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High-throughput tandem mass spectrometry multiplex analysis for newborn urinary screening of creatine synthesis and transport disorders, triple H syndrome and OTC deficiency

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ABSTRACT

Background: Creatine synthesis and transport disorders, Triple H syndrome and ornithine transcarbamylase deficiency are treatable inborn errors of metabolism. Early screening of patients was found to be beneficial. Mass spectrometry analysis of specific urinary biomarkers might lead to early detection and treatment in the neonatal period. We developed a high-throughput mass spectrometry methodology applicable to newborn screening using dried urine on filter paper for these aforementioned diseases.
Methods: A high-throughput methodology was devised for the simultaneous analysis of creatine, guanidineacetic acid, orotic acid, uracil, creatinine and respective internal standards, using both positive and negative electrospray ionization modes, depending on the compound.
Results: The precision and accuracy varied by <15%. Stability during storage at different temperatures was confirmed for three weeks. The limits of detection and quantification for each biomarker varied from 0.3 to 6.3 μmol/l and from 1.0 to 20.9 μmol/l, respectively. Analyses of urine specimens from affected patients revealed abnormal results. Targeted biomarkers in urine were detected in the first weeks of life.
Conclusions: This rapid, simple and robust liquid chromatography/tandem mass spectrometry methodology is an efficient tool applicable to urine screening for inherited disorders by biochemical laboratories.

1. Introduction

The Provincial Mass Urinary Screening Program for inherited metabolic disorders was initiated in 1971 in the Province of Quebec and the Territory of Nunavut as part of a preventive genetic medicine program [1–3] supported by the Quebec Ministry of Health and Social Services. The main goal of the program is the early detection and prevention of morbidity and mortality due to late-onset genetic metabolic diseases, some detectable only by analysis of urine, before the onset of clinical symptoms. More than 3,000,000 babies have been screened for 25 inherited Mendelian disorders of amino acids and organic acids (500 samples per day, totalizing 76,000 samples in 2013). Urine samples from infants at 21 days of age are collected on filter paper by parents and submitted to a central laboratory for analysis (CHUS). Voluntary compliance has averaged 90% per year. Disorders detected are divided into 2 groups: 1) urea cycle disorders and organic acidurias; 2) disorders of amino acid metabolism and transport [1]. Samples are analyzed using a multiplex thin layer chromatography technique with a sequential-four reagent staining methodology [1].

The screening program is a dynamic model that has evolved throughout the years to screen as many treatable disorders as possible [1–7]. The work presented here reports a plan to screen disorders of creatine synthesis and transport, as well as ornithine transcarbamylase (OTC) deficiency and Triple H (Hyperornithinemia, hyperammoniemia, and homocitrullinuria) syndrome using tandem mass spectrometry technology. These disorders were selected for many reasons: 1) creatine synthesis and transport disorders are underdiagnosed and present non-specific symptoms with tremendous variability [8,9], thus are good candidates for treatment with possible better outcomes, if started early. Clinical manifestations, among others, are mental retardation (moderate to severe), speech and growth delay, epilepsy, autistic traits, and hyperactivity [10,11]; 2) severe clinical manifestations are associated with OTC including but not limited to neonatal hyperammonemia, ataxia, seizures, growth and intellectual delay, and progressive liver damages [12,13]; 3) Triple H syndrome exhibits marked phenotypic heterogeneity, including variability in the age of onset of the disease, protein intolerance, vomiting, growth delay, ataxia, hypotonia, persistent or

Abbreviations: Triple H, hyperornithinemia, hyperammoniemia, and homocitrullinuria; OTC, ornithine transcarbamoylase; CHUS, Centre hospitalier universitaire de Sherbrooke; AGAT, arginine:glycine amidino-transferase; GAA, guanidineacetic acid; GAMT, guanidinoacetate *N*-methyltransferase; ERNDIM, European Research Network for evaluation and improvement of screening Diagnosis and treatment of Inherited disorders of Metabolism; CRTR, creatine transporter; ACN, acetonitrile; FA, formic acid.

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recurrent liver dysfunctions, hepatocellular necrosis, and seizures in the neonatal period which may lead to coma and death in the most severe forms [14]. Urine is the matrix of choice for early detection considering that dried blood spots collected in the neonatal period were found to be unreliable [14]. Moreover, a "founder effect" in the province of Quebec has resulted in an extraordinarily high incidence of the disease [15].

Many reasons support the feasibility and importance of the early diagnosis of these conditions. First, a deficiency in creatine synthesis due to arginine–glycine amidinotransferase (AGAT) led to abnormal metabolite excretion in a 15 day-old baby who presented decreased urinary concentrations of creatine and guanidineacetic acid (GAA) [16]. Moreover, a defect of guanidinoacetate methyltransferase (GAMT) led to increased concentrations of guanidineacetic acid easily detectable in a urine specimen from a 21 day-old baby [17]. The authors mentioned that urinary GAA was already increased on the second day of life with even greater urinary excretion at 21 days of age. It was reported that urinary creatine was increased in the creatine transporter disorder (mutated SLC6A8 gene) [18,19] whereas it was found to be normal in plasma [19]. This study by van de Kamp et al. reported that 81 patients with creatine transporter defect had abnormal urinary creatine/creatinine concentrations while analyses of all the available plasma specimens (children <10 years) tested were normal. Urine would appear to be the matrix of choice for the early detection of affected infants. The incidence of OTC is quite variable with 1:14,000 [20], 1:70,000 in Italy [21], 1:62,000 in Finland [22], and 1:77,000 in Australia [23]. Many women with late-onset manifestations are often underdiagnosed [24]. In both OTC and Triple H syndrome, orotic acid and uracil are increased in urine [25,26]. A recent study showed that newborn blood screening at 2 days of age revealed normal ornithine results in Triple H patients with F188Δ mutation [14]. Another study showed that a patient diagnosed early with Triple H syndrome had increased urinary excretion of orotic acid detectable at 4 days [27].

Our study had 2 main objectives: 1) to develop and validate a robust high-throughput multiplex tandem mass spectrometry methodology for the analysis of urine metabolites collected on filter paper related to creatine synthesis and transport disorders, OTC and Triple H syndrome which might be applicable to a mass urine screening program; in order to complete the validation of this method, we will perform the analysis of known affected clinical patients as well as samples obtained from the *European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders* (ERNDIM) for comparison; and 2) to assess the usefulness of these biomarkers for screening (mass or high-risk) newborn babies.

2. Materials and methods

2.1. Ethics approval

Institutional Research Board approval will be obtained prior to starting the project. This project involves the addition of treatable inborn errors of metabolism in a voluntary newborn urine screening program. Therefore, an informed consent (a leaflet of explanation will be distributed to parents) will be obtained from parents, mainly as an opting-out procedure. All samples from positive clinical cases, as well as anonymized controls analyzed in this project were part of a thorough investigation, required by physicians, aiming to improve high-risk screening or confirm the diagnosis of patients.

2.2. Urine specimens collected on filter paper

Random urine specimens collected on filter paper (Whatman-GE 903) from two hundred 21-day-old anonymized full-term newborns, as well as premature newborns, who had normal amino acid and organic acid profiles, were selected as healthy controls to establish normal reference values at this particular age for the analysis of creatine, guanidineacetic acid, orotic acid, uracil, and creatinine. It is noteworthy to mention that preterm newborn values were included in the healthy reference range established, considering that approximately 8% of babies are premature in the province of Quebec, with a birth weight of <2000 g (at ≤37 weeks of gestation). All samples were received by regular postal service and stored at room temperature until analysis. Liquid urine specimens were also available from 4 patients in whom the diagnosis was confirmed with creatine synthesis and transport disorders (3 patients with GAMT and 1 with CRTR), 2 patients with Triple H syndrome and 1 patient with OTC deficiency. The specimens were stored at −20 °C until analysis. They were then thawed and deposited on filter paper for analysis.

2.3. Preparation of standard solutions

Optima® LC-MS grade water (H_2O), ACS grade ammonium hydroxide (NH_4OH) 29%, and ammonium formate were from Fisher Scientific. Solvents such as HPLC grade methanol (MeOH) and LC-MS grade acetonitrile (ACN) were from EMD Chemicals Inc. Formic acid (FA) 99% purity was from Acros Organics. All calibration standards (creatine, guanidineacetic acid, orotic acid, uracil, and creatinine) were from Sigma-Aldrich. [1,3-^{15} N_2] uracil and [1,3-^{15} N_2] orotic acid:H_2O were from Cambridge Isotope Labs. Creatine-d_3 H_2O, creatinine-d_3 and guanidineacetic-2,2-d_2 acid internal standards were from C/D/N Isotopes (Pointe-Claire, QC). The molecular structures of the 5 metabolites and their respective internal standards are shown in Fig. 1.

2.3.1. Standard curve stock solutions

Stock solutions of creatine, guanidineacetic acid, orotic acid, uracil, and creatinine were prepared in water at concentrations of 50, 5, 2.5, 2.5 and 100 mmol/l, respectively. All solutions underwent sonication with care taken to ensure that dissolution was complete. Stock solutions were stored at −20 °C and were stable for at least 3 months.

2.3.2. Standard curve and quality control working solutions

Working solutions were a mixture composed of creatine, guanidineacetic acid, orotic acid, uracil, and creatinine prepared by diluting stock solutions in volumetric flasks with water. The final concentrations are available in Table 1. A total of 6 standard curve and 3 quality control working solutions (low, medium and high concentrations) were prepared. Working solutions were stable at −20 °C for at least 3 months.

2.3.3. Internal standard (IS) stock solutions

Stock solutions of the 5 internal standards [1,3-^{15} N_2] orotic acid: H_2O, guanidineacetic-2,2-d_2 acid, creatinine-d_3, creatine-d_3 H_2O, and [1,3-^{15} N_2] uracil, were prepared in water at a concentration of 5 mmol/l. All solutions underwent sonication with care taken to ensure that dissolution was complete. Stock solutions were stored at −20 °C and showed no sign of degradation for at least 4 months.

2.3.4. Internal standard working solution

A mixed internal standard working solution composed of [1,3-^{15} N_2] orotic acid:H_2O at 8 μmol/l, guanidineacetic-2,2-d_2 acid at 6 μmol/l, creatinine-d_3 at 1.5 μmol/l, creatine-d_3 H_2O at 1.5 μmol/l, and [1,3-^{15} N_2] uracil at 16 μmol/l was prepared by diluting stock solutions in a volumetric flask with water. This solution was stored at 4 °C and was stable for at least 4 months.

2.4. Sample preparation

2.4.1. Processing urine samples from patients and healthy reference controls

Urine samples were collected on 10 × 10 cm No. 903 virgin filter papers (Whatman-GE) (about 4 ml of urine will saturate the filter paper). Briefly, urine collection was performed at random, but preferably in the morning, by parents at home as part of a voluntary compliance to the

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