notes on methodology

An improved technique for separation of neutral glycosphingolipids by high-performance liquid chromatography

Akemi Suzuki, Samar K. Kundu, and Donald M. Marcus

Albert Einstein College of Medicine, New York NY 10461

Summary We have developed a high-performance liquid chromatographic (HPLC) procedure for separation of *O*-acetyl-*N*-p-nitrobenzoyl derivatives of six neutral glycosphingolipids: glucosylceramide, lactosylceramide, globotriaosylceramide, lactotriaosylceramide, globotetraosylceramide, and neolactotetraosylceramide. The recoveries of glucosylceramide and globotetraosylceramide for the derivatization procedure and HPLC analysis were approximately 75%, and one nanomole of glycolipid could be detected. The procedure was used for analysis of human erythrocyte neutral glycolipids.—**Suzuki, A., S. K. Kundu, and D. M. Marcus.** An improved technique for separation of neutral glycosphingolipids by high-performance liquid chromatography. *J. Lipid Res.* 1980. **21:** 473–477.

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Current techniques for quantitative analysis of tissue glycosphingolipids require isolation of individual glycolipids by chromatographic techniques, methanolysis, derivatization of the sugars, and analysis by gas–liquid chromatography (GLC). High-performance liquid chromatography (HPLC) offers the possibility of analyzing mixtures of glycolipids without hydrolysis, and two methods for separation of neutral glycosphingolipids containing one to four sugar residues have recently been published (1–7). Many tissues contain two or more different glycolipids that have the same number of sugar residues, such as globotetraosylceramide and neolactotetraosylceramide, or globotriaosylceramide and lactotriaosylceramide (8), and neither of the published techniques affords a clear separation of these compounds. In this paper we report a new procedure for the separation of certain neutral glycolipids that contain equal numbers of sugar residues, and the application of this method to the analysis of human erythrocyte neutral glycolipids.

Abbreviations: HPLC, high-performance liquid chromatography; GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Glycosphingolipids

Neutral glycolipids were purified from human erythrocytes as described previously (9). All preparations of erythrocyte globotriaosylceramide are contaminated with lactotriaosylceramide and these two compounds were purified by chromatography of the acetylated glycolipids on a column of Iatrobeads (Iatron Lab., Tokyo). Acetylated globotriaosylceramide was eluted with dichloroethane–acetone 8:2 (v/v) and acetylated lactotriaosylceramide was eluted with dichloroethane–acetone 7:3 (v/v). Unless otherwise indicated, all solvent mixtures are volume/volume (v/v).

Derivatization of standard glycolipids

The purified glycolipids (about 2 mg) were converted to *O*-acetyl-*N*-p-nitrobenzoyl derivatives and the derivatives were purified on a Sephadex LH-20 column (Pharmacia Inc., Piscataway, NJ) by the same procedure reported previously (2).

Thin-layer chromatography

The purity of the derivativized standards was determined by TLC with the solvent system: 7% isopropanol in hexane–dichloroethane 2:1 (v/v) or dichloroethane–methanol 40:1 (v/v). The compounds were detected under a UV light (254 nm) and with the α-naphthol reagent (10).

High-performance liquid chromatography

The apparatus included Altex model 100A pumps, a model 420 microprocessor, and model 153UV detector (254 nm) (Altex Scientific Inc., Berkeley, CA). Data were integrated by a Spectra Physics SP4000 (Santa Clara, CA) chromatography data system. A column (Zorbax SIL, 4.6 mm ID × 25 cm, DuPont, Wilmington, DE) was used and the separation was carried out by programmed gradient elution as follows: flow rate; 0.5 ml per min, 5 min with 1% isopropanol in hexane–dichloroethane 2:1, 55 min with linear gradient from 1% to 5% isopropanol

in hexane–dichloroethane 2:1, and 10 min with 5% isopropanol in hexane–dichloroethane 2:1. Before the next analysis, the column was reactivated with the initial solvent for 15 min.

Calibration curve

The concentration of each derivative was calculated from hexose content which was determined by an anthrone–sulfuric acid assay (11) using galactose and glucose as standards. Varying quantities of each derivative were analyzed by HPLC and peak areas were determined by integration.

Recovery of radioactive glycolipids

Tritiated glucosylceramide and globotetraosylceramide were prepared as described by Schwartzmann (12) except that Pd on $BaSO_4$ was substituted for $PdCl_2$ (Hakomori, personal communication), and the radiolabeled derivatives were purified by preparative TLC with the solvent chloroform–methanol–water 60:30:5. After the purity of the glycolipids was determined by autoradiography, the glycolipids were acetylated.

The acetylated neutral glycolipid fraction of human erythrocytes was prepared by acetylation and Florisil column chromatography (13) as reported previously (9). To triplicate samples, each containing acetylated glycolipids equivalent to 0.5 ml packed red cells, radiolabeled acetylated glucosylceramide (2.3×10^4 cpm/1.06 nmoles) or acetylated globotetraosylceramide (1.70×10^4 cpm/1.08 nmoles) was added. The samples were dried overnight in vacuo over fresh P_2O_5 and were then incubated with 0.3 ml of pyridine and 15–20 mg of p-nitrobenzoyl chloride at 60°C for 6 hr. The solid p-nitrobenzoyl chloride was dissolved in pyridine by sonication for a few minutes in an ultrasonic bath (Heat Systems-Ultrasonics, Plainview, NY). After the incubation period, 0.3 ml of a 3% $NaHCO_3$ solution was added and the mixture was kept overnight at room temperature. Four ml of 3% $NaHCO_3$ and 4 ml of chloroform were added to the mixture, the $NaHCO_3$ layer was removed, and the chloroform phase was washed two times with 4 ml of 3% $NaHCO_3$ and three times with 4 ml of distilled water. The aqueous phase was removed after centrifugation at 600 *g* for 5 min, and the chloroform layer was evaporated to dryness under N_2 at room temperature. The residue was dissolved in 4 ml of methanol by sonication for a few minutes and 1 ml of distilled water was added with vigorous mixing. The solution was applied to a Sep-Pak C18 cartridge (Waters Associates Inc., Milford, MA) which was prewashed with 10 ml of chloroform–methanol 1:1 and then 10 ml of methanol–water 8:2. The cartridge was washed with 10 ml of methanol–water 8:2 to remove impurities, the *O*-acetyl-*N*-p-nitrobenzoyl derivatives were eluted with 10 ml of chloroform–methanol 1:1 and concentrated by evaporation under N_2 at room temperature. The derivatives were dissolved in 400 μl of hexane–dichloroethane 2:1 by sonication and triplicate analysis of 100-μl aliquots was performed. Recovery of the glycolipids was determined by measuring the radioactivity in the glucosylceramide and globotetraosylceramide peaks obtained from the HPLC column.

Analysis of erythrocyte glycolipids

Erythrocytes obtained from one individual were washed three times with phosphate-buffered saline and the packed cells (1.5 ml) were extracted once with 50 ml of chloroform–methanol 2:1 and once with 50 ml of chloroform–methanol 1:1. The total lipid was fractionated on a DEAE-Sephadex column (Pharmacia, 1 cm × 17 cm) (14). The neutral lipids were dried, acetylated, and fractionated on a Florisil column (Fisher Scientific Co., 6 g) (13). Acetylated neutral glycolipids were obtained in fraction 3. The triplicate samples, each containing acetylated glycolipids equivalent to 0.5 ml packed red blood cells, were derivatized by the same procedure described above. Finally, the derivatives were dissolved in 400 μl of hexane–dichloroethane 2:1 and aliquots (40, 100, and 200 μl) were injected on to the HPLC column. The quantity of each glycolipid was calculated from the integrated area by reference to the calibration curve.

RESULTS

Purity of the standard glycolipid derivatives

The *O*-acetyl-*N*-p-nitrobenzoyl derivatives were analyzed by TLC to evaluate the completeness of the derivatization and the separation of the reaction products from the glycolipid. Each derivative migrated more rapidly than its acetylated glycolipid precursor.

Development of a new gradient program

We tested several solvent systems for their ability to separate the six glycolipid derivatives. Globotetraosylceramide and neolactotetraosylceramide were not separated by an isopropanol gradient in hexane–chloroform 2:1. These two compounds were resolved by a gradient of isopropanol in dichloroethane, but globotriaosylceramide and neolactotetraosylceramide were not well separated under these conditions. Separation of these three compounds is dependent

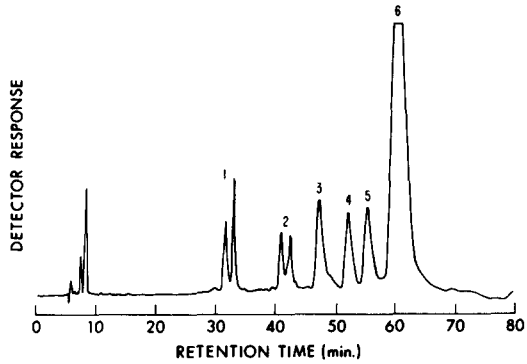

Fig. 1. Separation of six glycolipid derivatives. Column, Sorbax SIL. Flow rate: 0.5 ml per min. Elution was performed as follows: 5 min with 1% isopropanol in hexane–dichloroethane 2:1, 55 min with linear gradient from 1% to 5% of isopropanol in hexane–dichloroethane 2:1 and 10 min with 5% isopropanol in same hexane–dichloroethane mixture. Glucosylceramide (peak 1, 4.4 nmol), lactosylceramide (peak 2, 4.0 nmol), lactotriaosylceramide (peak 3, 2.8 nmol), globotriaosylceramide (peak 4, 4.1 nmol), neolactotetraosylceramide (peak 5, 2.4 nmol), and globotetraosylceramide (peak 6, 6.9 nmol) were injected.

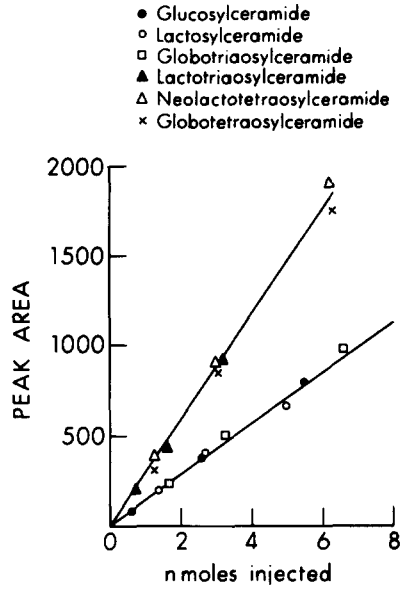

Fig. 2. Calibration curves for six glycolipid derivatives. HPLC was performed under the same conditions as in Fig. 1.

upon the hexane–dichloroethane ratio and a complete separation was achieved by a gradient of isopropanol from 1 to 5% in hexane–dichloroethane 2:1 (**Fig. 1**).

Calibration curve

The calibration curves for the six glycolipids are presented in **Fig. 2.** Derivatives of glycolipids that contain a single hexosamine residue, such as globotetraosylceramide, have a molar absorbance twice that of glycolipids containing only hexoses. This observation confirms our previous results (2) and is consistent with the concept that the p-nitrobenzoyl group is introduced into the acylamide group of ceramide and the acetamide group of the hexosamine.

Recovery

To evaluate the recovery of glycolipids obtained with this procedure, we added tritiated acetylated glucosylceramide and globotetraosylceramide to a sample of erythrocyte glycolipids. In these experiments we used the same amount of unlabeled glycolipids that we routinely use in analyses of small samples of human erythrocytes. From the derivatization through the HPLC analysis, the recovery of glucosylceramide was 77.1% ± 0.6 (mean ± S.D., n = 9) and that of globotetraosylceramide was 75.2% ± 3.4 (n = 9).

Analysis of erythrocyte glycolipids

Data on the analysis of glycolipids equivalent to 0.5 ml of packed erythrocytes from a single individual are presented in **Fig. 3** and **Table 1.** Aliquots of 40 to 100 μl, which are equivalent to 0.05–0.125 ml of packed red blood cells, are sufficient for measurement of major glycolipids, such as lactosyl-, globotriaosyl-, and globotetraosyl-ceramide; a 200 μl aliquot was needed for the determination of minor glycolipids such as glucosyl-, lactotriaosyl-, or neolactotetraosyl-ceramide.

DISCUSSION

A procedure used by many investigators for isolation of neutral glycolipids involves DEAE-Sephadex column chromatography (14), which removes gangliosides and acidic lipids, acetylation of DEAE fraction I, and separation of the acetylated neutral glycolipids from other classes of lipids by Florisil column chroma-

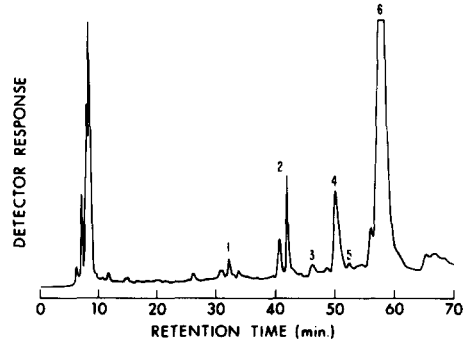

Fig. 3. Analysis of erythrocyte glycolipids. HPLC was performed under the same conditions as in Fig. 1.

TABLE 1. Glycolipid composition of human red blood cells

	Ullman and McCluer (6)	Present Analysis[a]	Yamakawa and Nagai (15)
Glucosylceramide	0.16	0.83 ± 0.13	0.35
Lactosylceramide	0.76	3.37 ± 0.40	2.28
Globotriaosylceramide	1.40	3.01 ± 0.16	0.93
Lactotriaosylceramide	n.d.[b]	0.24 ± 0.04	0.04
Globotetraosylceramide	9.40	12.63 ± 0.13	7.38
Neolactotetraosylceramide	n.d.[b]	0.34 ± 0.05	0.43

[a] The values are determined from the integrated data of three injections for each triplicate sample and corrected by 0.75, the mean recovery value.
[b] Not determined.
The values are expressed as μmoles per 100 ml packed cells.

tography (13). In our procedure the acetylated glycolipids are then derivatized with a chromophore that makes it possible to use a UV detector for their measurement. Derivatization of the glycolipids appears to be complete since none of the starting materials was detected by TLC. The loss of approximately 25% of glucosylceramide and globotetraosylceramide occurred during the partition and chromatographic procedures used to separate the p-nitrobenzoyl derivatives from impurities that interfere with the HPLC analysis. Ullman and McCluer reported a recovery of 77–90% for benzoylated glycolipids in their HPLC procedure (6).

Current analytical methods do not permit accurate measurement of lactotriaosylceramide or neolactotetraosylceramide in the presence of a much larger quantity of the globoside series of compounds that are found in erythrocytes and other tissues. The concentration of lactotriaosylceramide in erythrocytes is higher than appreciated previously (15). This procedure will be useful for analyzing human neutrophilic leucocytes, which contain larger quantities of these two glycolipids than erythrocytes, and for analysis of genetic abnormalities such as HEMPAS erythrocytes (16), which contain abnormally large quantities of these two glycolipids.

Another advantage of this method is more effective removal of the excess derivatization reagents and by-products by the small Sep-Pak column that contains octadecyl alkyl groups. This reverse phase separation was used previously in the separation of molecular species of glycolipids based on their hydrophobic portions (1, 2). This procedure is especially useful for microscale analyses because of its reproducibility and high recovery. The limitations of this method are that we have not devised conditions for effective derivatization of glycolipids that contain hydroxy fatty acids, or for the separation of gangliotriaosyl-, and gangliotetraosylceramides from the other neutral glycolipids. These problems are currently under investigation, and neither of these limitations is of any consequence in the analysis of human erythrocytes.

In Table 1 our data are compared with those of Ullman and McCluer (6), who analyzed erythrocytes from a single individual by HPLC, and analyses of pooled erythrocytes, summarized by Yamakawa and Nagai (15). Discrepancies among these data are attributable to several factors: inaccuracies inherent in the use of "packed cells", as opposed to actual numbers of erythrocytes, as the basis for calculation; different recoveries for each method; and variation between individuals. Fletcher, Brewer, and Schwarting (17) have found that erythrocytes of blood group P_1 individuals contain more globotriaosylceramide than lactosylceramide, whereas the reverse situation was found in individuals of blood group P_2. Our unpublished data confirm these findings and also indicate that there is considerable variation between individuals in the absolute quantities of several erythrocyte neutral glycolipids. The normal range of erythrocyte neutral glycolipids needs to be redefined with new analytical techniques and taking into account the influence of the P blood group system.

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