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GAS CHROMATOGRAPHY OF METHYL GLYCOSIDES AS THEIR TRI-METHYLSILYL ETHERS

THE METHANOLYSIS AND RE-N-ACETYLATION STEPS*

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SUMMARY

Gas chromatographic procedures for the analysis of carbohydrates in glycoproteins frequently include methanolysis and re-N-acetylation steps. The usual methanolysis conditions were found to result in almost complete conversion of the methanolic HCl into methyl chloride. A neutral solution of methyl chloride in methanol was found capable of releasing fucose, sialic acid, and a portion of the galactose from α_1-acid glycoprotein.

During the re-N-acetylation of the amino sugars O-acetylation of the alditols commonly employed as internal standards occurs. Addition of the standards after the re-N-acetylation step avoided the problem. The complete procedure for the determination of both neutral and amino sugars has been simplified to involve only one transfer step prior to final gas chromatography.

INTRODUCTION

The application of gas–liquid chromatography to the determination of carbohydrate composition is critically dependent upon the quantitative conversion of the component monosaccharide units into suitable volatile derivatives. Presently the most common derivatives used for this purpose are the trimethylsilyl ethers, although acetates and trifluoroacetates are also widely used.

Sweeley *et al.*[1] originally described the preparation of the trimethylsilyl ethers of a large number of different sugars and modified sugars. Their method for preparing the derivatives using hexamethyldisilazane and trimethylchlorosilane in pyridine is still in general use. These workers also described a general procedure for the determination of sugars in glycolipids, which employed both methanolysis and a step to re-N-acetylate the amino sugars[2]. Clamp *et al.*[3] adapted the procedure of Sweeley and Walder[2] for the routine analysis of the carbohydrate portion of glycoproteins.

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There are several advantages of using methanolysis rather than hydrolysis to release the monosaccharide components of glycoproteins. Methanolysis gives higher recoveries of neutral sugars than most hydrolytic methods, and certain sugars, such as galactose and glucose, are more readily separated as their methyl glycosides[4]. While considered a disadvantage by some, the fact that methanolysis gives rise to several characteristic peaks for each sugar makes it possible to identify a sugar with increased confidence. Finally, undesirable side reactions are minimized since the reducing groups of the liberated sugars are immediately protected as the methyl glycosides.

The procedure of Clamp *et al.*[3] is rather lengthy and involves numerous sample transfer steps. As a result, many authors have tried to improve upon the basic method. A problem common to all these procedures is the extensive de-N-acetylation occurring during the methanolysis step. The amino groups of the hexosamines are not silylated under the silylation conditions of Sweeley *et al.*[1], and the amino sugars have retention times overlapping those of neutral sugars. More vigorous silylation conditions have been described to replace one or both of the amino hydrogens with trimethylsilyl groups[5], but these silylation methods frequently give mixtures of products. Moreover, other substances present in glycoprotein digests, such as amino acids, are also silylated and complicate the gas chromatograms. To avoid these problems, it is common to derivatize the free amino groups after the methanolysis step. The most common procedure is to re-N-acetylate the amino groups although the N-carboethoxy derivatives also have been used[6].

The re-N-acetylation step introduces other problems. While not originally recognized by Clamp *et al.*[3], partial O-acetylation occurs in this method. In the procedure of Reinhold[7], O-acetylation is reversed by a short second methanolysis step. Subsequently Etchison and Holland[8] described a very rapid procedure for the quantitative N-acetylation of the methyl glycosides of amino sugars. In their procedure two equivalents of pyridine were added to the methanolysate to neutralize the HCl and to serve as a catalyst for the N-acetylation reaction with acetic anhydride.

The selection of a suitable internal standard is very important if good precision in the gas chromatographic analysis is to be obtained. Alditols have become very widely used as internal standards. Mannitol was chosen by Chambers and Clamp[4], who claimed that it was completely stable under their conditions. This has not been the experience of other workers. Jamieson and Reid[9] studied the stability of mannitol to methanolysis and found that extensive degradation occurred. They recommended, therefore, that mannitol not be added until after the methanolysis step. Furthermore, it has been the experience of our laboratory that mannitol will be extensively O-acetylated unless it is added after the re-N-acetylation step. Other workers have employed the more stable, but chemically dissimilar, long-chain alkanes as internal standards[9,10].

EXPERIMENTAL

Reagents

Carbohydrates were obtained from Sigma (St. Louis, Mo., U.S.A.). Tri-Sil "Z", acetic anhydride, and pyridine were purchased from Pierce (Rockford, Ill., U.S.A.). The cyclic alditol, 2,5-anhydro-D-mannitol, was synthesized as described by

Horton and Philips[11] and further purified by silica gel chromatography. Crystallization was initiated using a crystal kindly supplied by Dr. D. Horton from the Department of Chemistry, The Ohio State University (Columbus, Ohio, 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.).

Anhydrous methanol was prepared by refluxing 1 l of reagent grade methanol with magnesium turnings (10 g) and iodine (1 g) for 1 h followed by distillation in a 3-ft. spinning band still. Dry hydrogen chloride gas was then passed through the methanol until the concentration was 1.5 *N*. Care was taken to keep the solution cool during the addition of the hydrogen chloride gas. The methanolic HCl was promptly sealed in glass ampules under nitrogen and stored at —70°.

Mass spectrometry

Mass spectra were obtained using a DuPont Model 21-492B double-focusing mass spectrometer operated at an ionizing potential of 70 eV. Scans were taken every 9 sec, and the spectra were processed using a DuPont Model 21-094B disc-based data system.

Sample preparation

The analytical procedure employed was a modification of the method of Etchison and Holland[8]. Samples were placed in 1-ml all-glass ampules and dried over phosphorus pentoxide in a vacuum desiccator. Methanolic HCl (0.5 ml) was then added to the ampules, which were sealed under nitrogen and placed in a heating block at 80° for 24 h. The ampules were cooled, broken open, and 0.15 ml pyridine followed by 0.1 ml acetic anhydride was added. The ampules were sealed with silicone rubber caps and allowed to remain at room temperature for 30 min. They were then taken to dryness in a vacuum desiccator over KOH pellets. At this point 50 μl of an internal standard mixture comprising arabitol, mannitol and perseitol (all 2 m*M*) were added. They were then taken to dryness under vacuum over phosphorus pentoxide. The samples were trimethylsilylated by adding 100 μl Tri-Sil "Z" to the ampules followed by mixing and transferring rapidly to 0.1-ml automatic sampler vials.

Gas chromatography

These vials were placed in the automatic sample injector of a Hewlett-Packard Model 7620A gas chromatograph. A 6-ft. glass column (2 mm I.D.) packed with 3.8% UCW-98 on Gas-Chrom Q, 100–120 mesh (Applied Science Labs., State College, Pa., U.S.A.) was employed. The carrier gas was chromatographic grade nitrogen at a flow-rate of 60 ml/min. The chromatograph was equipped with a flame ionization detector and was temperature programmed from 100° to 145° at 1°/min. It was held at 145° for 10 min and then heated to 230° at the rate of 1°/min. Peaks were automatically integrated using an Autolab System IV computing integrator.

RESULTS AND DISCUSSION

Conversion of methanolic HCl to methyl chloride

Early in this study it was observed that the concentration of methanolic HCl in glass ampules stored at room temperature steadily decreased with time. The

possibility that this effect might be greatly accelerated under the usual heating conditions employed for methanolysis was therefore tested. The results shown in Fig. 1 clearly indicate that the acid is largely consumed during the heating step. The small amount of HCl remaining in a sample of heated methanolic HCl was neutralized by the addition of solid silver carbonate. When silver nitrate crystals were dissolved in this solution there was no apparent reaction at room temperature, but upon heating large amounts of silver chloride precipitated. This is the expected reaction if methyl chloride was dissolved in the methanol. The presence of a high concentration of methyl chloride in the methanol was confirmed by mass spectrometry.

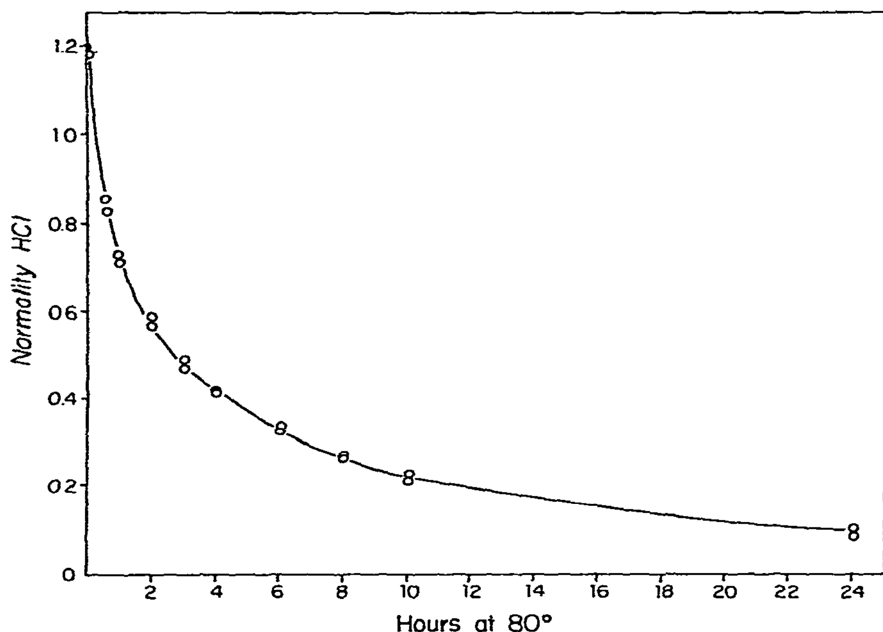

Fig. 1. Decrease in the concentration of methanolic HCl at 80°. A total of 20 sealed glass ampules each containing 2.00 ml of 1.2 *N* methanolic HCl was placed in an oven at 80°. The ampules were removed at various times, and the acid concentration was determined by titration with standard NaOH solution.

Although methanolysis procedures are widely employed in studies on glycoproteins, most investigators apparently are unaware that the acid is largely consumed during the procedure. This fact has obvious implications for those who are careful to neutralize the acid they assume to be still present, or those who unduly prolong methanolysis. Etchison and Holland's observation[8] that all reaction byproducts of their N-acetylation procedure are volatile (despite the fact that the pyridine hydrochloride that should have formed has a melting point of 82° and sublimes only very slowly) can simply be explained by the fact that there was virtually no HCl present in the first place.

It is also evident that methanolysis is never really carried out under strictly anhydrous conditions, since an equimolar amount of water is formed along with the methyl chloride. However, the presence of water in the methanolic HCl before the

heating step would be expected to cause an increase in the final HCl concentration.

Deterioration of methanolic HCl solutions could be prevented by storage at a low temperature. The acid concentration of methanolic HCl in sealed ampules stored at −70° showed no detectable change in 3 months.

An additional consideration is the method for preparing the methanolic HCl. The absorption of HCl gas in methanol is strongly exothermic. Unless precautions are taken to cool the mixture during preparation, it is likely that considerable amounts of methyl chloride would be formed but not initially determined as HCl. This would be expected to increase the final acid concentration.

Release of sialic acid and fucose by methyl chloride

The possibility that a neutralized methanol solution of methyl chloride could itself cause the release of monosaccharides from glycoproteins was investigated. A sample of α_1-acid glycoprotein was heated in a solution of 1.4 *M* methyl chloride in methanol for 24 h at 80°. A similar sample was analyzed by the standard procedure using 1.5 *N* methanolic HCl. The results are shown in Table I. Virtually complete release of the sialic acid and fucose residues was observed along with a portion of the galactose residues.

TABLE I

MONOSACCHARIDES RELEASED FROM α_1-ACID GLYCOPROTEIN USING EITHER 1.5 *N* METHANOLIC HCl OR 1.4 *M* METHYL CHLORIDE IN METHANOL

Samples (250 μg) of α_1-acid glycoprotein were placed in glass ampules and heated at 80° for 24 h with 0.5 ml of either 1.5 *N* methanolic HCl or 1.4 *M* methyl chloride dissolved in methanol. The samples were then re-N-acetylated, trimethylsilylated, and chromatographed as described in the text.

Monosaccharide	*Percent of dry weight*	
	1.5 N HCl	*1.4 M Methyl chloride*
Fucose	0.74	0.69
Mannose	6.15	<0.2
Galactose	6.44	1.3
N-Acetylglucosamine	9.16	<0.2
N-Acetylneuraminic acid	12.6	11.3

The potential utility of the above procedure for the routine removal and determination of sialic acid and fucose in glycoproteins is currently under investigation. Such methanolysis conditions would be expected to provide a low but roughly constant concentration of HCl due to the reservoir of methyl chloride present.

Re-N-acetylation of hexosamines

The procedure for the re-N-acetylation of hexosamines was also examined carefully. The re-N-acetylation procedure of Etchison and Holland[8] had definite advantages over the procedures of Clamp *et al.*[3] and of Reinhold[7]. It was much more rapid, the number of sample transfer steps were greatly reduced, and less O-acetylation occurred than with the other two methods. Unfortunately, O-acetylation of the alditol internal standards did occur. This was verified by combined gas chromatography–mass spectrometry. Table II shows typical results observed for the 4 alditols

TABLE II

PARTIAL O-ACETYLATION OF ALDITOLS DURING THE RE-N-ACETYLATION PROCEDURE

The alditols were subjected to the re-N-acetylation conditions described in the text and were analyzed as their trimethylsilyl derivatives. A 6-ft. glass column (2 mm I.D.) packed with 3.8% UCW-98 on Gas-Chrom Q (100–120 mesh) was maintained isothermally at 175°, and retention times were determined relative to D-mannitol.

Alditol	*Percent alditol O-acetylated*	*Relative retention time*	
		Alditol	*O-Acetylated alditol*
2,5-Anhydro-D-mannitol	5.6	0.39	0.47
D-Arabitol	10.8	0.40	0.48
D-Mannitol	10.2	1.00	1.24
Perseitol	7.9	3.46	3.83

examined. The extent of O-acetylation was variable. It is noteworthy that the cyclic alditol, 2,5-anhydro-D-mannitol, was least O-acetylated under these conditions.

The solution to the problem was simply not to add the internal standards until after the re-N-acetylation step. This later addition of internal standards does not introduce any appreciable error, since the samples are still in their original ampules at this point.

Trimethylsilylation of samples

In the procedure of Etchison and Holland[8], samples were trimethylsilylated using bis(trimethylsilyl) trifluoroacetamide. This very powerful silylating reagent has all the disadvantages discussed previously. The trimethylsilylation procedure of Sweeley *et al.*[1] invariably resulted in the formation of a precipitate, which necessitated a centrifugation and an additional sample transfer step.

The use of N-trimethylsilylimidazole in pyridine (Tri-Sil "Z") was found to be very advantageous in our laboratory. The reagent has properties similar to the silylation mixture of Sweeley *et al.*[1] but does not give rise to a precipitate. Despite the claims of the manufacturer, it has been our experience that small amounts of water in the sample do, in fact, seriously interfere with the quantitation of sugars.

The re-N-acetylation step can be carried out after the trimethylsilylation with Tri-Sil "Z" by the addition of 10 μl of acetic anhydride to the sample and allowing it to react for at least 30 min. This procedure is similar to a procedure for the simultaneous trimethylsilylation and re-N-acetylation of free amino sugars described by Hara and Matsushima[12]. Occasionally, pyridine acetate crystallizes out in the silylation vial, but this is seldom a problem. The major disadvantage of re-N-acetylation after trimethylsilylation is that sialic acid is generally lost in the process.

CONCLUSION

The analytical procedure described has been successfully employed in our laboratory for the routine analysis of glycoprotein samples. It involves considerably less sample manipulation than other published procedures and is well suited for the analysis of a large number of samples.

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