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Separation of some hydroxycarboxylic acids by capillary isotachophoresis in the presence of neutral cyclodextrins

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Abstract. The aim of this work was to study the various electrolyte systems in which hydroxycarboxylic acids can be separated by capillary isotachophoresis. Using β-cyclodextrin as additive to the leading electrolyte, the complete separation of hydroxycarboxylic acids was achieved. The results confirmed the significant influence of the cyclodextrin concentration and pH on the resolution of hydroxycarboxylic acids.

Key words. Isotachophoresis – hydroxycarboxylic acids – cyclodextrins – inclusion complexes.

Introduction

Complex formation in isotachophoresis is an important tool for improvement in the separation of sample ions. This possibility is mainly used for inorganic ions where the effective mobilities are less sensitive to pH changes. The possibility of influencing the effective mobilities of organic ions through complex formation may be useful in the separation of structurally related compounds with similar acid-base behaviour where the resolution cannot be achieved by varying the pH. The use of cyclodextrin as a neutral macrocyclic complex-forming agent seems to be advantageous for the resolution of similar organic compounds [1-6].

The analysis of carboxylic acids, such as mono-, poly, and hydroxy-carboxylic acids, is extremely important and nearly all separation techniques have been applied to them. Good results have been obtained by various research workers who analysed these substances by liquid chromatography [7-14], electrophoresis [15-18] and isotachophoresis [19-26]. Many references can easily be found and they are not cited here because only incomplete list could be given. So far, little attention has been paid to the separation of hydroxycarboxylic acids by isotachophoresis.

In this work, we discuss various electrolyte systems in which hydroxycarboxylic acids can be analysed by isotachophoresis. Several operational systems are considered below in order to show complex formation and the variations in the effective mobilities.

Experimental

Chemicals

Deionized and redistilled water was used in the preparation of the solutions of the electrolytes and compounds investigated. All chemicals were of the highest quality commercially available. The solutes investigated (Tab. I) were provided by Pharmaceutical Research Institute, Warsaw, Poland.

Sample solutions (2 mmol/L) were prepared by dissolving each substance in water and were stored in a refrigerator. β-cyclodextrin (βCD) was obtained from Merck (Darmstadt). Heptakis (2,3,6-tri-O-methyl)-β-cyclodextrin (TMβCD) was synthesised by the method of Nowotny et al. [27].

Apparatus

Isotachophoretic experiments were performed using a Villa Labeco ZKI 02 column-coupling isotachophoretic analyser (Slovak Republic) equipped with capillaries made of a fluorinated ethylene-propylene copolymer.

Operating conditions

The operating conditions are given in table II. In the case of one-dimensional isotachophoresis, the preseparation and the analytical capillary was filled with leading

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Table I. Hydroxycarboxylic acids investigated.

No.	Compound	pK_1 [a] pK_2 [a]	μ_o [a] ($cm^2V^{-1}s^{-1}.10^5$)
Group A			
1	tartaric acid	3.036	32.6
		4.366	60.7
2	malic acid	3.460	32.6
		5.050	59.0
3	glyceric acid	3.737	36.4
4	2-methylmalic acid		
5	lactic acid	3.860	36.5
6	2-hydroxybutyric acid	3.979	34.3
7	2-hydroxy-3-methylbutyric acid		
8	2-hydroxy-2-methylbutyric acid		
9	2-hydroxy-4-methylvaleric acid		
Group B			
10	mandelic acid	3.411	28.3
11	atrolactic acid		
12	p-hydroxymandelic acid		
13	3-phenyllactic acid		
14	3-(4-hydroxyphenyl)lactic acid	4.76 [b]	
15	2-hydroxy-4-phenylbutyric acid		
16	hexahydromandelic acid		
17	tropic acid		

μ_o absolute mobility; (a) Ref. [28]; (b) Ref. [29].

Table II. Electrolyte systems and conditions for isotachophoresis.

Parameter	*Conditions*
Leading electrolytes (LE)(1)	
LE1	10 mmol/L HCl adjusted with β-alanine to pH 3.0
	LE1 + 5 mmol/L βCD
	LE1 + 10 mmol/L βCD
	LE1 + 5 mmol/L TMβCD
	LE1 + 10 mmol/L TMβCD
LE2	10 mmol/l HCl adjusted with β-alanine to pH 3.5
	LE2 + 5 mmol/L βCD
	LE2 + 10 mmol/L βCD
	LE2 + 5 mmol/L TMβCD
	LE2 + 10 mmol/L TMβCD
LE3	10 mmol/L HCl adjusted with creatinine to pH 4.0
LE4	10 mmol/L HCl adjusted with creatinine to pH 4.5
LE5	10 mmol/L HCl adjusted with creatinine to pH 5.0
LE6	10 mmol/L HCl adjusted with histidine to pH 5.5
Terminating electrolyte	10 mmol/L caproic acid, pH 3.4
Capillaries	
I (preseparation)	90 mm × 0.8 mm I.D.
II (analytical)	90 mm × 0.3 mm I.D.
Current	
I	200 μA
II	40 μA
Detection	Conductivity
Injection	10-μL microsyringe
Temperature	22 ± 2 °C

(1) 0.1% methylhydroxyethylcellulose was added to all leading electrolytes in order to suppress the electroosmotic flow.

electrolytes LE1 - LE6. In the case of two-dimensional isotachophoresis, the preseparation capillary was filled with LE2. The analytical capillary was run with CD-modified leading electrolyte (either LE1 or LE2).

Calibration

Calibration analysis were carried out in the analytical capillary where six calibration points were measured within the range 0.08 – 4.0 mmol/L (injection 1 μL). The upper calibration limits were those concentrations where mixed isotachophoretic zones occurred.

Results and discussion

The ITP resolution was determined on the bases of the step height differences of the compounds. In all system the chloride ion (leading ion) has a step height of 0. The step heights of the analytes are given in mV from the level of the leading electrolyte zone. These values are given for comparison of the various electrolyte systems. According to the accuracy and reproducibility of the measurements, a value of 1% of caproic acid step height (terminating ion) was specified as the lowest step height difference limit in order to achieve practically significant solute resolution. Owing to the fact that caproic acid interacted very strongly with βCD and TMβCD, respectively, the difference limit varied from system to system. Differences lower than 1% were considered to be negligible.

For a clearer understanding of the experimental data and easier description of the role of cyclodextrins in structural differentiation, the compounds were divided into two groups. The solutes with a common aliphatic chain were included in group A and the solutes with aromatic and aliphatic ring form group B.

One-dimensional isotachophoresis

As not all pK values and mobilities of the hydroxycarboxylic acids are known we have to use the experimental method in order to find a suitable electrolyte system. We have chosen some leading electrolyte systems with pH values of 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5 (these values were chosen because several hydroxycarboxylic acids have pK_a

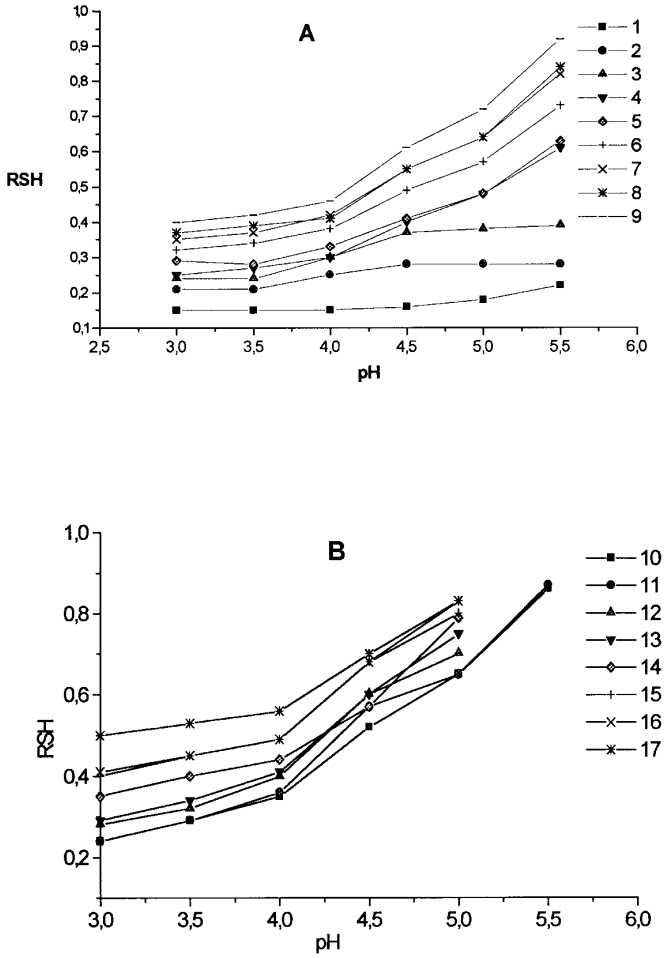

Fig. 1. The relative step height (RSH) values of hydroxycarboxylic acids as a function of pH of the leading electrolyte, RSH = $(h_x - h_l)/(h_t - h_l)$. Group A: 1- tartaric acid, 2- malic acid, 3- glyceric acid, 4- 2-methylmalic acid, 5- lactic acid, 6- 2-hydroxybutyric acid, 7- 2-hydroxy-3-methylbutyric acid, 8- 2- hydroxy-2-methylbutyric acid, 9- 2-hydroxy-4-methylvaleric acid. Group B: 10- mandelic acid, 11- atrolactic acid, 12- p-hydroxymandelic acid, 13- 3-phenyllactic acid, 14- 3-(4-hydroxyphenyl)lactic acid, 15- 2-hydroxy-4-phenylbutyric acid, 16- hexahydromandelic acid, 17- tropic acid. Foe electrolyte systems and conditions, see table II.

values between 3.0 and 5.0 [28]). The conditions for the electrolyte systems are given in table II and the measured relative step heights are shown graphically in figures 1A and 1B. It can be seen from Fig. 1A that tartaric, malic, 2-hydroxybutyric and 2-hydroxy-4-methylvaleric acid are separated from the other acids over the full pH range. From the Fig. 1B it is apparent that 3-(4-hydroxyphenyl)lactic and tropic acid are separated from the other anions in the pH range of the leading electrolyte between 3.0 and 4.0. It can also be seen from Fig. 1A and 1B that maximal differences in relative step heights are obtained at lower pH values. Therefore, for the separation of the mixture of hydroxycarboxylic acids we used the leading electrolyte at pH 3.0 and 3.5, resp. The isotachopherograms of these separations are shown in figures 2a and 2d (group A) and figures 3a and 3d (group B). From the conductivity detector record (Fig. 2a and Fig. 3a), it is appeared that at pH 3.0 of the leading electrolyte, nearly all hydroxycarboxylic acids are likely to be separated; glyceric + 2-methylmalic acid (group A) and p-hydroxymandelic + 3-phenyllactic acid and mandelic + atrolactic acid (group B), however, will comigrate in the mixed zones. At pH 3.5, four mixed zones were obtained (Figs. 2d and 3d). In the pH range between 3.0 and 5.5 the migration order changes for several solutes and the probability of the occurrence of mixed zones is rather high.

The most important information obtained by these measurements is that the complete resolution cannot be achieved by varying the pH of the leading electrolyte, therefore the structural differences of these solutes were taken as a tool for the adjustment of the separation selectivity.

Two-dimensional isotachophoresis

Cyclodextrins are known to be useful in the area of structurally related organic compounds and also inorganic ions. In this work, different amounts of βCD and TMβCD (5 and 10 mmol/L) were added to the leading electrolyte to propose isotachophoretic conditions suitable for effective resolution of hydroxycarboxylic acids. The resulting measurements were carried out two-dimensionally. Preseparation capillary was filled with the unmodified leading electrolyte pH 3.5. The pH of the CD-modified leading electrolyte of the analytical capillary was either 3.0 or 3.5.

ITP resolution of group A solutes

Effect of βCD

The step height difference of glyceric acid and 2-methylmalic acid with the unmodified electrolyte system pH 3.0 is not significant and the system does not resolved them. Figure 4a shows the effect of the concentration of βCD added to the leading electrolyte at pH 3.0 on the step height of the analyte compounds. By increasing the amount of βCD in the leading electrolyte the step height of all the analytes increased (decreased effective mobilities). This effect was most evident for 2-hydroxy-3-methylbutyric, 2-hydroxy-2-methylbutyric and 2-hydroxy-4-methylvaleric acid. The addition of βCD to the leading electrolyte at pH 3.0 significantly improves the resolution of glyceric acid and makes it possible to differentiate glyceric acid and 2-methylmalic acid from lactic acid. The best resolution of glyceric, 2-methylmalic and lactic acid was achieved at a βCD concentration of 5 mmol/L (Fig. 2b).With this system the stability of the separated zones was verified by construction of calibration lines (see tab. III). The zone lengths were evaluated from the differential conductivity signal of conductivity detector in the analytical capillary. From the correlation coefficients of the calibration equation it can be concluded that the isotachophoretic zones are stable. Higher concentrations lead to a loss in resolution of 2-methylmalic acid

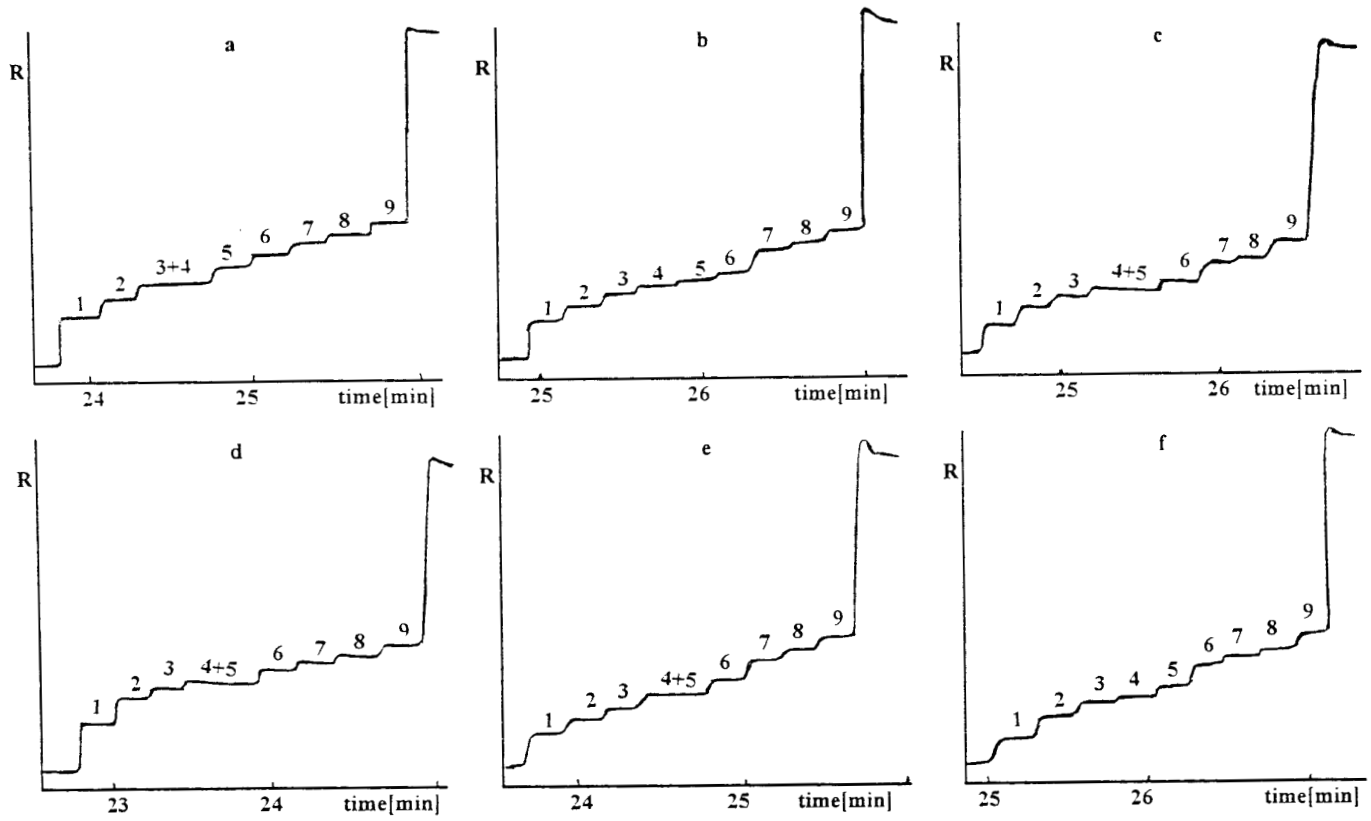

Fig. 2. Isotachophoretic separation of a group A model mixture (2 mmol/L each) by coupling the preseparation capillary filled with system LE2, pH 3.5 and the analytical capillary filled with system LE1, pH 3.0 (a,b,c) or with LE2, pH 3.5 (d,e,f). Electrolyte in the analytical capillary contains (a,d) 0 mmol/L, (b,e) 5 mmol/L and (c,f) 10 mmol/L βCD. The record (R) from the contact conductivity detector of the analytical capillary is shown. 1- tartaric acid, 2- malic acid, 3- glyceric acid, 4- 2-methylmalic acid, 5- lactic acid, 6- 2-hydroxybutyric acid, 7- 2-hydroxy-3-methylbutyric acid, 8- 2- hydroxy-2-methylbutyric acid, 9- 2-hydroxy-4-methylvaleric acid.

Table III. Linearity of the method in the concentration range 0.08 – 4.0 mmol/L. The equation for the straight line is $y = a + b.x$, where y is the zone length (mm), a is the intercept, b is the slope (absolute injected amount, nmol); r is the correlation coefficient.

Compound	*a*	*b*	*r*
tartaric acid	0.03	17.44	0.9997
malic acid	–0.02	14.26	0.9995
glyceric acid	0.01	16.11	0.9998
2-methylmalic acid	0.15	13.28	0.9993
lactic acid	0.11	15.04	0.9994
2-hydroxybutyric acid	0.04	13.11	0.9992
2-hydroxy-3-methylbutyric acid	–0.01	10.28	0.9996
2-hydroxy-2-methylbutyric acid	–0.09	11.78	0.9996
2-hydroxy-4-methylvaleric acid	–0.16	12.54	0.9993

and lactic acid (Fig. 2c). By performing similar isotachophoretic experiments with the leading electrolyte at pH 3.5 an increase in the step heights with increasing amount of βCD was observed in all instances (Fig. 4b) except tartaric and glyceric acid; however, this increase was less significant than that obtained at pH 3.0 may be due to the higher dissociation of the acids at pH 3.5. The resolution increased with increasing amount of βCD; complete separation was obtained at a βCD concentration of 10 mmol/L (Figs. 2e and 2f).

Effect of TMβCD

The effect of TMβCD on the step height of group A solutes is shown in figures 4c and 4d. Compared with the βCD, the TMβCD provides a weak retardation effect of hydroxycarboxylic acids. In Fig. 4d it is appeared that most acids cannot be separated with the leading electrolyte pH of 3.5 modified with TMβCD; only tartaric, 2-hydroxybutyric and 2-hydroxy-4-methylvaleric acid were separated at this pH. A better separation can be achieved by decreasing the pH of the leading electrolyte. However, the selectivity in the leading electrolyte pH 3.0 with 5 (10) mmol/L TMβCD

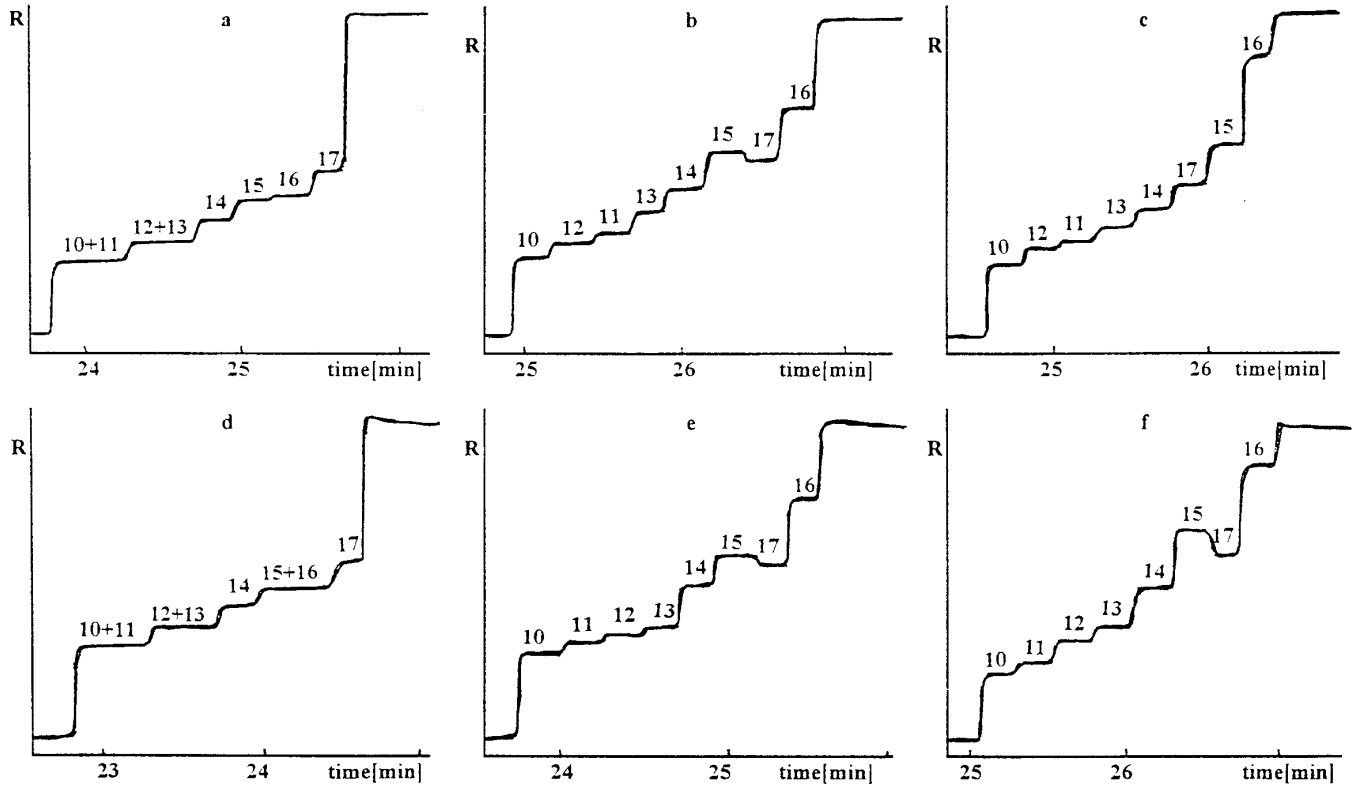

Fig. 3. Isotachophoretic separation of a group B model mixture (2 mmol/L each) by coupling the preseparation capillary filled with system LE2, pH 3.5 and the analytical capillary filled with system LE1, pH 3.0 (a,b,c) or with LE2, pH 3.5 (d,e,f). Electrolyte in the analytical capillary contains (a,d) 0 mmol/L, (b,e) 5 mmol/L and (c,f) 10 mmol/L βCD. The record (R) from the contact conductivity detector of the analytical capillary is shown. 10- mandelic acid, 11- atrolactic acid, 12- p-hydroxymandelic acid, 13- 3-phenyllactic acid, 14- 3-(4-hydroxyphenyl)lactic acid, 15- 2-hydroxy-4-phenylbutyric acid, 16- hexahydromandelic acid, 17- tropic acid.

(see Fig. 4c) is either insignificant (glyceric and 2-methylmalic acid) or completely absent (2-methylmalic and lactic acid). Generally, the addition of TMβCD has no positive separation effect.

ITP resolution of group B solutes

Effect of βCD

The effect of βCD on the step height of group B solutes is shown in figures 5a and 5b. By increasing amount of βCD in the leading electrolyte at pH 3.0 all the step heights increased (Fig. 5a) and complete resolution was obtained with 10 mmol/L βCD. With this system the calibration was carried out and from the correlation coefficients of the calibration equation (see tab. IV) it can be stated that the isotachophoretic zones are stable. An increase in the step height with increasing amount of βCD was also observed at pH 3.5 but this increase was less significant than that obtained at pH 3.0. Separation of the mixture of group B solutes with 5 and 10 mmol/L βCD at pH 3.0 and 3.5 is shown in figure 3. Fairly large changes in the effective mobilities and migration orders are obtained, but the leading electrolyte at pH 3.5 with 5 mmol/L βCD gives identical separation as a 10 mmol/L βCD(Figs. 3e and 3f). Large decreases in effective mobilities are observed for hexahydromandelic and 2-hydroxy-4-phenylbutyric acid. The most interesting feature of the leading electrolyte at pH 3.5 modified with βCD is the enforced migration [30] of 2-hydroxy-4-phenylbutyric acid ahead of tropic acid.

Effect of TMβCD

Figures 5c and 5d shows the relationship between the amount TMβCD added to the leading electrolyte at pH 3.0 (3.5) and the step height of the group B solutes. By adding TMβCD to the leading electrolyte, a weak reduction in effective mobility was observed for all compounds, but an increase in the amount of TMβCD did not result in complete resolution. Generally, the effective mobilities of the eight examined compounds were reduced more effectively by using βCD than TMβCD.

The main parameters affecting separations with CD-based electrolytes are the nature and concentration of the CD and leading electrolyte pH. Concerning the nature of the CD,

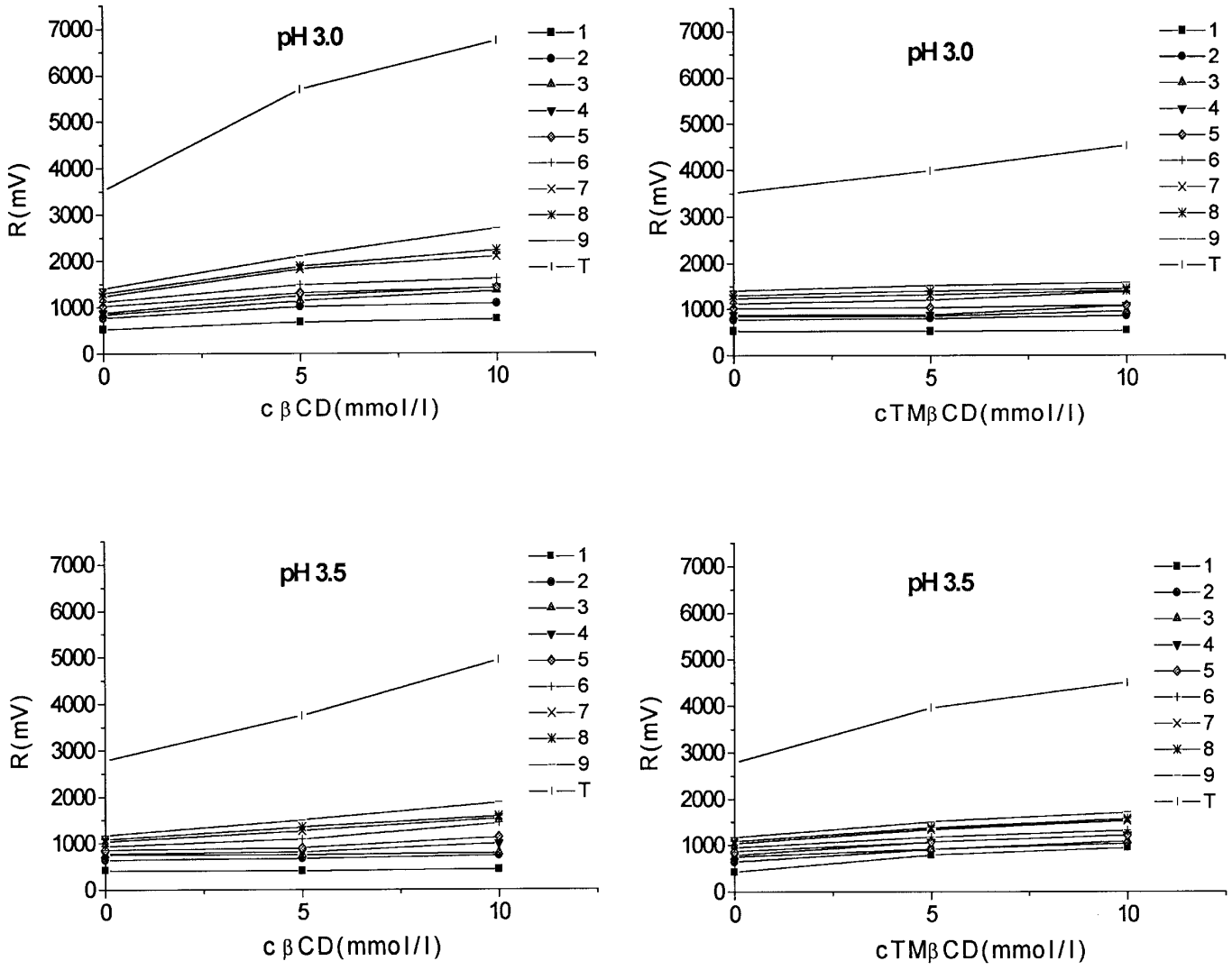

Fig. 4. Effect of βCD (a,b) and TMβCD (c,d) concentration on the step height of the group A solutes. Experimental conditions and symbols as in Fig. 2 (T - terminating ion).

Table IV. Linearity of the method in the concentration range 0.08 – 4.0 mmol/L. The equation for the straight line is $y = a + b.x$, where y is the zone length (mm), ,a is the intercept, b is the slope (absolute injected amount, nmol); r is the correlation coefficient.

Compound	*a*	*b*	*r*
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3-phenyllactic acid	0.11	10,19	0,9996
3-(4-hydroxyphenyl)lactic acid	0.12	11.59	0.9996
2-hydroxy-4-phenylbutyric acid	0.08	11.22	0.9995
hexahydromandelic acid	0.02	10.99	0.9994
tropic acid	0.01	10.72	0.9995

βCD leads to more stable inclusion complexes than TMβCD. TMβCD is characterised by the presence only of methyl groups and the absence of primary and secondary hydroxyl group on rim. The methylation of all hydroxyl groups makes the βCD more flexible and should lead to a better fit with analytes. However, βCD appeared to be better complexing agent than TMβCD. This indicates that hydroxyl groups of βCD are involved in the complexation of hydroxycarboxylic acids and the hydrogen bonding occurs. When the length of the alkyl chain of group A acids was increased, the retardation increased with the βCD, whereas it remained almost constant with the TMβCD. This trend also supports hydrophilic interactions. The higher complexation for group B solutes in case βCD could be attributed to the aromatic ring in the structure of group B, which is more likely fit in the hydrophobic cavity of the CD than the aliphatic chain of

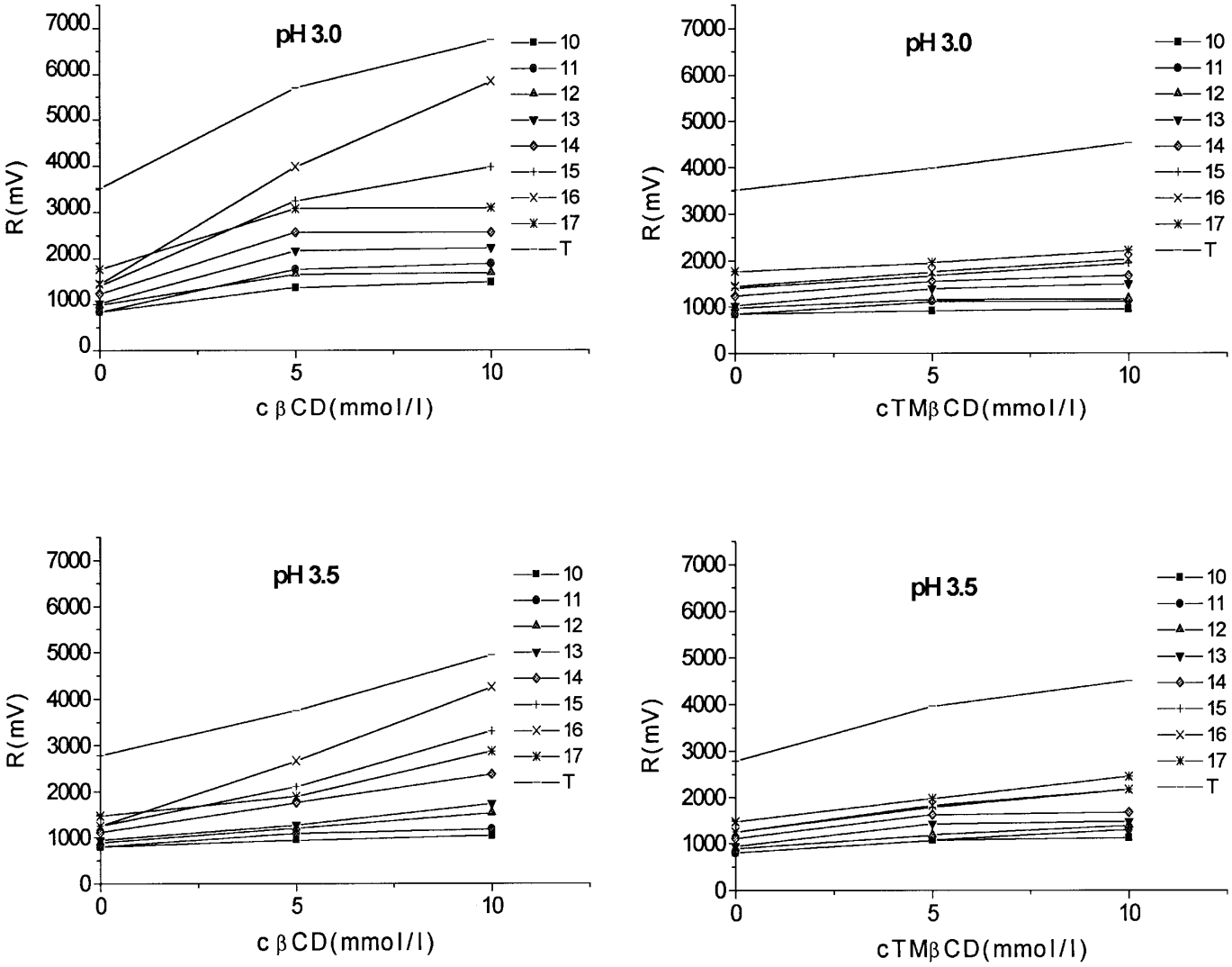

Fig. 5. Effect of βCD (a,b) and TMβCD (c,d) concentration on the step height of the group B solutes. Experimental conditions and symbols as in Fig. 3 (T - terminating ion).

group A solutes. With both the CDs studied, the hydroxycarboxylic acids formed more stable inclusion complexes at pH 3.0 than at other pH values probably due to the higher inclusion of the solutes when they are in their less dissociated form. These results indicated that hydrophobic interactions and hydrogen bonding occur in combination in the separation mechanisms.

Conclusions

The comparative measurements with βCD and its trimethyl derivative showed that the selectivity is highly influenced by the size and the hydrophobicity of the cavity. It can be concluded that the βCD is able to form relatively stable complexes with the compounds studied, but the effective resolution depends on the choice of the suitable pH of leading electrolyte.

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