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THE APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN ENZYMATIC ASSAYS OF CHONDROITIN SULFATE ISOMERS IN NORMAL HUMAN URINE

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SUMMARY

A study of the urinary excretion of isomeric chondroitin sulfates in normal individuals by high-performance liquid chromatographic (HPLC) determinations of the unsaturated disaccharides produced by digestion with chondroitinases is described. The composition of the HPLC mobile phase was systematically varied in order to select the optimal conditions for separation.

The data show that chondroitin 4-sulfate is the major component of the chondroitin sulfate isomers in normal urine, and that chondroitin 6-sulfate is a lesser component. It is also evident that dermatan sulfate is present in small quantities in normal urine.

INTRODUCTION

Chondroitin 4-sulfate (C-4S) and chondroitin 6-sulfate (C-6S) have been reported to be the principal urinary glycosaminoglycans (GAG) in normal subjects [1—4]. However, the assays for chondroitin sulfate isomers vary according to the method used [5—9]. An enzyme method based on the quantitative determination of the unsaturated disaccharides produced by the digestion of urinary isomeric chondroitin sulfates with chondroitinase ABC and AC is specific for the quantitative measurement of each of the isomeric chondroitin sulfates [10, 11]. A high-performance liquid chromatographic (HPLC) method for the rapid and sensitive quantification of these unsaturated disaccharides has been developed [12] which is applied to elucidate the isomeric chondroitin sulfate distribution in normal urine in the present study.

EXPERIMENTAL

Materials

The C-4S, C-6S, dermatan sulfate (DS), the unsaturated disaccharides 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-OS), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose (ΔDi-6S), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose (ΔDi-4S), chondroitinase ABC and chondroitinase AC were products of Seikagaku Kogyo (Tokyo, Japan) and were purchased from Miles Laboratories (Elkhart, IN, U.S.A.). Methanol and acetonitrile, distilled in glass, were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.).

Instruments

The apparatus used was a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery system equipped with a Waters Assoc. Model 440 UV detector (254 nm) and Model U6K injector. Peak heights, peak areas and retention times were measured by an on-line Columbia Model Supergrator-1 integrator (Columbia Scientific Industries, Austin, TX, U.S.A.). A Partisil-10 PAC (10 μm, 25 cm × 4.6 mm I.D.) column (Whatman, Clifton, NJ, U.S.A.) was employed.

Isolation of urinary GAG

The procedure used was essentially the method described earlier [13]. The urine specimens were filtered through Whatman No. 2 paper. Ten milliliters of the filtered urine were adjusted to pH 5.0 with 0.5 *M* acetic acid and mixed with 150 μl of 5% cetylpyridinium chloride (CPC) in 0.9% sodium chloride. The GAG were precipitated as CPC—polysaccharide complex after standing at 4°C overnight. The resulting precipitate was washed three times with 0.1% CPC and dissolved in 0.5 ml 2.0 *M* sodium chloride. The insoluble material was centrifuged and discarded. Four volumes of absolute ethanol were added to the supernatant and the GAG were precipitated at 0°C for 4 h. The precipitate was collected by centrifugation and washed successively with 80% ethanol, absolute ethanol and diethyl ether. After drying in air the GAG obtained were dissolved in 0.3 ml distilled water and precipitated at 0°C for 4 h in four volumes of absolute ethanol saturated with sodium acetate. The last step was repeated once. The precipitate was washed with absolute ethanol and diethyl ether and dried at reduced pressure over phosphorus pentoxide. The GAG isolated from 10 ml urine were dissolved in 180 μl distilled water and separated into two tubes for digestion with chondroitinase ABC and AC, respectively.

The total uronic acid in CPC fraction was determined using glucuronolactone as a standard by the borate—carbazole method [14]. Creatinine was determined by the method of Tausskey [15].

Enzymatic procedure

The digestion mixture contained 90 μl of a solution of urinary GAG in water (0.015—0.22 μmole of GAG as uronic acid), 30 μl of enriched Tris buffer (pH 8.0) and 30 μl of an aqueous solution of either chondroitinase ABC or chon-

droitinase AC (10 units/ml). The exhaustive digestion was carried out at 37°C for 5 h. Standard chondroitin sulfates were also digested in the same manner to check the enzyme activity. Four volumes of absolute ethanol were added after incubations and the mixture left overnight at 4°C. The clear supernatant obtained by centrifugation was dried under a stream of nitrogen and the residue, dissolved in 90% methanol, was applied to the chromatograph for analysis.

Recovery of added isomeric chondroitin sulfates

Solutions were prepared which contained 1 mg/ml of standard C-4S, C-6S and DS in distilled water. Aliquots of 50 μl of each of these solutions were added separately to 2 ml of normal urine. The GAG in these augmented urines were precipitated with 100 μl of 5% CPC in 0.9% sodium chloride and isolated as described above. Reproducibility of recovery was measured by duplicate isolations of GAG of each spiked normal urine sample. Enzyme reaction conditions and sample treatment for HPLC analysis were the same as described above.

HPLC

In the HPLC separations, the eluent used was acetonitrile—methanol—ammonium formate buffer. A Whatman Partisil-10 PAC, a bonded cyano-amino-type column was used at a flow-rate of 2.0 ml/min.

RESULTS AND DISCUSSION

Ratio of acetonitrile to methanol concentration in the eluent

The capacity ratios (k') of the disaccharides (ΔDi-0S, ΔDi-6S and ΔDi-4S) were determined as a function of the acetonitrile and methanol content of the mobile phase, while keeping the content of ammonium formate buffer constant at 20% (Fig. 1). Benzene was used as the inert peak in the calculations of the k' values. For sulfated disaccharides the k' values first decrease with increasing acetonitrile content and decreasing methanol content, pass through a minimum at an acetonitrile—methanol ratio of about 3.0 and then increase sharply. The k' value of nonsulfated disaccharide increases slightly in the region in which k' values of sulfated disaccharides decrease and then increases sharply as did the sulfated disaccharides. Fig. 1 demonstrates that the acetonitrile and methanol content is a valuable parameter for adjusting the retention. It is found that at a ratio of acetonitrile to methanol of 3.0, effective separation and good resolution of the three disaccharide isomers in the enzyme digest of urinary GAG is achieved. Thus, in further investigations of the influence of parameters in this aqueous buffer, this ratio for the two organic solvents was used.

Effect of pH, concentration and content of aqueous ammonium formate

The dependence of the k' values on the pH of ammonium formate buffer for the three unsaturated disaccharides was studied. For sulfated disaccharides the k' values decrease with an increasing pH of the buffer. However, the k' value of ΔDi-0S only changes slightly when the pH was varied from 3.5 to 5.75. Peak shapes of the three compounds become more symmetrical as the pH is

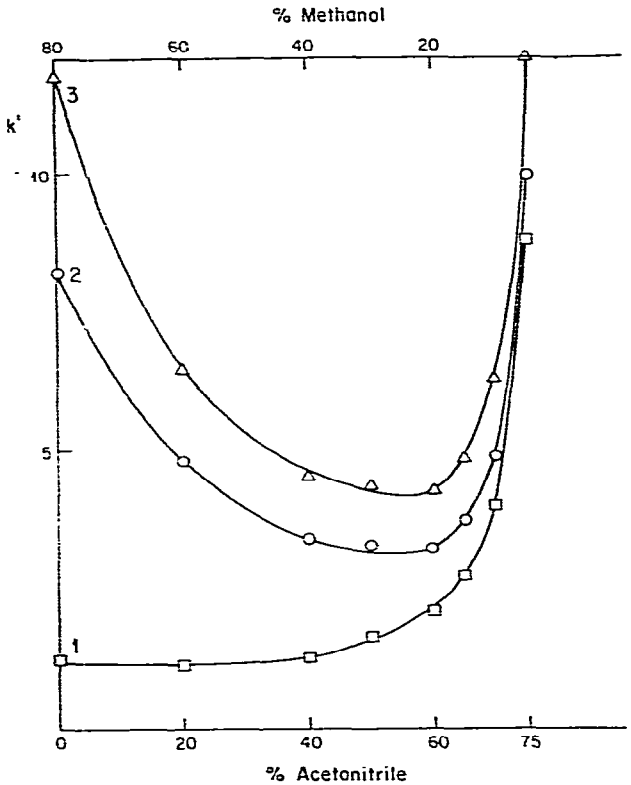

Fig. 1. Capacity ratios (k') of the three unsaturated disaccharides as a function of the acetonitrile and methanol content of the mobile phase. 1 (□), ΔDi-0S; 2 (○), ΔDi-6S; 3 (△), ΔDi-4S; solvent system, acetonitrile—methanol—0.5 *M* ammonium formate pH 4.5 (*X*:*Y*:20, v/v/v) (*X*, % acetonitrile; *Y*, % methanol; *X* + *Y* = 80%). Column, Whatman Partisil-10 PAC; flow-rate, 2.0 ml/min.

increased. Although the degree of retention can be affected by the pH of aqueous buffer, good resolution (selectivity factors for any two of the disaccharides are at least 1.22) is observed in the pH range tested. The three disaccharides are more retained as the ionic strength decreases. The baseline separation for the three compounds can be obtained when the concentration of ammonium formate is below 0.6 *M*. The effect of buffer content in the mobile phase on the k' values of the three disaccharides is shown in Fig. 2. As expected, the k' values decrease as the amount of aqueous buffer is increased. This phenomenon is probably due to the increase in the overall ionic strength of the mobile phase and the increased solvation of the solutes as the solvent becomes more polar.

All of the parameters in the mobile phase described above have a significant effect on separation. In particular, the ammonium formate buffer content in combination with variation of pH can be used to adjust the retention and selectivity of these unsaturated disaccharides. These studies demonstrate the flexibility of the HPLC system and serve as a guide for the selection of the best conditions for chromatography.

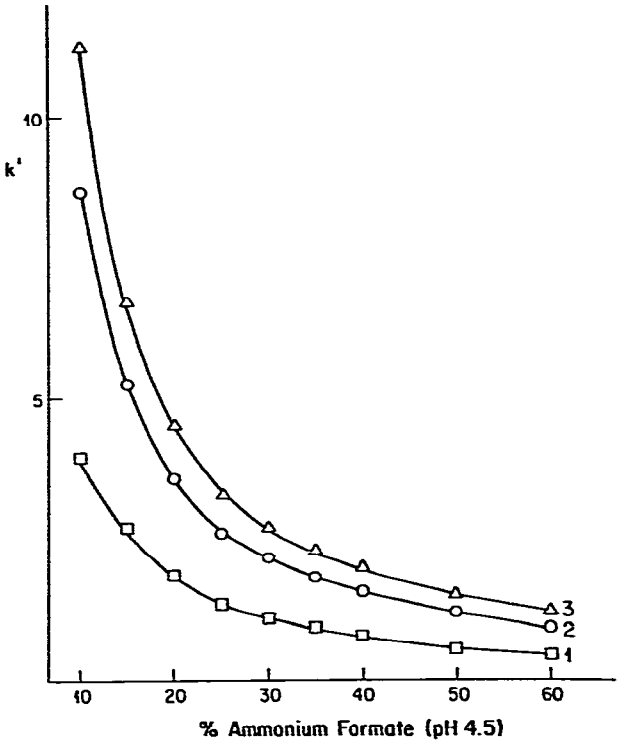

Fig. 2. Capacity ratios (k') of the three unsaturated disaccharides as a function of the ammonium formate content of the mobile phase. 1 (□), ΔDi-0S; 2 (○), ΔDi-6S; 3 (△), ΔDi-4S; solvent system, acetonitrile—methanol (3:1, v/v) with different amounts of 0.5 *M* ammonium formate (pH 4.5) added. Column, Whatman Partisil-10 PAC; flow-rate, 2.0 ml/min.

Linearity

The linearity of the HPLC response to the unsaturated disaccharides was evaluated by analyzing enzymatic digestion products derived from both fixed and varying amounts of normal urinary GAG. Duplicate urinary GAG (equivalent to a 8-ml normal urine in each) was subjected to enzyme reaction with chondroitinase ABC and AC respectively. The resulting disaccharide products, dissolved in 200 μl 90% methanol, were injected onto the HPLC column in increasing amounts (from 5 μl to 20 μl). It was established that for the range of 100 ng to 10 μg a linear relationship existed between the peak heights of disaccharides and amounts of digestion products injected. This wide linear range is more than sufficient for the determination of disaccharide isomers in enzymatic digests of normal urinary GAG.

Fig. 3 illustrates the linear relationship between the amounts of isomeric chondroitin sulfates in mixtures with other urinary GAG and the peak heights of disaccharide products. The GAG isolated from 200 ml normal urine was dissolved in 2 ml distilled water. Aliquots of different volumes were subjected to enzymatic degradation with chondroitinase ABC (Fig. 3A) and AC (Fig. 3B) and products were determined by HPLC. The difference in the slopes of ΔDi-4S lines between Fig. 3A and 3B is insignificant which indicates that little DS is present.

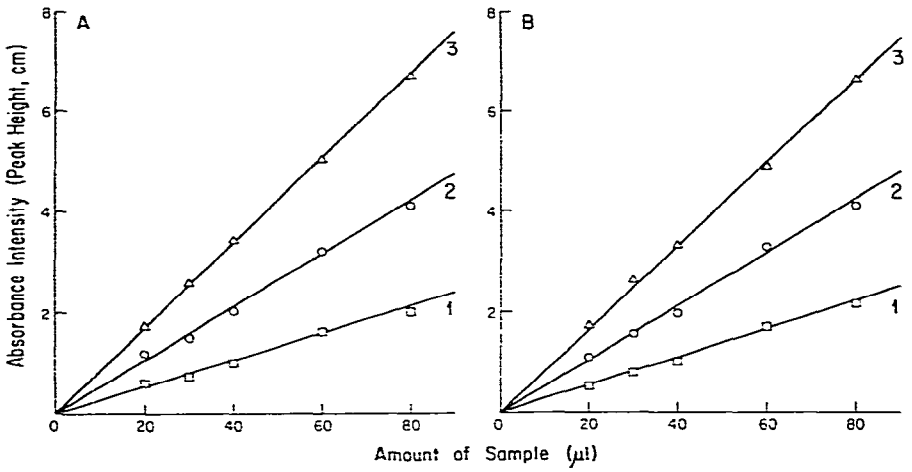

Fig. 3. Linear relationship between the amounts of chondroitin sulfate isomers in a normal urine and the peak heights of disaccharide products by HPLC. The amounts of sample are described in text. Ten percent of the degradation products from each sample were injected on the HPLC column. (A) Digestion with chondroitinase ABC; (B) digestion with chondroitinase AC. Compounds: 1 (□), ΔDi-0S; 2 (○), ΔDi-6S; 3 (△), ΔDi-4S.

Recoveries

Recovery studies were made by adding known quantities of standard C-4S, C-6S and DS to normal urine as described in the Experimental section. Chromatograms in Fig. 4 present a graphical illustration of recovery studies. All chromatograms are from independently prepared aliquots of enzymatic digestion products of GAG from normal urine with and without added standards. HPLC separations of disaccharides from normal urinary GAG after digestion with chondroitinase ABC and AC are shown in Fig. 4A and 4B, respectively. These blanks determine the amount of chondroitin sulfates in normal urine. The ΔDi-4S and ΔDi-6S are produced in similar amounts by treatment with either enzyme. Non-sulfated disaccharides from chondroitin and hyaluronic acid which are present in trace amounts appear at 4.2 min and are not completely separated. The recovery of added C-4S standard as its disaccharide fragments is illustrated in Fig. 4C and 4D, the effect of added C-6S standard is shown in Fig. 4G and 4H. The major peaks in these chromatograms correspond to the expected disaccharides, that is, ΔDi-4S in C-4S spiked urine and ΔDi-6S in C-6S spiked urine. The height increase of the ΔDi-6S peak in the enzyme digests of C-4S spiked urine and of the ΔDi-4S peak in the digests of C-6S spiked urine compared to the blanks (Fig. 4A and 4B), may be due to the presence of copolymers [16] and/or contaminants in the standard chondroitin sulfates. The added DS gave a large increase in amount of ΔDi-4S when digested with chondroitinase ABC (Fig. 4E), but did not when digested with chondroitinase AC (Fig. 4F).

Table I shows the recovery of the standard chondroitin sulfates added to normal urine, which represents the recoveries following three consecutive procedures: urinary GAG isolation, enzyme reaction and HPLC quantitation. The apparent lower recovery of DS in the chondroitinase ABC digest may be

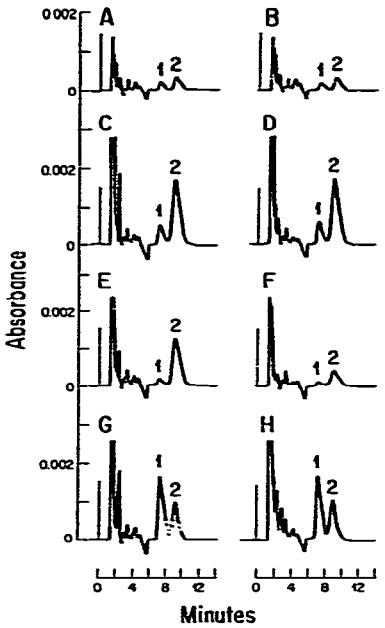

Fig. 4. Chromatograms of enzymatic degradation products of GAG from normal urine with and without standard isomeric chondroitin sulfates added. GAG from a 2-ml normal urine (A) degraded by chondroitinase ABC; (B) degraded by chondroitinase AC; (C) with standard C-4S spike degraded by chondroitinase ABC; (D) with standard C-4S spike degraded by chondroitinase AC; (E) with standard DS spike degraded by chondroitinase ABC; (F) with standard DS spike degraded by chondroitinase AC; (G) with standard C-6S spike degraded by chondroitinase ABC; (H) with standard C-6S spike degraded by chondroitinase AC. Peaks: 1, ΔDi-6S; 2, ΔDi-4S. Column: Partisil-10 PAC, 10 μm, 25 cm × 4.6 mm I.D. Solvent system, acetonitrile—methanol—0.5 *M* ammonium formate (pH 4.5) (60:20:20, v/v/v); flow-rate 2.0 ml/min; pressure, 700 p.s.i. Injection amount, 10% of the degradation products for (A) and (B), 7.5% of the degradation products for others. UV detection at 254 nm, 0.01 a.u.f.s.

due to oversulfated disaccharide products [17] which were not measured in this study. About 4% recovery of DS is found in the chondroitinase AC digest which could be due to contamination of the DS preparations by other chondroitin sulfates. Duplicate isolation of GAG and enzyme treatment from urines containing chondroitin sulfates as internal standards indicated that recovery had an experimental error of less than 7%.

Determination of isomeric chondroitin sulfates in normal urine samples

Table II represents the data for C-4S, C-6S and DS in twelve normal urine samples. The amount of C-6S was calculated from the mean values of ΔDi-6S by digestion of urinary GAG with chondroitinase ABC and AC. The C-4S was determined from the value of ΔDi-4S by digestion of urinary GAG with chondroitinase AC. The DS was calculated by subtracting the ΔDi-4S obtained by digesting GAG with chondroitinase AC from that with chondroitinase ABC. The data show that C-4S is the major component of normal urinary chondroitin sulfate isomers and comprises at least 72% of total urinary chondroitin sulfates in the cases examined. The C-6S is present in a lesser amount

TABLE I

RECOVERY OF ISOMERIC CHONDROITIN SULFATES ADDED TO NORMAL URINE

Fifty micrograms of standard C-4S, C-6S and DS were added separately to 2-ml samples of normal urine. The urinary GAG were isolated from these augmented urines and incubated separately with chondroitinase ABC and AC as described in Materials and methods. The resulting disaccharide products were analyzed by HPLC.

Enzyme	Sources of GAG	Products detected (μg)			Recovery	
		ΔDi-6S	ΔDi-4S	Total	μg	%
Chondroitinase ABC	urine alone	2.91	6.72	9.63		
	urine + C-4S	9.05	39.88	48.93	39.3	78.6
	urine + DS	2.91	37.2	40.11	30.48	60.96
	urine + C-6S	29.09	18.05	47.14	37.51	75.02
Chondroitinase AC	urine alone	2.76	6.55	9.31		
	urine + C-4S	9.03	38.97	48	38.69	77.38
	urine + DS	2.6	8.85	11.45	2.14	4.28
	urine + C-6S	29.03	20.12	49.15	39.84	79.68

TABLE II

RELATIVE PROPORTIONS OF THE THREE CHONDROITIN SULFATES IN NORMAL URINE SAMPLES BY ENZYMATIC AND HPLC ANALYSES

Case No.	Initials	Age (years)	Sex	Isomeric chondroitin sulfates (by HPLC)				Total uronic acid in CPC fraction, by carbazole (nmole/mg creatinine)	Molar ratio*
				Total amount (nmole/mg creatinine)	Percent				
					C-4S	DS	C-6S		
1	L.S.	2	M	34.36	87.5	1.4	11.1	44.62	77
2	W.L.	3	F	42.33	86.8	2.7	10.5	57.99	73
3	T.C.	3	F	41.22	83.4	3.8	12.8	55.03	74.9
4	M.J.	4	M	26.71	91.7	—	8.3	40.84	65.4
5	J.C.	5	M	33.9	81.5	1.5	17	50.22	67.5
6	A.W.	11	M	21.33	91.8	—	8.2	30.87	69.1
7	H.L.	25	F	5.29	72.8	6.4	20.8	6.09	86.8
8	J.G.	29	M	3.91	69.4	7.1	23.4	4.75	82.4
9	L.L.	32	F	3.85	79.7	3.7	16.6	5.13	75.1
10	H.K.	34	M	3.82	74.9	12.5	12.6	5.21	73.3
11	J.Y.	38	M	3.82	75.6	4.2	20.2	4.85	78.8
12	M.K.	65	F	5.21	78.6	6.7	14.7	8.26	63.1

*Total amount of sulfated disaccharide produced from urinary chondroitin sulfate detected by HPLC was divided by the uronic acid content in CPC fraction.

whereas DS is found to be only a minor component and negligible in certain cases. Several reports have indicated that C-6S is excreted in the largest amounts among normal urinary chondroitin sulfate isomers [7—9]. However, our values of C-4S in normal urine are essentially in agreement with that reported by Linker and Terry [5]. The presence of a small amount of DS is consistent with most reported values [4, 7, 18]. The total amounts of chondroitin sulfate isomers per mg of excreted creatinine determined by the combined enzymatic and HPLC methods indicate that with an advancing age

there is a trend to decreased excretion of urinary chondroitin sulfates. It is also evident that the amounts of urinary GAG precipitated by CPC and measured as uronic acid by the carbazole method exhibit a similar trend. It has been reported that in the measurement of normal urinary GAG the uronic acid: creatinine ratio is high in children, falling to a low level in adults [19—21]. The present results are in accord with these data. The molar ratio of total chondroitin sulfate isomers to total uronic acid indicates that normal urinary GAG consists mainly of chondroitin sulfate isomers. Analysis of the relative urinary chondroitin sulfate levels may reflect the metabolic states of connective tissues in the human body. The HPLC separation used with these specific enzyme reactions is convenient and reliable for these studies.

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