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Laser induced fluorescence and photochemical derivatization for trace determination of camptothecin

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ABSTRACT

Laser-excited fluorescence was used for the selective determination of camptothecin in samples containing anti-cancer camptothecin-analogs (irinotecan and topotecan). The selectivity of the method was based on the UV photochemical derivatization in basic solution which increased the analyte fluorescence (337/450 nm) and eliminated fluorescence from the two campthotecin-analogs. The influence of UV exposure time and sodium hydroxide concentration was studied using an experimental design. Limit of detection was 4×10^{-10} mol L^{-1} with linear fluorescence response up to 1×10^{-6} mol L^{-1}. Average recoveries of camptothecin (added to the samples to simulate a contamination) were 92 ± 4 and $94 \pm 6\%$ ($n = 3$) respectively in irinotecan and topotecan based pharmaceuticals.

1. Introduction

Camptothecin (Fig. 1A) is a quinoline-based alkaloid and it is one of the few known naturally occurring DNA topoisomerase-I inhibitors which decrease the ability of cells to use the genetic information to synthesize proteins [1]. Because cancer cells grow and reproduce at a much faster rate than normal cells, they are more vulnerable to topoisomerase-I inhibition than are normal cells. Camptothecin has a pentacyclic indole moiety which appears to be a requirement for its anti-cancer activity. Camptothecin is primarily in the lactone form in solutions set to pH values below 5 and undergoes totally reversible hydrolysis to the ionized carboxylate form at pH values greater than 8 [2,3].

The application of camptothecin as an anti-cancer drug is limited due to its poor water-solubility and toxic side effects [4]. Therefore, camptothecin derivatives (irinotecan and topotecan) have been developed and successfully applied in cancer therapy with reduced side effects. Irinotecan (Fig. 1B) and topotecan (Fig. 1C) based drugs are commercially available but due to their high cost, they are potential targets for counterfeiting. The trade of counterfeit drugs is widespread and affects both developing and developed countries [5]. Treatment of diseases with counterfeit or substandard medicines may lead to deterioration of health and even the death of patients. According to the World Health Organization, 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 (containing similar and cheaper ingredient) or without active ingredient and the products containing traces of undesired contaminants [6]. Therefore, ability to quantify camptothecin in anticancer drugs based on irinotecan and topotecan is important in order to detect counterfeit drugs that have been intentionally made to be substandard using camptothecin as a similar and cheaper ingredient or to identify drugs with high levels of camptothecin as impurity or as a contaminant.

Camptothecin and camptothecin-analogs have similar spectral characteristics including intense natural fluorescence [7], therefore, determination of camptothecin in the presence of camptothecin-analogs has been almost exclusively achieved by reversed-phase high performance chromatography (HPLC) using either molecular absorption or fluorescence detection. In 2001, the application of HPLC for the determination of camptothecin and camptothecin-analogs in biological fluids and plant extracts have been described and compared in three revision articles [3,8,9]. Buffered systems, at pH values around 5, provide separations and simultaneous detection of camptothecin in both the lactone and the carboxylic forms [10]. Alternatively, camptothecin is detected only in its lactone form (conversion of the carboxylate form to the lactone form) using mobile phases adjusted to pH values around 2. In latter works, camptothecin has been determined in seeds and in plasma (human and rat) using optical detection (absorption and fluorescence) and two-dimensional mass spectrometry MS/MS [11–14]. For more complex samples, previous analyte separation from the matrix was achieved by procedures such as solid phase

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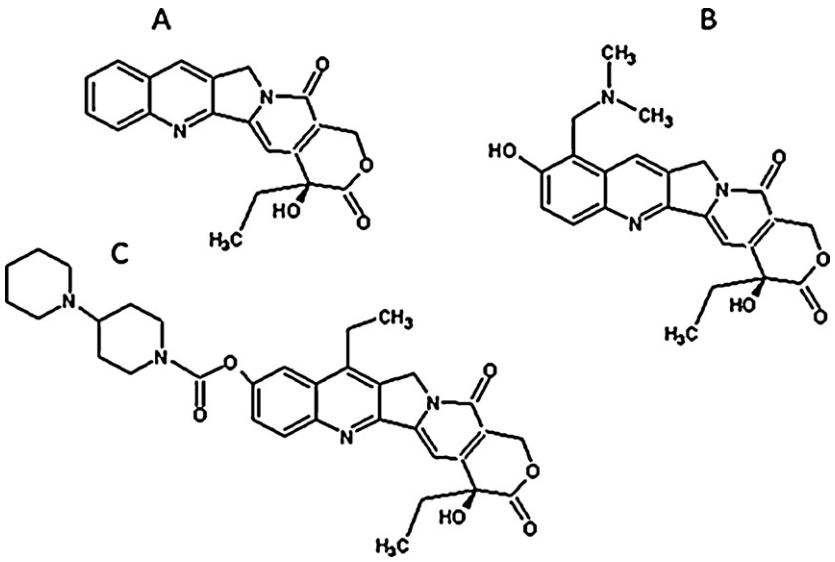

Fig. 1. Molecular structures: (A) camptothecin, (B) topotecan and (C) irinotecan.

extraction [12]. Limits of detection (LOD) as low as 10^{-9} mol L^{-1} are reported for camptothecin using HPLC with fluorescence detection.

In mixtures containing either irinotecan or topotecan, selective determination of camptothecin was achieved without previous physical separation of components by measuring the characteristic camptothecin signal (isodifferential wavelength at 267 nm) obtained from second-order derivatization of the synchronized ($\Delta\lambda = 80$ nm) fluorescence spectra. LOD at the 10^{-8} mol L^{-1} level was achieved [15]. Solid surface room-temperature phosphorimetry (using $TlNO_3$ as phosphorescence inducer) allowed selective determination of camptothecin in the presence of topotecan with LOD in the order of 10^{-6} mol L^{-1} (2.9 ng absolute limit of detection with 5 μL sample volume). Selective determination in the presence of irinotecan was not possible [16].

Photochemical derivatization is used to obtain derivative species with higher luminescent quantum yield that improves detectability of the species of interest. Such procedure may also be used to generate non-luminescent derivatives, suppressing luminescence from potential interferent species when complex mixtures are analyzed. Rodriguéz-Caceres et al. have shown that photochemical derivatization of two topoisomerase-I inhibitors (irinotecan and leucovorin) in solution (at pH 4 and in the presence of H_2O_2) enabled significant improvements of fluorescence [17]. Selective determination of them, including the complex samples such as biological fluids, was achieved by applying multivariate calibration methods. Post column photochemical derivatization has also been used to improve (14-fold) the fluorescence from lurtotecan (another topoisomerase-I inhibitor) when analyzing plasma samples by HPLC [18].

In the present work, photochemical derivatization (in strongly basic solution) was used to significantly improve fluorescence from campthothecin in basic medium and to minimize fluorescence from campthotecin-analogs (irinotecan and topotecan). In addition, excitation using the 337 nm nitrogen laser line improved sensitivity and selectivity in the determination of campthotecin in samples containing highly amounts of irinotecan and topotecan.

2. Experimental

2.1. Instrumentation

Fluorescence measurements were made on a Perkin-Elmer LS-45 luminescence spectrophotometer (Beaconsfield, UK) using 10 nm spectral bandpass and 1500 nm mm^{-1} scan velocity. Fluorescence spectra were acquired on a PC using the FLwinlab® software. All optimizations were made using the standard configuration of the luminescence spectrophotometer with the pulsed xenon arc lamp. Laser excitation was employed to establish the figures of merit and for camptothecin determinations. In this case, the external cover of the sample compartment was removed and replaced by a box, covered in black foil. The top and the back sides of the box were removed and one small role was made on the left lateral side. The box was positioned with the removed lateral in front of the sample compartment, its bottom part up and the side with the hole flipped to the right side. In such way, the box minimizes ambient light entering the spectrometer and the laser could be pointed to the sample compartment through the hole on the right. The laser (a NL 100 nitrogen laser with 170 μJ of pulse energy, Stanford Research System, CA, USA) was fixed on a jack platform and placed on an optical table in front of the luminescence spectrophotometer. The 337 nm laser beam was adjusted to pass through the hole on the side of the box, targeting the middle of one of the sides of the quartz cuvette (1 cm optical pathlength) placed in the sample compartment. The laser incidence was at a 90° angle with the emission monochromator window. The nitrogen laser was operated at 20 Hz and the luminescence spectrophotometer was set in bioluminescence mode (continuous signal acquisition) using a delay time of 3 ms, a large gate time (200 ms) and cycle of 2000 ms in order to avoid electronic triggering between the laser source and the detector. When using the standard xenon lamp as excitation source, camptothecin was detected at 368/450 nm. For laser excitation (337 nm) the detection was also made at 450 nm.

A pHmeter (MS Tecnopon, model MPA-210, Sao Paulo, Brazil) was employed. Liquid chromatographic analysis was made on a Waters Breeze HPLC system (Waters, MA, USA) equipped with a Model 1525 binary pump and a Model 2478 multi λ fluorescence detector set at 368/450 nm. Sampling was made manually using a Reodyne injector and a 20 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 mm × 150 mm X-Terra RP C18 (Waters) with 5 μm particle size. The column was kept inside an oven set at 35 °C.

In order to compare the recoveries achieved by the proposed method, samples were also analyzed by HPLC using the method proposed by Guo et al. [19] in which the mobile phase was composed by two components: acetonitrile and 20 mmol L^{-1}

ammonium acetate (pH 3.5). The mobile phase at 1 mL min^{-1} was pumped in the isocratic mode with 24% acetonitrile (v/v) for 2.7 min. Then an elution gradient was applied by increasing the proportion of acetonitrile from 24 to 29% in 2.3 min followed by the increasing of acetonitrile from 29 to 34% in 2 min, and then from 34 to 90% in 1 min. Finally the proportion of acetonitrile was decreased back to 24% in 1 min. The Retention time for camptothecin was at 7.8 min.

A laboratory made photochemical reactor was employed for the treatment of samples and standards. The photochemical reactor was constructed using an oven unit cabinet that was adapted by placing on the top of its internal part, six sterilization mercury vapor lamps (6 W each) with most intense UV wavelengths at 253 and in the 296–313 nm range. During operation, the internal temperature did not surpass 30 °C. Samples and blanks were placed in test tubes made of quartz.

2.2. Reagents

All chemical reagents were of analytical grade. *Ultrapure water* (18.2 MΩ cm) *from an ultrapurifier water system* (Milli-Q system, gradient A10, Millipore, MA, USA) was used to prepare all aqueous solutions. Campthotecin was from Fluka (MO, USA) irinotecan was from Sigma–Aldrich (MO, USA) and topotecan was obtained from their injectable pharmaceutical Hycamtin (sterile lyophilized powder containing topotecan hydrochloride equivalent to 4 mg of topotecan as free base, Glaxo SmithKline, Parma, Italy). Methanol, ammonium acetate, acetic acid, boric acid, phosphoric acid and sodium hydroxide were obtained from Merck (Darmstadt, Germany). Irenax (injectable solution containing 20 mg mL^{-1} irinothecan hydrochloride from Sandoz, Buenos Aires, Argentina) was the chosen irinotecan pharmaceutical formulation.

2.3. Procedures

2.3.1. Preparation of standard solutions and samples

Camptothecin stock solutions (1.0×10^{-3} mol L^{-1}) were freshly prepared in methanol/water 30/70% (v/v). Irinotecan hydrochloride solutions (1.0×10^{-3} mol L^{-1}) were prepared in water and topotecan hydrochloride stock solutions (1.0×10^{-3} mol L^{-1}) were prepared by dissolving a known amount of its pharmaceutical formulation directly in water. Standard solutions with lower concentrations were made by further dilution of the stock solutions with water/Britton–Robinson buffer or sodium hydroxide aqueous solution. Britton–Robinson buffer solutions (0.04 mol L^{-1}, from pH 2 to 12) were prepared by mixing acetic acid, boric acid and phosphoric acid aqueous solutions. The pH of the buffer was adjusted by adding concentrated sodium hydroxide solution. In buffered solutions, a final buffer proportion of 40%, in volume, was used as part of the water content of the solvent system used to prepare the working solutions. The simulated substandard pharmaceutical formulation samples were prepared by mixing a specific amount of camptothecin into a mass of the irinotecan or the topotecan pharmaceutical formulation. The acetate buffer (20 mmol L^{-1}) was prepared by dissolving ammonium acetate in water and adjusting the pH (3.5) with acetic acid. HPLC studies were also used to confirm the derivatization of camptothecin. In such studies, experiments were performed by injecting 5 μL of analyte solution prepared in NaOH 1 mol L^{-1} (before and after 10 min of exposition to UV) using isocratic flow with water/acetonitrile 75/25% (v/v).

2.3.2. Sample photochemical treatment

Camptothecin and its derivatives (topotecan and irinotecan) solutions, all prepared in sodium hydroxide 1 mol L^{-1}, were transferred to quartz test tubes (diameter of 1.9 cm and 13.7 high) and UV irradiated to produce the photochemical derivatives.

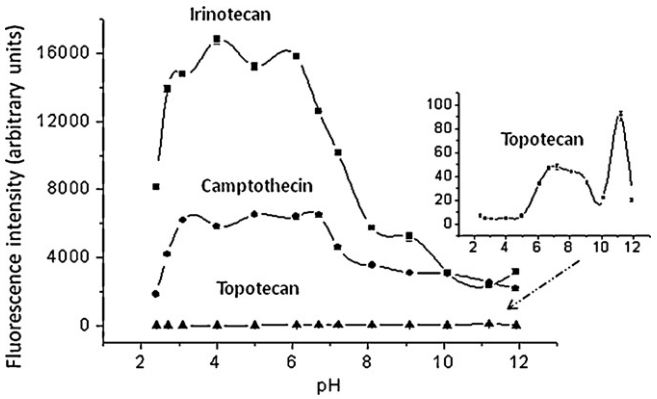

Fig. 2. Influence of pH in the fluorescence from camptothecin (1×10^{-6} mol L^{-1}), irinotecan (1×10^{-6} mol L^{-1}) and topotecan (5×10^{-6} mol L^{-1}) after UV irradiation (30 min).

3. Results and discussion

3.1. Photochemical induced fluorescence of camptothecin

Camptothecin and its derivatives (irinotecan and topotecan) present natural fluorescence which are associated with the extended conjugation of their quinoline ring system [3]. Fluorescence from these three substances has the same profile with maximum wavelengths of excitation (λ_{exc}) and emission (λ_{em}) at 360/440 nm with more intense signal for irinotecan followed by camptothecin and then, topotecan. When dissolved in 1 mol L^{-1} sodium hydroxide, fluorescence from camptothecin (368/450) is strongly decreased when compared to the one at the original pH of the aqueous solution (pH 6). In basic media, the lactone ring hydrolyzes producing the carboxylate form of camptothecin which, in turn, reduces its fluorescence quantum yield because the loss of rigidity of the molecular structure [4]. In a similar way, spectral characteristics from the camptothecin-analogs also changes. Fluorescence from topotecan is less intense than the one of camptothecin over the wide range of pH values. Irinotecan fluorescence decreases in solutions at basic pH values [20]. Besides decreasing in intensity, the fluorescence emission band of irinotecan shifts (λ_{em} changing from 450 to 550 nm). This is an indication of a more drastic molecular structural change. For instance, the fluorescence at 380/550 nm coincides with the one reported for CPT-11 derivatives such as SN-38 [21,22]. In spite of both the fluorescence intensity decreasing and band shifting, the contribution of this residual fluorescence as a spectral interference on camptothecin is still relevant, especially in samples originally containing higher amounts of irinotecan [15].

Studies involving the treatment of solutions with UV radiation have indicated no changes in the fluorescence characteristics of camptothecin and their two derivatives in solutions with pH values from 2 to 6 (Fig. 2). As the pH increased (from 7 to 12), fluorescence from these alkaloids, especially from irinotecan and from camptothecin, decreased significantly. Under strongly basic conditions (sodium hydroxide 1 mol L^{-1}) fluorescence from campthothecin is significantly increased (ten-fold after 30 min exposure to UV) with no significant modification in the $\lambda_{em}/\lambda_{exc}$ pair. Such results indicated the formation of a camptothecin derivative(s) with higher fluorescence quantum efficiency than the original analyte molecule (Fig. 3). In contrast, under these same conditions, irinotecan and topotecan fluorescence are eliminated. The excitation fluorescence band (with λ_{exc} = 368 nm) from camptothecin after exposition to UV is broad, therefore, a significant intensity is still measured at 337 nm, indicating the possibility of taking advantage from a higher radiance excitation source (nitrogen laser), which could improve

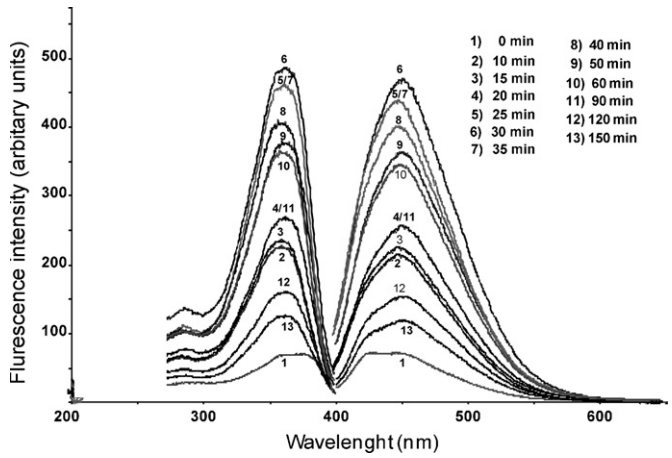

Fig. 3. Effect of the UV exposition time on the fluorescence of camptothecin (1×10^{-7} mol L^{-1}) prepared in NaOH 1 mol L^{-1}.

significantly the detectability and selectivity (due to the narrow line excitation) of the determination.

HPLC studies have shown that new derivatives from camptothecin are generated after UV irradiation (Fig. 4). When camptothecin is prepared in acetate buffer (pH 3.5), a main fluorescence (268/450 nm) peak (lactone form) is observed at 7.8 min and a small shoulder at 9.3 min. As this solution is exposed to UV (10 min), the fluorescence intensity of the peak at 7.8 min decreases while fluorescence of the peak at 9.3 min increases. In addition a third smaller peak appears at 8.3 min. Results also indicates some degree of photodegradation of camptothecin under ambient light (the small shoulder at 9.3 min) even before the UV photochemical treatment. Similar observations are found for camptothecin prepared in NaOH solution. Before UV exposition intense fluorescence is observe at 1.9 min (structure with opened lactone ring [4]). Three smaller peaks are found at 4.9, 6.2 and 6.6 min. The peaks at 6.2 and 6.6 min are produced by photodegradation of camptothecin due to the ambient light since the intensities of these peaks increase significantly (with decreasing of intensity of the peak at 1.9 min) after 15 min of exposition to UV.

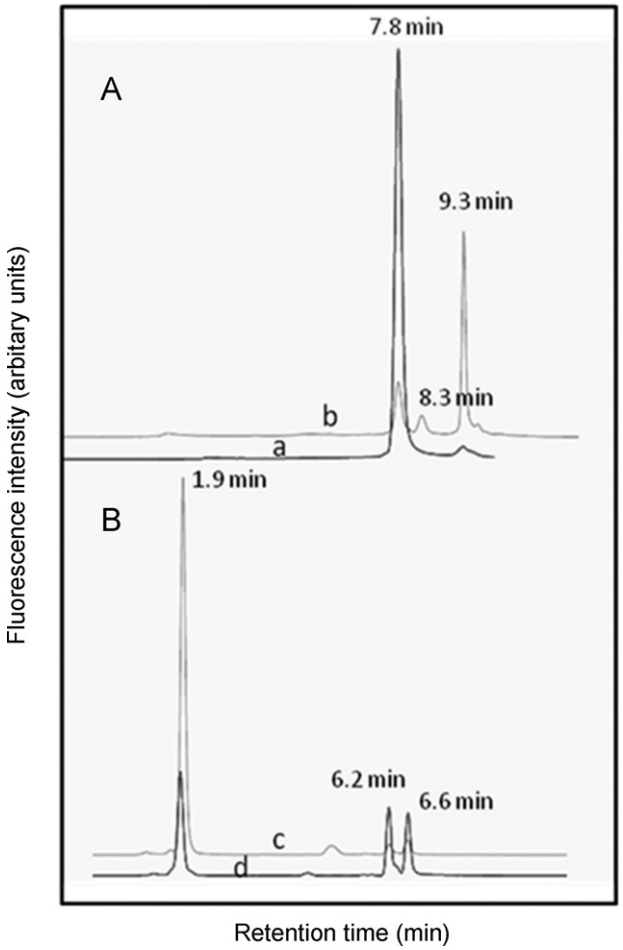

Fig. 4. HPLC chromatograms of (A) campthotecin prepared in acetate buffer (pH 3.5) and (B) camptothecin prepared in NaOH 1 mol L^{-1}. Chromatograms a and c are from samples non-exposed to UV and chromatograms b and d are from solutions previously exposed (15 min) to UV.

3.2. Optimization of experimental parameters

In order to optimize the photochemical derivatization of camptothecin, the univariate study of parameters was employed. Then, a factorial design was applied in order to establish the hierarchy of the importance of the experimental factors, identify any antagonic or synergic interaction among the main experimental factors, and to achieve final adjustment of conditions. The application of the 2^2 central composite design allows the investigations of the two main experimental parameters of the photochemical derivatization procedure. Each parameter is evaluated in five levels (calculated from the two original levels) covering the experimental range in a way that a more structured surface response is obtained [23].

3.2.1. Univariate studies

An evaluation of the influence of the UV exposition time was performed using camptothecin dissolved in sodium hydroxide 1.0 mol L^{-1}. Fluorescence from these solutions was measured using UV exposition in the time range from 0 to 150 min. Fluorescence intensity (368/450 nm) was higher at 30 min of UV treatment. In such condition, the fluorescence was about ten-fold higher than the one measured from a solution non-exposed to UV (Fig. 3). No significant modification in the $\lambda_{em}/\lambda_{exc}$ pair was observed. At this $\lambda_{em}/\lambda_{exc}$, fluorescence from irinotecan and topotecan are minimized after UV exposition.

The influence of the sodium hydroxide concentration in the UV treated solution was also studied. The fluorescence increased as the concentration of NaOH was increased from 0.4 to 1.0 mol L^{-1} remaining constant to up to at least 2.0 mol L^{-1} which indicated a robust range for this factor.

3.2.2. Multivariate studies

A central composite design was performed by choosing the amplitude for each of the parameters (UV exposure time and sodium hydroxide concentration) based on the results achieved from the univariate studies. The chosen levels for concentration of sodium hydroxide with codified level in parenthesis were: 1.35 mol L^{-1} ($+\sqrt{2}$), 1.25 mol L^{-1} (+1), 1.00 mol L^{-1} (0), 0.75 mol L^{-1} (-1) and 0.65 mol L^{-1} ($-\sqrt{2}$). For the UV exposition time, the chosen levels were: 44 min ($+\sqrt{2}$), 40 min (+1), 30 min (0), 20 min (-1) and 16 min ($-\sqrt{2}$). Replicates ($n = 5$) were made only at the central point (0,0). Fig. 5 is the resultant response surface modeled by Eq. (1) (using codified coefficients), where I_F is the fluorescence from camptothecin, [NaOH] is the concentration of sodium hydroxide and UV is the exposition time to ultraviolet radiation. Quadratic contributions from UV and [NaOH] are both relevant and responsible by the curvature of the response surface. The study indicated that the best values for UV and [NaOH] were respectively 26 min and 1.02 mol L^{-1}.

$$I_F = 3741.5 - 334.5(\mathrm{UV}) - 420.7(\mathrm{UV})^2 - 929.6([\mathrm{NaOH}])^2 \quad (1)$$

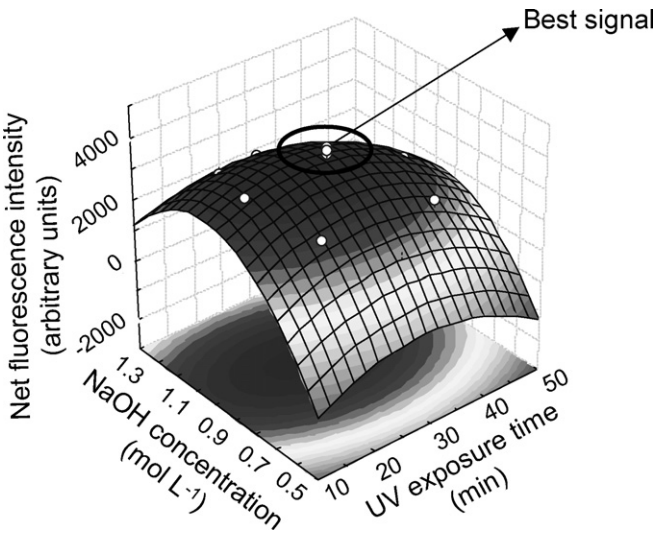

Fig. 5. Response surface from the optimization of the photochemical derivatization of camptothecin.

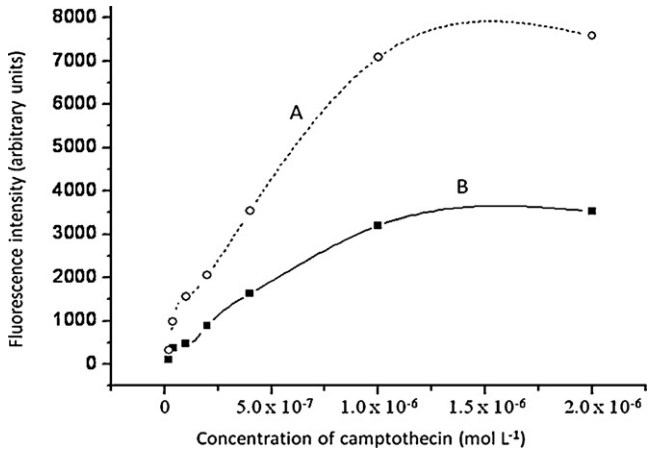

Fig. 6. Fluorescence from camptothecin measured at 450 nm after photochemical derivatization using: (A) laser excitation at 337 nm) and (B) pulsed xenon arc lamp at 368 nm.

3.3. Interference tests

Interference tests were performed to evaluate the feasibility of the selective determination of camptothecin. Synthetic mixtures containing different molar ratios between camptothecin and the camptothecin-analogs (irinotecan or topotecan) were used and the $I_0/I_{\text{irinotecan}}$ and $I_0/I_{\text{topotecan}}$ values indicate the ratios between the fluorescence measured from a camptothecin standard solution (I_0) and the one measured from a solution containing camptothecin standard of the same concentration mixed with increasing concentrations of either irinotecan ($I_{\text{irinotecan}}$) or topotecan ($I_{\text{topotecan}}$). Ratio values from 0.90 to 1.10 implied in no significant interference on the camptothecin signal. No interference was observed in mixtures containing topotecan in concentrations up to 50 times higher than the one of the analyte. When the topotecan/camptothecin proportion is increased to 100, the $I_0/I_{\text{topotecan}}$ value decreased to 0.79 indicating spectral interference. On the other hand, irinotecan imposed no interference in the measured analyte fluorescence in mixtures containing this camptothecin-analog in concentrations up to 10 times higher. However, analyte signal decreased ($I_0/I_{\text{irinotecan}} = 1.68$) when the irinotecan/camptothecin proportion is increased to 50. However, even in such conditions, quantification of camptothecin is feasible if analyte addition technique is employed to correct the interference.

3.4. Analytical parameters of merit

In Fig. 6, two profiles (fluorescence versus concentration of camptothecin) are compared. One curve was obtained measuring fluorescence at 368/450 nm using the pulsed xenon-arc lamp of the luminescence spectrometer while the other curve was constructed by measuring fluorescence at 337/450 nm using nitrogen laser as the excitation source. Despite the fact that the laser employed presents one of the lowest energy available in the market and that the 337 nm line is not the maximum wavelength of the excitation band of camptothecin, a significant improvement in sensitivity is found. The analytical figures of merit for the determination of camptothecin using the photoderivatization approach was, therefore, obtained using laser excitation. Three analytical curves were constructed within the linear fluorescence response (up to 1×10^{-6} mol L^{-1}) and the analytical curve equation (confidence limit of 95%) is described by $Y = 3.3 \times 10^{9}\ X + 66.1$ with a coefficient of determination (r^2) of 0.9989. The limit of detection (LOD) of 4×10^{-10} mol L^{-1} was estimated using the $3s_b/m$ criteria, where s_b is obtained from the standard deviation of ten fluorescence measurements of the lowest concentration of the analytical curve and m is the sensitivity of the curve. The limit of quantification (LOQ) was based on the $10s_b/m$ criteria and the result was 1.2×10^{-9} mol L^{-1}. The repeatability was estimated through the relative standard deviation (RSD) based on ten consecutive measurements of a 5.0×10^{-7} mol L^{-1} camptothecin standard solution under the same experimental conditions and each of the measurements was performed by replacing the measured solution by a new volume from the same solution added to the same cuvette. RSD from the fluorescence measurements was 8% (higher than the 1.8% obtained using the instruments pulsed xenon arc lamp).

3.5. Application of the method

The method was tested through recovery studies in samples with a higher content of irinotecan or topotecan. In order to do that, commercial anticancer drugs were fortified with camptothecin. The relative amounts (molar proportion) of camptothecin in the samples were fixed to be 5 times smaller than the topotecan content and 10 times smaller than topotecan content. Analyzed sample solutions contained 5×10^{-7} mol L^{-1} of camptothecin while the concentrations of the camptothecin-analogs were 2.5×10^{-6} mol L^{-1} (irinotecan) and 5×10^{-6} mol L^{-1} (topotecan). These samples were analyzed using the proposed method and compared to results achieved using a reference analytical method based on HPLC.

The average recoveries for camptothecin were 92 ± 4 and $94 \pm 6\%$ ($n = 3$) respectively for samples containing irinotecan and topotecan. These results were in the acceptable range and in agreement (at 95% confidence level) with the ones achieved using the reference HPLC method ($98 \pm 3\%$ for samples containing irinotecan and $99 \pm 3\%$ for samples containing topotecan).

4. Conclusions

In this work, a sensitive and selective determination of camptothecin is proposed. Selectivity was achieved using photochemical derivatization of samples prepared in basic media. The photochemical derivatization procedure is simple, clean, improved fluorescence from the analyte and eliminated fluorescence from campthothecin analogs (irinotecan and topotecan). Fluorescence from camptothecin was dependent upon the sodium hydroxide concentration and UV irradiation exposure time. The 337 nm line

excitation using a nitrogen laser greatly improved detectability of camptothecin and may have also contributed to minimize interferences. In the analysis of complex samples containing camptothecin analogs, for instance, the limit of detection for the spectrofluorimetric method, which is based on the use of the second derivative spectra [15], was two orders of magnitude poorer than the one reported using the present method. Further improvements in sensitivity may be achieved by using a powerful nitrogen laser. Recovery studies indicated satisfactory results for camptothecin in samples containing high content of camptothecin-analogs.

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