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RAPID AND SENSITIVE DETERMINATION OF ENZYMATIC DEGRADATION PRODUCTS OF ISOMERIC CHONDROITIN SULFATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation and quantitative analysis of enzymatic degradation products of isomeric chondroitin sulfates by high-performance liquid chromatography (HPLC) are described. The substituted unsaturated disaccharides which result from digestion of chondroitin sulfates with chondroitinase are quickly separated on polar adsorbents such as silica gel. The UV absorption properties of these unsaturated disaccharides permit UV measurement with detection limits of approximately 100 ng. Their separation by HPLC facilitates the use of enzymatic methods for the determination of chondroitin sulfates A, B and C.

The potential of this method in clinical application is demonstrated by quantitative assays of glycosaminoglycans from a normal urine and urine from a patient with Hunter syndrome. The results are consistent with amount of isomeric chondroitin sulfates found in comparable urines by others.

INTRODUCTION

Chondroitin sulfates A, B and C** are isomeric mucopolysaccharides containing alternating hexuronic acid and N-acetylgalactosamine residues as the

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**The abbreviations used are: ChS-A, ChS-B, and ChS-C = chondroitin sulfates A (chondroitin 4-sulfate), B (dermatan sulfate), and C (chondroitin 6-sulfate); GAG = glycosaminoglycan(s) [mucopolysaccharide(s)]; CPC = cetylpyridinium chloride; ΔDi-4S = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose; ΔDi-6S = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose; ΔDi-OS = 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosylurohic acid)-D-galactose; ΔDi-diS_B = Δ4.5-sulfoglucuronido-acetylgalactosamine-4-sulfate.

characteristic structural backbone. The sulfate residue is located at C-4 of the galactosamine residue in ChS-A and B and at C-6 in ChS-C [1, 2]. The hexuronic acid residue is derived from glucuronic acid in ChS-A and C and from both iduronic acid and glucuronic acid in ChS-B [3—5]. Because of the similarity in structure of the three isomeric chondroitin sulfates, few convenient methods have been reported for the quantitative analysis of the individual isomers in mixtures [6—9]. An enzymatic method for the determination of these chondroitin sulfates was developed by Saito et al. [10], who purified chondroitinase ABC from *Proteus vulgaris* and chondroitinase AC from *Flavobacterium heparinum* which both specifically degraded chondroitin sulfates into the unsaturated disaccharides [11].

The same 4,5-unsaturated disaccharide 4-sulfate (ΔDi-4S) is formed from both ChS-A and ChS-B. The 4,5-unsaturated disaccharide 6-sulfate (ΔDi-6S) is produced only from ChS-C [10]. The assay of the unsaturated disaccharides in the enzymatic degradation product mixture by analytical procedures leads to the identification and measurement of glycosaminoglycans. This enzymatic method has been widely used to estimate glycosaminoglycans in tissues and in serum, urine and other body fluids [12—16].

Methods for the determination of the unsaturated disaccharides from the enzymatic degradation mixture have usually employed paper chromatography [10, 17, 18], and have required up to 36 h for the development of the chromatograms. The detection limit is approximately 25 μg. Recently, thin-layer chromatography has been employed for these assays [19]. Although the analysis was improved by shortening the chromatograph developing time and increasing detection sensitivity, the method required 12 h including a desalting step. These procedures did not provide detailed quantitative data. Colorimetric methods for the assays of sulfated disaccharides in the enzymatic digest by forming chromogens with both sulfated disaccharides and with only ΔDi-6S were also reported recently [20]. This method appears to be sensitive but does not effectively differentiate between the individual disaccharides. The HPLC method described here is rapid and sensitive and can be used to estimate quantities of each of the different chondroitin sulfates.

EXPERIMENTAL

Materials

Chondroitin sulfates A and B and the unsaturated disaccharides were purchased from Miles Labs. (Elkhart, Ind., U.S.A.). Chondroitin sulfate C was purchased from Calbiochem (San Diego, Calif., U.S.A.). Chondroitinase ABC and AC were obtained from both Miles Labs. and Sigma (St. Louis, Mo., U.S.A.).

Prepacked HPLC columns, Partisil PXS, 10 μm, 25 cm × 4.6 mm I.D. (Whatman Labs., Clifton, N.J., U.S.A.) and SI-5A, 5 μm, 25 cm × 4.6 mm I.D. (Brownlee Labs., Berkeley, Calif., U.S.A.) were employed. All solvents used were distilled in glass (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.).

Instruments

For HPLC determination, a Waters Assoc. Model 6000A liquid chromato-

graph equipped with a Waters Assoc. Model 440 UV monitor (254 nm) and a Waters Assoc. Model U6K injector were used. Peak heights and peak areas were determined with a Columbia Model Super 1 integrator (Columbia Sci. Ind., Austin, Texas, U.S.A.).

Isolation of urinary glycosaminoglycans

The urine specimens were filtered through Whatman No. 2 filter paper and adjusted to pH 5.0 with 0.5 *M* acetic acid. Two milliliters of the treated urine was mixed with 30 μl of 5% cetylpyridinium chloride (CPC) in 0.9% NaCl. The urinary GAG required 12 h equilibration at 4° with CPC for maximum precipitation as a CPC-polysaccharide complex [21]. After standing overnight at 4° the precipitate which formed was washed three times with 0.1% CPC and dissolved in 1 ml 2.0 *M* NaCl. The insoluble material was centrifuged. Four volumes of absolute ethanol were added to the supernatant and GAG were precipitated at 0° for 4 h. The precipitate was washed successively with 80% ethanol, absolute ethanol and ether, and dried under a stream of nitrogen. The GAG obtained were further dissolved in 0.5 ml distilled water and precipitated overnight at 0° in four volumes of absolute ethanol saturated with sodium acetate. The precipitate was washed with absolute ethanol and ether and dried in vacuum over P_2O_5. The GAG isolated was then used for enzymatic digestion.

Enzymatic digestion

The digestion mixture contained 10 μl of a solution of 100 μg standard GAG or GAG from 2 ml of urine in water, 10 μl of enriched Tris buffer (pH 8.0) [10] and 20 μl of an aqueous solution of either chondroitinase ABC or chondroitinase AC (10 units/ml). After incubation at 37° for 2.5 h, another 10-μl portion of enzyme solution was added and incubation continued for another 2.5 h at 37° [19]. The enzymes were omitted in the blank. Four volumes of absolute ethanol were then added and the mixtures left overnight at 4°. The clear supernatant obtained by centrifugation was dried under a stream of nitrogen and the residue, dissolved in 100 μl of 90% methanol, was applied on the HPLC instrument.

HPLC Separation of the disaccharides in the enzymatic digest

Columns packed with Partisil 10 (10 μm silica gel particles) and LiChrosorb SI-100 (5-μm silica gel particles) were used. The mobile phase consisted of a three-component mixture: dichloromethane—methanol—ammonium formate buffer. The enzymatic degradation products in 90% methanol were injected directly onto the column.

The ultraviolet absorption spectra of the products of chondroitinase action on chondroitin sulfates as a function of pH were studied by Nakada [22]. At a pH 1.8, an absorption maximum at 232 nm was recorded. Because of relatively strong and broad absorption, the unsaturated disaccharides can be measured with good sensitivity at 254 nm.

Separations were carried out isocratically at room temperatures. Details are given separately with each chromatogram.

RESULTS AND DISCUSSION

Chromatography

A variety of solvent systems were tested. The best separations were achieved with the ternary solvent dichloromethane—methanol—0.5 *M* ammonium formate buffer pH 4.8 (60:34:6, v/v/v). A separation of the three standard unsaturated disaccharides ΔDi-0S, ΔDi-6S and ΔDi-4S requires 20 min and is shown in Fig. 1.

The capacity ratio (k') values of the three disaccharides with different content of buffer are shown in Table I. The data demonstrate that a change of 1% in buffer content has a significant effect on peak resolution. It is apparent that the resolution of ΔDi-0S and ΔDi-6S increases and resolution of ΔDi-6S and ΔDi-4S decreases with increasing buffer content.

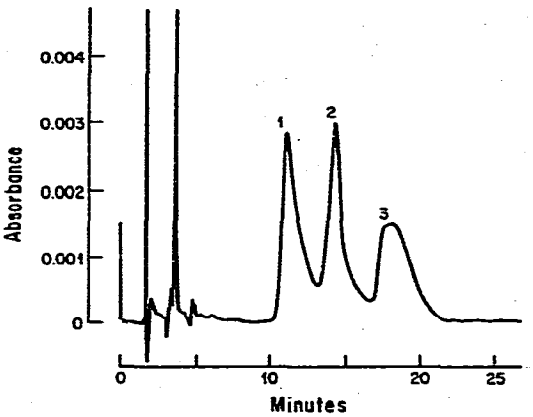

Fig. 1. HPLC of the three standard unsaturated disaccharides. 1 = ΔDi-0S; 2 = ΔDi-6S; 3 = ΔDi-4S; Solvent system: dichloromethane—methanol—0.5 *M* ammonium formate (pH 4.8) (60:34:6, v/v/v); Column: LiChrosorb SI-100, 5 μm, 25 cm × 4.6 mm I.D. Flow-rate: 2.0 ml/min. Pressure: 1800 p.s.i.; Injection port amount: 5 μg of each disaccharide. UV detection at 254 nm, 0.01 a.u.f.s.

TABLE I

CAPACITY FACTORS MEASURED FOR THE THREE STANDARD UNSATURATED DISACCHARIDES AT DIFFERENT AMOUNTS OF AQUEOUS BUFFER IN THE MOBILE PHASE

Values given are mean k' values determined from five separate chromatograms. Column: LiChrosorb SI-100, 5 μm, 25 cm × 4.6 mm I.D. Flow-rate: 2.0 ml/min. Pressure: 1800 p.s.i. UV detection at 254 nm, 0.01 a.u.f.s.

Disaccharide	Ratio of dichloromethane—methanol—0.5 *M* ammonium formate (pH 4.8)		
	60:34:4	60:34:6	60:34:7
ΔDi-0S	5	5.17	4.61
ΔDi-6S	6.11	6.89	6.39
ΔDi-4S	9.11	9.17	7.78

The pH of the ammonium formate buffer also plays an important role in the separation. When the pH was below 3.8, two peaks were observed for each disaccharide which suggests that tautomeric forms were separated. The phenomenon is reversible. At a higher pH of the buffer, only one peak for each disaccharide was observed. Peak shapes become more symmetric as the pH is increased. Above pH 4.0, the characteristic resolution of the three disaccharide peaks is nearly constant.

The dependence of the k' values on methanol content for the three unsaturated disaccharides is shown in Fig. 2. Ternary solvent systems containing less than 30% methanol separated into two phases. With an increase in the methanol content, ΔDi-6S was shifted toward ΔDi-0S, which was eluted first and eventually overlapped with it. The k' values decrease with increasing methanol concentration and asymptotically approach a limit.

Quantitation

For quantitative analysis, calibration curves were established at 254 nm for ΔDi-0S, ΔDi-4S and ΔDi-6S. The linearity is excellent over a large concentration range and the plots go through the point of origin. The advantage

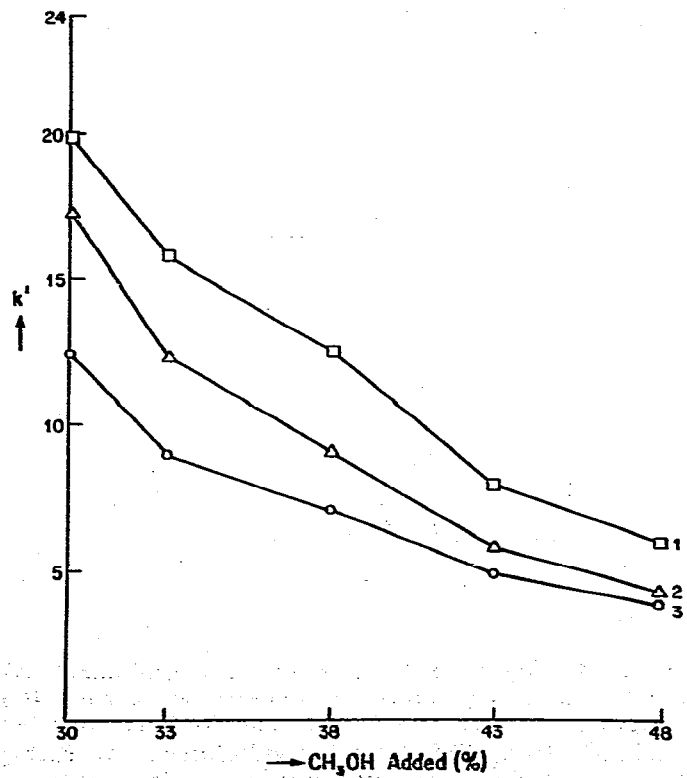

Fig. 2. k' Values of the three standard disaccharides as a function of the methanol content of the mobile phase. 1 = ΔDi-4S; 2 = ΔDi-6S; 3 = ΔDi-0S; Solvent system: dichloromethane—1 *M* ammonium formate (pH 4.8) (55:7) with different amounts of methanol added. Column: Whatman Partisil PXS, 10 μm, 25 cm × 4.6 mm I.D. Flow-rate: 2.0 ml/min. Pressure: 800 p.s.i. Injection volume: 15 μl. UV detection at 254 nm, 0.01 a.u.f.s.

of HPLC for quantitative separation is that it is possible to determine products directly from a calibration curve. In paper or thin-layer chromatography, several steps are required after developing the chromatogram to recover and assay the disaccharides [10, 19].

Application to enzyme digest

The potential of the HPLC separations of enzymatic degradation products is demonstrated in Fig. 3. Figs. 3A and 3C illustrate separations of the degradation products obtained in the incubation of ChS-A and ChS-C respectively with chondroitinase ABC. Figs. 3D and 3F illustrate separations after digestion of these with chondroitinase AC. The major peaks correspond to the expected products. Because of structural similarities, it is difficult to achieve a homogeneous preparation of one chondroitin sulfate to give a single disaccharide upon exhaustive digestion with chondroitinase. Thus, degradation of ChS-A

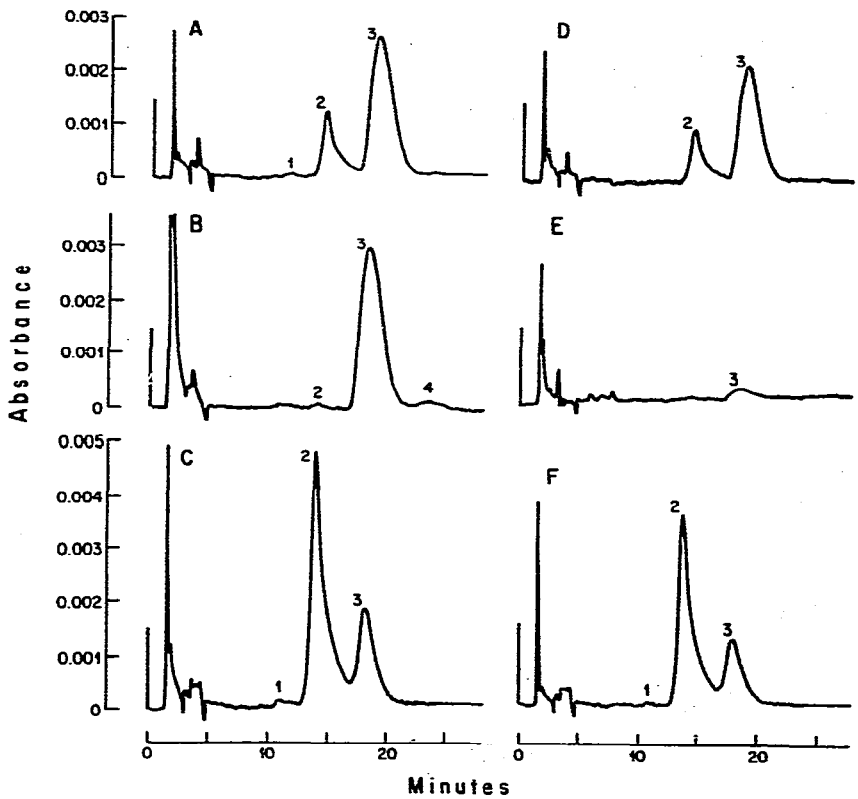

Fig. 3. HPLC of degradation products of chondroitin sulfates with chondroitinase. (A) 15 μg ChS-A with chondroitinase ABC; (B) 15 μg ChS-B with chondroitinase ABC; (C) 15 μg ChS-C with chondroitinase ABC; (D) 10 μg ChS-A with chondroitinase AC; (E) 10 μg ChS-B with chondroitinase AC; (F) 10 μg ChS-C with chondroitinase AC. Peaks: 1 = ΔDi-0S; 2 = ΔDi-6S; 3 = ΔDi-4S; 4 = ΔDi-diS$_B$. Solvent system: dichloromethane—methanol—0.5 *M* ammonium formate (pH 4.8) (60:34:6). Column: LiChrosorb SI-100, 5 μm, 25 cm × 4.6 mm I.D. Flow-rate: 2.0 ml/min. Pressure: 1800 p.s.i. UV detection at 254 nm, 0.01 a.u.f.s.

with chondroitinase ABC or AC gave some ΔDi-6S represented by additional minor peaks, which indicated possible contamination with ChS-C. Likewise, contamination with ChS-A in ChS-C preparations was suggested by the chromatogram.

Fig. 3B shows that ChS-B gave, as expected, ΔDi-4S as the major product following incubation with chondroitinase ABC. The last peak in this chromatogram (peak 4) is probably a disulfated disaccharide (ΔDi-diS_B) reported to occur in ChS-B [12, 23]. It has a higher k' than the monosulfated disaccharides as expected because of the polarity of the sulfate group. Positive identification of this peak is in progress. A chromatogram of the products of digestion of ChS-B with chondroitinase AC is shown in Fig. 3E. A small ΔDi-4S subunit is detected. Since chondroitinase AC is reported to have no activity toward ChS-B [11], this ΔDi-4S subunit could be due to contamination by ChS-A. A small peak corresponding to ΔDi-6S is observed when a large amount of enzyme digest (30% of 100 μg substrate) is injected which indicates contamination by a small amount of ChS-C is also possible. Blank runs indicate that disaccharides are not produced by this procedure in the absence of enzymes.

The recoveries of disaccharides from the enzymatic digest of 100 μg of chondroitin sulfates measured by HPLC are given in Table II. At least 70% of ChS-A can be recovered as disaccharides by digestion with chondroitinase ABC and up to 80% of ChS-A is recovered by action of chondroitinase AC-II, an enzyme of chondroitinase AC type.

TABLE II

RECOVERY OF CHONDROITIN SULFATES AFTER INCUBATION WITH CHONDROITINASE BY USING HPLC SEPARATION

Chondroitin sulfates (100 μg) A, B and C were incubated separately with chondroitinase ABC and AC as described in Experimental. The resulting products were dissolved in 90% methanol and injected onto the HPLC column. Values obtained are the mean of four sets from each digestion product.

Enzyme	Substrate	Product detected (μg)				Recovery (%, w/w)
		ΔDi-0S	ΔDi-6S	ΔDi-4S	ΔDi-diS_B	
Chondroitinase ABC	ChS-A	1.1	17.1	52.9	—	71.1
	ChS-B	—	1.1	59.2	N.M.*	> 60.3
	ChS-C	1.7	63.2	35.5	—	100.4
Chondroitinase AC-II**	ChS-A	1.4	19.5	63.1	—	84
	ChS-B	—	0.5	3.9	—	4.4
	ChS-C	1.7	70.8	36.2	—	108.7

*N.M. = Not measured.
**An enzyme of chondroitinase AC type.

Identification of urinary GAG

The application of HPLC to clinical assay is demonstrated by identification of urinary GAG in normal individuals and in a patient with Hunter syndrome.

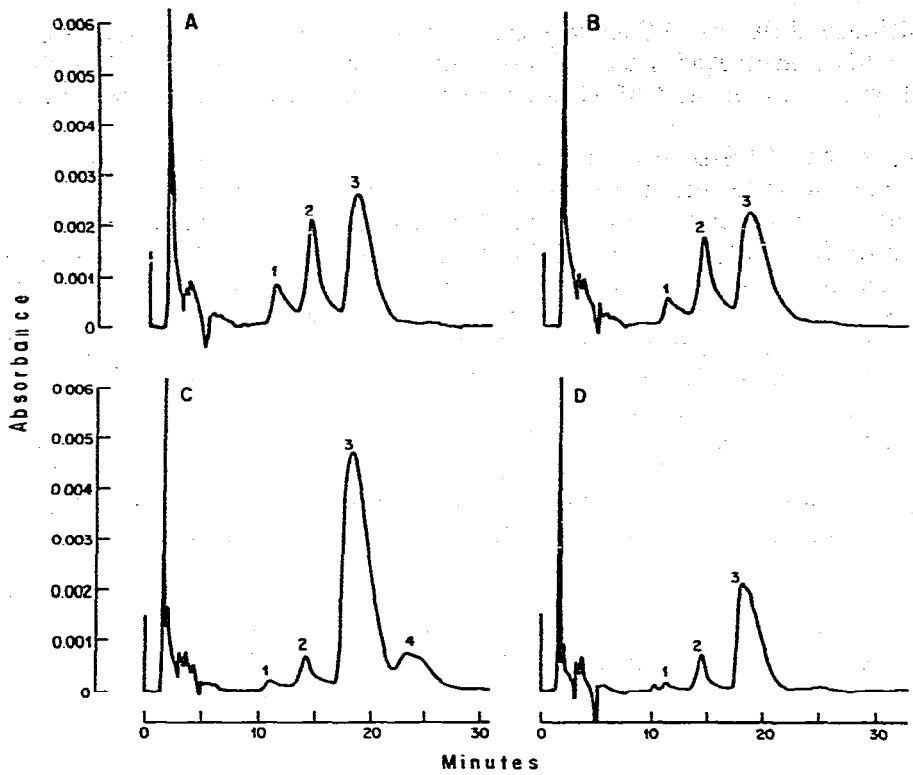

Fig. 4. HPLC of enzymatic degradation products of urinary GAG. (A) GAG from normal urine digestion with chondroitinase ABC; (B) GAG from normal urine digestion with chondroitinase AC; (C) GAG from Hunter's urine digestion with chondroitinase ABC; (D) GAG from Hunter's urine digestion with chondroitinase AC. Peaks: 1 = ΔDi-0S; 2 = ΔDi-6S; 3 = ΔDi-4S; 4 = ΔDi-diS. Solvent system: dichloromethane—methanol—0.5 *M* ammonium formate (pH 4.8) (60:34:6). Column: LiChrosorb SI-100, 5 μm, 25 cm × 4.6 mm I.D. Flow-rate: 2.0 ml/min. Pressure: 1800 p.s.i. UV detection at 254 nm, 0.01 a.u.f.s. Injection amounts are equivalent to 1.548 mg creatinine for normal urine and 0.234 mg creatinine for Hunter's urine.

Fig. 4 shows HPLC separations of disaccharides from urinary GAG after digestion with chondroitinase ABC and AC. The ΔDi-4S or ΔDi-6S obtained from normal urinary GAG are formed in the similar amounts in treatments with either enzyme chondroitinase ABC or AC (Figs. 4A and 4B). The results indicate that ChS-A and ChS-C predominate in the urinary GAG of normals and ChS-B is present in smaller amounts. This is consistent with the values obtained by other chromatographic methods. On the other hand, the patient with Hunter syndrome excreted a large amount of ChS-B [24]. The ΔDi-4S from Hunter's urinary GAG by digestion with chondroitinase AC are exclusively liberated from ChS-A (Fig. 4D), whereas ΔDi-4S obtained by digestion with chondroitinase ABC is liberated from ChS-A and B (Fig. 4C). The large difference in amounts of ΔDi-4S obtained from Hunter's urinary GAG in treatments with each to the two enzymes verifies that the excretion of large amounts of ChS-B is the characteristic of Hunter syndrome. Furthermore,

the appearance of the ΔDi-diS subunit in digestion of Hunter's urinary GAG with chondroitinase ABC (Fig. 4C) demonstrates the presence of ChS-B in the urine. Heparin sulfate is not detected by our chromatogram because of its resistance to digestion by the chondroitinase enzymes.

Table III shows the amounts of each disaccharide derived from enzymatic digestion of normal and Hunter's urinary GAG compared to excreted creatinine. The ratio of ΔDi-4S and ΔDi-6S from ChS-A and C are approximately 2:1 in normal urine. The urinary excretion of ChS-A and C from the patient with Hunter syndrome is significantly greater. These are essentially in agreement with previously reported enzymatic values [12].

TABLE III

ANALYSIS OF CHONDROITIN SULFATES IN URINE BY HPLC

Glycosaminoglycans were isolated from a normal child and a patient with Hunter syndrome and subjected to enzymatic digestion with chondroitinase ABC and AC as described in Experimental. The amount of disaccharides obtained in the enzymatic digest is given as μg disaccharides per mg of urinary creatinine.

Sources of urinary GAG	Enzyme used for degradation	Product	Amount of products (μg disaccharides per mg of urinary creatinine)	Type of chondroitin sulfate previding products
Normal urine	Chondroitinase ABC	1. ΔDi-0S	0.92	Non-sulfated chondroitin
		2. ΔDi-6S	2.72	ChS-C
		3. ΔDi-4S	4.98	ChS-A, ChS-B
	Chondroitinase AC	1. ΔDi-0S	0.62	Non-sulfated chondroitin
		2. ΔDi-6S	2.26	ChS-C
		3. ΔDi-4S	4.34	ChS-A
Hunter's urine	Chondroitinase ABC	1. ΔDi-0S	1.59	Non-sulfated chondroitin
		2. ΔDi-6S	6.11	ChS-C
		3. ΔDi-4S	60.15	ChS-A, ChS-B
		4. ΔDi-diS	N.M.*	ChS-B
	Chondroitinase AC	1. ΔDi-0S	1.51	Non-sulfated chondroitin
		2. ΔDi-6S	6.45	ChS-C
		3. ΔDi-4S	26.96	ChS-A

*N.M. = Not measured.

CONCLUSION

Effective separation and quantitation of the unsaturated disaccharides resulting from digestion of chondroitin sulfates with chondroitinase can be carried out by HPLC. This is a more rapid and sensitive determination than previously reported methods. The strong UV absorption of these disaccharides makes possible their detection at 254 nm without derivatization. The sensitivity can be increased by using a UV monitor at 232 nm.

Since it is possible to identify and distinguish between many of the individual mucopolysaccharidoses by the pattern of urinary GAG excretion we believe the present procedure could be usefully employed in such diag-

noses. Further work on the application of this technique to the quantitative determination of isomeric chondroitin sulfates is in progress.

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