Determination of ganglioside non-hydroxy fatty acid and long chain base by analysis of perbenzoylated derivatives

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Summary The non-hydroxy fatty acid and long chain base compositions from as little as 2.7 nmol of ganglioside were ascertained from perbenzoylated ganglioside derivatives. Non-hydroxy fatty acids were determined by mild alkaline methanolysis of the derivatives, followed by gas–liquid chromatography (GLC) of the methyl esters. N-acyl and N-benzoyl "gangliosides" that were generated by the methanolysis were hydrolyzed by a standard procedure that utilized aqueous acetonitrile–HCl, followed by high performance liquid chromatography (HPLC) determination of the biphenylcarbonyl derivatives with ultraviolet (UV) detection at 280 nm. A critical aspect of this procedure is a modified workup for the isolation of the biphenylcarbonyl derivatives which eliminates by-products that otherwise interfere with their separation by HPLC, especially when high sensitivity is required. — **Ullman, M. D., and R. F. Ventura.** Determination of ganglioside non-hydroxy fatty acid and long chain base by analysis of perbenzoylated derivatives. *J. Lipid Res.* 1987. **28:** 878–882.

Supplementary key words gangliosides • non-hydroxy fatty acid • long chain base • perbenzoylation • HPLC

It is desirable to have a sensitive method that can be used for the determination of the non-hydroxy fatty acid and long chain base composition of gangliosides. Such a procedure is described in this report. It utilizes perbenzoylated derivatives of gangliosides. The perbenzoylated derivatives of ganglioside mixtures or of individual derivatives that have been isolated after their separation by HPLC and quantification by their UV absorption are utilized. Thus, the procedure provides an efficient use of isolated gangliosides.

The hydrophobic fatty acid and long chain base (ceramide) portion of gangliosides is embedded in the lipid matrix of membrane bilayers and plays a significant role in the aggregation of gangliosides in neuronal plasma membranes (1). Thus, the fatty acid and long chain base composition of gangliosides must be determined to obtain a full understanding of the mechanisms by which gangliosides contribute to membrane and cellular function. The effectiveness of such studies is increased as the sensitivity of structural and quantitative analyses are increased because gangliosides from smaller, better defined brain regions can be characterized and quantified.

In the past, individual gangliosides have been isolated from ganglioside mixtures by laborious thin-layer or column chromatographic techniques and the fatty acid composition has been determined by acid methanolysis followed by GLC of the generated methyl esters (2). The long chain base composition has been determined by acid degradation of the gangliosides in aqueous methanolic-HCl followed by GLC analysis of the silylated derivatives (3–6).

More recently the long chain bases have been released by acid hydrolysis of the glycolipids in aqueous acetonitrile–HCl (7) followed by derivatization of the bases with biphenylcarbonyl chloride and HPLC of the biphenylcarbonyl derivatives (8). The products and side-products formed by this hydrolysis procedure have been characterized by mass spectrometry (9). Alternatively, the *p*-nitro-phenylacetyl derivatives have been used to quantify long chain bases (10).

This report describes a sensitive method for the determination of the non-hydroxy fatty acid and long chain base composition of gangliosides. It utilizes perbenzoylated gangliosides directly (when the gangliosides have been isolated and purified prior to the derivatization) or after they have been collected as they emerge from the UV detector after their separation by HPLC (11). The diacylamines (N-acyl and N-benzoyl) formed by the perbenzoylation reaction are unstable in mild alkali as are the benzoyl esters so that either the N-acyl *or* N-benzoyl group is released during mild alkaline methanolysis, as are the benzoyl esters. Thus, the perbenzoylated gangliosides are exposed to mild alkaline methanolysis and the released non-hydroxy fatty acids (as their methyl esters) are determined by GLC. This methanolysis procedure generates a mixture of "gangliosides" that contains the native fatty acid or a benzoyl group. The mixture of N-acyl and N-benzoyl "gangliosides" that results from the mild alkaline methanolysis (12, 13) is then exposed to aqueous acetonitrile–HCl hydrolysis. The released long chain bases are separated by HPLC of their biphenylcarbonyl derivatives (8). The ability to release a random sample of the non-hydroxy fatty acids from the diacylamines in mild alkali is the principal reason this procedure is so convenient. A modified procedure for the isolation of the biphenylcarbonyl derivatives is also reported. The modification eliminates by-products that otherwise comigrate with the desired products. The procedure works equally well with neutral glycolipids (data not shown).

Abbreviations: HPLC, high performance liquid chromatography; GLC, gas–liquid chromatography; BBG, bovine brain gangliosides; LCB, long chain base(s); the LCB designations are from Breimer et al. (16).

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MATERIALS AND METHODS

Chemicals and reagents

Bovine brain gangliosides (fraction III, Sigma Chemical Co., St. Louis, MO, and Analabs, Inc., North Haven, CT) and human peripheral nerve GT_{1b} were used in this study. All solvents were HPLC grade and chloroform was ethanol-free (Burdick and Jackson Laboratories, Muskegon, MI). Each solvent was tested by GLC analysis for fatty acid contamination prior to its use. Benzoyl chloride was purchased (Eastman Kodak, Rochester, NY) and used directly from the bottle with minimum exposure to atmospheric moisture. Pyridine and toluene (Fisher Scientific, Inc., Medford, MA) were stored over 4A molecular sieves at least 1 day prior to use. Biphenylcarbonyl chloride was stored refrigerated and used as provided by the manufacturer. Silicic acid (Clarkson Chemical Co., Williamsport, PA) columns (in Pasteur pipets), which were used for the isolation of biphenylcarbonyl derivatives of long chain bases, were prewashed with methanol and chloroform just prior to their use.

C-18 reversed-phase cartridges with stainless-steel frits (Bond Elut, Analytichem International, Harbor City, CA) were prewashed with 5 ml of methanol, 10 ml of chloroform, and 5 ml of methanol to minimize fatty acid contamination.

Fatty acid and long chain base determinations

The determinations were performed on individual gangliosides or ganglioside mixtures with 2.7 to at least 90 nmol of the compounds of interest. The gangliosides were perbenzoylated in 5% benzoyl chloride in 25% toluene in pyridine (v/v) at 45°C for 16 hr (11). Perbenzoylated gangliosides or the individual ganglioside derivatives that were separated by HPLC were subjected to mild alkaline methanolysis in 1.5 ml of 0.21 M methanolic NaOH–chloroform 1:2 (2). The reaction mixture was partitioned with 0.3 ml of 0.35 N acetic acid and the upper phase was removed and saved. The lower phase was washed twice with 1 ml of methanol–water 1:1. The fatty acids from the lower phase were analyzed by GLC. The mixture of N-acyl and N-benzoyl "gangliosides" that resulted from the mild alkaline methanolysis was extracted by twice passing the upper phase through a reversed-phase (Bond Elut) cartridge (14). The cartridge was then washed with 10 ml of water and the "gangliosides" were eluted with 1 ml of methanol followed by 4 ml of chloroform–methanol 2:1. The long chain bases were released from the "gangliosides" with aqueous acetonitrile–HCl hydrolysis.

The "gangliosides" were dissolved in 0.3 ml of aqueous acetonitrile–HCl reagent (0.5 N HCl and 4 M H_2O in acetonitrile). The tubes were flushed with nitrogen, capped, and heated at 75°C for 2 hr with periodic shaking. The long chain bases were isolated by first drying the reaction mixture with a stream of nitrogen. The dried sample was suspended in 1 ml of methanol–0.9% saline–chloroform 48:47:3 containing 0.05 N NaOH (solvent A). The suspension was extracted with 5 ml of chloroform–methanol–water 86:14:1 (solvent B). The lower phase was washed three times with 1 ml of solvent A and three times with solvent A without NaOH. The lower layer was then dried with a stream of nitrogen.

The biphenylcarbonyl derivatives of long chain bases were prepared by dissolving the bases in a mixture of 50 μl of 1% biphenylcarbonyl chloride in tetrahydrofuran and 100 μl of a saturated solution of sodium acetate in water. The biphasic reaction mixture was vigorously shaken at room temperature for 1.5 hr. The isolation procedure for the derivatives was modified to improve yields and to decrease by-product contamination. The derivatives were first isolated by adding to the reaction mixture 5 ml of the lower phase from a partition of chloroform–methanol 2:1 with 0.2 vol of 0.9% saline, and then 1 ml of methanol–0.9% saline–chloroform–2.5 N NaOH 48:47:3:2 (8). The upper phase was removed and the lower phase was dried with a stream of nitrogen. The derivatives were then further purified by silicic acid chromatography. They were placed on a 10-mg silicic acid column with 1 ml of hexane–chloroform 1:1 and the column bed was washed with an additional 4 ml of the same solvent and then with 4 ml of chloroform. The derivatives were eluted with 4 ml of chloroform–acetone 8:2. The collected fraction was dried with a stream of nitrogen and exposed to mild alkaline methanolysis in 0.21 M methanolic NaOH–chloroform 1:2 as described above. The isolated derivatives were dissolved in methanol, separated by reversed-phase HPLC, and detected by their UV absorption at 280 nm.

Chromatography

Fatty acid methyl esters were analyzed with a gas chromatograph (model 5710A) equipped with a computing integrator (model 3380A) (Hewlett-Packard, Mt. View, CA). The esters were separated with a 3% OV-1 glass column (6 ft × 2 mm i.d.) and a linear temperature gradient from 170°C to 220°C at 2°C/min.

Biphenylcarbonyl derivatives of long chain bases were separated on a reversed-phase (ODS, 5 micron mean particle diameter) column (4.6 mm i.d. × 250 mm) (Beckman, Altex Scientific, Berkeley, CA) with a mobile phase of methanol–water 94:6 (v/v) and a flow rate of 1 ml/min (7, 8). The derivatives were detected by their UV absorption at 280 nm and they were identified by their comigration with known standards previously identified by liquid chromatography–mass spectrometry (9).

RESULTS

Attempts to determine the non-hydroxy fatty acid and long chain base composition of perbenzoylated gangliosides (and neutral glycolipids) by direct acid hydrolysis in aqueous acetonitrile–HCl were unsuccessful. It was, therefore, necessary to devise a method that would first yield the fatty acid composition and second yield the long chain base composition.

We successfully ascertained the non-hydroxy fatty acid composition from as little as 5 μg of ganglioside(s) by mild alkaline methanolysis of their perbenzoylated derivatives. A comparison of the non-hydroxy fatty acids obtained from mild alkaline methanolysis of the perbenzoylated derivatives with those obtained from acid methanolysis (HCl–methanol) of the native glycolipids showed no significant differences in the percentages of non-hydroxy fatty acids obtained by the two procedures (**Table 1**). Peripheral nerve GT_{1b} was used because it contained a more complex fatty acid pattern than the bovine brain gangliosides, which contain mostly C:18 fatty acid. Either the N-benzoyl or N-acyl group of the diacylamine (perbenzoylated ganglioside) was randomly released by the alkaline methanolysis. Thus, the products of the alkaline methanolysis were methylbenzoate, acyl methyl esters, and a mixture of N-acyl and N-benzoyl "gangliosides."

After a mild alkaline methanolysis, the resultant mixture of N-acyl and N-benzoyl "gangliosides" was extracted by a reversed-phase sample preparation cartridge. The "gangliosides" were then hydrolyzed with aqueous acetonitrile–HCl and the resultant long chain bases were analyzed by HPLC of their biphenylcarbonyl derivatives (8). A comparison of the ganglioside long chain base compositions obtained by this procedure with those obtained by direct acid hydrolysis of the native gangliosides revealed no significant differences between the two procedures (**Table 2**). The compounds examined contained d18:0, d18:1, d20:0, and d20:1 long chain bases. LCB d19:1 and d20:1 have been reported as minor components of bovine brain gangliosides (9). In these analyses, all d19:1 products are included with d18:1 and d21:1 products with d20:1 because the *threo* form of the odd-numbered bases comigrated with the d18:1 and d20:1 bases, respectively. Further, the 5-hydroxy-d20:1 derivative, formed as a minor by-product during the hydrolysis (9), comigrated with the d18:0 derivative.

DISCUSSION

This report describes a sensitive method for the determination of the non-hydroxy fatty acid and long chain base composition of gangliosides. It utilizes perbenzoylated gangliosides directly (when the gangliosides have been isolated and purified prior to the derivatization) or after they have been collected as they emerge from the UV detector subsequent to their separation by HPLC (11). The procedure is sensitive (analyses can be performed on as little as 2.7 nmol of ganglioside) and results correspond to those obtained by direct analysis of the native gangliosides. The procedure is advantageous because it can be used on samples that have been quantified by HPLC. Thus, it provides an efficient use of isolated gangliosides. The procedure can also be utilized for the analysis of neutral glycolipids (data not shown).

Attempts to ascertain the non-hydroxy fatty acid and long chain base composition of gangliosides by the direct hydrolysis of their perbenzoylated derivatives (with aqueous acetonitrile–HCl) were unsuccessful. Apparently, the perbenzoylated derivatives possess a unique chemistry which promotes their degradation or derivatization to various uncharacterized products. Because it had been ascertained in an earlier study of neutral glycolipids (15) that fatty acids of perbenzoylated glycolipids are ran-

TABLE 1. Fatty acid composition of gangliosides

Fatty Acid	Bovine Brain		GM_1		GD_{1a}		GT_{1b}	
	Native	Perbenzoylated	Native	Perbenzoylated	Native	Perbenzoylated	Native	Perbenzoylated
	% of total fatty acids							
16:0	1.16 ± 0.37	2.41 ± 0.83	3.97 ± 0.48	1.66 ± 0.72	ND	ND	1.79 ± 0.96	1.09 ± 0.14
18:0	91.18 ± 2.79	90.35 ± 3.95	90.84 ± 0.98	93.36 ± 1.16	94.98 ± 0.06	96.05 ± 0.44	35.61 ± 0.98	36.27 ± 0.48
20:0	4.10 ± 0.18	4.24 ± 0.20	2.54 ± 0.42	2.35 ± 0.14	3.79 ± 0.07	3.24 ± 0.12	24.56 ± 1.24	25.38 ± 0.73
22:0	ND	ND	ND	ND	ND	ND	15.18 ± 0.65	15.24 ± 0.48
22:1	ND	ND	ND	ND	ND	ND	1.32 ± 0.04	1.32 ± 0.01
23:0	ND	ND	ND	ND	ND	ND	2.15 ± 0.09	2.18 ± 0.07
24:0	ND	ND	ND	ND	ND	ND	8.24 ± 0.34	7.82 ± 0.07
24:1	ND	ND	ND	ND	ND	ND	8.40 ± 0.37	8.26 ± 0.09

Fatty acid composition of representative mono-, di-, and trisialogangliosides and a bovine brain ganglioside mixture. Bovine brain GM_1 and GD_{1a} were analyzed after they were collected from the HPLC. Their values are the average of two determinations expressed as the percent of the total fatty acids. GT_{1b} was from human peripheral nerve. Both the GT_{1b} and the bovine brain gangliosides were analyzed after the perbenzoylation workup. Their values are percent of total fatty acids ± SEM; ND, not determined. Values less than 1% are not included.

TABLE 2. Ganglioside long chain bases

Sample	Long Chain Bases			
	d18:0	d18:1	d20:0	d20:1
	% of total long chain bases			
BBG				
N	2.95 ± 0.71	45.98 ± 0.51	3.31 ± 0.58	47.74 ± 0.88
P	3.63 ± 0.22	45.43 ± 0.71	3.29 ± 0.29	47.65 ± 0.27
GM_1				
N	2.50	53.1	1.60	43.0
P	2.85	54.3	1.45	41.4
GD_{1a}				
N	2.65	44.4	2.95	50.0
P	2.30	43.8	2.90	50.1
GD_{1b}				
N	2.25	40.2	3.40	54.1
P	2.25	39.3	3.45	55.0
GT_{1b} + GQ_{1b}				
N	16.2	57.8	2.50	23.6
P	16.1	58.1	2.30	23.4

Ganglioside long chain bases. Values are expressed as the percentage of total long chain base. Each set of values represents the average of two determinations except for the bovine brain ganglioside mixture which is the average of triplicate determinations. N, long chain bases acquired from acid methanolysis of native ganglioside(s); P, long chain bases acquired from acetonitrile–HCl hydrolysis of perbenzoylated ganglioside(s).

domly released by mild alkaline methanolysis, we successfully attempted to determine the fatty acid composition of gangliosides by mild alkaline methanolysis of their perbenzoylated derivatives. The procedure is easy to perform because the collected individual perbenzoylated gangliosides need no further purification. It requires, however, that the solvents used to comp.se the mobile phases are checked for fatty acid contamir ation prior to their use with the HPLC. The procedure i: effective on gangliosides that possess both medium and ong chain non-hydroxy fatty acids. It is not effective for the analysis of hydroxy-fatty acids because gangliosides that contain hydroxy-fatty acids do not form diacylamines with benzoyl chloride (13). We prefer this procedure for the determination of ganglioside and neutral glycolipid non-hydroxy fatty acids because: *1*) small amounts of tissue can be used (high sensitivity), *2*) fatty acid contamination is minimized because the perbenzoyl derivatives are purified during their quantification by HPLC, and *3*) efficient use of isolated gangliosides is provided.

After the mild alkaline methanolysis, the resultant N-acyl and N-benzoyl "ganglioside" mixtures are isolated by the use of a reversed-phase sample preparation cartridge with stainless-steel frits. The long chain base composition of the mixture is then determined by the use of aqueous acetonitrile–HCl hydrolysis, followed by formation of the biphenylcarbonyl derivatives of the released bases. The derivatives are isolated by a modified procedure. They are extracted by solvent partition, partially purified on a silicic acid column, and then subjected to mild alkaline methanolysis. The modification eliminates contaminants that otherwise comigrate with the desired products.

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