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PROBLEMS ASSOCIATED WITH THE COLUMN PACKINGS USED IN THE CHARACTERISATION OF DEXTRAN BY AQUEOUS GEL PERMEATION CHROMATOGRAPHY

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SUMMARY

A number of rigid column packings (Spherosil–Porasil and DuPont SEC) and semi-rigid packings (two types of Spheron gel and Hydrogel) have been used for the molecular-weight characterisation of dextran by aqueous gel permeation chromatography. Our experience with these packings is reported and the various problems encountered, and their possible causes, are discussed. Our criteria for an acceptable packing have been fractionating range, efficiency, short analysis time and, of particular importance, long-term stability. None of the column packings considered was found to be ideal.

INTRODUCTION

The molecular-weight characterisation of dextran was one of the first applications of gel permeation chromatography (GPC) and in the past it has been carried out on chromatographic packings of cross-linked dextran[1], polyacrylamide[2] and agarose[3]. The high-porosity grades of these packings have poor mechanical stability and in recent years a number of organic and inorganic packings have been introduced which are more rigid and should therefore be more suitable for use in the modern chromatographic systems now available.

General liquid chromatographic techniques have improved considerably in the last few years. Faster analyses and high efficiencies have been produced by using columns as short as 10 cm packed with small particles, typically 10 μm diameter. The column packings for this high-performance liquid chromatography (HPLC) must be rigid enough to withstand the consequential high pressures involved.

The Chemical Engineering Department at the University of Aston in Birmingham have been involved in the continuous preparative GPC of dextran for some time[4–7]. Recently up to 600 g/h of dextran has been fractionated on equipment consisting of ten columns (70 cm × 5 cm diameter) packed with porous silica beads (200–500 μm diameter). The mode of operation of this type of apparatus has been described previously[6,7]. This preparative work has required an ancillary analytical

system, able to characterise dextran over a wide molecular-weight range (ideally 10^3 to $2 \cdot 10^6$ daltons).

"Fine" particles (95–105 μm), sieved from the preparative packing, mentioned above, were used as a chromatographic packing for one of the earlier analytical systems[7]. This system was based on an Auto-Analyser used both as a pump and as a detector using a cysteine–sulphuric acid colourmetric assay[8]; this was used successfully for 18 months. The major problem with this analytical system was the 2.5 h required for each analysis, and attempts were made to shorten this time.

The analytical system has now been improved considerably; the introduction of positive displacement pumps, better sample application and use of a differential refractometer as the detector were relatively straightforward modifications, but there have been considerable problems associated with the column packings used. Shorter analysis times and higher efficiencies require the use of smaller chromatographic particles. A number of rigid and semi-rigid GPC packings have been reported as suitable for dextran analysis[9–14], but there have been very few reports of these packings being used routinely for quality control and molecular-weight characterisation[15,16].

We have now subjected various rigid and semi-rigid packings to long-term use and our experiences are reported here.

EXPERIMENTAL

A relatively simple chromatographic system was used, consisting of a pump, sample-introduction device, column(s), detector and recorder. All the results were manually measured and calculated from the chromatograms.

The eluent was pumped with positive displacement pumps Normados Model, Bran and Luebbe; Series II Model, Metering Pumps). The samples were applied using sample-injection valves (Spectroscopic Accessory Co.; Types 30.100 and 30.501), fitted with 20-μl sample loops. The samples were detected with differential refractometers (Laboratory Data Control; Model 1107LJ) and displayed on flat-bed recorders (Smiths, Venture Servoscribe, Types 2 and 1b).

The low-pressure chromatography (below 20 bar) was carried out using glass HPLC columns (1 m $\times$ 4 mm diameter) (Corning, Corning, N.Y., U.S.A.) and the higher pressure work was with pre-packed stainless-steel columns (8 $\times$ 200 mm) (DuPont, Wilmington, Del., U.S.A.) or stainless-steel HPLC columns (8 $\times$ 250 mm) (Shandon, London, Great Britain). Various column-packing techniques were attempted for each chromatographic packing.

Throughout the work, the eluent was either distilled or deionised water Occasional checks on the pH found the water to be slightly acidic, typically pH 5.5. No buffers, electrolytes or bacteriostats were used with the eluent although the samples were often saturated with chloroform or had sodium azide present at 0.02% w/w as bacteriostats.

The efficiency of each column was calculated, for glucose as the solute, by the equation:

$$N = 8 \cdot \left(\frac{t_R}{w_{h/e}}\right)^2$$

where N is the number of theoretical plates, t_R is the peak retention time and $w_{h/e}$ is the peak width at the peak height, h, divided by e, the base of the natural logarithm

The packed columns were calibrated using dextran "T" fractions (Pharmacia, Uppsala, Sweden). The elution of the solutes was compared in terms of the Wheaton and Bauman[17] distribution coefficient, K_d:

$$K_d = \frac{V_e - V_0}{V_t}$$

where V_e is the elution volume of the solute, and V_0 is the void volume, measured by the elution of a totally excluded solute, Dextran 2000 (Pharmacia). V_t is the internal pore volume, measured as the difference between the elution volume of a totally permeable solute, such as glucose, and the void volume. The column calibration was related to K_d rather than elution volume, to avoid the necessity of re-calibration if a column was re-packed.

Spherosil XOB 075 and Porasil C

Porasil is the trade name under which Spherosil is marketed by Waters Assoc., Milford, Mass., U.S.A.[18]. The XOB 075 and the C grades are of equivalent porosity.

In the earlier work, where an Auto-Analyser was used as the pump and as the detector, the Spherosil XOB 075 (95–105 μm), sieved from the preparative packing, had been a reasonably successful analytical packing. One of the early attempts at improving the analytical system was to replace the Spherosil XOB 075 with Porasil C (37–75 μm). The efficiencies, analysis times and pressure drops associated with each column packing are listed in Table I.

TABLE I

CHROMATOGRAPHIC COLUMNS USED

Packing	*Particle size (μm)*	*Column dimensions (mm)*	*Efficiency (No. of plates)*	*Analysis time (min)*	*Pressure drop (bar)*
Spherosil XOB 075*	95–105	4 × 1200	1200	150	—**
Porasil C***	37–75	4 × 1000	1000	40	10
Hydrogel VI***	37–75	4 × 1000	1300	25	10
DuPont SEC SI500§	8	8 × 200	13,000§§	10§§	100
DuPont SEC SI100§	8	8 × 200			
Spheron P1,000§§§	20–40	4 × 1000	2200	20	35
Spheron P1,000†	17 ± 2.4	8 × 250	2250§§	10	65

* Obtained from Rhone-Progil, Neuilly-sur-Seine, France.
** Very low and not measured.
*** Obtained from Waters Assoc., Stockport, Great Britain.
§ Obtained from DuPont, Hitchin, Great Britain.
§§ Initial value.
§§§ Obtained from Koch-Light Labs., Colnbrook, Great Britain.
† Obtained from Prof. J. Kalal, Institute of Macromolecular Chemistry, Prague, Czechoslovakia.

The Porasil C column was in use for several months and neither this column nor the Spherosil XOB 075 column showed any sign of deterioration. However, an inconsistency observed with these columns was the occasional extension beyond K_d 1.0 of dextran peaks on the Porasil C, but not on the Spherosil XOB 075.

Hydrogel VI

Hydrogel is reported to be a highly cross-linked ethylene glycol dimethacrylate polymer packing[16].

We have used the high-porosity grade Hydrogel VI, packed into glass HPLC columns. Better efficiencies were obtained by dry packing rather than by aqueous slurry packing. No deterioration in efficiency or change in calibration were observed over the 10 months continuous use and an even longer term stability has been reported[16].

The properties of Hydrogel VI appear to be very suitable for a wide range of aqueous GPC applications, particularly where long-term stability is required, such as in quality control. For our particular application, a chromatographic packing with a higher exclusion limit would be preferred as it is necessary to characterise polymers with molecular weights greater than 10^6 daltons.

Unfortunately, Waters Assoc. seem to have withdrawn the Hydrogel range of GPC packings from the market, apparently due to poor batch reproducibility and the possibility of hydrolytic breakdown of the packing matrix.

DuPont size exclusion chromatography (SEC) columns

We have used a pair of pre-packed DuPont SEC columns, SI500 and SI100, arranged in series with the higher porosity grade SI500 first. Initially, these columns appeared to be ideal, with a high efficiency (Table I), fast analysis time and a very wide fractionating range, 10^3–10^6 daltons. The deteriorating condition of this pair of columns, over 6 months intermittent daytime use, is shown in Table II. The variation in flow-rate and pressure reflects the intermittent cleaning of the column pre-filters.

TABLE II

CHANGE IN EFFICIENCY OF THE DUPONT SEC COLUMNS WITH USE

Solutes: day 1, glucose and Dextran 2000; day 6–76, glucose and Dextran 40

*Day**	*Flow-rate (ml/min)*	*Pressure (bar)*	*Efficiency (plates)*
1	0.918	130	13,000
6	1.363	105	8000
7	1.245	105	8300
14	0.640	150	5000
20	0.780	115	3200
43	1.015	90	2400
64	1.242	105	900
76	1.084	110	1100

* Estimated days of use.

The first part of the drop in efficiency was accompanied by the appearance of a shoulder on the trailing side of the glucose peaks. The shoulder gradually developed into a large tail as the efficiency continued to drop.

The final efficiencies of the individual columns were found to be 500 plates for the SI500 column and 1600 plates for the SI100 column (measured at flow-rates of 0.20 and 0.30 cm^3/min respectively). Opening up the columns revealed that the top of the SI500 column bed had dropped *ca.* 5 mm and in the SI100 column the space

above the packing was *ca.* 1 mm. Thus, the SI500 column had deteriorated more than the SI100 column.

Scanning electron photomicrographs showed that both of the used column packings still consisted of spherical unbroken beads.

Spheron P1,000

Spheron is described as poly(2-hydroxyethyl-methacrylate–co-ethylene dimethacrylate)[19].

Initially poor efficiencies were obtained for the 20–40-μm packing when dry packing and slurry packing in water were attempted. However, when the column was slurry packed using aqueous methanol a reasonable efficiency was achieved. Compared with Hydrogel VI, which has a larger bead size, the 20–40-μm Spheron P1,000 was a more efficient packing with a slightly lower exclusion limit of *ca.* $5 \cdot 10^5$ daltons. This packing has not been used as extensively as the others, but it would appear to be reasonably stable.

The 17-μm bead size Spheron P1,000 was packed as an aqueous slurry, by upward delivery, into a Shandon HPLC column. The density of polymeric packings is much lower than that of the inorganic packings and therefore there would appear to be little to be gained by using a balanced density-packing technique.

Although good efficiencies could be obtained for the 17-μm packing, a small front was observed when glucose was eluted. The size of the front was decreased by an increase in flow-rate. Knox and Parcher[20] have described an "infinite-diameter effect" where, given a small bead size, a wide and short column, central sample injection and a sufficiently high flow-rate, the solutes are eluted before they can diffuse to the column walls. The combination of the sample-introduction loop and the relationship between the bead size and column dimensions is probably such that an "infinite-diameter effect" is almost achieved with this Spheron P1,000 column. A small amount of glucose is able to diffuse to the column wall from where it is eluted more quickly as a front. Solutes with a lower diffusion coefficient, such as the oligomers of glucose and dextran, will be less likely to diffuse to the column wall and an "infinite-diameter effect" is probably achieved for the dextran.

The good efficiency displayed by the 17-μm Spheron P1,000 could not be maintained for very long. The packed bed was found to compress and the efficiency was rapidly reduced. The rate at which the packed bed compressed appeared to be proportional to the number of dextran samples analysed and not necessarily to the amount of water passed through the column. After the column packing had compressed, it could be unpacked, redispersed and then re-used to give a column with the same initial efficiency to that previously achieved. The same packing, with a small amount of "topping-up" material, has been re-packed approximately twelve times over a period of 6 months without any deleterious results.

The re-packing of a column did not require a re-calibration because, as the elution is expressed in terms of K_d, it is only necessary to re-measure the void volume and the internal pore volume of each re-packed column. However, dextran "T" fractions were used occasionally to check that no change had occurred in the calibration.

DISCUSSION

To characterise dextrans with broad molecular-weight distributions we do not require a very high chromatographic column efficiency; about 1000 plates would suffice, but we have found 2000 plates to be much more acceptable when using the chromatogram to calculate molecular-weight parameters. What is required is long-term stability, a wide fractionation range (ideally 10^3–$2 \cdot 10^6$ daltons) and preferably a short analysis time.

When considering the fractionating range of a packing, it should be noted that the shape of the plots of the logarithm of the molecular weight *versus* K_d is more important than the fractionation limits. Most of these curves are asymptotic to the molecular-weight axis and it is impossible to be precise about the exclusion limit; therefore the exclusion limits mentioned in this paper are the approximate useful upper limits. A packing with a steep calibration curve will be more useful in this analytical application than the frequently encountered shallow ogive curve[21,22].

None of the chromatographic packings used in this work was ideal. Generally, the small bead-size packings, less than 20 μm, were unsatisfactory in their stability and the larger bead-size packings either had insufficient fractionating range or long analysis times.

The instability of the small bead-size packings could be due to chemical breakdown, mechanical breakdown or a combination of the two. Apparently one of the major reasons for withdrawing Hydrogel VI from the market was the possibility of hydrolytic breakdown of the packing. Hydrolytic breakdown of the chemically similar Spheron packings might also be expected. We have not observed any deterioration in the larger bead-size polymeric packings and while the small bead-size Spheron packing does compress, it can be re-packed with no apparent impairment. While we have not met with any problems of chemical breakdown with the polymeric packings, we have only used these packings in near-neutral water, pH 5.5, at ambient temperature. It is possible that raised temperatures or non-neutral conditions could cause hydrolytic attack to occur.

Except for problems of adsorption, it has generally been assumed that porous silica and glass are equally applicable in aqueous and organic solvents[23], and they can be deactivated to avoid any adsorption problems[13,24]. However, the equilibrium solubility for silica in neutral water is reported[25] to be *ca.* 0.01 %. If in fact equilibrium solubility was achieved, then our preparative packing should have shown signs of deterioration after a few days use and this was not the case. Corning Controlled Pore Glass (C.P.G.), which is 96 % silica, is reported to lose 1 % of its weight in 24 h under reasonable chromatographic conditions[26]. The stability of our preparative packing and our larger bead-size analytical silica packings has therefore been better than expected. Despite the stated rate of loss of silica with C.P.G., Basedow[15] has reported using this material for a considerable length of time.

Iler[27] has calculated the increased silica solubility with decreasing bead size and this has been demonstrated by Alexander[28]. Hetherington and Bell[29] have reported that the "solubility" of silica in water is 0.01 mg/dm^2·h, implying that the surface area is an important factor in the rate of silica loss. Although the structure of DuPont SEC is not reported, micro-particular silica packings are usually conglomerates of colloidal size particles[30,31] and for this structure it is probably the colloidal

"sub-bead" size that should be used in equilibrium solubility estimation. Particles of this structure also have large surface areas and therefore the evidence would suggest that the micro-particular silica packings may be expected to be less stable. However, any calculations based on equilibrium solubility or surface-area-related solubility predict a much greater breakdown than is consistent with the observed drop in efficiency. Also both the solubilities related to the bead size and to the surface area predict a greater breakdown for the lower porosity SI100 column whereas it is the higher porosity SI500 which has shown the greatest deterioration. The SI500 column may have been "protecting" the SI100 column, in series after it, but this argument is dubious when applied to silica dissolution because the equilibrium solubility is a long way from being reached.

The pulsation caused by the positive displacement pump was minimised by using a large pressure gauge followed by a long, narrow diameter tube. Despite this, the high pressures and high flow-rates used with the small bead-size packings results in significant pulsing at the column top. However, it has already been mentioned that the 17-μm Spheron compresses when samples are analysed and not when water alone is pumped through the column.

The switching of a sample-injection valve will produce sudden changes in flow-rate and pressure. Also the migration of a viscous slug of polymer will cause local changes in pressure drop across sections of the column packing. The deformability of the elastic polymer be adscould cause the bed to compress under the combined effect of the local pressure changes and the continuous overall pulsing.

The local pressure changes in the packed column, and the continuous pulsing, could also cause mechanical attrition in the micro-particular silica packings. The electron photomicrographs showed that the used packings consist of unbroken beads but this is not inconsistent with the "shock waves" stripping off the outer colloidal sub-beads. Assuming that the conglomerate structure of the micro-particular silica is that of close-packed spheres and that the bonding between the spheres is approximately the same, regardless of sphere size, then it would be easier to remove the larger outer sub-beads of the higher porosity SI500 and hence a greater deterioration would be expected for the higher porosity packing. Despite the loss of efficiency with the DuPont SEC columns, there was no change in the calibration and this implies that there was no change in the pore size of the packing. This is consistent with the mechanical removal of the outer sub-beads of these packings. It also seems plausible that the SI500 column could "protect" the SI100 column from mechanical damage. The strength of the pulses would be less and the concentration of any viscous polymer plug would be reduced.

It is of interest to note that DuPont have replaced their SEC columns with a new micro-particular silica packing (Zorbax-SIL) claimed to be suitable for aqueous GPC[32,33]. Also Waters Assoc. have introduced a micro-particular silica GPC packing to replace the Hydrogel packings. This packing is described as a controlled-porosity silica, deactivated with a bonded ether phase[10].

DuPont SEC has been successfully applied to routine analysis in acidic conditions[33]. Therefore while our evidence suggests that the deterioration in the DuPont SEC columns is mechanical, the dissolution of silica probably contributes to the ease of mechanical breakdown.

The two larger bead-size polymeric packings used, Hydrogel VI (37–75 μm)

and Spheron P1,000 (20–40 μm), did not show any signs of deterioration. Both of these packings were reasonably efficient and the analysis time was quite acceptable, but both had an exclusion limit which was too low, *ca.* $5 \cdot 10^5$ daltons, to allow complete molecular-weight characterisation for our application. Another hydrophilic polymeric GPC packing has recently been reported with a maximum exclusion limit of $7 \cdot 10^5$ daltons[34] and a more porous Spheron, P100,000, is also sold although no chromatographic separations have been reported with this packing. The polymeric GPC packings are prepared by suspension polymerisation and this technique is likely to show a significant batch variation, particularly with respect to exclusion limit.

The larger bead-size silica packings, Spherosil XOB 075 and Porasil C, did not show any sign of deterioration. Also these packings had acceptable exclusion limits, *ca.* 10^6 daltons, but in both cases the analysis time and efficiency were far from ideal. It is interesting to compare the similarly sized Porasil C and Hydrogel VI in terms of efficiency and analysis time. With the polymeric packing the deformability of the beads allows a more closely packed column to be achieved and this is reflected in a higher efficiency. In order to obtain an acceptable efficiency with the Porasil C, a much slower flow-rate must be employed and this is reflected in a long analysis time.

CONCLUSION

Our criteria for an acceptable GPC analytical packing, in order of importance, have been long-term stability, satisfactory molecular-weight fractionation range, good efficiency and short analysis time. We have found the best compromise to be the 17-μm Spheron P1,000 despite the continued need to re-pack the column.

The larger bead-size polymeric packings, Hydrogel VI and Spheron P1,000, appear to be very stable and are probably excellent packings for applications where the low exclusion limit is not a problem.

There is a lot of evidence to suggest that the silica columns should suffer from severe silica dissolution. In practice we have found the larger bead-size silica packings to be very stable although their very rigidity prevents the realisation of the good efficiency and short elution time desirable for analytical application. Unfortunately, the micro-particular silica packing that we have used, although it initially appeared to be ideal, has not been at all satisfactory with respect to stability. While we are dubious about the possibility of any micro-particular silica column packing showing long-term stability when used for aqueous GPC, we intend to investigate the new DuPont packing Zorbax-SIL.

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