Journal of Chromatography, 363 (1986) 217–222
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 718

HIGH-PERFORMANCE AQUEOUS SIZE-EXCLUSION CHROMATOGRAPHY WITH DIOL-BONDED POROUS GLASS PACKING MATERIALS

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(Received April 10th, 1986)

SUMMARY

Diol-bonded porous glass packing materials with average particle diameters of 5 μm and pore sizes of 170 Å and 500 Å were packed into a 25 cm × 7.2 mm I.D. column by a high-viscosity slurry-packing procedure. The number of theoretical plates (N) was 13 000 per column. The slurry solvent was methanol–ethylene glycol (70:30, v/v) and the packing solvent was *n*-heptane. The reason for using a viscous slurry solvent (2.8 cSt at 20°C) was explained by using the equation for the Reynolds number. The viscosity of the slurry solvent should be increased in proportion to the column diameter when porous glass materials are packed in a wider bore column. The value of N increased with decreasing injection volume and the optimum injection volume was 25 μl. The value of N also increased with increasing mobile phase velocity from 0.3 ml/min and the maximum was obtained around 1–2 mm/s (N = 14 500 per column and height equivalent to a theoretical plate = 17.2 μm). Chromatograms and calibration graphs for polyethylene oxides, pullulans and proteins were obtained.

INTRODUCTION

Columns packed with hydrophilic polymer gels and diol-bonded silica gels are currently used for high-performance aqueous size-exclusion chromatography (HPASEC). Porous glass packing materials and other organic materials can also be considered for HPASEC. Porous glass is rigid, mechanically stable and easy for controlling pore size, with a distribution that is relatively monodisperse. However, particle sizes available commercially are between 35 and 75 μm and are inadequate for HPASEC.

Recently, porous glass with an average particle diameter of 5 or 10 μm and a relatively narrow particle size distribution has been developed, although it is still at the stage of trial manufacture. This paper is concerned with this packing material. The packing procedure, determination of the operational variables and the construction of calibration graphs are discussed.

0021-9673/86/$03.50

EXPERIMENTAL

Packing materials and packing procedure

The porous glass materials used in this experiment were FPG 170 and 500 (Fuji Photo Film), which have average pore diameters of 170 Å and 500 Å, respectively. These materials are irregularly shaped particles obtained by crushing the original larger materials. The average particle diameter of the porous glass in this experiment was 5 μm ($\pm$ 2 μm).The surface of the porous glass was treated with γ-glycidyloxypropyltrimethoxysilane to form glyceropropyl groups on the surface[1].

Column packing was performed on a Chemco high-pressure slurry-packing apparatus with an air-driven pneumatic amplifier pump, Model 124PP + 124 A (Chemco, Osaka, Japan). The column dimensions were 25 cm × 7.2 mm. The slurry reservoir (packer) was an empty tube (40 cm × 16 mm I.D.) with an inner volume of 80 ml. Between the packer and the column blank, an extension tube the same size as the column blank was attached.

The high-viscosity slurry solvent was methanol–ethylene glycol (70:30, v/v), which has a viscosity of 2.8 cSt at 20°C. An 8-g portion of porous glass was slurried in 70 ml of the slurry solvent. This slurry was sonicated with an ultrasonic cleaner at 26 kHz (Kokusai Denki) for 5 min. A 25-ml aliquot of carbon tetrachloride and the sonicated slurry were poured into the top of the column–reservoir assembly, in that order. The remainder of the reservoir was then filled with *n*-heptane.

The packing solvent was *n*-heptane. The packing pressure was increased gradually so as to maintain the packing flow-rate between 15 and 20 ml/min, and was finally increased to 500 kg/cm^2. Porous glass was packed in 5 min under a constant flow. After about 80 ml of *n*-heptane had passed through the column–reservoir assembly and at the same time the packing flow-rate had been increased rapidly, the packing pressure was regulated so as to maintain a flow-rate of 30 ml/min. After a further 200 ml of *n*-heptane had flowed out of the column, pumping was stopped and the slurry reservoir was disconected from the extension tube. Equilibration of the columns was performed by pumping chloroform for 5 min, methanol for 5 min and water for 30 min in succession, each at 3.0 ml/min. Finally, the column was disconnected from the extension tube.

HPASEC

SEC measurements were carried out on a Jasco TRIROTAR-V high-performance liquid chromatograph (Japan Spectroscopic Co., Tokyo, Japan). A UVIDEC-100 ultraviolet absorption detector (Jasco) and an SE-11 differential refractometer (Showa Denko, Tokyo, Japan) were used. Sample injection was effected with a Model VL-611 variable lop injector.

The number of theoretical plates (N) was determined by injecting 25 μl of a 1% ethylene glycol solution at a flow-rate of 0.5 ml/min. The mobile phase was water. The value of N was calculated by measuring the width of the peak at half-height. The operational variables were also measured by injecting a 1% ethylene glycol solution on a column of FPG 170.

Measurements of SEC chromatograms and the construction of calibration graphs for polyethylene oxides (Toyo Soda, Tokyo, Japan), pullulans (Showa Denko) and polyethylene glycols were made on a set of columns of FPG 170 and FPG 500.

The flow-rate of the mobile phase (water) was 1.0 ml/min. Chromatograms of proteins were measured by using 0.1 *M* phosphate buffer at pH 7.0 including 0.2 *M* NaCl as the mobile phase.

RESULTS AND DISCUSSION

Preparation of high-performance columns

A high-viscosity slurry-packing procedure was applied. The viscosity of the slurry solvent and the packing speed (i.e., slurry velocity in a column) were the most important factors. A viscous solvent was used in order to prevent sedimentation of particles during packing, but an excessive packing time caused particle segregation during packing and a higher speed of packing resulted in a heterogeneous bed in the column. The optimum packing procedure described under Experimental gave N = 13 000 plates per column (25 cm).

Several packing procedures using viscous slurry solvents for about 4 mm I.D. columns have been reported (*e.g.*, ref. 2). Satisfactory packing results were obtained by these methods when porous glass materials were packed into 4.6 mm I.D. columns. However, the same packing conditions (same slurry solvent and slurry linear velocity in a column) did not afford good results with 7.2 mm I.D. columns. Increases or decreases in the packing speed had insignificant effects. Dense and sparse regions near the wall and at the centre of the column were observed by visual inspection of the inside of the column.

Disordering of the column bed by turbulent flow during packing can be considered to be one of the causes of insignificant effects on column performance. The Reynolds number, *Re*, predicts the transition from laminar flow to turbulent flow:

$$Re = DU\rho/\mu$$

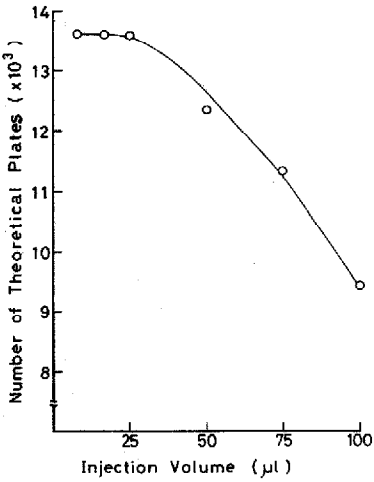
where D is the inner diameter of the tube, U the linear velocity, ρ the density of the fluid and μ the fluid viscosity coefficient.

Laminar flow changes to turbulent flow above a certain value of *Re*. The linear velocity of a slurry in a 7.2 mm I.D. column during packing should be similar to that in a 4.6 mm I.D. column which gives satisfactory packing results and, therefore, the value of *Re* of a fluid in a 7.2 mm I.D. column should be the same as that in a 4.6 mm I.D. column. This means that the viscosity of a slurry for packing of a 7.2 mm I.D. column is 1.5 times that of a slurry for a 4.6 mm I.D. column.

Intermixing of the slurry with the packing solvent in the slurry reservoir or in the column should be considered as a second reason[3] of insignificant effects. Therefore, *n*-heptane was used as the packing solvent in this experiment to prevent mixing of the slurry with packing solvent. Detailed preliminary experiments have been reported elsewhere[4].

Operational variables

The effect of the injection volume on N is shown in Fig. 1. The value of N remained unchanged up to an injection volume of 25 μl (8–25 μl) and subsequently decreased with increasing injection volume, although the values of N were still acceptable.



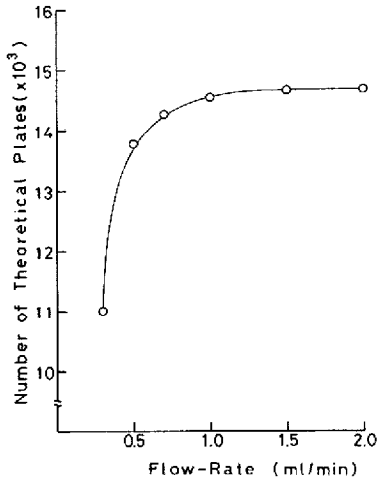


Fig. 1. Effect of injection volume on the number of theoretical plates. Column, FPG 170 (25 cm); sample, 1% ethylene glycol; flow-rate, 0.5 ml/min; mobile phase, water; number of theoretical plates, calculated at half-height for a 25 cm column.

Fig. 2. Effect of flow-rate on theoretical plates. Column, FPG 170 (25 cm); sample, 1% ethylene glycol, 25 μl; mobile phase, water; number of theoretical plates, calculated at half-height for a 25 cm column.

The effect of mobile phase velocity expressed as flow-rate on N is shown in Fig. 2. The value of N increased with increasing flow-rate up to 1.0 ml/min and remained steady between 1.0 ml/min and 2.0 ml/min, which was the highest flow-rate studied. The linear velocity of the mobile phase at a flow-rate of 1-0 ml/min is 0.96

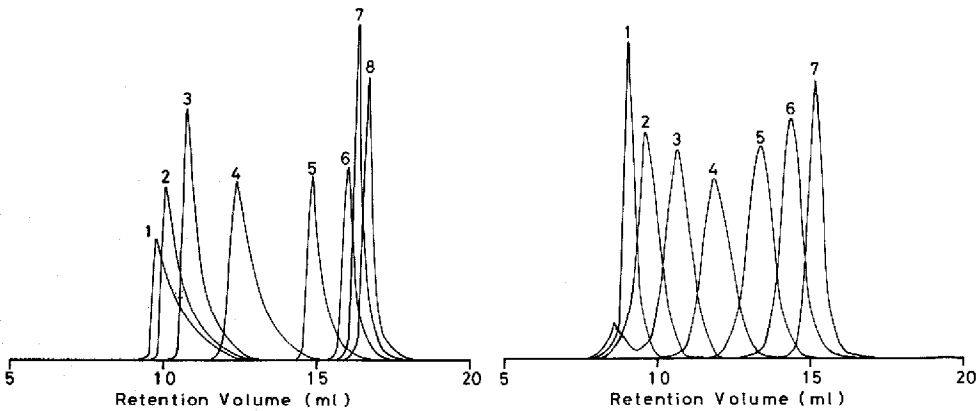


Fig. 3. Combined chromatograms of polyethylene oxides. Column: FPG 170 (25 cm) + FPG 500 (25 cm). Samples: 1–4, 0.2% polyethylene oxide, refractive index (RI) × 4; 5–7, 0.5% polyethylene glycol, RI × 16; 8, 1.0% ethylene glycol, RI × 32. Injection volume, 25 μl; detector, RI; flow-rate, 1.0 ml/min; mobile phase, water. Molecular weight: 1 = $2.8 \cdot 10^5$; 2 = $1.5 \cdot 10^5$; 3 = $7.3 \cdot 10^4$; 4 = $2.5 \cdot 10^4$; 5 = 5000; 6 = 1000; 7 = 300; 8 = 62.

Fig. 4. Combined chromatograms of pullulans. Column, FPG 170 (25 cm) + FPG 500 (25 cm); sample, 0.2%, 25 μl; detector, RI (× 4); flow-rate, 1.0 ml/min; mobile phase, water. Molecular weight: 1 = $3.8 \cdot 10^5$; 2 = $1.8 \cdot 10^5$; 3 = $1.0 \cdot 10^5$; 4 = $4.8 \cdot 10^4$; 5 = $2.37 \cdot 10^4$; 6 = $1.22 \cdot 10^4$; 7 = $5.8 \cdot 10^3$.

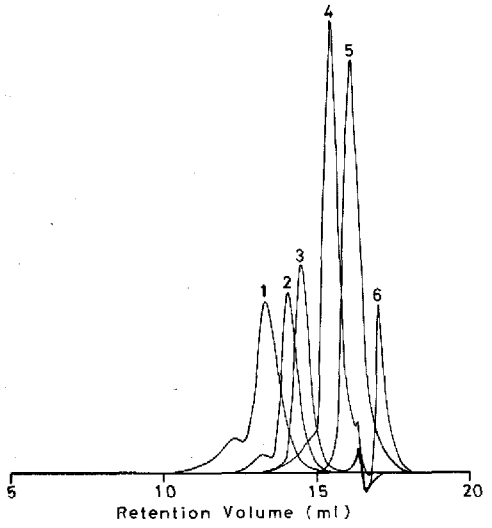


Fig. 5. Combined chromatograms of proteins. Column, FPG 170 (25 cm) + FPG 500 (25 cm); sample, 0.1%, 25 μl; detector, UV at 280 nm (×0.08); flow-rate, 1.0 ml/min; mobile phase, 0.1 *M* phosphate buffer (pH 7.0) + 0.2 *M* NaCl. Samples: 1 = human serum γ-globulin; 2 = bovine serum albumin; 3 = egg albumin; 4 = myoglobin; 5 = cytochrome *c*; 6 = D,L-phenylalanine.

mm/s, which was calculated by dividing the flow-rate by the effective cross-sectional area of a 7.2 mm I.D. column (0.174 cm^2), which was obtained as the product of the cross-sectional area of the column and the ratio of the interstitial volume, V_0 (= 4.35 ml), in the column and the column volume, V_t (= 10.2 ml). The value of V_0 was assumed to be the retention volume of pullulan, having a molecular weight of about $1 \cdot 10^6$, substracted from the dead volume of the SEC system.

Our result of a maximum value of N of 14 500 obtained at *ca.* 1–2 mm/s were similar to the results obtained on a 7.8 mm I.D. column of silica gel by Kirkland[5] and on an 8 mm I.D. column of polystyrene gel by Mori and Suziki[6]. A value of N of 14 500 corresponds to a height equivalent to a theoretical plate (HETP) of 17.2 μm.

Chromatograms and calibration graphs for various samples

Chromatograms of polystyrene oxides, pullulans and proteins are shown in Fig. 3, 4 and 5, respectively. Samples were measured separately and combined. Although the chromatograms of polyethylene oxides and some of polyethylene glycols showed tailing, those of pullulans and proteins had symmetrical peaks. The tailing peaks of polyethylene oxides probably resulted from hydrophobic interactions with both hydrophobic sites of gels and samples, because polyethylene oxides are less polar than pullulans and proteins.

Calibration graphs of molecular weight *vs.* retention volume for these polymers are shown in Fig. 6. These calibration graphs show that non-ionic polymers such as polyethylene oxides and pullulans up to a molecular weight of $1 \cdot 10^6$ can be separated by using this column system, which can also be applied for the determination of the molecular weight of proteins. The points for proteins such as human serum γ-glo-

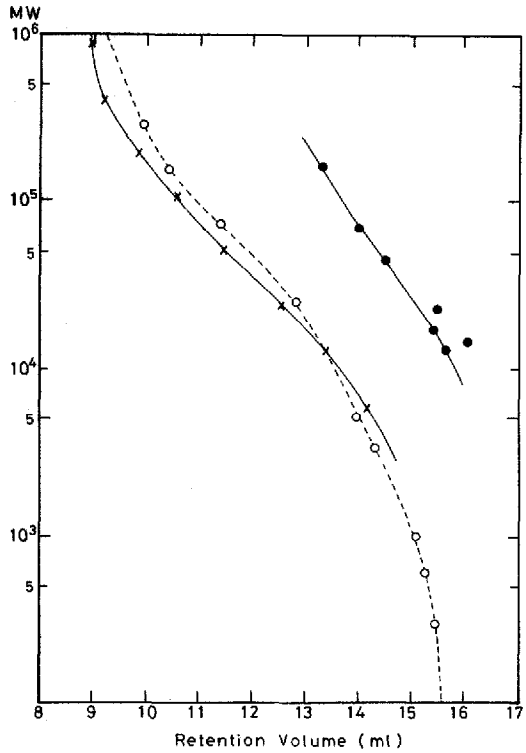


Fig. 6. Calibration graphs for polyethylene oxides, pullulans and proteins. Column: FPG 170 (25 cm) + FPG 500 (25 cm). (○) Polyethylene oxides and polyethylene glycols in water; (×) pullulans in water; (●) proteins in 0.1 *M* phosphate buffer (pH 7.0) + 0.2 *M* sodium chloride. MW = Molecular weight.

bulin, bovine serum albumin, egg albumin, myoglobin and cytochrome *c* fell on the line, but two points, for trypsin and lysozyme (egg white), deviated from the line. These results will be discussed elsewhere.

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