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Quantitative analysis of amino acids and acylcarnitines combined with untargeted metabolomics using ultra-high performance liquid chromatography and quadrupole time-of-flight mass spectrometry

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ABSTRACT

Metabolomics is an "omic" technique being increasingly used in epidemiological and clinical studies. We developed a method combining untargeted metabolomics with the quantitative determination of eight amino acids (AA) and eight acylcarnitines (AC) in plasma using ultra-high pressure liquid chromatography (UHPLC), electrospray ionization (ESI) and quadrupole time-of-flight mass spectrometry (QTOFMS). Separation of metabolites is performed by ion-pair reverse phase UHPLC using a HSS T3 column (2.1 × 100 mm, 100 Å, 1.8 μm particle size) and formic acid–ammonium acetate-heptafluorobutyric acid in water and formic acid–ammonium acetate in methanol as mobile phases. Metabolite identification and quantification are achieved using a QTOFMS operating in ESI-positive and full-scan mode along with MS^E acquisition of fragmentation patterns. Targeted metabolites are quantified using the appropriate labeled standards and include branched-chain AA (leucine, isoleucine, valine), aromatic AA (phenylalanine, tyrosine) as well as acetylcarnitine and propionylcarnitine, which have been identified as biomarkers of future cardiometabolic disease risk. The inter-day precision (relative standard deviation) for the targeted method was <15% for all but one metabolite and accuracy (bias) of amino acids ranged from 0.5% to 13.9% using SRM 1950 as the external standard. Untargeted metabolomics in 30 plasma samples from the general Canadian population revealed 5018 features, of which 48 metabolites were identified using the MZmine 2.19 software including 23 by our in-house library that comprises 671 annotated metabolites. SRM 1950 analysis revealed 11,684 features, among which 154 metabolites were identified. Our method is currently applied in several epidemiological studies to better characterize cardiometabolic diseases and identify new biomarkers for disease prevention.

1. Introduction

Recent developments in liquid-chromatography tandem mass spectrometry (LC–MS) now enable sufficient throughput for metabolite profiling in large prospective epidemiological studies [1–4]. A LC coupled to a high-resolution time-of-flight mass spectrometer operating in full-scan mode can acquire data on thousands of features – retention time (RT) and exact mass pairs – belonging to various chemical classes, due to the heightened mass resolution and sensitivity [2]. Hence metabolite profiling has begun to shed light on metabolic disturbances that accompany complex human diseases. Recent applications to epidemiological studies have led to the discovery of biomarkers of metabolic wellness and diabetes [5,6]. Cardiometabolic diseases (CMD) include type 2 diabetes and cardiovascular diseases that are characterized by disturbed biochemical pathways occurring years before their diagnosis [7]. There is great interest in identifying biomarkers of early biochemical changes in biological fluids that are predictive of future CMD risk, in order to implement early-on and monitor the effects of lifestyle changes such as increased physical activity, smoking cessation, caloric intake restriction and diet composition. These modifications would permit a significant reduction in CMD risk or a delayed onset of the diseases [8].

Circulating levels of acylcarnitines (AC) and some amino acids (AA), especially branched-chain AA (BCAA) and two aromatic AA

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(ArAA), namely tyrosine and phenylalanine, have been associated with fat-free mass index [1] and linked with cardiovascular health as well as increased diabetes risk [5,9,10]. AC profiles are used for identification of mitochondrial dysfunction [11] and as a diagnostic tool for metabolic diseases in newborns [12–14]. Carnitine (C0) can be synthesized endogenously from lysine and methionine; red meat, fish and dairy products are its main dietary sources. C0 is involved in the transport of free fatty acids into the mitochondria and therefore plays a major role in fatty acid oxidation. C0 also enhances utilization of carbohydrate as an energy source by providing acetyl-CoA to feed the tricarboxylic cycle [15]. Cardiac and skeletal muscles use fatty acid oxidation and AA catabolism as complementary sources of energy which produces AC as byproducts [16]. In addition to being the building blocks for protein synthesis, AA are used as energy fuel and are precursors of various metabolites; their circulating levels reflect a wide range of physiological states [17]. Moreover, they are known to modulate insulin resistance at high plasma concentration [18].

Recent untargeted metabolomic studies revealed additional biomarkers that belong to different chemical classes [2,19]. In order to more fully decipher the metabolic changes occurring early in the pathophysiological sequence leading to CMD, there is a need for a metabolomic method that allows both the quantification of recently discovered biomarkers (i.e. AC, BCAA and ArAA) and the semi-quantitative determination of metabolites in interacting biological pathways [20], including other AA and AC, organic acids, nucleosides, fatty acids, vitamins, steroids, neurotransmitters and their derivatives.

The techniques used for sample preparation and analysis in untargeted metabolomics must not induce metabolite bias by narrowing the range of compound analyzed [21]. A common technique for metabolite separation is reverse-phase (RP) LC, because it suits a wide range of compounds found in biological matrices with minimal sample pre-treatment [22]. Detection using a hybrid quadrupole time-of-flight mass spectrometer (QTOFMS), in addition to providing appropriate sensitivity and resolution, yields a large quantity of information on fragmentation through the MS^E acquisition technique that alternates between low and high collision energies. MS^E allows acquiring untargeted fragmentation on multiple metabolites at the same time without the use of transitions [23].

Using a UHPLC-QTOFMS platform, we set out to develop a combined metabolomic method that allows the semi-quantitative determination of a wide range of metabolites, while accurately quantifying concentrations of key metabolites recently associated with CMD risk: Ile, Leu, Val, C2 and C3. Additional AA and AC were added to the list of targeted metabolites on an opportunistic basis, pending (1) the presence of isotope-labeled analogues (or an appropriate surrogate) in the commercial mixtures used in our laboratory, and (2) compatibility with the analytical conditions optimized for the key targeted metabolites. Hence the quantitative method covers the following analytes: L-arginine [Arg], L-glutamic acid [Glu], L-isoleucine [Ile], L-leucine [Leu], L-methionine [Met], L-phenylalanine [Phe], L-tyrosine [Tyr] and L-valine [Val], L-carnitine [C0], L-acetylcarnitine [C2], L-propionylcarnitine [C3], L-butyrylcarnitine [C4], L-isobutyrylcarnitine [IsoC4], L-glutarylcarnitine [C5DC], L-hexanoylcarnitine [C6] and L-octanoylcarnitine [C8].

Here we present the complete validation of the quantitative method covering eight AA and eight AC, including data on trueness and inter-day precision obtained following repeated analyses of the metabolomics standard reference material 1950 (SRM 1950) from the National Institute of Standards and Technology (NIST) over 133 days. The use of an ion-pairing reagent allowed the separation and quantification of two sets of isomers which are not readily separated by conventional LC techniques. Full-scan data was used for quantification, without the need for targeted scans, thereby enabling simultaneous untargeted metabolomics. We also describe the identification of unknowns using the MZmine software and our in-house library that comprises retention time (RT) and accurate mass to charge ratio (m/z) of the pseudomolecular ion and fragments for more than 671 compounds belonging to different metabolite families. Finally, we present results obtained when applying our hybrid metabolomic method for the analysis of 30 plasma samples from Canadian adults who participated to the pilot phase of the Canadian Longitudinal Study of Aging (CLSA) [24].

2. Material and methods

2.1. Chemicals

HPLC grade solvents were purchased from EM Science (Gibbstown, NJ). TraceCert™ AA standard mix solution #79248 was purchased from Sigma-Aldrich (St-Louis, MO) and AC standards from Toronto Research Chemicals (Toronto, ON), Santa Cruz biotechnology (Dallas, TX), Sigma-Aldrich or Cayman Chemical (Ann Arbor, MI) (see Table A.1 in Appendix A in Supplementary material). Internal standard (IS) mixtures of isotope labeled AA (NSK-A) and AC (NSK-B) were obtained from Cambridge Isotope Laboratories (Tewksbury, MA) (see Table 1). Standards for the in-house library were acquired from Sigma-Aldrich, MP Biomedicals, Combi-Blocks, Bio-Rad, Toronto Research Chemicals, Santa Cruz Biotechnology and Roche Applied Science. The origin of compounds acquired for the elaboration of our in-house library is presented in Table A.1 (see Appendix A in Supplementary material). Leucine enkephalin (Leu-enk) (≥95%), formic acid (FA), ammonium acetate (AmAc; ≥99%) and heptafluorobutyric acid (HFBA) were purchased from Sigma-Aldrich. Milli-Q water was purified by the Advantage A10 ultrapure water system (Merck Millipore, Billerica, MA).

2.2. Stock solutions and calibration curves

The TraceCert™ AA standard mix solution contains 2.50 mmol/L of each AA. Stock solutions of each AC (1000 mg/L) were prepared by dissolving neat compounds in water except for C8 which was dissolved in methanol (MeOH). The working AC solution was created by adding the proper volumes of stock solutions to yield final analyte concentrations of 20 mg/L of C0, 10 mg/L of C5DC, 5 mg/L of C2, C4 and Iso C4 and 2 mg/L of C6 and C8. The AC stock solution was aliquoted and stored at −30 °C and that of AA at 4 °C, as specified by the manufacturer. Because a plasma sample free of AA and AC is not available, we prepared calibration curves in mobile phases used for chromatographic separation (98:2 A:B; see Section 2.5). A mixture comprising 60 μL of the AC working solution, 181.8 μL of the AA TraceCert™ AA mix and 358.2 μL of 98:2 A:B mobile phases was serially diluted to generate the calibration curve. For each point, a 150 μL aliquot of the appropriate dilution was transferred into a chromatographic tube and mixed with a 10-μL aliquot of labeled IS. Neat IS were dissolved in 1 mL of 50:50 (v:v) H_2O:MeOH to yield stock solutions that were kept at 4 °C. Working solutions were obtained by further diluting stock solutions by a factor of 5 with Milli-Q water.

Stock solutions of standards (1000 mg/L) for the in-house metabolomic library were prepared either in water or MeOH depending on the compound solubility and stored at −30 °C. Working solutions (1 mg/L) were obtained by diluting stock solutions with the chromatographic mobile phase 98:2 (A:B) (see Section 2.5).