BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **22**: 1108–1114 (2008)
Published online 27 May 2008 in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/bmc.1032

ORIGINAL RESEARCH

Rapid and sensitive liquid chromatography tandem mass spectrometry method for the quantification of ambroxol in human plasma

Wanqun Hu, Yu Xu,* Fei Liu, Aixiang Liu and Qingxiang Guo*

Department of Chemistry, University of Science and Technology of China, Hefei 230026, People's Republic of China

Received 11 January 2008; revised 2 February 2008; accepted 4 February 2008

ABSTRACT: A sensitive, specific and rapid high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) method was described and validated for the quantification of ambroxol in human plasma using enalaprilat as the internal standard (IS). Chromatographic separation was performed on a Lichrospher CN column with a mobile phase of methanol and water (containing 0.1% formic acid) (70:30, v/v). The total run time was 5.0 min for each sample. The analytes was detected by mass spectrometry with electrospray ionization source in positive selected reaction monitoring mode. The precursor-fragment ion reaction for ambroxol was *m/z* 378.9 → 263.8, and for IS was *m/z* 349.0 → 205.9. The linearity was established over the concentration range of 1.56–400.00 ng/mL. The inter-day and the intra-day precisions were all within 10%. A simple protein precipitation with methanol was adopted for sample preparation. The extraction recoveries of ambroxol and IS were higher than 90.80%. The validated method was successfully applied in pharmacokinetic study after oral administration of 90 mg ambroxol to 24 healthy volunteers. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: ambroxol; pharmacokinetic; LC-MS/MS

INTRODUCTION

Ambroxol, *trans*-4-(2-amino-3, 5-dibromobenzyl)-aminocyclohexanol hydrochloride, is a compound with potent mucolytic activity, for which it is used as a broncho-secretolytic and expectorant in therapeutics. It stimulates the transportation of the viscous secretion in the respiratory organs and increases its mobility. It is administered as hydrochloric salt in daily doses of 30.00–120.00 mg using mostly oral formulations like tablets and syrups (Hwang *et al.*, 2005).

Several methods have been published for determination of ambroxol in pharmaceutical preparations and biological materials, including spectroanalysis (Hwang *et al.*, 2005; Szostak and Mazurek, 2004; Perez-Rui *et al.*, 1996; Dincer *et al.*, 2003), high-performance liquid chromatography (HPLC) with UV detection and potentiometric detection (Dincer *et al.*, 2003; Qi *et al.*, 2004; Heinanen and Barbas, 2001; Bazylak and Nagels, 2003), sequential injection chromatographic (SIC) method (Satinsky *et al.*, 2005, 2006), capillary isotachophoresis (ITP; Pospisilova *et al.*, 2001) and capillary electrophoresis (CE; Perez-Rui *et al.*, 2000; Perez-Rui *et al.*, 1997). These reported methods are not ideal for pharmacokinetic studies beacause of their high detection limits, time-consuming sample preparation procedures and long chromatographic run times. Recently new methods based on liquid chromatography tandem mass spectrometry have been reported (Kim *et al.*, 2003; Su *et al.*, 2007) with a high sensitivity. The limits of quantitation (LOQ) of the assays were 0.2 and 1.0 ng/mL using 1 and 0.5 mL plasma aliquots, respectively. However, they needed a relatively large amount of extraction solvent to extract the analyte from the plasma. In addition, multi-step processing steps are not suitable for high-throughput analysis in pharmacokinetic studies. Therefore, a simple and sensitive method for quantification ambroxol in human plasma is needed.

Herein we establish a highly selective, sensitive and powerful LC-MS/MS method, which has some advantages such as a single-step sample preparation procedure, lower plasma sample volume (0.1 mL) and better ability to analyze large numbers of samples. The validated method is simple, rapid and the specifications are suitable for ambroxol quantification in human plasma and application in pharmacokinetic studies.

*Correspondence to: Yu Xu and Qingxiang Guo, Department of Chemistry, University of Science and Technology of China, Hefei 230026, People's Republic of China.
E-mail: xyu@ustc.edu.cn; qxguo@ustc.edu.cn

Abbreviations used: CE, capillary electrophoresis; ITP, capillary isotachophoresis; SIC, sequential injection chromatographic.

Contract/grant sponsor: NSFC; Contract/grant number: 20332020.
Contract/grant sponsor: University of Science and Technology of China.

EXPERIMENTAL

Chemicals and reagents. Ambroxol and enalaprilat (IS) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol of HPLC grade was obtained from Merck (Darmstadt, Germany). Formic acid of HPLC grade was purchased from TEDIA Corporation (Fairfield, USA). Distilled water, prepared from demonetarized water, was employed throughout the study. Blank plasma was provided by The First Affiliated Hospital of Anhui Medical University (Hefei, China).

Apparatus. A TSQ Quantum Ultra AM triple-stage quadrupole tandem mass spectrometer (Thermo Finnigan, USA), together with electrospray ionization (ESI) source, a Finnigan Surveyor LC pump and Finnigan Surveyor autosampler, was used for LC-MS/MS analysis. Data acquisitions were performed with Xcalibur 1.4 software (Thermo Finnigan, USA). The LC-MS/MS system was operated at ambient temperature 20°C.

LC-MS/MS conditions. Chromatographic separation of the analytes from potential interfering material was performed on a Lichrospher CN column (150 × 4.6 mm i.d., 5 μm; Jiangshu Hanbang Science & Technology Co. Ltd, China) with a mixture of methanol and water (containing 0.1% formic acid) (70:30, v/v) as the isocratic mobile phase which delivered at a flow-rate of 1.0 mL/min. The mobile phase was further divided approximately to a rate of 0.2 mL/min prior to ESI source.

MS detection with ESI source was performed in positive ion mode, using selected reaction monitoring (SRM). The precursor-fragment ion reaction for ambroxol was *m/z* 378.9 → 263.8 and for enalaprilat was *m/z* 349.0 → 205.9. The product ion spectra of $[M + H]^+$ ions of ambroxol and IS are shown in Fig. 1(A) and (B), respectively. The tuning parameters were optimized for both ambroxol and IS by infusing a mixture solution (containing 1 μg/mL of both analyte and IS) into the mass spectrometer. Parameters were summarized in the following: spray voltage, 4100 V; capillary temperature, 320°C; nitrogen was used as both sheath and auxiliary gas at pressures (arbitrary units) of 38 and 2, respectively. Argon was used as collision gas at a pressure of 1.6 mTorr. Collision energies of 25 eV were used for both ambroxol and IS. The scan width for SRM was 0.2, and scan time was 0.5 s. The peak width settings (full width at half maximum; FWHM) for both Q1 and Q3 were 0.7 Th.

Sample processing. The IS solution (10 μL; 300 ng/mL enalaprilat in methanol) was added to a 0.1 mL aliquot of plasma. The samples were briefly mixed. Then 300 μL of methanol was added to precipitate proteins. The mixture was vortex-mixed for 3 min and centrifuged for 10 min at 13,400***g***. The upper clean solution layer was collected, and a 10 μL aliquot of solution was injected into the LC-MS/MS system for analysis.

Calibration standards and quality control samples. A stock solution of ambroxol at a concentration of 4 μg/mL was prepared in methanol as a stock solution; IS was also prepared (6 μg/mL) in methanol and was further diluted with methanol to give a concentration of 300 ng/mL during all analyses. Calibration curve samples at concentrations of 1.56, 3.13, 6.25, 12.50, 25.00, 50.00, 100.00, 200.00 and 400.00 ng/mL of ambroxol were freshly prepared by spiking blank plasma and the analyses were performed in triplicate for each concentration.

The quality control (QC) samples were prepared in quintuplicate using a different stock solution of ambroxol to obtain plasma concentrations of 3.13, 25.00 and 350.00 ng/mL, representing low-, medium- and high-concentration QC samples, respectively. The spiked plasma samples (standards and quality controls) were prepared freshly for each analytical batch along with the unknown samples. All standards stock solutions were prepared once a month and stored at –20°C.

Calibration curves. For the calibration curves, peak area ratios (drug: IS) were plotted against ambroxol plasma concentrations. Linear standard curves were calculated using weighted least-squares ($1/x^2$) linear regression. To evaluate linearity, plasma calibration curves were prepared and assayed in triplicate on three separate days. In addition, blank plasma was also analyzed to confirm absence of interferences.

Precision and accuracy. Precision and accuracy were assessed in conjunction with the linearity studies by determining QC samples using quintuplicate ($n = 5$) preparations of spiked plasma samples at three concentration levels on three separate days. The accuracy, i.e. percentage concentration deviation, was expressed by (mean observed concentration – spiked concentration)/(spiked concentration) × 100%, and the precision was assessed in terms of the relative standard deviation (RSD) of the measured concentrations. The acceptable criterion was 15% or better.

Recovery and matrix effect. For the evaluation of recovery, blank human plasma was processed according to the sample preparation procedure described above. The supernatants were solvents with the addition of appropriate standards at concentrations corresponding to the final concentration of the pretreated plasma samples. These spike-after-precipitation samples represented 100% recovery. The precipitation recoveries for ambroxol were determined by comparing the mean peak areas of nine precipitated low-, medium- and high-QC samples to mean peak areas of nine spike-after-precipitation samples at the same concentrations.

Owing to the components of the sample matrix, signal suppression or enhancement may occur. Matrix effect was evaluated by comparing peak areas of ambroxol obtained from the spike-after-precipitation samples with those from the pure standard solutions at the same concentrations.

Stability. Ambroxol stability in plasma was assessed by analyzing QC samples in quintuplicate ($n = 5$), after exposure to different conditions of time and temperature. The results were compared with those of freshly prepared QC samples, and the percentage concentration deviation was calculated.

Pharmacokinetic study. The study was planned and performed according to the current GCP guidance. Twenty-four healthy male Chinese volunteers (aged 18–24, body weight 62–75 kg) were selected as subjects for the study. Subjects were included based on their medical history, clinical examination results and routine laboratory test results. All eligible subjects provided written informed consent for participation

DOI: 10.1002/bmc

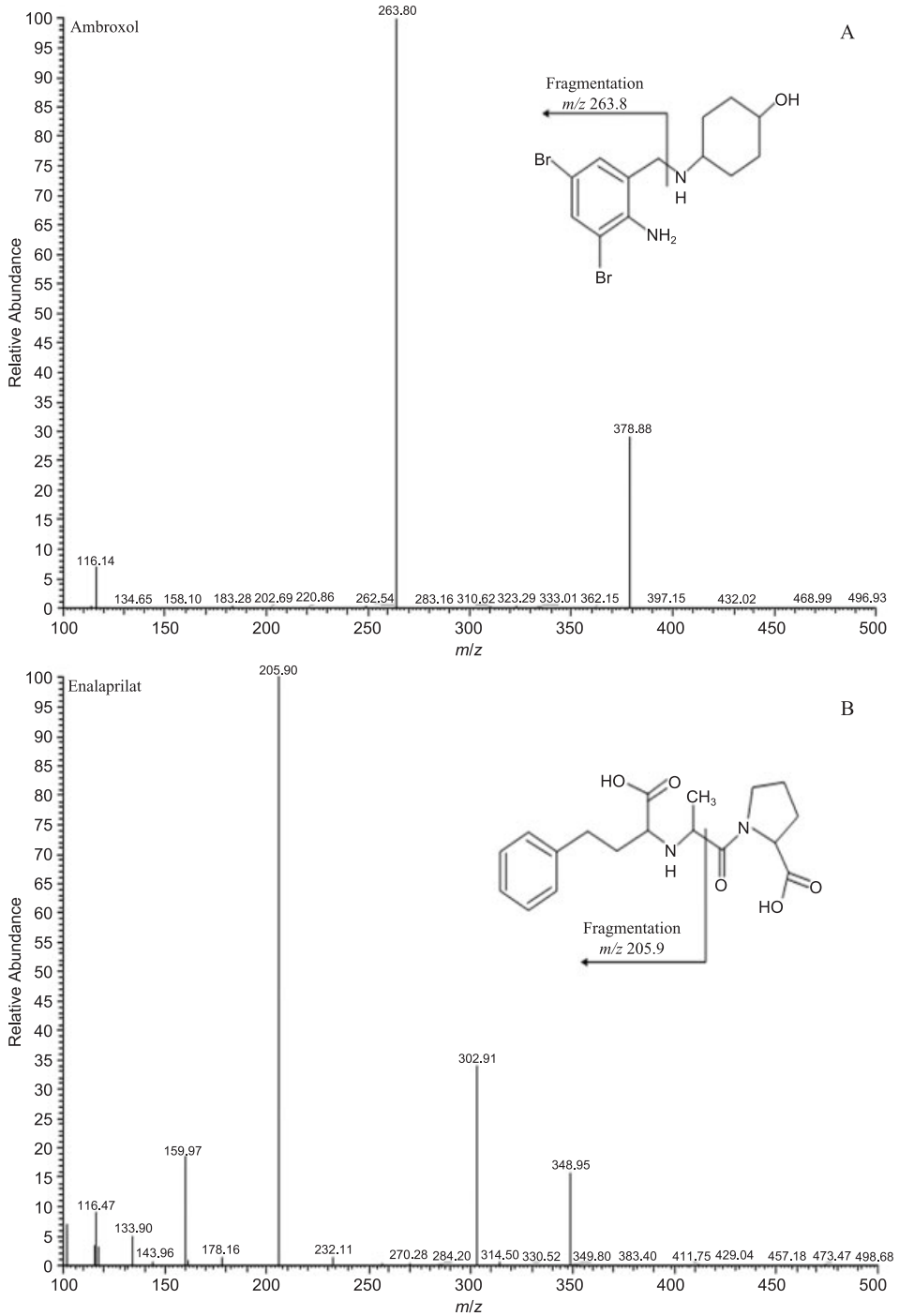

Figure 1. Production spectra of the $[M + H]^+$ ions of (A) ambroxol and (B) enalaprilat.

in the study. A 3 × 3, crossover, randomized, open-label design was used. Subjects were randomly assigned to receive reference formulation followed by test formulation with a 3-week washout period among three doses. After a 12 h (overnight) fast, subjects received a single dose of 90 mg oral dose of ambroxol with 200 mL of water. Venous blood samples (3 mL) were collected in heparinized tubes pre-dose 0 h and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 9.0, 12.0, 15.0 and 24.0 h, thereafter. Plasma was immediately separated by centrifugation at 4000***g*** for 10 min and stored at –20°C.

RESULTS AND DISCUSSION

Sample preparation

There are complex matrices such as fluids, tissues and organs in biological samples. In order to remove possibly interfering matrix components and increase the selectivity and sensitivity, sample preparation is essential before samples are analyzed.

DOI: 10.1002/bmc

Liquid–liquid extraction and direct protein precipitation with methanol for sample preparation of ambroxol in human plasma were investigated. Two sample preparation methods could give satisfactory linearity over the range 1.56–400.00 ng/mL. Direct protein precipitation with methanol was chosen as sample preparation method since it can save considerable time and simplify the operating process. Different volumes of methanol were evaluated for efficiency of protein precipitation. It was found that three times the plasma volume can precipitate the plasma proteins completely, and the chromatographic behavior of the analytes was not deteriorated by this procedure with satisfactory recovery for the analyte from 95.62 to 97.81% at levels of 3.13, 25.00 and 350.00 ng/mL (QC).

LC-MS/MS conditions

Ambroxol has nitrogen and oxygen atoms in its structure. Those heteroatoms easily obtain a proton which can give stronger signal intensity in the positive mode. Because of strong polarity of ambroxol, ESI yielded higher signals for *m/z* 378.9 compared with APCI. The collision-induced dissociation (CID) of protonated ambroxol (*m/z* 378.9) resulted in loss of the 4-aminocyclohexanol and produced the main fragment ion at *m/z* 263.8, the signal intensity of which was highest of all fragment ions. Therefore, the fragment ion at *m/z* 263.8 was chosen as quantitative ion of the measurement. Similarly, the fragmentation pathway for IS was also searched. The most abundant fragment ion was *m/z* 349.0. In order to improve the sensitivity, the ESI source and CID parameters for transition *m/z* 378.9 $\rightarrow$ 263.8 (ambroxol) and *m/z* 349.0 $\rightarrow$ 205.9 (IS) were further tuned.

A mixture of methanol–water (containing 0.1% formic acid) (70:30, v/v) was chosen as the isocratic mobile phase. Formic acid (0.1%) added to water can accelerate the ionization of ambroxol and IS in positive ion mode. The analyte was separated on a Lichrospher CN column (150 $\times$ 4.6 mm i.d., 5 μm). The mobile phase delivered at a flow-rate of 1.0 mL/min and was further divided approximately to 0.2 mL/min prior to ESI source. As a result, the maximum peak responses and symmetrical chromatographic peaks were obtained.

Selectivity

The selectivity of the method was examined by comparing the chromatograms of six individual human blank plasma samples before and after spiking with ambroxol and IS. The six individual human blank plasma samples were prepared and analyzed, and there were no endogenous substances in blank plasma at the same retention times as ambroxol and IS. A typical chromatogram for blank human plasma is shown in Fig. 2(A). Figure 2(B) shows the chromatogram of blank human plasma spiked with ambroxol (1.56 ng/mL) and IS (300 ng/mL). Figure 2(C) shows the chromatogram for a volunteer plasma sample 2 h after an oral dose of 90 mg ambroxol.

Linearity and the LLOQ

A calibration curve was constructed from a blank plasma sample (a plasma sample processed without drug and IS), and nine plasma samples (the plasma samples processed with drug and IS) over the concentration range 1.56–400.00 ng/mL for the analyte. Peak area ratios (drug:IS) were plotted against ambroxol plasma concentrations, and fitted by weighted ($1/x^2$) least-squares linear regression. To evaluate linearity, plasma calibration curves were prepared and assayed in triplicate on three consecutive days. A typical standard curve was $y = (0.035 \pm 0.004)x + (0.003 \pm 0.011)$. Correlation coefficients ranged from 0.998 to 0.999.

The lower limit of quantitation (LLOQ), defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy and precision, was established at 1.56 ng/mL (S/N > 10) in human plasma. The intra-day precision (RSD) at the LLOQ ($n = 5$) was 9.17 %, and the intra-day accuracy (RE %) was –2.98%. The inter-day precision was 10.38%, and the accuracy (RE %) was –1.05%.

Precision and accuracy

The intra-day precision and accuracy of the method were evaluated by analyzing QC samples in quintuplicate at three levels (3.13, 25.00 and 350.00 ng/mL). The inter-day precision and accuracy were observed over three separate batches by analysis of 45 QC samples. Precision and accuracy of the assay, described as relative stand deviation (RSD) and relative error (RE%), are summarized in Table 1. The intra-day precision ranged from 4.46 to 9.24% and accuracy ranged from –4.99 to 0.80%. The inter-day precision was in the range 4.52–7.12%, and accuracy was in the range of –5.71–2.87%.

Recovery and matrix effect

Direct precipitation protein with methanol was chosen for processing plasma samples and gave satisfactory recovery. The recoveries of ambroxol were 95.62, 96.50 and 97.81% at three QC concentrations (3.13, 25.00 and 350.00 ng/mL). The recovery was 90.80% for IS (300 ng/mL).

For LC-MS/MS analysis with ESI source, signal suppression or enhancement may occur because of co-eluting endogenous components existing in the sample matrix. In this assay, these potential matrix effects were evaluated by comparing peak areas of ambroxol

DOI: 10.1002/bmc

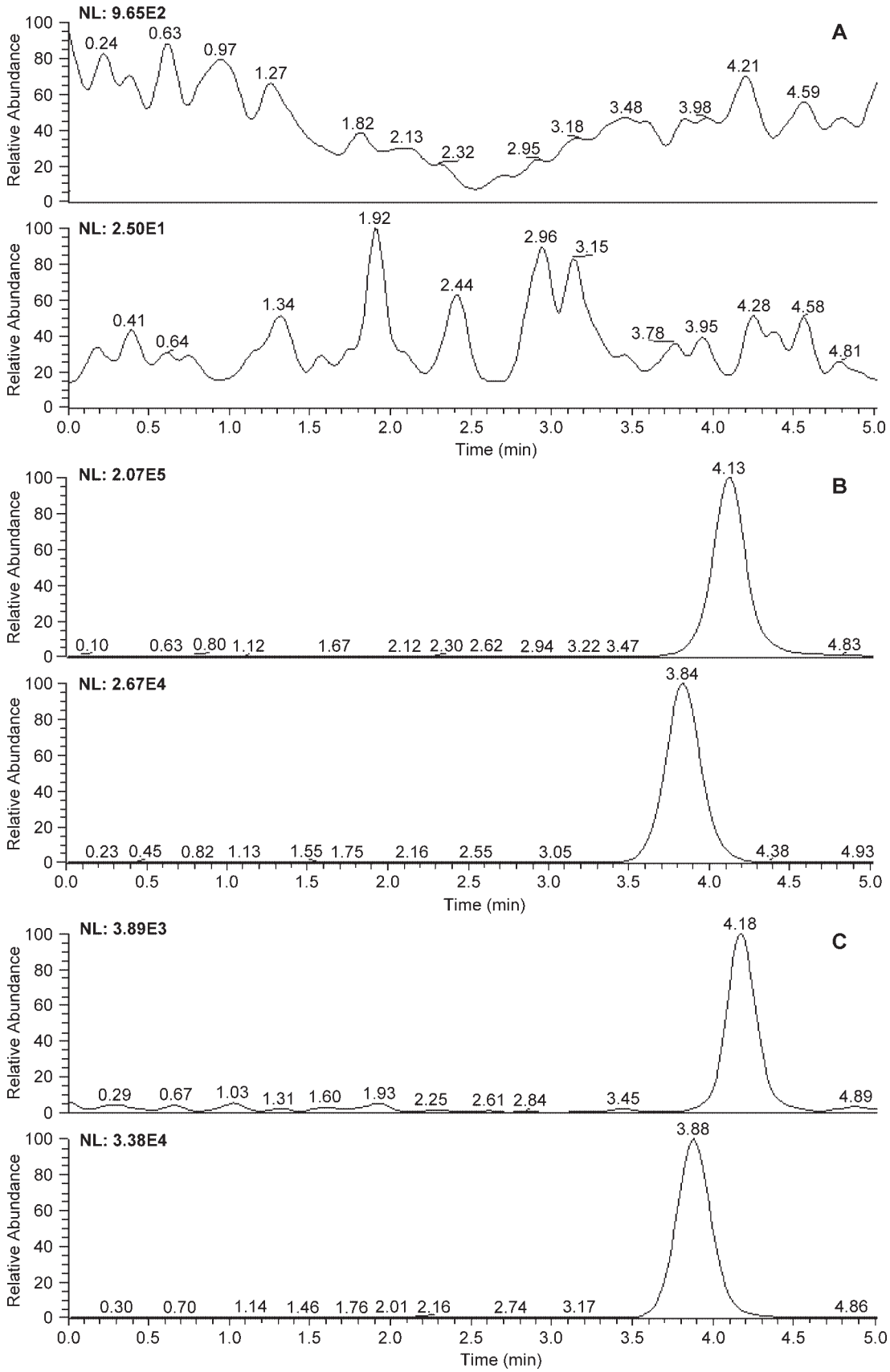

Figure 2. SRM chromatograms for ambroxol and enalaprilat (IS) in human plasma: (A) blank plasma, (B) blank plasma spiked with standard ambroxol (1.56 ng/mL) and IS (300 ng/mL), (C) human plasma sample after administration of ambroxol (2 h) and spiked with IS.

DOI: 10.1002/bmc

Table 1. Precision and accuracy for assay of ambroxol in human plasma

Nominal C (ng/mL)	Intra-day (n = 5)			Inter-day (n = 5)		
	Found C (ng/mL)	RSD (%)	RE (%)	Found C (ng/mL)	RSD (%)	RE (%)
3.13	3.15 ± 0.29	9.24	0.64	3.21 ± 0.20	6.12	2.56
25	23.75 ± 1.73	7.30	–4.99	23.57 ± 1.07	4.52	–5.71
350	339.63 ± 15.15	4.46	–2.96	340.82 ± 24.28	7.12	–2.62

RSD, relative standard deviation; RE, relative error.

Table 2. Stability for assay of ambroxol in human plasma (n = 5)

Conditions	Nominal C (ng/mL)	Found C (ng/mL)	Precision (RSD %)	RE (%)
Short-term Stability at room temperature for 24 h	3.13	3.23 ± 0.20	6.20	3.19
	25	24.85 ± 1.51	6.08	–0.58
	350	356.14 ± 17.87	5.02	1.75
Three freeze–thaw cycles	3.13	3.45 ± 0.22	6.33	10.22
	25	24.13 ± 1.28	5.32	–3.48
	350	357.43 ± 17.96	5.03	2.12
Long-term stability at –20°C for 30 days	3.13	3.21 ± 0.17	5.16	2.56
	25	25.60 ± 2.34	9.12	2.38
	350	387.36 ± 11.99	3.10	10.67
Autosampler stability at 20°C for 12 h	3.13	2.90 ± 0.14	4.88	–7.33
	25	24.00 ± 1.00	4.16	–4.01
	350	353.21 ± 9.64	2.73	0.92

RSD, relative standard deviation; RE, relative error.

Table 3. Pharmacokinetic parameters of three oral formulations of single-dose ambroxol 90 mg in healthy Chinese volunteers (n = 24)

Parameter	Test A (T_A)	Test B (T_B)	Reference (R)	T_A/R	T_B/R
C_{max} (ng/mL)	162.29 ± 55.21	183.92 ± 79.10	183.23 ± 66.65	0.98 ± 0.44	1.07 ± 0.48
T_{max} (h)	1.92 ± 0.96	2.29 ± 1.01	2.13 ± 0.81	1.11 ± 0.79	1.19 ± 0.58
AUC_{0-t} (ng/mL h)	171.44 ± 332.02	1418.07 ± 573.64	1244.06 ± 352.40	0.99 ± 0.33	1.15 ± 0.33
$AUC_{0-\infty}$ (ng/mL h)	1281.31 ± 359.04	1572.87 ± 645.46	1422.03 ± 441.25	0.95 ± 0.31	1.11 ± 0.31
$T_{1/2}$	6.69 ± 1.54	7.08 ± 2.08	7.48 ± 1.98		

obtained from the spike-after-precipitation samples with those from the pure standard solutions at the same concentrations. Five independent plasma samples were detected in three QC concentrations. The respective ratios were 98.41, 95.53 and 96.63% for ambroxol at the three QC levels (3.13, 25.00 and 350.00 ng/mL), while the value was 94.31% for IS at 300 ng/mL. The results indicated that there were no significant matrix effects under optimal conditions.

Stability

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 24 h). The long-term stability of ambroxol in human plasma was evaluated by storing plasma samples at below –20°C for one month. The samples underwent three freeze–thaw cycles and were examined to study the freeze–thaw stability. The post-preparative stability of ambroxol was investigated by placing prepared samples into the autosampler at 20°C for 12 h. The results for the stability of ambroxol in different conditions are given in Table 2. These tests indicated reliable stability behavior under the experimental conditions of the regular analytical procedure.

Application

The developed and validated method has been successfully applied to quantification of ambroxol concentration in human plasma after the administration of a 90 mg oral dose. The major pharmacokinetic parameters are listed in Table 3. The mean plasma concentration–time curve is presented in Fig. 3.

DOI: 10.1002/bmc

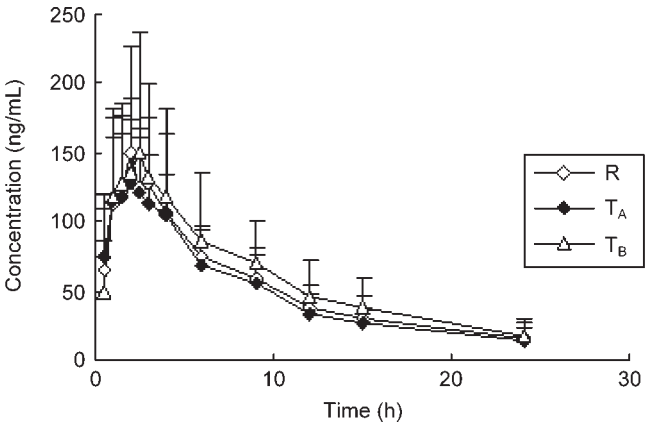

Figure 3. Mean plasma concentration–time profile of ambroxol from 24 healthy volunteers following a single oral dose of 90 mg. T_A, T_B = test formulation, R = reference formulation.

CONCLUSION

An LC-MS/MS method has been described for the determination of ambroxol in human plasma. Compared with earlier published methods, the current method offered interesting features: an excellent sensitivity, simple sample preparation procedure and relatively short chromatographic run time. The proposed method was readily applicable to the determination of ambroxol in human plasma in pharmacokinetic study.

Acknowledgements

The authors gratefully acknowledge the NSFC (no. 20332020) and University of Science and Technology of China for financial support.

REFERENCES

Bazylak G and Nagels LJ. Simultaneous high-throughput determination of clenbuterol, ambroxol and bromhexine in pharmaceutical formulations by HPLC with potentiometric detection. *Journal of Pharmaceutical and Biomedical Analysis* 2003; **32**: 887–903.

Dincer Z, Basan H and Göger NG. Quantitative determination of ambroxol in tablets by derivative UV spectrophotometric method and HPLC. *Journal of Pharmaceutical and Biomedical Analysis* 2003; **31**: 867–872.

Heinanen M and Barbas C. Validation of an HPLC method for the quantification of ambroxol hydrochloride and benzoic acid in a syrup as pharmaceutical form stress test for stability evaluation. *Journal of Pharmaceutical and Biomedical Analysis* 2001; **24**: 1005–1010.

Hwang MS, Cho S, Chung H and Woo YA. Nondestructive determination of the ambroxol content in tables by Raman spectrosocopy. *Journal of Pharmaceutical and Biomedical Analysis* 2005; **38**: 210–215.

Kim H, Yoo JY, Han SB, Lee HJ and Lee KR. Determination of ambroxol in human plasma using LC-MS/MS. *Journal of Pharmaceutical and Biomedical Analysis* 2003; **32**: 209–216.

Perez-Ruiz T, Martinez-Lozano C, Sanz A and San Miguel MT. Automatic extraction-spectrophotometric method for the determination of ambroxol in pharmaceutical preparations. *Talanta* 1996; **43**: 1029–1034.

Perez-Ruiz T, Martinez-Lozano C, Sanz A and Bravo E. Determination of bromhexine and ambroxol in pharmaceutical dosage forms, urine and blood serum. *Journal of Chromatography B* 1997; **692**: 199–205.

Perez-Ruiz T, Martinez-Lozano C, Sanz A and Bravo E. Sensitive method for the determination of ambroxol in body fluids by capillary electrophoresis and fluorescence detection. *Journal of Chromatography B* 2000; **742**: 205–210.

Pospisilova M, Polasek M and Jokl V. Determination of ambroxol or bromhexine in pharmaceuticals by capillary isotachophoresis. *Journal of Pharmaceutical and Biomedical Analysis* 2001; **24**: 421–428.

Qi ML, Wang P, Cong RH and Yang JJ. Simultaneous determination of roxithromycin and ambroxol hydrochloride in a new tablet formulation by liquid chromatography. *Journal of Pharmaceutical and Biomedical Analysis* 2004; **35**: 1287–1291.

Satinsky D, Dos Santos ML, Sklenarova H, Solich P, Montenegro M and Araujo AN. Sequential injection chromatographic determination of ambroxol hydrochloride in pharmaceutical preparations. *Talanta* 2005; **68**: 214–248.

Satinsky D, Huclova J, Ferreira RLC, Montenegro M and Solich P. Determination of ambroxol hydrochloride, methylparaben and benzoic acid in pharmaceutical preparations based on sequential injection technique coupled with monolithic column. *Journal of Pharmaceutical and Biomedical Analysis* 2006; **40**: 287–293.

Su FL, Wang F, Gao W and Li HD. Determination of ambroxol in human plasma by high performance liquid chromatography–electrospray ionization mass spectrometry (HPLC-MS/ESI). *Journal of Chromatography B* 2007; **853**(1–2): 364–368.

Szostak R and Mazurek S. FT-Raman quantitative determination of ambroxol in tablets. *Journal of Molecular Structure* 2004; **704**: 229–233.