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Quantification of theobromine and caffeine in saliva, plasma and urine via liquid chromatography–tandem mass spectrometry: A single analytical protocol applicable to cocoa intervention studies

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ABSTRACT

Targeted analyses of clinically relevant metabolites in human biofluids often require extensive sample preparation (e.g., desalting, protein removal and/or preconcentration) prior to quantitation. In this report, a single ultra-centrifugation based sample pretreatment combined with a designed liquid chromatography–tandem mass spectrometry (LC–MS/MS) protocol provides selective quantification of 3,7-dimethylxanthine (theobromine) and 1,3,7-trimethylxanthine (caffeine) in human saliva, plasma and urine samples. The optimized chromatography permitted elution of both analytes within 1.3 min of the applied gradient. Positive-mode electrospray ionization and a triple quadruple MS/MS instrument operated in multiple reaction mode were used for detection. $^{13}C_3$ isotopically labeled caffeine was included as an internal standard to improve accuracy and precision. Implementing a 20-fold dilution of the isolated low MW biofluid fraction prior to injection effectively minimized the deleterious contributions of all three matrices to quantitation. The assay was linear over a 160-fold concentration range from 2.5 to 400 μmol L^{-1} for both theobromine (average R^2 0.9968) and caffeine (average R^2 0.9997) respectively. Analyte peak area variations for 2.5 μmol L^{-1} caffeine and theobromine in saliva, plasma and urine ranged from 5 and 10% (intra-day, $N = 10$) to 9 and 13% (inter-day, $N = 25$) respectively. The intra- and inter-day precision of theobromine and caffeine elution times were 3 and <1% for all biofluids and concentrations tested. Recoveries for caffeine and theobromine ranged from 114 to 118% and 99 to 105% at concentration levels of 10 and 300 μmol L^{-1}. This validated protocol also permitted the relative saliva, plasma and urine distribution of both theobromine and caffeine to be quantified following a cocoa intervention.

1. Introduction

Robust, rapid and validated analytical techniques are essential for further elucidating the potential therapeutic benefits of cocoa consumption. Several examples of this emerging area of health research can be found in literature describing the potential holistic benefits of consuming this food product [1–9]. Such studies have ranged from a reduction in the likelihood of preeclampsia [10] to the inhibition of atherosclerotic plaque progression [11]. This work however, is preliminary and further research is needed to elucidate the underlying mechanism of these effects and to identify the active cocoa components responsible for the observed health benefits [12–14]. To this end, the contribution of cocoa's methylxanthine fraction has garnered much research attention. 3,7-Dimethylxanthine (theobromine) and 1,3,7-trimethylxanthine (caffeine) respectfully represent the major and minor physiologically active methylxanthine components of the food product. Each has been implicated in the observed beneficial health impacts of nutritional cocoa intervention. From an analytical perspective, these previous studies illustrate the importance of accurately measuring the levels of both of these metabolites in human saliva, plasma and urine. A single platform capable of quantitating these small molecules in multiple biofluids matrices would be attractive to health researchers investigating the effects of cocoa intervention on disease manifestation.

Directed metabolite analyses within complex biological matrices presents significant analytical challenges. Conventional approaches often include distinct off-line sample pretreatments including preconcentration, de-salting and protein removal. Although these procedures isolate and enhance the response of the target analyte, they are often time consuming, difficult to automate, reduce precision and incur sample loss. Several different pre-analytical protocols have been employed to minimize the deleterious effects of complex biofluid matrices, with liquid–liquid extraction (LLE) [15–24] and solid-phase extraction (SPE) [25–30] being the most common for these analytes. Once isolated from

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the matrix, numerous analytical techniques have been used for multiplex methylxanthine analyses including radioimmunoassay (RIA) [31], thin-layer chromatography (TLC) [26], capillary gas chromatography (GC) [32] and micellar electrokinetic electrophoresis (MEKC) [33,34]. However liquid chromatography (LC) with spectrophotometric detection [15–20,22,25,27,35–41] remains the most widely applied technique, despite the possibility of spectral interferences from co-eluting endogenous compounds. This limitation can be avoided by coupling LC with mass spectrometric detection (LC–MS), although there are relatively fewer reports using this methodology for methylxanthine analyses [28,29]. The selectivity and sensitivity of this technique may be further enhanced by employing compound specific mass transitions with tandem mass spectrometry (MS/MS) [10,42]. The LC–MS/MS analysis of eleven different methylxanthine and methylurate urine metabolites communicated by Schneider et al. demonstrates this benefit as their extractionless sample preparation and total LC run time of 60 min, including analyses in both positive and negative electrospray ionization (ESI) modes, permitted baseline resolution and measurements of all isomeric species [43].

In this report, a rapid LC–MS/MS procedure has been developed and validated for the quantification of μmol L^{-1} levels of theobromine and caffeine in human saliva, plasma and urine. Fast separations (3 min total run time) coupled with a single ultracentrifugation based sample preparation for all three matrices provided interference-free quantitation of both metabolites in all biofluids. This universal protocol circumvents the multiple conventional pre-analytical treatments typically required for their targeted measurement and provides a single platform for which cocoa intervention studies may be performed.

2. Methods and materials

2.1. Chemicals

1,3,7-Trimethylxantine (caffeine), 3,7-dimethylxantine (theobromine), 1,7-dimethylxanthine (paraxanthine), and 1,3-dimethylxanthine (theophylline) were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Double-distilled water and HPLC-grade acetonitrile (Sigma–Aldrich) were used with analytical-grade formic acid (Fisher-Scientific, Fair Lawn, NJ, USA) for mobile phase preparations. A 624 μmol L^{-1} stock solution of $^{13}C_3$ caffeine from Isotec (99 atom% ^{13}C, Miamisburg, OH, USA) was used as an internal standard (IS) to improve quantitation. Individual 10 mmol L^{-1} standard solutions of caffeine, theobromine, paraxanthine, and theophylline were prepared in distilled water and serially diluted to create all standard solutions used in the study. 41 g Hershey's Special Dark chocolate bars (The Hershey Company, Hershey, PA, USA) were used in the cocoa intervention studies.

2.2. Biological fluid sample preparations

All biological fluids used in the study were obtained from healthy volunteers, who were free of dietary restrictions. Plasma was isolated from whole blood via 7 min of centrifugation at 1800 × *g* and 5 °C. Untreated saliva, plasma and urine samples were stored at −80 °C and processed using the protocol outlined below upon thawing at room temperature. All samples were initially inoculated with the $^{13}C_3$ caffeine internal standard and centrifuged at 10,000 × *g* for 5 min to remove any particulate matter from the fluid. 500 μL of the supernatant was then transferred to a 10 kDa molecular weight cut-off (MWCO) filter (Millipore Corporation, Billerica, MA, USA) and centrifuged for 10 min at 10,000 × *g*. The low MW filtrate was then diluted and used for LC–MS/MS analysis.

2.3. LC–MS/MS protocol

An Acquity Ultra-Performance LC system with a diode-array UV spectrophotometer was coupled to a Waters Micromass® Quattro Premier triple quadruple instrument (Waters, Milford, MA, USA) and used throughout the study. The ESI source of the mass spectrometer was operated in positive ion mode, with a capillary voltage of 3 kV. The MS parameters and optimization protocol outlined by Mensch et al. for ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) analyses was implemented in this study [44]. Briefly, the source and desolvation temperatures were set to 120 and 350 °C respectively. Flow rates for the cone and desolvation gases, as well as source and desolvation temperatures were maintained at 100 and 800 L h^{-1} and 120 and 350 °C respectively. Multipliers were set at 650 V, with argon used as the collision gas at a regulated flow rate of 0.35 mL min^{-1}.

Multiple reaction monitoring (MRM) for each of the target compounds required both the cone voltage and collision energy to be respectively optimized for the metabolites precursor and product ions. 5 μmol L^{-1} of the test compounds were infused into the mass spectrometer via a syringe pump running 0.1% (v/v) formic acid in doubly distilled water at a constant flow rate of 20 μL min^{-1}. The derived optimal conditions were incorporated into the final MRM method for sample acquisition.

0.7 μL of all standards and samples were injected into a LC C18 bridged-ethyl hybrid (BEH) column (1.7 μm particles × 2.1 mm × 50 mm, Waters) maintained at a thermostatted 30 °C. The metabolites were separated using a gradient elution with mobile phases of 0.1% (v/v) formic acid in double-distilled water (phase A) and acetonitrile (phase B) respectively. A constant flow rate of 0.6 mL min^{-1} was employed throughout, with phase B initially maintained at 2% from 0 to 0.5 min. The sample was eluted with increasing the % of solvent B to 10, 13, 14 and 50% at 0.5, 0.7, 1.25 and 1.5 min respectively. The column was then equilibrated to 2% phase B over 0.1 min where it was held for an additional 1.5 min to recondition the column and eliminate any potential sample carry-over prior to the next injection. All compounds were detected by the in-line photodiode array UV detector (λ_{abs}: 280 nm) and mass spectrometer using their individually optimizing MRM methods. For all analyses the MS dwell time was 0.1 s and the spectrometer paused between mass transitions 0.005 s. All data acquisition and processing was performed using MassLynx™ 4.1 and QuanLynx™ software (Waters) with retention times (RT) and peak areas (PA) given in minutes and arbitrary units (au) respectively. All figure schematics presented were constructed using Igor Pro 5.0 (Wavemetrics Inc., Lake Oswego, OR, USA).

3. Results and discussion

3.1. Methylxanthine ESI-MS/MS behavior

Accurate assessment of theobromine biofluid levels via LC–MS/MS requires either chromatographic or mass spectrometric separation from both paraxanthine and theophylline. These isomeric alkaloid metabolites represent the primary *N*-demethylated metabolism products of caffeine and cannot be distinguished based on their precursor masses alone. Thus to facilitate accurate theobromine quantitation, compound specific multiple reaction monitoring (MRM) was employed with ESI-MS/MS. This protocol required two sample infusion experiments to optimize the cone voltage and collision energy for precursor and product ions of each compound respectively.

When infused into the mass spectrometer, operated in positive-ion mode, each of the three methylxanthine metabolites produced stable $[M+H]^+$ ions. Mass spectra of each compound were obtained