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QUANTITATIVE ANALYSIS OF CARBOHYDRATE RESIDUES OF GLYCOPROTEINS AND GLYCOLIPIDS BY GAS–LIQUID CHROMATOGRAPHY

AN APPRAISAL OF EXPERIMENTAL DETAILS

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SUMMARY

Many investigators have reported problems with the use of D-mannitol and other alditols as internal standards for gas chromatographic quantification of trimethylsilyl ether derivatives of methyl glycosides. Quantification of carbohydrate residues of glycoproteins and glycolipids requires neutralization of the products of dry acidic methanolysis. Silver carbonate and Amberlite IR-45 ion exchange resin (OH^-) were compared as neutralization agents for 0.75 *N* HCl methanolysates (80°, 20 h). Only 4–17% of the D-mannitol internal standard was recovered when a 50 nmole equimolar mixture of D-mannitol and α-methyl glycosides was neutralized with silver carbonate. Addition of 0.1 ml of acetic anhydride or glacial acetic acid after silver carbonate neutralization did not prevent significant loss of D-mannitol, *myo*-inositol and perseitol at the 50 nmole level. There was no appreciable loss of D-mannitol internal standard, when compared to α-methyl glycosides, after IR-45 resin (OH^-) neutralization. Resin neutralization allowed 10–200 nmoles of D-mannitol internal standard to be added to acidic methanolysates immediately after methanolysis and carried directly through the analytical procedure.

INTRODUCTION

Quantification of sub-microgram amounts of carbohydrates from glycolipids and glycoproteins requires reproducible and accurate assay procedures. Currently, many investigators substitute silver carbonate for ion-exchange resins for the neutralization of acidic methanolysates because it appeared to be a fast and easy method for obtaining theoretical ratios of methyl glycosides by gas–liquid chromatography (GLC). This modification has been widely accepted without verification of yields and reproducibilities. Specific examples of investigations employing silver carbonate neutralization have been cited later in the text of this paper. This report concerns

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re-examination of earlier analytical techniques for carbohydrate analysis, and comparison of the ion exchange and silver carbonate procedures.

The development by Sweeley and Walker[1] of specific procedures for analysis of the products by dry acidic methanolysis made the determination of the composition of glycolipids and gangliosides by GLC relatively straightforward. Specifically, retention indices of the methyl glycosides of galactose and glucose, galactosamine, and neuraminate as trimethylsilyl (TMSi) derivatives were reported. Relative yields of the anomeric forms of TMSi-methyl galactosides and glucosides were also noted, but a recovery study of the carbohydrates and quantification was not attempted. Prior to derivatization, acidic methanolysates were either taken to dryness with nitrogen or neutralized with Amberlite CG-45 ion exchange resin (OH^-).

Gas–liquid chromatography rapidly became a popular tool for identification and quantification of glycolipids. Penick and McCleur[2] outlined a procedure for quantitating the galactose and glucose content of aliquots of aqueous ganglioside solutions. They noted that solubility of the mannitol internal standard in pyridine seemed to be the most troublesome aspect of the procedure. Methanolysates were not neutralized prior to derivatization for GLC.

A review by Sweeley and Vance[3] outlined methodology for TMSi derivatization, identification and quantification of carbohydrates from glycolipids. The methanolysis procedure for formation of methyl glycosides was extensively discussed, and factors affecting galactose/glucose ratios were noted. Concentrations of neutral glycosylceramides of human plasma and erythrocytes were subsequently reported by Vance and Sweeley[4]. Methanolysates were neutralized by an ion exchange resin prior to derivatization, since low galactose/glucose ratios were obtained if samples were simply evaporated with nitrogen. Identification of individual glycolipids was based on migration with preparative thin-layer chromatography, as compared with reference standards, and galactose/glucose ratios after GLC.

Clamp *et al.*[5] were interested in a rapid method for quantitating carbohydrates from glycoproteins. Gas–liquid chromatography of the TMSi-methyl glycosides was attempted after neutralization of acidic methanolysates with solid silver carbonate followed by re-N-acetylation of hexosamines and neuraminate with acetic anhydride. This re-N-acetylation step was necessary to separate hexosamines from hexoses as their TMSi-methyl glycosides. The authors confirmed previous findings by Sweeley and Walker[1] that N-acetylneuraminic acid was most stable as the TMSi derivative of its methyl glycoside.

The purpose of this paper is to demonstrate differences in the recovery of sugar alcohol internal standards and calculated glycolipid concentrations that arise solely from the choice of neutralization procedure for acidic methanolysates.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 402 gas chromatograph, equipped with a hydrogen flame ionization detector, was used for all analyses. The chromatograph was operated with a U-shaped glass column (6 ft. × 2 mm I.D.) which had been packed with 3% SP-2100 silicone liquid phase on Supelcoport (100–120 mesh) (Supelco, Bellefonte, Pa., U.S.A.). Samples were assayed as TMSi derivatives by continuous temperature

programming from 140 to 240° at 3°/min, with nitrogen carrier gas. Glass columns, with a 30 ml reservoir and 11.5 cm stem (7 mm I.D.), were purchased from Supelco. Columns were fitted with 3-way polyethylene stopcocks from Pharmaseal (Toa Alto, Puerto Rico). Stainless steel hypodermic needles (2 in., 17 gauge) were attached to the collecting end of the stopcocks and were obtained from Becton, Dickinson and Co. (Rutherford, N.J., U.S.A.).

Reagents

Samples of methyl-α-D-mannopyranoside (α-Me-Man) were obtained from Calbiochem, San Diego, Calif., U.S.A.). Methyl-α-D-galactopyranoside (α-Me-Gal) and methyl-α-D-glucopyranoside (α-Me-Glc), hexamethyldisilazane, trimethylchlorosilane, N-acetylneuraminic acid and *myo*-inositol were purchased from Sigma (St. Louis, Mo., U.S.A.). D-Mannitol and perseitol were obtained from Pfanstiehl Labs. (Waukegan, Ill., U.S.A.). GM_1 ganglioside standard was purchased from Supelco. All solvents were reagent grade and redistilled prior to use. Dry pyridine was prepared by refluxing for 1 h with barium oxide, followed by distillation and storage over KOH pellets. Dry methanol was prepared by refluxing with magnesium turnings and iodine crystals for 1 h, followed by distillation into a glass reagent bottle containing a molecular sieve (Davison Type 3A; Fisher Scientific, FairLawn, N. J., U.S.A.). Technical grade HCl gas was purchased in lecture bottles from Matheson (East Rutherford, N.J., U.S.A.). Silver carbonate, resorcinol and Amberlite IR-45 ion exchange resin (OH^-) were obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). The resin was washed with the following reagents in sequence: 2 volumes of 0.75 *N* methanolic HCl, glass-distilled water to neutrality, 2 volumes of 0.75 *N* NaOH and glass-distilled water to neutrality. Resin-washing procedures were done in glass bottles that were shaken horizontally for 30 min. The washing sequence was repeated twice. Finally, the resin was rinsed with redistilled methanol in a büchner funnel, air-dried and stored dry at room temperature. Prior to use, 0.75 g of resin were checked for ability to neutralize acidic methanolysates [3 ml of 0.75 *N* HCl (80°, 20 h)], and for extraneous peaks when concentrates of the eluate from 3 ml of 0.75 *N* HCl and 35 ml of methanol were analyzed using GLC at high sensitivity.

The TMSi derivatizing reagent was prepared by combining dry pyridine, hexamethyldisilazane and trimethylchlorosilane (10:4:2). The reagent was centrifuged to remove ammonium chloride and stored in screw-cap septum vials for up to 2 weeks.

Procedure

Preparation and titration of 0.75 N methanolic HCl. Methanolic HCl was prepared by bubbling HCl gas through dry redistilled methanol in a glass-stoppered flask until the final concentration of HCl was approximately 1.0 *N*. A 5 ml aliquot of this solution was added to a 50 ml volumetric flask containing 5 drops of 1 % bromothymol blue in methanol and the solution was diluted to 50 ml with methanol. Standard 1.0 *N* NaOH was diluted 1:10 with glass-distilled water and used to titrate the diluted methanolic HCl. Methanolic HCl was always titrated and diluted with dry methanol to a final concentration of 0.75 *N* immediately prior to use. If left at room temperature (25°) for 2 weeks, the concentration of HCl would drop to *ca.* 0.5 *N*.

Preparation of samples. Samples of α-Me-Man, α-Me-Gal, α-Me-Glc and

programming from 140 to 240° at 3°/min, with nitrogen carrier gas. Glass columns, with a 30 ml reservoir and 11.5 cm stem (7 mm I.D.), were purchased from Supelco. Columns were fitted with 3-way polyethylene stopcocks from Pharmaseal (Toa Alto, Puerto Rico). Stainless steel hypodermic needles (2 in., 17 gauge) were attached to the collecting end of the stopcocks and were obtained from Becton, Dickinson and Co. (Rutherford, N.J., U.S.A.).

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Preparation of samples. Samples of α-Me-Man, α-Me-Gal, α-Me-Glc and

silver carbonate were required to achieve neutrality (pH 7.0) with pH paper. Sample tubes were then centrifuged and the clear methanolic supernatant was removed to 1 dram vials. The procedure employed was a modification of that outlined by Clamp, Dawson and Hough[5]. The silver carbonate pellet was washed 3 times with 2 ml methanol. After each addition of methanol, the sample was sonicated for 15 sec in a small ultrasonic bath at 25°, mixed and centrifuged. Wash supernatants were pooled with the original supernatant, and taken to dryness with nitrogen gas. Absolute ethanol–dry benzene (1:1) was added as above and samples were derivatized and analyzed by GLC in the same manner as outlined above.

Neutralization with silver carbonate followed by acetic anhydride or acetic acid. Acidic methanolysates were neutralized with silver carbonate, after which 0.1 ml of acetic anhydride (Clamp *et al.*[5]) or glacial acetic acid was added. No apparent change in pH was produced by these additions, as indicated by pH paper. Samples were mixed well and allowed to sit at room temperature for 16 h. At the end of this time, samples were mixed and centrifuged and methanolic supernatants were removed to 1 dram vials. Silver carbonate pellets were washed with methanol and prepared for GLC, as earlier described.

RESULTS AND DISCUSSION

Effect of IR-45 resin (OH^-) and silver carbonate neutralization on recovery

Figs. 1 and 2 illustrate the effect of α-methyl glycoside and D-mannitol concentration on recovery after neutralization of acidic methanolysates with 0.75 g IR-45 resin (OH^-) or 20 mg silver carbonate. It is important to note that, throughout the range of sample concentrations assayed, resin neutralization recoveries were markedly consistent for each compound assayed. The silver carbonate neutralization procedure,

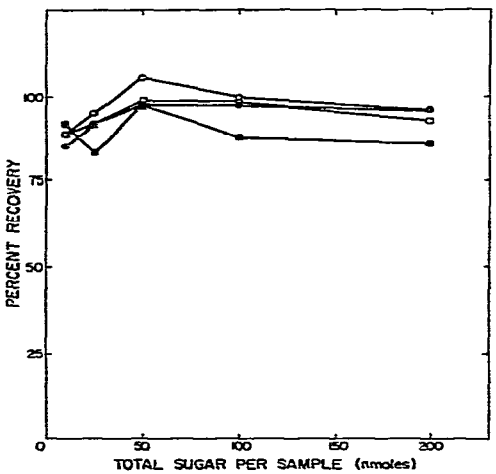

Fig. 1. Percentage recovery of D-mannitol and α-methyl glycosides after IR-45 ion exchange resin (OH^-) neutralization. Equimolar mixtures (10–200 nmoles) of D-mannitol (■), α-Me-Man (●), α-Me-Gal (□) and α-Me-Glc (○) were added to 3 ml of 0.75 *N* methanolic HCl that had been heated to 80° for 20 h. Samples were neutralized with 0.75 g of resin, analyzed as TMSi ether derivatives by GLC and percentage recovery calculated by comparison to TMSi derivatives of untreated standards.

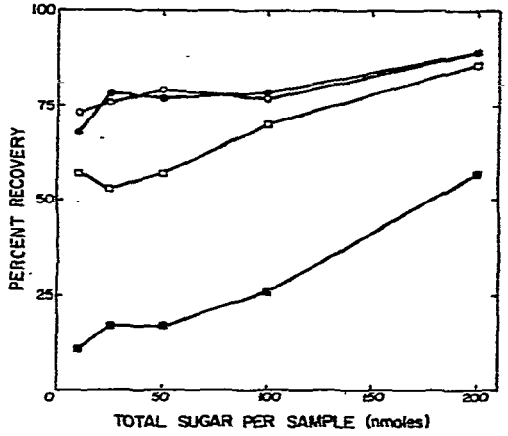

Fig. 2. Percentage recovery of D-mannitol and α-methyl glycosides after silver carbonate neutralization. Equimolar mixtures (10–200 nmoles) of D-mannitol (■), α-Me-Man (●), α-Me-Gal (□) and α-Me-Glc (○) were added to 3 ml of 0.75 *N* methanolic HCl that had been heated to 80° for 20 h. Samples were neutralized with 20 mg of silver carbonate, analyzed as TMSi ether derivatives by GLC and percentage recovery calculated by comparison to TMSi derivatives of untreated standards.

on the other hand, produced wide divergence in recoveries between methyl glycosides and the D-mannitol internal standard, especially at low sample concentrations. There appears to be a tendency for sample recoveries to converge with increasing sample concentration when using a constant amount of silver carbonate for neutralization. This may explain why investigators who assay milligram quantities of sample have not detected a problem with silver carbonate as a neutralization agent.

Effect of increasing amounts of silver carbonate on recovery

Some investigators who use silver carbonate, and have evaluated methanolysis conditions or the use of mannitol as a suitable internal standard, have not published the amounts of silver carbonate added per sample (Chambers and Clamp[6]; Jamieson and Reid[7]). Other investigators have reported using from 100 to 500 mg of silver carbonate for neutralization (Desnick *et al.*[8]; Dawson and Clamp[9]; and Sweeley and Tao[10]); however, in most of these cases a re-N-acetylation step with acetic anhydride was employed after neutralization.

The amount of silver carbonate needed for neutralization of acidic methanolysates depends upon the final HCl concentration after methanolysis and the amount of trituration during silver carbonate addition. The final HCl concentration is affected by oven temperature and the time period of methanolysis. Since the rate of reaction between methanol and HCl increases with increasing temperature, as little as 3.7% of the titratable HCl remains after 5 h at 100°, according to Kishimoto and Radin[11]. Therefore, samples containing 3 ml of 0.75 *N* methanolic HCl, after 20 h at 80°, would contain less than 0.08 mequiv. of HCl. Theoretically, only 11 mg of silver carbonate should be required for neutralization provided the initial concentration of methanolic HCl, time and temperature of methanolysis have been strictly controlled. Pritchard and Todd[12] recently showed that the normality of 2 ml of 1.2 *N* methanolic HCl decreased to *ca.* 0.1 *N* after 24 h at 80°. When using these conditions, *ca.* 28 mg of silver carbonate would be necessary for neutralization.

The effects of increasing the amount of silver carbonate used to neutralize these acidic methanolysates are shown in Fig. 3 for an equimolar mixture of α-methyl glycosides and D-mannitol (50 nmole each). It was initially predicted that 20 mg of silver carbonate would give a lower mannitol recovery than 30 or 50 mg, due to the earlier (and again, reproducible) bright yellow color of the TMSi-derivatization reaction with 20 mg samples. It was supposed that the color was due to incomplete neutralization, as all unneutralized acidic methanolysates, which were simply taken to dryness with nitrogen gas prior to derivatization, turned bright yellow upon addition of TMSi reagent. However, in fact, the highest mannitol recovery was found in samples neutralized with only 20 mg of silver carbonate, with mannitol recovery decreasing steadily up to 75 mg and finally appearing to plateau between 75 and 200 mg. The α-methyl glycoside recovery was clearly higher than that of D-mannitol by approximately a 4 to 5-fold margin throughout the range of silver carbonate used.

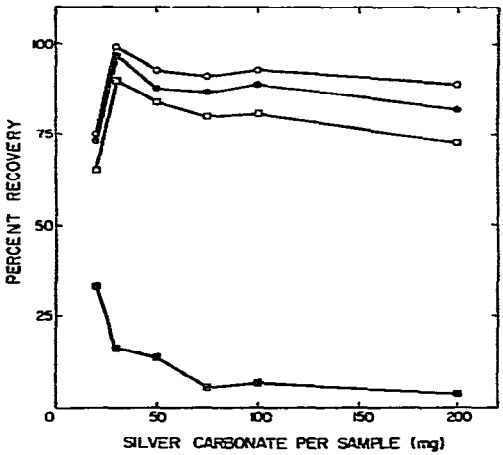

Fig. 3. Percentage recovery of D-mannitol and α-methyl glycosides after neutralization with increasing amounts of silver carbonate. A 50 nmole equimolar mixture of D-mannitol (■), α-Me-Man (●), α-Me-Gal (□) and α-Me-Glc (○) was added to 3 ml of 0.75 *N* methanolic HCl that had been heated to 80° for 20 h. Samples were neutralized with 20–200 mg of silver carbonate, analyzed as TMSi ether derivatives by GLC and percentage recovery calculated by comparison to TMSi derivatives of untreated 50 nmole standards.

Effect of addition of acetic anhydride or glacial acetic acid after silver carbonate

Because of problems encountered with the recovery of the D-mannitol internal standard after silver carbonate neutralization of acidic methanolysates, two additional sugar alcohols commonly used as internal standards were examined. Chambers and Clamp[6] recommended the addition of acetic anhydride or acetic acid to prevent loss of mannitol internal standard after silver carbonate neutralization. They supposed that this loss was not due to insolubility of mannitol in methanol, but rather to adsorption onto the neutralization agent complex.

The results of an experiment where α-methyl glycosides, D-mannitol, *myo*-inositol and perseitol recoveries were compared after several commonly used post-methanolysis procedures are given in Table I. There was no problem with use of

mannitol as an internal standard for quantitating methyl glycosides whenever IR-45 (OH^-) column neutralization was employed. The addition of acetic anhydride and acetic acid after silver carbonate did not appear to prevent significant loss of mannitol at the 50 nmole level. Mannitol recovery of silver carbonate neutralized samples was only slightly higher than samples that were directly dried with nitrogen gas and not neutralized.

TABLE I

RECOVERY OF α-METHYL GLYCOSIDES AND SUGAR ALCOHOL INTERNAL STANDARDS AFTER METHANOLYSATE NEUTRALIZATION AND RE-N-ACETYLATION

All samples contained 50 nmoles of each compound. Silver carbonate samples were neutralized with 30 mg Ag_2CO_3. Recoveries were calculated as percentage of untreated compounds recovered.

Neutralization procedure	*Recovery (%)*						*Ratio α-Me-Gal*
	α-Me-Man	*α-Me-Gal*	*α-Me-Glc*	*D-Mannitol*	*myo-Inositol*	*Perseitol*	*α-Me-Glc*
IR-45(OH^-) column	98.2	97.9	100.1	96.8	87.3	83.5	0.93
Ag_2CO_3	95.7	91.7	98.4	12.8	47.5	3.2	0.89
Ag_2CO_3 + acetic anhydride	93.8	92.0	99.4	48.5	80.1	31.1	0.88
Ag_2CO_3 + acetic acid	86.6	83.2	91.1	42.5	74.1	30.5	0.87
N_2 dried (unneutralized)	22.8	17.2	22.5	12.5	35.5	7.8	0.73
Untreated standards							0.95

Our results neither support nor refute the claim by Jamieson and Reid[7] that mannitol internal standard should not be added prior to methanolysis. We can, however, suggest the possibility that their measured loss of mannitol after nitrogen drydown and silver carbonate neutralization may have been due to the nearly equal effects of degradation and adsorption of sugar alcohols. In addition, since galactose/glucose ratios appear to remain fairly constant regardless of experimental manipulation, it seems probable that many investigators would not expect sugar alcohol internal standards to be so drastically affected by different neutralization procedures.

Quantification of GM_1 ganglioside standard

The GM_1 ganglioside concentration was initially determined by an 8-fold microreduction of a resorcinol colorimetric assay for sialic acids, as outlined by Svennerholm[13]. As little as 5 nmoles of N-acetylneuraminic acid could be detected accurately if sialic acid and hexose standard curves were run simultaneously with all samples, and absorbance measurements at 580 nm were corrected for the hexose contribution to the absorbance as described by Spiro[14]. GM_1 ganglioside sialic acid was also quantitated by selective cleavage with mild acid and conversion to the methyl-β-ketoside methyl ester of neuraminic acid by the method of Yu and Ledeen[15]. The TMSi-derivatized methyl ester was quantitated using TMSi-phenyl-N-acetyl-α-D-glucosaminide as the internal standard and by comparison with a standard curve of reference N-acetylneuraminic acid that had been simultaneously run through the procedure. The calculated concentrations of GM_1 by the resorcinol assay and the sialic acid GLC assay were 50.0 and 60.3 nmoles, respectively.

Table II shows that a wide variation in the experimentally determined GM_1

concentration can occur when mannitol internal standard is used to calculate glucose content after three different acidic methanolysate treatments prior to GLC quantification. Each GM_1 sample contained 40 nmoles of mannitol added after methanolysis. The methanolysates were extracted 3 times with 2 ml of hexane to remove fatty acid methyl esters prior to neutralization. Column neutralization with IR-45 resin (OH^-) resulted in values for GM_1 concentration that agreed with those obtained with the resorcinol colorimetric method. Assuming this to be the correct concentration, neutralization with 30 mg of silver carbonate produced an erroneous 4-fold increase in the calculated GM_1 concentration. Addition of 0.1 ml acetic anhydride resulted in a 72% increase above the actual level.

TABLE II

QUANTIFICATION OF GM_1 GANGLIOSIDE AFTER THREE DIFFERENT POST-METHANOLYSIS TREATMENTS

Silver carbonate samples were neutralized with 30 mg Ag_2CO_3. GM_1 concentration was calculated as 50.0 nmoles when analyzed using the resorcinol colorimetric assay and 60.3 nmoles when analyzed for TMSi-N-acetylneuraminic acid by GLC (see text). Flame ionization detector response correction factors of 1.23 for glucose and 1.26 for galactose were determined experimentally, relative to D-mannitol. GM_1 concentration after GLC analysis was based on glucose content using D-mannitol as internal standard.

Procedure	*Gal / Glc*	*D-Man / Glc*	*Calculated GM_1 (nmoles)*
IR-45(OH^-) column neutralization	1.88	0.99	50.32
Ag_2CO_3 neutralization	1.92	0.24	212.70
Ag_2CO_3 + acetic anhydride	1.88	0.54	86.50

The re-N-acetylation procedure is necessary for accurate quantification of both galactose and aminohexoses after IR-45 (OH^-) column neutralization. Methanolic effluents are in this case evaporated to dryness to remove traces of water which may interfere with the reaction. 3 ml of methanol and 10 mg of silver acetate or silver carbonate are added, followed immediately by 0.1 ml of acetic anhydride. The procedure employed is a modification of Procedure C outlined by Sweeley and Walker[1]. Samples are mixed and allowed to react at room temperature for a minimum of 6 h. Methanolic supernatants are removed and dried with nitrogen prior to TMSi derivatization as described above.

CONCLUSIONS

Our results indicate that GLC quantification of 200 nmoles or less of the carbohydrate residues of glycolipids and glycoproteins can be accomplished most accurately when IR-45 resin (OH^-) is used to neutralize acidic methanolysates. Mannitol internal standard can be added immediately after methanolysis and successfully carried directly through the neutralization procedure only when resin neutralization is employed. In no case was simple nitrogen drying or silver carbonate neutralization (with or without acetic anhydride or acetic acid) desirable when mannitol or perseitol were added as internal standards. When using silver carbonate, it would be possible to add

sugar alcohols just prior to TMSi derivatization; however, this procedure violates an important criterion in the choice of an internal standard for carbohydrate quantification, that it should be stable throughout all conditions of the analytical procedure.

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