Journal of Chromatography B, 870 (2008) 121–125

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

High-performance liquid chromatography/tandem mass spectrometry method for the simultaneous determination of cytarabine and its valyl prodrug valcytarabine in rat plasma

Yongbing Sun[a], Jin Sun[a], Bing Wen[a], Shiliang Shi[b], Youjun Xu[b], Ying Chen[c], Yongjun Wang[a], Changqi Pan[a], Chunyu Zhang[a], Tianhong Zhang[a], Zhonggui He[a,*]

[a] Department of Biopharmaceutics, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China
[b] Department of Medicinal Chemistry, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China
[c] Department of Pharmacology, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China

ARTICLE INFO

Article history:
Received 16 November 2007
Accepted 19 May 2008
Available online 18 June 2008

Keywords:
High-performance liquid chromatography/tandem mass spectrometry
Cytarabine
Valcytarabine
Solid-phase extraction
Rat plasma

ABSTRACT

A sensitive, specific and rapid HPLC–MS/MS method has been developed and validated for the simultaneous determination of cytarabine and valcytarabine (valyl prodrug of cytarabine) in rat plasma in the present study. The analytes were separated on a C_{18} column (50 mm × 2.1 mm, 1.7 μm) and a triple-quadrupole mass spectrometry equipped with an electrospray ionization (ESI) source was applied for detection. Cation exchange solid-phase extraction cartridge was employed to extract the analytes from rat plasma, with high recovery of cytarabine (>85%). The method was linear over the concentration ranges of 10–20,000 ng/mL for cytarabine and 25–1000 ng/mL for valcytarabine. The lower limit of quantitation (LLOQ) of cytarabine and valcytarabine was 10 and 25 ng/mL, respectively. The intra-day and inter-day relative standard deviation (RSD) were less than 15% and the relative error (RE) were all within 15%. Finally, the method was successfully applied to support the prodrug pharmacokinetic study after valcytarabine and cytarabine were orally administrated to the Sprague–Dawley rat, respectively.

1. Introduction

1-β-D-Arabinofuranosylcytosine (cytarabine, ara-C, Fig. 1), is a pyrimidine nucleoside analogue employed for the treatment of both acute and chronic myeloblastic leukaemias [1,2]. But cytarabine has a very short plasma half-life and a very low oral bioavailability (F = 20%) due to low permeability across the intestine and extensive metabolism to inactive 1-β-D-arabinofuranosyluracil (ara-U) [3]. It was found that amino acid ester derivatives had been a well-established method of overcoming the undesirable properties of medicinal agents and successfully employed as prodrug forms of some nucleoside analogues, such as acyclovir [4,5], and ganciclovir [6]. Following this idea, we synthesized the 5′-*O*-L-valinyl ester derivative (valcytarabine, Fig. 1) of cytarabine with the aim to improve the oral bioavailability of cytarabine. Therefore, a sensitive and accurate analytical method for the simultaneous determination of cytarabine and valcytarabine is required to support prodrug pharmacokinetic (PK) study.

High-performance liquid chromatography/ultraviolet method (HPLC/UV) has been reported for the determination of cytarabine in dog and rat plasma [7,8]. However, these methods were not highly sensitive. Recently, Hsieh et al. developed a series of liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) methods to determine cytarabine in mouse plasma, with a lower limit of quantification (LLOQ) being 50 ng/mL [9–12]. In these studies, the analyte was separated from the endogenous component by using a mixed-mode column [9], a porous graphitic carbon chromatography (PGC–HPLC) [10], an ion-pairing liquid chromatography (IP–HPLC) [11], or a packed-column supercritical fluid chromatography (pSFC) [12]. In the present research, we applied a C_{18} column with 1.7 μm particle size to determine simultaneously cytarabine and valcytarabine in rat plasma under the reversed-phase conditions. The developed HPLC/MS/MS method was more sensitive and convenient, and a cation exchange solid-phase extraction (SPE) for plasma pretreatment generated higher recoveries and provided much cleaner samples than protein precipitation method. The LLOQ of 10 ng/mL for cytarabine in the present study corresponded to an on-column sensitivity (the quantity of drug injected on the column per injection) of 2.5 pg cytarabine, which was three times

* Corresponding author. Tel.: +86 24 23986321; fax: +86 24 23986321.
E-mail address: hezhonggui@gmail.com (Z. He).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.05.033

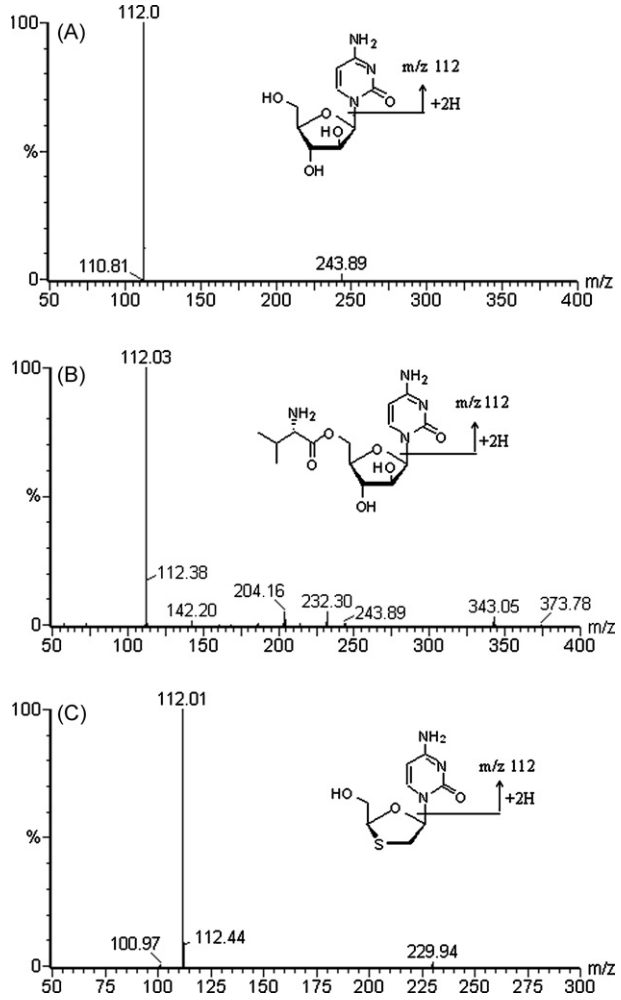

Fig. 1. Product ion mass spectra of $[M+H]^+$ ions of cytarabine (A), valcytarabine (B), and lamivudine (C).

lower than 8.3 pg reported in the literature [9–12]. The method was validated and successfully applied to the prodrug PK studies after valcytarabine and cytarabine were orally administrated to the Sprague–Dawley rat at 30 mg/kg (prodrug calculated as cytarabine), respectively.

2. Experimental

2.1. Chemicals and reagents

Cytarabine (99.3% purity) was purchased from Surui Chemical Corp. (Suzhou, China). Valcytarabine (99.2% purity) was synthesized in Department of Medicinal Chemistry, Shenyang Pharmaceutical University (Shenyang, China). Lamivudine (internal standard, IS >99.0% purity) 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 (FA) was purchased from Concord Chemical (Tianjin, China). Waters Oasis MCX cation exchange solid-phase extraction cartridges (1 cc/30 mg) were obtained from Waters Corporation (Milford, MA, USA). Ultrapure water was prepared by EASYPUREIIRF/UV system (Boston, MA, USA).

2.2. Instrumentation

A Waters ACQUITY TQD system was employed for the determination of the analytes of interest, 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 spectrometric detection with an electrospray ionization (ESI) interface (Waters Corp., Milford, MA, USA). An ACQUITY UPLC BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm, Waters Corp., Milford, MA, USA) was used to separate the analytes. All data were acquired and processed using MassLynx 4.1 software with QuanLynx program (Waters Corp., Milford, MA, USA).

2.3. HPLC/MS/MS conditions

A gradient elution programme was conducted for chromatographic separation with the mobile phase A (water–methanol, 98:2, v/v), and the mobile phase B (methanol containing 0.1% formic acid) as follows: 0 (100% A), 1.5 min (100% A), 3.0 min (70%, A), 3.01 min (100% A) and 6.0 min (100% A) and finished at 6 min. The flow rate was 0.2 mL/min and column temperature was 40 °C. Injection wash solvents were methanol–water–0.1% formic acid (5:95:0.1, v/v/v) and methanol–water–0.1% formic acid (50:50:0.1, v/v/v) for weak and strong wash, respectively. For MS detection, positive ESI was used as the ionization mode. Nitrogen was used as the desolvation and cone gas with 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 2.5 kV, source temperature 110 °C and desolvation temperature 380 °C. Cone voltage was 25, 28 and 25 V for cytarabine, valcytarabine and lamivudine, respectively. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of *m*/*z* 244–112 with collision energy (CE) of 15 eV for cytarabine, *m*/*z* 343–112 with CE of 20 eV for valcytarabine and *m*/*z* 230–112 with CE of 15 eV for lamivudine, respectively (dwell time of 0.1 s).

2.4. Preparation of standard and quality control samples

Standard stock solutions of cytarabine and valcytarabine were prepared individually in water at the concentrations of 400.0 and 20.0 μg/mL, respectively. A series of standard solutions of 10, 50, 100, 200, 1000, 5000, 10,000, 20,000 ng/mL for cytarabine and 25, 50, 100, 200, 400, 600, 800, 1000 ng/mL for valcytarabine were prepared by serially diluting the stock solution. The working solution of internal standard (2.0 μg/mL) was also prepared in water. All the solutions were stored at 4 °C.

Calibration curves were prepared by spiking 30 μL of blank rat plasma containing THU (0.1 mM) with 30 μL of cytarabine standard solution, 30 μL of valcytarabine standard solution and 20 μL of internal standard solution. The quality control (QCs) samples at low, medium, high concentration levels were prepared in the same way as the calibration samples. The nominal plasma concentrations of QC samples were 50, 1000, 16,000 ng/mL for cytarabine and 50, 400, 800 ng/mL for valcytarabine, respectively.

2.5. Sample preparation

To a 30-μL aliquot of plasma sample, 20 μL of internal standard solution (2.0 μg/mL) and 60 μL water were added. The sample was briefly mixed following the addition of 400 μL water and then centrifuged at 2500 × *g* and 4 °C for 5 min. The supernatant was transferred to a solid-phase extraction cartridge which was conditioned and equilibrated by washing with 1 mL methanol and then 1 mL water before use. The samples were washed by 1 mL of 0.05 M

hydrochloric acid solution containing 5% methanol twice and then by 1 mL of methanol. Cytarabine and valcytarabine were eluted with 1 mL of ammonium hydroxide in methanol (5:95, v/v) twice. The eluates were evaporated to dryness under N_2 at 37 °C and then reconstituted in 600 μL of water by vortex mixing (1 min). The resulting solution was transferred to an autosampler vial at 4 °C, and injected (5 μL) into HPLC/MS/MS system.

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 and high QC samples ($n = 3$) in three complete freeze/thaw cycles (−80 to 22 °C), long-term sample storage (−80 °C for 30 days), and bench-top (23 °C for 2 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. Pharmacokinetic (PK) study in rats

Male Sprague–Dawley rats weighing from 210 to 240 g were used for PK study. All animal experiments were performed in accordance with institutional guidelines and were approved by the University Committee on Use and Care of Animals, Shenyang Pharmaceutical University. The aqueous solutions of cytarabine and valcytarabine were separately administrated to a rat by gavage at 30 mg/kg (all calculated as cytarabine). Serial blood samples (0.2 mL) were obtained at 5, 15, 30, 45 min and 1, 1.5, 2, 3, 4, 6, 8, 12, 24 h after oral administration separately. During sampling, rats were anesthetized with ether. All samples were placed into heparinized tubes containing THU (final concentration 0.1 mM). After centrifugation at 800 × *g* and 4 °C for 10 min, plasma was collected and frozen at −80 °C until analysis.

3. Results and discussion

3.1. Method development

Cytarabine, valcytarabine and internal standard have strong mass responses under positive ionization mode. The ESI source provided a better response over the APCI source for the two analytes, especially for cytarabine. The product spectrum of the $[M+H]^+$ ion of cytarabine is highly depended on the CE. At high CE (35 eV), a major fragment ion at *m*/*z* 69 was formed. At lower CE (12–15 eV), a major fragment ion was at *m*/*z* 112 (Fig. 1). The transition of *m*/*z* 244–112 gave a considerably better response and a higher signal-to-noise ratio than that of *m*/*z* 244–69. As a result, the transition of *m*/*z* 244–112 at CE 15 eV was chosen for use in MRM. In a similar fashion, *m*/*z* 343–112 with CE of 20 eV for valcytarabine and *m*/*z* 230–112 with CE of 15 eV for lamivudine were selected for the MRM analysis.

An endogenous compound cytidine, whose structure is very similar to cytarabine, was present in rat plasma [13] and it could not be eliminated by pretreatment. So, it was imperative to separate cytarabine from the endogenous compound using HPLC since they have the same precursor and product ions in mass spectrometry. A reversed-phase C_{18} (20 cm × 0.46 cm, 5 μm, Diamosil, DIKMA) was even tried to separate cytarabine and the endogenous compound under highly aqueous mobile phase. However, cytarabine was not retainable, as reported by other studies [9]. When a hydrophilic interaction (HILIC) column (50 mm × 2.1 mm, 1.7 μm) was employed, it also could not achieve a satisfactory resolution between cytarabine and the endogenous compound, though the retention of two compounds was improved. A C_{18} column (ACQUITY UPLC BEH C_{18}, 50 mm × 2.1 mm, 1.7 μm) was then applied under the reversed-phase conditions, a base-line separation between cytarabine and cytidine was achieved (Fig. 2). But a C_{18} column (ACQUITY X-Bridge, 50 mm × 2.1 mm, 3.5 μm) could not achieve

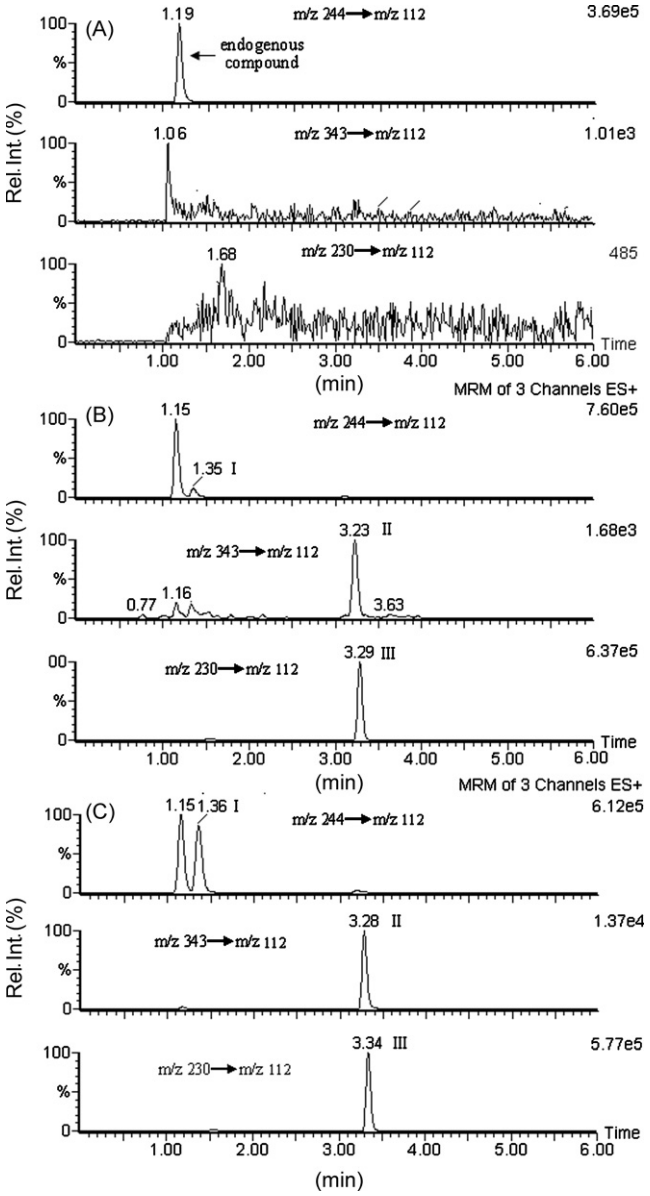

Fig. 2. Representative MRM chromatograms of cytarabine (I), valcytarabine (II) and lamivudine (IS, III) in rat plasmas: (A) a blank rat plasma sample; (B) a blank rat plasma sample spiked with cytarabine (10 ng/mL), valcytarabine (25 ng/mL), and lamivudine (2.0 μg/mL); (C) a rat plasma sample containing 2211 ng/mL of cytarabine and 301 ng/mL of valcytarabine following an oral dose of valcytarabine at 30 mg/kg (calculated as cytarabine) to a Sprague–Dawley rat.

Table 1
Accuracy and precision for the analysis of cytarabine and valcytarabine in rat plasma (in three validation days, six replicates at each concentration level per day)

Concentration (ng/mL)		RSD (%)		Relative error (%)
Added	Found (mean)	Intra-day	Inter-day	
Cytarabine				
50	53.2	3.2	4.6	6.4
1,000	1026	5.7	3.4	2.6
16,000	16202	4.6	3.9	1.3
Valcytarabine				
50	52.9	5.1	3.8	4.8
400	407.6	3.9	2.3	1.9
800	790.2	4.8	4.4	−1.2

this result under the same conditions although it has the same stationary phase chemistry as the ACQUITY UPLC BEH C_{18} column. We deduced a higher column performance with the smaller particle size in ACQUITY UPLC BEH C_{18} brought the good separation between cytarabine and cytidine [14].

The protein precipitation using methanol or acetonitrile was commonly employed to extract cytarabine from animal plasma in the previous reports [9–12]. But during our exploration, we found that this protein precipitation method resulted in a very low extraction recovery for cytarabine (about 10%). Because cytarabine, valcytarabine and lamivudine are basic solutes, cation exchange solid-phase extraction cartridges were accordingly used for their extraction, which gave recovery above 85% for all analytes. Furthermore, this solid-phase extraction procedure can provide cleaner samples than the protein precipitation method, thus reduced the background noise and increased the sensitivity. So it was selected as plasma pretreatment method.

3.2. Method validation

Fig. 2 shows the typical chromatograms of a blank, a spiked plasma sample with cytarabine (10 ng/mL), valcytarabine (25 ng/mL) and the internal standard (2.0 μg/mL), a plasma sample from a rat after an oral administration of valcytarabine. No interference from the endogenous compound with the analytes and the internal standard was detected.

The matrix effects calculated were in the range of −9.0% to 5.2%, which was within the acceptable limits.

Calibration curves were obtained between the mass responses and the plasma concentration over the range of 10–20,000 ng/ml for cytarabine and 25–1000 ng/ml for valcytarabine. The typical regression equation was as follows: cytarabine, $y = (1.84 \pm 0.61) \times 10^{-4} + (9.63 \pm 1.78) \times 10^{-4}x$; valcytarabine, $y = (6.97 \pm 2.44) \times 10^{-4} + (2.34 \pm 0.36) \times 10^{-4}x$, where y is the peak-area ratio of cytarabine or valcytarabine to the internal standard, x is the concentration of cytarabine or valcytarabine (ng/mL). All correlation coefficients (r) exceeded 0.99, showing a good linearity over the concentration range. The lower limit of quantitation (LLOQ) was 10 ng mL^{-1} for cytarabine and 25 ng mL^{-1} for valcytarabine in rat plasma. The intra- and inter-RSD were both less than 9.5% and the RE were within 8.3% for two analytes at LLOQ level, which were within the acceptable limits.

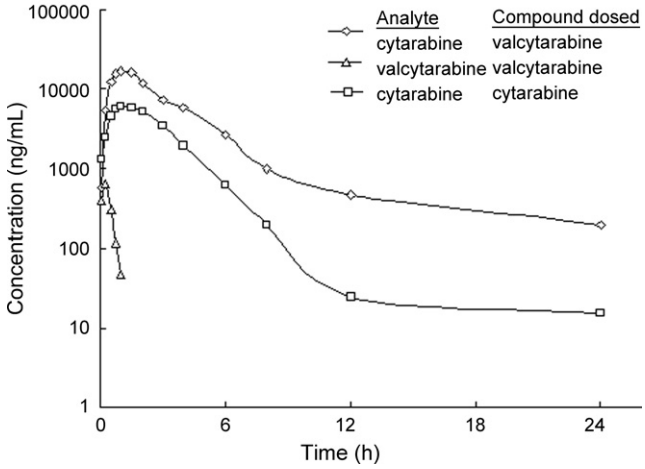

Fig. 3. Plasma concentration–time profiles of cytarabine and valcytarabine in the Sprague–Dawley rats. (◊): cytarabine and (△): valcytarabine following oral administration of valcytarabaine to a rat (30 mg/kg, calculated as cytarabine); (□): cytarabine following oral administration of cytarabine to a rat (30 mg/kg).

The intra- and inter-day precision and accuracy for cytarabine and valcytarabine at three QC concentration levels were shown in Table 1. The results indicated that all the values were within the acceptable range of ±15% and the method is accurate and precise.

The mean extraction recovery were 85.3 ± 4.2%, 90.3 ± 4.5%, and 87.0 ± 5.3% for cytarabine at 50, 1000, and 16,000 ng/mL, and 90.7 ± 6.2%, 94.4 ± 6.7%, and 88.1 ± 5.8% for valcytarabine at 50, 400, and 800 ng/mL, respectively. The mean recovery of the internal standard was 89.7 ± 6.1%. All recoveries had RSD less than 8% at three different concentrations, showing good assay consistency.

Table 2
Stability data of cytarabine and valcytarabine in rat plasma under different conditions

Storage conditions	Concentration (ng/mL)					
	Drug	Added	Found	SD (ng/mL)	RSD (%)	RE (%)
Bench-top for 2 h	Cytarabine	50	47.3	1.9	4.0	−5.4
		16000	15216	1252	8.1	−4.9
	Valcytarabine	50	48.4	1.2	2.5	−3.2
		800	868	48.6	5.6	8.5
Three freeze/thaw cycles	Cytarabine	50	53.4	3.9	7.3	6.8
		16000	15504	698	4.5	−3.1
	Valcytarabine	50	54.1	1.6	2.9	8.2
		800	834	43.4	5.2	4.3
Autosampler rack for 24 h at 4 °C	Cytarabine	50	52.1	1.1	2.2	4.2
		16000	17008	1054	6.2	6.3
	Valcytarabine	50	51.9	3.5	6.7	3.8
		800	744	43.9	5.9	−7.0
Freezing for 30 days at −80 °C	Cytarabine	50	47.2	3.0	6.4	−5.6
		16000	16736	853	5.1	4.6
	Valcytarabine	50	53.5	2.1	3.9	7.0
		800	782	16.4	2.1	−2.2

Stability results in Table 2 demonstrated that cytarabine and valcytarabine were stable in rat plasma under the indicated conditions.

3.3. Application to PK study in the Sprague–Dawley rats

This validated method was successfully applied to PK studies of cytarabine and valcytarabine following oral administration of cytarabine and valcytarabine to a Sprague–Dawley rat at 30 mg/kg (all calculated as cytarabine), respectively. The typical plasma concentration–time profiles of cytarabine and valcytarabine are shown in Fig. 3. Since valcytarabine was rapidly hydrolyzed into cytarabine by the esterase *in vivo*, its concentration in plasma was low. It is clear that oral administration of valcytarabine is able to enhance oral bioavailability of cytarabine.

4. Conclusions

A sensitive, specific and rapid HPLC–MS/MS method was developed for the simultaneous analysis of cytarabine and valcytarabine in rat plasma. It was very convenient to use a C_{18} column with 1.7 μm particle size for chromatographic separation under the reversed-phase conditions and it was more sensitive than the existing method, with the LLOQ being 10 ng/mL for cytarabine compared to the reported 50 ng/mL. The cation exchange solid-phase extraction procedure resulted in higher extraction recovery (>85%) for cytarabine and cleaner sample solutions for analysis. It has been successfully applied to the PK study of valcytarabine in the rat.

References

[1] M.G. Pallavicini, Pharmacol. Ther. 25 (1984) 207.
[2] M. Rustum, R.A. Raymakers, Pharmacol. Ther. 56 (1993) 307.
[3] R.L. Capizzi, J.C. White, B.L. Powell, F. Perrino, Semin. Hematol. 28 (1991) 54.
[4] K.R. Beutner, Antivir. Res. 28 (1995) 281.
[5] L.M. Beauchamp, G.F. Orr, P. De Miranda, T. Burnette, T.A. Krenitsky, Antivir. Chem. Chemother. 3 (1992) 157.
[6] M.D. Pescovitz, J. Rabkin, R.M. Merion, C.V. Paya, J. Pirsch, R.B. Freeman, J. O'Grady, C. Robinson, Z. To, K. Wren, L. Banken, W. Buhles, F. Brown, Antimicrob. Agents Chemother. 44 (2000) 2811.
[7] J.C. Scott-Moncrieff, T.C. Chan, M.L. Samuels, J.R. Cook, G.L. Coppoc, D.B. DeNicola, R.C. Richardsonl, Cancer Chemother. Pharmacol. 29 (1991) 13.
[8] C. Gomez, M.D. Blanco, M.V. Bernardo, R. Olmo, E. Muniz, J.M. Teijon, Eur. J. Pharm. Biopharm. 57 (2004) 225.
[9] Y.S. Hsieh, C.J. Duncan, M. Liu, J. Chromatogr. B 854 (2007) 8.
[10] Y.S. Hsieh, C.J. Duncan, J.M. Brisson, Rapid Commun. Mass Spectrom. 21 (2007) 629.
[11] Y.S. Hsieh, C.J. Duncan, Rapid Commun. Mass Spectrom. 21 (2007) 573.
[12] Y.S. Hsieh, F.B. Li, C.J. Duncan, Anal. Chem. 79 (2007) 3856.
[13] J.A. Sinkule, W.E. Evans, J. Chromatogr. 274 (1983) 87.
[14] G.F. Wang, Y.S. Hsieh, X.M. Cui, K.C. Cheng, W.A. Korfmacher, Rapid Commun. Mass Spectrom. 20 (2006) 2215.