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Note

Molecular weight distribution of hyaluronic acid by high-performance gel-permeation chromatography

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Sodium hyaluronate is a polysaccharide polyelectrolyte having high conformational rigidity[1]. It has been fractionated by conventional methods[2–4], and

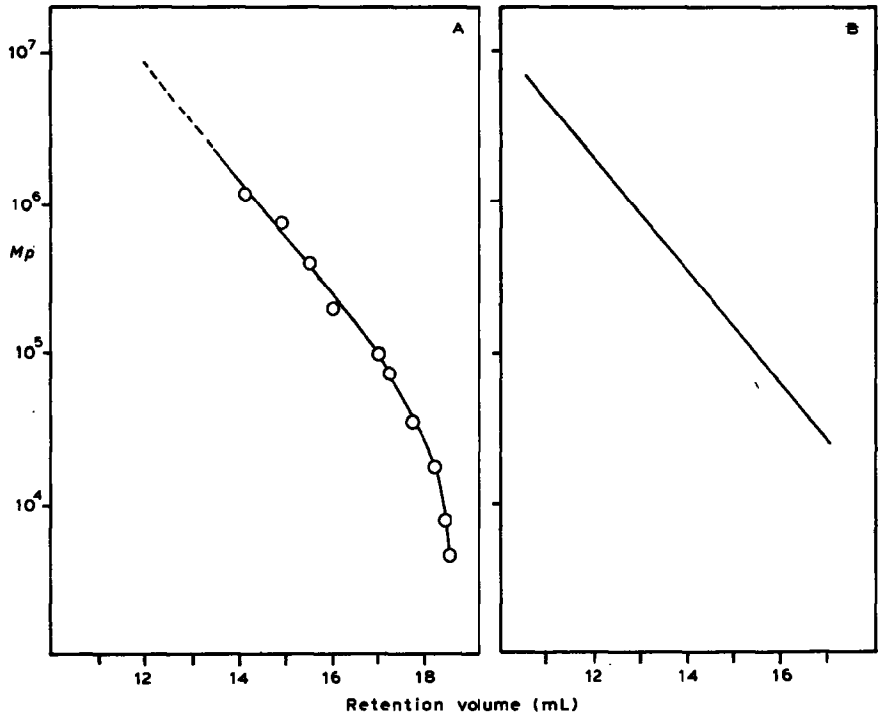

Fig. 1. Calibration curves for A, sodium polystyrene sulfonate in 0.2M NaCl; and B, hyaluronic acid in 0.2M NaCl; the curve in B is drawn by means of universal calibration (see text).

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its conformational properties have been examined both in solution and in the solid state[5–9].

Little has been reported[10–13] on gel-permeation chromatography (g.p.c.) or high-performance gel-permeation chromatography (h.p.g.p.c.) of hyaluronic acid. Few reports[14,15] are available on the determination of molecular weight using h.p.g.p.c. and no attempt has been reported on the evaluation of molecular weight distribution.

We now report an h.p.g.p.c. method for evaluating the above-mentioned parameters for samples of hyaluronic acid having average molecular weights in the range 10^5–10^6 (Table I)

The h.p.g.p.c. column was calibrated by the peak position method[16], using aqueous solutions of standards of sodium polystyrene sulfonate of narrow molecular-weight distribution. The results are reported in Fig. 1A.

The calibration curve for hyaluronic acid (Fig. 1B) was obtained from the calibration curve of the standards by applying the universal calibration technique (intrinsic viscosity × mol. wt., proportional to the hydrodynamic volume of the polymer molecules, is a universal parameter in g.p.c. calibration which governs size-separation[16]). Once the a and K viscometric parameters are known, the molecular weights of different polymers can be related directly to the retention volumes in g.p.c. In our work, the a and K values were 0.65 and 1.8×10^{-4} for the standard[17], and 0.72 and 7.3×10^{-4} for hyaluronic acid, respectively.

The elution patterns for two representative samples of hyaluronic acid are shown in Fig. 2. Exclusion peaks for salt and D_2O were observed and their elution

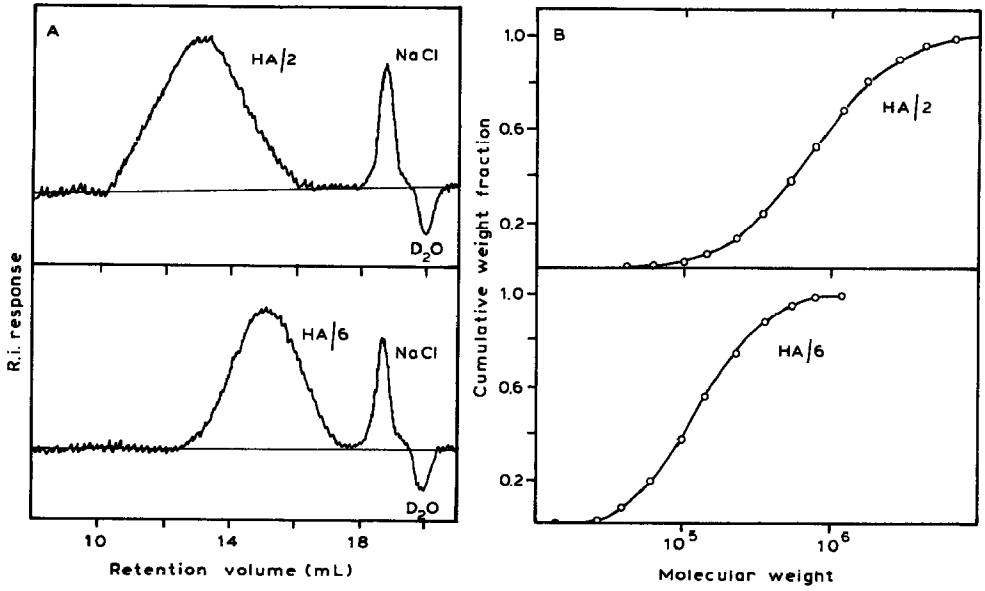

Fig. 2. A, H.p.g.p.c. elution patterns; and B, uncorrected integral distribution curves; for hyaluronic acid samples HA-2 and HA-6.

TABLE I

COMPARISON OF MOLECULAR WEIGHTS OBTAINED FOR VARIOUS SAMPLES OF HYALURONIC ACID BY LIGHT-SCATTERING (L.S.) AND G.P.C. TECHNIQUES

Sample	$(M_w)_{l.s.}$	$(M_w)_{g.p.c.}$[a]
HA-1	1.1×10^6	1.0×10^6
HA-2	9.5×10^5	9.6×10^5
HA-3	8.0×10^5	7.5×10^5
HA-4	5.6×10^5	5.4×10^5
HA-5	2.5×10^5	2.4×10^5
HA-6	1.5×10^5	1.5×10^5

[a]Peak-broadening effects are accounted for using the appropriate A parameters[24], obtained from elution curves for the standards.

is in agreement with the g.p.c. theory of polyelectrolytes[18]. The corresponding integral distribution curves are also presented in Fig. 2. The weight-average molecular weights obtained from the g.p.c. distribution data for all the samples of hyaluronic acid are compared with weight-average molecular weights obtained by light-scattering in Table I. The agreement is good.

These findings support the use of universal calibration as an adequate method for relating elution volumes in g.p.c. to the molecular weights of charged polymers under appropriate experimental conditions. Neutral standard polymers, such as dextrans, are not useful due to their small hydrodynamic volumes.

EXPERIMENTAL

Materials. — Highly purified hyaluronic acid[19], extracted from rooster comb and provided by Fidia Research Laboratories (Italy), typically contained only ~0.2% of other mucopolysaccharides, <0.2% of proteins, and <1% of sodium chloride.

Sodium polystyrene sulfonate standards with molecular weights (*Mp*) in the range 4.6×10^3–1.2×10^6 were obtained from Polymer Laboratories Ltd. (Great Britain). *Mp* corresponds to the peak molecular weight as determined by h.p.g.p.c. The M_w/M_n values were <1.1.

Solvent (0.2M NaCl containing 0.01% of NaN_3) and solutions were filtered using 0.45-μm Millipore filters (HAWPO1300).

The injected volume was always 100 μL. According to literature[18] suggestions, the polymer concentrations did not exceed the critical concentration (c*) at which the hydrodynamic volume of polymer chains equals the solution volume; c* has been obtained from both light-scattering and viscosity data, following the procedures previously described[20].

Liquid chromatography. — A Perkin–Elmer Series 3B HPLC apparatus was used, with a refractive index detector (1×10^{-5} r.i. units full-scale) and a flow rate of 1 mL/min which did not cause mechanical degradation of the samples[21].

The Shodex OHPAK B 806 column (50 × 0.8 cm i.d.) had a maximum volume (D_2O) of 20 mL. The excluded volume could not be determined experimentally, due to unavailabity of well characterised, water-soluble, high-molecular-weight standards. According to the manufacturers, the exclusion limit of this column corresponds to a mol. wt. of 25×10^6 for dextran. It can be assumed safely that it does not exceed 10 mL, since symmetrical elution patterns were obtained for the hyaluronic acid samples of highest molecular weight available for the above retention volume.

Experimental data were treated according to literature procedures[22].

Light-scattering. — These measurements were performed at 25° using a SOFICA Model 42000 photometer with cylindrical cells immersed in toluene. Non-polarised light (546 and 436 nm) was used, covering scattering angles θ between 30° and 150°. A Rayleigh ratio $R_{90} = 15.8 \times 10^{-6}$ cm^{-1} at 546 nm was used for calibration of the instrument with benzene. Before measurements were taken, the solutions and solvent were vigorously shaken for 1 h with 5:1 chloroform–isopentyl alcohol. The aqueous phase was separated and centrifuged for 2.5 h at 13,000 r.p.m. in a Beckmann L8-70 centrifuge.

The data were treated as previously reported[23]. The dn/dc value was 0.166 $mL.g^{-1}$, in agreement with literature data[2] for hyaluronic acid.

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