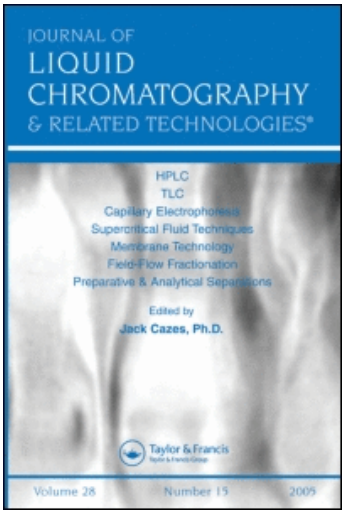

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DIRECT THIN-LAYER CHROMATOGRAPHIC METHOD FOR ISOLATION OF GANGLIOSIDES

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ABSTRACT

A simple procedure is developed for obtaining a ganglioside mixture. The scheme of the method is as follows: the sample was homogenized and extracted twice with cyclohexane (first extraction). The residue was reextracted with a mixture of chloroform /methanol = 1:3 (v/v) (second extraction). The extracts were collected after solvent evaporation. The residue was dissolved and applied onto a thin layer of silica gel according to the requirements of the preparative thin-layer chromatography. After the zone separation the visualized spots were scrapped and transferred to a column and eluted with a mixture of chloroform/methanol with increasing polarity. The eluate was concentrated and controlled for purity and composition by HPTLC. This procedure has been successfully applied to fractionation of ganglioside mixtures from human, calf and rat brains.

INTRODUCTION

Several procedures have been described for isolation of gangliosides. Most of them deal with the

isolation from brain tissue (1, 2, 3, 4, 5, 6, 7). Because of the amphiphilic nature of the gangliosides, the so called Folch`s solvent partitioning procedure is used for their separation from the other lipid components. It is expected that the upper water-methanolic phase contains the major quantity of gangliosides; although some other lipid groups may contaminate the upper layer. After a multistep purification (ion exchange chromatography, dialysis, reversed phase chromatography, etc.) a pure ganglioside fraction could be obtained. By another method the total lipid extract could be separated by means of silicic acid column chromatography (7). Most of the procedures are multistep and time-consuming. The direct separation of the total lipid extract using TLC is a perspective approach (5). Such an attempt followed by purification of the gangliosides obtained is described in this article. This method is directly applicable for preparative as well as for analytical purposes.

MATERIALS AND METHODS

A. Preparative Method

Human or calf brain tissue (10 g) was homogenized with 50 ml 0.25% acetic acid. After a 10 min

centrifugation at 5000 min^{-1} the supernatant was discarded and the tissue was transferred on a plastic fine sieve and centrifuged again. A cotton tampon was placed on the bottom of the tube in order to partially remove the water content. The sample was stirred with 1 g of pure quartz sand and kieselguhr followed by addition of 50 ml of cyclohexane. After a vigorous stirring (10 min) at room temperature and centrifugation at the conditions mentioned above the upper phase was transferred in a distillation flask and the pellet was reextracted with 50 ml of cyclohexane. The combined extracts (I) were evaporated to dryness at 40°C under vacuum in a rotary evaporator. The rest was dissolved in 10-15 ml of cyclohexane (eventually ultrasonication could be used). The second extraction was carried out twice with 50 ml portions of chloroform/methanolic mixture (1:3 v/v). The pooled supernatants (II) were evaporated at 35°C using rotary vacuum evaporator until 10-15 ml of solution were left in the flask. The residue formed was dispersed by ultrasonication after addition of 3-5 ml of chloroform. The lipid solutions obtained from the first and second extractions were submitted to TLC fractionation (20x20 cm glass plates; 0.75 mm silica gel G layer thickness). The solution were applied onto the layer on both sides of the plate. Mobile phase: chloroform/methanol/0.3% KCl = 30:18:4 (v/v/v) (A).

After 8 cm run distance the plate was dryed, turned at 180° and developed again. A chromatographic chamber with profiled bottom (Camag, Muttenz, Switzerland) was used. The zones were detected by spraying with orcinol reagent (0.5% solution in 20% sulfuric acid) on a small path at the edge of the plate followed by local heating at 80-90°C until purple spots on a white background could be seen on the plate. The sorbent of the corresponding not colored zones was scrapped and placed into a small glass column and a mixture of chloroform/ methanol = 1:2 (v/v) was added for column washing. After dropping out the sample was eluted with methanol (two sorbent volumes). The eluate was concentrated under nitrogen at 30°C and the composition was checked out by analytical HPTLC against standard mixture. Mobile phase: chloroform/ methanol/ 0.25% $CaCl_2.2H_2O$ = 55:45:10 (v/v/v) (B). The eluate could be desalted, if necessary by means of Sep-Pak C18 Cartridge (Waters Associates, Milford, Massachusetts, USA), before applying on the start. The spots visualization was done by treatment with orcinol spray reagent. Densitometry was used for quantitation. The purified ganglioside mixtures obtained from the first and second extracts were combined and kept dry or in solution in a sealed ampule. Isotonic salt solutions (pH 7.0) could be used as solvents. The mixture is suitable for isolation of

desired individual molecular species. Furthermore, this preparative variant was used for ganglioside isolation from milk powder (mainly GD3 fraction).

B. Analytical Method

It slightly differs from the procedure already described. The pooled extracts were applied on a silica gel layer (0.5 mm layer thickness) and developed with mobile phase A followed by column chromatography elution after visualizing and scrapping the zones. The eluate was concentrated and an aliquote was applied on the start of a HPTLC plate against test mixture. Two-dimensional chromatography was carried out for better identification. Mobile phase B (first direction), B + 1% concentrated ammonia (second direction).

RESULTS AND DISCUSSION

The successful separation of the lipid extract on a thin layer depends on the selection of suitable chromatographic conditions. Cyclohexane draws out (first extract) about a half of the total sample lipids

Table 1

Total Lipid Content from 1 g Brain Tissue.

	n	X	±S
extract I	21	47.2	9.1
extract II	21	46.0	6.1

and a predominant part of the cholesterol. The first extract contains about one third of the gangliosides. Probably this is due to the gangliosides solubilization by the cholesterol. The remaining lipids and gangliosides are contained in the second extract. The total lipid content from 1 g brain tissue is reported in Table 1. Four successive extractions with two different solvents are accomplished by this procedure allowing a total lipid yield. The brain tissue weight solvent volume ratio is 1:20. The chromatographic composition of both extracts (I and II) is given in Figure 1 (see text below). The gangliosides are well-separated from the other lipid classes (phospholipids). It is supposed that separation on a thin layer prevents the associate formation between the gangliosides and the other lipid groups.

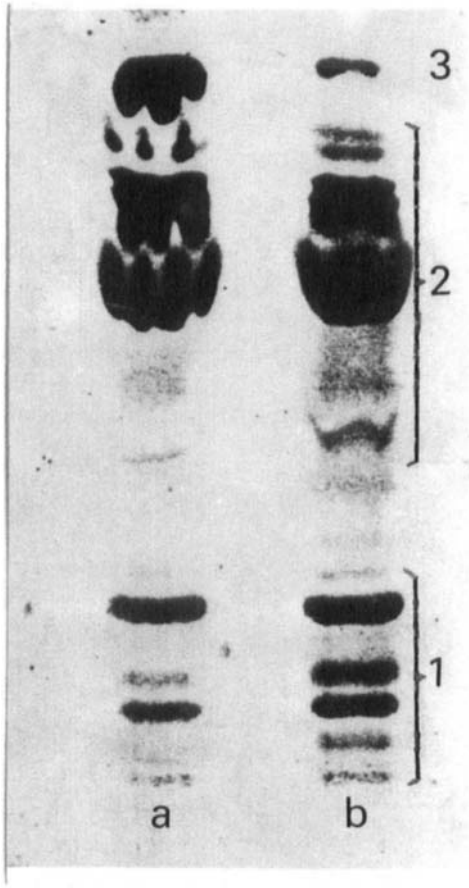

Fig. 1. Thin-layer chromatogram of total brain lipids. lane a: extract I; lane b: extract II; 1. gangliosides; 2. phospholipids; 3. cholesterol; silica gel 60 (Merck, Darmstadt, FRG), mobile phase A (see text), visualization by spraying with orcinol reagent.

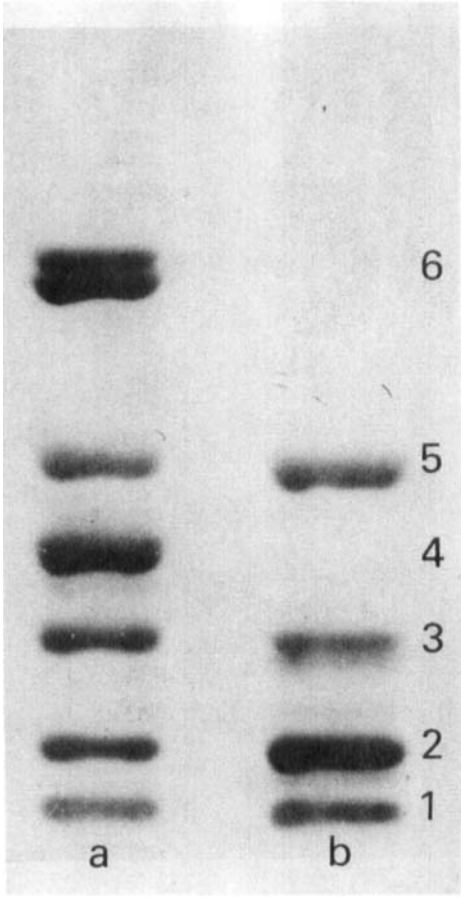

Fig. 2. HPTLC analytical chromatogram. lane a: standard mixture; lane b: purified ganglioside mixture; 1. GTb; 2. GD1b; 3. GD1a; 4. GD3; 5. GM1; 6. GM3 (N-acetyl- and N-glycolyl- isomers); HPTLC plate 10x10 cm, mobile phase B (see text), visualization as in Fig. 1.

Table 2

(n = 6)	GTb	GD1b	GD1a	GM1
standard mixture	18.0±2.5	13.3±2.2	44.0±1.6	24.8±3.9
sample	23.0±3.7	14.0±2.7	32.6±8.1	29.6±6.5
sample + standard mixture	22.0±3.3	13.2±2.1	35.0±4.3	29.4±3.0

An example of HPTLC analytical separation by this procedure compared with a standard test mixture (Sigma, St. Louis, Missouri, USA) is shown in Figure 2. Besides the four major fractions (GM1, GD1a, GD1b and GTb) four other minor ones (GQ, GD3, GM2 and GM3) could be seen on the chromatogram, but they were not identified.

We have accomplished an additional experiment in order to determine the overall recovery. Standard mixture (0.5 ml) containing ganglioside fractions obtained from calf brain (Calbiochem, San Diego, California, USA) was added to 3.5 ml of total ganglioside extract from rat brain. Sample from rat brain without addition of standard mixture was processed separately. Preparative TLC, column chromatography and

eluate check-up using TLC were applied according to the procedure already described. Densitometry was used for quantitation (CAMAG TLC Scanner II + Integrator SP 4290, Camag, Muttenz, Switzerland). Results (rel. %; means ± SD) are summarized in Table 2.

The analytical method appears to be fairly suitable for quantitation of separated ganglioside molecular species and the preparative procedure is a powerful tool in ganglioside medium and large-scale preparation. The methods are suitable for preparing immunological substances as well.

The recovery data calculated by average are as follows: GTb - 98%; GD1b - 95%; GD1a - 97%; GM1 - 98%.

The mean yield of ganglioside mixture from 1 g human brain tissue is 1.7-1.8 mg.

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