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Quantification of 4 antidepressants and a metabolite by LC–MS for therapeutic drug monitoring

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ABSTRACT

A liquid chromatography method coupled to mass spectrometry was developed for the quantification of bupropion, its metabolite hydroxy-bupropion, moclobemide, reboxetine and trazodone in human plasma. The validation of the analytical procedure was assessed according to Société Française des Sciences et Techniques Pharmaceutiques and the latest Food and Drug Administration guidelines. The sample preparation was performed with 0.5 mL of plasma extracted on a cation-exchange solid phase 96-well plate. The separation was achieved in 14 min on a C18 XBridge column (2.1 mm × 100 mm, 3.5 μm) using a 50 mM ammonium acetate pH 9/acetonitrile mobile phase in gradient mode. The compounds of interest were analysed in the single ion monitoring mode on a single quadrupole mass spectrometer working in positive electrospray ionisation mode. Two ions were selected *per* molecule to increase the number of identification points and to avoid as much as possible any false positives. Since selectivity is always a critical point for routine therapeutic drug monitoring, more than sixty common comedications for the psychiatric population were tested. For each analyte, the analytical procedure was validated to cover the common range of concentrations measured in plasma samples: 1–400 ng/mL for reboxetine and bupropion, 2–2000 ng/mL for hydroxy-bupropion, moclobemide, and trazodone. For all investigated compounds, reliable performance in terms of accuracy, precision, trueness, recovery, selectivity and stability was obtained. One year after its implementation in a routine process, this method demonstrated a high robustness with accurate values over the wide concentration range commonly observed among a psychiatric population.

1. Introduction

Antidepressant treatments are widely prescribed for depressive and other psychiatric disorders. They are classified into five classes. Among them, moclobemide (MOC) is a reversible and selective monoamine oxydase A inhibitor, bupropion (BUP) and its main active metabolite 2-hydroxybupropion (OHBUP) are selective inhibitors of catecholamine recapture (noradrenalin and dopamine), reboxetine (REB) is a selective inhibitor of noradrenalin recapture and finally trazodone (TRAZ) inhibits the serotonin recapture and antagonises serotonin receptors [1,2].

Because of the risk of non-compliance, drug interaction and inter individual variability in dose–response, therapeutic drug monitoring (TDM) is of interest to optimise the pharmacological treatment. Although a therapeutic window has not been clearly defined for all antidepressants, some range indications were reported for the drugs of interest [3,4]. Additional studies are still required to better define the therapeutic window, in particular for new molecules with low plasma concentrations such as BUP. A low limit of quantification (LLOQ) close to the ng/mL, is therefore necessary to discriminate between low plasma concentrations due for example to a rapid metabolism status and non-compliance suggested by the total absence of the considered therapeutic agent. Furthermore, wide calibration ranges are necessary to cover the range of plasma concentrations measured in clinical practise including cases of potential overdose.

BUP, MOC, REB, and TRAZ are currently quantified separately in plasma by different approaches such as gas chromatography (GC) coupled to mass spectrometry (MS) or nitrogen phosphorus detection (NPD) and liquid chromatography (LC) coupled to

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ultraviolet detection, MS or MS/MS [2,5,6], generally in positive electrospray ionization (ESI) mode. Some stereoselective methods were reported for BUP and REB, using either LC–MS/MS [7,8] or capillary zone electrophoresis [9]. New approaches were described for the simultaneous dosage of several antidepressants [10–14], in which one quantifies three of the five targeted molecules (REB, MOC, and TRAZ) with high reported LLOQ (10 ng/mL for REB and 100 ng/mL for MOC and TRAZ) [14]. High throughput and time saving methods are increasingly necessary for TDM laboratory. However, to our knowledge, no method was reported for BUP with simultaneous quantification of other antidepressants in plasma.

The aim of this work was, to provide for the routine use in a TDM laboratory, a simultaneous quantification of selected antidepressants by LC–MS with respect to the current validation procedures.

2. Experimental

2.1. Chemicals and reagents

Bupropion hydrochloride and its metabolite 2-hydroxybupropion were kindly provided by GlaxoSmithKline (Franklin Plaza, PA, USA), moclobemide by Roche (Basel, Switzerland), and trazodone hydrochloride by Vifor (Villars-sur-Glâne, Switzerland). Reboxetine mesylate hydrate was purchased at Sigma–Aldrich (St. Louis, MO, USA). Litracen (LIT) from Lundbeck (Glattburg, Switzerland) and remoxipride (REMO) from Astra Zeneca (London, England) were used as internal standards (IS).

A hydrochloric acid (HCl) solution at 0.01 N was prepared with 37% HCl provided by Merck (Darmstadt, Germany) diluted with ultrapure water obtained from a Milli-Q® RG with a QPAQ2 column system from Millipore (Billerica, MA, USA). The mobile phase was acetonitrile (ACN) from J.T. Baker (Deventer, Netherland) and ammonium acetate buffer (pH 9, 50 mM) from ammonium acetate puriss p.a. for MS provided by Sigma–Aldrich (Steinheim, Germany). Methanol (MeOH), formic acid, 25% ammonium hydroxide and isopropanol from Sigma–Aldrich and 85% ortho-phosphoric acid from Merck were used for the solid phase extraction. For the preparation of calibration and control samples, human plasma of different origins ($n > 10$) were tested and pooled. All plasma samples were obtained from the Hospital's blood transfusion centre (CHUV, Lausanne, Switzerland).

2.2. Working solution

Working solutions of 1 mg/mL of BUP, OHBUP, MOC, REBOX and TRAZ were prepared in MeOH. Drugs were divided into two groups ranging from 1 to 400 ng/mL for REB and BUP and 2–2000 ng/mL for OHBUP, MOC, and TRAZ, according to their therapeutic and observed plasma concentration ranges [3,4]. Plasma samples were spiked at the appropriate concentration by freshly prepared subsequent dilutions of the working solution. Calibration standards (CS) and validation standards (independent seeded controls, VS), were obtained by using different batches. CS and VS were independently prepared. Finally, internal standard (IS) solutions of REMO and LIT were prepared at 1 μg/mL in HCl 0.01 N. Working solutions, IS solution and spiked plasma were all stored at −20 °C prior to analyses. No degradation was observed for the target drugs at −20 °C after one year of storage for the working solutions and IS solution and after two months storage for the spiked plasma samples.

2.3. Equipment

2.3.1. Sample pre-treatment and extraction

A solid phase extraction was performed on plasma samples after thawing and storage at room temperature. The sample extraction was carried out onto a 10 mg SPE 96-well plate Oasis MCX support from Waters (Milford, MA, USA). 50 ng of REMO and LIT were added to 500 μL of plasma sample before a dilution with 4% H_3PO_4 (1:1). After vortexing, the sample was loaded onto a SPE 96-well plate previously conditioned with 500 μL of MeOH and 500 μL of H_2O. Three successive washing steps consisting of 500 μL of 2% formic acid in H_2O (v/v), and two times 250 μL of MeOH were applied on the sample. The elution step was achieved with two times 125 μL of 5% ammonium hydroxide in MeOH/isopropanol (1:1, v/v) followed by 250 μL of H_2O prior to injection into the HPLC–MS system. Between each extraction step, the wells were slowly dried.

2.3.2. HPLC–MS analysis

The liquid chromatography system consisted of an Agilent HP1100 binary pump equipped with a 100-vial autosampler from Agilent Technologies (Santa Clara, CA, USA) coupled to a MSD Agilent simple quadrupole mass spectrometer equipped with an ESI source working in the positive ionization mode. The system was controlled by the Chemstation 8.01.01 from Agilent Technologies. The system was maintained at 20 °C in an air conditioned room. Optimal chromatographic conditions were determined using Osiris software version 4.1 from Datalys (Saint Martin d'Hères, France) as previously described [15], and MS signal response was optimised thanks to an experimental design strategy obtained with StatGraphics Plus 5.1 from Statistical Graphics Corp. (Herndon, VA, USA). Drying gas flow, nebulizer pressure, drying gas temperature, capillary voltage, were set at 13 L/min, 40 psig, 350 °C, 1250 V, respectively. The m/z ratios used were: 240, 256, 269, 314, 372 for BUP, OHBUP, MOC, REB and TRAZ, respectively. Fragmentor voltage was set at 80 V except for TRAZ and LIT which was at 60 V.

Separation was carried out (5 μL of sample) on a XBridge C18 column (2.1 mm × 100 mm, i.d. 3.5 μm) equipped with a XBridge guard cartridge (2.1 mm × 10 mm, i.d. 3.5 μm) provided by Waters (Milford, MA, USA). Ammonium acetate 50 mM adjusted to pH 9 with 25% ammonium hydroxide (A) and ACN (B) were used as the mobile phase with a flow rate of 300 μL/min with the following gradient program: 26% to 60% of B from 0 to 4.3 min, then 60% B was maintained from 4.4 to 7.4 min. Finally, a washing step at 90% of B was applied until 8.4 min followed by 5 min of reconditioning with the initial mobile phase condition. All analytes were quantified by the peak area ratio between the drug and the IS (LIT) in the single ion monitoring mode (SIM).

2.4. Method validation

The method validation was performed according to the recommendations of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) regarding total error concept [16,17] and the last Food and Drug Administration (FDA) proposal [18]. The selectivity, trueness, repeatability and intermediate precision were evaluated on three different validation series. Accuracy profile based on tolerance intervals was used to select the best calibration function and to determine the validated concentration ranges. The tolerance probability β was set at 80% and the acceptance limit at ±30% [17]. Matrix effects were qualitatively and quantitatively analysed. The qualitative part of the matrix effect evaluation was performed by the post-infusion of a standard solution of the analytes at 2 μL/min in the HPLC–MS system while different blank plasma ($n = 6$) were injected according to the HPLC–MS method described herein [19]. A concentration of 0.1 ng/mL was used corresponding to the lower end concentration signal response (approximately 5 ng/mL). Any alteration of the signal in the detection window of the studied antidepressants was considered as a harmful matrix effect as it leads to a modification of the peak area of the compound of interest [20].

The quantitative assay was inspired from the procedure reported by Matuszewski et al. [21,22]. Low (10 ng/mL) and high concentrations (340 ng/mL for REB, BUP, OHBUP and 1700 ng/mL for MOC, TRAZ) were prepared in triplicate with a pool of six different batches of blank plasma. Extraction recoveries were established as the ratio between plasma samples at the same corresponding levels spiked before and after the extraction. Matrix effect was determined by using the peak area ratio between plasma spiked after extraction and a standard solution directly analysed. The process efficiency was defined in the Matuszewski procedure as the ratio between peak areas from the plasma spiked before extraction and from a standard solution at the corresponding concentration directly analysed [21,22]. It incorporates the effects of both the extraction recoveries and the matrix effect [22,23]. A maximum relative standard deviation (RSD) value of 15% was accepted for each concentration.

For each validation series, the selectivity was assessed by injecting two different extracted blank plasma samples at the beginning of the series and after the highest concentration standards. Thus, any eventual carryover effect was detected. Potential drugs ($n = 60$) which could be prescribed together with the studied antidepressants as well as some of their metabolites were investigated (see Supplementary Materials). These molecules, spiked at 100 ng/mL in human plasma, were extracted and analysed with regards to their retention factor. In case of co-elution, the MS signal alteration was carefully studied by comparing peak area of the antidepressant alone and together with the potential interference spiked at two different concentrations.

CS at three concentrations were performed in duplicate at the following concentrations: 1, 200, 400 ng/mL for BUP, REB and 2, 1000, and 2000 ng/mL for MOC, TRAZ, and OHBUP. VS were prepared in quadruplicate at the five following concentrations: 1, 4, 10, 100, and 400 ng/mL for BUP, REB and 2, 25, 100, 1000, and 2000 ng/mL for MOC, TRAZ, and OHBUP. It can be mentioned that OHBUP is the main metabolite of BUP and could present a concentration 3 to 14 fold higher than BUP [24]. These standards were independently prepared for each series using a pool of six different human plasma samples. The concentrations were chosen to cover the plasma therapeutic range and expected patients' plasma levels [3,25].

The concentrations of the VS were back-calculated using the best calibration function and analysed during the same run. The lowest and the highest levels included in the VS with respect to the latest criteria [16–18,26] were considered as the limit of quantification. A dilution step with ultrapure water (1:1) was performed during each validation series to demonstrate the possibility to dilute a sample presenting a concentration above the validated range.

For the following stability tests, five different batches of blank plasma were selected and spiked at different concentrations depending on the drug calibration range. Low stability control was set at 6 ng/mL for all target drugs and high stability control at 320 ng/mL for BUP, REB and 1600 ng/mL for MOC, TRAZ, and OHBUP. They were immediately separated into eight aliquots of 0.5 mL for each concentration. One set of aliquots was directly analysed and was considered as the nominal concentration. The other sets of aliquots were then quantified after storage at room temperature for 24 h, 72 h, at 4 °C for 72 h and after one and three freeze–thaw cycles. The long term stability was also investigated by keeping the two remaining sets of aliquots at −20 °C for two months. The extracted samples were also analysed after 36 h at room temperature in order to evaluate the post-preparative stability. For all stability tests, the variation in antidepressant concentration was determined by the ratio between the level after storage and the nominal level.

2.5. Confirmation ions

In order to detect an influence on the quantification process from an unknown interference, a confirmation ion is used for each drug when required. The presence of an interference (i.e. comedication) with similar retention factor and m/z ratio will potentially lead to an overestimation of the drug concentration. The confirmation run allowing a peak identification/discrimination in HPLC–MS was performed using the same HPLC–MS as previously described, with the addition of the confirmation ions (m/z ratios: 184 for BUP, 238 for OHBUP, 182 for MOC, 176 for REB, 373 for TRAZ). The mean relative ion intensity was calculated as a ratio between the peak areas of the confirmation and the original ions, with an accepted range of ±30%.

3. Results and discussion

3.1. Sample preparation

Co-elution of endogenous compounds was reported to be responsible for erroneous results especially with an ESI source [20,22,23,27–30]. A fast and easily automatable SPE process was chosen among other purification procedures. All analytes have a pKa above 6 and so were extracted in acidic conditions (pH < 2) in their ionised form using a mixed mode cation exchange 96-well plate (MCX). An optimal recovery of the analytes was obtained by using a mixture of ammonium hydroxide 5% in MeOH/isopropanol (1:1, v/v) followed by an addition of the same volume of water prior to injection into the LC–MS.

3.2. HPLC–MS

Improvement of selectivity, signal response and peak feature were reported in alkaline pH for basic compounds due to the relatively large amount of organic solvent in the mobile phase [31,32]. Therefore, the separation was developed with various alkaline pH buffers and different ammonium solution concentrations. The optimal separation was found at pH 9. It was particularly important to obtain a baseline separation of the analytes because the loss of the hydroxyl group (m/z 16) of OHBUP (m/z 256) gives a peak contribution on the BUP channel (m/z 240). Moreover, TRAZ (m/z 372) and REMO (m/z 373) gave an isotopic peak contribution to each other. Since the single quadrupole has a low mass resolution, these two pairs of compounds should be baseline separated by the LC for an unambiguous discrimination.

The run time was 14 min including a washing step at 90% ACN which allowed to maintain assay robustness and HPLC performance. A reconditioning step of 5 min with the initial mobile phase composition achieved the run. A typical single ion monitoring (SIM) of the selected antidepressants is presented in Fig. 1. No interferences were observed in the total ion current chromatograms (TIC) of blank plasma during the selectivity and matrix effect assays.

Analogue molecules with similar physico-chemical properties and ionisation fractionation, which may also compensate for matrix effects, were tested as IS [23,33]. Litracen (m/z 278) and remoxipride (m/z 373), both antipsychotic agents, which present the advantage that they cannot be present in the patients' blood as they were withdrawn from the market several years ago, were found the best fit for the purpose.

3.3. Method validation

A clean baseline was recorded for each compound m/z ratio for all the blank plasma tested, without carryover between injections. The selectivity was further investigated by analyzing plasma samples spiked with sixty drugs susceptible to be taken together

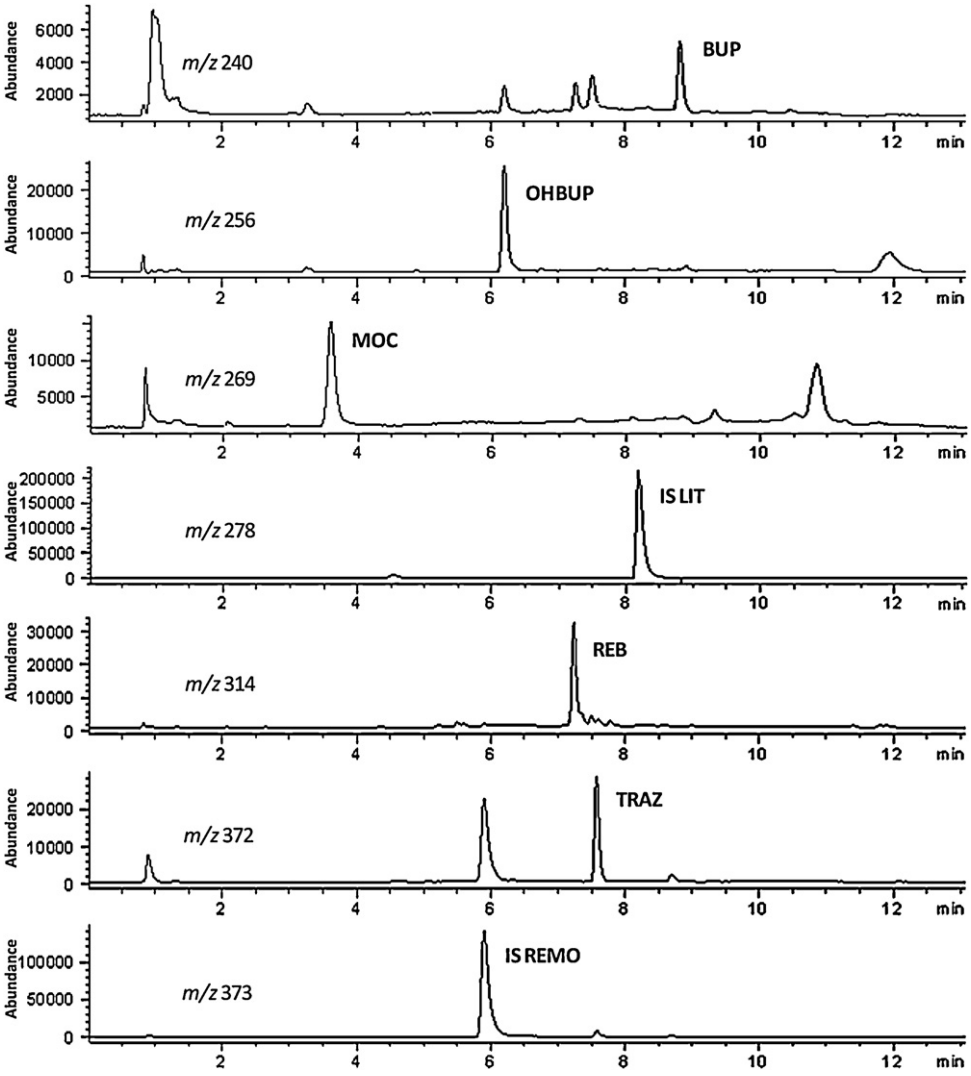

Fig. 1. Single ion monitoring chromatogram of a human plasma sample containing 10 ng/mL of bupropion (BUP, Rt = 8.9), reboxetine (REB, Rt = 7.3), moclobemide (MOC, Rt = 3.7), hydroxybupropion (OHBUP, Rt = 6.3), trazodone (TRAZ, Rt = 7.7), and 50 ng/mL of IS remoxipride (IS REMO, Rt = 5.6), IS litracen (IS LIT, Rt = 8.3). Note the contribution peak of IS REMO and OHBUP on TRAZ and BUP, respectively. Rt: retention time.

with the drugs of interest as well as some of their metabolites. No significant interference was observed for all the tested molecules (range: −4% to 2%). The highest variation was observed for BUP with methadone (−15%) which remains largely inferior to the clinical significance. Regarding matrix effect, no qualitative interferences were observed at the retention time of interest during the post-infusion test. Furthermore, on the different batches of blank plasma incorporated in every validation series either at the beginning of the series or after the highest CS, no interfering peaks were noticed for the selected antidepressants (data not shown).

The outcome of quantitative assessment for extraction recovery, matrix effect, and process efficiency is presented in Table 1. The process efficiency was highly repeatable (RSD ≤ 5%, $n = 3$) and ranged from 89 to 106% at high concentration and from 108 to 121% at low concentration. This increase at low concentration is predominantly due to the matrix effect measured between 116 and 130%. However, no clinically significant influence should be expected since the enhancement leads to an average maximum overestimation in absolute concentration of about 2 ng/mL. The matrix effects were observed with a maximum RSD of 8%. Finally, extraction recoveries remain very consistent at low and high concentration with a range between 88 to 96% and RSD ≤ 10%.

The best model was selected through the most favourable accuracy profile. The calibration curves were transformed with a quadratic regression by three calibration levels at 1, 200, 400 ng/mL for BUP, REB and 2, 1000, 2000 ng/mL for MOC, TRAZ, and OHBUP. LLOQ were set at 1 for BUP, REB and 2 ng/mL for MOC, TRAZ, and OHBUP. Good linearity was observed between calculated concentration and concentration of the analyte in the sample for the three validation series with a mean slope and determination coefficient higher than 0.95 and 0.99, respectively. All the validation standards remain in the acceptance criteria in terms of trueness, repeatability, and intermediate precision within tolerance interval ($\beta = 80\%$) and acceptance limit (±30%) as shown in the accuracy profiles presented in Fig. 2. The results for accuracy profiles are presented in Table 2.

No significant difference was found neither between a twofold dilution of 400 ng/mL and 200 ng/mL for BUP and REB nor between a twofold dilution of 4000 ng/mL and 2000 ng/mL for MOC, TRAZ and OHBUP ($n = 12$, with all t-test p values above 0.3). A twofold dilution with water was therefore found to be in the accepted range of the accuracy profile. Thus, when needed, a dilution of patient plasma with ultrapure water could be performed. Narrow accuracy profile with better LLOQ was found with LIT than REMO. Therefore, LIT was

Table 1
Recoveries, matrix effects and process efficiencies. Low concentration is defined as 10 ng/mL and high concentrations are defined as 340 ng/mL for BUP, OHBUP, and REB, and 1700 ng/mL for MOC and TRAZ. The IS are tested at the concentration used in the method namely 50 ng/mL for LIT and 100 ng/mL for REMO.

	Extraction recovery (%)		Matrix effect (%)		Process efficiency (%)	
	Low	High	Low	High	Low	High
BUP	93 ± 9	91 ± 1	116 ± 9	98 ± 3	108 ± 2	89 ± 4
OHBUP	94 ± 9	95 ± 0.3	125 ± 10	110 ± 1	117 ± 3	105 ± 1
MOC	95 ± 9	96 ± 1	128 ± 8	110 ± 3	121 ± 4	106 ± 2
REB	92 ± 9	93 ± 1	130 ± 11	109 ± 2	120 ± 3	102 ± 1
TRAZ	88 ± 8	95 ± 1	128 ± 9	109 ± 5	113 ± 3	104 ± 4
LIT (IS)	108 ± 1		96 ± 1		104 ± 3	
REMO IS)	109 ± 2		94 ± 4		103 ± 3	

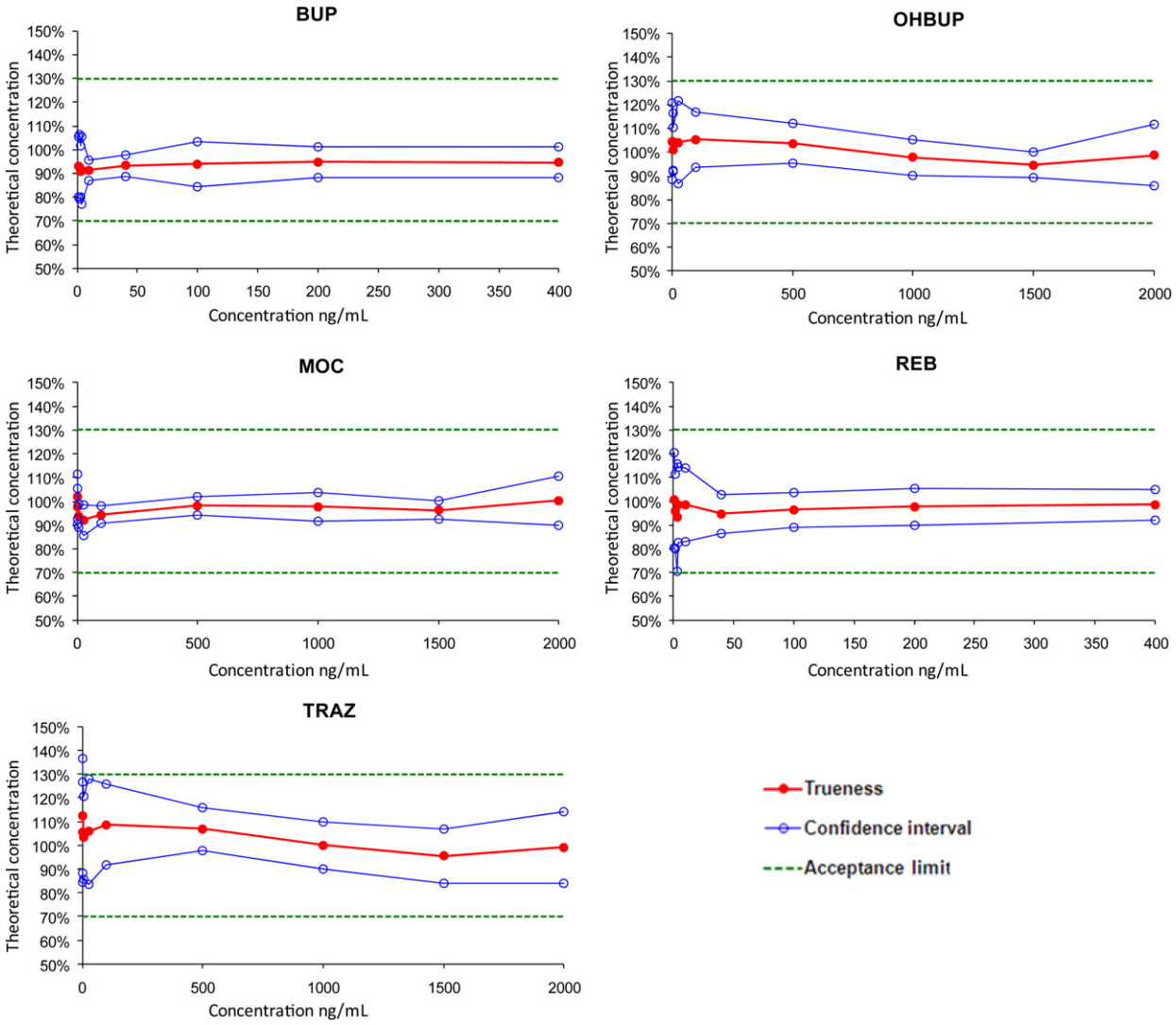

Fig. 2. Accuracy profiles.

chosen as IS for all the selected drugs and REMO remained in case of an unexpected event with LIT. REMO can also constitute a good alternative in case of supply problem of the former IS.

Low and high concentrations of each antidepressant underwent the stability tests as reported in Table 3. All tests were performed in quintuplicate for each antidepressant and at two concentrations. The target drugs remained stable (bias ≤ 15%) at room temperature for 24 h and 72 h as well as at 4 °C for 72 h with the exception of BUP. After 72 h at room temperature, more than 30% of BUP is lost. Special caution should thus be taken for the pre-analytic part for this drug and consequently BUP should be analysed within 48 h following blood sampling if the sample cannot be stored at 4 °C. Another study found that REB was stable at room temperature for seven days [34]. No significant difference was observed after one freeze–thaw cycle for all drugs. At low concentration, BUP presented a bias of 14% after three cycles. TRAZ remained stable (within 15% of the nominal concentration) for a maximum of two freeze–thaw cycles (data not shown) while presented a bias of −20% after three cycles. MOC, REB, OHBUP remained in the stability criteria. The long stability test of two months at −20 °C was successfully completed for all the drugs. Finally, extracted plasma remained stable for 36 h at room temperature for each target drug.

Table 2
Validation assay parameters.

Validation criterion	BUP	REB	Validation criterion	OHBUP	MOC	TRAZ
Calibration range (ng/mL)	[1; 400]	[1; 400]	Calibration range (ng/mL)	[2; 2000]	[2; 2000]	[2; 2000]
LLOQ (ng/mL)	1	1	LLOQ (ng/mL)	2	2	2
Trueness			Trueness			
Relative bias (%)			Relative bias (%)			
1 ng/mL	−7.2	0.6	2 ng/mL	4.6	2.0	12.5
4 ng/mL	−8.8	−1.6	25 ng/mL	4.2	−7.9	5.8
10 ng/mL	−8.7	−1.5	100 ng/mL	5.4	−5.7	8.7
100 ng/mL	−6.1	−3.6	1000 ng/mL	−2.2	−2.2	0.0
400 ng/mL	−5.3	−1.4	2000 ng/mL	−1.2	0.4	1.0
Precision			Precision			
Repeatability (%)/intermediate precision (%)			Repeatability (%)/intermediate precision (%)			
1 ng/mL	5.8/6.4	7.0/9.3	2 ng/mL	7.0/8.1	5.3/6.3	10.6/12.0
4 ng/mL	4.3/6.2	6.9/8.0	25 ng/mL	2.2/5.9	2.2/3.6	2.6/7.2
10 ng/mL	2.2/2.3	5.6/7.3	100 ng/mL	2.0/4.2	2.0/2.3	2.9/6.2
100 ng/mL	4.8/4.9	3.9/3.9	1000 ng/mL	4.1/4.1	4.3/4.3	5.2/5.2
400 ng/mL	2.0/2.9	3.3/3.3	2000 ng/mL	6.9/6.9	7.2/7.2	7.6/7.9
Accuracy			Accuracy			
Lower and upper confidence limits of total error (%)			Lower and upper confidence limits of total error (%)			
1 ng/mL	[−16.6; 2.2]	[−14.2; 15.3]	2 ng/mL	[−7.5; 16.7]	[−7.5; 11.5]	[−5.7; 30.6]
4 ng/mL	[−19.0; 1.5]	[−13.5; 10.3]	25 ng/mL	[−7.5; 16.0]	[−14.2; −1.6]	[−8.9; 20.4]
10 ng/mL	[−11.9; −5.4]	[−13.0; 10.1]	100 ng/mL	[−2.7; 13.4]	[−9.2; −2.1]	[−3.0; 20.4]
100 ng/mL	[−13.1; 1.0]	[−9.2; 2.0]	1000 ng/mL	[−8.0; 3.6]	[−8.3; 3.9]	[−7.4; 7.4]
400 ng/mL	[−10.0; −0.6]	[−6.2; 3.4]	2000 ng/mL	[−11.0; 8.6]	[−9.9; 10.7]	[−12.4; 10.5]

Table 3
Stability assay. The bias/RSD are presented in percent. The low levels are defined as 6 ng/mL for each molecule and high levels are defined as 320 ng/mL for BUP and REB and 1600 ng/mL for OHBUP, MOC, and TRAZ according to the validated range.

Stability assay	Level	BUP	OHBUP	MOC	REB	TRAZ
Short term stability, bias %(RSD)						
20 °C, 24 h	Low	−7 (5)	6 (3)	−5 (1)	4 (3)	−10 (4)
	High	−3 (2)	−2 (4)	−3 (3)	−3 (1)	−6 (4)
20 °C, 72 h	Low	−45 (9)	8 (0)	−5 (3)	7 (1)	−8 (3)
	High	−37 (14)	−13 (2)	1 (1)	0 (1)	11 (1)
4 °C, 72 h	Low	−15 (5)	8 (6)	−1 (4)	8 (6)	−10 (6)
	High	−5 (1)	−9 (2)	0 (1)	0 (1)	7 (2)
Long term stability, bias %(RSD)						
−20 °C, 2 months	Low	3 (4)	12 (3)	4 (5)	14 (5)	13 (4)
	High	−11 (4)	−15 (2)	−12 (2)	5 (2)	−11 (2)
Autosampler, bias %(RSD)						
20 °C, 24 h	Low	−15 (9)	0 (3)	−5 (3)	4 (3)	−14 (1)
	High	−14 (4)	1 (5)	−2 (3)	−3 (2)	−1 (2)
Freeze/thaw stability, bias %(RSD)						
1 cycle	Low	−11 (6)	9 (2)	0 (3)	13 (1)	−6 (2)
	High	−4 (2)	−10 (2)	5 (2)	3 (1)	15 (1)
3 cycles	Low	−14 (6)	9 (3)	−1 (2)	12 (2)	−4 (2)
	High	−10 (4)	−8 (1)	9 (4)	3 (3)	20 (6)

4. Clinical application

After the validation process, the method was applied on a routine basis for TDM. A quality control chart (QC) was also established. In each batch, three QC at low, medium and high concentrations were randomly inserted between patient samples. According to the therapeutic and validated range, the concentrations corresponded to 5, 80, 300 ng/mL for BUP, REB and 10, 700, 1500 ng/mL for MOC, TRAZ, and OHBUP. The mean bias and the RSD during the first year (50 series) for the three QC levels of all target drugs were at a maximum of 7% and 10%, respectively. All QC at any levels showed stable results throughout the year (Table 4).

The presence of an interference with a similar retention factor and m/z ratio would potentially lead to an increase of the target drug peak area which could consequently result in an overestimation of drug concentration. The mean relative ion intensities were based on the ratios between the peak areas acquired with the quantification method and with the confirmation method. The ratios were calculated on the data obtained during the validation procedure and the first year of IC and were of 9% for BUP, 18% for OHBUP, 2% for MOC, 10% for REB, 29% for TRAZ. The ratio was considered in acceptance range because it was within ±30% of the mean relative ion intensities.

The quantifications of BUP and TRAZ were the two most requested analyses. In total, the method was used to analyse these drugs in the plasma of 199 patients (Table 4). In eight cases (5%), plasma samples were diluted twofold before the extraction to be within the calibration range. No concentration above 800 ng/mL for BUP, REB and 4000 ng/mL for MOC, TRAZ, and OHBUP was observed, the patient plasma levels were then all within the validated range. As expected, the concentrations range of the drug of interest measured in the samples of patients receiving these drugs was found to be large (Table 4). External quality control samples were provided by two quality service centres (Arvecon, Gesellschaft für Toxicologische und Forensische Chemie, Walldorf, Germany and UTAK Laboratories, SL Marketing GbR, Utak Generalvertretung Radolfzell-Bohringen, Germany). These external controls were successfully quantified (data not shown). In addition, patients' plasma samples provided by another hospital laboratory working on a GC with nitrogen phosphorus detection were also analysed. The

Table 4
One year analysis with uncertainty assessment of internal control (IC) and drug plasma concentrations from TDM requests. Bias and RSD are presented in percent. Number of patients (n) is reported with the median (min–max) drug concentrations.

	BUP	OHBUP	MOC	REB	TRAZ
Relative bias, %(RSD)					
Low IC	4.3 (10.0)	7.5 (7.7)	0.8 (8.6)	5.0 (9.0)	8.0 (8.0)
Medium IC	−3.1 (4.4)	3.6 (5.2)	1.9 (5.9)	−0.6 (6.2)	3.6 (4.6)
High IC	−4.4 (4.0)	−1.6 (5.7)	−0.4 (5.0)	−1.3 (4.5)	−2.4 (5.6)
Real samples					
n	127	127	5	18	49
Drug levels	16 (1–119)	718 (2–1970)	1083 (51–1434)	144 (4–297)	621 (70–1851)

plasma concentrations obtained by the two methods were found similar (bias $\leq$ 15%, data not shown).

5. Conclusion

A quantification method based on an SPE cation exchange extraction followed by an analysis by HPLC–MS of several antidepressants (BUP and it main active metabolite OHBUP, MOC, REB, and TRAZ) was developed. The analytical procedure was validated using the latest recommendations. Moreover, the method was successfully implemented in a therapeutic drug monitoring laboratory for routine quantification of the drug of interest in patients' plasma. One year of analysis demonstrated a high robustness of the method and shows a wide range of plasma concentrations as measured in a psychiatric population.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.03.049.

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