

# A rapid UPLC–MS/MS method for simultaneous separation of 48 acylcarnitines in dried blood spots and plasma useful as a second-tier test for expanded newborn screening

Antonina Gucciardi · Paola Pirillo ·  
Iole Maria Di Gangi · Mauro Naturale ·  
Giuseppe Giordano

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**Abstract** Acylcarnitine profiling in dried blood spots (DBS) is a useful method for high-throughput newborn screening of metabolic disorders, but differentiation of isobaric and isomeric compounds is not achievable. Chromatographic methods for separation have already been reported but are specific for short-chain acylcarnitines or time-consuming. The aim of this work was to develop a fast ultraperformance liquid chromatography (UPLC)–tandem mass spectrometry (MS/MS) method for separation and quantification of a large number of acylcarnitines, including dicarboxylic acylcarnitines and hydroxyacylcarnitines, in DBS and plasma samples. Acylcarnitines from DBS and plasma were converted to their butyl esters and analyzed by electrospray ionization MS/MS. Chromatographic separation was achieved using a UPLC system equipped with an ethylene-bridged hybrid C<sub>18</sub> column. The correlation coefficients of the calibration curves ( $r^2$ ) ranged from 0.990 to 0.999. The limit of detection ranged from 0.002 and 0.063  $\mu\text{M}$  for all compounds, and the limit of quantification ranged from 0.004 and 0.357  $\mu\text{M}$ . Precision ranged from 0.8 to 8.8 % and the mean recovery was 103 %. Profiles of acylcarnitine isomers were investigated in specimens obtained from patients diagnosed with different inborn

errors of metabolism. Acylcarnitine concentrations were also measured in 58 term newborns and compared with flow injection analysis measurements. With this newly developed UPLC–MS/MS method, the simultaneous detection of 61 (13 of these labeled) acylcarnitines in DBS and plasma can be achieved in 15 min including post-run equilibration. The method has been validated and can be used as an important component of newborn screening methods as a second-tier test for discrimination and to confirm diagnosis.

**Keywords** Acylcarnitines · Newborn screening · Second-tier test · Ultraperformance liquid chromatography · Mass spectrometry · Metabolic disease

## Introduction

Acylcarnitine profiling in dried blood spots (DBS) by flow injection analysis (FIA) electrospray ionization (ESI) tandem mass spectrometry (MS/MS) is a useful method for high-throughput newborn screening, which is important in the diagnosis of defects related to fatty acid  $\beta$ -oxidation and branched-chain amino acid metabolism [1]. However, this method cannot differentiate acylcarnitine as isomers, isobaric compounds, and contaminants. Although several high-performance liquid chromatography–MS/MS methods have been developed to separate isomers in serum and urine, they are time-consuming [2–5], need different derivatization steps [3, 5], or are focused on specific acylcarnitines [6, 7]. Moreover, isomeric separation has been performed especially for short-chain acylcarnitines [8, 9], and has not been applied to DBS samples.

Recently a new ultraperformance liquid chromatography (UPLC)–MS/MS method has been developed, but only for identification of C<sub>4</sub> and C<sub>5</sub> acylcarnitine isomers [10].

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A. Gucciardi (✉) · P. Pirillo · I. M. Di Gangi · M. Naturale ·  
G. Giordano (✉)

Mass Spectrometry Laboratory, Department of Women's  
and Children's Health, University of Padua,  
Via Giustiniani 3,  
35128 Padua, Italy  
e-mail: antonina.gucciardi@unipd.it  
e-mail: giordano@pediatria.unipd.it

A. Gucciardi  
e-mail: antogucc@yahoo.it

The method consists of two diagnostic tests targeted at either C<sub>4</sub> or C<sub>5</sub> isomers performed in around 10 min to obtain information about disease related to only these specific acylcarnitines.

The aim of our work was to develop a UPLC–MS/MS method for the separation and quantification of a large number of butylated acylcarnitines, including dicarboxylic acylcarnitines and hydroxyacylcarnitines found in DBS and plasma samples, for use as a second-tier test in newborn screening.

This method was successfully applied to analyze DBS and plasma samples of term infants. We also investigated acylcarnitines in patients with different metabolic diseases to assess the identification of characteristic isomers, which are indistinguishable by conventional screening methods.

The concentrations of butyrylcarnitine and isobutyrylcarnitine (C<sub>4</sub> and isoC<sub>4</sub>) are increased in patients with short-chain acyl-CoA dehydrogenase (SCAD) deficiency (OMIM #201470) and with isobutyryl-CoA dehydrogenase deficiency (OMIM \*604773), respectively. Five-carbon acylcarnitine has three isomers: isovalerylcarnitine, a diagnostic marker for isovaleryl-CoA dehydrogenase deficiency (OMIM #243500), 2-methylbutyrylcarnitine—concentration elevated in patients with short/branched-chain acyl-CoA dehydrogenase (SBCAD) deficiency, also known as 2-methylbutyryl-CoA dehydrogenase deficiency (OMIM #610006)—and 2,2-dimethylpropionylcarnitine (also known as pivaloylcarnitine), which is generated after administration of pivalate-conjugated antibiotics.

For C<sub>5:1</sub> acylcarnitine there are two isomers—tiglylcarnitine and 3-methylcrotonylcarnitine—whose concentrations increase in β-ketothiolase deficiency (OMIM #203750) and in 3-methylcrotonyl-CoA carboxylase (MCC) deficiency (OMIM #210200).

Three C<sub>5</sub>OH isomers are involved in different forms of organic acidemias: 3-hydroxyisovalerylcarnitine, which appears in patients with MCC deficiency or 3-hydroxy-3-methylglutaryl-CoA lyase deficiency (OMIM #613898) in different types of 3-methylglutaconic aciduria (OMIM #250950, #302060, #258501, 250951), and two diastereoisomers of 2-methyl-3-hydroxybutyrylcarnitine, which appear in disorders of isoleucine metabolism such as 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency (OMIM #300438) and β-ketothiolase deficiency (OMIM #203750). C<sub>5</sub>OH is also an indicator for disorders of biotin metabolism such as multiple carboxylase deficiency, both early onset (holocarboxylase deficiency, OMIM #253270) and late onset (biotinidase deficiency, OMIM #253260).

With respect to dicarboxylic acid modified carnitines, separation of methylmalonylcarnitine and succinylcarnitine is important to discriminate methylmalonic aciduria

(MMA) from succinyl-CoA synthase deficiency. The discrimination between glutarylcarnitine (C<sub>5</sub>DC) and 3-hydroxydecanoylcarnitine (C<sub>10</sub>OH) is important in establishing the diagnosis of glutaric acidemia type 1 (OMIM #231670) as reported by Moore et al. [11].

The identification of all of these isomers is not feasible without chromatographic separation; thus, with our method, a rapid analysis is possible and can be applied to samples previously analyzed by FIA, making this method useful as a confirmatory test for current screening methods and follow-up of disease.

## Experimental

### Reagents and materials

Stable-isotope-labeled acylcarnitine internal standards—<sup>2</sup>H<sub>9</sub>-DL-free carnitine (*d*<sub>9</sub>-C<sub>0</sub>), <sup>2</sup>H<sub>3</sub>-DL-acetylcarnitine (*d*<sub>3</sub>-C<sub>2</sub>), <sup>2</sup>H<sub>3</sub>-DL-propionylcarnitine (*d*<sub>3</sub>-C<sub>3</sub>), <sup>2</sup>H<sub>7</sub>-DL-butyrylcarnitine (*d*<sub>7</sub>-C<sub>4</sub>), <sup>2</sup>H<sub>9</sub>-L-isovalerylcarnitine (*d*<sub>9</sub>-C<sub>5</sub>), <sup>2</sup>H<sub>6</sub>-DL-glutarylcarnitine (*d*<sub>3</sub>-C<sub>5</sub>DC), <sup>2</sup>H<sub>3</sub>-DL-hexanoylcarnitine (*d*<sub>3</sub>-C<sub>6</sub>), <sup>2</sup>H<sub>3</sub>-DL-octanoylcarnitine (*d*<sub>3</sub>-C<sub>8</sub>), <sup>2</sup>H<sub>3</sub>-DL-decanoylcarnitine (*d*<sub>3</sub>-C<sub>10</sub>), <sup>2</sup>H<sub>3</sub>-DL-dodecanoylcarnitine (*d*<sub>3</sub>-C<sub>12</sub>), <sup>2</sup>H<sub>3</sub>-DL-myristoylcarnitine (*d*<sub>3</sub>-C<sub>14</sub>), <sup>2</sup>H<sub>3</sub>-DL-hexadecanoylcarnitine (*d*<sub>3</sub>-C<sub>16</sub>), and <sup>2</sup>H<sub>3</sub>-DL-octadecanoylcarnitine (*d*<sub>3</sub>-C<sub>18</sub>)—were purchased from ChromSystems Instruments & Chemicals (Munich, Germany).

Unlabeled acylcarnitines—L-carnitine·hydrochloride (C<sub>0</sub>), acetyl-L-carnitine·hydrochloride (C<sub>2</sub>), propionyl-L-carnitine·hydrochloride (C<sub>3</sub>), isobutyryl-L-carnitine·hydrochloride (iso-C<sub>4</sub>), butyryl-L-carnitine·hydrochloride (C<sub>4</sub>), isovaleryl-L-carnitine·hydrochloride (iso-C<sub>5</sub>), valerylcarnitine·hydrochloride (C<sub>5</sub>), hexanoyl-L-carnitine·hydrochloride (C<sub>6</sub>), octanoyl-L-carnitine·hydrochloride (C<sub>8</sub>), decanoylcarnitine hydrochloride (C<sub>10</sub>), dodecanoylcarnitine hydrochloride (C<sub>12</sub>), tetradecanoylcarnitine hydrochloride (C<sub>14</sub>), hexadecanoyl-L-carnitine·hydrochloride (C<sub>16</sub>), octadecanoyl-L-carnitine·hydrochloride (C<sub>18</sub>), and pivaloylcarnitine were generous gifts from Piero Rinaldo (Mayo Clinic, Rochester, MN, USA).

Acetonitrile, methanol, and *n*-butanol were from Carlo Erba (Milan, Italy). Trifluoroacetic acid (TFA) and acetyl chloride were from Sigma-Aldrich (Milan, Italy). All solvents were high-performance liquid chromatography grade, and water was Milli-Q water (Millipore, Billerica, MA, USA). Filter paper (S&S 903) was purchased from Schleicher & Schuell (Dassel, Germany). Eight-strip tubes and dome caps were from Explera (Ancona, Italy), and 96-well plates were purchased from Abgene (part of Thermo Scientific, Epsom, UK).

DBS control level 1 and DBS control level 2 were purchased from ChromSystems Instruments & Chemicals (Munich, Germany).

## Sample collection

DBS and plasma were collected, on the third day after birth, from 58 healthy term newborns (mean gestational age  $38.8 \pm 0.7$  weeks) admitted to the Neonatology Unit of the Pediatric Hospital of Padua. Patient compliance was expressed by written informed consent before the newborns were enrolled in the study.

DBS or plasma was also obtained from patients of different ages admitted to the Pediatric Hospital of Padua diagnosed with different mitochondrial enzyme deficiencies: SCAD deficiency ( $n=2$ ), medium-chain acyl-CoA dehydrogenase deficiency ( $n=3$ ), very long chain acyl-CoA dehydrogenase deficiency ( $n=2$ ), long-chain hydroxyl acyl-CoA dehydrogenase deficiency ( $n=3$ ), glutaric acidemia type 2 ( $n=2$ ), and carnitine palmitoyltransferase II deficiency ( $n=2$ ). Patients with organic acidemias such as MMA ( $n=6$ ), succinyl-CoA synthase deficiency ( $n=2$ ), MCC deficiency ( $n=3$ ), 3-hydroxy-3-methylglutaryl-CoA lyase deficiency ( $n=1$ ), propionic aciduria ( $n=2$ ), and isovaleric acidemia ( $n=2$ ) and with other diseases such as SBCAD deficiency ( $n=2$ ) were also enrolled. In all cases, diagnoses were confirmed by performing characteristic urine organic acid patterns, tissue enzyme assays, and/or mutation analysis.

Whole blood was drawn by heel prick and spotted on Guthrie filter paper, dried at room temperature, and stored at  $4^\circ\text{C}$  until analysis.

Neonatal plasma was collected (about  $10\ \mu\text{L}$ ) using heparinized capillary tubes to harvest blood from heel. Plasma aliquots were stored at  $-20^\circ\text{C}$  until analysis.

## Preparation of standard solutions and calibrations curves

Internal standard solutions were prepared by reconstituting ChromSystems Instruments & Chemicals standards with  $50\ \text{mL}$  of methanol and adding  $100\ \mu\text{L}$  of this solution to each sample. To assess linearity, calibration curves were obtained by applying the stable isotope dilution method. Standard stock solutions at  $5\text{--}10\ \text{mmol/L}$  unlabeled acylcarnitines ( $\text{C}_2$ ,  $\text{C}_3$ ,  $\text{isoC}_4$ ,  $\text{C}_4$ ,  $\text{isoC}_5$ ,  $\text{C}_5$ ,  $\text{C}_6$ ,  $\text{C}_8$ ,  $\text{C}_{10}$ ,  $\text{C}_{12}$ ,  $\text{C}_{14}$ ,  $\text{C}_{16}$ ,  $\text{C}_{18}$ ) were prepared individually in water. A combined acylcarnitine spiking solution was prepared by diluting the respective stock solutions and this was diluted again to obtain five points in the range of concentration optimized for each acylcarnitine (Table S1). All dilutions were made with methanol, and  $30\ \mu\text{L}$  of each solution was added to DBS or plasma.

The endogenous concentrations of the analytes in all matrices were calculated from the value of the intercept with the  $y$ -axis of the calibration curves, obtained from peak area ratios of the analytes to the internal standard.

## Sample preparation

A  $3.2\text{-mm}$  DBS was punched into the strip tube and  $100\ \mu\text{L}$  of the reconstituted internal standard was added, the tube was sealed with a cap, and the mixture was sonicated for  $20\ \text{min}$ .

Plasma ( $6\ \mu\text{L}$ ) was added to  $100\ \mu\text{L}$  of the reconstituted internal standard, the mixture was frozen at  $-20^\circ\text{C}$  for  $10\ \text{min}$ , and was then centrifuged at  $13,000\ \text{rpm}$  for  $10\ \text{min}$  in a Biofuge Fresco centrifuge (Heraeus, Germany) to remove the precipitated proteins.

The supernatant obtained from DBS or plasma was transferred to a 96-well plate and evaporated under nitrogen flow at  $60^\circ\text{C}$ . Acylcarnitines were derivatized to their butyl esters by adding  $100\ \mu\text{L}$  of freshly prepared butanol solution containing  $5\ \%\text{v/v}$  acetyl chloride and heating the mixture at  $60^\circ\text{C}$  for  $20\ \text{min}$ . Samples are evaporated to dryness under nitrogen flow, reconstituted in  $50\ \mu\text{L}$  acetonitrile/water ( $80:20$ ,  $\text{v/v}$ ), and injected into the UPLC analytical column for chromatographic separation.

The controls were handled in the same manner as patient specimens.

## UPLC–MS/MS

MS/MS analysis was performed using a Micromass Quattro Ultima triple-quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with an ESI source operating in positive mode. Data were acquired with MassLynx 4.1 and processed for calibration and quantification of the analytes with QuanLynx supplied by Waters Technical Services (Etten-Leur, The Netherlands). Optimal fragmentation patterns were previously determined for each acylcarnitine at a concentration of approximately  $10\ \mu\text{M}$ .

Data were acquired in multiple reaction monitoring (MRM) mode, with source temperature  $90^\circ\text{C}$ , desolvation temperature  $200^\circ\text{C}$ , capillary voltage  $3.5\ \text{kV}$ , and desolvation gas flow  $400\ \text{L/h}$ . The cone voltage and collision energy were determined for each acylcarnitine (Table 1). Each acylcarnitine concentration was calculated on the basis of the corresponding calibration curve. Compounds for which specific calibration curves were not available were quantified by applying the curve of a metabolite with similar structure or retention time. All acylcarnitines and corresponding labeled internal standards are reported in Table 1.

Sample introduction ( $7\ \mu\text{L}$ ) into a UPLC ethylene bridged hybrid  $\text{C}_{18}$  column ( $15\ \text{cm long} \times 2.1\text{-mm}$  inner diameter with  $1.7\text{-}\mu\text{m}$  particles) from Waters was accomplished using an Acquity UPLC system (Waters, Manchester, UK). The chromatographic run was performed at  $400\ \mu\text{L/min}$  with a gradient elution of a mobile phase which consisted of solution A ( $\text{H}_2\text{O}$  with  $0.1\ \%\ \text{TFA}$ ,  $\text{v/v}$ ) and solution B

**Table 1** Ultraperformance liquid chromatography–electrospray ionization tandem mass spectrometry analytical parameters for the simultaneous determination of acylcarnitine butyl esters using relative labeled internal standards. The fragment ion was  $m/z$  85.2 for all acylcarnitines

Short name	Function	Measured acylcarnitine	Internal standard	Retention time (min)	Precursor ion ( $m/z$ )	IS precursor ion( $m/z$ )	Cone voltage (V)	Collision energy (eV)	Dwell time (s)
Free	1	Free carnitine	$d_9$ -Free carnitine	1.80	218	227	40	25	0.020
C <sub>2</sub>	1	Acetyl	$d_3$ -Acetyl	2.28	260	263	40	25	0.020
C <sub>4</sub> OH	1	3-Hydroxybutyryl	$d_3$ -Butyryl	2.12	304	288	120	25	0.020
C <sub>5</sub> OH	1	3-Hydroxyisovaleryl	$d_9$ -Isovaleryl	2.60	318	311	120	25	0.020
C <sub>5</sub> OH	1	2-Methyl-3 hydroxybutyryl	$d_9$ -Isovaleryl	NI	318	311	120	25	0.020
C <sub>3</sub>	2	Propionyl	$d_3$ -Propionyl	2.92	274	277	40	20	0.020
isoC <sub>4</sub>	2	Isobutyryl	$d_3$ -Butyryl	3.77	288	291	40	20	0.020
C <sub>4</sub>	2	Butyryl	$d_3$ -Butyryl	3.89	288	291	40	20	0.020
C <sub>6</sub> OH	2	3-Hydroxyhexanoyl	$d_3$ -Hexanoyl	3.37	332	319	40	20	0.020
C <sub>5:1</sub>	3	Tiglyl	$d_9$ -Isovaleryl	4.26	300	311	40	20	0.020
C <sub>5:1</sub>	3	3-Methylcrotonyl	$d_9$ -Isovaleryl	4.36	300	311	40	20	0.020
C <sub>5</sub>	3	Pivaloyl	$d_9$ -Isovaleryl	4.59	302	311	40	20	0.020
C <sub>5</sub>	3	2-Methylbutyryl	$d_9$ -Isovaleryl	4.86	302	311	40	20	0.020
C <sub>5</sub>	3	Isovaleryl	$d_9$ -Isovaleryl	5.05	302	311	40	20	0.020
C <sub>8</sub> OH	3	3-Hydroxyoctanoyl	$d_9$ -Isovaleryl	5.12	360	311	40	20	0.020
C <sub>3</sub> DC	4	Malonyl	$d_3$ -Hexanoyl	5.89	360	319	100	25	0.020
C <sub>4</sub> DC	4	Succinyl	$d_3$ -Hexanoyl	6.23	374	319	100	25	0.020
C <sub>4</sub> DC	4	Methylmalonyl	$d_3$ -Hexanoyl	6.35	374	319	100	25	0.020
C <sub>5</sub> DC	4	Glutaryl	$d_6$ -Glutaryl	6.41	388	319	100	25	0.020
C <sub>6</sub> DC	4	Adipyl	$d_3$ -Hexanoyl	6.64	402	319	100	25	0.020
C <sub>10</sub> OH	4	3-Hydroxydecanoyl	$d_3$ -Hexanoyl	6.92	388	319	100	25	0.020
C <sub>6</sub>	5	Hexanoyl	$d_3$ -Hexanoyl	6.32	316	319	40	20	0.020
C <sub>8:1</sub>	6	Octenoyl	$d_3$ -Octanoyl	6.66	342	347	100	25	0.020
C <sub>8</sub>	6	Valproyl	$d_3$ -Octanoyl	6.86	344	347	100	25	0.020
C <sub>8</sub>	6	Octanoyl	$d_3$ -Octanoyl	7.05	344	347	100	25	0.020
C <sub>10:2</sub>	7	Decadienoyl	$d_3$ -Decanoyl	7.03	368	375	100	25	0.020
C <sub>10:1</sub>	7	Decenoyl	$d_3$ -Decanoyl	7.37	370	375	100	25	0.020
C <sub>10</sub>	7	Decanoyl	$d_3$ -Decanoyl	7.64	372	375	100	25	0.020
C <sub>12</sub> OH	7	3-Hydroxydodecanoyl	$d_3$ -octanoyl	7.53	416	347	100	25	0.020
C <sub>8</sub> DC	8	Subaryl	$d_3$ -Decanoyl	7.09	430	375	120	25	0.020
C <sub>14</sub> OH	8	3-Hydroxymyristoyl	$d_3$ -Myristoyl	8.30	444	431	120	25	0.020
C <sub>10</sub> DC	8	Sebacyl	$d_3$ -Decanoyl	7.45	458	375	120	25	0.020
C <sub>12:1</sub>	9	Dodecenoyl	$d_3$ -Dodecanoyl	7.92	398	403	120	25	0.020
C <sub>12</sub>	9	Dodecanoyl	$d_3$ -Dodecanoyl	8.32	400	403	120	25	0.020
C <sub>16:1</sub> OH	9	3-Hydroxypalmitoleyl	$d_3$ -Palmitoyl	8.49	470	459	120	25	0.020
C <sub>14:2</sub>	10	Tetradecanedi-enoyl	$d_3$ -Myristoyl	8.15	424	431	120	25	0.020
C <sub>14:1</sub>	10	Tetradecenoyl	$d_3$ -Myristoyl	8.59	426	431	120	25	0.020
C <sub>14</sub>	10	Myristoyl	$d_3$ -Myristoyl	9.06	428	431	120	25	0.020
C <sub>16</sub> OH	10	3-Hydroxypalmitoyl	$d_3$ -Myristoyl	8.85	472	431	120	25	0.020
C <sub>18:2</sub> OH	11	3-Hydroxylinoleyl	$d_3$ -Stearoyl	8.25	496	487	120	25	0.020
C <sub>18:1</sub> OH	11	3-Hydroxyoleyl	$d_3$ -Stearoyl	9.11	498	487	120	25	0.020
C <sub>18</sub> OH	11	3-Hydroxystearoyl	$d_3$ -Stearoyl	9.66	500	487	120	25	0.020
C <sub>16:1</sub>	12	Palmitoleyl	$d_3$ -Palmitoyl	9.17	454	459	120	25	0.020
C <sub>16</sub>	12	Palmitoyl	$d_3$ -Palmitoyl	9.74	456	459	120	25	0.020
C <sub>18:2</sub>	13	Linoleyl	$d_3$ -Stearoyl	9.34	480	487	120	25	0.020
C <sub>18:1</sub>	13	Oleyl	$d_3$ -Stearoyl	9.84	482	487	120	25	0.020
C <sub>18</sub>	13	Stearoyl	$d_3$ -Stearoyl	10.47	484	487	120	25	0.020
C <sub>20</sub>	13	Arachidoyl	$d_3$ -Stearoyl	11.17	512	487	120	25	0.020

IS internal standard, NI not identified (the 2-methyl-3-hydroxybutyrylcarnitine peak was not verified)



(acetonitrile with 0.1 % TFA, v/v). The gradient began with 99 % solution A, then was programmed as follows: 0–0.1 min, gradient to 83 % solution A; 0.1–0.28 min, gradient to 76 % solution A; 0.28–0.35 min, gradient to 74 % solution A; 0.35–0.80 min, gradient to 71 % solution A; 0.80–1.71 min, gradient to 69 % solution A; 1.71–2.96 min, gradient to 66 % solution A; 2.96–4.50 min, gradient to 64 % solution A; 4.50–5.44 min, gradient to 44 % solution A; 5.44–6.37 min, gradient to 30 % solution A; 6.37–8.01 min, gradient to 18 % solution A; 8.01–11.30 min, gradient to 7 % solution A; 11.30–12.50 min, gradient to 5 % solution A; 12.50–13.50 min, gradient back to 99 % solution A; 13.50–15 min, hold at 99 % solution A to reequilibrate the column. The total run time was 15 min. The column temperature was 50 °C and the sample temperature was 5 °C. Flow to the mass spectrometer source was reduced by splitting 1:10 with a split tee positioned after the column in order to optimize ionization. Fig. 1 shows a representative total ion chromatogram of mixed standard solution. The chromatograms for different MRM functions, for a newborn DBS specimen, are shown in Fig. S1.

#### Statistical analysis

For statistical analysis, Prism 4.0 (GraphPad Software), STATISTICA 6.0 (StatSoft), and SPSS 15 (SPSS, Chicago, IL, USA) were used. Significance was defined as  $p < 0.05$ .

## Results and discussion

#### Validation studies

##### Linearity

For the determination of linearity in DBS and plasma, blood from a healthy volunteer was spiked with unlabeled acylcarnitines to achieve the final concentration ranges reported in Table S1. For regression analysis, the ratio of the analyte signal versus the internal standard signal was plotted against the analyte concentration. The mean slope,  $y$ -intercept, standard deviation (SD) of residuals, and coefficient of regression ( $r^2$ ) are reported in Table S1. Correlation coefficients ( $r^2$ ) of the calibration curves ranged from 0.990 to 0.999.

The limit of detection (LOD) was determined by a signal-to-noise ratio of 10:1 and the limit of quantification (LOQ) was estimated from the concentration of analyte that can be measured with an imprecision lower than 20 % and a deviation from the target below 20 %. Water and the sample (DBS or plasma) were spiked with decreasing concentrations of analytes to obtain the LOD and LOQ, respectively. The LOD ranged from 0.002 and 0.063  $\mu\text{M}$  for all compounds,

and the LOQ ranged from 0.004 and 0.357  $\mu\text{M}$ . Detailed values are also given in Table S1.

##### Precision, accuracy, and recovery

Interassay and intra-assay precision were assessed over a period of 20 days by measuring DBS controls obtained from ChromSystems Instruments & Chemicals (quality control level 1 and quality control level 2) and two internal quality controls (quality control plasma 1 and quality control plasma 2) at two different concentrations of acylcarnitines. The within-run precision was determined by injection of a duplicate of the same sample in the same analytical session for two different concentrations for each metabolite. The within-day precision was obtained by measuring five replicates on the same day of a duplicate of the same specimen for two different concentrations of each metabolite. The between-day precision on 20 consecutive days was also determined for all metabolites at the same concentrations. The precision ranged from 2.7 to 8.8 % for DBS and from 2.9 to 15.2 % for plasma. All data are reported as the mean, SD, and percent coefficient of variation in Table S2.

Accuracy for DBS was estimated by comparison of the measured concentration ( $n=80$ ) in quality control material with expected values given by ChromSystems Instruments & Chemicals. Also, quality control material and proficiency testing specimens from the Newborn Screening Quality Assurance Program of the Centers for Disease Control and Prevention were analyzed and used as internal quality controls.

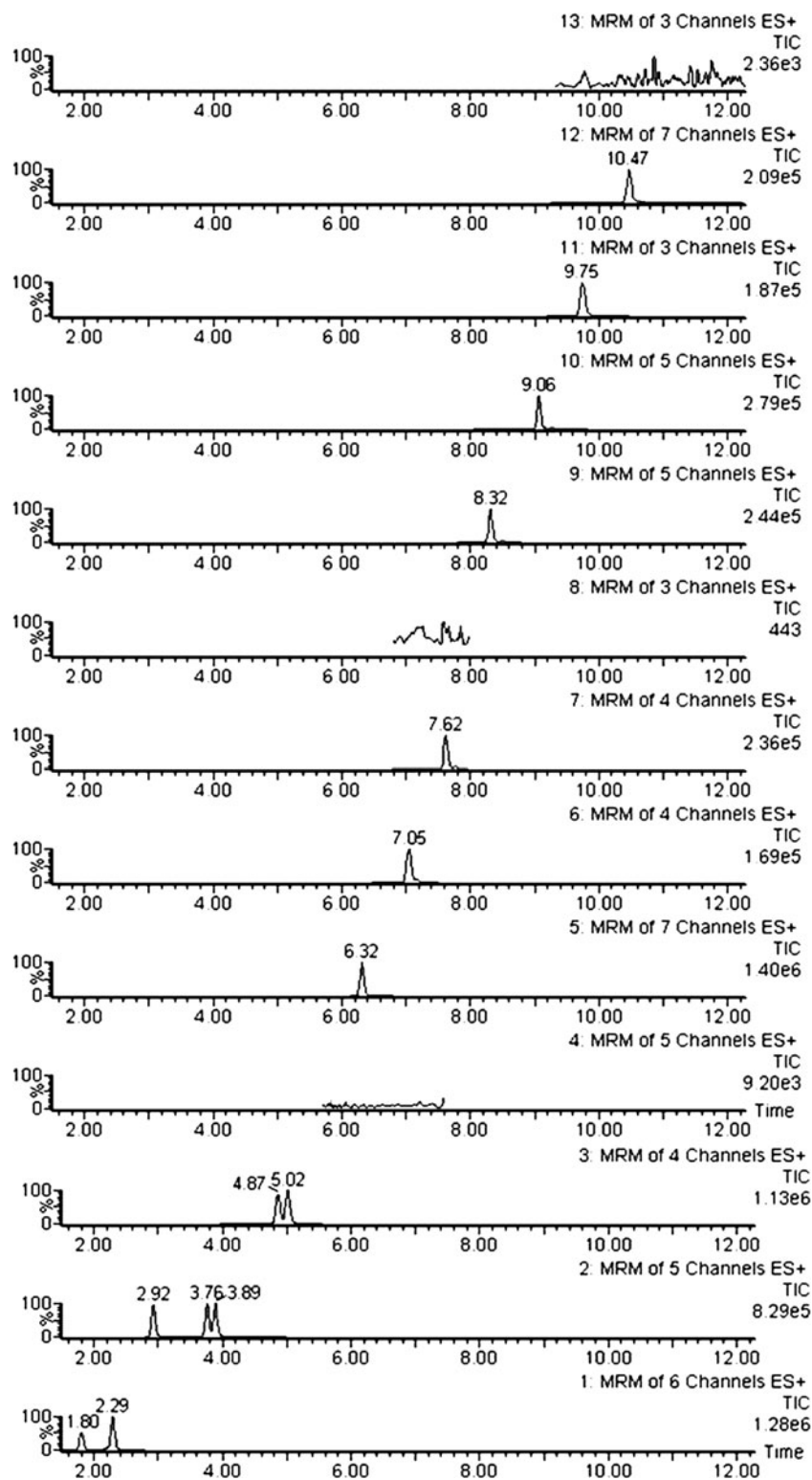
Since reference material for plasma is not available, accuracy for plasma samples was not estimated by referring to certified values. Accuracy for plasma was calculated by comparison of the measured levels of the spiked analyte with the expected concentration. Accuracy is reported as the percent bias of (measured value - expected value)/expected value (Table S3). The bias was lower than 20 % for all acylcarnitines in DBS controls. In plasma controls, the bias was lower than 30 %.

Recovery was determined from DBS ( $n=12$ ) and plasma ( $n=20$ ) samples spiked with unlabeled acylcarnitines, and was calculated as the percentage of the difference between the final and the initial concentration divided by the added concentration (Table S3). The mean recovery was 103 % and 102 % for DBS and plasma, respectively.

#### Concentrations of acylcarnitine in DBS and comparison with FIA

We investigated whether our method could be correlated with FIA. Acylcarnitine concentrations in DBS and plasma obtained from UPLC measurements were compared with those obtained using FIA. FIA measurements were performed

**Fig. 1** Total ion chromatograms (TIC) of acylcarnitine mixed standard solution (1  $\mu$ M). No peaks are detected in chromatograms 4, 8, and 13 because corresponding metabolites are not present in the mix. MRM multiple reaction monitoring



on the same derivatized samples, which were introduced into the ESI source of a Quattro Ultima triple-quadrupole instrument by an Alliance HT 2795 separations module (Waters, Manchester, UK). Injections of 10  $\mu$ L per sample were made into the mobile phase (acetonitrile/water/formic acid; 70:30:0.01, v/v/v) and a flow rate of

0.015 mL/min. The analysis time was 1.9 min per injection. The acylcarnitine profile was obtained by a parent scan of the ion at  $m/z$  85.

The concentrations of metabolites measured in DBS and plasma from healthy newborns by FIA and UPLC, expressed as the mean $\pm$ SD, are reported in Table S4.

Similar values were obtained in DBS measurements, with agreement between the average values confirmed by a bias lower than 20 % and always within two SD of the mean. Bland–Altman and  $x$ - $y$  plots were generated and the data showed a good fit ( $r^2$  ranged from 0.86 to 0.95) for free carnitine, C<sub>2</sub>, C<sub>3</sub>, C<sub>12</sub>, C<sub>14</sub>, C<sub>16</sub>, and C<sub>18</sub>. The values for C<sub>4</sub> and C<sub>5</sub> obtained by FIA were higher, as expected, owing to the contribution of isomers, isobutyrylcarnitine and 2-methylbutyryl carnitine, respectively. The concentrations of C<sub>6</sub>, and C<sub>8</sub> acylcarnitines were also higher in FIA measurements, probably owing to background signal. The FIA and UPLC values obtained for all acylcarnitines in DBS of healthy newborns are plotted in Fig. 2.

Comparison of the values obtained by FIA and UPLC analysis from plasma specimens showed that only C<sub>2</sub> data were well correlated, whereas data for the other acylcarnitines were slightly or not correlated, and if correlation was present, the  $r^2$  values obtained were low or very low (ranging from 0.8 to 0.1). FIA values were generally higher than UPLC values, denoting a background signal probably due to a matrix effect. The FIA and UPLC values obtained for all acylcarnitines in plasma of healthy newborns are reported in Fig. 3.

#### Separation of acylcarnitine isomers

DBS and plasma of patients with confirmed disease were examined to obtain isomer profiles to distinguish

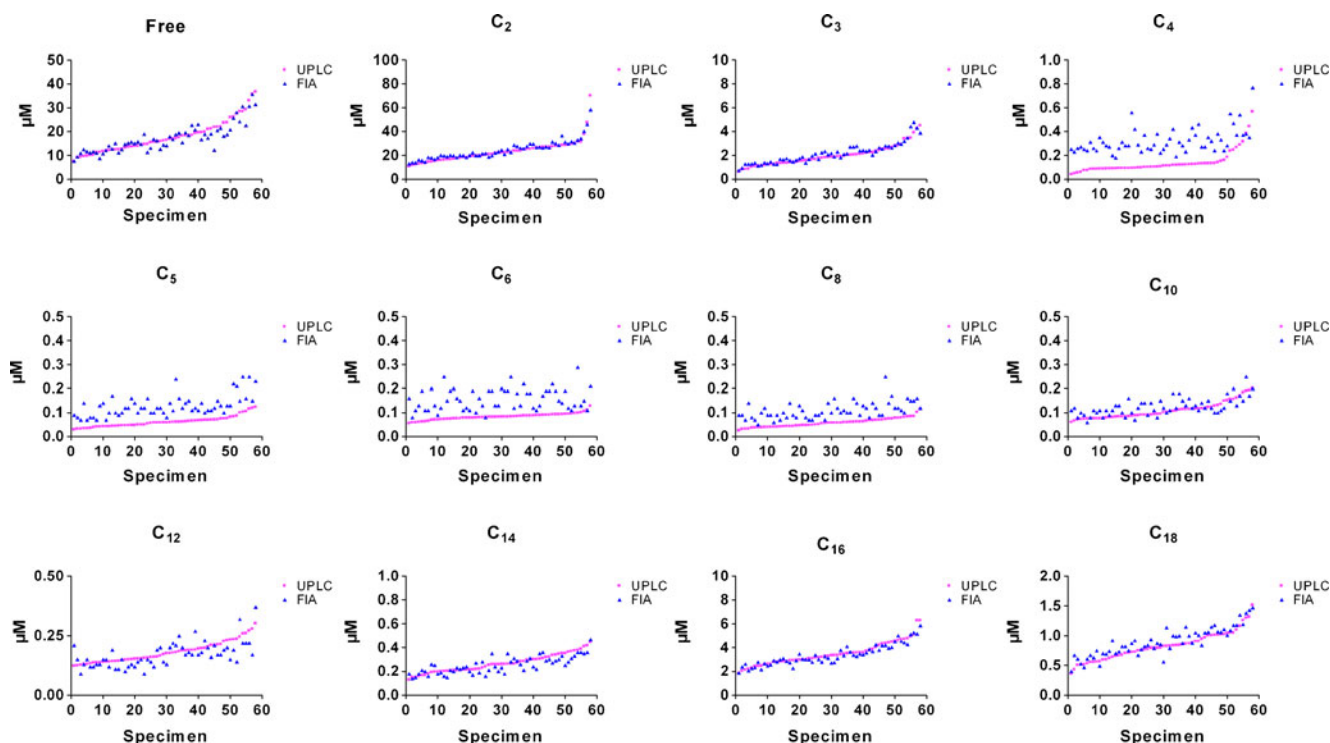
different types of disease. The identification of metabolites was confirmed against a standard. No interfering compound corresponding to metabolite masses was observed.

Figure 4 shows MRM chromatograms for separation of C<sub>4</sub> isomers in DBS and plasma of a healthy newborn, in DBS of a patient with SCAD deficiency, and a corresponding labeled internal standard. The good separation provides rapid discrimination of SCAD deficiency from isobutyryl-CoA dehydrogenase deficiency.

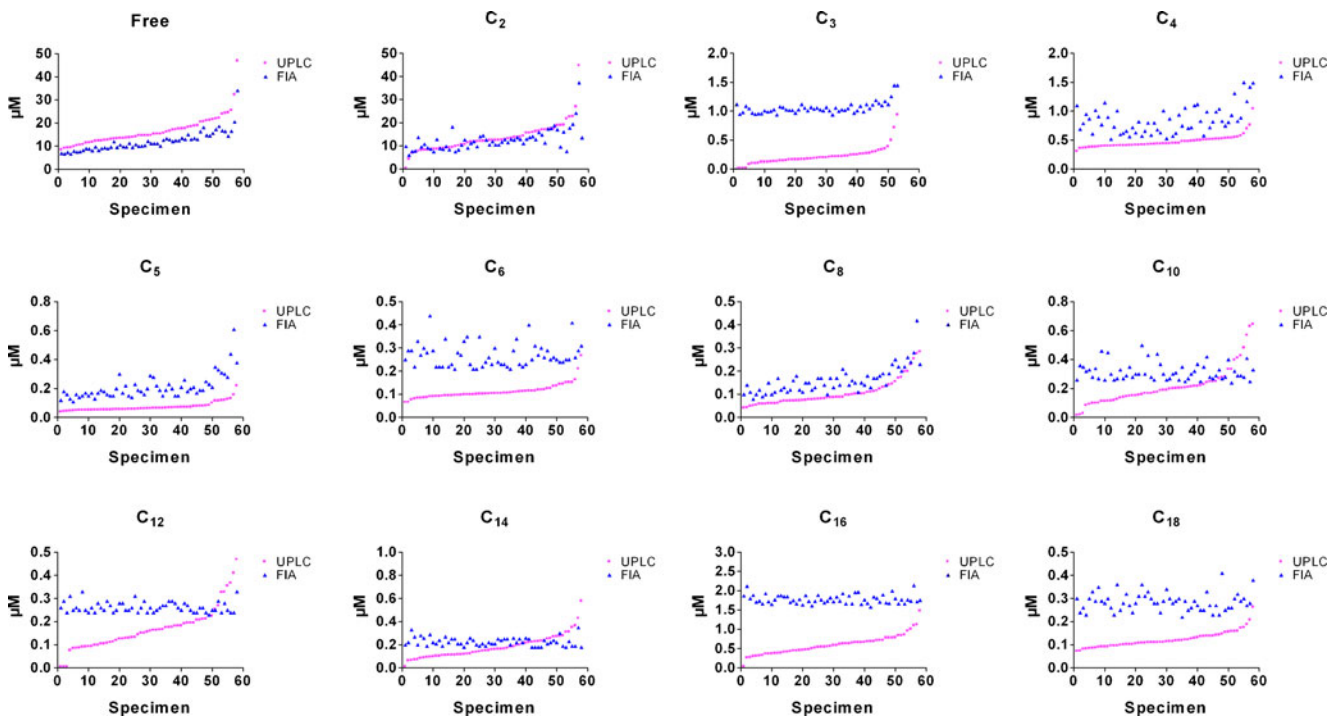
The appearance of isomers was different in DBS and plasma of the healthy newborn: the isobutyrylcarnitine to butyrylcarnitine ratio was greater than 1 (mean 1.4) in DBS, whereas in plasma it was less than 1 (mean 0.9).

Isomeric forms of C<sub>4</sub>DC are significant for discrimination of MMA from succinyl-CoA synthase deficiency [12]. Figure 5 shows succinylcarnitine and methylmalonylcarnitine separation (retention times 6.23 and 6.35 min) in DBS and plasma of a healthy newborn, in DBS of a patient with MMA, and in DBS of a patient with succinyl-CoA synthase deficiency.

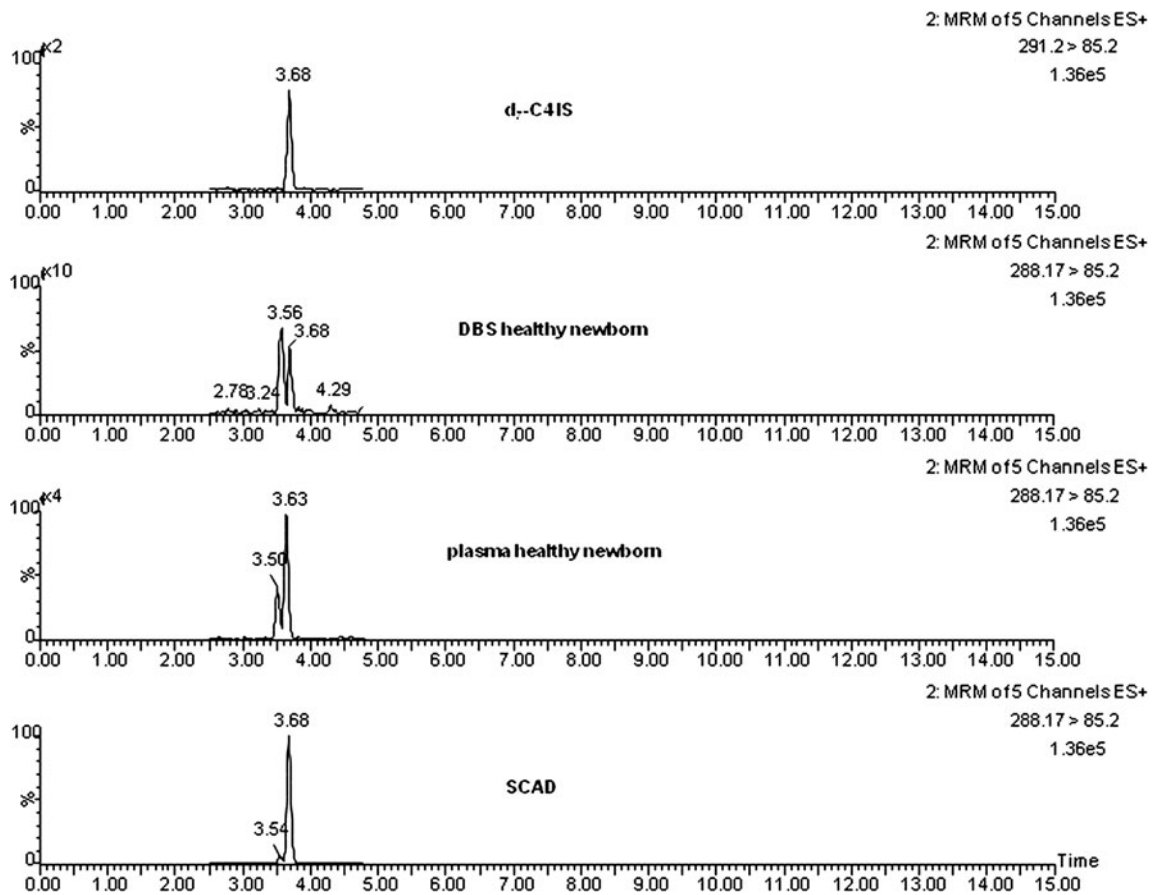
C<sub>5</sub> isomers—pivaloylcarnitine (retention time 4.59 min), 2-methylbutyrylcarnitine (retention time 4.86 min), and isovalerylcarnitine (retention time 5.05 min)—are well separated, as shown in Fig. 6. The MRM chromatogram of  $m/z$  311 shows the labeled internal standard of C<sub>5</sub>, whereas  $m/z$  302.2 represents 2-methylbutyrylcarnitine and isovalerylcarnitine in DBS and plasma of a healthy newborn, in a patient with isovaleric acidemia, and in a patient with



**Fig. 2** Flow injection analysis (FIA) and ultraperformance liquid chromatography (UPLC) values obtained for acylcarnitines in dried blood spots (DBS) of healthy newborns, expressed as micromoles per liter of blood, sorted for UPLC values in ascending order

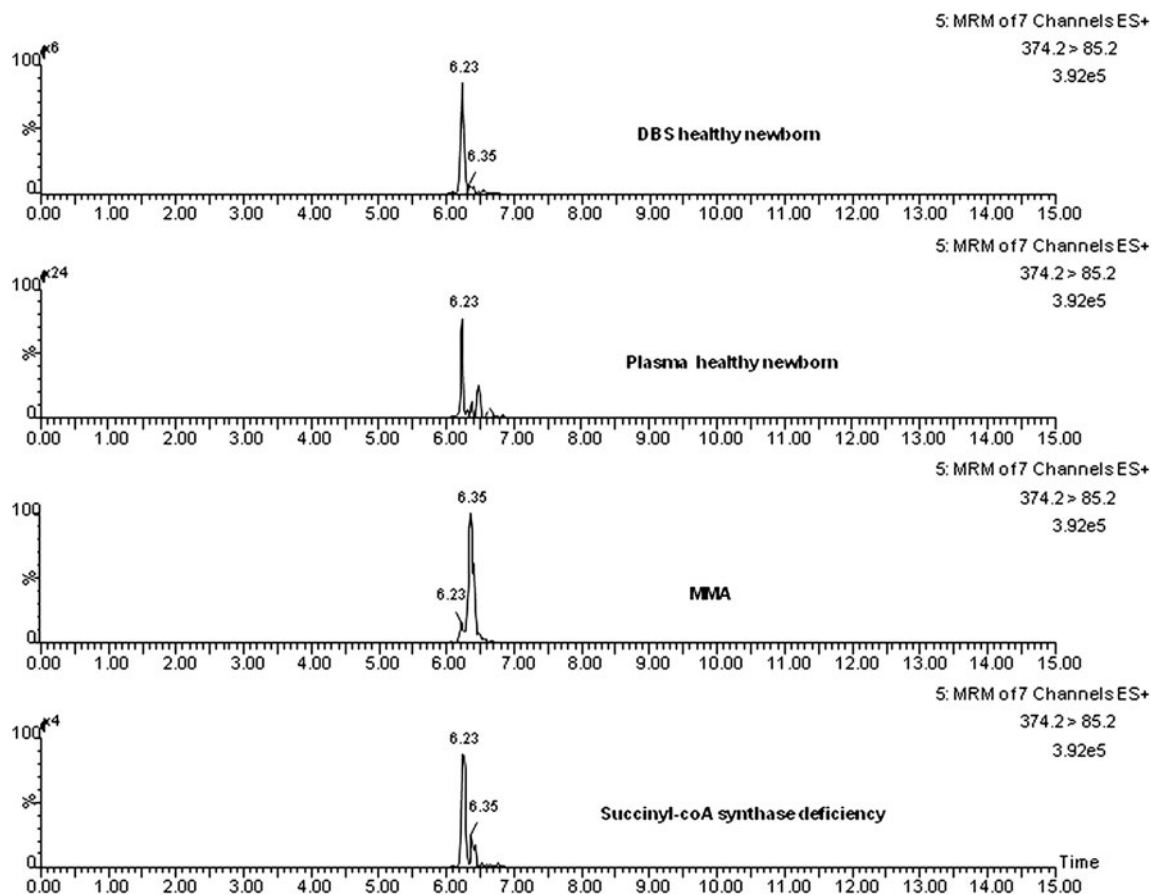


**Fig. 3** FIA and UPLC values obtained for acylcarnitines in plasma of healthy newborns, expressed as micromoles per liter of plasma, sorted for UPLC values in ascending order



**Fig. 4** Representative chromatograms of  $C_4$  acylcarnitine isomers obtained from healthy newborns and a patient with short-chain acyl-CoA dehydrogenase (*SCAD*) deficiency. *IS* internal standard





**Fig. 5** Representative chromatograms of  $C_4DC$  acylcarnitine isomers in DBS and plasma of healthy newborns and affected patients. *MMA* methylmalonic aciduria

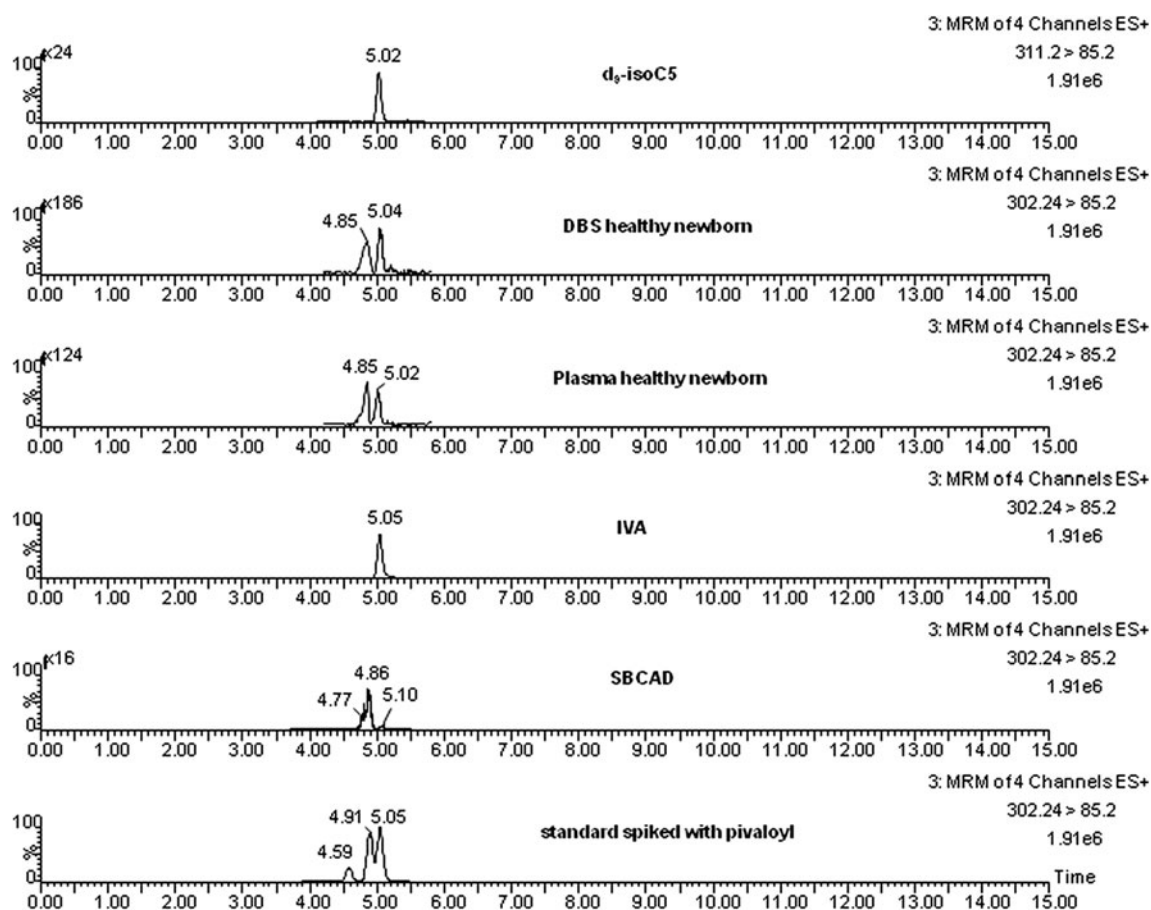
SBCAD deficiency. 2-Methylbutyrylcarnitine in the last patient is present as two unresolved peaks corresponding to *R* and *S* isomers [10]. The chromatogram at the bottom of Fig. 6 is for a standard solution containing  $C_5$  isomers spiked with pivaloylcarnitine. Isomeric separation of  $C_5$  isomers was useful in our laboratory for early identification in a case of suspected newborn DBS screening.

Acquired data are routinely compared with cutoff values by software. If an analyte crosses the cutoff value, a flag automatically appears. Following the standard procedure, the sample DBS sample is analyzed. A sample is classified as true-positive only if the first and second test results are positive. Then another DBS specimen is obtained from the infant. Here we report a case of a borderline result, identified early by UPLC analysis. The FIA spectrum showed a small increase ( $0.41 \mu\text{M}$ ) of the peak at  $m/z$  302 (cutoff  $0.40 \mu\text{M}$ ); the  $C_5/C_2$  ratio was 0.04 (cutoff 0.03), but the  $C_5/C_3$  ratio was below the cutoff limit. To exclude a false-positive result, with a concentration so close to the cutoff, UPLC analysis was performed and showed the presence of an abnormal quantity of 2-methylbutyrylcarnitine for a healthy newborn. Then a definitive diagnosis of SBCAD deficiency was made. The DBS spectrum and the chromatographic separation of the peak at  $m/z$  302 are reported in Fig. S2.

Moreover, discrimination between  $C_5DC$  and  $C_{10}OH$ , which share the transition  $m/z$  388 to 85, is also possible because of different retention times, 6.41 and 6.82 min, respectively (seen in Fig. S1, function 4, channel 388.2>85.2).

Chromatograms related to other isomeric peaks are reported in the [electronic supplementary material](#). Figure S3 depicts ion chromatograms of  $m/z$  318.2, corresponding to  $C_5OH$  isomers. 3-Hydroxyisovalerylcarnitine present in specimens of plasma and DBS of a healthy newborn, and DBS of a patient with MCC deficiency, a patient with SBCAD deficiency, and a patient with 3-hydroxy-3-methylglutaryl-CoA lyase deficiency is shown. No specimens from a patient with 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency were available; therefore, the peak of 2-methyl-3-hydroxybutyrylcarnitine was not verified. But for the patient with SBCAD deficiency, a shoulder before the peak at 2.60 min was identified at 2.55 min, presumably due to an isomeric form.

Hydroxyacylcarnitines were well identified and separated in DBS, whereas in plasma the peaks are near the LOD. In all samples,  $C_4OH$  is present as two peaks, at 2.12 and 2.19 min (Fig. S4). Other hydroxyacylcarnitines were well identified in three patients with long-chain hydroxyl acyl-CoA



**Fig. 6** Representative chromatograms of  $C_5$  acylcarnitine isomers in DBS and plasma of healthy newborns and affected patients. *IVA* isovaleric acidemia, *SBCAD* short/branched-chain acyl-CoA dehydrogenase

dehydrogenase deficiency, and we found two isomeric forms, *R* and *S*, for each of  $C_{16}OH$ ,  $C_{18:2}OH$ ,  $C_{18:1}OH$ , and  $C_{18}OH$ . Minkler et al. [3] demonstrated the presence of isomeric forms of hydroxyacylcarnitine.

Moreover, in one of these patients (Fig. S5) we found an additional peak at  $m/z$  470, presumed to be a  $C_{16:1}OH$  isomer, and two additional peaks at  $m/z$  496, related to  $C_{18:1}OH$ . Because it was established that the patient had not been treated with cefotaxime at the time of blood sampling, we excluded this peak as being a metabolite of this antibiotic [13]. The identification of these extra peaks and correlation with diagnosis are in progress.

The chromatogram in Fig. S6 shows peak separation of  $C_8$  acylcarnitine from valproylcarnitine, which can be produced after treatments with the anticonvulsant drug valproic acid, at 7.05 and 6.86 min, respectively.

## Conclusions

We have described the development of a rapid method for identification and quantification of a large number of

acylcarnitines (48 metabolites and 13 labeled internal standards), including hydroxyacylcarnitines and dicarboxylic acylcarnitines in DBS and plasma. The use of UPLC–ESI-MS/MS enabled us to separate and quantify all these acylcarnitines in a 15-min chromatographic run. Analysis can be performed with a very small sample volume (one punch of DBS or 6  $\mu$ L of plasma) and directly on derivatized samples used for newborn screening.

The method is capable of high precision and accuracy, and the separation of isomers makes it clinically suitable for accurate diagnoses as a second-tier test for newborn screening of metabolic diseases.

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