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Acidolysis-based component mapping of glycosaminoglycans by reversed-phase high-performance liquid chromatography with off-line electrospray ionization–tandem mass spectrometry: Evidence and tags to distinguish different glycosaminoglycans

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ABSTRACT

Diverse monosaccharide analysis methods have been established for a long time, but few methods are available for a complete monosaccharide analysis of glycosaminoglycans (GAGs) and certain acidolysis-resistant components derived from GAGs. In this report, a reversed-phase high-performance liquid chromatography (RP–HPLC) method with pre-column 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization was established for a complete monosaccharide analysis of GAGs. Good separation of glucosamine/mannosamine (GlcN/ManN) and glucuronic acid/iduronic acid (GlcA/IdoA) was achieved. This method can also be applied to analyze the acidolysis-resistant disaccharides derived from GAGs, and the sequences of these disaccharides were confirmed by electrospray ionization–collision-induced dissociation–tandem mass spectrometry (ESI–CID–MS/MS). These unique disaccharides could be used as markers to distinguish heparin/heparan sulfate (HP/HS), chondroitin sulfate/dermatan sulfate (CS/DS), and hyaluronic acid (HA).

Glycosaminoglycans (GAGs)[1] are linear polyanion natural carbohydrates composed of repeating disaccharide units of a uronic acid (glucuronic acid or iduronic acid) and an amino sugar (glucosamine or galactosamine) [1]. GAGs are mainly classified as heparin/heparan sulfate (HP/HS), chondroitin sulfate/dermatan sulfate (CS/DS), or hyaluronic acid (HA) based on their monosaccharide compositions and glycosidic linkages [2,3], as shown in Fig. 1. GAGs play a vital role in various biological processes such as cell growth and adhesion [4], coagulation and thrombosis [5,6], tumorigenesis and metastasis [7,8], and inflammation [9] through interactions with different functional proteins. Various GAGs' functions are influenced by their monosaccharide composition, charge density, and molecular size [3,10]. The monosaccharide composition of GAGs plays a critical role in the specificity of their protein-binding activity; for example, basic fibroblast growth factor binds specifically to iduronic acid-containing GAGs [11–13]. The identification of these GAGs needs not only the analysis of the amino sugars [14] but also the confirmation of the uronic acids [15]. However, in a typical disaccharide composition analysis of GAGs, different uronic acids are converted to the same unsaturated uronic acid after being digested by GAG lyases [16,17], and the composition information about uronic acids is lost. Thus, it is necessary to develop a reliable and sensitive method for the monosaccharide profiling of GAGs.

Monosaccharide analysis of GAGs is conventionally performed by gas chromatography (GC) [18], capillary electrophoresis (CE) [19], high-performance anion exchange chromatography–pulsed amperometric detection (HPAEC–PAD) [15], or high-performance liquid chromatography (HPLC) [20], but so far a complete monosaccharide profiling can be performed only by HPAEC–PAD [15]. Nevertheless, HPAEC–PAD is limited by unstable baselines, high pH values, and high salt concentrations [21]. Moreover, all of the

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[1] *Abbreviations used:* GAG, glycosaminoglycan; HP/HS, heparin/heparan sulfate; CS/DS, chondroitin sulfate/dermatan sulfate; HA, hyaluronic acid; HPAEC–PAD, high-performance anion exchange chromatography–pulsed amperometric detection; HPLC, high-performance liquid chromatography; RP–HPLC, reversed-phase HPLC; PMP, 1-phenyl-3-methyl-5-pyrazolone; ESI–CID–MS/MS, electrospray ionization–collision-induced dissociation–tandem mass spectrometry; Man, mannose; Glc, glucose; Gal, galactose; Xyl, xylose; GlcN, glucosamine; ManN, mannosamine; GalN, galactosamine; GlcA, glucuronic acid; GalA, galacturonic acid; IdoA, iduronic acid; ES, enoxaparin sodium; TFA, trifluoroacetic acid; PBS, phosphate buffer solution; OSCS, oversulfated CS.

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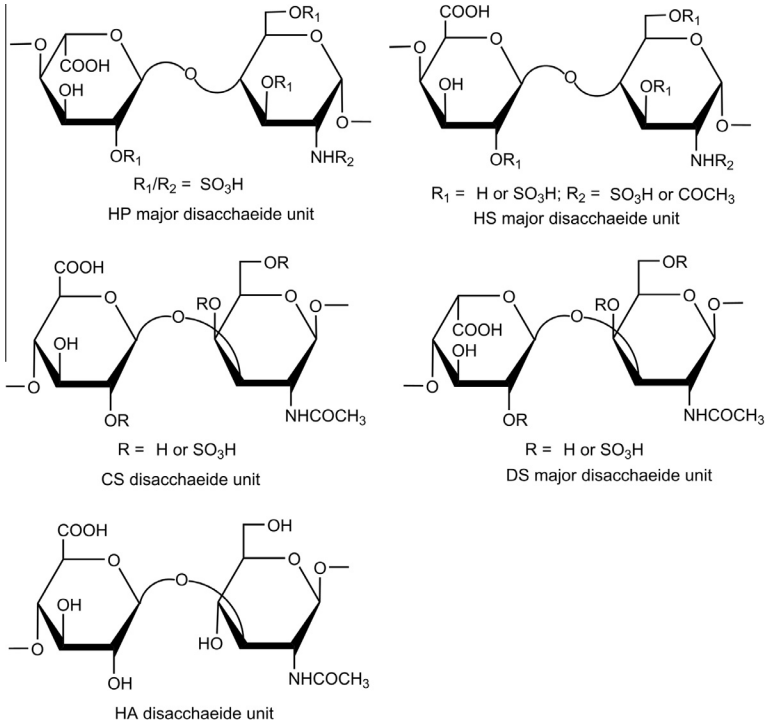

Fig.1. Structures of heparin (HP), heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), and hyaluronic acid (HA).

analysis methods listed above analyze only the monosaccharides, and little emphasis is placed on the analysis of acidolysis-resistant components of GAGs [15,22].

In this work, we have established an acidolysis component mapping of GAGs by a reversed-phase HPLC (RP–HPLC) method with pre-column 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization and gradient elution. The common monosaccharides in GAGs are identified, and five acidolysis-resistant disaccharides derived from GAGs are separated by RP–HPLC and analyzed by positive electrospray ionization–collision-induced dissociation–tandem mass spectrometry (ESI–CID–MS/MS).

Materials and methods

Materials

Monosaccharide standards—including mannose (Man), glucose (Glc), galactose (Gal), xylose (Xyl), glucosamine (GlcN) hydrochloride, mannosamine (ManN) hydrochloride, galactosamine (GalN) hydrochloride, glucuronic acid (GlcA), and galacturonic acid (GalA)—were purchased from Sigma–Aldrich (St. Louis, MO, USA). Iduronic acid (IdoA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HP from porcine intestinal mucosa, HS from bovine kidney, DS from porcine intestinal mucosa, CS from shark cartilage, and HA from rooster comb were purchased from Sigma–Aldrich. Enoxaparin sodium (ES) standard (lot no. FOI110) was purchased from Adhoc International Technologies (Beijing, China). PMP was purchased from Alfa Aesar (Ward Hill, MA, USA). All of the other chemicals and reagents were of chromatographic grade.

Hydrolysis of GAGs

Each of the GAGs was put into an ampoule, dissolved in 3 M trifluoroacetic acid (TFA) to obtain a concentration of 400 μg/ml, sealed and incubated in a 110 °C oven for 1, 2, 3, 4, 5, 6, 8, and 10 h, respectively. The hydrolysates were dried using a Büchi R-3 rotary vacuum evaporator (Flawil, Switzerland) to remove the excess TFA. Then, the hydrolysates were redissolved in 50 μl of H_2O and adjusted to pH 7.0 with 0.3 M NaOH solution.

PMP labeling

The derivation process was carried out as described previously [20]. Briefly, the GAG hydrolysates or the mixture of monosaccharide standard solutions (30 μl) were diluted to 50 μl with water, and 100 μl of 0.3 M NaOH and 120 μl of 0.5 M PMP in methanol were added, followed by incubation at 70 °C for 1 h. The reaction mixture was neutralized with 3 M HCl after it was cooled to room temperature and was then extracted with chloroform (700 μl each) 5 times. The collected supernatant solution was filtered through a 0.22-μm membrane filter and preserved at −20 °C.

RP–HPLC conditions

The PMP-labeled saccharides were separated on an Agilent 1260 LC system equipped with a ZORBAX Eclipse XDB-C18 guard column (4.6 × 10 mm, 5 μm, Agilent) and a ZORBAX Eclipse XDB-C18 analytical column (4.6 × 150 mm, 5 μm, Agilent) at a constant flow rate of 1.0 ml/min, and the column temperature was maintained at 30 °C. Solvent A was composed of 0.1 M phosphate buffer solution (PBS, pH 6.0), and solvent B was composed of 100% CH_3CN. The elution program is shown in Table 1.

Table 1
Elution program for PMP-labeled monosaccharide separation.

Time (min)	Solution A (%)	Solution B (%)	Elution
0–25	85	15	Isocratic
25–26	85 → 83	15 → 17	Linear
26–50	83	17	Isocratic
50–51	83 → 85	17 → 15	Linear
51–60	85	15	Isocratic

Desalting of PMP-labeled sugars after RP–HPLC separation

A Sep-Park classic C18 column (WAT051910, Waters, Milford, MA, USA) was pretreated as described in the product directions and equilibrated with 1% aqueous methanol before use. The PMP-labeled sugars collected from RP–HPLC were dried and redissolved in 100 μl of 1% methanol. The sample was loaded onto the column and eluted with 10 column volumes of 1% methanol to remove salts, and then it was washed with 6 column volumes of 100% methanol to obtain the desalted sugar derivative. The desalted sugar derivative was vacuum-dried and preserved at −20 °C.

ESI–MS analysis

Experiments were performed on a Thermo Scientific LTQ Orbitrap XL hybrid Fourier transform mass spectrometer (FTMS) equipped with an ESI source. The sheath gas flow rate was set at 8 arb. The capillary temperature was 275 °C. The spray voltage, capillary voltage, and tube lens were maintained at 3 kV, 17 V, and 80 V, respectively. The mobile phase was 50% CH_3CN at a flow rate of 8 μl/min. All of the samples were dissolved in mobile phase before injection. For CID–MS/MS analysis, the collision energy was adjusted to 20 eV.

Deamination reaction

The hydrolysate (5 μl) was transferred to a 1.5-ml glass tube and mixed with 5 μl of H_2O. Then, 5 μl of 1.5 M H_2SO_4 and 5 μl of 5.5 M $NaNO_2$ were added. After 0.5 min, the reaction was stopped with 10 μl of 1.5 M Na_2CO_3 [23]. The mixture was filtered with a 0.22-μm centrifuge tube filter, and the filtrate was preserved at −20 °C.

HPAEC conditions

The HPAEC analysis was performed as described previously by Zhang and coworkers [15] with some modifications. Experiments were performed on a Dionex ICS 3000 system (Dionex, Sunnyvale, CA, USA) with a Carbopac PA 20 guard column (3 × 30 mm, Dionex) and a Carbopac PA 20 analytical column (3 × 150 mm, Dionex). The temperature and flow rate were set at 30 °C and 0.4 ml/min, respectively. A two-step isocratic elution process was used, including 15 mM NaOH for 10 min and then 15 mM NaOH–150 mM NaOAc for another 10 min. The entire process was monitored by PAD (Au working electrode and Ag/AgCl reference electrode) using the Dionex default program of carbohydrate quadruple waveform.

Results and discussion

Optimization of RP–HPLC conditions

The PMP labeling technique, reported by Pitt and Gorman [24], has been widely used for carbohydrate analysis due to its mild reaction conditions and simple cleanup procedure. PMP-labeled monosaccharides were satisfactorily separated by 17% CH_3CN in 0.1 M PBS (pH 6.7) in our laboratory's previous work [20,25]. However, ManN–PMP and IdoA–PMP had identical retention times with GlcN–PMP and GlcA–PMP, respectively, under the same chromatographic conditions. Hence, several experiments were performed to optimize the separation conditions of PMP-labeled Gal, Glc, Man, Xyl, GalN, GlcN, ManN, GalA, GlcA, and IdoA.

The separation conditions of GlcN–PMP and ManN–PMP were first optimized by 0.1 M PBS with different pH values, as shown in Fig. S1 of the online supplementary material. They were eluted faster at higher pH values; the elution times went from 14 min at pH 6.0 to 3.5 min at pH 8.0. The resolutions of GlcN–PMP and ManN–PMP at pH values of 6.0 and 7.2 were above 1.0. As shown in Figs. S2 and S3, the higher the percentage of CH_3CN, the worse the resolution of GlcN–PMP and ManN–PMP. The resolutions of GlcN–PMP and ManN–PMP at 15% CH_3CN and 14% CH_3CN were both above 1.5, but the derivatives were eluted faster at 15% CH_3CN. Therefore, the optimal mobile phase was determined to be 15% (v/v) CH_3CN in 0.1 M PBS (pH 6.0). The separation of GlcA–PMP and IdoA–PMP was optimized using five elution conditions (see Table S1 in supplementary material), as shown in Fig. S5, and the resolution of GlcA–PMP and IdoA–PMP under the second set of elution conditions was the best.

Based on the above conditions, the following two-step isocratic gradient was selected to separate 10 PMP-labeled monosaccharides: 15% CH_3CN in 0.1 M PBS (pH 6.0) to separate PMP-labeled Man, GlcN, and ManN and 17% CH_3CN in 0.1 M PBS (pH 6.0) to separate PMP-labeled GalN, GlcA, IdoA, GalA, Glc, Gal, and Xyl subsequently. These monosaccharide derivatives were well separated in 60 min (Fig. 2A).

Mapping of PMP-labeled acidolysis components of GAGs

The composition profiles of HP, HS, and ES (low-molecular-weight heparin prepared by a β-elimination method) after hydrolysis at 110 °C for 3 h are shown in Fig. 2B–D, and GlcN, GlcA, and IdoA were observed as expected. The GalN was from DS, which was a common contaminant in HP/HS [26], and Gal and a little Xyl were from the tetrasaccharide linker [27,28]. Both the repeating disaccharide units of HP/HS and DS contain IdoA, so the IdoA in Fig. 2B–C may be derived from HP/HS or DS. In addition, ManN was found in ES, which was derived from GlcN by base-catalyzed C-2 epimerization [29] during the preparation process, making it distinguishable from HP/HS. The peak areas of the monosaccharides were calibrated using linear equations (Fig. S6). The monosaccharide compositions were calculated and are listed in Table 2. On the whole, the ratio of uronic acids to GlcN was not detected as 1:1, which may be due to the degradation of uronic acids and also the incomplete hydrolysis of these GAGs. Two unknown peaks in HP, HS and ES (a and b in Fig. 2B–D), were also observed between 28 and 30 min. The peak areas of a and b varied with the hydrolysis time, with component a exhibiting a maximum peak area at 8 h and component b exhibiting its maximum response at 3 h (Fig. 3A), indicating that peaks a and b corresponded to acidolysis-resistant components derived from HP/HS. To elucidate their structures, MS analysis was performed after desalting PMP-labeled components a and b with a C18 SPE column. The molecular mass of component b was 685 Da, validated by a mono-charged molecular ion in the positive (m/z 686) and negative (m/z 684) ESI–MS modes, allowing the conclusion that the structure of component b was a PMP-labeled disaccharide likely composed of one GlcN, one GlcA/IdoA, and two PMP according to the repeating unit of HP/HS. The positive ESI–CID–MS/MS spectrum (Fig. 4B) of the parent molecular ion (m/z 686) illustrated that the main product ions at m/z 668, 512, and 525 and 507 were assigned as the losses of H_2O, PMP, and glycosidic bond cleavages, respectively, showing the existence of GlcN at the nonreducing terminus. Component a also had a molecular mass of 685 Da and a similar structure to that of component b, as illustrated in Fig. 4A. However, the structure of the uronic acid at the reducing end of a and b could not be determined. A deamination reaction with nitrous acid [23] was a method used to aid in the hydrolysis of the disaccharide GlcN-IdoA/GlcA of HP/HS into free 2,5-anhydro mannose and IdoA/GlcA (Fig. S7), which can be separated and determined by HPAEC [15]. The peak areas of GlcA and IdoA in the HP hydrolysate after the deamination reaction increased by

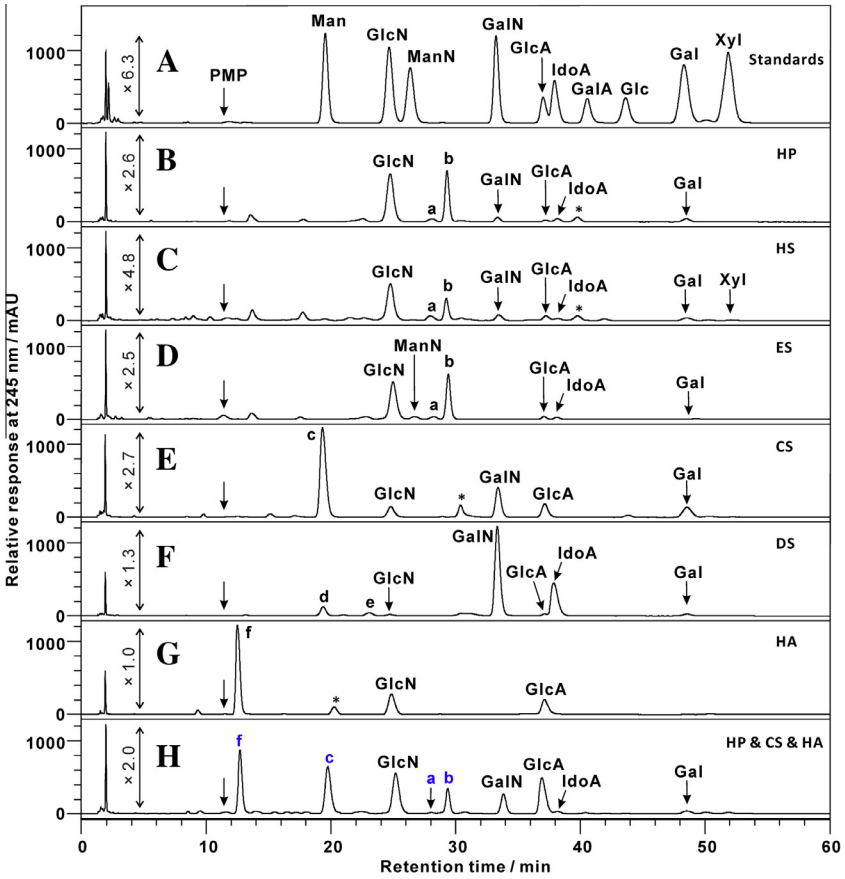

Fig.2. Chromatograms of the acid hydrolysates of GAGs that were hydrolyzed in 3 M TFA for 3 h at 110 °C to ensure a higher response value of the resistant disaccharide: (A) standards; (B) HP; (C) HS; (D) ES; (E) CS; (F) DS; (G) HA; (H) mixture of HP, CS, and HA. Peaks a, b, c, d, e, and f correspond to acidolysis-resistant disaccharides in GAGs. Peaks labeled with an asterisk (*) correspond to unknown components.

Table 2
Monosaccharide compositions of different GAGs after hydrolysis at 110 °C for 3 h.

Monosaccharide	Composition (mol%)[a]					
	HP	HS	ES	CS	DS	HA
GlcN	79.9	67.8	82.8	11.7	1.6	47.0
ManN			2.4			
GalN	5.1	8.2		30.7	64.3	
GlcA	0.6	3.7	4.7	45.9	0.5	53.0
IdoA	8.9	6.4	8.5		32.1	
Gal	5.4	4.8	1.6	11.8	1.5	
Xyl		9.1				

[a] The monosaccharide compositions of GAGs are calculated based on the linear equations of monosaccharide standards, as shown in Fig. S6 of the supplementary material.

0.47 fold and 4.05 fold separately compared with those before the deamination reaction. This increase indicated that IdoA-containing resistant disaccharide was the major unhydrolyzed component in the HP/HS/ES hydrolysate. Considering that component b was the major resistant product illustrated in Fig. 2B–D and component a was more stable than component b during the acid hydrolysis process (Fig. 3A), the structures of the resistant disaccharides a and b were identified as GlcN-GlcA and GlcN-IdoA, respectively. In view of the known structure of HP/HS disaccharide units, the glycosidic bond should be α1 → 4 between GlcN and GlcA/IdoA.

Similarly, the composition profiles of other GAGs—including CS, DS, and HA—after hydrolysis at 110 °C for 3 h were analyzed, as shown in Fig. 2E–G. GalN and GlcA/IdoA were the major components in CS and DS, whereas GlcN and GlcA were the major components in HA. Gal from the tetrasaccharide linker [30] and a little GlcN were observed in CS/DS, suggesting the presence of a GlcN-containing impurity. The monosaccharide compositions of HA were consistent with the structural information of HA, as shown in Table 2. However, the GalN content did not match that of uronic acids in CS and DS, and the degradation of uronic acids and the partial acidolysis may account for this inconsistency. In addition, components c and d (~ 20 min), e (~ 23 min), and f (~13 min) were observed in Fig. 2E–G, and their peak areas decreased with the hydrolysis time (Fig. 3B), indicating that the hydrolysis patterns of CS/DS and HA were different from that of HP/HS. The molecular mass of all the components (c, d, e, and f) is 685 Da, as determined by ESI–MS, showing that these components were PMP-labeled disaccharides. The positive ESI–CID–MS/MS spectra (Fig. 4C–F) of the parent molecular ion (*m*/*z* 686) illustrated that these disaccharide derivatives had the same fragment ions, and the disaccharide section was composed of a uronic acid at the nonreducing end and an amino sugar at the reducing end. Considering that these disaccharides were derived from different GAG types and DS contains a minor GlcA, as shown in Fig. 2E and previous work [31], disaccharides c and d were identified as the same structure of GlcA-GalN from CS and disaccharides e and f were identified as IdoA-GalN from DS and GlcA-GlcN from HA, respectively. The glycosidic bonds should be β1 → 3 between GlcA and GalN/GlcN and α1 → 3 between IdoA and GalN, according to the disaccharide units of CS, HA, and DS.

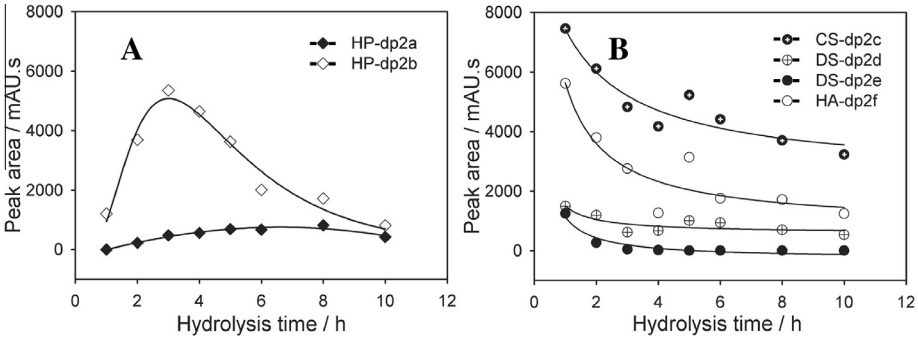

Fig.3. Effect of the hydrolysis time on the peak areas of PMP-labeled acidolysis-resistant disaccharides from GAGs: (A) HP disaccharides a and b; (B) CS disaccharide c, DS disaccharides d and e, and HA disaccharide f.

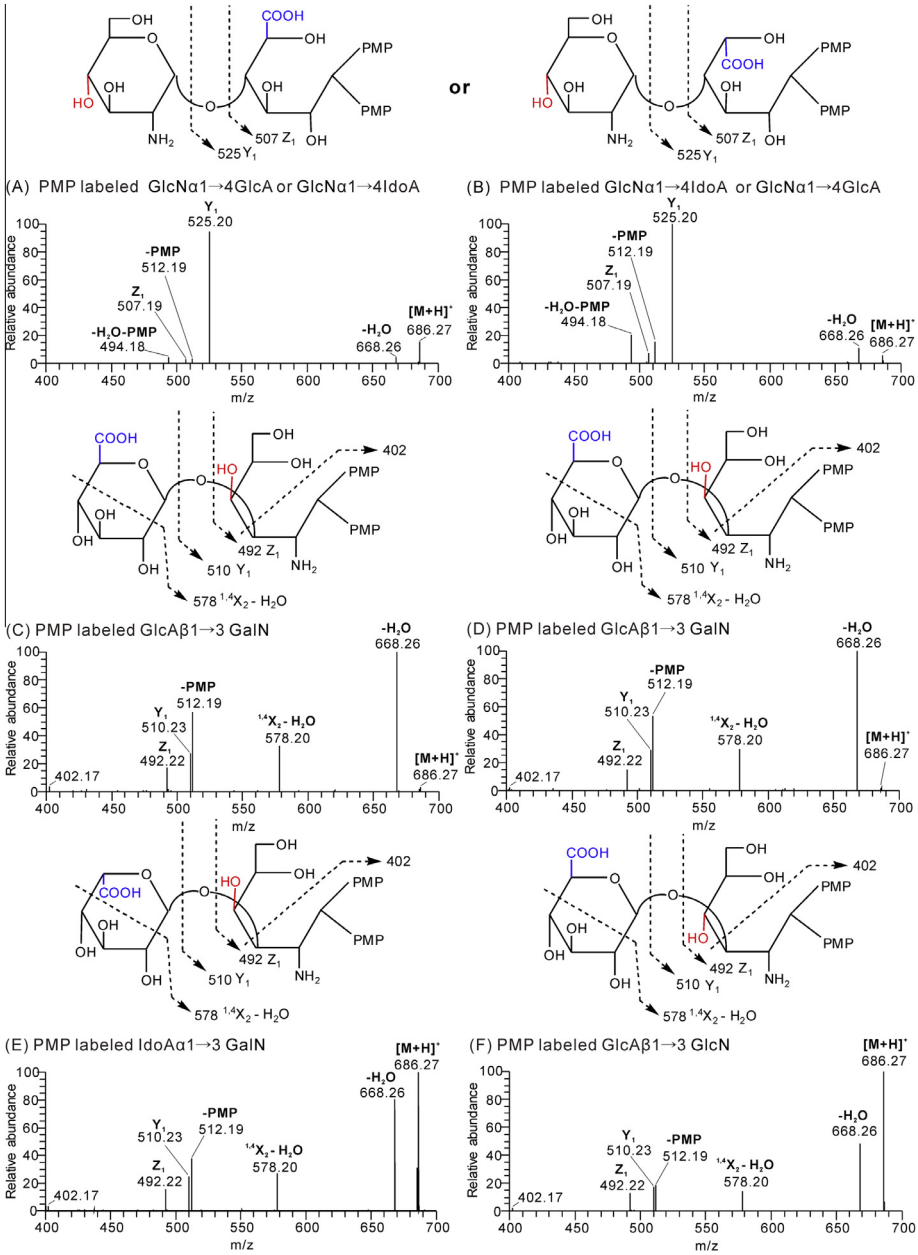

Fig.4. Positive ESI–CID–MS/MS spectra of PMP-labeled acidolysis-resistant disaccharides in GAGs: (A) HP disaccharide a; (B) HP disaccharide b; (C) CS disaccharide c; (D) DS disaccharide d; (E) DS disaccharide e; (F) HA disaccharide f.

Table 3
Structures of acidolysis-resistant disaccharides derived from HP/HS, CS/DS, and HA.

GAG	Disaccharide unit	Resistant disaccharides	
		Retention time[a] (min)	Structure[b]
HP/HS	-4IdoAα/GlcAβ1 → 4GlcNα1-	28.5	GlcNα1 → 4GlcA
		29.5	GlcNα1 → 4IdoA
CS	-4GlcAβ1 → 3GalNβ1-	20.0	GlcAβ1 → 3GalN
DS	-4GlcAβ1 → 3GalNβ1- (minor)	20.0	GlcAβ1 → 3GalN
	-4IdoAα1 → 3GalNβ1- (major)	23.0	IdoAα1 → 3GalN
HA	-4GlcAβ1 → 3GlcNβ1-	13.0	GlcAβ1 → 3GlcN

[a] PMP-labeled disaccharides.
[b] The glycosidic bond is determined based on the structure of disaccharide units.

These resistant disaccharides can be classified into two categories, as shown in Table 3; one is composed of an amino sugar (A) at the nonreducing end and a uronic acid (U) at the reducing end (A1 → 4U), and the other is composed of a uronic acid at the nonreducing end and an amino sugar at the reducing end (U1 → 3A). The acid stability of those disaccharides may result from the acid resistance of glycosidic linkages. For HP/HS, the 1 → 4 linkage between GlcN and IdoA/GlcA is more stable to acid hydrolysis than the 1 → 4 linkage between IdoA/GlcA and GlcN. However, for CS/DS and HA, the 1 → 4 linkage between GalN/GlcN and GlcA/IdoA is more labile to acid hydrolysis than the 1 → 3 linkage between GlcA/IdoA and GalN/GlcN.

In addition, three peaks—b, c, and f—corresponding to resistant disaccharides were observed in the component mapping of GAG mixtures after hydrolysis at 110 °C for 3 h (Fig. 2H). The three disaccharides could be used as tags to distinguish HP, CS, and HA, as shown in Table 3. The signal-to-noise (S/N) ratios of acidolysis-resistant disaccharide derivatives for HP, CS, and HA at 2.6 ng/μl were 10 to 16 in this study, indicating that an RP–HPLC method with pre-column PMP derivation has good sensitivity for detecting GAGs. In previous reports, different GAGs were usually confirmed by disaccharide analysis [16,17], but those methods needed specific enzymes and expensive standards. In contrast, the RP–HPLC approach is more accessible to distinguish diverse GAGs due to simple handling and low cost. OSCS is an oversulfated CS [32] and can generate the same acidolysis-resistant disaccharide as CS during the acidolysis process. Therefore, this method may have a potential application in detecting OSCS in contaminated heparin.

Conclusions

The RP–HPLC method with pre-column PMP derivatization has been established for a complete monosaccharide analysis of GAGs, especially for the good separation of GlcN/ManN and GlcA/IdoA. This method can also be applied to analyze the acidolysis-resistant disaccharides derived from HP/HS, CS/DS, and HA. The resistant disaccharides are identified as GlcNα1 → 4GlcA (a), GlcNα1 → 4IdoA (b), GlcAβ1 → 3GalN (c/d), IdoAα1 → 3GalN (e), and GlcAβ1 → 3GlcN (f) based on positive ESI–CID–MS/MS analysis and the disaccharide unit structures of GAGs. More important, the disaccharides b, c, and f can be used as tags to distinguish HP/HS, CS/DS, and HA.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2014.07.021.

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