ELSEVIER

Journal of Chromatography A, 979 (2002) 115–122

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

# Separation of aromatic aminophosphonic acid enantiomers by capillary electrophoresis with the application of cyclodextrins

Ewa Rudzińska[a], Paweł Dżygiel[b], Piotr Wieczorek[b,*], Paweł Kafarski[a,b]

[a]*Institute of Organic Chemistry, Biochemistry and Biotechnology, Wroclaw University of Technology, Wybrzeże Wyspiańskiego 27, 50-370 Wroclaw, Poland*

[b]*Institute of Chemistry, University of Opole, Oleska 48, 45-052 Opole, Poland*

**Abstract**

The detailed studies concerning capillary electrophoresis separation of aminophosphonic acid enantiomers with various commercially available cyclodextrins are presented. The obtained results show that the separation of these stereoisomers is dependent on pH of background electrolyte, concentration of cyclodextrin as well as on the type of applied chiral selector. The separation mechanism is based on the co-operative effect of hydrogen bond type interactions enhanced by hydrophobic forces and sterical constrains between aminophosphonate and cyclodextrin. With application of elaborated method, enantiomeric baseline or partial separation of 18 α-aminophosphonic acids was achieved. This separation can be successfully used for routine aminophosphonic acids enantiopurity determination.


*Keywords:* Enantiomer separation; Aminophosphonic acids; Organophosphorus compounds; Cyclodextrins

## 1. Introduction

Aminophosphonic acids are the compounds characteristic in that they possess amino and phosphonic acid group in their structure. The most popular among them are α-aminophosphonic acids, which might be considered as mimetics of amino acids and are obtained by substitution of their carboxylic group by phosphonic or phosphinic acid moiety. The structural similarity to amino acids causes that they exhibit interesting biological activity usually acting as amino acid antimetabolites and implicates their further promising usefulness in medicine and agriculture [1]. It is widely recognised that the biologically active compounds are active if applied in the form of certain stereoisomer. Therefore, it is important to have a simple and versatile method for the determination of enantiopurity of aminophosphonates. These compounds are usually obtained either by resolution of their enantiomers by classical methods, or prepared by asymmetric synthesis including the use of biocatalysis. The most common manner of the determination of the optical purity of these acids relays on comparison of their specific rotation with the highest value given in the literature. This method is, however, completely not useful if considering new chemical structures. In such cases, it is necessary to use NMR in chiral media or chromatography with application of chiral phases. The latter method usually requires derivatisation of aminophosphonates prior to analysis [2–7] and the direct determination of their enantiopurity is rather seldom [8–10]. Capillary electrophoresis

*Corresponding author. Fax: +48-77-441-0740.

*E-mail address:* piotr.wieczorek@uni.opole.pl (P. Wieczorek).

0021-9673/02/$ – see front matter 
PII: S0021-9673(02)01248-7

seems to be a new and promising method although it was used only for limited number of specific cases [7,11–14].

The main aim of this work was to determine if capillary electrophoresis with the application of commercially available cyclodextrins used as chiral selectors might be considered as a fast and simple method for the determination of the optical purity of aminophosphonates. The presented results are extension and continuation of our previous work [13] that concerned the separation of aromatic aminophosphonates by means of capillary zone electrophoresis with α-cyclodextrin (α-CD) as chiral selector (CS). In the preceding paper we reported the usefulness of this system for separation of several aminophosphonic acids from the set of 20 compounds differing mainly in the position and character of substitution of the benzene ring. The application of α-CD brought about the baseline separation for only four racemates, with six of them being partially resolved and ten not separated at all. Therefore, we have decided to apply other types of cyclodextrin as chiral selectors for enantioseparation of enlarged set of aromatic aminophosphonates. Several, commercially available cyclodextrins were used for this purpose, namely: β-cyclodextrin (β-CD), heptakis(2,3-*O*-dimethyl)-β-cyclodextrin (DM-β-CD), (2-hydroxypropyl)-α-cyclodextrin (HP-α-CD) and (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD). Additionally, some efforts were undertaken in order to optimize the conditions of separation regarding the influence of cyclodextrin concentration and of pH of background electrolyte (BGE). The degree of enantioseparation of aromatic aminophosphonates (the structures of the studied compounds are shown in Fig. 1) is expressed as separation factor ($\alpha$) and resolution ($R_s$) calculated form standard literature equations [13].

## 2. Experimental

### 2.1. Chemicals

Racemic mixtures of aminophosphonic acids, were either available from previous studies [15–17] or were obtained by standard methods [18,19]. Water used was purified with a Milli-Q system (Milipore, Bedford, MA, USA). The phosphorus salts were obtained from Sigma, Poland. β-CD was from Sigma (Poland), DM-β-CD was from Aldrich (Poland), and HP-α-CD and HP-β-CD was from Fluka (Poland). Hydroxypropylated cyclodextrins are characterized with degree of substitution 0.6.

### 2.2. Apparatus, background electrolyte and experimental procedure

Electrophoretic experiments were performed on P/ACE 5000 capillary electrophoresis system (Beckman, Palo Alto, CA, USA). Capillary zone electrophoresis was performed in a fused-silica capillary 57 cm (50 cm to the detector)×75 μm I.D.×375 μm O.D. obtained from Polymicro Technologies (Phoenix, AZ, USA). The capillary was cooled with fluorocarbon liquid and the temperature was set at 25±0.1 °C. UV detection was used with a deuterium lamp operated at 214 nm (bandpass filter).

To obtain a required background electrolyte solution, an appropriate amount of cyclodextrin to reach final concentration was dissolved in 0.1 *M* phosphate buffer or in 0.1 *M* borate buffer and adjusted to required pH with 0.1 *M* NaOH (one or two drops per 100 ml). Experimental solutions were prepared by dissolving appropriate amount of aminophosphonic acid in water in order to obtain its 1 m*M* final concentration. Experimental procedures were identical to those described earlier [16] and were as follows: at the start and end of each working day, the capillary was washed with 0.1 *M* NaOH solution (40 min) and water (2 min). Prior to every run, the capillary was washed with 0.1 *M* NaOH (2 min), water (2 min) and BGE (1 min). The sample was injected hydrostatically (3-s duration) by applying pressurized nitrogen. The electrophoretic separation was performed at applied voltage 12.5 kV.

## 3. Results and discussion

### 3.1. The influence of background electrolyte pH

Influence of background electrolyte pH on the received separation factor $R_s$ of compounds **1** and **10** in the presence of β-CD is shown in Fig. 2. These two compounds were chosen due to the striking difference in their structure. Compound **1** bearing

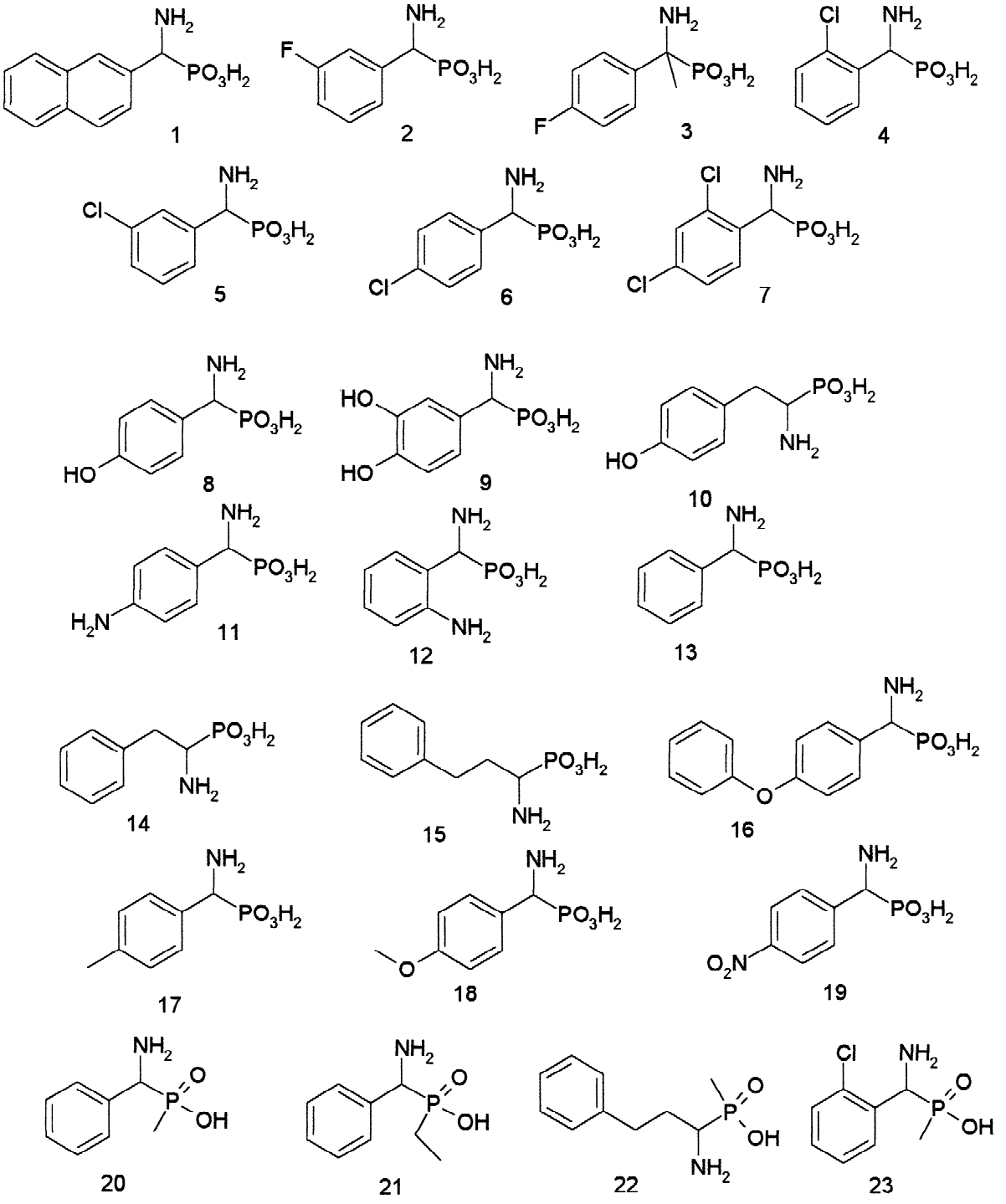


Fig. 1. Structures of α-aminophosphonates.

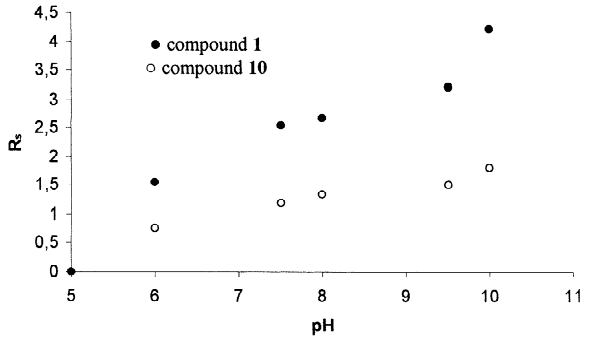


Fig. 2. The influence of the background electrolyte pH on the resolution of compounds **1** and **10**. Conditions: for pH 5.0, 6.0, 7.5, 8.0, BGE=0.1 *M* phosphate buffer; for pH 9.5 and 10.0, BGE=0.1 *M* borate buffer, 15 m*M* β-CD. Voltage: 12.5 kV; injection time: 3 s.

naphtyl moiety was one of the most hydrophobic in the examined set of aminophosphonates, whereas compound **10** is less hydrophobic due to the presence of hydroxyl moiety in *para* position of benzene ring, which is able to dissociate in certain conditions. As seen from the Fig. 2 the aminophosphonates began to be separated into enantiomers when background electrolyte pH value exceeded 6. This corresponds to the dissociation of the second hydroxyl of phosphonate moiety as indicated by the calculated p$K_a$ values. They are as follows: for compound **1** are 0.62, 5.73 for phosphonic acid group and 9.16 for amino group, whereas for compound **10** are 0.62, 5.60 for phosphonic acid group, 9.90 for phenolic one and 10.25 for amino group [20]. It suggests that for the resolution of α-aminophosphonic acids, it is crucial for the phosphonic group to be fully dissociated with two hydroxy groups being negatively charged. Such a deprotonation could increase the possibility of the formation of hydrogen bonds between phosphonate fragment of the complexed molecule and hydroxyl groups of cyclodextrin and by this manner the enantioselectivity is amplified. Raise of pH results in better resolution and we assume that this might be a result of the deprotonation of positively charged amino group (at pH higher than 9). The change of protonation status of phenol group of compound **10** failed to affect the resolution. It may be, therefore, assumed that specific interactions of aromatic fragment of aminophosphonate with hydrophobic cyclodextrin cavity typical to inclusion–complexation mechanism and network of hydrogen bonds formed between phosphonate and amino groups of the molecule and hydroxyl groups of cyclodextrin are vital for enantioseparation. This assumption was additionally supported by experiments performed with the same chiral selector (namely β-CD) and the pH of background electrolyte set to 7.5 and 10 with compounds **8** (containing one hydroxyl group at para position and shorter aliphatic chain) and **9** (the same as **8** but with two hydroxyl moieties). In the case of these two compounds the lack of resolution was observed showing that the shortening of the distance between phenolic moiety and aminophosphonate fragment disturbed the structural features required for the formation of inclusion complex.

Although it is evident that the most appropriate pH value for the resolution of α-aminophosphonates is set at 10, we have selected pH equal 7.5 for the further experiments. The reason for this was that at high pH the electrophoretic mobility was significantly small and caused long retention times (around 45–80 min depending on the compound) and thus resulted in significantly prolonged time of analysis.

### *3.2. Effect of cyclodextrin concentration in background electrolyte*

In order to achieve proper resolution of enantiomers optimal concentration of each kind of cyclodextrin is required. Thus, we have studied the influence of varying chiral selector content in BGE on $R_s$. As a model aminophosphonate compound **1** was selected. Fig. 3 shows that the resolution depended typically on the concentration of cyclodextrin nearly in the same manner despite the kind of chiral selector used. Initially, the resolution increased with the increase of chiral selector concentration reaching the saturation equilibrium between free aminophosphonate and diastereoisomeric aminophosphonate–cyclodextrin complex. As a result, amount of the bound aminophosphonate did not increase and the resolution reached plateau. The same dependence might be observed for β-CD, however, due to limited solubility of this cyclodextrin in water the maximal concentration possible to obtain in this background electrolyte was 15 m*M*.

For further experiments the following concentra-

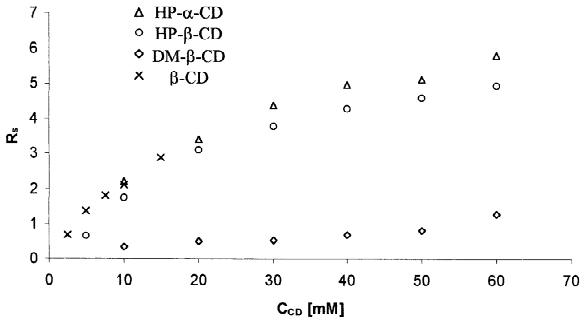


Fig. 3. Resolution dependence of compound **1** on the cyclodextrins concentration in background electrolyte. Conditions: BGE=0.1 *M* phosphate buffer; pH 7.5; voltage: 12.5 kV; injection time: 3 s.

tions of chiral selectors were chosen: β-CD 15 m*M*, DM-β-CD 30 m*M*, HP-α-CD 60 m*M* and for HP-β-CD 40 m*M*, again keeping in mind compromise between resolution and analysis time as well as the maximal possible concentration of cyclodextrin available under conditions of analysis.

### *3.3. The influence of type of cyclodextrin*

The type of chiral selector applied in enantioseparation is a very important factor that influences the enantioseparation. The varying strength of binding of enantiomers and creation of ''chiral selector–aminophosphonate'' diastereoisomeric complex is caused by the specific weak interactions of various origin. In our case two types of the interactions responsible for enantioseparation seems to be relevant. The first one is the formation of hydrogen bond network created between aminophosphonic fragment of the molecule and hydroxyl groups present at the surface of cyclodextrin. Their strength and geometry appear to be strongly dependent on the mode of inclusion of the aromatic part of aminophosphonic acid into hydrophobic cavity of cyclodextrin. In order to confirm this, the resolution of compound **1** by means of various cyclodextrins were carried out setting the pH of background electrolyte at 7.5 and cyclodextrin concentration at 15 m*M*. As shown in Table 1 (representative electropherograms shown at Fig. 4) the naphtyl moiety is preferably bound by β-cyclodextrin ring, which resulted in enhanced enantioselectivity. The situation had

Table 1
Enantiomeric separation of compound **1** at the same concentration of different cyclodextrins

| Cyclodextrin | $\alpha$ | $R_s$ |
|---|---|---|
| α-CD | 1.013 | 1.24 |
| β-CD | 1.029 | 2.80 |
| DM-β-CD | 1.008 | 0.54 |
| HP-α-CD | 1.025 | 2.33 |
| HP-β-CD | 1.021 | 1.95 |

Conditions: BGE=0.1 *M* phosphate buffer, pH 7.5, 15 m*M* of appropriate cyclodextrin, voltage: 12.5 kV, injection time: 3 s.

changed when used hydroxypropylcyclodextrins, where a slight reversal of enantioselectivity was observed. This indicates that the proper balance between hydrophobic interactions and network of hydrogen bonds formed decides about relative stability of diastereoisomeric complexes. The observed difference in binding may result from the formation of hydrogen bonds between hydroxy groups of hydroxypropyl moiety and aminophosphonate fragment of compound **1**, which resulted in partial removal of aromatic fragment of the aminophosphonate from the cyclodextrin hydrophobic cavity. Interestingly, quite opposite effect was observed when using α-CD and HP-α-CD. This surprising finding suggests the existence of different geometry of binding of naphtyl moiety by α- and β-cyclodextrins, although confirmation of this speculation requires more detailed studies.

The results of experiments concerning the potency of the used chiral selectors for the enantioseparation of remaining aminophosphonates are presented in Table 2. In the table are presented only these compounds for which enantioseparation was achieved, whereas compounds not included into the table were not separated. It can be seen, that only three compounds were completely resolved ($R_s$ value higher than 2.00) at pH 7.5 regardless of CD type. They were compounds **1**, **2**, **14**, which represent simple unsubstituted aromatic aminophosphonic acids. Seventeen compounds, namely **3–7**, **10**, **12–15**, **17–19**, **23**, were more or less separated into enantiomers. Unfortunately, there is no possibility to derive simple structure–resolution relationship in this case. Generally, the resolution was strongly selector-dependent with the best results of baseline separation obtained for β-CD and HP-β-CD and with DM-β-

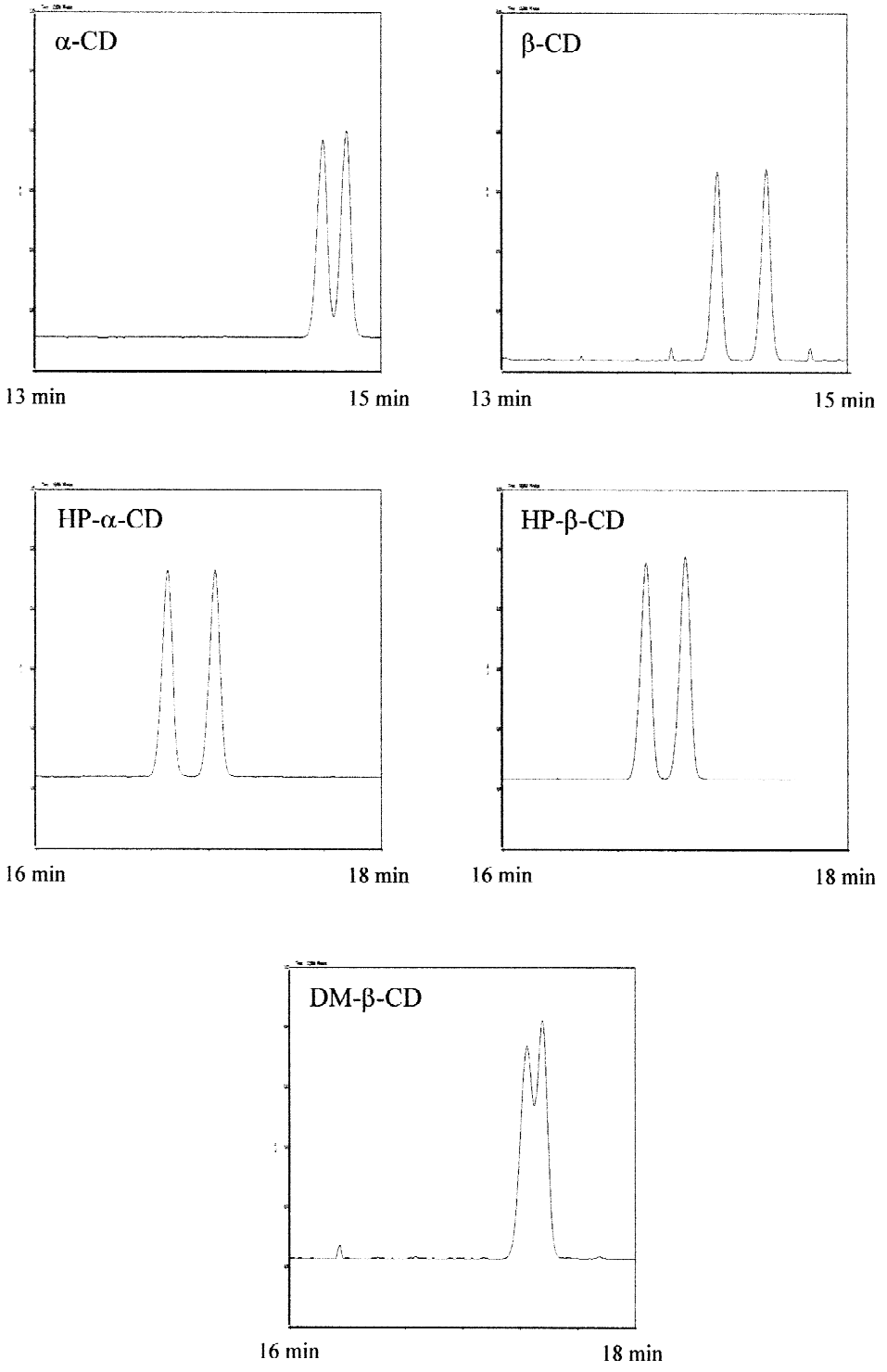


Fig. 4. Resolution of compound **1** by different cyclodextrins at the same 15 m*M* concentration in BGE. Conditions: BGE=0.1 *M* phosphate buffer; pH 7.5; 15 m*M* of cyclodextrin; voltage: 12.5 kV; injection time: 3 s.

Table 2
Compounds resolved with cyclodextrins

| Compound | $\alpha$ | $R_s$ |
|---|---|---|
| (a) 30 m*M* DM-β-CD | | |
| **1** | 1.014 | 0.52 |
| **6** | 1.009 | 0.90 |
| **7** | 1.009 | 0.83 |
| **12** | 1.008 | – |
| **14** | 1.007 | 0.37 |
| **15** | 1.011 | 1.08 |
| **17** | 1.003 | – |
| **19** | 1.020 | 1.83 |
| (b) 15 m*M* β-CD | | |
| **1** | 1.027 | 2.96 |
| **5** | 1.007 | 0.48 |
| **7** | 1.009 | 1.94 |
| **10** | 1.015 | 1.42 |
| **14** | 1.24 | 2.40 |
| **19** | 1.012 | 1.14 |
| **23** | 1.005 | – |
| (c) 60 m*M* HP-α-CD | | |
| **1** | 1.058 | 5.98 |
| **2** | 1.041 | 3.35 |
| **3** | 1.016 | 1.58 |
| **4** | 1.017 | 1.38 |
| **13** | 1.001 | 0.62 |
| **14** | 1.006 | 0.43 |
| **17** | 1.008 | 0.87 |
| **18** | 1.008 | 0.64 |
| (d) 40 m*M* HP-β-CD | | |
| **1** | 1.04 | 3.69 |
| **5** | 1.01 | 0.82 |
| **7** | 1.018 | 1.93 |
| **10** | 1.013 | 1.07 |
| **12** | 1.011 | 0.55 |
| **14** | 1.021 | 2.19 |
| **19** | 1.009 | 0.58 |
| **23** | 1.01 | 0.91 |

Conditions: BGE=0.1 *M* phosphate buffer; pH 7.5; voltage: 12.5 kV; injection time: 3 s.

CD being the least effective chiral selector. Compounds **8**, **9**, **11**, **16**, and **20–22** were not resolved despite of the kind of cyclodextrin used. Compounds **8**, **9** and **11** constitute analogues of phenylglycine substituted with polar groups in benzene ring and most probably this substitution effectively prevents their binding in hydrophobic cavities of the selectors. Compounds **20–21** constitute a group of P-alkyl phosphinic acids and most probably this fragment of the molecule is not able to form hydrogen bonds with hydroxyl groups of cyclodextrin, thus diminishing their effective binding. The only representative of this group, compound **23,** which was only slightly resolved by HP-β-CD and β-CD. It is worth to mention that its formal precursor, compound **4**, was not resolved by any of β-cyclodextrins studied indicating that the existence of the subtle equilibrium between bonding of hydrophobic and hydrophilic fragments of the aminophosphonate molecule is required for enantioseparation. The lack of resolution of compound **16** is somewhat surprising. It was, however, partially resolved by α-CD as it was shown in our previous paper (40 m*M* α-CD, $R_s$=0.75, from Ref. [13]). This compound is however of markedly different geometry in comparison to all other studied aminophosphonates, since the presence of ether bond causes that the molecule is bent, which may results in some constrains that counteract the fitting of aromatic part in the cavity of cyclodextrin.

Some more generalization could been made if considering enantioseparation potency of individual cyclodextrins. There is a group of seven compounds (Table 1a) resolved by β-CD (**1**, **5**, **7**, **10**, **14**, **19**, **23**) with two of them being completely resolved (**1** and **14**). Except of compound **10** (the only compound in this group with polar substituent in phenyl ring) they represent either simple aromatic aminophosphonates (compounds **1** and **14**) or analogues of phenylglycine with phenyl ring substituted by bulky groups of low polarity (compounds **5**, **7**, **19** and **23**). By using DM-β-CD (Table 1b) we were able to resolve only partially eight aminophosphonates (compounds **1**, **6**, **7**, **12**, **14**, **15**, **17** and **19**). In this case the position of substituents in aromatic ring played the most important role, since this selector was strongly preferential to analogues of phenylglycine substituted at *orto* and *para* position. If considering two remaining cyclodextrins, namely HP-α-CD (Table 2c) and HP-β-CD (Table 2d) no meaningful structure–resolution relationship could be draw suggesting more complicated fit of the aminophosphonates into binding sites of these cyclodextrins.

## 4. Conclusions

The results described in this paper indicate that separation of aminophosphonates into enantiomers is strongly dependent on the pH of background elec-

trolyte and cyclodextrin concentration independently on the kind of used selector. It was, however, difficult to set up any general rule explaining the dependence of extend of resolution of aminophosphonates on both their structure or the structure of the used cyclodextrin. It only could be concluded that compounds not substituted with polar, ionizable groups in phenyl ring are better resolved than those ones possessing such moieties. It can also be stated that the substitution at *para* position of the aromatic ring seems to be favorable regarding enantioseparation. It is also worth to note that the presence and of two dissociated hydroxyl group at phosphorus atom is crucial for enantioseparation, compounds without such groups generally failed to be resolved.

It can be assumed, that the differences in complexation between two enantiomers of aminophosphonate and therefore their separation can be attributed to creation of the network of hydrogen bonds between hydroxyl group of cyclodextrin and amino or phosphonic moieties of compound. This enantioseparation potency is additionally enhanced by penetration and specific undefined so far orientation of aromatic part of aminophosphonate inside cyclodextrin hydrophobic cavity. Those interactions can be additionally strengthened or weaken by the presence or absence of substituents in aromatic ring.

Since all the studied compounds were available only as racemates we were not able to determine whether elution order of enantiomers were the same and remain constant for the given selectors and among the individual species of the set of studied compounds. This additionally restricted the more detailed analysis of the experimental data.

Taking into account also the results presented in previous report [13] it was shown that among twenty six of aminophosphonate racemates, eighteen were separated completely or partially with only a few remained not resolved. Therefore, it can be concluded that cyclodextrin turned out to be efficient chiral selectors for enantiomeric separation of α-aminophosphonic acid but only for limited cases the baseline separation was achieved. This separation method although required the use of various selectors can be successfully used for routine enantiopurity determination due to its simplicity, minimal costs and robustness.

## Acknowledgements

This work was supported by The State Committee for Scientific Research in Poland (Komitet Badań Naukowych, KBN).

## References

[1] P. Kafarski, B. Lejczak, Phosphorus Sulphur Silicon 63 (1991) 193.
[2] U. Schölkopf, I. Hoppe, A. Thiele, Justus Liebig's Ann. Chem. 1 (1985) 555.
[3] U. Schölkopf, R. Schutze, Justus Liebig's Ann. Chem. 1 (1987) 45.
[4] R. Huber, A. Knierzinger, J.P. Obracht, A. Vasella, Helv. Chim. Acta 68 (1985) 1730.
[5] R. Huber, A. Vasella, Helv. Chim. Acta 70 (1987) 1461.
[6] A.I. Selim, Indian J. Chem. 33B (1994) 641.
[7] C. Fischer, U. Schmidt, T. Dwars, G. Oehme, J. Chromatogr. A 845 (1999) 273.
[8] Z. Glowacki, M. Topolski, E. Matczak-Jon, M. Hoffmann, Magn. Res. Chem. 27 (1889) 922.
[9] P. Camillieri, C.A. Reid, D.T. Manallack, Chromatographia 38 (1994) 771.
[10] E. Zarbl, M. Lämmerhofer, F. Hammerschmidt, F. Wuggenig, M. Hanbauer, N.M. Maier, L. Sajovic, W. Lindner, Anal. Chim. Acta 404 (2000) 169.
[11] C.J. Shaw, C.E. Silverman, Chirality 8 (1996) 84.
[12] F. Hammerschmidt, W. Lindner, F. Wuggenig, E. Zarbl, Tetrahedron Asymmetry 11 (2000) 2955.
[13] P. Dżygiel, E. Rudzińdska, P. Wieczorek, P. Kafarski, J. Chromatogr. A 895 (2000) 301.
[14] M. Lämmerhofer, E. Zarbl, W. Lindner, B. Peric Simov, F. Hammerschmidt, Electrophoresis 22 (2001) 1182.
[15] B. Lejczak, P. Kafarski, E. Makowiecka, Biochem. J. 242 (1987) 81.
[16] P. Kafarski, B. Lejczak, J. Szewczyk, Can. J. Chem. 61 (1983) 2425.
[17] P. Kafarski, B. Lejczak, R. Tyka, L. Koba, E. Pliszczak, P. Wieczorek, J. Plant Growth Regul. 14 (1995) 199.
[18] M. Soroka, Justus Liebig's Ann. Chem. 1 (1990) 331.
[19] M. Soroka, D. Jaworska, Z. Szczesny, Justus Liebig's Ann. Chem. 1 (1990) 1153.
[20] M. Rak, P. Dżygiel, P. Wieczorek, Anal. Chim. Acta 433 (2001) 227.