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Determination of ambroxol in human plasma using LC-MS/MS

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Abstract

A sensitive and selective liquid chromatographic method coupled with tandem mass spectrometry (LC-MS/MS) was developed for the quantification of ambroxol in human plasma. Domperidone was used as internal standard, with plasma samples extracted using diethyl ether under basic condition. A centrifuged upper layer was then evaporated and reconstituted with 200 μl methanol. The reconstituted samples were injected into a C_{18} XTerra MS column (2.1 × 30 mm) with 3.5 μm particle size. The analytical column lasted for at least 600 injections. The mobile phase was composed of 20 mM ammonium acetate in 90% acetonitrile (pH 8.8), with flow rate at 250 μl/min. The mass spectrometer was operated in positive ion mode using turbo electrospray ionization. Nitrogen was used as the nebulizer, curtain, collision, and auxiliary gases. Using MS/MS with multiple reaction monitoring (MRM) mode, ambroxol was detected without severe interferences from plasma matrix. Ambroxol produced a protonated precursor ion ($[M+H]^+$) at *m/z* 379 and a corresponding product ion at *m/z* 264. And internal standard (domperidone) produced a protonated precursor ion ($[M+H]^+$) at *m/z* 426 and a corresponding product ion at *m/z* 174. Detection of ambroxol in human plasma was accurate and precise, with quantification limit at 0.2 ng/ml. This method has been successfully applied to a study of ambroxol in human specimens.

Keywords: Tandem mass spectrometry; Liquid chromatography; Multiple reaction monitoring (MRM); Ambroxol; Domperidone

1. Introduction

Ambroxol, *trans*-4-(2-amino-3,5-dibromobenzylamino)cyclohexanol hydrochloride, is a compound with potent mucolytic activity that is used as an expectorant and bronchosecretolytic in therapeutics [1,2]. It is a pharmacologically active metabolite of bromhexine [3], *N*-cyclohexyl-*N*-methyl-(2-amino-3,5-dibromobenzyl)amino hydrochloride. Ambroxol stimulates the transportation of viscous secretion in the respiratory organs and reduces secretion stagnation.

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Previous studies have reported several different methods for the qualitative and quantitative detection of ambroxol in human plasma and pharmaceutical formulations, e.g. thin layer chromatography (TLC) [4,5], flow injection [6,7], gas chromatography (GC) with electron capture detection [8,9], high performance liquid chromatography (HPLC) with UV and amperometric detection [10–13], GC with mass spectrometry detection [14], and capillary electrophoresis (CE) [15–17]. However, these published methods are not ideal for pharmacokinetics work, because they are time-consuming, i.e. derivatization step, arduous sample preparation, and long chromatographic run times. Likewise, they need a relatively large amount of sample to reach a low quantification limit. In addition, detection of ambroxol using liquid chromatographic method coupled with tandem mass spectrometry (LC-MS/MS) has yet to be reported.

Therefore, this study established a novel quantification method for detecting ambroxol in human plasma using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS). This method is not only selective and reliable; it is also faster and simpler compared with other recently reported methods. Likewise, this method has been successfully applied to pharmacokinetic studies to determine the concentration of ambroxol in human plasma.

2. Experimental

2.1. Chemicals and solutions

Ambroxol and domperidone (internal standard) were obtained from Hanmi pharmaceutical Co., Ltd. (Kyunggi-Do, South Korea). HPLC grade methanol, acetonitrile, and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA), while sodium borate, diethyl ether, and ammonium acetate were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

To prepare standard stock solutions, appropriate amounts of ambroxol hydrochloride and domperidone were weighed accurately and dissolved separately in methanol in 50 ml methanol. From these stock solutions, working standard solutions of ambroxol and other analytes were prepared through sequential dilutions with mobile phase to produce varying concentrations.

2.2. LC-MS/MS conditions

Tandem mass spectrometry (MS/MS) was performed on API 2000 triple quadruple mass spectrometer equipped with turbo electrospray ion source (Perkin–Elmer Life Science, Inc., Boston, MA, USA). Twenty microliters of sample were delivered into the ESI source using the micro-LC (Series 200, Perkin–Elmer Life Science, Inc.) and autosampler (Series 200, Perkin–Elmer Life Science, Inc.) with C_{18} XTerra MS column (2.1×30 mm, 3.5 μm particle size). The mobile phase used after degassing was composed of 20 mM ammonium acetate in 90% acetonitrile (pH 8.8, using ammonium hydroxide solution), with total running time of 2.0 min and flow rate of 250 μl/min.

The following instrument settings were used: turbo ionspray interface maintained at 300 °C with nitrogen nebulization; nitrogen at a pressure of 50 psi; and turbo ion-spray drying gas (N_2) at a pressure of 80 psi. In detecting ambroxol using the MRM scan mode with positive ion detection, the following parameter settings were used: collision-activated dissociation gas (CAD) maintained at a pressure of 7 psi; curtain gas (CUR) at a pressure of 30 psi; turbo ionspray voltage at 5500 V; declustering potentials (DP) at 25 V; focusing potential (FP) at 360 V; entrance potentials (EP) at −11 V; collision cell entrance potentials (CEP) at 24 V; collision energies (CE) at 28 V; collision cell exit potentials (CXP) at 12 V; deflector (DF) at −150 V; channel electron multiplier (CEM) at 2500 V; and dwell time of 0.20 s. On the other hand, the detection of internal standard (domperidone) using MRM scan mode with positive ion detection had the following parameter settings: DP at 60 V; FP at 360 V; EP at −9 V; CEP at 21 V; CE at 43 V; CXP at 6 V; and dwell time of 0.20 s.

Mass calibration was performed through the infusion of a 10^{-4} M polypropylene glycols 2000 (PPG 2000) solution into the ionspray source. The peak widths of precursor and product ions were

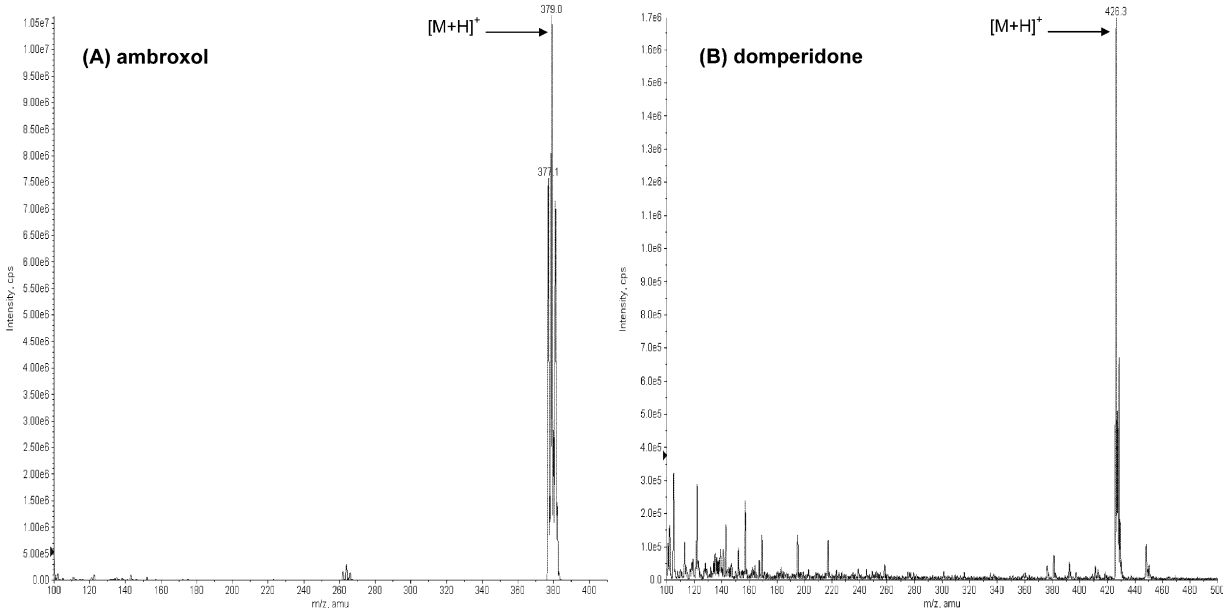

Fig. 1. Full scan first quadrupole spectrum of (A) ambroxol and (B) domperidone (internal standard).

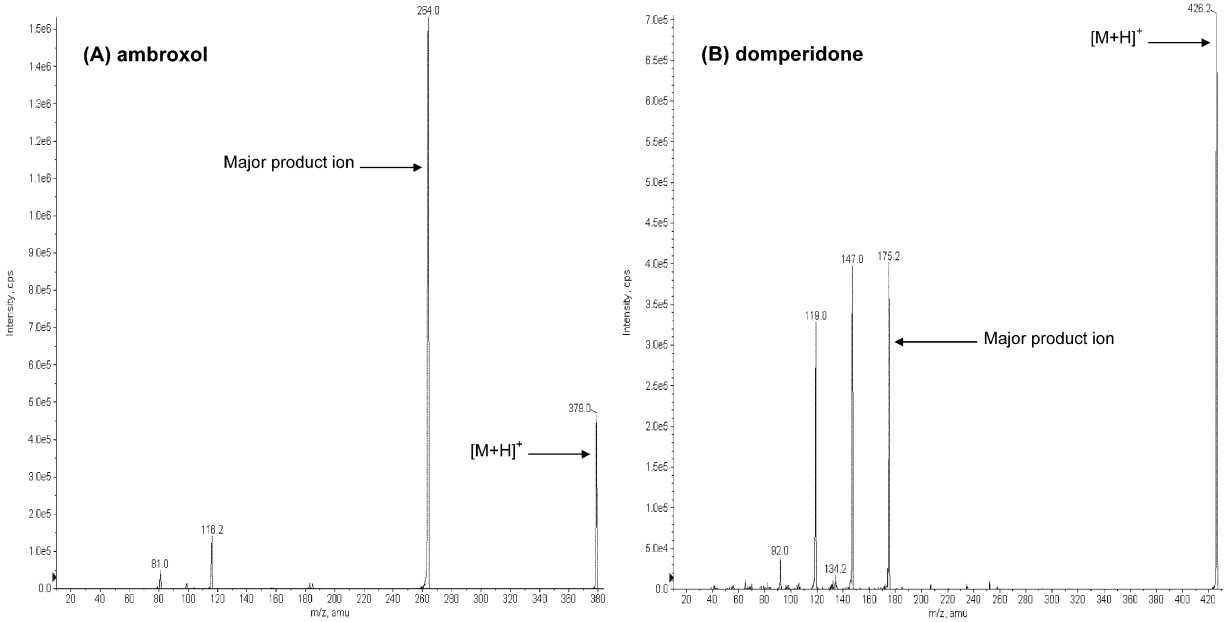

Fig. 2. Product ion spectrum of (A) ambroxol and (B) domperidone (internal standard). (A) Ambroxol (379/264 amu), time (min); (B) domperidone (426/175 amu), time (min).

maintained at ~0.7 um half-height in the MRM mode.

2.3. Sample preparation

1 ml plasma specimens were pipetted into conical glass tubes and spiked with 0.1 ml of 2 μg/ml internal standard solution. After adding 1 ml of 0.025 M sodium borate solution, the alkalinized plasma was vortex-mixed for 10 s and added to 6 ml diethyl ether. The sample was then shacked for 10 min. The two phases were separated through centrifugation at $2000 \times g$ for 10 min. The upper organic layer (diethyl ether layer) was transferred into another conical glass tube and completely evaporated at 40 °C under a stream of nitrogen. The dry residue was then reconstituted with 200 μl methanol and injected into the LC-MS/MS.

2.4. Validation procedures

To assess the intraday precision and accuracy of the method, five replicates of plasma standards at eight concentrations (0.2, 1.0, 2.0, 5.0, 20, 50, 100, and 200 ng/ml) were analyzed and used to construct the calibration curve. Similarly, five replicates of the quality control samples at varying concentrations of 0.2, 1.0, 2.0, 5.0, 20, 50, 100, and 200 ng/ml were analyzed to determine the initial

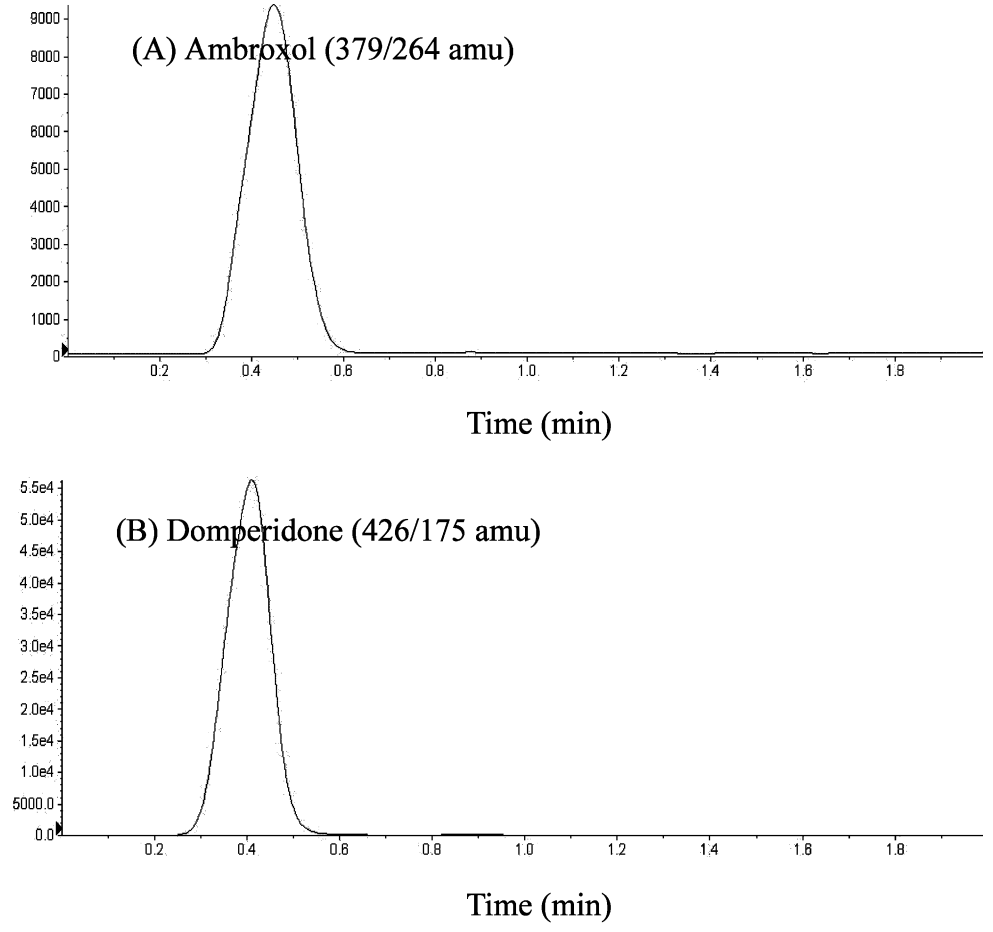

Fig. 3. LC-MS/MS chromatograms of (A) ambroxol and (B) domperidone in human plasma. Ambroxol (0.2 ng/ml, S/N ratio = 10), time (min).

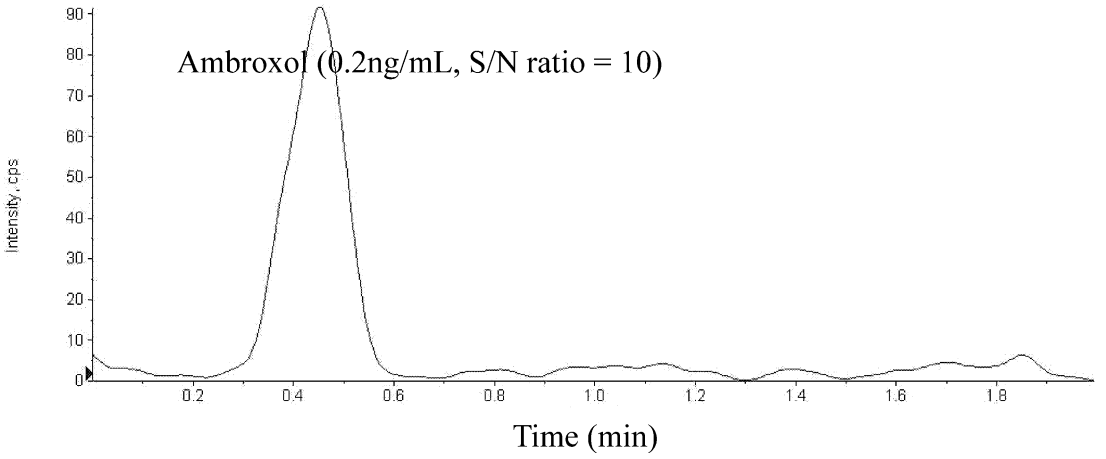

Fig. 4. Chromatogram of limit of quantification of ambroxol in human plasma.

interday precision and accuracy. The accuracy was expressed as (mean observed concentration)/(spiked concentration) × 100%, with the precision expressed using the relative standard deviation (R.S.D.).

For the plasma QC samples, 400 μl of the appropriate QC working solution (0.02, 0.1, 0.2, 0.5, 2, 5, 10, and 20 mg/ml) was added to 50 ml polypropylene tubes containing 39.6 ml human control plasma to yield QC concentrations of 0.2, 1.0, 2.0, 5.0, 20, 50, 100, and 200 ng/ml. The bulk QC plasma samples were then vortex-mixed, with 1.5 ml aliquots transferred into 2 ml microcentrifuge tubes and capped and stored at −70 °C.

2.5. Pharmacokinetic study

A single 30 mg dose of ambroxol was administered orally to 20 volunteers who were advised about the nature and purpose of the study. The volunteers possessed good health and have not taken any medication for at least 2 weeks prior to the study. The group consisted of healthy males with a mean age of 24.7±2.8, mean weight of 67.6±6.0 kg, and mean height of 173.5±3.6 cm. Blood samples were taken 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, and 24 h after ingestion using heparin vacutainer collection tubes. Plasma was obtained through centrifugation at 2000 × *g* for 10 min. Plasma specimens were then stored at −70 °C prior to analysis.

3. Results and discussion

Currently, ambroxol is assayed in human plasma and urine and pharmaceutical formulations using GC with electron capture detection [8,9] and HPLC with UV and amperometric detection [10–13]. However, these methods are time-consuming, i.e. derivatization step, arduous

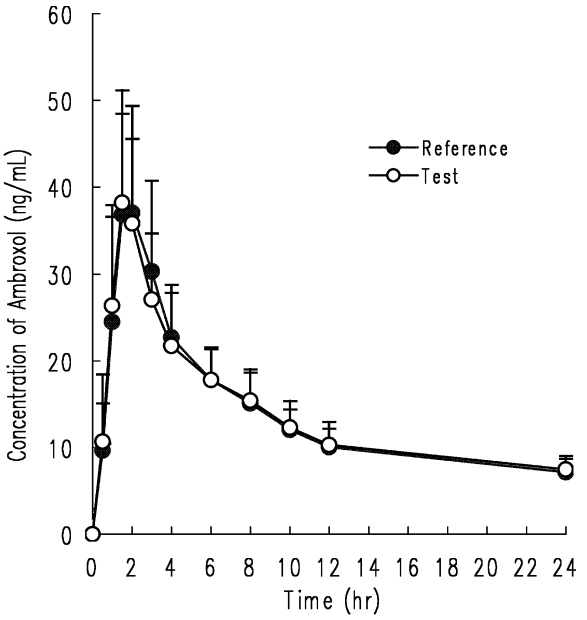

Fig. 5. Plasma concentration of ambroxol in human plasma–time curve.

Table 1
Intraday precision and accuracy of measurement of ambroxol

Ambroxol nominal concentration (ng/ml)	Ambroxol (mean ± S.D.)[a] calculated concentration (ng/ml)	Accuracy (%)	Precision (%R.S.D.)
0.20	0.22 ± 0.01	110.0	6.43
1.00	1.06 ± 0.06	106.4	5.74
2.00	2.02 ± 0.06	100.9	2.94
5.00	5.21 ± 0.16	104.1	3.00
20.0	22.5 ± 1.0	112.7	4.41
50.0	55.3 ± 1.4	110.5	2.48
100	115 ± 2	114.6	2.01
200	206 ± 5	102.9	2.46

[a] Five replicates at each concentration level ($n = 5$).

sample preparation, and long chromatographic run times. Likewise, they need a relatively large amount of sample to reach a low quantification limit. Therefore, the study established a highly sensitive and selective method for detecting ambroxol in human plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for pharmacokinetics studies.

Under turbo ionspray ionization condition, ambroxol and domperidone (internal standard) exhibited favorable sensitivity in positive ion detection mode. Fig. 1(A) shows the full scan first quadrupole positive ion spectrum of ambroxol, while Fig. 1(B) shows the full scan first quadrupole positive ion spectrum of internal standard. Both formed protonated molecules $[M+H]^+$ as major ion peaks. Ambroxol produced a protonated precursor molecule ($[M+H]^+$) at m/z 379 and a major product ion at m/z 264. On the other hand, domperidone produced a precursor molecule ($[M+H]^+$) at m/z 426 and a major product ion at m/z 174. Fig. 2(A and B) show the product ion spectrum of $[M+H]^+$ for ambroxol and domperidone. Multiple reaction monitoring (MRM) mode was used for quantification, thereby obtaining a very high sensitivity. Using MS/MS with MRM mode, ambroxol and internal standard were detected without severe interferences from plasma matrix (Fig. 3(A and B)).

For the chromatographic analysis and electrospray ionization of ambroxol, the study initially attempted to develop a reversed phase chromatographic system with methanol and phosphate buffer or methanol, acetonitrile, and the same buffer solution as that in the eluents. Such system yielded an assay where ambroxol and internal standard were co-eluted. Moreover, formation of precipitates was observed in the mobile phase in

Table 2
Interday precision and accuracy of measurement of ambroxol

Ambroxol nominal concentration (ng/ml)	Ambroxol (mean ± S.D.)[a] calculated concentration (ng/ml)	Accuracy (%)	Precision (%R.S.D.)
0.20	0.22 ± 0.01	112.0	2.45
1.00	1.06 ± 0.06	106.0	5.62
2.00	2.01 ± 0.07	100.5	3.37
5.00	5.11 ± 0.25	102.3	4.93
20.0	23.0 ± 0.5	114.9	2.35
50.0	55.9 ± 0.8	111.9	1.48
100	112 ± 6	111.8	5.04
200	208 ± 10	104.0	4.98

[a] Five replicates at each concentration level ($n = 5$).

Table 3
Pharmacokinetic parameters of reference ambroxol and test ambroxol

No	Reference			Test		
	AUC[a] (ng/ml per h)	C_{max}[b] (ng/ml)	t_{max}[c] (h)	AUC (ng/ml per h)	C_{max} (ng/ml)	t_{max} (h)
Mean	329	43.5	1.74	324	42.3	1.72
Mean−S.D.	276	33.9	1.36	267	33.8	1.33
Mean+S.D.	380	55.8	2.25	393	53.1	2.22

[a] AUC, area under the plasma concentration–time curve extrapolated to infinity.
[b] C_{max}, peak plasma concentration.
[c] T_{max}, time to reach C_{max}.

cases where the amount of organic solvent was increased. Ammonium acetate buffer was, therefore used, since it was easily miscible with organic solvents and improved peak symmetry and ionization. The amount of acetonitrile in mobile phase was then optimized at 90%. Likewise, the pH of the mobile phase was optimized at 8.8 using ammonium acetate and ammonium hydroxide solution. However, the chromatogram of ambroxol showed high sensitivity under these conditions. Fig. 3(A and B) show the LC-MS/MS chromatograms of ambroxol and internal standard.

In addition, the matrix effect was evaluated. The chromatographic conditions may cause co-elution with a number of endogenous interferences that are not detected by MS/MS. However, they may potentially affect the slightly high ionization efficiency of analytes. To solve the problem, plasma samples from different sources (subjects) ($n = 5$) were extracted and then spiked with the same amount of ambroxol. The MS/MS responses and precision were then compared with the same analysis repeated ($n = 5$) after spiking a single source of matrix. If the responses and precision vary among different sources and within a single source, then the matrix effect exists. Otherwise, there is no matrix effect with similar responses and precision. The study was not able to detect a matrix effect.

The intraday precision expressed as %R.S.D. was measured as 2.01–6.43% for 0.2, 1.0, 2.0, 5.0, 20, 50, 100, and 200 ng/ml standard concentrations, with five replicates at each concentration level. The intraday accuracy expressed as a percentage of nominal values was measured as 100.9–114.6% for four standard concentrations, with five replicates at each concentration level. Table 1 shows the intraday precision and accuracy of measurement of ambroxol. The interday precision was measured as 1.48–5.62% for four standard concentrations, with five replicates at each concentration level. Conversely, the interday accuracy was measured as 100.5–114.9% for eight standard concentrations, with five replicates at each concentration level. Table 2 shows the interday precision and accuracy for the measurement of ambroxol.

Standard calibration curves were constructed on different working days using the same human plasma. The response was linear throughout the concentration range of the study, with the coefficient of determination (r^2) greater than 0.9995 for all cases. And the calibration equations was $y = 0.0038x + 0.0037$ (± 0.0023) in human plasma. Based on a signal-to-noise level (S/N) of 9–10, the quantification limit for ambroxol was found to be 0.2 ng/ml upon injection of 20 μl of the sample into the LC-MS/MS system (Fig. 4).

Finally, determining the concentration of ambroxol in human plasma was applied to pharmacokinetic studies. Fig. 5 shows the concentration (mean ± S.D.) of ambroxol in human plasma (20 volunteers)–time curve after administration of a single dose of ambroxol. Table 3 shows the pharmacokinetic parameters of reference ambroxol and test ambroxol. Fig. 5 and Table 3 indicate that the proposed method has been successfully applied to pharmacokinetic studies

to determine the concentration of ambroxol in human plasma.

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