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LC-MS/MS method for rapid and accurate detection of caffeine in a suspected overdose case

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ABSTRACT

Excessive intake of caffeine, otherwise known to be a safe and mild central nervous system stimulant, causes nausea, vomiting, convulsions, tachycardia, and eventually fatal arrhythmias and death. Caffeine intoxication, a global problem, has been increasing in Japan since 2013. Thus, there is a need for rapid and accurate diagnosis of caffeine poisoning in forensic and clinical toxicology investigations. Herein, we demonstrate rapid and accurate caffeine quantitation by liquid chromatography tandem mass spectrometry using the standard addition method in a fatal case. Biological samples were diluted 500–100,000-fold and subjected to a simple pretreatment (adding caffeine standard and internal standard and passing through a lipid removal cartridge). The multiple reaction monitoring transitions were 195 → 138 for quantitation, 195 → 110 for the qualifier ion, and 204 → 144 for the internal standard (caffeine-d9). The standard plots were linear over 0–900 ng/mL (r^2 = 0.9994–0.9999) for biological samples, and the reproducibility (%RSD) of the method was 1.53–6.97% (intraday) and 1.59–10.4% (interday). Fatal levels of caffeine (332 μg/mL) and toxic to fatal levels of olanzapine (625 ng/mL), along with other pharmaceuticals were detected in the external iliac venous blood. The cause of death was determined to be multi-drug poisoning, predominantly caused by caffeine. Our method is useful for not only forensic cases but also the rapid diagnosis of caffeine overdose in emergency clinical settings.

1. Introduction

Caffeine is a derivative of xanthine, similar to theophylline and theobromine, and is a mild central nervous system stimulant (Baselt, 2017). Caffeine is present in various products that are used regularly, such as coffee, tea, cocoa, some soft drinks, and dietary supplements. However, the risks associated with caffeine at high doses are not well recognized in the general public. Because the chemical structure of caffeine is similar to that of adenosine, caffeine can bind to adenosine receptors (A_1 and A_{2A}) and exert its action. Normally, adenosine suppresses dopamine neuronal activity, but its action is blocked by caffeine, which exerts indirect excitatory effects and promotes myocardial inotropy and chronotropy. When caffeine is ingested at high amounts, phosphodiesterase (PDE) is inhibited and intracellular cAMP concentration increases, further strengthening myocardial contractions. This causes a variety of cardiac events, which can eventually lead to fatal arrhythmias and death (Banerjee, Ali, Levine, & Fowler, 2014; Lévy et al., 2019; Poussel et al., 2013; Rudolph & Knudsen, 2010).

Recently, in addition to the prevalent use of highly caffeinated energy drinks or energy shots among young individuals to stay awake, pure caffeine tablets or high-caffeine-containing supplements, marketed to combat sleepiness, are easily available in stores or via online shops in Japan. Therefore, in addition to accidental intoxication by consuming caffeine at large amounts, some caffeine-related suicide cases have been reported (Ishikawa, Yuasa, & Endoh, 2015; Nojima et al., 2019; Noto, Hanazawa, Yoshizawa, Murabayashi, & Morioka, 2019; Yamamoto et al., 2015). Kamijo et al. reported that caffeine intoxication cases have been increasing in Japan since 2013, and caffeine tablets were used in most of these cases (N = 101, 96%) (Kamijo, Takai, Fujita, & Usui, 2018). However, as the sales of caffeine products are not regulated in Japan, pure caffeine or highly concentrated caffeine products can be easily procured. Fatal and non-fatal intoxication by excessive caffeine

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intake is a major problem in not only Japan but also several other countries (Magdalan et al., 2017; Thelander et al., 2010). Therefore, a rapid, easy, and accurate diagnosis method of caffeine intoxication is needed for both forensic and clinical settings. Several analytical methods using gas chromatography, gas chromatography–mass spectrometry, high-performance liquid chromatography, and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) have been reported for the determination of caffeine in biological samples (Brooks & Smith, 1991; Chen, Hu, Parker, & Laizure, 2017; Koller, Zubiaur, Saiz-Rodríguez, Abad-Santos, & Wojnicz, 2019; Kumazawa, Sato, Seno, Ishii, & Suzuki, 1994; Lopez-Sanchez, Lara-Diaz, Aranda-Gutierrez, Martinez-Cardona, & Hernandez, 2018). However, most of these techniques require tedious and time-consuming pre-treatment processes before measurements or need relatively large amounts of biological samples. Although most of these methods are validated using normal human plasma/serum as biological matrix with the addition of an internal standard, it is difficult to completely correct the matrix effect (to obtain accurate values) when using forensic and clinical samples. Because most patients or victims in forensic and clinical poisoning cases usually take not only caffeine but also several other drugs at varying amounts, the various drugs themselves become a challenge in caffeine quantitation. This challenge can be overcome by using a standard addition method to eliminate the matrix effect in forensic and clinical samples. Herein, we report a rapid, simple, and accurate LC-MS/MS method for caffeine quantification using the standard addition method in a forensic case of a teenager who committed suicide by ingesting a large amount of pure caffeine tablets purchased online.

2. Methods

2.1. Reagents

Acetonitrile and methanol of LC-MS grade and caffeine (chemical purity >98%) were purchased from FUJIFILM Wako Pure Chemical Industries, Limited (Osaka, Japan). Caffeine-d9 (chemical purity >97%, isotopic purity >99.7%) was purchased from Toronto Research Chemicals (North York, ON, Canada). Diazepam-d5 methanol solution (1 mg/mL) was purchased from Cerilliant Corp. (Round Rock, TX, USA). Ammonium formate was purchased from Kanto Chemical Company Inc. (Tokyo, Japan). A QuEChERS pre-packed extraction packet (containing 6 g magnesium sulfate and 1.5 g sodium acetate), dispersive solid phase extraction kit (containing 25 mg primary secondary amine, 25 mg end-capped octadecylsilane, and 150 mg magnesium sulfate), and Captiva 3 mL Non-Drip Lipids were purchased from Agilent Technologies (Santa Clara, CA, USA).

2.2. Case presentation

A teenage boy who had been hospitalized for schizophrenia was permitted to temporarily return to his relative's home for an overnight stay. His prescriptions at the time were Lunesta (eszopiclone), Lexapro (escitalopram), risperidone, olanzapine, Akineton (biperiden), Abilify (aripiprazole), Cercine (diazepam), and flunitrazepam. Early next morning, he complained of malaise and groaned in pain while walking unsteadily. Within a minute, he vomited, lost consciousness, and collapsed. When paramedics arrived, he was in a supine position, was not breathing, and had no pulse. His Japan coma scale was III-300 (corresponding to a Glasgow coma scale of E1V1M1). He was experiencing ventricular fibrillation, and defibrillation with an automated external defibrillator resulted in asystole. At this point, no empty containers of pharmaceuticals or agrochemicals were found in the house. Adrenaline was administered intravenously, and cardiopulmonary resuscitation was continued, but his condition did not improve. Death was confirmed in the hospital where he was transported.

According to the subsequent police report, there was vomit along with traces a whitish powder at the scene, but the police did not collect this important evidence. The deceased had attempted suicide in the past; therefore, the police initially searched the living room for suspected drugs, but did not find any trace of pharmaceuticals, illegal drugs, or agrochemicals before the autopsy. The deceased was not a habitual drinker or smoker. Postmortem computed tomography imaging before the autopsy showed high X-ray absorption by the esophagus, stomach, duodenum, and small intestine. At autopsy, there was no external evidence of trauma or any specific findings in the pathological diagnosis. During autopsy, the following were observed: a small amount of bloody fluid in the oral cavity; approximately 45 mL of light brown, viscous liquid with whitish powder in the stomach; 41 mL of powdery, ocher-colored liquid in the duodenum; a small to moderate amount of pale-yellow liquid in the jejunum and ileum (powdery substances had adhered to the walls of the organs); and a moderate amount of yellowish liquid in the large intestine. Approximately 400 mL of heart blood and 300 mL of light-yellow transparent urine were collected. For the toxicological analysis, whole blood from the external iliac vein and right heart, urine, gastric contents, and duodenal contents were collected. Several days after the autopsy, the police investigated his internet browsing history and revealed that the deceased had purchased caffeine tablets (100 tablets, 200 mg each) via an online shop 3 days before his death. A reinvestigation of the scene by the police revealed an empty bottle of caffeine tablets, which the deceased had purchased via online shopping (Fig. 1). The empty bottle had been hidden behind a picture frame hanging on the wall.

2.3. Sample preparation

2.3.1. General targeted screening

Whole blood from the external iliac vein was extracted using the QuEChERS method (Usui, Hayashizaki, Hashiyada, & Funayama, 2012). Briefly, 0.5 mL of blood was diluted with 1.0 mL of ultrapure water. The diluted sample was then placed in a plastic tube with 0.5 g of the QuEChERS kit reagents, stainless steel beads (5 mm O.D.), and 1 mL of 50 ng/mL diazepam-d5 acetonitrile solution. The mixture was vigorously shaken for 30 s in a bead beater-type homogenizer (Beads crusher μT-12; TAITEC, Saitama, Japan) at 3200 rpm, and then centrifuged at 4400 ×*g* for 5 min at 4 °C. The supernatant was transferred to dispersive

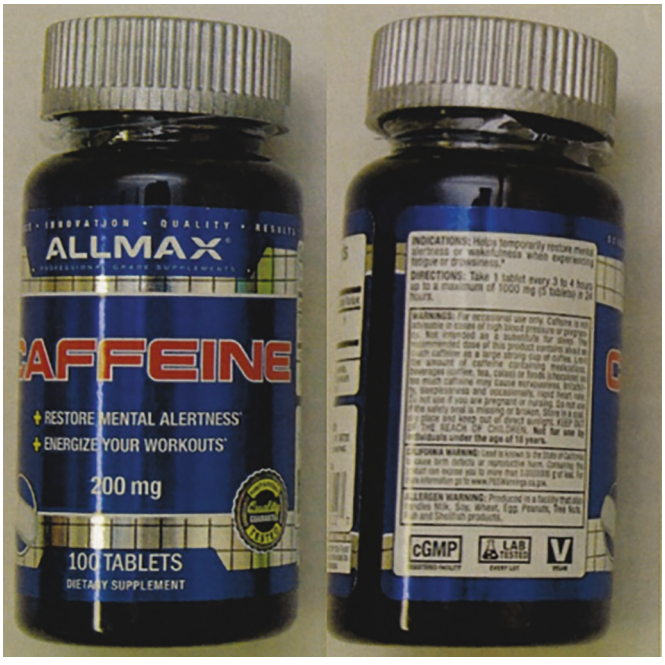

Fig. 1. Appearance of the bottle of caffeine tablets that the deceased purchased online.

solid-phase extraction kit reagents (2 mL centrifuge tube) for purification. The contents were mixed for 10 s and centrifuged at 4400 ×*g* for 1 min at 4 °C. Ten microliters of the upper layer was subjected to LC-MS/MS as described in section 2.4.1.

2.3.2. Caffeine quantitation

Whole blood and urine were diluted 1000-fold and 500-fold with deionized water, respectively.

The gastric and duodenal contents were also diluted 100,000-fold and 50,000-fold with deionized water, respectively. Ten microliters of the diluted sample, 10 μL of standard caffeine of appropriate concentration (0, 150, 300, 450, 600, 750, or 900 ng/mL), 180 μL of deionized water, and 300 μL of methanol containing 30 ng/mL caffeine-d9 as an internal standard (IS) were mixed vigorously. At this point, the samples were diluted 25,000–5 × 10^6 times. The mixed solution was then centrifuged at 15,000 ×*g* for 5 min. The supernatant was passed through a Captiva ND Lipids Cartridge (3 mL cartridge) to remove lipids, proteins, and fine particles. Subsequently, 1 μL of the eluent was subjected to LC-MS/MS analysis as described in section 2.4.2. Each seven-point standard curve was prepared three times a day for 3 consecutive days ($N = 9$). Intraday and interday precision values were calculated using the one-way analysis of variance, and they are expressed as relative standard deviations (%RSDs). The recovery rate of caffeine from the Captiva ND Lipids Cartridge was determined at three different concentrations (spiked concentrations, low: 150 ng/mL, medium: 450 ng/mL, high: 950 ng/mL) for each biological sample ($N = 3$).

2.4. LC-MS/MS conditions

2.4.1. General targeted screening

Liquid chromatography was carried out on a Shimadzu Prominence LC System (Kyoto, Japan). Chromatographic separation was achieved on an L-column ODS (150 mm × 1.5 mm i.d., 5 μm particle size; Chemicals Inspection and Testing Institute, Tokyo, Japan). Tandem mass spectrometry was performed on a Sciex 3200 QTRAP LC/MS/MS System (Framingham, MA, USA) equipped with an electrospray ionization (ESI) probe. The mobile phase was 95% 10 mmol/L ammonium formate–5% methanol (solvent A) and 5% 10 mmol/L ammonium formate–95% methanol (solvent B). The solvent gradient linearly increased from 0% to 100% solvent B in 20 min, and it was held at 100% solvent B for 5 min. The solvent flow rate was set at 0.1 mL/min. The product ion spectra were measured in the multiple reaction monitoring-enhanced product ion (MRM-EPI) scan mode, where the product ion spectra were measured as EPIs triggered by an MRM signal of threshold intensity. The threshold for EPI was set to 500 counts per second, and the EPI scan range was *m/z* 50–750. EPI spectra were acquired at three collision energy levels (20, 35, and 50 eV). Quantitative analysis was carried out in the MRM mode.

2.4.2. Caffeine quantitation

Liquid chromatography separation was performed using a Nexera LC System (Shimadzu, Kyoto, Japan). A CAPCELL PAC ADME column (100 mm × 2.1 mm i.d.; 3 μm particle size; Shiseido Co., Ltd., Tokyo, Japan) was used for chromatographic separation. The mobile phase consisted of 95% 10 mmol/L ammonium formate–5% methanol (solvent A) and 5% 10 mmol/L ammonium formate–95% methanol (solvent B). The separation was performed in the isocratic elution mode with 60% solvent B. The injection volume was 1 μL, cycle time was 3 min, flow rate of the mobile phase was 0.2 mL/min, and column temperature was maintained at 40 °C. Tandem mass spectrometry detection was performed using a QTRAP 5500 system (SCIEX, Framingham, MA, USA), in the MRM mode used to quantify caffeine. The MRM transition of 195 → 138 was used for quantitation, 195 → 110 was used as the qualifier ion, and 204 → 144 was used for caffeine-d9 (IS). Each dwell time was set at 150 ms. All experiments were conducted in the positive ion mode.

3. Results and discussion

In this forensic case, caffeine in biological samples was quantified using the standard addition method; Table 1 shows the results. The intraday and interday precisions (%RSD) were 1.53%–6.97% and 1.59%–10.4%, respectively. As standard addition plots are constructed in this method by adding standard caffeine of various concentrations to the real forensic samples, each sample matrix is identical. Therefore, the matrix effects, which cause variability in the quantification values in hyphenated chromatography-mass spectrometry techniques, can be negated to obtain accurate values using the standard addition method. Table 2 shows the recovery rate of caffeine from the Captiva ND Lipids Cartridge at three different concentrations. The absolute and relative recovery rates were 83%–94% and 99%–104%, respectively. Nearly constant recovery rates were obtained over the concentration range.

In general, the construction of standard addition plots increases the number of analyses, and therefore, more sample amount and analytical time are required. However, as caffeine concentrations in lethal/intoxication cases are usually over 80–100 μg/mL in blood samples (Cappelletti et al., 2018; Willson, 2018), the blood samples must be diluted at least 50,000-fold for the highly sensitive mass spectrometer used in our method. Because our method requires only 10 μL of 500–100,000-fold diluted sample, 0.5 μL of the original biological sample was more than sufficient for constructing the standard addition plots. Furthermore, pretreatment of biological samples is also simple and rapid, requiring only passage through a lipid-removal cartridge, and the chromatographic cycle time was only 3 min. This method is useful for not only forensic cases but also the rapid diagnosis of caffeine overdose in emergency clinical settings.

Jones reviewed 51 fatal caffeine poisoning cases and reported that the mean (± SD), median, and range of caffeine concentrations in postmortem blood were 187 ± 96, 180, and 33–567 μg/mL, respectively (Jones, 2017). The blood caffeine concentration in our case was 332–391 μg/mL, which is substantially higher than the mean reported by Jones (2017).

The toxicological analysis revealed that the external iliac venous blood contained citalopram (615 ng/mL), olanzapine (625 ng/mL), aripiprazole (393 ng/mL), nordiazepam (132 ng/mL), and biperiden (11 ng/mL), besides caffeine. It is well known that caffeine interacts with some pharmaceuticals that are also metabolized by CYP1A2, such as theophylline, olanzapine, and clozapine. In our case, olanzapine was detected, and its concentration (625 ng/mL) ranged from toxic to lethal levels (Molina, 2010). This suggests that competitive inhibition by combined use of caffeine and olanzapine had enhanced the pharmacological action of each drug.

Kamijo et al. reported that the mean caffeine intake in 101 patients who had been hospitalized for caffeine poisoning was 7.2 g (range, 1.2–82.6 g) (Kamijo et al., 2018). In their report, the caffeine intake in seven patients (6.9%) who experienced cardiac arrests during the clinical course was 6–36 g, and three of these patients died. The mean serum caffeine concentration upon admission in these seven patients was >232 μg/mL, and the minimum lethal dose of caffeine was 6 g. It is not clear how many caffeine tablets were ingested by our patient. However, considering that the bottle of pure caffeine tablets, which had been bought by the deceased 3 days before his death, was already empty the day after he returned to his relative's house and also because his blood caffeine level was very high, it is reasonable to surmise that the deceased ingested a large amount of caffeine in a short period. If all tablets had been ingested at once, the total intake of caffeine would have been 20 g. Alternatively, considering the weight of the deceased (67 kg), the volume of distribution of caffeine (0.4–0.6 L/kg) (Baselt, 2017), and the caffeine concentration in the biological samples, at least 8.9–13.4 g of caffeine would have been present in the body of the deceased. From these data, the cause of death of our patient was determined to be multidrug poisoning (the dominant substance could be caffeine), based on the high blood concentration of caffeine, olanzapine, and other drugs in

Table 1
Quantitation of caffeine using the standard addition method ($N = 9$).

Biological samples	Equations	Dilution factors	r^2	Mean concentrations (μg/mL)	Reproducibility (%RSD)	
					Intraday	Interday
External iliac venous blood	y = 9.09E-04 X+ 0.301	1000	0.9999	332	1.53	1.59
Right heart blood	y = 9.17E-04 X+ 0.359	1000	0.9994	391	1.99	3.72
Urine	y = 9.09E-04 X+ 0.027	500	0.9997	15.1	6.97	10.4
Gastric contents	y = 9.15E-04 X+ 0.126	100,000	0.9998	13,778	2.10	2.73
Duodenum contents	y = 8.92E-04 X+ 0.164	50,000	0.9996	9180	2.59	2.61

Table 2
Recovery rates of caffeine from the lipid cartridge (N = 3).

	External iliac venous blood		Right heart blood		Urine		Gastric contents		Duodenum contents	
Recovery rate %	Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative	Absolute	Relative
Low: 150 ng/mL	90 (3.8)	104 (4.0)	90 (2.4)	103 (1.6)	94 (1.2)	100 (9.4)	91 (3.2)	104 (1.6)	83 (4.2)	100 (0.29)
Medium: 450 ng/mL	91 (2.2)	101 (4.2)	88 (2.3)	100 (1.5)	89 (1.3)	102 (0.87)	86 (1.6)	99 (2.0)	83 (0.88)	101 (1.8)
High: 900 ng/mL	89 (2.8)	103 (2.9)	91 (4.6)	102 (1.0)	86 (1.7)	103 (2.1)	86 (1.7)	99 (1.7)	85 (2.9)	102 (1.0)

The values in parentheses are relative standard deviations %.

addition to no signs of fatal injuries and sickness. The mode of death was classified as suicide according to a forensic pathologist.

Sales restrictions on the amount of caffeine that can be purchased at one time have been imposed in some countries (Thelander et al., 2010). However, in several countries, it is still possible to purchase products, including caffeine, at more than the lethal dose, as pharmaceuticals or supplements of pure caffeine tablets/capsules without any restrictions. Some regulations on the sale of caffeine products should be implemented worldwide to prevent accidental intoxication or suicide.

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Declaration of Competing Interest

None.

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