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Short Communication

Reversed-phase chromatography of small molecules and peptides on a continuous rod of macroporous poly(styrene–co-divinylbenzene)

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Abstract

A continuous "molded" rod of porous poly(styrene–co-divinylbenzene) prepared by a bulk free radical polymerization within the confines of a chromatographic column has been used successfully for the reversed-phase HPLC of alkylbenzenes and peptides. An excellent rapid separation of bradykinin and [D-Phe^7]-bradykinin with a molecular mass of about 1000 was also achieved.

1. Introduction

Chromatographers are keenly aware of the inherent drawback of particulate separation media: the inability to completely fill the space within the chromatographic column. In theory, no more than *ca.* 74% of the space within a column can be occupied by a perfect array of monodisperse beads. Therefore, several attempts have been made to decrease the discontinuity of the column packings. For example, columns packed with a bundle of aligned porous silica hollow fibers can theoretically have an interstitial porosity of only 9% [1]. Macroporous membranes [2,3] and stacked [4] or rolled [5] cellulose sheets placed in a cartridge that simulates the function of a column, exhibit little or no interstitial porosity.

Yet another approach is to fill the column completely with the separation medium. For example, foams consisting of open-pore silica or polyurethane foams have been tested as normal phases but they did not provide sufficient chromatographic properties [6,7]. Hjertén and coworkers [8–10] developed a process for the preparation of swollen polyacrylamide gels that were compressed in the shape of a column and used successfully for the HPLC separation of proteins and peptides. Almost simultaneously, we have introduced a new HPLC medium consisting of a continuous "molded" rod of rigid macroporous polymer, prepared by a free-radical polymerization directly within the confines of a column, which does not have interstitial porosity and is essentially a single cylindrical particle [11,12]. This novel concept of molded continuous media was first verified with modified porous poly(glycidyl methacrylate–co-ethylene dimethacrylate) [11] and then with poly(styrene–co-divinylbenzene) rods [12]. These proved to be very efficient for the extremely fast reversed-phase HPLC separation of proteins. Subsequently,

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Matsui *et al.* [13] used our approach for the preparation of continuous rods of molecularly imprinted polymers and showed their capabilities for molecular recognition in a series of separations of positional isomers and enantiomers.

The absence of interparticular volume in the continuous column forces *all* of the mobile phase to flow *through* the separation medium rather than around it. According to theory [14], the mass transport of the molecules is enhanced by convection, a process that has a positive effect on the separation. A similar concept was used in perfusion chromatography [15–17] and in catalysis [18].

While the macroporous polymeric rods show excellent performance in the fast reversed-phase chromatography of proteins, their chromatographic properties for the separation of small and mid-size molecules have not yet been reported. This communication describes the first use of the poly(styrene–co-divinylbenzene) continuous rod columns for the reversed-phase separation of alkylbenzenes and peptides.

2. Experimental

2.1. Materials

The styrene (99%, Aldrich) and divinylbenzene (80% divinyl monomer, Dow Chemical) were extracted with 10% aqueous sodium hydroxide and water, dried over anhydrous magnesium sulfate, and distilled under vacuum. Azobisisobutyronitrile (AIBN) was obtained from Kodak, dodecyl alcohol and the substituted aromatic hydrocarbons from Aldrich. Bradykinin and [D-Phe^7]-bradykinin were purchased from Sigma. All solvents were HPLC grade.

2.2. Preparation of the continuous column

AIBN (1%) was dissolved in two volumes of a mixture of styrene–divinylbenzene (50:50), followed by the addition of eight volumes of dodecyl alcohol–toluene (70:30) mixture (porogenic solvent) to the monomers. After being purged with nitrogen for 15 min, a stainless-steel tube (50 × 8 mm I.D. or 100 × 8 mm I.D.) was filled with the above mixture and then sealed with rubber nut plugs. The polymerization was allowed to proceed at 70°C for 24 h. The rubber plugs were replaced by the column end fittings and the column was attached to the HPLC system. Tetrahydrofuran (100 ml) was pumped through the column at a flow-rate of 1 ml/min to remove the dodecyl alcohol, toluene and other soluble compounds present in the polymer rod after the polymerization was completed.

2.3. Characterization of pore properties

The specific surface area of the macroporous polymer rod was calculated from the BET isotherms of nitrogen; the pore size distribution in the dry state was determined by mercury porosimetry using an automated custom-made combined BET-Sorptometer and mercury porosimeter from Porous Materials, Ithaca, NY, USA.

2.4. Chromatography

Chromatography was carried out using a Nicolet LC 9560 ternary gradient liquid chromatograph equipped with a Hewlett-Packard 1050 UV detector. The samples were injected through a Rheodyne 7125 valve loop injector, and monitored at 254 nm (aromatic hydrocarbons) and at 218 nm (peptides). The column was thermostated at 30°C. The reversed-phase separations were performed either in an isocratic mode with the water–acetonitrile mobile phase or in a solvent gradient from 40 to 85% (v/v) acetonitrile in water. The retention factors k' and column efficiencies were calculated according to standard equations. The protein retention, defined as capacity factor k', was drawn against composition of the aqueous mobile phase containing different amounts of acetonitrile. The results obeyed the equation

$$\log k' = \log k_w - \varphi S \quad (1)$$

where k_w refers to the value of k' for water as a mobile phase, φ is the volume fraction of ace-

tonitrile in the mobile phase and S is a constant for a given compound and organic solvent [19]. The column efficiency (plate height) was estimated with bradykinin in aqueous acetonitrile and with benzene in tetrahydrofuran.

3. Results and discussion

3.1. Porous properties of the continuous rod column

The use of particulate polymer-based separation media for HPLC has grown steadily over the last decade [20,21]. The most popular packing beads, based on poly(styrene–co-divinylbenzene), are rather non-polar and are well suited for reversed-phase chromatography [22–26] of both low- and high-molecular-mass compounds such as hydrocarbons or proteins. Therefore, it was anticipated that a continuous rod prepared from an identical copolymer to that of classical HPLC beads would behave in a similar manner.

Molded rod columns were prepared by the polymerization of a mixture containing dodecanol and toluene (70:30) as the porogenic solvents. The average pore size diameter of canals (megapores) in this rod is 19.5 μm according to the mercury intrusion porosimetry. The total pore volume is 5.48 ml/g and represents a porosity of 84.5%. The volume within pores smaller than 100 nm only represents about 10% of the total pore volume and provides for a specific surface area of 23 m^2/g.

3.2. Separation of alkylbenzenes

Fig. 1 shows the effect of the flow-rate on the efficiency of the poly(styrene–co-divinylbenzene) rod for benzene in tetrahydrofuran (non-retentive conditions). The column exhibits about 13 500 plates/m at a low flow velocity and the efficiency does not decrease very much with increasing flow-rate; it still accounts for remarkable *ca.* 4200 plates/m at a flow-rate of 23 ml/min. It should be emphasized that the back pressure does not exceed 6.5 MPa even at this very high flow-rate and therefore the use of high-pressure pumps is not necessary.

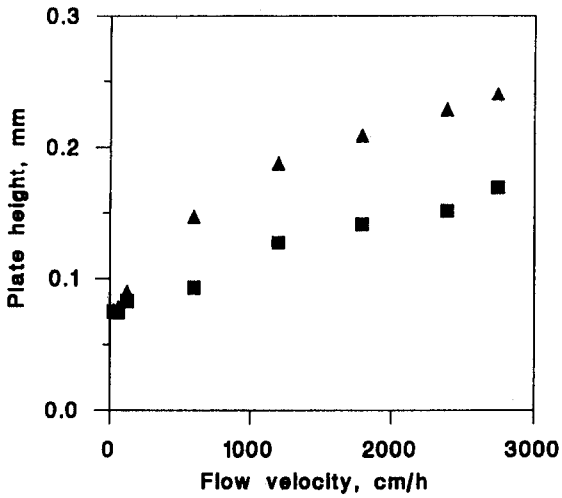

Fig. 1. Effect of flow velocity on efficiency of the continuous poly(styrene–co-divinylbenzene) rod column for benzene (▲) and bradykinin (■). Conditions: column 50 × 8 mm I.D., mobile phase tetrahydrofuran for benzene and acetonitrile–water (50:50, v/v) for bradykinin, temperature 30°C.

The size of the open space between beads packed in a column is typically about half of the particle diameter. The rod column with an average pore size of 19.5 μm should behave as a column packed with 40-μm beads. A simple calculation [27] reveals that a packed column of the same size as the size of the rod column would have a pressure drop of only 0.04 MPa. This is much lower than the actual back pressure of the rod that is 6.5 MPa. However, a column packed with 40-μm beads would have an efficiency of about 8 000 plates/m at a linear velocity of 1 cm/s [27] while the rod exhibits 13 000 plates/m, or almost twice as much. The reason for this discrepancy may be seen in the very different morphologies of the packed column and the continuous porous rods.

Because the poly(styrene–co-divinylbenzene) material of the rods and the standard beads typically used for reversed-phase chromatography of alkylbenzenes are essentially the same, the interactions between the surface chemistry within the rod and the solutes under the reversed-phase conditions should not differ from each

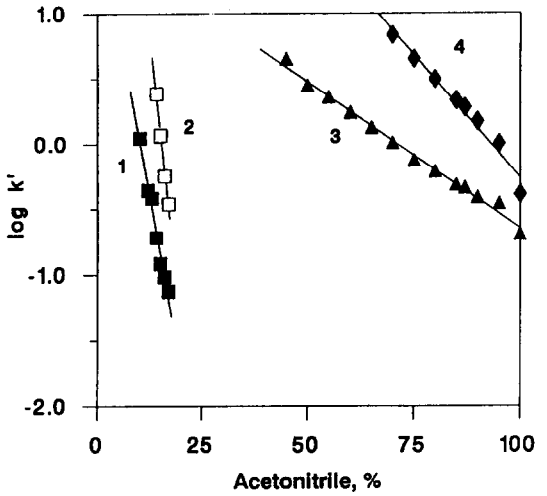

Fig. 2. Effect of the mobile phase composition on retention factors k' of bradykinin (1), [D-Phe^7]-bradykinin (2), benzene (3) and amylbenzene (4). Conditions: column 50 × 8 mm I.D., flow-rate 1 ml/min.

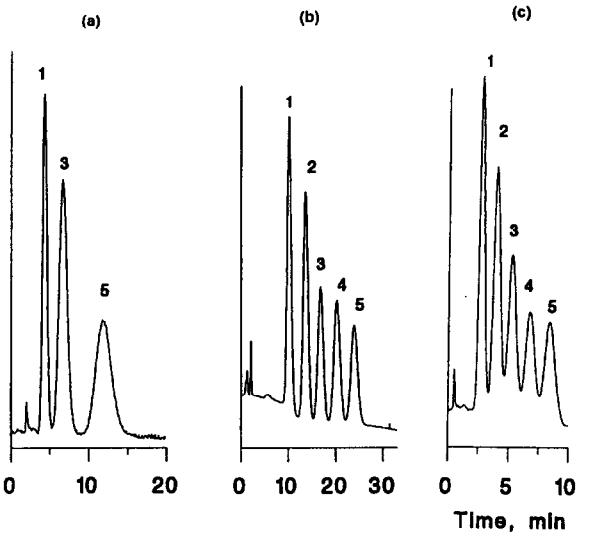

Fig. 3. Isocratic and gradient separation of alkylbenzenes by reversed-phase chromatography on continuous poly(styrene–co-divinylbenzene) rod columns. Conditions: (a) column 50 × 8 mm I.D., mobile phase acetonitrile–water (70:30), flow-rate 1 ml/min; (b) column 100 × 8 mm I.D., mobile phase linear gradient from 40 to 85% acetonitrile in water within 30 min, flow-rate 2 ml/min; (c) column 100 × 8 mm I.D., mobile phase linear gradient from 40 to 85% acetonitrile in water within 15 min, flow-rate 8 ml/min. Peaks: 1 = benzene; 2 = toluene; 3 = ethylbenzene; 4 = propylbenzene; 5 = butylbenzene.

other. The retention factor decreases with increasing concentration of acetonitrile in the mobile phase (Fig. 2) and, as expected, it is steeper ($S = -3.77$) for more non-polar amylbenzene than for benzene ($S = -2.24$). The slope of the logarithm of retention factor *vs.* number of carbon atoms in the alkyl of the alkylbenzenes dependency is 0.149 and the average separation factor of alkylbenzene homologues $\alpha(CH_2) = 1.42$. These data are comparable to those published in the literature for polystyrene-based beads [23,28]. Fig. 3a shows an isocratic separation of three alkylbenzenes at a flow-rate of 1 ml/min. A better separation can be achieved if the mobile phase contains more water; however, the retention times are longer. In order to carry out a faster separation, a gradient of the mobile phase was used to afford baseline separation of benzene and four alkylbenzenes within 25 min (Fig. 3b) and, using a steeper mobile phase gradient, within 8 min only (Fig. 3c).

3.3. Separation of peptides

Peptides are polyamino acids that have relatively low molecular masses while their chemical structures are similar to those of proteins. A number of peptides that are very important drugs such as insulin or somatostatin are produced by biotechnology processes. Chromatography is a very important part of both in-process control and downstream processing. Therefore, it was useful to check the suitability of the novel separation medium for the separation of peptides.

We used bradykinin (Arg–Pro–Pro–Gly–Phe–Ser–*Pro*–Phe–Arg) and [D-Phe^7]-bradykinin (Arg–Pro–Pro–Gly–Phe–Ser–*D-Phe*–Phe–Arg) that differ only in the 7th amino acid residue (L-proline and D-phenylalanine, respectively) as model peptides to demonstrate the separation ability of the rod. Fig. 1 shows the very good efficiency of the rod column for peptides with a molecular mass of about 1000 as determined with bradykinin in 50% aqueous acetonitrile. This efficiency is even higher than that measured for

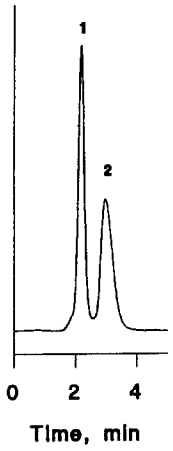

Fig. 4. Fast separation of bradykinin (1) and [D-Phe^7]-bradykinin (2) by reversed-phase chromatography on continuous poly(styrene–co-divinylbenzene) rod column. Conditions: column 50 × 8 mm I.D., mobile phase 16% acetonitrile in water, flow-rate 1 ml/min.

benzene. For example, at a flow-rate of 10 ml/min, the efficiency for bradykinin is 7900 plates/m while for benzene it is only 5300 plates/m. Moreover, the efficiency does not change very much when the flow-rate increases confirming the fast mass transfer that is promoted by the convection of all of the mobile phase through the separation medium. The very steep decrease of retention factors for bradykinin ($S = -17.30$) and for [D-Phe^7]-bradykinin ($S = -28.52$) within a narrow range of changes of the mobile phase composition, as well as the significant difference in retention factors at a particular composition (Fig. 2), confirm that even the isocratic mode can result in a good separation of the two peptides. Indeed, Fig. 4 shows the separation of the peptides achieved within only 3 min. This result is very encouraging because a gradient separation of bradykinin and [D-Phe^7]-bradykinin in a column packed with a beaded styrenic resin took 40 min [29].

4. Conclusions

The separations achieved with a poly(styrene–co-divinylbenzene) continuous rod column confirmed the suitability of the new medium even for the chromatography of small hydrophobic molecules and peptides. The separation of peptides compares favorably to that achieved with polystyrene beads. Further developments with rod columns, currently under investigation, are aimed at the preparation of optimized rods containing a larger volume of mesopores that should contribute positively to an increase in the rod column efficiency and help improve further the separation of small molecules.

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