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Indirect capillary electrophoresis with 8-anilino-1-naphthalenesulfonic acid as a fluorescence probe for determining the apparent stability constant of an inclusion complex formed between a cyclodextrin and a solute

Tianlin Wang, Huatao Feng, Sam Fong Yau Li*
Department of Chemistry, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore

Abstract

An indirect capillary electrophoresis (CE) method was developed based on two competitive chemical equilibria for determining the stability constant of an inclusion complex formed between a cyclodextrin and a solute. 8-Anilino-1-naphthalenesulfonic acid was employed as a fluorescence probe. A linear relationship between mobility difference and concentration of uncomplexed ligand was theoretically established and experimentally verified. The principle of the method was explained using an example of determining stability constant of an inclusion complex formed between a ligand of hydroxypropyl-β-cyclodextrin and a solute of amantadine. The stability constant was determined to be ~$2 \cdot 10^2$ M^{-1}. It was calculated without knowledge of the mobility of the complex measured at saturating ligand concentrations. This indirect method can be applied to solutes and ligands lacking signal response on the selected detector in the CE. In addition, the indirect method is valid for both charged and neutral solutes and ligands.
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Keywords: Stability constants; Complexation; Anilinonaphthalenesulfonic acid; Cyclodextrins; Amantadine

1. Introduction

Cyclodextrins (CDs) are a group of cyclic oligosaccharides composed of α(1,4)-linked glucopyranose units [1]. The most common have six, seven, and eight units with the common names α-, β-, and γ-cyclodextrin, respectively. Cyclodextrins are interesting molecules, appealing to investigators in both pure research and applied technologies. Biological chemists, physical chemists, and synthetic chemists all work shoulder to shoulder in their pursuit of the creation, analyses, and uses of these captivating molecules [2].

The utility of cyclodextrins stems from their molecular shapes [3], which are often described as ''turos-like''. The wide rim is composed of (carbon 2) C2-OH and C3-OH groups, while the narrow rim is composed of C6-OH groups. The molecules, in their most symmetrical forms, resemble truncated cones with a sizable inner cavity. CDs (hosts) form inclusion complexes with many kinds of solutes (guests) of molecules and ions, either in the solid phase or in solution. In solution, water molecules occupy the hydrophobic cavity in the absence of a solute molecule. However, a specific solute molecule that is more hydrophobic than the cyclodextrin, when

*Corresponding author. Tel.: +65-6874-2681; fax: +65-6779-1691.
E-mail address: chmlifys@nus.edu.sg (S.F.Y. Li).

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added into the cyclodextrin solution, drives water molecules out of the cavity by occupying the cavity itself and produces inclusion complex. This feature makes them suitable for an extremely large number of applications. Applications of cyclodextrin systems include biomimetic reactions, artificial enzymes, and drug carriers which dominate the industrial use of cyclodextrins.

The stability of an inclusion complex formed by the ligand of a cyclodextrin and a hydrophobic solute of suitable size is characterized by its stability constant. This constant is an important and widely usable thermodynamic parameter. Both theoretical and practical aspects of the stability of cyclodextrin complexes in solution were presented in a recently published review [4]. Various methods (solubility measurement, absorption spectroscopy, fluorescence spectroscopy, nuclear magnetic resonance, electroanalytical chemistry, reaction kinetics, calorimetry, chromatography, etc.) have been employed for determining this stability constant. A comprehensive review article covered the methodology and procedure of these methods in detail [5]. Unfortunately, these methods often lack accuracy and need large amounts of solute and/or ligand [6].

On the one hand, native and derivatized CDs have been proven to be useful selectors for the separation of a large number of molecules by capillary electrophoresis (CE) [7–9]. On the other hand, CE has been increasingly employed for determining stability constants of inclusion complexes between CDs and many kinds of solutes [6,10–24], as well as other chemical equilibrium constants [25–47]. The success of this approach may be attributed to a combination of separation and quantitation in one operation usually without requiring labeling reagents [48].

For the determination of stability constants of inclusion complexes by CE, general strategies are currently to measure the electrophoretic mobilities of free and complexed solutes. Then, the equilibrium constant can be graphically obtained by plotting μ_f (electrophoretic mobility of free solute), μ_c (electrophoretic mobility of complexed solute) and μ_i (electrophoretic mobility of the solute measured at ligand concentration of $[L]$). We refer these strategies to direct CE methods since both μ_f and μ_i are directly involved in the experimental measurements and calculation procedures. Although these direct CE methods have been established, there are shortcomings in several aspects. Measuring μ_c directly requires saturating ligand concentrations. The measurements can be difficult or even impossible in many systems [11]. Large errors in measuring μ_c may arise due to significant changes in viscosity, ionic strength and/or conductivity when buffer composition has been changed significantly. Solubility of the ligand may impose another constraint. Use of non-linear curve fitting was proposed to avoid these problems [10–12]. But the non-linear curve fitting still requires measurements at many levels spanning over a broad range of concentrations of the ligand to reduce the error in μ_c. In addition, analysis time and material consumption are not effectively utilized. A solute or its complex being able to generate a reasonable response on the selected detector in CE is an explicit requisite for the direct methods. When both the solute and ligand are neutral species, all the direct CE methods become invalid.

Previously, Shimura and Kasai reported on utilizing competitive equilibria for indirectly determining the equilibrium constant in affinity capillary electrophoresis [35]. Since a compound not commercially available was used as a probe in their experiments and data processing was not carried out in a straightforward way, the advantages of an indirect method could not be readily appreciated in their work. We present a simple and indirect CE approach to determine the stability constant of an inclusion complex based on competitive complexation equilibria in this report. When the indirect method is applicable, the above-mentioned drawbacks of the direct CE methods can be avoided. Moreover, the indirect method does not require suitable response on the detector employed from the solute and the ligand. It can be applied to both changed and neutral solutes and ligands. Consequently, applications of CE methods in determining the stability constants will be expanded.

For simplicity in elucidating the principle of the indirect CE method, we shall limit ourselves to a 1:1 complexation system. We shall consider apparent stability constant with omission of the effect of acid–base equilibria. Stability constant will be examined in terms of concentration instead of activity. We choose hydroxypropyl-β-cyclodextrin as the

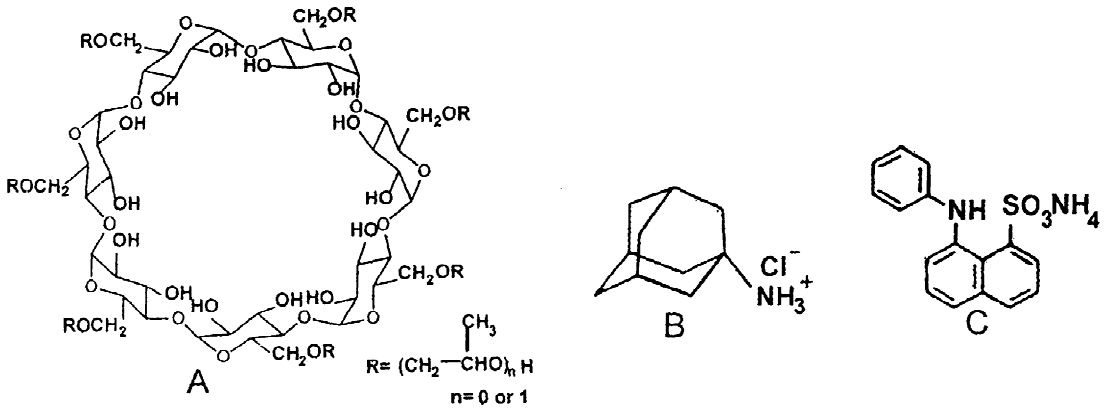

Fig. 1. Chemical structures of compounds used in this work: (A) hydroxypropyl-β-cyclodextrin (ligand); (B) amantadine hydrochloride (solute); (C) 8-anilino-1-naphthalenesulfonic acid ammonium salt (probe).

ligand, amantadine (an antiviral drug [49]) as the solute, and 8-anilino-1-naphthalenesulfonic acid as the probe in this work. Structures of these compounds are given in Fig. 1.

2. Materials and methods

2.1. Materials and solutions

Sodium dihydrogenphosphate and sodium monohydrogenphosphate were purchased form Fluka (Buchs, Switzerland). Amantadine hydrochloride was obtained from Merck (Darmstadt, Germany). 8-Anilino-1-naphthalenesulfonic acid (ANS) and dimethyl sulfoxide were supplied by Sigma (St. Louis, MO, USA). Hydroxypropyl-β-cyclodextrin (HP-β-CD) (MS = 0.8) was provided by Aldrich (Milwaukee, WI, USA). Fused-silica capillaries (75 μm I.D.) were products of Polymicro Technologies (Phoenix, USA). Total length of the capillaries was 75.0 cm, and effective length from the injection end to the detection window was 54.0 cm. Water (≥18 MΩ) used throughout the experiments was supplied by a NANOpure ultrapure-water-purification system (Barnstead, IA, USA).

The following solutions of pH 7.0 were prepared daily:

Solution A: 0.050 *M* phosphate buffer (buffer used for measuring mobility of ANS in the absence of the ligand of HP-β-CD).

Solution B: $5\cdot10^{-4}$ *M* ANS in solution A (buffer used for measuring electroosmotic flow (EOF) to correct for the mobility of ANS in the absence of the ligand of HP-β-CD).

Solution C: Adding 15% methanol to solution A (sample used for all measurements of electroosmotic flow).

Solution D series: HP-β-CD in solution A at respective concentrations of $5.0\cdot10^{-4}$, $1.0\cdot10^{-3}$, $1.5\cdot10^{-3}$ and $2.0\cdot10^{-3}$ *M* (buffers used for measuring mobility of ANS in the presence of the ligand of HP-β-CD).

Solution E series: $2.0\cdot10^{-5}$ *M* ANS in solution D series (buffer used for measuring EOF to correct for the mobility of ANS in the presence of the ligand of HP-β-CD).

Solution F: $1.0\cdot10^{-3}$ *M* amantadine hydrochloride and 1.0 m*M* HP-β-CD in solution A (buffer used for measuring mobility of ANS in the presence of both the ligand of HP-β-CD and amantadine).

Solution G: $2.0\cdot10^{-5}$ *M* ANS in solution F (buffer used for measuring EOF to correct for the mobility of ANS in the presence of both the ligand of HP-β-CD and amantadine).

2.2. Instruments

All CE experiments were carried out using the CE-L1 capillary electrophoresis system (CE Resources, Singapore) with an RF551 spectrofluorometric detector from Shimadzu (Kyoto, Japan). De-

tection was made at 365 nm for excitation and 495 nm for emission. Data acquisition and recording electropherograms were accomplished with CSW Chromatography Station (CE Resources, Singapore).

2.3. Procedure for CE experiments

A new capillary was rinsed with 0.1 *M* NaOH for 10 min, followed by the run buffer for another 10 min. Sample introduction was made in the hydrodynamic mode. After sample introduction, 12-kV voltage was applied for separation. Prior to changing a new buffer, the capillary was allowed to equilibrate with the buffer for 5 min by rinsing. The CE experiments were carried out at a temperature of 24.5±0.5 °C.

3. Results and discussion

3.1. Select a probe and plot a calibration curve for the ligand

The general equation for the equilibrium formation of any 1:1 complex is given by Eq. (1).

$$S + L \Leftrightarrow SL \quad (1)$$

where S refers to solute, L refers to ligand, and SL refers to complex formed between the solute and the ligand. The equilibrium constant, i.e. apparent stability constant, for the above process is denoted by K and is defined as:

$$K = [SL]/([S][L]) \quad (2)$$

The square brackets indicate molar concentrations of solute, ligand and complex.

Eq. (3) presented by Alberty and King [50] can be adapted for relating the stability constant to the electrophoretic mobilities of the species involved in the above equilibrium:

$$(\mu_S - \mu_i)/(\mu_i - \mu_{SL}) = K[L] \quad (3)$$

where K is the stability constant, $[L]$ is the equilibrium concentration of uncomplexed ligand, and μ_S, μ_{SL} are the electrophoretic mobilities of free and complexed solute; μ_i is the effective mobility of solute measured at ligand concentration $[L]$. Eq. (3) is an equivalent to Eq. (4):

$$\mu_i = (\mu_S + \mu_{SL}K[L])/(1 + K[L]) \quad (4)$$

Mobility difference between μ_S and μ_i can be represented by Eq. (5).

$$\mu_S - \mu_i = (\mu_S - \mu_{SL})K[L]/(1 + K[L]) \quad (5)$$

If $K[L] << 1$ for a specific system under given experimental conditions, Eq. (5) can be simplified to Eq. (6):

$$\mu_S - \mu_i = (\mu_S - \mu_{SL})K[L] \quad (6)$$

Eq. (6) indicates that mobility difference of $\mu_S - \mu_i$ changes proportionally with equilibrium concentration of uncomplexed ligand. If total concentration of ligand (C_L) is much greater than the total concentration of solute (C_S), the equilibrium free ligand concentration ($[L]$) will be approximated as total concentration of ligand (C_L), i.e. $[L] = C_L$. Then, Eq. (6) will be approximated as Eq. (7):

$$\mu_S - \mu_i = (\mu_S - \mu_{SL})KC_L \quad (7)$$

Eq. (7) shows that mobility difference of $\mu_S - \mu_i$ changes proportionally with total ligand concentration when the two requirements, $K[L] << 1$ and $[L] = C_L$, are satisfied. Eq. (7) is highly interesting because it indicates that a linear calibration line can be constructed for concentration of the uncomplexed ligand.

We selected ANS as a probe for the indirect measurement of stability constant of inclusion complex formed between a cyclodextrin and another solute. It was reported that fluorescence intensity emitted by ANS in water increased markedly when cyclodextrins were added to the aqueous solution. The fluorescence intensity has been considered to originate in ANS being including in the cavity of cyclodextrin molecules [51]. This feature makes ANS–CD complexes readily detectable and less spectrally interfered with in CE when a fluorospectrometric detector is employed. Other advantages of using ANS as a probe include its commercial availability and chemical stability in storage.

It is known that ANS forms 1:1 inclusion complex with β-CD in phosphate buffer of pH 7 with the stability constant of 70 M^{-1} being determined at 25 °C [52]. We set concentration of ANS at $2 \cdot 10^{-5}$ *M* in our experiments. This concentration was the

Table 1
Mobilities of 8-anilino-1-naphthalenesulfonate measured under different experimental conditions[a]

Electrophoresis buffer	0.050 *M* phosphate	$5\cdot10^{-4}$ *M* HP-β-CD in 0.050 *M* phosphate	$1\cdot10^{-3}$ *M* HP-β-CD in 0.050 *M* phosphate	$1.5\cdot10^{-3}$ *M* HP-β-CD in 0.050 *M* phosphate	$2.0\cdot10^{-3}$ *M* HP-β-CD in 0.050 *M* phosphate	$1\cdot10^{-3}$ *M* HP-β-CD and amantadine in 0.050 *M* phosphate
Mobility ($cm^2\ V^{-1}\ s^{-1}$)	$2.22\cdot10^{-4}$	$1.81\cdot10^{-4}$	$1.65\cdot10^{-4}$	$1.55\cdot10^{-4}$	$1.41\cdot10^{-4}$	$1.71\cdot10^{-4}$
Mobility difference ($\mu_S - \mu_i$)		$4.1\cdot10^{-5}$	$5.7\cdot10^{-5}$	$6.7\cdot10^{-5}$	$8.1\cdot10^{-5}$	$5.1\cdot10^{-5}$

[a] Mobilities were average of duplicate measurements calculated using Eq. (8).

lowest which would still be able to reliably generate a detectable peak in our experiments. Concentration of HP-β-CD was varied at four levels in a range of $5\cdot10^{-4}$to $2\cdot10^{-3}$ *M*. The two requirements, i.e. total concentration of ligand being much greater than total concentration of solute and $K[L]$ being much less than unity, are basically met for validity of Eq. (6). These concentration levels and the concentration range of HP-β-CD were experimentally confirmed to ensure measurable change in migration time of the peak of interest. Effective mobility of μ_i changes more rapidly with a ligand of low concentration. Use of possible low concentration of a ligand has the additional advantage of saving material and reducing operation cost.

Table 1 lists the mobility data obtained. Mobilities were calculated based on Eq. (8) [11]:

$$\mu = L_e L_t / V(1/t - 1/t_{EOF}) \quad (8)$$

where t is migration time of the peak for ANS (in the presence or absence of the ligand), t_{EOF} is migration time of electroosmotic flow marker, L_t is the total length of the capillary; L_e is the effective length of the capillary from the injection end to the detection end, and V is voltage applied for the separation. A calibration curve of the mobility difference of ($\mu_S - \mu_i$) versus uncomplexed ligand concentration of HP-β-CD can be made using the data in Table 1 (Fig. 2). The correlation coefficient (r^2) of 0.9908 indicates a good linear relationship between the mobility differ-

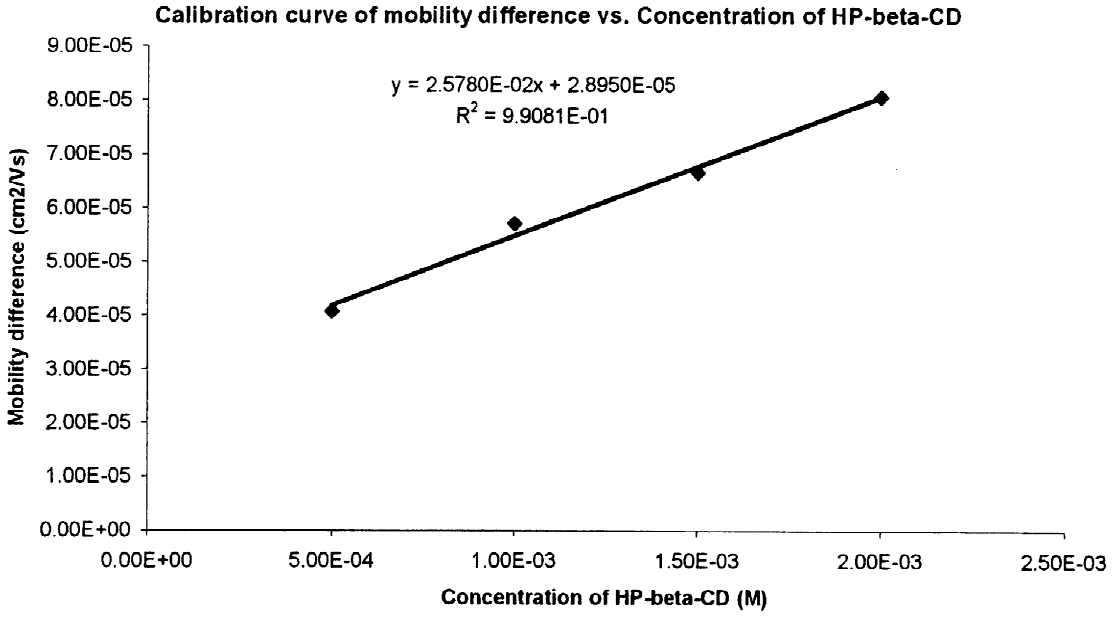

Fig. 2. Calibration curve of the mobility difference of ($\mu_S - \mu_i$) versus uncomplexed ligand concentration of HP-β-CD.

ence of $(\mu_S - \mu_i)$ and the uncomplexed ligand concentration of HP-β-CD.

For calculating the effective mobilities of ANS, both migration times of ANS and the neutral marker of methanol were measured, but in different runs. To measure the EOF with a fluorescence detector, a low concentration of ANS was added to the buffers to provide a fluorescence background. Methanol generated a disturbance, marking the EOF on the baseline of fluorescence background. The small amount of ANS introduced to the buffer did not affect the EOF. The validity of such a scheme was verified by agreement of an EOF measurement carried out in solution A employing a UV detector with dimethyl sulfoxide as neutral marker with that in the solution B employing a fluorescence detector with methanol as neutral marker.

3.2. Determine stability constant of inclusion complex formed between HP-β-CD and amantadine

When another solute is introduced to the system, two similar equilibria coexist. Uncomplexed ligand available to one solute is affected by the existence of the other. How much one equilibrium may be affected by another equilibrium depends on the relative competitiveness of the two solutes for the ligand. Fig. 3 shows the effect of competitive equilibria on migration time of ANS. When amantadine was introduced to the system of ANS and HP-β-CD, effective mobility μ_i of ANS shifted toward the mobility of the free solute, μ_S, because the concentration of HP-β-CD available to ANS was reduced. Consequently, migration time was prolonged in the experiments.

The effective mobility of ANS in solution F was measured to be $1.708 \cdot 10^{-4}$ cm^2 V^{-1} s^{-1}. The mobility difference of $(\mu_S - \mu_i)$ was $5.09 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}. The concentration of uncomplexed HP-β-CD was $8.51 \cdot 10^{-4}$ *M* by referring to Fig. 2. The concentration of the inclusion complexes formed between amantadine and HP-β-CD can be calculated using the following equation:

$$[\textit{Amantadine-HP-}\beta\textit{-CD}]_{\text{complex}} = \textit{total concentration of HP-}\beta\textit{-CD} - [\textit{HP-}\beta\textit{-CD}]_{\text{uncomplexed}} - [\textit{ANS–HP-}\beta\textit{-CD}] \quad (9)$$

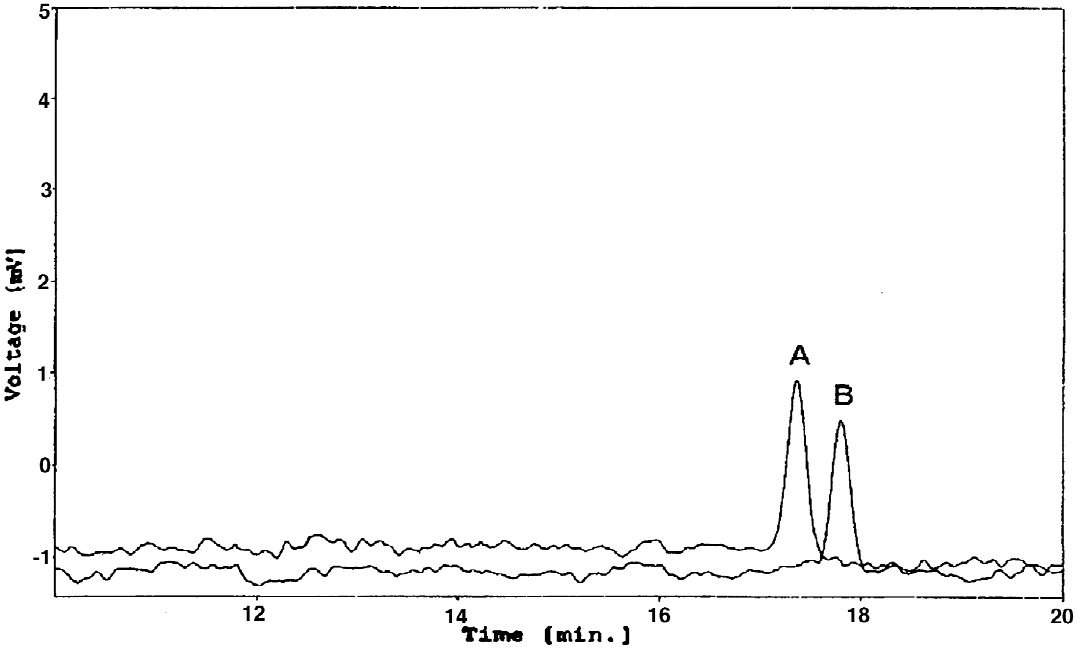

Fig. 3. Effect of competition for the ligand of HP-β-CD by amantadine on the effective mobility of ANS peaks: A = $2.00 \cdot 10^{-5}$ *M* 8-anilino-1-naphthalenesulfonic acid in 0.05 *M* phosphate buffer containing $1.00 \cdot 10^{-3}$ *M* hydroxypropyl-β-cyclodextrin; B = ANS in 0.05 *M* phosphate buffer containing $1.00 \cdot 10^{-3}$ *M* hydroxypropyl-β-cyclodextrin and $1.00 \cdot 10^{-3}$ *M* amantadine hydrochloride.

Approximation can be made to Eq. (9) as

$$[\textit{amantadine-HP-}\beta\textit{-CD}]_{\text{complex}} = \textit{total concentration of HP-}\beta\textit{-CD} - [\textit{HP-}\beta\textit{-CD}]_{\text{uncomplexed}} \quad (10)$$

since the concentration of inclusion complexes of ANS–HP-β-CD are negligible compared with other concentration terms. The concentration of inclusion complexes of amantadine-HP-β-CD was calculated to be $1.49 \cdot 10^{-4}$ M according to Eq. (10). The apparent stability constant for inclusion complex of amantadine-HP-β-CD was calculated to be $2.1 \cdot 10^{2}$ M^{-1} according to Eq. (2) with knowledge of corresponding concentrations. At respective concentrations of $1.5 \cdot 10^{-3}$ M and $2.0 \cdot 10^{-3}$ M of amantadine, the apparent stability constants were measured using the above described procedure to be $2.2 \cdot 10^{2}$ M^{-1} and $2.9 \cdot 10^{2}$ M^{-1}.

It may be worthwhile to explain the selection of concentration of the solute for which the stability constant is going to be determined. An appropriate concentration of the solute should ensure the mobility difference of $\mu_S - \mu_i$ is covered in the plot of Fig. 2 for accuracy. One way is by referring to data published for structurally similar solutes and ligands, and use Eq. (2) to help in selecting the concentration of the solute. If no relevant reference can be found, one may assume the stability constant to be $1 \cdot 10^{3}$ M^{-1}. So far reported stability constants of inclusion complexes of CDs with all kinds of solutes range between 10^{0} and 10^{6} [53]. After one measurement, the estimate for the stability constant may be varied, e.g. by 10 times, as another trial. We found that with a few trials, a suitable concentration of the solute can be found to ensure that the mobility difference of $\mu_S - \mu_i$ would be covered in the plot of Fig. 2.

4. Conclusions

The indirect CE method can be used for determining the stability constant of an inclusion complex of a CD and a solute. The indirect method is simple in experiment and data processing. The stability constant for a solute can be calculated without knowledge of the mobility of the complex at ligand saturating concentrations. The indirect CE method saves time and material. It can be applied to solutes and ligands lacking signal response on the selected detector in CE. Another advantage of the indirect method is its validity for both charged and neutral solutes and ligands.

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