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Identification of novel circulating coffee metabolites in human plasma by liquid chromatography–mass spectrometry

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ABSTRACT

This study reports a liquid chromatography–mass spectrometry method for the detection of polyphenol-derived metabolites in human plasma without enzymatic treatment after coffee consumption. Separation of available standards was achieved by reversed-phase ultra performance liquid chromatography and detection was performed by high resolution mass spectrometry in negative electrospray ionization mode. This analytical method was then applied for the identification and relative quantification of circulating coffee metabolites. A total of 34 coffee metabolites (mainly reduced, sulfated and methylated forms of caffeic acid, coumaric acid, caffeoylquinic acid and caffeoylquinic acid lactone) were identified based on mass accuracy (<4 ppm for most metabolites), specific fragmentation pattern and co-chromatography (when standard available). Among them, 19 circulating coffee metabolites were identified for the first time in human plasma such as feruloylquinic acid lactone, sulfated and glucuronidated forms of feruloylquinic acid lactone and sulfated forms of coumaric acid. Phenolic acid derivatives such as dihydroferulic acid, dihydroferulic acid 4′-*O*-sulfate, caffeic acid 3′-*O*-sulfate, dimethoxycinnamic acid, dihydrocaffeic acid and coumaric acid *O*-sulfate appeared to be the main metabolites circulating in human plasma after coffee consumption. The described method is a sensitive and reliable approach for the identification of coffee metabolites in biological fluids. In future, this analytical method will give more confidence in compound identification to provide a more comprehensive assessment of coffee polyphenol bioavailability studies in humans.

1. Introduction

Coffee is one of the most widely consumed beverages throughout the world and contains bioactive compounds [1] with antioxidant properties, which provide several potential health benefits. The major coffee polyphenolic components are chlorogenic acids (quinic acid esters of hydroxycinnamates) accounting for 6–10% of coffee on a dry-weight basis [2] as well as di-caffeoylquinic acids (diCQA) and caffeoylquinic lactone (CQAL) [3].

Coffee has often been studied in relation to health benefits and its consumption may reduce the risk of diabetes [4], cardiovascular diseases [5], and certain cancers [6,7]. However, human bioavailability and metabolism of coffee phenolics are not fully understood [8]. Chlorogenic acids can be cleaved prior to absorption [9,10], resulting in the appearance of phenolic acids such as caffeic acid derivatives. Up to now, identification and quantification of chlorogenic acid metabolites in human plasma have been performed after protein precipitation induced by the addition of methanol followed by enzymatic treatment with β-glucuronidase,

Abbreviations: ACN, acetonitrile; CA, caffeic acid; CE, collision energy; CID, collision induced dissociation; C_{max}, maximum plasma concentration; COMT, catechol-*O*-methyltransferase; CGA, chlorogenic acid; CoA, coumaric acid; CQA, caffeoylquinic acid; CQAL, caffeoylquinic acid lactone; diCQA, di-caffeoylquinic acid; diFQA, di-feruloylquinic acid; DHCA, dihydrocaffeic acid; DHDMCA, dihydro-dimethoxycinnamic acid; DHFA, dihydroferulic acid; DHiFA, dihydro-isoferulic acid; DMCA, dimethoxycinnamic acid; DMCQA, dimethoxycinnamoylquinic acid; EC, epicatechin; EDC, enhanced duty cycle; EDTA, ethylenediaminetetraacetic acid; EGC, epigallocatechin; EIC, extracted ion chromatogram; ESI, electrospray; EtOH, ethanol; FA, ferulic acid; FQA, feruloylquinic acid; (i)FQAL, (iso)feruloylquinic acid lactone; HDMS, high definition mass spectrometry; HRMS, high-resolution mass spectrometry; iFA, isoferulic acid; LC, liquid chromatography; LLOQ, lower limit of quantification; MeOH, methanol; MS, mass spectrometry; PA, peak area; RMS, root mean square; RS, relative sensitivity; R_t, retention time; ST, sulfuryl-*O*-transferase; T_{max}, time needed to reach maximum plasma concentration; UPLC, ultra-performance liquid chromatography.

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sulfatase and sometimes an esterase. The resulting free phenolic acids, also called aglycones, were then extracted with ethyl acetate [11,12] and separated using reversed-phase liquid chromatography with highly acidic eluents such as 1 mM trifluoroacetic acid [11,13] or 4% formic acid [14]. Detection and subsequent quantification of phenolics is most often performed using UV–VIS absorbance [13,14]. Bioavailability of the major green coffee constituents in humans has been studied by Farah et al. [15], including quantification of chlorogenic and cinnamic acids in plasma and urine after ingestion of green coffee extract capsules. Although semi-quantitative approaches based on caffeic- and ferulic acid equivalents have also been demonstrated [12], the lack of authentic standards hinders accurate calibration and absolute quantification of these metabolites. Indeed, to evaluate the amount of conjugated forms of phenolic acids, Azuma et al. [16] and Nardini et al. [17] compared the concentration of aglycone forms (e.g. caffeic acid (CA) and ferulic acid (FA)) in plasma with and without enzymatic treatment. Stalmach et al. [18] studied the bioavailability of coffee chlorogenic acids in volunteers by identifying conjugated forms of caffeic and ferulic acids in human plasma and urine. Stalmach et al. [18] proposed metabolic pathways of chlorogenic acids following the ingestion of instant coffee in human. This involved catechol-*O*-methyltransferase (COMT) for methylation, the sulfuryl-*O*-transferase (ST) for sulfation, the UDP-glucuronosyl transferase for glucuronidation, esterase for hydrolysis of the ester bond and reductases for conversion to dihydroforms.

Expanding our knowledge towards the metabolic fate of dietary antioxidants such as chlorogenic acids present in coffee is the key to understanding their actual health benefits. In the present paper, we report 19 novel circulating coffee metabolites in human blood as well as the occurrence of already reported conjugates. For this purpose, a methodology based on ultra performance liquid chromatography (UPLC) combined with high resolution mass spectrometry (HRMS) is proposed to detect, identify and quantify these metabolites.

2. Experimental

2.1. Chemicals

LC grade water, acetonitrile (ACN) and methanol (MeOH) were obtained from Chemie Brunschwig AG (Basel, Switzerland). LC–MS grade acetic acid was obtained from Fluka/Sigma–Aldrich Chemie GmbH (Buchs, Switzerland). Ethanol (EtOH, analytical grade) was purchased from Merck (Darmstadt, Germany). Dihydrocaffeic acid (DHCA), isoferulic acid (iFA), 3-caffeoylquinic acid (3-CQA), 5-caffeoylquinic acid (5-CQA), para-coumaric acid (*p*-CoA) were purchased from Extrasynthese (Genay Cedex, France). Phenolic acids: CA, FA and dimethoxycinnamic acid (DMCA) were purchased from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland). Dihydroferulic acid (DHFA) was purchased from alfa-chemcat (Ward Hill, USA).

Stable isotope labelled $d^{13}C_2$-caffeic acid was purchased from Orphachem S.A. (Clermont-Ferrand Cedex, France). All other available standards (mainly phenolic and chlorogenic metabolites) were synthesized as described previously [19].

2.2. Standard solutions

Stock solutions (named A–F) were prepared in methanol or water:acetonitrile (1:1) as given in Table 1. These were combined and diluted to obtain the working solution for instrument parameter optimization (10 μg/mL per each analyte) and the solution to optimize the chromatography (1 μg/mL per each analyte).

Internal standard $d^{13}C_2$-caffeic acid was dissolved in pure methanol to obtain 1 mg/mL of working solution.

2.3. Subject characteristics

Nine healthy subjects (four male, five female) were recruited for this study. Subjects were 34 ± 7 years of age, weighed 70 ± 10 kg and measured 170 ± 8 cm. Volunteers were informed of all the details of the study before giving their informed consent. The study was approved by the ethical committee of clinical research of the University of Lausanne (Protocol reference 136/07). Inclusion criteria were 18–45 years, healthy as determined by the medical questionnaire, average coffee consumption of 1–5 cups per day, BMI 18–25, non-smoker and given informed consent. Exclusion criteria were intestinal or metabolic diseases/disorders such as diabetic, renal, hepatic, hypertension, pancreatic or ulcer, food allergy, major gastrointestinal surgery, difficulty to swallow, regular consumption of medication, high alcohol consumption (more than four drinks/day), have given blood within the last 3 weeks or currently participating or having participated in another clinical trial during the last 3 weeks prior to the beginning of this study.

2.4. Study design

The original protocol was a 4-treatment crossover controlled study. Three of the treatments were considered for other objectives and will not be discussed in the present paper. One week prior to the first treatment, BMI was measured. Twenty four hours prior to treatment until the end of the sampling period, the ingestion of coffee, tea, cola, alcohol, whole grain cereal (white bread allowed) or any medication was not allowed. Only water could be drunk during the night and in the morning before the treatment. Subjects arrived fasted early in the morning at the metabolic unit. Baseline blood was sampled, and then subjects received 400 mL of instant coffee. Blood was collected at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 11, 12 and 24 h after drinking the beverage. A standard lunch and dinner were provided at the metabolic unit. Water was available *ad libitum*. Twenty four hours after receiving the treatment, a final blood sample was taken to assess return to baseline. From blood samples, EDTA plasma was collected and stored at −80 °C.

2.5. Sample preparation

Plasma samples of one subject (14 timepoints) were thawed, vortexed for homogenization and 400 μL was added dropwise to 1000 μL of acetonitrile (ACN) (containing 2 mg/mL of ascorbic acid and 100 ng/mL of $d^{13}C_2$-caffeic acid as internal standard). The mixture was vortexed for 10 s, left for 5 min at room temperature and vortexed again for 10 s to allow protein precipitation. The samples were centrifuged at $2500 \times g$ (3290 rpm) for 10 min at 4 °C in a Sigma 3–16 K centrifuge. The supernatant was transferred into amber vials and evaporated under nitrogen flow. Before analysis, samples were reconstituted in 100 μL of solvent A (water, 1% acetic acid (v/v)) and 10 μL was injected into the UPLC–MS system.

2.6. UPLC

LC separation of phenolic acid and chlorogenic metabolites was achieved on a reversed-phase column HSS T3, 150 mm × 1.0 mm, 1.8 μm at room temperature using a Waters Acquity UPLC system. Mobile phase A was water containing 1% acetic acid, mobile phase B was methanol/acetonitrile (1:3, v/v). Final mobile phase gradient is summarized in Table 2.