paper on methodology

Quantification of gangliosides by microbore high performance liquid chromatography

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Abstract A highly sensitive analytical method was developed that allows the separation of ganglioside mixtures and quantification of individual non-derivatized gangliosides in the concentration range between 2 pmol and 1 nmol. Gangliosides were separated with a gradient of acetonitrile/phosphate buffer on a 1 mm diameter microbore HPLC column packed with Spherisorb-NH_2. They eluted according to their number of sialic acid residues with increasing phosphate and decreasing acetonitrile concentrations. The separation of different gangliosides with equal sialic acid content is also described. The column effluent was monitored at the maximum of absorption at 197 nm. The sensitivity is higher than resorcinol staining of fractionated gangliosides by thin-layer chromatography, previously the standard method for ganglioside analysis. The separated gangliosides can be analyzed by further methods. The HPLC method described here has been applied to the analysis of serum and oligodendroglioma specimens.—**Wagener, R., B. Kobbe, and W. Stoffel.** Quantification of gangliosides by microbore high performance liquid chromatography. *J. Lipid Res.* 1996. **37:** 1823–1829.

Supplementary key words microbore HPLC • quantification of gangliosides • purification of serum and oligodendroglioma gangliosides

Gangliosides, first isolated from brain grey matter (1), are sialic acid-containing glycosphingolipids predominantly located in the outer leaflet of the plasma membrane (2). They are believed to have important function in cell-cell interaction. Gangliosides are also present in every body fluid. They consist of a hydrophilic oligosaccharide chain and a hydrophobic ceramide moiety. Structurally, their variability in the oligosaccharide moiety, their long-chain sphingosine base, and fatty acid composition are striking (3, 4). Ganglioside functions are mainly exerted by their oligosaccharide chains, but also by their hydrophobic part (5). Gangliosides play manifold physiological roles: they act as receptors for viruses, bacteria or toxins (6) and play a role in development and differentiation (7). During malignant transformation, alterations in ganglioside biosynthesis and metabolism lead to the expression of gangliosides that are qualitatively and quantitatively different from those found in normal progenitor cells (8) and may be of diagnostic value (9). Shed gangliosides of tumor cells have potent immunosuppressive activity (10). Recent studies show that metabolic products such as lysogangliosides and sphingosine (11, 12) or ceramide (13, 14) may function as second messengers.

Growing recent interest in the biological function of gangliosides has generated a need to improve their analysis especially in the nano and picomolar scales. TLC separation followed by resorcinol staining and densitometric scanning is the standard tool for the resolution, detection, and quantification of ganglioside mixtures (3). The detection of a specific color on a solid silica gel particle is, however, affected by various factors, e.g., quenching, conditions of TLC separation, and the method of visualization (15). To overcome these disadvantages several HPLC methods have been developed to combine high sensitivity and reproducibility. A sensitive HPLC analysis of non-derivatized gangliosides using UV-detection has proven rather difficult. The maximum absorption of gangliosides is at 197 nm, a wavelength at which solvents used in gradients may interfere. Therefore, most of the reported highly sensitive (pmol range) analytical HPLC methods use a precolumn derivatization with chromophores (15–18) or the cleavage of the molecules (19) for improved detection. Gazzotti, Sonnino, and Ghidoni (20) developed a NH_2–silica gel normal phase HPLC method with good separation properties, but with the drawback of monitoring the gangliosides at 215 nm far away from the absorption maxi-

Abbreviations: Abbreviations of gangliosides are according to the nomenclature of Svennerholm (32). MG-3/MG-4, sialosyl(2-3) lacto-N-neotetraosylceramid (according to Kundu et al. (27)); CM, chloroform–methanol; DIPE, diisopropylether; GalNAc, N-acetylgalactosamine; Fuc-GD1b, fucosyl-GD1b; Fuc-GM1, fucosyl-GM1; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; TLC, thin-layer chromatography; MS, mass spectroscopy; GLC, gas–liquid chromatography.

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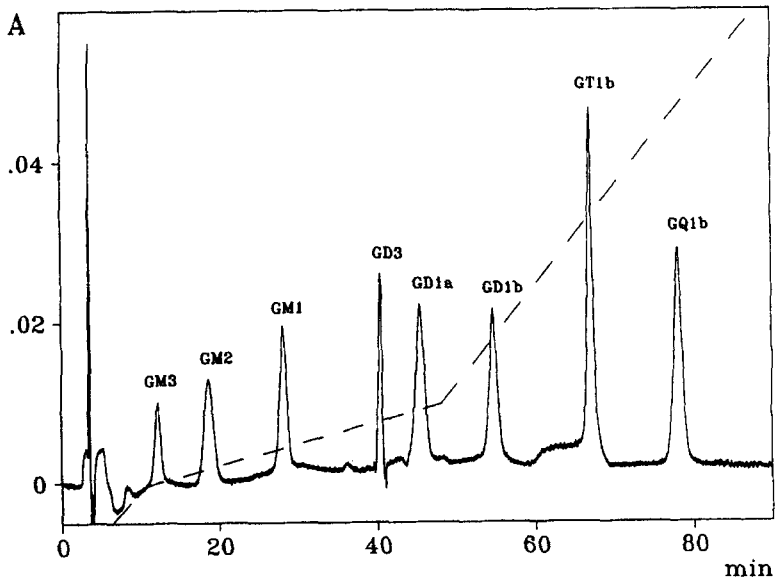

Fig. 1. Resolution of standard gangliosides by microbore HPLC. One hundred pmol of GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b, and GQ1b was applied. The elution gradient is given as a dashed line; it starts with 100% A, 0% B, and ends with 0% A, 100% B. Solvent A: acetonitrile–5 mM phosphate buffer, pH 5.6 (83:17); solvent B: acetonitrile–20 mM phosphate buffer, pH 5.6 (1:1).

mum. This limits the detection of GQ1b and GM3 to 0.08 and 0.260 nmol, respectively. Johnson, Masserini, and Alhadeff (21) in a modified method used isocratic elution, monitoring the eluate at 195 nm with a sensitivity of detection of GM1 and GD1b of 13 pmol and 11 pmol, respectively. Only the separation of GM1, Fuc-GM1, GD1b, and Fuc-GD1b was achieved.

In this report we describe an improved highly sensitive method using a microbore HPLC system, with detection at 197 nm that allows the separation of all gangliosides ranging from GM3 to GQ1b.

EXPERIMENTAL

Materials

Standard gangliosides (purity >98%) were obtained from Alexis, Grünberg, Germany. Chloroform and methanol were from Riedel-de Haen, Seelze, Germany and were analytical and HPLC grade, respectively. They were distilled before use. Diisopropylether (puriss. p.a.), 1-butanol (p.a.), ortho phosphoric acid, and sodium phosphate buffer (pH 6.5) were from Fluka, Neu-Ulm, Germany. HPLC grade acetonitrile was from Baker, Gross-Gerau, Germany and resorcin was from Sigma, Deisenhofen, Germany. Water was deionized and double distilled.

Purification of gangliosides

Gangliosides were isolated according to the partition method with diisopropylether(DIPE)/1-butanol/50 mM aqueous NaCl developed by Ladisch and Gillard (22). We adapted our method to that of Senn et al. (23). Gangliosides were extracted from 2 ml serum by stirring with 20 ml CM (1:1) for 1 h, followed by sonication for 5 min in a Branson bath sonicator.

Brain specimens were lyophilized. About 10 mg of the dried tissue was rehydrated in 1 ml water for 1 h and homogenized. The suspension was mixed with 10 ml CM (1:1) and treated further as described for serum samples.

The completely dispersed samples were centrifuged for 10 min (750 *g*) and the supernatant was filtered through a 3D filter funnel. The solvent was removed in a rotary evaporator; the residue was resuspended in 10 ml CM (1:1) by vortexing and sonication and incubated overnight at -20°C. The precipitate was removed by centrifugation at 750 *g* and -20°C. The supernatant was evaporated, desiccated in vacuum produced by an oil pump, and the remaining lipid extract was suspended in a mixture of 2.4 ml DIPE and 1.6 ml 1-butanol with sonication. For partition, 2 ml water was added and the suspension was vortexed, centrifuged for 10 min at 750 *g*, and the upper organic phase was carefully removed with a Pasteur pipette. The ganglioside-containing aqueous phase was re-extracted with the same volume of the organic solvents and evaporated to dryness in a Speed Vac concentrator (SVC100H, Savant). The residue was dissolved in 2 ml water–methanol 1:1 by sonication. Salts were removed by a C8-Plus™ cartridge (Millipore). The column was prepared according to the manufacturer's instructions. The column was loaded, washed with 20 ml water, concentrated in a stream of nitrogen, and eluted with 3 ml methanol and 6 ml chloroform–methanol 1:1. The remaining uncharged glycosphingolipids were separated by anionic exchange chromatography using a

QMA-Plus™ cartridge (Millipore) according to the manufacturer's instruction. Briefly, 3 ml chloroform was added to the C8-column eluate and mixed. The combined eluates were loaded on the prewashed QMA-column, washed with 10 ml methanol, and eluted with 15 ml 0.3 M sodium acetate in methanol. Fifteen ml water was added to the QMA-column eluat, mixed, and desalted by another C8-column. After loading, the column was washed with 30 ml water, purged with a stream of nitrogen, and gangliosides were eluted with 1.5 ml methanol and 3 ml CM 1:1. The eluate was dried in a Speedvac concentrator.

High performance liquid chromatography of ganglioside mixtures

An Applied Biosystems (Weiterstadt, Germany) high performance liquid chromatograph composed of a dual syringe solvent delivery system 140A and a photodiode array detector 1000S with a 6 µl flow-through cell was used. The system included a model 8125 injector (Rheodyne) with a 5 µl loop. The column (1 mm × 250 mm, MZ-Analysentechnik, Mainz, Germany) was packed with Spherisorb-NH_2 (Phase Separation, England), 3 µm particle size. The guard column (1 mm × 20 mm) was filled with the same material.

The flow-rate and temperature were 88 µl/min and 20°C, respectively. The separation of gangliosides is based on the method of Gazotti et al. (20). The solvents for gradient elution were solvent A: acetonitrile–5 mM phosphate buffer, pH 5.6 (83:17); solvent B: acetonitrile–20 mM phosphate buffer, pH 5.6 (1:1). The separation was performed with programmed gradient elution starting with solvent A for 6.4 min, followed by a linear gradient to 93% solvent A, 7% solvent B for 4 min, by a linear gradient to 77% solvent A, 23% solvent B for 37.6 min, and finally by a linear gradient to 100% solvent B for 40 min. The elution was monitored at 197 nm. The analytical data were processed using the LAB CALC software (Galactic Industries Corp., Salem, NH). The separation was completed within 90 min. For regeneration the column was washed for 20 min with solvent B and re-equilibrated for 20 min with solvent A.

RESULTS AND DISCUSSION

Separation of gangliosides

Gazzotti et al. (20) described an effective separation of gangliosides. To separate GM3 to GQ1b in an adequate time, we applied the 3 µm NH_2-Spherisorb phase and developed the elution gradient described below. Gangliosides are separated according to their sialic acid content (**Fig. 1**). This separation effect is presumably caused by the weak anionic exchange properties of the NH_2-phase in the presence of the phosphate buffer. There is also an efficient separation of gangliosides with an equal number of sialic acid residues. This is caused mainly by the polarity of the gangliosides such as the monosialogangliosides GM3, GM2, and GM1. Structural isomers like GD1a and GD1b are also separated with good resolution, but there is only an incomplete separation of gangliosides that differ from each other only in their fatty acid or long-chain base structure. The retention times of the gangliosides shown in Fig. 1 are reproducible with a maximum standard deviation of 1.5% (n = 8).

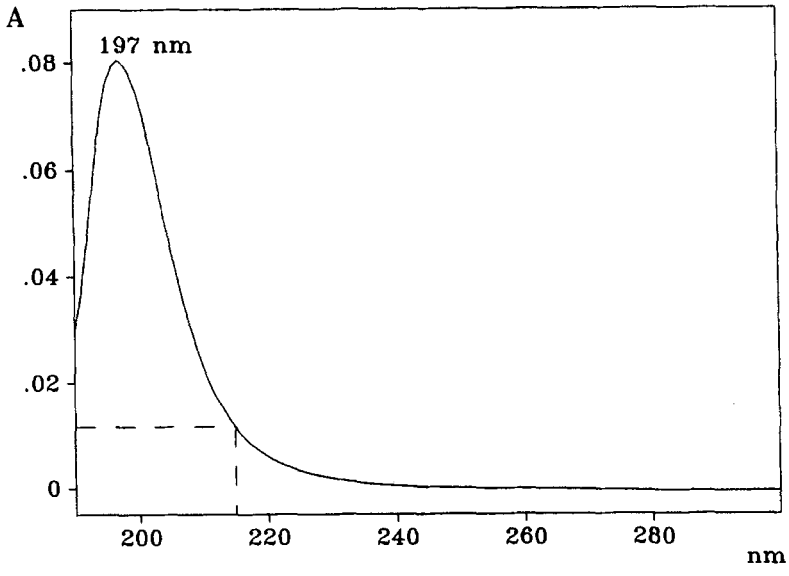

Fig. 2. UV absorption spectrum of GD1a. The spectrum was recorded during the HPLC separation of GD1a with the ABI 1000S diode array detector. The absorption at 215 nm is marked by a dashed line.

TABLE 1. Molar response and relative molar response (GM3 as 1) of gangliosides compared with chromophore content

Ganglioside	Molar Response (area/nmol)	Relative Molar Response (this work)	Relative Molar Response (Gazzotti et al. (20))	Number of N-acetyl Groups (sialic acid)	Number of N-acetyl Groups (GalNAc)	Number of N-acyl Groups
GM3	0.0537	1	1	1	0	1
GM2	0.1016	1.89	1.05	1	1	1
GM1	0.0946	1.76	1.13	1	1	1
GD3	0.0807	1.5	n.d.	2	0	1
GD1a	0.1222	2.28	1.86	2	1	1
GD1b	0.1245	2.32	1.94	2	1	1
GT1b	0.1432	2.67	2.80	3	1	1
GQ1b	0.1604	2.99	3.55	4	1	1

We bypassed the difficulties described by Gazzotti et al. (20) regarding baseline stability of the 195 nm absorption by applying a phosphate buffer of highest purity (from Fluka, Neu-Ulm, Germany), used for capillary electrophoresis. Thus it is possible to generate baseline-corrected chromatograms.

Sensitivity

The UV absorbance of the gangliosides has its maximum of about 197 nm (**Fig. 2**), with only a 1 nm deviation. It differs for standard gangliosides used here, from 196.5 to 197.1 nm. Recording the absorbance at 197 nm is therefore mandatory for maximal sensitivity. The absorbance at 215 nm drops to only 16% of the maximum. The amide bonds in the molecule are the main chromophores. The $\pi \rightarrow \pi^*$ transition of the related peptide bond in dipeptides has a molar absorption coefficient between 6820 and 8100 at an absorption maximum between 188 and 192 nm depending on the substituents (24). Gangliosides have three different types of amide bonds, the N-acyl bond of the ceramide moiety and the N-acetyl bonds of sialic acid and N-acetylgalactosamine (GalNAc), respectively. In **Table 1** the molar responses of the gangliosides are compared with the content of the different types of amide bonds in the molecules. The data indicate that not only the number of sialic acid residues is essentially responsible for the absorption as described by Gazzotti et al. (20), but also that of the ceramide N-acyl bond and the N-acetyl bond in GalNAc. The contribution of the ceramide N-acyl bond results in the molar response of the monosialoganglioside GM3 which has twice the value of an added sialic acid, e.g., from GM1 to GD1a. The contribution of the N-acetyl bond of GalNAc is clearly indicated by the increase of absorbance from GM3 to GM2 or from GD3 to GD1b. The increase in absorbance caused by N-acetyl bonds in sialic acid is not linear possibly because of quenching effects. Nevertheless, for gangliosides with known structures, it is possible to estimate their molar responses.

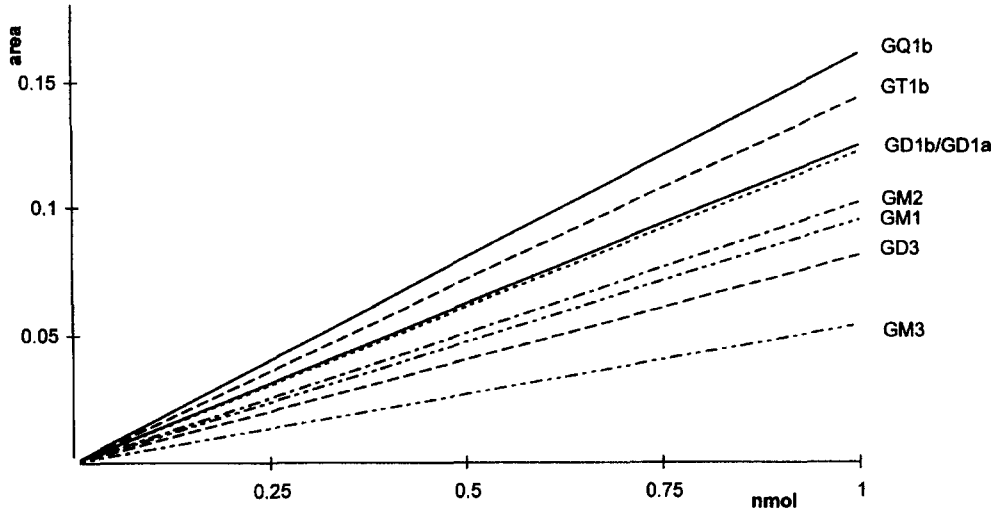

Fig. 3. Correlation of absorbance response and concentration of ganglioside species. The correlation coefficient r for every ganglioside is >0.992.

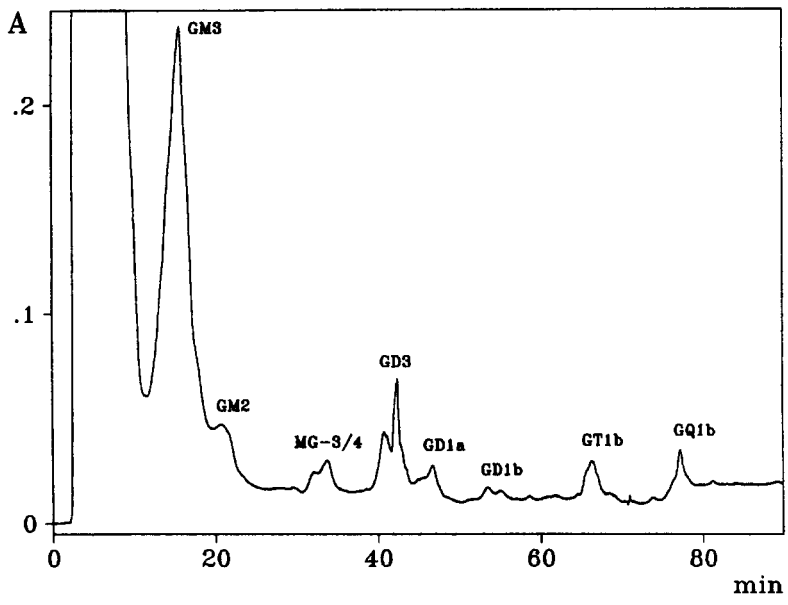

Fig. 4. HPLC separation of gangliosides from serum of a healthy human female. The gangliosides were prepared from 2 ml of serum. Gangliosides from 0.83 ml serum were applied to the HPLC. The concentrations of gangliosides in nmol/ml serum were: GM3 8.3(75.1), GM2 0.14(1.3), MG-3/4 (according to Kundu et al. (27)) 0.26(2.4), GD3 0.66(11.9), GD1a 0.07(1.3), GD1b 0.06(1.1), GT1b 0.11(3.0) and GQ1b 0.11(4.0). Percent lipid-bound sialic acid is given in brackets.

The lowest limit of detection (triple baseline noise) for all gangliosides lies in the range of 4 pmoles for GM3 and even lower for gangliosides with higher absorbance. The lowest amount of gangliosides that could be evaluated quantitatively (peaks 6-fold over baseline noise) is in the range from 8 pmol for GM3 to 2 pmol for GT1b. The response is linear up to 1 nmol (**Fig. 3**). Compared to hitherto published HPLC data, this is a 50-fold increase in sensitivity of the detection of non-derivatized gangliosides. The sensitivity is also superior to scanning densitometry of ganglioside bands in HPTLC stained with resorcinol where the lowest detection limit is between 10–25 pmol (25, 26) and about 10-fold better than the visible proof of HPTLC plates.

Sample preparation

The isolation of minute amounts of gangliosides from sources with a low ganglioside content requires a purification method that separates all UV-sensitive impurities of the samples prior to HPLC analysis. For the isolation of gangliosides from biological sources, we started with the partition method using diisopropylether/1-butanol/50 mM aqueous NaCl according to Ladisch and Gillard (22), who were able to obtain

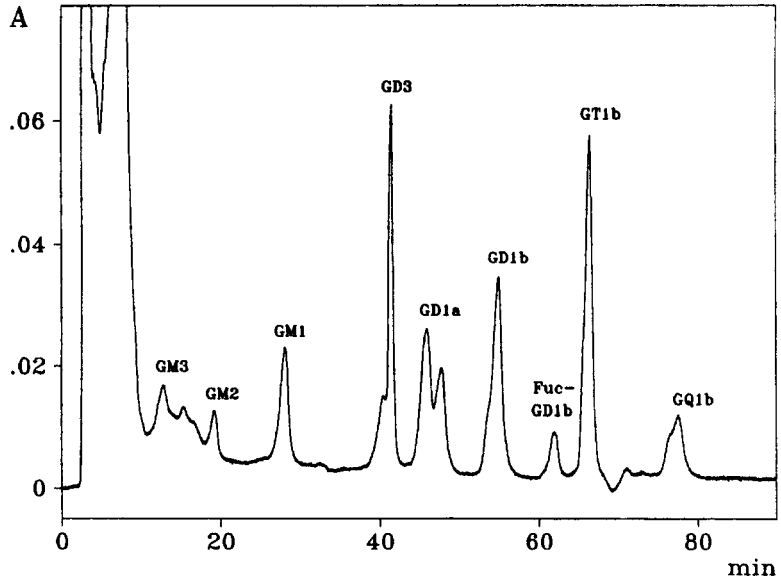

Fig. 5. HPLC separation of gangliosides isolated from human oligodendroglioma. The gangliosides were prepared from 19.1 mg (dry weight) of a human oligodendroglioma type II. An aliquot (1/19) of the ganglioside mixture was applied to the HPLC. The concentrations of gangliosides in nmol/mg dry weight were: GM3 0.27(8.1), GM2 0.04(1.2), GM1 0.15(4.4), GD3 0.35(21.0), GD1a 0.31(18.7), GD1b 0.26(15.8), Fuc-GD1b 0.04(2.6), GT1b 0.23(21.2) and GQ1b 0.06(7.1). Percent lipid-bound sialic acid is given in brackets.

sufficient gangliosides even from 1 ml of serum for HPTLC analysis with resorcinol staining. HPLC analysis of these samples detects impurities that cannot be seen on HPTLC plates because they are insensitive to resorcinol staining. Therefore we used, with two minor modifications, the method of Senn et al. (23), who expanded the method by an anion exchange chromatographic step to remove neutral glycosphingolipids. First, we replaced the Sep-Pak C_{18} cartridges by Sep-Pak C_8 cartridges, because they bind more hydrophobic gangliosides equally but can be eluted more easily. Two ml of serum instead of 14 ml was sufficient and tolerable for clinical studies. The recovery of gangliosides from this small volume is in the range of 70–80% as was examined with specimens spiked with bovine brain gangliosides and ^{14}C-radiolabeled GM1 (data not shown). The loss is nonspecific and approximately 1–2% in every step, due to nonspecific binding to the plastic and glass materials used. Higher losses occur only in the partition of GM3 (about 10%) which has also been described by Senn et al. (23). The sensitive HPLC analysis also detects an incomplete separation of phospholipids and cerebrosulfatides during the partition. Both negatively charged lipids elute at the beginning of the chromatogram and the resolution with respect to GM3 can be incomplete.

A typical HPLC chromatogram of serum gangliosides from a healthy human is seen in **Fig. 4.** The gangliosides were solely identified on the basis of their retention time and by comparison with the work of Kundu et al. (27). Because overlapping of gangliosides may exist (28), the identification is tentative and must be verified by further methods. This feature is shared with the standard HPTLC analysis of ganglioside mixtures. Additionally, the HPLC separation herein described provides intact ganglioside species suitable for structural analysis. The calculated total content of 11.1 nmol lipid bound sialic acid per ml serum is in the range that is described by others for serum or plasma (23, 29), except one (30) who has given a higher value than this work. The percent distribution is different from the published data. GM3, the main component, has a higher value and all the other components are below 10% total lipid bound sialic acid. The different evaluation methods may be responsible for the discrepancies.

We were able to isolate the gangliosides from brain tumor samples of only 2–4 mg (dry weight). As an example we present the HPLC analysis of a human oligodendroglioma specimen (**Fig. 5**). The ganglioside pattern is different and the concentration of lipid bound sialic acid is higher than in an oligodendroglioma examined by Shinoura et al. (31). In both cases GD3 has the highest concentration. As in the case of the serum gangliosides, the identification of peaks is tentative and based on the retention times. The existance of lacto and neolacto series gangliosides in glial tumors has been described (9) and therefore the structure of the fractionated gangliosides must be confirmed by appropriate methods.

We present here a useful and very efficient micromethod for the isolation and analysis of minute amounts of gangliosides, e.g., from serum samples or from minute amounts of tumor specimens with a low concentration of gangliosides. The great advantage of the HPLC procedure described here over HPTLC is that native non-derivatized gangliosides are separated, and can be analyzed further by RP-HPLC, GLC, and MS.

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