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Improved liquid chromatographic method for mitoxantrone quantification in mouse plasma and tissues to study the pharmacokinetics of a liposome entrapped mitoxantrone formulation

Jenifer L. Johnson, Ateeq Ahmad, Sumsullah Khan, Yue-Fen Wang, Aqel W. Abu-Qare, Jennifer E. Ayoub, Allen Zhang, Imran Ahmad*

Pharmacokinetics, Safety, and Efficacy Department, NeoPharm Inc., Research and Development, 1850 Lakeside Drive, Waukegan, IL 60085, USA

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Abstract

A simple, rapid HPLC method for quantification of mitoxantrone in mouse plasma and tissue homogenates in the presence of a liposome entrapped mitoxantrone formulation (LEM-ETU) is described. Sample preparation is achieved by protein precipitation of 100 μl plasma or 200 μl tissue homogenate with an equal volume of methanol containing 0.5 M hydrochloric acid:acetonitrile (90:10, v/v). Ametantrone is used as the internal standard (i.s.). Mitoxantrone and i.s. are separated on a C18 reversed phase HPLC column, and quantified by their absorbance at 655 nm. In plasma, the standard curve is linear from 5 to 1000 ng/ml, and the precision (%CV) and accuracy (percentage of nominal concentration) are within 10%. In mouse tissue (heart, kidney, liver, lung, and spleen) homogenates (5%, w/v), the standard curve is linear from 25 to 2000 ng/ml, with acceptable precision and accuracy. The method was used to successfully quantify mitoxantrone in mouse plasma and tissue samples to support a pharmacokinetic study of LEM-ETU in mice.
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1. Introduction

Mitoxantrone (Fig. 1) has been used extensively as a component of chemotherapeutic regimens for a number of fatal diseases, including leukemia, lymphoma, cancers of the breast and prostate, and to treat multiple sclerosis [1–4]. While it has been shown to be efficacious and is better-tolerated than other treatments, its use is limited by unwanted side effects, particularly dose-related cardiomyopathy [1–4]. In an effort to reduce the toxicity of mitoxantrone to normal cells, liposome entrapped formulations have been developed [3,5,6].

Liposomes have shown great promise for increasing the therapeutic efficacy of anticancer drugs. Many liposomes passively target the drug, accumulating in tumor tissue due to enhanced permeability of the tumor vascular system [7]. Liposomal delivery thereby decreases drug toxicity to normal tissues, and may outmaneuver the mechanisms of multi-drug resistance by tumor cells [7,8]. Preclinical studies of our liposome entrapped mitoxantrone formulation (LEM) have shown that it has improved pharmacokinetics and tissue distribution, and greater efficacy than free mitoxantrone [6]. Recently, we have developed a well characterized, easy-to-use liposome entrapped formulation of mitoxantrone (LEM-ETU) using our NeoLipid™ platform. To support pharmacokinetics and tissue distribution studies of the LEM-ETU formulation, we have developed a simple and rapid assay for quantification of mitoxantrone in mouse plasma and tissues in the presence of LEM-ETU.

Various methods have been published describing procedures for the extraction of mitoxantrone from plasma [9–13] and tissue homogenates [14]. HPLC using C18 reverse-phase columns is the most common means of separation, along with visible absorbance near 655 nm as the method of detection. Most of these methods require time-consuming sample preparation, including liquid–liquid extraction [14], SPE [12], and protein precipitation followed by solid-phase extraction [12] or by online column

* Corresponding author. Tel.: +1-847-887-0800; fax: +1-847-887-9281.
E-mail address: imran@neophrm.com (I. Ahmad).

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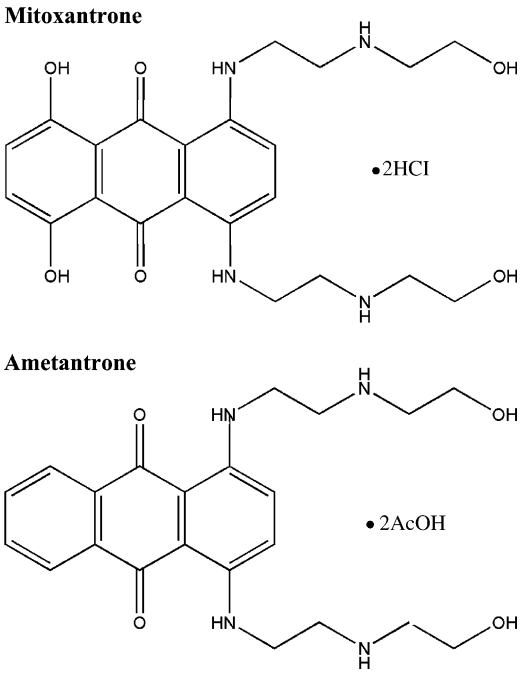

Fig. 1. Structures of mitoxantrone (top) and ametantrone, the internal standard (bottom).

switching [9]. One method uses a single step protein precipitation with sulfosalicylic acid [10], however it requires 250 μl of sample to achieve the reported lower limit of quantitation (LLOQ). We have used protein precipitation with organic solvents to extract mitoxantrone from just 100 μl of mouse plasma or 200 μl of mouse tissue homogenates containing LEM-ETU. Table 1 compares our method to those cited above. We have improved on previously-published assays by using only 100 μl of plasma to achieve a LLOQ of 5 ng/ml. In addition, we show this assay to be suitable for quantification of MTO tissue homogenates, and for matrices containing a liposome entrapped MTO formulation.

The method presented here is a simple, one-step protein precipitation for sample preparation using HPLC with visible detection for the quantitation of mitoxantrone in mouse plasma and tissue homogenates in the presence of LEM-ETU. Characteristics such as selectivity, linearity, LLOQ, extraction recovery, precision and accuracy, effect of dilution, and extracted sample stability are presented to show the method is reproducible and suitable for quantification of mitoxantrone in plasma and tissue samples containing LEM-ETU. The method was successfully used to support a pharmacokinetics and tissue distribution study of LEM-ETU in mice.

2. Experimental

2.1. Chemicals

The lipids 1,2-dioleoly-sn-glycero-3-phosphocholine, cholesterol, and 1,1′,2,2′-tetramyristoyl cardiolipin were obtained from Avanti Polar Lipids (Alabaster, AL). Mitoxantrone (MTO) dihydrochloride (100%) was purchased from the United States Pharmacopoeia (Rockville, MD, USA). Ametantrone diacetate (1,4-bis([2-(2-hydroxyethyl)amino]-ethylamino)9,10-anthracenedione; Fig. 1), the internal standard (i.s.), was a generous gift from the Drug Synthesis and Chemistry Branch, Development and Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD, USA). Ammonium formate, α-tocopherol acid succinate, and ascorbic acid were purchased from Sigma (St. Louis, MO, USA); HPLC-grade acetonitrile and methyl alcohol and USP-grade sodium chloride from EM Science (Gibbstown, NJ, USA); USP-grade ethanol from Aaper (Shelbyville, KY, USA); formic acid from Fisher Scientific (Hampton, NH, USA); hexanesulfonic acid sodium salt from Acros Organics (Pittsburgh, PA, USA); hydrochloric acid and sodium chloride from J.T. Baker (Phillipsburg, NJ, USA). Milli-Q water was obtained from an in-house Millipore Milli-Q™ Synthesis System (Millipore, Bedford, MA, USA).

2.2. Preparation of the easy-to-use liposome entrapped formulation of mitoxantrone (LEM-ETU)

LEM-ETU was prepared as detailed previously [6], without the sonication step. Briefly, lipids and tocopherol acid succinate were solubilized in ethanol, diluted into an

Table 1
Comparison of bioanalytical methods for quantification of MTO

Reference	Sample volume (ml)	Sample preparation[a]	LLOQ (ng/ml)	Amount MTO detected (ng)[b]	Matrix
[9]	0.650	PP + precolumn-switching	2.5	1.6	Human plasma
[10]	0.250	PP with sulfosalicylic acid	1.1	0.10	Human plasma
[11]	3.00	SPE (C18)	1.0	0.30	Human plasma
[12]	1.00	PP followed by SPE	4.0	0.40	Rat plasma
[13]	1.00	SPE (silica gel)	2.5	2.5	Human plasma
[14]	1.00	Liquid–liquid extraction	1.8	1.1	Mouse plasma and tissues
This issue	0.100 (plasma), 0.200 (tissue)	PP with organic solvents	5.0 (plasma), 25 (tissue)	0.25 (plasma), 1.25 (tissue)	Mouse plasma and tissues

[a] PP, protein precipitation; SPE, solid phase extraction.
[b] The amount detected was calculated by multiplying the initial sample volume and the injection volume, and dividing by the final sample volume (after reconstitution or dilution).

aqueous solution of mitoxantrone, sucrose, and saline, then lyophilized. Reconstitution of the lyophilized cake was accomplished by adding 5 ml of water and shaking well, to yield LEM-ETU with mean particle size less than 200 nm, and an approximate mitoxantrone concentration of 1 mg/ml.

2.3. Stock solutions

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 −70 ± 10 °C. Due to the known adherence of mitoxantrone to glass [11], all solutions were prepared and stored in polypropylene tubes. LEM-ETU was reconstituted in water, and then diluted to prepare test solutions of approximately 50, 100, 500 and 1000 ng/ml in diluent. The exact concentration of mitoxantrone in the LEM-ETU formulation was determined by comparing the peak areas of the test solutions to solutions made from USP standard mitoxantrone, using the chromatographic conditions specified below. A stock solution of LEM-ETU (100 μg mitoxantrone/ml) was then prepared in diluent and stored at −70 ± 10 °C.

2.4. Preparation of standards and quality control solutions

2.4.1. Plasma

Mouse plasma collected with sodium heparin from CD1 mice was obtained from Bioreclamation Inc. (Hicksville, NY, USA), and stored at −70 ± 10 °C until use. Thawed plasma was spiked with LEM-ETU stock solution to prepare a 1000 ng mitoxantrone/ml plasma standard. This solution was diluted further to prepare standards between 5 and 750 ng/ml. A separate LEM-ETU stock solution was used to prepare quality control (QC) samples between 12.5 and 800 ng mitoxantrone/ml plasma.

2.4.2. Tissue homogenates

Mouse tissues (heart, kidneys, liver, lungs, and spleen) were homogenized using an IKA-Ultra Turrax T25 Homogenizer (IKA-Works, Wilmington, NC, USA) with an 8 mm dispersing tool. A 5% (w/v) homogenate was prepared in 20% (w/v) ascorbic acid solution. Homogenates were aliquoted (200 μl) and spiked with 5 μl of an LEM-ETU standard solution to prepare homogenates with mitoxantrone concentrations from 25 to 2000 ng/ml. Quality control samples, with mitoxantrone concentrations from 75 to 1600 ng/ml, were prepared using a separate LEM-ETU stock solution.

2.5. Sample preparation

Plasma and tissue homogenates containing LEM-ETU were extracted by protein precipitation. One hundred μl of plasma or 200 μl of homogenate were mixed with 100 μl or 200 μl, respectively, of extraction solution (methanol containing 0.5 M hydrochloric acid:acetonitrile (90:10, v/v) with either 250 ng/ml or 500 ng/ml i.s.) After vigorous vortexing, the samples were placed on ice for approximately 10 min, and then centrifuged for 15 min at 17,000 × *g*. The supernatants were removed and 100 μl were injected onto the HPLC column for analysis.

2.6. Chromatographic equipment and conditions

An Agilent 1100 Series HPLC system controlled by ChemStation A.8.04 software was used for chromatographic analysis. The system was composed of a Model G1311A quaternary pump, vacuum degasser (Model G1322A), thermostated autosampler (Model G1329A or G1367A), thermostatted column compartment (Model G1316A), and a diode array detector (Model G1315B), all from Agilent Technologies (Palo Alto, CA, USA). The autosampler was maintained at 4 °C, and the column compartment at 30 °C.

The HPLC separations were achieved using a Nucleosil C18, 250 mm × 4 mm i.d., 5 μm particle size column from Macherey-Nagel (Easton, PA, USA). A precolumn filter with a 2 μm PEEK frit (Upchurch, Oak Harbor, WA, USA) and a guard column (Macherey-Nagel CC Nucleosil C18, 4 mm × 8 mm) were installed ahead of the analytical column. The isocratic mobile phase was 29:71 (v/v) acetonitrile:ammonium formate (160 mM) with hexanesulfonic acid (35 mM), adjusted to pH 2.7 with formic acid, running at a flow rate of 1.0 ml/min. The i.s. and mitoxantrone were detected by their absorbances at 655 nm.

2.7. Pharmacokinetic and tissue distribution study

Male CD2F1 mice weighing 18 to 24 g (approximately 12 weeks of age) were purchased from Charles River (Wilmington, MA, USA). The animals were used in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council, housed under standard conditions, and had ad libitum access to water and to a standard laboratory diet. Mice were randomized according to body weight, grouped three per time point, and administered a single 5 mg/kg i.v. dose of LEM-ETU. At each time point (5, 15, and 30 min, and 1, 2, 4, 8, 24, and 48 h), blood samples were collected by terminal retro-orbital bleeding under CO_2 anesthesia into pre-labeled, chilled micro tubes containing heparin as an anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA). Immediately after collection, each blood sample was gently inverted several times to ensure complete mixing with the anticoagulant, and placed on ice. The blood samples were centrifuged for 10 min at 4 °C and 3000 × *g* to separate plasma, and the latter was transferred to cryotubes containing 5 μl of 20% (w/v) ascorbic acid in 0.9% saline, to prevent oxidation of mitoxantrone. Heart, kidneys, liver, lungs, and spleen were rapidly excised following blood collection, and quickly placed on dry ice. All samples were stored at −70 ± 10° C until analysis.

Plasma and tissue concentrations of MTO versus time data were analyzed by a non-compartmental model using

the WinNonlin program (Pharsight, Mountain View, CA, USA). Plasma and tissue area under the curve (AUC_{0-x}) values, with x being the time of the last plasma concentration measured, were estimated from the linear trapezoidal method with uniform or with $1/Y^2$ weighting. $AUC_{0-\infty}$ in plasma was estimated by dividing the last plasma concentration value measured by the terminal plasma rate constant; extrapolated areas ($AUC_{x-\infty}$) accounted for less than 10% of the total $AUC_{0-\infty}$. The maximum MTO concentrations in plasma (C_{max}), the half-life of the plasma elimination phase ($t_{1/2}$), plasma volume of distribution at steady state (V_{ss}), and plasma clearance rate (C_l) were determined. For each tissue, the maximum concentration (C_{max}), time to reach maximum concentration (T_{max}), were determined, along with $AUC_{0-48\,h}$.

3. Results and discussion

3.1. Selectivity

Chromatograms obtained from blank, processed plasma and blank tissue (heart, as a representative sample) homogenate are shown in Figs. 2 and 3, respectively. No interfering peaks were observed in extracts of either matrix. Representative chromatograms of mouse plasma extract and mouse heart extract containing mitoxantrone and i.s. are also shown in Figs. 2 and 3, respectively. The i.s. and mitoxantrone elute at approximately 4 min and at approximately 5 min, respectively.

3.2. Linearity and LLOQ

Two complete sets of mitoxantrone standards, spiked as LEM-ETU (5, 10, 20, 50, 75, 125, 250, 500, 750, and 1000 ng/ml in plasma, and 25, 50, 100, 250, 500, 750, 1000, 1500, and 2000 ng/ml in tissue homogenate) were analyzed in each run, and the peak area ratios of MTO to i.s. were plotted against the MTO concentration to generate a calibration curve. Linear regression with $1/x^2$ (for plasma) or $1/x$ (for tissues) weighting was done to calculate the equation of the line. Standard curves generated acceptable data over the ranges 5–1000 ng mitoxantrone/ml in plasma and 25–2000 ng mitoxantrone/ml in tissue homogenates. All curves had correlation coefficients >0.99. The LLOQ is 5 ng mitoxantrone/ml in plasma, with coefficient of

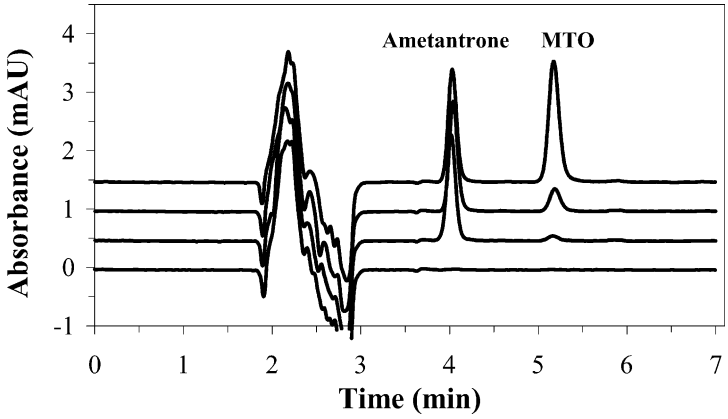

Fig. 2. Chromatograms of extracts of plasma spiked with (from top to bottom) 125, 25, 5, and 0 ng mitoxantrone/ml in the form of LEM-ETU. MTO elutes at approximately 5.2 min, and the internal standard at 4.0 min.

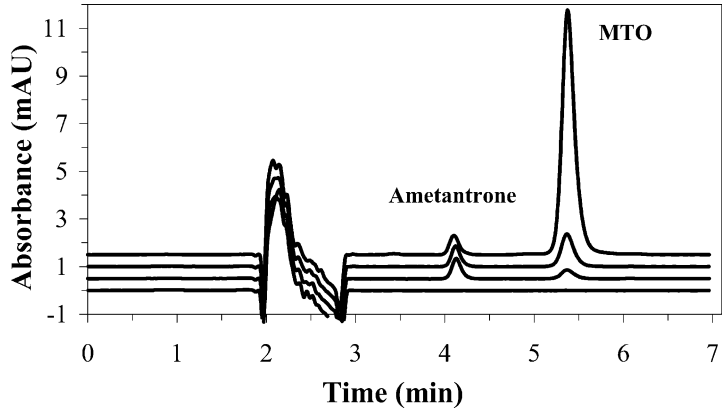

Fig. 3. Chromatograms of extracts of tissue homogenates spiked with (from top to bottom) 750, 100, 25, and 0 ng mitoxantrone/ml in the form of LEM-ETU. MTO elutes at approximately 5.3 min, and the internal standard at 4.1 min.

variation (%CV) equal to 4.3%, and accuracy (expressed as percent analytical recovery, %*AR* = [measured concentration/nominal concentration] × 100%) equal to 103%, which is comparable to that of other published methods [9–14]. The LLOQ in tissue homogenates is 25 ng mitoxantrone/ml, with %CV ranging from 3.08 to 14.1% and %AR from 95.0 to 104%. This LLOQ was sufficient for our tissue distribution studies. However, a comparison of the 25 ng/ml chromatograms in plasma and in tissue homogenate (see Figs. 2 and 3) indicates that higher sensitivity can be achieved in the homogenates.

3.3. Extraction recovery

The peak areas of unextracted analyte solutions were compared with those of extracted plasma and homogenate solutions to determine the recovery of MTO and i.s. Recovery of mitoxantrone from plasma was 93 ± 5%, and 82 ± 7% from tissue homogenate. The internal standard had similar recovery, 95 ± 5% in plasma and 85 ± 5% in tissue homogenates.

3.4. Precision and accuracy

Data presented in Table 2 show the between-run and within-run precision for plasma QC samples. The between-run precision and accuracy of QC samples in tissue homogenates are shown in Table 3. These data demonstrate that both precision and accuracy are within the acceptable limits of ≤20% at the LLOQ, and ≤15% at all other concentrations for %CV, and 100 ± 20 and 100 ± 15% for the LLOQ and all other concentrations, respectively, for %AR, as defined in the guidelines of the US Food and Drug Administration [15].

3.5. Dilution effect

The effect of diluting samples with blank matrix was examined. Plasma QC samples were diluted 10-, 50-, 100-, or 250-fold with blank plasma. The accuracy of these samples were within 5% of nominal, and the %CV at each dilution factor was less than 3% (n = 3 or 6, data not shown). Kidney, liver, and spleen QC samples were diluted 2-, 10-, or 20-fold with the appropriate blank matrix. The accuracy and precision of these samples were also within acceptable limits (data not shown) [15]. Study samples with MTO concentrations above the calibration range were therefore diluted with blank plasma or tissue homogenate into the calibration range, and QC samples with the same dilution factors were prepared in each of these runs.

Table 2
Between-run and within-run precision and accuracy of LEM-ETU mouse plasma QC samples

Nominal concentration (ng/ml)	N[a]	Mean measured concentration (ng/ml)	Precision (%CV)	Accuracy (%AR)
Between-run				
12.5	21	13.1	9.24	105
25	32	24.6	2.23	98.3
100	20	93.7	2.82	93.7
400	32	405	5.58	101
800	32	822	4.59	103
Within-run				
12.5	6	12.0	5.98	95.6
25	6	22.5	3.69	90.1
100	6	92.0	1.10	92.0
400	6	388	6.62	97.1
800	6	791	3.31	98.9

[a] N, number of replicate measurements.

Table 3
Between-run precision and accuracy of LEM-ETU mouse tissue QC samples

Tissue	Nominal concentration (ng/ml)	N[a]	Mean measured concentration (ng/ml)	Precision (%CV)	Accuracy (%AR)
Heart	75	12	70.6	8.91	94.2
	150	15	149	2.95	99.1
	350	12	350	3.52	100
	850	12	856	4.38	101
	1600	6	1605	2.13	100
Kidney	75	15	70.9	3.23	94.5
	150	12	144	2.69	96.1
	350	27	347	2.44	99.0
	850	15	834	5.19	98.1
	1600	3	1480	5.79	92.5
Liver	75	9	71.3	2.65	95.1
	150	6	147	2.29	98.1
	350	12	344	2.51	98.3
	850	9	822	1.61	96.7
	1600	3	1600	0.960	100
Lung	75	6	72.7	2.37	96.9
	150	9	146	1.781	97.4
	350	3	351	0.980	100
	850	6	835	4.89	98.3
	1600	6	1510	5.43	94.1
Spleen	75	9	70.8	1.76	94.3
	150	6	138	1.05	92.3
	350	15	331	9.44	100
	850	9	817	1.30	96.1
	1600	9	1540	2.29	96.1

[a] N, number of replicate measurements.

3.6. Stability

The peak area ratios from extracted samples were examined to ensure that MTO and i.s. are stable over the time needed to analyze a large number of samples. A comparison of the peak area ratios of the same sample solution at the beginning and at the end of 27 h (plasma extracts) and 20 h (homogenate extracts) runs was done. Maximum deviations of 7.7% in plasma, and 5.8% in tissue homogenates were observed.

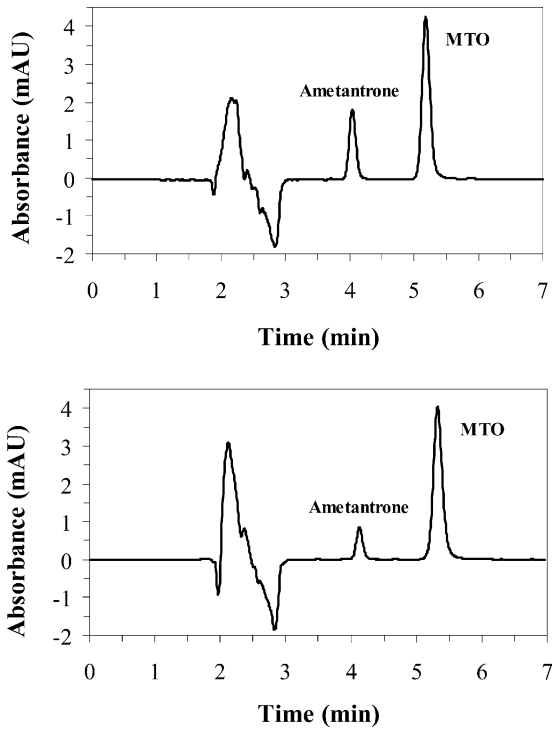

Fig. 4. Chromatograms of plasma (top) and kidney homogenate (bottom) from mice dosed with LEM-ETU at 5.0 mg/kg mitoxantrone. The plasma sample was spiked with 500 ng/ml internal standard, and the kidney homogenate with 250 ng/ml. MTO elutes at approximately 5.4 min, and the i.s. at approximately 4.0 min.

Table 4
Pharmacokinetic parameters of MTO in mouse plasma following an i.v. administration of LEM-ETU to male CD2F1 mice

$t_{1/2}$ (h)	C_{max}[a] (μg/ml)	V_{ss} (l/kg)	C_l (l/(h kg))	$AUC_{0-\infty}$ (μg h/ml)
2.29	67.4	0.172	0.300	16.6

Samples were collected from three mice per time point.
[a] Back-extrapolated to the *y*-axis at time zero.

Table 5
Pharmacokinetics parameters of MTO in mouse tissues following an i.v. administration of LEM-ETU to male CD2F1 mice

Tissue	T_{max}[a] (h)	C_{max}[a] (μg/g)	$AUC_{0-48\,h}$ (μg h/g)
Heart	0.0	11.1	112
Kidney	0.083	22.3	477
Liver	0.25	37.1	1020
Lung	0.0	11.7	171
Spleen	48	30.5	1280

Samples were collected from three mice per time point.
[a] Extrapolated values.

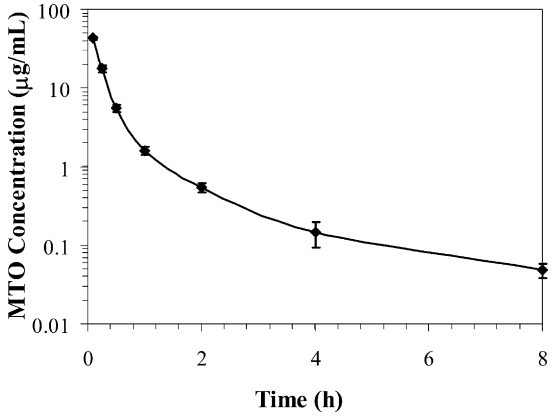

Fig. 5. Plasma concentration–time data for LEM-ETU. Male CD2F1 mice were injected i.v. with 5.0 mg/kg LEM-ETU, and plasma concentrations of mitoxantrone were determined by the method described in this paper. Samples with MTO concentration above 1000 ng/ml were diluted with blank plasma into the assay range. Each time point represents the mean ± standard error of the mean ($n = 3$).

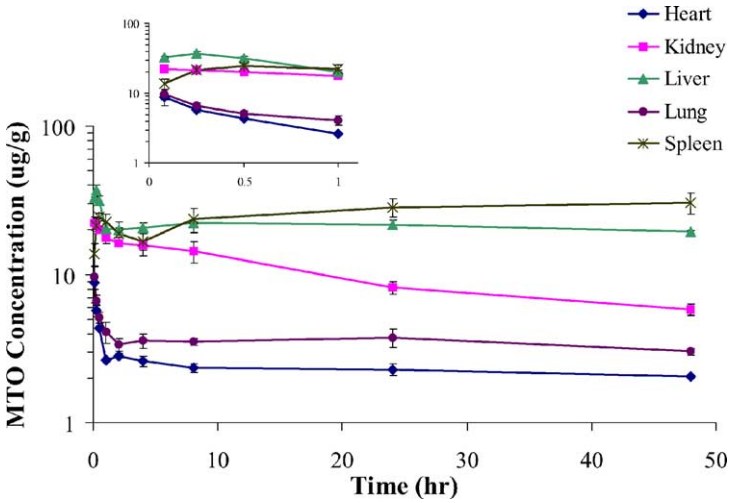

Fig. 6. Tissue concentration–time data for LEM-ETU. Male CD2F1 mice were injected i.v. with 5.0 mg/kg LEM-ETU, and tissue concentrations of mitoxantrone were determined by the method described in this paper. Samples with MTO concentration above 2000 ng/ml were diluted with blank tissue homogenate into the assay range. Each time point represents the mean ± standard error of the mean ($n = 3$). The inset is an expanded view of the 0–1 h range and is included for clarity.

3.7. Application—pharmacokinetics and tissue distribution study

The method presented here was successfully used to quantify mitoxantrone in plasma and tissue samples from a pharmacokinetics and tissue distribution study of LEM-ETU in male mice. Representative chromatograms for plasma and tissue (kidney) samples obtained from the study are shown in Fig. 4. MTO was measured up to 8 h post-dose in plasma, and up to 24 h in tissues. A non-compartmental model, linear trapezoidal method with $1/Y^2$ weighting was used to calculate the pharmacokinetic parameters from the raw data. These parameters in plasma and tissues are presented in Tables 4 and 5, respectively, and the concentration–time profiles in plasma and tissues are shown in Figs. 5 and 6, respectively. The plasma pharmacokinetic parameters of LEM-ETU are similar to those of our previous LEM formulation [6], with both liposomal formulations having total mitoxantrone exposure ($AUC_{0-\infty}$) more than 100-fold greater than free MTO. Both liposomal formulations also exhibit approximately two-fold less exposure of mitoxantrone to heart tissue. LEM-ETU improves on the original liposomal formulation by decreasing exposure to liver tissue by approximately 1/3, and to spleen tissue by more than 10-fold.

4. Conclusions

A majority of the published methods [9–14] for quantification of mitoxantrone in plasma require 250 μl to 3 ml of sample to detect 0.10–2.5 ng MTO. Such large sample volumes are unsuitable for quantification of MTO in mouse plasma, and may even be a limitation in other species, including human. The current method requires only 100 μl of mouse plasma to detect 0.25 ng mitoxantrone, allowing for quantification of 5 ng/ml mitoxantrone. This sensitivity is greater than [9,11–14] most other published methods that use visible absorbance. Moreover, it is a simple one-step protein precipitation, less time-consuming and tedious than methods employing multiple extraction steps [9,11–14], and allows for timely analysis of large numbers of samples. Mitoxantrone can also be quantified in tissue homogenates containing LEM-ETU using the same method. Preliminary data from our laboratory indicate the current method is suitable for mitoxantrone in biological matrices of other species, including human, and that even higher sensitivity may be achieved with minor modifications.

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