Enantiomeric separation and quantification of citalopram in serum by ultra-high performance supercritical fluid chromatography-tandem mass spectrometry

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Abstract

A method for enantiomeric separation and quantification of R/S-citalopram in serum was developed and validated using ultra-high performance supercritical fluid chromatography-tandem mass spectrometry (UHPSFC-MS/MS). Sample preparation prior to UHPSFC-MS/MS analysis consisted of protein precipitation with acidic acetonitrile and filtration through a phospholipid removal plate. The UHPSFC-MS/MS method used an UPC^2 Trefoil CEL2 column with a mobile phase consisting of CO_2 and methanol/acetonitrile (70:30, v/v) with 10mM ammonium acetate. The injection volume was 1 µl and run time was 4 minutes. MS/MS detection was performed with positive electrospray ionization and two multiple reaction monitoring transitions (*m/z* 325.1 > 262.0 and *m/z* 325.1 > 109.0). The calibration range was 5-500 nM for each analyte. The between-assay relative standard deviations were in the range of 3.4-4.5%. Recovery was 81-91% and matrix effects ranged from 96 to 101% (corrected with internal standard). After development and initial testing, the method has been successfully implemented in routine use in our laboratory for both separation and quantification of R/S-citalopram in more than 250 serum samples for therapeutic drug monitoring.

Keywords: UHPSFC-MS/MS, citalopram, enantiomeric, serum, therapeutic drug monitoring

1. Introduction

Many drugs are chiral compounds, most often produced and marketed as racemates consisting of an equimolar mixture of R- and S-enantiomers. The enantiomers of chiral drugs may exhibit marked differences both in their pharmacodynamic and pharmacokinetic properties. Citalopram is a selective serotonin reuptake inhibitor used for the treatment of depression and anxiety disorders. In many countries, the majority of citalopram is sold as the pure S-enantiomer (escitalopram) but in some therapeutic formulations it is sold as a racemic mixture. The S-enantiomer is more than twice as potent serotonin reuptake inhibitor as the racemic mixture and more than 100-200 times more potent than the R-enantiomer [1]. The enantiomers are metabolized by different pathways [2]. As the serum concentrations achieved by a certain dose are influenced by various factors such as sex, age, genetics etc., therapeutic drug monitoring (TDM) is an important tool to ensure that the patient's dose is optimized [3]. Due to the higher potency of S-citalopram than of racemic citalopram, the recommended therapeutic reference interval is lower for S-citalopram [3]. As it is sometimes not stated on the requisition form whether the patient is treated with S-citalopram or racemic citalopram, an enantioselective analysis may be a valuable tool to resolve this issue, thereby facilitating correct interpretation of the measured serum concentration and also a more adequate dosing of the drug.

In the pharmaceutical industry, preparative supercritical fluid chromatography (SFC) has been used for many years for chiral separation [4, 5]. In clinical and forensic toxicology, gas chromatography and high performance liquid chromatography (HPLC) are the techniques most commonly used for chiral analysis [6]. In order to determine the enantiomeric composition of citalopram in different matrices, various methods have been developed in recent years. Most of these involve the employment of HPLC equipped with chiral stationary phases [7-10]. These chiral HPLC-methods were reported to have undesirably long equilibration times -and analytical run times, varying from 12 to 45 minutes. As diffusion processes in HPLC can be relatively slow, significant peak broadening may occur and affect the quality of the separation. The increased diffusivity of SFC eluents leads to improved peak resolution and sharper peaks [11]. SFC also has other potential advantages including rapid separation of chiral analytes due to low viscosity and high diffusivity of supercritical CO_2, the main component in SFC mobile phase. As a result, high flow rate can be employed due to low pressure drop across the column, giving rise to shorter analytical run time with the same or higher resolving power [11, 12]. The usage of CO_2 also leads to reduction in the use of organic solvents compared to HPLC. The cost of pure CO_2 is relatively low, and it is virtually non-toxic and non-flammable. Only one SFC method for enantiomeric separation of citalopram by normal phase chromatography using a semi-preparative chiral column and UV-detection has been reported [13]. As this method had a run time of 35 minutes, it is less suitable for high-throughput routine purposes.

More robust SFC equipment has recently been developed in order to give better control of temperature and pressure. Accurate pressure control facilitates the use of gradient elution, which is a considerable advantage when the aim is to achieve short and efficient chromatography. These instruments, named Ultra High Performance SFC (UHPSFC), have

increased the popularity of SFC [14, 15]. Coupling SFC with mass spectrometry (MS) is a crucial step to increase sensitivity, selectivity and compound identity. Normal phase chromatography SFC has traditionally been used for chiral separation, but in the last years, more polar solvents such as methanol are used as co-solvent in SFC-MS, providing efficient electrospray ionization (ESI) [15]. In newer SFC models, an external pump delivers a make-up solvent to the column effluent to enhance ionization, increasing sensitivity and facilitating the usage of SFC-MS [14, 15]. Recently, new columns for chiral separation designed for the UHPSFC have been introduced (http://www.waters.com). These columns contain a modified polysaccharide-based stationary phase.Compared to other brands the particle size is reduced from 3.0 to 2.5 µm , providing high efficiency and shorter analytical run times.

The purpose of this study was to develop a robust and specific UHPSFC-MS/MS method for enantioselective quantification of R/S-citalopram in serum, suitable for routine use.

2. Materials and methods

2.1. Chemicals and reagents

R/S-citalopram was purchased from Carbosynth (purity 99.9%) (Berkshire, UK) and Toronto Research Chemicals Inc. (purity 98.0%) (Toronto, Canada). S-Citalopram-d_6 (purity 98%) was obtained from Toronto Research Chemicals Inc. and R/S-citalopram-d_4 (purity 99.5%) from Chiron (Trondheim, Norway). Enantiomeric distribution of the citalopram standards and internal standards was 50 % R-citalopram and 50 % Scitalopram. S-citalopram was obtained from Sequoia Research Products (Pangbourne, UK). LC-MS grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany), LC-MS chromasolve grade 2-propanol and ammonium hydroxide (ACS-reagent 28-30% NH_3 basis) were from Sigma-Aldrich (Saint Louis, MO, USA). CO_2 grade 5.2 was obtained from Aga (Oslo, Norway). Other chemicals were of analytical grade from various commercial sources. External quality control samples (n=8) were obtained from LGC Standards Proficiency Testing (Bury, UK). All citalopram concentrations are given in nM. The conversion factor from nM to ng/ml is 0.324 for R/S-citalopram. Human blank serum was obtained from healthy blood donors not using citalopram or escitalopram (St. Olav University Hospital, Trondheim, Norway).

2.2. Preparation of standard solutions

Two separate stock solutions were prepared in methanol (1mM), identified as calibrator and quality control (QC) stock solutions. Appropriate concentrations of calibrator and QC working solutions were prepared by dilution of stock solutions with methanol. The stock and working solutions were stored at 4-8 °C. The working solutions were used to prepare calibrator samples in blank serum with concentrations of 5, 10, 50, 100 and 500 nM for each enantiomer. QCs were prepared in blank serum at 7 nM (QC1), 75 nM (QC2) and 300 nM (QC3). A QC in blank serum containing S-citalopram at a concentration of 7 nM was used to ensure correct enantiomeric peak identification. The internal standards S-citalopram-d_6 (used

in method validation) and R/S-citalopram-d_4 were diluted with 20 % methanol in water to a concentration of 380 nM and 760 nM, respectively. The standards in serum were stored at -20 °C and the internal standard at 4-8 °C.

2.3. Sample preparation

Automatic sample preparation was performed using a Tecan Freedom Evo pipetting robot (Tecan, Männedorf, Switzerland). Aliquots of serum samples (100 µL) and internal standard (IS) (25 µL) were pipetted onto a phospholipid removal plate (Ostro Protein Precipitation & Phospholipid Removal Plate, 25 mg, Waters, Milford, MA, USA). Ice cold acetonitrile with formic acid (1% v/v, 375 µL) was added to the well. The mixture of serum samples and acetonitrile was mixed by aspirating thrice with the pipetting robot. The precipitate and supernatant were separated using a positive pressure unit (Positive pressure-96, Waters, Taunton, MA, USA) capturing the phospholipids and precipitated protein in the filtration plate. The eluates were collected in 2 mL sample collection wells (96-well Square collection plate, Waters, Milford, MA USA) and sealed with cap-mat square plugs (silicone/PTFE treated pre-slit, Waters, Milford, MA, USA).

2.4. UHPSFC system

The Waters Acquity UPC^2 system (Waters, Milford, MA, USA) with sample manager, binary solvent manager, column manager, convergence manager and isocratic solvent manager was used for separation A 10 µL loop was used and partial loop with needle overfill mode was selected. Chromatographic separation was performed on an Acquity UPC^2 trefoil CEL2 [cellulose tris-(3-chloro-4-methylphenylcarbamate), 3.0 mm x 150 mm, 2.5 µm particles] column (Waters, Taunton, MA, USA). The mobile phase consisted of CO_2 (A) and methanol/acetonitrile (70:30, v/v) with 10mM ammonium acetate (B). The gradient profile was: 25% B in 0.0-0.3 min, 25-30% B in 0.3-2.0 min, 30-40% B in 2.0-2.8 min, 40% B in 2.8-3.5 min, 40-25% B in 3.5-3.6 min and 25% B in 3.6-4.0 min. The column temperature was 35 °C,mobile phase flow rate was 2 mL/min, and injection volume was 1 µL. The autosampler temperature was set to 10 °C. The weak wash and strong wash were performed with 600 µL of methanol/2-propanol (50:50, v/v) and 200 µL of methanol, respectively. The automatic back pressure regulator (ABPR) was set to 2000 psi. The make-up solvent consisted of 2-propanol with 0.1% ammonium hydroxide and the flow rate was 0.3 mL/min. The co-solvent methanol/acetonitrile (70:30, v/v) with 10mM ammonium acetate (B) was stable for one week in ambient temperature and for two months at 4-8 °C.

2.5. MS/MS

A Xevo TQ-S tandem-quadrupole mass spectrometer (Waters, Manchester, UK) equipped with a Z-spray electrospray interface was used. Positive electrospray ionization (ESI) was

performed in the multiple reaction monitoring (MRM) mode. The capillary voltage was set to 3.0 kV, the source block temperature was 120 ºC, the desolvation gas (nitrogen) was heated to 500 ºC and delivered at a flow rate of 1000 L/h, and the cone gas (nitrogen) was set to 150 L/h. Dwell times were automatically adjusted with 15-20 data points per peak. The *m/z* 325.1 > 262.0 (cone voltage: 30 V, collision energy: 18 eV) and *m/z* 325.1 > 109.0 (cone voltage: 30 V, collision energy: 25 eV) transitions were monitored for R/S-citalopram. The *m/z* 331.1 > 262.0 (cone voltage: 30 V, collision energy: 18 eV) was monitored for S-citalopram-d_6 and *m/z* 329.4 > 266.0 (cone voltage: 30 V, collision energy: 18 eV) for R/S-citalopram-d_4. System operation and data acquisition were controlled using Mass Lynx 4.1 software (Waters, Manchester, UK). All data were processed with the Target Lynx quantification program (Waters, Manchester, UK).

2.6. Method validation

The validation was done according to guidelines given by Peters et al. [16] and the U.S. Department of Health and Human Services, Food and Drug Administration [17].

2.6.1. Quantification and calibration curves

The five-point calibration curves were evaluated with three replicates of each level of the calibrators. The calibration was based on peak area ratios of the analyte relative to the internal standard. Quadratic calibration curves were used with linearity weighing (1/x), excluding the origin. The coefficient of determination (R^2) of the curve was determined as a mean of three replicates at each level of the calibrators.

2.6.2. Limits of quantification and detection

To define the limit of quantification (LOQ), the signal to noise (S/N) ratios should be ≥ 10 for both transitions and the precision of the calculated concentrations should be within ± 20%. LOQ was determined by spiking blank serum with a concentration of 2 nM of each enantiomer and analyzed on ten different days. A standard sample with concentrations identical to the LOQ sample was included in the calibration curve. The limit of detection (LOD) was determined by dilution and evaluation of S/N ratio ≥ 3.

2.6.3. Precision and bias

Within-assay precision was estimated by analysis of ten separate replicates of QC samples at three concentrations in a single assay. The acceptance criterion for within-assay precision was a coefficient of variation (CV) ≤ 15%. Between-assay precision and bias were determined by analysis of one sample at three QC concentrations on ten different days. The acceptance criterion for between-assay precision was a CV ≤ 20%. The between-assay precision data were used to calculate the bias of the method. In addition, eight external quality controls from LGC (Bury, US) were included for validation. The bias of the external quality control samples was accepted with a $|Z| \leq 2$ for total citalopram.

2.6.4. Extraction recovery and matrix effects

Recovery was determined at two concentration levels (lowest and highest QC sample) with six replicates at each level. Total recovery was calculated dividing the analyte peak areas by the associated IS peak areas obtained when the analytes were added before sample preparation with those obtained when the analytes were added after phospholipid removal. In both cases, the internal standards were added after the extraction step. Matrix effects (ME) were evaluated at the lowest and highest QC level, the analyte signal in acetonitrile/water solution (80:20 v/v) was compared with the analyte signal in the matrix. ME was calculated as ME% = (Peak $area_{matrix}$/Peak $area_{acetonitrile\ /water}$) x 100 and when corrected with IS, ME %= [(Peak $intensity_{matrix}$/Peak IS $intensity_{matrix}$)/(Peak $intensity_{acetonitrile/water}$/Peak IS $intensity_{acetonitrile/water}$)] x 100 . Six replicates of serum samples (from six different individuals) were analyzed. Acceptance criteria for matrix effects were 75-125% with CV ≤ 15%.

2.6.5. Carry-over and retention time

Carry-over was investigated by injecting extracted blank samples after a high extracted standard corresponding to 1000 nM. Absolute retention time variations observed for the validation were calculated from the QC samples analyzed for determining between-day precision (n=10).

2.6.6. Stability

QC1 and QC3 (n=3 for each level) samples were prepared in serum and kept at -20 °C for three and 11 months to evaluate long-term stability. The stability of extracted samples were evaluated by analyzing one set of QC samples and samples from patients treated with citalopram (n=6) on the extraction day. After being kept at 10 °C in the autosampler for ten days the extracted samples were re-injected and analyzed. Patient serum samples (n=6) were kept at 30 °C and analyzed after three and eight days and then reanalyzed and calculated with freshly prepared calibrators.

2.6.7 Calibration and precision using R/S-citalopram-d_4 as internal standard

The calibration curves were based on peak area ratios of the analyte relative to R/S-citalopram-d_4. Quadratic calibration curves were used with linearity weighing (1/x), excluding the origin. The coefficient of determination (R^2) of the curve was calculated as a mean of ten between-day calibrations. Within-assay precision was estimated analyzing six separate replicates of QC samples at three concentrations in a single assay. Between-assay precision and bias were determined by analyzing one sample at three QC concentrations on ten different days. Data used for calculating the coefficient of determination, between-assay precision and bias were achieved by routine use of the method.

2.6.8 Application

In Norwegian, routine therapeutic drug monitoring of psychotropic drugs is well established, which provides access to samples obtained in a naturalistic setting. Serum samples sent to our laboratory for analysis of citalopram and escitalopram the first three months after adopting the method for routine use were included in the present study. After arrival at the laboratory the specimens were stored at 4°C for a maximum of one week until analysis.

3. Results and discussion

3.1 Method development

3.1.1 Chromatographic separation by UHPSFC

The separation of R/S-citalopram was optimized using two different columns, Acquity UPC^2 Trefoil CEL2 and Acquity UPC^2 Trefoil AMY1[amylose-tris-(3,5-dimethylphenylcarbamate)] with various mixtures of co-solvents (acetonitrile and methanol) and additives (NH_4OH and ammonium acetate). Both columns consist of polysaccharides coated on silica particles. CEL2 is based on cellulose whereas AMY1 is based on amylose-polysaccharide. As the chiral selectors are chemically diverse they will differ in chiral selectivity [5]. When using AMY1-column, no separation was achieved for citalopram with any of the co-solvents or additives tested (data not shown). In contrast, baseline separation of the two enantiomers was attained on the CEL2 column using methanol/acetonitrile (70:30, v/v) and 10mM ammonium acetate as additive.

Isocratic elution is commonly used in enantioselective methods [18]. However, gradient elution is also possible for UHPSFC due to more robust SFC equipment [14]. Only gradient separation was tested as the resolving power and sensitivity in this mode is often superior to the isocratic mode. . Thus, we could achieve a short cycle time of 4 minutes. The order of separation of the enantiomers was confirmed by analyzing only S-citalopram, which was found to elute after R-citalopram (data not shown). To elucidate possible co-elution between R/S-citalopram and the main metabolite R/S-desmethylcitalopram, the transition of *m/z* 311 >262 [10] was included in the MS-method. Analysis of routine therapeutic drug monitoring samples showed that R/S-desmethylcitalopram was eluted as one peak between R –citalopram and S-citalopram (data not shown).

Figure 1 shows the effect of changing column temperature for R/S-citalopram. The temperature was altered in steps between 35 and 60 °C, and the optimum separation of the enantiomers was achieved with a temperature at 35 °C. In contrast to a previous report [13] the retention times of R/S-citalopram in the present study decreased with higher temperature.

Yet another study reported that the influence of the temperature on enantiomer separation in SFC was limited [19]. Thus, a temperature change can clearly affect the properties of the compound, the supercritical fluid and the stationary phase, but its net effect on retention seems to be complicated to predict.

The influence of automatic back pressure regulator (ABPR pressure) was evaluated in the range of 1600-2100 psi (data not shown). The change in ABPR pressure did not improve enantiomer separation, thus all the analyses were further performed at 2000 psi. This observation is also in agreement with previously published reports [19].

3.1.2. Sample preparation

To minimize contamination of the MS interface from salts and other compounds using UPLC, the flow is usually diverted from the MS before and after elution of the analytes. Since the UPC^2-MS/MS instrument does not contain a divert valve prior to the MS the whole sample material will be introduced onto the system. Although the eluent from the UPC^2 is split (80 % in waste) prior the MS interface to reduce the level of CO_2 that is introduced onto the system, sample preparation prior to the analysis is important. Consequently, sample extraction before analysis is recommended to avoid phospholipid-related reduced column life and polluted MS interface [20, 21].

Two different sample preparation methods were evaluated. The first was solid phase extraction (SPE) using a Prime HLB well plate (Waters) whereas the second was a precipitation and filtration method using a 96-well Ostro Protein Precipitation & Phospholipid Removal Plate (Ostro plate). The Prime HLB plate was washed with water/methanol (95:5, v/v) and the analytes were eluted with 100 µL acetonitrile/methanol (90:10, v/v) and injected directly into the chromatographic system. For Ostro filtration, ice cold acetonitrile with formic acid was mixed with the sample prior to processing. These samples contained approximately 20% water. An injection volume of 1 µL gave satisfactory chromatographic performance. The two plates were then compared with regard to the analytical signal and the calibration curves achieved, and the phospholipid content. For evaluating the phospholipid content the MRM of *m/z* 184>184 transition using positive electrospray (Cone voltage 90 V, collision energy 7 eV) was employed [22]. The phospholipids were eluted after the analytes and the level was significantly (90%) lower using the Ostro plate (data not shown). Both methods worked well for the analytes in regard to analytical signal and calibration curves. Thus, as the Ostro plate had the lowest level of phospholipids, as it was less complicated in use, and as it was already used routinely at our laboratory for other analytical purposes, it was chosen for the sample extraction.

3.1.3. Internal standard

S-Citalopram-d_6 was used as an IS during the method development and validation process. Prior to method validation the external control samples from LGC had Z- scores of $|Z| \leq 1$ for the total amount of R/S-citalopram with assigned concentrations in the range of 30-440 nM. However, when analyzing these samples during method validation the Z-scores were outside the recommended interval of > |2|. Several attempts were made to clarify this issue. Since thecontrols were spiked in bovine serum, we assumed that the deviations in Z value were caused by matrix effects. External control samples (158-305 nM) from two other vendors ClinChek-control (Recipe, Chemical Instruments GmbH, Munich, Germany), and Instand (Society for Promoting Quality Assurance in Medical Laboratories, Düsseldorf, Germany) were purchased and tested. These were spiked in human serum, and the bias for R/S-citalopram related to the theoretical values was less than ±7%. In order to further clarify this issue, racemic deuterated R/S-citalopram (R/S-citalopram -d_4) was purchased and the bovine serum samples were subsequently tested three times on three different days. Using the racemic IS the external control samples had Z-scores of $|Z| \leq 1.3$. Finally, 21 serum patient samples and two external control samples (obtained from ClinChek) were run in parallel, using S-citalopram-d_6 in one of the series and R/S-citalopram-d_4 in the other series. The deviations in concentrations were less than 7.4%. These results support the assumption of matrix effects causing the high Z-scores in the control samples spiked in bovine serum. Figure 2 shows that when the number of deuterium-substitutes in the IS decreases from six to four the chromatographic separation between IS and S-citalopram also decreased. The concentrations of both analytes decreased to the same extent using racemic deuterated IS. We thus assume that by introducing a racemic deuterated IS, each enantiomer is eluted closer to its respective non-labeled enantiomer and thereby corrects the matrix effect to a greater extent. This demonstrates the importance of including racemic isotope-labeled IS when developing enantioselective analytical methods with a simple sample preparation technique.

3.2 Method validation

In the primary method validation S-citalopram-d_6 was used. However, supplementary validation data for calibration and precision are included for R/S-citalopram-d_4 as well.

3.2.1. Calibration curves

In the range of 5-500 nM the method showed a coefficient of determination (R^2) ≥ 0.997. Five calibrators were prepared .The weighted (1/x) residuals were found to distribute randomly around zero. The MRM chromatograms of the lowest calibration level with S-citalopram-d_6 as internal standard are shown in Figure 2.

3.2.2. Limit of detection and quantification, precision and bias

The LOD, LOQ, within-assay precision, between-assay precision and bias for R/S-citalopram are presented in Table 1. The precision was <15%, bias within ±10% and S/N ratio was ≥10 at LOQ for both analytes. The within-assay CVs were 1.1-5.7%, the between-assay CVs were 3.4-4.5% and bias for the QC samples varied between 0.6% and 8.3%.

3.2.3. Recovery and matrix effects

The extraction recoveries of the analytes were 81-91% (CV≤7.7%). Matrix effects were 96-98% (CV≤2.9%) without and 96-101% (CV≤4.0%) with internal standard correction (S-citalopram-d_6), indicating a negligible matrix effect on the ionization of R/S-citalopram in human serum.

3.2.4. Carry-over and retention time

No carry-over was observed in blank samples after an extracted sample with a concentration twice the highest standard. The absolute retention times (between-day; n=10) of QC samples were stable with CVs less than 0.3% for both analytes. The resolution (R_s) of R/S-citalopram was calculated at the first and last validation day. The R_s value was 1.4 throughout validation, indicating good stability for retention times and resolution of R/S-citalopram.

3.2.5. Stability

QC samples prepared in serum and kept at -20 °C were stable for at least 11 months. Extracted samples, standards and QC samples were stable for at least ten days at 10 °C. Stability of citalopram in patient samples (n=6) at 30 °C was tested to mimic a high summer temperature during postal shipment to the laboratory. After three days, the mean decrease was 14.8 % (range 5.0-18.7 %). After eight days, the mean decrease was 21.5 % (range 11.8-25.8 %). By applying a limit for stability at a mean of 15 % decrease, we conclude that samples should not be kept at 30 °C for more than three days.

3.3 Calibration and precision using R/S-citalopram-d_4 as internal standard

The calibration curves were reproducible with coefficients of determination (R^2) >0.999 (n=10, CV of R^2 < 0.1%). The within-assay and the between-assay CVs were 1.1-5.0% and 2.3-4.1%, respectively, and bias for the QC samples varied between 0.6% and 3.7%. These results are consistent, with the validation results obtained with S-citalopram-d_6 (Table 1). The MRM chromatograms of the lowest calibration level using R/S-citalopram-d_4 as internal standards are shown in Figure 2.

3.4. Application

In routine use, the racemic IS R/S-citalopram-d_4 was applied since a small difference in patient concentrations was observed related to whether S-citalopram-d_6 or R/S-citalopram-d_4 was used. The presented method has been in routine use for about three months. It runs on a thrice weekly basis and we have not detected any particular problems. Of the 276 routine therapeutic drug monitoring samples analyzed during this period, 33 (12%) were positive for both R- and S-citalopram and 243 (88%) were positive for S-citalopram only. The concentration range for R-citalopram was 39-380 nM, whereas the range for S-citalopram was 8-499 nM. Thus, for these routine samples, the concentrations found were within the calibrated range of 5-500 nM for the method. However, it might be desirable to include a higher upper standard to be able to quantify concentrations e.g. after therapeutic use of excessive doses or after intake in overdose without diluting the sample.

For the vast majority of samples, the findings were in concordance with the prescribed drug stated on the requisition form, but in five samples (2.0%), there was a discrepancy between the preparation allegedly used by the patient and the enantiomeric findings. Without enantioselective analysis this would have gone unnoticed, which could have led to an inappropriate interpretation of the results, since the therapeutic reference ranges differ for S-citalopram and R/S-citalopram [23].

In the 33 samples from patients using the racemate, the S-enantiomer on average amounted to 34% of the total concentration, with a range from 22% to 50%. This is consistent with the different metabolic pathways for the two enantiomers causing a longer elimination half-life and thereby a higher plasma levelof the R-enantiomer [2]. In the samples from subjects using escitalopram, only S-citalopram and no R-citalopram was found. The S-enantiomer is thought to convey all the pharmacological effect [1], which means that the therapeutic reference range for R/S-citalopram should be around three times higher than for escitalopram. However, this is not the case in much-cited compilations of therapeutic concentration ranges [3, 23], where the upper limit of the reference interval for R/S-citalopram is only about 40 % higher.

4. Conclusions

A robust and specific UHPSFC-MS/MS method for R/S-citalopram has been developed and validated. The method has been successfully implemented in routine use in our laboratory for several months for separation and quantification of R/S-citalopram. Its robustness has been demonstrated when analyzing more than 250 serum samples sent to our laboratory for routine therapeutic drug monitoring.

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Table 1. Calibration range, coefficient of determination, limit of detection (LOD), limit of quantification (LOQ), within-assay and between-assay precisions, bias and recovery for R/S-citalopram in serum.

Analyte	Calibration range (nM)	Coefficient of determination (R^2) (n=3)	LOD (nM)	LOQ (nM) (n=10)	Spiked QC sample concentration (nM)	Within-assay CV (%) (n=10)	Between-assay CV (%) (n=10)	Bias (%) (n=10)	Recovery (%) (n=6)
R-Citalopram	5-500	0.997	1.3	2.0	7.0	4.0	4.5	6.7	91
					75	2.4	4.1	0.6	-
					300	1.1	3.4	7.8	81
S-Citalopram	5-500	0.999	1.3	2.0	7.0	5.7	4.1	8.3	91
					75	2.0	3.9	0.9	-
					300	3.5	3.4	3.9	82

Table 2. Evaluation of matrix effects (ME) for R/S-citalopram in serum.

Analyte	Concentration (nM)	ME (%) (n=6)	Relative ME (CV %)	ME corrected with IS[a] (%)	Relative ME corrected with IS (CV %)
R-Citalopram	7.0	97	2.7	100	2.9
	300	96	1.8	96	2.0
S-Citalopram	7.0	98	2.9	101	4.0
	300	98	2.3	99	2.1

[a] IS = internal standard, S-citalopram-d_6

Figure legends

Figure 1

Chromatographic separations of R/S-citalopram at column temperatures of, 60, 50, 40 and 35 °C.

Figure 2

MRM-chromatograms of R/S-citalopram (R-citalopram in the left panel; S-citalopram in the right panel; S-citalopram-d_6 in the upper panel; R/S-citalopram-d_4 in the lower panel) of the lowest calibrator (5 nM of each enantiomer).

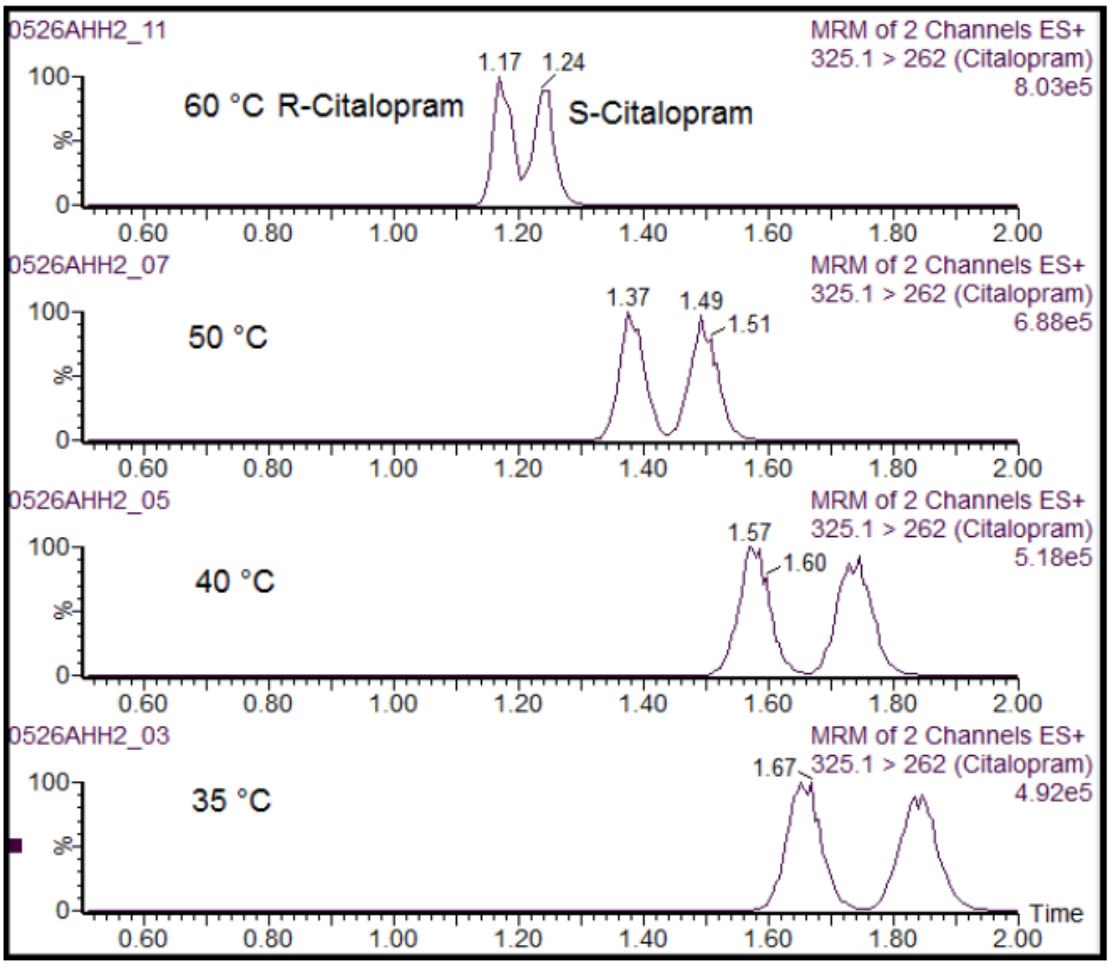
0526AHH2_11
MRM of 2 Channels ES+
325.1 > 262 (Citalopram)
8.03e5
1.17
1.24
60 °C R-Citalopram
S-Citalopram
100
%
0
0.60
0.80
1.00
1.20
1.40
1.60
1.80
2.00
0526AHH2_07
MRM of 2 Channels ES+
325.1 > 262 (Citalopram)
6.88e5
1.37
1.49
1.51
50 °C
0526AHH2_05
MRM of 2 Channels ES+
325.1 > 262 (Citalopram)
5.18e5
1.57
1.60
40 °C
0526AHH2_03
MRM of 2 Channels ES+
325.1 > 262 (Citalopram)
4.92e5
1.67
35 °C
Time

Figure 1

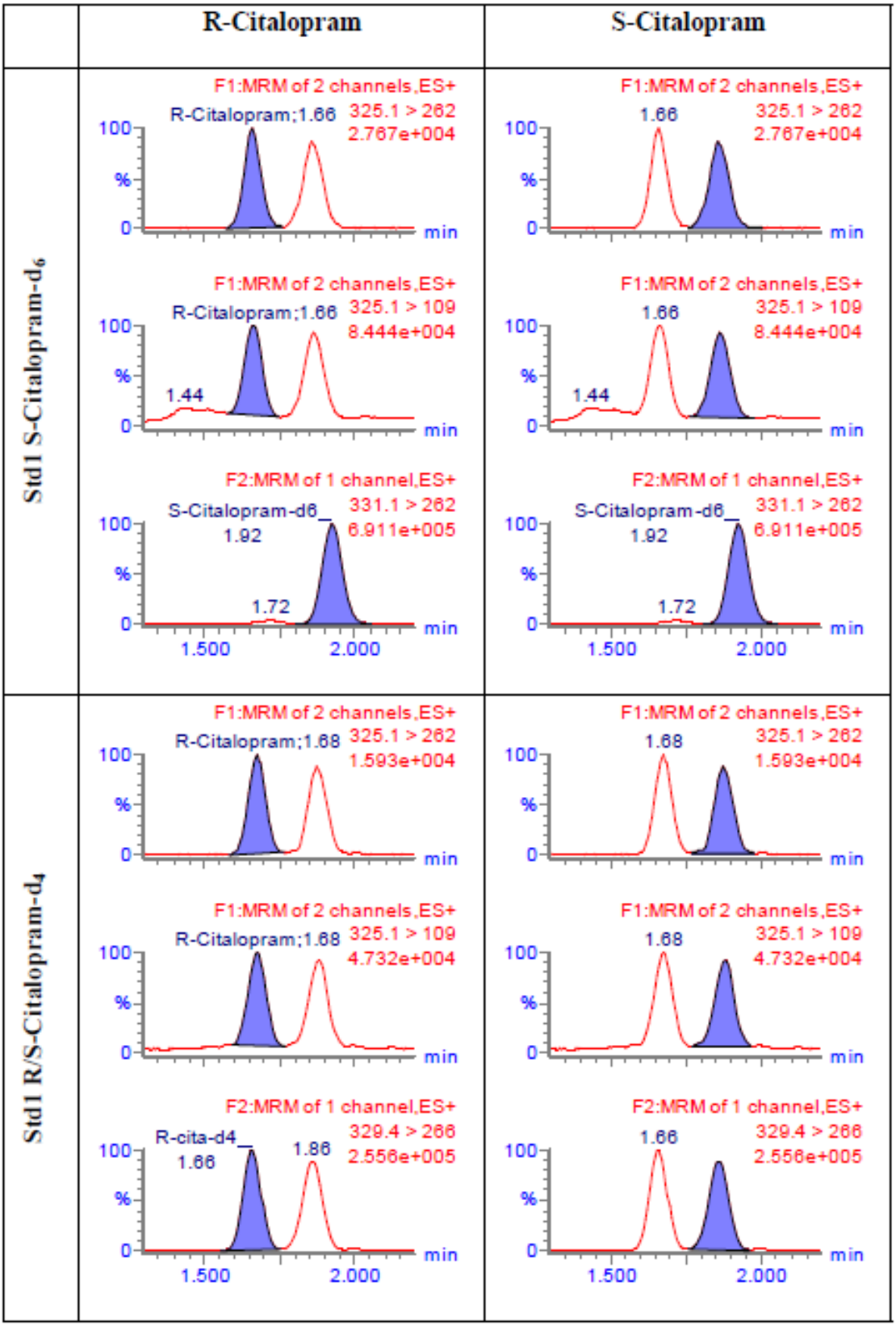
R-Citalopram
S-Citalopram
Std1 S-Citalopram-d6
Std1 R/S-Citalopram-d4
F1:MRM of 2 channels,ES+
R-Citalopram;1.66
325.1 > 262
2.767e+004
F1:MRM of 2 channels,ES+
R-Citalopram;1.66
325.1 > 109
8.444e+004
1.44
F2:MRM of 1 channel,ES+
S-Citalopram-d6
331.1 > 262
6.911e+005
1.92
1.72
F1:MRM of 2 channels,ES+
R-Citalopram;1.68
325.1 > 262
1.593e+004
F1:MRM of 2 channels,ES+
R-Citalopram;1.68
325.1 > 109
4.732e+004
F2:MRM of 1 channel,ES+
R-cita-d4
329.4 > 266
2.556e+005
1.66
1.86
100
%
0
min
1.500
2.000

Figure 2