Journal of Chromatography, 414 (1987) 156–160
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3419

Note

Rapid determination of disaccharides from chondroitin and dermatan sulphates by high-performance liquid chromatography

J. MACEK*, J. KRAJÍČKOVÁ and M. ADAM

Research Institute for Rheumatic Diseases, Na slupi 4, CS 128 50 Prague 2 (Czechoslovakia)

(First received June 1st, 1986; revised manuscript received September 26th, 1986)

Glycosaminoglycans form a part of proteoglycans and their amount is changing with ageing and under pathological conditions. Their determination, therefore, may give us a useful information in this respect.

Glycosaminoglycans are catabolized with specific enzymes to the characteristic unsaturated disaccharides 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose (ΔDi-6S) and 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-O-sulpho-D-galactose (ΔDi-4S). Chondroitin 4-sulphate (C-4S) and chondroitin 6-sulphate (C-6S) are digested with chondroitinase AC and chondroitinase ABC. The latter enzyme digests also dermatan sulphate (DS). High-performance liquid chromatography (HPLC) of the characteristic disaccharides provides a useful tool for quantification of respective glycosaminoglycans. Several HPLC methods have been developed for this purpose [1–8]. However, they suffer from low column efficiency [1–3, 6–8], long analysis times [4–7] or losses due to sample preparation [8]. The aim of this study was to develop a rapid, sensitive and accurate method for determination of C-4S, C-6S and DS.

EXPERIMENTAL

Chemicals

The unsaturated disaccharides, chondroitinase ABC and chondroitinase AC, were products of Seikagaku Kogyo (Tokyo, Japan) and were purchased from Miles Labs. (Elkhart, IN, U.S.A.). Sodium sulphate and acetic acid were of analytical-grade quality and were obtained from Lachema (Brno, Czechoslovakia).

0378-4347/87/$03.50 © 1987 Elsevier Science Publishers B.V.

For HPLC distilled water filtered through a 0.45-μm membrane filter (Supelco, Bellefonte, PA, U.S.A.) was used.

Apparatus

Chromatographic equipment was obtained from Spectra-Physics (San Jose, CA, U.S.A.). The SP-8100 liquid chromatograph equipped with SP-8110 autosampler was connected to an SP-8440 variable-wavelength UV–VIS detector and an SP-4200 computing integrator. The volume of the injection loop was 10 μl.

Chromatography

A stainless-steel analytical column (25 cm×4.6 mm I.D.), packed with aminopropyl-bonded silica stationary phase Separon SIX NH_2, particle size 10 μm, was obtained from Laboratorní Přístroje (Prague, Czechoslovakia). The presaturator column packed with the same packing as the analytical column was placed between the pump and the injector. The mobile phase was water containing sodium sulphate (0.01 mol/l) and acetic acid (0.001 mol/l). The flow-rate of the mobile phase was 2.2 ml/min and the pressure was 6 MPa. The column was operating at 50°C and equilibrated with 120 ml of the mobile phase; for storage the column was filled with methanol.

The absorbance was monitored at 232 nm. The range was 0.16 a.u.f.s. and the time constant 0.1 s. The integrator parameters were as follows: peak width = 12; peak threshold = 100; attenuation = 2; chart speed = 0.5 cm/min.

Sample preparation

For the determination of C-4S and C-6S 1–1000 μg of the lyophilized sample (in our case proteoglycans obtained by isopycnic centrifugation) was dissolved in 80 μl of enriched Tris buffer [9].

Chondroitinase AC (20 μl, 0.05 U) was added and the solution was incubated at 37°C for 16 h. After this time, 20 μl of 1 *M* trichloroacetic acid were added. When the autosampler was used, the volume of the mixture was increased to 320 μl by addition of 200 μl of deionized water at this step. The reaction vial was centrifuged for 8 min at 1000 *g* and 10 μl of supernatant were taken for the HPLC analysis.

The sample preparation for the DS analysis was essentially the same with one exception: the sample was dissolved in 160 μl of enriched Tris buffer, then divided into two equal portions, which were digested with either chondroitinase AC or ABC as specific above.

RESULTS AND DISCUSSION

Chromatography

The separation of ΔDi-6S from ΔDi-4S is shown in Fig. 1. When present, the non-sulphated disaccharide ΔDi-0S can also be detected in this system and it has a retention time of 1.81 min. However, the disaccharide resulting from enzymatic digestion of hyaluronic acid cannot be separated from ΔDi-0S in our chromatographic system. For the separation of these two disaccharides special separation

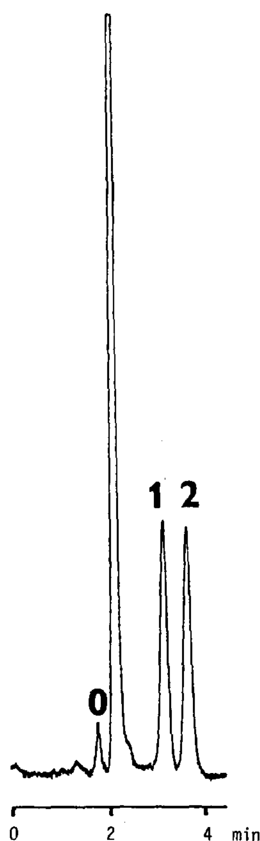

Fig. 1. Chromatogram of the unsaturated disaccharides from chondroitin sulphate isomers. Peaks: 0=ΔDi-0S; 1=ΔDi-6S (483 ng); 2=ΔDi-4S (588 ng). For chromatographic conditions, see text.

systems had been designated [5,10,11]. An organic modifier used in some previous methods proved to decrease column efficiency and therefore we used an aqueous buffer as a mobile phase. The concentration of sodium sulphate (0.01 mol/l) was chosen to give as short retention times as possible. The resolution between ΔDi-4S and ΔDi-6S was 1.80 at this concentration (Table I). The tem-

TABLE I

EFFECT OF MOBILE PHASE COMPOSITION ON RETENTION TIMES AND RESOLUTION

Sodium sulphate concentration (mol/l)	Retention time (min)		Resolution
	ΔDi-6S	ΔDi-4S	
0.02	2.74	3.02	1.20
0.01	3.21	3.70	1.80
0.005	5.32	6.32	2.15
0.002	10.57	13.1	2.67

TABLE II

EFFECT OF TEMPERATURE ON COLUMN EFFICIENCY AND RESOLUTION

Temperature (°C)	Retention time (min)		Number of plates		Resolution
	ΔDi-6S	ΔDi-4S	ΔDi-4S	ΔDi-6S	
25	3.39	3.97	1820	1460	1.6
40	3.33	3.84	2300	1950	1.64
50	3.21	3.7	2790	2360	1.8
60	3.11	3.55	2800	2700	1.73

perature should theoretically increase column efficiency due to lower viscosity of the mobile phase and faster solute exchange between the stationary and mobile phases. This was confirmed by experimental results, presented in Table II. A temperature of 50°C was used. This temperature gave the highest resolution between ΔDi-6S and ΔDi-4S and shortened the analysis time to 4 min, which is a substantial improvement compared to the previous methods.

Enzymatic digestion

With amounts of glycosaminoglycans not higher than 1 mg, the enzymatic digestion was completed in 8 h regardless of the enzyme used. No buffer-induced modification of ΔDi-6S [8] was observed.

The sample preparation is simple, because precipitation with ethanol followed by evaporation is not required. Using our procedure some high-molecular-weight material remains soluble. However, more than 400 samples were injected on the same column without signs of its deterioration and without a change in the retention behaviour. The final concentration of trichloroacetic acid in the sample is not as high to disturb the column equilibration.

Method parameters

The calibration curve was linear from 25 ng to 200 μg. The accuracy of the method was 2.1% ($n=4$). The precision is given in Table III ($n=3$). The detection limit was 25 ng (signal-to-noise ratio 3:1) for both disaccharides.

TABLE III

PRECISION OF THE METHOD

Precision is expressed as relative standard deviation (R.S.D.).

Enzyme	Di-6S		Di-4S	
	Amount (μg)	R.S.D. (%)	Amount (μg)	R.S.D. (%)
Chondroitinase ABC	490	1.15	123	0.47
	60	1.53	16.5	1.82
Chondroitinase AC	478	1.28	122	0.95
	60	0.29	13.3	2.3

Determination of dermatan sulphate

The DS amount was calculating by subtracting the ΔDi-4S amount obtained by digesting glycosaminoglycans with chondroitinase AC from that obtained with chondroitinase ABC [7]. The limitations of this procedure were studied: the enzymatic digestions with different enzymes are independent and, therefore, the variance of DS amount can be calculated as the sum of variances of both processes. From the results presented in Table II the standard deviation for the difference Di-4S (ABC) − Di-4S (AC) is 1.11 μg (1.06%) at 123-μg level and 0.43 μg (2.93%) at 16.5-μg level. That means that, e.g. for a mixture of 0.43 μg DS and 16 μg C-4S, the relative standard deviation of the determination is 100%. Therefore, the present procedure is not suitable for determination of small quantities of DS in the presence of a large amount of C-4S. In such a case, the method of Gurr et al. [5], which uses sequential ethanol precipitation, is to be preferred. We set the relative standard deviation at 20% as the limit for reliable results. This condition is met when the amount of DS is not less than 5.4% of C-4S at 123 μg and 13.4% of C-4S at 16.5-μg level. It may be concluded that some trace amounts of DS found in normal urine by Hjerpe et al. [7] could be caused by experimental error and not by presence of DS itself.

CONCLUSIONS

A rapid, sensitive and precise HPLC method has been developed for the determination of chondroitin sulphate isomers. The method can also be used for the determination of dermatan sulphate when the ratio C-4S/DS is not higher than 10:1.

ACKNOWLEDGEMENT

We are grateful to Mrs. Kadičová for the skilful technical assistence.

REFERENCES

1 G.J.-L. Lee and H. Tieckelmann, J. Chromatogr., 222 (1981) 23.
2 K. Murata and Y. Yokoyama, Anal. Biochem., 149 (1985) 261.
3 K. Murata and Y. Yokoyama, Anal. Biochem., 146 (1985) 327.
4 G.J.-L. Lee, D.-W. Lin, J.W. Pav and H. Tieckelmann, J. Chromatogr., 212 (1981) 65.
5 E. Gurr, G. Pallasch, S. Tunn, C. Tamma and A. Delbruck, J. Clin. Chem. Clin. Biochem., 23 (1985) 77.
6 N. Ototani, N. Sato and Z. Yosizava, J. Biochem., 85 (1979) 1383.
7 A. Hjerpe, C.A. Antonopoulos and B. Engfeldt, J. Chromatogr., 171 (1979) 339.
8 D.P. Norby, V.M. Goldberg, R.W. Moskowitz and C.J. Malemud, Anal. Biochem., 131 (1983) 324.
9 H. Saito, T. Yamagata and S. Suzuki, J. Biol. Chem., 243 (1968) 1536.
10 A. Hjerpe, C.A. Antonopoulos and B. Engfeldt, J. Chromatogr., 245 (1982) 365.
11 K. Murata and Y. Yokoyama, J. Chromatogr., 374 (1986) 37.