Journal of Chromatography B, 878 (2010) 2260–2265

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Short communication

Determination of mitoxantrone in rat plasma by liquid chromatography–tandem mass spectrometry method: Application to a pharmacokinetic study

Peng Zhang[a], Guixia Ling[a], Jin Sun[a,*], Yongbing Sun[b], Xiaohui Pu[a], Zhiyuan Wang[a], Zhonggui He[a,*]

[a] School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China
[b] National Engineering Research Center for Solid Preparation of Chinese Herbal Medicine, Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, China

ARTICLE INFO

Article history:
Received 10 March 2010
Accepted 16 June 2010
Available online 25 June 2010

Keywords:
HPLC–MS/MS
Mitoxantrone
Plasma concentration

ABSTRACT

A rapid, sensitive and specific high performance liquid chromatography–tandem mass spectrometric (HPLC–MS/MS) method has been developed for quantification of mitoxantrone in rat plasma. The analyte and palmatine (internal standard) were extracted from plasma samples with diethyl ether–dichloromethane (3:2, v/v) and separated on a C_{18} column. The chromatographic separation was achieved within 2.5 min using methanol–10 mM ammonium acetate containing 0.1% acetic acid as the mobile phase at a flow rate of 0.2 mL/min. The method was linear over the range of 0.5–500 ng/mL. The lower limit of quantification (LLOQ) was 0.5 ng/mL. Finally, the method was successfully applied to a pharmacokinetic study of mitoxantrone in rats following intravenous administration.

1. Introduction

Mitoxantrone, a synthetic anthracenedione anti-tumor drug, has been extensively used for the treatment of advanced breast and prostate cancer, lymphoma and leukaemia [1–4]. Since mitoxantrone is present in low concentrations in body fluids, a sensitive method that allows accurate quantification of low concentrations of mitoxantrone in biological samples is of great value in studying the pharmacokinetic behavior of mitoxantrone.

Previously published methods [5–16] for quantification of mitoxantrone in plasma employed high performance liquid chromatography equipped with ultraviolet detection (HPLC–UV). These methods had several disadvantages, for example, a long analytical time [5–16], large volumes of plasma were required [5–14], expensive extraction procedures were used (SPE [9–11] as well as online column switching [5–7]) and low sensitivity [5–16].

We have attempted to develop a novel, rapid, selective and highly sensitive method to determine mitoxantrone in rat plasma using HPLC–MS/MS. The validation results showed a higher sensitivity (an LLOQ as low as 0.5 ng/mL), a shorter analytical time (2.5 min per sample), used a lower plasma volume (50 μL) compared with the previous methods [5–16]. This method was then successfully applied to a preclinical pharmacokinetic study in rats after intravenous administration. It allows plasma drug monitoring for at least 8 h after intravenous administration in rats.

2. Experimental

2.1. Materials

Reference standard mitoxantrone dihydrochloride (99.4% purity) was provided by Beijing Xinze Tech. Co., Ltd. (Beijing, China) and palmatine (internal standard, I.S., 99.9% purity) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Methanol of HPLC-grade was purchased from Fisher Scientific (Pittsburgh, PA, USA). Water (>18 MΩ) was obtained using an EASYPURE® II RF/UV ultra pure water system (Barnstead International Corp., USA). Diethyl ether and dichloromethane of analytical grade were obtained from Concord Tech. Co. (Tianjin, China). Acetic acid and ammonium acetate (HPLC) were purchased from Dikma (Richmond Hill, NY, USA) and high-purity nitrogen (99.999%) was used.

2.2. LC–MS/MS conditions

Analyses were acquired on an ACQUITY UPLC™ system (Waters Corp., Milford, MA, USA) with a cooling auto-sampler and column oven allowing accurate temperature control of the analytical column. An ACQUITY UPLC™ BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm; Waters Corp., Milford, MA, USA) was used. The chromatographic separations were accomplished using gradient elution with a mobile phase composed of solvent B (methanol) and solvent A (10 mM ammonium acetate containing 0.1% acetic acid). The gradient conditions of the mobile phase were: 0 min 30% B, 0.3 min 30% B, 0.8 min 70% B, 1.25 min 70% B and 1.8 min 30% B. The column temperature was maintained at 40 °C with the flow rate set

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

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.06.016

at 0.2 mL/min. The auto-sampler temperature was conditioned at 10 °C. The injection volume was 5 μL using the partial loop mode for sample injection. The chromatographic run time per sample was 2.5 min. From 1.2 to 2.2 min, the eluent was injected into the detector, and the remainder was diverted to waste.

The separated compounds were detected by a Waters Tandem Quadrupole (TQ) Detector (Waters). The mass spectrometer was operated with an electrospray ionization (ESI) interface in positive ionization mode for mitoxantrone and I.S. The ionization source conditions were: capillary voltage 3.2 kV, cone voltage 40 V, source temperature 120 °C and desolvation temperature 350 °C. The optimized collision energy was 26 and 30 V for mitoxantrone and I.S., respectively. The cone and desolvation gas flow rates were 50 and 450 L/h, respectively, and were obtained from an in-house nitrogen source. Argon was used as collision gas at a pressure of approximately 3.62×10^{-3} mbar and the multipliers were set to 650 V. Under these HPLC–MS/MS conditions, the compounds were analyzed by multiple reaction monitoring (MRM) of the transitions of m/z 445 → 88 for mitoxantrone and m/z 352 → 336 for I.S., respectively. The scan time was set at 0.02 s per transition. Data were acquired using Masslynx 4.1 software.

2.3. Preparation of standards and quality control samples

Stock solutions of mitoxantrone and I.S. were prepared at 100 μg/mL in a saline/ascorbate solution ("diluent", 8.0 g/L sodium chloride and 10.0 g/L ascorbic acid) and stored at −80 °C. Because mitoxantrone was known to adhere to glass [9], all solutions were prepared and stored in polypropylene tubes.

Calibration standards were prepared by spiking 50 μL drug-free rat plasma with 10 μL of appropriate standard solutions. The effective concentrations in plasma samples were 0.5, 1, 2, 10, 50, 250 and 500 ng/mL. The quality control (QC) samples were prepared at concentrations of 1.0, 10 and 400 ng/mL in a similar way to the calibration standards. These calibration standard samples and QC samples were stored at −80 °C. Before processing each analytical batch, the appropriate standards and QCs were brought to room temperature and processed together with the biological samples.

2.4. Sample preparation

After spiking 10 μL saline/ascorbate solution (8.0 g/L sodium chloride and 10.0 g/L ascorbic acid), 10 μL I.S. solution (in saline/ascorbate solution, 500 ng/mL) and 50 μL 0.05 M borax-sodium carbonate buffer (pH 10.8), into 50 μL of plasma samples, the mixed samples were extracted with 3 mL diethyl ether–dichloromethane (3:2, v/v). The mixture was vortexed for approximate 1 min, then centrifuged at 13,000 × *g* for 10 min. The upper organic layer was removed and evaporated to dryness at 45 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL mobile phase and vortexed for 1 min. A 5 μL aliquot of the solution was then injected into the LC–MS/MS system.

2.5. Method validation

The method was validated for selectivity, matrix effect (M.E.), linearity, LLOQ, accuracy, precision, extraction recovery and stability. The different validation parameters and the values for acceptance of the range of validation parameters were in accordance with international guidelines [17] and US Food and Drug Administration guidelines (www.fda.gov/cvm).

Selectivity was assessed by comparing chromatograms of six different batches of blank plasma obtained from six rats with those of corresponding standard plasma samples spiked with mitoxantrone and I.S. and a plasma sample obtained after intravenous administration.

To evaluate the matrix effect, mitoxantrone at three concentration levels (1.0, 10 and 400 ng/mL) was added to the extract of 50 μL blank plasma from six different lots, then dried and reconstituted with 100 μL mobile phase. The corresponding peak areas (A) were compared with those of the mitoxantrone standard solutions dried directly and reconstituted with the same mobile phase (B). The ratio $(A/B \times 100)\%$ was used to evaluate the matrix effect. The same procedure was performed for the I.S.

Quantification of the plasma samples was based on the ratio of the detector response of mitoxantrone to that of the internal standard. Peak area ratios (Y) were plotted against mitoxantrone concentration (X) and standard curves in the form of $Y = A + BX$ were calculated using weighted ($1/x^2$) least squares linear regression. During routine analysis, each analytical run included a set of calibration standards, a set of QC plasma samples in duplicate at intervals per batch and the plasma samples to be analyzed.

Calibration curves were prepared using seven standard plasma samples over the range 0.5–500 ng/mL. 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, and the results should be less than 20%.

The precision and accuracy of the method were assessed using QC samples at three concentration levels (1.0, 10 and 400 ng/mL) performed on three separate days. On each day, six replicates of QC samples at each concentration level were analyzed. The accuracy was expressed by the relative error (R.E.) and the precision by the relative standard deviation (R.S.D).

The extraction recovery of mitoxantrone was assessed by comparing the mean peak area of the regularly prepared samples at three concentrations (1.0, 10 and 400 ng/mL) with the mean peak area of spike-after-extraction plasma samples. To prepare the spike-after-extraction samples, blank rat plasma was processed according to the sample preparation procedure described above. The supernatant was mixed with the appropriate standard solutions of mitoxantrone at concentrations corresponding to the final concentration of the pretreated plasma samples. The extraction recovery of I.S. was determined in a similar way using medium level of QC as a reference.

The stability of processing (three freeze–thaw cycles), sample storage (at room temperature for 4 h, at −80 °C for 21 days) and post-treatment (in the reconstituted extract at 10 °C for 24 h) was assessed by analyzing replicates ($n = 3$) of QC samples (at the concentration of 1.0 and 400 ng/mL). The results were compared with those of QC samples freshly prepared, and the percentage of concentration deviation was calculated.

2.6. Pharmacokinetic study

The developed method was used to determine the plasma concentrations of mitoxantrone in a preclinical study in six healthy adult male Wistar rats (Laboratory Animal Center of Shenyang Pharmaceutical University, Shenyang, Liaoning, China) weighing 220 ± 20 g (mean ± standard deviation). Before the day of administration via the tail vein, the rats were fasted for 24 h but allowed water *ad libitum*. The animal studies were approved by the Shenyang Pharmaceutical University Animal Care and Use Committee.

The injection was prepared with 0.9% NaCl to give a solution with a final concentration of 0.5 mg/mL. The rats received a single intravenous injection of 1 mg/kg, then, 250 μL blood samples were collected into heparinized cryotubes by puncture of the retro-orbital sinus according to the following time schedule: 0.083, 0.17, 0.33, 0.5, 1, 2, 4, 6 and 8 h post-dosing. The blood samples were centrifuged immediately at 13,000 × *g* for 10 min to obtain plasma which was transferred to cryotubes containing 5 μL 20% (w/v) ascorbic acid in 0.9% saline, to prevent oxidation of mitoxantrone.

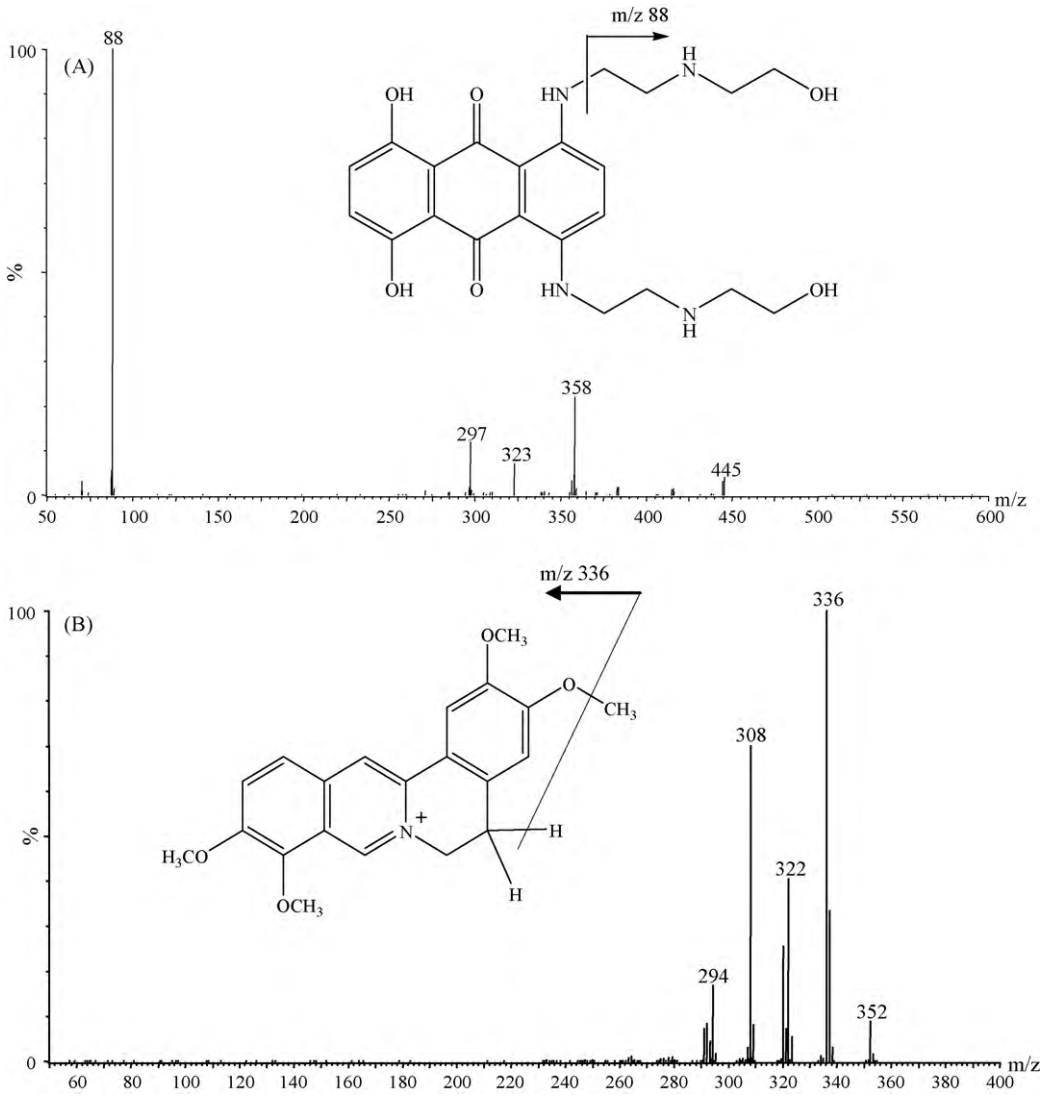

Fig. 1. Product ion scan spectra of mitoxantrone (A) and palmatine (B).

The plasma samples were labeled and kept frozen at −80 °C until analysis.

3. Results and discussion

3.1. Method development

In this work, LC–MS/MS operation parameters were carefully optimized for the determination of mitoxantrone and palmatine. A standard solution of mitoxantrone and palmatine was directly infused along with the mobile phase into the mass spectrometer. It was found that mitoxantrone and palmatine produce good mass spectrometric responses in positive electrospray ionization (ESI) mode. In the precursor ion full-scan spectra, the most abundant ions were protonated molecules $[M+H]^+$ m/z 445 for mitoxantrone and m/z 352 for palmatine. Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain highest intensity of protonated molecules. The product ion scan spectra showed high abundance fragment ions at m/z 88 and 336 for mitoxantrone and palmatine, respectively. The product-ion spectra of these compounds are shown in Fig. 1. The collision gas pressure and collision energy (CE) of collision induced decomposition (CID) were optimized for maximum response of the fragment of m/z 445 for mitoxantrone and m/z 352 for palmatine. The ion transitions of m/z 445 → 88 for mitoxantrone and m/z 352 → 336 for palmatine were chosen for MRM.

During the course of method development, we found that the use of methanol as the plasma protein precipitating reagent resulted in a relatively higher recovery (>90%), but at the cost of the presence of more interfering components which made the precision of the method unacceptable. So, plasma samples were subjected to a liquid–liquid extraction procedure which offers much cleaner samples making the method more robust and scalable.

Methanol produced a higher mass spectrometric response and lower background noise than acetonitrile and was chosen as the organic phase. Isocratic elution with mobile phase systems of methanol–buffer (10 mM ammonium acetate containing 0.1% acetic acid) in various proportions was tested. In order to avoid the influence of co-eluting components on analyte ionization, the proportion of methanol should be no more than 45%, which resulted in a longer analytical time (more than 8 min). To eliminate the matrix effect and to obtain a high sensitivity and sample throughput, gradient elution was finally employed in the study.

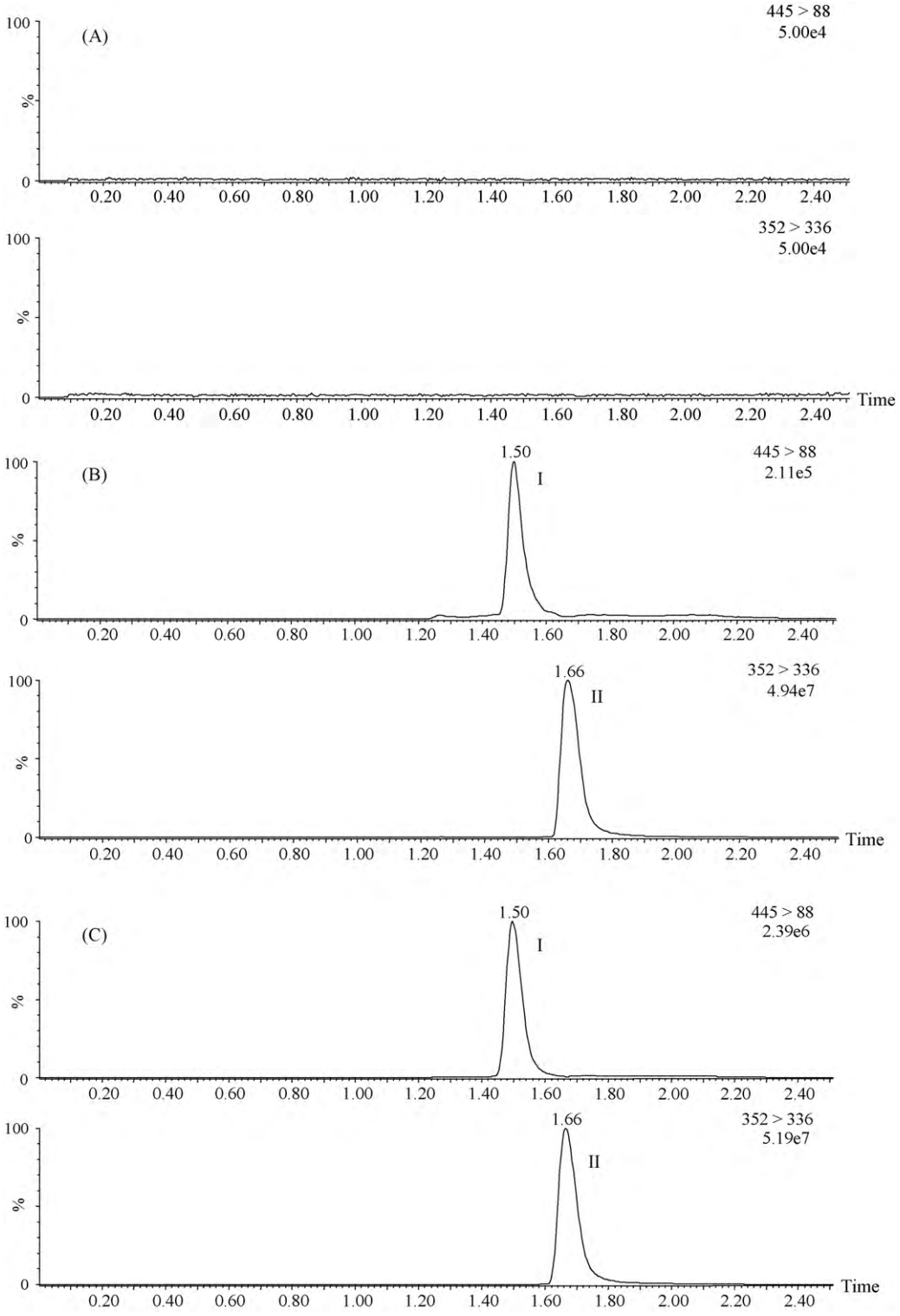

Fig. 2. Representative MRM chromatograms of mitoxantrone (peak I) and palmatine (peak II) in rat plasma samples. (A) A blank plasma sample; (B) a blank plasma sample spiked with mitoxantrone at an LLOQ of 0.5 ng/mL and I.S.; (C) plasma sample from a rat, 6 h after intravenous administration of mitoxantrone. The retention time for mitoxantrone and palmatine was 1.50 and 1.66 min, respectively.

The mobile phase used in the reported methods [13,14] contains high concentrations of expensive ion-pairing reagents (such as hexane sulfonic acid and 1-pentane sulfonic acid). The use of ion-pairing reagents makes the preparation of mobile phase more complicated and often leads to poor reproducibility, resulting in a longer time for column start-up, system equilibration and clean-up procedures. Compared with these methods, the method described here provides a simple, fast and economic alternative.

In the analysis of biological samples, the MS system was easily contaminated. In order to avoid contamination of the MS system by earlier eluted endogenous components of the sample matrix, a switch technique was developed. The first 1.2 min of the eluate was diverted away from the MS detector, and the eluate from 1.2

Table 1
Matrix effect (M.E.) for the LC–MS/MS method to determine mitoxantrone in rats plasma ($n = 6$).

Analytes	Con. (ng/mL)	A/Bmean ± S.D.	R.S.D. (%)
Mitoxantrone	1.0	96.5 ± 9.5	9.8
	10.0	106.2 ± 6.5	6.1
	400.0	102.5 ± 7.1	6.9
I.S.	100.0	105.8 ± 6.2	5.9

Table 2
Precision and accuracy of the LC–MS/MS method to determine mitoxantrone in rats plasma ($n = 3$ days, six replicates per day).

Added con. (ng/mL)	Found con. (ng/mL)	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	R.E. (%)
1.0	0.958 ± 0.095	10.0	9.6	−4.2
10.0	10.57 ± 0.54	5.2	4.2	5.7
400.0	404.2 ± 34.9	8.7	8.1	1.1

to 2.2 min was allowed to enter the MS system and be recorded. Under the chosen chromatographic conditions, both mitoxantrone and I.S. were rapidly eluted and the total run time was just 2.5 min per sample.

3.2. Method validation

3.2.1. Selectivity

Fig. 2 shows that there is no interference from endogenous substances observed at the retention time of the analytes.

3.2.2. Matrix effect

Regarding the matrix effect, all the ratios $(A/B \times 100)\%$ defined as in Section 2 were between 96.5 and 106.2% (Table 1), which means that there is no matrix effect for mitoxantrone and I.S. in this method.

3.2.3. Linearity and LLOQ

The linear regression of the peak area ratios versus concentrations was fitted over the concentration range 0.5–500 ng/mL in rat plasma. A typical equation for the calibration curve was as follows: $Y = 0.0018 + 0.7645\ X$ ($r = 0.9955$), where Y is the peak area ratio of mitoxantrone to palmatine, and X is the concentration of mitoxantrone. Good linearity ($r > 0.9952$) was seen over this concentration range in all analytical runs.

The present LC–MS/MS method provided an LLOQ of 0.5 ng/mL with an accuracy of 3.8% in terms of RE and a precision of 8.4% in terms of RSD ($n = 6$), which was more sensitive than the previously reported methods [5–16]. The concentration of mitoxantrone was capable of being determined in rat plasma samples up to 8 h after a single intravenous injection of 1 mg/kg with the validated method, which was sufficient to investigate the pharmacokinetic behavior of mitoxantrone.

3.2.4. Precision and accuracy

The intra- and inter-day precision and accuracy for mitoxantrone from QC samples are summarized in Table 2. The intra- and inter-day precisions were found to be below 10.0% and 9.6%, respectively. The inter-day accuracy ranged from −4.2 to 5.7%. The results, calculated using one-way ANOVA, were within the acceptable range [17] and the method was confirmed to be accurate and precise.

3.2.5. Extraction recovery and stability

The extraction recoveries of mitoxantrone at concentrations of 1.0, 10 and 400 ng/mL ($n = 6$) were found to be 70.0 ± 4.1%, 71.9 ± 3.7%, and 74.4 ± 7.6%, respectively. The extraction recovery of the I.S. was 77.2 ± 1.6%.

In the stability experiment, the relative errors of all samples were within 7.9%, which indicated that mitoxantrone exhibited no significant degradation under the conditions previously described.

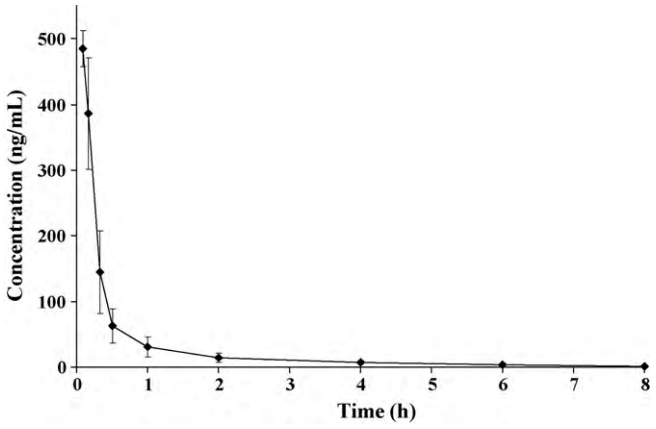

Fig. 3. Mean plasma concentration–time curve of mitoxantrone after a single intravenous dose of 1 mg/kg to six rats (each point represents mean ± S.D.).

3.3. Application

This validated analytical method has been successfully applied to determine the plasma concentrations of mitoxantrone to support preclinical pharmacokinetic studies in rats following intravenous administration. Serial blood samples were collected at predetermined times up to 8 h after intravenous administration. The profile of the mean plasma concentration of mitoxantrone versus time is shown in Fig. 3.

4. Conclusion

We have developed and validated an LC–MS/MS method for the determination of mitoxantrone in rat plasma. The method is rapid, sensitive and highly selective with an LLOQ of 0.5 ng/mL using only 50 μL rat plasma. It takes only 2.5 min to analyze one plasma sample and more than 280 samples can be assayed daily, including sample preparation, data acquisition and processing. It was shown to be superior in sensitivity, selectivity and speed of analysis to the previously reported methods. The validated method was successfully applied to preclinical pharmacokinetic studies of mitoxantrone in rats following intravenous administration.

Acknowledgement

We are grateful for financial support from the National Basic Research Program of China (973 Program), No. 2009CB930300.

References

[1] J.C. Doughty, E. Kane, T.G. Cooke, C.S. McArdle, Breast 11 (2002) 97.
[2] A. Khalaf, C. Pfister, M.F. Hellot, F. Dunet, J. Moussu, P. Grise, Prog. Urol. 12 (2002) 37.
[3] F. Hagemeister, F. Cabanillas, M. Coleman, S.A. Gregory, P.L. Zinzani, Oncologist 10 (2005) 150.
[4] A.V. Nowoselac, S. Reddy, J. Sanmugarajah, Leukemia 18 (2004) 1561.
[5] J. Catalin, A.F. Peloux, F. Coloma, B. Payet, B. Lacarelle, J.P. Cano, Biomed. Chromatogr. 8 (1994) 37.
[6] M.J. Czejka, A. Georgopoulos, J. Chromatogr. 425 (1988) 429.
[7] B. Payet, P. Arnoux, J. Catalin, J.P. Cano, J. Chromatogr. 424 (1988) 337.
[8] L. Slørdal, A. Andersen, D.J. Warren, Ther. Drug Monit. 15 (1993) 328.
[9] M.J. Priston, G.J. Sewell, J. Pharm. Biomed. Anal. 12 (1994) 1153.
[10] C.P. Luftensteiner, I. Schwendenwein, B. Paul, H.G. Eichler, H. Viernstein, J. Control. Release 57 (1999) 35.

[11] K.T. Lin, G.E. Rivard, J. LeClerc, J. Chromatogr. 465 (1989) 75.
[12] K.M. Rentsch, D.H. Horber, R.A. Schwendener, H. Wunderli-Allenspach, E. Hänseler, Br. J. Cancer 75 (1997) 986.
[13] K.M. Rentsch, R.A. Schwendener, E. Hänseler, J. Chromatogr. B 679 (1996) 185.
[14] N.J. Lacayo, B.L. Lum, D.L. Becton, H. Weinstein, Y. Ravindranath, M.N. Chang, L. Bomgaars, S.J. Lauer, B.I. Sikic, G.V. Dahl, Leukemia 16 (2002) 920.
[15] J.L. Johnson, A. Ahmad, S. Khan, Y.F. Wang, A.W. Abu-Qare, J.E. Ayoub, A. Zhang, I. Ahmad, J. Chromatogr. B 799 (2004) 149.
[16] G. An, M.E. Morris, J. Pharm. Biomed. Anal. 51 (2010) 750.
[17] V.P. Shah, K.K. Midha, J.W. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.