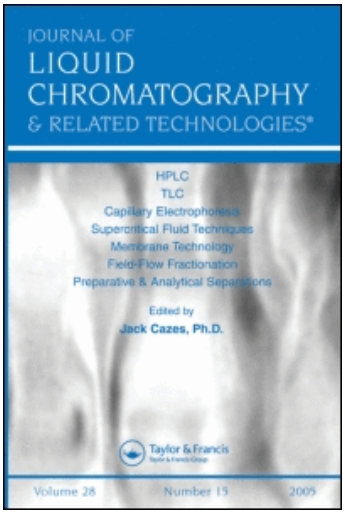

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Isolation and Purification of Lipids by Centrifugal Partition Chromatography

J. G. Alvarez[a]; J. Cazes[b]; J. C. Touchstone[a]; R. L. Grob[c]

[a] Department of Obstetrics and Gynecology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania [b] Sanki Laboratories Inc. Sharon Hill, Pennsylvania [c] Department of Chemistry, Villanova University Villanova, Pennsylvania

To cite this Article Alvarez, J. G. , Cazes, J. , Touchstone, J. C. and Grob, R. L.(1990) 'Isolation and Purification of Lipids by Centrifugal Partition Chromatography', Journal of Liquid Chromatography & Related Technologies, 13: 18, 3603 — 3614
To link to this Article: DOI: 10.1080/01483919008049558
URL: http://dx.doi.org/10.1080/01483919008049558

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(18), 3603-3614 (1990)

ISOLATION AND PURIFICATION OF LIPIDS BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

J. G. ALVAREZ[1]*, J. CAZES[2],
J. C. TOUCHSTONE[1], AND R. L. GROB[3]
[1]*Department of Obstetrics and Gynecology*
University of Pennsylvania
School of Medicine
Philadelphia, Pennsylvania 19104
[2]*Sanki Laboratories Inc.*
Sharon Hill, Pennsylvania 19038
[3]*Department of Chemistry*
Villanova University
Villanova, Pennsylvania 19085

ABSTRACT

Methodologies for the separation of various lipid classes including free fatty acids (FA) and fatty acid ethyl esters (FAEE), monoglycerides (MG), diglycerides (DG), triglycerides (TG), phosphoglycerides (PG), and glycosphingolipids (GSL) by Centrifugal Partition Chromatography are presented. The fatty acid ethyl esters, Hexadecanoate, Octadecanoate, cis-9-Octadecenoate; and cis,cis-9,12-Octadecadienoate, and all-cis-9,12,15-Octadecatrienoate, were separated at 800 rpm; flow rate of 2.0 ml/min; using n-hexane/acetonitrile (1:1, v/v) as the solvent system and normal ascending and reversed descending elution modes respectively. The fatty acid ethyl esters, all-cis-5,8,11,14,17-Icosapentaenoate and all-cis-4,7,10,13,16,19-Docosahexaenoate, were separated at 600 rpm; flow rate of 3.3 ml/min; using n-hexane/methanol/water (1/0.9/0.1, v/v/v) as the solvent system and normal ascending mode. FA and MG; and DG and TG were separated at 600 rpm; 10

ml/min; using n-hexane/methanol/water (1:1:0.05, v/v/v) as the solvent system and reversed descending and normal ascending modes respectively. PE and PC were separated at 600 rpm; flow rate of 5.0 ml/min; using heptane/ethanol (1:1, v/v) as the solvent system and normal ascending and reversed descending modes respectively. The peracetylated GalCer, SF, GB_4; and GM1, GD1a, and GT1b, were partially resolved at 800 rpm; flow rate of 4.0 ml/min; using heptane/acetonitrile/water (5:4:1, v/v/v) as the solvent system and normal ascending and reversed descending modes, respectively. These results indicate that CPC constitutes a powerful technique for the separation of large amounts of the aforementioned lipids. Its applications should prove most useful in the purification of these lipids and in facilitating their commercial availability

INTRODUCTION

Lipids, including fatty acids, triglycerides, diglycerides, monoglycerides, phosphoglycerides, and glycosphingolipids, are being increasingly recognized as an important group of biomolecules that play key roles in cell biochemistry. The significance of these roles has transcended to the field of biomedical research where they have recently emerged as therapeutic agents for several human diseases. Essential fatty acids have been used for the treatment of cardiac arrhythmias, atopic eczema, multiple sclerosis, hypertension, hepatic disorders, and coronary disease (1). Phosphoglycerides find one of their widest application in the pharmaceutical industry, for the manufacture of highly homogeneous liposomes utilized to encapsulate drugs and cosmetics. Glycosphingolipids have been utilized in the treatment of various neurological diseases.

Consequently, commercial availability of large amounts of the aforementioned biomolecules becomes an important limiting factor that will ultimately allow the implementation of their biomedical applications. Centrifugal partition chromatography (CPC) is a chromatographic mode of separation based on the partition of analytes in two-phase solvent systems. It does not require the use of expensive column packing resins, allows separation of gram amounts of analytes, and can be utilized in the automatic mode permitting unattended operation. Therefore, CPC emerges as the method of choice for the large scale purification of these lipids.

In this study we present data pertaining to the isolation and purification of fatty acids, as free fatty acids and fatty acid ethyl esters, according to the procedure reported by Murayama *et al.* (2,3); mono, di, and triglycerides; phosphoglycerides, including phosphatidylethanolamine and phosphatidylcholine.

Otsuka and Yamakawa (4) and Otsuka *et al* (5) reported a method for the separation of neutral and acidic glycosphingolipids, respectively, by Droplet Countercurrent Chromatography. In this report we also present preliminary data on the separation of peracetylated neutral and acidic glycosphingolipids, including GalCer, sulfatide (SF), GB_4, GM1, GD1a, and GT1b, by CPC.

MATERIALS AND METHODS

Reagents

The fatty acid standards including, hexadecanoic, octadecanoic, cis-9-octadecenoic, cis,cis-9,12-octadecadienoic, all-cis-9,12,15-octadecatrienoic, all-cis-5,8,11,14,17-icosapentaenoic, all-cis-4,7,10,13,16,19-docosahexaenoic acids; the monoglyceride, diglyceride, triglyceride, phosphoglyceride, and glycosphingolipid standards; and porcine pancreatic lipase (EC. 3.1.1.3), were purchased from Sigma Chemical Co. (St. Louis, MO). Precoated silica gel HP-K high performance plates (10 x 10 cm, 250 μm thickness) were purchased from Whatman Inc. (Clifton, N.J.). Solvents were EM Science chromatographic grade. Inorganic salts were from J.T. Baker (Phillisburg, N.J.) and of the highest purity available.

Sample Preparation

The fatty acid ethyl esters hexadecanoate, octadecanoate, cis-9-octadecenoate, cis,cis-9,12-octadecadienoate, and all-cis-9,12,15-octadecatrienoate, were obtained after alkaline ethanolysis of cereal oil. All-cis-5,8,11,14,17-icosapentaenoate, and all-cis-4,7,10,13,16,19-docosahexaenoate from fish oil were obtained from Gasukuro Kogyo, Ltd., Japan. Following ethanolysis, the fatty acid ethyl esters were extracted with 1 volume of n-hexane. This procedure was repeated twice.

The combined n-hexane extracts were evaporated to dryness and dissolved in 1 to 10 ml of either n-hexane or n-heptane to give a concentration of 1 gm/ml.

Free fatty acids, monoglycerides, and diglycerides were obtained following hydrolysis with pancreatic lipase of triglycerides from vegetable oil and extraction by partition with chloroform-methanol-water (C:M:W) (4:2:1, v/v/v) (6). The extract was evaporated to dryness and dissolved in 1 to 10 ml of n-hexane to give a concentration of 1 gm/ml.

Lipids from bovine brain cortex were peracetylated with acetic anhydride in pyridine (1:2, v/v) and the peracetylated glycosphingolipid fraction purified in a Florisil column according to the procedure described by Saito and Hakomori (7). The peracetylated glycosphingolipids were then evaporated to dryness, dissolved in n-hexane at 1mg/ml, and partitioned in n-hexane/acetonitrile (1:1) and n-hexane/acetonitrile/water (1:1:0.1, v/v/v). Following partition, the organic and aqueous phases were evaporated to dryness separately, dissolved in 200 µl of C-M (2:1), and applied to HP-K plates in 5 µl aliquots. The plates were first developed in solvent 1 consisting of C-M (100:4), dried thoroughly, and developed in solvent 2 consisting of C-M (70:30). Solvent 1 allowed separation of the peracetylated neutral glycosphingolipids, while solvent 2 permitted the separation of the peracetylated acidic glycosphingolipids. Following development the plates were thoroughly dried, sprayed with the orcinol ferric chloride reagent, and placed in a Frigidaire microwave oven for 5 min at setting 9 (8). The stained chromatograms were then scanned with a Shimadzu CS-9000 spectrodensitometer at 500 nm in the transmission mode. The partition coefficients for the different peracetylated glycosphingolipids were then calculated.

Gas Liquid Chromatography

The fatty acid ethyl esters, obtained following ethanolysis of cereal and fish oil, were analyzed and identified in a Shimadzu GC-9A gas liquid chromatograph equipped with a data processing unit c-R3A, and a FID detector. A packed column containing Advance -DS coated Chromasorb W (5%), 3.1 mm x 2.1 m, was used.

Centrifugal Partition Chromatography

Laboratory scale separations were carried out with a Centrifugal Partition Chromatograph, CPC Model LLN (Sanki Engineering Limited, Nagaokakyo, Kyoto, Japan). Partition microcells are contained in rectangular cartridges; one cartridge contains 400 micro cells with a total net volume of 21.3 ml. Twelve cartridges were connected in series around the rotor of the centrifuge giving a total of 4,800 partition microcells and a total volume of 256 ml.

For scale-up tests, a CPC Model MF-007 (Sanki Engineering Limited, Nagaokakyo, Kyoto, Japan) was used. The stacked-disk type rotor of the main centrifuge unit, 300 mm of diameter by 200 mm height, contains 1,200 partition cells and a total net volume of 6,800 ml. As much as 500 gm of crude materials have been processed with this configuration in a few hours.

Eluates from the CPC system were collected in 5 ml fractions. The fatty acid esters corresponding to each fraction were identified by GLC as described above. Monoglycerides, diglycerides, and triglycerides; and phosphoglycerides were separated and identified by quantitative high performance thin layer chromatography (HPTLC) following development in hexane-acetone (70:30, v/v), and chloroform-ethanol-triethylamine-water (30:34:30:8, v/v/v/v), respectively, staining with the copper sulfate reagent, and scanning at 400 nm in the reflectance mode with a Shimadzu CS-9000 spectrodensitometer (8).

Peracetylated glycosphingolipids were monitored by CPC-UV detection at 200 nm and HPTLC as described above.

Solvent Preparation

Two-phase partition solvent systems used for separation of the different lipid classes were prepared by mixing the corresponding organic and aqueous phases in a separatory funnel. The two-phase solvent systems were allowed to equilibrate for 5 min. Then the two phases were separated and placed in the corresponding reservoir.

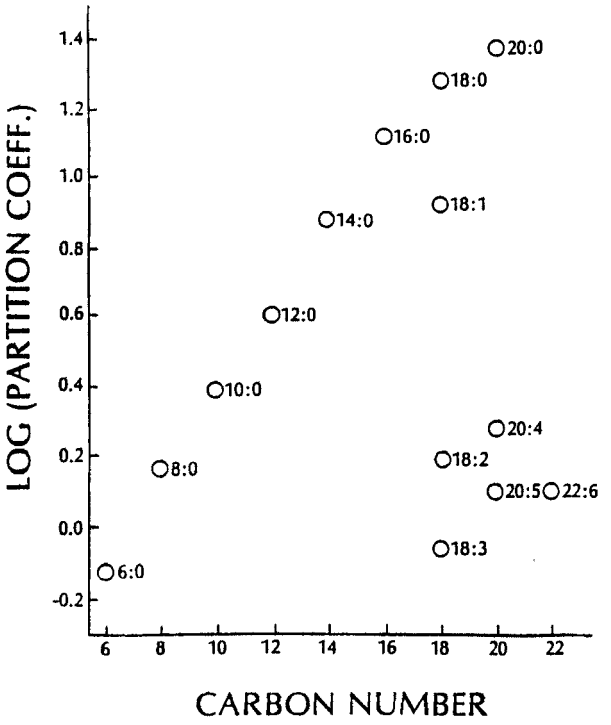

FIG. 1: Partition coefficients of fatty acid ethyl esters in n-Hexane/Acetonitrile (1:1) two-phase system.

RESULTS AND DISCUSSION

The partition coefficients for the different fatty acid ethyl esters in the three different two-phase solvent systems (n-hexane/acetonitrile; n-hexane/methanol/water; and n-hexane/ethanol/water) are shown in Fig. 1, 2, and 3 respectively. These partition coefficients were used to select the two-phase solvent systems utilized for CPC separation of the corresponding fatty acid ethyl esters. Selection of the two-phase solvent systems for the CPC fractionation of free fatty acids, monoglycerides, diglycerides and triglycerides, and glycosphingolipids was carried out in a similar fashion.

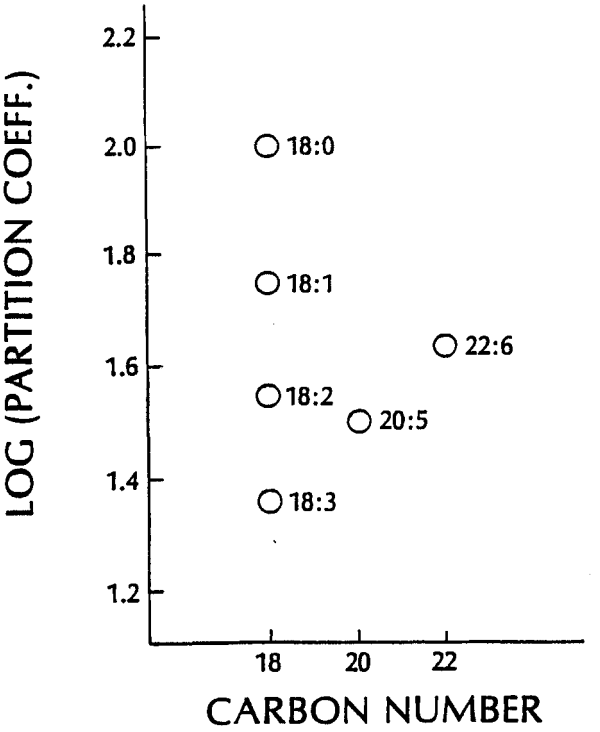

FIG. 2: Partition coefficients of fatty acid ethyl esters in n-Hexane/Methanol/Water (1:0.9:0.1) two-phase system.

The fatty acid ethyl esters, hexadecanoate, octadecanoate, cis-9-octadecenoate; and cis,cis-9,12-octadecadienoate and all-cis-9,12,15-octadecatrienoate, were separated at 800 rpm; flow rate of 2.0 ml/min; using n-hexane/acetonitrile (1:1, v/v) as the solvent system and normal ascending and reversed descending elution modes respectively (Fig 4). The two-phase solvent system, n-hexane/acetonitrile (1:1), is well suited for the separation of the C18 series of fatty acid esters given the wide range distribution of the corresponding partition coefficients. Elution in the ascending mode, where n-hexane and acetonitrile are the mobile and stationary phases respectively, allows separation

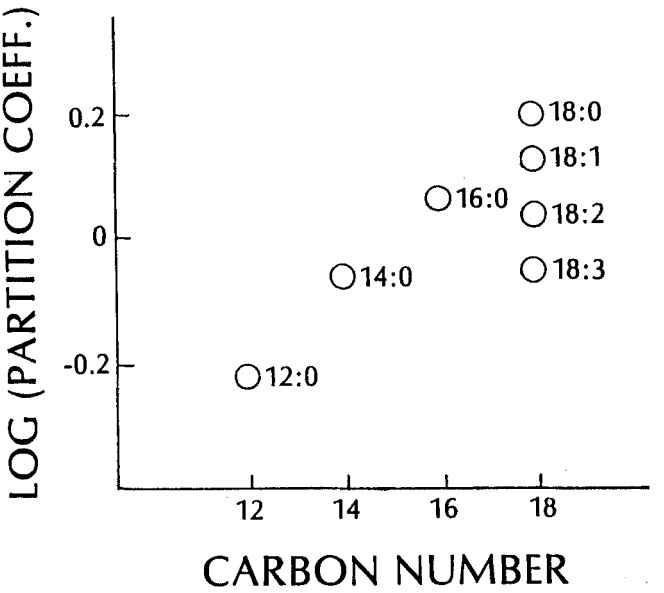

FIG. 3: Partition coefficients of fatty acid ethyl esters in n-Hexane/Ethanol/Water (1:0.9:0.1) two-phase system.

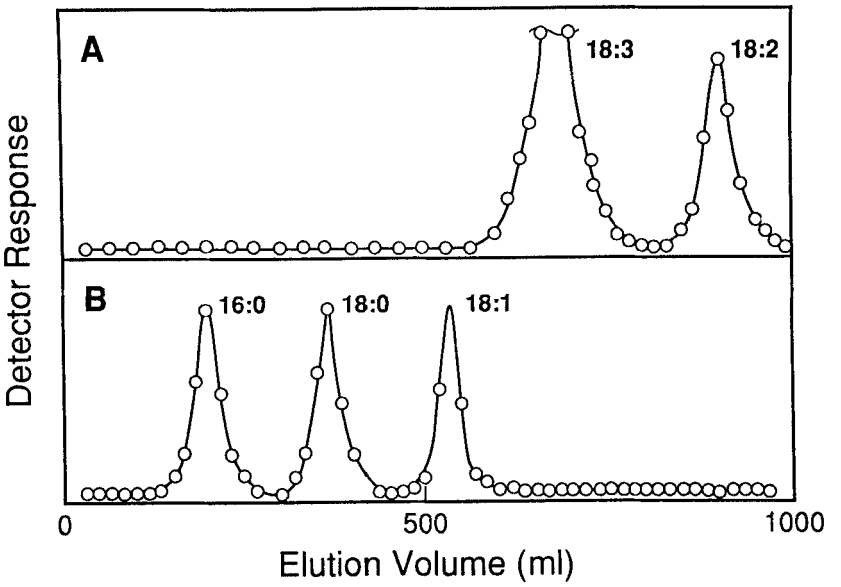

FIG. 4: Separation of fatty acid ethyl esters by Centrifugal Partition Chromatography. The fractions collected from the CPC separation were analyzed by GLC as indicated in Materials and Methods. A: normal ascending mode; B: reversed descending mode.

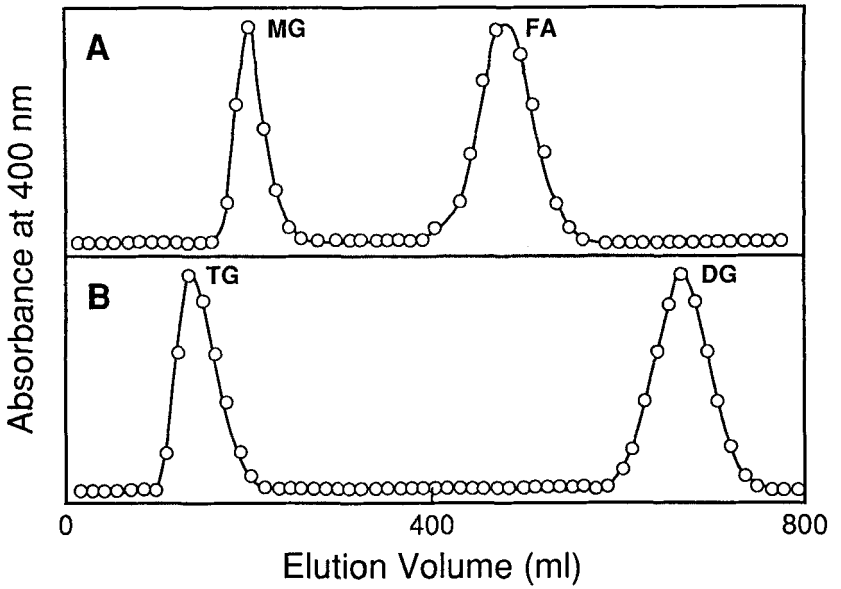

FIG. 5: Separation of free fatty acids, monoglycerides, diglycerides, and triglycerides by Centrifugal Partition Chromatography. MG, monoglycerides; FA, fatty acids; TG, triglycerides; DG, diglycerides. The fractions collected from the CPC separation were analyzed by reflectance-HPTLC at 400 nm after reaction with the copper sulfate reagent as indicated in Materials and Methods. A: reversed descending mode; B: normal ascending mode.

based on the number of double bonds. The fatty acid ethyl esters, all-cis-5,8,11,14,17-icosapentaenoate; and all-cis-4,7,10,13,16,19-docosahexaenoate, were separated at 600 rpm; flow rate of 3.3 ml/min; using n-hexane/methanol/water (1/0.9/0.1, v/v/v) as the solvent system and normal ascending mode (data not shown).

Free fatty acids and monoglycerides; and diglycerides and triglycerides were separated at 600 rpm; 10 ml/min; using n-hexane/methanol/water (1:1:0.05, v/v/v), as the solvent system and reversed descending and normal ascending modes respectively (Fig 5). In the reversed descending mode, n-hexane is the stationary

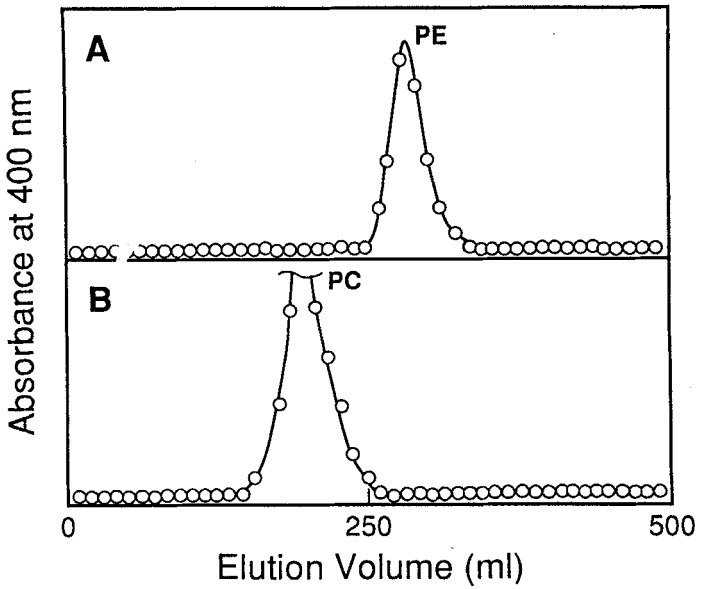

FIG. 6: Separation of phosphoglycerides by Centrifugal Partition Chromatography. PE, phosphatidylethanolamine; PC, phosphatidylcholine. The fractions collected from the CPC separation were analyzed by reflectance-HPTLC at 400 nm following spraying with the copper sulfate reagent as indicated in Materials and Methods. A: normal ascending mode; B: reversed descending mode.

phase and methanol-water the mobile phase. This configuration is analogous to that found in a C18-reversed-phase adsorption liquid chromatographic system. The main difference is that in the former the stationary phase is a liquid immobilized by a centrifugal force, and in the latter a solid matrix. Diglycerides and triglycerides were separated in the normal ascending mode where now hexane and methanol-water are the mobile and stationary phases respectively. This configuration is similar to that found in a normal phase adsorption liquid chromatographic system where the mobile phase is a non-polar solvent.

Phosphatidylethanolamine and phosphatidylcholine, were separated at 600 rpm with a flow rate of 5.0 ml/min, using heptane/ethanol (1:1, v/v) as the solvent

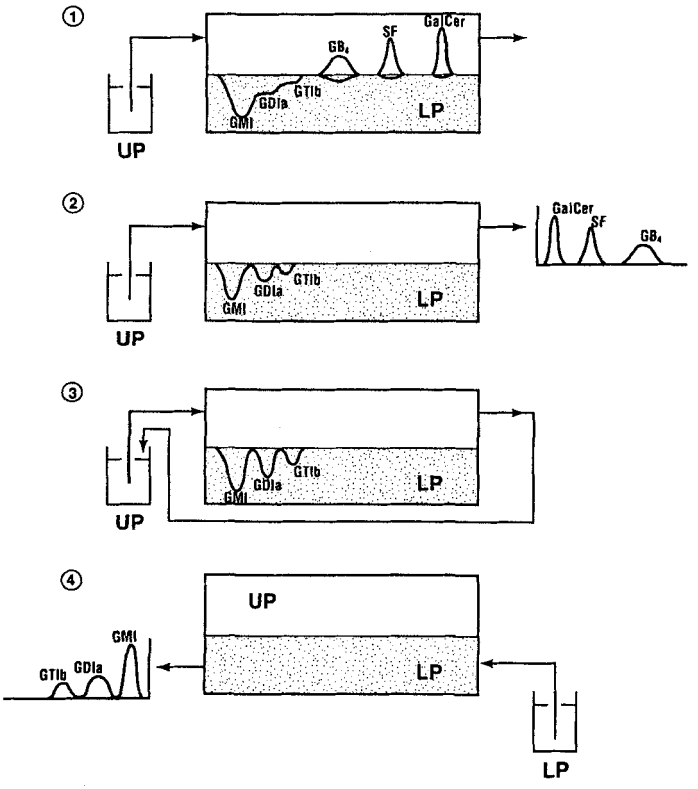

FIG. 7: Diagram illustrating the elution modes and separation of peracetylated neutral and acidic peracetylated glycosphingolipids by Centrifugal Partition Chromatography. UP: upper phase; LP: lower phase; GalCer, Gal(ß1-1) ceramide; SF, GalCer I^3-sulfate; GM1, II^3NeuAc-$GgOse_4$Cer; GD1a, IV^3NeuAc,II^3NeuAc-$GgOse_4$Cer; GT1b, $II^3(NeuAc)_3$-$GgOse_4$Cer.

system and normal ascending and reversed descending elution modes respectively (Fig 6).

The peracetylated glycosphingolipids, GalCer, SF GB_4; and GM1, GD1a, and GT1b, were partially resolved at 800 rpm; flow rate of 4.0 ml/min; using heptane/acetonitrile/water (5:4:1, v/v/v), as the solvent system and normal ascending and reversed descending elution modes respectively (Fig 7).

These results indicate that CPC constitutes a powerful technique for the separation of large amounts of the aforementioned lipids. Its applications should prove most useful in the purification of these lipids and in facilitating their comercial availability.

ACKNOWLEDGMENTS

This work was partially supported by NIH grants HD-15842 and HL-19737.

REFERENCES

1. Horrobin, D., F. and Manku, M., S. Clinical Biochemistry of Essential Fatty Acids. In Omega-6 Essential Fatty Acids: Pathophysiology and Roles in Clinical Medicine. (D. F. Horrobin, ed.); Wiley-Liss, New York, 1990.

2. Murayama, W., Kobayashi, T., Kosuge, Y., Yano, H., Nunogaki, Y., and Nunogaki, K. J. Chromatogr., 239: 343-349, 1982.

3. Murayama, W., Kosuge, Y., Nakaya, N., Nonogaki, Y., and Nunogaki, K. J. Liquid Chromatogr., 11(1): 283-300 (1988).

4. Otsuka, H., Suzuki, A., and Yamakawa, T. J. Biochem., 94: 2035-2041. (1983).

5. Otsuka, H. and Yamakawa, T. J. Biochem., 90: 247-254. (1981).

6. Alvarez, J.G., Touchstone, J.C., Storey, B.T., and Grob,R.L. J. Liquid Chromatogr., 12(16): 3115-3120, 1990.

7. Saito, T., and Hakomori, S-I. J. Lipid Res., 12: 257-259. 1971.

8. Alvarez, J.G., Storey, B.T., and Touchstone, J.C.: In situ determination of malondialdehyde on thin layer plates by fluorescence spectrodensitometry. In Planar Chromatography: Tha Analytical Method of Choice in the Life Sciences. (Touchstone, J.C., ed.); John Wiley and Sons, New York, 1990.