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Simultaneous quantification of selective serotonin reuptake inhibitors and metabolites in human plasma by liquid chromatography–electrospray mass spectrometry for therapeutic drug monitoring

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ABSTRACT

A simple and sensitive liquid chromatography–electrospray ionization mass spectrometry method was developed for the simultaneous quantification in human plasma of all selective serotonin reuptake inhibitors (citalopram, fluoxetine, fluvoxamine, paroxetine and sertraline) and their main active metabolites (desmethyl-citalopram and norfluoxetine). A stable isotope-labeled internal standard was used for each analyte to compensate for the global method variability, including extraction and ionization variations. After sample (250 μl) pre-treatment with acetonitrile (500 μl) to precipitate proteins, a fast solid-phase extraction procedure was performed using mixed mode Oasis MCX 96-well plate. Chromatographic separation was achieved in less than 9.0 min on a XBridge C18 column (2.1 × 100 mm; 3.5 μm) using a gradient of ammonium acetate (pH 8.1; 50 mM) and acetonitrile as mobile phase at a flow rate of 0.3 ml/min. The method was fully validated according to Société Française des Sciences et Techniques Pharmaceutiques protocols and the latest Food and Drug Administration guidelines. Six point calibration curves were used to cover a large concentration range of 1–500 ng/ml for citalopram, desmethyl-citalopram, paroxetine and sertraline, 1–1000 ng/ml for fluoxetine and fluvoxamine, and 2–1000 ng/ml for norfluoxetine. Good quantitative performances were achieved in terms of trueness (84.2–109.6%), repeatability (0.9–14.6%) and intermediate precision (1.8–18.0%) in the entire assay range including the lower limit of quantification. Internal standard-normalized matrix effects were lower than 13%. The accuracy profiles (total error) were mainly included in the acceptance limits of ±30% for biological samples. The method was successfully applied for routine therapeutic drug monitoring of more than 1600 patient plasma samples over 9 months. The β-expectation tolerance intervals determined during the validation phase were coherent with the results of quality control samples analyzed during routine use. This method is therefore precise and suitable both for therapeutic drug monitoring and pharmacokinetic studies in most clinical laboratories.

1. Introduction

Depressive disorders are a major cause of disability worldwide and affect up to 25% of women and 12% of men [1]. Selective serotonin reuptake inhibitors (SSRI), namely citalopram (CIT), escitalopram (ESCIT), fluoxetine (FLUOX), fluvoxamine (FLUVOX), paroxetine (PAROX) and sertraline (SERT) are commonly used to treat patients with depression. Others psychiatric uses include general anxiety disorder, panic disorder, obsessive-compulsive disorders, social phobia and post-traumatic stress disorder [2]. These drugs, which are mainly metabolized by cytochrome P450 enzymes, exhibit a high degree of pharmacokinetic variability, due to genetic and environmental factors [3]. Therapeutic drug monitoring (TDM) of antidepressants, which enable clinicians to adjust the dosage of drugs according to the characteristics of individual patients, has been accepted as a valid tool to optimize pharmacotherapy [4]. The uses of TDM include control of compliance, lack of clinical response or presence of adverse effects at recommended doses, pharmacokinetic drug–drug interactions, genetic particularity influencing drug metabolism, children and elderly patients. Therapeutic indexes in plasma have been proposed by the AGNP-TDM Expert Group Consensus Guidelines [4]

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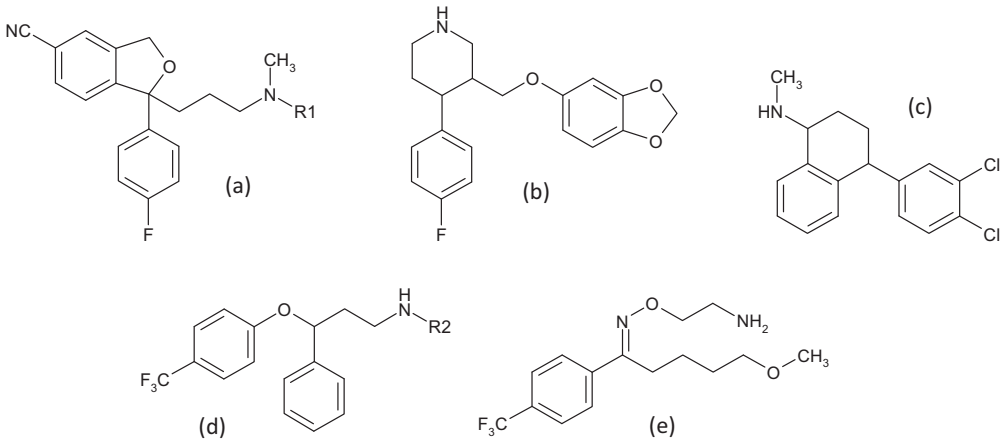

Fig. 1. Chemical structures of the selective serotonin reuptake inhibitors and their major active metabolites. Citalopram: R1 = CH_3, desmethyl-citalopram: R1 = H (a), paroxetine (b), sertraline (c), fluoxetine: R2 = CH_3, norfluoxetine: R2 = H (d), fluvoxamine (e).

for CIT (50–110 ng/ml), ESCIT (15–80 ng/ml), FLUOX plus its active metabolite norfluoxetine (NORFLUOX) (120–500 ng/ml), FLUVOX (60–230 ng/ml), PAROX (30–120 ng/ml) and SERT (10–150 ng/ml).

Matrix effects represent an important issue in high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS), particularly when dealing with complex matrices such as biological fluids. These phenomena can be reduced by an efficient sample preparation [5] and an adequate chromatographic separation with the elution of the analytes outside the matrix effect time window generally observed at the beginning of the chromatogram [6]. However, in the case of quantitative analysis, these conditions might be insufficient to reduce interferences and other approaches should be combined to compensate for residual matrix effects. The use of stable isotope-labeled internal standard (IS) minimizes the influence of matrix effects most effectively since the matrix effects observed for these IS are generally similar to those observed for the matching analytes. This approach is recommended by the American Association of Pharmaceutical Scientists (AAPS) and the Food and Drug Administration (FDA) [7].

Various chromatographic methods have been developed for the quantification of one or several SSRI in human plasma, including in some cases other antidepressants or antipsychotics. Methods using gas chromatography (GC) have been proposed [8–10], however, separation was mainly performed by HPLC coupled with ultraviolet (UV) [11–15], MS [16–21] or MS/MS detection [22–29]. Recently, ultra high performance liquid chromatography (UHPLC)–MS/MS methods have also been proposed [30,31]. Sample preparation was performed mainly by liquid–liquid extraction [8,9,11–13,16,17,22,23], solid-phase extraction (SPE) [10,14,18,19,24,30], protein precipitation [25,26,31] or on-line SPE using a column-switching system [15,20,21,27–29]. The main drawback of almost all of these methods is that multiple analytes were quantified using the same IS [8–16,18–20,22–29]. This approach could decrease the analytical precision, as it is difficult to have a single IS with physico-chemical properties and ionization behavior similar to several analytes. Furthermore, in many publications, a drug was chosen as IS [8,11,13–15,17–19,21–23,25,30], which could lead to an important interference if the patient had taken this compound. Although stable isotope-labeled IS were used in some methods [10,16,24,27–29,31], no method proposed the quantification of all SSRI with a stable isotope-labeled IS for each matching analyte.

The aim of this study was to develop and validate a method for the simultaneous plasma quantification of all SSRI and their major active metabolites, using stable isotope-labeled IS. To the best of our knowledge, this is the first method that allowed the quantification of these compounds with a stable isotope-labeled IS for each target analyte, to compensate for the global method variability, including extraction and ionization variations. The method consists of a simple and fast SPE procedure, followed by a HPLC separation coupled to electrospray ionization (ESI)-MS detection. The method was fully validated including function response, linearity, lower limit of quantification (LLOQ), recovery, matrix effects, process efficiency, trueness, repeatability, intermediate precision, accuracy profiles with β-expectation tolerance interval and stability. Finally, the performances of the method determined during the validation phase were compared with the results of quality control (QC) samples analyzed during routine use of the method for TDM.

2. Experimental

2.1. Chemicals and biologicals

Chemical structures of the analytes are shown in Fig. 1. Citalopram hydrobromide (purity, 99.1%) and its main metabolite desmethyl-citalopram hydrochloride (DCIT) (purity, >99.9%) were kindly provided by Lundbeck (Copenhagen, Denmark), fluoxetine hydrochloride (purity, 99.7%) and its main metabolite norfluoxetine hydrochloride (purity, 98.5%) by Eli Lilly (Indianapolis, USA), fluvoxamine maleate (purity, 99.4%) by Solvay Duphar (Weesp, Netherlands), paroxetine hydrochloride hemihydrate (purity, 99.4%) by SmithKline and Beecham (Thörishaus, Switzerland) and sertraline hydrochloride (purity, >99.9%) by Pfizer (Groton, USA).

The stable isotope-labeled IS citalopram-d6 base (CIT-d6), desmethyl-citalopram-d3 hydrochloride (DCIT-d3), fluoxetine-d6 oxalate (FLUOX-d6), norfluoxetine-d6 oxalate (NORFLUOX-d6), fluvoxamine-d3 maleate (FLUVOX-d3), paroxetine-d6 maleate (PAROX-d6) and sertraline-d3 hydrochloride (SERT-d3) were purchased from LGC Standards SARL (Molsheim, France).

Hydrochloric acid (HCl 37%) was purchased from Merck (Darmstadt, Germany), methanol (MeOH) in HPLC grade from J.T. Baker (Deventer, Holland), acetonitrile (ACN) in HPLC grade, ammonium acetate for MS, ammonium hydroxide 25% and citric acid monohydrate 99–102% from Sigma–Aldrich (Steinheim, Germany). All other reagents and solvents were of analytical grade. Ultra-pure water was supplied by a Milli-Q Water Purification System from Millipore (Molsheim, France).

Human blank plasma samples (more than 10 different batches) used for the preparation of calibration and validation standards were obtained from the Blood Transfusion Center of the Lausanne

University Hospital (Switzerland) and were stored at −20 °C until use.

2.2. Stock and working solutions

Stock solutions of each analyte at 1 mg/ml (calculated as base) were prepared in MeOH and stored at −20 °C. The stability of these solutions was verified over a period of 18 months (<6% bias). Further dilutions were made in HCl 0.01 N to prepare two working solutions at 100 ng/μl, the first one containing CIT, DCIT, PAROX and SERT (group 1) and the second one containing FLUOX, NORFLUOX and FLUVOX (group 2). These solutions were divided into aliquots (0.6 ml) and stored for a maximum of 1 year at −20 °C (<6% bias).

Further dilutions were performed to obtain working solutions at 10, 1 and 0.1 ng/μl in HCl 0.01 N for each group. Blank plasmas were then spiked with the appropriate working solutions of each group (maximum 4%) to prepare the calibration and the validation standards at the appropriate concentrations (see Section 2.6.3). Two different batches were prepared, one for the calibration standards and the other one for the validation standards. The spiked plasmas were divided into aliquots of 250 μl and stored for a maximum of 6 months at −20 °C.

For the IS, stock solutions of each compound (calculated as base) were prepared at 1 mg/ml in MeOH or were purchased directly at 0.1 mg/ml in MeOH and stored for a maximum of 2 years at −20 °C. These solutions were further diluted with MeOH to prepare one working solution containing CIT-d6, DCIT-d3, PAROX-d6 and SERT-d3 at 1 ng/μl, and FLUOX-d6, NORFLUOX-d6 and FLUVOX-d3 at 2 ng/μl. This solution was stored for a maximum of 6 months at −20 °C.

2.3. Equipment

The HPLC system included an Agilent Series 1100 LC system (Agilent Technologies, Palo Alto, USA), equipped with a binary pump and a 100-vial autosampler with a measured dwell volume of 1.15 ml. The chromatographic separation was performed on a XBridge C18 (2.1 × 100 mm; 3.5 μm) column from Waters® Corporation (Milford, USA), fitted with a XBridge C18 (2.1 × 10 mm; 3.5 μm) pre-column. The chromatographic system was coupled to an Agilent Series 1100 MSD single quadrupole mass spectrometer equipped with an orthogonal ESI interface. The whole system was maintained at 22 °C in an air-conditioned room. ChemStation Software Revision B.01.03 SR2 (Agilent Technologies) was used for the control of the instruments and data acquisition.

2.4. HPLC–MS conditions

The mobile phase for chromatography was ammonium acetate 50 mM adjusted to pH 8.1 with ammonium hydroxide 25% (solution A) and ACN (solution B). The stability of the buffer solution was checked and found to last at least 2 weeks at 4 °C. Before each analytical series, the mobile phases were degassed under vacuum in an ultrasonic bath for 5 min. The analytes were separated at room temperature on the analytical column using a stepwise gradient elution program with the proportion of solution B varying as followed: 33% at 0.0 min, 45% at 0.3 min, 45% at 4.5 min, 50% at 4.6 min and 50% at 8.0 min. This was followed by a washing step using 95% of B from 8.1 to 9.1 min and a re-equilibration step with the initial solvent composition from 9.2 min to 15.0 min. The flow rate was 0.3 ml/min and the maximum pressure of the system was 170 bar at the beginning of the run.

Nitrogen was used both as nebulizing gas at a pressure of 20 psi (1 psi = 6894.76 Pa) and as a drying gas at a temperature of 350 °C with a flow rate of 7 L/min. Capillary voltage was set to 1250 V in the positive ionization mode. MS detection was carried out in the selected ion monitoring (SIM) mode. Fragmentor voltages were optimized for each ion (Table 1). Molecular ions $[M+H]^+$ were used for quantification; *m/z* ratios are shown in Table 1. Dwell time was 18 ms for each ion.

Table 1
Mass spectrometer settings and chromatographic typical retention times.

Compound	$[M+H]^+$ (*m/z*)	Fragmentor voltage (V)	Retention time (min)
Citalopram	325.2	220	4.5
Citalopram-d6	331.2	80	4.4
Desmethyl-citalopram	311.2	190	3.3
Desmethyl-citalopram-d3	314.2	190	3.3
Fluoxetine	310.1	160	5.9
	148.0[a]	160	5.9
Fluoxetine-d6	316.2	150	5.9
Norfluoxetine	296.1	80	5.6
Norfluoxetine-d6	302.2	80	5.6
Fluvoxamine	319.2	150	5.3
Fluvoxamine-d3	322.2	150	5.3
Paroxetine	330.2	80	5.0
Paroxetine-d6	336.2	80	4.9
Sertraline	306.2	80	8.4
Sertraline-d3	309.2	80	8.3

[a] Confirmation ion.

2.5. Sample preparation

Sample preparation was performed by SPE using 96-well plate Oasis MCX support 10 mg (Waters, Milford, MA, USA). First, 25 μl of the working IS solution were added to 250 μl of plasma sample, which corresponds to a concentration in plasma of 100 ng/ml for CIT-d6, DCIT-d3, PAROX-d6 and SERT-d3 and 200 ng/ml for FLUOX-d6, NORFLUOX-d6 and FLUVOX-d3. After the addition of 500 μl of ACN to precipitate proteins, the mixture was vortexed and centrifuged at 3300 × *g* for 11 min at 8 °C. The supernatant (600 μl) was loaded onto the wells previously conditioned with 500 μl of MeOH and 1000 μl of citric acid 1 M. Two washing steps were performed, the first one with 1000 μl of citric acid 1 M and the second one with 500 μl of MeOH. The compounds were finally eluted with twice 100 μl of MeOH/NH_4OH 25% 90/10 (v/v). After each step, a weak vacuum was applied until the wells were dry. The extracted samples were directly transferred into glass HPLC microvials and 10 μl of the extract was injected into the HPLC–MS system.

2.6. Method validation

The method was fully validated based on the guidelines published on-line by the FDA [32], the Conference Report of the 3rd AAPS/FDA Bioanalytical Workshop [7], and the approach proposed by the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) [33,34]. The European Medicines Agency (EMEA) Guideline on Bioanalytical Method Validation was also considered [35].

2.6.1. Selectivity and carry-over

Method selectivity toward matrix was assessed by analyzing 10 different blank plasma samples injected at the beginning of the series, including lipemic and hemolyzed plasmas. Carry-over effect was assessed 6 times on each ion by injecting MeOH/NH_4OH 25% 90/10 (v/v) just after the highest calibration standard.

To assess possible interferences of drugs and their metabolites susceptible to be used as concomitant medications in psychiatric patients ($n = 63$, see Section 3.2.1), plasma spiked with these compounds in groups of five were extracted and analyzed with the developed method. The proportion of ACN in the mobile phase was

maintained at 50% from 8.0 to 30.0 min before the washing step. In case of similar retention times, a signal suppression or enhancement of the potential interfering compound on the analyte was assessed by comparing peak area of the analyte alone at 100 ng/ml and together with the potential interfering compound spiked at the maximum therapeutic level.

2.6.2. Matrix effect, extraction recovery and process efficiency

Matrix effect was qualitatively evaluated using the most current implemented technique proposed by Bonfiglio et al. [36]. Six different blank plasma processed by the extraction procedure were injected during the continuous post-column infusion (2 μl/min) of a solution containing the analytes and the IS at 1.5 μg/ml. Mobile phase and extracted water were also injected to assess if potential matrix effect was due to the injection system or the sample preparation procedure, instead of plasma compounds [5]. A decrease or increase in the MS signal at the retention time of the analytes and IS indicates the presence of a matrix effect.

Matrix effect was then quantitatively assessed using the method proposed by Matuszewski et al. [37] and according to the recommendations of the EMEA [35]. Experiments were performed at a concentration of three times the LLOQ (3 ng/ml for CIT, DCIT, FLUOX, FLUVOX, PAROX and SERT and 6 ng/ml for NORFLUOX) and at high concentration (400 ng/ml for CIT, DCIT, PAROX and SERT and 800 ng/ml for FLUOX, NORFLUOX and FLUVOX). The IS concentration were 100 ng/ml for CIT-d6, DCIT-d3, PAROX-d6 and SERT-d3 and 200 ng/ml for FLUOX-d6, NORFLUOX-d6 and FLUVOX-d3. Three different sets of solutions were prepared: 3 water samples spiked with the analytes and IS after extraction (A), 6 different blank plasmas spiked with the analytes and IS after extraction (B) and 6 different blank plasmas spiked with the analytes and IS before extraction (C). Matrix effect (ME) was estimated with the ratio of peak areas from the post-extraction spiked plasmas and the post-extraction spiked water (ME = B/A). Extraction recovery (ER) was evaluated by the ratio of peak areas from the pre-extraction spiked plasmas and the post-extraction spiked plasmas (ER = C/B). Process efficiency (PE), which takes into account ME and ER, was calculated as the ratio of peak areas from the pre-extraction spiked plasmas and the post-extraction spiked water (PE = C/A). For water spiked after extraction (A), the mean peak area of the 3 determinations was used as reference for the calculations. For the 6 different plasmas (B and C), the extractions were done in duplicate and the mean of each duplicate was considered. The inter-plasma variability of the parameters evaluated was assessed and expressed as relative standard deviation (RSD). The IS-normalized ME, ER and PE were also calculated by dividing the result of the analytes by the result of the respective IS.

2.6.3. Trueness, precision and accuracy profiles

For quantitative determination, validation experiments were repeated over 3 series ($j = 3$) to evaluate the trueness and precision of the method. For calibration standards in plasma, 6 levels ($k = 6$) initially in duplicate ($n = 2$) were independently prepared each validation day at the following concentrations: 1, 2, 10, 50, 250, 500 ng/ml for CIT, DCIT, PAROX and SERT and 1, 2, 20, 100, 500, 1000 ng/ml for FLUOX, NORFLUOX and FLUVOX. For validation standards in plasma, 7 levels independently prepared ($k = 7$) in quadruplicate ($n = 4$) each validation day were analyzed at the following concentrations: 1, 2, 4, 25, 100, 250 and 450 ng/ml for CIT, DCIT, PAROX and SERT and 1, 2, 4, 50, 200, 500, 900 ng/ml for FLUOX, NORFLUOX and FLUVOX. The concentration range was selected to include the therapeutic indexes [4] and the expected patients' plasma values. To include analysis outside the calibration range, a supplementary level of validation standard containing 900 ng/ml of CIT, DCIT, PAROX and SERT and 1800 ng/ml of FLUOX, NORFLUOX and FLUVOX was also included. This sample was diluted 1:2 (v/v) with blank plasma before analysis. Calibration curves were based on the peak area ratio between each analyte and its respective isotope-labeled IS.

Trueness, which represents the closeness of agreement between the mean value obtained from a series of measurements and the theoretical value (systematic errors), was expressed as the ratio between mean and theoretical measured concentration. Precision, which represents the dispersion level among a series of measurements from multiple sampling (random errors), was estimated with variances of repeatability (intra-day variances) and intermediate precision (sum of intra-day and inter-day variances), and calculated as described in the SFSTP 1997 report [38]. Precision parameters were finally expressed as RSD based on theoretical value at each concentration level as recommended by Rozet et al. [33]. As recommended by the FDA [32], the LLOQ was defined as the lower validation standard with an analyte response of at least 5 times the response to blank matrix and with acceptable validation performances. The trueness should be within 85–115% of the actual value on the calibration range, except at LLOQ, where it should be within 80–120%. The precision should not exceed 15%, except for the LLOQ, where it should not exceed 20% [32].

Accuracy profiles were used to evaluate the total error of the method including systematic and random errors. The approach based on β-expectation tolerance interval was chosen, which represents the area where β% of the future results are expected to lie [33,39]. This approach is based on the prediction of the future results that will be produced in routine, according to the results obtained during the validation phase.

2.6.4. Stability

Stability tests were performed in plasma and after extraction using 5 different plasmas at low (2 ng/ml for CIT, DCIT, FLUOX, FLUVOX, PAROX and SERT, and 4 ng/ml for NORFLUOX) and high (400 ng/ml for CIT, DCIT, PAROX and SERT, and 800 ng/ml for FLUOX, NORFLUOX and FLUVOX) concentrations, according to the calibration ranges. Stability in plasma was assessed at room temperature for 24 h and 72 h, at 4 °C for 72 h, at −20 °C for 2 and 6 months, and after 3 freeze/thaw cycles. Post-preparative stability in the injection vials was assessed after 24 h at room temperature and after an additional time of 48 h at 4 °C. For all experiments, concentration variations were expressed as a percentage of the initial concentration measured at the beginning of the stability study (T_0). The variability between the 5 different plasmas was expressed as RSD.

3. Results and discussion

In this report we describe the development and validation of a SPE HPLC–ESI-MS method for the simultaneous quantification of all SSRI drugs (CIT, FLUOX, FLUVOX, PAROX and SERT), and their major active metabolites (DCIT and NORFLUOX) in plasma. Excellent validation performances and method stability were obtained thanks to the use of a stable isotope-labeled IS for each analyte.

3.1. Method development

3.1.1. SPE and extraction recovery

A mixed mode polymeric sorbent (Oasis MCX) suitable for the extraction of basic compounds was selected for sample preparation. The selectivity of this SPE support is increased due to the combination of cation exchange and hydrophobic interactions. The operating conditions of a generic protocol proposed by the manufacturer were initially tested. Each step was then systematically optimized (conditioning, loading, washing and elution) to improve

Table 2
Extraction recovery (ER), matrix effect (ME), process efficiency (PE).

Compound	Concentration (ng/ml) (n = 6)	ER% (RSD%)	ME% (RSD%)	PE% (RSD%)
Analytes				
Citalopram	3	83 (2)	99 (4)	82 (4)
	400	84 (2)	99 (4)	83 (4)
Desmethyl-citalopram	3	76 (2)	103 (2)	78 (2)
	400	79 (2)	102 (3)	80 (2)
Fluoxetine	3	82 (4)	100 (3)	81 (3)
	800	85 (2)	99 (2)	84 (2)
Norfluoxetine	6	85 (1)	85 (2)	73 (2)
	800	84 (2)	101 (3)	85 (2)
Fluvoxamine	3	83 (1)	121 (4)	100 (4)
	800	84 (3)	108 (3)	91 (3)
Paroxetine	3	71 (2)	88 (1)	62 (3)
	400	74 (2)	99 (3)	73 (2)
Sertraline	3	80 (2)	101 (2)	81 (3)
	400	78 (2)	100 (3)	78 (2)
Internal standards				
Citalopram-d6	100	82 (3)	102 (5)	83 (5)
Desmethyl-citalopram-d3	100	78 (3)	101 (3)	79 (1)
Fluoxetine-d6	200	85 (2)	97 (2)	83 (2)
Norfluoxetine-d6	200	84 (2)	100 (3)	85 (3)
Fluvoxamine-d3	200	84 (3)	110 (3)	92 (3)
Paroxetine-d6	100	65 (3)	100 (3)	65 (3)
Sertraline-d3	100	79 (2)	101 (3)	80 (2)
Internal standard-normalization				
Citalopram/citalopram-d6	3	101 (2)	99 (2)	100 (1)
	400	102 (2)	98 (1)	100 (1)
Desmethyl-citalopram/desmethyl-citalopram-d3	3	101 (1)	102 (1)	103 (1)
	400	101 (2)	101 (1)	102 (1)
Fluoxetine/fluoxetine-d6	3	99 (3)	104 (2)	103 (2)
	800	100 (1)	102 (1)	101 (1)
Norfluoxetine/norfluoxetine-d6	6	106 (3)	87 (2)	92 (2)
	800	100 (0.4)	101 (0.3)	100 (0.5)
Fluvoxamine/fluvoxamine-d3	3	101 (3)	106 (5)	108 (4)
	800	101 (1)	98 (1)	99 (1)
Paroxetine/paroxetine-d6	3	112 (3)	94 (1)	105 (3)
	400	113 (2)	99 (1)	112 (1)
Sertraline/sertraline-d3	3	103 (2)	101 (3)	104 (2)
	400	99 (1)	99 (0.2)	97 (1)

the extraction performances (recovery, repeatability and selectivity). After a first conditioning step performed with MeOH, citric acid was investigated for the second conditioning step and the first washing step as previously reported [40]. For the second washing step and the final elution, different solution compositions and volumes were investigated: MeOH, ACN and isopropanol, pure or diluted with increasing proportions of alkaline solution at different concentrations. The best conditions were obtained with MeOH and citric acid as loading and washing solvents. The final elution of the analytes was performed with MeOH/NH_4OH 25% 90/10 (v/v). However, inadequate selectivity toward blank matrix was still present for NORFLUOX and PAROX. To overcome this problem, a protein precipitation step was added prior to the SPE to remove most endogenous compounds. Different acids, metal ions and organic solvents were investigated according to Polson et al. [41]. A sample pre-treatment with ACN gave the best extraction performances for the analytes in terms of selectivity, recovery (71–85%) and repeatability (RSD 1–4%) at low and high concentrations (n = 6 for each level, see Table 2). Due to a good sensitivity of the method, it was not necessary to evaporate and reconcentrate the sample prior to injection into the HPLC–MS system. Although the chromatographic gradient started with 33% of organic phase, no peak shape modifications were observed after the direct injection of the final elution solution composed of 90% of organic phase, probably due to the relatively low injected volume (10 μl) and the high retention of the compounds.

3.1.2. HPLC–MS

Chromatographic separation performed at basic pH for the analysis of basic compounds is associated with improved chromatographic resolution, due to higher retention of the compounds, which are under neutral forms, on the analytical support. A higher sensitivity is also expected, when the analytes are eluted with higher ACN proportion, due to better desolvatation and spray stability in the ESI source. Furthermore, an improved peak shape is expected, due to reduction of secondary interactions between the uncharged drugs and residual silanol groups [42,43]. Therefore, the chromatographic conditions were optimized at pH 8.1 using ammonium acetate as buffer, with molarity varying from 5 to 50 mM. A XBridge C18 column was chosen as analytical support, as different analytical methods were successfully developed on this column in our laboratory [44–46]. This approach allowed us to run different methods overnight on the same HPLC–MS equipped with a single column. The optimal chromatographic conditions were a stepwise gradient of ammonium acetate 50 mM pH 8.1/ACN for 8.0 min at a flow rate of 0.3 ml/min. This allowed a good resolution of the compounds on the analytical column and co-elution of each respective isotope-labeled IS in a relatively short time. After the separation of the compounds, the column was washed with 95% of ACN for 1.0 min to remove lipophilic compounds, and then re-equilibrated for 5.8 min with the initial solvent composition before the next sample injection. The total time for the analysis of one

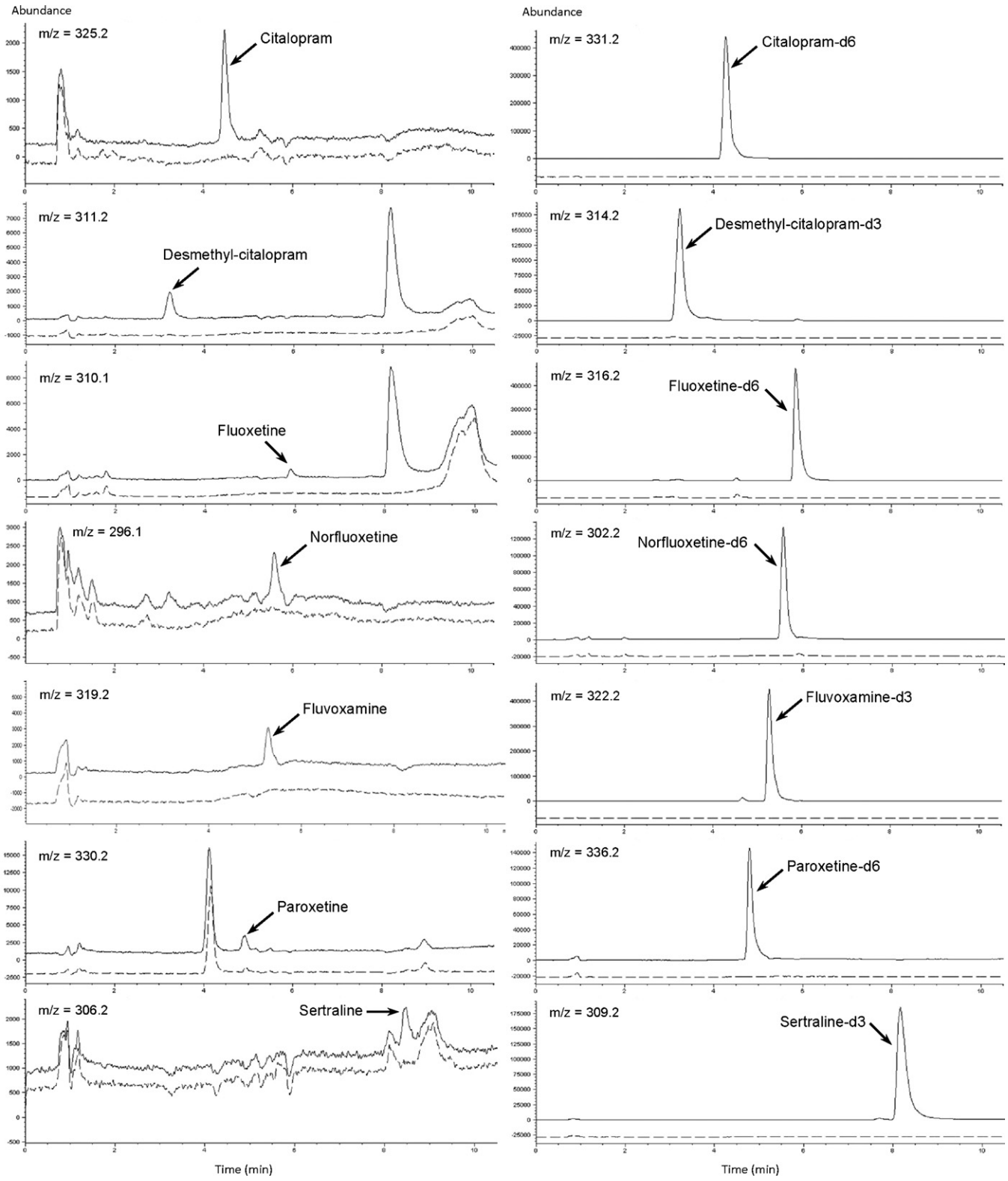

Fig. 2. Chromatograms of a blank plasma sample (dashed lines) and a quality control plasma sample (solid lines) containing the analytes at the lower limit of quantification (1 ng/ml for all compounds, except 2 ng/ml for norfluoxetine) and the internal standards at usual concentration (equivalent plasma concentration of 100 ng/ml for citalopram-d6, desmethyl-citalopram-d3, paroxetine-d6 and sertraline-d3 and 200 ng/ml for fluoxetine-d6, norfluoxetine-d6 and fluvoxamine-d3).

sample was 15.0 min. The typical retention times of the analytes and their respective IS are reported in Table 1. Typical SIM chromatographic profiles of a blank plasma sample and a QC plasma sample containing the analytes at the LLOQ and the IS at usual concentration are shown in Fig. 2.

Both CIT (racemic mixture of (*R*,*S*)-citalopram) and ESCIT (pure active enantiomer (*S*)-citalopram) are available on the market. The present chromatographic method did not allow the separation of each enantiomer due to the use of a non-chiral column. However, this was not necessary in the particular case of TDM, as the drug

taken by the patient is known from the prescriber. In the case of ESCIT prescription, the measured concentration corresponded directly to ESCIT (and not to CIT) as only this enantiomer was present in the sample.

Due to the co-elution of the stable isotope-labeled IS with the analytes, potential signal contaminations of the IS on the analytes (and inversely) were assessed. Five microliters of a solution containing only the IS (or the analytes) at very high concentration (10 ng/μl) were injected directly into the MS ($n = 3$), using a mobile phase composed of ammonium acetate 50 mM pH 8.1/ACN (60/40 v/v). After the injection of the IS, no significant signal was observed at the m/z ratio of the analytes (less than 0.2% of the signal of the IS). After the injection of the analytes, no significant signal was observed at the m/z ratio of the IS (<0.5%), except for SERT for which an isotopic contribution of 12% was observed. However, this interference was not considered problematic since it was taken into account in the calibration curve.

Potential signal modifications of the analytes on their respective IS were also evaluated. The signals of the IS at fixed concentration were measured in calibration samples during validation, with increasing concentration of the analytes. At the upper calibration level, a signal suppression of the analytes on the IS of −9%, −15%, −21%, −22%, −25% and −31% were observed for CIT-d6, PAROX-d6, DCIT-d3, FLUVOX-d3, NORFLUOX-d6 and FLUOX-d6, respectively. However, these phenomena did not decrease the performances of the method, since they were taken into account in the calibration. Due to the isotopic contribution of SERT on SERT-d3, a signal enhancement of 9% was observed on SERT-d3 at the highest SERT concentration. To compensate this phenomenon, a $1/x$ weighted quadratic regression was selected for SERT as calibration model (see Section 3.2.3).

MS operating conditions were systematically evaluated with standard solutions to optimize the ionization of the analytes and IS: nebulizing gas pressure (20–60 psi, selected 20 psi), drying gas flow rate (7–13 L/min, selected 7 L/min), drying gas temperature (200–350 °C, selected 350 °C), capillary voltage (1250–4000 V, selected 1250 V). Fragmentor voltage were tested between 80 and 220 V for each compound; the optimal conditions are presented in Table 1, together with the m/z ratios of the molecular ions $[M+H]^+$.

Repeatability of the signal after 6 successive injections of the same QC sample at medium concentration was found to be excellent, with variations of 0.5%, 0.4%, 0.4%, 0.6%, 0.6%, 0.3% and 1.4% for CIT, DCIT, FLUOX, NORFLUOX, FLUVOX, PAROX and SERT, respectively.

The robustness of the method was evaluated by analyzing QC samples (3 levels) with modifications of the chromatographic conditions (pH range 8.0–8.2, flow rate 0.285–0.315 ml/min, temperature ±5 °C and age of the column). Although slight variations in the retention times were observed, the results of the QC samples were not affected by the conditions tested. Furthermore, the method was found to be robust regarding the excellent results of QC samples analyzed during routine use over 9 months, which takes into account multiple variations of the operating conditions (see Section 4.1).

3.2. Method validation

3.2.1. Selectivity

The method was selective toward the matrix, as no interference was observed at the retention time of the analytes and IS with 10 different blank plasmas. The signal to noise ratios for the LLOQ were 14, 20, 6, 8, 10, 8 and 5 for CIT, DCIT, FLUOX, NORFLUOX, FLUVOX, PAROX and SERT, respectively, which were in accordance with the limit of at least 5 recommended by the FDA [32]. Based on a signal to noise ratio of 3, the corresponding limits of detection were 0.2, 0.15, 0.5, 0.75, 0.3, 0.4 and 0.6 ng/ml, respectively.

No carry-over (<0.05%) was observed when the eluant media of the SPE protocol was analyzed just after the highest calibration standard. This indicated that the needle wash procedure after the injection of the sample (continuous washing with the mobile phase during the run), and the washing step with 95% ACN for 1 min at the end of the run, were sufficient to remove potential residues of the analytes.

The influence of more than 60 common comedications in the psychiatric population on the quantification of the analytes was assessed (retention times in min; ND if not detected): amantadine (ND), amisulpride (1.4), amitriptyline (8.0), aripiprazole (14.1), dehydro-aripiprazole (11.6), atomoxetine (**4.9**), bupropion (7.2), hydroxy-bupropion (**3.2**), caffeine (ND), chlorpromazine (ND), clomipramine (9.6), desmethyl-clomipramine (9.6), clopenthixol (9.1), clozapine (7.1), norclozapine (4.1), N-oxide-clozapine (2.2), cocaine (ND), donepezil (6.3), duloxetine (**4.8**), cis-flupenthixol (10.5), galantamine (1.4), epigalantamine (9.8), haloperidol (**5.9**), imipramine (6.8), desmethyl-imipramine (**5.3**), loxapine (10.0), maprotiline (**5.5**), memantine (2.4), methadone (**5.9**), methadone primary metabolite (EDDP, 4.1), methadone secondary metabolite (EMDP, 14.0), mianserine (7.0), desmethyl-mianserine (**5.5**), midazolam (**5.8**), 1-hydroxy-midazolam (**4.6**), mirtazapine (ND), desmethyl-mirtazapine (ND), moclobemide (2.0), 3′-oxo-moclobemide (ND), N-oxide-moclobemide (1.2), morphine (ND), nicotine (0.9), nortriptyline (**5.5**), olanzapine (**4.9**), quetiapine (**6.1**), reboxetine (**4.8**), rispéridone (4.1), 9-hydroxy-risperidone (2.9), rivastigmine (2.8), sertindole (9.9), dehydro-sertindole (10.8), desmethyl-sertraline (7.4), sulpiride (ND), trazodone (**5.7**), trimipramine (10.3), desmethyl-trimipramine (**5.8**), valproic acid (0.9), varenicline (0.8), venlafaxine (2.9), O-desmethyl-venlafaxine (1.5), N-desmethyl-venlafaxine (1.9), N,O-di-desmethyl-venlafaxine (1.2) and ziprasidone (8.0). After extraction from the plasma, 16 compounds had a chromatographic retention time close to the analytes (±0.3 min, in bold). However, the m/z ratios were mostly different and after injection of the analytes with the co-eluting compounds, no signal modifications of the analyte higher than 15% were observed (data not shown). An interference was nevertheless identified between FLUOX and methadone which had the same retention time and m/z ratio (310). As these drugs could be prescribed together, particularly in psychiatric patients, the fragmentor voltage was increased to 160 V to obtain a confirmation ion for FLUOX (m/z ratio of 148). During routine use of the method, if the FLUOX concentration measured with the main ion was higher than 20% compared to the confirmation ion, an interference was considered and the confirmation ion was used for quantification. Due to a lower signal intensity of the confirmation ion, the LLOQ was only 20 ng/ml, compared to 1 ng/ml with the main ion. Although confirmation ions can increase the selectivity of the method, such approach can decrease its sensitivity. The formation of the confirmation ion generally requires higher fragmentor voltages, which can decrease the signal used for quantification. Furthermore, the monitoring of confirmation ions increases the number of ions monitored, which can be a critical issue when several drugs are quantified simultaneously and particularly when low LLOQ are required.

The main drawback of single quadrupole MS strategies is related to the fact that only one ion in SIM mode is generally used for drug quantification, which could be insufficient for method selectivity. Method selectivity can be improved with MS/MS, which use is increasing in bioanalytical chemistry. Several methods have recently been published for plasma quantification of SSRI drugs using MS/MS [22–31]. However, a compromise has to be found between method complexity and ease-of-use. For TDM, the use of single quadrupole MS method can be considered, as it is simple, less

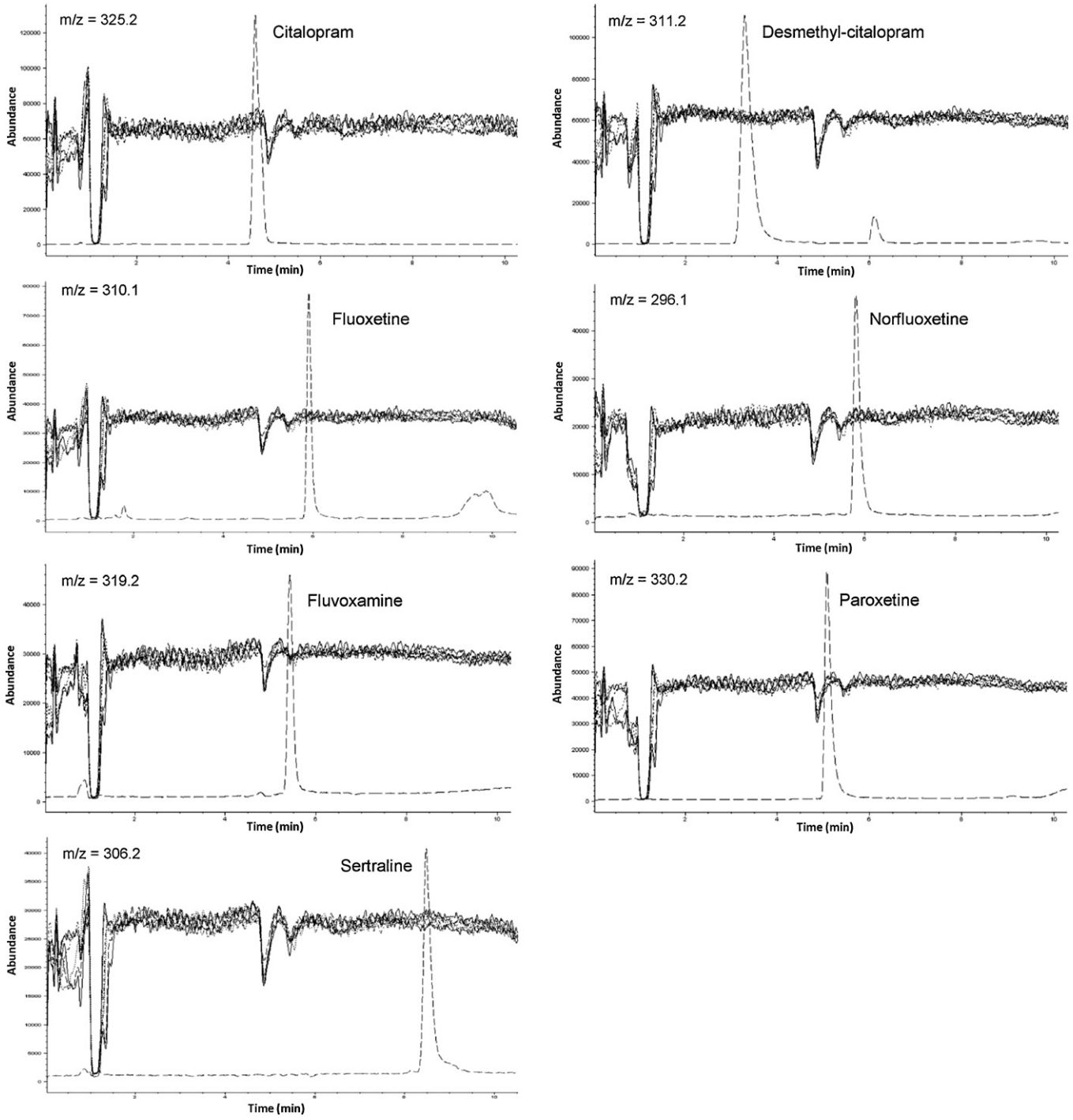

Fig. 3. Chromatograms of six blank plasma extracts with post-column infusion of the analytes and chromatograms of a calibration standard (dashed lines). Analytical conditions as described in Section 2.6.2.

expensive and can be used in most clinical laboratories with very good performance. When feasible, the monitoring of a secondary ion during the analysis can increase the selectivity of the method.

3.2.2. Matrix effect and process efficiency

Matrix effect is an important phenomenon influencing MS-based bioanalytical assay with possible suppression or enhancement of analytes' ionization by the presence of endogenous components of plasma, such as proteins, lipids, sugars or salts.

We used a post-column infusion system to qualitatively assess matrix effect [36]. The MS response was homogenous among the different sources of plasma investigated. No signal modification was observed at the retention times of the analytes and the IS (Fig. 3). An important signal suppression was observed at the beginning of the analysis (before 1.5 min) for extracted water and plasma, but not for mobile phase (data not shown). This indicated an effect due to polar compounds issued from the extraction procedure itself but not from the matrix. The first analyte (DCIT, retention time 3.3 min) was eluted outside this signal suppression time window, indicating that the retention of the analytes on the analytical support was sufficient. A weak signal suppression was also observed at 4.7 min with extracted plasma, but did not

Table 3
Trueness, repeatability and intermediate precision.

Compound	Concentration (ng/ml)	Trueness (%)	Precision	
			Repeatability (%)	Intermediate precision (%)
Citalopram	1	100.8	4.3	8.2
	2	104.7	3.3	5.6
	4	108.8	3.5	4.9
	25	109.6	4.0	4.2
	100	108.4	2.3	4.3
	250	102.8	1.6	4.2
	450	95.7	2.5	5.3
	900/2	91.4	1.9	3.7
Desmethyl-citalopram	1	105.8	5.9	5.9
	2	102.5	3.9	4.0
	4	101.1	3.2	3.9
	25	101.3	4.1	4.1
	100	105.2	2.7	5.1
	250	105.9	1.0	5.5
	450	107.4	2.2	6.7
	900/2	100.5	1.4	4.4
Fluoxetine	1	104.5	5.2	8.3
	2	101.8	3.2	8.9
	4	100.0	4.0	8.9
	50	103.2	4.3	5.4
	200	105.7	2.2	4.1
	500	103.0	1.4	5.6
	900	101.3	1.7	5.2
	1800/2	97.0	1.8	4.3
Norfluoxetine	2	101.6	11.9	18.0
	4	95.4	4.9	5.4
	50	100.9	3.9	4.1
	200	103.5	2.1	2.9
	500	102.1	1.6	2.6
	900	100.4	1.5	2.5
	1800/2	95.8	1.6	1.8
Fluvoxamine	1	108.1	14.6	17.8
	2	105.1	9.5	13.2
	4	97.9	3.5	6.5
	50	96.4	5.8	6.0
	200	101.2	3.2	4.7
	500	100.3	1.4	5.8
	900	98.1	1.6	5.0
	1800/2	93.1	1.6	3.8
Paroxetine	1	90.1	6.1	12.8
	2	84.2	3.4	9.4
	4	88.5	2.5	7.7
	25	99.2	4.6	5.2
	100	100.7	2.1	4.7
	250	102.1	1.4	4.6
	450	101.9	2.3	5.4
	900/2	95.3	1.8	3.8
Sertraline	1	100.7	5.3	6.5
	2	99.4	3.9	4.8
	4	99.9	2.9	5.9
	25	109.0	4.0	4.0
	100	104.1	2.2	3.2
	250	101.1	1.4	2.4
	450	101.3	0.9	2.3
	900/2	92.1	2.5	2.9

interfere with CIT (4.5 min) and PAROX (5.0 min). The baseline was stable during the rest of the chromatography, indicating that the sample preparation procedure was appropriate to remove endogenous compounds that could interfere with the quantification of the analytes.

Quantitative matrix effect was subsequently assessed using the approach described by Matuszewski et al. [37]. Matrix effect was expressed as a value above or below 100%, depending on the presence of a signal enhancement or suppression, respectively. Matrix effects, which ranged between 85 and 121% at low concentration (3× LLOQ) and between 99 and 108% at high concentration (Table 2), were considered of low importance. This indicated that residual co-eluting species were present in small quantities and did not interfere significantly with the analytes' ionization. Potential matrix effects should be reduced using an adequate extraction procedure and chromatographic separation, but most importantly, its variability must be decreased to the minimum. Using 6 different plasmas, the variability (RSD) of the matrix effects never exceeded 4% for the analytes, which is much lower than the limit of 15% proposed by recent recommendations [7].

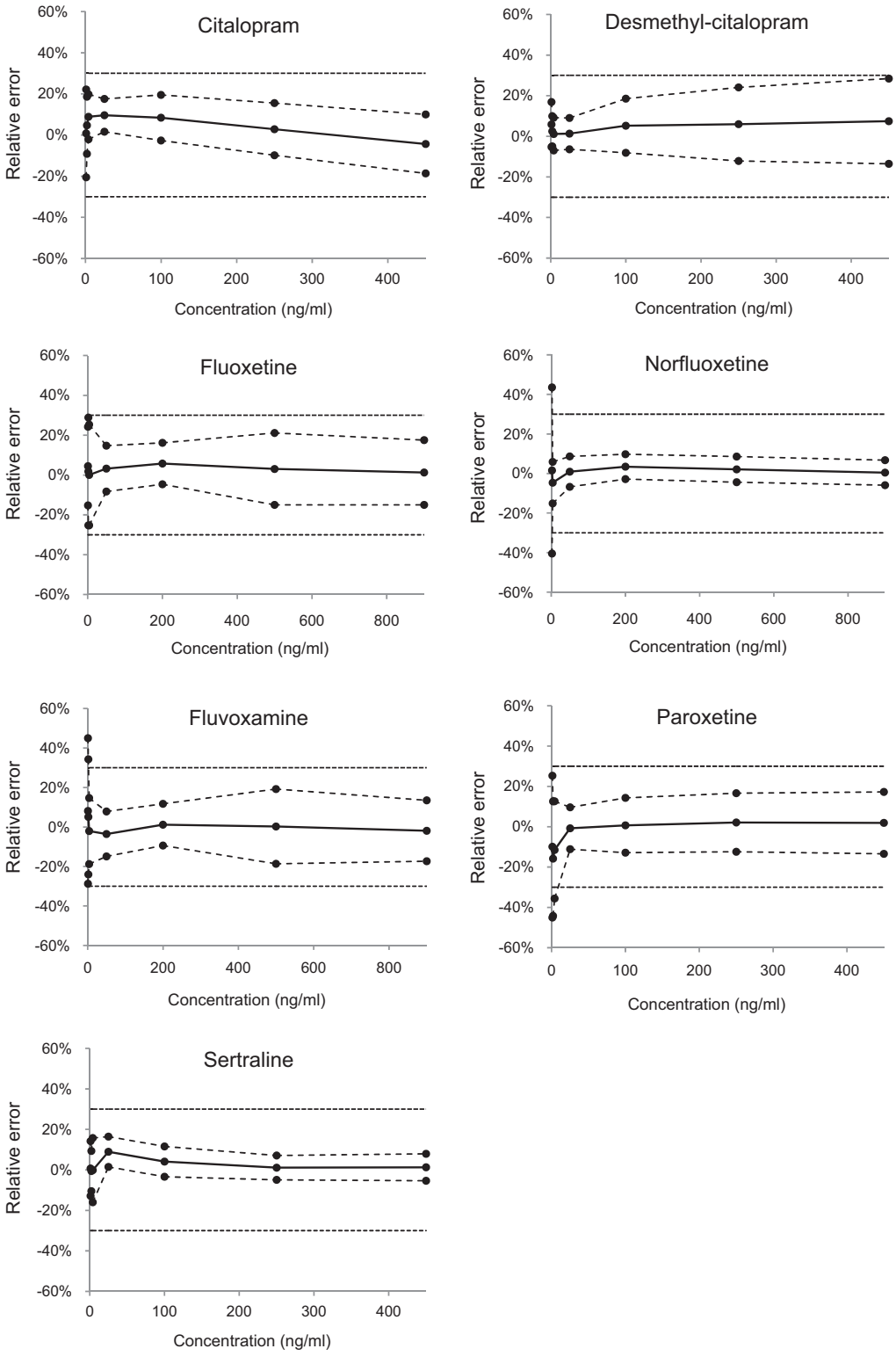

Fig. 4. Accuracy profiles for the studied compounds obtained with validation standard plasma samples. The solid lines indicate the bias, the dashed lines the upper and lower β-expectation tolerance intervals ($\beta = 90\%$) and the dotted lines the acceptance limits ($\lambda = \pm 30\%$).

As the degree of matrix effects may vary between samples obtained from diverse patients, the corrective capacity of the IS is essential. The use of stable isotope-labeled IS that co-elute with their respective analytes minimize the consequence of potential matrix effect and should be used whenever possible [7]. Indeed, the analytes and the IS will both experience the same interferences that might lead to modification of the MS response. The signal of the analytes and the IS will be affected in the same way, thus peak area ratios will still be consistent [36]. In a previous work on the quantification of methadone in human plasma, excellent stability of the method was obtained when the co-eluting methadone-d3 was used as IS, compared to previous tests with methadone-d9 which

Table 4
Plasma and post-preparative stability ($n = 5$).

Compound	Citalopram		Desmethyl-citalopram		Paroxetine		Sertraline	
Concentration (ng/ml)	2	400	2	400	2	400	2	400
Plasma RT								
24 h	105 (2)	96 (3)	92 (9)	91 (4)	100 (7)	93 (4)	104 (8)	100 (3)
72 h	105 (5)	98 (4)	98 (5)	95 (4)	94 (6)	95 (3)	100 (7)	99 (3)
Plasma 4 °C								
72 h	103 (6)	96 (5)	101 (4)	96 (5)	103 (13)	95 (4)	102 (3)	100 (2)
Plasma freeze/thaw								
3 cycles	103 (5)	101 (2)	103 (7)	96 (2)	108 (10)	95 (3)	97 (2)	98 (2)
Plasma −20 °C								
2 months	98 (5)	95 (3)	105 (5)	97 (2)	107 (4)	97 (4)	96 (3)	106 (2)
6 months	101 (2)	93 (3)	97 (8)	93 (2)	100 (10)	95 (3)	103 (8)	101 (2)
Post-preparative								
RT, 24 h	103 (5)	101 (3)	96 (6)	96 (2)	91 (4)	101 (1)	102 (2)	105 (1)
RT, 24 h + 4 °C, 48 h	108 (3)	97 (3)	106 (9)	98 (2)	108 (5)	94 (4)	102 (2)	102 (2)

Compound	Fluoxetine		Norfluoxetine		Fluvoxamine	
Concentration (ng/ml)	2	800	4	800	2	800
Plasma RT						
24 h	101 (5)	98 (3)	98 (4)	98 (4)	101 (8)	100 (1)
72 h	100 (5)	98 (2)	97 (9)	96 (4)	92 (13)	97 (2)
Plasma 4 °C						
72 h	98 (4)	98 (3)	99 (6)	98 (3)	95 (8)	100 (2)
Plasma freeze/thaw						
3 cycles	109 (4)	101 (3)	98 (10)	99 (4)	111 (11)	101 (1)
Plasma −20 °C						
2 months	101 (6)	97 (2)	96 (7)	93 (4)	100 (9)	100 (1)
6 months	102 (5)	95 (2)	101 (7)	96 (3)	97 (13)	96 (1)
Post-preparative						
RT, 24 h	94 (10)	97 (1)	104 (12)	102 (3)	103 (9)	100 (1)
RT, 24 h + 4 °C, 48 h	104 (6)	99 (3)	108 (4)	96 (4)	110 (14)	101 (2)

RT, room temperature; data are presented as percentage of concentration measured at T_0 (RSD%).

was chromatographically separated from methadone [47]. In the present work, the IS-normalized matrix effect ranged between 87 and 106%, with a variability (mean 1.5%) lower than the variability of the absolute matrix effect (mean 2.8%) (Table 2), indicating that the stable isotope-labeled IS were useful to compensate for residual matrix effects. Different analogs of the analytes were initially evaluated as IS, but were discarded due to inadequate precision of the results.

Finally, the process efficiency of the analytes, which described the combined effects of the extraction recovery and the matrix effect, ranged between 62 and 100%, with RSD never exceeding 4% (Table 2).

3.2.3. Trueness, precision and accuracy profiles

The method was validated over 3 series, using 6 levels of calibration standard initially in duplicate from 1 to 500 ng/ml for CIT, DCIT, PAROX and SERT and 1–1000 ng/ml for FLUOX, NORFLUOX and FLUVOX, and 7 levels of validation standard in quadruplicate from 1 to 450 ng/ml for CIT, DCIT, PAROX and SERT and 1 to 900 ng/ml for FLUOX, NORFLUOX and FLUVOX. To determine the best response function, different regression models were evaluated: linear regression, linear regression through 0, weighted linear regression (weighted factor $1/x$ or $1/x^2$), quadratic regression, quadratic regression through 0 and weighted quadratic regression (weighted factor $1/x$). The most suitable calibration model, based on the estimation of trueness and precision determined by recalculation of the validation standards with the daily calibration curves, was $1/x^2$ weighted linear regression for all analytes, except for SERT, where $1/x$ weighted quadratic regression was selected (due to the isotopic contribution of SERT on SERT-d3). A simplified calibration curve performed with 6 levels analyzed once was finally selected, as the validation performances were similar to those obtained with 6 levels in duplicate.

On the evaluated assay range including the LLOQ, trueness (84.2–109.6%), repeatability (0.9–14.6%) and intermediate precision (1.8–18.0%) were in accordance with the recommendations of the FDA [32], and consequently appropriate for the use of the method in routine TDM (Table 3). The LLOQ was set at 1 ng/ml for all compounds, except for NORFLUOX, for which it was set at 2 ng/ml due to a lower sensitivity. The upper limit of quantification (ULOQ) was 450 ng/ml for CIT, DCIT, PAROX and SERT and 900 ng/ml of FLUOX, NORFLUOX and FLUVOX. A dilution step 1:2 (v/v) before extraction of samples with blank plasma at concentrations of up to twice the ULOQ was also validated. Therefore, when out-of-range concentrations are expected during routine use of the method, a simple dilution of the samples can be performed.

The accuracy profiles with β-expectation tolerance intervals were built using a β value of 90%, which represents the percentage of the future results that is expected to fall within the obtained tolerance intervals during routine use of the method. In accordance with the most recent regulatory recommendations [7], the total error profiles were included in the acceptance limits of ±30% for biological samples (Fig. 4), except at concentrations near the LLOQ for NORFLUOX, FLUVOX and PAROX, where a relative bias up to 45% was observed. However, for the intended purpose of the method (TDM), these relative biases at low concentrations were not considered as clinically significant and the method was considered as valid on the entire investigated range.

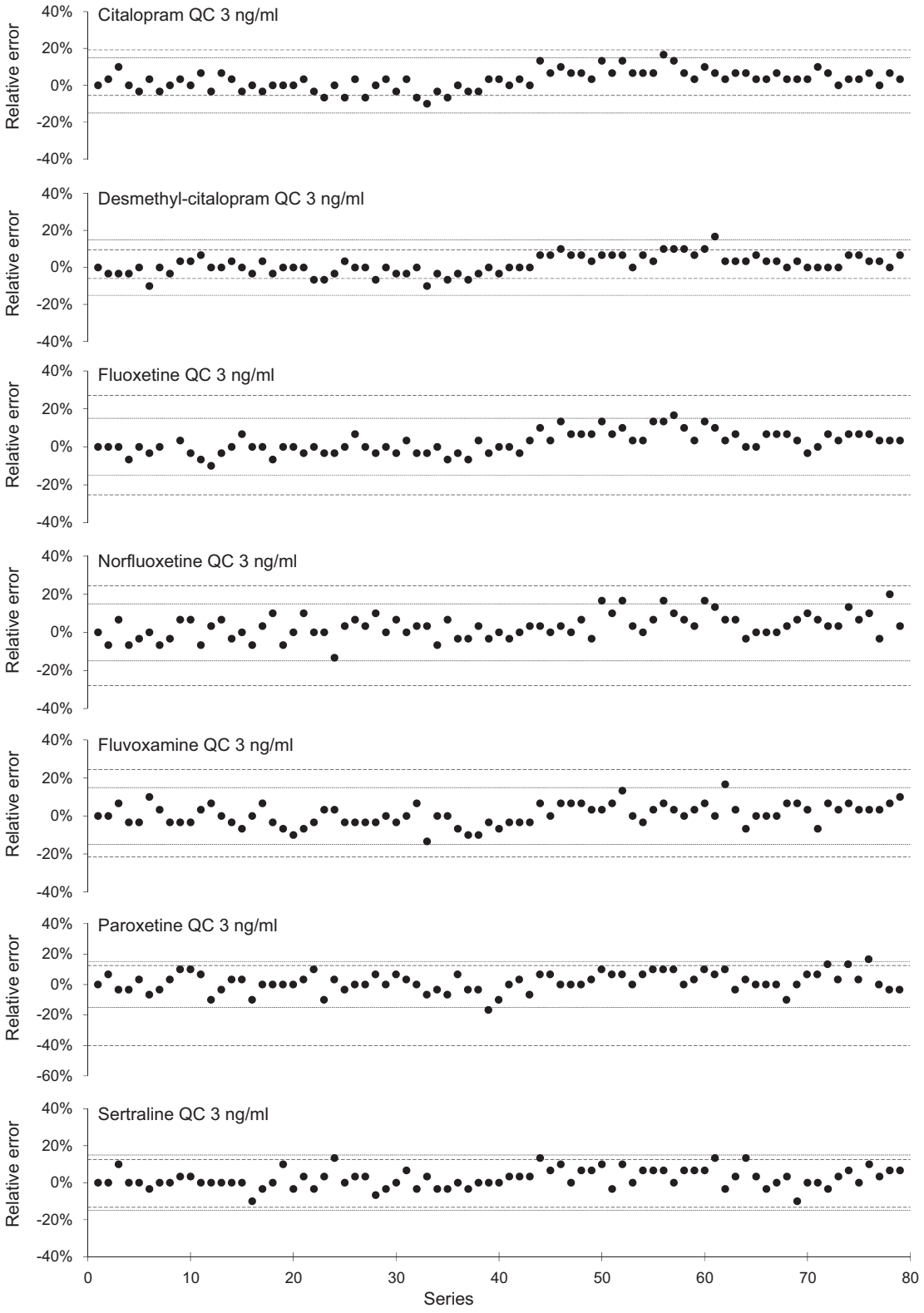

Fig. 5. Follow-up of quality control (QC) plasma samples during 79 runs in routine over 9 months. The dashed lines indicate the upper and lower 90%-expectation tolerance intervals estimated during validation. The dotted lines indicate the in-study validation FDA ± 15% acceptance limits. Only the results of the low QC samples (3 ng/ml) are shown. Between 92 and 100% of these QC were located within the 90%-tolerance interval, except for desmethyl-citalopram (84%). Between 94 and 100% of these QC were located within the FDA ± 15% acceptance limits. Similar profiles were obtained with the medium and high QC samples.

A linear regression model was applied to the recalculated validation standard concentrations versus theoretical concentrations. The following slopes 0.966, 1.073, 1.015, 1.007, 0.985, 1.021 and 1.011, and intercepts 3.855, −0.941, 2.062, 1.414, 1.441, −0.520 and 0.782 were found for CIT, DCIT, FLUOX, NORFLUOX, FLUVOX, PAROX and SERT, respectively. The corresponding determination coefficients were 0.995, 0.996, 0.997, 0.999, 0.997, 0.997 and 0.999, indicating that the developed

method was linear for the tested compounds over the investigated range.

The precision of the method was further investigated over 20 different series with reinjection of a calibration standard (level 5) at the end of the series. Mean of the absolute differences between the two injections were 1.0%, 1.8%, 3.2%, 1.4%, 1.4%, 0.8% and 3.3% for CIT, DCIT, FLUOX, NORFLUOX, FLUVOX, PAROX and SERT, respectively.

3.2.4. Stability tests

Drug stability experiments demonstrated a good stability of the analytes in plasma after 24 h and 72 h at room temperature and after 72 h at 4 °C. The plasma samples were also stable after 3 freeze/thaw cycles. The long-term storage stability in plasma was verified after 2 and 6 months at −20 °C. The post-preparative stability was verified for 24 h at room temperature and after an additional time of 48 h at 4 °C, indicating that the samples can be analyzed in case of unexpected delay in the analyses such as instrument failure. For all these conditions, deviations ranged from 91 to 111% (Table 4), which is comprised in the accepted range of 85–115% for drug stability studies [35], and corresponds probably mostly to analytical precision, rather than to analyte degradation.

4. Routine use and clinical application

4.1. Long-term evaluation of the method

The validity of an analytical method must be assessed at two levels. The "pre-study" validation aims to show, by an appropriate set of designed experiments, that the method is able to achieve its objectives. The "in-study" validation is intended to verify, by inserting QC samples in routine runs, that the method remains valid over time [39,48].

The validated method was released for routine analysis of plasma samples of patients receiving a SSRI drug. At the beginning of each analytical series, a system suitability test was performed to ensure the performances of the analytical system, by injecting a calibration standard (level 5). Acceptance criteria were defined for SERT, which is the last compound to be eluted and thus considered as the most sensitive to chromatographic variations: retention time 8.7 ± 0.7 min, signal intensity > 1,000,000 and tailing factor < 2.6, which include variations due to different column batches.

A total of 79 analytical series were performed for TDM over a period of 9 months. For each compound, three QC levels (low, medium and high) at 3, 100 and 400 ng/ml for CIT, DCIT, PAROX and SERT, and 3, 200 and 800 ng/ml for FLUOX, NORFULOX and FLUVOX were included in the beginning, middle and end of each analytical batch. A total of 21 control charts (3 levels for 7 compounds) were established using the 90% expectation tolerance intervals estimated during validation.

The results of these QC samples in routine were mostly observed within the 90%-expectation tolerance intervals estimated during validation (Fig. 5). For 76% (16/21) of the control charts, more than 90% (mean 97%) of the QC results were effectively included in the predicted 90% tolerance intervals. This indicated that in most cases, the β-expectation tolerance interval approach is suitable for the estimation of the performances of future assays. For 24% (5/21) of the control charts, less than 90% of the QC samples were within the expectation tolerance intervals: at low concentration for DCIT (84%), and at medium and high concentrations for SERT (70% and 57%, respectively) and NORFLUOX (78% and 61%, respectively), suggesting an under estimation during the validation process of the real variability. In these five situations, the lower and upper tolerance limits estimated during validation were very low (mean −4.6% and 9.1%, respectively) and far below the acceptance limits set at ±30% (Fig. 4). These discrepancies could be related to different column batches or to the operator, as the method was validated by a single operator and the routine analyses were performed by 9 different operators.

To verify that the method remained valid over time during routine use, we considered the in-study validation FDA acceptance criteria for a run, indicating that at least 67% of QC samples should be within 15% of their respective target value [32]. Using this approach, only a few runs were rejected: 1/79 for CIT, 1/79 for DCIT and 1/79 for SERT. In addition, a very high proportion of the QC (79 series × 3 levels per analyte) were within the FDA limit of ±15%: 97%, 94%, 98%, 96%, 99%, 96% and 98% for CIT, DCIT, FLUOX, NORFLUOX, FLUVOX, PAROX and SERT, respectively, which illustrates the very good performance of the method. Furthermore, none of the QC values measured (79 series × 3 levels × 7 analytes) was found outside ±22% of their target value.

4.2. Clinical application

During these 79 series performed in routine, a total of 1667 plasma samples of patient under SSRI treatment were successfully analyzed for TDM. The following concentrations were measured [median(range)]: CIT (n = 395): 63 (0–358) ng/ml; DCIT (n = 395): 22 (0–149) ng/ml; ESCIT (n = 644): 21 (0–211) ng/ml; DESCIT (n = 644): 8 (0–81) ng/ml; FLUOX (n = 198): 125 (0–824) ng/ml; NORFLUOX (n = 198): 140 (0–498) ng/ml; FLUVOX (n = 63): 96 (0–797) ng/ml; PAROX (n = 130): 41 (0–375) ng/ml; SERT (n = 242): 22 (0–208) ng/ml. No sample had to be diluted, as all measured values were included in the validated quantification range.

5. Conclusions

A simple, sensitive and precise HPLC–ESI-MS method was developed and validated according to FDA guidelines and SFSTP protocols for the simultaneous quantification of all SSRI drugs and their major active metabolites in human plasma. Matrix effects were strongly reduced thanks to a protein precipitation step followed by SPE and an adequate chromatographic separation. To the best of our knowledge, this is the first method described for the quantification of all SSRI with a stable isotope-labeled IS for each target analyte to compensate for the global method variability, including extraction and ionization variations. Using these conditions, very good validation performances were obtained, which were in line with the results of QC samples analyzed during routine use of the method. This method is therefore suitable both for routine TDM and pharmacokinetic studies in most clinical laboratories equipped with a single MS.

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