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Simultaneous determination of ipratropium and salbutamol in rat plasma by LC–MS/MS and its application to a pharmacokinetic study

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ABSTRACT

A novel, sensitive and specific LC–MS/MS method with silica-based solid-phase extraction was developed for simultaneous determination of ipratropium (IPR) and salbutamol (SAL) in rat plasma. Chromatographic separation was achieved on a Shiseido Capcell Pak CR column (SCX:C_{18} = 1:4, 150 mm × 2.0 mm, 5 μm) with a mobile phase consisting of methanol/water (85:15, v/v) containing 20 mmol/L ammonium formate and 0.1% formic acid at a flow rate of 0.3 mL/min. A tandem mass spectrometric detection with an electrospray ionization (ESI) interface was conducted via multiple reaction monitoring (MRM) under positive ionization mode. This method was validated in terms of specificity, linearity, accuracy (within ±115.4%), intra- and inter-day precision (<11.4%) over the concentration range of 8–1612 pg/mL for IPR and 50–10,000 pg/mL for SAL. In addition, stability and matrix effects of IPR and SAL in plasma were evaluated. This method has been successfully applied to the pharmacokinetic study of compound ipratropium bromide aerosol mainly containing ipratropium bromide (IB) and salbutamol sulphate (SS) after inhalation in rats.

1. Introduction

Chronic obstructive pulmonary disease (COPD) characterized by irreversible and slowly progressive airflow limitation is a major global health problem and causes a severe morbidity and mortality [1]. The lung function of patients with COPD could be improved by anticholinergic bronchodilators and β2-agonists for symptom relieved, which is recommended in the guideline of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [2].

Compound ipratropium bromide aerosol delivered in an aerosolized form via a pressurized metered dose inhaler (pMDI) is composed by ipratropium bromide (IB), an anticholinergic bronchodilator, and salbutamol sulphate (SS), a β2-adrenergic bronchodilator. The combination of IB and SS exhibits synergistic bronchodilatory effect because of their complementary effects on receptor signaling pathways [3]. Compared to concomitant use of separate IB and SS metered dose inhalers (MDIs), use of the combination therapy not only results in an additive bronchodilator effect, but also improves patients' compliance [4].

Because of the low sensitivity, there are few studies on the pharmacokinetics of IB or SS aerosols. Though several methods have been described in the literature for the determination of IPR or SAL, no assay for simultaneous determination of IPR and SAL has been reported, let alone the available pharmacokinetic data of compound ipratropium bromide aerosol via inhalation. For example, Ariffin and Anderson [5] developed a method for determining IPR in human whole blood with weak cation-exchange SPE and LC–MS/MS. The limit of quantitation was 19.5 ng/mL. Mazhar and Chrystyn [6] used a reversed phase HPLC-FD with two SPE methods for determining SAL in human urine after an inhalation. Ensing et al. [7] reported a radioreceptor assay to determine the bioavailability and pharmacokinetics of IB after different routes of administration. However, due to the detection limit of the method, no pharmacokinetic parameters after inhalation were obtained.

The polarity of IPR, which is a synthetic quaternary ammonium compound chemically related to atropine, is quite different from SAL belongs to salicylic alcohol compound [8]. The structures of two compounds are shown in Fig. 1. So it is difficult to determine of IPR and SAL simultaneously. This paper introduces a new bioanalytical LC–MS/MS method for simultaneous determination of the two drugs in rat plasma. A Shiseido Capcell Pak CR column used in the study contains a mixed-mode stationary phase with the mixture of sulfonic and C_{18} groups and thus the separation is governed by a multimode mechanism of ion-exchange and reversed phase. Silica-based solid phased extraction (SPE) cartridges were used for sample preparation. The low limit of quantitations (LLOQs) for IPR and SAL were 8 pg/mL and 50 pg/mL, respectively, with only 200 μL plasma. This sensitive method has been successfully applied to the pharmacokinetic study of compound ipratropium bromide aerosol delivered via inhalation in rats.

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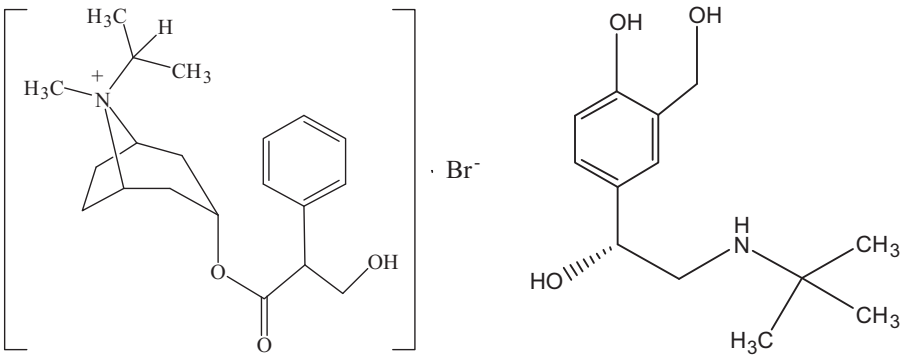

Fig. 1. Structures of ipratropium bromide and salbutamol.

2. Experimental

2.1. Chemicals and reagents

Ipratropium bromide (IB, purity: 100%, batch no. 100522-200601), salbutamol (SAL, purity: 99.5%, batch no. 100204-200702) were both obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ipratropium-d7 bromide (IB-d7, purity: 100%, batch no. 15-AZC-169-1) and salbutamol-d9 (SAL-d9, purity: 100%, batch no. 1-GJF-120-1) were both obtained from Toronto Research Chemical Inc. (Ontario, Canada). HPLC-grade methanol was purchased from Merck Co., Inc. (Darmstadt, Germany). Analytical grade formic acid was purchased from TEDIA Co., Inc. (Fairfield, USA). Analytical grade hydrochloric acid and ammonium acetate were both purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Analytical grade ammonium hydroxide was purchased from Shanghai Lingfeng Chemical Reagent Co. Ltd. (Shanghai, China). Analytical grade ammonium formate was purchased from Sigma–Aldrich (Steinheim, Germany). Purified water used throughout the study was commercially available (Wahaha®, Hangzhou Wahaha Co. Ltd., China).

Compound ipratropium bromide aerosol (batch no. GXX9210) was provided by Cipla Ltd. (Mumbai, India). The contents of IB and SAL per single puff dose were 20 μg for IB and 100 μg for SAL, respectively.

The SPE cartridges (200 mg/2.5 mL, SiO_2) were supplied from Dalian Institute of Chemical Physics, Chinese Academy of Sciences (Dalian, China).

2.2. Chromatographic conditions

A Shimadzu HPLC system, consisting of a CBM-20A system controller, LC-20AD_{XR} pump, a SIL-20AC_{XR} autosampler, a Rack Changer/c, a CTO-10Avp column oven and a Shiseido Capcell Pak CR column (multimode column with cation-exchange and reversed phase, SCX:C18 = 1:4, 150 mm × 2.0 mm, 5 μm, Shiseido Co. Ltd., Tokyo, Japan) equipped with a guard column (C18, 4 mm × 3.0 mm, Phenomenex Co. Ltd., Torrance, CA, USA), was used for the chromatographic separation of IPR, SAL and the internal standards.

The mobile phase was a solution of methanol/water (85:15, v/v) containing 20 mmol/L ammonium formate and 0.1% formic acid, and was delivered at a constant flow rate of 0.3 mL/min. The temperatures of the analytical column and autosampler were maintained at 45 °C and 4 °C, respectively. Under these conditions, the retention times for IPR, SAL, IPR-d7 and SAL-d9 were 3.9 min, 2.7 min, 3.9 min and 2.7 min, respectively. The total run time for a LC–MS/MS analysis was 5 min.

2.3. Mass spectrometric conditions

An API 3000 triple quadrupole mass spectrometer (AB/MDS-Sciex, Ontario, Canada) equipped with an electrospray ionization (ESI) interface in the positive ion mode was used for the mass spectrometric detection. The tandem mass spectrometer was operated under the multiple reaction monitoring mode (MRM) and Q1 and Q3 quadrupoles were set at unit mass resolution.

The operation conditions were optimized by infusing diluted stock solutions of each analyte into the mass spectrometer and were as follows: nebulizing gas flow was 12 kPa, curtain gas flow was 12 kPa, collision gas flow was 7 kPa, ion source voltage was 4500 V and source temperature was set at 475 °C. The specific parameters for each analyte are shown in Table 1. The quantification was performed via peak area ratio of interest analytes to internal standard (I.S.). The Applied Biosystems Analyst version 1.5.1 software was used to control the LC–ESI/MS/MS system and to collect and process the data.

2.4. Preparation of standards curves and quality control (QC) samples

Stock solutions of IPR and SAL were prepared in methanol at 201 μg/mL and 500 μg/mL, respectively. The IPR stock solution was then diluted with methanol to 81 μg/mL. The diluted IPR solution and SAL stock solution were serially diluted with methanol/water (40/60, v/v) to produce a series of standard or QC working solutions at the desired concentrations.

IPR-d7 and SAL-d9 stock solutions were prepared at 40 μg/mL and 100 μg/mL in methanol respectively, and diluted with methanol/water (40/60, v/v) to yield an I.S. working solution at the concentration of 2.0 ng/mL for IPR-d7 and 25 ng/mL for SAL-d9.

The calibration standards were freshly prepared by adding 20 μL of the appropriate standard working solutions to 200 μL blank plasma to provide the final concentrations of IPR at 1612, 1289, 645, 161, 81, 32, 8 pg/mL and SAL at 10,000, 8000, 4000, 1000, 500, 200, 50 pg/mL. Low, medium and high level of QC samples were prepared at the concentrations of 20, 161, 1209 pg/mL for IPR, and 125, 1000, 7500 pg/mL for SAL.

All solutions described above were stored at 4 °C.

Table 1
Optimized mass parameters for IPR, SAL and the internal standards.

Analyte	MRM (*m*/*z*)	Dwell time (ms)	DP[a] (V)	CE[b] (V)	FP[c] (V)	EP[d] (V)	CXP[e] (V)
IPR	332.3 → 166.2	200	38	36	225	10	10
IPR-d7 (I.S.)	339.3 → 173.2	200	38	36	225	10	10
SAL	240.2 → 148.1	200	25	27	225	10	10
SAL-d9 (I.S.)	249.2 → 148.1	200	25	27	225	10	10

[a] DP: declustering potential.
[b] CE: collision energy.
[c] FP: focallization potential.
[d] EP: entrance potential.
[e] CXP: collision cell exit potential.

2.5. Plasma sample preparation

An aliquot of thawed plasma (200 μL) sample was fortified with 20 μL of methanol/water (40/60, v/v) and 20 μL of I.S. working solution. The sample was briefly vortex-mixed and then centrifuged at 10,000 × *g* for 5 min.

The SPE cartridges were sequentially conditioned with 1 mL of 1% hydrochloric acid methanolic solution (containing 20 mmol/L ammonium formate) and 1 mL of ammonium acetate buffer (20 mmol/L, pH 8.0). The plasma samples were loaded and passed through the cartridges. The cartridges were then washed with 1 mL of water, followed by 1 mL of methanol/water (40/60, v/v). Finally the analytes were eluted with 1 mL of 0.2% hydrochloric acid methanolic solution (containing 4 mmol/L ammonium formate). The elutant was collected and evaporated to dryness under a gentle stream of nitrogen in water bath at 40 °C. The residue was reconstituted with 100 μL of methanol/water (40/60, v/v), and a 10 μL of aliquot was injected to the LC–MS/MS system for analysis.

2.6. Matrix effect and extraction recovery

Matrix effect (ME) including absolute matrix effect and relative matrix effect was estimated by the post-extraction addition method. Absolute matrix effect (aME) was used to evaluate the extent of MS signal suppression or enhancement. It was calculated by comparing the peak areas of analytes added post-extraction in six different lots of plasma (B) with mean peak areas of the standards at the same concentrations in the reconstitution solvent (A), and expressed as (B/A × 100%).

Relative matrix effect (rME) was used to evaluate the variations of different lots of plasma resulted from the matrix effect, and was calculated by the coefficients of variation [CV %] of peak area of analytes added post-extraction from six different lots of blank plasma.

Extraction recovery was calculated by comparing peak areas of QC samples (C) with the mean peak areas of analytes added post-extraction in five different lots of plasma (B), expressed as (C/B × 100%).

2.7. Method validation

The current LC–MS/MS assay method was validated for specificity, linearity, accuracy and intra-day and inter-day precision, recovery, and stability.

The specificity was confirmed by analysis six different lots of blank rat plasma.

Three validation batches were assayed to assess the linearity, accuracy and precision of the method. Each batch included a set of calibration standards and five replicates of QC samples at low, medium and high concentration levels, and was processed on three separate days. The linearity of each curve was assessed by plotting the peak area ratio (*y*) of the analyte to I.S. versus the corresponding concentration (*x*) of the analytes in the freshly prepared plasma calibrators. The accuracy of the assay was expressed by [(calculated concentration by the regression equations)/(spiked concentration)] × 100%, and the precision was evaluated by relative standard deviation (RSD).

The stability of IPR and SAL in spiked samples was investigated. The stability was evaluated under possible conditions that should reflect situations likely to be encountered during actual sample handling and analysis, including thawed plasma in room temperature for 5 h, frozen plasma in −20 °C for 36 days, plasma samples after three cycles of freeze and thaw, and the processed samples kept in 4 °C for 51 h. The stability of the analytes in stock solution was also evaluated.

2.8. Pharmacokinetics application

Five male Sprague-Dawley rats, weighing 300 ± 50 g, were supplied by the Experimental Animal Center of Shanghai Institute of Pharmaceutical Industry. The rat was fixed in a container and only its nose was exposed. Between the rat's nose and the aerosol, there was a connected tube with 10 cm in length and 2 cm in diameter. While the aerosol was puffed in one side of the tube, the rat was inhaling in the other side. Compound ipratropium bromide aerosol was administered as six puffs per rat and the animals were kept inhaling the aerosol for 1 min per puff. The inhaling procedure was lasting for 6 min. After administering, serial blood samples (~0.5 mL each at 0, 0.083, 0.17, 0.33, 0.5, 0.75, 1, 2, 4, 6 and 9 h after administration) were collected in heparinized tubes from jugular veins. The blood samples were centrifuged at 10,000 × *g* for 10 min and the plasma was separated and stored at −20 °C until assay. The study was approved by the Animal Ethical Committee of Shanghai Institute of Pharmaceutical Industry.

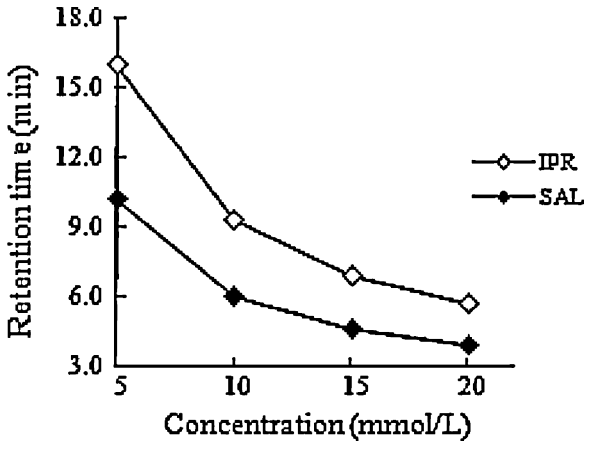

Fig. 2. The relationship between the concentration of ammonium formate and retention time of IPR and SAL.

Table 2
Matrix effects and extraction recovery of IPR, SAL and the internal standards in rat plasma.

Analyte	Analyte concentration (pg/mL)	Matrix effects (%, $n = 6$)	CV (%)	Extraction recovery (%, $n = 5$)	CV (%)
IPR	20.23	105.6 ± 5.7	5.4	83.98 ± 7.2	8.5
	161.8	90.25 ± 7.0	7.8	74.26 ± 3.0	4.1
	1214	92.55 ± 3.6	3.8	88.55 ± 3.1	3.4
SAL	126.5	89.21 ± 5.7	6.4	72.77 ± 2.3	3.1
	1012	98.15 ± 5.2	5.3	71.85 ± 2.5	3.4
	7590	76.93 ± 7.7	10	78.79 ± 3.5	4.5
IPR-d7	250.8	96.78 ± 3.7	3.8	83.33 ± 6.6	7.9
	250.8	82.29 ± 6.5	7.9	100.8 ± 3.9	3.8
	250.8	91.77 ± 3.4	3.8	102.7 ± 4.5	4.4
SAL-d9	3100	85.33 ± 5.5	6.5	71.28 ± 3.3	4.6
	3100	97.97 ± 5.2	5.3	73.25 ± 2.5	3.4
	3100	78.34 ± 8.1	10	82.69 ± 3.9	4.7

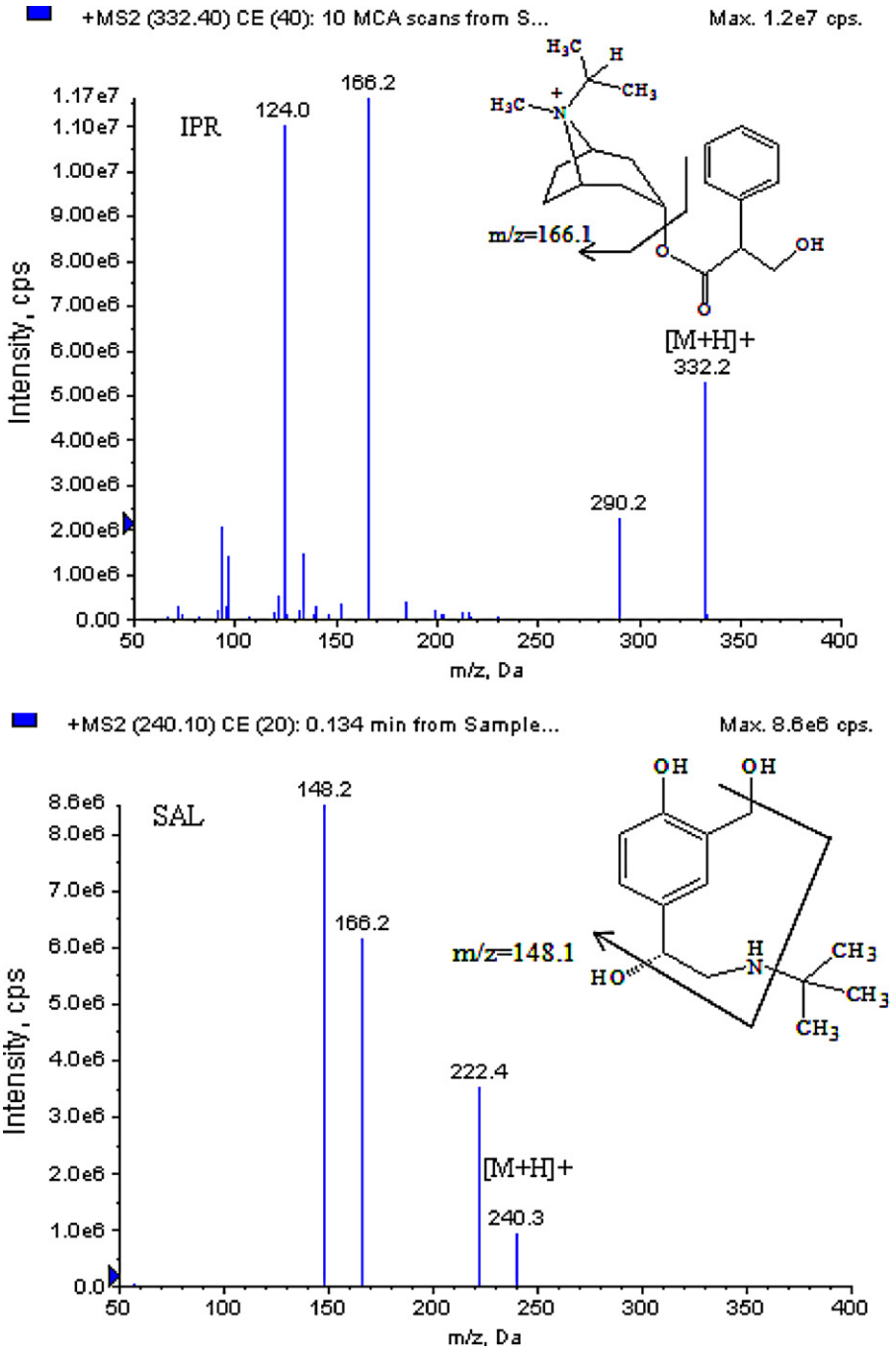

Fig. 3. MS/MS product ion mass spectra of the protonated molecules of IPR and SAL.

Table 3
Linearity for assay of IPR and SAL in rat plasma.

Analyte	Analytical batch	Linear range (pg/mL)	Regression equation	Correlation coefficient
IPR	1	8.090 –1618	$y = 0.00382x + 0.0579$	$r = 0.9993$
	2		$y = 0.00387x + 0.0350$	$r = 0.9990$
	3		$y = 0.00389x + 0.0330$	$r = 0.9990$
SAL	1	50.60 –10120	$y = 0.000984x + 0.00965$	$r = 0.9986$
	2		$y = 0.000995x + 0.0200$	$r = 0.9994$
	3		$y = 0.000983x + 0.0203$	$r = 0.9994$

3. Results and discussion

3.1. Optimization of chromatographic conditions

IPR was a quaternary ammonium base with stronger polarity and hardly retained on a C_{18} column, so a Shiseido Capcell Pak CR analytical column was chosen. This column with a mixed stationary phases of sulfonic and C_{18} groups made the separation basing on both ion-exchange and reversed phase separation process.

The retention of the analytes on the CR column mainly depends on the concentration of buffer salt in the mobile phase. A higher

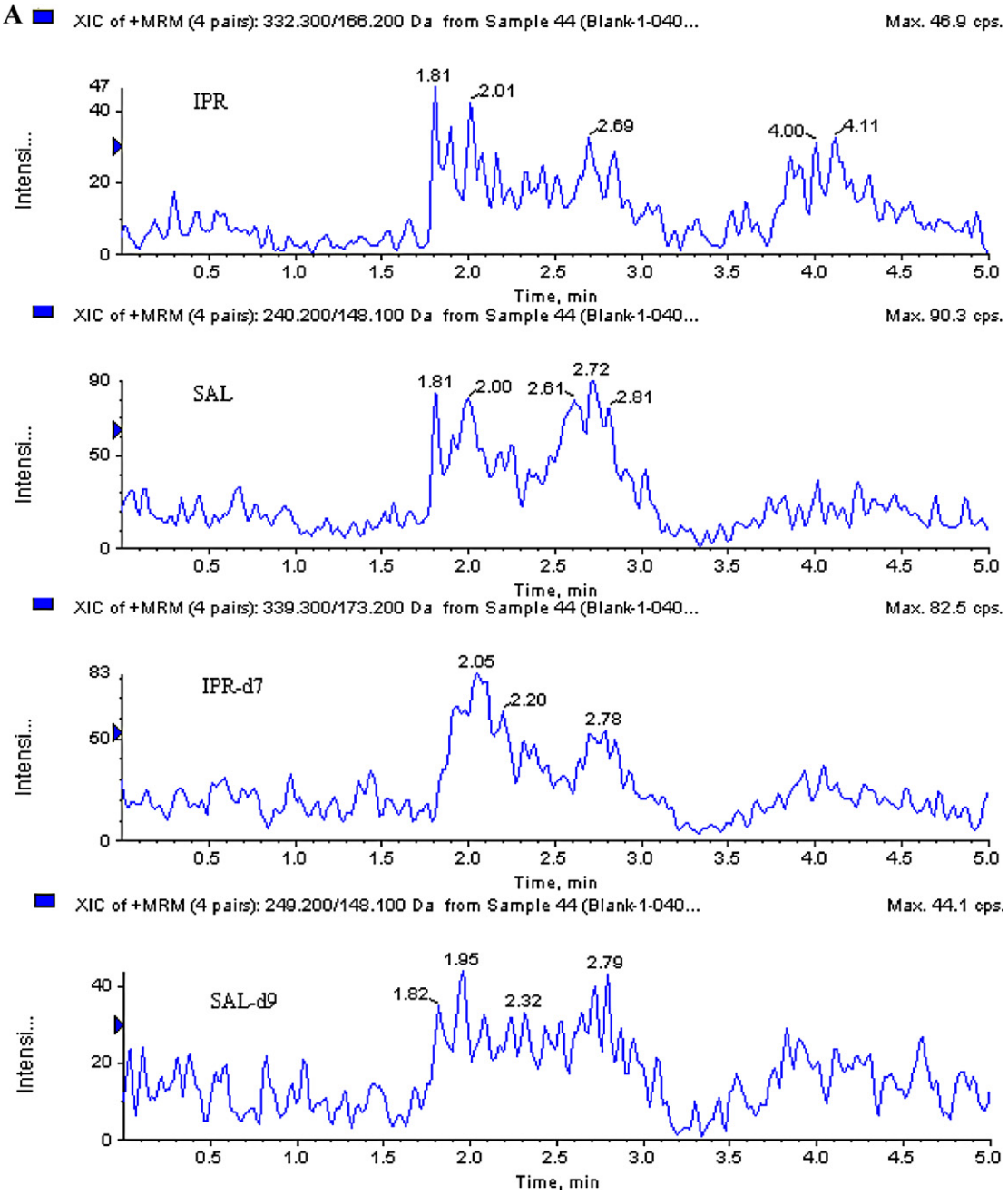

Fig. 4. Representative MRM chromatograms for (A) blank plasma, (B) LLOQ, 8 pg/mL for IPR and 50 pg/mL for SAL, (C) plasma sample, 0.083 h after administration.

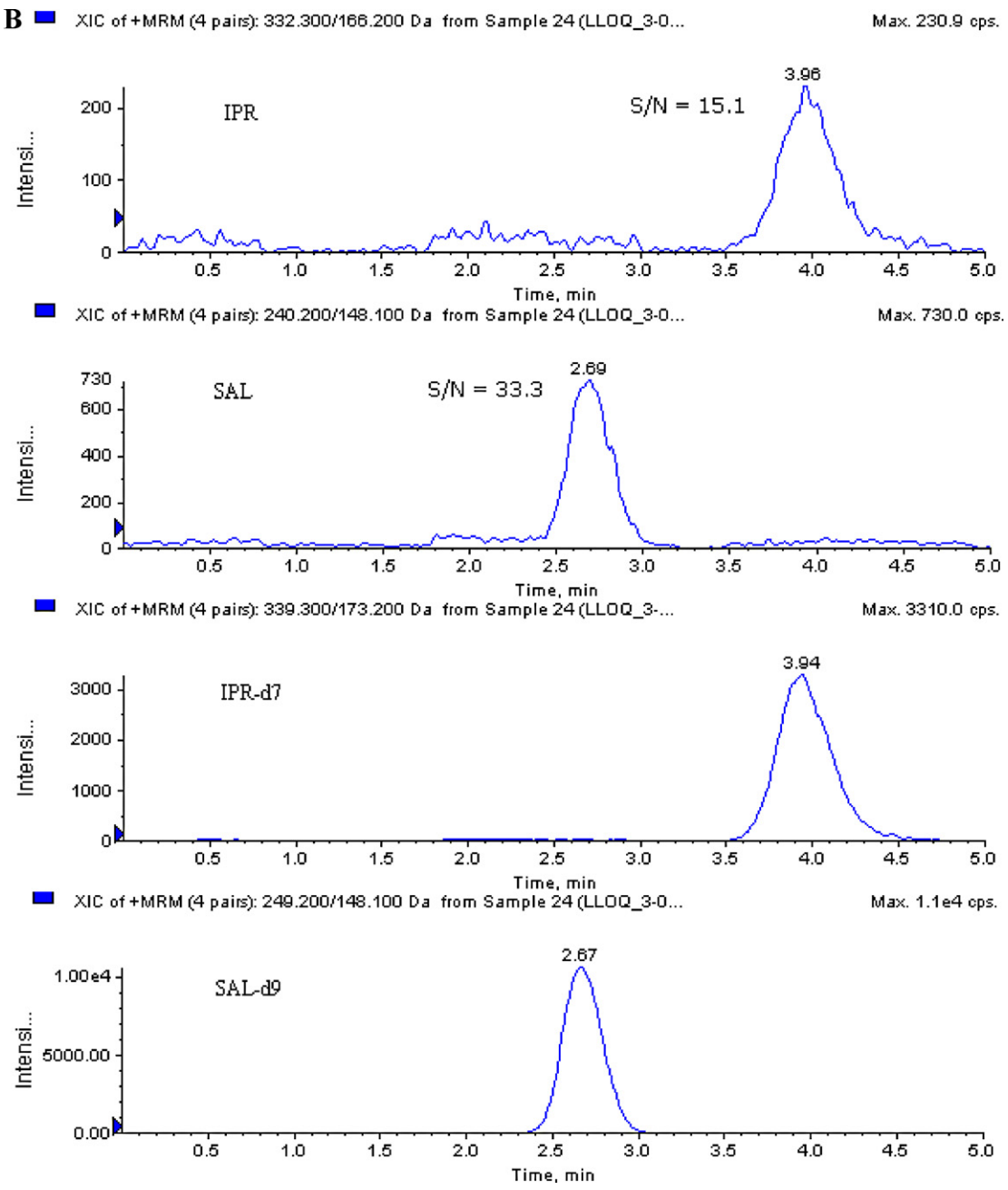

Fig. 4. *Continued.*

buffer concentration could decrease the retention. The ratio of methanol in the mobile phase had no significant effect on the chromatographic separation. However, higher concentration of organic phase and ammonium formate could improve the sensitivity of analysis. Fig. 2 sketches the relationship between the concentration of ammonium formate in the mobile phase and retention time of IPR and SAL. Thus, methanol/water (85:15, v/v) with 20 mmol/L ammonium formate and 0.1% formic acid was chosen as mobile phase.

3.2. Sample preparation

Protein precipitation with methanol was tried as a sample preparation technique, but the low extraction recovery was obtained and strong interferences from endogenous substances in plasma occurred. Liquid–liquid extraction cannot be employed in this study due to the low solubility of IPR in common organic solvents. Hence, solid-phase extraction was applied to extract IPR and SAL from rat plasma. The conditioning solvents and the elution solvents were the two key factors in improving the extraction recovery of the analytes.

Four kinds of conditioning solvents at different pH including water (pH 6.5), water with 0.1% formic acid (pH 3.0), water with 0.1% acetic acid (pH 4.0) and water with 20 mmol/L ammonium acetate (pH 8.0) were tested. Among these solvents, water with 20 mmol/L ammonium acetate (pH 8.0) showed the best results.

Elution solvents, which contained different proportions of hydrochloric acid and ammonium formate in methanolic solution, were also investigated. The results demonstrated that the ratio of hydrochloric acid and ammonium formate had

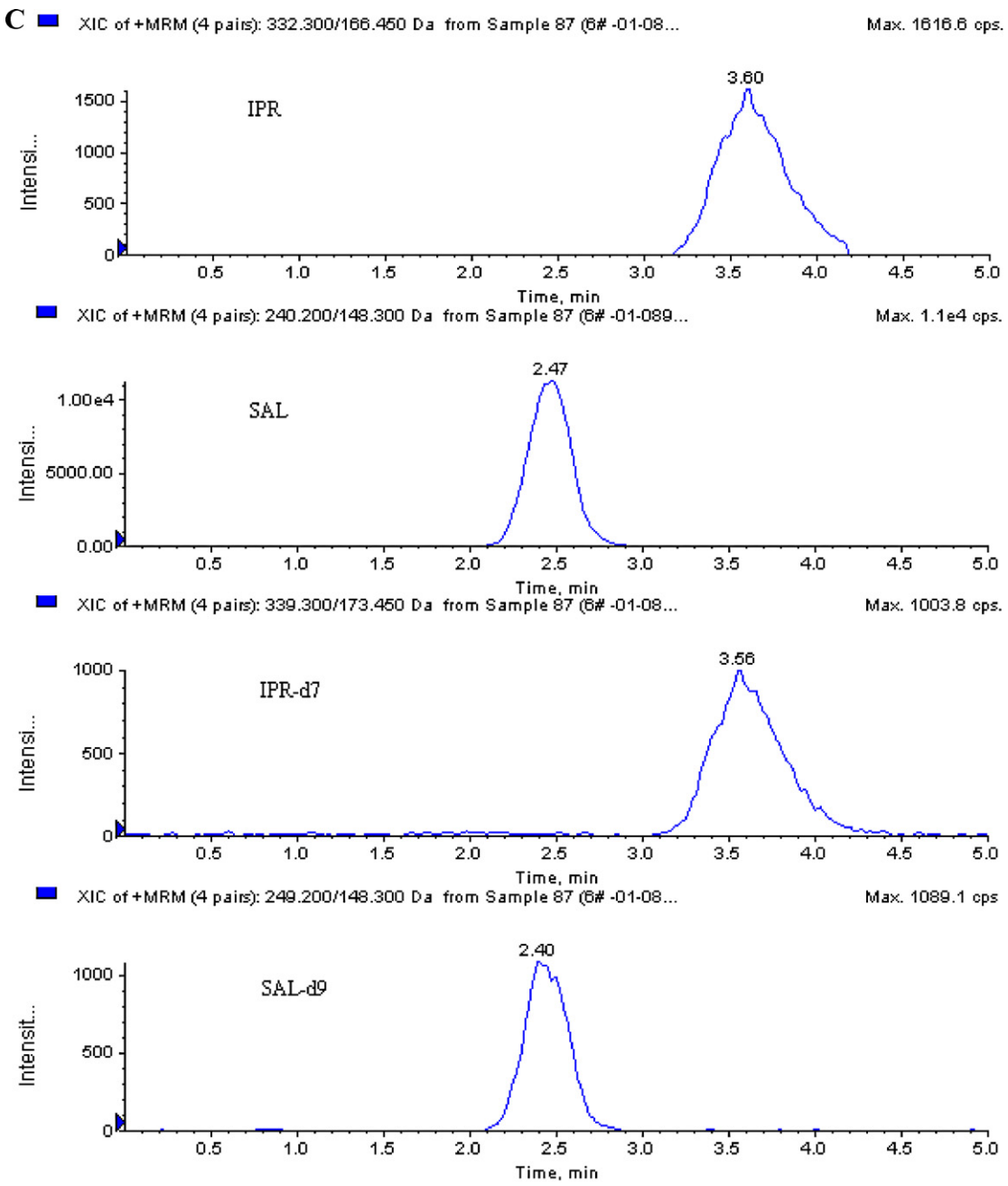

Fig. 4. *Continued.*

significant influences on the extraction recovery of IPR and SAL. 1 mL of 0.2% hydrochloric acid methanolic solution (containing 4 mmol/L ammonium formate) was the best elution solvent.

Washing the cartridges with methanol/water (40/60, v/v) before eluting could reduce plasma matrices. As a result, the sensitivity and precision was improved.

This extraction method can extract the two analytes simultaneously, meanwhile, the matrix effects were reduced and extraction recoveries were stable. According to the extraction procedures, we presumed that a weak cation-exchange interaction took place on the SPE cartridges at the condition of pH 8.0, when the silanols on the surface of silica formed anion retaining the analytes in cation form. The elution step was performed with an acidic salt solution which could replace the analytes from the sorbent. Therefore, a suitable pH was the most important factor in sample separation.

3.3. Matrix effect and extraction recovery

Due to the extreme low concentration of IPR, the endogenous substances and impurities from the SPE cartridges could interfere with the IPR determination. Conditioning the cartridges by 1% hydrochloric acid methanolic solution (containing 20 mmol/L ammonium formate) could realize the purpose to reduce the impurities. Washing the cartridges with methanol/water (40/60, v/v) before eluting could both greatly remove the endogenous substance and improve the sensitivity.

Choosing an appropriate internal standard is another approach to reduce the matrix effects. In this study, respective deuterium labeled standards of IPR and SAL were selected as internal standards. In Table 2, the data of matrix effects depicts signal suppression to analytes but little variance among different lots of plasma.

Table 4
Intra-day ($n = 5$) and inter-day ($n = 3$) precision and accuracy for assay of IPR and SAL in rat plasma.

Analyte	AC[a] (pg/mL)	Intra-day ($n = 5$)			Inter-day ($n = 3$)		
		CC[b] (pg/mL)	RSD (%)	A[c] (%)	CC[b] (pg/mL)	RSD (%)	A[c] (%)
IPR	20.23	23.35 ± 0.8404	3.6	115.4	20.73 ± 2.362	11.4	102.5
	161.8	165.9 ± 10.20	6.1	102.5	165.9 ± 7.027	4.2	102.5
	1214	1229 ± 76.73	6.2	101.3	1185 ± 64.83	5.5	97.59
SAL	126.5	126.2 ± 3.879	3.1	99.80	121.6 ± 4.040	3.3	96.12
	1012	993.7 ± 65.08	6.5	98.19	1021 ± 26.08	2.6	100.9
	7590	7691 ± 391.9	5.1	101.3	7638 ± 74.26	1.0	100.6

[a] AC: analyte concentration.
[b] CC: calculated concentration.
[c] A: accuracy.

The extraction efficiency for the two analytes as well as I.S. was consistent and reproducible according to the data presented in Table 2.

3.4. Method validation

3.4.1. Specificity

Abundant protonated molecules of IPR and SAL that formed the base peak of each mass spectrum were observed from Q1 scans during the infusion of the neat solution in positive mode. Two $[M+H]^+$ precursor ions, m/z 332.3 for IPR and m/z 240.2 for SAL, were subjected to collision-induced dissociation (CID). The product ion tandem mass spectra of the protonated IPR and SAL are shown in Fig. 3. Mass transition patterns, m/z 332.3 → 166.2 and m/z 240.2 → 148.1 were selected to monitor IPR and SAL, respectively. Two independent MS/MS channels of m/z 339.3 → 173.2 and m/z 249.2 → 148.1 were chosen to monitor the internal standard, IPR-d7 and SAL-d9.

Chromatograms of different lots of blank plasma showed no endogenous peak co-eluted with analytes. Representative chromatograms of blank sample, LLOQ sample, and a rat plasma sample (0.083 h after the aerosol applied) are shown in Fig. 4. In addition, the 'cross-talk' between channels used for monitoring the analytes and I.S. was evaluated by analysis of their individual solution at high concentration. The responses in all MRM mass transition channels used for quantification were monitored. No 'cross-talk' or interference between the analytes and I.S. was observed.

3.4.2. Linearity and sensitivity

Seven-point calibration curves were prepared ranging from 8 to 1612 pg/mL for IPR and from 50 to 10,000 pg/mL for SAL. The regression parameters of slope, intercept and correlation coefficient were calculated by $1/x$-weighted linear regression in Analyst 1.5.1 software. Excellent linearity was achieved with correlation coefficients greater than 0.9986 for all validation batches. The results are shown in Table 3.

The current assay offered a LLOQ of 8 pg/mL for IPR and 50 pg/mL for SAL, which is sensitive enough to investigate our pharmacokinetic behaviors of compound ipratropium bromide aerosol. Typical LC–MS/MS chromatogram of the LLOQ sample is shown in Fig. 4.

3.4.3. Accuracy and precision

The accuracy and the precision were analyzed by the QC samples at three concentrations. The assay accuracy and precision results are summarized in Table 4. The data obtained was within the acceptable limits to meet the guideline for bioanalytical methods [9].

3.4.4. Stability

The stability of IPR and SAL in plasma was investigated. And the results implied that no significant degradation occurred at room temperature for 5 h and at −20 °C for 36 days. The plasma samples after three freeze and thaw cycles and the processed samples kept in the autosampler (4 °C) for 51 h were stable. The stock solutions of IPR, SAL and I.S. in methanol were also stable at 4 °C for 36 days.

3.5. Pharmacokinetics

Following validation, the method was successfully applied to the pharmacokinetic study of the compound ipratropium bromide aerosol in rats. The mean plasma concentration–time profile of IPR and SAL after the application of the aerosol is shown in Fig. 5. The main pharmacokinetic parameters from a non-compartmental model analysis (Drug and Statistics, DAS version 2.0) are listed in Table 5. Applying the method, the drug concentration in plasma should be detected until 6 h after administration.

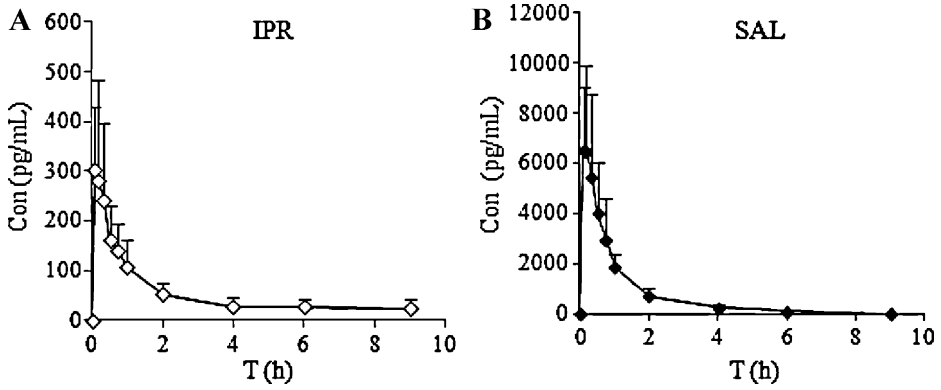

Fig. 5. Mean plasma concentration–time profile of (A) IPR and (B) SAL after administration of the aerosol in rats ($n = 5$).

Table 5
Main pharmacokinetic parameters of IPR and SAL in rat plasma ($n = 5$).

Parameters	Units	IPR	SAL
$AUC_{(0-t)}$	pg/(mL h)	427.1	6509
$AUC_{(0-\infty)}$	pg/(mL h)	450.8	6635
$MRT_{(0-t)}$	h	2.016	1.051
$MRT_{(0-\infty)}$	h	2.471	1.163
$t_{1/2z}$	h	1.894	1.029
T_{max}	h	0.333	0.116
C_{max}	pg/mL	349.0	7062

4. Conclusion

In this paper, a novel LC–ESI-MS/MS method for simultaneous determination of IPR and SAL in rat plasma is described. This method provides a low LLOQ (8 pg/mL for IPR and 50 pg/mL for SAL in 200 μL plasma), and the sample preparation can reduce the matrix effects and improve the sensitivity of the method. The fully validated method is simple, highly sensitive, specific and robust, and has successfully applied to pharmacokinetic study in rats. This method is suitable for routine analysis of large number of biological samples.

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