Journal of Chromatography, 299 (1984) 508–512
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,927

Note

Molecular weight determination of hyaluronic acid and its separation from mouse skin extract by high-performance gel permeation chromatography using a precision differential refractometer

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(Received May 29th, 1984)

Hyaluronic acid, a glycosaminoglycan, is ubiquitous in soft connective tissues such as the umbilical cord, synovial fluid and vitreous humour, and is also found in skin and cartilage. Its primary structure consists of repeating subunits of β-1,4-linked dissaccharides of glucuronic acid β-1,3-N-acetylglucosamine. A wide distribution of molecular weight has of the order of 10^4–10^7 been found. In biological fluids, hyaluronic acid is strikingly viscoelastic[1] but, in ageing or pathological fluid, loss of fluid viscosity and degradation of the hyaluronic acid occur[2,3]. Chromatography of hyaluronic acids has been studied using soft gels such as agarose gel[4,5] and ion-echange gel[6]. Their molecular weights have been determined by gel chromatography[5] and viscosity, ultracentrifuge and light-scattering measurements[7,8]. However, all of these methods require relative large amounts of material and long analysis times. For proteins, high-resolution separations and molecular weight determinations have become possible because of the development of pre-packed high-performance gel permeation chromatographic colums such as TSK-GEL SW type made from silica gel chemically bonded with hydrophilic compounds[9–13] and TSK-GEL PW type made from cross-linked hydrophilic polymers[12–15]. However, high-performance gel permeation chromatography of hyaluronic acid, a macromolecular glycosaminoglycan, has not yet been studied.

We have tried to achieve the separation of hyaluronic acid from other glycosaminoglycans and the determination of its molecular weight in a single run using small amounts of sample. This paper describes a rapid chromatographic procedure using the hydrophilic organic polymer gel column marketed by Showa Denko (Tokyo, Japan) as the Shodex OHpak B-800 series. As little as about 5 μg of hyaluronic acid can be detected on this support. This technique should serve as a powerful tool for examining the distribution of hyaluronic acid in connective tissues.

EXPERIMENTAL

Hyaluronic acid, grade I, from human umbilical cord was purchased from Sigma (St. Louis, MO, U.S.A.) and was used after purification through a Sephacryl S-500 column (45 × 2.5 cm I.D.) with 0.05 *M* phosphate buffer (pH 7.0) containing

0021-9673/84/$03.00

0.25 *M* sodium chloride at 4°C as it included about 25% of chondroitin sulphate, as shown in Fig. 1. Hyaluronic acid fractions were dialysed against deionized, distilled water and were lyophilized. The purified hyaluronic acid was dissolved in 0.1 *M* phosphate buffer (pH 6.8) containing 0.1 *M* sodium chloride, and the concentration was determined on the basis of its glucuronic acid content measured by the carbazole technique[16]. Hyaluronic acid from hog skin (sodium salt), chondroitin sulphate A from whale cartilage (sodium salt, mol.wt. 25,000–50,000), chondroitin sulphate B from hog skin (sodium salt, mol.wt. 11,000–25,000) and chondroitin sulphate C from shark cartilarge (sodium salt, mol.wt. 40,000–80,000) were obtained from Seikagaku Kogyo (Tokyo, Japan) and were used without further purification. Hog skin hyaluronic acid was free from chondroitin sulphates.

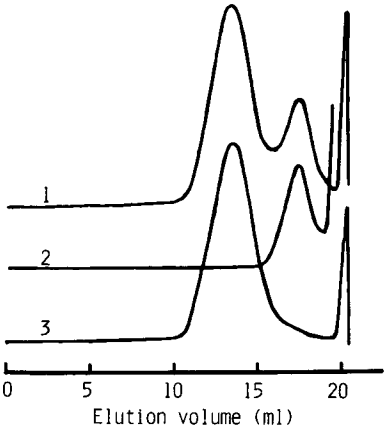

Fig. 1. Chromatograms of (1) commercial hyaluronic acid, (2) hyaluronidase-treated hyaluronic acid and (3) purified hyaluronic acid from human umbilical cord (Sigma, grade I). Column, Shodex OHpak B-806 (50 × 0.8 cm I.D.); eluent, 0.02 *M* NaCl; flow rate, 0.5 ml/min; injection volume, 200 μl; detector, refractive index $1 \cdot 10^{-5}$ refractive index units full-scale.

High-performance gel permeation chromatography was performed on a Shodex OHpak B-806 column (50 cm × 8 mm I.D.) preceded by a pre-column of Shodex OHpak 800p (50 × 6 mm I.D.). The column packing was a poly(hydroxyalkyl methacrylate) gel. The columns were attached to a Shimadzu LC-3A liquid chromatograph pump equipped with a Sil-1A injection valve and a 200-μl loop (Shimadzu, Kyoto, Japan). Detection was carried out at $1 \cdot 10^{-5}$ refractive index units full-scale with an Erma ERC-7520 precision differential refractometer thermostated at 40°C (Erma Optical Works, Tokyo, Japan). The height and areas of the peaks and the retention times were measured with a Shimadzu C-R2AX chromatograph integrator. Elution was performed with 0.02 *M* sodium chloride degassed with an Erma ERC-3310 degasser. The flow-rate was 0.5 ml/min.

Viscosity was measured in a calibrated, semimicro Cannon-Fenske viscometer at 25.0 ± 0.1°C. The flow time of distilled water was 200–800 sec at 30°C. The hyaluronic acid concentration was varried between 0.1 and 0.25 mg/ml for intrinsic viscosity [η] measurement. Dilutions were made with 0.1 *M* phosphate buffer (pH

6.8) containing 0.1 *M* sodium chloride. The molecular weights, *M*, were calculated from the intrinsic viscosities using the equation reported by Laurent *et al.*[7], $[\eta] = 3.6 \cdot 10^{-4} M^{0.78}$.

RESULTS AND DISCUSSION

The elution patterns of hyaluronic acids and chondroitin sulphates were investigated with distilled water and 0.01–0.2 *M* sodium chloride solution. Using distilled water, chondroitin sulphates were retained less than would have been expected on the basis of their molecular weights because of ionic repulsions with the stationary phase. In 0.02 *M* sodium chloride solution, all glycosaminoglycans tested were eluted satisfactorily, as shown in Fig. 2.

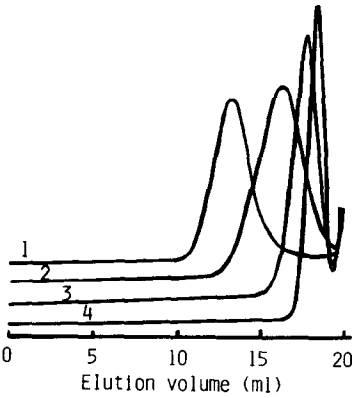

Fig. 2. Chromatograms of hyaluronic acid and chondroitin sulphates on a Shodex OHpak B-806 column. HPLC conditions as in Fig. 1. Sample concentrations: 200 μg/ml, except 100 μg/ml of 2. 1, Hyaluronic acid purified from human umbilical cord; 2, hyaluronic acid from hog skin; 3, chondroitin sulphate C from shark cartilage; 4, chondroitin sulphate B from hog skin.

Table I lists the elution volumes of umbilical cord hyaluronic acid obtained at various concentrations with the Shodex OHpak B-806 column. The viscoelastic property of hyaluronic acid increased the elution volume at concentrations of more than 267 μg/ml. At levels below 200 μg/ml, hyaluronic acid was eluted in the range 12.9–13.1 ml without the viscoelastic effect. Hence injection at a concentration of less than 200 μg/ml is recommended for the chromatography of hyaluronic acids. The detection limit measured refractometrically was 4.6 μg.

Intrinsic viscosity and molecular weight data for hyaluronic acids, are shown in Table II. The various molecular weights of hyaluronic acids were obtained by ascorbate- and Fe(III)–EDTA-induced degradation[17] under the conditions shown in Table II. The molecular weights of degraded hyaluronic acids were evaluated from the intrinsic viscosity data[7].

The elution positions of these hyaluronic acids using 0.02 *M* sodium chloride solution as the eluent are plotted against those of commercial chondroitin sulphates in Fig. 3. The elution volumes of hyaluronic acids from $9.8 \cdot 10^5$ to $1.1 \cdot 10^5$ were inversely correlated with the logarithms of their molecular weights. The results in

TABLE I

ELUTION VOLUMES OF VARIOUS CONCENTRATIONS OF UMBILICAL CORD HYALURONIC ACID ON A SHODEX OHpak B-806 COLUMN

Injection volume: 200 μl.

Concentration (μg/ml)	*Amount injected (μg)*	*Elution volume (ml)*
267	53.4	13.5
223	44.6	13.2
178	35.6	13.1
134	26.8	13.1
89	17.8	13.0
45	9.0	12.9
23	4.6	13.0

TABLE II

INTRINSIC VISCOSITY AND MOLECULAR WEIGHT DATA FOR HYALURONIC ACID DEGRADED UNDER VARIOUS CONDITIONS

Hyaluronic acids of various molecular weights were prepared by incubating hyaluronic acid solution (3 mg/ml) with ascorbic acid and Fe(III)–EDTA at 37°C for 16 h. Viscosity was measured between 100 and 250 μg/ml of hyaluronic acid. Dilution was made with 0.1 *M* phosphate containing 0.1 *M* NaCl (pH 6.8). Molecular weights were calculated using the equation $[\eta] = 3.6 \cdot 10^{-4} M^{0.78}$ (ref. 7).

Sample No.	*Sample*	*Intrinsic viscosity [η] (dl/g)*	*Molecular weight M (× 10⁵)*
1	Umbilical cord hyaluronic acid, purified	16.9	9.8
2	1 treated with 250 μ*M* ascorbic acid	9.6	4.7
3	1 treated with 375 μ*M* ascorbic acid	9.3	4.5
4	1 treated with 200 μ*M* ascorbic acid plus 2 μ*M* Fe(III)–EDTA	3.4	1.2
5	Hog skin hyaluronic acid	3.0	1.1

Fig. 3 show that the Shodex OHpak B-806 column can be used to determine the molecular weights of hyaluronic acids. The determination of molecular weights using the calibration graph in Fig. 3 could be performed even with one fiftieth of the amount (10 μg) of hyaluronic acid used in the semimicroviscosity measurement.

The elution curve of mouse skin extract is shown in Fig. 4. The chromatography was performed under the same conditions as for hyaluronic acid. Mouse skin extract was prepared during pronase digestion and deproteinization by adding trichloroacetic acid. The final buffer of the extract was 0.1 *M* Tris–HCl (pH 7.4) containing 0.15 *M* sodium chloride. The first broad peak was eluted in 13.5 ml and was positive in the uronic acid reaction using carbazole[16]. From these results and the calibration graph in Fig. 3, it was found to be hyaluronic acid of molecular weight 7×10^5. The method described here enabled us to separate hyaluronic acid from other glycosaminoglycans and to determine the molecular weight in about 40 min.

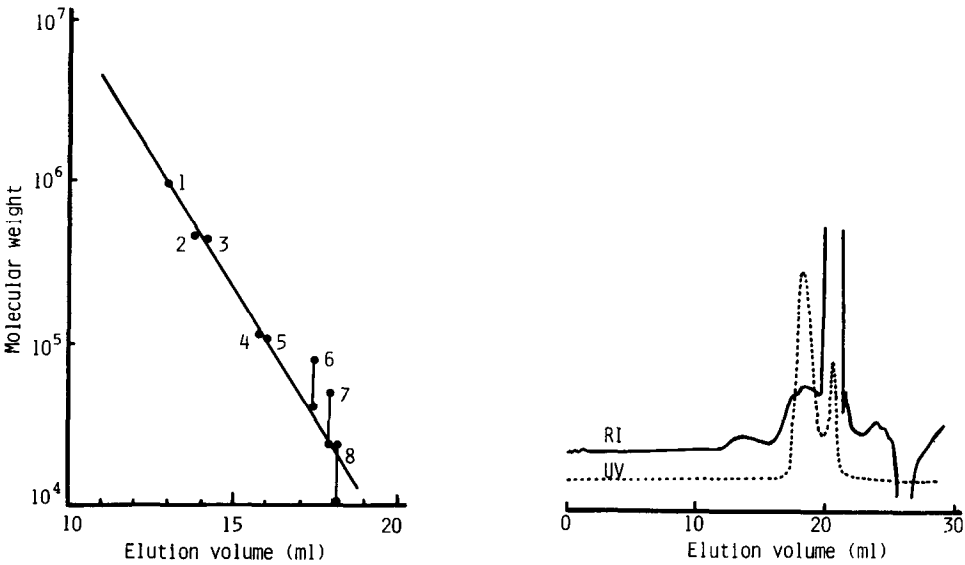

Fig. 3. Relationship between elution volume and logarithm of the molecular weight of hyaluronic acid using a Shodex OHpak B-806 column ($y = 10.15 - 0.32x$, $r = 0.993$). Hyaluronic acids were run at concentrations of 100–200 μg/ml with 0.02 *M* NaCl. Numbers 1–5 refer to sample numbers used in Table II; 6, chondroitin sulphate C (mol.wt. 40,000–80,000); 7, chondroitin sulphate A (mol.wt. 25,000–50,000); 8, chondroitin sulphate B (mol.wt. 11,000–25,000).

Fig. 4. Chromatogram of mouse skin extract after pronase digestion on a Shodex OHpak B-806 column. HPLC conditions as in Fig. 1. Detector: UV at 280 nm, 0.16 a.u.f.s.; RI, $1 \cdot 10^{-5}$ refractive index units full scale. The injection volume was 200 μl, corresponding to about 10 mg wet weight of skin of a 4-week-old male mouse.

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