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cis-3,4-Methylene-heptanoylcarnitine: Characterization and verification of the C8:1 acylcarnitine in human urine

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Abstract

Acylcarnitine profiles have been used to diagnose specific inherited metabolic diseases. For some acylcarnitines, however, the detailed structure of their acyl group remains a question. One such incompletely characterized acylcarnitine is *cis*-3,4-methylene-heptanoylcarnitine. To investigate this problem, we isolated the "C8:1" acylcarnitine from human urine, transesterified it to form its acyl picolinyl ester, and characterized it by GC/EI-MS. These results were compared to GC/EI-MS results from authentic standards we synthesized (*cis*-3,4-methylene-heptanoylcarnitine, *trans*-2-octenoylcarnitine, 3-octenoylcarnitine, *cis*-4-octenoylcarnitine, and *trans*-4-octenoylcarnitine). Only *cis*-3,4-methylene-heptanoylcarnitine matched the urinary "C8:1" acylcarnitine. The standards were then spiked in human urine, converted to pentafluorophenacyl esters, and detected by HPLC/MS. cis-3,4-Methylene-heptanoylcarnitine exactly matched the "C8:1" acylcarnitine in urine, whereas none of the other C8:1 acylcarnitine standards matched. Based on the data from GC/EI-MS and HPLC/MS, the "C8:1" acylcarnitine in human urine is shown to be *cis*-3,4-methylene-heptanoylcarnitine.

Keywords: Acylcarnitines; *cis*-3,4-Methylene-heptanoylcarnitine; GC/MS; HPLC/MS

1. Introduction

β-Oxidation of acyl-CoAs in mitochondria [1] produces various acylcarnitines that appear in blood and are excreted into urine. Acylcarnitine profiles in human blood, determined by tandem mass spectrometry [2], have been used to diagnose specific inherited metabolism diseases, such as medium-chain acyl-CoA dehydrogenase deficiency (MCAD) [3], multiple acyl-CoA dehydrogenase deficiencies (MADD) [4], very long-chain acyl-CoA dehydrogenase deficiency (VLCAD) [5], and long-chain hydroxyacyl-CoA dehydrogenase deficiency (LCHAD) [6].

Although many acylcarnitines have been identified, some acylcarnitines have been detected but not characterized. One of these is a so-called "C8:1" acylcarnitine, which Libert et al. identified as *cis*-3,4-methylene-heptanoylcarnitine [7]. They isolated an acylcarnitine fraction from urine, hydrolyzed the acylcarnitines, and derivatized the resulting acids to form picolinyl esters. However, this identification has not been generally recognized by laboratories that perform acylcarnitine analysis by tandem mass spectrometry, who do not list this paper in their citations, and label this compound simply as "C8:1" [4]. We feel that this lack of acknowledgement is based on two factors: (1) Libert et al.'s measurement of *cis*-3,4-methylene-heptanoylcarnitine was indirect, based on the identification of *cis*-3,4-methylene-heptanoate picolinyl ester, not the actual acylcarnitine, and (2) there has been no synthesis of *cis*-3,4-methylene-heptanoylcarnitine, which would allow for its validation and incorporation into acylcarnitine analysis studies.

We have shown that acylcarnitines can be transesterified to yield their corresponding acyl picolinyl esters, thereby avoiding the hydrolysis step and thus eliminating potential interferences from free organic acids. We also have shown that the GC/MS spectra of the resulting acyl picolinyl esters can be used to deduce the chemical structure of corresponding acylcarnitines [8]. Meanwhile, the fragmentation mechanism of the 3-picolinyl

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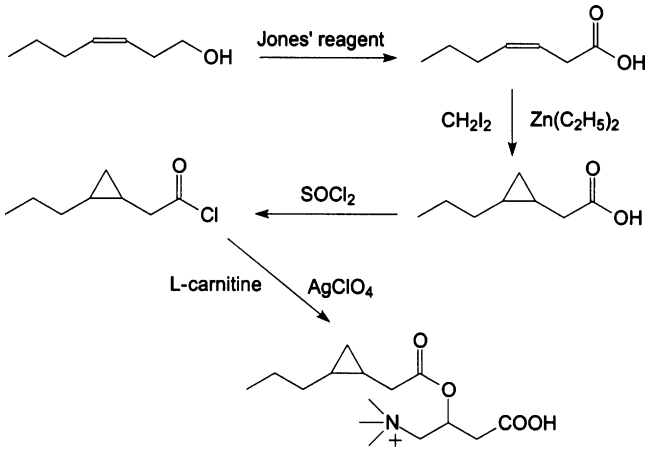
esters of fatty acids has been studied thoroughly by using tandem mass spectrometry [9] and applied to characterization of long-chain acids [10].

Herein, we describe the structural characterization of the "C8:1" acylcarnitine in human urine. Our approach is as follows: (1) synthesis of several candidate C8:1 acylcarnitines – *cis*-3,4-methylene-heptanoylcarnitine, *trans*-2-octenoylcarnitine, 3-octenoylcarnitine, *trans*-4-octenoylcarnitine, and *cis*-4-octenoylcarnitine. (2) Isolate the C8:1 acylcarnitine fraction from a normal human urine. (3) Transesterify the C8:1 acylcarnitine fraction, selectively converting acylcarnitines to picolinyl esters without first converting them to acids (Libert et al. did not use this process, and so their picolinyl esters were likely contaminated with endogenous acyl groups which were not originally from acylcarnitines). (4) GC/EI-MS. The picolinyl esters of transesterified acylcarnitines, when subjected to EI type ionization, yield rich fragmentation patterns which allows for the detailed characterization of the acylcarnitine acyl group [8]. (5) Acylcarnitine analysis by HPLC/MS. The derivative used in our HPLC/MS method contains the whole acylcarnitine molecule, not simply the acyl-group. We analyzed acylcarnitines in human urine using our procedure: isolation by cation-exchange SPE, derivatization to form acylcarnitine pentafluorophenacyl esters, HPLC separation, and ESI-MS detection [11]. Pentafluorophenacyl esters of acylcarnitines have excellent HPLC and ESI-MS characteristics, and therefore can be detected at high sensitivity with chromatographic resolution of constitutional isomers. In contrast to the butyl ester procedure used in Tandem MS, derivatization of acylcarnitines with pentafluorophenacyl trifluoromethanesulfonate does not hydrolyze acylcarnitines. By comparing the results from a urine specimen spiked with synthesized C8:1 acylcarnitine standards, we are able to demonstrate that the "C8:1" acylcarnitine in the urine specimen matches only *cis*-3,4-methylene-heptanoylcarnitine.

2. Materials and methods

2.1. Materials

Thionyl chloride, silver perchlorate, L-carnitine hydrochloride, anhydrous acetonitrile, *p*-toluenesulfonyl chloride, potassium *tert*-butoxide (1.0 M solution in tetrahydrofuran), anhydrous dichloromethane, and 3-pyridylcarbinol were purchased from Sigma–Aldrich (St. Louis, MO, USA). *Trans*-2-Octenoic acid was purchased from Lancaster (Pelham, NH, USA). 3-Octenoic acid was purchased from Alfa Aesar (Pelham, NH, USA). *cis*-3-Hepten-1-ol and ethyl *trans*-4-octenoate were obtained from Pfaltz & Bauer (Waterbury, CT, USA). *trans*-4-Octenoic acid was synthesized by hydrolysis of ethyl *trans*-4-octenoate [12]. *cis*-4-Octenoic acid was synthesized in three steps: first, *cis*-3-hepten-1-ol was converted into *cis*-3-heptenyl-4-methylbezenesulfonate [13]; then, *cis*-3-heptenyl-4-methylbezenesulfonate was reacted with potassium cyanide to form *cis*-4-octenenitrile; finally, *cis*-4-octenenitrile was hydrolyzed to form *cis*-4-octenoic acid [12]. The other chemicals used were of reagent grade.



Scheme 1. Total synthesis of *cis*-3,4-methylene-heptanoylcarnitine.

2.2. Chemical synthesis

2.2.1. Synthesis of cis-3,4-methylene-heptanoic acid

cis-3,4-Methylene-heptanoic acid was synthesized in two steps as depicted in Scheme 1. First, *cis*-3-hepten-1-ol was converted into *cis*-3-heptenoic acid by Jones's reagent, (Jones's reagent was made by combining 7.0 g CrO_3, 7 ml sulfuric acid, and 20 ml water). *cis*-3-Hepten-1-ol (5.0 g) in 25 ml acetone was cooled in an ice-water bath [12]. The above Jones's reagent was added over a period of 20 min until the solution turned from green to red. The reaction mixture was then stirred continually for 1 h at room temperature. This solution was evaporated with nitrogen at room temperature to remove acetone and extracted with ethyl ether. The ethyl ether solution was extracted with 2.0 M NaOH. The NaOH solution was acidified with 6 M HCl and extracted again with ethyl ether. This ethyl ether solution was dried with anhydrous sodium sulfate and the solvent was evaporated with nitrogen at room temperature to get *cis*-3-heptenoic acid (3.16 g, 56% yield). The product was 99% pure (TMS derivative determined by GC/MS) and its molecular weight was 128.

In the second step, *cis*-3-heptenoic acid reacted with diiodomethane to form *cis*-3,4-methylene-heptanoic acid. A two-necked flask was first cooled in an ice-water bath. While sparging with nitrogen, 1.0 M diethylzinc in hexane (12 ml) was added, followed by additions of anhydrous benzene (10 ml), *cis*-3-heptenoic acid (0.77 g), and diiodomethane (1.3 ml) [14,15]. The solution was stirred at room temperature for 40 h under nitrogen. 1.0 M HCl (10 ml) was added and the solution was stirred until it became clear, then extracted with ethyl ether. The ethyl ether solution was extracted with 1.0 M NaOH. The NaOH solution was acidified with 6.0 N HCl and then extracted with ethyl ether. This ethyl ether solution was dried with anhydrous sodium sulfate and the solvent was evaporated under nitrogen at room temperature to get *cis*-3,4-methylene-heptanoic acid (0.47 g, 55% yield). The product was more than 99% pure (TMS derivative determined by GC/MS) and its molecular weight was 142.

2.2.2. Synthesis of cis-3,4-methylene-heptanoylcarnitine

cis-3,4-Methylene-heptanoic acid (0.35 g) was reacted with thionyl chloride (0.30 g) at 60 °C for 1 h to produce *cis*-

3,4-methylene-heptanoyl chloride. Meanwhile, a mixture of L-carnitine hydrochloride (0.25 g), silver perchlorate (0.31 g), and 25 ml anhydrous acetonitrile was stirred at room temperature for 1 h [16]. The supernatant of the acetonitrile solution was then decanted into the flask with *cis*-3,4-methylene-heptanoyl chloride, and this mixture was stirred at room temperature for 20 h. After 0.5 ml water and 0.40 g sodium bicarbonate were added, the mixture was stirred at room temperature for another 30 min. The product was collected by centrifugation and evaporated to dryness with nitrogen gas at room temperature. The residue was washed with ethyl ether and dried with nitrogen gas at room temperature. The yield was 81%. The raw product was purified using preparative HPLC [17]. By electrospray ionization tandem mass spectrometry, *cis*-3,4-methylene-heptanoylcarnitine yielded fragment ions of *m/z* 286, 227, 144, 143, 125, and 85.

trans-4-Octenoylcarnitine, *cis*-4-octenoylcarnitine, 3-octenoylcarnitine, and *trans*-2-octenoylcarnitine were synthesized from their respective acids and purified using the same procedure as in the synthesis of *cis*-3,4-methylene-heptanoylcarnitine. Like *cis*-3,4-methylene-heptanoylcarnitine, these four acylcarnitines produced the fragment ions of *m/z* 286, 227, 144, 143, 125, and 85 in electrospray ionization tandem mass spectrometry.

2.3. Analytical methods

2.3.1. Extraction of acylcarnitines from human urine

The isolation of acylcarnitines from human urine was performed by reverse-phase solid phase extraction. An 200 mg Oasis HLB cartridge (Waters Corporation, Milford, MA) was conditioned with methanol (4 ml) and water (4 ml). Then 10.0 ml of human urine (spiked with 20 μg of *trans*-2-octenoylcarnitine) was loaded onto the cartridge. The cartridge was washed with 5% methanol/95% H_2O (4 ml). Acylcarnitines were eluted with methanol (4 ml). The collected methanol solution was evaporated to dryness with nitrogen at room temperature.

2.3.2. Transesterification of acylcarnitines

The method of Destaillats and Angers [18] was adapted for the transesterification of acylcarnitines [8]. Briefly, the residue containing acylcarnitines was reconstituted in 1.0 ml of anhydrous dichloromethane and reacted with a freshly prepared mixture of 100 μl of 1.0 M potassium *tert*-butoxide in tetrahydrofuran and 200 μl of 3-pyridylcarbinol. The reaction mixture was kept at 40 °C for 30 min, cooled to room temperature, and water (2 ml) and hexane (4 ml) was added. The organic phase was collected, passed through a disposable Pasteur pipette filled with anhydrous sodium sulfate, and evaporated to dryness with nitrogen at room temperature. The residue was reconstituted in 60 μl isooctane for GC/MS analysis.

2.3.3. Gas chromatography/electron ionization mass spectrometry

Analysis by GC/MS was performed on a system consisting of an HP6890 GC, HP5973 mass spectrometer, and HP7683 autosampler. Samples (1 μl) were injected into a split/splitless injector set at 280 °C using the splitless mode. Picolinyl esters were resolved on a capillary column of 30 m × 0.25 mm × 0.25 μm (HP-5 ms). The oven temperature was programmed to change from 80 to 150 °C at a rate of 30 °C min^{-1}, then from 150 to 280 °C at the rate of 5 °C/min, and held at 280 °C for 10 min. Helium was used as the carrier gas at the flow rate of 1.4 ml/min. The mass spectrometer was operated in the electron ionization mode at 70 eV with a source temperature of 230 °C, and spectra were acquired using a scan function from *m/z* 50 to 450.

2.3.4. High performance liquid chromatography/mass spectrometry

To characterize "C8:1" acylcarnitine in human urine by HPLC/MS, synthesized *trans*-2-octenoylcarnitine, 3-octenoylcarnitine, *trans*-4-octenoylcarnitine, *cis*-4-octenoylcarnitine, and *cis*-3,4-methylene-heptanoylcarnitine were individually spiked into a urine sample containing "C8:1" acylcarnitine. Acylcarnitines were isolated from urine by weak cation exchange solid phase extraction, derivatized with pentafluorophenacyl trifluoromethanesulfonate, separated by HPLC and detected by ESI-MS [11] (as in Fig. 1). However, ESI-MS analysis of acylcarnitines is not sufficient to characterize the structure of the acyl group of the acylcarnitine. This information comes from the acylcarnitine transesterification and GC/EI-MS analysis.

3. Results and discussion

3.1. "C8:1" acylcarnitine in human urine

Acylcarnitines, present in blood, urine, and various tissues, are mainly the metabolites of fatty acids or of amino acids. Determination of the chemical structure of the acyl group of an acylcarnitine could suggest the original source of the acylcarnitine's acyl group. One unusual acylcarnitine reported is *cis*-3,4-methylene-heptanoylcarnitine [7]. To determine if this identification is correct, a human urine sample containing "C8:1" acylcarnitine was collected. An HPLC/MS chromatogram of urinary acylcarnitine pentafluorophenacyl esters is shown in Fig. 1. Several major acylcarnitine species were identified by comparison with standard acylcarnitines. Peak 7, with a retention time of 34.5 min, corresponds to "C8:1" acylcarnitine pentafluorophenacyl ester. The MS/MS chromatogram of "C8:1" acylcarnitine pentafluorophenacyl ester is shown in Fig. 1B, and the inset picture is its fragment ion mass spectrum. "C8:1" acylcarnitine pentafluorophenacyl ester (*m/z* 494) yields fragment ions of *m/z* 435 and 293 in MS/MS spectrum. The *m/z* 435 ion is a product ion after "C8:1" acylcarnitine pentafluorophenacyl ester loses a neutral molecule of trimethyl amine ($N(CH_3)_3$, MW: 59). The *m/z* 293 ion is a product ion presumably generated after the fragment ion of *m/z* 435 loses a neutral molecule of C8:1 organic acid (M_W: 142), and is the ion common to all acylcarnitine pentafluorophenacyl esters. The acylcarnitine pentafluorophenacyl ester of *m/z* 494 corresponds to an acylcarnitine of *m/z* 286 before derivatization. Based on the *m/z* 286, this acylcarnitine contains eight carbons and one unsaturation in its acyl group. However, no

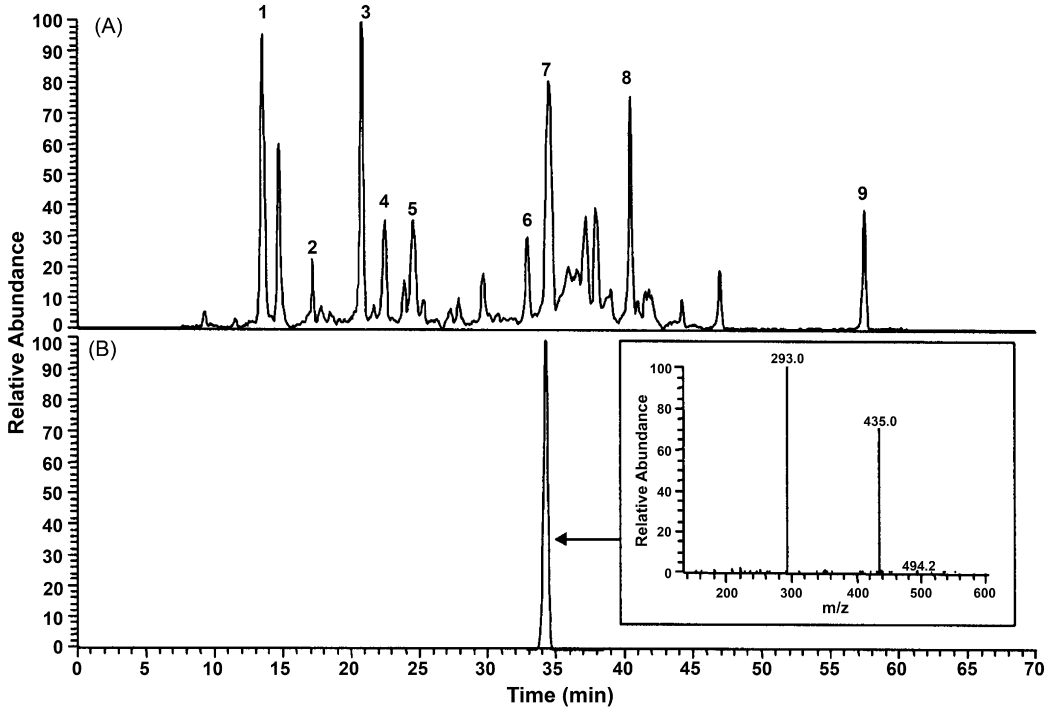


Fig. 1. HPLC/MS chromatogram of human urine. Acylcarnitines were detected as their corresponding pentafluorophenacyl esters. Full scan data in both the MS and MS/MS mode were collected and the chromatograms shown were generated post analysis by software selection of a specific ion mass to create a "reconstructed ion chromatogram" (RIC). (A) Full scan MS ($RIC_{m/z\ 293}$), acylcarnitine common ion chromatogram. (B) Full scan MS/MS on the *m/z* 494 ion ($RIC_{m/z\ 293}$). This ion corresponds to "C8:1" acylcarnitine pentafluorophenacyl ester. Also shown is the full scan MS/MS spectrum. Peak Identities: (1) acetyl- + d_6-acetylcarnitine pentafluorophenacyl esters, (2) propionyl- + d_3-propionylcarnitine pentafluorophenacyl esters, (3) isobutyrylcarnitine pentafluorophenacyl ester, (4) tigloylcarnitine pentafluorophenacyl ester, (5) 2-methyl-butyrylcarnitine pentafluorophenacyl ester, (6) succinylcarnitine pentafluorophenacyl ester, (7) "C8:1" acylcarnitine pentafluorophenacyl ester, (8) nonanoylcarnitine pentafluorophenacyl ester and (9) heptadecanoylcarnitine pentafluorophenacyl ester.

other structural information about the acyl group is available by HPLC/MS.

3.2. Characterization of urinary "C8:1" acylcarnitine by GC/MS

Trans-2-Octenoylcarnitine was used to evaluate "C8:1" acylcarnitine recovery. Fig. 2A shows a GC/MS chromatogram of acyl picolinyl esters produced from acylcarnitines in the urine sample. The spectra of the "C8:1" acyl picolinyl ester (Fig. 2B) and *trans*-2-octenoyl picolinyl ester (Fig. 2C) are illustrated. "C8:1" acyl picolinyl ester elutes more than 1 min earlier than *trans*-2-octenoyl picolinyl ester in this GC/MS system. The fragment ion pattern of "C8:1" acyl picolinyl ester (Fig. 2B) is different from that of *trans*-2-octenoyl picolinyl ester (Fig. 2C), although they have the same molecular ion of *m/z* 233 and some common fragment ions (*m/z* 190, 204, and 218). Because of their different retention times and mass spectra, "C8:1" acylcarnitine is not *trans*-2-octenoylcarnitine.

In the GC/MS spectrum of "C8:1" acyl picolinyl ester (Fig. 2B), the fragment ion of *m/z* 151 is consistent with a methylene group (–CH_2–) at C2 of the acyl group. The mass difference of 15 between *m/z* 218 ion and *m/z* 233 ion is consistent with a methyl group (–CH3) at C8 of the acyl group. The mass differences of 14 between fragment ions of *m/z* 190, 204, and 218 is consistent with methylene groups (–CH_2–) at C6 and C7 of the acyl group [19]. Thus, we conclude that "C8:1" acylcarnitine does not have a double bond between C2 and C3, C5 and C6, C6 and C7, or C7 and C8. There is a mass difference of 12 between *m/z* 178 ion and *m/z* 190 ion. Based on the '12 rule' interpretation for double bonds described by Christie [20], "C8:1" acylcarnitine may have a double bond between C4 and C5 of its acyl moiety.

To test this hypothesis, *trans*-4-octenoylcarnitine, *cis*-4-octenoylcarnitine, and 3-octenoylcarnitine were synthesized. These acylcarnitines were converted into their corresponding acyl picolinyl esters by transesterification, and their picolinyl esters were determined by GC/MS. In this GC/MS system, *trans*-4-octenoyl-, *cis*-4-octenoyl-, and 3-octenoyl picolinyl esters show almost identical retention times (around 9.9–10.0 min) to that of the "C8:1" acyl picolinyl ester. Their GC/MS spectra are shown in Fig. 3. "C8:1" acyl picolinyl ester (Fig. 2B) shows a different fragment ion pattern from those of *trans*-4-octenoyl-, *cis*-4-octenoyl- and 3-octenoyl picolinyl esters (Fig. 3A–C). Based on these GC/MS spectra, "C8:1" acylcarnitine is not *trans*-4-octenoyl-, *cis*-4-octenoyl-, or 3-octenoylcarnitine.

Libert et al. [7] reported that "C8:1" acylcarnitine in normal human urine is *cis*-3,4-methylene-heptanoylcarnitine, although only indirect evidence was presented. Our GC/MS spectrum of "C8:1" acyl picolinyl ester is similar to that shown by Libert [7]. The mass difference of 13 units between *m/z* 165 ion and *m/z* 178 ion in the GC/MS spectrum of "C8:1" acyl picolinyl ester is

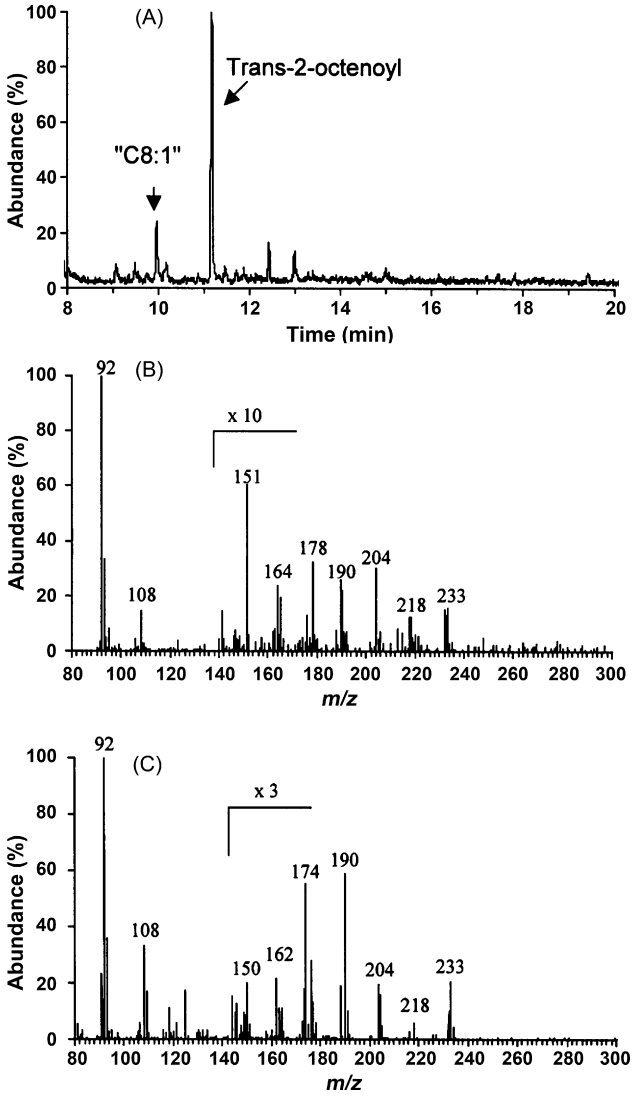


Fig. 2. GC/MS of acyl picolinyl esters. (A) Chromatogram of acyl picolinyl esters derived from acylcarnitines isolated from a human urine spiked with *trans*-2-octenoylcarnitine. (B) GC/MS spectrum of "C8:1" acyl picolinyl ester. (C) GC/MS spectrum of *trans*-2-octenoyl picolinyl ester.

similar in part to the observation reported for the picolinyl derivative of 11,12-methylene-octadecanoic acid [21]. We synthesized *cis*-3,4-methylene-heptanoylcarnitine (Scheme 1), converted it into the corresponding picolinyl ester, and analyzed the acyl picolinyl ester by GC/MS. In this GC/MS system, *cis*-3,4-methylene-heptanoyl picolinyl ester shows identical retention time to that of "C8:1" acyl picolinyl ester. The fragment ion pattern of *cis*-3,4-methylene-heptanoyl picolinyl ester is illustrated in Fig. 3D. The intensity of the MS spectrum of the "C8:1" acyl picolinyl ester in Fig. 2B is much weaker than that shown by Libert et al. and there is more noise in the signal in Fig. 2B. We attribute these causes to the differences. However, the general features of both spectra are identical: *m/z* masses 151, 164, 178, 190, 204, 218 and 233 are present in both, and the match between Libert et al.'s result and our transesterified *cis*-3,4-methylene-heptanoylcarnitine (Fig. 3D) is excellent.

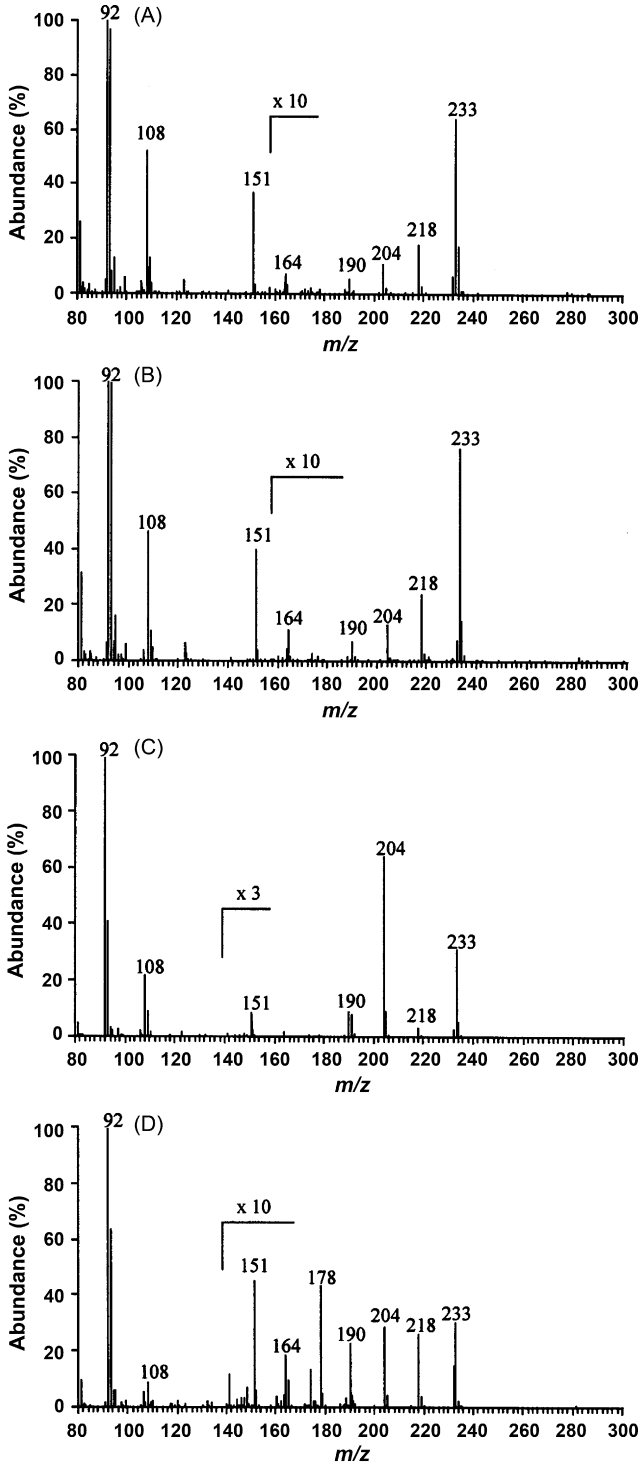


Fig. 3. The GC/MS spectra of acyl picolinyl esters derived from the synthesized C8:1 acylcarnitines. (A) *trans*-4-Octenoyl picolinyl ester. (B) *cis*-4-Octenoyl picolinyl ester. (C) 3-Octenoyl picolinyl ester. (D) *cis*-3,4-Methylene-heptanoyl picolinyl ester.

3.3. Characterization of synthesized cis-3,4-methylene-heptanoylcarnitine by MS/MS analysis

The electrospray ionization tandem MS spectrum of the synthesized *cis*-3,4-methylene-heptanoylcarnitine is shown in Fig. 4. The formation mechanism of the fragment ions is sug-

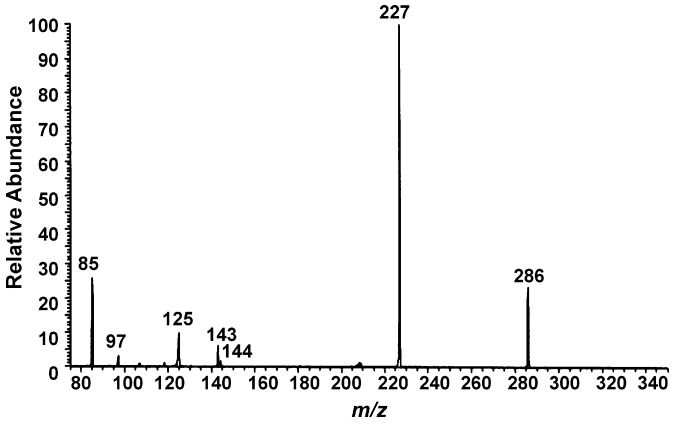


Fig. 4. The electrospray ionization tandem mass spectrum of synthesized *cis*-3,4-methylene-heptanoylcarnitine.

gested in Scheme 2. Fragment ions of *m/z* 85 and 144 are from the carnitine moiety, and fragment ions of *m/z* 97, 125, and 143 are from the acyl group. The *m/z* 227 ion is a fragment ion formed from the neutral loss of trimethylamine (M_W: 59).

3.4. Confirmation of cis-3,4-methylene-heptanoylcarnitine by HPLC/MS analysis

The HPLC/MS method developed in this laboratory [11] was used to show that "C8:1" acylcarnitine in human urine is consistent with *cis*-3,4-methylene-heptanoylcarnitine. Synthesized *trans*-2-octenoylcarnitine, 3-octenoylcarnitine, *trans*-4-octenoylcarnitine, *cis*-4-octenoylcarnitine, and *cis*-3,4-methylene-heptanoylcarnitine were spiked individually into urine. Acylcarnitines were isolated from urine, derivatized with pentafluorophenacyl trifluoromethanesulfonate, separated by HPLC, and detected by MS/MS. Fig. 5 shows the $RIC_{m/z\ 293}$ chromatograms generated from the full scan MS/MS spectra of *m/z* 494 for the derivatized "C8:1"

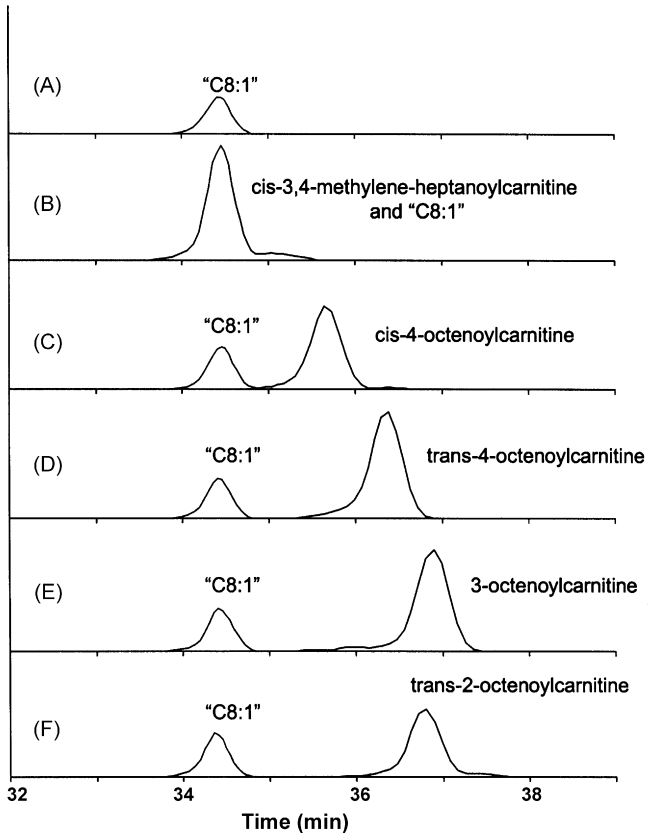
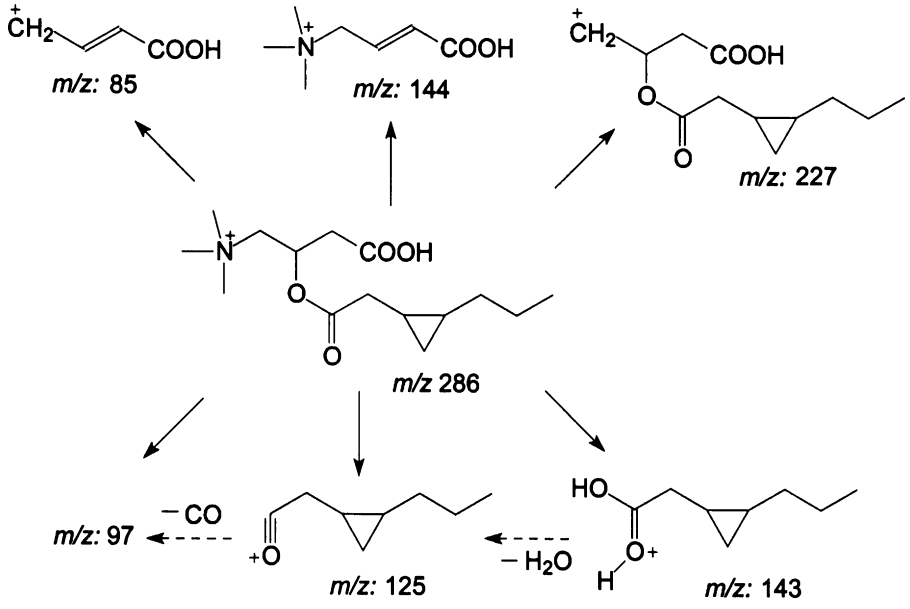


Fig. 5. HPLC/MS chromatograms of unspiked and spiked urine C8:1 acylcarnitine pentafluorophenacyl esters. Shown are $RIC_{m/z\ 293}$ chromatograms generated from full scan MS/MS on the *m/z* 494 ion. The *y*-axis is A/D counts with a full scale of 2.0E + 08. The samples are: (A) urine alone containing "C8:1" acylcarnitine; (B) the same urine spiked with *cis*-3,4-methylene-heptanoylcarnitine, or (C) *cis*-4-octenoylcarnitine, or (D) *trans*-4-octenoylcarnitine, or (E) 3-octenoylcarnitine, or (F) *trans*-2-octenoylcarnitine.



Scheme 2. Proposed structures of fragment ions of *cis*-3,4-methylene-heptanoylcarnitine in ESI-MS/MS spectrum.

acylcarnitine and the synthesized acylcarnitines. After derivatization, each spiked acylcarnitine exhibits the same molecular ion of *m/z* 494 and produces the same fragment ions of *m/z* 293 and 435, which are identical to the MS/MS spectrum of the urinary "C8:1" acylcarnitine pentafluorophenacyl ester shown in Fig. 1B. *trans*-2-Octenoylcarnitne, 3-octenoylcarnitine, *trans*-4-octenoylcarnitine, and *cis*-4-octenoylcarnitine elute about 1–2 min later than "C8:1" acylcarnitine in our HPLC/MS system. Thus, these data further confirm that "C8:1" acylcarnitine in human urine is not *trans*-2-octenoylcarntine, 3-octenoylcarnitine, *trans*-4-octenoylcarnitine, or *cis*-4-octenoylcarnitine. However, the peak of synthesized *cis*-3,4-methylene-heptanoylcarnitine co-elutes with that of "C8:1" acylcarnitine in human urine, further supporting the conclusion that "C8:1" acylcarnitine in human urine is *cis*-3,4-methylene-heptanoylcarnitine.

We applied the HPLC/MS method to the detection of *cis*-3,4-methylene-heptanoylcarnitine in human blood and plasma from a normal volunteer (Fig. 6). Please note that the chromatographic gradient was modified to improve the resolution, resulting in a slightly different retention time than that shown in Figs. 1 and 5. As shown in Fig. 6, *cis*-3,4-methylene-heptanoylcarnitine is observed in both human blood and plasma. In addition, a second minor C8:1 acylcarnitine is also present. We have excluded the possibility of the minor peak being *trans*-2-octenoylcarnitine, 3-octenoylcarnitine, *trans*-4-octenoylcarnitine or *cis*-4-octenoylcarnitine. We suggest most likely that this minor peak is *trans*-3,4-methylene-heptanoylcarnitine.

3.5. The likely source of cis-3,4-methylene-heptanoylcarnitine

Libert et al. reported that *cis*-3,4-methylene-heptanoylcarnitine is observed in human urine, except in urine from newborn patients. They further observed that the acylcarnitines displaying a cyclopropane ring in their fatty acid moieties disappeared in the urine of humans treated with adryamcine, an antibiotic [22]. Grogan and Cronan reported that fatty acids with cyclopropane rings can be synthesized from unsaturated fatty acids in bacterial membranes by cyclopropane fatty acid synthase [23]. Moreover, when *cis*-9,10-methylene-octadecanoate is administered to rats, an accumulation of *cis*-3,4-methylene-dodecanoic acid in their adipose tissue was found, suggesting the inability of the β-oxidation enzyme system to proceed past the cyclopropane ring in a fatty acid chain [24]. Odd-chain fatty acids such as pentadecanoic acid (C15:0) and heptandecanoic acid (C17:0) in milk are largely derived from bacteria leaving the rumen [25]. So, we propose that pentadecanoic acid (C15:0) and heptanoic acid (C17:0) in the membrane of some bacteria in human gut are converted to *cis*-11-pentadecenoic acid (C15:1) and *cis*-11-heptadecenoic acid (C17:1) by δ-11 desaturase, then *cis*-11-pentadecenoic acid (C15:1) and *cis*-11-heptadecenoic acid (C17:1) would be transformed into *cis*-11,12-methylene-pentadecanoic acid and *cis*-11,12-methylene-heptadecanoic acid by cyclopropane fatty acid synthase in bacteria. These two fatty acids

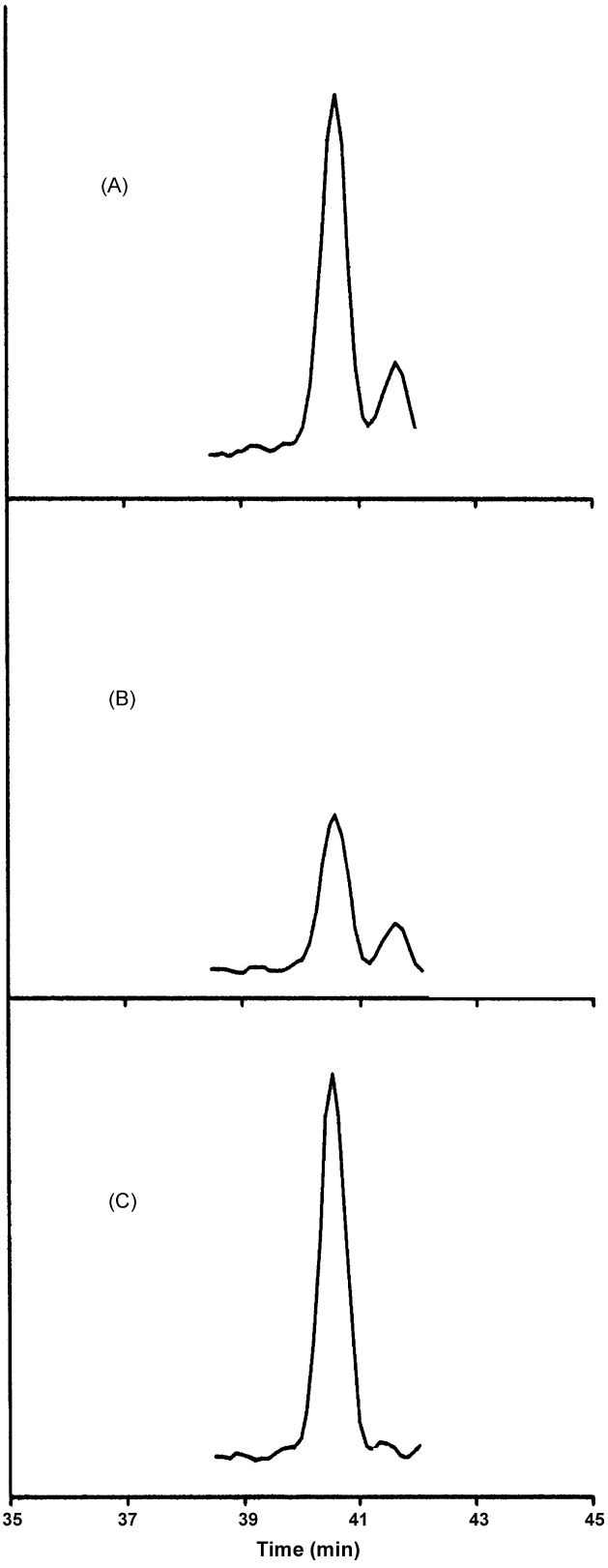


Fig. 6. HPLC/MS chromatograms of *cis*-3,4-methylene-heptanoylcarnitine in plasma and blood. Shown are total ion current chromatograms generated from full scan MS/MS spectra on the *m/z* 494 ion (pentafluorophenacyl ester). The *y*-axis is A/D counts with a full scale of 7.0E + 05. The chromatographic gradient was modified to improve the resolution, resulting in a slightly different retention time than that shown in Figs. 1 and 5. The samples are: (A) plasma; (B) blood, (C) standard.

would be absorbed and metabolized through β-oxidation in mitochondria to form *cis*-3,4-methylene-heptanoyl-CoA and *cis*-3,4-methylene-nonanoyl-CoA. These two compounds would then be converted into acylcarnitines and excreted. *cis*-3,4-Methylene-nonanoylcarnitine has been reported in human urine [7]. We also have observed this compound in human urine [unpublished data including comparison to synthesized standard], further supporting the above proposal.

If the proposed scenario is correct, following absorption of long-chain cyclopropane fatty acids and uptake into the liver, mitochondial or peroxisomal defects in long-chain fatty acid oxidation would be expected to result in the accumulation of long-chain cyclopropane acyl-CoAs or acylcarnitines. Therefore, in the future, we would like to obtain plasma samples from patients with defects of peroxisomal or mitochondrial long-chain fatty acid oxidation, to address which long-chain cyclopropane acylcarnitines accumulate.

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