Journal of Chromatography B, 935 (2013) 26–31

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Classification of type 2 diabetes rats based on urine amino acids metabolic profiling by liquid chromatography coupled with tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 29 March 2013
Accepted 18 July 2013
Available online 25 July 2013

Keywords:
Underivatized amino acid
LC–MS/MS
Type 2 diabetes
Rat urine

ABSTRACT

An analytical method for quantifying underivatized amino acids (AAs) in urine samples of rats was developed by using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). Classification of type 2 diabetes rats was based on urine amino acids metabolic profiling. LC–MS/MS analysis was applied through chromatographic separation and multiple reactions monitoring (MRM) transitions of MS/MS. Multivariate profile-wide predictive models were constructed using partial least squares discriminant analysis (PLS-DA) by SIMAC-P 11.5 version software package and hierarchical cluster analysis (HCA) by SPSS 18.0 version software. Some amino acids in urine of rats have significant change. The results of the present study prove that this method could perform the quantification of free AAs in urine of rats by using LC–MS/MS. In summary, the PLS-DA and HCA statistical analysis in our research were preferable to differentiate healthy rats and type 2 diabetes rats by the quantification of AAs in their urine samples. In addition, comparing with health group the seven increased amino acids in urine of type 2 rats were returned to normal under the treatment of acarbose.

1. Introduction

Type 2 diabetes is the most common form of diabetes and is the fourth leading cause of global death by disease, which its prevalence is increasing dramatically in both developed and developing countries [1]. As a group of endocrine metabolic diseases, diabetes would lead to the metabolic disorder of several kinds of endogenetic metabolites [2]. So, earlier identification of individuals at risk is particularly important for delaying or preventing the onset of type 2 diabetes and increasing the burden of the condition worldwide [3–5].

Recently it was demonstrated that amino acid metabolism has a potential key role in the pathogenesis of diabetes, and amino acid profiling is very helpful in diabetes risk assessment [6]. A combination of three amino acids (Ile, Leu, Val, Tyr and Phe) could predict future diabeters with a more than fivefold higher risk for individuals in top quartile by screening blood samples from human. These results prove that the amino acids play essential roles in energy metabolism as a cluster metabolite. It is necessary to search for biomarkers of type 2 diabetes with higher predictive values. Therefore, a good analysis assay of amino acids established is needed.

In previous studies, amino acids in body fluids are usually analyzed by classical ion-exchange liquid chromatography, which usually require an analysis time of 2–3 h per sample [7–9]. The combination of high performance liquid chromatography with tandem mass spectrometry (LC–MS/MS) offers a robust, reliable, and economical methodology for quantitative profiling of metabolite classes of a biological specimen. The LC–MS/MS assay based clinical metabolomic approaches has been most frequently applied method. Since most amino acids are very polar, LC flow phase containing ion-pair regents or derivatization (butylation) of sample were required usually when they were analyzed by means of LC–MS/MS [10–17]. Certainly, the combination of derivatization (butylation) of samples and ion-pair reagents used in flow phase of LC, the amino acids from body fluids also can be determined by LC–MS/MS [18]. Though the advances of LC–MS/MS method with using ion-pair reagents and sample derivatization for the analysis of amino acids are observed obviously, it also has some disadvantages, for example, ion-pair reagents may short the lifetime of the LC column and may eventually cause ion suppression in the mass spectrometry.

In this study, an analytical method for quantifying underivatized amino acids (AAs) in urine samples of rats was developed by using

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http://dx.doi.org/10.1016/j.jchromb.2013.07.016

LC–MS/MS, and this method did not use ion-pair reagents in flow phase. Multivariate profile-wide predictive models are constructed using partial least squares discriminant analysis (PLS-DA) and hierarchical cluster analysis (HCA). Statistical analysis results described here are preferable to differentiate the healthy rats and the type 2 diabetes rats through the quantification of AAs in urine samples.

2. Materials and methods

2.1. Reagents

The amino acid standards A9906 were obtained from Sigma Aldrich (St. Louis, MO, USA) which containing β-alanine (β-Ala), α-alanine (α-Ala), L-α-aminoadipic acid (Aad), γ-amino-n-butyric acid (GABA), D, L-β-aminoisobutryic acid (β-AIB), L-arginine (Arg), L-aspartic acid (Asp), L-citrulline (Cit), L-cystathionine (Hcy(Ala)), L-cystine ($(Cys)_2$), L-glutamic acid (Glu), glycine (Gly), L-histidine (His), 1(3)-methyL-histidine (1(3)Me-His), L-homocystine ($(Hcy)_2$), δ-hydroxylysine (Hyl), hydroxyl-L-proline (Hyp), L-isoleucine (Ile), L-leucine (Leu), L-lysine (Lys), L-methionine (Met), L-ornithine (Orn), L-phenylalanine (Phe), L-proline (Pro), L-sarcosine (Sar), L-serine (Ser), taurine (Tau), L-threonine (Thr), L-tryptophan (Trp), L-tyrosine (Tyr) and L-valine (Val). The other standards of creatinine (Cre), L-α-amino-n-butryic acid (Abu), homo-cysteine (Hcy), L-glutamine (Gln) and *N, N*-dimethylphenylalanine (*N, N*-Phe) were obtained from Sigma Aldrich (St. Louis, MO, USA), too. HPLC grade acetic acid was obtained from Tedia (USA). HPLC grade methanol was purchased from Fisher Scientific (Fairlawn, NJ). Water was obtained from a Milli-Q Ultra Pure Water Purification Systems (Millipore, Brussels, Belgium). Streptozotocin (STZ) and all the reference standards were purchased from Sigma Corporation (St. Louis, MO, USA).

2.2. Animal experiment

In order to develop a diabetic rat model, fifteen four-week-old male Wistar rats were purchased from Experimental Animal Center of Jilin University (China). Ten rats were fed with high-sucrose and high-fat chow (18% lard, 3% cholesterol, 20% sucrose and 59% standard rat chow) as the diabetic rat model group and the other five rats were fed with standard rat chow as the control group. After ten-week feeding, ten rats fed with high-sucrose and high-fat chow were injected intraperitoneal with STZ freshly prepared in citrate buffer (0.1 mol/L, pH 4.5) at a single dosage of 35 mg/kg body weight. The other five rats (healthy control group) were injected with citrate buffer only. One week later, tail-blood glucose value was detected with OneTouch Ultra Meter (Lifescan Inc., CA, USA). The ten rats injected intraperitoneal with STZ presenting blood glucose levels higher than 16.7 mmol/L were defined as diabetic rats [19]. Then all the fifteen rats were fed with the same standard rat chow and the five of ten diabetic rats were treated with acarbose for a month. Before the experiment, samples of urine were collected from rats fasted for 24 h.

2.3. Sample preparation procedure

The urine samples from rats were prepared as follows: 180 μL methanol solution containing 5 μmol/L *N, N*-Phe as internal standard was added to 20 μL urine sample. For precipitating protein, above samples were vibrated for 5 min at room temperature. Then they were centrifugated at 12,000 × *g* for 20 min, and the resulting supernatant was used for analysis.

2.4. Data processing

Data were acquired with MassLynx4.0 and processed for calibration and for quantification of the analytes with TargetLynx software (Micromass, UK). Data statistics were used by the SIMCA-P 11.5 version software and SPSS 18.0 version software.

2.5. LC–MS/MS conditions and instrumentation

LC–MS/MS analysis was performed using a Xevo TQ MS spectrometer combined with Acquity-Ultra Performance LC system (Waters, Manchester, UK). The column used for separation in LC–MS/MS analysis was a Venusil ASB C_{18} HPLC column (4.6 mm × 250 mm, 5 μm particle size, 150 Å and 300 Å pore size) from Agela Technologies.

The mass spectrometer with electrospray ion source (ESI) was operated in MRM under positive ion mode. (Table 1 was for detail). Our study started with using the Intellistart function optimized the cone voltages and the collision energies to find the most specific and sensitive detection parameters of each amino acid in MRM mode under appropriate instrumental conditions. The source voltage was 3 kV, the source temperature and desolvation temperatures were 150 °C and 350 °C, respectively. The desolvation gas flow was 800 L/h, and the collision gas argon was kept at a pressure of 1.7×10^{-3} mbar.

3. Results and discussion

3.1. Optimization of MS/MS Condition

Intellistart function was firstly used in study to find the best condition parameters of each amino acid in MRM mode. Each amino acid solution (the concentration of each analyte was 50 μmol/L) was directly injected into the mass spectrometer at a rate of 20 μL/min by a syringe pump. The tandem mass spectrometry conditions were as follows: lowest fragment ion mass was 40.00 Da, cone voltage ranges were 0–50 V, collision energy ranges were 0–40 eV, number of daughter transitions were 2, MS tune method was described as the "Tandem mass spectrometry conditions" in Section 2. The most abundant product ion of each analyte has been chosen as specific ion transitions for following the AAs in MRM under positive ion mode as described in Table 1. Among these specific ion transitions, most AAs lost neutral fragment of a formic acid and have a product ion at $[M+H-46]^+$ by a rearrangement. The transitions and corresponding optimal cone voltage and collision energies chosen for MRM of AAs are listed in Table 1.

3.2. Optimization of chromatographic condition

Even with special conditions of MS/MS in the study, amino acids separated by LC were evidently needed to diminish or eliminate the interferences from sample matrix. Therefore, the next step was to analyze the AAs in the LC–MS/MS method applying different mobile phase compositions to obtain the best separation of each analyte on column. We found that acetic acid as the modifier added to mobile phase was optimal for above purpose. The mobile phase consisted of two kinds of solvents: water containing 0.15% acetic acid (solvent A) and 30% methanol containing 0.9% acetic acid aqueous (solvent B) at a flow rate 0.5 ml/min. The optimized elution program was as follows: from 0 to 2.5 min a constant 0% B, from 2.5 to 5.0 min a linear gradient from 0% B to 17% B, from 5.0 to 8.0 min a linear gradient from 17% B to 33% B, from 8.0 to 8.5 min gradient from 33% B to 100%. The column was held at 100% B from 8.5 to 19 min. Then returned to 0% B from 19 to 20 min, and allowed to equilibrate for