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# Determination of gestrinone in human serum by liquid chromatography–electrospray tandem mass spectrometry

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**Abstract**

A rapid, sensitive and specific high-performance liquid chromatography–electrospray tandem mass spectrometric method has been developed for the determination of gestrinone (R 2323) in human serum using mifepristone (RU 486) as an internal standard. R 2323 was extracted from human serum by an ether extraction procedure. Multiple reaction monitoring was used to detect R 2323 and RU 486. The calibration curve was linear over the range of 3.5–177 ng/ml ($r^2 \geq 0.99$) with the limitation of detection of 0.8 ng/ml. The intra-day precision and accuracy, expressed as C.V. and RE, ranged from 2.3–13.7 to −4.8–3.0%. The inter-day precision and accuracy ranged from 5.5–14.8 to −6.7–3.1%. The mean recovery was 91.0% for R 2323, and 90.6% for the internal standard. The method was successfully applied to the pharmacokinetic study of R 2323. 

*Keywords:* Gestrinone

## 1. Introduction

R 2323, or D-18-ethyl-17α-ethynyl-17β-hydroxy-oestra-4,9,11-trien-3-one, is a new drug in the treatment of endometriosis and emergency contraception. For pharmacokinetic studies, Salmon et al. have developed a radioimmunoassay (RIA) method for R 2323 [1]. Since the radioactive tracers constitute a health hazard and are cumbersome to handle, a high-performance liquid chromatography (HPLC) method with UV detection at 342 nm was developed [2]. However, this HPLC assay needed relatively long run time and lacked the necessary sensitivity when low dosages were involved.

In recent years, liquid chromatography–mass spectrometry (LC–MS) or liquid chromatography–tandem mass spectrometry (LC–MS–MS) have become increasingly popular in the analysis of steroids and steroid conjugates in biological fluids [3–10]. Ma and Kim [3] evaluated the performance of on-line atmospheric pressure chemical ionization and electrospray ionization LC–MS methods, and the parameters such as the mobile phase composition and the mobile phase additives were optimized. Steroids have been classified into three major groups based on the spectra and the sensitivities observed. In the electrospray mode, the best sensitivity at about 5 pg was achieved for 3-one-4-ene steroids such as R

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2323 using pure methanol and water as mobile phase. Wong et al. [11] have described a LC–MS–MS method with atmospheric pressure chemical ionization interface for the determination of norgestimate and its metabolites in human serum. In this paper, we developed a rapid, sensitive and specific HPLC–MS–MS method employing an electrospray interface with multiple reaction monitoring (MRM) for the determination of R 2323 in human serum, and the method was successfully applied to the pharmacokinetic study of R 2323.

## 2. Experimental

### 2.1. Chemicals and reagents

R 2323 was provided by Beijing Third Pharmaceutical (Beijing, PR China), and RU 486 (I.S.) was provided by Roussel Uclaf (France). The structures of R 2323 and I.S. are shown in Fig. 1. HPLC-grade methanol was from Fisher Scientific (Shatin, Hong Kong), and other reagents were all of analytical grade.

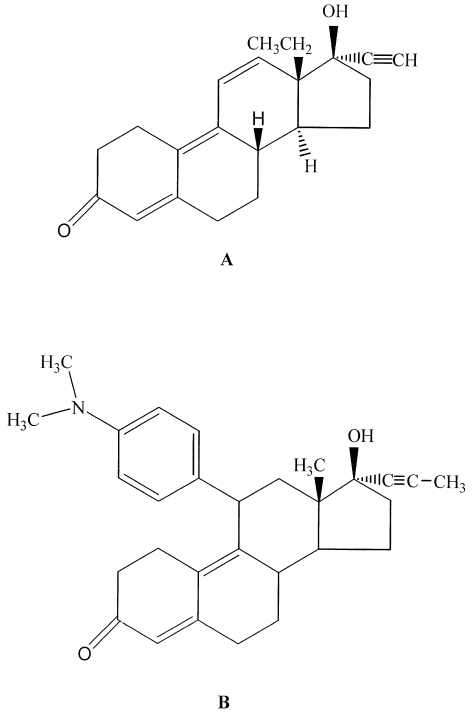


Fig. 1. Structure of (A) R 2323 and (B) I.S.

### 2.2. Sample preparation

Human blood samples were collected from healthy women of childbearing age by venipuncture after oral administration of 5 mg of R 2323. Blood samples were allowed to stay at room temperature for 2 h for clotting. After the clot was retreated, the serum was separated by centrifugation for 15 min at 1000 *g*. All sera were stored at −20°C until analysis.

Fifty μl of I.S. (100 ng/ml) in methanol was added into 200 μl serum samples followed by addition of 2 ml diethyl ether. The samples were vortexed for 2 min, and frozen in solid $CO_2$ in acetone. The ether phase was then decanted into a dry silanised tube. The thawed serum was re-extracted with 2 ml diethyl ether, and two ether extracts were combined and evaporated to dryness. The residue was reconstituted in 200 μl HPLC mobile phase and a 20 μl portion was injected into the HPLC–MS–MS system.

### 2.3. Preparation of calibration standards and QC samples

Stock solution of R 2323 (1 mg/ml) and diluted solutions were prepared in methanol and stored in 4°C. Calibration standards in serum over the range of 3.5–177 ng/ml were prepared by mixing aliquots (≤100 μl) of the diluted solutions of R 2323 and 50 μl of I.S. (100 ng/ml) with 200 μl of blank human serum. QC samples were also prepared at concentration of 4, 40, 100 ng/ml. These Calibration standards and QC samples were then treated using the same procedure as described in Section 2.2.

### 2.4. HPLC–MS–MS analysis

The HPLC system consisted of a PE Series 200 pump (Thornhill, Ont., Canada). Separation was achieved on a Kromasil $C_{18}$ column (250×4.6 mm I.D., 5 μm particle size, Turner, Tianjin, PR China) at ambient temperature (25±2°C). The HPLC was operated isocratically at 1 ml/min and the mobile phase consisted of methanol–0.2% formic acid. The column eluent was split and approximately 100 μl/

min were delivered into the electrospray interface (positive-ion mode, source temperature 200°C, ion-spray voltage +5.0 kV) of a PE SCIEX API 3000 mass spectrometer (Thornhill, ON, Canada). High pure nitrogen served as nebulizing gas at flow-rate of 1000 ml/min. Curtain gas was set at 1250 ml/min, and the orifice voltage was set at 45 V. Under these conditions, full-scan spectra of R 2323 and I.S. were recorded. For collisionally activated dissociation (CAD), high pure nitrogen was used as the collision gas at a pressure of $3\times10^{-5}$ Torr, with a collision energy of 45 eV. Quantification was performed by MRM with dwell time of 800 ms. For R 2323, $m/z$ 309 was used as precursor ion and $m/z$ 241 as

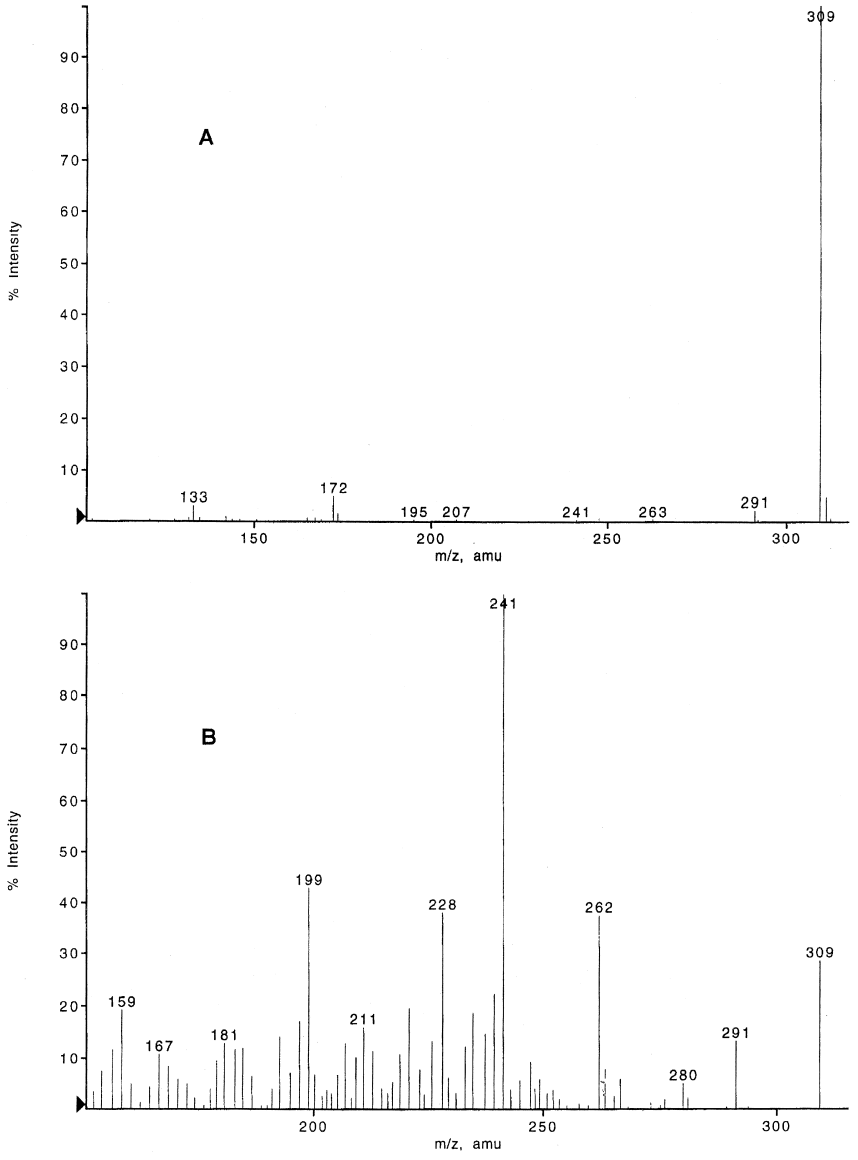


Fig. 2. (A) Positive-ion mass spectrum of R 2323; (B) product ion spectrum of R 2323 with its protonated molecular ion ($[MH]^+$) at $m/z$ 309 as the precursor ion; (C) positive-ion mass spectrum of I.S.; and (D) product ion spectrum of I.S. with its protonated molecular ion ($[MH]^+$) at $m/z$ 430 as the precursor ion.

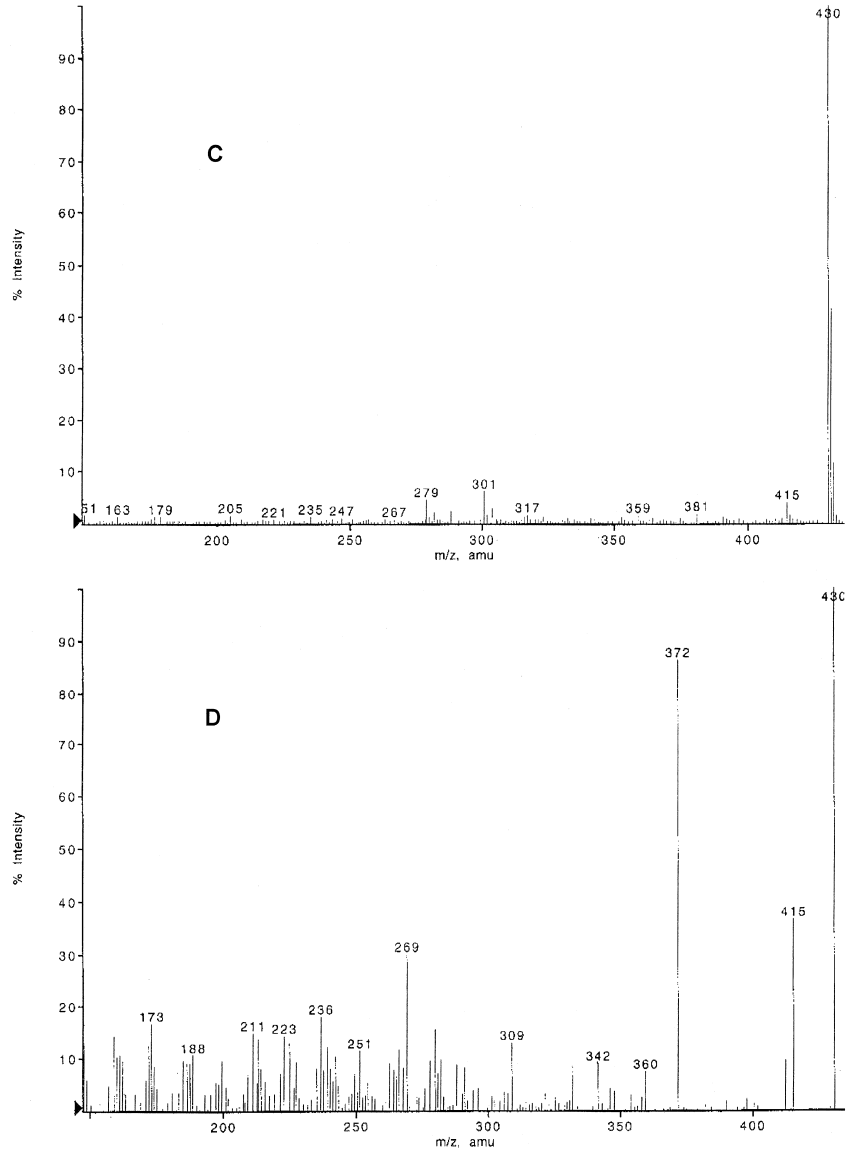


Fig. 2. (*continued*).

product ion, and the respective ions were $m/z$ 430 and $m/z$ 372 for I.S.

### 2.5. Recovery measurement

The recovery of R 2323 through extraction procedure was assessed at three different concentrations (4, 40, 100 ng/ml, $n=6$). The responses of R 2323 added to human blank serum prior to extraction were compared with those in which R 2323 was added after extraction. The recovery of I.S. was determined similarly.

### 2.6. Validation procedures

This HPLC–MS–MS method was validated for the linearity, limit of detection (LOD), recovery of R 2323 in human serum, respectively. The accuracy

and precision of the method were assessed by analyzing three QC samples (4, 40, 100 ng/ml, $n=6$).

## 3. Results and discussions

### 3.1. Method development

In order to detect R 2323 using MRM mode, precursor and product ions must be selected for both R 2323 and I.S. The selection criteria are: (1) the abundance of the selected ions should be as strong as possible, and (2) interference from the actual samples should be avoided. Similar with the results of Ma and Kim [3], we observed that the positive-ion mode was better than negative-ion mode for the detection of R 2323. The positive-ion mass spectrum of R 2323, obtained from a direct injection of 100 ng/ml standard solution, is shown in Fig. 2A. It can be seen that the most abundant peak is due to the protonated molecular ion ($[M+H]^+$) at $m/z$ 309. The product ion spectrum for $m/z$ 309 obtained from CAD shows that $m/z$ 241 is the most abundant product ion (Fig. 2B). According to Ma and Kim's report [3], the mass spectra of the 3-one-4-ene steroids, such as R 2323, were characterized by $[M+H]^+$, $[M+H]^+-H_2O$ and $[M+H]^+-2(H_2O)$ etc. In our experiments, the absolute abundance of $[M+H]^+-H_2O$ ion at $m/z$ 291 reached the maximum with the collision energy of 35 eV, and decreased significantly while the collision energy increased to 45 eV (Fig. 3). Since the absolute abundance of $m/z$ 241 was stronger than $m/z$ 291, $m/z$ 309→241 was chosen as the precursor to product combination for R 2323 in MRM detection. Under the same criterion, the combination for I.S. was $m/z$ 430→372 (Fig. 2C–D). Such selections were similar with that reported by Wong et al. [11]. As will be shown below, the selected ions were free from interference in the serum samples.

In the preliminary experiments, we optimized LC conditions using UV detection at 340 nm. R 2323 was almost resolved from other compounds in the extracted sample using pure methanol as mobile phase, and resolution improved slightly with the increase of water in the mobile phase. Base line resolution could be achieved with a mobile phase of methanol–water (70/30). However, in this case, it took about 30 min for one analysis. Therefore we chose pure methanol as mobile phase for LC–MS–MS to reduce the analysis time. When MRM detection was used instead of UV, it was also found that the sensitivity was improved when 0.2% formic acid was added to the mobile phase. Using acetonitrile–water as mobile phase gave similar separation but decreased the sensitivity.

In a previous report, Wu et al. [12] described a

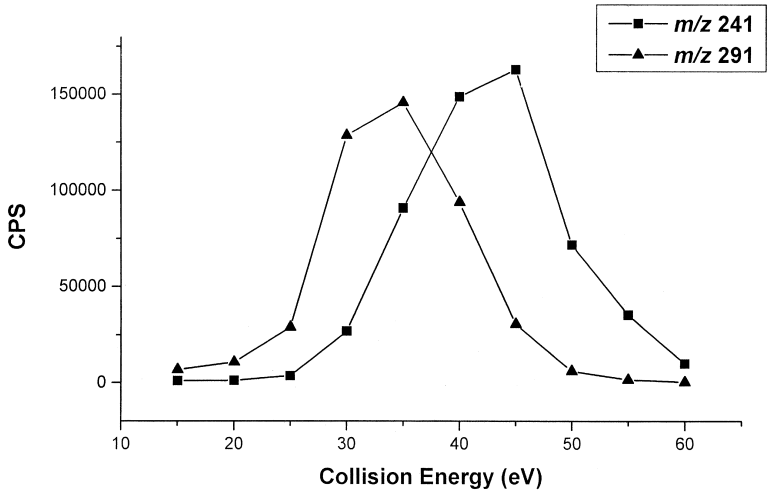


Fig. 3. The influence of collision energy on the absolute abundance of $m/z$ 241 and $m/z$ 291.

three-step procedure for optimizing LC–MS–MS conditions, and we followed this procedure in this work. The optimized conditions are listed in Section 2.4. Under these conditions, representative chromatograms are shown in Fig. 4. R 2323 and I.S. eluted at 3.0 and 2.7 min, respectively. The various blank peaks in blank serum sample (Fig. 4A) were background noise which was rather low since the scale of the *y*-axis in Fig. 4A was about 1/10–1/20 compared with that in Fig. 4C or 4D. Such low background noise has no apparent influence on the sensitivity of the method. It also can be seen from

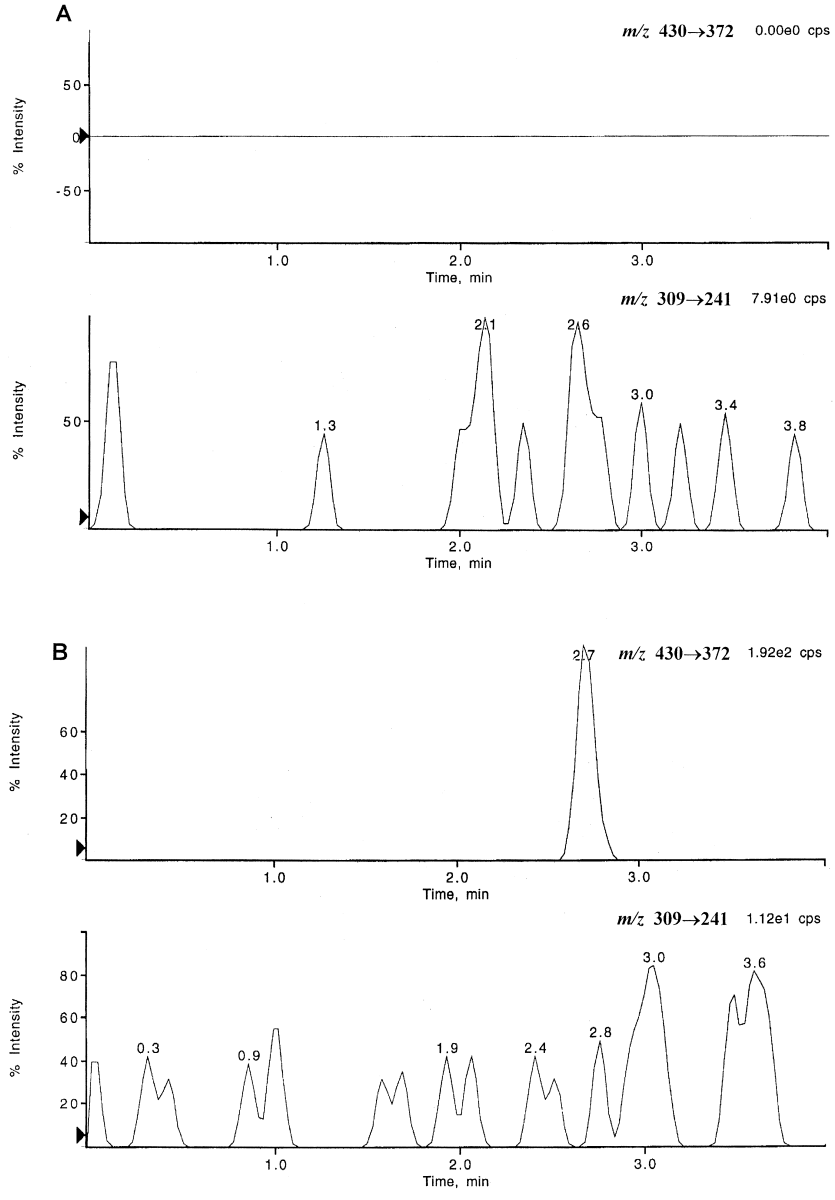


Fig. 4. Representative MRM chromatograms of R 2323 in human serum. (A) Blank human serum; (B) blank human serum spiked with I.S. (25 ng/ml); (C) blank human serum spiked with R 2323 (17.7 ng/ml) and I.S. (25 ng/ml); and (D) a serum sample containing R 2323 32.2 ng/ml spiked with I.S. (25 ng/ml).

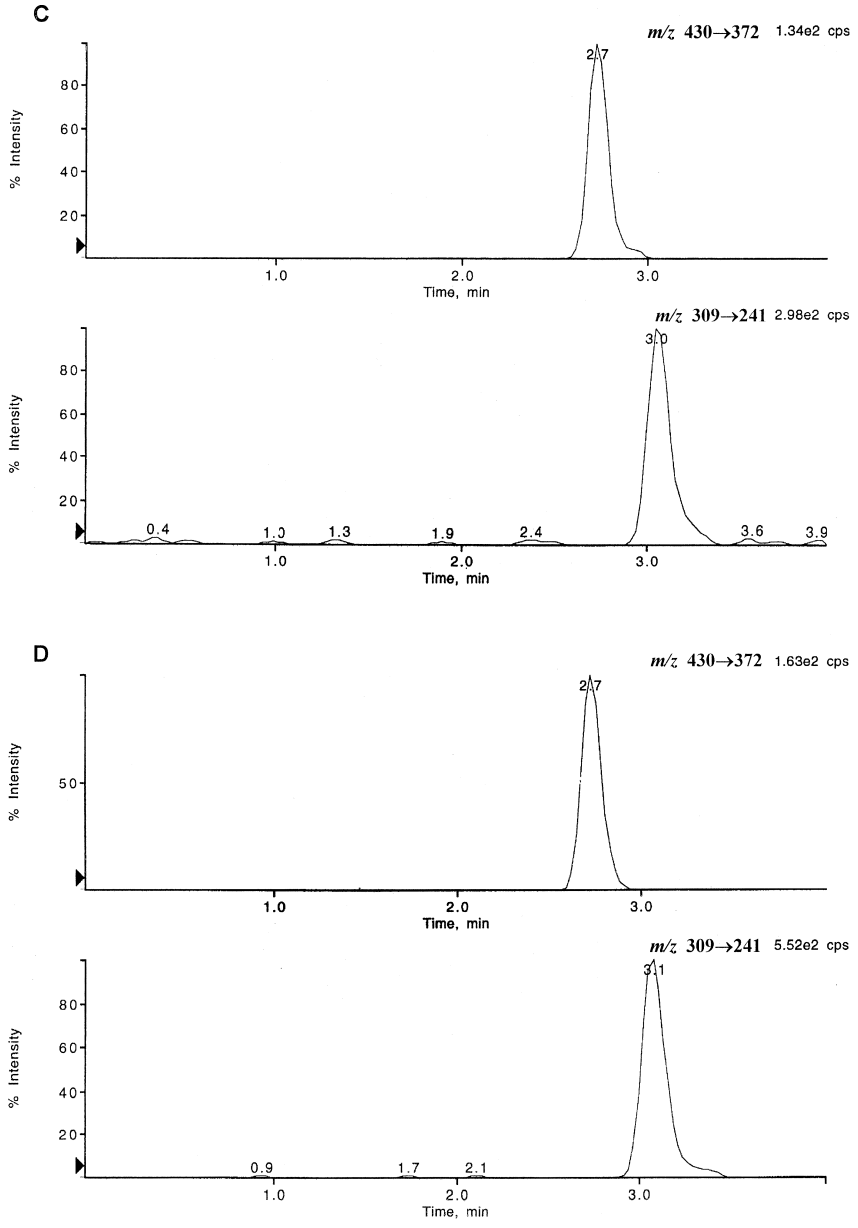


Fig. 4. (*continued*).

Fig. 4 that the chromatograms were free from interference for both R 2323 and I.S.

### *3.2. Quantitative characteristics of the method*

The calibration range was based on the concentrations expected in serum samples to be analyzed. Good linearity was found over the concentration range of 3.5–177 ng/ml. The regression line is $y=0.146x+0.129$ ($r^2\geq 0.99$, $n=6$), where $y$ is the peak area ratio (R2323/I.S.) and $x$ is the concentration of R2323. The C.V. values were less than 12%, and the RE values ranged from −4.1 to 4.4% (Table1). The LOD was 0.8 ng/ml ($S/N=3$).

Table 1
Calibration curve statistics for R 2323 in human serum[a]

| Normal conc. (ng/ml) | Calculated conc. (ng/ml) | C.V. (%) | RE (%) |
|---|---|---|---|
| 3.5 | 3.51 | 11.4 | 2.8 |
| 7.1 | 7.41 | 3.6 | 4.4 |
| 17.7 | 16.97 | 4.5 | 4.1 |
| 35.4 | 35.83 | 4.7 | 1.2 |
| 70.8 | 70.01 | 1.2 | 0.1 |
| 177 | 177.2 | 3.0 | 0.2 |

[a] $n=6$.

The precision and accuracy were checked by calculating the intra-day and inter-day variation of three QC samples (4, 40, 100 ng/ml, $n=6$). The results are shown in Tables 2 and 3. The intra-day C.V. values were less than 14% and RE values ranged from −4.8 to 3.0%, while the inter-day C.V. values were less than 15% and REs ranged from −6.7 to 3.1%.

The mean recovery of R 2323 from human serum ($n=6$) was 93.2% at 4 ng/ml, 88.5% at 40 ng/ml and 91.4% at 100 ng/ml. The mean recovery, including all concentrations, was 91.0%, and the value for I.S. was 90.6%.

### *3.3. Application in pharmacokinetic study*

Pharmacokinetic effect of R 2323 in human serum has been studied. Fig. 5 shows a profile of the mean serum levels ($n=5$) of R 2323. Quantifiable levels of R 2323 were detected for up to 48 h after oral administration of 5 mg of R 2323.

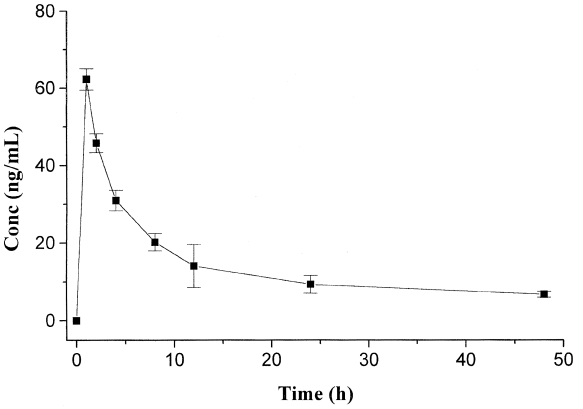


Fig. 5. Serum levels of R 2323 after oral administration of 5 mg R 2323.

Table 2
Intra-day precision and accuracy for R 2323[a]

| Normal conc. (ng/ml) | Calculated conc. (ng/ml) | C.V. (%) | RE (%) |
|---|---|---|---|
| 4 | 4.1 | 13.7 | 2.5 |
| 40 | 38.1 | 9.1 | −4.8 |
| 100 | 100.3 | 2.3 | 3.0 |

[a] $n=6$.

Table 3
Inter-day precision and accuracy for R 2323[a]

| Normal conc. (ng/ml) | Calculated conc. (ng/ml) | C.V. (%) | RE (%) |
|---|---|---|---|
| 4 | 3.8 | 14.8 | −5.0 |
| 40 | 37.3 | 7.5 | −6.7 |
| 100 | 103.1 | 5.5 | 3.1 |

[a] $n=6$.

## 4. Conclusions

A rapid, sensitive and specific HPLC–MS–MS method was established and validated. Each assay took about 4 min. The method was sensitive due to MRM detection mode and specific due to the inherent selectivity of tandem mass spectrometry. The method was successfully applied to the pharmacokinetic study of R 2323, and allowed for the determination of R 2323 in human serum up to 48 h after oral administration of 5 mg of R 2323.

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