Química Analítica[1], CONICET[2], Departamento de Física[3], Control de Calidad de Medicamentos[4], Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco y Pedernera. San Luis, Argentina

Determination of the binding constant of indomethacin-β-cyclodextrin complex by capillary electrophoresis: experimental optimization and temperature study

G. Acosta[1,2], D. Linares[3], R. Olsina[1,2], L. D. Martínez[1,2], M. R. Gomez[2,4]

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Dr. Mariá R. Gomez, Universidad Nacional de San Luis, Facultad de Química, Bioquímica y Farmacia, Area Quimica Analytica, Chacabuco y Pedernera, 5700 San Luis, Argentina
roxanag@unsl.edu.ar

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The apparent electrophoretic mobilities of indomethacin in β-cyclodextrin at a range of concentrations were measured directly by capillary electrophoresis. Three different linear plots and a non linear plot are proposed for the apparent binding constant calculations, based on the fact that the molar ratio of the inclusion complex was 1 : 1. K values obtained at 298 K were 421 M^{-1} (double reciprocal fit), 488 M^{-1} (x-reciprocal fit), 428 M^{-1} (y-reciprocal fit) and 490 M^{-1} (non linear fit). The corresponding K values at 313 K were 380 M^{-1} (double reciprocal fit), 355 M^{-1} (x-reciprocal fit), 366 M^{-1} (y-reciprocal fit) and 339 M^{-1} (non linear fit). Using the proposed methods, the binding constant of the indomethacin – β-cyclodextrin inclusion complex can be obtained easily. The methods have been applied to obtain the values of the constant K under different experimental conditions. Under optimized conditions the K constant is temperature dependent and non-arrhenian behaviour was observed.

1. Introduction

For analytes involved in dynamic equilibrium processes, capillary electrophoresis (CE) is a powerful method of determining binding constants. Equilibrium constants from capillary electrophoresis for the apparent binding of drugs to β-cyclodextrin (β-CD) show good agreement with literature values obtained using the reported methods, confirming that capillary electrophoresis is a viable technique for this purpose.

The main interest in cyclodextrins lies in their ability to form inclusion complexes with several compounds of pharmaceutical interest (Gutman et al. 1996; Wu and Ding 2005; Wang et al. 2006; Loftsson et al. 2005); this property and the recent biotechnological advances which have resulted in dramatic improvements in cyclodextrin production have led to their widespread use in pharmaceutical studies (Szejtli et al. 2005; Shimpi et al. 2005; Kanjickal et al. 2005). Complexation of poorly soluble drugs with cyclodextrins is a useful approach to improving drug dissolution and drug stability (Wu and Ding 2005). Indomethacin is an anti-inflammatory agent which is practically insoluble in water, and it has been reported that the bioavailability of this drug is improved by complexation with β-CD (Jambhekar et al. 2004).

Apparent equilibrium constants give us an approximate idea of drug-CD interactions. Many efforts have been directed to the development of new fast and reliable methods for determining apparent equilibrium constants. CE is a separation technique that takes advantage of changes in the electrophoretic mobility of the complexed solute in the background electrolyte (BGE) containing the complexation agent (ligand) (Berthod et al. 2002; Heegaard et al. 1998; Kajiwara et al. 1991; Chu et al. 1992; Honda et al. 1992; Heegaard and Robey 1992; Shimura and Kasai 1997; Heegaard 2003; Schultz and Kennedy 1993). CE allows the analysis of molecular interactions in free solution with relatively easy to use and simple instrumentation and can be used for the study of indomethacin-β-CD interactions.

The stability of the inclusion complex and the selectivity of the complexation process are determined by the fit of the whole or at least a part of the guest molecule in the hollow space in CD. In order to predict changes in the physico-chemical properties of the drug after inclusion in the CD cavity, it is important to determine the K value accurately.

The driving forces for complexation are both enthalpic and entropic in nature and are not fully understood. Fitting into the cyclodextrin torus is crucial for many biomedically relevant molecules, but is not the only consideration. The properties of both the part of the molecule interacting with the CD and the portion that is likely to be outside the cyclodextrin appear to be equally critical. Naturally occurring CDs, in particular β-cyclodextrin, have limited aqueous solubility, owing to CDs forming intramolecular hydrogen bonds between secondary OH groups, which detracts from hydrogen bond formation with surrounding water molecules, resulting in heats of hydration being less negative. Thus, intramolecular hydrogen bonding can result in relatively unfavourable enthalpies of solution and low aqueous solubilities.

In a previous study (Jambhekar et al. 2004) no correlations were found among the bioavailability, the binding constant measured by phase solubility analysis, and the dissolution of the indomethacin-β-CD complex. The apparent binding constant describes the extent of association, but provides insufficient information to predict the time required for dissociation of the complex. Although complexation efficacy depends on the binding constant of the inclusion complex, the "ultimate stability of complexation" is controlled by the entropy term (Rozou et al. 2005).

The entropic contribution to the association constant can be analyzed by its temperature behaviour. In order to analyze such dependence, in the present work we evaluated the temperature behaviour of the apparent association constants for the indomethacin-β-CD complex. Spectrophotometric and fluorescence methods have been reported previously and the molar ratio of the inclusion complex has been established as 1 : 1 (Jambhekar et al. 2004). The K values previously obtained were: 760 M^{-1}, 303 K (NMR) (Redenti et al. 2001); ~1000 M^{-1}, 298 K (phase solubility study) (Jambhekar et al. 2004) and 423 M^{-1} (double reciprocal fit), 486 M^{-1} (x-reciprocal fit), 452 M^{-1} (y-reciprocal fit), 298 K (capillary electrophoresis) (Bellini et al. 2001).

2. Investigations, results and discussion

2.1. Theory

As stated by Bellini et al. (2001) the following equation describes a system in which the solute (S) binds to a ligand (L) in a 1 : 1 ratio:

$$S + L = SL \tag{1}$$

The electrophoretic mobility (μ) of the solute in a background electrolyte (BGE) containing the ligand is the weighted average of the complexed (μ_c) and uncomplexed (μ_f) solute:

$$\mu_i = X_f\mu_f + X_c\mu_c \tag{2}$$

where μ_i is the experimentally measured mobility and X represents the molar fraction of the solute in the free (X_f) and complexed (X_c) state.

Introducing equilibrium concentrations the following equation is obtained,

$$\mu_i = \frac{[S]}{[S]+[SL]}\,\mu_f + \frac{[SL]}{[S]+[SL]}\,\mu_c \tag{3}$$

Using the expression for the equilibrium constant for the case considered:

$$K = \frac{[SL]}{[S]_f + [L]_f} \tag{4}$$

and combining this expression with Eq. (3), the following equation for the solute mobility at any given concentration of the complexing agent in the BGE can be derived,

$$K[L] = \frac{(\mu_f - \mu_i)}{(\mu_i - \mu_c)} \tag{5}$$

from which it follows that:

$$\mu_i = \frac{\mu_f + \mu_c K)[L]}{1 + K[L]} \tag{6}$$

Clearly the constant K obtained in this way refers to concentrations and is not the true thermodynamic equilibrium constant (Rundlett and Armstrong 1997).

To calculate the constant K, three different well-known linearization methods were used. The first method is obtained by rearranging the Eq. (6) as follows,

$$\frac{1}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f)\,K}\,\frac{1}{[L]} + \frac{1}{(\mu_c - \mu_f)} \tag{7}$$

Plotting $1/(\mu_i - \mu_f)$ versus 1/[L] we obtain the apparent binding constant as K = intercept/slope. This method is also called the double reciprocal plot.

The second method is also derived from Eq. (6) and has been named the y reciprocal plot, in which the apparent binding constant can be found as K = slope/intercept by plotting $[L]/(\mu_i - \mu_f)$ versus [L].

$$\frac{[L]}{(\mu_i - \mu_f)} = \frac{1}{(\mu_c - \mu_f)}\,[L] + \frac{1}{(\mu_c - \mu_f)\,K} \tag{8}$$

The following method is the × reciprocal plot, in which K is – slope from $(\mu_i - \mu_f)/[L]$ versus $(\mu_i - \mu_f)$.

$$\frac{(\mu_i - \mu_f)}{[L]} = -K(\mu_i - \mu_f) + K(\mu_c - \mu_f) \tag{9}$$

The three methods give similar values for the binding constant within the experimental error bars.

2.2. Method development

In order to propose a precise way to calculate binding constants using CE, it is necessary to find the best experimental conditions. The optimization was performed using a sample containing indomethacin and DMSO. The following parameters were consecutively optimized: sample conditioning, pH, BGE composition and concentration, and other electrophoretic parameters such as type and time of injection, pressure injection, etc.

2.2.1. Effect of pH

The buffer pH plays an important role in improving reproducibility in CE because it affects both the overall charges of the solute and the electro-osmotic flow.

The pK_a value for indomethacin is 4.5. Thus, the effect of the buffer pH was investigated within the range of 5.0–10.0 at a fixed buffer concentration, adjusted with 0.1 mol · l^{-1} NaOH and 0.1 mol · l^{-1} HCl. It was found that when the pH was lower than 6.5, the precision was poor. At pH 9.2 reproducible results were achieved.

2.2.2. Effect of buffer composition and concentration

Buffer concentration also has a significant effect on separation performance through its influence on the electro-osmotic flow (EOF) and the current produced in the capillary. Different BGEs were tested (sodium acetate, sodium phosphate, sodium tetraborate buffer and their combinations), and sodium tetraborate produced the best results with respect to reproducibility and current performance.

Keeping other parameters constant (pH: 9.2, 25 kV, 25 °C) the buffer concentration was varied from 10–75 mM. Increases in migration times because of decrease of EOF as well as increased current were observed when the buffer concentration buffer increased. The best performance was achieved with 25 mM sodium tetraborate buffer.

BGE solutions were prepared daily, and the β-CD concentration range studied was within the range 0.75–10 mM.

Voltage applied was varied between 10–30 kV, and 25 kV was chosen. The hydrodynamic and electrokinetic modes

of injection were evaluated; both methods are efficient, however the electrokinetic mode showed a higher % R.S.D. of time migration concerning precision, and consequently hydrodynamic injection was selected. The sample injection pressures used ranged between 0.1 and 2 psi; higher reproducibility was achieved when the pressure was 0.5 psi. Injection times tested were between 0.5 and 7 s; a time of 5 s was selected. In summary, the selected conditions were sodium tetraborate buffer 25 mM, pH 9.2; 25 kV; hydrodynamic injection at 0.5 psi for 5 s.

2.3. Determination of the binding constant

The binding constants of the inclusion complexes of β-cyclodextrin with indomethacin (IND) were determined on the basis of CE experiments. The change of electrophoretic mobility of IND was monitored as a function of β-CD added. Since changes in peak migration times may be due to non-specific effects, such as changes in the electro-osmotic flow, a neutral non-interacting EOF marker should be present in the sample to adjust the analyte migration time (Gomez et al. 2003). A typical electropherogram is reproduced in Fig. 1. The first peak corresponds to DMSO, which was selected as the EOF marker. The second peak corresponds to the IND ion. Upon complexation with β-CD (Fig. 1a), the indomethacin ion will move faster due to its incorporation in the CD cavity, and its peak will move closer to the DMSO peak.

Experimental values of the effective electrophoretic mobilities ($cm^2 \cdot V^{-1} \cdot s^{-1}$) were calculated as the difference between the apparent mobility in each buffer (with various quantities of CD) and that of the neutral marker. Three conventional linearization methods (y-reciprocal, x-reciprocal and double reciprocal) were employed to estimate the binding constants by least squares linear regression (Cirri et al. 2005).

When ligand concentration is lower than 0.2 or higher than 10.0, changes in mobility resulting from complexation are small compared to the random error. At high ligand concentrations, the analyte is, in fact, almost completely in the complexed form, while at low concentrations, the fraction of analyte in the complexed form is not high enough to give sufficient information about K. Thus, in order to minimize the error on the calculated constants and mobilities, the range of β-CD concentration was set at 0.75–10.0 mM (Lynen et al. 2001). Fig. 2 shows the mobilities (μ_i) obtained at different β-CD concentrations at 298 and 313 K. In Fig. 3 the double reciprocal, y-reciprocal and x-reciprocal calculated data are plotted for the selected range of concentrations at 298 and 313 K.

As already mentioned, in order to find the best experimental conditions we performed the experiments at three different values of pH, using phosphate and tetraborate buffer (pH 5, 7 and 9). Sodium tetraborate buffer, pH 9.2, showed the best performance in terms of reproducibility and time of analysis.

The values of the ligand constant K were similar to those obtained by other authors using different experimental conditions (Bellini et al. 2001). Table 1 shows apparent binding constants (K) of the indomethacin-β-CD complex estimated using the three types of linearization fit (double reciprocal, X- and Y-reciprocal) and non linear regression as well as the corresponding errors.

Next we present the performance of the method with respect to precision. The results are presented in Table 2, where we can see the % RSD for a given temperature and BGE concentration. Repeatability refers to the variability

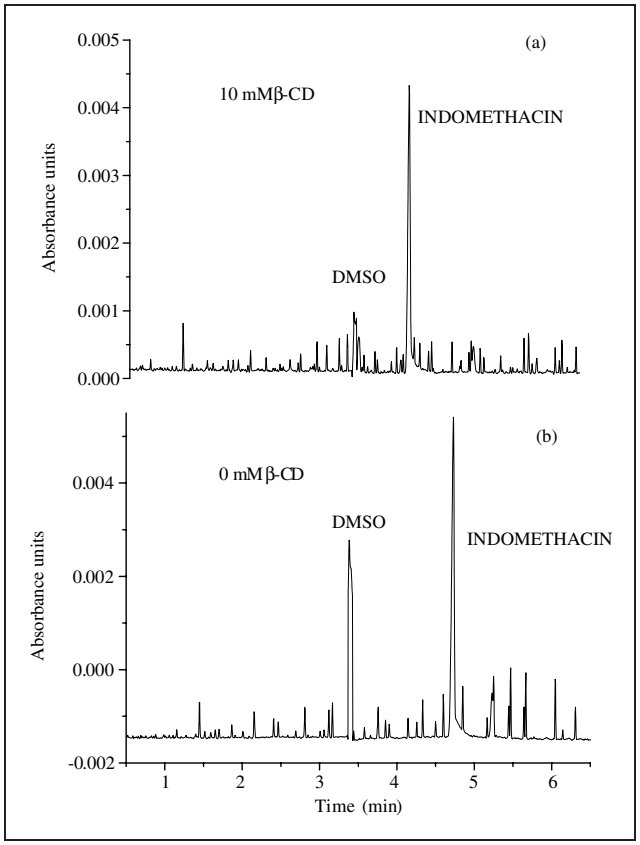

Fig. 1: Electropherograms of indomethacin (IND) in pH 9.2 sodium tetraborate buffer at 298 K in the presence of increasing concentrations of β-CD. 1a) 10 mM β-CD; 1b) 0 mM β-CD

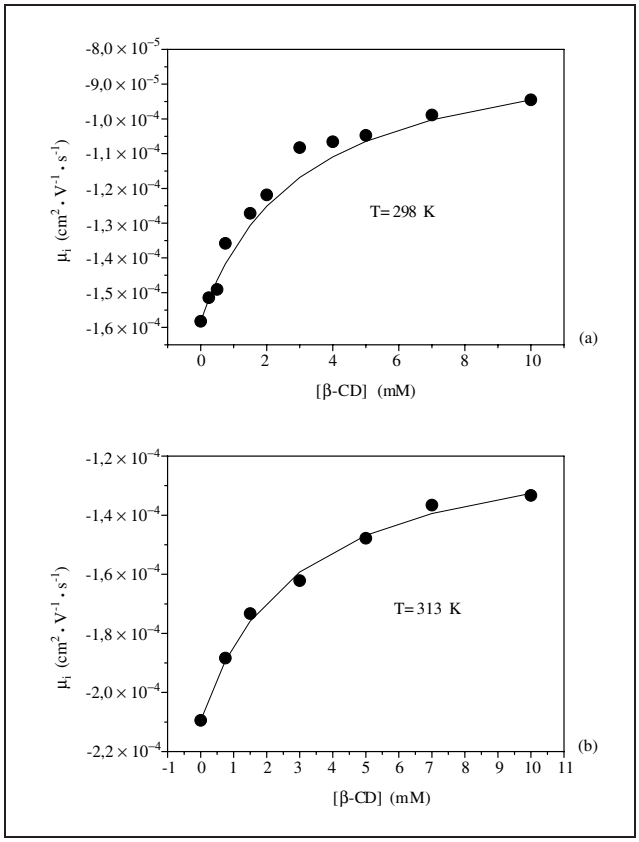

Fig. 2: Binding isotherms for a given range of additive (ligand) concentrations at 298 K (a) and 313 K (b)

Table 1: Apparent binding constants (K) of indomethacin-β-CD complex estimated from three types of linearization fit (double reciprocal, X- and Y-reciprocal) and non-linear regression in 25 mM tetraborate buffer, pH 9.2 at 298 K and 313 K

	298 K			313 K		
	K (M^{-1})	dK/K[a]	R^2	K (M^{-1})	dK/K[a]	R^2
Double reciprocal fit	421	0.13	0.976	380	0.09	0.995
X-reciprocal fit	488	0.12	0.995	355	0.12	0.993
Y-reciprocal fit	428	0.14	0.907	366	0.10	0.963
Non linear fit	490	0.17	0.989	339	0.17	0.995

[a] Error

Table 2: Precision results

Method Precision	
System repeatability (n = 10)	R.S.D. (migration time): 0.78%
Method repeatability (n = 10)	R.S.D. (migration time): 1.51%
Intermediate precision (n = 6)	R.S.D. operator (migration time): 1.41% R.S.D. inter-day (migration time): 0.84%

when different aliquots of the sample are tested on the same day (n = 6). Intermediate precision refers to the disparity among the data obtained under different experimental conditions (operator, day of operation). As is well known, when the measured mobility μ_i is too close to its μ_f value, poor precision of the $\mu_i - \mu_f$ term may be expected. This was observed at certain temperatures, particularly those higher than 323 K.

Moreover we have to take into account not only binding but also other effects which could affect analyte mobility. In fact, the viscosity and ionic strength of the BGE can be affected by changing the BGE concentration, as well as the adhesion to the capillary wall of solute or ligand molecules. At pH 6.5 there is a weak effect of pH changes on the variability of K values because of the acidic character of indomethacin; this compound behaves as an anion at pH 5.5. Under the optimized experimental conditions regarding pH, concentration and nature of the BGE as well as electrophoretic parameters, the standard deviation was minimal.

The equilibrium constant of a reaction changes when the temperature is changed. The easiest way to derive the magnitude and direction of the effect is to consider the effect of temperature on ΔG, the change in Gibbs free energy, and to do so we return to its expression in terms of $\Delta H - T\Delta S$ (where T is the temperature, ΔH and ΔS are the changes in the enthalpy and entropy, respectively). The relation between ΔH, ΔS and K is given by the well known van't Hoff equation,

$$R \ln K = -\frac{\Delta H}{T} + \Delta S \qquad (10)$$

A linear relationship of ln K vs 1/T indicates the independence of ΔH and ΔS of T, while the absence of such behaviour shows that these quantities are temperature dependent. In Fig. 4 we have plotted R ln K vs 1/T for indomethacin-β-CD complexes. We shall make the approximation that the standard reaction enthalpy and entropy are almost independent of temperature over the range of temperature studied. If we do so, the value of the reaction enthalpy and entropy can be determined as the slope and the intercept of the linear fit, giving $\Delta H \sim -14.7 \pm 0.75$ kJ and $\Delta S \sim -26.1 \pm 2.5$ J, which is in the acceptable range. Despite the approximation in the linear response of the van't Hoff equation, some general considerations can be made with respect to the temperature behaviour of the equilibrium constant. The process is exothermic, $\Delta H < 0$,

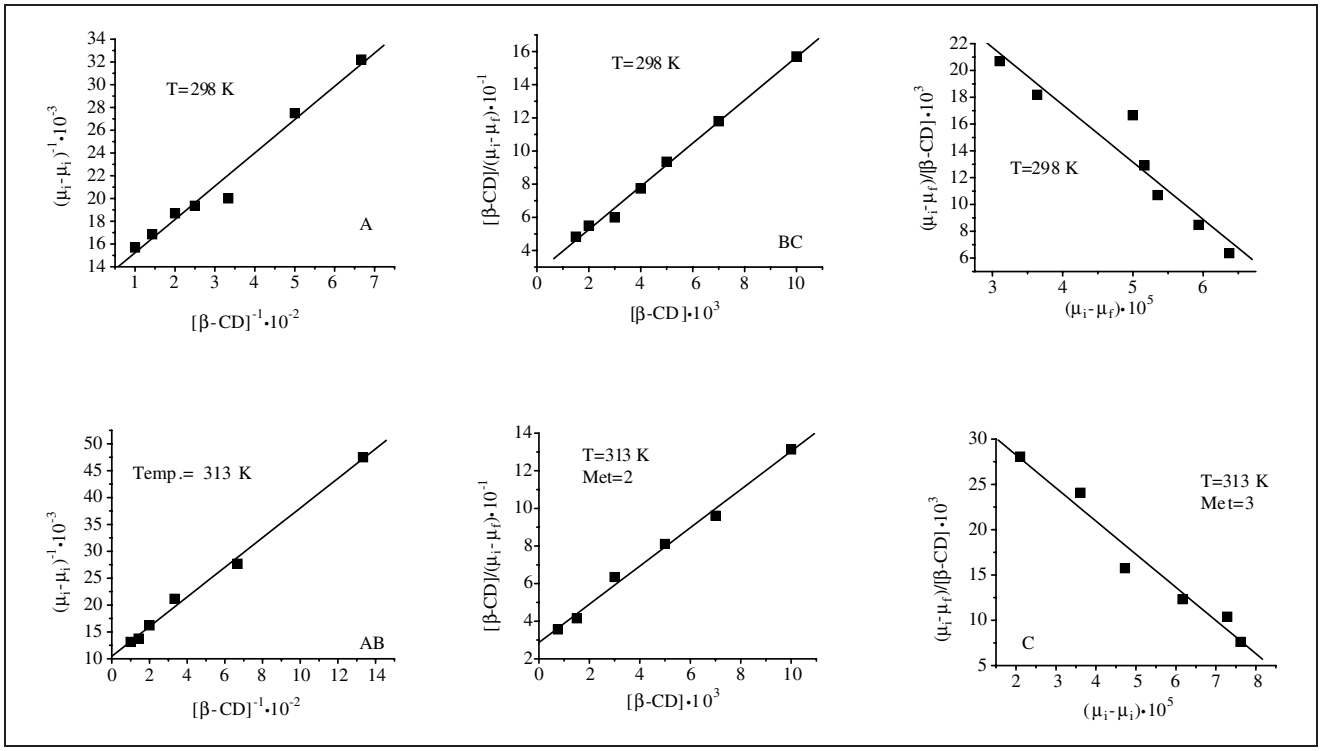

Fig. 3: Comparison of three different linearization methods of mobility data for indomethacin: (A) double reciprocal plot; (B) x reciprocal plot; (C) y reciprocal plot

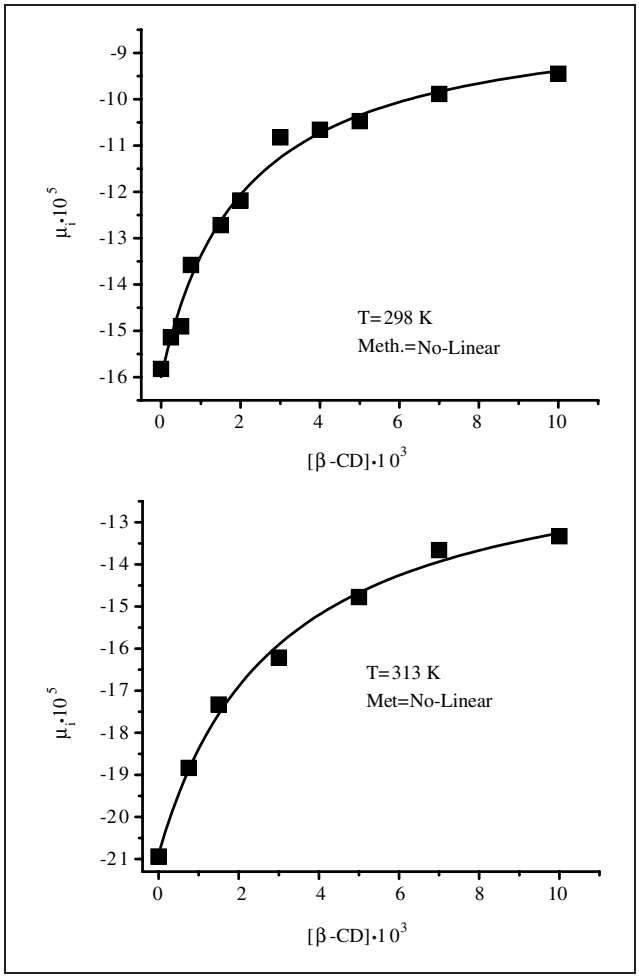

Fig. 4: Non-linear method at 298 K and 313 K

therefore the equilibrium constant decreases with temperature as is observed in Figure 5. We cannot conclude that the reaction is enthalpy driven ($|\Delta H| \sim T\,|\Delta S|$), as is usually observed for association between small guest molecules and the CD-cavity (Junquera and Aicart 1997; Rekharsky et al. 1994; Stauffer et al. 1990). Direct calorimetric measurements would be necessary to elucidate the thermodynamic behaviour.

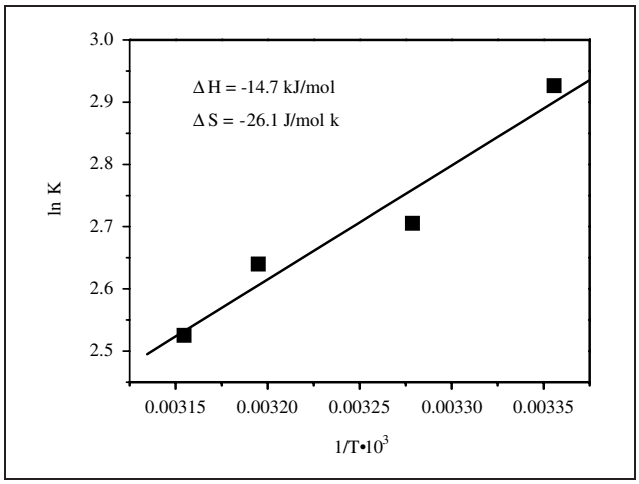

Fig. 5: Van't Hoff plot for CD-indomethacin complex. Experimental data as symbols (○), linear fit in solid line

3. Experimental

3.1. Instrumentation

A Beckman P/ACE MDQ instrument (Beckman Instruments, Inc. Fullerton, CA) equipped with a diode array detector and a data handling system comprising an IBM personal computer and P/ACE System MDQ Software was used. Detection was performed at 200 and 198 nm. The fused-silica capillaries were obtained from MicroSolv Technology Corporation (NJ, USA) and had the following dimensions: 57 cm total length, 50 cm effective length, 75 μm ID, 375 μm OD. The temperature of the capillary was kept within the range of 298 K–313 K, and the samples were stored at 298 K.

3.2. Chemicals

Indomethacin was purchased from Sigma Chemical Co. (St. Louis, MO); sodium tetraborate ($Na_2B_4O_7 \cdot 10\,H_2O$) and sodium dihydrogenphosphate (NaH_2PO_4) by Mallinckrodt (St. Louis, MO). Dimethylsulfoxide (DMSO) was supplied by Merck (Buenos Aires, Argentina). The water used in all studies was ultra-high-quality water obtained from a Barnstead Easy pure RF compact ultra pure water system. All other reagents and solvents were of analytical grade. All solutions were degassed by ultrasound (Testlab, Argentina). Running electrolytes and samples were filtered through a 0.45 μm Titan Syringe filter (Sri Inc., Eaton Town, NJ. USA).

3.3. Procedure

The electrolyte solution was prepared daily and filtered through a 0.45 μm Titan Syringe filter. At the beginning of the day, the capillary was conditioned with 0.1 mol · l^{-1} NaOH for 10 min, followed by water for 10 min, and then with running electrolyte for 10 min before sample injection. To achieve high reproducibility of migration times and to avoid solute adsorption, the capillary was washed between analyses with 0.1 mol · l^{-1} NaOH for 3 min, followed by water for 3 min, then equilibrated with the running buffer for 3 min. Samples were pressure-injected at the anodic side at 0.5 Psi for 5 s. A constant voltage of 25 kV was used for all the experiments.

Different organic solvents were tested as electro-osmotic flow (EOF) markers; the best results were obtained using DMSO. Stock standard solutions of indomethacin (1 mg · ml^{-1}) and DMSO (1%, v/v), were prepared in water, stored at 4 °C and used within one week. Working standard solutions were prepared daily by adding 50 and 250 μl of each of the stock solutions directly to a vial and diluting to 500 μl with 25 mM pH 9.2 tetraborate buffer, in order to obtain the desired final concentrations for indomethacin (0.1 mg · ml^{-1}) and for DMSO (0.1%, v/v). All solutions were filtered through a 0.45 μm membrane prior to injection.

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