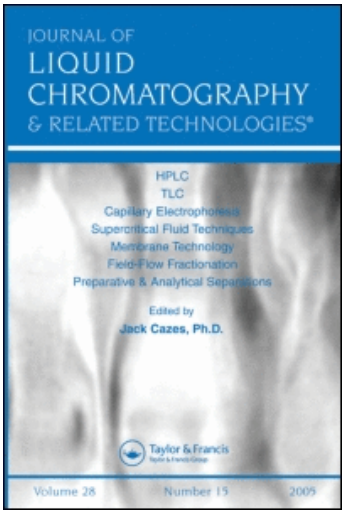

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ISOLATION OF LESS POLAR ALKALI-LABILE GLYCOLIPIDS OF HUMAN BRAIN BY HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

High-speed countercurrent chromatography (HSCCC), a liquid-liquid partition system, was used for the final purification of less polar alkali-labile glycolipids (ALGLs) of human brain. It has been reported that vertebrate brain contains ALGLs consisting of ester cerebroside and monoglucosyldiacylglycerol. ALGLs have alkali-labile ester bonds and are shown to be less polar than cerebroside.

First, ALGLs (ALGL-I, II, III and IV) were extracted and isolated by repeated silica gel column chromatography. Then, the mixture of ALGLs was subjected to the HSCCC in which a solvent system, hexane/ethanol/water (5:4:1, by volume), was used with the lower phase mobile. ALGL-IV and -III were clearly separated. ALGL-IV, which was resolved as a single band on high performance thin-layer chromatography, was further separated into several components (ALGL-IVa, b, c, d and e). This is the first application of HSCCC for the separation of human brain ALGLs. The availability of purified ALGLs provides an opportunity to determine their structure, metabolic pathway, and function in relation to human health and disease.

INTRODUCTION

The only glycolipids whose polarities are lower than that of cerebroside (glucosylceramide or galactosylceramide) reported in the vertebrate brain, are ester cerebroside and monoglucosyldiacylglycerol.[1-7] The mobilities of these less polar lipids on thin-layer chromatography are greater than cerebroside. The less polar glycolipids are alkali-labile because of the presence of ester bonds. The hexose component of these glycolipids was reported as galactose or glucose. Yasugi *et al.* isolated less polar glycolipids from human brain by silica gel, and showed the presence of 2-*O*-acyl-, 3- *O*-acyl-, 4-*O*-acyl- and 6-*O*-acylgalactosylceramides by methylation, gas chromatography and mass spectrometry.[7] Tamai *et al.*[5] purified less polar glycolipids in *Alaskan pollack* brain and other animal brain by stepwise elution with chloroform and methanol using silica gel. By methylation and mass spectrometry, it was shown that a fatty acid was bound to various hydroxy groups of cerebroside. Theret *et al.*[8] purified less polar glycolipids from rat brain using preparative TLC. They showed the presence of 2-*O*-acyl-, 3-*O*-acyl-, 4-*O*-acyl- and 6-*O*-acylgalactosylceramides and also that of galactosylcerebroside in which a fatty acid is esterified to the α-hydroxy group of the hydroxy acid. The presence of ester cerebrosides has also been reported in animal skin.

Hamanaka *et al.*[9] purified less polar lipids into molecular species by silica gel column and reverse phase column chromatography, and determined one of the structures as glucosyl β1-*N*-(α-*O*-linoleoyl)acyl-sphingosine. Until now, there is no report about the isolation of less polar glycolipids from human brain tissue and their precise structural analysis.

High-speed countercurrent chromatography (HSCCC) is a liquid-liquid partition system developed by Ito *et al.*[10] The first application of CCC to lipid purification was reported in the 1950's. Ohtsuka *et al.* reported the application of this system for the purification of glycosphingolipids and phospholipids.[11,12] In this study, we report the application of HSCCC as a final step in the purification of less polar glycolipids of human brain.

EXPERIMENTAL

Materials

Human brain tissue (frontal lobe) was obtained from schizophrenia patients at postmortem from NIMH Neuroscience Center at St. Elizabeth Hospital (Washington DC), and was stored at -80°C until the extraction of lipids.

Lipid Extraction and Purification

Human brain tissue (200 g wet weight) was homogenized, and total lipids were extracted with each 3 L of mixtures of chloroform:methanol, 2:1, 1:1, and 1:2, by volume, successively. The total lipid extract was evaporated to dryness in a rotary evaporator, suspended, dialyzed against distilled water, and then lyophilized.

Unbound (neutral) lipids and bound (acidic) lipids fractions were separated with a column packed with DEAE Sephadex A-25 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) (bed volume 200 mL) as described previously.[13,14] Then, the neutral (nonbinding) lipid fraction was dissolved in chloroform, applied to a column packed with Iatrobeads 6RS-8060 (Iatron Co., Ltd., Tokyo, Japan), which had been pre-equilibrated with chloroform, followed by elution with a linear gradient of chloroform-methanol. The elution profile of glycolipids was monitored by high-performance thin-layer chromatography (HPTLC).

By repeated Iatrobeads column chromatography, using linear gradient of chloroform : methanol, about 20 mg of mixture of the less polar glycolipids was obtained. Five mg of these lipids were subsequently subjected to HSCCC separation.

HSCCC Separation

HSCCC was performed with a two-phase solvent system composed of hexane/ethanol/water (5:4:1, by volume). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature, and the two phases were separated before use. The multilayer coil was filled with the upper stationary phase. The lower mobile phase was pumped into the inlet of the column at a flow rate of 3 mL/min, while the apparatus was rotated at 800 rpm. After the mobile phase front emerged and the two phases had established hydrodynamic equilibrium, the sample solution (5 mg of the lipids in 10 mL of the mobile phase) was applied through the injection valve. The effluent was collected in glass tubes with a fraction collector, and each fraction was subjected to HPTLC analysis.

High-Performance Thin-Layer Chromatography (HPTLC)

Lipids were separated on a high performance thin-layer chromatography (HPTLC) plate (Merck). The developing solvent was a mixture of chloroform, methanol, 0.2% aqueous $CaCl_2$ (90:12:1, by volume). Orcinol reagent (15) was used for the detection of glycolipids.

Alkaline Treatment

The glycolipids were treated with 1 mL of 0.6 N NaOH solution in methanol at 37°C for 1 hr. The reaction mixture was dried, and dissolved in 4 mL of solvent (chloroform:methanol=2:1, by volume) and 1 mL of water. The lower phase was collected and subjected to HPTLC analysis.

RESULTS AND DISCUSSION

After ion exchange and repeated silica gel column chromatography, the less polar glycolipids of human brain (ALGL's) were obtained. In Fig. 1a, the final silica gel column chromatography profile of ALGL's is shown. The ALGL's were clearly separated from cerebroside, however, the ALGL's could not be separated from each other. These ALGL's were alkali-labile and migrated close to cerebroside on HPTLC after alkali treatment (Fig. 1b). The mixture of ALGL's was then subjected to HSCCC. The elution profile is shown in Fig. 2. The lowest band (ALGL-IV) was separated from the other ALGL's. Furthermore, the lower band (ALGL-IV) was separated into

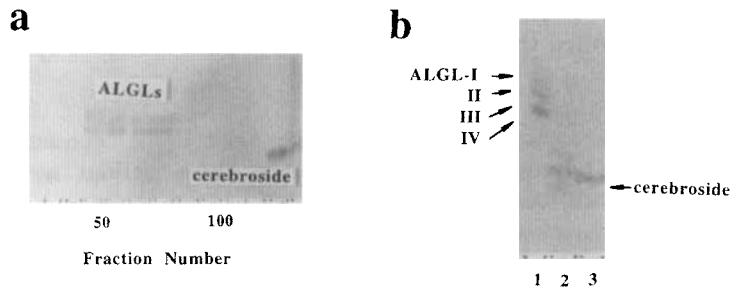

Figure 1. (a) Elution profile of glycolipids by Iatrobeads column chromatography (b) HPTLC of ALGLs, and their products after alkali treatment. The solvent system was of chloroform, methanol, 0.2% aqueous $CaCl_2$ (90:12:1, by volume) and orcinol reagent was used for the detection of bands.

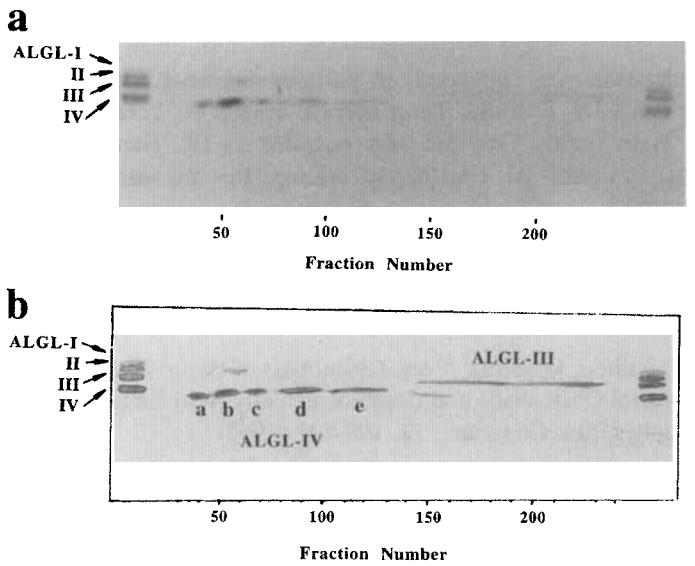

Figure 2. Profile of ALGLs separated by HSCCC (a) and representative scheme (b) ALGLs before HSCCC separation were applied on both sides of the HPTLC plate. The solvent system was of chloroform, methanol, 0.2% aqueous $CaCl_2$ (90:12:1, by volume) and orcinol reagent was used for the detection of bands.

at least five other components (ALGL-IVa, b, c, d and e). Although HPTLC has high resolution, the lower band could not be separated by this method. However, HSCCC clearly separated the ALGL-IV into several components. NMR analysis showed that these lipids were more than 95% pure (paper in preparation).

It has been reported that the less polar glycolipids, consisting of monoglycosyldiacylglycerol and ester cerebrosides, have various molecular species of fatty acids, ceramide and different sites of fatty acylation.[5,7-9,16] It was reported that ester cerebroside of skin contained long chain α-hydroxy fatty acid (30:0 or 32:1).[9,16] On the other hand, rat brain contains α-hydroxy fatty acid (16:0 or 18:0).[8]

Purification using HSCCC makes it possible to analyze the structures of these components more precisely by mass and NMR spectrometry, and also the study of metabolic pathway and its abnormality in human brain.

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