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Short communication

HPLC analysis of mitoxantrone in mouse plasma and tissues: Application in a pharmacokinetic study

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ABSTRACT

A simple, fast and economical HPLC assay for the determination of mitoxantrone in mouse plasma and tissue homogenates is described. Protein precipitation with sequential addition of sulfosalicylic acid and acetonitrile was used for sample preparation. The resolution of mitoxantrone and the I.S. were achieved by using acetonitrile and 10 mM sodium phosphate buffer with 0.1% TEA. The separation was performed on a Nucleosil C18, 250 mm × 4 mm I.D. column with UV detection at 610 nm. The inter-day and intra-day precision and accuracy of quality control (QC) samples, evaluated both in plasma and tissue homogenates, were all within 15%. The lower limit of quantification (LLOQ) was 5 ng/ml in plasma, 25 ng/ml in liver homogenate and 12.5 ng/ml in other tissue homogenates. This assay was successfully applied in a pharmacokinetic and tissue distribution study of mitoxantrone in mice.

1. Introduction

Mitoxantrone (MX), a synthetic anthracenedione derivative, is a clinically well-established anticancer agent which shows high efficacy in breast cancer, acute leukemia and non-Hodgkin's lymphoma. Several HPLC methods have been developed for the quantification of MX in biological fluids and the sample pretreatment procedures vary among different methods. Liquid–liquid extraction and solid-phase extraction, both of which are labor intensive and time consuming, are the most common extraction procedures [1–3]. The mobile phase used in most cases contains high concentrations of an ion-pairing reagent (such as hexane sulfonic acid and 1-pentane sulfonic acid) [4,5]. Using ion-pairing reagents adds complexity to the mobile phase preparation and often leads to poor reproducibility of sample analyses. The incorporation of an ion-pairing reagent in the mobile phase results in longer times for column start-up, system equilibration and clean-up procedures. In addition, the ion-pairing reagents are usually expensive. Here we report an improved HPLC assay which provides a simple, fast and economic alternative to published assays. The chromatographic conditions used in our method are based on a previous assay [6], but without the addition of ion-pairing reagents in the mobile phase. MX and its internal standard (I.S.) were well resolved by using acetonitrile and 10 mM sodium phosphate buffer. The extraction procedure proposed in our method is a simple protein precipitation procedure, which is modified from that of Slordal et al. [7]. Slordal et al. precipitated protein in human plasma using sulfosalicylic acid, and reported a recovery of 71.9%, but no accuracy and precision measurements were reported [7]. The mean recovery of MX using our assay is 92.3% in plasma, with the sequential addition of sulfosalicylic acid and acetonitrile for protein precipitation. Moreover, we validated this assay in various tissues (brain, lung, heart, liver, spleen and kidney), as well as plasma. Our results demonstrate the applicability of our assay in the evaluation of MX pharmacokinetics and tissue distribution following the i.v. administration of 5 mg/kg MX in mice.

2. Materials and methods

2.1. Chemicals

MX, ascorbic acid, sulfosalicylic acid and triethylamine were purchased from Sigma (St. Louis, MO). Ametantrone, the internal standard, was a generous gift from National Cancer Institute (Bethesda, MD, USA). HPLC-grade acetonitrile was obtained from J.T. Baker (Phillipsburg, NJ). All the other reagents or solvents used were commercially available and of reagent grade.

Abbreviations: I.S., internal standard; QC, quality control; TEA, triethylamine; LLOQ, lower limit of quantification.

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2.2. HPLC instrumentation and chromatographic conditions

The HPLC analysis was carried out on a system consisting of a Waters 1525 pump, 717 plus autosampler, a 2847 UV detector, and a Waters Breeze workstation. Chromatographic separation was achieved using a Nucleosil C18, 250 mm × 4 mm I.D. column (Macherey-Nagel, Easton, PA) with a guard column (Macherey-Nagel CC Nucleosil C18, 8 mm × 4 mm). A precolumn filter with a 2 μm PEEK frit (Upchurch, Oak Harbor, WA) was installed ahead of the guard column. The isocratic mobile phase consisted of 19:81 (v/v) acetonitrile:10 mM sodium phosphate (pH 2.3). Triethylamine (0.1%) was added to the mobile phase to prevent peak tailing. The I.S. and MX were detected at 610 nm.

2.3. Sample preparation

One hundred μl of plasma standards, quality control standards (QCs), or samples were spiked with 250 ng/ml I.S. (ametantrone). Twenty-five μl of sulfosalicylic acid and 75 μl of acetonitrile were then added sequentially to precipitate protein. Two hundred μL of tissue standards, QCs or samples were also spiked with 250 ng/ml I.S. and extracted by the sequential addition of sulfosalicylic acid (50 μL) and acetonitrile (150 μL). After vigorous vortexing, the samples were centrifuged for 15 min with 14,000 rpm at 4 °C. The supernatant was removed and a volume of 100 μL was used for HPLC analysis.

2.4. Standard and quality control solutions

Plasma calibration standards were prepared at concentrations of 5, 10, 50, 100, 500 and 1000 ng/ml, along with quality control samples at concentrations of 5, 25, 250 and 750 ng/ml. Mouse tissues (brain, heart, lung, liver, spleen and kidney) were homogenized using a Tissue Tearor Homogenizer (Biospec Products, Bartlesville, OK). The homogenizing buffer used in this experiment was 0.1 M citric buffer containing 100 mg/ml ascorbic acid at pH 3.0. Tissue homogenates were prepared with concentrations of 12.5, 50, 100, 500, 1000 and 2500 ng/ml. Quality control samples for tissue homogenates were prepared at concentrations of 25, 250 and 1500 ng/ml.

2.5. Recovery, precision and accuracy

MX recovery experiments were performed by comparing the peak area of added MX in plasma or tissue homogenates with the same amount of MX added to the mobile phase. Three concentrations (12.5, 25 and 250 ng/ml) were evaluated in recovery experiments. Extraction recovery of the I.S. was also determined at a concentration of 250 ng/ml.

To determine the intra-day precision and accuracy of the method, quality control samples in plasma and each tissue homogenate were analyzed three times on the same day. To determine the inter-day precision and accuracy, quality control samples were analyzed on three or four different days. The precision was assessed by determining the coefficient of variation (C.V.%). The accuracy was calculated by the percent of measured concentration to the nominal concentration.

2.6. Application in a pharmacokinetics study

Male Swiss–Webster mice weighing 24–32 g were purchased from Harlan Sprague–Dawley (Indianapolis, IN). Mice were administered an i.v. dose of 5 mg/kg MX through penile vein injection. One or two mice per time point were sacrificed after 5 and 30 min, and 1, 2, 4, and 48 h. Blood samples were centrifuged shortly after collection to separate plasma. To prevent oxidative degradation, 100 mg/ml ascorbic acid was added to each plasma sample. Brain, heart, lung, liver, spleen and kidneys were removed immediately after blood collection. All samples were stored at −80 °C until analysis. The area under the plasma concentration time curve (AUC) was calculated using the trapezoidal method with extrapolation to infinite time. The systemic clearance (CL) was determined as dose/AUC. Other basic pharmacokinetic parameters in plasma such as terminal slope (lambda_z), the apparent volume of distribution at steady state (Vss) and the terminal half-life ($t_{1/2}$) were also determined.

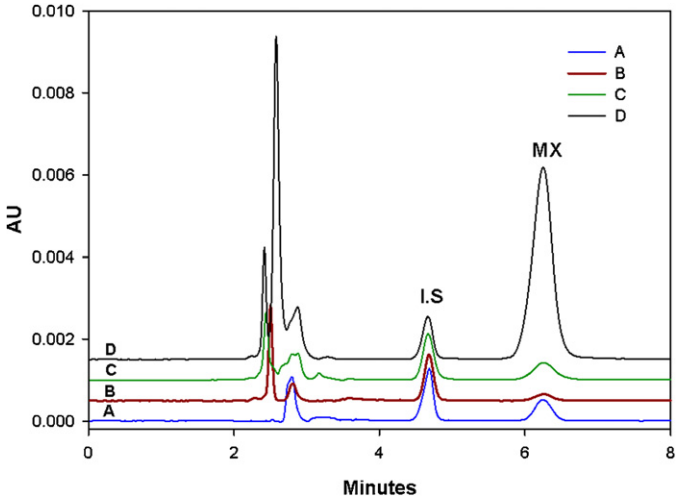

Fig. 1. Representative chromatograms of (A) plasma spiked with 100 ng/ml MX. (B) Plasma sample obtained 1 h after the i.v. administration of MX to mice. (C) Lung homogenate spiked with 100 ng/ml MX. (D) Lung sample obtained 4 h after the i.v. administration of MX. All the samples were spiked with 250 ng/ml I.S. The retention times of MX and ametantrone (I.S.) are ~4.6 and 6.2 min, respectively.

3. Results and discussion

3.1. Chromatograms in plasma and tissue (except liver) samples

Typical chromatograms of mouse plasma and tissue samples (heart was chosen as a representative tissue) are shown in Fig. 1. For the samples in plasma and most tissues (except liver), resolution of MX and I.S. was optimal using 81% mobile phase A (10 mM sodium phosphate with 0.1% TEA) and 19% mobile phase B (acetonitrile). MX and the I.S. were eluted at 6.2 and 4.6 min, respectively.

3.2. Chromatograms in liver samples

In addition to MX, three additional peaks, not present in blank liver homogenate samples and assumed to be MX metabolites, were observed when liver samples were analyzed. To obtain better resolution of those three metabolites and the parent molecule, the ratio of mobile phase A:B was adjusted to 83:17. The overall chromatographic run time was 11 min. An external standard method was used for these samples because one of the three metabolites had the same retention time as the I.S. (about 5.9 min). Chromatograms of blank liver homogenate (spiked with 2500 ng/ml MX) and liver samples obtained at 4 h after MX i.v. bolus administration (without an I.S.) are shown in Fig. 2A and B, respectively. The retention time of the three metabolites and parent compound were 4.9, 5.9, 6.6 and 8.3 min, respectively.

3.3. Calibration and LOQ

The calibration curves were linear over the MX concentration range of 5–1000 ng/ml in plasma, 25–2500 ng/ml in liver homogenate and 12.5–2500 ng/ml in other tissue homogenates.

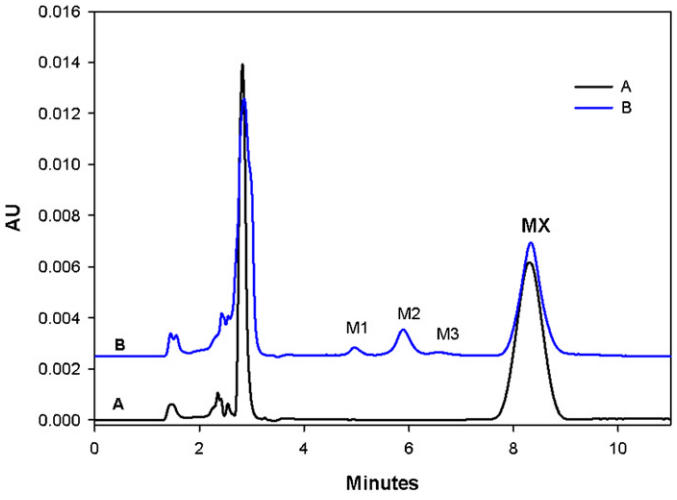

Fig. 2. Typical chromatograms of (A) liver homogenate spiked with 2500 ng/ml MX. (B) Liver sample obtained after 4 h after the i.v. administration of MX (5 mg/kg) to mice. The retention times of metabolites 1, 2 and 3 and MX are ~4.9, 5.9, 6.6 and 8.3 min, respectively.

The average slopes of the calibration curves determined in different tissues were close (ranging from 0.0071 to 0.0084) and the mean correlation coefficients were all above 0.99. Using our experimental conditions, the LLOQ was 5 ng/ml in plasma, 25 ng/ml in liver homogenate and 12.5 ng/ml in other tissue homogenates. The inter-day and intra-day precision and accuracy of the LLOQ in plasma were within 10.6 and 2.8%, respectively.

3.4. Recovery and stability

The overall mean extraction recoveries of MX were 92.3% in plasma and 85.8% in tissue homogenates. The average recovery of I.S. at the concentration of 250 ng/ml in plasma and tissue homogenates was 88.3%. MX and I.S. were stable in the presence of 100 mg/ml ascorbic acid in 0.1 M citrate buffer and no apparent oxidative degradation was observed during sample preparation and analysis.

3.5. Precision and accuracy

The inter-day and intra-day precision and accuracy of QC samples, evaluated both in plasma and tissue homogenates, are presented in Table 1. Based on the results for all the QC samples, which represent the low, medium and high concentrations of MX in seven different matrices, the precision and accuracy of the assay were all within acceptable limits (within 15% and 100 ± 15%, respectively), as defined in the Food and Drug Administration guidelines (www.fda.gov/cvm).

3.6. Application in a PK and tissue distribution study

Plasma and tissue samples were periodically collected up to 48 h following i.v. administration of 5 mg/kg MX to mice. PK parameters of MX are listed in Table 2. MX concentration–time profiles in plasma and tissues are presented in Fig. 3. As shown in Fig. 3, intravenously administered MX disappeared rapidly from plasma, with extensive tissue distribution. MX concentrations in brain samples were consistently low at all time points, indicating its poor penetration across the blood–brain barrier. The PK profiles obtained in our study were in a good agreement with previously reported results [5]. MX did not undergo extensive metabolism in the mouse,

Table 1
Inter-day and intra-day precision and accuracy of QC samples in plasma and tissue homogenates (n = 3 or 4).

QC conc (ng/ml)	Intra-day validation			Inter-day validation		
	Mean	Precision (%CV)	Accuracy (%)	Mean	Precision (%CV)	Accuracy (%)
Plasma						
5	4.91	10.6	98.2	4.86	6.61	97.2
25	25.7	14.2	103	25.7	11.9	103
250	262	4.79	105	271	7.18	109
750	749	3.57	99.9	742	7.98	99.0
Lung						
25	26.7	10.5	107	26.9	12.7	108
250	251	2.04	100	245	2.25	98.3
1500	1523	2.33	102	1530	2.49	102
Spleen						
25	23.3	3.62	93.4	24.2	1.99	96.9
250	263	5.02	105	249	2.43	99.7
1500	1572	0.27	105	1529	5.28	102
Kidney						
25	23.3	3.60	93.4	23.6	3.79	94.2
250	254	3.20	102	251	3.28	101
1500	1643	2.67	110	1596	4.18	106
Heart						
25	25.7	4.66	103	26.2	8.10	105
250	255	0.95	102	245	4.22	98.1
1500	1586	1.61	106	1599	8.58	107
Liver						
25	25.4	6.63	102	25.3	4.46	101
250	240	4.36	95.9	239	8.42	95.7
1500	1492	1.04	99.5	1501	3.51	100
Brain						
25	27.4	10.6	109	24.7	9.32	98.9
250	266	2.30	106	254	7.58	102
1500	1670	3.16	110	1597	5.53	106

Table 2
Pharmacokinetic parameters of MX in mouse plasma after i.v. administration of 5 mg/mg MX.

$t_{1/2}$ (h)	Cmax (μg/ml)	CL (L/h/kg)	Vss (L/kg)	Lambda_z (1/h)	$AUC_{0-8\,h}$ (h μg/ml)
3.83	2.12	6.39	6.88	0.176	0.749

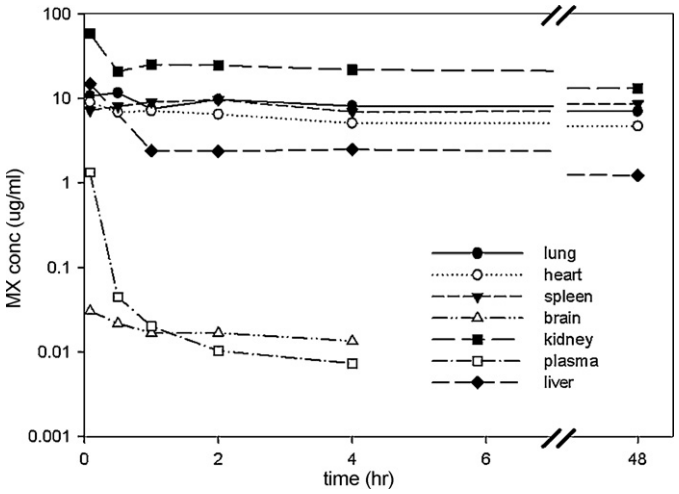

Fig. 3. The concentration–time profile of MX in plasma and tissues following the intravenous administration of 5 mg/kg MX in mice.

based on the peak area of the metabolites observed in our study. No metabolites were observed in other tissue samples except for liver, indicating that these metabolites have limited tissue distribution.

There are several HPLC assays that have been used to determine MX in plasma and different tissues including liver [5,8]; however, none of these reports indicated the presence of metabolites. One possible reason is that the retention time of the parent drug was too short to separate the co-eluted metabolites under the assay's chromatographic conditions. The choice of extraction method used in studies may be another possible reason contributing to the difficulty in determining MX metabolites. For example, during liquid–liquid extraction, which is a common extraction procedure used in many MX HPLC methods, MX is poorly extracted into water-immiscible solvents, and therefore it would not be expected that more polar metabolites would be extracted efficiently. With the sample preparation and chromatographic conditions used in our method, both parent drug and its metabolites are present in the sample following protein precipitation and can be separated.

4. Conclusion

The analytical method described here shows high selectivity, precision and accuracy for the determination of MX in mouse plasma and different tissue homogenates. Our sample preparation and chromatographic conditions make our HPLC assay a simple, rapid and economic procedure, suitable for studies that involve a large number of samples. The method was successfully applied in a MX pharmacokinetic and tissue distribution study. Three metabolites were detected in mouse liver samples following MX i.v. administration. Thus the method may also be suitable for studies which focus on the metabolites of MX.

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