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Anexo

Paper aprovado para publicação na Microchemical Journal intitulado como Room-temperature phosphorimetry for the determination of trace contaminations of camptothecin in anticancer drugs (doi:10.1016/j.microc.2010.02.007).

ARTICLE IN PRESS

MICROC-01183; No of Pages 6

Microchemical Journal xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Room-temperature phosphorimetry for the determination of trace contaminations of camptothecin in anticancer drugs

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ARTICLE INFO

Article history:
Received 20 November 2009
Received in revised form 11 February 2010
Accepted 18 February 2010
Available online xxxx

Keywords:
Camptothecin
Irinotecan
Topotecan
Room-temperature phosphorimetry
Measurement uncertainty

ABSTRACT

Campthotecin derivatives, irinotecan (CPT-11) and topotecan (TPT), have been used for the treatment of cancer and camptothecin (CPT) is a potential contaminant in these anticancer medicines. In this work, room-temperature phosphorimetry was used to quantify either CPT as trace contaminants in anticancer drugs and CPT-11 as the main ingredient in anticancer medicines. Phosphorescence from CPT-11 was induced using $Pb(NO_3)_2$ in SDS treated cellulose substrate while CPT was selectively detected using $TlNO_3$ as a phosphorescence enhancer in either cellulose or nylon substrates. The limit of quantification for CPT and CPT-11 was in ng order. A detailed metrological study was made to calculate the combined uncertainty associated to the CPT phosphorescence measurement. Satisfactory analyte recoveries were obtained for CPT-11 as a main active ingredient of a pharmaceutical formulation. The selective determination of CPT in a matrix containing TPT was made using the higher order (2nd) derivative of the excitation spectra. The results demonstrated the applicability of the method due its simplicity, cost effectiveness and selectivity.

1. Introduction

Cancer is the second leading cause of death worldwide. Although great advancements have been made for its treatment and control, chemotherapy remains one of the main approaches in cancer therapy despite a number of undesired side effects. [1]. Camptothecin (CPT) is a cytotoxic alkaloid from the *Camptoteca acuminata* [2] and among their synthetic derivatives, irinotecan (CPT-11) is currently used in the treatment of the cancer [3]. Its mechanism of action consists of the inhibition of the enzyme topoisomerase I, involved in the process of transcription of the DNA [2]. United States Food and Drug Administration approved CPT-11 for treatment of metastatic cancer of the colon or rectum and the recommended dosage of CPT-11 applied in the patient is 20 mg mL^{-1} [3]. Topotecan (TPT) is another topoisomerase I inhibitor approved by the FDA for the treatment of ovarian cancer.

According to the International Federation of Pharmaceutical Manufactures (IFPMA) and associations, the trade of counterfeit drugs is widespread and affects both developing and developed countries. Treatment of diseases with counterfeit or substandard medicines may lead to deterioration of health and even the death of patients [4]. The IFPMA also works in close partnership with the World Health Organization (WHO) to improve drug quality and fight counterfeiting around the world. Counterfeit drugs are found under different forms which include products with the correct ingredients but often containing incorrect quantities of these active ingredients, products with the wrong ingredients or without active ingredient and the products containing traces of undesired contaminants [5].

Only a few analytical methods are described in the literature for the determination of CPT-11 and CPT. Most of these methods employ HPLC with photometric (in the UV range), fluorimetric or mass spectrometry detection [6–8]. Phosphorimetric methods present great advantages in terms of labor and operational cost and enable the selective determination of very similar substances without previous separation by exploring small differences in their phosphorescence spectral characteristics. In solid surface room-temperature phosphorimetry (SSRTP) the analytical signal is measured from analytes immobilized in substrates, for instance, cellulose or nylon substrates. The right choices of the experimental and instrumental conditions when detecting traces of luminescent contaminants in a matrix containing other luminescent species improve selectivity and detectability [9,10]. However, the uncertainty of the luminescence measurement must be carefully evaluated in order to enable reliable results, in special, when inhomogeneous substrates like cellulose (high background fluctuations) is used [11].

Uncertainty is a metrological term which defines a parameter associated with the result of a measurement and characterizes the dispersion of the values that could be reasonably attributed to the measured parameter. In order to improve the quality of measurement, uncertainty sources must be identified and, if necessary, minimized. This approach requires the identification of all possible sources of uncertainty associated with the applied procedure, the estimation of their magnitude either from experimental or published data and,

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0026-265X/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.microc.2010.02.007

Please cite this article as: P.M.S. Maia, et al., Room-temperature phosphorimetry for the determination of trace contaminations of camptothecin in anticancer drugs, Microchem. J. (2010), doi:10.1016/j.microc.2010.02.007

finally, the combination of all individual sources to give the combined and expanded uncertainties for the whole measurement procedure [12].

In the present work, a SSRTP selective method was developed for the determination of the anticancer active principle CPT-11 and traces of the contaminant CPT in anticancer pharmaceutical formulations. Metrological aspects were evaluated aiming application to pharmaceutical formulations. The proposed method can be used as an alternative to HPLC based methods allowing rapid analysis and low operational costs.

2. Material and methods

2.1. Instrumentation

Phosphorescence measurements were made on a luminescence spectrometer LS-55 (Perkin-Elmer, Norwalk, USA) coupled to a solid surface analysis apparatus modified to allow a flow of purging dry nitrogen gas on the sample holder. The instrument was operated with 1500 nm min^{-1} scan rate, 0.10 ms delay time and 3 ms gate time. Spectral bandpass was set to 20 nm.

Chromatographic analysis was made on a high performance liquid chromatograph (model Breeze, Waters, USA) equipped with a model 1525 binary pump and a model 2478 multi λ fluorescence detector set at 355/515 nm. Sampling was made manually using a Reodyne injector and a 10 μL sample loop. Degassing of mobile phase solvents was made off-line in a 9 L ultrasonic bath, model NSC2800 (Unique, São Paulo, Brazil). Separation was made on a 4.6 × 150 mm X-Terra RP C18 (Waters, Massachusetts, USA) with 5 μm particle size. The column was kept inside an oven set at 35 °C. Statistical calculations were made using the Statistica 7.0 software package.

2.2. Reagents and materials

Camptothecin (CPT), irinotecan (CPT-11) hydrochloride were obtained from Sigma-Aldrich (USA). Camptosar (injectable solution containing 20 mg mL^{-1} CPT-11 hydrochloride from Pfizer) was the chosen CPT-11 pharmaceutical formulation. Topotecan (TPT) was obtained from their injectable pharmaceutical Hycamtin (sterile lyophilized powder containing topotecan hydrochloride equivalent to 4 mg of topotecan as free base, Glaxo SmithKline). Thallium and lead nitrates were from Across (USA). Methanol, acetic acid, boric acid, phosphoric acid, sodium dodecyl sulfate (SDS) and sodium hydroxide were obtained from Merck. Filter paper (Whatman No. 42) used as cellulose substrate was treated to reduction of background. The nylon substrate (Nylon 66 with 0.2 μm pore size) used was obtained from Whatman Ltd, UK. Ultrapure water (resistivity of 18.2 MΩ cm from a Millipore system) was used throughout. Nitrogen (99.996%) was from White Martins, Brazil and it was further purified passing it through an ammonium metavanadate solution and dried in a silica gel bed. Acetonitrile and methanol HPLC-grade were from Merck, Brazil. All reagents were of analytical grade and they were employed without further purification. Urine and saliva were obtained from a healthy volunteer.

2.3. General procedures

CPT stock solutions (4×10^{-4} mol L^{-1}) were prepared in methanol. Irinotecan hydrochloride and topotecan hydrochloride (4×10^{-4} mol L^{-1}) were prepared in ultrapure water. CPT standard solutions of lower concentrations were made by further dilution of the stock solutions with water/Britton–Robinson buffer (pH 10.5). The buffer solution (0.04 mol L^{-1}) was prepared by mixing acetic acid, boric acid and phosphoric acid aqueous solutions. The pH of buffer solution was adjusted by addition concentrated sodium hydroxide solution. Ultrapure water was used to prepare all heavy atom salts and SDS solutions. The concentration of these working solutions for the initial studies was $TlNO_3$ (0.25 mol L^{-1}), $Pb(NO_3)_2$ (0.25 mol L^{-1}), $AgNO_3$ (0.01 mol L^{-1}), $Cd(C_2H_3O_2)_2$ (0.50 mol L^{-1}), $HgCl_2$ (0.25 mol L^{-1}) and KI (0.20 mol L^{-1}). Five μL of analyte standard solutions or sample solutions was deposited on the solid substrates (cellulose or nylon). When necessary, the substrates were previously applied with 5 μL of surfactant (SDS) followed by 5 μL of the heavy atom solution. Solid substrates spotted with samples or blanks were dried at room temperature in a vacuum desiccator (120 min for cellulose and 40 min for nylon). Before signal measurement, the substrate was placed in a solid surface luminescence measurement apparatus. Excitation radiation was focused in the center of the substrate, where sample was spotted, and luminescence was collected at an angle of 90°. Before phosphorescence measurements, a dry nitrogen flow was passed over the substrate surface to minimize quenching effects from oxygen and air moisture. Urine and saliva samples were diluted in Britton–Robinson pH 10.5 buffer in a 1/1 v/v proportion before being placed onto the solid substrate.

Analyte recoveries achieved with the proposed phosphorimetric method were compared with the ones achieved by HPLC method with fluorescence detection adapted from the Tsai [8]. For HPLC, isocratic elution (1 mL min^{-1}) was used with mobile phase acetonitrile/10 mmol L^{-1} acetate buffer (pH 3.5) 30/70% v/v. Retention times for CPT-11 and CPT were 4.0 and 8.0 min, respectively.

3. Results and discussion

3.1. Preliminary studies

Six heavy atom salts were tested in order to evaluate their capacity to selectively induce room-temperature phosphoresce from CPT and CPT-derivatives. The heavy atom salts chosen were the ones most commonly used to induce room-temperature phosphorescence (RTP). The external heavy atom effect may induce or amplify phosphorescence of specific substances in a complex mixture to a few orders of magnitude by significantly enhancing both the rate of intersystem crossing (excited singlet state–excited triplet state transition) and the phosphorescence rate constant [13]. This study was made on cellulose substrates either with or without a previous spiking of SDS solution. Intense phosphorescence was achieved from the three alkaloids (CPT, CPT-11 and TPT) using either $Pb(NO_3)_2$ and $TlNO_3$, therefore, no selective heavy atom effect was observed (Fig. 1). However, some selective power may still be achieved by using resources such as synchronization and high order spectral derivatization.

3.2. Maximization of the phosphorescence from CPT and CPT-11

Two conditions were selected to be optimized based on the higher phosphorescence achieved. For CPT, the heavy atom enhancer was $TlNO_3$ while $Pb(NO_3)_2$ was selected for CPT-11 determinations. Studies were performed in order to maximize RTP from these two alkaloids in low background cellulose substrate.

A specific study was made to evaluate the influence of the pH of the analyte carrier solution used to deliver the analyte to the substrate. Buffered solutions at pH values between 2 and 12 (Britton–Robinson buffer) were used. From pH 2, where no phosphorescence was observed, to pH 3, a significant increasing of signal occurred. The signal remained constant up to pH 7 and then a further signal improvement (about two times) was achieved in basic range, where phosphorescence remained constant from pH 9 to 12. The effect of the amount of $TlNO_3$ deposited on the substrate was also evaluated (from 0 to 664 μg the $TlNO_3$). As the mass of the phosphorescence enhancer was varied from 0 to 266 μg a significant increasing of CPT signal was observed, which remained constant as the mass of $TlNO_3$ was increased up to 664 μg.

Please cite this article as: P.M.S. Maia, et al., Room-temperature phosphorimetry for the determination of trace contaminations of camptothecin in anticancer drugs, Microchem. J. (2010), doi:10.1016/j.microc.2010.02.007

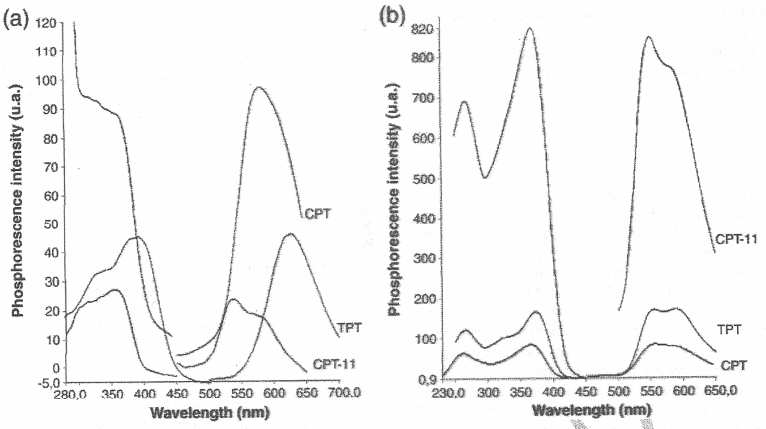

Fig. 1. Phosphorescence spectra for CPT, CPT-11 and TPT in (a) cellulose substrate with $TlNO_3$ and (b) SDS treated cellulose substrate with $Pb(NO_3)_2$.

The results obtained in the univariate study indicated that two factors influence the magnitude of the phosphorescence signal: i) the pH of the analyte solution and ii) the mass of the $TlNO_3$ deposited in the substrate. Therefore, a multivariate study was carried aiming to identify possible interactions between these factors, to evaluate the robustness for each factor and to obtain a final set of optimum experimental conditions. A circumscribed central composite design of two levels was used. A high value (+1) and a low value (−1) for each factor were chosen in function of the results displayed in the univariate study. The values set for pH were pH 9.0 (−1) and pH 12.0 (+1), therefore the other values set for the design were pH 10.5 (0); pH 8.4 ($-\sqrt{2}$); and pH 12.6 ($+\sqrt{2}$). For $TlNO_3$, mass values were 186 μg ($-\sqrt{2}$); 213 μg (−1); 266 μg (0); 332 μg (+1); and 346 μg ($+\sqrt{2}$). In this work the experimental design was performed with three genuine replicates for each point except the central one (0,0) where five replicates were used. Such strategy was used to minimize the influence of signal variations caused by the measurement of phosphorescence from solid substrates. In the statistical experimental analysis, the significance of the factors was evaluated *F*- and *t*-tests and any interaction among variables could be graphically detected by the Pareto's chart (Fig. 2). The results indicated no relevant interactions between the factors and the linear contribution of the amount of $TlNO_3$ as a significant factor in the model. In other words, the statistical result indicated that a further increase of the mass of $TlNO_3$ on the substrate was needed, however, since the previous univariate study indicated that CPT signal was constant over the phosphorescence enhancer mass range from 266 to 664 (robust condition), therefore, 332 μg of $TlNO_3$ was chosen as the optimum experimental condition. In terms of pH, results also indicated robustness of signal in the range from 9 to 12, therefore, the pH 10.5 was chosen for further experiments. Under the optimized conditions, CPT phosphorescence was amplified in one order of magnitude.

For CPT-11, the study to maximize the analytical signal involved an evaluation of the influence of the pH and the adjustment of the amount of $Pb(NO_3)_2$. In this case, CPT-11 phosphorescence was significantly more intense in SDS treated low background cellulose substrates (substrates with its center previously spiked with 5 μL of 0.40 mol L^{-1} SDS solution). SDS sometimes improves RTP from phosphors placed in solid substrates since it may establish a better interaction of the analyte with the solid substrate and may also improve the contact between the analyte and the heavy atom enhancer. The pH for CPT-11 was tested from 2 to 12 and the CPT-11 phosphorescence remained constant in the 5 to 12 pH range, therefore, no buffered CPT-11 solutions were used. The amount of the

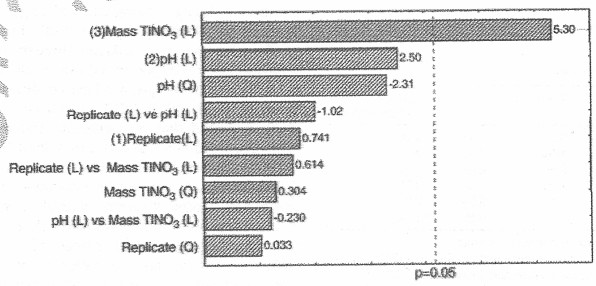

Fig. 2. Pareto's chart for the circumscribed central composite design using three replicates per point and five replicates for the central point (factors: amount of $TlNO_3$ and pH).

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heavy atom salt, $Pb(NO_3)_2$, deposited in the cellulose substrate was varied from 0 to 828 μg. The results indicated that robust condition with constant CPT-11 phosphorescence in substrates containing from 563 to 729 μg of $Pb(NO_3)_2$ were used. Therefore, 662 μg of $Pb(NO_3)_2$ was chosen for further experiments.

3.3. Selectivity studies

The feasibility of the selective CPT determination was performed using synthetic mixtures containing different CPT/CPT-derivatives. In Table 1, the ratios between phosphorescence measured from a CPT standard and from a CPT solution of the same concentration mixed with increasing concentrations of either CPT-11 (I_{CPT}/I_{CPT-11}) or TPT (I_{CPT}/I_{TPT}) are indicated. I_{CPT}/I_{CPT-11} or I_{CPT}/I_{TPT} values close to unity implied in no significant interference from CPT-11 or TPT in the measured CPT phosphorescence signal. Both CPT-11 and TPT presented phosphorescence under the same experimental conditions set for CPT. I_{CPT}/I_{CPT-11} value remained close to unity only for mixtures containing CPT-11 concentrations up to five times higher than CPT (measured at 570 nm of the emission spectra). As the proportion of CPT-11 increased, a spectral interference was observed ($I_{CPT}/I_{CPT-11}>1$). The use of second order derivative spectra brought no improvement in terms of selectivity.

In mixtures containing TPT, no spectral interferences on CPT was achieved only for TPT/CPT equimolar mixtures. By taking advantage of the small spectral differences, the higher order (2nd) derivative of the excitation spectra allowed selective determination of CPT in solutions containing concentrations of TPT up to 40 times higher. In this case, signal measurement was made at the isodifferential wavelength (λ_{iso}) at 367 nm as seen in Fig. 3.

3.4. Analytical figures of merit and calculation of the uncertainty using cellulose substrate

The analytical figures of merit were estimated using the optimized experimental conditions. Three analytical curves for each of the analytes were constructed in order to obtain the reported results. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to the equations: $3\times s_b\ a^{-1}$ and $10\times s_b\ a^{-1}$, where s_b is the standard deviation of 10 replicates of the blanks and "a" is the angular coefficient of the analytical curve. Detectability was also reported in terms of mass values in the substrate (absolute limits of detection, ALOD, and quantification, ALOQ). These values were calculated using the equations: ALOD = (LOD) × V × MM and ALOQ = (LOQ) × V × MM, where V is the volume of the analyte solution deposited in the cellulose substrate (5 μL) and MM is the molar mass of the analyte.

For CPT, LOD and LOQ calculated values were respectively 6.2×10^{-6} mol L^{-1} and 2.1×10^{-5} mol L^{-1}, which in terms of mass values in the substrate were 10.8 ng (ALOD) and 36.6 ng (ALOQ). These calculations were based on measurements at 570 nm of the regular emission spectra. When measuring the signal in the 2nd derivative excitation spectra ($\lambda_{iso}=367$ nm), LOD and LOQ values were respectively 2.7×10^{-6} mol L^{-1} and 9×10^{-6} mol L^{-1}. The ALOD and the ALOQ were respectively 4.8 and 15.9 ng. The linear response covered the range between the ALOD up to at least 348 ng of this analyte as indicated by $R^2=0.9936$ for the calibration curve ($y=3.9\times10^5\,x-26.6$) measured at 570 nm of the emission spectra and by $R^2=0.9932$ for the calibration curve ($y=7.2\times10^5\,x-1.2$) measured at the 367 nm of the 2nd derivative excitation spectra).

For CPT-11 the detectability parameters were LOD and LOQ of respectively 3.4×10^{-6} and 1.1×10^{-5} mol L^{-1} which enabled ALOD and ALOQ values of 11.5 and 38.6 ng. These measurements were made at 540 nm at the excitation spectra. The linear response covered the range between the ALOD up to at least 440.2 ng of the analyte as indicated by $R^2=0.9914$ for the calibration curve ($y=7.2\times10^6x-50.6$). The repeatability was estimated by the relative standard deviation of consecutive phosphorescence measurements, $n=10$ (CPT) and $n=7$ (CPT-11), of the same analyte solution spiked in different cellulose substrates. In such study, the precision was estimated through the relative standard deviation (RSD) in three different amounts of CPT (41.8; 83.7 and 139.3 ng) and a single one amount of CPT-11 (152.4 ng). The repeatability (RSD) values for CPT were 14; 6 and 6% in the increasing order of analyte mass. For CPT-11 the repeatability value was 4%. The intermediary precision was calculated based on the analysis of variance (ANOVA) comparing two different analysts, each one preparing ten different substrates with the same analyte solution before measuring their phosphorescence. For CPT, the result was below 3% no matter the amount of CPT in the substrate (41.8 ng; 83.7 ng and 139.3 ng). For CPT-11, intermediary precision was 1% using 152.4 ng of CPT-11 in the cellulose substrate.

The robustness was evaluated through either the univariate or the multivariate studies performed previously. Taking into consideration the influence of the pH, a robust condition was found for CPT (pH between 9 and 12) and CPT-11 (pH between 5 and 12). A further study indicated that no significant signal variation was observed when the parameter was varied by at least 10% of the optimized value.

Since CPT must be detected as a contaminant in a matrix containing a CPT-derivative, a more reliable evaluation of its measurement performance was needed, therefore, an evaluation of CPT phosphorescence measurement uncertainty was made. Potential sources of uncertainty were classified in four groups: repeatability (u_r); intermediary precision (u_R); solutions (u_s) and calibration curve (u_{curve}). The uncertainties were calculated using three CPT solutions with different concentrations (2.4×10^{-5}; 4.8×10^{-5} and 7.4×10^{-5} mol L^{-1}). The u_r and u_R results

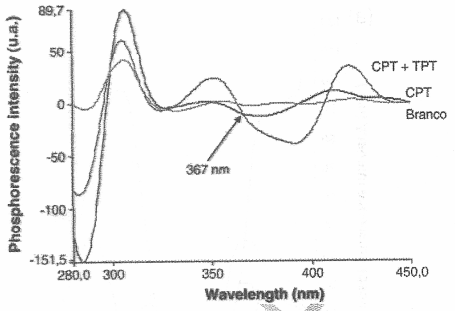

Fig. 3. Derivative of the CPT phosphorescence excitation spectra obtained from a CPT standard solution and from a CPT solution containing concentrations of TPT 40 times higher.

Table 1
Interference studies on CPT phosphorescence.[a]

CPT/CPT-11 or CPT/TPT (mol L^{-1})	Proportion	I_{CPT}/I_{CPT-11}[b]	I_{CPT}/I_{TPT}[c]	I_{CPT}/I_{TPT}[c] 2nd derivative
$4\times10^{-5}/4\times10^{-5}$	1 to 1	1.07	1.03	1.04
$4\times10^{-5}/2\times10^{-4}$	1 to 5	0.96	0.80	1.04
$4\times10^{-5}/4\times10^{-4}$	1 to 10	0.71	0.61	1.02
$4\times10^{-5}/6\times10^{-4}$	1 to 15	0.53	0.61	1.05
$4\times10^{-5}/1\times10^{-3}$	1 to 25	–	0.59	0.98
$4\times10^{-5}/1.6\times10^{-3}$	1 to 40	–	0.56	1.02

[a] Room-temperature phosphorescence in cellulose substrate.
[b] I_{CPT}/I_{CPT-11} – Ratio between the phosphorescence measured from a CPT standard and the phosphorescence measured from a mixture containing CPT and CPT-11.
[c] I_{CPT}/I_{TPT} – Ratio between phosphorescence measured from CPT standard and the phosphorescence measured from a mixture containing CPT and TPT.

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were obtained from the evaluation of repeatability (by the RSD calculation) and from the intermediary precision (ANOVA). The u_s value was calculated from the expanded uncertainties from the volumetric apparatuses (in this case the expanded uncertainty from the adjustable microliter pipettes, U_{mp} and the volumetric flask, U_{vf}) used in the preparation and dilution of the CPT solution. The adjustable pipettes (the 5 μL volume one used to deliver the solution to the substrate and the adjustable 100–1000 μL one used to prepare the analyte solutions) had their uncertainties established at specific volumes. The uncertainty was obtained from $u_{mp} = U_{mp}/k$ and from $u_{vf} = U_{vf}/k$ where U values were obtained from calibration certificates and $k = 2$ (the chosen coverage factor, 95.4%). The u_s value was calculated by the square root of the quadratic summation of these two uncertainty values (u_{vf} and u_{mp} of the microliter pipette with the highest uncertainty) multiplied by the uncertainty of a dilution factor indicated as $u_{f16.6}$ (dilution factor = 16.6) or u_{f8} (dilution factor = 8.0) or $u_{f5.4}$ (dilution factor = 5.4), depending on the CPT concentration used in the study. The u_{curve} was calculated using parameters of the analyte addition curve that was constructed using a restricted concentration range (from 2.4×10^{-5} to 7.4×10^{-5} mol L^{-1}) using four different CPT concentrations within this range. The standard deviations for both the sensitivity (m) and the linear coefficient (b) of the analyte addition curve were calculated in order to get their respective uncertainties u_m and u_b. A detailed description can be found in Cunha et al. [11]. Uncertainty contributions are listed in Table 2 where values in parenthesis were the ones obtained using measurements at 367 nm of the 2nd derivative spectra.

The combined uncertainty (u_c) is the square root of the quadratic summation of the four uncertainty values (u_r, u_R, u_s and u_{curve}), considering that the contributing groups are independent from each other on the overall variability of the measurement. The expanded uncertainty (U) which provides an interval within the measured value is believed to lie with a higher level of confidence. U is obtained by multiplying u_c by the coverage factor k whose value is chosen based on the desired confidence level ($k = 2$; 95.4%). The combined and expanded uncertainty values as well as the contribution from each of the groups of uncertainty can be observed in Table 2. The combined uncertainty (u_c) obtained for the CPT measurement for the more diluted analyte solution was 15% (28% when using the 2nd derivative spectra) which is a satisfactory value considering that this concentration is close to the LOD of the method. For the other two concentration levels, uncertainties associated to the CPT phosphorescence measurement were 6% (around 16% when using the 2nd derivative spectra). The major source of uncertainty was from the repeatability which is mainly affected by variation in solid substrate, due to, probably, the cellulose substrate non-homogeneity which, among other variations, produces fluctuations in phosphorescence background even in substrates from the same lot and cut from the same paper sheet.

Table 2
Uncertainty for the phosphorescence measurement of CPT in cellulose substrate with different concentrations (masses): A) 2.4×10^{-5} (42 ng); B) 4.8×10^{-5} (84 ng) and C) 7.4×10^{-5} mol L^{-1} (129.5 ng). Values in parenthesis are the ones obtained using the 2nd derivative spectra.

Uncertainty sources	Uncertainty values (mol L^{-1})		
	A	B	C
u_r	3.23×10^{-6} (1.94×10^{-6})	3.06×10^{-6} (2.24×10^{-6})	4.06×10^{-6} (3.82×10^{-6})
u_R	7.04×10^{-7} (2.25×10^{-7})	7.11×10^{-7} (1.28×10^{-6})	9.36×10^{-7} (6.04×10^{-6})
Volumetric flask 5 mL (u_{mp}, cert.*)	1.38×10^{-6}	1.38×10^{-6}	1.38×10^{-6}
Microliter pipette (100–1000 μL) (u_{mp}, cert.*)	3.45×10^{-7}	3.45×10^{-7}	3.45×10^{-7}
Microliter pipette 5 uL (u_{mp}, cert.*)	2.16×10^{-8}	2.16×10^{-8}	2.16×10^{-8}
Dilution factor (u_f)	1.4×10^{-1}	7.0×10^{-2}	4.0×10^{-2}
u_s	5.28×10^{-7}	3.73×10^{-7}	3.01×10^{-7}
Linear coefficient (u_b)	5.07×10^{2} (4.44×10^{1})	5.07×10^{2} (4.44×10^{1})	5.07×10^{2} (4.44×10^{1})
Sensitivity (u_m)	1.53×10^{11} (1.51×10^{10})	1.53×10^{11} (1.51×10^{10})	1.53×10^{11} (1.51×10^{10})
u_{curve}	1.45×10^{-6} (6.78×10^{-6})	1.45×10^{-6} (6.78×10^{-6})	1.45×10^{-6} (6.78×10^{-6})
u_c	3.65×10^{-6} 6.4 ng (7.08×10^{-6}) (12.33 ng)	3.48×10^{-6} 6.1 ng (7.27×10^{-6}) (12.66 ng)	4.43×10^{-6} 7.7 ng (9.86×10^{-6}) (17.17 ng)
$U_{(95\%,\ k=2)}$	7.30×10^{-6} 12.7 ng (1.42×10^{-5}) (24.7 ng)	6.96×10^{-6} 12.1 ng (1.45×10^{-5}) (25.3 ng)	8.85×10^{-6} 15.4 ng (1.97×10^{-5}) (34.3 ng)

* cert.: value obtained from the calibration certificate ($U_{mp(k=2,\ 95\%)}$). $u_{mp} = U_{mp}/k$.

3.5. Comparison between cellulose and nylon substrates

Since the major contribution in the uncertainty of the CPT phosphorescence measurement was the repeatability, caused by variations of CPT signal in the cellulose substrate, a comparison study was carried out in order to evaluate the performance of a more homogeneous nylon substrate with virtually no phosphorescence background.

The evaluation was made using the same experimental condition set for measuring CPT signal in the cellulose substrate (266 μg $TlNO_3$ and pH 10.5 analyte carrier solution) and using only measurements at 570 nm of the emission spectra. Analytical figures of merit were obtained with improvements in detectability, which also extended the operational linear range of the analytical curve. The LOD and LOQ values were 1.7×10^{-6} mol L^{-1} (2.9 ng ALOD) and 5.6×10^{-6} mol L^{-1} (9.7 ng ALOQ). This better performance was obtained though a lower background and also by the improvement of CPT phosphorescence signal in such substrate as can be seen in Fig. 4. Unfortunately the repeatability values using nylon were similar to the ones achieved using cellulose substrate, which indicated that the main signal variation was not from the fluctuations in background signal (in the case of cellulose paper) but caused by small variations in the deposition local of the sample in the substrate. The sample position in the substrate influences the amount of analyte interacting with the excitation radiation and also the impact in the collection of signal since part of the analyte could not be in the appropriate position to

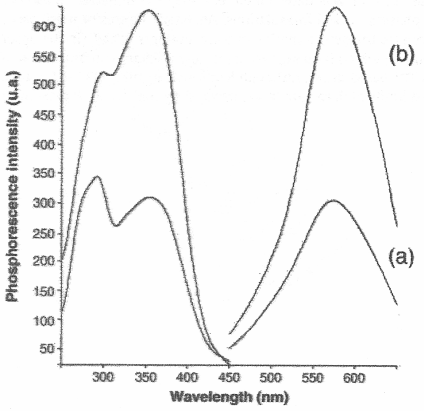

Fig. 4. CPT phosphorescence spectra in (a) low background cellulose and (b) nylon substrates.

have phosphorescence collected in the emission monochromator. This is the main reason why only 5 μL of sample is placed in the substrate. Larger volumes create large sample pool that would create a situation when part of the analyte would never interact with the excitation radiation.

3.6. Sample analysis

The applicability of SSRTP was evaluated by determining CPT-11 as the active ingredient in one pharmaceutical formulation and by determining CPT as a trace contaminant in a CPT fortified TPT based pharmaceutical formulation. In this case, CPT content was adjusted to be 40 times lower, in concentration, than TPT in the prepared sample solution. In this case, phosphorescence measurements were made at 367 nm of the 2nd derivative excitation spectra.

CPT recoveries as trace contaminant in TPT mixtures were 101.5 ± 3.5% and these results were satisfactory for the selective determination of the contaminant in pharmaceuticals. Further tests were made with CPT fortified urine and saliva. The results achieved were 102.5 ± 3.5% (urine) and 102.5 ± 4.5% (saliva).

The results obtained with the proposed phosphorimetric method were compared to the method based on HPLC-DF. The Student *t*-test was applied to compare the methods and the hypothesis test showed equivalent results. For CPT in TPT based medicine samples, the recovery was 111 ± 6%. In CPT fortified saliva samples, CPT recovery achieved by HPLC was 108 ± 3.5%. Those results indicated that the performance of the SSRTP method was satisfactory.

The quantification of CPT-11 as the major active ingredient in anticancer drugs was also performed. The average recovery was 92 ± 6%.

4. Conclusions

Room-temperature phosphorimetry was used to determine CPT in TPT based anticancer pharmaceutical formulations. The method allowed the selective detection of trace amounts of CPT in a matrix containing large amounts of TPT using the order (2nd) derivative (solutions containing concentrations of TPT up to 40 times higher). Derivative spectra have also improved LOD value since it minimized the influence of the blank signal on the chosen λ_{iso} for CPT. Results indicated that urine and saliva matrix, without any treatment but dilution, imposed no relevant interferences on the detection of CPT. In addition, CPT-11 was determined as a major component in CPT-11 based pharmaceutical formulations. Analytical figures of merit were adequate for the determination of trace amounts of CPT (in the order of μg mL^{-1}) which is enough to detect any relevant contamination of CPT in TPT based drugs. Although less sensitive than HPLC-DF, which enabled limits of detection in the order of ng mL^{-1}, SSRTP presents a much simpler procedure and requires no costly high-purity HPLC-grade solvents, filters and columns. A detailed metrological study was made in order to evaluate the reliability of the phosphorimetric CPT measurement. Nylon was found to be an advantageous substrate for the determination of CPT since it allowed the improvement in detectability and in the working linear range. Nylon also grant operational advantages such as a time considerably lower for sample drying in the vacuum dessicator at room temperature (40 min instead of the 120 min required for cellulose substrates) and no need for treatment for the reduction of background. The quantification of CPT-11, as the main ingredient of anticancer drugs, is also feasible by SSRTP since limits of detection are more than adequate to enable the detection this major component in pharmaceutical formulations.

Acknowledgments

The authors thank the Brazilian agencies FINEP, FAPERJ and CNPq for research grants. Scholarships from CNPq and FAPERJ are appreciated.

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Please cite this article as: P.M.S. Maia, et al., Room-temperature phosphorimetry for the determination of trace contaminations of camptothecin in anticancer drugs, Microchem. J. (2010), doi:10.1016/j.microc.2010.02.007