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Determination of bencycloquidium bromide in human urine using weak cation-exchange solid-phase extraction and LC–ESI-MS: Method validation and application to kinetic study of urinary excretion

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ABSTRACT

A sensitive LC–ESI-MS method has been developed and validated for the determination of bencycloquidium bromide (BCQB) in human urine samples. The method utilized a solid-phase extraction (SPE) procedure, choosing carboxy propyl phase (CBA) as the extracting sorbent for purification of BCQB, with better baseline and higher selectivity achieved. Sample preparation by this method yielded very good and consistent mean recovery of above 94.5%. Another major benefit of the present method was the high detectability, with a lower limit of quantification (LLOQ) of 0.02 ng/ml. The developed method was successfully applied to determine BCQB in human urine, and was proved to be suitable for use in Phase I clinical pharmacokinetic study of BCQB.

1. Introduction

Bencycloquidium bromide, chemically known as 3-{(2-cyclopentyl-2-hydroxy-2-phenyl) ethoxy}-1-methyl-1-azabicyclo [2,2,2] otane bromide, is a quaternary ammonium compound with anticholinergic properties. In a series of preclinical pharmacologic studies, BCQB was shown to be a potent muscarinic receptor antagonist, with a significant therapeutic effect on hypersensitive rhinitis, allergic rhinitis, anti-inflammation and anti-pruritus [1–3]. BCQB was developed to reduce the occurrence of side effects associated with systemic absorption from such compounds as atropine. BCQB is poorly absorbed across lipid membranes. Therefore, it minimally crosses the nasal and gastrointestinal membrane and the blood–brain barrier, resulting in a reduction of the systemic anticholinergic effects that are seen with tertiary anticholinergic amines [4].

Recently, we reported several LC–ESI-MS methods for determination of BCQB in rat urine, plasma and tissues, in which the LLOQ was 3 ng/ml [5–8]. Those methods were not appropriate for the pharmacokinetic study in human, in which a 45-μg nasal dose of BCQB was suggested for nasal administration to healthy volunteers. In the present work, a more reliable method was developed for determining BCQB in human urine using weak cation-exchange (WCX) solid-phase extraction (SPE) and LC–ESI-MS techniques. The method achieved an LLOQ as low as 0.02 ng/ml, and has been applied to evaluate the kinetics of urinary excretion of BCQB in humans.

2. Experimental

2.1. Chemicals and reagents

Both BCQB nasal spray and the reference substances of BCQB (99.7% purity, see Fig. 1(A)) and 1-ethyl-bencycloquidium bromide (99.9% purity, used as internal standard (I.S.), see Fig. 1(B)) were supplied by Beijing Shiqiao Biological and Pharmaceutical Co. Ltd. (Beijing, China). Methanol of HPLC grade was purchased from Merck KGaA (Darmstadt, German). Ammonium acetate, acetic acid, formic acid and ammonia water were of analytical grade, and purchased from Nanjing Chemical Reagent Co. Ltd. (Nanjing, China).

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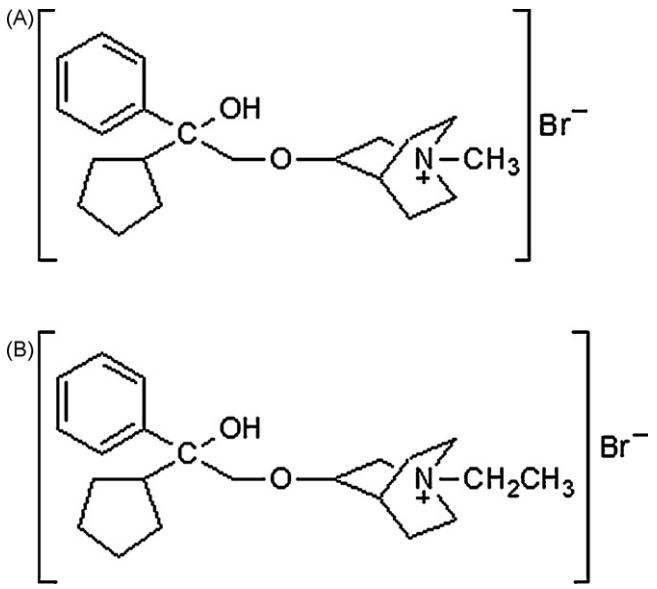

Fig. 1. Chemical structures of BCQB (A) and 1-ethyl-bencycloquidium bromide (B).

2.2. Instrumentation

The HPLC–ESI-MS methods were performed using an Agilent Technologies Series 1100 LC/MSD SL system (Agilent Technologies, Palo Alto, CA, USA), 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). The signal acquisition, peak integration and concentration determination were performed using the ChemStation software (10.02 A) supplied by Agilent Technologies. The solid-phase extraction of urine samples was carried out using a 12-position Visiprep™ DL manifold (Supelco, USA) and the Cleanert™ COOH SPE Tubes (100 mg/1 ml) from Agela Technologies (Beijing, China).

2.3. HPLC–ESI-MS conditions

A Zorbax Eclipse Plus C18 column, 3.5 μm, 150 mm × 2.1 mm i.d. (Agilent Technologies, Palo Alto, CA, USA) was used for the separation. The mobile phase was 20 mM ammonium acetate buffer solution containing 1% acetic acid–methanol (55:45, v/v) at a flow rate of 0.3 ml/min. The column temperature was maintained at 38 °C. The HPLC–ESI-MS method was carried out using nitrogen to assist nebulization. The quadrupole mass spectrometer equipped with an ESI source was set with the drying gas (N_2) flow of 10 L/min, nebulizer pressure of 40 psig, drying gas temperature of 350 °C, capillary voltage of 4.0 kV and the positive ion mode. The fragmentor voltage was 170 V. The ESI-MS was performed in the SIM mode using the target ions $[M-Br]^+$ at m/z 330.3 for BCQB and m/z 344.3 for the I.S.

2.4. Preparation of standard solutions

The stock solution of BCQB with a concentration of 1.0 mg/ml was prepared by dissolving BCQB in methanol. A series of standard working solutions with concentrations in the range of 1.0 ng/ml to 10 μg/ml for BCQB were obtained by further dilution of the stock solution with methanol. The I.S. working solution (2.0 ng/ml) was prepared by diluting the stock solution of 1-ethyl-bencycloquidium bromide with methanol. All the solutions were stored at −20 °C and were brought to room temperature before use.

2.5. Sample preparation

BCQB urine samples were prepared by spiking 100 μl of the I.S. solution (2 ng/ml) into 1 ml of urine sample. Then the urine samples were vortex mixed and centrifuged at 16,000 rpm for 3 min. Extraction cartridges were conditioned with 2 ml methanol followed by 1 ml water and 2 ml ammonium acetate buffer (pH 7.0; 20 mM). Each urine sample was dropwisely loaded onto the conditioned cartridge. It was then washed with 2 ml ammonium acetate buffer (pH 7.0; 20 mM) followed by 2 ml methanol. Finally, each cartridge was eluted with 2 ml mixture of methanol–formic acid (98:2, v/v). The eluate was evaporated to dryness under nitrogen at 45 °C and the residue was dissolved in 200 μl mobile phase. The reconstituted solution was transferred to an autosampler vial and an aliquot of 2 μl was injected into the chromatographic system.

2.6. Preparation of calibration curves and quality control samples

The calibration standards of BCQB were prepared by spiking appropriate amount of BCQB working solutions in 1.0 ml blank urine obtained from healthy volunteers. The standard curve was prepared for BCQB at concentration levels of 0.02, 0.1, 0.5, 2.0, 10, 30, 70, 120 ng/ml. And those standard urine samples were prepared according to the procedures described in Section 2.5. The calibration curve was prepared and assayed along with quality control (QC) samples. The QC samples for BCQB were prepared in 1.0 ml blank urine at concentration levels of 0.05, 3 and 100 ng/ml. The QC samples were assayed along with clinical samples to monitor the performance of the assay and to assess the integrity and validity of the result of the unknown clinical samples analyzed.

2.7. Assay validation [9]

2.7.1. Selectivity

The selectivity was assessed by comparing the chromatograms of six different blank human urines with the corresponding spiked urine. Each blank urine sample was tested using the proposed extraction procedure and the HPLC–ESI-MS conditions to ensure no interference of BCQB and the I.S. from the urine.

2.7.2. Linearity of calibration curves and lower limit of quantification

The linearity was assessed by assaying calibration curves in human urine. Calibration curves of eight concentrations of BCQB from 0.02 to 120 ng/ml were extracted and assayed. In addition, blank urine samples were run to discard the presence of interferences. The calibration curves were fitted by a weighted least squares linear regression method (weighting factor was $1/C^2$) through the measurement of the peak-area ratio of BCQB to the I.S. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value.

The lower limit of quantification (LLOQ) is the lowest concentration of the standard curve that could be measured with acceptable accuracy and precision. It was determined in five replicates. The precision should be equal or less than 20% and accuracy between 80% and 120% of the nominal concentration.

2.7.3. Precision and accuracy

Validation samples were prepared and analyzed on three separate runs which were performed on 3 consecutive days to evaluate the accuracy, intra- and inter-run precisions of the analytical method. The accuracy, intra- and inter-run precisions of the method were determined by analyzing five replicates at 0.05, 3 and

100 ng/ml of BCQB along with one standard curve on each of three runs. The assay precision was calculated by using the relative standard deviation (R.S.D.) and a one-way analysis of variance (ANOVA) [10]. It separates out the sources of variance due to within- and between-run factors.

The accuracy is the degree of closeness of the determined value to the nominal 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 (R.E.%). It was calculated using the formula: R.E.% = $(E - T)/T \times 100$. The intra- and inter-day precisions were required to be less than 15%, and the accuracy to be within ±15%.

2.7.4. Extraction recovery

The extraction recovery of BCQB was estimated at concentration levels of 0.05, 3 and 100 ng/ml by comparing two groups of control samples: (A) BCQB spiked after extraction of blank urine (post-extraction). (B) BCQB spiked to urine and prepared normally (pre-extraction). Extraction recovery was calculated as the response ratio of B/A.

2.7.5. Matrix effects

The matrix effect of the method for BCQB was evaluated at concentration levels of 0.05, 3 and 100 ng/ml. Five samples at each concentration level of the analytes were analyzed. The matrix effect (M.E.) was examined by comparing the peak areas of the analytes between two different sets of samples. In set 1, analytes were dissolved in the mobile phase, and the obtained peak areas of analytes were defined as A. In set 2, analytes were added to the reconstructed solutions of the blank urine samples which originated from five different donors and were submitted to the sample purification process, and the obtained peak areas of analytes were defined as B. The M.E. values was calculated by using the formula: M.E. (%) = B/A × 100. The M.E. value of the I.S. (0.2 ng/ml) was also evaluated. The inter-subject variability of matrix effect at every concentration level should be less than 15%.

2.7.6. Stability

The stability tests of the analytes were designed to cover anticipated conditions of handling of the clinical samples. The stability of BCQB was studied under a variety of storage and handling conditions.

2.7.6.1. Stock solution stability. The stability of stock solutions of BCQB and the I.S. was evaluated at room temperature for 4 h as well as in a refrigerator (−20 °C) for 10 days and 6 weeks. After completion of the desired storage time, the stability was tested by comparing the peak areas with that of freshly prepared solutions.

2.7.6.2. Freeze and thaw stability. The QC samples were stored at −20 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12 h under the same conditions. The freeze–thaw cycles were repeated three times, and then analyzed on the third cycle.

2.7.6.3. Short-term temperature stability. The QC samples at three concentration levels were kept at room temperature for a period that exceeded the routine preparation time of the samples (around 8 h).

2.7.6.4. Long-term stability. The QC samples at three concentration levels kept at low temperature (−20 °C) were studied for a period of 5 weeks and 11 weeks.

2.7.6.5. Post-preparative stability. The post-preparative stability (after extraction, in the mobile phase) was conducted by reanalyzing extracted QC samples kept under autosampler conditions (8 °C) for 15 h.

Following above treatments, those samples were analyzed with calibration curves obtained from newly prepared standards and the results were compared with those of obtained from the samples immediately processed at 0 h. The analytes are considered to be stable when the values of the precisions are less than 15% and the accuracies are in the range of 85–115%, respectively, for all QC levels.

2.7.7. Application

The developed HPLC–ESI-MS method was applied to determine the urine concentrations of BCQB from the Phase I clinical trial study in which 10 healthy volunteers were enrolled. The pharmacokinetic study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki. They all nasally administered a 45-μg dose of BCQB. The urine samples were collected pre-dose and over the intervals of 0–2, 2–4, 4–6, 6–8, 8–12, 12–24, 24–36, 36–48, 48–60, 60–72 h post-dose. The volume of urine collected in each interval was recorded and 5 ml of it was frozen and stored at −20 °C before analysis.

3. Results and discussion

3.1. Optimization of the mass spectrometric condition

Due to the presence of a basic quaternary amine in the molecule, BCQB exhibited favorable sensitivity in positive ion mode detection. Electrospray ionization (ESI) was found to be more sensitive than atmospheric pressure chemical ionization. In order to select the target ion for monitoring BCQB, the ESI mass spectra of BCQB at different fragmentor voltage obtained by the scan-monitoring mode were investigated. The results showed that the base peak (the highest ion peak in the mass spectrum) in the mass spectra of BCQB obtained at different fragmentor voltage was of the same ion at m/z 330.3, therefore, the target ion $[M-Br]^+$ at m/z 330.3 was selected as the target ion for BCQB in the SIM. In order to achieve the highest assay sensitivity for BCQB, the optimal fragmentor voltage of the ESI-MS was investigated. The intensity of the ion of BCQB at m/z 330.3 was compared at the different fragmentor voltages of 90, 110, 130, 150, 170, 190 and 210 V. The result showed that the highest sensitivity was obtained by using a fragmentor voltage of 170 V. Therefore the fragmentor voltage was set at 170 V in the ESI-MS assay for BCQB. At this fragmentor voltage, the base peak in the mass spectra of the I.S. was at m/z 344.3. Therefore, the positive ions at m/z 344.3 was selected as the target ion for the I.S.

3.2. Optimization of the chromatographic condition

1-Ethyl-bencycloquidium bromide was chosen as the I.S. because it is structurally similar to BCQB, and performed with similar retention to BCQB. Due to that BCQB and the I.S. both appear as tailing peaks in their chromatograms on the normal C18 columns, a Zorbax Eclipse Plus C18 column (3.5 μm, 150 mm × 2.1 mm i.d.), which could achieve superior peak shape with basic compounds, was used to resolve the tailing phenomena of chromatographic peaks of the analytes [11]. The selection of the mobile phase components was also critical in achieving good chromatographic peak shape and resolution. An ammonium acetate buffer solution was adopted in the mobile phase, the different concentrations of ammonium acetate buffer solution at levels of 10, 20, 30, 40 mM were tested to improve the chromatographic peak shapes of the analytes. The test results showed that the mobile phase of 20 mM ammonium acetate solution could sufficiently resolve the tailing problem and

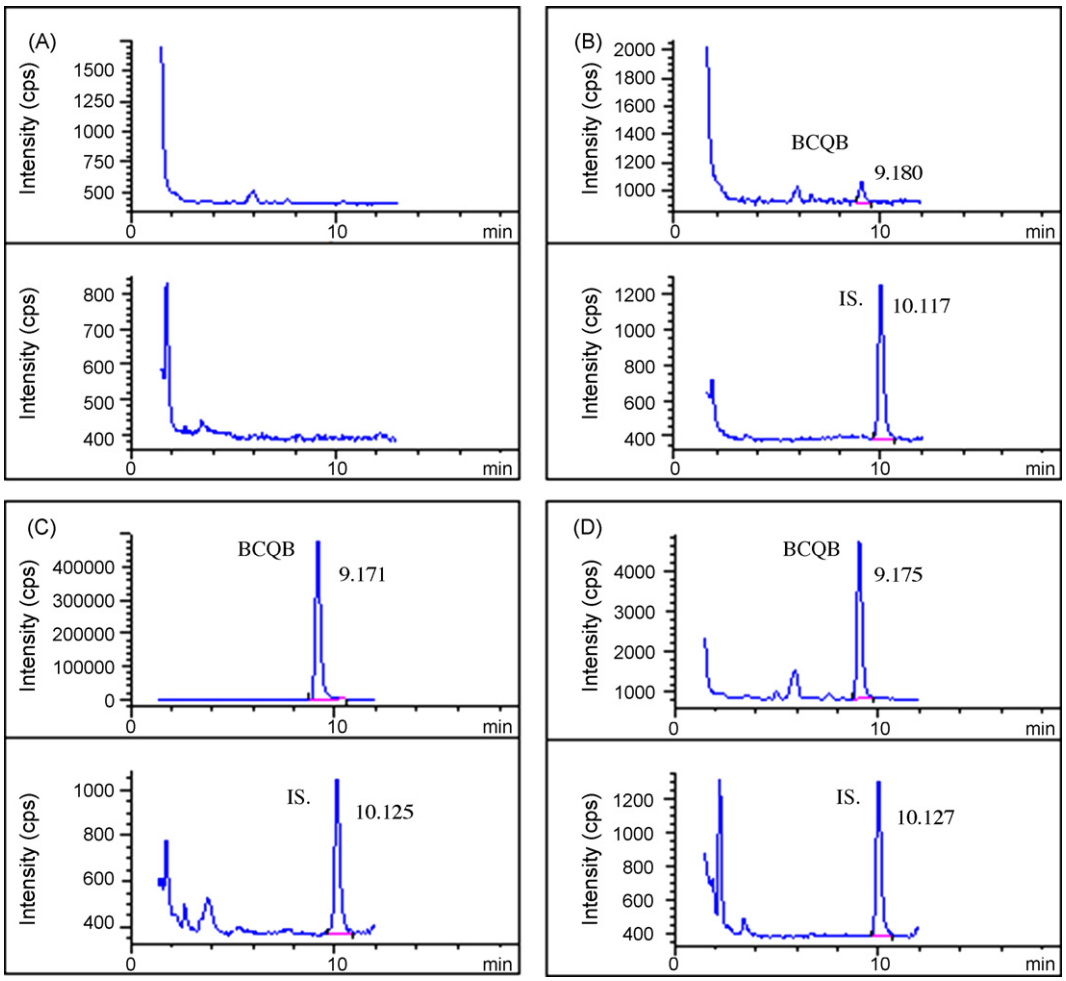

Fig. 2. Typical SIM chromatograms of blank urine (A), LLOQ for BCQB in urine (0.02 ng/ml) and I.S. (B), urine spiked with BCQB (120 ng/ml) and the I.S. (C), urine obtained from a volunteer at 24 h after a nasal administration of 45 μg BCQB, the urine concentration of BCQB was estimated to be 0.7689 ng/ml (D).

made the chromatographic peaks sharp and symmetric. Using a low pH mobile phase often results in the best peak shape for basic compounds on silica-based columns [11]. Further experiment results showed that acidifying the mobile phase with acetic acid could not only improve peak shapes of BCQB and the I.S., but also increase the MS sensitivity to the analytes. Therefore, a concentration of 1% acetic acid was finally added into the ammonium acetate solution of the mobile phase. The acceptable retention and separation of BCQB was obtained by using an elution system of 20 mM ammonium acetate buffer solution containing 1% acetic acid–methanol (55:45, v/v) as the mobile phase. Typical retention times were about 9.1 min for BCQB and 10.1 min for the I.S.

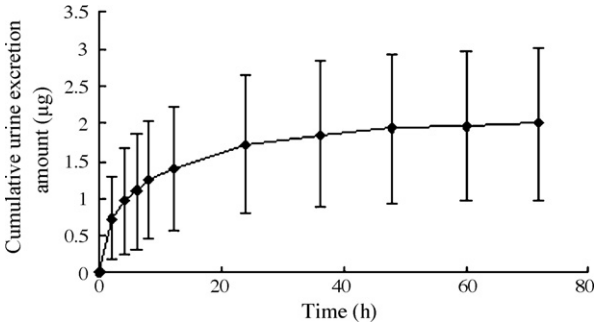

Fig. 3. Mean cumulative urine excretion amount–time curve of BCQB in 10 healthy volunteers after a single nasal administration of 45 μg BCQB ($n = 10$).

3.3. Sample preparation

Sample preparation is a critical step for accurate and reliable LC–MS assays. The widely employed biological sample preparation techniques are liquid–liquid extraction (LLE), protein precipitation (PPT) and solid-phase extraction (SPE). In the early stage of method development, a LLE method was employed in the sample preparation, however, resulted in poor recoveries. In order to overcome these problems, a solid-phase extraction (SPE) method was developed to separate and concentrate BCQB from the urine matrix. The WCX SPE cartridges were demonstrated to have much merit in comparison with the C18 and strong cation-exchange (SCX) cartridges. Compared with the C18 SPE and SCX SPE, the WCX SPE could remove almost all the matrix interferences in urine from BCQB. The WCX SPE cartridges applied in this method contains an aliphatic carboxylic acid (carboxypropyl acid, CBA) group that is bonded to the silica surface [12]. CBA is a weak acid, with a pK_a of about 4.8, and thus the carboxypropyl acid functional group does

Table 1
Precision and accuracy of the assay for the determination of BCQB in human urine ($n = 3$ runs, five replicates per run).

Concentration levels (ng/ml)		R.S.D. (%)		R.E. (%)
Added	Found	Intra-run	Inter-run	
0.051	0.049 ± 0.004	7.3	13.1	−3.9
3.045	3.017 ± 0.079	2.2	4.4	−0.9
101.5	100.4 ± 4.2	3.8	5.8	−1.1

Table 2
Stability data of BCQB in human urine under various storage conditions ($n = 3$).

Storage conditions	Added C (ng/ml)	Found C (ng/ml)	R.S.D. (%)	R.E. (%)
Room temperature for 8 h	0.051	0.051 ± 0.004	7.1	1.1
	3.045	2.950 ± 0.069	2.4	−3.1
	101.5	99.16 ± 4.18	4.2	−2.3
Three freeze–thaw cycles	0.051	0.052 ± 0.003	5.3	2.5
	3.045	3.042 ± 0.071	2.3	−0.1
	101.5	101.4 ± 3.5	3.4	−0.1
Freezing for 5 weeks (−20 °C)	0.051	0.051 ± 0.003	6.5	0.0
	3.045	3.016 ± 0.032	1.0	−1.0
	101.5	109.8 ± 2.8	2.5	8.1
Freezing for 11 weeks (−20 °C)	0.051	0.051 ± 0.003	5.9	−0.2
	3.045	3.109 ± 0.050	1.6	2.1
	101.5	109.5 ± 1.1	1.0	7.8
Autosampler for 15 h (8 °C)	0.051	0.050 ± 0.004	8.4	−2.2
	3.045	3.037 ± 0.023	0.8	−0.3
	101.5	102.2 ± 3.2	3.1	0.7

not require extreme basic conditions for elution that are required by other cation-exchange sorbents. For this reason, WCX SPE is often the best choice for the extraction of the analytes with a permanent positive charge, such as quaternary amines, which cannot be neutralized by pH control.

The degree of the retention of the analytes on the WCX ion exchange SPE cartridges depends mainly on the pH values of the samples and the conditioning solutions [12]. To ensure the total ionization of the sorbent is maintained during loading, the condition solution should be adjusted to pH 6.8 or higher (2 pH units above the pK_a of the sorbent). Buffering for pH control should be performed with the lowest strength buffer. As a result, an ammonium acetate buffer (pH 7.0; 20 mM) was chosen as the conditioning solution to make the majority of carboxypropyl acid groups carry negative charges to retain BCQB. Carboxypropyl acid functional group on the silica surface can be neutralized at lower pH values (2 pH units below the pK_a of the sorbent), and the test results showed that BCQB could be eluted with the mixture of methanol–formic acid (98:2, v/v).

3.4. Assay validation

3.4.1. Selectivity

Fig. 2 shows the typical chromatograms of a blank urine sample, a spiked urine sample with BCQB at the LLOQ of 0.02 ng/ml and the I.S., a spiked urine sample of 120 ng/ml and the I.S., and a urine sample from a healthy volunteer at 24 h after nasal administration of 45 μg BCQB. Typical retention times for BCQB and the I.S. were 9.1 and 10.1 min, respectively. There were no significant endogenous interferences observed at the retention times of the analyte and the I.S.

3.4.2. Linearity of calibration curves and lower limit of quantification

The calibration curve was linear over the range of 0.02–120 ng/ml in human urine with coefficient of correlation (r) > 0.999. The regression equation was as follow: $f = 0.08581 + 5.031 \times C$, where f represents the peak-area ratio of analyte to the I.S., and C represents the urine concentration of BCQB. The present LC–ESI-MS method offered an LLOQ of 0.02 ng/ml, which was established using five samples independent of calibrator standards. At 0.02 ng/ml, the R.S.D.% was 9.3% ($n = 5$), and the R.E.% ranged from −14.3% to 3.4%.

3.4.3. Assay precision and accuracy

Table 1 summarized the intra- and inter-run precision and accuracy for BCQB evaluated by assaying the QC samples. The intra- and inter-run precisions were measured to be less than 7.3% and 13.1%, respectively. The accuracy of the method presented as R.E. (%) was from −0.9% to −3.9%.

3.4.4. Extraction recovery

The WCX SPE was chosen as the extraction method for its higher extraction efficiency to the target compounds. The recovery of BCQB, determined at three concentration levels of 0.05, 3 and 100 ng/ml were 94.5 ± 2.9%, 95.8 ± 2.5% and 95.8 ± 1.5% ($n = 5$), respectively.

3.4.5. Matrix effects

The matrix effects for BCQB at concentration levels of 0.05, 3 and 100 ng/ml were 96.9 ± 2.0%, 101.4 ± 0.4% and 100.9 ± 1.0%, respectively. The matrix effects for the I.S. (0.2 ng/ml in urine) were 99.0 ± 3.1%. These results showed that ion suppression or enhancement from urine matrix was negligible in the present condition.

3.4.6. Stability

The stability results in Table 2 showed that no significant degradation occurred at room temperature for 8 h and during the three freeze–thaw cycles for BCQB in urine samples. BCQB in urine at −20 °C was stable for at least 11 weeks. The post-preparative samples of BCQB in autosampler were stable for at least 15 h. In addition, the stock solutions of BCQB and the I.S. in methanol were stable for at least 6 weeks at −20 °C, and at room temperature were stable for at least 4 h.

3.5. Application in the kinetic study of urinary excretion

The method described above had been applied successfully to the urinary excretion kinetic study of BCQB in healthy volunteers. This analytical method was able to measure the concentration of BCQB up to 72 h for all volunteers who nasally administered 45 μg BCQB. Fig. 3 is the mean cumulative urinary excretion amount–time curve of BCQB in 10 healthy volunteers after a single nasal administration of 45 μg BCQB. In the healthy volunteers who nasally administered 45 μg BCQB, the total amount of unchanged BCQB excreted in urine was 4.45 ± 2.28% ($n = 10$).

4. Conclusion

An HPLC–ESI-MS method combined with the solid-phase extraction for the quantification of BCQB in human urine was developed. The method achieved good sensitivity and specificity for the determination of BCQB in human urine. No significant interferences caused by endogenous compounds were observed. This reliable and

sensitive method is suitable for urinary excretion kinetic study of BCQB in humans.

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