Detection and Determination of Theobromine and Caffeine in Urine after Administration of Chocolate-Coated Peanuts to Horses

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Abstract

The objective of this study was to determine the urinary excretion of methylxanthines in horses following ingestion of chocolate over eight days. The study was performed in response to gas chromatography-mass spectrometry (GC-MS) confirmation of the presence of caffeine in a positive urine test in a racehorse. The trainer of the horse alleged that he often administered chocolate-coated peanuts as treats to his horses, and he believed that the ingestion of chocolate was responsible for the positive urine test. The urinary excretion of theobromine and caffeine after the ingestion of chocolate-coated peanuts was investigated in three horses. Enzyme-linked immunoassay (ELISA), high-performance liquid chromatography (HPLC), and GC-MS assays were performed on all urine specimens. Theobromine (HPLC) was detected for 72 h and caffeine (GC-MS) for 48 h after chronic ingestion of chocolate-coated peanuts. Methylxanthines were detected by ELISA for 120 h after administration of chocolate. 2 μg/mL detected in postrace urine samples would be sufficient evidence of previous theobromine administration that is distinct from innocent feeding of theobromine-contaminated feed. No threshold for caffeine has been established.

Introduction

The methylxanthines, caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine), have been detected in postrace urine samples of racehorses, and their qualitative detection is evidence of administration of caffeine or a caffeine/theobromine mixture. Because theobromine is a known metabolite of caffeine in horses (1), regulatory authorities have usually assumed that the detection of both caffeine and theobromine in urine is evidence that caffeine has been administered to the horse to alter performance. However, theobromine is a ubiquitous component of many pelleted horse feeds (2), and thus, the detection of theobromine in postrace urine samples raises questions about the source of theobromine. In order to control the illegal administration of theobromine in horses, a threshold of 2 μg/mL of theobromine in horse urine was established by the International Conference of Racing Authorities (2). Presumably, theobromine concentrations greater than

Materials and Methods

Urine samples collected from three mares that were administered chocolate-coated peanuts for eight days, were screened by ELISA. Selected samples were analyzed by HPLC (for theobromine and caffeine concentrations greater than 500 ng/mL) and by GC-MS (for caffeine concentrations less than 200 ng/mL).

Animal studies

Three healthy, Standardbred mares (aged 3–5 years and weighing 450–500 kg) were fed 20 chocolate-coated peanuts (peanut M&M’s®, Mars Inc, Hackettstown, NJ), containing approximately 19.6 g chocolate, that were crushed in sweet feed and crushed corn each morning for eight days. On Days 0, 7, 8, 9, 10, and 12, a Foley catheter was placed in the urinary bladder of each horse for urine collection. On Day 0, pre-administration urine samples were taken. On Day 7, before administration of the daily quantity of chocolate-coated peanuts, urine was drained from the bladder, and a urine sample was collected. After chocolate-coated peanut administration, hourly urine
samples were taken for 6 h. Urine samples were also taken at 24, 48, 72, and 120 h at the same time of the day (± 1 h) as previous samples. Aliquots of urine samples were frozen immediately and stored at −20°C until analysis less than 14 days later. Approximately three months later, urine samples were taken from two horses given sweet feed and crushed corn for five days to eliminate the possibility that feed alone lead to methylxanthine concentrations in urine. The experimental protocol was approved by and followed the guidelines of The Ohio State University Animal Care and Use Committee.

Laboratory studies

All thawed urine samples were subjected to ELISA, HPLC, and GC–MS analyses.

Stock solutions

A stock solution of caffeine (Sigma, St. Louis, MO) was prepared in methanol at a concentration of 1 mg/mL. A stock solution of theobromine (Radian, Austin, TX) was prepared in methanol at a concentration of 100 µg/mL. A stock solution of α-hydroxyethyltheophylline (Sigma) was prepared in methanol at a concentration of 100 µg/mL. A stock solution of 8-chlorotheophylline (Pfaltz and Bauer, Flushing, NY) was prepared in methanol at a concentration of 10 µg/mL. Stock solutions were stored in the dark at 4–6°C.

Urine calibration standards

Urine calibration standards were prepared by adding the appropriate volumes of the stock solution of caffeine (ELISA, HPLC, and GC–MS) or theobromine (HPLC) to drug-free horse urine to produce concentrations of 10, 20, 50, 100, and 200 ng/mL (for ELISA and GC–MS) or 0.50, 1.0, 2.0, 4.0, and 8.0 µg/mL (for HPLC). A set of urine calibration standards was processed with each set of unknown test samples.

Caffeine and theobromine content of chocolate

The average content of chocolate in chocolate-coated peanuts was measured in six candies. The amount of caffeine and theobromine in chocolate-coated peanuts was estimated by HPLC (3). Approximately 400 mg of chocolate was accurately weighed after removal of the sugar coating and peanuts. Fat was extracted from the chocolate by dissolving the chocolate in 5 mL of petroleum ether twice. After decantation of the petroleum ether, residual solvent in the defatted chocolate was evaporated under nitrogen at 65°C. The dry residue was weighed, and boiling chips were added with approximately 9 mL of water. The mixture was heated for 25 min at 100°C. After cooling to room temperature, sufficient water was added to give a total volume of 10.0 mL. The mixture was vortex mixed and centrifuged for 5 min at 2000 × g. The supernatant solution was filtered through a 0.2-µm membrane filter (Millipore Products Division, Bedford, MA) and submitted for HPLC analysis as described subsequently.

ELISA analysis of urine samples

Urine samples were tested with a commercially available Caffeine/Pentoxifylline ELISA test kit (product #106410, ELISA Technologies, Lexington, KY) using a robotic sample processor (Tecan US, Research Triangle Park, NC). All urine sample aliquots were diluted 1:5 with ELISA system buffer. If concentrations greater than 200 ng/mL were noted, samples were diluted 1:10, 1:50, 1:75, or 1:100 and re-assayed. The test was performed according to the manufacturer’s instructions. Urine calibrators containing caffeine at concentrations of 10, 20, 50, 100, and 200 ng/mL were processed with the test samples. The absorbance of each well was measured by a microplate reader (Kinetic Reader model 311, Bio-Tek Instruments, Winooski, VT) using a 650-nm filter. The absorbances of wells containing test samples were compared with the absorbances of wells containing urine caffeine calibrators using a four-parameter model (SigmaPlot, Jandel Scientific Software, San Rafael, CA) to determine apparent caffeine concentrations. The manufacturer reports that, in the ELISA test kit, theobromine has a cross-reactivity of approximately 24% relative to caffeine.

HPLC analysis of urine samples

Twenty-five microliters of α-hydroxyethyltheophylline stock solution was added to 100 µL of urine. If concentrations greater than 10 µg/mL were noted, 50 µL of sample was mixed with 50 µL of drug-free urine, and the analysis was repeated. Five milliliters of 5% isopropanol in chloroform was added. The mixture was rotated end-over-end for 20 min and centrifuged at 1500 x g for 6 min. The aqueous layer was removed, and the organic layer was evaporated to dryness at 65°C. The residue was dissolved in 250 µL of mobile phase, and 25 µL was injected into the chromatographic system. The liquid chromatographic system consisted of a Hewlett-Packard 1090 chromatographic system with a diode-array detector. The Phenomenex Prodigy chromatographic column (Phenomenex Torrance, CA) was 4.6 × 150 mm and packed with 5-µm ODS. The Uptight guard column (Upchurch Scientific, Oak Harbor, WA) was packed with 40-µm RP-18. The mobile phase was 5mM tetrabutyl ammonium hydrogen sulfate and 10mM sodium acetate (pH 5) in 12% methanol (v/v). The flow rate was 1.5 mL/min. The ratios of the peak areas of theobromine and α-hydroxyethyltheophylline (internal standard) at 280 nm were calculated and plotted against the concentration of theobromine in urine calibrators. The slope, intercept, and correlation coefficient for each calibration curve were calculated by linear regression with equal weighting of the data. The concentration of theobromine in test samples was calculated from the slope and intercept of the calibration curve. Retention times for theobromine, α-hydroxyethyltheophylline, and caffeine were approximately 3.0, 7.5, and 10.5 min, respectively. The retention times of other methylxanthines were not evaluated. The limit of quantitation for caffeine and theobromine (500 ng/mL) was determined by assessing the lowest drug concentration that resulted in accuracy of between 80 and 120% and a coefficient of variation of less than 20%.

GC–MS analysis of urine samples

Twenty-five microliters of 8-chlorotheophylline stock solution (internal standard) was added to 1.0 mL of urine. After vortex mixing, 1 mL of 0.1N sodium hydroxide was added to each tube, and the contents of each tube were vortex mixed and
allowed to stand at room temperature for 10 min. Five milliliters of 5% isopropanol in chloroform was added. The mixture was rotated end-over-end for 20 min and centrifuged at 1500 x g for 6 min. The aqueous layer was removed, and the organic layer was evaporated to dryness at 65°C. The residue was dissolved in 40 μL of ethyl acetate and 1.0 μL was injected into the chromatographic system. Extracted samples were analyzed by GC-MS operated under electron-impact ionization conditions in the selected ion monitoring mode. Instrumentation consisted of a GC with splitless injection, and helium was the carrier gas at a flow rate of 1 mL/min. A 15-m DB-5MS column of 0.25-mm internal diameter (J&W Scientific, Folsom, CA) was used. The injector temperature was maintained at 250°C. The oven temperature was initially 70°C and was increased to 280°C at 15°C/min; the interface was at 280°C. A Hewlett-Packard (Palo Alto, CA) mass selective detector equipped with Hewlett-Packard MS Chemstation operating software was used to obtain mass spectral data. The total ion chromatogram, the integrated ion areas and retention times for the following ions: m/z 67, 109, 194 (caffeine), and 228 (8-chlorocaffeine) were obtained. The concentration of caffeine in each test sample was determined by the internal standard method using the peak-area ratio. The peak-area ratios for calibrators and test samples were calculated using m/z 194 at the retention time of caffeine and m/z 228 at the retention time of 8-chlorocaffeine. The limit of quantitation for caffeine (10 ng/mL) was determined by assessing the lowest drug concentration that resulted in an accuracy of between 80 and 120% and a coefficient of variation of less than 20%.

Results

Chocolate obtained from chocolate-coated peanuts was weighed, extracted and analyzed for caffeine and theobromine content. Chocolate-coated peanuts contained a mean weight of chocolate of 0.98 g (+ 0.09 g) per candy (n = 6). The mean theobromine and caffeine concentrations of chocolate were 1.87 and 0.37 μg/mg of chocolate respectively (n = 2). Therefore approximately 37 mg of theobromine and 7.3 mg of caffeine was administered daily by administration of the candies.

No behavioral effects were observed after ingestion of chocolate-coated peanuts. Chocolate-coated peanuts were administered for eight days. Twenty-four hours after administration of the seventh dose and before administration of the last dose, apparent caffeine concentrations in urine measured by ELISA were between 1.1 and 1.6 μg/mL (Figure 1). These increased to 2.3–5.0 μg/mL approximately 4–6 h after ingestion of the last dose and decreased to between 50 and 83 ng/mL at 120 h. ELISA analysis of drug-free urine samples fortified with theobromine demonstrated the cross-reactivity of the ELISA kit to theobromine. Identical concentrations of theobromine and caffeine resulted in a more intense reaction to caffeine than to theobromine. Comparisons of test results for theobromine and caffeine each at concentrations at 200, 1000, 5000, and 10,000 ng/mL suggested that theobromine cross-reactivity was between 37 and 44% of that of caffeine. Apparent caffeine concentrations were indistinguishable from background in urine samples from two horses administered sweet feed and crushed corn without chocolate-coated peanuts for five days.

Urine samples were evaluated by HPLC to specifically measure the concentration of theobromine (Figure 2). Twenty-four hours after administration of the seventh dose and before administration of the last dose, theobromine concentrations in urine were between 3.3 and 3.7 μg/mL. These concentrations increased to a maximum of between 7.2 and 11.8 μg/mL approximately 5–6 h after ingestion of the last dose. At 120 h, theobromine in two horses was detectable, but the concentrations were less than the limit of quantitation. Over the 6 h after the last dose, total theobromine excreted was 7.82, 8.80, and

![Figure 1](image1.png)

**Figure 1.** Apparent caffeine concentration versus time data of ELISA screening of urine samples taken from three horses (#145, 146, and 147) after administration of chocolate-coated peanuts for eight days.

![Figure 2](image2.png)

**Figure 2.** Urinary theobromine concentration (determined by HPLC) and caffeine concentration (determined by GC-MS) versus time data in three horses (#145, 146, and 147) after administration of chocolate-coated peanuts for eight days.
10.67 mg. Caffeine concentrations were below 500 ng/mL and were not measurable using this HPLC method.

GC–MS was used to determine caffeine concentrations (Figure 2). Concentrations of caffeine in urine were much lower than those of theobromine. Twenty-four hours after administration of the seventh dose and before administration of the last dose, caffeine concentrations in urine were between 17 and 33 ng/mL. These concentrations increased to approximately 50 ng/mL about 5–6 h after ingestion of the last administration and decreased to between 11 and 17 ng/mL at 48 h. At 72 and 120 h, caffeine concentrations were not detected.

The ratios of urinary concentration of theobromine to caffeine at different time points were calculated. Twenty-four hours after administration of the seventh dose and before administration of the last dose, this ratio was between 112 and 198. The maximum ratio was 146–237, which occurred between 3 and 5 h after ingestion of the last administration. The ratio was 113–122 at 24 h and 82–88 at 48 h.

**Discussion**

The concentration of theobromine (1.87 μg/mg) in chocolate from the chocolate-coated peanuts used in our study was similar to concentrations measured in milk chocolate in a previous, collaborative study (theobromine, 1.90 μg/mg), although caffeine concentrations were approximately double that of the previous study (3).

We have shown that the administration of chocolate-coated peanuts to horses can result in detectable urinary concentrations of theobromine and caffeine and that such concentrations may result in positive ELISA tests for at least 120 h after the last of repeated administrations. The ELISA method is a screening method that alerts racing analysts to the possible presence of methylxanthines in urine. The confirmatory HPLC and GC–MS procedures demonstrated that theobromine was largely responsible for the prolonged detection of chocolate administration by ELISA testing. Indeed, urinary theobromine concentrations exceeded caffeine concentrations by 200-fold after administration of chocolate and were still over 80-fold greater at 48 h. We could not detect caffeine for longer than 48 h and were therefore unable to follow the ratio of theobromine/caffeine for a longer period of time.

Previously published information on the urinary excretion of caffeine and theobromine in horses is very limited. Caffeine pharmacokinetics have been described (1,4,5). Caffeine has a plasma clearance of approximately 0.7–1.0 mL/min/kg and a steady-state volume of distribution of approximately 700–900 mL/kg, which results in an elimination half life of approximately 10 h (4). Greene et al. (4) demonstrated measurable urinary concentrations of caffeine for nine days after single-dose, intravenous administration of 4 mg/kg caffeine. We could find no quantitative reports on the hourly excretion of theobromine after caffeine administration in horses, although plasma theobromine can be detected (1). Moss et al. (6) reported that 60% of a 7-mg/kg oral dose of [8-14C]-caffeine given to three horses was excreted in the urine over 5–8 days. Caffeine, dimethyl-xanthines (theobromine, theophylline, and paraxanthine), and methyl uric acids accounted for 1–4, 15, and 30% of the dose, respectively. This may be in contrast to dogs, in which caffeine is the predominant methylxanthine excreted in urine after caffeine administration (7). After chocolate administration to dogs, theobromine is the predominant methylxanthine excreted in urine, and only trace amounts of caffeine are detected (7). There are few quantitative reports of urinary excretion of theobromine after theobromine administration in horses. In horses fed cocoa husks, which contain a similar amount of theobromine to that given in our study, urinary concentrations of theobromine were similar to those in our study (8).

The numerous analytical methods used in this study to document the urinary excretion of methylxanthines after administration of chocolate are consistent with the procedures used in our laboratory for the screening and confirmation of methylxanthines in postrace horse urine samples. HPLC is frequently used for confirmation of the presence of theobromine and theophylline as these dimethylxanthines are polar and are therefore unsuitable for GC–MS analysis without appropriate derivatization. In contrast, confirmation of caffeine in urine is usually performed by MS.

Demonstrating that ingestion of chocolate by horses leads to persistent excretion of methylxanthines in urine has important practical and regulatory consequences. According to the guidelines recommended by the International Conference of Racing Authorities, our three horses would have exceeded the urine theobromine threshold of 2 μg/mL for over 24 h after chocolate administration. We would therefore advise that trainers avoid feeding chocolate to racehorses. When racing analysts detect caffeine and/or theobromine in a postrace urine sample, the ratio of theobromine to caffeine may be useful to determine the potential source of the methylxanthines in urine. Further research on the urinary excretion of methylxanthines after the administration of caffeine, theobromine, and their combination is required to determine the relevance of caffeine and theobromine concentrations in postrace equine urine samples.

**References**


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