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HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY OF WATER-SOLUBLE CELLULOSICS*

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SUMMARY

In order to determine the molecular-weight distribution of modified cellulosics, we have developed a high-performance chromatographic system using a hydrophilic coated silica (Synchropak) as the packing and a high ionic strength buffer as the mobile phase. By use of this system, various water-soluble cellulosics (carboxymethyl, hydroxyethyl, and carboxymethyl hydroxyethyl celluloses) were analyzed within 15 min.

By determining K_d values of carboxymethylcellulose as a function of mobile phase ionic strength, polymer contraction was followed. In a high ionic strength mobile phase, these polyelectrolytes were contracted to a reduced hydrodynamic volume. Not only does a contracted state reduce the effect of chemical heterogeneity of ionic groups along the chain, but it also greatly reduces the viscosity of injected solutions, thus eliminating chromatographic viscosity effects.

INTRODUCTION

Carboxymethylcellulose (CMC), hydroxyethyl cellulose (HEC) and carboxymethyl hydroxyethyl cellulose (CMHEC) are widely used polysaccharides in food and other industries[1]. As shown in Fig. 1, these polymers consist of a cellulose backbone in which some of the hydroxyl groups are derivatized[2,3] to form water-soluble ionic (CMC and CMHEC) or non-ionic (HEC) cellulosics.

Although average molecular weights or degrees of polymerization of these cellulosics are determined routinely from viscosity measurements[4–6], characterizing these polymers in terms of their molecular-weight distribution (MWD) is highly useful in production control and end-use performance evaluation. However, there

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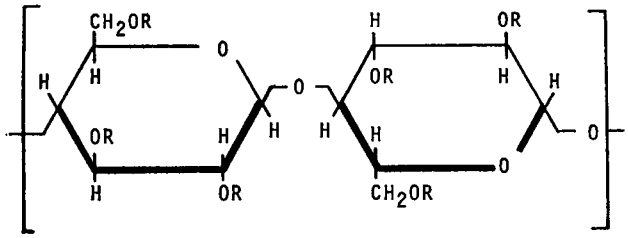

CMC R = -H or $-CH_2C(=O)ONa$

HEC R = -H or $-(CH_2CH_2O)_xH$

CMHEC R = -H, $-CH_2C(=O)ONa$, or $-(CH_2CH_2O)_xH$

Fig. 1. Structure of carboxymethyl, hydroxyethyl and carboxymethyl hydroxyethyl celluloses.

have been very few papers published concerning the MWD of these polymers. The MWD of CMC has been determined by gel permeation chromatography (GPC) using cadoxen as the mobile phase, and either agarose[7,8], or polyacrylamide[7,9] as the gel. According to a review by Segal[10], other cellulosic derivatives analyzed by GPC have been cellulose trinitrate, cellulose acetate, cellulose tricarbanilate and alkyl esters. Recently, GPC analysis of acetal derivatives of CMC and HEC have been reported using tetrahydrofuran (THF) as the mobile phase and, presumably, Styragel as the packing material[11].

During the past several years, high-performance GPC supports for water-soluble polymers have become commercially available. Because of their high efficiencies and short analysis times, these supports are superior to conventional packings. Except for several brief application examples[12–14], the use of high-performance GPC for hydrophilic modified cellulosics has not been extensively studied.

In this report, we describe the evaluation of Synchropak, a commercially available hydrophilic support which consists of a glycerylpropylsilyl layer covalently bonded to 10 μm porous spherical silica particles. These high-performance supports have previously been shown to be highly deactivated when used for the GPC analysis of a variety of proteins[15,16].

EXPERIMENTAL

Apparatus

A Varian 8500 liquid chromatograph and a Waters 401 differential refractometer were employed. The refractometer was thermostatted to 23–24° with a Haake FE water bath. Stagnant mobile phase was kept in the reference side of the refractometer. Samples were injected with a Rheodyne 70-01 injection valve.

Columns

The packing material consisted of a glycerylpropylsilyl layer covalently bonded to LiChrospher silica particles (10 μm) and was purchased prepacked in 25 cm $\times$ 4.1 mm I.D. long stainless-steel columns from SynChrom (Linden, Ind., U.S.A.). Nominal pore sizes used in this study were 100, 500, 1000 and 4000 Å. Columns were arranged in series with the smaller pore-sized support placed first.

Mobile phases

All mobile phases were prepared using distilled water and reagent grade chemicals. They were filtered under vacuum using a 0.22-μm membrane filter (Type GS; Millipore, Bedford, Mass., U.S.A.). Ionic strengths were calculated using:

$$I = \tfrac{1}{2} \Sigma\, C_i Z_i^2$$

where C_i is the molar concentration of an ion of charge Z_i. Degrees of dissociation for phosphate and acetate ions were taken from the literature[46].

Sodium sulfate solutions

Sodium sulfate solutions of 0.5, 0.1 and 0.01 *M* were prepared which had respective ionic strengths of 1.5, 0.3 and 0.03 *M*. The pH range of these solutions was about 7–7.5.

Phosphate buffers

Phosphate buffers of pH 6 and 7 were prepared by adjusting the pH of NaH_2PO_4 solutions with NaOH. Typically, about 1250 and 500 ml of 1 *M* NaOH were added to 2 l of 1 *M* NaH_2PO_4 to give pH 7 and 6 solutions, respectively. The respective ionic strengths of these solutions were 1.2 and 1.0 *M*. These solutions were diluted with appropriate amounts of water to give the required final ionic strengths.

Acetate buffers

A pH 5 acetate buffer of ionic strength 0.64 *M* was prepared by adding 1100 ml of 1 *M* acetic acid to 2 l of 1 *M* sodium acetate. A pH 6.0 acetate buffer of ionic strength 1.4 *M* was prepared by adding 480 ml of 3.1 *M* sodium acetate and 20 ml of 3.1 *M* acetic acid to a 1-l volumetric flask and filling to volume with water.

The recommended pH 3.7 mobile phase was prepared by first adding 60 ml of 4 *M* sodium acetate and 440 ml of 4 *M* acetic acid to a 1-l volumetric flask and filling to volume with water. This gives a pH 3.7 buffer of 0.22 *M* ionic strength. The ionic strength of this solution is then increased to 1.42 *M* by adding 0.4 moles of sodium sulfate to 1 l of the 0.22 *M* acetate solution. This solution is then diluted 1:1 with water and used as the mobile phase. The 1.42 *M* solution is used for sample preparation.

Standards

The following dextrans were obtained from Pharmacia (Uppsala, Sweden): 10T, 20T, 40T, 70T, 110T, 150T, 250T, 500T, and 2000T. These have weight-average molecular weights of $10 \cdot 10^3$, $20 \cdot 10^3$, $40 \cdot 10^3$, $70 \cdot 10^3$, $110 \cdot 10^3$, $150 \cdot 10^3$, $500 \cdot 10^3$ and $2000 \cdot 10^3$, respectively. Glucose was used to measure column efficiencies and to

determine the permeated column volume (V_p). Although dextran 2000T could be used to determine the exclusion volume (V_0) of the 100 and 500 Å columns, a calculated volume of 1.15 ml for each column was used in all partition coefficient (K_d) calculations. A list of all cellulosics mentioned in this study is given in Table I.

TABLE I

CELLULOSIC SAMPLES USED IN THIS STUDY

Last digits indicate aqueous Brookfield viscosities in cP (see refs. 28 and 29 for spindles sizes and speeds); 1% solutions were used for H CMC samples, 2% solutions were used for M and L CMC and K and G HEC samples, and 5% solutions were used for L and J HEC samples.

Cellulosic	*Designation*
Carboxymethylcellulose	7L1-8
(average degree of substitution, 0.7)	7L1-9
	7L2-12
	7L2-14
	7L2-18
	7L-27
	7L-34
	7M-450
	7M-570
	7M-1000
	7H-1100
	7H4-3000
Hydroxyethyl cellulose	250L-110
(average molar substitution, 2.5)	250JR-160
	250GR-260
	250KR-1800
Carboxymethyl hydroxyethyl cellulose	CMHEC-1800
(average degree of substitution, 0.37; average molar substitution, 1.89)	

Viscosity determinations

Relative viscosities at 25 and 40° were determined with standard Ubbelohde capillary viscometers. Intrinsic viscosities were determined at 25° with Ubbelohde capillary viscometers using at least six to eight concentrations and extrapolated to zero concentration. No shear rate corrections of the viscometers were made.

Recommended procedure

Sample preparation. A given amount (see Table V for guidelines) of sample (CMC, HEC, or CMHEC) was slowly added to 50 ml of distilled water. In order to avoid agglomeration and lumping, the sample was slowly sprinkled into the vortex of a rapidly stirred solution. Depending on the sample's viscosity, solutions were stirred for a minimum of two hours and quite often overnight. A set mixing speed was used so that samples had the same "shear rate history". After complete dissolution, 50 ml of 1.4 *M* ionic strength buffer (pH 3.7) was slowly added to a rapidly stirred solution. The solution was filtered through a 0.65-μm membrane (Type DA, 13 mm diameter; Millipore), using a Swinny holder. If excess microgels were present, a larger surface area membrane (47 mm) filter was used in which filtration was done under vacuum using a Millipore filtration apparatus. An alternative approach was to

prefilter samples through a 0.7-μm glass fiber filter (GF/F, 47 mm diameter; Whatman, Clifton, N.J., U.S.A.) with subsequent filtration, if necessary, on the 0.65-μm membrane filter. All samples were chromatographed within two days after preparation.

Chromatographic conditions. With the recommended column set and refractometer attenuation (see Table V), 20 μl of sample solution was injected in triplicate at a flow-rate of 0.5 ml/min. (To insure that chromatographic overloading effects were not occurring, several injections of lower concentration solutions were made and retention times and peak profiles compared.)

RESULTS AND DISCUSSION

Effect of mobile phase ionic strength

The influence of mobile phase ionic strength in aqueous GPC has been covered in several reviews[17,18], and recent papers[19–23]. Briefly, the ionic strength of the mobile phase must be sufficiently high to eliminate electrostatic interactions between ionic groups on the packing and on the polyelectrolyte to prevent ion exclusion, ion inclusion, ion exchange, and adsorption. Not only does the addition of electrolytes in the mobile phase eliminate these non-size-exclusion mechanisms, it also reduces the polyelectrolyte's hydrodynamic volume. This decreases the viscosity of injected solutions, thus minimizing chromatographic viscosity effects. Moreover, analyzing polyelectrolytes in their contracted state reduces the effect of chemical heterogeneity of ionic groups on the polymer.

Several examples of the effects of mobile phase ionic strength on the elution behavior of different CMC samples are shown in Figs. 2 and 3 for phosphate and sulfate mobile phases. (See Table I for interpretation of sample designations.) As the ionic strength is increased, the polyelectrolyte contracts, resulting in greater per-

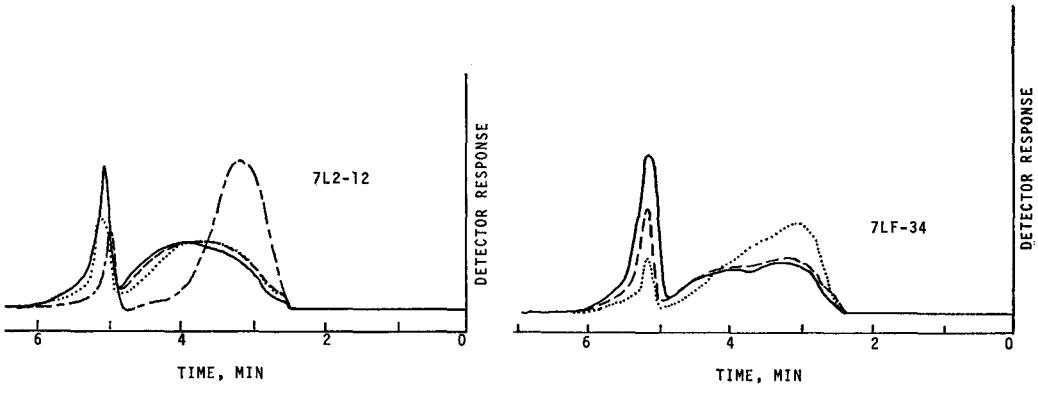

Fig. 2. Effect of mobile phase ionic strength on the elution of CMC using pH 7 phosphate buffers. Column, 500 Å SynChropak, 25 cm × 4.1 mm I.D.; flow-rate, 0.5 ml/min; injection volume, 20 μl of 2.5 mg/ml solutions; refractive index detector attenuation, ×8; chart speed, 1 in./min. Ionic strengths: — – — 0.012 *M*; ..... 0.12 *M*; -------- 0.23 *M*; ——— 0.58 *M*.

Fig. 3. Effect of mobile phase ionic strength on the elution of CMC 7L-34 using sodium sulfate solutions. Injection volume, 20 μl of a 1 mg/ml solution; refractive index detector attenuation, ×4. Ionic strength: ..... 0.03 *M*; -------- 0.3 *M*; ——— 1.5 *M*. See Fig. 2 for other conditions.

meation into the pores of the packing. At a low ionic strength of 0.012 *M*, CMC appears to be partially excluded from the pores of the packing. This could be caused by the expanded state of the molecule and/or ion exclusion. At increased ionic strengths, peak shapes remain essentially the same, but are shifted towards greater elution times. Since areas were not normalized, differences in peak heights can be attributed to concentration variations of injected samples. The origin of the totally permeated peak is not known, but could be the result of impurities introduced during sample preparation[24], solvent–mobile phase mismatch, and/or sodium ions that have been dissociated from CMC. Since permeated peaks also appear after injecting only mobile phase, they might also be caused by perturbing the (partition) equilibrium between electrolytes in the mobile phase, and those within the pores or adsorbed onto the packing. Evidence for this explanation is based on the absence of permeated peaks when mobile phase is injected when water or low ionic strength buffers are used as mobile phases. Injecting polyelectrolytes in low ionic strength mobile phases results in the establishment of Donnan equilibrium (ion inclusion), which gives rise to permeated peaks[13,17,23].

To quantify the influence of mobile phase ionic strength on polyelectrolyte contraction, K_d values were examined. These were calculated using the following equation:

$$K_d = \frac{V_r - V_0}{V_i}$$

where V_r is the elution volume of the sample as determined from its peak maximum, V_0 is the total interstitial volume, which was obtained by assuming that it is equivalent to 35% of the total column volume, and V_i is the total pore volume, which was determined from the elution volume on glucose (V_p) using the following equation:

$$V_i = V_p - V_0$$

The influence of ionic strength on K_d was determined on a number of different CMC samples using various pH buffers on 500, 1000, and 4000 Å columns. Only the results obtained on the 500 Å column are presented in Table II since this pore size

TABLE II

EFFECT OF MOBILE PHASE IONIC STRENGTH ON K_d*

Column, 25 cm × 4.1 mm I.D.; 500 Å SynChropak column (100–500 Å, column-set for pH 3.7 acetate buffer study); flow-rate, 0.5 ml/min; injection, 20 µl of 1 mg/ml.

Sample	K_d									
	Na_2SO_4 (pH 7–7.5)			*Phosphate buffer (pH 7.0)*				*Acetate buffer (pH 3.7)*		
	0.03 M	*0.3 M*	*1.5 M*	*0.012 M*	*0.12 M*	*0.23 M*	*0.58 M*	*0.06 M*	*0.11 M*	*0.41 M*
7L1-8	0.53	0.65	0.67	0.31	0.59	0.62	0.66	0.17	0.20	0.22
7L2-12	0.43	0.57	0.60	0.27	0.49	0.55	0.59	0.10	0.12	0.14
7L2-18	0.31	0.40	0.44	0.21	0.36	0.38	0.43			
7L-34	0.26	0.30	0.32							
7L-27								0.03	0.05	0.05

* Calculated from peak maximum; V_p from glucose and V_o = 1.15 ml per column.

gave the highest resolution. As indicated in Table II, K_d increased with increasing ionic strength using unbuffered sodium sulfate solutions, pH 7 phosphate buffers, and pH 3.7 acetate buffers as mobile phases. This effect was due to polyelectrolyte contraction. Also, for a given ionic strength, K_d values decreased with increasing sample viscosity, as expected.

In order to relate polyelectrolyte contraction as determined from K_d measurements, to hydrodynamic volumes, intrinsic viscosity ($[\eta]$) measurements were made on a number of samples as a function of ionic strength. As shown in Table III, CMC and CMHEC exhibited a large intrinsic viscosity decrease as the ionic strength was increased, while HEC, a non-ionic polymer, showed a small decrease.

TABLE III

EFFECT OF IONIC STRENGTH ON INTRINSIC VISCOSITIES AT pH 3.7

Ionic strength (M)	$[\eta]$		
	CMC 7M-450	*CMHEC-1800*	*HEC-250KR-1800*
0	80	90	6.8
0.01	7.5	18.0	
0.05	5.7	15.0	6.7
0.10	5.2	14.5	6.7
0.35	4.9	13.5	6.7
0.70	4.6 (4.9)*	13.2 (13.5)*	6.5 (6.8)*

* Determined in pH 5.9 acetate buffer.

Since $1-K_d$ is proportional to log (hydrodynamic volume) and $[\eta]$ is proportional to hydrodynamic volume, both K_d and $[\eta]$ can be directly compared as a function of ionic strength as shown in Fig. 4. The K_d values were obtained from

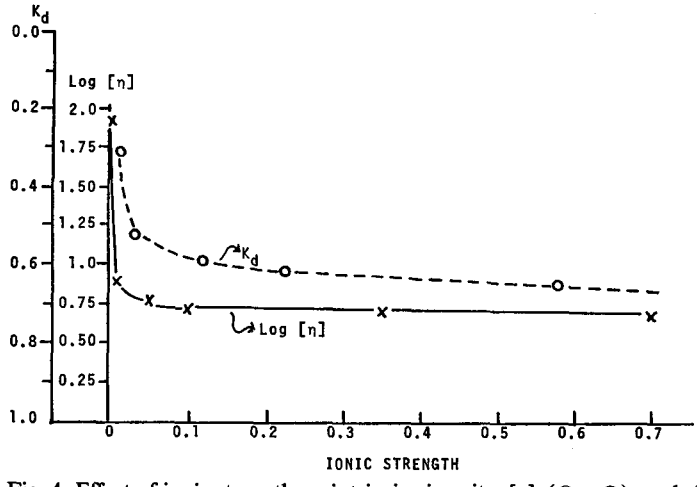

Fig. 4. Effect of ionic strength on intrinsic viscosity, $[\eta]$, (○—○); and chromatographic distribution coefficients, K_d (×—×), of CMC. Intrinsic viscosity measurements were made at 25° on CMC 7M-450 using pH 3.7 acetate buffers. Distribution coefficients were determined on CMC 7L1-8 using pH 7.0 phosphate buffers (for 0.03 *M* ionic strength, the measurements was made using Na_2SO_4 as the mobile phase). See Table II for chromatographic conditions.

CMC 7L1-8, using pH 7.0 buffered mobile phases (Table II), and $[\eta]$ values were obtained from 7M-450 in pH 3.7 buffers, (Table III). Because of differences in molecular weights of these two samples and pH (as will be discussed below, ionic strength rather than pH plays a major part in polyelectrolyte contraction), an accurate correlation cannot be made. However, both sets of measurements show similar trends. This demonstrates that the chromatographic separation appears to be based on molecular size (this has been subsequently verified, using a light scattering detector[25]), and more importantly, that a high ionic strength mobile phase is required for maximum contraction.

Figs. 5 and 6 graphically illustrate the influence of mobile phase ionic strength on K_d. In addition, these figures show that K_d is also a function of sample concentration and that this dependency is reduced with increased ionic strength. At the two lowest ionic strengths (0.012 and 0.03 M), the Na^+ from the CMC itself contributes significantly to the total ionic strength. This is reflected by the increased slope at these low ionic strengths. As discussed below in greater detail, the dependency of K_d on sample concentration can be explained in terms of viscosity effects.

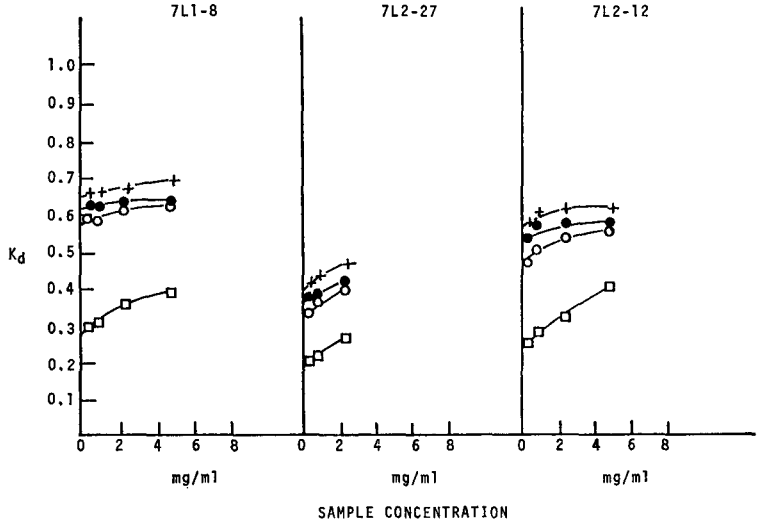

Fig. 5. Effect of sample concentration and mobile phase ionic strength on K_d of CMC using pH phosphate buffers. Ionic strength: □, 0.012 M; ○, 0.10 M; ●, 0.23 M; +, 0.58 M. See Table II for chromatographic conditions.

Effect of mobile phase pH

Several examples of the influence of mobile phase pH on peak shapes are given in Fig. 7. As shown, peak shapes were essentially independent of pH at ionic strengths greater than 0.3 M. The K_d values of these experiments are shown in Table IV, which also includes K_d values of CMC and HEC samples determined with pH 3.7 and 5.9 acetate buffers of 0.7 M ionic strength. These results demonstrate the pH independence of these mobile phases. Intrinsic viscosities measured in 0.7 M ionic strength solutions showed only a small decrease for CMC, CMHEC, and HEC, when

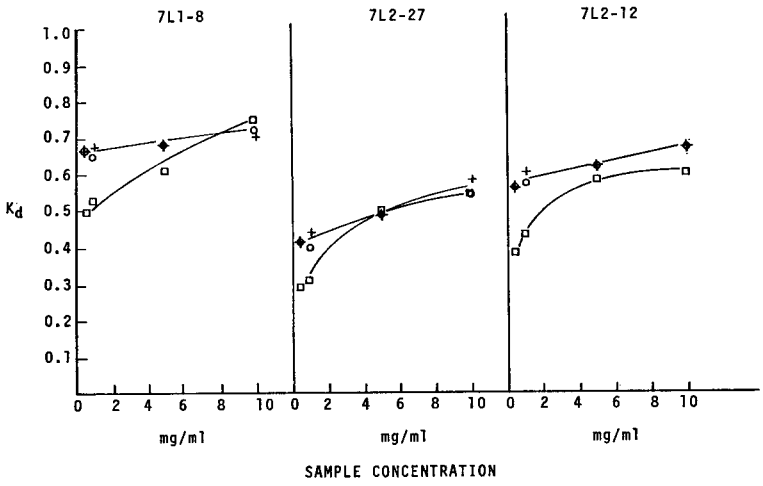

Fig. 6. Effect of sample concentration and mobile phase ionic strength on K_d of CMC using sodium sulfate solutions. Ionic strength: □, 0.03 *M*; ○, 0.30 *M*; +, 1.50 *M*. See Table II for chromatographic conditions.

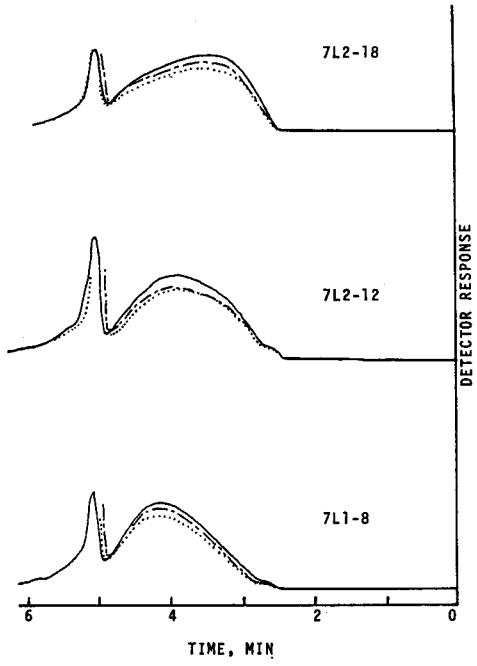

Fig. 7. Effect of mobile phase pH on the elution of CMC. Mobile phases: pH 5 acetate buffer, 0.32 *M* ionic strength, ———; pH 6 phosphate buffer, 0.51 *M* ionic strength, ---------; pH 7 phosphate buffer, 0.58 *M* ionic strength, ... See Fig. 2 for conditions.

the pH was increased from pH 3.7 to 5.9. Rinaudo and Milas[26] have also demonstrated that ionic strength, rather than pH, is the major factor which influences the hydrodynamic volume of CMC.

TABLE IV

EFFECT OF MOBILE PHASE pH ON K_d

Conditions: see Fig. 2; ionic strengths are given in parentheses; 100–500 Å column-set used with the pH 3.7 and 5.9 acetate buffer studies and 20 μl injection of 2.5 mg/ml.

Sample	*pH 5 (0.32 M)**	*pH 6 (0.51 M)***	*pH 7 (0.58 M)***	*pH ≈7.3 (0.30 M)****
CMC				
7L1-8	0.65	0.64	0.66	0.66
7L2-12	0.59	0.59	0.59	0.57
7L2-18	0.41	0.40	0.43	0.40
	*pH 3.7 (0.7 M)**	*pH 5.9 (0.7 M)**		
CMC				
7L1-9	0.18	0.18		
7L2-14	0.16	0.16		
7L-27	0.08	0.07		
HEC				
250L-110	0.07	0.07		
250JR-160	0.07	0.07		

* Acetate buffer.
** Phosphate buffer.
*** Sodium sulfate.

Recommended mobile phase

Based on Fig. 4 and Tables II and III, a sufficiently high ionic strength mobile phase must be employed ($\geqslant 0.3$ *M*) to insure maximum polyelectrolyte contraction. A 0.7 *M* mobile phase is being routinely used in our laboratory. At this high concentration, small variations in mobile phase composition do not change peak shapes or K_d values. A pH 3.7 buffer has been selected to increase column life by reducing silica dissolution. At this pH, the mobile phase can be stored for extended periods of time because of the absence of microbiological growth. This low pH also suppresses ionization of silanol groups. Moreover, this acetate–sulfate buffer is not corrosive. Solutions of CMC, CMHEC, and HEC are stable at pH 3.7 [27–29] and soluble in the mobile phase providing that they are first dissolved in water as explained in the Experimental section. Although ionic strength plays a major role in polyelectrolyte contraction[26,30], operating at a lower pH does contribute to a reduction in sample viscosity (see Table III).

Operational parameters

Overloading effects. As shown in Figs. 5 and 6, the concentration of injected samples can have a significant effect on K_d values. At low ionic strengths, this effect can be partially caused by the sample's contribution to the total ionic strength[17]; at higher ionic strengths, sample viscosity appears to play a major role.

Figs. 8 and 9 demonstrate the influence of sample concentration on peak shape. As sample concentration is increased, the elution volume of the peak increases and at higher concentrations, the peak becomes severely distorted. These overloading effects can be explained in terms of two phenomena[31], macromolecular crowding and viscous fingering. At high polymer concentration, individual chains become crowded, thus reducing their hydrodynamic volume. This leads to a peak shift towards a higher

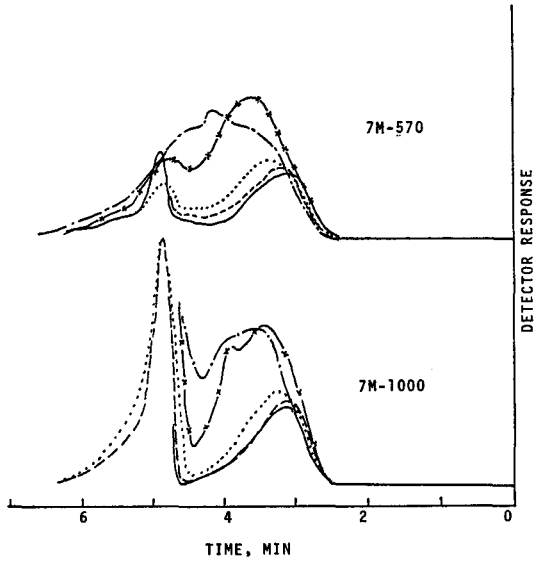

Fig. 8. Influence of sample concentration of CMC on peak shape. Column, 1000 Å SynChropak, 25 cm × 4.1 mm I.D.; flow-rate, 0.5 ml/min; mobile phase, 1.5 *M* ionic strength sodium sulphate; injection volume, 20 μl. Sample concentrations and attenuations: ——— 0.5 mg/ml (×2); ---------- 1 mg/ml (×4); ..... 2.5 mg/ml (×8); —×— 5 mg/ml (×8); —.—10 mg/ml (×16).

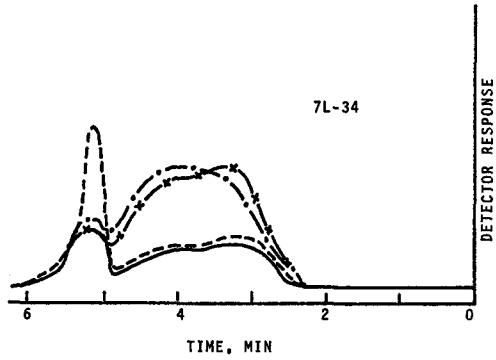

Fig. 9. Influence of sample concentration on peak shape. Column, 500 Å SynChropak, 25 cm × 4.1 mm I.D. See Fig. 8 for other conditions.

elution volume. Peak distortion was first explained by Moore[32] in terms of a phenomenon called viscous fingering: if the viscosity of an injected solution is significantly greater than the mobile phase, the mobile phase will push through creating "fingers" of sample resulting in a distorted peak.

According to our findings, if the relative viscosity of CMC is greater than approximately 1.5, K_d increases with sample concentration. At a relative viscosity of about 2.6, peak distortion occurs as shown in Figs. 8 and 9. Table V lists recommended sample concentrations of CMC, HEC, and CMHEC for the GPC procedure using a 0.7 *M* ionic strength mobile phase. Within a given concentration range, the relative viscosity is below 1.5. Included in this table are recommended refractive index attenuations based on a Waters 401 refractometer and a 20-μl injection.

TABLE V

RECOMMENDED GPC OPERATING CONDITIONS FOR CMC, HEC AND CMHEC

Viscosity type	*Brookfield viscosity* (cP)*	*Concentration range*	*Refractive index detector setting***	*Columns****
High	400–4500, 1%	0.02–0.05%	X1	100–4000 Å 100–1000 Å
Medium	400–3000, 2%	0.05–0.10%	X1–X2	100–1000 Å 100–500 Å
Low	<400, 2%	0.10–0.5%	X2–X4	100–500 Å

* See refs. 28 and 29 for viscosity determinations.

** Based on a 20-μl injection and a Waters 401 refractometer.

*** SynChropack, 25 cm × 4.1 mm I.D. Depending on the sample, other column combinations can be used.

These relative viscosity limits are comparable to values given by others. Samay and Kubin[33] have found that overloading effects occur at a relative viscosity of about 1.8 with polystyrene, polyacrylates and copolymers of these compounds using THF as the mobile phase and Styragel columns. Janča and Pokorný[34–39] have done an extensive investigation on concentration effects in GPC, and have shown that overloading for polystyrene appears at relative viscosities of 1.1 and severe peak distortion at 2.1, using THF as the mobile phase and Porasil B as the packing[35]. In an earlier paper by Flodin[40], peak distortion of hemoglobin was apparent at a relative viscosity of 4.2 using 0.1 *M* pH 7 phosphate buffer as the mobile phase and Sephadex as the support. In Flodin's experiments, the viscosity of the injected solutions was controlled by adding various amounts of high-molecular-weight dextran.

As demonstrated by Janča and Pokorný[35], overloading effects at high viscosities depended on mobile phase flow-rates; at high flow-rates, the overloading was less severe. This was explained by the non-Newtonian behavior of samples in which at higher shear rates, pseudoplastic solutions will exhibit a decrease in viscosity.

Several possible methods of reducing viscosity effects include operating at higher temperatures or matching the viscosity of the mobile phase to the sample's viscosity. However, at 40°, there was only a 2–6% viscosity reduction of several CMC samples in 0.12 *M* ionic strength phosphate buffer. Also, at high temperatures, there is the potential of decreasing column life. Increasing mobile phase viscosity will not only result in a greater pressure drop across the column, but also column efficiency will be decreased because of poor mass transfer in the mobile phase. Another approach of avoiding overloading effects is to inject larger volumes of more dilute solutions. However, as will be discussed below, column efficiency is sacrificed using larger injection volumes.

Increasing detector sensitivity to detect lower concentrations is the most ideal approach. In our laboratory we are now using a light scattering detector not only in conjunction with a refractometer to determine absolute MWDs, but also as a single detector to determine relative differences among high-molecular-weight samples[25]. Since the output of a light scattering detector is proportional to molecular weight times concentration, we are able to inject low concentrations of high-molecular-weight samples and thus avoid overloading effects.

Injection volume. With glucose as a test solute, efficiency of a 100–1000 Å column set was determined as a function of injection volume. In order to avoid interference from the extraneous permeated peak, water was used as the mobile phase. The results of this study are shown in Fig. 10. At constant glucose concentration, there was 5% efficiency loss from 10 to 20 μl, 8% loss from 20 to 50 μl, and 20% loss from 20 to 100 μl. Except for the 200-μl injection, the study consisting of injecting a constant amount of glucose gave similar results. Thus, for maximum column efficiency, injections of 50 μl or less is recommended.

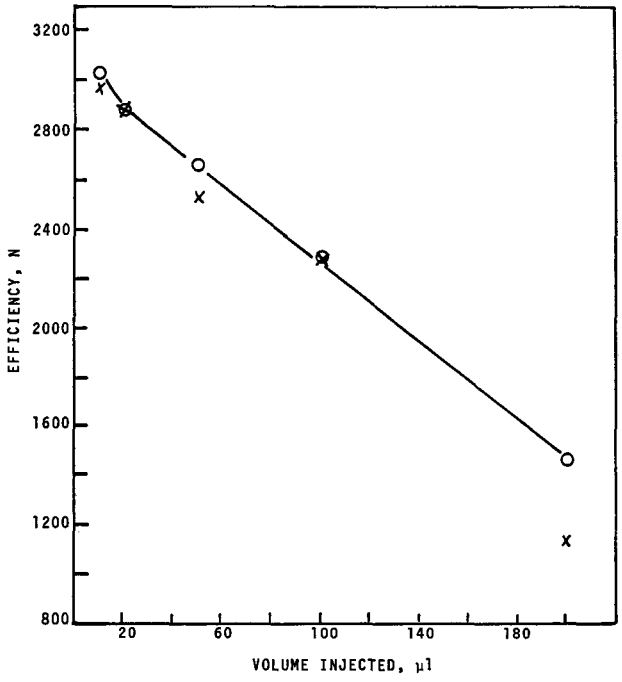

Fig. 10. Influence of injection volume on column efficiency, *N*. Column, 100–1000 Å SynChropak column-set; mobile phase, water; flow-rate, 0.5 ml/min; sample, glucose. ○, Injection of constant concentration, 1.0 mg/ml; ×, injection of constant amount, 20.6 μg.

Flow-rate. Typical height equivalent to a theoretical plate (HETP) *versus* flow-rate plots of 100, 500, 1000, and 4000 Å columns are shown in Fig. 11 using water as the mobile phase and glucose as the test solute. A flow-rate of 0.5 ml/min was used for most of our studies. At this flow-rate, analysis times of less than 15 min are obtained using a two-column set.

Column selection. Because of the appearance of the extraneous permeated peak, 100 Å SynChrom columns have been used in conjunction with other pore size columns to separate the permeated peak from the polymer envelope. Recently, we have also used a shorter commercially available 3 cm × 4.6 mm I.D. guard column (Brownlee Labs., Santa Clara, Calif., U.S.A.) for this purpose. Calibration curves of 100–500, 100–1000, and 100–4000 Å column sets are shown in Fig. 12 using dextran standards and 0.7 *M* pH 3.7 ionic strength mobile phase.

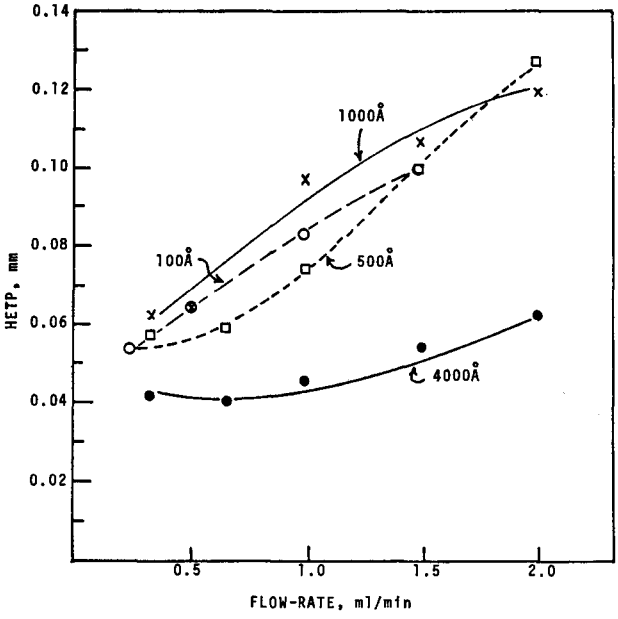

Fig. 11. HETP *versus* flow-rate. Mobile phase, water; injection volume, 20 μl of 1 mg/ml glucose; refractive index detection attenuation, $\times 4$.

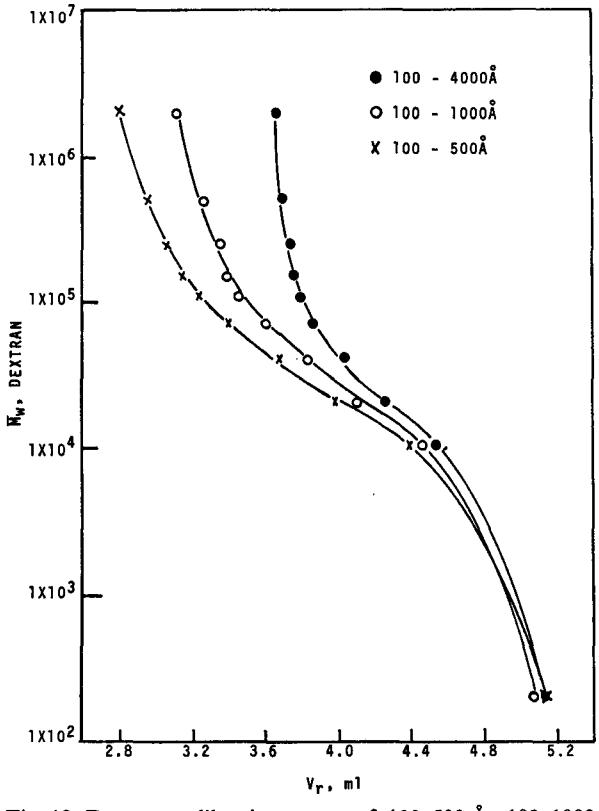

Fig. 12. Dextran calibration curve of 100–500 Å, 100–1000 Å, and 100–4000 Å on SynChropak column sets. Mobile phase, 0.7 *M* ionic strength pH 3.7 acetate buffer; flow-rate, 0.5 ml/min; injection volume, 20 μl of 1 mg/ml solutions. Samples; glucose: $10 \cdot 10^3$, $20 \cdot 10^3$, $40 \cdot 10^3$, $70 \cdot 10^3$, $110 \cdot 10^3$, $150 \cdot 10^3$, $250 \cdot 10^3$, $500 \cdot 10^3$ and $2000 \cdot 10^3$ weight-average molecular weight dextran standards.

In order to compare these calibration curves, separation factors, S, were calculated using the following equation:

$$S = \frac{V_{r1} - V_{r2}}{\log (V_{h2}/V_{h1})}$$

where V_r is the elution volume of a polymer of molecular weight V_h. Table VI lists these values for the middle and upper segments of the S-shaped curves. These two segments are presumably caused by the large pore-size distribution of the silica. As shown, the separation factors for the middle portion of the curve for the 100–500 and 100–1000 Å column-sets were significantly greater as compared to the 100–4000 Å column-set. The linear operating range of the 100–500 Å column set was significantly greater than for the other two column sets.

TABLE VI

CHARACTERISTICS OF SYNCHROPAK COLUMNS

Conditions: 1 mg/ml of glucose, $10 \cdot 10^3$, $20 \cdot 10^3$, $40 \cdot 10^3$, $70 \cdot 10^3$, $110 \cdot 10^3$, $150 \cdot 10^3$, $250 \cdot 10^3$, $500 \cdot 10^3$ and $2000 \cdot 10^3$ dextran standards; injection, 20 μl; flow-rate, 0.5 ml/min; mobile phase, 0.7 M ionic strength pH 3.7 buffer; column, 25 cm × 4.1 mm I.D.

Column set (Å)	*Middle segment of calibration curve*		*Upper segment of calibration curve*	
	*S (ml)**	*Linear range***	*S (ml)**	*Linear range***
100–500	1.06	$7 \cdot 10^4$–$1.1 \cdot 10^5$	0.30	$>1.1 \cdot 10^5$—5–$10 \cdot 10^6$***
100–1000	0.92	$9 \cdot 10^4$–$1 \cdot 10^5$	0.22	$>1 \cdot 10^5$—high
100–4000	0.76	$1 \cdot 10^4$–$7 \cdot 10^4$	0.09	$>7 \cdot 10^4$—very high

* Separation factor expressed as ml/decade of molecular weight.
** Molecular weight over linear portion of curve.
*** Extrapolated exclusion limit.

Concerning the upper segments of the curve, the 100–500 Å and 100–1000 Å column sets gave significantly higher separation factors than the 100–4000 Å set. Based on a total exclusion volume of 2.6 ml, the extrapolated exclusion limit for the 100–500 Å column set was estimated to be 5–$10 \cdot 10^6$. No attempt was made to determine the exclusion limits of the 100–1000 Å and 100–4000 Å column sets. Although these upper segments of the calibrations curves have low separation factors, they are still usable; the 100–4000 Å has limited use except for the highest molecular weight samples. These separation factors can be increased by adding more columns in series.

Table V lists recommended column sets to fractionate various types of modified cellulosics. Depending on the samples' composition, various column combinations can be used. Because of the high separation factor obtained with the 500 Å column, this column has been used to examine the MWD of low-molecular-weight fractions in high-molecular-weight samples as shown in Fig. 13. Although a 300 Å SynChropak column has not been evaluated in this present study, this column has been reported to give a higher separation factor than the 500 Å column[41].

Column calibration. Dextran can be used as a secondary standard to assign relative MWD to water-soluble cellulosic samples. However, these assignments must be used on a relative basis for sample comparison because of the cellulosic's greater

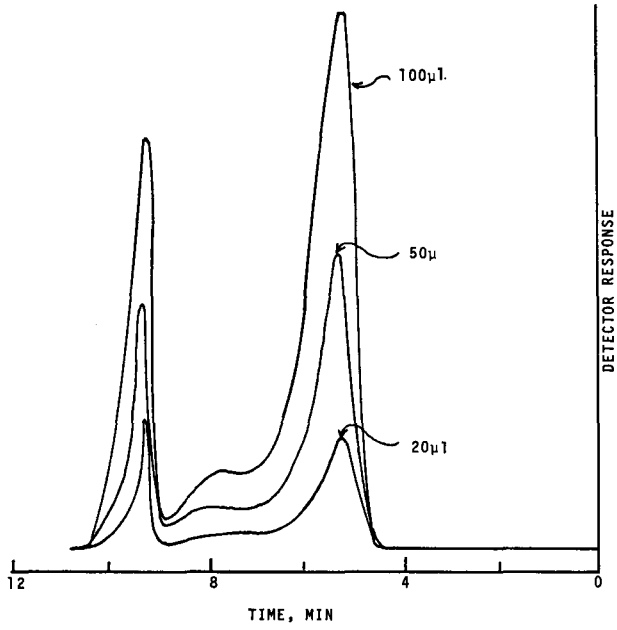

Fig. 13. GPC of CMC 7M-450. Column, 100–500 Å SynChropak column-set; mobile phase, phosphate buffer, 0.51 *M* ionic strength pH 6, sample size, 1 mg/ml; flow-rate, 0.5 ml/min; refractive index detector attenuation, ×4; chart speed, 0.5 in./min.

hydrodynamic volume than that of dextran of the same molecular weight[25]. In the past, we have been using this GPC method to compare peak profiles among samples. More recently, we have been using an on-line low-angle laser light scattering detector for absolute MWD[25].

Solution preparation. Depending on the sample, insoluble microgels, consisting of partially reacted cellulose, might be present. This can be easily removed by mem-

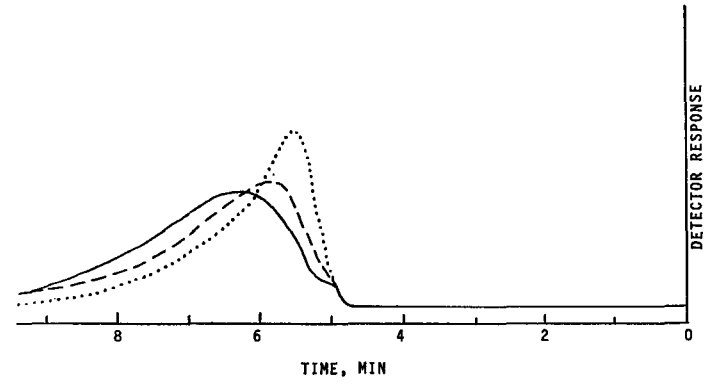

Fig. 14. GPC of CMC samples. Column, 100–500 Å SynChropak column-set; mobile phase, 0.7 *M* ionic strength acetate buffer pH 3.7; injection volume, 20 µl of 2.5 mg/ml solutions; flow-rate, 0.5 ml/min; refractive index detector attenuation, ×4; chart speed, 1 inch/min; samples: ....., 7L-27; - - - - - -, 7L2-12; ———, 7L1-8.

brane filtration (see Experimental section), and usually do not represent a significant portion of the sample[42]. If significant amounts of gel were present, there is the danger of concentration polarization occurring on the membrane, which might lead to ultrafiltration of the sample. Thus, the filtrate would not represent the soluble fraction of the sample[43]. If ultrafiltration is suspected, sample pre-treatment by centrifugation or use of a larger pore filter with a large surface areas can be tried. Occasionally, we have used depth filters (GF/F glass fiber filters; Whatman) for difficult samples.

Applications

Since this GPC method is reproducible and can give MWD within 15 min, it is being used on a routine basis in our laboratory to characterize CMC, HEC, and CMHEC samples. Several examples are given in Figs. 14–17; for clarity the permeated peaks have been omitted.

In addition to these water-soluble cellulosics, this method has also been applied to pectins[44] and polyacrylamide copolymers[45].

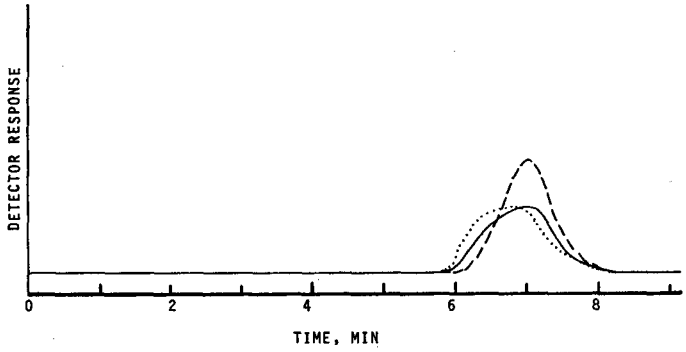

Fig. 15. GPC of CMC samples. Column, 100–4000 Å SynChropak column-set; injection volume, 20 μl of 0.5 mg/ml solutions; refractive index detector attenuation, $\times 2$. Samples: ———, 7H-1010; – – – – –, 7M-1000; ....., 7H4-3000.

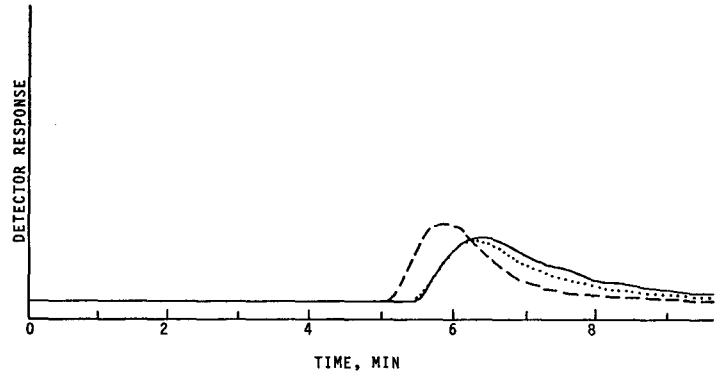

Fig. 16. GPC of HEC samples. Column, 100–1000 Å SynChropak column-set; injection volume, 20 μl of 1 mg/ml solutions; refractive index detector attenuation, $\times 4$. Samples: ———, 250L-110; – – – – –, 250GR-260; ....., 250JR-160. For other conditions, see Fig. 14.

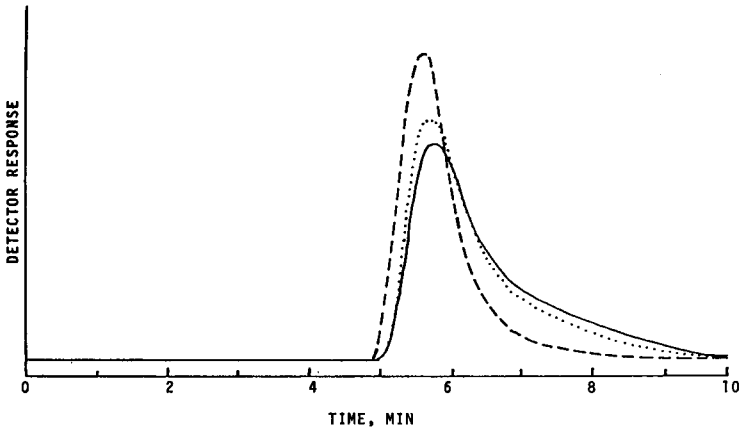

Fig. 17. GPC of HEC samples. Column, 100–500 Å SynChropak column set; injection volume, 20 μl of 2.5 mg/ml solutions; refractive index detector attenuation, $\times 4$. Samples: ———, 250L-110; – – – – –, 250GR-260; ....., 250JR-160. For other conditions, see Fig. 14.

CONCLUSIONS

This study demonstrates that high-performance GPC can be successfully used to determine the MWD of a number of water-soluble cellulosics; CMC, HEC and CMHEC. By using hydrophilically modified silica particles as the GPC packing (SynChropak) and a high ionic strength buffer (0.7 *M* pH 3.7), analyses were reproducible and no evidence of non-size-exclusion mechanisms was observed.

To prevent chromatographic overloading effects, the relative viscosities of injected solutions must be below 1.5. Because of the high viscosity of these cellulosics, low concentrations must be used. Injecting volumes greater than 50 μl results in significant loss of column efficiency. Because we are operating at the limits of the refractometer's sensitivity, we are presently investigating the use of a light scattering detector as a single detector for high-molecular-weight, high-viscosity samples.

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