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Deactivation rate of camptothecin determined by factor analysis of steady-state fluorescence and absorption spectra

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Camptothecin is a fluorescent compound exhibiting strong anticancer properties. A serious limitation to clinical application of this compound is its hydrolysis, when biologically active lactone form converts into inactive carboxylate. There are some differences in the shapes of both fluorescence and absorption spectra of the lactone and carboxylate forms of camptothecin. Therefore, during hydrolysis resultant fluorescence and absorption spectra evolve. Factor analysis of fluorescence/absorption spectra recorded during the hydrolysis process of camptothecin enables one to determine the temporary concentration of the lactone and carboxylate forms and obtain the deactivation rate of this compound.

Keywords: camptothecin, fluorescence, absorption, factor analysis.

1. Introduction

Camptothecin (CPT) is a plant alkaloid exhibiting anticancer properties [1]. The cellular target of CPT is nuclear protein topoisomerase I, an enzyme responsible for solving topological problems arising during the replication process of cells [2]. Cancerous cells replicate much more rapidly, so they can be killed with higher efficiency than healthy cells. Because CPT is a fluorescent compound, methods of fluorescence spectroscopy [3] can be used to study how CPT behaves in physiological conditions.

The lactone ring of CPT undergoes an opening due to hydrolysis in an aqueous solution at neutral and basic pH (Fig. 1). Only the lactone form (stable in an acid environment at pH < 5.5) has anticancer properties. In blood (pH 7.4) the lactone form easily hydrolyzes to the biologically inactive carboxylate form, which then immediately binds to human serum albumin (HSA), thus lowering the concentration of active

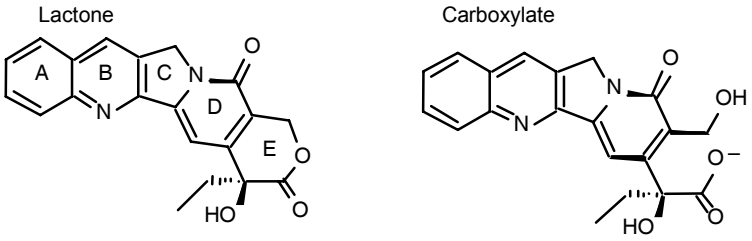

Fig. 1. Chemical structure of the lactone and carboxylate forms of camptothecin.

lactone. After about 2 hours the concentration of lactone in blood is equal to about 5% [4], while in plasma or HSA it practically decreases to zero [4, 5]. For the potential clinical use of this drug it is important to know the rate of hydrolysis reaction of the lactone form in different fluids, especially in physiological solutions, so as to find ways of keeping the lactone form active in human organisms for as long as possible.

High performance liquid chromatography (HPLC) is a method typically used to analyze the hydrolysis process of CPT [6]. The method allows direct measurement of the rate of hydrolysis, however it has some disadvantages [7]: 1) the solvents used for preparation of the liquid phase can interact with the lactone and carboxylate forms and introduce distortions in determining the rate of hydrolysis, 2) the time needed for sample analysis is quite long and can influence the results.

Optical methods of analysis are free of the above disadvantages and therefore they are worth trying. The base for application of optical methods is that there are differences in the shapes of both emission and excitation fluorescence spectra of the lactone and carboxylate forms. Absorption spectra of lactone and carboxylate forms also exhibit differences in shape. Because of the convertion of the lactone form into the carboxylate form during the hydrolysis process both fluorescence and absorption spectra evolve. CHOURPA *et al*. [7] proposed a spectroscopic non-invasive method of determining the hydrolysis rate of camptothecins. On the basis of variations of fluorescence intensity for two selected wavelengths during the hydrolysis process the change of concentration of the lactone form against time was determined [7]. The present paper reports results obtained with another approach for determining the rate of lactone hydrolysis. This approach is based on the analysis of the set of evolving fluorescence/absorption spectra with two methods well known in multivariate analysis, *i.e.*, principal component analysis (PCA) and factor analysis (FA) [8, 9]. Because PCA and FA make use of all information contained in spectra they are less sensitive to the random disturbances present in the spectra and it can, therefore, be expected that they should provide more precise results than the local method proposed by CHOURPA *et al*. [7]. This paper presents the results of application of PCA and FA to sets of fluorescence and absorption spectra that enable a determination of the rate of camptothecin deactivation in phosphate buffered saline (PBS) and HSA solutions. These results are compared with the results from HPLC.

2. Materials, experiment, method

2.1. Materials

The samples of camptothecin (National Cancer Institute, Betheseda, USA) were obtained from the laboratory of biotechnology, College of Pharmacy, University of Kentucky, Lexington (USA). A 2 mM stock solution of camptothecin was prepared in DMSO (dimethylsulfoxide C_2H_6OS). Such stock solution contains only a pure lactone form. A 1 mM stock carboxylate solution was obtained by dilution of stock lactone solution in PBS at pH 10 in a volume ratio 1:1. The PBS was adjusted to the desired value of pH using small quantities of 0.1 M KOH or HCl. Human serum albumin (95–97%) was purchased from Sigma-Aldrich (USA – Poland). A 40 μM solution of HSA in PBS was prepared. The pH of this solution was kept at 7.4 and temperature was kept at 37°C.

For fluorescence measurements, the concentration of camptothecin in final samples was equal to 1 μM. The desirable concentration was obtained by adding the stock solutions to PBS at pH 7.4 or to an HSA solution also at pH 7.4. For absorbance measurements the concentration of camptothecin in the sample was equal to 20 μM.

2.2. Instrumentation and measurements

To excite the fluorescence of camptothecin the following instrumentation was used: 150 W Xenon lamp, monochromator SPM2 (Carl Zeiss Jena, Germany) and quartz lens focusing a light of 370 nm on the sample. The fluorescence light was collected by a photographic lens (Sigma, Japan) at focal length 28–105 mm and F/2.8-4, into the entrance slit of an emission monochromator (SPM2 monochromator was used) equipped with a photomultiplier M12 FQ51. A PC with a measuring card (AMBEX, Poland) and software written in the MATLAB environment [10] was used for monochromator control and to manage data acquisition. The absorption spectra of camptothecin were collected with a spectrophotometer UV MINI 1240 (Shimadzu, Japan).

The time needed for recording single absorption or fluorescence spectrum was about 50 seconds. The fluorescence and absorbance measurements were carried out at room (23°C) and at elevated temperatures (37°C), in PBS solution at pH 7.4. The sample temperature during measurements was stabilized with an ultrathermostat U7^c (Medengen, Germany).

The spectra of the pure lactone and carboxylate forms were recorded immediately after mixing an adequate volume of the stock solutions in PBS. Single spectrum sweeps were repeated every 2 minutes during the hydrolysis process.

An SLM 8100 spectrofluorometer (SLM-Aminco, USA) was used for steady-state fluorescence anisotropy measurements. The measurements were performed with instrument in the "T-format". The 370 nm exciting light and 400 nm long-pass filters in each emission channel were used.

2.3. Principal component analysis and factor analysis

The principles of the PCA and FA methods were described in previous papers [11, 12]. PCA of the experimentally obtained sets of fluorescence emission, fluorescence excitation and absorption spectra, proved that only two independent components exist. These two factors can be identified as the spectra of the lactone and carboxylate forms of camptothecin. FA makes it possible to determine the contribution of each factor in each resultant spectrum. Time dependence of the contributions obtained from series of evoluting fluorescence/absorption spectra determine the deactivation rate of camptothecin in PBS and in HSA.

3. Results and discussion

Figure 2**a** presents fluorescence spectra of the lactone and carboxylate forms of camptothecin in PBS buffer at pH 7.4. As one can see the emission fluorescence spectrum of the pure carboxylate form exhibits a lower intensity and is red-shifted in comparison to the spectrum of the pure lactone form. Similar differences are observed in excitation fluorescence spectra of the lactone and carboxylate forms.

The shape of emission and excitation fluorescence spectra recorded during the hydrolysis process change over time. Figure 2**b** presents fluorescence spectra of the pure lactone form (characterized by the highest intensities in both series), selected

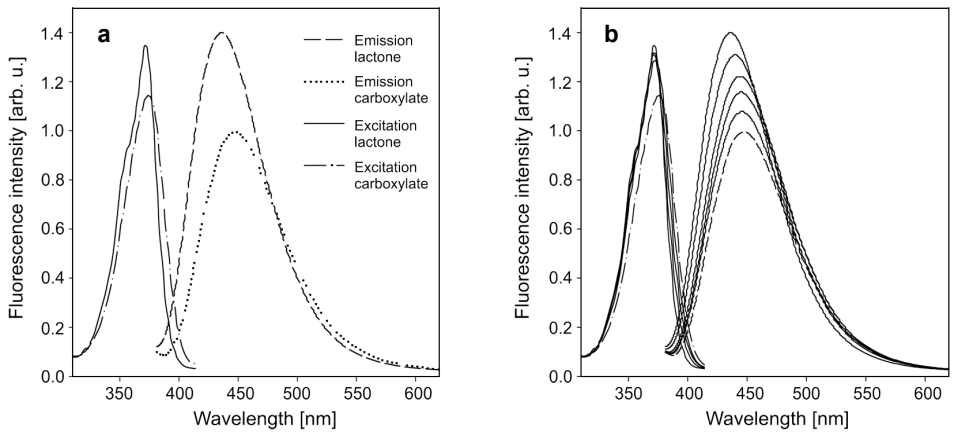

Fig. 2. Excitation and emission fluorescence spectra of pure lactone and pure carboxylate forms of camptothecin, recorded in PBS at pH 7.4 (**a**). Excitation and emission fluorescence spectra recorded during the hydrolysis process of camptothecin in PBS at pH 7.4 (**b**). The excitation spectra (maximum at about 370 nm): the highest intensity spectrum is of the lactone form, the subsequent ones are the spectra recorded 40, 90 and 180 minutes after sample preparation; the lowest spectrum (dash-dot line) is the excitation spectrum of the carboxylate form. The emission spectra (maximum at about 450 nm): the spectrum of highest intensity is for the lactone form, the subsequent ones are the spectra recorded 20, 50, 100 and 170 minutes after sample preparation; the lowest spectrum (dashed line) is the emission spectrum of the carboxylate form.

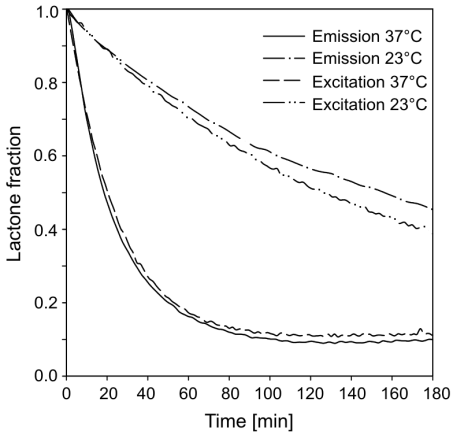

Fig. 3. Rate of deactivation process of camptothecin obtained by factor analysis of a series of excitation and emission spectra recorded during the hydrolysis process in PBS at pH 7.4 and temperature of 23 and 37ºC.

spectra recorded during the hydrolysis process and spectra of the pure carboxylate form (characterized by the lowest intensities in both series). As one can see the intensity of fluorescence decreases and the maximum of the spectra shifts during the hydrolysis process. The shape of evoluting spectra approaches the shape of spectrum of the pure carboxylate form but does not reach it. The observed changes are the result of the convertion of the lactone form into the carboxylate one.

Both emission and excitation fluorescence spectra recorded during the hydrolysis process were analyzed using FA, with the spectra of pure lactone and pure carboxylate forms as the factors. As a result, the fraction of the lactone form over time was obtained. The curves of decay of the lactone form obtained with FA are presented in Fig. 3. The measurements carried out at temperatures of 23 and 37°C were repeated several times, so each of the presented curves is the average of at least three independent measurements.

Figure 4**a** presents the absorption spectra of the pure lactone and pure carboxylate form of CPT in PBS at pH 7.4. The presented spectra were recorded immediately after adding a stock solution of lactone or carboxylate, respectively, into PBS at pH 7.4. There are some differences in the shape of absorption spectra of lactone and carboxylate. In the range near to ultraviolet the absorption spectrum of the pure lactone form exhibits two maxima (350 and 370 nm). The spectrum of the pure carboxylate form exhibits only one maximum (about 370 nm). Because of the convertion of lactone into carboxylate, the shape of absorption spectra recorded during the hydrolysis process changes over time. Figure 4**b** presents absorption spectra of the pure lactone form, some selected spectra recorded during the hydrolysis process and the spectrum of the pure carboxylate form. As one can see in Fig. 4**b**, the shape of absorption spectra evolves towards the shape of the spectrum of the carboxylate form, but does not reach it. The set of recorded absorption spectra was analyzed by FA, with the absorption

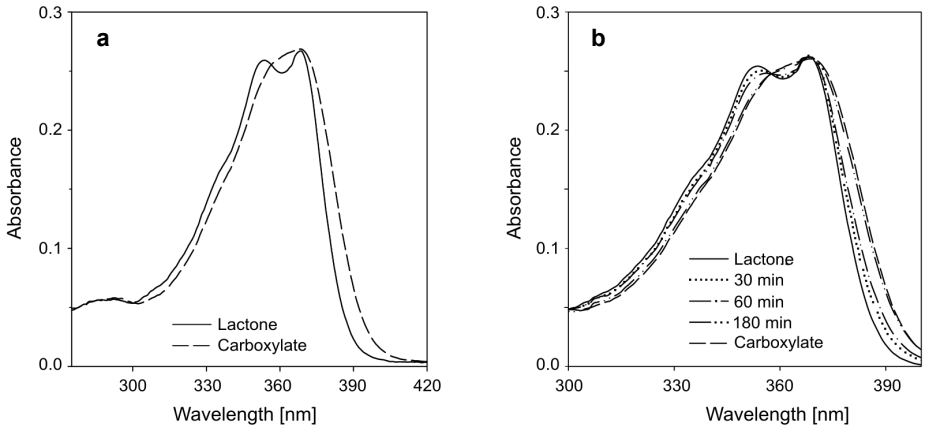

Fig. 4. Absorption spectra of lactone and carboxylate forms of camptothecin recorded in PBS at pH 7.4 (**a**). The series of absorption spectra of camptothecin recorded during the hydrolysis process in PBS at pH 7.4 (**b**). The first one is the lactone form spectrum (solid line), the subsequent spectra were recorded 30, 60 and 180 minutes after sample preparation. The last one is the spectrum of the carboxylate form (dashed line).

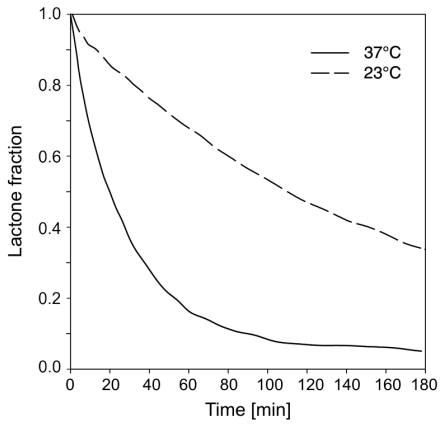

Fig. 5. Time dependence of the fraction of the lactone form obtained from factor analysis of a series of absorption spectra recorded during the hydrolysis process of camptothecin in PBS at pH 7.4 and temperatures of 23 and 37ºC.

spectra of pure lactone and pure carboxylate as the factors. Results of the analysis for two temperatures, 23 and 37°C, are presented in Fig. 5.

Figure 6**a** presents emission and excitation fluorescence spectra of lactone and carboxylate forms in 40 μM HSA solution at pH 7.4 and a temperature of 37°C. Both excitation and emission spectra of the carboxylate form exhibit lower intensity than respective spectra of the lactone form. Maximum of the excitation spectrum of the carboxylate form is red-shifted in comparison with the maximum of the excitation spectrum of lactone, however in the case of emission spectra no shift of maximum is

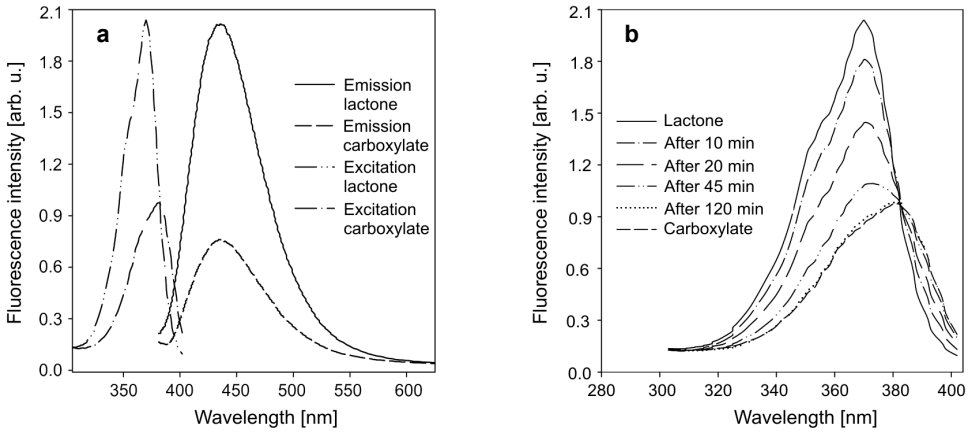

Fig. 6. Excitation and emission fluorescence spectra of pure lactone and pure carboxylate forms of camptothecin, recorded in an HSA solution at pH 7.4 (**a**). Excitation fluorescence spectra recorded during the hydrolysis process of camptothecin in an HSA solution at pH 7.4 (**b**). The spectrum of highest intensity is the emission of the lactone form (solid line), the subsequent ones are the spectra recorded 10, 20, 45 and 120 minutes after sample preparation; the last spectrum (short dashed line) is the emission spectrum of the carboxylate form.

observed. Figure 6**b** presents time evolution of spectra of the lactone form in HSA solution. One can see that after 2 hours the spectra attain the shape of the spectrum of pure carboxylate. Because there is no shift of maximum in emission spectra, only the excitation spectra were chosen for PCA and FA. The PCA of the set of excitation spectra proved the existence of only two factors. The factors were identified as the spectra of pure lactone and pure carboxylate forms. One could suspect that in an HSA solution 4 factors could exist because of the possibility of 4 fluorophores being present: free lactone, bound to HSA lactone, free carboxylate, bound to HSA carboxylate. However, the results of PCA suggest that only two fluorophores exist in an HSA solution of CPT. Results of PCA can be compared with the results of steady-state fluorescence anisotropy measurements. Fluorescence anisotropy defined as $r = (I_{||} - I_{\perp})/(I_{||} + 2I_{\perp})$ provides useful information about the binding of drugs to proteins or to membranes. The $I_{||}$ and $I_{\perp}$ are the fluorescence intensities of the vertically ($||$) and horizontally ($\perp$) polarized emission, when the sample is excited with vertically polarized light.

Figure 7**a** presents the time dependence of steady-state anisotropy of lactone and carboxylate forms of CPT in HSA solution. It can be seen from the figure that steady-state fluorescence anisotropy of the carboxylate form in an HSA solution is large (0.33) and does not change over time. The large value of steady-state anisotropy proves that the molecules of CPT carboxylate are bound to big HSA molecules. CPT lactone just after dilution in an HSA solution exhibits low steady-state anisotropy (0.013). This means that CPT lactone does not bind or binds poorly to HSA. The hydrolysis process, however, causes the anisotropy to rise over time. Free CPT lactone molecules convert into carboxylate and then immediately bind to HSA. After

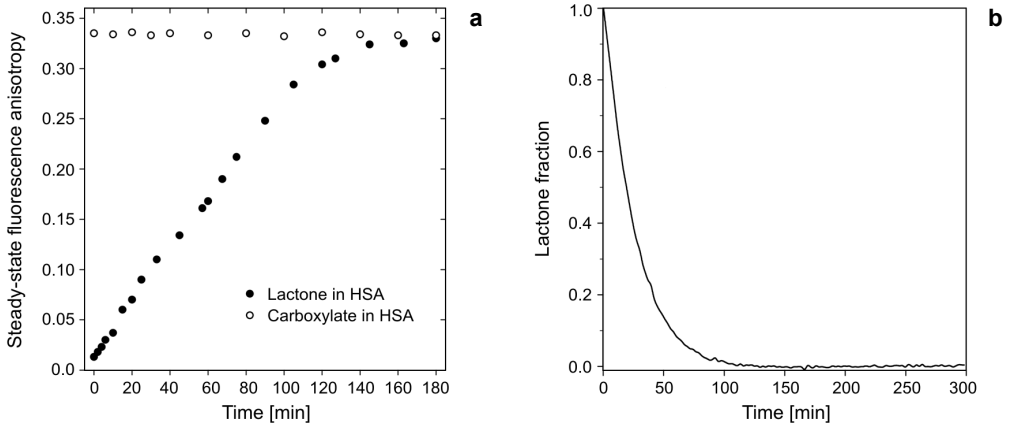

Fig. 7. Time evolution of steady-state fluorescence anisotropy of lactone and carboxylate forms of camptothecin in an HSA solution (**a**). The rate of the deactivation process of camptothecin obtained by factor analysis of a series of excitation fluorescence spectra recorded during the hydrolysis process in an HSA solution at pH 7.4 and a temperature of 37ºC (**b**).

about 2 hours the anisotropy approaches the value observed for the pure carboxylate form. This means that after 2 hours the lactone form has converted almost completely into the inactive carboxylate form. On the basis of the results presented in Fig. 7**a** one can conclude that two kinds of fluorophores exist in an HSA solution of CPT – a free lactone form and a bound to HSA carboxylate form. One can neglect the bound to HSA lactone form and free carboxylate form. This is consistent with the results of PCA, since PCA proved that only two independent components exist. We can state that the spectrum of free lactone is the first factor and the spectrum of bound to HSA carboxylate form is the second factor. Measurements of the life-time of fluorescence for both lactone and carboxylate forms in an HSA solution [13] as well as the affinity of lactone and carboxylate to HSA determined from these measurements also confirm that the factors were chosen correctly. Association constants of lactone and carboxylate to HSA are equal to 30 and 4700 M^{-1}, respectively [13]. The big difference in the affinity to HSA of the lactone and carboxylate forms shows that the former is practically free in an HSA solution, while the latter one is completely bound to HSA.

Series of spectra presented in Fig. 6**b** were analyzed using FA. The results of this analysis are presented in Fig. 7**b**. The figures show that the lactone form decays faster in an HSA solution than in PBS and after about 2 hours it is decayed completely. This means that after two hours the lactone form is converted to the carboxylate form, which is immediately and irreversibly bound to HSA molecules.

The rate of camptothecins hydrolysis in PBS and in HSA solutions obtained from PCA and FA of time dependent fluorescence/absorption spectra are consistent with the results obtained previously by the HPLC method [4, 6, 11]. This can be seen from Fig. 8, where the results obtained from FA of fluorescence/absorption spectra and from HPLC are set together. This figure shows the rate of camptothecin hydrolysis in

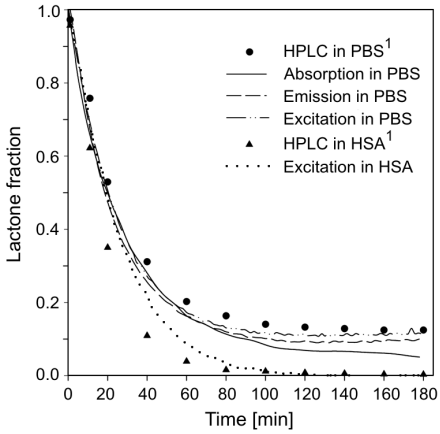

Fig. 8. Comparison of the results of the rate of camptothecin hydrolysis obtained from factor analysis of emission, excitation and absorption spectra and the HPLC method. The measurements were performed in PBS and HSA at pH 7.4 and a temperature of 37ºC ([1]Previously reported results [4, 5] obtained from HPLC measurements).

PBS at pH 7.4 and temperature of 37°C as well as the rate of camptothecin hydrolysis in an HSA solution at the same pH and temperature. Figure 8 confirms that FA of emission/excitation fluorescence and absorption spectra provides results consistent with those obtained from HPLC that we have reported previously [4, 11] as well as with the results reported by other authors [6, 14].

4. Conclusions

Application of PCA and FA to fluorescence/absorption spectra is an effective approach in investigations of the rate of camptothecin hydrolysis. Because both methods of analysis of the sets of spectra exploit information spread over the whole range of wavelengths, they are sensitive to even very subtle changes in the shape of the resultant line caused by small changes in contributions from constitutent factors. For this reason, the approach outperforms methods based on measurements of local intensities in the recorded spectra. When compared with HPLC, the approach offers some advantages as well, *i.e.*, it saves time necessary for measurements, needs no additional chemicals and permits distinguishig bound and free states of the molecules of the substance under study. Because of these advantages the approach presented in this work can be complementary to HPLC in research of camptothecin. In particular, based on the comparison of the rate of camptothecin hydrolysis obtained by FA of fluorescence/absorption spectra and HPLC one can conclude that FA may be successfully applied in seeking and studying camptothecin analogues [15–17] exhibiting properties more desirable than camptothecin itself.

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