Synthesis of novel internal standards for the quantitative determination of plasma ceramide trihexoside in Fabry disease by tandem mass spectrometry

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Abstract The concentration of globotriaosylceramide (ceramide trihexoside (CTH)) in the plasma of patients with Fabry disease has been determined quantitatively by tandem mass spectrometry (MS) using novel internal standards, [D4]C-16 CTH and C-17 CTH, which were synthesised enzymically from *lyso*-CTH using the reverse reaction of sphingolipid ceramide *N*-deacylase. C-17 CTH was also synthesised chemically from *lyso*-CTH. This strategy has also been used to prepare standards for the quantitative determination by MS of other glycosphingolipids. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Fabry disease; Mass spectrometry; Internal standard for glycosphingolipid; Quantitation

1. Introduction

Fabry disease is an X-linked lysosomal storage disease resulting from a deficiency of the lysosomal hydrolase, α-galactosidase A (EC 3.2.1.22), which leads to the progressive accumulation within lysosomes of glycosphingolipids with terminal α-galactosyl residues [1]. Affected hemizygous males develop various symptoms in childhood or early adolescence, including severe pain and paresthesias in the extremities, angiokeratoma and corneal opacities, and death usually occurs in the fifth decade from renal failure or cardiac disease. Atypical clinical variants with some residual activity may be asymptomatic or mildly affected with late onset and mainly cardiac manifestations. Heterozygous females either suffer from a milder form of the disease, or may be asymptomatic, but occasionally they may be as severely affected as the hemizygous males [2]. The major storage product is ceramide trihexoside (CTH) or globotriaosylceramide ($GbOse_3Cer$, Gb_3, GL-3) [1]. Galabiosylceramide is also found in certain tissues as are blood group B substances in patients with blood group B or AB [3]. Storage occurs predominantly in the endothelial, perithelial and smooth muscle cells of blood vessels but there is deposition in many other cell types and storage products are present in body fluids. The level of globotriaosylceramide in tissues, plasma or urine can be used to follow the course of the disease or conversely to monitor treatment [4,5]. Several methods (for reviews see [1,6]) have been used to measure globotriaosylceramide directly including thin-layer chromatography (TLC) [7], enzyme-linked immunosorbent assay (ELISA) [6] and mass spectrometry (MS) [8], or indirectly by release and measurement of the oligosaccharide moiety by GLC [9] or high-performance liquid chromatography [10]. Glycosphingolipids can be detected and identified readily by tandem MS (MS/MS) [11,12] but their quantitative determination has been handicapped by the lack of suitable internal standards [13,14]. In this paper we describe the synthesis and use of novel internal standards for the quantitative determination of globotriaosylceramide and other glycolipids in plasma using MS/MS.

2. Materials and methods

2.1. Materials

Plasma was obtained from male patients with the classic form of Fabry disease and from normal controls with informed consent. Human erythrocyte CTH (Galα-(1→4)Galβ-(1→4)Glc-ceramide) (globotriaosylceramide, $GbOse_3Cer$, Gb_3, GL-3 or CTH) was obtained from Sigma (Dorset, UK). *Lyso*-CTH (Galα-(1→4)Galβ-(1→4)Glc-sphingosine) and sphingolipid ceramide *N*-deacylase were obtained from Calbiochem, CA, USA. [D4]-palmitic acid labelled in the 7,7,8,8 positions was supplied by Cambridge Isotopes, MA, USA. All chemical reagents were of analytical grade from Sigma, Dorset, UK.

2.2. Chemical synthesis of C-17 CTH

50 μg of *lyso*-CTH was dried completely under N_2 in a glass vial and then dissolved in 100 μl of heptadecanoic anhydride in chloroform (10 mg/ml). Pyridine (300 μl) was added and the solution was vortexed thoroughly and left at room temperature for 30 min. After drying completely under nitrogen to remove all the excess pyridine, 100 μl of heptadecanoic anhydride in chloroform (100 mg/ml) and 300 μl of pyridine were added to the reaction mixture, which was again thoroughly mixed and left at room temperature for 30 min. The reaction mixture was dried under nitrogen and desalted using a C-18 solid-phase extraction cartridge (LiChrolute, Merck, Poole, Dorset, UK) [6].

2.3. Enzymic synthesis of C-17 CTH and [D4]C-16 CTH

50 μg (~50 nmol) of human erythrocyte CTH was incubated in 20 μl of sodium acetate buffer, pH 5.0 with 1 mU of sphingolipid ceramide *N*-deacylase for 16 h at 37°C essentially as described by Ito et al. [15]) except that Triton X-100 was replaced by fatty acid-free bovine serum albumin (BSA) (1%, w/v). The reaction mixture was freeze-dried and the lipid products were extracted in 5 μl of chloroform:methanol (1:2, v/v). The extent of the enzymatic deacylation was determined by TLC on silica gel plates (Silica Gel 60, Merck, Poole, Dorset, UK) using chloroform:methanol:acetic acid (10%) (5:4:1) as the mobile phase. The glycosphingolipids and *lyso*-glyco-

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sphingolipids were detected using iodine vapour and identified by comparison with standards. *Lyso*-CTH was scraped off the TLC plate and recovered from the silica using two washes of 500 μl of chloroform:methanol (2:1, v/v).

The reverse reaction of sphingolipid ceramide *N*-deacylase [16] was exploited to reacylate *lyso*-CTH to form [D4]C-16 CTH, a type 1 internal standard (an isotopically labelled analogue, which is chemically identical to but with a higher mass than the target analyte), and C-17 CTH, a type 2 internal standard (a structurally related compound with similar chemical characteristics to the analyte) for quantitation of CTH by MS. Purified or commercial *lyso*-CTH (75 nmol) was incubated with 75 nmol of heptadecanoic acid or [D4]-palmitic acid and 200 μU of sphingolipid ceramide *N*-deacylase in 20 μl of 25 mM sodium phosphate buffer, pH 6.5 (containing fatty acid-free BSA (1%, w/v)), for 20 h at 37°C. The products of the reacylation reaction were analysed using TLC as described above. Over 90% conversion of *lyso*-CTH to CTH was achieved. For larger-scale preparation of the internal standards, the reaction products were desalted using a C-18 solid-phase extraction cartridge (LiChrolute, Merck, Poole, Dorset, UK). The C-18 cartridge was first primed with 5 ml of methanol, followed by 5 ml of H_2O. The reaction mixture was added directly to the cartridge and the salts and residual detergent present in the enzyme preparation were removed by successive washing with 3 ml H_2O, 3 ml of 60% methanol and 3 ml of 70% methanol. The CTH internal standards were eluted from the column using 2 ml of methanol followed by 2 ml chloroform:methanol (2:1, v/v).

2.4. Measurement of CTH in plasma

1 μg of CTH internal standard was added to 100 μl of plasma, from which the total lipid was extracted by the addition of 2 ml of chloroform:methanol (2:1, v/v) with shaking on a multivortexer for 20 min. Precipitated protein was removed by centrifugation at $3000\times g$ for 10 min. The chloroform:methanol (2:1, v/v) layer was transferred to a vial and 400 μl of H_2O was added and the vial was shaken for a further 20 min. The two layers were separated by centrifugation for 10 min at $3000\times g$ and the lower layer containing the neutral glycolipids including the CTH was transferred to a new vial and dried under N_2. The CTH was desalted prior to MS by reconstitution in chloroform followed by solid-phase extraction on a C-18 column (LiChrolute, Merck, Poole, Dorset, UK) with elution with acetone:methanol (9:1) [6].

2.5. Electrospray ionisation–MS/MS (ESI–MS/MS)

MS of glycolipids was carried out using a triple quadrupole VG Quattro I instrument (MicroMass, Altrincham, UK). The instrument was operated in positive ionisation mode. Samples were directly infused into the electrospray source via a 25 mm (i.d.) fused silica transfer line by means of a Harvard syringe pump at a flow rate of 25 μl/min. The capillary voltage was maintained at 3.43 kV with a cone voltage of 173 V. The source temperature was held constant at 80°C and nitrogen was used as the nebulising gas at a flow rate of 30 l/h. The masses of all the CTH isoforms were determined by operating the mass spectrometer in scan mode over a mass range of *m/z* 700–1300. Product ions were determined over a mass range of *m/z* 50–1200 following collision-induced dissociation using argon as the collision gas. The optimum collision energy was determined to be 70 eV with an optimum gas cell pressure of 3.2×10^{-3} mbar. Data were acquired using neutral loss scanning of *m/z* 162.0, operating in multiple channel acquisition mode and with a dwell time for each ion species of 100 ms. For quantitative analyses samples were infused into the mass spectrometer using the electrospray ion source and CMA/200 refrigerated auto sampler.

2.6. Liquid secondary ionisation–MS/MS (LSI–MS/MS)

To confirm the structures of the individual CTH isoforms and the internal standard, the CTH isoforms were analysed using the tandem

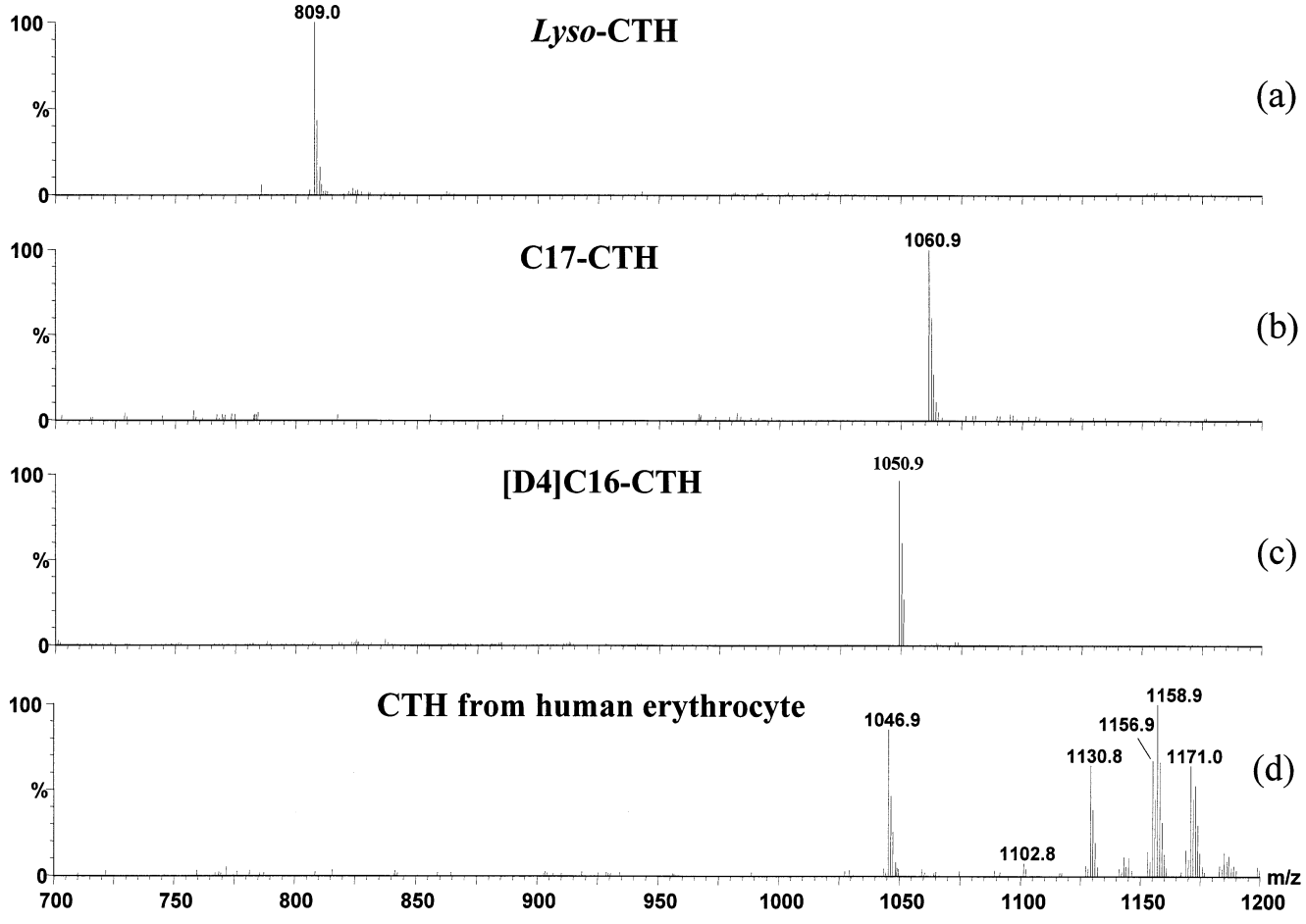

Fig. 1. ESI–MS spectra of (a) *lyso*-CTH, (b) C-17 CTH, (c) [D4]C-16 CTH and (d) CTH standard from human erythrocytes.

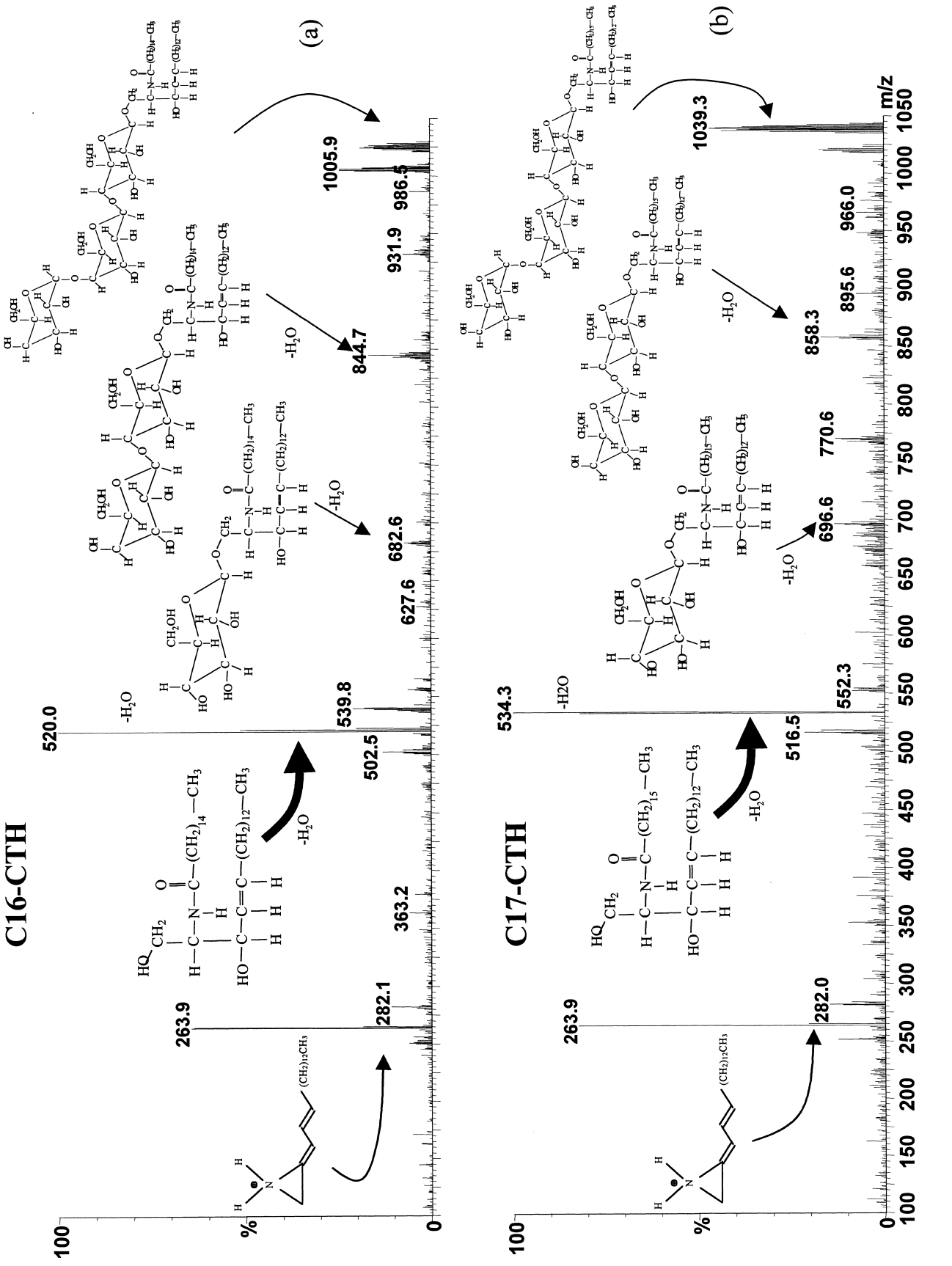

Fig. 2. LSI–MS/MS daughter ion spectra of (a) C-16 CTH and (b) C-17 CTH.

mass spectrometer operating with an LSI source (LSI–MS/MS). This ionisation mode produced more extensive fragmentation through the ceramide backbone of the CTH, which could not be achieved using the ESI source, and allowed definitive determination of the acyl group in each CTH isoform. LSI–MS/MS of glycolipids was carried out using a triple quadrupole VG Quattro I instrument (MicroMass, Altrincham, UK). The instrument was fitted with an LSIMS source (CsI) operating with an accelerating voltage of 9 kV in positive ionisation mode. MS/MS experiments were conducted using a collision energy of 30 eV and a gas cell pressure of 3.2×10^{-3} mbar (argon).

3. Results

3.1. Chemical synthesis of C-17 CTH and enzymic synthesis of C-17 CTH and [D4]C-16 CTH

The ESI–MS spectrum for *lyso*-CTH consisted predominantly of an ion of 809.0 *m/z*, which corresponds to a mono-sodiated Galα-(1→4)Galβ-(1→4)Glc-sphingosine (d-18) (Fig. 1a). The ESI–MS spectrum for the product of the chemical acylation of *lyso*-CTH had a molecular ion of 1060.9 *m/z*, consistent with the formation of C-17 CTH (CTH d-18:1 C-17) (Fig. 1b). The structure of the C-17 CTH was confirmed by LSI–MS/MS (Fig. 2b). The fragmentation pattern contained ions of 1039.3 *m/z*, 534.3 *m/z* and 264.0 *m/z* corresponding to the molecular ion and the daughter ions for the ceramide Cer (d-18:1 C-17–$1H_2O$) and sphingosine (d-18:1) moieties, respectively.

As the yield for the chemical synthesis of C-17 CTH was less than 10%, the specificity of enzymes was exploited to make C-17 CTH. Firstly, *lyso*-CTH was prepared from the commercially available erythrocyte CTH by using sphingolipid ceramide *N*-deacylase [14]. *Lyso*-CTH was purified from the reaction mixture by TLC and its structure established by a combination of ESI–MS/MS and LSI–MS/MS. C-17 CTH was synthesised enzymically from *lyso*-CTH, which had been prepared enzymically or commercially, using the reverse reaction of sphingolipid ceramide *N*-deacylase [15]. The C-17 CTH was purified from the enzymic reaction mixture as described above and analysed by ESI–MS/MS. The product ion spectrum was identical to that obtained for the chemically synthesised compound (Fig. 1b) and its structure was confirmed by LSI–MS/MS as for the chemically synthesised molecule (Fig. 2b). The yield for the enzymic acylation reaction was >90% as determined by the ratio of the *lyso*-CTH to

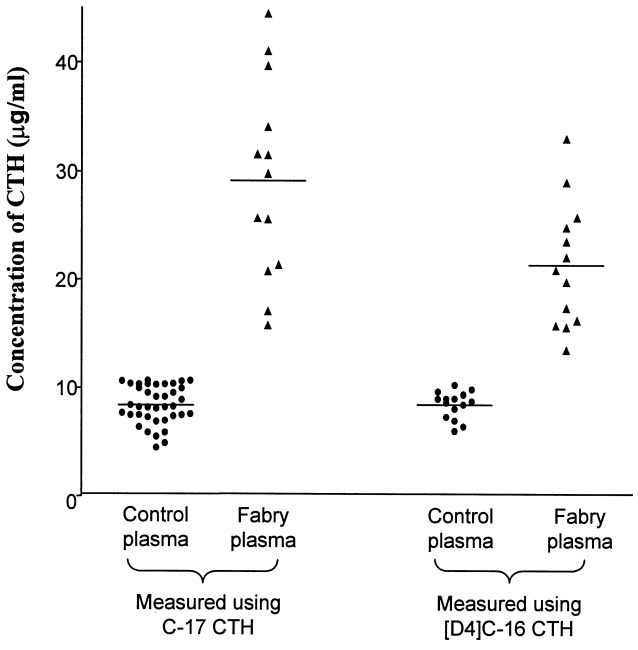

Fig. 4. Total plasma CTH (μg/ml) in control and Fabry patient plasma measured using two internal standards.

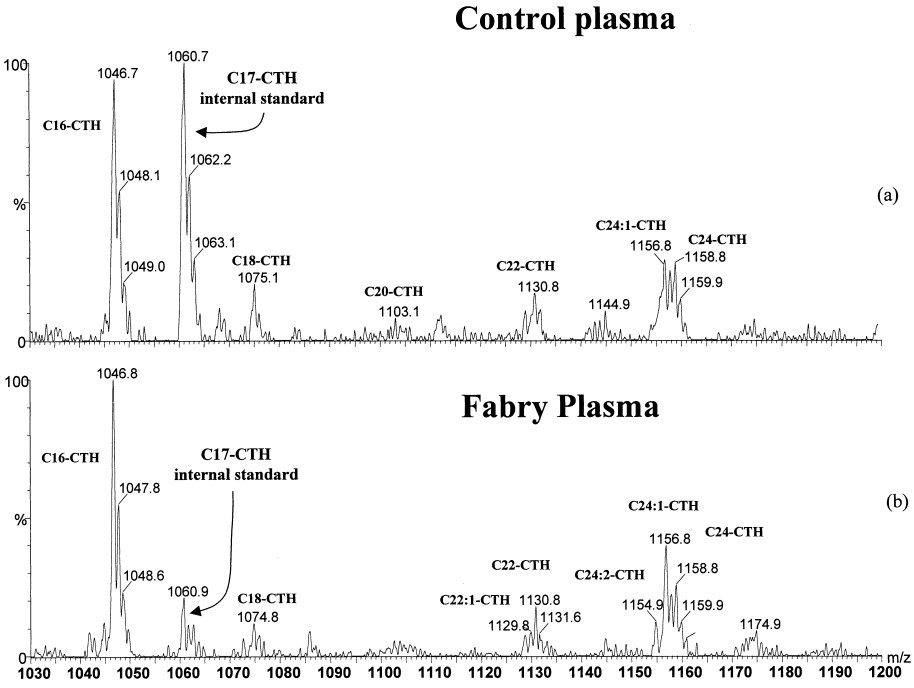

Fig. 3. ESI–MS/MS spectra of CTH in (a) control and (b) Fabry plasma obtained by neutral loss scanning of 162.0 *m/z*.

CTH after TLC. Therefore it was possible to make the C-17 CTH enzymically in very high yields (>90%) either directly from commercially available *lyso*-CTH or from human CTH by a two-step enzymic process. [D4]C-16 CTH was synthesised enzymically by substituting [D4]-palmitic acid for the heptadecanoic acid in the second step of the enzymic procedure, with a comparable yield. The structure of the [D4]C-16 CTH was confirmed by a combination of ESI–MS/MS (Fig. 1c) and LSI–MS/MS as for the C-17 CTH.

3.2. Calibration of C-17 CTH and [D4]C-16 CTH with authentic human erythrocyte CTH

Authentic human erythrocyte CTH consists of a mixture of isoforms with acyl chains of different masses (Fig. 1d). The most abundant forms contain lignoceric acid (C24:0) and nervonic acid (C24:1) with appreciable amounts of their hydroxylated derivatives. Other constituents are the C24:2 acid, behenic acid (C22:0) and its mono-unsaturated derivative (C22:1) and palmitic acid (C16:0) isoforms. All of the ions in the spectrum could be attributed to mono-sodiated CTH isoforms. The masses of the ions suggested that the sphingosine moiety is d-18:1, which was confirmed by LSI–MS/MS (Fig. 2a), which showed a characteristic fragmentation ion of 264 *m/z* corresponding to sphingosine d-18:1 minus 2. H_2O [11]. C-17 CTH and [D4]C-16 CTH were calibrated by mixing constant amounts with different amounts of the authentic CTH standard and analysing the mixtures by ESI–MS/MS. A linear relationship between the ratio of the responses for the standards and each molecular species of CTH was obtained, permitting construction of calibration curves.

3.3. Measurement of CTH in plasma from normal controls and patients with Fabry disease

The total CTH in a single normal plasma was measured in replicate using C-17 CTH ($n=12$) or [D4]C-16 CTH ($n=11$) as the internal standard with coefficients of variation of 9.9% and 7.9%, respectively. C-16 CTH was the major form of CTH in both normal and Fabry plasma but all the forms of CTH are elevated in the plasma from a male patient with classic Fabry disease (Fig. 3). The levels of the total CTH and of the individual isoforms were measured quantitatively by neutral loss scanning of *m/z* 162.0, operating in multiple channel acquisition mode and using C-17 CTH and [D4]C-16 CTH as the internal standards (Fig. 4). The mean levels of total CTH in the normal plasma were 8.4 ± 1.8 μg/ml (range 4.5–10.7, $n=38$) and 8.5 ± 1.3 μg/ml (range 6.0–10.3, $n=15$), respectively, and in the Fabry plasma 29.1 ± 9.9 μg/ml (range 15.8–44.5, $n=13$) and 21.4 ± 5.8 (range 13.5–33.1, $n=13$), respectively.

4. Discussion

The quantitative measurement of glycosphingolipids by MS/MS requires appropriate internal standards. The enzymic synthesis of a type 1 internal standard, i.e. [D4]C-16 CTH, and the enzymic or chemical synthesis of a type 2 internal standard, i.e. C-17 CTH, for the measurement of CTH are described in this paper. The yield for the chemical synthesis of C-17 CTH from *lyso*-CTH was very low but exploitation of the reverse reaction of sphingolipid ceramide *N*-deacylase to reacylate *lyso*-CTH gave very good yields. Furthermore, the hydrolase activity of ceramide *N*-deacylase can be used to deacylate CTH to prepare the *lyso*-CTH, which can then be reacylated enzymically or chemically to produce a type 1 or type 2 internal standard.

Using the novel internal standards, the levels of CTH were measured by MS/MS in plasma from patients with the classic form of Fabry disease and from normal control individuals. An approximately four-fold increase in total CTH was found in the plasma from Fabry patients. The predominant isoform of CTH in both normal and Fabry disease plasma is C-16 CTH whereas the predominant forms in human erythrocytes have C24:0 and C24:1 acyl groups. The ratio of C-16 CTH to C24:0 CTH only increased slightly from ~0.3 in normal plasma to ~0.4 in the Fabry plasma, suggesting that the majority of the circulating storage product is not derived directly from senescent erythrocytes. Higher levels of total CTH, particularly in normal controls, were observed with our method than by ELISA [6] and GLC [9] (12.6 ± 3.7 and 7.6 ± 2.1 μg/ml, respectively, for Fabry plasma and 0.9 ± 0.4 and 2.1 ± 0.7 μg/ml, respectively, for normal plasma). However the levels of C-16 CTH alone, measured with C-17 CTH and [D4]C-16 CTH, 4.1 ± 1.1 μg/ml (range 2.6–6.1, $n=38$) and 2.4 ± 0.3 μg/ml (range 2.0–3.0, $n=15$), respectively, for normal plasma, and 12.4 ± 3.4 μg/ml (range 7.5–17.4, $n=13$) and 8.4 ± 3.2 μg/ml (range 4.7–15.2, $n=13$), respectively, were comparable.

In principle the strategy of preparation of the *lyso*-compound followed by reacylation to synthesise an internal standard can be applied to any glycosphingolipid that is a substrate for the ceramide *N*-deacylase. We have prepared C-17, C-19, C-21, C-23 and C-25 CTH, chemically and enzymically, as standards for measuring the different forms of CTH found in different tissues. We have also prepared C-17-G_{M1}-ganglioside, -G_{M2}-ganglioside and -G_{M3}-ganglioside and [D4]C-16-galactocerebroside, -galactocerebroside-3-sulphate, lactosylceramide and -globoside as internal standards for measuring the storage products in tissues and organs from other glycosphingolipidoses by MS/MS (manuscript in preparation).

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