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Note

Sensitive detection of unsaturated disaccharides from chondroitin sulphates by thin-layer chromatography as their dansylhydrazine derivatives

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Paper chromatography[1] has been used for the analysis of unsaturated disaccharides after the digestion of chondroitin sulphates (ChS) by chondroitinase ABC or AC. Thin-layer chromatography (TLC) on cellulose plates has also been used by many workers[2–4]. Recently, high-performance liquid chromatography (HPLC) has been used for the determination of unsaturated disaccharides[5–12], but TLC is still useful as a simple analytical method.

Shimada and co-workers reported the application of TLC on silica gel for the separation of hyaluronate oligosaccharides[13] and unsaturated chondroitin sulphate disaccharides[14]. However, colorimetric detection on TLC plates with a carbazole reagent is not sensitive enough.

In this paper, we describe the TLC separation of small amounts of unsaturated disaccharides as their dansylhydrazine [1-naphthalenesulphonyl-5-(dimethylamino) hydrazide] derivatives, which have been used in fluorimetric precolumn derivatization in HPLC[10,11].

EXPERIMENTAL

Reagents and materials

The standard unsaturated disaccharides 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S), 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)-D-galactose (ΔDi-UA2S), 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$_E$), 2-acetamido-2-deoxy-3-O-(2-O-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4,6-di-O-sulpho-D-galactose (ΔDi-triS), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose (ΔDi-HA) and chondroitinase ABC were purchased from Seikagaku Kogyo (Tokyo, Japan) and dansylhydrazine from Fluka (Buchs, Switzerland). All other chemicals were of analytical-reagent grade.

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Kieselgel 60 thin-layer plates (plastic plate, 0.2 mm thickness) were obtained from Merck (Darmstadt, F.R.G.).

A UV lamp (Model UVGL-15 Mineralight Lamp; San Gabriel CA, U.S.A.) was used to detect the spots on the developed TLC plates.

Separation of glycosaminoglycans in rabbit plasma and urine

Plasma glycosaminoglycans (GAGs) were separated according to the method of Emura and Mukuda[15] with a 100-μl sample plasma.

Urinary GAGs were separated according to Poulsen's method[16].

Enzymatic digestion

Aliquots of 10 μl each of 0.2 *M* Tris–HCl buffer (pH 8.0) and an aqueous solution of 0.1 U chondroitinase ABC were added to 20 μl of plasma or a urinary GAG solution. The mixture was incubated at 37°C for 3 h and then lyophilized. The residue was dissolved in 10 μl of water. This solution was used for dansylhydrazine derivatization.

Fluorescent derivatization of unsaturated disaccharides

Aliquots of 20 μl of a 0.75% (w/v) solution of trichloroacetic acid in ethanol and 20 μl of a 1.0% (w/v) solution of dansylhydrazine in ethanol were added to 10 μl of an aqueous solution of unsaturated disaccharides produced enzymatically from plasma or urinary GAGs. The mixture was incubated at 40°C for 150 min, then cooled to room temperature. An aliquot of 5 μl of the resulting solution was submitted to TLC.

RESULTS AND DISCUSSION

Separation of unsaturated disaccharides

The separation of dansylhydrazine derivatives of unsaturated disaccharides was examined under various conditions using commercial silica plates. It was found that ΔDi-0S, ΔDi-4S, ΔDi-6S, ΔDi-UA2S, ΔDi-diS_B, ΔDi-diS_D, ΔDi-diS_E, ΔDi-triS and ΔDi-HA could be resolved satisfactorily with *n*-propanol–isopropanol–*n*-butanol–water (30:45:5:20, v/v) containing 0.04 *M* sodium chloride and 0.01 *M* ammonia (Table I).

The detection limits of ΔDi-0S, ΔDi-4S and ΔDi-6S were 90 pmol with observation under UV light with the naked eye.

TABLE I

R_F VALUES OF DANSYLHYDRAZINE DERIVATIVES OF UNSATURATED DISACCHARIDES

Unsaturated disaccharide	R_F	*Unsaturated disaccharide*	R_F
ΔDi-0S	0.72	ΔDi-diS_D	0.52
ΔDi-4S	0.56	ΔDi-diS_E	0.41
ΔDi-6S	0.61	ΔDi-triS	0.30
ΔDi-UA2S	0.66	ΔDi-HA	0.75
ΔDi-diS_B	0.47		

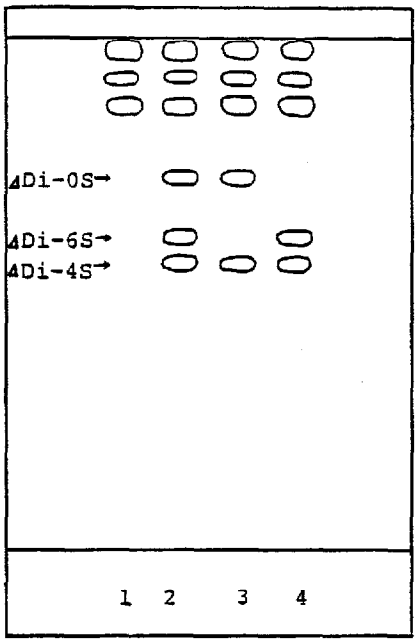

Fig. 1. Chromatogram of dansylhydrazine derivatives of unsaturated disaccharides produced from plasma and urinary chondroitin sulphates: 1 = blank; 2 = mixture of ΔDi-0S, ΔDi-4S and ΔDi-6S; 3 = plasma ChS; 4 = urinary ChS. Conditions: TLC plate, silica gel 60; developing solvent, *n*-propanol–isopropanol–*n*-butanol–water (30:45:5:20, v/v) containing 0.04 *M* sodium chloride and 0.01 *M* ammonia.

Although the TLC of ΔDi-0S, ΔDi-4S and ΔDi-6S has been studied previously[2–4,14], the separation of oversulphated unsaturated disaccharides such as ΔDi-diS_B, ΔDi-diS_D, ΔDi-diS_E and ΔDi-triS has not been reported. Using the method described here, all unsaturated disaccharides produced enzymatically from ChS can be separated satisfactorily with development under isocratic conditions.

Identification of unsaturated disaccharides from ChS in rabbit plasma and urine

The proposed method was applied to the identification of unsaturated disaccharides produced enzymatically from ChS in rabbit plasma and urine. Fig. 1 shows the chromatogram of dansylhydrazine derivatives of unsaturated disaccharides from rabbit plasma and urinary GAGs obtained by digestion with chondroitinase ABC.

ΔDi-0S and ΔDi-4S were detected in plasma ChS and ΔDi-4S and ΔDi-6S in urinary ChS.

In conclusion, the proposed method is useful for the identification of the unsaturated disaccharides produced from GAGs such as ChS by chondroitinases, and can be used for their structural characterization and as a screening test for ChS in biological samples because of its high sensitivity.

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