A new solvent system for the separation of neutral glycosphingolipids

K. Watanabe and Y. Arao

Shigei Medical Research Institute, Division of Biochemistry, 2117 Yamada, Okayama, 701-02, Japan

Summary A solvent system and a column for high performance liquid chromatography for the separation of glycosphingolipids without derivatization is described. A column packed with porous silica gel (Iatrobeads) and eluted with a mixture of isopropanol–hexane–water with increasing water content and decreasing hexane content was used. Glycosphingolipids with mono- to dodeca- or tetrakaidecasaccharides were separated within 60 min and the separation pattern was highly reproducible. The method was applied for preparative separation of highly complex glycolipids with blood group activity.—**Watanabe, K., and Y. Arao.** A new solvent system for the separation of neutral glycosphingolipids. *J. Lipid Res.* 1981. **22:** 1020–1024.

Supplementary key words glucosylceramide · lactosylceramide · globotriaosylceramide · globotetraosylceramide · IV^3-α-N-acetylgalactosaminylglobotetraosylceramide · blood group H-active glycolipids

In recent years, high performance liquid chromatography (HPLC) has been applied for analytical separation of glycosphingolipids as perbenzoylated (1–7), N-benzoylated and O-acetylated (8), and N-acetylated and O-benzoylated (9) derivatives. Gangliosides have been separated without derivatization on silica gel SI-60 column with a chloroform–methanol–aqueous HCl system, equipped with the moving wire flame ionization detector (10). Separation of glycosphingolipids on HPLC without derivatization is preferable because the separated lipids can be directly used in immunological analysis and structural characterization. In this paper, a new solvent system and a suitable column for separation of neutral glycosphingolipids from mono- to dodeca- or tetrakaideca-glycosylceramide are described. The system has been used for the preparation of blood group H-active glycolipids isolated from human O erythrocyte membranes.

Abbreviations: HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; glucosyl ceramide, Glcβ1 → 1Cer; $LcOse_2Cer$, lactosylceramide Galβ1 → 4Glcβ1 → 1Cer; $GbOse_3Cer$, globotriaosylceramide Galα1 → 4Galβ1 → 4Glcβ1 → 1Cer; $GbOse_4Cer$, globotetraosylceramide GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ1 → 1Cer; $nLcOse_4Cer$, neolactotetraosylceramide Galβ1 → 4GlcNAcβ1 → 3Ga1β1 → 4Glcβ1 → 1Cer; IV^{3}GalNAcα-$GbOse_4Cer$ (Forssman glycolipid), IV^3-α-N-acetylgalactosaminylglobotetraosylceramide GalNAcα1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ1 → 1Cer; H_1-glycolipid, IV^2-α-fucosylneolactotetraosylceramide L-Fucα1 → 2Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1Cer; H_2-glycolipid, L-Fucα1 → 2Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1→ 4Glcβ1 → 1Cer; H_3-glycolipid, L-Fucα1 → 2Galβ1 → 4GlcNAcβ1 → 6Gal(3 ← 1βGlcNAc4 ← 1βGal2 ← 1αFuc)β1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1Cer; H_4- and H_5-glycolipid, chemical structure has not been identified; PBS, phosphate-buffered saline, 0.9% NaCl in 0.16 M phosphate buffer, pH 7.2; solvent composition is indicated by volume ratio.

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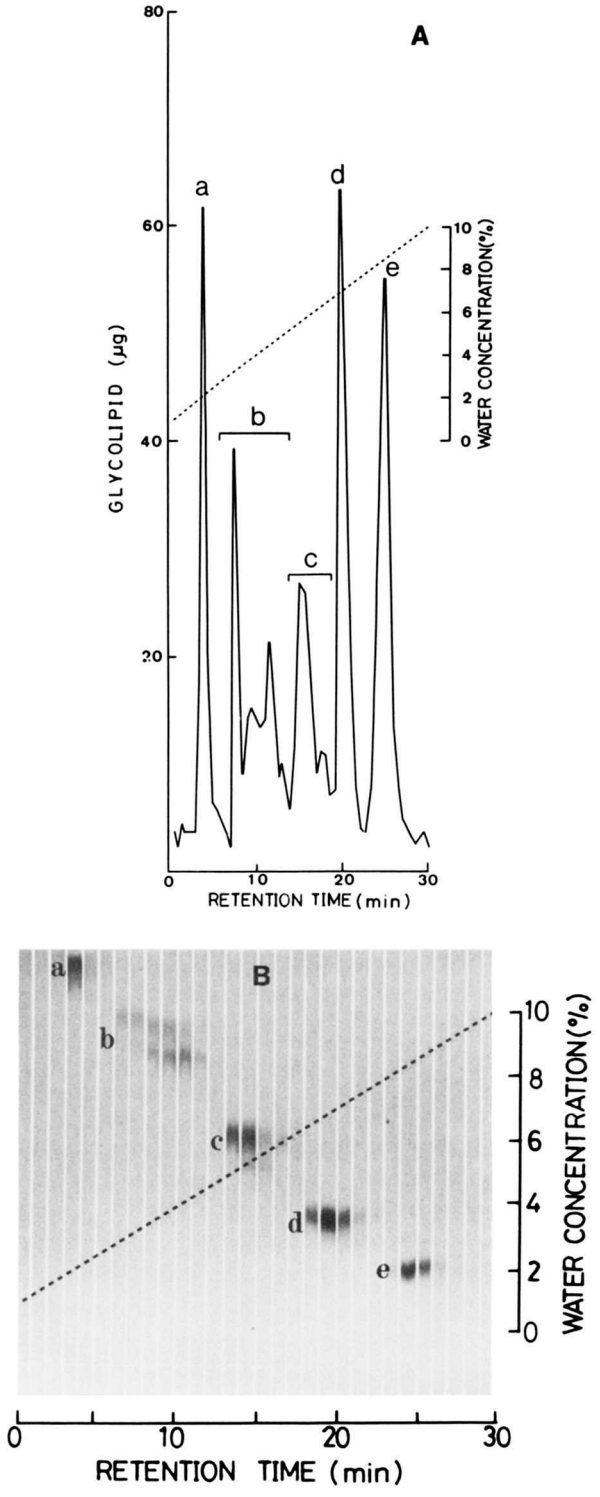

Fig. 1. A: Elution pattern of the standard glycolipid mixture (mono- to pentaglycosylceramide) by HPLC; column: 500 mm × 4 mm I.D., packed with Iatrobeads 6RS-8010 (10 μm, Iatron Lab., Inc.). A mixture of standard glycolipids (100 μg each) was dissolved in 20 μl of isopropanol–hexane–water 55:40:5 and applied to the column. The column was eluted with mixture of isopropanol–hexane–water by a linear gradient from the ratio of 55:44:1 to 55:35:10 at a flow of 2.0 ml/min. A dotted line indicates the concentration of water in isopropanol. Eluates were collected every 0.5 min by fraction collector. Glycolipid content in each fraction was determined by anthrone-sulfuric acid reaction and was calculated as globotetraosylceramide. a, glucosylceramide; b, $LcOse_2Cer$; c, $GbOse_3Cer$; d, $GbOse_4Cer$; e, $IV^3GalNAc\alpha$-$GbOse_4Cer$. B: Thin-layer chromatogram of the standard glycolipid mixture eluted from the Iatrobeads column by HPLC. The conditions were the same as in Fig. 1A. One hundred μl out of each fraction (2 ml) was evaporated, dissolved in chloroform–methanol 2:1, and spotted on a precoated silica gel G plate. The plate was developed with chloroform–methanol–water 65:25:4 and spots were visualized by spraying with orcinol reagent. a, glucosylceramide; b, $LcOse_2Cer$ (double spots); c, $GbOse_3Cer$ (double spots); d, $GbOse_4Cer$; e, $IV^3GalNAc\alpha$-$GbOse_4Cer$. The dotted line indicates a water concentration in isopropanol.

EXPERIMENTAL

Apparatus and method

HPLC[1] was performed with an Hitachi Liquid Chromatograph model 638 which was equipped with a solvent, flow, and pressure programmer. A stainless steel column (500 mm length and 4 mm I.D.) was slurry-packed with 10 μm porous silica spheres Iatrobeads 6RS-8010[2] (Iatron Lab., Inc., Tokyo) in a mixture of tetrabromoethane–tetrachloroethylene 60:40 at 400 kg/cm². The column was washed with isopropanol–hexane 55:45 and was equilibrated with the starting solvent (e.g., isopropanol–hexane–water 55:44:1). The elution was with a linear gradient of a mixture of isopropanol–hexane–water. The concentration of hexane and water varied depending on the glycolipid composition. The gradient was initiated immediately after the injection of the sample at a flow of 2.0 ml/min and eluate was collected every 0.5 or 1.0 min on a fraction collector. After use, the column was regenerated by washing it with isopropanol–hexane 55:45 at flow rate of 1 ml/min for 60 min, and it could be used repeatedly without loss of efficiency.

Glycolipid preparation

Glucosylceramide, lactosylceramide, globotriaosylceramide, and globotetraosylceramide were prepared from human erythrocyte membranes. $IV^3GalNAc\alpha$-$GbOse_4Cer$ (Forssman glycolipid) was isolated from goat erythrocyte membranes (11). The lower and upper neutral glycosphingolipid fractions were prepared from human type O erythrocyte membranes by the method described previously (12). Briefly, glycosphingolipids were extracted from erythrocyte membranes with hot ethanol followed by partition in chloroform–methanol 2:1. The glycolipids in lower

[1] On-line sample detection system is not utilized in this method, thus in the strict sense of the term, it is not HPLC.

[2] Iatron Lab., Inc., 1-11-4 Higashi-Kanda, Chiyoda, Tokyo, 101, Japan.

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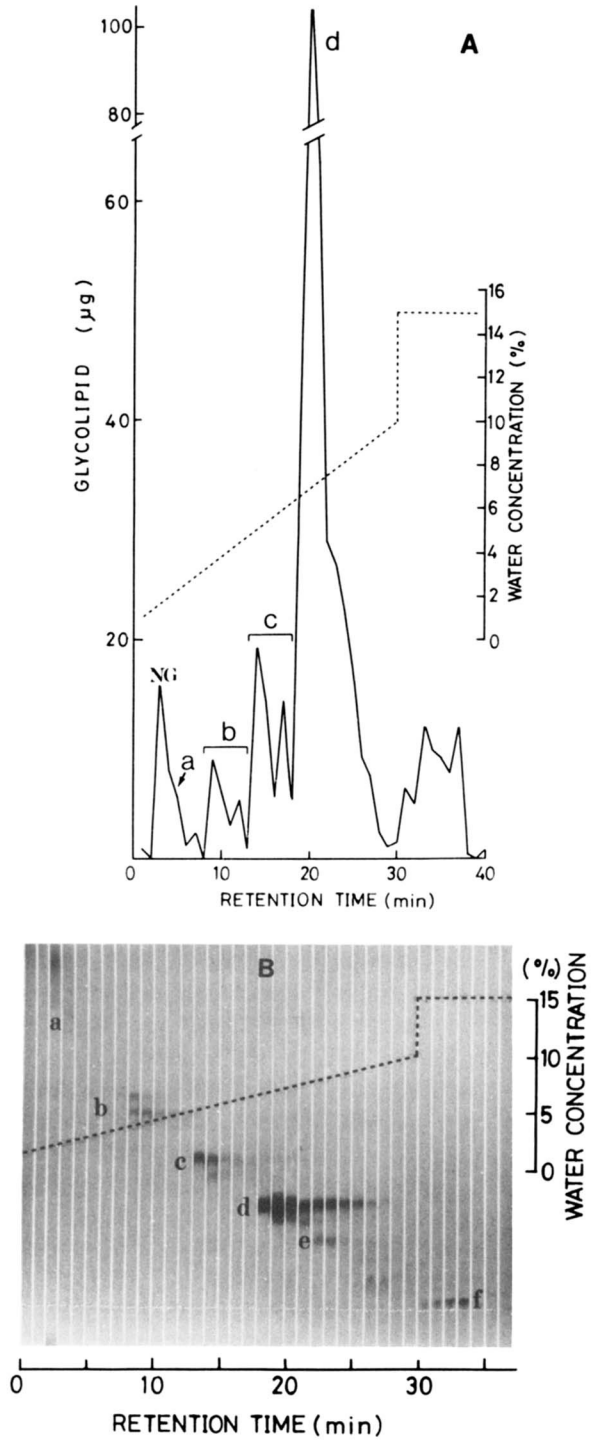

Fig. 2. A: Elution pattern of the lower glycolipids isolated from human erythrocyte membranes by HPLC; column: 500 mm × 4 mm I.D., packed with Iatrobeads 6RS-8010 (10 μm). Gradient elution: from isopropanol–hexane–water 55:44:1 to 55:35:10, and then eluted with 55:30:15, flow rate: 2.0 ml/min. Three hundred μg of the lower glycolipids was dissolved in 20 μl of starting solvent and applied to the column. Eluates were collected every 1 min, and 1 ml out of each fraction (2 ml) was evaporated to dryness, dissolved in 200 μl of water, and glycolipid content was determined by the anthrone-sulfuric acid reaction (calculated as $GbOse_4Cer$). a, glucosylceramide; b, $LcOse_2Cer$; c, $GbOse_3Cer$; d, $GbOse_4Cer$; NG, non-glycolipid contaminants. B: Thin-layer chromatogram of lower glycolipids isolated from human erythrocyte membranes eluted from the Iatrobeads column by HPLC. The conditions for HPLC were the same as in Fig. 2A. TLC: 100 μl of each fraction was evaporated and dissolved in 20 μl of chloroform–methanol 2:1 and spotted on TLC. The plate was developed with chloroform–methanol–water 65:25:4 and spots were visualized with orcinol reagent. a, glucosylceramide; b, $LcOse_2Cer$ (double spots); c, $GbOse_3Cer$ (double spots); d, $GbOse_4Cer$; e, H_1-glycolipid; f, H_2-glycolipid.

phase were purified by chromatography of the acetylated glycolipids on a column of Florosil (Floridin Co.) from non-glycosphingolipid contaminants, then deacetylated with 0.5% $NaOCH_3$ in methanol. The upper neutral glycolipids were separated from acidic glycolipids (gangliosides) by chromatography on DEAE-Sephadex A25 (acetate form) according to the procedure described by Yu and Ledeen (13).

Analytical method

TLC was performed on precoated silica gel G plates (Uniplate, Analtech Inc., Newark, DE), developed with chloroform–methonal–water 60:35:8 or 65:25:4, and glycolipids were detected by spraying with orcinol reagent. The glycolipid content in each fraction was determined by the anthrone-sulfuric acid reaction and calculated as globotetraosylceramide.[3]

Blood group activity

The blood group H-activities in each fraction with auxiliary lipids were determined by inhibition of hemagglutination caused by 3 hemagglutination doses of *Ulex europaeus* anti-H lectin as described previously (14). The hemagglutination inhibition was carried out after addition of auxiliary lipids. To each fraction (1.5 ml aliquots taken from 2-ml collection) 100 μg each of cholesterol and lecithin were added, the mixture was evaporated, suspended in 150 μl of PBS, and extensively sonicated for 10 min in an ultrasonic cleaning bath. From this solution 25-μl aliquots were taken for determination of blood group H-activities.

RESULTS AND DISCUSSION

As shown in **Fig. 1a** and **1b**, a standard mixture of mono- to pentaglycosylceramide (glucosylceramide to IV^{3}GalNAcα-$GbOse_4Cer$) was well separated within

[3] Response to the anthrone-sulfuric acid reaction of each glycolipid is different, even for glycolipids with the same number of sugars but having different types of sugars. Thus globotetraosylceramide was chosen for the standard; this is the representative glycolipid in human erythrocyte membranes and the chemical structure has been well characterized.

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30 min. The polarity of the solvent was increased by decreasing the proportion of hexane and increasing the proportion of water, whereas the concentration of isopropanol was kept at 55% (by volume). The required solvent composition for optimal separation depends upon the composition of the glycolipid mixture and is described in the legends for Fig. 1. An efficient separation of the glycolipid standard mixture (glucosylceramide, $LcOse_2Cer$, $GbOse_3Cer$, $GbOse_4Cer$, $IV^3GalNAc\alpha$-$GbOse_4Cer$) was achieved with a gradient of isopropanol–hexane–water from 55:44:1 to 55:35:10. Elution of glycolipids from the column was monitored by TLC and by determination of glycolipid content with anthrone-sulfuric acid reaction. Lactosylceramide and globotriaosylceramide gave several peaks, which are due to the differences in their ceramide composition. Glucosylceramide, globotetraosylceramide and IV^3-α-N-acetylgalactosaminylglobotetraosylceramide (Forssman glycolipid) gave, respectively, a single peak. Suzuki, Kundu, and Marcus (15) reported that neolactotetraosylceramide was eluted before globotetraosylceramide. In contrast, $nLcOse_4Cer$ was eluted immediately after elution of $GbOse_4Cer$ in this method; retention times were 20

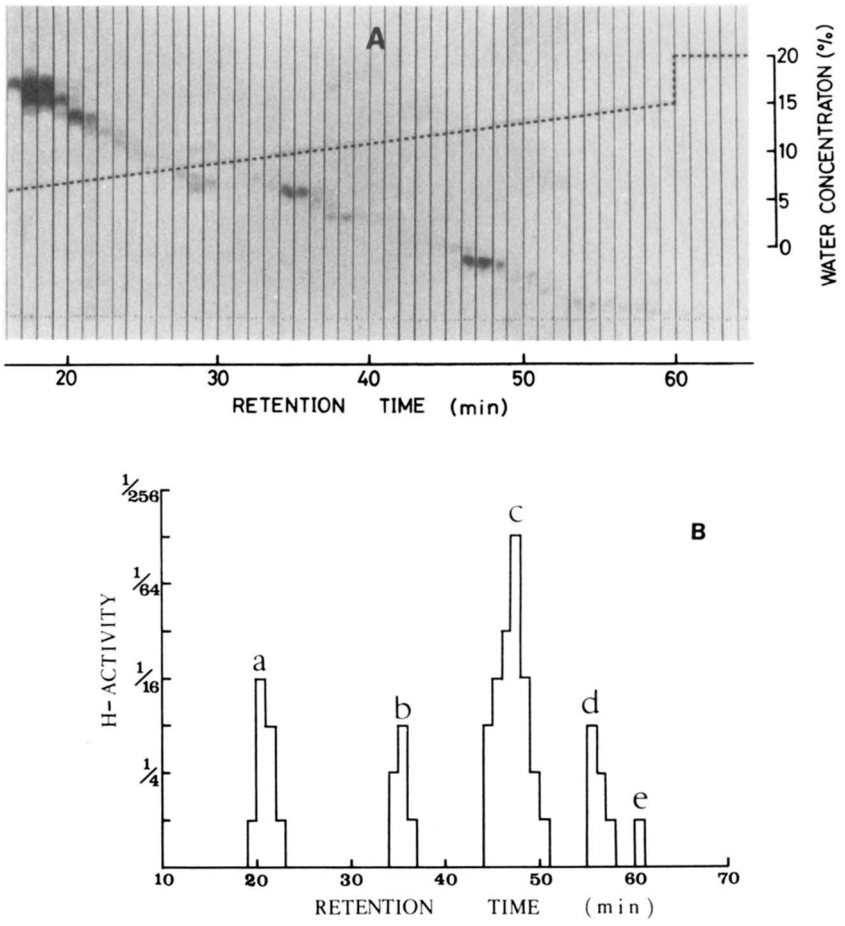

Fig. 3. A: Thin-layer chromatogram of the upper phase neutral glycolipids isolated from human erythrocyte membranes eluted from Iatrobeads column by HPLC. The conditions: column, 500 mm × 4 mm I.D., packed with Iatrobeads 6RS-8010 (10 μm), eluted with the mixture of isopropanol–hexane–water from 55:42:3 to 55:25:20 at a flow of 2.0 ml/min. The concentration of water in isopropanol is indicated by dotted line. Three mg (calculated as globotetraosylceramide) of the upper glycolipids was dissolved in 150 μl of the starting solvent and applied to the column. Eluates were collected every minute, 0.5 ml of each fraction (2 ml) was evaporated, and spotted on TLC. The plate was developed with chloroform–methanol–water 60:35:8 and glycolipids were visualized by spraying with orcinol reagent. B: Elution pattern of blood group H-activity by HPLC on an Iatrobeads column. An aliquot (1.5 ml) of each fraction in Fig. 3A was evaporated to dryness and mixed with auxiliary lipid, evaporated, and the residue was suspended in 150 μl of PBS. From this solution 25 μl was used for determination of blood group H-activities by hemagglutination inhibition with *Ulex europeaus*. Ordinate indicates the dilution of original glycolipid solutions which inhibited hemagglutination a, H_1-glycolipid; b, H_2-glycolipid; c, H_3-glycolipid; d, H_4-glycolipid; e, H_5-glycolipid.

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min and 19 min, respectively. Thus, those two glycolipids were hardly separated by this method. The chloroform–methanol–water mixture regularly used for the separation of glycolipids on classical column chromatography was also applied, but glycolipids were separated poorly by this solvent system under several different compositions of solvents (data are not shown).

The new solvent system was applied for separation of the lower and the upper phase glycosphingolipids from human type O erythrocyte membranes, isolated by partition in chloroform–methanol 2:1. The elution patterns of the lower and the upper phase glycolipid fractions recovered from the column are shown in **Fig. 2** and **Fig. 3**, respectively. The fractions glucosylceramide, lactosylceramide, globotriaosylceramide (with lactotriaosylceramide) and globotetraosylceramide were separated well and were recovered as homogeneous components. The upper phase neutral glycolipid fraction was applied to the same column. Glycolipids were eluted by a gradient of isopropanol–hexane–water from 55:45:3 to 55:25:20 as indicated in Fig. 3a. The glycolipid content and blood group H-activities in each fraction were determined by anthrone-sulfuric acid reaction and by hemagglutination inhibition with *Ulex europaeus* lectin. The thin-layer chromatogram of glycolipids eluted from the column (Fig. 3a) was similar to the pattern previously shown by silicic acid column chromatography (14, 16). However, the whole HPLC procedure was completed within 80 min, in contrast to the 1–2 days needed to complete column chromatography (at regular pressure operation). The whole elution pattern by HPLC was highly consistent and reproducible. The blood group H-activity was detected in five fractions (Fig. 3b). This is the first observation of the separation of highly complex glycosphingolipids by HPLC. HPLC separations of glycolipids have been limited to simpler glycolipids (2–7, 10, 15, 17). Another advantage of this solvent system is its nontoxicity as compared with the chloroform–methanol system which is highly toxic.

We wish to thank Dr. S-I. Hakomori for helpful suggestions and discussion throughout this work.

Manuscript received 24 November 1980 and in revised form 4 March 1981.

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