

Analysis of Citalopram in Plasma and Hair by a Validated LC – MS/MS Method

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Abstract

A simple method for quantification of citalopram in mice plasma and hair was developed and validated using liquid chromatography tandem mass spectrometry (LC–MS/MS). The procedure involves a protein precipitation extraction of citalopram and desipramine (internal standard) with methanol from mice plasma. On the other hand, hair samples were incubated overnight with methanol at 45°C followed by μ -SPE (OMIX Tip). The analysis was performed by resolving analytes in a Gemini® C18 column with a gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, at a flow rate of 250 μ L/min and with a total run time of 9 min. The mass spectrometer was operated in multiple reaction monitoring (MRM) by monitoring citalopram transition 325.3→109.0, and internal standard transition 267.3→72.2 for quantification. The qualifier transitions 325.3→83.1 and 267.3→190.8, respectively, were also monitored. Linearity was observed from 32.4 to 973.2 ng/mL and the limit of quantitation achieved was 32.4 ng/mL. Also, the intermediate precision, repeatability and accuracy were below the acceptance limits of 15%. This method was applied to plasma and hair samples that were collected from mice submitted to a treatment with citalopram for different days. The plasma concentration–time profile of citalopram showed a tendency to stabilize, approaching zero as samples were collected 24 hours after the last drug administration. In contrast, the concentration–time profile in hair increased over the period of 30 days.

Keywords: Citalopram; Plasma; Hair; LC-MS/MS; Analytical method validation

Introduction

Citalopram (1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-iso-benzofuran carbonitrile) is considered one of the most prescribed drugs being a selective and potent serotonin reuptake inhibitor [1]. This antidepressant is widely used because it is safer, less toxic and more tolerable than the tricyclic antidepressants and monoamine oxidase inhibitors. However it is worth noting that it may be involved in overdose deaths, particularly when combined with other drugs [2]. Liquid chromatography–mass spectrometry (LC–MS) has established itself as the clear leader in the quantification of antidepressant drugs in biological samples [3]. Some LC–MS [4,5] and LC–MS/MS [6,7] methods for the determination of citalopram have been developed in plasma samples. Nevertheless, in most cases they require plasma volumes by more than 200 μ L. Hair testing is a valuable way to increase the window of drug detection, and due to simple sample collection and storage, it has become an alternative matrix for drug testing. Methods regarding identification and screening of citalopram in hair have been published previously [8,9], with only two reports addressing quantification [10,11]. However these either entail amounts of hair greater than 10 mg or the procedure is demanding, requiring several extractions in opposition to incubation followed by a single solid phase extraction. Hence, we developed and validated a method for the determination and quantification of citalopram in mice plasma and hair with a small quantity of sample and with a simple extraction procedure, in contrast to the current literature which is mostly based on methods for humans. The small sample amount used for citalopram analysis in mice is significant for studies using this animal model. This method for both matrices was successfully used to characterize the time course of changes in plasma and hair concentrations of citalopram following intraperitoneal injection for different periods of time in mice.

Experimental

Reagents and standards

Citalopram hydrobromide (purity 99.8%) was purchased from BIOTREND® Chemicals AG. Desipramine hydrochloride (Internal Standard, IS) (purity \geq 98%) was purchased from Sigma-Aldrich®. Acetonitrile (ACN) and methanol (MeOH) (LC-MS Grade) were obtained from Biosolve. Formic acid (FA) and dichloromethane (LC Grade) were obtained from Sigma Aldrich® and water (LC Grade) from VWR®. Standard stock solutions for citalopram and desipramine were prepared in acetonitrile and were both stored at -20°C. In each working day, freshly diluted calibration standard solutions were prepared by diluting the stock solutions in 2% ACN: 0.1% FA.

Animal study

Young black male C57BL/6J mice (n=30, weight range 20-25 g), were purchased from Charles River Laboratories International, Inc., Spain. The animals were sacrificed after 1, 2, 4, 8, 15 and 30 days of daily intraperitoneal injections of citalopram at doses of 10 mg/kg, after 1 week of habituation to needle stick. They were divided into

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six groups according to treatment time, then they were weighed and anesthetised with a mixture of ketamine and xylazine 24 hours after the final injection.

Blood samples were collected by cardiac puncture and placed in EDTA-coated tubes (BD Vacutainer® tubes, Becton Dickinson). Followed by centrifugation at 12,000×g for 2 minutes, the plasma was recovered to another tube (BD Microtainer®, Becton Dickinson) with protease and phosphatase inhibitors (Roche). Regarding hair samples, they were pulled out with tweezers and placed into a microcentrifuge tube. Both plasma and hair samples were fast frozen in liquid nitrogen and stored at -80°C until further use. The thawed plasma and hair samples were spiked with IS solution and processed as mentioned in the sample preparation section.

Sample preparation

To each 70 µL of plasma, 10 µL of IS working solution (151.4 ng/mL) were added. After vortexing, 210 µL of MeOH (three times the plasma volume) were added and the samples were rehomogenized, followed by continuous agitation for 5 minutes at 1000 rpm's in a thermomixer (Comfort, Eppendorf). Samples were centrifuged at 14,000×g for 10 minutes, then the supernatant was collected, evaporated at 60°C during approximately 1 hour (Concentrator Plus, Eppendorf), reconstituted in 50 µL of 2% ACN: 0.1% FA in water.

To each sample of hair, containing between 0.7-9.7 mg (corrections were made to calculate the concentrations), 1 mL of dichloromethane was added in order to remove any blood residues or external contamination. Samples were mixed for 2 minutes at 750 rpm's in the thermomixer (Comfort, Eppendorf). Dichloromethane was discarded and this procedure was repeated. Then, the hair was incubated overnight (17 hours) in 1 ml of MeOH at 45°C. Methanol was recovered and evaporated to dryness at 60°C, for approximately 1 hour (Concentrator Plus, Eppendorf). Hair samples were cleaned up by using µ-Solid phase extraction (OMIX Tip C18) (Agilent Technologies). To the evaporated sample, 100 µL of 2% ACN: 1% FA were added and the samples sonicated (Sonicator VibraCell™ 75041, Sonics®) for 2 min using a cuphorn (20% amplitude with 1 sec on 1 sec off cycle). After conditioning of the C18 SPE-tip with 200 µL of 50% ACN, the tip was equilibrated with 300 µL of 2% ACN: 1% FA, subsequently the sample was allowed to interact with the tip for 5 cycles. The tip was rinsed with 100 µL of 2% ACN: 1% FA and eluted with 400 µL of 70% ACN: 0.1% FA. These samples were evaporated to dryness at 60 °C, then resuspended in 50 µL of 2% ACN: 0.1% FA and sonicated.

HPLC operating conditions

The chromatographic analysis was performed using an Ultimate 3000 LC system (LC Packings, Dionex™). Separation was performed with a Gemini C18 column (3 µm, 110 Å, 50 × 2 mm) coupled with a Security Guard™ cartridge Gemini C18 (4 × 2 mm).

The mobile phase was composed of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B). Gradient conditions were 10-20% B (0-0.3 min), 20-30% B (0.3-6.0 min) and 30-99% B (6.0-9.0 min), with a flow rate of 250 µL/min. This step gradient was chosen in order to separate other interferents, causing them to elute at former retention times than citalopram and desipramine.

Cleaning and re-equilibration steps (between samples) are performed by a blank injection with a chromatographic gradient 0-90% B (0-1.9 min) and 90% B (1.9-8 min). Also between batches, three blank injections were introduced (solution of 0.1% FA in ACN), with the same program and the same volume of injection as the one

used for the plasma samples. The volume that was injected was adjusted for each condition, 1 µL for hair samples and 20 µL for plasma samples. The blank injection between samples was of 10 µL.

Mass spectrometry operating conditions

A 4000 QTRAP® mass spectrometer (ABSciex™), equipped with a turbo V™ source was operated in electrospray positive mode (ESI+) and under the following optimized settings: curtain gas (CUR), 30 psi; ion source gas 1 (GS1), 30 psi; ion spray voltage, 5500 V; source temperature, 450°C. Compound's parameters were optimized for multiple reactions monitoring (MRM) mode by direct infusion of a standard solution of each analyte, with a concentration of 42.2 ng/mL for citalopram and 342.2 ng/mL for desipramine at 9 µL/min. A dwell time of 30 ms, entrance potential (EP) of 10 eV and collision gas (CAD) of 8 psi were used. The values of declustering potential (DP), collision energy (CE), collision exit potential (CXP), and transitions are presented in the Table 1. Data acquisition was performed by Analyst® 1.5.1 (ABSciex™).

Method validation

The method was validated for plasma and hair in terms of selectivity, linearity, accuracy, intra-day precision, intermediate precision, extraction efficiency, matrix effect, and carry-over. The different validation parameters and the values for accepting the range of validation parameters were in accordance with international guidelines, as International Conference on Harmonisation (ICH) and Food and Drug Administration (FDA).

Selectivity

Plasma's selectivity was determined by analyzing six individual blank samples and each one was divided in two aliquots (70 µL each). One aliquot was spiked with 20 µL of citalopram (8.1 ng/mL) and desipramine (7.6 ng/mL) and the other was not fortified with any compound. Similarly, to evaluate the selectivity in hair samples, six individual blank samples were divided in two aliquots (approximately with 3-4 mg each). One aliquot was spiked with 20 µL of citalopram (162.2 ng/mL) and desipramine (151.4 ng/mL) and the other was not fortified with any compound. Then, all samples were subjected to the analytical procedure developed for the extraction of drugs from plasma and hair, respectively.

Linearity and analytical limits

To study the linearity, the calibration curve was established between 32.4 and 1621.5 ng/mL (corresponding to 0.10 and 5 pmol/µL) and were prepared on five different occasions. Apart from other tests, the criteria for acceptance included a r² value of at least 0.99, and the calibrators accuracy within 80-120%.

According to the FDA, the limit of quantification (LOQ) is the lowest amount of an analyte in a sample that can be quantitatively

Compound	Transitions (m/z)	CE (eV)	CXP (eV)	DP (eV)
Citalopram	<u>325.3→109.0</u>	39	8	66
	325.3→261.9	27	24	
	325.3→83.1	91	4	
Desipramine	<u>267.3→72.2</u>	27	4	56
	267.3→208.0	33	16	
	267.3→190.8	83	14	

Collision energy (CE), collision exit potential (CXP) and declustering potential (DP). Transitions used for quantification are underlined.

Table 1: MRM parameters for each transition of citalopram and its internal standard.

determined with suitable precision and accuracy. Moreover, the signal-to-noise ratio should be investigated in order to determine whether it reaches a ratio of 10:1 [12].

Precision and accuracy

The precision of the method was evaluated in two levels: repeatability (or intra-day precision) and intermediate precision (or inter-day precision). To evaluate the repeatability, triplicates of quality controls (QCs) were prepared at three concentration levels (40.5, 243.2 and 891.8 ng/mL corresponding to 0.125, 0.75 and 2.75 pmol/ μ L) and were characterized in terms of percentage of coefficient of variation (%CV) after performing a one-way analysis of variance (ANOVA). The intermediate precision was also expressed in terms of %CV and it was determined by analyzing the QCs over five different occasions. Accuracy was evaluated in terms of percentage of mean relative error (%MRE) between the theoretical and the measured spiked concentrations for the calibrators [13,14]. An acceptable variability was set at 15% for all the concentrations, except at the LOQ, for which 20% was accepted [15,16].

Extraction efficiency

To evaluate the extraction efficiency of citalopram and IS in plasma, two levels of concentration were selected (162.2 and 973.2 ng/mL corresponding to 0.5 and 3 pmol/ μ L) and each concentration prepared in triplicate. For each level, two aliquots were prepared with 70 μ L: one was spiked with 20 μ L of the solution containing citalopram and desipramine, and then was subjected to the extraction procedure, and in parallel the other was subjected first to the extraction procedure and then spiked with 20 μ L of the citalopram and desipramine solution.

In an analogous way, to evaluate the analyte's extraction efficiency using the hair extraction protocol, two levels of concentration were selected (162.2 and 973.2 ng/mL) with each concentration in triplicate. For each level, two aliquots of hair weighing between 1.9–3.2 mg were prepared. One aliquot was spiked with 20 μ L of the spike solution containing citalopram and desipramine, and then was subjected to the extraction procedure. The other aliquot was first subjected to the extraction procedure and then was spiked with 20 μ L of the citalopram and desipramine solution.

In both plasma and hair, 10 μ L of sulfamethazine-D4 at 141.2 ng/mL were added, at the end of the procedure. The sulfamethazine-D4 was used to compensate injection losses as well as differences in the system sensitivity, since extraction efficiency of desipramine was also evaluated.

Matrix effect

The assessment of matrix effect was performed at two concentration levels (162.2 and 973.2 ng/mL) each one in triplicate for both plasma and hair. Blank samples were subjected to the respective extraction procedure and then spiked with 20 μ L of a solution with citalopram and desipramine. In parallel, pure solutions with citalopram and desipramine in mobile phase (2% ACN: 0.1% FA), at the equivalent levels of concentration, were also analysed.

Carry-over

To evaluate the carry-over phenomena, analytical blanks (2% ACN: 0.1% FA) were injected after a highest level of standard concentration (1622 ng/mL corresponding to 5 pmol/ μ L). This procedure was repeated in three different days.

Results

Method validation

Selectivity: The selectivity of the method was evaluated by analyzing blank samples of plasma and hair from six different animals to investigate the potential interferences at the peak region for citalopram and IS. The criteria for compound identification were the ones proposed by the World Anti-Doping Agency (WADA) [17]. In what concerns the chromatography, the acceptance criterion was the relative retention time (ratio between the RT of the interest compound and RT of the internal standard) that should not differ by more than \pm 1% when compared with the relative retention time of the control sample of the same compound.

Regarding the mass spectrometry identification in the MRM mode, it is required to monitor at least two precursor-product ion transitions. The second criterion to monitor is the relative abundance of a diagnostic ion that is expressed as a percentage of the intensity of the most intense fragment (base peak). The relative intensities should not differ by more than a tolerated range (Table 2) from those generated by the control sample of the same compound. Moreover, the signal-to-noise ratio should be greater than 3. Citalopram and desipramine were successfully identified in all spiked samples (Tables A.1 and A.2). In the non-spiked samples, for both plasma (Figure 1) and hair (Figure 2), no chromatographic peaks of citalopram were detected. Even though spiked samples display small differences in the retention times, these differences fulfill the criteria based on the WADA guideline (Tables A.1 and A.2). Therefore, the two described methods were considered selective for the determination of citalopram.

Linearity: Calibration curves were obtained by plotting the peak area ratio between each analyte and the internal standard, against analyte concentration. One of the assumptions of linear regression is a constant variance of measured values between the limits of the working range [18]. A test of homogeneity of variances (F-test) [19] showed that there is a significant difference between those limits (since $F_{\text{crit}} = 715.2 > F = 6.54$, $p\text{-value} = 1.87 \times 10^{-11}$), corresponding to heteroscedastic data. Usually when a homogeneous variance is not verified, a weighted least squares regression is used [20–22]. Therefore, six weighting factors were evaluated ($1/\sqrt{x}$, $1/x$, $1/x^2$, $1/\sqrt{y}$, $1/y$, $1/y^2$) and the one which originated the lowest sum of percentage of relative error (presenting simultaneously a mean r^2 value of at least 0.99) was selected taking into account the data relative to five independent calibration curves [23,24]. The chosen weighting factor for citalopram, given these criteria was $1/x$. The calibrators accuracy for the five days was within 80 and 120%. Calibration data is shown on Table 3. The LOQ was defined as the lowest concentration of analyte which could be measured reproducibly and accurately (%CV < 20% and accuracy of 80–120%). The value of LOQ achieved was 33.8 ng/mL.

Precision and Accuracy: To calculate the concentration of the QCs, prepared in three concentration levels (40.5, 243.3 and 892.1 ng/mL) each one in triplicate, calibration curves on five different days were used. First it was performed a Cochran's test to detect if the assumption of constant variance is accomplished [25]. Then the results obtained for the different levels of concentration for each compound were analysed by ANOVA [26,27].

The intermediate precision and the repeatability were expressed in terms of %CV and the acceptance criteria was set at 15% for all the concentrations, except at the LOQ, for which 20% was accepted.

Relative abundance (% of base peak)	Maximum tolerance ranges (%)
>50	± 10 (absolute range)
25 to 50	± 20 (relative range)
5 to <25	± 5 (absolute range)
<5	± 50 (relative range)

Table 2: Maximum Tolerance ranges for relative ion intensities to ensure appropriate confidence in identification [17].

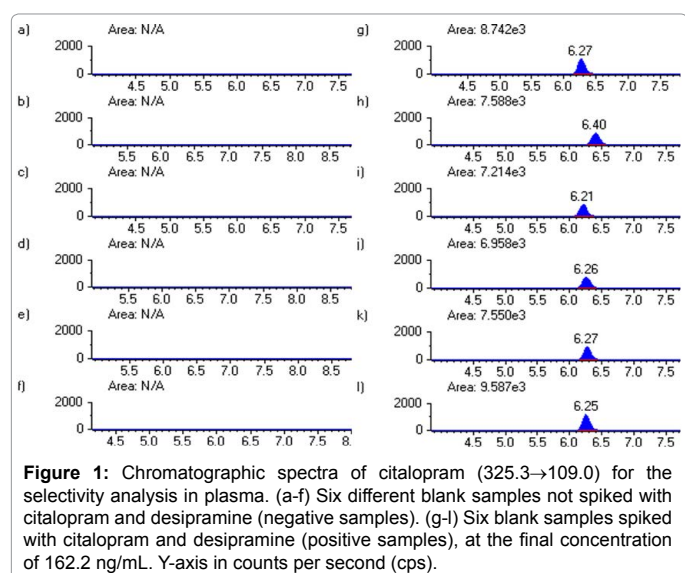


Figure 1: Chromatographic spectra of citalopram (325.3→109.0) for the selectivity analysis in plasma. (a-f) Six different blank samples not spiked with citalopram and desipramine (negative samples). (g-l) Six blank samples spiked with citalopram and desipramine (positive samples), at the final concentration of 162.2 ng/mL. Y-axis in counts per second (cps).

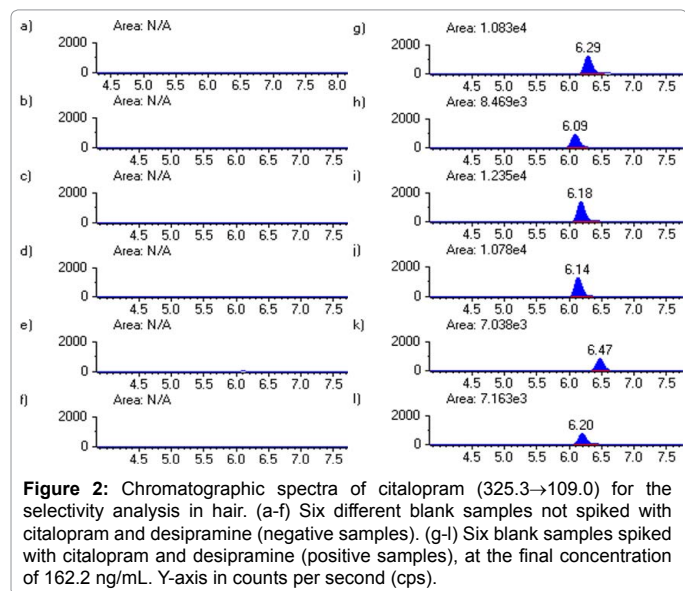


Figure 2: Chromatographic spectra of citalopram (325.3→109.0) for the selectivity analysis in hair. (a-f) Six different blank samples not spiked with citalopram and desipramine (negative samples). (g-l) Six blank samples spiked with citalopram and desipramine (positive samples), at the final concentration of 162.2 ng/mL. Y-axis in counts per second (cps).

Accuracy was evaluated in terms of percentage of mean relative error (%MRE) and as an acceptance criteria, the accuracy for each level of concentration should be within $\pm 15\%$ of the nominal concentration [15,16]. The obtained %CVs were lower than 15.1% for LOQ concentration level and 12.5 for the others concentrations, with an accuracy lower than 9.3% (Table 4).

Extraction efficiency: The extraction efficiency was determined by comparing the representative peak areas of extracted blank samples spiked before extraction with the peak area of blank samples fortified after the extraction at equivalent concentrations. The experiment was conducted in triplicate at two levels of concentration (162.2 and 973.2

ng/mL). The results showed that the extraction efficiency values for citalopram were 90.9 and 101.5% in plasma, and 33.9 and 38.9% in hair (Table 5). The extraction efficiency for desipramine was 68.9 and 92.2% in plasma, and 36.6 and 36.3% in hair respectively.

The method provided good extraction efficiency for citalopram and desipramine in plasma at both concentrations. In hair, lower recoveries were achieved, however Causon et al. [14], states that it is unlikely that recoveries of 50% or less will compromise the integrity of the method if adequate detection can be attained, which is the case of this method.

Matrix effect: Matrix effects can be described as the difference between the response of an analyte in pure solution and the response of the same analyte in biological matrix. A normalized matrix factor can be measured by dividing the peak response ratio (analyte/IS) in the presence of matrix ions with the peak response ratio in the absence of matrix ions [28]. Hence, a normalized matrix factor of one represents no matrix effects, a value lower than one suggests ionization suppression and a value greater than one may be due to ionization enhancement [28].

The results for plasma and hair (Table 6) at higher concentration showed a normalized matrix factor of one, indicating no matrix effects. At lower concentrations, there is possibly some ion suppression for plasma and ion enhancement for hair samples. Nevertheless a value of one is not necessary for a reliable bio analytical assay, because coefficient of variation for the lowest concentration were lower than 15% demonstrating that results are reproducible [28]. Overall, coefficients of variation obtained for plasma were below 10.33%, and for hair these were slightly higher than the defined criteria (16.9% in the highest concentration). However, the normalized matrix factor for the highest hair concentration presents a value close to one, despite being a more complex matrix. The intra-day precision of the ratio of citalopram with desipramine in the presence of matrix ions and in the absence of matrix ions, presented %CV values lower than 15%. Moreover, the normalized matrix effect showed values close to one and also presented lower values of %CV. Thus, it can be concluded that the use of the internal standard has a compensatory effect on variations between injections. Therefore, these results showed that ion suppression or enhancement from the plasma and hair matrix was negligible under the current conditions.

Carry-over: Carry-over is the amount of analyte retained, in this case in the LC system, from a preceding sample that carries over into the next injected sample. This phenomena can be measured by the response of the blank sample after the injection of a preceding sample at high concentration [29]. The results showed that no carry-over was observed for citalopram. In any case, a blank injection between samples, and three blank injections were introduced between batches.

Application of the analytical method to the animal study

The developed analytical method was applied in plasma and hair samples that were collected from mice daily injected with a solution of citalopram (10 mg/kg) for 1, 2, 4, 8, 15 and 30 days (five independent replicates per day). The plasma concentration time profile of citalopram in mice shows values closer to zero, as these samples were collected 24 hours after the last administration (Figure 3). On the other hand, the concentration time profile of citalopram in hair increases over the days (Figure 4), showing a direct correlation, probably explained by the fact that there is more compound incorporated into the hair shaft, providing evidence of longer term exposure of drugs.

Conclusion

A method for plasma based on protein precipitation and for hair

Transition (m/z)	Weight	Linear range (ng/mL)	Linearity		r ²	LOQ ^b (ng/mL)
			Slope ^a	Intercept ^a		
325.3→109.0	1/x	32.4–973.2	2.372 ± 0.657	0.020 ± 0.025	0.994 ± 0.003	32.4

^aMean values ± standard deviation
^bLimit of quantification

Table 3: Analytical parameters of the calibration curve for citalopram.

Nominal concentration (ng/mL)	Repeatability (%CV)	Intermediate precision (%CV)	Accuracy(%MRE)	Estimated concentration (ng/mL)
40.5	10.6	15.1	0.04	40.5
243.2	5.8	11.7	8.5	263.6
891.8	5.6	12.5	9.3	973.0

%CV - percentage coefficient of variation.
%MRE- Percentage of mean relative error.

Table 4: Precision and accuracy in three concentration levels for citalopram.

Matrix	Compound	% Extraction efficiency (mean ± C.I.)	
		162.2 ng/mL	973.2 ng/mL
Plasma	Citalopram	90.9 ± 14.6	101.5 ± 23.7
	Desipramine	68.9 ± 6.6	92.2 ± 24.5
Hair	Citalopram	33.9 ± 10.4	38.9 ± 4.2
	Desipramine	36.6 ± 12.7	36.3 ± 7.1

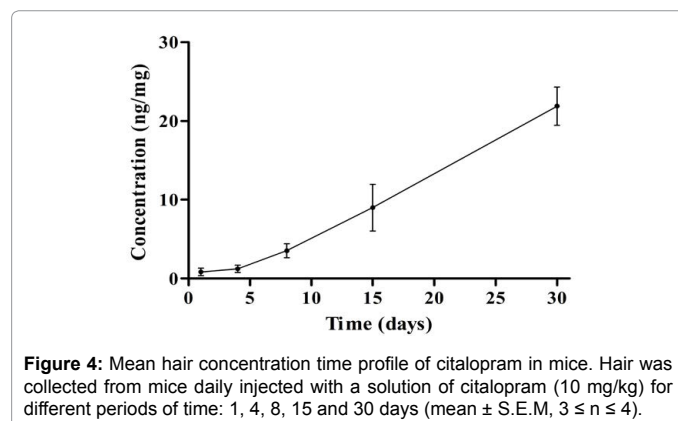
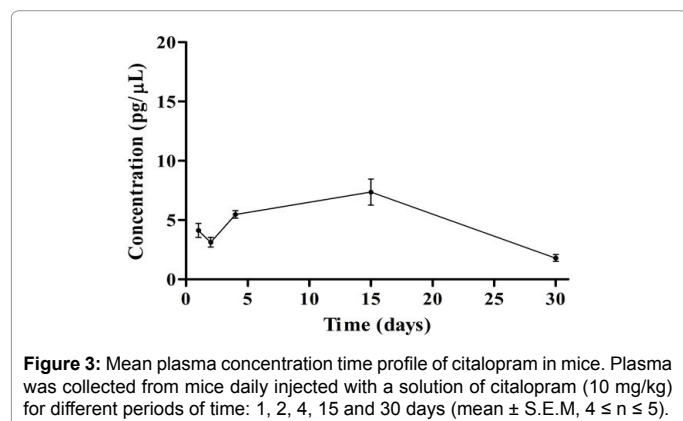
C.I: Confidence Interval

Table 5: Extraction efficiency, in percentage, of the extraction of the citalopram and desipramine in plasma and hair at two concentration levels.

Matrix	Nominal concentration (ng/mL)	Analyte with matrix ions peak area ratio ¹	Analyte pure solutions peak area ratio ¹	Normalized matrix factor ¹
Plasma	162.2	0.65 ± 1.43	0.76 ± 12.27	0.85 ± 1.43
	973.2	0.69 ± 10.33	0.68 ± 2.72	1.02 ± 10.33
Hair	162.2	1.81 ± 5.83	1.39 ± 5.56	1.31 ± 5.83
	973.2	1.53 ± 16.91	1.45 ± 7.46	1.06 ± 16.91

¹Mean values ± % Coefficient of variation

Table 6: Matrix effect of citalopram in plasma and hair at two concentration levels.



samples with MeOH incubation followed by solid phase extraction was developed and validated. In terms of plasma, this method uses smaller sample volumes in contrast to other published methods, which require sample volumes of more than 200 μL to achieve results with similar workload. Furthermore, the current methods in the literature for hair samples involve more extraction steps as well as a greater amount of hair (>10 mg) than the presented method.

The LC-MS/MS method developed for the determination of citalopram in mice plasma and hair fulfils the criteria generally required for bio analytical methods. The developed method was successfully applied for quantification of plasma and hair samples of mice injected daily with citalopram for 1, 2, 4, 8, 15 and 30 days.

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