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Note

Use of a flame thermionic detector in the determination of glucosamine and galactosamine in glycoconjugates by gas chromatography

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Gas chromatography (GC) is a convenient tool for the elucidation of carbohydrate constituents of glycoproteins or glycolipids. Recently, trifluoroacetic anhydride (TFAA) has been used as an acylating reagent in the GC separation and determination of alditols[1,2]. Acylated alditols have also been determined on a XF-1105 column in GC of human gastric mucopolysaccharides[3]. Tamura *et al.*[4] have developed this acylation method for an analysis of amino sugars. However, it seems likely that no satisfactory result has been obtained in the determination of amino sugars from natural origins, probably because of the instability to the trifluoroacetyl derivatives.

This paper describes a rapid determination method for glucosamine and galactosamine using a nitrogen-specific flame thermionic detector (FTD), and gives appropriate GC conditions for the simultaneous determination of the neutral and amino sugars in glycoconjugates of human origins.

EXPERIMENTAL

Materials

Standard neutral and amino sugars used were all commercial products: group A, L-fucose, L-arabinose, D-mannose, D-galactose, D-glucosamine hydrochloride, D-galactosamine hydrochloride; group B, L-rhamnose, D-xylose, D-glucose, D-mannosamine hydrochloride. The following biological materials were also investigated: crystalline ovomucoid (trypsin inhibitor from chicken egg white; Sigma, St. Louis, MO, U.S.A.); urinary crude glycopeptides[5] obtained from blood group O-secretor (U-SY), A-secretor (U-MS) and B-secretor (U-TS); partially purified glycoproteins[6] of blood group H-active (No. 5, Fr. I), A-active (No. 3, Fr. III) and B-active (No. 2, Fr. I) substances isolated from human ovarian cyst fluids according to the phenol extraction method[7]. *p*-Aminophenol hydrochloride was used as an internal standard.

A Shimadzu GC-7A gas chromatograph with FTD or a flame-ionization detector (FID) was used. The glass chromatographic columns were as follows: a, 2 m × 3 mm I.D., packed with 5% OV-101 on Chromosorb W AW DMCS (60–80 mesh); b, 1.5 m × 3 mm I.D., with 2% XF-1105 on Gas-Chrom P (60–80 mesh); c, 2 m × 3 mm I.D., with 2% QF-1 on Gas-Chrom P (60–80 mesh), modified by the chromatographic system[4].

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Methods

Preparation of derivatized sample solution for GC. To 1 ml of an aqueous solution containing 30–200 μg of each of the neutral and amino sugars (group A) was added 1 ml of 2% $NaBH_4$ containing 0.025 *M* NaOH in water, and the mixture was allowed to stand for 2 h at room temperature. After reaction, the excess of $NaBH_4$ was destroyed by adding 0.5 *N* HCl and the solution was concentrated, with several additions of methanol to remove methyl borate. The residue was dissolved in a small amount of water and the solution was applied to a column (8 × 1 cm I.D.) of QAE-Sephadex gels (borate form)[8], followed by elution with 25–30 ml of water. The eluent was discarded. The adsorbed alditols and amino alcohols on the gels were then eluted with concentrated HCl–methanol (1:24 v/v) until the eluent was completely replaced by this mixture, monitoring with a pH-test paper. The eluent was pooled and concentrated on an evaporator to yield a sugar alcohol fraction containing a considerable amount of methyl borate.

The concentrated residue was again dissolved in a small amount of methanol, followed by evaporation with several additions of the solvent to remove the borate. To the residue were added 0.2 ml of *p*-aminophenol in ethanol solution (360 μg/ml), and the mixture was completely transferred to a small capped glass tube (5 cm × 5 mm I.D.) by repeated washings with a small amount of methanol and water. The mixed solution was concentrated to dryness by evaporation and standing in a vacuum desiccator over P_2O_5. The dried matter was suspended in 50 μl of ethyl acetate, and 50 μl of TFAA were added with cooling on ice. The mixture was allowed to stand at room temperature for 30 min, and an aliquot of the reacted solution (1–2 μl) was applied to the chromatographic column.

The standard sugar mixture of group B was similarly treated and the prepared trifluoroacetate derivatives were analyzed by GC.

GC conditions. Standard calibration graphs for the hexosamine assays were made by using the OV-101 column under the following operating conditions in the FTD system: injector and detector temperature, 210°C; column temperature, 120°C (isothermal); nitrogen carrier gas flow-rate, 50 ml/min; hydrogen flow-rate, 6–8 ml/min; air flow-rate, 220 ml/min; electrical heating on an alkali-metal salt bed. A conventional FID system was also employed. The calibration graphs for the determination of the neutral sugars were obtained on the XF-1105 column with temperature programming from 100°C to 160°C at 2°C/min in the FID system under the operating conditions indicated in Table I. The internal standard (*p*-aminophenol hydrochloride) was used for both calibrations. Relative retention times (R_t, min) of the neutral or amino sugars on the chromatographic columns a–c were recorded with respect to the standard compound (Table I).

Determination of carbohydrate constituents of glycoconjugates. A 1–2 mg amount of each of the urinary crude glycopeptides, human ovarian cyst glycoproteins and ovomucoid were hydrolyzed in 4 *N* trifluoroacetic acid (TFA) for 16 h at 100°C in sealed tubes. After hydrolysis, TFA was removed by evaporation with repeated additions of water. The hydrolyzates were each treated with the reducing reagent, and the resultant alditols and amino sugar alcohols were subjected to clean-up on the QAE-Sephadex column, as described for the standard sugar alcohols. To the final methanol–HCl eluent was added an ethanol solution of *p*-aminophenol (36–180 μg). The eluent was evaporated with repeated additions of methanol to remove methyl

TABLE I

RELATIVE RETENTION TIMES IN SEPARATIONS OF SUGAR MIXTURES

Gas chromatograhhic conditions as in the text.

Sugar	*Column a, OV-101 120°C*	*Column b, XF-1105*		*Column c, QF-1 170°C*
		100–160°C	*170°C*	
Rhamnose	—*	0.60	—*	—*
Fucose	0.27	0.67	—	0.56
Arabinose	0.25	0.87	—	0.65
Xylose	—	0.95	—	—
Mannose	0.34	1.12	—	0.91
Glucose	—	1.27	—	—
Galactose	0.37	1.32	—	1.06
Glucosamine	0.63	dec.**	4.12	2.29
Galactosamine	0.71	dec.	4.71	2.62
Mannosamine	0.69	dec.	5.29	2.33
p-Aminophenol (min)	1.00 (11.5)	1.00 (16.2)	1.00 (1.7)	1.00 (3.4)

* Not examined.

** Considerably decomposed during the development.

borate. The residue was transferred to a small glass tube, followed by acylation with TFAA as described above. A portion of the resulting solution (1–2 μl) was used for chromatography. Determinations of the hexosamine contents of these biological samples were carried out on the OV-101 column (a) at 120°C in the FTD system. Determination of the content of neutral sugars were performed on the XF-1105 column (b) at 100–160°C (2°C/min) in the FID system.

RESULTS AND DISCUSSION

Fig. 1 shows the calibration graphs for glucosamine and galactosamine obtained by using FTD or FID. From these the detection limits for glucosamine or galactosamine were *ca.* 20 ng with the FTD system and *ca.* 100 ng with the FID. The sensitivity of FTD for the amino compounds may generally be varied by changing the detector arrangement; thus it is possible to raise the detector response at least ten times higher than the FID response by electrically heating the bed of an alkali source.

Table I gives the R_t values of the neutral and amino sugars on these chromatographic columns, with respect to *p*-aminophenol. The neutral monosaccharides were satisfactorily separated and determined on the XF-1105 column with temperature programming from 100°C to 160°C, using the FID system. However, amino sugars were considerably decomposed during the development under this conditions. There was little decomposition or adsorption of the amino sugars on this column in a rapid analysis and good separations were obtained at 170°C, with only slightly inferior reproducibility. Determination of glucosamine and galactosamine was satisfactorily performed with column a packed with the non-polar stationary phase OV-101 without degradation or adsorption on the column at the lower temperature (120°C). However, in the simultaneous separation of glucosamine, mannosamine, and galactosamine the R_t values obtained were 0.63, 0.69 and 0.71, respectively.

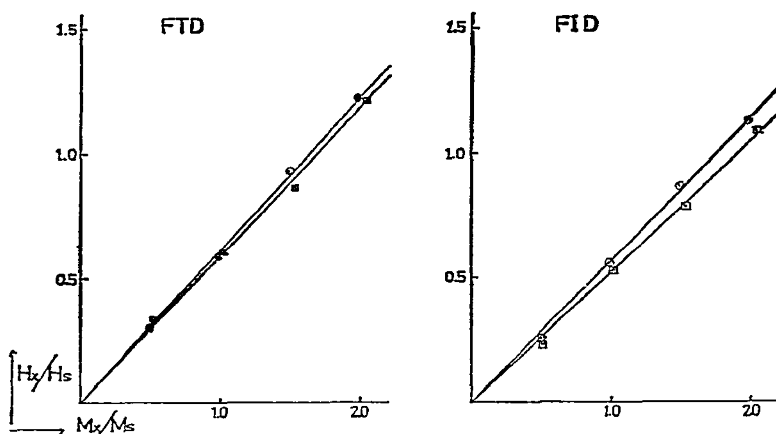

Fig. 1. Calibration graphs for determination of glucosamine (○) and galactosamine (□). Internal standard: *p*-aminophenol hydrochloride (72 μg). H_x/H_s, M_x/M_s = Peak height ratio and weight ratio of hexosamine to internal standard. Calibration was performed with a chromatographic column (2 m × 2 mm I.D.) packed with 5% OV-101 on Gas-Chrom P at 120°C. with either FTD (left graph) or FID (right graph).

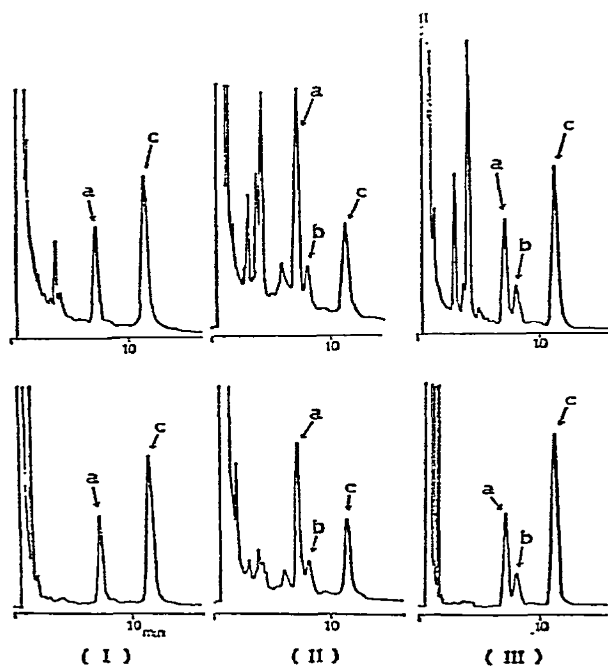

Fig. 2. Comparison of detector response to the hexosamine assays for ovomucoid (I), urinary glycopeptides (U-SY) (II) and ovarian cyst glycoprotein (No. 2, Fr. I) (III). Upper chromatograms: FID system. Lower chromatograms; FTD system. Analyses were accomplished with the OV-101 column at 120°C, and the data are indicated in Table II. Peaks: a = glucosamine; b = galactosamine; c = internal standard (*p*-aminophenol; I, II, 72 μg; III, 180 μg).

Fig. 2 shows the chromatographic profiles of ovomucoid (I), crude urinary fraction (U-SY) (II) and ovarian cyst glycoprotein (No. 2, Fr-I) (III) for comparison at the FID and FTD systems. The upper chromatogram was run with the FID and the lower with the FTD. In the FTD mode, glucosamine (a) and galactosamine (b) were selectively detected and well determined with the internal standard method. The standard *p*-aminophenol (c) was found to be suitable, having good stability and appropriate R_f value, for the determination of both amino sugars. Table II indicates the carbohydrate composition of the experimental biological materials. The monosaccharide composition of ovomucoid was estimated as about half that of the purified ovomucoid as reported in the literature[9]. This may be due to the heterogeneity of the mucoid or the difference in the hydrolysis conditions.

TABLE II

CARBOHYDRATE CONTENTS (%) IN GLYCOCONJUGATES

U.G. = Urinary crude glycopeptides; O.G. = ovarian cyst glycoproteins. The origins of the materials and the experimental conditions are as in the text. A minus sign indicates a value below the detection limit.

Material	*Fucose*	*Mannose*	*Galactose*	*Glucosamine (as hydrochloride)*	*Galactosamine (as hydrochloride)*
Ovomucoid	—	2.7	0.7	8.6	—
U.G. (U-SY)	1.2	1.9	3.1	7.0	1.5
U.G. (U-MS)	0.6	1.6	1.8	6.3	2.0
U.G. (U-TS)	0.6	1.0	2.1	4.2	0.9
O.G. (No. 5, Fr. I)	14.0	1.0	14.4	18.0	3.4
O.G. (No. 3, Fr. III)	12.0	—	24.6	30.1	7.7
O.G. (No. 2, Fr. I)	3.5	1.0	9.5	7.7	2.8

Acylation of neutral and amino sugar alcohols is often performed with acetic anhydride, as for the chemical characterization of glycoprotein[10] or glycolipid[11]. Acetylated amino alcohols are chemically more stable than the trifluoroacetylated compounds. However, they may lead to problems such as time-consuming preparation or column temperatures over 220°C in GC. Nevertheless, the trifluoroacetates of glucosamine and galactosamine were satisfactorily analyzed at the lower column temperature without any degradation. There are a few problems in the separation and determination of the amino sugars in glycoconjugates in this experimental system: (1) reduction to the amino alcohols requires the somewhat rigorous treatment with 2% $NaBH_4$ in 0.025 *M* NaOH; (2) impurities must be completely removed from the amino alcohols with QAE-Sephadex gel (borate form) filtration; (3) a suitable chromatographic column packed with a non-polar stationary phase such as the silicone OV-101 is required for a satisfactory determination.

The usefulness of FTD, already acknowledged in the determination of nitrogen- and phosphorus-containing compounds[12,13], has again been demonstrated in this hexosamine assay.

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