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Disaccharide Analysis of Heparin and Heparan Sulfate Using Deaminative Cleavage with Nitrous Acid and Subsequent Labeling with Paranitrophenyl Hydrazine

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Compositional analyses of heparin (Hep) and heparan sulfate (HS) have been undertaken with disaccharide units obtained by either enzymatic digestion with heparitinases or hydrazinolysis/deamination reaction of polysaccharides. Unsaturated disaccharide units generated by the enzymatic method are detectable on HPLC with a uv detector recording absorbance at 230 nm. On the other hand, disaccharide units generated by the chemical method possess a component of 2,5-anhydromannose (AnMan) bearing aldehyde groups in addition to intact iduronic acid (IdoA) or glucuronic acid (GlcA). The aldehyde groups of the disaccharide units are usually reduced with sodium borotritide, and detected by radiochromatography. Both of them, however, involve inevitable experimental problems, such as the use of costly enzymes and radioisotopes. In the present study, we have established a novel composition analysis system for Hep and HS essentially based on the chemical method. After hydrazinolysis/deamination treatment of Hep and HS, the aldehyde groups of AnMan in the disaccharide units generated were coupled with paranitrophenyl (PNP-) hydrazine instead of reduction with sodium borotritide, AnMan-CH=N-NH-PNP (AnMan-PNP) being formed. Then, the PNP-labeled disaccharides were pre-treated on a SepPak C-18 cartridge column, and subsequently separated and detected on ion-pairing reversed-phase HPLC with a detector recording absorbance at 390 nm. With the present system, the order of elution was GlcA-AnMan-PNP (GM), IdoA-AnMan-PNP (IM), IdoA(2S)-AnMan-PNP (ISM), IdoA-AnMan(6S)-PNP (IMS), and IdoA(2S)-AnMan(6S)-PNP (ISMS). As an application, the disaccharide compositions of heparin from bovine intestine and heparan sulfate from bovine kidney were analyzed by the present method, and the results were comparable to those obtained by a well-established enzymatic method. The present compositional analysis was demonstrated to be reliable and economical.

Key words: disaccharide analysis, heparan sulfate, heparin, ion-pairing reversed-phase HPLC, paranitrophenyl hydrazine-labeling.

Heparin (Hep) is a well-known glycosaminoglycans (GAGs) and has been used as an anticoagulant for over fifty years, and its biosynthesis is exclusively limited to mast cells. On the other hand, heparan sulfate (HS) is widely distributed in animal tissues. Although the biological roles of Hep and HS are highly diversified, it is believed that their biological

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Abbreviations: AnMan, 2,5-anhydromannose; AnMan-PNP, AnMan-CH=N-NH-PNP; $AnMan_R$, reduced AnMan; ⊿DiHS, unsaturated disaccharide unit from heparin and heparan sulfate; ⊿DiHS-0S, 2-acetamide-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-enopyranosyluronic acid)-D-glucose; ⊿DiHS-NS, 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; ⊿DiHS-6S, 2-acetamide-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose; ⊿DiHS-US, 2-acetamide-2-deoxy-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; ⊿DiHS-(6,N)S, 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose; ⊿DiHS-(U, N)S, 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; ⊿DiHS-(U, 6)S, 2-acetamide-2-deoxy-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose; ⊿DiHS-(U, 6, N)S, 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose; GlcA, glucuronic acid; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; GlcNS, *N*-sulfoglucosamine; GlcNS(6S), 6-*O*-sulfated GlcNS; GM, GlcA-AnMan-PNP; GMS, GlcA-AnMan(6S)-PNP; GMS_2, GlcA-AnMan(3, $6S_2$)-PNP; GSM, GlcA(2S)-AnMan-PNP; GSMS, GlcA(2S)-AnMan(6S)-PNP; Hep, heparin; HS, heparan sulfate; IdoA, iduronic acid; IdoA(2S), 2-*O*-sulfated IdoA; IM, IdoA-AnMan-PNP; IMS, IdoA-AnMan(6S)-PNP; IPRP-HPLC, ion-pairing reversed-phase HPLC; ISM, IdoA(2S)-AnMan-PNP; ISMS, IdoA(2S)-AnMan(6S)-PNP; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride; PNP-hydrazine, paranitrophenyl hydrazine; SAX-HPLC, strong anion exchange HPLC.

functions mostly depend upon interaction between polysaccharides and physiologically active molecules. For example, they are capable of attaching themselves to lipoprotein lipase (*1, 2*), antithrombin III (ATIII; *3, 4*), fibroblast growth factor (FGF; *5–8*), *etc.*

Disaccharide analyses of Hep and HS have been performed by means of strong anion exchange (SAX-) HPLC after digestion with a combination of heparitinase I, heparitinase II, and heparinase, affording unsaturated disaccharide units (*9, 10*). This technique enables the complete separation and quantitation of eight unsaturated disaccharide units as sharp peaks on SAX-HPLC, although a disaccharide unit containing *N*-sulfoglucosamine (GlcNS) bearing a 3-*O*-sulfate group is not detectable, which is a component of the ATIII binding domain of Hep (*11*). The enzymatic digestion of Hep and HS cleaves glycosidic bonds between the *N*-acetylglucosamine (GlcNAc) or GlcNS residue and the iduronic acid (IdoA) or glucuronic acid (GlcA) residue, resulting in disappearance of uronic acid because of the generation of unsaturated bonds between C-4 and C-5 of both the uronic acid residues. Furthermore, the enzymatic analysis of Hep and HS is expensive due to the costly enzymes.

An alternative method is utilized to analyze the disaccharide compositions of Hep and HS. With this method, Hep and HS are subjected to hydrazinolysis and the resultant deacetylated materials are then depolymerized by deaminative cleavage with HONO, resulting in disaccharide units composed of uronic acid (IdoA or GlcA) and 2,5-anhydromannose (AnMan) bearing an aldehyde group (*12–14*). This type of disaccharide unit can easily be labeled with tritium by reduction of the aldehyde group with NaB^3H_4, affording radiolabeled disaccharide units (IdoA-$AnMan_R$ or GlcA-$AnMan_R$) quantitatively. Then, the disaccharide units labeled with tritium can be analyzed by radiochromatography (*15*). The advantage of this chemical reaction is that the structure of uronic acid is retained even after depolymerization. However, the use of radioisotopes for this method has the inevitable problem of the requirement of a well-equipped analytical laboratory.

In the present study, we established a new analytical procedure for the disaccharide compositions of Hep and HS. The disaccharide units generated on deaminative cleavage with HONO were labeled with paranitrophenyl (PNP-) hydrazine, affording AnMan-CH=N-NH-PNP (AnMan-PNP). Then, the PNP-labeled disaccharides were pre-purified on SepPak C-18 cartridge columns, and subsequently separated and detected on ion-pairing reversed-phase (IPRP-) HPLC as to the absorbance at 390 nm. The elution order was GlcA-AnMan-PNP (GM), IdoA-AnMan-PNP (IM), IdoA(2S)-AnMan-PNP (ISM), IdoA-AnMan-(6S)-PNP (IMS), and IdoA(2S)-AnMan(6S)-PNP (ISMS). The compositions of these cold-labeled disaccharides were confirmed to be comparable to those obtained by the enzymatic method, showing the reliable qualification and quantitation.

MATERIALS AND METHODS

Materials—Hep from bovine intestine was obtained from Syntex. HS from bovine kidney was obtained from Seikagaku. 6-*O*-Desulfated Hep was prepared according to the method of Takano *et al.* (*16, 17*). 2-*O*-desulfated Hep was synthesized according to the method of Piani *et al.* (*18, 19*). The enzymes used for disaccharide analysis of Hep and HS by means of a well-established enzymatic method (*9, 10*) were heparitinase I [EC 4.2.2.8], heparitinase II (no EC number), and heparinase [EC 4.2.2.7], all of which were the products of Seikagaku. PNP-hydrazine (reagent grade) was purchased from Wako. All other reagents used were of analytical grade.

Depolymerization of Heparin and PNP-Labeling—At first, intact Hep was deacetylated by hydrazinolysis essentially according to Guo and Conrad (*14*). Briefly, 3 mg of intact Hep was dissolved in 100 μl of a 70% hydrazine solution containing 1% (w/w) of hydrazine sulfate as the catalyst, and then the reaction mixture was heated at 96°C for 6 h. After cooling the reaction mixture in an ice-cold bath, 100 μl of distilled water was added. The reaction mixture was evaporated to dryness under a flow of dry air and then 200 μl of distilled water was added, followed by subjection of the reaction mixture to BioRad AG 1-X4 column chromatography. The deacetylated material was eluted with 2 M NaCl after the elution of salts with distilled water. The recovered material was subjected to dialysis against distilled water and then to lyophilization, resulting in a freeze-dried powder.

Next, in order to perform deamination, pH 1.5 HONO reagent was prepared. To 56 mg of $BaNO_2$ was added 500 μl of ice-cold distilled water, followed by complete dissolution. Then to the solution was added 500 μl of ice-cold 1 N H_2SO_4, followed by thorough mixing. The resulting whitish turbidity was removed by centrifugation, resulting in pH 1.5 HONO reagent in the supernatant.

To 1 mg of deacetylated Hep was added 100 μl of the HONO solution (pH 1.5), prepared as above, and then the reaction mixture was allowed to stand for 10 min at room temperature to allow the deamination to proceed (*14*). The reaction was terminated by neutralization with 1 N Na_2CO_3, followed by evaporation to dryness under a flow of dry air. The dried residue was dissolved in 200 μl of distilled water and 200 μl of acetonitrile containing 1% PNP-hydrazine (w/v). The reaction mixture was incubated at 37°C for 30 min to allow the coupling reaction to proceed, followed by desalting on a SepPak C-18 cartridge column as described below.

Depolymerization of Heparan Sulfate and PNP-Labeling—Intact heparan sulfate was deacetylated by hydrazinolysis according to a slight modification of the method of Guo and Conrad (*14*). Briefly, 3 mg of HS was dissolved in 100 μl of a 70% hydrazine solution containing 1% (w/w) of hydrazine sulfate as the catalyst, and then the reaction mixture was heated at 96°C for 6 h. After cooling the reaction mixture in an ice-cold bath, 10 ml of distilled water was added. The reaction mixture was then subjected to dialysis against distilled water overnight. The dialyzate was evaporated down to 800 μl, followed by lyophilization. To the freeze-dried powder obtained was added 400 μl of ice-cold 0.25 N HIO_3, and then the hydrazide generated at uronic acid residues during hydrazinolysis was converted to the intact form of uronic acid (*12*). I_2 formed was removed by liquid partitioning between diethyl ether and distilled water. The water layer containing the deacetylated material was subjected to dialysis against distilled water overnight, followed by concentration of the dialyzate and lyophilization. To 1 mg of the freeze-dried powder was added

100 μl of the HONO solution (pH 1.5), and then the reaction mixture was stored for 30 min at room temperature to allow deamination (*14*). Then, to the reaction mixture was added an adequate amount of 0.1 N NaOH to adjust the pH to 4.0.

Next, in order to perform further deamination, pH 4.0 HONO reagent was prepared. To 368 mg of $NaNO_2$ was added 1 ml of distilled water. To 500 μl of the resultant solution was added 200 μl of 1 N H_2SO_4. The resultant solution was mixed to give the pH 4.0 HONO reagent.

To the reaction mixture was added 30 μl of the HONO solution (pH 4.0), prepared as above, and then the reaction mixture was allowed to stand for another 30 min at room temperature to complete the deamination (*14*). The reaction was terminated by neutralization with a sufficient amount of 1 N Na_2CO_3, followed by evaporation to dryness under a flow of dry air. The coupling reaction was performed as mentioned above for the PNP-labeling of Hep-derived disaccharides.

Confirmation of the Coupling Reaction and Desalting with a SepPak C-18 Cartridge Column—The yield of the coupling reaction between PNP-hydrazine and IdoA-AnMan or GlcA-AnMan was estimated from the difference in the contents of aldehyde groups in AnMan residues before and after the reaction by 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) colorimetry (*20*). Furthermore, suitable conditions for desalting of the coupling reaction mixture using SepPak C-18 cartridges were determined by HPLC in order to obtain the maximum purification. For this purpose, the coupling reaction mixture (400 μl) was applied to a SepPak C-18 cartridge column activated and equilibrated with 20 ml of MeOH, 2 ml of CH_3CN, and 8 ml of distilled water. Elution was performed with 400 μl of a 10% CH_3CN solution, and subsequently with 400 μl of a 20% CH_3CN solution. The flow-through fractions after application, and the two CH_3CN-eluted fractions were collected, respectively. A 5 μl aliquot of each of the three fractions was applied to a Jasco 880PU type HPLC equipped with a DaisoPak ODS column (ϕ4.5$\times$250 mm) equilibrated with distilled water. Elution was performed with a linear gradient of MeOH, from 0 to 100%, for 30 min, at the constant flow rate of 1.5 ml/min at 40°C. Detection was performed as to absorbance at 230 nm. Based on the HPLC patterns obtained, the most suitable fraction was selected for ion-pairing reversed-phase (IPRP-) HPLC analysis.

Analysis of PNP-Labeled Disaccharide Units on Ion-Pairing Reversed-Phase HPLC—IPRP-HPLC analysis was performed according to a partial modification of the method of Guo and Conrad (*15*). Two kinds of solvents (A and B) were used to form a linear gradient of CH_3CN for optimum elution of PNP-labeled disaccharides. Solvent A was composed of 38 mM $NH_4H_2PO_4$, 2 mM H_3PO_4, and 1 mM tetrabutylammonium phosphate in distilled water, while solvent B was composed of 38 mM $NH_4H_2PO_4$, 2 mM H_3PO_4, and 1 mM tetrabutylammonium phosphate in a 50% CH_3CN solution. Ten microliter aliquots of the coupling reaction mixture after desalting as described above were applied to a Shimadzu LC-6AD type HPLC equipped with a Cosmosil packed ODS column (ϕ4.6$\times$150 mm) equilibrated with 58% of solvent A and 42% of solvent B at the constant flow rate of 1 ml/min at 40°C. Elution was performed with a linear gradient, from 42 to 60%, of solvent B for 30 min, at the constant flow rate of 1 ml/min at 40°C. Detection was performed as to the absorbance at 390 nm.

Analysis of Unsaturated Disaccharide Units on Strong Anion Exchange HPLC—Strong anion exchange (SAX-) HPLC analysis was performed as reported previously (*9*). The enzymatic digestion of Hep and HS was performed using either enzyme mixture I (50 mU each of heparitinases I and II) for the digestion of 200 μg of HS or enzyme mixture II (50 mU each of heparitinases I and II, and heparinase) for the digestion of 200 μg of Hep. The unsaturated disaccharides generated were detected as to the absorbance at 230 nm on SAX-HPLC.

Composition Analysis of Tritium-Labeled Disaccharides Derived from Heparan Sulfate—This composition analysis of HS was performed by Chirazyme, IL, USA. Briefly, HS from bovine kidney was completely cleaved into disaccharides, followed by reduction with NaB^3H_4 according to Guo and Conrad (*14*). In order to compare the disaccharide compositions of heparan sulfate from bovine kidney with the PNP-labeling and tritium-labeling methods, tritium-labeled disaccharides were separated and quantitated according to their sulfate contents into zero-, mono-, and di-sulfated disaccharides by paper electrophoresis (*14*). On the other hand, tritium-labeled disaccharides were analyzed by paper chromatography in order to separate and quantitate GlcA-$AnMan_R$ and IdoA-$AnMan_R$ bearing no sulfate group according to Guo and Conrad (*14*). Finally, tritium-labeled disaccharides bearing sulfate group(s) were analyzed by IPRP-HPLC according to Guo and Conrad (*15*).

RESULTS

Yields of Coupling Products and Their Partial Purification—Figure 1 shows a schematic diagram of the synthetic procedure for PNP-labeled disaccharide units. In this scheme, the starting heparin is simplified as repeating disaccharide units exclusively composed of 2-*O*-sulfated IdoA [IdoA(2S)] and 6-*O*-sulfated GlcNS [GlcNS(6S)]. In this case, *N*-deacetylation by hydrazinolysis could be left out, since the sulfoamino group in GlcNS(6S) is susceptible to deamination with HONO as well as the amino group in GlcN. At first, deamination with HONO converts the GlcNS(6S) residue to an AnMan(6S) residue bearing an aldehyde group. The polysaccharide could be depolymerized by this treatment due to the cleavage of the glycosidic bonds between GlcN/GlcNS and IdoA/GlcA. Secondly, a coupling reaction between IdoA(2S)-AnMan(6S) bearing an aldehyde group and PNP-hydrazine was performed, resulting in the formation of a cold (PNP)-labeled disaccharide unit of IdoA(2S)-AnMan(6S)-CH=N-NH-PNP [IdoA(2S)-AnMan(6S)-PNP] called "ISMS."

The coupling yields of disaccharide units bearing aldehyde groups and PNP-hydrazine were estimated to be at least 90% on quantitation of aldehyde groups before and after the coupling reaction using MBTH colorimetry.

Partial purification of PNP-labeled disaccharide units was performed with a SepPak C-18 cartridge column. The HPLC patterns of the flow-through fraction (Fig. 2a), 10% CH_3CN-eluate (Fig. 2b), and 20% CH_3CN-eluate (Fig. 2c) were obtained in order to determine the preferred elution conditions. In Fig. 2a a single peak with a retention time of 4.7 min can be observed, which is possibly due to unretain-

ed salts. In Fig. 2c a single peak with a retention time of 25.7 min can be observed, which was acertained to be unreacted PNP-hydrazine. On the other hand, in Fig. 2b four peaks of coupling products eluted between 13–20 min in addition to a large peak of salts at 4.7 min and a small peak of unreacted PNP-hydrazine at 25.7 min can be seen. From these results, it was confirmed that the coupling products could be partially purified and collected on a SepPak C-18 cartridge column, being eluted in the 10% CH_3CN fraction without loss. There is no possibility that PNP-labeled disaccharides containing a GlcA moiety, which were shown to be minor components, were selectively removed by the cartridge column, because PNP-labeled disaccharides exhibit almost the same polarity regardless of the type of uronic acid moiety. Accordingly, coupling products were subjected to SepPak C-18 cartridge column purification in order to partially remove salts and unreacted PNP-hydrazine, before subjection to IPRP-HPLC.

Ion-Pairing Reversed-Phase HPLC Analysis of Intact, 2-O-Desulfated, and 6-O-Desulfated Heparin from Bovine Intestine—In Fig. 3 are shown the IPRP-HPLC patterns of intact, 2-*O*-desulfated, and 6-*O*-desulfated Hep from bovine intestine. These Hep samples were not subjected to hydrazinolysis before depolymerization and PNP-labeling in order to obtain simplified HPLC patterns (not taking the disaccharide units bearing *N*-acetyl group into consideration). In each pattern, there was a big peak at the retention time of 5.1 min, which was identified as unreacted PNP-hydrazine on comparison with the HPLC of authentic PNP-hydrazine (data not shown). In Fig. 3a, intact Hep from bovine intestine gave two peaks at the retention times of 11.8 and 16.7 min, whose area ratio was 1:2. It has been shown that the disaccharide units containing a GlcA moiety, such as GlcA(2S)β1→4AnMan-PNP (GSM), GlcAβ1→4AnMan(6S)-PNP (GMS), and GlcA(2S)β1→4AnMan(6S)-PNP (GSMS), are minor components of the intact heparin polymer. Since Hep from bovine intestine is composed of 26% of ΔDi-(U, N)S (mainly corresponding to IdoA(2S)α1→4GlcNS) and 55% of ΔDi-(U, 6, N)S (mainly corresponding to IdoA(2S)α1→4GlcNS(6S)), as shown by enzymatic disaccharide analysis (Table I), the peaks eluted at 11.8 and 16.7 min were assumed to be IdoA(2S)α1→4AnMan-PNP (ISM) and IdoA(2S)α1→4 AnMan(6S)-PNP (ISMS), respectively.

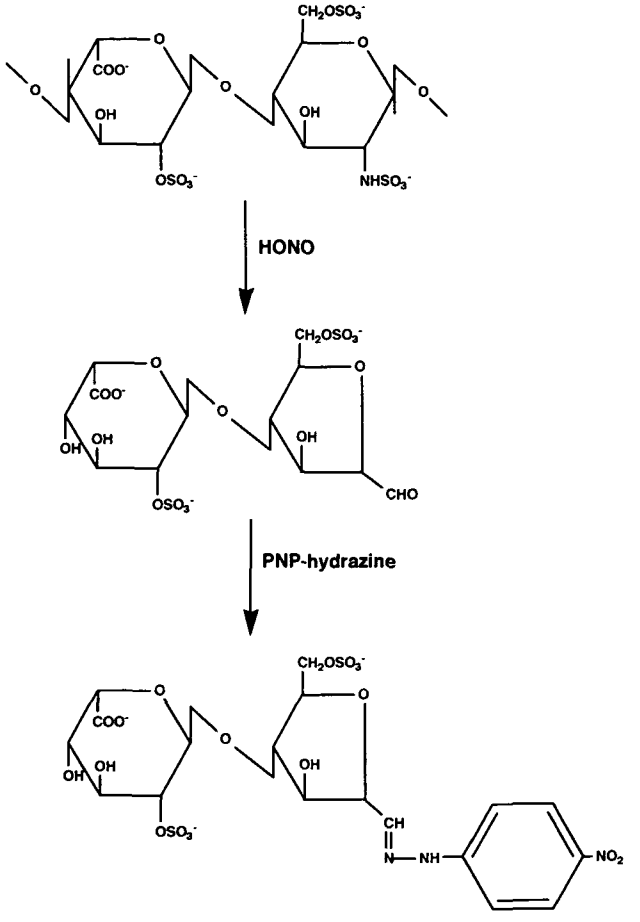

Fig. 1. **Schematic diagram of the synthetic procedure for obtaining PNP-labeled disaccharide units from Hep and HS, focusing on the tri-sulfated disaccharide unit of heparin.** Deamination with HONO converts the GlcNS(6S) residue to AnMan(6S) bearing an aldehyde group, followed by depolymerization of the intact polymer. The resulting IdoA(2S)-AnMan(6S) is coupled to PNP-hydrazine through the aldehyde group, forming the linkage, -CH=N-NH-.

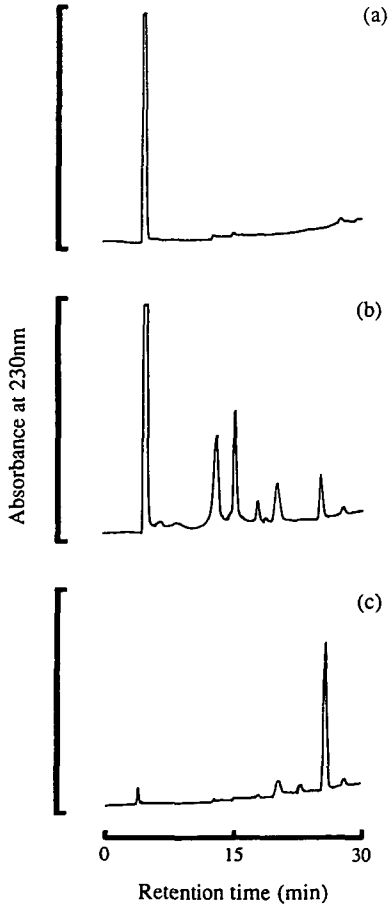

Fig. 2. **HPLC patterns of the coupling reaction mixture on a DaisoPak ODS column after treatment with SepPak C-18.** (a) Reversed-phase HPLC pattern of the flow-through fraction from a SepPak C-18 cartridge column. (b) Reversed-phase HPLC pattern of the 10% CH_3CN-eluate from the SepPak C-18 cartridge column. (c) Reversed-phase HPLC pattern of the 20% CH_3CN-eluate from the SepPak C-18 cartridge column.

In Fig. 3b, 2-*O*-desulfated Hep from bovine intestine gave two peaks with retention times of 6.7 and 12.8 min, with an area ratio of 1 : 2. The peak at 6.7 min could be IdoAα1→4AnMan-PNP (IM) derived on 2-*O*-desulfation of ISM, while the peak at 12.8 min could be IdoAα1→4AnMan(6S)-PNP (IMS) derived on 2-*O*-desulfation of ISMS.

In Fig. 3c, 6-*O*-desulfated Hep from bovine intestine gave one main peak at the retention time of 11.8 min. ISMS is converted to ISM by 6-*O*-desulfation, while ISM retains its form even after 6-*O*-desulfation. Accordingly, the main

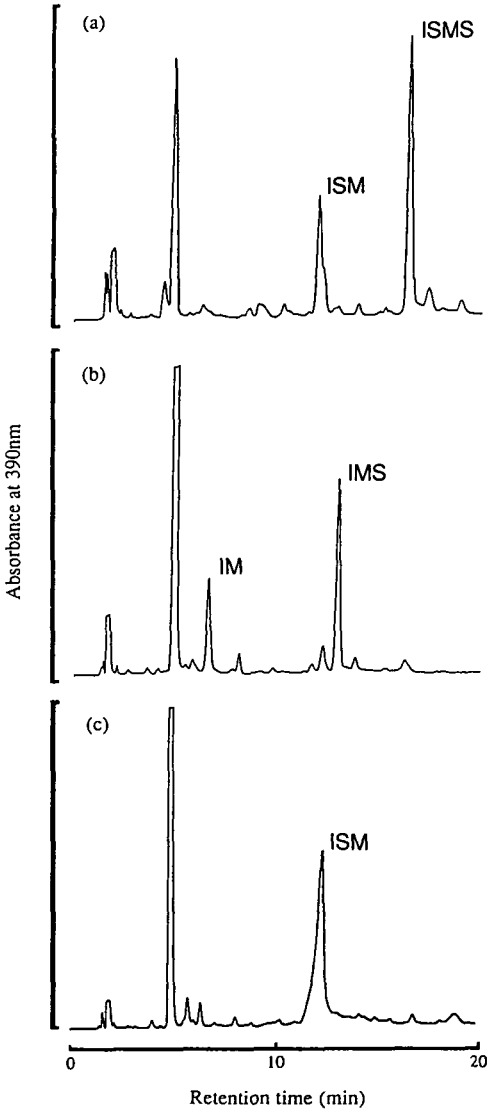

Fig. 3. **IPRP-HPLC patterns of intact, 2-*O*-desulfated, and 6-*O*-desulfated heparin.** (a) IPRP-HPLC pattern of intact heparin from bovine intestine, showing main peaks at 11.8 and 16.7 min corresponding to ISM and ISMS, respectively. (b) IPRP-HPLC pattern of 2-*O*-desulfated heparin from bovine intestine, showing main peaks at 6.7 and 12.8 min corresponding to IM and IMS, respectively. (c) IPRP-HPLC pattern of 6-*O*-desulfated heparin from bovine intestine, showing a main peak at 11.8 min corresponding to ISM.

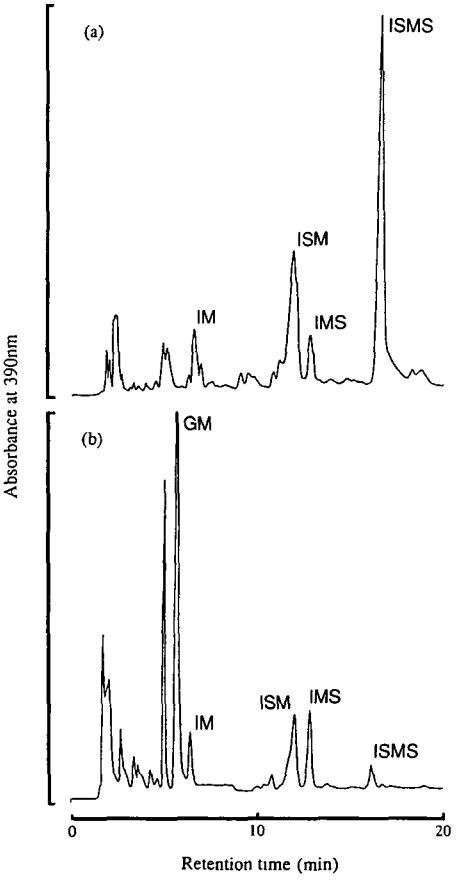

Fig. 4. **IPRP-HPLC patterns of heparin from bovine intestine and heparan sulfate from bovine kidney.** (a) *N*-Deacetylated heparin was depolymerized and coupled with PNP-hydrazine, followed by IPRP-HPLC analysis. There were four peaks with retention times of 6.7, 11.8, 12.8, and 16.7 min, corresponding to IM, ISM, IMS, and ISMS, respectively. (b) *N*-Deacetylated heparan sulfate was depolymerized and coupled with PNP-hydrazine, followed by IPRP-HPLC analysis. There were five peaks with retention times of 5.7, 6.7, 11.8, 12.8, and 16.7 min, corresponding to GM, IM, ISM, IMS, and ISMS, respectively.

TABLE I. **Comparison of the disaccharide compositions determined by chemical and enzymatic methods for heparin from bovine intestine and heparan sulfate from bovine kidney.**

Fraction	GM	IM	GSM+ISM		GMS+IMS		GSMS+ISMS
	⊿Di-OS	⊿Di-NS	⊿Di-US	⊿Di-(U, N)S	⊿Di-6S	⊿Di-(6, N)S	⊿Di-(U, 6, N)S
BI-Hep[a]	N.D.[b]	7.2	27.3		7.0		58.4
BI-Hep[c]	4.5	4.0	1.4	25.9	0.7	8.5	55.0
BK-HS[d]	61.7	8.5	10.8		14.9		4.1
BK-HS[c]	53.3	15.7	0.5	9.5	9.1	5.2	5.0

[a]Abbreviation used: BI-Hep, heparin from bovine intestine. [b]Not determined. [c]Data were obtained by enzymatic digestion, followed by SAX-HPLC. [d]Abbreviation used: BK-HS, heparan sulfate from bovine kidney.

TABLE II. **Disaccharide composition of heparan sulfate from bovine kidney analyzed with NaB^3H_4-reduced disaccharides by radiochromatography, paper electrophoresis, and with paper chromatography.**

Fraction	GM	IM	GSM	ISM	GMS	IMS	GSMS	ISMS
	GM+IM		GSM+ISM		GMS+IMS		GSMS+ISMS	
BK-HS[a]	62.1	7.2	0.0	9.8	2.4	13.8	0.0	4.7
	69.3		9.8		16.2		4.7	

[a]Abbreviation used: BK-HS, heparan sulfate from bovine kidney.

peak eluted at 11.8 min is supposed to be ISM.

Ion-Pairing Reversed-Phase HPLC Analysis of Deacetylated Heparin from Bovine Intestine and Heparan Sulfate from Bovine Kidney—In Fig. 4a is shown the IPRP-HPLC pattern of *N*-deacetylated Hep from bovine intestine. The peak of unreacted PNP-hydrazine at 5.1 min was greatly reduced. There were four peaks at the retention times of 6.7, 11.8, 12.8, and 16.7 min, corresponding to IM, ISM, IMS, and ISMS, respectively. The areas of these four peaks were comparable to those of the corresponding unsaturated disaccharides obtained on enzymatic cleavage (Table I). Since deacetylated Hep was only treated with pH 1.5 HONO, which was shown not to completely cleave unsubstituted GlcN residues, it is probable that a small amount of tetrasaccharides or higher oligosaccharides is included in the profile shown in Fig. 4a.

In Fig. 4b is shown the IPRP-HPLC pattern of *N*-deacetylated HS from bovine kidney. A new PNP-labeled disaccharide peak was eluted at 5.7 min in addition to the four peaks at 6.7, 11.8, 12.8, and 16.7 min. On comparison with the retention times of PNP-labeled disaccharide units as described above, these four peaks were identified as the disaccharide units of IM, ISM, IMS, and ISMS, respectively. The sum of the areas of the peaks eluted at 5.7 and 6.7 min is almost identical to that of zero-sulfated and *N*-sulfated unsaturated disaccharides obtained on enzymatic digestion of HS from bovine kidney (Table I). Since zero-sulfated and *N*-sulfated unsaturated disaccharides are only derived from IdoAα1→4GlcNAc (or GlcNS) and GlcAβ1→4GlcNAc (or GlcNS), the unknown peak eluted at 5.7 min is suggested to be GlcAβ1→4AnMan-PNP (GM). Alternatively, the disaccharide composition of HS from bovine kidney was analyzed by a combination of radiochromatography, paper electrophoresis, and paper chromatography. This method with tritium-labeled disaccharides afforded a quite comparable composition (Table II) to that with PNP-labeled disaccharides (Table I). Namely, it was clarified that the unsulfated disaccharide moieties were composed of 62.1% of GM and 7.2% of IM (Table II). Accordingly, the unknown peak eluted at 5.7 min with the present system was confirmed to be GM.

With the present analysis system, *ca.* 25 μg of a Hep- or HS-derived disaccharide sample is subjected to IPRP-HPLC. Since the area percentage of ISMS in Fig. 4b occupies 4.1% of the total peak area, at least *ca.* 1 μg (*ca.* 1 nmol) of PNP-labeled disaccharides could be detectable. Accordingly, the sensitivity of the present method is higher than that of the enzymatic method but lower than that of the tritium-labeling method.

DISCUSSION

In order to prepare cold-labeled materials targeting the reducing end of carbohydrates, many efforts have been made and some methods have become prevalent. For instance, the methods involving 2-aminopyridine (PA; *21, 22*), paraaminobenzoic acid ethyl ester (ABEE; *23*), and 7-amino-1,3-naphthalenedisulfonic acid (AGA; *24*) are useful for different types of analyses. The method involving PA is used for the two-dimensional mapping of oligosaccharides (*25*). The method involving ABEE is suitable for the FAB-MS analysis of oligosaccharides (*23*). The method involving AGA is used for the mapping of oligosaccharides after SDS-PAGE or capillary electrophoresis (*24*). The chemical reaction utilized to couple the reducing end of a carbohydrate with the above tags is reductive amination, in which a Schiff's base is formed between the aldehyde group of the reducing end of a carbohydrate and the amino group of the tag, followed by reduction with $NaB(CN)H_3$, the $-CH_2-NH-$ linkage being formed. On the other hand, the chemical reaction utilized in the present study to couple the aldehyde groups of AnMan in the disaccharide units with the tag, PNP-hydrazine, is more moderate and quantitative, resulting in the formation of a -CH=N-NH- group without the need for a reducing reagent (Fig. 1; *26*). In the present study, we have established novel method of disaccharide analysis of Hep and HS involving PNP-hydrazine coupling. The disaccharide compositions of Hep and HS obtained with the present method were comparable to those with the well-established enzymatic method (*9, 10*).

Previously, disaccharide units generated on deamination of Hep and HS were labeled with tritium by reducing the aldehyde groups of AnMan with NaB^3H_4. The tritium-labeled disaccharide units were then analyzed by SAX-HPLC (*13*) or IPRP-HPLC (*15*). When SAX-HPLC analysis is performed, unsulfated disaccharide units are eluted rapidly because they bear less charge, while sulfated disaccharide units are eluted slowly because of their higher charge, allowing them to bind to the anion exchange resin. When IPRP-HPLC analysis is performed, unsulfated disaccharide units are eluted rapidly, whereas sulfated disaccharide units are eluted slowly, resulting in almost the same elution order as on SAX-HPLC. In an IPRP-HPLC system the hydrophobic tetraammonium ions, which are coupled electrostatically to the sulfate groups, allow disaccharides to interact with the ODS resin. Accordingly, the more highly the disaccharide units are sulfated, the more slowly they are eluted from a IPRP-HPLC column.

Guo and Conrad (*14, 15*) reported that the aldehyde groups of AnMan in disaccharide units generated by deamination were reduced with 450 mCi/mmol of NaB^3H_4, but such a high level of radioactivity is difficult to use in many laboratories. Alternatively, we tried to prepare cold-labeled disaccharide units using the reaction between PNP-hydrazine and the aldehyde groups of AnMan in disaccharide units generated by deamination of Hep and HS. At first, we confirmed that the yield of the PNP-coupling reaction is high enough (more than 90%) and that a SepPak C-18 cartridge column is efficient for removing unreacted PNP-hydrazine (Fig. 2). Then we modified the conditions for the IPRP-HPLC separation, as reported by Guo and Conrad (*15*). They eluted tritium-labeled disaccharides on

IPRP-HPLC with a stepwise gradient of CH_3CN, from 1.8 to 18%, in 100 min, while we eluted PNP-labeled disaccharides on IPRP-HPLC with a linear gradient of CH_3CN, from 21 to 30%, in 30 min. Thus, we shortened the elution time from 100 to 30 min. Since Guo and Conrad (*15*) had to fractionate the eluate obtained on IPRP-HPLC in order to measure the radioactivity in the fractions using a liquid scintillation counter, the total elution time had to be at least 100 min to collect the necessary volume of each fraction for the radioactivity measurement. Since our PNP-labeled disaccharides are detectable as to the absorbance at 390 nm using a flow cell, we could shorten the elution time from 100 to 30 min, without considering the need for a certain elution volume.

The PNP-labeled disaccharide units derived from Hep and HS showed the elution order of GM, IM, ISM, IMS, and ISMS. In addition, the quantities of these five kinds of PNP-labeled disaccharides were comparable to those of the corresponding unsaturated disaccharides obtained by the enzymatic method (Table I). On the other hand, tritium-labeled disaccharide units from Hep and HS showed the same elution order of GM, IM, ISM, IMS, and ISMS (*15*).

Previously, the quantitation of GM and IM (tritium-labeled disaccharide forms) was performed by a combination of different methods, such as radiochromatography (HPLC), paper chromatography and paper electrophoresis, because tritium-labeled GM and IM are eluted in the flow-through fractions from a radiochromatography column. In the present study, GM and IM (PNP-labeled disaccharide forms) were successfuly separated and quantitated by merely IPRP-HPLC, allowing quite simple and reliable composition analysis of zero-sulfated disaccharides such as GM and IM composing HS obtained from various origins.

Four minor disaccharide units (GSM, GMS, GSMS, and GMS_2) found previously on analysis using tritium-labeling, however, have not yet been identified with the present analytical system using PNP-labeled form. Using the present system, we are now attempting to identify these four minor disaccharide units, and to distinguish ISM and GSM, IMS and GMS, and ISMS and GSMS, respectively. Regardless of such ambiguity, the present analytical method is a great tool for structural studies on Hep and HS instead of the costly enzymatic and radioisotopic methods.

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