Quantitation of Hyaluronic Acid and Chondroitin Sulphates in Rabbit Synovial Fluid by High-Performance Liquid Chromatography of Oligosaccharides Enzymatically Derived Thereof

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A high-performance liquid chromatographic (HPLC) method for quantifying unsaturated hexasaccharide and tetrasaccharide from *Streptomyces* hyaluronidase enzyme digestion products of hyaluronic acid was developed using a gel-permeation column packed with a sulphated polystyrene-divinylbenzene gel. For the oligosaccharides, the separation was accomplished in less than 7 min with a detection limit of 65 ng. An unsaturated non-sulphated disaccharide prepared from hyaluronic acid (ΔDi-HA) and an unsaturated sulphated disaccharide (ΔDi-4S) were analyzed by a HPLC method using a combination of two different gel-permeation columns. The separation of the disaccharides required less than 17 min at a flow rate of 0.7 ml/min with detection limits of as little as 4 ng for ΔDi-HA and 5 ng for ΔDi-4S. Both chromatographic methods were used for assay of a major component of hyaluronic acid and trace amounts of chondroitin sulphates in rabbit synovial fluid. The resulting contents of hyaluronic acid were compared to the values of polymeric hyaluronic acid directly measured by a HPLC method using two gel-permeation columns packed with a poly(hydroxyalkyl methacrylate) gel and the amounts of hyaluronic acid converted from uronic acid content determined by a colorimetric method.

Keywords hyaluronic acid; chondroitin sulphate; synovial fluid; high-performance liquid chromatography; oligosaccharide

Hyaluronic acid is a long-chain polysaccharide consisting of repeating units of glucuronic acid and *N*-acetylglucosamine. It exists as a major component in synovial fluid and participates in structural and regulatory functions. The functions are closely related to the acid's high molecular weight up to 10^7 and the viscoelastic property. In synovial fluid of inflamed joints, it is known[1–4] that the concentration of hyaluronic acid is reduced and the distribution of the molecular weight is also shifted to lower values. Moreover, it has been reported[5] that trace amounts of chondroitin sulphates also exist in synovial fluid.

We have recently reported[6] that polymeric hyaluronic acid of synovial fluid can be simultaneously analyzed for the molecular weight and the concentration by a high-performance gel-permeation chromatography using two hydrophilic organic polymer gel columns. The method was able to determine hyaluronic acid of molecular weight $>10^5$ in the range of 4 to 20 μg, but it also revealed lower molecular weights. To determine total amounts of hyaluronic acid in tissue, unsaturated hexasaccharide and tetrasaccharide from hyaluronic acid specifically digested with *Streptomyces* hyaluoronidase were separated by high-performance liquid chromatography (HPLC).[7–9] The analysis[7] on a silica gel (Zorbax SIL) column required 40 min with the detection limit of 5 μg. The chromatography[8] on an amino phase bonded silica gel (Zorbax NH_2) column permitted detection of as little as 0.1 μg in less than 12 min. However, neither of the methods was applied to the practical analyses of biological tissues and fluids. The ion-pair chromatography[9] using a linear gradient system caused the unsaturated hexasaccharide and unsaturated tetrasaccharide to clearly resolve in less than 18 min. It was applied to various types of biological samples. However, the detection limit was 0.5 μg.

The HPLC methods[8,10,11] have also been used for the analysis of unsaturated disaccharides from hyaluronic acid and chondroitin sulphate isomers using the absorbance at 232 nm. The method proposed by Murata and Yokoyama[10] did not permit the detection of disaccharides in the low nanogram range. The other methods reported by Gherezghiher *et al.*[8] and Zebrower *et al.*[11] enabled detection of sulphated disaccharides in amounts down to 25 ng. However, these methods lacked the desired sensitivity for the analysis of trace amounts of chondroitin sulphates in synovial fluid used here.

This paper describes rapid and sensitive chromatographic techniques for the quantitative analyses of enzymatic digestion products of hyaluronic acid and chondroitin sulphates and the practical assay for rabbit synovial fluid.

Experimental

HPLC For the direct separation and quantification of hyaluronic acid polymer in synovial fluid, the following apparatus[6] was used: An LC-3A HPLC instrument (Shimadzu, Kyoto, Japan) equipped with two Shodex OHpak B-806 and B-805 columns (50 cm × 8 mm i.d.) (Showa Denko, Tokyo, Japan), a guard column of Shodex OHpak 800P (50 × 6 mm i.d.) and a 200-μl sample loop. Peak elutions were monitored with an ERC-7520 precision differential refractometer (Erma, Tokyo, Japan) thermostated at 40 °C and a Shimadzu SPD-1 spectrophotometric detector set at 280 nm. Peak height, peak areas and retention time were recorded by a Shimadzu C-R4A chromatograph integrator. The system was run at a flow rate of 0.5 ml/min with 0.02 M sodium chloride degassed with an Erma ERC-3310 degasser.

The analysis of unsaturated tetrasaccharide and hexasaccharide from hyaluronic acid was performed using a Shimadzu LC-6A HPLC instrument equipped with a Shodex Ionpak KS-802 column (30 cm × 8 mm i.d.), a guard column of Shodex Ionpak KS-800P (50 × 6 mm i.d.) and a 20-μl sample loop. Peak elutions were monitored with a Shimadzu SPD-6AV UV-VIS spectrophotometric detector set at 232 nm and an Erma ERC-7510 precision differential refractometer. Peak heights, peak areas and retention times were measured by a Shimadzu C-R2AX chromatograph integrator. The system was run at 80 °C and a flow rate of 1 ml/min with 0.2 M sodium chloride degassed with an Erma ERC-3512 degasser. The HPLC analysis of unsaturated disaccharides was carried out as described above except using Ionpak KS-802 and KS-801 columns at a flow rate of 0.7 ml/min. The columns of the Ionpak KS-800 series are packed with a fully porous cation-exchange resin in the Na^+ form made from a sulphonated polystyrene-divinylbenzene gel and separate oligosaccharides and monosaccharides in a gel-permeation mode, that is, according to their molecular weights.

Materials Standard unsaturated disaccharides 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose (ΔDi-HA), 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose

(ΔDi-OS), 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose (ΔDi-4S), 2-acetamide-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose (ΔDi-6S) and 2-acetamide-2-deoxy-3-*O*-(2-*O*-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose (ΔDi-diS$_B$) were purchased from Seikagaku Kogyo (Tokyo, Japan), and hyaluronic acid, grade I, from human umbilical cord was from Sigma (St. Louis, MO, U.S.A.). *Streptomyces* hyaluronidase (EC 4.2.2.1) was purchased from Amano Seiyaku (Nagoya, Japan), and chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4) was from Seikagaku Kogyo. All other chemicals used were of the highest grade available. Water was purified with a Millipore Milli-Q system.

Unsaturated Hexasaccharide and Tetrasaccharide Standards from Hyaluronic Acid Hyaluronic acid (300 μg) was digested at 55 °C for 5 h with 80 turbidity reducing units (TRU) of *Streptomyces* hyaluronidase in 300 μl of 0.02 M acetate buffer, pH 6.0.[7,12] After incubation, the digested mixture was chromatographed through an Ionpak KS-802 column. No peak appeared in excluded volume, indicating the complete digestion of hyaluronic acid. The digested mixture was then diluted to 1.4 ml in 0.2 M sodium chloride. The diluted solution was filtered through a Millipore Molcut IIGC filter, which removes the components of molecular weight $>10^4$. The filter was pretreated with 1 ml of 50 μM myoglobin solution in order to avoid the adsorption of the resulting oligosacchardes. When the pretreated filter was tested for standard disaccharide solution, more than 95% of disaccharides applied were always recovered. When the digestion solutions were treated with ethanol in the place of a Molcut IIGC filter to remove proteins present, the resulting content of hyaluronic acid decreased to about 80%. The oligosaccharide-containing filtrate was analyzed by the carbazole reaction method as described by Bitter and Muir.[13] The uronic acid content obtained was converted into corresponding disaccharide units of hyaluronic acid. The unsaturated standardized oligosaccharide solution was employed for HPLC analysis using an Ionpak KS-802 column.

Rabbit Synovial Fluid A rabbit knee joint was intra-articularly injected with 1.0 ml saline and washed. The synovial fluid was drawn into a 1-ml syringe and immediately expelled into a polyethyrene tube in ice-water. An 100-μl volume of the fluid was diluted to 300 μl in saline and filtered. The solution was subjected to the HPLC system using OHpak B-806 and B-805 columns.

A 20-μl volume of the synovial fluid was digested at 55 °C for 5 h with 30 μl of Streptomyces hyaluronidase solution (250 TRU in 1 ml of 0.02 M acetate buffer, pH 6.0). Another 20-μl volume of the fluid was digested overnight at 37 °C with 20 μl of chondroitinase ABC (125 units in 1 ml of 0.02 M Tris–HCl buffer, pH 8.0). After individual incubations, each of the two kinds of digested solutions was diluted to 150 μl in 0.2 M sodium chloride, and then filtered through Molcut IIGC filters. The fluid digested with *Streptomyces* hyaluronidase was analyzed for oligosaccharide content by the HPLC system using an Ionpak KS-802 column. The other fluid digested with chondroitinase ABC was assayed for disaccharide content by a combination of Ionpak KS-802 and KS-801 columns.

The synovial fluid (30 μl) was also analyzed colorimetrically by the carbazole method[13] scaled down to one tenth. The color response was somewhat different from that of standard hyaluronic acid.

Results and Discussion

Characterization of Unsaturated Hexasaccharide and Tetrasaccharide from Hyaluronic Acid The elution profiles for the *Streptomyces* hyaluronidase digest of hyaluronic acid on an Ionpak KS-802 column are shown in Fig. 1. Though the two peaks could not be completely distinguished from each other, they appeared in less than 7 min. When the digestion products were further digested with chondroitinase ABC, the two peaks disappeared and a new peak appeared in larger elution volume; the elution profile was similar to that of standard ΔDi-HA (profile not shown). The Ionpak column separates oligosaccharides in a mode of gel-permeation. Accordingly, the two peaks were assigned to hexasaccharide for peak I and tetrasaccharide for peak II (Fig. 1). The unsaturated hexa- and tetrasaccharides and unsaturated non-sulphated disaccharide standards, ΔDi-HA as well as ΔDi-OS, were eluted in the order of their molecular weights through an Ionpak KS-802 column as shown in Fig. 2, whereas, less than would have been expected of three unsaturated sulphated disaccharide standards, ΔDi-diS$_\beta$, ΔDi-6S and ΔDi-4S, were retained on the basis of their molecular weights because of ionic repulsion with the stationary phase.

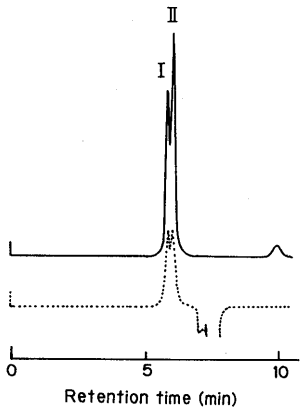

Fig. 1. Chromatograms of Standard Hyaluronic Acid Digested with *Streptomyces* Hyaluronidase on a Shodex Ionpak KS-802 Column

The injected load of the digestion products was 1.3 μg. The solid and dotted curves indicate the elution patterns obtained by monitoring the UV absorbance at 232 nm (0.16 a.u.f.s.) and refractive index (5×10^{-6} r.i.u.f.s.), respectively.

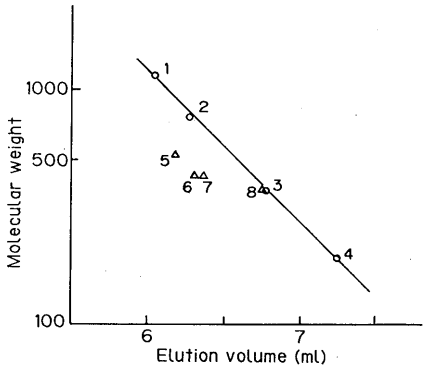

Fig. 2. Relationship between Elution Volume and Logarithm of the Molecular Weight of Unsaturated Oligosaccharides on a Shodex Ionpak KS-802 Column

1, unsaturated hexasaccharide from hyaluronic acid; 2, unsaturated tetrasaccharide from hyaluronic acid; 3, ΔDi-HA; 4, glucuronic acid; 5, ΔDi-diS$_B$; 6, ΔDi-6S; 7, ΔDi-4S; 8, ΔDi-0S.

A chromatogram of the *Streptomyces* hyaluronidase digestion products was also monitored with a refractive index detector to investigate the yields of the individual oligosaccharides (Fig. 1). The refractive index ratio of tetrasaccharide (peak II) to hexasaccharide (peak I) was 1.24, which was slightly higher than 1.22 obtained from an exhaustive digest of human hyaluronic acid.[14] The observed absorbance (232 nm) of hexasaccharide (peak I) and tetrasaccharide (peak II) arise from an unsaturated glucuronosyl residue at their nonreducing ends. The integral absorbance value of peak I and peak II, therefore, was tripled for peak I and doubled for peak II on the basis of a disaccharide unit. The absorbance ratio of tetrasaccharide to hexasaccharide was 1.20, close to the refractive index ratio. This result shows that hexasaccharide (peak I) and tetrasaccharide (peak II) are correctly assigned.

Calibration Curve of Standard Unsaturated Oligosaccharides Chemically determined unsaturated oligosaccharide standard was chromatographed through an Ionpak KS-802 column together with ΔDi-HA standard. The total

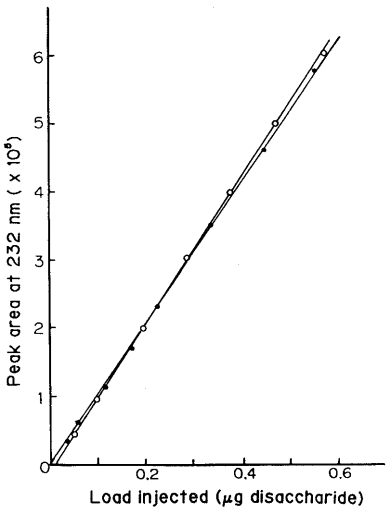

Fig. 3. Calibration Curves of Unsaturated Oligosaccharides from Hyaluronic Acid (○) and Standard ⊿Di-HA (●) on a Shodex Ionpak KS-802 Column

The curves were obtained as follows: $y=-2964+1066240x$ ($r=0.9999$) for oligosaccharides and $y=1462+1049139x$ ($r=0.9994$) for ⊿Di-HA.

absorbance value of oligosaccharides (T) was calculated from the observed abosrbances of hexasaccharide (peak I) and tetrasaccharide (peak II) as follows: T(areas)= peak I(areas)$\times 3$+peak II(areas)$\times 2$. In serial dilution of the oligosaccharide standard, the total absorbance value was plotted against applied load. For the correlation between the disaccharide content and the absorbance, the oligosaccharide standard solution indicated similar linearities to the ⊿Di-HA solution in the range of 65 ng to 2.5 μg (Fig. 3). This result shows that the calibration curve of the standard oligosaccharides represents the accurate content of disaccharide. Hyaluronic acid digested with *Streptomyces* hyaluronidase, therefore, can be determined using the calibration curve of oligosaccharides though the digested products, hexasaccharide and tetrasaccharide were not resolved completely into two peaks.

Resolution and Calibration Curves of ⊿Di-HA and ⊿Di-4S Standards The standard non-sulphated disaccharide (⊿Di-HA) and sulphated disaccharide (⊿Di-4S) were eluted clearly into peak I for ⊿Di-4S and peak II for ⊿Di-HA in less than 17 min by the HPLC method using two gel-permeation columns, Ionpak KS-802 and KS-801 (Fig. 4). The mixture of ⊿Di-4S and ⊿Di-HA was effectively separated into individual saccharides by the ionic repulsion between sulphated disaccharides and the stationary phase. Sulphated disaccharides, ⊿Di-4S and ⊿Di-6S, and non-sulphated disaccharides, ⊿Di-HA and ⊿Di-OS, of the individual groups could not be separated from each other because individual disaccharides of each group have the same molecular weight and similar structure.

The standard solutions of ⊿Di-HA and ⊿Di-4S were chromatographed at various concentrations through the Ionpak KS-802 and KS-801 columns. The correlation between each disaccharide amount injected and the absorbance is shown in Fig. 5. Each calibration curve of ⊿Di-HA and ⊿Di-4S shows a linear relationship in the amount up to 1.5 μg tested. The detection limit was as little as 4 ng for ⊿Di-HA and 5 ng for ⊿Di-4S. The high sensitivity was achieved by making the peaks sharply separate using a gel-permeation mode.

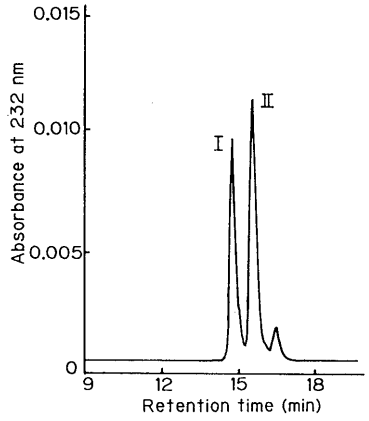

Fig. 4. Chromatogram of ⊿Di-4S (Peak I) and ⊿Di-HA (Peak II) on Ionpak KS-802 and KS-801 Columns

The injected loads of ⊿Di-4S and ⊿Di-HA were 190 and 150 ng, respectively.

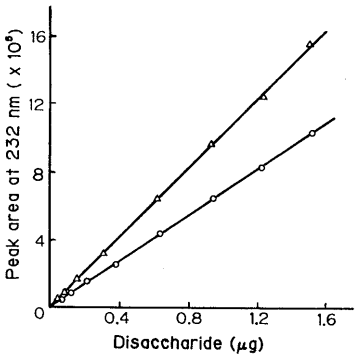

Fig. 5. Calibration Curves of ⊿Di-4S (○) and ⊿Di-HA (△) on Shodex Ionpak KS-802 and KS-801 Columns

The curves were obtained as follows: $y=-472+725500x$ ($r=0.9998$) for ⊿Di-4S and $y=3377+1019850x$ ($r=0.9994$) for ⊿Di-HA.

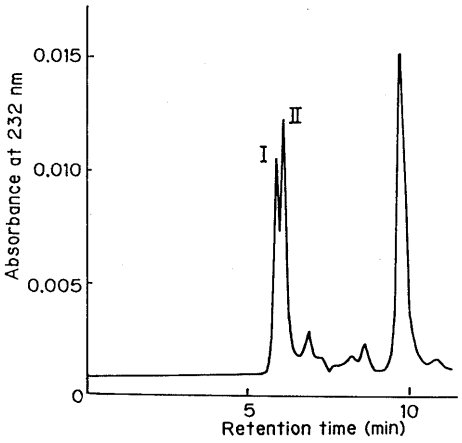

Fig. 6. Chromatogram of Rabbit Synovial Fluid Digested with *Streptomyces* Hyaluronidase on a Shodex Ionpak KS-802 Column

The flow rate was 1.0 ml/min. Peak I and peak II correspond to unsaturated hexasaccharide and unsaturated tetrasaccharide, respectively.

HPLC Analysis of Rabbit Synovial Fluid Representative chromatograms from HPLC analysis of rabbit synovial fluid are shown in Figs. 6 and 7. The unsaturated hexasaccharide and tetrasaccharide were eluted in peak I and peak II, respectively (Fig. 6). The unsaturated disaccharides were separated into sulphated disaccharides (peak I) and non-sulphated disaccharides (peak II) (Fig.

TABLE I. Measured Contents of Hyaluronic Acid (HA) and Chondroitin Sulphates in Rabbit Synovial Fluid

Rabbit	Carbazole reaction	HPLC				Content ratio
		B-806+B-805[a]	KS-802[b]	KS-802+KS-801[b]		
	HA (μg/ml)	HA (μg/ml)	Oligosaccharide (μg/ml)	ΔDi-HA (μg/ml)	ΔDi-4S[c] (μg/ml)	ΔDI-4S/ΔDi-HA
1	302.9	155.3	237.9	218.3	8.3	3.8
2	237.0	136.0	172.9	174.8	10.3	5.9
3	227.7	137.5	159.9	170.9	9.7	5.7
4	248.0	101.3	143.0	143.4	4.6	3.2
5	248.6	99.5	153.0	155.8	4.8	3.1
6	230.0	152.3	182.3	184.4	11.6	6.3
7	237.0	117.4	157.2	160.5	6.3	3.9

Each joint cavity was washed with 1 ml saline. *a*) The content of HA polymer in the synovial fluid was calculated using the equation, y(areas) $= -921 + 673x$ (μg/ml), $r = 0.99$.[6] *b*) The calibration curves from this work were used. *c*) Chondroitin sulphate isomers present in the synovial fluid were calculated as chondroitin 4-sulphate. The contents of HA and oligosaccharide are given in ΔDi-HA units.

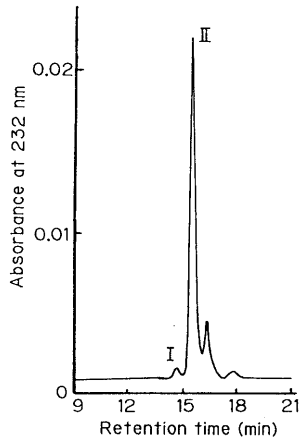

Fig. 7. Chromatogram of Rabbit Synovial Fluid Digested with Chondroitinase ABC on Shodex Ionpak KS-802 and KS-801 Columns

The flow rate was 0.7 ml/ml. Peak I and peak II correspond to unsaturated sulphated disaccharides and unsaturated non-sulphated disaccharides, respectively.

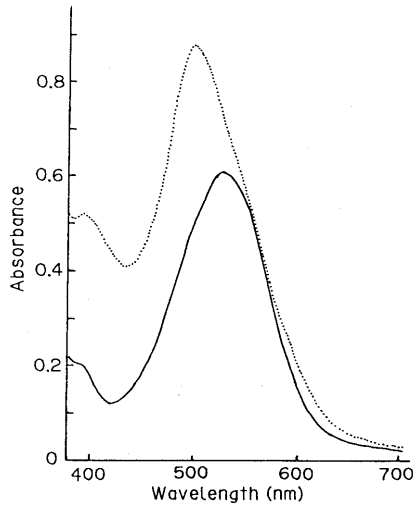

Fig. 8. Carbazole Spectra of Standard Hyaluronic Acid and Rabbit Synovial Fluid

Solid line indicates hyaluronic acid and dotted line synovial fluid.

7). Other peaks did not interfere with these. Table I summarizes the results for the hyaluronic acid contents of rabbit synovial fluid measured by the HPLC and carbazole methods. The total hyaluronic acid contents of the *Streptomyces* hyaluronidase and chondroitinase ABC digests from the same synovial fluid gave similar values. Since synovial fluid contains little chondroitin, the non-sulphated disaccharides are mostly induced from hyaluronic acid present. For a chondroitin-containing sample, the chondroitin content can be obtained by subtracting the amount of *Streptomyces* hyaluronidase digestion products from that of chondroitinase ABC digestion non-sulphated products. Trace amounts of chondroitin sulphates in the synovial fluid could be determined by the chromatography of the chondroitinase ABC digest. The content ratio of chondroitin sulphate to hyaluronic acid in the synovial fluid was similar to the value from ox synovial fluid reported by Silpananta *et al.*[5] Polymeric hyaluronic acid in the synovial fluid was directly resolved by the HPLC method using B-806 and B-805 columns.[6] The content determined was lower than that of total hyaluronic acid analyzed after enzyme digestions, indicating that the synovial fluid contained some lower molecular weights of hyaluronic acid. Further, this may be partially affected by the low sensitivity of the refractive index which is about one-fortieth the sensitivity at 232 nm.

The concentration of hyaluronic acid in synovial fluid was also chemically assayed by the carbazole method.[13] The concentration determined at 530 nm was higher than that of total hyaluronic acid analyzed by the HPLC system. A representative spectrum is shown in Fig. 8 together with that of standard hyaluronic acid and the resulting color response and the absorption spectrum are seen to significantly differ. The carbazole reaction was also carried out for the filtrate of enzyme digests through a Molcut IIGC filter, but the wavelength of absorption maximum was near 515 nm. The result demonstrates that some interfering components are present in the fraction of the molecular weight $< 10^4$ of synovial fluid. Thus, in using hyaluronic acid assay, synovial fluid must also be assayed by other methods besides the routine carbazole reaction technique.

For the determination of oligosaccharides from hyaluronic acid, the chromatography on the combination of two Ionpak columns was compared with that on the single Ionpak column. By the two column method, the two oligosaccharides were more clearly resolved from each other, but the separation required longer analysis time. Accurate amounts of oligosaccharides were satisfactorily obtained even by the single column method.

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