

Original Article

Pharmacokinetics of ambroxol and clenbuterol tablets in healthy Chinese volunteers

Yong-Ge Yang, Li-Xue Song, Nan Jiang, Xue-Ting Xu, Xiao-Hui Di, Mei Zhang

Department of Pharmacy, Beijing Military Region General Hospital, Beijing, China

Received March 14, 2015; Accepted May 20, 2015; Epub October 15, 2015; Published October 30, 2015

Abstract: Objective: To investigate the pharmacokinetics of Ambroxol and Clenbuterol Tablets in Chinese healthy volunteers after a single or multiple dosages oral administration. Methods: A total of 9 healthy adult subjects were given Ambroxol and Clenbuterol Tablets in a single dosage or multiple dosages respectively. LC/MS/MS were used for the determination of Ambroxol and Clenbuterol of in plasma. The important pharmacokinetic parameters were calculated by DAS 2.0 software (compartment model). Results: Single and multiple dosage groups of Ambroxol and Clenbuterol were all fitted two-compartment model. The pharmacokinetics fitted first order kinetics process. No difference in pharmacokinetics of Ambroxol in single and multiple dosage groups volunteers was observed, Which showed no marked changes, suggesting that multiple dosing did not influence the velocity of drug metabolism. Moreover, parameters of Clenbuterol had significant difference between the single and multiple dosage groups ($P < 0.05$), showing there was accumulation in the body. 9 subjects had completed single or multiple dosages oral administration test, with no adverse drug reactions appeared during the test. Conclusion: There was no obvious accumulation of Ambroxol after repeated dosing. But obvious accumulation of Clenbuterol was noted in multiple-dose administration. The established method is sensitive, accurate, reliable and specific, and it can meet the requirement of clinical pharmacokinetic trial.

Keywords: Ambroxol, clenbuterol, LC/MS/MS, pharmacokinetics

Introduction

Ambroxol, trans-4-(2-amino-3,5-dibromobenzyl)-amino-cyclohexanol, is an active ingredient of ambroxol hydrochloride which is usually used as bronchosecretolytic and expectorant drug [1]. In clinical practice, ambroxol hydrochloride is mainly used as a hydrochloric salt, and has been proved to facilitate the repair of bronchial epithelium and accelerates the mucociliary transport [2, 3]. In addition, it shows some other clinical properties such as the radial scavenger activity [4], and inhibition of doxorubicin induced lipid peroxidation in vivo [5].

Clenbuterol is a kind of β_2 -adrenergic receptor agonists, and clenbuterol hydrochloride is clinically used as anti-asthmatic agent that belongs to a broad group of drugs known as sympathomimetics [6]. It is commonly used because of its anabolic effects and the ability to promote lipolysis [7].

Ambroxol and clenbuterol are two drugs with potential pharmacological synergy. Previous study showed that ambroxol could improve the spasmolytic activity of clenbuterol in guinea-pig [8]. To date, several studies have been conducted to investigate the potential pharmacokinetic of ambroxol and clenbuterol after oral administration of single dosage of Ambroxol and Clenbuterol [9-11]. In this study, we investigated the pharmacokinetics of ambroxol and clenbuterol after oral administration of single dosage and multiple dosages of Ambroxol and Clenbuterol Tablets in healthy Chinese adults, based on which to provide the reference information for the clinical application of the medication.

Materials and methods

Subject selection

A total of 9 healthy Chinese adults (male: 4 and female: 5; age: 24.1 ± 2.8 years; body weight:

Pharmacokinetics of ambroxol and clenbuterol tablets

3.8±3.1 Kg) were enrolled in this study. The inclusion criteria were as follows: (i) those with normal electrocardiogram, blood pressure; (ii) those with no abnormality in the routine urine test, routine blood test and blood biochemical test. The exclusion criteria were as follows: (i) those with smoking habits; (ii) those participated in drug test within 3 months; (iii) those with medication of another drug 2 weeks before test. Informed consent was signed by all the subjects and the study was approved by Medicine Ethics Committee of the Military General Hospital of Beijing PLA.

Chemical and reagents

Ambroxol and Clenbuterol Tablets (lot No.: 100599-200502; 30 mg ambroxol hydrochloride and 20 µg clenbuterol hydrochloride per tablet) were provided by the Yangtze River Pharmaceutical group CO., Ltd. (Jiangsu, China). Chemical reference substances of ambroxol (100% purity) and clenbuterol (100% purity) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) with the lots No. of 100599-200502 and 0072-8501, respectively. Bisoprolol (internal standard, purity >99.5%) were provided by Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences (Beijing, China). Phentolamine (internal standard, purity >99.0%) were purchased from Shijiazhuang Great Pharmaceutical Co., Ltd. (Shijiazhuang, China). Methanol and methyl cyanides are of HPLC grade, water used for drug administration was Wahaha purified drinking water (Hangzhou Wahaha Group Co., Ltd.), and other solvents and reagents are of analytical grade.

Method validation

The specificity of the methods used for determination of ambroxol and clenbuterol in plasma were tested by using blank human plasma, blank human plasma plus ambroxol/clenbuterol and bisoprolol/phentolamine (internal standard, IS), and the plasma sample after the administration of Ambroxol and Clenbuterol Tablets. The calibration curve was constructed using plasma concentration of quality control (QC) sample (x) and peak area ratio (y) of QC sample vs IS. The concentration of QC samples were prepared in normal blank human plasma at the levels of 1, 2, 4, 16, 64, 128, 256 µg/L for ambroxol and 5, 10, 50, 250, 1000, 5000

ng/L for clenbuterol, respectively. The regression analysis was performed using weighted least squares method ($W=1/x^2$). The accuracy and precision and the extraction recovery of the assay in this study were determined by analysis of the QC samples. Three sequential assays (once per day) were performed with 6 replicates of QC samples (2, 16 and 128 µg/L for ambroxol and 10, 250, 4000 ng/L for clenbuterol) for each assay. The concentration of QC samples were calculated on the basis of calibration curve and compared with the control samples with the same concentration. On these basis, the extraction recovery, the intra-day accuracy and precision and the inter-day accuracy and precision were calculated.

Experimental design

Before administration, the subjects were fasted for 12 h after bland diet for the last supper. Each subject was orally given 2 tablets of Ambroxol and Clenbuterol Tablets (60 mg ambroxol and 40 mg clenbuterol) in the morning with 250 ml warm water. Then the same standard lunch was provided 4 h after administration. The blood collection from ulnar vein was performed before administration and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96 and 120 h post administration. Multidose administration test (2 tablets per morning for 9 days) was carried out in all 9 subjects after the end of single dose test. During the test, caffeine-containing beverages, alcohol and cigarettes were forbidden. Light daily activities were needed. The plasma concentrations of ambroxol and clenbuterol were determined on day 7, day 8 and day 9 respectively using venous blood collected from ulnar vein before administration to evaluate whether steady-state plasma concentrations of ambroxol and clenbuterol were achieved. On day 9, the blood collection from ulnar vein was performed after administration as in single dose test.

All the blood samples were immediately put in the heparinized tubes after collection. After vortex and 5 min standing, the samples were centrifuge for 10 min at 3000 rpm. The separated blood plasma was stored at -70°C.

Determination of plasma concentration of ambroxol

The plasma concentrations of amoxicillin was determined by using LC-MS/MS method [12]

Pharmacokinetics of ambroxol and clenbuterol tablets

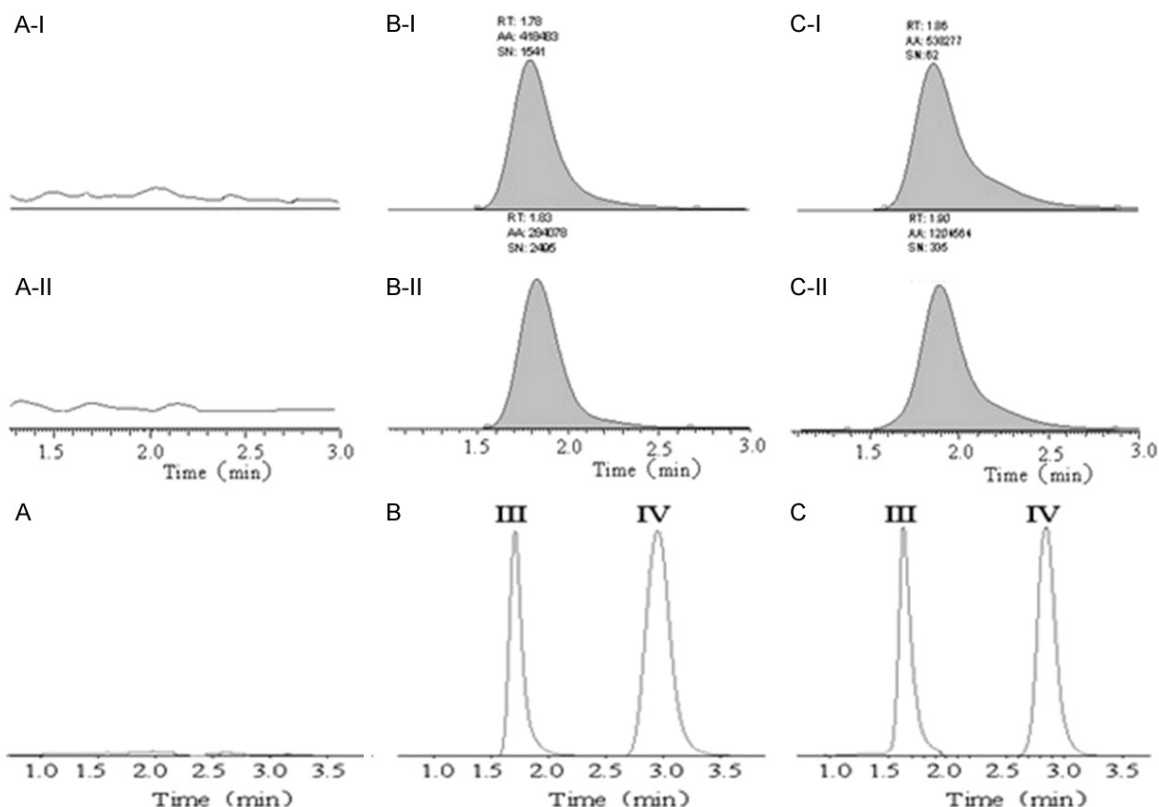


Figure 1. Typical chromatograms of (A) blank plasma, (B) plasma spiked with ambroxol/clenbuterol and IS, and (C) plasma sample obtained from a volunteer at 0.5 h after administration of Ambroxol and Clenbuterol Tablets (I: ambroxol; II: bisoprolol; III: clenbuterol; IV: phentolamine).

and the instrument was Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (Thermo Finnigan, USA). Briefly, 10 μ L bisoprolol solutions (50 ng/L, solved in methanol) were added to 0.2 ml plasma sample. After vortex mixture, 20 μ L sodium hydroxide-sodium dihydrogen phosphate buffer (0.1 M, pH=12) and 2 mL acetic ether were added and vortex mixed for 2 min, followed by centrifugation at 3000 rpm for 5 min. The supernatant was transferred to another glass tube and dried using stream of nitrogen. The dried sample was then dissolved in 100 μ L mobile phase consisting of methanol and water (25:75, v/v) containing 10% formic acid. After vortex mixture for 1 min and centrifugation at 10,000 rpm for 15 min, 10 μ L supernatant was used for concentration determination using Thermo Scientific BioBasic 8 Columns (5 mm 50 mm \times 2.1 mm) at room temperature. The flow rate of mobile phase was 0.2 mL/min. Mass spectrometry was operated in selected reaction monitoring (SRM) mode using electrospray ionization in positive-ion mode and the settings of the mass spectrometer were as fol-

lows: ion spray voltage, 4200 V; sheath gas (N_2) pressure, 11 arbitrary units; auxiliary gas (N_2) pressure, 15 arbitrary units. The transitions m/z 378.9 \rightarrow 263.8 and 326.3 \rightarrow 116.2 were used for quantitative analyses of ambroxol and bisoprolol, respectively.

Determination of plasma concentration of clenbuterol

The concentration of clenbuterol in plasma was measured using API 4000 liquid chromatograph/tandem mass spectrometer (Thermo Finnigan, USA). Fifty microliters distilled water and 50 mL phentolamine solution (IS, 10 μ g/L, solved in distilled water) were added to 0.5 mL plasma, followed by vortex mixture for 30 s. After centrifugation at 11,000 rpm for 5 min, the solid-phase extraction was performed using Oasis HLB 30 mg 1CC cartridges (Waters Corporations, USA). Finally, the sample was washed down using 0.5 mL washing solution (methyl cyanides: distilled water: formic acid=50:50:0.5, v/v/v). After vigorous mixing,

Pharmacokinetics of ambroxol and clenbuterol tablets

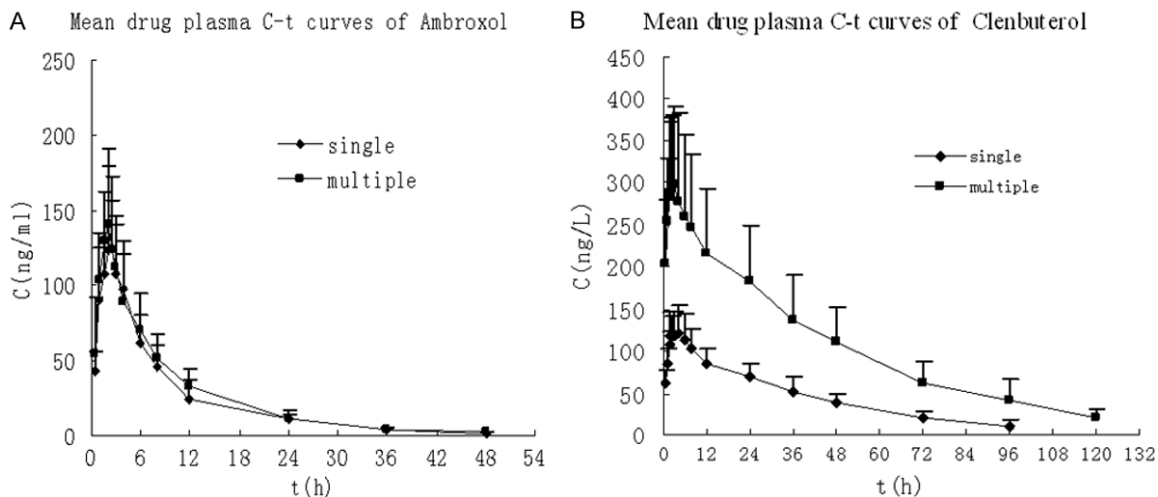


Figure 2. Mean drug plasma concentration-time curves of Ambroxol and Clenbuterol in 9 volunteers after oral administration of Ambroxol and Clenbuterol tablets (A: Ambroxol, B: Clenbuterol).

50 mL sample was loaded to the Restec Allure C18 column (150×4.6 mm; 5 μ m particle size; 60 Å pore size) for concentration determination at room temperature. The mobile phase, a mixture of 2 mmol/L ammonium acetate (containing 0.5% formic acid) and methyl cyanides (25:75, v/v), was run at a flow rate of 0.5 mL/min. Mass spectrometry was operated in multiple reaction monitoring (MRM) mode using electrospray ionization in negative-ion mode and the settings of the mass spectrometer were as follows: ion spray voltage, 5000 V; atomization temperature, 600°C; atomizing air flow rate, 30 L/min; curtain gas flow rate, 30 L/min; flow rate of collision-activated dissociation gas, 6 L/min; auxiliary gas flow rate, 50 L/min. The transitions m/z used for quantitative analyses of clenbuterol and phentolamine (IS) were 277.3→202.8 and 282.2→212.2, respectively.

Results

Method validation

Figure 1 showed the typical chromatograms of blank plasma, plasma sample spiked with ambroxol/clenbuterol and IS (bisoprolol/phentolamine), and the plasma sample from a volunteer after administration of. The retention time of ambroxol and bisoprolol was 1.78 min and 1.83 min respectively, and the retention time of clenbuterol and phentolamine was approximately 1.75 min and 3.0 min, respectively. No significant interfering peak was noticed at the

retention time of either the ambroxol/clenbuterol sample or IS. The calibration curve of ambroxol was linear over the range of 1-256 μ g/L, and the linear regression equation was $y=0.0404377C-0.024329$ ($r=0.9994$). The calibration curve of clenbuterol was also linear over the range of 5-5000 ng/L, and the linear regression equation was $y=0.315C+0.000878$ ($r=0.9998$). The lower limit of quantification of ambroxol and clenbuterol were 1 μ g/L and 5 ng/L respectively. The intra- and inter-day accuracy and precision of ambroxol were $\leq 7.3\%$ and $\leq 13.5\%$, respectively, and the intra- and inter-day accuracy and precision of clenbuterol were $\leq 5.8\%$ and $\leq 6.8\%$, respectively. The extraction recoveries of ambroxol were 71.2%, 77.4% and 73.3% at the concentration of 2, 16 and 128 μ g/L, respectively, and the extraction recoveries of clenbuterol were 87.2%, 92.6% and 89.6% at the concentration of 10, 250 and 4000 ng/L, respectively.

Pharmacokinetic study of ambroxol and clenbuterol tablets

Figure 2 showed the mean plasma concentration versus time profiles for ambroxol and clenbuterol after the single dose and multidose administration of Ambroxol and Clenbuterol Tablets. DAS 2.0 software analysis showed two-compartment model for both ambroxol and clenbuterol after the single dose and multidose administration of Ambroxol and Clenbuterol Tablets. The main pharmacokinetic parameters were shown in **Table 1**. Statistical analysis was

Pharmacokinetics of ambroxol and clenbuterol tablets

Table 1. Pharmacokinetic parameters of ambroxol and clenbuterol after single dose and multidose administration of Ambroxol and Clenbuterol Tablets in 9 healthy subjects

Parameter	Ambroxol		Clenbuterol	
	Single dose	Multidose	Single dose	Multidose
C_{max} ($\mu\text{g}\cdot\text{L}^{-1}/\text{ng}\cdot\text{L}^{-1}$)	(150.89±56.90)	(148.91±47.41)	(133.2±31.4)	(329.7±106.8)
T_{max} (h)	(2.4±1.0)	(1.9±0.5)	(2.6±0.9)	(2.4±0.9)
$t_{1/2}$ (h)	(8.72±1.52)	(8.56±1.64)	(26.56±7.38)	(33.15±9.70)
K_{10} (h^{-1})	(0.492±0.747)	(0.255±0.067)	(0.034±0.010)	(0.057±0.009)
CL/F ($\text{L}\cdot\text{h}^{-1}$)	(57.18±18.81)	(50.4±15.6)	(8.74±2.29)	(3.22±1.16)
V/F (L)	(707.5±199.3)	(612.3±205.5)	(323.7±76.1)	(147.8±49.6)
$MRT_{(0-t)}$ (h^{-1})	(9.6±0.6)	(9.8±0.9)	(30.0±5.0)	(36.6±3.2)
$MRT_{(0-\infty)}$ (h^{-1})	(10.6±0.7)	(10.9±1.3)	(37.9±6.9)	(46.7±7.1)
AUC_{ss} ($\mu\text{g}\cdot\text{L}^{-1}\cdot\text{h}/\text{ng}\cdot\text{L}^{-1}\cdot\text{h}$)	/	(858.2±241.5)	/	(5421.0±1846.4)
$AUC_{(0-t)}$ ($\mu\text{g}\cdot\text{L}^{-1}\cdot\text{h}/\text{ng}\cdot\text{L}^{-1}\cdot\text{h}$)	(1119.1±315.2)	(1267.6±364.8)	(4474.0±1271.8)	(12912.2±4751.5)
$AUC_{(0-\infty)}$ ($9 \mu\text{g}\cdot\text{L}^{-1}\cdot\text{h}/\text{ng}\cdot\text{L}^{-1}\cdot\text{h}$)	(1140.1±321.4)	(1292.3±379.7)	(4889.4±1384.5)	(14164.8±5691.7)
C_{ss-min} ($\mu\text{g}\cdot\text{L}^{-1}/\text{ng}\cdot\text{L}^{-1}$)	/	(13.44±6.95)	/	(116.0±54.9)
Cav ($\mu\text{g}\cdot\text{L}^{-1}/\text{ng}\cdot\text{L}^{-1}$)	/	(71.52±20.12)	/	(225.9±76.9)
DF	/	(1.90±0.30)	/	(0.95±0.29)

C_{max} : Maximum blood concentration; T_{max} : Time to reach C_{max} ; $t_{1/2}$: Elimination half-life; K_{10} : elimination rate constant; CL/F: Clearance; V/F: Apparent volume of distribution; $MRT_{(0-t)}$: Mean residence time from 0 to the last measured time point; $MRT_{(0-\infty)}$: Mean residence time from 0 to infinity; AUC_{ss} : Area under the plasma concentration versus time curve at steady state; $AUC_{(0-t)}$: Area under the blood concentration vs time curve from 0 to the last quantifiable time point; $AUC_{(0-\infty)}$: Area under the blood concentration vs time curve from 0 to infinity; C_{ss-min} : Minimum steady state concentration; Cav: Average concentration; DF: Fluctuation degree.

performed using Statistical Package for Social Sciences (SPSS, version 15.0).

Student's t test was carried out to compare the pharmacokinetic parameters of ambroxol and clenbuterol between the single dose and multidose administration, respectively. No statistical difference in ambroxol pharmacokinetic parameters ($P>0.05$). Nevertheless, the pharmacokinetic parameters of clenbuterol were statistically different except $t_{1/2}$ and T_{max} . For the pharmacokinetics of Clenbuterol, remarkable decrease was noticed in the clearance and apparent volume of distribution in the subjects received multiple doses of Ambroxol and Clenbuterol Tablets compared with those received single dose ($P<0.05$). Nevertheless, significant increase was noticed in the C_{max} , $AUC_{(0-t)}$, $AUC_{0-\infty}$, K_{10} , and MRT in subjects with multiple doses of Ambroxol and Clenbuterol compared with those received single dose ($P<0.05$). Therefore, accumulative action was observed in subjects with long-term oral administration of Ambroxol, however, no such effects were noticed in those with long-term oral administration of Clenbuterol. Moreover, no statistical difference was revealed in the pharmacokinetics in these two drugs in the male

subjects compared with those of the female, demonstrating that gender causing no effects on the metabolism of Ambroxol and Clenbuterol.

Discussion

Potential pharmacological synergy has been proved in Ambroxol and clenbuterol. Rare studies have been conducted to investigate the pharmacokinetics of multiple dosages of Ambroxol and Clenbuterol Tablets in healthy Chinese adults. We investigated the pharmacokinetics of these two drugs in vivo after multiple doses.

A majority of studies on Ambroxol and Clenbuterol have been focused on the single administration. For example, Bazylak and Nagels reported a high-throughput determination of clenbuterol and ambroxol in pharmaceutical formulations by HPLC [13]. In addition, Lin et al determined the levels of ambroxol and clenbuterol in human plasma using LC-MS/MS method after single administration [9]. To our best knowledge, rare studies have been conducted to investigate the pharmacokinetics after multiple administrations of Ambroxol and Clenbuterol. Similar with the previous studies

[14, 15], a two compartment model was observed in the metabolism of Ambroxol and Clenbuterol in Chinese subjects with single dose. In addition, no statistical difference was noticed in the major pharmacokinetics in the subjects received single dose medication compared with those received multiple doses. This indicated that the pharmacokinetics of Ambroxol and Clenbuterol were not affected by the administration duration, and no accumulation was noted even after long-term medication. For the Clenbuterol, no statistical difference was noticed in the $t_{1/2}$ and T_{max} in the patients with single dose compared with those with multiple doses, while statistical differences were noted in the other parameters. On this basis, we concluded that the pharmacokinetics of Clenbuterol were affected by the administration duration, and drug accumulation may be present after long-term medication.

LC-MS/MS has been frequently used for the determination of Ambroxol and Clenbuterol level in human plasma [12, 16, 17]. In 2007, Lin et al reported the linear concentration ranges of the calibration curves for ambroxol and clenbuterol were 0.08-400 microg/L and 5.0-5000 ng/L, respectively. The inter- and intra-day precision cross the validation process was no more than 7.5%, and the accuracy was within $\pm 2.5\%$ [9]. In our study, using LC/MS/MS, the calibration curve of ambroxol was linear over the range of 1-256 $\mu\text{g/L}$, and the linear regression equation was $Y=0.0404377C-0.024329$ ($r=0.9994$). For clenbuterol, the calibration curve of clenbuterol was also linear over the range of 5-5000 ng/L. The lower limit of quantification of ambroxol and clenbuterol were 1 $\mu\text{g/L}$ and 5 ng/L respectively. In addition, the intra- and inter-day accuracies ambroxol and clenbuterol were acceptable. This indicated that the LC/MS/MS was effective for the determination of ambroxol and clenbuterol in human serum. As the serum clenbuterol was comparatively lower, extraction was performed using solid phase column. On this basis, LC/MS/MS was performed and the detection limit was satisfactory compared with the previous data.

To monitor the potential adverse reactions, the blood pressure, heart rate and dynamic monitoring were performed to each subject under the guidance of qualified staff. The results revealed that no severe adverse reactions were noticed.

In conclusion, we investigate the pharmacokinetics of Ambroxol and Clenbuterol Tablets in Chinese healthy volunteers after a single or multiple dosages oral administration using LC/MS/MS method. Our results revealed no obvious accumulation of Ambroxol was noticed in healthy subjects after repeated dosing. However, obvious accumulation of Clenbuterol was noted in multiple-dose administration. The established method is sensitive, accurate, reliable and specific, and it can meet the requirement of clinical pharmacokinetic trial.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Mei Zhang, Department of Pharmacology, General Military Hospital of Beijing PLA, No. 5, Nanmencang, Dongcheng District, Beijing 100700, China. Tel: +86-10-66721926; Fax: +86-10-84043984; E-mail: zhangmeibj@sina.com

References

- [1] Satana E, Basan H and Goğer NG. Determination of ambroxol hydrochloride in tablets using flow-injection UV spectrophotometry and HPLC. *Journal of Analytical Chemistry* 2008; 63: 451-454.
- [2] Rojpibulstit M, Kasiwong S, Juthong S, Phadoongsombat N and Faroongsarn D. Ambroxol Lozenge Bioavailability. *Clin Drug Investig* 2003; 23: 273-280.
- [3] Qi M, Wang P, Cong R and Yang J. Simultaneous determination of roxithromycin and ambroxol hydrochloride in a new tablet formulation by liquid chromatography. *J Pharm Biomed Anal* 2004; 35: 1287-1291.
- [4] Štětínová V, Herout V and Květina J. In vitro and in vivo antioxidant activity of ambroxol. *Clin Exp Med* 2004; 4: 152-158.
- [5] Nowak D, Pierscinski G and Drzewoski J. Ambroxol inhibits doxorubicin-induced lipid peroxidation in heart of mice. *Free Radic Biol Med* 1995; 19: 659-663.
- [6] Hua D, Yuan ZN, Huang ZQ, Chen XH. (Hunan Extry-Exit Inspection and Quarantine Bureau, Changsha 410007, China); Determination of Clenbuterol Hydrochloride and Salbutamol in Feed by HPLC. *Journal of Instrumental Analysis* 2003; 3.
- [7] Daubert GP, Mabasa VH, Leung VW, Aaron C. Acute clenbuterol overdose resulting in supra-ventricular tachycardia and atrial fibrillation. *J Med Toxicol* 2007; 3: 56-60.
- [8] Pairet M, Engelmann P, Nicolai H, Champeroux P, Richard S, Rauber G and Engelhardt G.

Pharmacokinetics of ambroxol and clenbuterol tablets

- Ambroxol improves the broncho-spasmolytic activity of clenbuterol in the guinea-pig. *J Pharm Pharmacol* 1997; 49: 184-186.
- [9] Lin N, Chen XY, Song B and Zhong DF. [Determination of ambroxol and clenbuterol in human plasma by LC-MS/MS method]. *Yao Xue Xue Bao* 2007; 42: 308-313.
- [10] Zhao F, Ma Y, Jin X, Wang J and Cao D. Preparation of ambroxol hydrochloride push-pull osmotic pump controlled-release tablets and its pharmacokinetics in dogs. *Chinese Journal of Pharmaceuticals* 2011; 4: 013.
- [11] Faroongsarng D, Rojpibulstit M, Kasiwong S and Phadoongsombat N. Ambroxol Lozenge Bioavailability. *Clin Drug Investig* 2004; 24: 681-688.
- [12] Wen A, Hang T, Chen S, Wang Z, Ding L, Tian Y, Zhang M and Xu X. Simultaneous determination of amoxicillin and ambroxol in human plasma by LC-MS/MS: Validation and application to pharmacokinetic study. *J Pharm Biomed Anal* 2008; 48: 829-834.
- [13] Bazylak G and Nagels LJ. Simultaneous high-throughput determination of clenbuterol, ambroxol and bromhexine in pharmaceutical formulations by HPLC with potentiometric detection. *J Pharm Biomed Anal* 2003; 32: 887-903.
- [14] Duval V and Karlsson MO. Impact of omission or replacement of data below the limit of quantification on parameter estimates in a two-compartment model. *Pharm Res* 2002; 19: 1835-1840.
- [15] Bhat DS, Yajnik CS, Sayyad MG, Raut KN, Lubree HG, Rege SS, Chougule SD, Shetty PS, Yudkin JS and Kurpad AV. Body fat measurement in Indian men: comparison of three methods based on a two-compartment model. *Int J Obes (Lond)* 2005; 29: 842-848.
- [16] Dong X, Ding L, Cao X, Jiang L and Zhong S. A sensitive LC-MS/MS method for the simultaneous determination of amoxicillin and ambroxol in human plasma with segmental monitoring. *Biomed Chromatogr* 2013; 27: 520-526.
- [17] Du XL, Liu L and Yong XL. LC-MS/MS matrix effect and back-conversion neglected in study of biopharmaceutical methodology. *Chinese Journal of New Drugs* 2012; 22: 028.