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Enantiomeric separation of gemfibrozil chiral analogues by capillary electrophoresis with heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin as chiral selector

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Abstract

The enantiomeric separation of gemfibrozil chiral analogues was performed by capillary zone electrophoresis (CZE). Resolution of the enantiomers was achieved using heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) as chiral selector dissolved into a buffer solution. In order to optimize the separation conditions, type, pH and concentration of running buffer and chiral selector concentration were varied. For each pH value, the optimum chiral selector concentration that produced the resolution of the isomers was found. The migration order of labile diastereoisomers formed was valued at the optimum experimental conditions by adding a pure optical isomer to the racemic mixture. Data from ^{1}H NMR studies confirmed host–guest interaction between TM-β-CD and 5-(2,5-dimethylphenoxy)-2-ethylpentanoic acid sodium salt. The hypothesized stoichiometry host:guest was 1:1. An apparent equilibrium constant (K_a) was estimated monitoring the chemical shift variation as a function of TM-β-CD concentration. Salt effect on complexation equilibrium constant was also investigated.

Keywords: Chiral capillary zone electrophoresis; Enantiomeric separation; *heptakis*(2,3,6-Tri-*O*-methyl)-β-cyclodextrin; Gemfibrozil; NMR

1. Introduction

Enantiomeric separations represent an important topic of research in pharmaceutical analysis where a wide number of drugs with an asymmetric centre exists as couple of enantiomers. Very often the pharmacological activity and metabolism of two antipodes of a drug may be different. For this reason, rapid, selective and sensitive analytical methods are requested to verify the chiral purity of drugs. In particular, capillary gas chromatography (GC), high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) have been the most widely used methods in this area over the last decade [1].

Recently, the use of chiral capillary electrophoresis (CE) in enantiomeric resolution has been increasing because of its simplicity, rapidity, high efficiency and resolution; furthermore, CE is highly flexible and versatile, with a minimal use of expensive chiral reagents [2].

Enantiomeric resolutions in CE are based upon stereoselective interactions between the analyte and a chiral selector added in the background electrolyte (BGE). In this manner the electrophoretic mobility of the enantiomers is selectively modified [3].

Among various chiral reagents, cyclodextrins (CDs) and their derivatives have been successfully utilized in enantiomeric separations by CE. CDs are cyclic oligosaccharides consisting of six to eight D-(+)-glucose units linked by α-(1,4) bonds. Their simple geometry can be described as a hallow truncated cone with an hydrophobic inner cavity and an hydrophilic rim. Derivative forms of low soluble native

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β-CD have been synthesized with altered enantioselective capability. The chiral recognition occurs via formation of an inclusion complex between the alkyl or aryl function of solute and the hydrophobic cavity of CD; an additional requirement is the presence of lateral interactions, such as dipole–dipole interactions or hydrogen bonds between the hydroxyl groups at C-2 and C-3 at upper rim of the CD and the hydrophilic part near the stereogenic centre of the analyte [4,5].

Gemfibrozil, 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid, is a widely used hypolipidemic drug related to fibrates. Recent studies have shown that in addition to its effects on lipids, gemfibrozil may modulate the fibrinolytic system [6]. The synthesis and the pharmacological evaluation of gemfibrozil chiral analogues, the 5-(2,5-dimethylphenoxy)-2-ethylpentanoic acid (**1a**) and 5-(2,5-dimethylphenoxy)-2-methylpentanoic acid (**1b**) (Fig. 1), were described in a previous work [7]. The resolution of enantiomers **1a**–**b** was achieved after derivatization with a chiral reagent, separation by flash-cromatography and final hydrolysis to recover the enantiopure acids. The absolute configuration of each isomer of **1a**–**b** was determined by single crystal X-ray structural analysis. These compounds were tested to evaluate their antiplatelet activity, using the PFA-100® instrument. Data obtained showed a stereochemistry-dependent inhibitory effect.

The aim of this work was to develop a simple, fast and efficient method for the enantiomeric resolution of *rac*-**1a**–**b** by using chiral CE. In this study, *heptakis*(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) was selected as chiral resolving agent, referring to its ability to interact, in a selective manner, with arylalkanoic acids [8]. Resolution was optimized by examining the influence of some experimental variables such as type, pH and concentration of the running buffer, chiral selector concentration and addition of organic modifiers.

Structural characterization of supramolecular host–guest complexes, formed by TM-β-CD and compound **1a**, was achieved by NMR spectroscopy measurements. Nowadays NMR spectroscopy is one of the most powerful methods for structural elucidation of organic compounds in solid and solution state [9,10], allowing the determination of the stoichiometry of complexes under study, their binding constants, and also their spatial relationships [9,11]. Measurement of chemical shift change as a function of concentration – the so called NMR titration [12,13] – is a very useful method for the determination of the binding constants between molecules in solution, due to the sensitivity of nuclear chemical shift to specific and not specific interactions [14]. This method evaluates the differences between the chemical shift of molecular species in free and bound state [9,11].

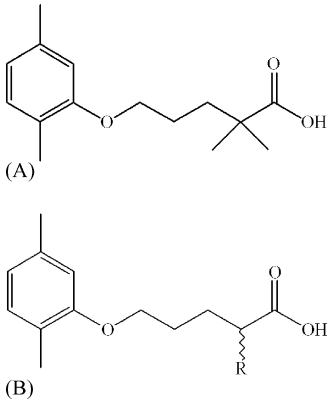

Fig. 1. Structures of gemfibrozil (A) and its chiral analogues (B): **1a**, R = ethyl; **1a**$_s$, sodium salt and **1b**, R = methyl.

In an aqueous solution the slightly non polar cyclodextrin cavity is not empty, but it is occupied by water molecules even though this condition is energetically unfavourable [10,15]. When less polar guest molecules are added, they readily displace water molecules from the non polar CD cavity and the energy gain obtained from the dispersive interaction guest–CD is the driving force of this process [15]. All complexation equilibria are generally described by apparent spectroscopic property [16,17], which here refers to a time averaged NMR shift. The apparent binding constant, K_a, for the hypothesized 1:1 complex formation was calculated from the change in the chemical shifts (c.s.) of the **1a**$_s$ (Fig. 1) signals measured as a function of TM-β-CD concentration, using the following equation [12,18]:

$$\Delta\delta_{obs} = (\delta_c - \delta_0)\frac{([A]_0 + [H]_0 + K_d) - \sqrt{([A]_0 + [H]_0 + K_d)^2 - 4[A]_0[H]_0}}{2[A]_0} \quad (1)$$

where $\Delta\delta_{obs} = (\delta_{obs} - \delta_0)$, δ_{obs} is the **1a**$_s$ actual measured c.s., δ_0 and δ_c are the c.s. of the free and complexed **1a**$_s$, respectively, $[A]_0$, and $[H]_0$ are the stoichiometric concentrations of **1a**$_s$, and TM-β-CD, respectively and $K_d = 1/K_a$.

By fitting the experimental data with Eq. (1), using a computer based non-linear least-square fitting which uses the Levenberg–Marquardt algorithm, the unknown δ_c and K_a were determined.

2. Experimental

2.1. Chemicals

The acids **1a**–**b** were synthesized by us [7]. TM-β-CD was purchased from Sigma (Sigma–Aldrich, Milan, Italy); phosphoric acid (85% w/w), acetic acid and tris-(hydroxymethyl)-methylamine (Tris) of analytical grade were from Aldrich (Sigma–Aldrich, Milan, Italy). HPLC-grade water (Milli-Q *plus* PF, Millipore, France) was used. All other chemicals and solvents were analytical grade from Aldrich.

2.2. Capillary electrophoresis

Experiments were performed on Biofocus 2000 CE instrument (Bio-Rad labs, Hercules, CA, USA) equipped with ultraviolet detection (deuterium lamp, 230 nm). A detection

window was created by burning off the polyimide coating on the capillary. The injection of the sample was done by pressure at the anode end (5 psi × s; 1 psi = 6894.7 Pa) into an uncoated fused-silica capillary (50 μm I.D., 40 cm overall length, 35.4 cm effective length) (Chemtek Analytica, Italy). The capillary, assembled in a cartridge (Bio-Rad), was thermostated by circulating water at 15 °C while carousel temperature was not fixed. The high-voltage power supply was operated in the constant-voltage mode, applying 20 kV. pH measurements were made by means of a pH-meter MP220 (Mettler Toledo). Data were collected using CE 2000 data acquisition software.

Buffer solutions were prepared dissolving an appropriate amount of phosphoric or acetic acid in Milli-Q water and the pH was adjusted with diluted NaOH or Tris to the selected values ranging within 5.5 and 6.5. When the BGE had to contain the chiral selector, TM-β-CD was dissolved in the BGE before running the electrophoretic experiments to yield concentrations in the range 5–40 mM. Solid analytes **1a**–**b** were dissolved in methanol at 1 mM. Sample solution was prepared mixing 25 μl of analyte solution, 75 μl of BGE solution and 400 μl of water. All solutions were freshly prepared and filtered on 0.2 μm syringe filters (Chemtek Analytica, Italy). Prior to each experimental batch and for BGE changeover, the capillary was flushed with 1 M NaOH, water and BGE for 5, 5 and 10 min, respectively.

2.3. NMR spectroscopy

For the measurement of cyclodextrin-induced shifts, an appropriate amount of the racemic mixture of **1a** with and without the cyclodextrin derivative was dissolved in deuterated water. ^{1}H NMR data were obtained on a Varian Mercury-300 FT NMR spectrometer operating at 300.06 MHz for ^{1}H. One hundred and twenty-eight scans with a frequency range of 3500 Hz were collected into 16 K data points. An appropriate Gaussian function was applied before Fourier transformation to enhance the spectral resolution. The temperature was controlled at 25 ± 1 °C and the residual protonated water signal was suppressed using a presaturation technique. The chemical shifts are reported in parts per million using HDO signal (δ 4.67) as an internal reference. Change on magnetic susceptibility, due to hydrogen bonding, affects equally solute and water, but we was interested only in the difference and not in absolute values. The Rotating Frame Overhauser Experiments (ROESY) were obtained with an applied mixing time of 400, 300, or 250 ms using a D_2O solution having ligand:TM-β-CD molar ratio 1:1.5 and [**1a**] = 4 mM at pD = 11.4 [15]. Combined mono-dimensional (^{1}H NMR) and bi-dimensional (COSY ^{1}H–^{1}H, and ^{1}H–^{13}C, ROESY) experiments allowed the unambiguous assignments of **1a** resonances in D_2O. COSY ^{1}H–^{1}H spectra were obtained on a Bruker Avance 600, operating at 600.13 MHz for ^{1}H, equipped with a BBI probe, and using for their acquisition a double-quantum-filtered COSY sequence, with gradient coherence pathway selection. The ^{1}H NMR spectra of free TM-β-CD in aqueous solution had already been assigned in detail [19].

For the determination of the binding constant, eight solutions, at constant [**1a**] = 1.4 mM, having ligand:TM-β-CD molar ratios 1:0, 1:3.1, 1:6.1, 1:9.2, 1:12.2, 1:15.3, 1:18.4 and 1:21.4, were prepared in deuterated water in order to measure the induced chemical shifts of the TM-β-CD and **1a**. The pD was adjusted to 11.4 by adding a concentrated solution of NaOD and correcting the pH-meter reading as reported [20]. In order to evaluate the salt effect, the previous solutions were added with a concentrated solution of NaCl in D_2O and eight solutions were obtained with the same ratio ligand:TM-β-CD and [**1a**] = 1.23 mM and [NaCl] = 0.67 M.

3. Results and discussion

To obtain the resolution of compounds **1a**–**b** using CZE, some experimental parameters, such as type, pH and concentration of the buffer and TM-β-CD concentration were varied. Organic modifiers were used to improve the dissolution of the analytes in the buffer and to evaluate their effects on resolution. Alternatively, one of the aforementioned parameters was varied while the others were kept constant. All experimental conditions are summarized in Table 1, considering that the CD concentration was varied from 5 to 35 mM for each pH value.

In absence of chiral selector no resolution of enantiomers was obtained, according to the fact that enantiomers possess similar physico-chemical properties and electrophoretic mobility.

Table 1
Experimental conditions for chiral separation of **1a** and **1b** compounds

Buffer type	[BGE] (mM)	pH
1a		
Sodium acetate	42	5.5
Tris acetate	25	5.5
		6.0
		6.5
	50	5.5
		6.0
		6.5
Tris phosphate	50	5.5
		6.0
		6.5
1b		
Sodium acetate	42	5.5
	100	5.5
		6.0
		6.5
Tris acetate	50	5.5
		6.0
		6.5
Tris phosphate	50	5.5
		6.0
		6.5

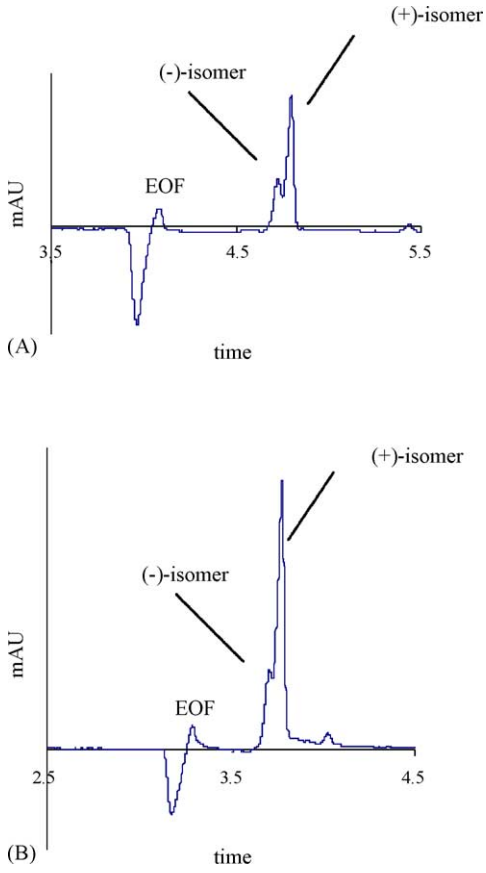

Fig. 2. Enantiomeric migration order of pure optical (*S*)-(+)-isomer enriched mixture, using 50 mM Tris phosphate, pH 6.0: (A) **1a**, 15 mM TM-β-CD and (B) **1b**, 25 mM TM-β-CD.

The enantiomer migration order was verified for **1a**–**b** adding a pure optical isomer in the racemic sample; in all instances, the (−)-isomers showed higher apparent mobility than (+)-isomers, indicating that (−)-isomers of **1a**–**b** form more stable inclusion complexes with TM-β-CD (Fig. 2). Cyclodextrin concentration and buffer pH did not alter the migration order.

To obtain more information on interaction magnitude and binding sites, we have undertaken NMR studies using the racemate of **1a**. We assumed that the obtained results could be equally applied to **1b** compound because the 2-ethyl group in **1a**, replaced by a methyl group in **1b**, did not show NMR relevant interactions with TM-β-CD.

3.1. Influence of BGE type and concentration

At first, we selected sodium acetate, Tris acetate and Tris phosphate as BGE because of their ability to be good buffer in enantioseparation of structurally related compounds; the Tris ion was especially useful because it is large and it can be used in high concentrations without generating significant current [2]. These three buffers were used at different concentrations for **1a**–**b** (Table 1).

Sodium acetate 42 mM at pH 5.5 did not give the resolution of **1a** while for **1b** it gave a better one. For **1b**, sodium acetate was also used at 100 mM at pH 6.0, but the isomers were not completely separated. So, the increase of concentration of sodium acetate did not produce an improvement in resolution.

Tris acetate 25 mM did not give a resolution for **1a** at three pH values studied; for this reason the buffer concentration was changed to 50 mM, but also in this case the resolution was not improved.

Very good resolution was obtained for **1a**, utilizing Tris acetate 50 mM at pH 6.0 and 6.5.

Tris phosphate was just used at 50 mM. For **1a**, the best resolution was obtained at pH 6.0, even if it was not a baseline one (Fig. 3A). For **1b**, Tris phosphate gave a nearly baseline resolution at pH 5.5 and 6.5, while at pH 6.0 it was totally baseline (Fig. 3B).

To resume, for compound **1a** the best separation was obtained with Tris phosphate 50 mM; for **1b**, sodium acetate

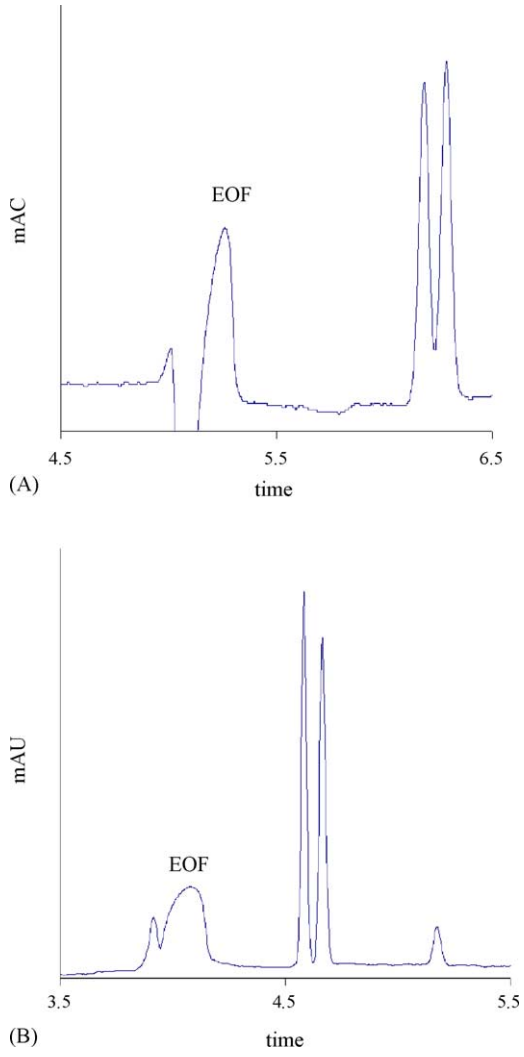

Fig. 3. Best separations using 50 mM Tris phosphate, pH 6.0: (A) **1a**, 15 mM TM-β-CD, MeOH 20% and (B) **1b**, 25 mM TM-β-CD.

42 mM gave a good resolution but the best one was again obtained with Tris phosphate 50 mM as for **1a**.

3.2. Effect of pH

pH is a critical parameter in CE separations because it influences the charge of analytes and the ionic state of the capillary wall, so the electroosmotic flow (EOF) [21].

Changes in the degree of solute ionization may alter the interaction at the CD binding site affecting chiral recognition. Chiral selectivity increases at lower pH values; however, a partial ionization of carboxylic group is necessary to give a negative charge to CD–analyte complex. This negative charge decreases the apparent mobility of the complex which migrates more slowly. The optimum buffer pH is around the value of pK_a +1 [22,23].

The enantiomeric separations of racemic **1a**–**b** were studied in the pH range 5–6.5 in which the carboxylic group (calculated pK_a for **1a**–**b** is 4.87, [24]) is partially dissociated and the compounds migrate as anions, in opposite direction to the EOF.

Fig. 4 shows the best electropherograms (for **1b**) obtained with Tris phosphate 50 mM at three pH values: as expected, the best resolution was obtained at pH 6.0 (pK_a +1). The same result was achieved for the compound **1a**, but without baseline separation.

3.3. Effect of chiral selector concentration

The resolution of enantiomers can be enhanced by optimization of the selector concentration [25].

TM-β-CD was used at concentrations between 5 and 35 mM for all chosen buffers, as shown in Table 1. For each buffer and also for each buffer pH, we found the optimal CD concentration to achieve the best resolution of each racemate: CD concentration up and down the optimal one decreased resolution. In Fig. 5 is reported the effect of CD concentration using Tris phosphate buffer 50 mM and pH 5.5: the best resolution was obtained at 10 mM, while at 8 or 15 mM the resolution decreased.

3.4. Effect of organic modifier

Organic modifiers were introduced to enhance the analyte solubilization in buffers and to evaluate their effect on resolution [23,26]. For all experiments, methanol (percentage added from 5 to 30) was used and its effect on resolution was not uniform.

For example, for **1a** using Tris phosphate buffer 50 mM, pH 5.5 and TM-β-CD 10 mM, an increase in methanol concentration decreased resolution, while at pH 6.0 and TM-β-CD 15 mM an increase in methanol concentration improved the resolution (Fig. 6). This result was in agreement with Wren principle [27].

The effect of methanol on analyte solubilization was remarkable only at lowest pH value.

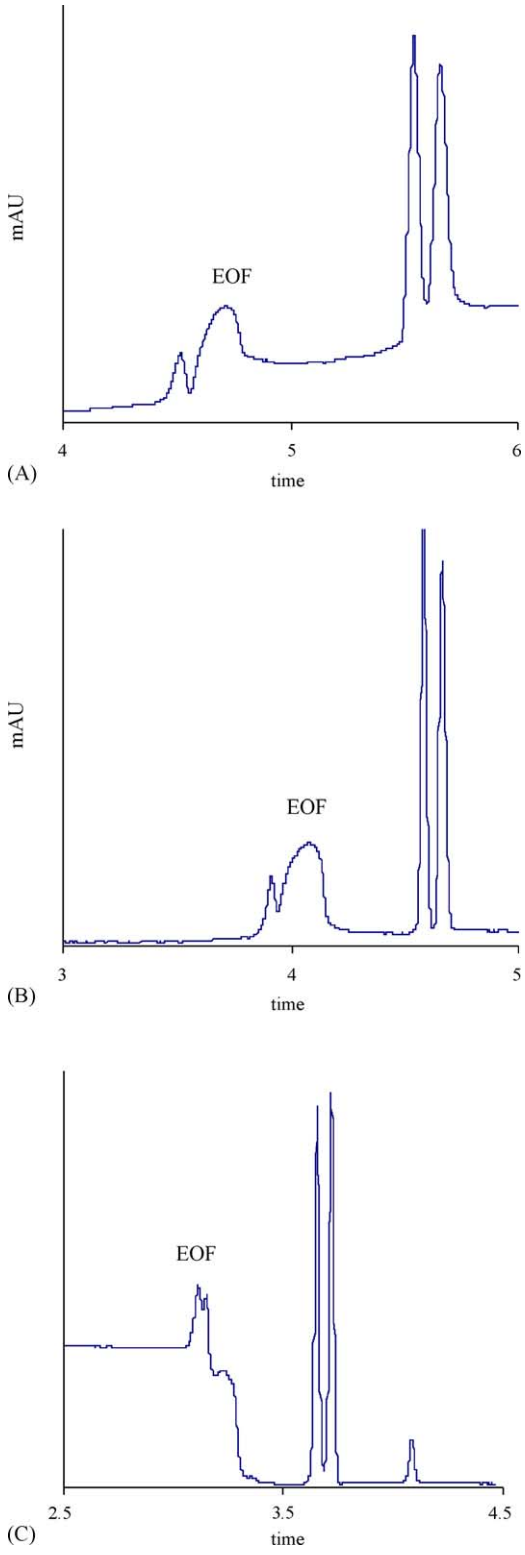

Fig. 4. Best electropherograms achieved for **1b** at three different pH values: 50 mM Tris phosphate: (A) pH 5.5, 20 mM TM-β-CD; (B) pH 6.0, 25 mM TM-β-CD and (C) pH 6.5, 30 mM TM-β-CD.

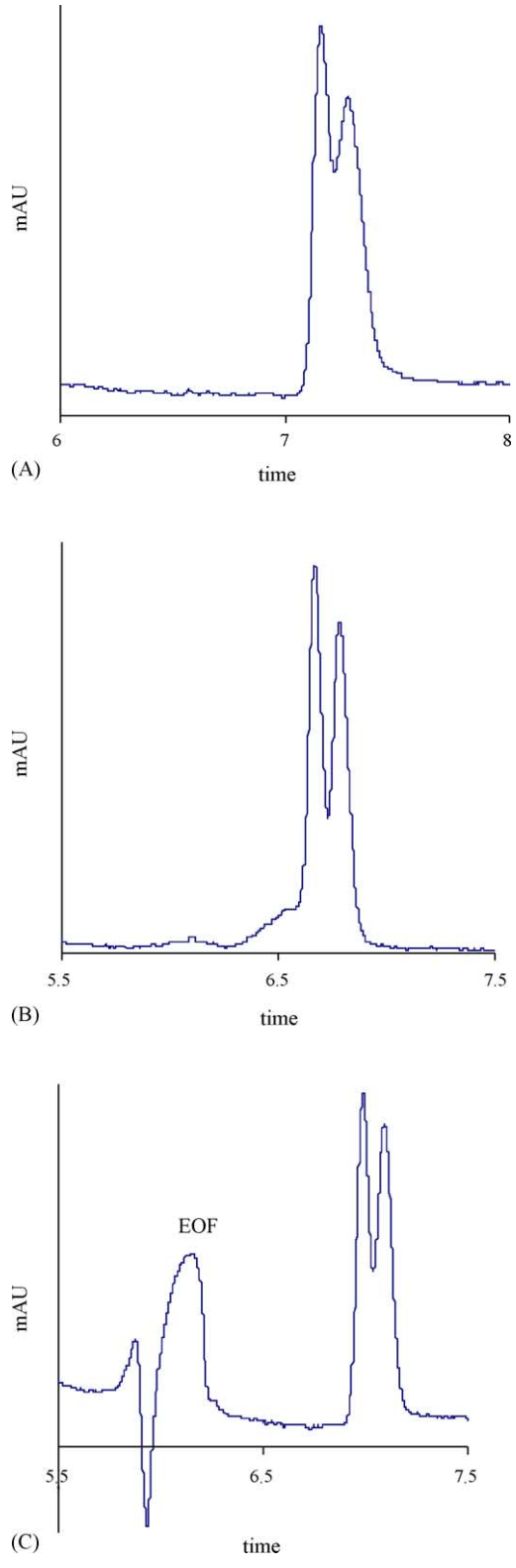

Fig. 5. Effect of TM-β-CD concentration on resolution for **1a**: 50 mM Tris phosphate, pH 5.5. TM-β-CD concentration: (A) 8 mM; (B) 10 mM and (C) 15 mM.

Other organic modifiers such as ethanol, isopropanol and acetonitrile were used to evaluate their effect on resolution, but they did not improve the resolution in any analysis, producing a high noisy baseline and, as expected, decreasing the electroosmotic mobility [28].

Since organic modifier is assumed to act as competitor to the analyte in its interaction with the CD molecule, these results indicate that hydrophobic interactions between analyte and host molecule play an important role for attaining enantioresolution.

3.5. NMR studies

The guideline that we have followed on selecting the NMR experiments was to find out the molecular explanation of CZE resolution. In order to evaluate separately each variable influence on the system, we have chosen to carry out NMR experiments on model solutions. The ^{1}H NMR spectra of **1a**$_S$ were measured at different TM-β-CD concentrations to find out the complex nature formed by host–guest interactions. The measured c.s. for each TM-β-CD concentration are shown in Table 2. Moreover, we also measured ^{1}H NMR spectra of **1a**$_S$ at different TM-β-CD concentrations adding to the solutions a constant amount of NaCl to evaluate the buffer ionic strength effect on host–guest interaction. The measured c.s. for each TM-β-CD concentration are shown in Table 3.

The analysis of the c.s. variations for the racemate of **1a**$_S$, as a function of the TM-β-CD added, showed that only the aromatic part and the proton at the chiral centre were affected. In fact, protons that have changed their c.s. were H4, H6 and H11, and the methyl Me14 and Me15 (for numeration see Fig. 7), but not the protons of aliphatic chain (except the one at the chiral carbon H11; H8 NMR signal was obscured by those of TM-β-CD). This fact clearly shows that only the 2,5-dimethylphenoxy part of the molecule was incorporated into the cyclodextrin cavity, maybe leaving the ether oxygen atom located at the rim (07, Fig. 7). A possible explanation for the observation that the meta H3 was not affected by the formation of the inclusion complex, is that changes on water solvation of ether oxygen atom affect differently *ortho*- and *para*-hydrogens *versus meta*-hydrogen chemical shifts.

The proton at the chiral centre (H11) was the only, in the aliphatic moiety, to be significantly affected by the presence of cyclodextrin, confirming the interaction between TM-β-CD and the chiral centre on **1a** and supporting the observed different interaction of the two enantiomers with the chiral selector on CZE separation. The NMR results could be explained assuming that the aromatic moiety was inside the CD cavity, and the flexible aliphatic part, at the rim, was folded, leaving only the carboxyl group and the hydrogen (H11) at the chiral centre, interacting with the CD rim, as shown in Fig. 7. This rationalization could also support the found dependence on pH of CZE. In fact the change on pH alters the amount of dissociated carboxylic acid modifying the host–guest interaction magnitude.

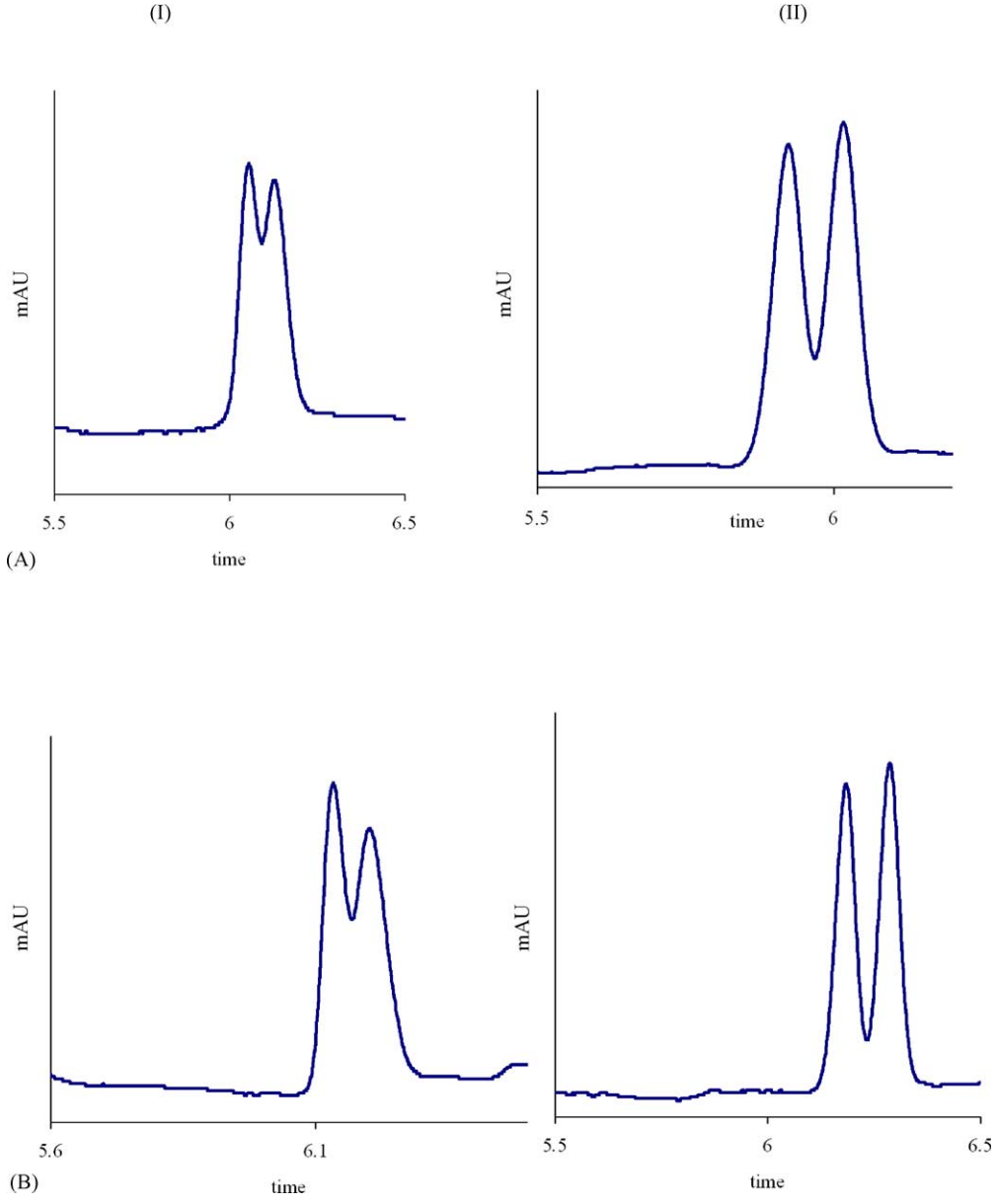

Fig. 6. Effect of methanol on resolution for **1a**: (I) 50 mM Tris phosphate pH 5.5, 10 mM TM-β-CD and (II) 50 mM Tris phosphate pH 6.0, 15 mM TM-β-CD. (A) No methanol and (B) 20% MeOH.

Table 2
^{1}H NMR chemical shifts (ppm) of 1.40 mM **1a** in D_2O with various TM-β-CD concentration at 25.0 °C and pD = 11.4

[TM-β-CD] (M)	H3	H6	H4	H8	Me15	Me14	H11	H9[a]	H10[a]	H12	Me13
0	7.122	6.910	6.808	4.064	2.290	2.168	2.161	1.750	1.608	1.464	0.859
4.30×10^{-3}	7.121	6.892	6.798	4.058	2.297	2.177	2.156	1.755	1.609	1.463	0.860
8.60×10^{-3}	7.116	6.873	6.790	[b]	2.304	2.185	2.151	1.757	1.611	1.463	0.861
1.29×10^{-2}	7.119	6.859	6.783	[b]	2.310	2.192	2.147	1.753	1.612	1.463	0.862
1.72×10^{-2}	7.117	6.844	6.776	[b]	2.315	2.199	2.143	1.750	1.613	1.462	0.862
2.15×10^{-2}	7.115	6.833	6.770	[b]	2.320	2.204	2.140	1.752	1.614	1.463	0.863
2.58×10^{-2}	7.114	6.823	6.766	[b]	2.324	2.208	2.138	1.753	1.616	1.463	0.864
3.01×10^{-2}	7.114	6.816	6.762	[b]	2.327	2.212	2.135	1.754	1.616	1.464	0.865

[a] Chemical shifts referred to the multiplet central value.
[b] Signal obscured by TM-β-CD.

Table 3
^{1}H NMR chemical shifts (ppm) of 1.23 mM **1a** in D_2O with NaCl 0.67 M and various TM-β-CD concentration at 25.0 °C and pD = 11.4

[TM-β-CD] (M)	H3	H6	H4	H8	Me15	Me14	H11	H9[a]	H10[a]	H12	Me13
0	7.130	6.914	6.817	4.067	2.295	2.170	2.160	1.750	1.618	1.466	0.860
3.76×10^{-3}	7.128	6.881	6.800	[b]	2.306	2.185	2.152	1.754	1.613	1.464	0.861
7.53×10^{-3}	7.126	6.859	6.788	[b]	2.314	2.195	2.146	1.756	1.612	1.463	0.862
1.13×10^{-2}	7.124	6.842	6.779	[b]	2.318	2.202	2.142	1.747	1.613	1.462	0.863
1.51×10^{-2}	7.124	6.830	6.773	[b]	2.324	2.209	2.139	1.750	1.617	1.462	0.865
1.88×10^{-2}	7.121	6.819	6.767	[b]	2.326	2.212	2.136	1.750	1.616	1.461	0.865
2.26×10^{-2}	7.121	6.812	6.763	[b]	2.329	2.216	2.134	1.752	1.618	1.463	0.866
2.63×10^{-2}	7.121	6.804	6.760	[b]	2.333	2.221	2.134	1.754	1.619	1.466	0.870

[a] Chemical shifts referred to the multiplet central value.
[b] Signal obscured by TM-β-CD.

However, TM-β-CD part of NMR proton spectra was not affected by $\mathbf{1a}_S$ presence, and ROE experiments gave very small cross pick signals; on such a basis, we were unable to decide which parts of the host and of the guest were in close proximity.

The binding constant K_a was determined by fitting the chemical shift of H4, H6, H11, Me14 and Me15 (22 ± 1.7, 20 ± 2, 20 ± 2, 21 ± 2, $18 \pm 1.5\ M^{-1}$, respectively) with Eq. (1) (Figs. 8 and 9). The results confirmed the assumed 1:1 stoichiometry, because these differ for less than 10% [9]. The small value obtained for the K_a indicates that $\mathbf{1a}_S$ enantiomers have a weak affinity with TM-β-CD: in fact, we were unable to find ROE correlation cross-pick signals for any interactions between TM-β-CD and the target compound, maybe due to the unfavourable ratio [complex]/[host].

Several aspects make important the investigation of salt effects on host–guest equilibria. A predictable change of complexation constant is useful to investigate such equilibria under varied conditions. In fact the change on pH alters the amount of dissociated carboxylic acid, modifying the host–guest interaction magnitude. The presence of inorganic salts can lead to a decrease of the binding constant [29,30] or to an increase of it [18,29], depending on whether a competing ion is present or if a salting-in and -out effects predominate. As we were interested in investigating salting-out effect, we have chosen an inert salt that does not affect other parameters such as the pH and that does not interact with the host or guest molecules (i.e. sodium chloride). In fact, NaCl gives relatively small and hard ions which increase water structuring and promote salting-out effects [31–33]. The measured K_a with 0.67 M NaCl added were 65 ± 2, 63 ± 2, 59 ± 3, 60 ± 5, $59 \pm 8\ M^{-1}$ for H4, H6, H11, Me14 and Me15, respectively. The expected increase of K_a, upon the addition of NaCl, very likely indicated a predominating hydrophobic interaction between host and guest, since NaCl is not a competing salt. This fact also suggests that only a salting-out effect occurs [13]. These results suggest that the buffer acts also as salting-out additive in the **1a** and **1b** resolution by CZE.

Comparing the NMR results obtained with and without the addition of salt, we could observe that the only difference was in the change of magnitude of chemical shift variation, whereas stoichiometry (1:1 because the calculated K_a's for each affected proton signal were within 10%), direction of change (upfield or downfield) and the affected protons remained the same. For these reasons, we believe that the host–guest adduct geometry is not affected by the change

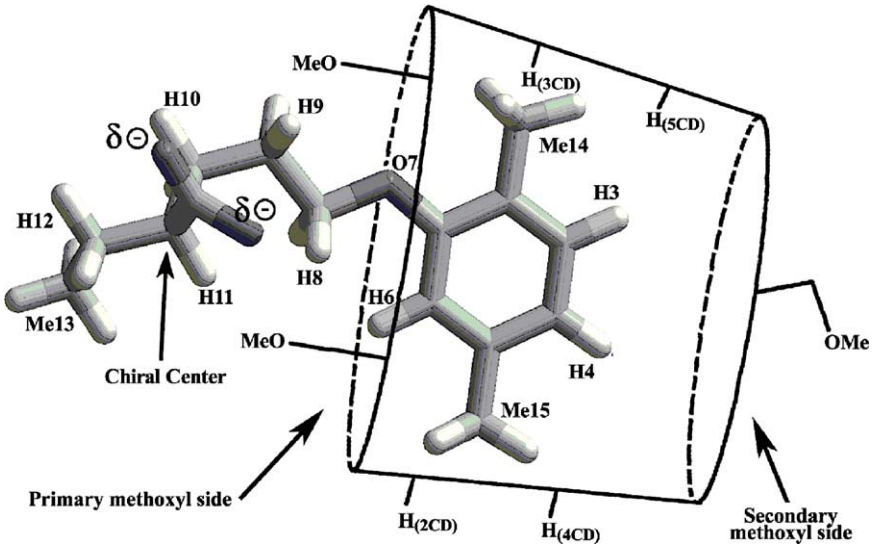

Fig. 7. Suggested host–guest geometry for the inclusion complex of **1a**, with TM-β-CD; see text.

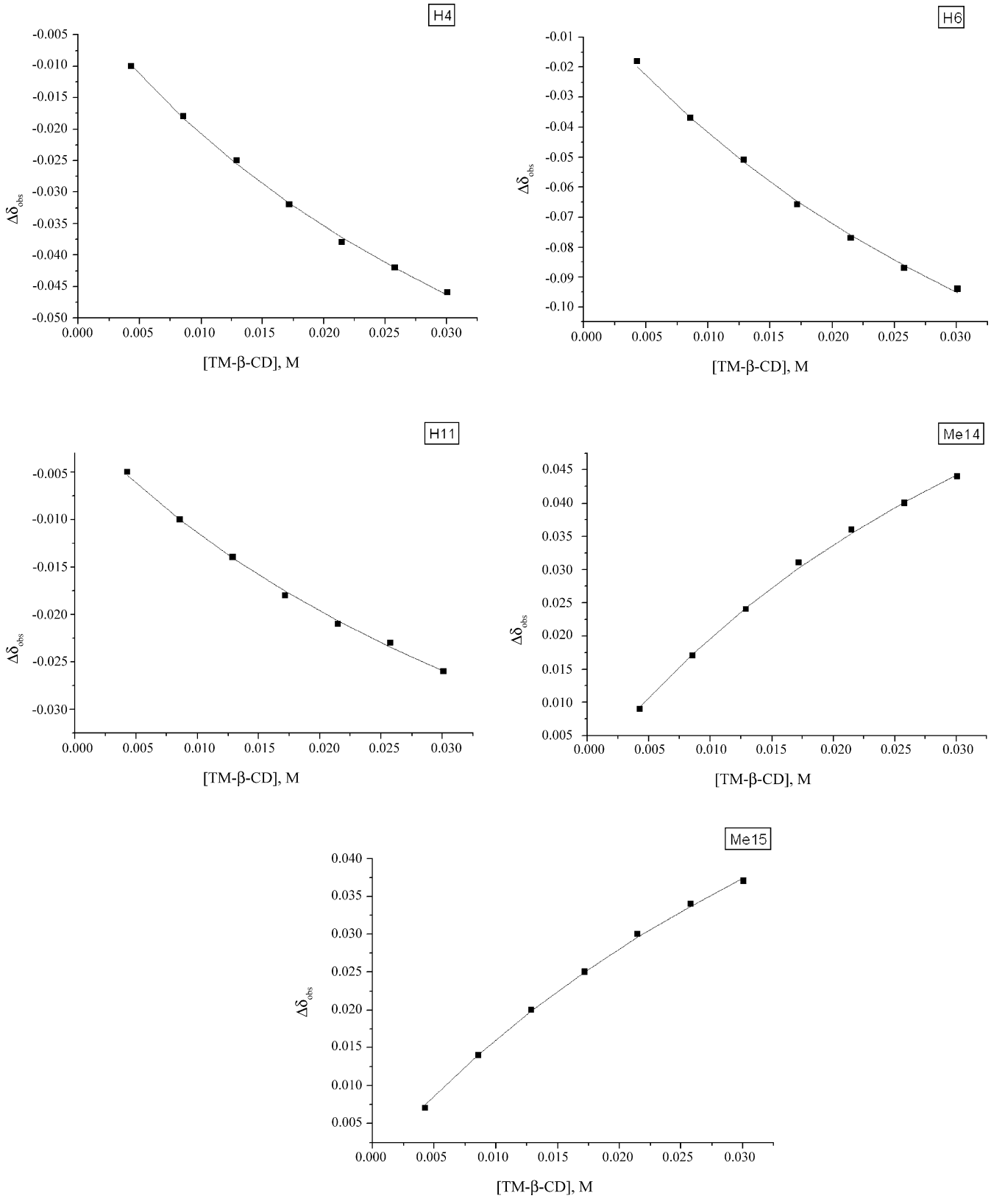

Fig. 8. Plot of the chemical shifts change ($\Delta\delta_{obs}$) as a function of TM-β-CD concentration for 1.40 mM **1a**$_s$ at pD 11.4. The line shows the fitting by Eq. (1).

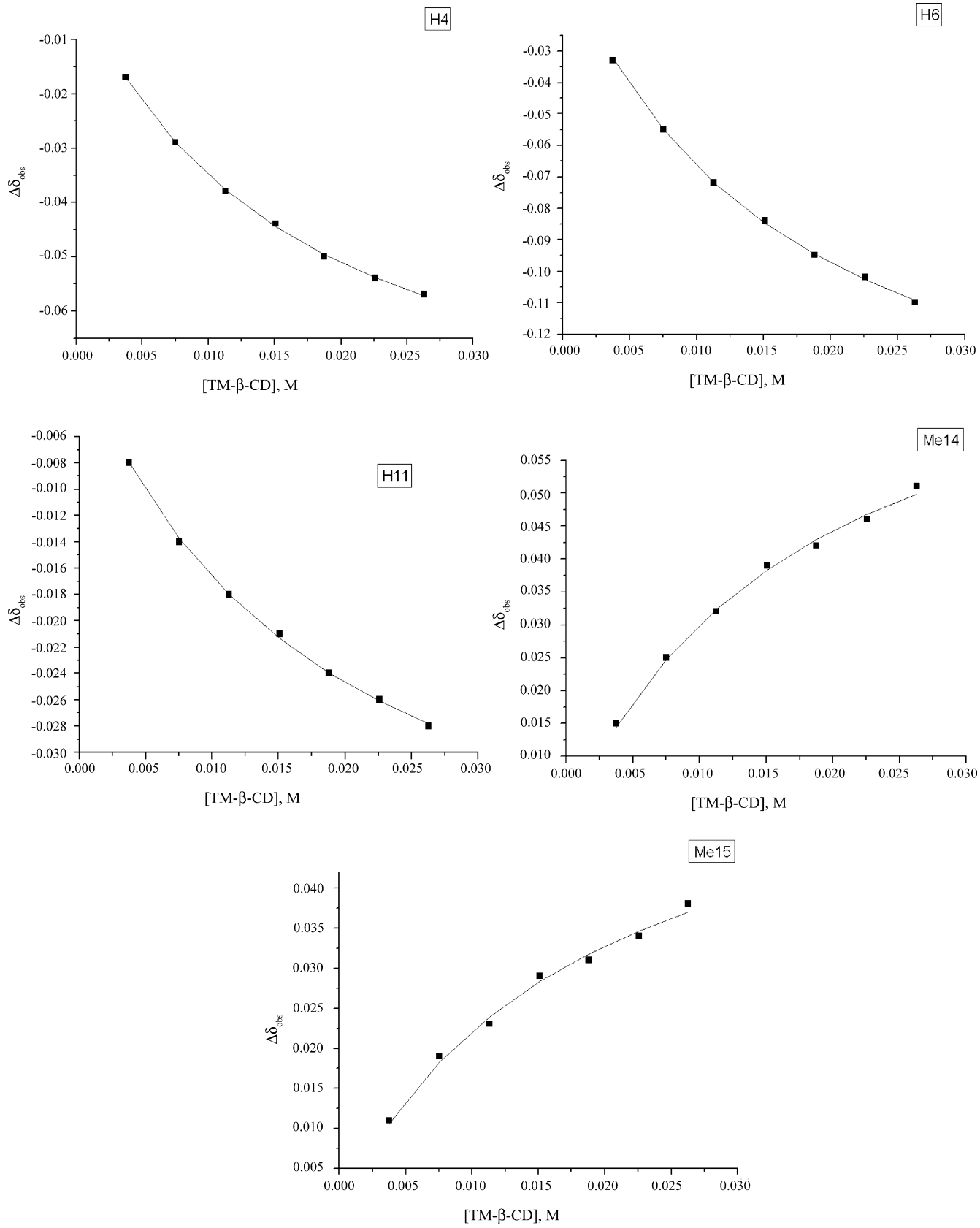

Fig. 9. Plot of the chemical shift change ($\Delta\delta_{obs}$) as a function of TM-β-CD concentration for 1.23 mM **1a**$_s$, with 0.67 M NaCl at pD 11.4. The line shows the fitting by Eq. (1).

on water activity induced by salts added, and that the only effect, on host–guest interaction, is the increase on the ratio [complex]/[host]. Furthermore the salting-out effect could provide a possible mean to increase the resolution efficiency with chromatographic techniques.

4. Conclusions

In this work we describe a simple tool for the separation of gemfibrozil chiral analogues using CZE. The enantiomeric separation of these compounds was accomplished using a modified β-CD, the TM-β-CD.

The variation of experimental parameters, e.g. concentration of chiral selector, organic additives, counter-ion, pH and ionic strength of BGE, was very important to improve the chiral separation. The best results for **1a**–**b** were obtained with Tris phosphate 50 mM at pH 6.0; the TM-β-CD concentration was 15 mM for **1a** and 25 mM for **1b**, respectively. The use of organic modifiers had a negative influence on resolution.

^{1}H NMR spectroscopy was used in order to demonstrate the complexation between the TM-β-CD and **1a**. The complex was characterized by NMR changes in the chemical shifts of some of the **1a** protons as a function of TM-β-CD concentration. However, the lack of change in the chemical shifts of the TM-β-CD protons, due to unfavourable ratio [complex]/[host], did not allow us to clearly define the exact location of the guest inside the host molecule. The addition of an inert salt increased the binding constant, but at the same time left the host–guest geometry interaction unaffected.

Current studies are in progress to evaluate the capability of different chiral selectors in enantiomeric resolution of new chiral aryloxyacids.

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