Research Paper

Development and pilot evaluation of an add-on orotic acid assay in dried blood spots for newborn screening of ornithine transcarbamylase deficiency

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Abstract Background: Ornithine transcarbamylase deficiency (OTCD) is the most common urea cycle disorder and can cause lifethreatening hyperammonemia. Biochemical diagnosis typically relies on elevated orotic acid (orotic acid (ORA)) and decreased citrulline (citrulline (Cit)) levels. However, in the newborn screening (NBS) setting, ORA measurement from dried blood spots (DBS) has been technically challenging and is not routinely implemented.

Methods: We developed a tandem mass spectrometry (MS/MS) method for quantifying ORA in DBS using negative electrospray ionization, performed concurrently with routine positive-mode amino acid and acylcarnitine analysis. Reference ranges and cutoffs were determined from retrospective analysis of 4,605 healthy newborn DBS specimens. A prospective pilot screening of 6,562 newborns (September 2018−March 2019) was conducted. DBS from OTCD patients in various clinical states were also analyzed.

Results: The assay showed excellent linearity (0.5−100 nmol/mL; R^2=0.9999) with acceptable precision (CV＜15% above 0.5 nmol/mL). In healthy newborns, the 99.5th percentile was 2.99 nmol/mL for ORA and 0.34 for the ORA/Cit ratio. No positives were detected in the pilot screening. Acutephase OTCD samples had markedly elevated ORA and ORA/Cit, while stablephase values often overlapped with normal ranges. Among four lateonset OTCD patients' newborn DBS, only one exceeded the cutoffs; this patient later developed severe hyperammonemia.

Conclusions: This ORAbased assay can be incorporated into existing NBS workflows with minimal modification. Our study suggests that ORAbased NBS might detect OTCD newborns who are already hyperammonemic as well as a subset of asymptomatic lateonset newborns.

Key words: orotic acid; urea cycle disorder; newborn screening; tandem mass spectrometry; ornithine transcarbamylase deficiency

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Introduction

Ornithine transcarbamylase deficiency (OTCD, MIM #300461) is the most common urea cycle disorder (UCD), and is inherited in an X-linked manner[1)]. In Japan, the overall incidence of UCDs is estimated to be 1 in 50,000 births, with OTCD accounting for approximately 60% of cases. Therefore, the incidence of OTCD is 1 in 80,000 births[2-4)].

The prognosis of OTCD depends on frequency and duration of hyperammonemic episodes, as well as the peak value of blood ammonia. Rapid and appropriate interventions to prevent and treat hyperammonemic episodes are essential to improve outcomes[4, 5]. Thus, implementing newborn screening (NBS) for OTCD during the presymptomatic neonatal period could significantly benefit patients. However, OTCD is not currently included in NBS panels in most countries due to the lack of a simple and reliable screening method, and only a few studies have reported on the feasibility of using orotic acid (ORA) measurement for NBS of OTCD[6–11].

The presence of ORA in urine is a useful diagnostic finding for OTCD. The enzymatic defect in ornithine transcarbamylase (OTC), which catalyzes the conversion of carbamyl phosphate (CP) and ornithine to citrulline, results in the accumulation of CP, which is subsequently converted to ORA[1]. Urinary ORA levels are typically high in symptomatic male OTCD patients, but can be variable in heterozygous female carriers.

In this study, we developed and validated a specific and sensitive method for quantifying ORA in dried blood spots (DBS) using tandem mass spectrometry (MS/MS) with negative electrospray ionization (ESI). By incorporating this assay into routine NBS, alongside conventional amino acid and acylcarnitine profiling conducted in positive ion mode, we aimed to enable effective screening for OTCD. We evaluated the assay performance through retrospective analysis of patient and control DBS specimens and subsequently conducted a small prospective pilot study in a general newborn population.

This study was conducted with the approval of the Institutional Review Board (IRB) at Shimane University Faculty of Medicine (approval number 2734, 2889, and 2908).

2. Materials and Methods

2.1. Reagents

ORA and stable-isotope-labeled ORA, [1,3-$^{15}N_2$] ORA, was purchased from Sigma-Aldrich (Darmstadt, Germany), and Cambridge Isotope Laboratories (Tewksbury, MA, USA), respectively. Acetonitrile, methanol, and deionized water of the LCMS grade were from Wako (Osaka Japan). NeoBase Non-derivatized MSMS Kit (PerkinElmer. Waltham, MA, USA) was used for analysis of routine amino acids except for ORA. Filter paper for NBS and for quality control were obtained from ADVANTEC (Tokyo, Japan).

2.2. Preparation of calibration samples

To prepare calibration standards for ORA in DBS, known amounts of ORA were spiked onto blank newborn DBS punches. Specifically, DBS samples with final ORA concentrations of 0 (blank), 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 nmol/mL were created by applying ORA solution onto blank filter paper blood spots. After air-drying, the spiked DBS samples were sealed and stored at −30°C until analysis. An internal standard (IS) stock solution of [1,3-$^{15}N_2$] ORA was prepared at 1 mg/mL (6.3 mM) in distilled water and stored at 4°C.

2.3. DBS extraction and MS/MS analysis

The assay was performed according to the NeoBase kit protocol for DBS, with modifications to include ORA measurement. In brief, a 3.2 mm disk punch from each DBS was placed into a well of a 96-well plate. A 100 μL volume of working extraction solution was added to each well. The working solution consisted of the NeoBase kit's internal standard mixture (for amino acids and acylcarnitines) supplemented with the [1,3-$^{15}N_2$] ORA internal standard at 0.1 mg/mL (approximately 0.63 mM). The plate was agitated on a shaker at 700 rpm for 45 min at 45°C. After extraction, the supernatant from each well was transferred to a fresh 96-well plate for MS/MS analysis.

MS/MS analysis was conducted by flow injection analysis on a Nexera MP System utilizing the SIL-30ACMP Multi-Plate autosampler and LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The system was programmed to perform sequential acquisitions in positive and negative ESI modes. The mobile phase provided in the NeoBase kit (a methanol-based solution) was used at a flow rate according to the kit's specifications. The injection volume was 1 μL per sample, and the total run time for each analysis was approximately 1 min.

The quantitative analysis in ORA was performed with multiple reaction monitoring (MRM) mode of the following transitions *m/z* 155.10＞111.05 for OA and *m/z* 157.10 ＞113.05 for OA IS in negative ESI mode. Other analytes were quantified in positive ESI mode. The dwell time for each MRM channel was 0.01 s. The source temperature was set to 250°C. Nitrogen was used as the nebulizing and drying gas, at a flow rate of 3 L/min.

2.4. Retrospective analysis of normal newborn DBS

4,605 of leftover DBS samples after NBS, which were presumably normal, were used as normal control in this study. DBS samples were collected between 4 days and 6 days after birth in accordance with the NBS program in Japan. These samples had been stored at −30°C after routine NBS analysis for at longest 2 years. Histograms of ORA and ORA/Cit ratio were illustrated in Fig. 1. We determined summary statistics (mean, standard deviation, and 99.5th percentile) to establish tentative cutoff values for prospective screening.

2.5. Prospective newborn screening pilot study

Using the cutoffs derived from the retrospective study, we conducted a prospective pilot screening. The study was carried out from September 2017 to March 2019. A total of 6,562 consecutive newborn DBS samples (collected for routine NBS) were analyzed with the expanded panel including ORA measurement. ORA and ORA/Cit values were measured for all samples (Fig. 2).

2.6. Analysis of OTCD patient DBS samples

To assess the method's ability to detect OTCD, DBS samples from 34 patients with OTCD were analyzed. They included 19 samples collected in acute metabolic decompensation (14 samples in neonatal onset form, 5 of late-onset form), 11 in stable condition (7 samples in neonatal onset form, 4 of late-onset form), and 4 NBS samples of

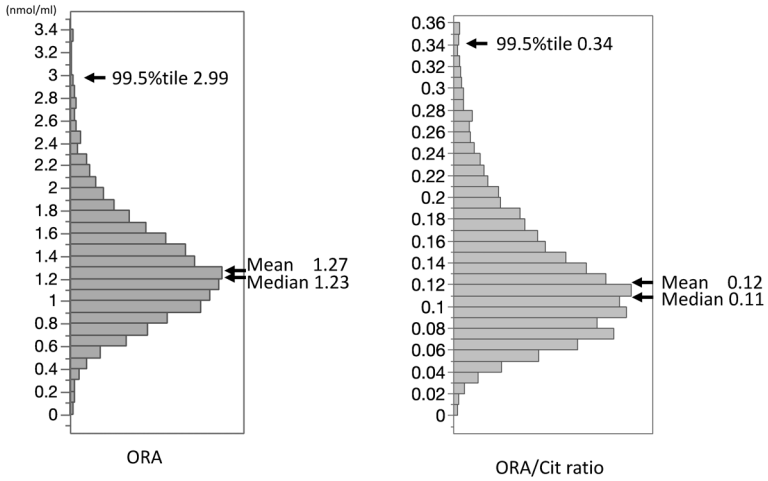

Fig. 1. Histogram of ORA and ORA/Cit ratio in 4,605 specimens of retrospective analysis.
Summary statistics (mean, standard deviation, and 99.5th percentile) were determined to establish tentative cutoff values for prospective screening.

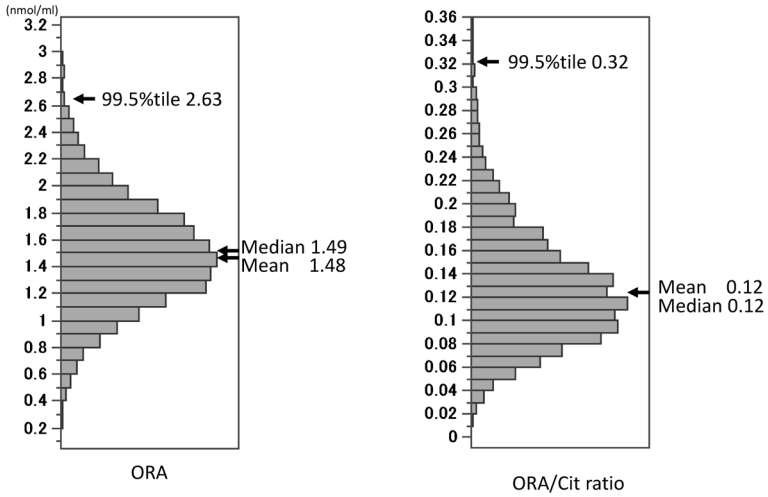

Fig. 2. Histogram of ORA and ORA/Cit ratio in 6,562 prospective pilot study.
The mean±SD of ORA was 1.49±0.40 nmol/mL. The mean±SD of ORA/Cit ratio was 0.124±0.052. The 99.5th percentile values of ORA and ORA/Cit ratio were 2.63 nmol/mL and 0.32, respectively.

patients with late onset form. All OTCD diagnoses were confirmed by either genetic testing of OTC gene or by specific biochemical examination. DBS samples of the late onset cases in NBS period were retrospectively identified and retrieved from leftover DBS storage after diagnosis later in life.

Two of the four NBS samples were obtained from sibling patients with late-onset form (Case 1 and 2 in Fig. 3). Since their older brother had been diagnosed with late-onset OTCD, they received dietary therapy and/or glucose infusion although they were asymptomatic when their NBS samples were collected. The other patient (Case 3 in Fig. 3) was a girl and was born in a member of late-onset OTCD family. She had not developed any acute metabolic decompensation such as hyperammonemia without any treatment until 3 years old. The other boy (Case 4 in Fig. 3) in one of the four cases developed hyperammonemia (highest NH_3 564 μM) when he had upper respiratory infection at the age of 1 year and 1 month, with consequent neurological sequelae.

3. Results

3.1. Validation of ORA quantification in DBS (Table 1)

Table 1 summarizes the validation results of the ORA quantification assay in DBS. The ORA assay showed a linear response over the tested range of 0.5−100.0 nmol/mL (spiked DBS concentrations). The regression equation was y=1.1172x+1.3558, with R^2=0.9999. At the low concentrations of 0 and 0.1 nmol/mL spiked), the coefficients of variation (CV) were 31.4% and 20.8%, respectively. For concentrations at or above 0.5 nmol/mL, CVs were all below 15%, which is generally acceptable on FDA guidance for industry, Bioanalytical Methods Validation.

3.2. Distribution of ORA and ORA/Cit in retrospective newborn samples

Fig. 1 shows the distributions of ORA concentrations and ORA/citrulline (Cit) ratios in 4,605 routine newborn DBS samples. Normal distribution was seen in ORA, with a

Table 1. Validation results of DBS sample spiked ORA using new method

Spiked concentration of ORA in DBS (nmol/mL)	mean (n=7)	S.D.*	CV** (%)
0	1.35	0.42	31.37
0.1	1.37	0.29	20.78
0.5	1.83	0.20	11.17
1	2.18	0.27	12.24
5	6.62	0.80	12.08
10	12.77	1.44	11.26
50	58.14	1.87	3.22
100	112.61	6.70	5.95

*; Standard deviation, **; coefficient of variation

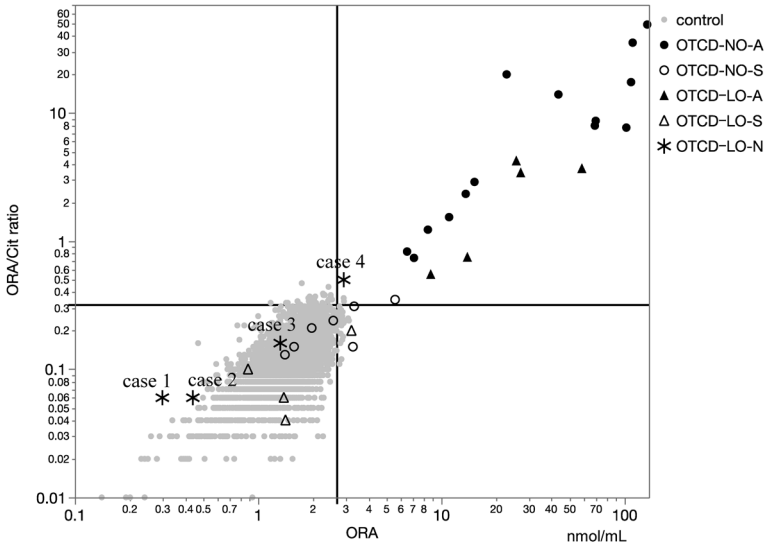

Fig. 3. Scatter plot of ORA versus ORA/Cit ratio in presumably normal newborns and patients with OTCD.
Both axes are plotted on a logarithmic scale. Each gray dot represents a newborn from the prospective cohort (n=6,562). Symbols denote OTCD patient samples: ●, neonatal-onset acute-phase; ○, neonatal-onset stable-phase; ▲, late-onset acute-phase; △, late-onset stable-phase. The four late-onset OTCD patient newborn DBS samples are indicated by asterisks (*). Case 4 lies above the normal cluster, exceeding both ORA and ORA/Cit cutoffs (dashed lines), indicating it would be positive by the screening. Cases 1−3 fall within the normal range and would not be identified by ORA screening alone.

mean of 1.27 nmol/mL and standard deviation (SD) of 0.48 nmol/mL. The 99.5th percentile was 2.99 nmol/mL. The ORA/Cit ratio also approximated a normal distribution, with a mean±SD of 0.12±0.06 and a 99.5th percentile of 0.34. Based on these findings, cutoff values for the prospective pilot study were set at 2.99 nmol/mL for ORA and 0.34 for the ORA/Cit ratio.

3.3. Prospective pilot newborn screening results

The distribution of OA and OA/Cit ratio in the prospective pilot study on 6,562 newborns are shown in Fig. 2, which closely mirrored that of the retrospective analysis. The mean±SD of ORA was 1.49±0.40 nmol/mL. For the ORA/Cit ratio, mean±SD was 0.124±0.052. The values in 99.5%tile of ORA and ORA/Cit ratio were 2.63 nmol/mL and 0.32, slightly lower than retrospective cutoffs.

3.4. ORA values in OTCD patient samples

Table 2 summarizes the ORA and ORA/Cit results for DBS from OTCD patients under various conditions, and Fig. 3 provides a visual comparison of select cases to the normal range. ORA values in the neonatal-onset OTCD cases ranged from 6.5 to 132.9 nmol/mL, and the median and mean values were 33.1 and 51.5, respectively. The ORA/Cit ratio was between 0.74 and 49.1 and the median and mean values were 7.7 and 11.6, respectively. Abnormal values exceeding the cutoff are underlined. In cases of acute phase of late-onset patients, the ORA ranged from 8.75 to 58.45, and the median and mean values were 25.64 and 26.76 nmol/mL, respectively. The OA/Cit ratio was from 0.55 to 4.23, and the median and mean values were 3.42 and 2.53, respectively. In the analysis in the stable metabolic condition in patients with late-onset type, ORA and ORA/Cit ratio were between 0.88 and 3.23 The median and mean values of ORA were 1.96, 2.42, respectively, and those of the ORA/Cit ratio were 0.15 and 0.18, respectively. In analysis of DBS samples collected in the neonatal period of 4 patients with late-onset type, OA was 030, 0.44, 1.32, and 2.96 nmol/mL, while the OA/Cit ratio of the 4 cases was 0.06, 0.06, 0.16, 0.50, respectively. These values reveal that only Case 4 exceeded the screening cutoffs (ORA 2.96 vs cutoff−3.0 nmol/mL, and ORA/Cit 0.50 vs cutoff−0.34). Cases 1, 2, and 3 all had ORA and ORA/Cit well within the normal newborn range. Fig. 3 plots ORA vs ORA/Cit for these cases against the distribution of the 6,562 pilot study newborns. Cases 1−3 fall squarely within the cluster of normal newborns, whereas Case 4 is an obvious outlier (high ORA and ORA/Cit), indicating a positive screen.

Discussion

In this study, we developed a novel screening method for

Table 2. Data are shown for acute-phase and stable-phase samples from neonatal-onset and late-onset OTCD cases, as well as neonatal DBS collected from late-onset patients

	ORA	ORA/Cit ratio
Normal control DBS in NBS (N=4,605)	1.27±0.48	0.124±0.061
[cut off value]	[>2.99]	[>0.34]
Prospective DBS in pilot study (N=6,562)	1.49±0.40	0.124±0.061
[cut off value]	[>2.63]	[>0.32]
Acute condition		
Neonatal onset form case (N=14)	6.5 to 132.9	0.74 to 49.1
Late onset form case (N=5)	8.75, 13.86, 25.64, 27.1, 58.45	0.55, 0.75, 3.42, 3.69, 4.23
Stable condition		
After neonatal samples from neonatal onset form case (N=7)	1.40, 1.57, 1.96, 2.57 3.29, 3.34, 5.59	0.13, 0.15, 0.15, 0.21, 0.24, 0.31, 0.35
After neonatal samples from late onset form (N=4)	0.88, 1.38, 1.41, 3.23	0.1, 0.04, 0.06, 0.2
NBS sample from late onset form (N=4)	0.30 (case 1), 0.44 (case 2), 1.32 (case 3), 2.96 (case 4)	0.06 (case 1), 0.06 (case 2), 0.16 (case 3), 0.50 (case 4)

For patient sample evaluation, cutoff values derived from the prospective cohort (ORA: 2.63 nmol/mL; ORA/Cit: 0.32). The normal control values are presented as mean±SD (standard deviation). Abnormal values exceeding the respective cutoffs are underlined.

OTCD using MS/MS without any derivatization steps. The quantification of amino acids and acylcarnitine in the NBS has been performed in the positive ESI mode, but this method allows the quantification of ORA by analyzing it separately in negative ESI mode[8]. Previously, detecting acidic compounds like ORA in the same run as basic amino acids was challenging because older MS/MS instruments could not rapidly switch polarities[9]. However, modern MS/MS systems have improved substantially and can alternate between positive and negative ion modes within a single injection[9]. This capability allows ORA assay to be incorporated into routine NBS assay at many NBS laboratories with minimal changes to the existing workflow.

Our method showed excellent linearity between 0.5 and 100.0 nmol/mL with a detection limit of less than 1.0 nmol/mL. The only modification to the routine NBS protocol was the addition of a labeled ORA internal standard to the extraction solution. No extra extraction or derivatization steps were needed, and the sample preparation remained simple. The cost implications are negligible—the labeled ORA standard adds a very small expense, and there are no additional consumables or instruments required. In addition, our method allows us to perform analysis without increasing the analysis time. This ORA assay is straightforward to implement in existing NBS laboratories: it does not appreciably increase labor, time, or cost, making it a practical addition to current screening markers.

Through the retrospective analysis of over 4,605 normal newborn samples, we established preliminary reference ranges for blood ORA and the ORA/Cit ratio in healthy neonates. Both distributions were approximately normal (bell-shaped), which allowed us to use a high percentile (99.5th) as a cutoff for defining abnormal results. We did observe that the stored DBS (up to 2 years old at −30°C) had slightly higher ORA and ORA/Cit values on average than the fresh samples from the prospective pilot. The exact reason for this difference is unclear. All measurements were done with the same instrument and protocol, so instrument drift is an unlikely factor. One possible explanation is that prolonged storage might cause a mild increase in measured ORA[12,13]. Strnadová et al. reported that citrulline concentrations decreased by approximately 18.5% per year in DBS stored under freezer conditions, and other amino acids likewise declined over several years[14]. Similarly, Dijkstra et al. reported that 19 out of 22 amino acids, including citrulline, significantly degraded in neonatal DBS stored at +4°C and ambient temperature over up to five years[11]. Taken together, gradual loss of citrulline over time may artificially elevate the ORA/Cit ratio, even if ORA remains stable or changes minimally. Although ORA-specific degradation data are limited, similar physicochemical mechanisms could explain the small increase in ORA units observed in older samples.

The results of retrospective analyses of DBS from OTCD patients revealed that the present method can detect biochemical abnormalities such as increased ORA and ORA/Cit ratio in the acute phase in both neonatal-onset and late-onset forms. On the other hand, the analysis of stable condition samples indicates that there are instances where ORA is not increased even in the neonatal-onset form, which is consistent with the fact that urinary ORA excretion is absent in well-controlled OTCD patients. Analysis of neonatal DBS in patients with late-onset OTCD showed that some patients could be identified by screening before their onset. The patient (Case 4) who was positive in the neonatal DBS analysis had subsequently developed severe hyperammonemia. Such patients represent a potential group that might benefit from NBS by this method. No biochemical abnormality was found in the other three patients. The results may have been influenced by their having received certain interventions at the time of blood collection because they were family cases, or by the fact that they were female. It is important to be aware that screening for OTCD using this method cannot identify all patients with mild forms. Lee et al. reported that pre-symptomatic blood citrulline concentrations measured by MS/MS-NBS were significantly lower in late-onset OTCD[15]. Further large prospective studies are needed to determine the severity spectrum of patients that can be detected in the neonatal period by screening with ORA and citrulline. In the present study, we were unable to evaluate screening performance using citrulline alone. This was because validation of citrulline measurements at low concentrations had not yet been performed in present study, making re-analysis of samples with low citrulline values impractical. Additional data including citrulline concentrations for both retrospective and prospective pilot studies are provided in the Supplementary Data (Table S1). With the advancement of high sensitivity mass spectrometers, low concentrations of citrulline measurement have become feasible. In future work, we plan to investigate a combined screening approach incorporating both low citrulline concentrations and elevated ORA levels.

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