HHS Public Access

Author manuscript

Clin Chim Acta. Author manuscript; available in PMC 2016 April 18.

Published in final edited form as:

Clin Chim Acta. 2014 September 25; 436: 149–154. doi:10.1016/j.cca.2014.05.016.

Development of an assay to simultaneously measure orotic acid, amino acids, and acylcarnitines in dried blood spots

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Abstract

Background—Orotic aciduria in the presence of hyperammonemia is a key indicator for a defect in the urea cycle, specifically ornithine transcarbamylase (OTC) deficiency. Current newborn screening (NBS) protocols can detect several defects of the urea cycle, but screening for OTC deficiency remains a challenge due to the lack of a suitable assay. The purpose of this study was to develop a high-throughput assay to measure orotic acid in dried blood spot (DBS) specimens as an indicator for urea cycle dysfunction, which can be readily incorporated into routine NBS.

Methods—Orotic acid was extracted from DBS punches and analyzed using flow-injection analysis tandem mass spectrometry (FIA–MS/MS) with negative-mode ionization, requiring <2 min/sample run time. This method was then multiplexed into a conventional newborn screening assay for analysis of amino acids, acylcarnitines, and orotic acid.

Results—We describe 2 assays which can quantify orotic acid in DBS: a stand-alone method and a combined method for analysis of orotic acid, amino acids, and acylcarnitines. Both methods demonstrated orotic acid recovery of 75–85% at multiple levels of enrichment. Precision was also comparable to traditional FIA–MS/MS methods. Analysis of residual presumptively normal NBS specimens demonstrated a 5:1 signal to noise ratio and the average concentration of orotic acid was approximately 1.2 μmol/l. The concentration of amino acids and acylcarnitines as measured by the combined method showed no significant differences when compared to the conventional newborn screening assay. In addition, retrospective analysis of confirmed patients and presumptively normal newborn screening specimens suggests potential for the methods to identify patients with OTC deficiency, as well as other urea cycle defects.

Conclusion—The assays described here quantify orotic acid in DBS using a simple extraction and FIA–MS/MS analysis procedures that can be implemented into current NBS protocols.

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Keywords

Orotic acid; Ornithine transcarbamylase deficiency; Urea cycle defects; Newborn screening

1. Introduction

A principal function of the urea cycle is to convert nitrogen waste, accumulated from either amino acid metabolism or intestinal bacteria, into urea, which can be safely excreted from the body. There are five catalyzed reactions in the urea cycle and defects of any step lead to the accumulation of ammonia with subsequent damage to the central nervous system [1]. In the most severe urea cycle defects, newborns will appear healthy during the first 24 h after birth, but will decompensate within days, develop vomiting and lethargy, worsening to coma and eventually death [1]. Early detection and treatment during the newborn period is critical to the prevention of these severe adverse outcomes [2].

The current US recommended uniform newborn screening (NBS) panel includes two urea cycle defects; argininosuccinic acid synthase (ASS) deficiency and argininosuccinic acid lyase (ASL) deficiency [3]. One of the most common and severe defect of the urea cycle, ornithine transcarbamylase (OTC) deficiency, was considered for inclusion in the panel, but it did not meet the assigned evaluation criteria, due to the lack of a screening test that had been validated in the general newborn population [4]. OTC deficiency, an X-linked disorder, has a wide range of clinical variability and can present in a severe neonatal-onset form that is life threatening within the first few days after birth or as a late-onset, typically milder, form of the disease. Female carriers can also experience symptoms related to increased ammonia levels [1]. The laboratory indications for OTC deficiency are elevated concentrations of glutamine and ammonia, low citrulline, and elevated excretion of orotic acid in the urine [2]. NBS laboratories have attempted to use glutamine and citrul-line to identify infants at risk for OTC deficiency, but have had limited success [5,6].

Orotic acid is an excellent marker for OTC deficiency. Orotic acid increases in urea cycle disorders due to the accumulation of carbamoyl phosphate when there is a mismatch between the fluxes through carbamoyl phosphate synthetase and the urea cycle steps. Carbamoyl phosphate enters the pyrimidine nucleotide synthesis pathway leading to markedly increased concentrations of orotic acid [7]. Orotic acid can be quantified in urine or plasma using liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods, which combine an easy extraction, not requiring derivatization, with a short analysis time per sample [8,9]. In 2010, D'Apolite et al., published a method for quantification of orotic acid in urine, plasma, and dried-blood spots using a hydrophilic interaction LC–MS/MS method [10,11]. This was the first report of using DBS specimens to measure orotic acid concentrations for the detection of urea cycle defects. However, shorter analysis times per sample (<2 min) using flow-injection analysis tandem mass spectrometry (FIA–MS/MS) would be advantageous for integration of OTC deficiency into the current NBS assays.

A recent publication by Janzen et al., stated that orotic acid can be measured simultaneously with amino acids and acylcarnitines extracted from DBS using FIA–MS/MS [12]. This present study provides the detailed FIA–MS/MS method and data demonstrating an accurate

quantification of orotic acid along with the traditional set of amino acid and acylcarnitine species. In addition, orotic acid was quantified in 1514 presumptively normal newborn DBS specimens and in 7 patients with confirmed OTC deficiency or other urea cycle defects.

2. Materials and methods

2.1. Dried-blood specimens

A protocol for use of residual, de-identified DBS specimens was approved by the Health Sciences Institutional Review Board (IRB) at the University of Wisconsin. All residual, presumptively normal, dried blood spot specimens were collected between 24 and 96 h after birth and were stored at 4 °C after routine NBS analysis. In addition, DBS specimens from seven patients diagnosed with either OTC deficiency or other urea cycle disorders were obtained from the California State Newborn Screening Laboratory and the Wisconsin State Newborn Screening Laboratory. These specimens were de-identified, according to IRB approved protocol, and used to evaluate the clinical performance of the assay. Samples were stored at −20 °C prior to analysis of orotic acid.

2.2. Reagents and standards

Orotic acid, amino acid, and acylcarnitine standards and ammonium hydroxide were purchased from Sigma-Aldrich. The isotopically labeled internal standards for amino acids, acylcarnitines, and orotic acid were purchased from Cambridge Isotope Laboratories, Inc. Acetonitrile and methanol HPLC–MS grade solvents were from J.T. Baker. Grade 903 filter paper for DBS preparation was from Whatman GmbH.

2.3. Quality control materials

Quality control materials for orotic acid were prepared by enriching whole blood from presumptively normal adult donors with varying amounts of orotic acid. Whole blood adjusted to a 50% hematocrit was enriched with 1 and 10 μmol/l orotic acid, and was spotted onto filter paper to prepare the low-enrichment and high-enrichment quality control materials, respectively. Additional quality control materials with enrichments ranging from 0.25 to 40 μmol/l of orotic acid were also prepared and used to assess the method's linearity and limit of quantification. The unenriched and enriched whole blood pools were dispensed onto filter paper, dried overnight under ambient conditions, and stored at −20 °C in zip-closure plastic bags containing desiccant packets. Quality control materials for amino acid and acylcarnitine analysis were obtained from the Newborn Screening Quality Assurance Program at the Centers for Disease Control and Prevention (CDC).

2.4. Stand-alone method for quantification of orotic acid

Orotic acid was extracted from DBS using 80:20 acetonitrile/water containing 0.5 μmol/l [1,3-$^{15}N_2$] orotic acid. To prepare the extraction solvent, 10 mg of [1,3-$^{15}N_2$] orotic acid was dissolved in 10 ml of 1 mol/l NaOH to create a 1 mg/ml stock solution, which was stable for 3 days at 20 °C based upon reproducible peak areas for [1,3-$^{15}N_2$] orotic acid in dilutions made on days 1 to 3. A working internal standard solution of 0.5 μmol/l [1,3-$^{15}N_2$] orotic acid was prepared in 80:20 acetonitrile/water. A 3 mm (1/8th inch) punch from the DBS was placed into a 96-well round bottom polypropylene plate. One hundred fifty micro-liters of

working internal standard solution were added to each punch followed by shaking at room temperature for 30 min. Finally, the extraction solution was transferred to a clean 96-well plate for FIA–MS/MS analysis.

Orotic acid was analyzed using an AB Sciex API 4000 tandem mass spectrometer with a TurboV electrospray ionization source. All data acquisition and processing was performed using Analyst 1.5.2 software (AB Sciex). The API 4000 (AB Sciex) tandem mass spectrometer was operated in negative-ionization mode with the following parameters: ion spray voltage at −4500 V, entrance potential at −10 V, declustering potential at −48 V, collision energy at −14 V, and collision cell exit potential at −9 V. The mobile phase of 80:20 acetonitrile/water containing 0.02% ammonium hydroxide (v/v) flowed at a rate of 0.08 ml/min and the analysis time per sample was 1.2 min. The injected sample volume was 10 μl. Orotic acid and its internal standard was analyzed using the following multiple reaction monitoring (MRM) pairs for the neutral loss of carbon dioxide: 154.9 → 110.9 and 156.9 → 112.9 (Fig. 1), respectively. Orotic acid concentrations were determined using the following calculation: (analyte peak area divided by internal standard peak area) multiplied by the internal standard concentration (0.5 μmol/l) multiplied by the dilution of DBS punch blood into the extraction solution.

2.5. Combined method for quantification of amino acids, acylcarnitine species, and orotic acid

A routine, non-derivatized FIA–MS/MS method for analysis of amino acid and acylcarnitine species was modified to include orotic acid. All analytes were extracted from 3 mm (1/8th inch) DBS by addition of 100 μl of methanol containing internal standards for amino acids, acylcarnitines, and orotic acid followed by shaking at room temperature for 30 min. The AB Sciex API 4000 tandem mass spectrometer with a TurboV electrospray ionization source was operated in both positive-and negative-ionization modes. The mobile phase of 80:20 acetonitrile/water was devoid of both formic acid and ammonium hydroxide and flowed at a rate of 0.06 ml/min for the first 30 s and then slowed to 0.03 ml/min for the remaining 1 min of the run. Amino acids, acylcarnitines, and orotic acid were quantified using ChemoView 2.0.2 software (AB Sciex).

3. Results

3.1. Validation of stand-alone method for orotic acid

Method precision for orotic acid was evaluated by analysis of three quality control materials (un-enriched, 1 μmol/l low enrichment, and 10 μmol/l high enrichment) on five different days (with n = 5 for each day). The inter-day CV for all three quality control enrichments was <15%. The limit of quantification (LOQ) for orotic acid was determined (by the y-intercept of the standard curve) to be 1 μmol/l, with a CV <15%. Analysis of unenriched DBS specimens demonstrated a 5:1 signal-to-noise ratio for orotic acid with an approximate concentration of 1.20 μmol/l. Apparent recovery was measured by comparison of the observed orotic acid concentrations to DBS quality control materials with two levels of enrichment (1 and 10 μmol/l) (Table 2). The slope and y-axis intercept of the standard curve, ranging from 5 to 40 μmol/l, were 0.73 and 1.02, respectively, with a coefficient of

correlation (R^2) of >0.999. Therefore, orotic acid recovery was estimated to be approximately 75% based upon both evaluations of quality control materials.

3.2. Quantification of orotic acid in presumptively normal NBS specimens and in seven patients with confirmed urea cycle defects

NBS specimens from 1514 presumptively normal newborns and 7 specimens from patients with urea cycle defects were analyzed for orotic acid using the stand-alone method described above. The orotic acid concentrations within the normal population ranged from 0.59 to 2.61 μmol/l, with an average concentration of 1.20 ± 0.23 μmol/l (median = 1.18 μmol/l; 1st–99th percentile 0.72–1.84 μmol/l) (Fig. 2).

Table 1 shows orotic acid concentrations from 7 patients with confirmed urea cycle disorders. Citrulline values were obtained through the original routine NBS results. Patients one and 2 are both males with OTC deficiency. The specimens were collected at 32 and 52 h after birth and routine NBS revealed below-normal citrulline concentrations (5 μmol/l) for both specimens. The orotic acid concentrations using the described method were 11.0 and 21.8 μmol/l for each sample, respectively. Patients 3 and 4 both have argininosuccinic acid synthetase deficiency, which is characterized by elevated citrulline concentrations (2796 and 1205 μmol/l, respectively). These specimens were collected at 18 and 4 days after birth and the orotic acid concentrations were 71.1 and 38.3 μmol/l, respectively. Patients 5, 6, and 7 have argininosuccinic acid lyase deficiency, which is characterized by accumulation of argininosuccinic acid and increased citrulline levels (130, 199, and 107 μmol/l, respectively). The orotic acid concentrations in these 3 samples ranged from 2.58 to 10.6 μmol/l.

3.3. Validation of combined method for amino acid, acylcarnitine, and orotic acid analysis

Precision of orotic acid quantification using the combined method was determined by analyzing three quality control materials (unenriched, 1 μmol/l low enrichment, and 10 μmol/l high enrichment) over 5 different days (with N = 10 each day). The inter-day CV for the high enrichment was <10%, however the low enrichment and unenriched specimens both had <25% CV. Recovery was determined to be approximately 85% by comparing the observed orotic acid concentration to the low and high enrichment values of the quality control material (Table 2). Five hundred and sixteen presumptively normal newborn screening specimens collected at 24–96 h after birth were analyzed for orotic acid. The concentrations ranged from 0.38 to 3.36 μmol/l, with an average concentration of 1.29 ± 0.45 μmol/l (median = 1.23 μmol/l; 1st–99th percentile 0.50–2.85 μmol/l) (Fig. 2).

Precision of the combined method for amino acid and acylcarnitine species was determined by analyzing three quality control materials (un-enriched, low enrichment, and high enrichment) over 5 different days (with n = 10 each day). The average inter-day CVs for both low and high enrichments of amino acids and acylcarnitine species were <10% and <15%, respectively. The average inter-day CVs for unenriched amino acids and acylcarnitine species were M <10% and <25%, respectively.

To demonstrate a similarity between the combined method and the current, routine, non-derivatized assay for amino acid and acylcarnitine analysis, the same 636 presumptively normal newborn screening specimens were analyzed by both methods and the results were

compared. Fig. 3 shows a box–whisker plot for each analyte analyzed by the two methods. The majority of analytes had a <15% difference in normal patient means between the methods, with the exception of C3DC/C4OH, C4DC/C5OH, tyrosine, and citrulline. Overall, no significant measurement differences were observed.

4. Discussion

The clinical presentation of hyperammonemia is common to the majority of urea cycle disorders and treatment prior to the onset of symptoms is critical to the prevention of devastating outcomes, including permanent neurological injury [1,2]. Most NBS programs have implemented testing for two urea cycle defects (argininosuccinic acid synthetase deficiency and argininosuccinic acid lyase deficiency) and studies have suggested favorable clinical outcomes associated with early diagnosis [13,14]. NBS for the most common urea cycle disorder, OTC deficiency, however, has not been uniformly implemented largely due to the lack of a high-throughput assay.

Elevated orotic acid is a key diagnostic finding in patients with OTC deficiency. Advances in LC–MS/MS have enabled rapid detection of orotic acid in plasma and urine without the use of derivatizing agents and long run times associated with other analytical methods. Detection of orotic acid in DBS was demonstrated using a hydrophilic interaction LC–MS/MS method with a run time of 5 min/sample [11]. Although this method identified patients with OTC deficiency, shorter run times are necessary for a high-throughput NBS laboratory [10].

The objective of our study was to develop a FIA–MS/MS method that incorporates orotic acid into routine newborn screening assays. To accomplish this goal, a FIA–MS/MS stand-alone method for orotic acid was first developed as a benchmark to which a combined FIA–MS/MS method (including amino acids, acylcarnitines, and orotic acid) could be compared. Using the stand-alone method, orotic acid was extracted from DBS and analyzed by FIA–MS/MS requiring approximately 1 min/sample. Validation parameters such as precision and recovery were comparable to previous reports of analytes measured by FIA–MS/MS [15–18]. Linearity was demonstrated up to a 40 μmol/l enrichment of orotic acid in DBS. Although patient samples may have orotic acid values greater than the highest concentration tested here (40 μmol/l), for NBS purposes this linear range encompasses both normal and significantly elevated orotic acid values. DBS from presumptively normal newborns had an average orotic acid concentration of 1.20 ± 0.23 μmol/l, which was similar to previous reports [10,12]. Analysis of DBS from two patients with OTC deficiency and five patients with other distal urea cycle disorders (argininosuccinic acid synthetase deficiency and argininosuccinic acid lyase deficiency) showed a significant increase in orotic acid concentration, greater than the 99th percentile above the presumed normal average.

Next, we chose to incorporate orotic acid into our current NBS assay for amino acids and acylcarnitines, which uses a methanol extraction and FIA–MS/MS (<2 min run time); amino acids and acylcarnitines were quantified using positive ion mode scans (neutral loss of 46 Da and precursor scans of m/z 85, respectively) while orotic acid and its internal standard were quantified using negative ionization mode MRMs. Switching the mass spectrometer between positive and negative ionization modes during analysis of one sample [19,20] can be an

effective way to quantify multiple metabolites when some of the metabolites (amino acids and acylcarnitines) readily generate positive precursor ions while others (orotic acid) readily generate negative precursor ions; however consideration must be given to the ability to quantify all analytes accurately. A comparison of amino acid and acylcarnitine quantification with and without polarity switching indicated that including the negative ionization mode did not significantly change the results. For implementation however, minor adjustments to analyte cutoffs may be necessary. In addition, the orotic acid concentrations resulting from polarity switching analyses were not significantly different from the results of the FIA–MS/MS stand-alone method, although the standard deviation was larger, which is likely a result of the decreased number of scans obtained by the mass spectrometer. Given the degree of orotic acid elevation in patients with confirmed urea cycle defects, particularly OTC deficiency, as compared to the normal population, it is unlikely that the wider range within the normal population will affect the ability to detect disease states.

There are multiple ways to implement either method into NBS workflows. First, laboratories could adopt the FIA–MS/MS stand-alone method for orotic acid quantification, which would necessitate a second sampling and analysis of the DBS specimen. Consideration could also be given to the use of the stand-alone method as a second tier test when traditional amino acid markers of the urea cycle (such as citrulline, glutamine, or arginine) are outside the normal range. Second, laboratories could simultaneously measure amino acids, acylcarnitines, and orotic acid using a methanol extraction and the combined FIA–MS/MS method with polarity switching.

The projected benefit from this research would be identification of patients with OTC deficiency through routine newborn screening however, before either assay can be implemented, the clinical utility needs to be demonstrated by the analysis of orotic acid in additional specimens from OTC deficient patients covering a range in clinical severity. Males affected with OTC deficiency can have a neonatal onset or present with a mild or late-onset [21]. Likewise, the severity in carrier females depends on which mutation is present and on the proportion of hepatocytes expressing the mutant allele [22,23]. It is unknown whether orotic acid is elevated in the milder forms of the disease or in symptomatic carriers, especially within the first few days of life. It is also probable that orotic acid measurements in combination with existing amino acid markers and ratios may assist with the detection of these milder cases. Future studies will also include a more extensive evaluation of orotic acid concentrations within the presumptively normal newborn population for comparison to affected individuals.

Elevated orotic acid is not unique to OTC deficiency. Patients with hereditary orotic aciduria, HHH syndrome (hyperornithinemia–hyperammonemia–homocitrullinuria syndrome), lysinuric protein intolerance, and other urea cycle disorders such as ASS and ASL deficiencies have elevated orotic acid levels [1]. Orotic acid may also be elevated as a result of certain treatments, or physiologic conditions [7]. In newborn screening, differential diagnoses resulting from out-of-range analyte concentrations are not uncommon. Planned population based prospective studies followed by a clinical evaluation will help elucidate the sensitivity of this assay to detect patients with OTC deficiency and other treatable disorders associated with elevated orotic acid.

In conclusion, this paper describes a high throughput assay quantifying orotic acid in DBS and demonstrates its compatibility with current FIA–MS/MS NBS assays for amino acids and acylcarnitines. Retrospective analysis of confirmed patient samples suggests a potential for the methods to identify patients with OTC deficiency, as well as other urea cycle defects.

Acknowledgments

The authors acknowledge Dr. Fred Lorey at the California Department of Public Health for providing residual, de-identified dried-blood spots from patients with confirmed OTC deficiency.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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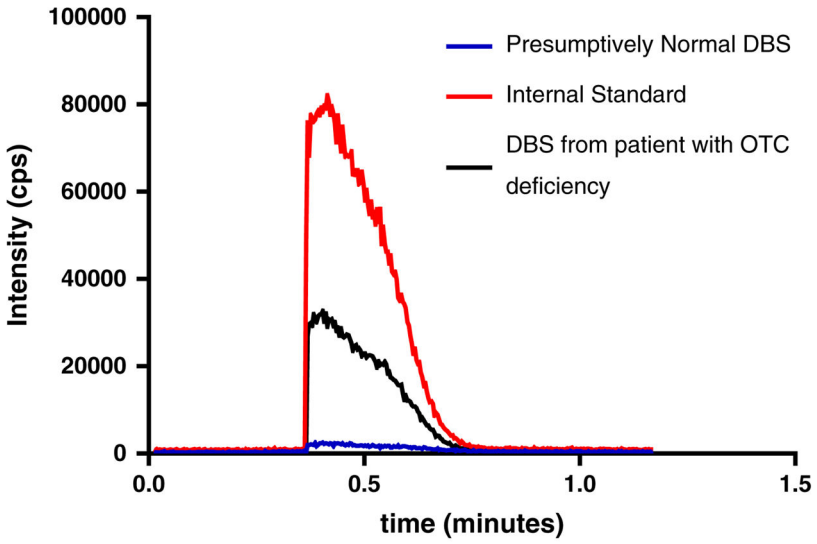

Fig. 1.

Total ion flow injection profile obtained by MRM of orotic acid and [1,3-$^{15}N_2$] orotic acid extracted from DBS specimens.

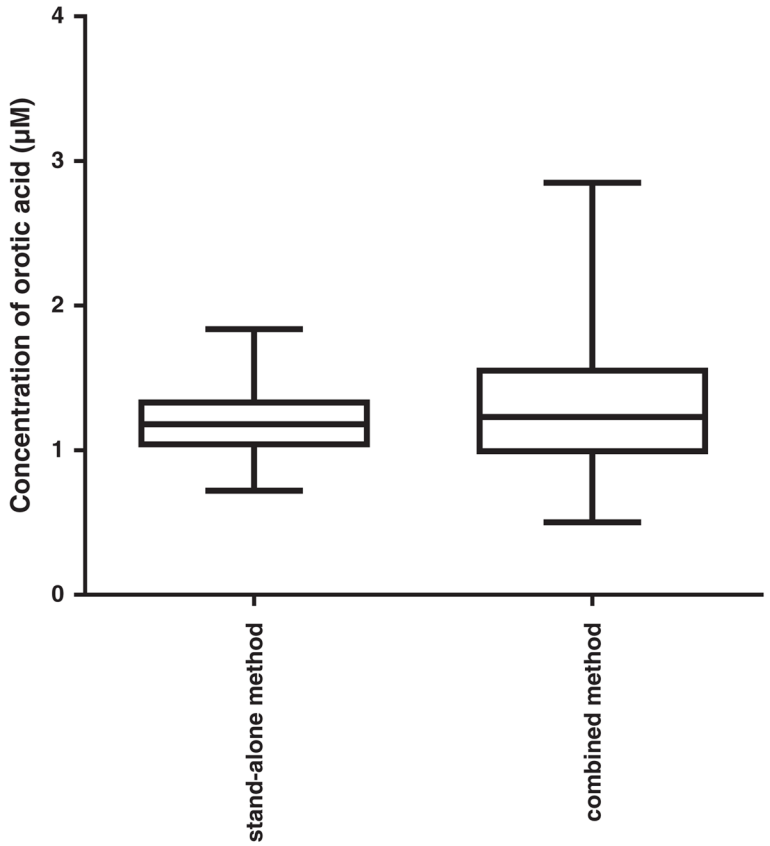

Fig. 2.

Box–whisker plot of orotic acid concentration measured in DBS using the 2 described methods: stand-alone method (n = 1514) and the combined method (n = 516). The box corresponds to the 25th to the 75th percentile. The whiskers are the 1st to the 99th percentile.

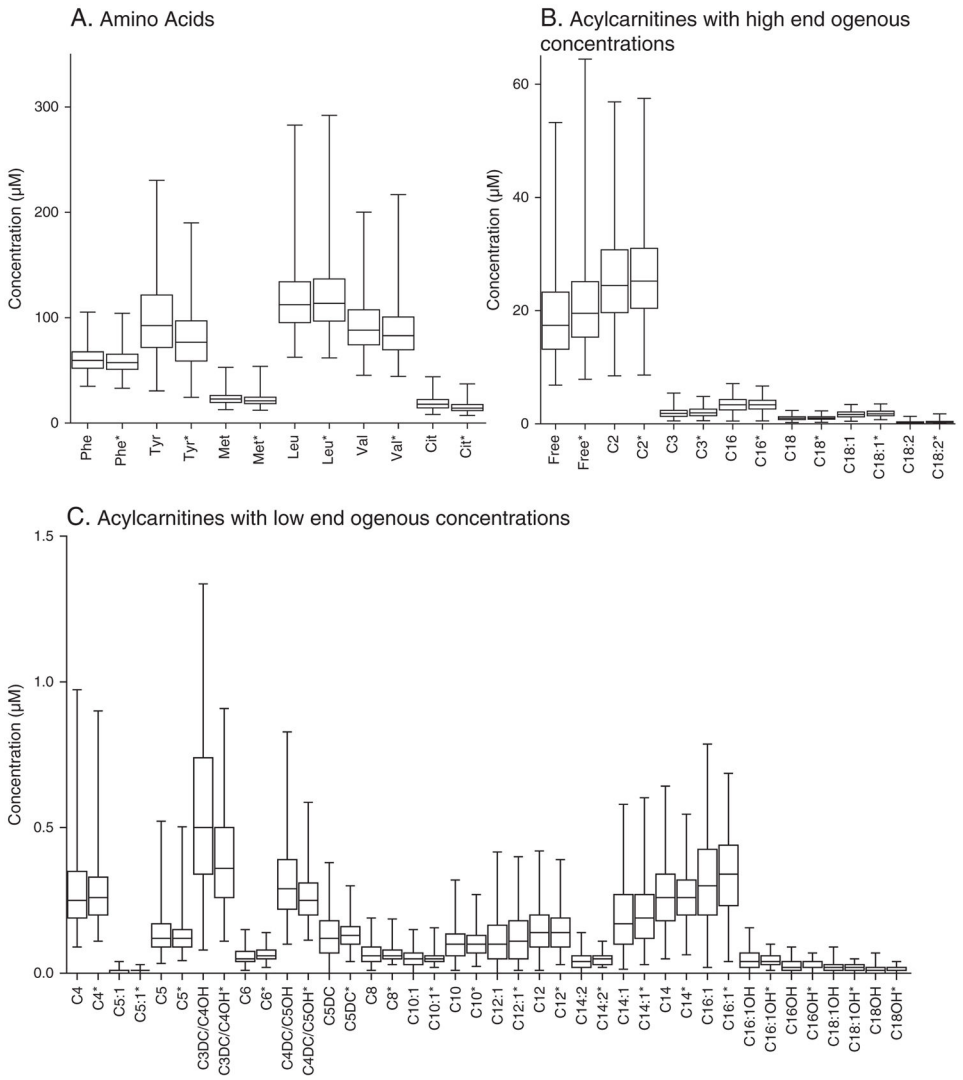

Fig. 3.

Comparison of analyte concentrations for 636 normal NBS specimens analyzed by the routine, non-derivatized method for amino acids and acylcarnitine analysis and the combined method which include orotic acid (*). The box corresponds to the 25th to the 75th percentile. The whiskers are the 1st to the 99th percentile.

Table 1

Orotic acid levels in DBS obtained from patients with a confirmed urea cycle defect.

Patient	Disease	Age at collection	Sex	Citrulline (μM)	Orotic acid (μM)
1	OTC	32 h	M	5	11.00
2	OTC	52 h	M	5	21.80
3	ASS	18 days	F	2796	71.10
4	ASS	4 days	M	1205	38.30
5	ASL	32 h	M	130	2.58
6	ASL	6 days	M	199	7.52
7	ASL	3 days	F	107	10.60

Table 2

Recovery of orotic acid from enriched DBS analyzed by both the stand-alone and combined methods.

	Stand-alone method (N = 25)		Combined method (N = 50)	
Expected (μmol/l)	**Obtained (μmol/l)**	**%CV**	**Obtained (μmol/l)**	**%CV**
1	1.05	11.2	0.89	21.4
10	7.41	3.6	8.18	9.9