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# Advances in the metabolic profiling of acidic compounds in children's urines achieved by comprehensive two-dimensional gas chromatography

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ABSTRACT

The main objective of this work was to evaluate a comprehensive two-dimensional gas chromatographic (GCxGC) coupled to quadrupole mass spectrometry (qMS) method in the field of biomarker candidates' discovery. To this purpose we developed a GCxGC-qMS method suitable for the separation of organic acids and other classes of compounds with silylable polar hydrogen such as sugars, amino-acids, and vitamins. As compared to those obtained by a widely used 1D-GC method, the urinary chromatographic profiles performed by the proposed 2D-GC method exhibit higher resolution and sensitivity, leading to the detection of up to 92 additional compounds in some urine samples including some well-known biomarkers.

In order to validate the proposed method we focused on three metabolites of interest with various functional groups and polarities including $CH_3$-malonic acid (MMA: biomarker of methylmalonic acidemia), 3-hydroxy-3-methyl-glutaric acid (3-OHMGA: biomarker of 3-hydroxy-3-methylglutaric acidemia), and phenylpiruvic acid (PhPA: marker of phenylketonuria). While these three metabolites can be considered as representative of organic acids classically determined by 1D-GC, they cannot be representative of new detected metabolites. Thus, we also focused on quinolic acid (QUIN), taken as an example of biomarker not detected at basal levels with the classical 1D GC-qMS method. In order to obtain sufficient recoveries for all tested compounds, we developed a sample preparation protocol including a step of urea removal followed by two extraction steps using two solvents of different polarity and selectivity. Recoveries with the proposed method reached more than 80% for all targeted compounds and the linearity was satisfactory up to 50 μmol/L. The CVs of the within-run and within-laboratory precisions were less than 8% for all tested compounds. The limits of quantification (LOQs) were 0.6 μmol/L for MMA, 0.4 μmol/L for 3-OHMGA, 0.7 μmol/L for PhPA, and 1 μmol/L for QUIN. The LOQs of these metabolites obtained by a classical GC-MS method under the same chromatographic conditions were 5 μmol/L for MMA, 4 μmol/L for 3-OHMGA, 6 μmol/L for PhPA while QUIN was below the limit of detection. As compared to 1D-GC, these results highlight the enhanced detectability of urine metabolites by the 2D-GC technique. Our results also show that for each new detected compound it is necessary to develop and validate an appropriate sample preparation procedure.



## 1. Introduction

Among inborn errors of metabolism (IEMs) organic aciduria (OA) is a large subgroup characterized by the presence or the increased level of organic acids in urine [1,2]. According to the Organic Acidemia Association [3], OA can affect approximately one out of 5000 births. OA is mostly associated with genetic conditions resulting in a specific step of amino acid catabolism dysfunction usually due to a deficient enzyme activity. Due to a block in the specific metabolic pathway, the resulting organic acids accumulation in biological fluids, namely blood and urine, irremediably leads to the alteration of the acid-base balance and the alteration of the

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pathways of the intermediary metabolism [4]. Such alterations can produce disease states that range from mild to lethal neurological involvement.

In some instances, the development and prognosis of affected children may depend on an early accurate diagnosis followed by a specific therapeutically intervention [5]. Sometimes clinical symptoms suggest a specific diagnosis that can be confirmed by routine metabolic screening tests or by more specific tests [4]. Nevertheless, gas chromatography coupled to mass spectrometry (GC-MS) remains the most used analytical technique for studying urinary organic compounds [5].

However, after analysing by GC-MS more than 1000 urine samples collected from children with neurological disorders in the Armand Trousseau-La Roche-Guyon hospital (Assistance Publique Hôpitaux de Paris), less than 0.5% of them were positive by this technique as to what concerns OA diagnosis [3,4]. It is therefore urgent to look for some new biomarker candidates that can help establish a reliable diagnosis for other metabolic disorders than OA.

Nowadays, profiling of urinary metabolome is an unavoidable approach for the discovery of new biomarker candidates in the field of metabolic disorders. Searching for new biomarker candidates by classical techniques of separation like GC-MS is a challenging task because human urine is a complex mixture of many types and classes of compounds at varying concentrations. In order to improve urine organic compounds profiling and to look for some new biomarker candidates a more efficient separation method based on multidimensional gas chromatography should be more suitable than single GC-MS.

Compared to GC comprehensive two-dimensional gas chromatography (GCxGC) shows great resolution power and high peak capacity because in this technique small portions of the first column effluents are sampled and introduced into a second column for an additional separation. For instance, in 1998 such a method has been successfully applied to the separation of D/L-enantiomers of lactic acid and alpha hydroxyl-carboxylic acid derivatives thus complementing the analysis of urinary organic acids [6]. More recently a GCxGC-MS method for searching and quantifying target pathological metabolites of five enzymatic deficiencies has been developed [7]. GCxGC-MS has been also used to identify crotonyl glycine as an accurate biomarker of 3-hydroxy-3- methylglutaryl CoA synthase deficiency [8]. In the same time, GCxGC coupled to time of flight mass spectrometric detection (GCxGC-TOFMS) has been proposed for the separation and semi- quantification of a metabolomic-type mixture with a microwave assisted silylation [9]. The same method was used for the quantitative analysis of amino acid enantiomers in serum and urine [10], as well as for the investigation of the volatile metabolites relative to oxidative stress in smokers' urine [11].

The main objective of this work is to evaluate the GCxGC–MS technique for the both qualitative and quantitative metabolic profiling of children's urines. To this purpose, we developed and compared a protocol using GCxGC–MS with the most widely used GC–MS method for OA diagnosis. In order to better evaluate the advantages of using GCxGC, we used the same detector namely a quadrupole mass spectrometer (qMS) for both procedures.

Furthermore, in order to evaluate the quantitative aspect of GCxGC for newly detected biomarker candidates, we focused on the quantification of quinolic acid (QUIN), taken as an example. QUIN is detected at basal levels by the proposed GCxGC–MS method while it is not detected by the classical GC–MS method. Quinolinic acid (QUIN) is a metabolite of the kinurenine pathway, which is one of the most important pathways of tryptophan metabolism. QUIN is involved in complex inter-relationships between inflammatory and apoptotic responses associated with neuronal cell damage. Depending on its concentration, QUIN can lead to neuronal death or to chronically neuronal dysfunction [12]. QUIN is also foreseen as a candidate factor in the complex and multifactorial cascade of Alzheimer disease neurodegeneration [13]. As QUIN is associated with neuro-inflammation, it could also be involved in the diagnosis of autism [14] and Huntington's disease [15]. Present findings provide new evidence that exposure to increased level of tryphtophan metabolites including QUIN during development can affect behavior in adulthood [16]. Such exposition may contribute to cognitive and behavioral deficits associated with various neuropsychiatric disorders, such as schizophrenia [17].

## 2. Material and methods

### 2.1. Sample collection

Urines samples were collected from children ($n = 8$, 1 to 9 years old) suffering neurological disorders with unknown etiology. Urine samples were collected at the Department of Pediatric Neurology of the Hospital Trousseau (Paris, France) as part of normal clinical management with the written informed consent of parents or legal representatives of each patient. This study was performed according to French Public health regulations (Code de la santé publique – Article L1121-3, modified by Law n°2011–2012, December 29 2011 – Article 5). Healthy children ($n = 30$, aged from 1 to 10) who participated to the study with the written consent of their parents were on a free and unrestricted diet, without symptoms of any disease and any supplemental vitamin or drug.

### 2.2. Sample preparation

The first morning urine was collected in sterile polystyrene containers. After centrifugation at 2000 g during 5 min for elimination of the urinary sediment, creatinine concentration was measured in each urine sample. Urine samples were then frozen at −20 °C until analysis.

In this work, two sample preparation procedures were performed as follow.

#### 2.2.1. Procedure 1

This procedure is derived from a commonly used sample preparation procedure for the GC–MS determination of urinary organic acids [18,19] with a minor modification. After thawing at ambient temperature and before extraction and silylation of organic acids, urea was removed from urine samples according to a previously described procedure [18] with some modifications. Briefly, 9 volumes of urine sample were buffered at pH 8 by adding 1 volume of 2 M potassium phosphate or bicarbonate. Urea is then removed from urine sample by incubation with TYPE IX urease (from *Canavalia ensiformis*, Sigma–Aldrich, 100 U per mL of urine sample) for 15 min at 25 °C. Extraction and silylation of organic acids from urea depleted urine samples were then performed as previously described [19]. Briefly, a liquid–liquid extraction was performed by adding 2 mL of ethyl acetate to the urea-depleted urine sample and centrifuged for 5 min at 5000 rpm. Then, ethyl acetic phase was collected and completely evaporated under a vacuum at room temperature (Savant SPD 1010, Speedvac concentrator, Thermo Fisher Scientific, USA). Finally, the silylation of the dry extract was performed using 50 μL of pyridine and 50 μL of BSTFA with 1% TMSC.

#### 2.2.2. Procedure 2

After extraction of organic acids with ethyl acetate following the procedure 1, the remaining aqueous phase was subjected to a second extraction step. The aqueous phase was first buffered at pH 9 with 32% NaOH. After adding 36 μL of triethylamine for a final concentration of 0.25 M, the resulting solution was incubated at 60 °C for 15 min. After adding 200 μl of 2.4 mol/L HCl, the resulting mixture was extracted with 2 mL of tetrahydrofurane (THF) after