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Application of delayed extraction–matrix-assisted laser desorption ionization time-of-flight mass spectrometry for analysis of sphingolipids in pericardial fluid, peritoneal fluid and serum from Gaucher disease patients

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Abstract

Gaucher disease is a glycolipid storage disorder characterized by the accumulation of glucosylceramide. Using delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry (DE–MALDI-TOF-MS), we analyzed sphingolipids in pericardial fluid, peritoneal fluid, and serum from two patients with Gaucher disease. Crude lipids were extracted from 1 ml each of pericardial fluid, peritoneal fluid, and serum 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) in all the specimens, peaks of ceramide monohexoside and sphingomyelin were detected in both the controls and Gaucher disease patients; (b) in pericardial fluid, peritoneal fluid, and serum, the ceramide monohexoside/sphingomyelin ratio was increased in the Gaucher disease patients compared with in the controls. It was indicated that the accumulation of ceramide monohexoside in such samples from Gaucher disease patients can be easily detected with this DE–MALDI-TOF-MS method.

Keywords: Sphingolipids

1. Introduction

Matrix-assisted laser desorption ionization (MALDI) was first reported in 1990 as a useful technique for determination of the molecular masses of proteins and glycoproteins [1,2], as well as neutral glycosphingolipids [3], and gangliosides [4,5]. Delayed ion extraction (DE) was later introduced to matrix-assisted laser desorption ionization time-of-flight mass spectrometry, the technique being abbreviated as MALDI-TOF-MS, which dramatically improved the resolution and accuracy of the mass spectra. DE–MALDI-TOF-MS was recently applied to the precise identification of lysosphingolipids [6–8] and gangliosides [9].

Gaucher disease (GD) is caused by a deficiency of

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lysosomal acid β-glucosidase (glucocerebrosidase), which hydrolyzes glucosylceramide to ceramide and glucose. In patients with GD, therefore, glucosylceramide accumulates in visceral organs such as the liver, spleen and brain [10]. For the diagnosis of patients with GD, sphingolipids in tissues are analyzed by thin-layer chromatography or high-performance liquid chromatography.

Taketomi et al. reported the usefulness of DE–MALDI-TOF-MS for the analysis of glycosphingolipids in monkey brain tissues [11]. Previously, we reported that the analysis of sphingolipids in human visceral organs such as the liver, brain and spleen by DE–MALDI-TOF-MS was useful for the diagnosis of patients with Farber disease, GD, Niemann–Pick disease type C, and GM1-gangliosidosis [12]. Furthermore, we demonstrated the usefulness of DE–MALDI-TOF-MS for lipid analysis of cultured skin fibroblasts in some kinds of sphingolipidoses [13]. Some patients with GD develop pericarditis with pericardial fluid [14,15] and peritoneal fluid [15–20]. We report here analysis of sphingolipids in pericardial fluid, peritoneal fluid, and serum from two patients with GD by DE–MALDI-TOF-MS.

2. Materials and methods

2.1. Patients and specimens

Patient 1 was a 7-year-old girl with GD. She developed pericardial effusion at 7 years of age despite enzyme replacement therapy and died. Pericardial fluid and peritoneal fluid were collected after death [21].

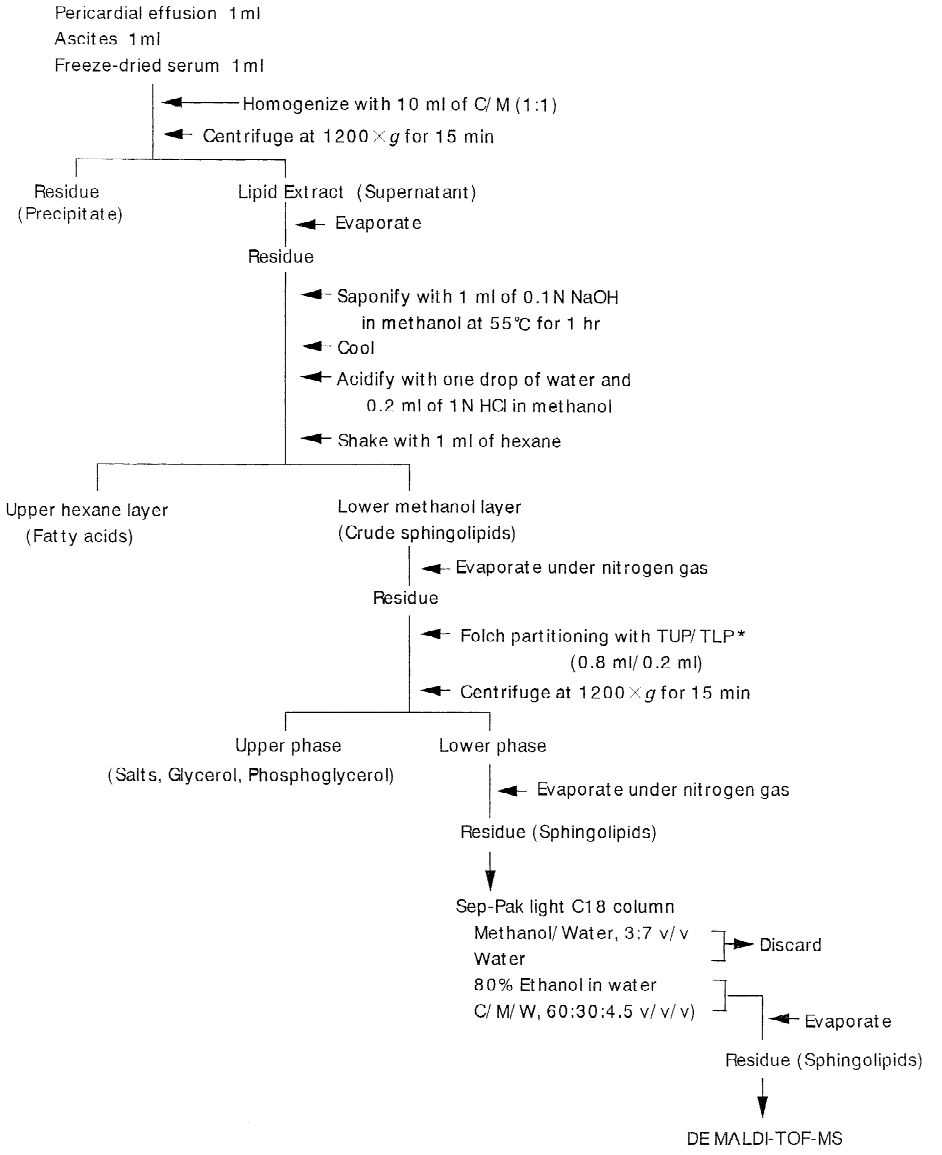
Patient 2 was a 6-month-old boy with GD. He developed severe central nervous system involvement with hepatosplenomegaly at 2 months of age. Enzyme assaying of lymphocytes confirmed this diagnosis and serum was collected for analysis.

Control specimens were collected, as follows: (a) pericardial fluid from a 6-year-old boy with acute leukemia. He had pericarditis and a large amount of pericardial fluid was drawn; (b) peritoneal fluid from three women with benign ovarian tumors, aged 32, 35 and 47 years, and from a 47-year-old women with a myoma uterus; and (c) serum from five children with common upper respiratory infection, aged from 2 to 5 years.

All materials were collected from the patients with their informed consent and/or that of their parents. Each sample was frozen and stored in −30 °C until examination.

2.2. Preparation of sphingolipids

The preparation of sphingolipids is schematically depicted in Fig. 1. In practice, 1 ml each of pericardial fluid and peritoneal fluid, and the residue of 1 ml of freeze–dried serum were homogenized in 10 ml of chloroform–methanol (1:1, v/v), ultrasonicated, and then centrifuged at 1200 *g* for 15 min. This procedure was performed twice, the crude lipids being obtained in the supernatant. The total crude lipids obtained were evaporated to dryness in a rotary evaporator and then treated with 1 ml of 0.1 *M* 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 1200 *g* for 5 min. After the upper phase had been removed, the lower phase was evaporated under a nitrogen stream. A 0.2-ml volume of the theoretical lower phase (chloroform–methanol–water, 86:14:1, v/v) and 0.8 ml of the theoretical upper phase (chloroform–methanol–water, 3:48:47, v/v) were mixed for Folch partitioning, and the resulting mixture was centrifuged at 1200 *g* for 15 min. After the upper phase had been discarded to remove salts, the lower phase was evaporated under a nitrogen stream. The resulting residue was dissolved in 1 ml of chloroform–methanol (1:1, v/v) and then applied to a Sep-Pak light C_{18} column (Waters, Milford, MA, USA) to remove salts. After washing of the column with 10 ml of methanol and 10 ml of methanol–water (3:7, v/v), each sample solution was applied to the column, followed by elution with 5 ml of methanol–water (3:7, v/v), 5 ml of water, 5 ml of 80% ethanol in water, and 5 ml of chloroform–methanol–water (60:30:4.5, v/v), in that order. Each eluate was collected for 15 min. The 80% ethanol in water and chloroform–methanol–water (60:30:4.5, v/v) fractions were collected and mixed. To completely elute



*TUP: theoretical upper phase solution (Chloroform/Methanol/Water, 3:48:47 v/v/v)
TLP: theoretical lower phase solution (Chloroform/Methanol/Water, 86:14:1 v/v/v)

Fig. 1. Schema of preparation of the sphingolipid fractions from body fluids and serum. Abbreviations: C/M, chloroform/methanol; C/M/W, chloroform/methanol/water.

various polar sphingolipids, which had been absorbed to the column, the two different polar solvent systems mentioned above were used for elution and collection. The collected fractions were evaporated to dryness in a rotary evaporator. The crude sphingolipids in the residue were dissolved in 0.1 ml of chloroform–methanol (1:1, v/v) for DE–MALDI-TOF-MS.

Through the above procedure, crude lipids, 5.2 to 24.6 mg, were obtained from each sample.

2.3. DE–MALDI-TOF-MS analysis

To 1.5 μl of each crude sphingolipid solution in a 1.5-ml Eppendorff tube, 1.5 μl of the matrix solution (10 mg of 2,5-dihydroxybenzoic acid 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 with a microcentrifuge (Chibitan; Tomy Kogyo, Fukushima, Japan) at 2000 *g* for 1 min. A 1-μl volume of the supernatant was loaded into a Voyager DE-RP (2.0 m flight length, reflector mode) Biospectrometry Workstation (Applied Biosystems, Framingham, MA, USA), and mass spectra of the 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), using the resolution calculator in the GRAMS/386 software (Galactic Industries, Salem, NH, USA) supplied with the instrument. 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 [6–9].

As matrices for MALDI-TOF-MS analysis, α-cyano-4-hydroxycinnamic acid (α-CHCA) and 2,5-DHB were purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

3. Results

The mass numbers of molecular species associated with sphingolipids are shown in Table 1 [22]. The mass spectra of sphingolipids in pericardial fluid, peritoneal fluid, and serum obtained by DE–MALDI-TOF-MS are presented in Figs. 2, 3 and 4, respectively (A: control, B: Gaucher disease). Sphingomyelin (SM) with various molecular ions corresponding to different carbon lengths of fatty acids and sphingosine moieties were observed as major peaks, and a small peak of ceramide monohexoside (CMH) was also detected.

The intensity ratios of CMH/SM calculated from the TOF-MS spectra were determined and the results were as follows. The ratios of CMH/SM in pericardial fluid from the controls ($n=2$) and GD patients ($n=2$) were 0.07 and 0.06, and 0.18 and 0.15, respectively, as shown in Fig. 5. The ratios in peritoneal fluid from the controls ($n=4$) and GD patients ($n=2$) were 0.04±0.014 (mean±SD, range, 0.03 to 0.06), and 0.08 and 0.085, respectively. The ratios in serum from the controls ($n=5$), and GD patients ($n=2$) were 0.023±0.016 (mean±SD, range, 0.0 to 0.045), and 0.075 and 0.070, respectively. These CMH/SM ratio results indicate that CMH was accumulated in pericardial fluid, peritoneal fluid, and serum in both the GD patients.

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

Sphingomyelin, m/z		Ceramide monohexoside*, m/z	
697	(d18:1C14) $[M+Na]^+$	698	(d18:1C16) $[M+H]^+$
699	(d18C14) $[M+Na]^+$	722	(d18:1C16) $[M+Na]^+$
701	(d18:1C16:1) $[M+Na]^+$	728	(d18:1C18) $[M+Na]^+$
703	(d18:1C16) $[M+Na]^+$	756	(d18:1C20) $[M+Na]^+$
705	(d18C16) $[M+Na]^+$	780	(d18C20) $[M+Na]^+$
721	(d18:1C16) $[M+H_2O+H]^+$		
723	(d18:1C16:1) $[M+Na]^+$		
725	(d18:1C16) $[M+Na]^+$		
727	(d18C16) $[M+Na]^+$		
741	(d18C17) $[M+Na]^+$		
745	(d18:1C19) $[M+H]^+$		
751	(d18:1C18:1) $[M+Na]^+$		
753	(d18:1C18) $[M+Na]^+$		
781	(d18:1C20) $[M+Na]^+$		
803	(d18C23) $[M+H]^+$		
807	(d18:1C22:1) $[M+Na]^+$		
809	(d18:1C22) $[M+Na]^+$		
813	(d18:1C24:1) $[M+H]^+$		
823	(d18:1C23) $[M+Na]^+$		
833	(d18:1C24:2) $[M+Na]^+$		
835	(d18:1C24:1) $[M+Na]^+$		
853	(d18C25) $[M+Na]^+$		

*Ceramide monohexoside includes glucosylceramide. ''d'' indicates dihydroxy-sphingosine.

4. Discussion

GD comprises a heterogeneous spectrum of clinical signs and symptoms in patients with defective

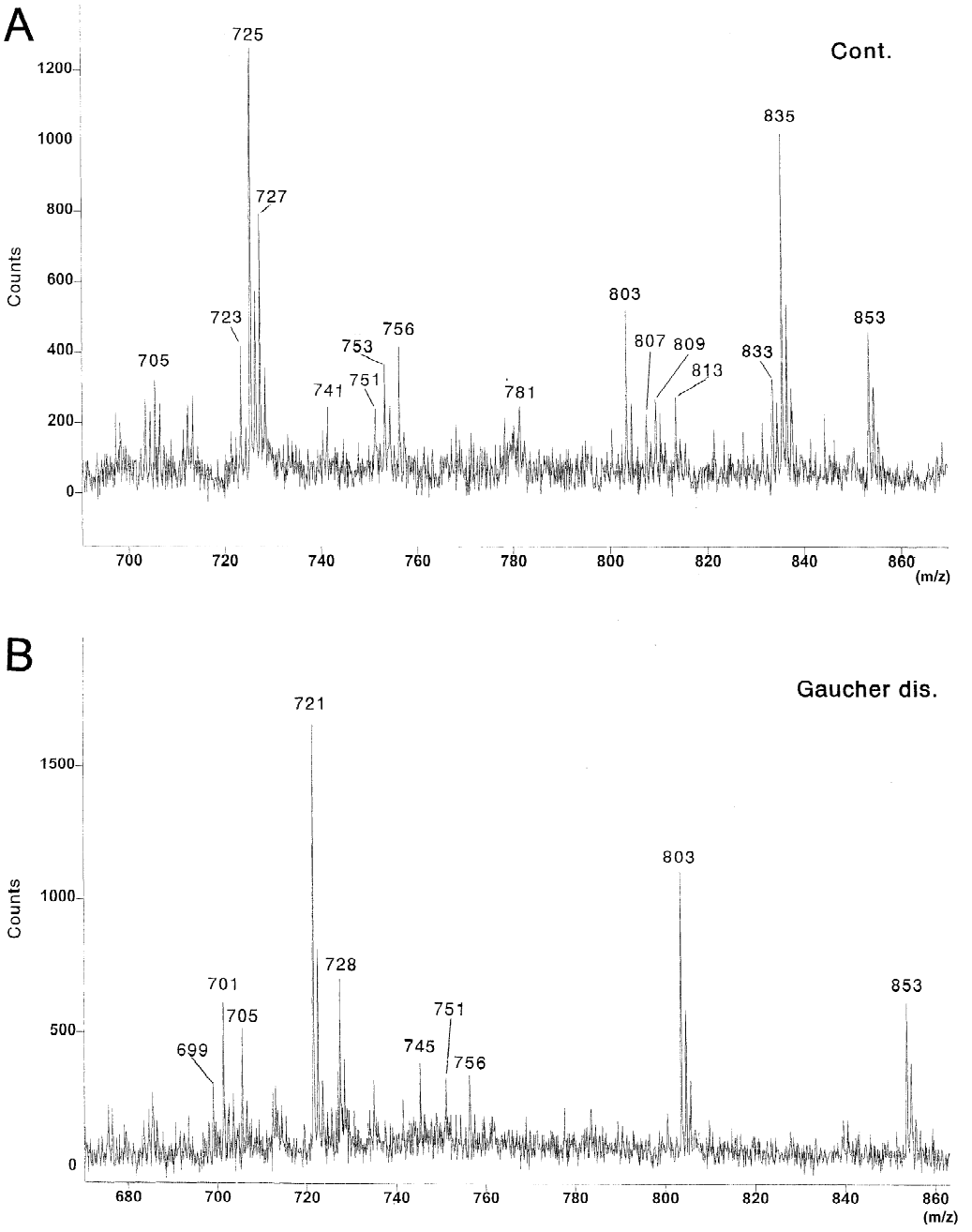


Fig. 2. TOF-MS mass spectra of pericardial fluid from a control (A) and a patient with Gaucher disease (B) in the positive ion mode. The proposed molecular species are shown in Table 1. The matrix was 2,5-dihydroxybenzoic acid (2,5-DHB).

intracellular hydrolysis of glucosylceramide and related glucosphingolipids [10]. At the molecular level, mutations of the gene that encodes lysosomal hydrolase, acid β-glucosidase or glucocerebrosidase have been reported.

Classically, the diagnosis of GD has been made

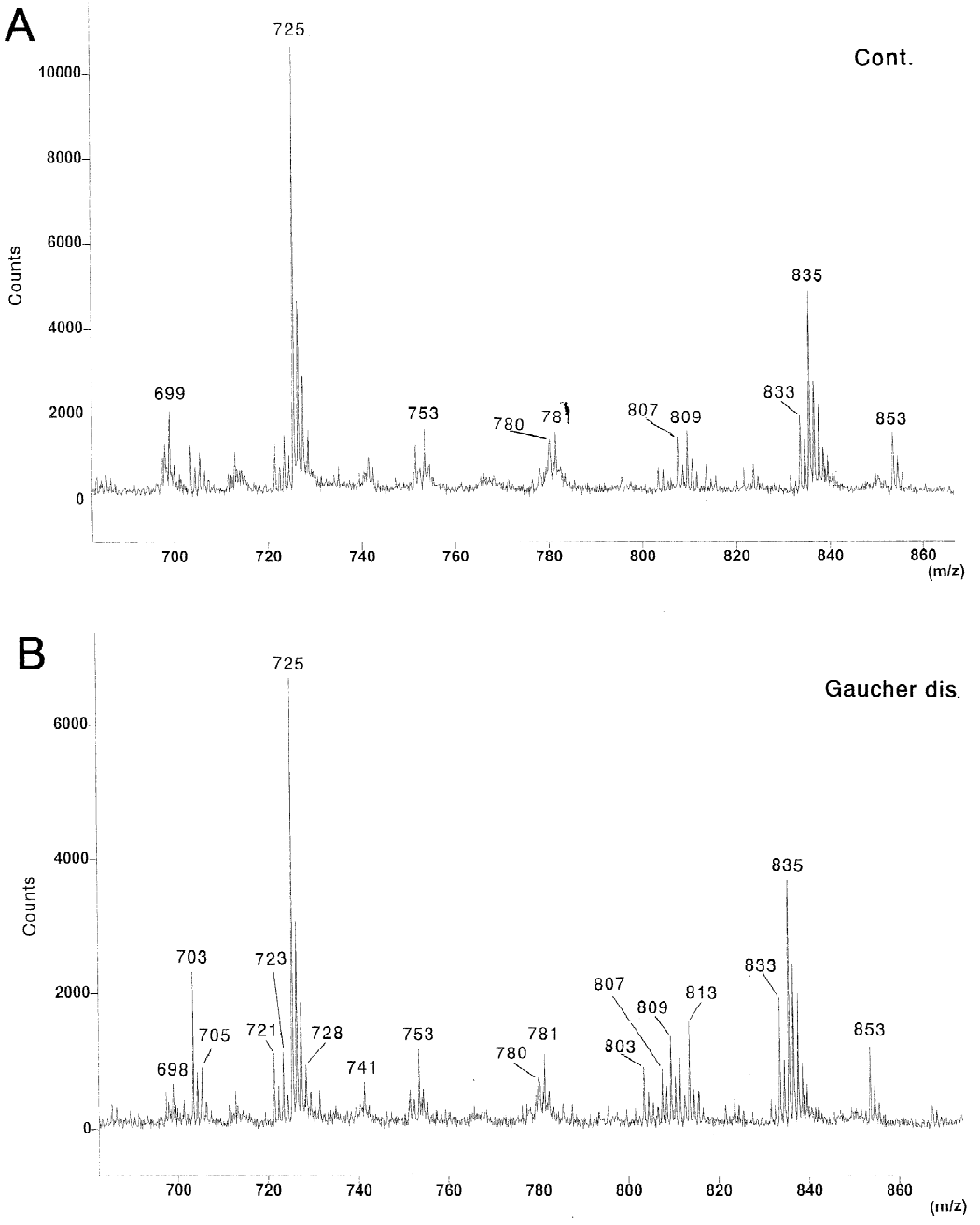


Fig. 3. TOF-MS mass spectra of peritoneal fluid from a control (A) and a patient with Gaucher disease (B) in the positive ion mode. The proposed molecular species are shown in Table 1. The matrix was 2,5-dihydroxybenzoic acid (2,5-DHB).

through the detection of a specific storage cell, the Gaucher cell. These cells are derived from the monocyte–macrophage phagocyte system and contain accumulated glucosylceramide [10]. The largest numbers of such cells are found in the spleen, sinusoids of the liver, bone marrow, and parenchyma

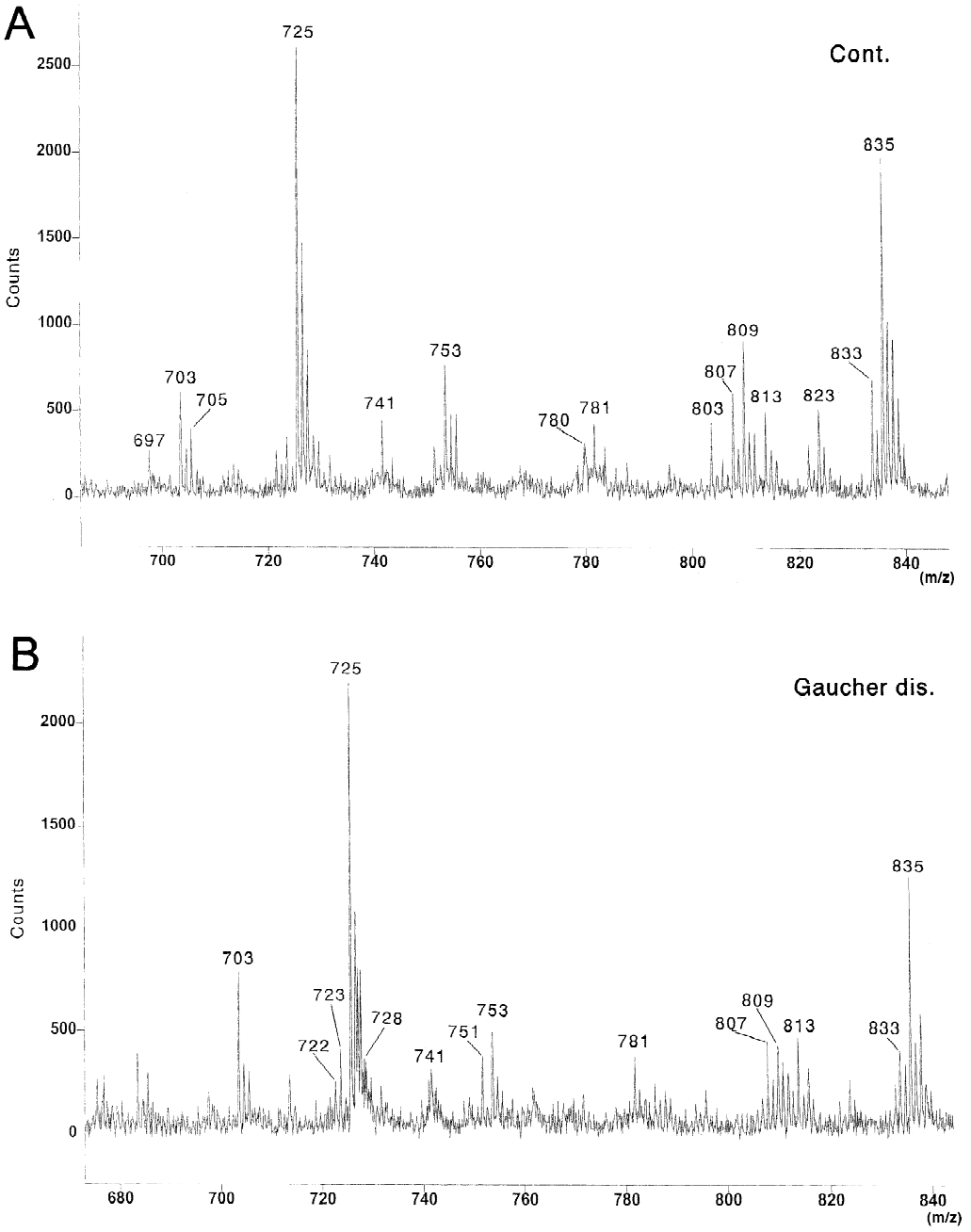


Fig. 4. TOF-MS mass spectra of serum from a control (A) and a patient with Gaucher disease (B) in the positive ion mode. The proposed molecular species are shown in Table 1. The matrix was 2,5-dihydroxybenzoic acid (2,5-DHB).

of the lymph nodes. Gaucher cells have also been detected in body fluids such as pericardial fluid [21], and cerebrospinal fluid [23] of patients with GD. Glucosylceramide released from lysed Gaucher cells into the extracellular space of the spleen [24] causes an elevated plasma level of glucosylceramide [10].

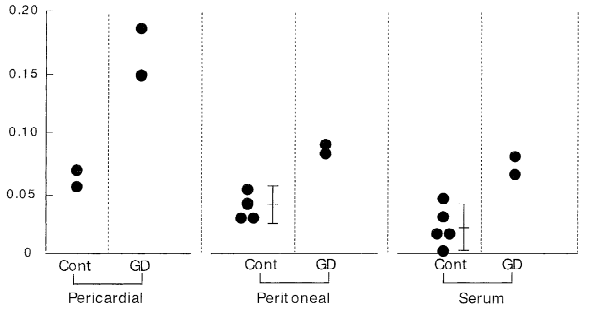


Fig. 5. Ceramide monohexoside/sphingomyelin (CMH/SM) peak intensity ratios. The data plotted are the averages of duplicate measurements. The bars in the control columns indicate the means and standard deviation. Ceramide monohexoside includes glucosylceramide. Abbreviations: Cont, control; GD, Gaucher disease.

These findings are consistent with the elevated levels of glucosylceramide in the pericardial fluid, peritoneal fluid, and serum from our patients with GD.

In previous investigations [12,13], we performed large-scale DE–MALDI-TOF-MS (Voyager Elite XL, 6.6 m flight length in reflector mode), but in this study we used a small-scale popular edition apparatus. Mass spectrometry including MALDI-TOF-MS has been widely used for the identification and confirmation of glycosphingolipids as well as other chemical compounds. Furthermore, Sugiyama et al. applied DE–MALDI-TOF-MS for the first time to determination of the sulfatide content of normal human sera [25]. They reported that this new approach involving DE–MALDI-TOF-MS for such quantitation was sensitive, convenient, and reliable. In this study, we determined the CMH/SM intensity ratio to evaluate the accumulation of CMH. It was revealed that this method involving DE–MALDI-TOF-MS is practical and convenient for the semi-quantitation of CMH in human body fluids.

There have been some reports of the quantitation of glucosylceramide in plasma from patients with GD [26–28]. In those studies, plasma glucosylceramide was measured by gas–liquid chromatography, total lipids being extracted from 50 ml of plasma [26–28]. In comparison with those methods, the volume of serum was small in our study, i.e., only 1 ml. Previously, it was reported that plasma glucosylceramide is associated with lipoproteins [26,27]. As lipoproteins exist in serum, elevation of CMH in the serum from the patients with GD in our study was observed, which is consistent with previous reports.

We conclude from our data that sphingolipids in the three kinds of body fluid examined from patients with GD can be evaluated by means of DE–MALDI-TOF-MS using only small amounts of the body fluids. This method is simple, convenient, and practical for the diagnosis of this disease. Some improvement of the sample preparation and analytical conditions may be needed to make this method more sensitive.

Nomenclature

α-CHCA	α-cyano-4-hydroxy-cinnamic acid
2,5-DHB	2,5-dihydroxybenzoic acid
DE–MALDI-TOF-MS	delayed extraction–matrix-assisted laser desorption ionization time-of-flight mass spectrometry
GD	Gaucher disease
CMH	ceramide monohexoside
SM	sphingomyelin
‘‘d’’	in the sphingolipid compositions indicates dihydroxy-sphingosine

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