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Short communication

Bioequivalence study of bromhexine by liquid chromatography–electrospray ionization-mass spectrometry after oral administration of bromhexine hydrochloride tablets

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ABSTRACT

A simple, sensitive and specific liquid chromatography–electrospray ionization-mass spectrometry method was developed for the quantitative determination of bromhexine in human plasma. Sample preparation involved simple liquid–liquid extraction. Simvastatin was used as internal standard. The separation of the analyte, internal standard and possible endogenous compounds were accomplished on a Shim-pack ODS column (150 mm × 4.6 mm i.d., 5 μm) with methanol–water (98:2, v/v) as mobile phase. Detection was performed in positive selected ion monitoring (SIM) mode at *m/z* 264.1 (for bromhexine) and *m/z* 441.7 (for IS). The method was validated over the range of 0.5–60 ng/mL and the results were acceptable. The method could offer the advantages of shorter run time (5.5 min) and lower LLOQ (0.5 ng/mL) with a decreased plasma volume requirement (250 μL) and it had been successfully applied to a bioequivalence study in healthy Chinese volunteers after single oral administration of 16 mg bromhexine.

1. Introduction

Bromhexine, *N*-cyclohexyl-*N*-methyl-(2-amino-3,5-dibromobenzyl)-amine, was proved to be antitussive and mucolytic because of promotion of bronchial secretion and splitting of polysaccharide fiber [1,2]. Researches on the excretion and metabolism showed that the plasma concentration decreased rapidly with extensive and rapid dealkylated or hydroxylated metabolism [3,4]. Low dosage and rapid biotransformation resulted in low plasma levels of bromhexine, and thus, it was not easy to get detailed pharmacokinetic character of bromhexine. Many investigations were performed to determine concentration of bromhexine in human plasma. Bechgaard and Nielsen [5] described a high-performance liquid chromatographic (HPLC) assay with ultraviolet (UV) detector. Though 3 mL of plasma volume was used, the sensitivity was not high enough and the LLOQ was 4 ng/mL. Another try was done by Johansson and Lenngren [6] who also used a 3-mL aliquot of plasma and the LLOQ was 5 ng/mL. Capillary gas chromatography (GC) with nitrogen-selective detector or electron capture detector could improve the sensitivity [4,7,8]. But in the methods reported by Nadongo et al. [7] and Yang et al. [8], baseline drift was observed and the separation was not very well. Schmid and Koss [4] extract bromhexine from human plasma by 5 repetitions of extraction, freezing and transfer, and silanized glass tubes were essential. The tedious procedures, followed a long analytical time (35 min for one sample), made the method inconvenience and inefficiency especially when it was applied to analyze large number of samples. Recently, a study on pharmacokinetic interaction between cefaclor and bromhexine was performed and a LC–MS method was utilized to determine bromhexine in plasma samples [9]. But there were not any information provided about evaluation of the analytical method, such as sensitivity, selectivity, accuracy and precision, etc. In this paper, a simple, sensitive and specific LC–MS method was developed and validated to meet the requirement of pharmacokinetic studies. Compared with those methods reported, this method has advantages of: (1) less need of plasma volume (250 μL), (2) higher sensitivity with LLOQ 0.5 ng/mL, and (3) shorter run time, 5.5 min for one sample. The application of this method was demonstrated for the analysis of bromhexine plasma samples in a Phase-I human pharmacokinetic study.

2. Experimental

2.1. Chemicals and reagents

Bromhexine (99.1%) was obtained from Jiangsu Qingjiang Medicine Co., Ltd. (Jiangsu, China). Simvastatin (99.7%) was pur-

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chased from Jiangsu Institute for Drug Control (Jiangsu, China). Methanol of HPLC grade was purchased from Merck KGaA (Darmstadt, Germany). Potassium dihydrogen phosphate and dibasic potassium phosphate were of analytical grade purity and purchased from Nanjing Chemical Reagent Co., Ltd. (Jiangsu, China). Cyclohexane of analytical grade purity was purchased from Shanghai No. 4 Reagent & H.V. Chemical Co., Ltd. (Shanghai, China). Redistilled water was used throughout the study.

The phosphate buffer solution was a mixture in which concentrations of KH_2PO_4 and K_2HPO_4 were 60 and 5 mM, respectively, with the pH approximate 5.8.

2.2. Instrumentation and conditions

Waters Alliance 2690 HPLC/Micro Quattro Micro™ MSD system (Milford, MA, USA) was used for LC–MS analysis. Data acquisition and peak integration were carried out using MassLynx V3.5.

Chromatographic separation was performed on a Shim-pack ODS column (150 mm × 4.6 mm i.d., 5 μm) protected by a Phenomenex ODS guard column (4.0 mm × 3.0 mm i.d., 5 μm). The mobile phase of methanol–water (98:2, v/v) was filtered and degassed. A constant flow-rate of 1.0 mL/min was maintained and a post-column split ratio of 1:1 was adequate. The column temperature was set at 35 °C.

LC–ESI-MS was performed in SIM mode. Target ions $[M+H-N$-methyl-cyclohexyl$]^+$ for bromhexine and $[M+Na]^+$ for simvastatin (IS) were monitored at m/z 264.1 and m/z 441.7, respectively. Nitrogen was used as desolvation gas with a flow-rate of 300 L/h and desolvation temperature was set 300 °C. The other critical parameters for ESI were: capillary voltage, 3.56 kV; cone voltage, 50 V; extractor, 6.4 V; source temperature, 100 °C; LM 1 resolution, 10; HM 1 resolution, 10.

2.3. Preparation of stock and working solutions

Stock solutions of bromhexine and IS were prepared individually by dissolving the accurately weighed reference compounds in methanol to get a final concentration of approximate 500 μg/mL. And the working solutions for bromhexine at the concentrations of 250, 50 and 5 ng/mL and for IS at that of 50 ng/mL were obtained further by gradually diluting the stock solutions with methanol. All the solutions were stored at −20 °C when not in use.

2.4. Sample preparation

A 250 μL aliquot of human plasma was introduced into a 10-mL glass centrifuge tube followed by 30 μL IS working solution. After briefly vortex-mixing, the mixture was added 250 μL of phosphate buffer (pH 5.8) and then 3 mL of cyclohexane, vortex-mixed for 3 min, then centrifuged for 10 min at 4000 rpm. The upper organic layer was transferred and evaporated to dryness at 40 °C under a gentle stream of nitrogen. Dry residues were dissolved in 100 μL mobile phase and 20 μL of reconstituted sample was injected into the LC–MS system.

2.5. Calibration curves and quality control samples

Calibration samples were prepared by spiking control plasma samples (250 μL) with bromhexine at 0.5, 1, 5, 10, 20, 30, 40, and 60 ng/mL and IS at 6 ng/mL on the day of sample work-up. To each batch of sample, a calibration curve covering the whole analytical working range was run in duplicate with the unknown samples. The calibration curve was constructed by plotting the peak area ratios of bromhexine to the IS versus the concentrations of bromhexine.

Quality control (QC) samples at the concentrations of 1, 10, and 40 ng/mL were prepared in the same way as calibration standards based on an independent weighing of reference substance. They were analyzed in each analytical batch along with the unknown samples.

2.6. Clinical study design

The bioequivalence study was approved by the Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. Twenty healthy Chinese male volunteers were selected. All volunteers had not taken any drugs within 2 weeks before the study. Tobacco, alcohol and caffeine were not allowed during the study. All volunteers were given written informed consents to participate in the study according to the principles of the Declaration of Helsinki.

The bioequivalence study was performed by a 2 × 2, crossover, randomized design. After an overnight fast, half of the volunteers received a single dose of test tablets (2 × 8 mg bromhexine), and others received reference formulation (2 × 8 mg bromhexine). The wash-out time between two periods was 7 days. The blood samples were collected into heparinized tubes pre-dose and 0.167, 0.333, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16 and 24 h after administration. The blood samples were centrifuged at 3500 rpm for 15 min and plasma samples were separated and stored at −20 °C until analysis.

3. Results and discussion

3.1. Selection of LC and MS conditions

According to the results of MS scan in positive or negative mode, the peak intensity got from positive mode was higher than that from negative one. Scanning in the range of m/z 200–500 showed that the positive ion with m/z 264.1 was of most abundant. Considering that it was produced from bromhexine molecular ion by a loss of N-methyl-cyclohexyl group ($C_7H_{15}N$), the ion with m/z 264.1 was selected for the determination of bromhexine. Critical tandem MS parameters were optimized and set to maximize the intensity of the ion. The target ion for IS is $[M+Na]^+$ with m/z 441.7.

The conditions of chromatography, especially the composition of mobile phase, were explored by several trials to achieve good resolution, high sensitivity, symmetrical peak shape, as well as short analytical time. Methanol, acetonitrile and water were mixed at different ratios. Addition of mobile phase modifiers such as ammonium acetate was also tested. The results revealed that a mixture of methanol and water (98:2, v/v) could satisfy the acquirement of resolution, peak shapes and analytical efficiency.

3.2. Selection of IS

The candidate IS included fenfluramine hydrochloride, benzhydramine, sibutramine hydrochloride, lovastatin and simvastatin. Based on the chromatographic and extract character of each compound, simvastatin was selected as IS. Under the chromatographic conditions selected above, retention time of IS and bromhexine were approximately 2.5 and 5 min, respectively. The HPLC run time for each sample was 5.5 min.

3.3. Optimization of sample preparation

Four different extraction solvents were tested, including hexane, cyclohexane, cyclohexane–ethyl acetate (3:2, v/v) and cyclohexane–ethyl acetate (9:1, v/v). These extraction solvents were used alone or combined with phosphate buffer (pH 5.8). After analysis of the recovery results, combination of cyclohexane and phosphate buffer (pH 5.8) was chosen. The volumes of cyclohex-

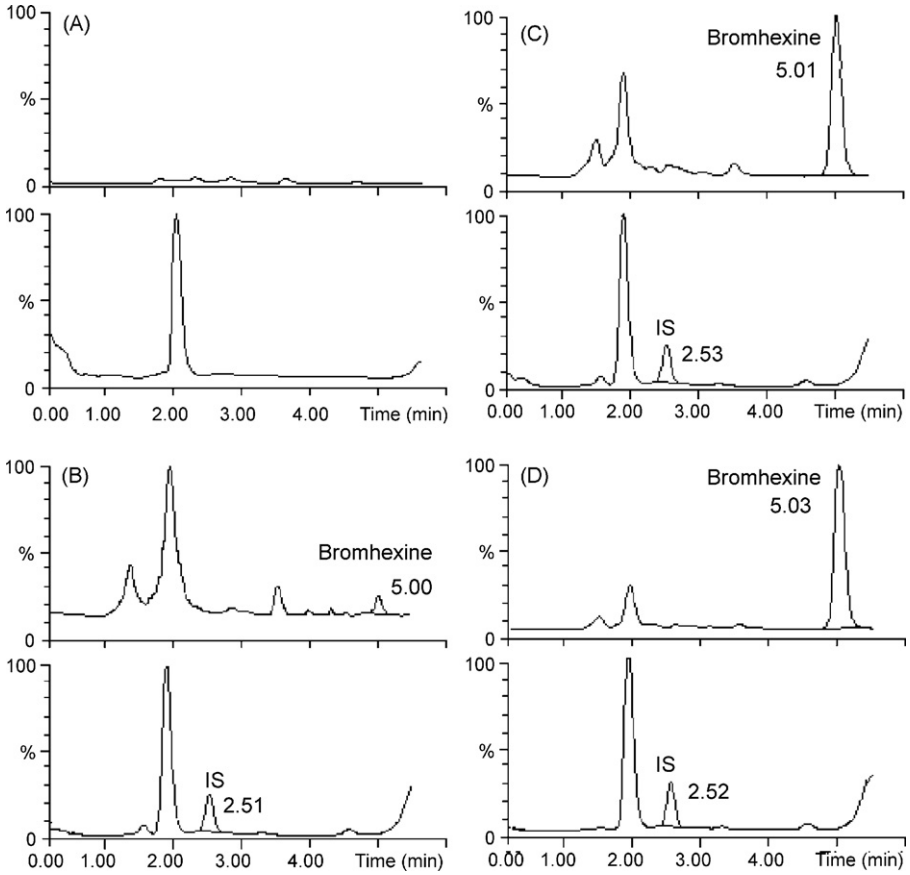

Fig. 1. Typical chromatograms of blank plasma (A) LLOQ for bromhexine (0.5 ng/mL) and IS (B), plasma spiked with bromhexine (30 ng/mL) and IS (C), plasma of a volunteer at 1 h after a single oral dosage of text formulation (D).

ane and phosphate buffer (pH 5.8) were adjusted to 3 and 0.25 mL, respectively.

3.4. Method validation

To confirm the reliability of the proposed method, validation assays were fulfilled according to the U.S. Food and Drug Administration (FDA) Guidance for Industry (Bioanalytical Method Validation) [10].

3.4.1. Selectivity

It was essential to certify that the signals measured were related only to the analytes. Selectivity of the method was examined by analyzing blank human plasma from six different sources using the selected extraction procedure and LC–MS conditions. Chromatograms (Fig. 1) showed that the method was able to discriminate the analytes from all potential interfering substances.

3.4.2. Matrix effect

To determine whether a significant matrix effect existed, the postextraction addition technique was used. Two groups of samples at concentrations of 1, 10, and 40 ng/mL were prepared as follow: in group A, blank plasma from five different sources were extracted, solutions of analytes were added and evaporated to dryness, then the residues were resolved in 100 μL mobile phase, the peak areas of analytes in group A were defined as *A*; in group B, analytes were evaporated to dryness and resolved in 100 μL mobile phase, the peak areas were defined as *B*. Five samples were tested at each concentration and the matrix effect was calculated by the formula: ME = $A/B \times 100\%$. IS was tested at the concentration of approximately 6 ng/mL. No interferences were detected and the ME values were within the range of 85–115%. It indicated that there were no co-eluting components interfering with the ionization of the analytes.

3.4.3. Linearity and lower limit of quantification

Calibration curve was determined by plotting the peak area ratios of bromhexine to IS versus the nominal concentrations of bromhexine. Good linearity was observed over the range of 0.5–60 ng/mL. Typical calibration curve had a slope of 0.493 and an intercept of 0.008 with coefficient correlation of 0.9996.

The lower limit of quantification (LLOQ) of bromhexine was defined as the lowest concentration of the calibration curve and it was determined to be 0.5 ng/mL in human plasma using five samples independent of calibration curves. The precision and accuracy at LLOQ was within 80–120% at LLOQ in this method.

3.4.4. Precision and accuracy

The results of spiked plasma samples in three consecutive runs were used to evaluate the accuracy and precision. Five replicate samples containing known amount of bromhexine (approximate 1, 10, and 40 ng/mL in plasma) were prepared and analyzed along with each calibration curve. Concentrations were determined by back-calculation of peak area ratios from the corresponding calibration curve. Accuracy was calculated by the formula: accuracy (%) = (measured concentration)/(nominal concentration) × 100. Precision was expressed by relative standard deviation (R.S.D.%). The results were presented in Table 1.

Table 1
Precision and accuracy for bromhexine QC samples (3 runs, 5 replicates per run)

Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	Accuracy (%)
1.0	1.11 ± 0.14	9.1	12.3	111.3
10.0	9.53 ± 0.60	5.5	6.3	95.3
40.0	40.30 ± 1.90	3.0	4.7	100.8

3.4.5. Extraction recovery

The extraction recovery was determined at the concentrations of 1, 10, and 40 ng/mL by comparing the peak areas of bromhexine extracted from plasma samples with those from standards. The extraction recovery at three concentrations was 95.5%, 97.9% and 98.3%, respectively. The results showed both good extraction efficiency and repeatability.

3.4.6. Stability

Stability studies were carried out at concentrations of 1, 10 and 40 ng/mL with three replicate samples for each level. For the freeze and thaw stability, spiked plasma samples were stored at −20 °C for 24 h and thawed at room temperature and refrozen at −20 °C for 24 h. After repeating the freeze–thaw cycles twice, samples were analyzed on the third cycle. For stability at room temperature, spiked plasma samples were thawed and kept at room temperature for 12 h. The long-term stability was assessed after storage of spiked plasma samples at −20 °C for 5 days. To test stability of post-prepared samples, spiked plasma samples were stored at −20 °C for 5 days after being evaporated. The stability was evaluated by comparing the results of stored samples with freshly prepared samples. The results were summarized in Table 2 which confirmed that bromhexine was stable under the investigated handling and storage conditions, and no stability related problems would be expected during routine analysis for clinical trial samples. The stability of stock solutions was tested at room temperature and at −20 °C. The results showed reliable stability behavior.

3.5. Bioequivalence study

The mean plasma concentration–time profiles of bromhexine in 20 healthy Chinese male volunteers were presented in Fig. 2. The main pharmacokinetic parameters of bromhexine summarized in Table 3 were calculated by noncompartmental model using the software of BAPP (programmed by China Pharmaceutical University). The bioequivalence of drugs was determined on basis of AUC_{0-24}, $AUC_{0-\infty}$ and C_{max} by analysis of variance (ANOVA) and two one-side *t*-test. T_{max} was evaluated by Wilcoxon test. The results showed that the test formulation was bioequivalent to the reference drugs based on the exposure to bromhexine.

Table 2
Stability of bromhexine under various storage conditions ($n = 3$)

Storage conditions	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)
Freeze and thaw stability	1.0	1.03 ± 0.01
	10.0	9.90 ± 0.81
	40.0	41.51 ± 1.85
Room temperature for 12 h	1.0	1.10 ± 0.01
	10.0	9.70 ± 0.05
	40.0	39.74 ± 1.62
−20 °C for 5 days	1.0	1.08 ± 0.02
	10.0	10.10 ± 0.02
	40.0	41.05 ± 1.17
Post-prepared and −20 °C for 5 days	1.0	1.13 ± 0.07
	10.0	10.40 ± 0.57
	40.0	41.07 ± 2.57

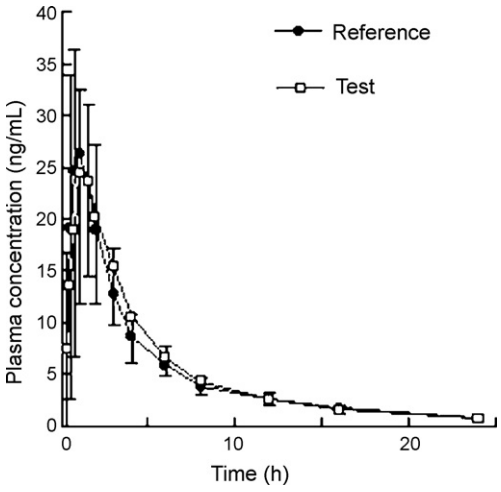

Fig. 2. Mean plasma concentration–time profiles of bromhexine in 20 healthy Chinese male volunteers.

Table 3
Main pharmacokinetic parameters of bromhexine in healthy Chinese volunteers after a single oral administration containing 16 mg bromhexine ($n = 20$)

Parameters	Mean ± S.D.	
	Test formulation	Reference formulation
C_{max} (ng/mL)	34.30 ± 7.02	35.06 ± 8.39
t_{max} (h)	1.4 ± 0.6	1.0 ± 0.5
MRT (h)	6.72 ± 0.66	6.85 ± 0.53
$t_{1/2}$ (h)	5.75 ± 0.82	6.12 ± 0.62
CL/*F* (L/h)	123.9 ± 13.3	129.2 ± 16.4
AUC_{0-24} (h ng/mL)	125.2 ± 16.1	119.8 ± 16.1
$AUC_{0-\infty}$ (h ng/mL)	130.8 ± 15.7	125.8 ± 16.6

Note: S.D., standard deviation; R.S.D., relative standard deviation; *n*, number of replicates.

4. Conclusions

A bioanalytical method for the determination of bromhexine in human plasma was developed. A LLOQ of 0.5 ng/mL was achieved based on 250 μL of plasma samples. This method showed good sensitivity and accuracy over the range of 0.5–60 ng/mL. No significant interference and matrix effect caused by endogenous compounds or other drug were observed. A LC–MS run time of 5.5 min per sample made the method a pragmatic one in bioanalysis of bromhexine. The results confirmed the bioequivalence of test and reference formulations.

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