Article type: *Short Communications*

Simultaneous determination of heparan sulfate, chondroitin/dermatan sulfates, and hyaluronan glycosaminoglycan disaccharides by high-performance liquid chromatography using a reverse-phase column with adamantyl groups

Hisatoshi Hanamatsu[a,b,*], Satoshi Makino[c], Masatsugu Ohara[a], Goki Suda[a], Ikuko Yokota[d], Shoko Nishihara[e,f], Naoya Sakamoto[a], Jun-ichi Furukawa[b,d,*]

a Department of Gastroenterology and Hepatology, Graduate School of Medicine, Hokkaido University, Sapporo, Hokkaido 060-8638, Japan

b Department of Orthopaedic Surgery, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Hokkaido 060-8638, Japan

c Sanyo Fine IRICA Technology Co., Ltd. Kyoto, Kyoto 601-8037, Japan

d Institute for Glyco-core Research (iGCORE), Nagoya University, Nagoya, Aichi 464-8601, Japan

e Department of Bioinformatics, Graduate School of Engineering, Soka University, Hachioji, Tokyo, Japan.

f Glycan & Life System Integration Center (GaLSIC), Soka University, Hachioji, Tokyo,

Japan.

Correspondence: h_hanamatsu@med.hokudai.ac.jp (H.H.); j-furu@med.nagoya-u.ac.jp (J.-i.F.);Tel.: +81-(0)11-706-9477 (H.H.); +81-(0)52-744-2943 (J.-i.F.)

ABSTRACT

Glycosaminoglycans (GAGs), which are one of the major components of proteoglycans, play a pivotal role in physiological processes such as signal transduction, cell adhesion, growth, and differentiation. Characterization of GAGs is challenging due to the tremendous structural diversity of heteropolysaccharides with numerous sulfate or carboxyl groups. In this present study, we examined the analysis of 2-aminobenzamide (2-AB) labeled GAG disaccharides by high-performance liquid chromatography (HPLC) using a reverse-phase (RP)-column with adamantyl groups. Under the analytical conditions, 17 types of 2-AB labeled GAG disaccharides derived from heparan sulfate, chondroitin/dermatan sulfates, and hyaluronan were sequentially separated in a single analysis. The analysis time was fast with high retention time reproducibility. Moreover, the RP-HPLC column with adamantyl groups allowed the quantification of GAGs in various biological samples, such as serum, cultured cells, and culture medium.

Keywords: RP-HPLC, Adamantly group, Δ-Disaccharide GAG analysis, heparan sulfate detection, chondroitin/dermatan sulfate detection

1. Introduction

Glycosaminoglycans (GAGs) are linear heteropolysaccharides that are highly-negatively charged due to numerous sulfate or carboxyl groups [1]. GAG chains are constructed from different repeating disaccharide units, uronic acid (glucuronic acid [GlcA] or iduronic acid [IdoA]), and an amino sugar (*N*-acetylglucosamine [GlcNAc] or *N*-acetylgalactosamine [GalNAc]). GAGs are classified into three categories, namely, heparan sulfate (HS), chondroitin sulfate (CS)/dermatan sulfate (DS), and hyaluronan (HA). HS and CS/DS are major components of proteoglycans (PGs) and modified with sulfate groups. PGs play various important roles in physiological processes such as cell signaling, cell–cell adhesion, growth, and differentiation [2, 3] and these GAGs have been reported to be associated with the immune response, inflammation, infection, and cancer [4-7]. GAGs consist of different disaccharide units with or without sulfate groups, and consequently possess extensive diversity in polysaccharide sequence. HA consists of a simple repeating disaccharide without sulfate groups (GlcA β1-3 GlcNAc) and is not covalently linked to proteins. For this reason, characterization of whole GAGs is

55 challenging due to the tremendous structural diversity.

56 The fine structures of GAGs are generally assessed by analyzing the Δ-disaccharide
57 composition after enzymatic depolymerization, fluorescent labeling, followed by high-
58 performance liquid chromatography (HPLC) analysis; however, there are 17 types of
59 structures of repeating disaccharides derived from HS, CS/DS, and HA. Various
60 analytical techniques have been developed [8, 9]. CS/HA disaccharides derivatized with
61 2-aminoacridone could be separated by reversed-phase (RP)-HPLC analysis [10].
62 Previously, we reported the simultaneous analysis of 17 GAG disaccharides derived from
63 HS, CS/DS, and HA by zwitterionic type of hydrophilic interaction chromatography
64 (ZIC-HILIC) separation and glycoblotting glycan purification [11]. However, separation
65 of 17 GAG disaccharides required HPLC method by two-step gradient elution, resulting
66 in poor retention time reproducibility.

67 In this present study, we investigated the separation of 17 GAG disaccharides using
68 various RP-HPLC columns and established the analytical conditions for the qualitative
69 and quantitative analysis of GAGs.

70 **2. Experimental**

71 *2.1. Cell cultures*

72 LX-2 cells (Billerica, MA, United States), an immortalized human hepatic stellate

cell line, were grown in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 2% fetal bovine serum (Moregate Biotech, Bulimba, QLD, Australia) and 1% penicillin/streptomycin (Wako Pure Chemical Industries) at 37°C and 5% CO_2. LX-2 cells were seeded in a 10 cm dish at a density of 1.5×10^6 cells/dish, cultured in DMEM with supplements and treated with or without 2 ng/mL TGF-β (PeproTech, Rocky Hill, NJ, United States) for 72 h [12, 13]. After replacement with FBS-free medium containing TGF-β, the LX-2 cells were cultured for 24 hours, and then collected by centrifugation. Corrected cells were subjected to GAG analysis, as described below.

2.2. Protein precipitation of cultured cell, cell culture medium, and serum

The protein extraction procedure was the same as a previously reported method [14]. Briefly, a 100 μL of phosphate-buffered saline (PBS) was added to cell pellets and they were homogenized by ultrasonication. A 4-fold volume of ethanol was added to the homogenized mixture or cell culture medium and incubated at -30°C for 3 h. After separation of protein pellets and supernatants, precipitates were dried at 37°C and re-dissolved in water. Protein concentration was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific).

2.3. Precipitation of glycosaminoglycanopeptides and hyaluronan

A 100 μg sample of cellular or cell culture medium protein, or 10 μL of serum, was dried *in vacuo* and resuspended in 90 μL of 50 mM $AcONH_4$ pH 7.5/10 mM $CaCl_2$. Samples were subjected to nonspecific protease digestion by adding 10 μL of 10 mg/mL pronase (Merck, Darmstadt, Germany) at 37°C overnight. Subsequently, 20 μL of 30% aqueous sodium acetate and 480 μL of cold ethanol were added, and pellets containing glycosaminoglycanopeptides and hyaluronan were recovered by centrifugation at 14,000 *g*.

2.4. Preparation of GAG disaccharides by enzymatic digestion

Ethanol-precipitated glycosaminoglycanopeptides were digested to disaccharides by treatment with 100 mU each of a mix of heparinase, heparitinase, hyaluronidase SD, and chondroitinase ABC (Sigma-Aldrich, St. Louis, MO) in 100 μL of 0.1 M ammonium acetate containing 5 mM calcium acetate, pH 7.5, at 37°C overnight. A known amount of maltose (typically, 20 pmol) was added as an internal standard, followed by a 4-fold volume of ethanol, and samples were incubated at -30°C for 3 h. After centrifugation at 14,000 *g*, supernatants were collected and dried *in vacuo*.

2.5. 2-Aminobenzamide (2-AB) labeling of glycosaminoglycan disaccharides

GAG disaccharides were labeled with 2-Aminobenzamide (2-AB) by the previously reported methods with minor modification [15, 16]. A 20 μL of 0.4 M 2-methylpyridine

borane complex (Junsei Chemical, Tokyo, Japan) was added to 1 mL of 2-AB in 45% methanol containing 10% AcOH to prepare the reaction solution. A 50 μL of the reaction solution was added to dried GAG disaccharides and incubated at 50°C for 2.5 h. After 2-AB labeling, excess reagent was removed using a G-10 column [11].

2.6. GAG disaccharide analysis by RP-HPLC column with adamantyl groups

Experiments were performed using an HPLC system consisting of a NANOSPACE SI-2 instrument with a fluorescence detector (Osaka soda, Osaka, Japan). Separation of 2-AB labeled GAG disaccharides was performed with the a7damantly column (3 μm, 4.6 mm × 250 mm; CAPCELL PAK ADME-HR, Osaka soda) in a column oven at 25°C. A gradient elution was applied at a flow rate of 500 μL/min using 10 mM ammonium acetate, pH 6.6–6.8 (solvent A) and 50% (v/v) acetonitrile containing 10 mM ammonium acetate, pH 7.1–7.3 (solvent B) as follows: A/B = 100/0 (0 min) → 70/30 (40 min) → 0/100 (50 min) → 100/0 (60 min)). Disaccharides were detected by in-line fluorescence (excitation at 330 nm, emission at 420 nm). The column was regenerated by washing with 100% solvent B before equilibrating in solvent A. Peak identification was confirmed through co-injection of 18 different standard disaccharides. The structures of GAG disaccharides were shown in **Table 1**. Quantitation was performed using maltose as an internal standard.

3. Results and Discussion

3.1. Separation of 2-AB labeled GAG disaccharide standards by RP-HPLC column with adamantyl groups

Volpi reported that chondroitin sulfates (CS)/hyaluronan (HA) disaccharides derivatized with 2-aminoacridone could be separated by RP-HPLC using ammonium acetate buffer and acetonitrile as eluents [10]. Previously, we also demonstrated that 17 kinds of GAGs derived from heparan sulfate (HS), CS/dermatan sulfates (DS), and HA were well separated using a ZIC-HILIC column and 2-AB labeling [11]. This eluent composition is similar to that used for the analysis of 2-aminoacridone labeled GAGs by RP-HPLC: however, the retention time reproducibility was poor as shown in **Fig. S1**. To overcome this problem, GAG standards mixture was analyzed using an ODS-RP-column with eluent A (10 mM ammonium acetate) and eluent B (50% (v/v) acetonitrile containing 10 mM ammonium acetate) with fluorescence detection. The mixture contained equal amounts of each of the 17 types of 2-AB labeled glycosaminoglycan disaccharides, including eight disaccharides comprising HS, eight disaccharides comprising CD/DS, and one HA disaccharide (**Fig. 1**). 2-AB labeled isomaltotriose was included for relative quantitation. First, the mixture of GAG standards (10 μL) was analyzed on a ODS-RP-

column with a linear gradient (0–40% eluent B over 40 min) at a flow rate of 500 μL/min. As shown in **Fig. 1a**, GAGs with two or three sulfate groups were sufficiently separated within 24 min. In contrast, GAG disaccharides with less than one sulfate group were not separated after 25 min. Other gradient elution and concentration of ammonium acetate were not effectives for the separation. Second, we examined whether commonly used phenyl and pentafluorophenyl (PFP)-RP-columns could separate the 17 types of GAGs. Using a phenyl RP-column, GAGs were eluted after 15 min, probably with less than one sulfate groups, could be separated. On the other hand, GAG disaccharides within 15 min could not be separated. Almost no GAG disaccharides could be separated using the PFP-RP column (**Fig. S2**). Therefore, we investigated RP-HPLC using a column with adamantyl groups (CAPCELL PAK ADME-HR) for GAGs separation. As shown in **Fig. 1d**, 17 kinds of peaks were successfully separated using the linear gradient (0–30% eluent B over 40 min). In the case of the ADME-HR column, strongly polar GAGs (HS-2SNS, HS-NS6S, and HS-2SNS6S) eluted earlier than with the ODS column (**Fig. 1a** and **d**); however, weakly polar GAGs (CS-0S, CS-2S, CS-4S, CS-6S, HS-0S, and HA) eluted slower. Peaks for CS-0S and isomaltotriose (IS) were not separated, but we solved this problem by replacing IS with maltose (**Fig. 1e**). In other words, HPLC analysis using an ADMR-HR column provided better separation of GAG disaccharides with few sulfate

groups than that with an ODS column and three or two sulfate groups than that with phenyl and PFP columns. A wide retention range was achieved with the ADME-HR column, whose hydrophobic adamantyl functional groups likely facilitate the simultaneous determination of GAG disaccharides. Retention time reproducibilities were less than 3.2% for 17 kinds of GAGs (**Table 1**). These results demonstrated that RP-HPLC with an ADME-HR column can be used for the quantitative analysis of 17 GAGs with good retention time reproducibility.

3.2. Analysis of GAGs in biological samples

After establishing HPLC conditions for 17 kinds of GAG analysis by RP-HPLC using an ADME-HR column, we performed quantitative glycosamino-glycomic analysis of LX-2 cells and their culture medium with/without TGF-β. In our other *N*- and *O*-linked glycans analysis of LX-2 cells,, we revealed that the terminal galactose of *N*- and *O*-glycans was elevated by the treatment with TGF-β. Jiang et al. reported that up-regulation of galectin-3 binding to galactose modulates phagocytosis-induced stellate cell activation and liver fibrosis [17]. These results suggested that fibrosis is appropriately induced by TGF-β stimulation in LX-2 cells. First, we measured 2-AB labeled GAGs derived from LX-2 cells and their culture medium under optimized conditions. As shown in **Fig. 2a**

and **b**, 12 GAG peaks were detected for both LX-2 cells with and without TGF-β stimulation. Expression of CS-4S was increased by the treatment with TGF-β, and the high expression of CS-4S in culture medium following addition of TGF-β was correlated with that of LX-2 cells. Expression of HA was increased only in the culture medium following TGF-β treatment (**Fig. 2c** and **d**). By contrast, GAG disaccharides derived from HS were not altered by TGF-β. Koshiishi et al. reported that expression of CS-4S were increased in fibrotic lesions of cirrhotic rat liver compared to non-fibrotic lesions [18]. Expression of HA was also increased in chronic liver diseases with fibrosis [19]. These results were consistent with these previous reports.

Next, we analyzed GAGs in human serum. Peaks for CS-4S and CS-0S were mainly detected at 28.0 and 34.7 min, respectively, following separation using the ADME-HR column (**Fig. 3**). These results were consistent with those of our ZIC-HILIC analysis. Huang et al. reported that CS-0S and CS-4S were the major GAG derivatives in human plasma [20]. Although human serum was treated similarly to samples prepared from cells, the peaks derived from HS were barely detectable. Together, these results demonstrate that HPLC using a RP-ADME-HR column is applicable for the qualitative and quantitative analysis of GAGs in various biological samples, such as cultured cells, cell culture medium, and serum. The work flow for GAG analysis of the various biological

199 samples is summarized in **Fig. 4.**

200

201 **4. Conclusion**

202 In this study, we assessed various RP-column for separation of 2-AB labeled GAGs
203 and established a HPLC method for the determination of GAG disaccharides using a
204 column containing adamantyl groups. The RP-ADME-HR column achieved quantitative
205 separation of GAGs derived from HS, CS/DS, and HA in a single analysis. In addition,
206 this analysis time was faster and achieved higher retention time reproducibility than ZIC-
207 HILIC column. Combined with ethanol precipitation, our RP-HPLC method was used
208 successfully for analysis of GAGs derived from various biological samples. RP-HPLC-
209 ESI-MS was reported for the analysis of CS/DS and HA disaccharides derivatized with
210 2-aminoacridone [10]. Yang et al. reported the analysis of 2-aminoacridone-tagged
211 CS/HS/HA disaccharides of GAGs using RP-UPLC-ESI-MS, which revealed the
212 composition and structures of GAGs in various biological samples, such as cells and
213 tissues [21]. The novel method using RP-ADME-HR column could easily be adapted to
214 LC/MS analysis when used with 10 mM ammonium acetate and it will be easily
215 applicable in the near future. GAG analysis by ADME-HR column is a powerful tool for
216 the discovery of diagnostic markers, and for understanding the roles of

glycosaminoglycans in various biological physiological processes.

Author contributions

H.H. designed the experimental plan. H.H., M.O., and S.M, performed the experiments. H.H, and I.Y. analyzed and interpreted the data. J-i.F., S.N., G.O., and N.S. conceived the study and provided funding. H.H. and J-i.F. drafted the manuscript. All authors critically reviewed the manuscript in its final form.

CRediT authorship contribution statement

Hisatoshi Hanamatsu: Investigation, Methodology, Formal analysis, Writing - original draft, Writing- review & editing. **Satoshi Makino**: Methodology, Formal analysis, Writing - review & editing. **Masatsugu Ohara**: Formal analysis, Writing – review, & editing. **Goki Suda**: Writing - review & editing. **Ikuko Yokota**: Formal analysis. **Shoko Nishihara**: Writing – review & editing, Funding acquisition. **Naoya Sakamoto**: Writing – review & editing, Funding acquisition. **Jun-ichi Furukawa**: Conceptualization, Writing - review & editing, Formal analysis, Funding acquisition.

Acknowledgements

We thank Dr. Utsugi and Fujie from Osaka Soda Co., Ltd. for valuable comments and discussion. This work was supported in part by JSPS KAKENHI (Grant Number 22H03502, J-i.F.) and AMED (Grant Number JP22ek0109446, J-i.F and S.N.; JP22fk0210072, J-i.F. and N.S.)

Funding

Competing interests

Table 1. List of GAG disaccharides

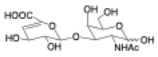
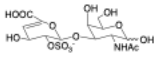
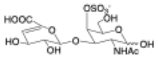
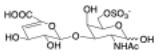
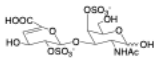
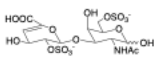
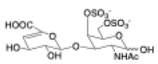
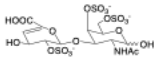
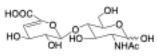
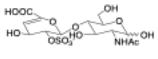
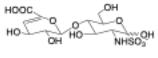
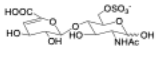
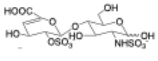
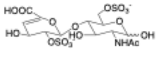
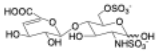
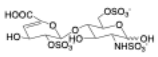
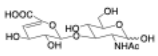
Disaccaride type	Glycan structure	Theoretical mass	Retention time %RSD	Area %RSD
CS-0S		379.3	0.47	3.53
CS-2S		459.4	0.77	4.05
CS-4S		459.4	0.78	5.62
CS-6S		459.4	0.82	4.69
CS-2S4S		539.4	0.71	5.17
CS-2S6S		539.4	0.91	5.48
CS-4S6S		539.4	0.90	5.35
CS-2S4S6S		619.5	0.86	8.07
HS-0S		379.3	0.57	5.04
HS-2S		459.4	0.85	5.39
HS-NS		417.3	1.08	7.20
HS-6S		459.4	0.84	4.95
HS-2SNS		497.4	2.84	4.87
HS-2S6S		539.4	0.95	5.94
HS-NS6S		497.4	2.91	4.38
HS-2SNS6S		577.5	3.19	4.58
HA		379.3	0.60	6.03

Figure 1

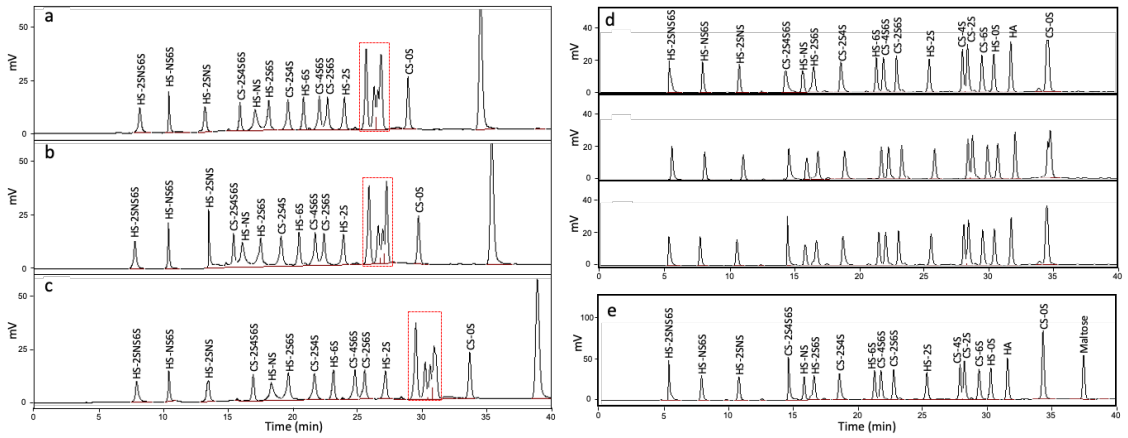


Figure 1. Separation of 2-AB labeled GAG disaccharide standards by ODS and ADME-HR columns. The liquid chromatogram from an ODS column using the following gradients: (A) Solvent A/ Solvent B = 100/0 (0 min) → 60/40 (40 min); (B) Solvent A/ Solvent B = 100/0 (0 min) → 65/35 (40 min); (C) Solvent A/ Solvent B = 100/0 (0 min) → 60/30 (40 min). The liquid chromatograms from an ADME-HR column using the following gradient: (D) Solvent A/ Solvent B = 100/0 (0 min) → 60/30 (40 min). (E) The same gradient as (D) but with maltose added.

Figure 2

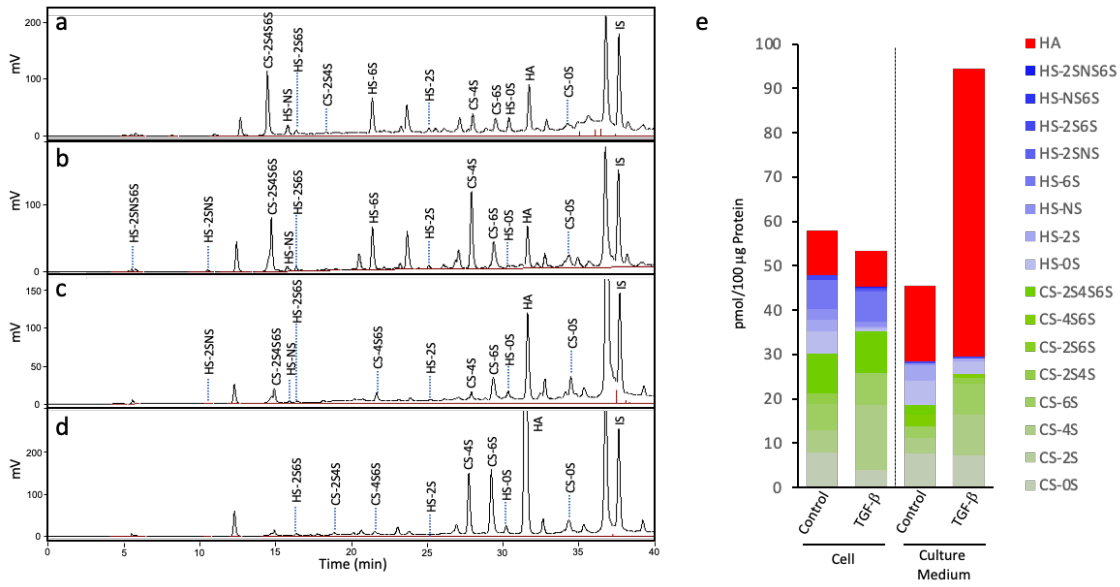


Figure 2. GAG analysis of LX-2 cells and its culture medium by ADME-HR column.

The liquid chromatograms of GAG in (a) LX-2 cells and (b) TGF-β-treated cells. The liquid chromatograms of GAG in (c) LX-2 cell culture medium and (d) TGF-β treated cell culture medium. e) Absolute quantification of detected GAG disaccharides. Absolute quantification was performed by comparative analyses between the areas of the HPLC signal derived from each GAG disaccharides and the internal standard (maltose).

Figure 3

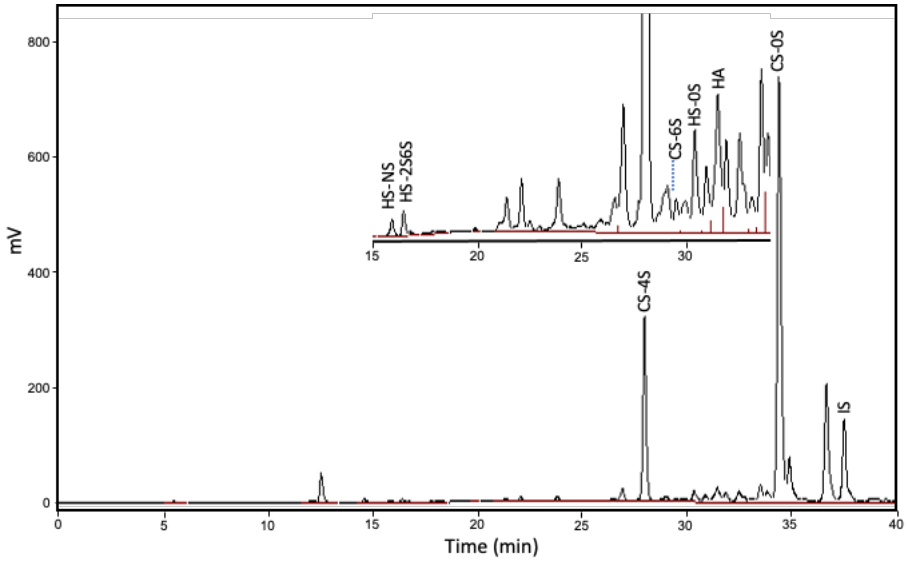
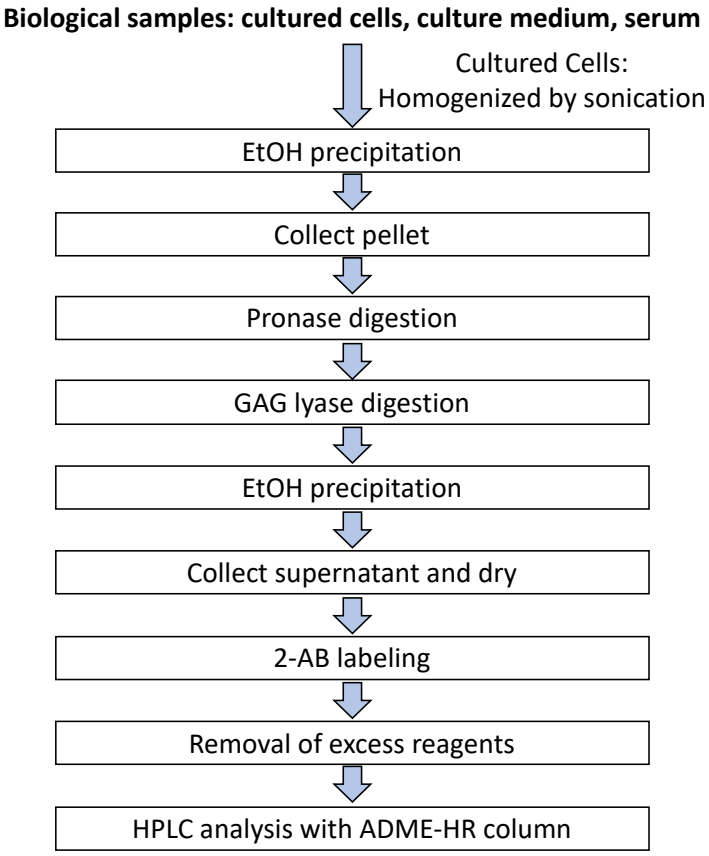


Figure 3. Human serum GAG analysis by ADME-HR column.

The liquid chromatograms of GAG in human serum with an ADME-HR column. 20 pmol of maltose is added as internal standard (IS).



286 **Figure 4. The experimental work flow for GAG analysis derived from various**

287 **biological samples.**

288

289

290

291

References

[1] A. Varki, R.D. Cummings, J.D. Esko, P. Stanley, G.W. Hart, M. Aebi, D. Mohnen, T. Kinoshita, N.H. Packer, J.H. Prestegard, R.L. Schnaar, P.H. Seeberger, Essentials of Glycobiology, 2022. https://doi.org/NBK579918.

[2] R.A. Smith, K. Meade, C.E. Pickford, R.J. Holley, C.L. Merry, Glycosaminoglycans as regulators of stem cell differentiation, Biochem Soc Trans 39(1) (2011) 383-7. https://doi.org/10.1042/BST0390383.

[3] A. Purushothaman, K. Sugahara, A. Faissner, Chondroitin sulfate "wobble motifs" modulate maintenance and differentiation of neural stem cells and their progeny, J Biol Chem 287(5) (2012) 2935-42. https://doi.org/10.1074/jbc.R111.298430.

[4] S. Hatano, H. Watanabe, Regulation of Macrophage and Dendritic Cell Function by Chondroitin Sulfate in Innate to Antigen-Specific Adaptive Immunity, Front Immunol 11 (2020) 232. https://doi.org/10.3389/fimmu.2020.00232.

[5] T.M. Clausen, D.R. Sandoval, C.B. Spliid, J. Pihl, H.R. Perrett, C.D. Painter, A. Narayanan, S.A. Majowicz, E.M. Kwong, R.N. McVicar, B.E. Thacker, C.A. Glass, Z. Yang, J.L. Torres, G.J. Golden, P.L. Bartels, R.N. Porell, A.F. Garretson, L. Laubach, J. Feldman, X. Yin, Y. Pu, B.M. Hauser, T.M. Caradonna, B.P. Kellman, C. Martino, P.L.S.M. Gordts, S.K. Chanda, A.G. Schmidt, K. Godula, S.L. Leibel, J. Jose, K.D. Corbett, A.B. Ward, A.F. Carlin, J.D. Esko, SARS-CoV-2 Infection Depends on Cellular Heparan Sulfate and ACE2, Cell 183(4) (2020) 1043-1057.e15. https://doi.org/10.1016/j.cell.2020.09.033.

[6] J. Ling, J. Li, A. Khan, Å. Lundkvist, J.P. Li, Is heparan sulfate a target for inhibition of RNA virus infection?, Am J Physiol Cell Physiol 322(4) (2022) C605-C613. https://doi.org/10.1152/ajpcell.00028.2022.

[7] J. Wei, M. Hu, K. Huang, S. Lin, H. Du, Roles of Proteoglycans and Glycosaminoglycans in Cancer Development and Progression, Int J Mol Sci 21(17) (2020). https://doi.org/10.3390/ijms21175983.

[8] N. Volpi, M. Cusmano, T. Venturelli, Qualitative and quantitative studies of heparin and chondroitin sulfates in normal human plasma, Biochim Biophys Acta 1243(1) (1995) 49-58. https://doi.org/10.1016/0304-4165(94)00123-f.

[9] M. Ambrosius, K. Kleesiek, C. Götting, Quantitative determination of the glycosaminoglycan Delta-disaccharide composition of serum, platelets and granulocytes by reversed-phase high-performance liquid chromatography, J Chromatogr A 1201(1) (2008) 54-60. https://doi.org/10.1016/j.chroma.2008.06.007.

[10] N. Volpi, High-performance liquid chromatography and on-line mass spectrometry detection for the analysis of chondroitin sulfates/hyaluronan disaccharides derivatized with 2-

aminoacridone, Anal Biochem 397(1) (2010) 12-23. https://doi.org/10.1016/j.ab.2009.09.030.
[11] Y. Takegawa, K. Araki, N. Fujitani, J. Furukawa, H. Sugiyama, H. Sakai, Y. Shinohara, Simultaneous analysis of heparan sulfate, chondroitin/dermatan sulfates, and hyaluronan disaccharides by glycoblotting-assisted sample preparation followed by single-step zwitter-ionic-hydrophilic interaction chromatography, Anal Chem 83(24) (2011) 9443-9. https://doi.org/10.1021/ac2021079.
[12] J.P. Carson, M.W. Robinson, G.A. Ramm, G.N. Gobert, RNA sequencing of LX-2 cells treated with TGF-β1 identifies genes associated with hepatic stellate cell activation, Mol Biol Rep 48(12) (2021) 7677-7688. https://doi.org/10.1007/s11033-021-06774-3.
[13] M. Ohara, S. Ohnishi, H. Hosono, K. Yamamoto, Q. Fu, O. Maehara, G. Suda, N. Sakamoto, Palmitoylethanolamide Ameliorates Carbon Tetrachloride-Induced Liver Fibrosis in Rats, Front Pharmacol 9 (2018) 709. https://doi.org/10.3389/fphar.2018.00709.
[14] F. Pecori, H. Hanamatsu, J.I. Furukawa, S. Nishihara, Comprehensive and Comparative Structural Glycome Analysis in Mouse Epiblast-like Cells, Methods Mol Biol 2490 (2022) 179-193. https://doi.org/10.1007/978-1-0716-2281-0_13.
[15] A. Kameyama, S.K. Dissanayake, W.W. Thet Tin, Rapid chemical de-N-glycosylation and derivatization for liquid chromatography of immunoglobulin N-linked glycans, PLoS One 13(5) (2018) e0196800. https://doi.org/10.1371/journal.pone.0196800.
[16] L.R. Ruhaak, E. Steenvoorden, C.A. Koeleman, A.M. Deelder, M. Wuhrer, 2-picoline-borane: a non-toxic reducing agent for oligosaccharide labeling by reductive amination, Proteomics 10(12) (2010) 2330-6. https://doi.org/10.1002/pmic.200900804.
[17] J.X. Jiang, X. Chen, D.K. Hsu, K. Baghy, N. Serizawa, F. Scott, Y. Takada, H. Fukada, J. Chen, S. Devaraj, R. Adamson, F.T. Liu, N.J. Török, Galectin-3 modulates phagocytosis-induced stellate cell activation and liver fibrosis in vivo, Am J Physiol Gastrointest Liver Physiol 302(4) (2012) G439-46. https://doi.org/10.1152/ajpgi.00257.2011.
[18] I. Koshiishi, M. Takenouchi, T. Imanari, Structural characteristics of oversulfated chondroitin/dermatan sulfates in the fibrous lesions of the liver with cirrhosis, Arch Biochem Biophys 370(2) (1999) 151-5. https://doi.org/10.1006/abbi.1999.1396.
[19] O.H. Orasan, G. Ciulei, A. Cozma, M. Sava, D.L. Dumitrascu, Hyaluronic acid as a biomarker of fibrosis in chronic liver diseases of different etiologies, Clujul Med 89(1) (2016) 24-31. https://doi.org/10.15386/cjmed-554.
[20] Y. Huang, H. Toyoda, T. Toida, T. Imanari, Determination of chondroitin sulphates in human whole blood, plasma and blood cells by high-performance liquid chromatography, Biomed Chromatogr 9(2) (1995) 102-5. https://doi.org/10.1002/bmc.1130090210.
[21] B. Yang, Y. Chang, A.M. Weyers, E. Sterner, R.J. Linhardt, Disaccharide analysis of glycosaminoglycan mixtures by ultra-high-performance liquid chromatography-mass

363 spectrometry, J Chromatogr A 1225 (2012) 91-8. https://doi.org/10.1016/j.chroma.2011.12.063.

364