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Relative Molecular Weight and Concentration Determination of Sodium Hyaluronate Solutions by Gel-Exclusion High-Performance Liquid Chromatography

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Gel-exclusion chromatography coupled with HPLC instrumentation can be used to determine the molecular weight of highly purified sodium hyaluronate in solution. The method is very reproducible, precise, and rapid, and allows molecular weight determinations up to 2 million to be done in the presence of considerable impurities. This technique offers considerable advantages over traditional light-scattering, sedimentation equilibrium, and viscometry methods for molecular weight determinations, in that HPLC–gel exclusion is rapid and not subject to errors arising from impurities. Simultaneous with molecular weight measurements, sodium hyaluronate concentrations can be determined with a lower range of 0.1 to 0.3 mg/ml dependent upon the sensitivity of the refractive index-detecting system. Unlike the carbazole reaction, this technique is unaffected by low-molecular-weight impurities such as monosaccharides or substances with relative molecular weights less than 18,000.

KEY WORDS: hyaluronate; HPLC; mucopolysaccharide; degenerative joint disease; arthritis.

Sodium hyaluronate is a long-chain biological polymer composed of repeating disaccharide units of *N*-acetylglucosamine and D-glucuronic acid joined by alternating $\beta(1-3)$-glucuronidic and $\beta(1-4)$-glucosaminidic bonds. It is found in many mammalian tissues and ranges in molecular weight from 5.0×10^5 to approximately 8×10^6 (1). Over the past 10 years there have been a number of studies reporting potential therapeutic applications of purified sodium hyaluronate. Indications of pharmacologic activity have been demonstrated in the treatment of inflammatory and degenerative joint diseases in both man and animals (2,3), prevention of postoperative tendon sheath adhesion formation (4), and facilitation of wound healing (5). Additionally, sodium hyaluronate preparations have been used as a replacement of the vitreous fluid during ophthalmic surgeries (6).

In all of these potential indications, the authors of the papers referenced above have suggested that part of the therapeutic efficacy of sodium hyaluronate is related to the extent of polymerization or the molecular weight of the repeating disaccharide chains. In general the greater the molecular weight the more effective the therapeutic response. Research in this area has, however, been hindered due to the difficulties in measuring the molecular weight of highly polymerized sodium hyaluronate fractions. For this reason relationships between pharmacologic activity and molecular weight have yet to be investigated in detail.

While spectral and chemical properties of sodium hyaluronate are easily obtained by traditional methods, determinations of molecular weight have been approached using a variety of complicated techniques. Molecular weight estimations have been reported using light scattering (7–10), sedimentation velocity and equilibrium ultracentrifugation (11,12), and viscometry (13). All of these procedures yield weight-average molecular weight data

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and considerable variations in molecular weights have been reported for equivalent preparations measured by different techniques.

Intrinsic viscosity measurements, one of the more widely reported methods of estimating molecular weights of hyaluronate preparations, are inherently difficult due to the non-Newtonian behavior of the polymer. Calculations of molecular weight from intrinsic viscosity data require assumptions of molecular configuration of the polymer in solution which may not be totally correct. Changes in the pH, or ionic strength, of the solution can produce significant variations in molecular configuration resulting in widely divergent molecular weights of equivalent preparations. Although such studies have been useful in establishing relative molecular weights or sizes of different sodium hyaluronate preparations, considerable uncertainties exist in these measurements due to the necessary extrapolations to ideality.

Exclusion chromatography, which separates molecules according to their effective size in solution, offers a convenient alternative method for determining relative molecular masses or sizes of biological molecules ranging from a few thousand to several hundred thousand daltons. With the development of new exclusion matrices for high-performance liquid chromatography systems, the effective range of molecular sizing has been extended to several million daltons, which encompasses most sodium hyaluronate preparations. The use of HPLC instrumentation coupled with exclusion matrices has greatly improved the speed and precision of such measurements. The technique is rapid, reproducible, and free from many of the problems associated with light scattering, sedimentation equilibrium, and viscometry. The data presented here demonstrate the use of HPLC–gel-exclusion chromatography for measuring the molecular weight and concentration of highly polymerized sodium hyaluronate. The method provides a high degree of precision and offers the advantages of rapid, reproducible measurements of molecular weight and concentration even in the presence of considerable lower molecular weight impurities. It should be noted that the well-known limitations with regard to interpretation of apparent molecular weight calculations inherent to gel-exclusion chromatography should be considered.

MATERIALS AND METHODS

High-performance liquid chromatography. Exclusion chromatography was performed on a Varian 5000 HPLC instrument equipped with a Toyo Soda TSK G6000 PW exclusion column (7.5 mm × 30 cm), with a guard column of the same material, and a 10-μl sample loop. Peak elutions were monitored with a Varian series RI-3 refractive index detector. Retention times and peak integration were recorded by a Spectra Physics 4270 integrator. The HPLC elution buffer was 150 mM NaCl, 3 mM NaH_2PO_4 (pH 7.0), and 0.02% NaN_3 as a preservative. Flow rates were 1.0 ml/min at 4.0 atmospheres. All measurements were made at room temperature.

Precise determination of the excluded volume of the column (V_i) is not presently possible because of the inavailability of well-characterized water-soluble high-molecular-weight standards. The literature provided by Toyo Soda for the TSK G6000 PW column shows an exclusion limit of 8 million using polyethylene glycols. The limit of the included volume (V_i) is identified on all chromatograms by the solvent peak.

Because the excluded volume of these columns cannot at present be clearly defined, the upper limit of molecular weight determination is unknown. The possibility exists that a non-log-linear relationship may exist near the void volume. For these reasons, caution should be exercised in extending the linear relationship between retention time and molecular weight as described herein beyond 2 million. Because the relationship is logarithmic and the void volume has been placed at about 8 million, the linear relation-

ship most likely continues through 4 to 6 million.

Molecular weight standards. Polyethylene oxides (PEO types 4 and 5 million) were obtained from Aldrich Chemical Company, Milwaukee, Wisc. and standardized by Modchrom, Inc. of Ohio. Additional polyethylene oxide standards were obtained from Toyo Soda. These latter standards have been characterized by light scattering, gel-permeation chromatography, and viscometry. The standards were dissolved in the HPLC elution buffer at 2 mg/ml prior to injection. Polyethylene oxide was chosen as the standard because it is one of the few, stable water-soluble polymers with fractions available in the appropriate molecular weight range.

Sodium hyaluronate and hyaluronidase. Sodium hyaluronate (NaHA) was prepared from rooster combs by extraction into 0.15 M NaCl and repeated cetylpyridinium chloride precipitation. Impurities were removed by ultrafiltration using an Amicon DC-30A diafiltration apparatus equipped with a 100,000-Da hollow-fiber cartridge (Amicon, Inc., Danvers, Mass.). The purified sodium hyaluronate was collected by precipitation in 95% ethanol and vacuum dried over silica gel. Three stock sodium hyaluronate solutions (NaHA-1, NaHA-2, NaHA-3) were prepared at 5 mg/ml in 0.9% w/v saline and sterilized by 0.22-μm membrane filtration. Molecular weight variations were produced by limited thermal shearing in an autoclave (i.e., NaHA-3 was produced by autoclaving NaHA-2 at 121°C for 60 min).

Concentrations of sodium hyaluronate in solution were determined by the carbazol method (14). Dilutions of known sodium hyaluronate concentrations were made by weight to four decimal places on a Mettler AE100 digital analytical balance using 1.00 as the solution density for all sodium hyaluronate concentrations. Concentration measurements made before and after thermal shearing showed no change in the glucuronate content, indicating no substantial degradation of the component sugars.

Elemental analyses of the hyaluronate preparations were performed by Galbraith Laboratories, Inc. These showed 36% carbon, 5.89% hydrogen, 2.86% nitrogen, and 49.46% oxygen. Sodium was not analyzed. Assuming one sodium per dimer, and 4.04 waters of hydration per dimer, the measured weight percentages divided by the theoretical weight percentage yield percentage ratios as follows: C = 101.5%, H = 99.4%, N = 96.8% and O = 97.4%. Protein was undetectable in these samples using a micro-Bio-Rad (15) or Lowry assay. Electrophoresis on cellulose showed the HA to migrate as a single alcian blue staining spot, indicating no chondroitin sulfates or other glycosaminoglycan contamination (16). Additionally, chondroitin sulfates and other nonhyaluronate glycosaminoglycans are separated by this procedure.

Enzymatic degradation of the sodium hyaluronate samples was done by incubation at 37°C with 50 TRU of *Streptomyces* hyaluronidase (Calbiochem-Behring, La Jolla, Calif.), a fungal, eliminase-type hyaluronidase which is specific for hyaluronic acid. In limit digests, it produces Δ-4,5-unsaturated tetra- and hexasaccharides (17).

Intrinsic viscosity. The following definitions of viscosity are taken from Tanford (18). Specific viscosity is $(n/n_0 - 1)$, where n is the measured macroscopic viscosity of the sample, and n_0 is defined as the corresponding viscosity of the pure solvent. Reduced viscosity is specific viscosity/concentration. The limiting value for reduced viscosity at the limit of zero concentration is defined as intrinsic viscosity. Since concentration of sodium hyaluronate is determined in milligrams per milliliter and viscosity units are cancelled from the definition, the units of intrinsic viscosity are milliliters per milligram. To be consistent with the previous literature, the units of intrinsic viscosity reported here are milliliters per gram (1 ml/mg = 1000 ml/g).

Relative viscosities of the sodium hyaluronate solutions were measured with a calibrated Cannon-Manning semimicro viscometer (No. 100, Cannon Instrument Co.,

Boalsburg, Pa.) in a constant-temperature water bath at 25°C. Intrinsic viscosities were obtained from a series of sodium hyaluronate dilutions (9).

While intrinsic viscosity is generally determined from reduced viscosity data extrapolated to both zero shear rate and zero concentration, the shear rate of the semimicro viscometer used in these studies was sufficiently close to zero (9.7 s^{-1}) to accept the limit value of reduced viscosity at zero concentration as a measure of intrinsic viscosity. To check this, a Ubbelohde type-75 viscometer was used on several sodium hyaluronate samples to directly determine the limiting viscosity value at both zero shear and zero concentration. Both instruments yielded the same values for intrinsic viscosity.

Because sodium hyaluronate is a non-Newtonian fluid, the reduced viscosity is strongly concentration dependent. Over the range of dilutions used in the determination of intrinsic viscosity. Furthermore, the plot of reduced viscosity versus concentration can be described by an equation which approximates a single exponential of the form $y = A + Be^{Ax}$. In practice, the determination of intrinsic viscosity was made by plotting reduced viscosity (ordinate) versus concentration (abscissa), and obtaining a least-squares best fit to the exponential function. Then at zero concentration the intrinsic viscosity is given as the sum of coefficients A and B.

RESULTS

Relative Molecular Weight

Under the conditions employed all molecular weight standards were eluted within the included volume of the column. The minimum molecular weight which could be resolved with this system was 18,000. Retention times of each polyethylene oxide (PEO)[1] standard were proportional to the log of molecular weight. Figure 1 shows the linear relationship between log molecular weight and retention time of the PEO standards. Measurements made over 10 consecutive days showed excellent reproducibility. The largest variation was less than 5 s in 8 to 12 min of run time. Mean and standard deviations for retention times for each standard at 2 mg/ml are given in Table 1. Comparison of the standard deviation with mean retention times revealed no more than a 0.8% variance among replicate determinations. Retention times were also found to be independent of PEO concentration between 0.5 and 5.0 mg/ml (data not shown).

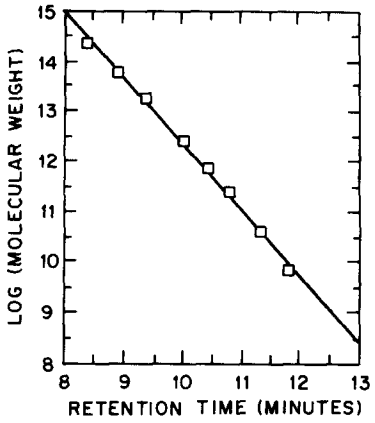

FIG. 1. HPLC–gel-exclusion chromatography standard curve for polyethylene oxides. Polyethylene oxides of varying mean molecular weights from 18,000 to 1.71 million were chromatographed as described under Materials and Methods. Each point represents the retention time of the PEO peak from a 17-min chromatogram. The column flow rate was 1.0 ml/min. The ordinate is in log (base e) units and the line drawn through the points is a linear least-squares fit showing the linearity of the procedure throughout this range. The equation of this fitted line was used to obtain the relative molecular weights of unknowns from their retention times. $\text{Log}_e (M_r)$ = slope × retention time + intercept; slope = 1.33, intercept = 25.67, coefficient of correlation = −0.9978.

Sodium hyaluronate (NaHA) eluted as a single peak within the included volume of the column (Fig. 2). The peak at 13.20 minutes represents the sample solvent peak and marks the end of the sample volume which is included by the column. The retention times for repeated injections of the three NaHA preparations showed excellent repro-

[1] Abbreviation used: PEO, polyethylene oxide.

TABLE 1

RETENTION TIMES OF POLYETHYLENE OXIDE STANDARDS

Type	M_r	Run 1	Run 2	Run 3	Run 4	Mean ± SD
SE2	18000	11.93	11.31	11.77	11.78	11.82 ± 0.07
SE5	39000	11.36	11.32	11.34	11.32	11.34 ± 0.02
SE8	86000	10.81	10.81	10.81	10.77	10.80 ± 0.02
SE15	140000	10.44	10.44	10.42	10.45	10.44 ± 0.01
SE30	250000	10.01	10.01	10.10	10.04	10.04 ± 0.04
SE70	590000	9.37	9.38	9.39	9.37	9.38 ± 0.01
SE150	990000	8.90	8.90	8.94	8.90	8.91 ± 0.02
SE250-4	1710000	8.45	8.33	8.45	8.34	8.39 ± 0.07

[a] Retention times of polyethylene oxide standards by HPLC–gel-exclusion chromatography. The eight standardized polyethylene oxides are listed in ascending order according to their molecular weight. For each standard, four injections were made to obtain the mean and standard deviations of the retention times. The average standard deviation over all molecular weight sizes is 0.0325 min or less than 2 s.

ducibility: NaHA-1, $R_T = 8.18 \pm 0.02$; NaHA-2, $R_T = 8.22 \pm 0.06$; NaHA-3, $R_T = 8.74 \pm 0.03$ (Table 2). Brief treatment of either of the three sodium hyaluronate preparations with (*Streptomyces*) hyaluronidase produced the expected increases in retention times resulting from enzymatic digestion of the sodium hyaluronate polymer. Prolonged incubation with hyaluronidase (>2 h), before chromatography, completely eliminated the high-molecular-weight peak from the included volume of the column.

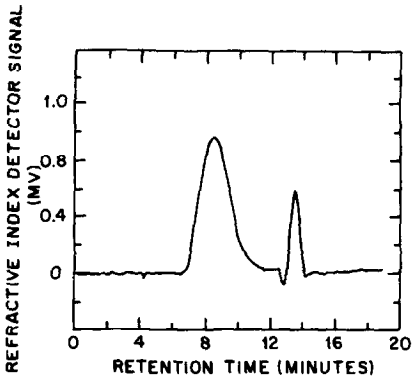

FIG. 2. HPLC–gel-exclusion chromatography of sodium hyaluronate. A 10-μl sample of sodium hyaluronate at 1.5 mg/ml was chromatographed as described under Materials and Methods. The larger peak at 8.22 min is the sodium hyaluronate; the smaller peak at 13.20 min is due to the refractive index difference between the sample solvent and the chromatographic buffer solvent.

Figure 3 illustrates the effects of hyaluronidase digestion on the chromatographic behavior of sodium hyaluronate. Prior to enzymatic digestion two peaks are observed (Fig. 3a). The broad peak at 8.49 min (Peak A) corresponds to the sodium hyaluronate; the smaller sharper peak (Peak B) is due to the difference in the refractive index between the sample buffer and the elution buffer. This latter peak also represents the lower limit of migration for molecular sieving and is thus the position of migration for the hyaluronidase-digested sodium hyaluronate (Fig. 3b). After hyaluronidase digestion, the high-molecular-weight sodium hyaluronate peak is absent and the digestion fragments appear at the solvent peak, thereby increasing the area of the latter.

The retention times reported here are unique for the specific instrument and column configuration. Any changes in operating parameters, column length and composition, and elution buffer (pH and ionic strength) can be expected to result in different retention times.

Dependence of HPLC Retention Time on Sample Concentration

The rate of flow of solutions of sodium hyaluronate through the small-bore capillaries

TABLE 2

REPRODUCEABILITY OF HPLC-GEL EXCLUSION CHROMATOGRAPHY USING SODIUM HYALURONATE

Type	Run 1	Run 2	Run 3	Run 4	Mean ± SD
NaHA-1	8.17	8.17	8.17	8.20	8.18 ± 0.02
NaHA-2	8.22	8.29	8.15	8.21	8.22 ± 0.06
NaHA-3	8.75	8.69	8.76	8.74	8.74 ± 0.03

Reproducibility of the HPLC–gel-exclusion chromatography method using sodium hyaluronate. Three hyaluronate preparations of different mean molecular weights were chromatographed four times each to obtain mean and standard deviation retention times. The average deviation reported here is 0.0367 min or 2.2 s. This corresponds closely to the deviations observed for polyethylene oxide standards and suggests that the method is very reproducible.

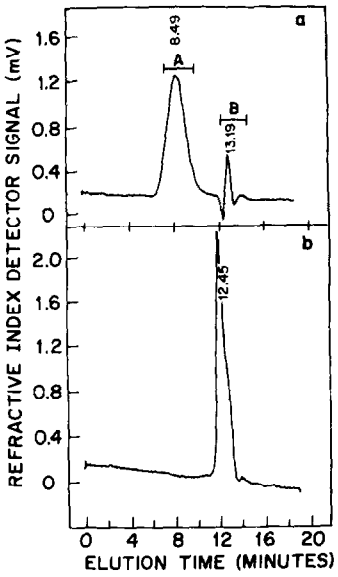

FIG. 3. Degradation of hyaluronate by *Streptomyces* hyaluronidase. To 1.0 ml of a standardized sodium hyaluronate sample (2 mg/ml) was added 1.0 ml of *Streptomyces* hyaluronidase containing 50 international units of activity. The top panel (a) shows a chromatogram of the sodium hyaluronate (2 mg/ml) prior to the enzyme addition. The peak retention of 8.49 corresponds to a molecular weight average of 1.7 million. The bottom panel is a chromatogram of the enzyme–sodium hyaluronate mixture after 3 h at 37°C. At that time the sodium hyaluronate is displaying an average molecular weight of approximately 9000, demonstrated by a retention time of 12.45 min. If the integration of the sodium hyaluronate peak in (a), is calculated for a 1-mg/ml sample size and is then added to the integration of the solvent peak, the value obtained matches that of the integration of peaks shown in (b). This indicated that the small size of the molecules after digestion did not effect the determination of concentration. The amount of enzyme added as hyaluronidase is so small that its contribution to refractive index is negligible.

typical of modern HPLC systems was found to be inversely proportional to concentration in the range 1–5 mg/ml, and thus the elution times from the sizing column are affected by sample concentration. This anomaly becomes more pronounced with increasing molecular weight. The behavior is predicted by a statistical treatment of the physical properties of flexible polymers, because the excluded volume which establishes the radius of gyration and, therefore, the apparent molecular weight by exclusion chromatography, is a function of the molecular concentration. The intermolecular interaction of the sodium hyaluronate polymer molecules increases with increasing concentration so that the flow through the small-bore tubing of the HPLC system is reduced by increasing sodium hyaluronate concentration. Further development of this principle can be found in Tanford (18).

The retention times observed for different sodium hyaluronate preparations injected at various concentrations are consistent with these theoretical considerations. Figure 4 illustrates the dependence of retention time on both concentration and molecular weight for two sodium hyaluronate preparations. Since retention time is a logarithmic function of molecular weight, the slope of the line is significant, and it is necessary to extrapolate to a retention time at zero concentration for the purpose of determining molecular weight. The effect of concentration on retention time is more pronounced in the larger molecular

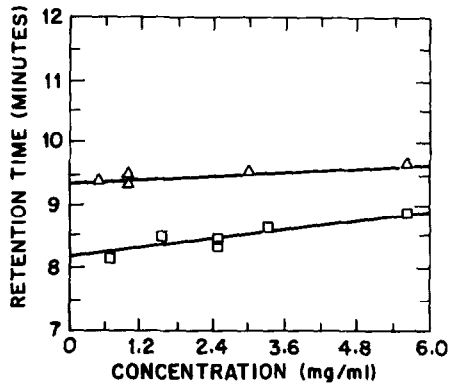

FIG. 4. Effect of concentration on retention time of sodium hyaluronate. Samples of sodium hyaluronate were prepared at various concentrations and chromatographed as described under Materials and Methods. Each point represents a separate chromatogram of the sample at the concentration indicated. □, A typical high-molecular-weight sodium hyaluronate preparation (NaHA-2). △, Data points obtained after autoclaving (thus thermally degrading) the sodium hyaluronate sample at 121°C for 1 h (NaHA-3).

weight preparation. This effect is not observed for the polyethylene oxide standards because they behave ideally at the concentration used. In order to determine an accurate relative molecular weight for sodium hyaluronate solutions, an extrapolation from several concentration points between 1 and 5 mg/ml to zero concentration is required to remove the effect of molecular concentration on the retention time and subsequently determine molecular weight.

Correlation of Molecular Weight with Intrinsic Viscosity

Comparison between the HPLC-gel-exclusion method described here and traditional viscometry show that the relative molecular weight calculated from intrinsic viscosity and gel exclusion are in agreement. The empirical relationship between molecular weight and intrinsic viscosity for polymers like sodium hyaluronate is given by Tanford as $[n] = K'M^a$ where [n] is intrinsic viscosity in units of milliliters per gram, M is molecular weight, and the constants K' and a are to be determined by a particular set of physical conditions (18). The relationship between intrinsic viscosity and molecular weight as determined by HPLC-gel exclusion is shown in Fig. 5. Using the empirical relationship described above, the constants K' and a were derived by least-squares fitting to these data ($K' = 0.3562 \times 10^{-3}$; $a = 1.0699$). Comparison of molecular weights calculated from intrinsic viscosity measurements with that determined by HPLC-gel-exclusion chromatography are in excellent agreement.

Determination of Sodium Hyaluronate Concentration from HPLC Data

Simultaneous with the determination of molecular weight, HPLC-gel-exclusion chromatography can provide accurate determinations of sodium hyaluronate concentration. The area under the sodium hyaluronate peak was found to be linearly proportional to concentration in the range of 1 to 6 mg/ml (Figure 6). These data were collected over a 2-week period, demonstrating the reproducibility of the measurement with time. By establishing the relationship between the area under the curve and the concentration of a

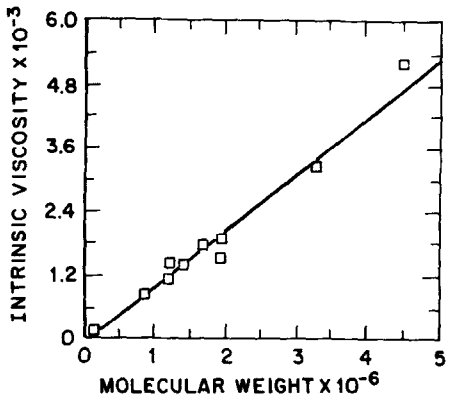

FIG. 5. The relationship of intrinsic viscosity to molecular weight as determined by high-performance exclusion chromatography. Sodium hyaluronate samples of varying molecular weight were produced by thermal degradation. For each sample the intrinsic viscosity (ml/g) and molecular weight were determined as described under Materials and Methods. The line is a nonlinear least-squares fit to the empirical formula $[n] = K'M^a$. For the data shown $K' = 0.3562 \times 10^{-3}$ and $a = 1.0699$.

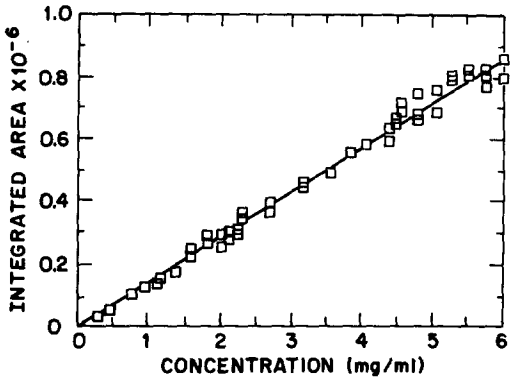

FIG. 6. Standard curve for the determination of sodium hyaluronate concentration by high-performance exclusion chromatography. Each point represents a different concentration of sodium hyaluronate. These concentrations were prepared by dilution from a carbazole standardized sodium hyaluronate preparation. The integration was performed digitally by an automated integrator attached to the refractive index detector. For the column configuration described here, the relationship between concentration and integrated area is linear and passes through zero (slope = 142793). The units of integration are μm^2.

known sodium hyaluronate preparation, the concentration of any unknown sample can be determined easily. For routine work only three to four concentrations of known sodium hyaluronate are necessary to construct the standard curve. Duplicate chromatograms may then be run in a short period of time, and the coefficient of correlation for linearity is invariably better than 0.990. Unknown hyaluronate concentrations may thus be determined from the relationship

[HA]
= (integration number − intercept)/slope.

Additionally, since gel-exclusion chromatography separates the sodium hyaluronate from most common impurities, the measurements of molecular weight and concentration can be made with relatively impure samples. This is a considerable advantage over traditional methods which require highly purified preparations for accurate results.

DISCUSSION

The results presented here demonstrate that HPLC–gel-exclusion chromatography provides a rapid, reproducible method for the simultaneous determination of concentration and molecular weight of sodium hyaluronate in solution. Although the procedure requires standardization of the column by a known concentration of sodium hyaluronate and externally standardized polyethylene oxides, the standardization is rapid and remains constant for several months. Compared to alternative methods for the determination of molecular weight and concentration, this method is rapid and also free from many experimental difficulties associated with impurities in the sodium hyaluronate sample.

Several applications of the method are important to the field of polymeric carbohydrate chemistry. HPLC–gel-exclusion chromatography offers an inexpensive and very precise method of monitoring stability of hyaluronate, since degradation of long-chain polymers is based on the average molecular weight of the sample. In practice as many as 15 to 20 determinations can be made in a single day. With the addition of automated injection equipment, the number of analyses could be increased considerably.

In the area of arthritis research, there is considerable interest in the degradation of endogenous sodium hyaluronate in synovial fluid as it relates to the pathogenesis of the disease process. Since the sample size is often limited in such studies, the 10-μl requirement for HPLC–gel-exclusion chromatography is a significant advantage. Moreover, there is no need to isolate and purify the sodium hyaluronate prior to chromatography.

Although the results shown here indicate the ability of the chromatographic method to separate hyaluronate from other impurities, possible interference by high-molecular-weight proteoglycans, nucleic acids, or hyaluronate-associated proteins has not been examined. Furthermore, treatment of impure hyaluronate preparations by protease or nuclease digestion may be necessary.

For the determination of hyaluronate concentrations in solution, HPLC–gel exclusion has an important advantage in being able to

give accurate data in the presence of considerable impurities. Traditional dye binding or carbazole methods are very sensitive to impurities, which greatly limit their utility in this application. Additionally, the method suggests a rapid and convenient assay for hyaluronidase enzymes, which would permit more detailed studies of these systems.

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