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Determination of the Stoichiometry and Stability Constants of the β-Cyclodextrin–Dextromethorphan Inclusion Complexes by Liquid Chromatography and UV Spectroscopy

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Abstract. The inclosion of dextromethorphan (DMN) by β-cyclodextrin (β-CD) was studied by using chromatography, UV spectroscopy and circular dichroism methods at 25 °C, pH 7.4 and 4.2. It was found that the βCD : DMN complex has 1 : 1 stoichiometry. It is more stable at pH 7.4 than at pH 4.2. with constants respectively equal to 8000 ± 800 M^{-1} and 5750 ± 500 M^{-1}, as determined by chromatography. The stability of the complex at pH 7.4 decreases as the temperature increases. From the van 't Hoff dependence the standard entropy and enthalpy changes were determined at this pH.

Key words: Dextromethorphan, β-cyclodextrin, complex, HPLC, UV spectroscopy.

1. Introduction

Cyclodextrins (CDs) are oligoglycosides of six (α-CD), seven (β-CD) or eight (γ-CD) glucose units forming a ring. This circular configuration results in a donut-shaped molecule in solution that has a hydrophilic exterior and a hydrophobic interior. Consequently this cavity is capable of including guest molecules of the appropriate size, shape and polarity [1, 2] to form inclusion complexes in the solid state and in aqueous solutions. The inclusion phenomenon has been extensively applied in the pharmaceutical field because of its ability to improve aqueous solubility, dissolution and release rates, bioavailability, chemical stability and modification of the pharmacokinetics of various drug molecules [2].

A wide variety of techniques have been used to study such complexes, including UV-visible spectroscopy [3–5], circular diebroism [6, 7], NMR [8, 9], competitive inhibition of catalytic reactions [10], microcalorimetry [11, 12], solubility [13],

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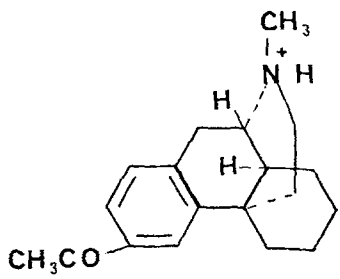
solvent partition [14], gel filtration [15, 16], thin layer chromatography [17], HPLC [18–21], pH potentiometry [22, 23] and competitive indicator methods [24–27].

The present study is concerned with the complexation by β-CD of an antitussive drug, dextromethorphan, the bitter taste of which is strongly attenuated when β-CD is added in large excess. Recently, Aumatell and Wells [28] have used β-CD complexation properties to separate the enantiomers of 3-methoxy-*N*-methylmethorphan, levomethorphan and dextromethorphan, using capillary zone electrophoresis with β-CD as an additive chiral agent, but, to our knowledge, the constants and the stoichiometry of such complexes have not been yet reported. Our main purpose was to determine these parameters and to investigate the effect of temperature and pH. HPLC, UV spectroscopy and circular dichroism were used.

2. Experiniental

2.1. Reagents

Dextromethorphan hydrobromide was a gift from Hoffmann Ia Roche & Co. (Switzerland), β-CD was a gift from Orsan (France). Acetic acid and sodium phosphate salts were obtained from Prolabo (France).



Formula of dextromethorphan

Dextromethorphan is present in its cation form, since its pKa is equal to 8.9, as determined by potentiometry [29], and the studies were carried out at pH 4.2 or 7.4.

2.2. Apparatus

2.2.1. *Chromatographic System*

The chromatographic equipment included a Shimadzu Model LC-9A pump, a Spectra Physics Model Spectra 100 variable-wavelength detector, monitoring between 260 and 280 nm and a Rheodyne Model 7125 injection valve with a loop of 50 μL (or 100 μL in the case of Hummel and Dreyer method experiments). Two columns were used: a (25 cm × 4.6 mm I.D., porosity 100 Å) Lichrospher Diol column (Merck) for the pH 4.2 and 7.4 *Hummel and Dreyer method* studies and the pH 7.4 *retention method* study, and a (10 cm × 4.5 mm I.D., porosity 300 Å) polysulfoethylaspartamide column (Poly LC) for the pH 4.2 *retention method*. The

columns were thermostated at 25 °C. Experiments were made respectively in a 0.1 M sodium phosphate buffer at pH 7.4, in 0.1 M sodium acetate buffer for the studies on the Lichrospher Diol column and in a 0.02 M sodium acetate (plus 0.01 M NaCl) buffer at pH 4.2 for the study on the Poly LC column (retention method).

2.2.2. *Spectroscopic UV System*

Ultraviolet spectra were recorded on a Varian Cary 100 spectrophotometer in 1 cm cuvettes. The DMN and β-CD solutions were prepared separately and mixed just prior to the measurements. The buffers used were 0.1 M sodium phosphate and 0.1 M sodium acetate at pH 7.4 and 4.2 respectively.

2.2.3. *Circular Dichroism*

Measurements were made with a dichrograph Jobin Yvon spectropolarimeter (Mark V) using a 1.0 cm cylindrical quartz cell. A total of three scans were averaged for each run.

2.2.4. *Theoretical*

Most binding studies assume a 1 : 1 stoichiometry between the cyclodextrin host and the guest molecule. However some cases of multiple β-CD complexes have been found: two or more β-CDs can bind to a single solute, and there are also cases where two guest molecules bind to a single cyclodextrin host, as reviewed by Armstrong *et al.* [19].

A 1 : 1 stoichiometry is described by the equilibrium:

$$L + CD \rightleftarrows LCD \quad \text{with } K_1 = [LCD]/[L][CD] \tag{1}$$

where [L] is the concentration of free guest, [CD] is the concentration of the free β-CD and [LCD] is the concentration of the solute β-CD complex. The appropriate Scatchard plot [30] equation is:

$$\bar{r}/[L] = K_1(1 - \bar{r})$$

where $\bar{r}$ is the number of moles of guest bound per mole of CD. The linearity of the $\bar{r}/[L]$ versus $\bar{r}$ plot indicates a 1 : 1 complexation. Such diagrams have been observed for drug: β-CD (1 : 1) complexes, as measured by HPLC [20, 21].

In cases where two or more β-CDS bind to a solute, appropriate expressions which take into account multiple β-CD complexation are easily formulated. For a 1 : 2 (drug: CD) complex, two supplementary equilibria must be considered:

$$LCD + CD \rightleftarrows L(CD)_2 \quad \text{with} \quad K_2 = [L(CD)_2]/[LCD][CD] \tag{2}$$

$$L + 2\,CD \rightleftarrows L(CD)_2 \quad \text{with} \quad K_1K_2 = [L(CD)_2]/[L][CD]^2 \tag{3}$$

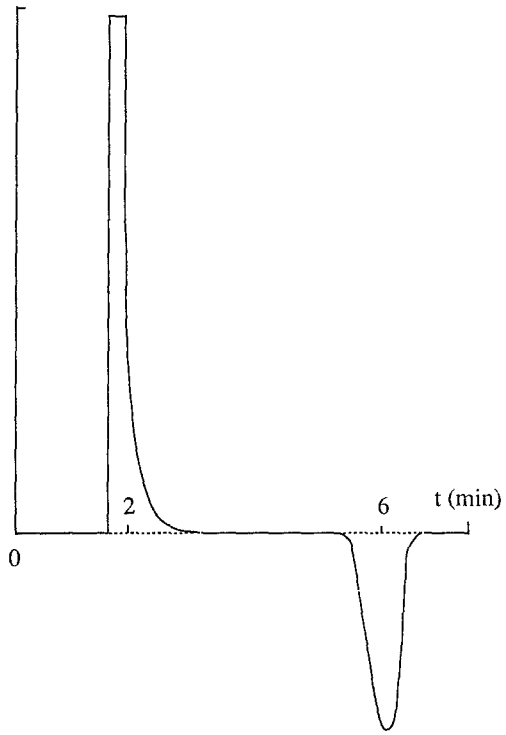


Figure 1. Hummel and Dreyer method elution profile on the Lichrospher Diol column eluted by a 0.33 mM DMN solution (in 0.1 M, pH 7.4 phosphate buffer). Experimental conditions: Flow rate = 1 mL/mn, sample = 100 μL of β-CD (2 g/L).

where $L(CD)_2$ is a 1 : 2, solute: β-CD complex. Non linearity of the Scatchard plot is then observed, and appropriate mathematical treament leads to the binding parameters. Thus, the K_1 and K_2 constants of the 2,6-TNS-β-CD complexes have been recently measured using potentiometry and construction of Scatchard plots with simulated data [31].

3. Results and Discussion

3.1. CHROMATOGRAPHY

Chromatography has been widely applied to obtain the parameters characterizing molecular complexes, as reviewed recently [32]. Different methods are available. We have chosen to use two of them as described further.

3.1.1. *Hummel and Dreyer Method*

This ingenious gel filtration chromatographic method [33], widely used to measure drug–protein interactions, and also employed to measure formation constants of

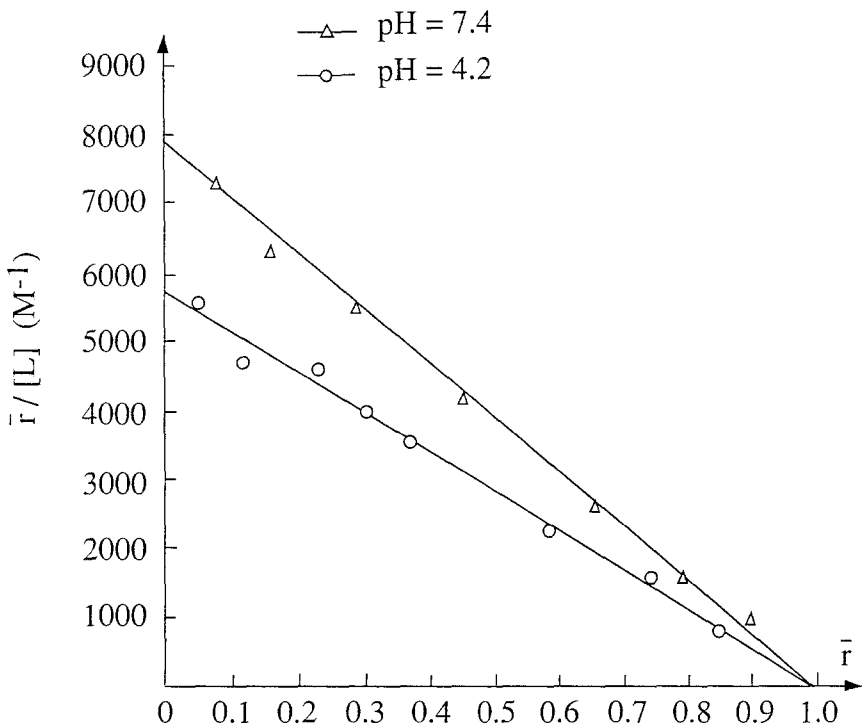


Figure 2. Scatchard diagrams obtained for the β-CD–DN/IN interaction at pH 7,4 and 4.2. Experimental conditions; Hummel and Dreyer method with eluents containing various concentrations of DMN (0.01–1.0 μM), at 25 °C.

guest substances with CD [15, 16], was applied by us to the HPLC study of β-CD–drug complexes [20]. The technique uses an eluent containing the drug at a given concentration (imposing the free drug concentration [L]), and a small amount of β-CD is injected onto the column.

A typical chromatogram is shown in Figure 1. First, a positive peak appears at the β-CD retention time, corresponding to β-CD and the β-CD-DMN complex formed, and a negative one emerges at the guest retention volume, corresponding to the deficiency in guest. The area of this last peak depends directly on the amount of bound guest, which is measured using internal calibration consisting of successive injections of increasing amounts of guest, as previously described [33], then $\bar{r}$ can be calculated. By varying the guest concentration in the eluent in the range 10^{-5} to 10^{-3} M, the plot of $\bar{r}$/[L] as a function of $\bar{r}$ allows the determination of the parameters of the complexation.

The results obtained at pH 7.4 and 4.2. are reported in Figure 2 (Scatchard plots). It appears that the stoichiometry of the complex is clearly of the 1 : 1 type, since the plots are linear and converge towards the value 1. The constants measured at 25 °C are respectively 8000 ± 800 M^{-1} and 5750 ± 500 M^{-1} at pH 7.4 and 4.2.

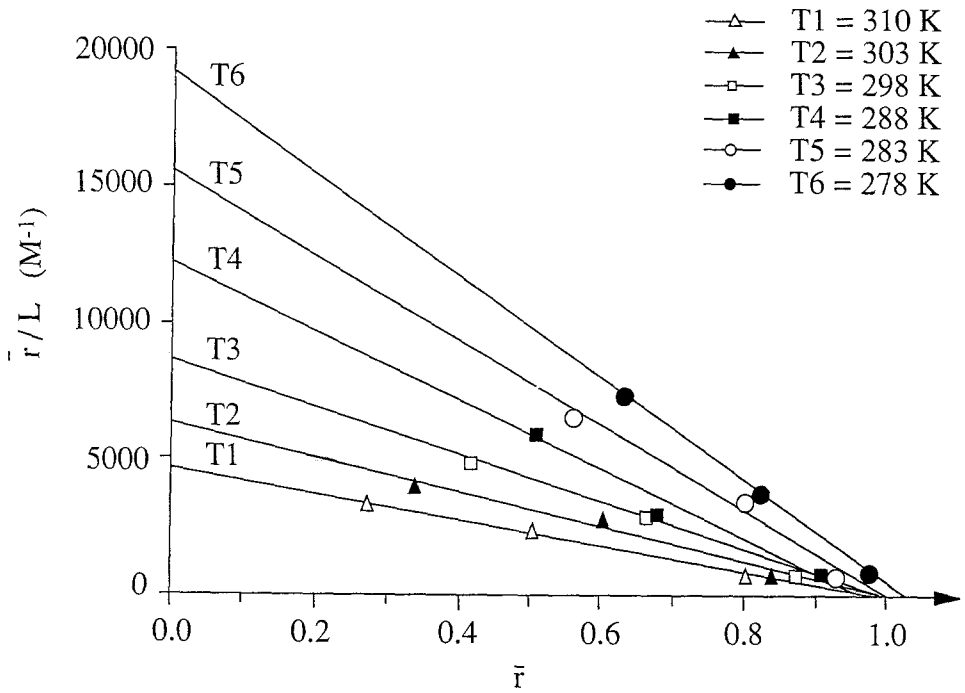


Figure 3. Scatchard diagrams obtained at different temperatures (pH 7.4).

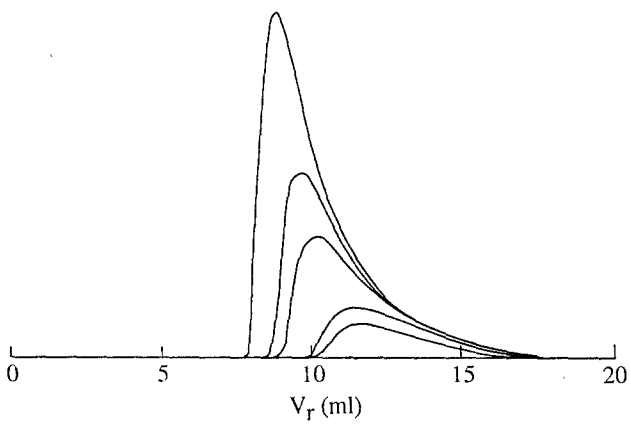


Figure 4. Retention method elution profiles of DMN on the Lichrospher Diol column with a pH 7.4 mobile phase containing 3.17 mM β-CD. Samples: 20 μL of (50, 30, 20, 8, 5 μM) DMN.

The stability of this complex with temperature was studied at pH 7.4 between 5 °C and 37 °C. Figure 3 illustrates the Scatchard plots so obtained. As previously, all the straight lines converge towards $\bar{r} = 1$. From the linear van 't Hoff plot obtained,

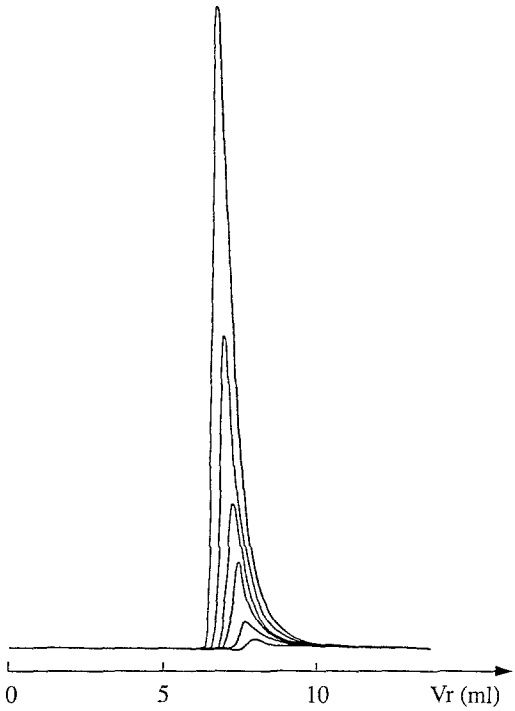


Figure 5. Retention method elution profiles of DMN on the Poly UC column with a pH 4.2 mobile phase containing 1.31 mM β-CD. Samples: 20 μL of (200, 100, 50, 30, 10, 8 μM) DMN.

the thermodynamic parameters of the formation of the DMN-β-CD complex were calculated $\Delta H_0 = -30.9 \pm 2.9$ kJ/mol and $\Delta S_0 = -29.3 \pm 1.0$ J/mol K.

In this chromatographic method, the amount of β-CD injected onto the column is low (50 μL of 2 g/L solutions) and its concentration decreases during the elution process. To examine the case where the [β-CD]/[DMN] ratio is higher, we have used another method in which the β-CD concentration is constant during the elution as it is present in the mobile phase.

3.1.2. *Chromatographic Method with β-CD in the Mobile Phase: Retention Method*

This method involves the measurement of the retention of DMN injected onto a chromatographic column eluted by mobile phases containing increasing concentrations of β-CD. The theoretical aspects of this method were described by Armstrong *et al.*, first for the evaluation of partition coefficients of solutes between micelles (or additives such as cyclodextrins) and aqueous phases via gel filtration [34], liquid chromatography [35], planar chromatography [17] and then for the evaluation of binding constants and stoichiometry of β-CD complexes [19, 36]. Uekama *et al.*

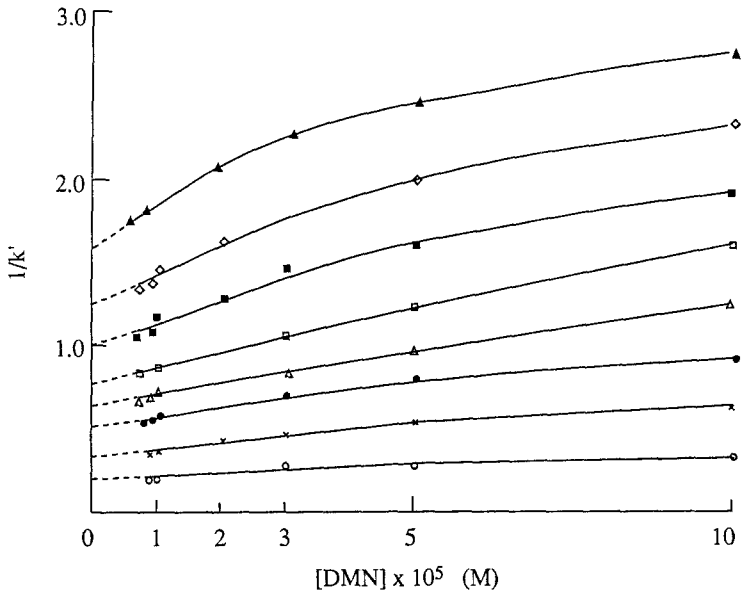


Figure 6. Determination of zero sample size $1/k'_0$ on the Lichrospher Diol column: extrapolation of plots representing the variation of $1/k'$ vs. sample DMN concentration, at varying concentrations of β-CD in the doting pH 7.4 buffer. β-CD concentrations: ▲ 14.2, ◇ 9.0, ■ 8.7, □ 7.1, △ 6.3, ● 4.7 × 3.2, ○ 1.6 mM.

[18] independently arrived at analogous equations, but they studied only single binding constants.

The following equilibrium between the guest and the stationary phase must be considered, according to [19]:

$$L + A \rightleftarrows LA \qquad K = [LA]/[L][A] \tag{4}$$

where A is the stationary phase adsorption site and K the association constant of the solute to the stationary phase binding site.

The retention equation for the system described by the system of Equations (1) to (4), according to [19], is:

$$\frac{1}{k'} = \frac{1}{\Phi K[A]} + \frac{K_1[CD]}{\Phi K[A]} + \frac{K_1 K_2[CD]^2}{\Phi K[A]} \tag{5}$$

where $k' = (V_r - V_0)/V_r$ is the capacity factor of the solute, Φ is the phase ratio (volume of stationary phase/volume of mobile phase or void volume (V_0).

Systems containing higher complexes are described by equations containing additional terms [36].

A graphical solution for Equation (5) is easy for a complex of the 1 : 1 type ($K_2 = 0$) since the plot of $1/k'$ versus [CD] is linear. It is also possible for a 1 : 2 stoichiometry, but somewhat complicated as some assumptions must be made

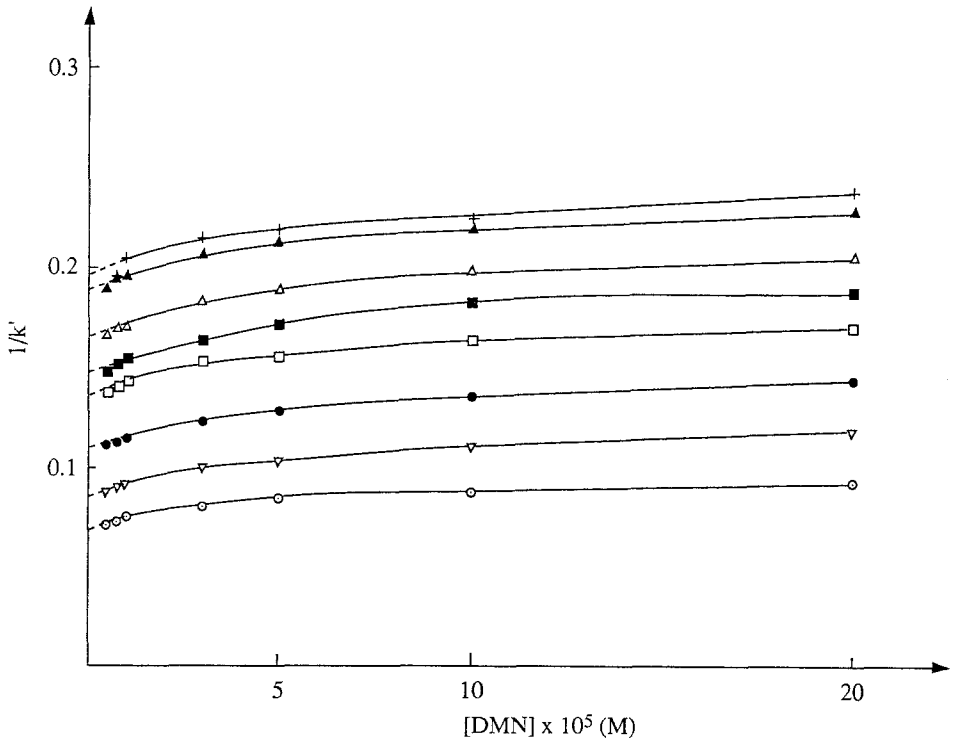


Figure 7. Determination of zero sample size $1/k'_0$ on the Poly LC column: (done as on Figure 6). β-CD concentrations: + 1.31, ▲ 1.23, △ 1.0, ■ 0.85, □ 0.62, ● 0.45, ▽ 0.29, ○ 0.17 mM.

[19]. Appropriate nonlinear least squares programs must be used to solve complex equilibria problems [36].

The existence of an L_2CD complex, i.e. the case where two guest molecules bind to a single cyclodextrin host, was not considered by us, because under the experimental conditions used, β-CD is in great excess relative to DMN.

Two types of columns were used for the study of DMN complexation by β-CD: a Lichrospher Diol column and a Polysulfoethyl column, respectively, for the studies at pH 7.4 and pH 4.2, since the retention time of DMN at pH 4.2 on the Lichrospher Diol column was too short. On both columns, in pure buffer, DMN is retained due to its interaction with the stationary phase. With an eluent containing β-CD, the drug retention is smaller due to its affinity for β-CD. But, in both cases, the peaks showed strong tailing, and concentration-dependent retention, indicating nonlinear binding isotherms to the stationary phase, as illustrated in Figures 4 and 5 for the Lichrospher Diol and the Poly LC columns, respectively.

As the retention volume depends on the amount of the solute injected, one has to extrapolate the retention volumes to zero sample size, for further consideration of the linear equilibrium isotherm [32]. Thus small amounts of DMN have to be injected, near the limit of the detector sentivity. Figures 6 and 7 show the plots

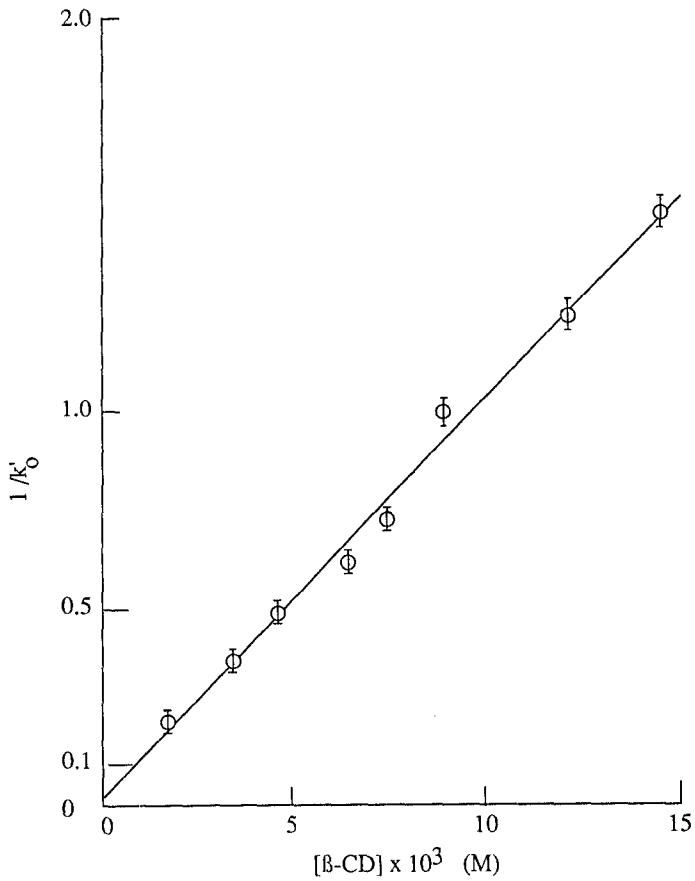


Figure 8. Retention method: plot of $1/k'_0$ vs. the concentration of β-CD in the eluent at pH 7.4.

of the variation of $1/k'$ as a function of DMN concentration respectively on each column, at various concentrations of β-CD. Extrapolation of the curves to zero DMN concentration allowed the determination of the $1/k'_0$ values.

Figures 8 and 9 present the linear variations of $1/k'_0$ versus the β-CD concentration obtained respectively at pH 7.4 on the Lichrospher Diol column and at pH 4.2 on the Poly LC column. These linear graphs are consistent with a 1 : 1 stoichiometry, in accordance with the result of the Hummel and Dreyer method. If $K_2 = 0$, Equation (5) is reduced to the first two terms and K_1 can be calculated from the ratio of the slope over intercept. But the error found on the determination of the Figure 8 intercept is of the same order of magnitude as the value itself which is near zero, and therefore K_1 determination is not as accurate as with the Hummel and Dreyer method. Its value is in the range 6000–11000 M^{-1}. The value found at pH 4.2 is $1900 \pm 1500\ M^{-1}$.

The advantage of this method is the possibility of using high concentrations of β-CD, until its solubility limit, and the linearity of Figure 8 over the whole

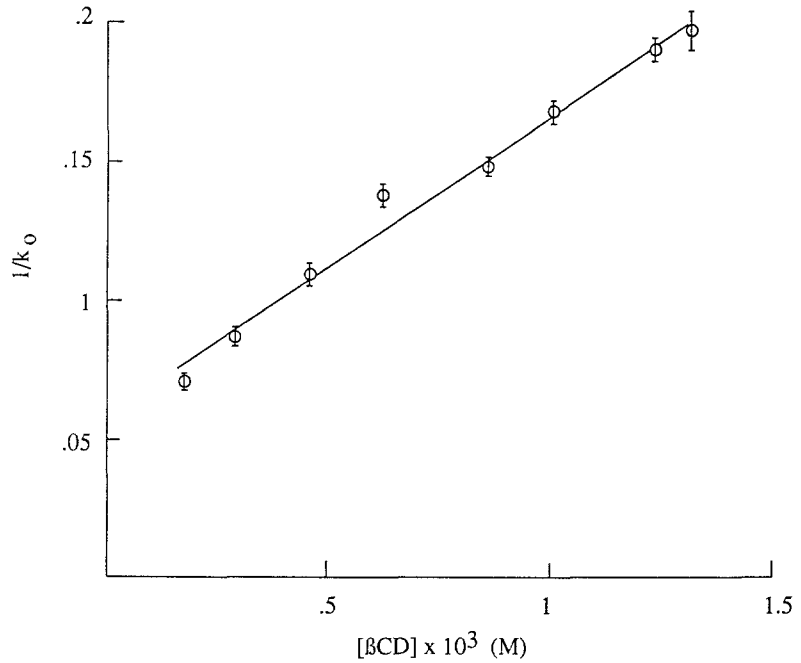


Figure 9. Retention method: plot of $1/k'_0$ vs. the concentration of β-CD in the eluent at pH 4.2.

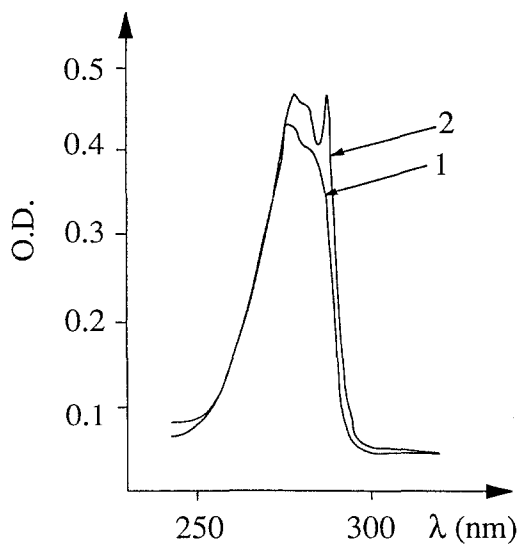


Figure 10. UV spectra in pH 7.4 phosphate buffer. Spectrum 1: (0.2 mM) DMN spectrum 2; (0.2 mM) DMN + (1.0) mM) β-CD.

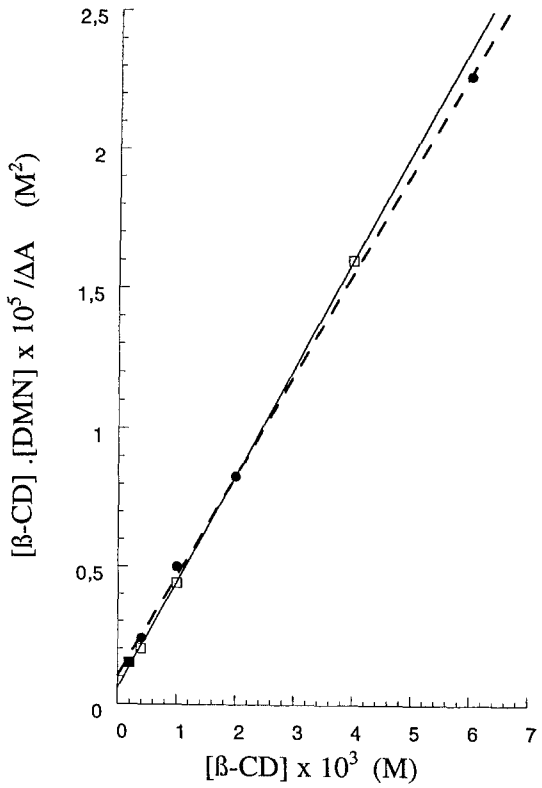


Figure 11. Benesi–Hildebrand diagrams (λ = 280 nm). - -□- - pH 7.4, - -●- - pH 4.2. Experimental conditions: mixtures of (0.2 mM) DMN and (0.1–6.0 mM) β-CD.

range of β-CD concentration, has proven that the stoichiometry of the β-CD-DMN complexation is 1 : 1.

3.2. UV Spectroscopy

The DMN UV spectrum shows a maximum absorbance at 277 nm and a shoulder at 283 nin at pH 7.4 in 0.1 M phosphate buffer (spectrum 1, Figure 10). The addition of β-CD involves a bathochromic effect, i.e. a displacement of the maximum to 280 nm, as is usually observed when a ligand is complexed by β-CD, and the appearance of another absorption peak at 287–300 nm according to the concentration of β-CD (spectrum 2, Figure 10). The variations of absorbance observed at 280 nm, at pH 7.4 and 4.2 respectively, when increasing concentrations of β-CD are added, are plotted in Figure 11, according to the Benesi and Hildebrand relation [37]:

$$[\text{DMN}][\beta\text{-CD}]/\Delta\text{A} = 1/K_{\text{ass}} \times 1/\Delta\varepsilon + [\beta\text{-CD}] \times 1/\Delta\varepsilon$$

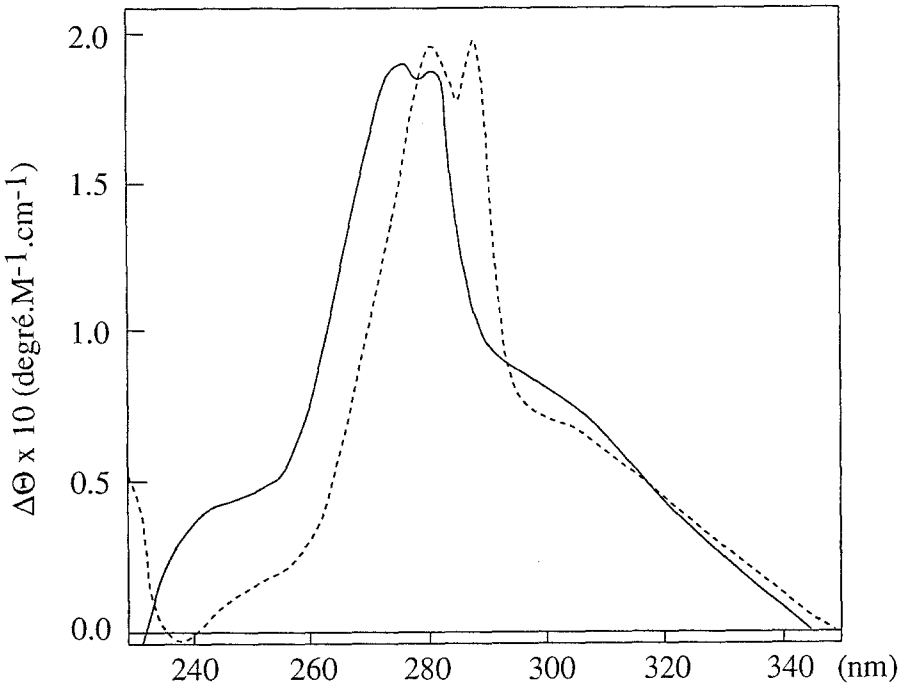


Figure 12. Circular dichroism spectra in pH 7.4 phosphate buffer. Spectrum 1: – (0.225 mM) DMN, spectrum 2: – (0.225 mM) DMN + (1.0 mM) β-CD.

where $\Delta\varepsilon$ is the difference of the molar extinction coefficients for free and complexed drug, ΔA is the change in the absorbance of the guest solution on adding β-CD, K_{ass} is the association constant of the complex, and [DMN] and [β-CD] are the total concentrations of the drug and of β-CD, respectively.

The linear plots obtained correspond to 1 : 1 complex formation and the association constants at pH 7.4 and 4.2 are calculated from the ratio of slope to intercept of the respective straight lines. Their values are 6600 ± 1000 M^{-1} at pH 7.4 and 3500 ± 800 M^{-1} at pH 4.2. These results are slightly lower than the chromatographic ones, but the measured absorbance variations, ΔA, are relatively low, which therefore suggests that these experiments lead to more imprecise results than the Hummel and Dreyer method values for the determination of association constants.

On the other hand we have noticed that the complex formation is nearly instantaneous. In fact, the spectra registered at 45 s, 5 min, 10 min, 20 min, 25 min and 40 min are quite similar.

3.3. Circular Dichroism

DMN is the D enantiomer of 3-methoxy-*N*-methylmethorphan and possesses an inherent circular dichroism spectrum, with two maxima at 276 nm and 280 nm,

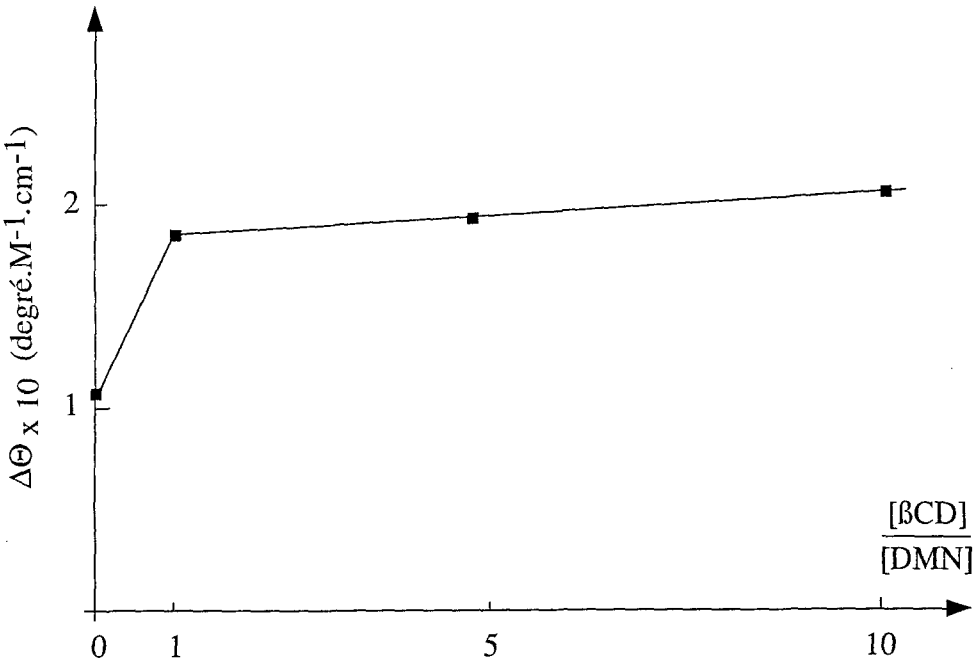


Figure 13. Variation of ellipticity $\Delta\theta$ at λ = 288 nm versus [βCD/DMN] ratio at pH 7.4, [DMN] = 0.225 mM.

approximately the same wavelengths as in UV, as shown on Figure 12 (spectrum 1). β-CD complexation induces spectral change with a maximum displacement towards higher wavelengths, to 280 nm and 288 nm respectively (spectrum 2, Figure 12). An enhancement of signal intensity with increasing concentration of β-CD, was observed (Figure 13). These results confirm the existence of a 1 : 1 complex since the ellipticity varies only slightly above a [β-CD]/[DMN] ratio equal to 1.

4. Conclusion

The different techniques used in the present work confirm the existence of a βCD-DMN complex of the 1 : 1 type. It seems that the formation constant of this complex was more precisely determined by the Hummel and Dreyer method, due to the amplitude of the measured signal compared to the other method. The complex is more stable at pH 7.4 than at pH 4.2. Moreover the study of the stability of this complex with temperature showed that the K_1 value decreased to one half from 20 °C to 37 °C.

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