Journal of Clinical Laboratory Analysis 16:115–120 (2002)

Analysis of 3-Hydroxydodecanedioic Acid for Studies of Fatty Acid Metabolic Disorders: Preparation of Stable Isotope Standards

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Current diagnostic tests to detect disorders of fatty acids metabolism, such as long-chain hydroxyacyl CoA dehydrogenase deficiency (LCHAD), are hampered by insensitivity or a long delay time required for results. Children with LCHAD deficiency are known to excrete 3-hydroxydicarboxylic acids with chain lengths of 10–16 carbons, but a quantitative method to measure excretion of these potentially diagnostically important compounds has not been reported. We report synthetic schemes for synthesis of 3-hydroxydodecanedioic acid and a di-deuterated analog, suitable for use in a stable-isotope dilution mass spectrometric analytical approach. Evaluation of several common derivatization protocols to produce a volatile derivative for gas chromatography determined that trimethylsyl derivatives produced the best efficiency and stability. Positive-ion chemical ionization mass spectrometry provided the greatest yield of characteristic ions. These results indicate the basic reagents needed to develop sensitive and accurate 3-hydroxydodecanedioic acid measurements for diagnosis of LCHAD deficiency and other fatty acid oxidation disorders. J. Clin. Lab. Anal. 16:115–120, 2002. © 2002 Wiley-Liss, Inc.

Key words: metabolic genetic disease; mass spectroscopy; diagnosis

INTRODUCTION

Among the most prevalent inherited metabolic disorders are deficiencies of the chain-length specific enzymes catalyzing the beta oxidation of fatty acids (for review, see Ref. 1). Recognition of these diseases was delayed (probably due to the episodic nature of the symptoms, which made elevations of diagnostically important metabolites transient) until the last two decades, when specific diagnostic tests were developed. Such tests include the measurement of acylcarnitine species in plasma (2), the quantitation of acylglycine conjugates in urine (3), and activity measurements of the oxidation of radiolabeled fatty acids by fibroblasts or cellular homogenates. However, these tests are not generally performed in the routine laboratories of most institutions, such as pediatric hospitals, where children with these deficiencies will likely be examined; instead, most institutions refer specimens for these tests to specialized laboratories. The first line in diagnosing fatty acid disorders is analysis of organic acids in urine. This test is much more readily available and is performed in many pediatric hospitals. When a child is symptomatic, a characteristic pattern of fatty acid metabolites (elevated dicarboxylic acids, with little or no 3-hydroxybutyric acid) will frequently be observed. However, the presence of dicarboxylic acids in urine is a nonspecific marker, with elevations occurring in prolonged fasting or when the diet contains a high percentage of calories as triglycerides. Moreover, several of the fatty acid disorders lead to elevation of dicarboxylic acids, and further testing is needed to distinguish between the possible specific deficiencies.

Deficiency of long-chain hydroxyacyl CoA-dehydrogenase (LCHAD) is among the most common fatty acid oxidation defects (4). Children with this deficiency develop symptoms in the first 2 years of life; hypoglycemia (with little or no ketosis), cardiomyopathy, muscle hypotonia, and hepatomegaly are the most important symptoms (5). Most patients have large amounts of dicarboxylic acids, including 3-hydroxy sebacic acid, in their urine when they are symptomatic, but the qualitative pattern of urinary metabolites is variable and

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Received 16 February 2001; Accepted 29 November 2001

DOI: 10.1002/jcla.10033
Published online in Wiley InterScience (www.interscience.wiley.com)

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difficult to distinguish from those seen in other fatty acid metabolic defects. Definitive diagnosis relies on measurement of LCHAD activity in fibroblasts, which usually requires many weeks of preparation, and may thereby delay definitive treatment.

We have sought to develop a means of diagnosing LCHAD deficiency with high specificity, relying on methods that are readily available in laboratories that commonly perform organic acid analyses, which avoids the delays associated with activity assays. One attractive approach is to quantitate the excretion of 3-hydroxydicarboxylic acids, which are alternative metabolic products of fatty acid metabolism. These metabolites likely accumulate in large quantities in LCHAD deficiency because of the inability of affected children to metabolize 3-hydroxy fatty acids, which are known to accumulate in the blood of LCHAD-deficient children (6). A quantitative method for measurement of long-chain 3-hydroxy dicarboxylic acids has not been reported.

In order to quantitate the excretion of 3-hydroxy long-chain dicarboxylic acids, suitable standards must be available. This work reports the synthesis of the 12-carbon compound and a di-deuterated analog, which possesses properties that may facilitate quantitation of this metabolite in the urine of LCHAD-deficient children.

METHODS

Analytical Methods

Unless otherwise indicated, ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra and carbon nuclear magnetic resonance (^{13}C NMR) spectra were obtained in $CDCl_3$ solution on a Varian XL-300 (Varian, Palo Alto, CA) mHz NMR spectrometer. The ^{1}H chemical shifts are reported in δ units downfield from tetramethylsilane, while carbon spectra are referenced to the center line of the chloroform-d triplet at 77.00 ppm. Nuclear magnetic data are reported as follows: chemical shift; multiplicities abbreviated as follows: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet; and number of protons. All melting points were determined on a Thomas Hoover capillary melting point apparatus (Philadelphia, PA) and are not corrected. Infrared spectra, reported as υ_{max} wavelengths, were obtained with a Perkin Elmer 1600 FTIR (Shelton, CT).

Gas chromatographic-mass spectrometric analysis of the principle compounds was performed with both electron impact and positive chemical ionization devices. Electron impact analysis was performed on a Varian 3700 chromatograph and an ion-trap detector (Finnigan, San Jose, CA) fitted with an XP-1 0.32 mm × 15 m column (0.5 μ coating of dimethylpolysiloxane (P.J. Cobert, Assoc., St. Louis, MO)), and a split/splitless injector. Separation was obtained using temperature programming from 80–280°C (ramped at 6°C/min). Some spectra were obtained with a Hewlett-Packard 5890 gas chromatograph/mass detector (Hewlett-Packard, Wilmington, DE). Positive chemical ionization analysis was performed on a Hewlett-Packard 5890 gas chromatograph fitted with an HP-1 column (0.31 mm × 15 m), using the same elution program as above. Eluted compounds were detected with a Hewlett-Packard 5988 detector, with the source temperature at 200°C.

Methyl-10-Undecylenate

10-Undecylenic acid (29.0 g (0.157 mol); Aldrich Chemical Co., Milwaukee, WI) was placed in a 500-ml round-bottomed flask equipped with magnetic stirrer, heating mantle, and condenser. Methanol (132 mL) and concentrated sulfuric acid (5 mL) were added and the mixture was then allowed to reflux gently for 24 hr. The reaction mixture was cooled to room temperature and the solvent was removed on a rotatory evaporator. Ether (200 mL) was added and the mixture was extracted with saturated sodium bicarbonate. Evaporation of the ether and vacuum distillation of the residue at 80°C (10 Pa) afforded the methyl ester of 10-undecylenic acid (29.8 g, 95.5% yield). ^{1}H NMR: 5.7–5.9 (m, 1H), 4.8–5.0 (m, 2 H), 3.6 (s, 3 H), 2.25 (t, 2H), 1.95 (m, 2H), 1.5–1.65 (m, 2H), 1.2–1.4 (m, 10H). ^{13}C NMR: 174.1, 138.1, 114.2, 52.5, 34.2, 34.0, 29.5, 29.4, 29.3, 29.2, 29.1, 25.1. IR: υ_{max} (neat): 3100, 2926, 2854, 1743, 1640 cm^{-1}.

Methyl ω-Aldehydo-Nonanoate

The procedure used was similar to that reported by Noller and Adams (7). A commercial laboratory ozone generator (OZONOSAN PM80, Dr. J. Häsler, Gmbh, Iffezheim, Germany) was used to generate ozone, which was immediately bubbled through an inlet tube into a three-neck, 100-mL round-bottom flask containing methyl-10-undecylenate (10.0 g (0.05 mol)), glacial acetic acid (50 mL), a magnetic stirrer, and a reflux condenser connected to a trap containing a saturated solution of KI. Ozone was gently bubbled through the solution until a sample no longer decolorized a solution of bromine in glacial acetic acid. A cool-water bath was used to prevent the reaction flask from overheating. The reaction mixture liquid was diluted with ether (100 mL) and transferred to a 500-mL round-bottomed flask fitted with a magnetic stirrer and reflux condenser. Zinc dust (25.0 g) was added to the stirred solution in small portions over a period of 1 hr. A vigorous reaction occurred upon the addition of the zinc dust. The zinc acetate sludge was then filtered through a Buchner funnel and washed thoroughly with ether. The residue was washed with water to prevent ignition of the excess zinc dust. The filtrate was then extracted with two portions of water (75 mL each), followed by an extraction with 10% sodium carbonate solution (40 mL), then another with water, and finally dried over calcium chloride. The ether was removed under vacuum and the residue was distilled at 80–84°C (16-20 Pa) to give methyl aldehydo-nonanoate (8.0 g, 78% yield). ^{1}H NMR: 9.5 (s, 1H), 3.4 (s, 3H), 2.2 (t, 2H), 2.1 (t, 2H), 1.3–1.5 (m, 4H), 1.1–1.2 (m, 8H). ^{13}C NMR: 201.7,

173.3, 50.9, 43.4, 33.6, 28.8, 28.7, 24.5, 21.6. IR: υ_{max} (neat) 2927.7, 2700, 1732 cm^{-1}.

Condensation of Methyl ω-Aldehydo-Nonanoate With Methyl Bromoacetate

Granulated zinc (4–5 g) was measured into a three-neck, 250-mL round-bottom flask containing a magnetic stir bar and equipped with an argon gas inlet, a reflux condenser connected to an oil bubbler, and an additional funnel (50 mL). Several drops of methyl bromoacetate were added to the reaction flask and heated in a hot-water bath to initiate the reaction. A solution of freshly distilled aldehyde ester (6.0 g, 0.03 mol) and methyl bromoacetate (5.5 g) in dry ether (40 mL) was added dropwise under argon flow with rapid stirring. Vigorous bubbling and immediate reflux of the reaction mixture characterized initiation of the reaction. Once addition was complete, gentle heat was provided by a water bath, and the reaction mixture was refluxed for about 1 hr. Hydrochloric acid (10 drops, 6 M) was added next, followed by additional ether (100 mL). The mixture was extracted with 5% sodium bicarbonate (2 × 200 mL). The solution was dried and the ether removed under vacuum; the residue was vacuum distilled. The methyl 3-hydroxydodecanedioate distilled at 131°C (5 Pa) (2.1 g isolated, 30% yield; the yield varied from 30% to 60%). Upon cooling, the ester solidified, mp 40°C. ^{1}H NMR: 3.9–4.05 (br-s, 1H), 3.7 (s, 3H), 3.6 (s, 3H), 2.9 (br-s, 1H), 2.35–2.5 (m, 2H), 2.25 (t, 2H), 1.1–1.7 (m, 8H), ^{13}C NMR: 174.2, 173.3, 68.1, 51.8, 51.5, 41.4, 36.7, 34.2, 29.6, 29.5, 29.3, 29.2, 25.6, 25.1 IR: υ_{max} (neat): 3503.2, 2929.4, 1739.6 cm^{-1}.

3-Hydroxydodecanedioic Acid

Hydrolysis was achieved by stirring methyl 3-hydroxydodecanedioate (2.0 g) with 10% KOH (ester: KOH, 1:3 molar ratio) at room temperature. After 1 hr only one phase remained. The solution was acidified with 6 M HCL and the compound extracted from the reaction with ether. Ether was removed under vacuum, leaving behind a white solid. The 3-hydroxydodecanedioic acid was recrystallized from ethyl acetate (1.0 g, 60% yield, mp 107-109°C). Purity, estimated from the absence of other NMR signals, was greater than 95%. ^{13}C NMR (DMSO): 174.4, 172.9, 67.1, 42.8, 37.0, 33.7, 29.1, 29.0, 28.8, 28.6, 25.1, 24.6. IR: υ_{max} (KBr): 3564, broad band from 3400–2300, 2926, 2850, 1694 cm^{-1}.

Preparation of Bromoacetic-d_2 Acid

The compound was prepared according to the published method of Goddard and Ward (8). Acetic acid-d_4 (20 g), was added to a round-bottom flask equipped with a reflux condenser, and red phosphorus (0.4 g) was added. The mixture was treated with bromine (60 g, 20 mL) and heated in a steam bath for 6 hr. The pale brown liquid was distilled. After bromine, acetic acid, and bromoacetyl bromide were removed, a clear liquid remained

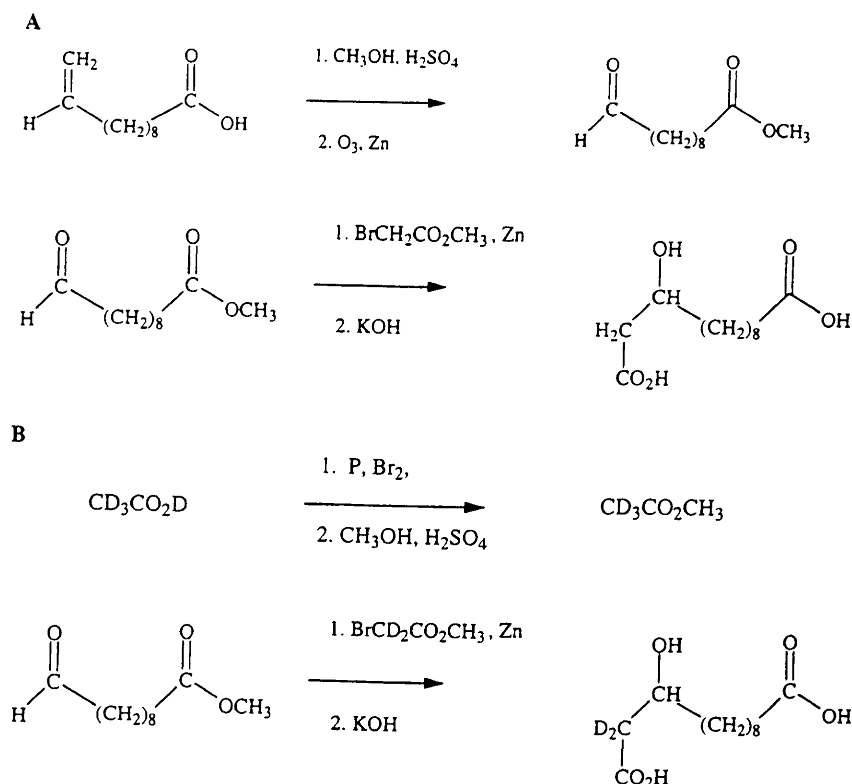

Fig. 1. Organic synthetic schemes for synthesis of (**A**) 3OHDD and (**B**) 3OHDD-D2.

in the reaction flask, which solidified on cooling (mp 48–50°C). The yield was about 80% (35 g). ^{13}C NMR: 169.0 (s), 27.7 (q).

Methyl Bromoacetate-d_2

A mixture of bromoacetic acid-d_2 (15 g), methanol (75 mL), and concentrated sulfuric acid (1 mL) were refluxed for 6 hr. The solution was cooled and then ether (100 mL) was added. The ether solution was washed with water and then extracted with 2% sodium bicarbonate solution. After removal of the ether, the solution was heated up to 100°C at atmospheric pressure to remove any remaining ether. The methyl bromoacetate-d_2 was used without any further purification. ^{1}H NMR: 375 (s, 3H). ^{13}C NMR: 167.8, 52.3, 25.4 (q).

3-Hydroxy-2,2-Dideuteriododecanedioic Acid

The methyl bromoacetate-d_2 and methyl ω-aldehydo-nonanoate were reacted with zinc as described above for the unlabeled material. The methyl 3-hydroxy-2,2-dideuterio-dodecanedioate that was isolated was characterized by ^{1}H. The ^{1}H NMR spectrum was similar to the unlabeled material, with the exception that the multiplet at 2.35–2.5 ppm was absent. Hydrolysis of the diester afforded the diacid that was characterized by ^{13}C NMR in DMSO-d_6. The ^{13}C spectrum of 3-hydroxy-2,2-dideuteriododecanedioic acid was identical to the unlabeled acid, with the exception of the resonance at 42.8 ppm, which was barely observable. Purity, estimated from the absence of other NMR signals, was greater than 95%.

RESULTS

Schemes were developed for the synthesis of 3-hydroxy-dodecanedioic (3OHDD) (Fig. 1A) and for a di-deuterated analog, 3-hydroxy-2,2-dideuterododecanedioic acid (3OHDD-D2) (Fig. 1B). Analysis of newly synthesized or recrystallized samples of these compounds (trimethylsilyl

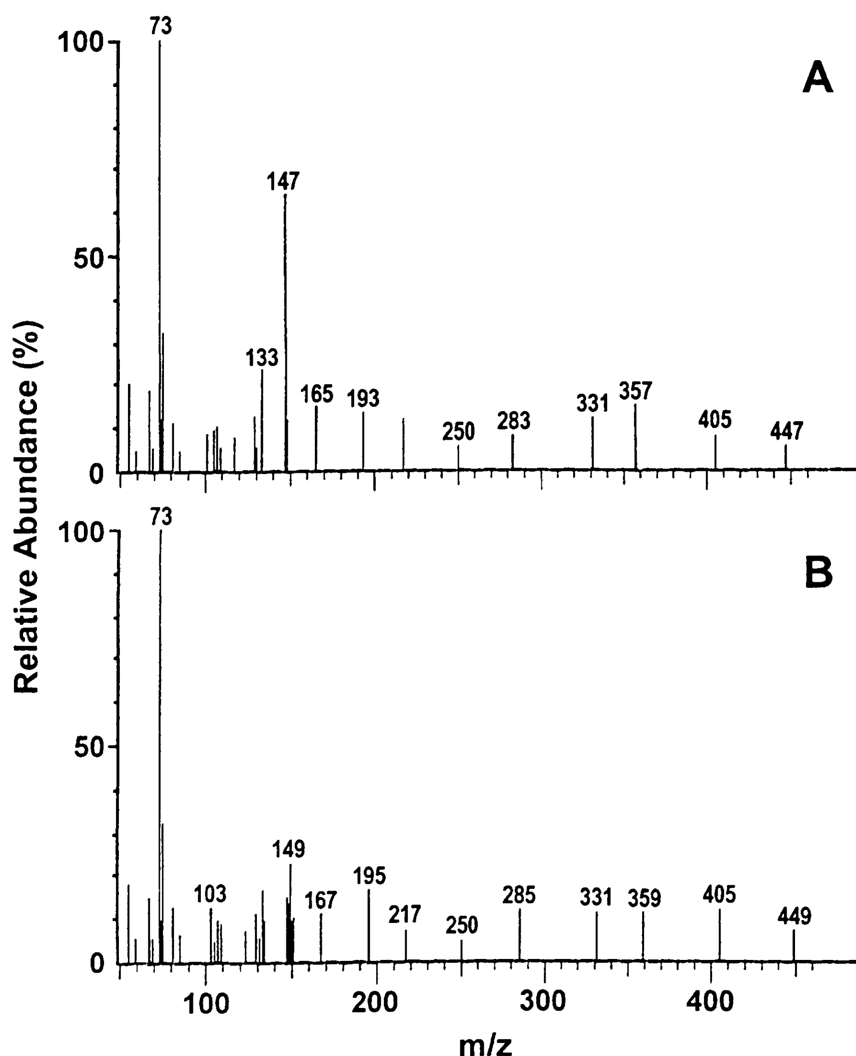

Fig. 2. Electron-impact mass spectrometric fragmentation pattern of the TMS derivatives of (**A**) 3OHDD and (**B**) 3OHDD-D2.

derivative) by gas chromatography on a dimethylpolysiloxane-coated 0.53 mm × 15 m column produced a single peak (data not shown). Native 3OHDD and 3OHDD-D2 produced similar chromatograms. However, additional peaks appeared when the compound was stored for weeks at room temperature or at 4°C (data not shown). Several derivatization schemes were attempted with the goal of producing mass spectral patterns that yielded high-intensity large mass ions that incorporated the deuterated carbon, and thus were suitable for use as internal standard in stable-isotope analysis of body fluids. Derivatization with diazomethane, methyl-bis(trifluoroacetamide), N,N-dimethylformamide dimethyl acetal, and N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide(MTBSTFA) all appeared to be unsuccessful, resulting in no detectable volatile products (first three agents) or a reduced yield (MTBSTFA). Only N,O-bis(trimethylsilyl) trifluoroacetamide yielded apparently quantitative derivatization, based on equivalent peak area (flame ionization detection) compared with an internal standard (o-hydroxyphenylacetic acid) present at the same weight concentration. Trimethylsilyl (TMS) derivatives appeared to be stable for periods of up to a week. Mass-spectral analysis of the TMS derivative of 3OHDD with ion-trap electron impact mass spectroscopy produced a characteristic pattern of ions (Fig. 2A), but the M-15 ion (m/z 447) and other large m/z ions were obtained in low quantities. Analysis with a quadropole device yielded somewhat better abundance of larger ions, and an additional ion of m/z 233 was prominent (data not shown).

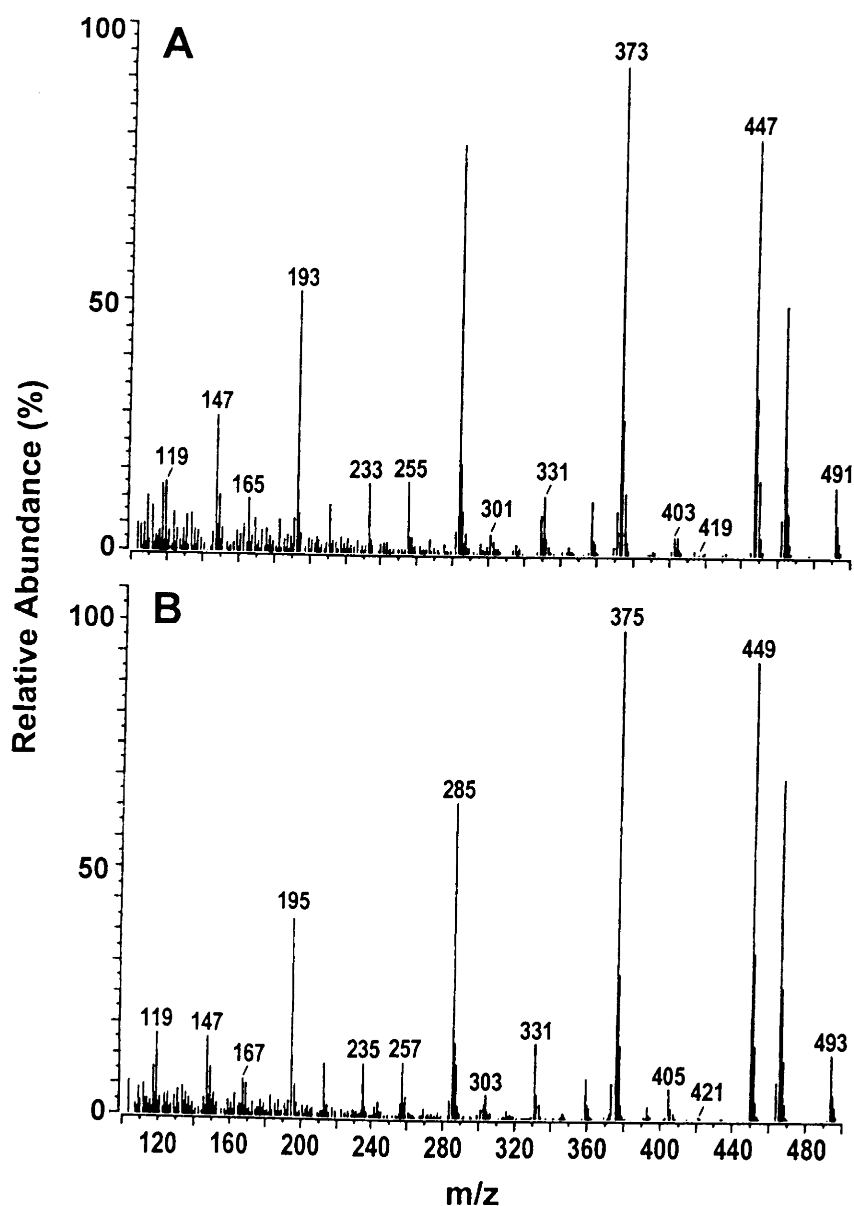

Fig. 3. Chemical ionization positive ion mass spectrometric fragmentation patterns of the TMS derivatives of (A) 3OHDD and (B) 3OHDD-D2.

The spectrum of 3OHDD-D2 showed several ions raised in mass by 2 (Fig. 2B), in comparison to 3OHDD—notably the M-15 ion (m/z 449) and m/z 359 (M minus CH_3-TMSOH). Analysis by a quadropole spectrometer produced a prominent additional ion of m/z 235 (data not shown). Analysis of 3OHDD with positive chemical ionization mass spectrometry produced spectra with a much greater yield of larger ions (Fig. 3A), with the M-15 ion (m/z 447) and the M minus TMSO ion (m/z 373) most prominent; the molecular ion and M+15 ion were also present. The spectra for 3OHDD-D2 contained analogous ions, with mass increased by 2 (Fig. 3B).

DISCUSSION

This work reports the first synthetic scheme for synthesis of 3-hydroxydodecanedioic acid (3OHDD). The development of methods to synthesize 3-OHDD and a di-deuterated analog (3OHDD-D2) offers new opportunities to quantitate, by stable-isotope dilution mass spectroscopy, the excretion of metabolites of 3-hydroxy fatty acids (3-OHFAs), which are known to accumulate in the body fluids of children with LCHAD (6). The principal alternative metabolic pathway for fatty acids in humans, where mitochondrial fatty acid metabolism is blocked by enzymatic deficiency, is omega-oxidation (9). This pathway, located in microsomes, forms dicarboxylic acids which are rapidly and quantitatively excreted in urine. Because of the known accumulation of the precursor 3-OHFAs, and the documented efficiency of omega-oxidation, diagnostically important elevations in excretion of 3-OHDD will be formed by this alternative pathway, which likely offer a specific and practical means of diagnosing LCHAD deficiency.

The synthetic schemes for 3OHDD and 3OHDD-D2 are likely to be effective for shorter and longer carbon chain lengths; chain lengths can be varied by varying the chain length of the unsaturated carboxylic acid starting material (see scheme 1). But because the 14-carbon and 16-carbon 3-OHFAs are known to accumulate in LCHAD children at the highest magnitude in relation to normal levels (6), and because omega-oxidation to produce dicarboxlic acids results in chain shortening by two carbons, the 12-carbon 3-OHDD is likely to be produced in very large amounts in children with LCHAD deficiency. These facts taken together suggest that 3-OHDD will be diagnostically the most sensitive elevation in fatty acid-derived metabolites in urine from LCHAD children.

Current diagnosis of LCHAD rests on appropriate clinical history, an abnormal profile of fatty acid metabolites in urine and serum, and demonstration of impaired fatty acid metabolism in fibroblasts cultured from skin biopsy. The signs and symptoms of LCHAD can occur in other diseases, particularly other metabolic errors of the respiratory chain, fatty acid metabolism, and carnitine metabolism. Patterns of fatty acid metabolites also can be qualitatively similar in these disorders (10,11). Measures of oxidation of fatty acids, and specifically long-chain fatty acids, are impaired in mitochondria from LCHAD children, but the culture of fibroblasts from biopsy requires weeks, and equipment to measure fatty acid oxidation is available at only a few locations worldwide. Development of a more specific and quantitative method, using stable isotope dilution mass spectroscopy and deuterated 3OHDD, may considerably lessen the time required to make a reliable diagnosis.

ACKNOWLEDGMENT

The authors thank Dr. John Turk for his help in the mass spectrometric studies, and Barbara Hartman for her assistance in preparing this manuscript. Mass spectrometric analyses were aided by NIH grant 2P41RR00954.

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