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Quantification of carbamazepine and its active metabolite by direct injection of human milk serum using liquid chromatography tandem ion trap mass spectrometry

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ABSTRACT

This work reports the use of a liquid chromatography ion trap tandem mass spectrometry (LC–IT-MS/MS) system for quantification in human milk samples of both carbamazepine (CBZ) and its active metabolite, carbamazepine 10,11-epoxide (CBZE). An octadecyl restricted-access media bovine serum albumin column (RAM-BSA C_{18}) was used in single-column mode. Selectivity, extraction efficiency, accuracy and precision were achieved employing 100 μL of the sample, without preparation, with detection limits of 20.0 ng/mL for CBZ and 40.0 ng/mL for CBZE. The matrix effect was investigated for the compounds by post-column infusion (qualitative) and by on-line extraction (quantitative). It was observed suppression effect for CBZ and CBZE by post-column infusion, ion suppression of 0.80 for CBZ, and enhancement of 1.28 for CBZE by on-line extraction. The developed method was validated and applied to analyze breast milk samples from one nursing mother. CBZ and CBZE were quantified in the concentrations of 2.26 μg/mL and 1.54 μg/mL, respectively. To our knowledge, this is the first report on the simultaneous determination of CBZ and its active metabolite by direct injection of human milk serum.

1. Introduction

Human milk is the most suitable food to the infant during the first 6 months of its life. The benefits of breastfeeding are well known. Among them, one can mention the ideal composition of nutrients and a better immunological protection, which leads to decrease mortality and morbidity in infants from infectious [1–3]. Moreover, breastfeeding facilitates the normal progression of neurocognitive development, also providing the opportunity for bonding between infant and mother [4].

However, women on medication may prematurely wean their infants for fear of exposing them to the medication through breastfeeding. Although the majority of drugs is considered safe for lactating mothers and their children, information on drug transfer into human milk and how this may affect the child health and development are necessary [5–7].

Carbamazepine (CBZ) is an anticonvulsant and mood-stabilizing drug used primarily in the treatment of epilepsy and bipolar disorder. Their use during breastfeeding is generally considered safe; however, observation for adverse effects is recommended. In the plasma, the half-life elimination of the unchanged drug is approximately 36 h following a single oral dose [8,9]. In addition, the major metabolite carbamazepine 10,11-epoxide (CBZE) has similar pharmacological activity and may be partly responsible for the side effects of the therapy [10,11].

During the last decade, a series of methods for quantifying CBZ and its main metabolites in biological matrices have used liquid chromatography coupled with mass spectrometry. The sample clean-up procedures used in these works were solid phase extraction (SPE), protein precipitation (PP) and liquid–liquid extraction (LLE) [9,11,12].

The use of restricted-access media (RAM) supports in column-switching or single-column mode has allowed the quantification of a diverse class of compounds by direct injection of a variety of biological fluids [13]. However, there are no reports on the use of RAM columns for sample clean-up of human milk. Thus, the present work reports a liquid chromatography ion trap tandem mass spectrometry (LC–IT-MS/MS) method using an octadecyl RAM bovine serum albumin (BSA) column, in single mode, for the simultaneous quantitation of the CBZ and CBZE in human milk serum.

2. Experimental

2.1. Reagents and materials

All the organic solvents were HPLC grade from Mallinckrodt Baker (St. Louis, USA). The water used for the mobile phase was

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purified using a Milli-Q system (Millipore, São Paulo, Brazil). CBZ were donated by Vita Nova Institute (Hortolândia, Brazil), CBZE was synthesized and provided by the same institution. [$^{13}C_1$, $^{15}N_1$]-Carbamazepine was obtained from Sigma–Aldrich (Steinheim, Germany). Standard and deuterated stock solutions were stored at 4 °C. All other reagents were of analytical grade.

Pooled human milk was supplied by the Breast Milk Bank from the Maternity Dona Francisca Cintra Silva of the São Carlos, SP, Brazil. The use of these samples was approved by Ethical Committee of Human Research of Federal University of São Carlos. The milk samples were stored in aliquots of 2.0 mL at −20 °C.

2.2. General procedure for the synthesis of epoxy carbamazepine derivative

2.2.1. Materials

Unless otherwise stated, all commercially available reagents were from Aldrich Chemical Co. (St. Louis, USA). Reagents and solvents were purified when necessary according to the usual procedures described in the literature. The CBZE infrared (IR) spectra refer to films and were measured on a Nicolet 4700 FT-IR spectrometer. ^{1}H and ^{13}C spectra were recorded on a Bruker DRX-200 (200 and 50 MHz, respectively) and ARX-400 (400 and 100 MHz, respectively). Analytical thin-layer chromatography was performed on a 0.25 μm film of silica gel containing fluorescent indicator UV_{254} supported on aluminum sheet (Fluka Analytical, St. Louis, USA). Purification was performed using silica gel column chromatography and Combi*Flash* Companion system.

2.2.2. Synthesis of epoxy carbamazepine derivative

In a round bottom flask with magnetic stirring at 0 °C, chloroperbenzoic acid (380 mg, 1.91 mmol) was added portionwise to a solution of CBZ (300 mg, 1.27 mmol) in anhydrous dichloromethane (50.0 mL). After standing at room temperature for 4 days, the mixture was cooled to 0 °C and the remaining chloroperbenzoic acid was filtered off. The solution was washed with 5% aqueous $NaHSO_3$ and with saturated $NaHCO_3$ solutions, dried using anhydrous $MgSO_4$ and evaporated in vacuo. The crude residue was purified using Combi*Flash* Companion system with hexane and ethyl acetate gradient, leading to the oxidized compound in 30% yield (96.0 mg). IV (λ_{max}) cm^{-1}: 3482, 3207, 1732, 1676, 1593, 1392, 1249, 1040, 767. RMN ^{1}H (400 MHz, $CDCl_3$) δ: 7.54–7.48 (m, 2H), 7.43–7.41 (m, 4H), 7.37–7.33 (m, 2H), 4.5 (slg, 2H), 4.28 (s, 2H). RMN ^{13}C (100 MHz, $CDCl_3$) δ: 157.87, 138.3, 131.61, 131.4, 130.46, 130.19, 128.38, 58.59.

2.3. Instrumentation

The LC–IT-MS/MS system consisted of an Esquire 6000 IT mass spectrometer fitted with an electrospray ionization (ESI) source (Bruker Daltonics, Bremen, Germany) and a Shimadzu LC system (Kyoto, Japan) equipped with two LC-20AD pumps, a SIL-20A autosampler, a DGU-20A5 degasser, a CBM-20A interface and a HPLC 7000 Nitronic EA (Sulpelco, St. Louis, USA) six-port valve. The Data Analysis software (Bruker Daltonics, Bremen, Germany) was employed to data acquisition. The expert tune mode was used for the optimization of the ionization source, voltages on the lenses and trap conditions.

2.4. Chromatographic parameters

A RAM-BSA C_{18} column (50.0 mm × 4.60 mm, LUNA®, 10.0 μm, 100 Å), which was prepared following Menezes and Felix [14] protocol, was used in the single mode for simultaneous size-exclusion of high-molar-mass matrix components and analysis of CBZ and CBZE. The LC system used for coupling the RAM column to the IT-MS/MS is illustrated in Fig. 1A. The time sequence and chromatographic conditions applied are schematized in Fig. 1B.

2.5. Depletion of human milk proteins by RAM-BSA C_{18} column

The depletion of proteins from human milk by the RAM-BSA C_{18} column was determined by Bradford's method [15], using aliquots of pool of samples (50.0, 100 and 200 μL) as previously described for plasma and bovine milk [16]. Aliquots of human milk were injected (n = 3) and eluted with water through the column at a flow rate of 1.00 mL/min and then collected in a 2.00 mL volumetric flask. A 500 μL portion of the eluent was analyzed.

The reference solution consisted of aliquots (50.0, 100 and 200 μL) of human milk dissolved in water to a total volume of 2.00 mL. The total amount protein present in the eluent was estimated from the absorbance ratio of the reference solutions of eluted samples milk.

To evaluate the molecular mass cut-off of the RAM-BSA C_{18} column for the human milk proteins, the elution profile of β-casein (24,000 Da) and α-lactalbumin (14,178 Da), at concentrations of 5.00 g/L, were also examined at the same conditions used for the human milk samples.

2.6. Mass spectrometry parameters

For the optimization of ionization conditions, full scan acquisitions were made to the specific mass ranges for individual compounds. For that, standard solutions of each compound (100 ng/mL) were continuously infused at a flow rate of 10.0 μL/min by a syringe pump into the mobile phase stream. The IT-MS/MS was operated in the Selected Reaction Monitoring (SRM) mode in which the protonated molecular ion was isolated and the fragment ions were monitored. For both analytes, two SRM transitions were respectively used one to quantification and the other one for confirmation: 237 > 194 and 237 > 192 for CBZ; 253 > 210 and 253 > 180 for CBZE. The parameters established for the analysis were the following: nebulizer pressure, 25.0 psi; drying gas flow, 6.00 L/min; temperature, 325 °C; capillary voltage, 3.50 kV; fragmentation amplitude, 0.28 V.

2.7. Working solutions

A stock solution of CBZ (25.0 μg/mL) and CBZE (50.0 μg/mL) were prepared in MeOH. For the calibration and the quality control (QC) samples, nine working solutions were prepared in MeOH using this stock solution, at the following concentrations to calibration: 0.250, 0.750, 1.50, 2.25, 4.50 and 6.00 μg/mL for CBZ and 0.500, 1.00, 3.00, 4.50, 9.00, 12.0 μg/mL for CBZE; and to QC samples: 0.300, 2.50 and 5.00 μg/mL for CBZ and 0.600, 5.00 and 10.0 μg/mL for CBZE. For the deuterated standard (IS), working solution of 1.00 μg/mL in MeOH was prepared. All solutions were stored at 4 °C.

2.8. Sample preparation

To prepare the spiked human milk samples, 50.0 μL of the appropriate working solution and 50.0 μL of the IS were placed in a culture tube. The solvent was evaporated under a stream of nitrogen and then 500 μL of human milk were added to the tube containing the dried analytes. The spiked samples were vortex-mixed for 30 s and left to stand for 10 min to allow a steady-state with the matrix components and then centrifuged (13,420 × g for 15 min at 4 °C), resulting in a thin upper fat layer, a middle aqueous layer and a small cell pellet at the bottom of the centrifuge tube. Aliquots of the middle layer (250 μL) were transferred to autosampler vials and 100 μL was injected into LC–IT-MS/MS system.

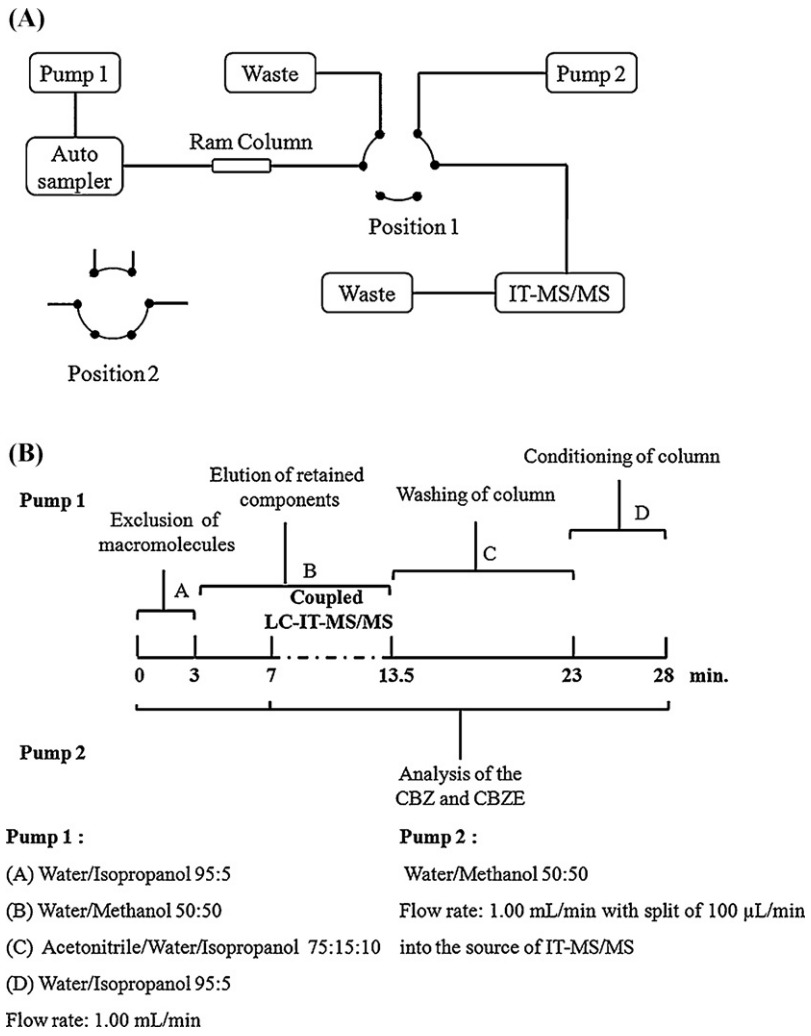

Fig. 1. (A) Schematic LC–IT-MS/MS single column system and (B) time sequence and chromatographic conditions used.

Calibration samples were prepared in triplicate while QC samples were prepared in quintuplicate.

The sample from nursing mother (10.0 mL) was collected 8 h after an oral dose administration of CBZ (200 mg) and it was prepared by dilution of 100 μL in human milk blank to furnish a 1.00 mL sample. Three replicates of the sample were prepared and the IS (100 ng/mL) was added to all replicates before analysis.

2.9. Method validation

The method validation was carried out in accordance with internationally accepted criteria [17], considering the selectivity, linearity, accuracy, precision, recovery, limit of quantification (LOQ), limit of detection (LOD), matrix effects and stability.

To determine linearity, six blank milk samples were spiked in triplicate at six different concentration level (25.0, 75.0, 150, 225, 450, 600 ng/mL for CBZ; 50.0, 100, 300, 450, 900, 1200 ng/mL for CBZE) and then analyzed.

For the determination of selectivity drug-free milk samples and milk spiked with CBZ and CBZE were evaluated. At all analytical runs, blank samples were analyzed to evaluate the selectivity of the method.

Intra- and inter-batch precision and accuracy were determined by analysis at five replicates of each QC samples (30.0, 250, 500 ng/mL for CBZ; 60.0, 500, 1000 ng/mL for CBZE). Precision was expressed as coefficient of variation (CV) of the replicate measurements and the accuracy of the method was evaluated as the percentage between the found and the nominal concentration of each compound.

The extraction efficiencies were calculated using the QC samples. The percentage of recovery was obtained comparing the peak-area ratios of QC samples to the ones prepared at the same concentration in ultrapure water.

The LOD and LOQ values were determined using spiked milk samples prepared in triplicate. The LOD were assumed as the minimum detectable amount of compound, with a signal-to-noise (S/N) ratio of three while LOQ as the lowest calibration level. The accepted criterion for the limit of quantification was that the precision and accuracy for the three samples should have a CV $\leq$ 20%.

Matrix effects were evaluated by on-line post-column infusion and by on-line extraction using the column-switching system with samples prepared with ultrapure water and a human milk sample. In the post-column infusion, 100 μL of each sample was injected into the RAM-BSA column at the established chromatographic conditions (Fig. 3). A 100 ng/mL aqueous solution of the analytes was infused, using a syringe pump at a flow rate of 10.0 μL/min, after the column and before the mass spectrometer ionization source [17]. For the on-line extraction procedure, milk sample and ultrapure water were spiked with the analytes (100 ng/mL). The comparison of the peak areas obtained from the milk and the ultrapure water samples was used to evaluate the enhancement or ion suppression effect.

The confirmation and identification of CBZ and CBZE in the sample were performed by the ratio between two transitions monitored and the retention times, in according to the FDA when using LC tandem MS as an instrumental technique for confirmation of the identity of animal drugs residues [18].

The stability of the CBZ and CBZE was evaluated by comparing assay results of spiked samples at three different concentrations (QC samples) and by analyzing aliquots of the same samples after storage at −20 °C and after two freeze–thaw cycles of 24 h each one. All stability determinations were assessed by preparing a set of samples from a freshly made stock solution of human milk.

3. Results and discussion

3.1. Method development

A variety of RAM columns are commercially available and their usefulness has been already demonstrated [13,19,20]. The immobilized BSA alkyl columns (RAMs-BSA) have for long time attracted our attention by the simplicity of their preparation [14,21]. Their efficiency has been reported for the quantification of a variety of drugs in different biological matrixes [16,19,22,23] and aqueous environmental samples [24,25].

Human milk has been used to assess neonatal exposure to drugs, with the advantage that it is collected easily and non-invasively. However, the extraction of drug from breast milk is a great analytical challenge given that the transfer of drugs from plasma into breast milk is related to factors such as capacity for plasma protein binding, lipophilicity, degree of ionization, molecular weight, bioavailability, plasma concentration, among others [1,26,27].

There are few works reporting the quantification of drugs and their metabolites in human milk, and most of them use extraction procedure, such as SPE, PP or LLE to evaluate the drug transfer [27]. RAM columns are a useful tool for direct injection of complex matrix allowing the extraction/concentration of analytes.

To permit its use in biological sample cleanup, the capability of efficiently removing macromolecule is an important asset to a RAM column. Thus, a series of RAM-BSA columns were prepared, as previously described [16], and percentage of protein depletion from human milk evaluated.

The percentages of proteins depletion by the RAM columns were ≥92.0% for three different aliquots ($n = 3$). The elution profile evaluated at $\lambda = 280$ nm using the RAM-BSA C_{18} column showed a exclusion time of 3 min (Fig. 2) for the milk serum samples. Furthermore, the elution profile of β-casein and α-lactalbumin, the two major human milk proteins [28], demonstrated that both proteins were eluted with water within 1.5 min at 1.00 mL flow rate.

After that, the RAM-BSA C_{18} column was selected to the method development.

Its ability to remove proteins and other macromolecules, combined with the selective retention and resolution obtained between CBZ and CBZE, through conventional hydrophobic interactions, allowed its use in the single-column mode. It is important to stress that very few methods make use of RAM column in the single mode [13,19].

The chromatographic conditions were optimized with the macromolecules exclusion time of 3 min, at the flow rate of 1.00 mL/min, and a 100 μL sample injection using ultrapure water with 5% ISO (v/v) as the mobile phase (Fig. 1B). Under this condition, CBZ, CBZE and IS were retained by the RAM column. The analytes were separated and analyzed using MeOH/H_2O (50:50, v/v) as solvent. To obtain the separation and estimate the time of LC–IT-MS/MS coupled (7.00 and 13.5 min), a blank matrix was fortified with a high concentration of compounds (1.00 μg/mL).

In order to avoid undesired absorption of substances on the column, the RAM column was cleanup, between injections, with ACN/H_2O/ISO (75:10:15, v/v/v).

3.2. Matrix effect

Lipids and proteins (casein, lactalbumin, and lactoferrin) are the main components of breast milk which can interfere in the extraction efficiency of analytes from the matrix and also in its detection. The atmospheric pressure ionization (API) source has as the major drawback the negative impact caused by matrix effect and since there are no universal protocol to overcome this problem, matrix effect should always be evaluate in a quantitative method development involving MS/MS detection [29].

The matrix effect was qualitatively measured by post-column infusion and by on-line extraction for quantitative analysis. The post-column infusion procedure showed suppression effect for CBZ and CBZE (Fig. 3). However, the on-line sample extraction system demonstrated ion suppression of 0.80 for CBZ and enhancement of 1.28 for CBZE, obtained by analytes peak area ratio of spiked human milk and aqueous solution samples. As Trufelli et al. [30] point out, information regarding the signal enhancement is still lacking. The importance of chemical structure on the matrix effect can be acknowledged by comparing the results observed for CBZ and its metabolite.

Representative chromatograms of the analysis of blank and spiked human milk samples analyzed at the established conditions are shown in Fig. 4. The chromatograms show that no endogenous compounds are interfering with CBZ and CBZE.

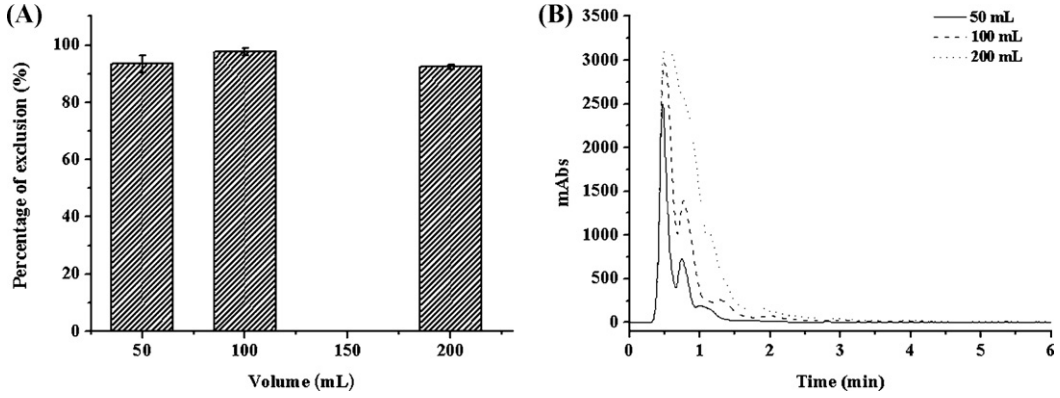

Fig. 2. Percentage of exclusion (A) and representative chromatogram (B) of macromolecules from human milk, using RAM-BSA C_{18} column at three different injection volumes. Chromatographic conditions: water/isopropanol (95:5), flow rate: 1.00 mL/min, and $\lambda = 280$ nm.

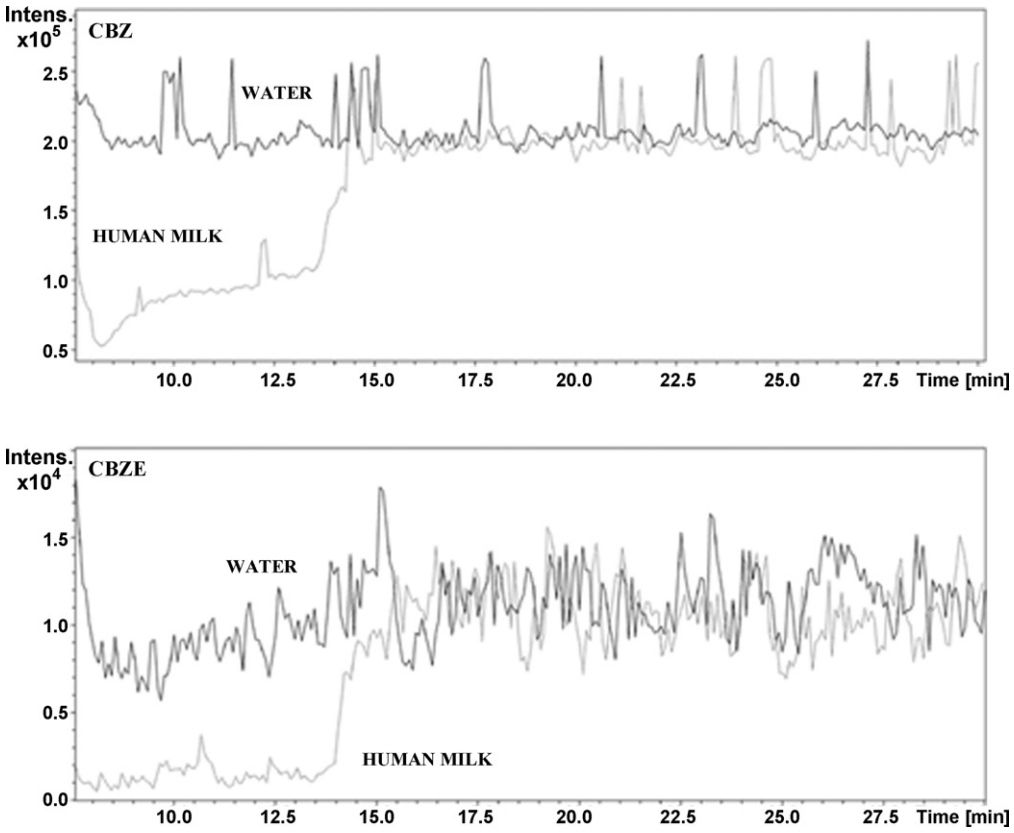

Fig. 3. LC–IT-MS/MS extracted ion chromatograms from the on-line post-column infusion: CBZ and CBZE – 100 ng/mL.

3.3. Method validation

The calibration curves were generated by polynomial regression analysis by plotting of the peak area ratio of the compound signal (CBZ or CBZE) to the IS versus nominal concentration [31].

The mean correlation coefficients ($n = 3$) were $R^2 \geq 0.995$ and the regression equations are given in Table 1.

Inter and intraday precision and accuracy obtained for CBZ and CBZE are given in Table 2. The values obtained are in accordance with the established criteria [17] especially considering that sample preparation procedure included a standby step which allowed the steady-state be reached between the analytes and the matrix [32].

The extraction efficiency data presented also in Table 2 concerns the ranged from 78.2 to 105.3%, for both compounds. These results were determined by comparing the peak areas of aqueous solutions at three different concentrations with those the QCs samples, and demonstrated that there is a slight influence of the extraction for the lower concentrations. The LOQs are the first calibration concentrations and the values obtained were 25.0 ng/mL for CBZ and 50.0 ng/mL for CBZE. The LOD values were of 20.0 and 40.0 ng/mL, respectively. These concentrations levels are similar to

Table 1
Calibration parameters for the method.

Compound	Range (ng/mL)	Calibration equation	R^2
CBZ	25.0–600	$Y = 0.02961 + 0.00463X + 1.10071E^{-6}X^2$	0.995
CBZE	50.0–1200	$Y = 0.10069 + 8.60123E^{-4}X + 2.10757E^{-7}X^2$	0.999

Table 2
Accuracy, average of intra-day ($n = 5$), variability and extraction efficiency.

Compound (ng/mL)	1st day		2nd day		3rd day		Average ($n = 15$)		Extraction efficiency (%)	CV% ($n = 5$)
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)		
CBZ										
30	86.1	13.4	112	16.4	97.7	5.4	98.4	11.7	78.2	9.50
250	96.4	10.5	82.5	7.00	88.1	9.30	89.0	8.90	105	5.20
500	85.9	10.9	91.2	11.4	83.1	11.2	86.7	11.2	104	3.50
CBZE										
60	122	18.0	104	20.0	95.5	20.7	98.1	19.6	87.2	9.00
500	114	12.6	116	14.3	118	9.50	110	12.1	102	5.70
1000	115	17.1	107	7.70	85.2	5.90	116	10.2	91.9	7.40

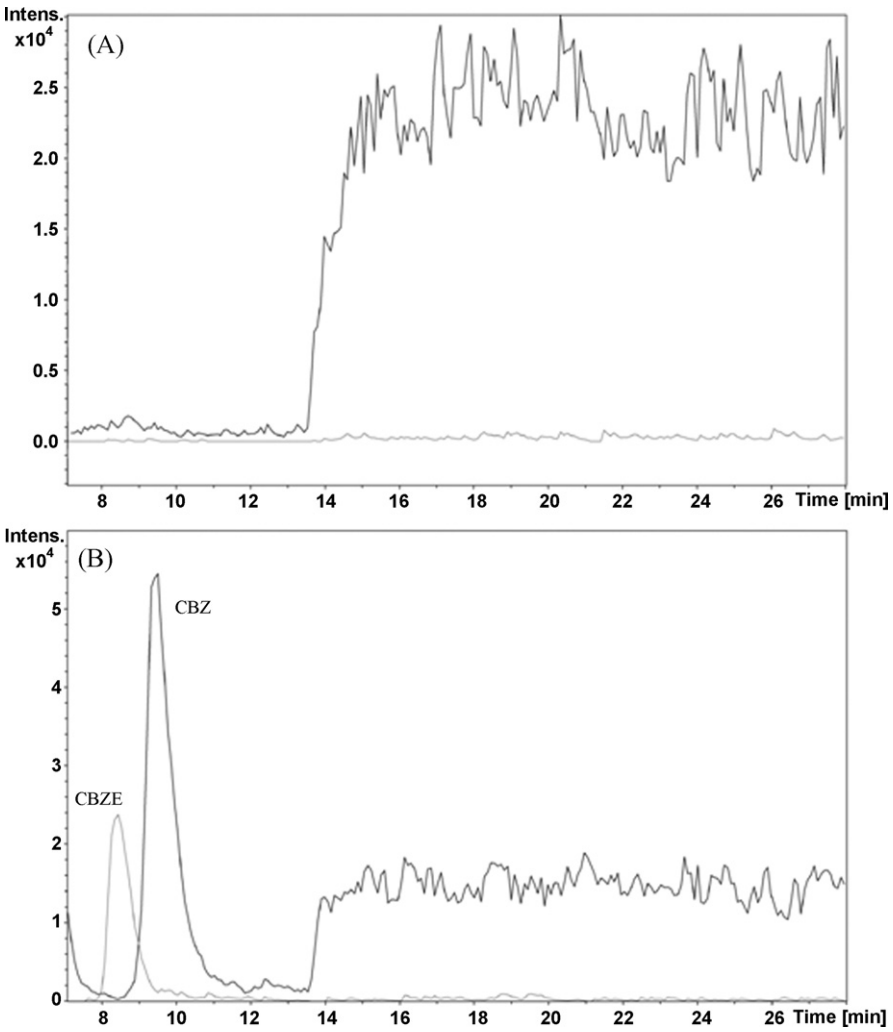

Fig. 4. Chromatograms of the analysis (A) of blank and (B) spiked human milk samples (CBZ 250 ng/mL; CBZE 500 ng/mL).

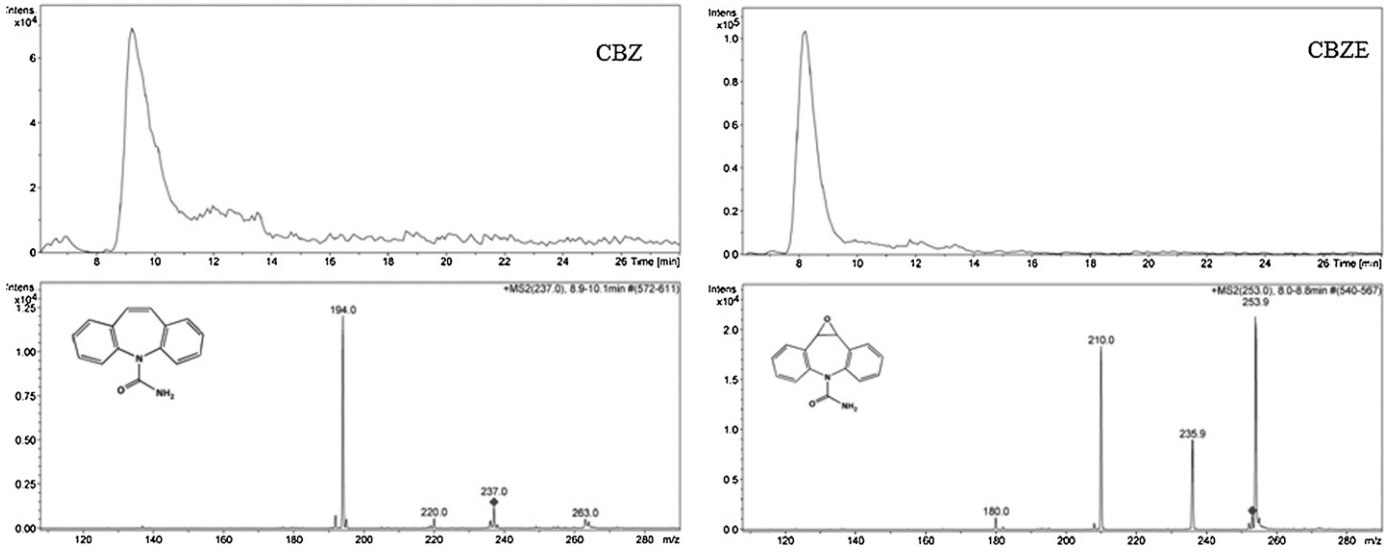

Fig. 5. LC–IT-MS/MS extracted ion chromatograms of the milk sample collected from a nursing mother after an oral dose (200 mg) with the respective mass spectrum.

reported on the literature for these compounds in plasma samples [33]. However, it is important to call attention that the method was developed without sample pre-concentration with direct injection into chromatographic system.

The stability of the spiked samples was evaluated by two freeze–thaw cycles and it was found that samples were stable for 48 h.

3.4. Application to nature milk sample

The validated method was used to analyze a human milk sample obtained from a nursing mother 8 h after an oral dose administration of CBZ (200 mg). After dilution, CBZ were quantified at the concentration of 226 ng/mL (CV = 5%) and CBZE 154 ng/mL (CV = 15%) corresponding to absolute concentrations of 2.26 μg/mL and 1.54 μg/mL, respectively. Based on these results, it is possible to calculate the amount of drug ingested by the infant. The positive confirmation of CBZ and their major metabolite (CBZE) was based on the SRM transition of the ions in accordance with European criteria (Fig. 5) [16].

4. Conclusions

The present work reports the development, validation and application of the LC–IT-MS/MS method for simultaneous quantification of the CBZ and its active metabolite, CBZE, in nature human milk using a RAM-BSA column in the single mode. The quality of the performance of the RAM-BSA column was maintained with over 500 human milk serum injections of 100 μL each. The method has been successfully applied to the analysis of human milk sample obtained from a nursing mother thus demonstrating that the method described herein can be used for routine antiepileptic drug monitoring.

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