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Determination of bencycloquidium bromide in rat plasma by liquid chromatography–electrospray ionization-mass spectrometry

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Abstract

A liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) assay for the determination of bencycloquidium bromide (BCQB) in rat plasma was firstly developed and validated. After addition of 1-ethyl-bencycloquidium bromide as an internal standard (I.S.), the plasma samples were deproteinized with methanol and the supernatant was assayed by LC–ESI-MS. Chromatographic separation was achieved with a Hanbon Lichrospher 5-C18 column. The mobile phase consisted of methanol–40 mM ammonium acetate buffer–formic acid (75:25:0.25, v/v/v) and delivered at the flow rate of 1.0 ml/min. LC–ESI-MS was carried out on a single quadrupole mass spectrometer using electrospray ionization (ESI) and positive selected-ion monitoring (SIM). Target ions were monitored at $[M]^+$ *m/z* 330.2 for BCQB and $[M]^+$ *m/z* 344.2 for I.S. Calibration curve was linear over the range of 3–1500 ng/ml. The lower limit of quantification (LLOQ) was 3.0 ng/ml. The intra- and inter-run relative standard deviations (R.S.D.%) of the assay were less than 7.1 and 12.3%, respectively. The accuracy determined at the concentrations of 3.0, 100.0, 500.0 and 1500 ng/ml for BCQB were within ±15.0%. The established method has been applied successfully to study the pharmacokinetics of BCQB in rats after intranasal administration.

Keywords: Bencycloquidium bromide; LC–MS; Pharmacokinetics

1. Introduction

Bencycloquidium bromide (BCQB) (3-{(2-cyclopentyl-2-hydroxy-2-phenyl)ethoxy}-1-methyl-1-azabicyclo[2,2,2]octane bromide, see Fig. 1) is a novel anticholinergic compound and acts as an anticholinergic bronchodilator. It was developed by Beijing Shiqiao Biological and Pharmaceutical Co. Ltd. and granted a China Patent [1]. The pharmacological effect of BCQB is similar to ipratropium. However, it has no effect on cholinergic receptors of the central nerve system [1]. Muscarinic (M) receptor antagonists such as ipratropium have been used for many years in the treatment of chronic obstructive pulmonary disease (COPD) and asthma. The existence of several subtypes of muscarinic receptors in airways has suggested that more selective muscarinic antagonists might have advantages over the existing nonselective drugs, such as ipratropium bromide and oxitropium bromide [2,3]. The selectivity of BCQB for M1 and M3 cholinergic receptor subtypes has been confirmed and thus BCQB has no adverse effects such as tachycardia and uroschesis. BCQB is a perfect anti-rhinitis candidate drug for the treatment of chronic allergic rhinitis, chronic non-allergic rhinitis and acute rhinitis as a result of common cold [1]. Entrusted by Beijing Shiqiao Biological and Pharmaceutical Co. Ltd., we carried out the study to determine the concentration of BCQB in biological fluids and investigate the pharmacokinetics of BCQB in animals after intranasal administration.

The quinoline analogues containing quaternary ammonium group such as BCQB and acting as anticholinergics for blocking cholinergic receptors have not been reported [1]. BCQB is similar to ipratropium bromide and tiotropium bromide that has a quaternary ammonium group in its structure. Earlier publication has described a method for the analysis of tiotropium bromide and ipratropium bromide in plasma and urine samples of different animal species by LC–MS/MS [4]. In this paper, a new liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) method was established for pharmacokinetic study of BCQB using 1-ethyl-bencycloquidium bromide as the

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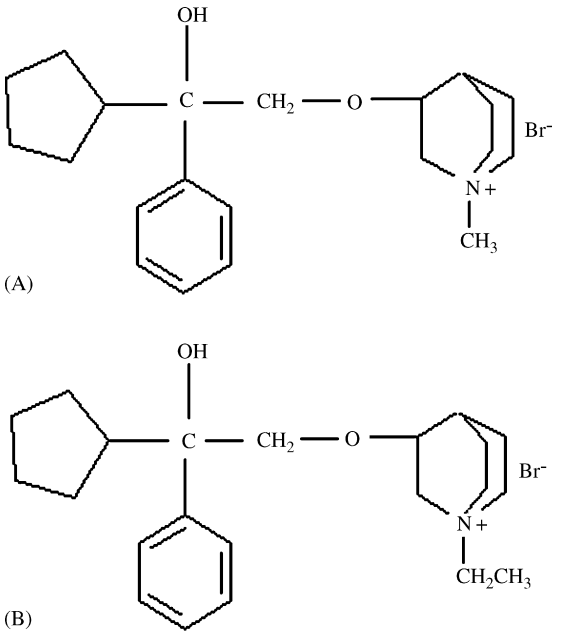

Fig. 1. Chemical structures of bencycloquidium bromide (A) and 1-ethyl-bencycloquidium bromide (B).

internal standard (I.S., see Fig. 1). The method was validated and successfully applied for the evaluation of pharmacokinetic profiles of BCQB in rats after intranasal administration of BCQB.

2. Experimental

2.1. Chemicals and reagents

The reference substance of BCQB (99.5% purity, Lot 031120) and the reference substance of I.S. (98.0% purity, Lot 041205) were supplied by Beijing Shiqiao Biological and Pharmaceutical Co. Ltd. (Beijing, China); BCQB for pharmacokinetic study was obtained from Beijing Shiqiao Biological and Pharmaceutical Co. Ltd.; methanol (HPLC grade) was purchased from Merck KGaA (Darmstadt, German). Ammonium acetate and formic acid were of HPLC grade, and purchased from Nanjing Chemical Reagent Co. Ltd. (Nanjing, China). Water was prepared with double distillation.

2.2. Instrumentation and conditions

An Agilent Technologies Series 1100 LC/MSD SL system (Agilent Technology, Palo Alto, CA, USA) was utilized for analysis, which included an Agilent 1100 G1312A binary pump, vacuum degasser (model G1322A), G1316A injection temperature controlled column compartment, Agilent 1100 autosampler (model G1313A), and an Agilent 1100 MSD single quadrupole mass spectrometer equipped with an electrospray source (model G1956B). Signal acquisition and peak integration were performed using the ChemStation Software (10.02 A) supplied by Agilent. A Hanbon Lichrospher 5-C18 column, 5 μm, 250 × 4.6 mm i.d. analytical column from Jiangsu Hanbon Science & Technology Co. Ltd. (Jiangsu, China) was used for analyte separation. The mobile phase was consisted of methanol–40 mM ammonium acetate buffer solution–formic acid (75:25:0.25; v/v/v). The mobile phase was delivered isocratically at a flow rate of 1.0 ml/min and the column temperature was maintained at 25 °C. The sample injection volume was 50 μl and the run time of each sample was 5 min. The MS system was operated in the positive ion mode. The MS conditions were as follows: a drying gas (N_2) flow 10 l/min, nebulizer pressure 45 psi, drying gas temperature 350 °C, capillary voltage 3000 V, fragmentor 150 V. Nitrogen was used as a nebulizing and drying gas. Selected-ion monitoring was accomplished at $[M]^+$ m/z 330.2 for BCQB and $[M]^+$ m/z 344.2 for I.S.

2.3. Preparation of standard solutions

The stock solutions of BCQB and the internal standard each with known concentration of 1 mg/ml were accurately prepared in methanol and stored at −20 °C. The stock solution was diluted consecutively with methanol to prepare a series of working solutions of 10 μg/ml, 1.0 μg/ml, 100 ng/ml and 10 ng/ml for BCQB. A solution of I.S. of 1.5 μg/ml was also prepared by diluting the I.S. stock solution with methanol. All the solutions were stored in refrigerator at −20 °C.

2.4. Sample preparation

All frozen standards and samples were allowed to thaw at room temperature and homogenized by vortexing. Aliquots of 30 μl of spiked sample standards, quality control samples (QC) or unknown plasma samples were placed into a 250 μl centrifuge tube, to which 10 μl of I.S. (1.5 μg/ml) was added. The sample mixture was mixed with 120 μl of methanol and vortex-mixed for approximate 3 min, then allowed to stand for 15 min to deproteinize and the precipitate was removed by centrifugation at 16,000 rpm for 5 min. Aliquots of 120 μl supernatant were transferred to injection vials, 30 μl aliquots of 40 mM ammonium acetate buffer solution containing 1% formic acid were added and then vortex-mixed. A 50 μl aliquot was injected into the LC–ESI-MS system.

2.5. Calibration curves

The calibration standards were prepared as follows: the proper amounts of each BCQB working solutions were separately added to eight 250 μl centrifuge tubes and evaporated to dryness under a gentle flow of nitrogen at room temperature. Then a 30 μl aliquot of blank plasma was added to each centrifuge tube and vortexed to result in calibration concentrations of 3.0, 10.0, 30.0, 100.0, 200.0, 500.0, 1000 and 1500 ng/ml for the calibration curve. The calibration curve was prepared and assayed along with quality control samples and each run of unknown plasma samples.

2.6. Preparation of quality control samples

QC samples were prepared at concentration levels of 3.0, 100.0, 500.0 and 1500 ng/ml for BCQB using the same method

of preparing the calibration standards and stored at −20 °C. QC samples were analyzed with processed test samples at intervals in each run. The results of the QC samples provided the basis of accepting or rejecting the run.

3. Method validation

3.1. Linearity and lower limited of quantification

Calibration standards of BCQB concentration levels at 3.0, 10.0, 30.0, 100.0, 200.0, 500.0, 1000 and 1500 ng/ml were prepared and assayed. The calibration curve was constructed by plotting the peak-area ratios of BCQB to the I.S. versus the concentrations of BCQB, using weighted least squares linear regression (weighting factor was 1/C). The LLOQ was defined as the lowest concentration on the calibration curve at which precision was within 20% and accuracy was within ±20% [5], and it was established using five samples independent of standards.

3.2. Precision and accuracy

Validation samples were prepared and analyzed on three separate runs to evaluate the accuracy, intra-run and inter-run precisions of the analytical method. The accuracy, intra-run and inter-run precisions of the method were determined by analyzing five replicates at 3.0, 100.0, 500.0 and 1500 ng/ml of BCQB along with one standard curve on each of three runs. Assay precision was calculated using the relative standard deviation (R.S.D.%). The accuracy is the degree of closeness of the determined value to the nominal or known true value under prescribed conditions. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage (RE%). It was calculated using the formula: $\mathrm{RE}\% = (E - T)/T \times 100$.

The accuracy of the assay was checked by preparation of QC samples at the start of the pharmacokinetic study. These QC samples were assayed along with unknown samples in each run to monitor the performance of the assay and to assess the integrity and validity of the result of the unknown plasma samples analyzed.

3.3. Extraction recovery

The extraction recoveries of BCQB were evaluated by analyzing five replicates of BCQB plasma samples at each concentration level of 3.0, 100.0, 500.0 and 1500 ng/ml, respectively. Recovery was calculated by comparing the peak areas of BCQB prepared in plasma with those obtained from direct injection of standards dissolved in the supernatant of the processed blank plasma.

3.4. Stability

Stability tests were performed to verify the stability of BCQB during handling procedures. Samples were assayed at the two QC concentrations of 3.0 and 1500 ng/ml for BCQB in triplicate. The long-term stability was performed at −20 °C in plasma for 21 days. The stability at ambient temperature was tested for 10 h. The samples were subjected to the three freeze–thaw cycles with each freeze cycle lasting at least 24 h. The post-preparative stability of processed samples under autosampler conditions was evaluated, where samples were prepared and placed in injection vials for 24 h at room temperature before injection into the LC–MS system. The stability of the stock solutions of BCQB and I.S. were determined by placing the stock solution at −20 °C for 1 month. The results were compared with the solutions freshly prepared.

3.5. Pharmacokinetic study

All studies in animals were in accordance with the guidelines for the Care and Use of Laboratory Animals in Jiangsu province, China. Sprague–Dawley rats (half male and half female, $n = 12$, 205–250 g, Grade II, Certificate No. 20020031) were purchased from the experimental animal center of Nanjing Medical University. The rats were fasted overnight before administration of drug with free access to water. For intranasal administration, the rats were anesthetized with ether lightly. Doses of 3.0 mg/kg BCQB in 0.1 ml of 0.9% saline were administered bilaterally through the nasal cavity using PVC tubing connected to a microliter syringe. A total volume of 20 μl of BCQB was administered for intranasal administration. Blood samples (0.1 ml) were collected from the tail vein in 0.25 ml sodium heparinized tubes before and 5, 15, 30, 60, 90, 120, 180, 300, 420, 540, 720 and 1440 min after intranasal administration of BCQB. Plasma was separated by centrifugation and stored at −20 °C until analysis. Aliquots of 30 μl plasma samples were processed and analyzed for BCQB concentration.

Pharmacokinetic parameters were determined using the plasma concentration–time data. The maximum plasma concentrations ($C_{\max}$) and the time to those ($t_{\max}$) were noted directly. The elimination rate constant (k_e) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated from the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration–time curve from zero to the last measurable plasma concentration (AUC_{0-t}) was calculated by the linear trapezoidal method. The area under the plasma concentration–time curve to time infinity ($\mathrm{AUC}_{0-\infty}$) was calculated as follows: $\mathrm{AUC}_{0-\infty} = \mathrm{AUC}_{0-t} + C_t/k_e$, where C_t was the last measurable plasma concentration and k_e was the elimination rate constant.

4. Results and discussion

4.1. Conditions for ESI-MS

BCQB is a surfactant because of a highly polar quaternary ammonium and the hydrophobic groups in its structure. The electrospray ionization is hypothesized as a surface-ionization technique in which the highly surface active chemical compounds such as BCQB always occupy the surfaces of the spraying droplets and has priority to form gas phase ions than others. Thus, high sensitivity would be observed for compounds with

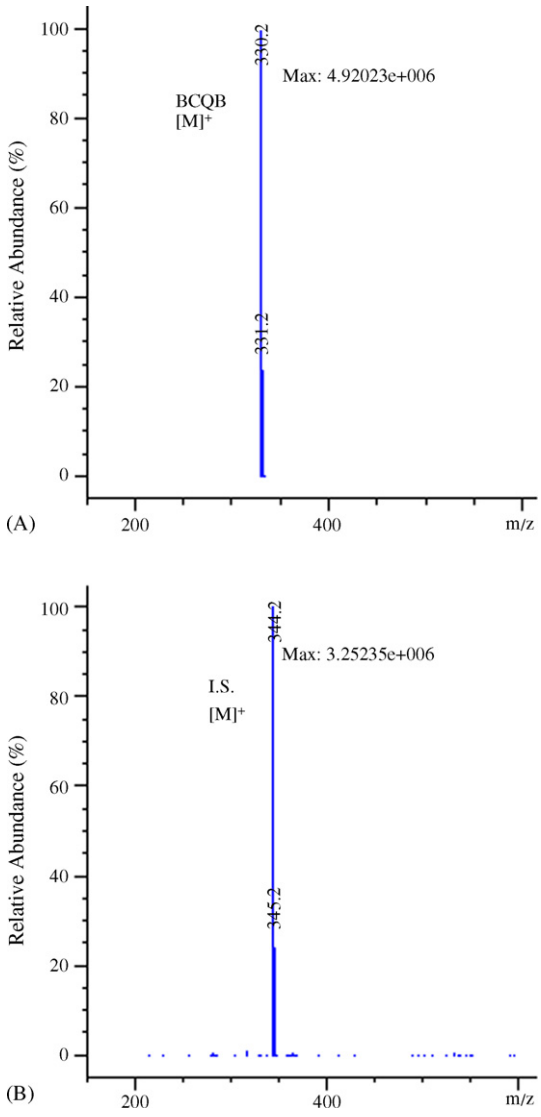

Fig. 2. Mass spectra of the positive ion of BCQB (A) and I.S. (B) at 150 V fragmentor voltage.

high surface activity. Consequently, the quaternary ammonium compound BCQB offers abundant ions in electrospray ionization. Those factors are beneficial to increasing the sensitivity of BCQB. Therefore, ESI in positive ion mode was adopted for the assay of BCQB. Fig. 2 shows a typical full-scan ESI mass spectrum of BCQB. ESI produced abundant molecular ions of $[M]^+$ at *m/z* 330.2 for BCQB in the scan mode. By monitoring this ion, a highly sensitive assay for BCQB was developed. The intensity of the ion of BCQB at *m/z* 330.2 was compared at fragmentor voltages of 30, 50, 70, 90, 110, 130, 150, 170 and 200 V in order to determine the optimal collision energy. The result showed that the highest sensitivity was obtained by using a fragmentor voltage of 150 V. Therefore, a fragmentor voltage of 150 V was used to carry out LC–ESI-MS in the scan mode. At this collision energy the most intensive ion of I.S. was the molecular ion $[M]^+$ at *m/z* 344.2 (Fig. 2). Therefore, the positive molecular ion at $[M]^+$ *m/z* 344.2 was selected as the target ion of I.S. in the SIM.

4.2. Chromatography

Selection of mobile phase components was also a critical factor. Because phosphate buffer cannot be used with MS, we employed ammonium acetate to supply the ionic strength. Increasing the percentage of buffer in the mobile phase enhanced analyte peak symmetry and resolution. The concentrations of ammonium acetate buffer ranged from 10 to 40 mM were investigated. The mobile phase of 10 mM ammonium acetate buffer yielded tailing peaks. We increased the concentration of ammonium acetate buffer to 40 mM, the chromatographic peaks became sharp and symmetrical. The ionization of samples at the LC–MS interface is affected by the mobile phase. Hence, a mobile phase containing a volatile acid or salt is used frequently. In this case, the responses were measured using 0.2, 0.5, 1 and 2% formic acid in aqueous phase. The response to BCQB was maximal by addition of 1% formic acid to the mobile phase. Finally, the acceptable retention and separation of BCQB were obtained by using an elution system of 40 mM ammonium acetate buffer–methanol–formic acid (25:75:0.25, v/v/v) as the mobile phase. Under the present chromatographic conditions, the run time of each sample was 5.0 min. The retention times were 3.88 and 3.97 min for BCQB and I.S., respectively. Representative selected-ion chromatograms are shown in Fig. 3.

4.3. Preparation of plasma samples

Sample preparation is a critical step for accurate and reliable LC–MS–MS assays. The most widely employed biological sample preparation methodologies currently are liquid–liquid extraction (LLE), protein precipitation (PPT) and solid-phase extraction (SPE). BCQB is a highly polar compound because of a quaternary ammonium in its structure, which makes it difficult to be extracted from plasma by LLE. The extraction recoveries were below 20% whether ethyl acetate, diethyl ether or cyclohexane used as the extraction solvents. BCQB also had a low extraction recovery below 50% using SDS as ion-pair reagent and ethyl acetate as extraction solvent. PPT often provided higher recovery compared with LLE for those compounds of high polarity. Thus, the BCQB plasma samples were prepared by protein precipitation procedure. Three kinds of precipitation reagents (methanol, acetonitrile and ethanol) were investigated during the experiment. In the case of acetonitrile and ethanol as precipitation reagents, when the supernatant of prepared samples was directly injected into the LC–MS system, the results showed peak fronting of the BCQB and I.S. By using methanol as a precipitation reagent sharp peak shape and higher extraction recovery of BCQB were obtained. In order to obtain symmetric chromatogram, a 30 μl aliquot of 40 mM ammonium acetate buffer solution containing 1% formic acid was added to supernatant of the treated plasma samples.

4.4. Method validation

4.4.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma with the corresponding

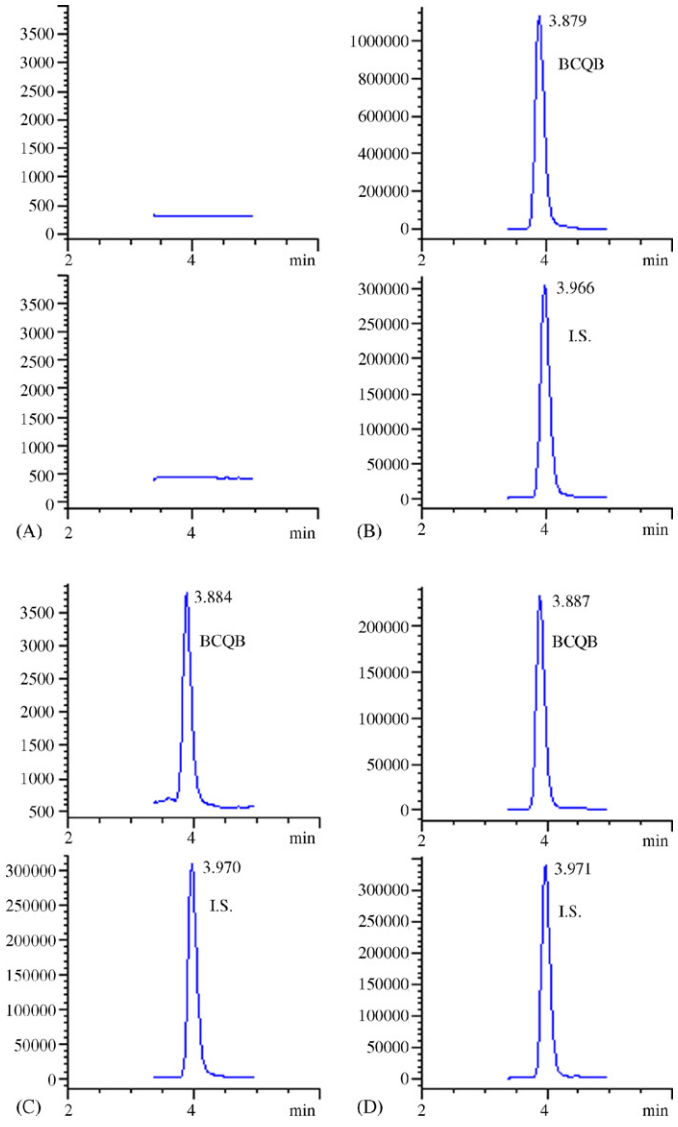

Fig. 3. Typical SIM chromatograms of blank plasma (A), the highest concentration of BCQB in plasma (1500 ng/ml) (B), LLOQ for BCQB in plasma (3.0 ng/ml) and I.S. (C), plasma obtained from a rat at 30 min after intranasal administration of 3.0 mg/kg BCQB, the plasma concentration of BCQB was estimated to be 267.4 ng/ml (D).

spiked plasma. Fig. 3 showed the typical chromatograms of a blank, a spiked plasma sample with BCQB and I.S. and a plasma sample from a rat 30 min after an intranasal administration of BCQB. Interferences from the matrices at the expected retention times of the target ions were not observed and typical retention times for BCQB and I.S. were 3.88 and 3.97 min, respectively. With short analysis time the interferences with metabolites are a potential issue and should be investigated. BCQB is a novel compound and its metabolites have not been reported before. Recently, the metabolites of BCQB have been investigated in our laboratory. The result shows that the major metabolites of BCQB are its monohydroxylated and bishydroxylated metabolites (phase I metabolites) and the glucuronides and sulfates of its monohydroxylated and bishydroxylated metabolites (phase II metabolites). The retention times of these metabolites were shorter than BCQB in our chromatographic conditions for determination of BCQB in rat plasma. There was no interference from the metabolites observed at the retention times of the BCQB and I.S.

Table 1
Matrix effect data for BCQB at 3.0, 100.0, 500.0 and 1500 ng/ml in six batches of rat plasma ($n = 6$)

Concentration of BCQB (ng/ml)	ME (mean ± S.D., %)
3.0	94.2 ± 9.2
100.0	97.4 ± 6.2
500.0	98.6 ± 5.1
1500	101.3 ± 3.1

4.4.2. Matrix effect

To evaluate the matrix effect, i.e., the potential ion suppression or enhancement due to co-eluting matrix components, six different batches of blank plasma were precipitated by methanol and then spiked with the analyte at four concentrations of 3.0, 100.0, 500.0 and 1500 ng/ml. The corresponding peak areas of the analytes in spiked plasma post-extraction (A) were then compared to those of the standards in mobile phase (B) at equivalent concentrations. The ratio ($A/B \times 100$) was defined as the ME. A ME value of 100% indicated that the responses for analytes in the mobile phase and in the plasma extracts were the same and that no ME was observed. A value of >100% indicated ionization enhancement, and a value of <100% indicated ionization suppression. The ME data at the four concentrations of BCQB in six different batches of rat plasma are presented in Table 1. The results showed there was no ME in this study.

4.4.3. Linearity of calibration curves and lower limits of quantification

Visual inspection of the plotted duplicate calibration curves and correlation coefficients >0.99 confirmed that the calibration curves were linear over the concentration ranges of 3.0–1500 ng/ml for the BCQB. Typical standard curve was $f = 0.002999 + 0.002823C$, where f represented the BCQB to I.S. peak area ratio and C represented the plasma concentration of BCQB.

The present LC–ESI-MS method offered a LLOQ of 3.0 ng/ml in 30 μl plasma sample. Under present LLOQ of 3.0 ng/ml, the BCQB concentration could be determined in plasma samples until 24 h after a single intranasal administration of 3.0 mg/kg BCQB, which was sensitive enough to investigate the pharmacokinetic behaviors of BCQB.

4.4.4. Precision and accuracy

BCQB plasma samples at four concentration levels of 3.0, 100.0, 500.0 and 1500 ng/ml were analyzed for accuracy and precision. The data obtained for BCQB is shown in Table 2. The precision was calculated by using one-way-ANOVA [6]. For the four concentration levels of BCQB, the intra-run precision was less than 7.1%, the inter-run precision was less than 12.3% and the accuracy was within ±15.0%. The data obtained for BCQB was within the acceptable limits to meet the guidelines for bioanalytical methods.

Table 2
Precision and accuracy of the assay for determination of BCQB in plasma ($n = 3$ runs, five replicates per run)

Spiked concentration (ng/ml)	Mean found concentration (ng/ml)	RE (%)	Intra-assay R.S.D.%	Inter-assay R.S.D.%
3.0	2.639	−10.9	7.1	12.3
100.0	98.89	−1.1	3.5	6.4
500.0	489.2	−2.2	2.4	5.1
1500	1496	−0.3	2.0	2.7

Table 3
Recovery data of BCQB in rat plasma ($n = 5$)

Spiked concentration (ng/ml)	Mean found concentration (ng/ml)	R.S.D. (%)	Extraction recovery (%)
3.0	2.745	7.4	91.2
100.0	92.90	4.2	92.9
500.0	478.5	3.4	95.7
1500	1457	1.1	97.1

4.4.5. Recovery

The mean extraction recoveries were measured at four different concentration levels for BCQB (3.0, 100.0, 500.0 and 1500 ng/ml) by comparing the peak areas of BCQB prepared in plasma with those obtained from direct injection of standards dissolved in the supernatant of the processed blank plasma. The data of recovery obtained are shown in Table 3.

4.4.6. Stability

The results of stability experiments showed that no significant degradation occurred at ambient temperature for 10 h and during the three freeze–thaw cycles for BCQB plasma samples. The accuracy values of low (3.0 ng/ml) and high (1500 ng/ml) concentration of BCQB in rat plasma were 97.7 and 98.0% after three freeze–thaw cycles, and 103.2 and 98.3% at −20 °C for 21 days. The post-preparative stability test showed that the prepared samples under autosampler conditions were stable for 24 h at least before injection into the LC–MS system. The stock solutions of BCQB and I.S. in methanol were shown to remain stable for 1 month at −20 °C.

4.4.7. Pharmacokinetic studies

The described method was successfully applied to a pharmacokinetic study in rats. The mean plasma concentration–time curve of BCQB is shown in Fig. 4. The main pharmacokinetic parameters of BCQB were calculated. After intranasal administration of 3.0 mg/kg BCQB, the mean values of T_{max} and C_{max} were 0.2 h (range 0.08–0.25 h) and 312.5 ng/ml (range 116.1–596.2 ng/ml), respectively. The elimination half-life of BCQB was 11.7 ± 7.1 h. The AUC_{0-t} and $AUC_{0-\infty}$ values obtained were 373.6 ± 175.4 and 429.0 ± 186.1 μg h/ml, respectively. The result indicated that BCQB was absorbed rapidly and eliminated slowly in rats.

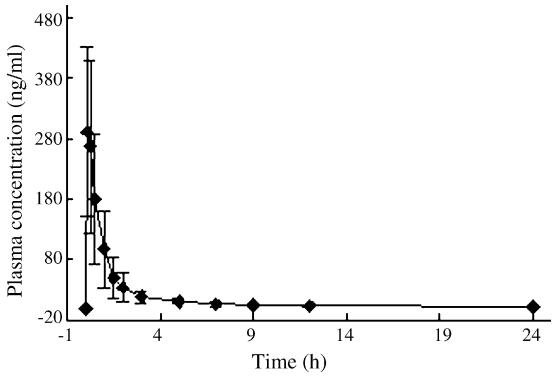

Fig. 4. Mean plasma concentration–time profile of ECQB after intranasal administration of BCQB 3.0 mg/kg in rats.

5. Conclusion

A sensitive LC–MS–MS method for the quantification of BCQB in rat plasma was firstly established and developed for pharmacokinetic study of BCQB in rats after intranasal administration. The pharmaceutical parameters of intranasal administration of BCQB in rats were obtained for the first time. The assay used 1-ethyl-bencycloquidium bromide as internal standard. The results of validation have shown that the method is rapid, sensitive and accurate. This LC–ESI-MS method required only 30 μl of plasma with a LLOQ of 3.0 ng/ml for BCQB and the determination of one plasma sample needed only 5 min. These results indicated that it was suitable for routine analysis of large number of biological samples.

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