Separation and Characterization of Dermatan Sulfate in Normal Human Urine

Guoning Qiu, Masahiko Tanikawa, Hiroaki Akiyama, Toshihiko Toida, Ichiro Koshiba and Toshio Imanari*

Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263, Japan.

Received October 15, 1992

Dermatan sulfate excreted in normal human urine was isolated and characterized by TLC and cellulose acetate strip electrophoresis after cetylpyridinium chloride precipitation and pronase digestion. In these separation methods, dermatan sulfate and chondroitin sulfate were extracted and then monitored by sensitive HPLC methods with post column fluorometric derivatization coupled with chondroitinase ABC, ACII and B digestion. From the results, we demonstrated that human urinary dermatan sulfate contains iduronic acid as its major uronic acid (80–90% of total uronic acid), and is composed mainly of repeated mono-sulfated disaccharide units [Di-4S (structure shown in Fig. 1), 89%] and small numbers of di-sulfated disaccharide units (Di-diSβ, 7% and Di-diSα, 1%).

Keywords: dermatan sulfate; human urine; glycosaminoglycan; chondroitin sulfate; cellulose acetate strip electrophoresis; TLC

Introduction

The dermatan sulfate (DS) polysaccharide chain is an extracellular matrix component in many tissues, such as skin,1) gastric mucosa,2) aorta,3) liver,4) etc. DS is considered to be produced through the epimerization of glucuronic acid (GlcUA) residues into iduronic acid (IdoUA) on the biosynthesis of its structural isomer, chondroitin sulfate (ChS).5) DS is usually present as proteoglycans (PGs) which are reported to have a high affinity to collagen,6) so it has been believed that DS is important in the formation of connective tissues. DS is also found in normal human urine,7) and it is well known that a large amount of DS is excreted in the urine of patients with cases of Hurler and Schles mucopolysaccharidosis.8)

In normal human urinary glycosaminoglycans (GAGs), the presence of ChS is 10–20 times greater than DS.9) Therefore, separation of DS from ChS was so difficult that the characteristics of human urinary DS were still poorly understood.

In the present paper we studied the separation of DS from ChS by the application of TLC followed by cellulose acetate strip electrophoresis. The separated urinary DS was then characterized by HPLC with post column fluorometric derivatization co-operating with the specific enzymatic digestion.

Materials and Methods

Reagents and Materials Unsaturated disaccharides [2-acetamido-2-deoxy-3-O-β-D-glucopyranosyl (uronic acid)-6-O-sulfate-α-galactose (Di-4S), 2-acetamido-2-deoxy-3-O-β-D-glucopyranosyl (uronic acid)-4-O-sulfate-α-galactose (Di-4S), 2-acetamido-2-deoxy-3-O-β-D-glucopyranosyl (uronic acid)-4,6-O-disulfate-α-galactose (Di-diSβ)] and chondroitinase ABC (Chase-ABC, EC 4.2.2.4), chondroitinase ACII (ChaseACII) arthrotin (EC 4.2.2.5) and chondroitinase B (ChaseB, EC 4.2.2.2) were purchased from Seikagaku Kogyo Co. (Tokyo, Japan), TSK gel NH2-60 (particle size, 5 μm) for HPLC column packing was obtained from Tosoh Ltd. (Tokyo, Japan). Silica gel 60 pre-coated plastic back TLC plates (without fluorescent indicator) were purchased from Merck (Darmstadt, Germany), and cellulose acetate strips (Separax) were purchased from Jokkoo Co., Ltd. (Tokyo, Japan). All other chemicals were of analytical grade. Reagent solutions were made up freshly in water purified by deionization and distillation.

Apparatus The determination of unsaturated disaccharides of ChS and DS was carried out on the HPLC systems described previously,11,12 and the conditions were as follows: A 10–20 μl portion of sample solution was loaded via a sample injector with a 50 μl loop. A TSK gel NH2-60 column (4.6 mm i.d. x 100 mm) was eluted with 10 mm ammonium formate and 16 mm sodium sulfate in 50% methanol at a flow-rate of 0.6 ml/min. To the eluate were added 0.3 m sodium hydroxide solution and aqueous 1% 2-eyanoacetamide solution at a flow-rate of 0.25 ml/min controlled by a double plunger pump. The mixture was then pass through a poly tetrafluoroethylene (PTFE) reaction coil (0.5 mm i.d. x 10 m) set in a dry reaction bath thermostated at 105 °C followed by a PTFE cooling coil (0.25 mm i.d. x 2 m) in a water bath at 25 °C. The effluent was monitored by the fluorometer (Ex. 346 nm, Em. 410 nm).

Preparation of Human Urinary GAGs The preparation procedure of urinary GAGs was similar to that described previously13–15 as follows: Aqueous 137 mm cetylpyridinium chloride (CPC) solution (10 ml) was added to fresh human urine (150 ml) and the mixture was allowed to stand at 4 °C for 4h. After centrifugation at 2300 x g for 15 min, the precipitate was collected and washed with aqueous 2.7 mm CPC solution, then dissolved in 2.5 m NaCl solution. Four volumes of ethanol were added to the solution and the mixture solution was allowed to stand for 1 h at 4 °C. After it was centrifuged again at 2300 x g for 15 min, the GAGs were collected and dried in vacuo.

The crude urinary GAGs were further treated with pronase digestion as follows: The sample was dissolved in 100 μl of water and the solution was heated in a boiling water bath for 5 min and then cooled in water. To the solution, 40 μl of 0.05 m Tris—HCl buffer (pH 8.0) containing 1% actinase was added, and the mixture was incubated at 45 °C for 3h. Then 340 μl of aqueous 12% sodium chloride solution and 60 μl of 0.1 m acetic acid were added to the sample solution, and the mixture was heated in a boiling water bath for 5 min. After cooling the solution in a water bath, it was centrifuged at 2300 x g for 15 min. To 360 μl of the supernatant, 40 μl of 0.1 m sodium hydroxide solution and 1.6 ml of cooled ethanol saturated with sodium acetate were added. The mixture was left to stand at 4°C for 1 h and centrifuged at 2300 x g for 15 min. The precipitate was washed twice with 0.6 ml of ethanol saturated with sodium chloride and was then dried in vacuo.

TLC of GAGs TLC separation of GAGs was performed on silica gel plates (25 mm width x 100 mm length) with the following solvent system; (η-propanol/25% NH4OH/acetone, 31:4.2:68/22.0, by vol). TLC plates were pre-activated at 105 °C for 15 min in a dry oven before being used. The sample was dissolved in 20 μl of water and carefully applied on the silica gel plate, then dried in vacuo. The chromatography was performed at 25 °C in the vapor saturated chamber, and stopped after the solvent advanced 7 cm from the origin. After the silica gel plate was dried in vacuo again, it was cut into 1 mm wide pieces from theorigin. GAGs were extracted from each silica gel plate with 0.5 ml of water for 1 h and lyophilized for analysis. The extraction efficiency of standard ChS, DS and urinary GAGs from a silica gel plate was about 90% (n=4).

Electrophoresis of GAGs Crude GAGs (approximately 2 μg) were dissolved in 5 μl of water, and this solution was submitted to electrophoresis on a cellulose acetate strip (20 mm width x 120 mm length) using 0.1 m
calcium acetate at a constant current of 1.0 mA/cm for 4.5 h. The strip was then cut into 1 mm wide pieces from the origin. GAGs in each strip were extracted by 100 µl of water for 1 h at room temperature. To the extraction solution, 10 µl each of 2% Na2CO3 was added to remove the calcium ion contamination caused by the electrolyte. After centrifugation, the supernatant was lyophilized for analysis of GAGs. The extraction efficiency of standard and urinary GAGs from the cellulose acetate strip was about 80%. Enzymatic Digestion of GAGs GAGs were digested by ChaseABC, ChaseACII and ChaseB under the conditions reported previously. Typically, 20 µl of sample solution was mixed with 20 µl of buffer solution containing 0.05 unit of enzymes, then the mixture was allowed to incubate for 3 h at 37°C. Next, the amount of the unsaturated disaccharides in a 10–20 µl portion of each sample was monitored by post column HPLC system.

Results and Discussion

DS, one of the structural isomers of ChS, is constituted of several types of disaccharide units which are distinguishable according to the IdoUA-content and the position of the sulfate group (Fig. 1).

In order to elucidate the biological origin and metabolic pathways of urinary DS, it is necessary to compare the structural characteristics of DS isolated from tissues and from urine. However, it has been very difficult to isolate DS from human urine so far because DS exists as a minor component in human urinary GAGs. In our preliminary experiments on the purification of urinary DS, we used ion-exchange chromatography and gel filtration chromatography on DEAE-Cellulose, DEAE-Sepharose and Sephadex G-50 and G-100 as column packings for GAGs fractionations. Although a DS-rich fraction could be obtained from human urine, a variation in the contents of IdoUA and/or sulfate ester was observed due to the incomplete separation (data not shown).

It is also well known that a part of GAGs in normal human urine binds covalently to various sizes of peptide chains. This may be one of the major reasons why it is difficult to isolate urinary DS from other GAGs. Therefore, we tried initially to avoid the influences of peptide chains on the separation methods by pronase digestion, then we examined the separation of DS from ChS by methods such as TLC and electrophoresis. TLC and electrophoresis have been reported to be very effective methods to separate GAGs isomers, but the amount of GAGs separated by these methods is too low to be analyzed by the traditional methods. Thus, we established methods for the micro-determination of GAGs in the form of their corresponding unsaturated disaccharides by HPLC systems coupled with specific enzyme digestion. Therefore, we tried to use these sensitive methods to monitor the chromatographic and electrophoretic behaviors of urinary ChS and DS.

Figure 2 shows the distribution of molecular weight of human urinary GAGs by gel filtration chromatography. DS overlapped with other GAGs. Apparent molecular weights of urinary DS together with other GAGs were distributed at around 10 kDa, calculated as the chromatographic behaviors of standard molecular markers. These results
suggest that the limitation of the molecular size of excreted GAGs depends on the renal filtration system.

The profiles of human urinary DS and CHS on TLC analysis are shown in Fig. 3. Under the established TLC condition (see experimental section), DS ($R_f = 0.03$) was separated from almost all urinary CHS ($R_f = 0.30$), yet small amounts of CHS were still detected in the DS fraction.

The fractions indicated by arrows in Fig. 3 were pooled and lyophilized for further electrophoretic investigation. The electrophoretogram of the DS-rich fraction obtained from TLC fractionation is shown in Fig. 4. The urinary DS migrates as a compact spot without overlapping with CHS.

Therefore, human urinary DS has been almost completely separated from CHS by a combination of TLC and electrophoretic separation methods.

The fractions indicated by arrows in Fig. 4 were collected as human urinary DS to study its structural characteristics. The disaccharide units of the human urinary DS were examined by HPLC methods after ChaseABC, ChaseACII and ChaseB digestions (Table I). The results clarified that repeated disaccharide units of DS chains excreted in normal human urine are mainly DI-4S (structure shown in Fig. 1, 89%), and also small amounts of DI-diS$_4$ (7%) and DI-diS$_5$ (1%). The responsiveness of DS to ChaseACII and ChaseB indicates that IdoUA content (IdoUA/total uronic acid) in urinary DS is approximately 85%. Furthermore, no difference between individual urinary DSs was recognized in IdoUA content (80–90%).

As described above, we demonstrated that preparative TLC and electrophoretic separation techniques served as excellent tools for the purification of human urinary DS. Through these studies, we found that the characteristics of urinary DS differ from those of tissue DS which is reported to have higher molecular weight and lower IdoUA content. Therefore, more knowledge on the metabolism of DS is necessary to understand its physiological significance.

References