Clinica Chimica Acta 485 (2018) 236–242

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Urine metabolome analysis by gas chromatography–mass spectrometry (GC–MS): Standardization and optimization of protocols for urea removal and short-term sample storage

Francesco Palmas[a], Michele Mussap[b,*], Claudia Fattuoni[a]

[a] Department of Chemical and Geological Sciences, University of Cagliari, I-09042, Italy
[b] Department of Surgical Sciences, University of Cagliari, Italy

ARTICLE INFO

Keywords:
Metabolomics, urea removal, gas chromatography–mass spectrometry (GC–MS)
Urease
Urine sample storage protocols
Pre-analytical phase

ABSTRACT

Background: Before derivatization, urine analyzed by gas chromatography–mass spectrometry (GC–MS) requires the complete removal of urea to avoid interferences. We aimed at establishing the most effective sample pre-treatment for urea removing; moreover, we explored the impact of two short-term sample storage conditions on urine metabolome.

Methods: 92 aliquots were obtained from a single sample collected from a healthy adult; they were divided into 6 groups. Group 1 consisted of untreated aliquots while groups 2–6 differed from each other for the addition of various defined urease solution volumes combined with either 30 min or 1-hour sonication time. Urine sample storage was tested by comparing 20 fresh aliquots analyzed after collection with 20 aliquots frozen at −80 °C for 72 h.

Results: the most effective protocol consisted of the combination between 200 μL urease solution with 1-h sonication time; urease solution volumes > 200 μL increase the risk to underestimate metabolite peaks because of sample dilution. Short-term storage of samples at −80 °C pointed out significant changes in the urine metabolic profile compared with that of fresh samples.

Conclusions: our study confirms the importance of urea removal for a reliable recognition and quantitation of metabolites; urine short-term storage at −80 °C should be carefully reconsidered.

1. Introduction

Metabolomics is a high-throughput 'omic' strategy for the evaluation and the monitoring of the global metabolic profile in biological fluids and tissues. Urine is a popular fluid in metabolomics [1], mostly in neonatology and pediatrics [2–7]; both exogenous and endogenous metabolites are abundant in urine, reflecting prompt changes in metabolic pathways, as a result of the interplay between genotype and environmental factors: namely the phenotype [8,9]. The most common analytical techniques for metabolomics studies are hyphenated techniques; in particular, gas chromatography–mass spectrometry (GC–MS) requires an extensive pre-analytical phase consisting of sample derivatization [10]. Two crucial steps for urine metabolomics performed by GC–MS are urease pre-treatment and sample storage. Urine should be pre-treated by urease to remove the significant urea amount; this metabolite may interfere with the chemical derivatization, leading to an incomplete chemical transformation. Further interferences are mainly instrument-related, such as chromatographic column overloading, peak distortions, and the likelihood of co-eluting metabolites peaks. Urease pre-treatment was described early in the literature [11]; later, various studies investigated the effects of pre-treatment on GC–MS results, originating controversial results [12–14]. Urine storage is also a pivotal pre-analytical step: under different storage conditions, urine metabolic profile may exhibit unexpected variability, ascribable to chemical and physical changes of metabolites [15,16]. Samples storage requires a rigorous standardization, especially in large-scale studies on urine either locally collected and stored until analysis or taken from biobanks. A group of experts has previously recommended to store urine at

Abbreviations: ASD, Autism Spectrum Disorder; BPD, Bronchopulmonary Dysplasia; ^{1}H NMR, Proton Nuclear Magnetic Resonance; GC, Gas Chromatography; LC, Liquid Chromatography; CE, Capillary Electrophoresis; MS, Mass Spectrometry; TCA, Tricarboxylic Acid; MSTFA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; AMDIS, Automated Mass Spectral Deconvolution and Identification System; NIST08, National Institute of Standards and Technology Mass Spectral Database; PCA, Principal Component Analysis; PLS-DA, Partial Least Squares Discriminant Analysis; LOOCV, Leave One Out Cross Validation; DTT, Dithiothreitol

* Corresponding author at: Laboratory Medicine Department of Surgical Sciences, University of Cagliari, Italy.
E-mail address: mumike153@gmail.com (M. Mussap).

https://doi.org/10.1016/j.cca.2018.07.006
Received 12 April 2018; Received in revised form 25 June 2018; Accepted 5 July 2018
Available online 06 July 2018

−80 °C or below without additives unless specified for a specific downstream analysis; however, cells and particulate matter should be removed by centrifugation before sample storage [17]. Recently, long-term urine storage at −22 °C revealed a high stability for several substances [18]. Despite recommendations, the lack of univocal results on the ideal urine storage protocol for metabolomic analysis calls for further investigations. Aims of this study were: to assess whether urine pre-treatment by urease entails more benefits than disadvantages; to improve the urease pre-treatment effectiveness by modulating the combination between the volume of urease solution and sonication time; to assess any effect and potential limitation of short-term urine storage at −80 °C.

2. Materials and methods

2.1. Study design

Effects of urea solution volume and sonication time on the effectiveness of urease pre-treatment were evaluated by collecting a urine sample from a healthy adult and then by splitting the sample into 92 aliquots; aliquots were grouped 1–6. Groups differed each other for different combinations of the two variables (Table 1, panel A). Group 1 consisted of untreated aliquots ($n = 19$); in group 2, we added 800 μL of urease solution combined with a sonication time of 30 min ($n = 15$); in group 3, 400 μL for 30 min ($n = 15$); in group 4, 400 μL for 1 h ($n = 14$); in group 5, 200 μL for 30 min ($n = 14$); in group 6, 200 μL for 1 h ($n = 15$). The effects of different storage conditions were tested on 40 aliquots prepared by using a urine sample collected from a healthy adult. Twenty aliquots were analyzed immediately after collection (group 7) and 20 aliquots were analyzed after storage at −80 °C for 72 h (group 8). Both groups 7–8 were pre-treated by adding 800 μL urease solution sonicated for 30 min (Table, 1 panel B). The study was conducted according to the World Medical Association Declaration of Helsinki on ethical conduct of research involving human subjects.

2.2. Specimen processing for GC–MS

Urease solution (1 mg/mL in water) was prepared by using a lyophilized commercially available enzyme (Urease type III, Sigma-Aldrich® catalogue number U1500). The solution was added in variable volumes (depending on the group) to 150 μL of urine sample into Eppendorf® vials. After samples sonication, 800 μL cold methanol were added and then samples were centrifuged 10 min at 14,000 rpm. Depending on the group, 1200, 900 or 750 μL supernatant were transferred in glass vials and evaporated to dryness overnight in an Eppendorf® vacuum centrifuge. Thirty microliters of methoxylamine hydrochloride in pyridine solution 0.24 M were added to each vial; samples were vortex mixed and then left to react for 17 h at room temperature. Subsequently, 30 μL *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were added, followed by a reaction time of 1 h at room temperature. Finally, the derivatized samples were diluted with 600 μL tetracosane in hexane (0.034 g/100 mL) solution, just before GC–MS analysis.

Table 1
Panels of the experimental design for evaluating the pre-treatment with urease solution (panel A) and storage conditions (panel B). Group 1: aliquots without urease pre-treatment; Group 2: aliquots treated with 800 μL urease, sonicated for 30 min; Group 3: aliquots treated with 400 μL urease, sonicated for 30 min; Group 4: aliquots treated with 400 μL urease, sonicated for 1 h; Group 5: aliquots treated with 200 μL urease, sonicated for 30 min; Group 6: aliquots treated with 200 μL urease, sonicated for 1 h; Group 7: aliquots analyzed immediately after sample collection; Group 8: aliquots analyzed after storage at −80 °C for 72 h.

Group	Aliquots (n)	Urease solution (μL)	Methanol (μL)	Sonication time (min)	Supernatant volume (μL)
Panel A					
1	19	–	–	–	–
2	15	800	800	30	1200
3	15	400	800	30	900
4	14	400	800	60	900
5	14	200	800	30	750
6	15	200	800	60	750
Panel B					
7	20	800	800	30	1200
8	15	800	800	30	1200

2.3. GC–MS analysis

Samples were analyzed using a Agilent 5975C interfaced to the GC 7820 (Agilent Technologies, Palo Alto, CA, USA) equipped with a DB-5 ms column (Agilent J&W Scientific, Folsom, CA, USA); the injection temperature was set at 230 °C and the detector temperature at 280 °C. Carrier gas Helium flow rate was 1 mL/min. The GC oven starting temperature program was 90 °C with 1 min hold time and ramping at 10 °C per minute until to a final temperature of 270 °C with 7 min hold time. Then, 1 μL was injected in split (1:20) mode. After a solvent delay of 3 min, mass spectra were acquired in full scan mode using 2.28 scans per second, with a mass range of 50–700 Amu. All chromatograms were analyzed by using the free software Automated Mass Spectral Deconvolution and Identification System (AMDIS, available at http://chemdata.nist.gov/mass-spc/amdis). Metabolites were identified by comparing retention times and mass spectra with those stored in an in-house made library including > 255 metabolites. Other metabolites were identified by using the National Institute of Standards and Technology mass spectral database (NIST08) [19] and the Golm Metabolome Database (GMD) [20], available at http://gmd.mpimp-golm.mpg.de (last access, January 15, 2018). This strategy allowed for the identification of 127 metabolites and the detection of other 23 compounds. Each sample analysis produced a file report with an integrated signal value for each metabolite. All the data were combined to generate a data matrix required for statistical analysis.

2.4. Statistical analysis

We evaluated the intermediate precision, expressed as coefficient of variation (CV, %), by scanning the mass spectra and then by using raw data originated from the intensity measurement of the area under the metabolite peaks, in order to avoid any manipulation/transformation of the original measurements. Intermediate precision was computed for each protocol tested for urea removal and included all the metabolites identified and quantified in this study, including those identified as unknown. Following normalization by sum, either unpaired (urease test) or paired (freezing effects) two-tailed homoscedastic Student's *t*-test were applied to observe significant differences in metabolic concentrations among the groups under study. The control of the error rate was done by applying the false discovery rate (FDR) correction; data were considered statistically significant when $P < 0.05$ [21]. Univariate statistical analysis was performed by using a matrix spreadsheet (Microsoft Excel®, Microsoft Co, Redmond, WA, USA); this was a preliminary step for the following chemometric analysis. Principal component analysis (PCA) was then performed to identify any potential outlier, hence to verify the coherence of samples in separated groups. Partial least squares discriminant analysis (PLS-DA) was applied to produce predictive models for the above-mentioned separations. Normalization by sum, log transformation, and auto scaling were set for calculations. Models were then validated by the Leave One Out Cross Validation (LOOCV) method. Multivariate analysis was developed by means of the web platform MetaboAnalyst 3.0, available at http://www.metaboanalyst.ca [22,23].