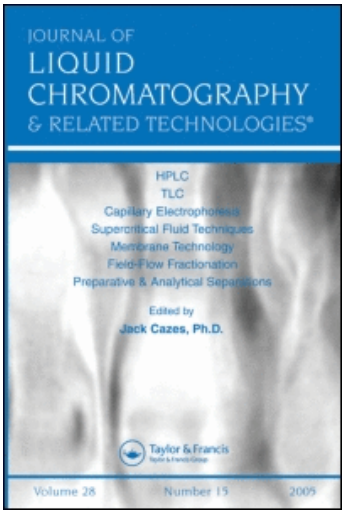

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SEPARATION OF PHOSPHOLIPIDS AND GLYCOLIPIDS USING ANALYTICAL TOROIDAL-COIL COUNTERCURRENT CHROMATOGRAPHY. I. SEPARATION OF HUMAN BRAIN LIPIDS

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ABSTRACT

We demonstrated the separation of human brain lipids using the toroidal-coil countercurrent chromatography (TC-CCC). It became possible to select the suitable two-phase solvent systems, because retention of a stationary phase is much more stable in the TC-CCC than in high-speed countercurrent chromatography (HS-CCC). Optimizing the solvent systems, we succeeded in

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separating major brain lipids. The two-phase solvent of chloroform : methanol : water (5 : 4 : 3) was suitable for the separation of acidic phospholipids (phosphatidic acid, phosphatidylserine, phosphatidylinositol, lysophosphatidylinositol, and lysophosphatidylserine). Using hexane : ethylacetate : ethanol : 0.1% aqueous ammonia (5 : 5 : 5 : 4), neutral phospholipids (phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine) were separated. Non-polar lipids (cholesterol, alkali-labile glycoglycerolipids and cerebrosides) were separated using the solvent of hexane : ethanol : water (10 : 15 : 4). Sphingomyelin (SPM), cerebrosides, and phosphatidylcholine are each reported to have more than 100 molecular species, which are derived from variations of the hydrophobic tail group in mammalian. For this reason, SPM was further separated into two groups (SPM-I and SPM-II). Cerebrosides were separated into several groups using hexane : ethanol : water (5 : 4 : 3). It was clearly shown that synthesized PC (distearoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol) was completely separated. Phosphatidylserine and phosphatidic acid were also separated in some groups. Because the partition behavior of molecules in the two-phase solvent system can be measured, the TC-CCC could be useful not only for the separation but also for the biological analysis of mammalian cell-membrane lipids.

INTRODUCTION

Phospholipids, glycolipids, and cholesterol are major components of mammalian cell-membrane lipids. They play important roles in cell signaling transduction, and cell-to-cell recognition or modification of enzyme functions.[1–8] All of these lipids have an amphipathic property, and contain hydrophobic and hydrophilic regions. In the cell-membrane, the hydrophobic and hydrophilic residues and their behavior play an important role in cell functions. The amphipathic property disturbs the separation of these compounds by means of a countercurrent distribution method because the vigorous mixing of two solvent phases causes the formation of an emulsion.

Countercurrent chromatography (CCC) is a liquid partition chromatography, which eliminates the use of a solid support.[9–11] CCC utilizes two immiscible solvent phases. The partition process takes place in an open column in which one phase (the stationary phase) is retained while the other phase (the mobile phase) is continuously equilibrating with the stationary phase. In order to retain the stationary phase in the column, the system uses various combinations of column

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configuration and force fields (gravitational and centrifugal). Liquid partition chromatography is a system without a solid phase. Therefore, it may become a useful method for analyzing the hydrophobicity and the behavior of lipids, avoiding the influence of a solid phase or spacers.

Previously, we have shown that HS-CCC could separate alkali-labile glycolipids (ALGLs), which had been isolated from the human brain, into several groups.[12] In that experiment, HS-CCC could finely separate the molecular species in the final step of purification. Nevertheless, the retention of a stationary phase is poor in HS-CCC. Therefore, the available two-phase solvent systems are limited. Also, it was actually difficult to separate them from crude materials due to severe emulsification and the subsequent loss of the stationary phase from the column. To overcome this problem, we developed an analytical scale TC-CCC, which has the advantage of using centrifugal force to retain the stationary phase.[13] In this system, a stable two-phase separation can be obtained by increasing the revolution speed (centrifugal force) and/or decreasing the flowrate to avoid emulsification. We can use most of the two-phase solvent systems for this TC-CCC.

In this study, the TC-CCC system was applied in order to solve the problem of emulsification, and a satisfactory stationary phase retention was obtained. Human brain lipids could then be separated. We established the solvent systems that are used for the separation of most of the phospholipids, glycolipids, and less-polar lipids of the human brain. Furthermore, we demonstrated that the molecular species, which are derived from variations of the hydrophobic tail group, were separated by optimizing the composition of the solvents. The TC-CCC is available for use in the analysis of the hydrophobicity of various lipid molecules in biomembranes.

EXPERIMENTAL

Apparatus

The present studies employ a commercial model of the toroidal coil centrifuge (TC-CCC 1000) purchased from Pharma-Tech Research Corporation (Baltimore, MD, USA). The apparatus is a compact tabletop unit measuring 30 cm × 30 cm × 40 cm. It is equipped with a flow-through device without the use of rotary seals, according to a principle described previously.[14] The rotation speed is continuously adjustable up to 3000 rpm with a speed regulator equipped with a digital display. The toroidal coil separation column was prepared by winding a 0.4 mm ID, 15–100 m PTFE (polytetrafluoroethylene) tubing (Zeus Industrial Products, Raritan, NJ, USA) onto a nylon pipe of 1.5 mm OD, thus making a right-handed coil. Then the coiled tube was affixed to the inner wall of the cylindrical centrifuge bowl (12 cm in diameter and 5 cm in height), thus

forming a doughnut-shaped configuration (toroidal coil) consisting of two to three coiled layers. The toroidal coil measures about 6 m in length (made from 60 m long PTFE tubing) and consists of 12,000 helical turns with a total capacity of about 8 mL.

The inlet and outlet flow lines were made from thick-wall PTFE tubes (0.35 mm ID) to withstand constant flexing movements. A chromatographic metering pump (model 515 HPLC pump, Waters, USA) was used for pumping in the mobile phase, and a fraction collector (Ultrorac, LKB Instruments, Stockholm, Sweden) was used to collect the eluate into test tubes.

Reagents

Phospholipid standards (Phospholipid kit) were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). Cerebroside (bovine), D-a-phosphatidylcholine dipalmitoyl, and D-a-phosphatidylcholine distearoyl were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chloroform and methanol, both of glass-distilled chromatographic grades, were purchased from Burdick–Jackson Labs. (Muskegon, MI, U.S.A), and a reagent grade of glacial acetic acid, water, hexane, and ethyl acetate from Fisher Scientific Co. (Fair Lawn, NJ, USA). Ethanol was purchased from Pharmaco Products Inc. (Brookfield, CT, USA). Trifluoroacetic acid (TFA) was purchased from Pierce Chemical Co. (Rockford, IL, USA). Ammonium hydroxide was purchased from J. T. Baker (Philipsburg, NJ, USA).

Lipid Extraction and Purification

Human brain tissue (200 g wet weight) was homogenized, and the total lipids were successively extracted with 3 liters each of mixtures composed of chloroform : methanol, 2 : 1, 1 : 1, and 1 : 2, by volume. The lipid extracts were combined and evaporated to dryness in a rotary evaporator, then suspended, dialyzed against distilled water and lyophilized. Unbound (neutral) lipids and bound (acidic) lipid fractions were separated with a column packed with DEAE Sephadex A-25 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) (bed volume 200 mL).

Preparation of Two-Phase Solvent System and Sample Solution

Each solvent system was thoroughly equilibrated in a funnel to separate at room temperature. The sample solution was prepared by dissolving 1–5 mg of lipids in the 0.05 mL each of the upper and lower phase.

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CCC Procedure

In each separation, the toroidal coil was first entirely filled with a stationary phase (either the upper or the lower phase), and a sample solution was injected into the coil. Then the mobile phase (organic phase) was pumped into the column while the column was rotated at the desired rate. The effluent from the outlet of the column was collected in test tubes at a rate of 0.2 mL/tube at a flow rate of 0.1 mL/min. After the desired peaks eluted, the centrifuge run was terminated and the column contents were fractionated into test tubes at 0.5 mL/tube by eluting the column with the solvent initially used as the stationary phase, at a flow rate of 0.25 mL/min.

High-Performance Thin-Layer Chromatography (HPTLC)

Lipids were separated on a high-performance thin-layer chromatography (HPTLC) plate. The developing solvent was a mixture of chloroform, methanol, and 0.2% aqueous $CaCl_2$. Orcinol reagent[15] and Dittmer's reagent[16] were used for the detection of glycolipids and phospholipids.

RESULTS

Figure 1 shows the procedure for the acidic and neutral lipid preparation. Human brain tissue was homogenized and extracted using a mixture of chloroform and methanol. Then, the lipid extract was dialyzed and applied to

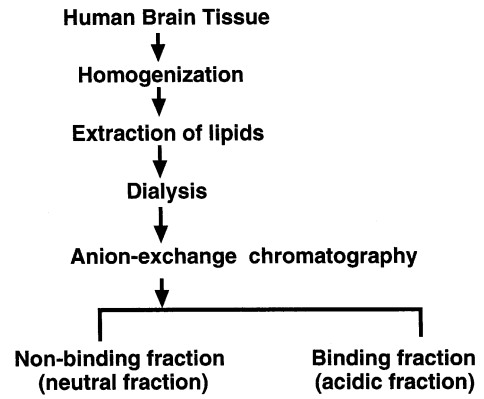


Figure 1. Procedure to obtain acidic and neutral fractions.

the anion exchange column. The binding fraction (acidic fraction) and the non-binding fraction (neutral fraction) were separated. Cholesterol, phospholipid, and glycolipids were the main components of these fractions. The general structures of these lipids are summarized in Figure 2. In glycolipids and phospholipids, there are two types of lipids. One is a sphingo-type and the other is a glycero-type. In human beings, sphingomyelin is only one sphingo-type phospholipid. In glycosphingolipids, the X represents various carbohydrates. The carbohydrate that contains sialic acid is called the ganglioside, and the ganglioside is eluted as a binding fraction using anion-exchange column chromatography. We tried various solvent systems and various compositions for each solvent system. We then determined that the following conditions are the most appropriate for the separation of the lipids.

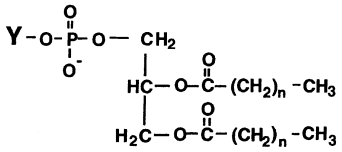
Separation of Acidic Fraction

For the separation of an acidic fraction, a chloroform/methanol/water system is appropriate. If the solvent compositions are not optimized, the phospholipids and glycolipids are eluted at the solvent front or at the column contents. When we chose a suitable solvent, acidic phospholipids (phosphatidic acid, phosphatidylserine, phosphatidylinositol, lysophosphatidyl-inositol, and lysophosphatidylserine) were separated satisfactorily between the solvent front and the column contents (Figure 3A). Two mg samples of the acidic fraction of human brain lipids was applied to the TC-CCC using the chloroform : methanol : water (5 : 4 : 3) solvent system. Aliquots of each fraction were spotted and developed on an HPTLC.

Phospholipids were visualized as blue-colored bands using Dittmer's reagent, which specifically stains phospholipids. Phosphatidylinositol (PI) is separated completely from phosphatidic acid (PA) and phosphatidylserine (PS) (Figure 2). The PA, which is the most hydrophobic phospholipid in this solvent system, eluted first, followed by PS, PI, lysoPI and lysoPS. These phospholipids were identified using phospholipid standards. The minor phospholipid components (fr. 65–68) may be either PIP or PIP2. Each phospholipid (e.g. PS or PI) was further separated in some groups, because each phospholipid has various molecular species, which are derived from variations of hydrophobic tails (ceramide or diacylglycerol). We will show the data of further analysis in Figure 6 later. Glycolipids were visualized as pink- or purple-colored bands using orcinol reagents (data not shown). Sulfatides (frs. 40–64) and gangliosides (GM1, GD1a and GD1b) (frs. 68–70) were eluted. The abbreviations for gangliosides (GM1, GD1a, and GD1b) are according to Svennerholm's nomenclature.[27] The sulfatides and phospholipids were eluted at the same fraction by the TC-CCC.

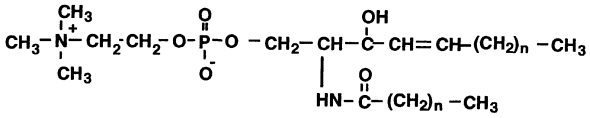
glycerolipids

glycerophospholipids

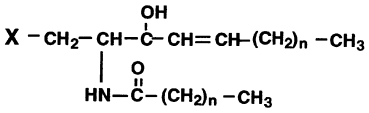


sphingolipids

sphingomyelin



glycosphingolipids



cholesterol

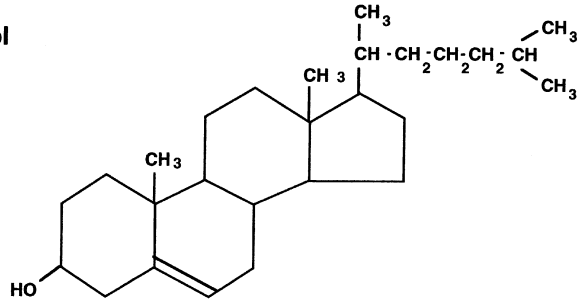


Figure 2. General structures of lipids analyzed in this study. Glycosphingolipid is composed of a ceramide (a hydrophobic-tail group) and a carbohydrate (X). X represents various sugar chains. If the X contains sialic acid, it is called a ganglioside. The gangliosides are eluted as acidic fraction in anion-exchange column chromatography. A glycerophospholipid is composed of diacylglycerol (a hydrophobic-tail group) and Y. Y is a hydrophilic head group. Phospholipid classes show differences in Y residue as follows: —H, PA; $-CH_2CH_2NH_3^+$, PE; $-CH_2CHN^+(CH_3)_3$, PC; $-CH_2CH(NH_3^+)COO^-$, PS; myo-inositol, PI.

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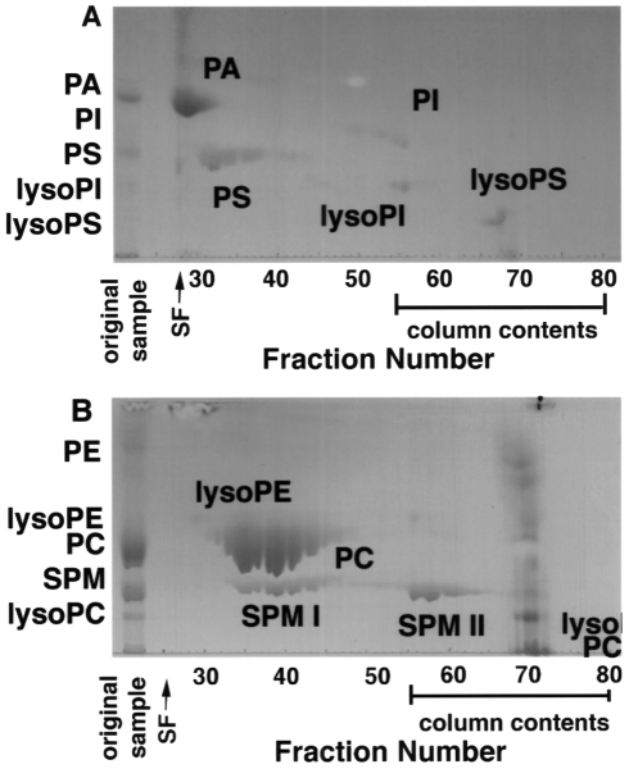

Figure 3. Separation of acidic phospholipids in the human brain. (A) The solvent system used for TC-CCC was chloroform : methanol : water (5 : 4 : 3). The lower phase (organic phase) was mobile. The revolution speed was controlled to run from 1500 rpm to 700 rpm. The highest column pressure was 350 psi. Two milligrams of the acidic fraction of human brain lipids were loaded. Each fraction was spotted and developed on the HPTLC using chloroform : methanol : 0.2% $CaCl_2$ (60 : 32 : 4). Phospholipids were stained with Dittmer's reagent. PA, phosphatidic acid; PS, phosphatidylserine; PI, phosphatidylinositol; lysoPI, phosphatidylinositol; lysoPS, lyso phosphatidylserine. SF, solvent front. (B) Separation of neural phospholipids in the human brain. The solvent system used for the TC-CCC was hexane : ethyl acetate : ethanol : 0.1% aqueous ammonia (5 : 5 : 5 : 4). The upper phase (organic phase) was mobile. The revolution speed was controlled to run from 1500 rpm to 700 rpm. The highest column pressure was 350 psi. Five milligrams of a neutral fraction of human brain lipids were loaded. Each fraction was spotted and developed on the HPTLC using chloroform : methanol : 0.2% $CaCl_2$ (60 : 32 : 4). Phospholipids were stained with Dittmer's reagent. PC, phosphatidylcholine; SPM, sphingomyelin; PE, phosphatidylethanolamine; lysoPE, lysophosphatidylethanolamine; lysoPC, lysophosphatidylcholine. SF, solvent front.

Separation of Neutral Fraction

Most of the lipids of the neutral fraction were eluted at the solvent front when the chloroform/methanol/water solvent system, which is suitable for the separation of the acidic fraction, was used. We then tried solvent systems, which can separate more hydrophobic lipids. Using the hexane : ethyl acetate : ethanol : 0.1% aqueous ammonia solvent system, we could separate phospholipids (Figure 3B) and glycolipids (data not shown). A 5 mg amount of a neutral fraction from human brain lipids was applied to the TC-CCC using hexane : ethyl acetate : ethanol : 0.1% aqueous ammonia (5 : 5 : 5 : 4). Phosphatidylcholine (PC), sphingomyelin (SPM), and lysophosphatidylcholine (lysoPC) were eluted successively. Phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (lysoPE) and other minor phospholipid components were retained as the column contents with this solvent system. Cerebroside (CS) (fr. 28–36) and some other neutral glycolipids (frs. 35–43 and frs. 55–63) were visualized on HPTLC when we used orcinol staining (data not shown). Cholesterol (Chol) is eluted at the solvent front (frs. 28–32) and stained non-specifically (a brown color). It should be noted that SPM, which is usually difficult to separate into two groups with a silica-bead column, is completely separated into two groups (SPM I and SPM II) (Figure 3).

Separation of Less-Polar Neutral Lipids

Cholesterol and cerebrosides were difficult to separate with the hexane : ethyl acetate : ethanol : 0.1% aqueous ammonia system. Therefore, we used another solvent system to separate less hydrophobic lipids. A 5 mg amount of a neutral fraction from human brain lipids was applied to the TC-CCC using hexane : ethanol : water at a volume ratio of 10 : 15 : 4 (Figure 4A). In Figure 4, the orcinol staining shows the specific purple color for cerebroside (CS) and ALGLs,[12] and a non-specific brown color for other lipids (not identified). Most of the phospholipids and glycolipids were retained in the column contents. Cholesterol was isolated from other lipids (frs. 38–43) (Figure 4A). Cerebroside is eluted at frs. 60–72.

We changed the composition of the solvent system to see whether the TC-CCC could separate molecular species of CS. Figure 4B shows the result when we used hexane : ethanol : water at a volume ratio of 5 : 4 : 3. It has been reported that CS is composed of more than 100 molecular species. The difference in intensity in the two bands represents the different molecular species. To show more clearly that the TC-CCC can separate molecular species, the following studies were carried out.

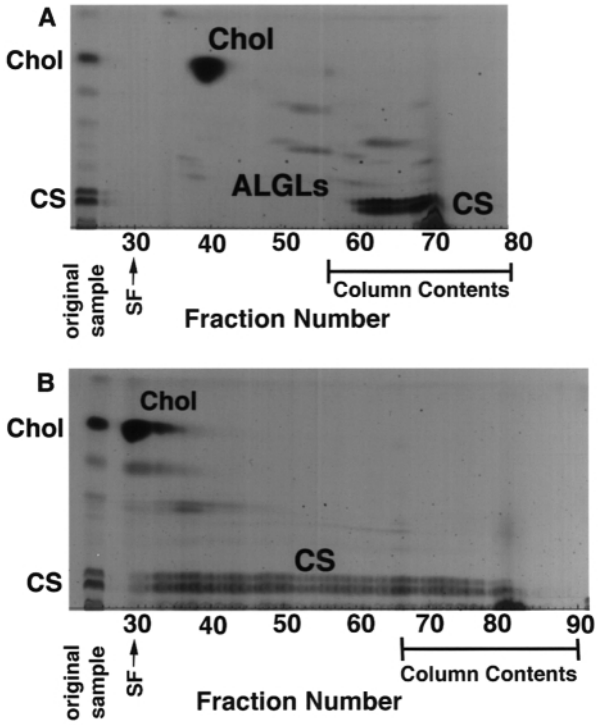

Figure 4. Separation of highly non-polar neutral glycolipids in the human brain. The solvent system used for the TC-CCC was hexane : ethanol : water (10 : 15 : 4) (A) and (5 : 4 : 3) (B). The upper phase (organic phase) was mobile. The revolution speed was controlled to run from 1500 rpm to 700 rpm. The highest column pressure was 350 psi. Five milligrams of a neutral fraction of human brain lipids were loaded. Each fraction was spotted and developed on the HPTLC using chloroform : methanol : 0.2% $CaCl_2$ (90 : 12 : 1). Chol, cholesterol; CS, cerebroside. SF, solvent front.

Separation of Molecular Species

To assess the ability of the TC-CCC molecular species, two molecular species of phosphatidylcholine, which were synthesized, were subjected to the TC-CCC. Dipalmitoyl phosphatidylcholine (PC C16 : 0) and distearoyl phosphatidylcholine (PC C18 : 0), two of the major molecular species of phosphatidylcholine, were completely separated as shown in Figure 5. Distearoyl phosphatidylcholine contains 2 moles of esterified stearic acids and dipalmitoyl phosphatidylcholine contains 2 moles of esterified palmitic acids. The structures of these compounds are shown in Figure 5. These two compounds were

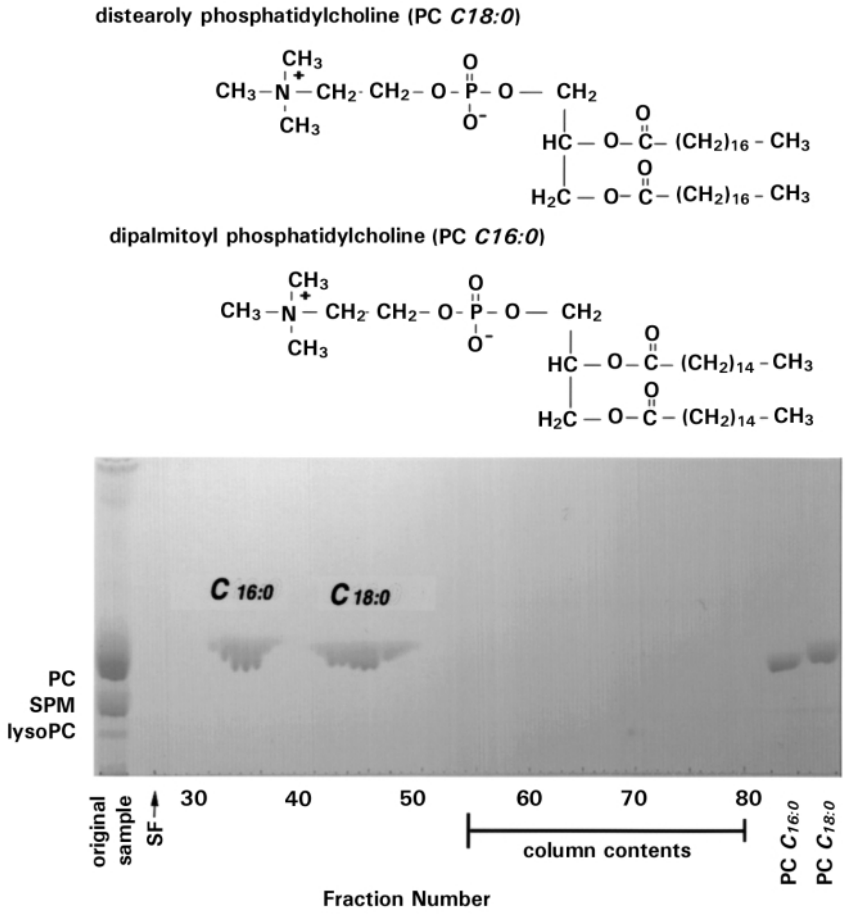

Figure 5. Separation of dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine. The solvent system used for the TC-CCC was hexane : ethyl acetate : ethanol : 1% trifluoroacetic acid (5 : 5 : 5 : 4). The upper phase (organic phase) was mobile, the revolution speed was controlled to run from 1500 rpm to 700 rpm. The highest column pressure was 360 psi. One hundred μg each of phosphatidylcholine dipalmitoyl and phosphatidylcholine distearoyl were loaded. Each fraction was spotted and developed on the HPTLC using chloroform : methanol : 0.2% $CaCl_2$ (60 : 32 : 4). Distearoyl phosphatidylcholine contains 2 moles of esterified stearic acids and dipalmitoyl phosphatidylcholine contains 2 moles of esterified palmitic acids. PC C16 : 0, phosphatidylcholine dipalmitoyl; PC C18 : 0, phosphatidylcholine distearoyl. SF, solvent front.

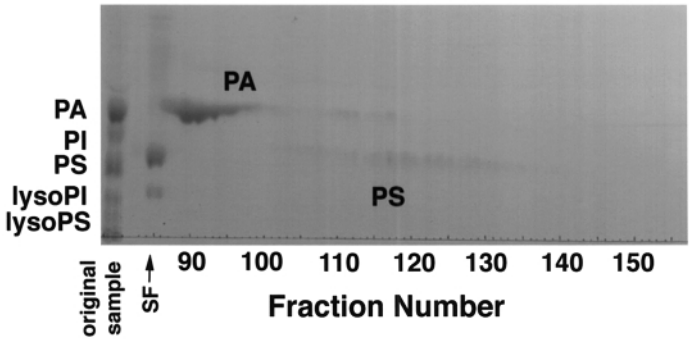

Figure 6. Separation of molecular species of phosphatidic acid and phosphatidylserine. The solvent system used for the TC-CCC was chloroform : methanol : water (5 : 4 : 3). The lower phase (organic phase) was mobile. The revolution speed was controlled to run from 1500 rpm to 700 rpm. The highest psi was 350. Two milligrams of an acidic fraction of human brain lipids were loaded. Each fraction was spotted and developed on the HPTLC using chloroform : methanol : 0.2% $CaCl_2$ (60 : 32 : 4). The elution speed was 0.05 mL/min, and 0.1 mL/tube was collected. SF, solvent front.

completely separated. This result indicates that the TC-CCC system can separate molecular species in both phospholipids and glycolipids.

The other data seen in Figure 6 show the potential of the TC-CCC to separate molecular species of each lipid. Previously, we reported that better separation can be obtained by decreasing the flow rate.[13] Therefore, both the flow rate and the fraction volume were reduced from 0.1 mL/min to 0.05 mL/min, and from 0.2 mL/tube to 0.1 mL/tube, respectively. Phosphatidic acid and phosphatidylserine were separated in some bands (a–e); this is not clear in Figure 3A. LysoPI and lysoPS have not yet been eluted.

DISCUSSION

Advantage of the TC-CCC

The applications of countercurrent distribution to lipid purification were already reported in the 1950s. These included the isolation of PC, SPM, and cerebrosides from the brain[17,18] or the placenta.[19] It was then mentioned that lipids easily emulsify, and this adversely affects their separation. Therefore, these methods were only used for coarse separation. The method also requires a long time for phase separation before each phase transfer, and this procedure needs to be repeated 500 to 3000 times. Otsuka et al. reported the application of droplet

countercurrent chromatography to the purification of phospholipids and glycolipids.[20,21] Because the stationary phase retension is much more stable in the TC-CCC than HS-CCC, it has become possible to select appropriate two-phase solvent systems. In this study, we showed the successful separation of human brain lipids using the TC-CCC. Additionally, if a isolated band can be seen on HPTLC, the lipid can be purified using silica column chromatography after TC-CCC separation.

Reverse phase column chromatography using HPLC can be used to separate these compounds according to their hydrophobicity. However, the reported solvent systems contain salt. The TC-CCC system has an advantage in that lipids can be separated without the use of salted buffer. The salt-free fractions facilitate mass spectrometric analysis. We plan to monitor the eluted lipids from the TC-CCC using a mass spectrometer, and this can be achieved using a salted-buffer-free solvent systems.

Separation of Molecular Species

Human phospholipids and glycolipids have many forms (molecular species) because of the variation in fatty acids or ceramides. Phosphatidylcholine, sphingomyelin, and cerebroside are each reported to have more than 100 molecular species.[22,23] As shown in Figures 5 and 6, it is possible to obtain better resolution by changing the flow rate and the amount of fractions. Also, better conditions can be optimized by changing the composition of the solvents to target one lipid as shown. One example is shown for cerebrosides in Figure 4B. In this paper, we have shown that this system can separate molecular species.

It should be emphasized that the SPM was completely separated into at least two groups (SPM I and SPM II). It was reported, that there are two forms of dihydrosphingomyelin (DHS) and sphingomyelin (SPM) in commercially available bovine brain SPM;[24] the SPM I and SPM II may correspond to these.

The TC-CCC is still in the prototype stage because there are many things that still have to be improved, e.g., the flow volume, the flow rate, and the tube length, as described previously. In addition, the injection system and the detection system could be improved. If these things were to be improved, resolution may improve dramatically.

Applications for Biological Analysis

The authors think there is a possibility that TC-CCC can be useful not only for the separation of lipids but also as an instrument for showing the hydrophobic behavior of lipids in cell membranes. Both phospholipids and glycolipids are

important components of cell membranes in which molecular interactions between hydrophilic or hydrophobic molecules play an important role in a cell's physiology. Avoiding the effects of hydrophobicity in a solid phase system, we are able to study molecular interactions between lipids and hydrophobic molecules. The advantage of the present system is that the net partition behavior of molecules in the two-phase solvent system can be measured by monitoring the retention time of eluted molecules. As a result, we can obtain the partition coefficient (K) of molecules in the two-phase solvent system. This may provide a method for measuring the lipid–protein interaction in a cell membrane. This method may be useful for the purification and analysis of caveolae, lipoprotein, or such hydrophobic microdomains in biomembranes[2,6,25,26).

We determined appropriate solvent systems and showed the hydrophobicity of lipids. In other words, most of the major human brain lipids were mapped on the HPTLC using TC-CCC. Calcium plays an important role in a biomembrane. It is interesting that calcium improves the separation of lipids on the HPTLC.[28] In this system, it is possible to see the effect of either calcium or pH on the elution profile of lipids.

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