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ORIGINAL ARTICLE

Effects of solvent, pH and β-cyclodextrin on the fluorescent behaviour of lomustine

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Abstract Fluorescent behaviour of lomustine, a DNA cross-linking agent, was investigated in different solvents, pH and in the presence of β-cyclodextrin (β-CD). The solvents in which fluorescence spectra were observed play a major role in determining the spectral intensity of fluorophore, since it was found to exhibit new fluorescent properties essentially influenced by intermolecular interactions, particularly by intermolecular H-bonding formed with solvents. The pH-dependence profile was typically U-shape with a maximum at pH between 3.51 and 6.58. It was corroborated that the fluorescence emission band of lomustine is significantly intensified in the presence of β-CD. From the changes in the fluorescence spectra, it was concluded that β-CD forms a 1:1 inclusion complex with lomustine and its association constant was calculated.

Keywords Lomustine · β-cyclodextrin · Inclusion complex · Fluorescence · Solvent effect · pH effect

Introduction

Studies on photophysical properties are one of the keystones in the elucidation of the chemical behaviour and electronic orientation of organic and biomolecules [1]. It is well established that electronic charge redistribution does take place at each atom (especially on the acid and basic centres) of the molecule when it is photoexcited [2]. Due to this the acidic and basic properties of these basic centres or groups are quite different in the S_0 and S_1 states [3]. Although it is an elementary, but a very important process, generally described as excited state intramolecular proton transfer (ESIPT), is observed in molecules containing both the proton donor (NH_2, OH) groups and the proton acceptor [–N=, carbonyl (C=O)] groups provided the two groups are connected by the intramolecular hydrogen bonding in the ground state. This is because, the former becomes stronger acid and the latter becomes stronger base on excitation. The position of proton transfer is associated with the change in the charge densities at the respective atom and this is linked to the acid–base properties of the two ionizable groups. Depending upon the positions of these two ionizable groups, the proton transfer may be or may not be solvent-dependent [4].

The main feature that makes cyclodextrins (CDs) of interest is their ability to form inclusion complexes with a wide diversity of molecules, either in solution or in solid phase, a property that offers many attractive applications described extensively in the literature [5–9].

The formation of an inclusion complex with CD can alter the photochemical and photophysical properties of guest molecules [10, 11]. In the case of fluorescent guests, the inclusion can affect the ground and/or excited electronic states of the fluorophore, consequently changing the spectral properties of the substrate. Fluorescence enhancement is the most common situation, which has found a number of interesting analytical applications, and can be attributed to factors such as the protection against collisional quenching, changes in the polarity of the microenvironment or an increase in the rigidity of the guest, amongst others [12].

A considerable literature was devoted to β-CD and its applications especially in the pharmacological area [6, 13–20]. Several medicinal substances have been successfully complexed by CD, such as antineoplastic agents

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[8, 21–24]. These complexes can increase the stability and improve the solubility and bioavailability of the substance and modify the pharmacokinetics of the resulting drugs with a subsequent reduction in adverse effects [6, 13–20].

Lomustine (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea) is an alkylating antineoplastic agent, which has been used to treat human cancers since the mid seventies [25]. It is an extremely potent nitrosourea, a class of anticancer agents which have a wide range of effects on various leukemias and solid tumors [26–31].

Inclusion complexes of lomustine with CDs have already been studied [32], however, to the best of our knowledge even lomustine or inclusion complexes of lomustine with CDs have not been studied by methods based on fluorescence before.

The aim of this study was to investigate how the nature of the media (solvent and pH effects) and the presence of β-CD affect the fluorescent behaviour of lomustine. Other investigations on inclusion complexes of lomustine and other nitrosourea derivatives with CDs including further details on the complexes structure obtained by ^{1}H NMR measurements and semiempirical calculations are underway. The present study serves as basis for further investigations.

Materials and methods

Apparatus and reagents

Lomustine was synthesized according to Ref. [26]. Melting point was determined in Kofler bench and is uncorrected. Solvents used in experimental measurements have been used without further purification. Distilled water was used in the preparation of all aqueous solutions. ^{1}H NMR (400 MHz) spectrum was recorded on a Bruker spectrometer AC 280. Lomustine was dissolved in methanol (about 1.00×10^{-4} M), after stirring at room temperature, the solution was directly analyzed by ES-MS electron ionization (30 and 20 eV); mass spectrum was recorded in positive or negative on a Water Micro Mass ZQ. All the spectrofluorimetric measurements were obtained on a Shimadzu RF-5301 PC spectrometer, equipped with a xenon lamp and using 1.00 cm quartz cell. The slit width (3.00 nm) and other conditions were set up as recommended by the manufacturer. The fluorescence spectra were obtained at room temperature and the optimum excitation and emission wavelengths were selected from these spectra. For the pH measurements, a Crison 2000 micro-pH-meter, equipped with a combined glass electrode was employed.

Procedures

All the solutions were prepared just before taking measurements to minimize the decomposition and loss of lomustine, and well mixed by using an ultrasonic processor G.S.: Geprufte Sicherheit type UP 50 H. All measurements were made in triplicate.

The concentration of 1.00×10^{-5} M for lomustine was used in all measurements. Stock solutions of lomustine (1.00×10^{-2} M) were prepared by dissolving the same calculated amount of lomustine in one of the following solvents: methanol, ethanol, dimethylsulfoxyde (DMSO), acetonitrile, dimethylformamide (DMF), acetone, dichloromethane (CH_2Cl_2), tetrahydrofuran (THF) and ethyl acetate. Working solutions with final concentration of 1.00×10^{-5} M were obtained by appropriate dilution of each stock standard solution with the proper solvent. In aqueous solutions, addition of acetonitrile was necessary to solubilize lomustine; therefore, lomustine was first dissolved in acetonitrile and diluted with water or buffer (2 vol%).

pH effect

The influence of pH in the fluorescence spectra of lomustine was studied recording the corresponding spectra at different pH values ranging from 1.8 to 9, prepared by adding stock solution of the drug in acetonitrile to aqueous buffer solutions (phosphate and citrate).

β-CD effect

Acetonitrile may not compete preferentially the hydrophobic cavity of β-CD due to its high polarity and therefore is chosen as organic solvent to prepare the lomustine stock solution. The working solution of lomustine was almost entirely aqueous and the percentage of acetonitrile present in the sample solution (2 vol%) was considered to be too low to have any significant influence on the complex formation between β-CD and lomustine. The pH was adjusted by addition of phosphate-citrate buffer (pH 5). The concentration of lomustine was kept constant at 1.00×10^{-5} M, while the CD concentration was varied from 0.00 to 1.00×10^{-4} M.

Continuous variation method (Job's method)

The stoichiometry of the inclusion complex was determined by the continuous variation method [33]. The continuous variation (Job's) plot was determined from spectrofluorimetric data, according to the continuous variation method. Equimolar 1.00×10^{-5} M solutions of lomustine and β-CD were mixed to a standard volume (1 ml: 4 ml; 3 ml: 2 ml; 2.5 ml: 2.5 ml and so on) varying the molar ratio but keeping the total concentration of the species constant. After stirring, the emission was measured

for all solutions and the fluorescence intensity was plotted against R (R = [lomustine]/{[lomustine] + [β-CD]}.

Characterization of lomustine

N-nitroso, *N*-(2-chloroethyl), *N′*-(cyclohexyl) urea: M = 233.5 g/mol [$C_9H_{16}ClN_3O_2$]; Rf: 0.78, Dichloromethane; Pf °C: 88-90; ^{1}H NMR (400 MHz, CD Cl_3) δ (ppm) : 6.79 (d, 1H, NH), 4.17 (t, 2H, j = 7,04 Hz, CH_2NN), 3.90 (m, 1H, CH–N), 3.49 (t, 2H, j = 7.02 Hz, CH_2Cl), 2.04–1.93 (m, 10H, CH_2 cycle); MS ESI$^+$ (30 eV) m/z : 258.70 [M+Na$^+$ + H], 227.4 [M−NO + Na$^+$ +H].

Results and discussion

Solvent effect

Effect of solvent on the fluorescence spectra

In order to investigate the effect of solvent on fluorescence intensity and wavelengths of excitation and emission of lomustine (Fig. 1), we have selected ten solvents with different properties. Using the intermolecular interactions that act between the solvent molecules and solutes, one may classify solvents into three categories [34]. Depending on the ability of solvent molecules to form hydrogen bonds with the solute molecules, we can classify the solvents used as polar protic, polar aprotic and non-polar aprotic. Polar protic solvents are hydrogen bond donors with strong polarities, having a dielectric constant (ε) greater than 15 and $E_T(30)$ (Mole Transition Energy) ranging from 47 to 63 (Table 1). Water, methanol and ethanol are all protic solvents. DMSO, acetonitrile, DMF and acetone are polar aprotic solvents with a dielectric constant greater than 15 and $E_T(30)$ range of 40–47. This kind of solvent cannot be used as a hydrogen bond donor, but can be a good electron donor. CH_2Cl_2, THF and ethyl acetate are non-polar aprotic solvents with a dielectric constant less than 15 and $E_T(30)$ from 30 to 40, which cannot be hydrogen bond donor, and only has very weak interaction with solute.

The fluorescence spectral data of lomustine in various solvents are compiled in Table 1 and its fluorescence spectra are displayed in Fig. 1.

As shown in Fig. 1, fluorescence behaviours of lomustine were largely dependent on the solvent properties.

The geometry of lomustine (Fig. 2) is in favor of the formation of an intramolecular hydrogen bonding between the –NH group and an electronic pair of nitroso group [37].

The existence of an intramolecular hydrogen bond N=O···HN in lomustine [38] determines specific features of its fluorescence spectra.

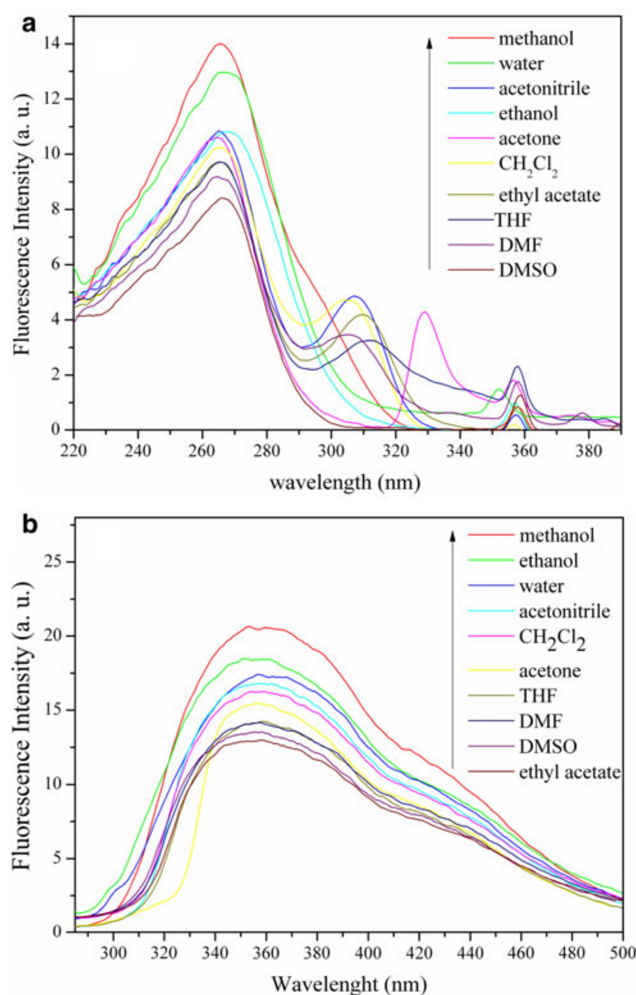

Fig. 1 Fluorescence spectra of lomustine in different solvents. **a** Excitation spectra; **b** Emission spectra

Table 1 Fluorescence spectral data (nm) and Stokes shifts (cm^{-1}) of lomustine in selected solvents with $E_T(30)$ [35] and Δf [36] values

Number	Solvent	Lomustine			$E_T(30)$	Δf
		λ_{ex}	λ_{em}	Δv		
1	Water	268	357	9302	63.1	0.319
2	Methanol	266	353	9265	55.4	0.308
3	Ethanol	267	351	8963	51.9	0.288
4	DMSO	266	358	9661	45.1	0.263
5	Acetonitrile	265	357	9724	45.6	0.304
6	DMF	265	357	9724	43.2	0.274
7	Acetone	265	357	9724	42.2	0.284
8	CH_2Cl_2	266	359	9738	40.7	0.211
9	THF	266	358	9661	37.4	0.209
10	Ethyl acetate	265	358	9803	38.1	0.199

The main reason for the fluorescence intensities increase of lomustine in protic solvents compared with those of aprotic solvents is due to the weakness of the intramolecular

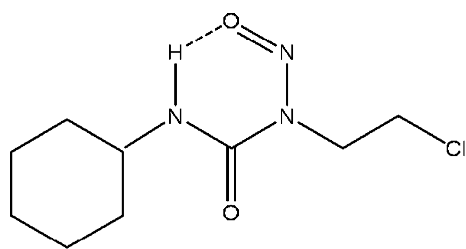

Fig. 2 Structure of lomustine showing intramolecular hydrogen-bonding formation

hydrogen bond, caused by increased solute–solvent interactions, due to the competition between inter- and intramolecular hydrogen bonding.

The characteristics of the intramolecular hydrogen bond in lomustine can be essentially influenced by intermolecular interactions, particularly by intermolecular hydrogen bonds formed with protic solvents. The intramolecular hydrogen bonding was largely affected by protic solvents; the fluorescence spectrum is regularly red shifted as the polarity and hydrogen bonding capacity of the solvent increase, a bathochromic shift is observed along from ethanol to water.

The relative indifference of lomustine in some solvents, especially aprotic solvents, to its environment can be explained by the fact that intramolecular hydrogen bonding is already taking place. This bonding is assumed to be satured so that the intermolecular hydrogen bond is not formed because of the existence of stable intramolecular hydrogen bond between the amino (NH) and nitroso (NO) groups, which is stable enough even in the basic solvents. Hence, when the compound is dissolved in aprotic solvents only a minimal effect such as a slight loss in spectral resolution is observed.

Correlation of solvatochromic shift with the solvent parameters

Polarity is one of the most important properties of organic solvents traditionally expressed by some physical parameters such as the dielectric constant (ε) and the permanent dipole moment (μ). In fact, it is not a precise definition, because the interactions between the solute and solvent molecules are much more extensive and complicated: in addition to the non-specific Coulombic, directional, inductive, and dispersion interactions, there is also a specific hydrogen bond, electron pair donor (EPD)/electron pair acceptor (EPA), and solvophobic interactions. The solvent polarity as thus defined generally cannot be described quantitatively by a single physical parameter. For the estimation of solvent polarities, resort is taken to empirical parameters obtained for certain standard substances used as probes, [39]. Of the many empirical polarity parameters or indexes that have been proposed, only a few remain viable, in the sense that they are currently more or less widely used to describe the polarity of solvents for various purposes. Some such parameters that are commonly used describe better other, more specific, properties than polarity: e.g., hydrogen bond or electron-pair donation ability. Dimroth and Reichardt's $E_T(30)$ [40] solvatochromic parameter, which was number 30 in a series of compounds studied, hence the designation $E_T(30)$ [41], generally provides a more comprehensive understanding of the over-all solvation ability of the solvent in the microscopic region than individual physical data. So $E_T(30)$ is an overall manifestation of the interactions between the solvent and the solute [42]. Values of $E_T(30)$ are known for several hundred solvents, and are based on the solvatochromic band of pyridinium-*N*-phenolbetaine. This value gives the transition energy (kcal.mol^{-1}) of the intramolecular charge transfer of pyridinium-*N*-phenolbetaine [42–44].

In this study, the Stoke's shifts ($\Delta\nu$, cm^{-1}) of the fluorescence spectra of lomustine determined in the solvents employed, have been correlated with $E_T(30)$ [40] and the Lippert–Mataga solvent polarity parameter Δf, as estimated using Eq. 1 [1, 45–47].

$$\Delta f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \quad (1)$$

where, ε is the static dielectric constant and n is the refractive index of the solvent. The ε and n values of pure solvents were obtained from literature [36]. Such correlations of Stokes shift with any one of these parameters are very interesting to elucidate the binding mode and the type of interaction between the solute and the solvent.

Table 1 gives the list of solvents, corresponding polarity parameters and the stokes shifts.

The resulting plot of stokes shift against Δf is shown in Fig. 3. From this plot it is clear that a linear relation is not obtained. The deviations are particularly large in hydrogen-bonding solvents, but since they are also obtained in non-hydrogen-bonding solvents, i.e. THF, a second form of hydrogen bond is expected, possibly an intramolecular hydrogen bond might occur.

The $E_T(30)$ parameter incorporates both hydrogen bonding and solvent polarity effects whereas the Δf parameter represents only solvent polarity effects.

The fluorescence solvatochromic shifts reveal that the solvent interactions of hydrogen bonding solvents are predominant. This is confirmed by the correlation of the Stokes shifts of lomustine with $E_T(30)$ ($r = 0.761$) and Δf ($r = 0.549$) values (Table 1). The better correlation of Stokes shifts of lomustine with $E_T(30)$ indicates predominance of hydrogen bonding interactions over dipolar interactions. Although the hydrogen bonding interactions

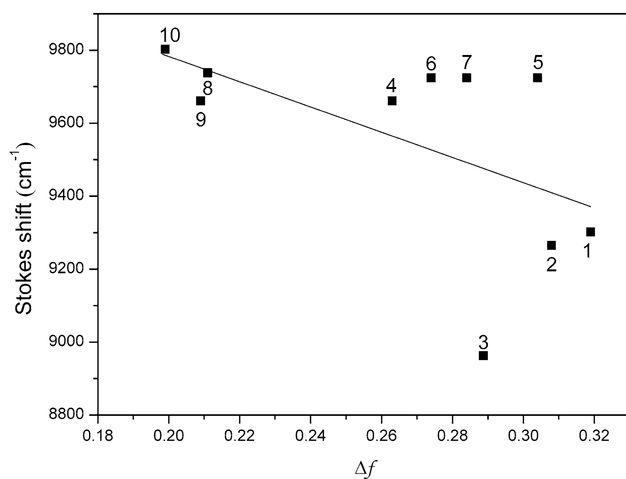

Fig. 3 Stokes shift versus Δf. *1* water, *2* methanol, *3* ethanol, *4* DMSO, *5* acetonitrile, *6* DMF, *7* acetone, *8* CH_2Cl_2, *9* THF, *10* ethyl acetate

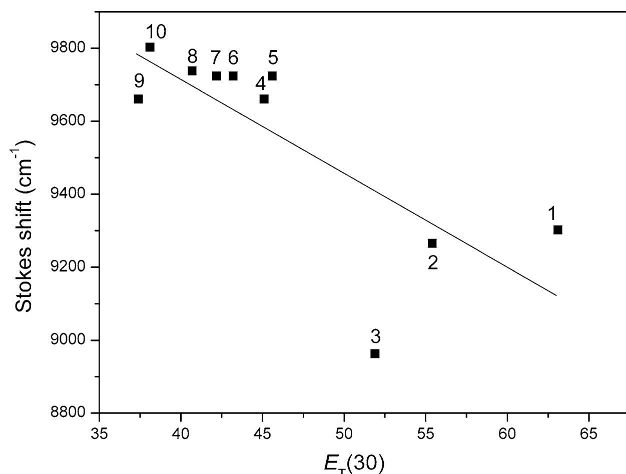

Fig. 4 Stokes shift versus $E_T(30)$. *1* water, *2* methanol, *3* ethanol, *4* DMSO, *5* acetonitrile, *6* DMF, *7* acetone, *8* CH_2Cl_2, *9* THF, *10* ethyl acetate

are predominant in the solvatochromic shifts of the compound, the correlation between the Stoke's shifts and the E_T (30) parameter is poor ($r = 0.761$). This is due to the presence of both intra- and intermolecular hydrogen bonding in protic solvents. So in hydrogen bonding solvents a large deviation from linearity is observed (Fig. 4).

pH effect

Since it was revealed that stability of lomustine depends on conditions such as pH [32], studies of the influence of pH were carried out before calculating the magnitude of its interaction with β-CD.

The effect of pH on the emission intensity is shown in Fig. 5. As can be seen, the study showed that fluorescence intensity of lomustine was dependent on the solution's pH values.

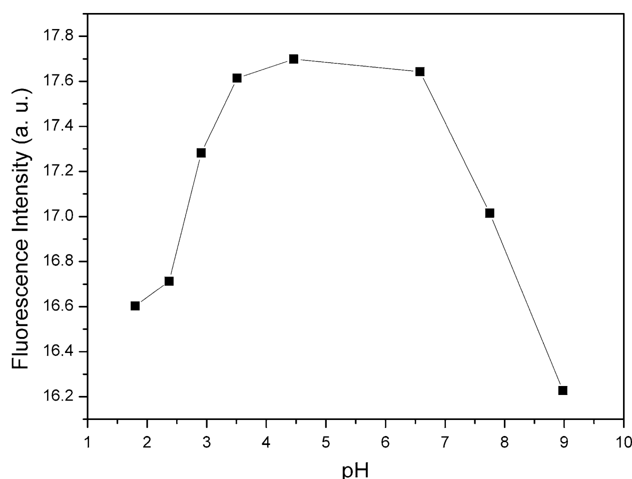

Fig. 5 pH effect on the fluorescence intensity of lomustine

The fluorescence emission increased with some pH values and then decreased with higher ones. The greatest fluorescence intensity was found between pH 3.51 and 6.58.

The fluorescence intensity of lomustine was affected greatly by the acidity of the medium. In strong acidic solutions, the fluorescence intensity of the molecule was low. Upon an increase of pH value, the fluorescence intensity increased.

The decrease of the fluorescence intensity with the increase of the solution acidity may be explained as follows: the lone pair on the nitrogen interacts with the delocalized system in the molecule. As is known, delocalization makes molecules exhibit fluorimetric properties. So, at higher pH, –NH moiety has the greater fluorescence signal. The increase of acidity leads to a decrease of fluorescence. The reason for this behaviour is the fact that under these conditions, the lone pair of nitrogen is used to join to a hydrogen ion (supplied by buffer solution) and it is no longer available to contribute to the delocalization. Thus, disruption of the delocalization with increasing acidity causes the fluorescence of lomustine to decrease.

The fluorescence intensity is relatively stable and strong in the range of pH value from 3.51 to 6.58, because the drug is not decomposed at this pH range.

The inflection of the latter curve at pH approximately 6.58 and the decrease for higher pH values can be attributed to the decomposition of lomustine, since it was shown experimentally that lomustine is not stable and suffer a significant decomposition in this pH range [32, 48].

β-CD effect

pH has influence on lomustine, further more pH effect on fluorescence of lomustine has been examined. Thus, during

this experiment, the pH value was controlled to be 5, which could accordingly maintain lomustine without decomposition and ensure the maximum fluorescence intensity.

As can be seen in Table 2 and Fig. 6, the fluorescence emission of lomustine is significantly increased with increasing concentrations of β-CD.

The possible reason for the fluorescence enhancement in the presence of β-CD was as follows. Lomustine molecule can enter the hydrophobic cavity of β-CD under the effect of noncovalent bonds such as Van der Waals bond and hydrogen bond. In the cavity, the degree of freedom of the molecule and the nonradiative decay process decreased, so the probability of the radiationless transition decreased. On the other hand, the cavity can shield the signal of the excited drug from quenching. So the fluorescence intensity

Table 2 Fluorescence intensities of lomustine at different concentrations of β-CD

Number	Concentration of β-CD (M)	Fluorescence Intensity of lomustine (a.u.)
0	0	19.03
1	2.00×10^{-5}	20.86
2	3.00×10^{-5}	21.75
3	4.00×10^{-5}	22.2
4	5.00×10^{-5}	22.79
5	6.00×10^{-5}	23.43
6	7.00×10^{-5}	23.86
7	8.00×10^{-5}	23.47
8	9.00×10^{-5}	25.91
9	1.00×10^{-5}	27.15

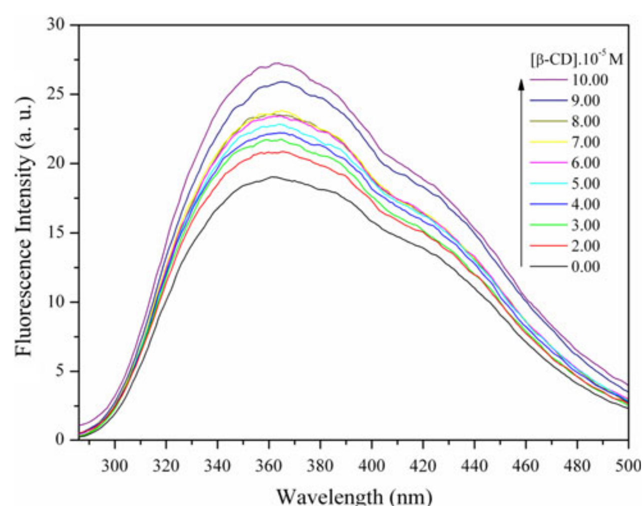

Fig. 6 Emission enhancement of lomustine in the presence of increasing concentrations of β-CD. [lomustine] = 1.00×10^{-5} M; [β-CD] = $0 - 1.00 \times 10^{-4}$ M

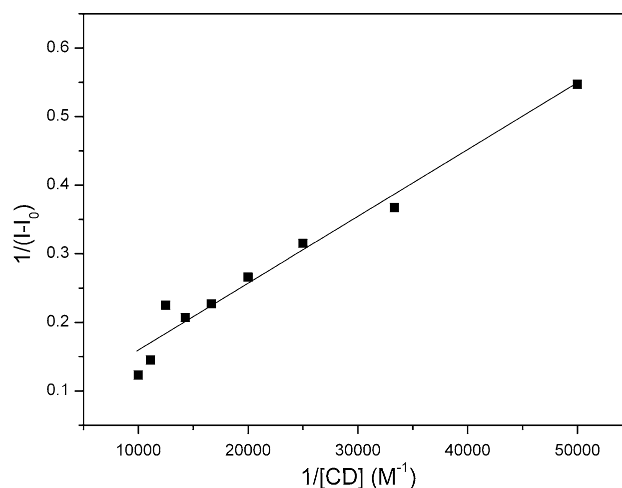

Fig. 7 Plot of $1/(I - I_0)$ versus 1/[CD]

increased with the formation of new species, i.e., the host–guest inclusion complex.

It is reasonable to consider the 1:1 complex formation between β-CD and lomustine because the drug is suitable to be complexed by one β-CD cavity according to the dimensions.

From the change in the fluorescence intensity of the system, the apparent association constant value (K_1) and the stoichiometry of the inclusion complex can be determined by the Benesi-Hildebrand method [49].

Assuming a 1:1 stoichiometry, the following Eq. 2 was used:

$$\frac{1}{I - I_0} = \frac{1}{(I_1 - I_0)} + \frac{1}{(I_1 - I_0)K_1[\mathrm{CD}]_0} \quad (2)$$

where $[\mathrm{CD}]_0$ represents the initial concentration of β-CD, I_0 and I are the fluorescence intensities in the absence and presence of β-CD, respectively, and I_1 is the limiting intensity of fluorescence. The K_1 value was obtained from the slope and the intercept of the plot. The Benesi–Hildebrand plot (Fig. 7) shows excellent linear regression ($r = 0.9838$) supporting the assumed 1:1 lomustine-β-CD inclusion complex. From the plot, $K1$ is evaluated as $6000\ \mathrm{M}^{-1}$.

In order to effectively check the existence of 1:2 inclusion complex in the solution, the corresponding Benesi–Hildebrand relation (3) of such kind of complex system can be expressed as [49]:

$$\frac{1}{I - I_0} = \frac{1}{(I_1 - I_0)} + \frac{1}{(I_1 - I_0)K_1K_2[\mathrm{CD}]_0^2} \quad (3)$$

where K_2 is the association constant for the 1:2 complex formation.

If a plot of $1/(I - I_0)$ versus $1/[\mathrm{CD}]_0^2$ is performed according to Eq. 3 and Fig. 8, a downward concave curvature is obtained and, hence the possibility of formation of

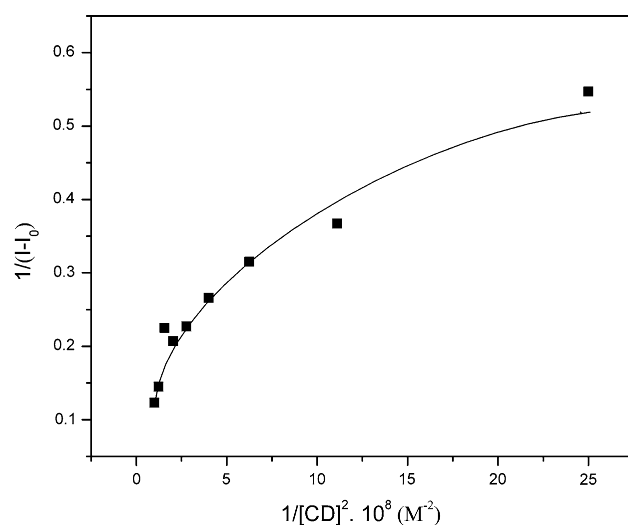

Fig. 8 Plot of $1/(I-I_0)$ versus $1/[CD]^2$

a 1:2 inclusion complex between lomustine and β-CD is ruled out.

The formation of 1:1 inclusion complex is further assisted by using continuous variation method (Job's method) [33]. According to the continuous variation method, when a physical parameter directly related to the concentration of the complex can be measured for a set of samples with continuously variable molar fraction of the components. The maximum concentration of complex will be present in the sample where the molar ratio R corresponds to the complexation stoichiometry. Job's plot (Fig. 9) shows a maximum value at $R = 0.5$ and high symmetrical shape indicating the existence of a complex with a 1:1 stoichiometry, within the range of the investigated concentrations.

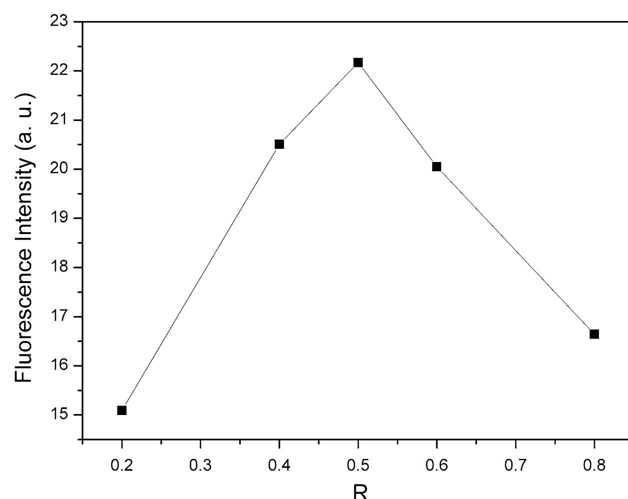

Fig. 9 Continuous variation plot of lomustine-β-CD inclusion complex

Conclusion

This study clarifies some aspects concerning the fluorescence properties of lomustine, possessing potentially useful antitumor therapeutic properties, the solvent and pH effects, as well as β-CD effect are examined. We have found that the fluorescent behaviour of lomustine is critically solvent and pH dependent; and the crucial role of the intermolecular hydrogen bonding was indicated. In protic solvents the intermolecular hydrogen bond is responsible for the observed fluorescence spectral behaviour of the drug, whereas in aprotic solvents an intramolecular hydrogen bond type is postulated to rationalize the data found. On the other hand, we have showed that the fluorescence intensity of lomustine was largely enhanced in the presence of β-CD because of the formation of an inclusion complex. The stability constant of the inclusion complex of lomustine with β-CD was determined to compare the corresponding inclusion capacity, and the 1:1 stoichiometry of the inclusion complexation was confirmed by the continuous variation method.

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