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Detection and characterization of cyclodextrin complexes with β-carboline derivatives by spectroscopic techniques

L. Martín [a], A. León [a,1], M.A. Martín [a], B. del Castillo [a,*], J.C. Menéndez [b]

[a] *Laboratorio de Técnicas Instrumentales, Sección Departamental de Química Analítica, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain*

[b] *Departamento de Química Orgánica y Farmacéutica, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain*

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Abstract

β-Carboline alkaloids exhibit a great variety of pharmacological activities. The solid inclusion complexes of harmane and harmine with β-cyclodextrin and also with hydroxypropyl-β-cyclodextrin, have been prepared following different procedures. IR and NMR spectroscopies were employed to verify the interaction of the guest molecules with the cyclodextrin cavities. The differences observed in the IR and NMR spectra are in agreement with those described in the literature for other guest molecules. The shifts in the ^{13}C- and ^{1}H-NMR spectra confirm the existence of the inclusion complexes. The fluorescence emission spectra of these complexes dissolved in buffered aqueous solution (pH 7.3) exhibit the characteristic peaks of the cationic form for harmane alkaloids. The neutral bands are not present for the free alkaloids in aqueous solutions. Fluorescence quenching emission of the complexes is compared to that of the corresponding free alkaloids.

Keywords: β-carboline and harmane alkaloids; Cyclodextrin complexes; IR; NMR; Fluorescence spectroscopic techniques

1. Introduction

β-Carboline alkaloids such as harmane and harmine are natural products which are present in a great variety of medicinal plants and also they are endogenously produced in human and animal tissues as a product of secondary metabolism [1]. They possess diverse biological properties such as hypotensive, hallucinogenic or antimicrobial actions [2] and tremorogenesis [3,4]. They are able to bind to benzodiazepine receptors [5] and they have been also proposed as the endogenous ligands for imidazoline receptors [6]. It has been recently demonstrated that harmane and other β-carbolines interfere the action of reactive oxygen species, protecting the nervous system [7] and that this behaviour is due to their antioxidative properties [8].

* Corresponding author. Tel.: +34-91-394-1756; fax: 34-91-394-1754.
E-mail address: mantonia@farm.ucm.es (B. del Castillo).
[1] On leave from Laboratorio de Análisis de Medicamentos, Departamento de Análisis y Control, Facultad de Farmacia, Universidad de Los Andes, Merida, Venezuela.

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The analysis of β-carboline alkaloids can be carried out by HPLC with fluorimetric [9,10], UV–vis spectrophotometric [11] and mass spectrometry detection [12]. Since harmine can be a metabolite of harmane [4], their separation is of interest. However, the application of conventional HPLC techniques to this separation is frequently difficult and micellar electrokinetic chromatography has been proposed as an alternative [13]. Natural and chemically modified cyclodextrins (CDs) have been profusely used to increase the resolution of chromatographic separations [14] and capillary electrophoresis [15]. On the other hand CDs provide an enhancement of the sensitivity in the luminescence techniques for the detection of fluorophores [16,17].

CDs have been used as chiral stationary phases [18] and also as additive to the mobile phase for the separation of aminoacids by CZE [19] and β-adrenolytics [20]. In both cases it is important to know the nature and the strength of the interactions between the CDs and the analytes. Thus, in many cases the enhancement in the separation is a consequence of the formation of inclusion complexes; in other separations the analytes interact with the hydroxy groups oriented outside CD cavities [21]. Many different techniques have been employed to elucidate the nature and the type of the interaction but only NMR is capable to differentiate the part of the analyte molecule involved in the interaction with the CD cavity [22,23].

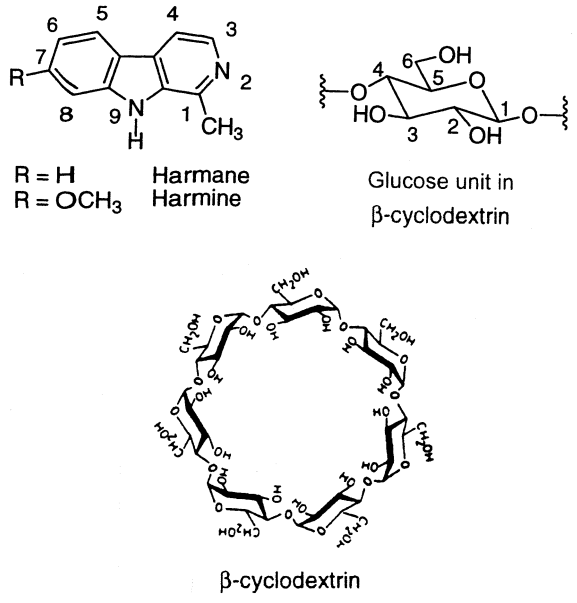


Fig. 1. Chemical structures and numbering of the β-carbolines studied and the βD-glucopyranose units present in the CDs.

In the present work we describe the existence of complexes between β-cyclodextrin (β-CD) and hydroxypropyl-β-cyclodextrin (HPβ-CD) and harmine and harmane (Fig. 1). The behaviour of the inclusion complexes towards the fluorescence quenching by halide ions is different than that of the free β-carboline bases in buffered aqueous solutions. The existence of the complexes is demonstrated by the changes produced in the ^{1}H-NMR and ^{13}C-NMR, IR and UV–vis absorption spectra.

2. Experimental

2.1. Apparatus and reagents

UV–vis 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 1 cm of path length were employed. IR absorption spectra were obtained with a Perkin-Elmer FT-IR, model Paragon 1000. NMR spectra were recorded with a Bruker AC 250 spectrometer (Servicio de Resonancia Magnética Nuclear, UCM). A thermostated water bath with multimagnetic stirring was used to prepare the inclusion complexes.

All the reagents and solvents employed were analytical or spectroscopic grade. Water was doubly distilled prior to use. Harmine and harmane were purchased from Sigma. β-CD was obtained from Merck and HPβ-CD was a generous gift from Rhône–Poulenc. Deuterium oxide was obtained from Aldrich and d_6-DMSO was purchased from SDS. Sodium [4,4-dimethyl-4-silopentane-1-sulfonate] (DSS) used as an internal reference standard for the spectra in D_2O was obtained from Aldrich.

2.2. Procedures

2.2.1. Preparation of the inclusion complexes

Solid complexes were prepared using two different procedures.

2.2.1.1. Procedure 1. The complexes were prepared at stochiometric ratios of 1:1 and 1:2, harmane:CD, as follows. An appropriate amount of harmane was weighed. A 10^{-2} M solution of β-CD or HPβ-CD was prepared in buffered aqueous solution (0.2 M KH_2PO_4 with the desired volume of 0.2 M NaOH to obtain pH ≅ 7.3–7.8). Then the appropriate volume of CD solution was added to the solid harmane and the mixture was magnetically stirred in a water bath at 50 °C during 1 week. The mixture was then cooled to 8 °C and the solid complexes obtained were filtered and washed with ethanol followed by water to eliminate free harmane. Finally, the complexes were freeze-dried during 48 h.

2.2.1.2. Procedure 2. The complexes were prepared in a similar way to the complexes obtained in solution [24]. Thus, ethanolic solutions of harmane (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 aqueous solutions (10^{-1}M), as described in procedure 1, were added and magnetically stirred for 24 h in a water bath at room temperature (22 °C). The fine precipitates thus obtained were freeze-dried during 48 h. The final concentration of harmine or harmane was 1.10^{-3} and 1.10^{-1} M for HPβ-CD.

The complexes obtained in the procedures 1 and 2 were dissolved in buffered aqueous solutions (0.2 M KH_2PO_4 with the desired volume of 0.2 M NaOH to obtain pH ≅ 7.3–7.5) giving a CD concentration of 10^{-2} M). These solutions were employed for obtaining the UV–vis absorption and fluorescence spectra.

These complexes were also ground with dry KBr (0.5+99.5, complex+KBr, w/w) and compressed at 10 kg cm^{-2} for 5 min; the pellets obtained were used to record the IR absorption spectra. A reference physical mixture of harmane+CD was prepared at the adequate proportions of host and guest compounds in order to verify the changes obtained in the IR spectra.

The complexes obtained in procedures 1 and 2 were dissolved in d_6-DMSO or deuterium oxide containing a trace of DSS at a concentration 1.10^{-2}M for β-CD and 1.10^{-1} M for HPβ-CD in order to obtain the ^{1}H-NMR and ^{13}C-NMR spectra. When the spectra were obtained in deuterium oxide, phosphate buffer was used to adjust the pH value of the solution at 7.5–7.8.

2.2.2. Fluorescence quenching study

The complexes obtained in procedures 1 and 2 were dissolved in aqueous buffered solutions (0.2 M KH_2PO_4 with the desired volume of 0.2 M NaOH to obtain pH ≅ 7.5–7.8) and different aliquots of 4 ml from these solutions were taken. Appropriate volumes of KBr and NaCl aqueous solutions were added to obtain a constant ionic strength of 1.0 M. The final volumes were 4 ml of the complex solutions and 500 μl combined volume of KBr and NaCl solutions. The concentration of bromide ion in these solutions varied from 1.10^{-3} to 0.1 M.

3. Results and discussion

Although a variety of spectroscopic techniques can be successfully used to detect the formation of the inclusion complexes with CDs, the most direct evidence for the inclusion of a guest molecule is obtained by means of different NMR techniques. Thus UV–vis absorption and fluorescence spectroscopies are adequate for measuring the complexation energies and association constants of the complexes, but they usually provide only qualitative information about inclusion modes and geometries [23]. Nevertheless, the sensitivity range of these techniques is different and it is necessary to prepare the samples considering the measurement technique employed. Besides, reactions (proton transfer) in excited states are usually detected by fluorimetry and therefore the information obtained by the use of different techniques should be complementary. Another point to be considered

is that the way of preparing the inclusion complexes depends on the sensitivity of the technique. Thus, for the detection of the complexes by fluorescence a concentration of 1.10^{-4} to 1.10^{-6} M of the guest fluorophore molecule is employed, but this range of concentration is not detected by NMR or IR spectroscopy. For these reasons we assayed two experimental procedures to obtain the solid complexes of β-carboline alkaloids and CDs. Procedure 2 is the same described for studying the complexes in solution but the final concentration of the alkaloid is higher than for the complexes in solution [24].

Fig. 2 shows the IR absorption spectra of the solid complex harmane/HPβ-CD, obtained using procedure 1. The spectra of the complexes (Fig. 2) with 1:1 and 1:2 stoichiometry were compared with those of free native HPβ-CD and the physical mixture. Unfortunately, the characteristic N-H band (3000–3500 cm^{-1}) from the heterocycle is completely overlapped with the broad band of the hydroxyl groups of the CD. On the other hand, the bands at 1500 and 1560 cm^{-1}, which correspond to the C=C stretching vibrations of harmane, are not overlapped by the CD and this allows to detect shifts of approximately 1 cm^{-1} with regard to the spectra of pure harmane. The physical mixture shows shifts of 0.5 cm^{-1} or less. The intensity of these bands does not significantly increase for the complexes with 1:1 harmane/CD stoichiometry. The changes detected in the IR spectra for the complexes obtained according to the procedures 1 and 2 are very similar. For the complexes harmane/β-CD, harmine/β-CD and harmine/HPβ-CD the behaviour observed is close to that described in Fig. 2. Although these changes are small, they can be considered as proof of the formation of a harmane/CD complex.

The ^{1}H-NMR spectra were obtained in d_6-DMSO and also in deuterium oxide for compar-

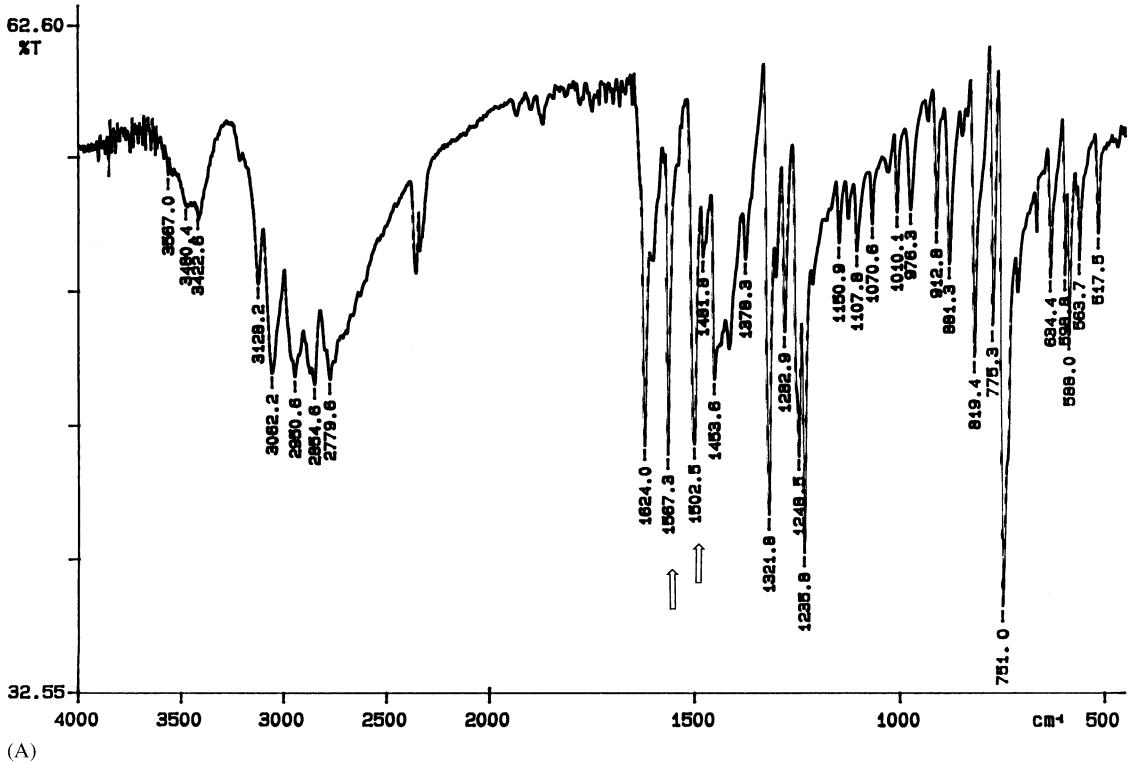
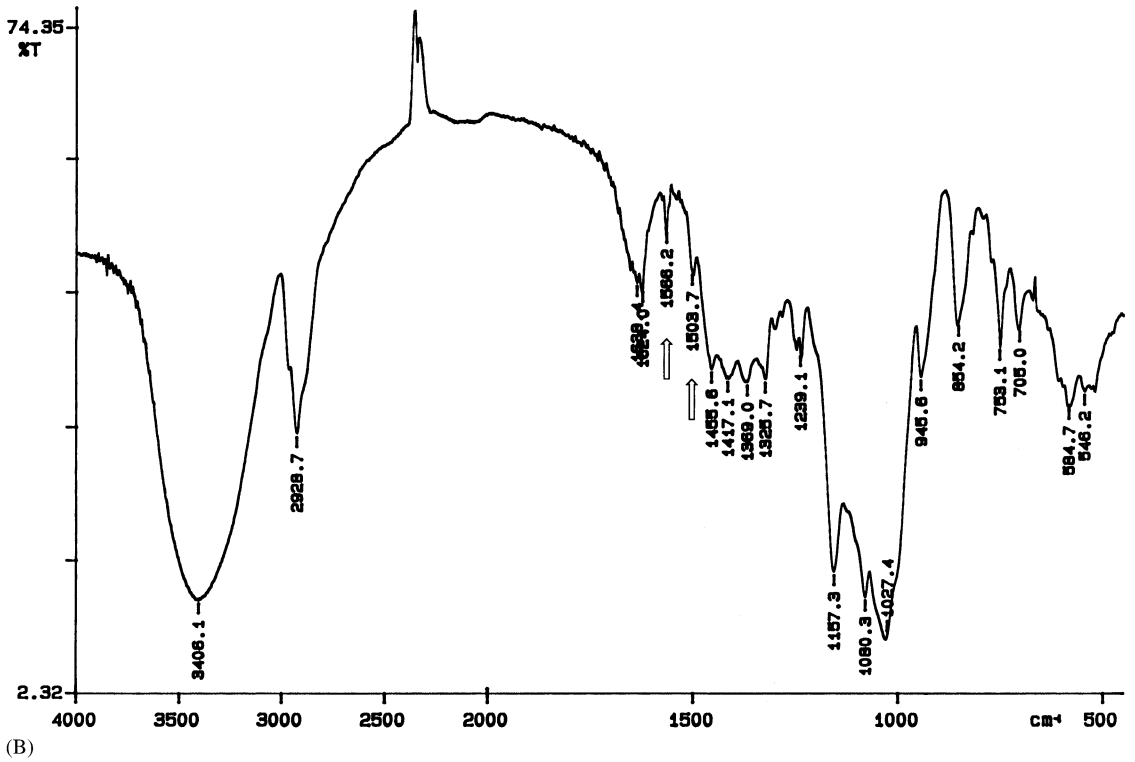


Fig. 2. FT-IR absorption spectra of (A) harmane in KBr (0.5%, w:w) and (B) harmane/HPβ-CD (1:2) complex (procedure 1) in KBr (0.5% w:w).



(B)

Fig. 2 (*Continued*)

ison purposes. Owing to the limited solubility of the complexes in D_2O, ^{13}C-NMR spectra could only be registered in d_6-DMSO. Significant changes were observed for both ^{13}C- and ^{1}H-NMR when the spectra in DMSO of the complexes and the corresponding CDs (β-CD or HPβ-CD) were compared, while the changes observed in deuterium oxide were smaller.

Fig. 3 shows a comparison of the ^{13}C-NMR spectra of harmane/HPβ-CD complex and the free HPβ-CD. Experimental NMR data for HPβ-CD are scarce, possibly due to the presence of mixtures of several species with the hydroxypropyl group at O-2, O-3 or both positions, which prevents a precise assignment of all signals. Nevertheless, when the spectra of the complexes were compared with those of the free CD, shifts could be appreciated for the signals corresponding to the carbons C_2, C_3 and C_5, which appear between 70 and 75 ppm (Table 1). A similar behaviour was observed for the complexes with β-CD, which were obtained by procedure 1 (Table 2). The alterations found in the ^{13}C-NMR spectra prove the existence of complexes, but ^{1}H-NMR affords more significant information concerning the moieties involved in the interaction of the guest and the host molecules.

Fig. 4 shows the ^{1}H-NMR spectra obtained in DMSO for the inclusion complexes of β-CD/harmane (1:1) (procedure 1) and also for β-CD, where notable changes in the spectral shape of the region between 3.5 and 3.2 ppm can be observed. This region corresponds to the signals due to the H_4 and H_2 protons, which are oriented outside the cavity of the CD in the secondary hydroxylic rim of the CD. The changes in the chemical shifts are presented in Table 3. Also, a significant variation of the J_{21} coupling constant was observed (Table 4), indicating that the CD cavity is deformed as a consequence of the host-guest interactions. Considering also that the signals corresponding to the protons H_3 and H_5 are not significantly altered, it

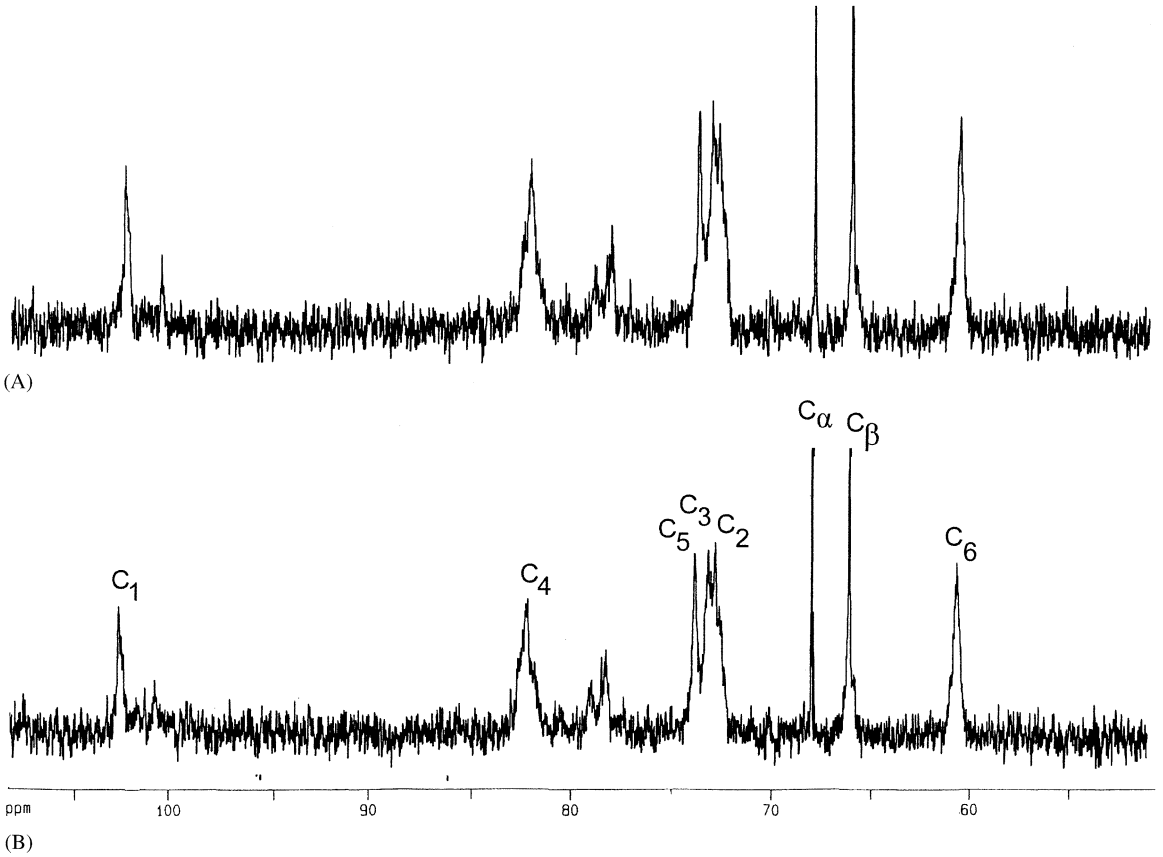


Fig. 3. ^{13}C-NMR spectra of (A) the harmane/HPβ-CD (1:100) complex obtained according to the procedure 2, and (B) the HPβ-CD. The spectra were obtained in d_6-DMSO and the spectral region corresponds to the signals of the CD carbons.

can be concluded that harmane or harmine molecules interact with the protons outside the CD cavity. The same conclusion can be drawn by examination of the region between 5.8 and 4.4 ppm, corresponding to H-1 and the three hydroxyl

Table 1
^{13}C chemical shifts in d_6-DMSO of HPβ-CD and changes observed for the complexes harmane/HPβ-CD ($\Delta\delta_1$) and harmine/ HPβ-CD ($\Delta\delta_2$)

Signal	δ_{CD}	$\Delta\delta_1$	$\Delta\delta_2$
C-1	102.254	0.002	−0.030
C-2	72.387	−0.019	−0.030
C-3	72.692	0.013	0.023
C-4	81.731	0.092	0.104
C-5	73.371	−0.012	0.000
C-6	60.237	−0.041	−0.046
C-α	67.511	−0.003	–
C-β	65.650	−0.004	–
C-γ	20.186	0.002	–

Both complexes were obtained by procedure 2.

Table 2
^{13}C-NMR, chemical shifts in d_6-DMSO of β-CD and changes observed for the complexes harmane/β-CD (1:1) and harmane/ β-CD (1:2)

Signal	δ	$\Delta\delta$ (CD/Harmane; 2:1)	$\Delta\delta$ (CD/Harmane; 1:1)
C-6	60.162	0.011	0.012
C-2	72.301	0.005	−0.024
C-3	72.662	0.008	−0.017
C-5	73.318	−0.001	−0.043
C-4	81.776	−0.008	0.019
C-1	102.205	0	−0.029

Both complexes were obtained by procedure 1.

signals (C_2-OH, C_3-OH and C_6-OH). Decoupling of the hydroxy hydrogens is observed in the complex, showing the existence of fast proton exchange, probably associated with the alteration of the hydrogen-bonding network at the wider rim of the CD. Accordingly, the most significant

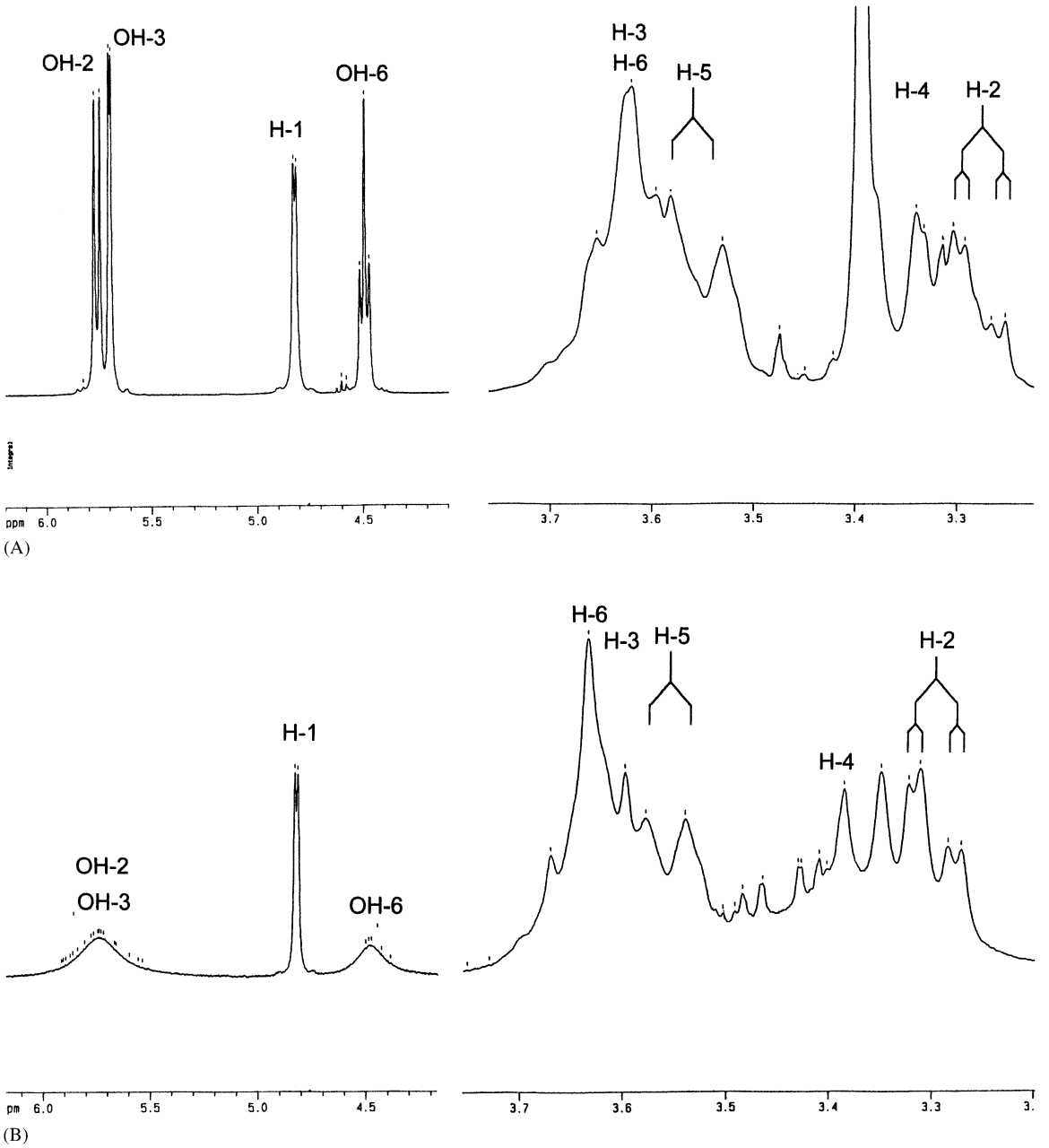


Fig. 4. Expansions of the: (A) ^{1}H-NMR spectrum of β-CD and (B) ^{1}H-NMR spectrum of the complex harmane/β-CD (1:1), obtained according to procedure 1. The spectra were obtained at 250 MHz in d_6-DMSO. The spectral region corresponds to the CD protons both on carbon and oxygen.

Table 3
^{1}H-NMR, chemical shifts in d_6-DMSO of β-CD and changes observed for the complex harmane/β-CD (1:1)

	OH-2	OH-3	OH-6	H-1	H-2	H-3	H-4	H-5	H-6
β-CD	5.759	5.698	4.494	4.822	3.279	*	3.342	3.557	*
Complex	5.781	5.781	4.491	4.827	3.296	*	3.384	3.558	*
$\Delta\delta$	0.022	0.183	−0.003	0.005	0.017	*	0.042	0.001	*

This complex was obtained by procedure 1.

Table 4
Coupling constants (Hz) measured in the ^{1}H-NMR spectrum of the 1:1 harmane β-CD complex (procedure 1)

	H-2		H-4	H-5	H-1
	J_{23}	J_{21}	$J_{45}=J_{43}$	J_{54}	J_{12}
β-CD	9.3	3.5	9.5	9.5	3.5
Complex	9.4	3.0	9.0	9.5	3.3

The coupling constant values for β-CD are taken from Ref. [23].

alterations in the chemical shifts correspond to the secondary hydroxyls C_2-OH ($\Delta\delta = 0.022$ ppm) and C_3-OH ($\Delta\delta = 0.183$ ppm). These changes are similar to those found for the complexes obtained according to procedure 1 but with a 1:2 stoichiometry ratio harmane:CD.

The changes in the protons corresponding to the guest molecules (harmine or harmane) were also determined. Only slight changes in the chemical shifts were observed when the data for the heterocyclic portions of the complex and the free guest molecule were compared. Thus $\Delta\delta = -0.005$ ppm for the proton corresponding to the pyrrolic nitrogen (N_9–H) and 0.001 ppm for the protons corresponding to the methyl substituent on the position 1 of harmane. However, for the aromatic protons placed at the C-6 position of harmane $\Delta\delta = 0.025$ ppm. Similarly, the most significant change in the ^{13}C-NMR chemical shifts for the harmane carbons corresponded to C-7 ($\Delta\delta = 0.050$ ppm). These observations can be explained considering that the hydrophobic part of the molecule, corresponding to the benzene ring, interacts with the CD cavity and partially penetrates inside it, but the most polar part of the molecule corresponding to the pyridine and pyrrolic rings remains outside the cavity (Fig. 5).

In the case of the complexes obtained by procedure 2, HPβ-CD /harmane and HPβ-CD / harmine with a molar ratio 100:1, the spectral changes detected by NMR are small due to the low amount of the guest molecule with regard to the CD. The percentage of free molecules of CD in solution is greater compared to the complexes obtained by procedure 1 and therefore the spectra resemble those of the free CD. The spectral changes for the complexes obtained by procedures 1 and 2 and measured in deuterium oxide are very slight due to the low solubility of the complexes.

In order to compare the results obtained for the solid complexes and the complexes obtained in solution according to the procedure described in Ref. [24], where the complexation is favoured due to the large excess of CD molar concentration (1:10 000, harmane:CD), the UV–vis absorption and fluorescence spectra were obtained. Fig. 6 shows the absorption spectra of the solid complexes obtained by procedures 1 and 2 as well as for harmane dissolved in buffered aqueous solution (pH 7.3–7.5). The spectra correspond to the neutral form, which is present at this pH value in the ground state. No significant changes are detected for the complexes with regard to the reference harmane. It is well known that the β-carboline ring exhibits singular luminescence properties [25] and that the proton transfer in the excited state is very fast [26,27]. For this reason, in

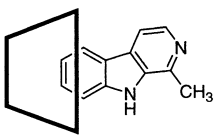


Fig. 5. Proposed model for the harmane-CD complexes.

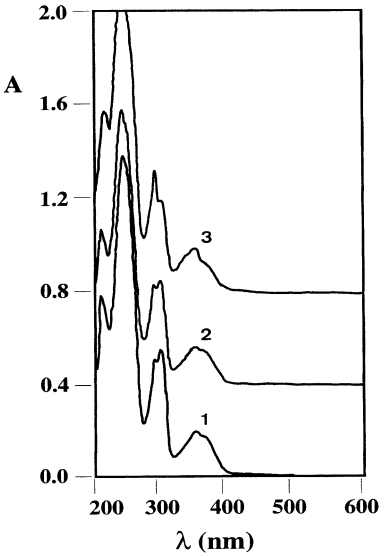


Fig. 6. UV–vis absorption spectra of: (1) harmane in buffered aqueous solution; (2) complex harmane/β-CD (1:2), obtained by procedure 1; (3) complex harmane/β-CD (1:100), obtained by procedure 2. The spectra are shifted in order to avoid overlapping.

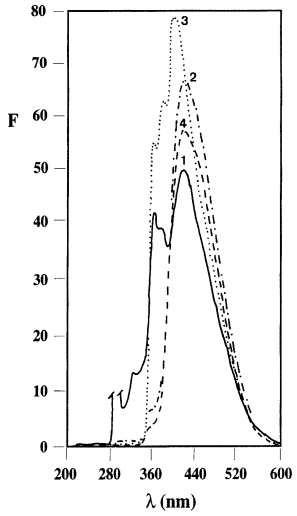


Fig. 7. Uncorrected emission spectra of the following complexes in buffered aqueous solutions (pH 7.3–7.5): (1) harmane/HPβ-CD (1:10 000), obtained according to Ref. [24]; (2) harmane/βCD (1:2), obtained by procedure 1; (3) harmane/β-CD (1:100), obtained by procedure 2; (4) harmane in buffered aqueous solution at pH 7.3–7.5.

ethanol the only observed band is the one corresponding to neutral harmane, with maxima at 370 and 390 nm. However, in aqueous solution at neutral pH only the band at 430 nm corresponding to the cationic form can be observed. This behaviour can be explained in terms of the existence of different equilibria in ground and excited states which involve the proton transfer among the neutral, cationic, zwitterionic and anionic forms [26,28]. Our group has demonstrated that the compartmentalization of these compounds in lower polarity environments such as surfactants [29] or CDs [24,30] allows to observe the emission corresponding to the neutral form in buffered aqueous solutions at neutral pH. Fig. 7 shows the fluorescence emission spectra for the different complexes with harmane obtained by different preparation procedures and also at different molar ratios harmane/CD. In the solid complexes obtained by the procedures 1 and 2 the band corresponding to the cationic form of harmane is higher than the peaks observed for the neutral form. This behaviour contrasts that observed for the complexes obtained in solution [24,30] where the emission corresponding to the neutral form is more intense than for the cationic form. These observations can be explained through our previously discussed hypothesis that β-carbolines interact with the wider rim of the CD leaving the basic nitrogen ring outside the cavity and hence facilitating its protonation and allowing to observe the emission corresponding to the cationic form. In order to verify the partial inclusion of the guest molecules by fluorimetric techniques, a study of the fluorescence quenching by bromide ions was carried out. When the guest molecules are included into the CD cavity a good protection against the quenchers has been previously described for β-carbolines [24] and related alkaloids [31], but in the case of the solid complexes described in the present work this protection is not efficient. Thus, the Stern-Volmer constants (K_{SV}, Table 5) obtained for the different complexes are higher than those for free β-carboline in buffered aqueous solution. This observation indicates that the environment is different from that in the aqueous solution, but it also shows that

Table 5
Fluorescence quenching of the β-carboline/CD complexes by bromide ions

Compound	K_{sv}	r
Harmane	1.82	0.9921
Harmine	10.38	0.9992
Harmane/βCD (1:1)	2.04	0.9990
Harmane/βCD (1:2)	2.09	0.9977
Harmane/HPβCD (1:1)	1.93	0.9984
Harmane/HPβCD (1:2)	2.13	0.9580
Harmane/HPβCD (1:100)	2.44	0.9408
Harmine/HPβCD (1:100)	4.74	0.9183

$SF_O/SF_A = 1 + K_{sv}[A]$.

the association with the CD does not significantly protect the excited state of harmane or harmine. This behaviour is also related to the different procedures employed for preparing the complexes because for the complexes in solution (1:10 000, harmane:CD molar ratio) the alkaloid is associated to one or two molecules of CD as has be shown through stoichiometry calculations, that will be reported elsewhere [32].

In conclusion, CDs play an important role in the separation techniques for discrimination of chemically related compounds. The discrimination is based in the interactions between the host and the guest molecules and depending on the experimental procedures and the techniques employed in the study the information obtained can be significantly different. In such cases NMR techniques offer the highest expectations for investigating the interactions between host and guest molecules. On the other hand, the sensitivity of other quantitative spectroscopic techniques allows the examination of the interactions and the calculation of association constants and free energies, which are difficult by NMR techniques [33].

Acknowledgements

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