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Liquid chromatography/tandem mass spectrometry for the determination of carbamazepine and its main metabolite in rat plasma utilizing an automated blood sampling system

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Abstract

A liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the simultaneous determination of carbamazepine and its main metabolite carbamazepine 10,11-epoxide in rat plasma is described. The method consists of a liquid–liquid extraction procedure and electrospray LC/MS/MS analysis. The chromatographic separation was achieved within 5 min using a C_8 (150 mm × 2.1 mm) 5 μm column with a mobile phase composed of water/acetonitrile/acetic acid (69.5:30:0.5, v/v/v) at a flow rate of 0.4 ml/min. D_{10}-carbamazepine is used as the internal standard for all compounds. Analytes were determined by electrospray ionization tandem mass spectrometry in the positive ion mode using selected reaction monitoring (SRM). Carbamazepine was monitored by scanning *m/z* 237 → 194, carbamazepine 10,11-epoxide by *m/z* 253 → 210 and d_{10}-carbamazepine by *m/z* 247 → 204. The lower limit of quantitation (LLOQ) is 5 ng/ml for each analyte, based on 0.1 ml aliquots of rat plasma. The extraction recovery of analytes from rat plasma was over 87%. Intra-day and inter-day assay coefficients of variations were in the range of 2.6–9.5 and 4.0–9.6%, respectively. Linearity is observed over the range of 5–2000 ng/ml. This method was used for pharmacokinetic studies of carbamazepine and carbamazepine 10,11-epoxide in response to two different blood sampling techniques (i.e., manual sampling versus automated sampling) in the rat. Several differences between the two sampling techniques suggest that the method of blood collection needs to be considered in the evaluation of pharmacokinetic data.

Keywords: Carbamazepine; Metabolite; LC/MS/MS; Autosampling

1. Introduction

Carbamazepine (CBZ) (5-H-dibenz[b,f]azepine-5-carboxamide) (Fig. 1) is an anticonvulsant used in clinical practice as first-line treatment for generalised tonic-clonic and partial seizures [1]. Over the last two decades, 33 metabolites of CBZ have been isolated and identified in the urine from patients on an oral dose [2]. Of these metabolites, carbamazepine 10,11-epoxide (CBZ-E) is the most important one from a clinical point of view (Fig. 1). CBZ-E is pharmacologically as active as the parent compound in experimental animals.

A number of LC methods with UV detection for the determination of CBZ and its metabolite in drug products and human plasma have been described [3–13]. Capillary electrophoresis has also been reported for the separation CBZ and its metabolites [14] and monitoring the concentrations of CBZ and its metabolites in plasma [15,16]. LC/MS method has been proved powerful for the characterization of the metabolites of carbamazepine in patient urine [17]. A few methods using LC/MS for the determination of CBZ in drug samples and plasma have been described [18–20].

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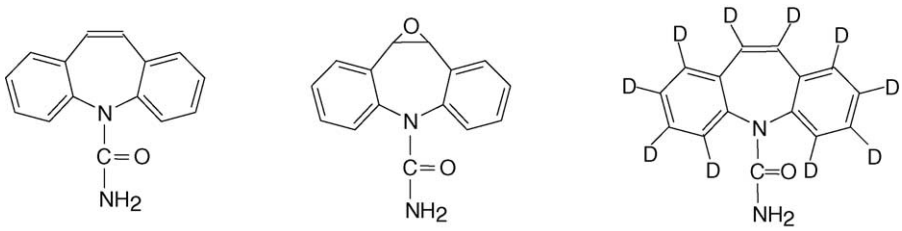

Fig. 1. Structures of carbamazepine, carbamazepine 10,11-epoxide and d_{10}-carbamazepine.

LC/MS/MS and LC-Q-TOF MS have recently been reported for the determination of CBE in aquatic environment [21,22]. But for all above LC/MS/MS methods, there is no report on the determination of CBZ and its metabolites using an isotope internal standard, though this was used for GCMS on the determination of CBZ in human whole blood [23].

This study describes the development and validation of an LC/MS/MS method for the determination of CBZ and CBZ-E in rat plasma with an isotope labeled internal standard. A recent study demonstrated that this method can be used for the analysis of CBZ and CBZ-E in rat brain microdialysis samples [24]. The present study also describes the determination of CBZ and CBZ-E in rats following oral administration of CBZ in two different blood collection experiments. The objective of the two experiments was to determine if blood sampling technique affected the pharmacokinetics following CBZ administration. Therefore blood was collected via a jugular catheter either manually (i.e., using a syringe and placing rats in a restrainer) or using an automated blood sampling system. Inherently, the stress associated with restraint and conventional blood sampling from conscious rodents influences rodent physiology (i.e., blood pressure and blood flow) and biochemistry (i.e. release of hormones and immune factors in response to stress), metabolism and protein expression [25,26]. Therefore, automated blood sampling of conscious cannulated animals in a stress-free environment may result in more meaningful pharmacokinetic data. Automated blood sampling combined with LC/MS/MS also provide a mean to determine CBZ and CBZ-E in rat blood and rat brain microdialysate simultaneously [24].

2. Experimental

2.1. Chemical and reagents

Carbamazepine and carbamazepine 10,11-epoxide were purchased from Sigma (St. Louis, MO, USA). Internal standard D_{10}-carbamazepine was purchased from C/N/D Isotopes (Pointe-Claire, Quebec, Canada). Acetonitrile and ethyl acetate were of HPLC grade (Burdick & Jackon, Muskegon, MI, USA). Acetic acid was of analytical reagent grade (Mallinckrodt, Paris, KY, USA). Reagent grade water was prepared from in-house deionized water using a NANOpure system (Barnstead/Thermolyne, Dubuque, IA, USA).

2.2. LC/MS/MS system

The LC/MS/MS system was equipped with a BASi PM-80 pump (BASi, West Lafayette, IN, USA) coupled to a Finnigan LCQ Deca ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA) equipped with an ESI source. The analytical column was a Discovery C_8, 5 μm, 150 mm × 2.1 mm (Supelco, Bellefonte, PA, USA). The mobile phase was water/acetic acid/acetonitrile (69.5/0.5/30, v/v/v). The flow rate was set at 0.4 ml/min. Samples were injected by an autosampler (Sample Sentinel, BASi), which was set at 10 °C and fitted with a 20 μl loop. The mass spectrometer was operated in ESI positive ion mode. Nitrogen was used as both the sheath and auxiliary gas at a pressure of 80 and 20 units, respectively. The spray and capillary voltage were set at 5.0 kV and 22 V, respectively. The capillary temperature was set at 275 °C.

2.3. Automated blood sampling system

The blood collecting system consisted of a robotic automated blood sampler (Culex™, BASi), and a freely moving rat containment device (Raturn, BASi). The blood samples were collected into a refrigerated fraction collector (HoneyComb, BASi) with vials maintained at 4 °C.

2.4. Sample preparation

A total of 0.25 ml of rat blood was collected and was centrifuged at 2000 g for 10 min. A volume of 0.1 ml of plasma was transferred to 1.7 ml Eppendorf tubes. D_{10}-CBZ was used as the internal standard. A volume of 0.1 ml of 500 ng/μl of internal standard was added to the plasma and mixed. Ethyl acetate (0.8 ml) was added to the plasma, vortex-mixed for 2 min, and centrifuged at 5600 × *g* for 6 min. Following centrifugation, the clear supernatant was transferred to another tube and dried under nitrogen at room temperature and the dried residue was reconstituted in 0.1 ml of mobile phase. A 0.02 ml aliquot of the reconstituted sample was injected into the LC/MS/MS system.

Table 1
Positive product ion mass parameters of CBZ, CBZ-E and D_{10}-CBZ

Compounds	$[M+H]^+$	Product ions (*m/z*)	Isolation width (*m/z*)	Collision energy (%)	SRM ion combination
CBZ	237	220, 194	1	30	237 → 194
CBZ-E	253	236, 210	1	22	253 → 210
D_{10}-CBZ	247	230, 204	1	26	247 → 204

2.5. Calibration curve

Stock solutions of CBZ and CBZ-E (1 mg/ml) were individually prepared in methanol and stored at −20 °C. Dilutions of these solutions were made in order to prepare the plasma standards as needed to construct the calibration curves. Calibration standards were prepared fresh for each batch analysis by adding aliquots of the spiking solutions in drug-free rat plasma. The concentration of the standards at respective points on the concentration graphs were 5, 10, 50, 100, 500, 1000 and 2000 ng/ml in rat plasma based on 0.1 ml of plasma with D_{10}-CBZ as internal standard. The quality control (QC) samples at concentration of 5, 50 and 1000 ng/ml were prepared similarly in pooled rat plasma for these two analytes. Peak integration, regression and calculation of concentration were computed using ThermoQuest's Xcalibur (Version 1.0) software. The calibration curve was constructed using a weighted (1/*x*) linear regression of peak-area ratios versus concentrations of analyte in rat plasma.

2.6. In vivo experiments

A total of seven male Sprague–Dawley rats (between 302 and 369 grams) supplied by Harlan, Indianapolis were used. Rats were surgically implanted with a jugular (CX-2010, BASi) and a femoral (CX-2020, BASi) vein catheter. Blood sampling was through the jugular catheter. Femoral catheters were only for use in the event of failure of the jugular catheter. Following surgery, all rats were housed in a metabolic cage (MR-1523, BASi) placed on a Raturn™, and allowed to recover for 1 day. Following surgery, rats were given free access to food and water. A solution of 5 mg/ml of CBZ was prepared in a 10% of 2-hydroxypropyl-β-cyclodextrin saline solution. Before oral dosing with CBZ, rats were fasted overnight. Rats were dosed with 5 mg/kg of CBZ. Following dosing, a volume of 0.25 ml blood sample was withdrawn either via manual sampling or using the Culex automated blood sampler at 0.25, 0.5, 0.75, 1, 1.5, 2.0, 3.0, 4.0, and 7.0 h, meanwhile a volume of 0.25 ml of saline was given to the rat via culex after each sampling for keeping body balance.

Blood was placed in a vial containing heparin and kept refrigerated until processing. During manual sampling, blood was collected into a syringe and stored in an Eppendorf tube in the refrigerator. During sampling with the Culex automated blood sampler, blood was kept in a refrigerated fraction collector.

3. Results and discussion

3.1. MS/MS optimization

Quantitation was conducted using the selected reaction monitoring (SRM) mode. Table 1 summarizes the product ion spectra of the two analytes and D_{10}-carbamazepine. The best sensitivities and minimum interferences were achieved by monitoring the transitions stated in Table 1 and conditions stated in Section 2.2. Here D_{10}-carbamazepine was used as an internal standard.

3.2. Chromatographic method development

Various bonded phase including ODS (C_{18}), Octyl (C_8) and CN were evaluated in an attempt to get better separation of analytes in a short run time. Among the various bonded phases, the Supelco Discovery C_8 (2.1 mm × 150 mm, 5 μm) provided good separation of CBZ and CBZ-E in the plasma sample and was satisfactory for the assay under the LC conditions used: water/acetonitrile/acetic acid (69.5:30:0.5, v/v/v), flow rate at 0.4 ml/min.

3.3. Validation of assay

3.3.1. Selectivity

The specificity of the method is documented by the absence of interference from endogenous substances from drug-free rat plasma. Typical chromatograms of an extracted drug-free rat plasma spiked with IS, plasma spiked with all analytes at 5 ng/ml (LOQ), and plasma spiked with all analytes at 500 ng/ml are illustrated in Figs. 2–4, respectively. No endogenous rat plasma components were observed at the retention times corresponding to both analytes and D_{10}-cabamazepine (internal standard).

3.3.2. Linearity

A weighed linear regression of the peak area ratio versus concentrations was performed for CBZ and CBZ-E. The observed peak area ratios were linear over the concentration range of 5–2000 ng/ml in rat plasma. The standard curves were fitted to a linear regression, $y = ax + b$, where y is the peak area ratio of analyte to internal standard, a is the slope of calibration curve, b is the intercept, and x is the analyte concentration (ng/ml). The mean values (±S.D.) ($n = 4$) for slope, intercept and r^2 were 0.0138 ± 0.0015, 0.0344 ± 0.0086 and 0.995 ± 0.0005

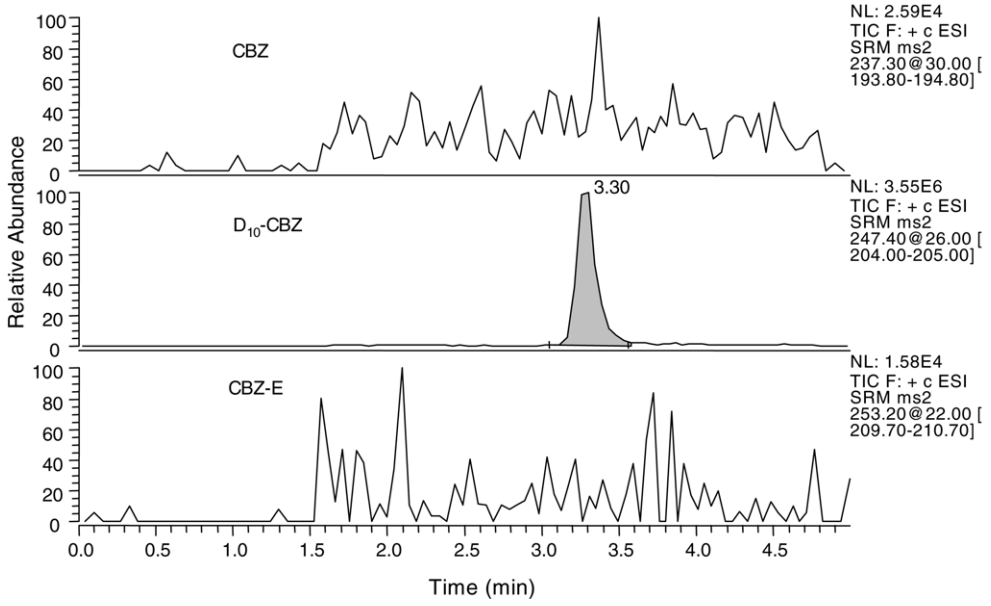

Fig. 2. Representative SRM chromatogram of extracted blank rat plasma spiked with IS.

for CBZ, and 0.0039 ± 0.0004, 0.0098 ± 0.0031 and 0.996 ± 0.002 for CBZ-E, respectively, for four calibration curve plots.

3.3.3. Lower limit of quantitation (LLOQ)

The lower limit of quantitation (LLOQ) is defined as the lowest concentration on the calibration graph for which acceptable accuracy (nominal ±20% and precision of 20% CV) were obtained. The current assay has a LLOQ of 5 ng/ml in rat plasma for each analyte. A modest ion trap spectrometer was used. With the latest triple quadruple instrumentation a much lower LOQ can be expected.

3.3.4. Accuracy and precision

To test the accuracy and precision of this assay, the quality control rat plasma samples were prepared and interpolated against the respective calibration curve. The intra- and inter-day accuracy and precision values for QC samples are present in Table 2. The precision values (coefficient of variation) at the three concentrations in the intra-day study varied between 2.6 and 5.2% for CBZ and 4.9 and 9.5% for CBZ-E while

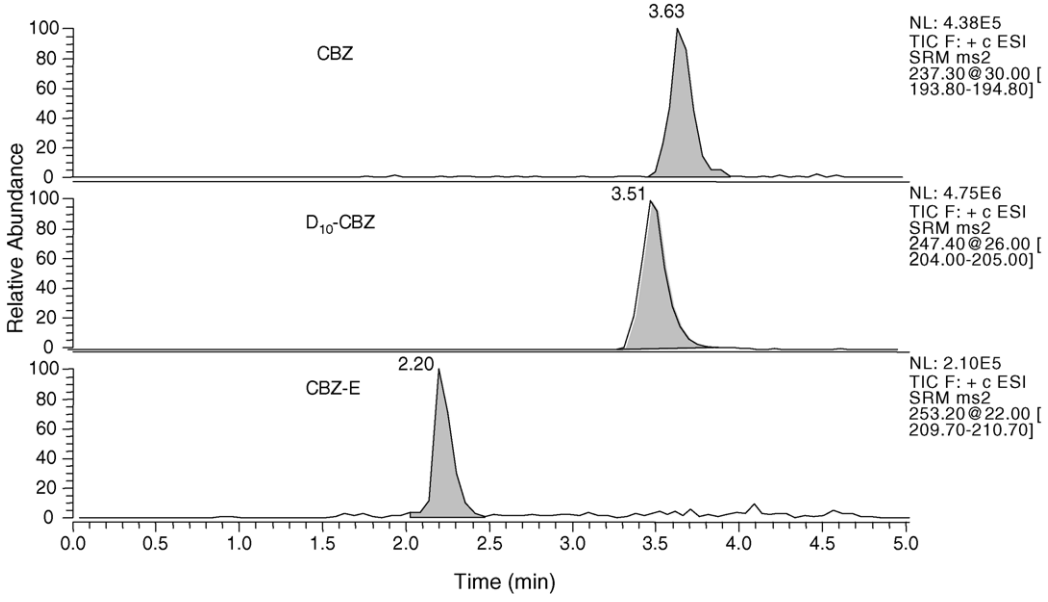

Fig. 3. Representative SRM chromatogram of two analytes from an extracted rat plasma at the LLOQ (5 ng/ml).

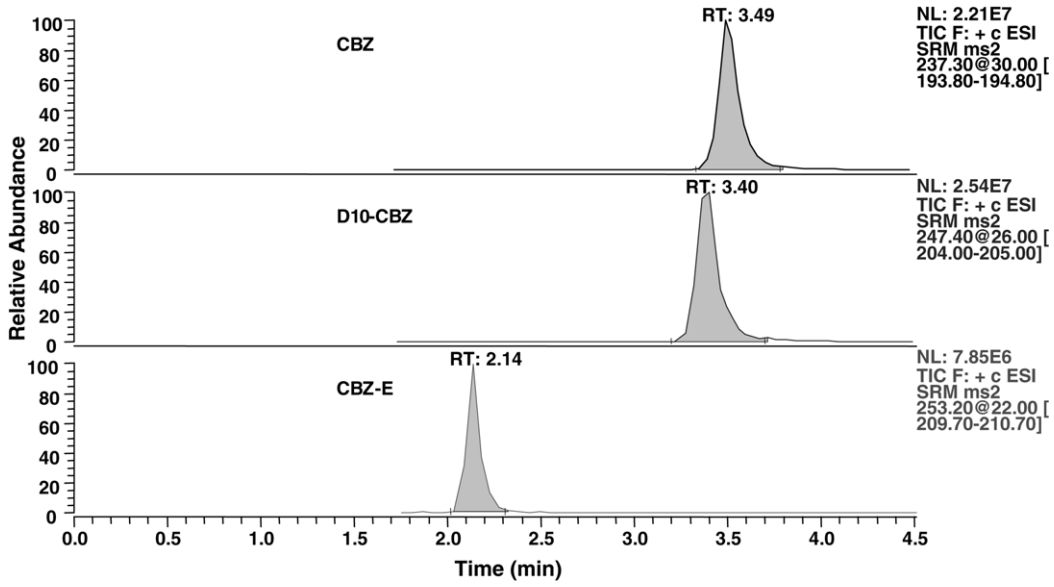

Fig. 4. Representative SRM chromatogram of two analytes from a blood sample taken 30 min after oral administration of CBZ at a dose of 5 mg/kg to rat.

that of inter-day study varied between 4.0 and 4.7% for CBZ and 8.7 and 9.6% for CBZ-E. The accuracy (relative error) values for all three concentrations deviated less than 4.7% for CBZ and 5.2% for CBZ-E from the corresponding nominal concentrations.

3.3.5. Extraction recovery

A comparison of the peak area ratio of analyte to the internal standard (D_{10}-carbamazepine) in a rat plasma sample that had been spiked with both analytes prior to extraction with samples to which both had been added after extraction was carried out. The internal standard was added to both sets of samples after extraction to correct for instrument drift and injection volumes. The recovery was calculated using the area ratios (analyte/internal standard) in both sets of samples. The overall extraction recoveries from rat plasma were 90% for CBZ and 87% for CBZ-E, respectively from three concentrations (10, 100 and 1000 ng/ml).

3.3.6. Matrix effect

The matrix effect was examined by using postcolumn infusion of the two analytes while injecting extracted rat plasma blank as reported [27,28]. There was no signal suppression observed due to matrix effect during the ionization process by postcolumn infusion test.

3.3.7. Stability

All stability experiments were performed with two different concentrations of analyte in plasma (10 and 1000 ng/ml) in triplicate. Carbamazepine and its metabolite carbamazepine-10,11-epoxide were found to be stable in rat plasma for at least 4 h at ambient temperature and in the reconstitution solution at 10 °C for at least 20 h. The stability criteria is within ±15% deviation.

3.4. Application

Traditional pharmacokinetic studies involve intermittent blood sampling and subsequent determination of drug concentrations in blood or plasma. Because of difficulties associated with collecting blood over time from a single rodent, often multiple animals are used to generate a single PK curve, reflecting the inter-animal variation. In the experiments described below, a complete blood sample sequence was taken over time for seven rats individually. This was

Table 2
Accuracy and precision for CBZ and CBZ-E from rat plasma

Compounds	Nominal concentration (ng/ml)	Inter-day variation ($n = 12$)			Intra-day variation ($n = 4$)		
		Measured concentration (ng/ml)	CV (%)	Relative error (%)	Measured concentration (ng/ml)	CV (%)	Relative error (%)
CBZ	5	4.9	4.5	2.0	4.8	5.2	4.5
	50	50.7	4.0	1.4	51.8	3.0	3.6
	1000	953	4.7	4.7	969	2.6	3.1
CBZ-E	5	5.2	9.6	4.0	5.1	6.3	1.5
	50	47.7	9.1	4.6	49.5	9.5	1.1
	1000	1052	8.7	5.2	990	4.9	1.0

Table 3
Mean PK parameters (±S.D.) of carbamazepine and its epoxide metabolite in plasma from rats in ($n = 7$) obtained by noncompartmental pharmacokinetics data analysis

Compound	Sampling	C_{max} (ng/ml)	T_{max} (h)	AUC (ng h/ml)	CL (ml/h)
CBZ	Manual	814 ± 345	0.5 ± 0.3	1818 ± 548	1005 ± 300
	Automated	888 ± 448	0.3 ± 0.1	1465 ± 774	1724 ± 1309
CBZ-E	Manual	867 ± 303	2.9 ± 2.3	3580 ± 1155	
	Automated	732 ± 367	2.3 ± 1.8	3074 ± 1587	

possible because both manual and automated blood sampling were carried out using a jugular catheter. Unlike manual sampling via a catheter, the automated blood sampler provides a means for blood withdrawal at preprogrammed intervals without the stress associated with handling the animal [29–31].

The method was used for the determination of carbamazepine and its metabolite carbamazepine-10,11-epoxide in rats following oral administration 5 mg/kg of CBZ. Blood samples were collected by one of two methods (i.e., manually or using the Culex automated blood sampler) following administration of CBZ. The rats were crossed over for the two sampling experiments with plasma collected at programmed time points post dose. Fig. 5 illustrates data of automated and manual sampling for a single oral dose administration of carbamazepine to rats ($n = 7$). Noncompartmental pharmacokinetics data analysis (PK Solutions 2.0) was used for PK data analysis. The pharmacokinetic parameters derived from the two sampling techniques are summarized in Table 3. It is interesting to see that Tmax of CBZ and metabolite CBZ-E obtained from automated sampling is less than for manual sampling. The difference of the concentration ratio of parent drug and metabolite from the two sampling techniques has also been demonstrated to be significant as determined by general linear model procedures using SAS. The observation of differences for PK parameters obtained by the two techniques can be attributed to physical stress, which influenced blood flow and distribution impacting drug absorption, distribution and metabolism. Blood flow to the extremities (away from the gut) is an attractive first order explanation, but is likely too simplistic.

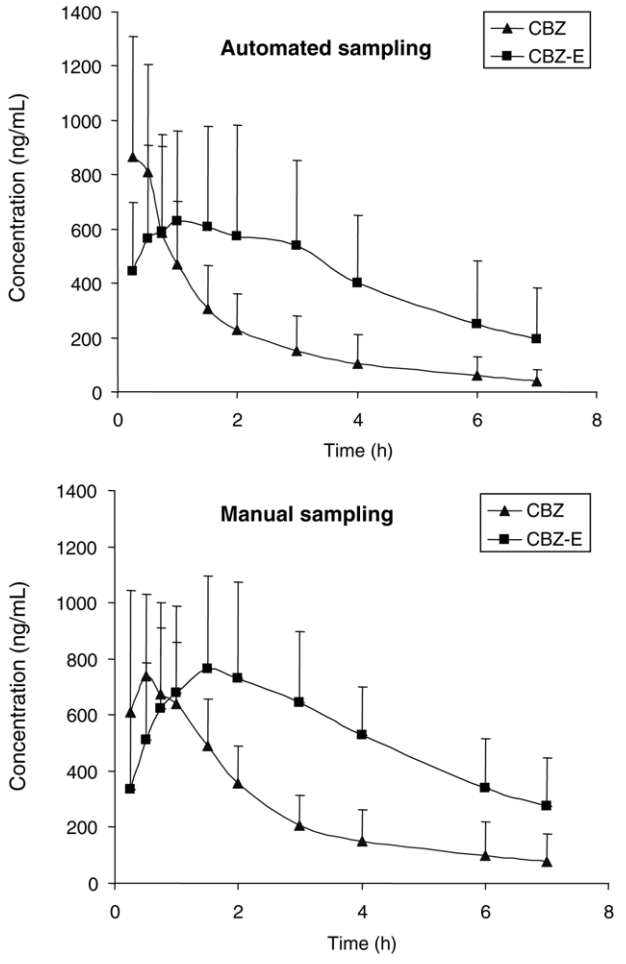

Fig. 5. Mean (±S.D.) plasma concentration-time profiles of CBZ and metabolite CBZ-E in rats ($n = 7$) after oral administration of 5 mg/kg of CBZ. Automated sampling on the top and manual sampling at the bottom.

4. Conclusion

A liquid chromatography/tandem mass spectrometry method was developed and evaluated for the determination of carbamazepine and its main metabolite in rat plasma. The automated blood sampling device and the reported method offer several advantages, such as an easy and accurate blood draw, low animal stress, a rapid and clean extraction and more reliable results. Automated blood sampling combined with LC/MS/MS shows promise for high throughput pharmacokinetic studies.

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