Quantitative analysis of monosialogangliosides by high-performance liquid chromatography of their perbenzoyl derivatives

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Abstract A quantitative high-performance liquid chromatographic method for the analysis of monosialogangliosides as their perbenzoyl derivatives has been devised. Samples containing as little as 3 nmol were converted to their perbenzoyl derivatives by reaction with 0.1 ml of 10% benzoyl chloride in pyridine at 60°C for 1 hr. The products were purified by silicic acid chromatography and analyzed by high-performance liquid chromatography (HPLC). The HPLC analysis was performed with a 50 cm × 2.1 mm LiChrosphere SI 4000 column and a linear gradient of 7–23% dioxane in hexane in 18 min. Detection was at 230 nm. The detector response was found to be proportional to the amount of monosialoganglioside analyzed. As little as 50 pmol of injected material could be conveniently quantitated. The overall yield from derivatization and chromatography, as determined with radiolabeled G_{M1}, was found to be 86%. To take advantage of the high sensitivity of the HPLC, a small-scale isolation method for gangliosides was devised. This method coupled with HPLC isotope dilution analysis was used to analyze the G_{M3} content of 1 ml of human plasma. —**Bremer, E. G., S. K. Gross, and R. H. McCluer.** Quantitative analysis of monosialogangliosides by high-performance liquid chromatography of their perbenzoyl derivatives. *J. Lipid Res.* 1979. **20:** 1028–1035.

Supplementary key words gangliosides · glycolipids

Gangliosides are defined as glycosphingolipids that contain sialic acid. They were first identified by Klenk (1) and have since been found outside the nervous system in all organs studied (2, 3). Since they were first described, most of the work on gangliosides has centered on the analysis of their chemical structures (3, 4). Recently a variety of specific functions have been associated with this interesting class of compounds. Among these is the involvement of gangliosides in the binding of viruses, toxins, and hormones (5). Gangliosides have also been implicated in cell–cell recognition (5) and at least four enzymopathies are known to involve ganglioside metabolism (6). In order to further elucidate the biological role of gangliosides, it would be desirable to have available a highly sensitive and convenient method for quantitative analysis.

In the past, the quantitative analysis of gangliosides has been carried out by preparative TLC with subsequent measurements of monosaccharides by destructive colorimetric and GLC methods (7, 8). In order to develop a more sensitive and convenient means of quantitation for gangliosides we applied HPLC. HPLC techniques can provide rapid and nondestructive separation of components which can then be analyzed by sensitive flow-through ultraviolet detectors. Previous investigations in this laboratory have shown the practicality of the use of perbenzoyl derivatives and HPLC for the quantitation of pmol amounts of neutral glycolipids (9–11). The feasibility of extending this approach to the quantitation of gangliosides has also been previously explored (12, 13).

The monosialogangliosides are of specific interest for several reasons. G_{M3} is the major ganglioside of extra neural tissues. Tay-Sachs disease, which is due to an inborn error of metabolism, gives rise to an accumulation of G_{M2}. It has been reported that the conversion of G_{M2} to G_{M1} may be important in the recognition mechanism of the chick retinal-tectal projections (14). G_{M1} is known to bind cholera toxin (15, 16) and it has been suggested that it may also be part of the binding site of human chorionic gonadotropin (17). The monosialogangliosides represent a different chromatographic problem than consideration of all the gangliosides. The major monosialogangliosides are characterized by an increasing hexose

Abbreviations: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; GLC, gas–liquid chromatography. The ganglioside nomenclature of Svennerholm (30) is used throughout. G_{M4} is used to designate NeuAc(α2 → 3)Gal(β1 → 1)Cer. IUPAC-recommended nomenclature is used for other glycosphingolipids (31).

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chain length whereas the polysialogangliosides include structural isomers. This report describes a convenient and sensitive quantitative HPLC analysis of the most common monosialogangliosides.

MATERIALS AND METHODS

Chemicals

The solvents used for HPLC were spectral grade (Burdick and Jackson Laboratories, Inc., Muskegon, MI) and were degassed by boiling briefly under reduced pressure. Benzoyl chloride (Eastman Kodak Co., Rochester, NY), pyridine (dried over 4 Å molecular sieves), and solvents used for extraction and TLC were reagent grade (Fisher Chemical Co., Fairlawn, NJ). They were used without prior distillation. The monosialoganglioside standards G_{M3} and G_{M1} were isolated and purified by the method of Svennerholm (18) from beef brain. The G_{M2} was a gift from Dr. E. Kolodny and was isolated from a Tay-Sachs brain. The G_{M4} standard was a gift from Dr. G. Schwarting and was isolated by the method of Li et al. (19) from egg yolk. The purity of each standard was determined by TLC and by comparison of resorcinol assays (20) with gravimetric measures. The standards were dried in an Abderhalden pistol under vacuum over refluxing methanol until a constant weight was obtained. Standards with a purity greater than 80% were selected for HPLC. For isotope dilution studies, a portion of the G_{M3} standard was labeled with tritium in the sphingosine and fatty acid moieties by the method of Schwarzmann (21). Galactose-labeled G_{M1} was a gift from Dr. S. Raghavan.

Thin-layer chromatography

The TLC was performed on Merck Silica Gel 60 or Silica Gel 60 F-254 fluorescent indicator plates (E. Merck, Darmstadt, Germany) with chloroform–methanol–0.25% $CaCl_2$ 60:35:8 as the developing solvent (22). Gangliosides were detected with resorcinol spray reagent and contaminating materials were detected with 5% H_2SO_4 in methanol.

Large-scale isolation of gangliosides

Gangliosides were isolated from 1 g (dry weight) of human liver or from 50 ml of plasma. The plasma ganglioside fraction was isolated by the method of Yu and Ledeen (23). The liver ganglioside fraction was isolated according to Seyfried, Ando, and Yu (24) with modifications. Human liver was lyophilized and ground to powder in a mortar and pestle. A 1-g portion of the powder was taken and 5% (v/w) water was added before extraction of the lipids. The lipids were extracted with 25 ml of chloroform–methanol 1:1 and stirred at room temp for 1 hr. The mixture was then filtered through filter paper in a sintered glass funnel. The retentate was re-extracted with chloroform–methanol 1:1, filtered, and the filtrates were combined. The combined filtrates were evaporated to dryness, redissolved in 50 ml of chloroform–methanol–water 30:60:8, and applied to a 2.0-g column of DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, NJ). After sample application, the column was first eluted with 150 ml of chloroform–methanol–water 30:60:8. The gangliosides were then removed from the column with 180 ml of chloroform–methanol–0.8 M sodium acetate 30:60:8, and the resulting solution was taken to dryness. The dried residue was treated with 10 ml of 0.2 N NaOH in methanol and incubated at 37°C for 1 hr. This was then evaporated to near dryness, taken up in 15 ml of water, and dialyzed for 2 days in the cold against distilled water. The bag contents were lyophilized, the sample was dissolved in 2 ml of chloroform–methanol 4:1 and was applied to a 1-g silicic acid column (Unisil, Clarkson Chemical Co., Inc., Williamsport, PA). The column was eluted with 20 ml of chloroform–methanol 4:1 and the gangliosides were recovered with 150 ml of chloroform–methanol 2:3. The chloroform–methanol 2:3 fraction was evaporated to dryness and the Unisil column chromatography was repeated with chloroform–methanol 85:15 and chloroform–methanol 2:3 as the eluting solvents. The ganglioside fractions recovered from the second Unisil column were evaporated to dryness and either benzoylated or spotted on TLC plates.

Small-scale isolation of gangliosides

For quantitative analysis, 8 ml of plasma was divided into 1-ml portions. To each of these ^{3}H-labeled G_{M3} (1.04×10^5 cpm, sp act 60.8 cpm/pmol) was added as an internal standard. The lipids were extracted with 10 ml of chloroform–methanol 1:1 and the solution was shaken vigorously for 15 min with intermittent mild sonication. The mixture was then centrifuged to form a pellet of the chloroform–methanol–insoluble material. The chloroform–methanol supernatant was removed and the pellet was re-extracted in 5 ml of chloroform–methanol 2:1 with the aid of sonication. This mixture was again centrifuged as before, then the chloroform–methanol 2:1 was removed and combined with the chloroform–methanol 1:1 extract. To the combined extracts, 5 ml of chloroform was added to obtain a solvent ratio of chloroform–methanol 2:1. A solvent partition was then formed by the addition of 0.2 volumes of water. The solvents were mixed well and the two phases were separated by centrifugation.

The upper phase was removed and the lower phase was washed three times with 5 ml of methanol–water 1:1. All the upper phases were combined and made 0.2 M in KCl for reverse-phase chromatography with a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA). The Sep-Pak cartridge was fitted with a three-way stopcock and conditioned with three alternate 20-ml washes of methanol and chloroform–methanol–0.2 M KCl in water 3:48:47. Just prior to sample application, the column was equilibrated with 20 ml of chloroform–methanol–0.2 M KCl in water 3:48:47 and the sample was applied to the Sep-Pak through a 50-cc Leur lock syringe. The eluate was collected and passed through the cartridge again. The column was washed with 10 ml of water and gangliosides were eluted with 15 ml of methanol. The methanol fraction was taken to dryness under nitrogen. The ganglioside fraction was dissolved in chloroform–methanol 85:15 and applied to a 0.04-g Unisil column. This column was washed with 8 ml of chloroform–methanol 85:15 and the gangliosides were eluted with 6 ml of chloroform–methanol 2:3.

Perbenzoylation conditions

Samples containing 3–20 nmol of monosialoganglioside standards or gangliosides from human plasma or liver were dried with nitrogen in 1-ml Reacti-vials (Pierce Chemical Co., Rockford, IL) and desiccated in vacuo over P_2O_5 for at least 1 hr. A 0.1-ml aliquot of freshly prepared 10% (v/v) benzoyl chloride in pyridine was then added. The vials were flushed with nitrogen, capped tightly, and incubated in a heating block at 60°C for 1 hr. After incubation, the reaction mixture was dried under a stream of nitrogen, dissolved in benzene, and again taken to dryness. The residue was then redissolved in less than 1 ml of benzene and placed on a 0.1-g Unisil column (constructed with glass wool, sea sand, and Unisil in a Pasteur pipet). The Reacti-vial was repeatedly washed with benzene until a total of 5 ml of benzene had passed over the Unisil column. The perbenzoylated products were recovered from the column with 2.5 ml of 10% (v/v) methanol in benzene and collected in a 13 × 100 mm screw-cap culture tube. The solvent was evaporated under a stream of nitrogen and the purified products were dissolved in 0.5 ml of CCl_4. Appropriate portions (5–25 μl) were then injected onto the HPLC column.

HPLC

HPLC analyses were performed with two reciprocating pumps (Model 6000, Waters Associates) controlled by a solvent programmer (Model 660, Waters). Samples were introduced into the chromatographic system by a universal liquid chromatograph injector (Model U6K, Waters) equipped with a 250-μl injection loop.

The chromatographic adsorbent was LiChrosphere SI 4000 (E. Merck) with an average particle size of 10 μm. It was packed into a 2.1 mm × 50 cm stainless steel column, with the stirred slurry method (25) and a Model 705 column packer (Micromeritics, Norcross, GA). The column effluent was monitored with a variable wavelength UV spectromonitor equipped with high-pressure (1500 psi) stainless steel cells (Schoeffel Instrument Corp., Westwood, NJ) and detector output was coupled in series to a single-channel computing integrator (Auto Lab System I, Spectro-Physics, Santa Clara, CA) and a strip chart recorder. The components of the chromatographic system were arranged so that the solvent from the pumping system first passed through the reference cell of the detector, next through the injector, the column, and finally through the sample cell of the detector as described by Ullman and McCluer (11). Separation of perbenzoylated monosialogangliosides was accomplished by gradient elution. Gradient elution was performed with an 18-min linear gradient of 7–23% dioxane in hexane or 10–25% in 10 min with a flow rate of 2 ml/min and absorbance was measured at 230 nm. After each gradient run was completed, the gradient was reversed over 3 min and the solvent was allowed to flow through the system for at least 8 min.

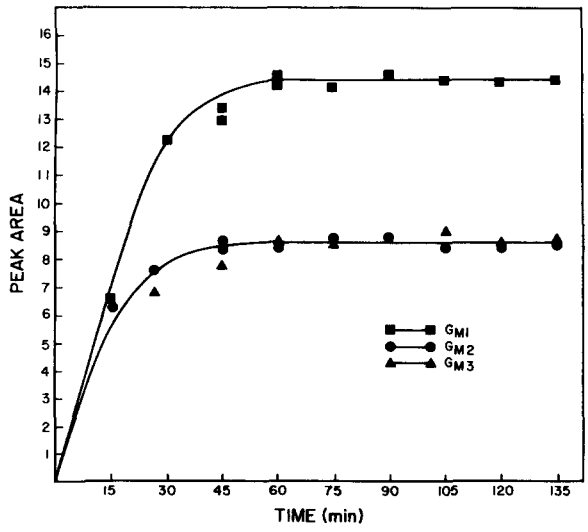

Fig. 1. Time course of benzoylation. Gangliosides were reacted with 10% benzoyl chloride for various periods of time at 60°C and chromatographed on a 50-cm SI 4000 column with a 10-min linear gradient of 10–25% dioxane in hexane. The area of the major peak was measured and plotted against time. The peak area increased up to 1 hr and then remained constant through 2 hr.

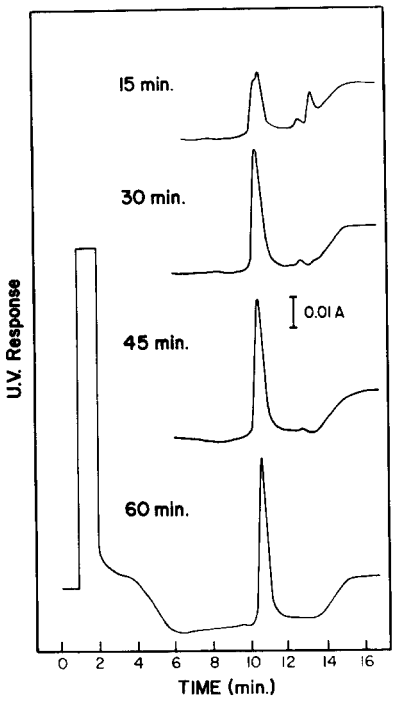

Fig. 2. HPLC of G_{M1} reacted at 60°C with 10% benzoyl chloride for various periods of time. Chromatography was with a 50-cm SI 4000 column and a 10-min linear gradient of 10–25% dioxane in hexane. Detection was at 230 nm.

RESULTS

Perbenzoylation conditions

The time course of the perbenzoylation for G_{M1}, G_{M2}, and G_{M3} was investigated with 10% benzoyl chloride in pyridine at 60°C and the products formed were measured by HPLC and UV absorption. The yield of the perbenzoylation product of each of these standards was maximal at 60 min and no degradation products were detected up to 135 min (**Figs. 1** and **2**). Derivatization at 60°C for 1 hr was chosen as the standard reaction conditions. HPLC of perbenzoylated G_{M1}, G_{M2}, and G_{M3} standards showed that in each case only one peak was produced (**Fig. 3**). Previous investigations have established the structure of perbenzoylated neutral glycolipids (9, 10). These data demonstrate that perbenzoylated GlcCer when subjected to mild alkaline methanolysis yields two compounds: the original GlcCer with the sphingosine *N*-acyl amide and a GlcCer with a *N*-benzoyl amide. Thus, perbenzoylation of neutral glycolipids that contain only nonhydroxy fatty acids results in benzoylation of the *N*-acyl amide nitrogen as well as of the hydroxyl groups. Similar evidence was obtained for the monosialoganglioside perbenzoates. G_{M1} and G_{M3} standards were benzoylated and the derivatives were isolated by HPLC. Each was subjected to mild alkaline methanolysis followed by TLC and visualization of the products with UV light and resorcinol spray. G_{M3} produced four spots and G_{M1} produced six spots. It can be predicted from the neutral glycolipid data that G_{M3}, which contains two amide nitrogens, should produce four products and that G_{M1}, containing three amide nitrogens, should produce nine products upon alkaline methanolysis. The data obtained for G_{M3} are consistent with this prediction. G_{M1} produced only six spots; the remaining three products may well have been obtained in insufficient quantity to be visualized or may not have been separated from some of the other products.

Chromatographic conditions

An 18-min linear gradient of 7–23% dioxane in hexane on a 50-cm LiChrosphere SI 4000 column were found to be the most satisfactory chromatographic conditions for the separation of monosialoganglioside perbenzoates. Previous results from this laboratory (12) indicated that acetic acid improved the chromatography of ganglioside perbenzoates but did not allow detection at 230 nm. By addition of phosphoric acid, which is transparent at 230 nm, monosialoganglioside perbenzoates were separated isocratically with 7.2% dioxane in hexane on a 10-cm

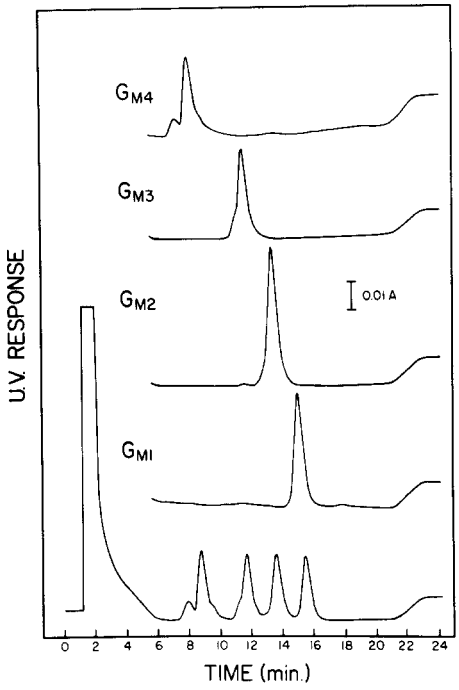

Fig. 3. HPLC of the major monosialoganglioside standards. Approximately 0.5 μg of each standard was used. The chromatography was with a 50-cm SI 4000 column and an 18-min linear gradient of 7–23% dioxane in hexane. Detection was at 230 nm. G_{M4} and G_{M3} multiple peaks and asymmetry are presumably due to the molecular heterogeneity in the lipid moiety of these compounds.

SI 4000 column. However, this system was not as satisfactory as the gradient elution for analysis of biological samples because of the marginal solubility of H_3PO_4 in the hexane dioxane mixtures.

Interference from polysialogangliosides

To determine if there was interference from polysialogangliosides, G_{D3}, G_{D1a}, and G_{D1b} were benzoylated under the conditions reported above. HPLC of these derivatives showed that G_{D3} chromatographed with a retention time greater than that of G_{M1} and did not interfere with G_{M1} quantitation. Both G_{D1a} and G_{D1b} chromatographed with a greater retention time than G_{D3} and did not interfere with the analysis of any monosialogangliosides. With the benzoylation conditions used here, G_{D1a} and G_{D1b} did not yield a single peak but, rather, yielded multiple or broad asymmetrical peaks.

Yields

The absolute yield of the perbenzoylation product of G_{M1} was determined with radiolabeled G_{M1}. Radiochemically pure [^{3}H]G_{M1}, labeled in the terminal galactose by the galactose oxidase method (26) (sp act 6.66 × 10^3 cpm/nmol), was benzoylated in triplicate and injected onto the HPLC column. The peak corresponding to the G_{M1} derivative was collected and radioactivity was detected with a liquid scintillation counter. The yield for G_{M1} perbenzoylation was then calculated from the amount of radioactivity recovered in the G_{M1} peak from the HPLC column. The yields of perbenzoylation products of G_{M2} and G_{M3} standards were then calculated relative to G_{M1} with the following assumptions: *1*) *N*-benzoyl groups have the same extinction coefficient as do *O*-benzoyl groups at 230 nm; *2*) the UV absorption of benzoyl groups are additive; and *3*) the final products formed are completely benzoylated (10). Considering all the available sites for benzoylation, a completely benzoylated G_{M3} molecule would have 13 benzoyl groups; G_{M2} would have 16 and G_{M1} 19. Therefore the molar absorptivities of G_{M3} and G_{M2} relative to G_{M1} were calculated to be 0.68 and 0.84, respectively. By injection of known amounts of G_{M3} and G_{M2} and by use of the above relative responses, the yields for these monosialogangliosides were calculated. The yields for G_{M3}, G_{M2}, and G_{M1} were found to be 83%, 87%, and 86%, respectively.

Linearity sensitivity and reproducibility

The detector response relative to the amount of derivative injected was found to be linear over the entire range studied. The relative standard deviation, determined from at least four injections at each point, was not more than 2% for G_{M1} and G_{M2} and not more than 3.5% for G_{M3}. The smallest amount conveniently quantitated with this reproducibility was about 50 pmol.

The relationship between peak area and amount of monosialoganglioside standard benzoylated was also found to be linear with relative standard deviations of 4%, 3%, and 7% for G_{M1}, G_{M2}, and G_{M3}, respectively (**Fig. 4**). This relationship was determined by benzoylation of various amounts of each monosialoganglioside standard and HPLC quantitation of a constant fraction. The variation obtained is therefore a reflection of both injection and benzoylation variability. For G_{M3}, the variability is larger with small sample sizes. For the formation of the G_{M3} perbenzoyl derivative with less than 5 nmol and injection of 150 pmol, the relative standard deviation was 8.5% but injection of larger quantities gave a relative standard deviation of 4%. For G_{M2} and G_{M1} the variation was constant over the range studied.

Analysis of monosialogangliosides from human plasma and liver

Ganglioside preparations from 1 g (dry weight) of human liver and 50 ml of plasma were isolated as described above and analyzed by HPLC and TLC. The HPLC analyses of these fractions by gradient elution (**Fig. 5**) compared favorably with the results seen by TLC analysis which utilizes resorcinol detection (**Fig. 6**). In both systems a component migrating as G_{M3} is the predominant ganglioside with minor

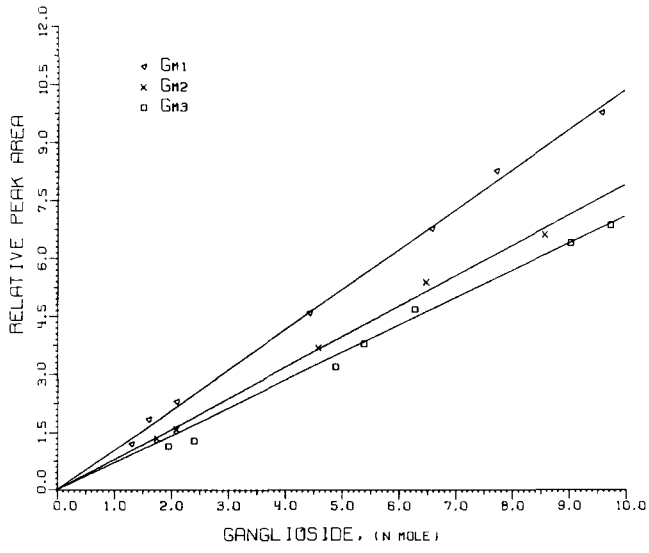

Fig. 4. Linearity of the UV response at 230 nm to the amount of monosialoganglioside analyzed. Different amounts of each monosialoganglioside standard were benzoylated and 3% of each benzoylated standard was injected. An individual data point represents the average of repeated injections.

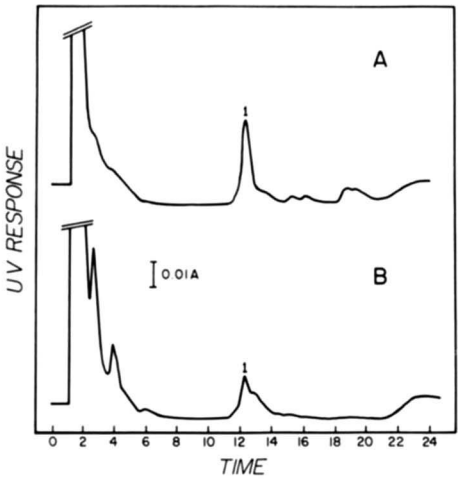

Fig. 5. HPLC gradient elution of the monosialogangliosides from human plasma (*A*) and human liver (*B*). The ganglioside fractions were isolated from the equivalent of 4 ml of human plasma and 0.01 g (dry weight) of human liver, as described in the text. The benzoylated samples were dissolved in 500 μl of CCl_4 and 50 μl of this was injected. The major peak (*1*) in *A* and *B* co-chromatographed with G_{M3} standard. The four minor peaks in plasma (*A*) did not correspond to any monosialoganglioside standards. HPLC conditions were as described in Fig. 3.

components migrating closely but not exactly with G_{M2} or G_{M1} standards. Two of these minor components have been reported to be sialosylparagloboside (IV^3-NeuAc-nLcOse$_4$-Cer) and disialosyllactosyl ceramide (G_{D3}) (27). Quantitative data were collected only for G_{M3}.

Analyses of three 1-ml equivalents of the plasma ganglioside preparation gave values of 8.7 ± 0.2 σ nmol of G_{M3}. Analysis of 10-ml equivalents of the same plasma preparation by TLC, as described by MacMillan and Wherrett (28), gave values of 7.6 ± 0.8 σ nmol G_{M3} per ml plasma. The HPLC and TLC determined G_{M3} values were compared with the Student's *t* test and found not to be significantly different ($P > 0.10$).

Small-scale isolation and HPLC isotope dilution analysis of plasma G_{M3}

The sensitivity of the HPLC method is such that the analysis of small biological samples (<1 ml of plasma) should be possible. However, the purification of small quantities of gangliosides to a state of purity that allows accurate HPLC led to large and variable losses when methods, as described above for larger samples, were applied to 1 ml of plasma. An alternative procedure suitable for the processing of small samples, which employs isotope dilution measurement to correct for losses, was therefore devised. This procedure which involves chloroform–methanol extraction, solvent partition, Sep-Pak and Unisil chromatography, as described in the methods section, was validated by the analysis of eight 1-ml identical plasma samples. Prior to extraction, [^{3}H]G_{M3} (1.04×10^5 cpm) was added to each sample and, during the HPLC analysis, the peaks corresponding to G_{M3} were collected and the radiospecific activities were calculated from the peak areas and radioactivities. The amount of plasma G_{M3} was calculated from the decrease in the specific activity of the added [^{3}H]G_{M3} internal standard. The measured specific activities of the samples, S.A.(detm), are defined as:

$$\text{S.A.(detm)} = \frac{\text{cpm(std)}}{\text{pmol(std.)} + \text{pmol(p1G}_{M3})}$$

where cpm(std) is the number of counts of standard [^{3}H]G_{M3} added and pmol(std) is the quantity of standard added. Thus the pmol of plasma G_{M3} (p1G_{M3}) from the sample can be derived:

$$\text{pmol(p1G}_{M3}) = \frac{\text{cpm(std)} - [\text{S.A.(detm)} \times \text{pmol(std)}]}{\text{S.A.(detm)}}$$

The concentration of G_{M3} as determined from the eight samples was found to be 7.1 ± 0.3 σ nmol per ml of plasma. The percentage recovery of the added [^{3}H]G_{M3} was found to be 59.8% ± 5.6 σ for these samples.

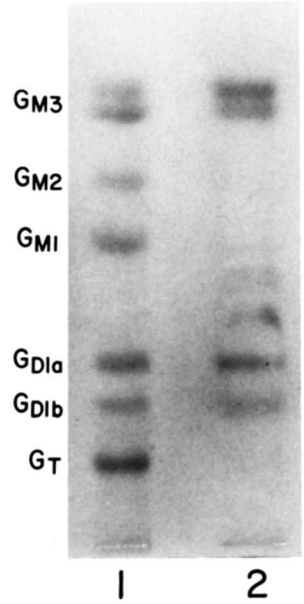

Fig. 6. Thin-layer chromatogram of human plasma. Lane *1*, mixed ganglioside standard; lane *2*, ganglioside fraction equivalent to 5 ml of plasma. The plasma ganglioside fraction was part of the large-scale isolation described in the text. TLC was performed on Merck Silica Gel 60 with chloroform–methanol–0.25% $CaCl_2$ 60:35:8 as the developing solvent. Gangliosides were visualized with resorcinol spray reagent.

DISCUSSION

Our results show that HPLC and UV detection of ganglioside perbenzoates provide a convenient and highly sensitive method for the quantitation of monosialogangliosides. The high sensitivity results from the large extinction coefficient of benzoyl derivatives at 230 nm. The reaction conditions used for derivatization were those that gave a single major product by HPLC for each standard in high yield.

HPLC conditions were designed with detection of the perbenzoyl derivatives at 230 nm as the primary goal. Of the several chromatographic supports and solvent systems investigated, the hexane–dioxane gradient elution on a 50-cm SI-4000 column has to date been found to be the most satisfactory. Because the chromatographic efficiency (theoretical plates) obtained is low compared to that usually seen in HPLC systems, we feel better resolution of these ganglioside derivatives is possible and are continuing efforts to define superior systems. Nevertheless, the conditions described here provide baseline resolution of the most common monosialogangliosides and have been applied successfully for the analysis of gangliosides from several sources.

With these chromatographic and benzoylation conditions, peaks corresponding to G_{M3} and G_{M4} had shoulders. These shoulders could be the result of incomplete benzoylation or degradation of the products but the recovery and time-course data suggest that derivatization is complete. A likely explanation is that the shoulders reflect the molecular heterogeneity of these gangliosides. Differences in the fatty acid composition of G_{M3} have been shown to alter TLC behavior. G_{M3} from neural tumors splits into two bands on TLC, based on the difference between long ($C_{22,24}$) and short ($C_{16,18}$) chain fatty acids (29). Our TLC results show standard and plasma G_{M3} to be split into two bands (Fig. 6). The exact cause of band splitting from plasma G_{M3} is not known, but the fatty acid profile indicates the presence of both long and short chain fatty acids (23). The splitting of these bands on TLC and the occurrence of shoulders on HPLC may be due to long and short chain fatty acid differences. Human liver G_{M3} has also been reported to separate by fatty acid differences on TLC (24). In this case, the upper band contains mainly long chain nonhydroxy fatty acids while, in the lower band, a mixture of hydroxy fatty acids and short chain nonhydroxy fatty acids predominated. Our HPLC results from human liver show the peak corresponding to G_{M3} (Fig. 5) to have two shoulders. Further work is needed to identify the components responsible for these peak shapes.

The analysis of human plasma has demonstrated the usefulness of this procedure for the quantitation of monosialogangliosides. The value for plasma G_{M3} levels determined by HPLC was close to that determined independently by the TLC–resorcinol method. Statistical analysis, with the Student's t test, showed the difference between the values to be insignificant ($P > 0.10$). This indicates that the UV detector response is due to ganglioside without interference by other material.

The small-scale isolation method was developed to exploit the potential of the quantitative HPLC procedure. With this isolation method it is possible to conveniently prepare ganglioside fractions from tissue or body fluids. The major problems encountered in the analysis of small tissue samples are large losses and high variability. This variability and loss have been compensated for by the use of $[^3H]G_{M3}$ as an internal standard. The use of the isotope dilution technique not only gives accurate information about the amount of an individual component but also allows the calculation of losses through the entire procedure. The analysis of eight 1-ml plasma samples has shown these procedures can be used to obtain highly reproducible results.

The HPLC value for plasma G_{M3} obtained from repeated analysis of a single large-scale preparation (8.7 ± 0.2 nmol/ml) and that obtained by the small-scale isotope dilution analysis of 1-ml plasma samples (7.1 ± 0.3 nmol/ml) compare favorably with previously published values. Yu and Ledeen (23) have reported data for total ganglioside sialic acid (11.3 nmol/ml) and the ratio of hematoside (G_{M3}) to hexosamine-containing gangliosides (2:1), thus a value of 7.5 nmol G_{M3} per ml of plasma can be calculated. Portoukalian et al. (27) has also reported ganglioside levels for human plasma. Their data include a direct determination of G_{M3} levels. They find the amount of G_{M3} to be 10.8 ± 2.1 nmol per ml of plasma and report the total lipid-bound sialic acid to be 12.3 ± 0.2 nmol per ml of plasma. The differences between these G_{M3} levels may represent biological variability. The plasma we used for the large-scale isolation was obtained from a different individual than the plasma used for small-scale isotope dilution analysis.

We believe the isolation and quantitation procedures reported here will be useful for the analysis of monosialogangliosides from a variety of sources. HPLC and UV detection of ganglioside perbenzoates provide about a 100-fold increase in sensitivity over microresorcinol methods. The small-scale isolation method simplifies isolation of ganglioside fractions for analysis and, with the addition of internal standard,

allows for precise quantitation. The convenience and sensitivity of these methods for analysis of monosialogangliosides may provide the opportunity for gaining new insights into the biological functions of gangliosides. Currently work is underway for the development of similar HPLC methods for the more complex gangliosides.

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