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Preparation of various silica-based columns for capillary electrochromatography by in-column derivatization

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Abstract

Chemically bonded silica gels were prepared in a capillary by pumping an ethanolic solution of a silylating reagent, such as octadecyltrimethoxysilane, 3-aminopropyltrimethoxysilane and dimethyloctadecyltrimethoxysilylpropylammonium chloride into a heated capillary packed with bare silica particles. The silylation reactions were completed in a short time and thus-prepared columns showed high column efficiency and high reproducibility. Examples are shown for the separation of 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives of aldopentoses on a 3-aminopropylated silica column and benzoate homologues as well as PMP derivatives of the component monosaccharides of glycoproteins on an octadecylammonium column. Since the presence of frit filters hampers high efficiency separation, an attempt was made to fix the bed of modified silica gel particles to the capillary inner wall by a cross-linking technique. The results indicated that this technique is promising.

Keywords: Electrochromatography; Capillary columns; Derivatization, electrochromatography; Carbohydrates

1. Introduction

Capillary electrochromatography (CEC) is a micro method for separating a wide range of compounds with high resolution power, and has been applied to various compounds as summarized in some reviews [1–7]. In CEC sample introduction as well as elution are performed using electroosmotic flow (EOF) generated by the surface of stationary phase particles and capillary inner wall [8]. The flat flow profile of EOF is favorable for enhancement of column efficiency, but variability of the velocity of EOF has hampered popularization of CEC for routine analysis. Dittman and Rozing [9] and Zimina et al. [10] reported that the velocity of EOF in some commercial ODS columns varied in a range as wide as 0.01–2 $cm^2/V\,s$ depending on the density of silanol groups remaining after modification with the silylating reagent. In liquid chromatography the remaining silanol functions are not favorable for separation of basic compounds due to undesired ionic and/or hydrophilic interactions, hence they are usually endcapped by trimethylsilylation. However, such end capping is undesirable in CEC, since the decrease of the density of the silanol group causes reduction of

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the velocity of EOF due to the decrease of negativity on the particle surface. Commercially available CEC columns are rare and the usability of CEC is practically limited. In 1991 Knox and Grant reported tentative preparation of reversed-phase columns by chemical modification of drawn packed capillaries [11]. This pioneering work has encouraged us to explore the possibility of preparing high-quality, laboratory-made columns of variously modified stationary phases by in-column derivatization.

First we prepared a few kinds of columns by in-column derivatization of bare silica-packed columns with silylating reagents and compared the capabilities of the obtained columns with those prepared by packing with commercially available modified silica particles. Since the higher capability of the in-column derivatized columns was demonstrated, we further attempted preparation of a new type of column based on the following concept. If a bare silica-packed capillary is treated with a bifunctional silylating reagent having both ionic and hydrophobic groups, the resultant bifunctional column may allow reversed-phase separation with sufficiently rapid velocity of EOF over a wide pH range. From this expectation we attempted preparation of a unique column by in-column derivatization with dimethyloctadecyltrimethoxysilylpropylammonium chloride, which has cationic (the quaternary ammonium) and hydrophobic (octadecyl) groups. The introduction of the quaternary ammonium group enhanced the resolution of basic compounds by eliminating unfavorable ionic and hydrophilic interactions of cationic analytes with the silanol groups. In addition derivatization with this reagent brought forth another advantage. It modified the surface of not only the silica gel particles but also of the capillary inner wall to yield positive charge, resulting in rapid separation due to faster EOF than in columns packed with silica gels similarly modified by the batch method.

In this paper, we describe some results of in-column derivatization using various silylating reagents.

We also present the result of attempted omission of column filters by fixing the bed of the stationary phase to the capillary inner wall using a cross-linking technique.

2. Materials and methods

2.1. Reagents

1-Phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Kishida (Osaka, Japan) and recrystallized from methanol before use. 3-Aminopropyltrimethoxysilane, *n*-octadecyltrimethoxysilane, dimethyloctadecyltrimethoxysilylpropylammonium chloride and bis[3-(trimethoxysilyl)propyl]ethylenediamine were received from ShinEtsu Silicon (Tokyo, Japan). Deionized and glassware-distilled water was filtered through a membrane filter (0.45 μm) for the preparation of eluents. Other chemicals and solvents were of the highest grade commercially available.

2.2. Apparatus

CEC was performed on a Hewlett-Packard ^{3D}CE apparatus, equipped with a power supply, a column pressurizing system, a column temperature control system, and a UV detector. The operation of the CEC system and data acquisition were performed using Chem-Station, a Windows program. A capillary column was mounted on an accessory cartridge and the cartridge installed in the apparatus. It was preconditioned with an eluent to be used for analysis, with application of a voltage of 5 kV and a pressure of 7.5 bar to the inlet for 1 h. Before each run the column was equilibrated with an eluent by flushing it with applying a voltage of 5 kV. A sample was introduced electrokinetically by applying a voltage of 2 kV for 3 s. Both ends were pressurized at 7.5 bar to prevent bubble formation, and analysis was performed under the conditions given in figure captions. The temperature of the column cartridge was controlled at 20°C throughout the work.

2.3. Columns and packing materials

A roll of polyimide-coated fused-silica capillary (100 μm I.D.×375 μm O.D.) was obtained from GL Science (Tokyo, Japan). Samples of Develosil silica (3 nm pore, 3 μm) and Develosil NH_2 (10 nm pore, 3 μm) were supplied by Nomura Kagaku (Aichi, Japan). Nucleosil silica (10 nm pore, 3 μm) was

from Machery–Nagel (Düren, Germany). A specimen of DaisoGel (30 nm pore, 3 μm) was kindly gifted by the manufacturer (Osaka, Japan).

2.4. Preparation of bare silica columns and in-column derivatization

One end of a capillary (40 cm long) was dipped in a water glass and taken out. The capillary end was gently heated by a nickel–chrome wire wound around it to fabricate a temporary frit [12], then the capillary was flushed with isopropanol at a pressure of 100 kg/cm^2 for 10 min. The other end of the capillary was connected to a stainless steel packer filled with a slurry of bare silica gels, prepared by suspending 200 mg of silica gel particles in 1 ml of isopropanol with sonication under reduced pressure. The packer was then pressurized to 400 kg/cm^2 until the bed height reached ca. 30 cm. After pumping ethanol the column was connected to the solvent delivery system and placed in an oven thermostated at 110°C, and an ethanolic solution containing a silylating reagent (2%, w/v) and acetic acid (0.5%, v/v) was flowed through the column for 1 h at 400 kg/cm^2. The column was then washed with ethanol followed by water commonly at 500 kg/cm^2 for 3 h. Before switching off the pump, two positions in the bed (4 and 29 cm from the temporary frit) were heated by a nickel–chrome wire for the fabrication of retaining frits so as to make up a 25-cm bed. The temporary frit was removed by cutting the column just beside the newly prepared retaining frit at the outlet and excess silica gel was removed by pumping water from the opposite side. The column was equilibrated sequentially with acetonitrile followed by the run eluent for 3 h each. The polyimide coat at the 25.5-cm position (the 9-cm position from the outlet of the column) was peeled off by a razor blade to make a detection window. The capillary column thus obtained was immediately mounted on a cartridge, which was then installed on the CEC apparatus.

2.5. Derivatization of monosaccharides with 1-phenyl-3-methyl-5-pyrazolone [13]

Aqueous 0.3 *M* sodium hydroxide and methanolic 0.5 *M* PMP solutions (50 μl each) were added consecutively to a mixture of monosaccharides (50 nmol each) placed in a 1.5-ml PTFE tube, and the tube heated for 30 min at 70°C. The solution was neutralized by adding 50 μl of 0.3 *M* HCl and the mixture evaporated to dryness by a centrifugal evaporator. The dried residue was dissolved in 0.2 ml of water and excess reagent was removed by three iterative extractions with 0.2 ml of chloroform. Then the aqueous layer was taken out and evaporated to dryness, and the residue dissolved in 100 μl of an eluent to make an analytical sample solution.

2.6. Preparation of a fritless column

Packing bare silica gel into a capillary and washing with ethanol was carried out in the same manner as described in Section 2.4. Then an ethanolic solution of bis[3-(trimethoxysilyl)propyl]-ethylenediamine (2%, w/v) was delivered to the silica packed capillary thermostated at 110°C for 1 h at 400 kg/cm^2. After washing extensively with ethanol the capillary was cut to obtain a bed height of 25 cm. The subsequent equilibration with eluent and mounting to the cartridge was also in the same manner as described in Section 2.4.

3. Results and discussion

3.1. Preliminary studies of in-column derivatization

Derivatization of silica gels in the batch method is usually performed by heating silica gel particles suspended in toluene containing a silylating reagent up to a concentration of a few percent. Since the most typical example is the derivatization with octadecyltrimethoxysilane (ODTMS), we modified small particles of silica gels packed in a capillary by supplying a stream of an ODTMS solution through the capillary with constant heating in order to allow in-column preparation. The solubilities of some other substituted alkoxysilanes in toluene are, however, too low to prepare solutions of such high concentrations as a few percent, and excess reagents sometimes

cause clogging of particles in the course of in-column derivatization. Therefore, we used ethanol slightly acidified with acetic acid instead of toluene throughout this work.

The time dependence of in-column silylation with ODTMS was observed using a capillary packed with Develosil silica gel having a pore size of 3 nm with 10 m*M* Tris–HCl (pH 7.0)–acetonitrile (1:1, v/v) as eluent. The introduction of the nonpolar ODS group caused suppression of the velocity of EOF, and the magnitude of EOF reduction observed from the delay of the thiourea peak can be used as a measure of the efficiency of in-column derivatization. After flowing an ethanolic ODTMS solution through the capillary for 15 min at 110°C a remarkable delay of the thiourea peak was observed. After 15 to 30 min conversion of separation mode from hydrophilic to hydrophobic interaction was observed as the change of the elution order of the thiourea and dimethylphthalate peaks. After 60 min the velocity of EOF was minimized, and no peaks of thiourea were observed within 60 min at 15 kV. Consequently we used this reaction time also for silylation with other reagents.

Fig. 1 shows examples of the analysis of a mixture of dimethylphthalate and thiourea using three different columns consecutively prepared by packing silica gels of the same batch into capillaries of the same

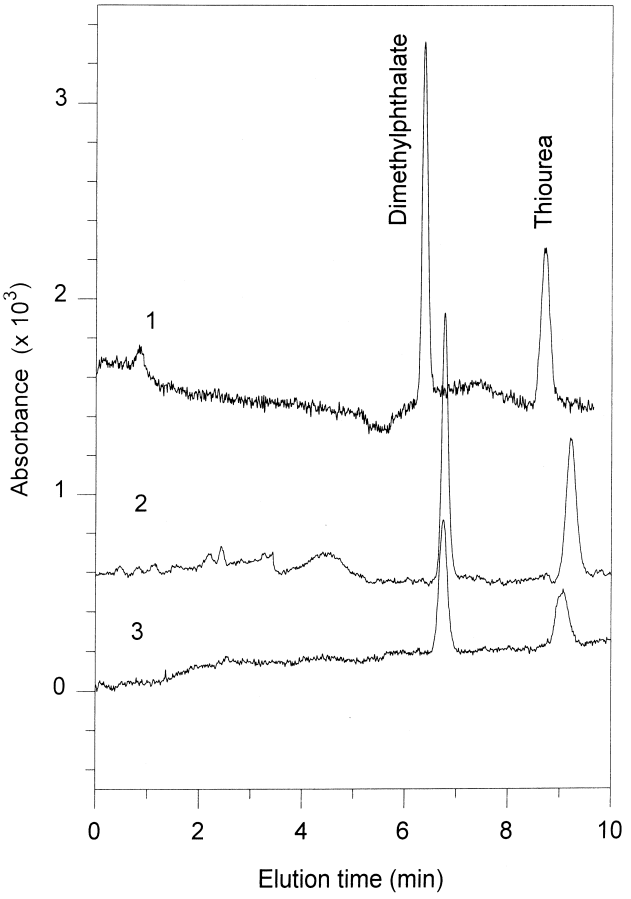

Fig. 1. Separation of dimethylphthalate and thiourea using three consecutively prepared columns by in-column derivatization with APTMS. Capillary, fused-silica [345 mm (250 mm effective length)×100 μm I.D.×365 μm O.D.] packed with Nucleosil silica (10 nm, 3 μm), in-column derivatized with APTMS; eluent, 10 m*M* Tris–HCl (pH 7.0)–acetonitrile (1:4, v/v); sample concentration, 0.05% each; injection, −1 kV for 2 s (from the cathodic end); cartridge temperature, 20°C; applied voltage, −15 kV; detection, UV absorption at 254 nm.

size, followed by in-column derivatization with 3-aminopropyltrimethoxysilane (APTMS).

In this case EOF was directed from the cathode to the anode because of the cationic nature of the introduced aminopropyl group, and migration obeyed its hydrophilic nature. The migration times of dimethylphthalate and thiourea ranged from 6.4 to 6.7 min, and 8.7 to 9.2 min, respectively. The separation factor (α) changed in a narrow range of 1.35 to 1.36. Recently Reynolds et al. [14] reported a relative standard deviation (RSD) value of ca. 9% for migration time reproducibility of gravimetrically packed ODS columns. Our RSD data (dimethylphthalate 2.9%, thiourea 3.3%) showed considerable improvement despite addition of the derivatization process. This reproducibility was a bit lower than that generally observed in high-performance liquid chromatography (HPLC). It may be due to variations in the porosity of the fabricated frit and the length of the bed of packed particles.

3.2. Simultaneous introduction of ionic groups to both packing materials and capillary inner wall, an advantage of in-column derivatization

It should be noticed that the in-column derivatization with a silylating reagent having a cationic group can introduce positive charge to both surface of silica gel particles and capillary inner wall yielding rapid EOF flowing to the anode, because both have the silanol groups that can be modified. In contrast, in uncoated capillaries packed with commercial cationic silica gels such as aminopropylated silica gels, the EOF component generating on the capillary inner wall flows from the anode to the cathode because the inner wall has unmodified silanol groups, whereas the EOF component resulting from the surface of modified silica gel particles flow in the opposite direction because the surfaces are protonated due to the introduction of the cationic group. Thus, these EOF components counteract each other.

This big difference was demonstrated by the separation of four PMP derivatives of aldopentoses on 3-aminopropylated columns (Fig. 2).

In CEC using a capillary packed with commercial Develosil NH_2 (Fig. 2c), these analytes moved to the anode, since they have negative charge under the operating conditions, though the velocities were not high because EOF was considered to be only slow toward the anode, as the thiourea peak did not appear within 20 min. The separation of xylose and ribose derivatives was incomplete and the analysis time was as long as ca. 20 min. The peaks at ca. 4 min and 18 min were thought to be due to impurities. However, when a capillary packed with bare silica gels was treated with APTMS to obtain an in-column derivatized column, all pentose derivatives were completely separated from each other and appeared in only ca. 10 min (Fig. 2a). This significant shortening of analysis time is obviously a result of the increased overall velocity toward the anode due to the reversion of the EOF component generating on the surface of capillary inner wall. When an uncoated capillary was aminopropylated with APTMS and the resultant capillary was packed with Develosil NH_2 of the same batch as used in the analysis in Fig. 2c, the analysis time was shorter than in Fig. 2c, though not to such an extent as in Fig. 2a (Fig. 2b). This also provides an evidence that the velocity of EOF generated on the capillary inner wall was changed by this treatment with APTMS. The separation, especially of the peaks of PMP–xylose and PMP–ribose, was somewhat improved. The improvement of separation in Fig. 2a and 2b is presumably due to the difference in the magnitude of silylation and packed state of the gel bed between these differently prepared stationary phases. Regarding the speculation of separation mechanism we must take into account the intramolecular ring formation between the carbonyl group in the PMP portion and the hydroxyl groups at C-2 and C-3 of the pentose moiety. Due to the different magnitude of this unusual intramolecular interaction depending on carbohydrate configuration, the PMP derivatives of aldopentoses were well separated from each other by capillary zone electrophoresis (CZE) in phosphate buffer at pH 7 filled in an uncoated capillary [15]. The migration order (arabinose, xylose, lyxose, ribose) is reverse to the order of negativity. Although the CZE separation was rather worse at pH 6, which was employed in the present CEC analysis, the migration order and accordingly negativity order, was unchanged from those at pH 7. If we think about ion-exchange mechanism in the present analysis, the elution order should be the same as the migration order in the CZE separation. The experimental result (arabinose,

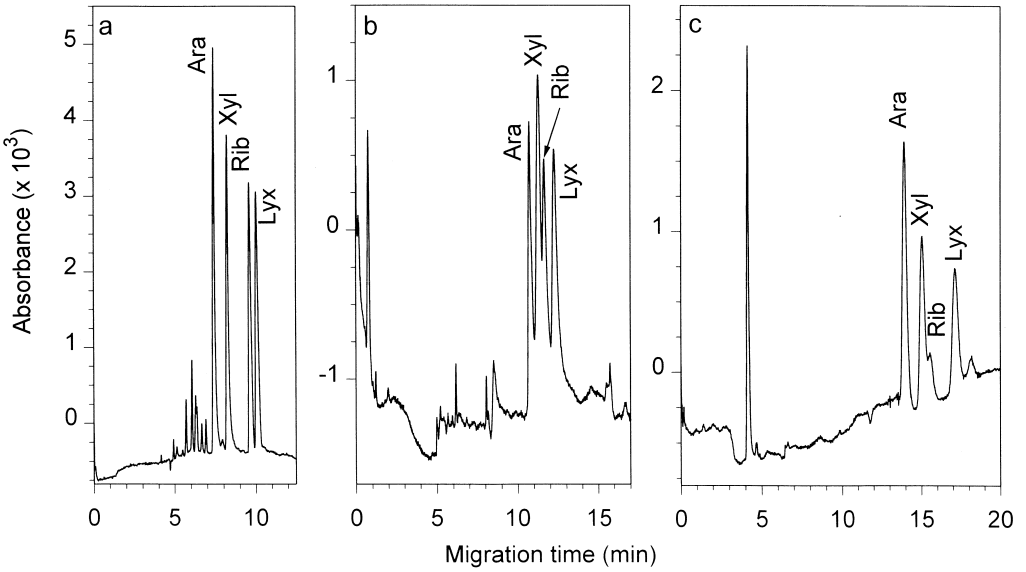

Fig. 2. Comparison of the separation of PMP derivatives of aldopentose isomers on various columns. (a) A column prepared by in-column 3-aminopropylation of Nucleosil silica gel, (b) a column prepared by packing a commercial sample of amino silica, Develosil NH_2, in an APTMS-treated capillary, (c) a column prepared by packing Develosil-NH_2 in an uncoated capillary. Eluent, (25 m*M* HEPES–NaOH, pH 6.0)–acetonitrile (2:1, v/v); sample concentration, 50 nmol in 100 μl of eluent; injection, −2 kV for 3 s (from the cathodic end); applied voltage, −20 kV; detection, UV absorption at 245 nm. Peaks: Ara=D-arabinose, Xyl=D-xylose, Rib=D-ribose, Lyx=D-lyxose, all as PMP derivatives.

xylose, ribose, lyxose) agreed with this inference, except for the reversion of the ribose/lyxose elution order. Based on these observations the major mechanism of the separation of PMP–aldopentoses is considered to be the ion exchange of these analytes on the protonated aminopropyl function on the surface of the stationary phase. Minor contribution of hydrophilic interaction should also be taken into account to understand the reversion of the elution order of the PMP–xylose/PMP–ribose.

3.3. A new type of hydrophobic column capable of generating rapid EOF

A capillary packed with 30-nm pore, 3 μm DaisoGel was treated with dimethyloctadecyltrimethoxysilylpropylammonium chloride. The resultant gels were hydrophobic by the presence of the octadecyl groups and could generate rapid EOF flowing to the anode over a wide pH range due to the presence of the quaternary ammonium group. The obtained column (ODN^+ column) was evaluated for the separation of homologues of alkylbenzoates (Fig. 3a).

The obtained theoretical plate numbers of benzoate homologues were as high as 49 000 to 57 000. This separation was compared to that by an ODS column prepared also by the authors by the in-column technique using ODTMS (Fig. 3b). Since EOF was too slow with the Tris–HCl (pH 7.0)–acetonitrile eluent, sodium dodecyl sulfate (SDS) was added to the minimum concentration in order to make it faster, based on a report of Seifar et al. [16] that the dodecyl groups in SDS were strongly adsorbed on ODS and the sulfate groups were exposed to the mobile phase resulting in the increase of negative charge on the particle surface. The theoretical plate numbers of the benzote homologues on the ODS column under these conditions ranged from 16 000 to 27 000. Though peak shape and resolution varied dependent on eluent and injected sample amount, the separation on the ODN^+ column was better than that on the ODS column.

Fig. 4 shows another application of the ODN^+

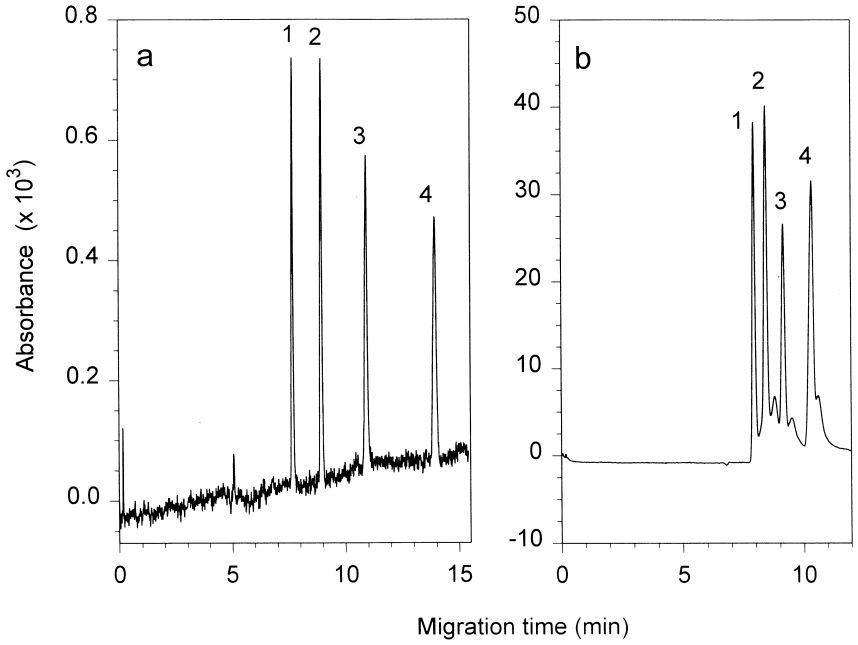

Fig. 3. Comparison of the separation of alkylbenzoate homologues on an ODN$^+$ column (a) and an ODS column (b). The columns were prepared by in-column derivatization of DaisoGel silica (30 nm, 3 μm) with dimethyloctadecyltrimethoxysilylpropylammonium chloride (a) or ODTMS (b) in ethanol. Eluent, (a) 10 m*M* Tris–HCl (pH 7.0)–acetonitrile (55:45, v/v), (b) 2.5 m*M* phosphate buffer containing 5 m*M* SDS (pH 7.0)–acetonitrile (6:4, v/v); injection, (a) −2 kV, for 3 s from the cathodic end, (b) 2 kV for 3 s from the anodic end; applied voltage, −23 kV (a) or 23 kV (b); detection, UV absorption at 254 nm. Peaks: 1=methyl benzoate, 2=ethyl benzoate, 3=*n*-propyl benzoate, 4=*n*-butyl benzoate.

column, i.e., separation of PMP derivatives of some monosaccharides.

In a previous paper [17] we reported that in CEC of these compounds on a Hypersil ODS column they were completely resolved but quantitative analysis was hampered by heavy tailing. The tailing was considered to be due to intermolecular hydrogen bonding between the silanol function on ODS and the keto groups in PMP derivatives. Though the separation of PMP derivatives of monosaccharides on a 30-nm pore DaisoGel ODN$^+$ column was not sufficient, resolution between neighboring peaks increased by 0.5–1 on a 10-nm pore Nucleosil ODN$^+$ column, as shown in Fig. 4. In this separation 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer mixed with acetonitrile was used as eluent. In our experiment for other purpose a concave line was observed for EOF against eluent pH (data not shown), which suggests weak adsorption of HEPES to ODS particles. The poor resolution of the PMP derivatives of these monosaccharides might be further improved by selection of more suitable eluent with a relevant pH.

3.4. An attempt to prepare fritless columns

One of the most serious problems encountered in CEC, even with the in-column derivatization technique, is bubble formation in the column bed, which occurs most often near the heat-fabricated frits [18]. To overcome this problem we attempted to prepare a fritless column by cross-linking the silanol groups between silica gel particles and also between the particles and capillary inner wall, using a silylating reagent having bifunctional groups. The result indicated that the use of bis[3-(trimethoxysilyl)propyl]ethylenediamine as a silylating reagent was promising. The gel bed was immobilized and there was no leakage of packed gels even at a high pressure of 200 kg/cm^2. Fig. 5 shows an example of

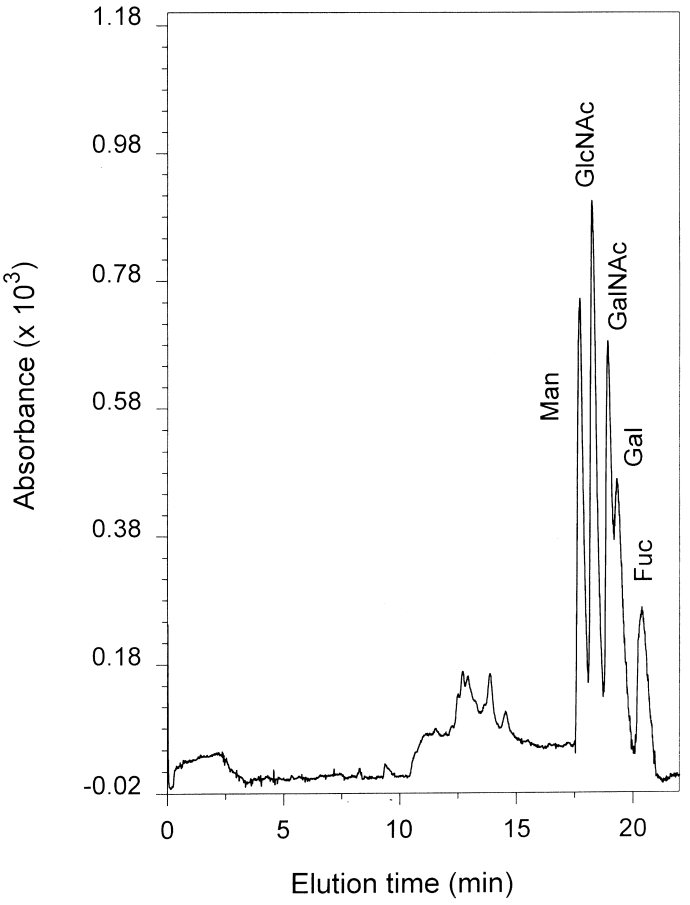

Fig. 4. Separation of the monosaccharides commonly found in glycoproteins as their PMP derivatives, on an in-column prepared ODN^+ column. The column was prepared by in-column derivatization of Nucleosil silica (10 nm, 3 μm) with dimethyloctadecyltrimethoxysilylpropylammonium chloride. Eluent, (100 m*M* HEPES–NaOH, pH 6.5)–acetonitrile (1:2, v/v); sample concentration, 50 nmol in 100 μl of eluent; injection, −2 kV for 3 s (from the cathodic end); applied voltage, −15 kV; detection, UV absorption at 245 nm. Peaks: Man=D-mannose, GlcNAc=*N*-acetyl-D-glucosamine, GalNAc=*N*-acetyl-D-galactosamine, Gal=D-galactose, Fuc=L-fucose, all as PMP derivatives.

a separation using a thus-prepared column packed with 3-μm Nucleosil, 10-nm pore size.

Since dimethylphthalate eluted near the void volume, the separation on this fritless column is considered to be based on hydrophilic interaction mode. The separation of dimethylphthalate and thiourea was rather good as compared to that on a column with frits (Fig. 1). The minor peaks are considered to be due to the impurities in the solutes, because they did not disappear on repeated analyses. After applying a high pressure at 200 kg/cm^2 for 1 h, the durability of the column was examined. The result indicated that there was no change of elution profile after 20 repetitions.

4. Conclusion

In this paper a survey of in-column derivatization is presented, and a few ideas are proposed for new types of CEC columns. It also reports a preliminary result for omission of column filters. All the results suggest the possibility of in-column derivatization to be widely used for CEC. Establishment and further

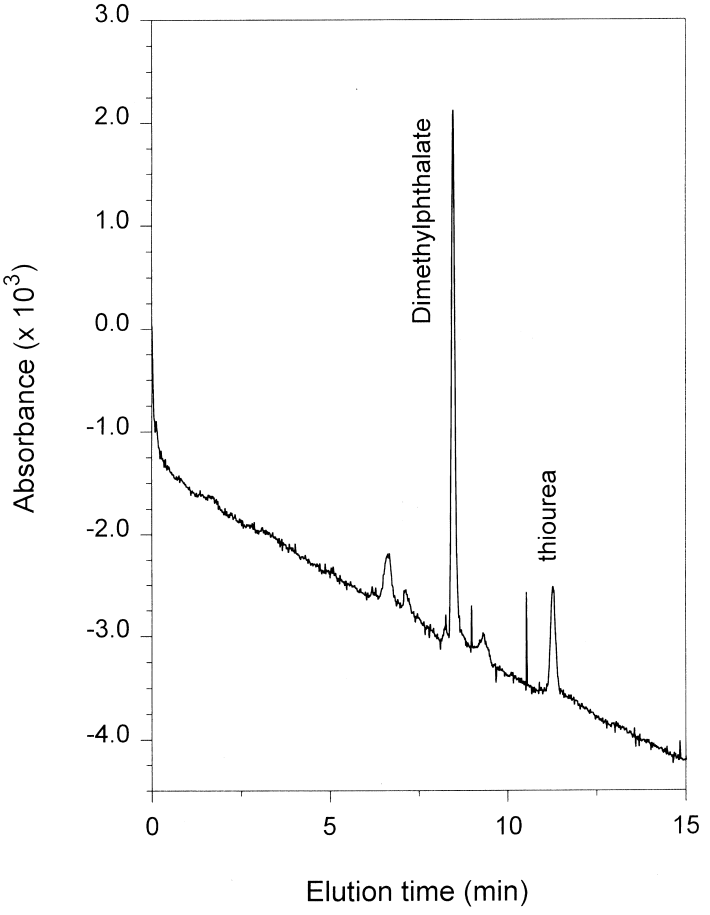

Fig. 5. Separation of dimethylphthalate and thiourea on a fritless column tentatively prepared by immobilization of the column bed by a cross-linking technique. Stationary phase, Nucleosil silica (10 nm, 3 μm); immobilization, treatment with bis[3-(trimethoxysilyl)propyl]ethylenediamine; eluent, 10 m*M* Tris–HCl, pH 6.0–acetonitrile (1:1, v/v); sample concentration, 0.05% each; injection −2 kV for 3 s (from the cathodic end); cartridge temperature, 20°C; applied voltage, −12 kV; detection, UV absorption at 254 nm.

extension of this technique will make CEC much more handy and reliable. Programmed derivatization using various reagent kits will provide any desired columns in short periods. If bare silica gel-packed columns of the same quality are constantly supplied as a starting material, we will be able to obtain other types of columns by simple and routine procedures of in-column derivatization, just prior to analysis. However, a number of problems must be solved, for example possible swelling and shrinkage of column bed during derivatization (though not observed in the present work), the effect of heterogeneous pore shape and size, durability of columns, etc., before we acquire perfect reliability of this technique. Studies on such fundamental problems are now going on in our laboratory.

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