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Reversed-phase high-performance liquid chromatography of diastereomers of some phosphonodipeptides

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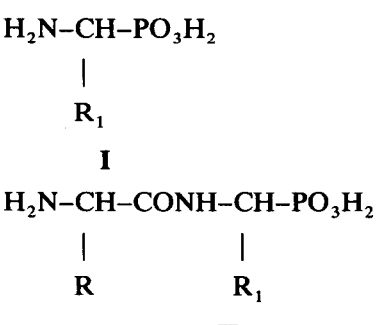
Abstract

The separation of diastereomers of four phosphonodipeptides, derivatives of 1-aminoethanephosphonic acid (AEP), was studied on different reversed-phase sorbents. The influence of the sorbent character and mobile phase composition on the retention was investigated. It was found out that the capacity factors of the *S,R* and *S,S* diastereomers were considerably different and strongly dependent on the hydrophobicity of the stationary phase. The capacity factors of both diastereomers were influenced by pH and the presence of a cation in the mobile phase in a certain pH range. All the above facts are closely connected with the dissociation constants and conformations of the phosphonodipeptides. An explanation of observed behaviour of the compounds studied is proposed.

1. Introduction

Aminophosphonic acids (**I**) and phosphonodipeptides (PDs) (**II**) differ from naturally occurring amino acids and dipeptides by the replacement of the carboxylic moiety with a phosphonic group ($-PO_3H_2$):



I

II

This class of compounds shows promising biological activity [1–6]. PDs containing P-terminal aminoalkylphosphonic acids have shown bacteriostatic [1,7] and herbicidal [8] effects.

PDs synthesized from optically pure amino acids can exist as four isomers, divided into two pairs. The *S,R* (L,L) and *R,S* (D,D) isomers are optically active isomers, whereas the *S,S* (L,D) and *R,R* (D,L) isomers are *meso* isomers. The members of each pair are enantiomeric and the two pairs are diastereomeric. It is well known that physico-chemical properties of diastereomers of PDs are different and, consequently, the fates of these isomers in organisms also differ considerably as well. Many of PDs have not been thoroughly studied.

The first step necessary in studying diastereomers of PDs with known absolute structure is the synthesis of the compounds of interest in a pure form. There are many methods for the preparation of PDs [9]. Unless an optically active aminoalkylphosphonic acid is used as a starting material for the reactions, a mixture of diastereomers

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of PDs is obtained that has to be separated in the next step. For this purpose, separation methods [10] such as crystallization, precipitation or chromatography are frequently used.

The aim of this work was to verify a method for the separation of diastereomers of PDs by reversed-phase high-performance liquid chromatography (RP-HPLC) [11] and to study the influence of the stationary phase character and mobile phase composition on the chromatographic behaviour of four different diastereomers, derivatives of 1-aminoethanephosphonic acid (AEP).

2. Experimental

2.1. Chromatographic conditions

The experiments were performed on an Knauer (Bad Homburg, Germany) liquid chromatographic system consisting of a Model 64 HPLC pump, a Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 20-μl sample loop and an UV–Vis spectrophotometer (Knauer). The results were processed on a Model 700 chromatography workstation (Bio-Rad Labs., Richmond, CA, USA). CGC compact glass columns (3.3 mm × 150 mm I.D.) (Tessek, Prague, Czech Republic) were packed by the slurry method in the laboratory. The sorbents used are listed in Table 1. Samples of the phosphonodipeptides were prepared by dissolution of a mixture of the respective diastereomers in the eluent at a concentration of 0.5 mg/ml of each diastereomer. The mobile phase compositions are indicated in the text and figure captions. The flow-rate was 0.5 ml/min and detection was at 225 nm.

The capacity factors were calculated as an average from three runs; the relative standard deviation was better then 5%. As a dead volume the refractive index disturbance caused by injection of water into methanol–water (70:30) eluent was used.

2.2. Materials

The PDs were prepared as described elsewhere [12]. Four PDs were used: (1) N-(*S*)-alanyl-(*S*,*R*)-AEP (*S*-Ala-*S*,*R*-AEP), (2) N-(*S*)-methionyl-(*S*,*R*)-AEP (*S*-Met-*S*,*R*-AEP), (3) N-(*S*)-leucyl-(*S*,*R*)-AEP (*S*-Leu-*S*,*R*-AEP) and (4) N-(*S*)-phenylalanyl-(*S*,*R*)-AEP (*S*-Phe-*S*,*R*-AEP). Orthophosphoric acid and sodium hydroxide (both from Lachema, Brno, Czech Republic), ammonia solution (Odzynninki Chemizcne, Lublin, Poland), dimethylamine (DMA) and diethylamine (DEA) (both from Merck, Darmstadt, Germany) were of analytical-reagent grade. Water was redistilled and filtered through a 0.45-μm membrane filtered (Schleicher & Schüll, Dassel, Germany).

3. Results and discussion

The influence of the stationary phase on the separation of PDs by RP-HPLC was studied using silica-based sorbents with different functional groups. The mobile phase used in these experiments was 10 m*M* orthophosphoric acid adjusted with ammonia solution to pH 3–7.

The hydrophobicity of the stationary phases based on the retention of an aromatic solute (*e.g.*, toluene) increased in the order SGX Phenyl < SGX C_8 < SGX C_{18}. The hydrophobicity of PDs (assumed from the hydrophobicity of the corresponding amino acids [13]) increased in the order *S*-Ala-*S*,*R*-AEP < *S*-Met-*S*,*R*-AEP < *S*-Leu-*S*,*R*-AEP < *S*-Phe-*S*,*R*-AEP. The retention behaviour of the four PDs confirmed the main role of hydrophobic interactions in the separation, as shown in Table 2. The results confirmed that the retention of the PDs increased in the order SGX Phenyl < SGX C_8 < SGX C_{18} and also with increasing hydrophobicity of the PDs. Addition of methanol to the mobile phase led to a decrease in the capacity factors of the PDs whereas the use 2.0 *M* ammonium sulphate as the mobile phase considerably increased the capacity factors of all the PDs. It is important to stress that both diastereomers of PDs are influenced to similar extents by changes in the eluent composition.

A very good separation of diastereomers of the four PDs confirmed the published results [11] (Fig. 1). The separation of diastereomers of PDs on the reversed-phase sorbents may be explained

Table 1
Reversed-phase sorbents used for experiments

Sorbent	Matrix	Bonded group	Particle size (μm)	Pore diameter (nm)	Efficiency[c] (theoretical plates)
Tessek[a] Separon SGX Phenyl	Silica	Phenyl	7	8	6000
Tessek Separon SGX C_8	Silica	Octyl	7	8	6400
Tessek Separon SGX C_{18}	Silica	Octadecyl	7	8	6400
Tessek Separon HEMA-BIO 1000 C_{18}	Hydroxyethyl methacrylate	Octadecyl	10	30	3000
Tessek Separon EDMA 2000	Ethylene dimethacrylate	None	7	>30	700
Serva[b] Octadecyl-Si 100	Silica	Octadecyl	10	10	4800

[a] Tessek, Prague, Czech Republic.
[b] Serva, Heidelberg, Germany.
[c] The efficiency (plate number, N) was determined for cGC columns (150 × 3 mm I.D.) for toluene as the solute and methanol–water (70:30) as the eluent at a flow-rate 0.5 ml/min. The equation used for calculation was $N = (t_R/w_{1/2})^2 \cdot 5.545$, where t_R = retention time of toluene and $w_{1/2}$ = toluene peak width at half-height.

Table 2
Capacity factors (k) of *S,S* diastereomers and resolution (R_s) for *S,R* and *S,S* diastereomers of the studied PDs on SGX Phenyl, SGX C_8 and SGX C_{18} columns with different compositions of the mobile phase

Phosphonodipeptide	Mobile phase composition	pH	Capacity factor of *S,S* diastereomer (k)			Resolution (R_s)		
			SGX Phenyl	SGX C_8	SGX C_{18}	SGX Phenyl	SGX C_8	SGX C_{18}
S-Ala-*S,R*-AEP	10 m*M* H_3PO_4–MeOH (9:1)	7.0	−0.09	−0.08	−0.05	0.07	0.14	0.21
	10 m*M* H_3PO_4	7.0	−0.08	−0.03	−0.07	0.20	0.57	0.54
	10 m*M* H_3PO_4	6.0	0.04	0.12	0.19	0.49	1.05	1.15
	10 m*M* H_3PO_4	5.0	0.10	0.19	0.24	0.66	1.44	1.40
	10 m*M* H_3PO_4	4.0	0.11	0.19	0.23	0.65	1.80	1.70
	10 m*M* H_3PO_4	3.0	0.10	0.19	0.21	0.70	1.61	1.49
	2 *M* $(NH_4)_2SO_4$	5.6	0.39	0.73	0.86	2.55	3.63	2.77
S-Met-*S,R*-AEP	10 m*M* H_3PO_4–MeOH (9:1)	7.0	0.09	0.20	0.37	0.67	1.65	1.10
	10 m*M* H_3PO_4	7.0	0.19	0.76	1.79	0.90	2.94	2.83
	10 m*M* H_3PO_4	6.0	0.63	1.63	3.14	2.24	6.60	6.82
	10 m*M* H_3PO_4	5.0	0.82	1.95	3.64	3.09	7.55	9.68
	10 m*M* H_2PO_4	4.0	0.83	1.96	3.59	2.81	9.67	8.69
	10 m*M* H_3PO_4	3.0	0.81	1.95	3.45	3.40	10.20	8.68
	2 *M* $(NH_4)_2SO_4$	5.6	4.53	10.93	20.58	6.27	12.25	10.26
S-Leu-*S,R*-AEP	10 m*M* H_3PO_4–MeOH (9:1)	7.0	0.16	0.53	1.00	1.16	3.00	2.43
	10 m*M* H_3PO_4	7.0	0.33	1.95	3.97	1.35	6.92	4.72
	10 m*M* H_3PO_4	6.0	0.87	3.79	7.21	3.26	12.56	11.66
	10 m*M* H_3PO_4	5.0	1.11	4.81	8.67	4.51	15.66	14.25
	10 m*M* H_3PO_4	4.0	1.13	4.76	8.43	5.03	15.12	12.26
	10 m*M* H_3PO_4	3.0	1.15	4.73	8.43	5.60	15.02	12.21
	2 *M* $(NH_4)_2SO_4$	5.6	7.74	32.20	62.02	8.91	17.64	12.22
S-Phe-*S,R*-AEP	10 m*M* H_3PO_4–MeOH (9:1)	7.0	0.73	1.25	2.66	2.11	3.26	2.37
	10 m*M* H_3PO_4	7.0	1.30	4.44	12.69	2.19	7.83	6.29
	10 m*M* H_3PO_4	6.0	2.77	9.16	21.82	5.53	13.84	10.73
	10 m*M* H_3PO_4	5.0	3.39	11.11	24.83	6.73	15.53	15.17
	10 m*M* H_3PO_4	4.0	3.39	11.13	24.36	7.06	15.34	13.05
	10 m*M* H_3PO_4	3.0	3.35	10.73	23.06	7.32	15.40	10.31
	2 *M* $(NH_4)_2SO_4$	5.6	19.8	–	–	9.39	–	–

Adjustments of pH were made with ammonia solution. The capacity factor was calculated according the equation $k = (t_{R_2} - t_0)/t_0$, where t_{R_2} = retention time of *S,S* diastereomer and t_0 = dead time of column; and resolution $R_s = 2 \cdot (t_{R_2} - t_{R_1})/(w_{R_2} + w_{R_1})$, where t_{R_2} = retention time of *S,S* diastereomer, t_{R_1} = retention time of *S,R* diastereomer, w_{R_2} = peak width of component 2 and w_{R_1} = peak width of component 1.

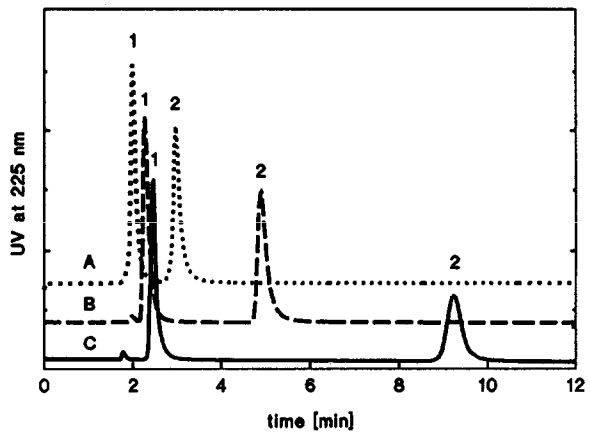

Fig. 1. Chromatograms of diastereomers of *S*-Met-*S,R*-AEP on cGC columns (150 × 3 mm I.D.): (C) SGX C_{18}, 7 μm (——), (B) SGX C_8, 7 μm (– – –) and (A) SGX Phenyl, 7 μm (·····). Mobile phase 10 m*M* orthophosphoric acid adjusted with ammonia solution to pH 4.0; flow-rate, 0.5 ml/min; detection, UV at 225 nm. Peaks: 1 = *S,R* diastereomer; 2 = *S,S* diastereomer.

by their different absolute structures. The *S,S* diastereomer can probably fold more easily than the *S,R* diastereomer [14]. In the folded conformation of a zwitterion the oppositely charged $-NH_3^+$ and $-PO_3H^-$ groups are nearer in the case of *S,S* diastereomers, which leads to a higher stabilization of this isomer. On the basis of ref. 14, tentative structures of *S,R* and *S,S* phosphonodipeptides in aqueous solution [15] are suggested in Fig. 2.

For all the PDs tested, the *S,S* diastereomers were more strongly retained than the *S,R* diastereomers. From the proposed structure it can be seen that in the *S,S* diastereomer both hydrophobic side-chains are closer to each other than in the *S,R* diastereomer. The plane of the peptidic bond divides the molecule into two halves. The hydrophilic charged part of the *S,S* diastereomer is located under the plane and the two hydrophobic chains are spread above the plane. The space under and above the plane is evenly filled with charge and hydrophobic arms, respectively. This conformation is better suited for an interaction with the hydrophobic surface of a reversed-phase sorbent. In contrast, in the *S,R* diastereomer the hydrophobic chains are oriented oppositely to each other, and this orientation is less suitable for interactions with the sorbent.

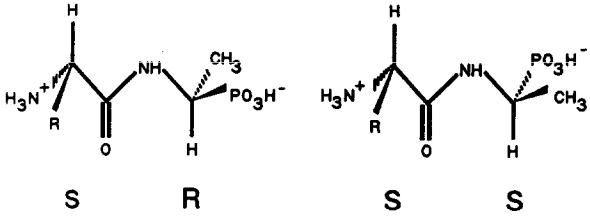

Fig. 2. Tentative structures of *S,R*- and *S,S*-phosphonodipeptides in aqueous solution.

Changes in the pH of mobile phase in the range 3–7 did not influence significantly the retention times of the *S,R* diastereomers, but above pH 5 a considerable decrease in the retention times of the *S,S* diastaereomers was observed. These dependences were very similar for all the PDs (Fig. 3). This behaviour can be explained on the basis of the dissociation constants of PDs; an example for *S*-Met-*S,R*-AEP [15] is presented in Table 3. As was mentioned above, the *S,S* diastereomers have a very stable structure if they exist in the form of neutral zwitterions, which according to the distribution diagram [15] exist in the pH range 3–5. An increase in pH above 5 leads first to the dissociation of the second proton from the phosphonic group and above pH 7 to the loss of a proton from the amino group. These processes are accompanied by a decrease in conformational

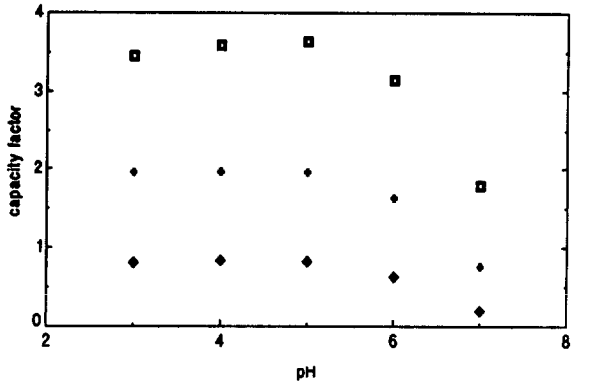

Fig. 3. Dependences of capacity factors for *S,S*-Met-AEP diastereomer of *S*-Met-*S,R*-AEP on mobile phase pH. Sorbents used: ◇ = SGX Phenyl; + = SGX C_8; □ = SGX C_{18}. Mobile phase, 10 m*M* orthophosphoric acid with pH adjusted with ammonia solution.

Table 3
pK_a values for *S*-Met-*S*,*R*-AEP

pK_a	*S*,*R*-Met-AEP	*S*,*S*-Met-AEP
p$K(NH_3^+)$	7.44	7.86
p$K(PO_3H^-)$	6.61	6.22
p$K(PO_3H_2)$	1.4	1.1

stability of the *S*,*S* diastereomer, resulting in a decrease in the capacity factors of all the *S*,*S* diastereomers studied at pH > 5. The *S*,*R* diastereomers are generally retained on reversed phases much more weakly than the *S*,*S* diastereomers even at pH 3–5, and therefore the effect of pH changes on the retention is less predictable and, as shown by the experimental results, much less pronounced.

The influence of the cation in the mobile phase on the separation and the retention times of PDs was also studied. For that purpose the pH of 10 m*M* orthophosphoric acid was adjusted in the range 3–8 with ammonia solution, sodium hydroxide, dimethylamine (DMA) and diethylamine (DEA). These dependences are very close for the three more hydrophobic PDs, and small differences can be seen only with *S*-Ala-*S*,*R*-AEP. The results obtained with *S*-Ala-*S*,*R*-AEP and *S*-Leu-*S*,*R*-AEP on SGX C_{18} are shown in Figs. 4 and 5. As expected, in the pH range 3–5 there is little influence of the cation in the mobile phase on the capacity factors of neutral zwitterions of PDs. At pH >5 the presence of DMA or DEA in the mobile phase leads to an increase in the retention times of both diastereomers of PDs in comparison with sodium hydroxide. The observed effect was stronger for DEA than for DMA. The negatively charged PDs will probably form ion pairs with hydrophobic cations at pH> 5. The retention increases in the order of increasing cation hydrophobicity, *i.e.*, ammonia solution <DMA < DEA. A decrease in the capacity factors not only for the *S*,*S* but also for the *S*,*R* diastereomers was observed for all the cations at pH 8 in comparison with pH 7. A shift of pH to 8 leads to an increase in the total negative charge of the diastereomer, which becomes *ca.* 2−, and this negative charge is seemingly not compensated for by forming of an ion pair of the diastereomer with two molecules of the ion-pairing reagent. Although the reproducibility of the capacity factors within one series of measurements was excellent, owing to the deterioration of the sorbent bonded phase after use in several different eluents the capacity factors were slightly shifted (compare corresponding data in Table 2 and Figs. 4 and 5). The observed dependences were unchanged, however.

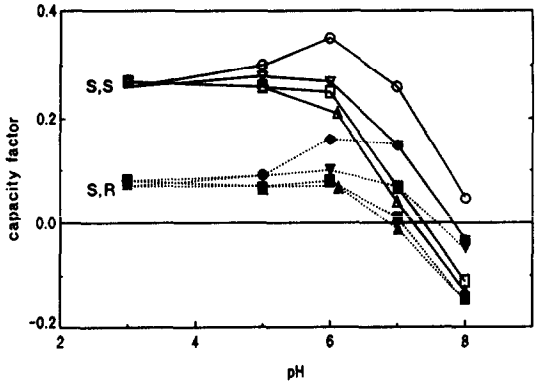

Fig. 4. Dependences of capacity factors for diastereomers of *S*-Ala-*S*,*R*-AEP on pH and eluent composition on SGX C_{18}. Mobile phase, 10 m*M* orthophosphoric acid with pH adjusted with (△, ▲) sodium hydroxide, (□, ■) ammonia solution, (▽, ▼) DMA and (○, ●) DEA.

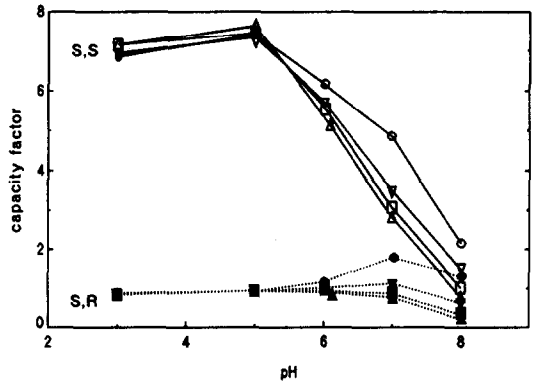

Fig. 5. Dependences of capacity factors for diastereomers of *S*-Leu-*S*,*R*-AEP on pH and eluent composition on SGX C_{18}. Mobile phase, 10 m*M* orthophosphoric acid with pH adjusted with (△, ▲) sodium hydroxide, (□, ■) ammonia solution (▽, ▼) DMA and (○, ●) DEA.

Because the differences in capacity factors were small, we checked if they are statistically significant. Using confidence intervals for a difference in means [16], we verified that the measured capacity factors at pH 7 were different for the 0.99 confidence level.

As the SGX silica-based reversed-phase sorbents are not totally end-capped, comparison was made with sorbents without silanol group activity. As a reference silica-based sorbent material Serva Octadecyl-Si 100 was used. The comparison of results obtained on SGX C_{18} and Octadecyl-Si 100 showed only one significant difference: the capacity factors for the *S,R* diastereomers in the pH range 3–7 (adjusted with ammonia solution) were nearly constant for SGX C_{18} and increased with increase in pH for Octadecyl-Si 100. The explanation may be connected with residual silanol groups on the surface of SGX C_{18}, which deliver a negative charge to the surface of the sorbent.

On the polymer-based sorbents HEMA-BIO 1000 C_{18} and EDMA 2000 the retention times achieved under the same conditions were much more smaller for the *S,S* diastereomers (*e.g.*, in 10 m*M* orthophosphoric acid, pH 7, adjusted with ammonia solution, the capacity factor for *S*-Phe-*S*-AEP was 12.7 on SGX C_{18} and 2.0 on HEMA-BIO 1000 C_{18}). These results are surprising because the capacity factors for some aromatics such as benzene and toluene are very similar for SGX C_{18} and the polymer-based sorbents HEMA-BIO 1000 C_{18} and EDMA 2000. The behaviour can probably be explained by the different structures of the sorbents: the silica-based sorbent has a more or less uniform layer of C_{18} moieties bonded on the surface, whereas on the organic polymer-based sorbents the hydrophobic groups form or are located on a net of cross-linked chains. As the differences in the retentions of the *S,R* and *S,S* diastereomers are based only on different steric orientations of hydrophobic groups, this difference is more significant in the case of silica-based RPs with a better defined interface between a non-polar stationary phase and a polar mobile phase.

4. Conclusions

Diastereomers of four PDs were successfully separated on silica-based reversed-phase sorbents and the influence of different bonded functional groups, eluent pH and cations was evaluated. The resolution of diastereomers achieved was very good. RP-HPLC was verified as a suitable method for analytical or preparative-scale separations of diastereomers of PDs. Optimization of the analysis can easily be achieved based on the observed dependences.

5. References

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