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SIMPLE MICRO-METHOD FOR THE ISOLATION OF GANGLIOSIDES BY REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

A simple and convenient technique has been developed for the isolation of gangliosides from small amounts of tissues or cells. A ganglioside fraction obtained by chromatography of the total lipid extract of DEAE-Sephadex was subjected to alkaline hydrolysis and salts and other non-lipid contaminants were removed by reversed-phase chromatography on a C_{18} Sep-Pak cartridge. The purified gangliosides were then obtained by chromatography on a small Iatrobeads or Unisil column. This procedure yields a quantitative recovery of gangliosides that are free of contaminants which interfere with thin-layer chromatographic analysis. The procedure was used for the quantitative isolation of gangliosides from human brain white matter and human erythrocytes.

INTRODUCTION

Current techniques for the separation of salts, sugars and other low-molecular-weight contaminants from gangliosides do not yield a consistently good recovery. Reversed-phase chromatography on octadecyl-coated silica gel has been used to separate molecular species of glycolipids [1, 2]. Williams and McCluer [3] recently used reversed-phase chromatography on C_{18} Sep-Pak cartridges to isolate gangliosides from Folch upper-phase fractions, but gangliosides isolated by this procedure contain contaminants that interfere with thin-layer chromatographic (TLC) analysis. In this report we describe a simple procedure for the quantitative isolation of pure gangliosides. This method combines the method of Ledeen et al. [4] with reversed-phase chromatography on a C_{18} Sep-Pak cartridge.

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EXPERIMENTAL

C_{18} Sep-Pak cartridges were obtained from Waters Assoc. (Milford, MA, U.S.A.). DEAE-Sephadex A-25 was purchased from Pharmacia (Piscataway, NJ, U.S.A.). DEAE-silica-gel (total capacity 150 μmol/g dry weight; pore diameter 200 Å; 120—200 mesh) was prepared as described previously [5, 6] and was also available commercially from Bio-Supports (Clifton, NJ, U.S.A.). Unisil (200—325 mesh) and Iatrobeads (6RS-8060) were procured from Clarkson Chemical Company (Williamsport, PA, U.S.A.) and Iatron Laboratory (Tokyo, Japan), respectively.

Human brain was obtained at autopsy from a patient without neurological disease and samples of human blood were obtained in acid—citrate—dextrose anticoagulant. The purified gangliosides used as standard components have been described previously [7, 8]. Tritiated beef brain ganglioside mixture was prepared by mild periodate oxidation followed by reduction with [^{3}H] $NaBH_4$ (specific activity 282 mCi/mmol; obtained from New England Nuclear, Boston, MA, U.S.A.) according to the procedure described by Veh et al. [9]. The specific activity of the tritiated beef brain ganglioside mixture was $2 \cdot 10^4$ cpm/μg sialic acid [10]. TLC—autoradiography revealed that all ganglioside species were labeled and unchanged in migratory rate. N-[^{14}C] Acetylneuraminic acid (specific activity 256 mCi/mmol) and [^{14}C] glucose (specific activity 59 mCi/mmol) were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). N-Acetylneuraminic acid was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.) and glucose from Fisher Scientific (Fairlawn, NJ, U.S.A.).

The ganglioside yield from each preparation was determined by gas—liquid chromatographic analysis of the lipid-bound sialic acid [11]. TLC was carried out on pre-coated silica gel 60 plates (E. Merck, Darmstadt, G.F.R.) with chloroform—methanol—water (55:45:10) containing 0.02% (w/v) of $CaCl_2 \cdot 2H_2O$ or chloroform—methanol—2.5 *N* ammonia solution (60:40:9) as solvent systems. Gangliosides were revealed by spraying the plate with resorcinol reagent [12] and heating the covered plate at 100°C for 10 min.

Lipid extraction from human brain white matter and human erythrocytes

A portion of human brain white matter (30 g, wet weight) was extracted three times with 10 volumes of chloroform—methanol (1:1) at room temperature. The total lipid obtained after evaporation was dissolved in 1200 ml of chloroform—methanol—water (30:60:8). Aliquots of 5, 10, 20, 40, 60 and 80 ml, which are equivalent to 0.125, 0.25, 0.5, 1.0, 1.5 and 2.0 g of white matter, respectively, were diluted to 100 ml with chloroform—methanol—water (30:60:8) and used for isolation of gangliosides as described below.

A 400-ml volume of freshly drawn blood in acid—citrate—dextrose anticoagulant was washed with phosphate-buffered saline (pH 7.3) and the packed erythrocytes (ca. 200 ml) were extracted with chloroform—methanol as described previously [8]. The total lipid was dissolved in 800 ml of chloroform—methanol—water (30:60:8). Aliquots of this solution equivalent to 0.5, 1.0, 5.0, 10 and 20 ml of packed erythrocytes were diluted to 100 ml with chloro-

form—methanol—water (30:60:8) and used for the isolation of gangliosides as described below.

Isolation of gangliosides from human brain white matter and human erythrocytes using a C_{18} Sep-Pak cartridge

The diluted lipid extract (100 ml) was applied to a DEAE-Sephadex (2 g, acetate form, dry weight) or DEAE-silica gel (10 g, acetate form, dry weight). After washing with 100 ml of chloroform—methanol—water (30:60:8) and 50 ml of methanol to remove the uncharged and zwitterionic lipids, acidic lipids were eluted with 50 ml of 0.2 *M* sodium acetate in methanol. The methanol was removed in a rotary evaporator and the residue was treated with 15 ml of 0.1 *N* sodium hydroxide in methanol and incubated at 37°C for 2 h to destroy the alkali-labile acidic phospholipids. The solvent was evaporated to dryness without heat and the residue was dissolved in about 50 ml of ice-cold water and neutralized to pH 4.5 by dropwise addition of 0.5 *N* hydrochloric acid. The solution was diluted to 115 ml with water to adjust the salt concentration to 0.1 *M*; this solution was then passed through a C_{18} Sep-Pak cartridge fitted tightly to a 25-ml glass disposable pipet at a flow-rate of approximately 1 ml/min under a slight positive pressure. [The cartridge was pre-washed with 25 ml of chloroform—methanol (1:2), 25 ml of methanol and 50 ml of water before use.] The cartridge was washed with 25 ml of water and the gangliosides were then eluted with 5 ml of methanol followed by 25 ml of chloroform—methanol (1:2). The cartridge can be used again after washing with methanol and equilibration with water (washing with methanol is necessary to remove chloroform from the cartridge).

Iatrobeads or Unisil column chromatography

The gangliosides eluted from the C_{18} Sep-Pak column were dried, dissolved in 2 ml of chloroform—methanol (85:15) and then chromatographed on an Iatrobeads [13] or Unisil [14] column to remove sulfatides and other colored impurities. For a 2-g column, 40 ml of chloroform—methanol (85:15) were used to remove impurities and gangliosides were quantitatively eluted with 40 ml of chloroform—methanol (1:2).

Isolation of gangliosides from human brain white matter and human erythrocytes using a dialysis method

The gangliosides from human brain white matter and human erythrocytes were isolated according to the procedure described previously [5, 14]. The method included DEAE-Sephadex column chromatography, treatment with base, dialysis against cold water and finally Unisil column chromatography.

Recovery of gangliosides from C_{18} Sep-Pak cartridge

Tritiated beef brain ganglioside mixture (1 μg of sialic acid, $2 \cdot 10^4$ cpm) was added to 100 ml of diluted lipid extract solution of human brain white matter (0.125—2.0 g wet weight) or human erythrocytes (0.5—20 ml of packed erythrocytes). The gangliosides were then isolated as described above and the amount of gangliosides recovered were measured.

Separation of gangliosides from glucose and sialic acid

[^{14}C] Glucose was dissolved in 25 ml of 0.1 *N* sodium chloride solution and allowed to pass through the C_{18} Sep-Pak cartridges as above. The cartridge was washed with 25 ml of water and the combined eluates (fraction 1) were counted to measure free glucose. The cartridge was then washed with methanol and chloroform—methanol as described above (fraction 2) and the percentage recovery of glucose was measured. Similarly, in separate experiments, N-[^{14}C]-acetylneuraminic acid was subjected to the separation procedure.

In another series of experiments, [^{14}C] glucose and N-[^{14}C] acetylneuraminic acid were separately mixed with tritiated beef brain ganglioside (1 μg of sialic acid, $5.3 \cdot 10^4$ cpm) and subjected to the same separation procedure as above. The percentage recoveries of glucose and N-acetylneuraminic acid in fraction 1 were 98% and 94%, respectively. Gangliosides were recovered from fraction 2 in almost quantitative yields (93—96% recovery; data not shown).

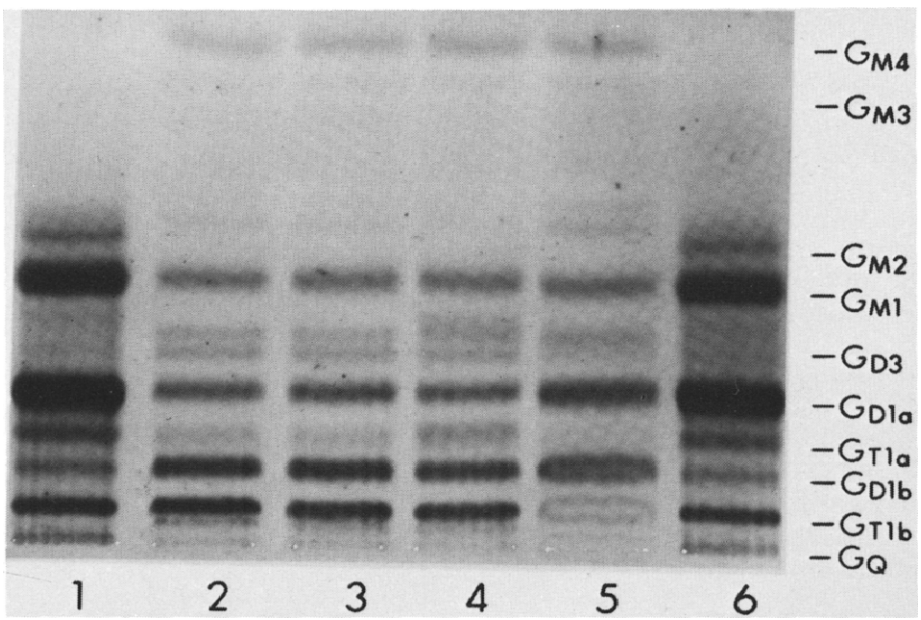

Fig. 1. Thin-layer chromatogram of gangliosides from normal human white matter: (1) and (6), standard beef brain ganglioside mixture; (2), white matter gangliosides by dialysis method; (3) and (4), white matter gangliosides by Sep-Pak method (overnight retention). Each of the lanes 2—5 contained 7 μg of sialic acid. Solvent system, chloroform—methanol—water (55:45:10) with 0.02% (w/v) of $CaCl_2 \cdot 2H_2O$. Plate, Merck pre-coated silica gel 60. All bands were purple after spraying by resorcinol reagent [12]. The ganglioside nomenclature of Svennerholm [15] is depicted: G_{M_4} = [NeuAc(α2-3)]Gal-Cer, G_{M_3} = [NeuAc(α2-3)]Gal(β1-4)Glc-Cer, G_{M_2} = GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer, G_{M_1} = Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer, G_{D_3} = [NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc-Cer, $G_{D_{1a}}$ = [NeuAc(α2-3)]Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer $G_{D_{1b}}$ = Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc-Cer, $G_{T_{1a}}$ = [NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc-Cer, $G_{T_{1b}}$ = [NeuAc(α2-3)]Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc-Cer, G_{Q_1} = [NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)Glc-Cer, where Gal = D-galactose, Glc = D-glucose, GalNAc = N-acetyl-D-galactosamine, NeuAc = N acetylneuraminic acid, Cer (ceramide) = N-acylsphingosine.

RESULTS

The recovery of the lipid-bound sialic acid from human brain white matter and normal human erythrocytes by the C_{18} Sep-Pak method and the dialysis method are presented in Table I. The values were similar and the yields agreed

TABLE I

LIPID-BOUND SIALIC ACID OF HUMAN BRAIN WHITE MATTER AND HUMAN ERYTHROCYTES

The values were determined by gas—liquid chromatography [11] and are expressed as mean ± standard deviation (n = 5).

Sialic acid	Sep-Pak method	Dialysis method
Sialic acid per gram wet weight of human brain white matter (mg)	296.8 ± 0.98	299 ± 1.50
Sialic acid per 100 ml (mg)	752.2 ± 1.25	751 ± 2.20

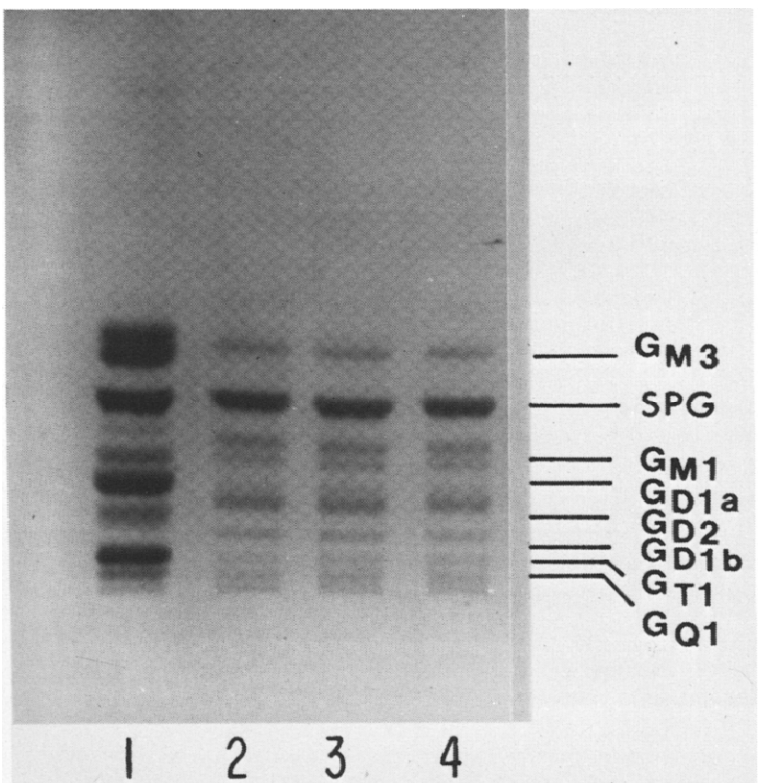

Fig. 2. Thin-layer chromatogram of gangliosides from normal adult human erythrocytes: (1), standard mixture of beef brain ganglioside, sialosylparagloboside (SPG) and G_{M_3}; (2), human erythrocyte gangliosides by dialysis method; (3) and (4), human erythrocyte gangliosides by Sep-Pak method. Solvent system, chloroform—methanol—2.5 N ammonia solution (60:40:9). Each of the lanes 2—4 contained 8 μg of sialic acid. SPG = [NeuAc(α2-3)]Gal(β1-4)-GlcNAc(β1-3)Gal(β1-4)Glc-Cer, G_{D_2} = GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal(β1-4)-Glc-Cer, where GlcNAc = N-acetyl-D-glucosamine. $G_{T_1} = G_{T_{1a}} + G_{T_{1b}}$; $G_{Q_1} = G_Q$.

well with previous data [5]. The TLC patterns of gangliosides from human brain white matter and human erythrocytes isolated by both methods were also identical, as shown in Figs. 1 and 2, respectively. It should be pointed out that the plates did not give any positive band for amine and phospholipid on spraying with ninhydrin and phosphorus spray reagent [7].

The recovery of gangliosides from C_{18} Sep-Pak cartridges, ascertained by adding known amounts of the tritiated beef brain ganglioside mixture to the total lipid extracts from human brain white matter and human erythrocytes, is presented in Table II. For human brain white matter the recovery of gangliosides was nearly quantitative between 0.125 and 1.5 g wet weight. Similarly, an excellent recovery of gangliosides was obtained from 0.5 to 20 ml of packed human erythrocytes (Table II).

TABLE II

RECOVERY OF GANGLIOSIDES FROM SEP-PAK CARTRIDGES

Human brain white matter sample (g)*	Unlabeled ganglioside sialic acid recovered (μg)**	Recovery of added [^{3}H]ganglioside (%)***
0.125	37.0	98
0.25	74.3	97
0.50	148.0	100
1.0	298.2	95
1.5	448.2	96
2.0	530.8	92
Packed human erythrocytes sample (ml)*	**Unlabeled ganglioside sialic acid recovered (μg)****	**Recovery of added [^{3}H]gangliosides (%)*****
0.5	3.6	99
1.0	7.4	98
5.0	36.5	100
10.0	74.0	95
20.0	144.2	96

*Known amounts of tritiated beef brain ganglioside mixture (1 μg of sialic acid, $2 \cdot 10^4$ cpm) were added to each sample before passing through Sep-Pak cartridges, to check the recovery of gangliosides.

**Determined by gas–liquid chromatography [11].

***The percentage recovery of ganglioside was determined from the recovery of tritium in fraction 2 (see Experimental for details). The values given here are the means of two separate experiments.

The results also showed that free sugars and free sialic acid are not absorbed by C_{18} Sep-Pak cartridges. As an illustration, [^{14}C]glucose and N-[^{14}C]acetylneuraminic acid were separately passed through C_{18} Sep-Pak cartridges. [^{14}C]-Glucose and N-[^{14}C]acetylneuraminic acid were recovered in fraction 1 (Table III).

We also observed from the double-labeling experiments with mixtures of [^{14}C]glucose or N-[^{14}C]acetylneuraminic acid and tritiated beef brain ganglio-

TABLE III

SEPARATION OF GLUCOSE AND SIALIC ACID FROM GANGLIOSIDES ON A SEP-PAK CARTRIDGE

Triplicate samples were utilized in each experiment and the results are means ± standard deviations.

Sample	Amount applied (cpm)*	Fraction 1**		Fraction 2**	
		cpm recovered	recovery (%)	cpm recovered	recovery (%)
[^{14}C]Glucose	$1.14 \cdot 10^5$	$1.11 \cdot 10^5 \pm 380$	98	500 ± 200	0.4
N-[^{14}C]Acetylneuraminic acid	$5.9 \cdot 10^4$	$5.64 \cdot 10^4 \pm 300$	96	260 ± 100	0.4

*Column blanks were processed to determine the cpm applied to the Sep-Pak cartridge.
**Fractions 1 and 2 represent the unabsorbed and absorbed fractions, respectively.

side that the recoveries of glucose and N-acetylneuraminic acid in fraction 1 were over 94% and gangliosides were isolated in fraction 2 in almost quantitative yield.

In addition, we have shown that during Iatrobeads or Unisil column chromatography, which is needed as a final purification step, the recovery of gangliosides was over 93%. This recovery was ascertained by adding known amounts of tritiated beef brain ganglioside to unlabeled human brain white matter and human erythrocyte ganglioside mixtures prepared by the dialysis method described above. The amounts of gangliosides recovered in the chloroform—methanol (1:2) eluates were 94.2 ± 3.9% (mean ± standard deviation, $n = 9$) and 93.2 ± 4.0% ($n = 9$) for white matter and erythrocytes, respectively.

DISCUSSION

A major problem in the isolation of small amounts of gangliosides has been the removal of salts and other non-lipid contaminants. Dialysis is the procedure most commonly used for this purpose, but at concentrations below 150 μg/ml of sialic acid there is considerable loss of gangliosides [16]. Other methods, such as gel filtration on Sephadex G-50 [13] or precipitation by trichloroacetic acid—phosphotungstic acid [17], are cumbersome and do not provide good recoveries when small amounts of gangliosides are used. The reversed-phase chromatographic procedure reported here provides a simple and efficient means for separating this type of impurity from gangliosides. It should be emphasized that the ganglioside solution should be passed through the Sep-Pak cartridge at a flow-rate of 1—2 ml/min because if the gangliosides remain on the column for long periods of time, the polysialo-gangliosides may form inner esters. The sample shown in lane 5 in Fig. 1 was passed through the cartridge over a period of 15 h. The G_{T1a} and G_{T1b} bands appear to be low in this sample, but treatment of this fraction with base hydrolyzed the inner esters and the chromatographic pattern became similar to those depicted in lanes 2—4.

While this work was in progress, a similar chromatographic procedure using a Sep-Pak cartridge was published by Williams and McCluer [3]. Their method

uses a Folch partition procedure in which most gangliosides are found in the aqueous phase but some G_{M3} and G_{M2} remain in the organic phase and the more polar neutral glycolipids are also found in the aqueous phase. The series of procedures used in our work provide a highly efficient micro-method for the total purification of small amounts of gangliosides and it should have wide applicability.

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