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ORIGINAL RESEARCH

Determination of Total and Free Plasma Concentration of Liposomal Mitoxantrone and Clinical Application in Adult Patients with Relapsed or Refractory Peripheral T-Cell Lymphoma

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Purpose: Currently reported methods for determining plasma concentration of mitoxantrone primarily measure total drug concentration. However, methodologies for quantifying liposomal mitoxantrone, particularly the free drug fraction, are lacking. Since the free drug represents the pharmacologically active moiety responsible for therapeutic effects, this study aimed to establish a detection method for total and free concentration of liposomal mitoxantrone in human plasma, while also exploring the temporal changes in drug concentration throughout the dosing cycle for liposomal mitoxantrone.

Patients and Methods: For total mitoxantrone, the mobile phase consisted of 10 mmol/L KH_2PO_4 and methanol, UV detection was performed at a wavelength of 610 nm and protein precipitation was utilized as a pretreatment method. For free mitoxantrone, a gradient elution program in 6.5 min using the mobile phase that made up by 0.2% formic acid and 10mM ammonium acetate water (A) and acetonitrile (B) after a simple pretreatment by solid phase extraction. Sixty-eight total mitoxantrone samples from 35 patients and 61 free mitoxantrone samples from 31 patients with relapsed or refractory peripheral T-cell lymphoma who received intravenous infusions of liposomal mitoxantrone-based chemotherapy regimen were determined.

Results: The linear calibration range of total and free mitoxantrone were 0.1–5 μg/mL and 5–500 ng/mL ($r^2 > 0.99$), respectively. Both intra- and inter-batch precision were less than 10.15%, and the accuracy ranged from 91.07% to 116.82%. The average total concentration in T-cell lymphoma patients after 12 hours of administration was 10.47 μg/mL, while the average free concentration was 272.22 ng/mL.

Conclusion: In this study, we established UPLC and UPLC-MS/MS methods for determining the total and free concentrations of liposomal mitoxantrone which were highly efficient, accurate, specific and suitable for clinical application and investigating the pharmacokinetics. Correlation analysis revealed that gender might be an important factor influencing the concentration of liposomal mitoxantrone in patients with relapsed or refractory peripheral T-cell lymphoma.

Keywords: liposomal mitoxantrone, UPLC, total concentration, free concentration, UPLC-MS/MS, relapsed or refractory peripheral T-cell lymphoma

Introduction

Liposomes are defined as a colloidal spherical structure formed by self-assembly of amphiphilic lipid molecules in solution, such as phospholipids which were first published in the 1960s. Initially, this was focused on examining the physical properties of biological membranes.[1] Phospholipids could assemble into closed bilayer structures when in aqueous environments, with an aqueous core that could be encased by one or multiple lipid bilayers. Subsequent studies demonstrated that the aqueous core of liposomes could encapsulate polar molecules within the confined aqueous space, while lipophilic compounds could become embedded in the lipid bilayer according to their affinity toward phospholipids. This property of liposomes rendered them highly appealing for delivering pharmaceuticals in vivo. Over the last sixty years, liposomes have emerged as some of the most

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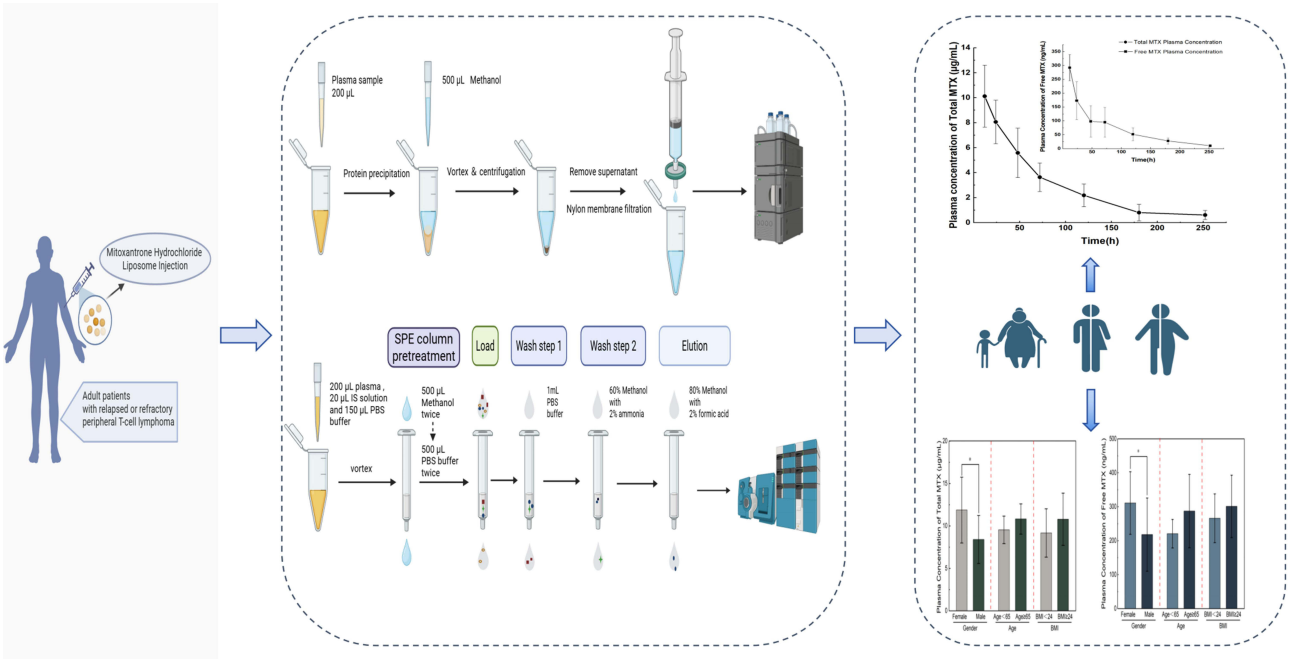
Graphical Abstract



effective nanoparticles for drug delivery, due to their structural versatility as well as their biocompatibility, biodegradability, non-toxic and non-immunogenicity nature.[2] Presently, a wide array of liposomal medications has gained approval and entered the market. Since their inception as a means of pharmaceutical delivery, liposomal formulations had advanced from basic and traditional types, which were comprised solely of neutral or cationic phospholipids, to more complex varieties such as PEGylated liposomes and ligand-targeted liposomes. These advanced formulations could deliver small molecules, peptides/proteins, and oligonucleotides/mRNAs, each with distinct therapeutic effects. Among these applications, utilizing liposomal drugs for cancer treatment stands out due to minimization of its exposure to healthy tissue, reducing the undesirable side effects compared with the free drug form.[3]

Mitoxantrone (MTX) is a broad-spectrum anthracycline chemotherapy drug primarily used in clinical settings for the treatment of malignant lymphoma, breast cancer, and various acute leukemias.[4] The drug intercalates between DNA bases, which inhibits DNA synthesis and transcription, resulting in cross-linking of DNA strands and subsequent disruption of chain structures. Common adverse reactions associated with mitoxantrone include cardiotoxicity and bone marrow suppression,[5] which significantly restrict its clinical use. Mitoxantrone hydrochloride liposomal injection is a novel nano-drug that was launched in China in 2022. It was approved for adult patients with relapsed or refractory peripheral T-cell lymphoma who have previously undergone at least first-line standard treatment. Due to its broad applicability, this drug can selectively accumulate at target sites and sustainably release therapeutic agents within the tumor microenvironment.[4] Liposomal mitoxantrone (Lipo-MTX) are administered via intravenous drip, allowing for direct entry into the body without absorption phase. The addition of polyethylene glycol (PEG) to the surface of the liposomes inhibits their binding to macromolecules in plasma, reduces phagocytosis by macrophages, and extends circulation time.[6] Liposomal mitoxantrone have been available for a relatively short period, and clinical experience with their use remains limited. A Phase 2 trial was evaluated the efficacy and safety of Lipo-MTX as single-agent chemo therapy in patients with relapsed or refractory mature T-and NK-cell lymphoma, the median progression-free survival was 8.5 months and overall survival was 23.3 months.[7] The results showed that Lipo-MTX monotherapy demonstrated robust efficacy in patients with relapsed or refractory mature T-and NK-cell lymphoma. In recent years, clinical studies on breast cancer and head and neck squamous cell carcinoma have demonstrated that Lipo-MTX possesses significant

clinical application value.[4,8] Furthermore, clinical research on multiple myeloma and other solid tumors is also ongoing. Unlike conventional drugs, liposome formulations consist of both encapsulated and free drug forms within the body, which undergo a dynamic release process. It is widely accepted that the pharmacokinetics of encapsulated drugs are influenced by the surface characteristics of the liposome carrier, and these drugs are not metabolized or excreted by the liver and kidneys. Consequently, there are notable differences in the pharmacokinetics of released free drugs compared to liposome-encapsulated drugs.[9] Additionally, the efficacy and toxic side effects associated with liposomes are positively correlated with the concentration of free drug released into the body. However, current pharmacokinetic studies of Lipo-MTX primarily focus on the total drug concentration in blood or tissue, which may not yield reliable data for clinical applications. Therefore, there is an urgent need to establish detection methods that can separately quantify the total and free concentrations of the drug in plasma.

This study aimed to establish an efficient method for determining both the total and free concentrations of Lipo-MTX. A satisfactory separation method should enable rapid and simple high-throughput analysis, provide immediate separation to prevent drug leakage from liposomes during sample preparation and storage, and achieve sufficient recovery of free drugs to ensure the accuracy of quantification. To analyze the factors influencing the in vivo exposure levels of liposomal mitoxantrone in clinical practice, we monitored the total and free drug concentrations in patients with relapsed or refractory peripheral T-cell lymphoma receiving Lipo-MTX during routine medical appointments, as well as any associated adverse reactions. The analysis seeks to provide a foundation for therapeutic drug monitoring and to support rational and effectively the clinical application of Lipo-MTX. In this study, we developed an efficient and convenient solid-phase extraction (SPE) method with controlled drug release functionality for the separation of free drugs from plasma and applied it to investigate factors of pharmacokinetic variability in patients with peripheral T-cell lymphoma.

Materials and Methods

Chemicals and Reagents

Reference standards and chemicals were obtained as follows: mitoxantrone hydrochloride (purity >99%; Lot:52010301) and mitoxantrone hydrochloride liposome injection (10 mg/10 mL; Lot: 069220807) were gifted by the CSPC Pharmaceutical Group (Shijiazhuang, China); Apatinib (purity>99%; Lot:668160504) was obtained from Innawei Biological Technology Co (Qingdao, China). HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific (Waltham, MA, USA). Formic acid and ammonium acetate were obtained from Mreda Technology Inc. (Dallas, TX, USA). Ultra-pure water was provided by the Watson and Company (Guangzhou, China). The blank plasma utilized was sourced from healthy volunteers at the Fourth Hospital of Hebei Medical University (Shijiazhuang, China).

Standard, QC and Internal Standard Preparation

For total mitoxantrone (T-MTX) assay, prepare MTX lipid with concentration of 1, 3, 5, 7.5, 10, 15, 30, and 50 μg/mL, begin with a 1 mg/mL mitoxantrone hydrochloride liposome injection and dilute it stepwise with distilled water. Calibration standard samples were prepared by spiking 20 μL MTX working solutions into 180 μL drug-free plasma to final concentrations of 0.1, 0.3, 0.5, 0.75, 1, 1.5, 3, and 5 μg/mL. The lower limit of quantitation (LLOQ; 0.1 μg/mL), low quality control (LQC; 0.25 μg/mL), middle quality control (MQC; 0.8 μg/mL), and high quality control (HQC; 4 μg/mL) were prepared in the same way.

For free mitoxantrone (F-MTX) assay, weigh precisely 10 mg of the MTX reference substance and transfer it to a 10 mL volumetric flask. Add water to dissolve the substance and adjust the volume to the mark, thereby preparing a reference substance stock solution with a concentration of 1 mg/mL. Prior to use, dilute this stock solution stepwise with water to create a standard series of working solutions with concentration of 50, 100, 200, 500, 1000, 2000, 3000, and 5000 ng/mL. Similarly, prepare MTX quality control working solutions with mass concentrations of 50, 80, 800, and 4000 ng/mL. Apatinib (10 mg of apatinib in a volumetric flask, dissolved in methanol, and fixed to obtain a 1 mg/mL standard stock solution). Dilute an appropriate amount of this stock solution stepwise with methanol/water (1:1, v/v) to obtain a 30 ng/mL apatinib internal standard (IS) working solution. In a 1.5 mL centrifuge tube, combine 180 μL of blank plasma with 20 μL of the calibration curve or quality control series working solution to obtain the calibration solutions and quality control samples.

UPLC and LC-MS/MS Conditions

T-MTX was performed using a Waters ACQUITY UPLC H-Class (UPLC) system (Waters, USA). Data acquisition and quantification were conducted using Empower data processing system (Waters, USA). MTX was analyzed using a Kinetex C18 column (100 mm × 2.1 mm, 2.6 μm, Phenomenex Corporation, MA, USA) at 30°C, with a flow rate of 0.2 mL/min. The detection wavelength was set at 610 nm, while the sample pan temperature was maintained at 8°C, and the injection volume was 10 μL. The mobile phase A was composed of 10 mM KH_2PO_4 (H_3PO_4 adjusts pH to about 2.5), and phase B was methanol. The mobile phase B in the LC gradient profile was 28% at beginning and linearly increased to 60% at 1.0 min, maintained at 60% from 1.5 to 5.0 min, and returned to 28% at 7.0 min, and maintained at 28% until 8.0 min. The retention time of MTX was 3.73 min.

LC–MS/MS analysis of F-MTX was performed using an ExionLC analytical (UPLC) system (AB Sciex, USA.) and an AB Sciex Qtrap 5500 (Applied Biosystems Inc, USA). Data acquisition and quantification were conducted using MultiQuant MD 3.0.3 (AB Sciex, USA). Both instruments are equipped with an electrospray ionization (ESI) source operating in the positive ion mode. The F-MTX and apatinib (IS) were analyzed using a Kinetex C18 column (100 mm × 2.1 mm, 1.7 μm, Phenomenex Corporation, MA, USA) at 40°C, with a flow rate of 0.3 mL/min. The mobile phase A was composed of 10 mM ammonium acetate with 0.2% formic acid and phase B was acetonitrile. The mobile phase B in the LC gradient profile was 15% at beginning and linearly increased to 90% at 1.5 min, maintained at 90% from 1.5 to 3.5 min, and returned to 15% at 4.5 min, and maintained at 15% until 6.5 min. The retention times of MTX and apatinib were 1.57 and 2.09 min respectively. MS/MS conditions were optimized as follows: source temperature, 500°C; ion spray voltage, 5500V; Gas1, 40 psi; Gas2, 50 psi; curtain gas, 35 psi and dwell time, 100 ms. The ion pairs for the positive multiple reaction monitoring (MRM) were m/z 445.0 to 88.1 for MTX, m/z 398.5 to 211.8 for IS. The declustering potential was 90and 70 V for MTX and IS, respectively. The collision energy was set at 28.6 and 41.0 eV for MTX and IS, respectively. The typical secondary mass spectrograms were shown in Figure 1.

Plasma Sample Treatment

In T-MTX method, a total of 500 μL methanol was combined with 200 μL of plasma in 1.5 mL centrifuge tubes. After vortexing for 3 min and centrifugation at 13,000 rpm for 10 min, pass the solution into nylon filter membrane with 0.22 μm to obtain a clear filtrate. Finally, an aliquot of 5 μL supernatant of prepared samples was injected into the LC-MS/MS system for analysis.

For F-MTX method, add 500 μL of methanol twice to activate the Oasis HLB solid-phase extraction column. Following this, equilibrate the column with 500 μL of phosphate-buffered saline (PBS) twice, and then set aside. A total of 150 μL PBS buffer was combined with 200 μL of plasma and 20 μL of the IS solution in 1.5 mL centrifuge tubes. After vortexing the mixture for 3 minutes, transfer 350 μL of the supernatant to a pretreated SPE column. Subsequently, add 1 mL PBS buffer solution, followed by 1 mL 60% methanol solution containing 2% ammonia. And then, add 300 μL

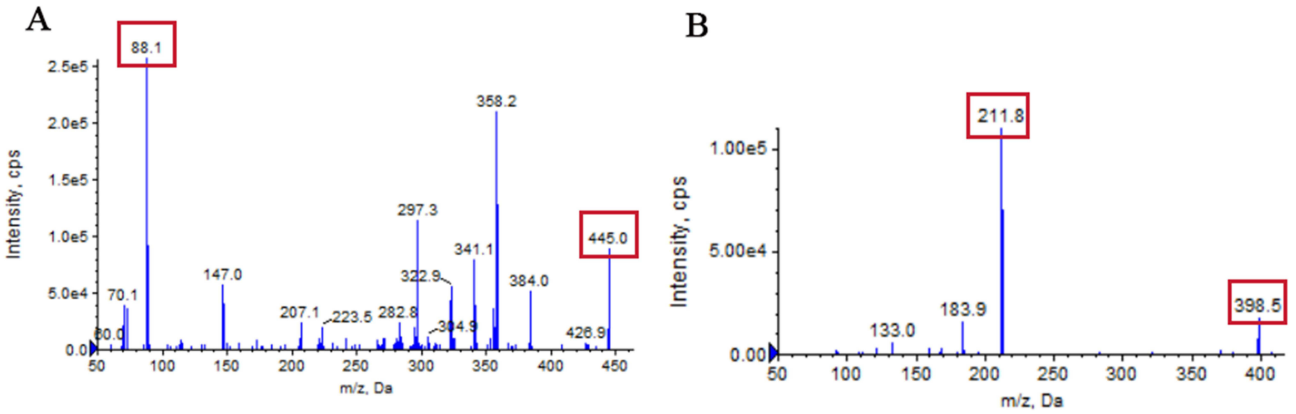


Figure 1 Product ion spectra of the protonated molecule [M + H] + ions of (**A**) mitoxantrone, (**B**) apatinib, the red boxes represent quantitative ion pairs.

of an 80% methanol solution containing 2% formic acid for the final elution. All solutions used during centrifugation were pre-chilled at 4°C before use, and the entire experimental process was performed on ice. Collect the eluate, vortex to mix thoroughly, and inject 2 μL for analysis.

Method Feasibility for Free Mitoxantrone

Method feasibility of SPE to separate free drugs was evaluated to ensure that the sample pretreatment method separates F-MTX from liposomes while maintaining the integrity of liposomes. This study verified the feasibility of the method by measuring the F-MTX content (F%) in the sample. A known concentration of MTX liposome solution was added to the blank plasma to determine the free concentration. Calculate the content of F-MTX according to the calculation formula $F\%=C_F/C_N\times100\%$, where C_F is the measured free concentration of MTX, and C_N is the MTX liposome added to the quality control sample. If the liposomes are not destroyed during solid phase extraction, the F% value after solid phase extraction should be equal to the formulation free rate.[10] A known amount of MTX liposome solution was added to human blank plasma with concentrations of 150, 250 and 4000 ng/mL for the determination of free drugs by solid phase extraction. It is known that the encapsulation rate of MTX liposomes is 92%, and the measured F-MTX in the eluate should also be about 8%, which proves that the established pretreatment method can accurately determine the F-MTX in plasma. Additionally, the release of the drug in vitro under different conditions (4°C, lucifuge vs 37°C) were investigated. The release extent of free drug was assessed by determining the percentage released (F%) at various time points.

Method Validation

According to the US Food and Drug Administration (FDA) Guidelines on the Bioanalytical Method Validation (2018) and the Chinese Pharmacopoeia (2020).[11] The method for detecting T-MTX and F-MTX in the plasma samples were validated through selectivity, carryover, linearity, lower limit of quantification, precision, accuracy, extraction recovery and stability. For the free concentration determination method using UPLC-MS/MS, the influence of matrix effect must also be investigated.

Selectivity

The evaluation of selectivity was conducted by analyzing human plasma samples from six different donors to investigate potential peaks of interference at the retention time of the analytes. Analytes were added to blank plasma samples at the lowest concentration of quantification. The responses of the blank plasma samples were confirmed to be selective and specific, with values ≤20% for LLOQ and 5% for the IS.

Linearity and Lower Limit of Quantitation

The calibration curve was generated using a weighted least squares method with the concentration of the analyte as the x-axis, the peak area ratio of the analyte as the y-axis for UPLC method and the peak area ratio of the analyte to the IS as the y-axis for UPLC-MS/MS method. The weighting factor used was $1/x^2$. The regression coefficient (r^2) of the standard curve must be greater than 0.99. The acceptable range for the difference between the back-calculated and nominal concentrations of each standard was found to be less than 15%. The difference for the LLOQ should be under 20%, with the LLOQ defined as the lowest concentration of the calibration curve having a signal-to-noise ratio of 10 or higher.

Precision and Accuracy and Dilution

The intra- and inter-day accuracy and precision were evaluated within two consecutive days at four concentration levels (LLOQ, LQC, MQC and HQC) with three analytical batches. Acceptable accuracy was defined as REs falling within ±20% of the nominal value for LLOQs and within ±15% for all other QC concentrations. Precision was deemed acceptable when the RSDs did not exceed 20% for the LLOQs and 15% for the remaining concentrations. Analyzing the samples at a concentration beyond the upper quantification limit (ULOQ), five-fold dilutions were carried out using blank plasma as a diluent with five replicates for each. In terms of quality control samples, precision should be within <15% RSD, while accuracy should stay within ±15% of the nominal concentrations for each QC level.

Extraction Recovery and Matrix Effect

To evaluate extraction recovery, the mean peak areas from QC samples spiked at low and high levels prior to extraction were compared to those spiked in plasma post-extraction. The matrix effect was assessed through the IS-normalized matrix factor (MF) at LQC, MQC, and HQC concentrations by comparing the responses of six individual blank plasma samples. The post-extracted blank plasma samples from six different individual spiked with corresponding amounts of analyte and IS, and six replicates post-extracted water spiked with equivalent amount of analyte and IS. The MF was calculated by the ratio of the peak area in the presence of matrix (blank matrix spiked after extraction) to the peak area in the absence of matrix (neat aqueous samples). The normalized MF of mitoxantrone was calculated by dividing their respective MF by the MF of the IS. Subsequently, the RSDs (%) of the normalized MF were compared to assess consistency.

Stability

Due to the instability of MTX under standard temperature and light conditions, concentration measurements may be affected. Consequently, we investigated the stability of working solutions at both low and high concentrations under varying lighting conditions (illuminated or lucifuge) and temperatures (room temperature or ice-bath).

The stability of T-MTX in human plasma samples at LQC and HQC levels was examined across four varying storage conditions. These included: samples left at room temperature for 4 hours, processed samples within the auto-sampler tray for 24 hours, subjected to three freeze-thaw cycles (initially frozen at −80 °C for 24 hours and then undergoing three cycles), as well as a prolonged storage at −80 °C for 4 weeks to assess long-term stability. And for F-MTX, the stability of QC plasma samples containing Lipo-MTX was examined under lucifuge and ice-bath for 5 hours.

Clinical Application

Thirty-five patients at the Fourth Hospital of Hebei Medical University from December 2022 to January 2024 with lymphoma, including T-cell lymphoma, NK/T cell lymphoma and other lymphoma subtypes who received intravenous infusions of MTX liposome-based chemotherapy regimen ware enrolled. All the patients had progressed following a first-line chemotherapy regimen, such as ABVD or CHOP. These patients received mitoxantrone-based chemotherapy regimens, for second-line and subsequent multi-line treatment. The dosage of mitoxantrone administered was 20 mg/m^2. 68 blood samples were gathered during routine medical appointments at varying time intervals using EDTA -K2 tubes and immediately placed them in a low-temperature transport box and transported them to the laboratory. Following centrifugation at 2000 g for 10 min at 4 °C, the supernatant was separated. 200 μL of the plasma sample and used it directly to determine the free concentration. The remaining samples should be stored at −80 °C for the determination of total concentration. All the procedures were undertaken by the Declaration of Helsinki, and the study protocol was approved by the Medical Ethics Committee of Ethics Committee of the Fourth Hospital of Hebei Medical University (No. 2022ky123, Hebei, China). Informed consent was obtained from all individual participants included in the study.

Statistical Analysis

The SPSS 21.0 statistical software was utilized for data analysis. Paired t-tests were employed for comparisons of measurement data that followed a normal distribution. Conversely, the Wilcoxon signed-rank test was applied for before-and-after comparisons of measurement data that did not adhere to a normal distribution.

Results

Method Feasibility for Free Mitoxantrone

Table 1 summarized the method feasibility results for F-MTX determination. To quantify the F-MTX released during the extraction process, samples spiked with Lipo-MTX solution (n=5) were subjected to SPE. The average F% values in the eluate of Lipo-MTX QC samples at the concentration of 150, 250 and 4000 ng/mL were 11.58%, 11.42% and 8.35%, respectively. These results indicated that the encapsulation rates of Lipo-MTX, as measured in QC samples at the three concentrations, were 88.42%, 88.48% and 91.65%, respectively. Furthermore, the in vitro studies demonstrated that under lucifuge condition at 4°C, liposomal F% values (representing the percentage of free drug released) averaged

Table 1 The Result of Method Feasibility for Free Mitoxantrone

	LQC	MQC	HQC
C_N (ng/mL)	150	250	4000
C_F (ng/mL)	17.37	28.55	334.15
F%	11.58	11.42	8.35
RSD%	8.79	0.12	5.52

Notes: C_F: the measured free concentration of MTX; C_N: the MTX liposome concentration added to the quality control sample; F%: $C_F/C_N \times 100\%$.
Abbreviations: LQC, low quality control; MQC, middle quality control; HQC, high quality control.

between 9.92% and 13.60% over 24 hours. In contrast, at 37°C, significant liposomal drug release was observed after 4.5 hours, as shown in Figure 2.

Analytical Method Validation

Selectivity

To detect T-MTX and F-MTX in human plasma, the chromatographic conditions were optimized. This included adjusting the type of chromatographic column and mobile phase based on previously published methods.[12–14] The goal was to attain optimal chromatographic peak shape, response intensity, retention time, and minimal carryover. Figures 3 and 4 display the typical chromatograms of the analytes extracted from human serum.

Calibration Curve and LLOQ

The concentration ranges tested for T-MTX and F-MTX were as follows: 0.1–5 μg/mL and 5–500 ng/mL respectively. The corresponding calibration curve equations for the three analytes were: y= 27.74x-452.32 (r^2 = 0.9992) and y= 0.00334x-0.00129 (r^2 = 0.9959), respectively. The linearity of each calibration curve was demonstrated across the concentration ranges, typical calibration curves were shown in Figures S1 and S2. The LLOQ for total and free mitoxantrone were 0.1 μg/mL and 5 ng/mL, respectively. Precision and accuracy were verified through the analysis of five consecutive LLOQ samples for each analyte. The concentrations of the calibration standards were back-calculated within ±15%, and the LLOQ values were within ±20%.

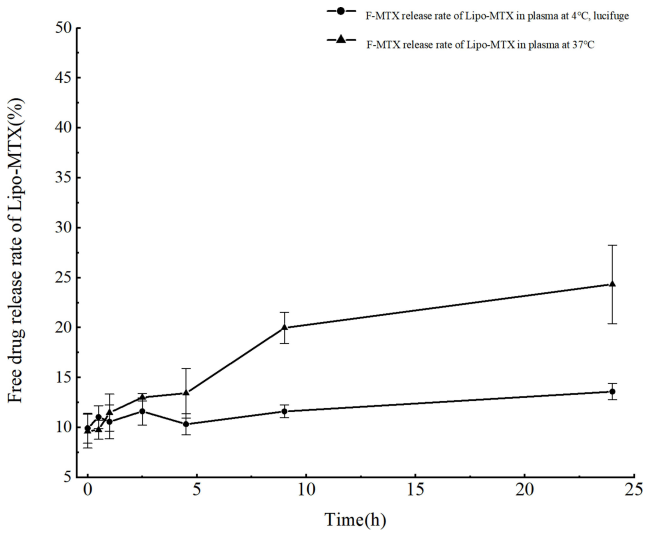


Figure 2 The release of the drug in vitro under different conditions (4°C, lucifuge vs 37°C).

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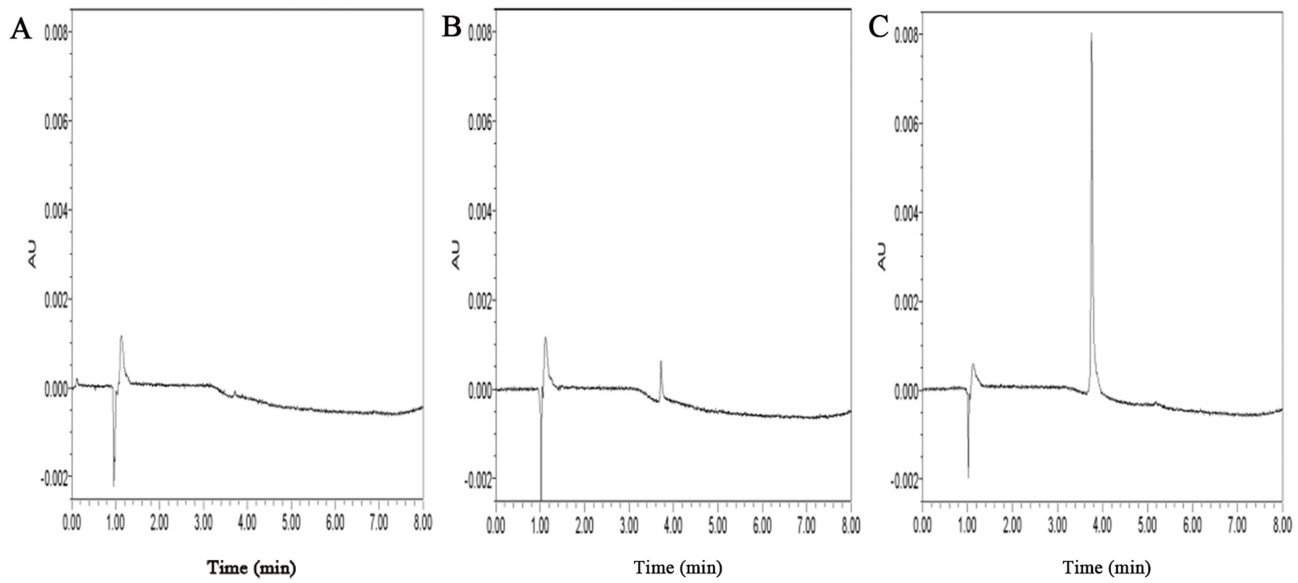

Figure 3 Chromatograms of MTX by UPLC. (**A**) Chromatograms of blank plasma; (**B**) Chromatograms of blank plasma with Mitoxantroen (100 ng/mL); (**C**) Chromatogram of patient plasma.

Precision and Accuracy and Dilution

Tables 2 and 3 displayed the precision of quality control (QC) and LLOQ samples for all analytes, both intra-day and inter-day, meeting the necessary criteria. The accuracy of these samples also fell within the acceptable range. The mean accuracy values were from 88.62% to 97.76% for the 5-fold dilutions. The RSD% values for the precision attained were <4.33%. The clinical samples were diluted with blank plasma without affecting the accuracy and precision of this method.

Extraction Recovery and Matrix Effect

For T-MTX assay, the relative recoveries of QC samples with low, medium, and high concentrations of MTX were 71.81%, 76.33%, and 72.04%, respectively, while the RSDs were 7.74%, 2.54%, and 8.95%, respectively.

In the determination of F-MTX method, the IS-normalized MFs (RSD%) at MTX concentration of LQC (8 ng/mL), and HQC (400 ng/mL) were 1.25 (3.82%), and 1.14 (2.50%), respectively. The recovery of MTX at LQC (8 ng/mL), MQC (80 ng/mL), and HQC (400 ng/mL), were observed as 88.84%, 84.39% and 80.48%, respectively. The recovery rate of the IS was 78.42%. These results indicated that the recovery rates of the two methods are stable and have minimal impact on the quantitative analysis of samples. Furthermore, endogenous substances did not interfere with the detection of F-MTX.

Stability

The stability results of MTX solution under varying conditions are presented in Figure 5. The findings indicated that the working solution of MTX remains stable when exposed to lucifuge at ice-bath, thereby fulfilling the requirements for concentration determination.

Table 4 presents the stability of MTX plasma QC samples using the total concentration determination method. The samples were subjected to a series of conditions: they were placed at room temperature for 4 hours, repeatedly frozen and thawed three times at −80 °C, and subsequently stored at −80 °C for 40 days. Additionally, the stability of Lipo-MTX-containing plasma samples was evaluated under lucifuge at ice-bath for 5 hours in the free concentration determination method. The results indicated that all back-calculated concentrations fall within ±15% of the labeled value, with the RSD of less than 15%. The findings demonstrate that the QC samples exhibited satisfactory stability for each analyte during the tested storage conditions.

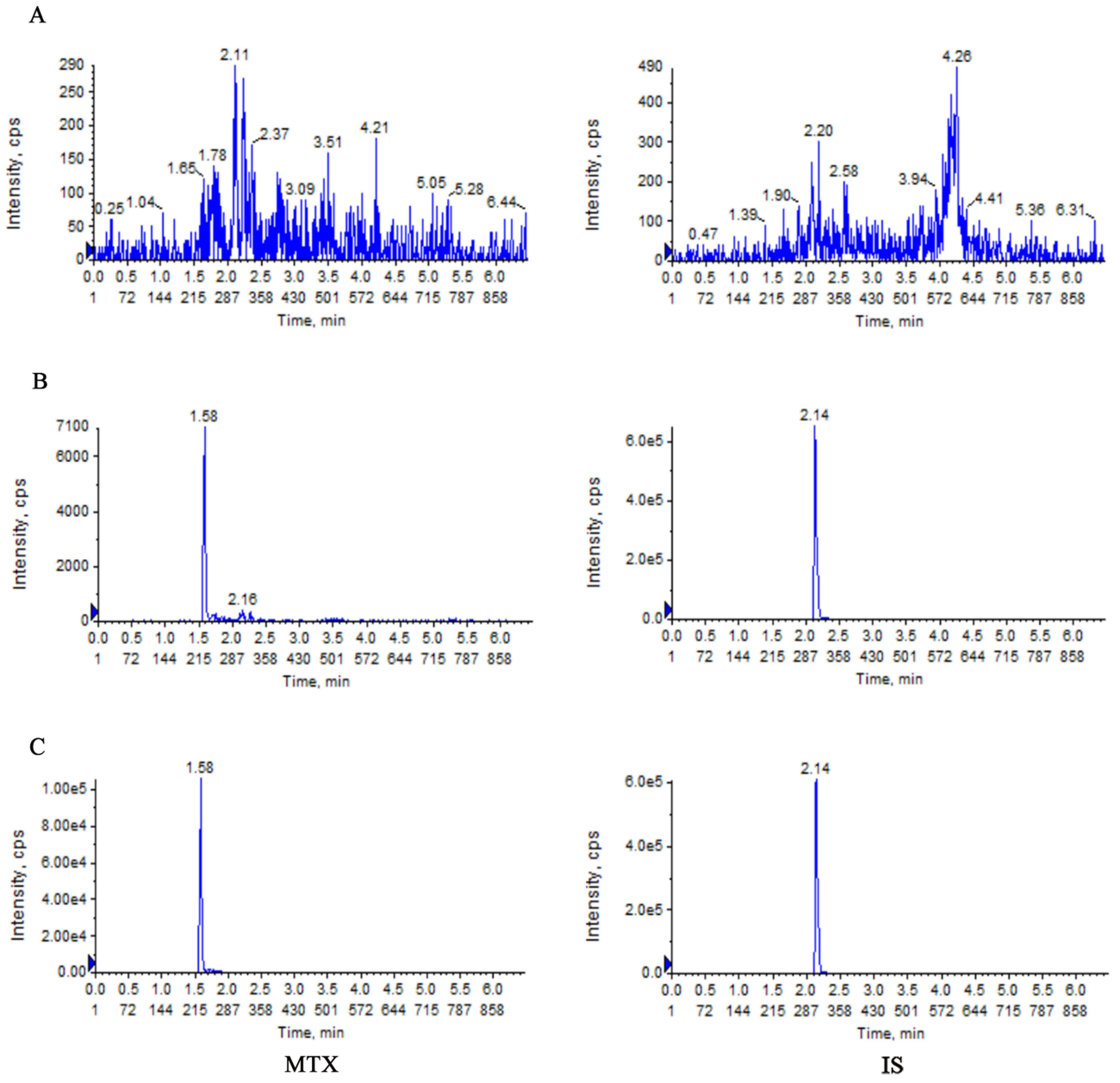

Figure 4 Chromatograms of Mitoxantroen and Apatinib (IS) by UPLC-MS/MS. (**A**) Chromatograms of blank plasma; (**B**) Chromatograms of blank plasmawith Mitoxantroen (5 ng/mL) and Apatinib (30 ng/mL); (**C**) Chromatogram of patient plasma.

Clinical Application

This study included a total of 68 T-MTX samples from 35 patients and 61 F-MTX samples from 31 patients, the characteristic of patients in this study were shown in Table S1. All plasma samples were collected during routine medical appointments, with a requirement that the blood collection time should be within ±2 hours. Based on the grouping results, the drug-time curves for the patient's T-MTX concentration and F-MTX concentration were generated, as illustrated in Figure 6 and Table S2. In addition, we further studied the effects of gender, age, and BMI on T-MTX and F-MTX concentrations, and the results were shown in Figures 7 and 8.

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Table 2 Precision and Accuracy Data of T-MTX Method

Spiked Conc. (μg/mL)	Intra-Day (n=5)			Inter-Day (n=15)		
	Mean±SD (μg/mL)	Precision (RSD%)	Accuracy (RE%)	Mean±SD (μg/mL)	Precision (RSD%)	Accuracy (RE%)
0.10	0.12±0.01	5.43	116.82	0.11±0.01	7.97	114.35
0.25	0.25±0.01	3.15	98.18	0.24±0.02	7.70	96.99
0.80	0.84±0.07	8.75	105.46	0.85±0.07	7.82	105.75
4.00	4.35±0.30	7.00	108.86	4.23±0.35	8.34	105.64

Table 3 Precision and Accuracy Data of F-MTX Method

Spiked Conc. (ng/mL)	Intra-Day (n=5)			Inter-Day (n=15)		
	Mean±SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)	Mean±SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)
5	4.97±0.33	6.64	99.44	5.43±0.45	8.38	108.53
8	8.25±0.43	5.23	103.13	8.25±0.47	5.69	103.13
80	78.62±3.53	4.48	98.28	77.13±7.83	10.15	96.41
400	381.74±11.83	3.10	95.44	364.28±18.42	5.06	91.07

Discussion

For the type of chromatographic column and organic phase in the UPLC-MS/MS analysis, C_{18} column with acetonitrile functioned well for both the peak response and peak shape. The addition of formic acid and ammonium acetate to the water phase could further enhance the ionization efficiency and peak shape in positive ion mode. In this study, 0.1–0.2% formic acid and 2–10 mM ammonium acetate was tested separately. Finally, 0.2% formic acid and 10 mM ammonium acetate in water was found to be the optimal mobile phases for MTX and IS separations.

Upon entering the human body, the liposome formation undergoes a dynamic release of free drugs, exhibiting pharmacokinetic behavior that is distinct from that of conventional drugs and is inherently more complex. In the case of anti-tumor drug liposomes, the free drug present in the blood and at target sites is responsible for the therapeutic effects, while its toxicities and side effects are closely associated with the concentration of the free drug. Consequently, it

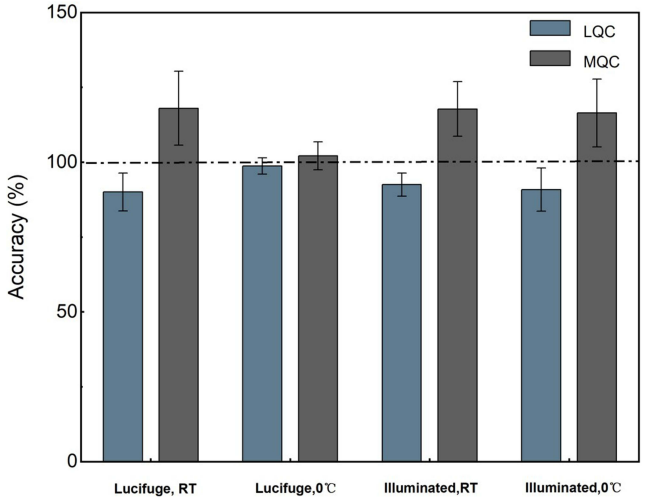

Figure 5 Mean value of mitoxantrone working solution (LQC, MQC) under different conditions (n=3).
Abbreviation: RT, Room temperature.

Table 4 Stability of T-MTX and F-MTX in Human Plasma Under Various Storage Conditions

Storage Conditions		Nominal Conc. (n=5)	Measured Conc. Mean±SD (n=5)	Accuracy (%)	RSD (%)
T-MTX (μg/mL)	Room Temperature for 4h	0.25	0.24±0.02	95.71	7.98
		4	4.15±0.12	103.74	2.85
	3 Freeze-Thaw Cycles, −80°C to Room Temperature	0.25	0.23±0.02	93.52	6.85
		4	4.16±0.04	103.99	1.02
	Long-term stability −80°C for 40 days	0.25	0.26±0.01	102.70	5.82
		4	4.23±0.38	105.83	8.91
F-MTX (ng/mL)	Lucifuge at 0°C for 5 h	8	7.50±0.38	93.73	5.10
		400	392.62±18.12	98.16	4.61

is essential to develop a device capable of separating liposomes from free drugs. This work innovatively presented a method for determining both the total and free concentrations of Lipo-MTX using UPLC-MS/MS and optimized the pretreatment process to achieve a stable and reliable separation of free drugs in plasma, the details were as follows.

The sample pretreatment method plays a critical role in accurately determining the concentration of free drugs. Solid-phase extraction materials can selectively adsorb free drugs without capturing liposomes.[12–14] Typically, the equilibrium solvent for SPE cartridges is water. However, this study demonstrated that utilizing water as the equilibrium solvent could lead to the rupture of liposomes. The average F% values in the eluate of Lipo-MTX QC samples at the concentrations of 150, 250 and 4000 ng/mL were 15.28%, 19.37% and 14.35%, which were significantly higher than the encapsulation rate of the drug. Conversely, activating the cartridge with a PBS buffer solution significantly mitigated this issue. It is speculated that the packing of the solid-phase extraction column might exhibit strong hydrophobicity,

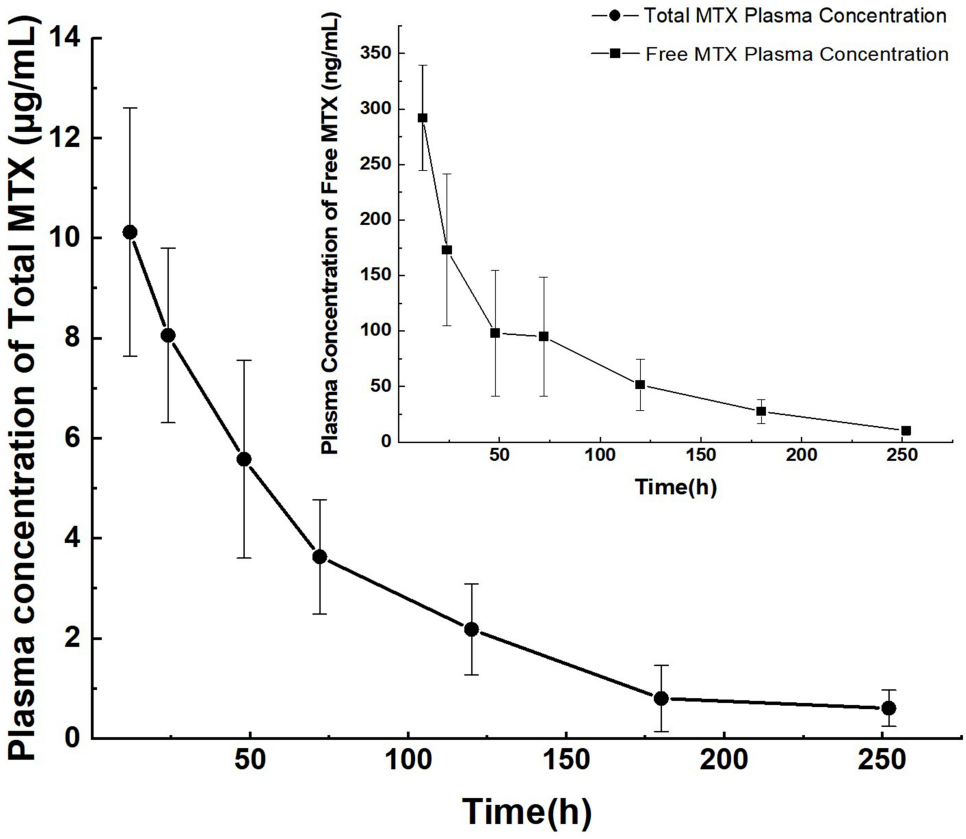

Figure 6 Plasma concentration-time profiles of T-MTX after Lipo-MTX infusion. Insert: Plasma concentration-time profiles of F-MTX after Lipo-MTX infusion.

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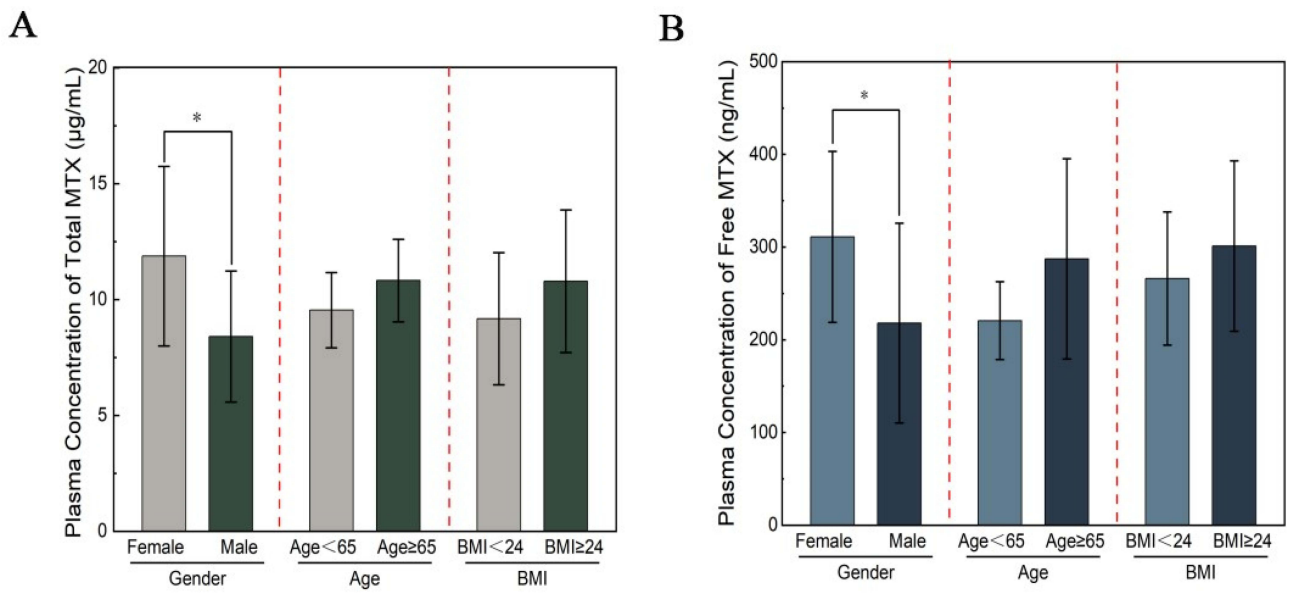

Figure 7 The relationship between physiological factors and T-MTX concentration after 12 hours of Lipo-MTX infusion (**A**); The relationship between physiological factors and F-MTX after 12 hours of administration of Lipo-MTX infusion (**B**).
Note: *P<0.05, indicates significant differences.

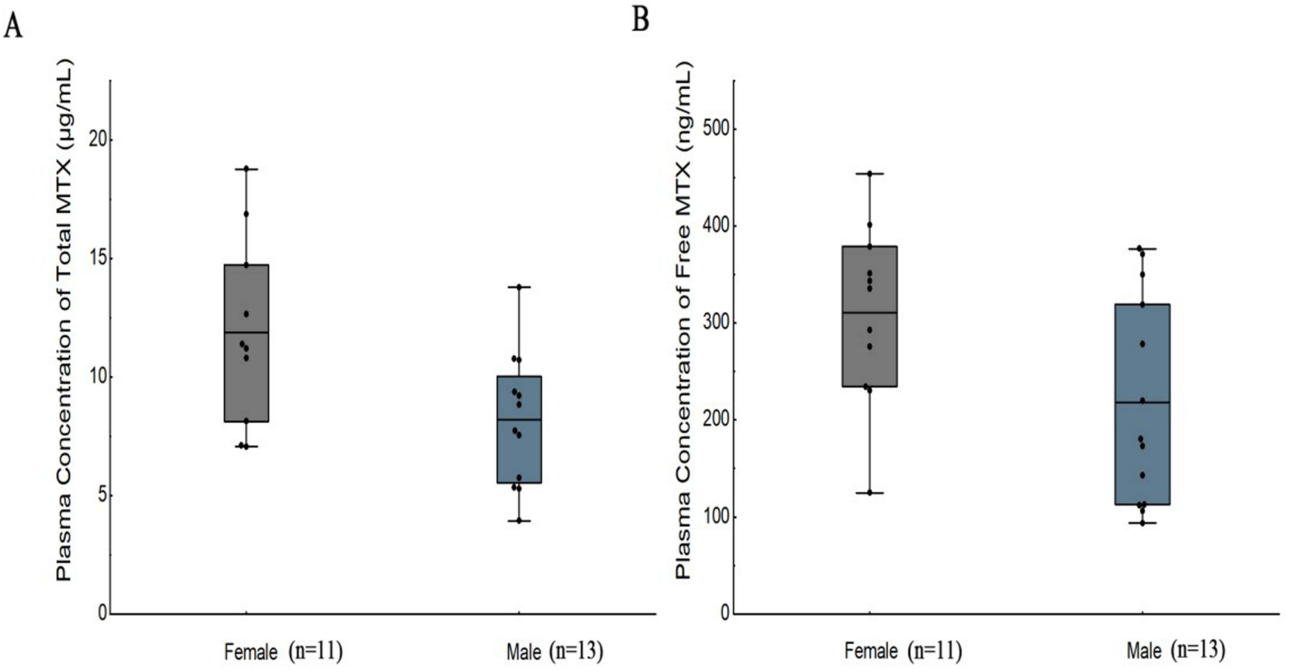

Figure 8 The T-MTX (**A**) and F-MTX (**B**) distribution in patients of different genders after 12 hours of Lipo-MTX infusion.

allowing it to adsorb liposomes through hydrophobic interactions. This adsorption could result in the fragmentation of uneluted liposomes during the extraction process, leading to an overestimation of free concentration. By adjusting pH and ionic strength, PBS indirectly influenced the interaction between the target substance and the packing, effectively mitigating this phenomenon.[15] Moreover, previous research had indicated that pre-equilibrating of the solid-phase extraction column with blank plasma or calf serum could minimize the damage to liposomes caused by the hydrophobic packing of the column, thereby preventing an increase in free drug concentration.[16] Nonetheless, our experiment revealed

a significant increase in the measured free drug concentration following treatment with blank plasma or BSA exceeds the labeled value by 3 to 4 times (accuracy range: 363%-415%, n=5). This observation might be attributed to the higher viscosity of plasma and its greater adsorption effect on liposomes, which resulted in an elevated free drug concentration. Additionally, the saturated phospholipid structure of PEG material improved its binding capacity with plasma, creating a long-circulating and tumor-targeting carrier system, while also leading to increased retention on solid-phase extraction columns.[10,17]

Co-eluting matrix components can suppress or enhance ionization, and variations in individual matrices also lead to quantitative inaccuracies. To ensure method ruggedness, appropriate IS normalization and matrix effect testing across multiple batches were employed to mitigate this variability and obtain reliable bioanalytical results, necessitating step-by-step SPE procedure optimization. During the extraction process, the drip washing step is crucial for removing plasma proteins and other impurities.[18] Hence, this study had fully optimized this process, the scrubbing steps in this study were divided into three phases. The first phase involved the use of a 1 mL PBS buffer dilution, which minimized the rupture of lipid bodies. Concurrently, the weak alkaline conditions enhanced the adsorption of the liberated free drug. The second phase was to wash away endogenous substances in the plasma as thoroughly as possible to mitigate the interference of the matrix effect. The pH of both the leaching solution and the elution liquid significantly influenced the recovery rate and the matrix effect. In this study, the addition of formic acid to the leaching solution was found to increase the recovery rate, with the effect rising from 48% to 82%; However, the matrix effect increased from 119% to 196%. Consequently, we investigated the irritation effect in an alkaline environment, and the results demonstrated that the incorporation of 2% ammonia in the elution liquid solution yielded the optimal elution effect, as illustrated in Figure 9

This approach maximized the retention of MTX while effectively removing foreign substances that might cause interference. MTX is a weak alkaline compound (pKa=8.1), an acidic environment (0.5%-2% formic acid) will induce the ionic state of MTX (pH range 2.06–2.36), facilitating its elution from the solid-phase extraction column. Previous studies have demonstrated that for basic compounds, the use of a methanol/water solution with an appropriate proportion of formic acid enables effective elution of analytes.[12,14] In this study, an eluent mixture consisting of 80% methanol and 2% formic acid yielded the highest recovery rate of MTX, while also improving the peak shape. The SPE process after full optimization was shown in Figure 10.

In this study, we included a total of 35 adult patients diagnosed with relapsed or refractory T-cell lymphoma. All participants had previously undergone at least one line of standard treatment, which included a regimen containing

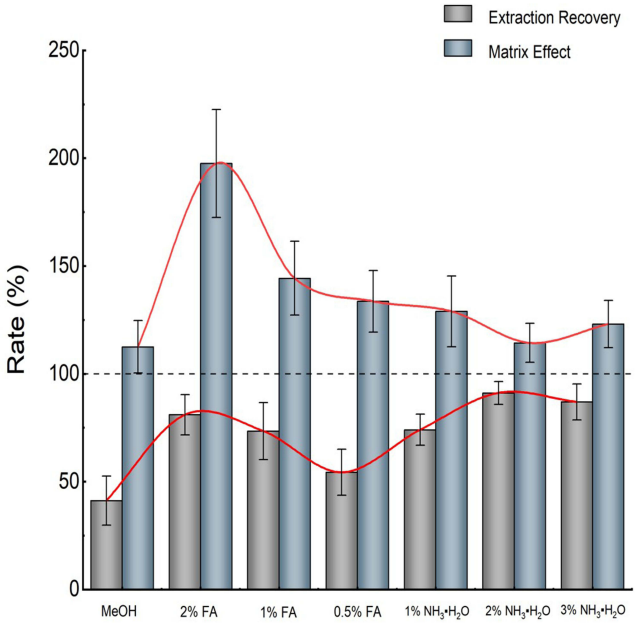

Figure 9 Extraction recovery and matrix effect of different pH methods of leaching solution for determination of free mitoxantrone (n=3).

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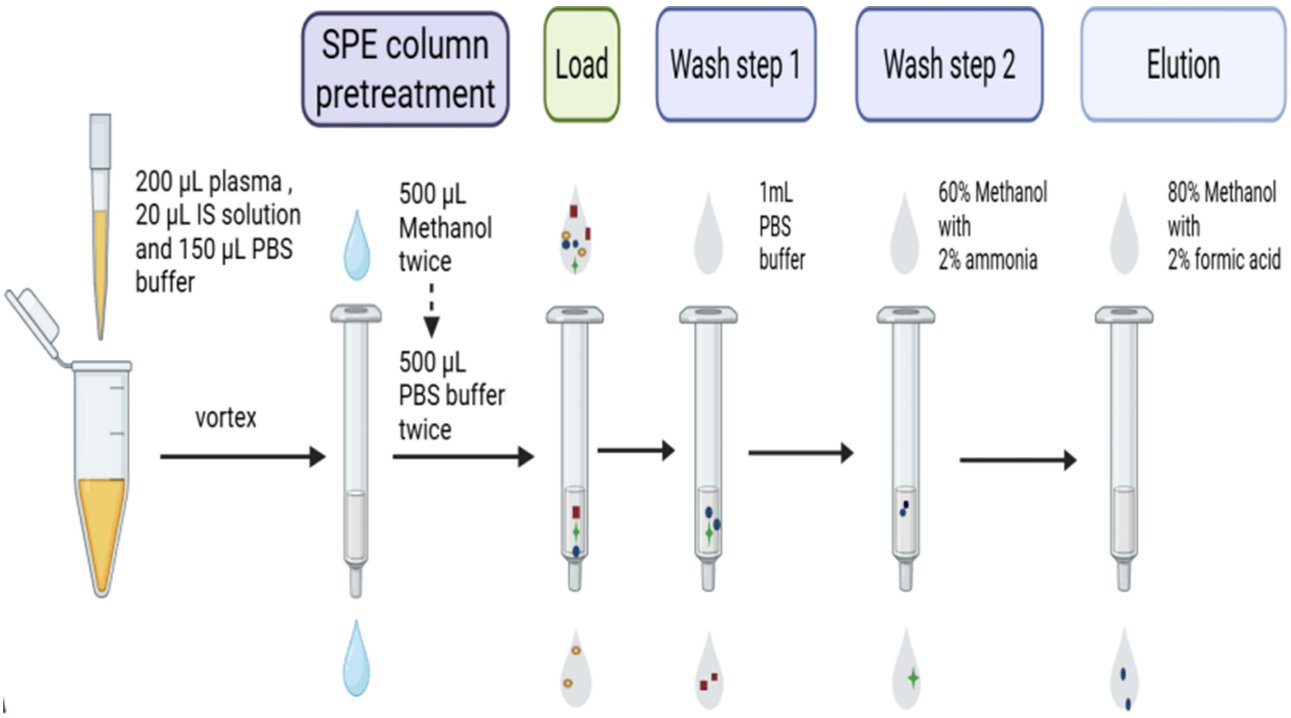

Figure 10 Graphical representation of the solid-phase extraction (SPE) approach to measuring the concentration of free mitoxantrone.

mitoxantrone liposome injection. Among these patients, the free concentration points for 4 individuals were found to be lower than the lower limit of quantification in 7 instances. The results indicated that the average total concentration after 12 hours of administration was 10.47 µg/mL, while the average free concentration was 272.22 ng/mL. Following MTX infusion, the ratio of the concentration of free drugs to the total concentration (F-MTX/T-MTX) ranges from 1.98% to 3.21%. As drug reservoirs, liposomes can continuously release free drugs at a controlled rate, indicating that there is a dynamic equilibrium between the release of F-MTX from the liposomes and its elimination from the tissues, thereby extending their circulation time.

In another Phase I clinical trial,[6] 17 non-Hodgkin lymphoma and advanced physical tumors were treated with MTX liposomes. Within 10 mg/m^2 dosage, the average C_{max} of the T-MTX in the patient was 6.69 µg/mL, and the C_{max} with the F-MTX was 27.11 ng/mL. The total concentration observed in our study was slightly higher than those reported in previous research, while the free concentration of MTX was significantly elevated compared to that in the phase I clinical trial. This discrepancy may be attributed to the larger dosage administered to patients in our study, along with physiological factors that could potentially influence blood concentration levels.

This study analyzed the relationship between the concentrations of mitoxantrone liposomes (T-MTX and F-MTX) and various physiological factors, as illustrated in Figure 7. The results indicated a significant correlation between gender and both the F-MTX (310.96 ng/ mL vs 217.99 ng/ mL) and T-MTX (11.87 µg/mL vs 8.40 ng/ mL) concentrations of mitoxantrone liposomes ($p< 0.05$). In contrast, body weight and age did not demonstrate statistically significant differences. Therefore, we proposed that gender might influence the blood concentration of mitoxantrone in vivo. Bavli et al found that the pharmacokinetic behavior of Pegylated liposomal doxorubicin was affected by gender.[19] Among 70 patients with physical tumors or Kaposi's sarcoma, the average removal rate for female patients was significantly lower than that for male patients (23.7±18.8 mL/h/m^2 vs 55.6±26.8 mL/h/m^2, $p< 0.0001$).[20] Estrogen could regulate the activation of monocytic phagocytosis (MPS), which in turn affected the removal of liposomes by MPS, subsequently influencing the blood concentration of lipid bodies and potentially leading to gender differences.[9,21] This study included a higher proportion of female patients (15/35), which was higher than the proportion of female patients in the previous study (4/17).[6] This might be one of the reasons for the increase in MTX concentration in the blood of

patients in this study. Liposuction demonstrates effective fat solubility and tissue penetration, while obese patients typically possess a greater body fat content.[22] Consequently, the distribution of liposomes differs between obese and normal patients.

Additionally, the influence of Body Mass Index (BMI) on the concentration of lipid body hemorrhage may be associated with the MPS, which can absorb anti-cancer drugs administered during liposuction.[16,23] Obesity is known to induce inflammation, leading to a more pronounced MPS function in obese patients. Although the correlation analysis revealed no significant relationship between mitoxantrone drug concentration and BMI ($p>0.05$), the free and total concentration levels were observed to be slightly higher in patients with BMI of 24 or greater compared to those with BMI of less than 24. We proposed that BMI might also serve as an influencing factor on the concentration levels of mitoxantrone liposomes. In this study, the physiological state of some patients deteriorated due to disease progression, resulting in lower fat content and subsequently higher drug concentrations. In contrast to the Phase I study that utilized MTX liposomes alone, the chemotherapy regimen administered to patients in this study comprised a diverse array of drugs. This variation introduced the potential for drug interactions and drug-related adverse reactions, which could also result in alterations to drug concentration levels.

This study has certain limitations. Due to the significant variability among individuals with tumors, there might be deviations in the measurement of blood concentration. Additionally, this study relied on opportunistic blood collection and few data from patients within 12 hours post-administration. Future research should aim to expand the sample size to enhance the robustness of the findings.

Conclusion

In conclusion, we established UPLC and UPLC-MS/MS methods for determining the total and free concentrations of mitoxantrone liposomes, respectively. The results indicated that these determination methods were highly efficient, accurate, and specific, making them suitable for monitoring blood concentrations and investigating the pharmacokinetics of mitoxantrone liposomes in patients with T-cell lymphoma. Correlation analysis revealed that gender might be an important factor influencing the concentration of mitoxantrone liposomes.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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