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Chemiluminescence high-performance liquid chromatography for the determination of hyaluronic acid, chondroitin sulphate and dermatan sulphate

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ABSTRACT

A sensitive chemiluminescence high-performance liquid chromatographic method has been developed for the determination of hyaluronic acid, chondroitin sulphate and dermatan sulphate as their unsaturated disaccharide–dansylhydrazine derivatives involving an effective sample clean-up system. The dansylhydrazones of the unsaturated disaccharides derived from the hyaluronic acid, chondroitin sulphate and dermatan sulphate by chondroitinase ABC and/or chondroitinase ACII, were separated by reversed-phase chromatography using a mixture of 0.1 *M* sodium acetate buffer (pH 6.0) and 80% acetonitrile on a column (250 mm × 4.0 mm I.D.) packed with amido-80 silica beads (5 μm diameter). For post-column elution in the chemiluminescence system, 1 m*M* bis[2-(3,6,9-trioxadecanyloxycarbonyl)-4-nitrophenyl]oxalate and 3 m*M* hydrogen peroxide in acetonitrile were used. The detection limit of each glycosaminoglycan was 100 fmol. The method was applicable to the determination of the levels of hyaluronic acid, chondroitin sulphate and dermatan sulphate in rat peritoneal mast cells.

INTRODUCTION

Glycosaminoglycans (GAGs), such as hyaluronic acid (HA), chondroitin sulphate (CS) and dermatan sulphate (DS) have been identified and determined by digesting GAGs to the corresponding unsaturated disaccharides with chondroitinase ABC and chondroitinase ACII. Because the unsaturated disaccharides contain one terminal 4,5-unsaturated glucopyranosyluronic acid, which absorbs at 232 nm, the unsaturated disaccharides have conveniently been determined by high-performance liquid chromatography (HPLC) with UV detection [1–5]. However, it has proved difficult to make this method sufficiently sensitive to use in the analysis of biological samples. Recently, several pre- and post-column derivatization methods, each with certain limitations, have been employed to determine GAGs in the extracellular matrix [6–8]. On the other hand, for the analysis of intracellular trace amounts of GAGs, a further increase in the sensitivity of the method is needed.

We have already reported a sensitive chemiluminescence HPLC system for the determination of HA levels in rabbit blood plasma [9]. In this paper, we report a sensitive, specific chemiluminescence HPLC system for the determination of unsaturated disaccharides produced enzymically from HA, CS and DS using dansylhydrazine as a pre-labeling reagent, and its application to the

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determination of the amounts of GAGs isolated from rat peritoneal mast cells.

EXPERIMENTAL

Materials

Standard unsaturated disaccharides (2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose (ΔDi-HA), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S), 2-acetamido-2-deoxy-3-O-(2-O-sulpho-β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-UA2S), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose (ΔDi-4S), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose (ΔDi-6S), 2-acetamido-2-deoxy-3-O-(2-O-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose (ΔDi-diS$_B$), 2-acetamido-2-deoxy-3-O-(2-O-sulpho-β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose (ΔDi-diS$_D$), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-O-sulpho-D-galactose (ΔDi-diS$_E$) and 2-acetamido-2-deoxy-3-O-(2-O-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4,6-di-O-sulpho-D-galactose (ΔDi-triS), and chondroitinase ABC (EC 4.2.2.4) and chondroitinase ACII (EC 4.2.2.5) were purchased from Seikagaku Kogyo (Tokyo, Japan). TSKgel NH_2-60 (particle size 5 μm) and TSKgel amido-80 (particle size 5 μm) for the HPLC column packings were obtained from Tosoh (Tokyo, Japan). TDPO {bis[2-(3,6,9-trioxadecanyloxycarbonyl]-4-nitrophenyl] oxalate} and acetonitrile were purchased from Wako (Osaka, Japan), and acetonitrile was used after distillation. All other chemicals were of the highest grade available commercially, unless otherwise stated. All solutions were made up freshly in water purified by deionization and distillation.

Apparatus and conditions

A flow diagram of the HPLC system is shown in Fig. 1 and the conditions are listed in the caption.

The system consisted of two pumps (L-6000; Hitachi Seisakusho, Tokyo, Japan), a sample injector with a 20-μl loop (VMD-350; Shimamura Instruments, Tokyo, Japan), a chemiluminescence detector (JASCO 825-CL; Japan Spectroscopic, Tokyo, Japan), a reaction coil (stainless-steel tubing, 400 mm × 0.25 mm I.D.), a mixing device (Kyowa Seimitsu, Tokyo, Japan) and an integrator (D-2500; Hitachi Seisakusho). It was important that 1 m*M* TDPO [10] in acetonitrile and 3 m*M* hydrogen peroxide in acetonitrile, as the chemiluminescence reagents, were thoroughly degassed and sonicated before use.

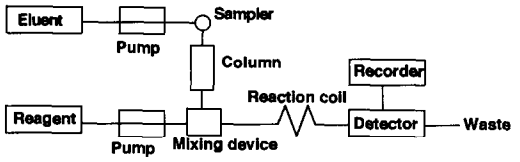

Fig. 1. Flow diagram of the chemiluminescence HPLC system. HPLC condition 1: column, TSKgel amido-80 (250 mm × 4.0 mm I.D.); column temperature, 50°C; reaction coil, 400 mm × 0.25 mm I.D.; eluent, acetonitrile–0.15 *M* acetate buffer (pH 5.0) (80:20, v/v) at a flow-rate of 0.8 ml/min; reagent, 1 m*M* TDPO and 3 m*M* hydrogen peroxide in acetonitrile at a flow-rate of 0.5 ml/min. HPLC condition 2: column, TSKgel NH_2-60 (250 mm × 4.6 mm I.D.); column temperature, 45°C; eluent, acetonitrile–0.1 *M* acetate buffer (pH 6.0) (80:20, v/v) at a flow-rate of 1.0 ml/min; reagent, 1 m*M* TDPO and 3 m*M* hydrogen peroxide in acetonitrile at a flow-rate of 0.7 ml/min.

Preparation of GAGs obtained from rat peritoneal mast cells

Mast cells were separated from the peritonial cavity fluid of Wistar rats by the method described by Nakagomi *et al.* [11]. The mast cells was suspended in 1 ml of Tyrode solution. The solution was frozen and thawed six times, then rat peritoneal cellular GAGs were prepared according to the method described previously [12].

Enzymic digestion

A 20-μl portion of GAGs solution prepared from rat peritoneal mast cells, 10 μl of 0.1 *M* Tris–HCl buffer (pH 8.0) and 10 μl of an aqueous solution containing chondroitinase ABC (0.1 U per 10 μl) and chondroitinase ACII (0.1 U per 10 μl) were mixed and incubated at 37°C for 3 h, and then lyophilized. The residue was dissolved in 20

μl of water. To another 20-μl portion of the sample solution were added 10 μl of 0.1 *M* acetate buffer (pH 6.0) and 10 μl of an aqueous solution of chondroitinase ACII (0.1 U per 10 μl). The mixture was incubated at 37°C for 3 h and lyophilized. The residue was dissolved in 20 μl of water. These solutions were used for dansylhydrazine derivatization.

Derivatization procedure

The derivatization procedure of the unsaturated disaccharide with dansylhydrazine was as follows. To 20 μl of the unsaturated disaccharide (from 500 fmol to 5 nmol) solution in a screw-cap sealed tube, 10 μl of 3% (w/v) trichloroacetic acid in ethanol, 10 μl of 2 nmol/ml ΔDi-UA2S as an internal standard, and 10 μl of 2% (w/v) dansylhydrazine in acetonitrile were added, successively. The tube was kept at 45°C in the dark for 150 min. The excess reagent was extracted from the tube by three extractions with 1.0 ml of toluene.

RESULTS AND DISCUSSION

Preparation of derivatives

The dansylhydrazone (Dns) derivative is suitable for the peroxyoxalate chemiluminescence reaction, but excess pre-labeling reagent generally prevents the rapid and sensitive determination of the substances on chromatogram. In particular, the reagent must be removed from the high sensitive chemiluminescence system.

Therefore, a clean-up procedure was designed to extract the reagent with an appropriate solvent. We examined the effect of several solvents by thin-layer chromatography (TLC) (Fig. 2). Among the solvents evaluated, benzene and toluene gave excellent results compared with aliphatic solvents, such as hexane. Because of the carcinogenic nature of benzene, toluene was selected, and the excess reagent was extracted from the pre-labeling reaction mixture by three extractions with 1 ml of toluene. Then, 10 μl of the lower aqueous phase containing the resultant hydrazone derivatives were subjected to HPLC. The extraction step is sufficient and effective not only for removing the fluorogenic reagent, which causes large background peak(s), but also for concentrating the unsaturated disaccharide solution.

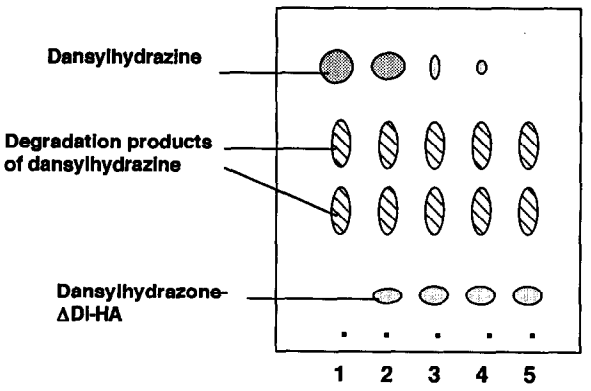

Fig. 2. Thin-layer chromatograms of the reaction mixtures in dansylhydrazine derivatization of ΔDi-HA. Lanes: 1 = blank; 2 = derivatized ΔDi-HA without extraction with toluene; 3 = derivatized ΔDi-HA extracted once with toluene; 4 = derivatized ΔDi-HA extracted twice with toluene; 5 = derivatized ΔDi-HA extracted three times with toluene. TLC conditions: plate, Kieselgel 60; solvent system, *n*-propanol–2-propanol–*n*-butanol–water (30:45:5:20, v/v) containing 0.04 *M* NaCl and 0.01 *M* ammonia (final concentrations).

Chromatographic separation of the derivatives

We have already reported the separation of Dns-unsaturated disaccharides (Dns-ΔDi-HA, Dns-ΔDi-0S) on an amino-modified silica gel column, TSKgel NH_2-60 [9]. However, the isocratic separation of all the Dns-unsaturated disaccharides (Dns-ΔDi-HA, 0S, 6S, 4S, UA2S, diS_D, diS_B, diS_E and triS) on this column is a lengthy process. Various eluents for an amido-modified silica gel column, TSKgel amido-80, which is usually used for the separation of neutral sugars, were examined. As a result, the satisfactory separation of the eight unsaturated disacharides, except the separation of Dns-ΔDi-6S and Dns-ΔDi-4S, was achieved with acetonitrile–0.15 *M* acetate buffer (pH 5.0) (80:20, v/v) on a TSKgel amido-80 column at 50°C (condition 1) (Fig. 3A). However, for analysis of Dns-ΔDi-6S and Dns-ΔDi-4S, the HPLC conditions were determined with acetonitrile–0.1 *M* acetate buffer (pH 6.0) (80:20, v/v) on a TSKgel NH_2-60 column at 45°C (condition 2) (Fig. 3B).

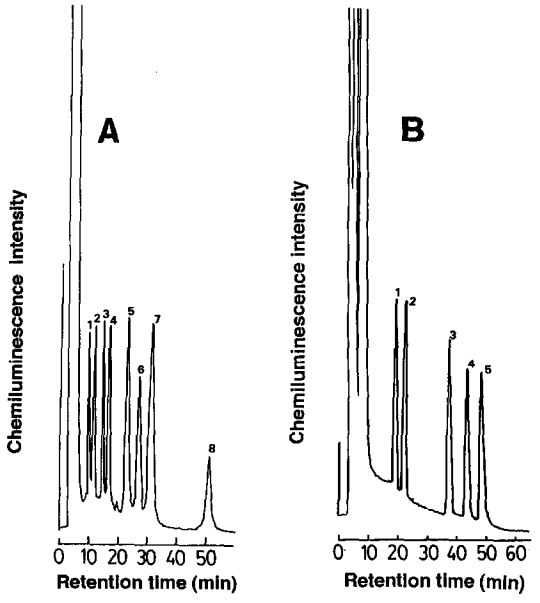

Fig. 3. Chromatograms of dansyl derivatives prepared from unsaturated disaccharide standards. (A) HPLC condition 1. Peaks: 1 = Dns-ΔDi-HA; 2 = Dns-ΔDi-0S; 3 = Dns-ΔDi-UA2S (internal standard); 4 = Dns-ΔDi-4S; 5 = Dns-ΔDi-diS$_D$; 6 = Dns-ΔDi-diS$_B$; 7 = Dns-ΔDi-diS$_E$; 8 = Dns-ΔDi-triS. (B) HPLC condition 2. Peaks: 1 = Dns-ΔDi-HA; 2 = Dns-ΔDi-0S; 3 = Dns-ΔDi-UA2S (internal standard); 4 = Dns-ΔDi-6S; 5 = Dns-ΔDi-4S.

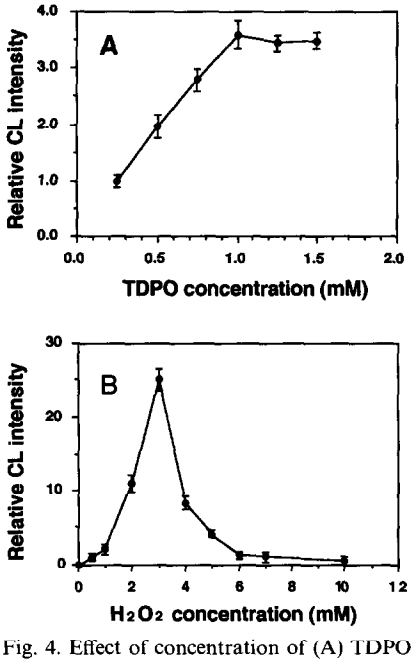

Fig. 4. Effect of concentration of (A) TDPO and (B) hydrogen peroxide on the chemiluminescence intensity.

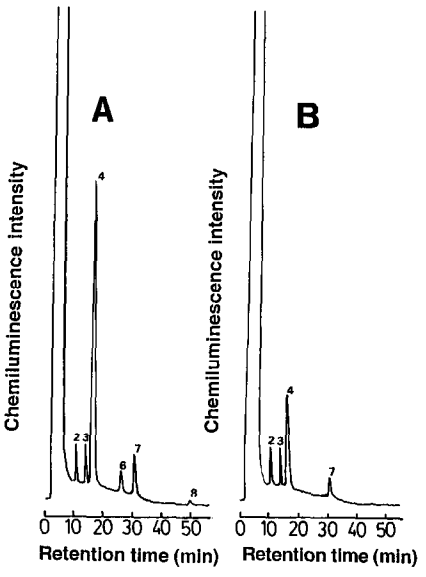

Fig. 5. Chromatographic patterns obtained from glycosaminoglycans in rat peritoneal mast cells by digestion with the enzymes: (A) digested with chondroitinase ABC together with chondroitinase ACII; (B) digested with chondroitinase ACII alone. HPLC conditions as in Fig. 3A.

Conditions for chemiluminescence in the detection system

In order to investigate the optimal conditions for the chemiluminescence detection system, some parameters were tested. The dependence of relative chemiluminescence intensity on the concentrations of hydrogen peroxide and TDPO is shown in Fig. 4. The chemiluminescence was observed at TDPO concentrations greater than 1 m*M* and the most effective concentration for hydrogen peroxide was 3 m*M*. The flow-rate of the chemiluminescence reagent was optimized at 0.5 ml/min. The calibration curves for Dns-unsaturated disaccharides were linear in the range from 500 fmol to 5 nmol, with a practical detection limit (at a signal-to-noise ratio of 3) of 100 fmol. The coefficients of variation (C.V.) at 60 pmol of each unsaturated disaccharide were less than 4% (n = 5).

Analysis of GAGs isolated from rat peritoneal mast cells

Fig. 5 shows typical chromatograms of unsaturated disaccharides obtained from the GAGs in rat peritoneal mast cells by combined digestion

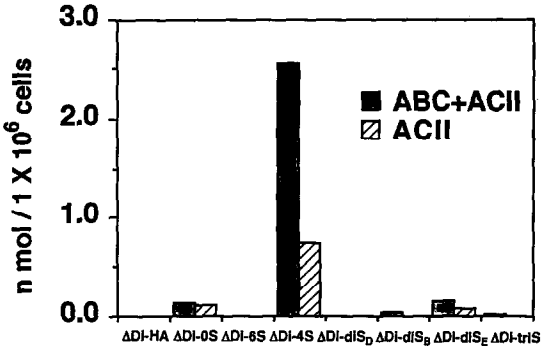

Fig. 6. Analysis of unsaturated disaccharides produced from glycosaminoglycans in rat peritoneal mast cells after digestion with the enzymes: (ABC + ACII) digested with chondroitinase ABC together with chondroitinase ACII; (ACII) digested with chondroitinase ACII.

with chondroitinase ABC and ACII (Fig. 5A) and by chondroitinase ACII only (Fig. 5B). The differences in the peak heights in the two chromatograms obtained by HPLC condition 1 (Fig. 5A and B) correspond to the amounts of DS in these GAGs.

Fig. 6 summarizes the results obtained for the composition of disaccharide units from the GAGs of rat peritoneal mast cells. The results represented in Fig. 6 indicate that DS is a dominant component of the minor GAGs of the cells. Generally, distinct subpopulations of rat mast cells have been identified by the differences in the types of proteoglycan that are synthesized and stored in secretory granules [13,14], by their differential histochemical staining properties [15].

It is well known that rat mucosal mast cells obtained from the gastrointestinal mucosa synthesize predominantly oversulphated chondroitin sulphate proteoglycans, and that rat connective tissue mast cells obtained from the serosal cavity synthesize heparin proteoglycan. Recently, in rat peritoneal mast cells, trace amounts of highly sulphated chondroitin sulphate proteoglycans have also been identified [16], but the report did not mention other minor GAGs, such as DS. We have demonstrated that the DS chain is the major component of the GAGs chains in rat peritoneal mast cellular fractions. DS may play some biological roles in mast cells and this chemiluminescence method is expected to be applied to investigate in detail the differences of GAGs between mucosal mast cells and connective tissue mast cells.

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