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Liquid chromatographic study of two methoxypsoralen isomers with β-cyclodextrin

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The inclusion into β-cyclodextrin (β-Cyd) by the two methoxypsoralen isomers (5-methoxypsoralen (5-MOP) and 8-methoxypsoralen (8-MOP)) has been extensively studied by reverse phase liquid chromatography (RP-HPLC). Some important quantitative data concerning the complexation in a dynamic flow system have been drawn. The stoichiometry of the complex formation (1:1 in all instances), and the affinity constants with the β-Cyd cavity (K_f) of the two guest compounds, have been established. By taking into account the competitive effect of the organic modifier of the eluent (methanol), the K_fs of both compounds have been extrapolated to pure water (233 M^{-1} and 106 M^{-1} for 5-MOP and 8-MOP, respectively). On the other hand, the established stoichiometry and affinity constants have been interpreted in terms of enthalpy and entropy changes. Finally, the importance of the position of the methoxy substituent has been underlined and seems to rule the quality of the fit of each isomer within the inner cavity of β-cyclodextrin.

INTRODUCTION

Cyclodextrins (Cyds) are well known for their ability of forming complexes with a large variety of organic compounds.[1-3] This characteristic is often used in analytical chemistry, especially in liquid chromatography (LC) due to the hydrophobicity of the inner cavity of Cyds. This makes them capable of including closely related compounds such as structural or chiral isomers.[4-6] This particular feature has drawn our attention to the possibility of inclusion into Cyds of two linear furocoumarin isomers also called psoralens: the 5-methoxypsoralen (5-MOP) and the 8-methoxypsoralen (8-MOP). From a chemical point of view, psoralens are linear heterocyclic compounds extracted from plants such as Psoralea corylifolia Wart. or Amni majus. L. The interest attached to psoralens is due to their large use in the therapeutic treatment of skin diseases, specially psoriasis.[7] Among them, 5-MOP and 8-MOP appear as the most important with respect to their clinical use.[8]

As a consequence, much attention has been paid to their determination in the body fluids,[9] and numerous analytical techniques were developed for trace level determination, especially by HPLC.[10] The aromaticity of the psoralen nucleus gives 5-MOP and 8-MOP some modest fluorescence properties (fluorescence quantum yield ($\phi f \simeq 10^{-2}$) that we have attempted to magnify in order to observe lower levels of drugs in complex biological matrices.[11] Among all the possible approaches to reach this goal, we chose to explore the supramolecular interaction between methoxypsoralens and β-Cyd. Indeed, literature reports

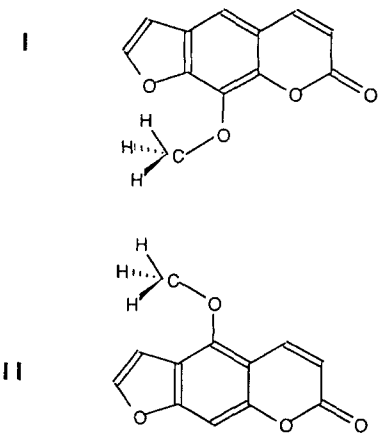

Figure 1 Formulae of the two methoxy psoralen studied. 1 = 8-methoxypsoralen (8-MOP); 2 = 5-methoxypsoralen (5-MOP).

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that the fit between organic species and the inner cavity of Cyd derivatives gives rise in some particular cases to an extensive modification of the spectroscopic characteristics of the guest.[12] More precisely in our case, previous work on 5-MOP and 8-MOP showed a significant increase in the fluorescence signal of 5-MOP (× 6) using a mobile phase spiked with β-Cyd, whereas the 8-MOP fluorescence remained unaffected.[13] Thus, attempts were made to explain this striking spectroscopic difference between 5-MOP and 8-MOP in the presence of β-Cyd. Unfortunately, the efforts made to isolate the β-Cyd/psoralen complexes in the solid state (for X-ray measurements) and in solution (for NMR studies) failed, due to the difficulty of crystallization, as well as the insufficient solubility of psoralens in aqueous media ($<10^{-5}$ M at 25 °C). Therefore, we attempted to perform a more convenient approach concerning the complexation in order to gain precise data on the psoralen/Cyd interaction in solution by using reverse phase liquid chromatography (RP-HPLC).

As state above, the solubility characteristics of 5-MOP and 8-MOP entail the use of a nonaqueous environment in order to perform measurements for the complexation studies. As a consequence, reverse phase HPLC seems to be an interesting alternative because of the use of water/organic solvent mixtures (e.g. water/methanol; water/acetonitrile). Such eluents are compatible within certain limits with the solubility of the introduced β-Cyd. Lastly, referring to previous work,[14] the study was performed only with β-cyclodextrin, essentially for practical reasons of cost and supply.

Thus, the stoichiometry of the complex formation as well as the affinity, K_f of the guest compounds towards β-Cyd were established. The possible correlation between the enthalpy and entropy changes associated to the inclusion were also studied. In addition, some attempts to underline the role of the methoxy substituent with respect to the quality of the fit between host and guest molecules were made.

RESULTS AND DISCUSSION

General considerations

Figure 2 shows a typical chromatogram of a mixture of 5-MOP and 8-MOP in the absence of β-Cyd at 25 °C under the conditions described in the experimental section.

As seen, the 8-methoxy isomer is more rapidly eluted than the 5-isomer, indicating a significant difference in interaction of the two derivatives with the C_{18} hydrophobic stationary phase. This feature classically

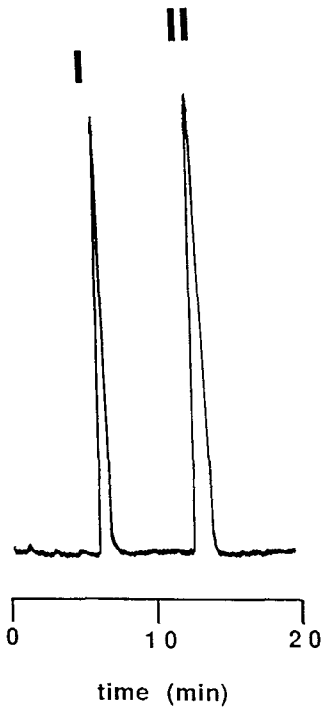

Figure 2 Typical chromatogram of the mixture of 5-MOP (II) and 8-MOP (I). Mobile phase methanol water (40 + 60 v/v). For detailed chromatographic conditions, see text.

suggests, in reverse phase chromatography (RP-HPLC), a relatively greater polarity of 8-MOP versus 5-MOP, findings well supported by solubility measurements which indicate the greater water solubility of the 8-MOP.[15] Moreover, it should be pointed out that the addition of β-Cyd in the eluent reduces the k' of each compounds whereas the elution order remains unchanged. Methanol was the organic modifier used in this study, because of its wide use in the L.C. determination of psoralens.[16-17] Despite its small association constant with β-Cyd ($K_M = 0.32\ M^{-1}$),[18] the amount present in the eluent must be taken into account because of its high molar ratio. Experimentally, the methanol content of the eluent was chosen to be as low as possible (40% by volume) compatible with an acceptable time of analysis (i.e. <20 min) and a correct selectivity (i.e. α_s only slightly decreases in the presence of added β-Cyd to a value of 3.39 at 50 °C. After introduction of β-Cyd in the eluent, if the interaction between β-Cyd and the apolar C_{18} stationary phase is neglected owing to the polarity of the outer hydroxyl groups of β-Cyd, the total concentration of the β-Cyd added to the system $[CD]_T$ can be expressed by:

$$[CD_T] = [CD_m] + [CD \,.\, M]_m \qquad (1)$$

where $[CD_m]$ is the available cyclodextrin concentration in the mobile phase (available for the inclusion process) and $[CD \,.\, M]_m$ is the methanol-complexed cyclodextrin

in the mobile phase. From the equilibrium constant K_m of the equilibrium:

$$[CD_m]_m + [M] \rightleftharpoons [CD.M]_m$$

and from eqn. (1), $[CD_m]$ can be calculated:

$$[CD_m] = [CD_T] \times (1/K_m[M] + 1)$$

As a consequence, in RP-HPLC when a concentration $[CD_T]$ of β-Cyd is introduced into the eluent, only $[CD_m]$ of β-Cyd can interact with the injected solute (S) and must be taken into account. Moreover, because of the very low concentration of the solute with respect to $[CD]_m$ and [M], we can consider that the fraction of β-Cyd complexed with S (i.e. [CD . S]) is negligible.[19] Therefore, using an eluent spiked with β-Cyd, and after injection of a solute (S), the complexation involves a decrease in the measured capacity factor owing to the modification of the mass transfer process in comparison with the same eluent without cyclodextrin.[20] Therefore, it can be shown that the formation constant of the complex (K_f) can be obtained from the following equation:[21-22]

$$1/k' = 1/k'_0(1 + K_f [CD_m]) \quad (2)$$

where k′ is the capacity factor of the solute in the presence of a concentration $[CD_m]$ of β-Cyd, k'_0 is the capacity factor in the same eluent in the absence of β-Cyd and $K_f = [CD.S]_m/\{[CD]_m \times [S]_m\}$, $[CD.S]_m$ being the concentration of solute complexed in the eluent and $[S]_m$ the concentration of the free solute. Thus, eqn. (2) assumes a 1:1 stoichiometry in complex formation between the solute and β-Cyd.[23]

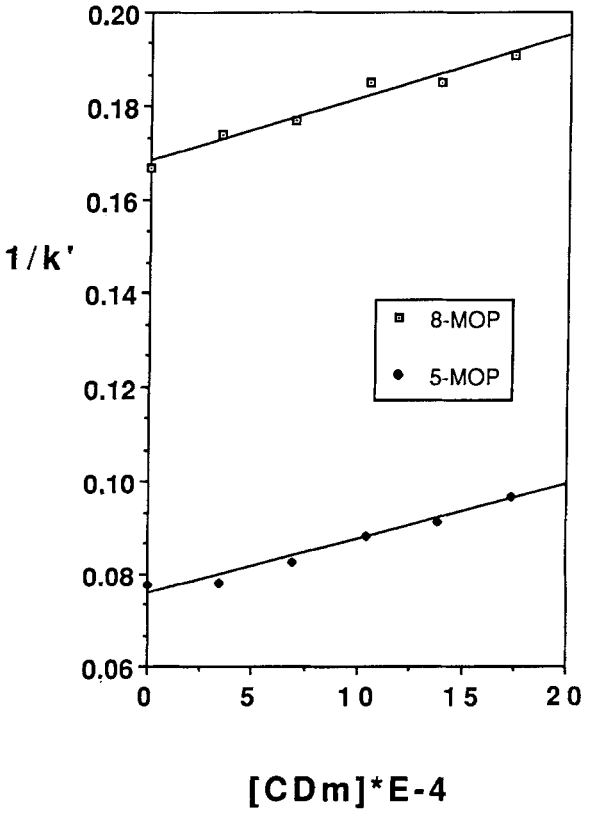

Figure 3 Plot of 1/k′ versus equilibrium available concentration of β-Cyd $[CD_m]$ with methanol water eluent. For detailed chromatographic conditions, see text. 8-MOP: $1/k' = 0.168 + 0.01$ $[CD_m]$, $r = 0.988$; 5-MOP: $1/k' = 0.075 + 0.01$ $[CD_m]$, $r = 0.989$.

The stoichiometry and the affinity constants of the psoralen-β-Cyd complexes

Equation (2) demonstrates that K_f can be determined by measuring k′ as a function of the concentration of the β-Cyd added to the mobile phase as shown in Fig 3 for the two isomers at 25 °C with a concentration of added β-Cyd ranging from 1.44 to 7.2×10^{-3} M (i.e. from 3.46×10^{-4} to 17.3×10^{-4} M available $[CD_m]$.

As already mentioned, the elution order 8 MOP < 5 MOP remains the same as in the absence of β-Cyd.

The linear plot of 1/k′ fits well with eqn. (2) for the two isomers ($r = 0.989$ for the 5-MOP; $r = 0.988$ for the 8-MOP). This satisfactory linear relationship between [β-Cyd] and 1/k′ confirms a 1:1 complexation ratio between the two compounds and β-Cyd. Indeed if two or more cyclodextrins bind to one molecule of psoralen, curvature in the plot of eqn. (2) should be observed rather than a straight line. Hence, the assumption of a 1:1 stoichiometry seems reasonable for 5-MOP and 8-MOP. Moreover, the plot of eqn. (2) allows the determination of the affinity constant (K_f) of each psoralen towards β-Cyd. Calculations give values of $154\ M^{-1}$ and $79\ M^{-1}$ for 5-MOP and 8-MOP, respectively. Thus, the order of elution in the chromatographic system should be associated with the K_f of the eluted compounds: the more rapid the elution, the lower the K_f. So, the affinity of the solute (5-MOP or 8-MOP) towards β-Cyd strongly influences the repartition process between the stationary phase and the mobile phase. This indirectly shows the importance of the position of the methoxy group on the psoralen nucleus, the sole difference between the two compounds. Thus, the decrease of the 5-MOP and 8-MOP capacity factors in the presence of β-Cyd should be attributed to the modification of the partition coefficient of the solute by increasing their solubility into the mobile phase. On the other hand, it should be mentioned that the K_f values determined by HPLC are lower than those already established for 5-MOP and 8-MOP by solubility methods[24] (300 and $86\ M^{-1}$, respectively), suggesting a non-negligible competitive role played by the methanol content towards the complexation of psoralens.

Figure 4 shows the evolution of the complex formation constant (K_f) at 25 °C for 5-MOP and

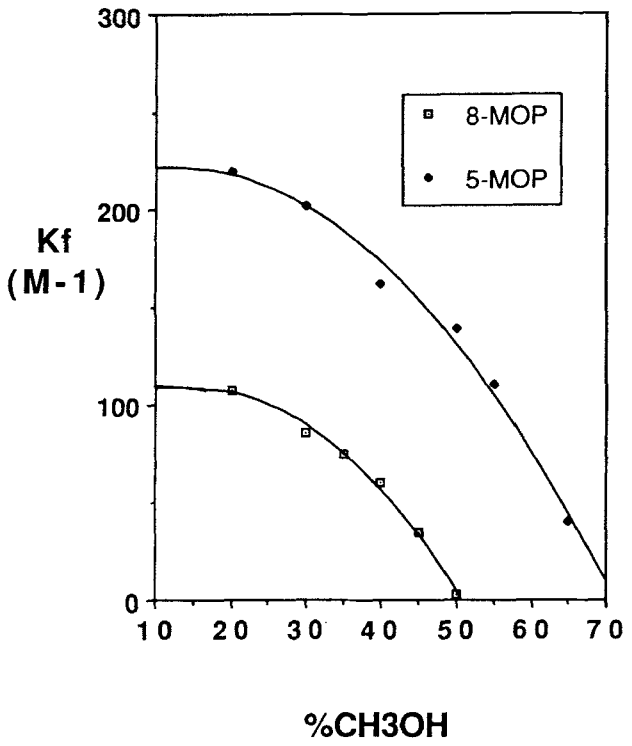

Figure 4 Plot of K_f ($L.mol^{-1}$) versus methanol content (% v/v) of the eluent established at 25 °C for the two isomers. β-Cyd introduced = 3.6×10^{-3} M.

8-MOP for a methanol content ranging from 20 to 65% by volume. As seen, an increase in methanol content dramatically decreases K_f, clearly emphasizing the importance of the role played by methanol as a competitor with regard to inclusion into the internal cavity of β-Cyd. Moreover, the plots of K_fs versus methanol content are non-linear but parallel, suggesting a progressive change in the strict 1:1 stroichiometry. Non-linear fitting allows an estimation of the two K_fs in pure water: 233 M^{-1} and 106 M^{-1} for 5-MOP and 8-MOP, respectively. These values are closer to the affinity constant determined by solubility experiments especially for 5-MOP. This indicates the reliability of the HPLC procedure for the determination of the K_f for a certain degree of complexation (in the case of 5-MOP). On the other hand, it strengthens the hypothesis of an approximate fit of 8-MOP into β-Cyd due to the presence of methoxy in the 8-position, entailing, in both HPLC and solubility methods, a greater imprecision in the determination of K_f.

The enthalpy and entropy changes associated with the complexation

Basically, the binary mixture CH_3OH/H_2O used as eluent in L.C. and the heterogeneous system (mobile phase and immiscible stationary phase) seems to be rather complicated for the determination of the thermodynamic parameters of the inclusion. Nevertheless, the RP-HPLC used here can offer a useful tool to achieve this goal, due to the relation linking the partition coefficient (K) of a solute and the absolute temperature T, according to[25]

$$\ln K = -\Delta G/RT \quad (3)$$

So,

$$\ln K = -\Delta H/RT + \Delta S/R \quad (4)$$

where ΔG is the Gibbs free energy for solute/stationary phase interaction, R is the gas constant and ΔH and ΔS are the enthalpy and entropy changes respectively associated with the transfer of a solute from the mobile to the stationary phase.[25] As the capacity factor (k') of the solute is related to K by $k' = \phi K$ where ϕ is the phase ratio of the stationary to mobile phase ($\phi = Vs/Vm$). The Van't Hoff equation describes the relationship between the capacity factor and the temperature of the system:

$$\ln k' = -\Delta H/RT + \Delta S/R + \ln \phi \quad (5)$$

When the eluent is, then, spiked with β-Cyd, the partition coefficient (K) is modified by the competitive effect of the introduced β-Cyd and the affinity constant (K_f) of the solute must be taken into account. Taking the logarithms of eqn. (2) gives

$$\ln k'_\beta = \ln k' - \ln K_f - \ln [1/K_f + [CD_m]) \quad (6)$$

where k'_β is the capacity factor of the solute in the presence of β-Cyd. Combining eqns (6) and (5), the variation of the capacity factor in the presence of β-Cyd can be evaluated by

$$\ln k'_\beta = (\Delta H_\beta - \Delta H)/RT + (\Delta S - \Delta S_\beta)/R + \ln \phi - \ln \{1/K_f + [CD_m])\} \quad (7)$$

Therefore, simple measurements of k' as a function of the temperature, in absence and in presence of β-Cyd, can allow a correct determination of enthalpy and entropy changes (ΔH_β and ΔS_β) associated with the transfer of the solute from the mobile phase to the stationary phase.

In such instance, Table 1 summarizes the k' values of each isomers in absence and in presence of β-Cyd in the eluent. The concentration chosen (i.e. 3.6×10^{-3} M introduced; i.e. 8.6×10^{-4} M available β-Cyd) corresponds to, approximately, the middle of the range of above studied variation of k' versus $(CD]_m$.

Figure 5 shows the plot of k' and k'_β versus 1/T for the two isomers, according to eqns (5) and (7). For both equations, according to refs. 26–27, with the assumption that Vm can be obtained from non-retained solutes, e.g. sodium nitrite (see Material and methods) ln ϕ can be calculated to be equal to −0.853.

From this plot, the enthalpy change ΔH associated with the pure chromatographic partition process (i.e. without β-Cyd added) can be obtained by the slope $= -\Delta H/R$ and the entropy change (ΔS) by the intercept ($\ln \phi + \Delta S/R$). Therefore, the introduction of β-Cyd in the eluent leads, in a second step, to the determination of ΔH_β and ΔS_β according to eqn. (7). As seen, the data agree satisfactorily with the model for both isomers with and without β-Cyd, indicating that the mechanism of the chromatographic partition process remains unchanged upon β-Cyd. As a consequence, the difference between ΔH, ΔH_β and, on the other hand, between ΔS and ΔS_β should be largely influenced by the inclusion of the solute in the dissolved β-Cyd owing to the negligible adsorption of β-Cyd molecules into the C_{18} support. The influence of added β-Cyd on enthalpy and entropy changes is summarized in Table 2.

Concerning the enthalpy change (ΔH), it can be said that in the presence or absence of β-Cyd, the more negative the value of ΔH, the longer the retention time, so the more efficient is the transfer of the solute to the stationary phase. So the interpretation of the elution order of the solutes stated above in the absence of β-Cyd remains valid upon addition of β-Cyd. This underlines that the introduced β-Cyd has a negligible impact on the stationary phase, as already mentioned, and, on the other hand, that the decrease of k' upon β-Cyd results from the competitive effect of β-Cyd towards the solute with respect to the stationary phase. This latter point is well supported by the ΔH_β values being significantly higher than those of ΔH, indicating a decrease in the interaction between the solute and the stationary phase. Moreover, as in absence of β-Cyd,

Table 1 Variation of k' of 8-MOP and 5-MOP as a function of temperature in the absence and in the presence of 3.6×10^{-3} M added β-Cyd

	without β-Cyd		*with β-Cyd*	
To(c)	*8-MOP*	*5-MOP*	*8-MOP*	*5-MOP*
25	16.976	34.119	14.319	25.990
30	14.618	28.972	12.573	22.380
35	12.786	25.798	11.114	19.489
40	11.554	21.530	10.331	17.874
45	10.754	20.145	9.659	16.664
50	9.585	17.560	8.781	14.507

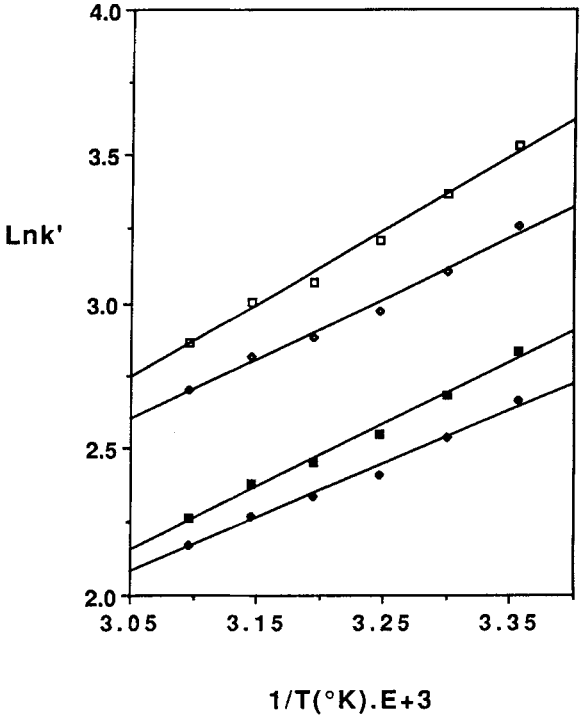

Figure 5 Plot of the ln k' and ln k'_β of 5-MOP and 8-MOP in the absence and in the presence (3.6×10^{-3} M added) of β-Cyd. ■: 8-MOP without β-Cyd: $\ln k' = -4.375 + 2.141$ (1/T) (r = 0.999)/◆: 8-MOP with β-Cyd: $\ln k'_\beta = -3.488 + 1.826$ (1/T) (r = 0.998). □: 8-MOP without β-Cyd: $\ln k' = -4.914 + 2.509$ (1/T), (r = 0.999)/◇: 5-MOP with β-Cyd: $\ln k'_\beta = -3.697 + 2.064$ (1/T) (r = 0.997).

Table 2 ΔH, ΔH_β, ΔS and ΔS_β of the two methoxy psoralen isomers in methanol-water (40 + 60) and methanol water (40 + 60) + 3.6×10^{-3} M added β-Cyd

	without β-Cyd		*with β-Cyd*	
Compounds	ΔH (kcal.mol^{-1}) (KJ.ml^{-1})[a]	ΔS (cal.mol^{-1}.K^{-1}) (J.mol^{-1}.K^{-1})[a]	ΔH_β (kcal.mol^{-1}) (KJ.ml^{-1})[a]	ΔS_β (cal.mol^{-1}.K^{-1}) (J.mol^{-1}.K^{-1})[a]
8-MOP	−4.088	−7.522	−0.602	+5.891
	(−17.11)	(−31.48)	(−2.52)	(+24.65)
5-MOP	−4.793	−8.550	−0.851	+6.448
	(−20.06)	(−35.78)	(−3.56)	(+26.98)

[a] Values in parenthesis.

ΔH_β remains strictly correlated to the k' and so with the structure of the isomer.

Concerning the entropy changes in absence of β-Cyd, the data (Table 2), are correlated with the elution order. ΔS, in such a dynamic flow system, can be associated with the change in ordering of the solvated solute during its transfer between the mobile phase and the stationary phase.[23] Thus, the position of the $-OCH_3$ group on the psoralen nucleus seems to play a crucial role in the organisation of the molecules in the both phases as suggested by the lower value of 5-MOP (ΔS) relative to 8-MOP (ΔS). In the presence of β-Cyd, the entropy changes of both isomers (ΔS_β) undergo a drastic increase, becoming highly positive. It should be noted that the calculated values of ΔS_β are in good agreement with some reported data on the interaction with hydroxyaromatic compounds and β-Cyd.[23] This strongly emphasizes the increasing perturbation generated by β-Cyd in the transfer process.

More interestingly, the ΔS_β of 5-MOP becomes higher than the ΔS_β of the 8-MOP. This appears to be extremely important with regard to the interpretation of the inclusion process with β-Cyd. Strictly, ΔS_β should be associated, in the presence of β-Cyd in the eluent, with the transfer process of the solute between the two phases. Nevertheless, if we assume that β-Cyd is negligibly adsorbed on the C_{18} support (see earlier), the transfer process is mainly altered by the β-Cyd present in the eluent. Therefore, ΔS_β can also conveniently represents the degree Δ of disorder in the whole mobile phase. Thus from the S_β measurements, the introduction of β-Cyd provokes a greater perturbation in the organisation of the eluent with 5-MOP than with 8-MOP. This could be attributed to the existence of two distinct populations of 5-MOP (complexed and uncomplexed), whereas the population of 8-MOP remains less perturbated because it is less complexed.

In the studied case, k', K_f, ΔH and ΔS seem to be well correlated, it should be noted that a high value of K_f (5-MOP) is associated to a high value of ΔS_β and for the 8-MOP, a low K_f with a low ΔS_β.

In conclusion, inclusion with 1:1 stoichiometry appears to be common to both isomers, certainly by moving the psoralens molecules in the cylindrical axis of the β-Cyd cavity as suggested by the length of the psoralen molecule (9.1 Å)[28] with respect to the internal diameter of β-Cyd (7.2 Å). Moreover, as shown by the K_f determination and the measure of ΔS_β, the importance of the position of the methoxy group on the quality of the fit is clearly underlined. In such a way, the fit of 5-MOP appears better than 8-MOP; this may be related to the magnified fluorescence signal observed upon β-Cyd with 5-MOP, whereas the 8-MOP emission remains unaffected under the same conditions.

Nevertheless, the stereochemistry of the respective 5-MOP/β-Cyd and 8-MOP/β-Cyd complexes can not be determined by this HPLC approach. This latter point appears to be the major limitation of the proposed method of investigation of the host-guest interaction in solution. Nevertheless, these experiments have demonstrated the interest in the use of LC for the determination of complex formation constants as well as some important associated thermodynamic parameters between cyclodextrin derivatives and various organic species, especially costly and potentially toxic compounds such as psoralens.

MATERIALS AND METHODS

Chemicals

β-Cyclodextrin (β-Cyd) was purchased from Roquette (Lestrem, France) and recrystallized once from hot water before use. 5-methoxypsoralen (5-MOP) and 8-methoxypsoralen (8-MOP) were from Sigma (St. Louis, Mo, USA). All solvents used were of LC grade (Merck, Darmstadt, Germany) and water was triply distilled before use.

Apparatus

All LC measurements were made using a Shimadzu LC 9A metering pump equipped with a Rheodyne Model 7185 5 μL loop injector and the mobile phase flow-rate was 1.4 mL.min^{-1}. The analytical column was a 5-μm reversed phase C_{18} Spheri-5 (10 × 4.6 mm i.d.) from Browlee-Labs (USA). A Shimadzu CTO 6A over was used to keep the column temperature constant with an accuracy of $\pm$0.1 °C. For temperature effect studies (enthalpy and entropy calculations), six temperatures from 25 to 50 °C were investigated. A Kratos FS 970 fluorimetric detector was used with an excitation wavelength set at 309 nm and a 417 nm cut-off emission filter. The chromatograms were recorded on a Shimadzu LC 5A integrator.

Solutions

– Stock solutions (10^{-3} M) of 5-MOP and 8-MOP were prepared in methanol and kept protected from light at +4 °C, for no more than 3 days. Successive dilutions were made with methanol and then, with mobile phase in order to give a working concentration of 2×10^{-5} M for the two isomers.

– Stock aqueous solutions (1×10^{-2} M) of β-Cyd were kept at room temperature for 2 days.

Chromatography

Throughout this study a basic methanol-water (40 + 60) eluent was used after careful degassing [helium, Ultrapur (Air Liquide, France)] and filtering (Waters-Millipore 0.45 μm filter).

With each change in the composition of the mobile phase or in temperature, the void volume (as t_{Ro}) was verified by injection of a methanol solution of sodium nitrite (UV detection at 220 nm with a Shimadzu SPD 6A UV detector), and the mean of three measurements of t_{Ro} was used in subsequent calculations of the capacity factors (k'; $k' = t_R - t_{Ro}/t_{Ro}$ with t_R = retention time of the solute).

When β-Cyd was added to the eluent the appropriate amount of β-Cyd was dissolved in water and then methanol was added to give the final desired concentration (from 10^{-3} to 10^{-2} M).

The poor water solubility of psoralens ($< 10^{-5}$ M at 25 °C), their spectroscopic properties and the low concentrations to be used in the study, led to the adoption of fluorimetric detection according to the conditions described above.

For the determination of the complex formation constant (K_f), different eluents characterized by an increasing concentration of β-Cyd (in the range $10^{-3} - 10^{-2}$ M) were used. In order to study the influence of methanol on the complex formation, six eluents were tested with methanol contents ranging from 20 to 65% (v/v). Higher concentrations were not tested in order to avoid the risk of precipitation of the fixed β-Cyd concentration (3.6×10^{-3} M) in the eluent.

All capacity factor (k') measurements were made systematically in triplicate. All the experimental data reported here refer to the average of these three measurements with an estimated error never higher than 0.5%.

Enthalpy and entropy changes in the absence and presence of β-Cyd (ΔH, ΔS and ΔH_β, ΔS_β, respectively) were calculated according to Van't Hoff equations and using a R value of 1.99 cal K^{-1} mol^{-1} (8.314 J K^{-1} mol^{-1}).

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