Food Chemistry 141 (2013) 2735–2742

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

# Simultaneous quantification of caffeine and its three primary metabolites in rat plasma by liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

*Article history:*
Received 29 October 2012
Received in revised form 29 April 2013
Accepted 16 May 2013
Available online 24 May 2013

*Keywords:*
Caffeine
Theobromine
Paraxanthine
Theophylline
Energy drinks
Rat plasma
Liquid chromatography–tandem mass spectrometry

ABSTRACT

A rapid, sensitive, simple and accurate LC–MS/MS method for the simultaneous quantitation of caffeine, and its three primary metabolites, theobromine, paraxanthine, and theophylline, in rat plasma was developed and validated. Chromatographic separation was performed on an Agilent Poroshell 120 EC-$C_{18}$ column using 1 μg/mL acetaminophen as an internal standard. Each sample was run at 0.5 mL/min for a total run time of 7 min/sample. Detection and quantification were performed using a mass spectrometer in selected reaction-monitoring mode with positive electrospray ionization. The lower limit of quantification was 5 ng/mL for all analytes with linear ranges up to 5000 ng/mL for caffeine and 1000 ng/mL for its metabolites. The coefficient of variation for assay precision was less than 12.6%, with an accuracy of 93.5–114%. The assay was successfully applied to determine plasma concentrations of caffeine, theobromine, paraxanthine, and theophylline in rat administered various energy drinks containing the same caffeine content. Various energy drinks exhibited considerable variability in the pharmacokinetic profiles of caffeine and its three primary metabolites, even containing the same caffeine. Different additives of energy drinks might contribute to these results.



## 1. Introduction

Caffeine (1,3,7-trimethylxanthine) is one of the most important naturally occurring methylated xanthine alkaloids. It is a constituent of coffee, tea, sodas, chocolate, various energy drinks, and is the most widely consumed behaviourally active substance in the world (Avisar, Avisar, & Weinberger, 2002). After rapid and complete absorption from gastrointestinal tract, caffeine undergoes extensive hepatic metabolism, mediated primarily by cytochrome P450 1A2 N-demethylation, to form three primary metabolites, paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxatnhine) (Jodynis-Liebert, Flieger, Matuszewska, & Juszczyk, 2004). In humans, the formation of paraxanthine accounts for 83.9 ± 5.4% of caffeine metabolism, while theobromine and theophylline account for 12.2 ± 4.1 and 3.7 ± 1.3%, respectively (McLean & Graham, 2002). These primary metabolites are further metabolized to form a variety of xanthenes, uric acids, and uracils, which are excreted in the urine (Kalow & Tang, 1991; Arnaud, 1993).

Caffeine and its primary metabolites have varying effects on the body. Caffeine itself increases the basal metabolic rate and acts as a mild central nervous system stimulant, myocardial stimulant, and smooth-muscle relaxant (Christian & Brent, 2001; Zagnoni & Albano, 2002). Paraxanthine is a central nervous stimulant with similar activity, but lower toxicity and less anxiogenic effects than caffeine (Okuro et al., 2010). Theobromine, which is highly prevalent in cocoa and chocolate, also shows similar stimulating effects, although to a lesser degree than caffeine (Benowitz, Jacob, Mayan, & Denaro, 1995). Theophylline is a widely used bronchodilating agent with a narrow serum therapeutic range (Shannon, 1999).

The mean caffeine intake in the overall population is approximately 210 mg/day, but 6% of the population ingests more than 500 mg/day and are considered to be heavy caffeine users (Hughes, McHugh, & Holtzman, 1998). Even though caffeine is widely consumed throughout the world, excessive caffeine intake is linked to undesired effects such as anxiety, tremors, headache, and gastrointestinal irritation (Okuro et al., 2010). Energy drinks are a

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http://dx.doi.org/10.1016/j.foodchem.2013.05.069

convenient way to consume caffeine and may be an effective supplement for increasing upper-body strength (Beck et al., 2006). In Korea, beverages containing a wide range of caffeine doses are marketed by various manufacturers. Hot Six® is a carbonated drink, similar to Red Bull® increasingly consumed among students during exam periods in Korea. A 250 mL can contains 80 mg of caffeine, 1000 mg of taurine, herbal extracts such as red ginseng and *Acanthopanax senticossus*, multi-vitamin B complex, and sugar derivatives, including glucuronalactone. Bacchus®, which is a non-carbonated drink, is specially formulated with diverse energy sources to provide a positive energy boost and is the most popular pick-me-up beverage in Korea. A 100 mL bottle includes 30 mg caffeine, 1000 mg taurine, 50 mg inositol, 20 mg nicotinamide, royal jelly, multi-vitamin B complex and other ingredients. Since the three primary metabolites of caffeine also have a stimulant effect (Okuro et al., 2010), it is important to obtain insight into the pharmacokinetics of these compounds in various energy drinks containing caffeine.

The majority of studies have reported simultaneous detection of caffeine and its metabolites using high-performance liquid chromatography (HPLC) with UV detection (Aresta, Palmisano, & Zambonin, 2005; Bispo et al., 2002; Jodynis-Liebert et al., 2004; McLean & Graham, 2002; Srdjenovic, Djordjevic-Milic, Grujic, Injac, & Lepojevic, 2008), despite the possibility of spectral interferences from co-eluting endogenous compounds (Ptolemy, Tzioumis, Thomke, Rifai, & Kellogg, 2010). This limitation can be resolved by coupling LC with tandem mass spectrometry (LC–MS/MS), although there few reports using this methodology for methylxanthine analyses (Hieda, Kashimura, Hara, & Kageura, 1995). These isomeric alkaloid metabolites cannot be distinguished based on their precursor masses alone, particularly caffeine and theobromine (Ptolemy et al., 2010). Furthermore, the interference of theophylline to paraxanthine is overlooked for the reported LC–MS/MS method (Noh et al., 2011; Ptolemy et al., 2010) although theophylline and paraxanthine have the same MS/MS transition at *m*/*z* 181.16/124.1. It is necessary to separate the two peaks chromatographically to avoid the interference. Several studies have successfully separated paraxanthine and theophylline peaks. However, these reported methods required time-consuming sample preparations procedures like solid phase extraction (Aresta et al., 2005; Caubet, Comte, & Brazier, 2004) or liquid–liquid extraction (Jodynis-Liebert et al., 2004) or a relatively large sample volume (Aresta et al., 2005; Huang et al., 2012) and also long chromatographic run times, i.e. 25 min (Schneider, Ma, & Glatt, 2003) and 45 min (Caubet et al., 2004; Huang et al., 2012), which lowers sample throughput capacity and sensitivity.

The aim of the present study was to develop a rapid, simple, and sensitive LC–MS/MS method for the simultaneous determination of caffeine and its three primary metabolites, paraxanthine, theobromine, and theophylline, in biological samples and to make a comparison of pharmacokinetic properties after oral administration of Hot Six®, Bacchus®, and caffeine to rats.

## 2. Materials and methods

### 2.1. Chemicals

Caffeine, paraxanthine (98%), theobromine (99%), theophylline anhydrous (⩾99%), acetaminophen (⩾99%), formic acid, trichloroacetic acid (TCA, 99%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Hot Six® (Lotte ChilSung, Seoul, Republic of Korea) and Bacchus® (Dong-A Pharmaceutical, Yongin, Republic of Korea) were purchased from local supermarkets. All solvents were of HPLC grade and obtained from Burdick & Jackson Company (Morristown, NJ, USA). All other chemicals were of the highest quality available.

### 2.2. Animals

Male Sprague–Dawley rats (8 weeks, 250–280 g) were purchased from Orient Bio (Sungnam, Gyeonggi-do, Korea) and were randomized and housed three per cage in a conditioned environment with 12-h light/dark cycles. Before the experiment, rats were fasted overnight and food was given 2 h after oral administration of the three solutions. The protocol for this animal study was approved by the Catholic University College of Medicine (Bucheon, Republic Korea).

### 2.3. Preparation of standards and quality controls

Stock solutions of caffeine (10 mg/mL), theobromine (300 μg/mL), paraxanthine (1 mg/mL), theophylline (1 mg/mL), acetaminophen (1 mg/mL, internal standard [IS]) were prepared by dissolving in distilled water. Serial dilutions were also prepared in distilled water and were added to drug-free rat plasma to produce final concentrations of 5, 20, 100, 500, 1000, 2000, and 5000 ng/mL for caffeine and 5, 10, 50, 100, 200, 500, and 1000 ng/mL for all three metabolites. The acetaminophen stock solution was diluted further to 1 μg/mL in 20% trichloroacetic acid (TCA) for routine use as an IS. Stock solutions for quality control (QC) samples were serially diluted with distilled water and added to drug-free rat plasma to achieve final concentrations of 5 (lower limit of quantification; LLOQ), 25 (low QC), 400 (medium QC), and 4000 (high QC) ng/mL for caffeine and 5 (LLOQ), 25 (low QC), 125 (medium QC), and 800 (high QC) ng/mL for all three metabolites.

On analyses days, calibration graphs for caffeine, theobromine, paraxanthine, and theophylline in rat plasma were derived from their peak area ratios relative to that of acetaminophen regression with $1/x$ for the three metabolites or $1/x^2$ for caffeine as a weighting factor. The respective QC samples were assayed along with each batch of plasma samples to evaluate the intra- and inter-day precision and accuracy of the method. All prepared plasma samples and stock solutions were stored at –80 °C.

### 2.4. Sample preparation

A 50 μL aliquot of 20% TCA containing 1 μg/mL acetaminophen (IS) was added to a 50 μL aliquot of rat plasma sample and mixed by vortexing for 5 min. After the mixture was centrifuged at 13,000*g* for 15 min at 4 °C, a 50 μL supernatant was transferred to another vial and diluted by adding 100 μL distilled water. The diluted sample was passed through a 1.2-μm syringe filter (Acrodisc, Pall Gelman Laboratory, Ann Arbor, MI, USA) and a 5-μL aliquot was injected into the LC–MS/MS system. All prepared samples were kept in an autosampler at 4 °C until injection.

### 2.5. LC–MS/MS analysis

The samples were analysed using a API 5500 Q-Trap mass spectrometer (AB SCIEX, Foster City, CA, USA) equipped with a 1260 HPLC system (Agilent Technologies, Wilmington, DE, USA) in electrospray ionization mode used to generate positive $[M+H]^+$ ions. The compounds were separated on a $C_{18}$ reversed-phase column (Poroshell 120 EC-$C_{18}$, 50 × 4.6 mm i.d., 2.7 μm particle size; Agilent) with an isocratic mobile phase consisting of 0.2% formic acid in distilled water and methanol (80:20, v/v) at a flow rate of 0.5 mL/min. The column temperature was maintained at 35 °C. The total run time was 7 min for each sample.

The optimized ion spray voltage and temperature were set at 4500 V and 600 °C, respectively. The operating conditions were

optimized by flow injection of caffeine, paraxanthine, theobromine, theophylline, and acetaminophen at concentrations of 10 ng/mL in distilled water. Nitrogen gas was used for the nebulizer, curtain, and collision-activated dissociation gas, which were set at 20, 60, and 60 psi, respectively. The individually selected reaction-monitoring with their transition parameters are listed in Table 1. The analytical data were processed using Analyst software (version 1.5.2; Applied Biosystems, Foster City, CA, USA).

### 2.6. Method validation

The validation parameters were selectivity, linearity, sensitivity, precision, accuracy, matrix effects, recovery, and stability of caffeine and its three metabolites in rat plasma, in accordance with United States (US) Food and Drug Administration (FDA) guidance for the validation of bioanalytical methods.

#### 2.6.1. Selectivity

Selectivity was evaluated by comparing chromatograms of six different batches of drug-free plasma obtained from six different sources to ensure that no interfering peaks were present at the retention time of caffeine and the three metabolites at the LLOQ.

#### 2.6.2. Linearity and sensitivity

Calibration curves in rat plasma were obtained by plotting the peak ratios of caffeine and its metabolites to the IS against the nominal concentrations of the calibration standards at 5, 20, 100, 500, 1000, 2000, and 5000 ng/mL for caffeine and 5, 10, 50, 100, 200, 500, and 1000 ng/mL for all three metabolites. The calibration curves were fitted using a linear least-squares regression with a weighing factor of $1/x$ for the three metabolites and $1/x^2$ for caffeine. The LLOQ for caffeine and the three metabolites in rat plasma were defined as the lowest concentration giving a signal-to-noise ratio of at least 10, acceptable accuracies of 80–120%, and sufficient precisions within 20%. These were verified by 10 replicate analyses.

#### 2.6.3. Precision and accuracy

Intraday precision and accuracy were determined by analysing six replicates of the LLOQ samples and three different QC samples (5, 25, 400, and 4000 ng/mL for caffeine and 5, 25, 125, and 800 ng/mL for the three metabolites) on the same day. Inter-day precision and accuracy were also evaluated by analysing 10 replicates of the LLOQ sample and three different QC samples (5, 25, 400, and 4000 ng/mL for caffeine and 5, 25, 125, and 800 ng/mL for the three metabolites) on five different days (two replicates/day). Precision was expressed as the relative standard deviation (RSD, %) and the accuracy was expressed as [(mean observed concentration)/(nominal concentration) × 100%]. The concentrations of QC, including LLOQ, samples were determined from the standard calibration curve and analysed on the same day.

**Table 1**
Selected reaction-monitoring transitions for MS/MS analysis.

| Analyte | Transition ($m/z$) | CE (V) | DP (V) | EP (V) | CXP (V) |
|---|---|---|---|---|---|
| Caffeine | 195.19/138.12 | 25 | 73 | 5 | 9 |
| Theobromine | 181.16/138.12 | 25 | 72 | 15 | 10 |
| Paraxanthine | 181.16/124.1 | 28 | 93 | 9 | 11 |
| Theophylline | 181.16/124.1 | 28 | 93 | 9 | 11 |
| Acetaminophen | 151.9/110.1 | 40 | 60 | 8 | 12 |

CE, collision energy; DP, declusting potential; EP, entrance potential; CXP, collision cell exit potential.

#### 2.6.4. Matrix effect and extraction recovery

Two different QC samples (25 and 4000 ng/mL for caffeine and 25 and 800 ng/mL for all three metabolites) and drug-free plasma were used to evaluate matrix effects and extraction recoveries of caffeine and its three metabolites. All assays were performed in triplicate. Taking the analyte peak areas obtained by direct injection of diluted (or neat) standard solutions as $A$, the corresponding peak areas of diluted (or neat) standard solutions spiked into plasma extracts after extraction as $B$, and the peak areas of diluted (or neat) standard solutions spiked into plasma prior to extraction as $C$, the matrix effects and extraction recovery were calculated as (Matuszewski, Constanzer, & Chavez-Eng, 2003):

$$\text{Matrix effect } (\%) = B/A \times 100$$

$$\text{Extraction recovery } (\%) = C/B \times 100$$

The matrix effects and extraction recovery of the IS were evaluated using the same method.

#### 2.6.5. Stability

The stability of caffeine and its three metabolites in rat plasma were assessed by analysing three replicate samples spiked with 25 and 4000 ng/mL for caffeine and 25 and 800 ng/mL for all three metabolites, respectively, under four conditions: (1) short-term storage for 8 h at room temperature; (2) long-term storage for (3 months at −80 °C; (3) three freeze–thaw cycles; (4) post-treatment storage for 48 h at 4 °C. The concentrations obtained were compared with the nominal values of the QC samples. The stabilities of stock solutions of caffeine, theobromine, paraxanthine, theophylline, and the IS evaluated after 4 weeks at 4 °C and after 3 months −80 °C, were evaluated by comparison with freshly prepared solutions of the same concentrations.

### 2.7. Animal treatment

The rats were divided randomly into three groups, Hot Six® (H, $n = 6$), Bacchus® (B, $n = 6$), and caffeine dissolved in distilled water (C, $n = 6$). For blood sampling, the carotid arteries of each rat were cannulated with polyethylene tubes (Clay Adams, Franklin Lakes, NJ, USA). Approximately 5 mL of the three solutions, equal to 1.5 mg of caffeine, was orally administered using a gastric gavage tube to rats in each group after fasting for 12 h. Approximately 0.12 mL of blood from each rat was collected into an Eppendorf tube at 0, 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, 480, and 600 min after oral administration. The blood samples were immediately centrifuged at 13,000 rpm for 5 min and the plasma samples (50 μL) were stored at −80 °C until LC–MS/MS analysis. Other procedures were complied reported previously (Bae, Bae, & Lee, 2009). After the experiments, the rats were euthanized with $CO_2$.

### 2.8. Pharmacokinetic parameters and statistical analysis

Pharmacokinetic parameters were calculated by a non-compartmental analysis using WinNonlin Professional, version 2.1 (Pharsight, Mountain View, CA, USA): the total area under the plasma concentration–time curve from time zero to infinity ($AUC_{0-\infty}$) or the last measured time ($AUC_t$), and terminal half-life ($t_{1/2}$). The peak plasma concentration ($C_{max}$) and time to reach $C_{max}$ ($T_{max}$) were read directly from the experimental data. The results were analysed using Student's $t$-tests, with $p$ values < 0.05 considered to indicate statistical significance. All data are expressed as means ± standard deviation (SD), except median (range) for $T_{max}$.

## 3. Results and discussion

### 3.1. LC–MS/MS optimization

In positive ion mode, all analytes yielded protonated molecular ions, $[M+H]^+$, as the major species. Fragmentation patterns of the protonated molecular ions were evaluated by increasing the collision energy. The product ion spectra and fragmentation patterns for caffeine, thoebromine, paraxanthine, theophylline, and IS are shown in Fig. 1. The most intense peaks were observed at *m*/*z* 138.12 for caffeine and theobromine, 124.1 for paraxanthine and theophylline, and 110.1 for IS. The mass parameters were optimized by observing the maximal response of the product ions. Since paraxanthine and theophylline have the same MS/MS transition at *m*/*z* 181.16/124.1, it was necessary to separate the two peaks chromatographically to avoid the interference.

To separate paraxanthine and theophylline peaks in a short run time, several different columns, including $C_8$, $C_{18}$, phenyl-hexyl, cyano, and hilic columns, and various compositions of mobile phase were evaluated. The Agilent Poroshell 120 EC-$C_{18}$ column (50 × 4.6 mm i.d., 2.7 μm particle size) and a mobile phase consisting of 0.2% formic acid in distilled water and methanol (80:20, v/v) yielded successful separations and good peak shapes. Although a stable isotope-labelled IS would have been ideal (Stokvis, Rosing, & Beijnen, 2005), an appropriate compound was not commercially available and not economical. Thus, several compounds were evaluated to identify a suitable IS. Acetaminophen was chosen because it showed no obvious matrix effects and exhibited a high extraction ratio.

### 3.2. LC–MS/MS method validation

#### 3.2.1. Selectivity

No interfering peaks were observed at the elution times of caffeine (5.0 min), theobromine (2.0 min), paraxanthine (2.8 min), theophylline (3.3 min), and the IS (2.2 min). Representative

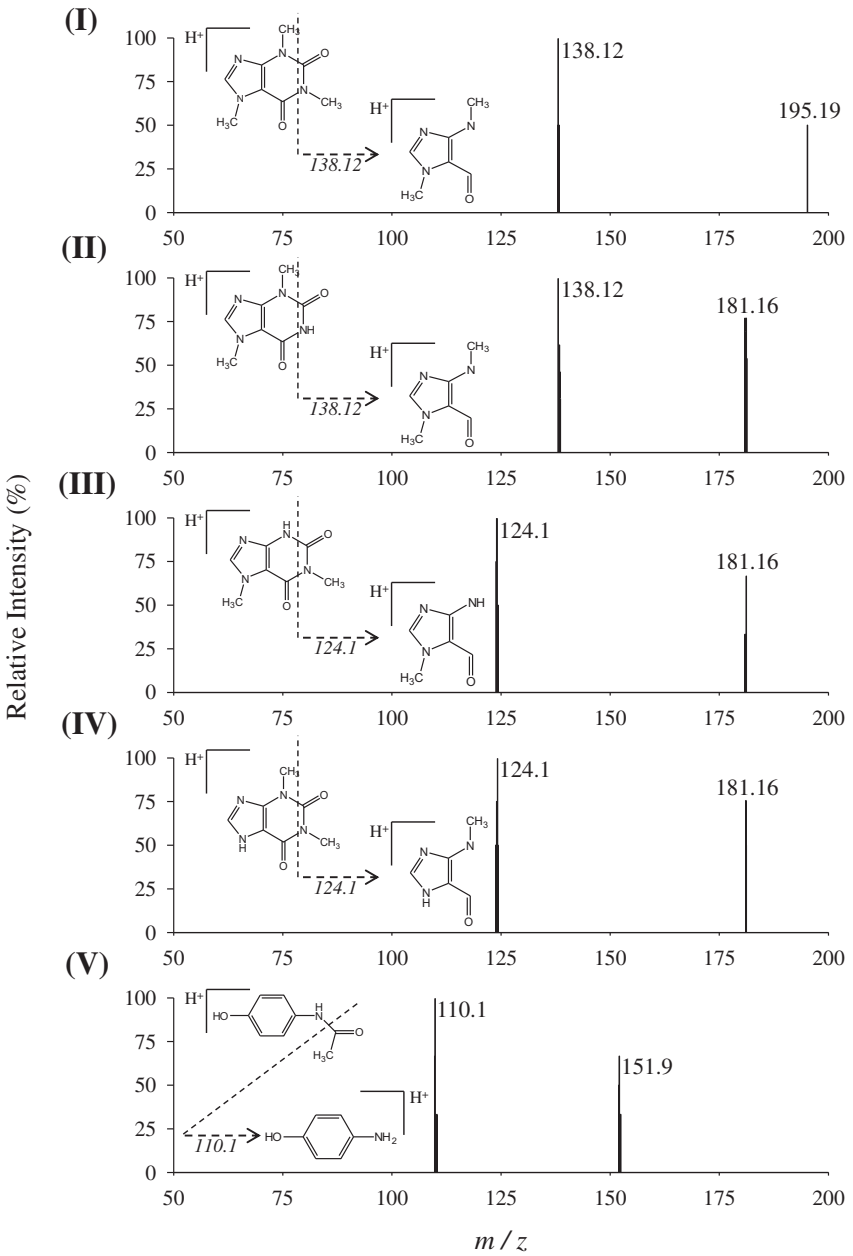


**Fig. 1.** Product ion mass spectra of caffeine (I), theobromine (II), paraxanthine (III) theophylline (IV), and acetaminophen (IS) (V) with $[M+H]^+$.

chromatograms of drug-free rat plasma, a plasma sample at LLOQ (5 ng/mL for caffeine and three metabolites), and a plasma sample from 480 min (caffeine, 164 ng/mL; theobromine, 222 ng/mL; paraxanthine, 115 ng/mL; theophylline, 218 ng/mL) after oral administration of Hot Six® are shown in Fig. 2. The total run time/sample was 7 min.

*3.2.2. Linearity and sensitivity*

Calibration curves were established using double blank (blank plasma with neither caffeine and three metabolites nor IS), zero blank (blank plasma with the IS only), and seven calibration standards with concentrations of 5, 20, 100, 500, 1000, 2000, and 5000 ng/mL for caffeine and 5, 10, 50, 100, 200, 500, and 1000 ng/mL for the three metabolites. During validation in rat plasma, the mean correlation coefficient ($r$) of the calibration curves was 0.9980 (range, 0.9954–0.9993; $n = 5$) for caffeine, 0.9999 (range, 0.9997–0.9999; $n = 5$) for theobromine, 0.9998 (range, 0.9996–0.9999; $n = 5$) for paraxanthine, and 0.9997 (range, 0.9994–0.9998; $n = 5$) for theophylline. The accuracies and precisions of all calibrators were within 89.7–106% and below 6.03% for caffeine, 97.9–102% and below 6.31% for theobromine, 94.9–103% and below 10.7% for paraxanthine, and 93.2–104% and below 3.91% for theophylline. The LLOQs for all analytes were 5 ng/mL, which were sufficient for pharmacokinetic studies of caffeine in rats.

*3.2.3. Precision and accuracy*

Intra- and inter-day precision and accuracy of the method were measured by analysing LLOQ and QC samples (5, 25, 400, and 4000 ng/mL for caffeine and 5, 25, 125, and 800 ng/mL for the three metabolites) on 5 different days and summarized in Table 2. Both precision and accuracy were well within the 15% acceptance range. The coefficients of variation for intra- and inter-day precision were <9.50% and <12.6% for caffeine, <4.60% and <8.14% for theobromine, <5.88% and <10.7% for paraxanthine, <4.48% and <7.94% for theophylline, respectively. The intra- and inter-day accuracies were 91.6–103% and 94.1–99.0% for caffeine, 93.9–102% and 94.7–102% for theobromine, 95.2–111% and 93.4–97.2% for paraxanthine, 96.0–114% and 95.7–100% for theophylline, respectively.

*3.2.4. Matrix effect and extraction recovery*

Two different QC samples (25 and 4000 ng/mL for caffeine and 25 and 800 ng/mL for the three metabolites) and drug-free plasma were used to evaluate the effects of the sample matrix on the ionization of caffeine, theobromine, paraxanthine, and theophylline; i.e., the degree of ion suppression or enhancement caused by matrix components. As defined in Section 2.6.4, the matrix effects were between 85% and 115%, indicating no significant differences between any of the analytes (data not shown).

The extraction recoveries in rat plasma were >86.7% for all analytes (data not shown). For the IS, the extraction recovery at an initial concentration of 1 μg/mL was 91.1 ± 5.16% (data not shown). Low matrix effects and highly reproducible recovery results demonstrate the reliability of the current method for bioanalyses.

*3.2.5. Stability*

Stock solutions of caffeine and its three metabolites in distilled water were stable for at least 4 weeks at 4 °C and for 3 months at –80 °C. More than 95.7% caffeine, 97.6% theobromine, 94.3% paraxanthine, and 101% theophylline were recovered from the initial amount of samples spiked with stock solutions stored under those respective conditions.

No significant degradation (within ±15% deviation between the predicted and nominal concentrations) of caffeine and its three metabolites occurred in rat plasma under the following conditions: short-term storage for 8 h at room temperature, long-term storage for 3 months at –80 °C, three freeze–thaw cycles, and post-treatment storage for 48 h at 4 °C (data not shown).

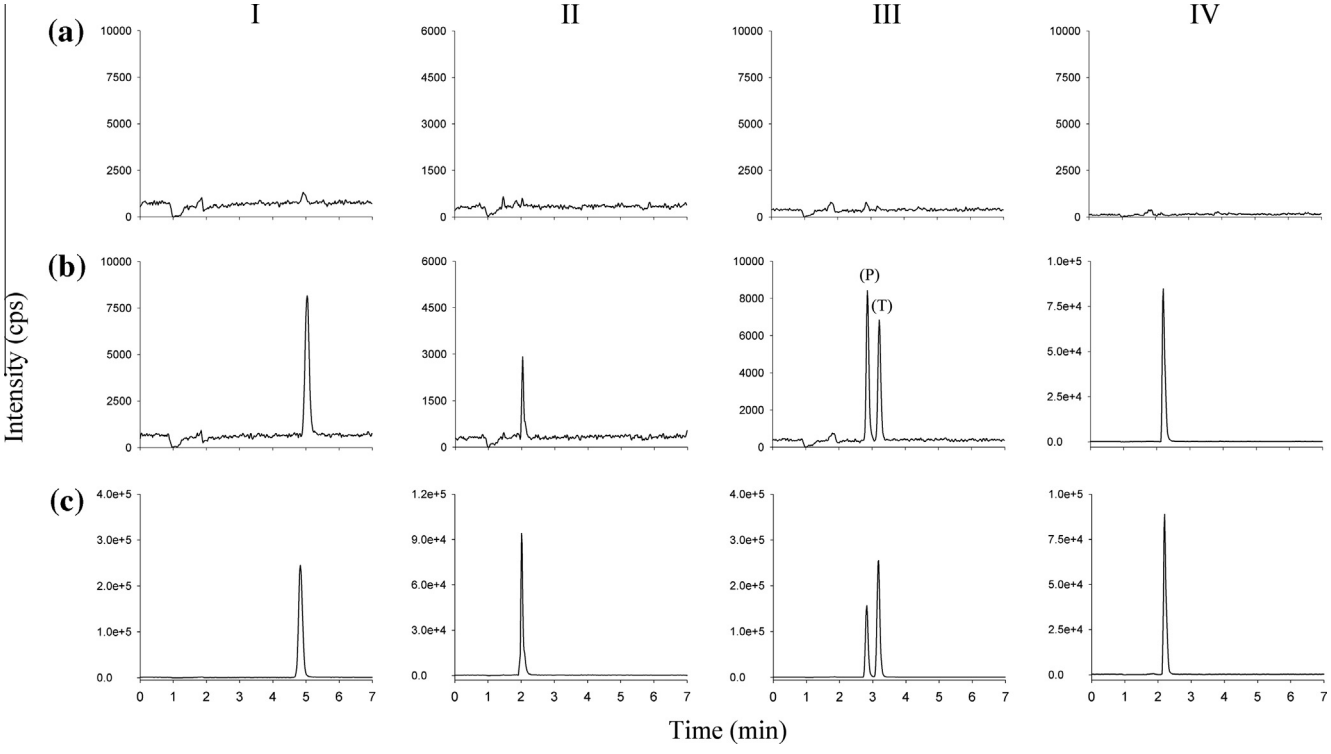


**Fig. 2.** Representative chromatograms of caffeine (I), theobromine (II), paraxanthine and theophylline (III), and acetaminophen (IS) (IV): (a) blank plasma, (b) blank plasma spiked with LLOQ (5 ng/mL) of caffeine, theobromine, paraxanthine, theophylline, and the IS (1 μg/mL), and (c) a plasma sample from 480 min after oral administration of Hot Six® in rats. (caffeine: 164 ng/mL, theobromine: 222 ng/mL, paraxanthine: 115 ng/mL, and theophylline: 218 ng/mL); (P), paraxanthine; (T), theophylline.

**Table 2**
The intra- and inter-day precision and accuracy data for assay of caffeine, theobromine, paraxanthine, and theophylline in rat plasma.

| Compounds | Nominal concentrations (ng/mL) | Intra-day ($n$ = 6) | | Inter-day ($n$ = 10) | |
|---|---|---|---|---|---|
| | | Precision (RSD, %) | Accuracy[a] (%) | Precision (RSD, %) | Accuracy[a] (%) |
| Caffeine | 5 | 9.50 | 103 | 12.6 | 94.1 |
| | 25 | 2.11 | 91.6 | 4.18 | 99.0 |
| | 400 | 2.04 | 93.5 | 2.20 | 96.7 |
| | 4000 | 1.76 | 101 | 4.02 | 97.5 |
| Theobromine | 5 | 4.60 | 102 | 8.14 | 94.7 |
| | 25 | 2.57 | 95.4 | 3.43 | 102 |
| | 125 | 1.92 | 93.9 | 1.89 | 99.6 |
| | 800 | 2.14 | 96.2 | 2.73 | 97.4 |
| Paraxanthine | 5 | 5.88 | 111 | 10.7 | 93.4 |
| | 25 | 2.70 | 96.5 | 4.02 | 96.8 |
| | 125 | 3.07 | 95.2 | 2.54 | 96.6 |
| | 800 | 2.01 | 95.5 | 2.97 | 97.2 |
| Theophylline | 5 | 4.48 | 114 | 7.97 | 95.7 |
| | 25 | 2.59 | 96.9 | 1.52 | 98.3 |
| | 125 | 1.41 | 96.0 | 2.00 | 100 |
| | 800 | 2.11 | 98.6 | 2.87 | 97.7 |

[a] Expressed as (mean calculated concentration)/(nominal concentration) × 100.

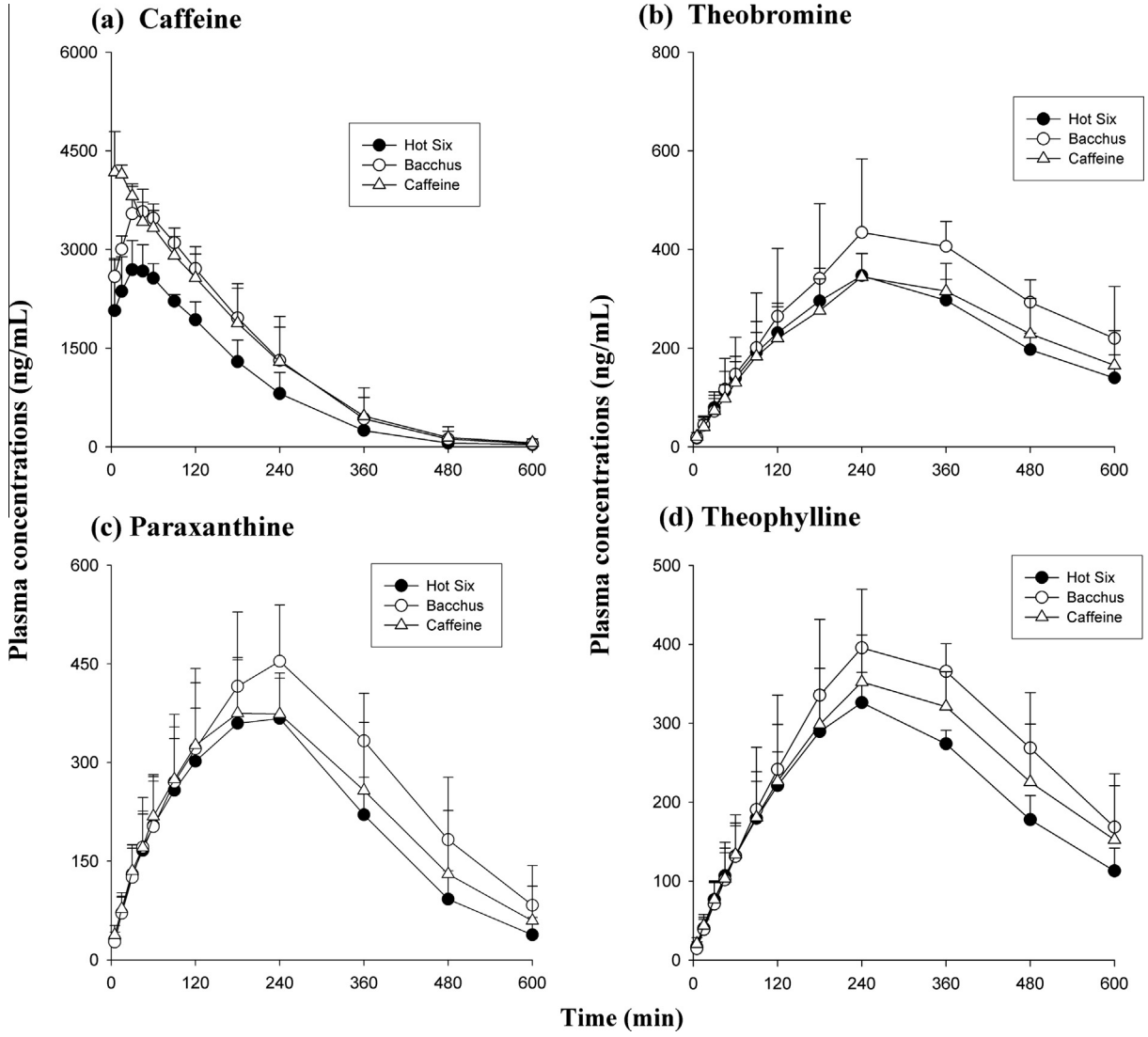


**Fig. 3.** Plasma concentrations of caffeine (a), theobromine (b), paraxanthine (c), and theophylline (d) after oral administration of Hot Six® (●, $n$ = 6), Bacchus® (○, $n$ = 6), and caffeine solution (△, $n$ = 6) in rats. Vertical bars represent standard deviation.

### 3.3. Pharmacokinetic properties of three different energy drinks containing caffeine

The LC–MS/MS method described herein was successfully applied to pharmacokinetic studies of caffeine after oral administration of two different energy drinks containing caffeine and caffeine solution to rats. The mean plasma concentration–time profiles for caffeine and its primary three metabolites, theobromine, paraxanthine, and theophylline, after oral administration of Hot Six®, Bacchus®, and a caffeine solution at a dose of 1.5 mg caffeine to rats are shown in Fig. 3. Relevant pharmacokinetic parameters are summarized in Table 3. The sensitivity and specificity of the method was sufficient for characterizing the pharmacokinetics of caffeine and its three primary metabolites. The QC samples ranged within 15% of the nominal concentrations, meeting the acceptance criteria of the US FDA for the validation of bioanalytical methods. As described earlier, there have not been any reports of chromatographic separation between paraxanthine and theophylline in a short run time. In this study, the two peaks having the same mass transition, paraxanthine and theophylline, from pharmacokinetic samples are clearly separated within 7 min per sample.

After oral administration of Hot Six®, the $AUC_t$ (32.8%) and $AUC_{0-\infty}$ (32.4%) was significantly smaller, and the $C_{max}$ of caffeine (36.6%) significantly lower. Additionally, the $AUC_t$ (25.6%) and $C_{max}$ (11.9%) of theophylline was significantly smaller and lower, respectively, that those of the caffeine solution (Table 3). Terminal half-life and $T_{max}$ of caffeine and theophylline, and other pharmacokinetic parameters of theobromine and paraxanthine were not changed compared with those of caffeine solution (Table 3).

With oral administration of Bacchus®, the $C_{max}$ (32.4%) and $T_{max}$ (280%) of caffeine were significantly lower and longer, respectively. Additionally, the $AUC_{0-\infty}$ of theobromine (35.9%) was significantly greater than that of caffeine solution (Table 3). The other pharmacokinetic parameters of theobromine, paraxanthine, theophylline were not changed compared with those of the caffeine solution (Table 3). In the Hot Six® group, the $AUC_t$ (31.5%) and $AUC_{0-\infty}$ (37.6%) was significantly greater and $C_{max}$ of caffeine (25.9%) significantly higher. For theophylline, the $AUC_t$ (38.8%) and $AUC_{0-\infty}$, (30.2%) was also significantly greater and the $C_{max}$ (18.7%) significantly higher than those of Hot Six® group (Table 3). Although the energy drinks contained the same caffeine content, different additives of energy drinks may influence the pharmacokinetic properties of caffeine and its three primary metabolites.

There are some limitations to our study. First, various energy drinks were administered to rats not humans. Second, in both species caffeine is mainly catalysed by CYP1A2 but by using different reactions: 3-N-demethylation in humans and C-8-hydroxylation in rats (Walton, Dorne, & Renwick, 2001). However, it was reported that the absorption, bioavailability, and route of excretion of caffeine generally similar between humans and rats (Walton et al., 2001). This study was not focused on drug/food-drug interactions with CYP1A2 but only compared the pharmacokinetics among same caffeine-containing drinks. Therefore, our findings may provide some useful information on the pharmacokinetic differences of caffeine in humans.

## 4. Conclusions

A rapid, sensitive, simple and accurate LC–MS/MS method for the simultaneous quantitation of caffeine, and its three primary metabolites, theobromine, paraxanthine, and theophylline, in rat plasma was developed and validated. This method allows a high sample throughput due to the short run time, 7 min and simple protein precipitation. This short analysis time may meet the requirement for high sample throughput in bioanalyses. The analysis time for caffeine and its three primary metabolites in the literature was about 25 min (Schneider et al., 2003) and 45 min (Caubet et al., 2004; Huang et al., 2012), which lowers sample

**Table 3**
Comparison of pharmacokinetic parameters of caffeine, theobromine, paraxanthine, and theophylline after single oral administration of Hot Six®, Bacchus®, and caffeine solution at a dose of caffeine 1.5 mg in rats.

| | Hot Six® ($n$ = 6) | Bacchus® ($n$ = 6) | Caffeine ($n$ = 6) |
|---|---|---|---|
| *Caffeine* | | | |
| $AUC_t$ (μg min/mL) | 507 ± 66.3$^{*,\int\int}$ | 740 ± 146 | 755 ± 203 |
| $AUC_{0-\infty}$ (μg min/mL) | 514 ± 72.9$^{*,\int}$ | 744 ± 150 | 760 ± 209 |
| $C_{max}$ (μg/mL) | 2.80 ± 0.353$^{**,\int\int}$ | 3.78 ± 0.203$^{**}$ | 4.42 ± 0.331 |
| Terminal half-life (min) | 61.3 ± 16.8 | 58.2 ± 117.1 | 58.7 ± 13.5 |
| $T_{max}$ (min)[a] | 30 (5–120) | 38 (30–60)$^{***}$ | 10 (5–15) |
| *Theobromine* | | | |
| $AUC_t$ (μg min/mL) | 139 ± 11.5 | 198 ± 61.7 | 179 ± 42.1 |
| $AUC_{0-\infty}$ (μg min/mL) | 188 ± 33.1 | 261 ± 68.7$^{*}$ | 192 ± 31.0 |
| $C_{max}$ (μg/mL) | 0.359 ± 0.0410 | 239 ± 129 | 0.366 ± 0.0319 |
| Terminal half-life (min) | 230 ± 64.3 | 228 ± 69.8 | 207 ± 21.6 |
| $T_{max}$ (min)[a] | 240 (180–360) | 300 (240–360) | 300 (240–360) |
| *Paraxanthine* | | | |
| $AUC_t$ (μg min/mL) | 130 ± 23.2 | 163 ± 24.0 | 137 ± 17.2 |
| $AUC_{0-\infty}$ (μg min/mL) | 135 ± 28.3 | 180 ± 35.9 | 150 ± 27.5 |
| $C_{max}$ (μg/mL) | 0.382 ± 0.0763 | 0.472 ± 0.0880 | 0.409 ± 0.0578 |
| Terminal half-life (min) | 97.5 ± 27.6 | 119 ± 47.1 | 113 ± 42.6 |
| $T_{max}$ (min)[a] | 240 (180–240) | 240 (180–360) | 210 (180–360) |
| *Theophylline* | | | |
| $AUC_t$ (μg min/mL) | 128 ± 6.10$^{*,\int\int}$ | 209 ± 31.9 | 172 ± 36.1 |
| $AUC_{0-\infty}$ (μg min/mL) | 162 ± 16.1$^{\int}$ | 232 ± 3361.9 | 187 ± 25.3 |
| $C_{max}$ (μg/mL) | 0.334 ± 0.0253$^{*,\int}$ | 0.411 ± 0.0577 | 0.379 ± 0.0244 |
| Terminal half-life (min) | 194 ± 43.5 | 201 ± 49.7 | 178 ± 24.3 |
| $T_{max}$ (min)[a] | 240 (180–360) | 240 (240–360) | 240 (240–360) |

[a] Median (ranges).
$^{*}$ $p < 0.05$ compared with caffeine group.
$^{**}$ $p < 0.001$ compared with caffeine group.
$^{\int}$ $p < 0.05$ compared with Bacchus group.
$^{\int\int}$ $p < 0.001$ compared with Bacchus group.

throughput capacity and sensitivity. This assay exhibited a sensitivity (LLOQ, 5 ng/mL) using small plasma volume (50 μL), and successfully applied to determine plasma concentrations of caffeine, theobromine, paraxanthine, and theophylline after oral administration of various energy drinks containing the same caffeine content to rats. Various energy drinks exhibited considerable variability in the pharmacokinetic profiles of all analytes, even containing the same caffeine. Different additives of energy drinks might contribute to these results.

## Acknowledgements

The authors thank all of second-year undergraduate students in the College of Pharmacy, The Catholic University of Korea who participating in preliminary studies. This study was supported by the Global Leading Technology Program of the Office of Strategic R&D Planning (OSP) funded by the Ministry of Knowledge Economy, Republic of Korea (No. 10039320) and the Research Fund of The Catholic University of Korea (2012).

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