Journal of Chromatography, 413 (1987) 9–15
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3372

RAPID AND SENSITIVE METHOD FOR MEASUREMENT OF HYALURONIC ACID AND ISOMERIC CHONDROITIN SULFATES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

TSEGGAI GHEREZGHIHER*, MICHAEL C. KOSS, ROBERT E. NORDQUIST and CHARLES P. WILKINSON

Dean A. McGee Eye Institute and The Department of Ophthalmology, The University of Oklahoma, 608 Stanton L. Young Boulevard, Oklahoma City, OK 73104 (U.S.A.)

(First received May 29th, 1986; revised manuscript received August 7th, 1986)

SUMMARY

A high-performance liquid chromatographic method for the separation and analysis of the unsaturated tetrasaccharide and hexasaccharide from Streptomyces hyaluronidase (S.HAase) enzyme digestion products of hyaluronic acid (HA) and standard unsaturated disaccharides 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose (ΔDi-4S) and 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose (ΔDi-6S) is described. An amino phase chemically bonded to silica with a particle diameter of 6 μm was used as the column. The composition and the pH of the mobile phase were systematically varied to determine the optimal chromatographic conditions for separation and analysis of the compounds. For HA, a complete separation was accomplished in less than 12 min with a practical detection limit of 100 ng. Separation of the disaccharides also required less than 15 min with detection limits of 10 ng for ΔDi-0S and 25 ng each for ΔDi-4S and ΔDi-6S. This chromatographic method represents a significant improvement over existing methods. It allows the simultaneous separation and analysis of HA and chondroitin sulfate isomers (after digestion of the latter with chondroitinase) at a higher speed, and with more sensitivity and efficiency.

INTRODUCTION

Selective analysis of hyaluronic acid (HA) from other glycosaminoglycans (GAG) became feasible only after the discovery of Streptomyces hyaluronidase (S.HAase) which is specific to HA but not to isomeric chondroitin sulfate [1]. The interaction of HA with S.HAase yields Δ-4,5 unsaturated tetrasaccharide and hexasaccharide with Δ-4,5 unsaturated uronic acid moiety at the non-reducing end which have a UV absorption maximum at 232 nm. The application of high-performance liquid chromatography (HPLC) to separate and quantitate S.HAase

0378-4347/87/$03.50

degradation products of HA has been reported [2]. However, in this method [2], the chromatographic elution time per sample was 40 min and produced relatively broad non-symmetrical peaks. The detection limit for HA was reported to be approximately 5 μg.

Estimation of chondroitin sulfate isomers has been attempted following enzymatic digestion with chondroitinase [3,4] and by assaying the resulting unsaturated disaccharide fragments using HPLC methods [5–8]. The chromatographic conditions reported for the separation and measurement of the unsaturated disaccharides 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose (ΔDi-4S) and 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose (ΔDi-6S) do not permit the detection of the compounds in the low nanogram range. Furthermore, none of the HPLC methods for the analysis of HA or chondroitin sulfate isomers cited above has proven useful for the simultaneous separation and measurement of the enzymatic digestive products from both HA and chondroitin sulfate.

Other investigators have reported various methods for the simultaneous determination of HA and chondroitin sulfate isomers. A periodate–thiobarbituric acid assay technique proposed by Adams and Muir [9] was admittedly time-consuming and less precise. In addition, the color response for each of the unsaturated disaccharides was different and much less sensitive. Kleine and Merten [10] reported a procedure for analysis of chondroitinase enzyme digestion products of HA and chondroitin sulfate using paper chromatography followed by the modified carbazole reaction [11]. Similarly, Hjerpe et al. [12] used size exclusion gel chromatography for the analysis of dentine GAG. Both methods lack the desired specificity, selectivity and sensitivity for the analysis of trace amounts of HA and isomeric chondroitin sulfates.

This paper describes a more rapid and sensitive chromatographic technique for the simultaneous separation and quantitative analysis of enzymatic digestion products of HA and standard unsaturated disaccharides.

EXPERIMENTAL

HPLC apparatus

HPLC analyses were performed using a Model PM-30A solvent delivery system (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a sample injection valve fitted with a 50-μl loop (No. 7125, Rheodyne, Cotati, CA, U.S.A.) and a Lambda Max Model 481 variable-wavelength UV detector (Waters Assoc., Milford, MA, U.S.A.). The column was a Zorbax NH_2 (25 cm × 4.6 mm I.D., 6 μm particle size) obtained from Dupont (Wilmington, DE, U.S.A.).

The eluting solvent consisted of 0.5 *M* ammonium formate and methanol made up to 1 l with double-distilled, deionized water prepared using a Milli-Q water system (Millipore). The pH was adjusted to the desired level by adding concentrated formic acid. The final solution was filtered through a 0.22-μm vacuum filtering unit (Millipore) and degassed by sparging with helium gas. Elution of compounds from the column was carried out at room temperature in an isocratic

mode. Chromatograms were recorded on an Omniscribe strip-chart recorder (Houston Instruments, Houston, TX, U.S.A.) at 10 mV full scale. Peak heights, peak areas and retention times were measured by an on-line Hewlett-Packard Model 3393A computing integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The computing integrator was interfaced with a Rainbow computer (Digital Equipment, Bedford, MA, U.S.A.) for storage, retrieval and analysis of data.

Chemicals

Standard unsaturated disaccharides ΔDi-0S, ΔDi-4S and ΔDi-6S were purchased from Miles Labs. (Elkhart, IN, U.S.A.), hyaluronic acid from bovine vitreous (H-7630; Grade IV) and hyaluronidase from Streptomyces hyalurolyticus (H-1136) were purchased from Sigma (St. Louis, MO, U.S.A.).

Enzymatic digestion of HA with Streptomyces hyaluronidase

Standard HA (10 mg/ml) was dissolved in 0.05 *M* phosphate buffer, pH 5.0. An aliquot was diluted 1:10 (v/v) with 0.02 *M* acetate buffer (pH 6.0) to 1 μg/μl. The digestion mixture then contained varying amounts of HA, each in 100 μl of acetate buffer, and 10–30 μl of hyaluronidase enzyme [one turbidity reducing unit (TRU) per microliter dissolved in the same acetate buffer]. In one batch of standards ($n=10$) the enzymatic digestion mixture contained a total of 50 μg HA per sample. The mixture was incubated at 60°C for 2 h [1,2]. After incubation, four volumes of pure ethanol were added to each sample. The mixture was briefly vortexed and left overnight at 4°C. The clear supernatant obtained after centrifugation was dried under a stream of nitrogen and the residue was reconstituted in 25–50 μl of the mobile phase solution for chromatographic analysis. Post-column eluates corresponding to chromatographic peaks 1 and 2 were pooled separately from the 50-μg HA sample batch to determine uronic acid content by the carbazole reaction method as described by Bitter and Muir [11]. The eluate from the disaccharide ΔDi-0S was used as a reference standard. The ratio of uronic acid per unit UV absorbance for peaks 1 and 2 as compared to ΔDi-0S is indicative of S.HAase enzyme digestion products of HA [1,2]. The standard disaccharides were dissolved in eluting solvent prior to chromatographic analysis.

RESULTS AND DISCUSSION

The S.HAase enzyme digestion products of HA (unsaturated tetrasaccharide and hexasaccharide) were separated by HPLC. Similarly, standard unsaturated disaccharides ΔDi-0S, ΔDi-4S and ΔDi-6S were separated using the same chromatographic technique. A complete separation was obtained in less than 12 min with a practical detection limit of 100 ng for HA. Separation of the disaccharides was completed in less than 15 min with detection limits of 10 ng for ΔDi-0S and 25 ng each for ΔDi-4S and ΔDi-6S.

Unlike the plain silica column [2], the amino functionality of a bonded-phase silica column becomes protonated under acidic conditions to an extent proportional to the pH of the mobile phase. Since retention is controlled mainly by ionic strength, and selectivity is controlled mainly by pH, careful control of the solvent

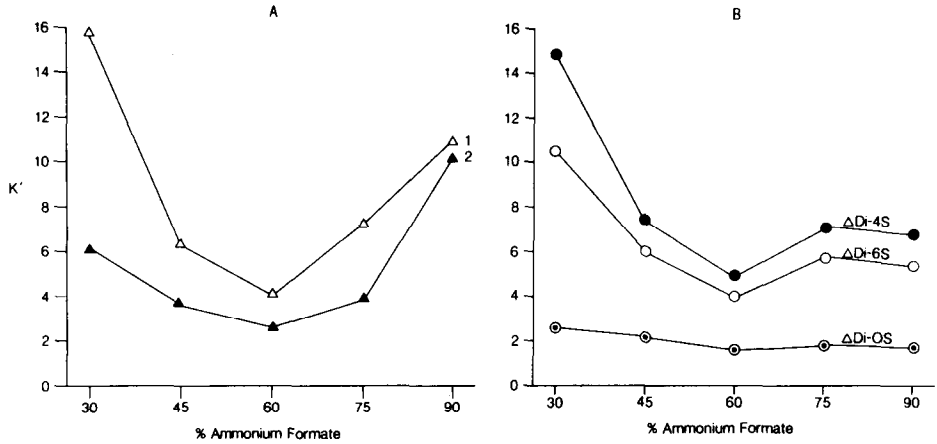

Fig. 1. Capacity ratios (k') of the unsaturated tetrasaccharides (1) and hexasaccharides (2) from S.HAase enzyme digestion products of HA (A) and standard unsaturated disaccharides (B) as a function of increasing amounts of ammonium formate added to the mobile phase. Solvent system: 4% methanol, 30–90% 0.5 *M* ammonium formate, pH 5.5; column: Zorbax NH_2, 6 μm particle size, 25 cm×4.6 mm I.D.; flow-rate: 0.9 ml/min; UV detection at 232 nm.

composition and pH can provide more flexibility for the analyst to optimize the chromatographic conditions for the separation of weakly acidic polysaccharides by ion-exchange chromatography. Thus, a variety of solvent compositions at various pH values were tested to determine the optimal chromatographic conditions.

The capacity ratio (k') values for the unsaturated tetrasaccharide and hexasaccharide from HA, as well as the standard unsaturated disaccharides, are shown in Fig. 1A and B, respectively, as a function of the amount of 0.5 *M* ammonium formate in the mobile phase at a constant pH of 5.5 and 4% methanol. The k' values of all compounds except for ΔDi-0S initially decreased significantly as the amount of ammonium formate was increased to 60%. This was followed by an increase in k' values for HA products and the disaccharides, respectively (Fig. 1A and B). No significant change in k' value was noticed for ΔDi-0S. The k' values for these compounds were also analyzed as a function of increasing amounts of methanol (0–48%) at constant pH of 5.5 and 60% 0.5 *M* ammonium formate. No significant change was found in the k' values. However, there was a steady decrease (up to 50%) in the number of theoretical plates (N) for all the compounds as a function of increasing amounts of methanol. This was accompanied by a steady increase in the column pressure.

When a mixture of all test compounds was injected into the column using 4% methanol and 60% 0.5 *M* ammonium formate at pH 5.5 as a mobile phase, the selectivity factor (α) for ΔDi-6S and the tetrasaccharide was 1.02. In an attempt to improve the resolution of these two compounds, the k' values were determined as a function of pH (as shown in Fig. 2). The value of α for ΔDi-6S and the tetrasaccharide was increased to 1.09, which may be adequate for qualitative analysis. The separation was reasonably stable with no significant change in the k' values in the pH range tested. There was, however, a mean decrease of 10% in the peak heights of all the compounds when run at pH 6.0 compared to that of

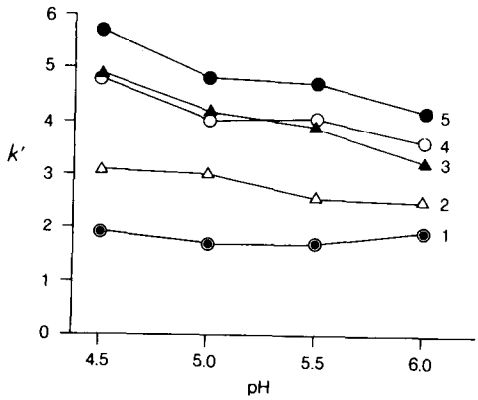

Fig. 2. Capacity ratios (k') of unsaturated tetrasaccharide (2) and hexasaccharide (3) from HA and standard unsaturated disaccharides ΔDi-0S (1), ΔDi-6S (4) and ΔDi-4S (5) as a function of pH of the mobile phase. Solvent system: 4% methanol, 60% 0.5 *M* ammonium formate. Other chromatographic parameters as in Fig. 1.

pH 5.5, which was accompanied by a mean decrease of 30% in the number of theoretical plates (N) for ΔDi-0S and the tetrasaccharide. Below pH 4.5 there was too much background absorbance due to the mobile phase. Thus it became apparent that when using the amino-bonded silica column, the optimal composition and pH of the mobile phase for the separation and analysis of the unsaturated tetra- and hexasaccharides from HA as well as the analysis of the three disaccharides was 4% methanol, 60% 0.5 *M* ammonium formate at pH 5.5. Typical chromatograms for the respective compounds are shown in Fig. 3A and B. In Fig. 3A, post-column analysis for peaks 1 and 2 for their relative contents of uronic acid was performed as described in the Experimental section. The results indicated that the ratio of uronic acid content for peaks 1 and 2 was 2.1:3.2 ($n=10$), respectively, using the disaccharide ΔDi-0S as a reference standard. This suggests that peak 1 is a tetrasaccharide and peak 2 is a hexasaccharide, which supports the conclusions of others [1,2].

Standard calibration curves demonstrating linear relationships between the amounts of HA digestion products and the chromatographic peak heights in UV absorbance units were established. The linear regression equations for the unsaturated tetrasaccharides and hexasaccharides were $y=0.603x-0.004$ and $y=0.338x-0.004$, respectively. Similar calibration curves were also determined for the unsaturated disaccharides and the regression equations were $y=4.847x-0.002$ (ΔDi-0S), $y=1.980x-0.001$ (ΔDi-4S) and $y=1.735x-0.006$ (ΔDi-6S). Such linear calibration curves facilitate direct quantitation of unknown samples.

CONCLUSION

The HPLC method described for HA and the unsaturated disaccharides represents a significant improvement over the existing methods. It allows qualitative and quantitative analysis of trace amounts of HA and isomeric chondroitin sul-

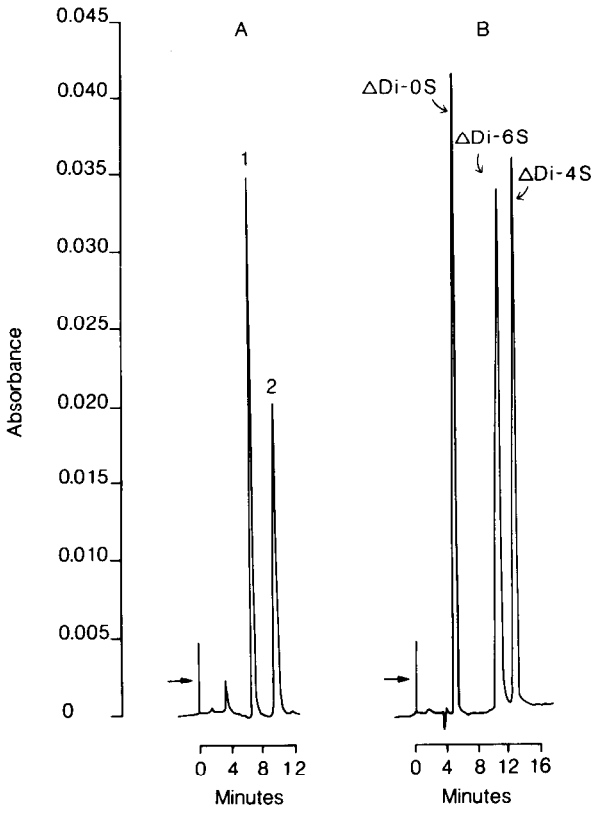

Fig. 3. Typical chromatograms from enzyme digestion products of HA (A) and standard unsaturated disaccharides (B) under optimal chromatographic conditions. (A) A 15-μg amount of HA from bovine vitreous was digested with 20 TRU S.HAase as described in the text. The final residue was reconstituted in 30 μl of the mobile phase with 10 μl injected. The volume injected corresponds to a total of 5 μg of the degradation products of HA. Peaks: 1 = unsaturated tetrasaccharide; 2 = unsaturated hexasaccharide. (B) The disaccharides were dissolved in the mobile phase. A 10-μl aliquot containing 0.9 μg of ΔDi-0S and 1.8 μg each of ΔDi-4S and ΔDi-6S was injected into the column. Mobile phase: 4% methanol, 60% 0.5 *M* ammonium formate, pH 5.5. Column: Zorbax NH_2, 6 μm particle size, 25 cm $\times$ 4.6 mm I.D.; flow-rate: 0.9 ml/min; UV detection at 232 nm, 0.05 a.u.f.s.

fates, side by side by measurement of the UV absorbances of tetra- and hexasaccharides and the absorbances of the disaccharides (ΔDi-0S, ΔDi-4S and ΔDi-6S), respectively.

This chromatographic technique may be useful to determine trace amounts of HA levels in the vitreous and/or aqueous humor of human eyes. Alteration in HA concentrations in the vitreous gel is associated with the vitreous changes leading to posterior vitreous detachment and/or rhegmatogenous retinal detachment [13]. Furthermore, since chondroitin sulfate is believed to be the principal urinary GAG in normal subjects [5,7,14], urinary levels of chondroitin sulfate isomers might be used to study connective tissue metabolism [14] and to diagnose genetic mucopolysaccharidoses [15]. The present method can also be used to analyze unsaturated disaccharides in order to determine the level of chondroitin sulfate (after digestion of the compound with chondroitinase enzyme).

ACKNOWLEDGEMENTS

This work was funded by Research to Prevent Blindness. The authors thank Robert Adams for his technical help and Nancy Ryan for her assistance in preparing the figures and manuscript.

REFERENCES

1 T. Ohya and Y. Kaneko, Biochim. Biophys. Acta, 198 (1970) 607.
2 I. Takazono and Y. Tanaka, J. Chromatogr., 288 (1984) 167.
3 H. Saito, T. Yamagata and S. Suzuki, J. Biol. Chem., 243 (1968) 1536.
4 T. Yamagata, H. Saito, O. Habuchi and S. Suzuki, J. Biol. Chem., 243 (1968) 1523.
5 G.J.L. Lee, J.E. Evans and H. Tieckelmann, J. Chromatogr., 146 (1978) 439.
6 G.J.L. Lee and H. Tieckelmann, Anal. Biochem., 94 (1979) 231.
7 G.J.L. Lee and H. Tieckelmann, J. Chromatogr., 222 (1981) 23.
8 I. Takazono and Y. Tanaka, Kurume Univ. J., 30 (1981) 165.
9 M.E. Adams and H. Muir, Anal. Biochem., 109 (1980) 426.
10 T.O. Kleine and B. Merten, Anal. Biochem., 118 (1981) 185.
11 T. Bitter and H.M. Muir, Anal. Biochem., 4 (1962) 330.
12 A. Hjerpe, C.A. Antonopoulos, B. Engfeldt and B. Wikstrom, Calcif. Tissue Int., 35 (1983) 496.
13 F.W. Newell, Opthalmology: Principles and Concepts, C.V. Mosby, St. Louis, MO, 5th ed., 1982, pp. 303-311.
14 K. Murata, T. Ishikawa and Y. Oshima, Clin. Chim. Acta, 28 (1970) 213.
15 N. Taniguchi, S. Koizumi, K. Masaki and Y. Kobayashi, Biochem. Med., 14 (1975) 241.