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Acidified acetonitrile and methanol extractions for quantitative analysis of acylcarnitines in plasma by stable isotope dilution tandem mass spectrometry

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Abstract

We have compared two sample preparation methods for the analysis of plasma acylcarnitines by tandem mass spectrometry. Extraction from liquid plasma using acetonitrile was compared with the widely used methanol extraction from plasma spotted on filter paper. The recovery and reproducibility of the acetonitrile extraction were improved by acidification with 0.3% formic acid. The acidified acetonitrile and methanol extractions have the same limit of detection and upper linearity limit for all acylcarnitine species studied. The correlation coefficients between the two methods were greater than 0.988 and the slopes of the linear regressions ranged from 0.901 to 1.070. The extraction of acylcarnitines by acidified acetonitrile from liquid plasma yielded results comparable to those obtained by methanol extraction from plasma spotted on filter paper.

Keywords: Acylcarnitine analysis; Organic acidemia; Defects of fatty acid oxidation; Inherited disorders; Tandem mass spectrometry

1. Introduction

The analysis of acylcarnitines in blood and/or in plasma by MS/MS has become a routine diagnostic tool in the investigation of metabolic disorders such as organic acidemias and disorders of fatty acid oxidation. In these disorders, L-carnitine plays a key role in removing accumulated acyl-CoA intermediates from mitochondria through the formation of acylcarnitine esters. This leads to the increased concentrations of circulating acylcarnitines, increased excretion of acylcarnitines in urine, and secondary carnitine deficiency. Therefore, analysis of free carnitine and acylcarnitines in plasma, blood spots, or urine is of diagnostic value for these disorders [1].

The application of MS/MS to the analysis of acylcarnitines in blood or plasma was first pioneered by Millington et al. [2,3] at Duke University using fast atom bombardment (FAB) MS/MS. The "soft ionization" of FAB permitted the analysis of acylcarnitines as their butyl or methyl esters. The precursor ion scans of *m*/*z* 85, the major fragment produced by collision induced dissociation of all butylated acylcarnitines, produced a profile of all acylcarnitines present in the sample. Later, electrospray ionization MS/MS for acylcarnitine analysis was introduced as a more sensitive alternative to FAB [4]. Automation of the MS/MS analysis and data processing were also developed to facilitate large-scale screening of samples [5]. Since then, this technique has become a standard for the analysis of acylcarnitines [6–16]. The preparation of blood or plasma samples for acylcarnitine analysis involves deproteinization/extraction with an organic solvent containing labeled internal standards (ISs). A widely used method involves extraction by methanol from dried blood or plasma spotted on filter paper [2,17–21]. Liquid plasma can also be used for acylcarnitine analysis with a suitable solvent for extraction/deproteinization. There have been studies reported in the literature, in which acetonitrile [14] or ethanol [22] were employed as extraction solvents for analysis of selected acylcarnitines from liquid plasma. Acetonitrile is a suitable solvent for plasma deproteinization, but it is not the best solvent for all acylcarnitine species. In our initial experiments we observed reduced recovery of deuterated free carnitine and acetylcarnitine when they were prepared in acetonitrile. We have overcome the problem by acidifying the acetonitrile with 0.3% formic acid. This method for extraction of acylcarnitines was

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compared to methanol extraction from plasma spotted on filter paper, using solutions of standards as well as plasma samples from patients with known metabolic disorders.

2. Experimental

2.1. Reagents and materials

High purity grade methanol and acetonitrile were obtained from Fisher Scientific (Houston, TX, USA). Filter paper cards for plasma spots were purchased from Schleicher & Schuell BioScience (Keene, NH, USA). The 3 N butanolic HCl (3 N HCl in butanol) was purchased from Regis (Morton Grove, IL, USA). Deuterated carnitine and acylcarnitine standards were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). This kit contained d_9-C0 and the following L-acylcarnitines, d_3-C2, d_3-C3, d_3-C4, d_9-C5, d_3-C8, d_9-C14, and d_3-C16. Additional ISs, d_3-C6, d_3-C10, d_3-C12, and d_3-C18, and all unlabeled L-acylcarnitines were purchased from Dr. Herman J. ten Brink (Amsterdam, The Netherlands). C0 was obtained from Life Science Resources (Milwaukee, WI, USA). The 96-well extraction plates were purchased from Waters Corporation (Milford, MA, USA). Dialyzed plasma used for the validation of the assay was prepared by the Reagent Lab at ARUP Laboratories, Inc.

2.2. Plasma specimens

Heparinized plasma samples, previously submitted to our laboratory for clinical studies, were used for this study. The samples were stored at −70 °C and de-identified before use according to a protocol approved by the Institutional Review Board of the University of Utah Health Science Center.

2.3. Sample preparation

Two IS solutions were prepared for this study, one in acetonitrile acidified with 0.3% formic acid and the other in methanol. The concentration of each IS was the same in the two solutions, 0.3040 μmol/L for d_9-C0, 0.0706 μmol/L for d_3-C2, 0.0152 μmol/L for d_3-C3, d_3-C4, d_9-C5, d_3-C6, d_3-C8, d_3-C10, d_3-C12, and d_9-C14, and 0.0304 μmol/L for d_3-C16 and d_3-C18. For acetonitrile extraction, we added 600 μL of the acidified acetonitrile IS solution to 20 μL of plasma in a 96-well extraction plate. For methanol extraction, we spotted 20 μL of plasma onto three filter paper disks (4.8 mm in diameter) in a 96-well plate. After drying under nitrogen, 600 μL of methanol IS solution were added to each well. Both plates were shaken for 30 min and centrifuged. The supernatant, 450 μL from each well, was transferred into a second 96-well plate. The solvents were removed by heated nitrogen at 50 °C. The dry extract in each well was derivatized with butanolic HCl, dried, and re-suspended in 150 μL of mobile phase for MS/MS analysis.

2.4. Instrument method

A Waters Alliance 2795 system equipped with an autosampler accommodating 96-well plates was used for sample and mobile phase delivery. A 3 ft long Peek tubing with 0.0025″ inside diameter was placed between the LC and the MS/MS to help maintain a stable flow for flow injection. The sample injection volume was 20 μL. Each sample was retained in the ionization source of mass spectrometer for ∼1 min by varying the flow rate between 20 and 500 μl/min. The mobile phase consisted of acetonitrile and water at 80:20 (v/v) ratio, acidified with 0.05% formic acid.

MS/MS analysis was performed on a Waters Quattro Micro™ tandem mass spectrometer in positive ion electrospray mode. The ionization source and desolvation temperatures were set at 120 and 250 °C, respectively. The instrument was operated through MassLynx software. Free carnitine and acetylcarnitine were monitored in multiple reaction monitoring (MRM) mode. All other acylcarnitines were monitored using parent scan of ion m/z 85 with a sample cone voltage of 35 V, collision energy of 25 eV, and a mass scan range from 270 to 580. Forty-four acylcarnitine species were monitored for quantitation using NeoLynx software. Twelve deuterated acylcarnitines were used as references for quantitation. The concentration of each species was calculated by comparing peak intensities of the species to the respective IS.

3. Results

3.1. Assay linearity and limit of detection

Dialyzed plasma samples spiked with 12 acylcarnitine standards, were prepared at eight different concentrations. Each sample was processed using the acidified acetonitrile extraction and the methanol extraction. All samples were analyzed by MS/MS under the same instrument conditions.

The linearity range of each analyte was obtained from the analysis of the eight samples at different concentrations, in triplicate. The two methods showed the same linearity range for each acylcarnitine species. The upper limit of linearity was 228 μmol/L for C0 and C2, 45.60 μmol/L for C3 and C5, 13.68 μmol/L for C4, C6, C8, C10, C12, and C14, and 18.24 μmol/L for C16 and C18. The limit of detection (LOD) was determined as the concentration corresponding to a signal to noise ratio of 10:1. The two methods reached the same LOD for each acylcarnitine, 0.1 μmol/L for C0 and C2, 0.02 μmol/L for all other acylcarnitines studied.

3.2. Accuracy and precision

We have studied the assay accuracy at three different concentrations within the linearity range for each acylcarnitine. The amount ratio of the measured versus the added analyte was calculated from the average of three replicates at each concentration to assess the accuracy of the measurements. For all the acylcarnitines studied, these ratios were greater than 88% with both methods. No significant discrepancy has been observed between the two methods. The assay precision was also studied at three different concentrations for each analyte. Inter- and intra-assay CVs were calculated from nine analyses at each concentration, three runs with three replicates in each. Both inter-

Table 1
Intra-assay and inter-assay precision (CV%) of acylcarnitine analysis by acidified acetonitrile extraction from liquid plasma and methanol extraction from plasma spotted on filter paper disks

Acylcarnitines	Extraction by acidified acetonitrile		Extraction by methanol	
	Intra-assay ($n = 3$)	Inter-assay ($n = 3$)	Intra-assay ($n = 3$)	Inter-assay ($n = 3$)
C0	1.7	3.0	9.7	3.7
C2	3.9	1.7	5.3	2.8
C3	13.0	3.8	6.8	1.4
C4	7.4	6.2	7.8	7.7
C5	3.7	1.8	8.2	1.7
C6	12.3	4.0	7.9	5.1
C8	8.4	8.6	3.4	3.2
C10	3.9	3.3	4.3	5.3
C12	5.7	3.6	9.7	6.7
C14	4.8	0.5	6.0	2.5
C16	3.7	6.6	5.0	9.2
C18	4.2	5.2	5.9	0.6

The data were obtained from samples prepared at the following concentrations: 22.8 μmol/L for C0 and C2; 4.56 μmol/L for C3 and C5; 1.368 μmol/L for C4, C6, C8, C10, and C14; and 1.824 μmol/L for C16 and C18.

and intra-assay CVs were less than 10% for most of the species at all three concentrations studied with both methods. Table 1 shows the results obtained at one of the three concentrations studied.

3.3. Comparison of the two extraction methods

We have studied the correlation of the two extraction methods for 12 acylcarnitines with solutions of standards prepared in dialyzed plasma at concentrations within the linearity range. The data were compared using linear regression analysis. The acidified acetonitrile extraction data plotted against methanol extraction data produced slopes ranging between 0.901 and 1.070 with R^2 greater than 0.989 (Table 2). Fig. 1 illustrates the correlation of the two methods for octanoylcarnitine (C8). In addition, the correlation between the two methods was evaluated using samples from patients with known metabolic disorders. Fifteen samples with elevated C0, C2, C4, C5-OH, and/or C5-DC were processed with the two methods. The regression analysis for these species yielded a slope of 1.0093 for C0 (concentration range: 0.18–102.43 μmol/L), 1.0383 for C2 (0.03–87.85 μmol/L), 1.0227 for C4 (0.03–1.58 μmol/L), 0.9441 for C5-OH (0.00–2.28 μmol/L), and 1.0186 for C5-DC (0.10–5.29 μmol/L). The correlation coefficients were all greater than 0.988.

Fig. 2 shows the acylcarnitine profiles, parent scan of ion 85, obtained using the acidified acetonitrile extraction from liquid plasma of a healthy control (2A) and methanol extraction from the same plasma spotted on filter paper disks (2B). There is no significant difference observed between the profiles obtained with the two methods.

Fig. 3 shows the plasma acylcarnitine profiles of a patient with Glutaric Acidemia Type I (GA I) obtained with acidified acetonitrile extraction (3A) and methanol extraction (3B). The profiles obtained using the two methods were consistent. The quantitation of glutarylcarnitine (C5-DC, $m/z = 388$), the diagnostic analyte in glutaric acidemia type I, was 1.32 μmol/L from

Table 2
Correlation of acylcarnitine concentration in plasma determined by MS/MS analysis from samples processed by acidified acetonitrile extraction and by methanol extraction

C0	$y = 0.9657x + 0.7598$	$R^2 = 0.9996$
C2	$y = 1.0696x - 0.1408$	$R^2 = 0.9952$
C3	$y = 1.0352x - 0.0880$	$R^2 = 0.9967$
C4	$y = 1.0526x - 0.0115$	$R^2 = 0.9887$
C5	$y = 0.9965x - 0.0355$	$R^2 = 0.9964$
C6	$y = 1.0029x + 0.0826$	$R^2 = 0.9890$
C8	$y = 0.9818x - 0.0075$	$R^2 = 0.9941$
C10	$y = 1.0066x + 0.0213$	$R^2 = 0.9951$
C12	$y = 0.9459x - 0.0185$	$R^2 = 0.9889$
C14	$y = 0.9777x + 0.0724$	$R^2 = 0.9978$
C16	$y = 0.9709x - 0.0190$	$R^2 = 0.9991$
C18	$y = 0.9010x + 0.0569$	$R^2 = 0.9954$

Twenty-four samples were prepared at eight different concentrations and extracted with both methods. Measured concentrations for each acylcarnitine species were compared by regression analysis with data obtained by acidified extraction method on the *y*-axis and data obtained by methanol extraction method on *x*-axis. The concentration range was 1.43–228 μmol/L for C0 and C2, 0.29–45.6 μmol/L for C3 and C5, 0.09–13.68 μmol/L for C4, C6, C8, C10, C12, C14, and 0.11–18.24 μmol/L for C16 and C18.

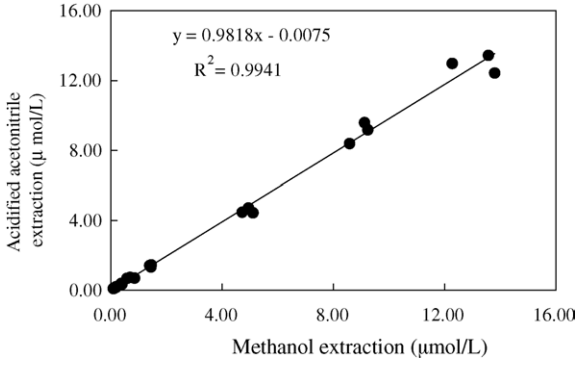

Fig. 1. Correlation of C8 concentration in dialyzed plasma determined by MS/MS analysis from samples processed by acidified acetonitrile extraction and by methanol extraction. Solutions of C8-canitine at eight different concentrations (range 0.09–13.68 μmol/L) were analyzed in triplicates, $n = 24$.

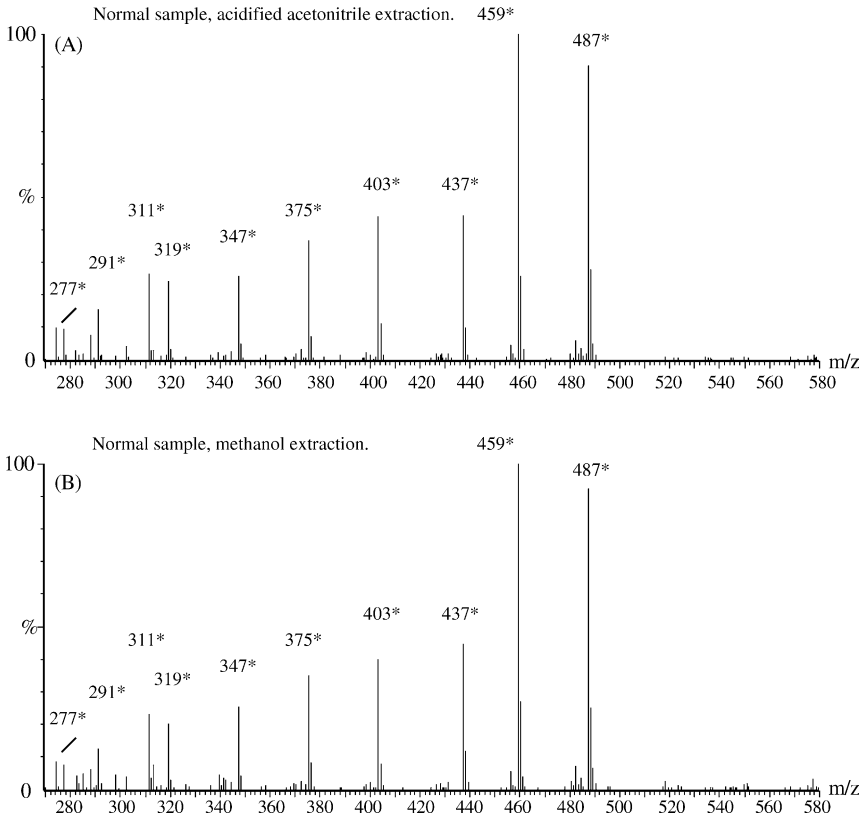

Fig. 2. Normal plasma acylcarnitine profile by LC/MS/MS using Parent Scan of ion 85. (A) Liquid plasma was extracted using acetonitrile acidified with 0.3% of formic acid. (B) Plasma was spotted on filter paper disks first and then extracted using methanol. All IS ions are marked by asterisks. They are the protonated molecular ions of deuterated acylcarnitine butyl esters with the following *m*/*z* ratios: d_3-C3, 277; d_3-C4, 291; d_9-C5, 311; d_3-C6, 319; d_3-C8, 347; d_3-C10, 375; d_3-C12, 403; d_9-C14, 437; d_3-C16, 459; d_3-C18, 487.

acidified acetonitrile extraction and 1.30 μmol/L from methanol extraction. Similar results were obtained when we compared samples from patients with other metabolic disorders (data not shown).

4. Discussion

Plasma acylcarnitine analysis is important for the diagnosis and management of patients with metabolic disorders. While the use of dried blood spots is most convenient for screening purposes [17], diagnostic methods require a more accurate quantitation. The use of liquid plasma allows accurate measurement of sample volume for analysis, eliminating the variability in plasma spots. In this study, we have used methanol extraction as a reference method for comparison. To control the volume of plasma used in the analysis, we have spotted a measured volume of liquid plasma onto filter paper disks for methanol extraction. The same volume of liquid plasma has been used for the analysis by acidified acetonitrile extraction. All samples, standards and patient specimens, have been analyzed with the two methods. The quantitative results for the acylcarnitines studied, short chain, medium chain, and long chain species, one hydroxylated (C5-OH) and one dicarboxylic (C5-DC) species, have been compared between methods. The correlation between the two methods using standards and patient samples yielded slopes between 0.901 and 1.070 with R^2 greater than 0.988. This has demonstrated that acidified acetonitrile extraction from liquid plasma is comparable to methanol extraction from dried plasma spots and suitable for analysis of acylcarnitine profiles by MS/MS.

In comparison with methanol extraction, we have found that acetonitrile itself is not a good solvent for all the acylcarnitine species. In our experiments we observed significant positive biases in the quantitation of C0 and C2 when deuterated acylcarnitines standards were prepared in acetonitrile for isotope dilution (Fig. 4). This was due to the significantly reduced intensities of d_9-C0 and d_3-C2 (by more then 50%). The intensities of d_9-C0 and d_3-C2 were restored by addition of 0.3% formic acid to the acetonitrile solution of deuterated standards. Fig. 4 shows the MRM mass spectra obtained from a solution of free carnitine extracted using acetonitrile based IS solution before acidification with 0.3% of formic acid (A) and after acidification with 0.3% of formic acid (B). Addition of 0.3% of formic acid into the IS solution did not increase the acid catalyzed hydrolysis of any acylcarnitine species and did not cause additional ion suppression. We have monitored, for each specimen, the quantity of d_3-C0, the hydrolysis product from all d_3 labeled acylcarntines, and the absolute intensities of a short chain, d_3-C2, a medium chain, d_3-C8, and a long chain, d_3-C18, acylcarnitine, with both methods. The average d_3-C0 concentration in the analyses of all dialyzed plasma standards, controls, and patient samples (a total of 208 samples) studied was 1.13 ± 0.11 μmol/L in acidi-

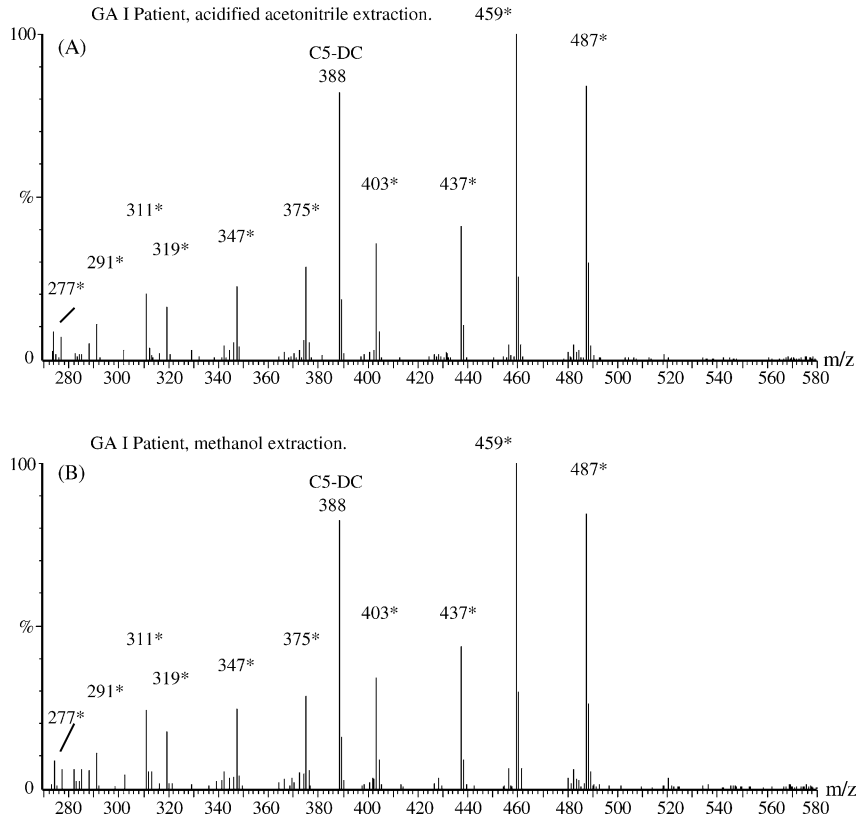

Fig. 3. Plasma acylcarnitine profile of a patient with glutaric acidemia type I (GA I). (A) Liquid plasma was extracted using acetonitrile acidified with 0.3% of formic acid. C5-DC = 1.32 μmol/L. (B) Plasma was spotted on filter paper disks first and then extracted using methanol. C5-DC = 1.30 μmol/L. The IS ions in the profile are marked with asterisks.

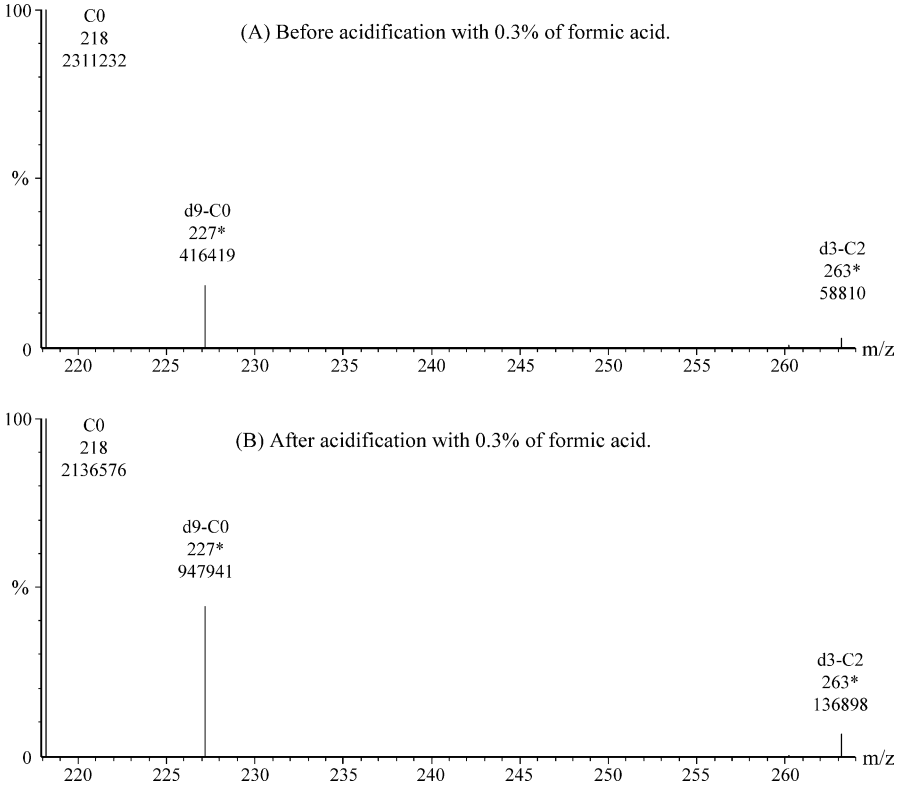

Fig. 4. Combined mass spectra of MRM channels from a solution of C0 extracted using the same acetonitrile based IS solution (A) before the acidification with 0.3% of formic acid and (B) after the acidification with 0.3% of formic acid. The concentration of C0 was the same (18.2 μmol/L) in both samples. Note the relative intensities of d_9-C0 and d_3-C2 to C0 are more than 50% lower in (A) than in (B).

fied acetonitrile extraction and 1.36 ± 0.23 μmol/L in methanol extraction. The average intensities of d_3-C2, d_3-C8, and d_3-C18 were (6.2 ± 1.7) E(+4), (6.7 ± 1.7)E(+5), and $(3.0E \pm 0.5)$ (+6) in acidified acetonitrile extraction, and (4.2 ± 0.6) E(+4), (4.5 ± 0.6) E(+5), and $(2.3E \pm 0.4)$ (+6) in methanol extraction, respectively. These data demonstrate that the addition of 0.3% of formic acid does not cause additional hydrolysis of acylcarnitines nor ion suppression, comparing to methanol extraction.

From this study we can conclude that the acidified acetonitrile extraction from liquid plasma is suitable for plasma acylcarnitine analysis by MS/MS. This method is simple and allows the direct use of liquid plasma with accurate measurement of sample volume for quantitation. The easier sample preparation makes this method particularly appealing and suitable for automation.

5. Nomenclature

C0	carnitine
C2	acetylcarnitine
C3	propionylcarnitine
C4	C4-canitine
C5	C5-carnitine
C5-DC	glutarylcarnitine
C6	hexanoylcanitine
C8	octanoylcarnitine
C10	decanoylcarnitine
C12	dodecanoylcarnitine
C14	myristoylcarnitine
C16	palmitoylcarnitine
C18	stearoylcarnitine
d_9-C0	$^2H_9$-carnitne
d_3-C2	$^2H_3$-acetylcarnitne
d_3-C3	$^2H_3$-propioylcarnitne
d_3-C4	$^2H_3$-butyrylcarnitne
d_9-C5	$^2H_9$-isovalerylcarnitne
d_3-C6	$^2H_3$-hexanoylcanitine
d_3-C8	$^2H_3$-octanoylcarnitine
d_3-C10	$^2H_3$-decanoylcarnitne
d_3-C12	$^2H_3$-dodecanoylcarnitne
d_9-C14	$^2H_9$-myristoylcarnitine
d_3-C16	$^2H_3$-palmitoylcarnitine
d_3-C18	$^2H_3$-stearoylcarnitine

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References

[1] J. Bremer, Physiol. Rev. 63 (1983) 1240.
[2] D.S. Millington, N. Kodo, D.L. Norwood, C.R. Roe, J. Inherit. Metab. Dis. 13 (1990) 321.
[3] D.S. Millington, N. Kodo, N. Terada, C.R. Roe, D.H. Chace, Int. J. Mass Spectrum. 111 (1991) 211.
[4] M.S. Rashed, P.T. Ozand, M.P. Bucknall, D. Little, Pediatr. Res. 38 (1995) 324.
[5] D.H. Chace, D.S. Millington, N. Terada, S.G. Kahler, C.R. Roe, L.F. Hofman, Clin. Chem. 39–1 (1993) 66.
[6] D.S. Millington, D.H. Chace, in: D.M. Desiderio (Ed.), Mass spectrometry: Clinical and Biochemical Applications, vol. 1, Plenum Press, New York, 1992, p. 299.
[7] L. Sweetman, Clin. Chem. 42 (1996) 345.
[8] H.L. Levy, Clin. Chem. 44 (1998) 2401.
[9] J. Charrow, S.I. Goodman, E.R.G. McCabe, P. Rinaldo, Genet. Med. 2 (2000) 267.
[10] M.S. Rashed, J. Chromatogr. B 758 (2001) 27.
[11] D.H. Chace, J.C. DiPerna, T.A. Kalas, R.W. Johnson, E.W. Naylor, Clin. Chem. 47 (2001) 1166.
[12] D.H. Chace, S.L. Hillman, J.L.K. Van Hove, E.W. Naylor, Clin. Chem. 43 (1997) 2106.
[13] P.T. Clayton, M. Doig, S. Ghafari, C. Taytor, J.V. Leonard, M. Morris, A.W. Johnson, Arch. Dis. Child. 79 (1998) 109.
[14] P. Vreken, E.M. van Lint, A.H. Bootsma, H. Overmas, R.J.A. Wanders, A.H. van Gennip, in: P.A. Quant, S. Eaton (Eds.), Current Views of Fatty Acid Oxidation and Ketogenesis: from Organelles to Point Mutations, vol. 327, Kluwar/Plenum, New York, 1999, p. 37, Chapter 38.
[15] K.G. Sim, J. Hammond, B. Wilchen, Clin. Chim. Acta 323 (2002) 37.
[16] D.H. Chace, J.C. DiPerna, B.L. Mitchell, B. Sgroi, L.F. Hofman, E.W. Naylor, Clin. Chem. 47 (2001) 1166.
[17] D.H. Chace, J.C. DiPerna, E.W. Naylor, Acta. Paediatr. Suppl. 432 (1999) 45.
[18] M. Rashed, M.P. Bucknall, D. Little, A. Awad, M. Jacob, A. Alamoudi, M. Alwattar, P.T. Ozand, Chin. Chem. 43 (1997) 1129.
[19] K. Carpenter, V. Wiley, K.G. Sim, D. Heath, B. Wilcken, Arch. Dis. Child Fetal Neonatal Ed. 85 (2001) 105.
[20] T.H. Zytkovicz, E.F. Fitzgerald, 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.
[21] A. Schulze, M. Lindner, D. Kohlmüller, K. Olgemöller, E. Mayatepek, G.F. Hoffmann, Pediatrics 111 (2003) 1399.
[22] W. Röschinger, D.S. Millington, D.A. Gage, Z.H. Huang, T. Iwamoto, S. Yano, S. Packman, K. Johnston, S.A. Berry, L. Sweetman, Clin. Chim. Acta. 240 (1995) 35.