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Talanta 60 (2003) 493–503
Talanta
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Spectrofluorimetric determination of stoichiometry and association constants of the complexes of harmane and harmine with β-cyclodextrin and chemically modified β-cyclodextrins☆

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Received 17 June 2002; received in revised form 11 December 2002; accepted 11 December 2002

Abstract

The association characteristics of the inclusion complexes of the β-carboline alkaloids harmane and harmine with β-cyclodextrin (β-CD) and chemically modified β-cyclodextrins such as hydroxypropyl-β-cyclodextrin (HPβ-CD), 2,3-di-*O*-methyl-β-cyclodextrin (DMβ-CD) and 2,3,6-tri-*O*-methyl-β-cyclodextrin (TMβ-CD) are described. The association constants vary from 112 for harmine/DMβ-CD to 418 for harmane/HPβ-CD. The magnitude of the interactions between the host and the guest molecules depends on the chemical and geometrical characteristics of the guest molecules and therefore the association constants vary for the different cyclodextrin complexes. The steric hindrance is higher in the case of harmine due to the presence of methoxy group on the β-carboline ring. The association obtained for the harmane complexes is stronger than the one observed for harmine complexes except in the case of harmine/TMβ-CD. Important differences in the association constants were observed depending on the experimental variable used in the calculations (absolute value of fluorescence intensity or the ratio between the fluorescence intensities corresponding to the neutral and cationic forms). When fluorescence intensity values were considered, the association constants were higher than when the ratio of the emission intensity for the cationic and neutral species was used. These differences are a consequence of the co-existence of acid–base equilibria in the ground and in excited states together with the complexation equilibria. The existence of a proton transfer reaction in the excited states of harmane or harmine implies the need for the experimental dialysis procedure for separation of the complexes from free harmane or harmine. Such methodology allows quantitative results for stoichiometry determinations to be obtained, which show the existence of both 1:1 and 1:2 β-carboline alkaloid:CD complexes with different solubility properties.

☆ Paper presented at X International Symposium on Luminescence Spectrometry, Granada, Spain, 4–7 June.

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doi:10.1016/S0039-9140(03)00066-3

Keywords: β-Carboline alkaloids; Cyclodextrin complexes

1. Introduction

The β-carboline ring exhibits singular luminescence properties [1] and the proton transfer in the excited state is very fast [2,3]. The existence of ionized and neutral forms is a consequence of the presence of the acidic pyrrolic nitrogen, which loses its proton in alkaline media. The pyridinic nitrogen behaves as a base and is easily protonated in neutral or acidic media. The pK_a values obtained in the ground state differ from those obtained in excited state (pK_a^*). In the excited state it is also possible to observe the emission corresponding to the zwitterionic forms [2] and this behaviour can be explained considering that the excitation of neutral species in ground states produces the corresponding cationic species due to the rapid proton transfer in the excited state. When the solvents employed are not proton donors it is possible to observe the emission corresponding to the neutral forms. Thus, in ethanol the only observed band is the one corresponding to the neutral form. However, in aqueous solution (pH 7.2–7.9) only the band corresponding to the cationic form is observed. These species are stronger bases in excited state than in ground state and the protonation of pyridinic nitrogen in the excited state is very fast compared to the deactivation of the excited state by fluorescence emission. The existence of different equilibria in ground and excited states allows the proton transfer among the neutral, cationic, zwitterionic and anionic forms when these species are irradiated under the experimental conditions to measure or detect the fluorescent emission [2,4]. Our group has demonstrated that the compartmentalization of these compounds in lower polarity environments such as surfactants [5] or cyclodextrins [6,7] allows the observation of the emission corresponding to the neutral form in buffered aqueous solutions at neutral pH.

β-Carboline alkaloids such as harmane and harmine are present in a great variety of medicinal plants and they are also endogenously produced in human and animal tissues as a product of secondary metabolism [8]. They possess diverse biological properties such as hypotensive, hallucinogenic or antimicrobial actions [9] and tremorogenesis [10,11]. They are able to bind to benzodiazepine receptors [12] and they have also been proposed as the endogenous ligands for imidazoline receptors [13]. It has been recently demonstrated that harmane and other β-carbolines interfere with the action of the reactive oxygen species, protecting the nervous system [14] and that this behaviour is due to their antioxidative properties [15]. The analysis of β-carboline alkaloids can be carried out by HPLC with fluorimetric [16,17], UV–Vis spectrophotometric [18] and mass spectrometry detection [19]. Since harmine can be a metabolite of harmane [11], their separation is of interest. However, the application of conventional HPLC techniques to this separation has frequently proved difficult and micellar electrokinetic chromatography has been proposed as an alternative [20].

Naturally occurring and synthetically modified cyclodextrins have found relevant applications in analytical chemistry [21,22]. The formation of the inclusion complexes between cyclodextrins and the analytes increases the solubility and the stability of the latter and therefore enhances the sensitivity of the detection of these analytes by spectroscopic techniques. For the luminescence based techniques [23], an increase in the fluorescence emission intensity or in the fluorescence quantum yield is produced as a consequence of the complex formation. This is the case for several flavonoids [24,25]. The forces involved in the cohesion of the inclusion complexes hamper some of the torsional modes and the free rotation of the guest analytes so that for the excited states, the radiationless deactivation processes are notably decreased. In these conditions, fluorescence quantum yields increase. Furthermore the restricted access to the analyte of quenchers present in the solution may promote room temperature phosphorescence [26–28].

Natural or chemically modified β-cyclodextrins bound to the stationary phases, in chromatography, allow the resolution of both geometrical and optical isomers. Chemically modified cyclodextrins enhance the chiral recognition of the analytes due to their flexibility and to the enlargement of the effective length of their cavities. Naphtylethylcarbamoxylated-β-cyclodextrin [29] or sulfated-β-cyclodextrin [30] have been used as chiral stationary phases in HPLC. Methyl- and propyl-β-cyclodextrin derivatives have been used in the separation of pharmaceutically interesting compounds [31]. Neutral [32] and charged [33] derivatives of cyclodextrins were used for chiral separations in capillary zone electrophoresis (CZE). The differences in the inclusion complex association constants for isomers or compounds with close chemical structures is the base of the discrimination and separation of this kind of compound. In the case of harmane and harmine both are compounds with a β-carboline structure that differ in the methoxy group on the C-7 position of the β-carboline ring as can be observed in Fig. 1. The present paper describes the association characteristics of the inclusion complexes of harmane and harmine with β-cyclodextrin (β-CD) and modified β-cyclodextrins such as hydroxypropyl-β-cyclodextrin (HPβ-CD), 2,3-di-*O*-methyl-β-cyclodextrin (DMβ-CD) and 2,3,6-tri-*O*-methyl-β-cyclodextrin (TMβ-CD). The association constants obtained for the different complexes showed a weak interaction between the host and the guest molecules. The differences in the values obtained varied depending on the experimental variable chosen for the determination. The magnitude of the association constants for two guest molecules and the same type of CD allows discrimination of these compounds on the basis of the geometrical characteristics of the inclusion complexes and therefore CDs could be used as an additive to the mobile phases in chromatography in order to increase the selectivity of the separation and the sensitivity of the fluorimetric detection.

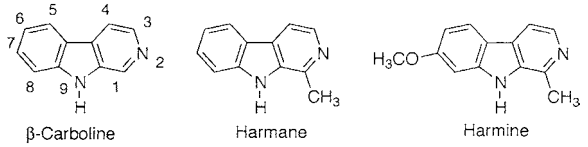


Fig. 1. Chemical structures of the β-carboline derivatives studied.

2. Experimental

2.1. Apparatus and reagents

UV–Vis absorption spectra were obtained with a Kontron Uvikon 810 spectrophotometer. Uncorrected fluorescence excitation and emission spectra were recorded with a Perkin–Elmer MPF-2A spectrofluorimeter. In both cases quartz cells with a path length of 1 cm were employed. A thermostated water bath with multimagnetic stirring was used to prepare the inclusion complexes. Dialysis equilibrium was carried out with Spectra/Por dialysis tubing MWCO 500 which allows the free movement of the molecules with molecular weights below 500 g mol^{-1}.

All the reagents and solvents employed were of analytical or spectroscopic grade. Water was doubly distilled prior to its use. Harmine and harmane were purchased from Sigma. β-CD was obtained from Merck and HPβ-CD was a generous gift from Rhône-Poulenc. DMβ-CD and TMβ-CD were obtained from Sigma.

2.2. Procedures

2.2.1. General procedure for preparing the inclusion complexes β-carboline/CDs

The inclusion complexes were prepared as described in Ref. [6]. Ethanolic solutions of harmane or harmine (1×10^{-2} M) were freshly prepared and appropriate volumes of these solutions were placed in a round-bottomed flask. The solvent was then evaporated at room temperature under reduced pressure, leaving a thin film of harmane in the bottom. Adequate volumes of the HPβ-CD in buffered aqueous solutions (0.2 M KH_2PO_4 with the desired volume of 0.2 M NaOH to obtain a pH ≅ 7.2–7.9) were added and magnetically stirred for 24 h in a water bath at room temperature (22 °C). The final concentrations of the harmine or harmane in the solution was $1.0 \times$

10^{-6} M and the concentration of CDs were 1.0×10^{-2} M.

2.2.2. Experimental procedure for the stoichiometric determinations

For the stoichiometric calculations the continuous variation method (Job's Method) was employed [34]. The concentrations of β-carboline and CD were changed to give a final constant value of 1×10^{-2} M, i.e. [β-carboline]+[CD] = 1.0×10^{-2} M. The solutions were filtered using Millipore 0.22 μm. The fluorescence spectra of the filtered liquors and those of the solutions obtained from the dissolution of the filtered solid were recorded. In other series of experiments the solutions of the complexes prepared according to the above mentioned procedure were dialysed during 24 h in 10 ml of buffered aqueous solution (0.2 M KH_2PO_4 with the desired volume of 0.2 M NaOH to obtain pH ≅ 7.2–7.9). In this case the free guest molecules were dialysed and the complexes remained inside the dialysis tube.

The experiments were carried out at least in duplicate sets.

2.2.3. Experimental procedure for the association constants determination

For the calculation of the association constants, inclusion complexes with different CDs were prepared as described in Section 2.2.1. The concentrations of harmine or harmane were fixed at 1×10^{-6} M and the concentration of β-CD changed from 1×10^{-3} M to 1×10^{-2} M. After the complex formation the fluorescence intensity was measured at the excitation wavelengths corresponding to the neutral and cationic forms of harmine and harmane. The experimental data obtained were used for calculating the corresponding association constants of the inclusion complexes according to the Benesi–Hildebrand treatment [35] (see Appendix A). For the guest molecules a blank was prepared without CD, using the same procedure employed for the inclusion complexes. These blanks were also considered in the calculations and to obtain better values. The experiments were carried out at least in duplicate sets.

3. Results and discussion

The changes induced in the absorption and fluorescence emission spectra of harmane and harmine in the presence of different cyclodextrins were used to calculate the association constants and stoichiometry of the β-carboline derivatives/CD complexes. For this kind of heterocycles many experimental difficulties are found because of the existence of acid–base equilibria in ground and excited states [2]. Thus at pH values close to the pK_a of harmane (7.7) or harmine (8.0) [3], only the emission corresponding to the cationic forms at 430 nm for harmane and 418 nm for harmine should be observed. The excitation of the neutral species detected by the UV–Vis absorption spectra leads to the formation of the corresponding cations of harmane or harmine by a rapid proton transfer with the solvent. However, inclusion into cyclodextrin cavities hampers the proton transfer in the excited states and consequently the emission corresponding to the neutral form (360, 380 nm for harmane and 350, 370 for harmine) [4] can be detected together with changes in the spectral shape [6] as in ethanolic solution. This behaviour can be appreciated in Fig. 2 and can be explained considering that the CD cavities provide a microenvironment with a polarity similar to that of ethanol or other alcohols [36,37]. The spectral curve 3 in Fig. 2 corresponds to the harmane/HPβ-CD inclusion complex. The maxima at 360 and 380 nm as in the case of ethanolic solutions, confirm the inclusion process. The emission band corresponding to the cationic form is also present, but it is shifted to 420 nm with regard to the characteristic emission of the cationic form at 430 nm present in buffered aqueous solutions at pH 7.2–7.9. The emission intensity corresponding to the neutral and cationic bands in cyclodextrin solutions are very close and therefore the spectral changes confirm the existence of the inclusion complexes for both neutral and cationic species. Fig. 3 shows the UV–Vis absorption spectra of the solutions of the complexes of harmane and different cyclodextrins. These spectra correspond to the liquors inside the dialysis tube, where the inclusion complexes (but not the free molecules) are present. The inclusion into the different cyclodextrins can

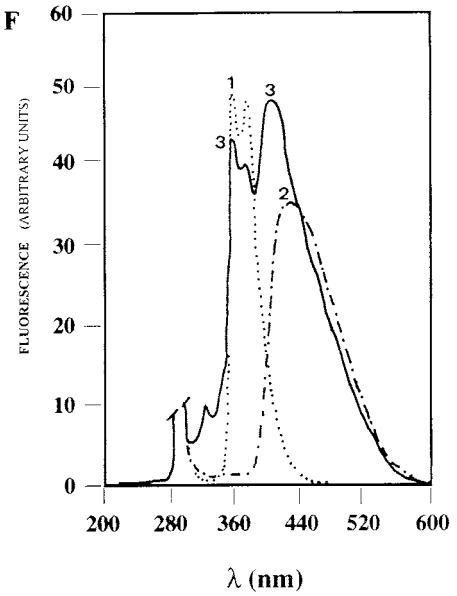


Fig. 2. Fluorescence emission spectra of different solutions of harmane(1×10^{-6} M) in: (1) ethanol, (2) buffered aqueous solution (0.2 M KH_2PO_4+0.2 M NaOH, pH 7.8), (3) harmane HPβ-CD complex, ([HPβ-CD] = 1×10^{-2} M). F: fluorescence intensity in arbitrary units, λ: wavelength, nm.

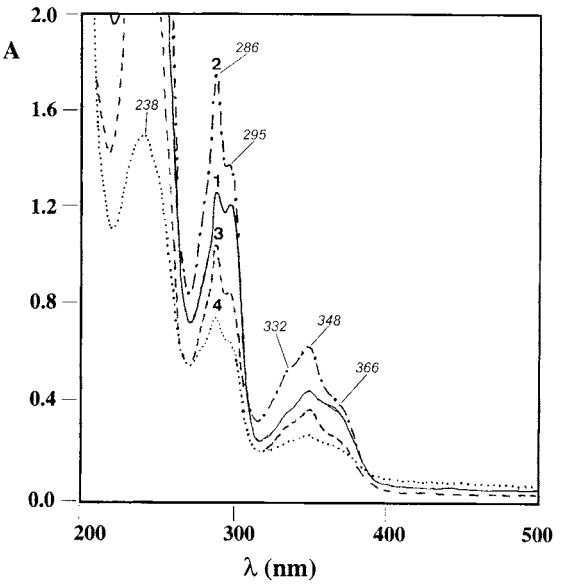


Fig. 3. UV–Vis absorption spectra of the dialysed solutions (liquors from inside the dialysis tube) corresponding to the complexes of harmane (1.0×10^{-5} M) with different cyclodextrins. (1) β-CD, (2) DMβ-CD, (3) TMβ-CD, (4) γ-CD. The concentrations of the cyclodextrins were 5×10^{-3} M. A: absorbance, λ: wavelength, nm.

also be detected by UV–Vis spectrophotometry because the maxima corresponding to the neutral form (240, 288, 300, the shoulder at 334 and 348 nm for harmane as can be seen in Fig. 3, and 240, 298, 326 and the shoulder at 388 nm for harmine) are present [4]. The shoulder which appears at 366 nm for harmane and at 362 nm for harmine can be attributed to the cationic species [4]. Considering the pK_a value of these compounds, at the working pH value (7.2–7.9) neutral and cationic forms coexist in the ground state but the spectral shape corresponds to the neutral species and also is better resolved than in the case of buffered aqueous solutions. This can be explained considering the effect of the cyclodextrins as "organized media" where the compartmentalization of the included species is favoured [23].

The effect of the addition of increasing amounts of CD on the emission spectra of harmane can be observed in Fig. 4. Initially (curve 1 in Fig. 4) the cationic band (430 nm) predominates with regard to the emission corresponding to the neutral form (360, 380 nm). However, when CD concentrations were increased the emission of both bands was increased and also the emission of the cationic form was shifted from 430 to 420 nm. The absence of an isoemissive point demonstrates the existence of different species in the equilibria and that the neutral/CD species arises not only from neutral harmane but also the cationic harmane which are present in the buffered aqueous solutions. It also indicates the existence of the proton transfer reaction in the excited state for the harmine or harmane inclusion complexes as can be seen in Scheme 1. It could be considered that the neutral form is included at this pH value in the ground state and that the pyridinic nitrogen is oriented outside of the cavity, a feasible mode of inclusion considering that the pyridine ring is the most polar moiety of the molecule; this orientation has been confirmed by ^{1}H-NMR studies [38]. In such an arrangement the proton transfer reaction in excited states for the included neutral harmane or harmine is possible, but this reaction is clearly more difficult in comparison with the homogeneous buffered solutions. The presence of CDs

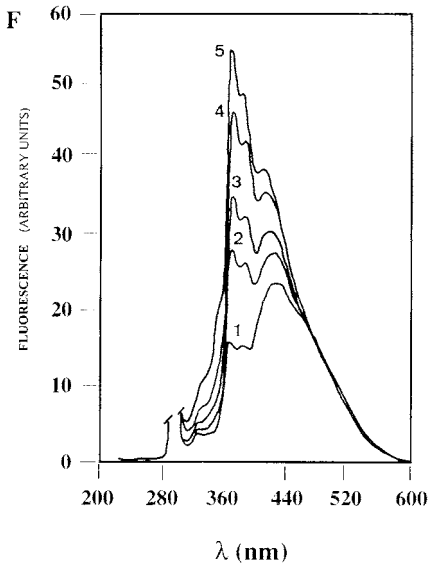
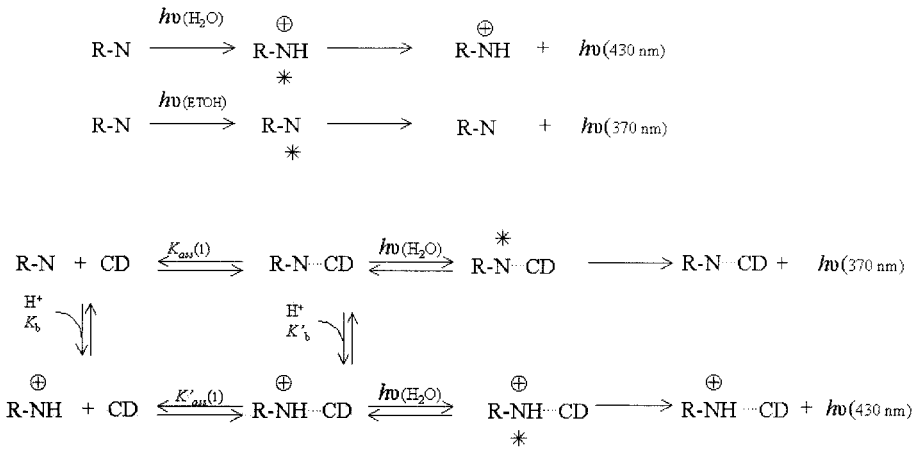


Fig. 4. Effect of the addition of increasing concentrations of HPβ-CD on the fluorescence spectra of harmane in buffered aqueous solutions (pH 7.8). The solutions were prepared according to the procedure of preparation of the inclusion complexes. [Harmane] = 1.0×10^{-6} M, [CD] (1) 2×10^{-3} M, (2) 6×10^{-3} M, (3) 1×10^{-2} M, (4) 2×10^{-2} M, (5) 4×10^{-2} M.

favours the inclusion of cationic and neutral forms but it hampers the protonation of pyridinic nitrogen in the excited state. Similar changes in the acid–base properties of different compounds as a consequence of the inclusion process have been reported [39] with notable changes in the pK_a values.

Due to the different CD complexation equilibria and proton transfer reactions in the excited state, the calculation of the stoichiometry and association constants with the different CDs presents many experimental difficulties. For the stoichiometry calculations, the Job's continuous variation method was employed. The concentrations of β-carboline derivative and CD were changed in order to obtain a final constant value of [CD]+[harmane] = 1.0×10^{-2} M and [CD]+[harmine] = 1.0×10^{-2} M. When the concentration range was 1.0×10^{-3} M, the formation of the complexes was not clearly observed because the emission of the cationic band overlapped with the emission corresponding to the neutral form. Considering the concentration range, the solutions have a turbid appearance possibly due to the existence of insolubilized harmane or harmine, or the harmane/CD or harmine/CD complexes and therefore the spectrophotometric measurements of these solutions produced a bad correlation. For this reason the complex solutions were filtered (Millipore 0.22 μm) and the UV–Vis absorption spectra of the filtered liquors were recorded, together with the solutions obtained by dissolving the filtered solid in an appropriate volume of buffered solution. All of the complexes prepared for this assay were adjusted at pH 11.0 in order to



Scheme 1. Different species and equilibria imply in the β-carboline derivatives–CD complexation, and proton transfer reaction in ground and in excited states.

ensure the shift of the acid–base equilibria to the neutral form in the ground state. The filtered solutions show stoichiometry ratios 2:1 CD:β-carboline in all CDs studied. However the solid obtained and dissolved in aqueous solution at pH 11 shows a stoichiometry ratio 1:2 CD:β-carboline indicating that the precipitates correspond to the free molecules of harmane or harmine which have not been included. Finally, the spectrofluorimetric determination of the stoichiometry was carried out at pH 7.8 because at pH 11 the zwitterionic emission band predominates with regard to the neutral emission band. In order to enhance the quantitative measurements a dialysis procedure was applied to achieve the separation of the complexes from the free β-carboline derivative. As can be seen in Fig. 5 the UV–Vis absorption spectra show the difference between the complexes and the free β-carboline derivative present in the dialysed liquors. Thus in the case of the solution that remains inside the dialysis tube a notable increase in the absorption band is produced as a consequence of the existence of the inclusion complexes. A similar behaviour was detected for the fluorescence emission of the neutral band (Fig. 6) and therefore the values of the fluorescence intensity for the liquors inside the dialysis tube were considered for the calculations. The spectra of the liquors outside the dialysis tube exhibited only the cationic band corresponding to the free β-carboline in homogeneous buffer solution at neutral pH. Fig. 7 shows the results of the stoichiometry corresponding to the complexes for DMβ-CD and TMβ-CD, the same behaviour being observed for all cyclodextrins studied. The maximum for the polynomial treatment corresponds to a 0.4 molar ratio harmane/CD or harmine/CD. These data allow to consideration that in solution complexes with 1:1 and 1:2 harmane/CD or harmine/CD stoichiometry coexist. It is important to remark that the dialysis process removes the free harmane or harmine from the solution.

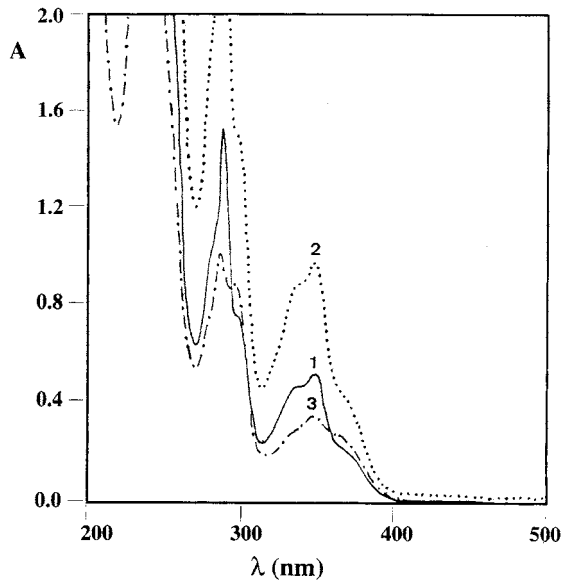


Fig. 5. UV–Vis absorption spectra of the dialysed solutions harmane/HPβ-CD (liquors from inside the dialysis tube). Complexes prepared for the stoichiometry calculations. (1) [harmane] = 1×10^{-3} M, [CD] = 9×10^{-3} M, (2) [harmane] = 5×10^{-3} M, [CD] = 5×10^{-3} M, (3) [harmane] = 9×10^{-3} M, [CD] = 1×10^{-3} M. A: absorbance, λ: wavelength, nm.

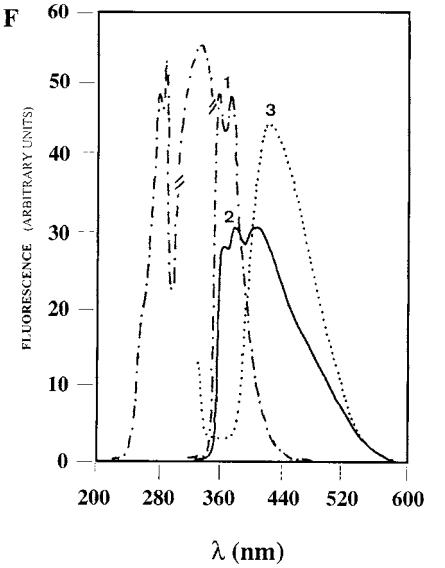


Fig. 6. Fluorescence excitation and emission spectra of the dialysed solutions harmane/HPβ-CD. Complexes prepared for the stoichiometry calculations. (1) Ethanolic solution of harmane (1×10^{-6} M). (2) Harmane HPβ-CD complex (5×10^{-3} M), liquors inside dialysis tube. (3) Harmane HPβ-CD complex (5×10^{-3} M), liquors outside dialysis tube. F: fluorescence intensity in arbitrary units, λ: wavelength, nm.

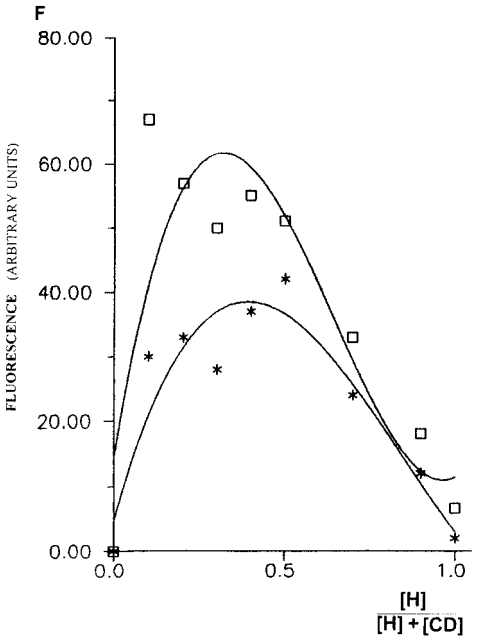


Fig. 7. Determination of the stoichiometry of the complexes by the continuous variation method from the fluorescence data. * harmane/TMβ-CD complex. □ harmine /DMβ-CD complex.

The tendency of a guest molecule to be included inside the cyclodextrin cavities is assessed by the affinity constants. Many instrumental techniques and classical methods can be used to evaluate the values of these constants [40,41]. In the present study the fluorescence was the variable employed. The concentration range of cyclodextrin ensures the inclusion of the guest molecules and makes it possible to detect the neutral band of the harmine or harmane without the need for a dialysis procedure. The double reciprocal treatment shows good correlation coefficients ($r = 0.90–0.99$) for the variables $1/F$ against $1/C$. In the cases where the correlation coefficients are close to 0.90 and considering the results of the stoichiometry study, we also tested the behaviour for the complexes with 1:2 (harmane/CDs) stoichiometry, the variables being $1/F$ against $1/C^2$; however, the correlation coefficients were below 0.90. Other authors consider a non-linear regression treatment more appropriate [42] for the calculation of the association constants because in the "classical" double reciprocal treatment the lower concentrations are more significant compared with the higher concentrations of the host molecules.

Table 1 shows the stability constant values obtained for the different complexes. For all guest molecules a blank was prepared without CD, using the same procedure as for the inclusion complexes. The emission fluorescence intensity of these blanks was considered for the calculations (F_0). The experiments were carried out in at least duplicate sets. For these calculations we considered the absolute variable F (fluorescence intensity) and also the ratio, R, of the emission intensity corresponding to the emission band of the neutral species and the emission band of the cationic species. In both cases important differences in the association constants can be observed if the emission corresponding to the blank solution is not considered ($1/F$ or $1/R$), and this behaviour results of the significance of the emission corresponding to the free guest molecules.

The values obtained for the association constants are similar to those obtained for other related heterocycles, e.g. indole where the association constants for β-CD vary from 142 to 236 depending on the temperature [43]. In different CDs studied the association constants obtained for harmane are higher than those obtained for harmine except for the complexes harmine/TMβ-CD. Considering that the polarity properties are similar for both compounds, the geometrical and steric interactions must play an important role in the inclusion processes. Thus in case of harmine the presence of the methoxy group on the position 7 of the heterocycle hampers the penetration of the guest molecule inside the cyclodextrin. The complexation through the pyridine ring is difficult due to the presence of a methyl group on position 2 and also by the presence of the basic pyridinic nitrogen with higher polarity that the benzene ring. Thus the hydrophobic part of the molecules corresponding to the benzene ring interacts with the CD cavity and partially penetrates inside it. This type of interaction is more difficult in the case of harmine due to the presence of methoxy group attached to the benzene ring and therefore the interaction should be weaker than for the complexes of harmane with different cyclodextrins

Table 1
Association constants obtained for harmane/CDs and harmine/CDs complexes

Inclusion complex	K			
	$1/F$	$1/F-F_0$	$1/R$	$1/R-R_0$
Harmane/HPβCD	602±46 (0.9676)	418±30 (0.9786)	1144±135 (0.9090)	745±66 (0.9527)
Harmine/HPβCD	353±14 (0.9953)	291±7 (0.9982)	346±21 (0.9897)	218±6 (0.9987)
Harmane/βCD	345±42 (0.9501)	*	364±41 (0.9559)	*
Harmine/βCD	201±14 (0.9923)	*	188±20 (0.9836)	*
Harmane/DMβCD	207±50 (0.9069)	116±42 (0.9221)	356±31 (0.9737)	213±22 (0.9811)
Harmine/DMβCD	148±27 (0.9687)	112±22 (0.9775)	254±5 (0.9991)	199±6 (0.9990)
Harmane/TMβCD	121±29 (0.9609)	23±14 (0.9886)	58±8 (0.9967)	*
Harmine/TMβCD	195±14 (0.9923)	208±34 (0.9529)	148±13 (0.9927)	28±7 (0.9972)

F: fluorescence intensity in arbitrary units, at the emission wavelength of the neutral form. F_0: fluorescence intensity in arbitrary units corresponding to the blank solution. R: ratio of the fluorescence emission intensity corresponding to the emission bands of the neutral and cationic forms. R_0: ratio of the fluorescence emission intensity corresponding to the emission bands of the neutral and cationic forms for the blank solutions. The correlation coefficients appears in parentheses.

* The data did not allow a satisfactory computation of the values.

studied. The strongest interactions are produced in the case of HPβ-CD and can be explained considering that the HPβ-CD is flexible compared to the β-CD cavity allowing the fit of guest molecules. In the case of TMβ-CD the association constants are lower, which can be explained considering that the methyl group on position 6 of the ring of the CDs makes difficult the adjustment of the guest and the host molecules. The effective hydrophobic interaction takes place inside the cavity. When the association constant values obtained for the complexes of harmine with the different cyclodextrins are compared (Table 1), the behaviour is similar to that described for the harmane complexes. The association constants are higher for HPβ-CD followed by β-CD, DMβ-CD and TMβ-CD. In the case of harmine/TMβ-CD complexes the constant was higher than for β-CD and DMβ-CD complexes, especially if the variable considered is $1/F-F_0$. The behaviour can be explained considering that TMβ-CD and harmine present a stronger hindrance for the penetration inside the cavity, but harmine can be partially accommodated on the rim of TMβ-CD producing a stable complex with a higher association constant. This behaviour has been described for the complexes with amino acids and α-CD and TMα-CD; thus, with the trimethylated α-CD the association constants are substantially greater than those formed with the parent α-CD [44]. In spite of the fact that complexes with 1:2 and 1:1 carboline may coexist in solution and that in the case of 1:2 complexes the encapsulation of the guest molecules is complete, complexes with 1:1 stoichiometry predominate, as shown by the calculated association constants. In such a case, the hydrophobic part of the molecule interacts with the wider rim of the CD leaving the basic nitrogen outside the cavity and allowing its protonation and therefore the emission band of the cationic species is observed. The equilibria in which these different species (protonated and non protonated harmine or harmane together with the corresponding inclusion complexes) are involved can be summarized in the Scheme 1.

In conclusion two alkaloids with very similar chemical and solubility properties can be molecularly recognized and discriminated by their inclusion in the cyclodextrin cavities. These phenomena should have important consequences on the separation of these compounds by HPLC or CZE, considering specially that the emission of these

compounds depends on the pH of the media and that the presence of cyclodextrins also modifies these acid–base equilibria.

Appendix A

According to Catena and Bright [42], H represents the fluorescent substrate (harmane or harmine) and C represents the cyclodextrin, and the equilibrium can be written:

$$H + C \Leftrightarrow HC \tag{1}$$

The association constant, K can be expressed as:

$$K = \frac{[HC]}{[H][C]} \tag{2}$$

and considering the mass balance expressions:

$$K = \frac{[HC]}{(c_H - [HC])(c_C - [HC])} \tag{3}$$

where c_H and c_C are the analytical concentrations of fluorescent substrate and the cyclodextrin respectively, and considering that $c_C \gg HC$, then:

$$K = \frac{[HC]}{(c_H - [HC])c_C} \tag{4}$$

The fluorescence quantum yield of the complex is given by:

$$\phi = \frac{F_{HC}}{k_{HC}[HC]} \tag{5}$$

where F_{HC} is the fluorescence intensity of the complex and k_{HC} is an instrumental constant. Multiplication of the latter equation by the former yields:

$$\frac{c_H}{F_{HC}} = \frac{1}{Kk_{HC}\phi}\frac{1}{c_C} + \frac{1}{k_{HC}\phi} \tag{6}$$

and dividing by c_H, a reasonable value of K can be obtained from a plot of $1/F_{HC}$ versus $1/c_C$ by simply dividing the intercept by the slope:

$$\frac{1}{F_{HC}} = \frac{1}{Kk_{HC}\phi}\frac{1}{c_H}\frac{1}{c_C} + \frac{1}{k_{HC}\phi}\frac{1}{c_H} \tag{7}$$

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