Spectrophotometric determination of etoposide from polymeric implant and application in the study of in vitro release profile

Determinação espectrofotométrica de etoposide de implante polimérico e aplicação no estudo do perfil de liberação in vitro

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RESUMO

Um método espectrofotométrico rápido, simples e econômico foi desenvolvido para a quantificação de etoposídeo em implantes poliméricos e em amostras obtidas a partir do estudo de liberação *in vitro*. As amostras foram quantificadas a 285 nm. O método foi linear ($r^2 > 0,99$) na faixa entre 5 e 100 µg/ml, preciso (DPR < 5%), exato (valores de recuperação próximos de 100%), seletivo em relação aos excipientes das amostras, e apresentou limite de quantificação igual a 1,68 µg/ml. O método validado foi empregado com sucesso para análises de rotina de controle de qualidade. Não houve diferença significativa entre os resultados obtidos pelos método espectrofotométrico e HPLC para a determinação de etoposídeo incorporado em implantes biodegradáveis.

Palavras chave: Estudos de Validação, Espectrofotometria, Polímero.

ABSTRACT

A rapid, economical, and simple UV spectrophotometric method was developed for quantification of etoposide in polymeric implants and samples derived from in vitro release study. The samples were quantified at 285 nm wavelength. The method was linear ($r^2 > 0.99$) over the range of 5 to 100 µg/ml, precise (RSD < 5%), accurate (recovery values close to the 100%), selective regarding excipient of the sample, and had a quantitation limit equal to 1.68 µg/ml. The validated method can be successfully employed for routine quality control analyses. There was no significant difference between the spectrophotometric method and HPLC method for determination of etoposide incorporated into biodegradable devices.

Keywords: Validation Studies, Sprectrophotometry, Polymer.

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INTRODUTION

The implant is a sustained drug delivery system designed for the treatment of several diseases, including solid tumors. The implants can be inserted in the region where the tumor is located or within the tumor itself. Despite the invasive characteristics of the implantation technique, the implants present several advantages that overlap the inconveniences. These advantages include: the increase of the tumor exposure to drug, the decrease of systemic toxicity, the local maintenance of therapeutic levels for a long period of time and the optimization of the chemotherapy scheme by reducing the number of doses to be administered (Weinberg *et al*., 2008).

The implants can be prepared from the biodegradable polymers, such as poly(ε-caprolactone) (PCL), an aliphatic polyester widely used owing to its high permeability to several drugs and the possibility of a sustained and controlled drug release rate (Woodruff & Hutmacher, 2010). The implants based on biodegradable polymers can be loaded to antineoplastic drugs, such as etoposide (Figure 1). Etoposide is a semisynthetic derivative of podophyllotoxin widely used in chemotherapy of various solid tumors including lung cancer, testicular tumor, gastric tumor, ovarian cancer, and retinoblastoma (Shah *et al*., 2013).

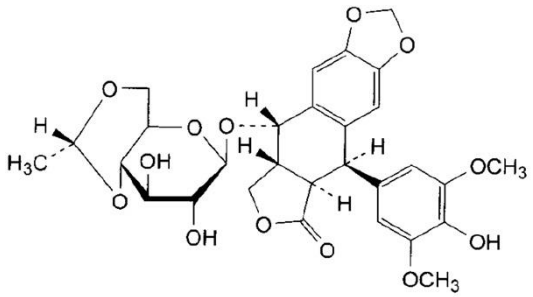

Figure 1. Chemical structure of etoposide.

Many methods are reported in literature for the measurement of etoposide in different samples. The high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) was employed for determination of etoposide in human serum and plasma (Chen & Uckun, 2000). However HPLC-MS does not represent viable analytical methods to quantify the drug in routine quality control analysis.

The official compendia recommend a method using HPLC with gradient mobile phase for assaying etoposide in raw material, soft capsules and injections (British, 2011; United, 2012; Japanese, 2012). However, this analytical method has some disadvantages when compared with the spectrophotometric method. These disadvantages including: higher cost, increased complexity and higher analysis time (between 40 and 60 min.). The development of a simple UV-spectrophotometric method can provide a very useful alternative for routine analysis of pharmaceutical formulations. Thus, the main goal of this study was the development and validation of a simple and reliable UV spectrophotometric method to quantify the etoposide incorporated into biodegradable implants. Also, the results obtained from spectrophotometric method were compared to a previously developed HPLC method to the same drug. Additionally, the analytical method was applied to assay the etoposide released from these polymeric devices.

MATERIALS AND METHODS

Materials and reagents

Etoposide was offered by Quiral Química (Brazil) and the etoposide chemical reference substance was purchased from The United Pharmacopoeia (USA) (lot H1K394, 99.7% purity). PCL (molecular weight of 14000) was purchased from Sigma-Aldrich Chemicals (USA). Acetonitrile HPLC grade was purchased from JT Baker. The other solvents and reagents used were of analytical grade.

Instruments and analytical conditions

The spectrophotometric analyses were carried out on a HP 8453 (HP, Agilent Technologies, EUA) (Shimadzu, Kyoto, Japan) spectrophotometer, in a 1 cm quartz cuvette. The detection was performed at 285 nm and the measurements were obtained against mixture of acetonitrile and phosphate buffered saline (pH 7.4) (PBS) as a blank.

Preparation of implant

The etoposide-loaded PCL implants were prepared by the melt method as described by Cheng *et al*. (2009). Briefly, PCL was melted at 60 ºC in a water bath and etoposide was thoroughly dispersed in the polymer melt. The mixture of PCL and etoposide (1:1) was allowed to cool at room temperature and molded into cylinders (6.4 mm in length, and 0.6 mm in diameter) at 60 ºC. The etoposide-loaded PCL implants contained approximately 50.0% (w/w) of the drug corresponding to 1.2 mg of etoposide.

Standard solution

Approximately 20 mg of etoposide reference standard were accurately weighed and transferred to a 100 ml volumetric flask. Acetonitrile (30 ml) was added to ensure complete solubilization and the solution was diluted to volume with PBS. The solution was filtered and aliquots of the filtrate were diluted in mixture of acetonitrile and PBS (3:7) to obtain the concentrations of 5.00, 20.0, 40.0, 60.0, 80.0 and 100.0 μg/ml.

Sample solution

Four etoposide-loaded PCL implants were weighed and transferred to a 25 ml volumetric flask. An aliquot of 7.5 ml of acetonitrile was added to ensure complete solubilization, and the volume adjusted with PBS. The mixture was filtered and an aliquot of 8 ml of filtrate was transferred to a 25 ml volumetric flask and the volume adjusted with mixture of acetonitrile and PBS (3:7).

Validation

The method was validated by determining the parameters of selectivity, calibration curve, precision, accuracy and quantitation limit (Brasil, 2003; ICH, 1996).

Selectivity

The selectivity was evaluated by the determination of etoposide concentration in the sample solution and in standard solution prepared as described above. The average concentration of etoposide (n = 6) of the two groups (sample and standard solutions) was compared using Student's t test ($\alpha = 0.05$). The F test (Snedecor) was applied to evaluate homoscedascity (INMETRO, 2003).

Calibration curve

The calibration curve was obtained using six reference standard concentrations (5.00, 20.0, 40.0, 60.0, 80.0 and 100.0 μg/ml) in three independent replicates run in random order. The calibration curve constructed was assessed using residue analysis (homoscedascity, normality, and independence of residues) and linear regression analysis was done by the ordinal least squares method (Souza & Junqueira, 2005).

Precision

The intra-day precision was assessed through the assay of sample solutions at concentrations of 5.00, 60.0, and 100.0 μg/ml on the same day. The solutions were prepared in triplicate for the incorporation of etoposide in placebo solution (PCL, 60.0 μg/ml) and subsequent filtration. Similarly, the inter-day precision was evaluated by same analyst in two consecutive days.

Accuracy

Standard solutions at concentrations of 5.00, 60.0 and 100.0 μg/ml were prepared in triplicate by the incorporation of etoposide reference standard in placebo solution (PCL, 60.0 μg/ml) and subsequent filtration. The solutions were assayed by the spectrophotometric method on two different days.

Quantitation limit

The limit of quantitation value (LOQ) was calculated directly from the calibration curve and can be expressed as:

$LOQ = 10\ \sigma/b$ [1]

where, σ is the standard deviation of the response and b is the slope of the calibration curve (Brasil, 2003; ICH, 1996).

Determination of released etoposide from polymeric implants

The in vitro release study was carried out in quintuplicate in the release medium (PBS) under sink conditions. The sink conditions are "*defined as the volume of medium at least three times that required in order to form a saturated solution of drug substance*" (United…, 2012). Each implant was immersed in 30 ml of PBS to ensure that the sink conditions were achieved, since the solubility of etoposide in PBS at 37 °C is 125.93 μg/ml (Shah *et al*., 1998). The tubes containing the implant and PBS were kept in an incubator at 37 ºC and 30 rpm for six months. At predetermined time points, 15 ml of

the release medium were taken out and replaced with 15 ml of fresh medium. The etoposide concentration in the release medium was determined by spectrophotometric method described above and expressed as the cumulative percentage of etoposide released in the medium.

Instrumentation and chromatographic conditions

The samples were analyzed by HPLC method (Solano *et al*., 2012) using a Thermo Surveyor System (USA) which included a quaternary pump, autosampler, diode array detector (DAD), and ChromQuest 4.2 software. The Ace C18 column (250 x 4.6 mm i.d.; 5 µm particle size) from ACT was used and maintained at 25 °C. The mobile phase was comprised of acetic acid 4% (v/v) and acetonitrile (70:30), at a flow rate of 2 ml/min. The injection volume was 25 µL and the detection was performed at 285 nm. The HPLC method was compared to the proposed spectrophotometric method using the Student's t-test (α=0.05).

RESULTS AND DISCUSSION

In this study, an UV spectrophotometric method was developed and validated for determination of etoposide content incorporated into PCL implants and released from these implantable devices. Initially, the UV spectra of etoposide and of PCL, in the range of 200–400 nm (Figure 2), were evaluated and the wavelength of 285 nm was selected for detection due to the adequate molar absorptivity of etoposide at this wavelength, and higher selectivity regarding possible interference of PCL in the sample. This fact was confirmed by the absence of the significant difference ($p > 0.05$) between the average concentrations of standard solution (60.15 ± 0.14 µg/ml) and sample solution (60.09 ± 0.15 µg/ml) as determined by the spectrophotometric method. Considering the previous results, the method had adequate selectivity for the determination of etoposide in polymeric implants.

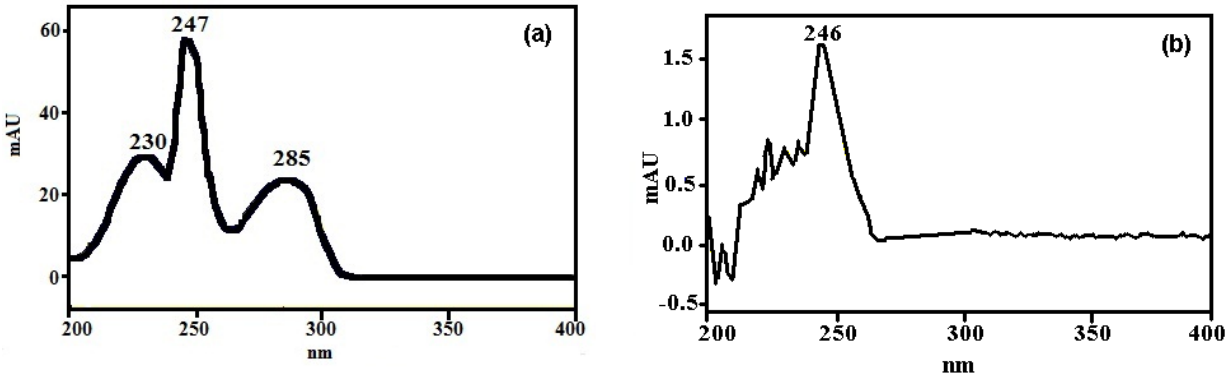

Figure 2. UV spectra of (a) etoposide solution at 60 µg/ml and of (b) PCL solution at 60 µg/ml, both in acetonitrile.

The regression between etoposide concentration and absorbance, within the range of 5.00 to 100.0 µg/ml, was considered significant. The linear model proved to be adequate as it could be shown that the residues followed a normal distribution pattern and were independent, while homoscedasiticity was evident and lack of fit was not significant. In addition, high determination coefficient (r^2) value was obtained (Table 1). The limit of quantitation was calculated as 1.68 µg/ml.

Table 1. Parameters of the calibration curve for etoposide within the range of 5.00 to 100.0 µg/ml.

Regression Parameter	Curve
Slope ± standard error	0.0067± 0.00008
Intercept ± standard error	0.0184 ± 0.005
Coefficient of determination (r^2)	0.998
Coefficient of correlation (r)	0.999
Number of points	6

The precision data obtained for the evaluated methods are demonstrated in Table 2. All levels of concentrations presented relative standard deviation (R.S.D.) values lower than 5.0%, assuring a good precision (Brasil, 2003).

Table 2. Validation parameters of the spectrophotometric method for etoposide determination.

Level (µg/ml)	Intra-day precision, n = 3 (R.S.D., %)	Inter-day precision, n = 6 (R.S.D., %)	Accuracy (mean recovery ± R.S.D., %)	
			Intra-day (n=3)	Inter-day (n=6)
5.00	1.18	1.62	99.66 ± 1.38	100.02 ± 1.31
60.0	0.20	0.31	99.56 ± 0.42	99.72 ± 0.34
100.0	0.23	0.37	99.65 ± 0.44	99.83 ± 0.34

According to the trueness parameter, there was no evidence indicating systematic errors in the results using the spectrophotometric method. Upon plotting the concentrations determined experimentally versus the theoretical values, a line was obtained. The experimental values were approximate to the true values, thus the line did not shift away from the ideal line, in which the intercept was equal to zero and the slope was equal to one, in turn proving the absence of systemic errors (INMETRO, 2003; Rozet *et al*., 2007).

The method had appropriate accuracy, as can be seen by the values calculated for the β tolerance interval (Figure 3) for each concentration level, which showed a maximum variation of 5% (Rozet *et al*., 2007). Accuracy is represented by the combination of the random (precision) and systematic (trueness) errors, which were considered in the β tolerance interval calculation. This represents the interval in which β% of the future individual results is expected (Solano *et al*., 2013).

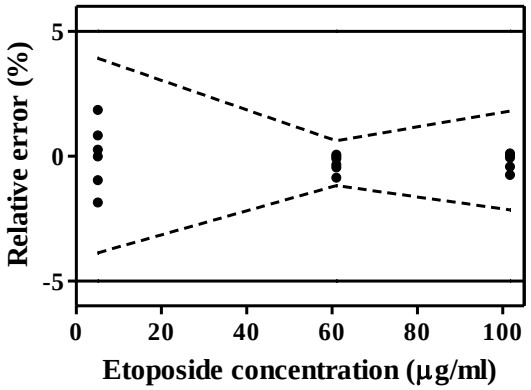

Figure 3. Accuracy profile obtained for the spectrophotometric method. The continuous lines represent the acceptance limits (-5%, 5%) whereas the dashed lines represent the 95% tolerance interval reached. When the tolerance intervals are included in the acceptance limits, the assay is able to be quantified accurately.

The validated method described was applied to assay etoposide content in the biodegradable devices. The mean etoposide content incorporated into the polymeric implant was 99.82 ± 0.22% (n = 6) of the pre-indicated value (50% w/w). The results obtained by spectrophotometric method were compared to HPLC method (100.07 ± 0.41%) and there was no significant difference between the methods ($p > 0.05$). Although both methods showed to be adequate to quantificate etoposide incorporate into PCL implant, the spectrophotometric method was more practicable (sample preparations for UV method was more simple), faster (HPLC analysis time was 15 minutes), and relatively less expensive (lower cost of instrumentation and operation). Thus, this method was also used to quantify the etoposide released from the polymeric implants.

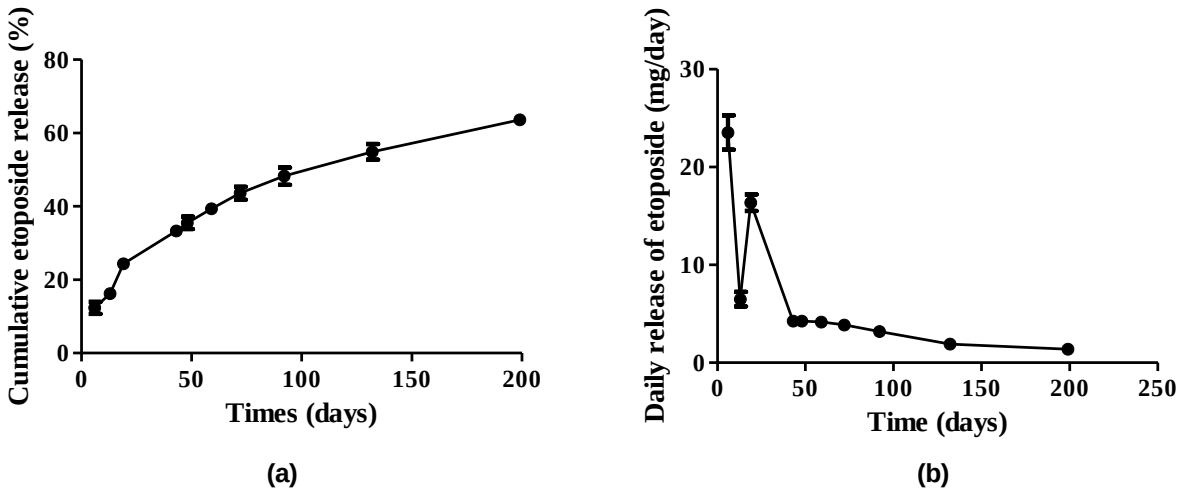

Figure 4. Cumulative release of etoposide from biodegradable implant in PBS (a), daily release of etoposide (mg/day) as a function of time (b). Results represent mean ± standard deviation (n = 5 for each time).

The Figure 4 shows *in vitro* release profile of etoposide from biodegradable implants. During the 200-days period, a small burst effect phase, followed by slow release over a prolonged period was observed. Drug release from monolithic device (system that has the drug dispersed within a polymer) can occur by diffusion, degradation of the polymer, or a combination of these two mechanisms (Dash & Konkimalla, 2012). During the first 20 days, approximately 24% of the etoposide was released from the implant (Figure 4a). This initial fast release was considered to be a result of the fast dissolution and diffusion of the drug at the solid liquid interface. In the second phase, the drug release rate gradually slowed down (Figure 4b), and approximately 63% of the etoposide was released from the implant. The slow rate of drug release probably was dominated by the diffusion of etoposide from polymer since the PCL is characterized by a very low hydrolysis rate, which can extend over a period of more than one year (Dash & Konkimalla, 2012). In addition, the low water solubility of etoposide may make its release to the medium difficult, making its diffusion very slow. Cheng *et al*. (2009) and Fialho *et al*. (2008) obtained similar results for lipophilic drugs (praziquantel and dexamethasone, respectively) incorporate into PCL implants. In these studies, a biphasic release pattern also was obtained for the drugs.

The development of modified release drug delivery system requires the evaluation of *in vivo* and *in vitro* performances of these systems. Thus, it is necessary to develop analytical methodologies to enable the realization of this evaluation. The UV spectrophotometric method developed in this study was applied in the *in vitro* release study and may be applied to *in vivo* evaluation in the future since there are reports of spectrophotometric quantification of etoposide in biological matrix (Dandagi *et al*., 2011).

CONCLUSION

Currently there is vast interest in the development of drug delivery systems due to their advantages. However, to ensure the quality of produced systems it is necessary to develop analytical methods for application in the routine quality control analysis. The spectrophotometric method showed to be adequate to quantify etoposide incorporated into PCL implant, and released from them. This method offers advantages over other analytical methods due to its rapidity, simplicity, and lower cost. In addition, there was no significant difference between the previously validated HPLC method and UV-spectrophotometric method.

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