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Towards newborn screening for ornithine transcarbamylase deficiency: Fast non-chromatographic orotic acid quantification from dried blood spots by tandem mass spectrometry

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ABSTRACT

Background: Orotic acid (OA) is the key parameter in the detection of ornithine transcarbamylase deficiency (OTC-D). Inclusion of OA into newborn screening compatibility with existing analytical procedures is necessary.
Methods: OA was eluted from dried blood spots with methanol containing deuterated [1,3–$^{15}N_2$] OA as internal standard. Quantification by tandem mass spectrometry was accomplished without chromatographic separation. Samples were measured in MRM mode for the masses *m/z* 155.1 → 111 for OA and 157.1 → 113 for d2 OA.
Results: OA was determined in a wide range of concentrations with high precision, LOD and LOQ being 0.21 and 0.65 μmol/L, respectively. Values correlated well with those obtained after chromatography. Pretreatment of samples with HCl–butanol regularly used for acylcarnitine measurement did not significantly affect quantitative results. Inclusion of the new method into the standard newborn screening procedure did not alter the results for acylcarnitines or amino acids; the total time per analysis, however, was increased from 1.15 to 1.85 min.
OA levels of 707 unaffected newborns ranged from 0.28 to 3.73 μmol/L. Five newborns with OTC-D showed concentrations of 89.7–211.1 μmol/L. In newborns with severe citrullinaemia we found values in the range of 4.99–127.7 μmol/L.
Conclusions: This new method can be used as a standalone measurement of OA but it can also easily be implemented into standard newborn screening techniques as a useful supplement. In this case the method allows detection of newborns with OTC deficiency without an extra analytical run.

1. Introduction

Ornithine transcarbamylase deficiency (OTC-D) is one of several urea cycle defects (UCD). The incidence of all UCD is estimated to be 1:35,000–1:8000 [1,2]. UCDs often manifest in the neonatal period with severe metabolic decompensation frequently associated with severe brain damage. The neurological outcome critically depends on the duration of hyperammonaemic coma [3]. Rapid diagnosis and specific treatment including glucose and lipid infusion, scavenger drugs and supplementation of arginine may considerably improve the clinical outcome. Haemodialysis is a further option to eliminate ammonia if conservative treatment fails. Once the acute neonatal crisis has been overcome, liver transplantation is a long term treatment option, especially in male OTC-deficiency (OTC-D). Hepatocyte transplantation is a more experimental procedure to compensate hepatic enzyme deficiency [4]. Obviously, these therapeutic options are not perfectly suitable for every child with UCD; however they may improve the outcome in this group of diseases which are lethal if left untreated.

The most severe UCD is male ornithine transcarbamylase deficiency (OMIM 311250), which is X-chromosomally inherited. Shortly after birth, this dysfunction in the urea cycle leads to severe hyperammonaemia and to elevated orotic acid (OA) concentrations in plasma and urine [5]. After only a few hours or days, the illness can be lethal in hemizygous male newborns [6], but late-onset forms in boys and girls have been described as well [7].

The amino acid pattern is usually unrevealing. In principle OTC-D could be indirectly detected on the basis of elevated glutamine levels, which reflect high ammonia concentrations. However, glutamine is hydrolysed during butylation, and consequently cannot be reliably quantified [8]. Elevated levels of citrulline indicate citrullinaemia, but levels below normal are an inconsistent finding in OTC-D [9], since it

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can be decreased in the blood of newborns due to other reasons such as short bowel syndrome [10]; in addition extraction of the amino acids from dried blood depends on many factors such as time, pH, etc. [11]. In contrast high levels of OA in blood or urine are highly suspicious of OTC-D although this finding is not specific. Other UCDs like citrullinaemia type I (OMIM 215700), argininosuccinate lyase deficiency (OMIM 207900) and arginase deficiency (OMIM 207800) also show increased concentrations of OA in blood and urine [12]; Sass et al. outlined that OA concentration in serum is elevated in argininosuccinic acid synthetase deficiency and leads to increased renal clearance [13].

OA is a pyrimidine (6-carboxyuracil; CAS [65-86-1]) and is an intermediate product in the biosynthesis of pyrimidines. It is usually quantitatively measured, primarily in urine, in the context of purine and pyrimidine determination by means of HPLC [14], but can also be identified in plasma [5,13]. Determination by LC-MS/MS has also been described [15,16]. Furthermore, recovery from urine by means of solid phase extraction (SPE) and subsequent derivatisation for analysis by GC/MS has been reported [17]. None of these methods would be suited for mass newborn screening.

D'Apolito et al. [18,19] developed a chromatographic method for OA in urine, plasma and dried blood. The substance was identified by means of hydrophilic interaction (HILIC) liquid chromatography coupled to tandem mass spectrometry (MS/MS). This procedure cannot easily be integrated into standard newborn screening either, because it needs an extra run of 4 min per sample. It was our goal to develop a method which on the one hand is as simple as possible for selective measurement of OA but which on the other hand could be part of an already existing tandem mass spectrum run of newborn metabolic screening.

2. Materials and methods

2.1. Reagents

OA was supplied from Sigma-Aldrich (Deisenhofen, Germany). [1,3–$^{15}N_2$] OA was purchased from Cambridge Isotope Laboratories (LCG EURISO-TOP, Saarbruecken, Germany). Methanol, acetonitrile and de-ionised water were used in the highest purified quality made for liquid chromatography and mass spectrometry delivered by Biosolve BV (Valkenswaard, The Netherlands). Filter paper cards were from Munktell & Filtrak (Bärenstein, Germany).

2.2. Calibrators and controls

A stock solution of 2.5 mmol/L OA was prepared. This was diluted further and used to spike whole blood from a healthy donor so that the added OA concentrations amounted to 0 (blank), 0.25, 0.5, 1.25, 2.5, 5, 10 and 20 μmol/L. Similarly, three control samples with concentrations of 0.25, 0.75 und 5.0 μmol/L were produced. The calibrators and control samples were spotted onto standard filter cards (25 μL per drop) and dried overnight at room temperature in the dark. The cards were stored until use at −20 °C in the refrigerator.

2.3. Internal standard preparation

For internal standards stock solutions of [1,3–$^{15}N_2$] OA at a concentration of 350 μmol/L were dissolved in methanol and stored at −20 °C. Immediately before use 30 μL of the stock solution was diluted with methanol to give a final concentration of 105 nmol/L (extraction solution).

2.4. Sample preparation for tandem mass spectrometry

Discs of 3.2 mm diameter from the dried blood spots were extracted for 30 min in microtiter plates (MTP) with 100 μL extraction solution while shaking the plate gently. The supernatant was placed into a new MTP and dried in a stream of nitrogen at 65 °C. The residue was resolved in 200 μL methanol/water (80:20 vol/vol) and subsequently centrifuged for 10 min at 1,280 rcf to remove any residues from the blood spot material for MS/MS analysis.

2.5. MS/MS analysis

To measure OA analyses were done on a Quattro Micro MS/MS system (Waters, Eschborn, Germany). The device was equipped with an ESI interface. Argon at a pressure of 3.3×10^{-3} mbar was used for collision; nebulising gas was nitrogen. The source temperature was set at 120 °C. Capillary voltage was 3.0 kV. OA was detected in ESI negative mode (ESI−) by multiple reaction monitoring (MRM). Collision energy was 10 eV, cone voltage 20 V, and dwell time 0.1 s. The analysed transitions were m/z 155.1 → 111 (daughter fragment) for OA and 157.1 → 113 for d2 OA. For analysis 20 μL of the sample solution was directly injected into the sample loop adjusted to a constant flow of 35 μL/min acetonitrile/water (80:20 vol/vol).

For full integration into newborn screening described by Chace et al. [20] and Sander et al. [21] some adjustments of the method were necessary. The flow rate was held constant at 60 μL/min to 1.05 min and was increased to 350 μL/min. The entire duration time between injections was 1.85 min each. The concentrations of acylcarnitines and amino acids of the respective samples were compared with the results from the routine screening without implementation of orotic acid determination.

2.6. Additional tests for method comparison

Chromatographic separation

In order to evaluate our new method by comparison we also analysed 41 samples (Fig. 1) spanning a range of 0.5 to 120 μmol/L OA using the chromatographic separation described by D'Apolino et al. [18,19]. Chromatography was performed on an Atlantis HILIC column (Silica, 3 μm, 2.1 × 150 mm, Waters, Eschborn, Germany) at a flow rate of 0.30 mL/min using an isocratic solvent of 85:15 (vol/vol) acetonitrile and aqueous 10 mmol/L ammonium acetate adjusted to pH 2.8. A volume of 10 μL was injected. Quantification was done by MS/MS as described above; the argon pressure, however, was adjusted to 4.5×10^{-3} mbar and capillary voltage was 0.25 kV.

Sample derivatisation with butanolic HCl

As in standard MS/MS analysis of many newborn screening programmes amino acids and acylcarnitines are derivatised to butanol esters by treatment of the residues of the methanolic extracts with 3 N butanolic HCl, we also tested the influence of that reagent on OA measurement. For this purpose calibrators and controls were extracted in triplicates with 100 μL internal standard solution and dried under

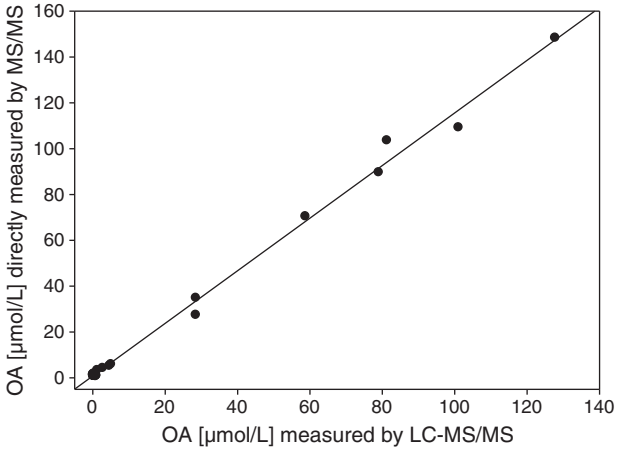

Fig. 1. Method comparison: MS/MS versus LC-MS/MS.