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Development and Validation of a Highly Sensitive HPLC Method for Determination of Paclitaxel in Pharmaceutical Dosage forms and Biological Samples

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Abstract: A new simple and highly sensitive HPLC-UV method for the analysis of paclitaxel in Taxol® and Abraxane® has been developed which also allows the quantification in biological samples of pharmacokimnetic studies with a very easy sample preparation. The proposed method has advantages over previously reported methods, such as an increase in sensitivity up to 20 times, good accuracy in terms of recovery that allows to work without addition of internal standard and a simple sample preparation in one step.

The analysis was carried out using a Zorbax Eclipse XDB-C18 (4.6 × 150 mm, 3.5 µm) with a column guard. The mobile phase consisted of acetonitrile: water (50: 50) and the flow rate was 1 mL/min, the detection wavelength was 227 nm, the injection volume 5 uL and the analysis was performed at room temperature.

The method was validated according ICH guidelines and FDA guidelines for validation of bioanalytical methods. Linearity was evaluated in the range from 0.005 – 50.0 µg/mL, with a regression coefficient (R^2) of 0.9967. LOD and LOQ were 0.001 and 0.005 µg/mL respectively.

Keywords: Paclitaxel, HPLC-UV detection, pharmaceutical formulation, pharmacokinetic studies, validation.

1. INTRODUCTION

Paclitaxel (PTX) (Fig. **1**) is one of the most effective antineoplastic drugs used for the treatment of breast and ovarian cancer [1]. PTX is also being studied as adjuvant, neoadjuvant and first-line therapy for others malignant tumors [2]. It is a hydrophobic drug with poor solubility in water (0.3-0.5 µg/mL); for this reason, it is commonly formulated as a mixture of Cremophor EL® (Cr EL) and dehydrated alcohol (1:1, v/v) (Taxol®) [3, 4]. Unfortunately, this vehicle has been responsible for serious side effects some of which have clinical implications [5]. A few years ago, the Food and Drug Administration (FDA) was approved a Cr EL free formulation based on nanoparticles of PTX albumin-bound (Abraxane®) for recurrent metastatic breast cancer [6]. This nanoparticle (NP) formulation can be administrated without the use of conventional excipients. In addition, various PTX formulations such as liposomes [7], solid lipid NPs (SLNPs) [8], polymeric NPs [9] and polymeric micelles [10] have been examined to achieve certain improvements in PTX solubility and are still in preclinical or clinical phase. The emergence of new pharmaceutical dosage forms of PTX has led to the need for new analytical methods applied to quality control, pharmacokinetic and stability studies. Although US pharmacopeia describes an assay for PTX injection analysis [11], this method is only suitable for formulations based on Cr EL as vehicle but is not adequate for the quantification of nanoparticles of PTX albumin-bound. On the other hand, numerous methods have been published for the determination of PTX in biological matrixes, including microbiological assays, HPLC-UV, HPLC coupled to mass spectrometric (LC-MS) and capillary electrophoresis [12-15]. Despite

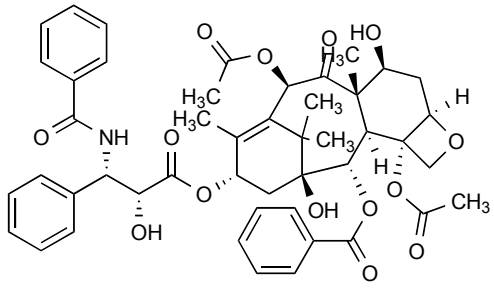

Fig. (1). Chemical structure of PTX.

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HPLC methods have been most frequently used due to their simplicity, efficiency, precision and low cost, most of them demand complex sample preparations that imply many tedious, expensive and time-consuming steps (evaporation to dryness, solid-phase extraction, liquid-liquid extraction) especially in biological matrixes [16-17].

In the present study, we have validated a new, simple and highly sensitive method with an easy sample preparation, first applied to the analysis of PTX in nanoparticles of PTX albumin-bound (Abraxane®) and Taxol. Moreover, the increase in sensitivity compared to previously published studies, allows to work with smaller amounts of serum and to reduce the injection volume up to twenty times, which means a lower LOD and LOQ (mass fewest analyte per injection). Other advantages of the proposed method include the analysis of PTX in biological matrices with good accuracy in terms of recovery that allows to work without addition of internal standard and a simple technique of sample preparation in a single step. These advantages make the method suitable for pharmacokinetics and biodistribution studies of PTX after a single intravenous injection of Taxol® or Abraxane® in animals.

2. MATERIALS AND METHODS

2.1. Chemical and Reagents

Paclitaxel ((1S,2S,3R,4S,7R,9S,10S,12R,15S)-4,12-bis(acetyloxy)-1,9-dihydroxy-15-{[(2R,3S)-2-hydroxy-3-phenyl-3-(phenylformamido)propanoyl]oxy}-10,14,17,17-tetramethyl-11-oxo-6-oxatetracyclo[11.3.1.0^{3,10}.0^{4,7}]heptadec-13-en-2-yl benzoate) (PTX) 99.9% was purchased from Rhenochem AG (Basel, Switzerland); Taxol® (30 mg paclitaxel in 6 mL of ethanol:CremophorEL, 50:50) and Abraxane® were supplied by Aventis Pharmaceuticals, USA. Cremophor EL® was purchased from BASF (Florham Park, NJ, USA). Acetonitrile (HPLC grade) was purchased from Sintorgan (Buenos Aires, Argentina). Ultrapure water was obtained from the Barnstead Easypure II (Thermo Scientific, Waltham, Massachusetts, USA). All solutions were filtered through a 0.45 µm nylon membrane (Nylaflo™, Pall Corporation, Ann Arbor, MI, USA) and degassed before use.

2.2. Equipment

The HPLC system consisted of a Shimadzu LC-A10 isocratic pump, SCL-10A controller, equipped with a SIL-10A autosampler, and a SPD-10A UV-detector (Shimadzu Corporation, Tokyo, Japan). Chromatograms were processed using Class-VP 4.2 software (Shimadzu Corporation, Tokyo, Japan).

2.3. Chromatographic Conditions

Separations were achieved using a Zorbax Eclipse XDB-C18 analytical column (4.6 x 150 mm, particle size 3.5 µm), (Agilent Technologies, Santa Clara, CA, USA) with a guard column. The isocratic mobile phase consisted of acetonitrile-water (50:50 v/v) and the flow rate was 1 mL/min. The column was kept at room temperature. The detection wavelength was 227 nm and the injection volume was 5 µL.

2.4. Preparations of Standard Solutions

A stock solution of PTX (1 mg/mL) was prepared in acetonitrile. Standard solutions were prepared by diluting the stock solution with acetonitrile.

2.5. Sample Preparation

Taxol® Samples

The contents of five Taxol® ampoules (6 mg/mL) were poured into a volumetric flask and well mixed. 5 milliliters of this solution were transferred to 100.0 mL volumetric flask, made up to volume with acetonitrile. 5.0 mL of this solution were transferred to 50.0 mL volumetric flask and were appropriately diluted with acetonitrile to get 30 µg/mL of PTX.

Abraxane® Samples

The contents of five vials of lyophilized powder Abraxane® (each single-use vial contains 100 mg of paclitaxel and approximately 900 mg of human albumin) were combined and reconstituted with water to a final concentration of 5 mg/mL PTX. 3 mL of this suspension were transferred to a 50 mL volumetric flask, made up to volume with acetonitrile-water (40:60 v/v) and mixed well. An adequate portion was centrifuged at 13000 rpm for 3 min and an aliquot (5.0 mL) taken from the supernatant was diluted with acetonitrile in a 50.0 mL volumetric flask to obtain a final concentration of 30 µg/mL of PTX.

Rat Serum Samples

PTX serum concentrations were determined after intravenous administration of Taxol® or Abraxane® in rats (N=3). In vivo studies were performed in male Wistar rats weighing 300–350 g. Briefly, 150 µl of acetonitrile were added to 75 µl of serum and vortexed for five minutes. Then, samples were centrifuged at 13.000 rpm for 4 min (MiniSpin® plus™, Eppendorf AG, Hamburg, Germany). 5 µl of supernatant were injected for analysis.

2.6. Validation

The validation of the developed method was accomplished according to the International Conference on Harmonization (ICH) guideline [18] and FDA bioanalytical method validation guidance [19], with respect to specificity, linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy, robustness and stability.

2.6.1. Specificity

Separation of the PTX from its main related impurity and degradation products formed under different stress conditions was evaluated. Also chromatograms of blank excipients of each pharmaceutical dosage form and a rat serum blank sample were analyzed. As resolution standard 10-deacetyl-7-epipaclitaxel (PTX related compound B) listed in the drug monograph of US pharmacopeia was used.

The selected stress conditions were:

Acidic: 10 mg of PTX were diluted with 10 mL of acetonitrile-HCl 0.1 M (50:50 v/v) and refluxed at 60 °C for 1 hour.

Alkaline: 10 mg of PTX were diluted with 10 mL of acetonitrile-NaOH 0.1 M (50:50 v/v) and refluxed at 60 °C for 1 hour.

Oxidation: 10 mg of PTX were diluted with 10 mL of acetonitrile-hydrogen peroxide 30 v/v (80:20 v/v) and left to stand for 1 hour.

Light: 10 mg of PTX were diluted to 10 mL with acetonitrile and left to stand in the light for 24 hours.

After exposure to stress conditions, each solution was diluted in acetonitrile until a final concentration of 30 µg/mL was reached and injected in triplicates.

2.6.2. Linearity, LOD and LOQ

In order to evaluate the matrix effect in the response of the analyte, three solutions of PTX were prepared and compared with a standard solution at the same concentration. For this, a blank of excipients of each dosage form and blank rat serum were spiked with PTX at a final concentration of 30 µg/mL. No differences were observed between the spiked samples and the standard solution.

The linearity of the method was evaluated by regression analysis of the calibration curve. Calibration samples of PTX standard were prepared at seven levels (0.005, 0.2, 1.0, 5.0, 20.0, 35.0 and 50.0 µg/mL) and analyzed in three replicates on three different days.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated as three times the signal to noise ratio (3 S/N) and ten times the signal to noise ratio (10 S/N), respectively.

2.6.3. Precision and Accuracy

Precision was evaluated for intraday (n=6) and interday assays (n=18) and it was expressed as %RSD for retention times and peak areas. Accuracy was evaluated from recovery studies of PTX from the three different matrixes.

Taxol samples:

Mixtures of PTX and excipients corresponding to three concentration levels of the drug (80, 100 and 120%) were prepared in triplicates. Peak area ratios of the samples and standard at the same concentration were compared.

Abraxane samples:

Due to the impossibility of replicating the abraxane matrix, the recovery study was performed by standard addition. An abraxane sample of 300 µg/mL was spiked with standard solution at three different levels. The spiked samples were then diluted (1:10 v/v) to yield a concentration of 38, 40 and 42 µg/mL of PTX. The samples were assayed in triplicates.

Rat serum samples:

Rat blank serum samples were spiked with PTX standard solution at three different levels to yield a final concentration of 0.2, 20.0 and 50.0 µg/mL of PTX and processed as described in the sample preparation. The samples were prepared in three replicates in three different days. Peak area ratios of the extracted and non-extracted samples at the same concentration were compared.

2.6.4. Robustness

The method robustness was assayed by changing the following parameters: composition of the mobile phase ± 2%, flow rate ± 0.2 mL/min, and wavelength ± 2 nm. The influence of the variations in the chromatographic parameters and the quantification of the pharmaceutical formulation was evaluated for each case.

2.6.5. Stability

Stability of PTX in serum samples was evaluated under different conditions. A pool of blank serum sample was spiked with PTX standard, separated in different vials and stored for 24 hs at room temperature, 8ºC and -20 ºC and after that, they were processed and injected. Additionally, fresh serum sample was analyzed immediately (reference value). Stability was expressed in terms of % recovery compared to the reference value. The acceptance criterion was set as no more than 5 % loss of recovery with respect to the reference value.

2.6.6. Application

Pharmaceutical Dosage form

The contents of PTX in pharmaceutical formulation, paclitaxel solution injection (30 mg/5 mL, Taxol®) and paclitaxel albumin-bound particles (100 mg/500 mg, Abraxane®) were assayed. The samples were prepared by triplicate according to the preparation sample section. The result was compared to the label content.

Animal Study

The two formulations of PTX used were diluted with saline at a PTX concentration of 2 mg/mL. PTX, at a dose of 6 mg/kg (n = 4 for each group) was injected intravenously (i.v.) during 15 seconds. Blood samples were collected from the arterial cannulae at different times (0.08, 0.25, 0.5, 1, 2, 4, 6, and 24 h) and collected in polypropylene microcentrifuge tubes. The samples were immediately centrifuged at 9,000 rpm for 3 min to isolate the serum. The collected serum was stored at -70°C in an ultra-low temperature freezer until analysis by HPLC.

3. RESULTS AND DISCUSSION

3.1. Validation

3.1.1. Optimization

A Zorbax Eclipse XDB-C18 (4.6 x 150 mm, particle size 3.5 µm) with an isocratic mobile phase was used instead of the reference method that uses of a L43 (Pentafluorophenyl groups chemically bonded to silica particles) with gradient elution [11]. The reduction in the column particle size led to a higher sensitivity which is reflected in the lower LOD and LOQ of the method (5 and 25 pg on column) compared to other methods previously reported (LOD and LOQ around 300 pg and 1000 pg on column respectively [16, 20-22]. Other HPLC methods coupled to a MS detector are described, achieving better selectivity and sensitivity, however this expensive equipment requires special installation and qualified operators also.

3.1.2. Specificity

PTX and its related compound B were well separated. The resolution between PTX and related compound B was 1.5, which fully complies with USP requirements (no less than 1.2) (Fig. **2**). Comparing the chromatograms of the blanks (Fig. 3b) and PTX solutions after being subjected to stress conditions (Fig. **4**) no interferences from endogenous or exogenous peaks with the PTX peak were detected. These experiments indicate that the proposed method is suitable for screening PTX in biological matrices and pharmaceuticals, and can also be used as a stability indicating study.

3.1.3. Linearity, LOD and LOQ

The analysis of the data obtained from the calibration curve showed that the method is linear in a range from 0.005 µg/mL to 50.0 µg/mL with a regression coefficient (R^2) of 0.9967. LOD and LOQ were 0.001 µg/mL and 0.005 µg/mL, respectively (Table 1), which makes it suitable for the pharmacokinetic evaluation of PTX in biological samples.

Table 1. Linearity, LOD and LOQ.

Parameter	
Linearity	y = 8911 x (RSD= 0,83)
***LOD* (3 S/N)**	0.001 µg/mL
***LOQ* (10 S/N)**	0.005 µg/mL

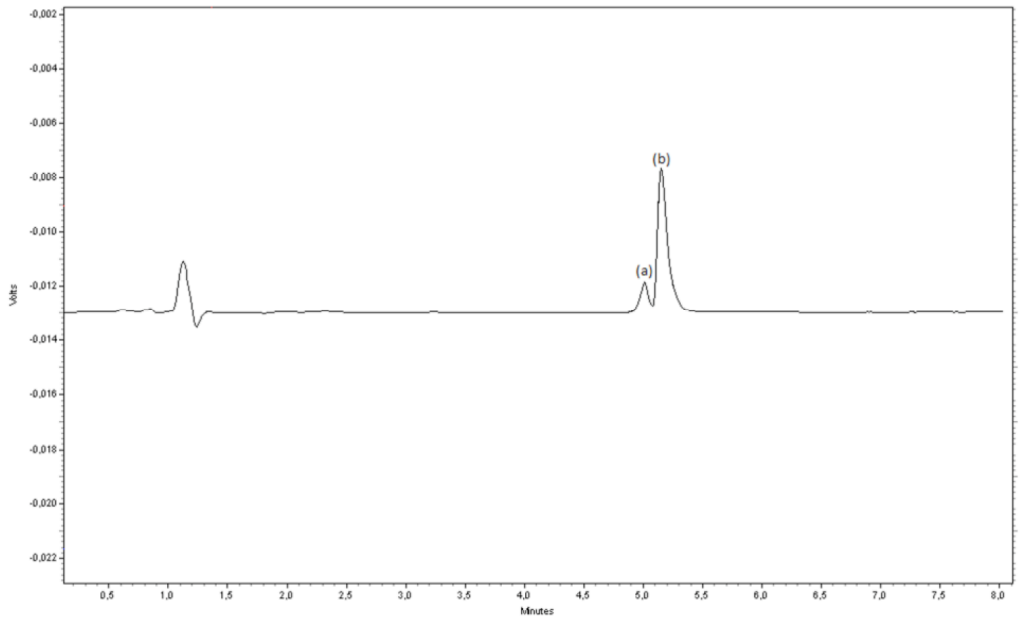

Fig. (2). Chromatogram of (**a**) 10-deacetyl-7-epipaclitaxel (PTX related compound B) and (**b**) PTX.

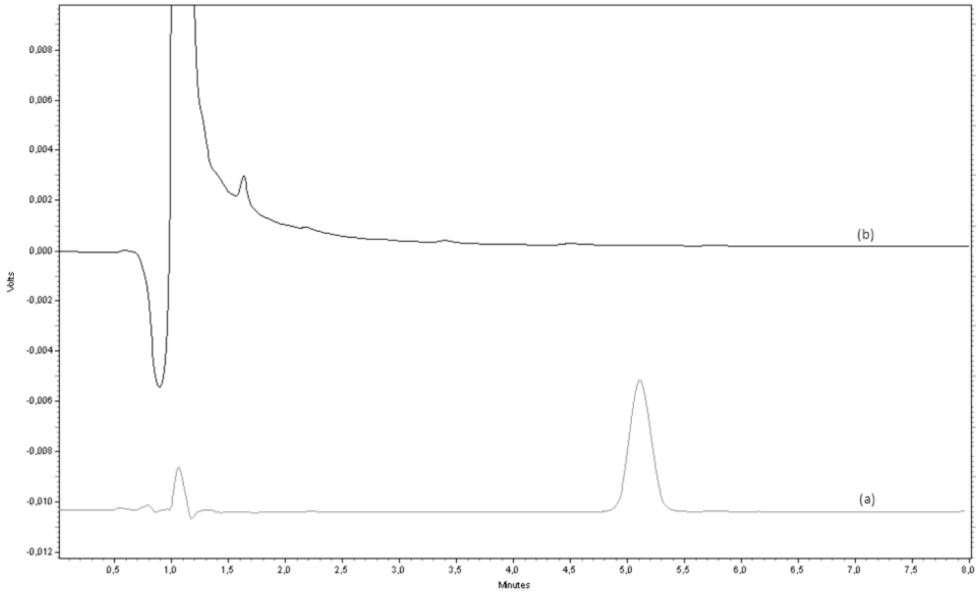

Fig. (3). Chromatograms of (**a**) PTX standard at 10 µg/mL and (**b**) blank serum rat sample.

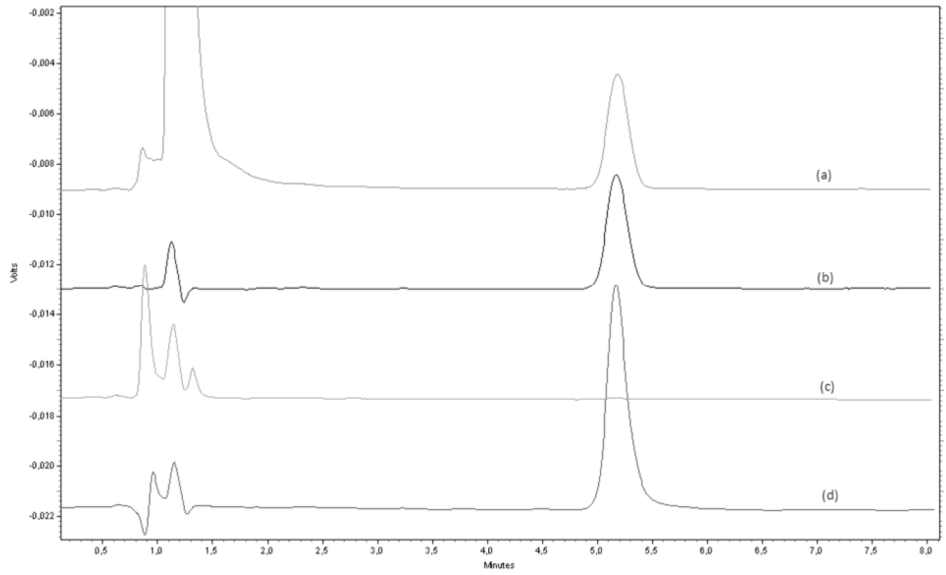

Fig. (4). Chromatograms of PTX solutions after being subjected to stress conditions (**a**) Oxidation (**b**) Photolysis (**c**) alkaline hydrolysis and (d) acidic hydrolysis.

3.1.4. Precision and Accuracy

The results for intraday (n = 6) and interday precision (n = 18) assays are presented in Table **2**, showing low variability in the retention time and peak area (% RSD below 2 %). Results from recovery studies were in agreement with the requirements of ICH guidelines [18] and FDA bioanalytical method validation guidance [19] (Table **2**).

3.1.5. Robustness

The robustness was statistically analyzed after evaluating the effect of varying the following operational parameters: flow rate, wavelength and composition of the mobile phase. Retention factor (k´), tailing factor (T) and theoretical plates numbers (N) calculated for each condition are presented in Table **3**. The changes in the operational pa-

Table 2. Precision and Accuracy of HPLC assay for PTX.

Precision (%RSD)				
Intraday (n=6)				
	Retention time		0.1	
	Peak area		0.3	
Interday (n=18)				
	Retention time		2.0	
	Peak area		1.5	
Accuracy				
Serum sample				
	Spiked levels	Low (0.2 µg/mL)	Medium (20 µg/mL)	Upper (50 µg/mL)
	Recovery %	82.4	95.1	92.7
	Intra-day (RSD)	4.0	4.5	4.3
	Inter-day (RSD)	5.9	4.1	4.0
Taxol samples				
	Spiked levels*	80	100	120
	Recovery %	100.0	98.2	99.5
	RSD	2.0	0.2	0.1
Abraxane samples				
	Spiked levels*	80	100	120
	Recovery %	101.0	100.9	100.3
	RSD	1.1	1.2	1.4

* % respect to labeled claim.

Table 3. Influence of varying operational parameters in the chromatographic performance.

	k´			T			N			Found Label					
										Abraxane (%)			Taxol (mg/mL)		
Flow rate	**1.2**	**1.0**	**0.8**	**1.2**	**1.0**	**0.8**	**1.2**	**1.0**	**0.8**	**1.2**	**1.0**	**0.8**	**1.2**	**1.0**	**0.8**
value	3.85	3.68	5.10	0.85	0.97	0.96	6100	6167	6859	10.3	10.2	10.1	6.4	6.3	6.5
Wavelength	229	227	225	229	227	225	229	227	225	229	227	225	229	227	225
value	3.68	3.68	3.68	0.95	0.97	0.94	6405	6167	6172	10.3	10.2	10.3	6.3	6.3	6.3
MP (ACN-H_2O)	52:48	50:50	48:52	52:48	50:50	48:52	52:48	50:50	48:52	52:48	50:50	48:52	52:48	50:50	48:52
	3.38	3.68	9.96	0.98	0.97	0.86	5864	6167	5310	10.3	10.2	10.3	6.4	6.3	6.0

rameters do not lead to significant difference on the chromatographic performance. Only significant difference for k´ was observed when the mobile phase composition was modified but remains, however, within USP specifications [11].

3.1.6. Stability

The stability data showed that the sample was stable when stored at -20ºC for 24 hours (recovery loss below 5 %), but the PTX recovery in the samples stored at 8ºC and room temperature were below 95%.

3.1.7. Application

Pharmaceutical Dosage form

The method was used in order to determine the concentration of each pharmaceutical formulation and compare it with the label content. Results are presented in Table **4** (Fig **5**).

Animal Study

The method was applied to the quantification of PTX in rat serum in order to compare the pharmacokinetic profile

Table 4. Analysis of PTX in pharmaceutical formulations.

	Abraxane (%)	Taxol (mg/mL)
Labeled claim	10.0	6.0
Found label*	10.2 (1.2)	6.3 (0.8)

*n=3, RSD values between parenthesis.

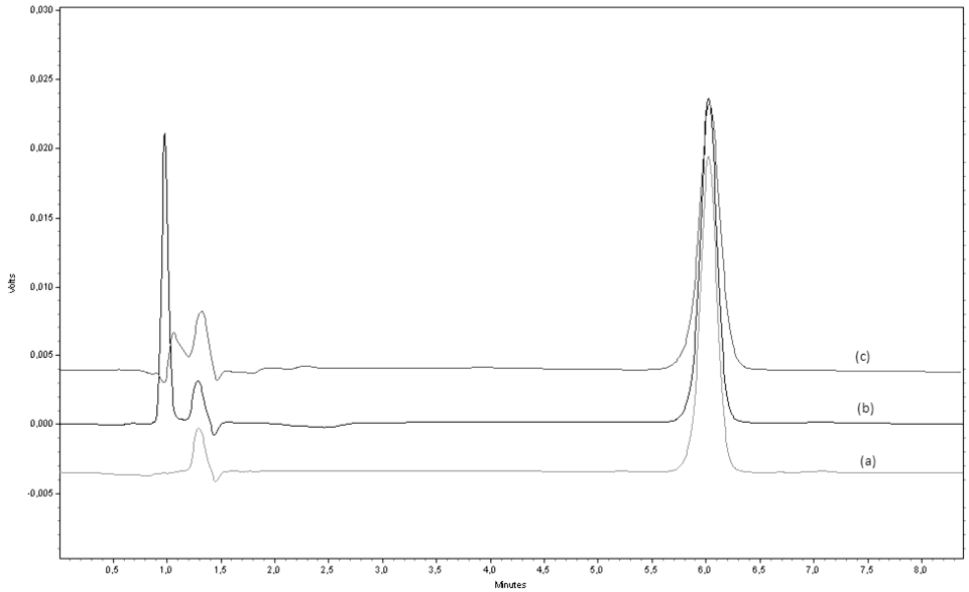

Fig. (5). Chromatograms of (**a**) PTX standard at 30 µg/mL, (**b**) Abraxane® formulation and (**c**) Taxol© formulation.

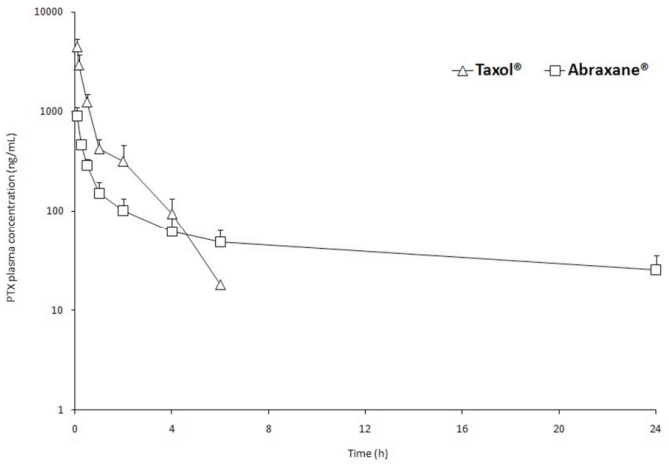

Fig. (6). Pharmacokinetic profile of Taxol® and Abraxane®. Data are reported as mean ± S.E. (n = 5).

after intravenous administration of Taxol®, Abraxane® and paclitaxel-loaded polymeric nanoparticles. For this, a serum concentration-time profile of PTX was constructed with the serum concentration media values obtained from the rats at different times according to what is described in section 2.7.1 (Fig. **6**)[23].

CONCLUSION

The proposed HPLC method constitutes a simple and highly sensitive method (20 fold times than HPLC-UV methods previously reported) for the analysis of PTX in different matrices. The developed method is the first one described for PTX determination in nanoparticles of PTX albumin-bound (Abraxane®) and it is also useful for the analysis of Taxol®. Validation parameters were exhaustively evaluated according to ICH guidelines, thus the method can be used for the routine quality control of pharmaceutical dosage form with a short analysis time. In addition, the present method is suitable for the determination of PTX in serum samples, with the advantage that plasma samples can be directly analyzed after a simple one step sample preparation. In this sense, the method was applied to the pharmacokinetic study in rats after administration of a new formulation of paclitaxel-loaded polymeric nanoparticles.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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