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CRITICAL STUDY OF THE ALDITOL ACETATE METHOD FOR QUANTITATING SMALL QUANTITIES OF HEXOSES AND HEXOSAMINES IN GANGLIOSIDES

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SUMMARY

An extensive investigation of and improvement in the method for quantitating the carbohydrates of glycolipids by the gas–liquid chromatography of their alditol acetate derivatives is described. The effects of duration and temperature of hydrolysis, neutralization after hydrolysis, and acetylation time and temperature on the relative detector responses of mixtures of free as well as ganglioside hexoses and hexosamines were extensively studied. It is concluded that optimum results are obtained with the following conditions: hydrolysis at 100°C for 8 to 12 h, a 40–60 min reduction, acetylation at 100°C for 30 min. When quantitating the carbohydrate components of gangliosides, the most reliable results will be obtained using as an external standard a glycolipid whose chemical structure is similar to that of the sample. Using this procedure, reliable results can be obtained with the initial glycolipid sample containing as little as 1 μg of each sugar.

INTRODUCTION

Glycosphingolipids comprise a class of cell membrane constituents which are the source of much speculation and investigation. They have been implicated as being cell surface receptors[1], blood group antigens[2], and recognition molecules involved in intercellular communication and growth control[3]. The major differences among these molecules are the number and types of monosaccharide units within their carbohydrate chains. Therefore, investigations of their functions must allow for identification and quantification of their carbohydrate units.

Methods for analyzing the carbohydrate moieties in glycosphingolipids include colorimetry[4–6], gas–liquid chromatography (GLC)[7–10] and mass spectroscopy[11,12]. GLC has the advantages of being more sensitive and specific than colorimetry while being less costly and simpler to interpret than mass spectroscopy. GLC methods for

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glycosphingolipids require chemical derivatization of the monosaccharides obtained from hydrolysis of the parent compound. Commonly used carbohydrate derivatives include trifluoroacetates (TFA)[10], alditol acetates[8,9] and trimethylsilyl (TMS) derivatives[7]. While TMS has been widely employed, inconsistencies during quantitation of hexosamines and problems with the $AgCO_3$ neutralization step resulting in loss of the internal standard[13–16] have been reported. Furthermore, these problems are enhanced when working in the microgram range. We report here an extensive examination of the alditol acetate derivatization procedure and present improvements in the method which allows reproducible quantitation of as little as one microgram (approx. 5 μmol) of glucose, galactose and hexosamine derived from glycosphingolipids.

EXPERIMENTAL

Materials

Diethyl ether was obtained from Chemical Samples, Columbus, OH, U.S.A. Benzene and acetic anhydride were purchased from Drake Bros., Menomonee Falls, WI, U.S.A. Hexane and sodium borohydride were procured from Fisher Scientific, Fairlawn, NJ, U.S.A. Phosphorus pentoxide was purchased from Matheson, Coleman and Bell, Norwood, OH, U.S.A. Sigma, St. Louis, MO, U.S.A., was the source of D-xylose. D-Galactose, D-glucose and N-acetyl-D-glucosamine were obtained from Calbiochem, San Diego, CA, U.S.A. N-Acetyl-D-galactosamine and GM_2* were purchased from Supelco, Bellefonte, PA, U.S.A. All other gangliosides were prepared from normal human cerebral cortex[18]. Chloroform and methanol were distilled prior to use and glacial acetic acid, hydrochloric acid and ammonium hydroxide were of reagent grade. All glassware was acid washed prior to use. Reacti-vials, total capacity 3 ml, were purchased from Pierce, Rockford, IL, U.S.A. and Tufbond PTFE–silicone septums for the reacti-vials' screw caps were obtained from Supelco.

Gas chromatography

GLC analyses were carried out on a Hewlett-Packard No. 5710A gas chromatograph with a flame-ionization detector and a dual differential electrometer (range 1, attenuation 32). The temperature program was set for 32 min at 190°C, followed by 16 min at 230°C (rate, 4°C/min). Glass columns, 6 ft. × 1/4 in. O.D., were obtained prepacked with 1% OV-225 (ref. 19) on 100–120 mesh Gas-Chrom Q from Supelco. Peak areas were either determined by weighing, or electronically, using a Hewlett-Packard No. 3385 integrator. Injection port temperature was maintained at 200°C and the detector temperature was set at 300°C. The carrier gas was nitrogen which flowed at a rate of 15 ml/min.

Optimized derivatization procedure

Standard sugars. D-Galactose (Gal), D-glucose (Glc), N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc) (4 sugars predominant in glycosphingolipids) and xylose (Xyl) (internal standard) were desiccated overnight before weighing and being brought to a concentration of 100 μg/ml in methanol. Equal amounts of each sugar, except xylose, (3 μg usually, but also 1, 5, 10, 25 and 50 μg) were placed in

* Nomenclature of gangliosides is according to Svennerholm[17].

a methanolysis tube and taken to dryness under a stream of nitrogen. A 0.5-ml volume of 1 *M* aqueous hydrochloride was added to each tube[20]. The volumes of reagents used were as mentioned when the amount of each monosaccharide varied between 1 and 5 μg. When derivatizing 10 to 50 μg of a monosaccharide, each reagent volume was doubled. The tubes were capped tightly with PTFE-lined screw caps and placed in a heating block at 100°C for 8 h[20,21]. The tubes were then removed from the heating block, cooled and an equivalent amount of xylose was added. The samples were then dried under nitrogen at 60–80°C. A 0.5-ml volume of freshly made $NaBH_4$ in 1 *M* NH_4OH (2 mg/ml) was added and each tube capped, vortexed and allowed to stand at room temperature for 40 min[22]. The excess reducing reagent was then destroyed by dropwise addition of glacial acetic acid[9] until all effervescence ceased (6–7 drops from a Pasteur pipet). The samples were then taken to near dryness under nitrogen at 60–80°C. The viscous boric acid remaining in each tube was removed by adding 1 ml of methanol–benzene (5:1), capping tightly, vortexing vigorously and then heating 5 min at 90°C. The samples were allowed to cool slightly, then evaporated to near dryness again. This procedure was repeated five more times using pure methanol[23]. By the end of the third repetition, the samples evaporated to complete dryness and could then be loosely capped and desiccated over P_2O_5 (no vacuum) overnight if the rest of the procedure could not be completed in the same day. Acetic anhydride, 0.75 ml, was added to each tube which was capped tightly, vortexed and placed in a heating block at 100°C for 30 min[22,23]. Following acetylation, the samples were cooled to room temperature and dried under a light stream of nitrogen at 38°C. Each sample was reconstituted with 0.5 ml chloroform and the salts removed by partitioning against 0.5 ml distilled water 5 times[21]. The desalted samples were taken to dryness under nitrogen at 38°C and transferred to reacti-vials with 3 additions of 0.5 ml chloroform. The contents of the reacti-vials were taken to dryness and the samples tightly capped and stored in the P_2O_5 desiccator until analyzed (never longer than 4 days). When analyzed, the samples were reconstituted to a volume of 10–50 μl, depending upon the amount of starting sugar; the volume equivalent to 1 μg of each sugar was injected (except for 1 μg samples where 0.5 μg was injected). Triplicate runs were performed on each sample (except 1 μg samples which had duplicate runs).

Gangliosides. Sets of gangliosides were prepared to correspond to 1, 3 and 5 μg of their glucose content. This was done by assigning their identity via thin-layer chromatographic mobilities and quantitating their sialic acid content colorimetrically[5,24]. Gangliosides were hydrolyzed in 0.5 ml of 1 *M* aqueous hydrochloride for 8 h at 100°C. Xylose, the internal standard, was added after hydrolysis, and the fatty acids extracted by partitioning against 0.5 ml of hexane 3 times. The pH was then adjusted to between 10 and 12 with 2 drops of 7.4 *M* NH_4OH and the sphingosines were extracted with 3 × 0.5 ml of diethyl ether[25]. (When sphingosines and fatty acids were not to be saved, pH adjustment was the initial step followed by the hexane extraction. No diethyl ether was used.) The samples were then subjected to the same steps as described above for the standard sugars. Standard sugars and GM_1 standard were always derivatized at the same time as the ganglioside samples.

Changes in derivatization procedure

Various conditions of the method such as hydrolysis time, reduction time, acetylation time and temperature, were examined using sugar standards and ganglio-

sides. Details denoting how these experimental protocols differed from the optimized derivatization procedures already described are noted under the appropriate heading within the Results section.

Statistics

Analyses included Student's *t*-test, one way analysis of variance, two way analyses of variance and Duncan's multiple range test[26].

RESULTS

Chromatogram

Fig. 1 shows a typical chromatogram of alditol acetates of xylose, glucose, galactose, N-acetylglucosamine and N-acetylgalactosamine. Xylose, the internal standard, is well separated from galactose, and there is little overlap of either the glucose and galactose peaks or of the N-acetylglucosamine and N-acetylgalactosamine peaks.

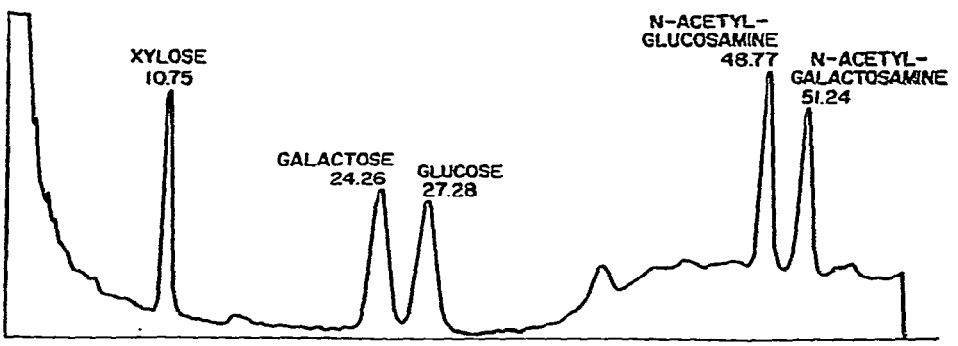

Fig. 1. Chromatogram of alditol acetates prepared from 3 μg each of xylose (internal standard), galactose, glucose, N-acetylglucosamine and N-acetylgalactosamine, chromatographed on a Hewlett-Packard No. 5710A GLC with a flame-ionization detector, dual differential electrometer (range 1, attenuation 32), and Hewlett-Packard No. 3385 integrator. Columns (1% OV-225 on 100–120 mesh Gas-Chrom Q) were 6 ft. × 1/4 in. O.D. Temperature program was 32 min at 190°C, then 16 min at 230°C (rate, 4°C/min). Injection port temperature was 200°C and detector temperature was 300°C. Carrier gas, nitrogen, flowed at 15 ml/min. A slight upward drift of the baseline occurred during temperature programming but did not affect electronic peak integration. The numbers represent the retention time of each sugar in minutes. Sample amount injected corresponds to 1 μg of each sugar. The ordinate is detector response; the abscissa is time in minutes.

Effects of varying the amounts of starting sugars upon the sugar ratios

All sugars relative to xylose decreased significantly (Table I) as the amount of starting sugar decreased from 50 to 25 ($P < 0.05$) and from 25 to 10 μg ($P < 0.01$), but only GalNAc decreased significantly from 3 to 1 μg ($P < 0.01$). The only sugar to change significantly relative to glucose (Table I) was GalNAc which decreased from 50 to 25 μg ($P < 0.05$).

Changes from the optimized derivatization procedure included a 16-h hydrolysis wich xylose added before hydrolysis, a 60-min $NaBH_4$ reduction and a 3-h acetylation.

Set to set variation in sugar ratios

All sugar ratios (Table I), except Glc:Xyl at 50, 25, 10 μg levels, varied significantly ($P < 0.01$) when prepared on different days (set number). The coefficient of variation (standard deviation/mean $\times$ 100) of data from multiple sets for Gal:Xyl averaged $\pm 12\%$; for Glc:Xyl, $\pm 9\%$; for GlcNAc:Xyl, $\pm 22\%$; for GalNAc:Xyl, $\pm 19\%$; for Gal:Glc, $\pm 11\%$; for GlcNAc:Glc, $\pm 22\%$; and for GalNAc:Glc, $\pm 18\%$.

Addition of the internal standard before or after hydrolysis

Fig. 2 shows the advantage of adding xylose after the hydrolysis step. Gal:Xyl is 290% greater ($P < 0.025$); Glc:Xyl, 200% greater ($P < 0.01$); GlcNAc:Xyl, 150% greater ($P < 0.05$); and GalNAc:Xyl, 130% greater ($P < 0.005$) when xylose is added before hydrolysis. Derivatization changes were as described under *Effects of varying the amounts of starting sugars upon the sugar ratios.*

Fig. 2. Effects of addition of the internal standard, xylose, before and after 16 h of acid hydrolysis. A 3-μg amount of each sugar was used to prepare alditol acetate derivatives. Each sugar relative to xylose is represented as a bar graph with the mean of 3 separate derivatizations $\pm$ standard deviation shown. Notice how much greater the ratios are when xylose is also hydrolyzed indicating xylose degradation. Significance was determined by Student's *t*-test.

Effects of length of hydrolysis on sugar standards

Fig. 3 graphs the decrease in each sugar (relative to xylose) with increasing length of hydrolysis. Gal:Xyl decreased 26% ($P < 0.001$), Gly:Xyl decreased 20% ($P < 0.005$), GlcNAc:Xyl decreased 14% ($P < 0.005$) and GalNAc:Xyl decreased 4% ($P < 0.01$) between 0 and 4 h. Glc:Xyl also decreased between 8 and 12 h ($P < 0.01$). No further changes of significance were noted. Xylose was added following a 16-h hydrolysis. Other changes included a 60-min $NaBH_4$ reduction and a 3-h acetylation. Each time point (0, 4, 8, 12 and 16 h) was run in triplicate.

TABLE I

VARIATION IN ALDITOL ACETATE SUGAR RATIOS AS A FUNCTION OF AMOUNTS OF STARTING MATERIAL AND OF PREPARATION ON DIFFERENT DAYS (SET NO.)

Derivatives were prepared from standard sugar as described in the optimized derivatization procedure except that xylose was added before hydrolysis, hydrolysis time was 16 h, $NaBH_4$ reduction time was 60 min and acetylation time was 3 h. Statistical analyses included a two-way analysis of variance and a Duncan's multiple range test. Each number represents the mean of three samples ± standard deviation.

Set No.	*Sugar (μg)*	*Gal:Xyl*	*Glc:Xyl*	*GlcNAc:Xyl*	*GalNAc:Xyl*	*Gal:Glc*	*GlcNAc:Glc*	*GalNAc:Glc*
1	50	1.077 ± 0.026	1.592 ± 0.118	0.738 ± 0.112	1.051 ± 0.167	0.678 ± 0.032	0.464 ± 0.049	0.659 ± 0.057
1	25	1.024 ± 0.042	1.564 ± 0.084	0.726 ± 0.026	0.882 ± 0.106	0.657 ± 0.057	0.465 ± 0.037	0.566 ± 0.091
1	10	0.906 ± 0.076	1.346 ± 0.121	0.635 ± 0.111	0.776 ± 0.077	0.675 ± 0.057	0.470 ± 0.044	0.576 ± 0.016
2	50	1.014 ± 0.143	1.663 ± 0.102	1.077 ± 0.163	1.051 ± 0.144	0.586 ± 0.068	0.631 ± 0.082	0.614 ± 0.049
2	25	0.839 ± 0.047	1.428 ± 0.027	1.018 ± 0.070	0.824 ± 0.110	0.583 ± 0.033	0.709 ± 0.056	0.570 ± 0.068
2	10	0.760 ± 0.038	1.456 ± 0.046	1.094 ± 0.072	0.836 ± 0.055	0.521 ± 0.011	0.743 ± 0.048	0.573 ± 0.022
3	50	1.153 ± 0.111	1.705 ± 0.097	1.295 ± 0.105	1.493 ± 0.092	0.676 ± 0.112	0.771 ± 0.029	0.853 ± 0.083
3	25	0.960 ± 0.031	1.567 ± 0.091	0.866 ± 0.259	1.078 ± 0.227	0.616 ± 0.053	0.548 ± 0.140	0.685 ± 0.113
3	10	0.827 ± 0.028	1.385 ± 0.147	1.022 ± 0.124	0.987 ± 0.080	0.613 ± 0.099	0.739 ± 0.044	0.743 ± 0.080
4	50	0.980 ± 0.029	1.652 ± 0.086	1.039 ± 0.133	1.590 ± 0.172	0.595 ± 0.016	0.625 ± 0.034	0.961 ± 0.056
4	25	1.136 ± 0.056	1.608 ± 0.178	0.958 ± 0.205	1.522 ± 0.235	0.711 ± 0.047	0.593 ± 0.077	0.948 ± 0.102
4	10	0.911 ± 0.059	1.258 ± 0.014	0.675 ± 0.120	1.037 ± 0.141	0.724 ± 0.040	0.536 ± 0.092	0.824 ± 0.106
5	5	1.076 ± 0.070	1.751 ± 0.088	0.967 ± 0.055	1.163 ± 0.129	0.620 ± 0.028	0.552 ± 0.011	0.666 ± 0.048
5	3	1.053 ± 0.043	1.651 ± 0.098	0.988 ± 0.110	1.216 ± 0.069	0.638 ± 0.022	0.597 ± 0.036	0.736 ± 0.003
5	1	1.250 ± 0.372	1.670 ± 0.190	0.901 ± 0.033	1.021 ± 0.094	0.738 ± 0.149	0.543 ± 0.049	0.613 ± 0.046
6	5	1.070 ± 0.077	1.394 ± 0.148	0.701 ± 0.078	1.171 ± 0.080	0.770 ± 0.032	0.505 ± 0.056	0.844 ± 0.030
6	3	0.968 ± 0.146	1.355 ± 0.216	0.783 ± 0.177	1.198 ± 0.200	0.715 ± 0.027	0.575 ± 0.038	0.876 ± 0.006
6	1	0.843 ± 0.030	1.218 ± 0.223	0.727 ± 0.065	1.023 ± 0.205	0.706 ± 0.112	0.613 ± 0.139	0.876 ± 0.149
7	5	0.878 ± 0.043	1.380 ± 0.105	0.606 ± 0.013	0.912 ± 0.048	0.640 ± 0.076	0.441 ± 0.039	0.665 ± 0.082
7	3	0.973 ± 0.084	1.415 ± 0.239	0.625 ± 0.085	0.991 ± 0.065	0.694 ± 0.061	0.454 ± 0.119	0.716 ± 0.143
7	1	0.942 ± 0.107	1.265 ± 0.071	0.706 ± 0.043	1.052 ± 0.121	0.717 ± 0.054	0.530 ± 0.049	0.794 ± 0.073
8	5	0.939 ± 0.089	1.508 ± 0.175	1.080 ± 0.074	0.934 ± 0.030	0.618 ± 0.025	0.699 ± 0.065	0.681 ± 0.079
8	3	0.890 ± 0.071	1.295 ± 0.157	1.112 ± 0.148	1.069 ± 0.028	0.671 ± 0.028	0.876 ± 0.123	0.835 ± 0.104
8	1	0.620 ± 0.139	1.237 ± 0.172	0.999 ± 0.313	0.583 ± 0.189	0.498 ± 0.054	0.801 ± 0.178	0.502 ± 0.120

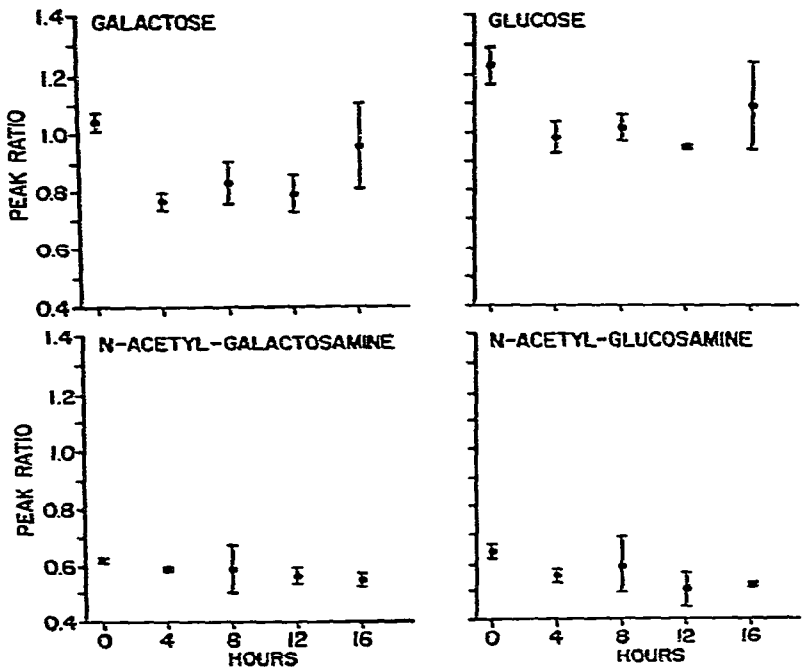

Fig. 3. Effects of hydrolysis time on standard sugars relative to xylose. A 3-μg amount of each sugar was derivatized; xylose was added after the hydrolysis step. Each time point represents a mean ± S.D. of three derivatizations. See Results (section *Effects of length of hydrolysis on sugar standards)* for significance.

Effects of length of $NaBH_4$ reduction on standard sugars

Fig. 4 graphs each sugar relative to Xyl *vs.* reduction time and shows that maximum ratios are obtained by 40 min. Relative to xylose, Glc and GlcNAc increased significantly between 30 and 40 min ($P < 0.01$) and GalNAc increased significantly between 10 and 20 min ($P < 0.05$). No hydrolysis was performed. Each time point (10, 20, 30, 40, 50 and 60 min) was run in triplicate. Acetylation was for 3 h.

Effect of acetylation temperature upon standard sugars

Fig. 5 shows that optimum acetylation in terms of maximum sugar ratios relative to xylose is achieved at 100°C. Gal:Xyl increased significantly between 80 and 90°C ($P < 0.05$) and GalNAc:Xyl increased significantly ($P < 0.01$) between 90 and 100°C. No hydrolysis was performed. $NaBH_4$ reduction was for 60 min. Each temperature point (80, 90, 100, 110, 120°C) was run in triplicate for 3 h.

Effects of length of acetylation on standard sugars

Fig. 6 shows that the optimum acetylation time in terms of maximum sugar ratios relative to xylose is 30 min. Gal:Xyl increased significantly between 5 and 10 ($P < 0.01$) and 15 and 30 min ($P < 0.05$). GlcNAc:Xyl decreased between 5 and 10 min ($P < 0.05$) while GalNAc:Xyl increased between 5 and 10 min ($P < 0.01$), 10 and 15 min ($P < 0.01$) and 15 and 30 min ($P < 0.05$). No hydrolysis was performed.

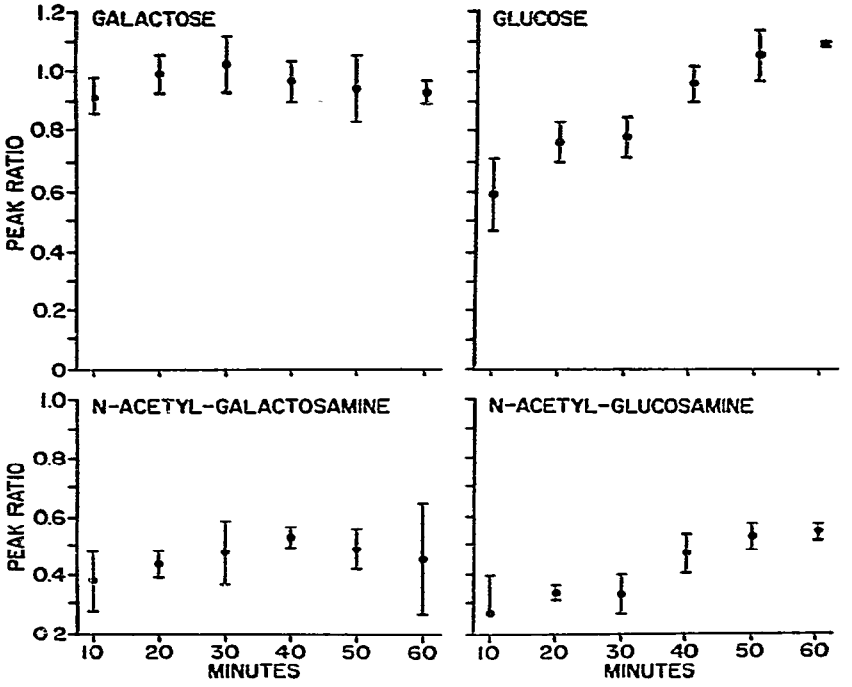

Fig. 4. Effects of varying $NaBH_4$ reduction time on peak ratios of standard sugars relative to xylose. A 3-μg amount of each sugar was derivatized with no hydrolysis step. Each time point represents the mean $\pm$ S.D. of three derivatizations. Significance was determined by a one way analysis of variance followed by Duncan's Multiple Range Test. See Results (section *Effects of length of $NaBH_4$ reduction on standard sugars)* for significance.

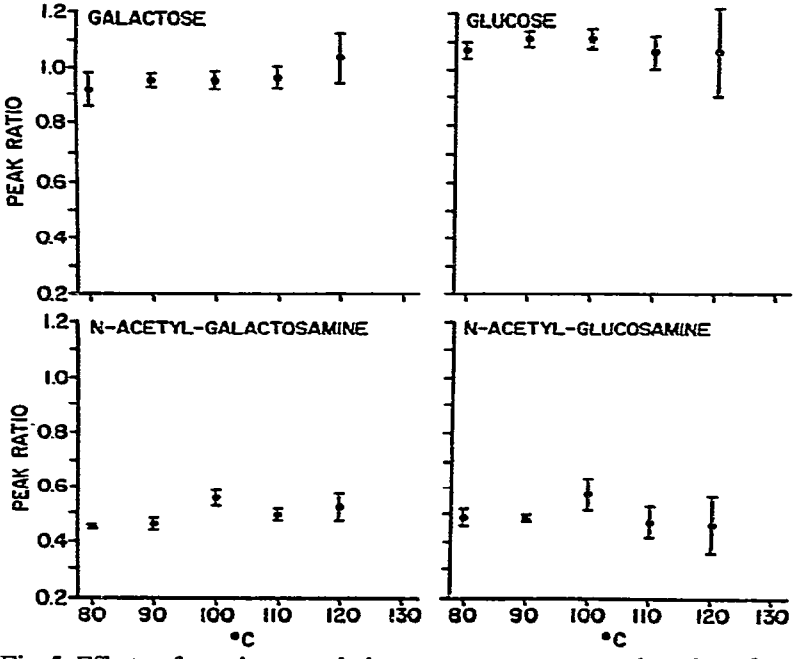

Fig. 5. Effects of varying acetylation temperatures on peak ratios of standard sugars relative to xylose. See Fig. 4 for details. Acetylation time was 3 h. See Results (section *Effect of acetylation temperature upon standard sugars)* for significance.

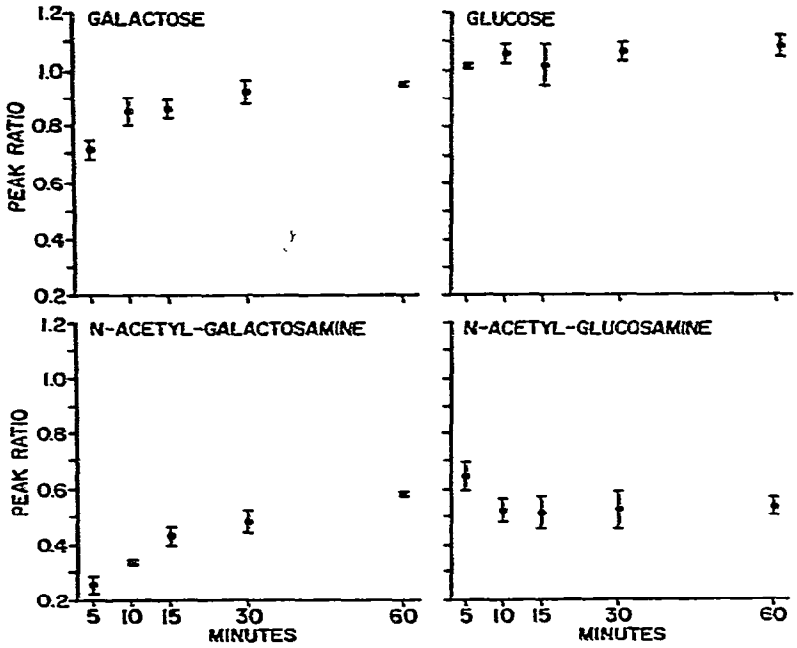

Fig. 6. Effects of varying acetylation time on peak ratios of standard sugars relative to xylose. See Fig. 4 for details. Acetylation temperature was 100°C. See Results (section *Effects of length of acetylation on standard sugars)* for significance.

$NaBH_4$ reduction was 60 min. Each time point (5, 10, 15, 30, 60 min) was run in triplicate at 100°C.

Effects of NH_4OH neutralization and hexane extraction on standard sugars

Table II shows that the only effect of neutralization and hexane extraction was a 34% decrease in GlcNAc relative to Xyl ($P < 0.005$). Three samples were treated normally (16-h hydrolysis, 60-min reduction, 3-h acetylation); three samples were neutralized with 2 drops of 7.4 *N* NH_4OH following hydrolysis and then extracted three times with 0.5 ml of hexane.

TABLE II

EFFECTS OF NH_4OH NEUTRALIZATION AND HEXANE EXTRACTION ON RATIOS OF STANDARD SUGARS RELATIVE TO XYLOSE

A 3-μg amount of each standard sugar was hydrolyzed 16 h. Xylose was added following hydrolysis. 3 samples were untreated; 3 samples were neutralized with 2 drops of 7.4 *N* NH_4OH and then extracted 3 times with 0.5 ml hexane. Significance was determined by Student's *t*-test.

Treatment	*Gal: Xyl*	*Glc: Xyl*	*GlcNAc: Xyl*	*GalNAc: Xyl*
Neutralized and extracted	0.806 ± 0.041	1.009 ± 0.055	0.471 ± 0.076*	0.570 ± 0.052
Untreated	0.823 ± 0.058	0.988 ± 0.094	0.714 ± 0.040	0.648 ± 0.046

* $P < 0.005$.

Effects of length of hydrolysis on the sugar ratios of GM_1

Table III demonstrates the phenomenon of sugar release from GM_1 *vs.* sugar degradation with time. Relative to xylose, Gal increases up to 4 h, then declines substantially between 12 and 16 h; Glc increases up to 12 h; GalNAc shows an initial increase at 2 h followed by a decline and then a rise again from 3 to 12 h.

TABLE III

EFFECTS OF LENGTH OF HYDROLYSIS ON THE SUGAR RATIOS OF GM_1

Duplicate samples per time point were prepared from an amount of GM_1 corresponding to 3 μg of its glucose content. Hydrolysis time varied between 1 and 16 h. Xylose was added following hydrolysis. Reduction was 60 min; acetylation was 3 h. Each time point represents 2 samples only, so no statistics were performed. Theoretical Gal:Glc and GalNAc:Glc ratios for GM_1 are 2:1 and 1:1, respectively.

Hydrolysis time (h)	*Gal: Xyl*	*Glc: Xyl*	*GlcNAc: Xyl*	*Gal: Glc*	*GalNAc: Glc*
1	1.080	0.171	0.500	6.384	2.924
2	1.268	0.324	0.654	4.290	2.225
3	1.322	0.418	0.638	3.186	1.538
4	1.351	0.498	0.694	2.764	1.424
8	1.302	0.638	0.756	2.040	1.205
12	1.298	0.708	0.880	1.846	1.240
16	1.180	0.685	0.770	1.725	1.112

Gal:Glc falls continuously from 1 through 16 h. It most closely approximates theoretical values of 2.00/1.00 at 8 h. GalNAc:Glc also declines between 1 and 16 h and most closely approximates theoretical values of 1.00/1.00 at 16 h. Changes from the optimized procedure included a 60-min reduction and 3-h acetylation. Duplicate samples of GM_1 were hydrolyzed for 1, 2, 3, 4, 8, 12 and 16 h.

Effects of nitrogen vs. air in the reaction mixture during GM_1 hydrolysis

Table IV shows no significant differences in the sugar ratios between samples treated normally (16-h hydrolysis, 60-min reduction, 3-h acetylation) and those having their ambient air displaced by bubbling nitrogen gas through the reactants for 30 sec before hydrolysis.

TABLE IV

EFFECTS OF AMBIENT NITROGEN *VS.* AIR DURING HYDROLYSIS UPON SUGAR RATIOS OF GM_1

Samples were treated as in Table III with 16 h of hydrolysis. Three samples had their air displaced for 30 sec by nitrogen bubbling before sealing the tube for hydrolysis; three samples were untreated. No significant differences were seen with Student's *t*-test.

Treatment	*Gal:Xyl*	*Glc:Xyl*	*GalNAc:Xyl*	*Gal:Glc*	*GalNAc:Glc*
GM_1 + air	1.390 $\pm$ 0.018	0.861 $\pm$ 0.030	0.789 $\pm$ 0.060	1.622 $\pm$ 0.055	0.926 $\pm$ 0.082
GM_1 + nitrogen	1.325 $\pm$ 0.118	0.855 $\pm$ 0.033	0.770 $\pm$ 0.055	1.589 $\pm$ 0.071	0.903 $\pm$ 0.056

Comparison of standard sugar ratios vs. those of GM_1

Table V shows that Gal:Xyl, Glc:Xyl and GalNAc:Glc ratios of standard sugars were significantly different from those of GM_1 ($P < 0.01$, $P < 0.001$, $P < 0.05$, respectively). In particular, the GalNAc:Glc ratio of standard sugars was 24% lower than that of GM_1. Three samples of GM_1 and three of standard sugars were hydrolyzed for 16 h, neutralized and extracted with hexane, had 60 min of reduction and a 3-h acetylation.

TABLE V

COMPARISON OF STANDARD SUGAR RATIOS WITH THOSE OBTAINED FROM GM_1

Three samples of standard sugars were prepared as described for untreated samples in Table II. Three samples of GM_1 were prepared as described in Table III. Significance was determined by a Student's *t*-test. Using these standard sugars, the corrected sugar ratios for GM_1 are Gal:Glc, 2.02:1 (theoretical 2:1) GalNAc:Glc, 1.23:1 (theoretical 1:1).

	Gal:Xyl	*Glc:Xyl*	*GalNAc:Xyl*	*Gal:Glc*	*GalNAc:Glc*
GM_1	$0.660 \pm 0.069^{**,\S}$	$0.744 \pm 0.040^{***}$	0.745 ± 0.077	$0.886 \pm 0.116^{\S}$	$1.010 \pm 0.114^{*}$
Sugar standards	0.860 ± 0.034	0.981 ± 0.034	0.758 ± 0.102	0.877 ± 0.023	0.772 ± 0.092

$^{*}P<0.05$.
$^{**}P<0.01$.
$^{***}P<0.001$.
§ Divided by 2 for comparison.

Sugar ratios of known gangliosides

Table VI demonstrates that use of a GM_1 standard for ratio correction of sugars from GD_{1a}, GM_2, GT_{1b} and GD_3 brings these ratios closer to theoretical values than does the use of standard sugars which yield Gal:Glc and GalNAc:Glc

TABLE VI

COMPARISON OF CORRECTED GANGLIOSIDE SUGAR RATIOS USING GM_1 AND STANDARD SUGARS

Each ganglioside was treated as in Table III. Duplicate sets were run; each set contained samples of both GM_1 and standard sugars. Ganglioside sugar ratios were corrected using both standard sugars and GM_1. These corrected ratios were compared to theoretical ratios using a paired *t*-test. Ganglioside sugar ratios corrected by using GM_1 were not significantly different from theoretical values. The GalNAc:Glc ratio varied significantly from theoretical ($P<0.005$) as did the Gal:Glc ratio ($P<0.05$) when corrected by using standard sugars.

Ganglioside	*Sugars*	*Theoretical ratio*	*Corrected ratio using GM_1*	*Corrected ratio using standard sugars*
GD_{1a}	Gal:GalNAc:Glc	2:1:1	1.9:1.0:1	2.2:1.7:1
GD_{1a}	Gal:GalNAc:Glc	2:1:1	2.0:1.2:1	2.2:1.8:1
GT_{1b}	Gal:GalNAc:Glc	2:1:1	2.0:1.1:1	2.2:1.8:1
GT_{1b}	Gal:GalNAc:Glc	2:1:1	1.9:1.0:1	1.9:1.2:1
GM_2	Gal:GalNAc:Glc	1:1:1	1.0:0.9:1	1.1:1.4:1
GM_2	Gal:GalNAc:Glc	1:1:1	1.0:0.8:1	1.2:1.3:1
GD_3	Gal:Glc	1:1	1.2:1	1.4:1
GD_3	Gal:Glc	1:1	0.9:1	0.8:1

ratios considerably different from theoretical values ($P < 0.05$ and $P < 0.005$, respectively). Variation from theoretical ratios in GM_1 corrected ratios was never more than 20% and averaged 7%. Variation from theoretical ratios corrected by using sugar standards was as high as 80% and averaged 32% due principally to the GalNAc:Glc ratios of the standard sugars being less than those of gangliosides. Changes from optimized conditions included a 16-h hydrolysis, 60-min reduction and a 3-h acetylation.

DISCUSSION

Analyses of microgram quantities of carbohydrates from glycosphingolipids requires both a sensitive and a reliable method. Sensitive techniques measuring nanogram amounts of sugars have been reported but require elaborate and expensive modifications of the simpler flame-ionization detector GLC (*e.g.* mass spectroscopy[11,12], electron-capture detectors[27,28], radiogas chromatography[29]. Reproducibility has been a persistent problem in the use of GLC to quantitate carbohydrates. The TMS method has been fraught with inconsistencies in hexosamines (from decomposition, absorption to columns and acidic resins, and de-N-acetylation during hydrolysis[14,30]). There have also been disparities in the $AgCO_3$ neutralization step with loss of the internal standard[13,15], an effect which is potentiated in the microgram range[16]. Interpretation of multiple peaks, especially the hexosamines, also enhances the difficulties.

The alditol acetate method offers a simpler spectrum. However, working in the 1–50-μg range requires some modifications of existing procedures in order to increase sensitivity of the method and to reduce baseline noise from residual reagents. Furthermore, glycosphingolipids present a special problem in that the glucose to ceramide bond is not readily cleaved[10,20] causing a situation in which there are differential rates of sugar release and destruction during hydrolysis. We have carefully investigated this alditol acetate micromethod with the goal of optimizing sugar ratios for glycolipid analysis while minimizing the length of time required for the procedure. In doing so, we have attended to anecdotal reports in the literature which speculate on causes of sugar loss.

We chose xylose as our internal standard since its peak separates well from galactose and since it is not known to be a component sugar of glycolipids (particularly gangliosides). As is clearly demonstrated by Table I and Fig. 2, xylose proves to be somewhat unstable in acid (high sugar ratios relative to xylose) especially as hydrolysis time approaches 16 h (data not shown). This confirms Albersheim's observation of low xylose stability in acid after 2 h of pinto bean cell wall hydrolysis[23]. Therefore, we have begun adding the internal standard after the hydrolysis step. Jamieson and Reid, after showing a loss of mannitol during the $AgCO_3$ neutralization step in TMS derivatization, suggested adding mannitol after hydrolysis[13] and Levvy *et al.*[14] put this suggestion into practice. We have reduced our coefficient of variation from a maximum of 32% (average of 20%) when xylose was hydrolyzed to a maximum of 15% (average 11%) when it was added following hydrolysis. Free sugars in acid are especially labile (see below).

Fucose-containing glycolipids require a different internal standard since fucose often appears as two peaks, one major peak (retention time, $t_R = 5.8$ min) and one minor peak ($t_R = 9.6$ min). The latter overlaps xylose. Therefore, we recommend

using deoxyglucose or ribose as an internal standard when dealing with fucose as these two sugars have distinguishable peaks with retention times of 11.9 and 7.0 min, respectively.

Table I shows that sugar ratios relative to glucose are consistent between 25 and 10 μg or between 5 and 1 μg. However, they vary significantly when expressed relative to xylose. Xylose was hydrolyzed in these experiments and its susceptibility to degradation is borne out by the consistency of these sugar amounts when expressed in terms of glucose rather than xylose. The 5, 3 and 1 μg results were more consistent in terms of xylose than the 50, 15 and 10 μg samples, possibly due to the closer amounts of material.

Set to set variation in sugar ratios exists as is demonstrated by Table I. This requires that each set of samples be derivatized with standards since no one set of correction terms will be reliable for every derivatization performed (due to reagent and column aging and possibly unidentified side products of the derivatization reactions).

Instability of free sugars is aptly demonstrated in Fig. 3. Xylose was added after hydrolysis so that the denominator of each ratio would remain constant with time. Large (up to 26%) decreases occurred within the first 4 h of hydrolysis with further, but insignificant, decreases in the hexosamines up to 16 h. Table III shows the results of this same experiment upon sugars released from GM_1. Here, two competing processes occur: sugar release from the lipid and sugar degradation. Glucose and N-acetylgalactosamine reach their peak values by 12 h. Kannan *et al.*[20] noted that 12 h was required for complete release of glucose from ceramide in glucocerebroside, and Zanetta *et al.*[10] claimed 16 h were required for GM_1 and GD_{1a} to completely break their glucose–ceramide bonds. However, by 16 h galactose is degrading causing a less than ideal sugar ratio for Gal:Glc (1.725:1). This could be accounted for if the values were corrected by a factor calculated from simultaneously run sugar standards to bring the Gal:Glc ratio back to the theoretical 2:1 value. However, as Table V demonstrates, there is significantly less galactose and glucose at 16 h in GM_1 than in free sugar standards. Concurrently, N-acetylgalactosamine is relatively stable. Therefore, the correction factor computed from sugar standards bring the Gal:Glc ratio to a value of 2:1 but elevates the GalNAc:Glc ratio to a value greater than 1:1 (1.31:1). These three experiments suggest that ganglioside hydrolysis differs from free sugar degradation and therefore free sugars are not legitimate standards for correcting sugar ratios of gangliosides and probably other glycolipids. Perhaps the difference is due to side products formed during lipid hydrolysis and/or micellar existence of gangliosides in aqueous HCl.

Table VI lends further support to this conclusion. Here ganglioside sugar ratios corrected by using GM_1 were not significantly different from theoretical ratios. However, ratios corrected by using standard sugars did vary significantly from theoretical values, especially in giving a faulty elevation of GalNAc:Glc ($P < 0.005$). Therefore, we suggest using pure, known gangliosides as standards when quantitating unknown gangliosides obtained from thin-layer or column chromatography. Clarke[31], Yu and Ledeen[32], and Holm *et al.*[21] have also reported using the gangliosides GM_3, GM_2, GM_1, and GD_{1a} as standards for determining correction factors in GLC analyses of ganglioside sugars (including N-acetylneuraminic acid).

The problem of choosing a hydrolysis time for gangliosides is difficult to resolve

due to the differential rates of sugar release and degradation. Our data and others[10,20] suggest that the peak of released glucose and N-acetylgalactosamine occurs at 12 h whereas that of galactose occurs at 4 h. Thus, there could be significant breakdown of galactose by 12 h[19]. Also, the most ideal Gal:Glc ratio occurs at 8 h but the less than ideal Gal:Glc ratio at 12 or 16 h can be corrected by use of appropriate standards. This problem can be resolved in several ways. If it is convenient to hydrolyze overnight, 12 to 16 h can be utilized, but under other circumstances, 8 h should be sufficient when coupled with the use of appropriate standards.

The effects of reduction on standard sugars are shown in Fig. 4. The Glc:Xyl and GlcNAc:Xyl ratios increase with time but are not significant past 40 min. Gal:Xyl and GalNAc:Xyl tend to decrease past 40 min, but this was not statistically significant. Since both trends prove insignificant, any time between 40 and 60 min of $NaBH_4$ reduction can be considered appropriate. The best acetylation temperature (Fig. 5) appears to be 100°C since glucose, N-acetylglucosamine and N-acetylgalactosamine peak at this temperature (only GalNAc:Xyl does so significantly). We routinely use 100°C which simplifies temperature control of our heating block since both hydrolysis and acetylation can be run at the same temperature. Sugar ratios increased with the amount of time spent acetylating up to 30 min after which there were no significant differences (data for 2, 3, 4, 5 h of acetylation not shown). This differs from Niedermeier's claim that acetylation is complete within 15 min[34] although his was done in a boiling water bath which provides more uniform initial heating and thus may slightly speed up the acetylation.

Many reports have appeared claiming neutralization of the hydrolysis mixture prior to evaporation prevents sugar loss. This is especially true of TMS where $AgCO_3$ and resin have been used to remove H^+ (refs. 15, 33), but has also been reported for alditol acetates[34]. Table II shows the results of an experiment we conducted to investigate these claims. NH_4OH and hexane extraction were chosen since these are used to remove fatty acids and sphingosines from ganglioside hydrolysates before evaporation. We used NH_4OH rather than NaOH to avoid introducing a new cation into the derivatization procedure. The only significant difference between the two treatments was a 34% decrease in GlcNAc:Xyl with neutralization and hexane extraction. This could become significant when dealing with an N-acetylglucosamine-containing glycolipid and therefore an appropriate N-acetylglucosamine-containing standard should be used.

Kim *et al.*[35] advised excluding oxygen from the hydrolysis mixture of glycoproteins since oxidation side products could possibly result in a loss of sugars. We investigated this claim using GM_1. As shown in Table IV, there was no significant difference between sugar ratios obtained from hydrolysis vials containing air *vs.* those containing nitrogen. This does not exclude an effect on glycoproteins but appears to make no difference upon ganglioside sugar ratios.

We originally investigated this method with the purpose of optimizing sugar ratios and increasing the sensitivity for routine work in the 1 to 10 μg range. This required first a scaling down and an integration of pre-existing alditol acetate methods. We then established that consistent ratios can be obtained for gangliosides if an appropriate ganglioside standard is used for determining the correction factor. We found that an 8-h ganglioside hydrolysis is as appropriate as a 12- to 16-h hydrolysis based on the differential rates of sugar release and breakdown and use of standards.

Xylose, our internal standard, proved to be unstable in acid requiring its addition after hydrolysis. (We have not investigated the acid susceptibilities of ribose or 2-deoxyglucose so both may be better internal standards for future use.) Nitrogen in the hydrolysis mixture had no influence upon sugar ratios compared with air. Neutralization after hydrolysis did not prevent sugar loss and for N-acetylglucosamine actually caused a loss although the hexane extraction may also have been a factor. A 40-min reduction and a 30-min acetylation were both compatible with good ratios and 100°C proved to be the best acetylation temperature. The alditol acetate method now presents a reliable and sensitive procedure for analyzing glycolipid sugars in the 1–10-μg range.

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