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Separation of oligonucleotides on novel monolithic columns with ion-exchange functional surfaces

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Abstract

Porous monolithic columns have been prepared by the direct free radical copolymerization of glycidyl methacrylate and ethylene dimethacrylate within the confines of a 50×8 mm I.D. chromatographic column in the presence of porogens. The epoxide groups of these monoliths were modified to different extents by reaction with diethylamine to afford 1-*N*,*N*-diethylamino-2-hydroxypropyl functionalities useful for ion-exchange chromatography. Following characterization of the monoliths, the columns were tested in the chromatographic separation of a homologous series of oligodeoxyadenylic [$pd(A)_{12-18}$] and oligothymidylic acids [$d(pT)_{12-24}$] at different flow-rates. Very good separations of the oligonucleotides were achieved even at the high flow-rate of 4 ml/min.

Keywords: Monolithic columns; Stationary phases, LC; Oligonucleotides

1. Introduction

The field of synthetic oligonucleotides is developing rapidly since these relatively low-molecular-mass compounds are finding numerous applications in experimental medicine (antisense and antigen drugs), molecular biology (probes for gene isolation), and biochemistry (diagnostics) [1–3]. They are also used as primers in the polymerase chain reaction for the amplification of DNA, and as linkers for gene splicing and DNA ligation [4,5]. Their large-scale preparation using solid-phase synthesis is fully automated and can easily produce multigram quantities in a relatively short period of time. Despite its high efficiency, this procedure affords products that always contain closely related impurities, mostly oligonucleotides with sequences shorter than the desired length, and, therefore, an additional purification step is required to obtain the pure oligomer. Additionally, current drug discovery efforts using the methods of combinatorial chemistry result in libraries of oligonucleotides and their analogs that also must be separated from impurities and identified [6]. Clearly, efficient, selective and fast separation methods are needed to meet the challenge of this rapidly growing area.

Currently, two major techniques are primarily used for the separation of oligonucleotides: electrophoresis in both capillary zone and capillary gel modes (CZE and CGE) [7,8] and high-performance liquid chromatography (HPLC) [9–20]. Although electrophoresis is a very powerful separation method, it is not well suited for larger scale separations. In contrast, HPLC has an intrinsic potential to be performed on a large scale.

Oligonucleotides are actually polyesters with up to about 50 repeat units. The phosphoric acid moieties render these polymers anionic while the pendant heterocyclic bases contain hydrophobic sites. These properties also determine the chromatographic modes

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best used for their separation: ion-exchange [13,17], mixed mode [12,14,16,20] and ion-pair reversed-phase chromatography [9,10]. Because of the very small differences in the hydrodynamic volumes and the short chain length of oligonucleotides, the technique of size-exclusion [11] and ''slalom'' chromatography [15] useful in the separations of DNA both appear to be less suitable for oligonucleotides. Numerous columns for the separation of oligonucleotides are commercially available. These are often packed with specifically designed separation media, typically modified porous silica beads.

In the early 1990s, we have developed an entirely new format of HPLC separation medium: rigid porous polymer monoliths consisting of a single piece of a separation medium [21–27], now produced by Isco (Lincoln, NE, USA), featuring high speed separation ability, large binding capacity, and very low back pressure. A few other monolithic media well suited for rapid separations of biopolymers have also emerged [28] and have been commercialized. They include convective interaction media in the shape of discs and tubes placed in a cartridge (CIM, Bia, Ljubljana, Slovenia) [29–31], compressed hydrophilic gel columns (UNO column, Bio-Rad, Richmond, Ca, USA) [32,33], and silica based monolithic rods (SilicaRod, Merck, Darmstadt, Germany) [34,35].

Our monoliths are prepared by a simple, one-step, in-situ free-radical polymerization ''molding'' process directly within the confines of a chromatographic column. Some monolithic columns such as those prepared from styrene and divinylbenzene [23,24] can be used in reversed-phase separations immediately after the polymerization is completed. In other cases, a subsequent chemical functionalization of the pore surface is necessary to obtain the chemistries required for other chromatographic modes such as affinity, hydrophobic interaction and ion-exchange chromatography. This functionalization can be achieved either by the modification of reactive groups localized at the surface with specific reagents [22,25] or by grafting with functional monomers at the pore surface [26].

Our previous reports demonstrated the utility of these monolithic columns for the rapid separations of two classes of macromolecular compounds: synthetic polymers [27], peptides and proteins [22–26]. Following our success in the separations of these large molecules, we had anticipated that this new generation of chromatographic columns would be well suited for the separation of oligonucleotides. This short communication describes the first use of a monolithic column for the ion-exchange chromatography of oligothymidylic and oligodeoxyadenylic acids.

2. Experimental

2.1. Chemicals

Glycidyl methacrylate, ethylene dimethacrylate (both Sartomer, Exton, PA, USA), azobisisobutyronitrile (AIBN, Kodak), cyclohexanol, dodecyl alcohol and diethylamine (all Aldrich) were used as obtained. The oligodeoxyadenylic [$pd(A)_{12-18}$, lot 8037975011) and oligothymidylic acids [$d(pT)_{12-18,}$ lot 87H9051 and $d(pT)_{19-24,}$ lot 57H6659] were purchased from Pharmacia Biotech and Sigma, respectively. All solvents used for chromatography were of HPLC grade.

2.2. Preparation of the monolithic column

The monolithic columns were prepared by a direct in situ polymerization within stainless steel tube of a 50×8 mm I.D. chromatographic column. A mixture consisting of 24 g glycidyl methacrylate, 16 g ethylene dimethacrylate (monomers), 54 g cyclohexanol, 6 g dodecanol (porogenic diluents), and 0.4 g azobisisobutyronitrile (initiator, 1% (w/v), with respect to monomers) was purged with nitrogen for 15 min. The stainless steel tubes sealed at the bottom were filled with the polymerization mixture and then sealed at the top. The polymerization was allowed to proceed at 55°C for 24 h. The seals were removed, the tube was provided with column fittings, attached to a syringe pump and washed with tetrahydrofuran pumped through the column at a flow-rate of 0.4 ml/min for 60 min to remove the alcohols and other soluble compounds present in the polymer monolith after the polymerization was completed.

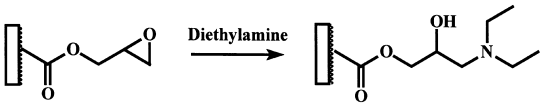

Fig. 1. Reaction of epoxide groups with diethylamine.

2.3. Preparation of 3-diethylamino-2-hydroxypropyl-functionalized porous monolith

The epoxide groups of the poly(glycidyl methacrylate–co-ethylene dimethacrylate) monoliths were allowed to react with diethylamine according to the reaction in Fig. 1. Diethylamine (10 ml) was pumped through the column for 2 h at a flow-rate of 5 ml/h using a syringe pump. The column was then removed from the pump, its fittings sealed with plugs and either heated in a bath or left at room temperature. Specific reaction conditions are shown in Table 1. The modified columns were then reattached to the pump and washed first with water at a flow-rate of 0.4 ml/min for 2 h and then with a 10 mmol/l phosphate buffer solution at pH 7 for another 1 h.

2.4. Characterization of properties

After the chromatographic experiments had been completed, the rod was washed with water (2 h at 0.5 ml/min), the bottom column fitting was removed and the polymer rod was pushed out of the tube using the pressure of the methanol–water (7:3) mobile phase at a flow-rate of 5 ml/min. The cylindrical monolith was cut with a razor blade to small pieces and dried under vacuum at 70°C. The pore size distribution in the dry state was determined by mercury intrusion porosimetry (Micromeritics, Norcross, GA, USA). The contents of amino groups were determined by elemental analysis (Table 1).

Table 1
Reaction conditions used for modification of poly(glycidyl methacrylate–co-ethylene dimethacrylate) monoliths and their contents of ion-exchange functionalities[a]

Column	Reaction temperature (°C)	Time[b] (h)	Nitrogen content	
			%	mmol/g
I	55	3	1.77	1.26
II	55	1	0.94	0.67
III	20	5.5	0.38	0.27

[a] For other reaction conditions see Section 2.

[b] This reaction time does not include 2 h at room temperature during which the monoliths are flushed with diethylamine at a flow-rate of 5 ml/h.

2.5. Chromatography

A Waters HPLC system consisting of two 501 HPLC pumps, a 707plus autosampler, and a 486 UV detector, was used to carry out all the chromatographic tests. The data was acquired and processed with Millenium 2010 software. Solutions consisting of 20% (v/v) acetonitrile and 80% (v/v) 20 mmol/l aqueous phosphate buffer (pH 7.0 adjusted after addition of acetonitrile) and 1 mol/l sodium chloride solution in the previous mixture were used as mobile phase A and B for the gradient elutions, respectively.

3. Results and discussion

3.1. Functionalization of the monolithic columns

A series of identical monolithic poly(glycidyl methacrylate–co-ethylene dimethacrylate) columns was prepared under conditions previously optimized for the separation of proteins [25]. These reactive monoliths can be modified easily to afford separation media with ionizable groups that are well suited for chromatography. Fig. 1 shows the simple reaction of the epoxide groups with diethylamine that results in 1-*N*,*N*-diethylamino-2-hydroxypropyl functionalities very similar to the ''classical'' diethylaminoethyl (DEAE) chemistry. Obviously, some of these amino groups are available on the surface of the large pores while others are buried inside the polymer matrix and in the small pores. These buried groups are not likely to participate in the separation process. Unfortunately, there is no simple method currently available that allows an exact direct determination of the distribution of functionalities in pores of various sizes as well as within the bulk matrix. We reasoned that some information about the effect of the spatial distribution of functionalities within the separation medium could be obtained if identical monoliths were functionalized to different degrees. The overall content of ionizable groups can be controlled easily by varying both reaction temperature and time. Table 1 shows that the extent of the modification is

relatively small even after almost 8 h of reaction time (2 h saturation+5.5 h reaction) at room temperature and only 0.27 mmol/g amine functionalities is found to be present in the monolith. In contrast, higher degrees of functionalization can be achieved at an elevated temperature of 55°C in only 1 and 3 h of reaction time (0.67 and 1.26 mmol/g, respectively).

3.2. Porous and hydrodynamic properties of the functionalized monolithic columns

Based on our extensive studies of in-situ polymerizations [21], we have chosen conditions that afford monoliths with a pore size distribution that enables good permeability. Fig. 2 shows the pore size distribution profiles for all three monoliths prepared at 55°C and subsequently modified with diethylamine. As expected, they are essentially identical, confirming the good reproducibility of the preparation process. Their total pore volume is 1.08 ml/g and translates to a porosity of 58%. According to mercury intrusion porosimetry, pores in the range of 50–500 nm and those smaller than 50 nm represent 14.5% and 24% of the total pore volume, respectively. The majority of the total pore volume, 61.5%, corresponds to pores with diameters in the range of 500–3000 nm thus enabling the high permeability of the monoliths.

This porous structure is designed to ensure only modest flow resistance in the 50 mm long monolithic columns. For example, a back pressure of only 6 MPa is obtained at a flow-rate of 6 ml/min for the modified columns, equaling a linear flow velocity of 1200 cm/h. The linearity of the back pressure vs. flow-rate dependency found with water as the mobile phase clearly documents the rigidity of the monolith, enabling it to withstand even higher back pressures.

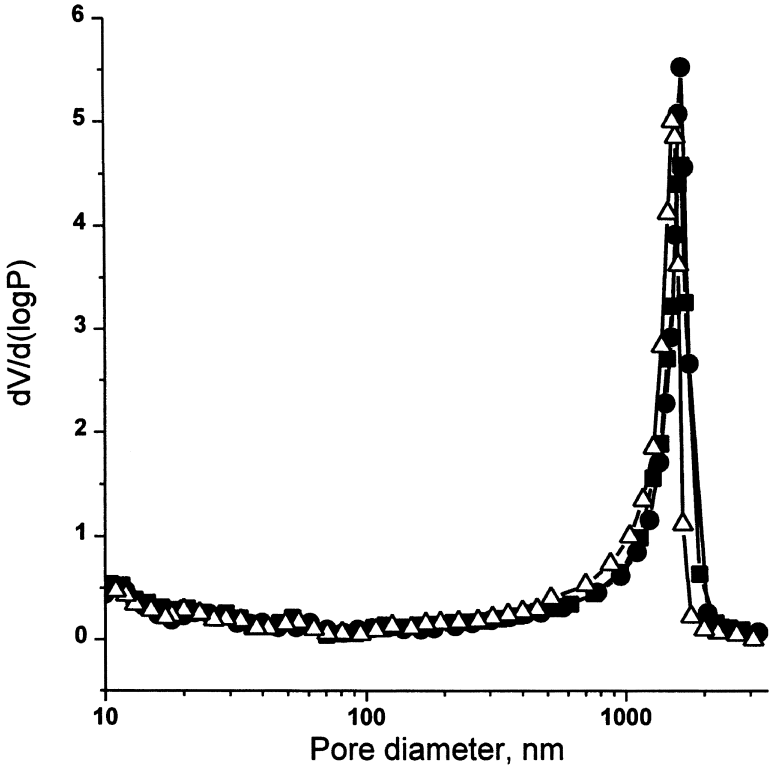

Fig. 2. Differential pore size distribution curves of the modified poly(glycidyl methacrylate–co-ethylene dimethacrylate) monoliths measured by mercury intrusion porosimetry. Columns I (■), II (●), III (△).

3.3. Chromatographic properties

The ultimate goal of developing a separation medium is its eventual use to effect the desired separation. The ability of the monolithic columns to separate oligonucleotides was tested first with a mixture of oligodeoxyadenylic acids with 12–18 repeat units. Fig. 3a shows that an almost baseline separation is achieved on column II in about 100 min using a very shallow gradient from 23 to 33% mobile phase B in A at a flow-rate of 1 ml/min. This time scale is also typically recommended by manufacturers for the separations of oligonucleotides on other commercial columns.

The major advantage of the monolithic media is their ability to effect separations even at extremely high flow-rates without the concomitant decrease in separation power due to the beneficial contribution of convection to the overall mass transport. The benefits of this improvement in mass transport has been demonstrated clearly in our previous reports [23,27]. Gradient elution separations can be accelerated simply by effecting simultaneous increases in both flow-rate and gradient steepnes, since the average retention factor in gradient elution, k^*, depends on the gradient time, t_G, the flow-rate, F, the change in composition of the mobile phase $\Delta\phi$, and the column dead volume V_m, according to the equation [36]:

$$k^* = t_G F/\Delta\phi V_m S \quad (1)$$

The constant S calculated for each solute from the retention data in isocratic systems, characterizes the strength of the interaction between the solute and the stationary phase. For both a constant range of mobile phase composition $\Delta\phi$ and a specific column and

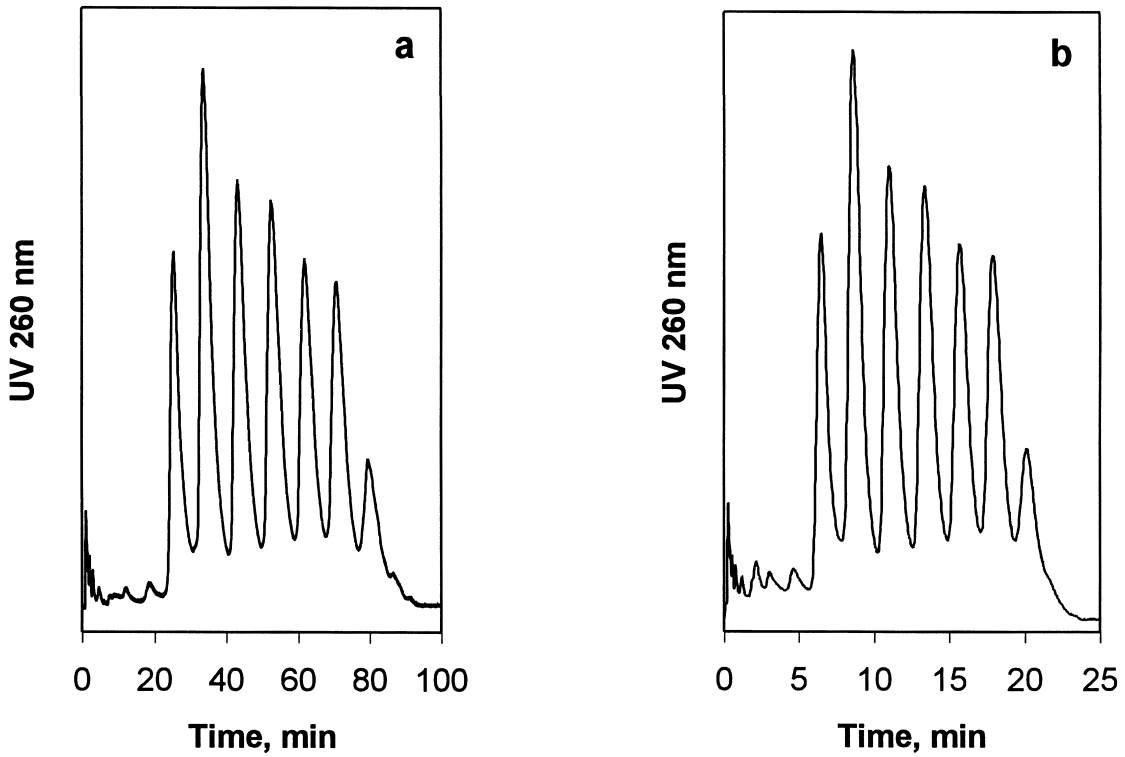

Fig. 3. Separation of oligodeoxyadenylic acids [pd(A)$_{12-18}$] on modified monolithic poly(glycidyl methacrylate–co-ethylene dimethacrylate) column II. Conditions: column 50×8 mm I.D.; mobile phase gradient from 23 to 33% buffer B in A (A=20% acetonitrile and 80% 20 mmol/l phosphate buffer, pH 7.0; B=1 mol/l sodium chloride solution in A) in 90 (chromatogram a) and 22.5 min (chromatogram b); flow-rate 1 (a) and 4 ml/min (b); UV detection at 260 nm.

solute, the denominator in the above equation remains constant, and the average retention factor, k^*, depends only on the gradient time and the flow-rate. Since the product of these variables is the gradient volume, V_G, equal peak capacities should be achieved within the same gradient volume, regardless of flow-rate and gradient steepness. Fig. 3b shows the separation of the deoxyadenylic acid oligomers mixture using a gradient time of 22.5 min at a flow-rate of 4 ml/min. Since the gradient volume of 90 ml is the same for both separations in Figs. 3a and b, no significant differences are observed between these two chromatograms. However, the second separation is achieved in only one quarter of the time of the first. In contrast to classical chromatographic columns packed with micrometer sized beads for which the use of high flow-rate is restricted by the rapid increase in back pressure with flow-rate, molded monolithic columns allow the use of very high flow-rates at reasonable back pressures without any concomitant loss in selectivity, making faster chromatographic runs possible.

Although the separation of seven oligonucleotides was easily achieved with our monolithic column, a mixture comprising 13 oligothymidylic acids with 12–24 repeat units provides a more challenging problem. Fig. 4 shows that an almost baseline separation again can be achieved with column II using a gradient from 28 to 41% mobile phase B in A, and that an identical separation also can be achieved using a flow-rate four times higher in conjunction with a corresponding steeper gradient.

Retention in ion-exchange chromatography depends on the density of interactive functionalities

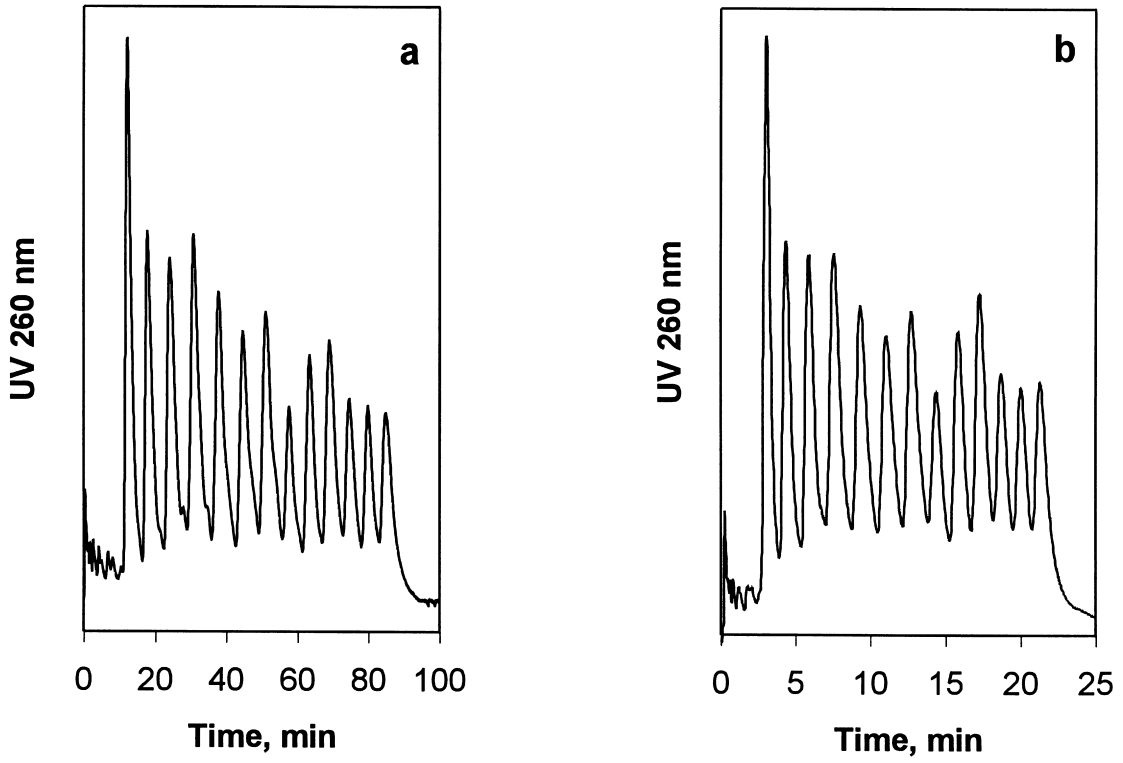

Fig. 4. Separation of oligothymidylic acids [$d(pT)_{12-24}$] on modified monolithic poly(glycidyl methacrylate–co-ethylene dimethacrylate) column II. Conditions: column 50×8 mm I.D.; mobile phase gradient from 28 to 41% buffer B in A (A=20% acetonitrile and 80% 20 mmol/l phosphate buffer, pH 7.0; B=1 mol/l sodium chloride solution in A) in 90 (chromatogram a) and 22.5 min (chromatogram b); flow-rate 1 (a) and 4 ml/min (b); UV detection at 260 nm.

localized on the surface of pores accessible to the analytes. The modification reaction used for the preparation of 1-*N*,*N*-diethylamino-2-hydroxypropyl groups is not very specific. Essentially each epoxide group exposed to diethylamine can react. As a result, a number of the tertiary amine functionalities are buried within the solid matrix and in small pores that are impermeable for larger molecules such as oligonucleotides. Obviously, those epoxide groups that are located in the most accessible parts of the matrix such as larger pores, react preferentially because they are more easily approached by the reagent. This difference in reactivity can be used to a certain extent to influence the distribution of site functionalization by optimizing reaction conditions. Unfortunately, there is no better method to specifically assess only the number of reactive sites that exist in pores sufficiently large to be operative in the specific separation beyond the preparation of materials modified to various extent and their use in the actual separation. Fig. 5 shows the separations of 13 oligothymidylic acids with monoliths modified to different extents using gradients optimized specifically for each monolithic column. The separations on columns I and II containing 1.26 and 0.67 mmol/g of functional groups, respectively, are virtually identical. Column I is more retentive since a higher starting salt concentration is required to achieve the separation. While the gradient range remains the same for both column I and II (the difference between the starting and final percentage of B in A is always 13%), 35% of B must be present in the original mobile phase used for the separation on column I, while only 28% B is required to achieve the separation on column II. This indicates that this increase in the level of functionalization resulted in the existence of more interacting functionalities at the accessible surface, suggesting that the surface is not completely functionalized at the degree of substitution of 0.67 mmol/g. The separation of oligonucleotides on column III with only 0.27 mmol/g substitution is poorer. Obviously, this degree of functionalization does not afford sufficient surface coverage of interactive sites required to achieve a good separation. This is further supported by the much lower salt concentration in the initial mobile phase required to achieve sufficient retention compared to column I (only 15% B in A). Further, the gradient range narrowed to a mere 10% difference in B between the initial and final mobile phase composition is necessary to separate the oligonucleotides.

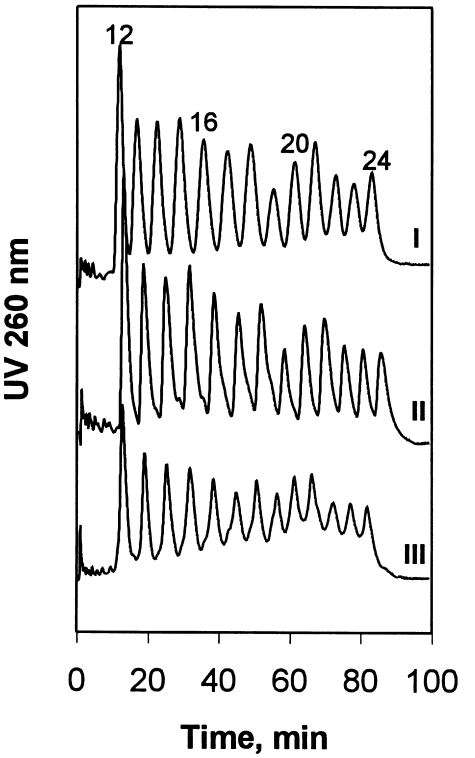

Fig. 5. Separation of oligothymidylic acids $[d(pT)_{12-24}]$ on modified monolithic poly(glycidyl methacrylate–co-ethylene dimethacrylate) columns I, II and III. Conditions: column 50×8 mm I.D.; mobile phase gradient from 35 to 48% B in A (A=20% acetonitrile and 80% 20 mmol/l phosphate buffer, pH 7.0; B=1 mol/l sodium chloride solution in A) (column I); 28 to 41% B in A (column II), and 15 to 25% B in A (column III) in 90 min; flow-rate 1 ml/min; UV detection at 260 nm.

4. Conclusions

We have demonstrated that ''molded'' monolithic columns can be used in an ion-exchange mode for the separation of oligonucleotides. In addition to their ease of preparation, their separation ability hardly changes over a broad range of flow-rates that are easily accessible due to the intrinsically low pressure drop of the monolithic columns.

Although the monolithic columns may have currently somewhat lower efficiencies than some other specialized packed columns, they do not lag in selectivity. This early work demonstrates the promising chromatographic properties of monolithic media and their great potential for the rapid separation of oligonucleotides. Further development of the monolithic columns in our laboratory is aimed at an increase in efficiency that can be achieved using the capillary format. Our initial results with capillary electrochromatography are very encouraging [37].

Acknowledgements

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