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Comparison of zirconia- and silica-based reversed stationary phases for separation of enkephalins

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Abstract

In this study, the separation of biologically active peptides on two zirconia-based phases, polybutadiene (PBD)-ZrO_2 and polystyrene (PS)-ZrO_2, and a silica-based phase C_{18} was compared. Basic differences in interactions on both types of phases led to quite different selectivity. The retention characteristics were investigated in detail using a variety of organic modifiers, buffers, and temperatures. These parameters affected retention, separation efficiency, resolution and symmetry of peaks. Separation systems consisting of Discovery PBD-Zr column and mobile phase composed of a mixture of acetonitrile and phosphate buffer, pH 2.0 (45:55, v/v) at 70 °C and Discovery PS-Zr with acetonitrile and phosphate buffer, pH 3.5 in the same (v/v) ratio at 40 °C were suitable for a good resolution of enkephalin related peptides. Mobile phase composed of acetonitrile and phosphate buffer, pH 5.0 (22:78, v/v) was appropriate for separation of enkephalins on Supelcosil C_{18} stationary phase.

Keywords: RP-HPLC; Octadecyl silica-based stationary phase; Zirconia-based stationary phase; Peptides; Enkephalin

1. Introduction

Chemical analysis of biologically active compounds is an important field in now a days analytical chemistry. Biologically active compounds often possess basic functional groups. They can be successfully separated by reversed-phase mode (RP) HPLC [1–3].

The widespread of RP-HPLC is due to its convenience, versatility and high efficiency of silica-based chromatographic supports [4–6]. The silica surface chemistry and the surface modification reactions are also well understood [7]. Despite the numerous positive features, silica gel has several limitations, such as low thermal and pH stability [8,9]. Silica dissolves at higher pH values (pH > 8) and the siloxane bond is unstable at acidic pH (pH < 2). The restricted pH range is a limiting factor mainly if separation of basic compounds should be performed. The presence of residual ionized silanol groups on the silica gel surface greatly complicates the retention process [10]. Increased separation temperature leads to gradual loss of the bonded phase [9–12]. Although the majority of analyses in RP-HPLC has been done using modified silica gel, a variety of other support materials has been investigated in an effort to develop alternatives to silica and silane chemistry to overcome the above mentioned limitations [13]. Many new types of silica-bonded stationary phases have been developed, including polymer-encapsulated phases [14], polymerized [15], polar embedded [16], bidentate [17] and monolithic phases [18].

The recent trend is pursuit of replace the silica-based reverse phases with phases of improved stability. The quest for such phases has encompassed various metal oxides such as zirconia, titania, alumina and to a lesser extent thoria and ceria [19–22]. Among them mainly zirconia-based stationary phases have shown promising quality [4,23–27]. Zirconia, i.e. zirconium dioxide, is useful for separation of solutes over a wide pH range (1–14) and at temperature up to 200 °C [20,22,28–33]. Zirconia is an amphoteric metal oxide, which exhibits both anion- and cation-exchange properties depending on the solution pH and the nature of the buffer [21,33,34].

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One of the most chemically challenging aspects of using zirconia support is to understand their surface chemistry, which is radically different from that of the silica support [19,20]. A large number of strong Lewis acid sites on zirconia surface can interact with Lewis bases as R–SO_3^-, R–PO_3^-, R–COO^- groups, etc. [29,34–37]. These interactions can be troublesome sometimes, for which reason the zirconia surface is often modified [19,38]. There are various ways of modification, such as addition of competing Lewis bases to used mobile phase, or permanent covering of the surface with polymers or carbon [19,29,35]. Polybutadiene (PBD) [4,24,39,40], polystyrene (PS) and polyethyleneimine (PEI) [41] have been successfully used as the polymers to modify the zirconia surface.

Pentapeptides, leucine^5-enkephalin, methionine^5-enkephalin, and related peptides have significant biological functions in human body and are termed as endogenous opiates, naturally synthesized in human body [42]. In human brain, they interact with receptors designed for morphine [43] and therefore they decrease sensitivity to pain. Enkephalins belong to group of neurotransmitters, transporting signals on short distances. Any disturbance in their synthesis can cause serious mental illnesses. Enkephalins are therefore intensively studied in connection with autism, Alzheimer and Parkinson diseases.

In this work, modern separation media, PBD- and PS-coated zirconia stationary phases, were used to study the separation behaviour of pentapeptides. The influence of composition of mobile phases (buffers, pH, organic modifiers), as well as temperature variations were studied as parameters affecting retention, separation efficiency, resolution and symmetry of peaks. The results obtained with the zirconia phases were compared to those for octadecyl silica-bonded reversed phases, and the differences and similarities between them were discussed.

2. Experimental

2.1. Instruments

All chromatographic measurements were performed on a Pye Unicam (Cambridge, Great Britain) chromatographic system, equipped with a LC-XPD pump, UV detector, and computer-based CSW32 v.1.3 software (Data Apex, Prague, Czech Republic). Manual injection was performed using a Rheodyne Model 7125 injection valve (Cotati, CA, USA) with 10 μL internal injection loop. Column thermostat LCO 101 (Ecom, Prague, Czech Republic) was used to control the column temperature.

Table 2
Structures of the studied pentapeptides

Peptide	Amino acid sequence
Methionine^5-enkephalin	Tyr-Gly-Gly-Phe-Met
Leucine^5-enkephalin	Tyr-Gly-Gly-Phe-Leu
Leucine^5-enkephalinamide	Tyr-Gly-Gly-Phe-LeuNH_2
D-Alanine^2, leucine^5-enkephalin	Tyr-D-Ala-Gly-Phe-Leu

2.2. Analytical columns

All columns, Discovery Zr-PBD, Discovery Zr-PS, and Supelcosil C_{18}, used in this study were 25 cm × 0.46 cm I.D., particle size 5 μm. They were obtained from Supelco (Bellefonte, PA, USA). Parameters of all the columns are summarized in Table 1.

2.3. Reagents

Acetonitrile and methanol (HPLC grade) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium phosphate monobasic and sodium phosphate dibasic were purchased from Lachema Chemapol (Brno, Czech Republic), orthophosphoric acid was obtained from Lachema (Neratovice, Czech Republic). All chemicals used in this study were reagent grade or better. Uracil (99%), used as the dead time marker, was supplied by Sigma–Aldrich. Deionised water was prepared using a Milli-Q water system (Millipore, Milford, MA, USA).

Separated pentapeptides (for structures see Table 2) were products of Sigma–Aldrich.

2.4. Chromatographic conditions

Reversed-phase separations were performed using buffer–acetonitrile or buffer–methanol mobile phases. Buffers were prepared by dissolving the appropriate amounts of orthophosphoric acid and sodium phosphate (monobasic, dibasic) in water for desired pH (2–6) and concentration (1×10^{-4} mol/L to 1×10^{-3} mol/L). Detection wavelength

Table 1
Parameters of the columns used (250 mm × 4.6 mm, 5 μm)

Column	Support material	Bonded phase	Particle shape	Pore size (Å)	Surface area (m^2/g)	Temp. limits (°C)	pH range
Supelcosil LC-18-DB	Silica gel	Octadecyl-silane	Spherical	120	170	60	2–7
Discovery Zr-PBD	Zirconia	Poly-butadiene	Spherical	300	30	up to 100	1–13
Discovery Zr-PS	Zirconia	Poly-Styrene (cross-linked)	Spherical	300	30	up to 100	1–13

was set to 214 nm and flow rate of the mobile phase was adjusted to 1 mL/min. The column temperature was controlled in the range from 25 to 80 °C with precision of ±0.5 °C. The column dead time was determined by injecting of uracil (0.1 mg/mL). Peptide samples were prepared as 1 mg/mL aqueous solutions. From the stock solutions, a mixture of all the peptides was prepared. The final concentration of each peptide was 0.22 mg/mL. Volume of 10 μL of this mixture was injected. More details can be found in the Section 3.

3. Results and discussion

Separation of a set of four pentapeptides (Met^5-enkephalin, Leu^5-enkephalin, Leu^5-enkephalinamide and D-Ala^2, Leu^5-enkephalin) was studied on two zirconia-based and one silica-based stationary phases, Discovery PBD-ZrO_2, Discovery PS-ZrO_2 and Supelcosil C_{18}. Effect of the type of organic modifier, the buffer pH and concentration, the ratio of organic modifier to buffer, and temperature variation was studied.

3.1. Zirconia-based columns

3.1.1. Effect of the type of organic modifier

In the first experiments, the effect of acetonitrile (ACN) and methanol (MeOH) on retention of pentapeptides was examined. A great difference between these organic modifiers was observed. Mobile phases containing MeOH as organic modifier were found to yield long retention times (∼40 min) and very bad peak shapes. ACN offered low viscosity, low UV transmittance, and high separation efficiency (around 30,000 plates per meter) on the zirconia-based columns. For these reasons acetonitrile was chosen as the organic modifier.

3.1.2. Effect of the type of buffer, buffer pH and concentration

Interactions between analytes and the modified zirconia-based columns are strongly dependent on the buffer type used. Acetate and phosphate buffers were investigated as potential mobile phase constituents for the separation of enkephalins. Retention of the analytes increased if phosphate buffer was used due to enhanced ion-exchange interactions. Another advantage of this buffer was its low absorbance compared to acetate.

Subsequently, mobile phases composed of ACN and 20 mM phosphate buffer were used. The ACN to buffer ratio was kept constant, i.e. (50:50, v/v). This starting buffer concentration and the ratio of ACN to buffer were chosen based on recommendation of the producer (Sigma–Aldrich). The effect of pH of the phosphate buffer was studied in the range 2.0–6.0, i.e. below and above pK_a of separated pentapeptides (pK_a ∼4.5). The retention increased with increasing pH value in the range of 2.0–3.5 and 2.0–3.0 on PBD-ZrO_2 and PS-ZrO_2 stationary phases, respectively. Leu^5-enkephalinamide showed different behaviour on both the stationary phases. Between pH 4.0 and 6.0, the retention factors of enkephalin derivatives, D-Ala^2, Leu^5-enkephalin, Met^5-enkephalin and Leu^5-enkephalin, were dramatically reduced. An increase in pH from 2.0 to 3.5 leads to an increase in dissociation of the phosphate ions adsorbed onto the surface of PBD-ZrO_2 and PS-ZrO_2 stationary phases. Thus a higher charge of the adsorbed phosphate ions is generated and the surface of these stationary phases becomes more negatively charged. It was found that the amount of phosphate adsorbed on the PBD-ZrO_2 surface will reach a maximum at pH 2.0 since the maximum adsorption occurs when the pH is equal to the pK_a—for H_3PO_4 pK_a = 2.1 [44]. The separated analytes are positively charged that results in domination of ion-exchange interactions with adsorbed phosphate on the zirconia surface (over the hydrophobic interactions with PBD coating). In the higher pH range (3.5–6.0) we assume that on the well-ionised phosphate modified stationary phase surface a competiting interactions of analytes and counterions present in the buffer takes place. In addition the increase of pH causes a decrease of ionisation of the separated enkephalins. As a result a decrease of retention factors was observed due to suppression of the Coulombic interactions between the negatively charged surface and peptides.

The effect of pH on resolution of analytes is strongly related to the change of peak symmetry. The better peak symmetry was observed at lower pH values. The most difficult pair of analytes to be separated was Met^5-enkephalin/Leu^5-enkephalin. Based on the results presented above, the following mobile phases were used in the subsequent set of experiments: ACN–20 mM phosphate buffer, pH 2.0 (50:50, v/v) on PBD-ZrO_2 and ACN–20 mM phosphate buffer, pH 3.5 (50:50, v/v) on PS-ZrO_2.

In the next step, the influence of the phosphate buffer concentration in the range 10–60 mM on PBD-ZrO_2 stationary phase and 40–100 mM on PS-ZrO_2 was tested (Figs. 1 and 2). A wider range of buffer concentration, i.e. higher than 60 mM on PBD-ZrO_2 and 100 mM on PS-ZrO_2, and lower than

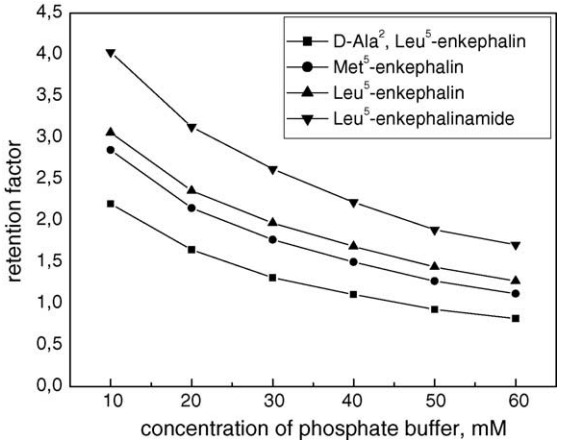

Fig. 1. Dependence of retention factor on concentration of phosphate buffer on Discovery PBD-ZrO_2; mobile phase ACN–phosphate buffer, pH 2.0 (50:50, v/v); temperature 25 °C.

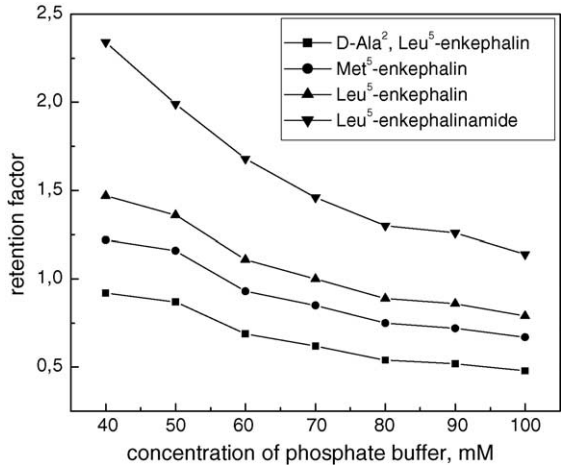

Fig. 2. Dependence of retention factor on concentration of phosphate buffer on Discovery PS-ZrO_2; mobile phase ACN–phosphate buffer, pH 3.5 (50:50, v/v); temperature 25 °C.

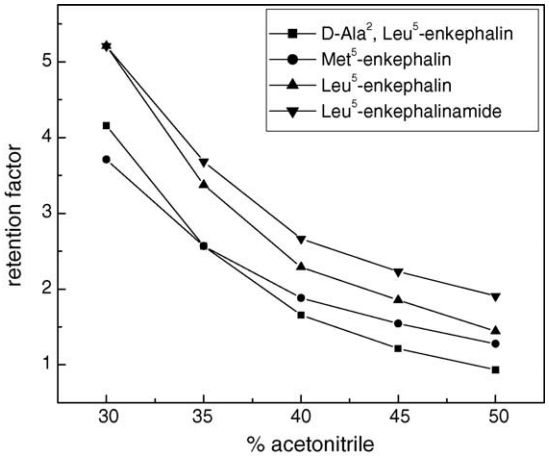

Fig. 3. Dependence of retention factor on content of acetonitrile in the mobile phase on Discovery PBD-ZrO_2; mobile phase ACN–50 mM phosphate buffer, pH 2.0; temperature 25 °C.

40 mM on PS-ZrO_2, could not be tested due to either elution of analytes with the dead time or too long retention times. Increase of buffer concentration led to decreased retention and improved peak symmetry. Better resolution between Met^5-enkephalin and Leu^5-enkephalin, the critical pair for separation, was found at higher buffer concentrations on PBD-ZrO_2 stationary phase ($R_{1,2}$ = 1.31 at buffer concentration of 50 mM) as well as on PS-ZrO_2 ($R_{1,2}$ = 0.98 at buffer concentration of 80 mM). Small decrease of resolution with increasing concentration of phosphate buffer was observed for pairs D-Ala^2, Leu^5-enkephalin/Met^5-enkephalin and Leu^5-enkephalin/Leu^5-enkephalinamide on PBD-ZrO_2 and for the latter also on PS-ZrO_2.

3.1.3. Effect of the ratio of organic modifier to buffer

The phosphate buffer concentrations of 50 mM (pH 2.0) and 80 mM (pH 3.5) were used on PBD-ZrO_2 and PS-ZrO_2, respectively, in the experiments, where the effect of the ratio of organic modifier to buffer was examined. Mobile phases with different content of ACN, i.e. 50, 45, 40, 35 and 30% (v) were tested (Figs. 3 and 4). Higher content of acetonitrile increased the elution power of mobile phases. Decrease of the organic modifier content resulted in an increase of retention of all the analytes, which was accompanied by a change of elution order on PBD-ZrO_2 for certain pairs of analytes (see Fig. 3). Neither improvement of resolution of analytes nor of peak symmetry was achieved by decreasing the ACN content using this stationary phase. On PS-ZrO_2 column, no change of elution order and no improvement of resolution were observed by varying the ACN to buffer ratio. The retention factors followed the same trend as on the previous column; they decreased with increasing the organic modifier content (see Fig. 4). From the separation systems tested, the following ones were selected as the most suitable for separation of the studied enkephalin derivatives: (1) PBD-ZrO_2 stationary phase and mobile phase composed of ACN–50 mM phosphate buffer, pH 2.0 (45:55, v/v); and (2) PS-ZrO_2 stationary phase and ACN–80 mM phosphate buffer, pH 3.5 (45:55, v/v).

3.1.4. Effect of temperature

The zirconia-based columns are designed for work at higher temperatures. In general, elevated temperature speeds up the mass transfer during the separation process and thus it can positively influence the separation. The effect of temperature on separation of pentapeptides was studied in the range from 25 to 80 °C. The obtained results are summarized in Tables 3 and 4. Increasing the temperature caused reduction of retention factors of the enkephalins on PBD-ZrO_2 while their retention was only slightly affected on PS-ZrO_2. Resolution of the individual pairs of analytes was influenced by temperature in a different way. Resolution of D-Ala^2, Leu^5-enkephalin and Met^5-enkephalin increased with temperature

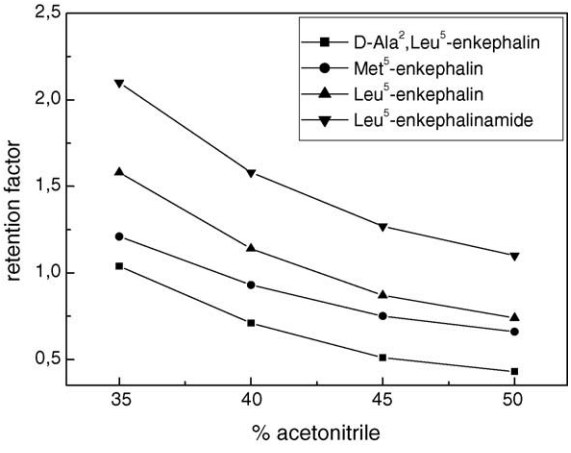

Fig. 4. Dependence of retention factor on content of acetonitrile in the mobile phase on Discovery PS-ZrO_2; mobile phase ACN–80 mM phosphate buffer, pH 3.5; temperature 25 °C.

Table 3
Retention factors, resolution and peak symmetry of the studied enkephalins under various temperature on PBD-ZrO_2; mobile phase ACN–50 mM phosphate buffer, pH 2.0 (45:55, v/v); flow rate 1 mL/min; injection 10 μL; UV detection 214 nm

Factor	Pentapeptide	Temperature (°C)					
		25	40	50	60	70	80
k	D-Ala, Leu	1.20	1.12	1.08	1.03	0.98	0.95
	Met	1.52	1.40	1.35	1.29	1.23	1.20
	Leu	1.84	1.64	1.58	1.50	1.43	1.39
	Leu amide	2.20	2.20	1.95	1.88	1.73	1.67
$R_{1,2}$	D-Ala, Leu/Met	2.38	2.43	2.46	2.64	2.79	2.86
	Met/Leu	1.97	1.85	1.86	1.90	1.94	1.90
	Leu/Leu amide	2.55	2.85	3.18	2.48	2.68	2.65
A_s	D-Ala, Leu	1.50	1.32	1.14	1.05	1.00	1.00
	Met	1.31	0.97	0.88	0.90	0.96	0.96
	Leu	0.94	0.88	0.74	0.70	0.66	0.64
	Leu amide	1.09	0.90	0.85	0.73	1.07	0.82

on PBD-ZrO_2 while on PS-ZrO_2 a decrease of resolution was observed when the temperature raised. Resolution of Met^5-enkephalin and Leu^5-enkephalin, the most difficult pair of analytes to resolve, was almost temperature independent on both the zirconia-based columns. No clear trend of resolving Leu^5-enkephalin and Leu^5-enkephalinamide could be traced on PBD-ZrO_2, while using PS-ZrO_2 column an improvement of their resolution was achieved at elevated temperature. No general trend of temperature effect on peak symmetry was found. On PBD-ZrO_2 column a decrease of asymmetry factors with increasing temperature was obtained. As the result an improvement of peak shape for D-Ala^2, Leu^5-enkephalin and Met^5-enkephalin while fronting peaks for Leu^5-enkephalin and Leu^5-enkephalinamide were observed. Contribution of axial molecular diffusion that is more significant at elevated temperatures can contribute to deformation of elution profiles of later eluting analytes.

Based on the results gained by evaluation of the temperature data, the separation temperature was set to 70 and 40 °C on PBD-ZrO_2 and PS-ZrO_2, respectively. Applying these temperatures, good resolution of all the analytes in a reasonable time was achieved on Discovery PBD-ZrO_2. Unfortunately, no baseline resolution could be obtained on Discovery PS-ZrO_2. Chromatograms measured under optimized conditions on PBD-ZrO_2 and PS-ZrO_2 stationary phases are depicted in Figs. 5 and 6, respectively.

Table 4
Retention factors, resolution and peak symmetry of the studied enkephalins under various temperature on PS-ZrO_2; mobile phase ACN–80 mM phosphate buffer, pH 3.5 (45:55, v/v); flow rate 1 mL/min; injection 10 μL; UV detection 214 nm

Factor	Pentapeptide	Temperature (°C)					
		25	40	50	60	70	80
k	D-Ala-Leu	0.50	0.45	0.43	0.42	0.39	0.35
	Met	0.71	0.61	0.56	0.53	0.49	0.46
	Leu	0.86	0.75	0.69	0.65	0.60	0.56
	Leu amide	1.27	1.15	1.08	1.03	0.96	0.91
$R_{1,2}$	D-Ala-Leu/Met	1.51	1.29	1.20	1.03	0.87	0.88
	Met/Leu	0.91	1.02	1.05	1.04	1.05	0.99
	Leu/Leu amide	2.48	2.77	2.88	2.90	2.96	3.19
A_s	Leu amide	1.19	1.00	0.97	0.94	0.90	0.91

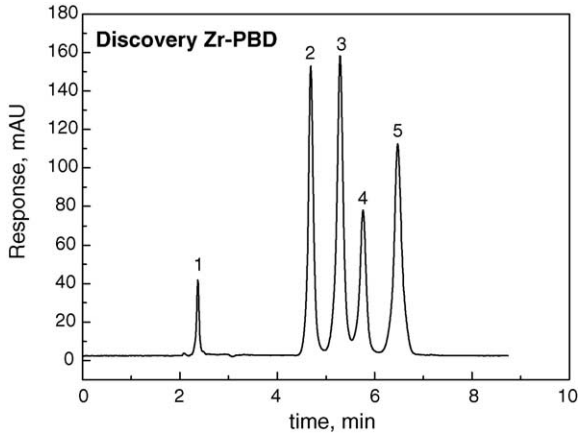

Fig. 5. Optimized separation of enkephalins on Discovery PBD-ZrO_2; mobile phase ACN–50 mM phosphate buffer, pH 2.0 (45:55, v/v); temperature 70 °C; flow rate 1 mL/min; injection 10 μL; detection 214 nm.Peak identification: (1) Uracil; (2) D-Ala^2, Leu^5-enkephalin; (3) Met^5-enkephalin; (4) Leu^5-enkephalin; (5) Leu^5-enkephalinamide.

3.2. Silica-based column

The optimized separation conditions designed for zirconia-based phases were not suitable for the silica-based stationary phase Supelcosil C_{18}. The mixture of enkephalins eluted with column dead volume. It was necessary to tune the pH and concentration of the phosphate buffer as well as the ratio of organic modifier to buffer.

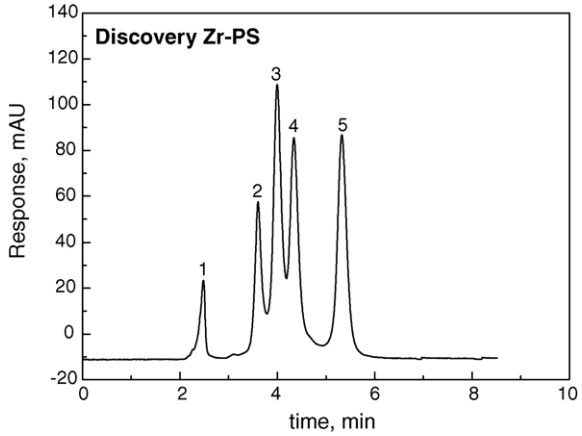

Fig. 6. Optimized separation of enkephalins on Discovery PS-ZrO_2; mobile phase ACN–80 mM phosphate buffer, pH 3.5 (45:55, v/v); temperature 40 °C; flow rate1 mL/min; injection 10 μL; detection 214 nm.Peak identification: (1) Uracil; (2) D-Ala^2, Leu^5-enkephalin; (3) Met^5-enkephalin; (4) Leu^5-enkephalin; (5) Leu^5-enkephalinamide.

The elution order of enkephalins varied on the zirconia-based and silica-based phases, implying that the retention mechanism differs. While ion-exchange and donor–acceptor interactions predominate on the PBD-ZrO_2 and PS-ZrO_2 phases [21,33,34,44], mainly hydrophobic interactions are responsible for separation on the C_{18} phase. Strong effect of the buffer concentration (sodium phosphate) on retention on the ZrO_2-based columns (Figs. 1 and 2) and in contrary, almost no change of retention on silica-based stationary phase (Fig. 8) clearly support this theory.

3.2.1. Effect of the buffer pH and concentration

Mobile phases composed of ACN and 50 mM phosphate buffer were used with the silica gel column. The ratio of ACN to phosphate buffer was set to (22:78, v/v) based on the preliminary experiments. The effect of buffer pH was studied in the range 3.0–6.0 (Fig. 7). The retention decreased with increasing pH value of the phosphate buffer in the whole range studied. Peak symmetry was found to be almost pH independent on the Supelcosil C_{18} column. This fact shows on well-shielded silica surface and good homogeneity of the silica gel particles. Resolution of the pairs of analytes was also evaluated. Due to the change of elution order, different pairs of pentapeptides, than had been examined on the zirconia-based columns, were established and compared. As can be seen from Fig. 7, decreasing pH implied significant improvement of resolution of Leu^5-enkephalin and D-Ala^2, Leu^5-enkephalin and also small improvement for Met^5-enkephalin and Leu^5-enkephalin. The pair D-Ala^2, Leu^5-enkephalin and Leu^5-enkephalinamide was better resolved either at high pH, i.e. pH 6.0, or at low pH, i.e. pH 2.0.

In the next step, the influence of phosphate buffer concentration in the range 10–100 mM was tested. The mobile phase of the following composition was used in the subsequent set of experiments: ACN–phosphate buffer, pH 5.0 (22:78, v/v). Increase of buffer concentration led to almost no change of retention (see Fig. 8); neither peak symmetry nor resolution was affected. Different behaviour was found for Leu^5-enkephalinamide. Its retention markedly decreased with increasing buffer concentration, especially in the range 10–60 mM, and an improvement of peak symmetry was observed in the same concentration range.

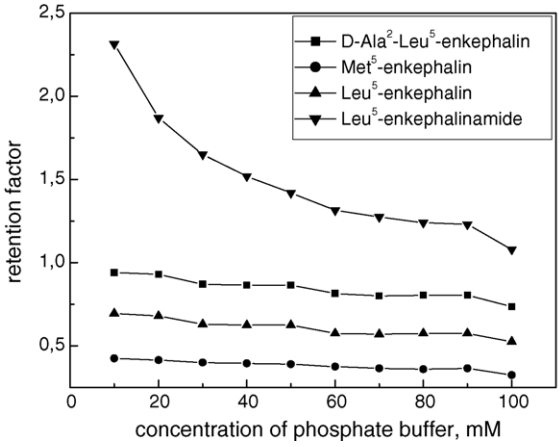

Fig. 8. Dependence of retention factor on concentration of phosphate buffer on Supelcosil C_{18}; mobile phase ACN–phosphate buffer, pH 5.0 (22:78, v/v); temperature 25 °C.

3.2.2. Effect of the ratio of organic modifier to buffer

Based on the above-presented results, mobile phases consisting of ACN and 50 mM phosphate buffer, pH 5.0 in ratios (40:60; 35:65; 33:67; 30:70; 27:73; 26:74; 25:75; 24:76; 23:77 and 22:78, v/v) were examined (Fig. 9). The retention of enkephalins markedly increased with increasing buffer content, and also resolution was improved in mobile phases with lower ACN content. The mixture of pentapeptides was well separated on silica-based Supelcosil C_{18} column using the mobile phase consisting of ACN–50 mM phosphate buffer, pH 5.0 (22:78, v/v). The representative chromatogram is shown in Fig. 10.

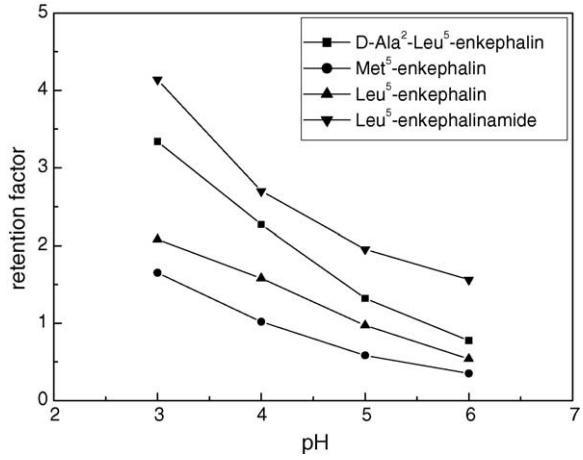

Fig. 7. Dependence of retention factor on phosphate buffer pH on Supelcosil C_{18}; mobile phase ACN–50 mM phosphate buffer (22:78, v/v); temperature 25 °C.

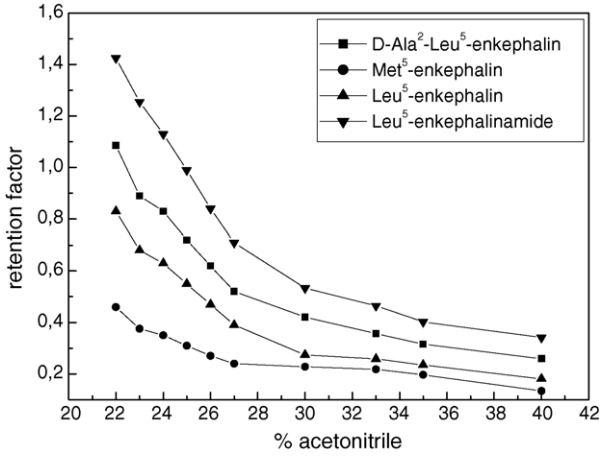

Fig. 9. Dependence of retention factor on content of acetonitrile in the mobile phase on Supelcosil C_{18}; mobile phase ACN–50 mM phosphate buffer, pH 5.0; temperature 25 °C.

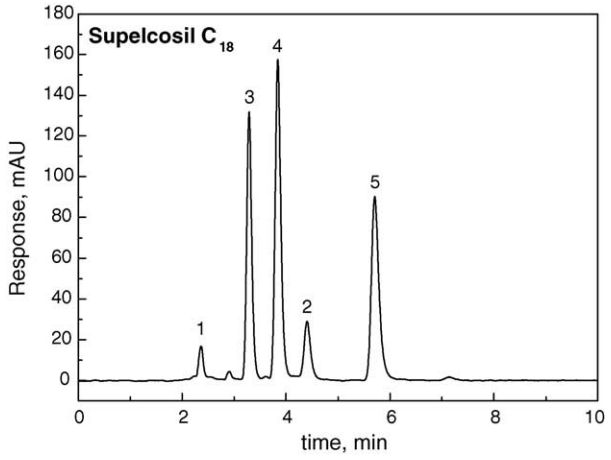

Fig. 10. Optimized separation of enkephalins on Supelcosil C_{18}; mobile phase ACN–50 mM phosphate buffer, pH 5.0 (22:78, v/v); temperature 25 °C; flow rate 1 mL/min; injection 10 μL; detection 214 nm.Peak identification: (1) Uracil; (2) D-Ala^2, Leu^5-enkephalin; (3) Met^5-enkephalin; (4) Leu^5-enkephalin; (5) Leu^5-enkephalinamide.

4. Conclusion

We have found substantial differences in the chromatographic behaviour of the set of pentapeptides on the zirconia-based columns, Discovery Zr-PBD and Discovery Zr-PS, and on the silica-based stationary phase, Supelcosil C_{18}. Variation of retention and separation of the enkephalins with the conditions of measurement was obvious on all the studied columns. (i) The acetonitrile content in the mobile phase showed similar trends on both types of the columns, i.e. decrease of retention with increasing ACN content, while a change of the elution order was observed only on the PBD-ZrO_2 stationary phase. A tentative explanation of this behaviour can be connected with the change of apparent pK_a^* (or pI^*) of the analytes (as well as of the buffer) under addition of organic modifier to the mobile phase [45]. (ii) Buffer concentration is a very important factor affecting the ion-exchange mechanism on the zirconia-based columns. From the dependencies obtained it could be clearly seen that the increase of the buffer concentration significantly decreased the retention on the ZrO_2-based phases while it had almost no effect on the retention of enkephalins, except of Leu^5-enkephalinamide, on the silica-based C_{18} phase. Another positive effect of the higher buffer concentration was the improved peak symmetry on the zirconia-based columns. There is a competition between the buffer and the analyte for the interaction sites on the ZrO_2 surface. If the surface is preferentially covered with the buffer component it positively affects the ion-exchange separation process. (iii) Variations of the buffer pH widely influenced the separation of pentapeptides on all the columns tested. Low pH was advantageous for good resolution and peak symmetry on the zirconia-based phases. On the other hand peak symmetry was almost independent on pH if the silica gel-based column was used. This could be an indication of well-shielded and homogenous silica surface. (iv) From the peak shape we could deduce that the mass transfer was faster on the silica gel based column under comparable temperature. The possibility to work at elevated temperature on the zirconia-based stationary phases allows to overcome this disadvantage by using higher temperature for the analysis there.

Zirconia-based columns were proved to be a good alternative to silica-based stationary phases for separation of enkephalins. Different interaction mechanism and a wide possibility of surface modification of zirconia columns destine these stationary phases for further successful applications in separation and purification of biomolecules.

We believe that the application potential of zirconia-based stationary phases has not been fully discovered yet. Zirconia as a base-stable material not only allows separation of biomolecules at high pH values but also provides an easy-to-clean surface. Their stability offers many chromatographic advantages such as use for the downstream processing of proteins because the use of hot alkaline media is a routine sanization procedure in these applications. In this sort of aggressive environment zirconia offers a distinct advantage over silica-based materials, which are destroyed under such conditions; polymer-based materials are limited by their mechanical stability. Thus there is a considerable interest in the development of zirconia-based supports for protein chemistry. Moreover, zirconia does not show the affinity to amines that leads to irreversible protein adsorption on silica-based supports. For these reasons zirconia-based materials are rather promising.

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