Gas–liquid chromatography of oligosaccharides released from red cell glycosphingolipids by ozonolysis

Masako Ohashi and Tamio Yamakawa

Department of Biochemistry, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan

Summary A method for the analysis of glycosphingolipids in mammalian erythrocyte membranes is described. It consists of ozonolysis and alkaline treatment of the crude lipid extract to obtain oligosaccharides from glycosphingolipids and then gas–liquid chromatography of trimethylsilyl derivatives of glycitols derived from the oligosaccharides. Typical gas–liquid chromatographic patterns of oligosaccharide components were obtained with various mammalian erythrocytes; these corresponded to the glycosphingolipid compositions. The analysis could be carried out on 10 ml of packed erythrocytes.

Supplementary key words glycitols · TMS derivatives

There are large differences in the chemical structure and concentration of the glycosphingolipids of erythrocytes of various species of mammals. According to Yamakawa and coworkers (1–3), erythrocyte glycosphingolipids are classified into two major types, "globosides," containing hexosamine, and "hematosides," containing sialic acid. However, there have been only a few quantitative studies on the glycosphingolipids in erythrocytes (3–5). In this paper, we describe a GLC method for the study of glycosphingolipid composition of erythrocytes.

Experimental

Materials. As the reference substances for GLC, we used glycosphingolipids from mammalian erythrocyte membranes; these lipids were isolated and purified in our laboratory. CMH (Glc-ceramide), CDH (Gal-Glc-ceramide), CTH (Gal-Gal-Glc-ceramide), and globoside I (GalNAc-Gal-Gal-Glc-ceramide) were isolated from human erythrocytes (6, 7). GalNAc-Gal-Glc-ceramide and its oligosaccharide were prepared from guinea pig erythrocytes and/or asialo G_{M2} from bovine brain (8). NGNA-Gal-Glc-ceramide was isolated from horse erythrocytes (9), and Forssman active glycosphingolipid was isolated from sheep erythrocytes (10).

Extraction of lipids. Lipids were extracted from dried erythrocyte ghosts (10–20 mg) or packed erythrocytes (10–20 ml) by successive treatments with $CHCl_3$–CH_3OH mixtures: 2:1, 1:1, and 1:9 (v/v) under reflux on a water bath. The combined extracts were dialyzed against water and evaporated to dryness. The residues were redissolved in a small amount of $CHCl_3$–CH_3OH 2:1, and insoluble materials were removed by filtration. In some cases when hematoside was absent, the lower phase obtained from the lipid extract (11) was used instead of the dialyzed total lipids.

Preparation of glycitols. The preparation of oligosaccharides of glycosphingolipids from crude erythrocyte lipids was carried out by ozonolysis and alkaline cleavage techniques according to procedures described by Wiegandt and Baschang (12) and Wiegandt and Bücking (13) with slight modifications. The dialyzed lipid dissolved in 0.5 ml of $CHCl_3$–CH_3OH 2:1 (v/v) was diluted with 15 ml of methanol–hexane 4:3 (v/v), and ozone was bubbled through the solution for 30 min at room temperature. The solution was evaporated to near dryness at 30°C in vacuo, and 15 ml of a freshly prepared aqueous solution of 0.1 N Na_2CO_3 was added; the mixture was then left overnight at room temperature. Oligosaccharides thus obtained were reduced to their glycitols after the addition of a few milligrams of $NaBH_4$, and the mixture was then left to stand for 3 hr. Dowex-50 (H^+) was added to the reaction mixture until the pH was slightly acidic. The mixture was then filtered through paper, and the filtrate was evaporated to dryness in vacuo. Boric acid was removed as volatile methyl borate by repeated evaporation of the residue with the addition of small portions of methanol. The residue was then redissolved in 1 ml of distilled water and washed with 5 vol of $CHCl_3$–CH_3OH 2:1 to remove some remaining lipid contaminants, which would result in many additional peaks in the gas chromatogram. Finally, the aqueous phase was evaporated and dried thoroughly over P_2O_5 in vacuo.

Trimethylsilylation. In order to make the silylation reaction complete, the two-step procedure was used. The glycitol fraction was first treated with 0.5 ml of TMS reagent I (mixture of pyridine–HMDS–TMCS 5:1:1) for 30 min at 50°C. The reaction mixture was diluted with $CHCl_3$ and washed three times with distilled water (14). The $CHCl_3$ layer, which contained the silylated derivatives, was dried completely, and 20–50 μl of TMS reagent II (pyridine–HMDS–TMCS 1.0:1.3:0.8) (15) was added to the residue. The mixture was allowed to stand for 15 min and it was then centrifuged; the clear supernatant was analyzed by GLC. The TMS derivatives of glycitols in the supernate were stable for a few weeks at room temperature when kept in a Teflon-stoppered tube.

Gas–liquid chromatography. GLC was carried out in a Hewlett-Packard F & M model 402, equipped with flame ionization detectors. For the separation of TMS glycitols of mono-, di-, tri-, tetra-, and pentasaccharides, glass columns (3 mm × 30 cm) packed with 1% OV-1 on Anakrom AB (90–100 mesh) were used; the temperature was programmed from 110°C to 350°C (10°C/min). The

Abbreviations: CMH, CDH, and CTH, ceramide monohexoside, dihexoside, and trihexoside, respectively; NANA, *N*-acetylneuraminic acid; NGNA, *N*-glycolylneuraminic acid; HMDS, hexamethyldisilazane; TMCS, trimethylchlorosilane; TMS, trimethylsilylated; GLC, gas–liquid chromatography.

TABLE 1. Relationship between the amounts of standard glycosphingolipid mixture and the peak areas obtained by GLC

Glycosphingolipids	CMH	CDH	CTH	Globoside I
Molar ratio of standard mixture (A)	3.30	1	1.66	1.89
Ratio of peak area[a] (B)	1.57 ± 0.08	1	2.02 ± 0.06	2.52 ± 0.14
Correction factors (A/B)	2.10	1	0.82	0.75

[a] The ratio of CMH, CTH, or globoside I area to CDH area. The values represent the means of five separate experiments using the original stock solution of known amounts of the standard glycosphingolipid mixture.

temperature of the injection port was maintained at 230°C and the detectors were at 300°C.

Results and discussion

Since the long-chain base of glycosphingolipids in erythrocytes consists predominantly of C_{18}-sphingosine and the content of dihydrosphingosine is less than 5% (16–18), the ozonolysis procedure is adequate for the splitting of the sugar moieties from glycosphingolipid molecules.

To quantitate the amount of the various glycosphingolipids, correction factors were applied. These factors were calculated from the amounts of standard mixture and the peak areas on the gas chromatogram (Table 1).

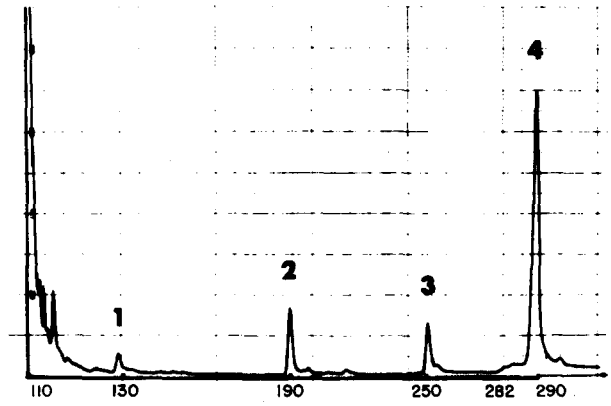

Fig. 1. Gas–liquid chromatogram of TMS derivatives of the reduced oligosaccharides derived from human erythrocyte glycosphingolipids in the dialyzed total lipids. Peak *1*, glucitol; *2*, Gal-glucitol; *3*, Gal-Gal-glucitol; and *4*, GalNAc-Gal-Gal-glucitol. The numbers on the abscissa are column temperatures (°C).

Fig. 1 shows the GLC pattern of TMS glycitols from lipid extract of human erythrocyte membrane. Four peaks were identified as glucitol, Gal-glucitol, Gal-Gal-glucitol, and GalNAc-Gal-Gal-glucitol from their elution temperatures and retention times. Using the correction factors in Table 1, the mean molar ratio of CMH, CDH, CTH,

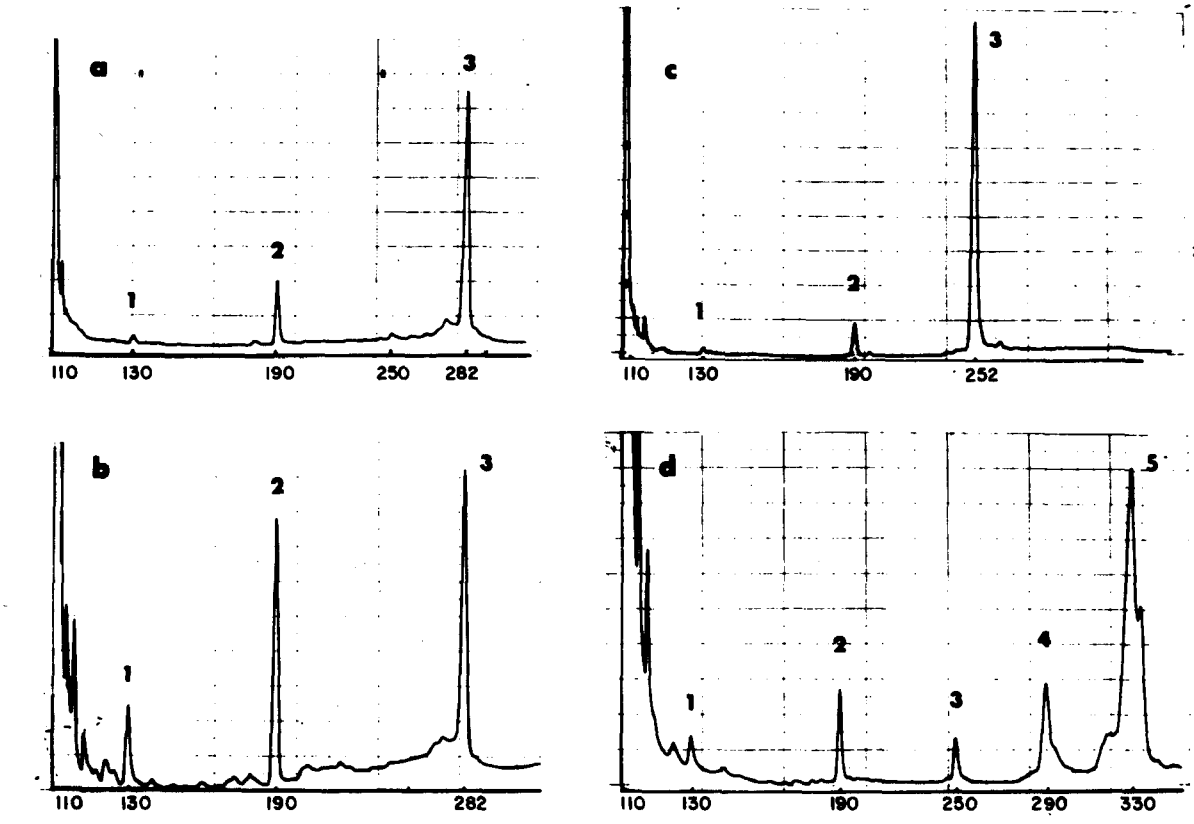

Fig. 2. Gas–liquid chromatograms of TMS derivatives of the reduced oligosaccharides derived from the erythrocyte glycosphingolipids of four mammalian species. (*a*) Horse: *1*, glucitol; *2*, Gal-glucitol; *3*, NGNA-Gal-glucitol. (*b*) Dog: *1*, glucitol; *2*, Gal-glucitol; *3*, mixture of NANA- and NGNA-Gal-glucitol. (*c*) Guinea pig: *1*, glucitol; *2*, Gal-glucitol; *3*, GalNAc-Gal-glucitol. (*d*) Goat: peaks *1–5* correspond to mono-, di-, tri-, tetra-, and pentasaccharides, respectively. In guinea pig erythrocytes (*c*), the lower phase of the lipid extract (11) was used for analysis instead of dialyzed total lipid. The numbers on the abscissa are column temperatures (°C).

and globoside I was 0.57:1:0.84:5.1. These values agree quite closely with the ratio 0.35:1:0.89:4.9 calculated from the data of Sweeley and Dawson (4), who determined the glycosphingolipid concentrations to be 0.50, 1.43, 1.27, and 7.05 μmoles/100 ml of erythrocytes, respectively.

Further examinations revealed that the glycosphingolipids from a given individual always had the same GLC pattern but the patterns were not always the same from person to person. This was also noted by Sweeley and Dawson (4). In some cases, the content of CTH was somewhat higher than that of CDH.

For the further determination of glycosphingolipid content in erythrocytes, it was found preferable to add a proper glycosphingolipid (a purified CDH or CTH) as an internal standard. The amounts of the glycosphingolipids can then be evaluated from the differences of the GLC peak areas before and after the addition of a known glycosphingolipid.

Erythrocyte glycosphingolipids of four other mammalian species, horse, dog, guinea pig, and goat (Fig. 2), were determined. In studies that utilized conventional separation methods such as silicic acid column chromatography, thin-layer chromatography, or a combination of both methods, it has already been reported (2, 4, 8–10, 19) that erythrocyte membranes of every species have their own characteristic glycosphingolipid compositions. The present GLC method offers a convenient method for obtaining the species-specific glycosphingolipid patterns with a small amount of erythrocyte materials. However, the peaks derived from oligosaccharides that have different sequences but are similar in their monosaccharide components are sometimes not clearly resolved under the GLC conditions employed, as in the case of peak 3 in Fig. 1 and peak 3 in Fig. 2*c*. In such cases, the combination of GLC and mass spectrometry may be utilized to obtain the exact identification of the oligosaccharides.

Further application of this method to the glycosphingolipid analysis of other organs and tissues seems to be possible. However, the nature of the long-chain base should be examined prior to the determination. Glycosphingolipids that contain phytosphingosine or dihydrosphingosine resist ozonolysis. Such glycosphingolipids occur in some tissues, and, indeed, the phytosphingosine content in CMH of human kidney is quite high (20).

Manuscript received 27 February 1973; accepted 30 July 1973.

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