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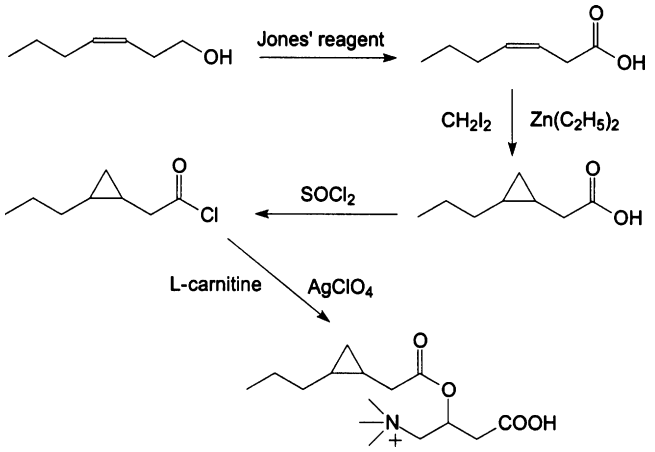
esters of fatty acids has been studied thoroughly by using tandem mass spectrometry [9] and applied to characterization of long-chain acids [10].

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*-