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Sensitive and specific method for the determination of josamycin in human plasma by liquid chromatography–mass spectrometry

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Abstract

A highly sensitive and specific method for the determination of josamycin in human plasma by LC–MS was developed and validated. Josamycin was extracted from human plasma by a single-step liquid–liquid extraction and analyzed by LC–MS via an electrospray ionization interface. Selected ion monitoring was used to detect josamycin and its internal standard. The intra-day precision and accuracy, expressed as C.V. and R.E., ranged from 2.8% to 13.5% and −10.3% to 7.6%, respectively. The lower limit of detection was 0.1 ng/ml and the lower limit of quantitation was set at 1 ng/ml when 0.5 ml of plasma was used. No endogenous interference was observed in human plasma obtained from drug-free volunteers.

Keywords: Josamycin

1. Introduction

Josamycin, (3*R*,4*R*,5*S*,6*R*,8*R*,9*R*,10*E*,12*E*,15*R*)-3-acetoxy-5-[*O*-2,6-dideoxy-4-*O*-isovaleryl-3-*C*-methyl-α-L-*ribo*-hexopyranosyl-(1→4)-3,6-dideoxy-3-dimethylamino-β-D-glucopyranosyloxy]-6-formylmethyl-9-hydroxy-4-methoxy-8,15-dimethyl-10,12-pentadecadien-15-olide, is a macrolide antibiotic, effective against mycoplasma, gram positive cocci and bacilli, and certain gram negative organisms [1–6]. It was developed by Yamanouchi Pharmaceutical Co., Ltd. and has been launched under licenses in many countries. Previously, josamycin concentrations in biological fluid have been determined mainly by microbiological assay [7–12]. Since josamycin is metabolized rapidly to produce metabolites possessing antimicrobial activity [13,14], the concentrations obtained by microbiological assay represent the sum of josamycin and its active metabolites [15,16]. Although high-performance liquid chromatography with UV detection (HPLC–UV) [17–20] and fluorescence detection [21] have been used to specifically determine unchanged josamycin, the methods lack sufficient sensitivity to obtain pharmacokinetic data from clinical trials.

Recently, liquid chromatography–mass spectrometry (LC–MS) has been recognized as a rapid, robust, sensitive and specific analytical method to determine concentrations of a wide variety of drugs and their metabolites in biological fluids. This paper reports the development and validation of a sensitive

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method specific for the determination of unchanged josamycin by LC–MS technique.

2. Experimental

2.1. Chemicals

Josamycin and the internal standard (IS), (4*S*, 5*R*, 6*R*, 8*R*,10*E*, 12*E*, 14*R*, 15*R*)-5-(3-dimethylamino - 3, 4, 6 - trideoxy- β -D - glucopyranosyloxy)-15-ethyl-6-formylmethyl-14 -hydroxymethyl-4, 8, 12-trimethyl-9-oxo-10,12-pentadecadien-15-olide hydrochloride, were synthesized by Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). The structural formulae of the compounds are shown in Fig. 1.

2.2. Reagents

Heparinized blank human plasma for the preparation of calibration standards and quality control (QC) samples was obtained from drug-free healthy male volunteers. Milli-Q SP TOC (Millipore Japan, Tokyo, Japan) distilled deionized water was used. Reagent grade diethyl ether, HPLC grade acetonitrile and methanol, were purchased from Kanto Chemical (Tokyo, Japan). Reagent grade ammonium acetate and sodium hydrogen carbonate were from Nacalai Tesque (Kyoto, Japan).

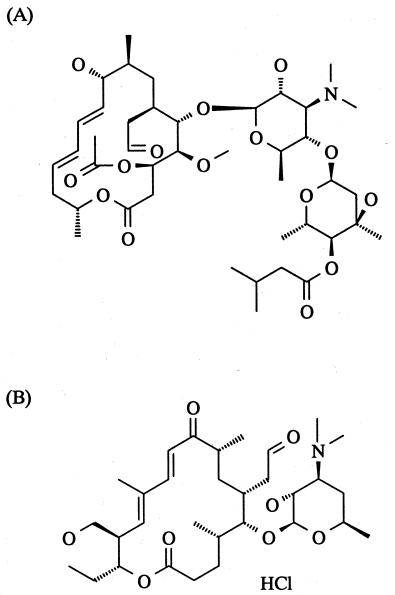

Fig. 1. Chemical structures of (A) josamycin and (B) internal standard.

2.3. Preparation of calibration standards and QC samples

Primary stock solutions (0.1 mg/ml) of josamycin were prepared in methanol for calibration standards and QC samples by separate weighing. The stock solution of josamycin was further diluted with ultra distilled deionized water to give a series of calibration and QC solutions. Calibration standards over the range 1–100 ng/ml were prepared by spiking calibration solutions (0.5 ml) into 0.5 ml of blank human plasma. QC samples (3, 40 and 80 ng/ml) and LOQ (limit of quantitation) samples (1 ng/ml) were also prepared using blank plasma. The samples were stored at −20°C prior to analysis.

An IS stock solution (0.1 mg/ml) was prepared in methanol. A portion of this IS stock solution was diluted with water to give a concentration of 1000 ng/ml.

2.4. Instrumentation and chromatographic conditions

The HPLC system consisted of a Model 616 pump, a Model 717 plus auto-injector, a Model 600s controller and a Model CHZ column oven (Waters Japan Co., Ltd., Tokyo, Japan). Separation was achieved on a J'sphere ODS-H80 (4 μm, 75 mm× 4.6 mm I.D.) reversed-phase column (YMC, Tokyo, Japan) at the column temperature of 40°C. The HPLC system was operated isocratically at a flow-rate of 0.4 ml/min. The mobile phase consisted of 20 m*M* ammonium acetate and acetonitrile (3:7, v/v).

A TSQ 7000 triple stage quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) was used with an electrospray ionization (ESI) in the positive ion mode at a spray voltage of 4.5 kV, multiplier voltage of 1.2 kV and heated capillary temperature of 250°C. High purity nitrogen served both as a sheath gas with an operating pressure of 70 p.s.i. and as auxiliary gas with a flow-rate of approximately 1–2 l/min. Quasi-molecular ions $(M+H)^+$ monitored via selected ion monitoring

(SIM) were m/z 828 for josamycin and m/z 566 for IS, respectively (Fig. 2).

2.5. Extraction procedure

Calibration standards (1–100 ng/ml, 0.5 ml) and QC samples (0.5 ml) were spiked with 0.2 ml of IS (1000 ng/ml), 0.5 ml of saturated sodium hydrogen bicarbonate and 5 ml of diethyl ether. After shaking for 15 min, the organic layer was separated from the aqueous layer by centrifugation at 2200 rpm for 10 min. The organic layer was then evaporated to dryness at 45°C and the residue was reconstituted in 0.3 ml of HPLC mobile phase. A 0.05 ml portion of the sample was injected into the LC–MS system.

2.6. Validation tests

2.6.1. Calibration curves

Calibration standards (range=1–100 ng/ml) at seven concentrations were extracted and assayed. A linear model was fit to the concentration vs. peak-height ratio (PHR) data using weighted ($1/y$) least-squares regression.

2.6.2. Specificity

Human blank plasma samples from six drug-free male volunteers were extracted and assayed. The chromatograms were visually inspected for peaks from endogenous substances which might correspond to josamycin or IS peaks.

2.6.3. Accuracy and precision

QC samples at each of three concentrations (3, 40 and 80 ng/ml, $n=6$) and LOQ samples (1 ng/ml, $n=6$) of josamycin were assayed to determine the intra-day accuracy expressed as mean relative error (R.E.) and precision expressed as coefficient of variation (C.V.).

QC samples ($n=2$) at each of three concentrations were assayed on nine separate occasions to determine the inter-day accuracy and precision of josamycin.

2.6.4. Extraction recovery

The recovery of josamycin through extraction procedures was assessed at three different concentrations (3, 40 and 80 ng/ml, $n=3$). The responses of josamycin added to human blank plasma prior to extraction was compared with those in which josamycin was added after extraction (control). The recovery of IS was similarly determined.

2.6.5. Stability

The stability of josamycin in human plasma stored at −20°C was investigated. QC samples at each of three concentrations (3, 40 and 80 ng/ml, $n=4$) were assayed after storage for 118 days. The stability expressed as percentage remaining of control was determined by comparing the observed josamycin concentrations with those obtained from samples which were prepared immediately before analysis (control).

3. Results

3.1. Mass chromatograms and specificity

The representative mass chromatograms of (A) blank plasma, (B) plasma spiked with josamycin at the LOQ (1 ng/ml) and IS, and (C) plasma obtained from a human subject 24 h after final oral administration of josamycin at a dose of 600 mg t.i.d., are shown in Fig. 3. IS was chosen as the appropriate internal standard due to its structural resemblance to josamycin. No endogenous substances were observed in human blank plasma from six different drug-free individuals. The retention times of josamycin and IS were at 5.4 min and 3.4 min, respectively.

3.2. Linearity and reproducibility

Good linearity was observed over the concentration range of 1–100 ng/ml ($y=0.0095x+0.0013$, $r=0.9954\pm0.042$, $n=13$). The C.V. and R.E. of back-calculated values at 1 ng/ml were 19.4% and 1.9%, respectively. The C.V. and R.E. at 2–100 ng/ml were not more than 15% and within ±6%, showing good reproducibility (Table 1).

3.3. Accuracy and precision

The intra- and inter-day precision and accuracy are assessed in Table 2. The C.V. and the R.E. at LOQ were 9.8% and 18.6%, respectively. The C.V. at 3, 40

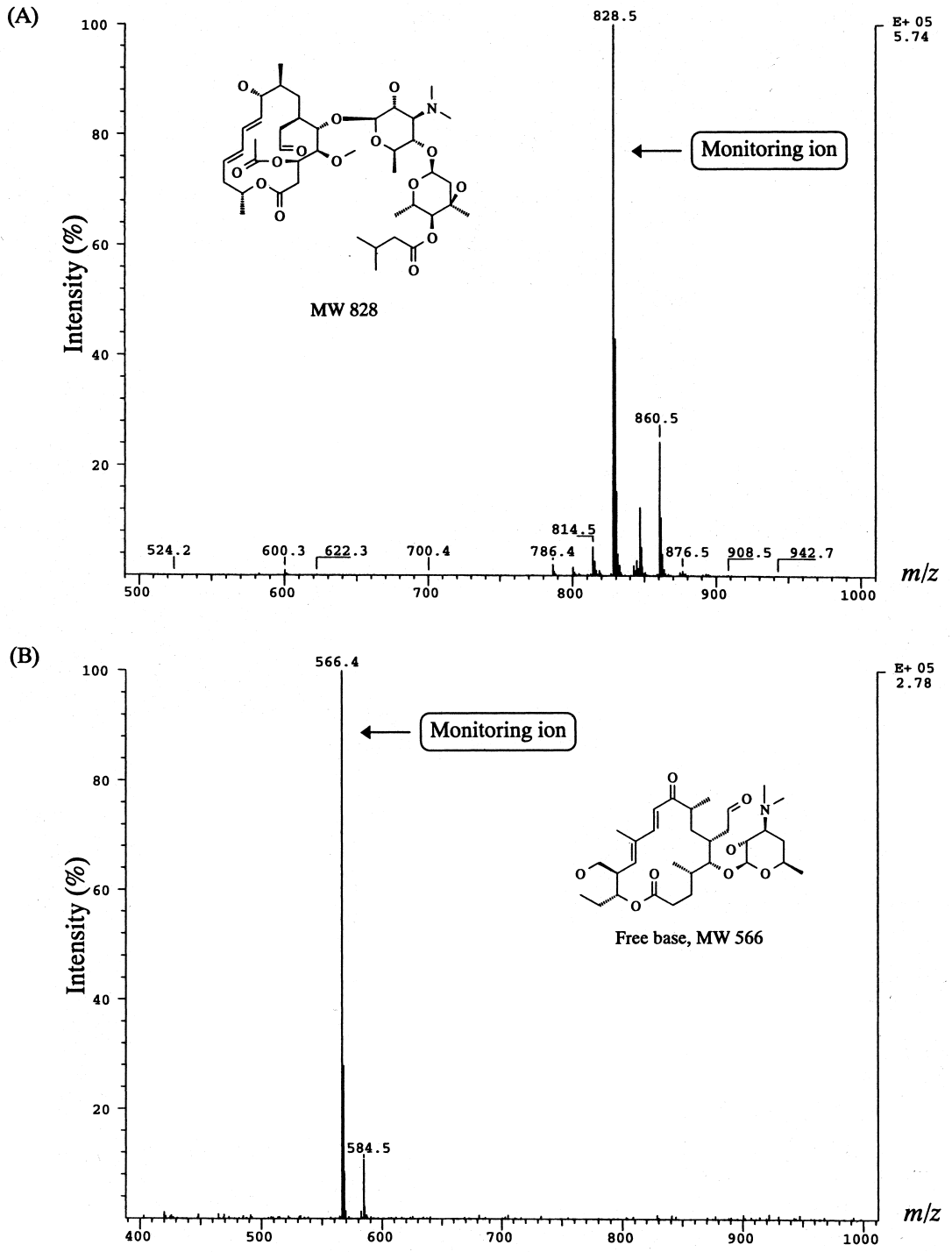

Fig. 2. ESI-positive mass spectra of quasi-molecular ions $(M+H)^+$ of (A) josamycin (m/z 828) and (B) internal standard (m/z 566).

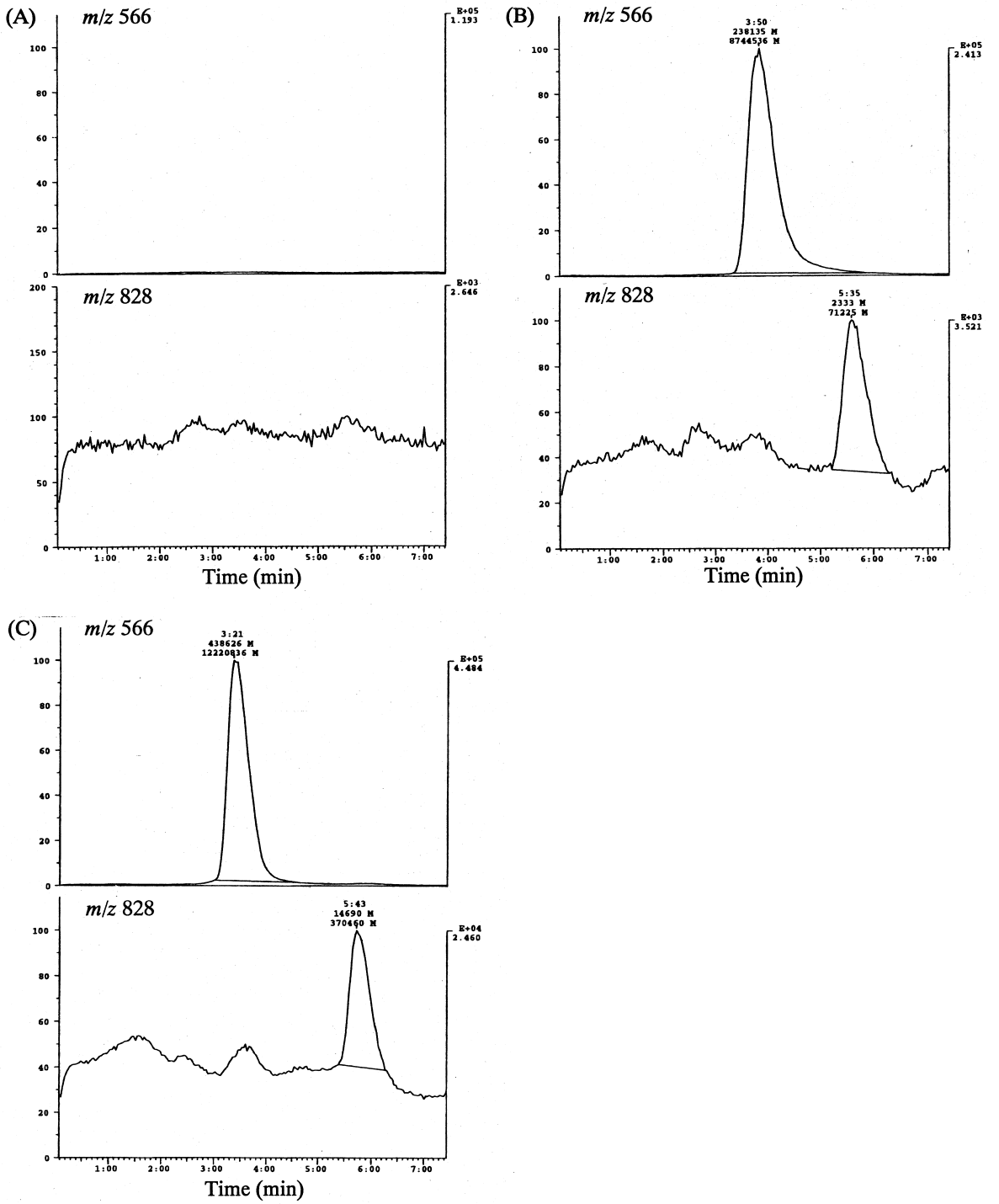

Fig. 3. Representative mass chromatograms of josamycin and its internal standard (IS) extracted from (A) blank plasma (without IS), (B) plasma spiked with josamycin (1 ng/ml) and IS, (C) plasma obtained from a human subject, 24 h after the final oral administration of josamycin at a dose of 600 mg t.i.d spiked with IS.

Table 1
Back-calculated values obtained from 13 different calibration curves

Concentration (ng/ml)	Back-calculated values (ng/ml) Mean±S.D.	C.V. (%)	R.E. (%)
1	1.0±0.2	19.4	1.9
2	2.0±0.2	12.1	1.6
5	4.7±0.3	7.4	−5.4
10	10.0±1.3	13.0	−0.1
20	21.1±3.1	14.8	5.6
50	51.0±4.2	8.2	1.9
100	99.3±4.2	4.2	−0.7

and 80 ng/ml ranged from 2.8% to 13.5%, and the R.E. ranged from −10.3% to 7.6% for intra-day. The values for inter-day were in the range from 9.6% to 11.4% and from −11.3% to −3.1%, respectively.

3.4. Extraction recovery

The mean recovery of josamycin from human plasma ($n=3$) was 80.3% at 3 ng/ml, 67.6% at 40 ng/ml and 75.2% at 80 ng/ml. The values for IS ($n=3$) was 70.7% (Table 3).

3.5. Stability

Josamycin was shown to be stable in human plasma when stored frozen at −20°C for up to 118 days. The observed values exhibited 99.4%, 106.5% and 98.5% of the control at 3, 40 and 80 ng/ml, respectively (Table 4).

Table 3
Extraction efficiency of josamycin and internal standard (IS) from human plasma[a]

Compound	Nominal concentration (ng/ml)	Recovery (%) Mean±S.D., $n=3$
josamycin	3	80.3±2.8
	40	67.6±24.4
	80	75.2±7.1
IS	400	70.7±8.0

[a] The extraction procedures are described in the method in Section 2.5.

Table 4
Stability of quality control samples stored frozen at −20°C for 118 days

Concentration (ng/ml)	% of control Mean, $n=4$
3	99.4
40	106.5
80	98.5

4. Discussion

A highly sensitive and specific method for the determination of josamycin in plasma by LC–MS has been established. The lower limit of detection was 0.1 ng/ml and the lower limit of quantitation (LOQ) was set at 1 ng/ml, where the S/N ratio was more than 20 (Fig. 3). The LOQ of this new method is 50 to 60-fold more sensitive than the HPLC method previously reported [20,21]. Furthermore,

Table 2
Precision and accuracy for the measurement of josamycin in human plasma[a]

	Nominal concentration (ng/ml)	Observed concentration (ng/ml) Mean±S.D.	C.V. (%)	R.E. (%)
Intra-day ($n=6$)	1 (LOQ)	1.2±0.1	9.8	18.6
	3 (QCL)	3.2±0.4	13.5	7.6
	40 (QCM)	36.0±1.8	5.0	−9.9
	80 (QCH)	71.8±2.0	2.8	−10.3
Inter-day[b] ($n=18$)	3 (QCL)	2.7±0.3	9.6	−11.3
	40 (QCM)	38.7±4.2	10.8	−3.1
	80 (QCH)	75.1±8.6	11.4	−6.1

[a] LOQ: Limit of quantitation, QCL: Low concentration quality control sample, QCM: Medium concentration quality control sample, QCH: High concentration quality control sample.
[b] Each of two quality control samples at 3, 40 and 80 ng/ml were measured on nine different occasions.

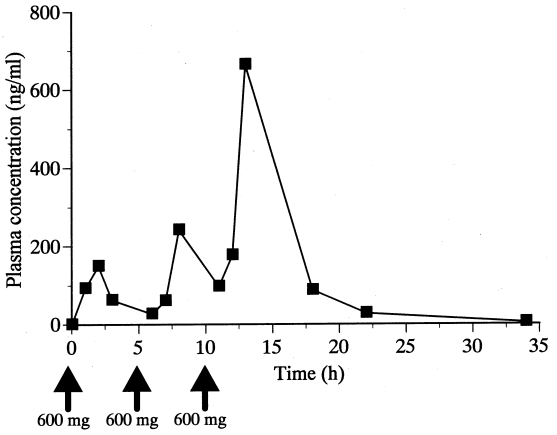

Fig. 4. Plasma concentration–time curve obtained from a human subject after oral administration of josamycin as a capsule at a dose of 600 mg t.i.d.

since the method requires only a simple single-step liquid–liquid extraction procedure and a short run-time, large sample batches (more than 50 samples) can be processed on a daily.

The LC–MS method has solved previous problems with low specificity and inaccuracy existing in both the microbiological and HPLC assay methods. This method also removes the long complicated extraction techniques such as derivatization and column-switching used in the previous methods.

Fig. 4 shows the representative plasma concentration–time curve after oral administration of josamycin as a capsule at a dose of 600 mg three times a day (t.i.d.) to a healthy male volunteer. Plasma concentrations of josamycin 24 h after the final dose was 4.2 ng/ml, demonstrating that the method has been successfully applied to determine josamycin concentrations in clinical trials.

5. Conclusions

A simple, rapid and robust method for the determination of josamycin in human plasma by LC–MS was developed and validated. Validation experiments have shown that the assay has good precision and accuracy over a concentration range of 1 to 100 ng/ml. No endogenous substances which could interfere with the assay were observed. The method is specific and sensitive enough to obtain pharmacokinetic data from clinical trials.

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