Determination of brain gangliosides by determination of ganglioside stearic acid

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ABSTRACT A new method is described for the determination of brain gangliosides by measuring stearic acid, the chief acid of gangliosides, in an appropriately purified brain extract. The method includes extraction of tissue with chloroform–methanol, extraction of gangliosides from the extract with 0.1 M KCl, evaporation of the aqueous phase, methanolysis, and gas–liquid chromatography of the resultant methyl esters with a double internal standard. The method depends on the simple composition of ganglioside fatty acids (80% stearic acid) and allows determination of less than 0.05 micromole of gangliosides. Interfering lipids are removed from the ganglioside extract by washing with chloroform–methanol–water. The effects of contamination with nonlipid *N*-acetylneuraminic acid are avoided.

KEY WORDS brain · gangliosides · determination · gas–liquid chromatography · stearic acid · double internal standard · rat · human · gray matter · white matter

THE GANGLIOSIDES ARE a mixture of closely related sphingolipids in which the characteristic side group is a polysaccharide containing NANA (1). Most methods of determining gangliosides, as a class, have been colorimetric, the reagents acting on the carbohydrate moiety. The NANA residue, most characteristic of this lipid class, has been the subject of most of these analytical procedures. Unfortunately, the methods as applied to tissues have not been free of serious defects owing in part to the following factors: unknown chromogens, interference by other carbohydrate residues, interference by unsaturated fatty acids, contamination of extracts by nonlipid NANA, and the lability of NANA. The difficulties have been reduced by some workers by purifying the gangliosides before analysis, by isolating the NANA after hydrolytic release, or by applying colorimetric corrections of questionable validity.

We have investigated the possibility of eliminating the various sources of interference by determining the fatty acid moiety of gangliosides. These fatty acids are very stable, in contrast to NANA, and can be determined with high sensitivity by GLC. The simple fatty acid distribution in gangliosides makes the analysis of the GLC data simpler and more precise than would be the case for lipids containing a wider spectrum of fatty acids. The primary question which had to be studied was whether other lipids containing fatty acids could be removed from the gangliosides completely. We investigated the simplest of the known methods of removing gangliosides from other lipids: the solvent partitioning method of Folch, Lees, and Sloane Stanley (2).

This paper describes a reasonably simple procedure, sensitive to 0.05 μmole, which is rather specific and gives, in addition, information about the composition of the different fatty acids in the gangliosides. A feature which makes the procedure particularly convenient is the use of a methanolysis tube sealed by means of an O-ring. The GLC step is made more reliable by the use of a double internal standard.

METHOD

Materials

All solvents were redistilled from a glass still and were reagent grade, except for chloroform (U.S.P.). A mixture of methyl 19:0 and 21:0 esters (Applied Science Laboratories, Inc., State College, Pa.) was used as a double internal standard. The esters were stored in benzene at concentrations of 19.4 μg of 19:0 per ml and 47.6 μg of 21:0 per ml. The ganglioside used for the recovery experiment was prepared from beef brain gray matter

Abbreviations: NANA, *N*-acetylneuraminic acid; GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

by a process involving solvent partition and Florisil chromatography. Pooled brains from 31- and 49-day old rats of the Sprague-Dawley strain were used for the study.

Equipment

Methanolysis was carried out in special test tubes which could be readily sealed and opened (3). GLC was carried out at 185° with a flame ionization detector (Model 609, F & M Scientific Corp., Avondale, Pa.) and glass column, 6 ft × 3.8 mm i.d., packed with 10% diethylene glycol succinate polyester on Gas-Chrom Z, 80–100 mesh. The column was enclosed in a special oven chamber and was connected to the metal Swagelok fittings by means of Kovar seals (4). A short reservoir column containing the same packing was an integral part of the column (5).

TLC was performed on Silica Gel G with hexane–ether mixtures used as developing solvents. Bromothymol blue solution was used to locate the spots (6).

Analytical Procedure

The first step involves preparation of the lipid extract and removal of interfering lipids. A chloroform–methanol extract of wet brain is prepared (2) and an aliquot corresponding to about 0.05–0.1 μmole of ganglioside is placed in a 13 × 100 mm screw cap test tube. The extract is shaken with 0.2 volume of 0.1 M KCl, then centrifuged briefly. The upper layer is transferred with a small Pasteur pipet to another tube and the lower layer is washed twice more with 0.48 volume (based on the original filtrate volume) of chloroform–methanol–0.1 M KCl 3:48:47 ("Folch upper phase") (2). The pooled upper layer is washed once with 0.25 volume (based on the pooled upper layer volume) of chloroform–methanol–water 86:14:1.

The upper layer is transferred to the methanolysis tube with a Pasteur pipet (rinsing with a little Folch upper phase) and evaporated with a stream of dried, filtered air. The tube is held in a 70° water bath to speed the evaporation. For final removal of water, the tube is left 1 hr in a 110° oven. A portion (2 ml) of internal standard solution is added to the residue and the solvent evaporated off at 37°.

Methanolytic cleavage of the crude ganglioside is carried out by adding 2 ml of 5% methanolic HCl, capping the test tube, and heating at 75° overnight. The tube is cooled with dry ice and uncapped, and 2 ml of hexane are shaken with the methanolysis mixture. Most of the upper layer is transferred to a 3 ml centrifuge tube and evaporated to dryness in a 37° bath. The esters on the walls of the tube are rinsed down with a little ether, which is then removed with nitrogen. Shortly before the GLC is to be performed, the residue is dissolved in 0.05 ml of carbon disulfide; 1–2 μl of solution is injected. Under the conditions used, the 21:0 peak emerges in about 10 min, so the entire GLC analysis takes only about 12 min of instrument time.

The amount of stearoyl ganglioside is calculated from the areas of the stearate and one of the internal standard peaks (for choice of which one, see next paragraph):

$$\mu\text{moles ganglioside} = \frac{\text{Weight of internal standard}}{298} \times \frac{\text{Area of stearate}}{\text{Area of internal standard}}$$

where 298 refers to the molecular weight of methyl stearate. The amounts of the minor fatty acid homologues can be calculated similarly.

RESULTS AND DISCUSSION

Internal Standard

Addition of the internal standards prior to methanolysis makes it unnecessary to extract the esters quantitatively. The use of two standards has a number of advantages over the more usual use of one. First, by calculating the ratio of the areas of the two standards, one is able to check the performance of the GLC apparatus in each run. Second, by using different amounts of each internal standard one can obtain different peak heights and thereby make it more likely that one of the standards will closely approximate the peak height of the ganglioside ester. This should improve the precision of the area measurements and, in the unfortunate event of a run which sends one standard peak off the recorder scale, may ensure having the other standard peak on scale and usable.

If the chromatograph available can separate the ethyl ester of 20:0 from the methyl ester, the former is to be preferred over methyl 21:0 as a standard since it is cheaper. Ideally one of the standards should elute early in the chromatogram and one late, since leakage (a common problem in high temperature GLC) produces a late peak which is too small.

Reproducibility

Nine aliquots of washed, pooled upper layer, each corresponding to 100 mg of rat brain, were analyzed by the above method, each preparation of methyl esters being subjected to two GLC analyses. The 18:0 ganglioside content was calculated from the 19:0 internal standard and the two values from each sample were averaged. The mean of the nine values was 102 mμmoles, the standard deviation was 1.8 mμmoles, and the coefficient of variation was 1.8%.

Much of this variability arose from the GLC step itself, as was shown by examining the duplicate GLC runs from 17 similar samples, using the 19:0 standard to calculate the 18:0 contents. The average difference between the duplicate values was 1.7 mμmoles (out of about 100 mμmoles). The same degree of variability was found when both internal standards were used in the calculation. Progress in GLC instrumentation may reduce this variability.

In developing this procedure we found that an important source of variation arose in the various evaporation steps. The washed upper layer contains KCl as its major solid component and this tends to flake off the methanolysis tube (with ganglioside) if the incoming air stream is too rapid. We standardized this step by using a pressure regulator and restrictor on the air line and a manifold of 5 mm o.d. glass tubes to conduct the air into the methanolysis tubes. The heating bath was thermostatically controlled and raised, together with the test tube rack, by a jack. Use of the jack to raise the test tubes during evaporation prevents sudden spurts of air from agitating the liquid excessively.

The evaporation of the hexane extract is performed similarly, but with Pasteur pipets in the manifold.

Recoveries

A recovery test was made for the steps following the extraction of the gangliosides into the upper solvent phase. Aliquots of upper phase, with and without added ganglioside, were analyzed for their content of ganglioside stearate. The data (Table 1) show that recovery is complete within the precision of the method.

The question of completeness of extraction in the first step was not studied. Judging by the findings of others (2, 7, 8), the extraction procedure is adequate for normal brain. In the case of Tay-Sachs brain, the washes with "Folch upper phase" do not extract every ganglioside, and a chromatographic step or washes with "Folch upper phase" not containing KCl are needed.

Analysis of the wash layer by the resorcinol method for NANA (9) showed that there was negligible loss of ganglioside in this step.

Specificity and Interference

The specificity of our method depends on the absence of appreciable amounts of stearate-containing lipids in the upper phase. The study by Folch et al. (2) showed that very little lipid other than ganglioside enters the upper phase when salt is present. We found by temperature-programmed GLC that there was no detectable amount (<1%) of 24:0 in the ganglioside acids extracted, so that contamination by sphingolipids is negligible. (This was true even if the washing step was omitted.) Moreover, TLC of the ganglioside acids after methanolysis showed the absence of hydroxy acids, which are characteristic of cerebrosides and sulfatides.

TABLE 1 RECOVERY OF ADDED GANGLIOSIDE AFTER WASHING, METHANOLYSIS, AND GLC PROCEDURES

Sample	Ganglioside Found	Recovery*
	mμmoles of 18:0	%
A. Rat brain upper phase	107	
B. Ganglioside alone	86	
C. Mixture of A + B	194	101

* Calculated as 100(C − A)/B.

TABLE 2 ESTIMATION OF CONTAMINATION OF GANGLIOSIDE FATTY ACIDS BY ESTER-LINKED FATTY ACIDS

Layer	16:0	18:0	18:1	20:0
		mμmoles/g		
Rat brain				
Washed upper layers	33	950	10	76
Ester-linked acids	14	24	10	0
Washings from upper layers	44	95	64	0
Human white matter				
Washed upper layers	18	243	31	26
Ester-linked acids	5	3	5	0
Washings from upper layers	116	99	124	0
Human gray matter				
Washed upper layers	22	1020	33	163
Ester-linked acids	6	6	7	0
Washings from upper layers	107	150	116	0

Data are calculated on the basis of 1 g of fresh brain. The washed, pooled upper layers were prepared as described in the text. The ester-linked fatty acids were derived from this layer by mild alkaline hydrolysis.

To test the possibility that there are contaminating lipids of the ester type in the pooled upper layers, we studied the effect of mild alkaline saponification. An aliquot corresponding to 300 mg of brain was evaporated to dryness, then heated for 24 hr at 37° with 1 ml of N KOH. The mixture was acidified and extracted with hexane, and the free acids in the hexane layer were esterified with dimethoxypropane–HCl (10) and analyzed by GLC. These fatty acids are shown in line 2, Table 2; the *total* fatty acid content of a similar aliquot of washed, pooled upper layers is shown in line 1. Comparison of the two lines shows that all of the 18:1 found in the washed, pooled layers is ester-linked, whereas all of the 20:0 occurs in the gangliosides. About 40% of the 16:0 and 2–3% of the 18:0 are ester-linked. Thus it is apparent that the washing step does not remove all of the contaminating esters from the pooled upper layers and that a mild saponification step would be required if one wished to determine ganglioside 16:0. The error in determining stearoyl ganglioside

seems rather small, and for routine purposes the saponification does not seem necessary.

Line 3 of Table 2 lists the fatty acids found in the washings from the upper layer. It is apparent that the washing step removes a significant amount of lipid and should be included in the procedure. A second washing did not change the ganglioside stearate value appreciably, although it did remove a small amount of lipid.

Although most studies of the ganglioside fatty acids have not mentioned oleate, Svennerholm reported that it constituted 5% of the C_{18} acids (11) and Saifer, Robin, and Volk (12) found the oleate content to be half of the stearate content, a remarkably high figure. It is likely that in the latter case the methanolysis conditions were much too mild, making a minor contamination with ester-linked oleate into a serious source of error. Stanacev and Chargaff (13) found less than 1% oleate in their preparations, and we found a similar level in the purified ganglioside used in our recovery experiment. Our alkali-treated ganglioside does show a trace of 18:1 (as well as traces of 14:0 and 22:0).

The fatty acids found in the hexane layer after saponification could not have come from partial breakdown of ganglioside. We tested this point by treating highly purified ganglioside as above, and found only 0.5% of the fatty acids in the hexane layer. This trace must have come from an impurity since the contents of 16:0, 18:0, and 18:1 in the hexane were similar. The hexane layer also tested negatively for NANA, showing that ganglioside does not enter hexane under these conditions.

Table 2 also shows the results of a similar experiment with human brain, obtained from a person who died of lung cancer and pneumonia. Gray matter was taken from the cerebral cortex and white matter from an adjacent area. The results are qualitatively similar to those found with whole rat brain and indicate that the method is applicable to human samples as well. The effectiveness of the washing step seems more satisfactory here and the need for it greater. Of incidental interest is the finding that the ganglioside fatty acids differ appreciably in their composition according to their origin. White matter gangliosides contain 4% palmitic, 79% stearic, 9% oleic, and 9% arachidic acids (correcting for the ester-linked fatty acids by means of the saponification data). The gray matter gangliosides contain 1% palmitic, 83% stearic, 2% oleic, and 13% arachidic acids. The largest differences are in the 16:0 and 18:1 contents.

The KCl used in the partitions and produced in the saponification experiment does not interfere with the subsequent steps. This was shown by the lack of effect of adding additional KCl to the washed upper layers.

The possibility was considered that some of the sphingosine produced by methanolysis might be extracted into the hexane layer, then react with part of the esters in the hot injection zone of the gas chromatograph. The hexane layer, on examination by TLC, showed only a minor spot at the origin (besides the methyl ester spot). While the color reaction with bromothymol blue was not characteristic of sphingosine, the possibility remains that the contaminant is a reactive derivative of the amine. Tests made by adding equimolar amounts of sphingosine triacetate to the ganglioside sample prior to methanolysis did not affect the GLC results.

The recent report (14) on the appearance of false ester peaks by use of methanol–HCl was not confirmed. Blank runs carried out with everything but the brain sample by using fresh or 2-month old methanol–HCl yielded no peaks in GLC.

Loss of shorter-chain fatty acid methyl esters during vacuum evaporation has been reported (15). Our procedure was checked for this possibility by evaporating a mixture of 16:0 (5.8 μg), 18:0 (41.3 μg), and 22:0 (57.3 μg) in 3-ml centrifuge tubes, then blowing air for 30 and 90 min at 37°. Analysis by GLC showed no change in composition in the 30 min sample, while the ratio of 16:0/18:0 dropped about 4% in the 90 min sample. Another test with shorter esters, 12:0, 14:0, and 18:0, showed that evaporation losses can be quite severe. The losses are reduced by stopping the evaporation of the hexane extract when its volume is about 0.05 ml and injecting this directly. Since rat brain gangliosides analyzed by this method still show only minor amounts of 14:0 we follow the more convenient practice of evaporating to dryness. The seriousness of a loss of this type would be greater with smaller samples.

Comparison with Other Methods

The difficulties inherent in other methods of ganglioside determination, discussed in the various papers on the subject, are rather strikingly brought out by comparing the actual values found. A summary of reports on rat brain ganglioside is shown in Table 3. Of course exact comparisons cannot be made because of differences in animal age, animal strain, and nature of the assay, but the values calculated to the same base (μmoles/g of fresh brain) do differ greatly.

The first value is even higher than it seems, since it was obtained from very young rats. At 12 days of age, rat gangliosides are about one-half their adult level (16, 17). The second value is even lower than it seems, as it was derived from gray matter, which has a much higher concentration than whole brain. It can be seen that four investigators found values close to ours. James and Fotherby (18) reported 74% recovery of added NANA in their procedure; values corrected to allow for this would be a little higher than ours.

TABLE 3 COMPARISON OF REPORTED GANGLIOSIDE CONTENTS IN RAT BRAIN

Investigator	Rat Age	Ganglioside Concentration
	days	*μmoles/g wet wt*
McCluer, Coram, and Lee (20)	12	1.58*†
Pritchard and Cantin (17)	25	0.65†
Sperry (21)	Adult	1.96‡
James and Fotherby (18)	21	0.94†
Häkkinen and Kulonen (22)	Adult	1.1†
Seminario, Hren, and Gómez (23)	Adult	1.0†
Burton, Garcia-Bunuel, Golden, and Balfour (24)	25	0.67
Hess and Rolde (19)	—	1.45†
Lowden and Wolfe (27)	Adult	1.1 ± 0.2†
This study	31	1.13§

* Calculated from the reported data for dry weight by dividing by 5.6 (24).

† Calculated from the reported NANA values assuming 1.7 moles of NANA per mole of ganglioside (25).

‡ Calculated from the reported gravimetric value using 1586 as the molecular weight (mol wt calculated from the assumed value of 1.7 moles of NANA per mole of ganglioside).

§ Corrected for the presence of ester-type impurities, and for the fact that gangliosides contain fatty acids besides stearic.

The values of Hess and Rolde (19) were obtained by a fluorimetric procedure in which the aqueous wash of the initial lipid extract was analyzed without further purification. Presumably the high values here and in other studies are due to the presence of nonlipid NANA in the extract (8).

In the procedure described in this paper, the sensitivity is approximately the same as that of the colorimetric methods. Both approaches are susceptible to increase in sensitivity, the latter by the use of microcuvettes, the former by changing the sensitivity setting of the gas chromatograph or by using less carbon disulfide to dissolve the methyl esters. A trial with one-tenth the amount of pooled upper layer (containing 0.01 μmole of ganglioside) and standards gave the same value for ganglioside 18:0 concentration of the brain.

A particular advantage of our method is that it gives additional information, i.e., the composition of the ganglioside fatty acids. Coupled with a reliable method for determining the NANA moiety, our method could reveal changes in the proportions of the different types of gangliosides.

Other Applications of the Method

Gangliosides or other water-extractable lipids occur outside the brain so that it is likely the method can be applied to their determination in a similar manner. The method can be scaled up to somewhat larger amounts and we have used it for isotopic studies of the biosynthesis of ganglioside stearic acid (26).

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