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Short communication

Determination of ambroxol in human plasma by high performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–MS/ESI)

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Abstract

A rapid, sensitive and specific method to determination of ambroxol in human plasma using high performance liquid chromatography coupled with electrospray ionization mass spectrometry (HPLC–MS/ESI) was described. Ambroxol and the internal standard (I.S.), fentanyl, were extracted from plasma by *N*-hexane-diethyl ether (1:1, v/v) after alkalinized with ammonia water. A centrifuged upper layer was then evaporated and reconstituted with 100 μl mobile phase. Chromatographic separation was performed on a BDS HYPERSIL C_{18} column (250 mm × 4.6 mm, 5.0 μm, Thermo electron corporation, USA) with the mobile phase consisting of 30 mM ammonium acetate (0.4% formic acid)–acetonitrile (64:36, v/v) at a flow-rate of 1.2 mL min^{-1}. The total run time was 5.8 min for each sample. Detection and quantitation was performed by the mass spectrometer using selected ion monitoring at *m/z* 261.9, 263.8 and 265.9 for ambroxol and *m/z* 337.3 for fentanyl. The calibration curve was linear within the concentration range of 1.0–100.0 ng mL^{-1} ($r = 0.9996$). The limit of quantification was 1.0 ng mL^{-1}. The extraction recovery was above 83.3%. The methodology recovery was higher than 93.8%. The intra- and inter-day precisions were less than 6.0%. The method is accurate, sensitive and simple for the study of the pharmacokinetics and metabolism of ambroxol.

Keywords: Ambroxol; HPLC–MS/ESI

1. Introduction

Ambroxol is a pharmacologically active metabolite of bromhexine. It is chemically designated as *trans*-4-(2-amino-3, 5-dibromobenzylamino)cyclohexanol hydrochloride (Fig. 1) [1]. It is a compound with potent mucolytic activity that is used as an expectorant and bronchosecretolytic in therapeutics. Ambroxol stimulates the transportation of viscous secretion in the respiratory organs and reduces secretion stagnation [2]. In addition to a mucolytic action, ambroxol has also been reported to have a cough-suppressing effect, antioxidant and anti-inflammatory action [1,3,4]. It is frequently used in the treatment of bronchial asthma and chronic bronchitis, and also used in pulmonary alveolar proteinosis and infant respiratory distress syndrome [5]. In recent studies, it found that ambroxol may be useful for preventing acute upper respiratory diseases (AURDs) [6].

To date, some assays for the determination of ambroxol in human plasma or serum have been reported, including capillary electrophoresis (CE) [7,8], gas chromatography with electron capture detection (GC–ECD) [9,10], GC with mass spectrometry detection (GC–MS) [11], high performance liquid chromatography (HPLC) with UV and electrochemical detection [12–15] methods. However, these published methods [6–14] are not ideal for large number of sample determination, because they are time consuming or costly, i.e. derivatization step, arduous sample preparation and long chromatographic run times. Kim et al. [2] described a liquid chromatography with tandem mass spectrometry (LC–MS/MS) method which achieved better sensitivity. The assay was found to be linear in the range 0.2–200 ng mL^{-1} with a LOQ of 0.2 ng mL^{-1}. However, it used 1 mL plasma aliquot to reach the low quantification limit. In addition, it needed a relatively large amount of extraction solvent to extract the analyte from the plasma (shake for 10 min and centrifuge for another 10 min). Therefore, a simple and valid method for concentration determination of ambroxol in plasma is needed.

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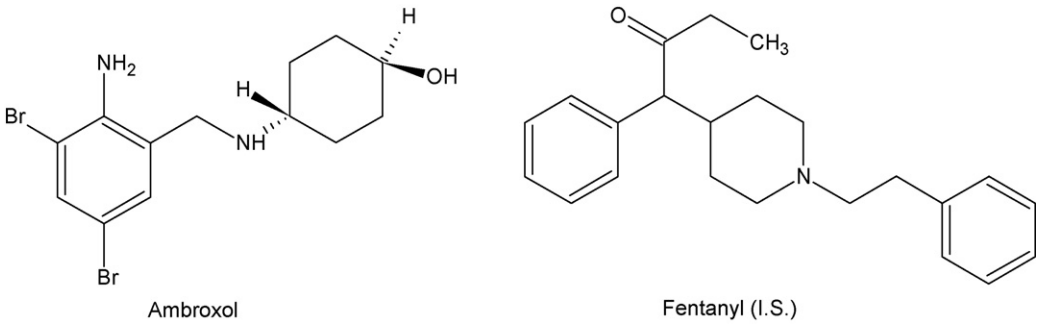

Fig. 1. The chemical structures of ambroxol and fentanyl (I.S.).

In the present article, we establish an innovative simple, rapid and sensitive high performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–MS/ESI) method for the determination of ambroxol in plasma using a simple liquid–liquid extraction. This method has been successfully applied to pharmacokinetic study of ambroxol in healthy subjects.

2. Experimental

2.1. Reagents

Ambroxol (>99.9%) and fentanyl citrate (>99.9%, I.S.) were purchased from National institute for the control of pharmaceutical and biological products (Beijing, China). The primary stock solutions were prepared separately in methanol (220 μg mL^{-1} for ambroxol and 604 μg mL^{-1} for fentanyl citrate). Working solutions of ambroxol were obtained by diluting the stock solutions with distilled water. The I.S. working solution (483.2 ng mL^{-1}) was obtained by diluting the stock solution with ammonia water. All the standard solutions were stored at −20 °C. Acetonitrile, methanol and *N*-hexane (HPLC grade) were purchased from Caledon Laboratories Ltd. (Georgetown, Canada). Ammonium acetate and formic acid were purchased from Tedia Company Inc. (Fair Field, USA). Other AR grade reagents were purchased from Chemical Reagent Factory of Hunan (Changsha, Hunan, China) or Shanghai Experiment Reagent Co. Ltd. (Shanghai, China). Control human plasma was obtained from the healthy subjects.

2.2. Calibration standards and control samples

Routine daily calibration curves were prepared in drug free plasma. Appropriate volumes of working solutions and drug-free human plasma were added to each test tube. Final concentrations were 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng mL^{-1}. Similarly, quality control samples that were run in each assay at concentrations of 2.0, 10.0 and 50.0 ng mL^{-1} were also prepared.

2.3. Chromatographic and Mass/ESI detection conditions

A system of HPLC (Waters 2690, USA)–MS with a Micromass ZQ mass spectrometer (Wythenshawe, Manchester, UK) with mass-selective detector equipped with an electrospray ionization (ESI) ion source was used. COMPAQ Deskpro Workstation and MassLynx^{TM} 3.5 software were utilized. The analytes were separated on a BDS HYPERSIL C_{18} Column (4.6 mm × 50 mm, 5 μm, Thermo electron corporation, USA) with column temperature 45 °C. The mobile phase was water (formic acid: 0.4%, ammonium acetate: 30 mM)–acetonitrile (64:36, v/v) and was filtered using 0.45 μm filters in a Millipore solvent filtration apparatus and was never recirculated. The flow-rate was 1.2 mL/min, and the postcolumn splitting ratio was 3:1. Ionization of the analytes was obtained by electrospray in the positive ion mode (ESI^+). The main working parameters set as follows: capillary voltage, 3.50 kV; cone voltage, 40.00 V; extractor voltage, 1.00 V; source temperature, 105 °C; desolvation temperature, 320 °C; cone gas flow, 70 L/h, desolvation gas flow, 400 L/h. Selected ion recording (SIR) mode was used for quantitation by the protonated molecular ions of each analyte (*m/z* 261.9, 263.8 and 265.9 for ambroxol and *m/z* 337.3 for fentanyl).

2.4. Sample preparation

0.5 mL plasma specimens were pipetted into10 mL conical glass tubes and spiked with 50 μl internal standard working solution. The alkalinized plasma was then vortex-mixed for 10 s and added 3 mL *N*-hexane-diethyl ether (1:1, v/v). After vortex-mixed for 1.5 min, the mixture was centrifuged at 3000 rpm for another 3 min. The upper organic layer was carefully transferred into another conical glass tube and evaporated to dryness at 60 °C under a gentle stream of nitrogen. The dry residue was then reconstituted with 100 μl mobile phase and vortex-mixed for 10 s; 30 μl solution was injected into the HPLC–MS/ESI.

3. Results and discussion

3.1. HPLC- MS/ESI

The HPLC–MS/ESI in the SIR mode provided a highly selective method for the determination of ambroxol and the I.S. The retention times were approximately 3.32 and 5.32 min, respectively. No endogenous substance was observed in the chromatograms of blank plasma. Compared with the published methods [7–15], the chromatographic run of this method was shortened; the complete elution was obtained in less than

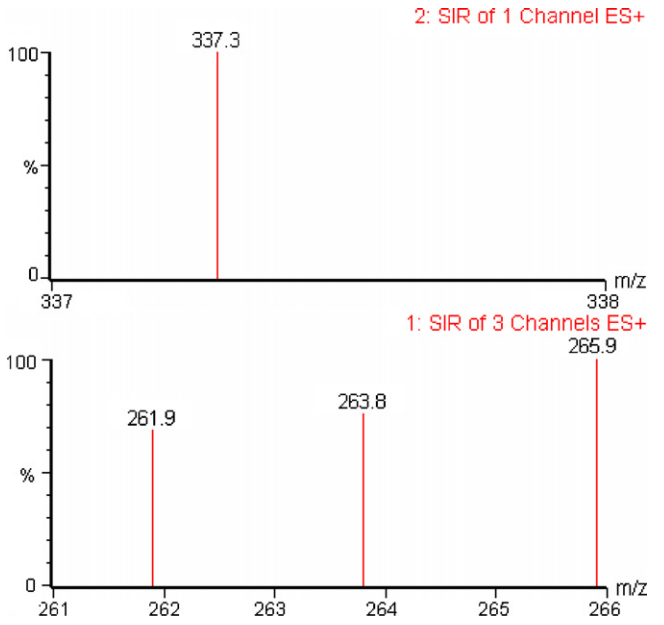

Fig. 2. Full scan ESI + mass spectra of amoroso and I.S. in control human plasma 1: SIR of 3 Channel: ambroxol; 2: SIR of 1 Channel: fentanyl (I.S.).

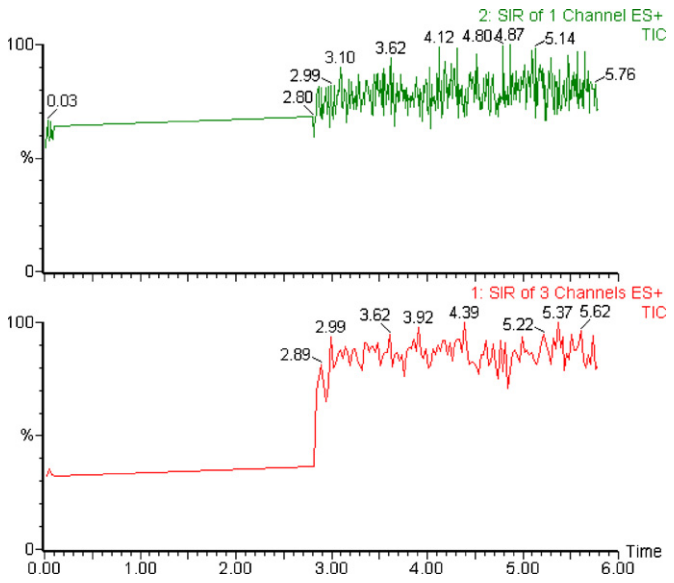

Fig. 3. HPLC-ESI/MS chromatograms of the control human plasma 1: SIR of 3 Channel: ambroxol; 2: SIR of 1 Channel: fentanyl (I.S.).

6.0 min. In our assessment of different mobile phase, 30 mM ammonium acetate (0.4% formic acid)–acetonitrile (64:36, v/v) provided the best effect which can eliminate the baseline noise completely during the chromatographic analysis. Formic acid was added in the mobile phase to adjust the pH, which can increase the sensitivity and shorten the retention times of the analytes. There are two bromines in the chemical constitution of anmbroxol, so ambroxol produced a protonated precursor ion ($[M+H]^+$) at *m/z* 265.9 and two corresponding product ions at *m/z* 263.8 and at *m/z* 261.9. As the ion abundance of the three was all very rich, all ions were chosen as the monitoring objects. The internal standard, fentanyl, produced a precursor molecule ($[M+H]^+$) at *m/z* 337.3. Full scan ESI^+ mass spectra of standards in control human plasma, the chromatograms of control human plasma, standards in control human plasma and samples were shown in Figs. 2–5, respectively. In this study, we utilized a simple one-step extraction method. Among the various extraction solvents, we found *N*-hexane-diethyl ether (1:1, v/v) can got a high recovery (>85%) and only just needed 3 mL. The time of extraction and centrifugation was also shortened. As demonstrated in this assay, this method is sensitive, specific, allowing for analyzing samples in batches and perfectly suitable for a high-throughput routine such as pharmacokinetic studies.

3.2. Matrix suppression

The matrix effect (i.e. potential ion suppression or enhancement effects of co-eluting and undetected matrix components in plasma) was investigated. It was evaluated by comparing the peak area of ambroxol spiked in pre-extracted plasma samples to that of ambroxol spiked in mobile phase at equivalent concentration. In addition, to assess the lot-to-lot matrix variation, plasma samples from different subjects were extracted and then spiked with the same amount of ambroxol. The precision in peak area ratio among the different plasma was calculated as an indicator of the inter-lot matrix variability. In the present study, no matrix components in plasma caused significant changes in the MS response of ambroxol. For the six lots of plasma spiked with 10.0 ng mL^{-1} of ambroxol, the inter-lot variation in peak area ratio (RSD) was 3.3%.

3.3. Validation of the method

The extraction recoveries were determined at three concentration levels by comparing the analyte peak areas obtained from the quality control samples ($n = 5$) after extraction to those obtained from the corresponding unextracted reference standards pre-

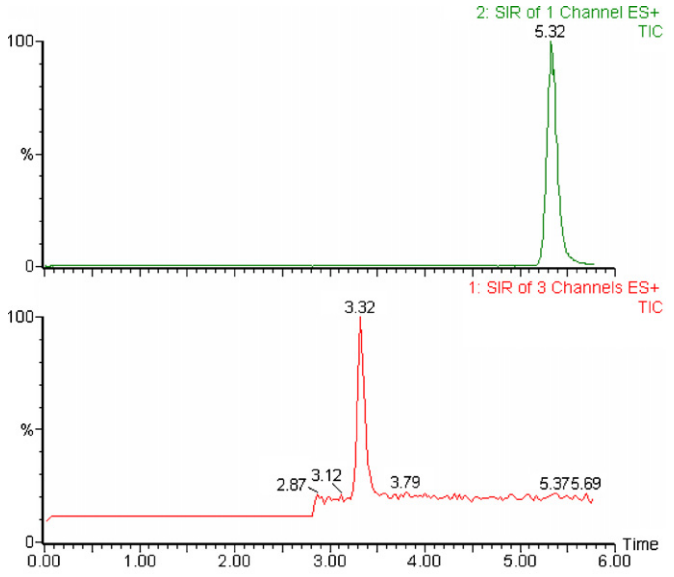

Fig. 4. HPLC–ESI/MS chromatograms of standards in control human plasma 1: SIR of 3 Channel: ambroxol; 2: SIR of 1 Channel: fentanyl (I.S.).

Table 1
Extraction recoveries, methodology recoveries, intra- and inter-day precision

Concentration (ng/mL)	Extraction recoveries ($n=5$)		Methodology recoveries ($n=5$)			Intra-day precision ($n=5$)		Inter-day precision ($n=5$)	
	Mean	RSD (%)	Mean	Recoveries (%)	RSD (%)	Mean	RSD (%)	Mean	RSD (%)
2.0	86.13	3.61	1.88	93.80	1.94	1.88	1.94	1.96	2.58
10.0	87.91	7.77	9.45	94.41	1.77	9.45	1.77	10.05	5.62
50.0	89.48	5.19	48.22	96.37	1.15	48.22	1.15	49.74	3.22

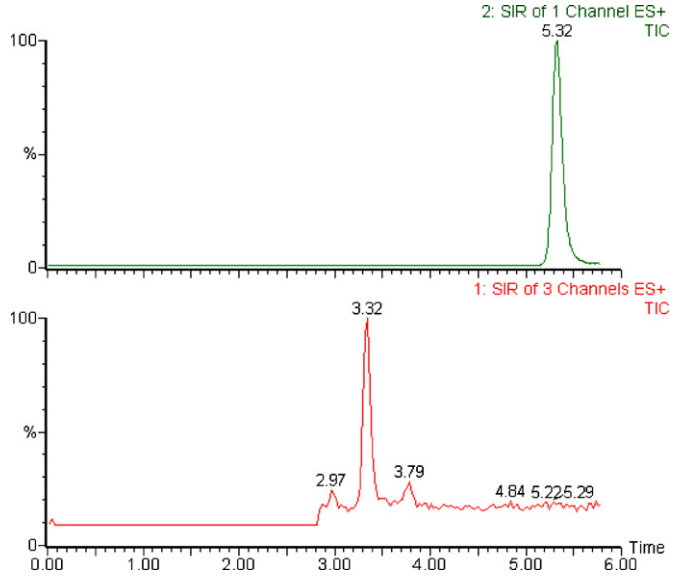

Fig. 5. HPLC–ESI/MS chromatograms of a plasma sample from a healthy subject 1: SIR of 3 Channel: ambroxol.

pared at the same concentrations. The methodology recoveries were measured as the percentage difference from theoretical according to the equation:

$$\text{Methodology recovery}\,(\%) = \left(\frac{\text{concentration}_{\text{measured}}}{\text{concentration}_{\text{theoretical}}}\right) \times 100$$

Precision assays were carried out five times using three different concentrations on the same day and over 5 different days. All the results are summarized in Table 1.

The calibration curve of ambroxol was linear over the concentration range of 1.0–100.0 ng/mL. The equation was $Y = 0.004991\,X + 0.001827$ ($r = 0.9996$). The limit of quantitation (LOQ) validated was 1.0 ng/mL (S/N = 9) and the assay only requires 0.5 mL of plasma. These features are important for clinical situations when blood volume is restricted and yet high assay sensitivity is required.

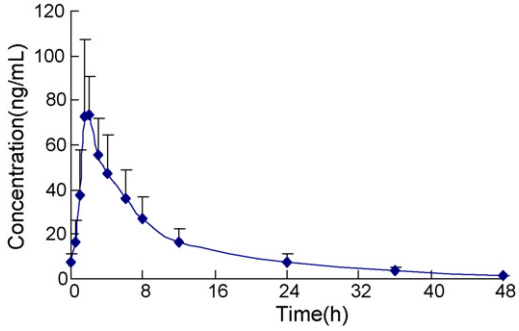

Fig. 6. Mean steady-state plasma concentration–time curve of ambroxol ($n = 24$).

3.4. Stability

Stability quality control plasma samples (2.0, 10.0 and 50.0 ng mL^{-1}) were found stable in plasma when placed in the short-term (32 h) room temperature, four freeze/thaw (−20–25 °C) cycles and stored at −70 °C for 40 days (Table 2).

3.5. Pharmacokinetic study

The assay method was used in a multiple dose pharmacokinetics study of ambroxol in 24 healthy subjects which was approved by the Ethical Committee of XiangYa Second Hospital of Central South University. The subjects possessed good health and have not taken any medication for at least 2 weeks prior to the study. All subjects provided written informed consent prior to participating in the study. The subject received a 60 mg oral daily dose at 7:00 am for 7 days. On day 7, after the 60 mg daily dose at 7:00 am, 4 mL of blood samples were taken before and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36 and 48 h. The mean plasma concentrations versus time profile of ambroxol after administration of a multiple dose of ambroxol were shown in Fig. 4. The maximal steady state plasma concentration of ambroxol averaged 81.5 ± 25.7 ng mL^{-1}, occurring around 1.7 ± 0.3 h post-dosing. The elimination half-life of ambroxol is 8.6 ± 1.5 h. These indi-

Table 2
Stability test of ambroxol

Concentration (ng/mL)	RT ($n=5$)		−70 °C ($n=5$)		Freeze/thaw ($n=5$)	
	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)
2.0	2.03	1.65	2.01	4.01	1.79	4.51
10.0	9.69	4.31	8.97	1.84	8.64	1.91
50.0	49.77	1.63	47.63	1.27	46.88	1.29

cated that the proposed method has been successfully applied to pharmacokinetic studies to determine the concentration of ambroxol in human plasma (Fig. 6).

4. Conclusion

Compared with other methods, HPLC–MS/ESI improved the specificity and sensitivity, shortened the analytical time of the samples and lowered the cost of the experiment. The main aim of the study was to establish a HPLC–MS method that was suitable for determining the concentration of ambroxol in human plasma. The method described here has been found to be specific and accurate in application, and meets the request of the present pharmacokinetic study.

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