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Short communication

An HPLC–MS/MS method for simultaneous determination of decitabine and its valyl prodrug valdecitabine in rat plasma

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ABSTRACT

A simple and sensitive HPLC–MS/MS method was developed and validated for the simultaneous determination of decitabine and valdecitabine in rat plasma. The analytes were separated on a C_{18} column (150 mm × 4.6 mm, 3.5 μm) and a triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source was applied for detection. A clean solid-phase extraction procedure with cation exchange cartridge was employed to extract the analytes from rat plasma with high recovery of decitabine (>82%). The calibration curves were linear over a concentration range of 10–10,000 ng/mL for decitabine and 5–500 ng/mL for valdecitabine. The lower limit of quantitation (LLOQ) of decitabine and valdecitabine was 10 and 5 ng/mL, respectively. The intra-day and inter-day precisions were less than 15% and the relative error (RE) was all within ±15%. The validated method was successfully applied to a pharmacokinetics study in rats after either decitabine or valdecitabine orally administrated to the Sprague-Dawley rats.

1. Introduction

Decitabine (5-aza-2′-deoxycytidine, DAC, Fig. 1A) is a DNA methyltransferase (DNMT) inhibitor for the treatment of myelodysplastic syndrome (MDS) [1,2], acute and chronic myeloid leukemia (AML and CML) [3,4]. However, it exhibits a low oral bioavailability (only 9% in mice) [5], because of low permeability across the intestine membrane and extensive metabolism to inactive metabolite. It has been reported that amino acid ester prodrug of nucleoside analog, especially L-valine, could increase oral absorption by targeting the intestinal proton-coupled peptide transporter. Following this idea, we synthesized the 5′-O-L-valinyl ester derivative (valdecitabine, Fig. 1B) of decitabine to enhance the oral bioavailability of decitabine. Therefore, a sensitive and selective analytical method for the simultaneous determination of decitabine and valdecitabine in biological fluids is necessary for supporting the preclinic pharmacokinetic (PK) study of the prodrug.

Decitabine is a deoxycytidine analog and the bioanalysis of decitabine is easily interfered by endogenous 2-deoxycytidine, especially in human plasma from breast cancer patients and in rat plasma. Some high-performance liquid chromatography (HPLC) methods have been developed for the determination of decitabine in biological fluids [6,7]. But these methods suffered from low sensitivity and poor selectivity. Patel et al. and Cashen et al. developed sensitive liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) methods to determine decitabine in human plasma [8,9]. However, these methods were not applicable for the determination of decitabine in plasma obtained from breast cancer patients and rats because of high level of 2-deoxycytidine. To date, only Liu et al. [10] and Xu et al. [11] have developed HPLC–MS/MS methods to quantify decitabine in rat plasma. Liu et al. reported the quantitative method for decitabine by HPLC–MS/MS using 100 μL rat plasma [10]. They conducted the complex pretreatment of plasma samples in a refrigerator at 4 °C to prevent degradation of decitabine, with single analysis time more than 40 min. Xu et al. obtained LLOQ of 5 ng/mL using 50 μL plasma with protein precipitation pretreatment [11]. But the samples prepared by protein precipitation cause contamination of the MS and column system by endogenous components of the sample matrix.

In the present study, we have attempted to develop a novel, rapid, selective and sensitive method to determine decitabine and

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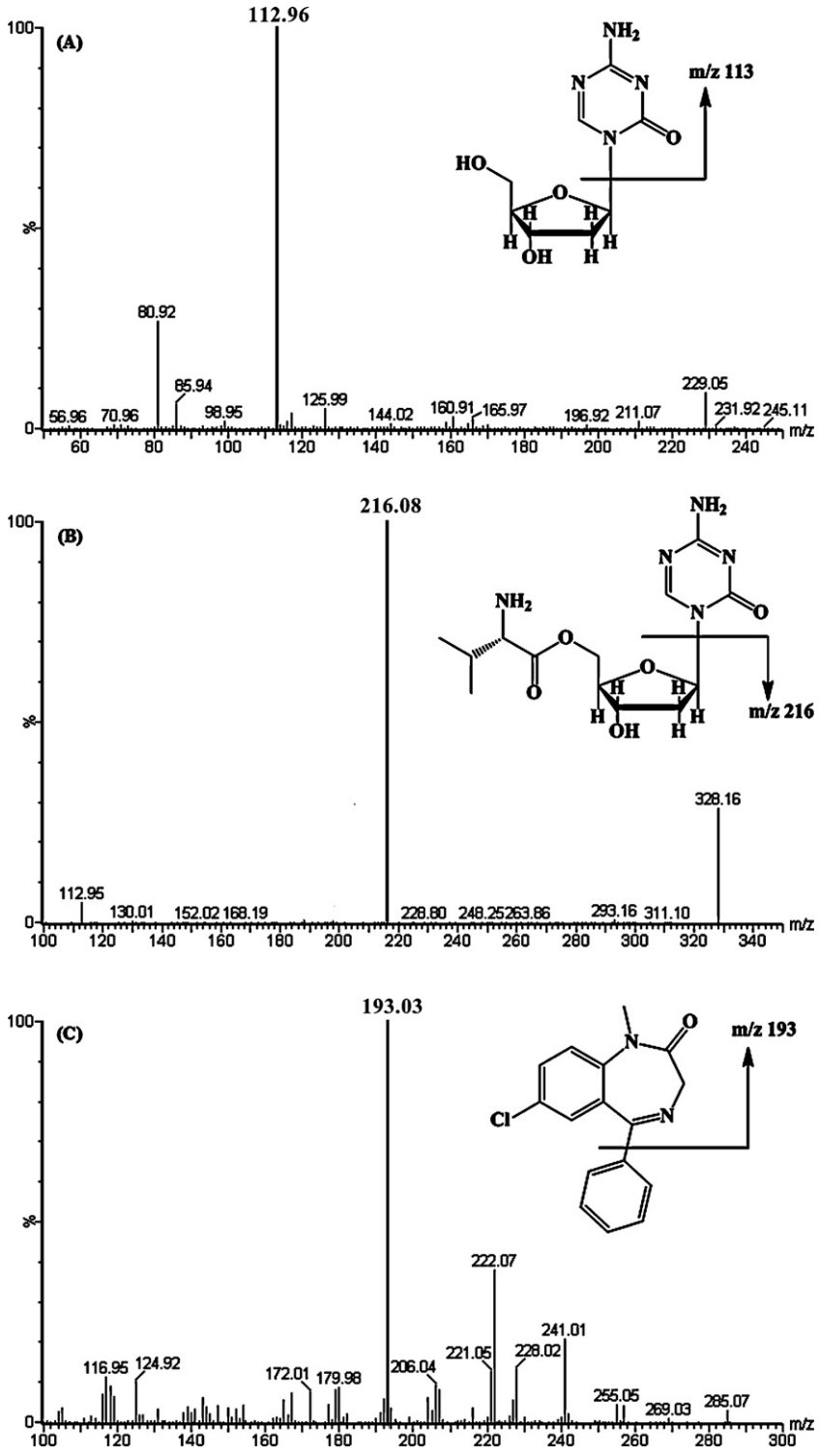

Fig. 1. Product ion mass spectra of $[M+H]^+$ ions of decitabine (A), valdecitabine (B) and diazepam (C).

valdecitabine in rat plasma using HPLC–MS/MS. The validation results showed simple sample preparation, shorter analysis time [10], less amount of plasma [9,10] and sufficient sensitivity [6–8,10] compared with the previous methods. The LLOQ was 10 ng/mL with a clean solid phase extraction (SPE) procedure using 50 μL plasma, which was sufficiently sensitive to measure relatively low concentration of decitabine in rat plasma. In addition, the single analysis time was about 15 min, which was much shorter than 40 min reported by Liu et al. Finally, this method was successfully applied to assess the PK characteristics after an oral administration of valdecitabine to Sprague-Dawley rats.

2. Experimental

2.1. Chemicals and reagents

Decitabine (98% purity) and valdecitabine (97% purity) were synthesized in Shenyang Pharmaceutical University (Shenyang,

China). Diazepam (purity >99.0%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Tetrahydrouridine (THU), the deaminase inhibitor, was purchased from Calbiochem (La Jolla, CA, USA). Methanol (HPLC-grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid and ammonium hydroxide solution (HPLC grade) were obtained from Dikma (Richmond Hill, NY, USA). Ultrapure water was prepared by EASYPURE® II RF/UV system (Boston, MA, USA).

2.2. Instrumentation

Analyses were acquired on a Waters ACQUITY Xevo TQD system, which consisted of an ACQUITY UPLC system and an ACQUITY triple-quadrupole tandem mass spectrometer with an electrospray ionization (ESI) interface (Waters Corp., Milford, MA, USA). Waters Oasis MCX cartridges (3 cc/60 mg) were applied for pretreatment of biological samples (Milford, MA, USA). A Symmetry C_{18} column (150 mm × 4.6 mm, 3.5 μm, Waters Corp., Wexford, Ireland) was used to separate analytes. All data were acquired and processed by MassLynx 4.1 software with QuanLynx program (Waters Corp., Milford, MA, USA).

2.3. HPLC–MS/MS condition

A gradient elution was conducted for chromatographic separation with the mobile phase A (acetonitrile containing 0.1% formic acid) and the mobile phase B (water containing 0.1% formic acid and 0.1% ammonium hydroxide) as follows: 0–8.2 min (5% A), 8.21–14 min (80% A) and 14.01–15 min (5% A). The flow rate was 0.2 mL/min and column temperature was 25 °C. Injection wash solvents were acetonitrile–water (10:90, v/v) and acetonitrile–water (90:10, v/v) for weak and strong wash, respectively. Nitrogen was used as the desolvation and cone gas at a flow rate of 500 and 50 L/h, respectively. High purity argon was used as the collision gas at a pressure of approximately 3.51×10^{-3} mbar. The optimal MS parameters were as follows: capillary 4 kV, source temperature 150 °C and desolvation gas temperature 380 °C. Cone voltage was 15, 15 and 47 V for decitabine, valdecitabine and diazepam, respectively.

The ESI source was operated in positive mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of *m/z* 229–113 with collision energy (CE) of 15 eV for decitabine, *m/z* 328–216 with CE of 10 eV for valdecitabine and *m/z* 285–193 with CE of 33 eV for diazepam, respectively. The scan time was set at 0.128 s per transition.

2.4. Preparation of standard and quality control (QC) samples

Standard stock solutions of decitabine, valdecitabine and diazepam (internal standard, IS) were prepared individually in methanol at the concentrations of 1 mg/mL, 50 μg/mL and 100 μg/mL, respectively. Standard solutions of decitabine and valdecitabine were freshly prepared by serially diluting stock solutions of decitabine and valdecitabine with water. And the IS solution was diluted with water to give a concentration of 100 ng/mL. All the solutions were stored at −20 °C and brought to room-temperature before use.

Calibration curves were prepared by spiking 50 μL of blank rat plasma containing THU (0.1 mM) with 100 μL of decitabine standard solution or valdecitabine standard solution, followed by the addition of 100 μL IS solution. Then the sample was mixed following the addition of 250 μL water. The final concentrations in standard plasma samples were 10, 20, 50, 200, 500, 2000, 5000 and 10,000 ng/mL for decitabine and 5, 10, 20, 100, 200 and 500 ng/mL for valdecitabine. The QC samples were prepared in the same way as the calibration samples. The plasma concentrations of QC samples were 20, 500 and 8000 ng/mL for decitabine and 10, 100 and 400 ng/mL for valdecitabine.

2.5. Plasma sample preparation

To a 50 μL aliquot of plasma sample, 100 μL of the IS solution (100 ng/mL) and 100 μL of water were added. The sample was briefly mixed following the addition of 250 μL water. After brief vortex, the mixture was loaded on a solid-phase extraction cartridge which had been pre-activated and equilibrated with 3 mL methanol and then 3 mL water before use. The sample was washed with 3 mL hydrochloric acid solution (0.05 M) containing 5% methanol and then by 3 mL methanol. Decitabine and valdecitabine were eluted with 3 mL 5% ammonium hydroxide in 95% methanol. The eluates were evaporated under a stream of nitrogen at 35 °C and then reconstituted in 100 μL water by vortex-mixing for 1 min. The resulting solution was then transferred to an autosampler vial at 4 °C and injected (5 μL) into HPLC–MS/MS system for analysis.

2.6. Method validation

Selectivity was assessed by comparing chromatograms of six different batches of blank rat plasma with the corresponding spiked rat plasma. Linearity was assessed by weighted ($1/x^2$) least-squares analysis of six different calibration curves. Intra- and inter-day precision (the relative standard deviation, RSD) and accuracy (the relative error, RE) were determined by analysis of low, medium and high QC samples ($n = 6$) on 3 different days. The matrix effect was investigated by comparing the peak areas of analytes in the post extraction spiked blank plasma at low and high concentrations with those of the corresponding standard solutions. The extraction recovery was determined by comparing the mean peak areas of six extracted samples at low, medium and high QC concentrations with the mean peak areas of spike-after-extraction samples. The stability of low, medium and high QC samples ($n = 3$) in three complete freeze/thaw cycles (−80 to 20 °C), long-term sample storage (−80 °C for 30 days) and bench-top (20 °C for 1 h) was assessed. The ready-to-injection stability of extracted samples in the autosampler rack at 4 °C for 24 h was also evaluated.

2.7. Application to a pharmacokinetic (PK) study

The validated assay was then applied to a PK study after oral administration of decitabine or valdecitabine to the male Sprague-Dawley rats (weighing from 220 to 250 g) at a dose of 15 mg/kg (calculated as decitabine). All animal experiments were performed in accordance with institutional guidelines and approved by Shenyang Pharmaceutical University Animal Care and Use Committee. Serial blood samples (about 0.2 mL) were obtained at 5, 10, 15, 30, 45 min and 1, 1.5, 2, 3, 5, 7, 9, 12 h separately. During sampling, rats were anesthetized with ether. All samples were collected into heparinized tubes containing THU (final concentration 0.1 mM). Blood samples were centrifuged at 800 × *g* for 10 min and plasma was separated and stored at −20 °C until analysis.

3. Results and discussion

3.1. Method development

The chemical structures (Fig. 1) of decitabine and valdecitabine (containing an amino group) indicate that they tend to capture a

proton and give a strong mass response in positive ion mode. Meanwhile, it was found that the ESI source provided a better response over the APCI source for both analytes. The response of $[M+H]^+$ of the analytes was highly depended on the capillary voltage. Therefore, the capillary voltage was optimized and 4.0 kV was chosen for the analysis of decitabine and valdecitabine according to the mass response of the precursor ions. The collision energy was optimized according to the response of the product ion. The mass transitions m/z 229 → 113 for decitabine with CE of 15 eV and m/z 328 → 216 for valdecitabine with CE of 10 eV were selected for the MRM analysis.

The chemical structure of decitabine is similar to an endogenous compound 2-deoxycytidine with a high level in rat plasma and it is difficult to obtain good separation between them. In our study, some Waters ACQUITY UPLC BEH C_{18} columns (50 mm × 2.1 mm, 1.7 μm; 100 mm × 2.1 mm, 1.7 μm) were originally utilized but failed to separate decitabine from 2-deoxycytidine. Then a Waters ACQUITY UPLCTM BEH HILIC column (100 mm × 2.1 mm, 1.7 μm) was tested, but low resolution and poor peak shape of the analytes were obtained. A C_{18} column (Waters Symmetry C_{18}, 150 mm × 4.6 mm, 3.5 μm) was then applied under a reversed-phase condition and a base-line separation between decitabine and 2-deoxycytidine was achieved.

The protein precipitation using methanol or acetonitrile was commonly employed to extract decitabine from the biological samples in the previous reports [9,11]. But in our preliminary study, we found that the protein precipitation method resulted in a low extraction recovery for decitabine (less than 50%). Since decitabine, valdecitabine and diazepam are basic, cation exchange solid-phase extraction cartridge was selected accordingly, which gave the recovery over 82% for decitabine. In addition, this solid-phase extraction procedure can provide cleaner samples than the protein precipitation method so that the background noise could be reduced and the sensitivity was increased.

3.2. Method validation

Fig. 2 shows the typical chromatograms of blank plasma, blank plasma spiked with decitabine (10 ng/mL), valdecitabine (5 ng/mL) and the IS (100 ng/mL) and plasma sample after oral administration of valdecitabine to rats. No interferences from the endogenous compounds with the analytes or the internal standard were observed.

The linear regressions of the peak area ratios *versus* concentration were fitted over the concentration range of 10–10,000 ng/mL for decitabine and 5–500 ng/mL for valdecitabine in rat plasma. Linear regressions were performed with $1/x^2$ as the weighting factor. Typical equations for the calibration curves were as follows: decitabine, $y = 0.00191x + 0.00216$; valdecitabine, $y = 0.00109x + 0.00885$, where y is the peak area ratio of decitabine or valdecitabine to IS and x (ng/mL) is the plasma concentration of decitabine or valdecitabine. The SD values of slope and intercept were 0.000113 and 0.000148 for decitabine and 0.0000654 and 0.000670 for valdecitabine. Both correlation coefficients (r) exceeded 0.995, which showed a good linearity over the studied concentration range.

The LLOQ was 10 ng/mL for decitabine and 5 ng/mL for valdecitabine in rat plasma. Assay precision was 6.05% for decitabine and 6.22% for valdecitabine at the LLOQ level, respectively.

Table 1 summarizes the intra-day and inter-day precision and accuracy for decitabine and valdecitabine from the QC samples. The intra- and inter-day precisions were all below 14% and maximum deviation of accuracy was 6.5%.

The extraction recovery in the plasma was excellent for decitabine: 95.70 ± 3.00%, 82.00 ± 8.68% and 90.56 ± 6.16% for the low, medium and high QC, respectively. Mean extraction recoveries were low but consistent for valdecitabine: 58.49 ± 1.54%, 59.06 ± 1.26% and 52.37 ± 0.68% for the low, medium and high QC, respectively. The extraction recovery of the IS was 56.27 ± 2.41% with the RSD value below 4.3%.

Table 1
Accuracy and precision for the analysis of decitabine and valdecitabine in rat plasma (3 validation days, six replicates at each concentration level per day).

Concentration (ng/mL)		RSD (%)		RE (%)
Added	Found	Intra-day	Inter-day	
Decitabine				
20	21.04	6.0	8.3	6.5
500	481.0	0.3	3.4	−3.0
8000	7892.1	6.1	10.5	0.4
Valdecitabine				
10	10.5	4.4	14.0	5.0
100	105.0	1.3	12.8	5.0
400	415.3	6.2	5.9	3.8

All the ratios of matrix effect for decitabine and valdecitabine were between 96% and 106%, suggesting that there was no significant matrix effect. A series of stability experiments were performed and the results are shown in Table 2. In the stability experiments, the relative errors of all samples were within 15%, which indicated that decitabine and valdecitabine exhibited no significant degradation under the conditions previously described.

3.3. Pharmacokinetic application of the developed method

This method was successfully applied to a pharmacokinetic study of decitabine and valdecitabine following a single oral dose of 15 mg/kg to the Sprague-Dawley rats (all calculated as decitabine). The typical mean plasma concentration–time profiles of decitabine and valdecitabine are shown in Fig. 3. Since valdecitabine was rapidly hydrolyzed into decitabine by the esterase *in vivo*, its

Table 2
Stability of decitabine and valdecitabine for in rat plasma exposed to various storage conditions ($n = 3$).

	Concentration (ng/mL)		RSD (%)	RE (%)
	Added	Found (mean)		
Autosampler rack at 4 °C for 24 h				
Decitabine	20	21.5	2.4	7.4
	500	487.6	6.4	−2.5
	8000	8689.5	0.9	8.6
Valdecitabine	10	9.9	6.0	−1.5
	100	100.3	5.8	0.3
	400	383.3	4.2	−4.2
Three freeze/thaw cycles (−80 to 20 °C)				
Decitabine	20	18.3	7.0	−8.4
	500	512.8	11.8	2.6
	8000	8584.5	9.1	7.3
Valdecitabine	10	10.6	5.2	6.2
	100	99.4	5.8	−0.7
	400	389.5	4.1	−2.6
Bench-top (20 °C for 1 h)				
Decitabine	20	21.5	1.6	7.3
	500	545.8	5.7	9.1
	8000	8764.4	6.7	9.6
Valdecitabine	10	9.6	5.9	−4.5
	100	99.4	4.1	−0.6
	400	371.5	6.8	−7.1
Freezing at −80 °C for 30 days				
Decitabine	20	18.8	3.1	−6.2
	500	505.7	8.7	1.1
	8000	7530.2	2.4	−5.9
Valdecitabine	10	9.7	7.4	−3.0
	100	88.6	2.4	−9.7
	400	356.4	5.7	−10.9

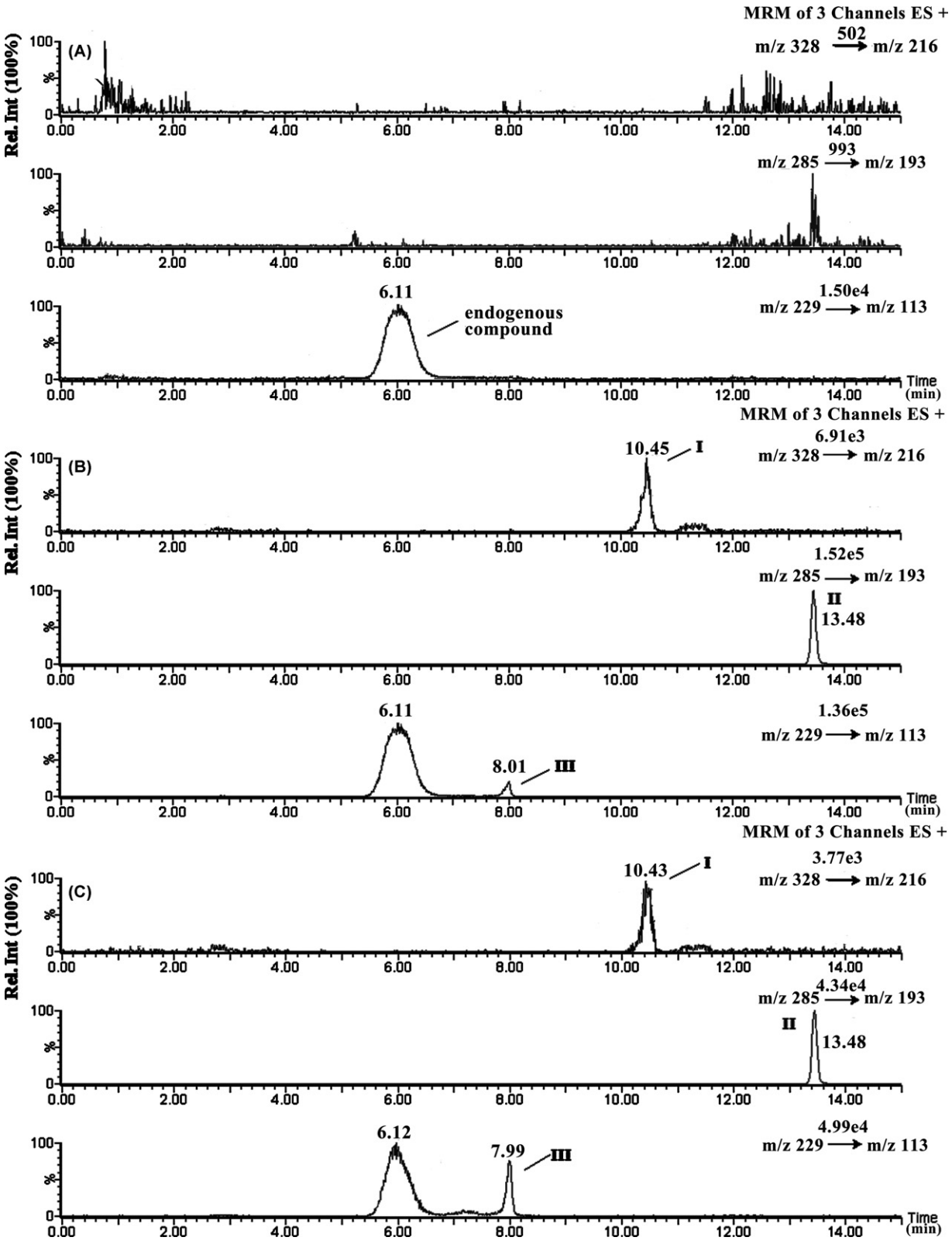

Fig. 2. Representative MRM chromatograms of valdecitabine (I), diazepam (IS, II) and decitabine (III) in rat plasmas: (A) a blank rat plasma sample; (B) a blank rat plasma sample spiked with decitabine (10 ng/mL), valdecitabine (5 ng/mL) and diazepam (100 ng/mL); (C) a rat plasma sample following an oral dose of valdecitabine at 15 mg/kg (calculated as decitabine) to a Sprague-Dawley rat.

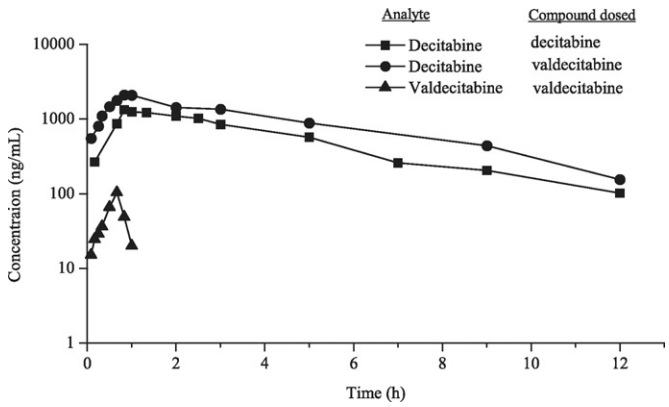

Fig. 3. Mean plasma concentration–time profiles of decitabine and valdecitabine in Sprague-Dawley rats ($n = 4$). (●) Decitabine and (▲) valdecitabine, following oral administration of valdecitabine to rats (15 mg/kg, calculated as decitabine); (■) decitabine, following oral administration of decitabine to rats (15 mg/kg).

concentration in plasma was very low. Additionally, it was clear that the oral bioavailability of decitabine was enhanced significantly after oral administration of valdecitabine.

4. Conclusions

A simple and sensitive HPLC–MS/MS method was developed and validated for the simultaneous analysis of decitabine and valdecitabine in rat plasma. It was very convenient to use a C_{18} column with 3.5 μm particle size for chromatographic separation with a single analysis time close to 15 min. The simplified solid-phase extraction pretreatment provided clean sample and high recovery for decitabine (>82%). It has been successfully applied to the pharmacokinetic study of valdecitabine after oral administration to rats.

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