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SENSITIVE DETERMINATION OF SUGARS UTILIZING PACKED CAPILLARY COLUMNS AND ELECTRON CAPTURE DETECTION

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SUMMARY

A sensitive method is described for carbohydrate analysis in the picomole range. Methanolysis and trifluoroacetylation were employed to generate methyl N,O-trifluoroacetyl glucosides of sugars, which were separated by gas chromatography on 7 m × 0.5 mm packed capillary columns and quantitated by an electron capture detector. Sample transfer steps are avaoided by performing both methanolysis and trifluoroacetylation in a single capillary tube. Routine analyses of α_1-acid glycoprotein, fetuin, and carcinoembryonic antigen have been achieved with amounts as low as 0.1 μg of glycoprotein.

INTRODUCTION

The solution of many current structural problems in biochemistry necessitates precise determinations on microsamples. Application of fluorescence techniques has made possible quantitation of amino acids in proteins in the picomolar range[1,2]. Comparable picomolar sensitivity for the carbohydrate components of glycoproteins can now be achieved by the techniques described here. This method employs methanolysis, conversion of the methyl glycosides to their N,O-trifluoroacetyl (TFA) derivatives, separation by gas chromatography in packed capillary columns, and quantitation by electron capture detection.

EXPERIMENTAL

Reagents

A sample of α_1-acid glycoprotein was obtained from Dr. Yu-Lee Hao of the American Red Blood Research Laboratory, Bethesda, Md., U.S.A. Carcinoembryonic antigen (CEA) was purified as described elsewhere[3]. Mesoinositol was purchased from Pierce (Rockford, Ill., U.S.A.). All other carbonydrates, fetuin, and TFA anhydride were obtained from Sigma (St. Louis, Mo., U.S.A.).

Anhydrous methanol was prepared as described elsewhere[4].

Sample preparation

A glycoprotein sample which contained 10–1000 pmoles of individual sugars

was placed in a capillary tube (1.6 × 50 mm; Kallestad Lab.) with 100 pmoles of mesoinositol as internal standard. After lyophilization, 30 μl of 1.5 *M* methanolic HCl was added. The capillary tube was sealed and heated at 80° for 18 h. The tube was opened, and the solvent was evaporated under vacuum. TFA anhydride (5–10 μl) was added to the sample. The capillary tube was sealed again and heated for 10 min at 145°. A 1–5-μl portion was injected into the gas chromatograph.

Gas chromatography

Standard columns (1.8 m × 2 mm I.D.) were packed with 3% OV-210 on Supelcoport, 80–100 mesh (Supelco, Bellefonte, Pa., U.S.A.).

Capillary columns (7.0 m × 0.5 mm I.D.) were drawn from soft glass tubing (8 mm O.D., 3 mm I.D.) on a Shimadzu GDM-1 glass-drawing machine. These columns were sealed at one end with a glass wool plug and packed with Supelcoport, 80–100 mesh. After packing, 10 ml of a 1% solution of OV-210 (Applied Science Labs., State College, Pa., U.S.A.) in dichloromethane–acetone (4:1) was passed through the column under a nitrogen pressure of 1.5 kg/cm^2. The solvent was evaporated by passing a stream of nitrogen through the column. The column was conditioned at 210° overnight with nitrogen at a flow-rate of 2 ml/min and installed into a Hewlett-Packard 5708 gas chromatograph equipped with a 5709 electron capture detector and a homemade stream splitting device. Argon–methane (95:5) was used as a carrier gas at a flow-rate between 1 and 2 ml/min. A stream splitting ratio of 1:1 was used. The same gas mixture was used as make-up gas (30 ml/min) at the end of the column. The oven temperature was programmed at 1°/min (2°/min for shorter runs) from 120–210°. The injection port was heated at 200° and the detector at 300°. The peak areas were calculated by an Autolab System IV computing integrator.

RESULTS

Methanolysis

The methanolysis step was carried out in capillary tubes small enough for picomolar samples. They offer the advantage that they can be sealed for methanolysis, opened for the evaporation of the solvent, and sealed again after addition of TFA anhydride. Thus, no transfer of the sample is necessary until it is injected into the gas chromatograph. Since methanolic HCl is converted to methyl chloride almost completely under these methanolysis conditions[4], the solvent was evaporated without prior neuralization. Because the precooled capillary tubes prevented bubbling out of the solvent, a strong vacuum pump could be employed, thus reducing this step to a few minutes and allowing less time for degradation reactions reported for this step[5].

Trifluoroacetylation

In our experiments, almost complete N-deacetylation of amino sugars occurred during methanolysis, which was shown by gas–liquid chromatography of their trimethylsilyl (TMS) ethers. N-Deacetylated amino sugars require rigorous derivatization conditions to obtain complete N-trifluoroacetylation of free amino groups[6,7]. Therefore, N,O-trifluoroacetylation was carried out at 145° for 10 min. We found that the N-acetyl group of N-acetylaminosugars and their methyl glycosides is exchanged with a TFA group under these conditions. When methyl N-acetylglucosaminide is re-

acted under milder trifluoroacetylation conditions (20 min at 60°), a mixture of methyl tris(O-trifluoroacetyl)-2-trifluoroacetamido-2-deoxyglucoside (retention time of main peak = 61.6 min; see Table I) and methyl tris(O-trifluroacetyl)-2-acetamide-2-deoxyglucoside (retention time of main peak = 77.0 min) is obtained. The latter compound is not obtained under the more rigorous reaction conditions (10 min, 145°) as is apparent from Fig. 1.

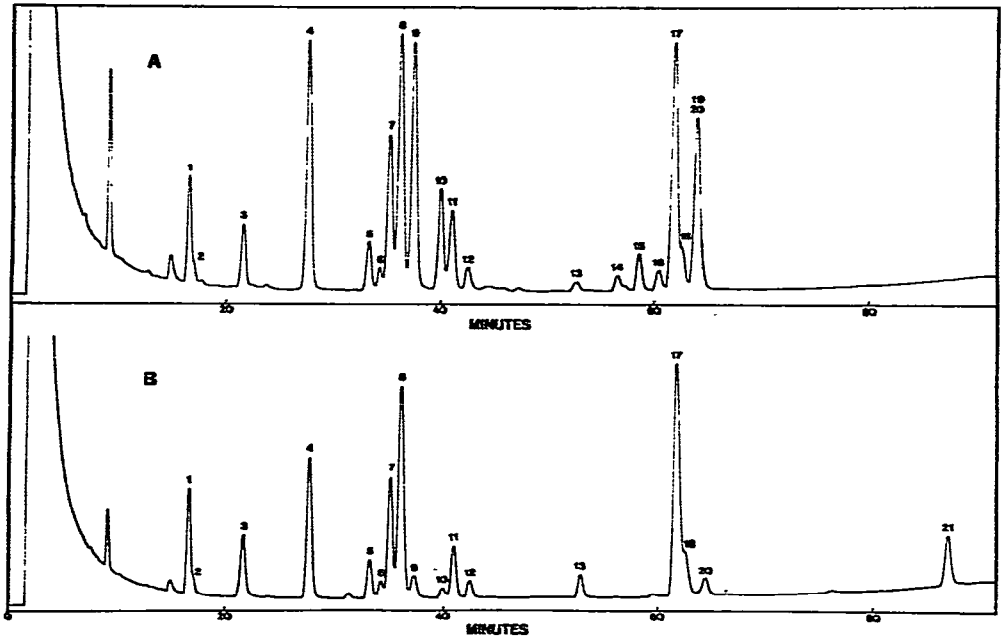

Fig. 1. Gas chromatograms of methyl N,O-TFA-glycosides on 7.0 m × 0.5 mm packed capillary column coated with a 1% solution of OV-210. Temperature program was 1°/min from 120 to 210°. (A) A sugar standard of 200 pmoles of each sugar and 100 pmoles of mesoinositol. Peaks are fucose (1, 2, 3); galactose (5, 6, 7, 11); mesoinositol (4); mannose (8, 12); glucose (9, 10); N-acetylglucosamine (13, 17, 18, 20); N-acetylgalactosamine (14, 15, 16, 19); and N-acetylneuraminic acid (21). (B) A 0.3-μg CEA sample with 50 pmoles mesoinositol as internal standard.

Gas chromatography

Standard columns packed with commercially available 3% OV-210 on Supelcoport, 80–100 mesh, and nitrogen as a carrier gas with a flame ionization detector gave poor separation of the TFA methyl glycosides of galactose, mannose, and glucose. Quantitation of these hexoses was possible only if they were present in ideal proportions. The much more sensitive electron capture detector required argon–methane (95:5) as a carrier gas. This gas mixture slightly broadened the peaks giving even worse separation.

Sharp peaks were obtained when a packed capillary column was employed. It was necessary to prepare such columns by packing them with the dry support and then coating the walls and support by passing OV-210 in dichloromethane–acetone through the packed column. Empty capillaries could not be packed with the sticky precoated support.

TABLE I

RETENTION TIME AND PERCENT ISOMER COMPOSITION OF O-METHYL GLYCOSIDES AND ALDITOLS ANALYZED AS N,O-TFA DERIVATIVES*

Results were obtained with a 7.0 m × 0.5 mm packed capillary column coated *in situ* with a 1% solution of OV-210. Temperature program was 1°/min from 120–210°. Response factors were linear over the entire analysis range.

Parent monosaccharide	*Retention time of isomer (min)*	*Percentage distribution*
Rhamnose	14.0	87.0
	18.7	13.0
Fucose	16.4	50.1
	16.5	15.3
	21.3	34.6
Ribose	18.6	18.9
	20.0	8.9
	20.6	15.8
	24.9	56.4
Arabinose	17.3	74.6
	17.5	
	19.9	25.4
Xylose	16.6	60.9
	17.4	3.6
	18.8	35.5
Lyxose	16.1	75.0
	17.6	19.0
	20.3	6.0
Galactose	33.1	14.7
	34.2	6.3
	35.0	50.2
	40.9	28.8
Mannose	36.0	92.2
	42.4	7.8
Glucose	37.2	72.5
	39.8	27.5
N-Acetylglucosamine*,**	52.7	6.4
	61.6	88.5
	61.8	
	64.2	5.1
N-Acetylgalactosamine**	56.3	5.2
	58.4	15.4
	60.2	8.5
	63.8	70.9
N-Acetylneuraminic acid	86.5	100.0
Arabitol	27.2	100.0
Mesoinositol	27.5	100.0
Fucitol	23.2	100.0
Mannitol	32.6	100.0
Sorbitol	33.7	100.0
Galactitol	35.2	100.0
Glucosaminitol	50.2	100.0
Galactosaminitol	53.3	100.0

* Ratio of isomers differed slightly for standard N-acetylglucosamine and glycoprotein sample.

** Entirely different ratio of isomers was obtained, when standard contained hexosamines instead of N-acetylhexosamines.

The relative retention times of the isomeric glycosides could be influenced by varying the concentration of the OV-210 in the coating solution. Elution at higher temperatures from columns with a higher concentration of coating even changed the order of elution for some peaks by comparison with that obtained at lower temperatures from columns with a lower concentration of coating. The best separation of the neutral hexoses was obtained from columns which were coated with a 1% solution of OV-210 in dichloromethane–acetone (4:1) and used with a temperature program from 120–210°.

Typical chromatograms obtained under these conditions for a sugar standard and a CEA sample are presented in Fig. 1. Retention times and percentage distribution of isomers are listed in Table I. Under these gas chromatographic conditions, two of the three fucose peaks (peaks 1 and 2) are not separated from each other. The second and third glucosamine peaks (peaks 17 and 18) elute at nearly the same time. These peak overlaps do not affect the calculations. However, the overlap of the fourth glucosamine peak (peak 20) (5.1% of the total glucosamine) with the main galactosamine peak (peak 19) must be considered in the calculations.

Since methanolysis and/or treatment with TFA anhydride remove both N- and O-acetyl groups from variously substituted sialic acids, only a single peak is observed on gas chromatography for these derivatives. Excellent yields and reproducibility could be obtained after methanolysis of bound sialic acids, whereas only low yields and reproducibility were obtained after methanolysis of standard solutions of free sialic acids. Therefore, the response factor for sialic acid was calculated from reference sialoglycoproteins of known compositions, α_1-acid glycoprotein and fetuin. Sialic acid was completely destroyed at temperatures higher than 210° before it could be eluted from columns coated with a 2% solution of OV-210 programmed from 140 to 230°. N,O-TFA-hexosamines (non-glycosylated sugars) were destroyed on the same column at temperatures above 190°.

DISCUSSION

Methanolysis

Quantitative carbohydrate analysis requires cleavage of the glycosidic bonds in oligosaccharide chains. Methanolysis is preferred over hydrolysis since minimal destruction occurs under conditions effecting complete cleavage of the glycosidic bonds. Anomeric methyl furanosides and pyranosides are formed in a constant ratio determined by the methanolysis conditions. The release of monosaccharides from biological materials and their stability on methanolysis have been carefully investigated by Chambers and Clamp[5]. They found monosaccharades generally stable for 24 h in 1 *M* and 2 *M* methanolic HCl at both 85° and 100°. Carbohydrates were completely released from glycopeptides and oligosaccharides within 3 h in 1 *M* methanolic HCl at 85°. We used 1.5 *M* methanolic HCl at 80° for 18 h to effect optimal release of sugars from glycoprotein. Peptide bonds are generally resistant to methanolysis[7]. Amino acid derivatives did not contribute to our chromatograms; however, the possibility of free amino acids complicating the chromatograms in certain cases should not be overlooked. In the analyses of glycoproteins, Zanetta *et al.*[7] found the TFA derivatives of the methyl ester of asparagine and the dimethyl ester of aspartic acid in their chromatograms.

Derivatization of monosaccharides

Gas chromatography has become the most widely used method for separation of the component monosaccharides. However, conversion to volatile derivatives is first required. The trimethylsilyl (TMS) ethers of methyl glycosides[4,5,8] and the alditol acetates[9,10] have been most widely used.

All these procedures are rather long and involve reaction steps which could lead to errors if not performed properly. For example, amino sugars require a re-N-acetylation step to ensure their separation from neutral sugars in gas–liquid chromatography of TMS ethers of methyl glycosides. During the reacetylation step both N- and O-acetylation can occur[4]. The alditol acetate procedure requires an ion-exchange step for the complete removal of borate ions generated during the borohydride reduction step. The ion-exchange step involves sample loss through transfer steps and introduces impurities from the ion-exchange resin, especially noticeably at low sample concentrations.

These problems do not exist for the preparation of the TFA derivatives of methyl glycosides. The early workers reacted their samples with TFA anhydride under rather mild conditions which only O-trifluoroacetylated completely[11]. Pyridine was found to have a catalytic effect on the reaction[12]. The presence of different solvents can influence the proportions of the anomeric and configurational isomers of the generated TFA derivatives of free sugars[13]. The derivatization conditions specified in this paper give uniform results for the methyl glycosides of both neutral and amino sugars. Both N-TFA and O-TFA derivatives are obtained simultaneously by treatment with TFA anhydride immediately after evaporation of the solvent from the methanolysis step. The reaction mixture is analyzed by gas chromatography without further treatment.

Gas chromatography

Adequate separation of the several anomeric and configurational isomers of methyl N,O-TFA-glycosides of neutral and amino sugars by gas chromatography is very difficult to achieve. Ando and Yamakawa[6] described the use of a mixed coated column of SE-30 and XE-60 for the separation of the methyl N,O-TFA-glycosides. Since these investigators were mainly interested in glycolipids. they focused on the separation of the various galactose and glucose peaks. The mannose peaks, which elute in the same area but are usually not present in glycolipids, were ignored.

Zanetta *et al.*[7] introduced the OV-210 phase to gas chromatography of TFA derivatives. With OV-210 acceptable separation of almost all sugars present in biological materials was achieved. Their procedure used a regular column (2 m × 2 mm) packed with 5% OV-210 on Varoport 30, nitrogen as a carrier gas, and a flame ionization detector for the gas chromatographic analysis of TFA derivatives of sugars and their methyl glycosides. Mesoinositol (200 nmoles) was used as an internal standard.

With similar equipment we encountered difficulties in the separation and quantitation of N,O-TFA derivatives of non-glucosylated sugars. Some peaks for the various isomers overlapped in the chromatograms. Single peaks alone could not be used for quantitation, since the relative proportions of the isomers were found not to be constant. For these reasons we turned our attention to the use of N,O-TFA derivatives of methyl glycosides. The separation of the various peaks of galactose, mannose, and glucose was of special interest, since galactose and mannose are frequently found

together in glycoproteins, and glucose is frequently present as a contaminant (Sephadex, cellulose) especially in preparations in the microgram range. As indicated in the Results section, this approach proved successful. An important aspect of this method is the *in situ* coating of prepacked small diameter columns of sufficient length to achieve the desired resolution.

Detection method

The advantage of TFA derivatives in gas chromatography is based on their ability to evoke a strong response in electron capture detectors. Whereas early prototypes possessed a limited range with a non-linear response, the electron capture detector used in this work (Hewlett-Packard Model 5709) is based on a feedback pulsing frequency in which the frequency is directly related to the sample content. This model can detect as little as 10^{-12} g of sample. This performance greatly exceeds that achieved by the more conventional flame ionization detectors used for detection of compounds lacking the high electron capture efficiency of fluorine.

Tamura and Imanari[13] reported preliminary results using electron capture detection for TFA derivatives of aldoses in the ng range. Later these studies were extended to the reduction of aldoses to the corresponding alditols followed by trifluoroacetylation[14–16]. Shapira[11] used the same reduction and TFA derivatization for sugar mixtures, Ando and Yamakawa[6] applied methanolysis and trifluoroacetylation to the analysis of glycolipids, and Zanetta *et al.*[7] analyzed glycoproteins the same way. In these three papers, the authors did not apply electron capture detection.

CONCLUSION

The success achieved in the present study results from the application of the new model linear response electron capture detector, *in situ* coating of support packed in small diameter columns, and the use of a derivatization technique performed without sample transfer in a single capillary tube. This method permits rapid carbohydrate analysis in the picomole range for glycoproteins in 0.1–1.0 μg amounts. The analytical procedure involves no loss or contamination of sample due to transfer steps. All frequently encountered sugars (neutral sugars, hexosamines, and sialic acid) are separated and quantitated in a single gas chromatography run.

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