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# Evaluation of sphingolipids in vitreous bodies from a patient with Gaucher disease, using delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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**Abstract**

Gaucher disease is a glycolipid storage disorder characterized by the accumulation of glucosylceramide in tissues. Using delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry (DE MALDI-TOF-MS), we analyzed sphingolipids in vitreous bodies from a patient with Gaucher disease who suffered from vitreous opacities. Crude lipids were extracted from the freeze-dried vitreous bodies with chloroform and methanol. After mild alkaline treatment of the crude lipids, a sphingolipid fraction was prepared and analyzed by DE MALDI-TOF-MS. The results were as follows: (a) the *m/z* values of the ions found in the mass spectra for both the control and the Gaucher disease patient corresponded to different sphingomyelin species. (b) The mass spectrum of the Gaucher disease patient showed additional ions with *m/z* values corresponding to different ceramide monohexoside (CMH) species. It was indicated that the accumulation of CMH in vitreous bodies from Gaucher disease patients could be easily detected with the DE MALDI-TOF-MS method.
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*Keyword:* Sphingolipids

## 1. Introduction

The laser desorption time-of-flight mass spectrometer was developed in order to analyze non-volatile, thermally labile and high mass organic molecules. Matrix-assisted laser desorption ionization (MALDI) was reported [1] as a useful technique for the detection of high mass molecules. Subsequently this technique was applied to proteins, glycoproteins, neutral glycosphingolipids, and gangliosides [2–6]. Delayed ion extraction (DE) was later introduced to MALDI time-of-flight mass spectrometry (MALDI-TOF-MS), which dramatically improved the resolution and accuracy of the mass spectra.

Gaucher disease (GD) is caused by a deficiency of lysosomal acid β-glucosidase (glucocerebrosidase), which hydrolyzes glucosylceramide to ceramide and glucose. In patients with GD, therefore, glucosylceramide accumulates in various organs. For the diagnosis of GD, sphingolipids in tissues are analyzed by thin layer chromatography or high-performance liquid chromatography.

Previously, we reported that the analysis of sphingolipids in human various tissues [7], cultured skin fibroblasts [8], and body fluids [9] by DE MALDI-TOF-MS was useful for the diagnosis of sphingolipidoses. We analyzed the sphingolipids in vitreous bodies from a patient with GD using DE MALDI-TOF-MS. We present this paper in order to show the potential of MALDI-TOF-MS analysis of sphingolipids from vitreous bodies as a method for the diagnosis and investigation of the

*Abbreviations:* DE MALDI-TOF-MS, delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry; GD, Gaucher disease; 2,5-DHB, 2,5-dihydroxybenzoic acid; CMH, ceramide monohexoside

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biochemical pathogeneses of ophthalmologic complications of GD.

## 2. Materials and methods

### 2.1. Patients and specimens

The patient was a 23-year-old male with type 1 GD (lack of primary central nervous system involvement type). Hepatosplenomegaly was noted at 9 months of age, and he was diagnosed as having GD based on reduced β-glucosidase activity in skin fibroblasts. He developed thrombocytopenia due to hypersplenism and bilateral vitreous opacities at 10 years of age. He underwent total splenectomy at 16 years of age. The vitreous opacity advanced slowly, extending to the macula region, and the patient suffered from metamorphopsia from 20 years of age. He started enzyme replacement therapy at 21 years of age. Subsequently, vitreoretinal and macula traction developed, and thus he underwent vitrectomy at the age of 23 years. A pair of vitreous bodies from a non-GD patient, a female patient with diabetes mellitus, who underwent vitrectomy at 62 years of age due to diabetic retinopathy, was used as controls. Each sample was steeped in an isotonic sodium chloride solution, frozen and then stored at −80 °C until examination. All materials were collected with the informed consent of the patients.

### 2.2. Preparation of sphingolipids

The preparation of sphingolipids is schematically depicted in Fig. 1. In practice, each unilateral vitreous body was homogenized with 5 ml of an isotonic sodium chloride solution and then freeze-dried. Each sample was shaken vigorously in 8 ml of chloroform–methanol, 2:1 (v/v), on a vortex mixer, ultrasonicated, and then filtered through filter paper, total crude lipids being obtained in the filtrate. We added 2 ml of water to the filtrate for Folch partitioning. Then, after centrifugation at 1,200 × *g* for 5 min, the lower phase was evaporated to dryness in a rotary evaporator, and then treated with 1 ml of 0.1 N NaOH in methanol at 55 °C for 1 h to decompose all esterified glycerolipids including glycerophospholipids and triacylglycerols. After acidification with 0.2 ml of 1 M HCl in methanol, one drop of water and 1 ml of hexane were added, followed by centrifugation at 1,200 × *g* for 5 min. After the upper phase had been removed, the lower phase was evaporated under a nitrogen stream. 0.2 ml of the theoretical lower phase, chloroform–methanol–water, 86:14:1 (v/v/v), and 0.8 ml of the theoretical upper phase, chloroform–methanol–water, 3:48:47 (v/v/v), were mixed for Folch partitioning. The resulting mixture was centrifuged at 1,200 × *g* for 15 min. After the upper phase had been discarded to remove salts, the lower phase was evaporated under a nitrogen stream. The crude sphingolipids in the residue were dissolved in chloroform–methanol (2:1 (v/v)) to give a certain concentration, 10 mg crude lipid/ml, for thin-layer chromatography and DE MALDI-TOF-MS. Through the above procedure, crude lipids, 5.6, 3.7 and 4.9 mg, were obtained from the GD patient sample and two control samples, respectively.

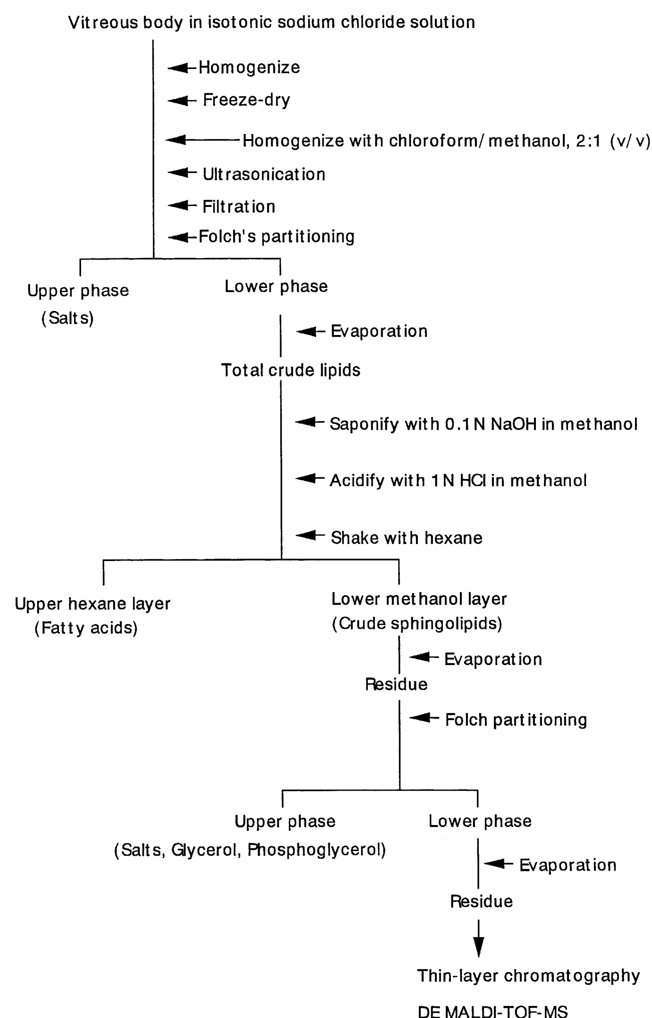


Fig. 1. Schema of preparation of sphingolipid fractions from vitreous bodies.

### 2.3. Thin-layer chromatography

Thin-layer chromatography was performed on silica gel 60 plates (Merck, Darmstadt, Germany) with a solvent system of chloroform–methanol–0.2% $CaCl_2$ in water, 60:30:6 (v/v/v). After development, spots were visualized with primuline reagent, under ultraviolet light at a wavelength of 365 nm [10], and orcinol reagent [11], followed by heating of the plates at 100 °C for 10 min [12]. The standard glucosylceramide used for the thin-layer chromatography analysis was purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

### 2.4. Delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry (DE MALDI-TOF-MS) analysis

To 5 μl of the crude sphingolipid solution in a 1.5 ml Eppendorff tube, 5 μl of the matrix solution (10 mg of 2,5-dihydroxybenzoic acid (2,5-DHB) in 1 ml of a 9:1 mixture of water–ethanol) was added. The tube was shaken vigorously on a vortex mixer, and then centrifuged in a microcentrifuge (Chibitan; Tomy Kogyo, Fukushima, Japan) at 2,000 × *g* for 1 min. One microliter of the supernatant was loaded into a Voyager$^{TM}$ DE-RP (2.0 m flight length, reflector mode) Biospectrometry$^{TM}$ Workstation (Applied Biosystems, Framingham, MA, USA), and mass spectra of samples were obtained in the positive ion mode with an $N_2$ laser (337 nm); delay, 100 ns; accelerating voltage, 25 kV; and scan average, 256. The resolution of the ion peak was more than 2000, and is represented as $M/\Delta M$ (peak centroid mass/right mass − left mass), the resolution calculator in the GRAMS/386 software (Galactic Industries Corp., Salem, NH) supplied with the instrument being used. Two point external calibration was performed each time. In the positive ion mode with 2,5-DHB as the matrix, angiotensin I ($[M+H]^+$: 1296.6853) and des-Arg-bradykinin ($[M+H]^+$: 904.4681) were used for calibration of the instrument. five-point Savitsky-Golay smoothing was applied to the mass spectra as described [13–16]. 2,5-DHB was purchased from Sigma Chemicals (St. Louis, MO, USA). All other reagents were of analytical grade.

## 3. Results

The lipid material of the vitreous bodies from the patient with GD gave one spot, which exhibited a similar $R_f$ value to that of the standard glucosylceramide and was not present in the control lanes, on thin-layer chromatography analysis (Fig. 2).

The mass spectra of sphingolipids in the vitreous bodies from the controls and the patient with GD obtained on DE MALDI-TOF-MS are presented in Fig. 3. Table 1 shows the *m/z* values of the different $[M+Na]^+$ and $[M+H_2O+Na]^+$ ions found in the mass spectra in Fig. 3, and the proposed

Table 1
Measured mass-to-charge ratios (*m/z*), and proposed molecular species associated with sphingolipids

| Ceramide monohexoside[a] (*m/z*) | Sphingomyelin (*m/z*) |
|---|---|
| 722 (d18:1C16) $[M+Na]^+$ | 721 (d18:1C16) $[M+H_2O+H]^+$ |
| 778 (d18:1C20) $[M+Na]^+$ | 725 (dl 8:1C16) $[M+Na]^+$ |
| 806 (d18:1C22) $[M+Na]^+$ | 727 (d18:1C16) $[M+Na]^+$ |
| 832 (d18:1C24:1) $[M+Na]^+$ | 751 (d18:1C18:1) $[M+Na]^+$ |
| 834 (d18:1C24) $[M+Na]^+$ | 839 (d18:1C23:l) $[M+H_2O+Na]^+$ |
| | 853 (d18:1C24:1) $[M+H_2O+Na]^+$ |

[a] Ceramide monohexoside (CMH) includes glucosylceramide. "d" indicates dihydroxy-sphingosine.

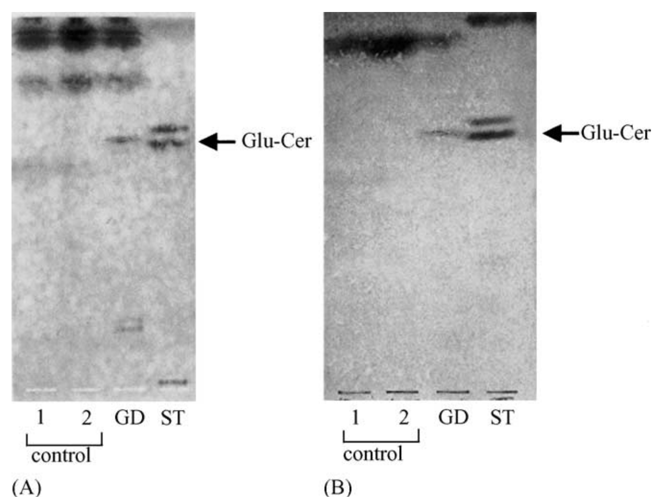


Fig. 2. Thin-layer chromatograms of sphingolipid fractions of vitreous bodies from a patient with GD and a control: (A) 70 μg of total crude lipids was applied and color was developed with primuline reagent under ultraviolet light; (B) 200 μg of total crude lipids was applied and color was developed with orcinol reagent. The solvent system was chloroform–methanol–0.2% $CaCl_2$ in water, 60:30:6 (v/v/v). Lanes 1 and 2: controls 1 and 2; GD: Gaucher disease; ST: standard glucosylceramide. The lipid material in vitreous bodies from the patient with GD gave one spot, which exhibited a similar $R_f$ value to that of the standard glucosylceramide and is not present in the control lanes.

sphingolipid species corresponding to these ions [17]. The *m/z* values of the $[M+Na]^+$ and $[M+H_2O+Na]^+$ ions observed in the mass spectrum corresponded to different sphingomyelin species in the lipid material of the vitreous bodies from a control specimen (Fig. 3A). The mass spectrum of the sphingolipids from the vitreous bodies of the GD patient showed sphingomyelin ions similar to those of the control patient and, in addition, $[M+Na]^+$ ions corresponding to ceramide monohexoside (CMH) species (Fig. 3B).

These thin-layer chromatography and DE MALDI-TOF-MS results were compatible, indicating that CMH was accumulated in the vitreous body from the GD patient.

## 4. Discussion

A heterogeneous spectrum of clinical symptoms and courses is noted in GD patients. Specific storage cells, Gaucher cells, are derived from the monocyte–macrophage phagocyte system and contain accumulated glucosylceramide [18]. Although some ophthalmologic complications of GD have been reported [19,20], to the best of our knowledge, advanced vitreous opacity, which requires vitrectomy in patients with GD, has not been reported previously. Vitreous opacity in GD has been reported in one case, and after 5 months of enzyme replacement therapy, the intermediate uveitis with vitreous opacity resolved almost completely and the visual acuity increased [21].

Vitreous bodies comprise transparent gel tissue that fills the vitreous cavity. Vitreous bodies are almost completely

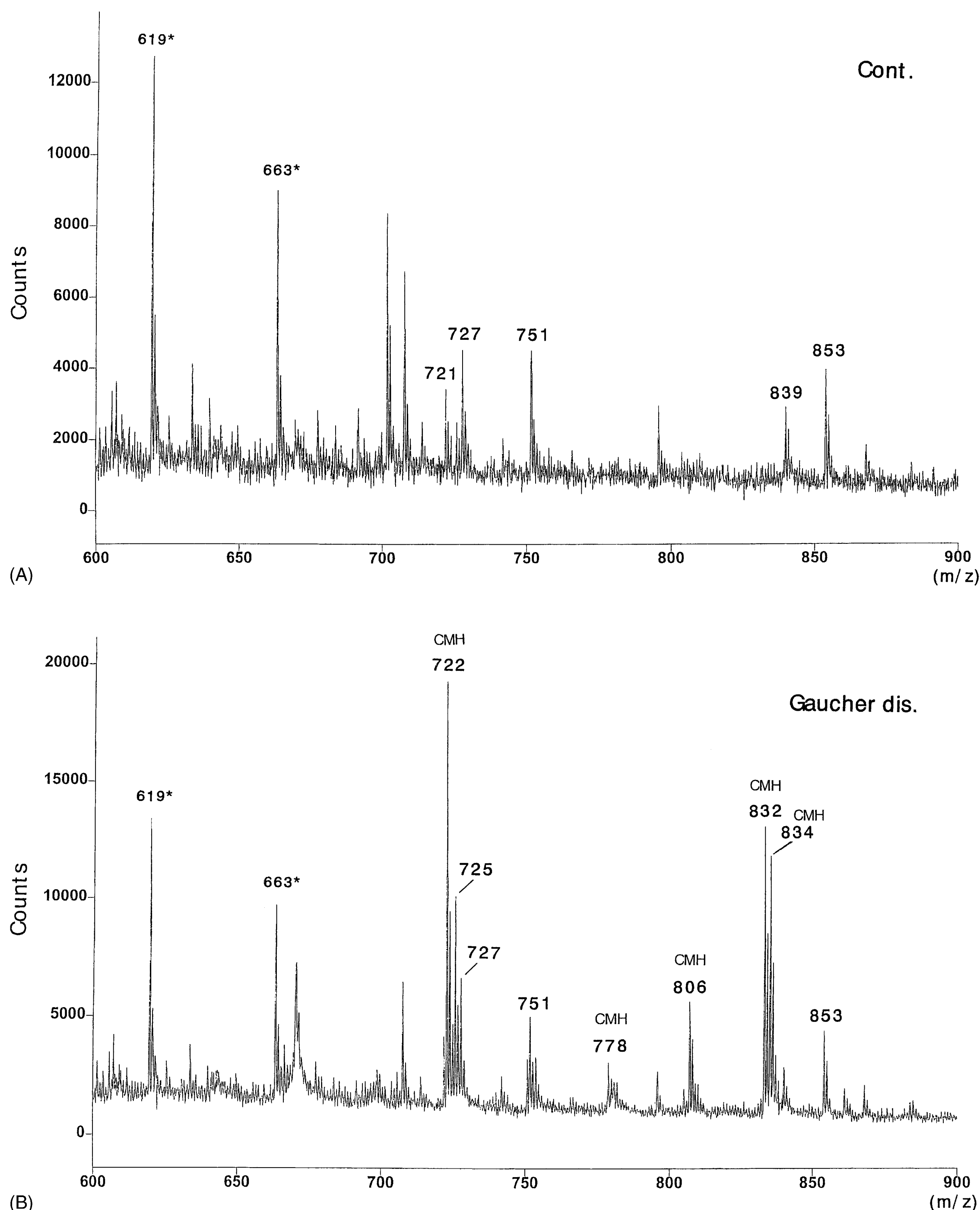


Fig. 3. TOF-MS mass spectra of vitreous bodies from a (A) control and (B) a patient with GD in the positive ion mode. The proposed molecular species are shown in Table 1. The matrix used was 2,5-DHB. The mass spectrum of the sphingolipids from the vitreous bodies of the GD patient showed sphingomyelin ions similar to those of the control patient and, in addition, $[M+\mathrm{Na}]^+$ ions corresponding to CMH species. (*) the molecular species of the mass peaks of *m/z* 619 and 663 are unknown, the authors speculate that these ions might be derived from the matrix.

composed of water. Vitreous opacity is commonly induced by uveitis, as a consequence of intrusion of effusive and infectious cells into vitreous bodies. These cells comprise neutrophils, lymphocytes, plasma cells, epithelioid cells and macrophages. Although the accumulation of glucosylceramide in the vitreous bodies of patients with GD has not been reported previously, the vitreous opacity in this patient might have been induced by infiltration of monocyte–macrophage phagocyte system cells, which contain much glucosylceramide, into the vitreous bodies.

We compared the molecular species of CMH accumulating in the vitreous bodies, which we analyzed this time, with those in the other lipid materials from liver, skin fibroblasts, and body fluids of GD patients, which we previously

reported [7–9]. The results were as follows: (a) while most of the molecular species of CMH identified in the vitreous bodies were the same as those identified in the liver, skin fibroblasts, and serum, a novel one, (d18:1C24:1) $[M+\mathrm{Na}]^+$, was identified in the GD vitreous bodies. (b) The molecular species of CMH in the vitreous bodies showed a wider spectrum, C16–C24, than in other tissues, i.e. liver, C16–C18; skin fibroblasts, C20–C24; and serum, C16–C18. (c) CMH in the vitreous bodies appeared as $[M+\mathrm{Na}]^+$ ions similarly to in other tissues on DE MALDI-TOF-MS analysis.

## 5. Conclusion

In this study, we first found the accumulation of CMH in the vitreous bodies in GD, using DE MALDI-TOF-MS as well as thin-layer chromatography. The former method is simple, convenient, and practical for the diagnosis of this disease. DE MALDI-TOF-MS analysis allows the accurate determination of small amounts of sphingolipids in tissues that contain small lipid components, and the identification of molecular species of accumulating sphingolipids. Some improvement of the sample preparation or analytical conditions might make this method more sensitive.

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