Charles University in Prague
Faculty of Science
Department of Analytical Chemistry

Modern Reversed Stationary Phases Based on Silicagel, Zirconium Dioxide and Organic Monoliths; Their Used in Separation of Biologically Active Compounds

Synopsis of PhD. Thesis

Prague 2008 Klára Soukupová

The PhD. Thesis was carried out at the Department of Analytical Chemistry, Faculty of Science, Charles University in Prague, in the period of 2003-2008.

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1. Introduction

High performance liquid chromatography (HPLC) is modern instrumental method used mainly in analytical chemistry. Most used separation mode is reversed phase liquid chromatography (RP HPLC). This method is very useful because of high separation efficiency, reliability and wide choice of stationary phases with different selectivity.

Most used stationary phases in RP HPLC are bonded phases, which have many advantages: good availability, wide application range, fast establishment of equilibrium and ability to use aqueous mobile phases. The most widely used carrier for RP HPLC stationary phases is silica with bonded C18 or C8 groups. Silica gel modified with pentafluorophenylpropyl ligands belongs among the recently introduced silica based stationary phases[1]. This new type of stationary phase is used as a sufficient alternative to the classical C18 phases.

Silica gel based HPLC phases have well known chemical and temperature limitations. Variety of other carrier materials has been investigated in an effort to overcome these limitations[2]. In attempt to replace the silica based reversed phases by more stable and selective carrier materials, various metal oxides such as zirconium, titanium and aluminum oxides were tested and characterized. Especially zirconia based stationary phases have exhibited special physical and chemical properties, namely good mechanical and chemical stability in a wide pH range (1-14) and temperature range up to 150°C[3,4].

The trend of the recent years is miniaturization of HPLC[5,6] columns. Capillary liquid chromatography (cLC) use the same stationary phases as classical HPLC but in smaller dimensions. New promising technique, which is an alternative to traditional particle based phases are monolithic columns[7]. These monolithic phases have become popular especially in cLC thanks to their easy preparation.

In this PhD. Thesis, retention behaviour of biologically active peptides with various stationary and mobile phases was tested. Basic analytical parameters on various columns were compared and the differences were discussed.

2. Objectives of the Thesis

The subject of this Thesis is a chromatographic behaviour of selected biologically active pentapeptides and nonapeptides (*Table 2.1*). Different reversed stationary phases (*Table 2.2*), based on silica gel, zirconium dioxide and organic monolith were tested using various mobile phase systems. Studied stationary phases were critically compared and practical use was assessed.

Table 2.1

Amino acid sequences of the studied biologically active peptides. The vasopressin analogues have disulphide bridge between Cys^1 and Cys^2.

Pentapeptides	**Amino acid sequence**
methionine^5–enkephalin	Tyr-Gly-Gly-Phe-Met
leucine^5–enkephalin	Tyr-Gly-Gly-Phe-Leu
D–alanine^2,leucine^5–enkephalin	Tyr-D-Ala-Gly-Phe-Leu
leucine^5–enkephalinamide	Tyr-Gly-Gly-Phe-LeuNH_2

Nonapeptides	**Amino acid sequence**
arginine^8–vasopressin (AVP)	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH_2
lysine^8–vasopressin (LVP)	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-GlyNH_2
oxytocin (OXT)	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-GlyNH_2
arginine^8–oxytocin (AVT, vasotocine)	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-GlyNH_2
isotocin (ISO)	Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Ile-GlyNH_2
deaminoCys^1,D–arginine–vasopressin (dDAVP, desmopresine)	Mpa*-Tyr-Phe-Gln-Asn-Cys-Pro-D-Arg-GlyNH_2

* 3–mercaptopropionic acid

Table 2.2

List of the reversed phase columns tested and their relevant physico-chemical properties.

Column	***Partical Platform***	***Bonded Phase***	***Partical Shape***	***Pore Size (Å)***	***Surface Area (m^2/g)***	***Temp. Limits (°C)***	***pH Range***
Supelcosil C18	silica	C18	spherical	120	170	60	2–7
Discovery HS F5	silica	pentafluoro–phenylpropyl	spherical	120	300	70	2–8
Discovery Zr PBD	ZrO_2	poly–butadien	spherical	300	30	150	1–13
Monolith	–	–	polymeric	200	24	–	–

Monoliths are new materials and all their properties are not available.

3. Results and Discussion

In this Thesis, four different reversed stationary phases were studied: two silica based stationary phases (Supelcosil C18 and Discovery HS F5), one zirconia based stationary phase (Discovery Zr PBD) and one monolithic column prepared in Department of Analytical Chemistry, Faculty of Science, Charles University in Prague. Columns were characterized and evaluated using common chromatographic parameters measured under Walters test conditions. The influence of mobile phase composition (pH, buffer, concentration, content of organic modifier) was tested as parameter affecting retention, separation efficiency, resolution and peak symmetry. Selected biologically important pentapeptides and nonapeptides were used as test compounds.

3.1 Walters test

The Walters test[8] is a simple test proposed for classification of reversed phases based on evaluation of two predominating retention mechanism in RP HPLC, hydrophobic and polar. The results obtained are summarized in *Fig. 3.1.1*. It was found that the selected stationary phases differ both in hydrophobic (HI) and polar (SI) indexes.

This choice of columns with different properties leads to different separation behaviour of tested peptides and makes possible to use to multidimensional separation.

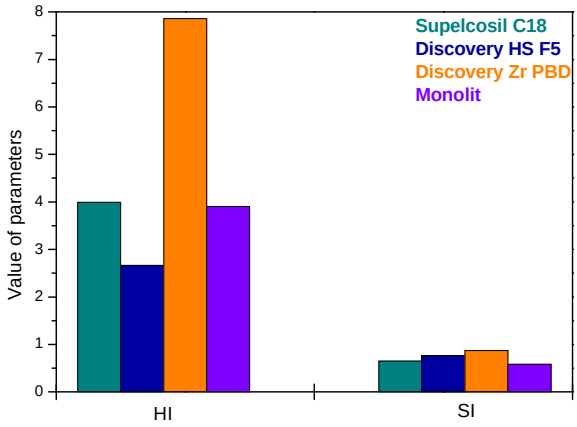


Fig. 3.1.1
Order of all studied columns according to the hydrophobicity (HI) and polarity (SI) parameters

To test column stability, Walters test was used repeatedly during optimization experiments, with differently buffered mobile phases. According to the results, the buffers did not affect the properties (HI, SI) of tested columns, except for Discovery Zr PBD column, where phosphate and acetate buffers caused change of hydrophobicity and polarity of this column.

3.2 Silica based stationary phases

During the first experiment with silica based stationary phases, Supelcosil C18 and Discovery HS F5, were used. Both columns were obtained from Supelco (Bellefonte, PA, USA). Supelcosil C18 column is classical C18 phase that is specially deactivated for analyses of basic compounds. Second column, Discovery HS F5, has stationary phase based on silica modified by pentafluorophenylpropyl ligands. The Discovery HS F5 bonded phase provides unique selectivity, excellent peak shape and good stability.

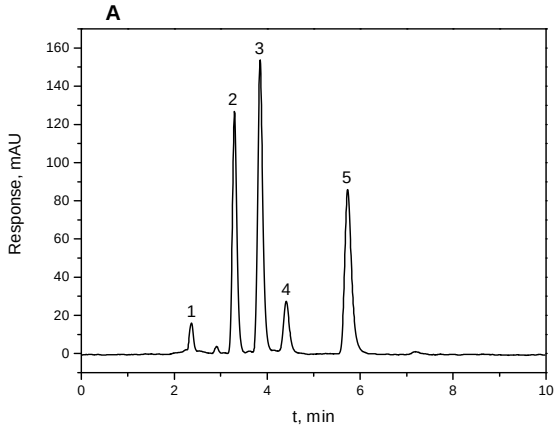
Various mobile phases composed of acetonitrile (ACN) and phosphate, acetate or formate buffer were tested. The goal was to reach a rapid and effective separation of tested analytes.

3.2.1 Pentapeptides

First tested group was mixture of four enkephalins (*Table 2.1*). The effect of concentration of acetonitrile, pH, the type and concentration of buffer were studied.

Based on the results obtained by optimization of parameters it was found out that mixture of pentapeptides is well separated on Supelcosil C18 column with mobile phase containing ACN - $5 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 5.0 22/78 (v/v). Separation of mixture was within 7 minutes (*Fig. 3.2.1.1 A*). All studied parameters were satisfactory; resolution (R_s) $\geq$ 2.8, symmetry of peaks (A_s) $\leq$ 1.3, column efficiency (tp/m) ~ 38 000. On Discovery HS F5 column the mixture of pentapeptides was best separated in mobile phase containing ACN - $8 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 4.5 22/78 (v/v) (*Fig. 3.2.1.1 B*). The separation of pentapeptides took 10 minutes with suitable resolution ($R_s \geq 2.8$), peak symmetry ($A_s \leq 1.7$) and column efficiency ~ 26 000 (tp/m).

Comparing the results, separation on Supelcosil C18 was unquestionably better than on Discovery HS F5 column in terms of analysis time, column efficiency and peak symmetry.



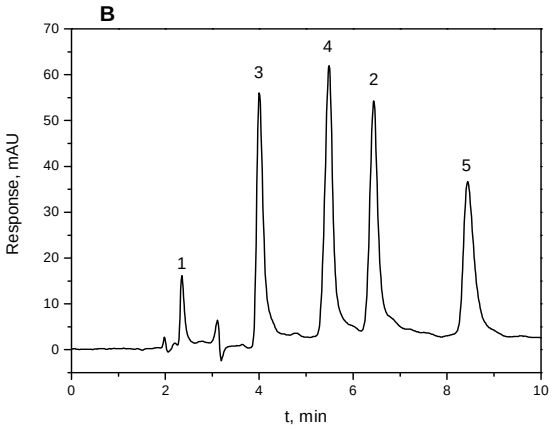


Fig. 3.2.1.1 A

Optimized separation of enkephalins on Supelcosil C18; mobile phase ACN – $5 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 5.0 22/78 (v/v)

Fig. 3.2.1.1 B

Optimized separation of enkephalins on Discovery HS F5; mobile phase ACN – $8 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 4.5 22/78 (v/v)

Temperature 25 °C; flow rate 1 ml/min; injection 10 µl; UV detection 214 nm. Identification of peaks: (1) uracil; (2) D–Ala,Leu–enkephalin; (3) Met–enkephalin; (4) Leu–enkephalin; (5) Leu–enkephalinamide

3.2.2 Nonapeptides

Second group of analytes tested were nonapeptides (*Table 2.1*). These compounds are structurally different from pentapeptides. Their structure is formed by cyclic part closed by disulfidic bridge. Remaining three aminoacids make a free side chain which is accessible for interactions.

As in experiments with enkephalins, the influence of pH and concentration of buffers, the ratio of acetonitrile to buffers on retention parameters were tested and differences were discussed. Based on the chromatographic parameters, suitable systems for separation of nonapeptides set were following:

Supelcosil C18 column

ACN - $1 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 4.5 18/82 (v/v)

ACN - $3 \cdot 10^{-2}$ mol·dm^{-3} acetate buffer pH 5.0 20/80 (v/v)

Discovery HS F5 column

ACN - $7 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 6.5 20/80 (v/v)

ACN - $5 \cdot 10^{-2}$ mol·dm^{-3} acetate buffer pH 6.5 21/79 (v/v)

Optimum mobile phases for Supelcosil C18 column had similar composition in terms of ACN concentration and pH. With ACN - phosphate buffer mobile phase analysis time of six nonapeptides was 26 minutes. Arg- and Lys-vasopressin were not separated from each other ($R_s \sim 0.6$). In system

ACN - acetate buffer system isotocin eluated in dead time and was not separated from uracil. The other analytes were separated in only 10 minutes ($R_s \geq 1.2$). It is interesting that the retention order of isotocin, oxytocin and uracil in ACN – phosphate system is different from retention order in ACN – acetate system. The change in buffer anion from inorganic to organic leads to significantly different system selectivity. The calculated separation efficiency was in range 15 000-22 000 tp/m in mobile phase ACN - phosphate buffer and 18 000-24 000 tp/m for ACN - acetate buffer.

Results obtained on Discovery HS F5 column show that in system with mobile phase ACN-phosphate buffer set of six vasopressins was separated in 18 minutes. Resolution ($R_s \geq 1.8$) and peak's symmetry ($A_s \leq 1.3$) were satisfactory. In second mobile phase consisting of ACN-acetate buffer separation of six nonapeptides was not successful. Isotocin, Arg-vasopressin and Lys-vasopressin were not separated to the baseline. In this system, separation order of isotocin changed compared to the system with ACN-phosphate buffer, being eluated first. Separation efficiency of system was ACN-phosphate buffer 37 000-47 000 tp/m and 17 000-30 000 tp/m for ACN-acetate buffer.

The most suitable system for separation of set of nonapeptides was with Discovery HS F5 column using mobile phase ACN - $7 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 6.5 20/80 (v/v). The representative chromatogram is shown in *Fig. 3.2.2.1*.

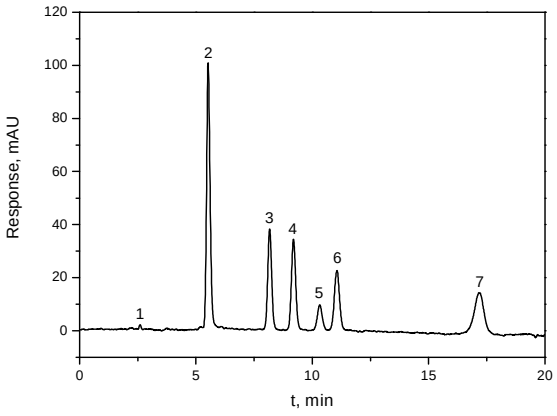


Fig. 3.2.2.1

Optimized separation of vasopressin analogues on Discovery HS F5; mobile phase ACN-$7 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 6.5 20/80 (v/v); temperature 25 °C; flow rate 1 ml/min; injection 10 μl; UV detection 214 nm. Peak identification: (1) uracil; (2) Arg-vasotocin; (3) Lys-vasopressin; (4) Arg-vasopressin; (5) isotocin; (6) oxytocin; (7) desmopresine.

3.3 Zirconia based stationary phase

Reversed-phase, zirconia based stationary phases have exceptional pH and thermal stability compared to silica based particles. The separation mechanism on zirconia based stationary phases is different from silica based phases and is based on Lewis acid-base chemistry.

Zirconia based Discovery Zr PBD column (Supelco, Bellefonte, PA, USA) was chosen for separation of set of pentapeptides and nonapeptides in different mobile phases. Mobile phases consisted of ACN or MetOH combined with phosphate or acetate buffer. The buffers used are strong Lewis bases, especially phosphate ion. Zirconia based columns are thermally stable so the influence of temperature was also tested among parameters affecting chromatographic behaviour of analytes.

3.3.1 Pentapeptides

Zirconia based columns are relatively new material on market. So minimum references about zirconia based columns and separation of peptides were found. Thus in first experiments, the effect of acetonitrile (ACN) and methanol (MetOH) on retention of analytes were examined.

A significant difference between MetOH and ACN can based mobile phases was observed (*Fig. 3.3.1.1*). Mobile phases containing MetOH were found to yield long retention times and very bad peak shapes. ACN in mobile phases offered low viscosity, low UV transmittance, and high separation efficiency (~ 30 000 tp/m). For these reasons ACN was chosen as organic part of mobile phase.

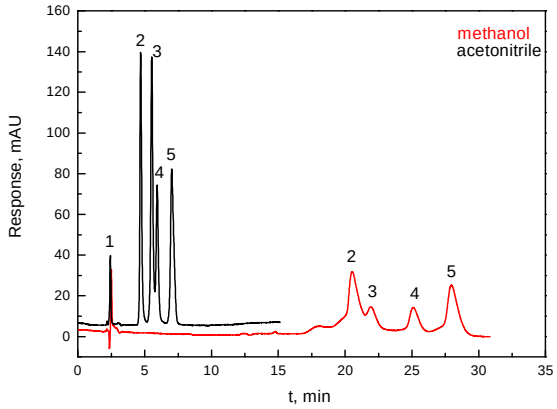


Fig. 3.3.1.1

Influence of organic modifier on retention behaviour of tested analytes. Mobile phase MetOH / ACN and $5 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 2,0 50/50 (v/v); temperature 25 °C; flow rate 1 ml/min; injection 10 µl; UV detection 214 nm. Peak identification: (1) uracil, (2) D-Ala-Leu-enkephalin, (3) Met-enkephalin, (4) Leu-enkephalin, (5) Leu-enkephalinamide

In following, effect of pH and concentration of phosphate buffer, ratio of ACN to buffer, and temperature variation were studied as parameters affecting retention behaviour of analytes.

Based on the optimized results, good resolution (R_s 1.9-2.9), good peak symmetry ($A_s \leq 1.4$) and good efficiency (tp/m ~ 20 000) in the reasonable time was achieved on Discovery Zr PBD in mobile phase consisting of ACN - $5 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 2.0 45/55 (v/v) and separation temperature was set to 70 °C (*Fig. 3.3.1.2*). The elution order of enkephalins varied on zirconia based and silica based phases, implying that the retention mechanism differs. The advantage of zirconia based stationary phase is that acidic mobile phase can be used without danger of column damage.

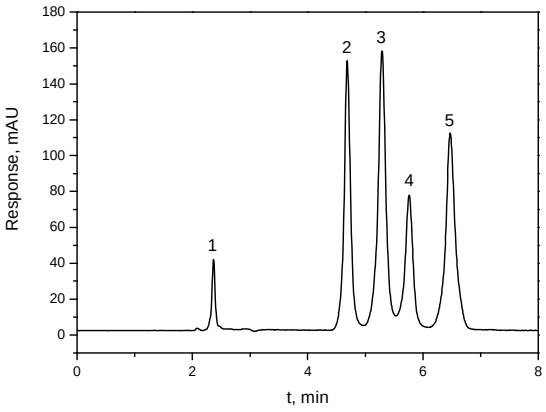


Fig. 3.3.1.2

Optimized separation of enkephalins on Discovery Zr PBD; mobile phase ACN - $5 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 2.0 45/55 (v/v); temperature 70 °C; flow rate 1 ml/min; injection 10 µl; UV detection 214 nm. Peak identification: (1) uracil; (2) Met-enkephalin; (3) Leu-enkephalin; (4) D-Ala,Leu-enkephalin; (5) Leu-enkephalinamide.

3.3.2 Nonapeptides

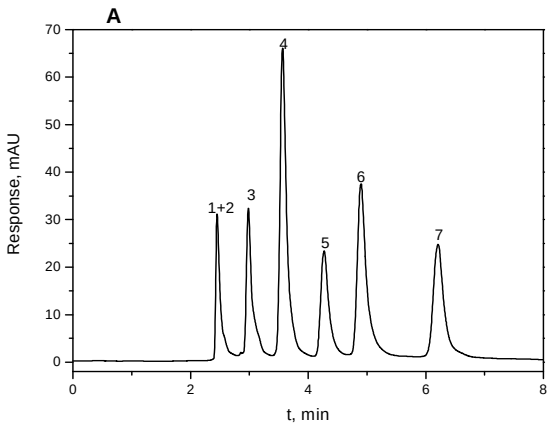
Retention behaviour of nonapeptides on Discovery Zr PBD was also studied. The influence of composition of mobile phase (buffer pH and concentration, content of organic modifier) and temperature was tested as variables affecting retention, separation efficiency, resolution and peak symmetry of selected nonapeptides. Mobile phases consisted of ACN as organic modifier and phosphate and acetate buffer.

Based on the results obtained by evaluation of chromatographic parameters, a successful attempt to separation of nonapeptides on Discovery Zr PBD was done in following mobile phase:

ACN-$7 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 10.0 18/82 (v/v), temperature 50 °C (*Fig.3.3.2.1 A*)

ACN-$6 \cdot 10^{-2}$ mol·dm^{-3} acetate buffer pH 6.5 18/82 (v/v), temperature 40 °C (*Fig. 3.3.2.1 B*)

When looking at *Fig. 3.3.2.1 A or B*, the separation of nonapeptides in mobile phase consisting of ACN-phosphate buffer was better then in mobile phase consisting of ACN-acetate buffer. Isotocin was eluated in dead time not separated from uracil. Despite this fact, separation within 7 min was done with good resolution (R_s min 2.9), good peak symmetry (A_s max 2.0) and separation efficiency in range 24 000 – 33 000 tp/m depending on analytes. In the mobile phase consisting of ACN-acetate buffer, separation of six vasopressins was done in 20 minutes and isotocin was separated from uracil (dead time marker). In this mobile phase, significant base line noise was observed and peak symmetry was not good (A_s 1.1 – 2.9). Separation efficiency was in range 5 000 – 22 000 tp/m.



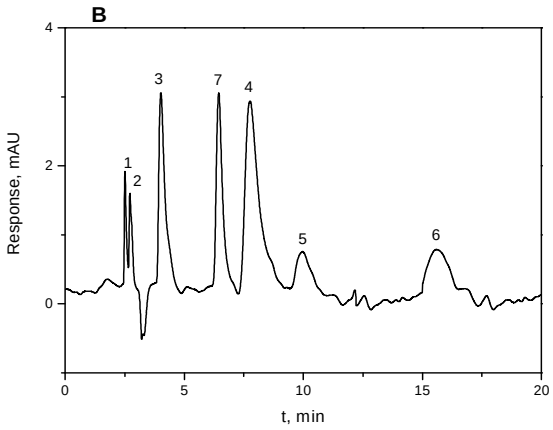


Fig. 3.3.2.1 A

Optimized separation of vasopressins on Discovery Zr PBD; mobile phase ACN-7·10^{-2} mol·dm^{-3} phosphate buffer pH 10.0 18/82 (v/v) temperature 50 °C.

Fig. 3.3.2.1 B

Optimized separation of vasopressins on Discovery Zr PBD; mobile phase ACN-6·10^{-2} mol·dm^{-3} acetate buffer pH 6.5 18/82 (v/v) temperature 40 °C.

Flow rate 1ml/min; injection 10 μl; UV detection 214 nm. Peak identification: (1) uracil; (2) isotocin; (3) oxytocin; (4) Arg-vasotocin; (5) Arg-vasopressin; (6) Lys-vasopressin; (7) desmopresine

3.4 Butyl-methacrylate monolithic stationary phase

In the following part of this Thesis, the retention behaviour of nonapeptides on monolithic column was tested. It is supposable that monolithic stationary phases are more chemically and thermally stable than silica based stationary phases. Disadvantage of these columns is their smaller separation efficiency.

The monolithic columns were prepared by thermally initiated radical polymerization of butyl methacrylate monomers in 320 μm i.d. fused silica capillaries according to procedure[9], developed at the Department of Analytical Chemistry, Faculty of Science, Charles University in Prague.

3.4.1 Nonapeptides

The same set of experiments that were used with silica and zirconia based columns was applied to on monolithic columns. The influence of composition of mobile phase (buffer pH and concentration, content of organic modifier) and temperature was tested as variables affecting retention, separation efficiency, and resolution and peak symmetry of selected nonapeptides. Mobile phases were consisted of ACN and phosphate, acetate or borate buffer.

The mixture of nonapeptides was well separated on monolithic phase using mobile phase consisting of ACN-phosphate buffer, but analysis time was 40 minutes. Phosphate buffer caused

damage of bonds between monolith and walls of capillary, resulting in monolith being pushed out of column. For this reason acetate and borate buffers were preferred.

Based on the results, mobile phase consisting of ACN - $3 \cdot 10^{-2}$ mol·dm^{-3} acetate buffer pH 4.5 3/97 (v/v) or ACN - $7 \cdot 10^{-2}$ mol·dm^{-3} borate buffer pH 7.7 8/92 (v/v) was suitable for separation of mixture of vasopressins. In mobile phase ACN-acetate buffer all six vasopressins analogues were well separated in 45 minutes, but significant noise of base line was observed. In second mobile phase, ACN-borate buffer, six analytes were separated in 25 minutes, but poor peak resolution and peak symmetry was observed. Separation efficiency for this monolithic column was lower than on other column only 2 400 to 10 000 tp/m. These monolithic stationary phases are experimental materials so there is chance for further improvement.

4. Conclusions

This Thesis was focused on study of retention behaviour of two selected group of peptides on different stationary and mobile phase. Based on the detailed experiments, suitable separation system for good separation of analytes was found.

Four different reversed stationary phases were tested; two silica based columns, one zirconia based column and one butyl-methacrylate monolithic phase. Selected stationary phases are relatively new materials and they promise new possibilities in separation of biologically active peptides.

In the first step, selected columns were tested by Walters test. The great differences in hydrophobicity and polarity of chosen columns were observed. Different character of selected columns leads to good characterization of separation behaviour of selected peptides.

Elution order of tested pentapeptides was similar on all tested stationary phases, except D-Ala-Leu-enkephalin that eluated first on Supelcosil C18 and third on Discovery HS F5 and Discovery Zr PBD. Different retention behaviour of this analyte was obviously related to polarity of stationary phase. D-Ala- Leu-enkephalin was most held on stationary phases high polarity. The mixture of pentapeptides was well separated on silica based Supelcosil C18 column using mobile phase consisting of ACN-$5 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer pH 5.0 22/78 (v/v).

Generalized information about retention behaviour of nonapeptides: isotocin eluated in dead time apart from separation system Discovery HS F5/phosphate buffer and Supelcosil C18/phosphate buffer. Second fact about separation was problematic separation of Lys-vasopressin and Arg-vasopressin. These analytes differ in one aminoacid (lysine or arginine), which is in position 8 of aminoacid chain. Both mentioned analytes were separated to baseline on Discovery HS F5 column using ACN - $7 \cdot 10^{-2}$ mol·dm^{-3} phosphate buffer o pH 6.5 20/80 (v/v) as mobile phase.

Finally, new reversed stationary phases modified silica based, zirconia based and monolithic phase, are a satisfactory and interesting alternatives to “classical” silica based stationary phases for separation of biologically active pentapeptides and nonapeptides.

Zirconia based reversed stationary phase provide little shorter retention times for nonapeptides, but separation of analytes is not better, compared to silica based columns.

The trend of recent years is miniaturization of HPLC. Capillary liquid chromatography (cLC) exploits the same stationary phases like in classical HPLC but dimensions are smaller. Promising new stationary phases used mainly in cLC are monolithic columns. Monolithic capillary columns are cheap and easy to prepare. The columns can be used to separate nonapeptides but efficiency is lower than with particle based columns, retention times longer and there are problems with baseline noise. The methods of preparation of experimental monolithic columns still need further improvement.

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Appendix 1: List of Publications, Lectures and Poster Presentations

List of Publications:

K. Soukupová, J. Suchánková–Sobotníková, L. Janečková, E. Tesařová: *Application of modern silica–based and zirconia–based reversed stationary phases for separation of selected vasopressins* – in preparation

K. Soukupová, E. Krafková, J. Suchánková, E. Tesařová: *Comparison of zirconia- and silica-based reversed stationary phases for separation of enkephalins.* Journal of Chromatography A 1087 (2005) 104 – 111

J. Suchánková, K. Soukupová, E. Tesařová, Z. Bosáková, P. Coufal: *Separation and Quantification of Enkephalin and Vasopressin Related Peptides in Reversed Phase Capillary Liquid Chromatography.* Chromatographia 60 (2004) S119 - S124

List of Lectures:

K. Soukupová: Comparison of zirconia-based and silica-based reversed stationary phases for separation of enkephalins, *2nd International conference „Modern Analytical Chemistry"*, Charles University in Prague, Faculty of Science, 25. 1. 2005

K. Soukupová: Separation of biologically active compounds on zirconia-based reversed stationary phases in RP HPLC, *SPE and HPLC seminar organized by Sigma-Aldrich*, ÚOCHB Prague, 22.10.2004

K. Soukupová: Separation of biologically active compounds in cLC, *SPE and HPLC seminar organized by Sigma-Aldrich*, ÚOCHB Prague, 21.11.2003

List of Poster presentations:

K. Soukupová, L. Janečková, J. Suchánková, E. Tesařová: New reversed stationary phases applied to separation of selected biologically active compound, 12*th Internation Symposium on Separation Sciences ISSS 2006*, Lipica, Slovenia, 27. – 29.9.2006

K. Soukupová, J. Kodeš, J. Suchánková, E. Tesařová: New reversed Stationary Phases and Their Use for separation of biologically active peptides, *11th Internation Symposium on Separation Sciences ISSS 2005*, Pardubice, Czech Republic, 12.– 14.9.2005

K. Soukupová, E. Krafková, J. Suchánková, E. Tesařová: Comparison of separation behaviour of some biologically active compounds on zirconia- and silica-based RP-HPLC stationary phases, *25th International Symposium on Chromatography*, Paris, France, 4.-8. 10. 2004

K. Soukupová, J. Suchánková, E. Tesařová, Z. Bosáková: Capillary liquid chromatography for separation of biologically active peptides, *5th Balaton Symposium on High-performance Separation Methods*, Siófok, Hungary, 3.-5. 9. 2003

J. Suchánková, K. Soukupová, E. Tesařová: Srovnání separace biologicky aktivních peptidů na různých reverzních stacionárních fázích, *IX. konferencia ACP 2002 Súčasný stav a perspektivy analytické chemie v praxi*, Bratislava, Slovakia, September 2002

Appendix 2: Curriculum Vitae

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