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Development and validation of a liquid chromatography–tandem mass spectrometry method for quantification of decitabine in rat plasma

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ABSTRACT

Decitabine is chemically unstable at physiological temperature and pH. In addition, the bioanalysis of decitabine is easily interfered by endogenous 2-deoxycytidine. A simple, sensitive and specific LC–MS/MS method was developed for the analysis of decitabine in rat plasma. No exogenous stabilizers were used to prevent the degradation of decitabine in rat plasma. After deproteinized with acetonitrile at room temperature, rat plasma samples were analyzed on a Hypersil APS-2 NH_2 column interfaced with a triple quadrupole tandem mass spectrometer in positive electrospray ionization mode. Decitabine was completely separated from 2-deoxycytidine using gradient elution of water (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL/min. To quantify decitabine and daidzin (internal standard), respectively, multiple reaction monitoring (MRM) transitions of m/z 251.1 → 134.7 and m/z 417.3 → 255.3 was performed. The assay was linear over the concentration range of 5.0–2000 ng/mL. The intra- and inter-day precision was within 12.0% in terms of relative standard deviation (RSD%) and the accuracy within 5.9% in terms of relative error. The LC–MS/MS method was fully validated for its sensitivity, selectivity, stability study, matrix effect and recovery. The data indicate that this LC–MS/MS method is a specific and effective method for the pharmacokinetic study of decitabine in rat plasma. Compared with the previously reported analytical methods, this method showed easy and economic sample preparation, good specificity and high sensitivity with less plasma (50 μL).

1. Introduction

Decitabine (5-aza-2′-deoxycytidine, Fig. 1), a DAN methyltransferase inhibitor, possesses anti-cancer activities by inhibiting of DAN methylation, leading to DNA hypomethylation and resulting in gene re-expression and cellular differentiation [1–3]. It has been widely used in clinic for the treatment of myelodysplastic syndrome, acute myeloid leukemia and chronic myeloid leukemia [4]. In the last 10 years, it was proposed to combine DNA methyltransferase inhibitors with other agents for the treatment of patients with refractory solid tumor malignancies [4,5].

Decitabine is a highly polar compound and is unstable at physiological temperature and pH. Since decitabine is a deoxycytidine analog, endogenous 2-deoxycytidine (Fig. 1), a potential prognostic marker for breast cancer, interferes its bioanalysis [6]. Therefore, it is a challenge for analysts to determine decitabine in biological matrixes. A bioassay based on L1210 cell killers [7] and a high performance liquid chromatography-ultraviolet (HPLC-UV) method [8,9] have been developed to determine decitabine in human plasma, however, these methods suffered from low sensitivity and poor specificity. Patel et al. and Cashen et al. [10,11] developed sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods to determine decitabine in human plasma. They used an expensive inhibitor of cytidine deaminase, tetrahydrouridine (THU), to stabilize decitabine in plasma. Their methods cannot be applied for the determination of decitabine in biological matrixes with high level of 2-deoxycytidine, such as plasma obtained from breast cancer patients.

Rat plasma contains high amount of 2-deoxycytidine (about 700 ng/mL as detected in this study) and is a good model to investigate the bioanalysis of decitabine. To date, only Liu et al. [6] reported the quantitative method for decitabine in rat plasma. They obtained a lower limit of quantification (LLOQ) of 10 ng/mL using 100 μL rat plasma by a very complex solid phase extraction (SPE) process in a refrigerator at 4 °C to prevent degradation of decitabine. Analytical methods of more economic and simpler sample preparation, as well as exclusion interference from 2-deoxycytidine are still required for determination of decitabine in biological matrixes. In this study, we developed a sensitive and specific LC–MS/MS method to analyze decitabine concentration in rat plasma with simple and

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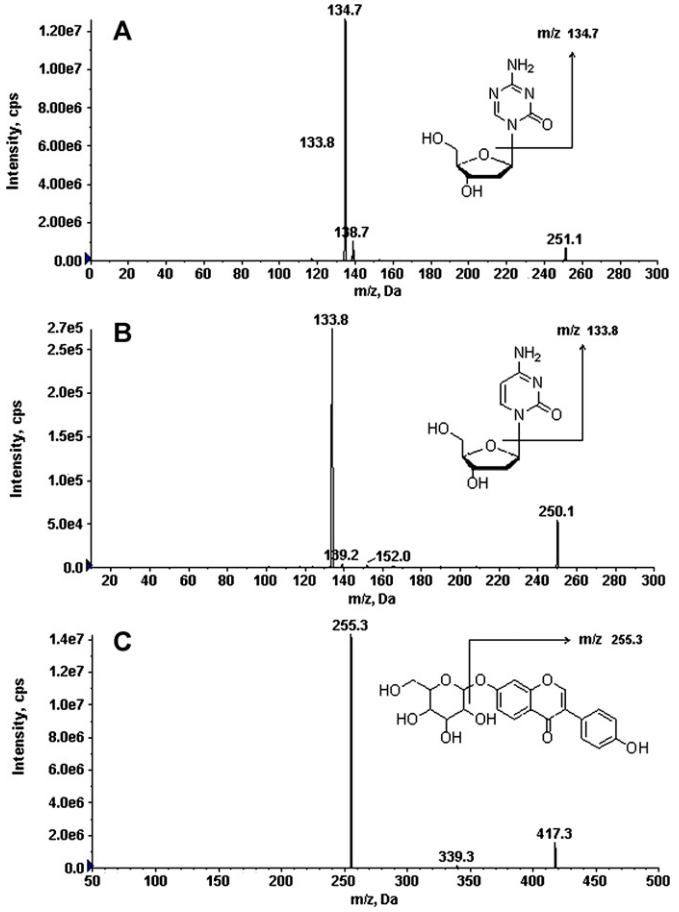

Fig. 1. Full scan MS/MS spectra for $[M+Na]^+$ of decitabine (A), 2-deoxycytidine (B) and for $[M+H]^+$ of daidzin (IS, C).

economic sample preparation. Without protection by stabilizers, decitabine was extracted from rat plasma by protein precipitation. Then it was separated with 2-deoxycytidine on a Hypersil APS-2 NH_2 column and detected by LC–MS/MS. The LLOQ of this method was 5 ng/mL using 50 μL rat plasma. This analytical method was proved to be accuracy and reliable and was successfully applied to the pharmacokinetic study of decitabine in Sprague–Dawley rats.

2. Experimental

2.1. Chemicals and reagents

Decitabine (>99% purity) and daidzin (>99% purity) were obtained from Chembest Bioscience Inc. (Shanghai, China) and Yuanye Bioscience Inc. (Shanghai, China), respectively. HPLC-grade acetonitrile was purchased from Yuwang industrial Co. Ltd. (Shandong, China). Distilled water, prepared from demineralized water, was used throughout the study. All other chemicals and solvents were of analytical grade and used without further purification.

2.2. Preparation of calibration standards and quality control (QC) samples

Stock solutions of decitabine and daidzin (internal standard, IS) were prepared to a concentration of 0.5 mg/mL using methanol. A series of working standard solutions of decitabine ranging from 12.5 to 5000 ng/mL and the IS solution at 4.0 μg/mL were prepared by diluting their stock with methanol. All solutions were stored at −20 °C until analysis. The plasma calibration standards of decitabine were prepared as follows: 20 μL of decitabine working solutions was spiked into 2-mL eppendorf centrifuge tubes and evaporated dryness by nitrogen, and then 50 μL of blank plasma was added to obtain the concentrations of 5.0, 10, 50, 200, 1000 and 2000 ng/mL. Quality control (QC) samples were prepared in a similar manner at 8.0, 160 and 1600 ng/mL.

2.3. Sample preparation

For sample preparation, 50 μL of each plasma sample, 20 μL of IS solution and 10 μL of 2 mmol/L ammonium acetate (NH_4AC) were mixed before spiking with 100 μL of acetonitrile for protein precipitation. The mixture was vortexed for 1 min and then frozen at −20 °C for 15 min. After centrifugation at 13,000 × *g* for 5 min at −5 °C, a 10 μL aliquot of the supernatant was injected for LC–MS/MS analysis.

2.4. LC–MS/MS conditions

The chromatographic separation was performed on a Hypersil APS-2 NH_2 column (250 mm × 4.6 mm, 5 μm, ThermoFisher, USA). Water (A) and acetonitrile (B) were used as mobile phase for elution. The gradient was controlled as follows: 0–5.0 min, 80–100% B, 5.0–8.0 min, 100% B, 8.0–9.0 min, 100–30% B, 9–10 min, 30% B, 10–11 min, 30–80% B, 11–16 min, 80% B. The flow rate was 1.0 mL/min. The outlet of the column was split and only 0.5 mL/min portion of the column effluent was carried into mass spectrometer.

An API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA, USA) with electrospray source (ESI) was operated in positive ion mode. The quantification was performed using MRM method with the transitions of m/z 251.1 → 134.7 for decitabine and m/z 417.3 → 255.3 for IS. The main working parameters were set as follows: ionspray voltage, 5.5 kV; ion source temperature, 500 °C; gas1, 50 psi; gas2, 60 psi; curtain gas, 20 psi. Analyte concentrations were determined using the software Analyst 1.5.

2.5. Method validation

A full validation according to the FDA guidelines for bioanalytical method validation [12] was performed for the assay of decitabine in rat plasma.

2.5.1. Specificity

The specificity of the method was evaluated by analyzing six different blank plasma samples to investigate the potential interferences at the LC retention times for the analyte and IS.

2.5.2. Calibration curve, accuracy, and precision

The linearity of the method was assessed by processing (in duplicate) a six-point calibration curve over the concentration range of 5.0–2000 ng/mL on 3 consecutive batches. Calibration curves were built by fitting the analyte concentrations of the calibrators versus the peak area ratios of the analyte to IS using least-squares non-linear regression analysis with a weighting factor of $1/x^2$. Lowest limit of quantification (LLOQ) was defined as the lowest plasma concentration in the calibration curve.

The precision and accuracy were evaluated by analyzing QCs at plasma concentrations of 5.0 (LLOQ), 8.0, 160 and 1600 ng/mL in six replicates on three separated days. The criteria for acceptability of the data included accuracy within ±15% relative error (%RE) of the nominal values and a precision of within 15% relative standard deviation (%RSD) except for LLOQ at which both precision and accuracy were within 20%.

2.5.3. Matrix effect and recovery

The matrix effect and extraction recovery for decitabine and IS were evaluated by assaying three groups of samples: neat standard solutions of decitabine and IS (group 1), blank plasma extracts from six different rats spiked with decitabine and IS after protein precipitation (group 2), and plasma extracts spiked with decitabine and IS before protein precipitation (group 3). Samples of each group were prepared at three decitabine levels of 8.0, 160 and 1600 ng/mL. The matrix effects were calculated as the ratio of peak area of an analyte spiked post-extraction (group 2) to its mean peak area from neat solution (group 1). The variability (RSD) of matrix effect at each concentration level should be less than 15% [13]. The recovery was calculated as the ratio of the peak area of an analyte spiked prior to extraction (group 3) to its mean peak area after extraction (group 2).

2.5.4. Stability

The stability of decitabine in rat plasma was assessed by analyzing triplicates of QCs at 8.0 and 1600 ng/mL, which were exposed to different temperatures and storage conditions. These QCs were analyzed after storage at room temperature for 2.0 h (bench-top), at −70 °C for 15 days and after three freeze (−70 °C)–thaw (room temperature) cycles. The stability of decitabine and IS in the injection solvent was determined periodically by re-injecting the processed QCs for up to 12 h (at 4 °C) after the initial injection. Samples were considered stable if assay values were within the acceptable limits of accuracy (±15% RE) and precision (15% RSD).

2.6. Pharmacokinetic study

The established method was applied to the determination of decitabine in plasma obtained from six rats following a single intravenous dose of decitabine. Male Sprague–Dawley rats (200 ± 10 g) were supplied by Laboratory Animal Center of Shenyang Pharmaceutical University (Shenyang, China). They were housed under standard conditions and had *ad libitum* access to water. All experimental procedures were performed in accordance with the guidelines of the Experimental Animal Care and Use Committee of Shenyang Pharmaceutical University (Shenyang, China). After fasted for 12 h, rats were given a dose of 1.0 mg/kg decitabine *via* the tail vein. Blood samples were collected into heparinized tubes before (0 h) and at 0.08, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 12 h after administration. Plasma was separated by centrifugation at 4000 × *g* for 5 min at −5 °C and stored at −70 °C until analysis.

The area under the plasma concentration–time curve (AUC) was calculated by the linear trapezoidal rule. The terminal elimination rate constant (k_e) was estimated by log-linear regression of concentrations observed during the terminal phase of elimination. The elimination half-life ($t_{1/2}$) was calculated as $0.693/k_e$.

3. Results and discussion

3.1. Optimization of the mass spectrometric condition

Decitabine was reported to be easily ionized in the positive ionization mode [6,10,11]. In the present study, decitabine formed predominantly adduct ion $[M+Na]^+$ at *m/z* 251.1 in Q1 full-scan mass spectra in positive ESI interface, while the protonated molecule ion $[M+H]^+$ at *m/z* 229.0 was less than 5% relative abundance of $[M+Na]^+$. The corresponding product ion mass spectrum of decitabine is depicted in Fig. 1, where $[M+Na]^+$ was selected as the precursor ion.

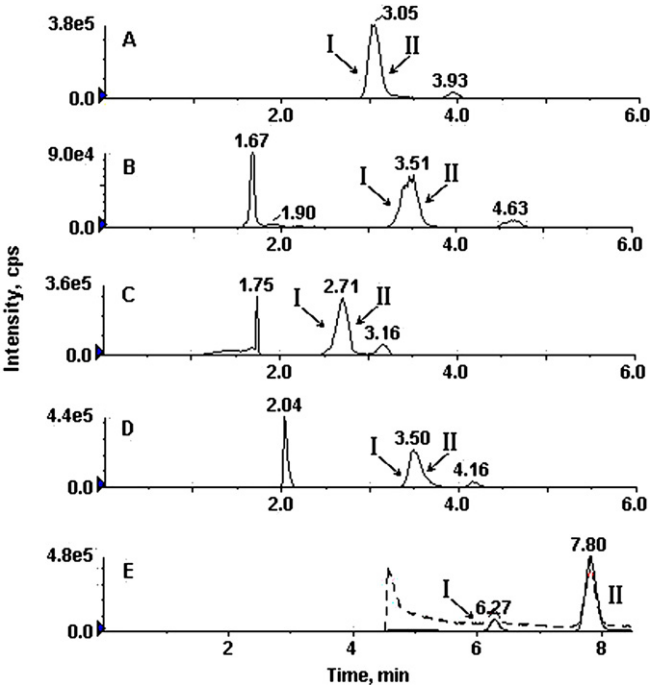

Fig. 2. Typical chromatograms of decitabine in rat plasma acquired at different columns to evaluate the separation between decitabine (I) and 2-deoxycytidine (II). Panel A: methanol–water (60:40, v/v) on a Diamonsil C18 column (200 mm × 4.6 mm, 5 μm, Dikma, China); panel B: methanol–water (10:90, v/v) on a Venusil ASB-C18 column (150 mm × 4.6 mm, 5 μm, Agela, USA); panel C: methanol–water (10:90, v/v) on a Hypersil BDS C18 column (150 mm × 4.6 mm, 5 μm, Elite, China); panel D: methanol–water (20:80, v/v) on a Hydro-RP C18 column (150 mm × 4.6 mm, 4 μm, Phenomenex, USA); panel E: acetonitrile–water (85:15, v/v) on a Hypersil APS-2 NH_2 column (250 mm × 4.6 mm, 5 μm, ThermoFisher, USA). Solid line: the first injection of a rat sample; dotted line: the repeated injection of the same sample as shown by the solid line.

3.2. Optimization of chromatographic condition and sample preparation

Decitabine cannot be distinguished from 2-deoxycytidine, the endogenous interference, by triple stage quadrupole mass spectrometry because they have the same precursor–product ion transition, although the molecular weight of 2-deoxycytidine is 1 Da less than that of decitabine (Fig. 1). Therefore, complete separation of decitabine with 2-deoxycytidine is necessary for the determination of decitabine in rat plasma using triple stage quadrupole mass spectrometry.

Decitabine is a strong hydrophilic compound. Several reversed-phase C18 columns which were suitable for analysis of high-polarity compounds were tested in the study, including Diamonsil C18 (Dikma, China), Hypersil BDS C18 (Elite, China), Hydro-RP C18 (Phenomenex, USA), and Venusil ASB-C18 (Agela, USA). Good peak shape and high signal response were observed for decitabine on all tested C18 columns. However, no matter how the mobile phase was comprised, it was hard to separate decitabine completely with 2-deoxycytidine on these columns (Fig. 2A–D).

Good separation between decitabine and 2-deoxycytidine was easily achieved on a Hypersil APS-2 NH_2 column (ThermoFisher, USA) using acetonitrile–water (85:15, v/v) as mobile phase at a flow rate of 1.0 mL/min. The retention times of decitabine and 2-deoxycytidine were 6.3 and 7.8 min, respectively (Fig. 2E). Nevertheless, the noise baseline obviously rose with the injection of samples. We speculated that the increasing noise was caused by matrix components accumulated in the column. Thus, in order to decrease the interference form matrix, 10 μL of 2 mmol/L NH_4AC were added into plasma samples (50 μL) and the supernatant was

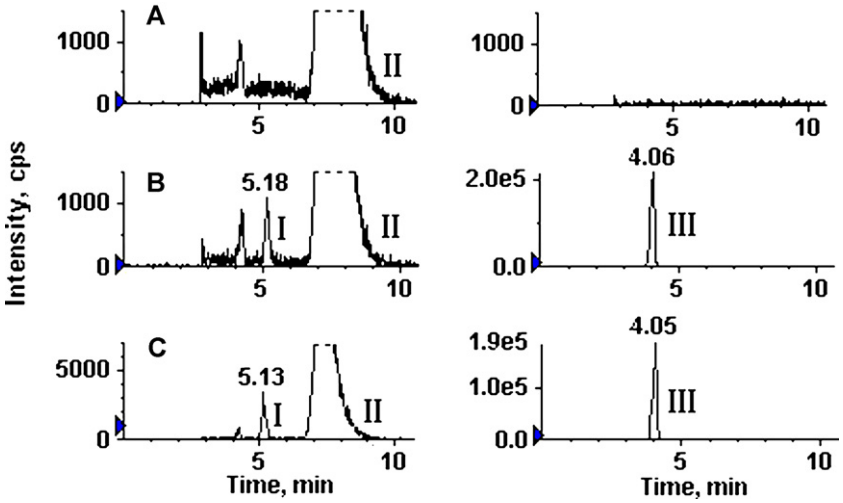

Fig. 3. Representative MRM chromatograms for decitabine (I), 2-deoxycytidine(II) and daidzin (IS, III) in rat plasma samples. Panel A: a blank plasma sample; panel B: a blank plasma sample spiked with decitabine at the LLOQ of 5.0 ng/mL; panel C: a rat plasma sample obtained at 12 h after a single intravenous administration of 1.0 mg/kg decitabine. The retention times for decitabine and IS were about 5.2 min and 4.1 min, respectively.

separated after the sample was frozen at −20 °C for 15 min. In addition, gradient elution with water (A) and acetonitrile (B) was performed to completely elute matrix components out of the column and consequently stable the noise. At last, the gradient was controlled as follows: 0–5.0 min, 80–100% B, 5.0–8.0 min, 100% B, 8.0–9.0 min, 100–30% B, 9–10 min, 30% B, 10–11 min, 30–80% B, 11–16 min, 80% B. Under this condition, decitabine was completely separated with 2-deoxycytidine and the noise was low and stable during sample analysis.

3.3. Method validation

3.3.1. Specificity

Fig. 3 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with decitabine at the LLOQ and a plasma sample obtained from a rat at 12 h after an intravenous administration of 1.0 mg/kg decitabine. No significant interferences from endogenous substances with decitabine or IS were detected.

3.3.2. Linearity and sensitivity

The plasma calibration curve was constructed using six calibration standards over the concentration range of 5.0–2000 ng/mL. A typical equation of the calibration curve on a validation batch was as follows: $y = 0.000894x + 0.000171$ ($r = 0.9994$), where y represents the peak-area ratio of decitabine to IS and x represents the plasma concentration of decitabine. Good linearity was obtained in this concentration range with a correlation coefficient (r) greater than 0.996. The LLOQ was confirmed to be 5.0 ng/mL for decitabine, at which the accuracy was in the range of 104.5–106.8% and the precision were below 17% (Table 1). The limit of detection (LOD) was 1.5 ng/mL with a signal to noise ratio of 3.

3.3.3. Precision and accuracy

Table 1 summarizes the results for intra- and inter-day precision and accuracy for decitabine measured by QCs. The intra- and inter-day precisions were all below 15% with a maximum RSD of 12.0%, and a maximum bias of 5.9% for accuracy was calculated.

3.3.4. Recovery

The recoveries of decitabine extracted from plasma were 84.7 ± 5.4, 79.9 ± 6.8 and 81.2 ± 7.3% at the concentrations of 8.0, 160 and 1600 ng/mL, respectively ($n = 6$). The recovery of IS was 102.8 ± 6.1% ($n = 18$).

3.3.5. Matrix effect

The matrix effects of decitabine from six different rat plasma samples at concentrations of 8.0, 160 and 1600 ng/mL were in the range of 97.8–109.3% with RSD values below 5.5%. The matrix effect of IS (1.6 μg/mL in plasma) was 92.0% and the RSD value was 4.3%.

3.3.6. Stability

The working solutions at the concentrations of 12.5, 20.0, 400 and 5000 ng/mL for decitabine in methanol and 4.0 μg/mL for IS

Table 1
Precision and accuracy of the LC/MS/MS method to determine decitabine in rat plasma (in 3 consecutive days, six replicates for each day).

	Theoretical concentration			
	LLOQ 5.0 ng/mL	Low 8.0 ng/mL	Medium 160 ng/mL	High 1600 ng/mL
Intra-day ($n = 6$)				
Mean	5.2	7.7	166	1592
SD	0.3	0.9	7	145
Precision (RSD%)	4.0	7.9	6.3	8.0
Accuracy (%)	104.5	96.3	103.6	99.5
Inter-day ($n = 18$)				
Mean	5.3	7.7	161	1506
SD	0.4	0.7	10	139
Precision (RSD%)	16.7	2.7	7.2	12.0
Accuracy (%)	106.8	95.7	100.3	94.1

Table 2
Stability of decitabine under various storage in rat plasma ($n = 3$).

Storage condition	Concentration (ng/mL)		RSD (%)	Accuracy (%)
	Added	Measured		
Room temperature for 2 h	8.0	7.6 ± 1.1	14.4	95.5 ± 13.8
(bench-top)	1600	1380 ± 90	6.5	86.3 ± 5.6
Three freeze–thaw cycles at −70 °C				
1	8.0	7.9 ± 1.1	14.5	99.0 ± 14.3
	1600	1517 ± 129	8.5	94.8 ± 8.0
3	8.0	7.4 ± 0.4	5.6	92.0 ± 5.1
	1600	1700 ± 50	2.9	106.2 ± 3.1
Frozen (−70 °C) for 15 days	8.0	7.1 ± 0.4	5.3	88.6 ± 4.8
	1600	1707 ± 140	8.2	106.7 ± 8.8
Post-pretreatment at 4 °C for	8.0	8.9 ± 0.3	2.9	111.4 ± 3.2
12 h	1600	1793 ± 129	7.2	112.1 ± 8.1

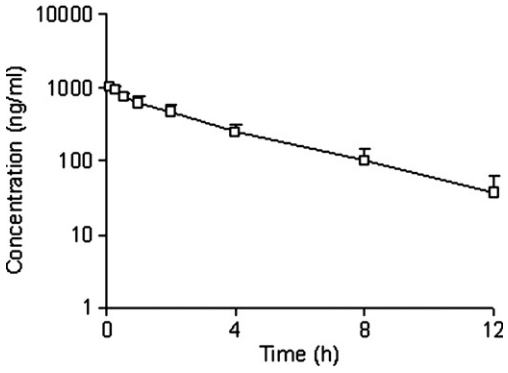

Fig. 4. Mean plasma concentration–time curve of decitabine in male Sprague–Dawley rats after a single intravenous administration of 1.0 mg/kg decitabine ($n = 6$).

in methanol were stable for at least 7 days at −20 °C. The plasma samples at two QC concentrations were found stable after storage at room temperature for 2.0 h (bench-top), at −70 °C for 15 days, after three freeze–thaw cycles at −70 °C. Decitabine in the injection solvent was also stable at 4 °C for 12 h. The data are summarized in Table 2.

3.4. Pharmacokinetic study

The validated LC–MS/MS method was successfully applied to a pharmacokinetic study of decitabine in male Sprague–Dawley rats following a single intravenous administration of 1.0 mg/kg decitabine. The mean plasma concentration–time curve of decitabine is shown in Fig. 4. The elimination half-life ($t_{1/2}$), clearance (CL) and apparent volume of distribution (V_z) were 3.6 ± 0.2 h, 0.31 ± 0.05 mL h^{-1} kg^{-1} and 1.64 ± 0.34 mL kg^{-1}, respectively. The results were similar to the previous data [6].

4. Conclusion

A simple, sensitive and specific LC–MS/MS method has been developed and validated for the analysis of decitabine in rat plasma. Compared with the previously reported analytical methods, this method showed easy and economic sample preparation, good specificity and high sensitivity with an LLOQ of 5.0 ng/mL using less rat plasma (50 μL). This method was successfully applied to characterize the pharmacokinetics of decitabine in rats.

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References

[1] C.M. Bender, M.M. Pao, P.A. Jones, Cancer Res. 58 (1998) 95.
[2] M. Daskalakis, T.T. Nguyen, C. Nguyen, P. Guldberg, G. Köhler, P. Wijermans, P.A. Jones, M. Lübbert, Blood 100 (2002) 2957.
[3] A. Pinto, V. Zagonel, V. Attadia, P.L. Bullian, V. Gattei, A. Carbone, S. Monfardini, A. Colombatti, Bone Marrow Transplant. 4 (1989) S28.
[4] M. Daskalakis, N. Blagitko-Dorfs, B. Hackanson, Recent Results Cancer Res. 184 (2010) 131.
[5] J. Gilbert, S.D. Gore, J.G. Herman, M.A. Carducci, Clin. Cancer Res. 10 (2004) 4589.
[6] Z. Liu, G. Marcucci, J.C. Byrd, M. Grever, J. Xiao, K.K. Chan, Rapid Commun. Mass Spectrom. 20 (2006) 1117.
[7] G.G. Chabot, G.E. Rivard, R.L. Momparler, Cancer Res. 43 (1983) 592.
[8] K.T. Lin, R.L. Momparler, G.E. Rivard, Ther. Drug Monit. 5 (1983) 491.
[9] K.T. Lin, R.L. Momparler, G.E. Rivard, J. Chromatogr. 345 (1985) 162.
[10] K. Patel, S.M. Guichard, D.I. Jodrell, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 863 (2008) 19.
[11] A.F. Cashen, A.K. Shah, L. Todt, N. Fisher, J. DiPersio, Cancer Chemother. Pharmacol. 61 (2008) 759.
[12] Guidance for Industry Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), May 2001. http://www.fda.gov/cder/guidance/4252fnl.htm.
[13] S. Bansal, A. DeStefano, AAPS J. 9 (2007) E109.