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Note

Rapid, quantitative method for the isolation and purification of gangliosides by LIPSEP gel chromatography

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Gangliosides are complex acidic lipids that are normal constituents of mammalian cell membranes, the highest concentrations being found in neuronal cell membranes [1] They are glycosphingolipids that contain at least one sialic acid moiety Isolation of gangliosides by the method of Folch et al [2] involves the chloroform–methanol extraction of tissues, followed by the partitioning of gangliosides in the methanol–saline upper phase. To remove salts and other low-molecular-mass, water-soluble substances, the upper fraction (methanol–saline) is dialysed [2,3] exhaustively against distilled water, followed by concentration and lyophilization. This makes the isolation of gangliosides a time-consuming and multi-step procedure. Moreover, loss of some less polar species of gangliosides has been reported [4] to occur if the methanol–saline fraction is dialysed without prior removal of organic solvents [5].

By taking advantage of the acidic properties of gangliosides, a different approach has been proposed for their isolation and purification This involves the use of anion exchangers [6–12], the total lipid extract of the tissues being applied to an anion-exchange column and the gangliosides eluted with solvents containing sodium acetate The resulting fraction is then subjected to mild alkaline hydrolysis and desalted by dialysis or gel filtration [13] and further purified by adsorption [14–20] chromatography. Recently, reversed-phase chromatographic methods [21–23] have been described for the concentration and isolation of gangliosides, involving the use of Sep-Pak C_{18} cartridges. The

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low capacity of these cartridges for gangliosides and their high cost restrict their use for the large-scale isolation and purification of gangliosides

The requirement for high-purity gangliosides has increased during last few years as new areas of research have developed. The neuronotrophic and neuritogenic properties of gangliosides [24–26] have led to the need for a preparation free from impurities such as peptides. Ganglioside metabolism and transport studies also require ganglioside preparations of the highest purity The methods reported so far were aimed at obtaining maximum yields rather than a high quality of gangliosides Data on the levels of peptides, phospholipids, nucleotide sugars and other contaminants presented in isolated gangliosides have not been reported

Ion-exchange methods [6–12] have been found to be satisfactory for small amounts of gangliosides However, for large amounts, the original method of Folch et al [2] for the extraction of total lipids and isolation of gangliosides, as applied to brain and other tissues, is still widely used despite the problems with partitioning methods. In this paper, we describe the use of LIPSEP[a], a novel gel matrix developed at this Centre for the quantitative isolation and purification of bovine brain gangliosides Gangliosides from the upper phase fraction (methanol–saline) can be desalted, concentrated and purified in a short period of time with a minimum number of steps using LIPSEP gel The high adsorption capacity of the gel for gangliosides and the low cost of its preparation make it a very attractive and suitable chromatographic support for the large-scale isolation and purification of gangliosides

EXPERIMENTAL

Materials

LIPSEP gel and gangliosides (sialic acid content ca 10 and 20% by weight) were products of the CSIR Centre for Biochemicals (Delhi, India). HPTLC silica gel 60 (200 μm) was obtained from Merck (Darmstadt, F.R.G) Buffalo brains were obtained from a local slaughter house Other chemicals and reagents used were of analytical-reagent grade and were used as such.

Analytical methods

Thin-layer chromatography (TLC) of gangliosides was performed under the following conditions· (a) precoated silica gel plates were heated for 2 h at 120°C before use, (b) solvent system, chloroform–methanol–water (55 45 10, v/v) containing 0 02% (w/v) calcium chloride dihydrate; (c) operation at room temperature, and (d) detection of spots with resorcinol–HCl reagent [27] by heating at 100°C for 15 min Ganglioside-bound N-acetylneuraminic acid

[a]Patent applied for

(NANA) was determined according to Svennerholm [27], phosphorus according to Fiske and Subba Row [28] and proteins by the method of Markwell et al [29]

Extraction of gangliosides

Acetone powder of bovine brains (bovine brains dehydrated with acetone) was prepared according to a standard procedure [30]. Air-dried acetone powder of bovine brain tissue (1 kg) was extracted by Suzuki's modification of Folch's method involving the extraction of the acetone powder with chloroform–methanol (2 1) followed by chloroform–methanol (1 2) containing 5% water [31,32] Partitioning was carried out twice with aqueous salt solution and once with water The upper phase (methanol–saline) was separated and subjected to LIPSEP gel column chromatography

LIPSEP gel column chromatography

Slurry packing of LIPSEP gel was carried out as follows LIPSEP gel (20 g) was suspended in 100 ml of methanol–water (1 1, v/v) and allowed to settle for 5 min The supernatant and fines were removed with a pipette The LIPSEP gel was treated in this manner twice further and finally suspended in 100 ml of methanol–water (1 1, v/v) A glass column (30 cm×3 cm I D), provided with a small glass-wool plug at the bottom, was packed under gravity The column was washed with 100 ml of methanol–water (1 1, v/v) under a slightly positive pressure of nitrogen to give a homogeneous packing of the column Crude ganglioside solution (2 0 l) obtained after Folch partitioning was applied to the column When all of the solution had been adsorbed on the column bed, the column was washed successively with methanol–water (1 1, v/v) and water to remove non-lipids and low-molecular-mass, water-soluble impurities The gangliosides were eluted with different solvent systems in order to obtain the maximum yield and purity of gangliosides as specified under Results and discussion Fractions of 10 ml were collected at a flow-rate of 1–1 5 ml/min

Regeneration of LIPSEP gel

Used LIPSEP gel was regenerated by washing the column successively with dichloroethane (DCE)–methanol (5 1, v/v) and DCE The column was then washed with methanol followed by equilibration with methanol–water (1 1, v/v) for re-use After using the same gel a number of times, complete regeneration was carried out by washing the gel successively with DCE, isopropanol and methanol and finally equilibrating the column in methanol–water (1 1, v/v)

Determination of the capacity of the LIPSEP gel for gangliosides

A column of LIPSEP gel (1 g) was packed in methanol–water (1 1, v/v) as described above and crude gangliosides (200 ml, 50 mg) were applied The

column was washed with one bed volume each of methanol–water (1 1, v/v) and water. The breakthrough and washings were collected and analysed for sialic acid content. The difference in the sialic acid content applied to the column and that found in the washings and breakthrough fractions gave directly the adsorption capacity of the LIPSEP gel.

Optimum conditions for the elution of gangliosides from LIPSEP gel

The mixture of bovine brain crude gangliosides (500 ml, 200 mg) was dissolved in 500 ml of methanol–water (1 1, v/v) and applied to a column of LIPSEP gel packed in methanol–water (1 1, v/v). The column was washed with one bed volume of each of methanol–water (1 1, v/v) and water at a flow-rate of 1–1.5 ml/min maintained with a peristaltic pump The breakthrough and washing fractions were collected The elution of gangliosides was then checked with a number of solvent systems and the recovery of gangliosides was calculated by sialic acid determination

RESULTS AND DISCUSSION

LIPSEP gel can be rapidly equilibrated in the desired solvent system and can be used a number of times without a significant decrease in capacity or resolving power

The adsorption capacity of LIPSEP gel for gangliosides was found to be very high: LIPSEP gel (1 g) can quantitatively adsorb and desorb 50 mg of gangliosides

A solution of crude gangliosides (200 ml) obtained after Folch partitioning

TABLE I

EFFECT OF COMPOSITION OF SOLVENT SYSTEM ON ELUTION OF GANGLIOSIDES FROM 5-g LIPSEP GEL COLUMN

The values presented are the means of at least two separate experiments

System No	Eluent[a]	Elution volume[b] (ml)	Recovery of gangliosides (%)
1	C-M (2 1)	100	72 7
2	C-M (3 1)	100	84 4
3	C-M (4 1)	100	39 6
4	DCM-M (2 1)	100	74 2
5	DCM-M (3 1)	100	81 5
6	DCE-M (2 1)	75	99 9

[a]C = Chloroform, M = methanol, DCM = dichloromethane, DCE = dichloroethane The solvent systems were prepared by mixing solvents in volume proportions

[b]Elution was continued until the eluate fractions were negative to the resorcinol test

TABLE II

CONTENT OF SIALIC ACID AND IMPURITIES IN CRUDE AND LIPSEP GEL-PURIFIED GANGLIOSIDES

The values presented are the means of two separate experiments

Sample	Neu Ac per 100 μg gangliosides (μg)	Phosphorus per μg Neu Ac (μg)	Peptide[a] per μg Neu Ac (μg)
Crude gangliosides[b]	10 0	0 03	2 8
Purified gangliosides	34 0	0 00	0 05

[a]Protein level in gangliosides isolated by the present method was found to be 0 0183 μg protein/μg ganglioside (1 83%)
[b]Crude gangliosides obtained by the method of Folch et al [2]

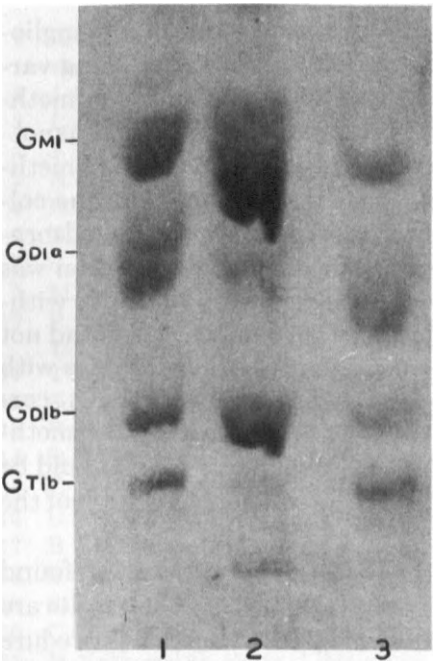

Fig 1 Thin-layer chromatogram of gangliosides on Merck HPTLC silica gel 60 (200 μm) The solvent was chloroform-methanol-water (55 45 10, v/v) containing 0 02% (w/v) calcium chloride dihydrate and the gangliosides were detected with resorcinol reagent Lanes 1, LIPSEP-purified bovine brain gangliosides, 2, ganglioside standard from bovine brain, 3, crude gangliosides before purification on LIPSEP gel Ganglioside nomenclature of Svennerholm [33] is used

was applied to a column of 5 g of LIPSEP gel packed in methanol–water (1 1, v/v) The column was further washed with one bed volume of methanol–water (1 1, v/v) and 25 ml of water. Gangliosides were then eluted with a number of

solvent mixtures as shown in Table I Recoveries in the range 40–85% were obtained when the column was eluted with solvent systems 1–5 (Table I) The results indicated that a relatively non-polar solvent system is required to elute the gangliosides quantitatively from LIPSEP gel. DCE–methanol (2 1) gave the best results, with recoveries >99% based on sialic acid content The sialic acid content in purified gangliosides was found to be 34% by weight The content of other impurities was found to be very much reduced (Table II)

Fig 1 shows the TLC patterns of crude and LIPSEP gel-purified gangliosides Both TLC patterns were identical with that of bovine brain gangliosides (BBG) standards No selective loss of any of the ganglioside species was observed in the LIPSEP gel-purified gangliosides This was further confirmed by loading known amounts of gangliosides (samples) and individual species of gangliosides (in terms of μM sialic acid) followed by elution with DCE–methanol (2 1, v/v), which resulted in a quantitative recovery (>99%) of both gangliosides and individual species of gangliosides

The effect of sodium chloride concentration on the adsorption of gangliosides on LIPSEP gel was also studied A ganglioside solution containing various concentrations of sodium chloride and without sodium chloride in methanol–water (1 1, v/v) was applied to a LIPSEP gel column packed in methanol–water (1 1, v/v) The column was washed with one bed volume each of methanol–water (1 1, v/v) and water, and gangliosides were eluted from the column with DCE–methanol (2 1, v/v) No significant difference in the adsorption and elution of gangliosides was found when the ganglioside solution was applied to a LIPSEP gel column with different concentrations of salt or without salt Adsorption of gangliosides on the LIPSEP gel column was found not to be salt-dependent This is in contrast, however, to the earlier findings with Sep-Pak C_{18} cartridge, where adsorption of gangliosides on a Sep-Pak C_{18} cartridge was found to be salt-dependent This is an important finding, the methanol–saline fraction of ganglioside obtained after Folch partitioning could be directly purified on LIPSEP gel without adjusting the salt concentration of the ganglioside solution

The purity of gangliosides isolated by the LIPSEP gel procedure was found to be better than that obtained by other methods [6,12,22,32] The results are given in Table II However, gangliosides isolated and purified by this procedure were still found to be contaminated with some non-ganglioside components, and further purification would be necessary to remove these materials

CONCLUSION

The LIPSEP gel procedure provides a useful alternative to dialysis and concentration during the isolation and purification of gangliosides All lipids are retained by the LIPSEP gel column and non-lipids are washed off in the breakthrough fraction Most of the glycoproteins are washed off when the LIPSEP

gel column is washed with water Gangliosides are quantitatively eluted with DCE–methanol (2 1, v/v) without the loss of any of the gangliosides Non-sialic acid lipids that were bound more tightly with LIPSEP gel required a higher concentration of DCE in the eluent and eluted later than gangliosides. In conclusion, LIPSEP gel was found to be an economical matrix for the large-scale isolation and purification of gangliosides

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