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Comparative Study of First-Derivative Spectrophotometry and High Performance Liquid Chromatography Methods for Quantification of Paclitaxel in Liposomal Formulation

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A simple and sensitive method using first-derivative ultraviolet spectrophotometry (DS-UV) was developed, validated, and compared to the high performance liquid chromatography (HPLC) method for quantification of paclitaxel (PTX) in a liposomal formulation. Different analytical performance parameters such as linearity, accuracy, precision, specificity, detection, and quantification limits were determined according to International Conference on Harmonization (ICH) guidelines. No interference from the lipid compounds was detected in the HPLC and the DS-UV methods at 246 nm. Linearity determined for paclitaxel concentrations ranging from 6.0 to 24.0 µg mL^{-1} presented a correlation coefficient higher than 0.999 for both methods. Relative standard deviation (RSD) values lower than 2% for intra- and inter-day precision data could be obtained. Accuracy mean values ranged from 98.9 to 102.0%. Robustness data showed that the PTX content was unaffected by the alteration proposed. Both methods were adequate to quantify the drug in the liposomal formulation. DS-UV proved to be rapid, accurate, selective, sensitive, and, therefore, an attractive tool for routine determination of PTX.

Keywords: paclitaxel, derivative spectrophotometric, validation, HPLC, liposome

Introduction

Paclitaxel (PTX) is a highly effective antineoplastic agent derived from natural sources. PTX covers a broad spectrum of antitumor activity and has been used to treat ovarian cancer, breast cancer, non-small cell lung cancer, head and neck tumors, Kaposi's sarcoma, and urologic malignancies.[1] One of the main problems associated with this drug is its low solubility in water. The pharmaceutical product commercially available, Taxol®, consists of micellar dispersion of PTX in Cremophor EL® (polyethoxylated castor oil used as a solubilizing surfactant) and dehydrated ethanol (1:1 v/v). However, Taxol® therapy is associated with severe toxic side effects such as hypersensitivity reactions, nephrotoxicity, and neurotoxicity.[2,3] In addition, PTX upon dilution with aqueous media can result in the

drug precipitation.[3,4] To overcome these disadvantages, efforts have been made to develop PTX delivery systems, including the use of liposomes.[5-8] Liposomes are currently being investigated to improve current cancer treatment regimens due to their capacity to increase the solubility of poorly water-soluble antitumor drugs.

The determination of PTX has been successfully carried out in raw material, biological matrices, and pharmaceutical products by high performance liquid chromatography (HPLC).[7-11] Despite its undisputed advantages, the HPLC technique also presents several disadvantages: the high cost of instrumentation and operation, the need of experience in handling the equipment and processing samples, the long time required for analysis, and the use of large quantities of solvents. Such disadvantages have encouraged the development of simpler and faster methods, with smaller amounts of solvents and without prior extraction steps.

The derivative spectrophotometric method with ultraviolet detection (DS-UV) has been widely used as

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a tool for quantitative analysis, characterization, and quality control in the pharmaceutical samples.[12-14] This technique is based on the so-called derivative spectra generated from conventional or zero-order scan. DS-UV offers various advantages over the conventional methods such as the discrimination of the sharp spectral features over large bands, the enhancement of the resolution of overlapping spectra, and the elimination of interference from other formulation components.[15,16] DS-UV is a simple, easily performed technique that provides a less expensive alternative when compared to HPLC. To our knowledge, no studies of PTX quantification in a liposomal formulation using the DS-UV method have been reported in the literature. Thus, the aim of the present study was to develop and validate an alternative analytical method, using DS-UV to quantify the PTX in a liposomal formulation and to compare the results obtained with HPLC, previously reported as the reference method for PTX determination.[3,10,17]

Experimental

Materials

Paclitaxel was supplied from Quiral Quimica do Brasil S.A. (Juiz de Fora, Brazil). Dioleoylphosphatidylethanolamine (DOPE), and distearoylphosphatidylethanolamine-polyethyleneglycol 2000 (DSPE-PEG$_{2000}$) were purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterylhemisuccinate (CHEMS) was supplied by the Sigma Chemical Company (St. Louis, USA). Sodium chloride was obtained from Merck (Rio de Janeiro, Brazil). Acetonitrile HPLC grade was obtained from Fischer Scientific (New Jersey, USA). Analytical grade methanol was obtained from Isofar or ProQuimios (Rio de Janeiro, Brazil). Water was purified using a Milli-Q apparatus (Millipore, Billerica, USA). All other chemicals and reagents used were of analytical grade.

Preparation of liposomes

The empty liposomes were made up of DOPE, CHEMS, and DSPE-PEG$_{2000}$ (in a 5.7:3.8:0.5 molar ratio, respectively), and were prepared by the lipidic hydration method. First, chloroform aliquots of lipids at 20 mmol L^{-1} total lipid concentration prepared in chloroform were transferred to a round bottomed flask and submitted to evaporation under reduced pressure until a thin lipid film was obtained. For preparation of PTX liposomes, a PTX equivalent at 0.1% (m/v) was added to the lipid solution. The resulting film was hydrated in 0.9% m/v

NaCl solution, followed by stirring with a vortex, until completely hydrated. This preparation was immediately submitted to the high-intensity probe sonication (20% amplitude) for 5 minutes, using a high-intensity ultrasonic processor (R2D091109 model; Unique® Instruments, Indaiatuba, Brazil). Nonentrapped PTX was eliminated by centrifugation (Sigma 4k-15 centrifuge, Sigma Laborzentrifugen GmbH, Osterode, Germany) at 3000 rpm at 4 ºC for 10 minutes.

Instruments and analytical conditions

Spectrophotometric analyses were carried out on an Allcrom® UV 6300 spectrophotometer, connected to the UV-Vis Analyst® software. The absorbance was determined within the range of 200-400 nm with a 2 nm bandwidth, using 1.00 cm quartz cells. Measurements were performed using the zero-crossing wavelengths in the first derivative of the absorbance spectra, measuring the amplitude at 246 nm. The chromatographic apparatus of the HPLC analysis consisted of a Model 515 pump, a Model 717 Plus auto-injector, and a Model 2996 variable wavelength UV detector (Waters Instruments, Milford, USA) connected to the Empower software. Separations were performed using a 25 cm × 4 mm, 5 μm Lichrospher® 100 RP-18 column (Merck Millipore, Darmstadt, Germany). The mobile phase consisted of an acetonitrile-water (55:45 v/v) mixture, filtered and degassed by suction-filtration through a nylon membrane, in isocratic flow. The flow rate was 1.2 mL min^{-1} and the injection volume was 20 μL. The eluate absorbance was monitored at 227 nm.

Method validation

The proposed method was validated for both techniques for selectivity, linearity, precision, accuracy, robustness, and limits of detection (LOD) and quantification (LOQ) according to the procedures described in the International Conference on Harmonization (ICH) guidelines Q2 (R1) for the validation of analytical methods.[18]

Specificity

The specificity of the method was evaluated by analyzing solutions of empty liposome and PTX solutions (15.0 μg mL^{-1}). Empty liposomes were disrupted using isopropanol in a volume ratio of 1:10 and then analyzed. The UV spectra of both solutions were recorded in the range of 200-400 nm. The system response was determined through the presence of interference or overlaps of liposome composition with the PTX response. For the HPLC

analysis, these same preparations were injected into the chromatograph, to evaluate possible interfering peaks.

Linearity

For linearity experiments, standard solutions containing 200.0 µg mL^{-1} of PTX in methanol were prepared in triplicate. Aliquots of these solutions were diluted in methanol or mobile phase for DS-UV or HPLC analysis, respectively, and seven different concentrations (6.0, 9.0, 12.0, 15.0, 18.0, 21.0, and 24.0 µg mL^{-1} of PTX) were obtained and analyzed on three different days. Calibration curves with concentration *vs.* absorbance or peak area were plotted for each method and the obtained data were subjected to regression analysis using the least squares method. The linearity was expressed as correlation coefficient, considering values higher than 0.99.

LOD and LOQ limits

These parameters were determined based on the standard deviation of the response and the slope, using the calibration curve data. Analysis was performed in triplicate.

Precision

The intra-day precision was evaluated by analyzing six samples of PTX for a concentration equal to 15.0 µg mL^{-1} (at 100% of the test concentration) during the same day under the same experimental conditions using the DS-UV and HPLC methods. The inter-day precision was assessed by the same sample and carried out on a different day. The PTX contents and the relative standard deviations (RSD) were calculated. The acceptable values for the RSD were lower than 2%.

Accuracy

The accuracy experiments were performed applying the method to quantify PTX in the presence of components of the formulations. Empty liposomes were spiked with known amounts of PTX at different concentration levels (6.0, 12.0, 15.0, 18.0, and 24.0 µg mL^{-1}). The samples were prepared in triplicate, appropriately diluted and analyzed by DS-UV and HPLC methods. The 98% to 102% were acceptable variables in measured concentrations.

Robustness

The robustness was determined in six samples (15.0 µg mL^{-1}) after small variations in the experimental

conditions. For the DS-UV method, two different methanol suppliers (Isofar and Proquimios) were used. For the HPLC analysis, the concentration of acetonitrile in the eluent system was altered (acetonitrile:water 55:45 or 53:47 v/v). Student's *t*-test was used to evaluate the difference between parameters. Differences were considered statistically significant when the *p* values were lower than 0.05.

Drug encapsulation percentage

The amount of PTX was determined in the liposomes before centrifugation (non-purified liposomes) and after centrifugation (purified liposomes). Briefly, the liposomes were disrupted using isopropanol in a volume ratio of 1:10 and later diluted in methanol or mobile phase for DS-UV or HPLC analysis, respectively. This dispersion was filtered in a 0.45 µm Millex HV filter and analyzed by DS-UV or HPLC method. The encapsulation percentage (EP) was calculated using the following equation 1:

$$EP = \frac{[PTX] \text{ in purified liposomes}}{[PTX] \text{ in non purified liposomes}} \times 100 \qquad (1)$$

Results and Discussion

The main purpose of this work was to establish and validate a simple, sensitive and accurate method based on ultraviolet spectroscopy followed by derivative analysis for PTX quantification in a liposomal formulation. In addition, the evaluation of the efficiency of the developed method compared to the HPLC method, previously reported for the determination of the studied drug,[3,10,17] was also investigated. In recent years, the development of spectrophotometry methods for determination of drugs has increased considerably due to their low cost and simplicity compared to the HPLC method.[12-15] On the other hand, liposomes have been studied extensively because of their capability to accommodate a large variety of drugs, their good biocompatibility, low toxicity, and lack of immune system activation or suppression.[19]

The first analytical experiments were performed in order to evaluate whether the components of the liposomal formulation could interfere with PTX quantification. For the HPLC method, no interference of the liposome excipients was noticed since no peak with the same retention time of the PTX was detected after injection of empty liposomes and detection at 227 nm (Figure 1). For the DS-UV method, the zero-order spectra of PTX and components of the liposomal formulation showed complete overlapping (Figure 2a). It was impossible to detect a wavelength where such interference was negligible; therefore, the classical

UV method cannot be applied. To assure no significance of these signals from lipidic components, nominal derivative values were assessed. The first-order derivatives obtained from zero-order spectra eliminated interferences with zero-crossing of all lipidic components at 246 nm, allowing PTX quantification in the liposomal formulation (Figure 2b). In addition, in order to optimize the first-order derivative method, different smoothing factors ($\Delta\lambda = 2.0$, 4.0, and 8.0) were tested and a suitable signal-to-noise ratio and spectra with good resolution were obtained when $\Delta\lambda = 2.0$ was used. Spectra with a higher order of derivation were also
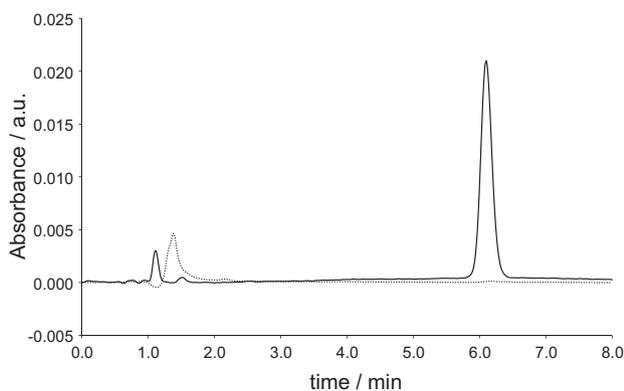
Figure 1. Representative chromatograms of specificity study. Empty liposome (dashed line) and PTX solution at 15.0 µg mL^{-1} (solid line).
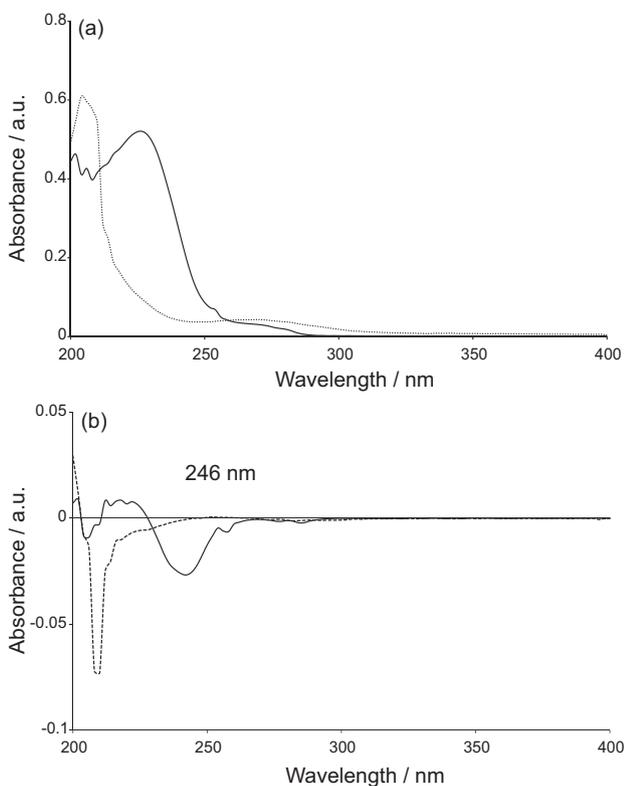
Figure 2. Zero-order absorbance spectra (a) and first-order derivative spectra (b) of empty liposome (dashed line) and PTX solution at 15.0 µg mL^{-1} (solid line).

analyzed; however, a significant reduction in sensitivity and an increase in the signal-to-noise ratio were observed (data not shown).

Table 1 shows the regression analysis data from the calibration curves of PTX for the DS-UV and HPLC methods. The least square regression showed excellent correlation, higher than 0.99, between the PTX concentration and the peak amplitude at 246 nm or peak area for DS-UV and HPLC, respectively, in the range of 6.0 and 24.0 µg mL^{-1}. The linear regression model obtained by the ordinary least squares method is the statistical method most applied to evaluate analytical procedures. This method requires the treatment of the outliers as well as the verification of the assumptions of normality, homoscedasticity, and independency of the residuals.[20] Both methods demonstrated adequate normality and homoscedasticity for *p* value less than 0.05. The linear regression equation was found to be y = 19495x (µg mL^{-1}) + 8052 and y = 0.00096x (µg mL^{-1}) + 0.0005 for HPLC and DS-UV, respectively. No significant difference was found among the slopes of the calibration curves prepared on three different days for HPLC (*p* = 0.10) and DS-UV (*p* = 0.06) methods. Significant linear regression and no deviation from linearity for both methods could be observed (Table 1).

The LOD were estimated at 0.6334 µg mL^{-1} and 0.9969 µg mL^{-1} while the LOQ found were equal to 2.1113 µg mL^{-1} and 3.3229 µg mL^{-1} for HPLC and DS-UV methods, respectively. The difference in LOD values found in comparison to previous studies[21,22] can be attributed to the methods used for the determination of this parameter. In previous studies, the LOD and LOQ were established by assessing the signal-to-noise ratio level in a proportion of 3:1. In spite of being the simplest path to determine the detection capabilities of a chromatographic method, this approach is not recommended because it is dependent on analyst interpretation since, there is no agreement on where to measure the noise and the extension of baseline that has to be measured. Therefore, the results show great variability among laboratories and analysts leading to difficulties in comparing the results. In this case, method based on the parameters of the calibration curve is statistically more reliable.[23,24] Based on these considerations, in the present study, LOD and LOQ were estimated by using the standard deviation of the response and the slope of the constructed calibration curve.

The results of intra- and inter-day precision, calculated as RSD, are reported in Table 2. Mean content of PTX intra- and inter-day were 100.9 and 101.3% for the HPLC method, whereas for the DS-UV the mean content obtained was 101.5% in both intra- and inter-day analysis. Relatively small RDS values, lower than 2.0%, for intra- and inter-day

Table 1. Overview of the linearity data, LOD, and LOQ obtained for PTX by HPLC and DS-UV methods

Regression parameter	HPLC	DS-UV
Slope ± standard deviation	19495 ± 255	$9.6 \times 10^{-4} \pm 0.2 \times 10^{-4}$
Intercept ± standard deviation	−8052 ± 4116	$5.5 \times 10^{-4} \pm 3.2 \times 10^{-4}$
Correlation coefficient (r)	0.9983	0.9991
Concentration range / ($\mu g\ mL^{-1}$)	6-24	6-24
Normality ($R_{critical}$)[a]	0.9835 (0.9437)	0.9826 (0.9569)
Homoscedasticity ($T_{critical}$)[b]	0.5707 (0.5720)	0.5117 (0.6702)
Regression ($F_{critical}$)[c]	23368.69 (4.54)	13740.24 (4.30)
Linearity ($F_{critical}$)[d]	0.32 (3.36)	1.91 (2.99)
LOD / ($\mu g\ mL^{-1}$)	0.6334	0.9969
LOQ / ($\mu g\ mL^{-1}$)	2.1113	3.3229

[a]Ryan-Joiner test; [b]Levene test; [c]Fisher test; [d]ordinary least squares method.

Table 2. Intra-day and inter-day determination of the precision of DS-UV and HPLC methods for PTX quantification

Analytical method	Mean concentration ± SD / ($\mu g\ mL^{-1}$)		RSD / %		
			Intra-day[a]		Inter-day[b]
	Day 1	Day 2	Day 1	Day 2	
HPLC	15.14 ± 0.07	15.35 ± 0.18	0.48	1.18	1.15
DS-UV	15.23 ± 0.11	15.10 ± 0.08	0.71	0.55	0.61

[a]Mean of six determinations; [b]mean of twelve determinations; SD: standard deviation; RSD: relative standard deviation.

analysis were considered adequate, assuring a good precision of the results of the two methods evaluated. Statistical analysis showed no significant difference for precision data between the DS-UV and HPLC methods ($p > 0.05$).

The accuracy of the method was determined by recovery studies of the standard addition procedure. The maximum percent recoveries for low, intermediate, and high concentrations were, respectively, 98.10, 102.0, and 98.26% for the HPLC method, and 101.40, 100.70, and 101.30% for the DS-UV method (Table 3). These results demonstrated a good agreement between amounts added and found; thus, any small change in the PTX concentration in the solution can be accurately determined by the proposed methods.

The results of the robustness study are summarized in Table 4. The results showed that the PTX content was unaffected by alteration of the concentration of acetonitrile in the eluent and attractive system for HPLC or reagent supplier in DS-UV. Although a mean recovery greater than 102.0% was observed for the HPLC method, no significant difference between the effects analyzed was detected ($p > 0.05$).

Finally, both methods were used to evaluate the PTX content in liposomal formulation. The evaluation of the drug encapsulation percentage is an essential physicochemical parameter in the development of a new drug delivery system. No significant difference was found between the previously validated HPLC method and the DS-UV method, and similar results for PTX quantification were obtained. The values obtained were equal to 87 ± 1% and 92 ± 9% for HPLC and DS-UV analysis, respectively ($p = 0.51$); thus, both methods were adequate for the determination of PTX in liposomal formulation. This

Table 3. Recovery tests of PTX in the presence of components of liposomes analyzed by HPLC and DS-UV methods

Analytical method	Amount added / ($\mu g\ mL^{-1}$)	Amount found Mean[a] ± SD / ($\mu g\ mL^{-1}$)	Recovery / %	RSD / %
HPLC	12.0	11.77 ± 0.01	98.09-98.12	0.01
	15.0	15.27 ± 0.03	101.64-101.98	0.17
	18.0	17.69 ± 0.02	98.20-98.302	0.06
DS-UV	12.0	12.05 ± 0.16	98.97-101.55	1.31
	15.0	15.11 ± 0.06	100.34-101.17	0.39
	18.0	18.23 ± 0.16	100.33-102.05	0.86

[a]Mean of three determinations; SD: standard deviation; RSD: relative standard deviation.

Table 4. Effects of the variation of analytical parameters in the PTX content

Analytical method	Effect[a]	Mean concentration[b] ± SD / (µg mL^{-1})	Mean recovery / %	RSD / %
HPLC	1	15.14 ± 0.07	100.91	0.48
	2	15.53 ± 0.17	103.79	1.07
DS-UV	1	15.33 ± 0.16	100.48	0.72
	2	15.18 ± 0.16	101.17	1.02

[a]For the HPLC method, the acetonitrile concentration was estimated at 55% (1) and 53% (2) in the mobile phase. For the DS-UV method, the methanol supplier, Proquimios (1) and Isofar (2), was evaluated; [b]mean of six determinations; SD: standard deviation; RSD: relative standard deviation.

finding confirms that the DS-UV method is valid and could be employed for the routine quality control of PTX in liposomal formulations. In addition, DS-UV is a very simple and rapid method that is advantageous in terms of cost in routine analysis.

Conclusion

In the present study, a new derivative spectrophotometric method was developed for the determination of PTX in the presence of compounds of the liposomal formulation. The DS-UV method is simple, rapid, precise, accurate, robust, and selective, and may be a useful tool for routine use in quality control laboratories for PTX determination.

Acknowledgements

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