

Application of principal component and factor analysis of fluorescence spectra in camptothecin studies

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The application of fluorescence spectroscopy methods in investigations of camptothecin (CPT) is presented in this paper. Fluorescence of CPT enables one to follow the process of hydrolysis, *i.e.*, the process of converting the biologically active lactone form into inactive carboxylate. The fluorescence spectra of CPT recorded during the hydrolysis were analysed using principal component analysis (PCA) and factor analysis (FA). The results obtained on the basis of fluorescence spectra analysis are compared with high performance liquid chromatography (HPLC) data.

Keywords: camptothecin, fluorescence, principal component analysis, 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 and so they can be killed with higher efficiency than healthy tissue. Because CPT is a fluorescent compound, then methods of fluorescence spectroscopy [3] can be used to study how CPT operates 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. The carboxylate form (stable at pH > 9), which arises in the hydrolysis process, is not only biologically inactive but also toxic. From the point of view of clinical use it is important to know the kinetics of hydrolysis reaction of the lactone form to find out ways of keeping the lactone form active in an organism for as long as possible.

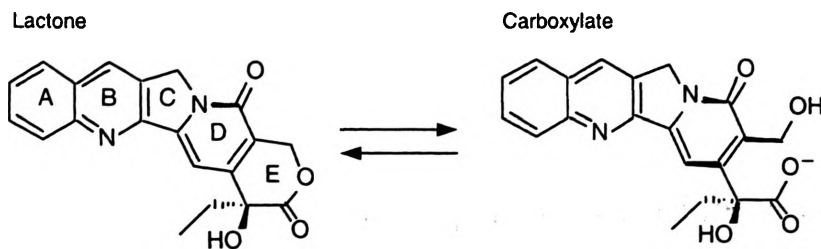


Fig. 1. Structures of the lactone and carboxylate forms of camptothecin.

To analyze the hydrolysis process of CPT the high performance liquid chromatography (HPLC) method is generally used [4]. This approach permits direct measurement of the rate of hydrolysis, but has some limitations [5]. CHOURPA *et al.* [5] proposed a spectroscopic non-invasive method of determining the hydrolysis rate of camptothecins. On the basis of variations of fluorescence spectra during the hydrolysis process the concentration of the lactone form in relation to time is determined.

In this paper, other methods of fluorescence spectra analysis are applied to determine the rate of lactone hydrolysis. The principal component analysis (PCA) and factor analysis (FA) of fluorescence spectra of CPT recorded during hydrolysis reactions are used here. They also permit determination of the rate of lactone hydrolysis. The results obtained are compared with those from HPLC.

Section 2 contains a description of the material and instrumentation used. The principles of PCA and FA methods of spectra analysis are described in Sec. 3. Section 4 presents a discussion on the fluorescence spectra of pure lactone and carboxylate forms that were prepared in different pH solutions as well as a series of spectra illustrating the hydrolysis process of CPT and the results of PCA and FA analysis of the above fluorescence spectra. The influence of temperature on the kinetics of CPT hydrolysis is discussed in this section. A comparison with HPLC results is also included.

2. Experimental

2.1. Materials

The CPT (produced by National Cancer Institute, Bethesda, USA) was obtained from the laboratory of Prof. T.G. Burke, University of Kentucky, Lexington (USA). Its purity was greater than 98%. 1 mM stock solution of CPT was prepared in dimethylsulfoxide (DMSO). Such a stock solution contains only a pure lactone form. For fluorescence measurements the stock solution was added to a phosphate buffered saline (PBS) at different pH (3, 7.4, 10) to obtain a final drug concentration equal to 1 μ M. PBS is an aqueous solution containing 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 and 1 mM KH_2PO_4 . Double distilled water was used for the preparation of PBS. The PBS was adjusted to desired value of pH using small quantities of 0.1 M

KOH or KCl. The stock solution of carboxylate form was equal to 0.5 mM and was obtained by dilution of stock lactone solution in PBS at pH 10 in a volume ratio 1:1.

2.2. Instrumentation and measurements

For recording the fluorescence spectra of camptothecin the following instrumentation was used: stabilised 50 W mercury lamp (Carl Zeiss Jena), optical system with band-pass filters and quartz lenses that focus light corresponding to a 366 nm line into a quartz cell placed in a thermostabilized sample compartment, an optical system collecting the fluorescence light into the entrance slit of a monochromator and double grating monochromator GDM-1000 (Carl Zeiss Jena) equipped with a photomultiplier. Using the 366 nm mercury line for excitation, instead of the excitation monochromator, ensures a sufficiently narrow spectral band of excitation (high excitation resolution). A high resolution of both the excitation system and the double grating monochromator allows precise detection of emission spectra and the distinguishing of very small changes in shape and position of spectra. A PC computer (Pentium 133) with measuring card (AMBEX Poland) and software SPEKTRUM [6] working in MATLAB environment were used for monochromator control and data acquisition. Samples of 1 μ M solution of camptothecin were prepared by mixing an adequate volume of stock camptothecin solution in PBS at 20, 25 and 37 °C. To record the fluorescence spectra the solution was placed immediately in a thermally stabilized compartment. The time needed for one spectrum recording was about 1.5 minutes. Solutions used for recording fluorescence spectra of pure lactone and pure carboxylate form were prepared by diluting respectively stock lactone and stock carboxylate solution in PBS at pH 7.4. Fluorescence spectra were recorded as quickly as possible. Because of this procedure fluorescence of either pure lactone or pure carboxylate form was observed at physiological pH 7.4. There were also detected fluorescence spectra of stable lactone form in a buffer at pH 3, 4.4, 5.5 and spectra of stable carboxylate form in a buffer at pH 9, 10, 12. To observe the hydrolysis process at physiological pH, the stock lactone solution was mixed in PBS at pH 7.4 and fluorescence spectrum was recorded as quickly as possible. Such recordings were repeated subsequently every few (about 5 to 15) minutes.

The hydrolysis kinetics of camptothecin in PBS was also determined by HPLC using a Waters Alliance 2690 separations module with a fluorescence detector. Separation of the lactone and carboxylate forms was achieved using an isocratic mobile phase consisting of varying mixtures of acetonitrile and a 2% triethylamine acetate buffer (pH 5.5).

3. Principles of PCA and FA methods

Any set of spectra recorded in digital form can be arranged as a data matrix \mathbf{X} , of dimensions determined by the number of spectra m , and the number of channels

(wavelengths) n , where intensities were probed. Rows in the matrix are subsequent spectra, while columns contain intensities. The data matrix can always be written as a product of two other matrices

$$\mathbf{X} = \mathbf{C} \mathbf{B}$$

where \mathbf{C} can be interpreted as a matrix of coefficients needed to reconstruct \mathbf{X} from rows of \mathbf{B} . There are an infinite number of ways to set up \mathbf{B} ; the choice depends on what one is interested in. For instance, if one wants to deal with physically meaningful constituents present in \mathbf{X} (*e.g.*, spectral lines), then these constituents can be put as rows of \mathbf{B} . In general, any rows of \mathbf{B} (the rows can also be thought of as n -dimensional vectors) are termed as components. The components that have a direct physical/chemical interpretation are called factors [7]. In other words, components are related to a mathematical description of a data matrix (set of spectra), while factors deal with physical interpretation of the spectra.

3.1. Principal component analysis

There exists a special set of components referred to as principal components (PCs), defined by their properties:

- PCs are mutually orthogonal,
- PCs are ordered in accordance with the amount of total variance present in \mathbf{X} and explained with a given PC,
- a full set of PCs restores completely a data matrix.

There is a unique set of PCs for a given \mathbf{X} , but it can be found in many ways. One of them is based on solution of the eigenproblem for covariance matrix \mathbf{Z} , for the original data matrix ($\mathbf{Z} = \mathbf{X} \mathbf{X}^T$). The values of non-negative eigenvalues resulting from the solution of the eigenproblem can be thought of as measures of the variance ascribed to particular PCs represented by eigenvectors. These PCs are some mathematical, abstract objects having no direct physical interpretation. Therefore, they are called abstract components or abstract factors. Abstract PCs can be transformed to n -dimensional space, the same as when the original spectra can be seen as n -dimensional vectors. The result of the transformation gives a set of m n -dimensional vectors called loadings on subsequent PCs. These vectors create a matrix of loadings \mathbf{P} , in which subsequent rows are ordered in accordance with the importance of particular PCs. The data matrix can now be written as

$$\mathbf{X} = \mathbf{T} \mathbf{P}$$

where matrix \mathbf{T} (scores) consists of coefficients which can be interpreted as co-ordinates in a co-ordinate system defined by rows of \mathbf{P} . In the full set of PCs some of them describe nothing but disturbances present in the original data. These PCs should be rejected from the set and only a number, say k , of important (essential) PCs should be saved. Using essential PCs one can reconstruct a model of a data matrix

$$\mathbf{X}_k = \mathbf{T}_k \mathbf{P}_k$$

having the property that all essential information is saved while contribution from disturbances is reduced.

There are many numerical criteria helpful in a proper determination of the number k of essential PCs [7], [8]. Of them two simple ones will be used in the following. Cumulative variance is defined as the ratio of the variance explained by a number, say j , of the first PCs to the total variance, *i.e.*,

$$cv(j) = \frac{\sum_{i=1}^j \lambda_i}{\sum_{i=1}^m \lambda_i}$$

where λ_i is the i -th eigenvalue. Inverse condition number (ICN) compares the importance of a given PC with the importance of the 1st PC, and is defined as

$$ICN(j) = \frac{\lambda_j}{\lambda_1}$$

Visual inspection of the plot of loadings on subsequent PCs is often used as an additional criterion in spectroscopy: when noise and other disturbances dominate over the regular structure seen in the plot, then the PC becomes inessential. In practice, however, comparison of several criteria is needed to reliably determine the number k of essential PCs.

One dimensional plots of loadings can be useful in making decision on k . Two dimensional plots of objects (spectra) can be even more useful in getting insights into the properties of the data matrix (set of spectra), especially when k equals 2 (or 3). For instance, if $k = 2$ then the first two columns of \mathbf{T} can be thought of as a pair of co-ordinates for subsequent objects (spectra) along two axes: one related to the 1st PC and the other to the 2nd PC. Such a plot gives a distribution of m points (each presents a particular object-spectrum) and the way the points are distributed often illustrates graphically properties of a given set of objects.

3.2. Factor analysis

PCA is followed by FA. The aim of FA is to construct a data matrix model that can be interpreted in terms of physically meaningful factors. In spectroscopy the factors typically are spectral lines ascribed to known or expected constituents. Factors can be thought of as rows of a matrix, say \mathbf{F} . If we replace \mathbf{B} with \mathbf{F} , then a new model of \mathbf{X} can be written as

$$\mathbf{X}_F = \mathbf{C}_F \mathbf{F}$$

where \mathbf{C}_F consists of coefficients needed to model particular spectra in \mathbf{X}_F from factors. A detailed discussion of problems that can appear in various situations can be found in [7]. In the following will be using a simple variant of FA, when factors are known and \mathbf{X}_F is replaced with a PC model of the data matrix. Then the matrix \mathbf{C}_F can be calculated according to

$$\mathbf{C}_F = (\mathbf{X}_F \mathbf{F}^T) (\mathbf{F} \mathbf{F}^T)^{-1}.$$

The result is known to be equivalent to the least square approximation of \mathbf{F} to $\mathbf{X}_F = \mathbf{X}_k$.

4. Results and discussion

There are some differences in the fluorescence spectra of pure lactone and pure carboxylate form. The emission spectra of the carboxylate form are slightly red-shifted and characterized by a lower intensity in comparison to the lactone form spectra. Figure 2 presents emission spectra of 1 μM pure lactone and carboxylate form prepared in a buffer at pH 7.4 and a temperature of 37 $^\circ\text{C}$. The spectra were recorded immediately after the dilution of stock lactone and carboxylate solutions in PBS at pH 7.4.

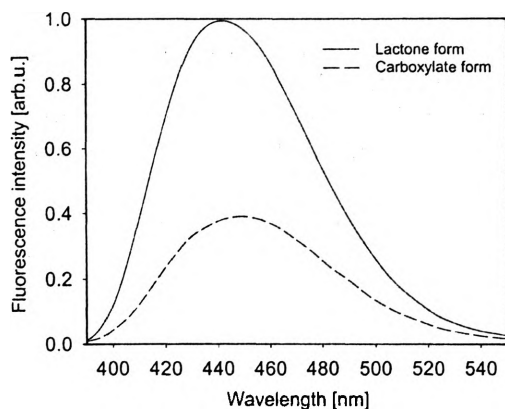


Fig. 2. Emission fluorescence spectra of 1 μM pure lactone (solid line) and pure carboxylate (dashed line) forms of camptothecin, recorded at pH 7.4 and a temperature of 37 $^\circ\text{C}$.

Fluorescence spectra of carboxylate form prepared in buffers at different pH are also slightly shifted. Figure 3 shows the emission spectra of carboxylate form prepared in PBS at pH 9, 10 and 12. All the presented spectra are normalized. There is no evident difference between the fluorescence spectra of pure lactone form prepared in buffers at pH 3, 4.4, 5.5 and 7.4. Because of the influence of pH on the shape of spectra of carboxylate form, for the purpose of determining the rate of CPT hydrolysis in buffer at pH 7.4, spectra of pure lactone and carboxylate forms have to be recorded also in buffer at physiological pH 7.4.

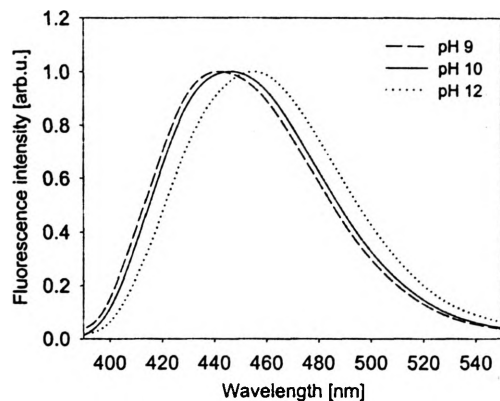


Fig. 3. Emission fluorescence spectra of pure carboxylate form of camptothecin recorded at pH 9, 10 and 12.

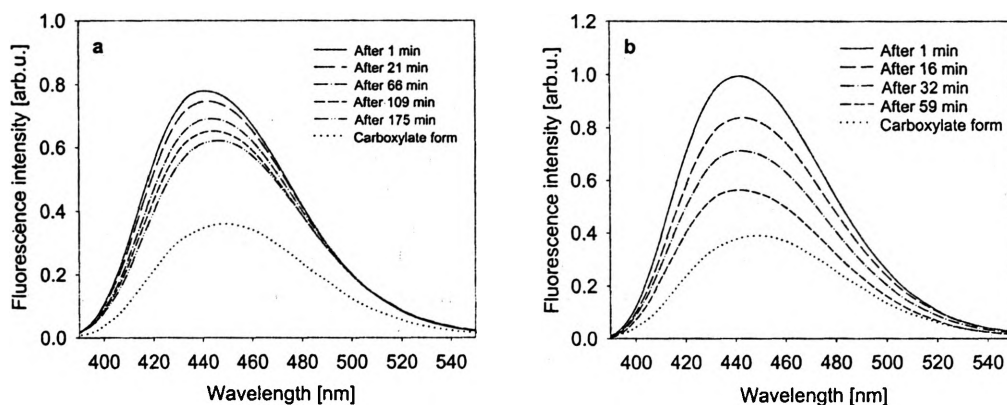


Fig. 4. Evolution of emission fluorescence spectra of 1 μM of camptothecin during the hydrolysis process in PBS at pH 7.4 and temperatures of 25 $^{\circ}\text{C}$ (a), and 37 $^{\circ}\text{C}$ (b). The first spectrum was recorded 1 minute after solution preparation (solid line) and subsequent ones after time given at curves (in minutes). The lowest spectrum in this figure is the spectrum of pure carboxylate form.

The fluorescence spectra recorded during the hydrolysis process, *i.e.*, in the case when the stock lactone form was diluted into PBS at pH 7.4 are presented in Fig. 4. Measurements were performed at temperatures of 20 $^{\circ}\text{C}$ (results obtained at this temperature have previously been published in [9]), 25 $^{\circ}\text{C}$ (Fig. 4a) and 37 $^{\circ}\text{C}$ (Fig. 4b). Spectrum of pure lactone form exhibits the highest intensity while the spectrum of carboxylate form is the least intensive. This figure shows that the fluorescence spectra of a sample change over time. Their shape is approaching the shape of spectrum of the carboxylate form, but it does not reach the spectrum of pure carboxylate form. The changes observed are the result of converting lactone into carboxylate.

In order to get a better insight into time dependent changes of fluorescence spectra, the full set of spectra was analysed with PCA. Figure 5 presents loadings on the first three PCs calculated for measurements at a temperature of 25 $^{\circ}\text{C}$. One can see from

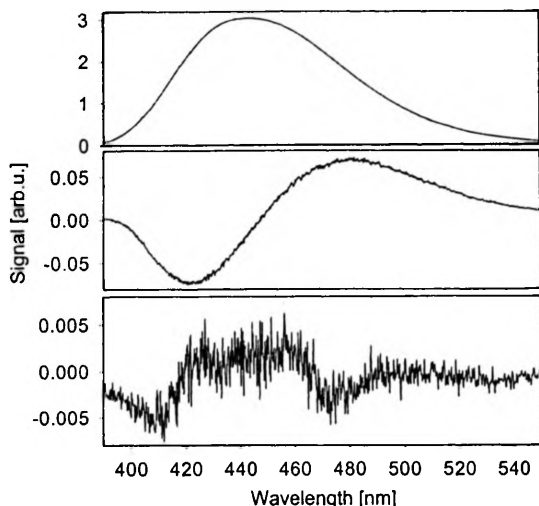


Fig. 5. Loadings on the first three PCs obtained from PCA of the set of spectra measured at pH 7.4 and a temperature of 25 °C.

the figure that all the curves presented contain some structure. However, if numerical criteria are calculated (see Tabs. 1–3 for temperatures of 20, 25, 37 °C, respectively), then it is seen that the first two PCs are much more informative when compared to the 3rd and subsequent ones. Therefore one can conclude that a two PC model of the set of analysed spectra describes the important information and that it describes the variance at a very satisfactory level (CV = 99.999%).

T a b l e 1. Values of numerical criteria selected to the first five PCs, obtained for a set of fluorescence spectra of camptothecin recorded at pH 7.4 and a temperature of 20 °C.

PCs	Eigenvalue	CV [%]	ICN $\times 10^3$
1	2309.2	99.985	1000
2	0.0290	99.998	0.1257
3	0.0085	99.998	0.0037
4	0.0074	99.999	0.0032
5	0.0071	99.999	0.0031

T a b l e 2. Values of numerical criteria selected to the first five PCs, obtained for a set of fluorescence spectra of camptothecin recorded at pH 7.4 and a temperature of 25 °C.

PCs	Eigenvalue	CV [%]	ICN $\times 10^3$
1	5346.4	99.941	1000
2	3.1123	99.999	0.5821
3	0.0102	99.999	0.0019
4	0.0050	99.999	0.0009
5	0.0048	99.999	0.0009

Table 3. Values of numerical criteria selected to the first five PCs, obtained for a set of spectra detected at pH 7.4 and a temperature of 37 °C.

PCs	Eigenvalue	CV [%]	ICN $\times 10^3$
1	4259.8	99.980	1000
2	0.6100	99.994	0.1432
3	0.1984	99.999	0.0466
4	0.0091	99.999	0.0021
5	0.0059	99.999	0.0014

A series of fluorescence spectra detected during hydrolysis reaction have been decomposed with FA, using the pure lactone and the pure carboxylate spectra as the factors. Results of FA of the sets of spectra recorded at temperatures of 20, 25 and 37 °C are presented in Fig. 6. It presents the values of coefficients (elements of C_{FA} matrix) which determine the contribution of each of two forms (factors) to a given mixture. One can assume that the elements of C_{FA} matrix are equal to the fraction of lactone and carboxylate form, then Fig. 6 illustrates the kinetics of CPT hydrolysis.

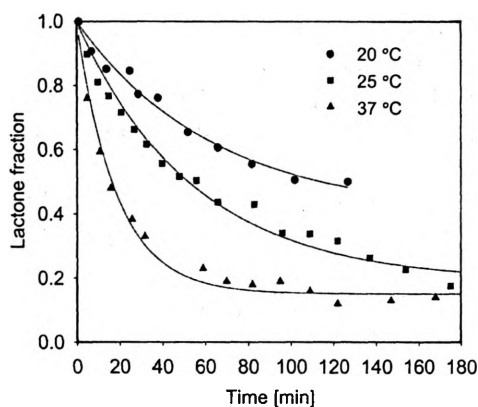


Fig. 6. Time dependence for relative contribution of lactone form of camptothecin obtained by factor analysis of fluorescence spectra. Comparison of results calculated from sets of spectra recorded at pH 7.4 and temperatures of 20, 25 and 37 °C.

The concentration of lactone form is decreasing over time. The fraction of lactone form $L(t)$ as a function of time can be described by the equation [5]

$$L(t) = L_0 + B \exp(-bt)$$

where $L_0 + B$ is the lactone fraction just after dilution in PBS (we assume that $L_0 + B$ is equal to 1 at $t = 0$) and L_0 is the fraction of lactone form in equilibrium, that is, in such condition when both forms coexist without changing concentration. Generally, L_0 , B and b depend on temperature and pH. The hydrolysis process of CPT is described by the equilibrium constant L_0 and the half-life of hydrolysis reaction $t_{1/2} = (\ln 2)/b$.

Table 4. Hydrolysis parameters of camptothecin obtained by factor analysis of fluorescence spectra. Measurements performed at pH 7.4 and temperatures of 20, 25 and 37 °C.

	Temperature		
	20 °C	25 °C	37 °C
L_0 [%]	38	19	15
$t_{1/2}$ [min]	48	37.7	13

As is shown in Fig. 6 and Tab. 4, the process of hydrolysis of CPT in PBS at pH 7.4 is faster if the temperature of the solution is higher (for the data reported the parameter $t_{1/2}$ is largest at 20 °C and smallest at 37 °C). At a temperature of 37 °C equilibrium is achieved earlier than at 20 °C and 25 °C and it is at the lowest level (among the temperatures considered here). This means that after achieving equilibrium there is less lactone form in the solution of 37 °C than in the solutions at lower temperatures.

To analyse the hydrolysis process of CPT the HPLC method is generally used. Figure 7 presents a comparison of results obtained using our spectroscopic approach and the HPLC method. As can be seen in Fig. 7, the kinetics of hydrolysis process obtained from these two methods are very similar. This is confirmed by values of the kinetic and equilibrium parameters, which are collected in Tab. 5 (13 min and 15% for spectroscopic data, 18.3 min and 12% for HPLC data). This table also contains results obtained by BURKE *et al.* [10], and MI and BRUKE [11] from HPLC data. Values calculated by the authors of this paper are especially comparable with data reported in [10].

Methods of fluorescence spectra analysis can be an alternative to the HPLC measurements of CPT. They can also be applied in cases when the HPLC method is failing, for example in determining free and bound CPT molecule concentration in liposomes or red blood cell suspensions. There are differences in fluorescence spectra of free and bound camptothecins [10], [12]. Free as well as bound CPT molecules exist

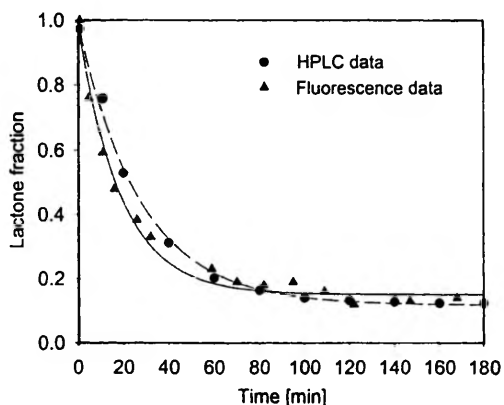


Fig. 7. Comparison of the kinetics of camptothecin hydrolysis obtained by factor analysis of fluorescence spectra and HPLC methods.

Table 5. Summary of the kinetic and equilibrium parameters for hydrolysis of CPT observed at pH 7.4 and a temperature of 37 °C.

	Method			
	HPLC ¹	HPLC ²	HPLC ³	Fluorescence ⁴
L_0 [%]	12	17	17	15
$t_{1/2}$ [min]	18.3	16.6	23.8	13

¹Hydrolysis values obtained from HPLC as described in Sec. 2.

²Previously reported values obtained from HPLC [10].

³Previously reported values obtained from HPLC [11].

⁴Hydrolysis values calculated from data of fluorescence spectroscopy as described in this paper.

in liposomes or red blood cell suspensions. HPLC does not permit determination of the concentrations of free and bound drugs, while this is possible by applying the fluorescence anisotropy titration method [10], [12]. We suppose that we will be able to determine these concentrations using the PCA and FA analysis of fluorescence spectra of a mixture of free and bound drugs.

5. Conclusions

PCA and FA methods of fluorescence spectra analysis are promising tools in investigations of the kinetics of camptothecin hydrolysis. These are very precise methods based on all the information contained in spectra. They enable one to see very subtle changes in the shapes of fluorescence spectra appearing in the hydrolysis process and then make it possible to observe the kinetics of CPT hydrolysis in physiological fluids. Because of the advantages of the approach presented in this work and some limitations of the HPLC method the two methodologies can be complementary in the research of camptothecin. Figure 7 confirms that the two approaches give similar results.

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