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ANALYSIS OF METHYL GLYCOSIDES AS THEIR TRIMETHYLSILYL ETHERS: ON-COLUMN RE-N-ACETYLATION AND IMPROVED GAS–LIQUID CHROMATOGRAPHIC SEPARATION

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SUMMARY

A rapid procedure is described for methyl glycoside derivatization for gas–liquid chromatographic analysis. Re-N-acetylation is carried out after the trimethylsilylation step directly in a gas chromatographic column by injecting acetic anhydride together with a sample. A quantitative acetylation of amino groups occurs, whereas trimethylsilylated hydroxyls remain intact. The entire procedure of treating a glycoprotein hydrolysate prior to the analysis takes *ca.* 40 min. The use of a 3.5-m column with OV-1 as stationary phase provides a complete peak separation for the derivatives of all of the neutral and amino sugars and N-acetylneuraminic acid commonly encountered in animal glycoproteins, and the analysis can be performed within 2.5 h.

INTRODUCTION

Recent progress in the structural investigations of carbohydrate moieties of glycoproteins is connected with development of analytical techniques and, primarily, gas chromatographic (GC) analysis of different sugar derivatives[1]. The methanolysis and subsequent GC determination of methyl glycosides as their trimethylsilyl (TMS) derivatives, as proposed by Sweeley and Walder[2] and applied to glycoproteins by Clamp *et al.*[3], served as a basis for a standard procedure for estimating the monosaccharide composition of glycoproteins and glycopeptides.

Proton-catalyzed methanolysis is undoubtedly the most reliable technique available for achieving a complete cleavage of glycosidic linkages[3]. In this case the solvolysis can be accomplished without any appreciable destruction of the monosaccharides. The stability of peptide bonds to methanolysis helps to avoid tedious procedures for freeing released sugars from peptide material and amino acids. Methyl glycosides of all sugars commonly encountered in glycoproteins can be analyzed in a single GC run. A specific chromatographic pattern consisting of several peaks obtained for each monosaccharide allows one to identify and quantify a sugar with increased confidence, even with biological samples contaminated with non-carbohydrate impurities.

The conventional procedures used for preparing volatile methyl glycoside derivatives[3,4] are time-consuming and involve sample transfer steps. This mainly concerns the steps of methanolysate neutralization and re-N-acetylation of methyl hexosaminides and the methyl ketoside of methylneuraminate, which lose their N-acetyl groups under the methanolysis[5]. On the one hand, these steps are essential for obtaining reproducible quantitative results and achieving satisfactory peak separation[6]. On the other hand, at the re-N-acetylation step the side reaction of O-acetylation of polyols, generally used as the internal standards, may occur[7]. Many workers have tried to find a rapid and efficient derivatization procedure[8-10].

We have found that the re-N-acetylation may be successfully carried out after the trimethylsilylation step, directly in the analytical GC column by injecting the sample together with a small amount of acetic anhydride. This amendment used in combination with the neutralization of the acid after methanolysis with pyridine[8] allowed us to reduce the time required for methanolysate treatment prior to the analysis to 40 min.

It is commonly found[8,11] that, in the GC analysis of TMS ethers of methyl glycosides, some peaks of the mannose and galactose derivatives are not sufficiently separated, which reduces the precision of determining related peak areas. Bearing in mind the possibility of analyzing the TMS ethers of methyl glycosides using columns with other than SE-30 non-polar stationary phases, as mentioned by Chambers and Clamp[6], we attempted to define chromatographic conditions which would provide a better peak resolution. The best results were obtained with 3% OV-1 on Gas-Chrom Q in a column of 3.5 m length.

EXPERIMENTAL

Materials

L-Fucose, D-mannose, D-galactose, D-glucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, N-acetylneuraminic acid, D-mannitol and D-arabitol of reinst grade were purchased from Serva (Heidelberg, G.F.R.). Stock solutions of monosaccharides were prepared by dissolving them in triply distilled water. Other chemicals used were of chemically pure or pure-for-analysis grade (Reakhim, Moscow, U.S.S.R.) and were subjected to additional purifications as described below.

Acetic anhydride was distilled over anhydrous sodium acetate. Pyridine was distilled and stored over potassium hydroxide pellets. Trimethylchlorosilane and hexamethyldisilazane were redistilled immediately before preparation of a silylating mixture. The latter was prepared according to Sweeley *et al.*[12] and stored in a refrigerator for up to 3 days.

Methanol was refluxed with magnesium turnings in the presence of iodine and then distilled over calcium hydride. Methanolic hydrochloric acid was prepared by slow bubbling of dry hydrogen chloride gas at 0° until the desired strength, namely 0.5 or 1.5 *N*, was reached.

Five-times recrystallized ovalbumin (Reakhim) was employed. The glycopeptide fraction of ovalbumin pronase digest was isolated according to Bogdanov *et al.*[13].

Methods

A standard analytical procedure developed and routinely used in our laboratory is described below. Whenever the techniques proposed by other workers were reproduced, the steps of methanolysis, neutralization and re-N-acetylation were carried out exactly according to the original descriptions (references are given at suitable places in the text).

Methanolysis. A solution of the standard monosaccharide mixture or of a carbohydrate-containing sample (0.1–5 μmole of monosaccharides) was placed in a glass ampoule. An appropriate amount of the internal standard solution (arabitol and mannitol, 0.5 μmole/ml of each) was added. The contents of the ampoule were then taken to dryness *in vacuo* at 35° and kept overnight in a vacuum desiccator over phosphorus pentoxide. 0.5–1 ml of 0.5 *N* methanolic HCl was added and a steady stream of argon was passed over the mixture. The dissolved air was removed by repeated freezing of the methanolic solution with liquid nitrogen and thawing out *in vacuo*, special attention being paid to complete freezing of the HCl before evacuation of the ampoule. The ampoule was then sealed and placed in a thermostat. The methanolysis was carried out at 100° for 24 h.

Neutralization and trimethylsilylation. The ampoule was cooled and the seal was broken. The acid was neutralized by the addition of pyridine (one equivalent of the initial amount of HCl) and the sample was then taken to dryness *in vacuo* at 35°. The silylating mixture (100 μl) was added and the ampoule was sealed with Parafilm. The contents were carefully mixed and left at ambient temperature for 30 min.

When the analysis could not be performed during the same day, the methanolysate was neutralized, taken to dryness and placed in a vacuum desiccator with P_2O_5. Samples could be stored for at least 1 week without any decomposition. Storage of the sealed ampoules in the refrigerator led to a substantial loss of N-acetylneuraminic acid (*ca.* 25% in the first 24 h).

Injection of the sample, re-N-acetylation. A syringe equipped with a long needle for on-column injection (Scientific Glass, North Melbourne, Australia) was filled with 1–3 μl of acetic anhydride and an equal volume of the solution of the sample in the silylating mixture. The two liquids were always separated by a small bubble of air. The plunger was pulled back to take the liquid off the needle and the combined sample was injected on to the upper layer of the column packing.

Chromatographic conditions

Gas–liquid chromatography (GLC) was performed with a Chrom 41 gas chromatograph (Laboratorní Přistroje, Prague, Czechoslovakia) equipped with flame ionization detectors, linear temperature programmer and electromechanic integrator. The stainless-steel injection-port liners were replaced by those of silanized glass. A coiled glass column (350 × 0.3 cm), silanized and packed with 3% OV-1 on Gas-Chrom Q (100–120 mesh) (Serva), was employed. The carrier gas was highest purity helium at a flow-rate of 65 ml/min. The temperatures of the injection port and the detector were 200° and 230°, respectively. After the sample injection the column was held at 110° for 15 min and then heated to 220° at a rate of 1°/min, the limit of 220° being held for 10 min.

RESULTS AND DISCUSSION

Methanolysis and neutralization

When heated with anhydrous methanolic HCl, monosaccharides are known[2,14] to undergo partial destruction, the extent of which increases with the hydrogen chloride concentration. Therefore, the methanolysis step was carried out under mild conditions (see Experimental). The analysis of glycoproteins of known composition showed that under these conditions glycosidic linkages could be cleaved quantitatively. The application of a more severe methanolysis (1.5 *N* HCl, 48 h)[15] resulted in partial destruction of all of the monosaccharides.

We introduced the internal standards in the sample prior to the methanolysis. According to Penick and McCluer[16], heating with 0.5 *N* methanolic HCl does not give rise to destruction of mannitol. The contradictory data of Jamieson and Reid[17] can be explained by the fact that these workers did not exclude the effect of atmospheric oxygen. Besides, during the employed procedure of neutralization with solid silver carbonate (without subsequent addition of acetic anhydride), mannitol adsorption undoubtedly occurred[6].

In accordance with data obtained by Ludowieg and Dorfman[5] and recently confirmed by Pritchard and Todd[9], during the methanolysis an appreciable part of the HCl reacted with the methanol so that after the methanolysis the apparent pH increased to 3.5–4. Moreover, with glycoprotein samples, the HCl was partially consumed in the salt formation with the protein amino groups: this sometimes led to an increase in pH up to 5–6.

In any case, prior to the evaporation of the methanolic HCl, remaining acid should be neutralized to avoid the loss of methyl glycosides[6,18]. Zanetta *et al.*[19] reported that no appreciable losses occurred when removing methanolic HCl by a stream of inert gas without preliminary neutralization. However, it is our experience, that even under such conditions large and irreproducible losses occur of methyl glycosides of neutral sugars and, especially, the methyl ketoside of neuraminic acid[20]. Thus, the neutralization step is indispensable.

From the data presented in Table I it can be concluded that the neutralizations with solid silver carbonate and with pyridine are equally effective. It is, however, more convenient to apply pyridine, since the use of solid carbonate necessitates centrifugation and transference of the sample to another container.

Trimethylsilylation

We employed the usual procedure for the preparation of the TMS ethers of sugars. The reaction time of 30 min is probably optimal. Shorter periods resulted in incomplete trimethylsilylation of mannitol, while longer times gave rise to some loss of the sialic acid. The centrifugation step for separating the NH_4Cl precipitate was omitted because, in accordance with Sweeley *et al.*[12], ammonium chloride did not interfere with the GLC analysis. Moreover, when applying the centrifugation, we sometimes observed a decrease of the mannitol peak and the peaks of N-acetylhexosamines, accompanied by the appearance of extraneous peaks in the mannitol region. Apparently, this is associated with the limited mannitol solubility in pyridine, leading to incomplete trimethylsilylation of the polyol[16], and with adsorption on the inorganic salt residue. It could have been the reason for the similar phenomenon

observed by Chambers and Clamp[6] in the presence of sodium chloride. It should be noted that the use of previously silanized glassware greatly decreases the formation of NH_4Cl, since the reagents are not consumed by the reaction with hydroxyl groups in the glass surface.

Thirty minutes are sufficient for the NH_4Cl residue and protein particles (in the analysis of glycoproteins) to precipitate, and it is reasonably easy to remove a few microlitres of supernatant without disturbing the precipitate.

Re-N-acetylation

From Table I, it can be seen that the on-column re-N-acetylation proceeds quantitatively. Under these conditions no acetylation of trimethylsilylated hydroxyls occurs; neither a decrease of the corresponding peaks nor the appearance of any extraneous peaks were observed by comparison with a chromatogram of an identical sample passed through the conventional derivatization procedure.

Moreover, the injection of acetic anhydride together with a sample subjected to the conventional procedure of the re-N-acetylation enhanced slightly the values of the relative molar response (RMR)* for N-acetylhexosamines and N-acetylneuraminic acid. In the course of chromatographic separation, small portions of these compounds are likely to be adsorbed irreversibly on the packing, whereas simultaneously injected acetic anhydride suppresses these processes.

When using the re-N-acetylation procedures by Clamp and co-workers[7] and by Etchison and Holland[8], the internal standards were introduced after the re-acetylation step in order to avoid O-acetylation[9]. In our procedure, the internal standards can be introduced at any convenient stage.

Gas chromatographic conditions

Fig. 1 shows a chromatogram of the standard monosaccharide mixture subjected to the methanolysis, neutralization with pyridine, trimethylsilylation and on-column re-N-acetylation. The complete separation of all of the mannose peaks from those of galactose permits a high-precision determination of these monosaccharides without any additional calculations. A shoulder on the methyl α-D-galactopyranoside peak (peak 8 in Fig. 1) appears to correspond to one of the galactofuranoside anomers. Under other chromatographic conditions these peaks coincide[7].

The temperature programme proposed was optimal for the completeness of separation and analysis duration. Both a higher initial temperature and faster programme rate resulted in considerable loss of resolution. Stopping of the programme at temperatures lower than 220°, and waiting for the last peak to emerge in the isothermal regime, did not increase the RMR of N-acetylneuraminic acid, but prolonged the analysis time.

As seen from Fig. 2, under the chosen chromatographic conditions the major peaks of deacetylated hexosamine derivatives did not coincide with those of methyl α-D-mannopyranoside and methyl α-D-galactopyranoside[7], but emerged immediately before these. This permitted the additional possibility of following the completeness of the re-N-acetylation.

* RMR values were determined as ratios of the total peak areas of the compounds analyzed to the peak area of the internal standard at equal concentrations in the sample.

TABLE I

CHROMATOGRAPHIC PARAMETERS OF THE TMS ETHERS OF METHYL GLYCOSIDES AND THE EFFECT OF VARIOUS DERIVATIZATION PROCEDURES ON RMR VALUES

The retention times were determined with respect to mannitol, and the RMR values with respect to arabitol (RMR_A) and mannitol (RMR_M).

Monosaccharide	*Relative retention time*	*Derivatization procedure*									
		Ref. 3		*Ref. 8*		*Ref. 3, co-injection of acetic anhydride*		*This paper*			
		RMR_A*	RMR_M*	RMR_A*	RMR_M*	RMR_A**	RMR_M**	RMR_A***	*S.D.****	RMR_M***	*S.D.****
Fucose	0.51										
	0.52	1.04	0.90	1.09	0.93	1.04	0.90	1.07	0.01	0.92	0.01
	0.55										
Mannose	0.77	1.03	0.89	1.04	0.89	1.03	0.90	1.06	0.01	0.91	0.01
	0.80										
Galactose	0.78										
	0.82	1.06	0.91	1.04	0.90	1.08	0.92	1.04	0.02	0.90	0.02
	0.87										
Glucose	0.89	1.08	0.94	1.08	0.92	1.08	0.93	1.08	0.02	0.93	0.01
	0.92										
N-Acetylglucosamine	1.03										
	1.09	0.85	0.73	0.84	0.72	0.89	0.77	0.89	0.02	0.76	0.02
	1.14										
N-Acetylgalactosamine	1.05	0.60	0.52	0.65	0.56	0.64	0.55	0.66	0.02	0.57	0.01
	1.10										
N-Acetylneuraminic acid§	1.46	0.45	0.40	0.46	0.40	0.53	0.45	0.52	0.01	0.45	0.01

* Based on three experiments with a mean relative error of *ca.* 3%.

** Based on four experiments with a mean relative error of *ca.* 5%.

*** Based on nine experiments.

§ The peak of the major isomer (92%)[7] only was taken in account.

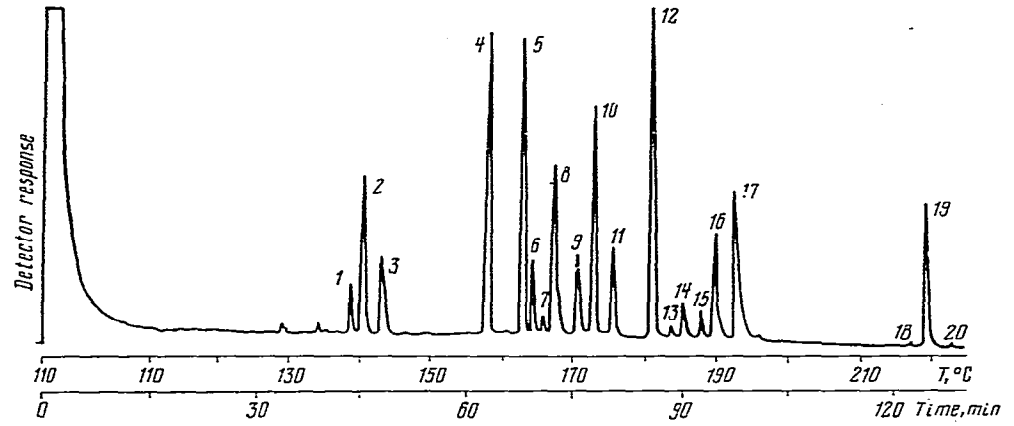

Fig. 1. Chromatogram of a standard monosaccharide mixture (*ca.* 5 nmoles of each sugar) subjected to the methanolysis, neutralization with pyridine, trimethylsilylation and on-column re-N-acetylation. Chromatographic conditions as given in Experimental. Peaks: 1, 2, 3 = fucose; 4 = arabitol; 5, 7 = mannose; 6, 8, 9 = galactose; 10, 11 = glucose; 12 = mannitol; 13, 15, 17 = N-acetylglucosamine; 14, 16 = N-acetylgalactosamine; 18, 19, 20 = N-acetylneuraminic acid.

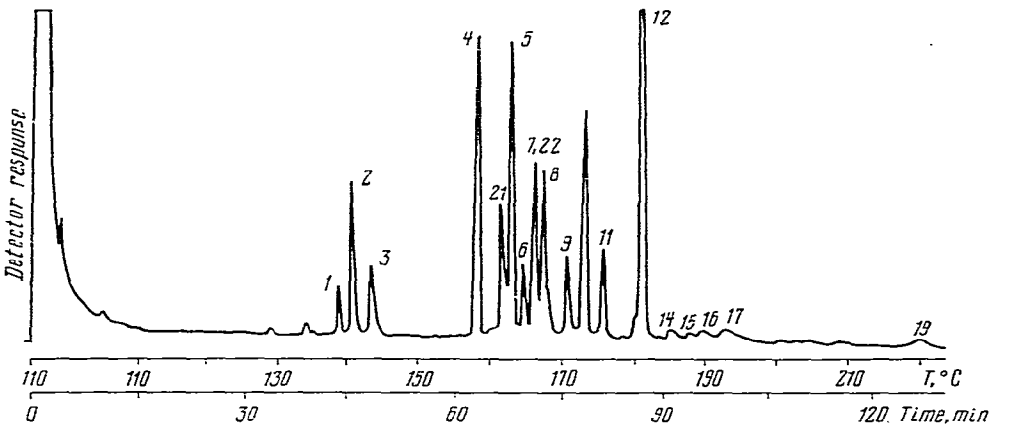

Fig. 2. Chromatogram of a standard monosaccharide mixture obtained as in Fig. 1 except that the re-N-acetylation was omitted. Peaks: 21 = galactosamine; 22 = glucosamine; other peaks designated as in Fig. 1.

Some factors affecting the reproducibility of results

Standard deviations of RMR values given in Table I correspond to an error of *ca.* 1.5% for the determination of the neutral sugars and N-acetylneuraminic acid and of *ca.* 2.5% for hexosamines. Such scattering is reasonable when estimating the monosaccharide compositions of glycoproteins and glycopeptides.

However, to obtain reliable results it is necessary to take into account some other factors. It is essential that the methanolysis should be carried out under anhydrous conditions and in the absence of oxygen. The preparation of the methanolic hydrochloric acid should proceed slowly and with cooling[9]. The acid must not be

stored for more than 2 or 3 weeks, because we have observed that, even at temperatures below 0°, the reactions which produce water may occur. It is necessary to prepare the fresh silylating mixture every 2–3 days, special attention being paid to the purity of hexamethyldisilazane. The presence of the hydrolysis products of this reagent interferes with the analysis of N-acetylhexosamines and N-acetylneuraminic acid.

We have also found that the RMR values decrease with long-term use of the column. This may be explained by the appearance of adsorption sites on the solid support. The reproducibility of results could be improved and the effective functioning of the column prolonged by applying occasional deactivation of the support. This was done as follows. Several portions (5 μl each) of acetic anhydride and the silylating mixture were successively injected (at short intervals) into the column maintained at 100–110° and with the carrier gas flow-rate of 20–30 ml/min; addition of the silylating mixture closed the sequence. The column was held at this temperature for another 10 min and then heated to 250–300°. In addition, the conventional conditioning procedure was repeated monthly. With such treatments the column properties remained unchanged and the chromatographic parameters of the TMS ethers of methyl glycosides remained constant for 1 year.

Analysis of ovalbumin

As seen from Table II, there is good agreement between our data and

TABLE II

MONOSACCHARIDE COMPOSITION OF OVALBUMIN IN MOLES PER MOLE OF PROTEIN

Monosaccharide	*Protein*		*Glycopeptide fraction*
	Found	*Ref. 21*	
Mannose	5.1	5.0	5.1
N-Acetylglucosamine	3.0	3.0	3.1

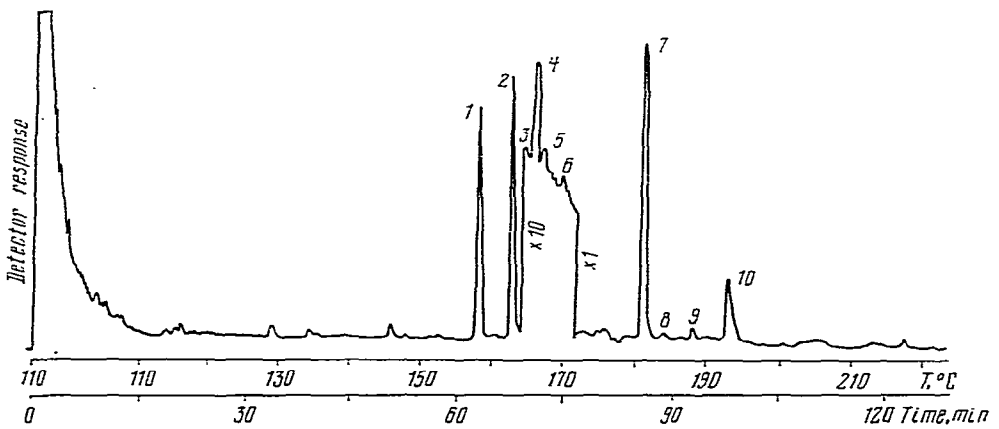

Fig. 3. Carbohydrate components of ovalbumin. Peaks: 1, 7 = internal standards; 2, 4 = mannose; 8, 9, 10 = N-acetylglucosamine; and 3, 5, 6 = galactose (impurity).

literature data[21] on the content of mannose and N-acetylglucosamine in ovalbumin. Fig. 3 shows a chromatogram of the TMS ethers of methyl glycosides obtained from 10 nmoles of ovalbumin. We have succeeded in detecting a trace impurity of galactose (*ca.* 0.5 nmoles). The characteristic pattern of the three peaks permitted a reliable identification of galactose and quantification with an accuracy of *ca.* 10%.

CONCLUSIONS

The derivatization procedure described is rapid and reproducible, which is essential in routine work when estimating the monosaccharide composition of carbohydrate-containing samples. It has been successfully used in our laboratory for investigating such complex glycoproteins as human transcortin and immunoglobulins. The possibility of performing trace analyses under the proposed GC conditions is of particular importance in connection with the known microheterogeneity of glycoproteins.

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