Gas–liquid chromatographic assay of lipid-bound sialic acids: measurement of gangliosides in brain of several species

ROBERT K. YU and ROBERT W. LEDEEN

The Saul R. Korey Department of Neurology and The Department of Biochemistry, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461

ABSTRACT A method is described for analysis of gangliosides by GLC assay of the sialic acid component. Mild acid treatment in methanol converted the latter to methyl ketoside methyl ester, which was then chromatographed as the TMS derivative. The major methanolysis product was shown to be the β-anomer, and its chromatographic peak was used for quantification. NANA and NGNA could be analyzed simultaneously, while an *O*-acetylated derivative of NGNA was detected qualitatively.

Standard curves were obtained for the three following representative samples: (*a*) a mixture of beef brain gangliosides, (*b*) Tay-Sachs ganglioside, and (*c*) hematoside-NANA. These had different slopes which reflected the variation in yield of β-NANA obtained from methanolysis. The smallest sample analyzed in the present study contained 0.3 μg of NANA. The advantage of GLC in solving the problem of false chromogens is illustrated in a comparative study with two colorimetric procedures. Two columns are described whose combined use is highly effective in establishing identity and in eliminating false peaks when they arise.

The GLC method has been applied to analysis of the total brain ganglioside content of several species, and a general trend was observed toward decreasing levels in the lower vertebrates. In addition, NGNA was detected and quantified in several of these samples.

SUPPLEMENTARY KEY WORDS hematosides · *N*-acetylneuraminic acid · *N*-glycolylneuraminic acid · colorimetric assay · false chromogens

Gangliosides are characterized by the presence of one or more sialic acids in their multicomponent oligosaccharide chains. Since gangliosides are the only glycolipids which contain sialic acid (by definition), their assay has usually been based on colorimetric determination of this sugar. The two most widely used reagents today are resorcinol (1) and thiobarbituric acid (2); their use allows measurement of approximately 10 μg and 5 μg of sialic acid, respectively. Less frequently employed are orcinol (3), diphenylamine (4), tryptophan–perchloric acid (5), and the direct Ehrlich assay (6). More recently a fluorometric method which can measure 0.3 μg or less was described, and is the most sensitive method to date (7).

A common limitation of all these procedures is their inability to differentiate the various types of sialic acid encountered in biological materials. There have been seven different sialic acids characterized to date, and while most of these are associated with glycoproteins (8, 9) at least three types have been identified in gangliosides (8, 10, 11). Another serious handicap of the colorimetric methods is interference from false pigments, particularly in the analysis of complex or crude mixtures. One illustration of this came from a comparative study of several biological materials (12) in which a number of samples gave widely varying sialic acid values with different colorimetric methods. Another study of purified products from plants reported a substance which gave the same absorption spectrum with thiobarbituric acid as NANA, but which reacted negatively with the resorcinol, orcinol, and direct Ehrlich assays (13). Similarly, in our

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; NANA, *N*-acetylneuraminic acid; NGNA, *N*-glycolylneuraminic acid; β-NANA, methyl β-ketoside methyl ester of NANA; β-NGNA, methyl β-ketoside methyl ester of NGNA; BBG, beef brain ganglioside mixture; TSG, Tay-Sachs ganglioside; TMS, trimethylsilyl; RDR, relative detector response.

own attempts to utilize resorcinol or thiobarbituric acid to assay gangliosides from visceral organs, we have encountered sizable errors with crude or only partially purified samples which we attributed to false chromogens.

Based on previous success in differentiating NANA and NGNA on a qualitative basis (10), GLC was considered a promising solution to these problems of identification and interference. The present study describes a quantitative procedure for measuring both NANA and NGNA in ganglioside preparations, and also indicates the feasibility of identifying other sialic acids. Interference is greatly diminished even with one GLC column, while the use of two different columns provides a highly effective means of eliminating artifacts and establishing identity. Finally, the ability to analyze submicrogram amounts places this procedure among the more sensitive assays for sialic acid.

EXPERIMENTAL PROCEDURE

Materials

Methyl β-ketoside methyl ester of NANA (β-NANA) was synthesized and crystallized as described previously (14). The same procedure was used to prepare crystalline methyl β-ketoside methyl ester of NGNA (β-NGNA); this appears to be the first synthesis of this compound which was characterized as follows: melting point, 203–206°C; analytical data (Schwartzkopf Microanalytical Laboratories Inc., Woodside, N.Y.) C: 44.04, H: 6.66, N: 3.39 (calculated for $C_{13}H_{23}NO_{10}$: C: 44.19, H: 6.56, N: 3.39). GLC analysis of the TMS derivatives indicated that both substances were at least 95% pure. The starting materials for each synthesis (NANA and NGNA) were obtained from Sigma Chemical Co., St. Louis, Mo. Phenyl *N*-acetyl-α-D-glucosaminide, used as internal standard, was obtained from the same source. It could be crystallized from water, but analysis and melting point comparison indicated that the original material was sufficiently pure.

For methanolysis, redistilled Baker analyzed reagent grade methanol was used. Concentrated hydrochloric acid was also Baker analyzed reagent, while the dry HCl employed in some experiments was from Matheson Co., Inc. (East Rutherford, N.J.). Merck reagent grade pyridine was distilled over BaO and stored over KOH. Hexamethyldisilazane and trimethylchlorosilane were obtained from Applied Science Laboratories, Inc., State College, Pa. GLC column packings were obtained from Supelco, Inc., Bellefonte, Pa. These packings included OV-1, 3% on 100–120 Chromosorb W HP, and OV-225, 3% on 100–120 Supelcoport.

A mixture of beef brain gangliosides (BBG) was prepared from bovine gray matter by the method of Folch, Lees, and Sloane Stanley (15); it could also be obtained commercially from Supelco, Inc. Each preparation was further purified by trypsin digestion followed by silica gel chromatography. The ganglioside mixture was converted to the sodium salt by dialysis of a solution containing excess NaOH and NaCl, and after lyophilization was crystallized from a small volume of methanol. Tay-Sachs ganglioside (TSG), which was isolated from the brain of a patient with Tay-Sachs disease and purified by silica gel chromatography (16), was similarly crystallized as the sodium salt. Two hematosides containing NANA and NGNA, respectively, were prepared from bovine adrenal medulla as previously described (10). A third hematoside containing *O*-acetylated-NGNA (11) was a gift from Dr. S. Hakamori.

Rat brain was obtained from stock laboratory animals following decapitation. Frog (*Rana catesbeiana*) and goldfish (*Carassius auratus*) brains were gifts from Mr. David Forman of Rockefeller University. Human brains were obtained from a 19 yr old female (16 hr postmortem) and a 51 yr old male (3 hr postmortem); both were neurologically normal. The remaining brains were from mature animals and were obtained from a local abattoir a few hours postmortem with the exception of calf which was approximately 6 months old. Ox was allegedly 1.5–2 yr of age, while bull was over 8 yr old.

Ganglioside and Sialic Acid Standards

Weighed portions of β-NANA and β-NGNA synthetic samples were dissolved in CH_3OH, 1 mg/ml; aliquots were diluted an additional 100-fold. The formula weight for β-NANA was calculated to include 1 mole of water, in accordance with analytical data (14), whereas the corresponding NGNA derivative had no water of hydration. Samples of BBG and TSG, prepared as the sodium salts, were dried, weighed, and dissolved in $CHCl_3$–CH_3OH 1:1 to make stock solutions of 1 mg/ml. Hematosides were dissolved in the same solvent but could not be weighed beforehand due to limited quantity and the presence of a small amount of silica gel. In addition to GLC analysis, these solutions were assayed by the modified resorcinol (17) and thiobarbituric acid (18) procedures. Since the resorcinol results for BBG and TSG agreed well with calculated values based on weight, it was assumed that resorcinol provided a reliable value for hematoside-NANA.[1]

Ganglioside Isolation

Brain tissue was homogenized with 10 volumes of $CHCl_3$–CH_3OH 1:1, filtered through a sintered glass funnel (medium porosity), and $CHCl_3$ was added to the filtrate

[1] The term hematoside-NANA is used here to designate the substance isolated from bovine adrenal medulla (10), while hematoside-NGNA is the corresponding substance with the other sialic acid. The term "hematoside" denotes the following general structure: ceramide–lactose–sialic acid.

to adjust the ratio of $CHCl_3$:CH_3OH to 2:1. Partitioning was carried out according to Folch et al. (15) using 0.2 volumes of 0.12 M NaCl. The lower phase was partitioned a second time with "pure solvents upper phase" containing NaCl, and the two upper phases were combined. Aliquots corresponding to about 10 μg of sialic acid were evaporated to dryness and methanolyzed directly (*vide infra*) to measure total lipid-bound sialic acid. The small amount of salt in such samples did not interfere. However, for determination of NGNA, which comprised a very small percentage when present at all, much larger aliquots were needed corresponding in most cases to 100–200 μg of sialic acid. Since this larger quantity of salt was found to interfere such samples were processed as follows: 2 ml of $CHCl_3$–CH_3OH 1:1 was added to the dried residue, and the mixture was warmed and mixed with a Vortex vibrator several times; an additional 1 ml of $CHCl_3$ was then added, and the sample was cooled in ice. Precipitated salt was removed by centrifugation and washed with 1.5 ml of cold $CHCl_3$–CH_3OH 2:1. The combined supernates, which contained the ganglioside, were evaporated to dryness with nitrogen in a 12 ml methanolysis tube and were analyzed by the procedure described below.

Methanolysis and GLC Analysis

The dried ganglioside samples were treated with 2.0 ml of 0.05 N methanolic HCl, freshly prepared by addition of one part concentrated HCl (12 N) to 240 parts of distilled CH_3OH. After mixing well the samples were heated for 1 hr at 80°C. The Teflon-lined screw caps employed usually prevented solvent evaporation, but any samples, which did lose volume during heating, were discarded. The cooled solutions were extracted three times with 3 ml of hexane (spectroquality; Matheson Co., Inc., East Rutherford, N.J.) to remove the small quantities of liberated fatty acid esters which interfered with GLC analysis. The methanolic solutions were then evaporated to dryness with a slow steam of nitrogen. Internal standard (phenyl *N*-acetyl-α-D-glucosaminide) was added in a small volume of methanol, and the tube walls were rinsed three times with 0.1 ml of CH_3OH. The mixture was carefully evaporated with nitrogen and finally dried in a vacuum desiccator for 10–15 min. TMS derivatives were formed by adding 50 μl of reagent which was prepared according to Carter and Gaver (19). The sample was agitated well with a Vortex vibrator and allowed to stand 15 min. Approximately 4 μl was injected per assay, and each sample was assayed in triplicate. The other substances present in the mixture besides β-NANA (asialoganglioside, small amounts of hexose and hexosamine, etc.), did not interfere.

Quantification was achieved with a standard curve or by concurrent analysis of a ganglioside standard of known composition. Best accuracy was attained when the standard was similar in structure to the sample being analyzed, but if standards were not available, it was possible to calculate ganglioside-NANA from the known yield of β-NANA and its relative detector response (*vide infra*).

Peak areas were measured with an Infotronics CRS-101 electronic integrator; in some analyses, areas were also calculated by triangulation. Agreement between the two procedures was generally within 5%. Standard mixtures of fatty acid methyl esters were chromatographed on the OV-1 column as an additional test of quantification, and good agreement was obtained between the two methods and the theoretical values.

The following two types of U-shaped glass columns were utilized: (*a*) 3% OV-1, 6 ft × 4 mm; (*b*) 3% OV-225, 4 ft × 4 mm (see Materials). They were operated isothermally at 205°C and 190°C, respectively. Helium carrier gas was employed at a flow rate of 70 ml/min. Most analyses were performed on OV-1, which gave the best separation of β-NANA and β-NGNA. OV-225 was useful as a check for highly contaminated samples, but required a smaller injection volume because of the longer time required to elute pyridine; it was often beneficial to evaporate the pyridine solvent with nitrogen and redissolve in hexane before injection. The Hewlett-Packard F & M 402 gas chromatograph, equipped with the standard flame ionization detector, was utilized as a single column instrument.

RESULTS

GLC Identification of NANA and NGNA

A typical chromatogram using the OV-1 column is depicted in Fig. 1. A mixture of hematosides from bovine adrenal medulla (10) containing NANA and NGNA was subjected to methanolysis followed by TMS derivatization. Peaks *C* and *F* were identified as β-NANA and β-NGNA, respectively, by comparison with synthetic standards; *B* and *E* represented the α-ketosidic anomers.[2] Peak *A* was shown to be the *N*-deacylated product (methyl β-ketoside methyl ester of neuraminic acid) which always appeared in small amounts despite the mildness of the acid treatment. Peak *D* was internal standard.

GLC analysis of the same sample on the OV-225 column is shown in Fig. 2. This is more polar than OV-1, and both β-NANA (*B*) and β-NGNA (*C*) emerged ahead of the internal standard. The small α-NANA peak (*A*) was partially resolved from β-NANA, but the latter apparently masked the small α-NGNA peak. This would in-

[2] These are designated herein as α-NANA and α-NGNA. Their identification was also based on comparison with synthetic standards.

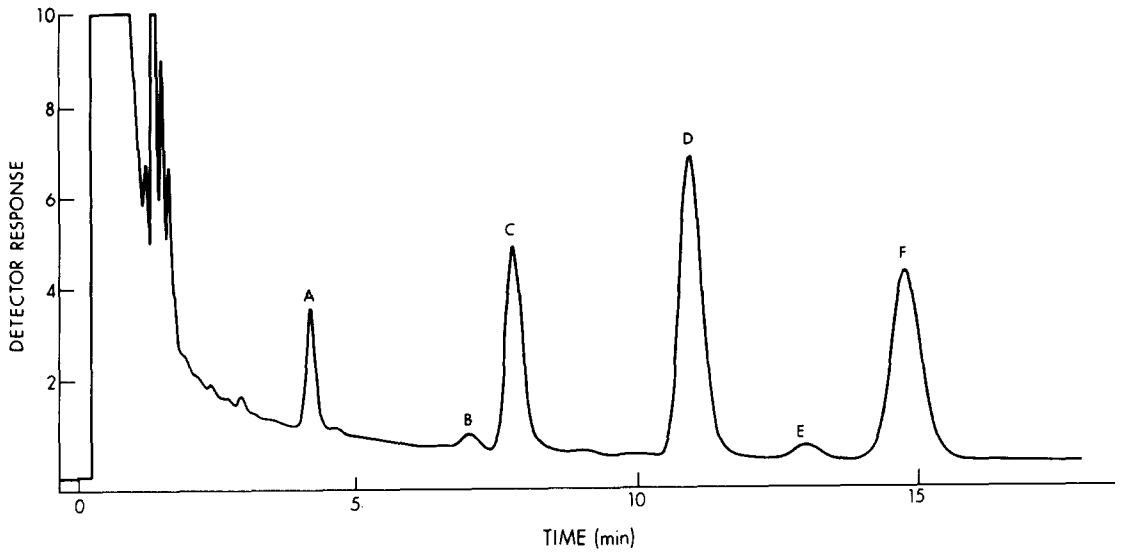

FIG. 1. Gas–liquid chromatogram of sialic acids on OV-1. A mixture of hematoside-NANA and hematoside-NGNA was heated 1 hr at 80°C in 0.05 N methanolic HCl, and the products were converted to TMS derivatives. Column was run isothermally at 205°C. Peak identification: *A*, methoxyneuraminic acid methyl ester (deacylated product); *B*, α-NANA; *C*, β-NANA; *D*, internal standard; *E*, α-NGNA; *F*, β-NGNA.

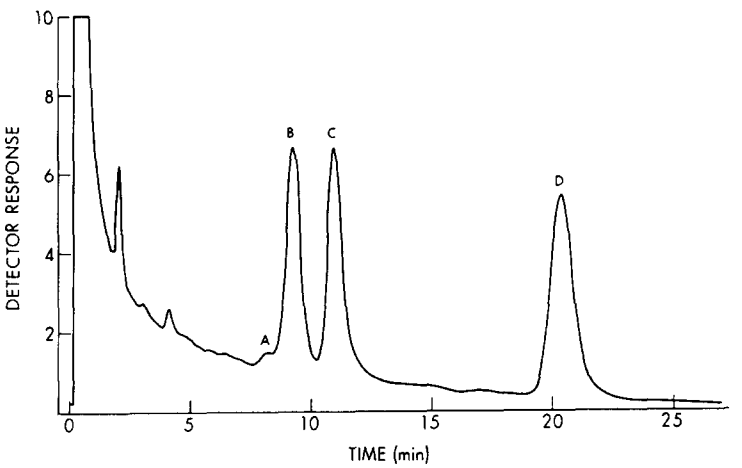

FIG. 2. Gas–liquid chromatogram of sialic acids on OV-225. Same samples as Fig. 1. Column was run isothermally at 190°C. Peak identification: *A*, α-NANA; *B*, β-NANA; *C*, β-NGNA; *D*, internal standard.

troduce a small error if this column were used for quantification of samples containing both types of sialic acid. Relative retention times of these various peaks are summarized in Table 1.

Relative Detector Response (RDR) of β-NANA and β-NGNA

Table 2 summarizes a series of experiments in which different amounts of pure β-NANA and β-NGNA were mixed with a constant amount of internal standard and analyzed on the OV-1 column. The samples were converted to TMS derivatives in 50 μl of reagent, and 5 μl was injected per analysis. The quantity of injected sialic acid of each type was 5.0–0.05 μg, while internal standard was constant at 0.5 μg. Relative detector response was calculated from the following equation, where β-SA represents β-NANA or β-NGNA, and IS is internal standard.

$$\text{RDR} = \frac{\text{Area (}\beta\text{-SA)}}{\text{Area (IS)}} \times \frac{\text{Weight (IS)}}{\text{Weight (}\beta\text{-SA)}}$$

The values did not change significantly between weight ratios of 10 and 0.6, and when all values within this range were averaged the difference between β-NANA and β-NGNA was found to be insignificant. However, with weight ratios below 0.6, the RDR values for both sialic acids decreased appreciably, indicating adsorptive losses on the GLC column. In another series the internal stan-

TABLE 1 RELATIVE RETENTION TIMES OF SIALIC ACIDS

Compound	Column	
	OV-1	OV-225
NANA: α-methyl ketoside methyl ester	0.71	0.41
NANA: β-methyl ketoside methyl ester	0.89	0.45
NGNA: α-methyl ketoside methyl ester	1.50	—
NGNA: β-methyl ketoside methyl ester	1.69	0.53
NGNA-*O*-acetyl methyl ketoside methyl ester	2.08	1.18
Phenyl *N*-acetyl-α-D-glucosaminide (IS)*	1.00	1.00

Retention times were measured relative to internal standard, whose absolute values were 10.0 min on OV-1 and 20.4 min on OV-225. Column conditions were: OV-1 3% on 100–120 Chromosorb W HP, 6 ft, 205°C; OV-225 3% on 100–120 Supelcoport, 4 ft, 190°C. Helium carrier gas was employed at a flow rate of 70 ml/min. All sialic acid samples were prepared synthetically except NGNA-*O*-acetyl, which was prepared by methanolysis of an hematoside (11) provided by Dr. Hakomori. All samples were chromatographed as TMS derivatives.

* Internal standard.

TABLE 2 RELATIVE DETECTOR RESPONSE (RDR) OF β-NANA AND β-NGNA*

Weight Ratio of Sialic Acid: Internal Standard	RDR	
	β-NANA	β-NGNA
10.0	0.932	0.950
8.0	0.937	0.950
6.0	0.925	0.949
4.0	0.938	0.949
2.0	0.912	0.913
1.0	0.924	0.919
0.8	0.918	0.920
0.6	0.900	0.897
Average ±2 SD	0.923 ± 0.028	0.931 ± 0.044
0.4	0.851 ± 0.016	0.824 ± 0.066
0.2	0.814 ± 0.030	0.765 ± 0.014
0.1	0.697 ± 0.010	0.648 ± 0.022

* Chromatographed as TMS derivatives on OV-1. See text for details. Three samples of each mixture were injected three times, and peak areas were measured by an electronic integrator. The two averages shown (average ±2 SD) were calculated for all samples having weight ratios between 10.0 and 0.6.

dard was also found to suffer adsorptive losses with quantities less than 0.3 μg per injection.

RDR for β-NANA was found to be approximately 4% lower on the OV-225 column than on OV-1. Although RDR was not utilized in the empirical standard curves employed for ganglioside sialic acid determination, it was a necessary constant for calculating the yield of β-NANA obtained by different methanolysis procedures (see below). RDR values were also measured periodically as a means of checking detector performance.

Optimal Methanolysis Conditions

Three ganglioside standards of known composition (BBG, TSG, hematoside-NANA) were methanolyzed by a variety of procedures to determine the conditions which gave optimal yield of β-NANA, the peak used for quantification. One such study, with time as the variable, is shown in Fig. 3. The yield was computed from the following equation, where T-NANA represents total NANA in the ganglioside sample, and IS is internal standard.

$$\text{Yield of } \beta\text{-NANA} = \frac{\text{Area } (\beta\text{-NANA})}{\text{Area (IS)}} \times \frac{\text{Weight (IS)}}{[\text{RDR}] \times [\text{T-NANA}]}$$

The conditions finally adopted were 0.05 N methanolic HCl at 80°C for 1 hr.

Although these conditions were suitable for the ganglioside standards used in this study (and for the large majority of gangliosides found naturally), they were too severe for *O*-acetylated sialic acids (relatively rare in gangliosides) which are easily deacylated in the methanolic medium. Thus *O*-acetyl NGNA in the hematoside of Hakomori and Saito (11) was converted almost entirely to β-NGNA. However, with milder conditions it was possible to obtain a peak for the acetylated form as well as β-NGNA; using 0.02 N methanolic HCl for 1 hr at 80°C, the ratio of areas for *O*-acetyl NGNA and β-NGNA was approximately 1:3. The ketoside ester of *O*-acetyl NGNA had a longer retention time than either β-NANA or β-NGNA (Table 1). It is probable that other *O*-acetylated sialic acids can be identified by this procedure, although quantification is not yet feasible with present methodology.

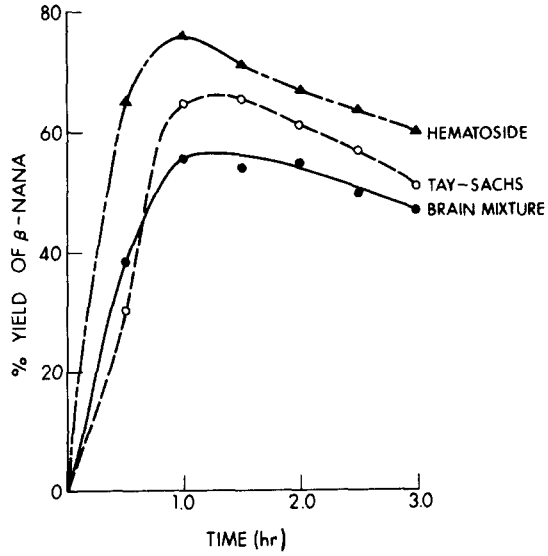

FIG. 3. Time study of ganglioside methanolysis. Ganglioside samples were heated at 80°C in 0.05 N methanolic HCl for different periods of time. Following evaporation of the solvent, internal standard was added, and the TMS derivatives were prepared. Yield of β-NANA was determined from peak areas (OV-1 column), and is expressed as percentage of total NANA.

In the initial trial runs the methanolic HCl reagent was prepared in two ways: with 12 N HCl and from dry HCl gas. Comparative studies revealed that the anhydrous reagent produced somewhat more deacylation product (peak *A*, Fig. 1), but the yield of β-NANA was not significantly different. Because of the greater convenience, 12 N HCl was used in all subsequent analyses.

An important observation in these experiments was that the yield of β-NANA from methanolysis was different for different gangliosides. Thus, the yield for hematoside (1 hr of methanolysis) was 76%, whereas TSG and BBG gave yields of 65% and 55%, respectively (Fig. 3). Quantification based on the β-NANA peak thus requires the appropriate ganglioside standard for maximum accuracy. It was acceptable to treat brain gangliosides as a unit for analytical purposes, despite the presence of several molecular species in the mixture, provided the composition of the sample did not differ drastically from that of the standard.

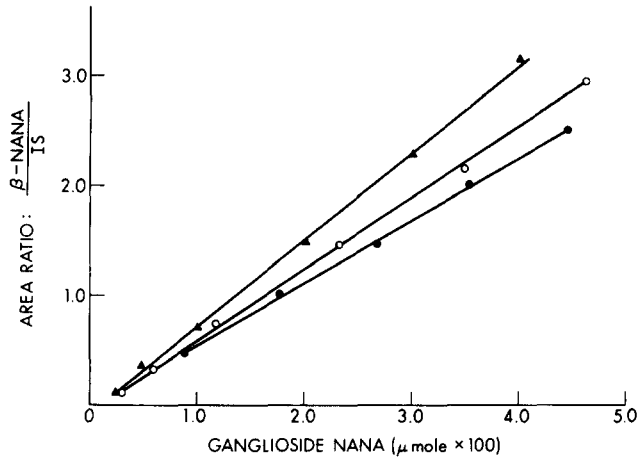

FIG. 4. Standard curves for three ganglioside samples. Range from 0.3×10^{-2} to 4.5×10^{-2} μmole NANA. See text for details. Abscissa represents total NANA in sample as determined by weight and resorcinol. ●, beef brain ganglioside mixture; ○, Tay-Sachs ganglioside; ▲, hematoside-NANA. IS is internal standard.

Standard Curves for Quantitative Analysis

Initial attempts at quantification of NANA were based on summation of the three peaks produced by methanolysis (Fig. 1), but eventually it was found more accurate and convenient to use the large β-peak alone. Standard curves for the three ganglioside samples are depicted in Fig. 4. The curves were linear over the indicated range, although there were different slopes for each sample. This was expected from the difference in yield observed in the time study (Fig. 3). The quantities of ganglioside NANA ranged from 0.9 to 14.4 μg (2.9–14.5 μg for BBG) with a constant amount of internal standard (3 μg) present in each sample. Derivatization was carried out in 50 μl of reagent, and 4-μl samples were injected on to the OV-1 column (205°C); analyses were done in triplicate.

Fig. 5 shows a similar series for BBG alone, however the samples were one-tenth as large as those in Fig. 4. The abscissa represents ganglioside NANA in each sample. In this series the methanolysis products were converted to TMS derivatives in 30 μl of reagent, and 5 μl was injected per assay (OV-1 column, 205°C). The analyses were carried out in triplicate. The smallest sample contained approximately 0.3 μg of NANA, and approximately 0.05 μg was injected on to the GLC column. The slope of the linear portion differed somewhat from that of the BBG series in Fig. 4, and in the lower region there was also some departure from linearity. This was probably due to the adsorptive losses mentioned previously. A third series (not shown) was carried out with intermediate amounts of BBG: 0.7–11.6 μg of sialic acid with 1 μg of internal standard. In this case the slope was identical to that of BBG in Fig. 4.

In establishing these empirical relationships, it was necessary to have an accurate measure of lipid-bound sialic acid in each ganglioside sample. This was provided by sample weight in the case of Tay-Sachs ganglioside, which was calculated to contain 21.8% sialic acid from its known composition (16). A similar calculation, slightly less accurate, was made for the mixture of beef brain gangliosides on the assumption of an average disialo composition corresponding to 32.8% sialic acid. Analysis of sialic acid in both samples by the resorcinol method were in close agreement with the calculated values. Hematoside-NANA, which could not be weighed, was sufficiently pure for accurate resorcinol assay.

GLC Analysis of Brain Gangliosides

The GLC method was used to estimate the amount of lipid-bound sialic acid in brain tissue of several species (Table 3). Gray and white matter were studied separately where possible, but whole brains were analyzed in the case of rat, rabbit, chicken, frog, and goldfish. The tabulated values were obtained with the OV-1 column, but some samples were also analyzed on OV-225 to check consistency; agreement was generally good.

The NGNA content of each sample was measured by analyzing large aliquots at high sensitivity. A typical GLC run of this type is shown in Fig. 6, and the quantitative results are summarized in Table 4. Bovine brain contained the highest level of NGNA, and gray matter had somewhat more than white in the case of calf and ox. There was no clear trend with age. Pig, sheep, and goldfish had detectable NGNA, but the levels were below 1% of the total sialic acid. Although quantitative accuracy was somewhat impaired at these low levels, qualitative identification could be made with considerable reliability by use of the two columns. No NGNA was detect-

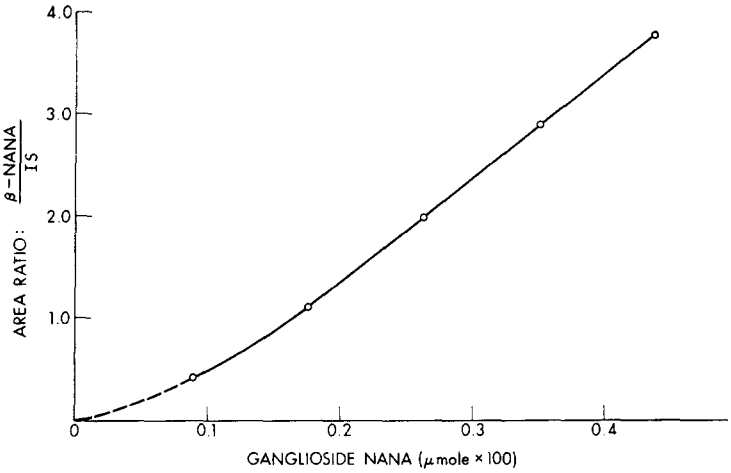

FIG. 5. Standard curve for beef brain ganglioside mixture. Range from 1×10^{-3} to 4.5×10^{-3} μmole NANA. See text for details.

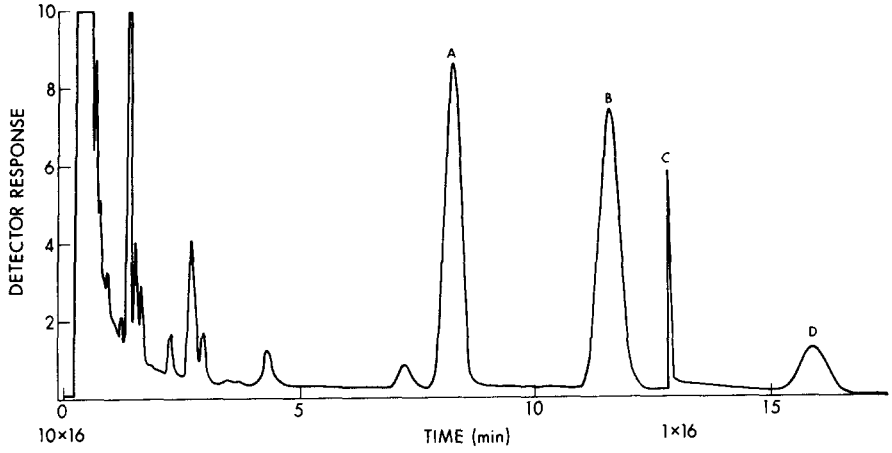

FIG. 6. Identification of NGNA in beef brain ganglioside. Purified ganglioside mixture (73 μg sialic acid) was heated 1 hr at 80°C in 0.05 N methanolic HCl. Following evaporation, 40 μg of internal standard was added, and the mixture was converted to TMS derivatives. Two-thirds of the total was injected on to the OV-1 column at 205°C. Range attenuation was changed from 10 × 16 to 1 × 16 after IS peak. Peak identification: *A*, β-NANA; *B*, internal standard; *C*, recorder indicating attenuation change; *D*, β-NGNA.

ed in gangliosides from human, rabbit, rat, chicken, or frog brain.

Comparison of Methods

The three ganglioside standards were analyzed by the following three different methods for comparison: GLC, thiobarbituric acid, and resorcinol (Table 5). The results for BBG and TSG were adjusted to correspond to 100 μg of NANA in GLC assay (which in turn was related to sample weight). For hematoside-NANA, the GLC value was based on resorcinol which was normalized to 100 μg of NANA. Whereas GLC and resorcinol showed good agreement in determination of BBG and TSG, thiobarbituric acid values were significantly lower for these two samples. Hematoside was unique in giving comparable values with the thiobarbituric acid and resorcinol methods. Free NANA was the standard employed for both colorimetric assays. From the results with BBG and TSG, it may be concluded that the resorcinol method, but not the thiobarbituric acid method, is as reliable as weight data in establishing the sialic acid content of pure gangliosides.

With impure samples, on the other hand, neither colorimetric method can be assumed a priori to be reliable. This is illustrated in Table 6 which shows results of experiments in which aliquots of Folch upper phase from brain were analyzed by the same three methods (GLC data taken from Table 3). The thiobarbituric acid values in this series were corrected for the 62% yield previously obtained with BBG (Table 5). The results

TABLE 3 GANGLIOSIDE SIALIC ACID CONTENT OF BRAIN*

Species	Gray Matter	White Matter	Whole Brain
Human (19 yr old)	925 ± 5†	277 ± 5	—
Human (51 yr old)	875 ± 32	257 ± 13	—
Calf (ca. 6 month old)	1069 ± 30	326 ± 8	—
Ox (1–1.5 yr old)	1016 ± 43	338 ± 13	—
Bull (>8 yr old)	992 ± 20	205 ± 5	—
Pig	987 ± 37	406 ± 12	—
Sheep	1073 ± 26	342 ± 7	—
Rat	—	—	849 ± 11
Rabbit	—	—	816 ± 8
Chicken	—	—	471 ± 9
Frog	—	—	264 ± 1
Goldfish	—	—	235 ± 10

* Samples containing 5–15 μg lipid-bound sialic acid were methanolyzed, converted to TMS derivatives, and analyzed by GLC on the OV-1 column.

† The values are expressed as μg sialic acid per g of fresh tissue ± SD, and represent the average of two samples analyzed in triplicate. Each value is from analysis of one brain.

TABLE 4 NGNA CONTENT IN BRAIN GANGLIOSIDES OF VARIOUS SPECIES

Species	Gray Matter	White Matter	Whole Brain
	% of total sialic acid ± SD		
Human	0	0	
Ox	1.55 ± 0.19	1.06 ± 0.10	
Bull	1.85 ± 0.02	2.31 ± 0.09	
Calf	2.07 ± 0.07	1.03 ± 0.06	
Pig	0.22 ± 0.01	0.26 ± 0.02	
Sheep	0.11 ± 0.02	0.29 ± 0.02	
Rat			0
Rabbit			0
Chicken			0
Frog			0
Goldfish			0.40 ± 0.10

Samples containing 100–200 μg of sialic acid were methanolyzed, converted to TMS derivatives, and analyzed by GLC on the OV-1 column; there were three or more GLC analyses per sample. The lowest detectable amount was approximately 20 ng. Zero result, indicating no detectable NGNA, signifies a level below approximately 0.05% of total sialic acid.

were noteworthy in two respects: colorimetric values were higher than GLC values for all samples, and most samples gave lower values following dialysis when analyzed by resorcinol or thiobarbituric acid. Both phenomena are consistent with the presence of false chromogens in these colorimetric assays. GLC analyses, on the other hand, showed virtually no change after dialysis, and these results are believed to reflect the true values. Goldfish brain was particularly striking in the quantity of false pigment manifested in both resorcinol and thiobarbituric acid assays.

DISCUSSION

A method for GLC assay of sialic acid in gangliosides has been described which has a number of advantages over colorimetric procedures. One is the capacity to determine simultaneously both NANA and NGNA, the two most prevalent types of sialic acid in gangliosides. A third sialic acid, *O*-acetylated NGNA (11), could be detected but not quantified due to partial removal of *O*-acetyl during methanolysis; very mild conditions were necessary to avoid complete removal of this highly labile group. The GLC procedure is potentially useful for detecting other types of sialic acid which may eventually be

TABLE 5 SIALIC ACID CONTENT OF PURIFIED GANGLIOSIDE STANDARDS: COMPARISON OF THREE METHODS

Sample	GLC	Resorcinol	Thiobarbituric Acid
		μg NANA	
BBG	100	105	62.1
TSG	100	99.7	43.1
Hematoside-NANA	100	100	95.5

BBG and TSG were weighed standards whose sialic acid contents were calculated from their known compositions and utilized as standards in GLC analysis. Hematoside-NANA was not weighed, but its GLC assay was related to the resorcinol value, hence, the two are in agreement. Values are given as μg NANA and were adjusted to correspond to samples containing 100 μg NANA on the basis of GLC. Resorcinol assays represent the average of three aliquots, while thiobarbituric acid represents the average of three aliquots from each of two samples.

TABLE 6 ANALYSIS OF BRAIN GANGLIOSIDE SIALIC ACID: COMPARISON OF THREE METHODS

Sample	GLC		Resorcinol		Thiobarbituric Acid	
	Non-dialyzed	Dialyzed	Non-dialyzed	Dialyzed	Non-dialyzed	Dialyzed
			μg NANA/g fresh tissue			
Human gray matter	925	925	1014	964	1030	1049
Human white matter	277	284	348	293	356	302
Rat whole brain	849	857	981	969	1052	1027
Goldfish whole brain	235	233	634	395	575	416

Folch upper-phase samples (see Table 3) were analyzed before and after dialysis. Values are the average of three aliquots from each sample. The average deviations were: GLC, 2.5%; resorcinol, 1.5%; thiobarbituric acid, 1.9%. The maximum deviation for a GLC sample was 4.2%.

discovered in gangliosides, but modifications will be necessary before the various *O*-acetylated structures can be quantified.

High sensitivity was demonstrated by the successful analysis of samples containing as little as 0.3 μg of ganglioside NANA. This sensitivity could be increased somewhat by partial evaporation of the pyridine used in derivatization, or by replacing it entirely with a smaller volume of hexane. However, some departure from linearity was observed at these lower levels, probably owing to adsorptive losses. The possibility of increasing sensitivity another order of magnitude by use of an electron capture detector is under investigation.

The GLC method also appears to alleviate the problem of interfering substances which frequently plague the colorimetric procedures. This was illustrated by comparative analysis of four crude ganglioside preparations (Table 6). The fact that GLC gave lower values than either resorcinol or thiobarbituric acid for all samples was attributed to false chromogens in both colorimetric assays. The samples from human gray and white matter lost most of this false color on dialysis (at least with the resorcinol assay), as judged by the closer agreement with GLC. Goldfish gangliosides showed a surprising degree of interference in both colorimetric assays, and although a substantial part of this was removed by dialysis, the values remained 70–80% above the GLC values.[3] The nature of all these interfering substances is not known, but their presence was apparently not detrimental to the GLC method since this assay showed virtually no change in any of the samples following dialysis.

The use of two different columns provided a powerful tool for eliminating false GLC peaks which occasionally appeared in some samples. Such interference was seldom encountered in the vicinity of the β-NANA, β-NGNA, or internal standard peaks when the samples were only moderately contaminated, such as the untreated Folch upper phases from brain (Tables 3, 4, and 6). However, when Folch partitioning was carried out in the absence of salt—a procedure known to result in greater lipid contamination of the upper phase (15)—a few relatively small foreign peaks were seen in these regions. Such interference was usually eliminated with the OV-225 column. For routine assays OV-1 was the column of choice and only when interference was suspected was it necessary to use both columns.

The utility of GLC for analyzing sialic acid was first demonstrated by Sweeley and Walker (20) who chromatographed all the ganglioside carbohydrates as TMS derivatives. Their procedure, which was designed to measure molar ratios of the various sugars within a given structure, employed vigorous methanolysis to ensure complete cleavage of glycosidic bonds. This treatment, however, also caused deacylation of amino sugars with resultant loss of sialic acid identity. For our purpose it was necessary to retain this identity, and this proved to be possible with the milder methanolysis conditions we employed. It was fortuitous that the relatively greater lability of the α-ketosidic bond of sialic acid permitted preferential cleavage of this linkage.

The major product of methanolysis was identified by comparison with synthetic material as the methyl β-ketoside methyl ester (β-NANA). This was previously shown to be the more stable of the two anomers, with the carboxyl group equatorial to the pyranoid ring (14). The less stable α-isomer appeared as a small band preceding the β-peak, and a small amount of deacylated product was also detected. These small peaks were useful markers for identification, but it was advantageous to ignore them in quantification since the area of the large β-peak alone was a linear function of ganglioside NANA over a wide concentration range. In addition, a constant and reproducible yield of β-NANA resulted from methanolysis of a given sample under fixed conditions.

However, it was also observed that the yield of β-NANA varied significantly with ganglioside structure. This was reflected in the different slopes obtained for the standard curves (Fig. 4) as well as the results of the time study (Fig. 3). The fact that hematoside gave a higher yield than TSG was probably the result of hindrance to acid catalyzed cleavage by the terminal hexosamine of the latter. This same unit also presented hindrance to neuraminidase cleavage of TSG (16). The lower yield of BBG might be thought to reflect the still more complex structures comprising this mixture. These results point to the need for standards whose structures resemble the sample being analyzed, where greatest accuracy is desired. Due to these variations in yield, β-NANA itself cannot be used as a standard unless the actual yield for a given ganglioside is known (*vide infra*). Free NANA, which is a suitable standard for colorimetric methods, cannot be employed with GLC.

It may be noted that this problem of differing yields is not unique to the present method, but is characteristic of any method which depends on a prior hydrolytic (or methanolytic) step to liberate sialic acid. The best known example is the thiobarbituric acid procedure which has been reported elsewhere to give substantially lower values than other colorimetric methods for brain ganglioside mixture (21, 22). This was further documented in the present study (Table 5) when this method was utilized to analyze BBG and TSG. The lower values are very likely

[3] TLC of dialyzed goldfish brain gangliosides showed marked differences from mammalian brain samples. In addition to a different ganglioside pattern, in which polysialogangliosides were increased at the expense of the monosialo types, other glycolipids were present which were not seen in mammalian brain samples. These undoubtedly accounted for at least part of the false color. (Ledeen, R., and D. Forman. Unpublished observations.)

due in large part to incomplete liberation of free sialic acid by hydrolysis. Hematoside was exceptional in showing only a small reduction compared with resorcinol assay. The fact that the order for BBG and TSG in this study was reversed in comparison with the methanolysis data shown in Fig. 3 is likely due to the different media employed for acid treatment.

In analyzing an unknown sample, semiquantitative estimation of ganglioside composition can often be obtained from TLC, and this facilitates a more accurate GLC determination. For example, if one estimated by TLC that a sample has 75% hematoside and 25% brain-type gangliosides, the peak area ratio y, (β-NANA):(internal standard) from GLC would be converted to μmole ganglioside NANA ($\times 10^{-2}$) by the following expression, where k_1 and k_2 are the slopes of the standard curves for hematoside and BBG, respectively (computed from Fig. 4 to be 0.76 and 0.55, respectively).

$$0.75\,\frac{y}{k_1} + 0.25\,\frac{y}{k_2}$$

If no correction were made, the error in this example would be 8.6% using the hematoside standard curve alone. In the absence of information on ganglioside composition, one may obtain a fairly good approximation using a value which is the average of hematoside and BBG (line slope 0.655). The maximum error in this case would be of the order of 15% if the ganglioside happened to be entirely one type or the other. Since many tissues are composed of brain-type gangliosides and hematosides in different proportions, these two preparations would serve as generally useful standards.

It is also possible to utilize the GLC method with reasonable accuracy without the use of standards, if necessary, by using the known yield of β-NANA for a given ganglioside (Fig. 3) together with RDR for β-NANA (Table 1). Thus, for an hypothetical sample containing predominantly hematoside-NANA,

$$\text{total NANA } (\mu g) = \frac{(y)\quad (\text{IS})}{(0.76)\,(0.923)}$$

where y is the measured area ratio β-NANA:internal standard, (IS) is the μg of added internal standard, 0.76 is the yield of β-NANA from methanolysis of hematoside (Fig. 3), and 0.923 represents RDR for β-NANA relative to internal standard. For a sample containing predominantly brain-type gangliosides the same expression would apply with the substitution of 0.55 for 0.76 (β-NANA yield from BBG). Mixtures may be similarly calculated using weighted averages.

In applying the GLC method to measure brain ganglioside levels of several species we employed the standard curve for BBG (Fig. 4). This was undoubtedly a suitable standard for all the mammalian brains since their ganglioside patterns did not differ significantly from that of beef. Goldfish brain, on the other hand, had a somewhat different pattern with relatively more polysialogangliosides,[3] but the error due to this difference was probably very small since BBG itself is composed of about 80% polysialo structures (21, 23). Frog brain was reported to have a pattern somewhat similar to that observed here for goldfish (24, 25), and it is possible this may be characteristic for other lower vertebrates as well.

The results (Table 3) show a general trend toward decreasing brain ganglioside concentration on descending the phylogenetic scale. This is particularly evident when comparing the last five species of the table for which whole brain was analyzed. Some differences were noted between the various mammalian species, and, contrary to the evolutionary trend, human gray matter appears to have a slightly lower concentration than beef, pig, or sheep. However, the significance of these small differences is difficult to ascertain since no effort was made to standardize the brain regions taken for analysis. White matter in particular has been shown to vary widely in ganglioside content from region to region (26).

The GLC results reported here correspond reasonably well to ganglioside levels previously reported for ox (7), human (27) and rat brain (7, 28). Several analyses of rat brain have been compiled in a report which also describes another method for ganglioside assay based on GLC measurement of stearic acid (29). Many values in the literature are significantly lower than those in Table 3, and this is believed to be due at least in part to difference in extraction procedure. Experiments have shown that the 1:1 mixture of $CHCl_3$–CH_3OH employed here effects more complete extraction of brain gangliosides than the widely used mixture of 2:1.

Another application of the GLC method has been the measurement of NGNA content of brain gangliosides for several species. The first evidence for the presence of this sialic acid came from a study of bovine brain gangliosides by Tettamanti, Bertona, Berra, and Zambotti (30), whose method of identification was based on detection of glycolic acid following vigorous acid hydrolysis. The present study confirms that finding and provides a quantitative estimate. Bovine brain contained more NGNA than any of the other species examined, although this still amounted to approximately two percent of total sialic acid. The fact that no NGNA-containing gangliosides have yet been isolated from bovine (or any other) brain indicates the presence of unknown minor species in this tissue. The absence of NGNA in human brain gangliosides is consistent with the prevailing view that NANA is the only sialic acid present in human tissues (8), although small amounts of NGNA have been reported recently in HeLa cells (31).

Additional studies are under way to determine whether the present method can be adapted to the analysis of protein-bound sialic acid. Clamp, Dawson, and Hough (32) employed GLC for this purpose after methanolysis with strong acid, while Craven and Gehrke (33) have described a method utilizing mild aqueous hydrolysis. Although neither of these studies demonstrated assay of different sialic acids from protein, they strongly suggest this as a feasible goal.

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