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ANALYSIS OF HYALURONIC ACID AND CHONDROITIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF THE CONSTITUENT DISACCHARIDE UNITS

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SUMMARY

Unsaturated non-sulphated disaccharides prepared from hyaluronic acid and chondroitin were analysed by an improved high-performance liquid chromatographic (HPLC) method using an ion-exchange resin made from a sulphonized styrene—divinylbenzene copolymer. This new HPLC method gave accurate and reproducible retention times for these unsaturated non-sulphated disaccharides, which appeared in order of derivation from hyaluronic acid and chondroitin. The contents of these unsaturated non-sulphated disaccharides could be determined with similar sensitivities by UV absorption. Good sensitivity and reproducible retention times, as well as good resolution, were obtained for these compounds at various ratios. The new HPLC microassay method can be used to determine amounts as small as 1—8 μg.

INTRODUCTION*

Hyaluronic acid is a glycosaminoglycan (GAG) that forms macromolecules and plays numerous important roles: a small amount of it forms aggregates with other proteoglycan monomers [1—3], it acts as a lubricant, has water-binding capacity and regulates water transportation [4, 5]. HA is present in small amounts in various tissues and organs [6], and it has been suggested

*Abbreviations used: ΔDi-0S_{HA} = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose; ΔDi-0S_{Ch} = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose; ΔDi-6S = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose; HA = hyaluronic acid (= N-acetyl-D-glucosamine, D-glucuronic acid); Ch = chondroitin (= N-acetyl-D-galactosamine, D-glucuronic acid); Ch-4S = chondroitin 4-sulphate (= N-acetyl-D-galactosamine 4-sulphate, D-glucuronic acid); Ch-6S = chondroitin 6-sulphate (= N-acetyl-D-galactosamine 6-sulphate, D-glucuronic acid).

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that it participates in regulating the metabolism of other GAGs [6—9]. On the other hand, chondroitin constitutes a non-sulphated part of repeating disaccharide chains of chondroitin sulphate isomers and is present as a major GAG component of under-sulphated chondroitin sulphates in body fluids [10—12]. Chondroitin (or very-low-sulphated chondroitin sulphate) in conjugation with ordinary sulphated GAG shows a certain heterogeneity as to chain length and constituent units [11, 12]. The separation and identification of non-sulphated GAGs, i.e. HA and chondroitin, are difficult by means of electrophoresis. Certain GAGs can be uniformly degraded to their disaccharide constituent units by specific GAG lyses [11—17]. This procedure facilitates identification of the constituent disaccharide units of non-sulphated GAGs in addition to those of sulphated GAGs. For quantitation of HA and chondroitin components, hyaluronidase and chondroitinase digests can be separated to a certain extent by paper chromatography [16, 17], but not by high-voltage electrophoresis [18].

High-performance liquid chromatography (HPLC) has been developed as a useful technique for the separation and quantitation of unsaturated disaccharides (ΔDi-S) obtained by digestion from chondroitin sulphate isomers with chondroitinases. With HPLC methods using silica compounds, it is possible to separate unsaturated non-sulphated disaccharides (ΔDi-0S) from unsaturated 4-sulphated and 6-sulphated disaccharides (ΔDi-4S, ΔDi-6S) [19—22]. However, the separation of the unsaturated non-sulphated disaccharide generated from HA (ΔDi-0S$_{HA}$) and that from chondroitin (ΔDi-0S$_{Ch}$) is not always efficient due to the less reproducible retention times, and a means of quantitative analysis has not yet been established [23]. Recently, we devised a new HPLC method for separating these ΔDi-S using a column of a sulphonized styrene—divinylbenzene copolymer [24]. This new method made it possible to identify ΔDi-S in alternative elution orders compared with those in the cases of ordinary HPLC systems using silica compounds. Now we can differentiate ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$ by this new technique. This method showed not only good resolution of both ΔDi-0S$_{Ch}$ and ΔDi-0S$_{HA}$, with constant times, but also very good and close sensitivity for both these ΔDi-0S.

EXPERIMENTAL

Standard ΔDi-0S

Two standards, ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$, were prepared from HA and chondroitin, respectively, by degradation with either chondroitinase-ABC or chondroitinase-AC. HA from cockscomb, chondroitin, chondroitin 4-sulphate, chondroitin 6-sulphate from shark cartilage, chondroitinase-ABC (EC 4.2.2.1) from *Proteus vulgaris* [14, 15] and chrondroitinase-AC (EC 4.2.2.5) from *Arthrobacter aurescens* [25] were obtained from the Tokyo Institute of Seikagaku Kogyo, 2-9, Honcho, Nihonbaski, Chuo-ku, Tokyo 103, Japan (available also from Miles Laboratories, U.S.A.). Standard ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$, which were separated from other ΔDi-S by high-voltage electrophoresis [15, 18], showed single spots on paper chromatography. These ΔDi-0S were dissolved in distilled water, quantitated by mean of the

borate carbazole reaction [26], and adjusted to a concentration of 25 μg per 100 μl. These ΔDi-0S showed broad UV absorption bands with a maximum at 232 nm. Methanol, acetonitrile, ammonium formate and other chemicals of HPLC grade were purchased from Wako Fine Chemicals, Tokyo, Japan.

HPLC apparatus

The HPLC apparatus employed was a Model 803D (Toyosoda, Tokyo, Japan) solvent-delivery system. A flat mini-pen type integrator recorder, Model 7000B (System Instrument, Tokyo, Japan), with an automatic computer system was used. The separation of each ΔDi-0S was performed on a Shodex RS (Type DC-613) ion-exchange chromatography column which contained a fully porous ion-exchange resin, Na^+, made from a sulphonized styrene–divinylbenzene copolymer. The resin, with a particle size of 10 μm, was prepacked in a stainless-steel column (150 × 6 mm I.D.) connected with a precolumn (70 × 6 mm I.D.) (Showa Denko America, 280 Park Avenue, West Bldg. 27th Fl., New York, NY 10017, U.S.A.; or Showa Denko Europe, 4000 Düsseldorf 11, F.R.G.).

Application of ΔDi-0S to HPLC

A 0.5–8 μg aliquot in 10 μl of each ΔDi-0S was injected into the Shodex RS column and chromatographed at a flow-rate of 1.0 ml/min with acetonitrile–methanol–0.5 *M* ammonium formate buffer, pH 4.5 (65:15:20). The ratio of constituents of the mobile phase was selected so as to obtain the optimal conditions for the separation of $\Delta Di\text{-}0S_{HA}$ and $\Delta Di\text{-}0S_{Ch}$, from among those of the other solvent ratios. The HPLC was carried out at 70°C and at 15 bar for approximately 10 min. The eluate was monitored by measuring the UV absorption at 232 nm, and the elution response was recorded at a chart speed of 2.5 or 5 mm/min for determination of retention times, peak height and peak areas.

RESULTS

The present study showed that $\Delta Di\text{-}0S_{HA}$ and $\Delta Di\text{-}0S_{Ch}$ have different retention times, so they can be separated from each other; they also have satisfactorily similar sensitivities. The possible appearance of extra peaks due to either impurities or the high temperature was examined for each ΔDi-0S with the HPLC method. $\Delta Di\text{-}0S_{HA}$ and $\Delta Di\text{-}0S_{Ch}$ showed only single peaks, not contaminated by other ΔDi-S or any impurities (Fig. 1). The best mobile phase composition by volume was found to be the ratio of 65:15:20 for the acetonitrile–methanol–0.5 *M* ammonium formate solvent at pH 4.5. It was confirmed that the retention time of each ΔDi-0S is quite reproducible even when the HPLC for ΔDi-0S is performed in different runs on different days. The number of theoretical plates (*N*) for $\Delta Di\text{-}0S_{Ch}$ and $\Delta Di\text{-}0S_{HA}$ with various mobile phase compositions is shown in Table I. The values were virtually constant (within 2% difference) when the experiment was carried out on different days. This value increased in proportion to the amount of acetonitrile.

The separation of each ΔDi-0S with the new HPLC system required only

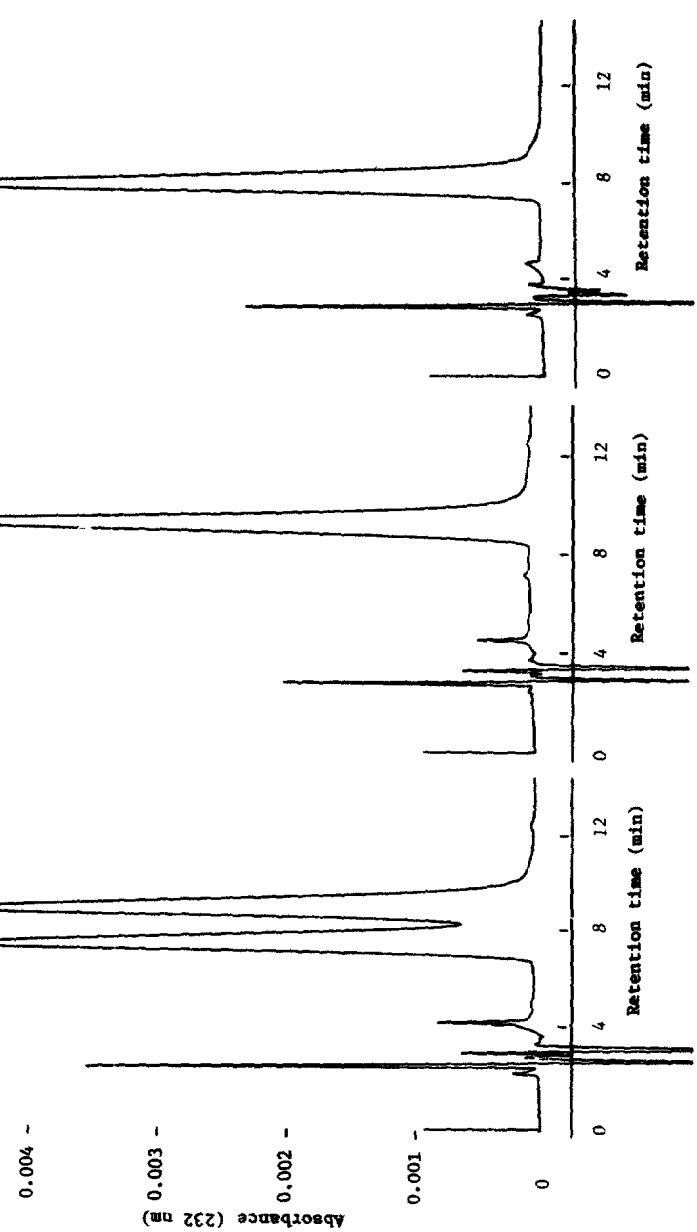

Fig. 1. HPLC profiles of individual ΔDi-0S on a Shodex RS column. ΔDi-0S_{HA} from HA and ΔDi-0S_{Ch} from chondroitin were purified by separation by high-voltage electrophoresis. Approximately 5 μg per 10 μl of the specimen was injected into the column (150 × 6 mm I.D.; particle size 10 μm) (Shodex RS, Type DC-613) connected to a pre-column (70 × 6 mm I.D.). The solvent system was acetonitrile—methanol—0.5 *M* ammonium formate (65:15:20) at pH 4.5; pressure, 15 bar; flow-rate, 1.0 ml/min; UV detection at 232 nm. 1, ΔDi-0S_{HA} plus ΔDi-0S_{Ch}; 2, ΔDi-0S_{Ch}; 3, ΔDi-0S_{HA}.

TABLE I

NUMBER OF THEORETICAL PLATES (N) WITH DIFFERENT BUFFER COMPOSITIONS (BY VOLUME) OF THE MOBILE PHASE USING A SHODEX-RS COLUMN

N is calculated by the half-peak-height method, with the formula $N = 5.54 \times (t_R/W)^2$, where t_R = retention time and W = peak width at half-peak-height. N values are the averages of four different chromatograms.

	Ratio of acetonitrile—methanol—0.5 M ammonium formate buffer (pH 4.5)			
	60:20:20	65:15:20	65:20:15	70:10:20
ΔDi-0S$_{HA}$	291	318	422	921
ΔDi-0S$_{Ch}$	315	458	529	958

10 min. It was found that the ΔDi-0S appeared in the elution order of ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$. The retention times of ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$ prepared from ten different preparations were 7.48 ± 0.07 and 8.84 ± 0.08 min (mean ± S.D.), respectively. The present study showed that ΔDi-0S$_{HA}$ can be clearly differentiated from ΔDi-0S$_{Ch}$ even when the present HPLC for separation of each ΔDi-0S is performed on different days and each ΔDi-0S is present in different proportions in mixtures (Fig. 2).

Following the digestion of either ΔDi-4S with chondro-4-sulphatase (EC 3.1.6.10) or ΔDi-6S with chondro-6-sulphatase (EC 3.1.6.9), both digests showed the same retention time as ΔDi-0S$_{Ch}$. ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$ showed good resolution because of their different constituents: the hexosamine moiety of the former comprises glucosamine and the latter galactosamine.

To evaluate the sensitivity and reproducibility of this procedure, 10-μl aliquots containing 0.5—8 μg of ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$ were chromatographed under the same conditions as used above for the efficient separation of each ΔDi-0S. The peak areas of these compounds were plotted against the corresponding uronic acid amounts of the compounds. The results showed that a linear relationship could be obtained in the calibration curve for peak area versus concentration from 1 to 8 μg of each ΔDi-0S. The contents of ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$ could be measured with very close sensitivities.

DISCUSSION

The new resin column used for the present HPLC method for the separation of ΔDi-0S provides precise, selective and highly reproducible data. The significantly different retention times of ΔDi-0S made good resolution for individual compounds possible with this HPLC system. The reproducible separation of these ΔDi-S in the present HPLC study was more efficient than in the cases of previous silica HPLC methods in which resolution of ΔDi-0S was not sufficient [24]. Both ΔDi-0S were eluted in a short period (< 10 min) and showed similar responses with equivalent sample amounts. The values obtained for different ΔDi-0S contents were within 1% difference at a concentration of 1 μg when the experiment was carried out under the same conditions, and a linear relationship was obtained between the uronic acid content and response area value.

The good resolution of ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$ with HPLC is possibly

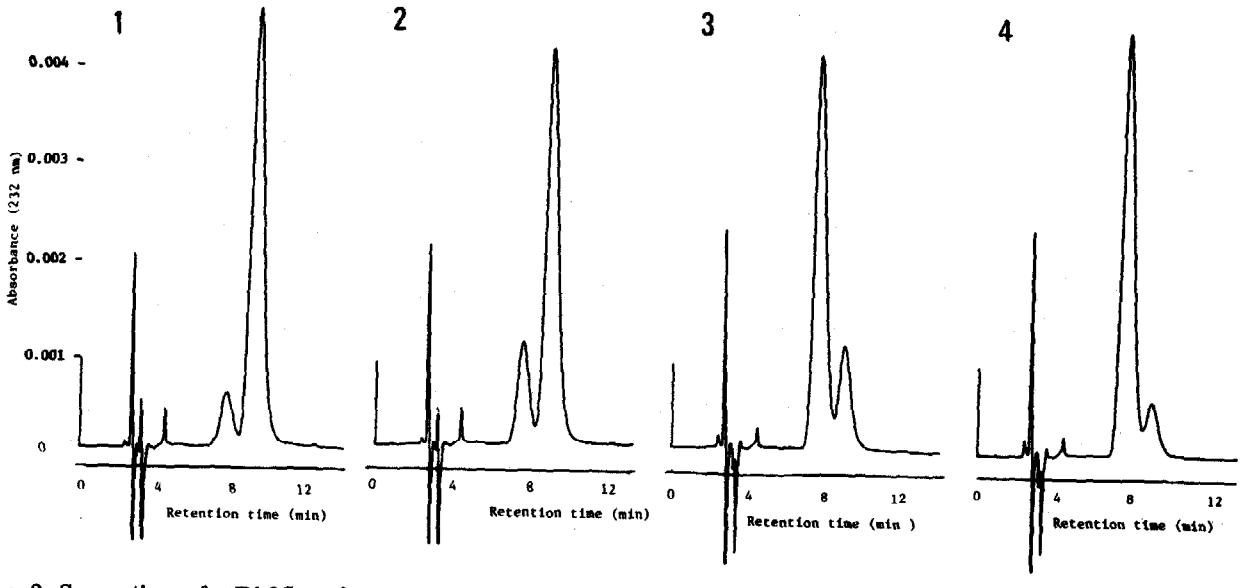

Fig. 2. Separation of ΔDi-0S$_{HA}$ from ΔDi-0S$_{Ch}$ in different ratios by HPLC on a Shodex RS column. The ratios of ΔDi-0S$_{Ch}$ to ΔDi-0S$_{HA}$ were 9:1 (1), 8:2 (2), 2:8 (3) and 1:9 (4). The conditions for this HPLC analysis were the same as those in Fig. 1.

due to the different hexosamine moieties of these compounds: i.e. glucosamine and galactosamine. With ordinary silica HPLC methods, ΔDi-0S$_{HA}$ generated from HA and ΔDi-0S$_{Ch}$ from chondroitin, have been shown to exhibit lower resolution compared with other representative ΔDi-monoS, i.e. ΔDi-4S and ΔDi-6S, and complete separation and quantitation cannot be achieved by ordinary HPLC. ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$ were separated to a certain extent by paper chromatography [11, 16] but it was not possible to separate them by electrophoresis or high-voltage electrophoresis [18]. Following drastic hydrolysis of non-sulphated GAG, the hexosamine moieties of HA and chondroitin can be separated, respectively, as glucosamine and galactosamine moieties by thin-layer chromatography [27]. The different migration distances of these ΔDi-0S using paper chromatography can be explained in the same way.

The UV absorbance sensitivity of the present method is the same for ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$ when they are applied in equivalent amounts. This makes their accurate quantitation possible. The retention times of ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$ were very selective with this new HPLC system, and ΔDi-0S$_{HA}$ appeared significantly faster than ΔDi-0S$_{Ch}$, even when they were applied on different days.

Since HA and chondroitin may coexist in various connective tissues, good resolution of these compounds is necessary. With the present HPLC method ΔDi-0S$_{HA}$ is eluted much faster than ΔDi-0S$_{Ch}$, so both ΔDi-0S$_{HA}$ and ΔDi-0S$_{Ch}$ in mixtures can be separated and quantitated. Analysis of GAGs in human urine and synovial fluid proved the practicability of the new system in that it makes determination of small amounts of ΔDi-0S possible [28]. The GAGs in these samples were prepared and digested with chondroitinase-AC and chondro-4- or -6-sulphatase, and then subjected to HPLC. The ΔDi-0S$_{HA}$ in human synovial fluid accounted for 92—98% of the total GAG and could be separated from ΔDi-0S$_{Ch}$, as shown in Fig. 2-4. The urinary GAGs in patients with Werner's syndrome contained HA accounting for 8—20% of the total GAG. With HPLC, the ΔDi-0S$_{HA}$ could be separated from other ΔDi-0S as seen in Figs. 2-1 and 2-2. Utilizing the HPLC method in our laboratories, studies are in progress to assess the various GAGs rich in non-sulphated HA and chondroitin; further details will be reported elsewhere.

In conclusion, the present HPLC method has the advantage that: (1) definite retention times of ΔDi-0S can be obtain in comparison to those with ordinary HPLC using silica and NH_2; and (2) the newly devised resin column can be used to obtain reproducible retention times, statistically within 5% error, for long periods.

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REFERENCES

1 W.D. Comper and T.C. Laurent, Physiol. Rev., 58 (1978) 255—313.
2 H. Muir, Biochem. Soc. Trans., 11 (1983) 613—622.
3 D. Heinegård and M. Paulsson, in K.A. Piez and A.H. Reddi (Editors), Connective Tissue Biochemistry, Elsevier, Amsterdam, 1984, pp. 277—327.
4 T.C. Laurent, in A. Balazs (Editor), Chemistry and Molecular Biology of the Intercellular Matrix, Academic Press, New York, 1970, pp. 703—732.
5 U. Lindahl and M. Höök, Ann. Rev. Biochem., 47 (1978) 385—417.
6 U.B.G. Laurent and A. Tenglad, Anal. Biochem., 109 (1980) 386—394.
7 D.A. Swann, in L. Sokoloff (Editor), The Joints and Synovial Fluid, Vol. 1, Academic Press, New York, 1978, pp. 407—483.
8 E.A. Balazs, in A.J. Helfet (Editor), Disorders of the Knee, J.B. Lippincott, Philadelphia, PA, 1982, pp. 61—74.
9 A. Maroudas, Ann. Rheum. Dis., 34 (Suppl. 2) (1975) 77—81.
10 K. Murata and Y. Horiuchi, Clin. Chim. Acta, 75 (1977) 59—69.
11 K. Murata, Experientia, 38 (1982) 313—314.
12 J.R.E. Fraser, T.C. Laurent, A. Engström-Laurent and U.G.B. Laurent, Clin. Exp. Pharmacol. Physiol., 11 (1984) 17—25.
13 K. Murata, Clin. Chim. Acta, 57 (1974) 115—124.
14 T. Yamagata, H. Saito, O. Habuchi and S. Suzuki, J. Biol. Chem., 243 (1968) 1523—1535.
15 K. Murata, in R.L. Whistler and J.N. BeMiller (Editors), Methods in Carbohydrate Chemistry, Academic Press, New York, 1980, pp. 81—88.
16 K. Murata and A.O. Bjelle, Connect. Tissue Res., 7 (1980) 143—156.
17 K. Murata and Y. Yokoyama, Atherosclerosis, 45 (1982) 53—65.
18 K. Murata, Biochem. Med., 23 (1980) 324—335.
19 G.J.-L. Lee, J.E. Evans and H. Tieckelmann, J. Chromatogr., 146 (1978) 439—448.
20 A. Hjerpe, C.A. Antonopoulos and B. Engfeldt, J. Chromatogr., 171 (1979) 339—344.
21 G.J.-L. Lee, D.-W. Liu, J.W. Pav and H. Tieckelmann, J. Chromatogr., 212 (1981) 65—73.
22 D.C. Seldin, N. Seno, K.F. Austin and R.L. Stevens, Anal. Biochem., 141 (1984) 291—300.
23 A.H. Hjerpe, C.A. Antonopoulos and B. Engfeldt, J. Chromatogr., 245 (1982) 365—368.
24 K. Murata and Y. Yokoyama, Anal. Biochem., 146 (1985) 327—335.
25 K. Hiyama and S. Okada, J. Biol. Chem., 250 (1975) 1824—1828.
26 T. Bitter and H.M. Muir, Anal. Biochem., 4 (1962) 330—334.
27 E. Moczar, M. Moczar, G. Schillinger and L. Robert, J. Chromatogr., 31 (1967) 561—563.
28 K. Murata, T. Mizoguchi, A. Mimori and T. Mitamura, Abstracts 29th Jpn. Soc. Rheumatol., Fukuoka, May 10th, 1985, p. 54.