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Improved separation of lysoglycolipids from solvolysates by reversed-phase high-performance liquid chromatography

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ABSTRACT

This paper describes the preparation of lysoglycosphingolipids by improved separation procedures. After desalting from the hydrazinolysate or alkaline hydrolysate of neutral glycolipids and sulphatide, the lipid mixtures were fractionated on a second reversed-phase high-performance liquid chromatography column to yield lysogalactosylceramide, lysolactosylceramide, lysoglobotriaosylceramide and lysoglobotetraosylceramide with a recovery rate of 50–68%. Lysosulphatide was separated from the desalted hydrazinolysate by DEAE-Sephadex column chromatography with a recovery rate of 75%. The purified lysoglycolipids were characterized by proton NMR spectrometry.

INTRODUCTION

Lysoglycosphingolipids (lysoGSLs), which are *N*-deacylated at the ceramide moiety in GSLs, accumulate in the brains of patients with glycosphingolipidoses [1–3] and in the twitcher mouse brain [4,5]. Such accumulations of lysoGSLs are suspected to be responsible for the pathological manifestations of these diseases [6], although they are also detected in minute amounts in the normal brain [7,8]. Cytotoxic lysoGSLs, such as sphingosine, are powerful inhibitors of protein kinase C [9].

LysoGSLs can be converted into artificial GSLs with a uniform carbon length that can be radiolabelled at the acyl moiety. For the laboratory preparation of lysoGSLs, intact GSLs are *N*-deacylated by saponification [10–13] or by hydrazinolysis with [14] or without [15] hydrazine sulphate as an effective catalyst. The hydrazinolysis of GSLs containing *N*-acetylhexosamine and/or sialic acid, however, yields various *N*-deacylated products, depending on the time and temperature of the reaction [14]. In many instances the reaction mixture is dialysed to remove low-molecular-mass non-lipid materials, followed by the isolation of lysoGSLs by silicic acid chromatography. With these procedures for the preparation of lysoGSLs, the recovery is generally low.

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In this paper, reversed-phase high-performance liquid chromatography (HPLC) is described as an effective procedure for the isolation of lysoGSLs from the hydrazinolysate or hydrolysate. The separated lysoGSLs were characterized by proton NMR spectrometry.

EXPERIMENTAL

Materials

Anhydrous hydrazine was purchased from Eastman Kodak (NY, USA). The Sep-Pak C_{18} cartridges and Wakosil C_{18} columns (5C18-200, 4.6 × 250 mm) were obtained from Waters (Milford, MA, USA) and Wako (Tokyo, Japan), respectively. Pre-coated thin-layer chromatography (TLC) plates (silica gel 60, 20 × 20 cm) were from Merck (Darmstadt, Germany). All other reagents were of analytical-reagent grade. Galactosylceramide (GalCer) and sulphated galactosylceramide (sulphatide) were purified from porcine brain. Lactosylceramide (LacCer) was isolated from equine erythrocytes as reported previously [16]. Globotriaosylceramide (Gb_3Cer) and globotetraosylceramide (Gb_4Cer) were prepared from porcine erythrocytes [17].

Hydrazinolysis and saponification of GSLs

The ratio of the solvent mixture is expressed by volume unless stated otherwise.

Hydrazinolysis was carried out according to the method of Suzuki *et al.* [14], except that the reaction temperature was 130°C. Briefly, 20–800 mg of GSLs, except for Gb_4Cer, were heated at 130°C for 16 h in 0.1–1.6 ml (200–500 mg/ml) of anhydrous hydrazine containing 2% (w/v) hydrazine sulphate in a glass tube (15 mm × 8 cm) with a cap sealed with PTFE tape. The hydrazinolysate was then supplemented with chloroform–methanol–water (3:48:47) until the solution became clear. Gb_4Cer (200 mg), however, was saponified with 1 *M* tetramethylammonium hydroxide in 10 ml of *n*-butanol at 100°C for 13 h according to the method of Sonnino *et al.* [13] The saponification mixture was diluted with an equal volume of water.

The diluted reaction mixture of hydrazinolysis or saponification was directly applied to Sep-Pak cartridges (15 mg or less of starting GSL per cartridge) and successively washed with the chloroform–methanol–water mixture and water [18] until the washings became negative to Nessler's reagent for hydrazinolysis, or the pH became neutral in saponification. LysoGSL and unreacted GSL were eluted completely from one cartridge with 1 ml of methanol and 5 ml of chloroform–methanol–water (60:30:4.5). The eluates were concentrated and developed by TLC with chloroform–methanol–water–acetic acid (60:40:8:1, solvent I), followed by staining with an orcinol–sulphuric acid reagent.

Isolation of lysoGSL by reversed-phase high-performance liquid chromatography or by DEAE chromatography

To isolate lysoGSL, although not lysosulphatide, the desalted sample was dried and dissolved in a minimum volume of chloroform–methanol–water (3:48:47) and applied to a reversed-phase column for HPLC. Elution was performed at a flow-rate of 1 ml/min in 1.5-ml fractions with methanol–water (6:4, 10 ml) using a linear gradient from methanol–water (6:4, 30 ml) to methanol (30 ml) for 60 min, and chloroform–methanol–water (60:30:4.5, 5 ml). Each fraction was concentrated *in vacuo* and

monitored by TLC developed with chloroform–methanol–water (60:35:8, solvent II), followed by staining with an orcinol–sulphuric acid reagent.

After the hydrazinolysate of the sulphatide (200 mg) was treated with the cartridge as described, the eluate was directly applied without concentration on a DEAE-Sephadex A-25 (1.0 × 5 cm, acetate form) column. Lysosulphatide passed through the column with 10 ml of chloroform–methanol–water (30:60:8), while unreacted sulphatide bound to the column. The unreacted sulphatide was eluted with 10 ml of 90 m*M* ammonium acetate solution in methanol followed by desalting with a Sep-Pak cartridge as described.

The fraction containing lysoGSL was combined and examined for purity on a TLC plate developed with solvent I, followed by staining with an orcinol–sulphuric acid or with ninhydrin reagent.

Proton NMR spectrometry

Proton NMR spectra of the isolated lysoGSLs (0.5–2 mg) were obtained in 0.4 ml of [$^2H_6$]dimethylsulphoxide containing 2% ^2H_2O at 90°C on a Varian JNM-GX500 spectrometer in the Fourier-transform mode. The apparatus was equipped with a JEC-980B computer which had a 48k memory capacity, and was used at the High Resolution NMR Laboratory, Hokkaido Unversity, as described previously [19]. The frequency was 500 MHz and the sweep width was 5 kHz. The chemical shifts were indicated by the distance (ppm) from tetramethylsilane as an internal standard.

RESULTS

Desalting from solvolysate

The reversed-phase cartridge technique resulted in the elution of most of the lysoGSLs with methanol–water (7:3 or 8:2) free from the bulk of hydrazine and salts, as seen when the bound and unbound fractions were examined by TLC and Nessler's

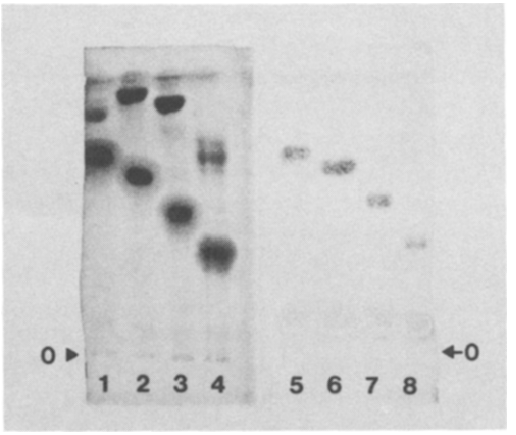

Fig. 1. TLC of hydrazinolysates of GSLs. Hydrazinolysates of GSLs were applied to a Sep-Pak C_{18} cartridge and then chromatographed on silica gel TLC developed with solvent I. Lanes: 1, 5 = hydrazinolysate of sulphatide; 2, 6 = of GalCer; 3, 7 = of LacCer; 4, 8 = of Gb_3Cer. Lanes 1–4 and 5–8 were separately stained by an orcinol–sulphuric acid reagent and a ninhydrin reagent, respectively. O = Origin.

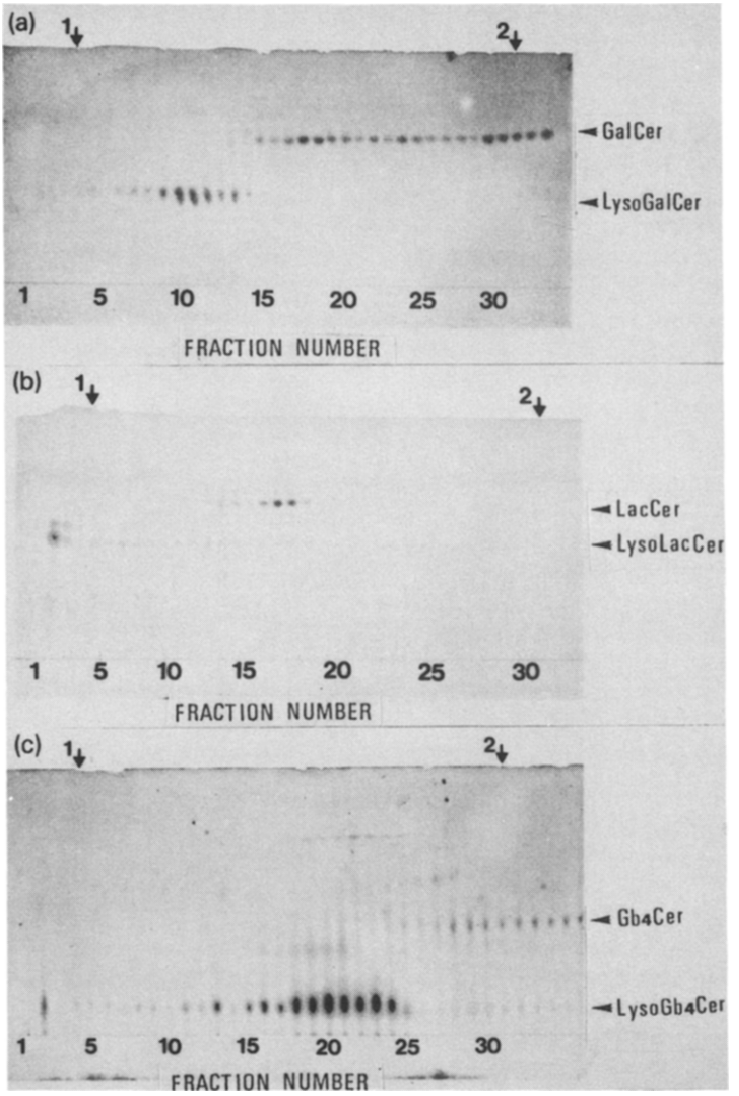

Fig. 2. Separation of lysoGSL from GSL using reversed-phase HPLC. The hydrazinolysates of sulphatide, GalCer, LacCer and Gb_3Cer, and the hydrolysate of Gb_4Cer were desalted using a Sep-Pak cartridge and were applied to a C_{18} column from HPLC as described under Experimental. After gradient elution, each fraction was concentrated and examined by TLC developed with solvent II, followed by staining with an orcinol–sulphuric acid reagent. (a) hydrazinolysate of GalCer; (b) of LacCer; (c) hydrolysate of Gb_4Cer. In each panel, the lower spots were positive for the ninhydrin reaction and the upper spots were negative. Arrows 1 and 2 indicate the start and finish points of gradient elution, respectively.

reagent. However, the co-elution of lysoGSLs with unreacted GSLs from the cartridge was unavoidable, as it was not possible to separate each of these lipids only by using the cartridge. Fig. 1 shows a thin-layer chromatogram of lipid fractions after treatment with the cartridges of hydrazinolysates of sulphatide, GalCer, LacCer and

Gb_3Cer. The slowly migrating, ninhydrin-positive spots can be assigned to the small amounts of remaining hydrazine or fatty acyl hydrazide as a result of the similar mobility of this species in every lane.

Separation of lysoGSL for GSL

When a mixture containing lysoGSL and GSL was subjected to reversed-phase HPLC, the best separation of the lipids was attained by gradient elution from methanol–water (6:4) to methanol. As shown in Fig. 2, lysoGalCer (a), lysoLacCer (b) and lysoGb$_4$Cer (c) were clearly separated from their unreacted, native GSLs, eluting faster than the respective unreacted GSL. However, with a large initial amount (more than 800 mg) of GalCer at the hydrazinolysis step, the lysoGSL was not completely eluted, and further elution with chloroform–methanol–water (30:80:8, Fig. 2a) was necessary. This solvent system was usually also employed for generation of the column.

From 200 mg each of the GSLs, 80 mg (64% of the calculated amount) of lysoGalCer, 95 mg (68%) of lysoLacCer, 87 mg (58%) of lysoGb$_3$Cer and 79 mg (50%) of lysoGb$_4$Cer were obtained using these separation procedures.

For the isolation of lysosulphatide, DEAE chromatography was used instead of a second reversed-phase chromatographic separation. The lysosulphatide was recovered in the unbound fraction on the DEAE column, whereas unreacted sulphatide bound to the column, from which sulphatide was eluted with 90 m*M* ammonium acetate in methanol [20], as shown in Fig. 3. The yield of the lysosulphatide was 75%, which was fairly high compared to the separation using reversed-phase chromatography.

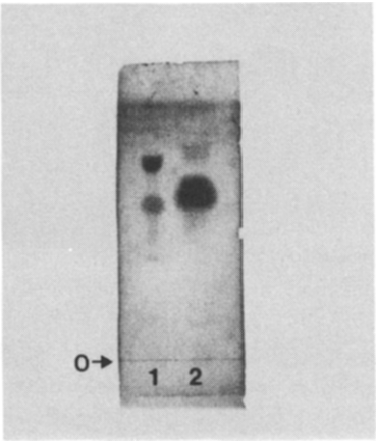

Fig. 3. Separation of lysosulphatide and sulphatide. The hydrozinolysate of sulphatide was desalted and applied directly to a DEAE–Sephadex column. The unbound and bound fractions were obtained as described under Experimental. These fractions were concentrated and separated by TLC developed with solvent I, followed by staining with an orcinol–sulphuric acid reagent. Lanes 1 and 2 demonstrate fractions bound and unbound to the ion exchanger, respectively. O = Origin.

Proton spectrometry of lysoGSLs

The lysoGSLs obtained were charaterized by proton NMR spectrometry, comparing the spectra obtained with the respective native GSLs. The spectrum of lysoGalCer shows the disappearance of a triplet either at 2.10 ppm due to the C–2 hydroxymethine proton or at 5.3 ppm due to cisoid olefinic protons of the fatty acyl group, which were predominantly α-hydroxy fatty acids of porcine brain GalCer (data not shown). Similar observations were made in the spectra of lysoLacCer, lysosulphatide and lysoGb$_4$Cer. In the lysoGSLs, although chemical shifts of the anomeric protons on the carbohydrate chains and transoid olefinic protons on the long-chain base resonated down field, these protons were retained, verifying the chemical structures of the lysoGSLs. Amide protons resonated at 7.5 ppm, and the doublet on the ceramide moiety of the GSLs [21] shifted to a higher field with a triplet in the lysoGSLs examined (data not shown).

DISCUSSION

As lysoGSLs lack one of two hydrocarbon chains and are dialysable in water, the dialysis of lysoGSLs against water is not favourable. The separation of a lysoGSL from the parent GSL by reversed-phase chromatography reported in this paper is based on the difference of hydrophobicity between these two compounds. As lysoGSLs have less mobility than intact GSLs in silicic acid chromatography, these lipids can be separated by this procedure. However, an amino group in the lysoGSL will interact with silicic acid to give tailed elution during the chromatographic separation and will consequently lead to a low recovery on the column. Therefore, reversed-phase chromatography appears to be more advantageous than silica gel chromatography for the separation of lysoGSLs. For the separation of lysosulphatide and sulphatide, the lysosulphatide, which has a neutral charge, did not bind to the DEAE column, whereas the sulphatide did bind to the column. However, the method could not be applied for the separation of monosialoganglioside and its lysoganglioside (data not shown).

Hydrazinolysis was advantageous compared to the standard procedure using alkaline hydrolysis to remove the fatty acyl group at the ceramide moiety of *N*-acetylhexosamine-lacking neutral GSL or sulphatide. The alkaline hydrolysis of sulphatide concomitantly gave a more than 20% of yield of lysoGalCer as a by-product. The hydrozinolysis of GSL containing *N*-acetylhexosamine and/or sialic acid, however, could not be used to obtain a corresponding homogenous deacylation product at the ceramide moiety, as the reaction gave a mixture containing a partially deacylated derivative at *N*-acetylhexosamine and/or the sialic acid moiety. Deacylation of *N*-acetylhexosamine could be avoided by the alkaline hydrolysis of Gb4Cer as demonstrated in this work and of gangliosides as reported previously [22].

The chemical characterization of lysoGSLs has been performed by fast atom bombardment mass spectrometry and carbon NMR spectrometry for lysoGalCer and lysoGlcCer [23] and for sulphatide [24], or by proton NMR for lysogangliosides [22,25]. These spectrometries were effective in confirming the absence of a fatty acyl group in native GSL. In the proton NMR spectum of lysoGalCer, the anomeric proton of the carbohydrate attached to sphingosine shifted to a lower field compared to that of the parent GSL, suggesting that sphingosine and ceramide have different electronegativities.

REFERENCES

1 O. Nilsson and L. Svennerholm, *J. Neurochem.*, 39 (1982) 709.
2 S. Neuenhofer, E. Conzelmann, G. Schwarzmann, H. Egge and K. Sandhoff, *Biol. Chem. Hoppe-Seyler*, 367 (1986) 241.
3 B. Rosengren, J.-E. Mansson and L. Svennerholm, *J. Neurochem.*, 49 (1987) 834.
4 H. Shinoda, T. Kobayashi, M. Katayama, I. Goto and H. Nagara, *J. Neurochem.*, 49 (1987) 92.
5 K. Tanaka, H. Nagata, T. Kobayashi and I. Goto, *Brain Res.*, 454 (1988) 340.
6 Y. A. Hannun and R. M. Bell, *Science*, 235 (1987) 670.
7 H. Igisu and K. Suzuki, *J. Lipid Res.*, 25 (1984) 1000.
8 B. Rosengren, P. Fredman, J.-E. Mansson and L. Svennerholm, *J. Neurochem.*, 52 (1989) 1035.
9 Y. A. Hannun, C. R. Loomis, A. H. Merrill and R. M. Bell, *J. Biol. Chem.*, 261 (1986) 12604.
10 T. Taketomi and T. Yamakawa, *J. Biochem.*, 54 (1963) 444.
11 G. Nonaka, Y. Kishimoto, Y. Seyama and T. Yamakawa, *J. Biochem.*, 85 (1979) 511.
12 K. M. Koshy and J. M. Boggs, *Lipids*, 7 (1982) 998.
13 S. Sonnino, G. Kirschner, R. Ghidoni, D. Acquotti and G. Tettamanti, *J. Lipid Res.*, 26 (1985) 248.
14 Y. Suzuki, Y. Hirabayashi and M. Matsumoto, *J. Biochem.*, 95 (1984) 1219.
15 H. Higashi and S. Basu, *Anal. Biochem.*, 120 (1982) 159.
16 S. Gasa, A. Makita and Y. Kinoshita, *J. Biol. Chem.*, 258 (1983) 876.
17 F. Sako, S. Gasa, A. Makita, A. Hayashi and S. Nozawa, *Arch. Biochem. Biophys.*, 278 (1990) 228.
18 M. A. Williams and R. H. McCluer, *J. Neurochem.*, 35 (1980) 266.
19 S. Gasa, M. Nakamura, A. Makita, M. Ikura and K. Hikichi, *Eur. J. Biochem.*, 155 (1986) 603.
20 S. Gasa, M.-T. Casl, A. Makita, N. Sakakibara, T. Koyanagi and T. Atsuta, *Eur. J. Biochem.*, 189 (1990) 301.
21 S. Gasa, T. Mitsuyama and A. Makita, *J. Lipid Res.*, 24 (1983) 174.
22 S. Neuenhofer, G. Schwarzmann, H. Egge and K. Sandhoff, *Biochemistry*, 24 (1985) 525.
23 A. Hara and T. Taketomi, *J. Biochem.*, 100 (1986) 415.
24 T. Taketomi, A. Hara, Y. Kutsukake and E. Sugiyama, *J. Biochem.*, 107 (1990) 680.
25 Y. Hirabayashi, M. Kimura, M. Matsumoto, K. Yamamoto, S. Kadowaki and T. Tochikura, *J. Biochem.*, 103 (1988) 1.