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ANALYSIS OF MATRIX GLYCOSAMINOGLYCANS (GAGS) IN URINARY STONES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Shizuka Iida[1]*, Isoko Takazono[2], Koji Suzuki[2], Akihiko Shimada[1], Junro Yahara[1], Shinici Yoshii[1], Kei Matsuoka[1] and Shinshi Noda[1]

[1]Department of Urology, [2]Department of Chemistry, Kurume University School of Medicine, Kurume 830, Japan
[3]Department of Urology, Kanazawa Medical University, Ishikawa 920-02, Japan

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Abstract

Glycosaminoglycans (GAGs) have been identified as one of the macromolecules present in the stone matrix. Although electrophoresis has been used to analyze the composition of GAGs, this method is laborious and insensitive. The aim of this study was to examine GAG profiles in the stone matrix from 4 types of stones using high-performance liquid chromatography (HPLC). This method has been shown to be rapid, accurate, and highly sensitive. We could isolate and quantitate the amounts of matrix GAGs in various stones. In the calcium and uric acid stones, the predominant GAG component was heparan sulfate (HS) followed by chondroitin 4 sulfate (Ch-4S) or chondroitin 6 sulfate (Ch-6S), respectively. Chondroitin (Chon) and hyaluronic acid (HA) were absent or present in small amounts. In the MAP stones, the predominant GAG component was ChS and Chon, followed by HA. HS was not detected in magnesium ammonium phosphate (MAP) stones. In conclusion, the process of selective inclusion of GAGs into calcium and uric acid stone matrix is more greatly related to binding affinity than to urinary GAG concentration. The GAGs present in MAP stone matrix differed from those in other types of stones which suggests that the inclusion of GAGs into MAP stone matrix is dependent up on the concentration of urinary GAGs.

Key words: Glycosaminoglycans (GAGs), stone matrix, high performance liquid chromatography (HPLC), heparan sulfate (HS), chondroitin 4 sulfate (Ch-4S), chondroitin 6 sulfate (Ch-6S), chondroitin (Chon), hyaluronic acid (HA).

*Address for correspondence:
Shizuka Iida
Department of Urology,
Kurume University School of Medicine,
Kurume 830, Japan

Telephone number: (942) 31-7572
FAX number: (942) 34-2605
E-mail: iidas@kurume.ktarn.or.jp

Introduction

Urinary stones contain organic matrix which accounts for 2-3% of their weight [5]. Many investigators have described that the organic matrix consists of various urinary macromolecules such as glycosaminoglycans (GAGs) [2, 3, 5, 9, 13, 22, 23, 25, 26, 27], glycoproteins [5, 7, 14, 21, 24] and other urinary proteins [8]. Baba *et al*. [3] and Nishio *et al*. [22] have reported that the GAGs in calcium-containing stone matrix consisted mainly of heparan sulfate (HS) and hyaluronic acid, while the GAG in magnesium ammonium phosphate (MAP) calculi was characterized by the presence of chondroitin sulfate (ChS) and low sulfated chondroitin. Roberts and Resnick [23] showed similar findings. Recently, Yamaguchi *et al*. [27] and Suzuki *et al*. [26] have reported that HS is selectively included in calcium oxalate crystals. In previous reports, matrix GAGs in urinary stones were analyzed by electrophoresis [3, 22, 23, 26, 27], but this technique is laborious and insensitive. High performance liquid chromatography (HPLC) has been used for analyzing various glycosaminoglycans (GAGs) [11, 15, 16, 18, 19, 20]. This method has been shown to be rapid, accurate, and highly sensitive [16]. In this study, we used HPLC to analyze GAG profiles in the stone matrix. We also examined the amount of these compounds in various stone composition groups.

Materials and Methods

Stone matrix extraction procedure

Urinary stones 800-1500 mg dry weight were selected and separated into groups by similar composition.

Seven calcium oxalate (CaOx) stone pools, seven calcium oxalate+calcium phosphate (CaOx+CaP) stone pools, four uric acid (UA) stone pools and four MAP stone pools were used as samples. The urinary stones in each 5 grams pool were pulverized and mixed well, then a portion was analyzed by infrared spectrometry. These pooled stone powders were put into a visking tube (Spectra/Por Membrane, molecular weight 3,500 cut off, SPECTRUM Medical Industries, Los Angeles, CA) and then decalcified with 0.25 M ethylene diammine tetraacetic acid (EDTA) (pH 8.0, 4°C) for 14 days with the exception of uric acid stones.

Uric acid stones were decalcified with 0.5 normal sodium hydroxide solution (pH 13.0) for 3 days. These decalcified materials were dialyzed further against distilled water for 3 days and lyophilized.

Proteolysis

Matrix extract was put into a visking tube and digested with 1/50 sample weight of actinase E in 0.1 M Tris-HCl buffer (pH 8.0) for 24 hours with constant shaking at 50°C. The suspension was cooled and 50% trichloroacetic acid (TCA) was added to obtain a final concentration of 10% TCA. After standing for 1 hour at 4°C, the suspension was centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was dialyzed against distilled water for overnight.

Purification of matrix GAGs

The dialyzed material was concentrated with a rotary evaporator and 1/10 volume of a 0.4 M NaCl-1% cetyl pyridinium chloride (CPC) solution was added. The suspension was incubated for 1 hour at 37°C, celite (Waco Pure Chemicals, Osaka, Japan) was added to obtain a final concentration of 10% celite and the solution was centrifuged at 3,000 rpm for 30 minutes. The precipitate was air-dried at room temperature, dissolved in 10 ml of 2.1 M sodium chloride, and stirred for 30 minutes to remove celite. After centrifugation at 3,000 rpm for 15 minutes, the supernatant was collected. These steps to remove celite were repeated for 3-4 times. Two volumes of ethanol and 1/10 volume of methanol were added. After standing overnight at 4°C, the mixture was centrifuged at 3,000 rpm for 15 minutes. The precipitate was dried and dissolved in distilled water to elute GAGs. The GAG solution was desalted and lyophilized.

Measurement of uronic acid

Uronic acid of each GAG sample was measured by the method of Bitter and Muir [4].

HPLC sample preparation

GAG samples were separated into 3 test tubes, each containing 10 mg of material. Subsequently, enzymatic digestion was performed to convert each GAG into unsaturated disaccharides. All enzymes were obtained from Seikagaku Kogyo (Tokyo, Japan). The first tube for ChS and Chon were digested in 30 µl of 0.1 M Tris-HCl buffer (containing 10^{-3}M calcium acetate, pH 7.3) with 0.1 unit of chondroitinase AC from *Flavobacterium heparinum* at 37°C for 2.5 hours. This sample, which was further treated with additional 0.1 units of chondroitinase AC for another 2.5 hours HA was digested with 0.1 unit of hyaluronidase SD from *Streptomyces hyalurolyticus* in 50 µl of 10 mM sodium phos-phate buffer (pH 6.5) at 37°C for 3 hours. HS was digested with 0.01 unit of heparitinase from *Flavobacterium heparinum* in 50 µl of 10^{-5} M calcium acetate buffer (pH 7.0) at 40°C for 1 hr.

After each digestion, four volumes of ethanol (99.5%) were added to each reaction and the mixture was allowed to stand overnight at 4°C. Subsequently, these mixtures were centrifuged at 3,000 rpm for 20 minutes to

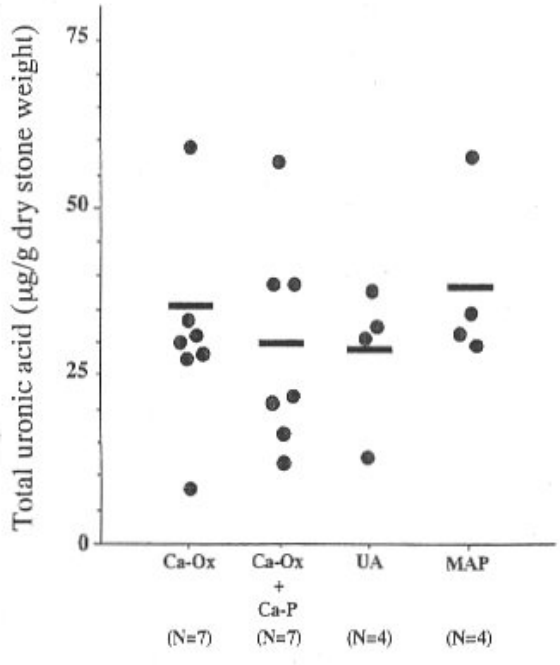
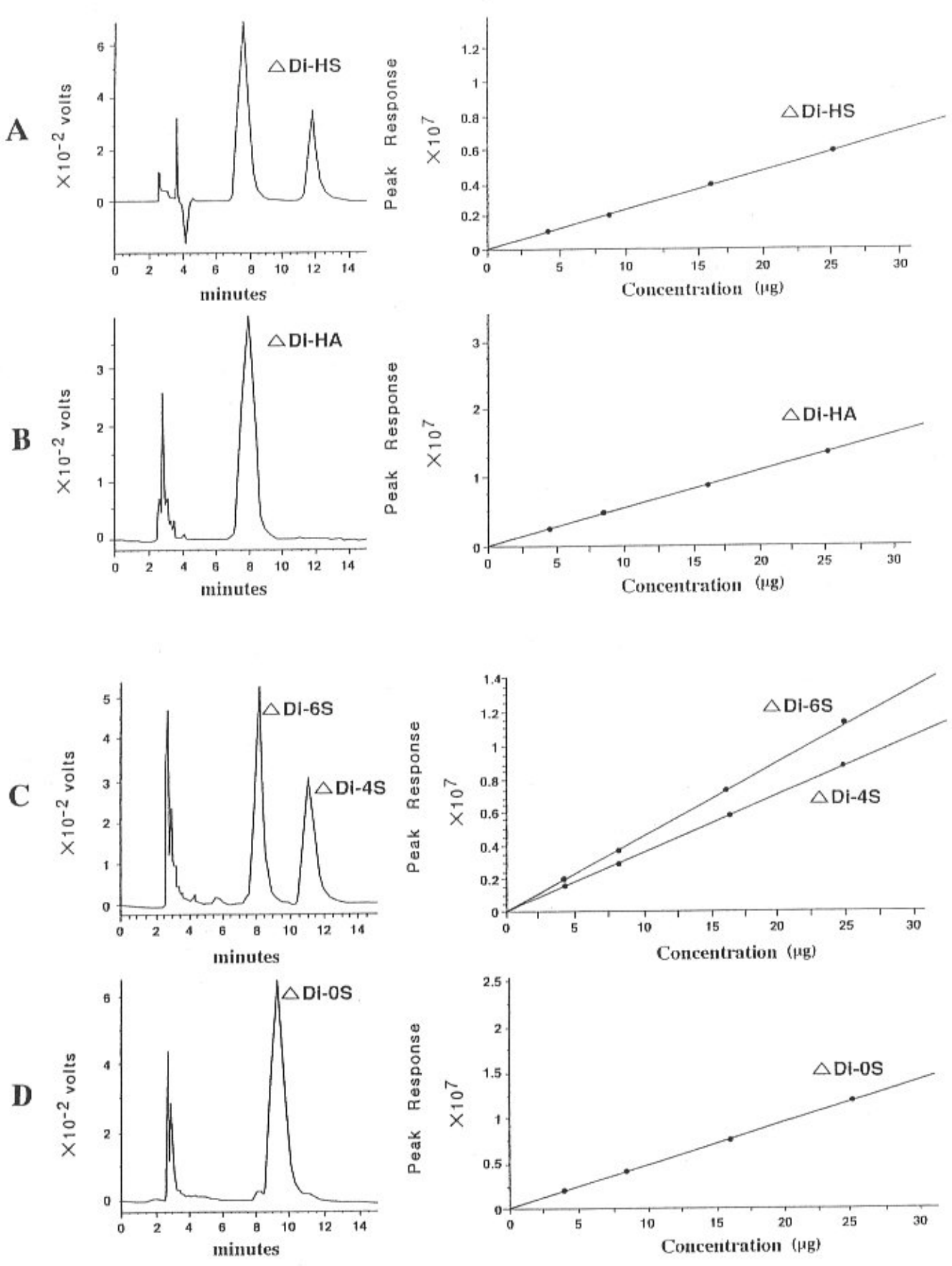


Figure 1. Total uronic acid levels in CaOx, CaOx +CaP, UA and MAP stones as measured by the methods of Bitter and Muir with glucuronic acid. Each concentration is expressed in µg/g dry weight of stone. The solid bars represent the mean value. CaOx: calcium oxalate stone, CaOx+CaP: calcium oxalate and calcium phosphate stone, UA: uric acid stone, MAP: magnesium ammonium phosphate stone.

Figure 2 (*on facing page*). The separation of each unsaturated disaccharide, which represent the degradation products of GAGs, and their corresponding calibration curves. The degradation products of heparitinase treatment are shown in panel A and the degradation products of HA are shown in panel B. Panel A shows double peaks for ΔDi-HS. The calibration curve is on the right. Panel B shows a single peak for ΔDi-HA along with the calibration curve. Panel C and D show separations of the degradation products of Ch-4S, 6S and Chon along with their calibration curves. ΔDi-HS: degradation products of HS, ΔDi-HA: degradation products of HA, ΔDi-4S, 6S, 0S: degradation products of Ch-4S, Ch-6S, chondroitin.


A
△Di-HS
×10⁻² volts
minutes
Peak Response
×10⁷
△Di-HS
Concentration (μg)
B
△Di-HA
×10⁻² volts
minutes
Peak Response
×10⁷
△Di-HA
Concentration (μg)
C
△Di-6S
△Di-4S
×10⁻² volts
minutes
Peak Response
×10⁷
△Di-6S
△Di-4S
Concentration (μg)
D
△Di-0S
×10⁻² volts
minutes
Peak Response
×10⁷
△Di-0S
Concentration (μg)

remove any residual proteins. The supernatant was then dried under N_2 gas [16], and the residue was dissolved in 100 µl of mobile phase solution (aceto-nitrile-methanol-0.5 M ammonium formate, 65:15:20, v/ v/ v, pH 4.5) for HPLC assay [18, 19, 20].

Preparation of GAG standard solutions for HPLC

All standards for HPLC analysis were purchased from Seikagaku Kogyo (Tokyo, Japan). Commercial HPLC standards which named "unsaturated GAG disaccharide kit (Seikagaku Co. #400572)" were used as the standards for Ch-4S, 6S, chondroitin and HA. The enzymatic degradation product of CDSNS-HS (completely desulfated N-sulfated heparan sulfate) from bovine kidney were used as HS standard. CDSNS-HS was treated by the same procedure used for the experimental samples described above. Standard solutions for each GAG were prepared in concentrations of 4, 8, 16 and 25 µg/ 100 µl.

HPLC apparatus and procedure

The HPLC system consisted of a Waters chromatography Model 626 LC system (Waters Ltd., Milford, MA) fitted with a Shodex RS Type DC-613 column (Showa Denko Co., Tokyo, Japan) [18, 19, 20]. This system successfully separated each ΔDi-4S, ΔDi-6S, ΔDi-0S, ΔDi-HA, and ΔDi-HS. All chemicals were HPLC grade and were purchased from Waco Fine Chemicals (Tokyo, Japan).

The sample size used for these analyses was 100 µl. Chromatography was carried out at 70°C at a flow rate of 1.0 ml/minutes. Each component was detected at 232 nm [18, 19, 20] with a UV detector (Waters 486 Tunable absorbance detector) and the elution response was monitored by computer system (NEC 98 note NS/R) and calculated by 805 data station software (Waters Ltd.). The values of each GAG composition were described as the percentage of unsaturated disaccharide in each sample.

Results

Concentration of uronic acid

The concentration of uronic acid in various stones is shown in Figure 1. While no significant difference existed among in each groups, the concentration of total uronic acid in CaOx+CaP stones and UA stones were slightly lower than in other.

Separation of unsaturated disaccharides by HPLC

HPLC separations of each unsaturated disaccharide are shown in Figure 2. Panel A shows double peaks for ΔDi-HS which represent the degradation products of heparitinase digestion. Panel B shows a single peak for ΔDi-HA which is the degradation product of hyaluronidase SD digestion. Panels C and D show chromatographs of the degradation products of Ch-4S, Ch-6S, and chondroitin after chondroitinase AC treatment. The retention times of unsaturated disaccharides were as follows: ΔDi-HA, 7.91 ± 0.05 minutes, ΔDi-HS, 7.80 ± 0.07 minutes, 12.30 ± 0.07 minutes, ΔDi-6S, 8.04 ± 0.08 minutes, ΔDi-0S, 9.16 ± 0.06 minutes, and ΔDi-4S, 10.98 ± 0.09 minutes.

Figure 3 (*on facing page, left*). Panel represent the chromatograph of degradation products in CaOx stone matrix. Five types of the unsaturated disaccharides were identified. HSase: digestion with heparitinase, HAase SD: digestion with hyaluronidase SD, Chase AC: digestion with chondroitinase AC, CaOx: calcium oxalate stone.

Figure 4 (*on facing page, right*). Panel represent the chromatograph of degradation products in CaOx+CaP stone matrix. CaOx+CaP: calcium oxalate and calcium phosphate stone.

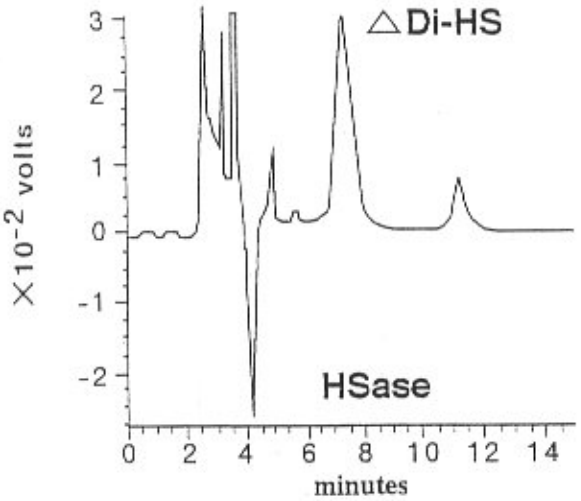
Figure 3 shows the matrix GAG profile in the CaOx stone sample. Five types of GAG constituents were identified. The matrix GAGs in the CaOx+CaP stone sample are shown in Figure 4. The results were similar to the findings in CaOx stones sample. Figure 5 shows the matrix GAG profile in the UA stone sample. Only ΔDi-HS, ΔDi-4S and ΔDi-6S were determined. Figure 6 shows the matrix GAG profile in the MAP stone sample. Although the peaks of ΔDi-4S, 6S, 0S and ΔDi-HA were detected in all MAP stones, ΔDi-HS was not determined.

Analysis of unsaturated disaccharides in matrix GAGs

Figure 7 shows the percentage of each unsaturated disaccharide in the four groups of stone samples. HS is a major GAG component in the matrix of CaOx, CaOx+CaP and UA stones. Interestingly, HS is not detected in all samples of MAP stone matrix. Although Ch-4S and Ch-6S were the second most commonly found components in CaOx, CaOx+CaP and UA stones, they were the predominant factors in MAP stone matrix. Although ΔDi-0S which is the degradation product of Chon tended to increase in MAP stone, only small amounts of that were detected in CaOx or CaOx+CaP stones.

Discussion

In past decades, various urinary macromolecules, such as glycosaminoglycans, glycoproteins and other urinary proteins, were investigated. These macromolecules were initially thought to act as promoters of stone formation and growth because they easily bind to calcium [3, 5, 8, 22]. However, such macromolecules have been shown to act as inhibitors of the aggregation or growth of calcium oxalate crystals [2, 9, 14, 17, 21, 24, 25, 26, 27]. Glycosaminoglycans, in the stone matrix, which may also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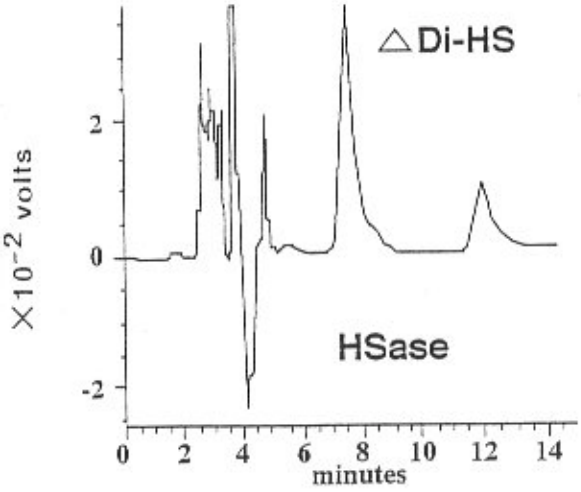
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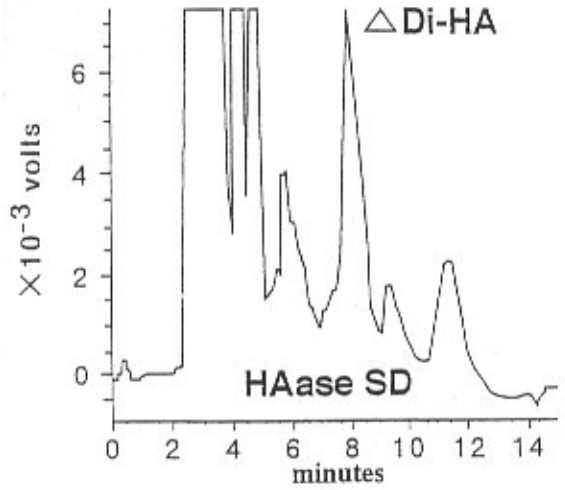
△Di-HA
HAase SD
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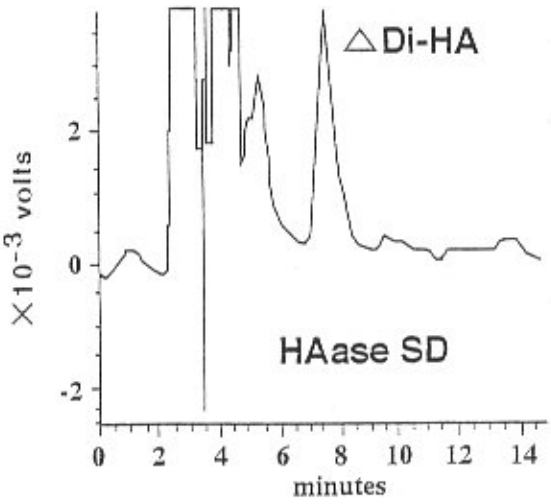
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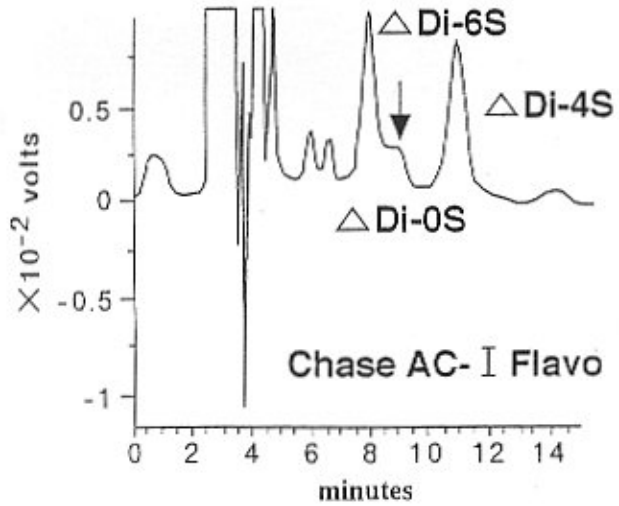
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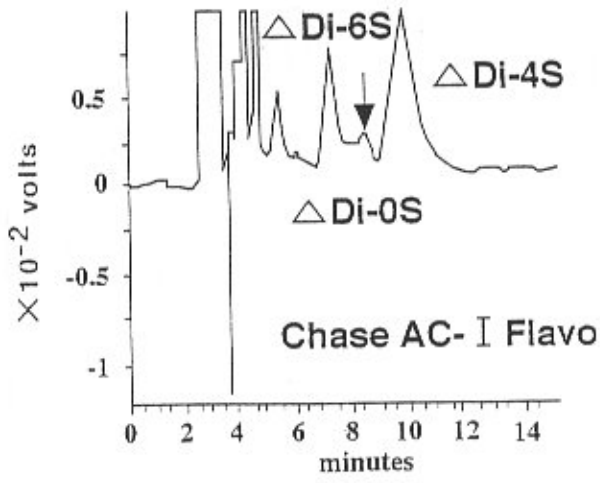
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minutes

have some important role in the crystal aggregation and growth, but their effect on the crystal formation still remains unsettled. Recently, the main GAGs found in stone matrix were identified as heparan sulfate and hyaluronic acid and they are thought to play an important role in CaOx crystallization [3, 22, 23]. Baba *et al*. [3] and Nishio *et al*. [22] have reported that the GAGs in calcium stone matrix consist mainly of HS and hyaluronic acid while the GAG in MAP calculi is characterized by the presence of ChS or low sulfated chondroitin. They concluded that these GAGs must be promoters of stone formation. On the basis of identical findings, Roberts and Resnick [23] have suggested an alternative interpretation. They have attributed the presence of HS and HA in stone matrix to the strong binding affinity of these GAGs to the surface of crystals in the stone. Since it is generally accepted that the magnitude of an inhibitor's effect is a function of the efficiency of its binding to the crystal surface, they implied in their discussion, that HS and HA normally function as inhibitors of stone formation. Yamaguchi *et al*. [27] and Suzuki and Ryall [25] have reported that heparan sulfate strongly inhibits CaOx crystal growth and aggregation, because HS was the only GAG present in soluble stone and crystal matrix, which exhibited strong inhibitory effects on CaOx crystallization. Moreover, Suzuki *et al*. [26] have reported that the relative binding affinity of HS to CaOx crystal surface was much stronger than that of ChS. They concluded that HS and ChS compete for specific binding sites on the crystal surface in the early stages of CaOx crystallization, ChS was included into the crystals only when HS was absent from the urine. Fellström *et al*. [9] have shown that the binding affinity of each GAG is directly related to its charge density. These four studies support the reason why the HS included selectively in the CaOx crystal or CaOx stone matrix. Generally, HS is present in tissues as a proteoglycan and in the urine as metabolic turnover product of tissue proteoglycan (PG). HS-PG is the major component of the glomerular and tubular basement membrane [10] and also its localized on the surface of urotherium [12]. In our present data, HS is also the major GAGs component in the UA stone matrix and these findings are similar to previous reports [13, 22]. However, we also found small amounts of ChS in the calcium-containing stone matrix or UA stone matrix.

Now we speculate the mechanism of the selective inclusion of HS into calcium stones or uric acid stones as follows: once the crystals have formed in the tubular lumen, these crystals injured epithelial layer and HS-PG layer. HS will bind to the crystal surface by its strong binding affinity in the early crystallization. When the crystals start to aggregate and growth, HS is included into the stone matrix. Moreover, if the binding of HS to the crystal surface is not sufficient, ChS, which is the major GAG component in the urine, may also bind to the crystal surface. Finally, the stone is formed in the urinary tract, other GAGs component will be included according to these own concentration in the urine.

Figure 5 (*on facing page, left*). Panel represent the chromatograph of degradation products in UA stone matrix. Only ΔDi-HS, ΔDi-4S and ΔDi-6S were determined in all samples. The peaks of ΔDi-0S and ΔDi-HA were not detected. UA: uric acid stone.

Figure 6 (*on facing page, right*). Panel represent the chromatograph of degradation products in UA stone matrix. Although the peaks of ΔDi-4S, 6S, 0S and ΔDi-HA were detected in all MAP stones, ΔDi-HS was not determined. MAP: magnesium ammonium phosphate stone.

In our study, although HS was the major GAG component in the matrix of calcium-containing stones and uric acid stones, HS was completely absent in MAP stones where ChS or chondroitin are the main GAGs. Generally, MAP stones or carbonate-apatite stones have been referred to as "infection stones" [6]. Other investigators have shown that a proteinaceous milieu is produced by urea splitting infections, and it is suspected that this milieu may important in the pathogenesis of struvite stones but not in the production of other stone types. "Matrix concretions" are large, soft, gel-like masses composed of bacteria, inflammatory cells, mucoid debris, and scattered crystals of magnesium ammonium phosphate and carbonate-apatite [1, 5]. Such conditions may not promote the urinary excretion of HS. Moreover, while the binding affinity of HS to calcium or uric acid is strong, binding of HS to other cations is weak. These facts suggest that HS is a minor component in the matrix of MAP stones.

In conclusion, HPLC offers a new technique for studying matrix GAGs in urinary stones. The isolation and quantification of small amounts of GAGs by electrophoresis are laborious and insensitive. The separation of Ch-4S and Ch-6S or the quantification of HA are particularly difficult with this method. Since we performed HPLC to analyze various matrix GAGs, we could isolate and quantify each GAG component, respectively. We strongly recommend the use of this method to analyze the matrix GAGs in urinary stones.

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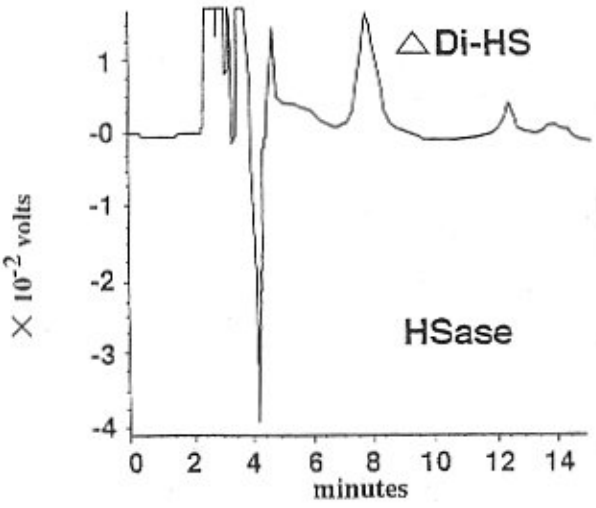
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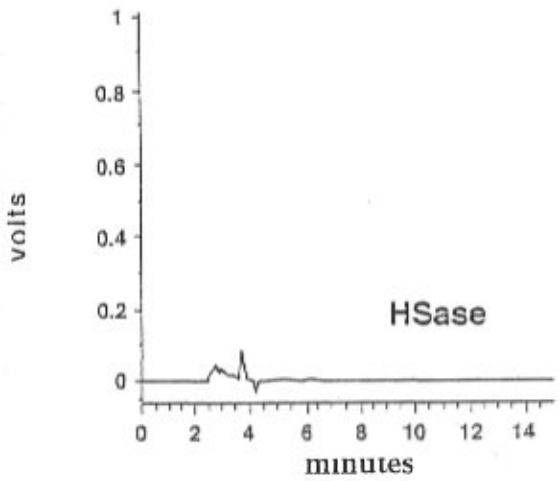
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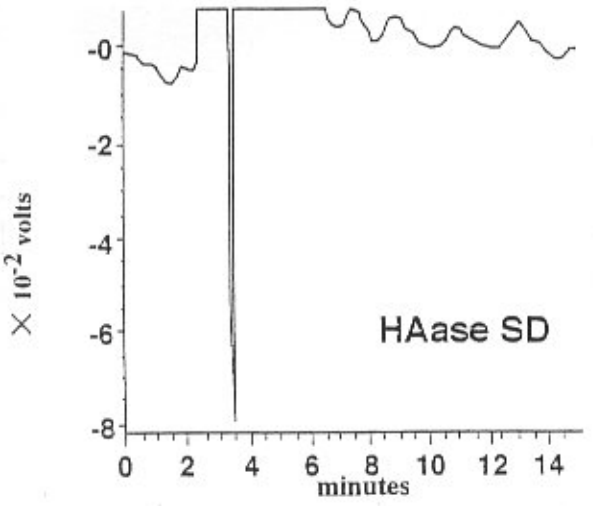
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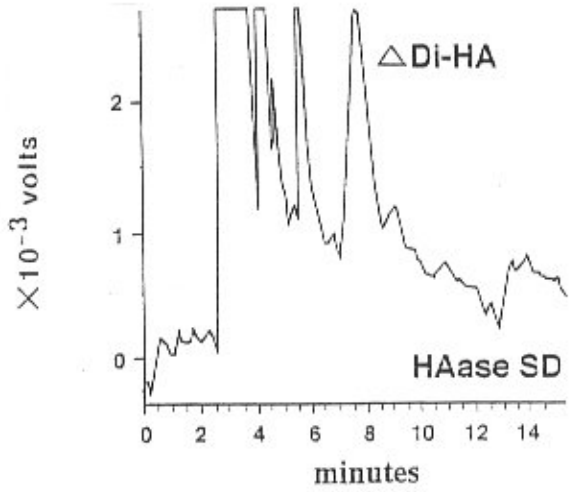
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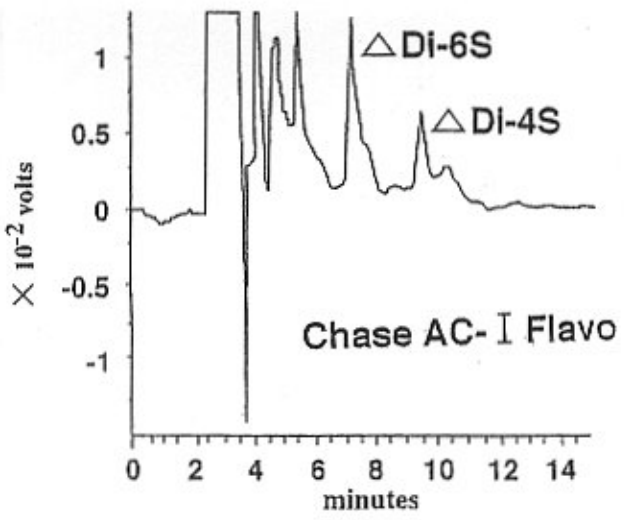
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△Di-4S
Chase AC- I Flavo
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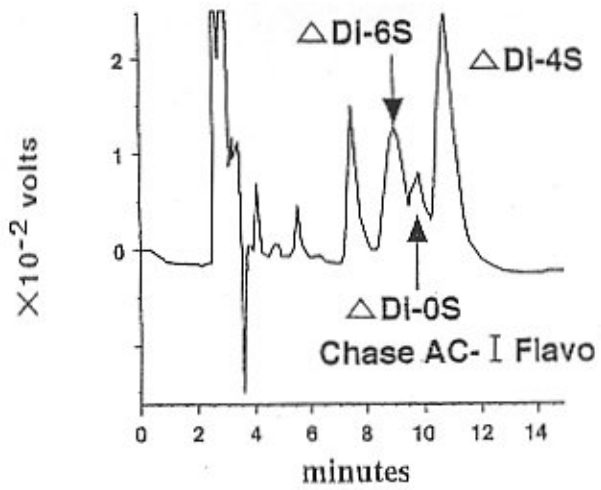
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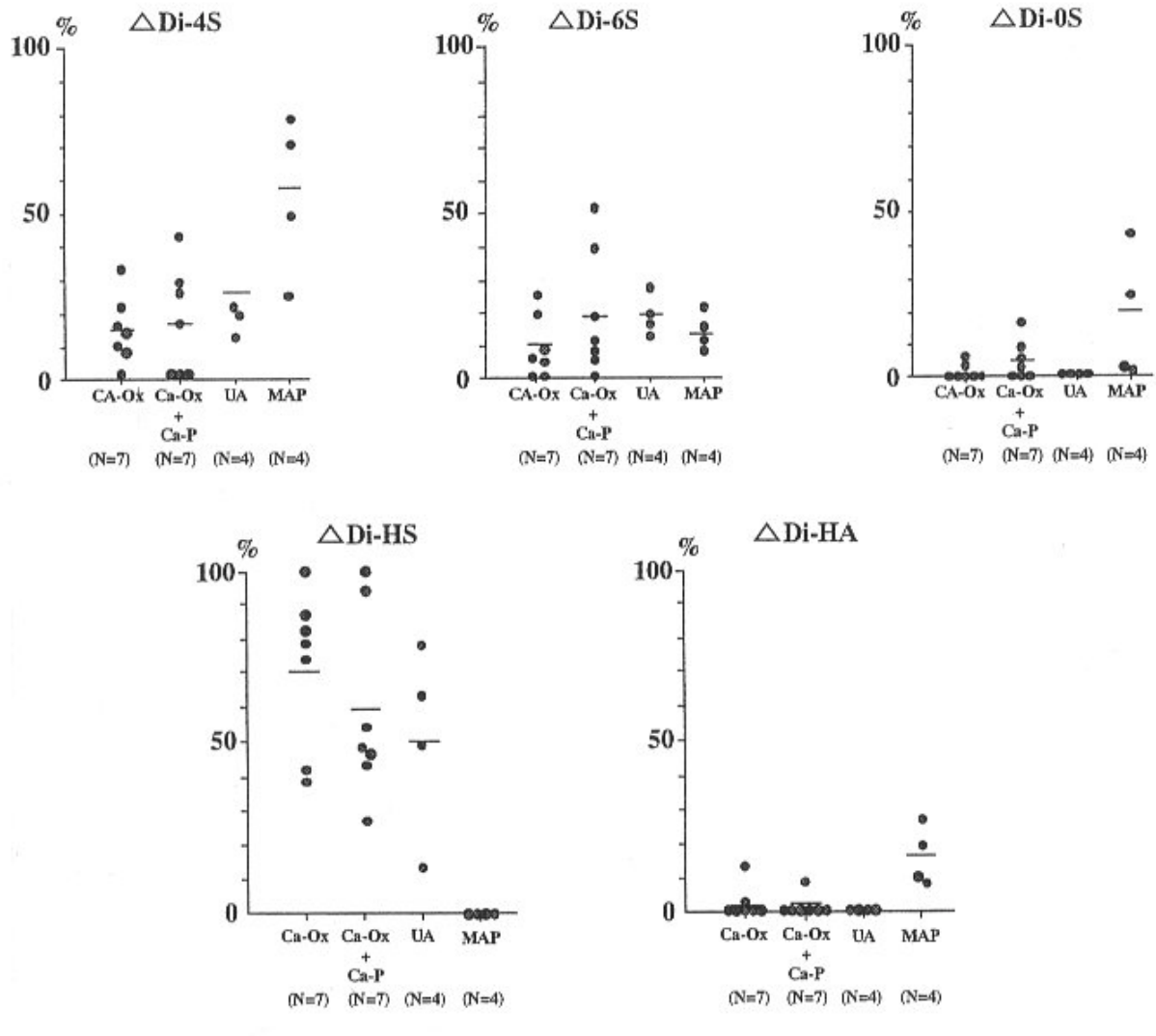

Figure 7. Percentage of unsaturated disaccharides in CaOx, CaOx+CaP, UA, and MAP stone matrix. In the calcium and uric acid stones, the predominant GAG component was HS followed by chondroitin 4 sulfate (Ch-4S) or chondroitin 6 sulfate (Ch-6S), respectively. Chondroitin (Chon) and hyaluronic acid (HA) were absent or present in small amounts. In the magnesium ammonium phosphate (MAP) stones, HS was completely absent and the predominant GAG component was ChS and Chon, followed by HA. The solid bars represent the mean value.

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