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Original Article

Development of a RP-HPLC method for analysis of docetaxel in tumor-bearing mice plasma and tissues following injection of docetaxel-loaded pH responsive targeting polymeric micelles

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Abstract

Background and purpose: A simple, rapid, and sensitive reversed-phase high performance liquid chromatography (RP-HPLC) method based on liquid-liquid extraction was developed and validated for determination of docetaxel (DTX) in plasma and homogenate tissues of tumor-bearing mice.
Experimental approach: Samples were spiked with celecoxib as the internal standard and separation was achieved on a µ-Bondapak C18 HPLC column. The mobile phase consisted of a mixture of acetonitrile/water (40/60 v/v) at flow rate of 1.2 mL/min and the effluent was monitored at 230 nm.
Results: Calibration curves were linear over the concentration range of 0.1-10 µg/mL of DTX in plasma and 0.25-50 µg/mL in tissue homogenates with acceptable precision and accuracy. The mean recoveries of the drug from plasma extraction was 94.6 ± 1.44% while those of tissue homogenates ranged from 73.5 ± 3.2 to 85.3 ± 2.8% depending on the type of tissues examined. DTX was stable in biological samples with no evidence of degradation during 3 freeze-thaw cycles and two months of storage at -70 ± 15 °C. The developed HPLC method was applied to quantify DTX in the mouse plasma and tissues after intravenous administration of 7.5 mg equivalent DTX/kg dose of DTX-loaded folic acid-polyethylene glycol-heparin-tocopherol (FA-PEG-HEP-CA-TOC) micelle formulation to female Balb/c mice.
Conclusion: A simple, sensitive, rapid, accurate, and prudent RP-HPLC method was developed, validated, and applied for DTX determination in plasma and tissues.

Keywords: Celecoxib; Distribution; Docetaxel; HPLC; Pharmacokinetics; Tissue.

INTRODUCTION

Docetaxel (DTX), a semisynthetic anticancer agent, stabilizes microtubule in G2-phase of cell cycling. DTX has been used in the treatment of breast, ovarian, and non-small cell lung cancers (1). Because of low aqueous solubility, DTX is commercially available as Taxotere® in a mixture of Tween® 80 and ethanol. Inappropriately, administration of Taxotere® often induces serious hypersensitivity reactions (2). Thus, many researches have been conducted so far to achieve surfactant free delivery systems to efficiently and selectively deliver DTX to the tumor site without considerable systemic adverse effects. To accomplish this goal, several drug delivery systems including macromolecular conjugation, liposomes, polymersomes, and polymeric micelles have so far been studied for more safe and efficient delivery of DTX to tumor tissues (3-6).

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Polymeric micelles, self-assembled nanoparticles with core-shell structure, have drawn much attention as one of the most desirable and effective drug carriers enabling to solubilize hydrophobic drugs in their inner cores. Encapsulation of DTX into the polymeric micelles offers many advantages such as improving drug solubility without incorporation of surfactants, prolonging carrier circulation time, sustaining drug release, and reducing the normal cell toxicity by passive targeting *via* the enhanced permeability and retention effect. Moreover, active targeting through conjugation of appropriate ligands on the micelle surface can increase drug concentration in tumor tissues leading to improving therapeutic efficacy.

We have recently developed a novel multifunctional pH-sensitive, targeted polymeric micelle formulation composed of alpha tocopherol (TOC), heparin (HEP), polyethylene glycol (PEG), and folic acid (FA), for targeted delivery of DTX in breast cancer cells. DTX-loaded micelles (DTX/FA-PEG-HEP-CA-TOC) exhibited higher *in vivo* antitumor activity in 4T1 tumor bearing Balb/C mice without systemic toxicity compared to Taxotere® (7).

In the present study, we describe a high performance liquid chromatography (HPLC) method for quantification of DTX in mouse plasma and tissues following intravenous administration of DTX/FA-PEG-HEP-CA-TOC micelles in tumor-induced mice. Thus far, sensitive methods for quantification of DTX in plasma using LC tandem mass spectrometry (LC-MS) have been validated using liquid-liquid extraction (LLE), polymer precipitation (PP) and/or solid phase extraction (SPE) as clean up procedures with limit of quantification (LOQ) ranging from 0.00025-0.003 µg/mL (8-10). Although, very sensitive and accurate with the short chromatographic time, these methods involve expensive equipment, which is not affordable in most laboratories and acceptable in routine HPLC analysis of DTX particularly in pharmacokinetic studies.

HPLC methods for quantitation of DTX in plasma samples have also been previously reported using SPE extraction and UV detection with LOQ ranging from 0.001-0.005 µg/mL (11-13). SPE technique is not only a high cost and time-consuming clean up procedure due to multiple washing procedures, but also require large volume of samples (1-4 mL) which does not meet the requirements of multi-sample analysis in pharmacokinetic studies or routine drug-monitoring programs. HPLC methods for measuring DTX in plasma samples using LLE with sensitivity of 0.01-0.015 µg/mL have been reported using large volume of plasma (1-2 mL) which is not applicable in routine pharmacokinetic studies in small animals and rodents (14-16). Recently, a rapid and precise RP-HPLC method for analysis of DTX in rat plasma with sensitivity of about 0.100 µg/mL has been reported. However, the method is not developed and validated for measurement of the drug in various tissues and organs (6). In addition to plasma, a sensitive analytical method for determination of DTX in tissues and various organs is of great importance because of its multi-compartmental behavior and large volume of distribution. In most studies discussed above, quantitation of DTX in tissue samples has not been performed. In two studies, DTX was extracted from tissue samples using LLE following LC-MS method with LOQ ranged from 0.003- 0.02 µg/mL. However, as pointed out earlier, the equipment requires is not available to most laboratories and these methods are suitable only for studies where higher sensitivity than HPLC-UV-based assays is required (17,18). Zhao *et al.* (19) developed a method for determination of DTX in tissues with good sensitivity (0.020 µg/g) using SPE technique as the cleanup procedure, which is not a reasonably priced method in routine HPLC analysis of DTX in tissues though for trace amount of drug in tissues sensitive analytical methods are needed.

In the present study, we, therefore, described a validated, rapid, simple, and yet sensitive HPLC method to quantitate DTX concentrations in mouse plasma and tissues. The described method utilizes neither SPE nor LC-MS and yet sensitive, making the method rapid, simple, and appropriate for pharmacokinetic and tissue distribution studies. We have used celecoxib as internal standard (IS) which is more available and

cheaper than paclitaxel used in previous HPLC assays (6, 14-16). This method was validated for its accuracy, precision, limit of detection (LOD) and LOQ as per ICH guidelines. The validated method is selective, sensitive and suitable for the measurement of DTX in the mouse plasma and tissues following intravenous administration of DTX/FA-PEG-HEP-CA-TOC micelle formulations to tumor bearing Balb/c mice.

MATERIAL AND METHODS

Chemicals and reagents

DTX from Indian Pharmaceutical and Biotechnology Company (India); celecoxib from Amin Pharmaceutical Company (Isfahan, Iran); methanol, acetonitrile, and diethyl ether from Caledon (Ontario, Canada). All reagents and solutions were either HPLC or analytical grades. DTX/FA-PEG-HEP-CA-TOC micelles were prepared in our laboratory.

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). The mobile phase consisted of water/acetonitrile at 60/40 eluted at a flow rate of 1.2 mL/min. Column effluent was detected at 230 nm with a UV detector.

Standard solutions of docetaxel and internal standard

Standard stock solution of DTX was prepared at 500 µg/mL in methanol. A series of working solutions at concentrations of 1, 1.25, 2.5, 5, 10, 50, 100 µg/mL were prepared by further dilution of the standard stock solution in methanol. Working solution of celecoxib (IS) 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 School of Pharmacy and Pharmaceutical Science (Isfahan, I.R. Iran) and stored at -70 ± 15 °C until the use. To construct a calibration curve, 20 µL of DTX working solutions were added to 200 µL of blank plasma to obtain DTX standard concentrations ranging from 0.1 to 10 µg/mL in plasma.

Tissue homogenates

Tumor, 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. Aliquots of 200 µL homogenates were spiked with 20 µL of DTX working solutions to prepare homogenates with DTX concentrations ranging from 0.25 to 50 µg/mL, which corresponded to 0.52- 104.1 µg/g tissue.

Sample preparation

Diethyl ether was used to extract the drug from plasma or tissues. Mouse standard plasma, standard tissue homogenates, and unknown samples (200 µL of plasma and tissue homogenates of mice received DTX/FA-PEG-HEP-CA-TOC micelles intravenously) were spiked with 20 µL of IS solution and mixed with 6 mL diethyl ether as the extracting solvent. Sample tubes were vortexed for 2 min followed by 10 min centrifugation at 5,000 rpm. The upper organic layer was transferred to clean tubes and evaporated to dryness under nitrogen gas. The residue was reconstituted in 100 µL of mobile phase and 60 µL aliquot was injected into the HPLC column.

Method validation

Linearity

Two calibration curves (un-weighted regression line) were obtained by linear least squares regression analysis by plotting peak area ratios (DTX/IS) versus two different ranges (low and high) of DTX plasma concentrations. Low and high DTX concentration ranges were 0.1-0.5 and 0.5-10 µg/mL, respectively. Percent differences between back-calculated (measured) concentrations from the calibration equation and nominal concentrations were

used to validate the correlation. The calibration model is accepted if percent accuracy is within ± 20% for LOQ and within 15% for all other standard concentrations (20).

Limits of detection and quantitation

LOD was determined using the signal-to-noise ratio by dividing the peak height of the test samples with known concentrations of analyte to the peak height of baseline noise of the blank samples. The analyte concentration that produces a signal-to-noise ratio of 3:1 is 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 and precision of 15 to 20% (21).

Precision and accuracy

The intra- and inter-day variation of the assay were determined by replicate analysis (n = 3) of samples at concentrations within the range of calibration curves in a single analytical run on the same day and four different days, respectively, using the same stock solutions and plasma batches. Percent coefficient of variance (CV%) or relative standard deviation (RSD) or accuracy were determined as the measure of precision and accuracy using following equations.

$$\text{Precision (\%)} = \frac{\text{Standard deviation}}{\text{Average concentration}} \times 100 \quad (1)$$

$$\text{Accuracy (\%)} = \frac{\text{Measured concentration}}{\text{Nominal concentration}} \times 100 \quad (2)$$

Extraction recovery

The extraction recovery of DTX was estimated at 0.25, 1, and 10 µg/mL concentrations in plasma and 5 µg/mL in tissues. DTX-containing plasma and tissue samples (in six replicates) were extracted and analyzed. Six samples containing identical 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 DTX solution with those obtained by plasma and tissue samples containing the same amount of DTX.

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 organic phase (acetonitrile) and the mobile phase flow rate. The effect on peak parameters and recovery were studied (22). Tailing and resolution factors were calculated using following equations:

$$Tailing\ factor = \frac{W_{5\%}}{2F} \quad (3)$$

where, $W_{5\%}$ is the peak width at 5% of the peak height and F is the front peak half-width.

$$Resolution\ factor = \frac{RT_2 - RT_1}{0.5 \times (W_1 + W_2)} \quad (4)$$

where, RT is peak retention time and W is peak width.

Stability

Stability studies of DTX in mouse plasma were also conducted in three concentration levels (0.25, 1, and 10 µg/mL) in several different storage conditions namely at room temperature for 12 h, after three freeze-thaw cycles and at -20 °C for 2 months. For each concentration and each storage condition, six replicates were analyzed. Samples were considered stable if the assay values were within the acceptable limits of accuracy and precision (23).

Application of the developed method in in vivo studies

All female Balb/c mice, 4-6 weeks' old weighing 15-20 g, were pathogen free and allowed to access to food and water freely. All animal experiments were approved by the Animal Research Ethics Committee of Isfahan University of Medical Science in I.R. Iran (ethical code: IR.MUI.RESEARCH.REC.393057) and performed in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals. Possible efforts were made to decrease animal number and distress. 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 50-80 mm^3, the mice were injected

intravenously through the tail vein with DTX/FA-PEG-HEP-CA-TOC micelles at an equivalent dose of 7.5 mg/kg DTX. Three mice were sacrificed at 0.08, 0.25, 0.5, 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 ± 15 °C. DTX plasma concentrations and tissue samples were measured using HPLC method developed in the current study.

RESULTS

Selectivity and specificity

Figure 1 shows typical chromatograms of blank plasma and plasma spiked with DTX and IS. As can be seen, two substances eluted quickly with good resolution as two separate resolved peaks within 10 min. The small peaks related to plasma matrix components appeared at 1-5 min, which did not interfere with the analyte peaks. DTX and the IS eluted at 5.9 min and 7.9 min, respectively.

Calibration curve

The calibration curves were constructed by plotting the ratio of DTX peak areas to that of IS versus standard DTX concentrations. The developed method demonstrated excellent linearity in both low (0.1-0.5 µg/mL) and high DTX concentrations (0.5-10 µg/mL) in plasma and 0.25-50 µg/mL DTX concentrations in tissue homogenates. The regression equations for the low and high concentrations of DTX in plasma were Y = 0.2492 X + 0.0995 and Y = 0.2161 X + 0.1632 respectively, where y indicates the ratio of peak area of DTX to IS and X indicates DTX concentration. Mean correlation coefficients of the linear regression analysis were 0.997 ± 0.004 and 0.994 ± 0.005 for low and high plasma DTX concentrations, respectively. The corresponding mean correlation coefficients of the linear regression analysis of different tissues studied are presented in Table 1.

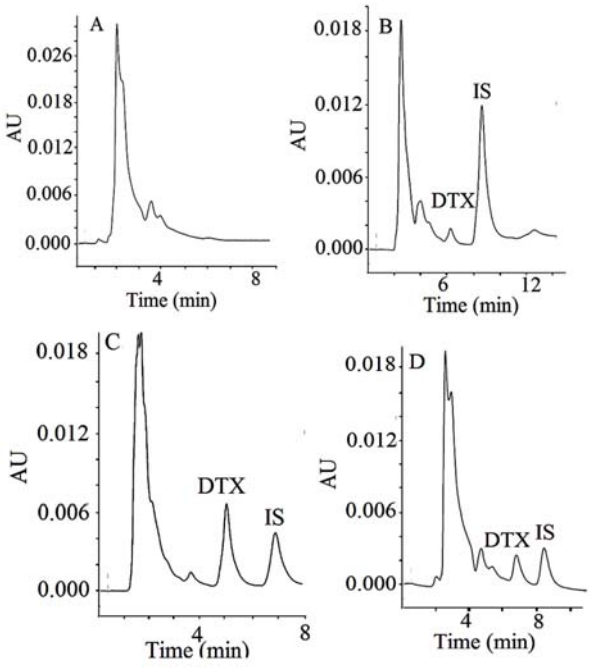

Fig. 1. Representative chromatograms of (A) blank plasma, (B) plasma containing 0.1 µg/mL DTX (LOQ) and 2.5 µg/mL IS, (C) plasma containing 10 µg/mL DTX and 2.5 µg/mL IS, and (D) plasma sample 0.5 h after intravenously administration of DTX/FA-PEG-HEP-CA-TOC micelles. DTX, Docetaxel; LOQ, limit of quantification, IS, internal standard; FA-PEG-HEP-CA-TOC, folic acid-polyethylene glycol-heparin-tocopherol.

Table 1. Intra- and inter-day precision and accuracy of docetaxel measurements in mouse tissue samples.

Nominal Concentrations (µg/mL)	Intra-day variability				Inter-day variability				Linear range (µg/mL)	R^2 (mean ± SD)
	Mean	SD	CV (%)	Accuracy (%)	Mean	SD	CV (%)	Accuracy (%)		
Liver										
0.25	0.254	0.014	5.48	101.8	0.262	0.024	9.19	104.7	0.25-50	0.993 ± 0.004
5	5.590	0.059	1.06	111.8	5.095	0.660	12.9	101.9		
25	22.60	0.443	1.96	90.41	22.87	0.825	3.61	91.49		
50	50.68	1.746	3.44	101.4	48.13	1.862	3.87	96.27		
Kidney										
0.25	0.255	0.033	12.8	102.2	0.248	0.022	9.07	99.39	0.25-50	0.989 ± 0.002
5	4.479	0.112	2.50	89.58	5.274	0.388	7.36	105.5		
25	26.80	2.731	10.2	107.2	25.03	1.577	6.30	100.1		
50	49.21	3.380	6.87	98.41	51.76	1.643	3.17	103.5		
Heart										
0.25	0.225	0.038	16.7	90.23	0.219	0.029	13.2	87.61	0.25-50	0.964 ± 0.028
5	5.325	0.135	2.54	106.5	5.253	0.238	4.53	105.0		
25	27.09	2.495	9.21	108.4	22.76	2.432	10.7	91.03		
50	49.04	2.866	5.84	98.08	48.20	1.672	3.47	96.39		
Spleen										
0.25	0.269	0.042	15.5	107.5	0.275	0.032	11.8	110.2	0.25-50	0.988 ± 0.002
5	4.755	0.466	9.80	95.10	4.727	0.427	9.03	94.55		
25	22.52	1.887	8.38	90.08	25.86	3.239	12.5	103.4		
50	51.75	3.357	6.49	103.5	52.60	2.163	4.11	105.2		
Lung										
0.25	0.229	0.012	5.36	91.58	0.252	0.020	7.94	100.7	0.25-50	0.996 ± 0.003
5	5.515	0.424	7.68	110.3	5.310	0.713	13.4	106.2		
25	23.64	2.851	12.1	94.56	23.17	2.813	12.1	92.69		
50	50.60	6.859	13.6	101.2	54.62	1.169	2.14	109.2		
Tumor										
0.25	0.239	0.038	15.7	95.72	0.216	0.005	2.48	86.59	0.25-50	0.995 ± 0.002
5	4.805	0.346	7.20	96.10	5.309	0.133	2.50	106.2		
25	22.67	1.386	6.11	90.68	23.14	2.139	9.25	92.55		
50	51.87	2.729	5.26	103.7	47.84	2.961	6.19	95.69		

SD, Standard deviation; CV%, percent coefficient of variance.

Limits of detection and quantitation

Representative chromatograms of the blank samples and DTX in plasma and in tissue homogenates are presented in Figs. 1 and 2. The LOQ for DTX in extracted plasma was 0.1 µg/mL with precision expressed as a CV% of 17.7% and accuracy of 117%. The LOQ for DTX in the tissue homogenates was 0.25 µg/mL, with respective precision and accuracy ranging from 2.14 to 13.4% and from 86.5 to 110% in various organ tissues tested. Representative chromatograms of different tissue homogenates at the LOQ concentration are presented in Fig. 3. The LOD, defined as a signal-to-noise ratio of 3:1 were 0.04 and 0.125 µg/mL in plasma and tissue homogenates, respectively.

Precision and accuracy

The intra- and inter-day precision and accuracy for tissue homogenates as well as plasma samples are presented in Tables 1 and 2, respectively. In plasma spiked with DTX, the intra- and inter-day precision (CV%) were 1.90-15.7% and 3.60 -17.7%, respectively. The intra- and inter-day precision in different types of tissues studied were 1.06-16.7 and 2.14-13.4%, respectively. Method accuracy was in the range 92.28-117% for plasma samples and 86.6-111% for different organ tissues.

Recovery

Recovery was determined according to the ratio of the areas of extracted samples with those in corresponding standard solutions. The overall mean recovery of DTX was 94.6 ± 1.44% after plasma extraction and ranged from 73.5 ± 3.2 to 85.3 ± 2.8% after extraction from different organ tissues.

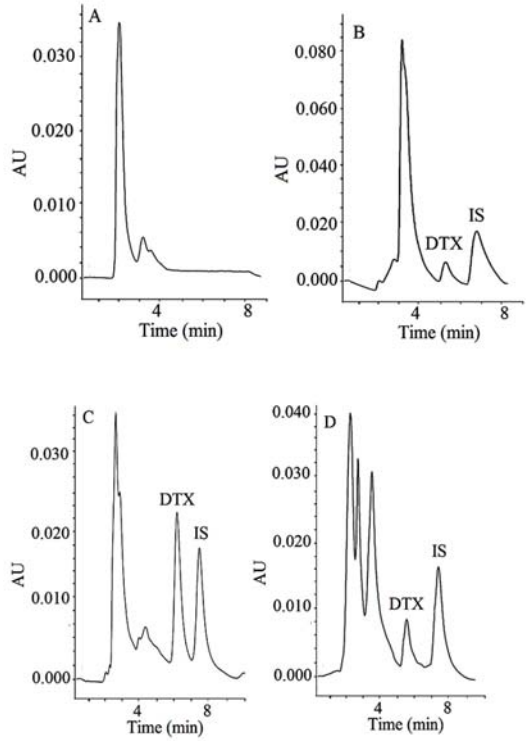

Fig. 2. Representative chromatograms of (A) blank tumor homogenate, (B) tumor homogenate containing 0.25 ng/mL DTX (LOQ) and 2.5 μg/mL IS, (C) tumor homogenate containing 50 μg/mL DTX and 2.5 μg/mL IS, and (D) tumor homogenate sample 0.5 h after intravenously administration of DTX/FA-PEG-HEP-CA-TOC micelles. DTX, Docetaxel; LOQ, limit of quantification, IS, internal standard; FA-PEG-HEP-CA-TOC, folic acid-polyethylene glycol-heparin-tocopherol.

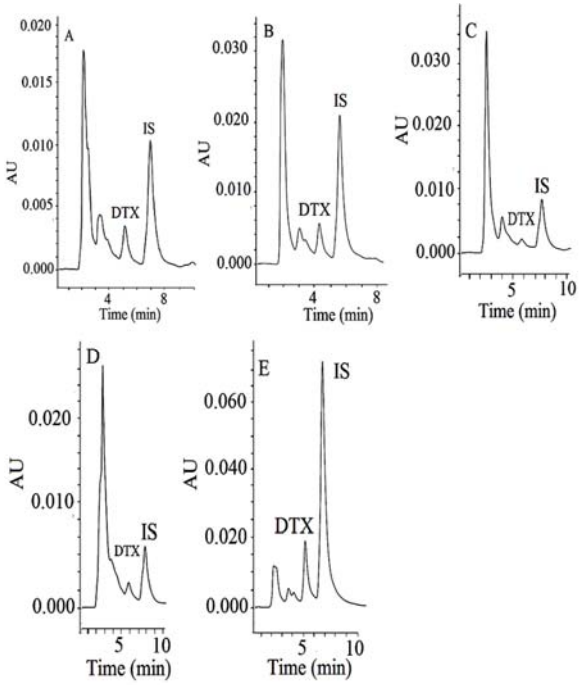

Fig. 3. Representative chromatograms of (A) liver homogenate, (B) kidney homogenate, (C) heart homogenate, (D) spleen homogenate, and (E) lung homogenate, standard samples containing 0.25 μg/mL DTX (LOQ) and 2.5 μg/mL IS. DTX, Docetaxel; LOQ, limit of quantification, IS, internal standard.

Table 2. Intra- and inter-day precision and accuracy of docetaxel measurements in mouse plasma samples.

Concentrations (µg/mL)	Intra-day variability				Inter-day variability			
	Mean	SD	CV (%)	Accuracy (%)	Mean	SD	CV (%)	Accuracy (%)
0.1	0.110	0.017	15.74	110.1	0.118	0.021	17.74	117.8
0.125	0.131	0.010	7.964	104.8	0.139	0.019	13.74	111.7
0.25	0.238	0.018	7.789	95.23	0.231	0.021	9.182	92.27
0.5	0.515	0.010	1.901	103.1	0.507	0.018	3.606	101.4
1	1.095	0.088	8.077	109.5	1.108	0.077	6.924	110.8
5	4.857	0.202	4.169	97.14	4.785	0.236	4.927	95.71
10	9.593	0.844	8.796	95.93	9.728	0.740	7.606	97.28

SD, Standard deviation; CV%, percent coefficient of variance.

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

Parameters	Concentrations (µg/mL)	Acetonitrile:water ratio (v:v)			Flow rate (mL/min)		
		45:55	**40:60**	**35:65**	**1.0**	**1.2**	**1.4**
Recovery	0.25	95.23	94.32	94.11	95.44	94.32	92.37
	1	96.14	97.21	91.56	97.63	97.21	91.94
	10	92.34	95.75	97.88	94.49	95.75	90.48
Tailing factor	0.25	1.06	1.01	1.11	1.05	1.01	1.02
	1	1.04	1.01	1.08	1.18	1.01	0.98
	10	1.12	1.20	1.24	1.25	1.20	1.09
Resolution factor	0.25	1.31	1.41	1.42	1.35	1.41	1.49
	1	1.23	1.37	1.42	1.30	1.37	1.41
	10	1.16	1.35	1.39	1.31	1.35	1.35

Robustness

Analysis were carried out with three levels of DTX (0.25, 1, and 10 µg/mL) using the developed method with slight changes in water/acetonitrile ratio and mobile phase flow rate. Significant changes in recovery, resolution, and tailing factors were taken into consideration for interpretation of the robustness assessment. The results of the assay robustness are listed in Table 3.

Stability

The results of the stability study determined at various storage conditions are summarized in Table 4. No significant degradation of DTX after all periods of storage was observed indicating the drug has acceptable stability in mouse plasma.

Application of the assay for measurement of DTX in plasma and tissues

The described method was applied to the pharmacokinetics and tissue distribution studies of DTX/FA-PEG-HEP-CA-TOC in tumor-bearing Balb/c mice. As shown in Figs. 1 and 2, no endogenous peaks were found interfering with DTX or IS. Figure 4 show the representative plasma and tumor concentration-time profiles of DTX following intravenous administration of DTX/FA-PEG-HEP-CA-TOC micelles in tumor-induced mice. The corresponding pharmacokinetic parameters are listed in Table 5.

DISCUSSION

In the present study, a sensitive, specific, and reproducible HPLC method was developed for the quantitation of DTX in plasma, cancer tumor, and various organs of tumor-bearing mice. In this method, DTX and IS were eluted approximately at 5.9 and 7.9 min, respectively, and overall separation run time lasted 10 min thereafter, the system was ready for the next injection. This time is shorter than that reported in some previous studies (11,16). For instance, in the study conducted by Zhao *et al.* (19) not only DTX was extracted from plasma samples with SPE, time consuming and expensive, technique but also eluted at 15 min. In our study, both DTX and IS were eluted as sharp symmetrical peaks and no interfering peaks were observed at the retention time of DTX or IS.

Table 4. Stability of docetaxel in mice plasma samples (n = 6).

Stability	Nominal concentrations (µg/mL)	Measured concentrations (µg/mL)	SD	CV (%)	Accuracy (%)
Twelve h in plasma	0.25	0.238	0.011	4.62	95.20
	1.00	1.126	0.044	3.91	112.6
	5.00	5.538	0.369	6.67	110.8
Three freeze-thaw	0.25	0.250	0.014	5.60	100.0
	1.00	0.976	0.114	11.7	97.60
	5.00	5.738	0.197	3.43	114.8
Two months at -70 ± 15 °C	0.25	0.251	0.020	7.97	100.4
	1.00	1.071	0.034	3.17	107.1
	5.00	5.244	0.644	12.3	104.9

SD, Standard deviation; CV%, percent coefficient of variance.

Table 5. Pharmacokinetic parameters after intravenous injection of DTX/FA-PEG-HEP-CA-TOC micelles containing 7.5 mg/kg DTX in tumor bearing Balb/c mice (mean ± SD, n = 3).

Parameters	$T_{1/2}$ α (h)	$T_{1/2}$ β (h)	V_d (mL/kg)	CL (mL/h/kg)	$AUC_{0-\infty}$ (µg. h/mL)	MRT (h)
Plasma	0.54 ± 0.04	5.38 ± 0.24	1228 ± 42.1	186.7 ± 8.72	40.16 ± 1.41	6.58 ± 0.06
Tissue	-	-	-	-	AUC_{0-12} 940.4 ± 44.6 (µg. h/g)	6.07 ± 0.86

DTX/FA-PEG-HEP-CA-TOC, Docetaxel-loaded folic acid-polyethylene glycol-heparin-tocopherol; $T_{1/2}$ α, distribution half-life;$T_{1/2}$ β, elimination half-life; V_d, apparent volume of distribution; CL, systemic plasma clearance; AUC, area under the plasma concentration-time curve; MRT, mean residence time.

Both substances were eluted completely without peak tailing indicating that the assay method involves high specificity and selectivity from endogenous substances and other associated agents. The precision, repeatability, and accuracy of the calibration standard concentrations for both plasma and tissue specimens were within the acceptable limits as defined in the ICH guidelines indicating that the developed method is accurate, precise and reproducible for measuring DTX.

The method robustness refers to ability of the assay to remain unaffected by small but deliberate changes to chromatographic conditions, which provides an indication of its reliability during routine usage (24). The robustness of the method checked after deliberate alterations of the mobile phase composition and flow rate 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 DTX ranged from 0.98 to 1.25 and the eluents were well separated under all changes carried out (resolution factor: 1.16-1.49). The percent recoveries of DTX 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.

To extract DTX from biological samples of plasma and tissues, most of analytical methods have used PP, SPE, LLE or multiple steps LLE followed by evaporation to cleanup and concentrate samples prior to injection into the HPLC column. In PP method, one-step PP followed by direct injection of samples make the procedure rapid and simple. However, analytical methods involve PP usually utilize acetonitrile or mixture of acetonitrile and methanol to precipitate the plasma proteins and this will result in low recoveries specially for drug such as DTX that are highly lipophilic and bounded to plasma proteins (~ 97%) and significant sample dilution reducing the assay sensitivity (25). Previously, Kim *et al.* (26) developed an UV-HPLC method based on PP with acetonitrile for quantification of DTX in rat plasma. The LOQ of the method was 3.1 µg/mL, which is much higher

than that obtained in our study (0.1 µg/mL). Lower sensitivity resulted from PP might be due to unavoidable dilution during sample preparation. In addition, PP may result in column deterioration, greater backpressure, and late eluting peaks due to endogenous compounds, which are considered as major drawbacks of PP cleanup methods (23).

SPE technique for drug extraction and sample cleanup has been considered as a more efficient approach for increasing method sensitivity. Zhao *et al.* quantified DTX in rabbit plasma using PP followed by SPE of samples and achieved a LOQ equal to 10 ng/mL (19). SPE method not only removed the interfering substances in rabbit plasma or rabbit tissue samples, but also offered good extraction recoveries of DTX in rabbit biological samples. Several HPLC assays for DTX determination in biological fluids have until now been conducted using SPE cleanup method (9,27-29) but required large volume of samples (1-4 mL) and long eluting run times. Moreover, SPE technique is an expensive procedure and suffers from sorbent drying between washing procedures resulting in the cracking of the packing materials.

One of the specific features of the present assay is one-step efficient LLE of DTX from plasma and tissue matrices making the method more convenient and facile for pharmacokinetic studies of DTX. Amongst different extracting solvents used in preliminary evaluation, diethyl ether achieved good recovery and volatility that greatly shortened sample preparation time. Lopez *et al.* used acetonitrile/ethyl acetate mixtures for extraction of DTX from plasma specimen (16). Under such a condition, an endogenous compound was extracted by ethyl acetate, which co-eluted with the IS, paclitaxel. Kharkar *et al.* extracted DTX from plasma with acetonitrile providing an extraction efficiency relatively lower than that obtained by other solvents such as diethyl ether used in our study (25). Interestingly, the recovery of DTX obtained in the current study is relatively higher than that reported in some previous studies (14,16,19).

Most of HPLC methods already been developed have employed paclitaxel as the IS which is an expensive drug whose routine use in analysis is not cost effective and is not as readily available as celecoxib which was used in our study (6,14-16). In the present work, celecoxib met all typical requirements of a compound, appropriate as an IS. This agent was stable during the analysis, is readily available, and well resolved from DTX.

In order to achieve highest resolution, sensitivity, and elution under an isocratic condition, the mixtures of acetonitrile with various proportions of water were assessed as mobile phase. Binary mixture of water/acetonitrile at 60/40 (v/v) proved to be the most effective combination as evidenced by more efficient resolution and lack of tailing. Previously, the gradient mode was used to resolve DTX from IS peak, in which composition of mobile phase should be continuously changed throughout the run (30). However, gradient methods are more complex and not sufficiently reproducible compared to isocratic methods.

The LOQ of the present assay in plasma and tissues is adequate for the pharmacokinetic studies of DTX in small animals from whom limited volumes (200 µL) of plasma can be withdrawn. The clinical pharmacokinetic of DTX have been reported. Its disposition profile is multiphasic, with rapid initial tissue uptake and a large volume of distribution. Thus, development of a sensitive analytical method for DTX quantitation in tissues is essential. Currently, a rapid and precise RP-HPLC method for analysis of DTX in rat plasma with sensitivity of about 0.100 µg/mL is reported (6), however, the method is not developed and validated for measurement of the drug in various tissues and organs. Some studies have been developed to quantitate DTX in tissues using UV detection. LOQ of 1.35 and 1 µg/mL have already been reported for DTX in tissues (31,32), which are higher than those obtained in the current study. Zhao *et al.* (19) also developed the method for determination of DTX in tissues with good sensitivity of 20 ng/g using SPE technique, however, as mentioned in earlier sections, the SPE method is not reasonably priced in routine HPLC analysis of DTX because it is costly

and time-consuming procedure due to multiple washing procedures.

The isocratic HPLC-UV methods using LLE described in the current study is particularly advantageous for routine therapeutic drug monitoring because it is rapid, sensitive, reproducible, and requires simple materials that are already available in most bioanalytical laboratories. The low sample volume required for the assay is reasonable, the 10-min run time is suitable for practical throughput of samples, and the LOQ of the assay is comparable with some reports that used more complicated chromatographic conditions and/or larger sample volumes. The HPLC assay developed in the present study was successfully used for the quantitation of DTX in plasma and tumor tissues of mouse-bearing cancer injected with DTX self-assembled FA-PEG-HEP-CA-TOC micelle formulation. Plasma and tissue collected from animals after DTX administration did not reveal the presence of interfering endogenous compounds. The plasma concentration-time profiles of DTX containing-micelles in mice were best fitted in a two-compartment open model. Rapid distribution of DTX took place after intravenous injection.

CONCLUSION

The present investigation describes a simple, sensitive, and selective HPLC method for analysis of DTX 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 water/acetonitrile at proportion of 60/40 (v/v) considered the most effective mobile phase as evidenced by more efficient resolution and lack of tailing. The method met the requirements of linearity, recovery, accuracy, and precision. This validated method was successfully used to quantitate DTX in plasma and tissue homogenates of tumor-bearing mice injected intravenously with FA-PEG-HEP-CA-TOC micelle formulation developed in our laboratory.

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CONFLICT OF INTEREST STATEMENTS

The authors declare that there is no conflict of interest in this work.

AUTHORS' CONTRIBUTION

J. Emami contributed in concept, study design, definition of intellectual content, data analysis, manuscript editing and review. M. Kazemi contributed in literature search, experimental studies, data acquisition, statistical analysis, and manuscript preparation. F. Hasanzedeh contributed in preparation and characterization of nanoparticles. M. Minaiyan and M. Mirian contributed in animal study design and tumor induction. A. Lavasanifar contributed in manuscript editing and review. All authors read and approved the final manuscript.

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