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Original Article

Caffeine analysis in urine by gas chromatography mass spectrometry: A non-derivatization detection and confirmatory method

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ABSTRACT

Background and Aims: Caffeine is a xanthine alkaloid found naturally in plants. Caffeine has cardiotonic and stimulant effects in humans and animals. For this reason, caffeine is on the monitoring list for human sports and is listed as a feed contaminant in horse racing. The aim of this study was to develop a rapid, practical, and specific method for the determination of caffeine in horse urine.

Methods: In the new method, the pH of the sample was adjusted by the addition of phosphate buffer, and after solid phase extraction, it was dissolved in methanol before being analysed by gas chromatography mass spectrometry without derivatization. The method was validated according to the European Commission's 2002/657/EC criteria.

Results: The effects of different cartridge brands, pH, and elution solution were determined. Intraday and interday CV% values are 2.8 and 5.2 for the International Residue Limit (IRL), respectively. Five levels (blank, 0.5xIRL, IRL, 1.5xIRL, and 2xIRL) were used in constructing the curve, and the R2 value was greater than 0.99. The analysis run was 11.8 min. The decision limit (CCα) was determined to be 56.7 ng/mL due to IRL. The detection limit of the method was calculated to be 3.3 ng/mL. The method was determined to be robust according to changes in extraction pH, phosphate buffer concentration, centrifugation time, hexane volume in the wash step, different grades of methanol, inlet temperature, and operator.

Conclusion: The applicability of the method was demonstrated by analysing positive and negative horse urine samples. Validation parameters showed the method to be selective, specific, and easy to apply.

Keywords: Caffeine, Gas chromatography-mass spectrometry, Urine

INTRODUCTION

Caffeine is a methylxanthine alkaloid that is naturally found in the environment (Greene, Woods, & Tobin, 1983). Due to its widespread use, caffeine can also be present in the metabolism as a result of environmental contamination. Pharmacologically, caffeine is an effective cardiotonic and diuretic for horses as it possesses pronounced effects on the central nervous system (Aramaki, Suzuki, Ishidaka, Momose, & Umemura, 1991). The International Federation of Horseracing Authorities (IFHA) has determined a 50 ng/mL residue limit for caffeine in horseracing due to the possibility that feed may contain caffeine contamination and environmental factors may affect its levels. In human sport, due to its positive effect on the cardiorespiratory system and brain functions, caffeine limit was determined above 12 µg/mL concentration from 1984 to 2004, but now it is on the World Anti-Doping Agency's monitoring list only (Büyüktuncel, 2010; Russo et al., 2018). Moreover, caffeine is listed chemicals with high production volumes of the United States Environmental Protection Agency and Food and Drug Administration regulations require beverage companies to list caffeine in the ingredients list on product labels ('CFR - Code of Federal Regulations Title 21', n.d.). Therefore, caffeine must be analysed in various matrices, and new and current methods are continually being developed.

Among the various sources of caffeine, tea leaves, coffee beans, cola nuts, and cocoa beans/leaves are widely consumed by humans (Shrivas & Wu, 2007). Caffeine is a polar characterised compound with the structure of xanthine (Figure 1). Because of its common use and presence in most plants, qualitative and/or quantitative analysis in biological samples (plasma, urine, etc), wastewater, feed, food products, and beverages is routinely performed. According to previous studies, by far the most common method of analysis has been the use of UV (ultraviolet) or MS (mass spectrometry) detectors coupled to liquid chromatography. However, the use of gas chromatography to analyse caffeine is quite limited notably in biological samples.

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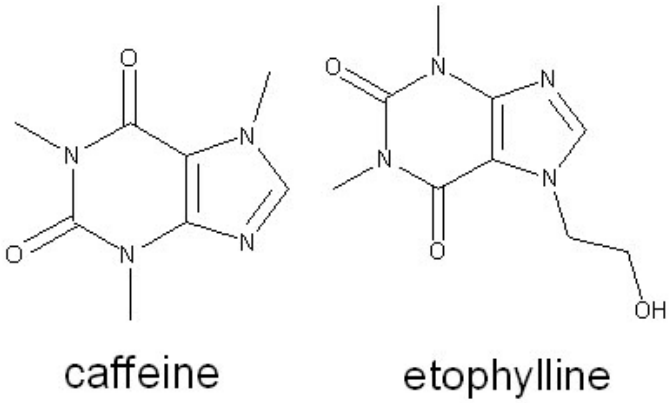

Figure 1. Structure of caffeine and etophylline

trometry allows trace-level, reproducible, and high-accuracy analyses.

Chromatographic determination requires the preparation of samples with liquid–liquid extraction (LLE) (Büyüktuncel, 2010; Del Coso et al., 2011; Ventura et al., 2003) or solid-phase extraction (SPE) (Thomas & Foster, 2004; Verenitch & Mazumder, 2008) as part of the sample preparation process, especially for biological samples. The presence of non-active substances such as salt, acids, bases, xenobiotics, proteins, nucleic acids, etc., which are factors that may make urine matrix complex, should be removed or minimised prior to chromatographic analysis. To achieve low detection levels, SPE is generally preferred due to its advantages in both extraction and enrichment.

By combining gas chromatography and mass spectrometry, high accuracy, reproducibility, and trace-level results can be obtained. Despite its powerful separation and identification properties, the use of gas chromatography mass spectrometry for the development of caffeine methods is limited. To demonstrate that gas chromatography is a useful alternative method for the analysis of caffeine without the need to derivatize the drug, a novel method has been developed. In this study, we propose a more practical, traceable, and validated caffeine analysis method in urine using gas chromatography mass spectrometry, compared with other available methods.

MATERIAL AND METHOD

Chemicals and Reagents

Reference standards for caffeine and etophylline (7-(β-hydroxyethyl)theophylline) (Internal Standard-IS) were purchased from Cayman Chemical (Michigan, USA). All standards of purity were ≥99%. The Elga-pure lab flex water purification system (Elga-Veolia Water Solutions&Technologies, UK) was used for water deionisation. n-hexane was purchased from VWR Chemicals (VWR International Fontenay Sous Bois, France). Chloroform and methanol were obtained from J.T. Baker (Gliwice, Poland). Potassium dihydrogen phosphate was purchased from Merck (Darmstadt, Germany). All chemicals were of analytical grade purity.

The automated solid-phase extraction (SPE) system was procured from Gilson (Gilson Aspec GX274 and operated by Trilution LH software). The SPE cartridge (UCT Xtract C18 Reverse Phase Hydrophobic Sorbent 500 mg/3 mL) was obtained from UCT (Philadelphia, USA). A vortex mixer (Allsheng MTV-100), laboratory centrifuge (Thermo Scientific Heraeus Cryofuge 5500i), and nitrogen evaporator (Biotage Turbo Vab LV) were used.

Urine samples (drug free samples), which were used for fortifying, were obtained from post-race samples that were free of the target analytes. The official samples were taken from post-race samples that were declared positive or negative after an accredited analysis. The Pendik Veterinary Control Institute Animal Experiments Local Ethics Committee approved this study (Approval no:08/2022-275).

Instrumental

GC-MS analyses were performed on an Agilent 7890A GC, 5975C mass spectrometer, and 7683 autosampler equipped with a bonded-phase fused-silica DB-5MS column (30 m length, 0.25 mm internal diameter, 0.25 µm film thickness, Agilent J&W, USA). Helium was used as the carrier gas at 1.0 mL/min, and the injection volume was 1 µL. The injection mode was splitless, the injector was heated at 300°C, and the transfer line temperature was 280°C. The temperature programme was 100°C (0.5 min), 100°C to 220°C (20°C/min), and 220°C to 300°C (15°C/min) and held for 5 min with a post temperature of 300°C (5 min). The run time was 11.8 min. The MS quadrupole and source temperatures were 150°C and 230°C, respectively. Screening analyses were performed in the Selective Ion Monitoring (SIM) acquisition mode with the monitoring

of characteristic ions for caffeine (m/z:194, 109, 82, 67) and etophylline (m/z:180, 224, 193, 95).

Preparation of the solutions

The primary stock standard solution was prepared in methanol at a caffeine concentration of 1 mg/mL of caffeine. A working solution of caffeine was prepared by diluting 100 µL of stock solution to 10 mL (10 µg/mL). Etophylline (IS) was prepared in methanol at a concentration of 50 µg/mL. All standard solutions were stored at 20°C in amber flasks.

Sample preparation

A total of 5 mL of urine sample was taken into a polypropylene tube. 25 µL of IS (50 µg/mL) and 4 mL phosphate buffer (1.0 M, pH 6) were added and mixed before centrifugation (4400 rpm, 15 min). The extraction procedure was modified according to a previously published method (Göktaş, Kabil, & Arıöz, 2020). The tubes were placed into an automated solid phase extraction device. Cartridges were preconditioned with 2 mL deionised water and 2 mL of methanol before loading the samples. The cartridge was then washed with 3 mL deionised water and 6 mL hexane and dried for 2 min. The analytes were eluted with 5 mL of chloroform and evaporated to dryness (N_2, 45°C). Before transfer to a vial, 50 µL of methanol was added to the sample, which was then mixed for 3 min. Subsequently, 1 µL of the sample was injected into the GC-MS system.

Parameters of validation

The method was validated according to the requirements of the guidelines of the European decision 2002/657/EC directive. The validation parameters for quantitation of confirmatory methods for authorised substances included precision, recovery, accuracy, linearity, decision limit (CCα), relative matrix effect, robustness and stability (Official Journal of the European Union, 2021).

For validation, linearity was calculated by preparing five levels (Blank, 0.5xIRL, IRL, 1.5xIRL and 2xIRL) of matrix-matched calibration curves. Drug-free samples were loaded with 25, 50, 75, and 100 ng/mL of caffeine and analysed on 3 separate days (n=6). These results were used to determine the precision, accuracy, and recovery.

For the calculation of LOD and LOQ, 10 samples were analysed after being fortified with 5 ng/mL (S/N>3) caffeine concentration and calculated according to the formula below.

$LOD=3x(sd/\sqrt{10})$

$LOQ=10x\ (sd/\sqrt{10})$

For the calculation of CCα, 20 blank samples were fortified at the IRL level (50 ng/mL) of caffeine and analysed. CCα was calculated by adding the obtained standard deviation multiplied by 1.64 to the mean value of the results. Six different blank and fortified urine samples analysed for selectivity. Robustness was determined by applying minor changes in the sections of analysis. In the stability study, a working solution was prepared and an appropriate volume of drug-free urine was fortified with 100 ng/mL caffeine. After the first analysis, fortified samples (containing 100 ng/mL caffeine) and working solutions (containing 5 µg/mL caffeine) were divided into three parts and stored at -20ºC (deep freezer), +4ºC (refrigerator) and +20ºC (room temperature). For calculation of the matrix effect, 20 blank samples were fortified after extraction with caffeine at the IRL concentration and compared with the same concentration of the pure solution of analyte. The matrix factor (standard normalised for IS) was calculated as follows.

MF (standard)=peak area of post-extraction/peak area of solution standard

MF (IS)= peak area of post-extraction IS/peak area of solution IS

MF (standard normalised for IS)=MF (standard)/MF (IS)

RESULTS AND DISCUSSION

Method Development

Selection of the internal standard was the first step in the study. Etophylline was chosen as the internal standard because of its structure similar to that of caffeine (Figure 1) and its ability to be detected without derivatization. After IS selection, a SIM method was developed using caffeine and etophylline standards by changing the gradient, flow rate, auxiliary temperature, gain factor, and other instrument parameters.

To select the appropriate cartridge for the extraction step, different brands of products were compared (UCT Xtract, CS-DAU503, and OASIS HLB). The same extraction method was applied to all cartridges. The results of the study showed that C18 cartridges (UCT Xtract) performed well in terms of abundance (Supplementary Material S1). Following this, the pH and eluted solution factors were examined (Supplementary Material S1). Based on the pKa of caffeine (10.4), the pH was adjusted to 6, 7, 8, and 9, followed by extraction after centrifugation. Additionally, acetone, dichloromethane, and chloroform elution solutions were tested and their abundances were compared (Supplementary Material S1). Previous studies have indicated that caffeine is more soluble in chloroform than in other solutions; therefore, chloroform was used for caffeine extraction (Shrivas Wu, 2007). The most effective results were obtained at pH 6 and elution with chloroform (Figure 2).

Validation

The validation parameters were calculated in accordance with the guidelines, and the data obtained are summarised in Table 1. Good linearity was achieved within the range with a correla-

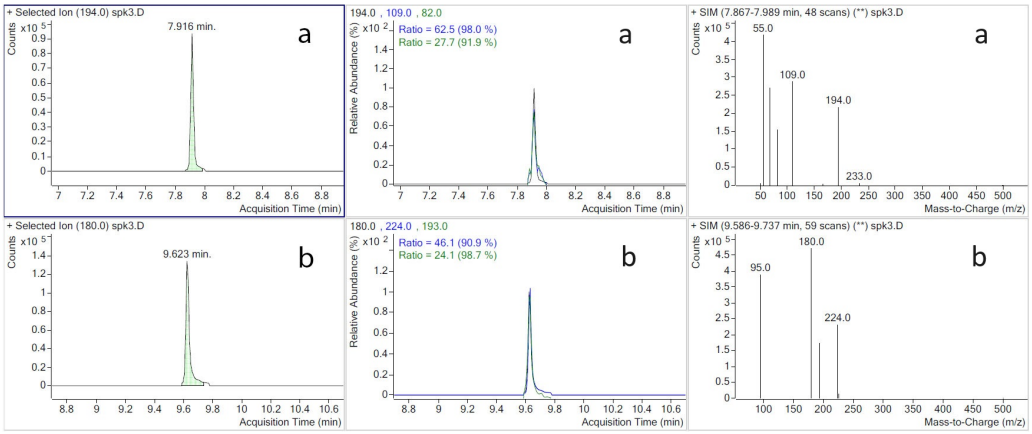

Figure 2. 50 ng/mL caffeine-fortified urine sample GC-MS chromatogram, characteristic ions, and spectra of caffeine (a) and etophylline (b).

tion coefficient (R2) of 0.9988 (Figure 3). The CV% values for precision were less than 16% and the recovery value deviated less than ±10%, which met the criteria of the guideline (Table 1). The low value of the calculated standard deviation for CC was an indication of the reproducibility and robustness of the method. In addition, the limit of detection (LOD) and limit of quantification (LOQ) were calculated to determine the performance of the method. LOD and LOQ were calculated as 3.3 and 10.9 ng/mL.

In a study using gas chromatography to quantify the caffeine concentration in urine doping control, it was concluded that 26.2% of urine samples were below 100 ng/mL, which is the LOD of the study (Del Coso et al., 2011). Therefore, it is important to detect caffeine at lower concentrations. When the current method was compared with other studies in urine (Del Coso et al., 2011; Xiong, Chen, He, & Hu, 2010), it was clear that the method was able to perform analyses with a lower detection limit.

Selectivity and specificity

Chromatograms of blank and fortified urine samples were compared, and no conflicts were detected. Theobromine (structurally similar), tripelennamine, pentoxifylline, tolmetin, pyrilamine, promazine, and zomepirac were fortified with caffeine to control specificity. No interference was detected on the chromatogram.

Robustness

Extraction pH (5 and 6), phosphate buffer concentration (1.0 M and 0.5 M), centrifugation time (15 min and 30 min), hexane volume (6 mL and 3 mL) in the washing step, different grades of methanol (HPLC and MS grade), inlet temperature (300°C and 200°C), and operator were determined and compared for robustness (Supplementary Material S2). According to the Youden test assessment, slight changes in parameter effects were insignificant for the method.

Relative matrix effect

For the evaluation of the matrix effect, the MF was calculated and found to be 1.01. The coefficient of variation of MF was less than 20%, and the relative matrix effect was not found to be significant for caffeine analysis.

Stability

To determine stability, samples were analysed at the end of week 2, week 4, month 3, and month 6 and compared with the results from fresh samples (Table 2). Data obtained were within the ±15% deviation criteria at +20°C after 1 month in the matrix. The results were in accordance with previous studies and revealed that the current method is compatible with other methods and that the results are mutually supportive (Göktaş et al., 2022; Ventura et al., 2003).

Real sample application

Official positive and negative samples were analysed using this method. According to the results, the concentration of real sample 1 was calculated to be 134.1 ng/mL (>56.7), and this sample was declared positive. On the other hand, the concentration of real sample 2 was calculated as 14.2 ng/mL (<56.7). Therefore, this sample was reported as negative. Another laboratory which is using a validated method confirmed these results.. Figure 4 shows a blank, a spiked sample at IRL (50 ng/mL), and the chromatograms of the positive and negative real samples, the product ion (m/z:194.0), two precursor ions (m/z:109.0 and 82.0), and the ion ratios. The results demonstrate the applicability of the method.

Table 1. Validation results for caffeine

Analytes	Fortified Con. (ng/mL)	Corr. coef.(R^2)	Interday (n=6) Measured concentration Mean±SD (ng/mL)	Interday (n=6) Precision CV (%)	Interday (n=6) Recovery (%)	Interday (n=6) Accuracy (%)	Intraday (n=6) Measured concentration Mean±SD (ng/mL)	Intraday (n=6) Precision CV(%)	Intraday (n=6) Recovery (%)	Intraday (n=6) Accuracy (%)	With-in Lab. Rep. (%) CV	CCα (ng/mL) (α=%5)
	25.0		26.2 ± 1.9	7.4	104.7	4.7	26.3 ± 1.7	6.6	105.1	5.1		
Caffeine	50.0	0.99	48.2 ± 1.3	2.8	96.3	-3.7	48.7 ± 2.5	5.2	97.3	-2.7	8.0	56.7
	75.0		76.5 ± 2.6	3.4	102.0	2.0	74.5 ± 3.3	4.4	99.3	-0.7		
	100.0		100.3 ± 2.1	2.1	100.3	0.3	101.1 ± 3.8	3.8	101.1	1.1		

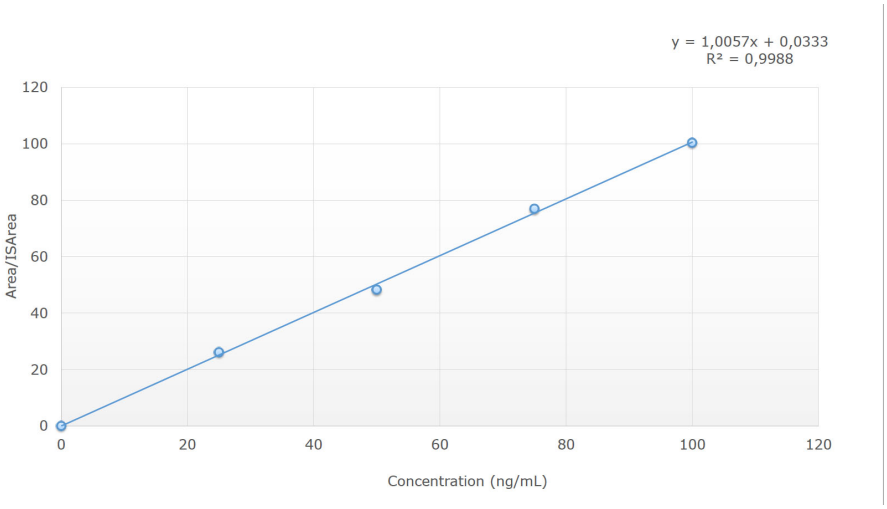

Figure 3. Matrix-matched calibration curve for caffeine

Table 2. Solution and matrix stability results of caffeine (Analyte Remaining (%) = $C_{\text{concentraion at time point}} \times 100 / C_{\text{fresh}}$)

	Solution stability			Matrix stability		
Time	**-20°C**	**+4°C**	**+20°C**	**-20°C**	**+4°C**	**+20°C**
2 week	9.9	1.8	7.9	6.4	11.1	1.3
4 week	-3.3	0.1	-2.7	1.4	-5.7	-0.2
3 month	-2.3	-2.6	-4.0	5.4	1.2	-26.7
6 month	3.6	6.3	2.3	2.0	1.2	-35.3

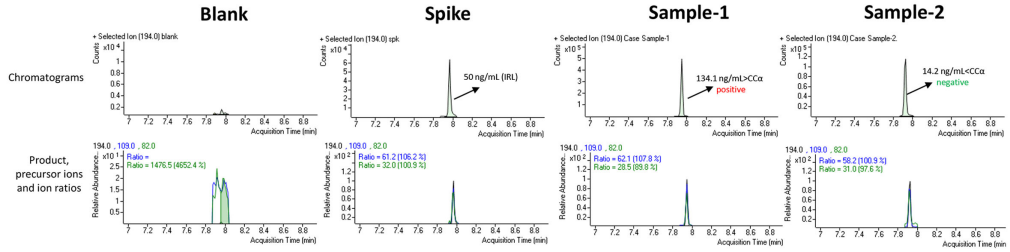

Figure 4. Blank, spiked sample at IRL (50 ng/mL), positive and negative real samples chromatogram, product ion (194.0), two precursor ion (109.0 and 82.0) and ions ratios

CONCLUSION

In summary, a sensitive, simple, and selective GC-MS method for the quantification of caffeine in horse urine has been developed and validated. A number of advantages distinguish this method from previously published methods, such as its low detection and quantification limits, short run time, simplicity, cost effectiveness, and selectivity. Additionally, since derivatization damages columns, the elimination of derivatization extends the column's lifetime and reduces analysis costs. The method has been validated for selectivity, linearity, precision, recovery, accuracy, relative matrix effect, robustness, and stability under various conditions in accordance with Decisions 2002/657/EC and 98/179/EC. Considering these advantages, this method shows promise for the detection of caffeine in various matrices such as wastewater, beverages, biological samples, and other environmental samples.

Ethics Committee Approval: The Pendik Veterinary Control Institute Animal Experiments Local Ethics Committee approved this study (Approval no:08/2022-275).

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