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Determination of three major components of bitespiramycin and their major active metabolites in rat plasma by liquid chromatography-ion trap mass spectrometry

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Abstract

The rapid, selective and sensitive liquid chromatographic–ion trap mass spectrometric (LC–MS^n) method was developed and validated for determination of three major components (isovaleryspiramycins, ISV-SPMs) of a novel macrolide antibiotic bitespiramycin and their major active metabolites (spiramycins, SPMs) in rat plasma. The analytes ISV-SPMs, SPMs, internal standard roxithromycin and azithromycin were extracted from plasma samples by liquid–liquid extraction, and chromatographed on a C_{18} column using two mobile phase systems. Detection was carried out on an ion trap mass spectrometer by selected reaction monitoring (SRM) mode via electrospray ionization (ESI). Three components (ISV-SPM I, II, III or SPM I, II, III) could be simultaneously determined within 6.5 min. Linear calibration curves were obtained in the concentration ranges of 4–200 ng/ml for ISV-SPM I and SPM I, 12–600 ng/ml for ISV-SPM II and SPM II, and 18–900 ng/ml for ISV-SPM III and SPM III. The intra- and inter-run precision (RSD), calculated from quality control (QC) samples were less than 8.8 and 10.4% for ISV-SPMs, and 9.3 and 11.2% for SPMs, respectively. The method was applied for the evaluation of the pharmacokinetics of bitespiramycin in rats following peroral/intravenous administration.

Keywords: Bitespiramycin; Isovaleryspiramycin; Spiramycin; Antibiotics

1. Introduction

Spiramycin (SPM) is a 16-membered macrolide antibiotic and mainly constituted of SPM I (over 85%) while SPM II, and III are minor derivatives (lower than 5 and 10%, respectively) [1]. SPM has been used to treat infections of the oropharynx, respiratory system and genito-urinary tract as well as cryptosporidiosis and toxoplasmosis [2–4].

Bitespiramycin (Shengjimycin) is a novel antibiotic, which was expected to have better pharmacokinetic behavior than that of SPM. It is a group of 4′-acylated SPMs with 4″-isovaleryspiramycins (ISV-SPMs) as the major components, produced by recombinant *Streptomyces spiramyceticus* F21 [5] (Fig. 1). Bitespiramycin consisted of ISV-SPM I (7.4%), II (22.5%) and ISV-SPM III (37.7%). Minor components in bitespiramycin include about 10 derivatives of spiramycin such as butanoylspiramycin and propionylspiramycin.

Pharmacokinetic (PK) screening currently plays

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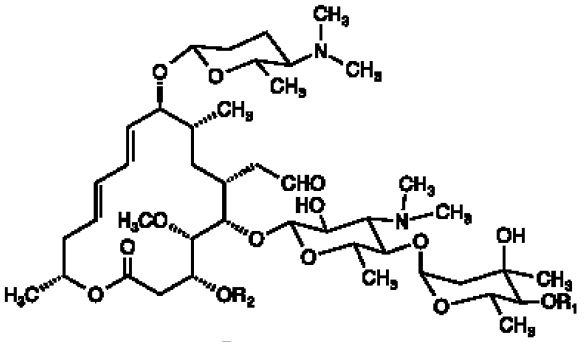

	R_1	R_2
ISV-SPM I	$COCH_2CH(CH_3)_2$	H
ISV-SPM II	$COCH_2CH(CH_3)_2$	$COCH_3$
ISV-SPM III	$COCH_2CH(CH_3)_2$	COC_2H_5
SPM I	H	H
SPM II	H	$COCH_3$
SPM III	H	COC_2H_5

Fig. 1. Chemical structures of ISV-SPM I, II, III and SPM I, II, III.

an important role in the screening paradigm on selecting lead compounds for most drug discovery projects. Rapid and effective ways to provide PK information are desirable to shorten the times required for drug discovery in the pharmaceutical industry. As the components of bitespiramycin are complex and more metabolites could be produced in vivo, a highly selective, rapid and sensitive analytical method is required. A variety of methods have been employed for the analysis of SPM I in plasma and tissue including radioactive assay [6], microbiological analysis [7,8] and HPLC with ultraviolet detection [9,10]. However, these methods had low specificity or sensitivity, and it was impossible to simultaneously determine ISV-SPM I, II, III or SPM I, II, III as the metabolites of a complex multicomponent drug.

A few LC–MS–MS methods have been employed to monitor SPM I residues in edible tissues, milk and eggs of livestock animals [11–13]. But these methods only focused on the determination of SPM I and to our knowledge, a method for simultaneous determination of ISV-SPM I, II, III or SPM I, II, III in biological samples has not been reported. In this paper we describe a rapid, selective and sensitive $LC–MS^n$ method for determination of ISV-SPM I, II, III and SPM I, II, III in rat plasma. The method developed was successfully applied to preclinical pharmacokinetic studies for a complex multicomponent antibiotic bitespiramycin.

2. Experimental

2.1. Reference compounds and chemicals

Bitespiramycin and ISV-SPM I, II, III were provided by the Institute of Medical Biotechnology (Beijing, China), and SPM I, II and III were isolated from commercial SPM (Chaoyang Pharmaceutical Factory, Liaoning, China) by semipreparative HPLC and identified by ^1H NMR and ^{13}C NMR spectroscopy. Roxithromycin and azithromycin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Methanol and acetonitrile were of HPLC-grade, and other chemicals used were of analytical-grade. Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Instrumentation

A Shimadzu LC-10AD pump (Kyoto, Japan) was used. Chromatography was carried out on a Kromasil C_{18} column (150×4.6 mm, 5 μm, Hi-Tech Scientific Instrument Corp., Tianjin, China), using a mobile phase of acetonitrile–10 m*M* ammonium acetate–acetic acid to determine ISV-SPMs (45:55:0.5, v/v/v) and SPMs (35:65:0.5, v/v/v). The flow-rate was isocratic at 0.5 ml/min. The column temperature was maintained at 25 °C.

A Finnigan LCQ ion trap mass spectrometer equipped with an electrospray ionization (ESI) source (San Jose, CA, USA) was used for mass analysis and detection. The settings for the ESI ion source were as follows: the capillary temperature was heated to 170 °C, the capillary voltage was set to 6.0 V and an ion-spray voltage of 4.5 kV was applied. Nitrogen was used as the sheath gas (0.75 l/min) and auxiliary gas (0.15 l/min) to assist with nebulization. A divert valve directed the HPLC flow to a waste container in the first 3.5 min of the chromatographic run and afterwards to the ion source. Helium was used as the collision gas at a

flow-rate of 0.2 ml/min. The instrument was operated in the positive ion detection mode, producing positive charged ions in the form of $[M+2H]^{2+}$ and $[M+H]^{+}$. Double-charged ions were trapped and then fragmented by collision induced dissociation. Quantitation was carried out using selected reaction monitoring (SRM) mode. The ions of analytes and internal standards dissociated at a relative collision energy of 23–32% into their main fragments: m/z 464.3 (ISV-SPM I)→m/z 350.2 and 699.2; m/z 485.4 (ISV-SPM II)→m/z 371.3 and 741.2; m/z 492.3 (ISV-SPM III)→m/z 378.2 and 755.2; m/z 837.3 (internal standard roxithromycin)→m/z 679.2; m/z 422.3 (SPM I)→m/z 350.2 and 699.2; m/z 443.2 (SPM II)→m/z 371.2 and 741.2; m/z 450.2 (SPM III)→m/z 378.2 and 755.2; m/z 375.3 (internal standard azithromycin)→m/z 591.3, respectively.

2.3. Preparation of standard and quality control solutions

The stock standard solutions of ISV-SPM I, II and III were prepared by dissolving the accurately weighed standard compounds in acetonitrile to give final concentrations of 100, 300, 450 μg/ml for each analyte in the same volumetric flask. The solutions were then successively diluted with acetonitrile to achieve standard working solutions at concentrations of 400/1200/1800, 320/960/1440, 80/240/360, 40/120/180, 20/60/90, and 8/24/36 ng/ml for ISV-SPM I/II/III. The standard working solutions at concentrations of 8/24/36, 40/120/180 and 320/960/1440 ng/ml were used as quality control solutions (low, medium, high) for ISV-SPM I/II/III, respectively. The standard working and quality control solutions of SPMs were prepared in the same process as ISV-SPMs. A 800 ng/ml roxithromycin and a 1200 ng/ml azithromycin working solution was prepared by diluting stock standard solutions of roxithromycin and azithromycin with acetonitrile, respectively.

The standard solutions (50 μl) were used to spike blank plasma samples (0.1 ml) either for calibration curves of ISV-SPMs and SPMs or for quality control in prestudy validation and during the pharmacokinetic study.

All solutions were stored at 4 °C and brought to room temperature before use.

2.4. Sample preparation

To a 0.1-ml aliquot of rat plasma were added 50 μl acetonitrile and 50 μl of the internal standard. The samples were vortexed for 15 s. The mixture was adjusted to pH 9–10 with 0.3 ml of 0.02 *M* Na_2CO_3 and extracted with 2 ml of ethyl acetate–isopropanol (95:5, v/v) for 20 min on a roller-shaker. Phase separation was achieved by centrifugation at 3000 *g* for 10 min. The organic layer was removed and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was dissolved in 100 μl of mobile phase, and vortex mixed. A 20-μl aliquot of the solution was injected into the LC–MS^n system for analysis.

2.5. Data acquisition and analysis

Data were collected and analyzed by the ThermoFinnigan Xcalibur software package, version 1.0. Peak integration and calibration were carried out using Finnigan LCQuan software. Peak area ratios of analytes to the internal standard curves were calculated using $1/x^2$ weighted linear least-squares regression of plasma concentrations and the measured peak area ratios. Concentrations of analytes were calculated by interpolation from the calibration curves.

2.6. Method validation

The calibration curves for ISV-SPMs and SPMs were prepared by analyzing spiked plasma samples. The spiked plasma samples at three concentration levels (low, medium and high concentrations) were used as QC samples and analyzed by the LC–MS^n system.

During prestudy validation [14], the calibration curves were defined in three runs based on triplicate assays of the spiked plasma samples, and QC samples were determined in replicates ($n=6$) on the same run. Overall assay performance was assessed by calculating the accuracy and intra- and inter-run precision of QC samples analyzed. During routine analysis each analytical run included a set of calibration samples, a set of QC samples in duplicate and unknowns.

3. Results and discussion

The LC–MS^n method for simultaneous determination of ISV-SPM I, II, III or SPM I, II, III in rat plasma was developed. The LC–MS^n procedure developed was used to investigate the plasma profile of the major components of bitespiramycin and their major active metabolites after a single peroral/intravenous dose.

3.1. Liquid chromatography

The chromatographic conditions were optimized to reduce analytical cycle time and to obtain good chromatographic peak shape. Although ISV-SPMs and SPMs are structural analogs, their polarities are significantly different due to the fact that ISV-SPM has one more lipophilic functional group (introduction of isovalery) than SPM. It was difficult to determine ISV-SPMs and SPMs within a short run time using the same chromatographic system. Therefore, two different mobile phase systems were used to determine ISV-SPMs and SPMs in rat plasma. Symmetrical peak shapes of the ISV-SPMs could not be easily obtained on many C_{18} or C_8 columns, due to highly lipophilic character and adsorption to column materials of ISV-SPMs. A Kromasil C_{18} (5 μm packing) HPLC column (150×4.6 mm) with a 4×3.0 mm Dikma guard column was eventually used for the analysis of ISV-SPMs and SPMs as only 6.5 min were required to elute all the analytes completely and achieved the symmetrical peak shapes. The selection of mobile phase components was also a critical factor; 10 m*M* ammonium acetate buffer (pH≈3.5, achieved with 0.5% acetate acid) was used as the element of mobile phase to supply the ionic strength, in order to obtain good chromatographic peak shape and resolution. Acetonitrile was used as organic modifier because a quantity of $[M+H+MeOH]^+$ adduct ions occurred which would decrease the sensitivity of the analytes.

To avoid contamination of the mass spectrometer, a divert valve was placed between the analytical column and the mass spectrometer, allowing the eluate to pass through the mass spectrometer only during analyte elution (3.0–6.5 min).

In addition, during sample preparation, it was necessary to reconstitute the residues with the mobile phase, otherwise the chromatographic behavior of the analytes would be seriously deteriorated.

3.2. Mass spectrometry

The electrospray interface was used and good sensitivity, fragmentation and linearity were obtained. We have tested atmospheric pressure ionization (APCI) interface and no obvious improvement was observed. The first step in developing the detection method was to select the precursor ion to be fragmented in the ion trap. In the full mass spectra of ISV-SPMs and SPMs, the protonated molecules $[M+H]^+$ and the most intensive double-charged ions $[M+2H]^{2+}$ were observed. The ions $[M+2H]^{2+}$ were chosen as the precursor ions for the analytes and this could increase the specificity of the LC–MS^n method because larger fragment ions could be produced which is different from the dissociation mechanism of protonated molecules. The ESI interface and mass spectrometer parameters were optimized to obtain maximum sensitivity of $[M+2H]^{2+}$. The collision energy was selected by observing the maximum response obtained for the fragment ion peak m/z (Table 1). Product ion spectra of the analytes and internal standards are presented in Fig. 2. The base peaks of product ions were observed at m/z 699.2, 741.2 and 755.2 in the product ion spectra of ISV-SPMs and SPMs. They were the fragment ions having lost a mycaminose moiety compared to the precursor ions of ISV-SPM I and SPM I; ISV-SPM II and SPM II; ISV-SPM III and SPM III, respectively. Minor product ions were also observed at m/z 350.2, 371.2 and 378.2, which are double-charged ions of the base peaks, respectively. Evidence for this proposed dissociation pattern is gained by the further fragmentation of the product ions in MS^3 experiments (data not shown). In the chromatograms, two major product ions produced from the double-charged ion were added up with regard to their parent ion to enhance the signal-to-noise ratio.

A good internal standard should mimic the analyte in the entire sample extraction, chromatographic elution, and mass spectrometric detection. It will compensate for any potential inconsistent response due to matrix effects and will not cause interference to the analyte and vice versa. We used roxithromycin

Table 1
Precursor ions and product ions of the analytes and internal standards in the positive ion mode

Molecule	Precursor ion (m/z)	Product ions for quantification (m/z)	CID (%)
ISV-SPM I	464.3 (d)	350.2 (d), 699.2 (s)	24
ISV-SPM II	485.4 (d)	371.3 (d), 741.2 (s)	24
ISV-SPM III	492.3 (d)	378.2 (d), 755.2 (s)	24
Roxithromycin	837.3 (s)	679.2 (s)	32
SPM I	422.3 (d)	350.1 (d), 699.2 (s)	23
SPM II	443.2 (d)	371.2 (d), 741.2 (s)	23
SPM III	450.2 (d)	378.2 (d), 755.2 (s)	23
Azithromycin	375.3 (d)	579.3 (s)	25

(d): double charged ion $[M+2H]^{2+}$; (s): single charged ion $[M+H]^{+}$.

as an internal standard for ISV-SPMs and used azithromycin as an internal standard for SPMs. Both roxithromycin and azithromycin are macrolide antibiotics structurally related to the analytes. Roxithromycin eluted close to ISV-SPMs and azithromycin eluted close to SPMs, as shown in Fig. 3. In particular, the full mass spectrometry of azithromycin was also dominated by double-charged ion $[M+2H]^{2+}$, so $[M+2H]^{2+}$ of azithromycin was chosen as the precursor ion and this will compensate for effects on the ratios of $[M+2H]^{2+}/[M+H]^{+}$ of the SPMs due to minor fluctuations of temperature, pH and flow-rate. The intra- and inter-run precision (RSD) calculated from QC samples proved both roxithromycin and azithromycin are suitable internal standards. Precursor ions and product ions of the analytes and internal standards are listed in Table 1.

3.3. Method validation

3.3.1. Assay specificity

The specificity of the method was demonstrated by comparing chromatograms of blank samples and spiked samples. Fig. 3 demonstrates that no interferences were detected from endogenous substances with the analytes and internal standards. Typical retention times for ISV-SPM I, II, III and roxithromycin were 4.09, 4.56, 5.45 and 5.44 min, respectively. Typical retention times for SPM I, II, III and azithromycin were 3.65, 4.14, 5.18 and 3.95 min, respectively.

It has been noted that co-eluting, undetected endogenous matrix components may suppress the ion intensity of the analytes and adversely affect the reproducibility and accuracy of the LC–MS^n assay [15]. To determine whether this matrix effect is present or not, the peak area of the compounds in the plasma samples spiked after extraction were compared with those of each compound in mobile phase injected into the system. The results indicated that no co-eluting endogenous species interfered with the ionization of the analytes and internal standards.

3.3.2. Linearity of calibration curve and lower limit of quantitation

Linear calibration curves were obtained over the concentration ranges of 4–200, 12–600 and 18–900 ng/ml for ISV-SPM I, II, III and SPM I, II, III in rat plasma. Typical equations of calibration curves are listed in Table 2.

The lower limit of quantitation (LLOQ), defined as the lowest concentration analyzed with acceptable accuracy and precision, was 4 ng/ml for ISV-SPM I and SPM I, 12 ng/ml for ISV-SPM II and SPM II, and 18 ng/ml for ISV-SPM III and SPM III, respectively.

3.3.3. Assay precision and accuracy

Intra- and inter-run precision were assessed from the results with QC samples. The mean values and RSD for QC samples were calculated over three validation runs. Six replicates for each QC level were determined in each run. These data were then used to calculate the intra- and inter-run precision (RSD) using a one-way analysis of variance (ANOVA).

The accuracy of the method was determined by

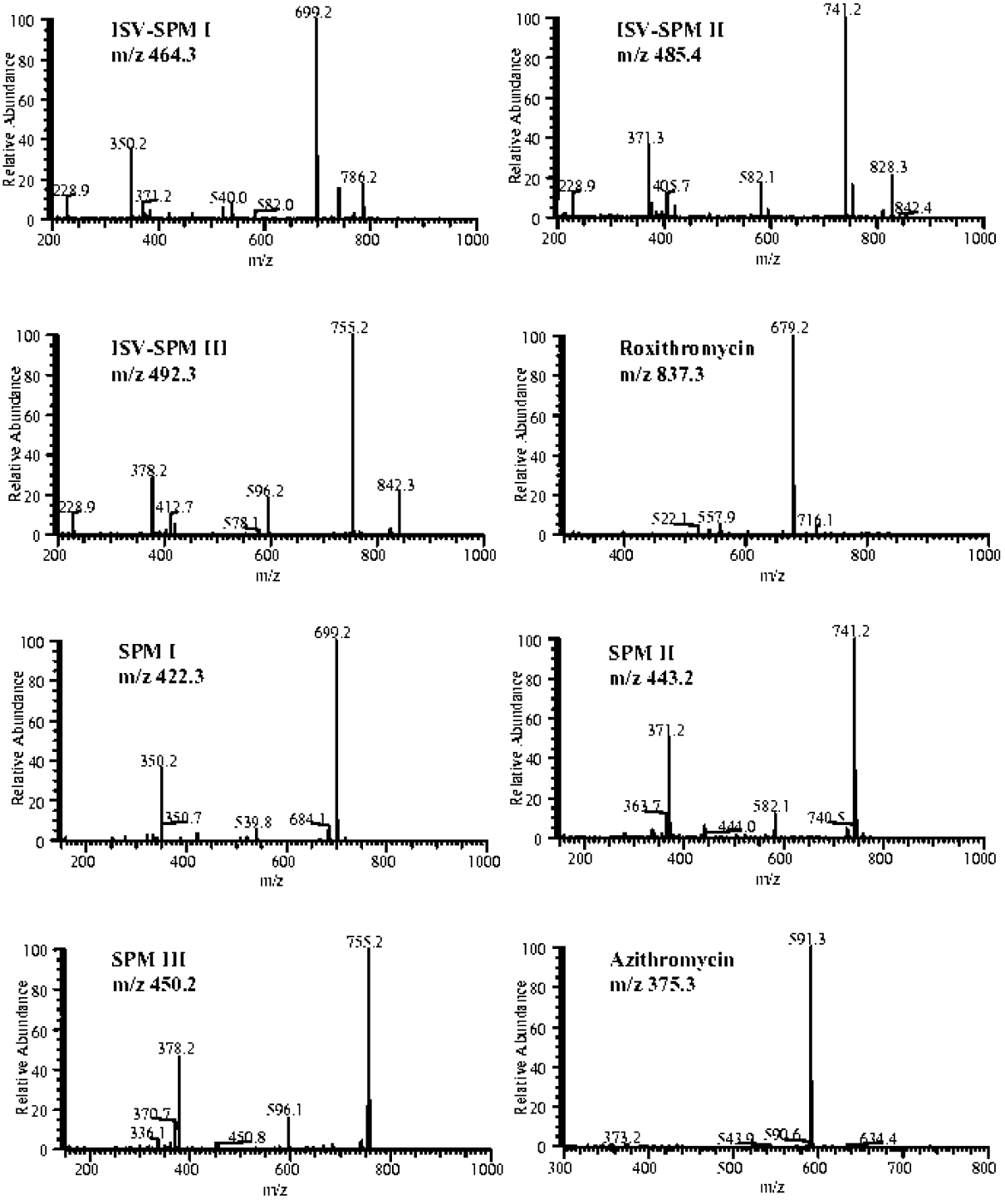

Fig. 2. Full scan MS^2 spectra of the analytes and internal standards.

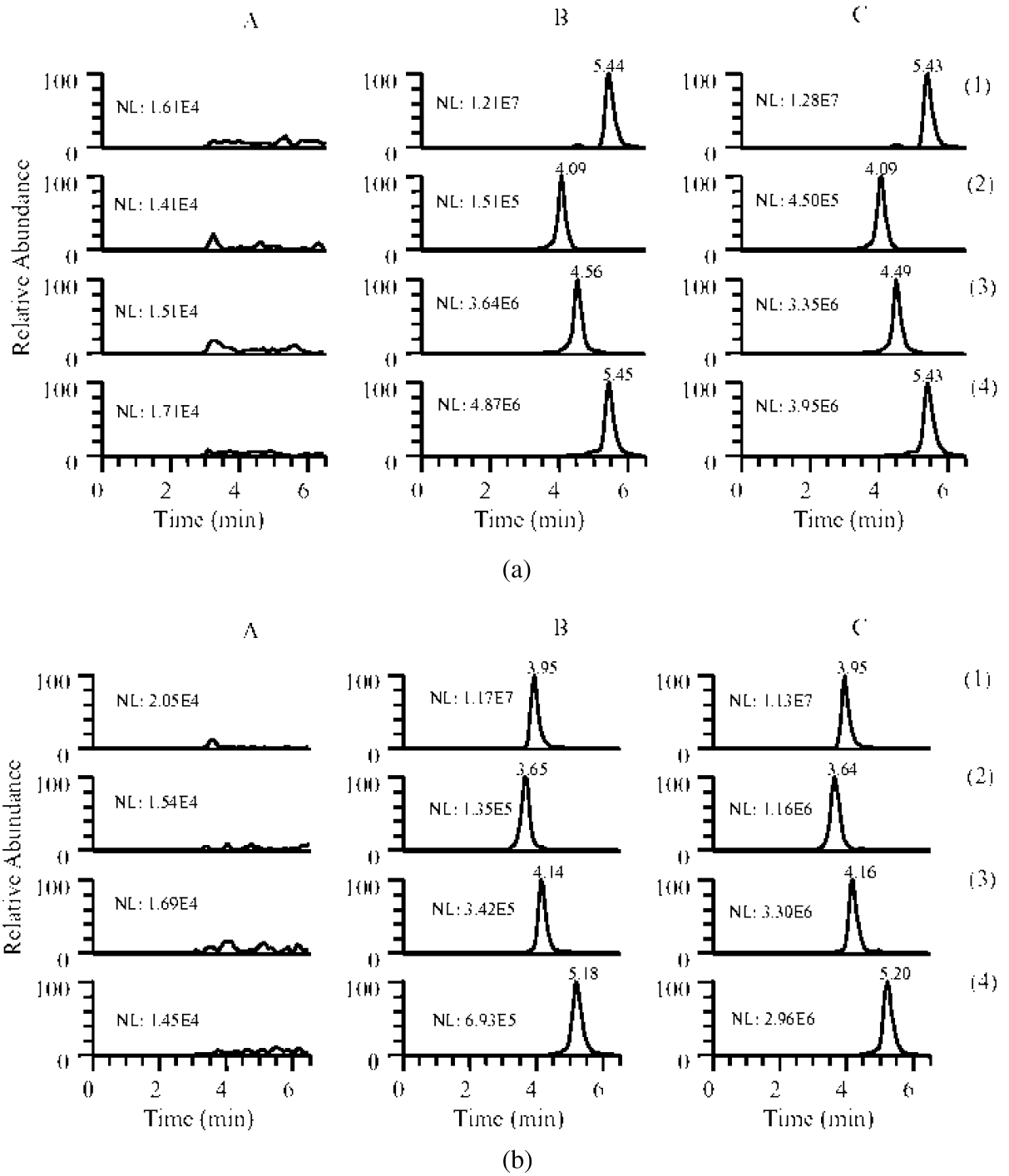

Fig. 3. (a) Representative LC–MS^n chromatograms of plasma extracts obtained by SRM mode: (A) blank plasma sample; (B) blank plasma spiked with 8 ng/ml ISV-SPM I, 24 ng/ml ISV-SPM II, 36 ng/ml ISV-SPM III and 400 ng/ml roxithromycin (internal standard); (C) plasma sample of the rat 0.75 h after oral administration of 80 mg/kg bitespiramycin. Channels 1–4 refer to the internal standard, ISV-SPM I, ISV-SPM II and ISV-SPM III, respectively. (b) Representative LC–MS^n chromatograms of plasma extracts obtained by SRM mode: (A) blank plasma; (B) blank plasma spiked with 8 ng/ml SPM-I, 24 ng/ml SPM-II, 36 ng/ml SPM-III and 600 ng/ml azithromycin (internal standard); (C) plasma sample of the rat 6 h after intravenous administration of 80 mg/kg bitespiramycin. Channels 1–4 refer to the internal standard, SPM I, SPM II and SPM III, respectively.

Table 2
Summary of precision and accuracy from QC samples of rat plasma extracts (in prestudy validation, $n=18$)

Analyte	Intra-run RSD (%)	Inter-run RSD (%)	Relative error (%)	Typical equation for calibration curve
ISV-SPM I	8.8	10.4	6.1	$y=9.028\times10^{-4}+6.634\times10^{-4}x$, $r=0.9954$
ISV-SPM II	8.5	8.5	5.0	$y=2.772\times10^{-3}+1.015\times10^{-3}x$, $r=0.9978$
ISV-SPM III	6.4	8.7	4.7	$y=5.376\times10^{-3}+1.071\times10^{-3}x$, $r=0.9989$
SPM I	9.3	11.2	5.3	$y=9.222\times10^{-4}+1.085\times10^{-4}x$, $r=0.9974$
SPM II	8.4	8.6	4.3	$y=8.498\times10^{-4}+1.895\times10^{-4}x$, $r=0.9988$
SPM III	7.5	9.3	4.5	$y=8.866\times10^{-3}+1.981\times10^{-3}x$, $r=0.9983$

calculating the percentage deviation observed in the analysis of QC samples and expressed in the relative error (RE).

Table 2 shows the intra- and inter-run precision and accuracy for ISV-SPMs and SPMs from the QC samples.

3.3.4. Extraction recovery

The extraction recoveries of ISV-SPMs and SPMs were determined by comparing peak areas obtained from plasma samples with those found by direct injection of standard solution at the same concentration. The results showed that the mean extraction recoveries of ISV-SPM I, II and III were 61.1±5.2, 63.7±6.5 and 69.8±4.2%, and mean extraction recoveries of SPM I, II and III were 58.2±5.9, 64.1±6.6 and 70.8±5.1%, respectively. The mean extraction recoveries of roxithromycin and azithromycin are 75.8±5.5 and 71.2±6.2%, respectively.

To determine SPM I in biological fluids, solid-phase extraction had been used, which needed a long drying time and was expensive [10]. In this study, the analytes and the internal standards were extracted from plasma samples by simple liquid–liquid extraction. There are two $-N(CH_3)_2$ groups in the structures of SPM derivatives, which had pK_a values close to 7.7. Therefore, 0.3 ml of 0.02 *M* Na_2CO_3 was added to the plasma samples adjusting the pH to 9–10 before extraction. After evaluating five kinds of organic extraction solvents, a mixture of ethyl acetate–isopropanol (95:5, v/v) proved to be the most efficient to extract ISV-SPMs and SPMs from rat plasma. More importantly, variation of extraction recoveries are small over the concentration range.

3.4. Application of the analytical method in pharmacokinetic studies

After a single peroral/intravenous administration of 80 mg/kg bitespiramycin to each of six rats, plasma concentrations of ISV-SPM I, II, III and SPM I, II III were determined by the LC–MS^n method described. Fig. 4 shows mean plasma concentration–time curves of ISV-SPMs and SPMs after administration ($n=6$).

4. Conclusion

The LC–MS^n method has been developed and validated for the determination of three major components of bitespiramycin and their major active metabolites in rat plasma. Three components (ISV-SPM I, II, III or SPM I, II, III) could be simultaneously determined within 6.5 min. The method has been shown to be rapid, sensitive, selective and reproducible. The method was validated for simultaneous quantitation of ISV-SPM I, II, III or SPM I, II, III and was shown to be successful in applications supporting preclinical studies of a novel macrolide antibiotic bitespiramycin, a complex multicomponent drug.

Acknowledgements

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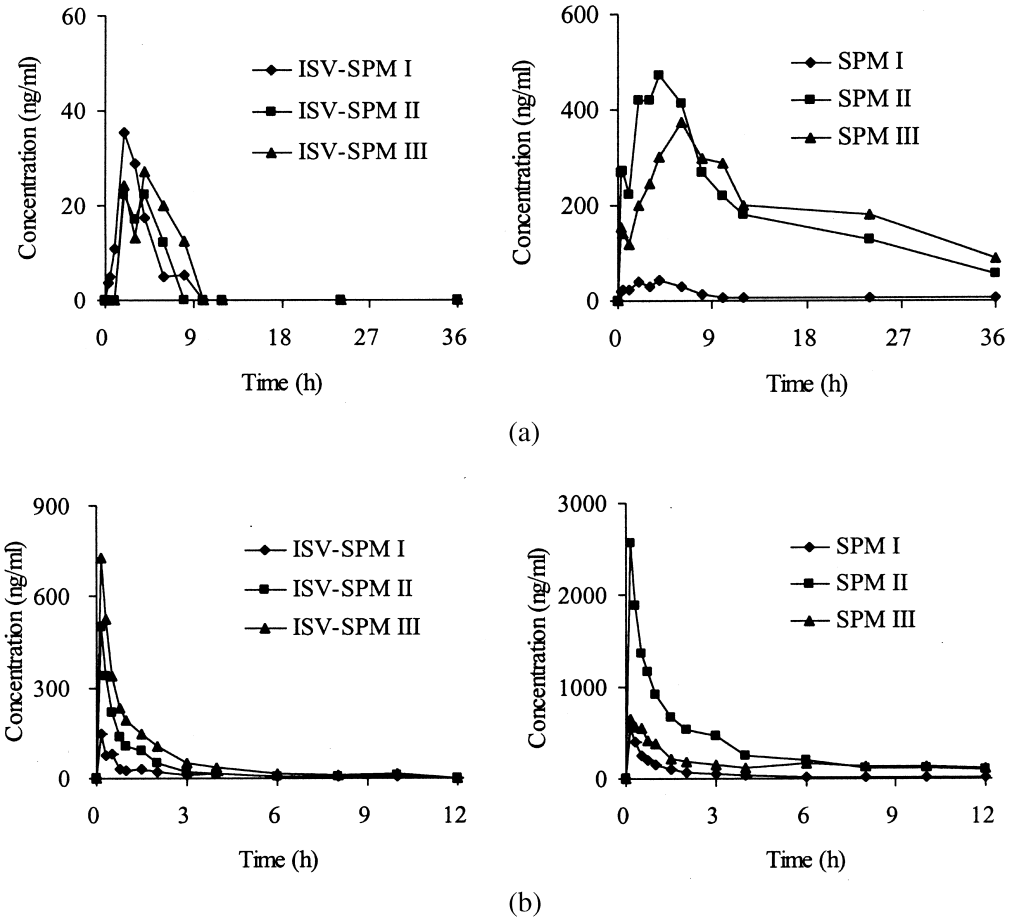

Fig. 4. (A) Average plasma pharmacokinetic profiles of ISV-SPM I, II, III and their main active metabolites SPM I, II, III from six rats after oral administration of 80 mg/kg bitespiramycin. (B) Average plasma pharmacokinetic profiles of ISV-SPM I, II, III and their main active metabolites SPM I, II, III from six rats after intravenous administration of 80 mg/kg bitespiramycin.

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