

A Rapid and Sensitive HPLC Method for Quantitation of Paclitaxel in Biological Samples using Liquid-Liquid Extraction and UV Detection: Application to Pharmacokinetics and Tissues Distribution Study of Paclitaxel Loaded Targeted Polymeric Micelles in Tumor Bearing Mice

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ABSTRACT - A simple, rapid, and sensitive reversed-phase HPLC method was developed and validated for determination of paclitaxel (PTX) in plasma, various organs and tumor tissues of tumor-bearing mice. Tissue specimens of liver, kidneys, spleen, lungs, heart and tumor were separately homogenized in normal saline. Plasma or tissue homogenate (250 μ l) containing PTX and internal standard (diazepam) were extracted by diethyl ether (6 ml). The separation was achieved on a μ -Bondapak C₁₈ HPLC column using sodium acetate buffer solution (0.01 M)/acetonitrile (58/42 v/v) at pH 5 \pm 0.1 and flow rate of 1.9 mL/min. The effluent was monitored at 227 nm and column temperature was adjusted at 58°C. The internal standard and PTX were eluted at 4.2 and 5.2 min, respectively and no interfering peaks were observed. Calibration curves were linear over the concentration range of 0.25-10 μ g/ml of PTX in plasma and 0.3-20 μ g/ml PTX in tissue homogenates with acceptable precision and accuracy (<15%). The mean recoveries of the drug after plasma extraction was 87.4% \pm 3.6 while those of tissue homogenates ranged from 62.1 \pm 4.5 to 75.5 \pm 3.2 depending on the type of tissues studied. PTX was stable in samples with no evidence of degradation during 3 freeze-thaw cycles and 3 months storage at -70 °C. The developed HPLC method was applied to quantify PTX in the mouse plasma and tissues after intravenous administration of 10 mg equivalent PTX/Kg dose of PTX-loaded tocopherol succinate-chitosan-polyethylene glycol-folate (TS-CS-PEG-FA) micelles formulation or Anzntax[®] (Cremophor[®] EL- based formulation of PTX) to female Balb/c mice.

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INTRODUCTION

Paclitaxel (PTX), a semisynthetic antineoplastic agent, has been successfully used in the treatment of a broad spectrum of human cancers, especially in advanced carcinomas of the lungs, breast and ovary (1). The drug is an inhibitor of microtubule depolymerization that blocks cell cycle transition from G₂-phase to M-phase (1, 2). Because of the low water solubility, PTX is commercially available in a solution composed of a 50:50 (v/v) mixture of Cremophor[®] EL (polyethoxylated castor oil) and dehydrated ethanol known as Taxol[®]. Unfortunately, Cremophor[®] EL induces some serious adverse reactions such as hypersensitivity, nephrotoxicity, and neurotoxicity after intravenous administration (1, 3). Hence, many attempts have so far been

conducted to replace Cremophor[®] EL-based formulations with alternative carriers enable to deliver PTX selectively to the tumor tissues without causing adverse effects to healthy tissues. In this direction, numerous drug delivery carriers including emulsions (4, 5), liposomes (6-8), nanoparticles (9-11), and polymeric micelles (12-15) have been extensively studied for delivery of PTX into the cancer cells via parenteral administration. We have recently developed a novel targeted polymeric micelle formulation, through synthesizing tocopherol succinate-chitosan-polyethylene glycol-

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folate (TS-CS-PEG-FA), for delivering of PTX into cancer cells (16). PTX-loaded micelles (PTX/TS-CS-PEG-FA) exhibited decreased toxicity and increased efficacy compared to Anzatax[®] (Cremophor[®] EL- based formulation of PTX) in a breast tumor model in Balb/c mice (16). The current study details a high performance liquid chromatography (HPLC) method to quantify PTX concentrations in mouse plasma and tissues following intravenous administration of PTX/TS-CS-PEG-FA micelles in tumor-bearing mice. The separation and analysis of PTX in biological fluids and tissues has been mainly performed by HPLC techniques using different clean-up procedures including protein precipitation (PP) (17-20), solid-phase extraction (SPE) (21, 22), and multiple step of liquid-liquid extraction (LLE) (23-28). Although direct injection of samples after precipitation of the plasma proteins does not require an evaporation step and make the procedure rapid and simple, this method could deteriorate the chromatographic column due to inadequate protein precipitation. Endogenous compounds can also overload the column which interfere with the peak of interest or appear as late eluting peaks and consequently lead to a long run time. Additionally, sample dilution, which occurs after deproteinization, reduces the sensitivity of the assay (29). To increase the column durability and efficiency and to remove the interferents coming from proteins, the use of SPE has been reported (21, 22). SPE technique is a fairly expensive procedure and suffers from sorbent drying between washing procedures resulting in cracking of the packing materials (22). In most instances, LLE has been considered as the main procedure for the isolation and determination of drug substances in biological matrices. Several publications have appeared with regard to HPLC determination of PTX in biological fluids after LLE (23-28). Some assays employ a relatively large volume (0.5-2 ml) of biological sample to maximize the sensitivity of the assays (24, 25). Some other techniques were not validated according to the International Conference on Harmonization (ICH) guidelines (26, 27). Kim et al, developed an HPLC method for PTX analysis that minimized the sample volume and also achieved a higher sensitivity with a limit of detection (LOD) of 5 ng/ml in plasma (28). They used a small volume of plasma sample (100 µl) and intra- and inter-day variation of the assay were within 9.35 and 8.33%, respectively. To the best of our knowledge, protein precipitation of tissue homogenates following liquid

chromatographic tandem mass spectrometric (LC-MS) method has been the only successful method for quantification of PTX in tissue samples (30-32). Though, LC-MS is more responsive and explicit than HPLC-UV, this technique involves expensive equipment which is not affordable at most laboratories. In the present study, we therefore describe a validated, rapid, simple, and yet sensitive HPLC method to quantitate PTX concentrations in mouse plasma and tissues to characterize pharmacokinetics and tissue distribution of the drug. This method was validated for its accuracy, precision, LOD and limit of quantitation (LOQ) as per ICH guide-lines. The validated method is selective, sensitive and suitable for the measurement of PTX in the mouse plasma and tissues following intravenous administration of PTX self-assembled TS-CS-PEG-FA micelle formulations or Anzatax[®] to tumor bearing Balb/c mice.

MATERIAL AND METHODS

Chemicals and Reagents

Paclitaxel (PTX) and diazepam were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol, acetonitrile and diethyl ether were procured from Caledon (Ontario, Canada). All reagents and solutions were either HPLC or analytical grades. PTX-loaded tocopherol succinate-chitosan-polyethylene glycol-folic acid (PTX/TS-CS-PEG-FA) micelles were prepared in our laboratory. Anzatax[®] (commercial formulation of PTX) (30 mg/5 ml) was provided by Hospira (Mulgrave, Australia).

Chromatographic conditions

The apparatus used was a Waters HPLC system model 746 (Milford, US) consisting of a model 515 intelligent solvent delivery pump, a 100 µl injection loop, a computerized system controller, and a Waters 2487 UV detector. Chromatographic separation was achieved using a µ-Bondapak C₁₈ column (3.9 mm × 250 mm Waters, Ireland) heated at 58°C. The mobile phase consisted of acetate buffer (0.01 M)/acetonitrile at 58/42 (pH, 5 ± 0.1) eluted at flow rate 1.9 ml/min. Column effluent was detected at 227 nm with a UV detector.

Standard solutions of paclitaxel and internal standard

Standard stock solution of PTX was prepared at 500 µg/ml in methanol. A series of working solutions at

concentrations of 2.5, 5, 10, 25, 50, 75, 100 µg/ml were prepared by further dilution of the standard stock solution in methanol. Stock solution of diazepam (internal standard) at a concentration of 25 µg/ml was prepared in methanol.

Standard samples of plasma and tissues

Plasma: Plasma specimen was collected from Balb/c mice obtained from Laboratory Animal Resource Center of Faculty of Pharmacy and Pharmaceutical Science (Isfahan, Iran) and stored at $-70\text{ }^{\circ}\text{C} \pm 15\text{ }^{\circ}\text{C}$ until the use. To construct a calibration curve, 25 µl of PTX working solutions were added to 250 µl of blank plasma to obtain PTX standard concentrations ranging from 0.25 to 10 µg/ml in plasma.

Tissue homogenates: heart, kidney, liver, lung, and spleen tissues of Balb/c mice were homogenized using a Silent Crusher S Homogenizer (Heidolph, Germany) with 2-fold weight of normal saline. 250 µl aliquots of homogenates were spiked with 25 µl of PTX working solutions to prepare homogenates with PTX concentrations ranging from 0.3 to 20 µg/ml.

Sample preparation

Liquid-liquid extraction with diethyl ether was used to extract the drug from plasma or tissue preparation. Mouse plasma standards, tissues homogenate standards, and unknown samples (250 µl of plasma and tissue homogenates of mice received Anzatak[®] or PTX/TS-CS-PEG-FA intravenously) were spiked with 25 µl of internal standard solution and 50 µl of sodium acetate buffer (pH: 5) and mixed with 6 ml diethyl ether as extracting solvent. Sample tubes were vortexed for 2 min followed by 10 min centrifugation at 5000 rpm. The upper organic layer was transferred to the clean tubes and evaporated to dryness under nitrogen gas. The residue was reconstituted in 100 µl of mobile phase and 80 µl aliquot was injected into the HPLC column.

Method validation

Linearity

Calibration plots were constructed by plotting the ratio of the PTX peak area to that of internal standard, using five different concentrations of each sample on five separate days. Distribution of the residuals (% difference of the back-calculated concentration from the nominal concentration) was determined to validate the correlation. The calibration model will be accepted if the residuals are

within $\pm 20\%$ for lower limit of quantification and within 15 % for all other calibration levels and at least 2/3 of the standards should meet this criterion. The calibration curves were evaluated by correlation coefficient, slope, and intercept.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The parameter LOD was determined using the signal-to-noise ratio by comparing the results of test samples with known concentrations of analyte to the blank samples. The analyte concentration that produced a signal-to-noise ratio of 3:1 was accepted as the LOD. The LOQ was identified as the lowest plasma and tissue concentrations of the standard curves that could be quantified with acceptable accuracy, precision and variability (33).

Precision, accuracy and recovery

The intra- and inter-day variation of the assay were determined by replicate analysis ($n = 5$) of samples at concentrations within the range of calibration curves in a single analytical run on the same day and at five different days, respectively, using the same stock solutions and plasma batches. Percentage coefficient of variance (% CV) was used as the measure of precision, and percentage accuracy [% Accuracy = (measured concentration/nominal concentration) $\times 100$] was also determined. The extraction recovery of PTX was estimated at 0.25, 5, 10 µg/ml and 0.3, 10, 20 µg/ml concentrations in plasma and tissues, respectively. Plasma and tissues samples (in six replicates) containing PTX were extracted and analyzed. Six samples containing similar concentrations of the compound in mobile phase were directly injected and peak areas were measured. Absolute recovery was calculated by comparing the peak areas for direct injection of pure PTX solution with those obtained by plasma and tissue samples containing the same amount of PTX.

Robustness

The robustness of the HPLC method was determined by analysis of samples under a variety of conditions such as small changes in the percentage of mobile phase acetonitrile, in the pH, in the mobile phase flow rate and in the temperature. The effect on retention time and peak parameters were studied (34).

Stability

Stability of PTX in mouse plasma containing PTX/TS-CS-PEG-FA or Anzatax[®] was examined by keeping replicates of plasma samples at room temperature for 12 h. Freeze–thaw stability of PTX in mouse plasma samples were studied over three freeze–thaw cycles, by thawing at room temperature for 2–3 h and refrozen for 12–24 h. Stability of the drug in plasma was also tested after storage at below –70 °C for 3 months (33). In all stability studies, plasma samples were spiked with PTX/TS-CS-PEG-FA or Anzatax[®] at three concentrations of PTX, low (0.25 µg/ml), medium (5µg/ml), and high (10 µg/ml). For each concentration and each storage condition, six replicates were analyzed. The concentration of PTX after each storage period was related to the initial analyte concentrations of freshly prepared samples. Samples were considered stable if the assay values were within the acceptable limits of accuracy and precision

Pharmacokinetics and tissue distribution study in Balb/c mice

Female Balb/c mice (5-6 weeks old, weighing 25±2 g) were obtained from Laboratory Animal Resource Center of Faculty of Pharmacy and Pharmaceutical Science of Isfahan University of Medical Science (Isfahan, Iran). All the animals were pathogen free and allowed to access to food and water freely. All the animal experiments were approved by the ethics committee of Isfahan University of Medical Science (permission number: 190084) and performed in accordance with Guide for the Care and Use of Laboratory Animals published by National Institute of Health. The mice were inoculated subcutaneously into the right flank with mouse 4T1 breast cancer cells (2×10^6 cells). When the tumor volumes reached to around 50-100 mm³, the mice were injected intravenously through the tail vein with Anzatax[®] or PTX/TS-CS-PEG-FA micelles at an equivalent dose of 10 mg/kg PTX versus the body weight. Three mice were sacrificed at 5 min, 30 min, 1, 2, 4, 8, and 12 h after the drug administration. Samples of plasma, liver, kidneys, spleen, lungs, heart, and tumor were harvested and stored at -70 °C ± 15 °C. The PTX plasma concentration-time data were analyzed by noncompartmental analysis with the aid of the program WinNonLin[®] (version 4.1, Pharsight

Co., Mountain view, CA). The peak plasma concentration (C_{max}) was obtained from experimental observations. Other pharmacokinetic parameters were calculated by standard methods (16).

RESULTS**Calibration curve**

The calibration curves were constructed by plotting the ratio of PTX peak areas to that of internal standard against standard PTX concentrations. Acceptable linear relationships were found over the concentration ranges 0.25-10 µg/ml PTX in plasma and 0.3-20 µg/ml PTX in homogenates, the latter corresponding to 0.88- 60.2 µg/g tissue. Since distribution of residuals was within ± 5%, no weight factor was applied. Although blank plasma samples (with a zero concentrations) were not selected in constructing the calibration curves, the 95 % confidence interval of the intercept encompassed the origin. Mean correlation coefficient of the linear regression analysis was 0.996 ± 0.014 for plasma and 0.987 ± 0.023 to 0.954 ± 0.031 for different tissues studied.

Limit of Detection and Limit of Quantitation

The LOQ was 0.25 µg/ml in plasma with precision, expressed as a coefficient of variation (CV%), of 8.23 % and accuracy of 105 %. The LOQ in the tissue homogenates was 0.3 µg/ml, with precision and accuracy ranging from 4.98 to 12.3 % and from 91.9 to 105%, respectively, in different types of tissues studied. The LOD were 70 ng/ml and 120 ng/ml at a signal to noise ratio of 3:1, in plasma and tissue homogenates, respectively.

Representative chromatograms of the extracted sample before drug administration and chromatograms of PTX at the LOQ levels in plasma and in tissue homogenates are presented in Figs. 1 and 2. No interfering substances were observed at the retention time of PTX and internal standard and both compounds were eluted completely and appeared as two separate resolved peaks (Resolution factor: 1.16) without peak tailing (tailing factor: 1.22, 1.08, respectively). An optimum flow rate of 1.9 ml/min for the mobile phase resulted in the retention times of 4.2 min for diazepam and 5.2 min for PTX.

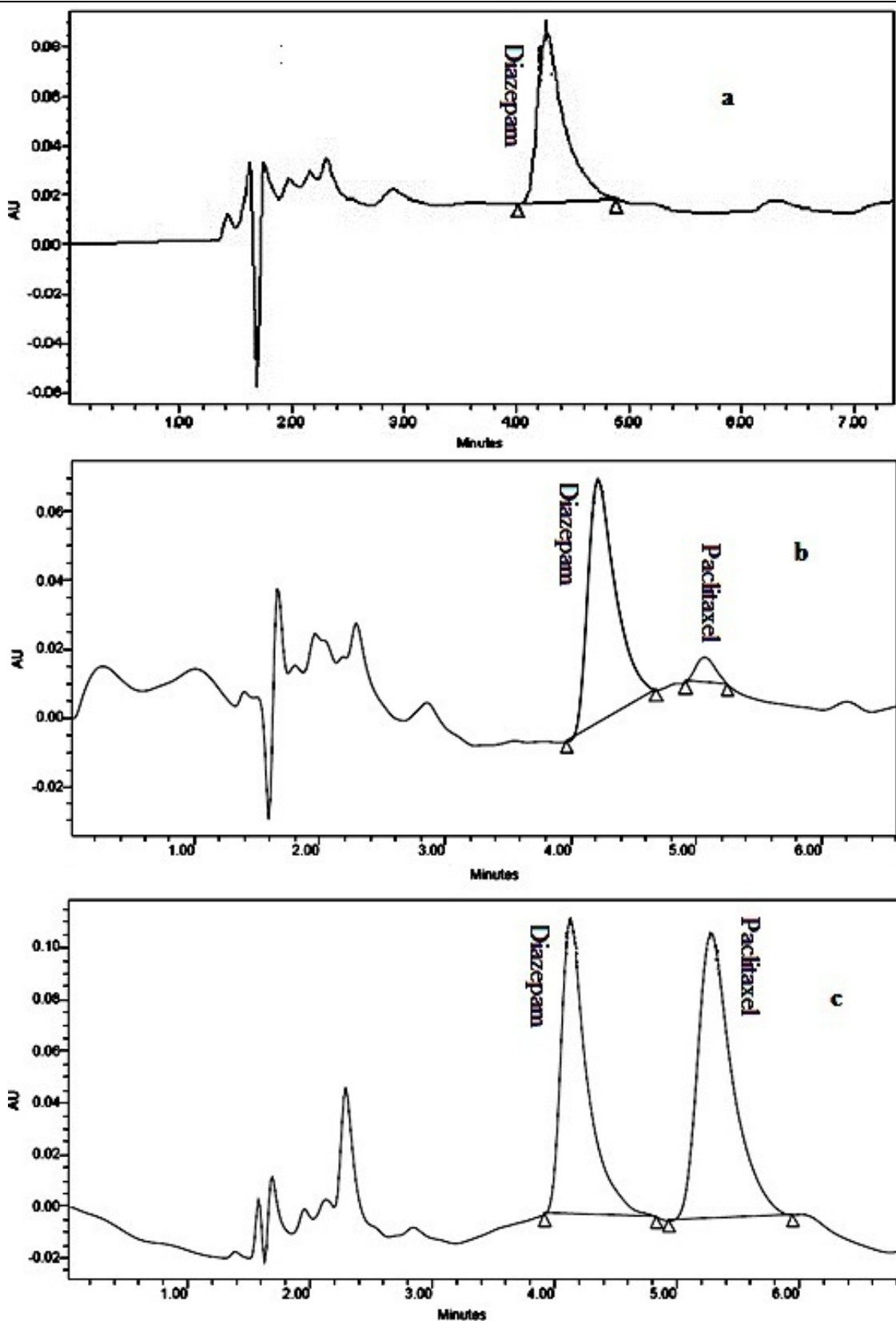


Figure 1. Representative chromatograms of (a) blank control plasma containing internal standard (I.S) at a nominal concentration of 2.5 µg/ml (b) plasma containing 0.25 µg/ml PTX (LOQ) and 2.5 µg/ml I.S, and (c) plasma containing 10 µg/ml PTX and 2.5 µg/ml I.S.

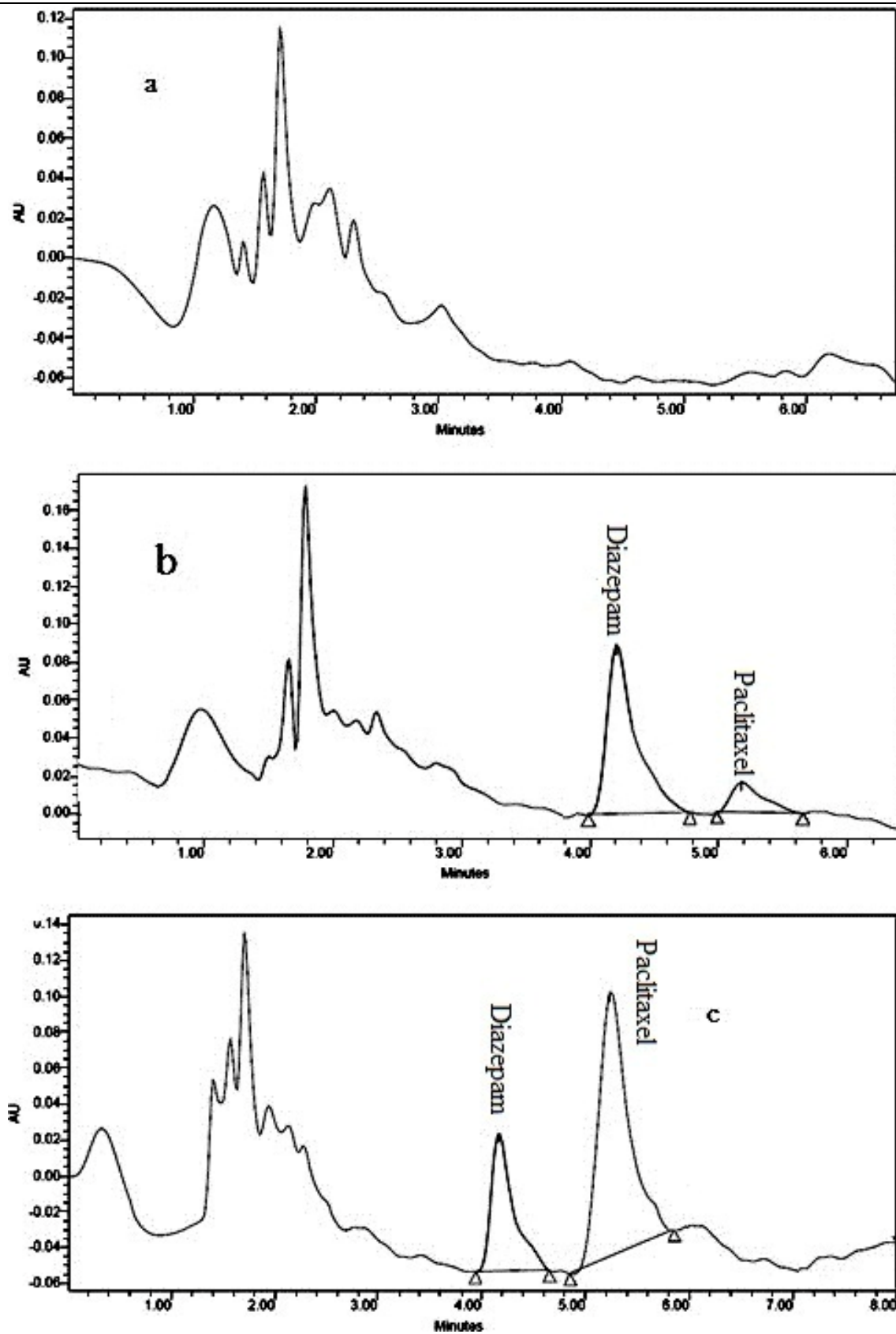


Figure 2. Representative chromatograms of (a) blank liver homogenate (b) liver homogenate containing 0.3 µg/ml PTX (LOQ) and 2.5 µg/ml I.S (c) liver homogenate containing 20µg/ml PTX and 2.5 µg/ml I.S.

Precision, accuracy and recovery

The precision and accuracy of calibration standard concentrations for both plasma and tissue homogenates were within acceptable limits as defined in the ICH guidelines. The inter- and intra-day precision and accuracy for plasma samples as well as tissue homogenates are presented in Tables 1 and 2, respectively. The mean recovery of PTX after plasma extraction was $87.4\% \pm 3.6$. The mean recoveries in tissue homogenates ranged from 62.1 ± 4.5 to 75.5 ± 3.2 in different types of tissues studied.

Robustness

The results of the robustness of the assay method are listed in Table 3. Method robustness that was checked after deliberate alterations of the mobile phase composition, flow, pH, and temperature showed that the changes of the operational parameters did not lead to any essential changes in the performance of the chromatographic system. The tailing factor for PTX and diazepam always ranged

from 1 to 1.3 and the eluents were well separated under all the changes carried out (resolution factor: 1.12-1.26). The percent recoveries of PTX were good under most conditions and did not show a significant change when the critical parameters were modified. Considering the result of modifications in the system suitability parameters and the specificity of the method, it would be concluded that the method conditions are robust.

Stability

The results of the stability study determined at various storage conditions are summarized in Table 4. No tendency of degradation of PTX after storage at room temperature was observed. In three freeze-thaw cycles, the drug was stable in plasma, indicating no significant substance loss during repeated thawing and freezing. Acceptable stability of PTX in mouse plasma spiked with PTX/TS-CS-PEG-FA or Anzatax[®] was demonstrated for all phases of storage and processing.

Table 1. inter- and intra-day precision and accuracy of PTX in mouse plasma samples.

Nominal concentration	day	Mean	SD ^a	CV% ^b	Accuracy%
Intra-day variation					
0.25	1	0.26	0.021	8.07	101
	2	0.23	0.024	10.4	92
	3	0.28	0.025	8.92	112
	4	0.24	0.018	7.50	96
	5	0.27	0.019	7.03	108
5	1	5.21	0.48	9.21	104
	2	5.93	0.51	8.55	119
	3	4.81	0.42	8.73	96
	4	5.63	0.51	9.08	112
	5	4.54	0.38	8.37	91
10	1	9.12	0.98	10.7	91
	2	10.4	0.86	8.25	104
	3	9.46	0.71	7.50	95
	4	10.9	1.31	11.9	110
	5	9.32	0.62	6.65	93
Inter-day variation					
0.25		0.26	0.02	8.23	105
5		5.16	0.51	9.88	103
10		9.72	0.82	8.43	97

Table 2. inter- and intra-day precision and accuracy of PTX in mouse tissue samples.

Nominal Concentrations (ug/ml)	Inter-day variability				Intra-day variability ^c			
	Mean	SD ^a	CV% ^b	Accuracy %	Mean	SD	CV%	Accuracy %
Liver								
0.3	0.29	0.025	8.57	97.7	0.28	0.03	12.3	94.4
10	9.93	0.41	4.19	99.3	10.2	0.71	6.86	102
20	19.8	1.32	6.66	99.2	19.7	0.68	3.44	98.8
kidney								
0.3	0.29	0.020	7.02	96.6	0.32	0.03	9.64	105
10	9.83	0.48	4.89	102	9.76	0.37	3.87	97.6
20	19.6	0.65	3.34	95.4	19.9	1.00	5.05	99.6
Spleen								
0.3	0.31	0.026	8.53	103	0.31	0.02	8.20	102
2	2.01	0.111	5.52	101	1.98	0.15	7.57	99.4
5	4.96	0.208	4.19	99.3	5.06	0.21	4.11	101
Heart								
0.3	0.30	0.015	4.98	102	0.31	0.02	8.20	102
5	4.83	0.251	5.20	96.6	5.03	0.20	4.13	100
10	9.83	0.152	1.55	98.3	9.95	0.36	3.64	99.5
Lung								
0.3	0.27	0.032	11.7	91.9	0.29	0.03	10.3	96.6
5	5.11	0.26	5.18	102	5.06	0.25	4.96	101
10	9.66	0.47	4.88	96.6	9.86	0.41	4.09	98.6

Pharmacokinetics and tissue distribution in Balb/c mice

The method described here was successfully employed to quantify total PTX in mouse plasma and tissue samples from pharmacokinetics and tissue distribution studies of PTX/TS-CS-PEG-FA in tumor-bearing mice. Fig.3 shows the representative chromatograms of PTX in mice plasma and different tissues four hours after intravenous injection of PTX/TS-CS-PEG-FA micelles at an equivalent dose of 10 mg/kg PTX versus body weight. The plasma and tissue concentration-time profiles of PTX following intravenous administration of PTX/TS-CS-PEG-FA micelles are presented in Figs 4 and 5, respectively. PTX level in plasma and tissues represents the total concentration of free and micelle-associated PTX. Peak plasma concentrations (C_{max}), terminal half-life ($t_{1/2\beta}$), area under the plasma concentration-time curve (AUC), apparent volume of distribution (V_d), and total body clearance (CL) of

PTX in the micelles were $8.41 \pm 1.63 \mu\text{g/ml}$, $4.64 \pm 0.38 \text{ h}$, $11.81 \pm 1.40 \mu\text{g h/ml}$, $5.60 \pm 0.91 \text{ L/kg}$, and $832.9 \pm 76.27 \text{ ml/h/kg}$, respectively.

DISCUSSION

To characterize pharmacokinetics and tissue distribution profiles of PTX, a sensitive, specific and reproducible HPLC method was developed and validated to quantitate PTX in plasma, various organs and tumor tissues of tumor-bearing mice. To extract PTX from biological samples such as plasma and tissues, most of analytical methods have made the use of multiple steps LLE, SPE or PP followed by evaporation to prepare and concentrate samples prior to injection into the HPLC column. A literature survey revealed that PP has widely been used as a clean-up procedure prior to HPLC analysis of PTX from plasma and tissue samples (17-20).

Table 3. Influence of changes in experimental parameters on the performance of chromatographic system.

Parameter	Modification	Recovery*	Tailing factor	Resolution factor
Mobile phase ratio (v/v)				
Acetonitrile: Buffer				
	47:53	85.6	1.08	1.14
	44.5:55.5	99.1	1.05	1.18
	42:58	87.4	1.08	1.21
	39.5:60.5	89.5	1.12	1.28
	37:63	89.1	1.29	1.26
PH				
	5.5	89.8	1.06	1.24
	5	87.4	1.08	1.21
	4.5	85.5	1.12	1.21
Flow rate (ml/min)				
	2.2	88.9	1.05	1.13
	1.9	87.4	1.08	1.21
	1.7	82.2	1.23	1.22
Temperature (°C)				
	65	88.8	1.05	1.28
	58	87.4	1.08	1.21
	50	91.7	1.25	1.23

*Recovery= [measured concentration/nominal concentration] ×100

Table 4. Stability of paclitaxel in mice plasma samples following PTX/TS-CS-PEG-FA micelles or Anzatax[®] injection (n = 6).

Stability	Concentrations (ug/ml)	PTX/FA-PEG-CS-TS			Anzatax [®]		
		Concentration (mean ±SD)	CV% ^a	Accuracy%	Concentration (mean±SD)	CV%	Accuracy %
Short term stability for 12 h in plasma	0.25	0.26 ± 0.02	8.66	106	0.24 ±0.02	8.43	98.6
	5	4.96 ± 0.49	9.93	99.3	5.06 ± 0.02	4.12	101
	10	9.62 ± 0.78	8.13	96	10.5 ± 1.18	11.1	105
Three freeze-thaw stability	0.25	0.23 ± 0.01	4.34	92.1	0.25 ± 0.01	5.95	102
	5	5.20 ± 0.26	5.29	100	4.89 ± 0.22	4.62	97.8
	10	10.3 ± 0.55	5.48	100	9.79 ± 0.64	6.53	97.9
3 months stability at < -70 °C	0.25	0.25 ± 0.02	10.6	101	0.23 ± 0.02	8.19	94.2
	5	4.98 ± 0.33	6.64	99.7	4.84 ± 0.36	7.48	96.8
	10	9.74 ± 0.45	4.58	97.4	10.2 ± 0.61	6.01	102

However, PP will result in low recoveries due to drug precipitation, low sensitivity because of unavoidable dilution, column deterioration, back pressure

increment, and late eluting peaks due to endogenous compounds. In the work conducted by Jain and coworker, total chromatography run time was

prolonged to 30 min to allow for late eluting peaks (17). In another similar study, the plasma sample containing PTX was deproteinized and the supernatant was directly injected to the HPLC column (20). Although, the LOQ was reported 0.05 µg/ml, the interfering peak from plasma in the chromatogram demonstrated inadequate protein precipitation which could diminish peak resolutions and sensitivity of the assay after a long run time. One of the prominent features of the present assay is one-step efficient liquid-liquid extraction of PTX from plasma and tissue matrices which renders the method more convenient and faster for pharmacokinetic and bioequivalence studies of PTX. Amongst different extracting solvents such as tetra-butyl methyl ether, diethyl ether, and ethyl acetate, only diethyl ether resulted in excellent recoveries of PTX and internal standard from plasma and tissue samples. Sparreboom *et al.* quantified PTX in human plasma after liquid extraction of samples with a mixture of acetonitrile and n-butyl chloride, however, it takes 45 min for the organic layer to evaporate (23). In some previous studies docetaxel have been used as an internal standard in HPLC analysis of PTX in plasma and tissue samples (24, 25, 27, 35). However, the peak of docetaxel was not adequately resolved from PTX peak (resolution factor < 1.1). Attempts have been made to explore a suitable internal standard that can be completely resolved from PTX peak. For instance, Kim and coworkers (28) used a synthesized analogue of PTX as internal standard, but this agent is not available to other laboratories. In a similar study, cephalomannine was employed as the internal standard, but this compound is not as readily available as diazepam and its routine use is not cost effective (30). In the present work diazepam fulfilled all typical requirements of a compound which is used as an internal standard. This agent was stable during the analysis, readily available, well resolved from PTX (resolution factor: 1.21), and its peak was sharp and symmetric (tailing factor: 1.22).

In order to achieve highest resolution and sensitivity under isocratic condition, the mixtures of methanol and/or acetonitrile with sodium acetate buffer solution at various combinations were assessed as the mobile phase. Binary mixture of acetate buffer/acetonitrile in proportion of 58/42 (v/v) proved to be the most effective combination as evidenced by more efficient resolution and lack of any tailing. In some previous works (1, 28, 31) the

gradient mode was used to resolve PTX peak from internal standard peak, in which composition of mobile phase should be changed throughout the run. However, gradient method is more complex technique and is not sufficiently reproducible compared to isocratic method. The LOQ of the present assay using plasma and tissue is adequate to pharmacokinetic studies in small animals, from which limited volumes (200-300 µl) of plasma can be withdrawn. Kim *et al* and song *et al* described a more sensitive methods but they used internal standards which is difficult to obtain commercially (28, 36). Moreover, the analysis run times were longer than 25 min makes the process laborious and time consuming which is not acceptable in routine HPLC analysis of PTX particularly it's pharmacokinetic and bioequivalence studies. Some other researchers achieved to the lower LOQ for PTX in plasma and tissues following liquid chromatography tandem mass spectrometric method (30-32). Although, these methods are sensitive and accurate with the short chromatographic time, they involve expensive equipment which is not affordable at most laboratories.

The HPLC assay developed in the current study was adequately sensitive, selective and suitable for quantitation of PTX in the mouse plasma and tissues containing PTX self-assembled TS-CS-PEG-FA micelle formulation or Anzatax[®]. As previously indicated (16, 37), PTX will not be released easily from the micelles leading to the circulation of PTX-loaded micelles rather than free form of the drug. Therefore, the plasma level of PTX when administered as PTX/TS-CS-PEG-FA would represent PTX associated in the micelle in plasma. The presence of PEG on the surface of the micelles could avoid reticuloendothelial system (RES) uptake and they would remain in circulation for longer period of time. According to the organ distribution in Fig. 5, the micelles were widely distributed in most tissues and the highest drug concentration was found in the tumor tissue. This might be attributed to the fact that tumor tissue is characterized by hypervascular permeability and impaired lymphatic drainage to facilitate significant accumulation of nanoparticles in the tumor tissue. In addition, folic acid presenting on the surface of micelles mediates active targeting and increases the intracellular concentration of PTX (38, 39).

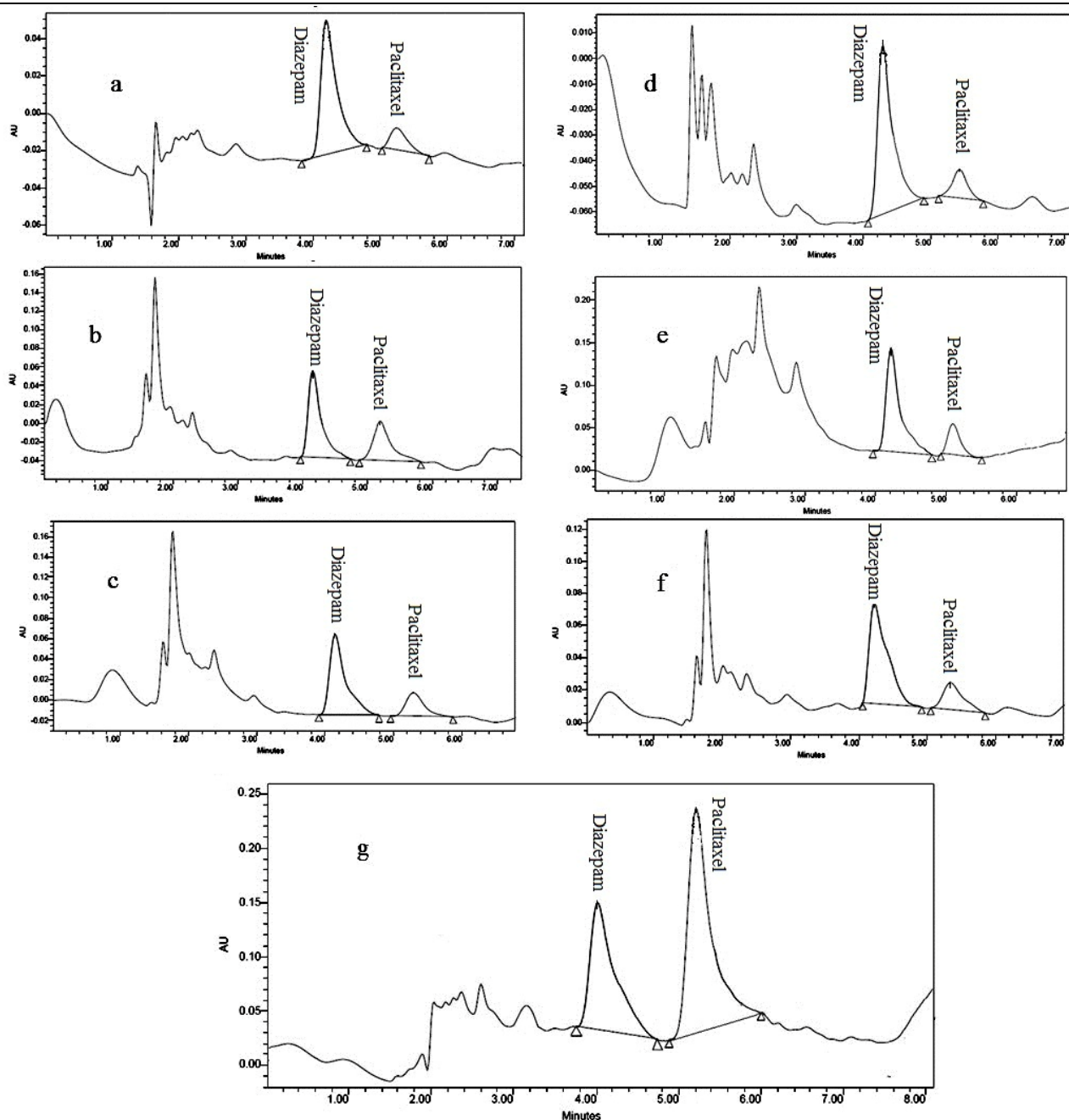


Figure 3. Representative chromatograms of (a) mice plasma (b) liver (c) kidney (d) spleen (e) lung (f) heart and (g) tumor, 4 hours after intravenous administration of 10 mg/kg PTX/TS-CS-PEG-FA micelles

CONCLUSION

The present investigation describes a simple, sensitive and selective HPLC method for analysis of PTX in plasma and tissue specimens with LLE and UV detection. Diethyl ether as the extracting solvent resulted in highly efficient recovery of the drug from plasma and tissue samples. The binary mixture of acetate buffer /acetonitrile in proportion of 58/42

(v/v) was the most effective combination of mobile phase as evidenced by more efficient resolution and lack of any tailing. The method was validated and met the requirements of linearity, recovery, accuracy, and precision. This validated method was successfully used to quantify PTX in plasma and tissue homogenates of tumor-bearing mice received PTX self-assembled TS-CS-PEG-FA micelles formulation by intravenous injection.

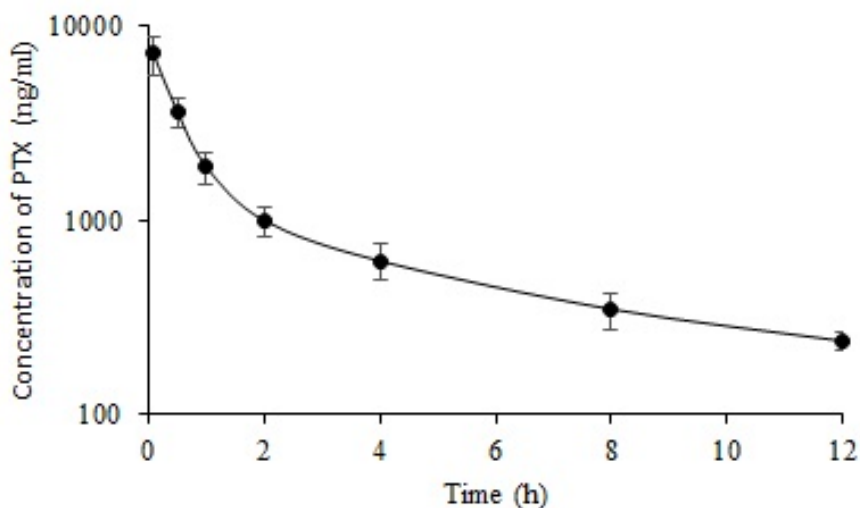


Figure 4. Plasma concentration–time profile of PTX (free and micelle associated) in tumor bearing Balb/c mice after intravenous administration of 10-mg/kg PTX/TS-CS-PEG-FA micelles. Each point represents the mean \pm SD (n=3)

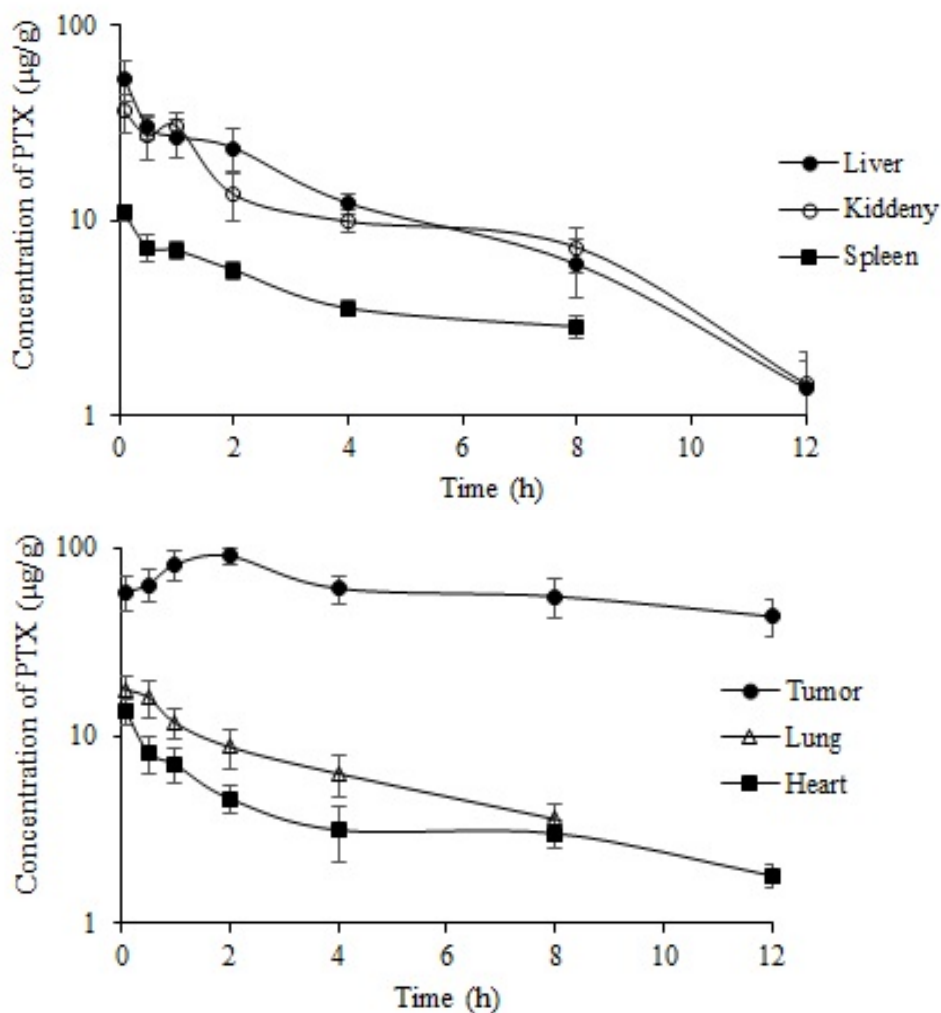


Figure 5. Tissue concentration–time profiles of PTX following intravenous injection of 10 mg/kg PTX/TS-CS-PEG-FA micelles. Each point represents the mean \pm SD (n=3)

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