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Quantification of the major metabolites of bromhexine in human plasma using RRLC–MS/MS and its application to pharmacokinetics

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ABSTRACT

(*E*)-4-hydroxydemethylbromhexine (*E*-4-HDMB) and (*E*)-3-hydroxydemethylbromhexine (*E*-3-HDMB) were found as major metabolites, while (*Z*)-4-hydroxydemethylbromhexine and (*Z*)-3-hydroxydemethylbromhexine as minor metabolites of bromhexine in human plasma. These compounds were identified in comparison with synthetic authentic samples. A sensitive and selective rapid resolution liquid chromatography tandem mass spectrometry (RRLC–MS/MS) method was developed to quantify the concentration of bromhexine and its two major metabolites (*E*-4-HDMB and *E*-3-HDMB) in human plasma. Following solid phase extraction, the analytes were separated on a Zorbax 1.8 μm particle size reversed-phase C_{18} column, using a gradient elution program with solvents consisting of 0.1% formic acid in acetonitrile and 0.1% formic acid in 5 mM ammonium acetate at a flow rate of 0.7 mL/min. Detection was carried out with an Agilent 6460 triple-quadrupole mass spectrometer operated with an electrospray ionization source mode operated in the positive ion mode. The recovery of bromhexine, *E*-4-HDMB, *E*-3-HDMB, and internal standard (IS) was 63.1–70.9%, 60.5–68.4%, 57.0–63.5%, and 87.8%, respectively. The matrix factors of bromhexine, *E*-4-HDMB, *E*-3-HDMB, and IS were 89.9–96.7%, 89.6–94.8%, 90.4–91.4%, and 103%, respectively. After an oral administration of 8.0 mg bromhexine to five healthy male subjects, $AUC_{0-24\,h}$ values of bromhexine, *E*-4-HDMB, and *E*-3-HDMB were found to be 93.5 ± 31.9, 34.0 ± 14.5, and 15.8 ± 6.89 ng h/mL, respectively; while C_{max} values were 24.6 ± 5.16, 3.11 ± 1.13, and 5.36 ± 2.55 ng/mL, respectively. Plasma concentration of bromhexine, *E*-4-HDMB, and *E*-3-HDMB declined with $t_{1/2}$ which gave 3.6 ± 0.5, 8.4 ± 2.7, and 6.4 ± 2.5 h, respectively.

1. Introduction

Bromhexine, 2-amino-3,5-dibromo-*N*-cyclohexyl-*N*-methylbenzylamine (Fig. 1), is a synthetic derivative of vasicine, one of the active ingredients of the Asian plant *Adhatoda vasica* [1]. Bromhexine has proven its effectiveness in normalizing mucus in the respiratory tract so that a natural cough response is able to clear the airway. Introduced for the first time in 1963 as a secretolytic or mucolytic medicine by Boehringer Ingelheim, bromhexine became one of the most frequently used cough remedies. Today, it is still widely used as an over-the-counter drug.

In clinical use, bromhexine is administered orally three times a day at dosages of 8 or 16 mg. In mammals and pig hepatocyte cultures, bromhexine is extensively converted to a number of metabolites [2–5]. Schraven et al. reported several metabolites of bromhexine, including (*E*)-4-(2-amino-3,5-dibromobenzylamino)cyclohexanol (ambroxol, *E*-4-HDMB), (*E*)-3-(2-amino-3,5-dibromobenzylamino)cyclohexanol (*E*-3-HDMB), and (*Z*)-3-(2-amino-3,5-dibromobenzylamino)cyclohexanol (*Z*-3-HDMB) (Fig. 1) in rabbit urine [3]. Kopitar et al. reported the differences in metabolism and excretion of bromhexine in mice, rats, rabbits, dogs, and humans, and found that the rabbit pattern appears most similar to humans, while the least similar is the rat [4]. *E*-4-HDMB, also confirmed to have an expectorant effect, was first introduced in 1978 by Boehringer Ingelheim.

By virtue of the activity of *E*-4-HDMB, it is very likely that its isomers may also have activity. Illuminating the pharmacokinetics of active metabolites is, therefore, important. Four reference standards of hydroxylated and dealkylated metabolites of bromhexine were synthesized for the pharmacokinetic study. A robust rapid resolution liquid chromatography tandem mass spectrometry (RRLC–MS/MS) method has been developed and validated for simultaneous quantification of bromhexine and its two major metabolites in human plasma. The pharmacokinetics of bromhexine and its major metabolites following oral administration of 8 mg bromhexine to healthy human subjects is reported for the first time.

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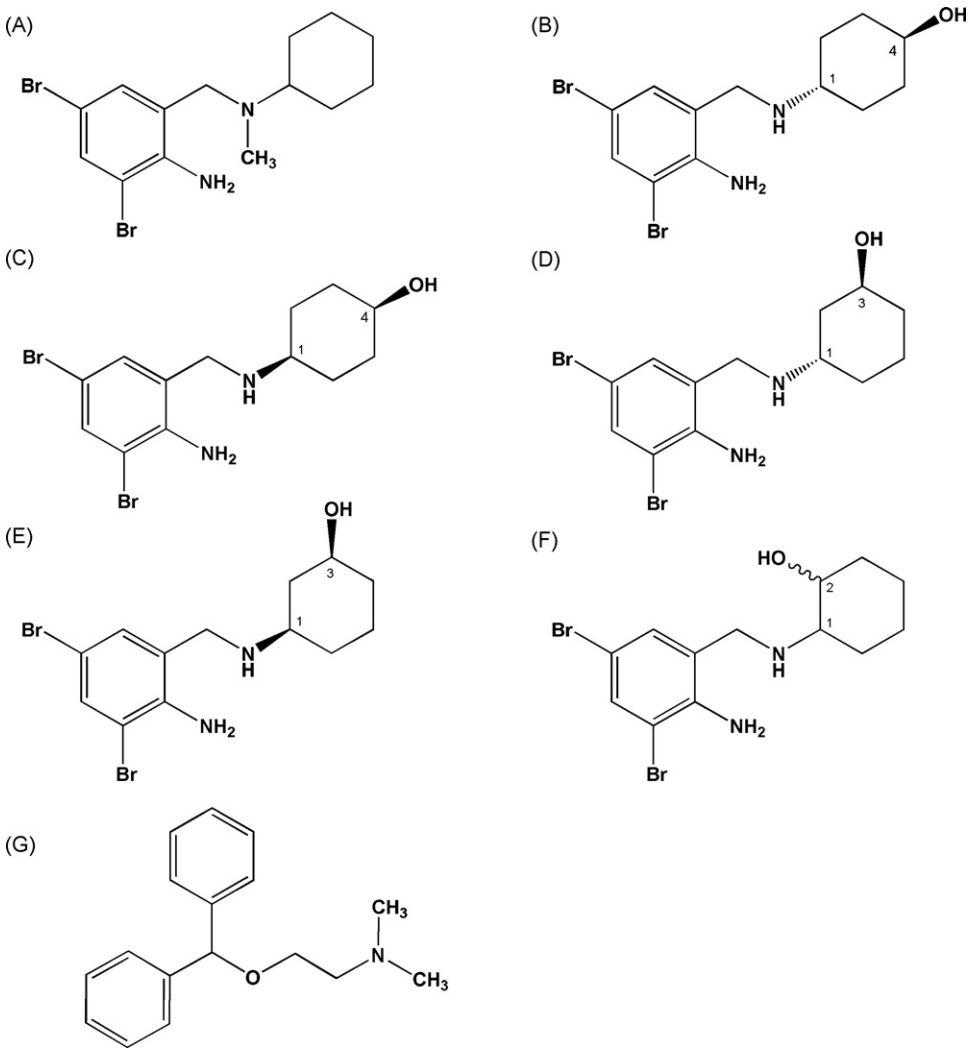

Fig. 1. Chemical structures of (A) bromhexine, (B) *E*-4-HDMB, (C) *Z*-4-HDMB, (D) *E*-3-HDMB, (E) *Z*-3-HDMB, (F) 2-HDMB, and (G) diphenhydramine (IS).

2. Experimental

2.1. Chemicals and reagents

Reference standard bromhexine hydrochloride was kindly provided by Nanchang Hongyi Pharmaceutical Factory Co., Ltd. (Jiangxi, China); *E*-4-HDMB hydrochloride and diphenhydramine hydrochloride (internal standard, IS) were both obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Authentic (*Z*)-4-(2-amino-3,5-dibromobenzylamino)cyclohexanol (*Z*-4-HDMB), *E*-3-HDMB, *Z*-3-HDMB, and 2-(2-amino-3,5-dibromobenzylamino)cyclohexanol (2-HDMB) were synthesized in our laboratory. The chemical structures of the synthesized compounds were confirmed by means of electrospray ionization tandem mass spectrometry (ESI-MS/MS), and ^{1}H nuclear magnetic resonance (^{1}H NMR) analysis. HPLC grade acetonitrile, methanol, formic acid, and ammonium acetate were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA) and Tadia (Fairfield, OH, USA). Heparinized blank (drug-free) human plasma was obtained from Shanghai Blood Donor Service (Shanghai, China). Ultrapure water (resistivity >18.2 MΩ) was generated by a MilliQ apparatus (Millipore, Bedford, MA, USA).

2.2. Apparatus

RRLC was carried out using an Agilent Technologies 1200 series system equipped with a G1322A degasser, a G1312B SL binary pump, a G1357D high-performance autosampler (HiP ALS SL+), and a G1316B SL thermostated column compartment. Separation of the analytes from the plasma was achieved on a Zorbax Extend-C_{18} column (50 mm × 2.1 mm i.d., 1.8 μm; Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a Security-Guard C_{18} guard column (4 mm × 3.0 mm i.d.; Phenomenex, Torrance, CA, USA). A gradient mobile phase was run at 10% A (0.1% formic acid in acetonitrile) and 90% B (0.1% formic acid in 5 mM ammonium acetate) for the first 7 min, then 70% A for 3 min. The column was re-equilibrated to initial conditions for 3 min. RRLC flow rate was 0.7 mL/min at 40 °C with a total run time of 13 min.

A 6460 triple-quadrupole mass spectrometer (Agilent Technologies, Inc.) was operated with an Agilent G1948B ionization source in positive ESI mode. An Agilent Mass Hunter workstation (Agilent Technologies, Inc.) was used for the control of equipment, data acquisition, and analysis. For the optimization of MS/MS parameters, the software's tune mode and standard solutions of bromhexine, *E*-4-HDMB, *E*-3-HDMB, and the IS solution prepared in methanol were infused into the mobile phase (0.7 mL/min) at

a flow rate of 20 μL/min using a syringe pump (Harvard Apparatus, Holliston, MA, USA). Finally, the instrument was operated with the capillary voltage at +3.5 kV, and charging voltage at +1 kV. Nitrogen was used as nebulizer gas of 0.31 MPa, a carrier gas of 10 L/min at 300 °C, and a sheath gas of 11 L/min at 350 °C. Multiple reaction monitoring (MRM) was employed for data acquisition. The optimized MRM fragmentation trasitions were *m/z* 377 → *m/z* 114, with a fragmenter voltage of 100 V and a collision energy (CE) of 15 V for bromhexine, *m/z* 379 → *m/z* 264 with a fragmenter voltage of 120 V and a CE of 20 V for both *E*-4-HDMB and *E*-3-HDMB, and *m/z* 256 → *m/z* 167 with a fragmenter voltage of 120 V and a CE of 15 V for IS. The dwell time for each trasition was 150 ms.

^{1}H NMR spectra were recorded on a Bruker AM 400. Chemical shifts are expressed in ppm with $CDCl_3$ as the IS (7.26 for ^{1}H), and coupling constants (*J*) are given in Hz. Assignments of the ^{1}H NMR signals were done using rotating frame Overhauser effect spectroscopy (ROESY) experiments.

2.3. Synthesis of Z-4-HDMB

Authentic *Z*-4-HDMB was synthesized according to synthetic routes of *E*-4-HDMB [1,3]. A solution of 2-amino-3,5-dibromobenzaldehyde (92 mg, 0.33 mmol) and *Z*-4-aminocyclohexanol (38 mg, 0.33 mmol) in ethanol (4 mL) was stirred at room temperature for 4 h, followed by the addition of $NaBH_4$ (15 mg, 0.40 mmol) with continued stirring for 4 h. The mixture was filtered, and the solvent was removed by vacuum. The resulting residue was purified by preparative thin layer chromatography (TLC) using $CHCl_3$–methanol (10:1, v/v), with a retardation factor value (*Rf* value) of 0.42, to give *Z*-4-HDMB (white amorphous powder, 30 mg, 24% yield). MS: *m/z* 377 $[M+H]^+$; ^{1}H NMR: δH (400 MHz; $CDCl_3$) 7.47 (d, *J* = 2.1 Hz, 1H), 7.09 (d, *J* = 2.1 Hz, 1H), 5.41 (brs, 2H), 3.89 (m, 1H), 3.79 (m, 2H), 2.59 (m, 1H), 1.71–1.18 (m, 10H).

2.4. Synthesis of E-3-HDMB and Z-3-HDMB

The same procedure was used as for *E*-3-HDMB and *Z*-3-HDMB, using 2-amino-3,5-dibromobenzaldehyde (123 mg, 0.44 mmol) and 3-aminocyclohexanol (51 mg, 0.44 mmol, a mixture, *E*:*Z* = 1:3). Synthesized products were purified by preparative TLC using $CHCl_3$–methanol (10:1, v/v), with *Rf* values of 0.30 and 0.35, respectively, to give *E*-3-HDMB (white amorphous powder, 10 mg, 8% yield) and *Z*-3-HDMB (white amorphous powder, 30 mg, 24% yield). *E*-3-HDMB, MS: *m/z* 377 $[M+H]^+$; ^{1}H NMR: δH(400 MHz; $CDCl_3$) 7.46 (d, *J* = 2.4 Hz, 1H), 7.09 (d, *J* = 2.4 Hz, 1H), 5.33 (brs, 2H), 4.14–4.05 (m, 1H), 3.83–3.71 (m, 1H), 2.94 (m, *J* = 4.2 Hz, 1H), 1.90–1.15 (m, 10H). No ROESY correlation was observed between H-1 (δ 2.94) and H-3 (δ 3.83–3.71), thus, suggesting that H-1 and H-3 were orientated on the different face (*E*) of the molecule. For *Z*-3-HDMB, MS: *m/z* 377 $[M+H]^+$; ^{1}H NMR: δH(400 MHz; $CDCl_3$) 7.46 (d, *J* = 2.4 Hz, 1H), 7.08 (d, *J* = 2.4 Hz, 1H), 5.25 (brs, 2H), 3.83 (s, 2H), 3.65 (m, 1H), 2.55 (m, 1H), 2.14 (d, *J* = 12 Hz, 1H), 1.93–0.96 (m, 9H), the ROESY correlation observed between H-1 (δ 2.55) and H-3 (δ 3.65) suggested that H-1 and H-3 were orientated on the same face (*Z*) of the molecule.

2.5. Synthesis of 2-HDMB

The same procedure was used as for 2-HDMB using 2-amino-3,5-dibromobenzaldehyde (92 mg, 0.33 mmol) and 2-aminocyclohexanol (38 mg, 0.33 mmol). Synthesized product was purified by preparative TLC using $CHCl_3$–methanol (10:1, v/v), with an *Rf* value of 0.5, to give 2-HDMB (white amorphous powder, 30 mg, 24%). MS: *m/z* $[M+H]^+$; ^{1}H NMR: δH(400 MHz; $CDCl_3$) 7.36 (d, *J* = 2.7 Hz, 1H), 7.05 (d, *J* = 2.7 Hz, 1H), 5.30 (brs, 2H), 3.61–3.89 (m, 2H), 3.21 (m, 1H), 2.23 (m, 1H), 2.03–1.00 (m, 8H).

2.6. Preparation of standard and quality control (QC) samples

Stock solutions of bromhexine, *E*-4-HDMB, and *E*-3-HDMB at concentrations of 400, 400, and 357 μg/mL, respectively, were prepared by dissolving accurately weighed reference substances in methanol. The stock solutions of bromhexine, *E*-4-HDMB, and *E*-3-HDMB were then serially diluted with a mixed solution of methanol–water (50:50, v/v) to give quantification working solutions at the following concentrations: 1.5, 3.0, 6.0, 12, 30, 75, 150, and 300 ng/mL; 0.50, 1.0, 2.0, 4.0, 10, 25, 50, and 100 ng/mL; and 1.0, 2.0, 4.0, 8.0, 20, 50, 100, and 200 ng/mL, for bromhexine, *E*-4-HDMB, and *E*-3-HDMB, respectively. The other three stock solutions were independently diluted in a similar way to achieve QC solutions at concentrations of 3.0, 30, and 270 ng/mL; 1.0, 10, and 90 ng/mL; and 2.0, 20, and 180 ng/mL, for bromhexine, *E*-4-HDMB, and *E*-3-HDMB, respectively.

IS solution (100 ng/mL) was prepared by diluting the 500 μg/mL stock solution of diphenhydramine with a mixture of methanol–water (50:50, v/v). All solutions were kept at 4 °C and were brought to room temperature before use.

Both the calibration standard samples and the QC samples, which were used in the pre-study validation and during the pharmacokinetic study, were prepared by spiking 200 μL blank plasma with 20 μL of the corresponding working solutions.

2.7. Sample preparation

To an aliquot of 200 μL of plasma samples, 20 μL of methanol–water (50:50, v/v) and 20 μL of the IS solution were added, then the mixture was diluted with 800 μL of Milli Q water and vortex-mixed for 30 s. This sample mixture was loaded on preconditioned (1 mL methanol followed by 2 mL water) Oasis HLB cartridges (1 cm^3, 30 mg), washed with 1 mL water, and then eluted with 2 mL 1% formic acid in methanol. The eluate was evaporated to dryness at 40 °C under a stream of nitrogen in a TurboVap evaporator (Zymark, Hopkinton, MA, USA). The residue was reconstituted in 100 μL of methanol–water (50:50, v/v) and vortex-mixed for 1 min. A 10 μL aliquot of the reconstituted extract was used for the RRLC–MS/MS analysis.

2.8. Method validation

The validation experiments were designed according to "Guidance for Industry–Bioanalytical Method Validation," recommended by the US Food and Drug Administration (FDA) [6], with consideration of the intended application of the assay for sample analysis.

The specificity of the method was evaluated by assaying human blank plasma samples from six different donors and the lower limit of quantification (LLOQ) samples, respectively. LLOQ was defined as the lowest concentration of analytes determined with acceptable precision and accuracy [six replicates on three validation days with relative standard deviation (RSD) below 20% and a relative error (RE) within ±20%]. The analyte responses at this concentration level should be >5 times the baseline noise.

Linearity was assessed by plotting calibration curves in human plasma in duplicate in three separate runs. The curves were fitted by a linear weighted ($1/x^2$) least square regression method through measurement of the peak area ratio of the analytes to the IS solution.

To evaluate the precision and accuracy of the method, QC samples at three concentration levels (0.30, 3.0, and 27 ng/mL; 0.10, 1.0, and 9.0 ng/mL; and 0.20, 2.0, and 18 ng/mL, for bromhexine, *E*-4-HDMB, and *E*-3-HDMB, respectively) were analyzed in six repli-

cates on three validation days. The assay precision was calculated using the RSD and a one-way analysis of variance. RSD separated out the sources of variance due to within- and between-run factors. The assay accuracy was expressed as the RE, or (observed concentration − nominal concentration)/(nominal concentration) × 100%. The accuracy was required to be within ±15%, and the intra- and inter-day precisions were not to exceed 15%.

The recoveries of bromhexine, *E*-4-HDMB, and *E*-3-HDMB at three QC levels ($n = 6$) were determined by comparing peak area ratios of the analytes to the IS in samples that were spiked with the analytes prior to solid phase extraction and in samples to which the analytes had been added post-extraction. The IS solution was added to both sets of samples post-extraction. The recovery of the IS was determined in a similar way, using the QC samples at medium concentration as a reference.

Using the method described by Matuszewski et al. [7], we assessed the matrix effects (MEs), or whether the potential ion suppression or enhancement owing to the co-eluting matrix components existed in the present experiment. The corresponding peak areas of the analyte from the spike-after-protein precipitation samples at low and high concentration levels were then compared to those of the standard solution at the same concentration in the mobile phase.

The stability of bromhexine, *E*-4-HDMB, and *E*-3-HDMB in human plasma was evaluated by analyzing replicates ($n = 3$) of plasma samples that were exposed to different conditions (time and temperature) at concentrations of 0.30 and 27 ng/mL, 0.10 and 9.0 ng/mL, 0.20 and 18 ng/mL, for bromhexine, *E*-4-HDMB, and *E*-3-HDMB, respectively. These results were compared with those obtained for freshly prepared plasma samples. The analytes were considered stable in the biological matrix when 85–115% of the initial concentration was retained.

2.9. Application to a clinical pharmacokinetic study

Five healthy Chinese male subjects (ages 20–24) received per oral administration of 8 mg dose of bromhexine. The blood samples were drawn at baseline (before drug administration) and at 0.33, 0.67, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 14.0, and 24.0 h after drug administration. The study protocol was approved by the Human Investigation Ethical Committee, and blood sampling was carried out at the Second Affiliated Hospital of Liaoning University of Chinese Medicine. Plasma samples were obtained by centrifugation at 2000 × *g* for 10 min, and frozen at −20 °C until analysis.

Time profiles for plasma concentrations of bromhexine, *E*-4-HDMB, and *E*-3-HDMB were acquired for each subject. The major non-compartmental pharmacokinetic parameters of bromhexine, *E*-4-HDMB, and *E*-3-HDMB were then calculated. The maximum plasma concentration (C_{max}) and the time of occurrence (T_{max}) were obtained directly from the measured data. The terminal elimination rate constant (k_e) was estimated by linear least square regression of the terminal portion of the plasma concentration–time curve, and the corresponding elimination half-life ($t_{1/2}$) was then calculated as $0.693/k_e$. The area under the plasma concentration–time curve (AUC) was calculated according to the linear trapezoidal rule to the last measurable point (AUC_{0-t}) or to infinity ($AUC_{0-\infty}$) by $AUC_{0-t} + C_t/k_e$, where C_t was the last measurable drug concentration.

3. Results and discussion

3.1. Mass spectrometry

The positive ionization mode was selected for the quantification of bromhexine and the metabolites by virtue of the presence of an amino group. ESI was chosen as the ionization source in the experiment, because ESI could offer better response intensity of the analytes than did atmospheric pressure chemical ionization.

Because there are two bromine atoms in the structure of bromhexine, the soft ionization process in the ESI source produced the protonated molecules $[M+H]^+$, $[M+H+2]^+$, and $[M+H+4]^+$, at the abundance ratio of 1:2:1, at *m/z* 375, 377, and 379, respectively. Similarly, the metabolites of bromhexine, which are isomers, were all produced as protonated molecules $[M+H]^+$, $[M+H+2]^+$, and $[M+H+4]^+$, at the abundance ratio of 1:2:1, at *m/z* 377, 379, and 381, respectively.

Under the experimental conditions, the product ion mass spectrum of $[M+H+2]^+$ of bromhexine showed intense fragments at *m/z* 264 and *m/z* 114, formed by the cleavage of an amide bond. The protonated molecule $[M+H+2]^+$ of the metabolites showed intense product ions at *m/z* 264 and *m/z* 116 resulting from similar fragmentation pathways to that of bromhexine. For bromhexine, the ions *m/z* 114 showed more intense signals than the ions *m/z* 264, while for the metabolites, the situation was the opposite. Finally, when quantifying the analytes, the MRM reactions at *m/z* 377 → *m/z* 114 were used for bromhexine, and *m/z* 379 → *m/z* 264 were used for both *E*-4-HDMB and *E*-3-HDMB.

In our case, diphenhydramine was used as the IS. The base peak $[M+H]^+$ ion at *m/z* 256 in the Q1 full-scan mode was selected as the precursor ion. Its fragment ion at *m/z* 167 proved to be steady and abundant and it was chosen for the MRM acquisition.

The full-scan and MS/MS mass spectra of bromhexine, *E*-4-HDMB, *E*-3-HDMB, and IS are shown in Fig. 2.

3.2. Chromatography

The isomers *E*-4-HDMB, *Z*-4-HDMB, *E*-3-HDMB, *Z*-3-HDMB, and 2-HDMB were not distinguishable by the mass spectrometry. Consequently, it was of vital importance that chromatographic separation could be achieved. Different columns and mobile phases were tested during the method development. Separation of the metabolites was achieved using a Capcell MG-C_{18} (100 mm × 4.6 mm i.d., 3 μm, Shiseido, Tokyo, Japan); however, the run time was 30 min. It was essential to develop a method to achieve complete isomer separation in a relatively short run time.

Reversed-phase chromatographic media with a 1.7 or 1.8 μm particle size offer significant advantages in resolution, speed, and sensitivity for analytical determinations. In our study, 1.8 μm particle size columns were selected, and the chromatographic conditions were optimized with mobile phases containing varying percentages of organic phase. Because of the polarity properties of the analytes, a gradient mobile phase consisting of 0.1% formic acid in acetonitrile and 0.1% formic acid in 5 mM ammonium acetate was finally chosen. With these selected chromatographic conditions, successful separation of the five analytes of interest was achieved in 9 min. A representative chromatogram for *E*-4-HDMB, *Z*-4-HDMB, *E*-3-HDMB, *Z*-3-HDMB, and 2-HDMB is shown in Fig. 3(A).

By comparison with reference substances, *E*-4-HDMB and *E*-3-HDMB were found to be the two major metabolites of bromhexine in human plasma, while small amounts of *Z*-4-HDMB and *Z*-3-HDMB were also observed. However, the concentrations of *Z*-4-HDMB and *Z*-3-HDMB were quite lower than those of *E*-4-HDMB and *E*-3-HDMB. Based on their relative high concentrations in human circulation, only the two major metabolites were chosen for quantification.

3.3. Method validation

3.3.1. Linearity of calibration standards

The plotted calibration curves and correlation coefficients >0.99 confirmed that the calibration curves were

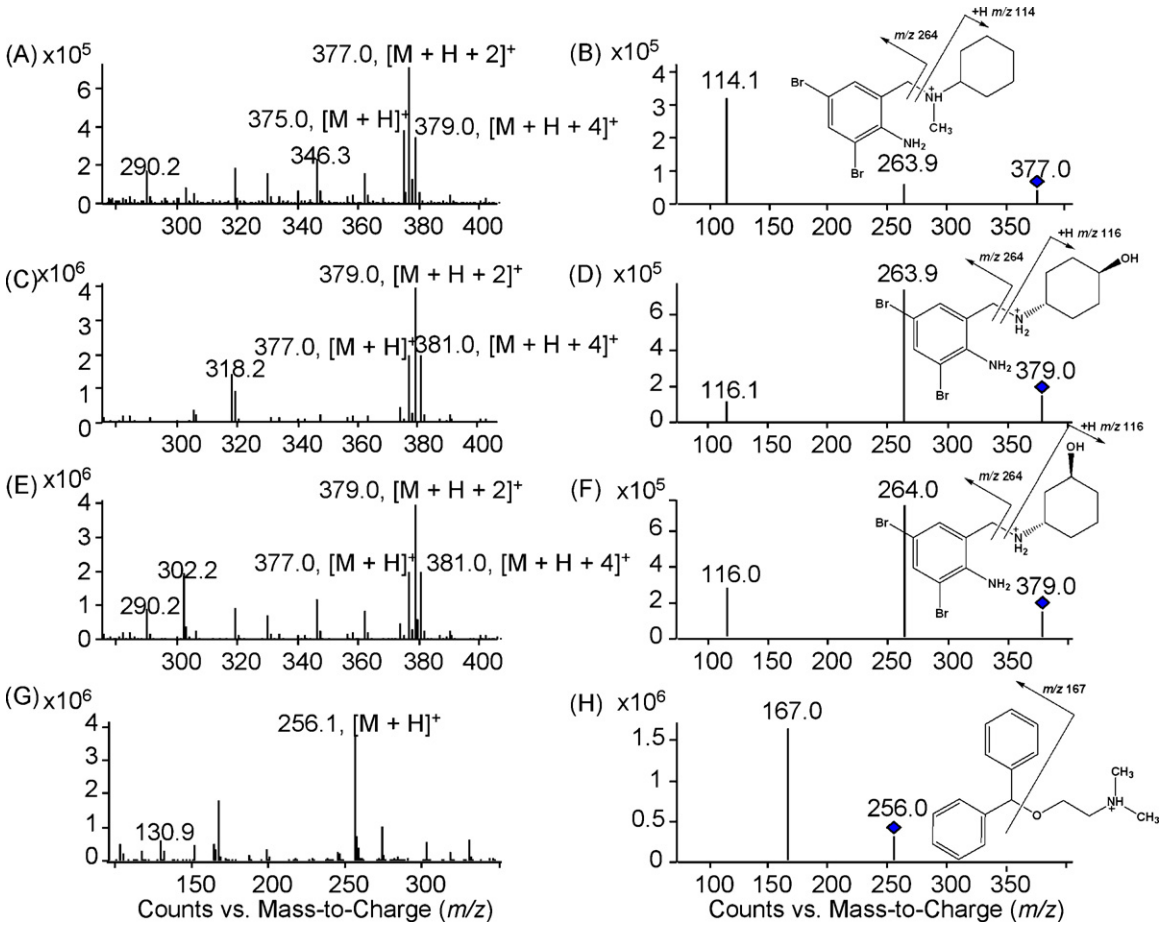

Fig. 2. Full-scan mass spectra and MS/MS mass spectra of (A) and (B) bromhexine, (C) and (D) *E*-4-HDMB, (E) and (F) *E*-3-HDMB, and (G) and (H) diphenhydramine (IS).

linear over the concentration range 0.15–30, 0.050–10, and 0.10–20 ng/mL for bromhexine, *E*-4-HDMB, and *E*-3-HDMB, respectively. Typical standard curves for bromhexine, *E*-4-HDMB, and *E*-3-HDMB were as follows: $y = 0.3254x + 2.90 \times 10^{-3}$, $r^2 = 0.9985$, $y = 0.1651x - 2.75 \times 10^{-4}$, $r^2 = 0.9982$, and $y = 0.1203x - 6.17 \times 10^{-4}$, $r^2 = 0.9988$, respectively, where y represents the ratio of analyte peak area to that of IS, and x represents the plasma concentration of analyte.

3.3.2. Assay specificity and LLOQ

Three typical MRM chromatograms from the study of bromhexine and its metabolites in human plasma are shown in Fig. 3. No interfering peaks were observed in blank plasma (Fig. 3(B)). The MRM chromatograms of blank plasma spiked with bromhexine (0.15 ng/mL), *E*-4-HDMB (0.050 ng/mL), *E*-3-HDMB (0.10 ng/mL), and IS (10 ng/mL) are shown in Fig. 3(C). A sample from a subject 1.5 h after an oral administration of 8 mg bromhexine tablets is shown in Fig. 3(D). For the analytes and the IS, the chromatograms were free from endogenous matrix interference at their respective retention times.

For bromhexine, *E*-4-HDMB, and *E*-3-HDMB, the present RRLC–MS/MS method offered an LLOQ of 0.15, 0.050, and 0.10 ng/mL, with the accuracy of −3.0%, 2.2%, and 4.0% in terms of RE and the intra- and inter-day precisions were less than 8.8%, 3.6%, and 7.2% in terms of RSD (3 days, $n = 6$), respectively.

3.3.3. Precision and accuracy

The intra- and inter-day precision and accuracy were calculated by analysis of variances, based on replicate analyses (3 days, three concentrations, each $n = 6$) of QC samples. In this study, the intra- and inter-day precisions were less than 8.3%, 12.8%, and 10.7% for each QC level, for bromhexine, *E*-4-HDMB, and *E*-3-HDMB, respectively. The inter-day RE were −3.1%, −0.9%, and −1.9% for bromhexine; 1.5%, 0.9%, and 0.1% for *E*-4-HDMB; and 2.7%, 2.2%, and 3.3% for *E*-3-HDMB. These data indicated that the RRLC–MS/MS results were reproducible, and that the assay was accurate and reliable. The accuracy and precision data are shown in Table 1.

3.3.4. Recovery and stability

The recovery of bromhexine, determined at three concentrations (0.30, 3.0, and 27 ng/mL), was 65.8 ± 3.2%, 70.9 ± 1.1%, and 63.1 ± 1.7% ($n = 6$), respectively. The recovery of *E*-4-HDMB, determined at three concentrations (0.10, 1.0, and 9.0 ng/mL), was 64.4 ± 2.7%, 68.4 ± 4.1%, and 60.5 ± 2.2% ($n = 6$), respectively. The recovery of *E*-3-HDMB, determined at three concentrations (0.20, 2.0, and 18 ng/mL), was 62.6 ± 2.8%, 63.5 ± 4.1%, and 57.0 ± 1.5% ($n = 6$), respectively. The recovery of IS was shown to be 87.8 ± 2.3% ($n = 6$). The recovery data are shown in Table 2.

The results of stability experiments showed that bromhexine, *E*-4-HDMB, and *E*-3-HDMB were stable for 24 h after preparation at 22 °C, for 2 h at 22 °C following three freeze/thaw cycles (−20 to 22 °C) on consecutive days, and for 3 months at −20 °C, as the RE values were within ±15% for both the low and high concentrations.

Taken together, the stability data indicated that bromhexine samples could be stored and prepared under routine laboratory conditions without special attention.

3.3.5. Matrix effect

The matrix factors of bromhexine, determined at two concentrations (0.30 and 27 ng/mL), were 89.9 ± 3.2% and 96.7 ± 0.9%

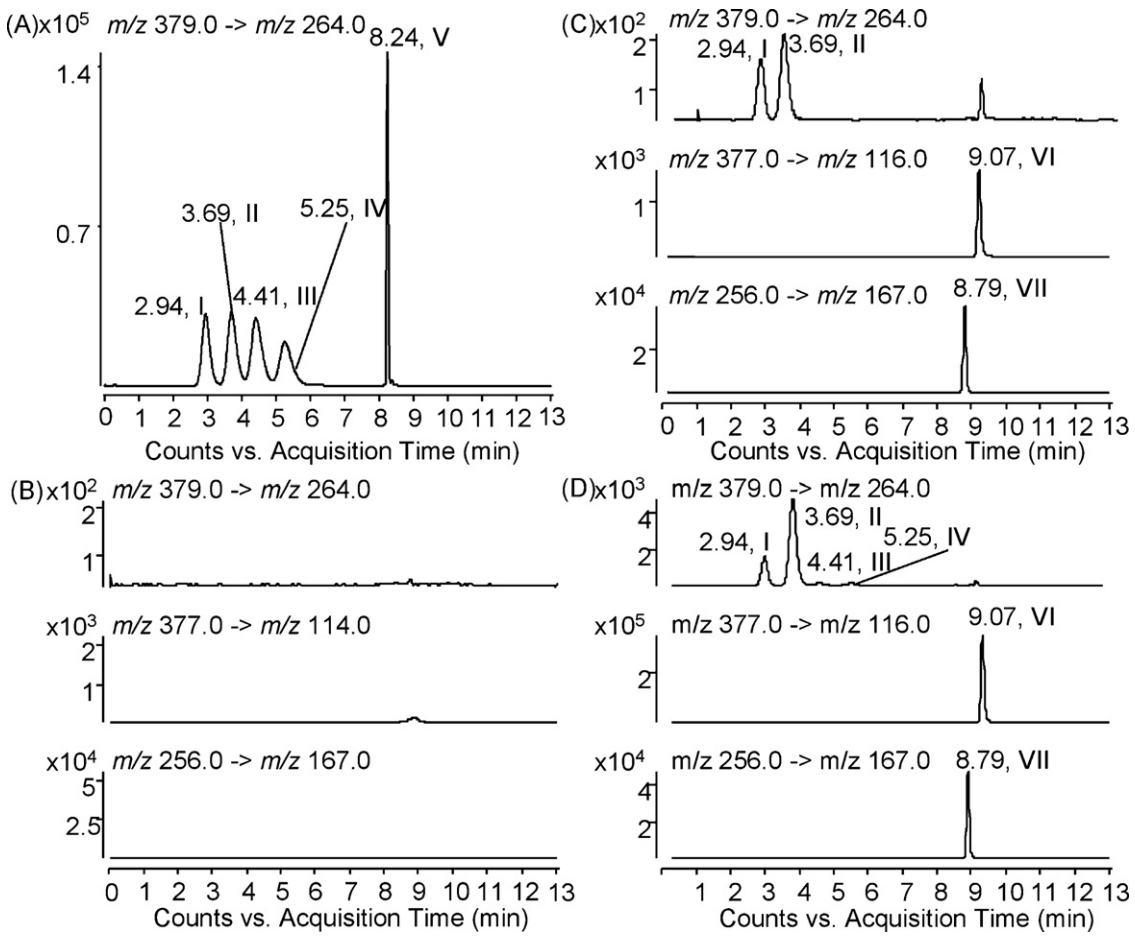

Fig. 3. Representative chromatograms for *E*-4-HDMB (I), *E*-3-HDMB (II), *Z*-3-HDMB (III), *Z*-4-HDMB (IV), 2-HDMB (V), bromhexine (VI) and IS (diphenhydramine, VII) in human plasma samples. (A) The MRM chromatograms of *E*-4-HDMB, *Z*-4-HDMB, *E*-3-HDMB, *Z*-3-HDMB, and 2-HDMB; (B) the MRM chromatograms of the blank plasma sample; (C) the MRM chromatograms of blank plasma spiked with bromhexine (0.15 ng/mL), *E*-4-HDMB (0.050 ng/mL), *E*-3-HDMB, (0.10 ng/mL), and diphenhydramine (IS, 10 ng/mL); (D) the MRM chromatograms of a plasma sample taken from a volunteer 2 h after oral administration of 8 mg bromhexine.

Table 1
Accuracy and precision for the analysis of bromhexine, *E*-4-HDMB, and *E*-3-HDMB in human plasma (in pre-study validation).

Compound	Nominal plasma concentration (ng/mL)	Mean measured concentration (ng/mL)	Relative error (%)	Intra-day RSD (%)	Inter-day RSD (%)
Bromhexine	0.150	0.146	−3.0	5.2	8.8
	0.300	0.291	−3.1	7.7	5.8
	3.00	2.97	−0.9	4.9	4.5
	27.0	26.5	−1.9	8.3	4.1
E-4-HDMB	0.0500	0.0511	2.2	3.6	3.6
	0.100	0.101	1.5	11.9	5.1
	1.00	1.01	0.9	2.4	5.3
	9.00	9.01	0.1	12.8	5.4
E-3-HDMB	0.100	0.104	4.0	7.2	6.9
	0.200	0.205	2.7	10.7	4.5
	2.00	2.04	2.2	3.3	5.4
	18.0	18.6	3.3	10.0	2.4

Table 2
Recovery (%) of bromhexine, *E*-4-HDMB, *E*-3-HDMB and diphenhydramine in human plasma (three concentrations, $n = 6$).

	Conc. of bromhexine (ng/mL)			Conc. of *E*-4-HDMB (ng/mL)			Conc. of *E*-3-HDMB (ng/mL)			Conc. of diphenhydramine (ng/mL)
	0.300	3.00	27.0	0.100	1.00	9.00	0.200	2.00	18.0	10.0
	70.2	70.1	61.6	63.7	71.9	57.1	62.5	67.6	57.3	91.2
	63.8	69.9	66.4	62.6	65.7	63.9	61.2	60.7	59.8	85.1
	69.2	69.9	62.7	69.2	63.2	59.7	67.5	58.6	55.7	87.3
	63.2	71.9	62.7	62.9	67.2	61.2	61.7	61.7	56.4	86.4
	62.5	72.1	63.0	65.9	68.4	60.3	63.6	63.3	56.2	87.1
	65.7	71.6	62.1	62.2	74.2	60.8	59.4	69.3	56.5	89.9
n	6	6	6	6	6	6	6	6	6	6
Mean (%)	65.8	70.9	63.1	64.4	68.4	60.5	62.6	63.5	57.0	87.8
SD (%)	3.2	1.1	1.7	2.7	4.1	2.2	2.8	4.1	1.5	2.3

Table 3
Matrix effect (Matrix factor[a] (%)) of bromhexine, *E*-4-HDMB, *E*-3-HDMB and diphenhydramine in human plasma (two concentrations, $n = 6$).

	Conc. of bromhexine (ng/mL)		Conc. of *E*-4-HDMB (ng/mL)		Conc. of *E*-3-HDMB (ng/mL)		Conc. of diphenhydramine (ng/mL)
	0.300	27.0	0.100	9.00	0.200	18.0	10.0
	93.8	97.5	83.8	90.4	84.3	87.0	106
	86.6	96.4	86.6	96.0	86.0	90.4	105
	93.5	96.5	96.1	96.1	92.7	93.8	104
	90.3	97.1	95.0	94.1	104	87.4	101
	87.0	97.7	89.1	97.6	87.8	96.5	100
	88.5	95.3	87.2	94.7	87.8	93.6	100
n	6	6	6	6	6	6	6
Mean (%)	89.9	96.7	89.6	94.8	90.4	91.4	103
SD (%)	3.2	0.9	4.9	2.5	7.3	3.8	2.7

[a] Matrix factor = (peak area of the analyte from the spike-after-protein precipitation sample/peak area of the standard solution) × 100%.

($n = 6$), respectively. The matrix factors of *E*-4-HDMB, determined at two concentrations (0.10 and 9.0 ng/mL), were 89.6 ± 4.9% and 94.8 ± 2.5% ($n = 6$), respectively. The matrix factors of *E*-3-HDMB, determined at two concentrations (0.20 and 18 ng/mL), were 90.4 ± 7.3% and 91.4 ± 3.8% ($n = 6$), respectively. The matrix factor of the IS was 103 ± 2.7%.

The results showed that using the present RRLC–MS/MS method, the MEs of bromhexine, *E*-4-HDMB, *E*-3-HDMB, and IS could be ignored. The matrix effect data are shown in Table 3.

3.4. Application of the method to a pharmacokinetic study in healthy subjects

The validated analytical method was applied to the assay of bromhexine and the major metabolites in human plasma after a single oral administration of 8 mg bromhexine to five healthy male human subjects. The plasma samples were processed based on the proposed extraction protocol for quantification. The method allows the determination of bromhexine, *E*-4-HDMB, and *E*-3-HDMB up to 24 h after an oral administration.

Profiles of the mean plasma concentration of bromhexine, *E*-4-HDMB, and *E*-3-HDMB versus time are shown in Fig. 4. The main pharmacokinetic parameters of bromhexine, *E*-4-HDMB, and *E*-3-HDMB in five male subjects are presented in Table 4.

After oral administration of 8.0 mg bromhexine to five healthy male subjects, the $AUC_{0-24\,h}$ values for bromhexine, *E*-4-HDMB, and *E*-3-HDMB were found to be 93.5 ± 31.9, 34.0 ± 14.5, and 15.8 ± 6.89 ng h/mL, respectively. The C_{max} values for bromhexine, *E*-4-HDMB, and *E*-3-HDMB were found to be 24.6 ± 5.16, 3.11 ± 1.13, and 5.36 ± 2.55 ng/mL, respectively. Plasma concentration of bromhexine, *E*-4-HDMB, and *E*-3-HDMB declined with $t_{1/2}$ of 3.6 ± 0.5, 8.4 ± 2.7, and 6.4 ± 2.5 h, respectively.

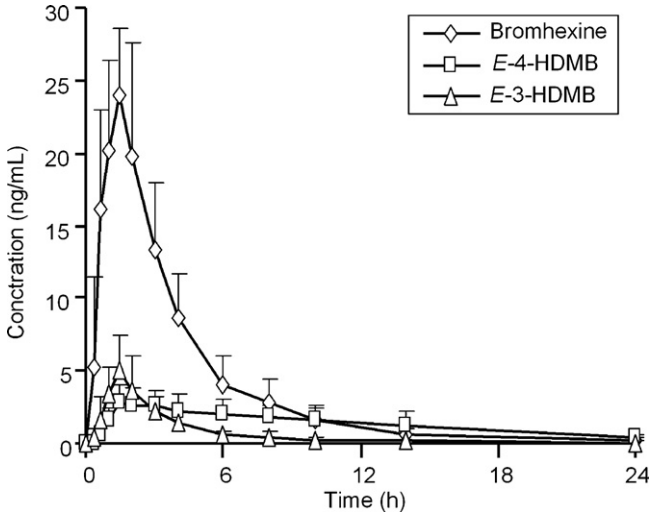

Fig. 4. Mean plasma concentration–time curve of bromhexine, *E*-4-HDMB, and *E*-3-HDMB in healthy subjects ($n = 5$, mean ± SD).

Table 4
The main pharmacokinetic parameters of bromhexine and the major metabolites after an oral administration of 8 mg bromhexine to five healthy subjects.

Parameter	Bromhexine	*E*-4-HDMB	*E*-3-HDMB
C_{max} (ng/mL)	24.6 ± 5.16	3.11 ± 1.13	5.36 ± 2.55
$AUC_{0-24\,h}$ (ng h/mL)	93.5 ± 31.9	34.0 ± 14.5	15.8 ± 6.89
$AUC_{0-\infty}$ (ng h/mL)	94.2 ± 32.2	39.3 ± 15.4	16.3 ± 7.33
T_{max} (h)	1.4 ± 0.5	2.8 ± 1.9	1.5 ± 0.4
$t_{1/2}$ (h)	3.6 ± 0.5	8.4 ± 2.7	6.4 ± 2.5

Kim et al. reported that after oral administration of 30 mg ambroxol (*E*-4-HDMB) to 20 volunteers, C_{max} was 43.5 ng/mL (33.9–55.8 ng/mL) and T_{max} was 1.74 h (1.36–2.25 h) [8]. Hu et al. reported that after oral administration of 90 mg ambroxol to 24 volunteers, C_{max} was 183.23 ± 66.65 ng/mL, T_{max} was 2.13 ± 0.81 h, and $t_{1/2}$ was 7.48 ± 1.98 h [9]. Compared with *E*-4-HDMB, which was orally administrated as an independent drug, the concentration of it as a metabolite of bromhexine of 8 mg oral dose was much lower, while $t_{1/2}$ was similar.

Comparison of bromhexine, *E*-4-HDMB and *E*-3-HDMB should be considered as major metabolites in human circulation ($AUC_{0-24\,h}$ ratios 64.7:24.2:11.1). According to the T_{max} and $t_{1/2}$ data, the formation and elimination of *E*-3-HDMB were more rapid than those for *E*-4-HDMS. The $t_{1/2}$ values for both *E*-3-HDMB and *E*-4-HDMB were longer than that for bromhexine.

In our study, the concentrations of *E*-hydroxylation metabolites were far higher than the corresponding *Z*-hydroxylation metabolites, probably because of the specificity of the enzymes involved in bromhexine metabolism.

4. Conclusion

Four metabolites of bromhexine were observed in human plasma. *E*-4-HDMB and *E*-3-HDMB were major metabolites in human plasma, while concentrations of *Z*-4-HDMB and *Z*-3-HDMB were quite low. Because *E*-4-HDMB was marketed, its isomers may also have activity. The pharmacokinetics of bromhexine, *E*-4-HDMB, and *E*-3-HDMB in healthy subjects after an oral administration of 8 mg bromhexine was characterized, which possesses a guiding significance in the clinical use of bromhexine. In the future, when studying the pharmacokinetics of bromhexine, its major metabolites should also be considered.

Acknowledgements

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