HHS Public Access

Author manuscript

Biomed Chromatogr. Author manuscript; available in PMC 2018 June 01.

Published in final edited form as:

Biomed Chromatogr. 2017 June ; 31(6): . doi:10.1002/bmc.3900.

Measurement of caffeine and its three primary metabolites in human plasma by HPLC-ESI-MS/MS and clinical application

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Abstract

Caffeine is a mild stimulant with significant potential for abuse being consumed in larger doses with the widespread availability of energy drinks and by novel routes of administration such as inspired powder, oral sprays, and electronic cigarettes. How these recent changes in caffeine consumption affecting caffeine disposition and abuse potential is of growing concern. In the study of caffeine disposition in humans, it is common to only measure the caffeine concentration; however, caffeine's three major metabolites (paraxanthine, theobromine, and theophylline) retain central nervous system stimulant activity that may contribute to the overall pharmacological activity and toxicity. Therefore, it would be scientifically more rigorous to measure caffeine and its major metabolites in the evaluation of caffeine disposition in human subjects. Herein, we report a method for the simultaneous quantification of caffeine and its three major metabolites in human plasma by high-performance liquid chromatography coupled to electrospray tandem mass spectrometry (HPLC-ESI-MS/MS). Human plasma samples were treated by simple protein precipitation and the analytes were separated using a 6-min gradient program. Precision and accuracy were well within in the 15% acceptance range. The simple sample preparation, short runtime, sensitivity, and the inclusion of caffeine's major metabolites make this assay methodology optimal for the study of caffeine's pharmacokinetics and pharmacodynamics in human subjects.

Keywords

Caffeine; metabolites; HPLC-ESI-MS/MS; formic acid; pharmacokinetics

Introduction

Caffeine, a naturally occurring component of coffee, tea, and chocolate, is the most widely consumed central nervous stimulant in the world. Coffee remains the primary source of

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Contributions of authors

L.S.C. and P.R.B. participated in the design of the study and reviewed the results. C.F. and H.Z.Y. conducted the experiments. C.F. and L.S.C. drafted the manuscript. H.Z.Y. helped to draft the manuscript. All authors have approved the manuscript and agree with submission to the *Biomedical Chromatography*.

Competing interests

There are no competing interests to declare.

caffeine intake, but high-caffeine content beverages such as energy drinks and new caffeine formulations such as orally inspired powders, oral spray mists, and electronic cigarettes are increasing sources of intake especially in young adults. In view of its widespread use, many epidemiological studies have been conducted to evaluate the public health consequences of caffeine intake (Arab, Khan and Lam 2013, Greenberg, Boozer and Geliebter 2006). A growing concern is caffeine abuse in young adults in combination with alcohol, which has been shown to increase binge drinking and the detrimental consequences associated with excessive alcohol intake (Reissig, Strain and Griffiths 2009).

Caffeine is completely absorbed from gastrointestinal tract, and undergoes hepatic metabolism by CYP1A2 to form three major metabolites: paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine) (Tang-Liu, Williams and Riegelman 1983). Paraxanthine and theobromine have similar stimulating effects as caffeine (caffeine > paraxanthine > theobromine), and theophylline is used therapeutically as a bronchodilator and is well known for its low therapeutic index with increasing risk of seizures, arrhythmias, and neurologic toxicities occurring at plasma concentrations exceeding 20 μg/mL (Benowitz, Jacob, Mayan and Denaro 1995, Carney 1982). Thus, investigations of caffeine pharmacokinetics and pharmacodynamics in humans should include quantification of caffeine's three major metabolites.

Historically, high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection has been the most common method to simultaneously detect caffeine and its metabolites in human plasma (Tanaka 1992, Wahllander, Renner and Karlaganis 1985, Zysset, Wahllander and Preisig 1984). More recently several HPLC coupled to electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) methods have been developed, which provide higher sensitivity and selectivity with smaller sample volume requirements compared to HPLC with UV detection. HPLC-ESI-MS/MS methods have been published for the quantification of caffeine and its metabolites in human milk (Pellegrini, Marchei, Rossi, Vagnarelli, Durgbanshi, Garcia-Algar, Vall and Pichini 2007), urine (Caubet, Comte and Brazier 2004, Ptolemy, Tzioumis, Thomke, Rifai and Kellogg 2010), saliva (Ptolemy, Tzioumis, Thomke, Rifai and Kellogg 2010) and plasma (Gassner, Schappler, Feinberg and Rudaz 2014, Ptolemy, Tzioumis, Thomke, Rifai and Kellogg 2010), using solid phase- or liquid-liquid extraction as sample clean-up approaches, typically requiring large sample volumes, long run times and large volumes of organic solvent (Gassner, Schappler, Feinberg and Rudaz 2014, Huang, Gao, Zhai, Liang, Wang, Bai and Luo 2012). We report the development and validation of an HPLC-ESI-MS/MS caffeine assay with protein precipitation sample preparation for the measurement of caffeine and its three main metabolites in human plasma samples. The validated assay provides a simple and sensitive method with high throughput capacity and low sample volume requirement. The method was applied to the study of caffeine pharmacokinetics in human subjects.

Experimental

Chemicals and materials

Caffeine (purity 99%, similarly hereinafter; CAF), paraxanthine (98%; PAR), theophylline (99%; THY), theobromine (99%; THM), as well as isotope labeled caffeine (98%; CAF-d9) and paraxanthine (98%; PAR-d6) (Figure 1) were purchased from Toronto Research Chemicals (North York, ON, Canada). HPLC-grade acetonitrile and methanol were the products of Fisher Scientific (Fair town, NJ, USA). Formic acid (HCOOH) was obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade water was prepared with an in-house Milli-Q Advantage A10 Ultrapure water purification system (Bedford, MA, USA). Drug free plasma was from human subjects participating in a clinical study.

HPLC-ESI-MS/MS analysis

An Agilent 1100 series liquid chromatography system (Waldbronn, Germany) connected to an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) was coupled to an AB SCIEX 3000 triple quadrupole mass spectrometer (Toronto, Canada) with an Ionics Hot Source Induced Desolvation (HSID) interface (Ontario, Canada). Data acquisition and processing was performed by Analyst® 1.5.1 software.

The chromatographic separation was achieved on a 3.5 μm Waters Symmetry C_{18} column: 75 mm × 4.6 mm i.d. (Waters, Milford, MA, USA) at 35°C. Mobile phase consisted of water for phase A and methanol for phase B, both containing 25 mM HCOOH. Separation was optimized using a fast gradient method with mobile phase A/B set to 95%/5% from 0.00 to 0.10 min and 60%/40% from 0.11 to 2.50 min and then back to 95%/5% from 2.51 to 6.00 min.

The mass spectrometer was operated in the positive ion mode. The nebulizer gas, curtain gas, ionspray voltage and source temperature were set at 12 psi, 15 psi, 5500 volts, and 550 °C, respectively. The collision gas flow (CAD) was set at level 4. The precursor-product ion pairs used for multiple reaction monitoring (MRM) of CAF, PAR, THY, THM, CAF-d9, and PAR-d3 were m/z 194.9→137.8 (DP, 26; FP, 20; EP, 7; CE, 29; CXP, 5.3), 181.0→124.2 (DP, 28; FP, 20; EP, 6; CE, 30; CXP, 4.8), 181.1→124.2 (DP, 30; FP, 14; EP, 6; CE, 28; CXP, 5), 181.1→137.8 (DP, 26; FP, 20; EP, 5; CE, 26; CXP, 5.4), 204.2→144.0 (DP, 26; FP, 25; EP, 6; CE, 29; CXP, 8), and 184.1→124.2 (DP, 26; FP, 25; EP, 6; CE, 29; CXP, 22), respectively. The scan time was 80 ms for each ion pair. DP, FP, EP, CE, and CXP denotes declustering potential, focusing potential, entrance potential, collision energy, and collision cell exit potential, respectively. Optimization of these values involved gradually changing the voltage range while monitoring the signal intensity of the compound. The HPLC eluent was introduced into the ESI source for analysis over the period of 2.4–4.4 min at a rate of 700 μL/min with flow splitting (split ratio 1:1) throughout the gradient program.

CAF-d9 was the internal standard (IS) used for quantification of CAF. Similarly, PAR-d3 was used for quantification of the three isomers, *i.e.*, PAR, THY, and THM. Matrix-matched calibration curves were constructed for CAF, PAR, THY, and THM, using weighted (1/X) linear regression of the target analytes/IS peak area ratio (Y) against the corresponding target analytes nominal concentrations (X, ng/mL).

Preparation of solutions, standards, and quality control samples

Stock solutions for CAF and CAF-d9 at 2 mg/mL were prepared in methanol and then diluted with methanol to 100 μg/mL working solutions. PAR (0.5 mg/mL), PAR-d3 (0.67 mg/mL), THM (0.2 mg/mL), and THY (2 mg/mL) were prepared in water as stock solutions. THY solution was further diluted with water to a 100 μg/mL working solution. All the solutions were stored in −70°C freezer before use.

Methanol solution containing CAF-d9 (600 ng/mL), PAR-d9 (400 ng/mL), and HCOOH (125 mM) was prepared before experiments and used to precipitate the plasma protein for sample clean-up.

To prepare standards in blank plasma, an intermediate 3000 ng/mL solution containing CAF, PAR, THY, and THM was prepared by combining appropriate volumes of each standard solution. Subsequently, the resulting solution was diluted with blank plasma to give drug concentrations of 1000, 333, 111, 37.1, 12.3, 4.12, and 1.37 ng/mL. Quality control samples at 1000, 111, and 12.3 ng/mL were prepared from separate stock solutions in the same way as calibration standards.

Plasma sample clean-up

Plasma samples (30 μL) were mixed with 100 μL methanol (containing 125 mM HCOOH, 600 ng/mL CAF-d9 and 400 ng/mL PAR-d3). The plasma-methanol mixture was vortexed for 5 min at 1,175 *rpm*. After centrifugation for 5 min at 17,900 × *g*, 10 μL of the supernatant was injected into the HPLC-ESI-MS/MS.

Assessment of varying formic acid concentration in mobile phase and assay validation

The influence of the HCOOH concentrations ranging from 0 to 25 mM in the mobile phase on the signal intensities of the analytes was studied. In order to assess the absolute and relative matrix effects (ME) for method validation, a post-extraction spike method was used (Matuszewski, Constanzer and Chavez-Eng 2003).

Assay validation was carried out according to the US Food and Drug Administration guidance (www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf) on bioanalytical method. Briefly, the within-run (n=3) and between-run (n=6) accuracy and precision were studied by using three concentration levels of quality control samples. Plasma stability was tested by preparing quality control samples at three different concentrations in triplicate and analyzing them after remaining on the bench for 4 hours at room temperature before clean-up. Quality control samples after extraction with the same concentration levels in triplicate stored in the autosampler at 4 °C for 12 hours were used to evaluate the post-preparative stability. Freeze-thaw stability was assessed after three freeze-thaw circles, which consisted of storage at −70 °C overnight, followed by thawing at room temperature in the morning. For long-term stability evaluation, the quality control samples were stored in freezer at −70 °C for 9 months and then thawed before clean-up and prepared, and tested against freshly prepared samples.

Human caffeine study

The disposition of caffeine and its three major metabolites was determined in healthy normal volunteer subjects who consumed 100 mg of caffeine by administration of an energy drink (Guru Lite; Guru Beverage Co., Irvine, CA, USA) and by oral inspiration of a fine powder (Aeroshot™; Breathable Foods, Inc., Cambridge, MA, USA) on separate study days in a randomized crossover design. Fifteen blood samples (including 1, 2, 5, 10, 15, 20, 30, 40, 60 minutes, and 2, 3, 4, 6, and 8 hours after the start of caffeine consumption) were collected over an 8-hour period after dosing. The plasma was separated and stored at −70 °C. This study protocol (13–02648-FB) was approved by the University of Tennessee Health Science Center Institutional Review Board.

Results and discussion

Sample preparation and liquid chromatography

A small volume (30 μL) of human plasma was precipitated with 100 μL methanol containing 600 ng/mL CAF-d9 and 400 ng/mL PAR-d3, and 125 mM HCOOH, which was used to increase the purity of caffeine and its metabolites in the supernatant. The background noise was found to significantly interfere with the determination of analytes especially caffeine if HCOOH was not added to the precipitation solvent during sample preparation.

Positive ion electrospray product ion tandem mass spectra for CAF, PAR, THY, and THM are shown in the upper panel of Figure 2. A chromatographic column packed with 3.5 μm particles and an optimized elution approach with a relatively high flow rate (700 μL/min) at 35 °C resulted in complete separation for all the analytes. Another key concern was the use of a splitter. Due to the high flow rate, the eluent was split (ratio 1:1) to prevent excessive eluent volume from interfering with the ionization efficiency in the ESI source. This allowed all the analytes to be adequately separated with an elution time that spanned only 0.8 min. The retention times for CAR, PAR, THY, and THM were 3.46, 2.98, 3.14, and 2.72 min, respectively (Figure 2 (a), (b), (c), (d), (e), and (f)). The resulting limits of detection of CAR, PAR, THY, and THM were 1.4, 0.7, 0.8, and 2.0 ng/mL, respectively.

The analytical approach provided efficient purification, reliable separation, and sensitive quantification of analytes in human plasma. The clean-up method required minimal preparation while purifying and enriching the analytes from a complex biological matrix thereby enabling their final instrumental measurement (Shipkova and Svinarov 2016). In the context of the present research, simple protein precipitation pretreatment with small sample volume was developed. Good chromatography was the key to avoiding isobaric interferences. The chromatographic conditions were optimized to separate caffeine and the three isomers, including a column packed with 3.5 μm particles, high flow rate, high column temperature and gradient elution program. Small particle size is attributed to lower theoretical plate heights, shorter column lengths, and higher optimum eluent velocities. High column temperature reduces viscosity, and allows fluid to move faster at the same pressure, which improves interphase mass transfer (Carr, Stoll and Wang 2011).

Formic acid in mobile phase influences the signal intensity and absolute matrix effects

The influences of HCOOH at different concentration levels on the signal intensity and absolute matrix effects are shown in Figure 3. Solid circles denote the intensity changes of the standard solutions containing CAF, PAR, THY, and THM in pure solvents (Set 1). Open circles depict profiles of the mass responses of blank human plasma matrix-based solution spiked with standard solutions (Set 2). Overall, HCOOH exerted minor effects on the signal intensity of the analytes in Set 1 samples except for THM. As for CAF, the relative standard deviations (RSD) for signal intensity changes ranged from 7.67% to 9.94%. The same was true for PAR and THY with RSD 12.0–17.1% and 13.4–16.3%, respectively. However, HCOOH enhanced THM signal intensity by 2.31–2.74 fold. HCOOH obviously influenced the signal intensities of analytes in Set 2 samples with CAF as an exception. Bell-shaped curves were observed for PAR, THM, and THY. In other words, the signal of the three metabolites increased initially, leveled off and then declined gradually along with increasing HCOOH concentration in the mobile phase. The differences in signal intensity from lowest to highest were between 1.58- and 4.25-fold.

The absolute matrix effects were evaluated by post-extraction spiked method (Hu, Laizure, Meibohm, Herring and Parker 2013) and were calculated by the following equation: ME (%) = $(\text{Mean peak area})_{\text{set 2}}/(\text{Mean peak area})_{\text{set 1}} \times 100$. There was significant signal suppression for PAR (about 68%), THY (about 35%), and THM (about 55%) without HCOOH in the mobile phase. The matrix effects of PAR and THY were nearly completely eliminated with HCOOH concentrations from 0.5 to 5 mM, and then signal suppression recurred when HCOOH concentration increased to 25 mM (Figure 3). THM followed a similar pattern, but the matrix effects were not overcome until the HCOOH concentration reached 5 mM (Figure 3). Significant signal suppression of PAR, THY, and THM occurred at the highest HCOOH concentration (25 mM). This can be explained partly by the fact that the higher concentration of HCOOH competes with the target analytes for the limited charge and surface available on the ESI droplets (Trufelli, Palma, Famiglini and Cappiello 2011). On the other hand, there were no matrix effects interfering with CAF analysis with or without any tested concentration level of HCOOH in the mobile phase (Figure 3). But it should be kept in mind that methanol containing 125 mM HCOOH was used for protein precipitation as it was a precondition for the analysis of caffeine and its metabolites. Similar results were observed for its effects on the signal intensity and absolute matrix effects of caffeine and its metabolites when the tested sample concentrations of 12.3 and 1000 ng/ml were performed in both Set 1 and Set 2 (data not shown).

We calculated the absolute and relative matrix effects of caffeine and its metabolites using blank plasma from three donors with 5 or 25 mM HCOOH in mobile phase, as shown in Table 1 and Table 2. Consistently, the absolute matrix effects of CAF, PAR, THY, and THM, as well as internal standards CAF-d9 and PAR-d3, were between 85.2% and 112% when the concentration of HCOOH was 5 mM in the mobile phase. The corresponding relative matrix effects ranged from 1.20% to 12.3% (Table 2). However, HCOOH took a toll on the absolute matrix effects (*i.e.*, 45.3%-75.2%) of PAR, THY, THM, and PAR-d3 when its concentration was increased to 25 mM, but the relative matrix effects were no more than 12.1% (Table 1).

In this context, the signal suppression was due to the higher concentration of HCOOH rather than the plasma background components.

HPLC-ESI-MS/MS-based quantitative analysis is often accompanied by signal suppression or enhancement, referred to as matrix effects, due to co-eluting matrix components from complex background matrices, which can detrimentally impact the detectability and efficiency of a method (Trufelli, Palma, Famiglini and Cappiello 2011). In this study, we evaluated strategies to overcome potential matrix effects. First, we optimized the liquid chromatography to achieve good chromatographic separation among the isomers, caffeine, and their internal standards. Secondly, we addressed the importance of HCOOH on signal intensity and matrix effects of the analytes, which has been largely ignored by the earlier reported assays (Choi, Bae, Park, Kwon, Jang, Zheng, Lee, Lee and Bae 2013, Noh, Nepal, Jeong, Kim, Um, Seo, Kang, Park, Kang, Jeong and Jeong 2015, Noh, Oh, Nepal, Jeong, Choi, Kang, Kang, Jeong and Jeong 2016). Thirdly, we utilized isotopic analogs as internal standards (*i.e.*, CAF-d9 for CAF, PAR-d3 for PAR, THY, and THM). The compensation for matrix effects by isotopic analogs depends on their co-elution with the unlabeled analytes. To our knowledge, this is the first assay paper using isotope labeled internal standards for determination of caffeine and its primary metabolites.

Method validation

The calibration curves for quantification of CAF, PAR, THY, and THM were linear ($r > 0.99$) from 4.1 to 3000 ng/mL. The within-run (n=3) and between-run (n=6) precision of the assay were acceptable for caffeine and its metabolites, *i.e.*, 0.55–6.24% and 1.96–9.12%, respectively. The assay accuracy was also satisfactory, *i.e.*, 97.7–109% and 98.1–108%, respectively (Table 3). The lower limit of quantification from an initial 30-μL plasma volume was 4.1 ng/mL for all the analytes.

The stability of CAF, PAR, THY, and THM was studied under various conditions to mimic situations likely to be encountered during actual sample handling and analysis (Table 4). The storage of plasma samples at room temperature for 4 h did not reduce the signal responses of the analytes as evidenced by the assay accuracy ranging from 94.4% to 114% and a precision no more than 13.3%. Similarly, processed samples were stable in the autosampler for 12 hours. The accuracy and precision for freeze-thaw stability evaluation was 97.4–113% and 1.68–11.2%, respectively. In addition, after > 6 months of storage at −70 °C, all analytes were within 15% of control values. Overall, both precision and accuracy were well within in the 15% acceptance range.

Application to human pharmacokinetic study

The HPLC-ESI-MS/MS assay described herein was successfully applied to quantify caffeine and its metabolites in human subjects. The plasma concentration-time profiles of caffeine and its metabolites from a single subject after taking a 100 mg dose of caffeine by oral inspiration (panel A) and energy drink (panel B) administration are plotted in Figure 4.

Conclusions

A method with low sample volume consumption, high sensitivity, and simplicity for the simultaneous quantification of caffeine, paraxanthine, theophylline, and theobromine in human plasma by HPLC-ESI-MS/MS was developed and validated. A fast 6-min gradient program using high flow rate and high column temperature achieved good separation for the three isomers and their parent drug without impacting the ionization efficiency through post-column splitting approach. The method is the first, to our knowledge, to evaluate different concentrations of formic acid in mobile phase to optimize the ionization and matrix effects. This straightforward and easy-to-use strategy can be transferred to other HPLC-ESI-MS/MS-based assays. The newly developed method was successfully applied to the analysis of caffeine and its metabolites in human plasma samples.

Acknowledgments

This work was supported by a grant from the National Institutes of Health (R03DA035347). We also acknowledge the staff at the University of Tennessee Clinical Research Center for their assistance with this study.

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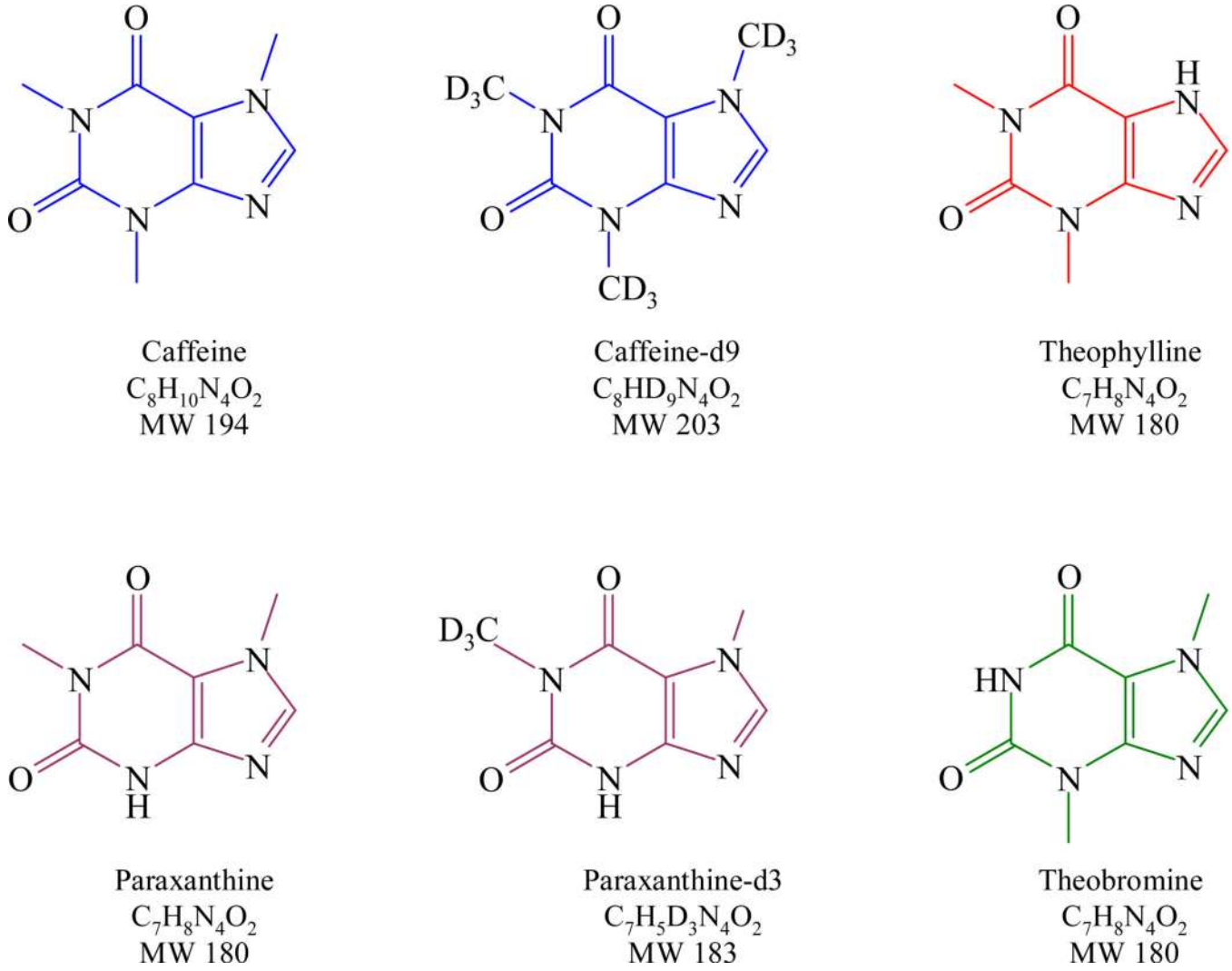

Figure 1.

Chemical structures of caffeine (CAF), paraxanthine (PAR), theophylline (THY), theobromine (THM), deuterated caffeine (CAF-d9), and deuterated paraxanthine (PAR-d3).

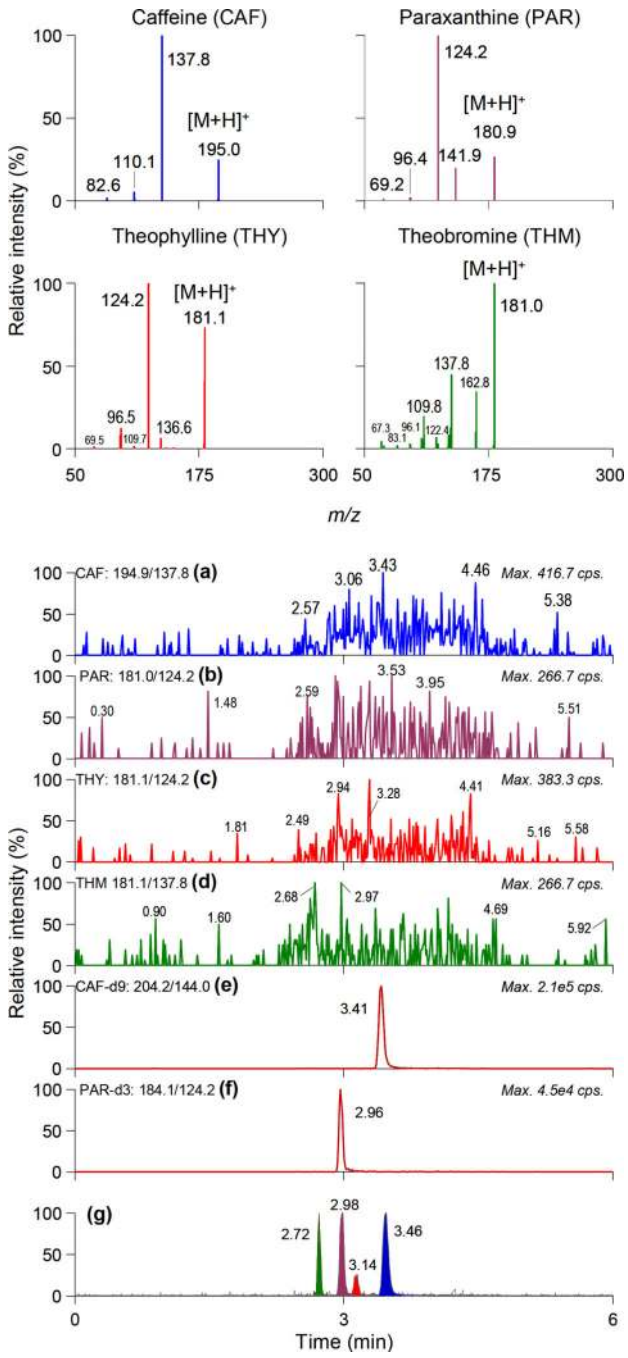

Figure 2.

Product ion mass spectra of the protonated molecules ([M+H]$^+$) of caffeine (CAF), paraxanthine (PAR), theophylline (THY) and theobromine (THM) (top panel); chromatograms of blank human plasma (bottom panel, **a** for CAF, **b** for PAR, **c** for THY and **d** for THM), blank human plasma spiked with deuterated standard solutions (bottom panel, **e** for CAF-d9, 600 ng/mL; **f** for PAR-d3, 400 ng/mL,) and a study subject plasma sample (bottom panel, **g**) for the caffeine and its three metabolites. The retention times (human

plasma sample) for CAF, PAR, THY, and THM were 3.46, 2.98, 3.14, and 2.72 min, respectively.

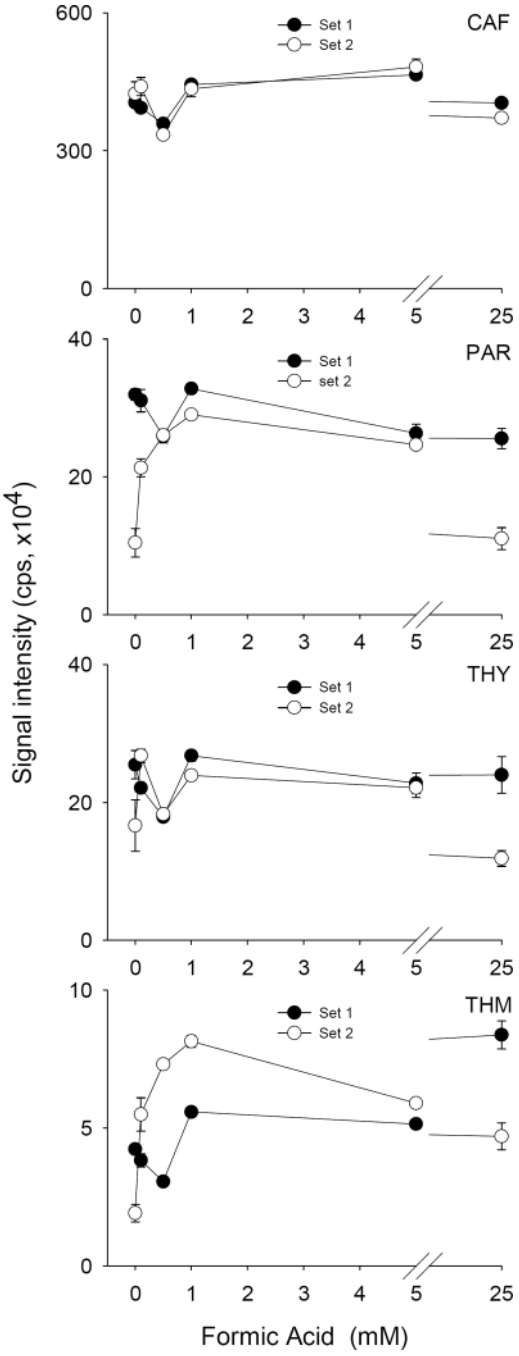

Figure 3.

The effect of formic acid concentration in mobile phase on the signal intensity and absolute matrix effects of caffeine (CAF), paraxanthine (PAR), theophylline (THY), and theobromine (THM). Each data point represents the mean ± standard deviation (n=3). Solid circles (Set 1) denote the standard solutions containing caffeine and its metabolites in pure solvents (methanol or water only for CAF and CAF-d9). Open circles (Set 2) denote the blank human plasma matrix-based solution spiked with standard solutions at the same concentration (111 ng/mL) as Set 1. Absolute matrix effect = $(\text{Mean peak area})_{\text{Set 2}}/(\text{Mean peak area})_{\text{Set 1}}$.

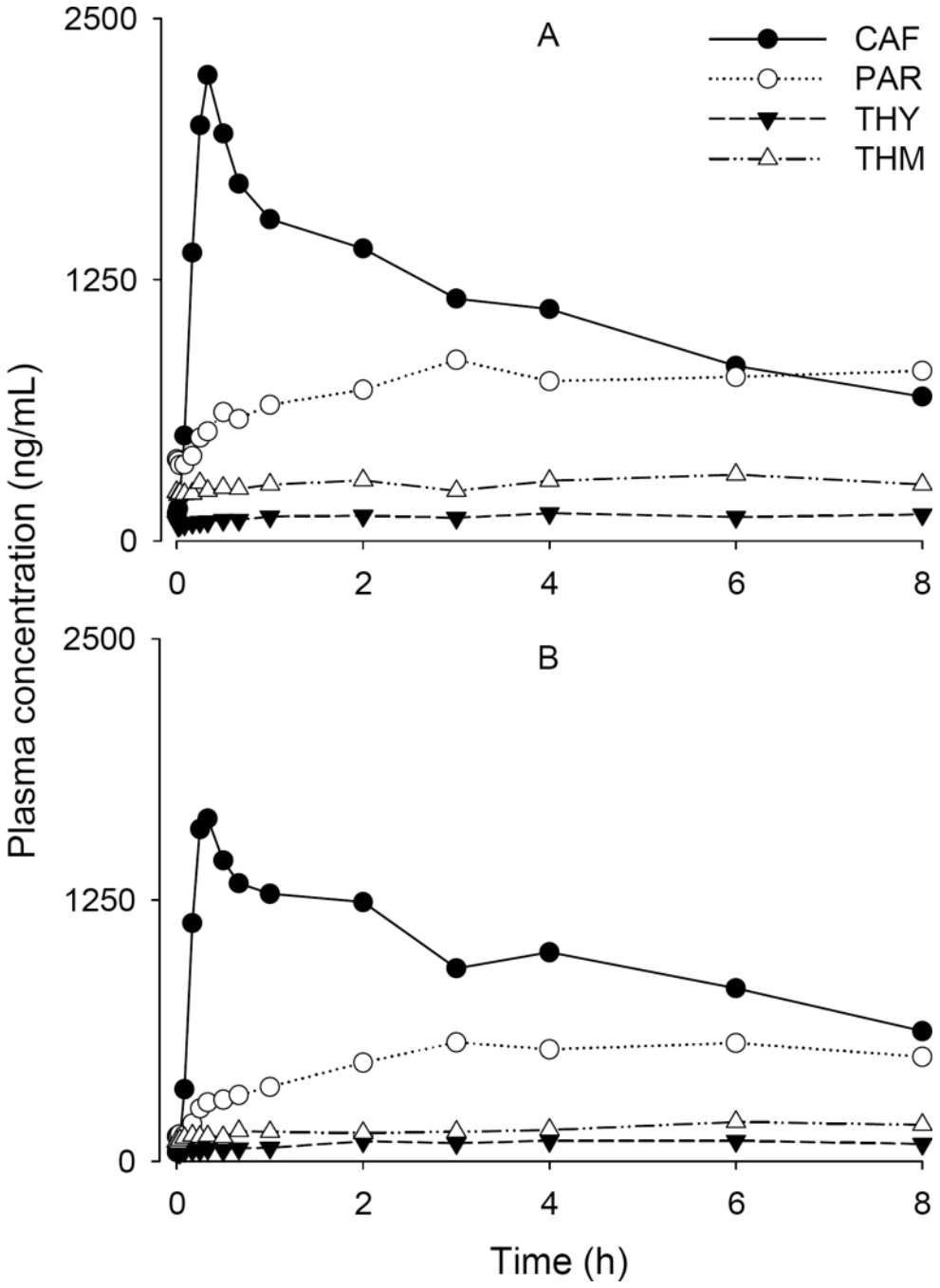

Figure 4.

Plasma concentration-time curves of caffeine and its three metabolites after administration by oral inspiration (AeroShot™; containing 100 mg caffeine; **A**) and energy drink (Guru Lite™; containing 100 mg caffeine; **B**) to a human subject on separate study days.

Table 1

Matrix effects of caffeine (CAF), paraxanthine (PAR), theophylline (THY), and theobromine (THM), as well as deuterated caffeine (CAF-d9) and deuterated paraxanthine (PAR-d3) in human plasma performed with 25 mM HCOOH in mobile phase.

Compounds	Concentration ng/mL	Blank A Absolute ME (%)	Blank B Absolute ME (%)	Blank C Absolute ME (%)	Average Absolute ME (%)	CV Relative ME (%)
CAF	12.3	109	95.7	85.4	96.7	12.3
	111	108	103	96.4	102	5.54
	1000	91.7	86.4	83.8	87.3	4.59
PAR	12.3	45.9	52.9	58.6	52.5	12.1
	111	53.8	55.7	49.8	53.2	5.55
	1000	46.7	45.3	52.1	48.0	7.50
THY	12.3	60.9	67.2	75.2	67.8	10.6
	111	68.2	67.7	64.6	66.8	2.88
	1000	62.6	62.2	56.2	60.3	5.95
THM	12.3	52.9	54.1	55.3	54.1	2.18
	111	60.1	61.5	61.1	60.9	1.20
	1000	49.8	53.6	53.4	52.3	4.09
CAF-d9	600	103	91.6	101	98.4	6.13
PAR-d3	400	45.5	50.9	56.5	51.0	10.8

Table 2

Matrix effects (ME) of caffeine (CAF), paraxanthine (PAR), theophylline (THY), and theobromine (THM), as well as deuterated caffeine (CAF-d9) and deuterated paraxanthine (PAR-d3) in human plasma performed with 5 mM HCOOH in mobile phase.

Compounds	Concentration ng/mL	Blank A Absolute ME (%)	Blank B Absolute ME (%)	Blank C Absolute ME (%)	Average Absolute ME (%)	CV Relative ME (%)
CAF	12.3	105	110	111	109	3.28
	111	85.2	101	96.9	94.2	8.01
	1000	94.2	91.9	96.2	94.1	2.15
PAR	12.3	112	102	93.5	102	9.10
	111	97.4	95.4	89.3	94.0	4.24
	1000	97.9	96.5	95.4	96.6	1.28
THY	12.3	85.5	101	101	95.9	9.02
	111	90.8	100	95.1	95.3	4.67
	1000	103	93.4	96.4	97.7	5.09
THM	12.3	104	111	109	108	3.26
	111	102	103	89.6	98.4	7.64
	1000	97.7	108	95.4	100	6.90
CAF-d9	600	105	103	97.3	102	4.02
PAR-d3	400	101	96.9	98.3	98.6	1.87

Table 3

Accuracy and precision (RSD) for assay of caffeine (CAF), paraxanthine (PAR), theophylline (THY), and theobromine (THM) in human plasma.

Compounds	Nominal Concentration ng/mL	Within-run (n=3)		Between-run (n=6)	
		Measured concentration (ng/mL), mean ± SD (RSD)	Accuracy	Measured concentration (ng/mL), mean ± SD (RSD)	Accuracy
CAF	12.3	12.0 ± 0.7 (5.69%)	97.7%	12.1 ± 0.7 (5.94%)	98.2%
	111	120 ± 5 (3.93%)	109%	119 ± 5 (3.80%)	107%
	1000	1057 ± 6 (0.55%)	106%	1055 ± 25 (2.38%)	106%
PAR	12.3	12.6 ± 0.7 (5.20%)	102%	12.0 ± 1.1 (9.12%)	97.2%
	111	110 ± 4 (3.64%)	99.1%	117 ± 3 (2.44%)	106%
	1000	1003 ± 6 (0.58%)	100%	1021± 42 (4.06%)	102%
THY	12.3	12.0 ± 0.8 (6.24%)	97.7%	11.9 ± 1.0 (8.32%)	97.0%
	111	112 ± 2 (2.07%)	101%	114 ± 8 (6.65%)	103%
	1000	991 ± 26 (2.63%)	99.1%	977 ± 24 (2.42%)	97.7%
THM	12.3	12.1 ± 0.7 (5.96%)	98.6%	12.1 ± 1.0 (8.37%)	98.1%
	111	110 ± 4 (3.28%)	99.0%	119 ± 2 (1.96%)	108%
	1000	1023 ± 15 (1.49%)	102%	1067 ± 24 (2.27%)	107%

Table 4

Stability for assay of caffeine (CAF), paraxanthine (PAR), theophylline (THY), and theobromine (THM) in human plasma.

Compounds	Nominal Concentration (ng/mL)	Plasma stability (4 hr, n=3)		Autosampler stability (12hr, n=3)		Freeze-thaw circle stability (n=3)		Long-term storage stability (9 months, n=6)	
		Measured concentration (ng/mL) mean ± SD (RSD)	Accuracy	Measured concentration (ng/mL) mean ± SD (RSD)	Accuracy	Measured concentration (ng/mL) mean ± SD (RSD)	Accuracy	Measured concentration (ng/mL) mean ± SD (RSD)	Accuracy
CAF	12.3	11.7 ± 0.9 (7.82%)	94.4%	11.4 ± 0.8 (7.23%)	92.9%	13.2 ± 0.7 (5.50%)	108%	12.7 ± 0.9 (7.44%)	103%
	111	115 ± 4 (3.86%)	103%	112 ± 2 (1.55%)	101%	126 ± 3 (2.48%)	113%	119 ± 5 (3.83%)	108%
	1000	971 ± 23 (2.38%)	97.1%	963 ± 17 (1.71%)	96.3%	1018 ± 17 (1.68%)	102%	1016 ± 39 (3.79%)	102%
PAR	12.3	12.4 ± 1.5 (12.3%)	101%	11.9 ± 1.0 (8.02%)	105%	12.4 ± 1.3 (10.3%)	101%	12.9 ± 0.9 (7.16%)	105%
	111	116 ± 6 (5.02%)	104%	121 ± 1 (0.79%)	109%	114 ± 8 (6.98%)	103%	112 ± 5 (4.03%)	101%
	1000	1053 ± 21 (1.96%)	105%	1063 ± 22 (2.09%)	106%	1050 ± 41 (3.89%)	105%	1003 ± 33 (3.31%)	100%
THY	12.3	14.0 ± 0.4 (3.05%)	114%	12.9 ± 1.3 (9.69%)	105%	13.4 ± 0.8 (5.93%)	109%	11.7 ± 0.9 (8.02%)	94.8%
	111	123 ± 7 (5.50%)	111%	126 ± 2 (1.64%)	113%	117 ± 8 (7.08%)	106%	115 ± 5 (4.21%)	103%
	1000	1053 ± 37 (3.50%)	105%	1048 ± 41 (3.93%)	105%	1016 ± 63 (6.20%)	102%	1030 ± 45 (4.38%)	103%
THM	12.3	12.7 ± 1.7 (13.3%)	103%	11.4 ± 1.5 (12.7%)	86.9%	12.9 ± 1.4 (11.2%)	104%	12.6 ± 1.4 (10.8%)	102%
	111	106 ± 4 (3.85%)	95.5%	101 ± 10 (10.3%)	90.9%	111 ± 11 (9.93%)	100%	106 ± 5 (4.80%)	95.2%
	1000	946 ± 44 (4.63%)	94.6%	943 ± 41 (4.29%)	94.3%	974 ± 40 (4.06%)	97.4%	992 ± 76 (7.64%)	99.2%