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Short communication

Simultaneous determination of didanosine and its amino acid prodrug, valdidanosine by hydrophilic interaction chromatography coupled with electrospray ionization tandem mass spectrometry: Application to a pharmacokinetic study in rats

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ABSTRACT

A rapid, sensitive and selective ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method with hydrophilic interaction chromatography has been developed and validated for the simultaneous determination of didanosine and valdidanosine (*L*-valine amino acid ester prodrug of didanosine) in rat plasma. Solid-phase extraction (SPE) column was employed to extract the analytes from rat plasma, with high extraction recovery (>85%) for both didanosine and valdidanosine. The analytes were then separated by hydrophilic interaction chromatography (HILIC column) and detected by a triple-quadrupole mass spectrometry equipped with an electrospray ionization (ESI) source. The method was linear over the concentration ranges of 2–20,000 ng/mL for didanosine and 4–300 ng/mL for valdidanosine. The lower limit of quantitation (LLOQ) of didanosine and valdidanosine was 2 and 4 ng/mL, respectively. The intra-day and inter-day relative standard deviation (RSD) were less than 15% and the relative errors (RE) were all within 15%. Finally, the validated UPLC–MS/MS method was successfully applied to the pharmacokinetic study after either didanosine or valdidanosine orally administrated to the Sprague–Dawley rats.

1. Introduction

Didanosine (Videx®, 5′-*O*-2′-3′-dideoxydidanosine, DDI, Fig. 1A), is nucleoside analog reverse transcriptase inhibitor (NRTIs) in the treatment of AIDS [1–3]. However, it exhibits a low oral bioavailability (F = 20–40%) because of poor permeability across the intestinal epithelium [4]. It has been reported that amino acid ester prodrug of nucleoside analogue especially *L*-valine could be transported across the intestine mediated by the intestinal proton-coupled peptide transporter for increasing oral absorption [5,6], such as acyclovir and ganciclovir [7–9]. Following this idea, we had synthesized the 5′-*O*-*L*-valinyl eater derivative (valdidanosine, Fig. 1B) of didanosine in an attempt to enhance the oral bioavailability of didanosine. Therefore, a specific and accurate analytical method for simultaneous determination of didanosine and valdidanosine is needed to support the valdidanosine pharmacokinetic study.

Most frequently used methods for determining plasma levels of didanosine were high-performance liquid chromatography/ultraviolet (HPLC–UV) and liquid chromatography–tandem mass spectrometry (LC–MS/MS). But HPLC–UV method was time-consuming (more than 10 min per single run) and had a low detection sensitivity of about 30 ng/mL [10–12]. LC–MS/MS was mainly used to determine the intracellular metabolites of didanosine [13–15]. And when it was applied to determine didanosine in biological sample, it had a lower limit of quantification (LLOQ) of around 5 ng/mL [16,17]. Other methodologies also have been described, such as radioimmunoassay [18] and capillary electrophoresis with MS/MS [19]. To our best knowledge, no methods have been published concerning the simultaneous determination of didanosine and valdidanosine in rat plasma using UPLC–MS/MS method.

UPLC–MS/MS is a powerful analytical technique combining the resolving power of UPLC and the high detection specificity and sensitivity of MS. In this study, we developed a facile hydrophilic interaction chromatography coupled with tandem mass spectrom-

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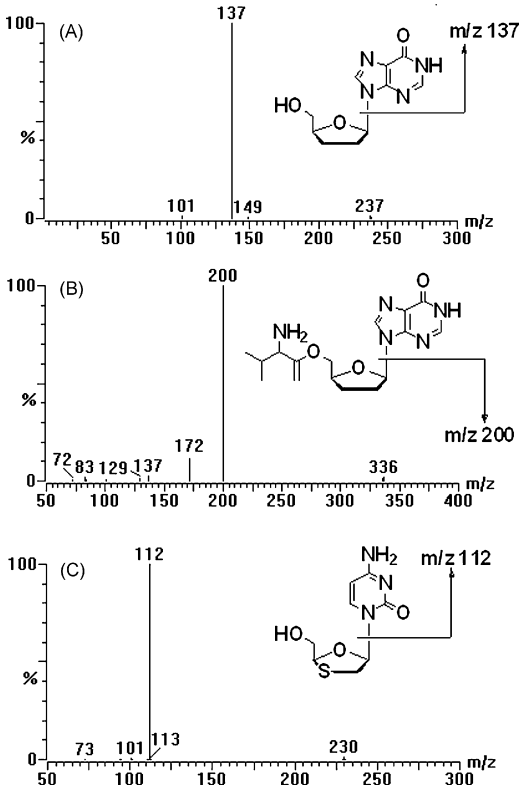

Fig. 1. Product ion mass spectra of $[M+H]^+$ ions of didanosine (A), valdidanosine (B) and lamivudine (C).

etry (HILIC–MS/MS) to simultaneously determine didanosine and valdidanosine in rat plasma. The developed HILIC–MS/MS method was more rapid and convenient with single run time less than 1.5 min and demonstrated a higher sensitivity with LLOQ of 2 ng/mL for didanosine and 4 ng/mL for valdidanosine. Also solid-phase extraction (SPE) cartridge was employed to extract the analytes from rat plasma, and presented higher recoveries, concentrated analytes and less matrix interference. The validated method was then successfully applied to the preclinical pharmacokinetic study of valdidanosine in Sprague–Dawley rats following oral administration at 30 mg/kg (valdidanosine dose calculated as didanosine).

2. Experimental

2.1. Chemicals and reagents

Didanosine was purchased from Wuhan Yuanchen Technology Development Co., Ltd. (Wuhan, China). Valdidanosine was synthesized in Department of Medical Chemistry, Shenyang Pharmaceutical University (Shenyang, China). Lamivudine (internal standard, IS >99.0% purity) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (HPLC-grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid (HPLC-grade) was obtained from Dikma (Richmond Hill, NY, USA). Triple deionized water was prepared by a Barnstead EASYPURE® II RF/UV system (Dubuque, IW, USA).

2.2. Instrumentation

A Waters ACQUITY TQD system was employed for the determination of didanosine and valdidanosine, which consisted of an ACQUITY UPLC system (Waters Corp., Milford, MA, USA) with cooling autosampler and column oven, and an ACQUITY triple-quadrupole tandem mass spectrometer with an electrospray ionization (ESI) interface (Waters Corp., Milford, MA, USA). Oasis HLB cartridges (1 cc, 30 mg) used were purchased from Waters Corporation (Milford, MA, USA). HILIC column (50 mm × 2.1 mm, 1.7 μm) was obtained from Waters Corporation (Wexford, Ireland). All data were acquired and processed by MassLynx 4.1 software with QuanLynx program (Waters Corp., Milford, MA, USA).

2.3. UPLC/MS/MS condition

An elution was performed for chromatographic separation with a mobile phase consisted of water containing 0.1% formic acid and methanol (85:15, v/v). The flow rate was 0.2 mL/min and column temperature was maintained at 40 °C. The autosampler was conditioned at 4 °C and the sample volume injected was 5 μL. Injection wash solvents were methanol–water (5:95, v/v) and methanol–water (50:50, v/v) for weak and strong wash, respectively.

The mass spectrometer was operated with an ESI interface in positive ionization mode. The cone and desolvation gas flow rates were 50 L/h and 550 L/h, respectively, and were obtained from an in-house nitrogen. High purity argon was used as collision gas at a pressure of 3.00×10^{-3} mbar. The optimal MS parameters were as follows: capillary voltage of 2.5 kV, source temperature 120 °C and desolvation temperature 350 °C. Cone voltage was 10, 20 and 22 V, collision energy was 10, 12 and 12 eV for didanosine, valdidanosine and lamivudine, respectively. Quantification was performed using multiple reaction monitoring (MRM) mode of the transitions m/z 236 → 137 for didanosine, m/z 336 → 200 for valdidanosine and m/z 230 → 112 for IS, respectively. The scan time was set at 0.02 s per transition.

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

Standard stock solutions of didanosine, valdidanosine and IS were separately prepared in water. Standard solutions of didanosine and valdidanosine were prepared by serially diluting stock solution of didanosine and valdidanosine with water. And the IS solution was diluted with water to give a concentration of 232 ng/mL. All the solutions were stored at 4 °C and brought to room-temperature before use.

Calibration curves were prepared by spiking 50 μL of blank rat plasma with 100 μL of didanosine standard solution or valdidanosine 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 2, 4, 20, 100, 200, 800, 1000, 2000, 4000, 10,000, 16,000, and 20,000 ng/mL for didanosine and 4, 10, 20, 100, 200, 240, and 300 ng/mL for valdidanosine. The QC samples were prepared in the same way as the calibration samples. The plasma concentrations of QC samples were 4, 1000 and 16,000 ng/mL for didanosine and 10, 100 and 240 ng/mL for valdidanosine.

2.5. Plasma sample preparation

To a 50 μL aliquot of plasma sample, 100 μL of the IS solution (232 ng/mL) and 100 μL of water were added. The sample was briefly mixed following the addition of 250 μL water and centrifugated at 10,000 rpm for 10 min. The supernatant was then transferred to a HLB cartridge which was individually activated and equilibrated by washing with 1 mL methanol and 1 mL water before use. The plasma samples were washed by 1 ml water containing 5% methanol once. Didanosine and valdidanosine were eluted with 1 ml methanol twice. The elutes were collected and dried under nitrogen gas flow at 40 °C. The residue was reconstituted with 100 μL of water, then vortexed for 1 min and centrifuged

at 10,000 rpm for 10 min. The supernatant was transferred into an autosampler vial and injected (5 μL) into the UPLC–MS/MS system for assay.

2.6. Method validation

Selectivity was assessed by comparing the chromatograms of six different batches of rat plasma with the corresponding spiked rat samples. Standard calibration graphs were constructed by linear least squares regression analysis on the analyte/IS area ratio plotted against the sample concentration with $1/x^2$ as the weighting factor and assayed in duplicate on three consecutive days. LLOQ was defined as a signal/noise ≥10, and the precision and accuracy were evaluated by analyzing six samples which were prepared in six replicates.

The accuracy, intra-day and inter-day precision were assessed by measuring six replicates of QC samples of each concentration level on three validation days. The precision was expressed as relative standard deviation (RSD) and the accuracy was determined by relative error (RE).

The extraction recoveries of didanosine and valdidanosine were determined by comparing the peak areas of six replicates of blank plasma samples spiked with QC samples at low, medium and high levels before extraction with those of blank plasma samples spiked with the corresponding QC samples after extraction. The extraction recovery of IS was also evaluated.

The matrix effect was investigated by comparing the peak areas of the extracted blank plasma from six different sources spiked with low, medium, and high concentrations with those of standard solutions that had been prepared in the same way as the QC samples, except that water was substituted for blank plasma. The corresponding peak areas of the analytes in spiked blood post-extraction samples (A) were then compared to those of the water-substituted samples (B) at equivalent concentrations. The absolute matrix effect (ME) was calculated as follows:

$$\mathrm{ME} = \frac{\mathrm{A}}{\mathrm{B}} \times 100$$

Stability was evaluated under conditions mimicking situations likely to be encountered during sample storage, preparation and the analytical process including room-temperature stability (20 °C for 6 h), freeze–thaw stability (3 cycles of freeze–thaw) and long-term stability (−80 °C for 30 days). The ready-to-inject stability (after extraction) was also evaluated in the UPLC autosampler at 4 °C for 24 h. All the stability studies were conducted with three QC samples.

2.7. Application to a pharmacokinetic (PK) study

The developed assay was then applied to a PK study after oral administration of didanosine or valdidanosine to male Sprague–Dawley rats (weighing from 250 to 300 g) at a dose of 30 mg/kg (prodrug dose calculated as didanosine). 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 2, 5, 10, 15, 20, 30, and 45 min and 1, 1.5, and 4 h separately. During sampling, rats were anesthetized with ether. All samples were placed into heparinized tubes. The rat plasma samples were centrifugated at 10,000 rpm for 10 min, collected and frozen at −20 °C until analysis.

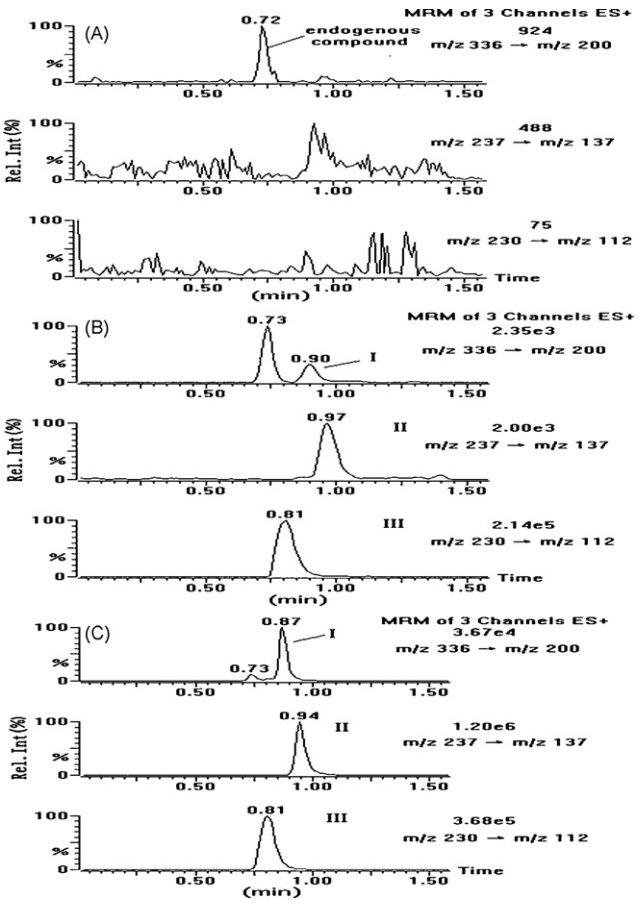

Fig. 2. Representative MRM chromatograms of valdidanosine (I), didanosine (II) and lamivudine (IS, III) in rat plasmas: (A) a blank rat plasma sample; (B) a blank rat plasma sample spiked with didanosine (2 ng/mL), valdidanosine (4 ng/mL), and lamivudine (232 ng/mL); (C) a rat plasma sample following an oral dose of valdidanosine at 30 mg/kg (calculated as didanosine) to a Sprague–Dawley rat.

3. Results and discussion

3.1. Method development

The chemical structures (Fig. 1) of didanosine and valdidanosine (containing basic group) indicate that they tend to capture a proton, and give a strong mass response in positive ion mode. The ionization mode was firstly investigated using atmospheric pressure chemical ionization (APCI) and ESI sources. The ESI source provided a better response over the APCI source for the two analytes. Therefore further analysis development in this study was employed to adopt the ESI source. The cone voltage was set at 10 eV for didanosine (m/z 236 → 137) because didanosine can give highly

Table 1
Accuracy and precision for the analysis of didanosine and valdidanosine 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	
Didanosine				
4.00	4.07	6.62	7.81	1.8
1000	1013	1.35	6.80	1.3
16,000	15,074	1.11	10.11	−5.8
Valdidanosine				
10.0	10.2	10.8	2.83	1.7
100.0	105.5	1.80	4.44	5.5
240.0	229.6	1.39	8.41	−4.3

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

Condition	Concentration (ng/mL)				Didanosine		Valdidanosine	
	Didanosine		Valdidanosine		RSD (%)	RE (%)	RSD (%)	RE (%)
	Added	Found	Added	Found				
Ambient, 6 h	4.00	3.97	10.0	10.3	3.85	−0.83	5.33	3.33
	16,000	16,841	240.0	236.1	1.11	5.26	1.47	−1.63
−80 °C, 30 days	4.00	3.70	10.0	10.5	4.68	−7.50	1.65	5.00
	16,000	16,615	240.0	224.1	2.31	3.84	5.04	−6.61
Three freeze–thaw	4.00	4.30	10.0	10.7	6.58	7.50	4.28	7.00
	16,000	16,581	240.0	254.1	3.18	3.63	1.41	−5.88
Sample rack for 24 h at 4 °C	4.00	4.20	10.0	9.37	8.25	5.00	7.11	−6.33
	16,000	14,417	240.0	223.2	0.41	−9.56	9.37	−7.01

protonated molecules $[M+H]^+$ at very low energy. Similarly, mass transitions (m/z 336 → 200) with CE of 12 eV for valdidanosine and (m/z 230 → 112) with CE of 12 eV for lamivudine were selected for the MRM analysis. It was found that the source temperature and the desolvation temperature did not significantly influence the MS behavior of these analytes, thus these parameters were conditioned at the recommended values of 120 °C and 350 °C, respectively.

The reported extraction methods of didanosine from biological matrix included protein precipitation (PP) [19,20] and SPE [21,22]. But during our exploration, we found that the PP method resulted in a very low extraction recovery for didanosine and valdidanosine (both less than 50%). Because didanosine, valdidanosine and lamivudine are basic compound with high polarity, SPE cartridge was accordingly used for their extraction, with the recovery over 85% for all analytes. SPE cartridge sorbent is a copolymer of hydrophilic N-vinylpyrroridone and lipophilic divinylbenzene, so it can provide reasonable retention capacity for most of hydrophilic compounds. Furthermore, this SPE procedure reduced the background noise and increased the sensitivity, therefore SPE was selected as plasma pretreatment method.

We found there was an endogenous compound in rat plasma that interfered valdidanosine mass detection due to the same fragment ion. It could not be eliminated by sample pretreatment because of the same high polarity as valdidanosine. Therefore we attempted to separate valdidanosine from the endogenous compound using chromatographic method. Previously, there were many successful studies to analyze and separate polar compounds using hydrophilic interaction chromatography (HILIC) [23–25]. HILIC has been described as an alternative to traditional chromatography to retain very polar analytes. In this mode, a polar stationary phase is used to achieve reasonable retention of very polar analytes with better peak shape. Therefore, a HILIC column was chosen to separate valdidanosine and the endogenous compound. And it could achieve a base-line separation between valdidanosine and the endogenous compound (Fig. 2).

3.2. Method validation

Representative chromatograms of blank plasma, blank plasma spiked with didanosine (2 ng/mL), valdidanosine (4 ng/mL) and the IS (232 ng/mL), and plasma sample after rat oral administration of valdidanosine were shown in Fig. 2. It was clear that there was no interference from endogenous substances in plasma at the retention times of the analytes and the IS.

All the ratios of matrix effect for didanosine, valdidanosine and IS were between 85% and 115%, suggesting that there was no significant matrix effect in this method.

The calibration curves were determined over the range of 2–20,000 ng/mL and 4–300 ng/mL for didanosine and valdidanosine, respectively. Linear regressions were preformed with $1/x^2$ as the weighting factor. Typical equation for the calibration curves were as follows: didanosine, $y = 0.0032x + 0.0051$; valdidanosine, $y = 0.0025x - 0.0009$ where y is the peak area ratio of didanosine or valdidanosine to IS, and x (ng/mL) is the plasma concentration of didanosine or valdidanosine. Both correlation coefficients (r) exceeded 0.99, showing a good linearity over the concentration range. The LLOQ was 2 ng/mL for didanosine and 4 ng/mL for valdidanosine in rat plasma. Assay precision and accuracy were 8.5% and 5.0% for didanosine, and 12.7% and −3.3% for valdidanosine at the LLOQ level, respectively, which were within the acceptable limits.

Table 1 summarizes the intra-day and inter-day precision and accuracy for didanosine and valdidanosine from the QC samples. The results indicated that all the values were within the acceptable range of ±15% and the method exhibited good precision and accuracy.

Mean extraction recoveries were 116.0 ± 4.7%, 102.8 ± 2.8% and 120.5 ± 4.6% at the concentrations of 4, 1000 and 16,000 ng/mL for didanosine, and 97.9 ± 5.1%, 88.4 ± 2.9%, and 85.2 ± 5.1% of 10, 100 and 240 ng/mL for valdidanosine, respectively. The extraction recovery of the IS was 88.0 ± 4.0%.

A number of stability experiments were performed and the results were shown in Table 2. No significant changes in the didanosine and valdidanosine concentrations were observed under the indicated conditions.

3.3. Pharmacokinetic application of the method

The presented method was successfully applied to quantify didanosine and valdidanosine following a single 30 mg/kg oral dose to the Sprague–Dawley rats (valdidanosine calculated as didano-

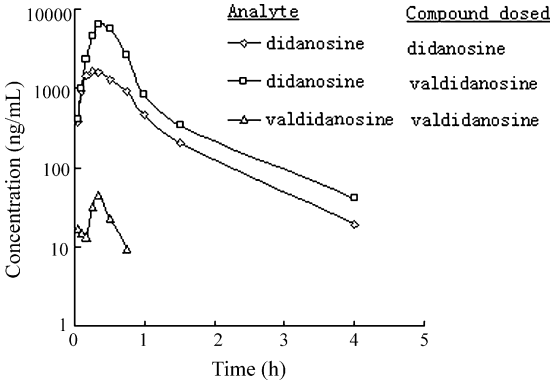

Fig. 3. Mean plasma concentration–time profiles of didanosine and valdidanosine in Sprague–Dawley rats ($n = 3$). (□) Didanosine and (△) valdidanosine, following oral administration of valdidanosine to rats (30 mg/kg, calculated as didanosine); (◊) didanosine, following oral administration of didanosine to rats (30 mg/kg).

sine). The mean plasma concentration–time profiles were shown in Fig. 3. Since valdidanosine was rapidly hydrolyzed into didanosine by the esterase in rat gastrointestinal tract, liver and plasma, its concentration in plasma was very low. Additionally, it was clear that oral bioavailability of didanosine was enhanced significantly after oral administration of valdidanosine.

4. Conclusion

A sensitive, selective and fast UPLC–MS/MS method for the simultaneous determination of didanosine and valdidanosine in rat plasma was developed and validated. The SPE procedure was adopted in plasma sample preparation, which provided clean sample and consistent high extraction recovery. The analytes were separated by hydrophilic interaction chromatography. The LLOQ of this method was low enough to meet the needs of pharmacokinetic study with good intra-day and inter-day reproducibility for the QCs. The applicability of the method was demonstrated in a pharmacokinetic study of didanosine and valdidanosine in rats.

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