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Note

Simultaneous determination of the alditol acetate derivatives of amino and neutral sugars by gas–liquid chromatography

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Gas–liquid chromatography (GLC) of alditol acetates has frequently been used for the identification and quantification of neutral sugars in biological materials. Amino sugars are important components of the carbohydrate portion of glycoproteins, glycolipids, mucopolysaccharides and the free oligosaccharides of milk and urine, which are usually present together with neutral sugars. N-Acetylalditol acetates of amino sugars have too long retention times and poor responses on gas chromatography[1,2]. The use of a column of Poly A-103[3] gives better results for the alditol acetates of amino sugars, but the alditol acetates of neutral sugars are not resolved. Hence an efficient method for the simultaneous determination of amino and neutral sugars as alditol acetates has not yet been established, although the derivative is stable and the alditol derivatives give fewer GLC peaks than do the trimethylsilyl derivatives of aldose, possessing two anomers of the pyranose and furanose rings.

To overcome these problems, we selectively methylated amino groups in the alditols of amino sugars by reaction with formaldehyde and sodium cyanoborohydride[4]. The mixture of amino sugars (glucosamine, galactosamine and mannosamine) and neutral sugars was reduced with sodium borohydride, and amino groups were treated with formaldehyde and sodium cyanoborohydride, then acetylated. The resulting derivatives were used as samples for GLC.

EXPERIMENTAL

Reagents

All chemicals were of analytical-reagent grade. Sodium cyanoborohydride was purchased from Kanto Chemicals (Tokyo, Japan), and the other reagents were obtained from Nakarai Chemicals (Kyoto, Japan).

Apparatus

GLC was performed with a Shimadzu 4CM chromatograph equipped with a hydrogen flame ionization detector, using a glass column (2 m × 0.3 cm I.D.) packed with 2% EGSS-X on Chromosorb W AW DMCS (60–80 mesh) at 195°C and a

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flow-rate of nitrogen of 45 ml/min. Peak areas and retention times were measured by use of a Shimadzu Chromatopac-E1A integrator. GLC–mass spectrometry (GLC–MS) was conducted with a JEOL JMS-D 300 instrument equipped with a glass column (1 m × 0.2 cm I.D.) packed with 3% ECNSS-M on Gas-Chrom Q (100–120 mesh) as described in a previous paper[5].

Evaporation

All evaporations were conducted under reduced pressure at bath temperatures not exceeding 40°C.

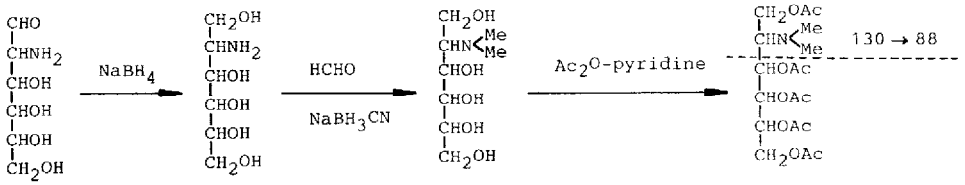
Preparation of amino and neutral sugars for gas chromatography

Monosaccharides (0.1–2 mg) were reduced with sodium borohydride (3 mg) in water (2 ml) for 2 h at room temperature, and the pH of the solution was adjusted to *ca.* 6 with 1 *M* acetic acid. Formaldehyde (0.5 ml of 0.7% aqueous solution) and sodium cyanoborohydride (3 mg) were added to the alditol solution and the mixture was allowed to stand for 3 h at room temperature. Excess of cyanoborohydride was destroyed by the addition of a few drops of concentrated hydrochloric acid and the solution was concentrated. Methanol (1 ml) was added and the solution was evaporated to remove the resulting boric acid as trimethyl borate, and then to dryness. After the last step had been repeated three times, the sample was stored *in vacuo* over potassium hydroxide pellets.

Peracetylation was carried out with acetic anhydride–pyridine (1:1) (2 ml) at 100°C for 2 h. Toluene (1 ml) was then added to the reaction mixture, which was evaporated to dryness. By this treatment, the residual acetic anhydride and pyridine were removed as an azeotropic mixture with toluene. The residue was dissolved in chloroform (5 ml) and the solution was washed twice with water (5 ml). The chloroform layer was evaporated to dryness, the residue was redissolved in chloroform (50 μl) and the solution was applied to the gas chromatograph.

RESULTS AND DISCUSSION

Fig. 1 shows the reaction scheme for an amino sugar. In the procedure, amino and neutral sugars were first converted into alditols with sodium borohydride, then the amino group of the alditols of amino sugars was methylated with formaldehyde and sodium cyanoborohydride at pH *ca.* 6. As an excess of formaldehyde causes by-products in the methylation, the aldehyde should be used as a dilute aqueous solution. Hydrochloric acid was added to the reaction mixture to destroy excess of cyanoborohydride, and the resulting boric acid was evaporated as trimethyl borate



(I)

Fig. 1. Reaction scheme for an amino sugar. Me = methyl; Ac = acetyl.

by adding excess of methanol *in vacuo*. The N-methylated alditols thus obtained were acetylated together with neutral alditols in the mixture of acetic anhydride and pyridine. After acetylation, acetic anhydride and pyridine in the reaction mixture were readily evaporated as an azeotropic mixture[6] by adding excess of toluene *in vacuo*, and the resulting residue was dissolved in chloroform. The chloroform layer was washed with water to remove a small amount of by-products arising from formaldehyde, and applied to the gas chromatograph for analysis.

On a column of 3% ECNSS-M[7,8], used for the alditol acetates of neutral sugars, the peaks of the three amino sugar derivatives in the sugar mixture were detected between those of xylitol and mannitol acetates, which had much shorter retention times than those of N-acetylalditol acetates. However, glucosamine and mannosamine derivatives gave almost the same retention time, so we made further investigations.

On a column of 2% EGSS-X[9], the derivatives of neutral sugars (L-rhamnose, L-fucose, D-ribose, L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose), D-glucosamine and D-galactosamine were separated satisfactorily, and the derivatives of D-glucosamine and D-mannosamine were able to be detected as a shoulder peak, as shown in Fig. 2. Table I gives the retention times. Amino sugar derivatives were shown to be in the N-dimethyl form (compound I in Fig. 1) by the characteristic mass fragments at m/z 130 and 88 ($130-CH_2=C=O$) using GLC–MS.

The response factors (peak area of each sample/peak area of internal standard)

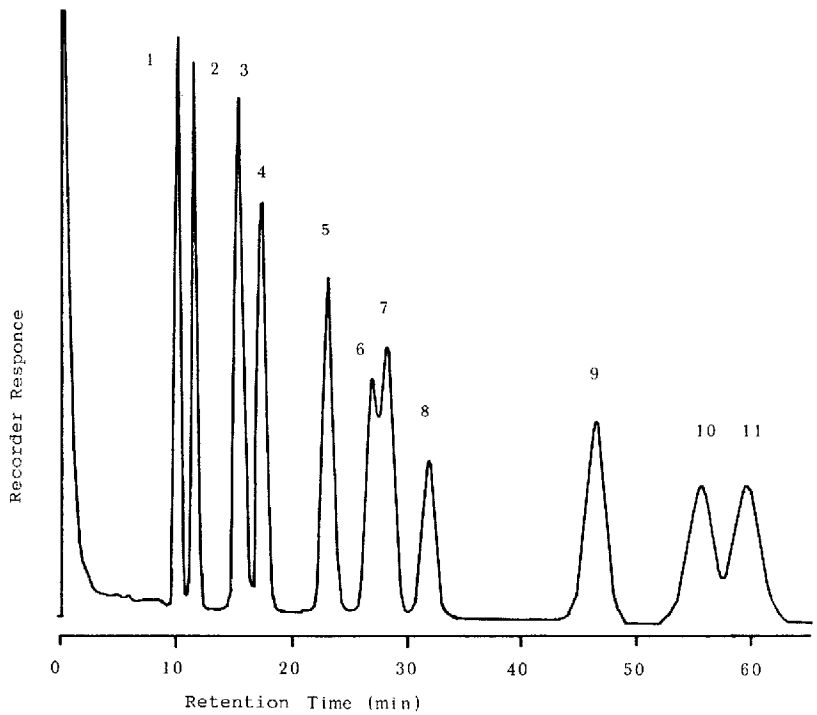


Fig. 2. Separation of the derivatives of amino and neutral sugars by GLC. Peaks as in Table I.

TABLE I

RETENTION TIMES OF ALDITOL ACETATES OF AMINO AND NEUTRAL SUGARS ON A 2% EGSS-X COLUMN

*Peak No.**	*Neutral sugar (as alditol acetate)*	*Retention time (min)*	*Peak No.**	*N-Dimethyl amino sugar (as alditol acetate)*	*Retention time (min)*
1	Rhamnitol	9.64	6	Glucosaminitol	24.81
2	Fucitol	10.98	7	Mannosaminitol	25.88
3	Ribitol	14.41	8	Galactosaminitol	29.08
4	Arabinitol	16.28			
5	Xylitol	21.34			
9	Mannitol	42.21			
10	Galactitol	50.08			
11	Glucitol	53.94			

* See Fig. 2.

of glucosamine, galactosamine and mannosamine to xylose as an internal standard were 0.97, 1.03 and 0.97, respectively.

In conclusion, the proposed method is suitable for the determination of mixtures of neutral sugars, glucosamine, galactosamine and mannosamine.

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