J Incl Phenom Macrocycl Chem (2007) 57:577–583
DOI 10.1007/s10847-006-9252-5

ORIGINAL ARTICLE

The role of β-cyclodextrin and hydroxypropyl β-cyclodextrin in the secondary chemical equilibria associated to the separation of β-carbolines by HPLC

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Received: 15 May 2006 / Accepted: 20 October 2006 / Published online: 9 February 2007

Abstract The use of cyclodextrins (CDs) in HPLC as mobile phase additives provides a flexible alternative for the separation of chemically related compounds because these separations can be performed on conventional columns and are economically advantageous over the use of chiral stationary phases. The present paper describes the influence of the presence of β-cyclodextrin (β-CD) and 2-hydroxypropyl-β-cyclodextrin (HPβ-CD) on the separation of the β-carboline alkaloids norharmane, harmane and harmine. The nature of the stationary phase (reverse phases C_1 and C_{18}) affects the chromatographic separations and also the stability of the inclusion complexes that are developed. The changes in the proportion of the organic solvents at constant concentration of CDs (3 mM for β-CD and 15 mM for HPβ-CD) modify the retention factors (k') for all alkaloids studied. The nature of the organic solvent in the mobile phase also changes the chromatographic parameters. The logarithm of the capacity factor (k') is linearly increased with the proportion of water in the hydro-organic mobile phase (ethanolic or methanolic) but the slopes obtained vary depending on the CD added to the mobile phase. The role of competitive equilibria, i.e., chromatographic distribution and inclusion complexes formation is discussed.

Keywords Cyclodextrins · β-carboline alkaloids · Mobile phase additives

Introduction

Cyclodextrins have been employed in a variety of separation techniques [1] as chiral stationary phases or added to the mobile phases in HPLC or to the buffers in capillary electrophoresis. The use of CDs as mobile phase additives in HPLC provides a flexible alternative for the separation of isomers and chemically related compounds because the separations can be performed on conventional columns with generally higher efficiencies than those obtained with the hydro-organic mobile phases usually employed in RP-HPLC. Thus, 10 aromatic isomeric naphthalenesulphonic acids [2] were separated in modified mobile phases containing β-CD in concentration 10 mM using C_{18} columns. The separations of polynuclear aromatic compounds (PAC) [3] or salicylic acid and their metabolites gentisic acid and salicylglycine improve as a consequence of the addition of β-CD to the mobile phase [4]. This methodology has also been applied for the chiral separation of pentazocine enantiomers [5] and for enantiomeric separations of amino acids [6]. However, due to the limited solubility of CDs in these mobile

This paper was presented at XIII[th] International Cyclodextrin Symposium. Torino, Italy, May, 14–17, 2006

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phases the benefits on the separation processes are not always evident [7].

β-Carboline alkaloids are widely distributed in medicinal plants, harmane being the first of them to be isolated from *Peganum harmala*. They are also endogenously produced in human and animal tissues as a product of secondary metabolism and therefore they are known as *mammalian indole alkaloids* [8]. They possess biological properties such as hypotensive, hallucinogenic, antimicrobial, tremorogenic and cardiovascular actions [9]. Small doses of harmine and related alkaloids act as cerebral stimulants, while large doses cause hallucinogenic and toxic effects. Different chromatographic methods have been developed for their quantitation in biological samples such as seeds of *Peganum harmala* [10], human plasma [11, 12], blood samples from rats [13], and coffee brews [14]. Harmane has also been isolated from the whole culture broth of *Pseudomonas* sp. K44-1 using preparative HPLC [15]. Norharmane, harmane and harmine (Fig. 1) differ in a methyl group on the C-1 position and a methoxy group on the C-7 position of the *β*-carboline ring and consequently they can be considered as good model structures to study the influence of CDs on the separation by RP-HPLC.

In the present study, the role of the addition of native *β*-CD and chemically modified HP*β*-CD to the mobile phase on the separation of the model alkaloids norharmane, harmane and harmine is considered. The influence of the nature and the proportion of organic solvent in the mobile phase was also studied. Special attention has been paid to the nature of the stationary phases (methyl–C_1– and octadecyl–C_{18}–) on the analytical separation of these alkaloids and to the study of the advantages associated to the low proportion of the organic solvents in the mobile phases.

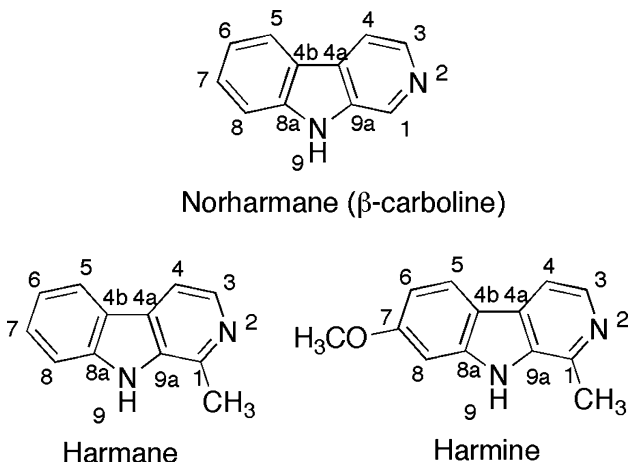

Fig. 1 Chemical structures of the *β*-carboline alkaloids studied

Experimental

Apparatus and reagents

UV-Vis absorption spectra were obtained with a Kontron (Zurich, Switzerland) Uvikon 810 double beam spectrophotometer.

Two Hitachi (Tokyo, Japan) liquid chromatographic systems were employed. One of them was equipped with a quaternary gradient pump model L-7100 and a fluorescence detector model L-7485. The second one was equipped with an isocratic pump model L-6000, and a F-1050 fluorescence detector. For all experiments the detection conditions were λ_{ex} = 290 nm, λ_{em} = 430 nm. Both chromatographic systems were equipped with a Rheodyne model 7725 injector (20 µl loop) and were under computer control through the HPLC System Manager software, version 4.1. The temperature was controlled with a column oven Merck Hitachi model L-2300. The experiments were carried out at 35°C. Separations were developed in reverse phase using C_{18} column (Spherisorb, ODS2, 5 µm, 150 × 4.6 mm) and C_1 (Chromasil, 5 µm, 125 × 4.0 mm). The analytical columns where purchased from Restek (Bellafonte, PA, USA).

All reagents and solvents were analytical, spectroscopic or chromatographic grade and were used without further purification. Water was doubly distilled prior to its use. Harmine, harmane and norharmane were purchased from Sigma (Steinheim, Germany). *β*-CD was obtained from Merck (Darmstadt, Germany) and HP*β*-CD was a generous gift from Rhône-Poulenc (Couverboi, France).

Procedures

Standard solutions

Stock solutions of harmine, harmane or norharmane 3.0×10^{-3} M in ethanol were freshly prepared. Aliquots of these stock solutions were taken to prepare solutions around 3.0×10^{-5} M of the alkaloids in 0.1 M HCl. These acidic solutions were spectrophotometrically measured and their molar absorptivities [16] were considered in order to adjust the final value of the concentration for the assays. Appropriate aliquots of the stock ethanolic solutions were taken to prepare the

solutions of the alkaloids in the mobile phase at concentrations 5.0×10^{-7} M.

Chromatographic conditions

Isocratic mobile phases methanol:phosphate buffer (pH 7.8) in the range studied from 60:40 to 40:60, v:v for a C_{18} column and in the range from 50:50 to 30:70 v:v for a C_1 column were employed. In the case of the isocratic mobile phases ethanol:phosphate buffer (pH 7.8) the proportion of the organic solvent changed in the range from 45:65 to 25:75, v:v for the C_{18} column and in the range from 35:65 to 15:85 v:v for the C_1 stationary phase. The phosphate buffer consists of a 0.02 M sodium dihydrogen phosphate-1-hydrate and its pH was adjusted to 7.6–7.8 with a solution of 20% (w:v) sodium hydroxide. The mobile phases were filtered through a 0,45 μm × 47 mm membrane filter (GPH, Waters, Bedford, MA, USA) under vacuum followed by the application of ultrasound during 5 min. These mobile phases were modified by the addition of appropriate amounts of CDs being the selected concentrations 3 mM for β-CD and 15 mM for HPβ-CD.

The influence of the presence of CDs in the mobile phase on the chromatographic parameters was considered. Thus increasing amounts of β-CD (0–3 mM) were added to the mobile phase methanol:phosphate buffer for C_{18} and C_1 stationary phases and the range of β-CD concentrations studied was 0–15 mM for the mobile phase ethanol:phosphate buffer and C_{18} and C_1 stationary phases. In the case of HPβ-CD the concentration range studied was 0–17 mM for C_1 and 0–11 mM for C_{18} stationary phases in both cases for ethanolic and methanolic mobile phases.

The columns were conditioned with the mobile phases containing the CDs during 30 min and the mixture of the β-carboline derivatives was then injected. The separations were performed at 35°C at flow rates of 0.9 ml/min.

All series of experiments were carried out in duplicate sets. For each set of experiments duplicate injections in the chromatrograhic system for a particular conditions (CD concentration, water percentage in the mobile phase) were injected.

Results and discussion

HPLC has been shown to be a promising tool for studying molecular interactions [17] such as the host-guest interactions in CD-analyte systems. Figure 2 shows the effect of the increasing amounts of CDs in the mobile phase on the chromatograms. For the two stationary phases and the two mobile phases a decrease in the retention time and retention factors was observed with the presence of increasing amounts of CDs. The decrease in the retention factors was higher in the presence of HPβ-CD than for β-CD. Thus comparing the same range of concentration of CD in the mobile phase, for ethanolic:buffer mobile phases the reduction in the retention time of harmine observed in the range of CD concentration from 0–9 mM was 4.49% (β-CD, C_{18}), 5.42% (β-CD, C_1), 13.35% (HPβ-CD, C_{18}) and 15.62% (HPβ-CD, C_1). In the case of methanolic phases the highest concentration of β-CD studied was 3 mM due to the low solubility of β-CD in methanol. The decrease in the retention time was around 1% for β-CD instead of the value found for HPβ-CD (3 mM), which was close to 5% depending on

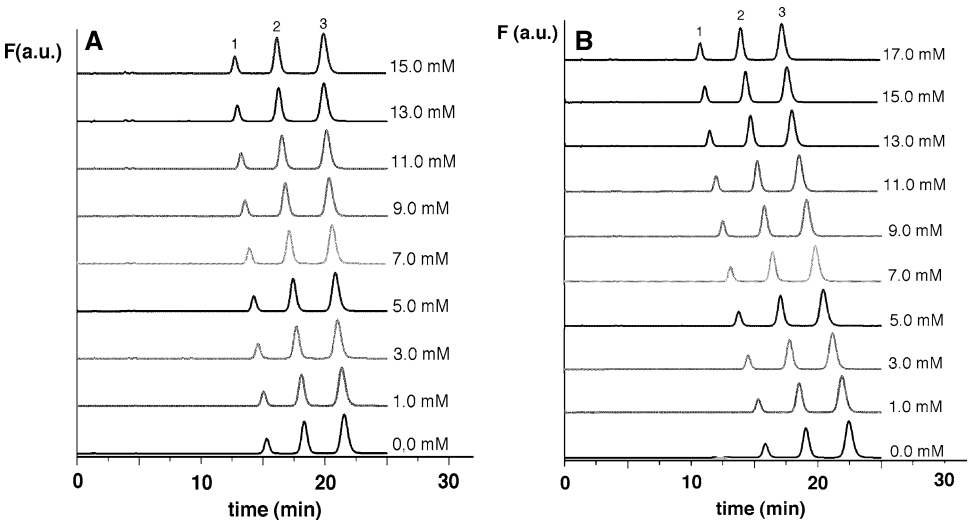

Fig. 2 Effect of increasing amounts of CDs on the chromatograms obtained in the separation of β-carboline alkaloids at 35°C. (**A**) β-CD, stationary phase: C_1, mobile phase: ethanol:buffered aqueous solution pH 7.8; 25:75, v:v. (**B**) HPβ-CD, stationary phase : C_1, mobile phase: ethanol:buffered aqueous solution pH 7.8; 25:75, v:v. F: fluorescence intensity in arbitrary units, retention time in minutes. (1) Norharmane, (2) harmane and (3) harmine

the stationary phase. This behaviour shows the significant effect of chemical nature of CD on the separation processes. Considering the same CD and stationary phase the decrease in the retention factor was higher when ethanol was present in the mobile phase than when the organic solvent was methanol. This behaviour can be explained by considering that in the presence of CDs in the mobile phase, inclusion complexes between the analytes and the CDs can be formed. Complexation increases the solubility of analytes in the mobile phases and then decreases the interaction with the stationary phases [18]. Therefore in ethanolic phases, where the proportion of organic solvent was lower than in methanolic phases, the solubility of the inclusion complexes in the mobile phases is increased and a decrease in the retention factor was observed. When the stationary phases C_1 and C_{18} are compared for the same CD as mobile phase additive and the same mobile phase, it can be concluded that the decrease in the retention factors was higher for C_1 than for C_{18} stationary phases. The lower polarity of the C_{18} phase causes stronger β-carboline-stationary phase interactions than in the case of C_1 and therefore the effect of CDs is better observed in C_1 stationary phase. The competitive equilibria analyte-stationary phase and analyte-CD inclusion favour the inclusion complexes formation for C_1 stationary phase. Besides the C_1 phase allows to increase the proportion of water in the mobile phase and consequently facilitates the solubilisation of the inclusion complexes in mobile phases reducing the retention factors. The reduction observed for the retention factors was smaller than that observed for other compounds with high lipophilicity and association constant i.e., polycyclic aromatic compounds [4] and camphor enantiomers [19]. This behaviour can be explained considering that the association constants for the carboline/CD complexes obtained by fluorimetry were not high (K_{ass} = 220–600) [20]. These alkaloids present association constant values similar to those obtained for other related heterocycles e.g., indole/β-CD (K_{ass} = 142) [21].

In the presence of a constant concentration of β-CD (3 mM) or HPβ-CD (15 mM) and when the organic solvent percentage is increased in the mobile phase, the retention factor decreases notably compared to the decrease produced as a consequence of the increasing concentrations of CDs in the mobile phases. This behaviour is observed for the different mobile and stationary phases studied and these results show that the principal equilibrium, the chromatographic distribution, predominates over the secondary equilibrium, the formation of inclusion complexes and the inclusion complexes partition/adsorption between mobile and stationary phases. Also the presence of increasing proportions of organic solvent in the mobile phase contributes to dissociate the inclusion complexes formed. However it is important to pay attention to the different behaviour of β-CD and HPβ-CD. When the organic solvent proportion in the mobile phase changes at constant CD concentration, the retention times for harmine change from 83 min to 12 min in the case of β-CD (3 mM) in the mobile phase (C_{18}, methanol:buffered aqueous solution), and in the case of HPβ-CD (15 mM) in the mobile phase (C_{18}, methanol:buffered aqueous solution) the retention times for harmine vary from 62 min to 9 min. The presence of

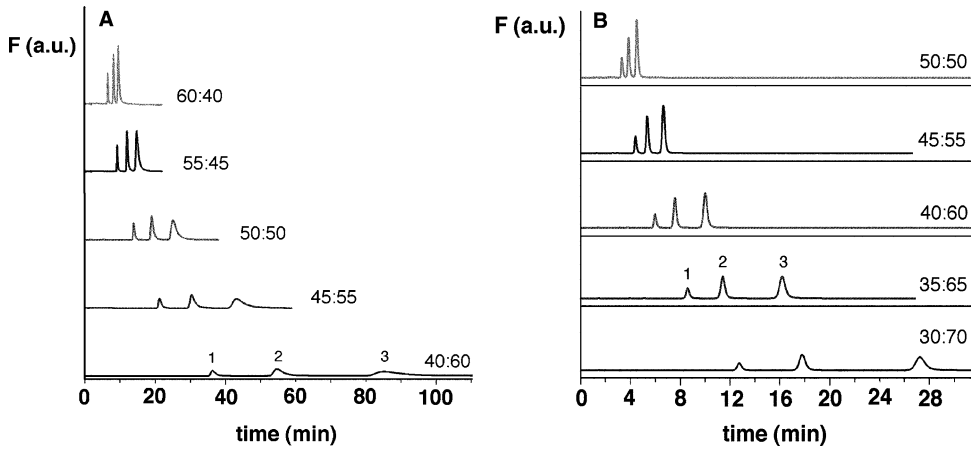

Fig. 3 Effect of the variation of water proportion in the mobile phases on the chromatograms obtained in the separation of β-carboline alkaloids at 35°C and at constant concentration of CD. (**A**) In the presence of 3 mM β-CD, stationary phase: C_{18}, mobile phase: methanol:buffered aqueous solution pH 7.8. (**B**) In the presence of 3 mM β-CD, stationary phase: C_1, mobile phase: methanol:buffered aqueous solution pH 7.8.F: fluorescence intensity in arbitrary units, retention time in minutes. (1) Norharmane, (2) harmane and (3) harmine

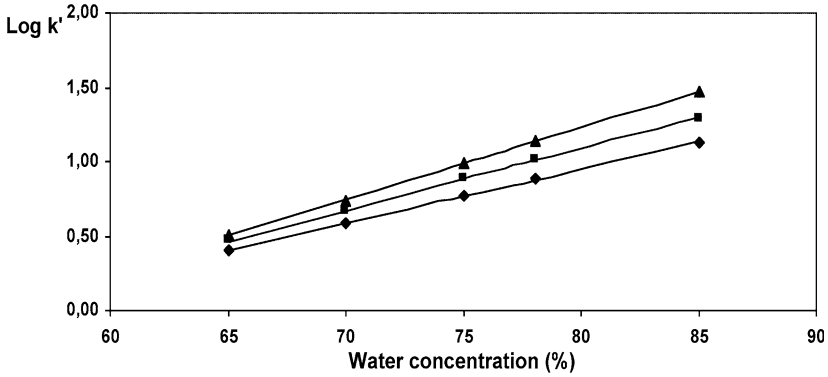

Fig. 4 Correlation between log *k'* (retention factor) and the percentage of water present in the mobile phase. Temperature 35°C. (◆)Norharmane, (■)harmane and (▲)harmine. HP*β*-CD 15 mM in the mobile phase ethanol:buffered aqueous solution pH 7.8 and stationary phase C_1

the organic solvent determines the chromatographic behaviour and the dissociation of the complexes, however HP*β*-CD competes with the organic solvent and exerts a positive influence on the chromatographic parameters (selectivity and efficiency). A similar behaviour was observed when the stationary phase employed was C_1. The retention times for harmine vary from 52 min to 8 min in the case of *β*-CD (3 mM, C_1, ethanol:buffered aqueous solution), and from 35 min to 5 min for HP*β*-CD (15 mM, C_1, ethanol:buffered aqueous solution). Figure 3 shows a representative example of this behaviour. The lower retention time observed in presence of HP*β*-CD and with a higher proportion of water in the mobile phases can be explained considering that the inclusion equilibrium is favoured for HP*β*-CD with regard to *β*-CD, but also it is important to consider that the higher concentration of HP*β*-CD in the mobile phases facilitates the inclusion equilibrium. As it is well known, the chemically modified CDs exhibit better solubility properties than native *β*-CD.

Figure 4 represents the logarithm of the retention factor (*k'*) of the *β*-carboline alkaloids studied as a function of the water percentage in the mobile phase and in the presence of HP*β*-CD in the mobile phase. In the absence of CDs in the mobile phase the retention factor of the *β*-carboline studied increased with increasing water percentage. Linear relationships between log *k'* and the water percentage in the mobile phase were observed, demonstrating that a classical reverse phase elution mechanism is operating. In the presence of *β*-CD or HP*β*-CD, a similar linear response

Table 1 Slopes (*m*) and correlation coefficients (*r*) obtained for linear regression treatment of log *k'* and the percentage of water present in the mobile phase at constant concentration of CDs

CYCLODEXTRIN	*β*-CARBOLINE	STATIONARY PHASE : C_{18}				STATIONARY PHASE : C_1			
		Methanol/Buffered Solution pH = 7.8		Ethanol/Buffered Solution pH = 7.8		Methanol/Buffered Solution pH = 7.8		Ethanol/Buffered Solution pH = 7.8	
		m	*r*	*m*	*r*	*m*	*r*	*m*	*r*
[CD] = 0 mM	Norharmane	0.0439 (0.0003)	0.9980	0.0524 (0.0003)	0.9965	0.0359 (0.0002)	0.9983	0.0479 (0.0001)	0.9998
	Harmane	0.0469 (0.0004)	0.9981	0.0557 (0.0004)	0.9959	0.0389 (0.0002)	0.9984	0.0515 (0.0001)	0.9996
	Harmine	0.0529 (0.0005)	0.9975	0.0629 (0.0007)	0.9943	0.0442 (0.0002)	0.9981	0.0592 (0.0003)	0.9989
β-CD 3 mM	Norharmane	0.0414 (0.0001)	0.9969	0.0527 (0.0001)	0.9941	0.0358 (0.0001)	0.9993	0.0449 (0.0006)	0.9985
	Harmane	0.0445 (0.0001)	0.9968	0.0562 (0.0003)	0.9935	0.0390 (0.0001)	0.9992	0.0493 (0.0005)	0.9988
	Harmine	0.0504 (0.0001)	0.9959	0.0640 (0.0004)	0.9915	0.0441 (0.0001)	0.9989	0.0577 (0.0007)	0.9987
HP*β*-CD 15 mM	Norharmane	0.0363 (0.0003)	0.9980	0.0471 (0.0001)	0.9975	0.0296 (0.0001)	0.9994	0.0367 (0.0001)	0.9999
	Harmane	0.0401 (0.0004)	0.9978	0.0519 (0.0001)	0.9966	0.0331 (0.0001)	0.9996	0.0415 (0.0001)	0.9998
	Harmine	0.0457 (0.0005)	0.9975	0.0603 (0.0001)	0.9949	0.0375 (0.0001)	0.9997	0.0486 (0.0001)	0.9997

The values of the standard deviation obtained for *m* appear in parenthesis. The experiments were carried out by quatriplicate

(log *k'* versus water percentage) was observed, with good correlation coefficients ($r > 0.99$). The data summarized in Table 1 indicate that no change in the inclusion mechanism was produced in the range of the organic solvent percentage studied. Thus when the proportion of organic solvent is increased the driving forces for the inclusion become weak due to the higher solubility of the guest molecules in the mobile phases. The slopes (Table 1) obtained in the linear regression treatment were lower in the presence of CDs than in the absence of CDs for both stationary phases C_{18} and C_1. Additionally, the slopes were lower for HPβ-CD compared to those obtained for β-CD. Considering the chemical structures of the alkaloids, the slopes obtained were always harmine>harmane>norharmane because harmine is the compound with lower polarity and therefore it is more influenced by the changes in the proportion of organic solvents in the mobile phases. These results are in agreement with the effect described for a series of aromatic compounds [22] in which the slopes obtained for the compounds with lower polarity were higher than those obtained for the compounds with higher polarity. This behaviour can be explained considering that chromatographic retention is essentially driven by a single mechanism involving hydrophobic interactions, a mechanism that is altered by the presence of CDs due to the secondary chemical equilibria introduced in the chromatographic process. These secondary equilibria can be shown to be relevant at high percentages of water in the mobile phases. In such case the formation of the inclusion complexes is favoured with regard to the retention on the stationary phases. These experimental conditions can be useful for studying the interaction mechanism although they are not satisfactory from the analytical point of view. An intermediate combination with an appropriate concentration of CD in the mobile phase allows to reduce the organic solvent proportion in the mobile phases without diminishing the chromatographic efficiency and resolution, with the consequent environmental benefits.

Acknowledgements Financial support from MCYT (Spain) through grant BQU-2003/04046 and UCM (Grupos de Investigación UCM-CAM 920234) is gratefully acknowledged. The authors are grateful to FONACYT (Venezuela) for a research fellowship for A. G. León.

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