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Caffeine and Theobromine Identifications in Post-Race Urines: Threshold Levels and Regulatory Significance of Such Identifications

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Caffeine is a widespread environmental substance, and testing for caffeine is now highly sensitive. Currently, many authorities in North America are not concerned about plasma caffeine concentrations of <100 ng/ml, equivalent to ~300 ng/ml in urine. This is because such low caffeine concentrations are likely to be associated with environmental exposure to caffeine and are also unlikely to be associated with pharmacological responses. Authors' addresses: Graduate Center for Toxicology, Health Sciences Research Building, University of Kentucky, Lexington, KY 40536 (Budhraja); Maxwell Gluck Equine Research Center, Farm Lane and Nicholasville Road, University of Kentucky, Lexington, KY 40546 (Camargo, Hughes, Tobin); U.K. Livestock Disease Diagnostic Center, 1490 Bull Lea Road, Lexington, KY 40512 (Lehner); Florida Horsemen's Benevolent and Protective Association, PO Box 1808 Calder race course, Opa-Locka, FL 33056 (Stirling); and Connolly's Redmills, Goresbridge, Kilkenny, Ireland (Brennan, Dowling); e-mail: ttobin@uky.edu (Tobin). *Presenting author.

1. Introduction

Caffeine and theobromine are commonly found in equine feeds and environments. They are well absorbed and may appear at microgram concentrations in equine blood and urine; enzyme-linked immunosorbent assay (ELISA) tests can detect low nanogram per milliliter (ppb) concentrations. Therefore, low-concentration identifications of caffeine and theobromine are not unusual and are most likely caused by environmental exposure or feed. Between 2000 and 2006, ~67 caffeine "positives" were called in the United States, and 2 were called in Canada.

In horses, caffeine is metabolized to theophylline, theobromine, and paraxanthine. These metabolites are excreted at high concentrations in equine urine. Caffeine identifications in the absence of these metabolites suggest post-collection entry of caffeine into the sample.

Behavioral studies suggest that performance-threshold plasma and urinary concentrations of caffeine are ~2000 and 10,000 ng/ml, respectively. Regulatory thresholds or "cut-offs" for caffeine and theobromine are in place in a number of jurisdictions worldwide. For caffeine, reported "cut-offs" vary from 10 ng/ml in plasma to 1000 ng/ml in urine.

NOTES

Theobromine is both a metabolite of caffeine and a medication in its own right. Theobromine is also a common component of equine feed, and there is a long established international threshold of 2000 ng/ml in urine of theobromine, although this threshold may be relatively conservative.

Theophylline is another metabolite of caffeine and a therapeutic medication in its own right. The Canadian urinary "withdrawal time" for theophylline is 96 h after therapeutic administration. The equivalent urinary cut-off seems to be ~1000 ng/ml.

2. Background

Caffeine is a central nervous-system stimulant and the most widely consumed psychoactive substance in the world. In humans, caffeine-containing beverages, at a dose rate of ~80–200 mg per cup of coffee[1,2] (special coffee brands may contain higher amounts), ward off drowsiness and restore alertness. Common human beverages containing caffeine include coffee, tea, soft drinks, and especially, "energy drinks." [1–3]

In nature, caffeine is a plant alkaloid that functions as a natural pesticide against insect predators. The most widely cultivated caffeine-containing plants are Coffea arabica (coffee), Camellia sinensis (tea), and to a lesser extent, Theobroma cacao (Cocoa). The primary source of caffeine is the coffee bean. The coffee plant is native to Africa. It was first introduced to Europe in about 1600 A.D., and the first cup of coffee consumed in England was in Balliol College, Oxford in 1637.[1] Tea is another common source of caffeine. The tea plant is originally native to Burma, reaching China by 300 A.D. and Japan by 1000 A.D.. In Europe, tea arrived as a medicinal in 1500 A.D. and became a common beverage by 1600 A.D.

Caffeine is now inextricably associated with humans and can be found in many unexpected places. For example, bee pollen has been found to contain sufficient caffeine to trigger "positive" identifications in racing dogs and horses in Florida in the 1990s.

3. General Pharmacology

In humans, caffeine is a central nervous-system and metabolic stimulant and is widely used to restore mental alertness and alleviate physical fatigue. Caffeine produces these effects by virtue of its purine structure acting on some of the same receptors as adenosine-related nucleosides and nucleotides, such as the cell surface G protein-coupled receptors (GPCRs) as well as the intracellular ryanodine receptor (RyR).[1] Physiologically, however, caffeine's action is unlikely to be caused by increased RyR opening, since this would likely require a lethal plasma concentration of caffeine; as such, its effects are most likely due to its actions on adenosine receptors.

After oral administration, caffeine is rapidly and completely absorbed from the stomach and small intestine and distributed to all tissues of the body.

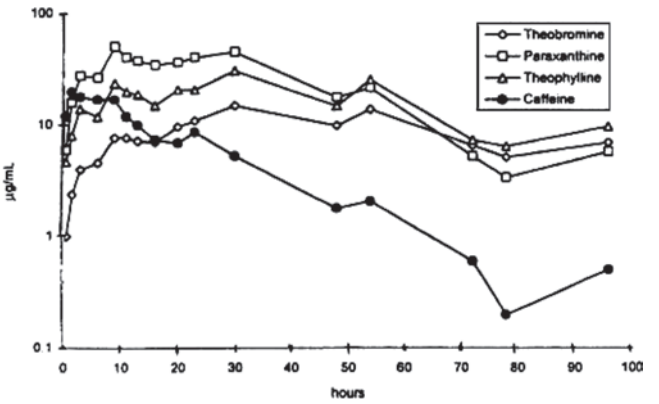

Fig. 1. Urinary concentrations of caffeine and its metabolites after administration of 2 g of caffeine orally to a horse. Reproduced with permission from the Canadian Pari-Mutuel Agency's *Analytical Methodology for Detection and Confirmation of Drugs in Body Fluids*, vol. 2.

Its actions are largely terminated by metabolism, and the decline of plasma concentrations follows simple first-order kinetics.[1,3] In the horse, the plasma half-life of caffeine is relatively long (~19 h).[4,5]

Caffeine in the Horse

Caffeine, at doses of up to 2 g/day, seems to produce broadly similar pharmacological affects in horses and humans. Orally administered caffeine is well absorbed, and urinary concentrations peak at ~21,000 ng/ml at ~1 h post-administration; it then declines to 1000 ng/ml at 96 h post-administration. Of considerable forensic significance, the concentrations of its metabolites, paraxanthine, theophylline, and theobromine, are always higher than those of caffeine by 24 h after administration and at least 10-fold higher at 96 h post-administration (Fig. 1).[6]

Caffeine and Its Metabolites in the Horse

Caffeine, a trimethylxanthine, is metabolized in the liver to yield three dimethylxanthines (Fig. 2), and each of these metabolites has a different spectrum of pharmacological effects on the body. Theophylline (1,3-dimethylxanthine) is a bronchodilator; theobromine (3,7-dimethylxanthine) is a less potent diuretic, but there are no reports of significant pharmacological activity for paraxanthine (1,7-dimethylxanthine).

4. Sensitivity of ELISA Testing for Caffeine, Theophylline, and Theobromine

Routine post-race testing for these agents is now highly sensitive. Commercially available ELISA tests have an I-50 of about 10 ng/ml for caffeine; as such, a 1 mg/horse dose of caffeine has the potential to give rise to detectable (10 ng/ml) plasma and urinary concentrations of caffeine. The IC-50s (i.e., the concentrations for one-half maximal inhibition of the tests) are caffeine at 10 ng/ml, theophylline at 7 ng/ml, and theobromine at 25 ng/ml (Figs. 3 and

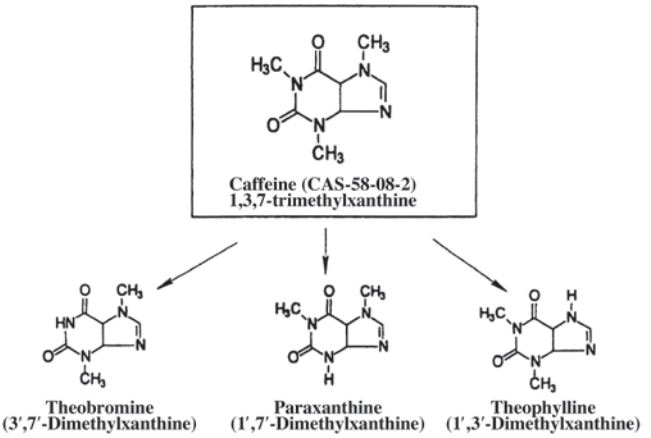

Fig. 2. Chemical structures of caffeine and its metabolites. Note that caffeine has three methyl groups, and its metabolites have two methyl groups each.

4).[7] Review of the urinary excretion concentrations of these agents after the administration of pharmacologically effective doses shows that concentrations in the low μg/ml range (1000 ng/ml) are readily attained urinary concentrations of these substances.

These findings give rise to the following general guidelines with reference to caffeine. If the concentration is <100 ng/ml in plasma or 300 ng/ml in urine, the finding is pharmacologically ineffective and of no forensic interest; if the expected metabolites of caffeine, theophylline, theobromine, and paraxanthine are not present, then it is unlikely that the caffeine in question "passed through the horse." Therefore, the caffeine identification is most likely caused by post-collection contamination, which is also the case if caffeine is not detectable in the blood.

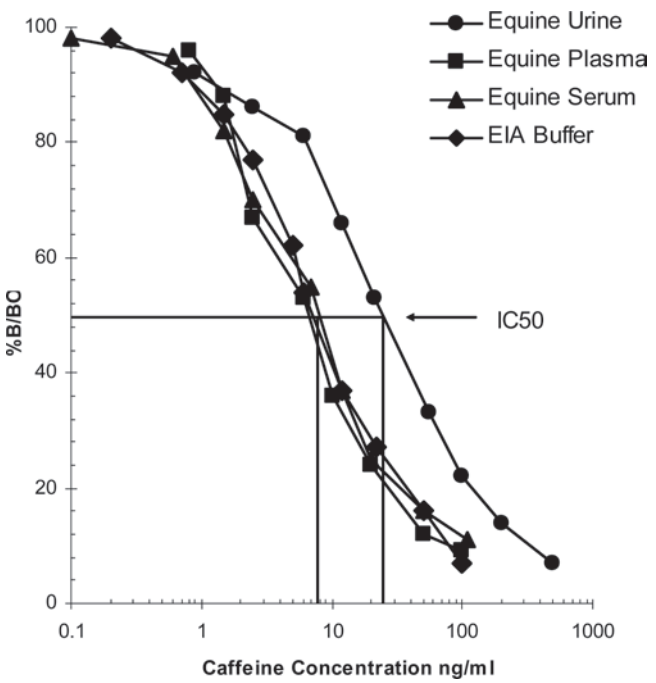

Fig. 3. ELISA standard curves for caffeine in several equine matrixes, such as urine, plasma and serum and also, in EIA buffer. The IC-50 represents the sensitivity of the test, which is ~9–10 ng/ml for serum and plasma and ~10–20 ng/ml for equine urine. Adapted with permission from Neogen Corp.

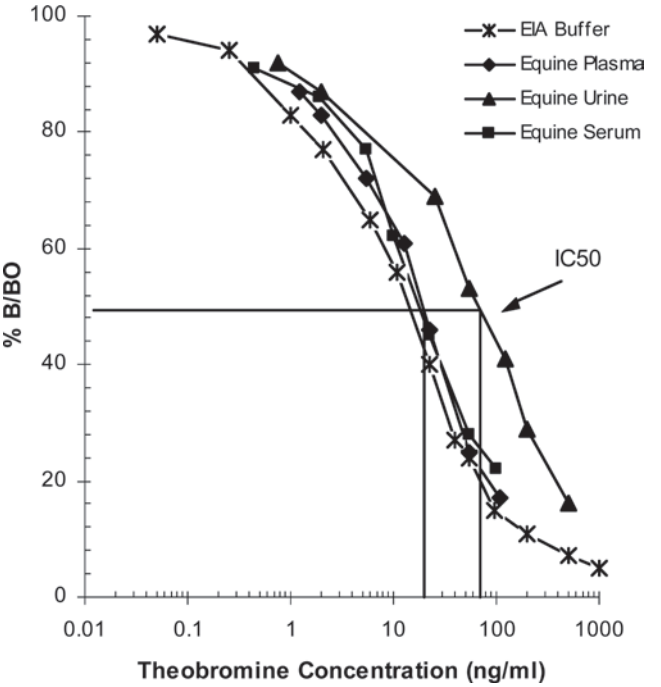

Fig. 4. ELISA standard curves for theobromine in several equine matrixes, such as urine, plasma, and serum and also, in EIA buffer. The IC-50 represents the sensitivity of the test, which is ~20 ng/ml for serum and plasma and ~70 ng/ml for equine urine. Adapted with permission from Neogen Corp.

With respect to post-collection contamination, caffeine may be present in unexpected places, including as a "binder" in pH test strips. In Australia ~20 yr ago, a laboratory was testing the pH of post-race urine samples by dipping pH strips into the urine samples, a violation of good laboratory procedure. The strips' caffeine binder contaminated many samples. This matter was eventually sorted out but not before a very large number of caffeine identifications were made and called in the absence of any metabolite or blood-caffeine information.[8]

Finally, these data also show a considerable reserve of detection sensitivity with regard to concentrations of these agents likely to be found in association with pharmacological administrations. As such, there is a clear need for "limitations" on the sensitivity of testing for these agents, and indeed, the first international horse-racing "threshold" ever introduced was for theobromine in horse urine.

5. Theobromine

The caffeine metabolite theobromine is also a plant alkaloid and a medication in its own right. Theobromine is commonly found in cocoa plants and as

such, is a common trace component of horse feed.[9] Like caffeine, theobromine is well absorbed orally and is excreted at high concentrations in equine urine. The first international regulatory threshold in racing for a pharmacologically active "foreign" substance was for theobromine, which was introduced by the English Jockey Club.[a10]

In England and Ireland in the early 1980s, numerous "positives" were being reported for theobromine, resulting from exposure of horses to small amounts of theobromine associated with cocoa husk in horse feeds. Reviewing this matter, the approach of eliminating traces of cocoa husk from horse feed was considered impractical, and a regulatory threshold or "reporting level" for theobromine in horse urine[b] was designated. In 1987, this first regulatory threshold of 2000 ng/ml of theobromine in urine was introduced,[9,10] although it was apparently based on data from experiments in only three horses. This threshold has since been adopted internationally.

Although seemingly generous, this regulatory threshold for theobromine may, in fact, be conservative. In an incident in the United States in the late 1990s, a high-concentration urinary theobromine identification was claimed by the trainer to be associated with the daily feeding of small numbers of chocolate-covered peanuts to the horse in question. Addressing this circumstance, one author (KS) organized the administration of chocolate-covered peanuts to test horses, 20/day for 8 days. These horses then duly produced urinary theobromine concentrations of as much as 12,000 ng/ml.[11] Clearly, exposure to relatively small amounts of theobromine can readily give rise to very substantial urinary concentrations of theobromine.

Although the urinary concentrations of theobromine reported are apparently large, administering 20 chocolate-covered peanuts/horse/day is, on a weight basis, equivalent to a human consuming ~1.5 chocolate covered peanuts/day, hardly a major "illicit medication" concern.

Therefore, the theobromine international threshold, although well established and long standing, is apparently extremely conservative and is also based on data from only three horses.

6. Regulatory Thresholds for Caffeine

Because caffeine is a well-recognized environmental substance, a number of thresholds for caffeine are now in place throughout the world. One of the longer standing and also one of the lowest reported regulatory thresholds for caffeine is 10 ng/ml in plasma, which is equivalent to ~30 ng/ml in urine (the Hong Kong Jockey Club and the Jockey Club Brasileiro). These are extremely conservative thresholds, approximately equivalent to the ELISA limits of detection (LODs) for caffeine.

In reviewing Table 1 of world regulatory thresholds for caffeine, it will be helpful to keep in mind that caffeine is extremely unusual in that there seems to be a relatively constant relationship between plasma and urinary concentrations of caffeine,[12] and the ratio is about three parts of caffeine in urine to one part in plasma. Table 1 presents the list of current published regulatory thresholds for caffeine in plasma and urine that are available to us.

Table 1. World Thresholds for Caffeine

Authority	Threshold	Matrix
Hong Kong Jockey Club	10 ng/ml	Plasma
Jockey Club Brasileiro	30 ng/ml	Plasma
Ohio	100 ng/ml	Urine
Washington	100 ng/ml	Plasma
New Jersey	100 ng/ml	Plasma
Louisiana	100 ng/ml urine	or 25 ng/ml blood
Florida	200 ng/ml	Urine
RMTC* (urine)	100 ng/ml plasma	Equivalent to 300 ng/ml
TOBA† (urine)	100 ng/ml plasma	Equivalent to 300 ng/ml
Canada	1,000 ng/ml urine	Published research

* Racing and Medication Testing Consortium.
† Thoroughbred Owners and Breeders Association.

In 1999, Ohio introduced a threshold of 100 ng/ml in urine for caffeine, and Florida currently has in place a 200 ng/ml threshold in urine.[13] Each of these thresholds is more conservative than the official Racing and Medication Testing Consortium (RMTC) threshold for caffeine of 100 ng/ml in plasma, which is equivalent to 300 ng/ml in urine. Similarly, 100 ng/ml plasma thresholds for caffeine are in place in Washington State and New Jersey, and the Thoroughbred Owners and Breeders Association (TOBA) "suggested minimum level of detection" for caffeine is set at 100 ng/ml in plasma.[14] Many of these thresholds are presented in the 2003 National Horsemen's Benevolent and Protective Association's "Proposed National Policy of Drug Testing and Therapeutic Medication."[15]

However, published research from Canadian sources suggest a Canadian threshold in urine of ~1000 ng/ml of caffeine, which may seem high until one considers the Canadian data presented previously.[6] Additionally, research presented by our colleague, Professor Tony Queiroz-Neto, on the minimum plasma concentrations of caffeine required for behavioral stimulation[16] (i.e., a "no-effect threshold" or NET) suggests that concentrations >2000 ng/ml of caffeine in plasma or 10,000 ng/ml in urine are required before any pharmacological effects are observed.

In good agreement with the research of our colleague, Professor Tony Queiroz-Neto, the human Olympic "threshold" for caffeine is 12,000 ng/ml in urine, a threshold that apparently recognizes the widespread social use of caffeine in humans.

In view of the above references and "thresholds" for caffeine, we will now review the number of pos-

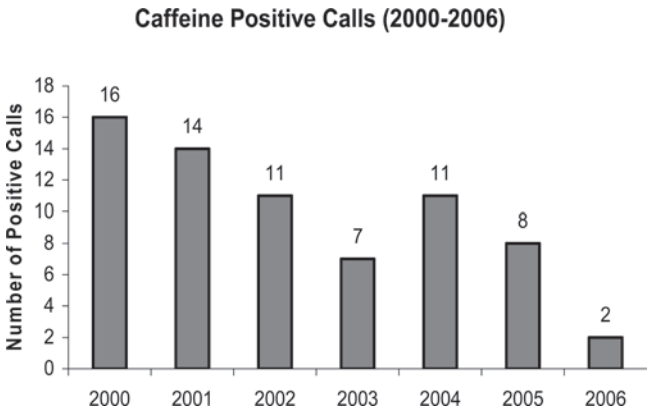

Fig. 5. Number of caffeine identifications between the years 2000 and 2006.

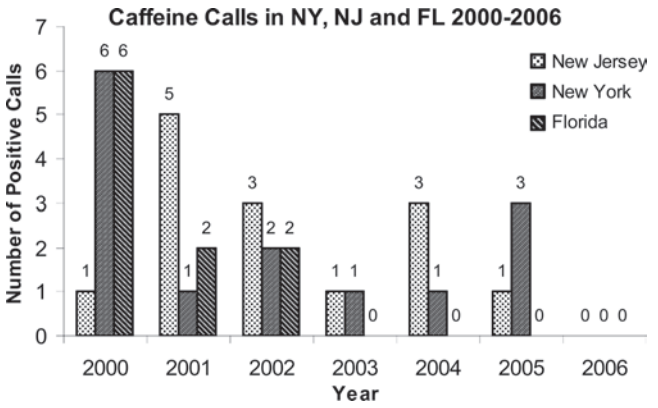

Fig. 7. RCI-positive calls for caffeine in 2000–2006 for the states of New Jersey, New York, and Florida. The "positive" identifications that were reported in Florida were between 2000 and 2002, after which Florida reported a 200 ng/ml urine threshold; subsequent to this, there have been no "positive" calls for caffeine in Florida through September of 2006.

itive calls for caffeine and its metabolites in horse racing in the United States between the years 2000 and 2006 courtesy of the Association of Racing Commissioners International (Figs. 5–7).

Since 2000 (Fig. 5), the number of caffeine identifications in North America dropped from 16 in the year 2000 to 2 in the year 2006, which is a large reduction in the number of reported caffeine identifications. Review of the distribution of positive calls by state (Fig. 6) shows that New York and New Jersey led in the number of identifications with 14 each, and Florida had 10 positive calls, providing about one-half of the total number of caffeine identifications. Outside these states, however, the numbers of identifications were small: five in Louisiana, four in Texas, and less than three in each of the remaining American states and Canada.

After the publication of a 2002 University of Florida report on caffeine (Fig. 7),[13] there has not been a reported caffeine identification in that jurisdiction. This suggests that at least some of the previous identifications may have been low-concentration identifications.

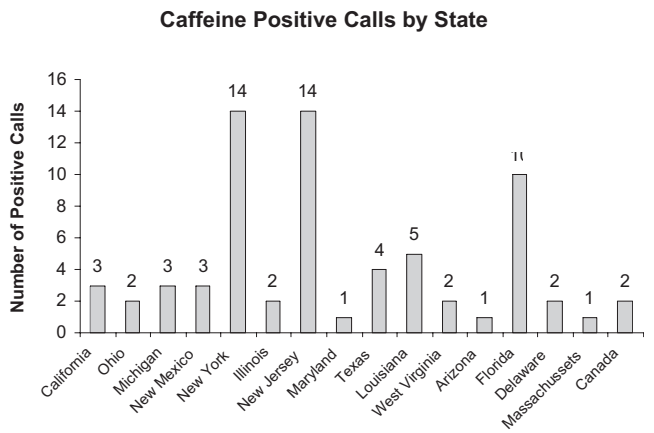

Fig. 6. Positive caffeine identifications distributed by state between the years 2000 and 2006.

7. Theophylline

Theophylline is probably the most pharmacologically active of the methylxanthines, and it is widely used as a bronchodilator in horses and humans.[17] It is generally recognized as a therapeutic agent in horses, and judging by its inclusion as a "withdrawal-time substance" in the Canadian schedule of drugs, theophylline is also recognized in Canada as a therapeutic medication, (i.e., a medication appropriate for use in horses in training but not at pharmacologically effective concentrations in racing horses). Published Canadian information[18] (Fig. 8) suggests a 96-h "detection time" for theophylline in the horse, and a review of the Canadian data showed that when theophylline was administered at a dose

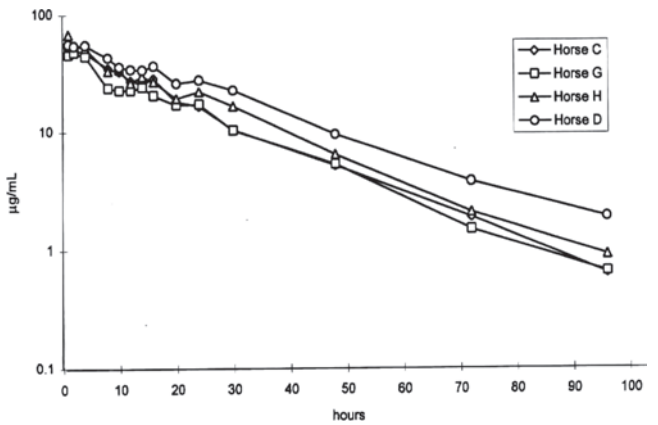

Fig. 8. Urinary theophylline concentrations after 1.5 g of IV theophylline administration. Note the very high urinary concentrations, 80,000 ng/ml of theophylline, and its long plasma half-life, which yield concentrations of >1000 ng/ml in urine at 96 h. Reproduced with permission from the Canadian Pari-Mutuel Agency's *Analytical Methodology for Detection and Confirmation of Drugs in Body Fluids*, vol. 2.

of 1.5 g/horse IV, the peak urinary concentrations were as high as 80,000 ng/ml in urine with a detection time of 96 h, which is approximately the equivalent to a "cut-off" for urinary theophylline of >1000 ng/ml. More recently (March of 2007) in the United States, the RMTC reported a "withdrawal time" for theophylline in Indiana of 120 h.[19]

8. Conclusion

Caffeine is inextricably associated with the presence of humans. Domestic horses are, therefore, inevitably exposed to varying concentrations of caffeine. Caffeine identifications in horse urines have been associated with a variety of sources, including the feeding of caffeine-containing bee pollen and the inappropriate use of caffeine-containing pH strips. If the concentration of caffeine reported is <100 ng/ml in plasma or 300 ng/ml in urine, there is little need for regulatory review. Behavioral data suggests that performance effects require caffeine concentrations of >2000 ng/ml in plasma or 10,000 ng/ml in urine; lesser concentrations are unlikely to be pharmacologically significant. Finally, any caffeine, theophylline, or theobromine identification should include full evaluation of all available scientific evidence for data suggesting innocent, inadvertent, or atypical sources associated with human activity.

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References and Footnotes

1. Fredholm BB, Battig K, Holmén J, et al. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol Rev* 1999;51:83–133.
2. Lovett R. Caffeine: the demon drink? *New Scientist* 2005;187:2518.
3. Harkins JD, Rees WA, Mundy GD, et al. An overview of the methylxanthines and their regulation in the horse. *Equine Pract* 1998;20:10–16.
4. Ohtake I, Murata M, Nagata S, et al. Caffeine in feed and feed additives: studies conducted in consideration of a caffeine threshold, in *Proceedings*. 9th International Conference of Racing Analysts and Veterinarians 1992;245–254.
5. Stenhouse AM, McLinden NJ. Caffeine levels in the blood of dosed horses, in *Proceedings*. 4th International Conference of Racing Analysts and Veterinarians 1981;25–28.
6. Stevenson AJ, Weber MP, Topper P, et al. *Analytical methodology for detection and confirmation of drugs in equine body fluids.* Volume II: Respiratory Drugs, CAFFEINE CMPA #10, p 21–33, Canadian Equine Drug Evaluation Program, Canadian Pari-Mutuel Agency, Agriculture and Agri-Food Canada. Copyright© Her Majesty the Queen, 1977.
7. Edgar T, Tobin T, Watt D, et al. ELISA assay for caffeine, in *Proceedings*. 11th International Conference of Racing Analysts and Veterinarians 1996;478–480.
8. Tobin T. Deception Bay, Australia: the world's record number of "false-positives." *Hub Rail* 1985;73–78.
9. Haywood PE, Teale P, Moss MS. The excretion of theobromine in Thoroughbred racehorses after feeding compounded cubes containing cocoa husk—establishment of a threshold value in horse urine. *Equine Vet J* 1990;22:244–246.
10. "Prohibited substances" New Rules Press Release, the Jockey Club News, 42 Portman Square, London W1H OEN. Communicated October 5, 1987 under 24 hour embargo to Mr. M. Connolly, Red Mills Racehorse Feeds, Goresbridge Co. Kilkenny, Ireland, Press Conference, October 6, Released to the press, Wednesday, October 7, 1987.
11. Dyke TM, Sams RA. Detection and determination of theobromine and caffeine in urine after administration of chocolate-coated peanuts to horses. *J Anal Toxicol* 1998;22:112–116.
12. Greene EW, Woods WE, Tobin T. Pharmacology, pharmacokinetics, and behavioral effects of caffeine in horses. *Am J Vet Res* 1983;44:57–63.
13. Colahan P, Ford P, Chou C-C, et al. The elimination of caffeine originating from human food sources from athletically conditioned Thoroughbred horses as determined by ELISA analysis, in *Proceedings*. 14th International Conference of Racing Analysts and Veterinarians 1999;308–314.
14. Thoroughbred Owners and Breeders Association. *Graded stakes post-race sample analysis.* Testing Requirements-RCI Class 2 drugs, undated document on TOBA letterhead communicated to TT by Mr. Calvert Bratton of Neogen, May 9, 2005.
15. Stirling KH, Bellocq R, Tobin T. New National Horsemen's Benevolent and Protective Association proposed medication rules. *J Equine Vet Sci* 2003;23:4–40.
16. Queiroz-Neto A, Zamur G, Carregaro AB, et al. Effects of caffeine on locomoter activity of horses: determination of the no-effect threshold. *J Appl Toxicol* 2001;21:229–234.
17. Todi F, Mendonca M, Ryan M, et al. The confirmation and control of metabolic caffeine in standard bred horses after administration of theophylline. *J Vet Pharmacol Ther* 1999; 22:333–342.
18. Stevenson AJ, Weber MP, Hopkins DL, et al. Analytical methodology for detection and confirmation of drugs in equine body fluids; Volume X: Pharmacokinetics, CMPA #10, p 47, Canadian Equine Drug Evaluation Program, Canadian Pari-Mutuel Agency, Agriculture and Agri-Food Canada. Copyright© Her Majesty the Queen, 1977.
19. Author: Racing Medication and Testing Consortium (RMTC), RMTCNET.com. Available online at http://www.rmtcnet-.com/content.asp?whatpage = withdrawl _show. Accessed on March 1, 2007.

[a]The Jockey Club London Telex. Personal communication. 1987.
[b]Connolly WV and Connolly J. Personal communication. 2007.