 0.2 and its predicted octanol–water partition coefficient (log P value) is 2.8 ± 0.4 [7].

In rats, RBX is rapidly absorbed (T_{max} 0.5–1 h) after oral administration. Its levels decline approximately monoexponentially without a noticeable distribution phase both after oral and intravenous dosing with an elimination half-life of approximately 1 h. The bioavailability after oral administration is 5% and the plasma protein binding is 85% [8,9]. Following subcutaneous administration, RBX plasma and brain homogenate concentration–time profiles have been reported [10]. The measured whole brain concentrations were on average 3 to 4-fold higher than the concentrations in the plasma samples, indicative of a more extensive distribution and protein binding of RBX in the brain. Rapid elimination of RBX from rat plasma and brain compartments was observed. These findings are consistent with high RBX in vivo clearance [10].

The biophase kinetics of a central nervous system (CNS) drug is an important determinant in the time course and intensity of its CNS effects. Plasma concentration–time profiles are generally used to determine the pharmacokinetic (PK) parameters of drugs. However, especially for CNS drugs, biophase PK may differ significantly from plasma PK, because blood–brain barrier (BBB) transport and brain distribution often do not occur instantaneously and to full

Abbreviations: ACN, acetonitrile; BBB, blood–brain barrier; CNS, central nervous system; ECF, extracellular fluid; FMOC-Cl, 9-fluorenylmethyl chloroformate; i.p., intraperitoneal; LC, liquid chromatography; LLOQ, lower limit of quantification; LOD, limit of detection; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; NBD-F, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; PK, pharmacokinetics; QC, quality control; RBX, reboxetine; SNRI, selective noradrenalin reuptake inhibitor.

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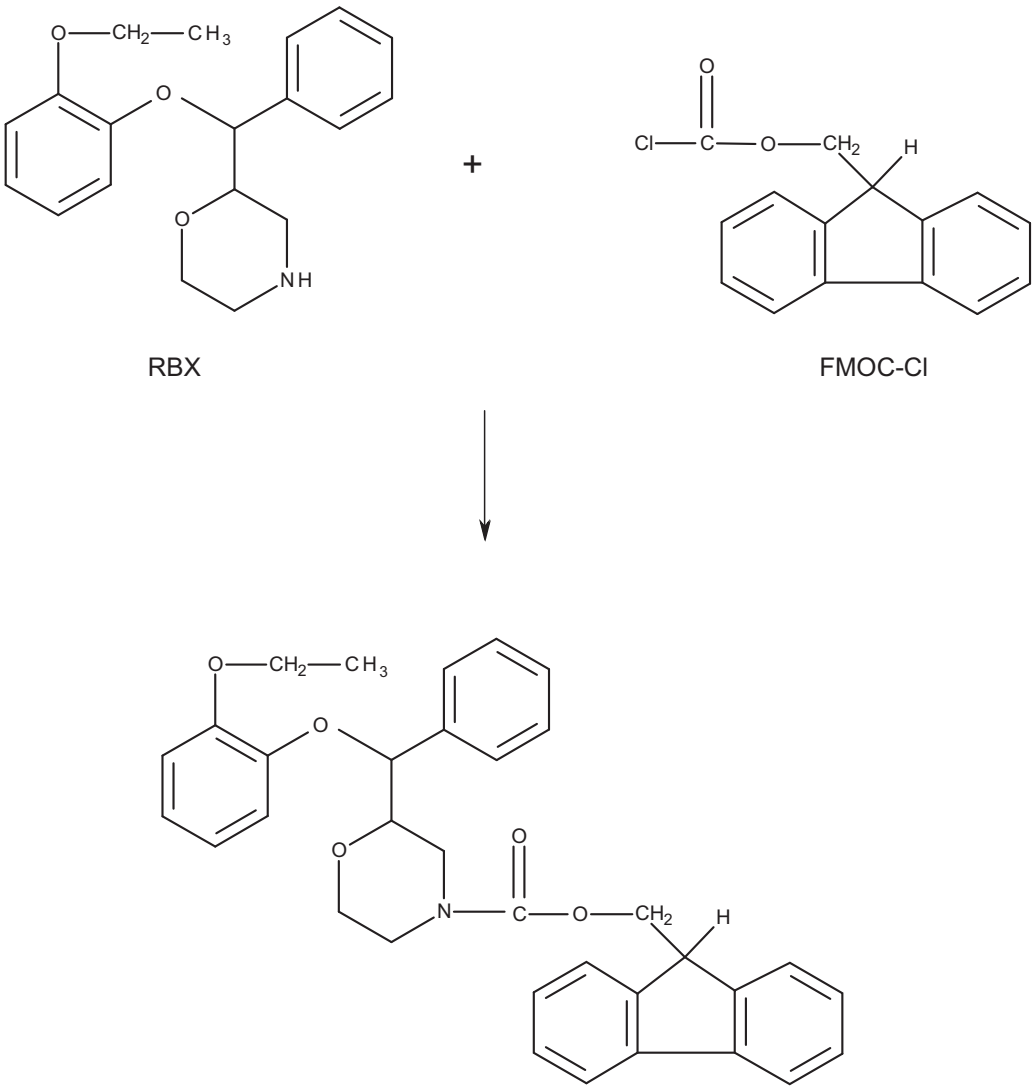

Fig. 1. Chemical reaction between RBX and FMOC-Cl.

extent [11,12]. In pathological conditions, such as during epileptic seizures, the delicate homeostasis of the BBB can be disturbed. As the BBB plays a crucial role in the availability and distribution of CNS drugs, it is even more relevant to study the biophase kinetics in these conditions [11,12]. For many CNS active drugs, it is accepted that the extracellular brain concentrations are most closely related to the biophase concentrations.

To define the brain PK of RBX in rats, sampling was performed using the in vivo cerebral microdialysis technique. This technique involves the insertion of a microdialysis probe into the selected area and continuous perfusion with a physiological solution. The mechanism of microdialysis is based on the passive diffusion of compounds down their concentration gradient over a semipermeable membrane. The obtained samples are free of high molecular weight components such as proteins; a sample clean-up prior to analysis is therefore not required. The concentration of the compound in dialysate samples reflects the free concentration (unbound) in the brain extracellular fluid (ECF). Besides, it only represents a fraction of the actual concentration, since equilibrium conditions are not reached. Microdialysis as a sampling technique allows continuous drug monitoring in different brain regions with the BBB remaining intact following probe implantation. However, microdialysis sampling with relevant temporal resolution yields small sample volumes of microdialysate. In addition, due to low probe recovery, low concentrations of analytes are attained. For these reasons, a highly sensitive analysis method is needed [13–16].

Several analytical methods to determine RBX in biological fluids have been reported in literature. Mostly RBX is measured in human plasma using liquid chromatography (LC) coupled to UV [4,17–20], fluorescence [4,5,21,22] or mass spectrometric detection [6,10,23–26]. Turnpenny and Fraier have determined RBX also in brain homogenates [10]. However, to our knowledge, no method for measuring RBX in brain microdialysis samples has been described in literature. The lowest limit of quantification (0.05 ng/mL) has been obtained using a chiral LC method coupled to tandem mass spectrometry [10]. Limits of quantification of approximately 2 ng/mL in plasma have been obtained using fluorescence detection [5,21,22]. Hartter et al. [19] were able to quantify 4 ng/mL RBX using a fully automated LC–UV method with column-switching.

Although RBX is marketed as racemic drug, its activity is primarily due to the (S,S)-enantiomer. Turnpenny and Fraier [10] have developed a chiral LC tandem mass spectrometry method for the analysis of both enantiomers in rat plasma and brain homogenates. Only a small change in the (S,S)/(R,R) enantiomeric ratio occurred in rat plasma and no change was observed within brain tissue following subcutaneous administration of RBX.

The analysis of RBX in biological samples is hampered by its weak UV absorption and its lack of native fluorescence. This is especially the case for small volume microdialysis samples containing low concentrations of the drug. Since RBX does not possess a native fluorescence, a pre-column derivatization step with a fluorophore was necessary. Several different fluorophores have been described in literature for the determination of RBX, i.e. FMOC-Cl (9-fluorenylmethyl chloroformate) [4], NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) [5] and NBD-Cl (7-chloro-4-nitro-2,1,3-benzoxadiazole) [21]. FMOC-Cl is widely used for derivatization of amines; it can react rapidly and quantitatively with both primary and secondary amino compounds under basic conditions to produce a highly fluorescent derivate [27,28]. FMOC-Cl offered superior reagent stability and detection sensitivity compared to other methods using alternative derivatizing agents [29]. Fig. 1 illustrates the chemical reaction between RBX and FMOC-Cl.

The aim of the present work was to develop a sensitive LC assay with fluorescence detection for the quantification of RBX in small volumes of hippocampal microdialysate and plasma samples of rat. Since only small or no changes in enantiomeric ratio were observed in rat plasma and brain homogenates respectively [10], we opted to develop a non-chiral method. The method included a pre-column derivatization with FMOC-Cl comprising a liquid–liquid extraction step. The obtained method was validated according to the EMA and FDA guidelines for bioanalytical method validation [30,31]. The method was sensitive enough for determining the brain and plasma concentration profiles in rats.

2. Experimental

2.1. Chemicals and reagents

Reboxetine mesylate was kindly donated by Pfizer (Groton, CT, USA). Acetone (analytical grade), acetonitrile (ACN, HPLC-grade) and tetrahydrofuran (THF, HPLC-grade) were obtained from Fisher Scientific (Loughborough, UK). Hexane was from Acros Organics (New Jersey, USA). The purified water used for preparing solutions was obtained from an Arium® pro UV purification system (Sartorius Stedim Biotech, Göttingen, Germany). Heparine Leo 5000 IU/mL was obtained from Leo Pharma (Wilrijk, Belgium) and Nembutal® was from Ceva (Brussels, Belgium). Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA), calcium chloride hexahydrate and FMOC-Cl were obtained from Fluka (Buchs, Switzerland). Sodium chloride and L-proline were obtained from Sigma–Aldrich (Bornem, Belgium). Potassium chloride, perchloric acid 70% (analytical grade) and sodium tetraborate were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation and chromatographic conditions

All chromatographic experiments were carried out using a Shimadzu LC system (Shimadzu, Antwerp, Belgium), which consisted of two LC-10 AD pumps and SCL-10AVP system controller, a RF-10AXL fluorescence detector, a DGU-20A5 degasser and a Gilson auto sampler (Gilson, Villiers le Bel, France). Separations were performed on an Alltima™ C_{18} column (150 mm × 2.1 mm ID, 5-μm particle size, Grace Davison Discovery Sciences, Lokeren, Belgium) equipped with an Alltima C_{18} guard column (7.5 mm × 2.1 mm ID, 5-μm particle size, Grace Davison Discovery Sciences) and column inlet filter (Rheodyne 3 mm; Grace Davison Discovery Sciences). The injection volume was 20 μL and the flow rate was set at 0.25 mL/min. Fluorescence detection was performed at excitation wavelength of 260 nm and emission wavelength 314 nm (medium sensitivity/gain 1 for microdialysates and low sensitivity/gain 2 for plasma samples). Gradient elution was performed using mobile phase A (water:ACN:THF 45:50:5, V/V/V) and mobile phase B (water:ACN:THF 20:75:5, V/V/V). The mobile phases were degassed for 15 min on a Branson 5200 ultrasonic bath (Danbury, CT, USA) after preparation. Elution started with 80% B for 9 min and then the linear LC gradient was 80–100% B in 1 min, 100% B for 5 min, 100–80% B in 1 min and 80% B for 4 min. The total run time was 20 min. Integration of the chromatograms was performed with Chromeleon® version 6.80 (Dionex, Amsterdam, The Netherlands).

2.3. Preparation of calibration standards and quality control (QC) samples

2.3.1. Stock and working solutions of RBX

The stock solution of RBX (free base, 1 mg/mL) was prepared in purified water. The in-between stock solution of 10 μg/mL was obtained by diluting 100 μL of the stock solution up to 10 mL with water. The stock solution and the in-between stock solution were made every 3 months. The working standard solutions of serial concentrations were made weekly by diluting the in-between stock solution with purified water. Stock and working solutions were stored at 4 °C. Intraperitoneal (i.p.) solution was prepared by dissolving 10 mg of RBX salt (i.e. 7.63 mg RBX free base) in 1 mL physiological saline to administer a dose of 20 mg/kg by i.p. injection.

2.3.2. Calibration standards and QC samples for microdialysate analysis

Calibration standards were prepared by mixing 25 μL of RBX working standard solutions (4, 10, 20, 50, 80, 120, 180, 240, 300 or 400 ng/mL) with 25 μL of purified water resulting in (2, 5, 10, 25, 40, 60, 90, 120, 150 and 200 ng/mL) final concentrations. QC samples were obtained by spiking 25 μL of blank dialysate with 25 μL (4, 10, 180 or 300 ng/mL) of RBX working solutions resulting in (2, 5, 90 and 150 ng/mL). Blank hippocampal microdialysates for QC sample preparation were obtained by continuous overnight microdialysis probe perfusion with modified Ringer's solution as a perfusion fluid (for further information see Section 2.6.2). The collected microdialysates were stored in the freezer at −20 °C.

2.3.3. Calibration standards and QC samples for plasma analysis

Calibration standards made in water were obtained by diluting 10 μL of RBX working solutions (100, 300, 500, 750, 1250, 1750, 2500 and 3500 ng/mL) with 40 μL purified water resulting in (20, 60, 100, 150, 250, 350, 500 and 700 ng/mL) final concentrations. For QC plasma samples 40 μL of pooled blank plasma was spiked with 10 μL RBX working standard solutions resulting in following nominal concentrations (20, 60, 350 and 500 ng/mL). Blank plasma for preparing the QC samples was obtained by withdrawing blood from the heart of the rat after being euthanized by CO_2 (see Section 2.6.1). The blood samples were collected in Na_2EDTA collection tubes and centrifuged at 13,700 × *g* for 3 min at 4 °C using a microcentrifuge (MIKRO 200 centrifuge, Hettich GmbH & Co., Tuttlingen, Germany). Plasma was kept at −20 °C in the freezer.

2.4. Derivatization procedure

2.4.1. Derivatizing working solutions

FMOC-Cl stock solution of 18 mM was obtained by dissolving 4.66 mg of FMOC-Cl in 1 mL acetone; the stock solution can be kept in the freezer for 2 months. 200 μL of the stock solution was diluted up to 10 mL with ACN to obtain the 0.36 mM FMOC-Cl working solution. Borate buffer (0.2 M, pH 9) was prepared by dissolving 0.7630 g of sodium tetraborate in 8 mL of purified water. The pH of the solution was adjusted to 9.00 ± 0.05 by a calibrated Metrohm

744 pH-meter (Metrohm Ltd., Herisau, Switzerland) using 2 M hydrochloric acid solution and the volume was completed to 10 mL with purified water. The L-proline solution (100 mM) was made by dissolving 0.1151 g of this amino acid in 10 mL purified water. All of these working solutions were prepared every week and were kept at 4 °C.

2.4.2. Derivatization procedure for brain microdialysis samples

A volume of 50 μL of calibration standard, QC sample or unknown sample was mixed with 25 μL of 0.2 M borate buffer pH 9 and 25 μL of 0.36 mM FMOC-Cl. The mixture was left at ambient temperature for 15 min before adding 25 μL of 100 mM L-proline to quench the chemical reaction. 2 min later 300 μL of n-hexane was added and the sample was vortexed for 1 min. After 20 min, the aqueous layer was discarded and the n-hexane layer was evaporated by exposing to a nitrogen stream at room temperature. The dried sample was re-dissolved in 50 μL of mobile phase B and injected into the LC system.

2.4.3. Sample preparation and derivatization procedure for plasma samples

Plasma protein precipitation was achieved by adding 150 μL of ACN to 50 μL of calibration standard, QC sample or unknown sample. After mixing for 1 min, the sample was centrifuged at 9500 g for 15 min. The supernatant was evaporated under nitrogen stream and re-dissolved in 50 μL of purified water. This sample is derivatized as described for microdialysis samples (Section 2.4.2).

2.5. Method validation

The optimized method was validated according to EMA and the FDA guidelines for bioanalytical method validation, in terms of selectivity, linearity, accuracy, precision and stability [30,31], both for microdialysates and plasma samples.

2.5.1. Selectivity

The selectivity of the method was assessed by examining the absence of potential interfering peaks. This was performed by comparing chromatograms of 6 blank microdialysates obtained from different rats with dialysate spiked at the lower limit of quantification. The same was done for plasma.

2.5.2. Limits of detection and quantification

The limit of detection (LOD) was estimated as the concentration yielding a signal to noise of 3. The lower limit of quantification (LLOQ) was defined as the lowest concentration which could be measured with a precision (RSD %) not exceeding 20% and with an accuracy between 80% and 120% ($n = 5$).

2.5.3. Linearity

Linearity of the calibration curves was evaluated using external calibration standards in water. All standard solutions were analyzed on 5 different days. The calibration curves were constructed using linear regression of the peak area versus the nominal concentration.

2.5.4. Analytical precision

Repeatability (intra-day precision) and intermediate or inter-day precision were determined by repeated measures of standard solution within 1 day and on 5 different days. Analytical precision was demonstrated for the LLOQ, low concentration level (about three times of the LLOQ), medium concentration level (about 50% of the highest calibration standard) and a high concentration level (at least at 75% of the upper calibration range).

2.5.5. Accuracy and method precision

Accuracy was assessed by analyzing 5 replicates of blank microdialysate or blank plasma spiked at four levels of RBX concentration. The percentage of recovery was determined comparing the experimental values, calculated using the calibration line constructed with standards in water, to the nominal values. Method precision was evaluated using the data of the accuracy testing. Method repeatability was assessed by analyzing 5 replicates of each sample within 1 day. Intermediate precision was obtained by analyzing each concentration level on 5 days.

2.5.6. Stability

The stability experiments were performed at three concentration levels, i.e. low, medium and high levels in both spiked brain microdialysates and spiked plasma samples. Four different stability experiments were performed. In the first set of experiments the stability of the derivate in mobile phase B was studied over 24 h after derivatizing the samples. During the second experiment, spiked underivatized samples were allowed to stand on the bench-top for 5 h and 24 h at room temperature. Thirdly, the freeze and thaw stability was assessed by storing the spiked samples of each concentration at −20 °C for 24 h, whereafter they were thawed unassisted at room temperature. 50 μL from each sample was derivatized and injected on the LC system while the rest was re-stored in the freezer. This procedure was repeated 2 times to obtain three freeze–thaw cycles. The fourth experiment was accomplished by storing the spiked samples at −20 °C for a month before being treated and analyzed to assess long term freezer storage stability.

2.6. Application of the method

2.6.1. Animals

All animal experiments were carried out according to the national guidelines [Belgian guideline on the protection of laboratory animals (KB November 1993) and the Revised European Guideline (appendix E to ETS123)] on animal experimentation and were approved by the Ethical Committee for Animal Experiments of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel. All efforts were made to minimize animal suffering. The experiments were performed on male albino Wistar rats (Charles River, Brussels, Belgium), weighing 270–340 g.

2.6.2. Brain microdialysis

2.6.2.1. Surgical procedures and stereotaxic implantation of the microdialysis probe. Rats were first anesthetized with a mixture of ketamine (66.7 mg/kg i.p.; Ketamine 1000 Ceva®, Ceva Sante Animale, Brussels, Belgium) and diazepam (5 mg/kg i.p.; Valium®, Roche, Brussels, Belgium) and placed on a Kopf stereotaxic frame (ear bars positioned symmetrically). The skull was exposed and two burr holes were drilled to implant the guide cannulae (MAB 2/6/9.14.IC, Microbiotech/se AB, Stockholm, Sweden) positioned 3 mm above the left and right dorsal hippocampi according to the atlas of Paxinos and Watson [32] (coordinates relative to bregma: L: ±4.6, A: −5.6 and V: +4.6). The guide cannula was fixed to the skull with dental acrylic cement. After surgery, a microdialysis probe (MAB 6.14.3, Microbiotech/se AB) with a membrane length of 3 mm and a molecular cut-off value of 15 kDa was introduced via the cannula in both hippocampi. The animals received ketoprofen (4 mg/kg i.p.; Ketofen®, Merial, Brussels, Belgium) as analgesic to assure post-operative pain control. The probes were continuously perfused with modified Ringer's solution (composition in mM: NaCl 147, KCl 4 and $CaCl_2.6H_2O$ 2.3) at a constant flow rate of 2 μL/min using a microdialysis pump (CMA 100; CMA Microdialysis, Solna, Sweden). Animals were allowed to recover from surgery overnight and dialysate sample collection was started the following day. During the actual experimental procedures, rats were placed

in experimental cages and allowed to move freely. All animals had access to tap water and standard laboratory chow ad libitum.

2.6.2.2. In vivo experimental protocol. Two dialysate samples were collected before the i.p. administration of 20 mg/kg RBX salt. In the first 2 h after RBX administration, microdialysis samples were collected every 20 min, followed by a second 2 h of 30 min intervals collection. Left side samples collection was started 10 min before the injection of RBX, while for the right hippocampus the collection was started immediately after administration of the dose. The sampling times were time averaged for collection time intervals. 25 μL of the collected samples was mixed with 25 μL of purified water before the derivatization step. Samples were stored in the freezer for maximum 24 h before analysis.

2.6.3. Plasma samples

2.6.3.1. Surgery. Rats were anesthetized with pentobarbital sodium (Nembutal®, 60 mg/kg i.p.) and were kept under anesthesia during the experiment. Serial blood samples were collected through an indwelling cannula in the right femoral artery. The cannula (ID = 0.58 mm; OD = 0.96 mm) was made from 20 cm pyrogen-free polyethylene tubing (Portex Limited, Hythe, Kent, UK). The arterial cannula was filled with saline containing 100 IU/mL heparin to prevent clotting.

2.6.3.2. Blood sampling protocol. For determination of plasma RBX levels, serial arterial blood samples (100 μL) were taken at predefined time points and drawn into 0.5 mL polypropylene tubes (Eppendorf, Hamburg, Germany) containing Na_2EDTA as an anticoagulant. Centrifugation was performed as described in Section 2.3.3. A pre-dosing blood sample was collected before RBX injection. Blood samples were collected over a 210 min sampling period in anesthetized animals according to the protocol demonstrated in Fig. 4B. The total blood sample volume collected did not exceed 2.0 mL. Samples were stored in the freezer for maximum 24 h before analysis. Upon analysis 40 μL of plasma was mixed with 10 μL of purified water. Protein precipitation was performed by adding ACN as indicated in Section 2.4.3 before derivatization of the samples. Plasma samples were diluted with mobile phase B when necessary.

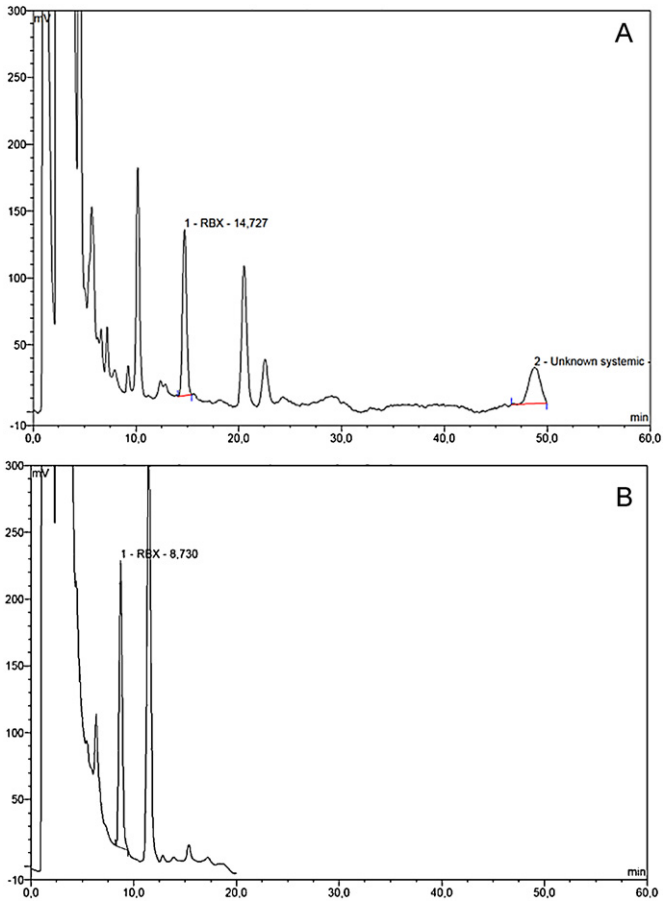

Fig. 2. (A) a typical chromatogram of 100 ng/mL RBX standard solution under optimized conditions obtained by isocratic elution using a mobile phase composed of water:ACN:THF (30:65:5, V/V/V) at a flow rate of 0.20 mL/min showing the unknown peak at ±50 min, (B) a representative chromatogram of 40 ng/mL RBX standard solution under the optimized conditions performing gradient elution at a flow rate of 0.25 mL/min. (Peak 1 = RBX, the detector sensitivity settings in A are lower than B.)

3. Results and discussion

3.1. Microdialysis samples

3.1.1. Method development and optimization

As discussed in the introduction, the analysis of RBX in biological samples is hampered by the weak UV absorption and its lack of native fluorescence. Indeed, the obtained sensitivity with a LC–UV system was too low for our purpose (LOD of approximately 20 ng/mL). Therefore, a LC method coupled to fluorescence detection was investigated. FMOC-Cl was chosen as fluorescent derivatization agent. The starting conditions of our method were based on the method developed by Raggi et al. [4]. However, several modifications were made as described below. First, small modifications to simplify the derivatization protocol were done. Since only small volumes of microdialysate samples (40 μL) are obtained, all volumes were reduced with a fraction of 1/10 (for protocol: see Section 2.4.2). For solubility reasons of tetraborate a 0.2 M borate buffer was used instead of the 0.8 M buffer used by Raggi et al. [4]. A higher response was observed when increasing the pH from 8 to 9. During the extraction step, the mixture was left for 20 min before being extracted in order to attain equilibration between the two phases. In addition, the further liquid–liquid extraction with ACN in order to eliminate interferences from the matrix as described in [4] was redundant in our case. Instead the n-hexane layer was evaporated to dryness under a nitrogen stream, whereafter the dried sample was re-dissolved in 50 μL mobile phase B (water:ACN:THF 20:75:5, V/V/V).

Secondly, the chromatographic parameters were further optimized. Initially, chromatographic separation was performed using isocratic elution on a reversed phase C_{18} column. A mobile phase composed of THF: 0.01 M acetate buffer, pH 7.5 (50:50, V/V) and a flow rate of 0.15 mL/min were selected. Due to instability and the corrosive effects of THF, a smaller percentage of THF was used in the mobile phase. Different combinations of mobile phases consisting of methanol or ACN, THF and acetate buffer were tested. In addition, the influence of the pH on the retention time of the derivatized RBX was investigated. No change in the retention time of the RBX derivate was observed when comparing mobile phases containing an acetate solution at pH 4.5 or pH 7.5 with water. Fig. 2A shows a chromatogram under optimized conditions, including a mobile phase consisting of (water:ACN:THF 30:65:5, V/V/V) and flow rate of 0.20 mL/min. The retention time of RBX under these conditions was 14.5 min. However, the total run time was around 60 min, since an unknown system peak was always eluted after approximately 50 min. In order to reduce the total run time, gradient elution was carried out. By applying gradient elution starting with 80% mobile phase B for 9 min, followed by 80–100% B in 1 min, 100% B for 5 min, 100–80% B in 1 min and 80% B for 4 min, and setting the flow rate at 0.25 mL/min, the total run time was reduced to 20 min. The retention time of RBX under the optimized conditions is 8.80 ± 0.10 min (mean ± SD, $n = 12$; Fig. 2B).

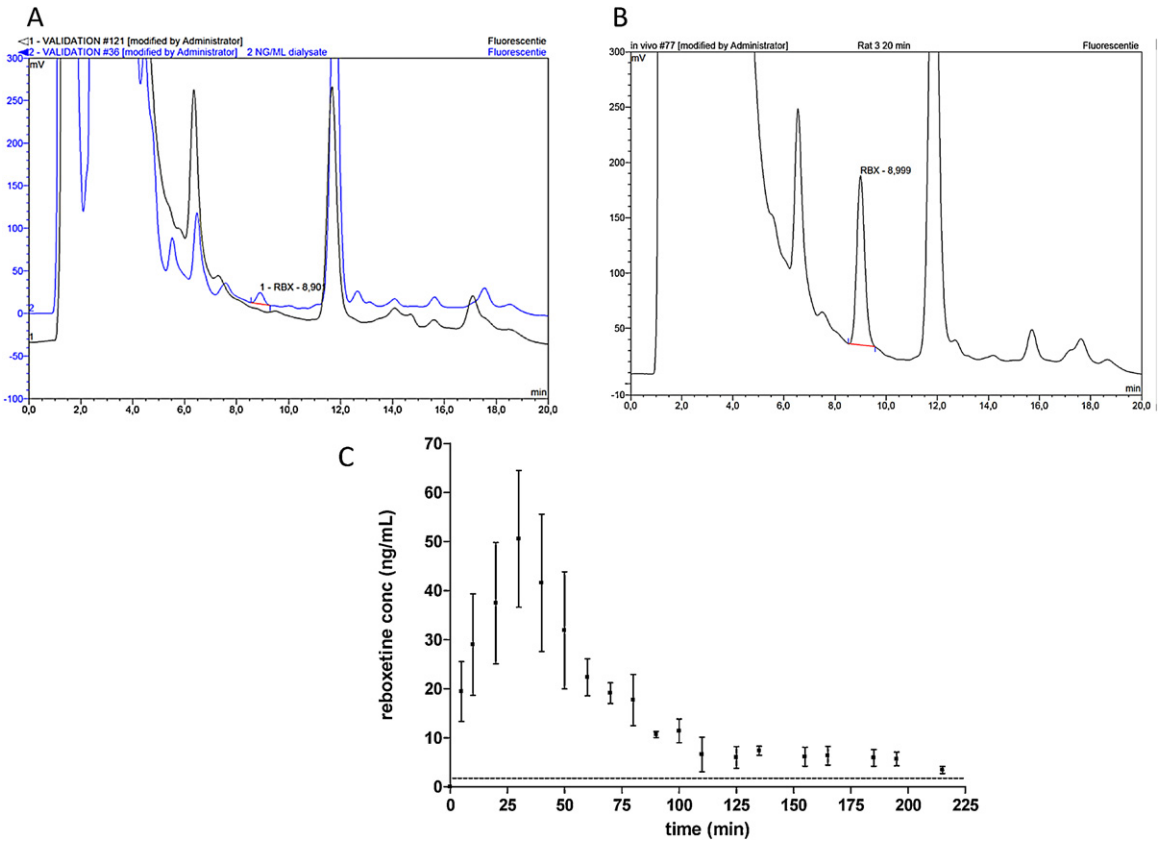

Fig. 3. Typical chromatograms of (A1) unspiked blank hippocampal dialysate; (A2) blank dialysate spiked with 2 ng/mL of RBX (LLOQ); (B) real sample collected from right hippocampus of the rat 20 min after i.p. injection of 20 mg/kg of RBX salt; and (C) brain microdialysate concentration–time profile following 20 mg/kg i.p. injection of RBX to rats ($n = 4$), the dashed line represents the LLOQ (peak 1 = RBX).

3.1.2. Method validation

3.1.2.1. Selectivity. Blank brain microdialysates and dialysate samples spiked at 2 ng/mL were evaluated for selectivity. As shown in Fig. 3A, no significant interference peaks appeared at the retention time of RBX, indicating good selectivity of this method.

3.1.2.2. Limits of detection and quantification. The LOD, estimated as concentration yielding signal to noise of 3, was found to be 0.6 ng/mL. The LLOQ, the lowest concentration that can be determined with acceptable accuracy and precision, was 2 ng/mL.

3.1.2.3. Linearity. Calibration curves were constructed using standards ranging from 2 to 200 ng/mL (with 10 concentration calibrants). A linear relationship within the studied concentration range was obtained. The linear regression equation was $y = (2.06 \pm 0.04)\ x + (0.47 \pm 1.69)$, ($r = 0.9992 \pm 0.0005$). The equation is expressed as mean ± SD ($n = 5$).

3.1.2.4. Analytical precision. The analytical repeatability ranged from 3.1% to 11.7% RSD. The inter-day precision ranged from 5.7% to 12.9% RSD ($n = 5$).

3.1.2.5. Accuracy and method precision. QC dialysate samples spiked at 2, 5, 90 and 150 ng/mL were used. An average recovery of 101.2 ± 8.2% (mean ± SD, $n = 40$) was obtained. Method repeatability was found to range from 1.5% to 11.4% RSD. The RSD values for intermediate precision ranged from 2.4% to 11.7%. The obtained values are within the acceptance criteria (not more than 20% at LLOQ and not more than 15% for the others). Therefore, the method can be considered accurate and precise for this application. Table 1 summarizes the obtained results.

Table 1
Method precision and accuracy for RBX in microdialysates. The observed concentration is expressed as mean ± SD ($n = 5$).

Nominal concentration (ng/mL)	Observed concentration (ng/mL)	Method precision RSD %	Accuracy %
Intra-day			
2	1.84 ± 0.23	11.4	91.7
5	5.38 ± 0.15	2.7	107.7
90	91.26 ± 1.33	1.5	101.4
150	156.91 ± 11.18	7.1	104.6
Inter-day			
2	1.98 ± 0.26	11.7	99.4
5	5.47 ± 0.15	6.0	98.2
90	91.12 ± 1.32	2.8	100.6
150	156.59 ± 11.15	2.4	105.8

3.1.2.6. Stability. The stability experiments were performed at three concentration levels (5, 90 and 150 ng/mL) in hippocampal microdialysis samples. The stability of the RBX–FMOC-Cl derivate in dialysate was examined over 24 h. The results show that the derivate is stable over the studied period. The QC microdialysate samples revealed reasonable stability (i.e. 85–115%) under all conditions tested (Table 2).

3.1.3. Application of the method

The validated LC method was applied to obtain the brain microdialysate RBX concentration–time profile following i.p. administration of RBX salt to the rats ($n = 4$). Fig. 3B shows a representative chromatogram for RBX 20 min after i.p. administration of the drug (20 mg/kg) to the rat. The average hippocampal dialysate concentration–time profile is shown in Fig. 3C.

Table 2
Stability of RBX in microdialysis samples. The % stability is expressed as the mean measured concentration ($n = 2$) at the indicated time divided by mean measured concentration ($n = 2$) at zero time × 100.

Nominal concentration (ng/mL)	Stability (%)							
	Derivate		Spiked samples (stored before derivatization)					
	Room temperature		Room temperature		Freeze–thaw			Long term freezer storage at −20 °C
	5 h	24 h	5 h	24 h	Cycle 1	Cycle 2	Cycle 3	Month
5	98.0	93.6	97.6	95.6	96.0	96.1	95.8	91.3
90	94.3	92.7	98.5	95.2	98.9	97.7	94.5	93.4
150	97.5	95.2	96.3	92.3	99.3	96.7	92.9	92.9

3.2. Plasma

3.2.1. Plasma protein precipitation

A sample preparation and pretreatment step needs to be included before plasma samples can be analyzed. A classical plasma protein precipitation step was chosen in this case. Different protein precipitating agents (ACN, methanol and perchloric acid) were evaluated, including different mixtures of ACN with methanol. The optimal results were obtained using ACN in a ratio of 3:1 ACN:plasma (V/V).

Furthermore, the supernatant was derivatized immediately or evaporated to dryness and reconstituted with water before derivatization. It was observed that the derivatization of the supernatant directly without evaporation led to buffer precipitation during the derivatization procedure. This can be explained by the increased ACN concentration in the reaction medium. Therefore, the supernatant was evaporated to dryness and reconstituted with water before being derivatized.

3.2.2. LC method coupled to fluorescence detection

A small interfering peak was eluted always very close to RBX after injection of blank plasma sample using the LC method described for brain microdialysis samples. Therefore further optimization procedures were performed in order to separate or to eliminate this interfering peak. The best results were obtained when the detector sensitivity settings were decreased 8 times; accordingly the linear range was adjusted. Since, higher concentrations of RBX were anticipated in plasma, this could be done without compromising the method.

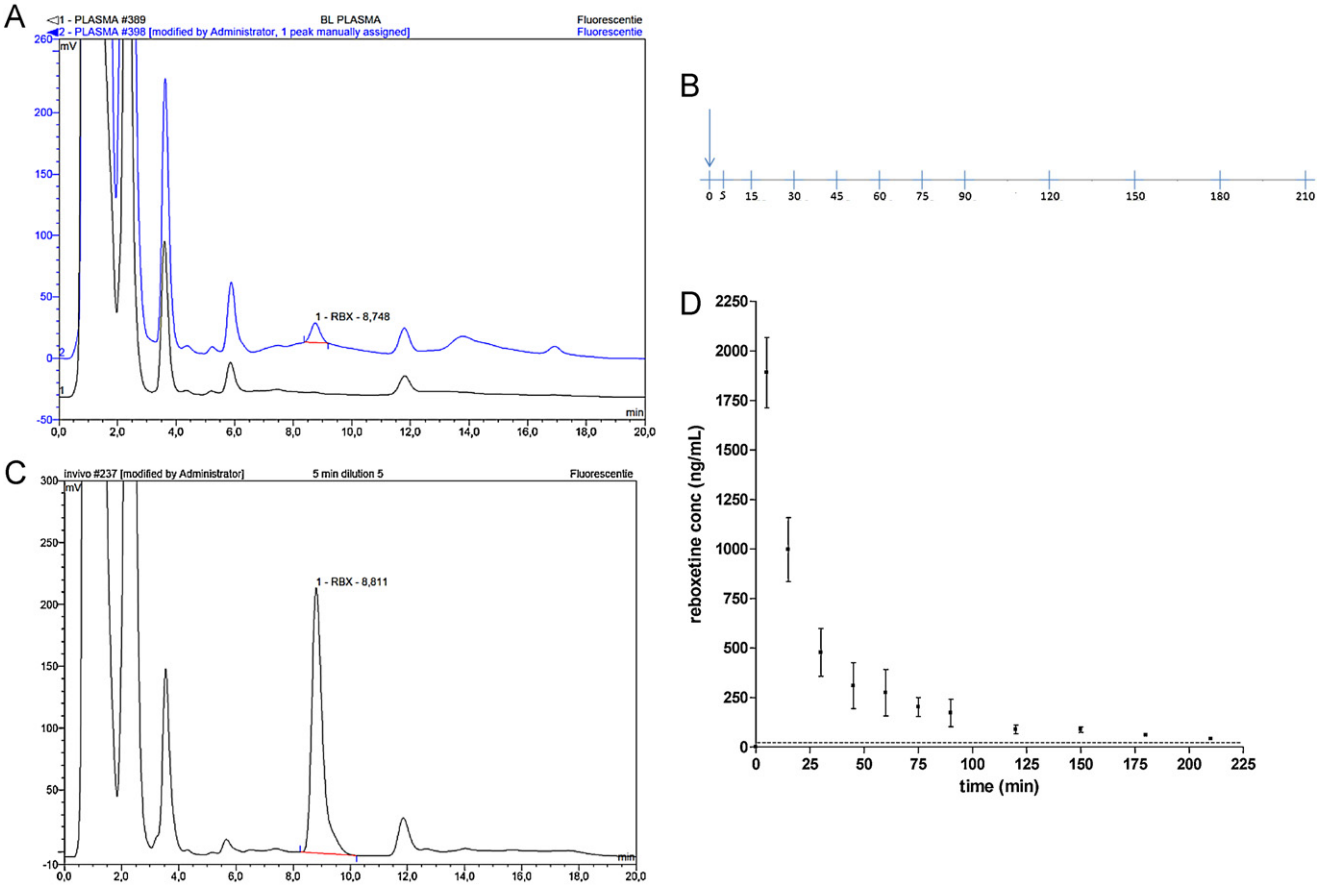

Fig. 4. Representative chromatograms of (A1) unspiked blank plasma; (A2) blank plasma spiked with 20 ng/mL of RBX (LLOQ); (B) timescale showing blood sample collection time in minutes, arrow indicates the i.p. injection of RBX; (C) real sample collected from arterial femoral artery 5 min after administration of 20 mg/kg i.p. dose of RBX to a rat, 5 times diluted with mobile phase B before LC analysis; and (D) plasma concentration–time profile after administration of 20 mg/kg i.p. dose of RBX to rats ($n = 2$), the dashed line represents the LLOQ (peak 1 = RBX).

Table 3
Method precision and accuracy for RBX in plasma. The observed concentration is expressed as mean ± SD ($n = 5$).

Nominal concentration (ng/mL)	Observed concentration (ng/mL)	Method precision RSD %	Accuracy %
Intra-day			
20	19.22 ± 2.15	11.0	96.1
60	57.64 ± 2.41	4.1	96.1
350	332.87 ± 5.82	1.8	95.1
500	541.27 ± 9.34	1.7	108.3
Inter-day			
20	18.54 ± 2.16	11.5	92.7
60	60.43 ± 4.41	8.3	100.7
350	345.39 ± 6.88	2.0	98.7
500	491.09 ± 11.77	2.4	98.2

Table 4
Stability of RBX in plasma samples. The % stability is expressed as the mean measured concentration ($n = 3$) at the indicated time divided by mean measured concentration ($n = 3$) at zero time × 100.

Nominal concentration (ng/mL)	Stability (%)							
	Derivate		Spiked samples (stored before derivatization)					
	Room temperature		Room temperature		Freeze–thaw			Long term freezer storage at −20 °C
	5 h	24 h	5 h	24 h	Cycle 1	Cycle 2	Cycle 3	Month
60	96.4	94.2	94.4	84.6	96.8	91.0	88.2	86.0
350	96.5	94.4	95.4	88.5	98.0	93.6	87.0	87.3
500	96.2	95.3	93.5	88.8	98.4	93.7	87.2	86.7

3.2.3. Method validation

3.2.3.1. Selectivity. The selectivity of the method was assessed by comparing chromatograms of blank plasma obtained from 6 different rats with plasma samples spiked at 20 ng/mL. As shown in Fig. 4A, no interfering peaks appeared at the retention time of RBX.

3.2.3.2. Limits of detection and quantification. As mentioned above the sensitivity of the detector settings was lower compared to the method used for quantification of RBX in brain microdialysis samples. Hence, higher values of LOD and LLOQ were obtained. The LOD and the LLOQ were found to be 6 and 20 ng/mL respectively.

3.2.3.3. Linearity. The calibration curve was linear over the range 20–700 ng/mL (with 8 concentration calibrants). The linear regression equation was $y = (0.262 \pm 0.003)\ x + (0.08 \pm 0.17)$, ($r = 0.9989 \pm 0.0004$). The equation is expressed as mean ± SD ($n = 5$).

3.2.3.4. Analytical precision. The repeatability and the inter-day precision ranged from 0.9% to 4.8% and from 1.6% to 5.4% RSD respectively ($n = 5$).

3.2.3.5. Accuracy and method precision. Accuracy was evaluated by analyzing 5 replicates of blank plasma spiked at 20, 60, 350 and 500 ng/mL of RBX. An average recovery of 98.5 ± 7.3% (mean ± SD, $n = 40$) was obtained. Method repeatability and intermediate precision were found to range from 1.7% to 11.0% and from 2.0% to 11.5% RSD respectively. Table 3 summarizes the obtained results.

3.2.3.6. Stability. The stability experiments were done on three concentration levels (60, 350 and 500 ng/mL). The derivate was stable over the 24 h time period. The stability of plasma QC samples revealed an acceptable stability under all tested conditions (within 85–115%) (Table 4).

3.2.4. Application

This validated method for quantifying RBX in plasma samples was applied to investigate the plasma concentration–time profile of this antidepressant drug in anesthetized rats ($n = 2$). For this purpose, a dose of 20 mg/kg RBX salt was administered i.p. to the rats. Plasma samples were taken over a time period of 210 min. Fig. 4C represents a typical chromatogram for RBX after administrating a 20 mg/kg i.p. dose to the rat at time 5 min post-injection. The average plasma concentration–time profile of RBX after administration of the drug to rats ($n = 2$) is shown in Fig. 4D.

4. Conclusion

In this study, a LC method with fluorescence detection was developed and validated to quantify RBX in small volume brain microdialysis samples and in plasma samples of the rat. The method was applied for the analysis of brain microdialysis and plasma samples after 20 mg/kg i.p. injection of RBX salt. The method was found to be suitable for PK profiling in both biological fluids.

Disclosure statement

The authors state no conflict of interest.

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