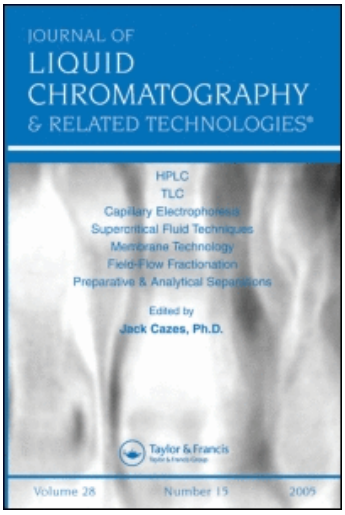

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HIGH PERFORMANCE REVERSED-PHASE LIQUID CHROMATOGRAPHY USING NOVEL CIM RP-SDVB MONOLITHIC SUPPORTS

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ABSTRACT

Monoliths have already proven to be efficient chromatographic supports for the separation of various types of molecules. In this paper, the characterization of the novel reversed-phase support, CIM® RP-SDVB disk monolithic column is presented.

Using a 3 mm long RP-SDVB disk monolithic column, excellent separation of proteins within a very short time was achieved. The pressure drop observed on the column was considerably low (few bars), even at flow rates of the mobile phase up to 30 mL/min. Due to the low pressure drop, the use of high flow rates was preferred since they did not influence the quality of the gradient separation. The separation of test proteins was performed within only 14 seconds; faster separations were limited by the configuration of the HPLC system.

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Furthermore, RP-SDVB disk monolithic columns were applied for fast separation of peptides. Five peptides of different lengths and composition were successfully separated in a very short time.

Finally, the preparative purification on the laboratory scale of the complex sample of oligodeoxynucleotide within a range of 1 minute demonstrates practical applicability of these columns.

INTRODUCTION

The need to achieve fast and reliable separation of different samples has stimulated the rapid development of new materials in the field of chromatographic supports. The continuing popularity of reversed-phase chromatography is reflected in an improvement of existing supports, as well as in the development of new types of supports.(1-5)

Silica-based materials dominate the market for reversed-phase supports, mostly packed as beads in a column. Porous polymers like *poly*(styrene-*co*-divinylbenzene) have also been utilized as a suitable material in reversed-phase separations.(3-6) In spite of the many advantages of silica-based materials, polymeric materials have less restriction on the mobile phase within a wide pH range, which makes them useful in numerous applications.(6,7)

Porous particles have been constantly improved over the last decades; however, they still show some limitations.(4,8,9). The absence of flow within the pores of particles results in slow diffusional mass transfer of samples. In addition, high pressure drop and the difficulty of packing the particles are two other problems.

Efforts to solve the problems of a stagnant mobile phase and, consequently, slow diffusional transport took many forms. One was to create non-porous particles. The problem of a small specific surface area has been solved by decreasing the size of the particles. Unfortunately, decreasing the size of the particles reflects in a considerable high pressure drop, which can be diminished only by reducing the column length.(10)

Yet another strategy, was to make the pores of the particles so large that some of the mobile phase could flow through them, as in the case of perfusion particles.(1,11,12) However, it was found that the major part of the mobile phase flows through a large void volume between the particles, resulting in reduced efficiency of the column.(12)

Superficially porous particles represent the next configuration of the stationary phase that has been of special interest in high-speed HPLC of large molecules.(13,14) A solid core with an outer layer of porous shell facilitates rapid mass transfer between the mobile and stationary phase. The current lack of commercial availability of these particles restricts their applications.(14)

Development of materials with monolithic structure is one of the approaches to overcome the problems of packed particles. These kinds of supports

consist of a single piece of a highly porous polymer with a bimodal pore size distribution forming flow-through channels.(5,15) Smaller pores provide a large surface area, needed for high binding capacity, while larger pores enable low pressure drop even when using very high flow rates. Since all of the mobile phase is forced to flow through the pores, molecules to be separated are transported to the active sites by convection.(16)

Reversed-phase monolithic supports based on styrene-divinylbenzene chemistry were first introduced in 1993 as a polymer prepared by a free-radical polymerisation.(16) The separations achieved with such a column have been found to be very efficient in the chromatography of proteins. Further investigations led to the optimisation of the porous properties and the dimensions of the monolithic rod to perform efficient separations of polystyrenes and peptides in an extremely short time.(17,18)

In the field of process monitoring and down-stream processing, rapid separations are of significant importance in large preparative scale and analytical scale chromatography.(18) Very short (2-3 mm length) disk monolithic columns can be effectively applied in this area.(19-21) They demonstrate advantages, as the time of analysis is within few seconds, exhibits low pressure drop, high flow, and unaffected binding capacity. Even more, by using short columns, unspecific binding and product degradation of larger biomolecules can be reduced.(22,23).

Despite many investigations in reversed-phase separations, properties and the applicability of very short (mm long) RP-columns were little studied. Since the column length does not play an important role in the gradient mode chromatography, short columns represent an alternative for the separation of various biomolecules. (24-26)

In this work, the ability of a novel semi-preparative reversed-phase support, CIM RP-SDVB disk monolithic column for separating proteins and peptides, has been investigated. For this purpose, short columns (3-12 mm) were used and the effect of decreasing column length on the resolution under the conditions of a linear gradient has been also studied.

Finally, fast purification of oligodeoxynucleotide from the synthetic mixture using 3 mm long column is presented, and the binding capacity of this column for the synthetic mixture of oligodeoxynucleotides has been determined.

EXPERIMENTAL

Chromatographic System

A gradient HPLC system (Knauer, Berlin, Germany) built of two pumps K-500, an injection valve with 20 μL, 200 μL, or 1 mL sample loop, a UV-VIS detector K-2500 set to response time of 0.1 s, with a 10 mm optical path, operated at 260 or 280 nm (depending on the sample), and with a 10 μL volume flow-

cell, connected by means of 0.25 mm I.D. PEEK capillary tubes and HPLC hardware/software (data acquisition and control station), were used in all separations.

A Knauer mixing chamber with its relatively large dead volume was replaced by the PEEK mixing tee with an extra low dead volume (VICI Jour Research, Uppsala, Sweden).

Chromatographic Columns

Semi-preparative CIM® RP-SDVB disk monolithic columns (BIA Separations d.o.o., Ljubljana, Slovenia) with the diameter of 12 mm and the length of 3 mm were used for the separation of proteins, peptides, and oligodeoxynucleotides. The base material was a macroporous styrene-*co*-divinylbenzene (SDVB) polymer.

Kromasil 100 C-18, 5 μm, 250 x 4.0 mm I.D. silica-based column (Eka Nobel, Nobel Industries, Bohus, Sweden) was used for testing the purity of oligodeoxynucleotide.

Mobile Phase

High-purity water and chemicals were used throughout the experimental work. HPLC grade acetonitrile and methanol were purchased from Rathburn (Walkerburn, Scotland), trifluoroacetic acid and triethylammonium acetate were purchased from Fluka (Buchs, Switzerland), and ammonium acetate was purchased from Merck (Darmstadt, Germany).

Samples

Ribonuclease A, Cytochrome C, and Chicken Egg Albumin were purchased from Sigma (St. Louis, MO, USA), while Bovine Serum Albumin was purchased from Fluka (Buchs, Switzerland).

The structure of the test peptides was as follows: Tyr-Gly-Gly-Phe-Met, Methionine Enkephalin from Sigma (St. Louis, MO, USA); Lys-Ser-Gly-Asp-Trp-Lys-Ser-Lys-Cys-Phe-Tyr, denoted as peptide A, donated by the Institute for Applied Microbiology, University of Agricultural Sciences (Vienna, Austria); Glu-Tyr-Ile-Lys-Trp-Glu-Glu-Phe-Lys, denoted as peptide B, donated by the Institute for Applied Microbiology, University of Agricultural Sciences (Vienna, Austria); Glu-Trp-Ile-Lys-Trp-Glu-Glu-Phe, denoted as peptide C, donated by the Institute for Applied Microbiology, University of Agricultural Sciences (Vienna, Austria); Polypeptide, Insulin from Bovine Pancreas from Sigma (St. Louis, MO, USA).

Oligodeoxynucleotide was synthesized on a controlled pore glass solid support using conventional phosphoramidite chemistry with the DNA synthesizer (Expedite Nucleic Acid Synthesis System 8909, PerSeptive Biosystem Inc., Framingham, MO, USA) at National Institute of Chemistry (Ljubljana, Slovenia).

Its structure was as follows:

No. of Bases	5'-3' Sequence	Notation
16	GCCG AGG TCC ATG TCT	16mer

RESULTS AND DISCUSSIONS

CIM (Convective Interaction Media)® RP-SDVB disk monolithic columns have been recently introduced on the market. The monolith shape is the same as for the other CIM® disk monolithic columns of different chemistries, which have already proven to be very efficient for the separation of large molecules like proteins and pDNA.(27, 28)

CIM monolithic materials combine the advantages of low pressure drop, high efficiency, and high binding capacity.(20) Since the separation of large molecules is almost exclusively limited to a gradient separation mode, shortening of the column length results in a lower pressure drop and a better yield of large unstable biomolecules. It has been shown, that the column length does not have a big influence on the resolution under linear gradient conditions in the case of large biomolecules.(29) In this way, also, very short (mm long) columns can be successfully applied for the separations of various biomolecules.(16,24-26).

In view of these theoretical foundations, we have tested the separation performance of the CIM RP-SDVB disk monolithic column, which is an extremely short semi-preparative support.

One of the advantages of CIM monolithic columns, is the possibility to vary the column length by simply placing a different number of CIM disk monolithic columns (disks) into the single housing.(20,30,31) In this way, the length of the column is equal to the number of disks placed in the housing, multiplied by the length of the individual disk. In order to decrease the pressure drop on the column, which allows increased flow rates and, consequently, higher productivity, the length of the column is optionally decreased.

To test the influence of the column length on the resolution of biomolecules, separation of four different proteins using a RP-SDVB monolithic column was performed. For this purpose, the RP-SDVB monolithic column was built of 1 to 4 disks in the single housing, thus, ranging the column length from 3 to 12 mm. The gradient of the mobile phase was adjusted to have a constant elution volume for each volume of the monolithic column and changed from 20% acetonitrile (MeCN) with 0.15 % trifluoroacetic acid (TFA), to 70 % MeCN with 0.15 % TFA, to achieve baseline separation of proteins.

Reversed-phase separation of a protein mixture, including Ribonuclease A (RNAse), Cytochrome C (Cyt C), Bovine Serum Albumin (BSA), and Chicken Egg Albumin (CEA) was first performed using a RP-SDVB monolithic column built of 4 disks in the housing with a total column length of 12 mm. As can be seen in Figure 1, the shortening of the RP-SDVB monolithic column to 9, 6, and finally 3 mm (3, 2 and 1 disks in the housing) does not affect the resolution of proteins. This further demonstrates that the column length has no influence on the quality of the gradient separation of proteins.

Besides, the pressure drop on a RP-SDVB monolithic column built with a single disk was considerably low (with 20 % MeCN at 10 mL/min, the pressure drop was only 3 bar), and was increasing linearly with the number of disks in the housing according to the equation:

$$\Delta P = N \times 3 \text{ bar}$$

where ΔP represents the overall pressure drop on the RP-SDVB monolithic column and N represents the number of disks in the housing.

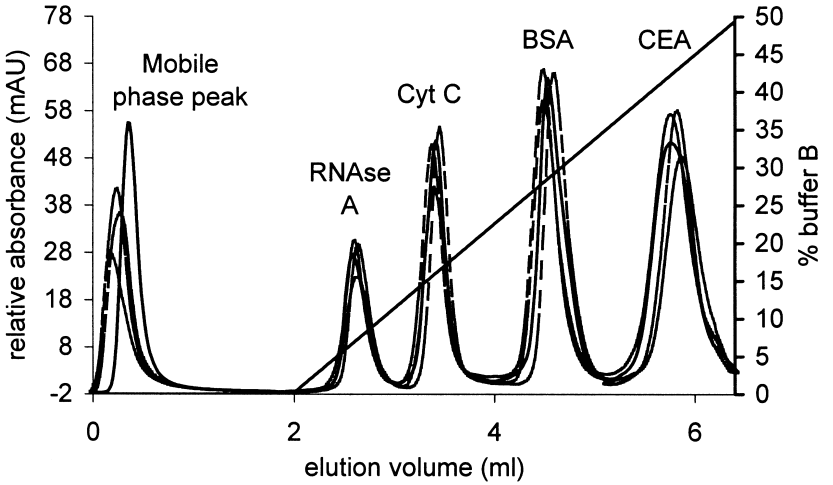

Figure 1. The influence of the column length on the quality of the gradient separation of proteins. Conditions: Stationary phase: RP-SDVB monolithic column of different length (3, 6, 9 and 12 mm length); Buffer A: 20 v/v% MeCN + 0.15 % TFA; Buffer B: 70 v/v% MeCN + 0.15 %TFA; Flow rate: 10 mL/min; Gradient: as shown in the Figure; Detection: UV at 280 nm; Temperature: room temperature; Injection volume: 20 μL; Flow monitoring: digital flow meter (K-3773, Phase Separations, UK); Sample: 1.5 mg/mL Ribonuclease A (RNAse A), 0.5 mg/mL Cytochrome C (Cyt C), 2.5 mg/mL Bovine Serum Albumin (BSA) and 3.0 mg/mL Chicken Egg Albumin (CEA), all dissolved in high-purity water.

Due to the low pressure drop on the column, it is possible to apply high mobile phase flow rates in order to reduce analysis time, which is especially important for downstream processing and in-process control. Using the RP-SDVB disk monolithic column (1 disk of 3 mm length in the housing), a wide range of flow rates was examined (Figure 2). The gradient of the mobile phase was adjusted to have a constant elution volume for each flow rate and changed from 20% MeCN with 0.15% TFA, to 70% MeCN with 0.15% TFA. Figure 2 presents the independence of the quality of the gradient separation using different flow rates of the mobile phase.

Figure 3 presents a very fast separation of proteins versus time, which was achieved in only 14 seconds at the mobile phase flow rate of 30 mL/min using a RP-SDVB disk monolithic column. As can be seen, all proteins are baseline separated. Since neither high pressure drop, nor the mechanical stability of the column, represent the limiting factor (with 20% MeCN at 30 mL/min, the pressure drop was only 9.1 bar); higher mobile phase flow rates can be applied. However,

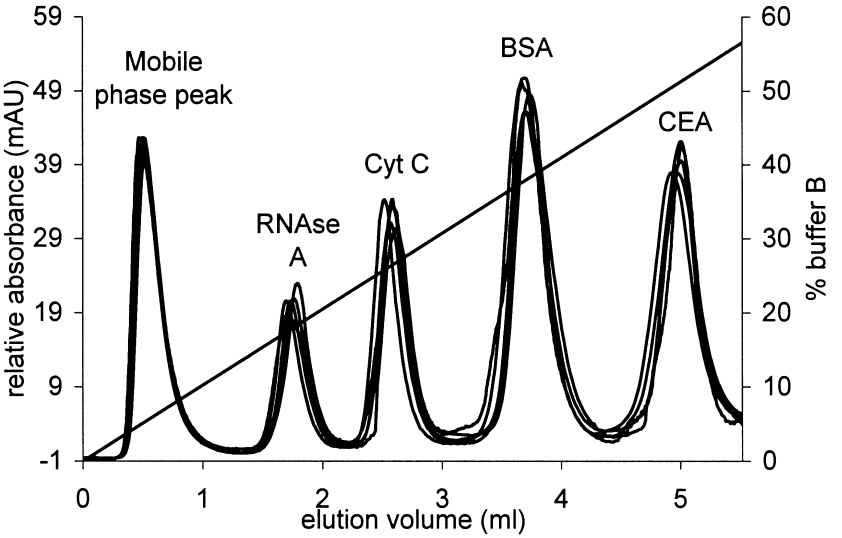

Figure 2. The influence of the flow rate of the mobile phase on the gradient separation of proteins. Conditions: Stationary phase: RP-SDVB disk monolithic column; Buffer A: 20 v/v% MeCN + 0.15 % TFA; Buffer B: 70 v/v% MeCN + 0.15 % TFA; Flow rates: 2, 5, 10, 20, or 30 mL/min; Gradient: as shown in the Figure; Detection: UV at 280 nm; Temperature: room temperature; Injection volume: 20 μL; Flow monitoring: digital flow meter (K-3773, Phase Separations, UK); Sample: 1.5 mg/mL Ribonuclease A (RNAse A), 0.5 mg/mL Cytochrome C (Cyt C), 2.5 mg/mL Bovine Serum Albumin (BSA) and 3.0 mg/mL Chicken Egg Albumin (CEA), all dissolved in high-purity water.

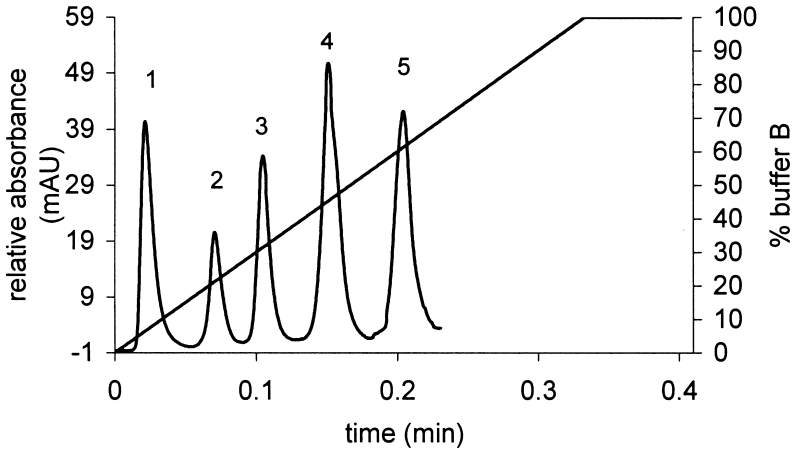

Figure 3. A 14 seconds linear gradient separation of proteins. Conditions: Stationary phase: RP-SDVB disk monolithic column; Buffer A: 20 v/v% MeCN + 0.15% TFA; Buffer B: 70 v/v% MeCN + 0.15 % TFA; Flow rate: 30 mL/min; Gradient: as shown in the Figure; Detection: UV at 280 nm; Temperature: room temperature; Injection volume: 20 µL; Flow monitoring: digital flow meter (K-3773, Phase Separations, UK); Sample: 1) Mobile phase peak, 2) 1.5 mg/mL Ribonuclease A (RNAse A), 3) 0.5 mg/mL Cytochrome C (Cyt C), 4) 2.5 mg/mL Bovine Serum Albumin (BSA) and 5) 3.0 mg/mL Chicken Egg Albumin (CEA). Samples are dissolved in high-purity water.

the maximum possible flow rate delivered by the HPLC system, the acquisition data rate, and detector response time of the HPLC system restricts further lowering of the analysis time without decreasing the resolution.(32)

Although short columns show many advantages in gradient separations, they might not be appropriate for the separations of smaller molecules, where the length of the column is important. Namely, better separation is obtained using columns with a higher number of theoretical plates.

Peptides represent one group of biologically important molecules of lower molecular masses compared to proteins, but have similar chemical structure. Xie et al. have already shown good separation of five peptides using a monolithic column of 50 x 4.6 mm I.D. based on styrene-divinylbenzene chemistry.(18) In order to achieve a fast analysis at a lower pressure drop, an RP-SDVB disk monolithic column was used for the separation of a mixture of five peptides with a different sequence of amino acid residues (the structure of peptides is presented in section 2.4.). Figure 4 presents a gradient separation of peptides that was performed at 8 mL/min. The gradient of the mobile phase was changed from 25% methanol (MeOH) with 1 % TFA, to 70% MeOH with 1 % TFA, to achieve baseline separation of peptides.

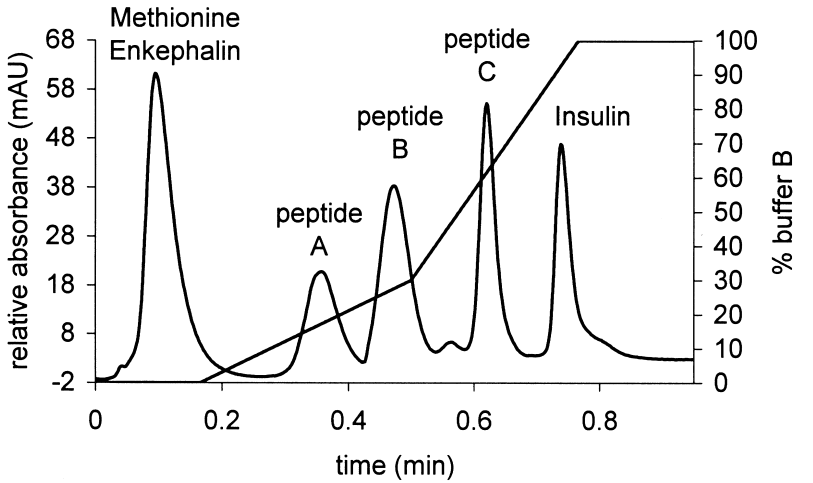

Figure 4. A 1 minute gradient separation of peptides. Conditions: Stationary phase: RP-SDVB disk monolithic column; Buffer A: 25 v/v% MeOH + 1 % TFA; Buffer B: 70 v/v% MeOH + 1 % TFA; Flow rate: 8 mL/min; Gradient: as shown in the Figure; Detection: UV at 280 nm; Temperature: room temperature; Injection volume: 20 μL; Flow monitoring: digital flow meter (K-3773, Phase Separations, UK); Sample: 0.1 mg/mL Methionine Enkephalin, 1.0 mg/mL peptide A, 7.0 mg/mL peptide B, 0.1 mg/mL peptide C and 0.1 mg/mL Insulin, all dissolved in high-purity water.

The sequence of peptides significantly affects their retention. Methionine Enkephalin, peptide A, peptide B, peptide C, and Insulin (sequence is presented in section 2.4.) were used as test peptides and eluted in this order. Methionine Enkephalin, a relatively short peptide, consists of 5 amino acid residues, among them 2 non-polar. Peptide A consists of 11 amino acid residues, 2 of them non-polar too. Peptide B is similar to peptide C, differing only in 2 amino acid residues. However, peptide C consists of 4 non-polar amino acid residues in contrast to peptide B, which consists of only 3 non-polar amino acid residues. Insulin binds strongly to an RP-SDVB disk monolithic column due to its long polypeptide structure, which consists of 51 amino acid residues (18 of them non-polar).

As shown in Figure 4, successful separation of peptides of different length and composition was accomplished using an RP-SDVB disk monolithic column. Furthermore, the separation of very similar peptides, peptide B and peptide C, was achieved.

CIM monolithic columns have already shown good separation performance of various complex mixtures.(21,33) To verify the usefulness of the new semi-preparative RP-SDVB disk monolithic column on complex samples, the purification of a crude mixture of oligodeoxynucleotides was carried out.

Synthetic oligodeoxynucleotides have a potential to become important therapeutic agents, mostly produced by solid-phase synthesis. While the synthesis is relatively fast, the purification of oligodeoxynucleotides represents a time-consuming step, frequently accomplished by reversed-phase or ion-exchange chromatography. The conventional HPLC columns, based on beads packed in the column, suffer from slow mass transfer of oligodeoxynucleotides in a chromatographic unit.(34) Accordingly, there is a need for rapid and efficient analyses and purifications of oligodeoxynucleotides.(35) Podgornik et al. have already presented separation of oligodeoxynucleotides using an ion-exchange CIM DEAE disk monolithic column.(30)

Our goal in this experiment, was to develop a fast method for the purification of desired oligodeoxynucleotide from the crude mixture with reversed-phase monolithic material using a RP-SDVB disk monolithic column.

For reversed-phase purification, 5'-deoxyribose hydroxyl group (5'-O-H group), located on the last deoxynucleotide in the chain of oligodeoxynucleotide, is mostly kept protected by 4,4'-dimetoxytrityl chloride (DMT). DMT utilized in 'trityl on' purification not only serves as a protecting group, but also significantly increases the hydrophobicity of oligodeoxynucleotide. The crude mixture of oligodeoxynucleotides contains 'trityl on' target oligodeoxynucleotide and impurities, including 'trityl on' and 'trityl off' failure sequences. After the purification, the trityl group can easily be cleaved from the target oligodeoxynucleotide with the acid.

16mer oligodeoxynucleotide (sequence is presented in section 2.4.) was prepared as a 'trityl on' oligodeoxynucleotide and purified using a semi-preparative RP-SDVB disk monolithic column. A crude sample of 16mer oligodeoxynucleotide was injected onto the RP-SDVB disk monolithic column in the isocratic mode, with 12% MeCN in 0.1 M ammonium acetate at 10 mL/min. After 30 seconds, the concentration of MeCN was linearly increased over 30 seconds to 100% MeCN. The conditions of the separation, the composition of the mobile phase, and the gradient of the mobile phase, were optimized to separate 16mer oligodeoxynucleotide from failure sequences (Figure 5).

Three fractions (fraction 1, 2 and 3) were collected to verify the purification of the crude mixture by using a Kromasil 100 C-18, 5 μm, 250 x 4.0 I.D. column. (Figure 6).

Fraction 1 contains a mixture of 'trityl off' oligodeoxynucleotides, which can be further purified using ion-exchange chromatography.(30) Fraction 2 consists of the pure 'trityl on' 16mer oligodeoxynucleotide, while fraction 3 comprises a mixture of 'trityl on' failure sequences of higher hydrophobicity.

By using a semi-preparative RP-SDVB disk monolithic column, in each separation run 20 μg of 16mer oligodeoxynucleotide were applied and successfully purified from the synthetic mixture. The main and very important feature observed, was the ability of the RP-SDVB disk monolithic column to separate the

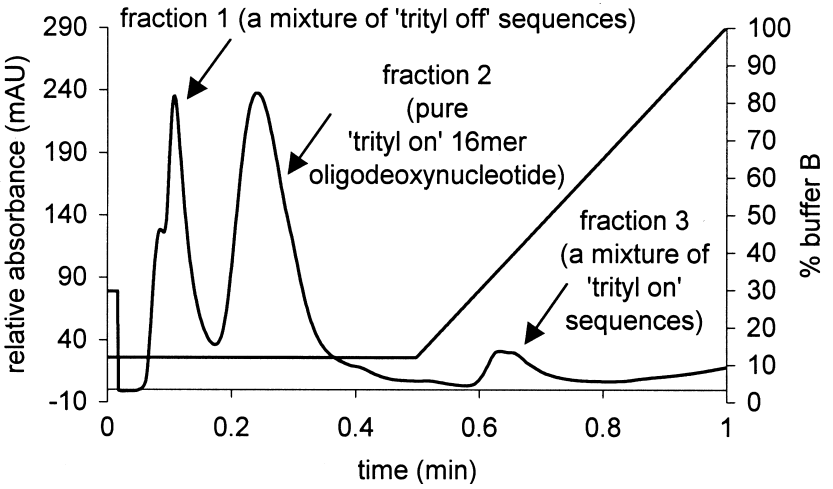

Figure 5. A 1 minute purification of 16mer oligodeoxynucleotide from a synthetic mixture. Conditions: Stationary phase: RP-SDVB disk monolithic column; Buffer A: 12 v/v% MeCN in 0.1 M ammonium acetate, pH=8.5; Buffer B: 100 % MeCN; Flow rate: 10 mL/min; Gradient: as shown in the Figure; Detection: UV at 260 nm; Temperature: room temperature; Injection volume: 200 µL; Flow monitoring: digital flow meter (K-3773, Phase Separations, UK); Sample: 30 µg of the crude 16mer, dissolved in buffer A.

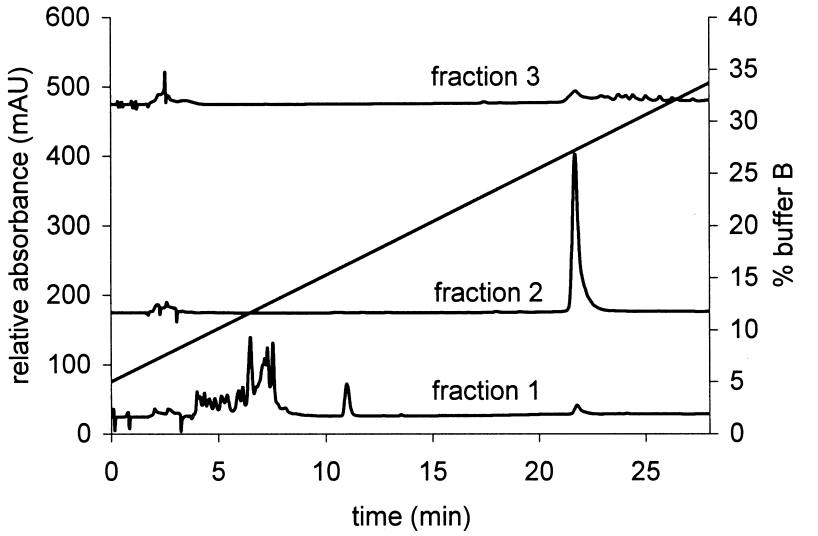

Figure 6. Verification of the purity of 16mer oligodeoxynucleotide fractions. Conditions: Stationary phase: Kromasil 100 C-18, 5 µm, 250 x 4.0 mm I.D. column; Buffer A: 5 v/v% MeCN in 0.050 M triethylammonium acetate, pH=7; Buffer B: 95 v/v% MeCN in 0.050 M triethylammonium acetate, pH=7; Flow rate: 1 mL/min; Gradient: as shown in the Figure; Detection: UV at 260 nm; Temperature: room temperature; Injection volume: 1 mL; Samples: 10 µg of fraction 1, 4 µg of fraction 2, 2 µg of fraction 3, all dissolved in buffer A.

desired oligodeoxynucleotide from the synthetic mixture, consisted of failure oligodeoxynucleotides, within only 1 minute.

The binding capacity of a RP-SDVB disk monolithic column was found to be equal to 1.8 mg of the crude sample of 16mer oligodeoxynucleotide per mL of the support volume. For the purification of larger amounts of oligodeoxynucleotide, the preparation of tube monolithic columns of larger volumes would be the appropriate approach. Such preparative radial columns, currently available only for ion-exchange and affinity mode, have already proven to be very useful for purification of larger amounts of the sample.(20,36)

CONCLUSIONS

CIM® RP-SDVB disk monolithic columns have demonstrated their suitability as chromatographic supports applicable in reversed-phase mode. In this work, their use for the efficient separation of various proteins, peptides, and oligonucleotides is reported.

The pressure drop is considerably low, and consequently, by applying higher flow rates, the analysis time is significantly diminished to a range of a few seconds.

RP-SDVB disk monolithic columns with the diameter of 12 mm and the length of 3 mm show their general application in the field of semi-preparative chromatography. In order to cover the analytical area, smaller units of these monolithic columns are in the course of development, which will be convenient for fast analysis and in-process control.

REFERENCES

1. Afeyan, N.B.; Gordon, N.F.; Mazsaroff, I.; Varady, L.; Fulton, S.P.; Yang, Y.B.; Regnier, F.E. Flow-through Particles for the High-Performance Liquid Chromatographic Separation of Biomolecules: Perfusion Chromatography. J. Chromatogr. **1990**, *519*, 1.
2. Bidlingmaier, B.; Unger, K. K.; von Doehren, N. Comparative Study on the Column Performance of Microparticulate 5-µm C_{18}-Bonded Reversed-Phase Columns in High-Performance Liquid Chromatography.(1) J. Chromatogr. A **1999**, *832*, 11.
3. Fulton, S.P.; Afeyan, N.B.; Gordon, N.F.; Regnier, F.E. Very High Speed Separation of Proteins with a 20-µm Reversed-Phase Sorbent. J. Chromatogr. **1991**, *547*, 452.
4. Unger, K.K. In *Packings and Stationary Phases in Chromatographic Techniques*; Unger, K.K., Ed.; Marcel Dekker Inc.: New York, 1990.

5. Wang, Q.C.; Švec, F.; Frechet, J.M. J. Macroporous Polymeric Stationary-Phase Rod as Continuous Separation Medium for Reversed-Phase Chromatography. Anal. Chem. **1993**, *65*, 2243.
6. Xie, S.; Švec, F.; Frechet, J.M. J. Porous Polymer Monoliths: Preparation of Sorbent Materials with High-Surface Areas and Controlled Surface Chemistry for High-Throughput, Online, Solid-Phase Extraction of Polar Organic Compounds. Chem. Mater. **1998**, *10*, 4072.
7. Mohammad, J.; Jäderlund, B.; Lindblom, H. New Polymer-Based Prepacked Column for the Reversed-Phase Liquid Chromatographic Separation of Peptides Over the pH Range 2-12. J. Chromatogr. A **1999**, *852*, 255.
8. Van Deemter, J.J.; Zuiderweg, F.J.; Klinkenberg, A. Longitudial Diffusion and Resistance to Mass Transfer as Causes Nonideality in Chromatography. Chem. Eng. Sci. **1956**, *5*, 271.
9. Snyder, L.R.; Kirkland, J.J. In Introduction to Modern Liquid Chromatography; Wiley: New York, 1990.
10. Huber, C.G.; Oefner, P.J.; Preuss, E.; Bonn, G.K. High-Resolution Liquid Chromatography of DNA Fragments on Non Porous Poly(styrene-divinylbenzene) Particles. Nucl. Acid. Res. **1993**, *21*, 1061.
11. Horvat, J.; Boschetti, E.; Guerrier, L.; Cooke, N. High-Performance Protein Separations with Novel Strong Ion Exchangers. J. Chromatogr. **1994**, *679*, 11.
12. Rodrigues, A.E.; Lopes, J.C.; Lu, Z.P.; Loureiro, J.M.; Dias, M.M. Importance of Intraparticle Convection in the Performance of Chromatographic Processes. J. Chromatogr. **1992**, *590*, 93.
13. Chen, H.; Horvath, Cs. High-Speed High-Performance Liquid Chromatography of Peptides and Proteins. J. Chromatogr. A **1995**, *705*, 3.
14. Kirkland, J.J. Ultrafast Reversed-Phase High-Performance Liquid Chromatographic Separations: An Overview. J. Chromatogr. Sci. **2000**, *38*, 535.
15. Tennikova, T.B.; Belenkii, B.G.; Švec, F. High-Performance Membrane Chromatography. A Novel Method for Protein Separation. J. Liquid Chromatogr. **1990**, *13*, 63.
16. Tennikova, T.B.; Švec, F. High-Performance Membrane Chromatography: Highly Efficient Separation Method for Proteins in Ion-Exchange, Hydrophobic Interaction and Reversed-Phase Modes. J. Chromatogr. **1993**, *646*, 279.
17. Petro, M.; Švec, F.; Frechet, J.M.J. Molded Continuous Poly(styrene-*co*-divinylbenzene) Rod as a Separation Medium for the Very Fast Separation of Polymers. Comparison of the Chromatographic Properties of the Monolithic Rod with Columns Packed with Porous and Non-Porous Beads in High-Performance Liquid Chromatography of Polystyrenes. J. Chromatogr. A **1996**, *752*, 59.

18. Xie, S.; Allington, R.W.; Švec, F.; Frechet, J.M.J. Rapid Reversed-Phase Separation of Proteins and Peptides Using Optimized 'Moulded' Monolithic Poly(styrene-*co*-divinylbenzene) Columns. J. Chromatogr. A **1999**, *865*, 169.
19. Mihelič, I.; Koloini, T.; Podgornik, A.; Štrancar, A. Dynamic Capacity Studies of CIM (Convective Interaction Media)® Monolithic Columns. J. High Resol. Chromatogr. **2000**, *23*, 39.
20. Štrancar, A.; Barut, M.; Podgornik, A.; Koselj, P.; Josić, Dj.; Buchacher, A. Convective Interaction Media: Polymer-Based Supports for Fast Separation of Biomolecules. LC-GC **1998**, *11*, 660.
21. Štrancar, A.; Koselj, P.; Schwinn, H.; Josić, Dj. Application of Compact Porous Disks for Fast Separations of Biopolymers and In-Process Control in Biotechnology. Anal. Chem. **1996**, *68*, 3483.
22. Coffman, J.L.; Roper, D.K.; Lightfoot, E.N. High-Resolution Chromatography of Proteins in Short Columns and Adsorptive Membranes. Bioseparation **1994**, *4*, 183.
23. Štrancar, A. Separation of Biopolymers with Different Techniques of Liquid Chromatography. Dissertation Thesis. University of Ljubljana, 1997.
24. Belenkii, B.G.; Podkladenko, A.M.; Kurenbin, O.I.; Mal'tsev, V.G.; Nasledov, D.G.; Trushin, S.A. Peculiarities of Zone Migration and Band Broadening in Gradient Reversed-Phase High-Performance Liquid Chromatography of Proteins with Respect to Membrane Chromatography. J. Chromatogr. **1993**, *645*, 1.
25. Tennikov, M.B.; Gazdina, N.V.; Tennikova, T.B.; Švec, F. Effect of Porous Structure of Macroporous Polymer Support on Resolution in High-Performance Membrane Chromatography of Proteins. J. Chromatogr. A **1998**, *798*, 55.
26. Dubinina, N.I.; Kurenbin, O.I.; Tennikova, T.B. Peculiarities of Gradient Ion-Exchange High-Performance Liquid Chromatography of Proteins.(1) J. Chromatogr. A **1996**, *753*, 217.
27. Giovannini, R.; Freitag, R.; Tennikova, T. High-Performance Membrane Chromatography of Supercoiled Plasmid DNA. Anal. Chem. **1998**, *70*, 3348.
28. Tennikova, T.B.; Freitag, R. An Introduction to Monolithic Disks as Stationary Phases for High Performance Biochromatography (Review). J. High Resol. Chromatogr. **2000**, *23*, 27.
29. Yamamoto, S.; Nakanishi, K.; Matsuno, R. In Ion-Exchange Chromatography of Proteins; Cazes, J., Ed.; Marcel Dekker Inc: New York, 1988; Vol. 43, 238.
30. Podgornik, A.; Barut, M.; Jancar, J.; Štrancar, A. Isocratic Separations on Thin Glycidyl Methacrylate-Ethylendimethacrylate Monoliths. J. Chromatogr. A **1999**, *848*, 51.

31. Vodopivec, M.; Podgornik, A.; Berovic, M.; Štrancar, A. Application of Convective Interaction Media (CIM®) Disk Monolithic Columns for Fast Separation and Monitoring of Organic Acids. J. Chromatogr. Sci. **2000**, *38* (11), 489.
32. Hahn, R.; Jungbauer, A. Peak Broadening in Protein Chromatography with Monoliths as Very Fast Separations. Anal. Chem. **2000**, *72*, 4853.
33. Branović, K.; Buchacher, A.; Barut, M.; Strancar, A.; Josić, Dj. Application of Monoliths for Downstream Processing of Clotting Factor IX. J. Chromatogr. A **2000**, *903*, 21.
34. Gilar, M.; Bouvier, E.S.P. Purification of Crude DNA Oligonucleotides by Solid-Phase Extraction and Reversed-Phase High-Performance Liquid Chromatography. J. Chromatogr. A **2000**, *890*, 167.
35. Oefner, P.J.; Bonn, G.K.; Huber, C.G.; Nathakarnkitkool, S. A Comparative Study of Capillary Zone Electrophoresis and High-Performance Liquid Chromatography in the Analysis of Oligonucleotides and DNA. J. Chromatogr. Biomed. Appl. **1992**, *625*, 331.
36. Štrancar, A.; Barut, M.; Podgornik, A.; Koselj, P.; Schwinn, H.; Raspor, P.; Josić, Dj. Application of Compact Porous Tubes for Preparative Isolation of Clotting Factor VIII from Human Plasma. J. Chromatogr. A **1997**, *760*, 117.

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