Journal of Chromatography B, 903 (2012) 142–149

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A quantitative method for acylcarnitines and amino acids using high resolution chromatography and tandem mass spectrometry in newborn screening dried blood spot analysis

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ARTICLE INFO

Article history:
Received 12 April 2012
Accepted 11 July 2012
Available online 20 July 2012

Keywords:
Newborn screening
Second-tier test
Acylcarnitines
Amino acids UHPLC

ABSTRACT

We have developed a high resolution liquid chromatographic separation with electrospray ionization (ESI) mass spectrometry detection for the combined analysis of twelve acylcarnitines and seven amino acids commonly measured in newborn screening heritable metabolic disorders. Samples were prepared by punching 3.2 mm disks out of dried blood spots and extracting with a mixture of methanol and 0.1% formic acid containing stable isotopically labeled internal standards. Analysis was performed on an UHPLC system using a HILIC amide, 2.1 mm × 50 mm, 1.7 μm column. A normal phase gradient, employing 10 mM ammonium acetate in 90:10 acetonitrile/water for mobile phase B and 0.1% formic acid in water for mobile phase A, was used. Optimized multiple reaction monitoring (MRM) was used for detection of amino acids and acylcarnitines on a Waters Premier mass spectrometer. Quantification of analytes was performed using internal calibration by fortification of sodium heparin whole blood with analytes at appropriate levels to encompass the range around the reported cut-off values. The method was fully validated with respect to precision, accuracy, recovery, linearity, matrix suppression and extraction robustness. Precision and accuracy were evaluated over 3 days and determined to be acceptable with an overall precision within 10% and accuracy within 15% of theoretical for all analytes except for acetylcarnitne at one fortified level, which quantitated 21.8% lower than the expected value. This method is suitable as a second-tier test for newborn screening of specific disorders associated with abnormal levels of acylcarnitines and amino acids, potentially reducing false positive cases and shortening the time to diagnosis.

1. Introduction

Newborn screening is claimed to be "one of the most successful public health programs" [1] ever initiated and originated with Robert Guthrie screening for phenylketonuria (PKU) [1,2]. Since its inception, additional markers have been identified for different disorders and added to the panel of screening tests. However, most of these methods required a separate analysis for each disorder. It was not until a method was developed by researchers at Duke University that multiple heritable metabolic disorders could be identified in a single test [3]. This approach relied on a technique called flow injection in which samples were directly injected onto the mass spectrometer without chromatographic separation. Mass spectra were extracted from the elution plug allowing for identification of metabolic disorders based on elevated levels of specific acylcarnitines. Over the years additional analytes were added to this screening method allowing for early detection of more than 20 different metabolism disorders in a single analysis [4,5]. Although this approach has proven to be successful and provides a rapid screening test for many disorders, the analysis is not fully quantitative [1], and screen positive results often need to be confirmed by a second-tier test [6].

One major disadvantage with this flow injection approach is its inability to differentiate isobaric species associated with different metabolic disorders requiring additional testing by a second method to identify the specific disorder [1,4,7,8]. Chromatographic separation is required to separate these isobars, however, current approaches involve lengthy analysis times [9], use ion pair reagents [10] or require additional instrumentation such as capillary electrophoresis [11]. One paper discusses the chromatographic separation of C4 and C5 isomers, but does not include separation of additional acylcarnitines or amino acids [12]. Another potential problem for a flow injection approach is quantification using a single point response factor with stable isotope labeled internal

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http://dx.doi.org/10.1016/j.jchromb.2012.07.008

standards. Assuming that the responses of the isotopically labeled internal standards are equivalent to their corresponding analyte can lead to inaccuracies in quantification and fluctuations in quantification limits. It has been reported, that the response factors of the analytes and internal standards must be evaluated to accurately quantify analytes [13]. Chace et al. have reported the quantification of leucine can be impacted by unresolved isobaric species that have different response factors [14]. Although this research allowed for the identification of maple syrup urine disease (MSUD), it also shows that response factors can be affected by unresolved isobars. Not previously discussed regarding newborn screening, is the potential for response factors to be affected by matrix effects. Although, stable isotope labeled internal standards reduce errors associated with matrix suppression there are reports that show analyte to internal standard response ratios can be effected [15–17]. In addition, since there is no chromatographic separation employed in the flow injection screening method, ionization can be reduced by the co-elution of matrix related components or by other analytes at higher concentrations [16]. This could result in the misidentification of samples close to the cut-off value, if the matrix suppression reduces or enhances the ionization of an analyte differently than its corresponding internal standard.

The original and still widely used approach for flow injection analysis involves derivatization of acylcarnitines and amino acids with n-butanol and HCl to form butyl esters which enhances the sensitivity of certain analytes [5]. However, the caustic nature of this derivatization is a safety hazard to laboratory personnel and hydrochloric acid is corrosive to laboratory equipment. More recently a method has been developed without derivatization, but this approach may result in lower sensitivity for specific dicarboxylic acid acylcarnitines [13]. Although both of these techniques have been shown to provide acceptable results for screening purposes there is still the potential for a high false positive rate for some disorders [1,7]. This has resulted in the need for implementation of second-tier tests to identify certain disorders [1,7]. The current approach to confirm specific disorders is one second-tier test for each disorder, resulting in multiple second-tier tests that have to be used by newborn screening laboratories and/or physicians. To date there is not a comprehensive second-tier test able to quantify acylcarnitines and amino acids in a single analysis. Common practice for quantitative analysis of acylcarnitines and amino acids profiles requires a second blood sample to be drawn from the infant and sent to an independent laboratory for analysis. This can result in unnecessary parental anxiety for false positive cases and further delaying the time to diagnosis. Therefore, there is a need for a rapid second-tier test that could be used to improve identification of the confounding disorders associated with results obtained by flow injection newborn screening methods.

Typical methods employed by independent laboratories for quantitative analysis of acylcarnitines and amino acids are not usually available within public health newborn screening laboratories. In addition, these methods are not commonly performed together, so samples are analyzed by separate procedures using different instruments. The reported methods for acylcarnitines are not practical for second-tier testing due to long analysis times [9,18,19], poor chromatography [20], or involve alternative instrumentation such as capillary electrophoresis [11]. There are many methods available for amino acid analysis; however, the majority involves derivatization of samples followed by HPLC-UV, FLD, LC/MS or GC/MS [21]. These methods typically have analysis times longer than 10 min and require costly derivatization techniques to enhance sensitivity and retention. One published method for the analysis of amino acids does not require derivatization; however, the analysis time is 50 min [22], which would be unacceptable as a rapid second-tier test. The method described here is a comprehensive method capable of chromatographically separating both acylcarnitines and amino acids in 2.2 min, with a total injection to injection cycle time of 3.1 min, a dramatic improvement over all other previously reported techniques.

The combination of high resolution chromatography and unique selectivity of HILIC stationary phase is ideal for fast chromatographic separation of both amino acids and acylcarnitines without the need for derivatization or ion pair reagents. It has been reported that ultra-high pressure liquid chromatography using smaller particles and higher linear velocities are able to achieve high efficiency separations in faster analysis times compared to conventional HPLC separations [23]. In addition, polar stationary phases such as hydrophilic interaction liquid chromatography (HILIC) are better suited to retain diverse polarity analytes and offer improved sensitivity over reverse phase chromatography [24]. Combining both these techniques, we have developed a complementary method that could be implemented within public health newborn screening laboratories to identify fatty acid oxidation disorders, amino acid metabolism disorders and organic acid disorders. In addition, the chromatographic separation resolves leucine, isoleucine and hydroxyproline allowing for confirmation of MSUD, which is not possible with the flow injection methods. This analysis is performed with the same sample extract used in the non-derivatized screening method to rapidly confirm abnormal screening results. In addition, quantification is based on a full calibration using reference standards prepared in whole blood and dried on newborn screening cards. Alternative conditions were used to resolve butyrylcarnitine (C4) from isobutyrylcarnitine (ISO C4) along with valerylcarnitne (C5) from isolvalerylcarninte (ISO C5) which would allow for differential diagnosis for short chain acyl-coenzyme A dehydrogenase deficiency (SCAD) and isobutyryl-CoA dehydrogenase deficiency (IBCD) or isovaleric academia (IVA). This methodology provides newborn screening laboratories with a complementary rapid second-tier test which is able to confirm multiple metabolism disorders in a single analysis.

2. Materials and methods

2.1. Materials and reagents

Acylcarnitine reference standards (purity > 95%) were purchased from VU Medical Center Metabolic Laboratory, (Amsterdam, The Netherlands) and amino acid reference standards (purity > 96%) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Stable isotope labeled amino acids and acylcarnitines reference materials (purity > 98%) were obtained from Cambridge Isotopes (Andover, MA, USA). Hydroxyproline obtained from ARCOS Organics (Morris Plains, NJ, USA) was used as a system check to confirm its retention time. HPLC grade acetonitrile, formic acid and ammonium formate were from Fisher Scientific (Pittsburgh, PA, USA). Control human sodium heparin whole blood was obtained from Biochemed (Winchester, VA, USA). Newborn screening cards, 903 Protein Saver, were obtained from Whatman (Piscataway, NJ, USA). A 22 Multiple Syringe Pump from Harvard Apparatus (Holliston, MA, USA) and a 250 μL Hamilton gas tight syringe from Fisher Scientific (Pittsburgh, PA, USA) was used for infusion tests.

2.2. Chromatographic instrumentation

Chromatographic separations were conducted with a Waters Acquity HILIC amide column (2.1 mm × 50 mm, 1.7 μm) Waters Corp., (Milford, MA, USA). Separation of C4 and C5 isomers was achieved by placing a Supelco Astec CYCLOBOND™ I 2000 Chiral HPLC guard column, 1.0 mm × 20 mm, 5 μm, from Sigma–Aldrich (Milwaukee WI, USA) prior to the HILIC amide column. A Waters Acquity UPLC pump and sample manager were used for analysis

Table 1
Acylcarnitines and amino acids analyzed and the mass spectral settings for MRM mode.

Analyte	ID	CV (V)	CE (eV)	MRM transition	I.S.	I.S. MRM transition	Time segment (min)
Carnitine	C0	22	20	161.9 → 84.6	C0-D9	170.9 → 84.6	1.73–2.03
Acetylcarnitine	C2	22	20	203.9 → 84.6	C2-D3	206.9 → 84.6	1.57–1.87
Propionylcarnitine	C3	22	20	217.9 → 84.6	C3-D3	220.9 → 84.6	1.43–1.73
Butyrylcarnitine	C4	22	21	231.9 → 84.6	C4-D3	235.0 → 84.6	1.31–1.61
Valerylcarnitine	C5	22	23	246.0 → 84.6	C5-D9	255.1 → 84.6	1.21–1.51
Hexanoylcarnitine	C6	22	23	260.0 → 84.6	C8-D3	291.1 → 84.6	1.10–1.40
Octanoylcarnitine	C8	22	23	288.1 → 84.6	C8-D3	291.1 → 84.6	0.97–1.27
Decanoylcarnitine	C10	22	28	316.1 → 84.6	C8-D3	291.1 → 84.6	0.80–1.10
Lauroylcarnitine	C12	22	28	344.1 → 84.6	C14-D9	381.2 → 84.6	0.63–1.01
Myristoylcarnitine	C14	22	28	372.1 → 84.6	C14-D9	381.2 → 84.6	0.63–1.01
Palmitoylcarnitine	C16	22	28	400.2 → 84.6	C16-D3	403.2 → 84.6	0.00–0.95
Stearoylcarnitine	C18	22	28	428.2 → 84.6	C16-D3	403.2 → 84.6	0.00–0.95
Phenylalanine	Phe	20	15	165.8 → 119.7	Phe-D6	171.8 → 125.7	1.37–1.67
Leucine/Isoleucine	Leu/Ile	18	11	131.8 → 85.7	Leu-D3	134.85 → 88.7	1.41–2.03
Methionine	Met	14	11	149.8 → 103.6	Met-D3	152.85 → 106.6	1.50–1.80
Tyrosine	Tyr	15	14	181.8 → 135.8	Tyr-D6	187.8 → 141.8	1.54–1.84
Valine	Val	20	8	117.8 → 71.8	Val-D8	125.8 → 79.7	1.60–1.90
Citrulline	Cit	16	10	175.8 → 158.7	Cit-D2	177.8 → 160.7	1.88–2.18

and gradient separation. A Waters Premier triple quadrupole mass spectrometer (Milford, MA, USA) equipped with electrospray ionization (ESI) source in positive ion mode was used. Optimization of the instrument parameters including desolvation temperature, source temperature, desolvation gas flow, cone gas flow, capillary voltage, cone voltage and collision energy was performed by direct infusion of a solution containing acylcarnitines, amino acids and their labeled internal standards. Optimal sensitivity was achieved using 3.8 kV for the capillary, 390 °C desolvation temperature with nitrogen as the desolvation gas at a flow of 800 L/h. Cone gas was nitrogen at a flow of 20 L/h with a source temperature of 110 °C. Argon was used for the collision gas at a flow of 0.4 L/h. Multiple reaction monitoring (MRM) was used for all analytes and internal standard, transitions shown in Table 1. Optimized MRMs were used based on the retention time of analytes to achieve a minimum 0.4 min window around each peak with a 0.02 s dwell time and a 0.02 s inter-scan delay.

2.3. Preparation of dried blood spots

For the validation portion of this study, sodium heparin human whole blood was fortified at 6 levels for a calibration curve and at 4 additional levels for quality control samples. Individually prepared reference standards containing acylcarnitines and amino acid were spiked into whole blood to fortify samples at the desired concentration to prepare the highest calibration standard and quality control sample. These samples were then serially diluted to prepare the additional calibration levels and quality control samples. Each dried blood spot was prepared by spotting 50 μL of sample onto Whatman, 903 Protein Saver newborn screening card. These samples were allowed to dry for at least 2 h at room temperature and then stored at 0–5 °C until analyzed.

3. Results and discussion

3.1. Response factor evaluation

The typical method used to calculate the concentration of analytes in a sample is determined based on Eq. (1) where the response factor is often not calculated and assumed to be equal to one. However, this assumption can lead to errors in calculated concentrations if the response factors deviate from 1.0. We evaluated the response factors for all analytes using both external solutions and extracted samples to determine if sample matrix had a significant effect on the response factors. The analyte response factors were initially determined for each analyte by flow injection analysis without derivatization using external solutions containing both analytes and internal standards. External solutions were prepared such that the final concentrations of the analytes and internal standards were equivalent. Serial dilutions were made to achieve five-point response curves with concentrations above and below target concentration of internal standard in the final extract. For flow injection analysis, 3 replicate injections of an external solution containing analytes and internal standards were injected onto the LC/MS/MS system and the response from 50 scans were averaged, which is the maximum number of scans across the center of the elution plug. The responses of each analyte and their respective isotopically labeled internal standard were evaluated. Data for carnitine and acetylcarnitine were plotted to show the relationship between response and concentration, supplementary data 1A and 1B, respectively. Eq. (2) was used to calculate the response factors for each analyte and its isotopically labeled internal standard at each concentration. The response factors for all the analytes were plotted versus concentration to evaluate if the response factors were consistent across the concentration range. Supplementary data section contains example plots for both carnitine and acetylcarnitine 2A and 2B, respectively. The average response factors were calculated by averaging the response factor across all concentrations and results are reported in Table 2.

Conventional calculation for concentration for analytes extracted from dried blood spots (Eq. (1)):

$$\text{Analyte conc.} = \frac{\text{Analytical Response}}{\text{I.S. Response}} \times \text{Conc. I.S.} \times \text{Response Factor} \times \frac{\text{Extraction Volume}}{\text{Sample Volume}} \quad (1)$$

Response factor determination for external solutions (Eq. (2)):

$$\text{Response Factor} = \frac{\text{Internal Standard Response}}{\text{Analyte Response}} \quad (2)$$

The response factors in extracted dried blood spot samples were evaluated using the flow injection analysis technique described above. Internal standard stock solutions were prepared in the appropriate solvent identified by Cambridge Isotopes and diluted 200-fold in the extraction solvent to achieve the appropriate concentration used in the established newborn screening method. Three replicates of dried blood spot samples from quality control samples were extracted in 100 μL of methanol with 0.1% formic acid containing labeled internal standards. These samples were

Table 2
Evaluation of response factors for externals and extracted samples.

Analyte	External response factor	S.D.	%RSD	*p*-Value	Extracted response factor	S.D.	%RSD	*p*-Value
C0	1.26	0.0238	1.89%	<0.05	1.09	0.0649	5.94%	<0.05
C2	0.642	0.0151	2.35%	<0.05	1.53	0.137	8.95%	<0.05
C3	0.792	0.0530	6.69%	<0.05	1.26	0.148	11.7%	<0.05
C4	0.906	0.0268	2.96%	<0.05	1.31	0.0616	4.70%	<0.05
C5	1.13	0.0344	3.05%	<0.05	1.27	0.0851	6.70%	<0.05
C6	1.19	0.0361	3.02%	<0.05	1.13	0.0772	6.83%	<0.05
C8	0.905	0.0267	2.95%	<0.05	0.931	0.120	12.9%	0.249
C10	0.728	0.0283	3.89%	<0.05	0.713	0.119	16.7%	<0.05
C12	0.963	0.0287	2.98%	<0.05	0.883	0.129	14.6%	<0.05
C14	0.897	0.0251	2.80%	<0.05	0.829	0.117	14.1%	<0.05
C16	1.01	0.0299	2.95%	0.0742	0.976	0.114	11.7%	0.315
C18	1.10	0.0471	4.26%	<0.05	1.16	0.141	12.1%	<0.05
Phe	0.931	0.00694	0.746%	<0.05	1.29	0.0841	6.50%	<0.05
Leu/Ile	0.932	0.00461	0.495%	<0.05	1.22	0.105	8.62%	<0.05
Tyr	1.16	0.0626	5.42%	<0.05	1.82	0.120	6.60%	<0.05
Val	1.15	0.0249	2.16%	<0.05	1.38	0.0893	6.46%	<0.05
Cit	1.09	0.0282	2.58%	<0.05	1.67	0.0503	3.00%	<0.05
Met	1.08	0.0465	4.32%	<0.05	1.43	0.0214	1.49%	<0.05

Three replicates at 5 levels were analyzed ($n = 15$) for externals. Three replicates at 4 levels were analyzed ($n = 12$) for extracted samples. *p*-Value was used to test if the mean is different than 1.0 using JMP 9.0.0 software.

analyzed by flow injection analysis for acylcarnitines and amino acids. The calculated concentration was determined using the response ratios with the appropriate labeled internal standard and then multiplying by the concentration of the internal standard and the dilution factor as shown in Eq. (1). The actual concentration of analytes in the samples was determined based on back-calculated concentrations using a calibration curve as described earlier. Using Eq. (3), the response factors were determined for each analyte and results are reported in Table 2.

Response factor determination for extracted dried blood spot samples (Eq. (3)):

$$\text{Response Factor} = \frac{\text{Actual Concentration}}{\text{Calculated Concentration}} \quad (3)$$

From our results, it is apparent that the response factors for external standards are different than extracted samples, indicating that matrix does affect the response factors. In addition, the response factors for most analytes and internal standards are significantly different from one using a two tailed *t*-test ($p < 0.05$). Overall, the response factors for external samples appear to be very consistent with %RSDs less than 6.69%; however, response factors for extracted samples are much more inconsistent with %RSDs as high as 16.7% for some analytes. Since the responses are not equal for analytes and internal standards, concentrations of analytes would not be proportional to the internal standard at the same concentration, thus resulting in values that are lower or higher than the actual concentration in the sample. Further, if the slope of the analyte response versus concentration is significantly steeper than that of the internal standard, then small changes in analyte concentration may result in relatively large quantitation errors. Therefore, when samples are analyzed by flow injection analysis using only response factors, there is a deviation from the actual value when analyte response factors are different from their internal standard. For instance, the response factor for C5 was determined to be 1.27 so the calculated concentration is 27% lower than the actual concentration. This indicates that using the concentration of the internal standard has the potential to inaccurately quantify analytes when the response factors are significantly different from one.

3.2. Direct analysis ionization suppression evaluation

One major problem reported with electrospray ionization is matrix related ionization suppression [16,25]. As shown previously, the response factors for external samples are different from extracted samples, which is a result of competing ionization between the analytes and other components found in the extracted sample. When analyzing samples by flow injection analysis there is no separation between matrix components and the analytes. In addition analytes at higher concentrations compete for ionization with other analytes reducing their response which could potentially cause an analyte to be undetected. Using labeled internal standards does reduce problems with quantification related to matrix effects; however, sensitivity can be dramatically affected [16].

In order to evaluate ionization suppression or enhancement, a post-injection infusion experiment for acylcarnitines and amino acids was conducted using a standard technique [16,25]. A syringe pump was used to continuously infuse (20 μL/min) of an external solution containing acylcarnitines and amino acids labeled internal standards, at a 200-fold dilution of the stock solution, in the flow path to the mass spectrometer prior to the electrospray source. This provided a high baseline response for transitions associated with each compound so that when matrix was eluted, ion suppression would appear as a negative response shift in this high baseline, whereas ion enhancement would appear as a positive shift. The time duration of these shifts indicates elution times where ion suppression and enhancement occur. Fortified and unfortified matrix blood spots were evaluated by flow injection analysis while monitoring for shifts in the specific mass transitions for the analytes. In order to quantify the degree of ion suppression, the average internal standard responses were evaluated during the elution plug and compared to the average internal standard response obtained by direct infusion with mobile phase.

A representative unfortified sodium heparin human blood sample analyzed by flow injection without infusion of internal standard solution is shown in Fig. 1a. A representative unfortified sodium heparin human blood sample analyzed by flow injection with infusion of internal standard solution is shown in Fig. 1b. It is apparent from this experiment that there is a dramatic decrease in ionization when the plug of sample is eluted. The overall decrease in ionization was calculated for each analyte at 4 different levels in triplicate and average overall suppression was determined to be 63%. For some analytes the matrix suppression was as high as 78% which could dramatically affect the detectability of analytes by flow injection resulting in potential misidentification of a metabolism disorder. In order to reduce this matrix effect and improve sensitivity, chromatographic separation is necessary.

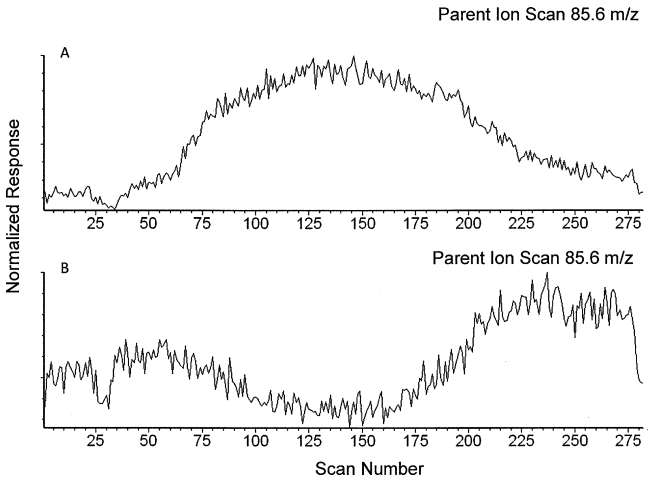

Fig. 1. Post-injection infusion evaluation for flow injection analysis: (A) flow injection analysis of an unfortified sodium heparin blood sample without infusion of internal standards; (B) flow injection analysis of a unfortified sodium heparin blood sample with infusion at 20 μL/min of labeled internal standards solutions at a 200-fold dilution.

3.3. Chromatographic separation of amino acids and acylcarnitines

Preliminary investigations involved optimization of chromatographic conditions to achieve acceptable retention of all analytes in a 3.1 min total injection cycle time. Mobile phase A contained water with 0.1% formic acid and mobile phase B contained a mixture of 90% acetonitrile and 10% water, such that the final buffer concentration was 10 mM ammonium formate. A gradient elution was employed from 96% mobile phase B to 55% mobile phase B over 1.65 min. An Acquity UPLC pump was used to control the mobile phase flow at 0.3 mL/min and mobile phase composition allowing for the gradient elution of analytes. Representative chromatograms of a single injection of an extracted sample using this gradient elution are presented in Fig. 2a and b. Optimized MRMs were determined based on the retention times of the analytes and then allowing for 0.2 min retention time shift in either direction. Acceptable separation of the isomers leucine, isoleucine and hydroxyproline was achieved; however, C4 and C5 isomers were not separated under these conditions.

3.4. Chromatographic separation of C4 and C5 isomers

Additional work was conducted to resolve the C4 and C5 isomers, since we were unsuccessful in achieving separation on the HILIC amide column alone. Several different chiral stationary phases were evaluated to improve this separation; however, the cyclodextrin phases provided the best resolution. Cyclodextrin phases provide unique selectivity in which interaction is based on inclusion complexation [26]. Therefore, smaller analytes with less steric hindrance will form a more stable complex with the cyclodextrin, whereas larger analytes with more steric hindrance will not complex as strongly. Combining the cyclodextrin phase and HILIC amide phase allowed approximately 43% resolution between the C4 and C5 isomers. The same mobile phase was used for this separation with a modified gradient from 0% to 5% mobile phase A over 10 min with a flow rate of 0.3 mL/min. The chromatographic separation of C4 and C5 isomers in an extracted sample that contains butyrylcarnitine, isobutyrylcarnitine, valerylcarnitine and isovalerylcarnitine is shown in Fig. 3. Although baseline resolution was not achieved, the separation described would allow identification and quantification of the analytes. Longer columns could be used to achieve full resolution of these isomers; however, the run

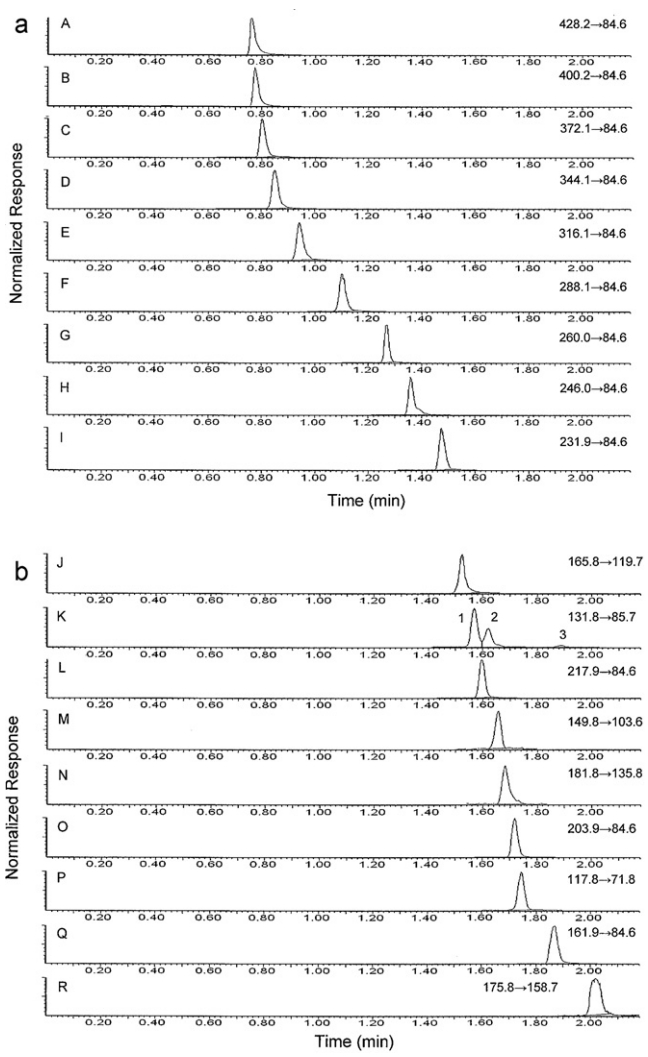

Fig. 2. (a) Chromatographic separation of an extracted lowest level sample using a HILIC amide column with gradient conditions as specified in Section 3.3 and optimized MRM transitions for each analyte. A, C18; B, C16; C, C14; D, C12; E, C10; F, C8; G, C6; H, C5; I, C4. (b) Chromatographic separation of an extracted lowest level sample using a HILIC amide column with gradient conditions as specified in Section 3.3 and optimized MRM transitions for each analyte: J, phenylalanine; K, (1) leucine, (2) isoleucine, (3) hydroxyproline; L, C3; M, methionine; N, tyrosine; O, C2; P, valline; Q, carnitne; R, citrulline.

time would be significantly longer. It was decided to employ the current separation, because a longer analysis time would not be desirable for a rapid second-tier test. Separation of these isomers allows for differential diagnosis for SCAD and IBCD or IVA, which is not possible with flow injection analysis.

3.5. Extraction optimization

An extraction optimization experiment was conducted in which samples were extracted using various concentrations of methanol and water containing 0.1% formic acid. Additional experiments were performed using acetonitrile and water containing 0.1% formic acid, and acetonitrile and water containing 10 mM ammonium acetate. A 3.2 mm spot was punched from a control sample and extracted in 100 μL of each extraction solution. Three replicates were analyzed for each extraction solution evaluated. The samples were injected using the chromatographic conditions described earlier. The response ratios with internal standard were plotted

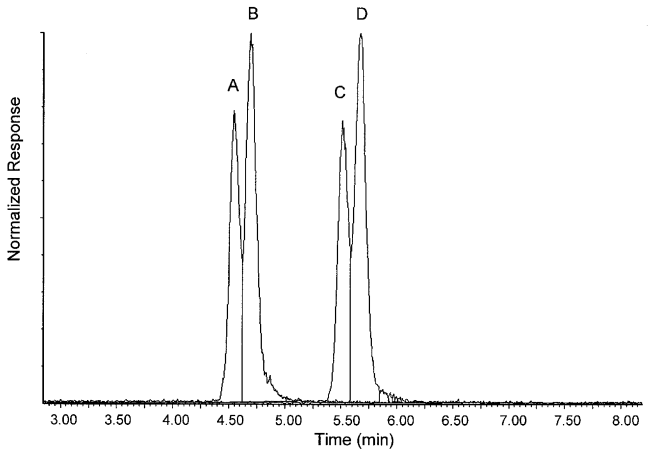

Fig. 3. Chromatographic separation of an extracted sample fortified with (A) valerylcarnitine (C5), (B) isovalerylcarnitine (ISOC5), (C) butylcarnitine (C4) and (D) isobutyrylcarnitine at 1.2 μmol/L, using a modified gradient with a HILIC amide column and Supelco Astec CYCLOBOND™ I 2000 Chiral HPLC guard column as specified in Section 3.4.

Table 3
Calibration range with average correlation coefficients from 3 days.

	Calibration range (μmol/L)	Average correlation coefficient
C0	36.4–636	0.9990
C2	16.7–257	0.9993
C3	1.28–41.3	0.9989
C4	0.210–10.2	0.9994
C5	0.160–8.16	0.9988
C6	0.048–8.05	0.9982
C8	0.090–8.09	0.9967
C10	0.090–8.09	0.9965
C12	0.055–10.1	0.9968
C14	0.052–8.052	0.9958
C16	0.076–40.8	0.9954
C18	0.570–16.6	0.9951
Phe	91.3–1291	0.9973
Leu	115–715	0.9952
Ile	64.4–664	0.9982
Tyr	61.4–1261	0.9971
Val	180–1180	0.9980
Cit	42.8–443	0.9966
Met	15.1–615	0.9973

for each analyte and extraction condition, typical extraction profile curves for acetylcarnitine (C2), palmitoylcarnitine (C16) and phenylalanine (Phe) using methanol containing 0.1% formic acid are shown in supplementary data section 3. The extraction profiles for all the other analytes are very similar to these three profiles and are therefore not presented. From these plots it is apparent that 100% methanol with 0.1% formic acid demonstrated the highest recovery as compared to the other conditions tested and is a common extraction solution used for flow injection analysis [27,28]. Although there was not a significant difference between some of the other extraction solutions, it was observed that more aqueous in the extraction solution caused the samples to appear cloudy. The samples extracted in acetonitrile mixtures were visibly clearer than the samples extracted with methanol mixtures; however, 20% aqueous is required in order to achieve comparable recovery to 100% methanol containing 0.1% formic acid. Since methanol containing 0.1% formic acid is commonly used in newborn screening analysis, we elected to employ this solvent for further investigations.

3.6. Ionization suppression evaluation for chromatographic separation

Ionization suppression was also evaluated for the chromatographic separation using the same technique as described earlier. Samples were injected and the labeled internal standards were infused post column to evaluate matrix effects. While it was apparent that some regions in the chromatogram showed some ionization suppression the complexity of the chromatogram makes it difficult to quantify the degree of matrix suppression using this approach. Therefore, an additional experiment was conducted by comparing internal standard responses in extracted samples to external samples. This experiment was performed for dried blood spots prepared from six different individuals. Dried blood spot samples were extracted in triplicate for each individual using an internal standard working solution prepared in methanol containing 0.1% formic acid. The samples were transferred to a clean 96-well plate and injected onto the LC/MS/MS system. The responses for the internal standard in extracted samples were compared to injections of the working internal standard solution. The results for this experiment showed minimal matrix effects based on the percent differences in responses of the internal standards in extracted and external samples, shown in Fig. 4. Overall there was less than 20% matrix suppression observed for all the compounds except C16 which showed ionization suppression of 21.5%. All analytes demonstrated less than 5% relative standard deviations from triplicate analysis of 6 different lots of sodium heparin human whole blood samples. This is a dramatic improvement over the flow injection analysis in which 63% ion suppression was observed. There was also a significant improvement for the relative standard deviation for this chromatographic analysis compared to the flow injection approach, which could further improve the positive predictive value (PPV) for metabolism disorders associated with elevated levels amino acids and acylcarnitines.

3.7. Validation of analysis with chromatographic separation

Validation of the chromatographic method included precision and accuracy, linearity and recovery. Three replicate samples were analyzed on three separate days in order to assess inter assay precision and accuracy. Prior to validation six different lots of sodium heparin human whole blood were analyzed to select a matrix that contained the lowest endogenous levels of analytes and to ensure the levels of analytes were below the reported cut-off values [29]. The endogenous levels of all analytes were determined using the method of standard additions to create a four point curve from the amount fortified versus the response ratios obtained. Acceptable linearity with correlation coefficients greater than 0.9950 was observed for all analytes using linear regression. From the linear regression $y = mx + b$, the concentration in the unfortified sample can be obtained at the x-intercept where $y = 0, x = -b/m$. After establishing concentration in the blank sample the calibration range for each analyte was established by adding the amount fortified to the blank concentration producing a six point calibration curve. A larger range was used for calibration curves to allow for quantification above and below the targeted cut-off values for each analyte. Different regression models and weightings were assessed for each analyte and the best model was chosen based on having the best correlation coefficient and lowest average percent difference from theoretical. After evaluating multiple models it was determined that a quadratic fit model with a 1/concentration weighting provided the best results for all analytes. The final calibration range based on the adjusted concentrations is shown in Table 3. Correlation coefficients for all runs were greater than 0.9950 for all analytes. Overall average precision for analysis of dried blood spots on three separate days ranged from 2.38% to 9.98%. The recoveries for all the acylcarnitines and amino acids were calculated at

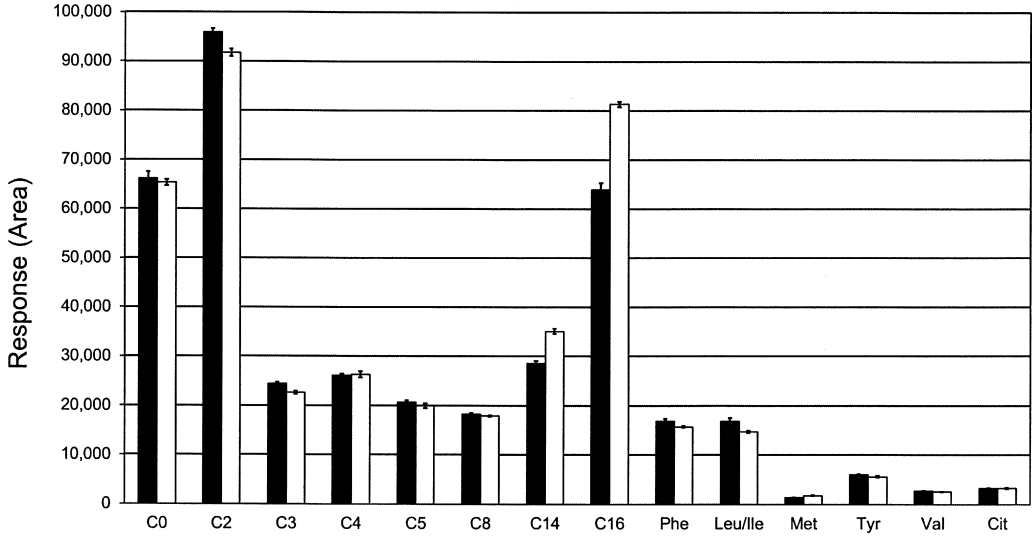

Fig. 4. Evaluation of matrix suppression for samples injected with chromatographic separation. Labeled internal standard responses for extracted dried blood spot samples and external samples were analyzed in triplicate. Black bars are extracted samples white bars are external samples. Error bars represent 1 standard deviation.

Table 4
Precision and accuracy over 3 days using chromatographic separation and MRM transitions.

	Level 1		Level 2		Level 3		Level 4	
	Mean (μmol/L)	%RSD	Mean (μmol/L)	%RSD	Mean (μmol/L)	%RSD	Mean (μmol/L)	%RSD
C0	59.4 (101)	3.10%	87.1 (99.6)	2.97%	240 (104)	3.03%	545 (105)	3.25%
C2	23.7 (98)	9.85%	33.7 (93.4)	7.89%	86.0 (89.5)	7.97%	169 (78.2)	5.05%
C3	2.50 (103)	10.9%	4.14 (98.3)	7.39%	12.7 (95.8)	4.83%	28.0 (89.6)	2.91%
C4	0.529 (103)	8.90%	0.934 (97.2)	3.42%	3.08 (95.8)	3.19%	7.20 (93.4)	4.23%
C5	0.412 (104)	8.48%	0.747 (98.9)	4.51%	2.47 (96.8)	2.65%	5.81 (94.4)	3.40%
C6	0.291 (99.8)	2.16%	0.617 (94.7)	0.29%	2.34 (95.4)	2.69%	5.77 (95.3)	2.17%
C8	0.333 (97.2)	6.00%	0.654 (93.0)	4.64%	2.47 (98.7)	2.92%	5.97 (97.8)	3.68%
C10	0.338 (95.3)	7.14%	0.658 (92.2)	3.64%	2.41 (95.9)	3.50%	6.06 (99.1)	3.36%
C12	0.335 (93.9)	3.62%	0.740 (91.4)	2.25%	3.07 (100)	2.07%	7.80 (103)	0.964%
C14	0.283 (91.4)	8.72%	0.604 (90.2)	3.35%	2.43 (98.4)	3.51%	6.18 (102)	1.57%
C16	2.43 (94.7)	13.1%	4.17 (88.4)	7.35%	15.2 (97.8)	1.53%	38.2 (103)	1.48%
C18	1.23 (97.1)	13.2%	1.78 (89.4)	8.43%	5.64 (101)	4.73%	13.3 (104)	2.48%
Phe	123 (98.9)	6.38%	170 (96.3)	5.62%	426 (97.7)	3.45%	903 (94.6)	4.89%
Leu	130 (99.3)	3.47%	151 (96.4)	2.25%	272 (95.2)	1.75%	513 (94.1)	3.87%
Ile	86.7 (101)	5.91%	111 (98.8)	5.10%	233 (96.4)	2.46%	498 (99.4)	4.91%
Tyr	124 (97.6)	4.35%	213 (97.3)	8.84%	719 (108)	5.90%	1614 (103)	2.50%
Val	233 (99.6)	7.38%	270 (97.3)	9.07%	488 (98.9)	6.22%	877 (94.7)	5.83%
Cit	51.1 (100)	6.77%	66.7 (97.7)	4.48%	154 (99.8)	3.09%	320 (97.6)	2.96%
Met	31.3 (91.6)	4.98%	51.5 (85.7)	3.79%	197 (104)	5.03%	473 (105)	7.80%

3 replicates for each level on 3 days ($n = 9$). % of target values indicated in parentheses.

each of the four concentrations in triplicate and the overall average recovery ranged from 78.2% to 108%, Table 4.

4. Conclusions

In conclusion, a high speed separation for quantification of twelve acylcarnitines and seven amino acids using the same extracted sample that is analyzed with the non-derivatized newborn screening method was developed. The method was validated with respect to linearity, precision and accuracy and recovery. Matrix related ionization suppression evaluated by post-column infusion showed less than 21.5% decrease in response for all the analytes, which is a significant improvement over the flow injection analysis. Furthermore, the chromatographic separation allows for assessment of MSUD by separating leucine, isoleucine and hydroxyproline. In addition alternative chromatography can be used to adequately resolve C4 and C5 isomers allowing for differential identification of additional disorders SCAD, IVA IBMC, which is not possible in the current newborn screening methods. The overall chromatographic separation can be conducted in approximately the same time frame as the established flow injection analysis and with the same sample, eliminating the need for additional sample preparation or instrumentation [4]. Overall, this method is a complementary technique that could be used in conjunction with the current screening method for identification of specific disorders.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.07.008.

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