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ORIGINAL RESEARCH

A Novel Strategy for Liposomal Drug Separation in Plasma by TiO_2 Microspheres and Application in Pharmacokinetics

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Purpose: Liposomes are nano-scale materials with a biofilm-like structure. They have excellent biocompatibility and are increasingly useful in drug delivery systems. However, the in vivo fate of liposomal drugs is still unclear because existing bioanalytical methods for quantitation of total and liposomal-encapsulated drugs have limits. A novel strategy for liposomal-encapsulated drug separation from plasma was developed via the specific coordinate binding interaction of TiO_2 microspheres with the phosphate groups of liposomes.

Methods: Liposomal-encapsulated docetaxel was separated from plasma by TiO_2 microspheres and analyzed by the UPLC-MS/MS method. The amount of TiO_2, pH of the dilutions, plasma dilution factors and incubation time were optimized to improve extraction recovery. The characterization of the adsorption of liposome-encapsulated drugs by TiO_2 microspheres was observed by electron microscopy. For understanding the mechanism, pseudo-first and the pseudo-second order equations were proposed for the adsorption process. The study fully validated the method for quantitation of liposomal-encapsulated in plasma and the method was applied to the pharmacokinetic study of docetaxel liposomes.

Results: The encapsulated docetaxel had a concentration range of 15–4000 ng/mL from the plasma sample using a TiO_2 extraction method. Successful method validation proved the method was sensitive, selective and stable, and was suitable for quantitation of docetaxel liposomes in plasma samples. Extraction recovery of this method was higher than that of SPE method. As shown in electron microscopy, the liposomes adsorbed on TiO_2 microspheres were intact and there was no drug leakage. The study proposed pseudo-first and the pseudo-second order equations to facilitate the adsorption of liposomal drugs with TiO_2 microspheres. The proposed strategy supports the pharmacokinetic study of docetaxel liposomes in rats.

Conclusion: TiO_2 extraction method was stable, reproducible, and reliable for quantitation of encapsulated docetaxel. Because of versatility of lipids, it is expected to a universal bioanalysis method for the pharmacokinetic study of liposomes.

Keywords: liposomal-encapsulated drug, docetaxel liposomes, TiO_2 microspheres, coordinate binding, bioanalysis

Introduction

By 2021, a total of 24 liposomal drugs had been approved by the Food and Drug Administration (FDA) [1] and European Medicines Agency (EMA) [2] to treat a variety of diseases, including cancer, fungal infections, and hepatitis A.[3] Liposomes are composed of phospholipids, which are self-assembled in aqueous solution to form a spherical lipid bilayer membrane with a water core; sizes range from 50 to 450 nm.[4] This strategy can increase the solubility of the drugs in aqueous solution[5] and also improve biocompatibility and biodegradability. Liposomal formulations have been developed to improve the relative safety of encapsulated drugs by altering the balance of the distribution of that drug to targets with which it is intended to bind and those with which it is not intended to bind.[6]

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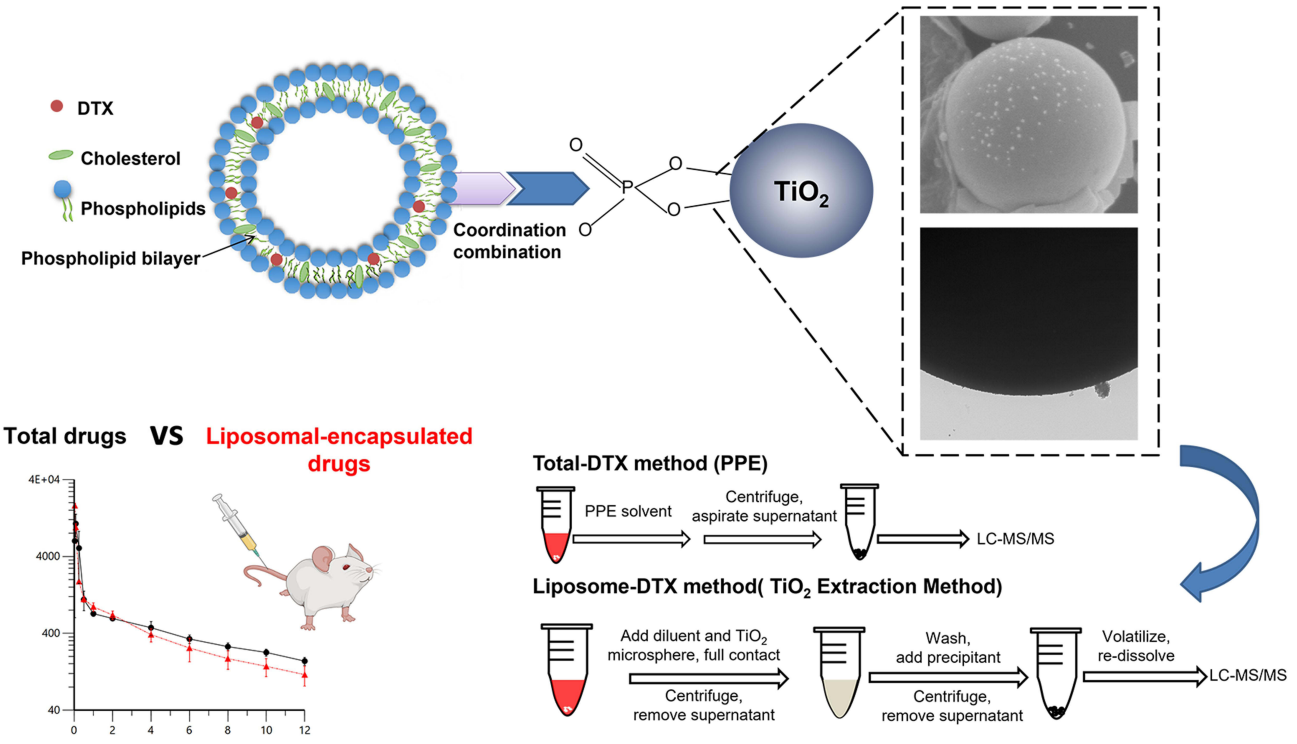
Graphical Abstract



The enhanced permeability and retention (EPR) effect enables the increased delivery of drugs to the tumor by liposomes.[6,7] Docetaxel (DTX) is a taxane and a semi-synthetic anticancer agent.[8] It plays a pivotal part in the treatment of solid tumors, those without cysts or liquid, and it has been applied in clinical trials to treat ovarian, breast, lung, and head/neck cancers.[9,10] Unfortunately, the clinical use of DTX is still limited owing to its low water solubility and serious adverse effects.[11,12] There are several patents on the preparation of docetaxel liposomes,[13] and these products are still in the preclinical phase because the in vivo fate of liposomal drugs is still unclear.

According the guidance of EMA,[2] FDA,[1] and National Medical Products Administration (NMPA) for liposomal drugs, a simple measurement of total drug substance concentration in plasma may not be reflective of bioavailability of the drug at the intended target organ as required for pharmacokinetic studies. Thus, bioanalytical methods specific to liposomes are needed to quantify encapsulated drugs in plasma. The most common methods to separate the total drug, liposomal-encapsulated drug, and free drug concentrations from plasma are manual solid phase extraction (SPE)[14] or ultrafiltration and protein precipitation extraction (PPE).[15] Unfortunately, these methods have limitations. Ultrafiltration of a free drug may bind to plasma proteins that can use a precipitate to carry down the drug with a liposomal-encapsulated drug. Ultrafiltration is also inconsistent owing to the loss of the drug by adsorption on the filter membrane. In the SPE procedure, free drugs are retained on the reversed-phase cartridges, but liposomes are not retained because of the liposomal surface's tendency to dissolve. Another major challenge in SPE is preventing a breakdown of the liposome structure during the separation of free drugs. Liposomal breakdown during SPE is mainly due to the low osmotic pressure in the cartridges. Liposomal breakdown leads to inaccurate results. In addition, the method requires multiple steps and a long separation time (up to 2 h). The complicated manufacturing procedure of SPE and the low throughput of these methods limit their large-scale applications in pharmacokinetic studies. The majority of SPE methods focus on the extraction of free drugs. Due to the small amount of free drug in liposomes, minor interferences induced during sample processing will lead to larger deviations of drug concentration. Thus, a rapid, efficient, and convenient encapsulated

drugs-based extraction sample processing technique is critical for bioanalysis of preclinical and clinical studies of liposomal drugs.

Here, we present a novel strategy for the easy separation of liposomal-encapsulated drugs from plasma. The strategy takes advantage of the coordinate binding between transition metals and the phosphate groups on the lipid bilayer of liposomes. Some metal oxides, such as titanium oxide (TiO_2), can reversibly bind with phosphate groups with high specificity.[16] By exploiting this property, TiO_2 can enrich phosphorylated peptides,[17] water-soluble organic phosphates,[18] and organophosphorus pesticides.[19] Thus, we used micron-sized TiO_2 particles (without modification) for enrichment of liposomal-encapsulated drugs in plasma for a pharmacokinetics study. The mechanism is coordinate binding between the phosphate groups on the surface of the lipid bilayer of liposomes and TiO_2. There was an improved rate of liposome extraction, lowered nonspecific adsorption, and a shorter sample processing time. Moreover, a biomimetic drug delivery system (C- TiO_2/TPZ@CM) exhibited remarkable biocompatibility without manifest damage and toxicity to the blood and major organs under the injection dose, which was showing that the use of TiO_2 was not only effective but also safe.[20] The process of TiO_2 microsphere and liposomal drugs absorption obeys pseudo-first-order kinetics, which is when a reaction is first-order in relation to two reactants, at high initial concentrations of the solute, and obeys a pseudo-second -order kinetics model, when a reaction is second-order in relation to two reactants, at lower initial concentrations of the solute.[21] The method was successfully applied in the pharmacokinetic study of DTX liposomes. This strategy focused on the phospholipid bilayer of encapsulated drugs; therefore, it can be developed into a general strategy for bioanalysis of liposomes.

Materials and Methods

Chemicals & Animal

Docetaxel liposomes were produced by TOT Biopharm Company Limited (Suzhou, China). This was a freeze-dried powder containing 7.5 mg pharmaceutical ingredient (API) per vial (p/n 100666–201704) with an encapsulation efficiency of >95%. The main components of the lipid bilayer of docetaxel liposomes (without modification) in this study are semi-synthetic lipid distearoylphosphatidylcholine (DSPC), cholesterone and a small amount of polyethylene glycol 2000-Distearoyl Phosphoethanolamine (PEG2000-DSPE, about two percent). The particle size, encapsulation efficiency, API content and the zeta potential were 183 nm, 91.7%, 7.5 mg per vial and −6.45mV, respectively. The internal standard (IS) stock solution and the chemotherapy drug paclitaxel (PTX,100382–201904, purity >99%) was purchased from the National Institute for Food and Drug Control (Beijing, China). Titanium dioxide microspheres (TiO_2) were purchased from GL Sciences Incorporated (pore diameter of 10 nm and particle size of 5 μm; Tokyo, Japan). Specific-pathogen-free (SPF)-grade Sprague Dawley male rats weighing 200–220 g were obtained from Beijing Charles River Laboratory Animal Technology Co., Ltd. (Beijing, China). Before drug administration, the rats fasted for 12 h with free access to water; 15 rats were then randomly placed into three groups. The experimental procedure was approved by the Animal Ethics Committee of Beijing Institute of Pharmacology and Toxicology (animal certification number was SCXK (Beijing) 2021–0011; the ethics number was IACUCDWZX-2021-764). All of the experiments involving animals observed the Guidelines for the Care and Use of Laboratory Animals.

Method Development

The liposomal-encapsulated DTX method (TiO_2 extraction method) was developed: Docetaxel liposomes plasma samples were mixed with TiO_2 microspheres. After incubation and washing, liposomal drugs were demulsified by MeOH. After the drug underwent centrifugation and ultrasound, the supernatant was dried under nitrogen gas, redissolved, and then transferred to the UPLC-MS/MS system for analysis. Various experimental conditions were optimized to improve the rate of liposome extraction from rat plasma including plasma sample dilution factors, ratio of TiO_2 to plasma, pH, and incubation time. Method validation used optimized conditions.

Preparation of Calibration Standards and Quality Control Samples

The DTX and IS stock solutions were prepared in dimethyl sulfoxide (DMSO) at 5 mg/mL. DTX liposome-suspended stock solutions had a concentration range of 1.27 mg/mL in sodium chloride. For the total DTX assay, the calibration standards were prepared in rat plasma at a concentration range of 7.5–4000 ng/mL. For the encapsulated DTX assay, the calibration standards were prepared in rat plasma at a concentration ranging from 15 to 4000 ng/mL. Calibration standards were prepared by spiking 95 μL of blank rat plasma with 5 μL of the corresponding working solutions to obtain final concentrations of 7.5, 15, 50, 250, 500, 1000, 2000, and 4000 ng/mL for total DTX as well as 15, 50, 250, 500, 1000, 2000, and 4000 ng/mL for liposomal-encapsulated DTX. Quality control (QC) samples, including the lower limit of quantification (LLOQ), low QC (LQC), medium QC (MQC), and high QC (HQC), were prepared similarly to how the calibration standards were prepared. The final concentrations of QCs were prepared at 7.5, 20, 1500, and 3000 ng/mL for total DTX and 15, 40, 1500, and 3000 ng/mL for liposomal-encapsulated DTX. In addition, the stock solution of IS was 5 μg/mL in MeOH.

Extraction Procedure

PPE was utilized for the total DTX extraction from rat plasma. Briefly, 20 μL of plasma samples underwent protein precipitation with 60 μL of MeOH. Following vortex-mixing for 2 min and centrifugation at 15,000 g for 10 min, the supernatant was transferred to the LC-MS/MS system for analysis.

TiO_2 extraction method was utilized for the DTX liposomal-encapsulated from rat plasma. First, 40 μL plasma samples were combined with 160 μL of buffer solutions (HAC or HAC-NaAC) and 3 mg TiO_2 microsphere. After the sample had been incubated at 4°C for 15 min, it was centrifuged at 2000 g for 15s. The precipitate was washed by PBS three times and centrifuged at 2000 g for 5 s each cleaning, the supernatant was discarded and the precipitate was TiO_2 microspheres adsorbed on liposomal-encapsulated drugs. The precipitate was then demulsified with 300 μL of MeOH. After the sample had been bath sonication for 10 min, liposomes adsorbed on precipitate burst and liposomal-encapsulated docetaxel dissolved sufficiently in MeOH, it was centrifuged at 15,000 g for 5 min. The supernatant was then evaporated to dryness and redissolved with 45 μL of 70:30 Acetonitrile: water (0.1% FA). The redissolved solution was then transferred to the LC-MS/MS system for analysis. The TiO_2 extraction method was applied to a wet ice bath for sample preparation and extractions. The principle of separation of liposome components with TiO_2 microspheres and schematic diagrams of the two extraction methods were followed by Figure 1.

UPLC-MS/MS Conditions

The UPLC-MS/MS (Agilent 1290–6410B, USA) conditions for the two methods were identical. A Shiseido C18 column (3 μm, 2.0 mm×50 mm) was used. The mobile phase was 0.1% FA in Water (A) and 0.1% FA in Acetonitrile (B) at the flow rate of 0.30 mL/min. The gradient elution was set as follows: 0–2.5 min, 25–95% B; 2.5–3 min, 95% B; 3–3.3 min, 25% B. The total time was 4.5 min. The sample volume was 10 μL. The optimized MS parameters included an ionization voltage of 4000 V, TEM 350°C, and atomizing air pressure 30 psi. Multiple reaction monitoring (MRM) mode for quantification of DTX was m/z 830.4 → m/z 304.1, PTX (IS) m/z 854.3 → m/z 286.1.

Characterization of TiO_2-Based Liposomes Extraction Samples

The characterization of the adsorption of liposome-encapsulated drugs by TiO_2 microspheres was observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM analysis, the TiO_2 microspheres were initially mixed with buffer solutions. Then, TiO_2 microspheres were mixed with buffer solutions and plasma sample. After incubation for 15 min and washed thrice with PBS, the samples were dried. The dried samples were spread on a double-sided conductive carbon tape and coated with gold utilizing a sputter device. Images were generated with a Hitachi S-3400N SEM. For transmission electron microscopy (TEM) analysis, the TiO_2 microspheres were mixed with buffer solutions and the plasma sample. After incubation for 15 min and being washed thrice with PBS, the samples were added onto copper grids for 5 min. The excess solution was eliminated by blotting the edge of every grid with filter paper. After that, the sample was

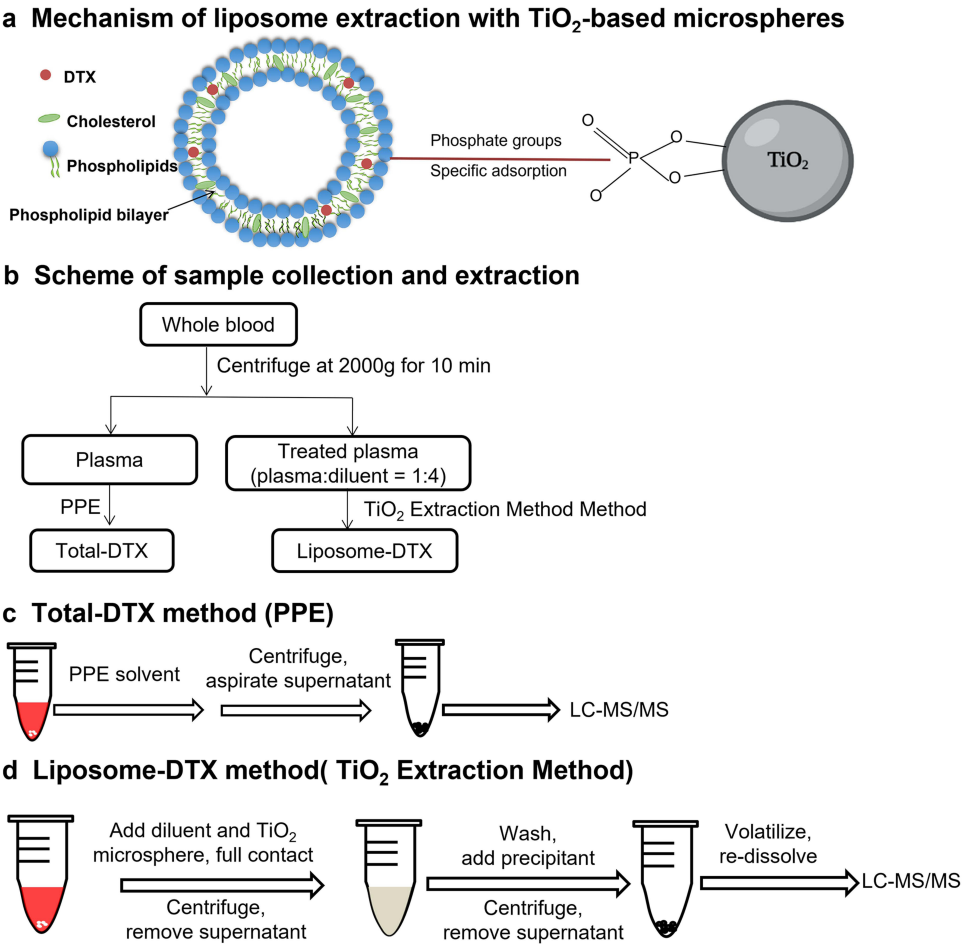


Figure 1 (**a**) Mechanism of liposome extraction with TiO_2-based microspheres and the plasma sample preparation procedures: (**b**) Sample collection and extraction; (**c**) Total DTX method (PPE); (**d**) Liposomal-encapsulated DTX method (TiO_2 extraction method).

negatively stained with phosphotungstic acid and incubated for 1 min. The unevaporated solution was absorbed with filter paper, and the grid was air-dried for TEM imaging. The sample was examined with a Hitachi HT7800 TEM.

Method Validation

Full validation of the two bioanalytical methods was performed according to the Chinese Pharmacopeia 9012 Quantitative Bioanalytical Method Validation Guidelines (2015).[22] The standard validation tests for each assay include sensitivity, linearity, intra- and inter-day accuracy and precision, rate of liposome extraction, the matrix effect, stability, and dilution reliability.

Pharmacokinetic Study

Briefly, after rats had adjusted to changes in the environment, they fasted for 12 h and then randomized to undergo treatment with iv 1, 2, and 4 mg/kg doses of DTX liposomes (n = 5/group, all were male animals). Blood samples were collected from the orbital vascular plexus. All DTX liposomes solutions were prepared in sodium chloride. Blood samples from these rats were collected in heparinized tubes at 0, 0.033,0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 h following iv. injection administration. Blood samples were centrifuged for 10 min at 1500 g after which plasma was collected and analyzed by the above methods.

Data Acquisition and Analysis

UPLC-MS/MS detection data was quantified via analysis software (Symantec, 15.0, USA). A $1/x^2$ weighted linear regression model was used to determine the relationship between concentrations and responses (peak area ratios of analyte to IS). Pharmacokinetic parameters were calculated by noncompartmental analysis using WinNonlin (version 8.1.0, Pharsight, USA). Data are given as mean ± standard deviation (S.D.) by Excel 2010. Statistical analysis was performed by Excel 2010.

Results

Characterization of TiO_2-Based Liposomes Extraction Samples

Before incubation with the liposomal solution, the TiO_2 microspheres have a clean surface and uniform spherical structure with a diameter of 5 μm followed by the SEM images (Figure 2a). After incubation, numerous vesicles with a particle size of approximately 100 nm (liposomes) were found adsorbed on the surface of the TiO_2 microspheres. TEM images showed that the captured vesicles have a typical intact spherical structure (Figure 2b). As shown in the SEM and TEM images, liposomes were obviously adsorbed on TiO_2 microspheres. The liposomes adsorbed on TiO_2 microspheres were intact and there was no drug leakage.

Mechanism of Liposomal-Encapsulated Drugs Separation from Plasma by TiO_2 Microspheres

Adsorption reactions were known to be important in many heterogeneous systems, and adsorption was a time-dependent process.[23] It was important to recognize the adsorption and desorption kinetics and determine the phenomenological coefficients characterizing their transport.[24] Various kinetics models have been suggested for adsorption.[25] The pseudo-first order equation and the pseudo-second order equation have been proposed for adsorption of drug cargo using TiO_2 microspheres for liposomal separation. The pseudo-first order rate equation of Lagergren was expressed as follows:

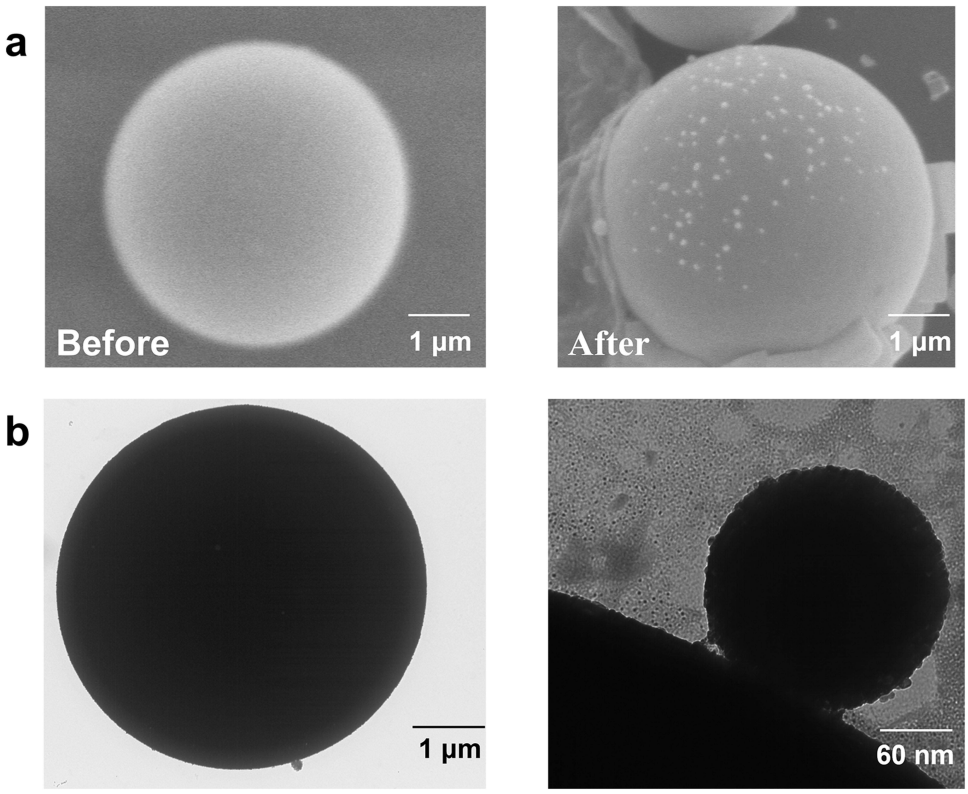


Figure 2 Characterization and validation of the TiO_2-based liposome samples: (**a**) SEM and (**b**) TEM images.

$$dq_t/dt = k_1(q_e - q_t) \quad (1)$$

Here, q_t and q_e are grams of liposomes adsorbed on each gram of TiO_2 at a specific time and at equilibrium, respectively (mg/g); and k_1 is the rate constant of first-order sorption (min^{-1}).

Following integration with the boundary conditions of t=0 to t=t and q_t=0 to q_t=q_t, Eq. 1 was rearranged to the following linear form:

$$\ln(q_e - q_t) = \ln q_e - k_1 t \quad (2)$$

The pseudo-second order rate equation is expressed as

$$dq_t/dt = k_2(q_e - q_t)^2 \quad (3)$$

Here, k_2 is the rate constant of second-order sorption (g/(mg ·min)).

After integrating with the boundary conditions of t=0 to t=t and q_t=0 to q_t=q_t, the integrated form of Eq. 3 can be re-written:

$$1/(q_e - q_t) = 1/q_e + k_2 t \quad (4)$$

Eq. 4 can be rewritten in linear form as

$$t/q_t = 1/k_2 q_e^2 + 1/q_e \quad (5)$$

Method Development

The study evaluated the optimal ratio of the volume of plasma and the amount of TiO_2 microspheres on the rate of liposome extraction as determined by the concentration of the QC samples of DTX liposomes (n = 3). As the amount of the TiO_2 microspheres increased from 1 mg to 50 mg, the maximum rate of liposome extraction was reached at 3 mg TiO_2 (Figure 3). Thus, the amount of the TiO_2 microspheres was held at 3 mg in 40 μL of plasma. Additional increases in the amount of TiO_2 led to a stable rate of liposome extraction, presumably owing to the difficulty of mixing a large amount of TiO_2 microspheres.

Acidic solvents are typically used to strengthen the binding between phosphate groups and TiO_2.[26] Therefore, the pH is a critical factor for the absorption of TiO_2 and liposomes. This study used acidic buffer solution to the improve the rate of liposome extraction with 3 mg TiO_2 microspheres in 40 μL of plasma. The pH value of the dilutions was from 2.5 to 7 and the rate of liposome extraction of the QC samples was highest at pH 3.

This study also evaluated the effect of the rate of liposome extraction on plasma sample dilution factors from 1 to 6. The maximum rate of liposome extraction from plasma was reached at a plasma sample dilution factor of four (using 3 mg TiO_2 microspheres in 40 μL of plasma).

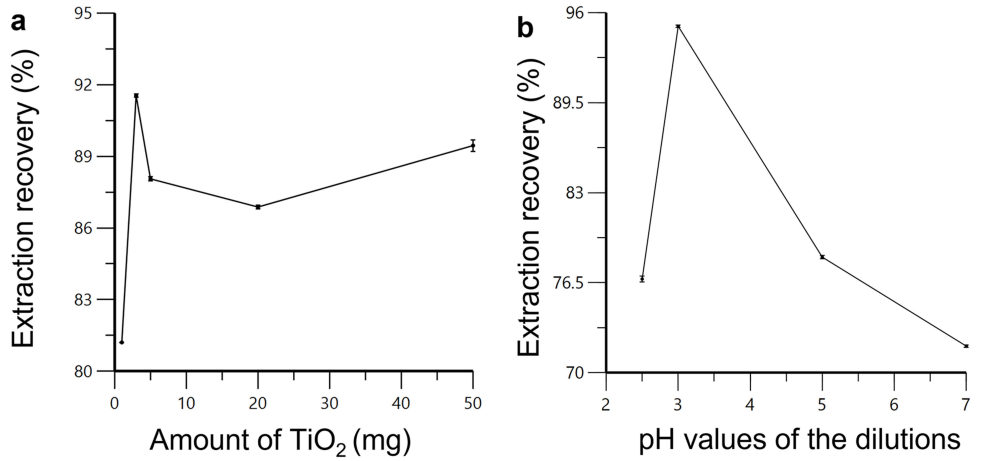


Figure 3 Optimization of the conditions for liposomes separation: (**a**) Rate of liposome extraction of DTX liposomes as a function of the amount of TiO_2; (**b**) pH values of the dilutions.

The rate of liposome extraction slightly increased as the incubation time was extended from 5 min to 60 min. Theoretically, a long incubation time led to high rate of liposome extraction, but it may also lead to a rupture of liposomes and a reduced rate of liposome extraction. Based on a comprehensive consideration of the time requirements and the rate of liposome extraction, 15 min was selected as the optimal incubation time, producing a sufficiently high rate of liposome extraction (Table 1).

Method Validation

Linearity and Sensitivity

Fresh calibration curves with samples of different concentration were prepared daily to validate the two assays. The LLOQ for total DTX and encapsulated DTX assays were 7.5 and 15 ng/mL in plasma, respectively. The representative chromatograms are shown in Figure 4. Linear regression with weighting at 1/concentration^2 was chosen for the analyte in the two assays. The calculated coefficient of determination (R^2) values were utilized to determine the fit of the curves. The calibration curve range for total DTX and encapsulated DTX was 7.5–4000 and 7.5–4000 ng/mL with the regression equation of $y = 0.014 \pm 0.002x + 0.082 \pm 0.014$ and $y = 0.007 \pm 0.002x - 0.078 \pm 0.015$, respectively. For all five assays, the R^2 were ≥0.99, the % CV were ≤9.93, and the % bias was −11.15–7.11 for total and encapsulated DTX as shown in Table 2.

Intra- and Inter-Day Accuracy and Precision

Four (for total and encapsulated DTX drugs) levels of QCs were extracted and analyzed in five parallel measurements during three accuracy and precision runs performed over two different days. This led to intra- and inter-day accuracy and precision evaluations. The precision was expressed as a percentage of the relative standard deviation (% CV). The accuracy was expressed as a percentage of the relative error from the nominal concentration (% bias). The % CV and % bias were all within the acceptance criteria (Table 3), thus demonstrating excellent inter- and intra-day accuracy and precision.

Selectivity and Matrix Effect

The method was evaluated for selectivity with five lots of matrix blanks and selectivity QCs prepared in different lots of plasma samples from five rats. None of the five selectivity blank samples had detectable peaks at the retention times of the analyte of interest. Matrix effect were evaluated conducted at three concentration levels (LQC, MQC, and HQC) using the same blank rat plasmas from five different rats to evaluate the suppression or enhancement of analyte ionization from components. IS-normalized matrix effect was calculated by comparing the peak area ratio of analyte/IS of the extracted samples with that of the corresponding post-extraction spiked samples. The matrix effect for docetaxel liposome QC samples ranged from 91.00% to 95.66% for total DTX and from 85.95% to 90.87% for encapsulated DTX. % CV values were <14.97 (Table 4), thus suggesting that

Table 1 Optimization of the Conditions for DTX Liposome Extraction

Optimized Conditions	Encapsulated DTX		
	LQC (40 ng/mL)	MQC (300 ng/mL)	HQC (3000 ng/mL)
Rate of Liposome Extraction (%) 1) plasma sample dilution factors			
1 4 6	53.18±3.58 52.25±5.33 46.23±4.00	92.1±8.87 93.84±6.02 98.23±6.04	86.9±6.43 97.67±3.48 80.56±4.67
2) Incubation time			
5 min 15 min 60 min	75.18±4.58 90.9±8.83 78.05±5.25	96.45±9.33 99.56±3.91 99.38±5.37	87.96±9.89 96.7±3.53 91.29±3.79

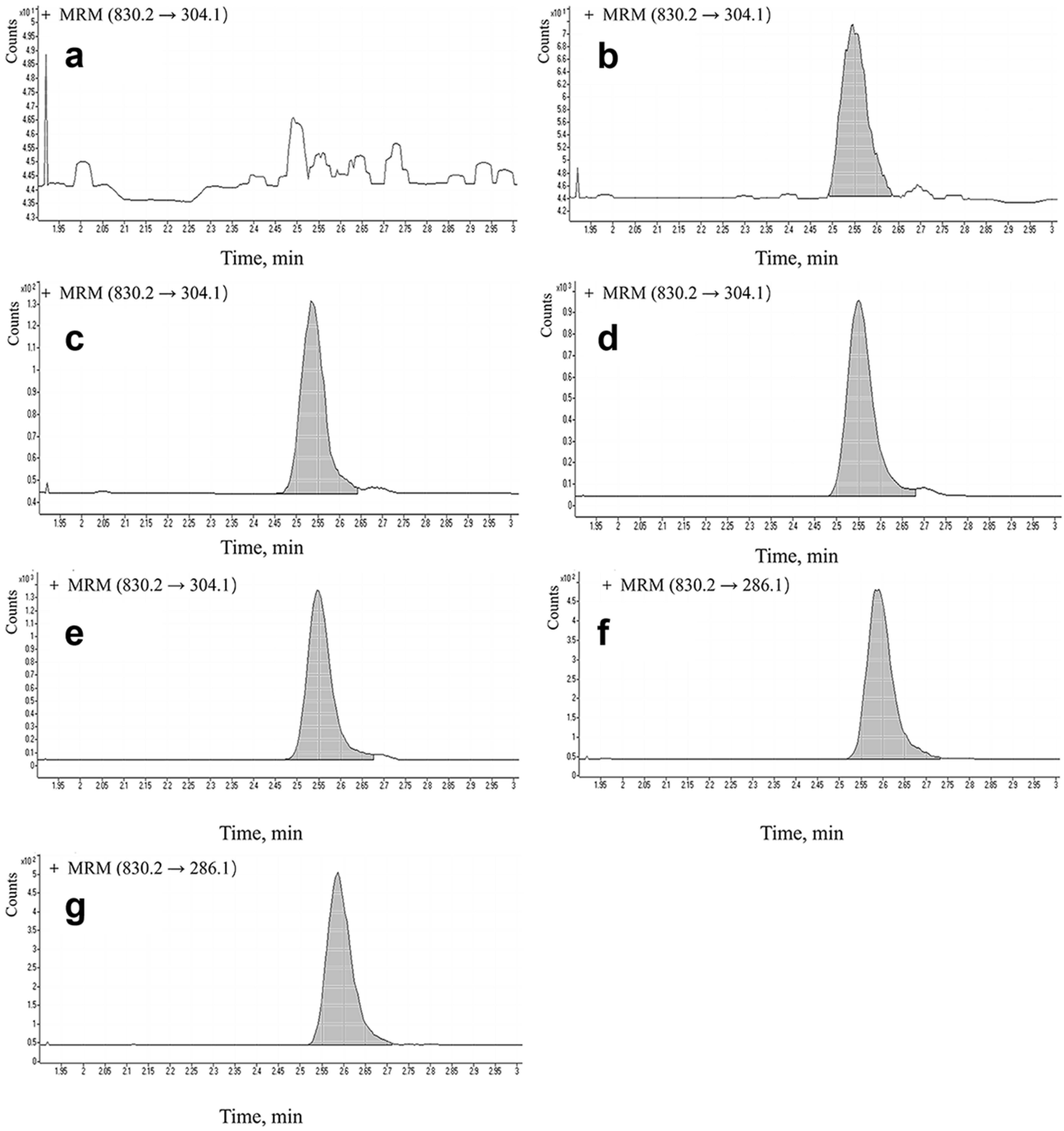


Figure 4 Representative chromatograms of all analytes in the two assays: (**a**) Blank sample; (**b**) LLOQ (7.5 ng/mL) of encapsulated DTX; (**c**) LLOQ (15 ng/mL) of total DTX; (**d**) Rat plasma sample of encapsulated DTX; (**e**) Rat plasma sample of total DTX; (**f**) IS of encapsulated DTX; (**g**) IS of total DTX.

every one of the assays was accurate, reproducible, and had no matrix effects for measuring the analytes in rat plasma.

Rate of Liposome Extraction

The rates of extraction of total and encapsulated DTX were evaluated at low, medium, and high QC concentration levels. The relative rate of extraction was evaluated by comparing the peak areas of test samples spiked with analyte and IS before to extraction versus after extraction. The average relative extraction recoveries for total DTX were all between

Table 2 Back-Calculated Concentrations of Calibration Standards for All Five Assays (All of the Concentrations are Expressed as Ng/mL)

1) Back-Calculated Concentrations of Calibration Standards for Total DTX (Linear Weighted $1/x^2$)								
Run Number	**7.5**	**15**	**50**	**250**	**500**	**1000**	**2000**	**4000**
1	7.18	16.37	46.62	244.83	549.65	981.44	2108.20	3592.99
2	8.11	16.85	45.62	232.79	470.26	1015.47	2263.63	3966.70
3	7.15	12.98	44.71	261.39	481.64	1110.29	2148.13	3864.57
4	7.23	16.07	52.92	248.31	437.69	1049.22	2053.83	3873.35
5	7.07	16.35	54.57	234.22	505.12	1104.28	1792.80	3712.03
Mean±SD	7.35±0.43	15.72±1.56	48.89±4.52	244.31±11.65	488.87±41.76	1052.122±55.77	2703.32±174.71	3801.93±148.25
% CV	5.85	9.93	9.25	4.77	8.54	5.30	8.43	3.90
% Bias	−2.03	4.82	−2.23	−2.28	−2.23	5.21	3.67	−4.95
2) Back-Calculated Concentrations of Calibration Standards for Encapsulated DTX (Linear Weighted $1/x^2$)								
Run Number		15	50	250	500	1000	2000	4000
1		15.61	43.59	244.84	449.93	1104.20	2207.22	4003.05
2		15.36	46.39	235.65	487.64	1088.37	2114.60	3937.87
3		15.6	42.97	261.58	465.99	1055.06	2164.18	3940.17
4		15.46	43.60	273.39	538.69	994.83	2070.80	3584.54
5		15.36	45.56	250.25	529.49	1052.10	2153.93	3503.42
Mean±SD		15.48±0.12	44.42±1.47	253.14±14.70	494.35±38.80	1058.91±42.09	2142.15±51.74	3793.81±231.34
% CV		0.79	3.31	5.81	7.85	3.98	2.42	6.10
% Bias		3.18	−11.15	1.26	−1.13	5.89	7.11	−5.15

Table 3 Intra- and Inter-Run Accuracy and Precision of QC Samples for All of the Analytes in Five Assays

Analyte	**Concentration (ng/mL)**		**% CV**		**% Bias**
	Added	**Measured Conc**	**Intra-Day**	**Inter-Day**	
Total DTX	LLOQ	7.58±0.44	6.41	5.84	1.07
	LQC	20.22±1.10	7.88	5.43	1.10
	MQC	301.13±16.11	4.04	5.35	0.38
	HQC	2938.77±113.67	3.53	3.87	−2.04
Encapsulated DTX	LLOQ	16.28±1.40	9.55	6.42	8.53
	LQC	41.14±3.67	8.10	8.41	2.85
	MQC	315.61±20.21	5.90	4.78	5.20
	HQC	3185.81±169.61	2.36	4.35	6.19

Table 4 Matrix Effect and Rate of Liposome Extraction for Total DTX and Encapsulated DTX in Rat Plasma (n = 5 per Assay)

Analyte	**Concentration (ng/mL)**	**Rate of Liposome Extraction (%)**		**Matrix Effect (%)**	
		Mean±SD	**% CV**	**Mean±SD**	**% CV**
Total DTX	LQC	96.81±9.30	8.59	95.66±10.21	9.55
	MQC	92.08±14.71	14.29	93.16±15.59	14.97
	HQC	94.06±8.36	7.95	91.00±8.38	8.24
Encapsulated DTX	LQC	95.87±4.01	3.74	85.95±2.82	3.28
	MQC	96.40±5.32	4.93	90.87±9.60	10.56
	HQC	93.39±4.47	4.28	89.72±7.95	8.86

92.08% and 96.81%; encapsulated DTX was between 93.39% and 96.4%. % CV values of the rate of extraction of DTX liposomes were within ± 15 and could meet the requirements of biological sample determination (Table 4).

Stability and Dilution Reliability

DTX liposomes stability in rat plasma samples was tested for three different QC sample concentrations (Table 5), indicating that all of the samples remained stable in plasma following 12 h of storage at 4°C, 15 days of storage at −20°C, or one round of freeze-thawing for total DTX and liposomal-encapsulated drugs. % CV and % Bias values of the rate of extraction of DTX liposomes were within ± 15 and could meet the requirements of sample bioanalysis. The result indicates that when the sample concentration exceeded the upper limit of the quantitation level, it was reliably diluted by 20-fold with blank plasma before extraction.

Pharmacokinetic Studies

The two fully validated methods were used to support a preclinical pharmacokinetic study of DTX liposomes in rats. The pharmacokinetic parameters were calculated using the non-compartment model (Table 6). The plasma concentration-time profiles of DTX liposomes after intravenous administration in rats are shown in Figure 5. The AUC increased in a nonlinear fashion with increasing dose for both total and encapsulated DTX liposomes. There were significant differences in most pharmacokinetic parameters for total and encapsulated DTX, but there were no significant differences in V_{ss} at 1 mg/kg and 2 mg/kg and $T_{1/2}$ for 1 mg/kg. The dose-proportionality of DTX liposomes is shown in Table 7.

Discussion

The TiO_2 microsphere extraction method is a new strategy to separate liposomal drugs in plasma. It can successfully separate encapsulated drugs from liposomes in plasma. The concentration of free drugs can be determined in the supernatant after washing with PBS and centrifuging, and the concentration of total drugs can also be measured in our study. It was fast, low cost, and convenient.

Table 5 Stability of DTX Liposomes in Rat Plasma (n = 5 per Assay)

Experiment	Added Concentration	4°C Stability for 12 h		Freeze-Thaw Stability for One Cycle		−20°C for 15 Days	
		Measured (ng/mL)	% Bias	Measured (ng/mL)	% Bias	Measured (ng/mL)	% Bias
Total DTX	LQC (20 ng/mL)	21.15±0.67	5.77	20.86±1.35	4.30	19.96±2.39	−0.21
	MQC (300 ng/mL)	318.64±20.59	6.21	296.21±22.70	−1.26	314.05±26.92	4.68
	HQC (3000 ng/mL)	2717.15±45.64	−9.43	2619.00±58.59	−12.70	2804.16±147.13	6.53
Encapsulated DTX	LQC (40 ng/mL)	39.16±1.96	−2.09	35.85±1.78	10.37	41.66±3.23	4.14
	MQC (300 ng/mL)	288.06±21.21	−3.98	282.95±25.22	−5.68	316.53±24.84	3.51
	HQC (3000 ng/mL)	2817.80±131.40	−6.07	2754.08±84.58	−8.20	3074.72±127.87	2.49

Table 6 Pharmacokinetic Parameters of DTX Liposomes in Rats (n = 5 per Group)

PK Parameters	Total DTX			Encapsulated DTX		
	1 (mg/kg)	2 (mg/kg)	4 (mg/kg)	1 (mg/kg)	2 (mg/kg)	4 (mg/kg)
$T_{1/2}$ (h)	5.93±2.03	4.94±1.16[b]	13.48±1.99[c]	3.79±1.59	2.78±0.50	5.81±0.52
Cmax (ng/mL)	6635.20±1519.51[a]	12,783.50±387.01[b]	18,382.21±1356.27[c]	5621.26±499.09	9201.27±1302.95	13,862.73±1247.71
$AUC_{0-\infty}$ (ng·h/mL)	3462.09±1020.50[a]	4373.71±922.13[b]	7788.37±552.33[c]	1351.12±191.70	2448.25±441.87	5765.83±226.00
AUC_{0-t} (ng·h/mL)	4195.16±954.26[a]	5053.47±1344.22[b]	9157.73±567.99[c]	2147.55±549.38	2943.16±455.66	7008.26±284.15
CL (mL/h·kg)	249.24±60.90[a]	417.83±106.19[b]	438.17±27.83[c]	493.71±142.38	693.23±111.08	571.50±22.87
MRT (h)	3.18±0.57[a]	2.69±0.50[b]	4.77±0.50[c]	0.89±0.28	1.15±0.23	3.23±0.11
Vss (mL/kg)	1641.24±849.84	1880.96±335.28	4639.83±598.03[c]	1755.24±457.34	1718.58±287.64	3583.92±185.63

Notes: [a]$p < 0.01$ was statistically significant versus liposomal-encapsulated DTX group at 1 mg/kg, [b]$p < 0.01$ was statistically significant versus liposomal-encapsulated DTX group at 2 mg/kg, [c]$p < 0.01$ was statistically significant versus liposomal-encapsulated DTX group at 4 mg/kg.

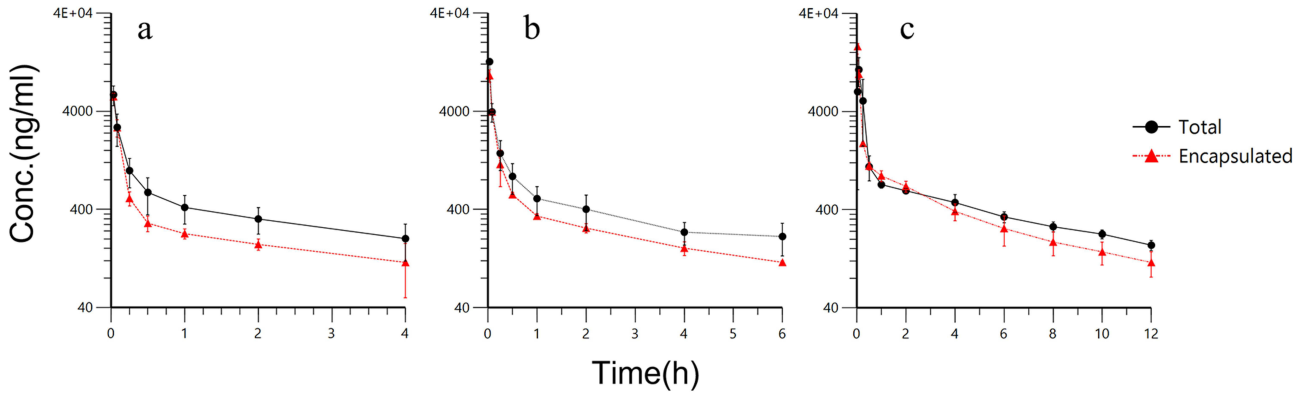


Figure 5 Concentration-time curves for total drugs and liposomal-encapsulated drugs after intravenous administration of DTX liposomes in rat. (**a**) 1 mg/kg; (**b**) 2 mg/kg, and (**c**) 4 mg/kg (n = 5).

Liposomes are composed of lipids and/or other amphiphilic molecules.[27] The lipid bilayer is composed of amphiphilic phospholipids with hydrophobic tails and hydrophilic phosphate heads. In biological systems, the hydrophilic phosphate head of the phospholipids is exposed on the outer surface of the lipid bilayer.[28] The extraction of liposomes from plasma by TiO_2 is based on the complexation of transition metals and phospholipids.[29] It is well known that some metal oxides such as TiO_2 (TiO_2 is a highly biocompatible material[30]) can reversibly bind with phosphate groups with high specificity. The phosphate groups in these lipids directly bind to the TiO_2 surface, thus forming strong chemical interactions. When liposomes were mixed with TiO_2 microspheres, the liposomes remain spherical and are only adsorbed —there is no fusion.[31] The pH is a critical factor for the absorption of TiO_2 and liposomes. The adsorption of phospholipid and TiO_2 increased dramatically in the acidic environment, and the pH of the dilutions were thus optimized. The isoelectric point of TiO_2 nanoparticles is between 4 and 6.[32] In the solution with pH of 3, TiO_2 was positively charged because the solution holds H^+ on the hydroxyl group on the particle surface. The phosphate group was negatively charged, which made it easier to combine with TiO_2. According to the pseudo-first and pseudo-second order rate equations, the absorption was independent of particle size. It was reported that titanium dioxide particles with different sizes, crystal forms, and surface roughness had a similar effect on the rate of liposome extraction.[29] In addition, the commoditized products are more stable. Therefore, the particle size of TiO_2 optimization was not performed here.

Existing bioanalytical methods to measure docetaxel liposomes in plasma normally use PPE or SPE extraction; only the total drug concentrations are determined. Luo[8] et al reported that total DTX of liposome in rat plasma could be evaluated with a calibration curve from 3.25 to 26,000 ng/mL. Wang[33] et al reported that the total DTX of liposomes in plasma could be evaluated with a calibration curve from 2 to 1000 ng/mL. Our study also developed SPE method to detect total and liposomal-encapsulated DTX, the rate of extraction of liposomal-encapsulated DTX was higher via TiO_2 microsphere extraction method which was between 93.39% and 96.4% for TiO_2 extraction method and between 70.20% and 72.43% for SPE method. The quantitation of total DTX used only one method, which was protein precipitation method. (Data on SPE method have been summarized in Supplementary Material (Table S1)).

Luo[8] et al reported a pharmacokinetic study after DTX or DTX liposome iv. administration at dose of 2.5 mg/kg in rats. The $T_{1/2}$ was 4.35 h, the AUC was 1677.76 μg/l·h, and the clearance from the body (CL) was 1.52 L/kg·h in 2.5 mg/kg for DTX injection. Our study $T_{1/2}$ was 4.94 h, AUC was 4373.71 μg/l·h, and CL was 0.42 L/kg·h in 2.0 mg/kg for total DTX of liposomes. Versus conventional injections, DTX liposomes could be removed more slowly from the

Table 7 Summary of Statistical Analysis for Dose-Proportionality for 1 Mg/Kg, 2 Mg/Kg, and 4 Mg/Kg of DTX Liposomes

	Parameter	Predicted Geometric Mean	Slope Estimate (90% CI)	Rdnm (90% CI)	Conclusion
Total DTX	Cmax (ng·mL^{-1})	(9228.02, 26,100.79)	0.75(−0.27, 1.78)	0.71(0.17–2.95)	Not conclusive
	AUC (ng·h·mL^{-1})	(3165.29, 7373.44)	0.61(0.43–0.78)	0.58(0.45, 0.74)	Not conclusive
Encapsulated DTX	Cmax (ng·mL^{-1})	(4914.77, 14,291.88)	0.77(0.56, 1.00)	0.73(0.51–1.00)	Inconclusive
	AUC (ng·h·mL^{-1})	(1274.11, 5462.21)	1.05(0.93–1.18)	1.07(0.91, 1.28)	Conclusive

circulation, which proved that liposomes prolong the active lifetime. Luo[8] et al reported that $T_{1/2}$ was 2.97 h, AUC was 2608.3 μg/l·h, and CL was 1.09 L/kg·h in 2.5 mg/kg for DTX liposomes. In our study, the $T_{1/2}$ was 2.78 h, AUC was 2448.25 μg·h/l, and CL was 0.69 L/kg·h in 2 mg/kg for liposomal-encapsulated DTX with the TiO_2 extraction method. The DTX liposomes in our study resulted in an increase in the AUC, $T_{1/2}$, and correlated with a decrease in the CL. This means that they have a longer circulation and greater proportion of the drug in circulation than the DTX liposomes in this study.

Here, the $T_{1/2}$ and AUC of total drugs in rat plasma increased in contrast to liposomal-encapsulated DTX, thus indicating the slow drug release and slow CL of DTX liposomes. This feature can increase the retention time of DTX in circulation in the body and improve the drug's effects. These properties demonstrate that liposomes offer continuous and stable drug release. Liposomes can release slowly, decrease toxicity, and lower side effects. These data described the disposal process of different morphologies of liposomes in vivo. Our pharmacokinetic study of docetaxel liposomes proved the reliability of this novel bioanalytical method for liposomal drugs.

Conclusion

This paper reported the direct analysis of total and liposomal-encapsulated drugs in plasma using a TiO_2 microspheres extraction method and protein precipitation. It was time-saving, low cost, and convenient, and the rate of liposome extraction of this method was higher than that of the SPE method. We elaborated on the mechanism of action underlying the separation of liposomal-encapsulated drugs via TiO_2 microspheres. As shown in the SEM and TEM images, the liposomes adsorbed on TiO_2 microspheres were intact and there was no drug leakage. The current method was obtained via optimization. The amount of the TiO_2, pH of the dilutions, plasma dilution factors, incubation time and liquid chromatography conditions were optimal to improve the rate of liposome extraction. Two methods were developed and fully validated for direct quantitation of encapsulated and total docetaxel concentrations in rat plasma according to relevant regulatory guidance. Full validations were carefully designed and executed with two methods. Excellent method validation results were obtained for the two methods, thus suggesting that the two methods were accurate, rugged, and reproducible. The methods were application in a pharmacokinetic study of DTX liposomes in rats. The method developed here focuses on extraction of the phosphate groups on the lipid bilayer of liposomes different from current methods. Therefore, this method was suitable for different liposomal drugs based on the coordination bonding principle between phospholipids and transition metals, therefore it can be developed into a universal method.

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Disclosure

The authors report no conflicts of interest in this work.

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