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Determination of 3-hydroxyisovalerylcarnitine and other acylcarnitine levels using liquid chromatography–tandem mass spectrometry in serum and urine of a patient with multiple carboxylase deficiency☆

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Abstract

Due to its increased concentration in blood, 3-hydroxyisovalerylcarnitine (C5OH-I) is an important indicator for the diagnosis of organic acidemias in newborns. However, C5OH-I has not been used as a standard in tandem mass spectrometric (MS/MS) assays because its isolation is difficult. We developed a new synthesis of C5OH-I and investigated its behavior by MS/MS. A method using the multiple reaction monitoring (MRM) mode of MS/MS with HPLC was developed which provides high accuracy, precision and reproducibility. Acylcarnitine profiles in the serum and urine of a patient with multiple carboxylase deficiency (MCD) showed increased levels compared to a healthy patient.

Keywords: Acylcarnitine; 3-Hydroxyisovalerylcarnitine; 3-Methylcrotonylcarnitine; Tiglylcarnitine; Multiple carboxylase deficiency; Holocarboxylase synthetase deficiency; Newborn screening; Tandem mass spectrometry; LC–MS/MS

1. Introduction

Analysis of acylcarnitines by tandem mass spectrometry (MS/MS) has recently been used to screen newborns for organic acidemias and fatty acid oxidation defects [1–5]. These diseases cause the accumulation of acyl-CoA, which is esterified to acylcarnitine by carnitine acyltransferase. Acylcarnitine is then eliminated in the urine, thus acylcarnitine concentration serves as an excellent indicator for these diseases [6]. 3-Hydroxyisovalerylcarnitine (C5OH-I; **4** in Scheme 1) is an indicator for diagnoses of multiple carboxylase deficiency (MCD), 3-methylcrotonyl-CoA carboxylase deficiency and 3-hydroxy-3-methylglutaryl-CoA lyase deficiency, and 3-hydroxy-2-methylbutyrylcarnitine (C5OH-M, an isomer of C5OH-I) is an indicator for β-ketothiolase deficiency [7–9]. The low activities of the enzymes in these disorders toward the metabolism of amino acids cause the accumulation of 3-hydroxyisovaleryl-CoA or 3-hydroxy-2-methylbutyryl-CoA which are then conjugated to C5OH-I or C5OH-M by carnitine acyltransferase. C5OHs have previously not been used as standards in the screening method for these diseases because of the difficulty in obtaining them.

We previously reported the determination method of 17 acylcarnitine isomers by the multiple reaction monitoring (MRM) mode of MS/MS coupled with HPLC [10]. For example, distinction between pivaloylcarnitine accumulated by treatment with antibiotics modified by the pivoxyl group and isovalerylcarnitine accumulated by isovaleric acidemia is impossible in current screening method using precursor ion mode analysis. The individual determination of acylcarnitine isomers is

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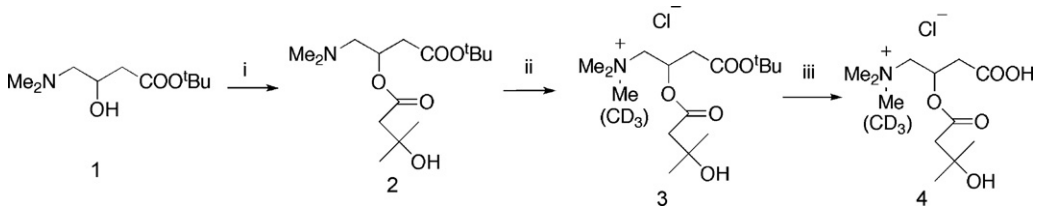

Scheme 1. The synthetic pathway for 3-hydroxyisovalerylcarnitine. Reagents: (i) 3-hydroxyisovaleric acid, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, 1-hydroxybenzotriazole; (ii) (1) MeI or CD_3I, (2) ion exchange resin (Dowex 1 × 2), (iii) 2% HCl.

important for diagnosis. Our method can be applied to detailed diagnostics of diseases indistinguishable by current screening methods and to monitoring of acylcarnitines in various clinical states of patients. A determination method using C5OH-I, 3-methylcrotonylcarnitine (C5:1-M), and tiglylcarnitine (C5:1-T; an isomer of C5:1-M) was newly developed in this study.

In this paper, we describe a new synthesis of C5OH-I and its behavior on MS. Moreover we measured the levels of 20 different acylcarnitine concentrations in the serum and urine of a patient with MCD by HPLC–MS/MS. These results will be discussed.

2. Experimental

2.1. Materials and synthesis of acylcarnitines

HPLC-grade acetonitrile was obtained from Nakarai Tesque (Kyoto, Japan). L-Carnitine hydrochloride was purchased from MP Biomedicals (Eschwege, Germany). C5:1s were prepared by reacting tiglylchloride or 3-methylcrotonylchloride with L-carnitine hydrochloride in trifluoroacetic acid. $^2H_3$-C5:1s, used as internal standards (IS), were prepared from $^2H_3$-carnitine hydrochloride [11] and the corresponding acylchloride. C5OH-I and $^2H_3$-C5OH-I (IS) were synthesized as shown in Scheme 1. All organic reagents were obtained from Tokyo Chemical Industry (Tokyo, Japan).

A solution of *t*-butyl 4-dimethylamino-3-hydroxybutyrate (**1**, 500 mg, 2.5 mmol) [11], 3-hydroxyisovaleric acid (2.5 mmol), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (2.5 mmol) and 1-hydroxybenzotriazole (2.5 mmol) in dimethylformamide (5 mL) was stirred for 18 h at 60 °C. The mixture was quenched with water, then washed with ether. The aqueous layer was adjusted to pH 8 by $NaHCO_3$ and extracted with ether. The extract was evaporated to give crude *t*-butyl 4-dimethylamino-3-(3′-hydroxyisovaleryloxy)butyrate (**2**).

Iodomethane or $^2H_3$-iodomethane (1.8 mmol) was added to a solution of **2** (480 mg, 1.6 mmol) in MeOH (5 mL) and the mixture was stirred for 3 h at room temperature. The solution was evaporated and the residue was washed with ether to give 3-hydroxyisovalerylcarnitine hydroiodide *t*-butylester. The iodide ion was exchanged to a chloride ion by an ion exchange resin (Dowex 1 × 2 chloride form, Sigma–Aldrich, St. Louis, MO, USA) to give 3-hydroxyisovalerylcarnitine hydrochloride *t*-butylester or its labeled analog (**3**).

A solution of **3** or its labeled product (270 mg, 0.7 mmol) in 2% HCl (12 mL) was stirred for 24 h at room temperature, then evaporated to give crude C5OH-I or $^2H_3$-C5OH-I (**4**). The mixture was purified by preparative HPLC (Inertsil ODS-80A column, 250 × 10 mm, GL Sciences, Tokyo, Japan) using water and MeOH (80:20). The eluted solution was lyophilized to give C5OH-I or $^2H_3$-C5OH-I (total yield 15%). The NMR spectrum matched the data in Ref. [12].

2.2. HPLC chromatographic and mass spectrometric conditions

The HP 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a gradient pump, a vacuum degasser and autosampler was used. A 10 μL sample solution was injected onto a Cadenza CD-C18 column (150 × 2.0 mm; Imtakt, Kyoto, Japan) at ambient temperature. Chromatography was performed at a flow rate of 0.2 mL/min using a step gradient alternating between acetonitrile (A) and 0.08% aqueous ion pair reagent (IPCC-MS3, GL Sciences, Tokyo, Japan) (B) for simultaneous analysis of the 20 acylcarnitines listed in Table 2. The gradient began with 6% A, then was programmed as follows: 0–1 min, gradient to 12% A; 1–7 min, hold at 12% A; 7–8 min, gradient to 20% A; 8–13 min, hold at 20% A; 13–14 min, gradient to 25% A; 14–17 min, hold at 25% A; 17–19 min, gradient to 40% A; 19–25 min, hold at 40% A; 25–25.1 min, gradient back to 6% A; 25.1–33 min, hold at 6% A to re-equilibrate the column.

A Quattro II tandem triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization (ESI) source was used for MS/MS analysis. Nitrogen was used as the nebulizing gas and argon was used as the collision gas at a pressure of 0.15 Pa. The source temperature was 130 °C and the capillary voltage used was 4.5 kV. The acylcarnitines were analyzed by multiple reaction monitoring (MRM) mode in positive ion mode. The cone voltage was 30 V, collision energy was 20 eV and transitions used were *m/z* 244.0 → 84.8 for C5:1, *m/z* 247.0 → 84.8 for $^2H_3$-C5:1, *m/z* 261.9 → 84.8 for C5OH and *m/z* 264.9 → 84.8 for $^2H_3$-C5OH.

2.3. Calibration curve

Standard stock solutions of acylcarnitines listed in Table 2 (10 mmol/L) for calibration curves were individually prepared in water. A mixed acylcarnitine spiking solution was prepared by diluting the respective stock solutions to 0.05, 0.1, 0.2, 0.4, 0.8, 1.5, 3, 6, 12 and 25 μmol/L. IS stock solutions of $^2H_3$-acylcarnitines (labeled analogs listed in Table 2; 10 mmol/L) were individually prepared in water. Mixed IS spiking solutions were prepared by diluting the respective stock solutions to

1 μmol/L. Calibration curves were prepared after mixing 50 μL of the respective spiking solution and 50 μL of the IS solution.

2.4. Sample preparation

Acylcarnitines were extracted from serum and urine using an Oasis MCX 30 mg/1 cc solid phase extraction cartridge (Waters, Milford, MA, USA). The cartridge was conditioned using 1 mL methanol and 1 mL water. The sample serum or urine (50 μL) and internal standard solution (50 μL) were loaded, washed with 1 mL 0.1% phosphoric acid and then 1 mL acetonitrile, and eluted with 2 mL 100 mmol/L pyridine in water–acetonitrile (1:1). The eluent was evaporated under nitrogen at 60 °C and the residue was dissolved in 50 μL water–acetonitrile (95:5). The solution was analyzed by HPLC–MS/MS.

3. Results and discussion

3.1. Synthesis of 3-hydroxyisovalerylcarnitine

It has been reported that only 1.2 mg of C5OH-I was obtained from17.1 kg of the mushroom *Suillus laricinus* [12]. The synthesis of this compound as a standard is necessary before its employment as an IS. Although the synthesis of 3-hydroxybutyrylcarnitine (C4OH), an analog of C5OH-I, has been reported [12], the synthesis of C5OH-I by a similar procedure gave a complex mixture and its purification by preparative HPLC was very difficult. In this method, the esterification of carnitine and 3-hydroxyisovaleric acid would occur at two positions. Accordingly, the carnitine analog protected carboxyl group, *t*-butyl 4-dimethylamino-3-hydroxybutyrate (**1**, Scheme 1), was selected for the starting material to minimize the complexity of the reaction. C5OH-I was obtained by deprotection followed by methylation of the obtained crude product. The synthetic C5OH-I was purified by preparative HPLC and dried over P_2O_5 under reduced pressure at 40 °C. The total yield of these three steps was 15%. MS and NMR spectra of the products indicated no contaminating organic products. Moreover, a labeled analog for IS is obtained easily and cheaply using iodomethane-d3 for the methylation. This method can be applied to the preparation of other acylcarnitines that contain hydroxy groups.

3.2. Analysis by MS/MS

Newborn screening using MS/MS has been carried out by the analysis of a precursor ion of *m/z* 85 for acylcarnitines. Each acylcarnitine concentration was determined by comparison with the peak intensity of a labeled acylcarnitine (IS). If there was no corresponding labeled product, an analog product was substituted. Since C5OH had not been used as a standard for newborn screening previously, the relationship between its concentration and its MS peak intensity must first be established. We determined that identical concentrations of C5OH-I and isovalerylcarnitine (C5) gave the same peak intensities. Carboxyl groups in acylcarnitines are generally butylated in order to obtain high sensitivity of acylcarnitines in the precursor ion mode.

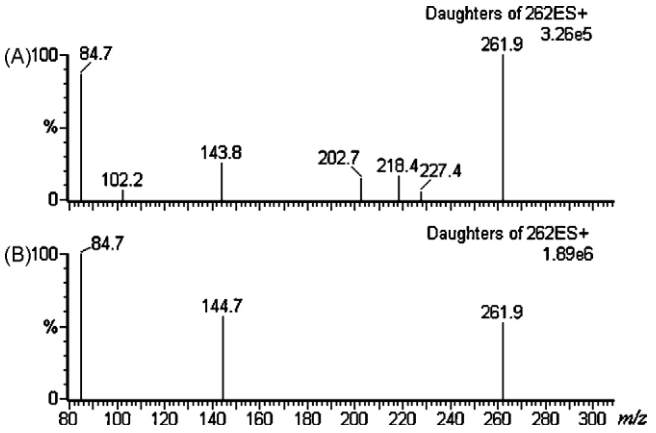

Fig. 1. Daughter ion spectra at a collision energy of 15 eV. (A) Methylmalonylcarnitine; (B) 3-hydroxyisovalerylcarnitine.

However, the butylation step in pretreatment needs time and effort. Moreover, it is questionable whether acylcarnitines were completely butylated. By using the more sensitive MRM mode rather than the precursor ion mode to analyze the underivatized acylcarnitines, the sensitivity became sufficiently high. The analysis of acylcarnitine without the derivatization has been reported [13–15]. Therefore, acylcarnitines were analyzed without the prior butylation in our HPLC–MS/MS method.

However, the new problem occurred by non-derivatized analysis. The *m/z* values of methylmalonylcarnitine (C4DC-M) and succinylcarnitine (C4DC-S, an isomer of C4DC-M) are both 262. Although C5OH is not an isomer of these compounds, the *m/z* value is also 262. Thus MRM analysis using same transition cannot distinguish these products. The daughter ion spectra of C4DCs and C5OH-I were examined (Fig. 1).

The spectra of the C4DCs were the same. The highest daughter ion peaks of C5OH-I and C4DCs are *m/z* 85 for all ranges of collision energy. The second highest daughter ion peak for C5OH-I was *m/z* 144, generated by elimination of the 3-hydroxyisovaleryloxy group at a collision energy of 15 eV. Although several daughter ion peaks were generated at a collision energy of 15 eV for C4DCs, these peaks were not useful for MRM analysis because of their very low intensities. Only daughter ion peak of *m/z* 85 was generated at collision energy more than 15 eV for both acylcarnitines. Therefore, separation due to the differences in daughter ion peaks is also impossible. So separation of these compounds was accomplished by HPLC. The separation of C5:1 isomers was also performed by HPLC. These chromatograms are shown in Fig. 2A.

3.3. Linearity

Calibration curves were obtained by linear regression with a weighting factor of $1/x$ for a plot of the analyte/internal standard peak–area ratio (y) vs. concentration (x) of analyte in water. The concentration ranges were 0.05–25 μmol/L. The equations for the linear calibration curves were $y = 1.024x - 0.00455$ ($R^2 = 0.9990$) for C5OH-I, $y = 1.12x - 0.00390$ ($R^2 = 0.9986$) for C5:1-T and $y = 1.072x - 0.00206$ ($R^2 = 0.9995$) for C5:1-M. The limit of quantitation (LOQ) was 0.05 μmol/L.

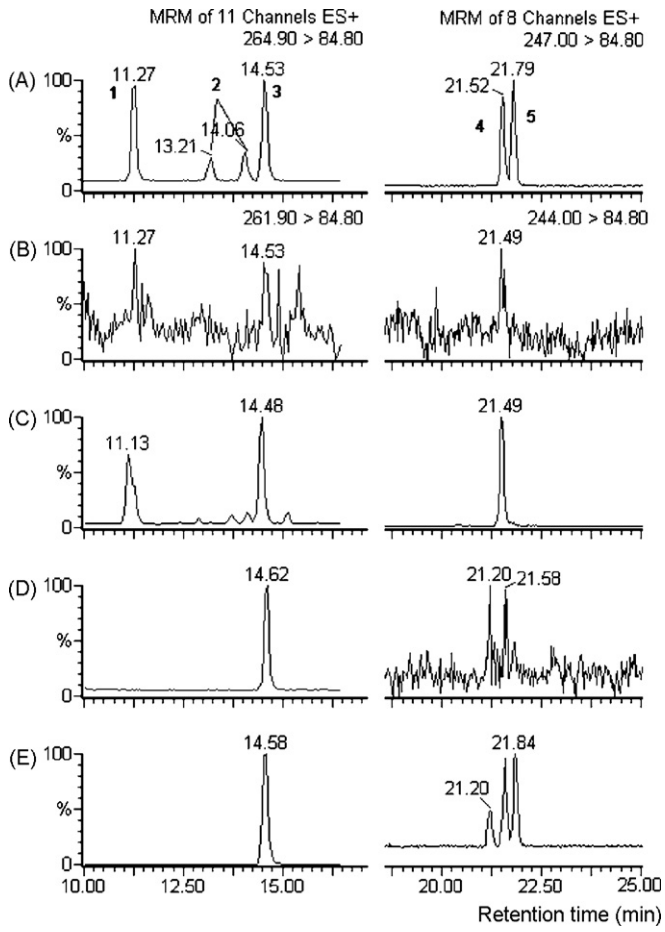

Fig. 2. LC–MS/MS spectra of acylcarnitines obtained by MRM mode. 1, Succinylcarnitine; 2, methylmalonylcarnitine; 3, 3-hydroxyisovalerylcarnitine; 4, tiglylcarnitine; 5, 3-methylcrotonylcarnitine. (A) IS; (B) control serum; (C) control urine; (D) patient serum; (E) patient urine.

3.4. Accuracy and precision

The imprecision data showed that intraday CVs and interday CVs at three concentrations were less than 8.3% and 9.1%, respectively, for the serum samples and less than 7.3% and 9.1%, respectively, for the urine samples (Table 1). Accuracies of intraday and interday were less than 8.6% and 9.4%, respectively, in serum samples and less than 9.9% and 10%, respectively, in urine samples.

3.5. Concentrations of carnitine and acylcarnitines in serum and urine of a patient with multiple carboxylase deficiency

MCD occurs in two forms: one type is holocarboxylase synthetase deficiency and the other type is biotinidase deficiency [16]. The activities of four carboxylase enzymes (propionyl-CoA carboxylase, 3-methylcrotonyl-CoA carboxylase, acetyl-CoA carboxylase and pyruvic acid carboxylase) are interrupted in MCD. Low activities of propionyl-CoA carboxylase and 3-methylcrotonyl-CoA carboxylase cause accumulations of propionyl-CoA and 3-methylcrotonyl-CoA, respectively. Propionyl-CoA is conjugated to propionylcarnitine (C3). After 3-methylcrotonyl-CoA is metabolized to 3-hydroxyisovaleryl-CoA by enoyl-CoA hydratase, C5OH-I is formed by carnitine conjugation [17]. The accumulation of C5:1-M in tissues and body fluids of MCD model rats has been reported [18]. We determined the level of C5:1-M concentrations, separately from C5:1-T, to investigate the accumulation of C5:1-M in serum and urine of patients.

Acylcarnitine concentrations in the serum and urine of a healthy infant as control and a patient with MCD were measured. The samples were collected after obtaining informed consent, in accordance with the Ethics Committee at the Graduate School of Medical Sciences, Nagoya City University. The age of the control boy is 4 years. The patient girl is 3 years old and was diagnosed with holocarboxylase synthetase deficiency. She had been treated with carnitine and biotin. The serum and urine samples were obtained when she was 1 and 3 years old. Twenty acylcarnitines in the control and patient samples were determined by the HPLC–MS/MS method. The chromatograms for C5OH and C5:1 are shown in Fig. 2 and summarized in Table 2.

The carnitine concentration in the serum and urine of the patient is higher than the control sample due to her treatment by carnitine. C5OH-I and C3 concentrations in patient serum are much higher than in the control and varied from day to day in the patient. These concentrations would be influenced by the condition of the patient. The C3 concentration was higher than that of C5OH-I. C5:1-M did not appear in the serum of either control or patient, because 3-methylcrotonyl-CoA would be conjugated to 3-methylcrotonylglycine rather than carnitine conjugation [17]. Moreover the metabolism of 3-methylcrotonyl-CoA to 3-hydroxyisovaleryl-CoA by enoyl-CoA hydratase is faster than carnitine conjugation. The concentrations of acylcarnitines in the urine of the patient are very high, and those of C5OH-I and C3 are especially high. A low concentration of C5:1-M appeared in urine, confirming the existence of 3-methylcrotonyl-CoA. Although C5:1-T, a metabolite of isoleucine, was also excreted in urine, it also existed in the control urine sample.

In a healthy individual, 3-methylcrotonyl-CoA is metabolized to 3-methylglutaconyl-CoA by 3-methylcrotonyl-CoA carboxylase in the leucine metabolism process [19]. When this enzyme activity is low, 3-methylcrotonyl-CoA is metabolized to 3-hydroxyisovaleryl-CoA by enoyl-CoA hydratase. In isoleucine metabolism, tiglyl-CoA is converted to 3-hydroxy-2-methylbutyryl-CoA by enoyl-CoA hydratase. As C5:1-T appeared in the urine of both healthy individual and patient and C5:1-M appeared in only the patient's urine, it appears that carnitine acyltransferase can compete with enoyl-CoA hydratase. The enzyme activity of 3-methylcrotonyl-CoA carboxylase must be higher than that of carnitine acyltransferase, because C5:1-M did not appear in the urine of the healthy individual. Moreover, a peak which is believed to be an isomer of C5:1 appeared on the chromatograph (Fig. 2E). This product has not yet been structurally characterized. Its identity would greatly clarify the metabolism process which occurs in MCD.

Table 1
Intra- ($n = 6$) and inter-assay ($n = 12$) CVs for pooled serum and urine samples enriched with acylcarnitines

Acylcarnitine	Added (μmol/L)	Serum				Urine			
		Imprecision CV (%)		Accuracy (%)		Imprecision CV (%)		Accuracy (%)	
		Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay
C5:1-T	0					3.4	7.7		
	0.2	6.6	9.1	1.9	−1.3	2.8	5.9	−9.9	2.8
	2.0	3.0	3.4	−4.5	−4.5	1.6	4.2	9.5	9.1
	20	3.3	4.3	−0.1	4.9	3.0	7.3	9.1	9.6
C5:1-M	0.2	2.8	7.9	5.3	−1.3	7.3	6.2	9.8	6.4
	2.0	3.7	6.8	−0.7	−9.4	3.1	8.9	9.0	7.8
	20	2.2	3.3	−8.3	−9.3	4.2	1.4	4.1	3.9
C5OH-I	0					3.9	7.5		
	0.2	8.3	8.3	8.6	8.9	3.3	9.1	−8.7	5.6
	2.0	4.3	8.8	−2.3	−8.5	4.5	6.6	8.8	10
	20	3.7	7.3	−2.6	−5.1	4.0	8.3	6.7	9.3

Table 2
Concentrations[a] of acylcarnitines in human serum and urine

Acylcarnitine	Serum (μmol/L)			Urine (μmol/g creatinine)		
	Control	Patient with MCD		Control	Patient with MCD	
		(1 year)	(3 years)		(1 year)	(3 years)
Carnitine	47.4	83.1	72.6	270	4710	731
Acetylcarnitine	16.9	6.14	7.28	60.6	8760	566
Propionylcarnitine (C3)	0.48	2.94	10.6	5.55	1380	105
Butyrylcarnitine	0.07	–[b]	0.06	–	6.44	0.30
Isobutyrylcarnitine	0.06	–	0.12	31.5	11.4	16.3
Tiglylcarnitine (C5:1-T)	–	–	–	2.55	1.91	2.77
3-Methylcrotonylcarnitine (C5:1-M)	–	–	–	–	2.54	2.49
Isovalerylcarnitine	–	–	0.08	0.65	6.06	2.60
2-Methylbutyrylcarnitine	0.05	0.07	0.08	19.2	23.2	7.70
3-Hydroxyisovalerylcarnitine (C5OH-I)	–	0.36	3.56	3.71	165	715
Hexanoylcarnitine	–	–	–	0.10	1.79	0.17
Octanoylcarnitine	0.08	0.12	0.07	0.23	5.26	0.46
Malonylcarnitine	–	–	–	1.34	2.17	1.18
Succinylcarnitine (C4DC-S)	–	–	–	4.80	9.60	3.17
Glutarylcarnitine	–	–	–	5.97	5.38	3.88

[a] Valeryl-, pivaloyl-, heptanoyl-, valproyl- and methylmalonyl-carnitine are below LOQ for all samples.
[b] Below LOQ.

3.6. Conclusion

In this study we developed a synthesis of C5OH-I and C5:1s and studied their behaviors by HPLC–MS/MS. This synthetic method is a general one for preparing acylcarnitines that contain hydroxy groups. Accuracy and imprecision of the HPLC–MS/MS method were less than 10%, and good reproducibility was obtained for the determination method. The correct determination of acylcarnitines is very important for the diagnosis of clinical conditions in patients. The acylcarnitine profiles of a patient with MCD showed that C5OH-I, C5:1-M and C3 concentrations were higher than those of a healthy individual, thus providing validation of our method.

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References

[1] M.S. Rashed, P.T. Ozand, M.P. Bucknall, D. Little, Pediatr. Res. 38 (1995) 324.
[2] M.S. Rashed, M.P. Bucknall, D. Little, A. Awad, M. Jacob, M. Alamoudi, M. Alwattar, P.T. Ozand, Clin. Chem. 43 (1997) 1129.
[3] D.H. Chace, J.C. Diperna, B.L. Mitchell, B. Sgroi, L.F. Hofman, E.W. Naylor, Clin. Chem. 47 (2001) 1166.
[4] Y. Shigematsu, S. Hirano, I. Hata, Y. Tanaka, M. Sudo, N. Sakura, T. Tajima, S. Yamaguchi, J. Chromatogr. B 776 (2002) 39.
[5] D.H. Chace, T.A. Kalas, E.W. Naylor, Clin. Chem. 49 (2003) 1797.
[6] J. Bremer, Physiol. Rev. 63 (1983) 1420.
[7] P. Vreken, A.E.M. van Lint, A.H. Bootsma, H. Overmars, R.J.A. Wanders, A.H. van Gennip, J. Inher. Metab. Dis. 22 (1999) 302.
[8] T.H. Zythovicz, E.F. Fitzgerand, D. Marsden, C.A. Larson, V.E. Shih, D.M. Johnson, A.W. Strauss, A.M. Comeau, R.B. Eaton, G.F. Grady, Clin. Chem. 47 (2001) 1945.
[9] L.S. Han, J. Ye, W.J. Qui, X.L. Gao, Y. Wang, X.F. Gu, J. Inher. Metab. Dis. 30 (2007) 504.
[10] Y. Maeda, T. Ito, A. Suzuki, Y. Kurono, A. Ueta, K. Yokoi, S. Sumi, H. Togari, N. Sugiyama, Rapid Commun. Mass Spectrom. 21 (2007) 799.

[11] M.O. Tinti, Eur. Pat. Appl. 208, 662 A1, January 14 (1987).
[12] H. Kawagishi, H. Murakami, S. Sakai, S. Inoue, Phytochemistry 67 (2006) 2676.
[13] A. Schulz, C. Schulz, D. Kohlmüller, G.F. Hoffmann, E. Mayatepek, Clin. Chim. Acta 335 (2003) 137.
[14] A.K. Ghoshal, T. Gut, N. Soukhova, S.J. Soldin, Clin. Chim. Acta 358 (2005) 104.
[15] A.K. Ghoshal, J. Balay, S.J. Soldin, Clin. Chim. Acta 365 (2006) 352.
[16] E.R. Baumgartner, T. Suormala, Int. Vitam. Nutr. Res. 67 (1997) 377.
[17] J.L.K. van Hove, S.L. Rutledge, M.A. Nada, S.G. Kahler, D.S. Millington, J. Inher. Metab. Dis. 18 (1995) 592.
[18] Y. Shigematsu, I.L. Bykov, Y.Y. Liu, A. Nakai, Y. Kikawa, M. Sudo, M. Fujioka, J. Inher. Metab. Dis. 17 (1994) 678.
[19] H.O. de Baulny, J.M. Saudubray, Semin. Neonatol. 7 (2002) 65.