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Note

Fluorometric analysis of neutral sugars as their glycamines by high-performance liquid chromatography and thin-layer chromatography

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The analysis of neutral sugars by high-performance liquid chromatography (HPLC) using a prelabelling method has been studied by many workers, because it generally requires only simple and inexpensive apparatus. Consequently, various methods have been developed by converting neutral sugars into their derivatives: benzoyl[1], 4-nitrobenzoyl[2] or phenyldimethylsilyl[3] derivatives due to the reactivity of the hydroxyl group, and dansylhydrazine[4,5], 4-amino-4-dimethylaminoazobenzene[6] or 2-aminopyridine[7] derivatives due to the reactivity of the aldehyde group.

On the other hand, Hara *et al.*[8] developed a method based on the separation of glycamines (1-amino-1-deoxyglycitols) derived from neutral sugars by reductive amination, using an amino acid analyzer with ninhydrin as a colour-developing reagent. Further, Perini and Peters[9] examined and improved this method by use of fluorometric detection with *o*-phthalaldehyde.

Recently, Watanabe and Imai[10] proposed an excellent fluorescent prelabelling reagent, 7-fluoro-4-nitrobenz-2-oxa-1,3-diazole (NBD-F) for amino acids and we have established a sensitive analytical method for amino sugars using this reagent[11].

In this work, the analysis of glycamines corresponding to neutral sugars has been investigated using NBD-F as a prelabelling reagent and HPLC and thin-layer chromatography (TLC). The proposed method was applied to the analysis of Tamm-Horsfall urinary glycoproteins.

EXPERIMENTAL

Reagents and materials

Sodium cyanoborohydride was obtained from Aldrich Chemical Company (Milwaukee, WI, U.S.A.), hexoses from Kanto Chemical Co. and NBD-F from Wako Pure Chemical Industries. All other chemicals were of reagent grade.

Silica Gel 60 thin-layer plates were obtained from Merck (Darmstadt, F.R.G.) TSK-gel LS-410A (Toyo Soda Co.) of particle size 5 μm was used as a stationary phase in HPLC.

Apparatus

The chromatographic equipment included a reciprocating pump (Type PSD-

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3 · 2; Seishin Pharmaceutical Co., Tokyo, Japan), a variable input recorder (SS-250F; Sekonic Co., Tokyo, Japan) and a fluorescence detector (Type FLD-1, cut-off filter EM-5; Shimadzu Seisakusho, Kyoto, Japan).

Sample preparation

The glycamines were prepared according to the method of Perini and Peters[9] as follows. A 100-μl volume of 0.2 *M* sodium cyanoborohydride in 1 *M* ammonium sulphate, pH 7.0, prepared daily, was added to each sample containing 2.0 nmol–2.5 μmol monosaccharide. The tubes were flushed with nitrogen, tightly capped and heated at 100°C for 90 min. The reaction was stopped by the addition of 100 μl of 0.2 *M* hydrochloric acid, and the reaction mixture was evaporated to dryness. To the residue, 300 μl of 1 *M* sodium hydroxide were added to remove the residual ammonia and the mixture was evaporated. Then, to the residue, 500 μl of water were added and the solution was evaporated to dryness. The residue was redissolved in 100 μl of water, and the solution was neutralized with 0.2 *M* hydrochloric acid and evaporated. The residue was dissolved in 100 μl of 0.1 *M* boric acid–sodium hydroxide buffer (pH 8.0). This solution was used for NBD-F derivatization.

The derivatization was performed as described for amino sugars[11]: to 20 μl of sample solution, 20 μl of 50 m*M* NBD-F in ethanol were added, and the mixture was heated at 60°C for 30 min. The reaction was stopped by the addition of 60 μl of 0.1 *M* hydrochloric acid and the reaction mixture was subjected to HPLC and TLC.

RESULTS AND DISCUSSION

Identification of NBD-glycamines by TLC

The separation of NBD-glycamines by TLC was examined under various conditions. Ultimately, commercial silica plates were used after treatment with 1% boric acid in methanol and drying. Glucose (Glc), galactose (Gal), mannose (Man), fucose (Fuc), ribose (Rib) and arabinose (Ara) could be resolved with chloroform–methanol–water (55:45:10) as a developing solvent (Fig. 1). However, Gal and Rib could not be separated from Xylose (Xyl) and rhamnose (Rham), respectively.

This method was not subject to interference from amino acids, except from arginine which coincided in R_F value with Rib and Rham.

The densitometric detection limit of Gal was 60 pmol.

TLC can be used to identify neutral sugars in biological samples with the advantage that many samples are analyzed simultaneously.

Determination of NBD-glycamines by HPLC

The identification and determination of NBD-glycamines was investigated by HPLC using a reversed phase ODS-C_{18} column. Gal, Glc, Man, Xyl, Fuc and Rham were eluted with 8% aqueous acetonitrile solution as a mobile phase (Fig. 2).

Calibration plots for Gal, Man and Fuc were linear in the range of 40 pmol to 50 nmol for injection volumes of 10 μl.

Trace amounts of amino acids in biological samples did not interfere with the separation of NBD-glycamines.

We applied the proposed methods to the analysis of Tamm-Horsfall urinary glycoproteins (T-H-GPs). The T-H-GPs were separated from human urine in the

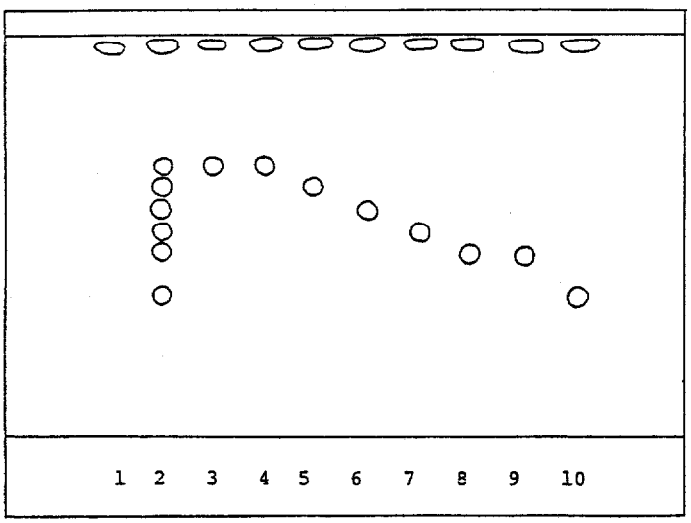

Fig. 1. Chromatogram of NBD derivatives of glycamines: 1 = blank; 2 = mixture of neutral sugars; 3 = Rham; 4 = Rib; 5 = Fuc; 6 = Ara; 7 = Man; 8 = Xyl; 9 = Gal; 10 = Glc. Conditions: Silica gel 60 treated with 1% boric acid in methanol; developing solvent; chloroform–methanol–water (55:45:10).

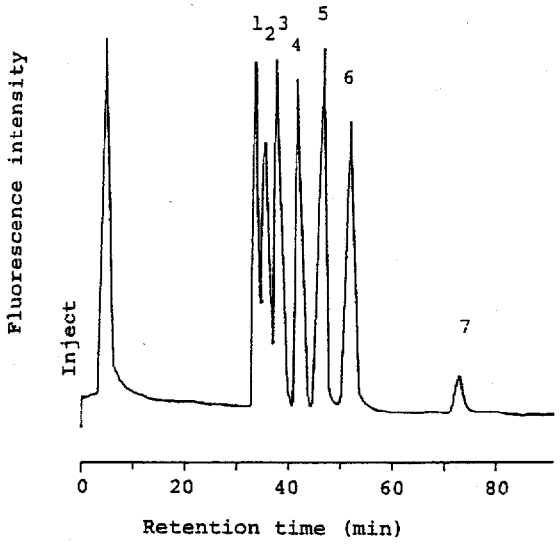

Fig. 2. Chromatogram of NBD derivatives of glycamines: 1 = Gal; 2 = Glc; 3 = Man; 4 = Xyl; 5 = Rib; 6 = Fuc; 7 = NBD-NH_2. Conditions: column; TSK-Gel LS-410A (250 mm × 4.6 mm I.D.); mobile phase, 8% (v/v) acetonitrile; flow-rate 1.0 ml/min; detetector, Shimadzu FLD-1 fluorometric detector (cut-off filter EM-5); sample size, 10 μl (5 nmol of each sugar).

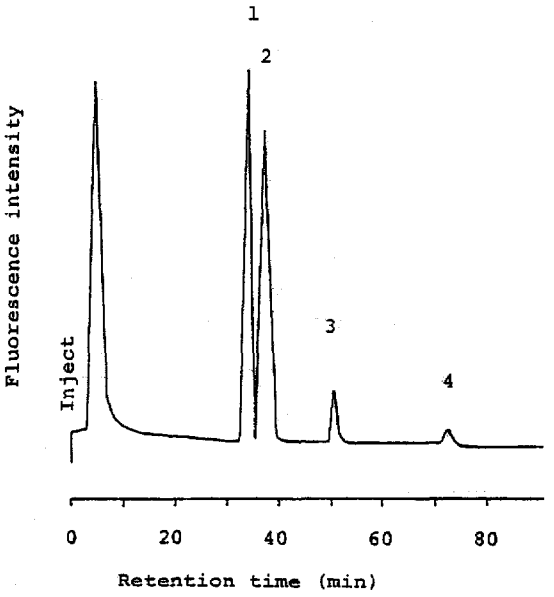

Fig. 3. Chromatogram of neutral sugars in Tamm-Horsfall urinary glycoproteins: 1 = Gal; 2 = Man; 3 = Fuc; 4 = NBD-NH_2. Conditions as in Fig. 2.

usual way and hydrolyzed by the method of Arakawa *et al.*[12]. As shown in Fig. 3, it was confirmed that T-H-GPs contained Gal, Man and Fuc as the component sugars. The composition of these sugars in human urinary T-H-GPs was 5.8:6.1:1.0, in agreement with results reported by Stevenson and Kent[13]

As described above, the results obtained suggest the possibility of the identification and determination of neutral sugars in microgram amounts of glycoconjugates.

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