Journal of Chromatography, 149 (1978) 489–500

CHROM. 10,898

GEL PERMEATION CHROMATOGRAPHY ON UNMODIFIED SILICA USING AQUEOUS SOLVENTS

F. A. BUYTENHUYS* and F. P. B. VAN DER MAEDEN

Akzo Research Laboratories Arnhem, Corporate Research Department, P.O. Box 60, Arnhem (The Netherlands)

SUMMARY

Chemically unmodified, totally porous, spherical silica particles (*e.g.*, LiChrospher from Merck, Darmstadt, G.F.R.) were found to be very suitable for the rapid gel permeation (size-exclusion) chromatography of water-soluble polymers. These materials permit the easy preparation of high efficiency columns. With 10-μm LiChrospher particles, plate numbers >30,000 per metre were obtained for glucose as a totally permeating solute and water as the mobile phase on 30 × 0.46 cm columns at a flow-rate of 0.5 ml/min.

The apparent molecular weight of polyelectrolytes depends on the concentration of the polyelectrolyte and the ionic strength of the mobile phase because of polyelectrolyte expansion and ion-exclusion effects.

Adsorption on the silica surface of non-ionic and cationic polymers can be prevented by using a tetramethylammonium salt as a physical modifier.

INTRODUCTION

Gel permeation chromatography (GPC) or size-exclusion chromatography (SEC) with apolar solvents is traditionally carried out with columns of about 40–70-μm porous, crosslinked polystyrene particles. Owing to the relatively large particle size and the broad particle size distribution of these materials, it normally takes several hours to obtain sufficient resolution[1].

More recently, columns of 10-μm polystyrene gels have become available (*e.g.*, μStyragel from Waters Assoc., Milford, Mass., U.S.A., and TSK gel from Toyo Soda, Tokyo, Japan). Columns with these small, porous gel particles give greatly reduced elution times, because they appear to possess the expected advantages of high rate of separation and high resolution. Although Chow[2] used Styragel for the separation of a series of dextran standards, polystyrene gels cannot, in general, be used in aqueous systems. Therefore, most of the molecular weight studies on water-soluble polymers and proteins have been carried out so far with polydextran (Sephadex from Pharmacia, Uppsala, Sweden) and polyacrylamide gels (Bio-Gel from Bio-Rad Labs.,

* To whom correspondence should be addressed.

Richmond, Calif., U.S.A.)[3]. Unfortunately, these gels cannot be used in high-performance GPC because of mechanical limitations.

According to Cooper and Matzinger[3], the ideal aqueous GPC column packing should be stable over a considerable pH range, should not be affected by variations in ionic strength and temperature and should not exhibit any adsorption properties. Controlled-pore glass (CPG) columns have the required mechanical strength for high-pressure work and can be used for separating water-soluble polymers[4]. To avoid adsorption effects, CPG columns were coated with Carbowax, but this was not very successful because of instability of the columns. Glycophase columns, in which glycerol is chemically attached to the CPG, were found to be more successful in characterizing several water-soluble polymers.

Wu and Bough[5] used Glycophase for establishing the molecular-weight distribution (MWD) of chitosan and Rodriguez[6] for the MWD of heparin. Persiani *et al.*[7] used Glycophase in molecular-weight studies on proteins and industrial glues, but adsorption occurred with this material when using poly(vinyl alcohol) (PVA). These samples could be better characterized with unmodified CPG. Sugisaka and Petracek[8] used Glycophase and porous silica for the rapid molecular-size separation of heparins.

As shown by De Vries *et al.*[9] and Scott and Kucera[10], porous silica is extremely well suited for GPC in organic solvents, because optimal pore-size distributions can be obtained. The same was observed by Belenkii and Gankina[11]. They used unmodified silica with organic solvents in thin-layer chromatography of polymers. In addition, silica has a high mechanical and thermal stability and is available as totally spherical particles of uniform size (*e.g.*, LiChrospher from Merck, Darmstadt, G.F.R.). The rigidity of the silica spheres ensures regular packing of the column.

Kirkland and co-workers[12,13] used porous silica for high-performance GPC in both organic and aqueous solvents; to prevent adsorption effects, the silica particles were deactivated with chlorotrimethylsilane. However, this modification decreases the water compatibility of silica and may lead to reversed-phase adsorption effects. This drawback can be overcome by applying μBondapak, a porous silica chemically modified with a polyether, which was recently introduced by Waters Assoc.

In this paper, it is shown that the MWD of various polyelectrolytes and neutral water-soluble polymers can be determined rapidly and reliably by GPC using chemically unmodified porous silica (LiChrosorb and LiChrospher) as the stationary phase.

EXPERIMENTAL

Equipment

The high-performance liquid chromatograph consisted of a high-pressure pump (Model M6000 A, Waters Assoc.), equipped with a differential refractometer (Waters R 401), a high-pressure sampling valve (Chromatronix HPSV-20), a linear potentiometric recorder (Kipp and Zonen B.D. 8) and a computing integrator system (Spectra Physics S.P. 4000).

Chemicals and materials

The LiChrospher packing materials (mean particle size 10 μm) were purchased from Merck. The buffers used were prepared from distilled water and reagents of analytical-reagent grade (Baker, Deventer, The Netherlands). Tetramethylammonium hydroxide (TMAH) was used as a 20% solution in methanol (Aldrich, Milwaukee, Wisc., U.S.A.).

The polystyrene standards were purchased from Pressure Chem. (Pittsburgh, Pa., U.S.A.) and ArRo Labs. (Joliet, Ill., U.S.A.) and the dextran standards (T series) from Pharmacia. Poly(acrylic acid) and poly(methacrylic acid) as 25% and 20% aqueous solutions, respectively, and PVA were supplied by BDH (Poole, Great Britain).

The heparin samples were obtained from Diosynth (Oss, The Netherlands).

Procedures

Stainless-steel columns (30 cm $\times$ 4.6 mm I.D.) were packed by the slurry method. For LiChrospher Si 300 and Si 500 the packing fluid was propanol-2, and for LiChrospher Si 100 and LiChrosorb Si 60 a mixture of 1,1,2,2-tetrabromoethane and chloroform (3:2). The columns were terminated with modified Swagelok reducing unions (1/8–1/16 in.) provided with 2-μm removable frits supplied by Chrompack (Vlissingen, The Netherlands). After packing, the columns were successively reconditioned with *n*-hexane, tetrahydrofuran (THF), methanol, methanol–water (1:1), water, and the aqueous buffer at a flow-rate of 0.5 ml/min (0.125 cm/sec). All of the solvents were de-gassed before use.

The efficiency of the columns was determined using glucose as the solute and water as the mobile phase. Mobile phase velocities were based on the elution of a totally excluded polydextran.

All of the columns were operated at ambient temperature. At a flow-rate of 0.5 ml/min, the pressure did not exceed 600 p.s.i. per column. The columns were stored in methanol–water (1:1). As LiChrospher was soluble in neutral salt solutions, buffers of pH > 6 should be avoided.

Molecular weight calibration graphs were made by plotting the logarithm of the weight average ($\bar{m}_w$) of the standards against the distribution coefficient, K, defined as

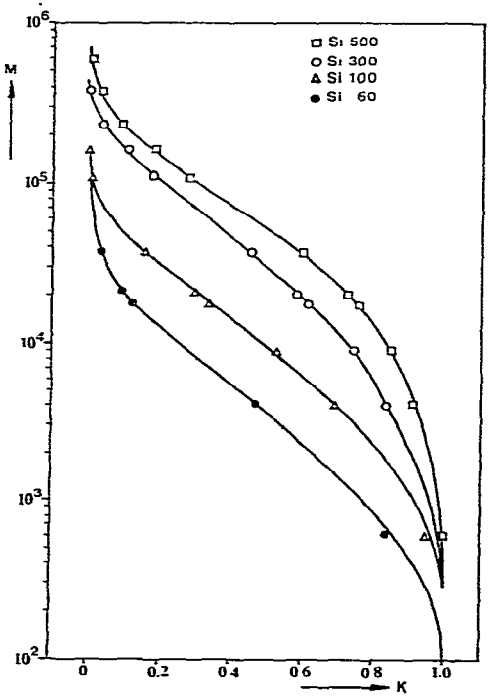
$$K = (V_r - V_0)/(V_t - V_0)$$

where V_r is the retention volume of the solute, V_0 the retention volume of a totally excluded polymer and V_t the retention volume of the totally permeating glucose.

RESULTS AND DISCUSSION

Molecular weight calibration graphs for LiChrospher columns with different exclusion limits for polystyrene standards in THF are shown in Fig. 1. The results are in close agreement with the results obtained for trimethylsilyl-modified porous silica microspheres recently described by Kirkland and co-workers[12,13].

Fig. 2 gives the calibration graphs for some of the LiChrospher columns with polydextran standards using 0.5 *M* sodium acetate solution (pH 5) as the mobile phase. The high resolution of these columns in aqueous solvents is demonstrated in Fig.



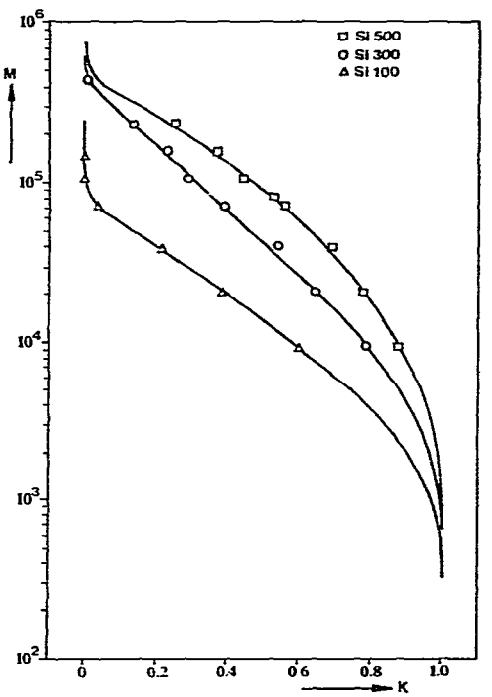


Fig. 1. Molecular weight calibration graphs for LiChrospher columns with polystyrene standards in tetrahydrofuran. Conditions: flow-rate, 1.0 ml/min; sample, 20 μl of 0.5% solution.

Fig. 2. Molecular weight calibration graphs for LiChrospher columns with dextran standards in 0.5 *M* sodium acetate solution (pH 5). Conditions: flow-rate, 0.5 ml/min; sample, 20 μl of 1% solution.

3. Even at relatively high mobile phase velocities, small plate heights are obtained for the totally permeating glucose. As glucose elutes at the same elution volume as toluene using THF as the mobile phase, glucose is assumed to migrate only by size effects ($K = 1$).

These results indicate that LiChrospher will be extremely useful as a substrate for the GPC of water-soluble polymers, provided that polyelectrolyte effects[14] can be suppressed and adsorption can be avoided.

Polyelectrolyte effects

Ion exclusion. As mentioned above, 0.5 *M* sodium acetate solution was used as the mobile phase for the polydextran calibration graphs. Although with pure water the maximum of the MWD of the polydextrans was obtained at the same elution volume as with 0.5 *M* sodium acetate solution, the use of an electrolyte solution gave slightly better results. In water a small part of the polydextrans is excluded from the pores and is eluted near the void volume, as can be seen from Fig. 4. This may be due to the presence of some negatively charged groups on the polydextrans, leading to repulsion effects. As described by Forss and Stenlund[15] for lignosulphonates, these charge repulsion effects arise when the pore surface contains charged groups of the same sign as the solute, which results in limited pore penetration. This ion exclusion effect is clearly demonstrated in Fig. 5. With water as the mobile phase,

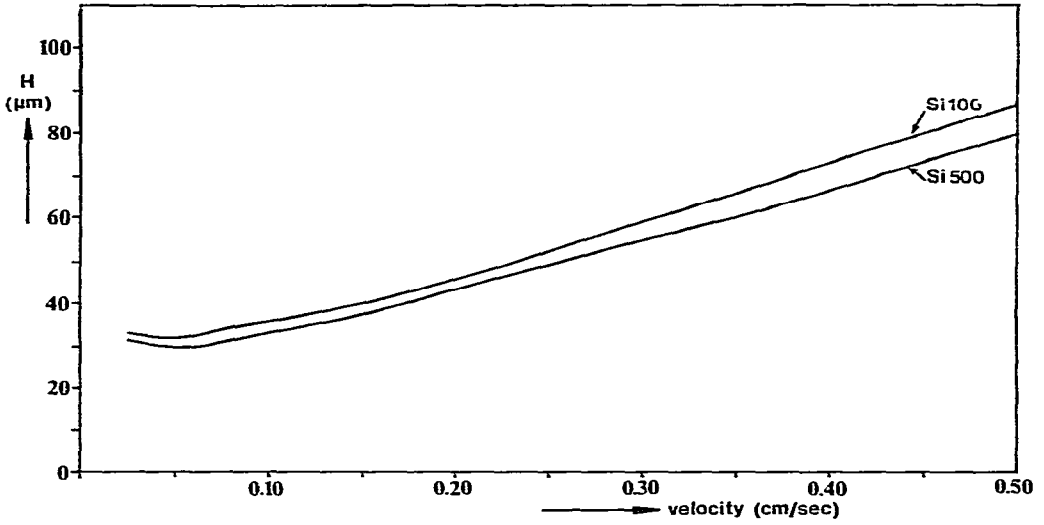


Fig. 3. Effect of flow-rate on the plate height (H) for LiChrospher columns. Conditions: mobile phase; water; sample, 20 μl of a 0.2% glucose solution.

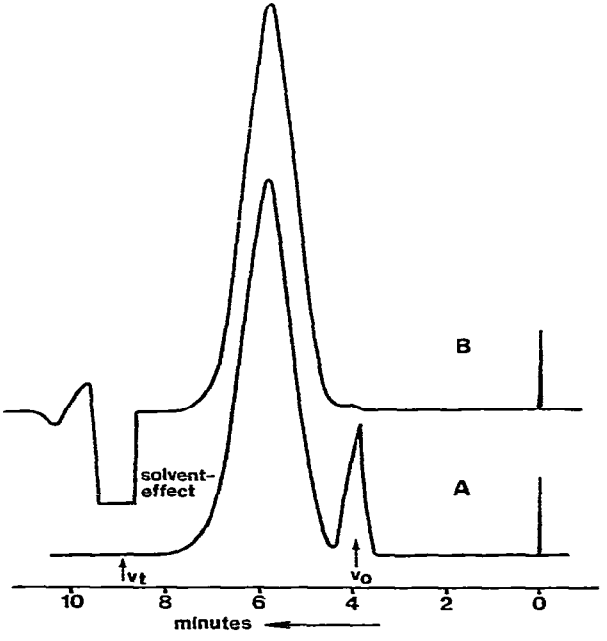


Fig. 4. Effect of mobile phase on the MWD of dextran T 20 on LiChrospher Si 100. Conditions: mobile phase, (A) water, (B) 0.5 M sodium acetate solution (pH 5); flow-rate, 0.5 ml/min; sample, 20 μl of a 10 mg/ml solution of dextran T 20 in water.

the negative pores of the silica are accessible, only to a small extent to the sodium acetate, which starts to elute at the void volume, but they can be fully penetrated by glucose (elution at V_t). This effect can be suppressed by using an aqueous buffer of sufficient ionic strength as the mobile phase, thus shielding the negative charges of the silica.

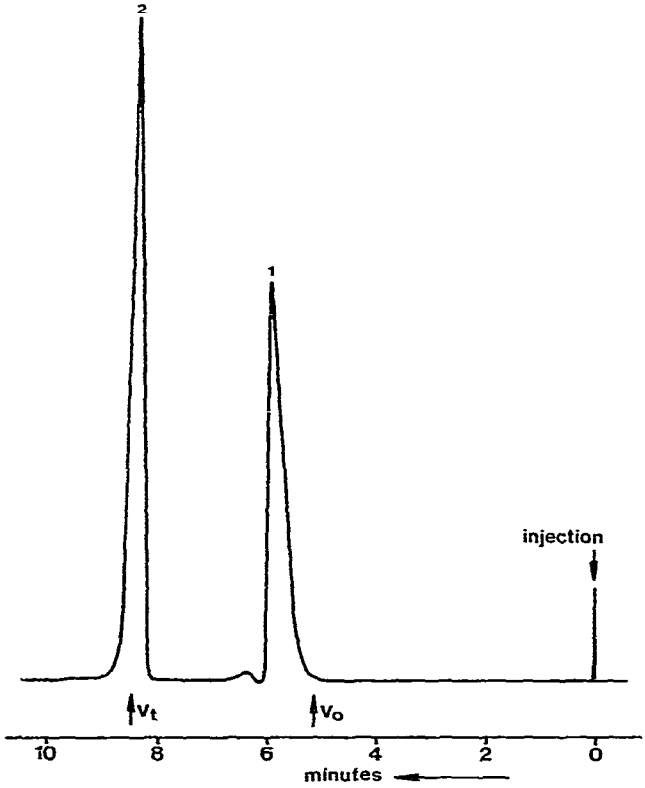


Fig. 5. Ion-exclusion effect on LiChrosopher Si 60. Conditions: column, 30 × 0.46 cm; mobile phase, water; flow-rate, 0.5 ml/min; sample, 20 μl of 2 mg/ml sodium acetate (1) and 2 mg/ml glucose (2) in water.

Polyelectrolyte swelling. In the MWD analysis of polydisperse polyelectrolytes, too low an ionic strength in the eluent may lead to incorrect distributions[16]. Fig. 6 shows the effect of the ionic strength of the mobile phase on the elution volume of the maximum of the MWD of sodium heparin, a sulphated mucopolysaccharide that is used as an anticoagulant. Increasing the ionic strength above 1.0 does not affect the apparent molecular weight.

The apparent molecular weight is also influenced by the polyelectrolyte concentration itself. This effect at constant ionic strength of the buffer is shown in Fig. 7. Heparin solutions in amounts of 20, 40 and 100 μl and concentrations up to 8% were injected into a set of three 30 cm × 4.6 mm I.D. columns packed with LiChrospher Si 100 (10 μm). As an increase in elution volume is related to a decrease in hydrodynamic volume, it can be concluded that the hydrodynamic volume de-

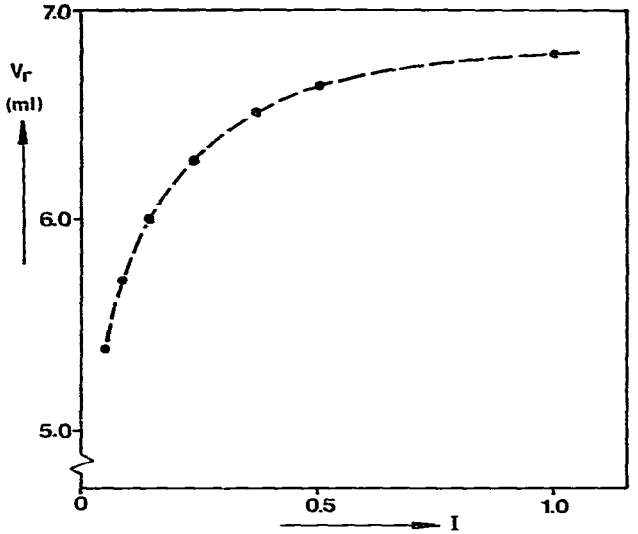


Fig. 6. Effect of ionic strength (I) on the elution volume of the maximum of the MWD of a heparin. Conditions: column, 60 × 0.46 cm LiChrospher Si 100; mobile phase, sodium acetate solution (pH 5); flow-rate, 0.5 ml/min; sample, 20 μl of a 2% heparin solution.

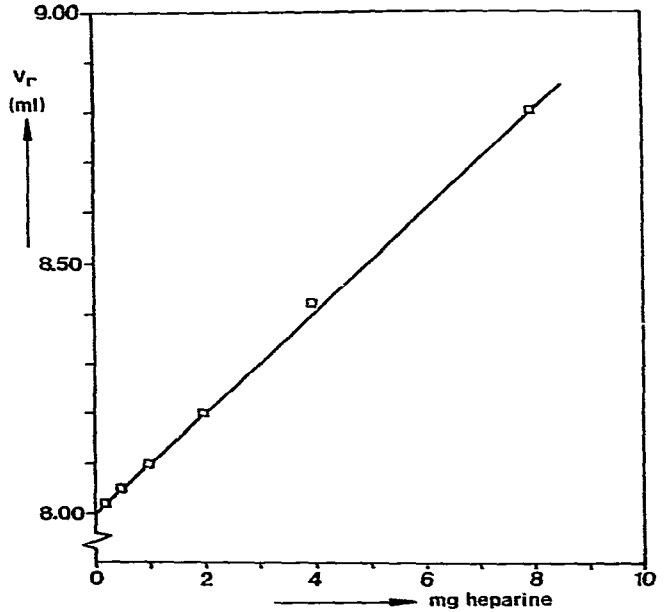


Fig. 7. Effect of amount of heparin on the elution volume of the maximum of the MWD. Conditions: column, 90 × 0.46 cm LiChrospher Si 100; mobile phase, 0.5 M sodium acetate solution (pH 5); flow-rate, 0.5 ml/min; sample volume, 20, 40 and 100 μl.

creases with increasing sample loading, which is presumably caused by a decrease in charge density with increasing concentration. This effect is more pronounced with a lower ionic strength of the mobile phase. No influence of the initial sample volume on the hydrodynamic volume could be observed.

Ion inclusion

The appearance of peak 3 at the elution volume of sodium acetate in the chromatogram of heparin (Fig. 8) is not due to the presence of inorganic salts, as suggested by Sugisaka and Petracek[8] and Rodriguez[6], but must be the result of the ion inclusion effect.

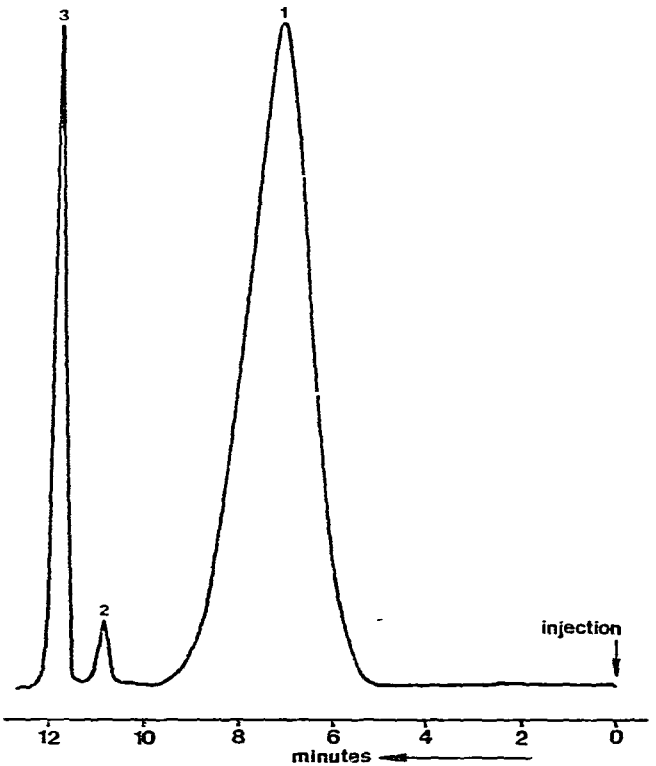


Fig. 8. Ion-inclusion effect on LiChrospher Si 100. *Conditions*: column, 90 × 0.46 cm; mobile phase, 0.5 *M* sodium acetate solution (pH 5); flow-rate, 10 ml/min; sample, 40 µl of a 2% heparin sample. Peaks: 1 = heparin; 2 = NaCl; 3 = NaAc.

When a solution contains two or more different ionic species and one of them is barred from certain regions (in a gel or membrane) penetrable by the other ionic species, a Donnan equilibrium will be established[17]. In the GPC of sodium heparin on silica with an aqueous sodium acetate buffer as the mobile phase, the silica behaves as a membrane that is fully accessible to sodium acetate and only partly to heparin. As on injection of the sodium heparin the sodium ions tend to level their activity inside and outside the silica particles, extra sodium acetate migrates into the silica

pores. Thus the sodium acetate concentration inside the silica pores will be higher than that outside at the beginning of the chromatographic process. As the heparin migrates faster than the sodium acetate through the column (by partial exclusion), the extra sodium acetate inside the silica will behave as a solute and migrate as such.

Applications

When these effects are taken into consideration, various anionic polyelectrolytes can be characterized using unmodified silica. Fig. 9 illustrates the use of LiChrospher for the determination of the MWD of a poly(methacrylic acid). Good results were also obtained for poly(acrylic acid). Whereas the characterization of this polyelectrolyte on the chemically modified silica, μBondagel, still requires 2% sodium dodecylsulphonate (SDS) to be added as a physical modifier to the mobile phase[18], we succeeded in a characterization with an aqueous buffer on chemically unmodified silica.

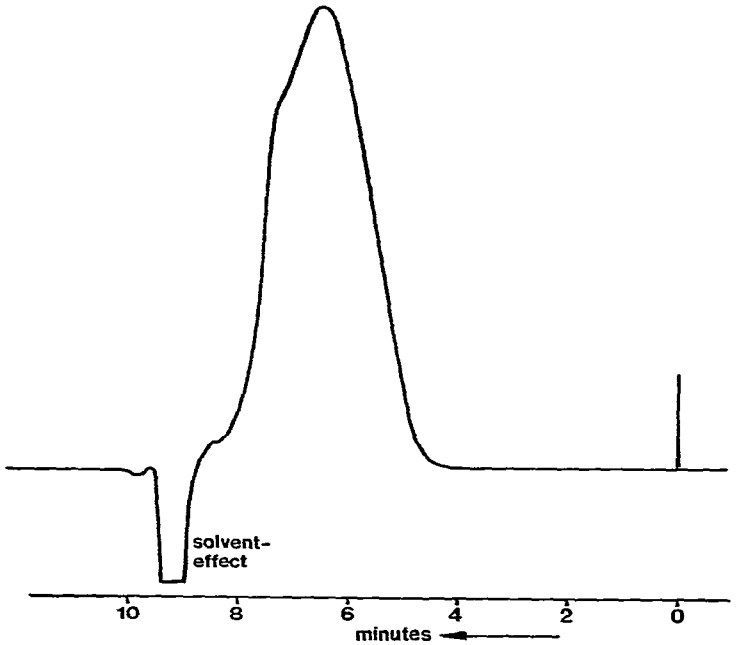


Fig. 9. MWD of a poly(methacrylic acid) on LiChrospher Si 100. Conditions: column, 30 × 0.46 cm; mobile phase, 0.5 *M* sodium acetate solution (pH 5); flow-rate, 0.5 ml/min; sample, 20 μl of 40 mg/ml 20% aqueous poly(methacrylic acid) solution in 0.5 *M* aqueous sodium acetate.

New possibilities are offered by the latter systems for the MWD determination of carboxymethylcellulose (CMC) and other soluble cellulose ethers and esters, because silica can be used at elevated temperatures to reduce the high viscosities of these polymer solutions.

Fig. 10 shows a chromatogram of a degraded CMC sample. Although a LiChrospher column with a higher exclusion limit would be preferable, it is obvious that the MWD of these samples can be determined rapidly and simply without the need for chemical modification prior to analysis.

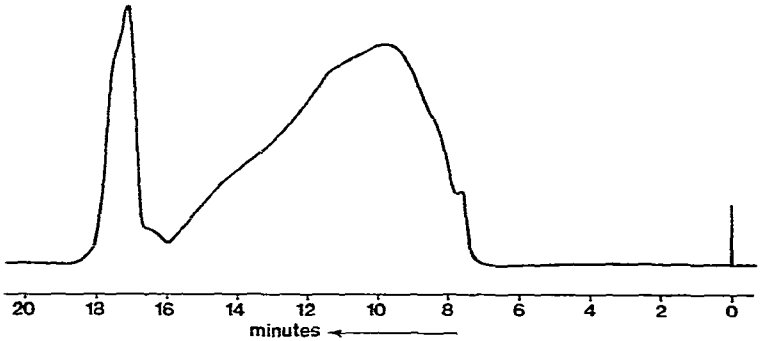


Fig. 10. MWD of a CMC sample on LiChrospher Si 100 + Si 500. Conditions: two columns, 30 × 0.46 cm; mobile phase, 0.5 *M* sodium acetate solution (pH 6); flow-rate, 0.5 ml/min: sample, 100 μl of a 0.5% solution.

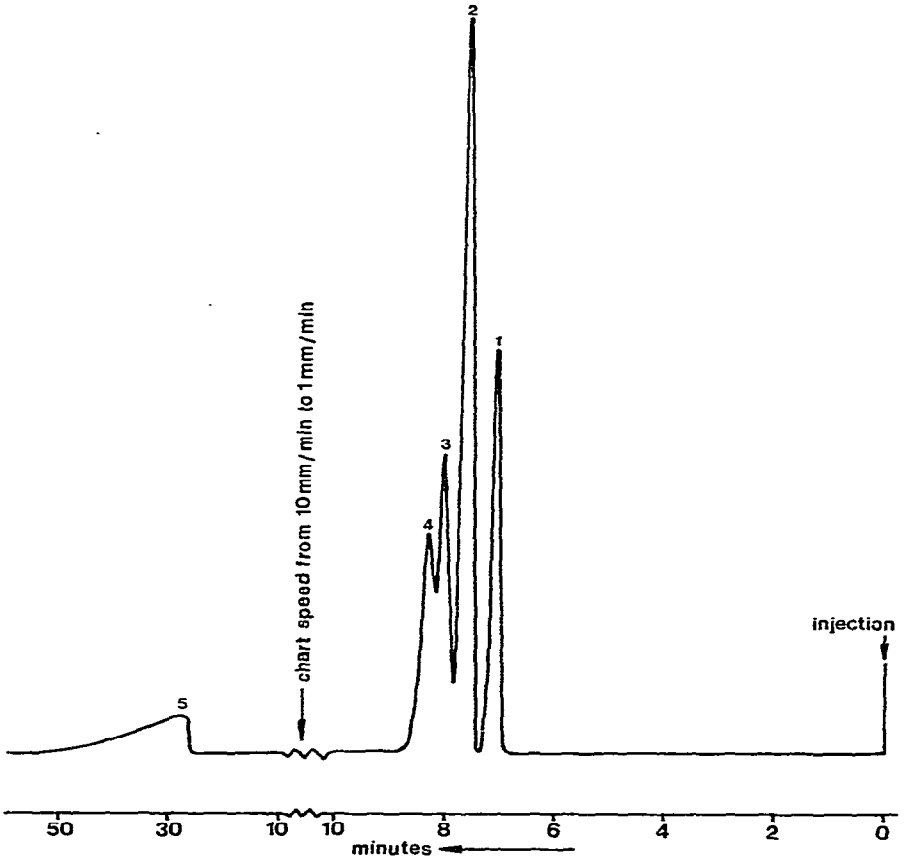


Fig. 11. Adsorption effects on LiChrospher Si 100. Conditions: column, 30 × 0.46 cm; mobile phase, 0.5 *M* sodium acetate solution (pH 5); flow-rate, 0.6 ml/min; samples, 20 μl of 3 mg/ml ammonium acetate, 3 mg/ml potassium acetate and 6 mg/ml TMA chloride solutions. Peaks: 1 = NaCl; 2 = NaOAc (solvent peak); 3 = NH_4OAc; 4 = KOAc; 5 = TMAOAc.

Adsorption effects

Although elution beyond the total permeating volume (V_0), was not observed for anionic and some neutral polymers, indicating no retention other than the SEC mechanism, a number of neutral and cationic polymers are permanently retained. Owing to its acid silanol groups, at pH $>$ 4–5 silica behaves as a weakly acidic ion exchanger[19]. This ion exchange behaviour is demonstrated in Fig. 11.

The Na^+, NH_4^+, K^+ and TMA^+ cations are increasingly adsorbed in this order at pH 5. The TMA^+ cation can be desorbed only by using high sodium concentrations. This result substantiates the explanation given by Knox and Pryde[19] for the decrease in the k' values of organic acids with increasing pH using the NH_2–silica packing: "at pH $>$ 4.5 when the silica becomes negatively charged the ion-exchange capacity of the propylammonium group is neutralized by the negative charges on the silica". At pH $<$ 4, where silica is neutral or even positively charged, TMA^+ is still retained, unlike Na^+, K^+ and NH_4^+.

Our findings suggest that of Na^+, NH_4^+, K^+ and TMA^+, the last cation is the most effective physical modifier in preventing adsorption on silica for a number of neutral and cationic species. As the TMA^+ cation renders the silica surface hydrophobic, reversed-phase effects may occur. This effect was observed for, *e.g.*, PVA, which is permanently retained on silica[20] when water or 0.5 *M* sodium acetate solution is used as the mobile phase. The use of 0.025 *M* TMA–phosphate buffer, adjusted to pH 3 in water–methanol (1:1), made it possible to determine the MWD of PVA. The low pH prevents dissociation of the silica, TMA serving for further deactivation of the silanol groups. Methanol is added to suppress reversed-phase effects[21].

The TMA^+ cation is also extremely effective in deactivating unreacted silanol groups in reversed-phase chromatography with chemically modified silicas[21]. This will be the subject of further investigations.

CONCLUSIONS

Chemically unmodified, totally porous silica can be used in GPC with aqueous electrolyte solvents.

With 10-μm LiChrospher particles, MWDs can be obtained within 2 min. The apparent molecular weight of polyelectrolytes is strongly dependent on the ionic strength of the mobile phase and the amount of the polyelectrolyte used for the determination. Hence the MWD of polyelectrolytes can only be determined comparatively and under strictly standardized conditions.

Elution of poly-dextrans and anionic polymers on silica, using aqueous mobile phases, beyond the total permeating volume has not been observed.

Irreversible adsorption of PVA and cationic polymers seems to be prevented by physical modification of the silica with a TMA salt. The net effect, however, may result in reversed-phase adsorption effects.

ACKNOWLEDGEMENTS

The authors thank Diosynth B.V., Oss, The Netherlands, for supplying heparin samples and Mr. P. C. G. M. Janssen for packing the high-resolution columns.

REFERENCES

1 J. L. Mulder and F. A. Buytenhuys, *J. Chromatogr.*, 51 (1970) 459.
2 C. D. Chow, *J. Chromatogr.*, 114 (1975) 486.
3 A. R. Cooper and D. P. Matzinger, *Amer. Lab.*, 9 No. 1 (1977) 15.
4 D. P. Matzinger and A. R. Cooper, *Polym. Prepr., Amer. Chem. Soc. Div. Polym. Chem.*, 17 (1976) 450.
5 A. C. M. Wu, W. A. Bough, E. C. Conrad and K. E. Alden, Jr., *J. Chromatogr.*, 128 (1976) 87.
6 H. J. Rodriguez, *Anal. Lett.*, 9 (1976) 497.
7 C. Persiani, P. Cukor and K. French, *J. Chromatogr. Sci.*, 14 (1976) 417.
8 N. Sugisaka and F. J. Petracek, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 36 (1977) 89.
9 A. J. de Vries, M. Le Page, R. Beau and C. L. Guillemin, *Anal. Chem.*, 39 (1967) 935.
10 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 125 (1976) 251.
11 B. G. Belenkii and E. S. Gankina, *J. Chromatogr.*, 141 (1977) 13.
12 J. J. Kirkland, *J. Chromatogr.*, 125 (1976) 231.
13 J. J. Kirkland and P. E. Antle, *J. Chromatogr. Sci.*, 15 (1977) 137.
14 J. C. Giddings, E. Grushka, J. Cazes and P. R. Brown, *Advan. Chromatogr.*, 14 (1976) 37.
15 K. G. Forss and B. G. Stenlund, *J. Polym. Sci.*, 42 (1973) 951.
16 M. Skalka, *J. Chromatogr.*, 33 (1965) 456.
17 P. A. Neddermeyer and L. B. Rogers, *Anal. Chem.*, 41 (1969) 95.
18 *Waters Assoc. Bull.*, F 68, December 1976, p. 1.
19 J. H. Knox and A. Pryde, *J. Chromatogr.*, 112 (1975) 171.
20 K. J. Bombaugh, W. A. Dark and J. N. Little, *Anal. Chem.*, 4 (1969) 1337.
21 F. P. B. van der Maeden, P. T. van Rens, F. A. Buytenhuys and E. Buurman, *J. Chromatogr.*, 142 (1977) 715.