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SENSITIVE GAS CHROMATOGRAPHIC DETERMINATION OF THE MONOSACCHARIDE COMPOSITION OF GLYCOPROTEINS USING ELECTRON CAPTURE DETECTION

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SUMMARY

A gas chromatographic procedure using electron capture detection has been developed for the monosaccharide analysis of submicrogram quantities of glycoproteins. Methanolysis of the glycoproteins and subsequent trifluoroacetylation of the resultant methyl glycosides using N-methylbis(trifluoroacetamide) is carried out in a single capillary tube. The carbohydrate composition of 0.1 μg of several glycoproteins has been quantitatively determined by this method.

INTRODUCTION

Gas chromatography (GC), because of its ability to resolve complex mixtures, has become the method of choice for the determination of the monosaccharide composition of glycoproteins. The GC procedures in current use generally require about 200 μg of glycoprotein for an analysis. Since many biologically important glycoproteins are obtainable in only very minute quantities, there is a need for more sensitive methods for monosaccharide analysis.

GC methods are based upon the quantitative conversion of sugars into suitable volatile derivatives. Trimethylsilyl ethers, acetates, and trifluoroacetates are the most commonly employed derivatives. The advantage of trifluoroacetates is that they are more volatile than either acetates or trimethylsilyl ethers and therefore chromatographic separation may be carried out more rapidly and at lower temperatures. Furthermore, the use of trifluoroacetates makes it possible to carry out GC using highly sensitive electron capture detectors.

Vilkas *et al.*[12] reported GC studies of trifluoroacetate derivatives of free sugars, methyl glycosides and oligosaccharides using a flame ionization detector. Tamura and Imanari[11] subsequently described a GC procedure for the analysis of free sugars using an electron capture detector. Separation of alditols as their trifluoroacetate derivatives has been reported by several authors[5,9]. Using a flame ionization detector, Zanetta *et al.*[13] described a GC procedure for the analysis of monosaccharides in glycoproteins and glycolipids as the trifluoroacetate derivatives of the methyl glycosides.

None of these procedures have been widely employed. Many investigators have had difficulty in achieving satisfactory quantitation of sugars as trifluoroacetate derivatives. For example, in a study of partially methylated O-methylglucitols as their trifluoroacetates, satisfactory resolution could be obtained but quantitatively the results were not reproducible[7].

A GC procedure for the analysis of monosaccharides as the trifluoroacetate derivatives of their methyl glycosides, which employs electron capture detection, apparently has not been reported previously. The use of methyl glycosides has several distinct advantages over the use of reducing sugars or alditols. Methyl glycosides are more stable than the reducing sugars[2-4]. The analysis of either reducing sugars or alditols necessarily involves a hydrolysis procedure. Different hydrolytic conditions are frequently required for neutral sugars, hexosamines, and sialic acids. By contrast, a single methanolysis step is capable of releasing all three classes of sugars in high yields with little or no degradative losses[2]. In methanolysis, the acid is largely consumed during the procedure, being converted into methyl chloride[8]. Methanolysis gives rise to several isomeric methyl glycosides in defined ratios for a particular sugar. This makes it possible to identify the sugar on chromatograms, with increased confidence, from its characteristic relative peak area ratios.

The preparation of alditols involves several steps including hydrolysis, reduction, desalting, and removal of borate ions. These numerous steps and sample transfers are a disadvantage in the routine analysis of nanogram quantities of sugars. Preparation of the trifluoroacetate derivatives of methyl glycosides, on the other hand, can be a relatively simple matter. In this report a procedure is described in which both methanolysis and derivatization are carried out in a single capillary tube with no intermediate sample transfer steps.

Several different trifluoroacetylation procedures have been reported. Vilkas *et al.*[12] prepared derivatives using trifluoroacetic anhydride and sodium trifluoroacetate in acetonitrile. Tamura and Imanari[11] reported the use of a 20% solution of trifluoroacetic anhydride in tetrahydrofuran accompanied by mild heating. Shapira[19] employed trifluoroacetic anhydride containing catalytic amounts of pyridine. Zanetta *et al.*[13] used a 1:1 mixture of trifluoroacetic anhydride and dichloromethane and subjected samples to two 5 min heating periods at 150°.

Sullivan and Schewe[10] recently described the use of a new reagent, N-methyl-bis-(trifluoroacetamide) (MBTFA) for the derivitization of free sugars. Equal volumes of this reagent and pyridine were used for derivatization. Such a procedure was claimed to result in more reproducible derivatizations of the sugars than either trifluoroacetic anhydride or trifluoroacetylimidazole. The present communication describes the use of this reagent for trifluoroacetylation of all the monosaccharides normally occurring in mammalian glycoproteins and their subsequent separation and quantitation by GC using an electron capture detector.

EXPERIMENTAL

Reagents

Fucose, mannose, galactose and glucose were obtained from Pfanstiehl Laboratories (Waukegan, Ill., U.S.A.). All other sugars and alditols were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). High purity methanol and dimethyl-

formamide were obtained from Burdick and Jackson Laboratories (Muskegon, Mich., U.S.A.). N-Methylbis(trifluoroacetamide) (MBTFA) was obtained from Pierce Chemical Co. (Rockford, Ill., U.S.A.). A sample of α_1-acid glycoprotein was obtained from Dr. Yu-Lee Hao of the American National Red Cross Blood Research Laboratory (Bethesda, Md., U.S.A.). Carcinoembryonic antigen (CEA) was purified according to the method of Coligan *et al.*[3].

Methanolic HCl was prepared by passing dry hydrogen chloride gas through methanol until the concentration was 1.4 *N* (determined by titration with standard base). A 10% (w/v) solution of MBTFA in N,N-dimethylformamide was prepared weekly. The derivatization mixture was prepared immediately before use by adding 10 μl of pyridine to 100 μl of the 10% MBTFA solution.

Special equipment

Capillary tubes, sealed at one end, 90 mm × 0.8 mm I.D., were obtained from Fisher Scientific Co. (Atlanta, Ga., U.S.A.). Microcapillary pipets were prepared by drawing 50 μl glass disposable pipets in a flame. Suitably prepared microcapillary pipets could be inserted completely to the bottom of the capillary tubes. A miniature oven for the capillary reaction tubes was constructed from two interlocking aluminium blocks designed to fit into a Supelco block heater (Bellefonte, Pa., U.S.A.). The lower block contained a milled depression to hold the capillary tubes.

Sample preparation

Aqueous samples (0.5–20 μl) were placed in the capillary reaction tubes followed by the addition of 5 μl of a 100 μM solution of a mesoinositol internal standard. The capillaries were briefly centrifuged in a clinical centrifuge to ensure that all liquid was at the bottom of the tubes. The samples were placed in a vacuum desiccator and rapidly brought to dryness using continuous pumping. Approximately 10 μl of 1.4 *N* methanolic HCl was added to each capillary tube using a glass micropipet. The capillaries were then immediately sealed in a small flame and placed in the heating block oven for 24 h at 80°. The capillary tubes were broken open and the samples rapidly taken to dryness under vacuum. Approximately 10 μl of freshly prepared derivatization mixture was added to each capillary which was then resealed and vigorously shaken. Derivatization was allowed to take place at room temperature in the dark for at least 2 h. A 5 μl syringe (Hamilton Syringe Co., Reno, Nev., U.S.A.) was used to inject a 0.5 μl sample into the gas chromatograph. Derivatized samples could be stored for several days in a freezer (−20°).

Gas chromatography

A Varian Model 3700 gas chromatograph equipped with a ^{63}Ni linear electron capture detector was employed for the analysis. A 6 ft. glass column (2 mm I.D.) packed with 2% XF-1105 on Gas-Chrom P, 80–100 mesh (Supelco, Bellefonte, Pa., U.S.A.) was used. The carrier gas was zero grade nitrogen (Union Carbide Corp., Linde Div., New York, N.Y., U.S.A.) at a flow-rate of 44 ml/min. The pneumatics of the instrument were altered to permit the introduction of a nitrogen make-up gas through the normal hydrogen inlet of the detector base. The make-up nitrogen gas flow-rate was 215 ml/min. The chromatograph injector temperature was 200° and the detector was operated at 280°. The chromatograph was temperature programmed

from 80° to 200° at 2°/min. Peaks were automatically integrated by a Varian Model CDS III chromatography data system.

RESULTS

Fig. 1 illustrates typical chromatograms obtained for 0.25 μg samples of α_1-acid glycoprotein (Fig. 1A) and carcinoembryonic antigen (Fig. 1B). In order to obtain chromatograms free of extraneous peaks, it was extremely important to use ultra pure reagents and to inject the samples very rapidly employing an "on column" injection technique.

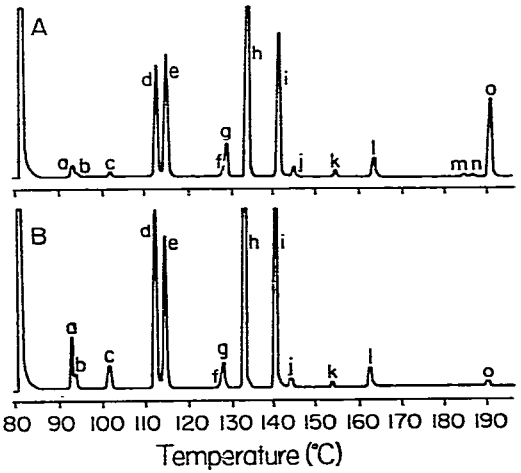

Fig. 1. Gas–liquid chromatograms of the methyl glycosides derived from 0.25 μg samples of α_1-acid glycoprotein (A) and carcinoembryonic antigen (B). A 6 ft. glass column (2 mm I.D.) packed with 2% XF-1105 on Gas-Chrom P, 80–100 mesh was employed. The carrier gas was zero grade nitrogen at a flow-rate of 44 ml/min. The flow-rate of the nitrogen make-up gas was 215 ml/min. The chromatograph injector temperature was 200° and the detector was operated at 280°. The chromatograph was temperature programmed from 80 to 200° at 2°/min. Peaks are as follows: (a) fucose-1; (b) fucose-2; (c) fucose-3; (d) mannose-1; (e) galactose-1; (f) mannose-2; (g) galactose-2; (h) meso-inositol (internal standard); (i) N-acetylglucosamine-1; (j) N-acetylglucosamine-2; (k) N-acetylglucosamine-3; (l) N-acetylglucosamine-4; (m) N-acetylneuraminic acid-1; (n) N-acetylneuraminic acid-2; (o) N-acetylneuraminic acid-3.

The retention times and relative peak areas of several methyl glycosides and alditols are given in Table I. Several sugars, including mannose, galactose, N-acetylglucosamine and N-acetylneuraminic acid, have one predominant peak. For these sugars, it was convenient to calculate relative response factors from the areas of single peaks. For other sugars, such as fucose and N-acetylgalactosamine, which have a number of relatively large peaks, it was more convenient to use the total peak area of several peaks for quantitation.

Standard response curves for 6 monosaccharides are given in Fig. 2. Response factors relative to mesoinositol were obtained from the slopes of each curve and are given in Table II along with the correlation coefficients for each curve. The values take into account the relative total peak area of the sugar peak or peaks used for quantita-

TABLE I

RETENTION TIMES AND PEAK AREA PROPORTIONS OF THE TRIFLUOROACETATE DERIVATIVES OF METHYL GLYCOSIDES AND ALDITOLS

Monosaccharide	*Peak No.*	*Retention time (min)*	*Percentage total peak area*
Fucose	1	6.43	56.4
	2	6.77	11.3
	3	10.73	32.3
Mannose	1	16.07	93.0
	2	23.55	7.0
Glucose	1	16.80	70.1
	2	20.67	29.9
Galactose	1	17.21	81.6
	2	23.89	18.4
N-Acetylglucosamine	1	30.18	82.5
	2	31.87	5.3
	3	36.63	2.5
	4	41.07	9.7
N-Acetylgalactosamine	1	30.87	14.8
	2	34.10	59.6
	3	35.93	17.7
	4	41.78	7.9
N-Acetylneuramic acid	1	51.28	2.1
	2	52.22	2.0
	3	54.35	95.9
Glucosamine-HCl	1	30.18	13.7
	2	31.87	34.6
	3	36.63	37.4
	4	41.07	14.3
Galactosamine-HCl	1	30.87	49.5
	2	34.10	16.9
	3	35.93	24.0
	4	41.78	9.6
Arabitol	1	18.50	100
Ribitol	1	17.06	100
Mannitol	1	23.03	100
Sorbitol	1	25.33	100
Galactitol	1	26.39	100
Mesoinositol	1	26.33	100

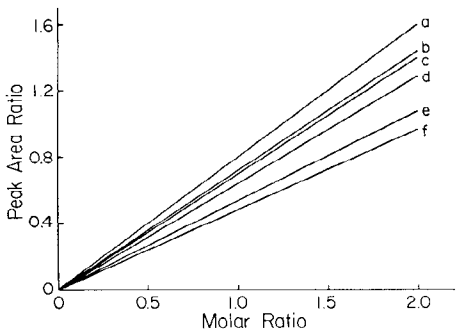

Fig. 2. Standard response curves for fucose, mannose, galactose, N-acetylglucosamine, N-acetylgalactosamine and N-acetylneuraminic acid. Twelve samples ranging from 50 pmoles to 2 nmoles of sugar were analyzed. All samples contained 0.5 nmoles of the mesoinositol internal standard. The curves correspond to (a) N-acetylgalactosamine (all peaks); (b) galactose-1; (c) mannose-1; (d) N-acetylglucosamine-1; (e) fucose (all peaks); and (f) N-acetylneuraminic acid-3. The slope of each curve and its respective correlation coefficient are given in Table II.

TABLE II

RELATIVE RESPONSE FACTORS OF THE TRIFLUOROACETATE DERIVATIVES OF THE METHYL GLYCOSIDE PEAKS USED FOR QUANTITATION

Peak(s) *	*Response factor* **	*Correlation coefficient*
Fucose-(Σ1,2,3)	0.54	0.995
Mannose-1	0.70	0.998
Galactose-1	0.72	0.997
N-Acetylglucosamine-1	0.65	0.997
N-Acetylgalactosamine-(Σ1,2,3,4)	0.80	0.996
N-Acetylneuraminic acid-3	0.49	0.997

* Peak assignments are those illustrated in Fig. 1.

** Response factors were calculated relative to mesoinositol and are the slopes of the lines shown in Fig. 2.

tion. A linear response was obtained for each sugar under the conditions described. It is important to note, however, that it was not possible to obtain a satisfactory linear response without employing a make-up gas as described in the Experimental section. The make-up gas reduced sensitivity by almost 80% and permitted analysis to be carried out in a linear region of electron capture detector response.

The precision of the procedure was demonstrated by analyzing several samples of α_1-aeid glycoprotein and carcinoembryonic antigen. The results of this experiment are given in Table III.

TABLE III

MONOSACCHARIDE COMPOSITION OF SAMPLE GLYCOPROTEINS

Monosaccharide	*Mole/10⁵ g glycoprotein*	
	*α_1-Acid glycoprotein** (mean ± S.D.)*	*Carcinoembryonic antigen*** (mean ± S.D.)*
Fucose	4.9 ± 0.7	56.9 ± 5.6
Mannose	32.1 ± 4.5	60.6 ± 6.5
Galactose	38.8 ± 5.0	73.7 ± 3.9
n-Acetylglucosamine	49.5 ± 4.8	105.5 ± 8.8
n-Acetylneuraminic acid	43.4 ± 3.9	4.1 ± 0.4

* A total of 18 samples (3 each of 4.5 μg, 2.5 μg, 1.0 μg, 0.5 μg, 0.25 μg, 0.125 μg) were analyzed.

** A total of 9 samples (3 each of 1.0 μg, 0.5 μg, 0.25 μg) were analyzed.

DISCUSSION

There are several advantages to the monosaccharide analysis procedure described in this paper. The method is rapid, simple to perform, and capable of very high sensitivity. Peaks arising from the protein portion of the glycoprotein were not observed. While the use of an electron capture detector greatly contributes to the sensitivity of the method, the microprocedure used for carrying out the methanolysis and trifluoroacetylation steps is also important. A particular advantage of using glass capillary tubes for sample preparation is that liquid samples may be taken to dryness

rapidly under vacuum without bubbling out of their tubes as happens with larger diameter tubes. This effect probably results from the relatively high surface tension of the sample liquid and its low air–liquid interfacial area. Capillary tubes that have been silanized are not satisfactory. Furthermore, the probability of sample contamination is greatly reduced by carrying out all reaction steps in a single capillary tube without any intermediate sample transfer steps. The ability to reseal a capillary tube in a flame several times is also an advantage.

Within rather wide limits, the volumes of methanolic HCl and trifluoroacetylating mixture used in this procedure are not critical and are generally estimated by the height of liquid in the capillary tube. However, it is important that these reagents be added rapidly with a minimum of exposure to air before sealing the capillary tubes.

Methanolysis, in principle, can yield four different glycosides for each sugar; the α- and β-anomers of both the methyl pyranosides and the methyl furanosides. The relative proportion of the different isomers for a given sugar is a function of the methanolysis conditions and usually is not altered by subsequent derivatization of the methyl glycosides. In some cases, the proportion of an isomer of a sugar may be so low as to be negligible. Often, however, two or more isomers may not be resolved under a particular set of chromatographic conditions and yield only a single peak. Thus, in the procedure described in this paper, galactose yields only two peaks whereas three peaks were observed when galactose was chromatographed as a trimethylsilylated methyl glycoside[4].

It was convenient to employ relative response factors for certain sugars based only upon their predominant peak. N-Acetylglucosamine and N-acetylgalactosamine gave almost the same relative molar response when the total peak areas of each sugar were used. Under the conditions employed, galactose always gave a slightly higher molar response than mannose. The relatively low molar responses of fucose and N-acetylneuraminic acid probably reflect partial destruction of these sugars during methanolysis. It is important, therefore, to analyze unknown samples under exactly the same conditions used for determining relative response factors. Relative response factors for a given column may not be identical with those obtained using another column, even though both were prepared from the same column packing material. It is therefore important to determine new response factors every time the GC column is replaced.

Zanetta *et al.*[13] claimed that no significant de-N-acetylation occurred during methanolysis and reported different retention times for N-acetylated and nonacetylated hexosamines. Other workers have reported almost complete de-N-acetylation during methanolysis[2,6,8]. In this work the same four peaks were obtained for a particular hexosamine whether or not it was originally N-acetylated. The relative peak area proportions, however, differed greatly. Presumably, protonation of the free amino group of an hexosamine strongly influences the proportion of different methyl glycosides formed.

It is possible that much of the difficulty experienced by earlier workers in achieving satisfactory quantitation resulted from metal-catalyzed decomposition of the trifluoroacetate derivatives in the injection port of the chromatograph. In this work, an "on-column" injection technique was employed. In addition, sample injection was carried out very rapidly in order to minimize exposure of the vaporized sample to the syringe needle.

This procedure is one of several methods being developed in this laboratory for the monosaccharide analysis of submicrogram quantities of glycoproteins.

CONCLUSION

The method described in this paper permits the qualitative and quantitative determination of the monosaccharide composition of submicrogram quantities of glycoproteins. The procedure is rapid and simple. Only the quantity of glycoprotein used and the addition of the internal standard need be carried out quantitatively.

The high sensitivity of this method makes it feasible to carry out monosaccharide analysis on the small amounts of material separated by polyacrylamide gel electrophoresis, gel isoelectric focusing and related techniques. Procedures for this are currently under development.

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