Stereoselective determination of citalopram and desmethylocitalopram in human plasma and breast milk by liquid chromatography tandem mass spectrometry

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Abstract

A high performance liquid chromatography (HPLC) tandem mass spectrometry (MS/MS) method was developed for the simultaneous, stereoselective quantification of the antidepressant citalopram and its active metabolite desmethylcitalopram in human plasma and breast milk. Sample preparation was performed by a two-step approach, including generic protein precipitation with acetonitrile followed by solid phase extraction. Enantiospecific separation of analytes was achieved on a Phenomenex[®] Lux Cellulose-2 column (4.6 mm x 150 mm; 5 μ m), using reversed phase chromatography conditions characterized by a gradient elution of ammonium acetate buffer (pH 9.0; 20 mM) and acetonitrile at a flow rate of 0.6 ml/min. The compounds were detected by a tandem quadrupole mass spectrometer equipped with an electrospray ionization source and operating in multiple reaction monitoring mode. The method was fully validated in both biological fluids over a large concentration range of 0.1-100 ng/ml for S-(+)- and R-(-)-citalopram, and 0.3-100 ng/ml for S-(+)- and R-(-)-desmethylcitalopram. Trueness (90.0-113.3% and 97.1-103.6%), repeatability (0.9-15.9% and 0.9-8.4%) and intermediate precision (1.3-17.8% and 0.9-9.6%) in plasma and breast milk, respectively, meet international guidelines for method validation. Internal standard-normalized matrix effects ranged between 99-101% and 98-105%, respectively. The accuracy profiles (total error of trueness and precision) were mostly within the acceptance limits for biological samples defined as $\pm 30\%$. The method was successfully applied to patient samples in a clinical trial setting.

Keywords: Citalopram; Enantiomer; LC-MS/MS; Plasma; Breast milk; Quantification.

1. Introduction

According to the World Health Organization, mental disorders figure among the leading causes of ill health and disability in the Western world and women are affected almost twice as often as men [1]. The prevalence rate of major depression during pregnancy and the postpartum period is estimated to be 6% and 9%, respectively [2, 3]. The use of an antidepressant medication is therefore not rare in this particular population, with up to 6% of pregnant women taking a selective serotonin reuptake inhibitor (SSRI) [4]. These drugs readily cross the placenta and enter breast milk [5, 6]. They may thus be associated with an increased risk of poor neonatal adaptation and may affect child long-term cognitive skills [7, 8]. Determination of drug concentrations in maternal blood, cord blood and breast milk allows an estimation of drug exposure of the fetus or the breastfed infant. Based on these concentrations and the daily milk intake, the relative infant dose (RID) can be determined in lactating women [9]. This weight-normalized parameter is widely used among clinicians and represents the most accurate measure of infant exposure through breast milk.

Citalopram (CIT) is a SSRI antidepressant metabolized in the liver by two succeeding N-demethylation steps to desmethylcitalopram (DCIT) via the cytochromes (CYP) CYP2C19 and CYP3A4, and to didesmethylcitalopram (DDCIT) via CYP2D6 [10]. These metabolic pathways play an important role in the interindividual variability in drug blood levels through genetic polymorphisms or drug-drug interactions. CIT and its major metabolites are chiral compounds, i.e. present in a racemic mixture of both S-(+) and R-(-) enantiomers. S/R ratio for CIT *in vivo* ranges from 0.32 to 1.25, thus pointing to a stereoselective metabolism [10, 11]. However, the S-(+) enantiomer of CIT and to a lesser extent of DCIT are the pharmacologically relevant moieties mainly responsible for the antidepressant effect [10, 12]. This led to the commercialization of new pharmaceutical formulations including only the active enantiomer S-

(+)-citalopram or escitalopram. Differences in concentration and activity of CIT enantiomers may impact maternal treatment response and infant drug exposure.

In 1995, Rochat et al. developed the first enantioselective methods based on high pressure liquid chromatography (HPLC) for the determination of CIT and its main metabolites in plasma [13, 14]. Following sample preparation by liquid-liquid extraction, samples were separated on a Chiralcel OD or an acetylated β -cyclodextrin column and detected by fluorescence (limit of quantification: 2-15 ng/ml). From this time on, different stationary phases (e.g. macrocyclic antibiotic- [15-17], protein- [18] or saccharide-based columns [19, 20]) have been used to separate CIT enantiomers from multiple biological matrices such as blood, wastewater, brain tissue or hair and coupled to a fluorescence [15, 20, 21], UV [16, 18] or tandem mass spectrometry detector (MS/MS) [17, 19, 22]. The addition of β -cyclodextrin to the mobile phase [23], the derivatization of the analytes with a chiral reagent [21] or the use of capillary electrophoresis [24-27] have also been proposed.

The aim of this work was to develop and validate a sensitive and selective HPLC-MS/MS method for the simultaneous quantification of CIT and DCIT enantiomers in human plasma and breast milk. To our knowledge, this is the first published method that allows a stereoselective determination of these analytes in breast milk. Subsequently, the method was successfully applied to patient samples in a clinical trial setting.

2. Experimental

2.1. Chemicals and reagents

CIT hydrobromide (purity, 99.1%) and DCIT hydrochloride (purity, 100%) were kindly provided by Lundbeck® (Copenhagen, Denmark), while stable isotope-labeled internal standards (IS)

citalopram-d6 (CIT-d6; purity, 98%) and desmethylocitalopram-d3 (DCIT-d3; purity, 95%) were purchased from LGC Standards[®] (Molsheim, France). All chemicals were provided as racemic mixtures of S-(+) and R-(-) enantiomers. Peaks were identified either by using single enantiomer solutions (S-(+)-CIT and R-(-)-CIT provided by Lundbeck[®]) or by measuring samples from patients treated with the pure enantiomer escitalopram (SSRI-Breast Milk study, see below).

Acetonitrile (ACN) HPLC grade used for protein precipitation was purchased from J.T. Baker[®] (Deventer, Holland); methanol (MeOH) HPLC grade from Macron[®] (Gliwice, Poland), ammonium hydroxide 25% and citric acid monohydrate >99% for solid phase extraction from Sigma Aldrich[®] (Steinheim, Germany). Ammonium acetate for buffer preparation and ACN ULC grade for mobile phase preparation were produced by Biosolve[®] (Dieuze, France) and bought from Chemie Brunschwig[®] (Basel, Switzerland). All other reagents and solvents were of analytical grade. Ultra-pure water was supplied by Elga Labwater Purification System from Labtec Services[®] (Villmergen, Switzerland).

Human blank plasma samples (7 different batches) were used for method development and validation, and obtained from the Blood Transfusion Center of the Lausanne University Hospital (Switzerland). A study evaluating antidepressant exposure in blood and in milk of lactating depressed patients treated with various antidepressants is presently underway at the Lausanne University Hospital and other maternities ("SSRI-Breast Milk study", NCT01796132, more information on clinicaltrials.gov). The study was approved by the corresponding ethics committee and all included subjects gave their informed consent to provide blood and milk samples. For the validation of the present method, breast milk samples obtained from 7 untreated female donors were analyzed. All products were stored at -20°C until use.

2.2. Stock and working solutions

Stock solutions of racemic CIT and DCIT were prepared once at 1 mg/ml (calculated as base) in MeOH and stored in a glass tube at -20°C (accuracy of weighing was confirmed by other independently prepared solutions). One single working solution containing both racemic CIT and DCIT at 100 µg/ml was then prepared in MeOH, divided into aliquots of 700 µl and stored in glass vials for a maximum of 1 year at -20°C. Starting from one new aliquot, the working solution was diluted on each validation day with HCl 0.01 N to obtain 6 different series of spiking solutions all containing concentrations at 10, 1, 0.1 and 0.01 µg/ml. Two series were then used for the preparation of calibration standards (duplicate) and four series for validation standards (quadruplicate) in plasma or breast milk (see Section 2.6.3) [28, 29].

Stock solutions of racemic CIT-d6 and DCIT-d3 were prepared at 1 mg/ml (calculated as base) in MeOH and stored in a glass tube at -20°C. These solutions were further diluted with ACN to obtain one working solution containing both internal standards at 250 ng/ml, which was directly added to plasma or to breast milk samples during protein precipitation (see Section 2.5). This solution was stored in a glass bottle for a maximum of 6 months at -20°C.

2.3. Equipment

The liquid chromatography system consisted of a Waters® Acquity UPLC I-Class instrument (Waters, Milford, USA) and was equipped with a binary pump and a 96-vial autosampler. The enantioselective separation was performed on a Phenomenex® Lux Cellulose-2 column (4.6 mm x 150 mm; 5 µm) fitted with a Phenomenex® SecurityGuard filter. Column temperature was set to +30°C and the autosampler remained stable at +10°C. The detector consisted of a tandem quadrupole mass spectrometer (TQS) from Waters®, equipped with an electrospray ionization source operating in positive ionization mode (ESI+). Control of the whole system and data acquisition were managed via MassLynx® software (Waters, version 4.1).

2.4. HPLC-MS/MS conditions

The mobile phase was composed of ammonium acetate buffer (pH 9.0; 20 mM) - ACN, and the flow rate was set to 0.6 ml/min. A gradient elution was used (changes in ACN %): 35% from 0.0 min to 11.0 min, then continuous increase to 45% at 23.5 min. This was followed by a washing step using 90% of ACN from 23.6 min to 25.6 min and a re-equilibration step with initial solvent proportions from 25.7 min to 27.5 min. Column flushing with ACN at the end of each run should ensure an efficient removal of possible strongly retained materials or matrix impurities, which may otherwise deteriorate separation and also column lifetime. Based on still limited experience, mean lifetime of this chiral column is approximately 850 injections or 400 hours of runtime, which can be considered as acceptable under these working conditions. A maximum pressure of 170-210 bars was measured at the beginning of the chromatographic run.

Nitrogen was used both as desolvation gas (flow rate 800 l/h, temperature 600°C) and as cone gas (flow rate 150 l/h). Source temperature was set to 150°C and capillary voltage to 3 kV. Argon was employed as collision gas at a flow rate of 0.20 ml/min. Detection was carried out in multiple reaction monitoring (MRM) mode, using two different time windows. Mass spectrometer settings including optimized cone voltages, collision energies, MRM transitions and dwell times are shown in Table 1.

2.5. Sample preparation

Sample preparation was performed by a two step approach, including generic protein precipitation followed by solid phase extraction as previously described [30]. Briefly, 50 µl of the IS working solution in ACN (corresponding to 50 ng/ml racemic CIT-d6 and DCIT-d3 in plasma/breast milk) were added to 250 µl of plasma or breast milk in a polypropylene tube

followed by the addition of 450 μ l of ACN. The mixture was thereafter vortexed and centrifuged at 3300 x g for 11 min at +8°C for plasma and 20160 x g for 10 min at room temperature for breast milk. Meanwhile, a 96-well plate Oasis[®] MCX support (Waters, Milford, USA) was conditioned first with 500 μ l MeOH and subsequently with 1000 μ l citric acid 1 M. After centrifugation, the clear supernatant was loaded onto the wells, and a first washing step with 1000 μ l citric acid followed by a second washing step with 500 μ l MeOH were performed. Finally, analytes of interest were eluted under a weak vacuum with twice 100 μ l MeOH/NH₄OH 25% 90:10 (v/v). Extracted samples were transferred from the collector plate to glass vials, and 2 μ l of the eluate were injected into the HPLC-MS/MS system.

2.6. Method validation

For both matrices, the method was fully validated according to the harmonized recommendations published by the “Société Française des Sciences et Techniques Pharmaceutiques” (SFSTP) [28, 29, 31]. Current guidelines from the Food and Drug Administration (FDA) [32], the European Medicines Agency (EMA) [33] and the 3rd AAPS/FDA Bioanalytical Workshop [34] were also considered.

2.6.1. Selectivity and carry-over

Method selectivity toward matrix was assessed by comparing blank plasma or breast milk samples from at least 6 different donors with a spiked sample, including internal standards. Carry-over effect was evaluated for each analyte by injecting a blank plasma or breast milk sample right after the highest concentration of the calibration curve.

2.6.2. Matrix effect, extraction recovery and process efficiency

First, matrix effects were assessed qualitatively using the internationally recognized technique proposed by Bonfiglio et al. [36]. While a mixture of analytes and IS was continuously infused post-column directly to the MS detector, extracts from seven different blank plasma or breast milk samples were injected into the HPLC-MS system. For plasma samples, all the compounds including IS were infused at a concentration of 0.4 ng/ml in HCl 0.01 N in combined mode at a flow rate of 8 µl/min. For breast milk samples, CIT/CIT-d6 were infused at 0.6 ng/ml in HCl 0.01 N and DCIT/DCIT-d3 at 0.4 ng/ml in combined mode at a flow rate of 8 µl/min. Extracted water and unextracted mobile phase (ammonium acetate 20 mM pH 9.0-ACN 65:35, v/v) were also analyzed to better determine the origin of potential matrix effects (e.g. sample preparation procedure, endogenous plasma or breast milk components). A signal suppression or enhancement at the retention time of the compounds suggests a matrix effect.

Subsequently, matrix effects were also assessed in a quantitative way, following the procedure proposed by Matuszewski et al. and the recommendations of the EMA [33, 37]. For this purpose, different solutions were prepared as follows: three water samples spiked at low and high concentration with analytes and IS after extraction (A), six blank plasma or breast milk samples spiked at low and high concentration in duplicate with analytes and IS after extraction (B) and six blank plasma or breast milk samples spiked at low and high concentration in duplicate with analytes and IS before extraction (C). Low concentration was defined as three times the anticipated lower limit of quantification ($3 \times \text{LLOQ} = 0.3 \text{ ng/ml}$ for S-(+)-CIT, R-(-)-CIT, S-(+)-DCIT and R-(-)-DCIT), whereas high concentration was prepared at 80% of the anticipated upper limit of quantification ($80\% \text{ ULOQ} = 80 \text{ ng/ml}$ for S-(+)-CIT, R-(-)-CIT, S-(+)-DCIT and R-(-)-DCIT). Internal standards were always at a constant concentration of 25 ng/ml. Matrix effects ($\text{ME} = \text{B/A}$), extraction recovery ($\text{ER} = \text{C/B}$) and process efficiency ($\text{PE} = \text{C/A}$) can finally be calculated from the mean peak areas obtained from the experiments A, B and C. Variability

between the six plasma or breast milk samples were expressed as relative standard deviation (RSD). The IS-normalized ME, ER and PE were also calculated.

2.6.3. Trueness, precision and accuracy profiles

Validation in plasma and breast milk was carried out on 3 different days/series to determine trueness and precision of the method. For this purpose, blank plasma or breast milk samples from a healthy donor were independently prepared with appropriate spiking solutions (see Section 2.2) at 6 concentration levels in duplicate (calibration standards) and quadruplicate (validation standards) [28, 29]: 0.1, 0.3, 2.5, 25, 50 and 100 ng/ml for S-(+)-CIT, R-(-)-CIT, S-(+)-DCIT and R-(-)-DCIT. The concentration range was chosen in order to include common therapeutic plasma levels [38, 39] and expected concentration values in breast milk [40]. An additional validation standard containing 200 ng/ml of S-(+)-CIT, R-(-)-CIT, S-(+)-DCIT and R-(-)-DCIT was also included, in case of outlying values requiring sample dilution. In plasma, this standard was first diluted 1:1 (v/v) with blank plasma followed by IS addition and then usual sample preparation (see Section 2.5). In breast milk, IS was first added to this standard before being precipitated and extracted following the described protocol (see Section 2.5). The sample extract was finally diluted 1:1 (v/v) with MeOH/NH₄OH 25% 90:10 (v/v) just before injection. Calibration curves were determined using the peak area ratio between each analyte and its respective IS.

Validation performances were described according to the recommendations of the SFSTP [31]. Briefly, the mean result of repeated measurement is composed of the true value, systematic errors and random errors related to the trueness and the precision of the method. Trueness (called accuracy in the FDA guidelines [32]) represents the closeness of the mean value obtained from a set of replicated measurements and the true or nominal value. The dispersion level among replicated measurements is defined as precision and was described by the

repeatability (intra-day or within-run variances) and the intermediate precision of an analytical method (sum of intra-day or within-run variances and inter-day or between-run variances). Both parameters were expressed as RSD based on the theoretical concentration at each level according to the recommendations of Rozet et al. [35]. The LLOQ was determined as the lowest concentrated validation standard, for which the signal to noise ratio between analyte and blank matrix was at least 5 [32] and the validation performances were acceptable. Trueness should be within 85-115% of the theoretical concentration level for each point of the calibration curve, except for the LLOQ (80-120%) [32]. Precision should not exceed 15%, except at the LLOQ (20%) [32]. The total error of the analytical method including systematic and random errors (trueness and precision) was presented as accuracy profiles with one-sided 95% confidence intervals.

2.6.4. Stability

Stability tests of CIT and its main metabolite in plasma have been published previously [30]. Therefore, only stability tests in breast milk were performed under different conditions at low and high concentration (0.3 and 80 ng/ml for S-(+)-CIT, R-(-)-CIT, S-(+)-DCIT and R-(-)-DCIT). Analyte stability in spiked breast milk from 5 different donors was assessed at room temperature up to 72h (protected or not from light), at +4°C for 72h, after 3 freeze/thaw cycles and after 4 months at -20°C. Post-preparative stability was evaluated by re-injecting extracted samples refrigerated at +10°C on the autosampler up to 72h. Results were expressed as observed variations in percentage compared to the mean initial concentration. Inter-donor variability was described by RSD.

3. Results and discussion

3.1. Method development

3.1.1. Sample preparation

A previously validated two step approach was chosen for sample preparation of plasma and breast milk [30], consisting of a simple protein precipitation with ACN (matrix to precipitant ratio of 1:2) followed by a solid phase extraction. An efficient removal of potential endogenous interference was particularly desirable as CIT enantiomers do not co-elute with their respective IS, and human milk composition differs from blood plasma (e.g. rich in lipids). Indeed, following breast milk centrifugation, the supernatant consisted of two immiscible liquid phases (organic and oily), which confirms visually the higher amount of fat molecules. Centrifugation speed was therefore set higher for breast milk preparation compared to plasma (20160 x g instead of 3300 x g), in order to obtain good phase separation. The organic phase only was loaded onto the SPE plate. Owing to the very good sensitivity of the MS instrument, no evaporation and re-concentration step was necessary after extraction. The possibility of simplifying this two step approach in order to reduce preparation time and costs has yet to be assessed.

Solid extraction performances were obtained at low and high concentration in both biological fluids. The extraction recovery of individual analytes and IS varied between 71-81% in plasma and 58-83% in breast milk, with RSD not exceeding 13% (see Table 2). Results after IS-normalization ranged between 96-114% for plasma and 99-107% for breast milk, with a maximal RSD of 14%.

3.1.2. HPLC optimization

Chromatographic separation of the four analyte enantiomers was tested on several columns in chiral screening programs offered by Chiral Technologies® (Illkirch, France) and Phenologix® (Torrance, USA), leading to the purchase of the best adapted column (i.e. Lux Cellulose-2). Best LC conditions were then determined in our laboratory in different experiments in terms of buffer composition (ammonium formate, ammonium bicarbonate and ammonium acetate),

buffer pH (4, 8 and 9), buffer molarity (10 and 20 mM), mobile phase flow rate (0.5 and 0.6 ml/min), injection volume (2, 5 and 10 μ l) and column temperature (+30 and +40°C). An obstruction of the electrospray nebulizer needle was observed with ammonium bicarbonate buffer (20 mM) after the injection of long sample series at a flow rate of 0.6 ml/min. This issue was solved by replacing the buffer salt by ammonium acetate. The best separation was achieved with a stepwise elution gradient of ammonium acetate buffer (pH 9.0; 20 mM) - ACN at a flow rate of 0.6 ml/min in a total runtime of 27.5 min (including washing and re-equilibration step). Although an ultra-high pressure liquid chromatography system was used, it was unfortunately not possible to take advantage of its superior efficiency over a conventional HPLC in terms of improved analysis time, resolution and sensitivity. In fact, chiral columns packed with sub-2 μ m or 2 μ m particles are not yet marketed, thus limiting pressure resistance and separation performance of the system. Nevertheless, the developed method shows a lower limit of quantification [13, 21, 23] or a shorter runtime [17, 19] compared to previously published methods, and uses the analytes' deuterated form as internal standards. Typical retention times (RT) of the four analytes and their respective IS are reported in Table 1, and an example of MRM chromatogram is shown in Figure 1. Slight changes in RT were observed on the same column between the beginning and end of column lifetime, as well as between two columns of different production batches. However, there is no matrix effect or signal interference in a large region around expected RT (see Section 3.2.2), and separation as well as elution order of analytes remained unchanged.

Stable isotope-labeled internal standards have similar physico-chemical characteristics to analytes. Hence, they minimize via normalization the variations resulting from sample preparation and compensate for potential matrix effects, as long as they co-elute with their respective analytes. In this case, S-(+)-DCIT-d3 and R-(-)-DCIT-d3 co-eluted only partially with DCIT enantiomers, while S-(+)-CIT-d6 and R-(-)-CIT-d6 were completely separated from their

respective analytes (see Figure 1). The difference in retention time was probably due to the presence of a higher deuterated IS for CIT enantiomers (d6 vs. d3), the enhanced selectivity of this column and the long runtime. However, given the extensive sample preparation selected for this method, matrix effects should be limited as demonstrated in Section 3.2.2.

3.1.3. MS/MS optimization

Mass spectrometry conditions yielding the highest signal intensities for CIT and DCIT were assessed using the IntelliStart[®] program implemented in MassLynx[®] or by manual tests. Briefly, each compound was infused at 25 ng/ml HCl 0.01 N directly to the MS detector at a flow rate of 5 µl/min in combined mode with the mobile phase set to 0.6 ml/min (ammonium acetate pH 9.0 20 mM-ACN 60:40, v/v). Optimal conditions were: source temperature 150°C, capillary voltage 3.0 kV, cone voltage 20 V, cone gas flow 150 l/h, desolvation gas temperature 600°C, desolvation gas flow 800 l/h, collision gas flow 0.2 ml/min, and collision energies 26, 24, 22 and 20 eV for CIT, CIT-d6, DCIT and DCIT-d3, respectively. Detection was performed in MRM mode using one specific function (time window) for both enantiomers of each couple analyte/IS. Repeatability of the MS instrument was evaluated by injecting 10 times the same extracted sample at low and high concentration (0.3 ng/ml and 80 ng/ml for S-(+)-CIT, R-(-)-CIT, S-(+)-DCIT and R-(-)-DCIT), while IS concentration remained the same. Excellent results in terms of peak areas were achieved, with RSD ranging in plasma between 1.2-1.5% for low and 0.5-0.9% for high concentration, in breast milk between 0.8-3.3% for low and 0.6-0.7% for high concentration (data not shown). IS area values varied between 0.4-1.0% in plasma and 0.6-0.8% in breast milk.

3.2. Method validation

3.2.1. Selectivity and carry-over

Comparing blank plasma or breast milk samples from 6 different donors with spiked samples at the LLOQ, no endogenous interference was observed in blank samples at the different retention times of analytes and IS. Furthermore, the signal to noise ratio at the LLOQ was > 5 for all compounds, in accordance with FDA guidelines [32]. Potential signal cross-contaminations of the IS on its respective analyte (and vice versa) were assessed only for S-(+)-DCIT and R-(-)-DCIT (S-(+)-CIT and R-(-)-CIT do not co-elute with their IS). No significant signal was measured on the analytes' MRM trace when a blank sample containing only IS was injected. Conversely, no significant signal was observed on the internal standards' MRM trace after the injection of the highest calibration standard without IS. Carry-over was assessed by analyzing a blank plasma or breast milk sample right after the highest calibration standard (i.e. 100 ng/ml). No influence on the signal was detected, suggesting that washing procedures of the injection needle and the column between two analyses were appropriate.

3.2.2. Matrix effects and process efficiency

Investigation of potential matrix effects is a key element for the validation and implementation of an analytical method. Indeed, the presence of endogenous plasma or breast milk components such as proteins, phospholipids or salts may either conduct to suppression or enhancement of the analytes' ionization. This phenomenon can lead to an under- or overestimation of the true concentration value. The use of a stable isotope-labeled IS that co-elutes with its respective analyte is one possibility to avoid such problems. In fact, signal of both the analyte and its IS will be affected in the same way, thus peak area ratios will remain constant. In the present work, S-(+)-CIT and R-(-)-CIT did not co-elute with their respective IS and S-(+)-DCIT and R-(-)-DCIT only partially, therefore potential matrix effects could theoretically lead to signal alterations of the

analyte but not its IS (or vice versa). However, this was demonstrated to be limited by an extensive sample preparation such as the two-step approach selected for this method and an efficient chromatographic separation of potential interferences.

Matrix effects were analyzed in a qualitative (post-column infusion) and quantitative manner (Matuszewski method). In plasma, the post-column infusion test showed a signal suppression at 3 and 4 min run time (not for extracted water or mobile phase), but distant from the first eluted peak and therefore unproblematic (see Figure 2). The MS response remained otherwise stable and constant, except at the end of the analysis due to column flushing. In breast milk, signal suppression was observed for several blank breast milk samples (but not for mobile phase or extracted water) at the beginning (from 3 to 6 min) as well as in the middle of the run (from 16 to 18 min), see Figure 3. However, these signal suppressions did not overlap with the retention times of the analytes or IS. A decrease in signal intensity was observed at the retention times of S-(+)-CIT and R-(-)-CIT, but only when injecting a mobile phase and was therefore considered of low importance. In summary, no significant signal was detected at the retention time and transition of the analytes. Absolute matrix effects varied between 96-103% in plasma and 91-97% in breast milk for all compounds, with very small variations of 2-4% and 3-4%, respectively (see Table 2). Performances were even better after IS normalization (99-101% and 98-105%, respectively). In conclusion, these results confirm the efficient removal of endogenous matrix components through sample preparation and suggest a low risk of signal interference.

Finally, process efficiency combining both matrix effects and extraction recovery was determined (see Table 2). Good performances were achieved after IS normalization in plasma (96-115%, RSD 0-13%) and breast milk (97-110%, RSD 1-3%).

3.2.3. Trueness, precision and accuracy profiles

Method validation was performed on three independent days, using 6 levels of calibration standards in duplicate and 6 levels of validation standards in quadruplicate from 0.1 to 100 ng/ml for S-(+)-CIT, R-(-)-CIT, S-(+)-DCIT and R-(-)-DCIT. Concentrations of the validation standards were recalculated, using the daily calibration curves and different mathematical regression models in order to determine the best response function. Linear regression, linear regression forced through 0, weighted linear regression ($1/x$ or $1/x^2$) were tested as well as quadratic regression, quadratic regression forced through 0, weighted quadratic regression ($1/x$ or $1/x^2$) and log-log transformed regression.

In plasma, validation criteria in terms of trueness and precision were met over the entire calibration range of 0.1-100 ng/ml for S-(+)-CIT and R-(-)-CIT by using different response function models. The LLOQ for S-(+)-DCIT and R-(-)-DCIT was determined at 0.3 ng/ml, due to stronger variations in terms of repeatability and intermediate precision at low concentrations (data not shown). However, this level was still considered sufficient for the determination of metabolite plasma concentrations in patient samples. The linear regression model forced through zero was finally chosen as the most suitable response function for all compounds, yielding acceptable performances in terms of trueness (90.0-113.3%), repeatability (0.9-15.9%) and intermediate precision (1.3-17.8%), see Table 3. These results were in agreement with the recommendations of the FDA [32], thus the method can be used in routine.

In conclusion, a six-point calibration curve was validated with a LLOQ set to 0.1 ng/ml for S-(+)-CIT and R-(-)-CIT, and a five-point calibration curve with a LLOQ of 0.3 ng/ml for S-(+)-DCIT and R-(-)-DCIT. The upper limit of quantification (ULOQ) was 100 ng/ml for all four compounds. A simple dilution step was also validated in case of outlying values (i.e. 100-200 ng/ml), see Section 2.6.3. The accuracy profiles (total error) were within the acceptance limits of $\pm 30\%$ for biological samples [34], except for S-(+)-DCIT and R-(-)-DCIT where an accuracy interval of -16% to +43% was observed at the LLOQ (see Figure 4). However, this deviation was

considered acceptable in this case, due to its limited clinical impact at this very low concentration level (in the worst case 0.30 ng/ml would be wrongly reported as 0.25-0.43 ng/ml).

In breast milk, several response functions were suitable to describe S-(+)-CIT and R-(-)-CIT in terms of trueness and precision over the complete calibration range of 0.1-100 ng/ml. Validation performances for S-(+)-DCIT and R-(-)-DCIT at 0.1 ng/ml were not acceptable, independently of the selected response function (data not shown). Similar to plasma, the LLOQ for these two enantiomers was therefore set to 0.3 ng/ml. Using the linear regression model forced through zero for all compounds, good validation results were achieved in terms of trueness (97.1-103.6%), repeatability (0.9-8.4%) and intermediate precision (0.9-9.6%), see Table 3.

In conclusion, a six-point and five-point calibration curve was validated with a LLOQ set to 0.1 ng/ml (S-(+)-CIT, R-(-)-CIT) and 0.3 ng/ml (S-(+)-DCIT, R-(-)-DCIT), respectively. The ULOQ for all four compounds was 100 ng/ml. The dilution method in breast milk had to be modified (post-extraction dilution of the eluate with MeOH/NH₄OH 25% 90:10 v/v), due to systematic undervaluation of the true value of S-(+)-CIT and R-(-)-CIT but not of DCIT enantiomers (trueness approximately 80%, data not shown). This might be explained by the difficulty of sample homogenization or a loss of analytes during transfers, but could not be confirmed in additional experiments. The accuracy profiles (total error) were within the acceptance limits of $\pm 30\%$ for biological samples (see Figure 4).

3.2.4. Stability tests

Drug stability of SSRI antidepressants in plasma has been demonstrated previously under several conditions [30]. Short term stability at low and high concentration was therefore assessed in breast milk only, up to 72h at room temperature when protected or not from light,

after 72h at +4°C, after 3 freeze/thaw cycles and after 4 months at -20°C (see Table 4). For all the conditions tested, deviations from initial concentrations ranged between 88-109% (RSD 1-13%), suggesting no significant sample deterioration. These results are compatible with recommendations for drug stability studies [33].

4. Practical applications

While abundant data are available on potential risks for the newborn associated with SSRI use during pregnancy, information on milk transfer and safety of these drugs in breastfed infants are often incomplete [41]. The analytical method presented in this article was developed and validated primarily for use in the SSRI-Breast Milk study (see Section 2.1). The main objective of this observational study is to describe antidepressant pharmacokinetics in pregnant and nursing women, and drug transfer into breast milk using a population pharmacokinetics approach (pop PK). Complete results from this study will be published elsewhere (analysis ongoing). An example of representative MRM chromatograms of plasma and breast milk samples (fore- and hindmilk) from one nursing mother taking CIT 20 mg/day are shown in Figure 5. All samples were collected during the same feeding occasion 13h after the last drug dose. This sampling design allows the determination of the milk-to-plasma ratio (M/P) for each analyte and the RID for the pharmacologically relevant S-(+)-CIT. RID was calculated as follows:

$$RID [\%] = \frac{C_{milk} * V_{milk} [mg/kg/day]}{D_{mat} [mg/kg/day]}$$

where C_{milk} represents drug concentration measured in milk, V_{milk} the daily milk intake (in average 150 ml/kg/day) and D_{mat} the weight-adjusted daily maternal dose.

The following concentrations were measured: in plasma 22.2 ng/ml for S-(+)-CIT, 25.5 ng/ml for R-(-)-CIT, 6.7 ng/ml for S-(+)-DCIT and 6.5 ng/ml for R-(-)-DCIT; in foremilk 30.0 ng/ml for S-(+)-CIT, 37.9 ng/ml for R-(-)-CIT, 9.9 ng/ml for S-(+)-DCIT and 9.0 ng/ml for R-(-)-DCIT; in hindmilk 39.0 ng/ml for S-(+)-CIT, 49.1 ng/ml for R-(-)-CIT, 13.2 ng/ml for S-(+)-DCIT and 12.4 ng/ml for R-(-)-DCIT. In this case, concentrations of R-(-)-CIT but not R-(-)-DCIT were slightly higher in both matrices than those of the active S-(+) enantiomer (S/R ratio for CIT: 0.8-0.9 in plasma or breast milk; S/R ratio for DCIT: 1.0-1.1 in plasma or breast milk). A similar stereospecific disposition in plasma has been described previously [10, 11]. Milk levels of both drug and metabolite were consistently higher than in plasma (M/P ratio for the active S-(+)-CIT: 1.4 in foremilk and 1.8 in hindmilk), confirming results from previous reports [42]. This may suggest possible drug accumulation in breast milk. However, the calculated RID for the active S-(+)-CIT does not exceed 3% in this case, meaning that a breastfed infant ingests daily via breast milk less than 3% of the weight-adjusted maternal dose. These amounts of drug can be considered as small and are in line with values reported in other studies [43]. Differences between fore- and hindmilk are probably due to changes in milk composition during breastfeeding (i.e. hindmilk rich in fat).

5. Conclusions

A sensitive and selective HPLC-MS/MS method was developed for the quantification of CIT and DCIT enantiomers in human plasma and breast milk. To our knowledge, this is the first published method that allows an enantioselective quantification of these analytes in breast milk. Sample preparation was performed by a two step approach consisting of protein precipitation and solid phase extraction. Despite a rather long and costly sample preparation, this approach provides clean samples free of endogenous interference, as confirmed by the lack of significant

matrix effects. Chiral separation was achieved with a good resolution on a cellulose-derived column. For both matrices, the method was fully validated over a large concentration range according to the latest international guidelines and is suitable for use in routine or in pharmacokinetic studies in pregnant or breastfeeding women.

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Figure legends

Figure 1: Representative MRM chromatogram of a calibration standard in plasma containing the analytes at the lower limit of quantification (0.1 ng/ml for S-(+)-CIT and R-(-)-CIT, 0.3 ng/ml for S-(+)-DCIT and R-(-)-DCIT; solid lines) and the internal standards at constant concentration (25 ng/ml for all compounds; dashed lines)

Figure 2: MRM chromatograms of seven blank plasma extracts, mobile phase and extracted water after post-column infusion overlaid with a MRM chromatogram of a calibration standard

Figure 3: MRM chromatograms of seven blank breast milk extracts, mobile phase and extracted water after post-column infusion overlaid with a MRM chromatogram of a calibration standard

Figure 4: Accuracy profiles of the investigated compounds obtained with validation standards in plasma (left) and breast milk samples (right). Trueness (solid lines), bias (dashed lines) and acceptance limits ($\lambda = \pm 30\%$, dotted lines) are shown

Figure 5: MRM chromatograms of plasma (A) and breast milk (fore- and hindmilk, B and C) from one nursing patient treated with citalopram 20 mg/d. Solid lines represent the analytes, dashed lines the internal standards

Figure 1

632 **Figure 3**

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635 **Figure 4**

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638 **Figure 5**

641 **Table 1:** Mass spectrometer settings and typical retention times

Compound	Time window (min)	Transition ions (m/z)	Cone voltage (V)	Collision energy (eV)	Dwell time (ms)	Retention time (min)
Desmethylocitalopram	0.0-15.0	311.2 → 109.1	20	22	250	11.5 (S) 12.3 (R)
Desmethylocitalopram-d3	0.0-15.0	314.2 → 109.1	20	20	250	11.1 (S) 11.8 (R)
Citalopram	15.1-27.5	325.2 → 109.1	20	26	250	23.5 (S) 24.3 (R)
Citalopram-d6	15.1-27.5	331.3 → 109.1	20	24	250	21.7 (S) 22.5 (R)

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644 **Table 2:** Extraction recovery (ER), matrix effect (ME) and process efficiency (PE) in plasma and
645 breast milk

Compound	Concentration (ng/ml) n=6	ER% (RSD% ^a)		ME% (RSD% ^a)		PE% (RSD% ^a)	
		plasma	breast milk	plasma	breast milk	plasma	breast milk
Analytes							
S-(+)-citalopram	0.3	77 (9)	78 (7)	100 (3)	91 (4)	77 (11)	72 (10)
	80	75 (6)	82 (2)	96 (2)	95 (4)	72 (5)	78 (4)
R-(-)-citalopram	0.3	77 (9)	78 (8)	100 (3)	92 (3)	78 (10)	72 (9)
	80	75 (6)	82 (2)	96 (2)	96 (4)	72 (5)	78 (2)
S-(+)-desmethylocitalopram	0.3	81 (12)	61 (11)	102 (4)	97 (4)	83 (9)	59 (8)
	80	75 (2)	68 (4)	97 (2)	97 (3)	73 (1)	65 (3)
R-(-)-desmethylocitalopram	0.3	81 (13)	61 (12)	103 (3)	99 (5)	83 (10)	60 (9)
	80	75 (2)	67 (5)	97 (2)	97 (4)	73 (1)	65 (3)
Internal standards (IS)							
S-(+)-citalopram-d6	25	80 (7)	79 (5)	101 (2)	92 (4)	80 (8)	73 (8)
	25	78 (4)	82 (2)	96 (2)	95 (3)	75 (3)	77 (3)
R-(-)-citalopram-d6	25	79 (7)	79 (5)	101 (2)	94 (4)	80 (8)	74 (7)
	25	79 (4)	83 (2)	96 (2)	95 (4)	75 (3)	79 (3)
S-(+)-desmethylocitalopram-d3	25	71 (3)	58 (5)	102 (2)	94 (3)	73 (3)	55 (8)
	25	73 (2)	63 (5)	97 (2)	97 (3)	71 (1)	61 (4)
R-(-)-desmethylocitalopram-d3	25	71 (3)	58 (5)	101 (2)	94 (3)	72 (3)	55 (8)
	25	73 (2)	63 (5)	96 (2)	97 (3)	70 (1)	61 (4)
IS-normalization							
S-(+)-CIT / S-(+)-CIT-d6	0.3	97 (2)	99 (3)	99 (1)	99 (2)	96 (3)	99 (3)
	80	96 (2)	100 (1)	100 (0)	100 (2)	96 (2)	100 (1)
R-(-)-CIT / R-(-)-CIT-d6	0.3	97 (2)	99 (4)	100 (1)	98 (1)	97 (2)	97 (3)
	80	96 (2)	99 (1)	100 (0)	101 (1)	96 (2)	100 (1)
S-(+)-DCIT / S-(+)-DCIT-d3	0.3	114 (14)	105 (7)	101 (2)	104 (6)	115 (12)	108 (1)
	80	104 (0)	107 (1)	100 (0)	100 (0)	103 (0)	106 (1)
R-(-)-DCIT / R-(-)-DCIT-d3	0.3	114 (14)	105 (8)	101 (2)	105 (7)	115 (13)	110 (3)
	80	103 (0)	106 (1)	101 (0)	100 (1)	104 (0)	106 (1)

646 ^a relative standard deviation

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648 **Table 3:** Trueness, repeatability and intermediate precision in human plasma and breast milk (for definitions refer to section 2.6.3)

Compound	Concentration (ng/ml)	Plasma			Breast milk		
		Trueness (%)	Precision		Trueness (%)	Precision	
			Repeatability (%)	Intermediate precision (%)		Repeatability (%)	Intermediate precision (%)
S-(+)-citalopram	0.1	93.8	4.6	5.9	103.6	8.0	8.4
	0.3	101.2	6.1	6.1	99.5	4.8	5.0
	2.5	95.9	3.1	3.9	98.1	1.1	1.7
	25	98.9	3.7	5.1	101.1	1.2	1.2
	50	99.9	3.5	5.1	102.1	1.3	1.3
	100	101.9	1.2	1.5	101.0	1.0	1.0
	200/2	96.0	3.3	7.9	102.4	1.9	3.4
R-(-)-citalopram	0.1	90.0	4.4	5.6	100.1	8.4	9.6
	0.3	99.2	6.6	6.8	97.1	4.3	4.3
	2.5	95.1	2.9	3.8	97.3	0.9	2.1
	25	98.6	3.7	5.4	100.8	1.1	1.1
	50	99.8	3.5	4.6	101.8	1.2	1.4
	100	101.9	1.2	1.4	100.9	0.9	0.9
	200/2	95.9	3.3	7.6	103.2	1.9	3.6
S-(+)-desmethylocitalopram	0.3	113.3	15.5	16.0	103.6	7.8	7.8
	2.5	99.2	1.5	2.4	100.0	4.3	4.4
	25	102.3	1.1	2.3	100.9	1.6	1.6
	50	101.9	1.4	1.4	101.8	1.1	1.1
	100	100.8	1.4	1.4	99.6	1.3	1.7
	200/2	97.2	2.2	3.9	103.3	2.1	4.6
R-(-)-desmethylocitalopram	0.3	111.5	15.9	17.8	99.8	8.3	8.3
	2.5	99.2	1.6	2.4	100.1	4.1	4.1
	25	103.1	0.9	2.3	101.1	1.4	1.4
	50	102.4	1.4	1.4	101.8	1.1	1.1
	100	100.6	1.1	1.3	99.2	1.1	1.7
	200/2	97.0	2.4	4.1	102.9	2.5	4.6

649 **Table 4:** Stability in breast milk and in post-preparative solvent (n=5)

Compound	S-(+)-citalopram		R-(-)-citalopram		S-(+)-desmethyl-citalopram		R-(-)-desmethyl-citalopram	
	0.3	80	0.3	80	0.3	80	0.3	80
RT, protected from light								
Breast milk, 72h	98 (3)	99 (3)	102 (4)	102 (5)	95 (6)	97 (3)	95 (3)	98 (3)
RT, light								
Breast milk, 72h	96 (3)	98 (2)	100 (4)	102 (5)	91 (6)	96 (3)	94 (9)	96 (4)
Fridge 4°C								
Breast milk, 72h	96 (4)	96 (4)	99 (3)	101 (3)	94 (2)	100 (2)	96 (3)	100 (2)
Autosampler 10°C								
Post-preparative, 72h	98 (3)	98 (1)	101 (3)	100 (1)	101 (1)	102 (2)	96 (4)	99 (2)
Freeze / thaw								
Breast milk, 3 cycles	95 (5)	96 (4)	97 (4)	98 (4)	93 (4)	98 (4)	95 (7)	98 (3)
Freezer -20°C								
Breast milk, 4 months	90 (5)	91 (4)	88 (4)	88 (5)	93 (4)	97 (4)	89 (3)	97 (4)

650

651

**Citalopram
racemate**

Blood

Breast milk

Sample preparation:
Protein precipitation
Solid phase extraction

Stereoselective separation:
Chiral HPLC column

Detection:
Electrospray tandem MS