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Determination of gangliosides as 2,4-dinitrophenylhydrazides by high-performance liquid chromatography

Katsumi MIYAZAKI,*†§ Nobuyuki OKAMURA,*†‖ Yasuo KISHIMOTO*†¶ and Yuan Chuan LEE‡
*John F. Kennedy Institute, †Department of Neurology and ‡Department of Biology, Johns Hopkins University, Baltimore, MD 21205, U.S.A.

A specific, sensitive and easily performed method for the determination of gangliosides in tissue was developed. After removal of water-soluble compounds, total lipids were extracted from tissue and then treated with 2,4-dinitrophenylhydrazine hydrochloride and dicyclohexylcarbodi-imide in dimethylformamide at 0 °C to form ganglioside hydrazides. After removal of excess reagents by column chromatography on silicic acid, the ganglioside 2,4-dinitrophenylhydrazides were eluted from the column and analysed by h.p.l.c. with the use of a silica-gel normal-phase column eluted with an isocratic chloroform/methanol/water/acetic acid system. The addition of $CaCl_2$ improved the separation of G_{M3} ganglioside containing *N*-acetylneuraminic acid from that containing *N*-glycollylneuraminic acid. 2,4-Dinitrophenylhydrazide peaks were measured by the absorbance at 342 nm. Quantification of G_{M3}, G_{M2}, G_{M1}, G_{D1a}, G_{D1b}, G_{T1b} and L_{M1} gangliosides was linear in a range 0.02–1.6 nmol. G_{M4}, G_{D3}, G_{T1a} and G_{Q1b} gangliosides also yielded distinct peaks, although the range of linearity was not examined. This method was applied to the analysis of the total lipids of rat brain and hepatocytes.

INTRODUCTION

Gangliosides are glycosphingolipids containing one or more *N*-acetyl- or *N*-glycollyl-neuraminic acid (sialic acid) residues as part of the carbohydrate chain. They are abundant in the central nervous system, especially in grey matter, but smaller amounts are also widely distributed in the plasma membranes of a variety of tissue cells (Ledeen & Yu, 1976; Sweeley & Siddiqui, 1977; Ledeen, 1983). Because of their complexity in isomeric composition, distribution pattern in tissues and unique amphipathic and ionic structures, gangliosides are thought to be important in various cellular functions. For example, it has been suggested that gangliosides are active as hormone receptors, neurotransmitters and neurotropic factors, and in cell recognition, cell-to-cell interaction and ion transport (Ando, 1983; Rahmann, 1983; Svennerholm, 1983). Despite these implications, definitive evidence proving ganglioside involvement in these cell functions has been difficult to obtain. A simple analytical technique that is sufficiently specific and sensitive for the identification and determination of individual gangliosides would be highly desirable for establishing the roles of gangliosides in various biological structures and functions.

Distribution of individual gangliosides has largely been studied by t.l.c. separation followed by densitometry (Ando *et al.*, 1978; Mullin *et al.*, 1984) or by g.l.c. (Yu & Ledeen, 1970). Several methods for ganglioside determination by h.p.l.c. have also been developed. Some methods assess gangliosides without formation of derivatives (Tjaden *et al.*, 1977; Kundu & Scott, 1982; Gazzotti *et al.*, 1984), whereas others involve preliminary treatments such as perbenzoylation (Bremer *et al.*, 1979; Ullman & McCluer, 1985), conversion into *p*-bromophenacyl derivatives (Nakabayashi *et al.*, 1984), hydrolytic removal of sialic acid followed by perbenzoylation (Lee *et al.*, 1982), or ozonolysis followed by reaction with *p*-nitrobenzyloxyamine (Traylor *et al.*, 1983). Although derivative formation before h.p.l.c. provides increased sensitivity, the above methods require lengthy steps for purification of the ganglioside before preparation of the derivative. These cumbersome pre-purification steps, though necessary for removal of interfering materials, may introduce quantification errors in the procedure. The high-performance t.l.c. densitometric determination of gangliosides currently in wide use is also subject to interference (Ando *et al.*, 1978). To overcome these problems, we have developed a simple method, which is specific and sensitive, for the determination of gangliosides in animal tissue.

EXPERIMENTAL

Materials

Silica Gel 60 extra pure, Fractogel and Silica Gel 60 t.l.c. plates were obtained from EM Science, Silica Gel G was from E. Merck, and Unisil was from Clarkson Chemical Co. 2,4-Dinitrophenylhydrazine, *NN'*-dicyclohexylcarbodi-imide and methanolic HCl were purchased from Sigma Chemical Co., Aldrich Chemical Co. and Supelco respectively. Horse blood was purchased from Pel-Freeze, and calf and chicken brains were obtained from local slaughterhouses. All other common chemicals and solvents were supplied by local distributors.

Abbreviations used: the nomenclature for gangliosides is in accordance with Svennerholm (1963).

§ Present address: Department of Pharmacy, Hokkaido University Hospital, Sapporo 060, Japan
‖ Present address: Faculty of Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729-02, Japan.
¶ To whom reprint requests and correspondence should be addressed, at: John F. Kennedy Institute, 707 North Broadway, Baltimore, MD 21205, U.S.A.

Preparation of ganglioside standards

Calf brain (2.13 kg) was homogenized in 10 vol. of chloroform/methanol (1:1, v/v) for 30 min and filtered. The filtrate, after the addition of more chloroform, was subjected to partitioning by the procedure of Folch *et al.* (1957) with 5% (w/v) NaCl. Organic solvents from the upper phase were removed with a rotary evaporator, and the remaining aqueous solution was dialysed and freeze-dried. The residue (7.38 g) was extracted with 1.3 litres of boiling methanol, and the extract was evaporated to dryness in a rotary evaporator. The residue was taken up in a small volume of chloroform/methanol (7:1, v/v) and placed on a silica-gel column containing 700 g of Silica Gel 60 extra pure (70–230 mesh). The column was eluted with 17.5 litres each of chloroform/methanol/water (7:3:0.5, by vol.), chloroform/methanol/water (6:4:1, by vol.) and chloroform/methanol (1:1, v/v). Gangliosides in the effluent were monitored by t.l.c. on a Silica Gel 60 plate with chloroform/methanol/0.2% $CaCl_2$ (4:3:0.7, by vol.) as the developing solvent followed by detection with resorcinol/HCl reagent (Svennerholm, 1957). All gangliosides were recovered in the second fraction. After removal of the solvent, the residue from the second fraction weighed approx. 3.4 g and was further fractionated by preparative high-pressure column chromatography on 200 g of Silica Gel G with a Jobin–Yvon Chromatospac Prep 100 apparatus. The column was eluted with 4 litres of chloroform/methanol/water (6:4:0.9, by vol.) followed by 4 litres of chloroform/methanol/water (4:4:1, by vol.). Fractions (25 ml) were collected, and those containing the individual gangliosides were pooled separately, dialysed and freeze-dried. Final yields were: G_{M1}, 276 mg; G_{D1a}, 794 mg; G_{D1b}, 34 mg; G_{T1b}, 38.4 mg. Each ganglioside showed a single spot on examination by t.l.c. on a Silica Gel 60 plate developed with chloroform/methanol/0.2% $CaCl_2$ (4:3:0.7, by vol.) and detected with resorcinol/HCl reagent.

Haematoside (G_{M3} containing *N*-glycollylneuraminic acid) was isolated from erythrocytes prepared from 2 litres of horse blood (purchased from Pel-Freeze). The erythrocytes were haemolysed and lipids were extracted from the stroma as described by Yamakawa *et al.* (1960). The total lipids were dissolved in a minimum volume of chloroform/methanol (2:1, v/v), mixed with enough Celite to form a cake, and then the solvent was evaporated to dryness *in vacuo*. The lipid-coated Celite was then suspended in chloroform, and the mixture was placed on top of a column containing 300 g of Silica Gel 60 extra pure in the same solvent. The column was eluted with 1 litre each of chloroform, chloroform/methanol (20:1, v/v), chloroform/methanol/water (7:3:0.3, by vol.) and chloroform/methanol/water (7:3:0.4, by vol.), and finally with 3 litres of chloroform/methanol (1:1, v/v). T.l.c. examination showed that all the haematoside was recovered in the final eluate. The chloroform/methanol (1:1, v/v) eluate was then evaporated to dryness and again subjected to Silica Gel 60 (100 g) column chromatography. The column was eluted isocratically with chloroform/methanol/water (7:3:0.5, by vol.), the fractions containing haematoside were pooled, and the solvent was removed. The residue obtained was finally purified by column chromatography on 10 g of Florisil (70–100 mesh; Fisher Scientific Co.) by eluting isocratically with methanol. The fractions containing pure haematoside were pooled and the solvent was evaporated. The residue weighed 75.2 mg.

G_{M4}, G_{M3} containing *N*-acetylneuraminic acid, and G_{D3} gangliosides were isolated from chicken brains. Total lipids were extracted as described above and fractionated by ion-exchange chromatography on a column of Fractogel TSK DEAE-650S. The conditions for chromatography were essentially similar to the method described by Ledeen & Yu (1978). Individual gangliosides were finally purified by preparative t.l.c. L_{M1}, G_{T1a}, G_{T1b} and G_{Q1b} gangliosides from human brain were generously given by Dr. S. Ando (Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan) and G_{M2} ganglioside from a Tay–Sachs-disease patient's brain was kindly supplied by Dr. Y. Hirabayashi (Shizuoka College of Pharmacy, Shizuoka, Japan).

Concentrations of ganglioside were determined by colorimetric assay as described by Svennerholm (1957) and modified by Miettinen & Takki-Luukkainen (1959).

Preparation of 2,4-dinitrophenylhydrazine hydrochloride

To a suspension of 5 g of dinitrophenylhydrazine in 50 ml of chloroform was added 125 ml of methanolic 1.5 M-HCl with vigorous stirring until all the red crystals of dinitrophenylhydrazine had disappeared. The reaction mixture was evaporated to dryness and then recrystallized from chloroform/methanol (5:1, v/v). Yellow crystals of 2,4-dinitrophenylhydrazine hydrochloride were obtained in almost quantitative yield.

Formation of ganglioside 2,4-dinitrophenylhydrazide derivatives

Tissue was homogenized in 10 vol. or more of water and centrifuged at 12000 *g* for 30 min. The pellet was mixed with 20 vol. of chloroform/methanol (1:1, v/v), and heated, with occasional shaking, at 70 °C for 20 min. After centrifugation at 900 *g* for 10 min, the insoluble material was extracted again with 10 vol. of the same solvent under identical conditions. The extracts were combined and then evaporated to dryness to obtain the total lipids. When the ganglioside concentration was extremely low, such as in hepatocytes or serum, an unidentified broad peak was sometimes observed on the h.p.l.c. chromatogram. Although the ganglioside peaks were clearly identified above the baseline area, it could be eliminated by passing the total lipid extract through a silica-gel column. The total lipid dissolved in a small volume of chloroform/methanol (1:4, v/v) was placed on a column containing 0.2 g of Unisil (100–200 mesh) and eluted further with a total of 6 ml of the same solvent. The eluent was evaporated to dryness.

A sample containing up to 5 mg of total lipids or a partially purified fraction was dried over P_2O_5 in an evacuated desiccator for at least 1 h and mixed with 60 μl of a solution of 0.1 g of dinitrophenylhydrazine hydrochloride in 3 ml of dimethylformamide. To the mixture was added 250 μl of a solution of 10 g of *NN'*-dicyclohexylcarbodi-imide in 3.5 ml of dimethylformamide, and the mixture was agitated until complete solution was obtained. This mixture was then immediately placed on ice and left to stand for 1 h, then diluted with 1.5 ml of chloroform and applied to 0.2 g of Unisil packed in a disposable glass Pasteur pipette by the use of chloroform. The column was rinsed with 20 ml of chloroform/methanol (15:1, v/v) to remove unused phenylhydrazine and most of the other lipids, and the

dinitrophenylhydrazide derivatives of gangliosides were eluted with 5 ml of methanol. The methanol eluate was evaporated to dryness, dissolved in a known small volume of chloroform/methanol (3:1, v/v), and then a portion was injected into the h.p.l.c. chromatograph.

H.p.l.c. analysis of ganglioside 2,4-dinitrophenylhydrazides

Two h.p.l.c. systems were used to obtain the results shown here. One consisted of two model 6000A pumps (Waters Associates) and a model 660 solvent programmer, with either a model SF 770 Spectroflow monitor (Schoeffel Instruments Corp.) or a model 440 Absorption Monitor (Waters Associates) for peak detection. A model 8500 mixer (Spectra-Physics) was placed between the pumps and the injector. The second h.p.l.c. system consisted of a model LC-4A system (Shimadzu) with a SPD-2AS spectrophotometer (Shimadzu). Both systems included a model 7125 sample injector (Rheodyne) with a 5 μl or 20 μl loop. An RCM-100 Radial Compression Module with a 0.8 cm × 10 cm Resolve Si 5 μ Spherical Silica column (Waters Associates) was used in both systems. Three variations of the h.p.l.c. were developed, involving different portions of two solvent mixtures and slightly different conditions as shown in Table 1, to obtain the complete range of gangliosides. Peaks were detected by measuring the absorbance at 342 nm with a sensitivity of 0.02 *A* unit/full scale. The peaks were integrated by use of an Apple II+ computer. An interface and software program (Chromatochart) were purchased from Interactive Microware and used for integration.

RESULTS AND DISCUSSION

Identification of ganglioside 2,4-dinitrophenylhydrazides

The 2,4-dinitrophenylhydrazide of standard G_{M1} ganglioside was prepared, and the product was fractionated on a column of Unisil as described in the Experimental section. The 2,4-dinitrophenylhydrazide was further purified by preparative t.l.c. on a Silica Gel G plate with chloroform/methanol/0.2% $CaCl_2$/acetic acid (7:3:0.5:0.2, by vol.). The yellow band obtained was scraped off and the pure 2,4-dinitrophenylhydrazide of G_{M1} ganglioside was eluted with methanol. This material gave a single yellow spot (R_F 0.30) on t.l.c. examination under the conditions described above. When the plate was sprayed with 10% (v/v) H_2SO_4 and heated, the spot first turned purple and then green; no other spots appeared.

The G_{M1} ganglioside dinitrophenylhydrazide thus purified has intense absorption in the 415–420 nm region in neutral chloroform/methanol (2:1, v/v). The molar absorption coefficient (ϵ) at 417 mm is calculated to be 8300 $M^{-1} \cdot cm^{-1}$. On adding acetic acid, this peak shifts to the lower-wavelength region (maximum 342 nm) with a molar absorption coefficient of 7000 $M^{-1} \cdot cm^{-1}$. Verification of the structure of the dinitrophenylhydrazide of G_{M1} ganglioside was obtained by the fast-atom-bombardment mass spectrum, which showed molecular ion $[M+Na]^+$ of 1749 (ceramide moiety of G_{M1} ganglioside composed of stearic acid and sphingosine) and 1777 (ceramide composed of stearic acid and C_{20}-sphingosine), together with other smaller ions attributable to sphingoid base and fatty acid homologues. The spectrum was obtained as described previously (Shigematsu *et al.*, 1982).

Quantitative determination of individual gangliosides as 2,4-dinitrophenylhydrazides by h.p.l.c.

Formation of the 2,4-dinitrophenylhydrazide derivatives of all standard gangliosides G_{M3}, G_{M2}, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} under the conditions described in the Experimental section was rapid and quantitative. A large excess of 2,4-dinitrophenylhydrazine hydrochloride and dicyclohexylcarbodi-imide was necessary for a complete reaction with higher gangliosides. When examined by t.l.c. on Silica Gel G plates with chloroform/methanol/0.2% $CaCl_2$ (7:3:0.5, by vol.), each ganglioside derivative, except that of G_{T1b} ganglioside, showed a single yellow spot in addition to a large spot of unused reagent on the solvent front. Spraying with 10% H_2SO_4 followed by heating showed only the ganglioside derivative spot. Under these conditions, as well as all other conditions examined, G_{T1b} ganglioside yielded two spots. The ratio of intensity of the two spots did not change on changing the reaction conditions. The detailed structures of these two compounds have not, as yet, been elucidated.

While the silica column was new, a neutral chloroform/methanol/water system provided excellent separation of individual ganglioside derivatives, each yielding a separate peak. However, the column deteriorated quickly and the sharpness of each peak was lost. We found that the addition of acetic acid to the eluting solvents prolonged the efficiency of the column at least for several months, although it shifted the absorption maximum from 417 to 342 nm.

Although gradient elution of the column with the chloroform/methanol/water/acetic acid system resulted in the separation of most ganglioside derivatives, except for those of G_{D1b} and of G_{T1b} gangliosides, which nearly completely overlapped (chromatogram not shown). In addition, the gradient elution was time-consuming and reproducible values for retention time were difficult to

Table 1. Conditions of elution for h.p.l.c. analysis of ganglioside 2,4-dinitrophenylhydrazide derivatives

Various ganglioside mixtures were analysed by h.p.l.c. by use of isocratic elution with one of the solvent mixtures described below. A special silica column was used. Solvent A consisted of chloroform/methanol/water (100:7:0.6, by vol.) containing 0.5 ml of acetic acid/l. Solvent B consisted of chloroform/methanol/water (7:7:0.6, by vol.) containing 0.5 ml of acetic acid/l.

Method	Solvent	Flow rate (ml/min)	For analysis of
1	65% A/35% B	1.0	Lower homologues typically present in extraneural tissues
2	35% A/65% B	0.5	Higher homologues typically present in nervous systems
3	42% A/58% B containing 10 ml of 0.1 M-$CaCl_2$/l	1.0	Separation of G_{M3} containing *N*-acetylneuraminic acid from that containing *N*-glycollylneuraminic acid

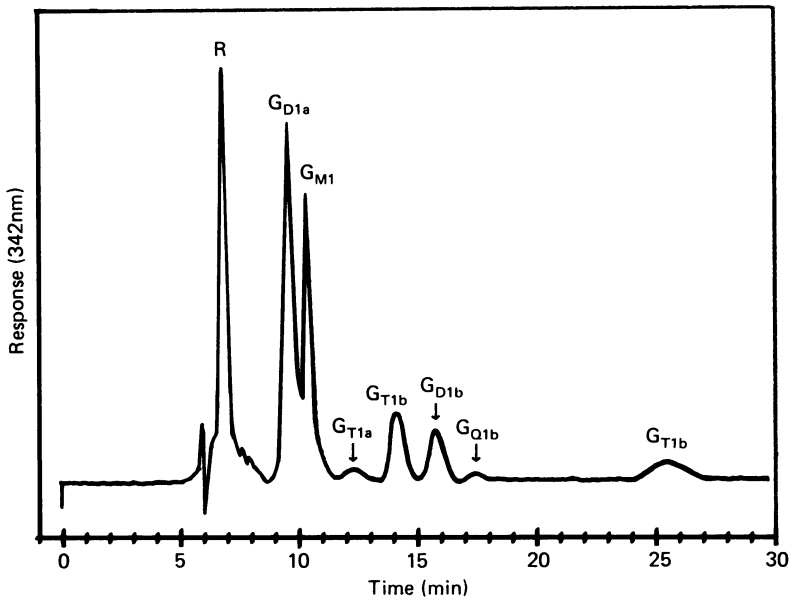

Fig. 1. H.p.l.c. chromatogram of 2,4-dinitrophenylhydrazide derivatives of total lipids of rat brain obtained by isocratic elution (method 2)

The amount of the sample injected into the h.p.l.c. chromatograph was equivalent to 0.53 mg of fresh brain from one 30-day-old Sprague–Dawley CD rat (Charles River Breeding). Details of h.p.l.c. conditions are described in the Experimental section.

obtain, especially for gangliosides with fewer sialic acid residues. Therefore we developed two simple isocratic elution methods (1 and 2) that gave shorter and more reliable retention times. Method 2 separated the derivatives of all gangliosides normally enriched in mammalian brain (shown in Fig. 1). L_{M1} ganglioside derivative, if present, showed a well-separated peak at 13 min of retention time. The G_{M3} ganglioside derivative, however, emerged in the void volume and could not be separated from residual reagent peak under this condition. On the other hand, method 1 permitted the separation of the G_{M3} ganglioside derivative peak from the reagent peak (shown in Fig. 2), but was less suitable for quantification of derivatives of gangliosides with higher sialic acid content. We recommend method 1 for the sample containing derivatives of gangliosides of lower carbohydrate content, such as those of visceral organs or cells, and method 2 for samples containing derivatives of gangliosides of higher carbohydrate content, such as mammalian brain. A sample containing derivatives of gangliosides with both low and high sialic acid content could best be analysed by using both methods. Although the retention times are slightly different, peaks of G_{M3} ganglioside derivative containing *N*-acetylneuraminic acid cannot be separated from that containing *N*-glycollylneuraminic acid. We found, however, that they can be separated by using method 3, as shown in Fig. 3. With this elution method, derivatives of G_{M4}, G_{D3} and G_{M2} gangliosides can also be separated, as shown in Fig. 3.

With the use of method 1, G_{M3} and G_{M2} gangliosides yielded a linear response with 20 pmol to 1.4 nmol of product (Fig. 4*a*). Similarly, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} gangliosides were quantitatively analysed in the range 20 pmol to 1.6 nmol by using method 2 (Figs. 4*b* and 4*c*). In agreement with the observation on t.l.c., the derivative of G_{T1b} ganglioside from calf brain yielded two separate

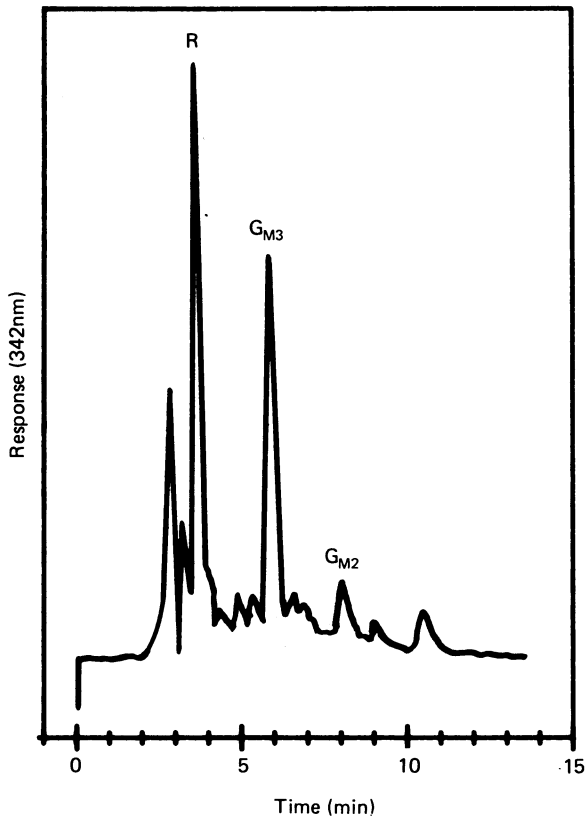

Fig. 2. H.p.l.c. chromatogram of 2,4-dinitrophenylhydrazide derivatives of rat liver hepatocyte lipids

An isocratic elution (method 1) was used. The amount of the product injected into the h.p.l.c. chromatograph was equivalent to 0.35 mg of protein content. Details of h.p.l.c. conditions are described in the Experimental section.

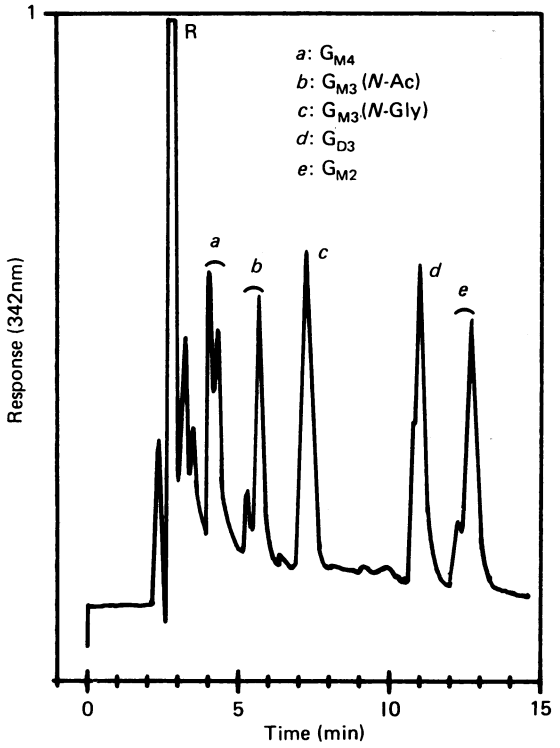

Fig. 3. H.p.l.c. chromatogram of 2,4-dinitrophenylhydrazides of a mixture of standard gangliosides with lower carbohydrate contents

Note the well-separated peaks of *N*-acetyl- and *N*-glycollyl-neuraminic acid isomers of G_{M3} gangliosides. Separate peaks or shoulder peaks in each isomer peaks may be due to different fatty acids. An isocratic elution (method 3) containing $CaCl_2$ was used. Details of h.p.l.c. conditions are described in the Experimental section.

peaks, the ratio of which was not altered by changing conditions for derivative formation. G_{T1b} ganglioside isolated from human brain also yielded the identical two peaks, but in a somewhat different ratio. The amount of this ganglioside was therefore calculated by adding the areas of those two peaks. Slopes, intercept points and correlation coefficients of each standard curve were calculated by linear-regression analysis and are presented in Table 2. These values indicate the high reliability of the present method. The absorbance at 342 nm is due to the dinitrophenylhydrazide group attached to each sialic acid residue of the gangliosides. Therefore the colour yield for sialic acids should be identical for all gangliosides. The slightly different slopes for the different ganglioside isomers may be due to variations in their peak shapes. Although the quantification of G_{T1a} and G_{Q1b} gangliosides was not tested because of the limited availability of the standard compounds, they showed single peaks at 12 min and 17.5 min respectively when eluted by method 2.

Evaluation of the method with tissues

Preliminary investigation with fresh rat brain showed a cluster of unexpected peaks near the G_{M3} ganglioside peak. Subsequent investigation established that these peaks were derived from amino acids. Therefore fresh tissues were first extracted with water to remove free amino acids before extraction of total lipids. Examples of h.p.l.c.

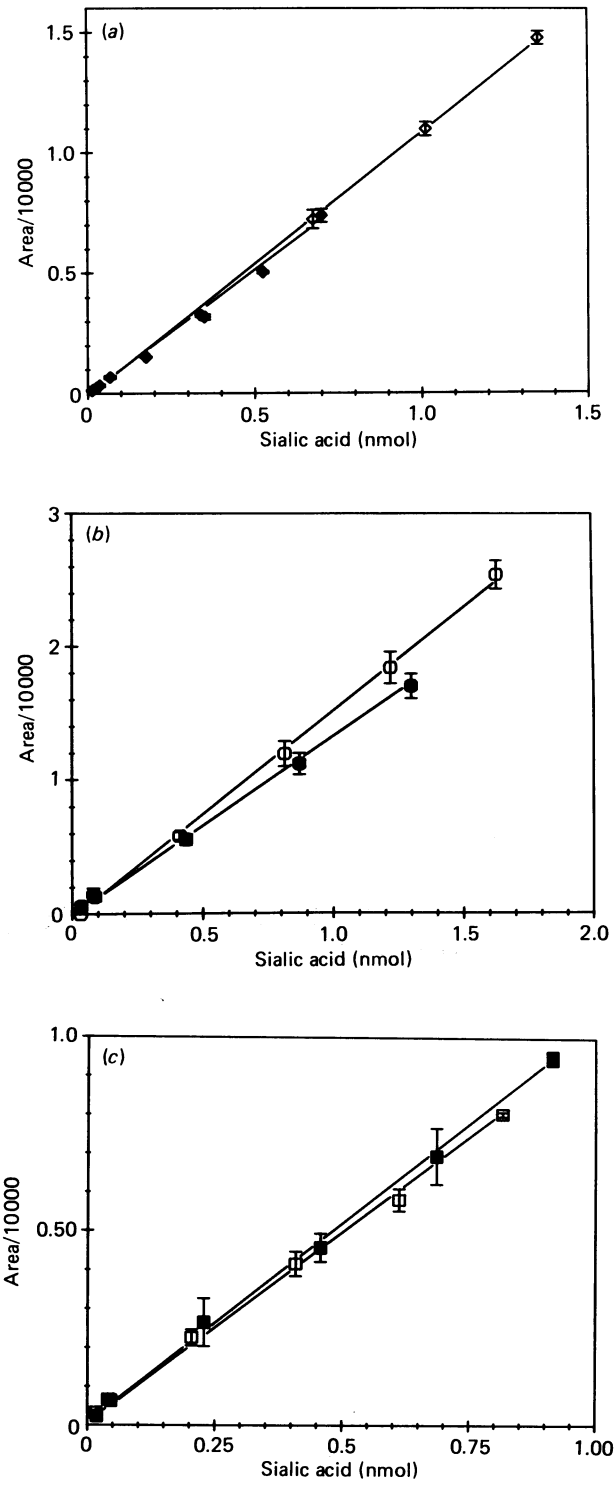

Fig. 4. Standard curves for determination of (*a*) G_{M3} (◇) and G_{M2} (◆) gangliosides, (*b*) G_{M1} (○) and G_{D1a} (●) gangliosides, and (*c*) G_{D1b} (□) and G_{T1b} (■) gangliosides

Various concentrations of these gangliosides in triplicate, ranging from 0.32 to 32.6 nmol, were permitted to react with 2,4-dinitrophenylhydrazine hydrochloride and dicyclohexylcarbodi-imide in dimethylformamide as described in the text, and one-twentieth of each reaction product was injected into the h.p.l.c. chromatograph. The values presented are means for three or four separate determinations with error bars indicating standard deviations.

Table 2. Calibration of detection of ganglioside 2,4-dinitrophenylhydrazides by h.p.l.c.

Values were calculated from data presented in Fig. 3 by linear-regression analysis.

Ganglioside	Slope (peak area/nmol of sialic acid)	Intercept point	Correlation coefficient
G_{M3}	11030	−172.2	0.9996
G_{M2}	10400	−157.3	0.9966
G_{M1}	15380	−119.7	0.9990
G_{D1a}	12890	+ 82.4	0.9998
G_{D1b}	9430	+243.6	0.9990
G_{T1b}	10110	+137.2	0.9990

chromatograms of rat brain lipids are shown in Fig. 1. Recovery of gangliosides during derivative formation and h.p.l.c. was examined by adding a mixture of standard gangliosides (G_{M3}, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}) to the water-washed whole-rat-brain particulate material. As shown in Table 3, the recovery of these gangliosides ranged from 93 to 101% throughout the procedure. Because of their carbohydrate content, gangliosides are known to be poorly extracted by organic solvents. We overcame this difficulty by extracting with chloroform/methanol at elevated temperatures. To verify the complete extraction of gangliosides, we compared the amounts of gangliosides extracted by our method with those obtained by the method developed and widely tested by Svennerholm & Fredman (1980). The results obtained by both methods agreed within 5% (detailed results not shown). Prior extraction of water-soluble materials and heating at 70 °C did not alter ganglioside quantification. This was confirmed by omitting tissue extraction by water or by heating a standard ganglioside mixture in chloroform/methanol.

Table 4 shows the amounts of individual gangliosides in young rat brain as determined by the present method. The values were compared with those previously published by various investigators. Although our values for G_{D1b} and G_{T1b} gangliosides agreed well with those published previously, the value for G_{D1a} ganglioside was in the upper high limit of previously published results. However, we obtained a 50–100% higher value for G_{M1} ganglioside, as shown in Table 4. Our value was

Table 4. Determination of rat brain gangliosides by h.p.l.c.

A young rat brain (Sprague–Dawley CD strain) weighing 1.68 g was homogenized in 10 ml of cold water, and portions (equivalent to 21.2 mg of brain) were analysed as described in the text. The data presented are means ± S.D. for five determinations.

Ganglioside	Sialic acid concentration (μmol/g of fresh brain)		
	This study	Ledeen & Yu (1976)*	Ando *et al.* (1978)
G_{M1}	0.74 ± 0.05	0.25–0.52	0.39
G_{D1a}	1.31 ± 0.04	0.75–1.30	0.83
G_{D1b}	0.45 ± 0.06	0.40–0.74	0.34
G_{T1b}	0.62 ± 0.05	0.55–1.04	0.65

* These values were calculated from data presented in the cited reference.

confirmed by repeated analysis with several other rat brains. Although the reason for this discrepancy should be further examined, the values obtained by previous workers were obtained with preparations that had undergone extensive purification steps. Therefore it is conceivable that some non-polar gangliosides were lost during partition, dialysis or the chromatographic steps.

Tissues containing relatively simpler gangliosides in much lower concentrations than in nervous tissues were examined next. These included rat hepatocytes (prepared from Sprague–Dawley rat liver in this laboratory based on the method of Seglen, 1976), a hybridoma of neuroblastoma and glioma NG 108 (provided by Dr. H. Singer) and mouse plasma (provided by Dr. A. Yeager). When total lipids of these tissues were subjected directly to the present procedure, a broad peak spanning nearly the whole area of ganglioside peaks was observed. Although the peaks of gangliosides, which showed on top of this broad peak, were clearly identifiable and measurable, we found that this impurity peak could be eliminated by first fractionating the total lipids by simple silica-gel chromatography. An example of a chromatogram obtained with the rat hepatocytes is shown in Fig. 2. The values for G_{M3} and G_{M2} gangliosides were shown to be 408 and 126 pmol/mg of protein respectively. Seyfried *et al.* (1978) reported the presence in human liver of 65.9 μg of lipid-bound sialic acid/g wet wt., which was almost

Table 3. Recovery of added gangliosides

A mixture of standard gangliosides was added to water-washed rat brain particulate material and their ganglioside values were compared with those in original particulate material and added standard mixtures.

Ganglioside	Rat brain lipids with added standard (nmol) (A)	Standard only (nmol) (B)	Rat brain lipids only (nmol) (C)	Added standard (nmol) ($A-C$)	Recovery of standard (%) $\frac{(A-C)}{B} \times 100$
G_{M3}	10.8	10.9	Not detected	10.8	99.1
G_{M1}	110.6 ± 5.2	34.9	78.1 ± 6.8	32.5	93.1
G_{D1a}	168.6 ± 3.2	30.3	138.4 ± 2.7	30.2	99.7
G_{D1b}	69.2 ± 1.7	21.3	47.8 ± 1.1	21.4	100.5
G_{T1b}	111.3 ± 3.2	44.8	66.1 ± 2.4	45.2	100.9

exclusively G_{M3} ganglioside. This value was equivalent to about 2 nmol/mg of protein and was significantly higher than the value that we obtained with rat hepatocytes. The G_{M2} ganglioside content in hepatocytes was much higher than that in human liver. Whether the difference is due to species or to other cells present in human liver requires further investigation.

Conclusion

Several advantages of the present method over previously published h.p.l.c. methods for ganglioside determination are as follows. First, total lipids can be taken directly for derivative formation and analysis by h.p.l.c. Instead of measuring the peak absorption in the u.v. range, the present method uses absorption at a higher wavelength (342 nm) to monitor the effluent. This decreases interference from contaminating material present in specimens. Secondly, since the 2,4-dinitrophenylhydrazide group attaches only to the carboxy group of the sialic acid moiety, the peak area is directly related to the number of sialic acid residues. Thirdly, the detection of peaks on h.p.l.c. in the higher-wavelength range allows the use of a wide choice of eluting solvents.

Because of the simplicity of the protocol for derivative formation, 20 or more total lipid extracts can be processed in a single day. The interference by amino acids, described above, was not expected, but was easily eliminated by removing the water-soluble compounds by homogenizing the tissue in water and centrifuging. However, small amounts of gangliosides in cytosol, reported to be present in brain (Sonnino *et al.*, 1979), will be lost by this procedure. Dialysis of total lipids or precipitation by $(NH_4)_2SO_4$ (as described by Sonnino *et al.*, 1979) could replace the water extraction. The removal of amino acids is not necessary for analysis of mammalian brain gangliosides because only a trace of GM_3 ganglioside is present. Nevertheless we found that the amounts of gangliosides in rat brain measured by the present method were virtually identical with and without the extra step of water extraction. We demonstrated linearity of measurement in the range 0.02–1.6 nmol for G_{M3}, G_{M2}, G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} gangliosides. In addition to the quantification of gangliosides, the present method should also provide a means for the isolation of minute quantities of these lipids for metabolic studies, similarly to the h.p.l.c. technique developed for cerebrosides and sulphatides (Yahara *et al.*, 1980; Shimomura *et al.*, 1984), other important glycosphingolipids in the nervous system.

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REFERENCES

Ando, S. (1983) Neurochem. Int. **5**, 507–537
Ando, S., Chang, N. C. & Yu, R. K. (1978) Anal. Biochem. **89**, 437–450
Bremer, G., Gross, S. K. & McCluer, R. H. (1979) J. Lipid Res. **20**, 1028–1035
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) J. Biol. Chem. **226**, 497–509
Gazzotti, G., Sonnino, S., Chidoni, R., Kirschner, G. & Tettamanti, G. (1984) J. Neurosci. Res. **12**, 179–192
Kundu, S. K. & Scott, B. D. (1982) J. Chromatogr. **232**, 19–27
Ledeen, R. W. (1983) Handb. Neurochem. 2nd Ed. **3**, 44–90
Ledeen, R. W. & Yu, R. K. (1976) in Glycolipid Methodology (Witting, L. A., ed.), pp. 187–214, American Oil Chemists' Society, Champaign
Ledeen, R. W. & Yu, R. K. (1978) Res. Methods Neurochem. **4**, 371–410
Lee, W. M. F., Westrick, M. A. & Bacher, B. A. (1982) Biochim. Biophys. Acta **712**, 498–504
Miettinen, T. & Takki-Luukkainen, I. T. (1959) Acta Chem. Scand. **13**, 856–858
Mullin, B. R., Poore, C. M. B. & Rupp, B. H. (1984) J. Chromatogr. **305**, 512–513
Nakabayashi, H., Iwamori, M. & Nagai, Y. (1984) J. Biochem. (Tokyo) **96**, 977–984
Rahmann, H. (1983) Neurochem. Int. **5**, 539–547
Seglen, P. O. (1976) Methods Cell Biol. **13**, 29–83
Seyfried, T. N., Ando, S. & Yu, R. K. (1978) J. Lipid Res. **19**, 538–543
Shigematsu, H., Okamura, N., Shimeno, H., Kishimoto, Y., Kan, L.-S. & Fenselau, C. (1982) J. Neurochem. **40**, 814–820
Shimomura, K., Yahara, S., Kishimoto, Y. & Benjamins, J. A. (1984) Biochim. Biophys. Acta **795**, 265–270
Sonnino, S., Chidoni, R., Marchesini, S. & Tettamanti, G. (1979) J. Neurochem. **33**, 117–121
Svennerholm, L. (1957) Biochim. Biophys. Acta **24**, 604–611
Svennerholm, L. (1963) J. Neurochem. **10**, 613–623
Svennerholm, L. (1983) Neurochem. Int. **5**, 549–552
Svennerholm, L. & Fredman, P. (1980) Biochim. Biophys. Acta **617**, 97–109
Sweeley, C. C. & Siddiqui, B. (1977) in The Glycoconjugates (Horowitz, M. I. & Pigman, W., eds.), vol. 1, pp. 459–540, Academic Press, New York
Tjaden, U. R., Krol, J. H., Van Hoeven, R. P., Oomen-Muelemans, E. P. M. & Emmelot, P. (1977) J. Chromatogr. **136**, 233–243
Traylor, T. D., Koontz, D. A. & Hogan, E. D. (1983) J. Chromatogr. **272**, 9–20
Ullman, M. D. & McCluer, R. H. (1985) J. Lipid Res. **26**, 501–506
Yahara, I., Singh, I. & Kishimoto, Y. (1980) Biochim. Biophys. Acta **619**, 177–185
Yamakawa, T., Irie, R. & Iwanaga, M. (1960) J. Biochem. (Tokyo) **48**, 490–507
Yu, R. K. & Ledeen, R. W. (1970) J. Lipid Res. **11**, 506–516

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