Quantitative analysis of brain gangliosides by high performance liquid chromatography of their perbenzoyl derivatives

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Abstract This report describes a convenient, highly sensitive, and reproducible HPLC procedure for the quantitative analysis of gangliosides from brain tissues. The procedure involves the conversion of gangliosides to their perbenzoyl derivatives, isolation of the derivatives on a C18-reversed-phase cartridge, separation of the derivatives on a column (3-micron silica) maintained at an elevated temperature, and UV detection of the derivatives at 230 nm. The convenience of the procedure, its sensitivity, reproducibility, and application to the analysis of gangliosides from tissue sources make it the method of choice for ganglioside quantification in our laboratories. Three aspects of the procedure contribute to its convenience: reaction conditions that lead to single products, a convenient isolation procedure for the derivatives, and chromatographic conditions that provide resolution of the derivatives. – **Ullman, M. D., and R. H. McCluer.** Quantitative analysis of brain gangliosides by high performance liquid chromatography of their perbenzoyl derivatives. *J. Lipid Res.* 1985. **26:** 501–506.

Supplementary key words polysialogangliosides • HPLC

A rapid, sensitive, quantitative HPLC technique would aid in studies of the function, distribution, and metabolism of gangliosides. This study reports such a technique that involves high performance liquid chromatography (HPLC) of perbenzoylated gangliosides.

Gangliosides are acidic glycosphingolipids that contain sialic acid. The high concentration of gangliosides in the CNS, as opposed to non-neural tissue, implies that gangliosides play an important role in CNS function.

The major gangliosides of mammalian brain are G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}. These, and other gangliosides, may function in the central nervous system as receptor site determinants (1) or modifiers (2), and in neural transmission (3). Although significant progress has been made in the delineation of ganglioside structure, distribution, and function, that progress has been limited by the available quantitative methods.

Current methods for ganglioside analysis include thin-layer chromatography (TLC) (4) or high performance thin-layer chromatography (HPTLC) (5, 6) followed by destructive densitometry, colorimetry, or gas–liquid chromatography (GLC) (7). The use of ozonolysis to form the *p*-nitrobenzyloxyamine derivatives, which are separated and quantitated by reversed-phase HPLC, has also been reported (8). The analysis times, limited sensitivity, and variability of these techniques (9), however, restrict the number, sample size, and reproducibility of the ganglioside determinations. Further, even though gangliosides have been quantitated in the picomolar range by HPTLC (6), the destructive detection does not allow further characterization of the separated gangliosides.

Convenient and sensitive HPLC methods for the quantification of monosialogangliosides (10) or monosialogangliosides and G_{D1a} (11) as their perbenzoyl derivatives have been reported. However, those techniques do not simultaneously quantify the mono- and polysialogangliosides. The method reported here is reasonably simple to perform, highly sensitive, reproducible, and measures mono- and polysialogangliosides in a single chromatographic run. It also permits collection of the derivatives for further characterization and analysis.

MATERIALS AND METHODS

Chemicals

Glass-distilled HPLC grade solvents (Burdick & Jackson Laboratories, Muskegon, MI) were used and were degassed under reduced pressure. Pyridine and toluene for the perbenzoylation reactions were dried over 4-A

Abbreviations: HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; The ganglioside nomenclature used here is according to the system of Svennerholm (1963. *J. Neurochem.* **10:** 613–623).

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molecular sieves for at least 1 day prior to use. Benzoyl chloride (Eastman Kodak, Rochester, NY) was used directly from the bottle with minimum exposure to atmospheric moisture.

Ganglioside mixtures and standards

Ganglioside fractions, suitable for isolation of standards or for perbenzoylation, were prepared from tissues by extraction and solvent partition (12) followed by reversed-phase chromatography on rapid sample preparation cartridges (Sep-Pak, Waters Associates, Milford, MA) (13). If gangliosides were isolated from tissues that contain significant quantities of complex neutral glycolipids, it was necessary to further purify the gangliosides on a DEAE-Sephadex column (14). The ganglioside fractions were used for the preparation of individual standards by chromatography on Iatrobeads (Iatron Industries, Inc., 1-11-4 Higashi-Kanda, Chiyoda-Ku, Tokyo, Japan) (14) and some gangliosides (G_{D2}, G_{D3}, G_{Tlb}, and G_{Qlb}) were generously provided by Dr. Robert Yu (New Haven, CT). Other ganglioside preparations used for analysis by perbenzoylation were purchased (bovine brain gangliosides, Analabs, North Haven, CT) or received as a gift (human brain gangliosides, from Dr. Michael Malone and Ms. Maria Szoke, VA Hospital, Bedford, MA). The concentration of each ganglioside standard was determined by the resorcinol-HCl assay (15). Assumed molecular weights were: G_{M1} = 1547; G_{Dla} = 1838; G_{Dlb} = 1838; G_{Tlb} = 2129.

Thin-layer chromatography

Thin-layer chromatography was performed on silica gel TLC or HPTLC plates (E. Merck, Darmstadt, Germany) with chloroform–methanol–0.25% $CaCl_2$ 60:35:8 as the developing solvent. Perbenzoylated ganglioside derivatives were separated on silica gel G or GF plates with benzene–methanol 8:2 as the developing solvent. Both gangliosides and perbenzoylated gangliosides were visualized with the resorcinol spray reagent. Perbenzoylated gangliosides gave a pink spot with the resorcinol. Perbenzoylated gangliosides were detected, prior to resorcinol spray, by their UV absorption on the silica gel GF plates and, after resorcinol spray, with a laser TLC scanner (Zeineh, LKB Instruments Inc., Gaithersburg, MD) (10).

Perbenzoylation conditions

Samples that contained 10 pmol to 8 nmol of each ganglioside standard, or gangliosides from tissue sources, were dried under nitrogen in 1-ml reaction vials (Reactivial, Pierce Chemical Co., Rockford, IL) and desiccated in vacuo over P_2O_5 for at least 4 hr prior to perbenzoylation. A 100-μl portion of 5% benzoyl chloride (v/v) in 25% toluene in pyridine (v/v) was then added to the vial. The vial was capped tightly and incubated in a heating oven at 45°C for 16 hr.

Isolation of perbenzoylated gangliosides

The reaction vial was placed in a water bath maintained at room temperature and the solvent was removed with a stream of nitrogen. The residue was then transferred with 0.8 ml of methanol to a reversed-phase sample preparation cartridge (C18-Bond Elut, Analytichem International, Harbor City, CA), which had been prewashed with 2 ml of methanol. The reaction vial was rinsed with an additional 0.8 ml of methanol and the rinse solvent was transferred to the sample preparation cartridge. The elutes, collected and combined, were then passed through the cartridge again. The cartridge was washed with 4 ml of methanol to remove reaction byproducts. Perbenzoylated gangliosides were eluted from the cartridge with 3 ml of methanol–benzene 8:2. The derivatives were collected in a 13 × 100 mm screw-capped test tube and the solvent was removed at room temperature with a stream of nitrogen. They were then dissolved in 100–500 μl of carbon tetrachloride and an appropriate aliquot was injected into the HPLC.

HPLC

HPLC analyses were performed with a microprocessor-controlled single-piston pump equipped with the manufacturer's column oven (model 5060, Varian Instruments, Walnut Creek, CA) coupled with a universal sample injector (model U6K, Waters Associates). The chromatographic columns (4.6 mm × 150 mm, Excaliber, Analabs, State College, PA or 4.6 mm × 100 mm, Short-one, Rainin Instruments, Woburn, MA) were packed with 3-micron mean particle size silica. The column oven temperature was maintained at 90°C and the column effluent was monitored at 230 nm with a variable wavelength UV detector (model 970A, Tracor Inc., Austin, TX). The detector output was coupled to a single-channel computing integrator/printer plotter (model SP4100, Spectra-Physics, Santa Clara, CA). Separation of the perbenzoylated gangliosides was accomplished with a 15-min linear gradient (flow rate, 2.0 ml/min) of 1.8% to 12% isopropanol in hexane. Ganglioside concentrations were calculated by the external standards method. The perbenzoylated gangliosides could be collected and used for molecular species analysis. However, it is important to note that the original gangliosides cannot be regenerated by mild alkaline hydrolysis because either N-benzoyl or N-acyl groups are removed during base treatment (16). Thus a mixture of gangliosides containing N-benzoyl or N-acyl groups is obtained.

Yields

The absolute yield of G_{M1} was determined in duplicate with the use of [^{3}H]G_{M1} (6.66 × 10^3 cpm/nmol) labeled

in the terminal galactose by the galactose oxidase method (17). The labeled derivative was injected into the HPLC column and the peak for G_{M1} was collected. After the solvent was evaporated, the residue was counted with a liquid scintillation counter. The yield of G_{M1} was then calculated from the amount of radioactivity recovered in the G_{M1} peak compared to the total number of counts in the original perbenzoylated sample. The yields of the other perbenzoylated gangliosides were estimated relative to G_{M1}. The estimations were made by assuming that: *1*) N-benzoyl groups had the same extinction coefficient as O-benzoyl groups; *2*) the UV absorption of benzoyl groups was additive; and *3*) the final products were completely perbenzoylated. Completely perbenzoylated G_{M1} possessed 19 benzoyl groups (9). Likewise, completely perbenzoylated G_{D1a} and G_{D1b} were calculated to possess 23 benzoyl groups, and G_{T1b} 27 benzoyl groups. Therefore, the molar absorptivities of G_{D1a}, G_{D1b}, and G_{T1b} relative to G_{M1} were calculated to be 1.21, 1.21, and 1.42, respectively.

RESULTS

Perbenzoylation conditions

The time course for the perbenzoylation of G_{T1b} was investigated using 5% benzoyl chloride (v/v) in 25% toluene in pyridine (v/v) at 45°C. G_{T1b} perbenzoylation was completed by 16 hr as judged by the detection of a single product peak. The perbenzoylation of the other gangliosides was completed prior to 16 hr, but no degradation of the derivatives occurred over the 16-hr time period of G_{T1b} perbenzoylation. Thus, 45°C was our standard perbenzoylation temperature and 16 hr was our standard reaction time.

Isolation of perbenzoylated gangliosides

After the 16-hr time period, the perbenzoylated gangliosides were isolated from excess reagents and reaction by-products with a C18-reversed phase sample preparation cartridge. When duplicate tubes of labeled G_{M1} (2.7 nmol each) were perbenzoylated, the recoveries of the radiolabeled perbenzoylated G_{M1} from the sample preparation cartridge were 90.5% and 91.1%. The absolute yields of G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b} were 87, 82, 82, and 77%, respectively.

The solvent partition procedure used for the isolation of perbenzoylated neutral glycolipids (16) and the silicic acid column used for the isolation of perbenzoylated monosialogangliosides (10) provided variable and excessive losses of the polysialogangliosides.

Chromatographic conditions

The perbenzoylated ganglioside standards were separated with a 15-min linear gradient (flow rate, 2 ml/min) of 1.8% to 12% isopropanol in hexane. The HPLC column was positioned in a heating oven maintained at 90°C. One major peak for G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b} was generated under these conditions (**Fig. 1**). Each major peak contained at least 95% of the total area. Perbenzoylated G_{D1b} eluted before perbenzoylated G_{D1a} with these conditions.

Linearity, sensitivity and reproducibility

The peak areas of perbenzoylated gangliosides were linear with the amount of ganglioside perbenzoylated between the range of 10 pmol to at least 8 nmol (**Fig. 2**). They were also linear with the amount of perbenzoylated ganglioside injected. The lowest limit of detection (twice baseline noise) for each ganglioside was approximately 5 pmol.

The relative standard deviations for the analysis of each ganglioside from quadruplicate analyses of human gray

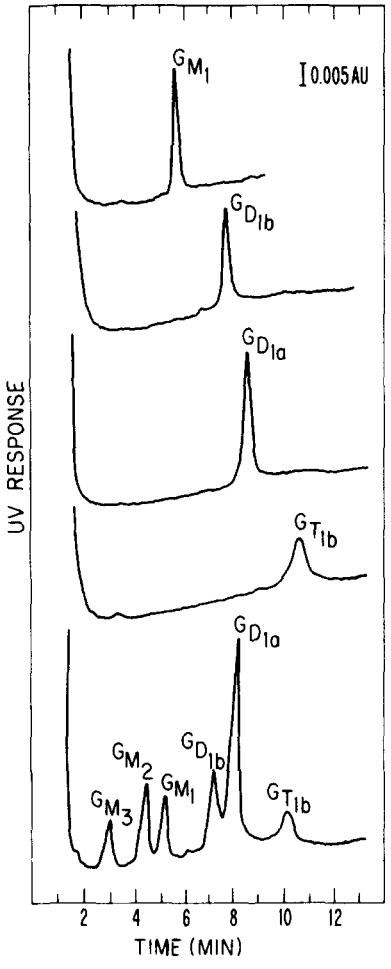

Fig. 1 HPLC of ganglioside standards. Chromatographic conditions were as described in Materials and Methods. Approximately 0.2 nmol of the individual ganglioside standards was injected into the HPLC. The mixed standard injection (bottom chromatogram) contained varying amounts of the standards.

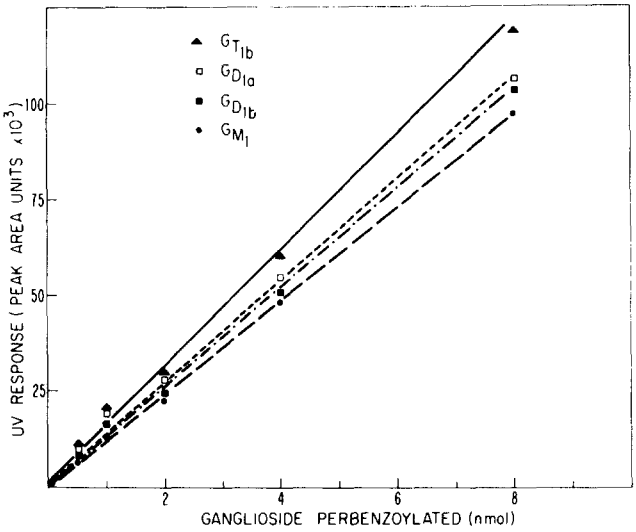

Fig. 2 Linear UV response of gangliosides perbenzoylated and analyzed by HPLC. Different amounts of each ganglioside standard were perbenzoylated and 10% of each perbenzoylated standard was injected into the HPLC. An individual data point represents the average of three injections.

matter (**Fig. 3**) were: G_{M1} = 2.28%, G_{D1a} = 2.76%, G_{D1b} = 1.83%, G_{T1b} = 1.61%. The same preparation of human brain gray matter gangliosides was also analyzed by HPTLC (5) followed by densitometric scanning with a laser scanner. The relative standard deviation for the analysis of each major ganglioside was: G_{M1} = 7.65%, G_{D1a} = 3.34%, G_{D1b} = 0.24%, G_{T1b} = 4.72%.

Tissue analyses

A ganglioside preparation from human gray matter was analyzed by HPLC and HPTLC (Fig. 3 and **Table 1**) and for comparative purposes the data for the individual major gangliosides were expressed as percent of total ganglioside sialic acid. In both systems the major gangliosides were shown to be: G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}. The minor components were only tentatively identified and not included in the calculations. The differences in the values of percent ganglioside sialic acid varied from 2% to 4% which further indicated that there was no significant by-product formation when the ganglioside fraction from this tissue was analyzed.

In addition to analyses of human gray matter and LS (long sleep) mouse cerebellum (Fig. 3 and **Fig. 4**, respectively), gangliosides from a variety of other tissues were analyzed, including those from bovine brain, mouse brain hippocampus, and cultured neuroblastoma cells. The analysis provided chromatograms of the quality equivalent to that obtained with the human brain gangliosides.

DISCUSSION

This report describes a convenient, highly sensitive, and reproducible HPLC procedure for the quantitative analysis of gangliosides. The procedure involves the conversion of gangliosides to their perbenzoyl derivatives, isolation of the derivatives on a C18-reversed-phase cartridge, separation of the derivatives on a column (3-micron silica) maintained at an elevated temperature, and UV detection of the derivatives at 230 nm. The convenience of the procedure, its sensitivity, reproducibility, and application to the analysis of gangliosides from tissue sources make it the method of choice for ganglioside quantification in our laboratories. Three aspects of the procedure contribute to its convenience: reaction conditions that lead to single products, a convenient isolation procedure for the derivatives, and chromatographic conditions that provide resolution of the derivatives.

The reaction conditions were selected for their ability to provide essentially single peaks for each ganglioside tested, in a time period that allowed the isolation of the perbenzoylated derivatives and ganglioside quantification on the same day. Many different reaction conditions were tested. The reaction conditions for neutral glycolipids (16) and monosialogangliosides (11) did not prove satisfactory because they generated an apparent by-product of G_{T1b} standard which comigrated with G_{D1a} under the chromatographic conditions described. Toluene, added to the reaction mixture, significantly diminished the formation of that by-product when G_{T1b} standard was perbenzoylated. The remaining by-product, (5%) from G_{T1b}, was an artifact from the storage of the standard in an organic solvent. Certainly the quantitative data obtained from freshly prepared brain gangliosides indicated that there was no by-product formation (Table 1). If there had been, the HPLC values for G_{D1a} would have been greater and

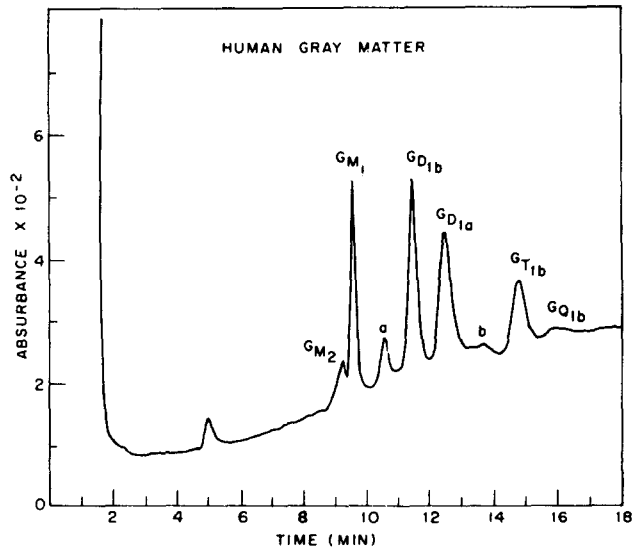

Fig. 3 HPLC of human gray matter gangliosides. The gangliosides from 50 mg of human gray matter were isolated as described in Materials and Methods and perbenzoylated. Peaks a and b have been tentatively identified as G_{D3} and G_{D2}, respectively.

TABLE 1. Human gray matter gangliosides (motor strip)

Method	N	Gangliosides			
		G_{M1}	G_{D1a}	G_{D1b}	G_{T1b}
		% ganglioside sialic acid ± SD[a]			
HPLC[b]	6	17.97 ± 0.41	23.17 ± 0.64	29.01 ± 0.53	29.85 ± 0.48
HPTLC[c]	6	19.35 ± 1.48	21.53 ± 0.72	33.08 ± 0.08	25.42 ± 1.20

[a]Total of G_{M1}, G_{D1a}, G_{D1b}, G_{T1b} only.
[b]The percentages of ganglioside sialic acid were calculated from the nmoles of each ganglioside (obtained by the external standard method) by conversion to nmoles of ganglioside sialic acid and dividing by the nmoles of total ganglioside sialic acid × 100.
[c]Values were obtained by the method of Ando, Chang, and Yu (5).

the G_{T1b} values smaller than those obtained by HPTLC.

The isolation of the derivatives upon completion of the reaction was simplified over previous procedures by the use of reversed-phase sample preparation cartridges. The use of these cartridges eliminated the time-consuming partition procedure and/or the use of silicic acid columns.

The isolated derivatives were separated on a silica (3-micron mean particle size) HPLC column. They were quantitated with UV detection at 230 nm. Several other silica columns were tested in attempts to separate the perbenzoylated gangliosides. The columns with 3-micron mean particle size silica were the only ones that provided the necessary selectivity and efficiency to effect the resolution of the perbenzoylated gangliosides. All of the silica columns generated a doublet or trailing shoulder for perbenzoylated G_{T1b} when the column oven was maintained at ambient temperature. A doublet configuration for various perbenzoylated gangliosides had been reported previously (18). It was surmised in those reports that the doublet represented the protonated and unprotonated carboxyl groups of the derivatives. Indeed, the formation of single peaks was accomplished, in those reports, by the addition of either glacial acetic acid (18) or phosphoric acid (10) to the respective mobile phases. The addition of either of those acids was not suitable for our purposes, however, because glacial acetic acid absorbed UV light at 230 nm and because phosphoric acid was not highly soluble in the hexane–isopropanol mobile phase.

Instead of the use of acids, we used elevated column temperature (column oven temperature at 90°C) to increase the interconversion rate of the protonated and unprotonated forms of the sialic acid moieties. The rapid association-dissociation of the carboxyl protons caused the protonated and unprotonated species to be indistinguishable in the HPLC column.

The increased column temperature also served to increase the sensitivity of the procedure since the narrower peaks, which resulted from the increased efficiency, were more easily detected. Thus, the lower limit of detection for each ganglioside tested was approximately 5 pmol. For reproducible quantitative data at least 10 pmol of each ganglioside was injected.

When small quantities of gangliosides were to be quantitated, the limiting step was the isolation of the gangliosides to be derivatized since much of the ganglioside was lost during the isolation (presumably through their binding to glass, etc.). We routinely conduct ganglioside analyses on 25 mg (wet weight) of human gray matter. We have also successfully performed single analyses on as little as 4 mg (wet weight) of mouse brain hippocampus. For the analysis of small quantities of gangliosides, a known amount of radiolabeled ganglioside internal standard should be added to the tissue at the time of extraction so that absolute yields can be calculated.

This HPLC procedure was shown to be very reproducible. The relative standard deviations for the analysis of the ganglioside standards or gangliosides from tissue sources were less than 3% under carefully controlled conditions. But clearly, the advantages of this HPLC procedure were most evident in the analysis of gangliosides from tissues. In this report, human gray matter gangliosides were quantified and the results were com-

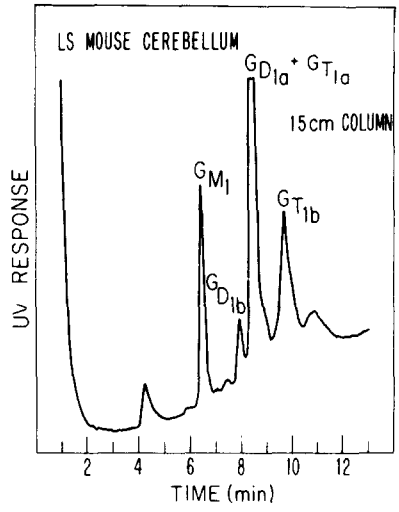

Fig. 4 HPLC of gangliosides of LS (long sleep) mouse cerebellum. One-half of the gangliosides from 30 mg of mouse cerebellum was perbenzoylated. The perbenzoylated gangliosides were then dissolved in 200 μl of CCl_4 and 40 μl were injected into the HPLC.

pared to those obtained by HPTLC and scanning. The HPLC and HPTLC values were in good agreement. Unlike published results, our sample utilized for this comparison contained only trace amounts of G_{M3}, G_{M4}, and G_{Q1b}. These differences could be explained by differences in the procedures by which the gangliosides were isolated or individual differences in gray matter ganglioside composition. The identification of the derivatives of some of the minor gangliosides remains to be accomplished.

Finally, this nondestructive analytical procedure allows the derivatives to be collected for further analyses, e.g., the separation and quantification of their individual ceramide molecular species on a reversed-phase HPLC column.

The authors wish to express their appreciation to Dr. Robert Yu for his generous gift of several ganglioside standards and to Ms. Maria Szoke and Dr. Michael Malone for their gift of human gray matter gangliosides and neuroblastoma gangliosides. The technical assistance of Ms. Marianna Berk and Ms. Hsiu-Chin J. Kuo is also gratefully acknowledged. This work was supported in part by the Veterans Administration and by NIH Grants NS 15037 and HD 04414.

Manuscript received 16 August 1984.

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