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Simultaneous determination of amoxicillin and ambroxol in human plasma by LC–MS/MS: Validation and application to pharmacokinetic study

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ABSTRACT

A rapid, simple and sensitive LC–MS/MS method was developed for simultaneous determination of amoxicillin and ambroxol in human plasma using clenbuterol as internal standard (IS). The plasma samples were subjected to a simple protein precipitation with methanol. Separation was achieved on a Lichrospher C_{18} column (150 mm × 4.6 mm ID, dp 5 μm) using methanol (containing 0.2% of formic acid) and water (containing 0.2% of formic acid) as a mobile phase by gradient elution at a flow rate of 1.0 mL/min. Detection was performed using electrospray ionization in positive ion multiple reaction monitoring (MRM) mode by monitoring the ion transitions from *m/z* 365.9 → 348.9 (amoxicillin), *m/z* 378.9 → 263.6 (ambroxol) and *m/z* 277.0 → 203.0 (IS). Calibration curves were linear in the concentration range of 5–20,000 ng/mL for amoxicillin, and 1–200 ng/mL for ambroxol, with the intra- and inter-run precisions of <9% and the accuracies of 100 ± 7%. The method has been validated and applied to pharmacokinetic studies of compound amoxicillin and ambroxol hydrochloride tablets in healthy Chinese volunteers.

1. Introduction

Amoxicillin (see Fig. 1) ((2*S*,5*R*,6*R*)-6-[(*R*)-(−)-2-amino-2-(*p*-hydroxyphenyl) acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3. 2. 0]heptane-2-carboxylic acid trihydrate) is a semisynthetic antibiotic, an analog of ampicillin, with a broad spectrum of bactericidal activity against many gram-positive and gram-negative microorganisms. It has been extensively used in the treatment of infections of the lower respiratory tract, ear, nose and throat [1,2]. Ambroxol (*trans*-4-(2-Amino-3, 5-dibromobenzyl)-aminocyclo-hexanol hydrochloride) is an expectorant and mucolytic agent, which could reduce the bronchial hyper-reactivity, stimulate the cellular surfactant production and increase the amount of antibiotic penetration [3–6]. And some articles reported that ambroxol could improve the penetration of amoxicillin into lung alveolar cells [4,6]. In view of their synergistic benefit, amoxicillin and ambroxol were often administered concomitantly for treatment of respiratory infection. However, it was still unclear about how and what mechanism of amoxicillin and ambroxol caused these effects. It was very necessary to investigate whether they interacted with each other in pharmacokinetics.

Several analytical methods for amoxicillin and ambroxol determination in human plasma have been reported, including liquid chromatographic coupled to UV detector [7–9] and liquid chromatography–mass spectrometric (HPLC–MS) [10–12]. However, no method is reported till date for simultaneous determination of these two drugs in human plasma. In this study, we report a simple, sensitive and specific LC–MS/MS assay for simultaneous quantification of both the drugs in human plasma and it was suitable for pharmacokinetic study.

2. Experimental

2.1. Chemicals and reagents

Compound amoxicillin and ambroxol hydrochloride tablets (500 mg amoxicillin and 30 mg ambroxol hydrochloride per tablet) were provided by Sichuan Changwei Pharmaceutical Group Co., Ltd. (Sichuan, China). Amoxicillin tablets (250 mg per tablet) were purchased from Harbin Pharmaceutical Group Co., Ltd. (Heilongjiang, China). Ambroxol hydrochloride tablets (30 mg per tablet) were purchased from Beijing Taiyang Pharmaceutical Industry Co., Ltd. (Beijing, China). Chemical reference substances of amoxicillin

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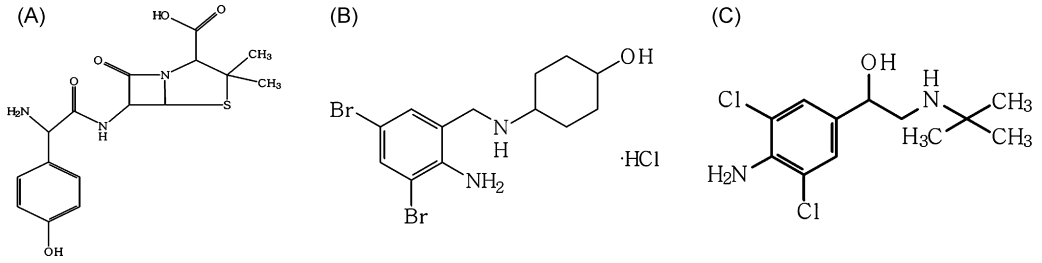

Fig. 1. Chemical structure of amoxicillin (A), ambroxol hydrochloride (B) and clenbuterol (C).

(99.5% purity), ambroxol hydrochloride (99.5% purity) and clenbuterol (IS, 99.7% purity) were provided by Sichuan Changwei Pharmaceutical Group Co., Ltd. (Sichuan, China). Methanol of HPLC grade was obtained from Merck Company (Darmstadt, Germany). Water was deionized and purified by using a Milli-Q system (Millipore, Milford, MA, USA) and was used to prepare all aqueous solutions.

2.2. Instrumentation and chromatographic conditions

The samples were analyzed on the Waters quattro micro LC–MS/MS using a Lichrospher C_{18} (150 mm × 4.6 mm ID, dp 5 μm) column. The mobile phase consisting of mixtures of methanol (containing 0.2 % of formic acid)–water (containing 0.2% of formic acid) was delivered at 1.0 mL/min using a gradient elution (see Table 1), and total run time was 6.5 min.

The instrument was operated in the positive ion mode. Ion scan mode was with the following settings: needle voltage (ISV), 3000 V; nebulizer gas flow rate, 550 L/h; curtain gas flow rate, 50 L/h; collision gas flow rate, 2.26×10^{-3} mbar; resolution, Q1 and Q3; unit dwell time, 200 ms. Analytes were quantificated by multiple reaction monitoring (MRM) employing the following precursor to product ion transitions and parameters: amoxicillin, m/z 365.9 → 348.9 with declustering potential (DP) 17 V and collision energy (CE) 8 eV; ambroxol, m/z 378.9 → 263.6 with DP 23 V and CE 16 eV; clenbuterol, m/z 277.0 → 203.0 with DP 20 V and CE 15 eV.

2.3. Preparation of stock and standard solutions

Stock solutions of amoxicillin and ambroxol were prepared by dissolving the drug in methanol at a concentration of 1 mg/mL and stored in 10 mL flask volumes at −20 °C. Serial (working) dilutions of amoxicillin were prepared at the concentrations of 80, 40, 32, 20, 8, 4, 2, 0.8, 0.4, 0.2, 0.08 and 0.02 μg/mL, respectively. Serial (working) dilutions of ambroxol hydrochloride were prepared at the concentrations of 800, 400, 320, 200, 80, 40, 20, 8, 4 ng/mL, respectively. Stock solution of IS was prepared by dissolving clenbuterol in methanol at a concentration of 800 ng/mL.

Table 1
The gradient elution for simultaneous determination of amoxicillin and ambroxol

Time (min)	A: 0.2% formic acid solution
0.0–1.0	95%
1.0–2.0	95–10%
2.0–4.8	10%
4.8–4.9	10–95%
4.9–6.5	95%

2.4. Sample preparation

The plasma concentrations of amoxicillin and ambroxol hydrochloride were simultaneously determined by using LC–MS/MS method. To an aliquot of 0.4 mL plasma sample in a 2.0 mL plastic centrifuge tube, 50 μL of IS solution (800 ng/mL clenbuterol solution) and 1.2 mL methanol were added and vortex-mixed for 1 min, followed by centrifugation at 16,000 rpm for 10 min. The upper clear layer solution was transferred to an auto-sampler vial for LC–MS/MS analysis.

2.5. Calibration curve

Calibration curves were prepared at the concentration levels of 5, 10, 20, 50, 100, 200, 500, 1000, 2000 5000, 8000, 10,000, and 20,000 ng/mL for amoxicillin and 1, 2, 5, 10, 20, 50, 80, 100 and 200 ng/mL for ambroxol by spiking an appropriate amount of the standard solutions in 0.4 mL blank plasma. The calibration curve was prepared and assayed along with quality control (QC) samples. QC samples were prepared in 0.4 mL blank plasma at three levels of 10, 200, 2000 ng/mL for amoxicillin, and 2, 20, 80 ng/mL for ambroxol, respectively. The plasma samples were stored at −80 °C.

2.6. Specificity

The specificity of the method was tested by screening six different batches of blank human plasma. Each blank sample was tested for interferences in the MRM channels using the proposed extraction procedure and chromatographic/MS–MS conditions, and the results were compared with those obtained for aqueous solution of the analytes at a concentration near to the lower limit of quantification (LLOQ).

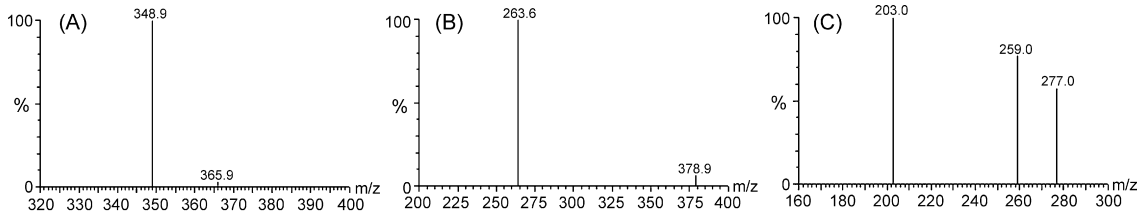

Fig. 2. Full-scan positive ion mass spectra of amoxicillin (A), ambroxol (B) and clenbuterol (C).

2.7. Precision and accuracy

The intra-run precisions and accuracies were estimated by analyzing five replicates containing amoxicillin and ambroxol at three different QC levels, 10, 200 and 2000 ng/mL for amoxicillin and 2, 20 and 80 ng/mL for ambroxol. The inter-run precisions were determinated by analyzing QC samples on three different runs. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal values and a precision of within ±15% relative standard deviation.

2.8. Extraction recovery

The recoveries of amoxicillin and ambroxol were determined by comparing the peak area obtained for QC samples that were subjected to the extraction procedure with those obtained from blank plasma extracts that were spiked post-extraction to the same nominal concentrations (10, 200 and 2000 ng/mL for amoxicillin; 2, 20 and 80 ng/mL for ambroxol).

2.9. Stability

The stability of amoxicillin and ambroxol in plasma under different temperature and timing conditions was evaluated. Plasma samples were subjected to short-term conditions, to long-term storage conditions (−80 °C), and to three freeze-thaw stability studies. The autosampler stability was conducted by re-analyzing extracted kept under the autosampler conditions for 10, 24 and 48 h. All the stability studies were conducted at three concentration levels (10, 200 and 2000 ng/mL for amoxicillin; 2, 20 and 80 ng/mL for ambroxol) with three determinations for each.

2.10. Pharmacokinetic study

Twelve healthy volunteers, including six males and six females, were recruited. All the volunteers were informed the experimental announcements, especially the potential risks. They all provided written informed consent. The study protocols were approved by the Hospital Ethical Review Committee in accordance with the principles of the Declaration of Helsinki and the recommendations of the State Food and Drug Administration of China.

The study was carried out in an open randomized four-way crossover design with blind determination of drug plasma concentrations. The washout period between treatments was one week. All volunteers received a single oral dose of one compound amoxicillin and ambroxol hydrochloride tablet (500 mg + 30 mg per tablet, test formulation, treatment R), two amoxicillin tablets (250 mg per tablet, reference formulation, treatment A), one ambroxol hydrochloride tablet (30 mg per tablet, reference formulation, treatment B), two amoxicillin tablets (250 mg per tablet) and one ambroxol hydrochloride tablet (30 mg per tablet) (combined reference formulations, treatment C), respectively.

Blood samples were collected into heparinized tubes before administration and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 24 and 36 h after administration. Plasma samples were separated immediately by centrifugation at 3500 rpm for 10 min at 4 °C and stored at −80 °C until analysis.

3. Results

3.1. Method development

The electrospray ionization of amoxicillin, ambroxol and the IS produced the $[M+H]^+$ ions at 365.9, 378.9 and 277.0 under positive ionization conditions. The product ion spectra ions at m/z 348.9, m/z 263.6, and m/z 203.0 were produced as the prominent product ions for amoxicillin, ambroxol and the IS (Fig. 2). The quantitative analysis was performed using the MRM mode due to high selectivity and sensitive of MRM data acquisition: m/z 365.9 → 348.9 for amoxicillin, m/z 378.9 → 263.6 for ambroxol, m/z 277.0 → 203.0 for the IS. Declustering potential and collision energy were determined by observing maximum response of the product ion.

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve symmetric peak shapes for amoxicillin, ambroxol and the IS as well as a short run time. It was found that a mobile phase containing a certain proportion of formic acid gave symmetric peak shapes for ambroxol and the IS and a mobile phase containing high proportion of methanol gave short run time for amoxicillin. Thus gradient elution was adopted. It was also found that the inclusion of 0.2% formic acid solution in the mobile phase was crucial to obtaining high signal intensity, and in order to keep constant pH of the mobile phase, 0.2% formic acid was added into water and methanol, respectively. A flow rate of 1 mL/min gave a short chromatographic run time.

3.2. Method validation

3.2.1. Specificity and selectivity

Fig. 3 showed the typical chromatograms of blank plasma, spiked plasma sample with amoxicillin, ambroxol and the IS, and the plasma sample from a volunteer after oral administration. The retention time of amoxicillin, ambroxol and the IS was 4.1, 3.8 and 4.0 min, respectively. No significant interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of amoxicillin, ambroxol or the IS.

3.2.2. Calibration curve

Calibration curves were characterized by two different linear segments for both amoxicillin and ambroxol. For amoxicillin, the regression equation was $y = 442.7C + 0.1518$ ($r = 0.9998$, $n = 5$) over the range of 5–500 ng/mL and $y = 429.9C + 59.31$ ($r = 0.9999$, $n = 5$) over the range of 500–20,000 ng/mL. For ambroxol, the regression equation was $y = 0.002438C + 0.000763$ ($r = 0.9996$, $n = 5$) over the range of 1–20 ng/mL and $y = 0.002654C - 0.003651$ ($r = 0.9999$, $n = 5$) over the range of 20–200 ng/mL. These analysis results revealed the good linear correlations in both of amoxicillin and ambroxol. The LLOQ of amoxicillin and ambroxol were established at 5 ng/mL and 1 ng/mL, respectively, and the RSD were all less than 20%.

3.2.3. Accuracy and precision

The intra- and inter-run precision and accuracy of the assay were assessed by running a single batch of samples containing a calibration curve and five replicates at each QC levels. The precision was calculated by using one-way ANOVA. The results, which were summarized in Table 2, demonstrated that the precision and accuracy values were within the acceptable range and the method was accurate and precise.

3.2.4. Extraction recovery and matrix effects

The extraction recoveries of the two analytes were: amoxicillin 94.7 ± 2.4%, 92.5 ± 1.4% and 96.4 ± 2.0% at the concentrations of 10, 200, 2000 ng/mL, respectively; ambroxol 87.5 ± 2.0%, 90.5 ± 4.9% and 94.5 ± 3.9% at the concentrations of 2, 20, 80 ng/mL, respectively.

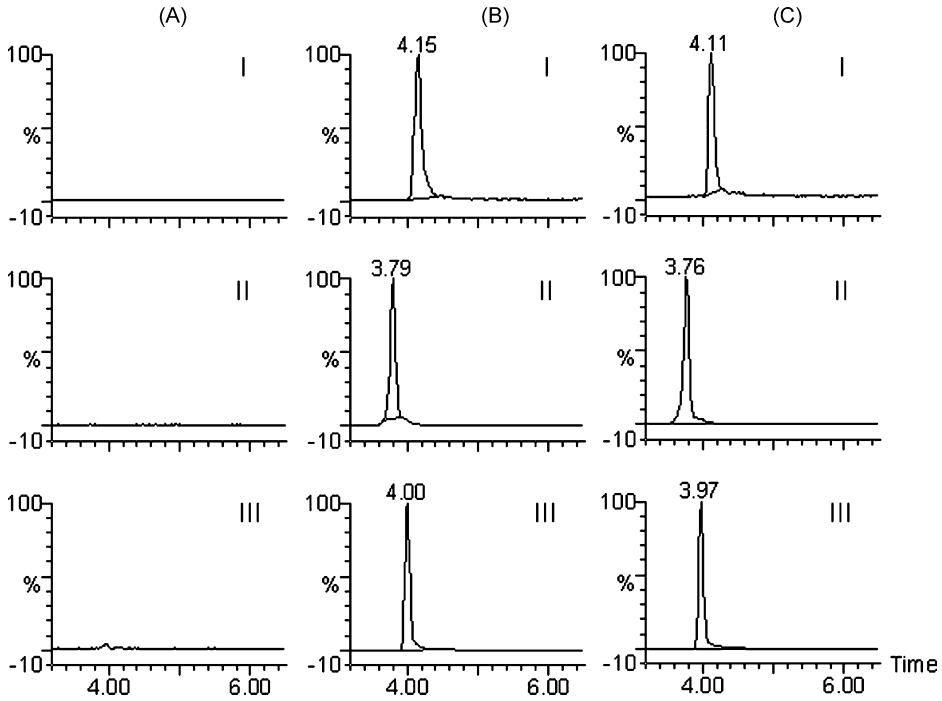

Fig. 3. Typical chromatograms of (A) blank plasma; (B) plasma spiked with ambroxol, amoxicillin and clenbuterol; (C) plasma obtained from a volunteer at 0.5 h after oral administration of ambroxol, amoxicillin and clenbuterol at 11.43 ng/mL, 1340 ng/mL and 160 ng/mL, respectively (I: ambroxol; II: amoxicillin; III: clenbuterol).

Table 2
Intra- and inter-run precision and accuracy of determination of amoxicillin and ambroxol in human plasma

Concentration (ng/mL)		RSD (%)	
Nominal	Found C	Intra-run	Inter-run
Amoxicillin			
10	9.447	3.0	5.7
200	201.4	1.8	4.0
2000	1948.8	3.3	3.8
Ambroxol			
2	2.084	6.4	8.7
20	19.20	2.7	4.6
80	80.81	2.9	3.1

The matrix effect was defined as the direct or indirect alteration or interference in respond due to the presence of unintended or other interfering substances in the samples. It was evaluated by comparing the peak area of the analytes dissolved in the blank plasma sample's reconstituted solution (the final solution of the blank plasma after extraction and reconstitution) with that dissolved in mobile phase. Three different concentration levels of analytes were evaluated by analyzing five samples at each level. The blank plasma used in this study was from five different batches of the blank plasma. If the peak area ratio is less than 85% or more than 115%, a matrix effect will be implied. In this study, the peak area ratios of the analytes were: amoxicillin 96.9 ± 1.8%, 87.0 ± 2.5% and 92.1 ± 3.1% at the concentration of 10, 200, 2000 ng/mL, respectively; ambroxol 85.4 ± 3.2%, 88.4 ± 4.9% and 91.7 ± 2.8% at the concentrations of 2, 20, 80 ng/mL, respectively; clenbuterol 94.8 ± 6.1% at the concentration of 800 ng/mL. There was no matrix effect of other substances on the amoxicillin and ambroxol detection.

3.2.5. Stability

Stability data were shown in Table 3. The analytes were stable in plasma under different temperature and timing conditions. Plasma samples were subjected to short-term room temperature conditions, to long-term storage conditions for 10, 20, 30 days (−80 °C), to three freeze-thaw stability, and to autosample stability for 10, 24, 48 h studies.

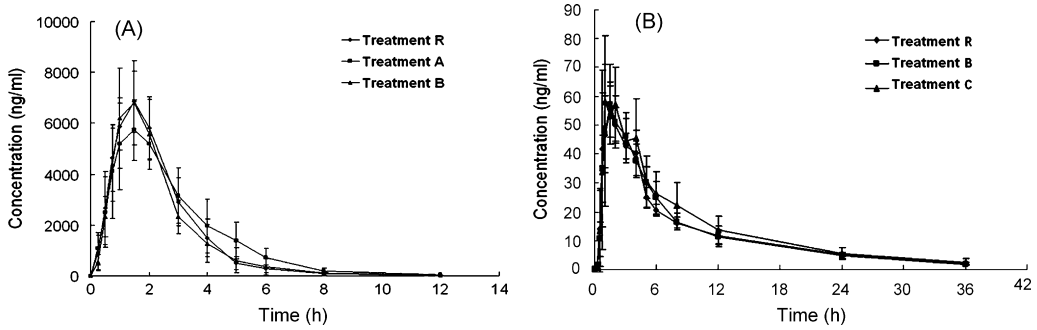

Fig. 4. Mean drug plasma concentration versus time curve of amoxicillin in 12 volunteers after oral administration of amoxicillin (A); mean drug plasma concentration versus time curve of ambroxol in 12 volunteers after oral administration of ambroxol (B).

Table 3
Stability of amoxicillin and ambroxol under different storage conditions ($n = 3$)

Storage conditions	Drug	Concentration (ng/mL)	
		Nominal	Mean found C
Stability at −80 °C for 10 days	Amoxicillin	10	9.165 ± 0.3
		200	206.8 ± 6.4
		2000	2166.3 ± 78.9
	Ambroxol hydrochloride	2	1.948 ± 0.2
		20	19.55 ± 2.7
		80	81.32 ± 6.3
Stability −80 °C at for 20 days	Amoxicillin	10	10.12 ± 0.4
		200	197.2 ± 7.8
		2000	2035.4 ± 86.9
	Ambroxol hydrochloride	2	2.051 ± 0.1
		20	19.32 ± 1.4
		80	80.34 ± 5.9
Stability −80 °C at for 30 days	Amoxicillin	10	9.165 ± 0.7
		200	211.5 ± 10.3
		2000	2046.1 ± 104.0
	Ambroxol hydrochloride	2	2.103 ± 0.08
		20	19.17 ± 0.5
		80	78.43 ± 6.5
Freeze-thaw stability	Amoxicillin	10	11.04 ± 0.8
		200	202.6 ± 5.6
		2000	2066.7 ± 155.4
	Ambroxol hydrochloride	2	2.009 ± 0.2
		20	19.52 ± 0.7
		80	82.38 ± 5.3
Autosampler stability at 4 °C for 10 h	Amoxicillin	10	9.538 ± 0.09
		200	211.6 ± 9.3
		2000	2097.1 ± 100.9
	Ambroxol hydrochloride	2	1.956 ± 0.13
		20	22.30 ± 1.8
		80	78.95 ± 6.7
Autosampler stability at 4 °C for 24 h	Amoxicillin	10	9.557 ± 0.4
		200	191.1 ± 1.3
		2000	2044.5 ± 88.7
	Ambroxol hydrochloride	2	2.223 ± 0.15
		20	20.18 ± 0.8
		80	81.49 ± 7.1
Autosampler stability at 4 °C for 48 h	Amoxicillin	10	10.29 ± 0.7
		200	219.0 ± 10.1
		2000	2085 ± 99.7
	Ambroxol hydrochloride	2	2.242 ± 0.12
		20	20.96 ± 1.0
		80	80.31 ± 7.9
Stability in plasma at 4 °C for 2 h	Amoxicillin	10	9.957 ± 0.8
		200	205.0 ± 8.2
		2000	2016.1 ± 55.3
	Ambroxol hydrochloride	2	2.115 ± 0.6
		20	20.55 ± 3.1
		80	79.13 ± 6.4

3.3. Pharmacokinetic study

Mean plasma concentration versus time profiles for amoxicillin and ambroxol were presented in Fig. 4. Multiple comparison ANOVA was used to evaluate the statistical difference of pharmacokinetic parameters of the amoxicillin and ambroxol among different treatment groups. The maximum plasma concentration (C_{max}), the time to it (T_{max}), the mean residence time (MRT), elimination half-life ($t_{1/2}$), $AUC_{(0-36)}$ (the area under the plasma concentration–time curve from 0 to 36 h) and $AUC_{(0-\infty)}$ (the area under the plasma concentration–time curve from 0 to infinity) of amoxicillin and ambroxol were similar among the different treatment groups (see Tables 4 and 5). The p values of C_{max}, T_{max}, $t_{1/2}$ and $AUC_{(0-36)}$ of amoxicillin among the groups of treatment R, treatment A and treatment C were 0.10, 0.76, 0.73 and 0.20, respectively. The p values of C_{max}, T_{max}, $t_{1/2}$ and $AUC_{(0-36)}$ of ambroxol among the groups of treatment R, treatment B and treatment C were 0.13, 0.85, 0.06 and 0.19, respectively. The p values were all greater than 0.05, and the results indicated

Table 4
Pharmacokinetics of amoxicillin in treatments R, A and C (mean ± S.D., $n = 12$)

Parameter	Treatment R	Treatment A	Treatment C
C_{max} (ng/mL)	6900.1 ± 1140.3	6150.5 ± 960.4	6860.7 ± 1750.6
T_{max} (h)	1.4 ± 0.2	1.5 ± 0.4	1.4 ± 0.2
MRT (h)	2.22 ± 0.2	2.68 ± 0.6	2.25 ± 0.4
$t_{1/2}$ (h)	1.4 ± 0.3	1.4 ± 0.1	1.3 ± 0.2
AUC_{0-36} (h μg/mL)	17.67 ± 3.54	18.88 ± 2.87	16.82 ± 3.78
$AUC_{0-\infty}$ (h μg/mL)	17.70 ± 3.56	18.95 ± 2.89	16.86 ± 3.78

Treatment R: test formulation; treatment A: reference formulation; treatment C: combined reference formulations.

Table 5
Pharmacokinetics of ambroxol hydrochloride in treatments R, B and C (mean ± S.D., $n = 12$)

Parameter	Treatment R	Treatment B	Treatment C
C_{max} (ng/mL)	60.75 ± 16.00	59.03 ± 12.57	68.96 ± 15.24
T_{max} (h)	1.5 ± 0.4	1.5 ± 0.5	1.6 ± 0.9
MRT (h)	9.22 ± 0.6	8.87 ± 0.5	9.15 ± 0.9
$t_{1/2}$ (h)	9.7 ± 0.8	8.8 ± 0.9	8.7 ± 1.3
AUC_{0-36} (h ng/mL)	450.6 ± 42.3	455.2 ± 73.4	522.6 ± 131.0
$AUC_{0-\infty}$ (h ng/mL)	480.7 ± 44.3	479.6 ± 80.2	551.9 ± 147.6

Treatment R: test formulation; treatment B: reference formulation; treatment C: combined reference formulations.

that the pharmacokinetics of both amoxicillin and ambroxol hydrochloride are not affected by their concomitant oral administration [12,13].

4. Conclusions

A rapid, simple and sensitive LC–MS/MS method for the simultaneous determination of amoxicillin and ambroxol in human plasma has been developed and validated. This method provided superior sensitivity with LLOQ as low as 5 ng/mL for amoxicillin and 1 ng/mL for ambroxol. The method was successfully applied to pharmacokinetic study of amoxicillin and ambroxol, and there were no obvious pharmacokinetic interactions between amoxicillin and ambroxol hydrochloride after oral administration.

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