Original Article

Development of a Rapid and Precise Reversed-phase High-performance Liquid Chromatography Method for Analysis of Docetaxel in Rat Plasma: Application in Single-dose Pharmacokinetic Studies of Folate-targeted Micelles Containing Docetaxel

Somayeh Taymouri, Jaleh Varshosaz, Shaghayegh Haghjooy Javanmard[1], Farshid Hassanzadeh[2]

From the Department of Pharmaceutics, School of Pharmacy and Novel Drug Delivery Systems Research Centre, Isfahan University of Medical Sciences, [1]Physiology Research Center, Isfahan University of Medical Sciences, [2]Department of Medicinal Chemistry, School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: A simple and sensitive reversed-phase high-performance liquid chromatography (HPLC) method based on liquid-liquid extraction was established and validated for determination of docetaxel (DTX) in plasma of rat. **Materials and Methods:** Samples were spiked with paclitaxel as the internal standard and the chromatographic separation was carried out using C18 HPLC column. The mobile phase consisted of a mixture of acetonitrile/water with the ratio of 60/40 v/v. The ultraviolet detector was operated at 230 nm, and the flow rate of mobile phase was 1 ml/min. The method was validated for linearity, precision, accuracy, recovery, and limit of quantification (LOQ). Then the method was applied to quantify DTX in the rat plasma after intravenous (IV) administration of the self-assembled micelles of folate-targeted Synpronic F127/cholesterol (FA-PF127-Chol) loaded with DTX and Taxotere® as the reference marketed solution of DTX. The blood samples were taken from the ophthalmic vein at predetermined time intervals after treatment. **Results:** Calibration curve was linear between the concentration ranges of 0.1–7.5 μg/ml with the relative standard deviation % and evaluating error % ranged from 2.263 to 15.53 and −12.75 to 12.7 for intra- and inter-day validity, respectively. The mean recovery of the drug after plasma extraction was 95.67 ± 0.99% for the concentration of 1 μg/ml. The LOQ and the limit of detection for DTX in serum were 100 ng/ml and 30 ng/ml, respectively. **Conclusions:** The results indicated that the developed method could be adopted for pharmacokinetic studies of DTX-loaded FA-PF127-Chol micelles and Taxotere® in rat.

Keywords: *Docetaxel, folate-targeted micelles, high-performance liquid chromatography method, pharmacokinetics*

Introduction

Docetaxel (DTX) is a microtubule stabilizing agent interfering with mitosis and cell division. DTX has considerable activity in a broad spectrum of cancer including advanced breast cancer, nonsmall cell lung cancer, head and neck cancer, gastric cancer, bladder cancer, and melanoma.[1] However, because of poor aqueous solubility, it is formulated using Tween 80 and is marketed as Taxotere® that often causes hypersensitivity reaction in patients after administration.[2,3] On the other hand, after dilution with a hydroalcoholic solvent, Taxotere® formulation is unstable and should be taken as soon as possible.[2] Thus, there is an urgent need to develop a new delivery system for systemic administration of DTX. Polymeric micelles, a multimolecular assembly of block copolymers with core-shell structure, have gained considerable attention as drug delivery systems to improve therapeutic efficacy and reduce drug toxicity. They have potential to solubilize hydrophobic drug in micelle core, increase blood circulation time, evade scavenging by the reticuloendothelial system, passive targeting of drug to tumor tissue by the enhanced permeability and retention (EPR) effect, and sustain drugs in tumor tissue.[4,5] Furthermore, the innovative idea of active targeted drug delivery has motivated to surface modification of polymeric micelles by ligands, targeting tumor tissue to increase drug accumulation not only in tumor tissue but also in tumor cells.[6] Our group recently developed a novel polymeric micelle consists of folic acid-targeted

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Address for correspondence:
Prof. Jaleh Varshosaz, Department of Pharmaceutics, School of Pharmacy and Novel Drug Delivery Systems Research Centre, Isfahan University of Medical Sciences, Isfahan, PO Box 81745-359, Iran. E-mail: varshosaz@pharm.mui.ac.ir

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Synpronic F127-cholesterol copolymers (FA-PF127-Chol) containing DTX for targeting the drug to melanoma tissue.[7] The separation and analysis of DTX in biological fluids and tissues have been mainly performed by high-performance liquid chromatography (HPLC) techniques using different cleanup procedures including protein precipitation using methanol,[8] acetonitrile (ACN),[9] the mixture of methanol/ACN,[10] and trichloroacetic acid,[11] solid-phase extraction,[12,13] liquid-liquid extraction,[14] and different internal standards (IS) such as butyl 4-hydroxybenzoate,[8] loratadine,[9] N-heptylbenzamide,[11] and paclitaxel (PTX).[14-16] Protein precipitation method can deteriorate the chromatographic column due to inadequate protein precipitation and decrease the sensitivity of assay.[17] None of these reported methods involved short retention time using a simple and economical process of liquid-liquid extraction. In the present study, we investigated the optimum conditions for the analysis of DTX and successfully established a rapid HPLC method for studying the pharmacokinetic characteristic of DTX-loaded FA-PF127-Chol following IV administration in rats and the results were compared with Taxotere®.

Materials and Methods

Materials

DTX and PTX were purchased from Cipla (India). DTX loaded in micelles of FA-PF127-Chol was prepared in our laboratory (Isfahan University of Medical Science). ACN and methanol (preparative HPLC grade) were purchased from Merck Chemical Company (Germany).

High-performance liquid chromatography system and chromatographic condition

HPLC study was carried out using a Waters HPLC system model 746 (USA) consisted of an HPLC pump model 515, a Waters 2487 Dual ultraviolet absorbance detector, Rheodyne 7725I autoinjector, and 746 chromatopac injectors.

Chromatographic condition

A C18 column (250 × 4.6 mm, 5 μm, Waters, Ireland) was used for chromatographic separation. The mobile phase was a mixture of water and ACN in a ratio of 40:60 v/v. Each run lasted 8 min. The flow rate of mobile phase was 1 ml/min and the effluent was monitored at 230 nm.

Experimental animal studies

Male Wistar rats were supplied by the Pasteur Institute (Iran). All rats were maintained under normal condition and allowed free access to water and food. All animal experiments were in accordance with Isfahan University Ethics Committee (Isfahan, Iran).

Standard solution

DTX stock solution with a concentration of 200 μg/ml was made by dissolving 10 mg of the drug in 50 ml of methanol. Working solutions at the concentrations of 1, 3, 5, 10, 20, 50, 75 μg/ml were prepared by dilution of the standard stock solution with methanol. PTX stock solution at concentration of 20 μg/ml was also prepared in methanol as the IS.

Specificity and selectivity

Specificity of analytical method was tested by optical evaluation of chromatogram extracted from blank plasma for the presence of interference from endogenous peak at retention time of DTX and the IS.

Calibration curve

20 μl of DTX solutions at concentrations of 1, 3, 5, 10, 25, 50, 75 μg/ml and 20 μl of IS at concentration of 20 μg/ml were added to 200 μl of blank plasma to obtain PTX concentration at 2 μg/ml and DTX concentrations ranging from 0.10 to 7.5 μg/ml. After 30 s mixing, 4 ml of the ether was added to each test tube and vortexed for 3 min. After that, the samples were centrifuged at 5000 rpm for 15 min. The total organic phase was then separated, transferred to a clean test tube, and dried under the nitrogen gas. The drug residue was next reconstituted in 100 μl of mobile phase, and 70 μl of the final solution was injected into the HPLC system. Calibration curve was obtained by plotting peak area ratios of DTX/IS versus different DTX plasma concentrations.

Recovery determination

The recoveries of DTX were determined by comparing the peak areas achieved after extraction of known amount of DTX from plasma with those obtained from the same amounts of DTX in mobile phase.[18]

Precision and accuracy

The precision and accuracy of the assay were determined by repeating analysis of samples of DTX at concentrations within the range of calibration curve in 3 days and 3 times in a day. The precision of an HPLC method was determined by evaluating intra- and inter-day relative standard deviations percent value (RSD%). The accuracy of the HPLC method was demonstrated by evaluating error percent value.

Limit of detection and limit of quantitation

Limit of detection (LOD) is defined as the lowest detectable concentration of analyte of signal to noise ratio was ≥3. Limit of quantification (LOQ) was identified as the lowest plasma concentration of the standard curve with an acceptable accuracy within ±20% and the precision below 20%.

Application of the developed high-performance liquid chromatography method

In the present study, two groups of rats each contained 3 animals were treated intravenously through tail vein with either Taxotere® or DTX loaded in folate-targeted micelles of Synpronic F127-cholesterol at a dosage of

7 mg/kg. Blood samples (0.5 ml) were withdrawn at predetermined time intervals through retro-orbital sinus vein using heparinized capillary. The blood samples were centrifuged at 10,000 rpm for 10 min. Then plasma was collected and frozen until analysis. 200 µl of plasma samples were spiked with 20 µl of internal standard solution (20 µg/ml) and vortexed for 30 s. The extraction was carried out as described in pervious section. Final sample concentrations were calculated by determination of the peak area ratio of DTX related to internal standard, and the ratio was determined with the standard curve. The plasma concentration-time profiles of the two preparations were fitted by a two-compartment model. The studied pharmacokinetic parameters included the plasma concentration-time profiles of the two preparations were fitted by a two-compartment model as follows:[19]

$$C(t) = Ae^{-\alpha t} + Be^{-\beta t}$$

Where $C(t)$ is the drug concentration at time t, A and B are intercept of ordinate axis, and α and β are the corresponding first-order disposition rate constants.

$AUC_{0-\infty}$ (area under the curve), CL (clearance), V_{dss} (volume of distribution at steady state) and $T_{1/2}^{\alpha}$ (distribution half-life) and $T_{1/2}^{\beta}$ (elimination half-life) were calculated with the following equations:

$$AUC_{0-\infty} = (A/\alpha + B/\beta)$$

$$CL = Dose/AUC_{0-\infty}$$

$$V_{dss} = MRT \text{ (mean residence time)} \times CL$$

$$MRT = AUMC_{0-\infty}/AUC_{0-\infty}$$

$AUMC_{0-\infty}$ is the area under the first moment of the concentration-time curves and was calculated according to the following equation:

$$AUMC_{0-\infty} = A/\alpha^2 + B/\beta^2 T_{1/2\alpha} = 0.693/\alpha$$

$$T_{1/2}^{\beta} = 0.693/\beta$$

Results

Figure 1 shows the typical chromatogram of blank plasma, spiked with PTX (internal standard) and DTX at concentrations of 0.1, 1, and 7.5 µg/ml, plasma sample obtained 2 min after IV administration of DTX (7 mg/kg) and plasma sample obtained 2 h after IV administration of DTX (7 mg/kg). The retention times of DTX and PTX were 5.9 and 6.5 min, respectively. The overall run time lasted

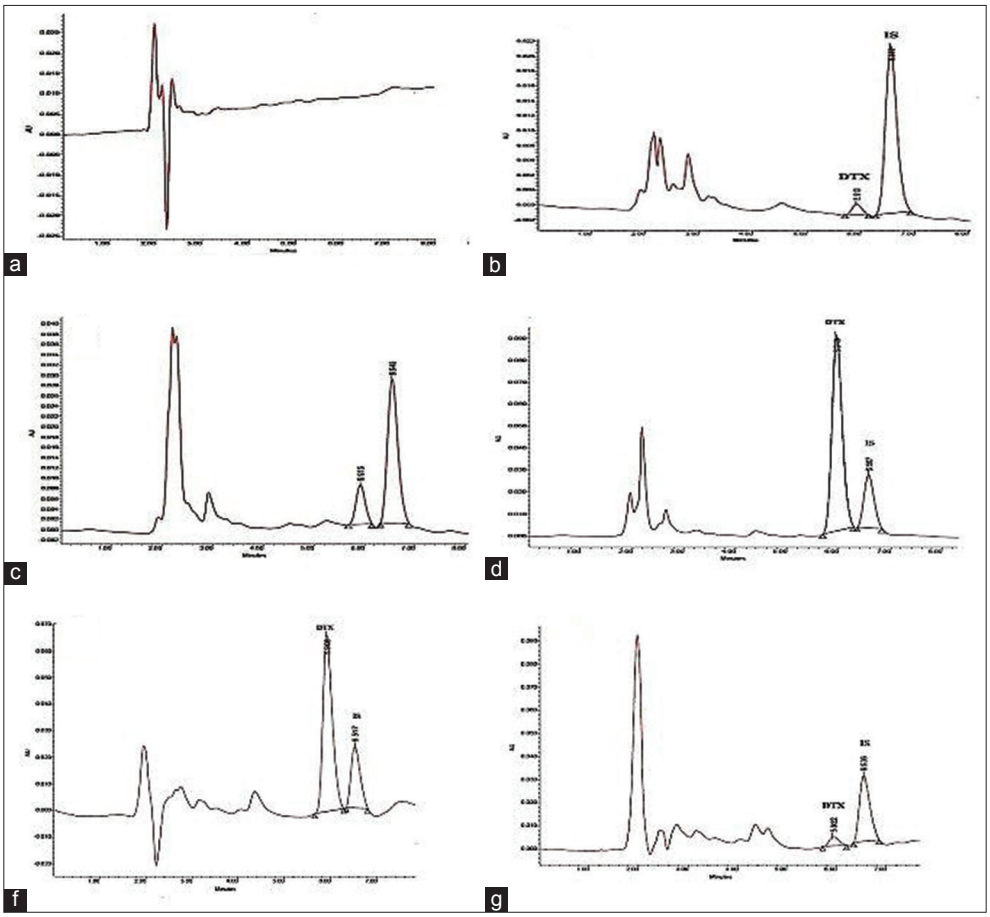

Figure 1: Typical HPLC chromatograms for DTX: (a) blank plasma obtained from Wistar rats, (b) blank plasma spiked with DTX (100 ng/ml) and PTX as internal standard (IS, 2 µg/ml), (c) blank plasma spiked with DTX (1 µg/ml) and IS, (d) blank plasma spiked with DTX (7.5 µg/ml) and IS, and (e and f) samples after iv administration of drug loaded FA-PF127-Chol nanomicelles 2 min and 2 h after injection, respectively

8 min. This retention time was less than similar studies for HPLC analysis of DTX in plasma in which the retention time of DTX was reported to be 7.7, 8.5, and 9.2 min.[14-16] No interfering peaks were observed in chromatogram at retention times of DTX and PTX in specified conditions.

The peak area ratio of DTX to PTX was plotted against DTX concentration in plasma. The good linearity was found in the range of 0.1–7.5 µg/ml. The typical linear regression equation in rat plasma obtained was Y = 0.487X−0.0058 with a correlation coefficient of 0.9997. The results of between days and within day variability are given in Table 1. The absolute value of percent of RSD and relative error% are <15.53 and 12.75, respectively, which are below the limits specified for precision and accuracy (i.e., <20%). These results indicate the method is reproducible between and within day. The LOD and LOQ were determined to be 30 and 100 ng/ml, respectively. The mean recovery with diethyl ether was about 94.26 ± 6.44%, 95.67 ± 0.99%, and 94.29 ± 3.22% at 0.5 µg/ml, 1 µg/ml, and 2 µg/ml for DTX, respectively. This indicates the recovery of DTX using the mentioned method was efficient and consistent.

The applicability of the method described here was evaluated by analysis the samples taken after administration of Taxotere® and DTX-loaded FA-PF127-Chol nanomicelles. Taxotere® and DTX-loaded FA-PF127-Chol were administered intravenously through tail vein at a dose of 7 mg/kg and samples were taken at the predetermined time. The plasma concentration of drug versus time is illustrated in Figure 2.

Discussion

In present study, the HPLC method developed is sensitive, specific and reproducible for the quantitative determination of DTX in human plasma requiring short retention time and small volumes of plasma for analysis. This simple analytical method based on liquid-liquid extraction and a total run time of 8 min permits the large number of samples in a short period of time. The HPLC assay developed in the present study was successfully used for studying the pharmacokinetic characteristic of DTX-loaded FA-PF127-Chol following IV administration in rats. A two-compartment model was used to evaluate the pharmacokinetic of the two formulations, and pharmacokinetic parameters were listed in Table 2. Plasma concentrations of DTX obtained at 0.033 h after IV injection of DTX-loaded FA-PF127-Chol was approximately twofold higher than that of Taxotere® (7233.705 ± 1730.34 vs. 3813.27 ± 870.25 ng/ml). The possible reason could be related to slow release behavior of micelles which could not distribute readily. As a result, they made higher concentration at the first sampling time.[20] The plasma AUC0−∞ of DTX-loaded FA-PF127-Chol micelles (2994.44 ± 418.3 ng/h/ml) increased significantly compared to that of Taxotere® (1227.58 ± 33.20 ng/h/ml). This was resulted from a significant decrease of Vdss and the CL values for DTX-loaded micelles compared to

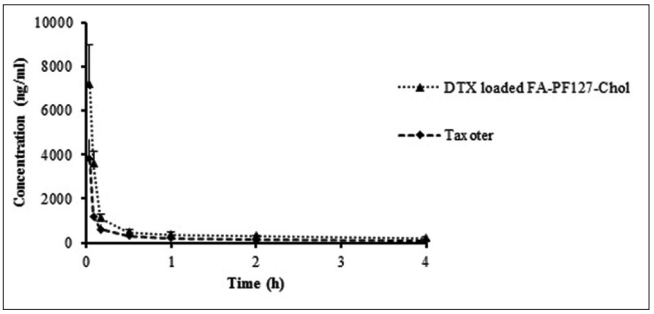

Figure 2: The mean plasma concentration-time profiles of Taxotere® and docetaxel-loaded folate-targeted Synpronic F127/cholesterol micelles (mean ± standard deviation, *n* = 3)

Table 1: Intra- and inter-day precision and accuracy for determination of docetaxel (*n*=3)

Concentration (µg/ml)	Between days				Within days			
	Mean concentration (µg/ml)	SD	RSD%	Error (%)	Mean concentration (µg/ml)	SD	RSD%	Error (%)
0.1	0.103	0.02	15.53	3.24	0.0991	0.004	4.07	−12.75
0.3	0.333	0.04	11.34	11.24	0.328	0.007	2.263	5.58
0.5	0.563	0.05	9.06	12.70	0.549	0.078	14.33	7.51
1	0.907	0.06	6.81	−9.25	0.885	0.066	7.483	−12.65
2	1.97	0.21	10.90	−1.09	2.03	0.195	9.614	0.87
5	5.004	0.33	6.79	0.08	5.013	0.383	7.656	0.040
7.5	7.509	0.79	10.57	0.13	7.493	0.49	6.538	−0.249

SD: Standard deviation, RSD: Relative standard deviation

Table 2: The plasma pharmacokinetic parameters of docetaxel-loaded folate-targeted Synpronic F127/cholesterol micelles and Taxotere®

Formulations	$AUC_{0-\infty}$ (µg/h/L)	CL (L/kg/h)	MRT (h)	V_{dss} (L/kg)	$T_{1/2\beta}$ (h)	$T_{1/2\alpha}$ (h)
Taxotere®	1227.58±33.20	5.70±0.15	2.24±0.01	12.79±0.44	2.06±0.16	0.041±0.02
DTX-loaded FA-PF127-Chol micelles	2994.44±418.3	2.37±0.33	3.55±0.35	8.52±2.08	3.20±0.26	0.04±0.00

DTX: Docetaxel, FA-PF127-Chol: Folate-targeted Synpronic F127/cholesterol, V_{dss}: Volume of distribution at steady state, AUC: Area under the curve, CL: Clearance, MRT: Mean residence time, $T_{1/2\beta}$: Elimination half-life, $T_{1/2\alpha}$: Distribution half-life

Taxotere®. Moreover, MRT and T1/2β for FA-PF127-Chol micelles were significantly longer than those of Taxotere® ($P < 0.05$). These results indicated FA-PF127-Chol could increase somewhat blood circulation time and decrease DTX elimination time.

Conclusions

In brief, simple, sensitive, and selective HPLC method based on liquid-liquid extraction method was developed for analyses of DTX in plasma samples. All the validation data, such as accuracy and precision, were within the required limits. Developed HPLC method is easy, fast to perform, and economic. This analytical procedure can readily be used for pharmacokinetic studies of DTX in rats.

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Conflicts of interest

There are no conflicts of interest.

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