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Method development and analysis of free HS and HS in proteoglycans from pre- and postmenopausal women: Evidence for biosynthetic pathway changes in sulfotransferase and sulfatase enzymes

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Abstract

Heparan sulfate (HS) is one of the most complex and informative biopolymers found on the cell surface or in the extracellular matrix as either free HS fragments or constituents of HS proteoglycans (HSPGs). Analysis of free HS and HSPG sugar chains in human serum at the disaccharide level has great potential for early disease diagnosis and prognosis, however, the low concentration of HS in human serum, together with the complexity of the serum matrix, limits the information on HS. In this study, we present and validate the development of a new sensitive method for in-depth compositional analysis of free HS and HSPG sugar chains. This protocol involved several steps including weak anion exchange chromatography, ultrafiltration and solid phase extraction for enhanced detection prior to LC-MS/MS analysis. Using this protocol, a total of 51 serum samples from 26 premenopausal and 25 postmenopausal women were analyzed. Statistically significant differences in heparin/HS disaccharide profiles were observed. The proportion of N-acetylation and N-sulfation in both free HS and HSPG sugar chains were significantly different between pre- and postmenopausal women, indicating changes in N-deacetylase/N-sulfotransferases (NDSTs), the enzymes involved in the initial step of the biosynthetic pathway. Differences in the proportion of 6-O-sulfation suggest that 6-O-sulfotransferase and/or 6-O-sulfatase enzymes may also be implicated.

INTRODUCTION

Heparin/heparan sulfate (HS) is a negatively charged linear polysaccharide, consisting of 50–200 repeating disaccharide units with various sulfation and acetylation patterns (Table 1).^{1–3} They are the most structurally complex glycosaminoglycans (GAGs) found on the cell surface and in the extracellular matrix (ECM). The repeating disaccharide units of hexuronic acid linked to glucosamine form the backbone of heparin/HS. The glucosamine may be 6-O-sulfated, N-sulfated, N-acetylated and sporadically 3-O-sulfated, while the hexuronic acid may be 2-O-sulfated. When HS undergoes exhaustive enzymatic digestion, it can generate typically 12 heparin/HS-derived disaccharides including some of the rare 3-O-sulfated disaccharides.^{4,5} (Table 1)

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The heterogeneous structures of heparin/HS are created on a protein core through a non-template-driven biosynthetic pathway with the cooperation of several enzymes. The biosynthetic pathway is initiated from the protein core through a tetrasaccharide linker, GlcUA β 1-3Gal β 1-3Gal β 1-4Xyl β 1, and elongated by adding GlcNAc and GlcUA alternatively to form a backbone. This heparin/HS backbone is initially modified by N-deacetylase/N-sulfotransferase (NDST) enzymes, which replace N-acetates with N-sulfates, producing NS (N-sulfated domain), NA (unmodified contiguous N-acetylated domain) and NS/NA (transitional domain with alternative N-acetates and N-sulfates) domains on the chain. These domains with various sulfation and acetylation patterns provide substrates for further enzymatic modification of the chain.⁶⁻⁸ Some of the GlcUA residues are then replaced with IdoUA by C-5 epimerase enzymes, creating additional diversity. Further modifications involve the introduction of 2-O-sulfates, 6-O-sulfates and more rarely, 3-O-sulfates by 2-O-sulfotransferase (2OST), 6-O-sulfotransferases (6OSTs) and 3-O-sulfotransferases (3OSTs), respectively.^{6,9-12} In addition to biosynthetic pathways, heparin/HS can be modified by the corporation of editing enzymes, including endosulfatase and endo- β -D-glucuronidase.¹³⁻¹⁵ There are two extracellular 6-O-endo-sulfatases, Sulf1 and Sulf2, which selectively remove sulfate groups from the carbon 6 positions along the heparin/HS chain. The specificity of Sulf1 and Sulf2 has been highly linked to the tri-sulfated disaccharide in the NS domain.¹⁶⁻¹⁹ Endo- β -D-glucuronidase, mainly present as heparanase, cleaves HS chains from the protein core, generating free HS fragments.^{20,21} The changes in disaccharide compositions of free HS and HSPG sugar chains are a direct result of these enzyme changes during biosynthetic pathway and post-synthetic modifications.

Heparan sulfate is involved in many physiological and pathological processes, such as inflammation, cell motility, differentiation, morphogenesis and tumorigenesis, via interactions with various proteins.^{3,11,21} Differential sulfation patterns have been shown to be a crucial factor determining the binding specificity of HS to biologically important molecules, including growth factors, cytokines and chemokines.^{22,23} For instance, 6-O-sulfation is an important modification required for HS and vascular endothelial cell growth factor (VEGF) interactions.²⁴ It has been suggested that the disaccharide compositional profiling of the free HS fragments and HSPG sugar chains, together with the relative editing enzymes are closely related to various disease states.²⁵⁻³⁵ Excess expression of heparanase enzymes has been associated with inflammation, cancer metastasis and rheumatoid arthritis progression.²⁵⁻²⁷ Diseases such as mucopolysaccharidoses, osteoarthritis and myeloma cancer have been reported to correlate with disaccharide compositional changes of HS.²⁸⁻³³ The sulfation structures of HS are also reported to vary with development, aging, and progression of colon adenoma.^{34,35} Therefore, the evaluation of the disaccharide compositional changes of free HS and HSPG sugar chains in different disease states may be crucial for disease diagnosis and prognosis. Menopause status closely associates with hormone levels, and therefore has dramatic health effects.³⁶ It has been reported that early rheumatoid arthritis may lead to a more serious progression of the disease in postmenopausal women.³⁷ Given the important roles of HS in many diseases, the evaluation of the HS composition changes in pre- and postmenopausal states may provide crucial information on early disease diagnosis and prognosis.

Glycosaminoglycans, including chondroitin sulfate, dermatan sulfate, keratan sulfate, hyaluronan and heparin/HS, have been found in human serum.³⁸⁻⁴¹ However, the low concentration of GAGs in human serum together with the complexity of the serum matrix limits the information obtained. Heparin/HS has been found in human serum and plasma in relatively low concentration (0.5 μ g/mL) compared to the other GAGs, making it even more challenging to perform purification and quantitative analysis.⁴² Current methods for the analysis of HS in serum is achieved by cleaving HS chains from the protein core by pronase enzymes, followed by extracting HS from biological matrix, and digesting long-chain HS

into disaccharide units. Various techniques have been developed, such as Cohn-Oncley fractionation, multiple proteolytic enzyme digestions, ethanol, trichloroacetic acid (TCA) or heating precipitation, dialysis, lipid extraction, chromatographic purification and gel electrophoretic separation.^{42–47} However, these methods require a large amount of samples (up to 10 mL) to extract sufficient HS for quantitative analysis. More importantly, there is no analytical method currently available that allows for the determination of GAG from HSPG.

In this study, we present a new method for the compositional analysis of HSPG sugar chains from human sera. We have formatted and combined this new method in a recently published method for analysis of free HS in sera.⁴⁸ This method involves weak anion exchange chromatography, ultrafiltration, exhaustive enzymatic digestion, solid phase extraction (SPE) and LC-MS/MS. Using the new protocol, 51 pre- and postmenopausal women sera were studied and statistically significant differences were detected in both free HS and HSPG fractions.

EXPERIMENTAL SECTION

Materials

Heparin/heparan sulfate-derived disaccharide standards Δ UA-GlcNAc (IV-A), Δ UA2S-GlcNAc (III-A), Δ UA-GlcNAc6S (II-A), Δ UA2S-GlcNAc6S (I-A), Δ UA2S-GlcNS (III-S), Δ UA-GlcNS6S (II-S), Δ UA2S-GlcNS6S (I-S), Δ UA2S-GlcN6S (I-H), Δ UA-GlcN6S (II-H), Δ UA2S-GlcN (III-H), Δ UA-GlcN (IV-H), and Δ UA2S-GlcNCOEt6S (I-P) were purchased from V-Labs (Covington, LA). Δ UA-GlcNS (IV-S) was obtained from Iduron (Manchester, UK). Protease from *Streptomyces griseus* was purchased from Sigma-Aldrich (St Louis, MO). Heparinase I (heparinase, EC 4.2.2.7), heparinase II (heparitinase II, EC 4.2.2.8), and heparinase III (heparitinase, EC 4.2.2.8) from *Flavobacterium heparinum* were obtained from Seikagaku Corporation (East Falmouth, MA). Commercial human serum was procured from Sigma-Aldrich (St Louis, MO).

Human Sera

Banked human sera from healthy individuals were provided from University of California San Francisco (UCSF) Medical Center. Donors were women who were confirmed as negative (benign) for breast cancer upon biopsy. A total of 51 serum samples were analyzed: 26 from premenopausal women (38 ± 8 years old) and 25 from postmenopausal women (57 ± 8 years old). Samples were coded and randomized to minimize analytical bias. The daily accuracy and precision of the purification and analysis method was validated with a commercially available human serum control (Sigma-Aldrich St Louis, MO) using published analytical procedures.^{2,48}

Purification of Free HS and HSPG Sugar Chains from Human Serum

Each of the 51 human serum samples (70 μ L) were centrifuged at $12,000 \times g$ for 15 min. The supernatant was diluted ($\times 10$) using 0.2 M NaCl and applied to a HiTrap DEAE FF column (GE Healthcare, Pittsburgh, PA) equilibrated in 0.2 M NaCl. The column was washed exhaustively with a low concentration of salt (0.3 M NaCl) to remove any weakly bound molecules. Subsequently, a high concentration of salt (2 M NaCl) eluted the strongly bound free HS and HSPGs. The free HS were separated from HSPGs using a 50-kDa molecular weight cut-off (MWCO) filter (Millipore, Billerica, MA). The HSPG fraction remained on the top of the filter and was buffer exchanged with 50 mM ammonium bicarbonate. The HSPGs were subsequently digested by pronase (0.1 mg/mL) overnight at 37 °C using gentle agitation. The HS chains released from HSPG protein cores were purified using the same DEAE method described above. All the DEAE-purified HS fractions were

desalted using two HiTrap desalting columns (GE Healthcare, Pittsburgh, PA) connected in series and dried *in vacuo*.

Digestion and Sample Clean-up

Free HS and HSPG sugar chains were resuspended in 25 μL of digestion buffer, containing 20 mM NH_4OAc (pH 7.5) and 1 mM $\text{Ca}(\text{OAc})_2$. A combination of heparinase enzymes I, II and III (1 mU each) was added to each HS sample and incubated at 37 °C for 20 hrs with gentle agitation. After digestion, each sample was spiked with 2 μL of 20 mM internal standard I-P ($\Delta\text{UA}2\text{S} \rightarrow \text{GlcNCOEt}6\text{S}$) and purified using a C18+carbon SPE TopTip cartridge (Glygen, Columbia, MD). The cartridge was conditioned 2 times with 50 μL of 0.1% trifluoroacetic acid (TFA) in 80% (v/v) ACN/ H_2O , followed by 100 μL of Milli-Q water and 100 μL of 0.5 M NaCl. Each digested sample was diluted five-fold with 0.5 M NaCl, and then applied to the cartridge. To remove NaCl and non-bound contaminants, each cartridge was washed 5 times with 100 μL of Milli-Q water. Heparin/HS-derived disaccharides retained by the cartridge were eluted with 250 μL of 0.05% TFA in 40% (v/v) ACN/ H_2O . All steps were performed in a swinging platform centrifuge at 2000 rpm for 1 min. The eluted fractions were collected and dried *in vacuo*. Finally, samples were redissolved in 20 μL of Milli-Q water before LC-MS/MS analysis.

LC-MS/MS Analysis

Mass spectra were acquired using a LTQ 2-D linear ion trap mass spectrometer equipped with an electrospray ionization (ESI) source and directly coupled to an HPLC system (Thermo Electron, San Jose, CA). The LC-MS/MS method was described previously.⁴⁸ Briefly, the purified disaccharide samples were loaded onto a Hypercarb column (2.1 mm ID $\times$ 150 mm, 5 μm) (Thermo Fisher Scientific, Waltham, MA) at a flow rate of 200 $\mu\text{L}/\text{min}$. The sample was desalted with 100% solvent A (5 mM $\text{NH}_4\text{HCO}_3/\text{H}_2\text{O}$) for 4 min, then eluted in 38% solvent B (5 mM NH_4HCO_3 in 80% ACN). The MS analysis was performed in the negative ion mode using a capillary temperature of 250 °C. The instrument was tuned using disaccharide standards for optimal performance before sample analysis. For MS/MS experiments, the precursor ions were selected using an isolation width of 3 Da and activated using 18–21% normalized collision energy for 100 ms. Data acquisition and analysis were performed using Xcalibur 2.0 software.

Statistical Analysis

Statistical tests were performed using OriginPro 8.0. One-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison tests were used to analyze the variation among three different race groups. Comparisons between pre- and postmenopausal women were performed using a two-way t-test for normally distributed data and a Mann-Whitney rank sum test for non-normally distributed data. A 95% confidence interval was used with the p value less than or equal to 0.05 being considered statistically significant. Error bars are expressed as the standard error of the mean (SEM) that accounts for the sample size.

RESULTS AND DISCUSSION

Method Development for HSPG Sugar Chains Purification from Serum

Herein, we present an optimized method that enables the compositional analysis of HSPG sugar chains using small quantities of human serum, as shown in Scheme 1. This method, in addition to that used for measuring free HS⁴⁸, was employed in the analysis of sera from 51 pre- and postmenopausal women. The new method introduces weak anion exchange chromatography on a DEAE column, which allows further purification of the negatively charged molecules from serum. Most of the neutral, positive and weakly bound negatively

charged species were removed from the DEAE column using a low concentration of salt, producing a purified fraction containing free HS and HSPGs.

The quantity of HS present in human serum is very low (0.5 $\mu\text{g/mL}$), making it more challenging for purification and quantitative analysis. Therefore, it was crucial to develop a method that reliably yielded high recovery. The recovery of HS through purification procedures was evaluated by spiking in 0.05 $\mu\text{g}/100\text{ }\mu\text{L}$ of commercially available HS from bovine kidney. The concentration of HS was chosen to simulate the actual quantities of HS present in human serum (0.5 $\mu\text{g/mL}$). Three control samples were analyzed, including (1) water + 0.05 $\mu\text{g}/100\text{ }\mu\text{L}$ bovine kidney HS, (2) serum + 0.05 $\mu\text{g}/100\text{ }\mu\text{L}$ bovine kidney HS, and (3) HS digested by pronase. Interestingly, the control sample digested with pronase showed significantly lower recovery (around 60% less) compared to the other two controls (HS spiked in water and HS spiked in serum). This result indicates that interactions between free HS and pronase enzymes occurred, leading to a dramatic reduction in HS recovery. To resolve this problem, a low concentration of salt (0.2 M NaCl) was added before loading pronase digested samples onto the DEAE columns. The low ionic strength of the NaCl solution helped eliminate the non-specific/low affinity interactions and increased the recovery of HS to approximately 91% (data not shown).

Free HS and HSPG sugar chains were digested exhaustively using a combination of Heparinase I, II and III. The digested samples were cleaned up by SPE cartridges containing C18 and graphitized carbon resins. (Scheme 1) The SPE cartridges were prewashed to remove any existing salts, precipitates, and any other interfering compounds, reducing the number of contaminants that could negatively impact the MS analysis. The heparinase-digested samples were suspended in 0.5 M NaCl before being applied onto the SPE cartridges. This low concentration of NaCl eliminates non-specific interactions between heparinase enzymes and disaccharide products, increasing the recovery of HS.

To validate the optimized method, a commercial serum sample (Sigma) was analyzed on three different days (Table 2). Results showed good reproducibility with errors less than 3%. This commercial serum sample was also analyzed with banked serum samples, serving as a control for inter- and intra-day accuracy and precision of purification and quantitative analysis.

Comparison of Free HS from Pre- and Postmenopausal Women

A total of 51 serum samples were analyzed from which 26 were premenopausal women (38 ± 8 years old) and 25 were postmenopausal women (57 ± 8 years old). Free HS and HSPGs were separated by a 50 kDa MWCO filter and analyzed separately. (Scheme 1) Blinding and randomization were used to control analytical bias. Commercial human serum (Sigma) and commercial HS from bovine kidney were used as quality control samples to ensure daily accuracy and precision of purification and analysis.

An overall comparison was first performed using two-way ANOVA. The results showed that various ethnic groups exhibited no statistical differences ($p=0.33$), however, there were significant differences in the results based on menopausal status (Figure 1). Therefore, the variable, ethnicity, is not a significant factor impacting HS changes. Further investigation of the free HS changes within the same variable (menopausal status) was conducted using two-way t-test for normally distributed data and Mann-Whitney rank sum test for non-normally distributed data.

Figure 1A shows a comparison of free HS from pre- and postmenopausal women, with statistically significant differences indicated for two of the twelve disaccharides, IV-A and I-S (See Table 1 for all twelve structures). In postmenopausal women, the amount of the N-

acetylated disaccharide, IV-A, was increased, while the fully-sulfated disaccharide, I-S, was decreased. The N-acetylated IV-A disaccharide ($\Delta\text{UA}\rightarrow\text{GlcNAc}$) should be located in the NA domain, while the sulfated I-S disaccharide ($\Delta\text{UA}2\text{S}\rightarrow\text{GlcNS6S}$) should be located in the NS domain. The heterogeneity of HS is a result of a non-template driven biosynthetic process involving various editing enzymes. The domains (NS, NA, and NS/NA domains) on HS chains are generated in the initial modification step during biosynthesis, where NDSTs catalyze the substitution of acetyl groups by sulfate groups on the glucosamine. Addition of further O-sulfate groups by 2-O-sulfotransferase, 6-O-sulfotransferases and 3-O-sulfotransferases increases HS complexity. Therefore, the free HS composition changes may be due to the change in the activity of NDSTs, the enzyme involved in the initial step in the biosynthetic pathway of HS.

In postmenopausal women, it was also observed that when the disaccharides were summed into groups of N-sulfates, 2-O-sulfates and 6-O-sulfates, there was an overall reduction by 10.5%, 9.7% and 9.3%, respectively. During HS biosynthesis, the sulfation of the C2 position and the C6 position tends to occur in the NS domain. Variations in N-sulfation, 2-O-sulfation and 6-O-sulfation appear to be initiated from the reduction of N-sulfation.

The total HS concentration was calculated through a cumulative total of each disaccharide. (Figure 1B) The statistical analysis showed that premenopausal women have a significantly higher amount of free HS compared to postmenopausal women ($p<0.05$). Upon menopause, the most significant change is the reduction in hormone levels.³⁶ These results raise the question as to whether there may be a possible relationship between the HS levels and hormone levels, the latter of which could affect endo- and sulfotransferases. Free HS present in the human body is created by heparanase cleavage of sugar chains from the proteoglycan. Therefore, the variation in the concentration of free HS might indicate the changes in either heparanase levels or heparanase activity upon menopausal status.

Comparison of HSPG Sugar Chains from Pre- and Postmenopausal Women

The purification and analysis methodology was applied to the HSPG fraction collected from the top of the 50kDa MWCO filter. (Scheme 1) The same statistical analysis steps were applied to the HSPG results. ANOVA analysis showed that the p value was greater than 0.05, indicating, once more, that race is not a contributing factor in the HSPG variation. On comparing pre- and postmenopausal women HSPG sugar chain compositions, four of the twelve heparin/HS-derived disaccharides showed significant differences (Figure 2A). Disaccharides IV-A (N-acetylated disaccharide) and I-S (fully-sulfated disaccharide) from HSPG sugar chains showed the same changes as those from free HS, where IV-A was increased ($p<0.001$) and I-S was decreased ($p<0.01$) in postmenopausal women. These disaccharide differences support our theory that the NDST activity changes upon menopause.

Disaccharides II-S (N-sulfated and 6-O-sulfated disaccharide) and I-H (2-O-sulfated and 6-O-sulfated disaccharide) also showed statistical differences in the HSPG sugar chain compositions between pre- and postmenopausal women. Analysis of the HSPG sugar chain compositions showed that in postmenopausal women, both II-S and I-H were significantly decreased, with p values less than 0.01 and 0.05, respectively. Decrease of the 6-O-sulfated containing disaccharides, II-S and I-H, contributed to a greater overall reduction in 6-O-sulfation (15.1%), while reduction in the proportion of N-sulfation and 2-O-sulfation (9.9% and 12.3%) was comparable to those in free HS. The reduction in 6-O-sulfation could indicate a disruption to enzymatic patterns involved in both HS biosynthetic and post-synthetic pathways during menopause. During the HS biosynthetic pathway, the 6-O-sulfotransferases are responsible for the sulfation at the C6 position. A decrease in enzyme activities could result in limited numbers of 6-O-sulfates synthesized on HS chains. Since

the addition of 6-O-sulfation is the latter stage in the HS biosynthetic pathway, it is also possible that prior modifications also result in a compositional change, leading to a reduction in the number of markers employed by the 6-O-sulfotransferases. During the post-synthetic pathway, there are two extracellular 6-O-endo-sulfatases, Sulf1 and Sulf2, which selectively remove sulfate groups from the C6 positions along the HS chain. It is possible that these sulfatases contribute to the postmenopausal compositional changes. However, this explanation alone is insufficient to explain our results, as increases in III-S (2-O-sulfated and N-sulfated disaccharide) were not observed.

The concentration of HSPG sugar chains and free HS both showed the same pattern in pre- and postmenopausal women. The concentration of HS chains on the PGs decreased significantly when women were menopausal ($p < 0.01$). (Figure 2B) HS chains are localized to cell surface membranes and extracellular matrix, where an abundance of biological interactions can occur. Some of these anchored HS chains are cleaved through enzymatic hydrolysis, producing free HS to the blood stream as a result. Therefore, the decrease in the concentration of HSPG sugar chains in postmenopausal women, would logically lead to the reduction of free HS in the blood stream, which agrees with our free HS findings.

CONCLUSIONS

In this study, we present and validate the development of a sensitive method for in-depth compositional analysis of free HS and HSPG sugar chains. This protocol involved several steps including weak anion exchange chromatography, fractionation and enrichment techniques for enhanced detection prior to LC-MS/MS analysis. Using this protocol, the comprehensive compositional analysis of both free HS and HSPG sugar chains was conducted. A total of 51 serum samples from 26 premenopausal and 25 postmenopausal women were analyzed. Results showed that two (IV-A and I-S) of the twelve disaccharides from free HS and four (IV-A, I-S, II-S, and I-H) from HSPG sugar chains are statistically different between these two populations. The proportion of N-acetylation and N-sulfation in both free HS and HSPG sugar chains were significantly different between pre- and postmenopausal women, indicating changes in NDSTs, the enzyme involved in the initial step of the biosynthetic pathway. Another notable difference was in the proportion of 6-O-sulfation, where less was observed in HSPG sugar chains from postmenopausal women, indicating 6-O-sulfotransferase enzymes and/or 6-O-sulfatase enzymes may also be crucial. This analysis will serve as a healthy control data set for further studies on disease states. Further studies are planned in which this method will be applied to the analysis of sera from pre- and postmenopausal women with rheumatoid arthritis (RA).^{49–51} Changes in HS disaccharide profiles, especially in 6-O-sulfated disaccharides, may provide crucial information about up or down regulation of HS editing enzymes involved in RA.

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Figure 1.

Comparison of free HS in pre- and postmenopausal women. (A) Compositional analysis of free HS in pre- and postmenopausal women. Individual disaccharides are represented as the average percentage identified in serum samples. The composition of free HS in premenopausal woman shows significant differences in disaccharides IV-A and I-S compared to postmenopausal women. (B) The concentrations of free HS in pre- and postmenopausal women. The concentration ($\mu\text{mol/mL}$) of each disaccharide is obtained from the mass spectrum¹. This value for each of the 12 disaccharides is then multiplied by the molar mass to produce the concentration in $\mu\text{g/mL}$. All 12 concentrations are then summed to produce Fig 1 B. The free HS concentration in premenopausal women is significantly higher than that in postmenopausal women. The data values are expressed as the mean $\pm$ SEM. P-values were determined through two-way t-test or nonparametric Mann-Whitney Rank Sum test. A 95% confidence interval was used, with the p value less than or equal to 0.05 being considered statistically significant.

Figure 2.

Comparison of HSPG sugar chain compositions in pre- and postmenopausal women. (A) Compositional analysis of HS chains on PGs in pre- and postmenopausal women. Individual disaccharides are represented as the average percentage identified in serum samples. The composition of HSPG sugar chains in premenopausal woman shows significant differences in disaccharides IV-A, II-S, I-S and I-H compared to postmenopausal women. (B) The HSPG sugar chain concentrations in pre- and postmenopausal women. The concentration ($\mu\text{mol/mL}$) of each disaccharide is obtained from the mass spectrum¹. This value for each of the 12 disaccharides is then multiplied by the molar mass to produce the concentration in $\mu\text{g/mL}$. All 12 concentrations are then summed to produce Fig 2 B. The HSPG sugar chain concentration in premenopausal women is significantly higher than that in postmenopausal women. The data values are expressed as the mean $\pm$ SEM. P-values were determined through two-way t-test or the nonparametric Mann-Whitney Rank Sum test. A 95% confidence interval was used, with the p value less than or equal to 0.05 being considered statistically significant.

Scheme 1.

Flowchart for the purification and analysis of free HS (LMW) and HSPG sugar chains (HMW) from human serum.

Table 1

Structures of 12 heparin/heparan sulfate-derived disaccharides and internal standard I-P.

	Disaccharides	R ₂	R ₆	Y
I-A	ΔUA2S→GlcNAc6S	SO ₃	SO ₃	COCH ₃
II-A	ΔUA→GlcNAc6S	H	SO ₃	COCH ₃
III-A	ΔUA2S→GlcNAc	SO ₃	H	COCH ₃
IV-A	ΔUA→GlcNAc	H	H	COCH ₃
I-S	ΔUA2S→GlcNS6S	SO ₃	SO ₃	SO ₃
II-S	ΔUA→GlcNS6S	H	SO ₃	SO ₃
III-S	ΔUA2S→GlcNS	SO ₃	H	SO ₃
IV-S	ΔUA→GlcNS	H	H	SO ₃
I-H	ΔUA2S→GlcN6S	SO ₃	SO ₃	H
II-H	ΔUA→GlcN6S	H	SO ₃	H
III-H	ΔUA2S→GlcN	SO ₃	H	H
IV-H	ΔUA→GlcN	H	H	H
I-P	ΔUA2S→GlcNCOEt6S	SO ₃	SO ₃	COC ₂ H ₅

Table 2

Reproducibility of the commercial serum control sample (Sigma).

	Trial 1	Trial 2	Trial 3	Mean$\pm$ SD
IV-A	40.1	41.4	42.9	41.5 $\pm$ 1.4
III-A	0.8	0.5	0.8	0.7 $\pm$ 0.2
II-A	6.5	6.4	5.7	6.2 $\pm$ 0.4
I-A	2.3	1.9	1.3	1.9 $\pm$ 0.5
IV-S	17.8	17.4	17.6	17.6 $\pm$ 0.2
III-S	10.8	11.4	9.8	10.7 $\pm$ 0.8
II-S	8.5	10.3	8.1	8.9 $\pm$ 1.2
I-S	8.5	6.8	10.3	8.5 $\pm$ 1.8
I-H	3.0	2.1	2.6	2.6 $\pm$ 0.4
II-H	0.3	0.3	0.0	0.2 $\pm$ 0.2
III-H	0.4	0.3	0.7	0.5 $\pm$ 0.2
IV-H	1.0	1.0	0.2	0.7 $\pm$ 0.4