Journal of Chromatography, 429 (1988) 293–313
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4153

REVIEW

METHODS FOR ANALYSIS OF URINARY GLYCOSAMINOGLYCANS

CHIE KODAMA*

Department of Biochemistry, Tohoku University School of Medicine, Sendai, Miyagi 980 (Japan)

TOHRU KODAMA

Department of Psychology, Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu City, Tokyo 183 (Japan)

and

ZENSAKU YOSIZAWA

Department of Biochemistry, Tohoku University School of Medicine, Sendai, Miyagi 980 (Japan)

(First received December 22nd, 1987; revised manuscript received February 9th, 1988)

CONTENTS

0378-4347/88/$03.50

LIST OF ABBREVIATIONS

CPC	Cetylpyridium chloride
CTAB	Cetyltrimethylammonium bromide
GAG	Glycosaminoglycan
HPLC	High-performance liquid chromatography
TLC	Thin-layer chromatography
⊿Di-	2-Acetamido-2-deoxy-3-O-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)
⊿Di-0S	⊿Di-D-galactose
⊿Di-HA	⊿Di-D-glucose
⊿Di-4S	⊿Di-4-O-sulpho-D-galactose
⊿Di-6S	⊿Di-6-sulpho-D-galactose
⊿Di-diS_B	2-Acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulpho-α-L-*threo*-hex-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose
⊿Di-diS_D	2-Acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulpho-α-L-*threo*-hex-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose
⊿Di-di$S_{E\text{ or }H}$	2-Acetamido-2-deoxy-3-O-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-4,6-di-O-sulpho-D-galactose
⊿Di-triS	2-Acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulpho-α-L-*threo*-hex-4-enepyranosyluronic acid)-4,6-di-O-sulpho-D-galactose
⊿Di-GlcNAc	2-Acetamido-2-deoxy-4-O-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-glucose
⊿Di-GlcNAc(6S)	2-Acetamido-2-deoxy-4-O-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-6-O-sulpho-D-glucose
⊿Di-GlcN(2S)	2-Deoxy-2-sulphamino-4-O-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-glucose
⊿Di-GlcN(2,2′-diS)	2-Deoxy-2-sulphamino-4-O-(4-deoxy-2-O-sulpho-α-L-*threo*-hex-4-enepyranosyluronic acid)-D-glucose
⊿Di-GlcN(2,6-diS)	2-Deoxy-2-sulphamino-4-O-(4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid)-6-O-sulpho-D-glucose
⊿Di-GlcN(2,6,2′-triS)	2-Deoxy-2-sulphamino-4-O-(4-deoxy-2-O-sulpho-α-L-*threo*-hex-4-enepyranosyluronic acid)-6-O-sulpho-D-glucose

1. INTRODUCTION

Glycosaminoglycans (GAGs) are long-chain polysaccharides composed of repeating disaccharides consisting of either D-glucosamine or D-galactosamine as hexosamine and either D-glucuronic acid or L-iduronic acid as hexuronic acid, with the exception of keratan sulphate, in which galactose is substituted for hexuronic acid. In vertebrates most GAGs exist in the extracellular matrix of the connective tissues, bound to proteins as proteoglycans. However, GAGs such as heparan sulphate are also found in non-connective tissues on the cell surface.

Urinary GAGs have long been investigated for detecting inheritable diseases widely known as mucopolysaccharidoses. Patients with various types of mucopolysaccharidoses are characterized by the excess amount of specific GAGs excreted into the urine [1–3]. Therefore, qualitative analysis and quantitation of urinary GAGs are effective for the differential diagnosis of mucopolysaccharidoses. Urinary GAGs have also been investigated in relation to bladder carcinoma, Werner syndrome, Weber Christian disease, Rothmund Thomson syndrome, epidermolysis bullosa and other diseases [4–9]. With further investigation, results useful for diagnosis of these diseases may be observed.

In this paper, methods used for or applicable to the identification or determination of urinary GAGs are reviewed, with emphasis on high-performance liquid chromatographic (HPLC) methods.

2. SAMPLE PREPARATION FOR GLYCOSAMINOGLYCAN ANALYSIS

2.1. Urinary glycosaminoglycans

The amounts of acidic glycosaminoglycans in the urine are small and many different types of impurities co-exist in urine. Therefore, the isolation of GAGs from urine sample is generally required prior to their assay. For the analysis, most commonly 24-h urine collected in a bag preserved with an antiseptic agent such as thymol and toluene is used. If the collected specimen is not treated at that time, it should be kept frozen at $-20\,^{\circ}C$ until the analysis.

As a pre-treatment, the impurities are first removed from collected urine by, e.g., (1) filtration through Whatman No. 2 filter paper, (2) centrifugation at 1500 *g* for 10 min at 25 °C, (3) dialysis against 0.03 *M* sodium chloride or (4) purification through a Sephadex G-25 column (50.0 cm × 2.0 cm I.D.) after concentration [10]. Filtration through filter paper or centrifugation is generally sufficient. Thereafter, to isolate the crude GAG sample from the urine, the pre-treated urinary samples are passed through an ion-exchange column or fractionated with ethanol or precipitated with salts such as cetylpyridinium chloride (CPC).

2.1.1. Ion-exchange chromatography

Wessler [11] reported the isolation of urinary GAGs using an ECTEOLA-cellulose (OH^-) column. One tenth of the 24-h urine dialysed against distilled water at 4 °C was applied to an ECTEOLA-cellulose column (2.0 cm × 0.8 cm I.D.) and washed with 100 ml of 0.01 *M* hydrochloric acid. Non-sulphated GAGs and gly-

coproteins were obtained by washing with 10 ml of 0.05 *M* hydrochloric acid, and then heparan sulphate, keratan sulphate, chondroitin sulphate and acidic glycoprotein were eluted with 3 *M* sodium chloride. Di Ferrante [12] also isolated GAGs using an ECTEOLA-cellulose (Cl^-) column. GAGs absorbed on this column were washed with 0.9% sodium chloride and collected by eluting with 2.0 or 4.0 *M* sodium chloride.

For further isolation of each different type of GAG, Schmidt [13] used a DEAE-Sephadex A-25 column and stepped gradient elution with 0.5 *M* sodium chloride and 1.25, 1.5 and 2.0 *M* sodium chloride in 0.01 *M* hydrochloric acid. Schiller et al. [14] used a Dowex 1-X2 (200–400 mesh) column with stepwise elution with 0.5, 1.25, 1.5, 2.0 and 3.0 *M* sodium chloride. GAGs were eluted in the order hyaluronic acid, heparan sulphate, chondroitin sulphate, heparin and keratan sulphate.

2.1.2. Precipitation with cetylpyridinium chloride or cetyltrimethylammonium bromide

Precipitation of GAGs with CPC or cetyltrimethylammonium bromide (CTAB) is the most commonly used method for the preparation of GAG samples [15–20]. The CPC precipitation method is more frequently used, as the recovery of urinary GAGs is better than that using CTAB. For example, specimens are adjusted to about pH 5.0 with acetic acid or with 0.2 *M* sodium citrate buffer, then 100 ml of the specimen is mixed with 2 ml of 5% CPC in 0.9% sodium chloride and the GAGs are precipitated as CPC–polysaccharide complexes after standing overnight at 4 °C. After centrifugation, the precipitate is washed two or three times with 100 ml of 0.1% CPC in 0.03 *M* sodium chloride. The CPC complexes are dissolved in 25 ml of 2.0 *M* sodium chloride and GAGs are precipitated by the addition of 100 ml of absolute ethanol to the solution. The mixture is kept at 4 °C for 4 h and centrifuged. The sediment is washed several times with 80% ethanol to remove sodium chloride and then washed successively with absolute ethanol and diethyl ether and dried in air. If the GAG sample contains much salt, the GAG is dissolved again in 15 ml of distilled water and precipitated at 4 °C with four volumes of absolute ethanol saturated with sodium acetate. Using these procedures, a recovery of 10–40% of the total GAGs is obtained.

2.1.3. Other methods of isolation

Whiteman [21,22] reported a method of precipitation with alcian blue and Di Ferrante [12] with aminoacridine. The rivanol precipitation method is suitable for precipitating keratan sulphate [23]. These isolation techniques are awkward and time-consuming. In 1984, Thorne and Resnick [24] modified the method of Hurst et al. [25] and ultrafiltered urine utilizing Diaflo membrane XM 300 (Amicon) followed by preparative gel permeation chromatography on Bio-Gel P-4. The macromolecular fraction was lyophilized, reconstituted and separated by electrophoresis.

For the further isolation of the different types of GAGs, ethanol precipitation methods have been used to separate chondroitin sulphate A and/or C and heparan sulphate [26–28].

2.2. Enzymatic digestion of glycosaminoglycans

Most HPLC methods for the analysis of GAGs require enzymatic digestion prior to the separation and determination. Enzymatic digestion is especially effective in the determination of chondroitin sulphate isomers. Many investigators have reported the quantitation of saccharides in chondroitinase ABC or AC digestion products. Moreover, the enzymatic digestion method provides much information on the structures and constituents of GAGs, which may be useful for the differential diagnosis of certain diseases. The enzymes that are most commonly employed in the assay procedures (Fig. 1) are described in Sections 2.2.1–2.2.4.

2.2.1. Chondroitinase ABC and AC [29,30]

Chondroitinase ABC (EC 4.2.2.4) from *Proteus vulgaris* functions at the N-acetylhexosaminidic linkages in GAGs such as hyaluronic acid, chondroitin, chondroitin sulphate A and C and dermatan sulphate, to produce unsaturated disaccharides, ΔDi-HA, ΔDi-0S, ΔDi-4S, ΔDi-6S and ΔDi-4S, respectively. This enzyme is, however, insensitive to heparin, heparan sulphate and keratan sulphate. Chondroitinase AC or ACII (EC 4.2.2.5), obtained from *Flavobacterium* or *Anthrobacter aurescens* [31], functions at the N-acetylhexaminidic linkages and produces unsaturated disaccharides. The characteristics of chondroitinase AC differ from those of chondroitinase ABC in that chondroitinase AC does not digest dermatan sulphate composed of N-acetylgalactosamine and L-iduronic acid. Chondroitinase ABC and AC used in combination are effective and are utilized for the qualitative analysis and quantitation of chondroitin sulphate isomers. The optimum pH values for the digestion with chondroitinase ABC and AC are 8.0 and 7.3, respectively, employing chondroitin sulphate A as a substrate. Digestion is usually carried out at 37°C. although these enzymes digest hyaluronic acid, their reactivities are relatively low when used to determine the amount of hyaluronic acid. Chondroitinase A, B and C, which react with chondroitin sulphate A, dermatan sulphate and chondroitin sulphate C, respectively, may be effective in the determination of these GAGs [32].

2.2.2. Chondro-4-sulphatase and chondro-6-sulphatase

Chondro-4-sulphatase (EC 3.1.6.9) and chondro-6-sulphatase (EC 3.1.6.10) desulphate the 4-O-sulphate and 6-O-sulphate, respectively, of sulphated unsaturated disaccharides [29,33]. Desulphation with these sulphatases of certain sulphated unsaturated disaccharides produced from chondroitin sulphate isomers by chondroitinase ABC and AC provide information of the site and degree of sulphation. However, these enzymes do not seem to react with saturated disaccharides [34,35].

2.2.3. Hyaluronidase

Bovine testicular hyaluronidase (EC 3.2.1.35) cleaves the N-acetylhexosaminidic linkages in hyaluronic acid, chondroitin and chondroitin sulphates. The digestion, carried out at pH 5.0 at 37°C, is useful for studying the structures of these GAG molecules. On the other hand, *Streptomyces* or leech hyaluronidase

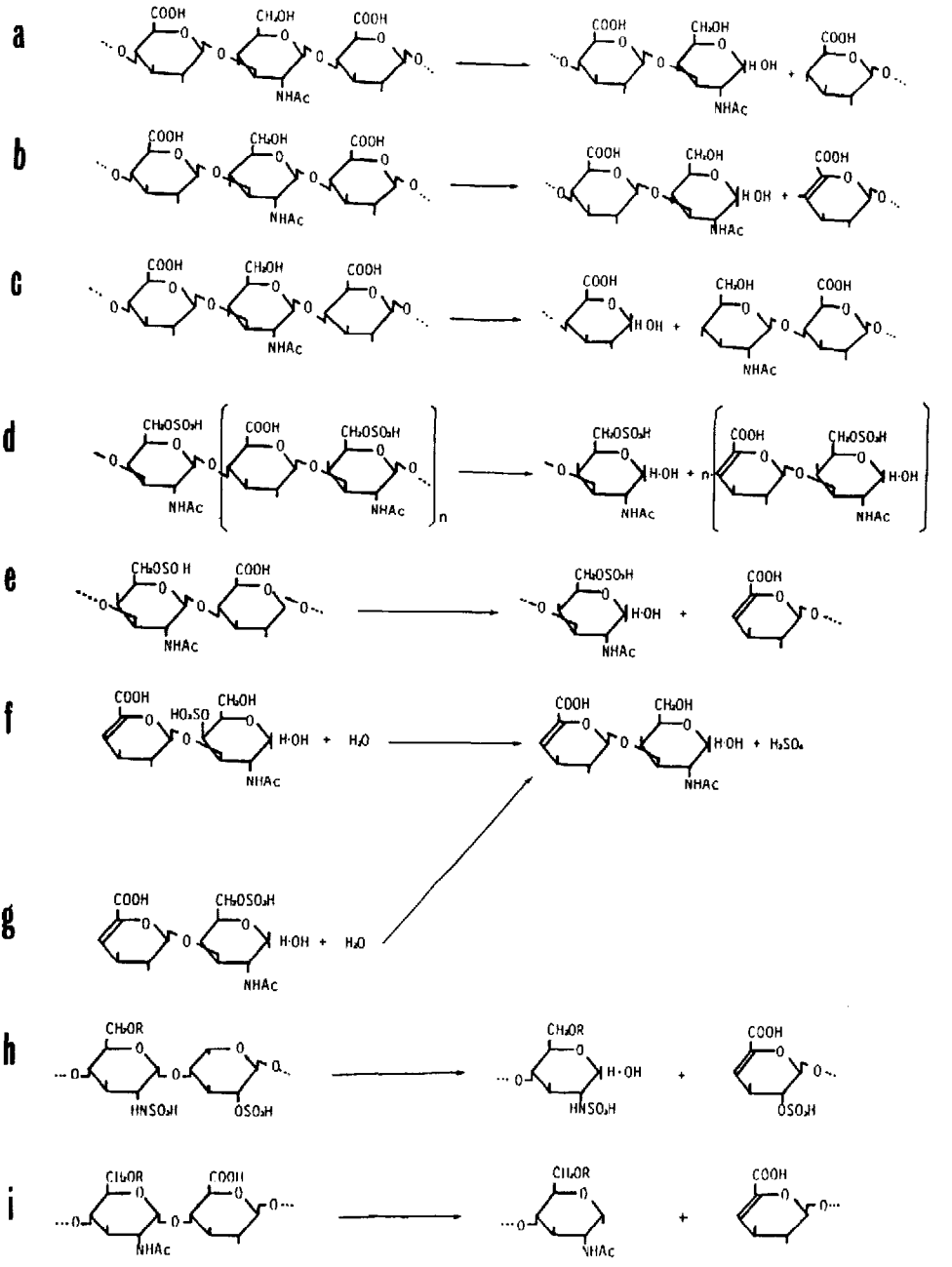

Fig. 1. Actions of enzymes: (a) bovine testicular hyaluronidase; (b) *Streptomyces* hyaluronidase; (c) leech hyaluronidase; (d) chondroitinase ABC; (e) chondroitinase AC; (f) chondro-4-sulphatase; (g) chondro-6-sulphatase; (h) heparinase; (i) heparitinase. Ac = $COCH_3$, R = H or SO_3H.

(EC 4.2.2.1, EC 3.2.1.36) is insensitive to chondroitin sulphates, but sensitive to hyaluronic acid [36]. Therefore, the enzyme is used to determine hyaluronic acid or to exclude hyaluronic acid from the sample. The digestion is performed at pH

5.0.

2.2.4. Heparinase and heparitinase

Ototani and co-workers [37,38] used a mixture of heparinase (EC 4.2.2.7) and heparitinase I and II (EC 4.2.2.8) to digest heparan sulphates in GAG samples at pH 7.0 at 37°C. Many different types of unsaturated disaccharides were obtained, and provided information of the structures of heparan sulphates.

3. METHODS APPLICABLE FOR ROUTINE GLYCOSAMINOGLYCAN ANALYSIS

3.1. Electrophoresis

Electrophoresis is the most common method for the qualitative analysis of the constituents of GAG samples. Among several types of supporting media such as cellulose acetate, paper and agarose gel, cellulose acetate membranes are the most popular and useful. We performed [39,40] (Fig. 2) electrophoresis with a cellulose acetate membrane by the method reported by Hata and Nagai [41], with 0.1 *M* pyridine–formate at pH 3.0, and by the method reported by Dietrich et al. [42], with 0.06 *M* sodium barbiturate buffer (pH 8.6), to identify GAGs in porcine thoracic aorta, etc. As these separations are based on the charge difference, chondroitin sulphate A and chondroitin sulphate C cannot be separated. The separation of GAGs by electrophoresis on a cellulose acetate membrane in 0.1 *M* hydrochloric acid is also based on the charge difference [43,44].

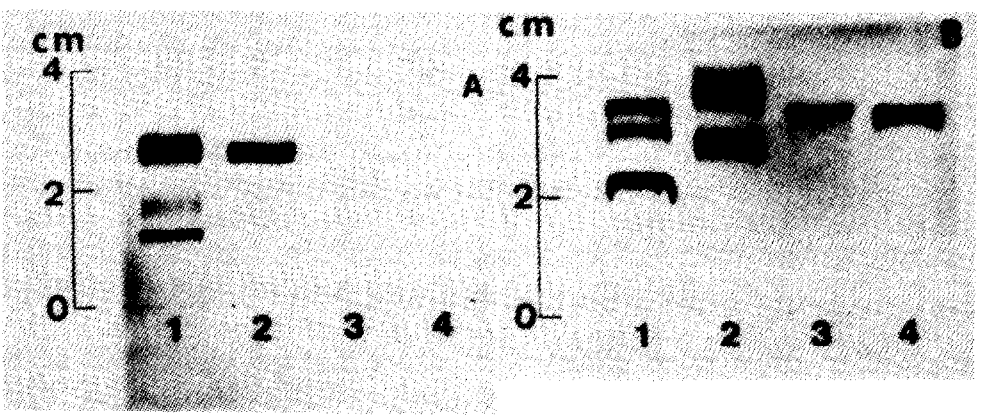

Fig. 2. Identification of glycosaminoglycans from the aortic proteoglycan by cellulose acetate membrane electrophoresis. The anode is at the top and the spots are rendered visible by alcian blue staining. (A) Electrophoresis was carried out in 0.06 *M* sodium barbiturate buffer (pH 8.6) at 1 mA/cm for 25 min. (1) Authentic samples of chondroitin 6-sulphate, heparan sulphate and hyaluronic acids (from top to bottom); (2) glycosaminoglycan fraction from porcine aortic proteoglycan; (3) glycosaminoglycan fraction after digestion with chondroitinase AC; (4) glycosaminoglycan fraction after digestion with chondroitinase ABC. (B) Electrophoresis was carried out in 0.1 *M* pyridine-formate (pH 3.0) at 1 mA/cm for 25 min. (1) Chondroitin 6-sulphate, dermatan sulphate and hyaluronic acid (from top to bottom); (2) heparin (top) and heparan sulphate (bottom); (3 and 4) glycosaminoglycan fraction from porcine aortic proteoglycan. (Reproduced from ref. 40 with permission.)

Wessler [45] reported an electrophoretic method employing cellulose acetate strips and 0.1 *M* barium acetate. In barium acetate, chondroitin sulphate C migrated slightly ahead of chondroitin sulphate A, and dermatan sulphate and hyaluronic acid migrated at the same rate behind chondroitin sulphates. Heparin and heparan sulphate migrated at the same rate with the smallest mobility. Wessler [45] stated that in this system, the mobility of GAGs depends more on the backbone structure than on the degree of sulphation. The system is utilized to identify GAGs in urine by changing the buffer concentration to 0.05 *M* [46]. Calcium acetate is also effective in separating GAGs by their backbone structure [47].

These electrophoretic methods are easy and suitable for the routine analysis of urinary GAGs, although their sensitivities and precision are relatively low for practical use. As it is difficult to identify all types of GAGs by using only one of the foregoing methods, analysis with two or more different solvent systems or two-dimensional electrophoresis is required in order to identify all types of GAGs. The discontinuous electrophoretic method of Cappelletti et al. [48,49] provided a sharp separation of heparin, heparan sulphate, hyaluronic acid, dermatan sulphate, chondroitin sulphate A, chondroitin sulphate C and keratan sulphate with 0.1 *M* barium acetate (pH 5.0) and a solvent containing ethanol. Hopwood and Harrison [50] applied this method for the analysis of urinary GAGs from control individuals and patients with mucopolysaccharidoses. In this method, heparan sulphate migrates as two discrete bands because of the heterogeneity existing in the standard sample used. The fraction which Cappelletti et al. [48,49] designated as highly sulphated heparan sulphate migrated as a sharp band, whereas the fraction designated as low sulphated heparan sulphate migrated as a broad band, suggesting a more heterogeneous nature of this fraction. Hopwood and Harrison [50] distinguished the two types of dermatan sulphate in urinary GAG samples from patients with Hurler's, Hunter's and Maroteaux–Lamy syndromes. They reported that the content of one type of dermatan sulphate designated as DS2 is generally higher in the urine of patients with Maroteaux–Lamy syndrome than in those of other samples. These heterogeneities in heparan sulphate and dermatan sulphate need to be further investigated, as they may provide new data and views on the composition of GAGs and new information related to urinary GAGs and diseases.

Most commonly, GAGs are rendered visible by staining with alcian blue or toluidine blue. With alcian blue staining, 0.1–1.0 μg of GAGs can be detected. Quantitation is performed with a densitometer or by analysing eluates of the stained bands. For more sensitive detection, Carlson [51] introduced a new reagent, ruthenium-103 red.

3.2. High-performance liquid chromatography

3.2.1. Hyaluronic acid

Hyaluronic acid is a long-chain biopolymer composed of N-acetylglucosamine and D-glucuronic acid repeating units. Several interesting reports have appeared on the analysis of hyaluronic acid by HPLC. These methods differ from the HPLC

methods used for the analysis of chondroitin sulphate or heparan sulphate in that they separate sodium hyaluronate or hyaluronate oligosaccharides according to their molecular mass.

Beaty et al. [52] applied HPLC–gel exclusion chromatography and reported that it is a rapid, reproducible method for determination of the molecular mass and concentration of sodium hyaluronate. Using a TSK G6000PW column (30 cm×7.5 mm I.D.) (Toyo Soda) with 150 m*M* sodium chloride and 3 m*M* sodium dihydrogenphosphate (pH 7.0) in the presence of 0.02% sodium azide, they determined simultaneously the concentration and molecular mass of sodium hyaluronate with a molecular mass ranging from $18 \cdot 10^3$ to about $2 \cdot 10^6$. Sodium hyaluronate, detected with a refractive index detector, showed a linear relationship between the peak area and concentration in the range of 1–6 mg/ml. As the existence of other molecules of equivalent molecular size interfered with the detection, digestion of the protein with a protease or nucleic acids with nuclease, etc., may be required in order to determine or detect hyaluronate in biological fluids.

Nebinger et al. [53] developed an HPLC system for separating and detecting hyaluronate oligosaccharides of different chain length. Even-numbered oligosaccharides were prepared by digesting hyaluronic acid with bovine testicular hyaluronidase or with leech hyaluronidase, and the even-numbered oligosaccharides were digested with β-glucuronidase of β-N-acetylglucosamidase to prepare odd-numbered oligosaccharides. They employed an amino-modified Ultrasil-NH_2 silica gel column (Beckman) and a LiChrosorb-NH_2 pre-column (Knauer) to separate such oligosaccharides. They separated the foregoing oligosaccharides up to the octasaccharides within 21 min, using 0.1 *M* potassium dihydrogenphosphate (pH 4.75) as the mobile phase. The separation of these odd-numbered oligosaccharides of the same chain length is based on the difference in the net charges of odd-numbered oligosaccharides produced by bovine testicular hyaluronidase and β-glucuronidase digestion and those of the same chain length produced by leech hyaluronidase and β-N-acetylglucosaminidase digestion. However, owing to their identical charge and molecular size, even-numbered oligosaccharides of the same chain length produced by different hyaluronidases cannot be separated by this method. The chromatogram showed an excellent separation of mono-, di-, tri-, tetra-, penta-, hexa-, hepta- and octasaccharides. The authors stated that the detection limit of the hyaluronate oligosaccharides was 0.01 nmol per sample when detecting at 206 nm, which was 10–100 times higher than that obtained with a refractive index detector.

3.2.2. Chondroitin sulphate and dermatan sulphate

Most HPLC methods for the analysis of chondroitin sulphate and dermatan sulphate involve the analysis of disaccharides produced by the digestion of these polymers with chondroitinase ABC and/or AC [54–58]. As stated in Section 2.2, the amount of ΔDi-6S in the chondroitinase ABC or AC digest represents the amount of chondroitin sulphate C. The amount of ΔDi-4S in the chondroitinase AC digest represents the amount of chondroitin sulphate A, and the difference in the amount of ΔDi-4S between the chondroitinase ABC and chondroitinase AC

digests represents the total amount of dermatan sulphate. In addition to these three major constituents, several minor components such as ΔDi-diS_B, ΔDi-diS_D and ΔDi-diS_E are also present in the digests.

The HPLC method presented by Lee and Tieckelmann [59] in 1979 employed a Partisil-10 PAC column (Whatman), a column packed with a bonded cyano-amino-type polar material, with acetonitrile–methanol–0.5 *M* ammonium formate buffer (pH 4.8) (60:15:25, v/v/v) as the mobile phase, or a weak anion-exchange column, LiChrosorb-NH_2 (Altex), with methanol–0.5 *M* ammonium formate buffer (pH 4.8) (35:65, v/v) as the separation buffer. Both methods separated ΔDi-0S, ΔDi-6S and ΔDi-4S within 30 min. The detection and quantitation were performed by measuring the UV absorbance at 254 nm. A linear relationship was obtained between 100 ng and 10 μg of disaccharides. As unsaturated disaccharides show maximum UV absorption at 232 nm, detection at 232 nm should give a higher sensitivity. The separation system with a Partisil-10 PAC column, which seemed to produce better results, was applied to the analysis of urinary GAGs from patients with mucopolysaccharidosis and for the analysis of brain GAGs by Ikeno et al. [56].

Ototani et al. [57] (Fig. 3) reported an ion-pair chromatographic method employing a μBondapak C_{18} column (Waters Assoc.) with 0.035 *M* tetrabutylammonium phosphate (pH 7.54) as the mobile phase to separate ΔDi-0S, ΔDi-4S and ΔDi-6S standards and applied it to the chondroitinase ABC digests of chondroitin sulphates from sturgeon notochord, sturgeon cranial cartilage and bovine tracheal cartilage. Using the ion-pairing reagent, mono- or disulphated unsaturated disaccharides can be retarded in the μBondapak C_{18} column. The mobile phase provides information on the solvent to separate unsaturated disaccharides with a reversed-phase C_{18} column.

Seldin et al. [58] employed a Partisil-10 PAC column for the analysis of the

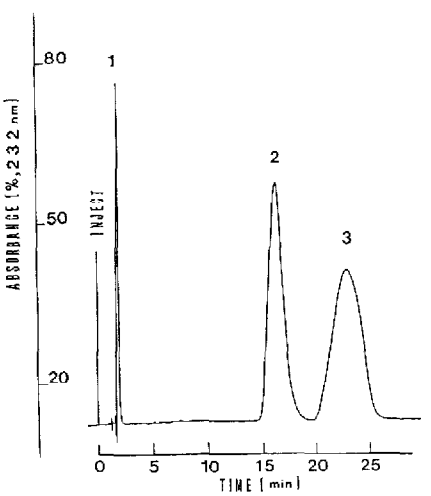

Fig. 3. Chromatogram of standard unsaturated disaccharides. Peaks: 1 = ΔDi-0S; 2 = ΔDi-6S; 3 = ΔDi-4S. Column, μBondapak C_{18}; eluent, 0.035 *M* tetrabutylammonium phosphate (pH 7.54). (Reproduced from ref. 57 with permission.)

non-, mono-, di- and trisulphated chondroitin disaccharides ΔDi-0S, ΔDi-4S, ΔDi-6S, ΔDi-diS$_E$, ΔDi-diS$_B$, ΔDi-diS$_H$, ΔDi-diS$_D$ and ΔDi-triS. The standard solvent employed for the separation had an apparent pH of 7.0 and was composed of 70% acetonitrile–methanol (3:1, v/v) and 30% 0.5 *M* ammonium acetate–acetic acid buffer (pH 5.3). All the above disaccharides except ΔDi-triS were resolved within 20 min. The retention times of the disaccharides were found to decrease with increasing ionic strength, pH and proportion of the aqueous component of the solvent. With another solvent containing 65% of acetonitrile–methanol (3:1, v/v) and 35% of 0.5 *M* ammonium acetate–acetic acid buffer (pH 5.3), ΔDi-triS appeared at a retention time of 13.1 min.

3.2.3. Heparan sulphate

Owing to the shortage of commercially available unsaturated disaccharide standards, few reports concerning the analysis of heparan sulphate by HPLC have appeared. Lee and Tieckelmann [60] reported a method for separating unsaturated disaccharides derived from heparin and heparan sulphate. A Partisil-10 PAC column and acetonitrile–methanol–0.5 *M* ammonium acetate (pH 6.5) (60:20:20, v/v/v) were used to separate ΔDi-GlcNAc, ΔDi-GlcNAc(6S) and ΔDi-GlcN(2S).

Using a reversed-phase column (Jasco SC-02) and 10 m*M* tetrabutylammonium phosphate (pH 7.0) containing 30% methanol, Ototani et al. [38] were able to separate ΔDi-GlcNAc, ΔDi-GlcN(2S), ΔDi-GlcNAc(6S) and ΔDi-GlcN(2,6- or 2,2′-diS) (Fig. 4). With a higher content of methanol in the mobile phase, ΔDi-GlcN(2,6,2-′triS) could also be separated. However, the retention times of ΔDi-GlcN(2,2'-diS) and ΔDi-GlcN(2,6-diS) were identical with this solvent system. Therefore, a different ion-pairing reagent, 35 m*M* triethylamine phosphate (pH 5.3), was employed for the separation of these unsaturated disaccharides. These systems are useful for the comparison of constituents of different types of heparan sulphate and heparin, or heparan sulphates from different sources, which may be diagnostically important.

3.3. Other methods

Methods such as paper chromatography or thin-layer chromatography (TLC) have often been used for GAG analysis [33,61,62]. The separation of unsaturated disaccharides produced by enzymatic digestion has been performed by paper chromatography followed by measurement of the UV absorbance of the eluates from the corresponding spots. Descending paper chromatography is performed in solvents such as isobutyric acid–0.5 *M* ammonia (5:3) or *n*-butanol–acetic acid–1.0 *M* ammonia (2:3:1) for 10–15 h to separate disaccharides produced by chondroitinase ABC or AC digestion. TLC methods for the separation of these saccharides followed by spectrophotometric quantitation has also been reported, employing the same solvent as in paper chromatography. Although TLC is much

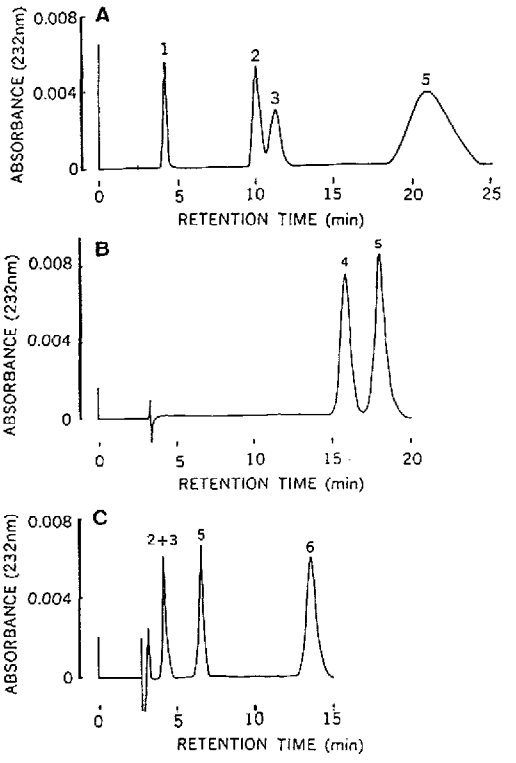

Fig. 4. Chromatograms of standard unsaturated disaccharides isolated from enzymatic digests of heparan sulphate. Peaks: 1=ΔDi-GlcNAc; 2=ΔDi-GlcN(2S); 3=ΔDi-GlcNAc(6S); 4=ΔDi-GlcN(2,2′-diS); 5=ΔDi-GlcN(2,6-diS); 6=ΔDi-GlcN(2,6,2′-triS). Column, Jasco SC-02. Eluent, buffers: (A) 10 m*M* tetrabutylammonium phosphate (pH 7.0) containing 30% methanol; (B) 35 m*M* triethylamine phosphate (pH 5.3); (C) 10 m*M* tetrabutylammonium phosphate (pH 7.0) containing 47% methanol. (Reproduced from ref. 38 with permission.)

faster than paper chromatography, the separation usually requires several hours. In this respect, HPLC is more rapid than TLC and more suitable for routine analysis.

For future detection and quantitation of each type of GAG, the use of antibodies to GAG may be a candidate method. Owing the low antigenicity of the saccharides, it is difficult to produce polyclonal antibodies against GAG. On the other hand, monoclonal and polyclonal antibodies against proteochondroitin sulphate or proteoheparan sulphate have been introduced, and in most instances epitopes were shown to exist in the core protein of proteoglycans. However, monoclonal antibodies such as S54 clone, obtained by Jenkins et al. [63], were shown to react with polysaccharide chains of proteochondroitin sulphate by the inhibition technique with chondroitin sulphate oligosaccharides. Moreover, Couchman et al. [64] introduced monoclonal antibodies which react with unsaturated disaccharides derived from chondroitin sulphate by digestion with chondroitinase ABC. Koike et al. [65] introduced monoclonal antibodies that recognize polysaccharide chains of proteoheparan sulphate. These monoclonal antibodies, which react specifically with each different type of GAG, may serve as an effective tool for the identification and determination of urinary GAGs in routine analysis in the near future.

4. APPLICATION OF HPLC TO URINARY AND TISSUE GLYCOSAMINOGLYCAN ANALYSIS

4.1. Analysis of urinary glycosaminoglycans

4.1.1. Hyaluronic acid

The amount of hyaluronic acid excreted in urine from normal individuals is usually very low. In 1967, Varadi et al. [66] reported the content of hyaluronic acid in urinary GAGs to be about 1%. However, changes in the urinary hyaluronic acid levels have been observed in several disorders, such as Werner syndrome and Hutchinson–Gilford syndrome [5,67,68]. Zebrower et al. [69] developed an HPLC system for separating and quantifying ⊿Di-HA, ⊿Di-0S, ⊿Di-4S and ⊿Di-6S in 25 min and applied the system to examine changes in the contents of urinary hyaluronic acid and chondroitin sulphate isolated from normal individuals and patients with Werner syndrome or Hutchinson–Gilford syndrome. The system was also capable of examining disorders in which under-sulphation of chondroitin sulphate may occur, such as Lowe syndrome. The separation was carried out with a solvent consisting of acetonitrile–methanol–aqueous buffer (0.5 *M* Tris–0.1 *M* boric acid pH 8.0) (52:12:36) with the addition of 200 μl of concentrated sulphuric acid per litre of the buffer, and a Partisil-5-PAC column (25 cm × 4.6 mm I.D.), protected by a Solvecon pre-column and a Copell PAC guard column, at a flow-rate of 1.2 ml/min. In this system, borate, which is known to form complexes with sugars at alkaline pH, is effective and provides a good separation of ⊿Di-0S and ⊿Di-HA. The authors reported also that this solvent provided such a low background that as little as 25 ng of disaccharides could be measured, which is applicable for the detection of hyaluronic acid or low-sulphated chondroitin sulphate, whose content is usually low in urinary GAGs. The HPLC system demonstrated the content of hyaluronic acid in urinary GAGs to be 11.5% in patients with Werner syndrome and 16.5% in patients with progeria.

4.1.2. Chondroitin sulphate and dermatan sulphate

Most studies of the determination of urinary GAGs by HPLC have involved the determination of chondroitin sulphates or dermatan sulphate in the urine of patients with mucopolysaccharidoses [36,69,70]. The genetic mucopolysaccharidoses, a group of disorders of GAG catabolism caused by genetic deficiency of specific hydrolases, are characterized by the storage of incompletely degraded GAGs [1]. As it is well known that an abnormally large amount of dermatan sulphate is excreted in the urine of patients with Hurler's, Scheie's, Hunter's and Maroteaux–Lamy syndromes, and that those with Morquio's disease excrete excess amounts of chondroitin sulphate C [2,3], the determination of chondroitin sulphate or dermatan sulphate in the urine of these patients by HPLC may be an effective means for the differential diagnosis of these mucopolysaccharidoses.

As stated already, Lee et al. [70] applied their method to assay chondroitin sulphate isomers in the urine of patients with mucopolysaccharidoses. They isolated GAGs from 2 ml of urine and digested it with chondroitinase ABC or chondroitinase AC. Enzymatic digest equivalent to 0.05 ml of urine was injected and

chromatographed by the method mentioned in Section 3.2.2, employing a Partisil-10 PAC column. The results obtained for five patients with Hurler's syndrome, three patients with Hunter's syndrome and three patients with Maroteaux–Lamy syndrome showed a large difference in the amount of ⊿Di-4S produced by chondroitinase AC and chondroitinase ABC, which indicated that a large amount of dermatan sulphate was excreted into the urine of these patients, which is consistent with the concept that there are disorders of dermatan sulphate catabolism in mucopolysaccharidoses. Lee et al. [70] also reported that peaks corresponding to ⊿Di-diS_B and ⊿Di-diS_E at retention times of about 21.5 and 25.0 min, respectively, could be detected by this method. In addition, a small amount of disulphated disaccharides was detected in the disaccharide samples from the above patients. The peak of ⊿Di-0S, whose retention time is reported to be 3.5 min by this method, was difficult to determine in normal urinary GAG samples, as ⊿Di-HA and other substances co-eluted at the same retention time. Nevertheless, the method is rapid and sufficiently sensitive for the determination of unsaturated disaccharides from urinary GAGs.

Fluharty et al. [71] employed an anion-exchange Partisil-10 SAX column, and separated ⊿Di-0S, ⊿Di-6S, ⊿Di-4S and ⊿Di-diS_E with 7.5 m*M* potassium phosphate buffer (pH 6.5) followed by gradient elution with an increasing buffer concentration to 500 m*M*. They applied this method to six samples of commercial chondroitin sulphate isomers and to urinary GAGs from patients with Hunter's, Hurler's, Scheie's and Sanfillipo's A syndromes, Morquio's disease and multiple sulphatase deficiency disorder. No discussion was provided regarding the elution of ⊿Di-HA. In the above systems and most separation methods for unsaturated disaccharides that have been reported so far a UV absorption detector was employed for detecting and determining the compounds.

The system that we have reported for separating unsaturated disaccharides derived from chondroitin sulphate isomers employs fluorescence labelling of the saccharides. The fluorescence labelling method of Hase and co-workers [72–74] was first applied to the detection of nineteen sulphated oligosaccharides in the deamination products of heparin [75]. The results suggested that it can be applied to the detection and quantitation of nanomole–picomole levels of unsaturated disaccharides derived from chondroitin sulphates. Unsaturated disaccharide standards were coupled with 2-aminopyridine in the presence of sodium cyanoborohydride. The resulting pyridylamino derivatives were separated on a μBondapak C_{18} column (300 mm×3.9 mm I.D.) with 8 m*M* KH_2PO_4–Na_2HPO_4 (pH 6.0)–methanol (30:1, v/v) as the mobile phase [76]. The method was applied to the analysis of urinary GAGs from patients with various types of genetically induced mucopolysaccharidoses and from normal individuals. The method is 10–100 times more sensitive than the UV absorption method but the fluorescence labelling procedure is time-consuming and complicated.

However, as has been stated, systems employing the UV absorption method are capable of determining the amount of chondroitin sulphate isomers excreted in urine but are unable to distinguish among those from Hunter's, Hurler's (Scheie's) and Maroteaux–Lamy syndrome samples, which have a common biological feature, viz., an abnormally large amount of dermatan sulphate excreted

in the urine. As our method included the fluorescence labelling of saccharides, detection of saturated disaccharides or monosaccharides derived from non-reducing terminal parts of GAGs by enzymatic digestion, which cannot be detected by the UV absorption method, was expected. Also, a possibility of distinguishing between these mucopolysaccharidoses by this method was suggested. By separating the pyridylamino derivatives of the chondroitinase ABC digests (0.7–0.9 μg of uronic acid equivalents) with a 20 cm$\times$0.6 cm I.D. column of reversed-phase (C_{18}) ERC-ODS-1171 and KH_2PO_4–Na_2HPO_4 buffer (6.5 mmol/l, pH 6.05)–methanol (100:1, v/v), Hunter's, Hurler's and Maroteaux–Lamy syndromes and Morquio's disease could be distinguished from the qualitative differences in the

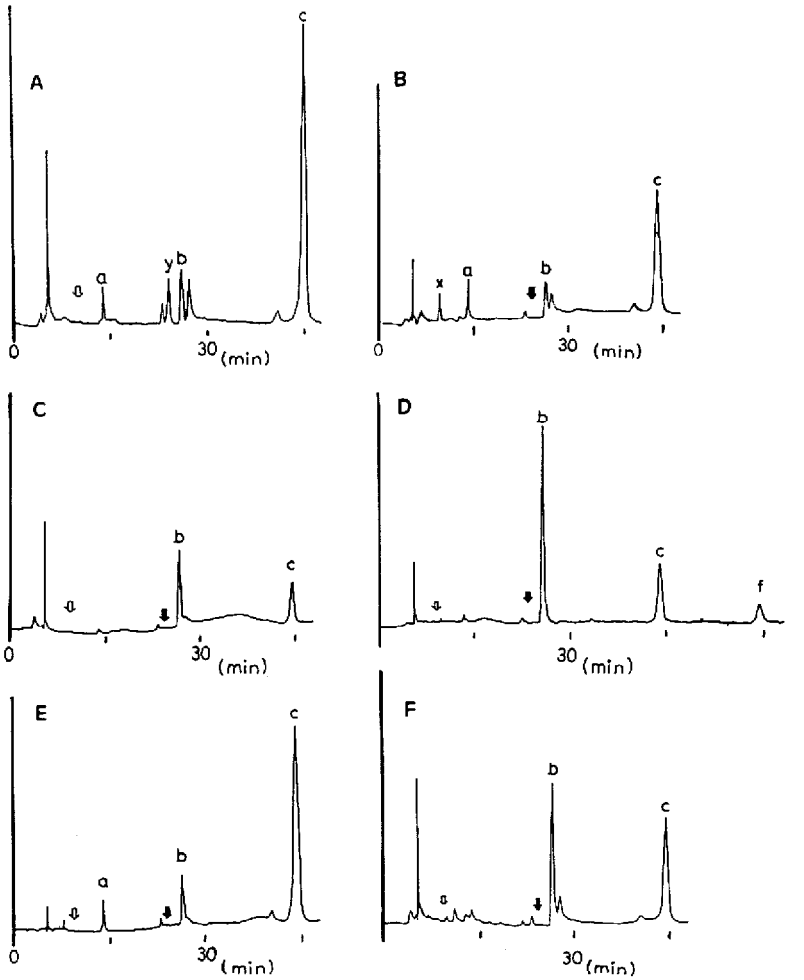

Fig. 5. Chromatograms of pyridylamino derivatives of chondroitinase ABC digests of urinary GAGs. (A) Scheie's syndrome; (B) Hunter's syndrome; (C) Sanfillipo's C syndrome; (D) Morquio's disease; (E) Maroteaux-Lamy syndrome; (F) normal individuals. Peaks a, b, c and f are pyridylamino derivatives of ΔDi-diS, ΔDi-6S, ΔDi-4S and ΔDi-GalNAc(6S), respectively. Peak x is specific for Hunter's syndrome and peak y is specific for Hurler's (Scheie's) syndrome. Ordinate: fluorescence responses. (Reproduced from ref. 76 with permission.)

TABLE 1

MOLAR RATIOS OF THE PYRIDYLAMINO DERIVATIVES OF ΔDi-diS AND ΔDi-4S IN THE CHONDROITINASE AC AND ABC DIGESTS OF URINARY GAGs FROM PATIENTS WITH MUCOPOLYSACCHARIDOSES AND CONTROLS

Syndrome	Sex	Age	ΔDi-diS/ΔDi-4S molar ratios* after digestion with		Peak x**	Peak y**
			Chondroitinase AC	Chondroitinase ABC		
Scheie	F	28	0.00/1.31	0.44/22.22	–	+
Hunter	M	4	0.00/1.59	0.69/11.76	+	–
Sanfilippo C	M	12	0.02/1.77	0.05/ 1.68	–	–
Morquio	M	8	0.01/0.81	0.02/ 0.96	–	–
Maroteaux-Lamy	F	4	0.00/1.68	0.33/11.98	–	–
Normal	F	11	0.03/2.01	0.04/ 2.02	–	–

*Taking ΔDi-6S as unity.

**Presence (+) or absence (–) on the urinary chromatogram after digestion with chondroitinase ABC.

chromatogram, i.e., by the presence or absence of specific peaks [35] (Fig. 5, Table 1). Experiments suggested that these specific peaks were derived from the non-reducing termini of GAGs. We were able to distinguish all the urinary GAG samples from patients with mucopolyccharidoses that we analysed. Samples from six patients with Hurler's syndrome, Hurler–Scheie compound and Scheie's syndrome, one with Maroteaux–Lamy syndrome, nine with Hunter's syndrome, one with Sanfillipo's C syndrome and one with Morquio's disease were distinguished by their chromatographic profiles. The specific peaks could also be observed with a μBondapak C_{18} column, which indicates that any reversed-phase C_{18} column may be employed with a small modification of the eluent. Application of a pre-column is also recommended.

For the separation and quantitation of urinary heparan sulphate, Ototani et al. [38] applied their method to the urine of patients with various types of mucopolysaccharidoses (unpublished data). Large amounts of heparan sulphate were excreted in the urine from patients with Hunter's and Sanfillipo's syndromes, but the distinction among the different types of Sanfillipo's, Hurler's and Hunter's syndromes was impossible. When this system was applied to tissue samples containing nucleic acid, digestion with nuclease was necessary because of the co-elution of nucleic acid with ΔDi-GlcNAc.

4.2. Analysis of tissue glycosaminoglycans

All the HPLC systems described can be applied to the analysis of GAGs in tissue after isolation of the GAGs. However, few reports have described the analysis of human tissue GAGs by HPLC. Ikeno et al. [56] used the method of Lee and co-workers [60,70] to analyse chondroitin sulphate isomers in brain samples from patients with mucopolysaccharidoses. Small peaks of other compounds were recognized in their chromatograms in addition to a poor resolution of ΔDi-4S and

ΔDi-0S. However, they succeeded in showing that a large amount of dermatan sulphate is accumulated in the brain.

Murata and Yokoyama [77,78] introduced an HPLC method for the separation of unsaturated disaccharides (0.5 μg in 10 μl of solution) from chondroitin sulphate isomers and hyaluronic acid with a Shodex RS column and acetonitrile–methanol–0.5 *M* ammonium formate (pH 4.5) (65:15:20, v/v/v) at 70°C, to analyse GAGs from human coronary arteries. We applied our method to investigate the age difference of the composition of GAGs of the human thoracic aorta (unpublished work). As GAGs exist as proteoglycans in tissues, digestion with proteolytic enzymes or alkaline extraction is performed prior to the analysis.

5. CONCLUSION

There are seven different types of GAGs and each may vary in its molecular mass, degree of sulphation and total charge. This heterogeneous nature is one of the disadvantages that makes it difficult to analyse urinary GAGs and to develop new methods for their analysis.

The most common and long-used method for the isolation of urinary GAGs is the CPC precipitation method. The separation is based on the negative charge of the molecule. Therefore, coprecipitation with other molecules bearing negative charge such as sulphated proteins should be taken in consideration. We employed digestion of the precipitate with pronase for further purification. Although the CPC precipitation method is most frequently employed and convenient, it is still much too laborious for routine use, and the development of a faster isolation method is desirable.

The preferred method for qualitative analysis or for identification may be cellulose acetate membrane electrophoresis. Solvents such as barium acetate, calcium acetate, barbital buffer and pyridine–formic acid are employed for the separation. However, by conventional one-dimensional electrophoresis, it is virtually impossible to separate all seven types of GAGs with a single solvent system. On the other hand, the discontinuous electrophoresis method introduced by Cappelletti et al. [49] provides a sharp separation of heparin, heparan sulphate, dermatan sulphate, hyaluronic acid, chondroitin sulphate A, chondroitin sulphate C and keratan sulphate in 30 min. The method is rapid and effective in identifying all seven different types of GAGs in urinary samples and urinary GAGs from patients with mucopolysaccharidoses were analysed. Further, heterogeneities of dermatan sulphate and heparan sulphate were also observed in GAG samples by this method. Investigation of the heterogeneities of these GAGs may provide new information related to the constitution of GAGs excreted in the urine.

Enzymatic digestion by chondroitinase AC and ABC has been utilized for the sensitive quantitation of chondroitin sulphate isomers by determining the UV absorbance of the resulting unsaturated disaccharides. Enzymes such as chondroitinase ABC, chondroitinase AC and heparitinase are also useful for investigating the constituents of GAG polymers. Profiling of enzymatic digests has been performed by paper chromatography. Although paper chromatography has the

TABLE 2

HPLC METHODS FOR GAG ANALYSIS

Reference	Column	Solvent	Compounds separated
Nebinger et al. [53]	Ultrasil-NH_2	0.1 *M* NH_2PO_4 (pH 4.75)	Hyaluronate Oligosaccharides
Beaty et al. [52]	TSK G6000PW	150 m*M* NaCl–3 m*M* Na_2PO_4 (pH 7.0)	Hyaluronic acid
Lee and Tieckelmann [59]	Partisil-10 PAC	Acetonitrile–methanol–0.5 *M* ammonium formate (60:15:25) (pH 4.8)	ΔDi-0S, ΔDi-6S, ΔDi-4S
	LiChrosorb-NH_2	Methanol–0.5 *M* ammonium formate (35:65) (pH 4.8)	ΔDi-0S, ΔDi-6S, ΔDi-4S
Ototani et al. [57]	μBondapak C_{18}	0.035 *M* Tetrabutylammonium phosphate (pH 7.54)	ΔDi-0S, ΔDi-4S, ΔDi-6S
Delaney et al. [55]	Partisil-10 SAX	40 m*M* KH_2PO_4–15% methanol	ΔDi-6S, Di-6S, ΔDi-4S, Di-4S
Fluharty et al. [71]	Partisil-10 SAX	7.5 m*M* potassium phosphate (pH 6.5)	ΔDi-0S, ΔDi-4S, ΔDi-6S, ΔDi-diS_E
Seldin et al. [58]	Partisil-10 PAC	70% Acetonitrile–methanol (3:1, v/v)–30% 0.5 *M* ammonium acetate–acetic acid buffer (pH 5.3)	ΔDi-0S, ΔDi-4S, ΔDi-6S, ΔDi-diS_E, ΔDi-diS_B, ΔDi-diS_H, ΔDi-triS
Kodama and co-workers [35,76]	μBondapak C_{18}	8 m*M* KH_2PO_4–Na_2HPO_4 (pH 6.0)–methanol (30:1)	Pyridylamino derivatives of ΔDi-diS_D, ΔDi-0S
	Erma ODS-1171	6.5 m*M* KH_2PO_4–Na_2HPO_4 (pH 6.05)–methanol (100:1)	ΔDi-4S, ΔDi-6S
Murata and Yokoyama [77,78]	Shodex RS (DC 613)	Acetonitrile–methanol–0.5 *M* ammonium formate (65:15:20) (pH 4.5)	ΔDi-diS, ΔDi-6S, ΔDi-0S, ΔDi-4S
Zebrower et al. [69]	Partisil-5 PAC	Acetonitrile–methanol–Tris (52:12:36) containing 0.1 *M* boric acid (pH 8.0)	ΔDi-HA, ΔDi-0S, ΔDi-6S, ΔDi-4S
Lee and Tieckelmann [60]	Partisil-10 PAC	Acetonitrile–methanol–0.5 *M* ammonium acetate (60:20:20)	ΔDi-GlcNAc, ΔDi-GlcN(2S), ΔDi-GlcNAc(6S)
Ototani et al. [38]	Jasco SC 02	Tetrabutylammonium phosphate–30% methanol	ΔDi-GlcNAc, ΔDi-GlcN(2S), ΔDi-GlcNAc(6S), ΔDi-GlcN(2,6/2,2′-diS), ΔDi-GlcN(2,6,2′-triS)

advantage of simple instrumentation, it is time-consuming, and quantitation of separated unsaturated disaccharides is laborious. On the other hand, the HPLC methods reported by Lee and Tieckelmann [59] gave good separations of unsaturated disaccharides within 30 min with a sensitivity at the nanomole level. More recently, several HPLC systems capable of analysing GAGs have been developed (Table 2).

The HPLC methods described give good separations of unsaturated disaccharides in a short time and provide quantitation with higher sensitivity and accuracy than paper chromatography or TLC, and are the most efficient methods for the quantitation of chondroitin sulphate isomers at present. Whereas most HPLC methods detect only unsaturated disaccharides, fluorescence labelling of saccharides made it possible to detect additionally monosaccharides and saturated disaccharides in the enzymatic digests. The method is effective for the investigation of the non-reducing end of chondroitin sulphate isomers. Thus, urinary GAGs from patients with Hurler's, Hunter's and Maroteaux–Lamy syndromes could be distinguished by the chromatographic profiles. Kosakai and Yosizawa [75] first applied this fluorescence labelling method for the separation and quantitation of oligosaccharides present in the deamination products of heparin. Fluorescence labelling of the deamination products or enzymatic digestion products may be efficient for the investigation of urinary heparan sulphate composition.

At present, cellulose acetate electrophoresis may be the most convenient method of identifying the different types of GAGs excreted in the urine, and HPLC may be the most effective for their sensitive determination.

6. SUMMARY

Methods for the analysis of urinary GAGs that can be used for or are applicable to routine assays are described. The most popular method for isolation of GAGs from a urine sample is CPC precipitation, in spite of the fact that it is time-consuming.

To identify the different types of GAGs excreted, separation by one-dimensional cellulose acetate electrophoresis followed by staining with alcian blue or toluidine blue may suffice for routine purposes. Solvents such as barium acetate, calcium acetate, barbital buffer and pyridine–formic acid are used for the separation. However, the separation of the seven types of GAGs by conventional one-dimensional electrophoresis is difficult, and a discontinuous electrophoretic method with barium acetate buffer and barium acetate buffer containing ethanol has proved effective for the separation.

HPLC separation methods are used for assaying the profiles of enzymatic digestion products of GAGs. Advanced HPLC methods for separating intact GAGs of different types are currently unavailable. Unsaturated disaccharides produced with heparitinase and/or heparinase from heparan sulphate and oligosaccharides produced by hyaluronidase digestion of hyaluronic acid can be separated by HPLC. For chondroitin sulphate isomers, unsaturated disaccharides produced by digestion of the samples with chondroitinase ABC or chondroitinase AC are separated by HPLC and determined by their UV absorbance or by fluorescence labelling.

Highly sensitive quantitation of chondroitin sulphate isomers is possible by these methods, which are also efficient for the investigation of the constituents of GAG polymers. Some of these methods have been applied to urine samples from patients with, e.g., mucopolysaccharidoses.

REFERENCES

1 A. Dorfman and R. Matalon, Proc. Natl. Acad. Sci. U.S.A., 73 (1976) 630.
2 D. Kaplan, Am. J. Med., 47 (1969) 721.
3 C.A. Pennock, J. Clin. Pathol., 29 (1976) 111.
4 P.T. Hennessey, R.E. Hurst, G.P. Hermstreet and G. Cutter, Cancer Res., 41 (1981) 3868.
5 M. Tokunaga, T. Fukushima, E. Wakamatsu, M. Endo and Z. Yosizawa, Clin. Chim. Acta, 62 (1975) 89.
6 Y. Murata, Y. Yukiyama and Y. Horiuchi, Clin. Chim. Acta, 49 (1973) 129.
7 M. Tokunaga, E. Wakamatsu and Z. Yosizawa, Tohoku J. Exp. Med., 126 (1978) 1.
8 M. Endo, R. Yamamoto, Z. Yosizawa, Y. Sasai and N. Saito, Clin. Chim. Acta, 57 (1974) 249.
9 K. Murata, T. Ishikawa and Y. Oshima, Clin. Chim. Acta, 28 (1970) 213.
10 A. Dorfman and R. Matalon, Am. J. Med., 47 (1969) 691.
11 E. Wessler, Clin. Chim. Acta, 16 (1967) 235.
12 N.M. Di Ferrante, Anal. Biochem., 21 (1967) 98.
13 M. Schmidt, Biochim. Biophys. Acta, 63 (1962) 346.
14 S. Schiller, G.A. Slover and A. Dorfman, J. Biol. Chem., 236 (1961) 983.
15 R. Humbel and N.A. Chamoles, Clin. Chim. Acta, 40 (1972) 290.
16 M. Endo, O. Namiki and Z. Yosizawa, Tohoku J. Exp. Med., 113 (1974) 65.
17 N. Taniguchi, Clin. Chim. Acta, 42 (1972) 221.
18 C.A. Pennock, F. White, D. Murphy, R.G. Charles and H. Kerr, Acta Paediatr. Scand., 62 (1973) 481.
19 N.G.G.M. Abeling, S.K. Wadman and A.H. van Gennip, Clin. Chim. Acta, 56 (1974) 297.
20 K.Y.T. Kao and J.G. Leslie, Biochem. Med., 9 (1974) 317.
21 P. Whiteman, Biochem. J., 131 (1973) 343.
22 P. Whiteman, Biochem. J., 131 (1973) 351.
23 A. Moretti and M.W. Whitehouse, Biochem. J., 87 (1963) 396.
24 I.D. Thorne and M.I. Resnick, J. Urol., 131 (1984) 995.
25 R.E. Hurst, J.M. Settine, W.M. and A.E. Lorincz, Clin. Chim. Acta, 70 (1976) 427.
26 K. Meyer, E. Davidson, A. Linker and P. Hoffman, Biochim. Biophys. Acta, 21 (1956) 506.
27 M. Endo, O. Namiki and Z. Yosizawa, Tohoku J. Exp. Med., 131 (1980) 23.
28 M. Endo, O. Namiki, H. Munakata and Z. Yosizawa, Tohoku J. Exp. Med., 131 (1980) 167.
29 T. Yamagata, H. Saito, O. Habuchi and S. Suzuki, J. Biol. Chem., 243 (1968) 1523.
30 H. Saito, T. Yamagata and S. Suzuki, J. Biol. Chem., 243 (1968) 1536.
31 K. Hiyama and S. Okada, J. Biol. Chem., 250 (1975) 1824.
32 Z. Yosizawa, N. Ototani and S. Satake, Anal. Biochem., 128 (1983) 250.
33 S. Suzuki, H. Saito, T. Yamagata, K. Anno, Y. Kanai and T. Furuhashi, J. Biol. Chem., 243 (1968) 1543.
34 A. Kimura, S. Hayashi and K. Tsurumi, Tohoku J. Exp. Med., 131 (1980) 241.
35 C. Kodama, N. Ototani, M. Isemura, J. Aikawa and Z. Yosizawa, Clin. Chem., 32 (1986) 30.
36 T. Ohya, and Y. Kaneko, Biochim. Biophys. Acta, 198 (1970) 607.
37 N. Ototani, M. Kikuchi and Z. Yosizawa, Carbohydr. Res., 88 (1981) 291.
38 N. Ototani, M. Kikuchi and Z. Yosizawa, J. Biochem. (Tokyo), 94 (1983) 233.
39 H. Munakata, M. Isemura, J. Aikawa, C. Kodama and Z. Yosizawa, Tohoku J. Exp. Med., 147 (1985) 77.
40 J. Aikawa, M. Isemura, H. Munakata, N. Ototani, C. Kodama, N. Hayashi, K. Kurosawa, K. Yoshinaga, K. Tada and Z. Yosizawa, Biochim. Biophys. Acta, 883 (1986) 83.
41 R. Hata and Y. Nagai, Anal. Biochem., 45 (1972) 462.

42 C.P. Dietrich, N.M. McDuffie and L.O. Sampaio, J. Chromatogr., 130 (1977) 299.
43 E. Wessler, Anal. Biochem., 41 (1971) 67.
44 J.H. Poulsen and M.K. Cramers, Clin. Chim. Acta, 130 (1983) 305.
45 E. Wessler, Anal. Biochem., 26 (1968) 439.
46 N. Taniguchi, J. Jpn. Chem., Suppl., 99 (1973) 181.
47 N. Seno, K. Anno, K. Kondo, S. Nagase and S. Saito, Anal. Biochem., 37 (1970) 197.
48 R. Cappelletti, M.D. Rosso and V.P. Chiarugi, Anal. Biochem., 93 (1979) 37.
49 R. Cappelletti, M.D. Rosso and V.P. Chiarugi, Anal. Biochem., 99 (1979) 311.
50 J.J. Hopwood and J.R. Harrison, Anal. Biochem., 119 (1982) 120.
51 S.S. Carlson, Anal. Biochem., 122 (1982) 364.
52 N.B. Beaty, W.P. Tew and R.J. Mello, Anal. Biochem., 147 (1985) 387.
53 P. Nebinger, M. Koel, A. Franz and E. Werries, J. Chromatogr., 265 (1983) 19.
54 G.J.-L. Lee, J.E. Evans and H. Tieckelmann, J. Chromatogr., 146 (1978) 439.
55 S.R. Delaney, H.E. Conrad and J.H. Glaser, Anal. Biochem., 108 (1980) 25.
56 T. Ikeno, R. Minami, S. Tsugawa and T. Nakao, Tohoku J. Exp. Med., 137 (1982) 253.
57 N. Ototani, N. Sato and Z. Yosizawa, J. Biochem. (Tokyo), 85 (1979) 1383.
58 D.C. Seldin, N. Seno, K.F. Austen and R.L. Stevens, Anal. Biochem., 141 (1983) 233.
59 G.J.-L. Lee and H. Tieckelmann, Anal. Biochem., 94 (1979) 231.
60 G.J.-L. Lee and H. Tieckelmann, J. Chromatogr., 195 (1980) 402.
61 N. Taniguchi, N. Moriya and I. Nanba, Clin. Chim. Acta, 50 (1974) 319.
62 L. Wasserman, A. Ber and D. Allalouf, J. Chromatogr., 136 (1977) 342.
63 R.B. Jenkins, T. Hall and A. Dorfman, J. Biol. Chem., 256 (1981) 8279.
64 J.R. Couchman, B. Caterson, J.E. Christner and J.R. Baker, Nature (London), 307 (1984) 650.
65 Y. Koike, K. Kimura, M. Kato and S. Suzuki, Seikagaku, 58 (1986) 785.
66 D.P. Varadi, J.A. Cifonelli and A. Dorfman, Biochim. Biophys. Acta, 141 (1967) 103.
67 M. Goto and K. Murata, Clin. Chim. Acta, 85 (1978) 101.
68 M. Tokunaga, E. Wakamatsu, K. Sato, S. Satake, K. Aoyama, K. Saito, M. Sugiyama and Z. Yosizawa, J. Am. Geriatr. Soc., 26 (1978) 296.
69 M.E. Zebrower, F.J. Kieras and W.T. Brown, Anal. Biochem., 157 (1986) 93.
70 G.J.-L. Lee, J.E. Evans, H. Tieckelmann, J.T. Dulaney and E.W. Naylor, Clin. Chim. Acta, 104 (1980) 352.
71 A.L. Fluharty, J.A. Glick, N.M. Matusewicz and H. Kihara, Biochem. Med., 27 (1982) 352.
72 S. Hase, T. Ikenaka and Y. Matsushima, Biochim. Biophys. Res. Commun., 85 (1978) 257-263.
73 S. Hase, T. Ikenaka and Y. Matsushima, J. Biochem. (Tokyo), 85 (1979) 989.
74 S. Hase, T. Ikenaka and Y. Matsushima, J. Biochem. (Tokyo), 90 (1981) 407.
75 M. Kosakai and Z. Yosizawa, J. Biochem. (Tokyo), 92 (1982) 295.
76 C. Kodama, N. Ototani, M. Isemura and Z. Yosizawa, J. Biochem. (Tokyo), 96 (1984) 1283.
77 K. Murata and Y. Yokoyama, Anal. Biochem., 146 (1985) 327.
78 K. Murata and Y. Yokoyama, J. Chromatogr., 374 (1986) 37.