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Simultaneous quantification of cardiovascular disease related metabolic risk factors using liquid chromatography tandem mass spectrometry in human serum

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ABSTRACT

Recent observations from metabonomic studies have consistently found that branched-chain amino acids (BCAAs), aromatic amino acids (AAAs), glutamine (Gln), glutamic acid (Glu), Gln/Glu ratio, carnitine, and several species of acylcarnitines and lysophosphatidylcholines (LPCs) are possible risk factors for metabolic diseases such as diabetes mellitus (DM) and cardiovascular diseases (CVD). We described here a simple and reliable method for simultaneous quantification of these metabolic risk factors by liquid chromatography tandem mass spectrometry (LC–MS/MS). Serum samples were extracted with isopropanol, and the extracted metabolites were separated by hydrophilic interaction liquid chromatography (HILIC) and detected with electrospary ionization (ESI) inpositive ion mode with multiple reaction monitor (MRM) mode. All the metabolites were effectively separated within 5.5 min. Analytical recoveries were in the range of 92.8–106.9%, with an average of 100.6%. The intra- run and total imprecisions for the measurement of these metabolites were 1.2–3.8% and 1.5–7.4%, respectively. Serum concentrations of the metabolites were analyzed in 123 apparently healthy volunteers. Significant associations between the metabolites and traditional CVD risk factors were observed. The newly developed LC–MS/MS method was simple, precise, and accurate and can be used as an efficient tool in CVD research and studies.

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1. Introduction

Diabetes mellitus (DM) is considered one of the most serious and prevalent metabolic disorders whose prevalence in adults has doubled over nearly 3 decades [1]. A portfolio of abnormalities of metabolic and macrovascular homeostasis accompanied DM is believed to conspire to lead to accelerated cardiovascular disease (CVD) and premature deaths [2,3]. Therefore, DM has been proposed as an independent risk factor for the development of CVD. Earlier identification of individuals at risk and effective interventions are particularly important for delaying or preventing the onset of DM and CVD [4].

Recent high-throughput metabonomic/metabolomic studies have allowed the characterization of hundreds of metabolites (low-molecular weight compounds) from human biospecimens [5]. These metabolites represent intermediates and end products of metabolic pathways that reflect physiological dysfunctions and may mirror earlier stages of diseases. Several studies have documented that branched-chain amino acids (BCAAs) and aromatic amino acids (AAAs) are strongly correlated with obesity, insulin resistance, and coronary artery diseases [6–8]. Meanwhile, the

Abbreviations: DM, diabetes mellitus; CVD, cardiovascular diseases; BCAA, branched-chain amino acid; AAA, aromatic amino acid; Phe, phenylalanine; Trp, tryptophan; Tyr, tyrosine; Leu, leucine; Ile, isoleucine; Val, valine; Gln, Glutamine; Glu, Glutamic acid; LPC, Lysophosphatidylcholine; LC–MS/MS, Liquid chromatography tandem mass spectrometry; HILIC, hydrophilic interaction liquid chromatography; ESI, electrospary ionization; MRM, multiple reaction monitor; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol; FFBG, fasting blood glucose; hsCRP, high sensitivity C-reactive protein.

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associations of Glutamic acid (Glu), Glutamine (Gln), and Gln/Glu ratio with DM and CVD were also reported [9]. In addition, some species of lysophosphatidylcholines (LPCs), for example, higher levels of LPC 18:0 (stearoyl) and lower value of LPC 18:1 (oleoyl), LPC 18:2 (linoleoyl) as well as higher values of acetylcarnitine have been found to predict impaired glucose tolerance before the onset of DM [10,11]. These observations are consistence and raise the possibility that alterations in plasma metabolite levels could presage the onset of DM and CVD and therefore aid in the identification of at risk individuals by adding information over standard clinical markers. However, the usefulness of these metabolites in CVD risk assessment needs to be confirmed and validated on external independent populations with simple and reliable methods. Analytical methods for respective measurement of species of amino acids, carnitine and acylcarnitines, and LPCs have been reported [12–14], however, it requires separate sample preparations and analyses. It is highly desirable to develop a simple, precise, and reliable method for simultaneous measurement of all the metabolites.

In this study, a liquid chromatography tandem mass spectrometry (LC–MS/MS) method for simultaneous measurement of CVD related metabolic risk factors including BCAAs, AAAs, carnitine, some of the acylcarnitines and LPCs has been established. The relationships between these metabolites and traditional CVD risk factors were analyzed in healthy subjects. The LC–MS/MS method uses small amount of serum samples, requires no sample derivation, separates all the metabolites in 5.5 min, and has high through put. This method has been validated to be simple, precise, and accurate, and can be used in CVD research and studies.

2. Materials and methods

2.1. Chemicals and reagents

Standards of amino acids [phenylalanine (Phe), tryptophan (Trp), tyrosine (Tyr), leucine (Leu), isoleucine (Ile), valine (Val), Gln, Glu], free carnitine, acetylcarnitine, high performance liquid chromatography (HPLC)-grade acetonitrile, ammonium formate and formic acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Standards of LPCs [palmitoyl (LPC 16:0), LPC 18:0, LPC 18:1], oleoylcarnitine (18:1acylcarnitine) and isotopically labeled internal standards of LPCs (LPC 16:0-D31, LPC 18:0-D35) were purchased from Avanti Polar Lipids (Alabaster, AL). LPC 18:2 was purchased from Larodan AB (Malmo, Sweden). Decenoylcarnitine (10:1acylcarnitine), isotopically labeled internal standards for each of the amino acids (Glu-D5, Gln-D5, Val-D8, Ile-D10, Leu-D3, Tyr-D4, Trp-D5 and Phe-D5), carnitine-D3 and acetylcarnitine-D3 with isotopic purities of 99%, were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Decanoylcarnitine (10:0 acylcarnitine)-D3, octadecanoylcarntine (18:0 acylcarnitine)-D3 with isotopic purities of 99% were obtained from C/D/N Isotopes Inc. (Quebec, Canada).

2.2. Serum samples

For method development and validation, fresh serum samples were collected from the leftovers of patient samples in the department of laboratory medicine of Beijing Hospital. Serum was pooled and re-aliquoted and stored at −80 °C until analysis. For analysis of the metabolites in healthy subjects, 123 apparently healthy volunteers (52 males and 71 females) aged 18–81 years were recruited. Fasting blood samples were taken by venipuncture into tubes containing clot-activator. Serum was isolated, frozen, and stored in 1 mL aliquots at −80 °C until analyses. This study had been reviewed and approved by the Ethics Committee of Beijing Hospital. All studied individuals were informed in writing of the intended use of their samples and each provided written consent.

2.3. Preparation of calibrators and internal standards

A series of standard mixture of the metabolites was prepared according to their serum concentration ranges reported in literatures [6–10]. Standards of Val, Ile, Leu, Phe, Tyr, Trp, Glu, Gln, LPC 16:0, LPC 18:0, LPC 18:1, LPC 18:2, carnitine, acetylcarnitine, 10:1 acylcarnitine, and 18:1 acylcarnitine were accurately weighed and transferred into a Class A 50 mL volumetric flask and then methanol was added to the scale to make the mixed stock standard (S1). Calibration curves (5 points for each compound) were prepared by diluting the stock calibrator S1 with methanol to the serial concentrations (S2–S5) as shown in Table 1. A mixed internal standard solution containing the corresponding internal standards for the metabolites was made in methanol. The mixed calibrators and the internal standards were aliquoted and stored in sealed glass tubes at −80 °C until analysis.

2.4. Sample preparation

Serum samples, calibrators and the internal standards were thawed, mixed and equilibrated to room temperature and then precisely transferred by an automatic diluter (Hamilton, Reno, NV, USA). Aliquots of 0.01 mL of the calibrators or serum samples were washed with 0.5 mL of isopropanol into 2 mL vials, followed by the addition of 0.01 mL of the internal standards washed with 0.5 mL isopropanol. The vials were shaken on a mechanical shaker for 20 min, and then centrifuged at $2342 \times g$ for 10 min. An aliquot of 0.2 mL of the supernatant was transferred to another vial and evaporated under a stream of nitrogen. The residue was reconstituted with 0.2 mL of the mobile phase (75% acetonitrile containing 7.5 mmol/L ammonium formate and 0.5% formic acid).

2.5. LC–MS/MS analysis

The LC separation was performed using an Agilent 1200 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) with a Kinetex hydrophilic interaction liquid chromatography (HILIC) silica column (150 mm × 2.1 mm, particle size 2.6 μm, Phenomenex, USA) maintained at 20 °C. Aliquots of 2 μL of the reconstituted samples were injected onto the column and eluted with a mobile phase of 75% acetonitrile containing 7.5 mmol/L ammonium formate and 0.5% formic acid and pH 3.0, at a flow rate of 0.25 mL/min.

MS/MS was carried out on an API 4000 triple quadrupole mass spectrometer (Sciex Applied Biosystems). The MS detection was performed with positive electrospary ionization (ESI) in multiple reaction monitor (MRM) mode at source temperature of 500 °C and a voltage of 5500 V. The curtain gas, nebulizer gas and collision gas were nitrogen at settings of 10, 70 and 12 psi, respectively. The dwell time was 0.08 s for MRM. Applied Biosystems Analyst version 1.6.2 software was used for system control, data collection, and processing.

2.6. Method validations

To evaluate the linearity of the calibration curve, peak area ratios (y) of metabolites to their internal standards were linearly regressed on the corresponding concentrations of the metabolites (x) (5 calibrators, each in duplicate). To test the matrix effect and the analytical recoveries, two levels of mixed standard solutions were added to a serum sample and the concentrations of the metabolites in the spiked and unspiked serum samples were analysed. Matrix effect was assessed by comparing the increase of the absolute responses of the metabolites in spiked serum with those in the

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