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Journal of Chromatography B, 832 (2006) 97–102
JOURNAL OF CHROMATOGRAPHY B
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Quantitative evaluation of sphingolipids using delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry with sphingosylphosphorylcholine as an internal standard
Practical application to cardiac valves from a patient with Fabry disease

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Received 1 January 2005; accepted 26 December 2005
Available online 20 January 2006

Abstract

Fabry disease is a glycolipid storage disorder caused by a defect of α-galactosidase A, and characterized by the systemic deposition of glycosphingolipids with terminal α-galactosyl moieties, mainly globotriaosylceramide, in tissues. Using delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry (DE MALDI-TOF-MS), we analyzed the sphingolipids in the cardiac valves from a 49-year-old male patient with Fabry disease who suffered from congestive cardiac failure. Crude lipids were extracted from the cardiac valves 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) ion peaks with *m/z* values corresponding to different ceramide trihexoside (CTH) species were detected; (b) with sphingosylphosphorylcholine (SPC) as the internal standard for semi-quantification of CTH, the relative peak height of CTH was calculated and plotted versus its amount loaded on the sample plate for MALDI-TOF-MS. The relative peak height of CTH with fatty acid C16:0 showed linearity between 0 and 50 ng CTH (regression coefficient, $r > 0.95$); (c) semi-quantitative analysis revealed striking accumulation of CTH in the cardiac valves from the patient with Fabry disease. It was indicated that the accumulation of CTH in cardiac valves from Fabry disease patients can be detected with the DE MALDI-TOF-MS method. SPC is commercially available, and this semi-quantitative method involving MALDI-TOF-MS was found to be convenient, reliable and useful for CTH. It is expected to be applied to the quantification of CTH in small amounts of body fluids or other tissues and to clinical examination. It is also expected to be applicable to the quantification of other glycosphingolipids.
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Keywords: Delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Fabry disease; Sphingolipid

1. Introduction

Matrix-assisted laser desorption ionization (MALDI) has been reported [1] to be a useful technique for the detection of high mass molecules, and subsequently for detecting glycosphingolipids including lysosphingolipids [2–4]. It was reported previously that delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry (DE MALDI-TOF-MS) is a useful technique for the quantitative analysis of biological materials with high sensitivity [5–8].

Fabry disease is an X-linked recessive inborn error caused by a deficiency of lysosomal hydrolase α-galactosidase, which hydrolyzes glycosphingolipids with terminal α-galactosyl moieties. This enzymatic defect leads to the systemic deposition of glycosphingolipids with terminal α-galactosyl moieties, predominantly globotriaosylceramide [Gal(α1 → 4)Gal(β1 → 4)-Glc(β1 → 1′)-ceramide], which is usually called ceramide trihexoside (CTH). The lipoidal moiety of amphipathic glycosphingolipids comprises a hydrophobic

Abbreviations: DE MALDI-TOF-MS, delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry; 2,5-DHB, 2,5-dihydroxybenzoic acid; CTH, ceramide trihexoside; SPC, sphingosylphosphorylcholine

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doi:10.1016/j.jchromb.2005.12.039

structure called ceramide, which consists of a mixture of sphingosine and related long chain aliphatic amines joined through amide linkages to various fatty acids. The fatty acid moieties are primarily saturated and monounsaturated compounds with chain lengths of C16 to C26. For the diagnosis of Fabry disease, sphingolipids in tissues have been analyzed by thin-layer chromatography or high-performance liquid chromatography. Previously, we reported that the analysis of sphingolipids in various human tissues [9], cultured skin fibroblasts [10], and body fluids [11] by DE MALDI-TOF-MS was useful for the diagnosis of sphingolipidoses. We analyzed the sphingolipids in the cardiac valves from a patient with Fabry disease using DE MALDI-TOF-MS. We also report in this paper a method for semi-quantitative analysis of sphingolipids, predominantly CTH, involving DE MALDI-TOF-MS with sphingosylphosphorylcholine (SPC) as an internal standard. This study demonstrates that the MALDI-TOF-MS method is useful and promising for the quantification of glycosphingolipids.

2. Materials and methods

2.1. Patients and specimens

The patient was a 49-year-old male with classical type Fabry disease. He suffered from exertional dyspnea, congestive cardiac failure, hypertrophic cardiomyopathy, aortic insufficiency, and right hypercardia at 46 years of age. He underwent cardiac valvular, aortic and mitral valves, displacement due to progression of the congestive cardiac failure. The α-galactosidase activity in lymphocytes in peripheral blood was only 3% of the control level. The aortic and mitral valve, which were resected from the patient on operation, were used as samples for analysis. Autopsy samples of the aortic and mitral valves of a 46-year-old female with hypertension and dissecting aneurysm of the aorta were 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 method used to prepare a sphingolipid fraction is schematically depicted in Fig. 1. Briefly, about 20 mg of each tissue sample was homogenized in 8 ml of chloroform–methanol, 2:1 (v/v), 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 $1200 \times g$ for 5 min, the lower phase was evaporated to dryness in a rotary evaporator, and then treated with 1 ml of 0.1N 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 1N HCl in methanol, one drop of water and 1 ml of hexane were added, followed by centrifugation at $1200 \times g$ 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 $1200 \times g$ 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 for thin-layer chromatography and DE MALDI-TOF-MS.

2.3. Thin-layer chromatography

For comparison with MALDI-TOF-MS analysis, thin-layer chromatography was performed on silica gel 60 plates (Merck, Darmstadt) 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 [12]. All reagents were of analytical grade.

2.4. 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 mixture was shaken vigorously on a vortex mixer, and then centrifuged in a microcentrifuge (Chibitan; Tomy Kogyo, Fukushima, Japan) at $2000 \times g$ for 1 min. One microliter 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 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 previously [2–4,13]. 2,5-DHB was purchased from Sigma Chemicals (St. Louis, MO, USA). All other reagents were of analytical grade.

2.5. Quantitative analysis of CTH

Various amounts of authentic CTH with fatty acid C16:0 and 50 μg of SPC, as an internal standard, were added to 20 mg of bovine aortic valve tissue to obtain a standard curve. A standard curve was obtained each time. Lipid extraction, preparation, and DE MALDI-TOF-MS analysis were performed by the methods mentioned above. The relative peak height of CTH as to that of SPC was calculated. Using the standard curve obtained, quantitative analysis of CTH in sample tissues from the patient and control was performed, i.e. from the ion peak height ratio for

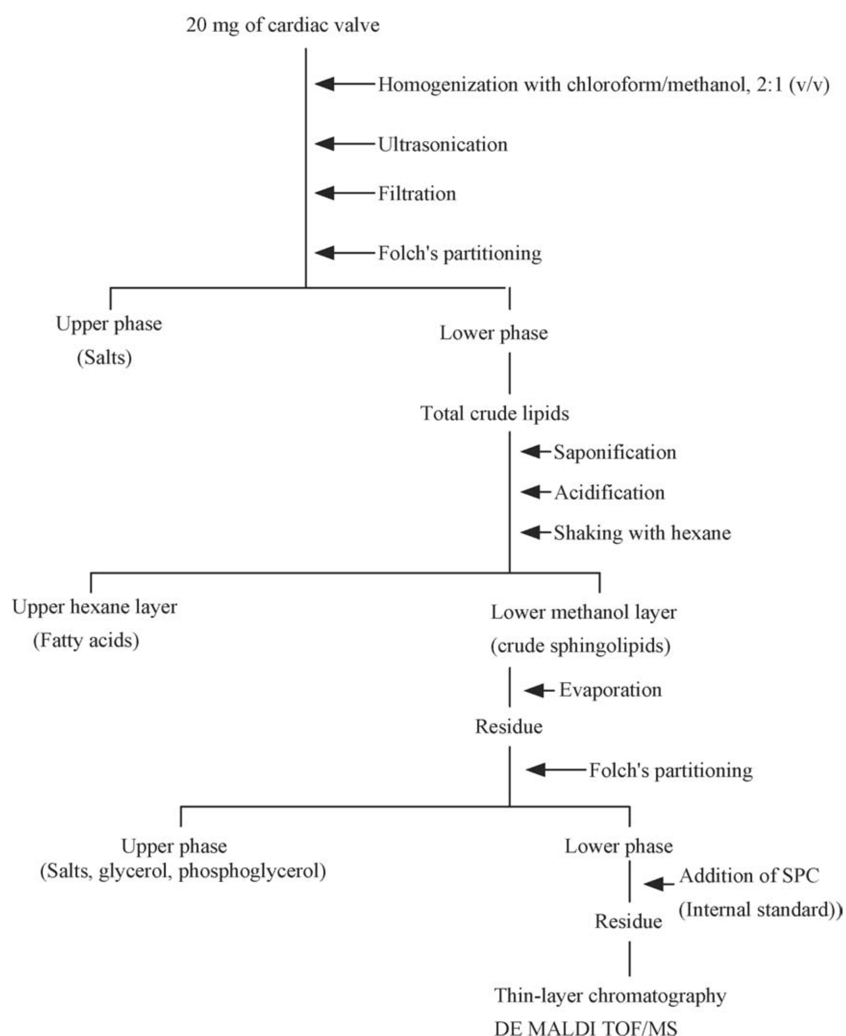

Fig. 1. Schema of preparation of sphingolipid fractions from cardiac valves.

each CTH species and SPC, the amount of CTH in each sample tissue was determined as CTH with fatty acid C16:0. SPC and authentic CTH with fatty acid C16:0 were purchased from Calbiochem-Novabiochem (San Diego, CA, USA) and Sigma Chemicals (St. Louis, MO, USA), respectively.

3. Results

The standard curve for CTH semi-quantification is shown in Fig. 2. Each point is the mean value of triplicate measurements of the relative peak height. The relative peak height value was plotted against the CTH content. A good linear relationship was obtained between 0 and 50 ng CTH, as illustrated in Fig. 2. The regression coefficient of these values was 0.975.

The mass spectra of sphingolipids in the cardiac valves from the control and patient with Fabry disease obtained on DE MALDI-TOF-MS are presented in Fig. 3. Table 1 shows the *m/z* values of the different $[M+Na]^+$ and $[M+H]^+$ ions in the mass spectra in Fig. 3, and the proposed sphingolipid species corresponding to these ions [14]. The *m/z* values of the $[M+Na]^+$ and $[M+H]^+$ ions in the mass spectrum corresponded to SPC in the lipid material of a control mitral valve specimen (Fig. 3A). The mass spectrum of control cardiac valves showed scanty ion peaks with *m/z* values, 1103, 1157, and 1159, corresponding to different CTH species (data not shown). The mass spectrum of the sphingolipids from the aortic valve of the Fabry disease patient showed SPC ions similar to those in the control one and, in addition, prominent $[M+Na]^+$ ions corresponding to CTH species (Fig. 3B). The CTH contents of the cardiac valves determined by MALDI-TOF-MS are presented in Table 2.

Thin-layer chromatography analysis gave a single band exhibiting a similar Rf value to that of the standard CTH that was

Table 1
m/z and proposed molecular species associated with sphingolipids

Ceramide trihexoside	Sphingosylphosphorylcholine
m/z 1047 (d18:1C18:0) $[M+Na]^+$	*m/z* 465 $[M+H]^+$
1075 (d18:1C18:0) $[M+Na]^+$	487 $[M+Na]^+$
1103 (dl8:1C20:0) $[M+Na]^+$	
1131 (dl8:lC22:0) $[M+Na]^+$	
1157 (dl8:lC24:l) $[M+Na]^+$	
1159 (dl8:lC24:0) $[M+Na]^+$	

“d” indicates dihydroxy-sphingosine.

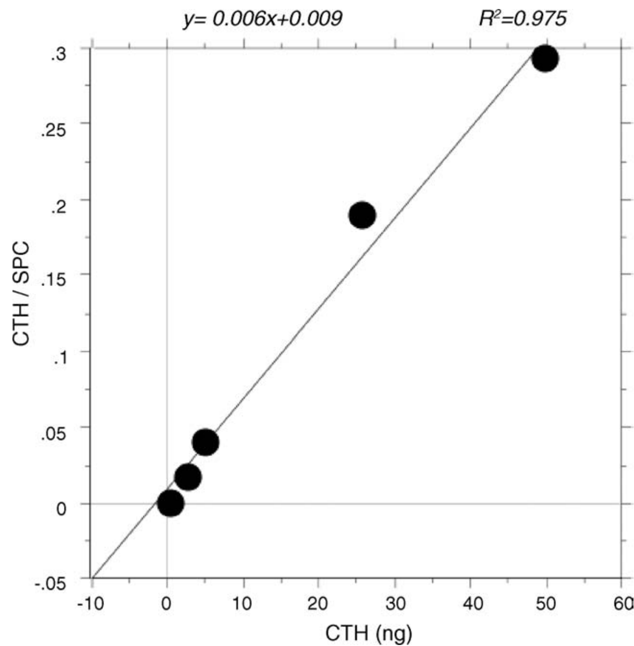

Fig. 2. A representative standard curve for the CTH values for bovine aortic valves. The transverse axis indicates the amount of CTH with fatty acid C16:0 loaded on the sample plate. Various amounts of authentic CTH with fatty acid C16:0 and 50 μg of SPC, as an internal standard, were added to 20 mg of bovine aortic valve, and subsequently lipid extraction, preparation, and DE MALDI-TOF-MS analysis were performed, and the relative peak height of CTH as to that of SPC was calculated. The averages of triplicate measurements are plotted. *R* indicates the regression coefficient.

Table 2
CTH contents of cardiac valves

Fabry disease		Control	
LCC	RCC	Mitral valve	Aortic valve
8.7 mg/mg protein	11.0 mg/mg protein	2.1 mg/mg protein	1.2 mg/mg protein

LCC: left coronary cusp of aortic valve; RCC: right coronary cusp of aortic valve.

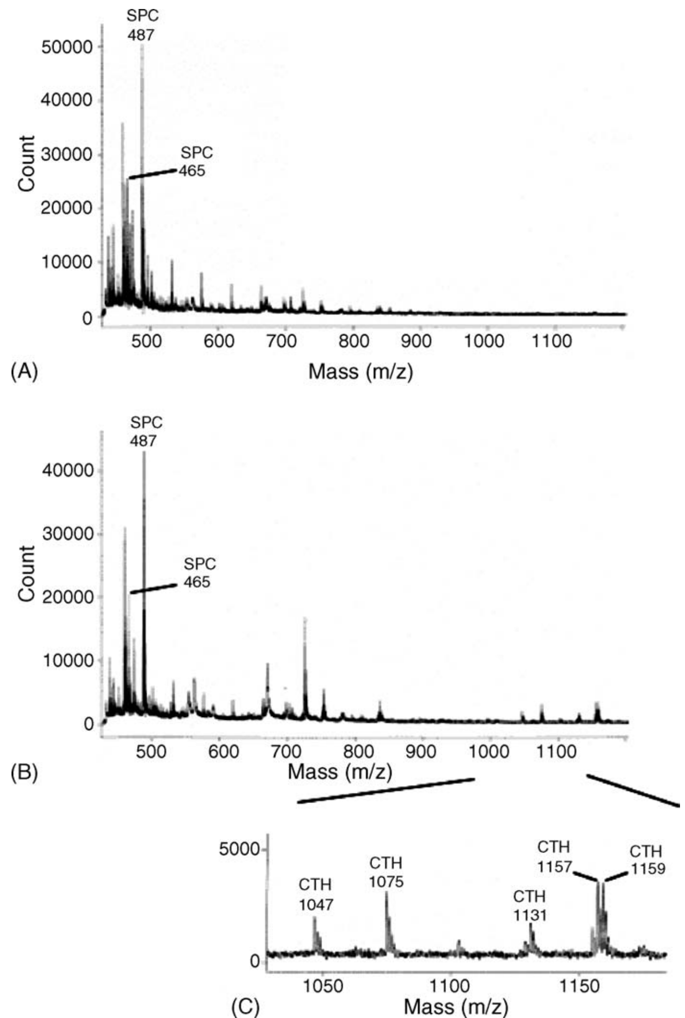

Fig. 3. TOF-MS mass spectra of a control mitral valve (A), the right coronary cusp of an aortic valve from the patient with Fabry disease (B), and a section of the CTH mass spectrum (C) in the positive ion mode magnified. The mass numbers (*m/z*) and proposed molecular species are shown in Table 1. The matrix used was 2,5-DHB. The mass spectrum of the sphingolipids from the aortic valve of the Fabry patient showed $[M + Na]^+$ ions corresponding to CTH species.

obviously observed for the valve tissues from the Fabry patient, but was not seen in the control lanes (Fig. 4).

Hence, these thin-layer chromatography and DE MALDI-TOF-MS results were compatible, indicating that CTH had accumulated in the cardiac valves from the Fabry disease patient.

4. Discussion

The α-galactosidase A activity deficiency in patients with Fabry disease leads to the progressive accumulation of glycosphingolipids with terminal α-D-galactosyl residues in the lysosomes in most non-neural tissues and in body fluids. The cardiac manifestations in Fabry disease are one of the major problems for the patients. With aging, the cardiac symptoms of the disease worsen with the progressive deposition of glycosphingolipids in the cardiovascular system [15]. The progressive deposition of glycosphingolipids in myocardial cells and valvular fibroblasts, and coronary vessels is a primary cause of cardiac disease in affected hemizygotes and some heterozygotes with Fabry disease [16–18].

In normal individuals, the highest concentration of globotriaosylceramide is found in the kidneys, followed by in the aorta, spleen, and liver [19–21]. In classic hemizygotes with Fabry disease, increased concentrations of globotriaosylceramide are found in all analyzed materials except erythrocytes [19,21–24], which indicates that most tissues are involved in the catabolism of these glycosphingolipids. In this study, the accumulation of CTH in the aortic valves, which were not fixed in formalin, from a live patient with Fabry disease was eight to nine times as high as that in normal control samples. The major fatty acid composition of CTH in this study was 16:0, 18:0, 22:0, 24:1, and 24:0, which was nearly the same as previously reported [19].

Sugiyama et al. first reported the quantitative analysis of glycosphingolipids, including serum sulfatide, using DE MALDI-TOF-MS [25]. In that study, hydrogenated *N*-acetyl lysosulfatide was used as an internal standard, and the results corresponded

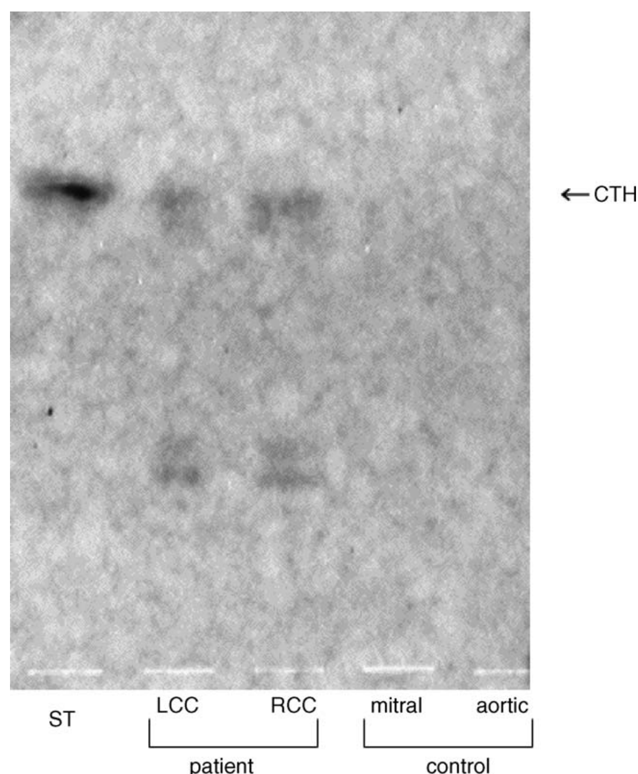

Fig. 4. Thin-layer chromatograms of sphingolipid fractions of cardiac valves from the patient with Fabry disease and a control. Crude lipids were applied and the visualized with primuline reagent under ultraviolet light. The solvent system was chloroform–methanol–0.2% $CaCl_2$ in water, 60:30:6 (v/v/v). ST: standard ceramide trihexoside; LCC: left coronary cusp of aortic valve; RCC: right coronary cusp of aortic valve; patient: patient with Fabry disease; mitral: mitral valve; aortic: aortic valve. The lipid materials in the cardiac valves from the patient with Fabry disease gave one spot, which exhibited a similar Rf value to that of the standard CTH and is not present in the control lanes.

well to the reported data obtained on gas–liquid chromatography. We used SPC as an internal standard. SPC is physiologically nonexistent in normal human tissues or body fluids except as an artifact. CTH with fatty acid C17:0, which is one of the candidate internal standards for quantitative analysis of CTH, might possibly exist in small amounts in organisms. Although CTH with fatty acid C17:0 needs to be prepared for use, SPC is commercially available and convenient to use. In this study, we extracted crude lipids from tissues and subsequently purified sphingolipids from the lipids prepared. Consequently, the standard curve of the relative peak height of CTH as to SPC showed good linearity between 0 and 50 ng CTH loaded on the sample plates for MALDI-TOF-MS. Within this CTH range, the influence of ionization of CTH due to interference by sample compounds other than sphingolipids (biological matrix) seemed to be little. CTH is a heterogeneous glycosphingolipid that consists of numerous isoforms with various fatty acid moieties. In this study, using the standard curve obtained for CTH with fatty acid C16:0, from the ion peak height ratio of each CTH species and SPC, the amounts of CTH in the sample tissues were determined as CTH with fatty acid C16:0. Because each isoform of CTH with a different fatty acids moiety might exhibit a different ionization tendency, the method involving DE MALDI-TOF-MS presented here only allows semi-quantification of CTH. Previously, Desnick et al. reported morphologic and biochemical studies on the cardiac valves of patients with Fabry disease. In their study, after silicic acid column chromatography and mild-acid hydrolysis of total lipids extracted from the patient's tissues, the glycosphingolipids were quantitated by gas–liquid chromatography [26]. In comparison with the quantitative method reported by Desnick et al., the MALDI-TOF-MS method we described in this paper has the following advantages: (a) although in the paper of Desnick et al., there is no mention of the volumes of every tissue sample from which lipids were extracted, the solvent volume with which lipids were extracted from tissues is smaller for our method. Eight milliliters and 300 ml of chloroform–methanol, 2:1 (v/v), were used in our study and that of Desnick et al., respectively; and (b) with the MALDI-TOF-MS method, it is possible to obtain the isoform profiles of CTH concurrently with quantification. Recently, liquid chromatography–tandem mass spectrometry (LC/MS-MS) has also been applied to the analysis of globotriaosylceramide in plasma from patients with Fabry disease [27]. Generally speaking, MALDI has advantages over the electrospray ionization (ESI) method, which is used for LC/MS-MS, for investigation of non-volatile and/or high mass molecules, and thus the two methods, MALDI-TOF-MS and LC/MS-MS, should be compared and investigated regarding sensitivity, reproducibility, convenience, and time of analysis for the quantification of glycosphingolipids.

Semi-quantitative analysis involving DE MALDI-TOF-MS with SPC as an internal standard is expected to be applicable to the quantification of various glycosphingolipids other than CTH from small amounts of body fluids or tissues, and to clinical examination.

5. Conclusion

We showed the accumulation of CTH in the cardiac valves in Fabry disease semi-quantitatively, using DE MALDI-TOF-MS as well as thin-layer chromatography. The former method was simple, convenient, and practical for the diagnosis of this disease. DE MALDI-TOF-MS analysis with SPC as an internal standard may allow the semi-quantification of even low levels of sphingolipids in tissues, and identification of the molecular species of accumulating sphingolipids.

Acknowledgements

We are indebted to Tamotsu Taketomi (Department of Biochemistry, Research Center on Aging and Adaptation, Shinshu University School of Medicine) for the helpful discussions. This study was partly supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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