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ANALYSIS OF MONOSACCHARIDES BY GAS–LIQUID CHROMATOGRAPHY OF THE O-METHYL GLYCOSIDES AS TRIFLUOROACETATE DERIVATIVES

APPLICATION TO GLYCOPROTEINS AND GLYCOLIPIDS

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SUMMARY

A quantitative gas–liquid chromatographic method has been developed for the analysis of carbohydrates. The method involves the conversion of free or covalently bound monosaccharides into their O-methyl glycosides and the analysis of the glycosides as their trifluoroacetate derivatives. The procedure has been tested on standard monosaccharides, glycolipids and glycoproteins. The results show that all commonly occurring pentoses, hexoses, hexosamines, N-acetylhexosamines and sialic acids are accurately determined by the use of mesoinositol as the internal standard.

INTRODUCTION

The analysis of mixtures of simple monosaccharides by colorimetric methods has traditionally been difficult because of the lack of specificity of colour development, and because of the different extinction coefficients of different sugar chromophores. The accurate analysis of monosaccharide mixtures required a time-consuming preliminary separation of the components by using paper, column or thin-layer chromatography. More recently, most of these difficulties have been overcome by the simultaneous separation and estimation of monosaccharide mixtures in one step by gas–liquid chromatography (GLC) of the acetate or trimethylsilyl derivatives of the monosaccharides[1–3].

The application of these methods to the analysis of the monosaccharides in oligosaccharide chains requires preliminary enzymatic or chemical cleavage. While the specificity of the glycosidases makes them extremely useful in the determination of

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carbohydrate sequences, the most common techniques involve acid hydrolysis (for reviews, see refs. 4 and 5). However, the stabilities of the various glycoside bonds differ, and under the conditions required for the complete hydrolysis of glycoside bonds, extensive destruction of certain sugars may take place. The complete analysis of oligosaccharide chains normally requires four different hydrolyses, followed by time-consuming procedures to eliminate acid and separate the sugars. The difficulties involved in controlling all the steps in these procedures, and in correlating several different hydrolyses, are obvious. An analogous technique[6] is based on hydrolysis catalyzed by an ion-exchange resin in the acid form, but this technique suffers from the same inherent difficulties as the classical acid-catalyzed hydrolysis.

Cleavage of glycoside bonds can also be catalyzed by protons in anhydrous methanol (for a review, see ref. 7), liberating the O-methyl glycosides. Methanolysis has been used to study glycolipids[7–11] and glycoproteins[12]. It does not produce extensive degradation under the conditions necessary for complete hydrolysis, although secondary reactions with galactose and deacetylation of N-acetyl derivatives have been reported[7,12,13]. As all the components can be liberated in one step in the presence of an internal standard, the method is very accurate, particularly if coupled with the GLC analysis of the sugars.

This paper describes a new technique for the separation of sugars by GLC of the trifluoroacetate derivatives of the O-methyl glycosides. All common pentoses, hexoses, hexosamines (both acetylated and non-acetylated) and hexuronic and sialic acids can be determined by using an internal standard. The method has been tested on glycolipids, glycopeptides and glycoproteins of known compositions.

MATERIALS

Reagents

Standard carbohydrates were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), except mesoinositol, which was bought from F. Hoffman-La-Roche et Cie (Paris, France). Ovalbumin (Grade V) was obtained from Sigma Chemical Co. Gangliosides (GM_1 and GD_{1a}) and ovine submaxillary mucin (OSM) were generously supplied by Dr. G. TETTAMANTI (Istituto di Chimica Biologica, Università di Milano, Italy). Cerebrosides were obtained from Drs. L. SARLIÈVE and L. FREYSZ (Centre de Neurochimie du CNRS, Strasbourg, France). Ovalbumin glycopeptides were prepared as described by BRECKENRIDGE *et al.*[14].

Trifluoroacetic anhydride was purchased from Fluka AG (Buchs, Switzerland) and sodium dodecyl sulphate (SDS) from Fisher Scientific Co. (Fair Lawn, New Jersey, U.S.A.). All other chemicals were of Uvasol grade and were purchased from Merck AG (Darmstadt, G.F.R.).

Methanol was refluxed in an all-glass apparatus with magnesium turnings for 2 h, and then distilled to yield anhydrous methanol.

Chromatographic materials and apparatus

Liquid and stationary phases were purchased from Applied Science Laboratories Inc. (State College, Pa., U.S.A.) and Varian Aerograph Co. (Quartier Courtaboeuf, 91-Orsay, France).

All analyses were carried out with a Varian-Aerograph gas chromatograph,

Model 2100, equipped with a temperature programmer, a four-column oven, four flame-ionization detectors and two differential electrometers. The chromatographic tracings were monitored on two dual pen Varian Aerograph recorders, Model 20.

METHODS

Methanolysis

The solution of glycoprotein, glycopeptide, glycolipid or standard carbohydrate (1–500 μg of carbohydrate) was placed in a 2-ml conical Pyrex reaction tube which was stoppered with a PTFE-lined screw-cap (Sovirel, 92-Levallois-Perret, France). Mesoinositol (40 μg) was added and the sample was lyophilised and placed overnight in a desiccator over P_2O_5. The residue was taken up in 250–500 μl of 0.5 *M* methanolic HCl (prepared by dissolving gaseous HCl, which had been dried with H_2SO_4, in anhydrous methanol). The tube was stoppered, shaken vigorously and left to stand at 80° for 20 h. The methanolic HCl was removed under a stream of nitrogen at 50°. As soon as the sample was dry, the residue was trifluoroacetylated.

Trifluoroacetylation

The dried sample, containing 1–200 μg of free carbohydrate or O-methyl glycoside was dissolved in a mixture of dichloromethane (100 μl) and trifluoroacetic anhydride (100 μl). The tube was quickly stoppered and placed for 5 min at 150° in a sand-bath, which was kept in a fume-hood behind a protective shield. After 5 min, the tube was cooled to room temperature and then placed again in the sand-bath for 5 min. After cooling, the sample was ready for injection into the gas–liquid chromatograph.

Conditions for gas–liquid chromatography

Columns of 5% (w/w) OV-210 on Varaport 30 were used for the analysis of the trifluoroacetate derivatives of sugars and O-methyl glycosides. Varaport 30 (9.5 g) was placed in a round-bottomed vacuum flask and covered with methylene chloride. The liquid phase (0.5 g) was dissolved in methylene chloride and quantitatively transferred to the flask. The solvent was then slowly evaporated at room temperature, using a rotary evaporator, until the residue was only slightly damp. Final traces of

TABLE I

CONDITIONS FOR GAS–LIQUID CHROMATOGRAPHY

Condition	*Value*
Column temperature:	
Initial	90°
Final	190°
Temperature programming rate	1°/min
Detector cell temperature	250°
Injector temperature	225°
Gas flow rates:	
Hydrogen	30 ml/min
Air	300 ml/min
Nitrogen carrier gas	7.5 ml/min
Sample volume	1–10 μl

solvent were removed under vacuum by heating the flask at 60°. Clean, dry, U-shaped Pyrex columns (2 m × 2 mm I.D.) were packed by gently tapping. Plugs of silanized glass-wool were placed at both ends of the column to hold the packing in position. The columns were conditioned overnight at 240° with a carrier gas (nitrogen) flow-rate of 40 ml/min.

The operating conditions of the GLC are given in Table I.

RESULTS

Chromatographic resolution of mixtures of trifluoroacetates of standard sugars

Most simple monosaccharides were readily resolved by GLC of their trifluoroacetate derivatives, as shown in Fig. 1. The retention temperature of each sugar is given in Table II. The different isomers of the monosaccharides were identified by their relative proportions at equilibrium in water and by comparison with literature values for the trimethylsilyl derivatives[15].

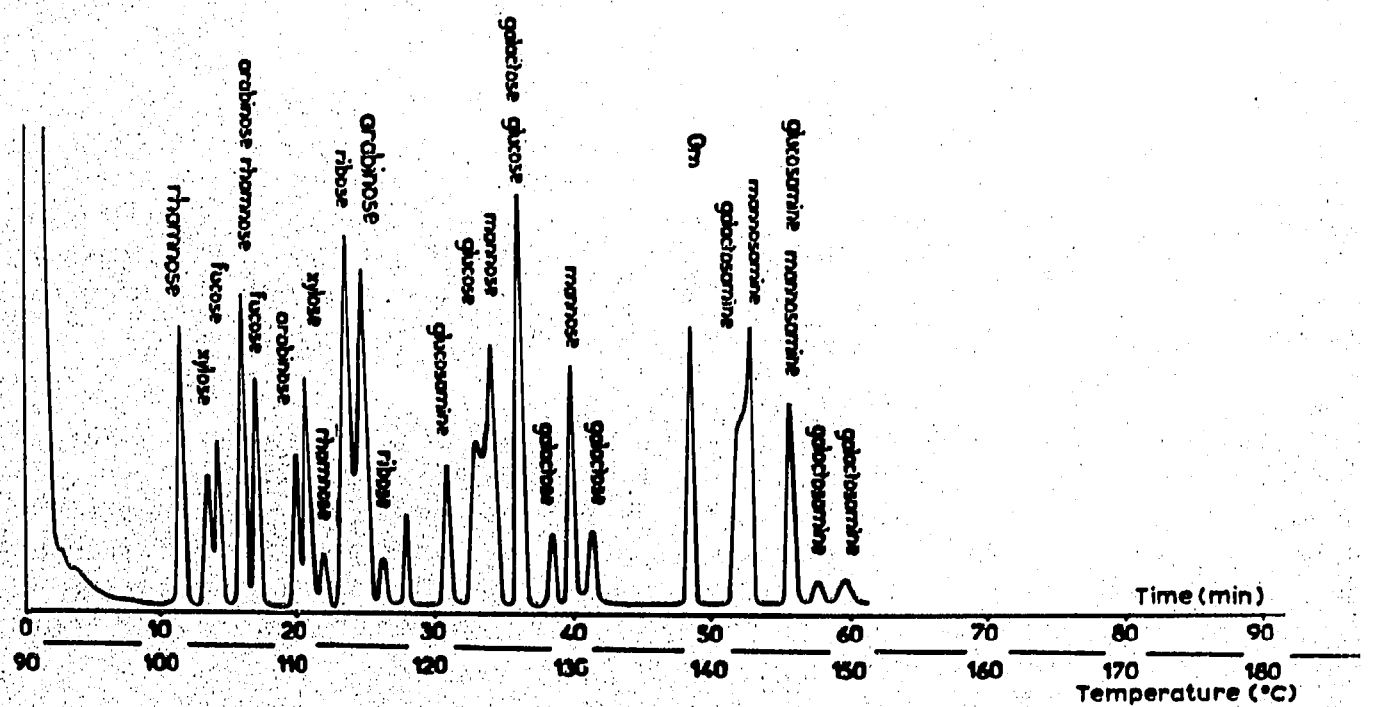

Fig. 1. GLC resolution of the trifluoroacetate derivatives of standard sugars. GLC conditions as defined in Table I. Orn = ornithine methyl ester used as internal standard.

A single analysis was sufficient to resolve a mixture of pentoses, hexoses and hexosamines. In most other methods that have been published[1-3], such a separation has not been achieved. However GHEORGHIU AND OETTE[15] have obtained similar separations using ethylene glycol succinate packings and trimethylsilyl derivatives. Using our system, the trifluoroacetates of α-glucose and of β-glucose overlapped with the trifluoroacetates of β-mannose and of α-galactose, respectively. Mannose was always revealed by the peak corresponding to α-mannopyranose while galactose was detected by the peak corresponding to β-galactopyranose and galactofuranose. Mannose and galactose could be quantitatively determined in the presence of glucose

TABLE II

RETENTION TEMPERATURE AND MASS DISTRIBUTION OF MONOSACCHARIDES ANALYZED AS TRIFLUOROACETATE DERIVATIVES

Monosaccharide	*Isomer*	*Retention temperature of isomer (°C)*	*Percentage distribution of isomer*[b]
Rhamnose	[a]	102	38.8
		107	56.0
		112	5.2
Arabinose	β	107	43.8
	α	110	50.5
	γ	115.5	5.7
Fucose	β	105	60.8
	α	108	39.2
Ribose	[a]	114	78.2
		118	21.8
Xylose	β	104	47.5
	α	111	40.2
	γ	117	12.3
Galactose	α	126	67.5
	γ	128	14.3
	β	132	18.2
Mannose	β	124	68.4
	α	130	31.6
Glucose	α	123	38.2
	β	126	61.8
Mannosamine	β	143	69.2
	α	146	30.8
Glucosamine	β	121	70.1
	α	148	29.9
Galactosamine	α	142	67.5
	γ	148	14.3
	β	150	18.2
Mannitol	—	118.5	100
Galactitol	—	122.5	100
Sorbitol	—	120	100
Inositol	—	114	100

[a] Configuration of isomers not defined.
[b] Based on the percentage area of the peaks on the chromatogram.

by taking into account the relative proportions of the isomers at equilibrium in water. Glucose could be identified and determined by difference. This problem was overcome when the trifluoroacetate derivatives of O-methyl glycosides were analyzed as described later. It should also be noted that hexoses could be resolved from the corresponding sugar alcohols after direct trifluoroacetylation (Table II).

Chromatographic resolution of the trifluoracetate derivatives of O-methyl glycosides

Superior resolution of the monosaccharides could be obtained by GLC of the trifluoroacetate derivatives of the O-methyl glycosides. O-Methyl glycosides of the monosaccharides were formed under conditions identical with those used for the methanolysis of oligosaccharide chains, and subsequently trifluoroacetylated. The resolution of the derivatives is shown in Fig. 2. The retention temperatures and percentage distributions of the isomers are given in Table III.

The separation in a single analysis was excellent for most naturally occurring pentoses, hexoses, hexosamines, N-acetylhexosamines, sialic acids (N-glycolyl- and

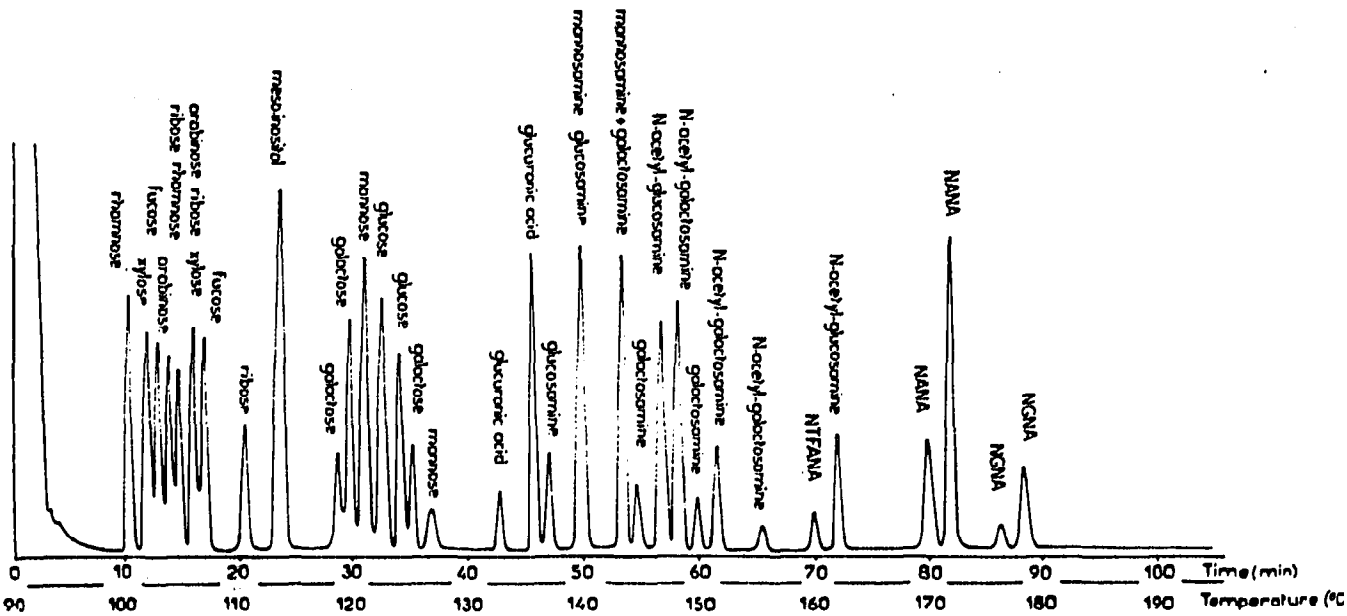

Fig. 2. GLC resolution of the trifluoroacetate derivatives of standard O-methyl glycosides. GLC conditions as defined in Table I. NANA = N-acetylneuraminic acid; NGNA = N-glycolylneuraminic acid; NTFANA = N-trifluoroacetylneuraminic acid.

N-acetylneuraminic acids) and glucuronic acid. The few instances of overlapping were not important as they involved sugars that usually do not occur in the same oligosaccharide chains. The present separation was considerably better than the reported separations of O-methyl glycosides as their trimethylsilyl derivatives[7,12,13] and superior to the separation of the trifluoroacetate derivatives of the simple sugars. However, galacturonic acid was not completely separated from mesoinositol. To determine galacturonic acid, mannitol could be used as the internal standard. It should be noted that O-methylhexosamines were separated from the corresponding N-acetylhexosaminyl derivatives.

Determination of the relative molar response

All molar responses were determined relative to mesoinositol, which was eluted between the pentoses and the hexoses and served as an excellent internal standard (see, however, Table III). An equimolar mixture (250 nmole) of mesoinositol and of each carbohydrate, or its O-methyl glycoside, was treated with trifluoroacetic anhydride, directly or after methanolysis. Aliquots of 5 μl were injected into the gas chromatograph. The area of each peak was determined by planimetry and the areas of the various peaks representing all the isomers of a monosaccharide were summed. The ratio between the area corresponding to the monosaccharide and mesoinositol was defined as the relative molar response (RMR). The results (the means of ten experiments) are given in Table IV.

The RMR of each sugar generally corresponded extremely well with the values expected on the basis of the number of carbon atoms. However, under all conditions, the RMRs for the N-acetylhexosamines and non-acetylated hexosamines were lower than expected.

A similar phenomenon has already been described for the trimethylsilyl deriva-

TABLE III

RETENTION TEMPERATURE AND MASS DISTRIBUTION OF O-METHYL GLYCOSIDES ANALYZED AS TRIFLUOROACETATE DERIVATIVES

Parent monosaccharide[a]	*Retention temperature of isomer*[b] *(°C)*	*Percentage distribution of isomer*[c]
Rhamnose	100.5	91.5
	105.0	8.5
Arabinose	104.0	75.5
	106.0	24.5
Fucose	103.0	59.1
	103.2	28.4
	107.0	12.5
Ribose	105.0	13.3
	106.0	20.9
	110.5	65.8
Xylose	102.0	57.0
	106.0	43.0
Mesoinositol	114.0	100.0
Mannitol	118.5	100.0
Galactitol	122.5	100.0
Sorbitol	120.0	100.0
Galactose	119.0	31.0
	120.0	52.5
	125.5	16.5
Mannose	121.5	92.0
	127.0	8.0
Glucose	123.0	61.5
	124.5	38.5
Mannosamine	140.0	62.0
	143.5	38.0
Glucosamine	140.0	76.0
	137.5	24.0
Galactosamine	143.5	78.5
	145.5	13.0
	150.0	8.5
N-Acetylglucosamine	147.0	62.5
	162.0	37.5
N-Acetylgalactosamine	148.5	78.0
	151.5	12.3
	155.0	9.7
N-Acetylneuraminic acid	170.0	25.0
	172.0	75.0
Glucuronic acid	133.0	84.7
	137.5	15.3
Galacturonic acid	115.0	86.3
	119.0	13.7

[a] Parent monosaccharide converted to O-methyl glycoside by methanolysis.
[b] Configuration of isomers not defined.
[c] Based on the percentage area of the peaks on the chromatogram.

tives of N-acetylhexosamines and O-methyl glycosides of N-acetylhexosamines[13,16,17]. It is possible that the nitrogen plays some role in the depression of the detector response. The detector response was linear between 0.1 and 10 μg of injected sugar.

Application to gangliosides

The gangliosides (GM_1 and GD_{1a} from rat brain) were separately subjected to methanolysis for various times between 1 and 24 h in the presence of mesoinositol as

TABLE IV

RELATIVE MOLAR RESPONSE OF THE TRIFLUOROACETATE DERIVATIVES OF MONOSACCHARIDES AND O-METHYL GLYCOSIDES

Parent monosaccharide	*Relative molar response of trifluoroacetates*[a]		
	Monosaccharides	*O-Methyl glycosides*	
		Time of methanolysis	
		1 h	*20 h*
Rhamnose	630	660	665
Arabinose	750	770	765
Fucose	830	810	810
Ribose	750	760	765
Xylose	750	750	750
Mesoinositol	1000	1000	1000
Mannitol	1000	1000	1000
Galactitol	1000	1000	1000
Sorbitol	1000	1000	1000
Galactose	890	920	932
Mannose	905	945	950
Glucose	910	950	945
Mannosamine	795	835	841
Glucosamine	810	825	830
Galactosamine	785	830	837
N-Acetylglucosamine	780	810	805
N-Acetylgalactosamine	790	805	815
N-Acetylneuraminic acid	[b]	1200	1130
Glucuronic acid	[b]	1100	1100
Galacturonic acid	[b]	1100	1100

[a] Results are the average of 10 determinations. The error was less than 2%.
[b] Not determined.

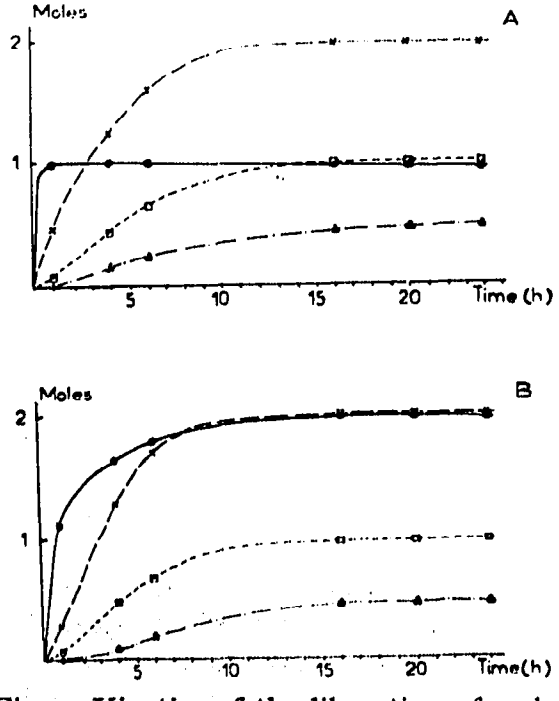

Fig. 3. Kinetics of the liberation of carbohydrates from gangliosides by methanolysis. Carbohydrates were analyzed by GLC of the trifluoroacetate derivatives of O-methyl glycosides in the presence of mesoinositol as internal standard. A, Ganglioside GM_1 from rat brain. B, Ganglioside GD_{1a} from rat brain. ●, NANA; ×, galactose; □, N-acetylgalactosamine; ▲, glucose. Values are expressed in moles of sugar/mole of ganglioside.

the internal standard. The liberation of monosaccharides was followed by GLC analysis of the trifluoroacetate derivatives of the products, as shown in Fig. 3.

The results obtained after methanolysis for 20 h showed that the number of moles of galactose, N-acetylgalactosamine and N-acetylneuraminic acid were in excellent agreement with the theoretical values for each ganglioside. This indicated that methanolysis for 20 h liberated these carbohydrates completely and that the RMRs determined on standard sugars were entirely reliable.

Sialic acid was liberated very rapidly and there was no significant degradation after liberation, even after methanolysis for 24 h. However, the liberation of glucose from GM_1 and in GD_{1a} was considerably less than the theoretical values (44% and 48%, respectively). This confirms the observation of SWEELEY AND VANCE[7] on the stability of the bond between sphingosine and glucose to methanolysis. The incomplete cleavage of this bond was shown by the presence on the chromatograph of a peak that had the same retention time as glucosylsphingosine (Fig. 4). The identity of this compound was confirmed by the isolation of glucosylsphingosine according to CARTER AND FUJINO[18] and gas chromatographic analysis of the trifluoroacetate derivatives before and after methanolysis. The products of methanolysis contained the trifluoroacetate derivatives of glucose, sphingosine and glucosylsphingosine. The trifluoroacetate derivative of glucosylsphingosine was eluted more rapidly than that of sphingosine owing to the greater number of groups available for trifluoroacetylation. When the same experiment was carried out on galactosylsphingosine, there was complete liberation of galactose and sphingosine.

When gangliosides were subjected to the standard conditions of methanolysis, trifluoroacetylation and gas–liquid chromatography, some peaks occurred on the chromatogram (Fig. 4), which were identified as being due to fatty acid methyl esters. This confirmed the observations of other workers[7,11] that the amide bond between sphingosine and fatty acids is broken during methanolysis. As the retention

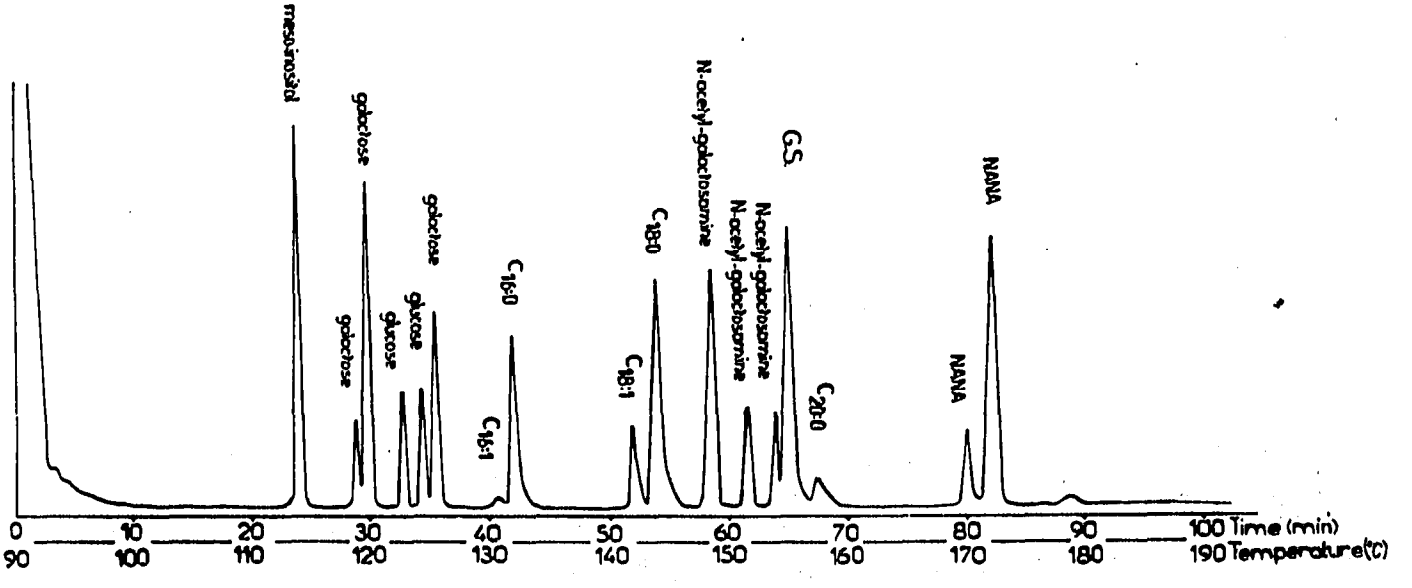

Fig. 4. GLC after trifluoroacetylation of the methanolysis residue from ganglioside GM_1 from rat brain. Conditions of chromatography described in the text. G.S. = glucosphingosine (psychosine).

TABLE V

RETENTION TEMPERATURE AND RELATIVE MOLAR RESPONSE OF FATTY ACID METHYL ESTERS

Fatty acid[a]	*Retention temperature (°C)*	*RMR*[b]
C14:0	116.0	810
C16:0	132.0	900
C16:1	130.5	895
C18:0	144.0	990
C18:1	142.0	990
C20:0	157.5	1150
C22:0	168.0	1300
C24:0	180.0	1420

[a] Fatty acids identified by total number of carbon atoms and double bonds.
[b] Relative molar response to mesoinositol taken as 1000.

temperatures of the fatty acid methyl esters were different from those of the trifluoroacetates of the O-methyl glycosides, sugars and fatty acids could be determined in a single chromatographic run. Retention temperatures and RMRs are given in Table V. From the kinetics of liberation of these fatty acid methyl esters during methanolysis (Fig. 5), it can be seen that they are completely liberated after methanolysis for 20 h. The precision of measurement of the fatty acids was about 5%, as the peaks of the fatty acid methyl esters showed some tailing on the polar column. Sphingosine also gave volatile trifluoroacetate derivatives, which were eluted at higher temperatures (200–220°). Work is now in progress to determine precisely the retention temperatures and the RMRs of the major sphingosines. This method of analysis should provide many advantages in the analysis of glycolipids, as it is possible, in one analysis, to identify and determine all the components of gangliosides and cerebrosides.

The analysis of gangliosides can be accomplished in a much shorter time by using a temperature programme of 4°/min and a carrier gas flow-rate of 35 ml/min. Galactose and glucose are still resolved under these conditions.

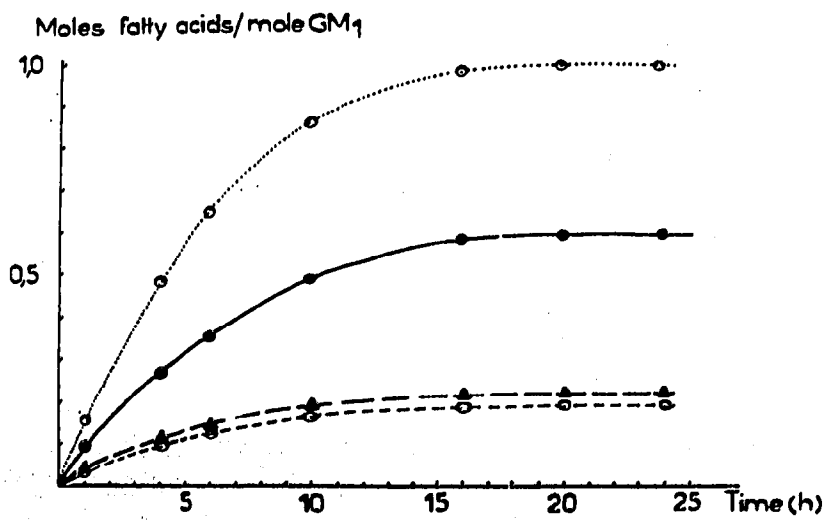

Fig. 5. Kinetics of liberation of fatty-acid methyl esters by methanolysis of ganglioside GM_1 from rat brain. Analysis by GLC in the presence of carbohydrates and mesoinositol as internal standard. ●, $C_{18:0}$; ▲, $C_{18:1}$; O---O, $C_{18:0} + C_{18:1}$; O...O, total.

Application to glycoproteins and glycopeptides

The method has been tested on two well defined glycoproteins. The carbohydrate composition of ovine submaxillary mucin (OSM), ovalbumin and ovalbumin glycopeptides, prepared as described elsewhere[14], are presented in Table VI. In all instances, the content of monosaccharides was very close to the theoretical value for the glycoproteins[12,20]. Most differences were probably due to the well known phenomenon of microheterogeneity of the carbohydrate chains[12].

TABLE VI

CARBOHYDRATE COMPOSITION OF GLYCOPROTEINS AND GLYCOPEPTIDES

Monosaccharide	*OSM*[a]		*Ovalbumin*[b]			
	Found	*Reported*[c]	*Protein*		*Glycopeptides*	
			Found	*Reported*[d]	*Fraction I*[e]	*Fraction II*[e]
Fucose	0.40	0.40	—	—	—	—
Mannose	0.72	0.15	5.10	5.0	5.10	5.10
Galactose	0.95	0.30	—	—	—	—
Glucose	Trace	—	—	—	—	—
N-Acetyl glucosamine	—	—	3.85	3.0	4.40	3.55
N-Acetyl galactosamine	14.70	15.0	—	—	—	—
N-Acetyl neuraminic acid	26.00	25.40	—	—	—	—

[a] Values are expressed as a percentage of dried ovine submaxillary mucin (OSM).
[b] Values are expressed in mole/mole of protein or glycopeptide.
[c] According to GRAHAM AND GOTTSCHALK[19].
[d] According to JOHANSEN *et al.*[20].
[e] Fraction I glycopeptides have a slightly higher molecular weight than fraction II glycopeptides, as they are eluted first during Sephadex G-50 gel filtration.

It should be noted that the analysis of the sugars of glycoproteins by the present techniques does not require the precipitation of the protein after the methanolysis. The chromatograms showed no extraneous peaks when glycoproteins that had been dialyzed and delipidized were used. Interference by free amino acids or small peptides is not serious, provided that such compounds are present at concentrations of less than 10% of those of the sugars. In the methanolysis, no free amino acids or peptides are liberated from the proteins, as the peptide bonds are not cleaved.

The kinetics of liberation of the monosaccharides from ovalbumin and the ovalbumin glycopeptides (Fig. 6) show considerable differences. The initial release of mannose and, in particular, N-acetylglucosamine are slower in the glycoprotein than in the glycopeptide, but after prolonged methanolysis the analyses of sugars were identical for both the glycoproteins and glycopeptides. This observation could indicate the occurrence of steric hindrance of the methanolysis by the protein. However, it is also possible that the glycoprotein is initially insoluble in the reaction mixture, as vigorous agitation is necessary to obtain a homogeneous suspension.

With the glycopeptides, two additional peaks were observed. They corresponded to the trifluoroacetate derivatives of the dimethyl ester of aspartate (retention temperature 95°; RMR to mesoinositol 0.40) and of the methyl ester of asparagine (retention temperature 105°; RMR to mesoinositol 0.40). These two peaks could be derived

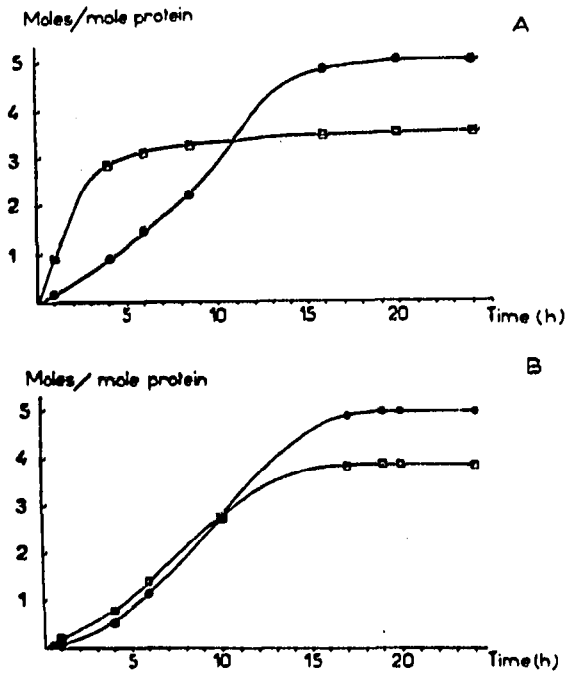

Fig. 6. Kinetics of liberation of carbohydrate from ovalbumin and ovalbumin glycopeptides by methanolysis. Products were analyzed by GLC of the trifluoroacetate derivatives of the O-methyl glycosides in the presence of mesoinositol as internal standard. A, glycopeptides of ovalbumin; B, ovalbumin. ●, mannose; □, N-acetylglucosamine.

from the asparagine linked to N-acetylglucosamine. The presence of these two components was verified by dansylation of the glycopeptides. After methanolysis, dansyl-aspartic acid and dansylasparagine were identified by using techniques that we have described previously[21].

DISCUSSION

The results indicate that the trifluoroacetate derivatives are preferable for GLC analysis to the acetyl or trimethylsilyl derivatives that have been used in other investigations[4–9,12]. The trifluoroacetates are more volatile, allowing the use of a more polar stationary phase without the risk of adsorption of material on the column. Trifluoroacetic anhydride blocks simultaneously the hydroxyl and free amino groups, whereas the classical trimethylsilylation blocks only the hydroxyl groups. However, the blocking of the free amino groups requires a relatively high temperature (3 h at 100° or 5 min at 150°).

It should also be noted that the trifluoroacetate derivatives are more easily injected into the chromatograph, as no precipitate is formed. It is also possible to store these derivatives in the reaction tube for a long period of time. We obtained the same results when using a sample that was analyzed immediately or one month after trifluoroacetylation. However, it is imperative to maintain the derivatives in a completely anhydrous environment and in the presence of trifluoroacetic anhydride.

The conditions that we used for the methanolysis were chosen on the basis of the thorough studies of SWEELEY AND VANCE[7]. There were no extensive differences between the RMRs (Table IV) of the trifluoroacetate derivatives of sugars that had

been subjected to methanolysis for 1 h or 20 h, and similar sugars that had been directly trifluoroacetylated. This indicated that methanolysis did not cause significant destruction of the sugars. In particular, methanolysis of either the simple monosaccharides or the gangliosides did not cause significant destruction of acidic sugars. Contrary to claims made by other investigators[7,12,13], the present conditions did not cause significant (less than 2%) deacylation of the O-methyl derivatives of the N-acetylhexosamines (or N-acetylneuraminic acid), as there was almost no peak of the O-methyl derivatives of free hexosamines (or neuraminic acid) present.

Satisfactory results for the methanolysis of the simple sugars and glycolipids were obtained only when experiments were carried out in an absolutely anhydrous medium. If the step of drying under P_2O_5 after lyophilization was omitted, a partial deacylation of the O-methyl derivatives of the N-acetylhexosamines and N-acetylneuraminic acid was observed. It was also necessary to prepare and conserve the 0.5 *M* HCl in methanol in absolutely anhydrous conditions. The presence of trace amounts of moisture probably explains the deacetylation observed by previous workers[7,12,13] for the silyl derivatives of the O-methyl sugars.

In contrast to the simple sugars and gangliosides, in analyses of the glycoproteins and glycopeptides partial deacetylation of the hexosamines occurred even though the conditions for the methanolysis were the same as those used for the gangliosides. Two explanations could be advanced to account for this observation. The first concerns a possible lability of the N-acetyl group of the hexosamine, which is linked with the amino acid. The other interpretation is that it is more difficult to completely eliminate all moisture from a glycoprotein sample.

A point of interest is the utility of this method for the analysis of the carbohydrate components of membrane glycoproteins that have been solubilized by detergents, in particular sodium dodecyl sulphate (SDS). When pure SDS was subjected to methanolysis and trifluoracetylation, four major peaks were obtained on the gas chromatogram in the proportions 3.1:72.5:17.4:7.0. Their retention temperatures were 90°, 97.5°, 111.5° and 125.5°, respectively. The last peak was eluted at the same temperature as one peak of galactose and its tailing interfered also with the last peak of mannose. However, the SDS could readily be eliminated by a differential extraction. After the methanolysis, when SDS is present in the form of methyl esters and the sugars are present in the form of O-methyl glycosides, extraction with hexane (3 × 1 volumes) removed the SDS, but left the sugars and the internal standard in the methanolic phase. In this way, the sugars of ovalbumin were analyzed in the presence of large amounts of SDS.

The trifluoroacetate derivatives of the monosaccharides are extremely volatile, as they are eluted in the solvent front on a 2-m column of 3% SE-30 at 90°. It therefore appears that oligosaccharides, and the products of partial methanolysis of glycoproteins and glycopeptides, might be resolved by GLC of their trifluoroacetate derivatives. Further experiments are in progress to test these possibilities.

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REFERENCES

1 C. T. BISHOP, *Adv. Carbohyd. Chem.*, 19 (1964) 95.
2 W. W. WELLS, C. C. SWEELEY AND R. BENTLEY, in H. A. SZYMANSKI (Editor), *Biomedical Applications of Gas Chromatography*, Plenum Press, New York, 1964, p. 169.
3 C. T. BISHOP, in D. GLICK (Editor), *Methods of Biomedical Analysis*, Vol. X, Interscience, New York, 1962, p. 1.
4 G. SPIRO, in S. P. COLOWICK AND N. O. KAPLAN (Editors), *Methods in Enzymology*, Vol. 8, Academic Press, New York, 1966, p. 3.
5 J. MONTREUIL AND G. SPIK, *Microdosage des Glucides*, Vols. I–III, Laboratoire de Chimie Biologique de la Faculté des Sciences, Lille, 1963, 1967 and 1968.
6 W. F. LEHNHARDT AND R. J. WINZLER, *J. Chromatogr.*, 34 (1968) 471.
7 C. C. SWEELEY AND D. E. VANCE, in G. V. MARINETTI (Editor), *Lipid Chromatographic Analysis*, Vol. I, Marcel Dekker, New York, 1967, p. 465.
8 D. E. VANCE AND C. C. SWEELEY, *J. Lipid Res.*, 8 (1967) 621.
9 R. K. YU AND R. W. LEEDEN, *J. Lipid Res.*, 11 (1970) 506.
10 G. DAWSON, S. F. KEMP, A. C. STOOLMILLER AND A. DORFMAN, *Biochem. Biophys. Res. Commun.*, 44 (1971) 687.
11 N. F. AVROVA AND S. A. ZABELINSKII, *J. Neurochem.*, 18 (1971) 675.
12 G. A. LEVVY, A. J. HAY, J. CONCHIE AND I. STRACHAN, *Biochim. Biophys. Acta*, 222 (1970) 333.
13 R. E. CHAMBERS AND J. R. CLAMP, *Biochem. J.*, 125 (1971) 1009.
14 W. C. BRECKENRIDGE, J. E. BRECKENRIDGE AND I. G. MORGAN, in A. N. DAVISON, I. G. MORGAN AND P. MANDEL (Editors), *Structural and Functional Proteins of the Nervous System*, Plenum Press, New York, in press.
15 TH. GHEORGHIU AND K. OETTE, *J. Chromatogr.*, 48 (1970) 430.
16 T. BHATTI, R. E. CHAMBERS AND J. R. CLAMP, *Biochim. Biophys. Acta*, 222 (1970) 339.
17 J. R. CLAMP, G. DAWSON AND L. HOUGH, *Biochim. Biophys. Acta*, 148 (1967) 342.
18 H. E. CARTER AND Y. FUJINO, *J. Biol. Chem.*, 221 (1956) 879.
19 E. R. B. GRAHAM AND A. GOTTSCHALK, *Biochim. Biophys. Acta*, 38 (1960) 513.
20 P. G. JOHANSEN, R. D. MARSHALL, A. NEUBERGER, *Biochem. J.*, 78 (1961) 518.
21 J. P. ZANETTA, G. VINCENDON, P. MANDEL AND G. GOMBOS, *J. Chromatogr.*, 51 (1970) 441.