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ELISA ASSAY FOR CAFFEINE

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Abstract

An enzyme linked immunosorbent assay was developed for use as a screening device to detect caffeine. The sensitivity of the assay to caffeine is a demonstrated I_{50} of 21 ng/ml in equine urine, 6.4 ng/ml in equine plasma and 35 ng/ml in canine urine. After administration of 250 mg caffeine by iv injection to one horse, its presence was detected for at least 72 h in urine and plasma.

Introduction

Caffeine (1,3,7-trimethylxanthine) is a central nervous system stimulant that reduces drowsiness, fatigue and produces mental alertness and decreased reaction time. Caffeine relaxes bronchial smooth muscle, produces diuresis and stimulates respiration. It has a stimulant action on the myocardium, increasing cardiac output and coronary vessel dilation (Goth *et al.* 1976; Greene *et al.* 1983; Gilman *et al.* 1990). It has been used to enhance the performance of horses and dogs. It is classified as a Class 2 drug by the RCI *Uniform Classification Guidelines for Foreign Substances*, revised February 1995, and is prohibited in racehorses in all United States jurisdictions.

Theobromine (3,7-dimethylxanthine) produces many of the same effects as caffeine but is less potent (Gilman *et al.* 1990). It was classified as a Class 4 drug by the RCI *Uniform Classification Guidelines for Foreign Substances*, revised February 1995.

Pentoxifylline (1-[5-oxohexyl]-3,7-dimethylxanthine) is a vasodilator used for treatment of navicular disease in horses by increasing the blood flow to the hypoxic tissues in the foot (Kwong *et al.* 1988). It has been classified as a Class 3 drug by the RCI *Uniform Classification Guidelines for Foreign Substances*, revised February 1995.

The most widely used screening method for caffeine has been thin layer chromatography with an approximate detection range of 1–5 µg/ml.

Materials and methods

Dosing and sample collection

A farm horse weighing approximately 450 kg was administered 250 mg caffeine iv. Plasma and urine samples were collected before administration. Post administration urine and plasma samples were collected at 1, 2, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168 and 192 h. All samples were frozen until time of testing.

Samples used for the background study were collected at the racetrack and had tested negative for a panel of drugs in a drug testing laboratory.

One-step enzyme linked immunosorbent assay (ELISA)

A caffeine hapten was conjugated to bovine serum albumin (BSA; Sigma Chemical Co, Missouri, USA) and injected into a New Zealand rabbit to produce a polyclonal antisera for caffeine and its metabolite, theobromine. Caffeine was derivatised to create a carboxyl group used to link the enzyme, horseradish peroxide (HRP; Boehringer Mannheim Corporation, Indiana, USA) to produce the drug:enzyme conjugate. An optimal dilution of the antisera was bound on Costar 96-well microplates (Costar Corporation, Massachusetts, USA). The drug:enzyme conjugate was optimised to the plates. This ELISA is a competitive assay in which the drug in the sample and the drug:enzyme conjugate compete for antibody binding sites. The first step is adding 20 µl of sample or standard to each well. Next, the diluted drug:enzyme conjugate is added at 180 µl/well. The solution is incubated for 45 min at room temperature.

The solution in the wells is discarded and washed 3 times with a mild wash buffer and tapped dry. Any sample or drug:enzyme conjugate bound to the antibody binding sites will not wash away. A 3,3',5,5', tetramethylbenzadine based substrate is added to each well at 150 µl/well. After 30 min incubation at room temperature, the assay is read

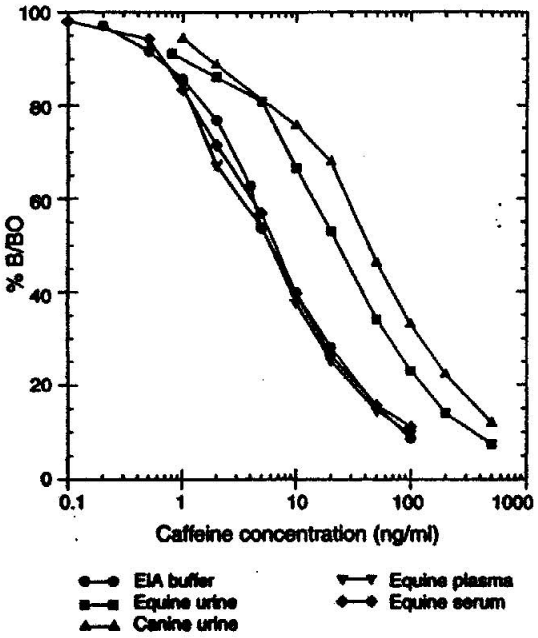

Fig 1: Caffeine standard curves in 5 media.

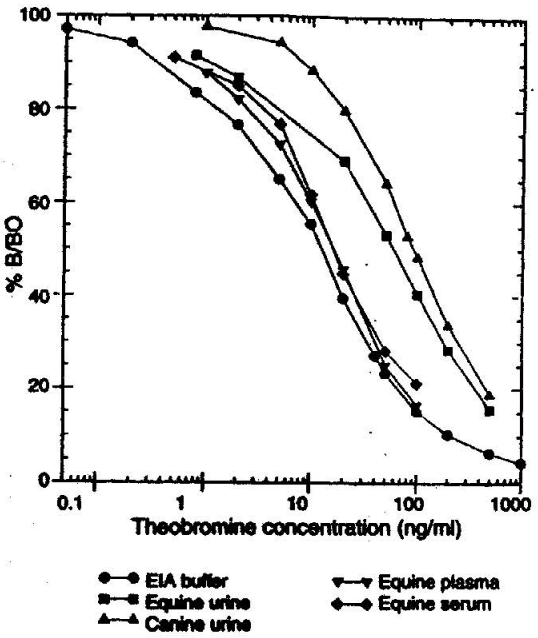

Fig 2: Theobromine standard curves in 5 media.

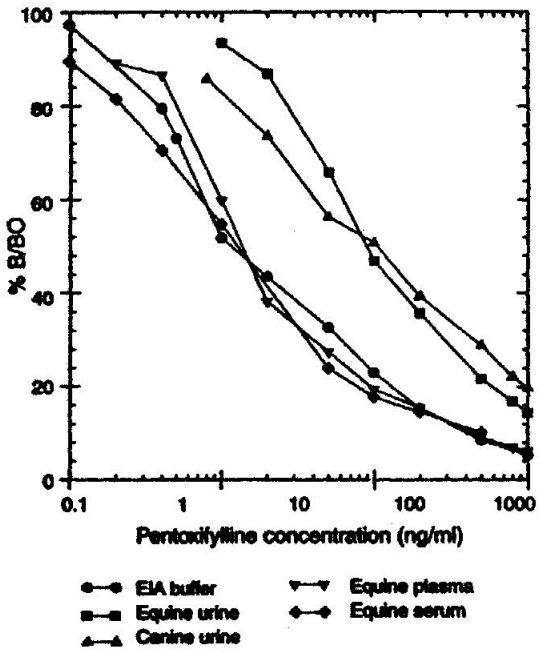

Fig 3: Pentoxifylline standard curves in 5 media.

on a microplate reader (Bio-Tek Instruments, Inc, Vermont, USA) at 650 nm. The substrate will turn blue in the presence of HRP. Therefore, the extent of colour development is inversely proportional to the amount of drug present in the sample. The absence of drug in the sample will result in a bright blue colour. Drug in the sample will result in a light blue colour to no colour as the concentration of the drug increases.

Results

Matrix effects

Matrix effects, also known as background, are common when testing raw urine. Endogenous components of equine urine will interfere with the assay. They will bind to the antibody which inhibits a portion of the drug:enzyme conjugate from binding. This gives the false appearance of a low concentration of drug in the sample. Dilution of the urine samples with buffer before testing can eliminate this problem.

Possible matrix effects were examined for equine urine and canine urine track samples. Results showed that a 1:5 dilution with buffer was necessary to eliminate the background interference.

Sensitivity

The antisera are capable of detecting caffeine, its metabolite (theobromine) and pentoxifylline. Standard curves were produced for all 3 drugs in 5 media: EIA buffer, equine urine, equine plasma, equine serum and canine urine. The I_{50} is a term used to indicate the sensitivity of an ELISA. The drug concentration that gives 50% less colour than the zero standard is considered to be the I_{50}.

Sensitivity of the assay to caffeine is demonstrated by an I_{50} of 21 ng/ml in equine urine, 6.4 ng/ml in equine plasma, 7.2 ng/ml in equine serum and 35 ng/ml in canine urine. Theobromine gave an I_{50} of 55 ng/ml in equine urine, 14 ng/ml in equine plasma, 16 ng/ml in equine serum and 95 ng/ml in canine urine. Pentoxifylline gave an I_{50} of 10 ng/ml

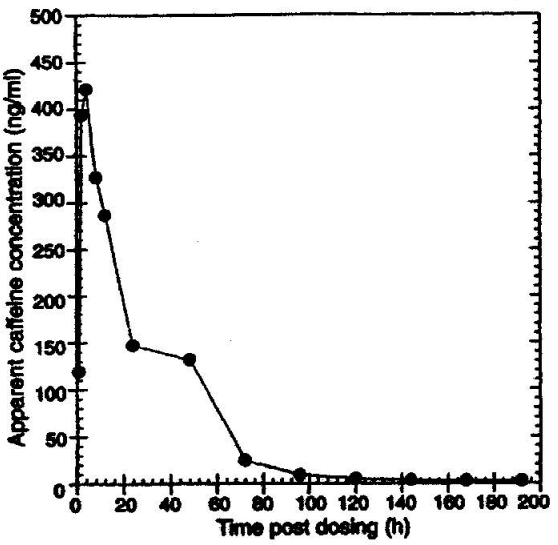

Fig 4: Duration of detection of caffeine in equine urine.

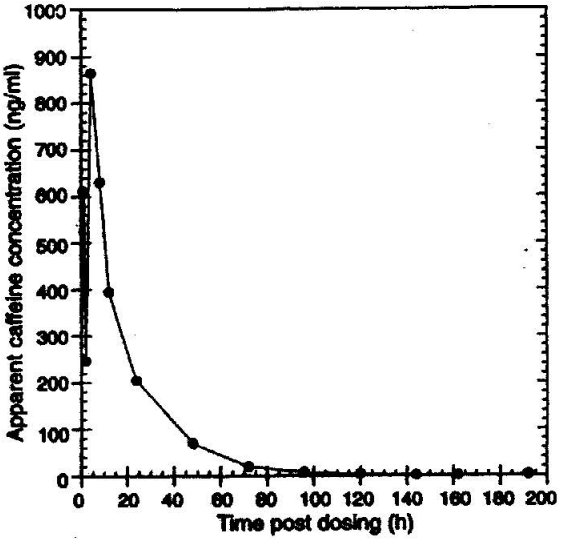

Fig 5: Duration of detection of caffeine in equine plasma.

in equine urine, 1.9 ng/ml in equine plasma, 1.3 ng/ml in equine serum and 10 ng/ml in canine urine. These values reflect the necessary dilution of 1:5 of equine and canine urine with buffer. See Figures 1 to 3.

Cross-reactivity

An extensive cross-reactivity study was performed with a variety of illegal drugs, therapeutic drugs, potential masking agents and drug vehicles. Other than the above mentioned drugs, only theophylline was found to have slight cross-reactivity at 0.06%.

Duration of detection

The pre- and post administration time points from administration of 250 mg caffeine iv indicated detectability of caffeine equivalents for at least 72 h in urine and plasma. The highest level of caffeine equivalents was observed 4 h post administration for urine and plasma. An anomalous decrease was observed 2 h post administration. This anomaly may have resulted from the assay detecting metabolites after the 2 h post administration, a solubility problem with the caffeine administration or entero-hepatic circulation. After 4 h, caffeine equivalent levels decreased gradually (Figs 4 and 5). All equine urine administration samples were diluted 1:5 pre-testing to reduce background interference. Plasma samples do not require dilution pre-testing. Haemolysed serum and plasma samples would require an extraction before use to eliminate interference from red blood cells.

Discussion

There are ongoing discussions within the racing industry about naturally occurring caffeine in foodstuffs. The problem occurs when animals ingest foodstuffs that naturally contain caffeine, causing them to test positive for trace levels of the substance. Some racing jurisdictions utilise unpublished caffeine thresholds. Most notably, the Malayan Racing Association and the Royal Hong Kong Jockey Club have unpublished thresholds of 0.01 and 0.03 µg/ml, respectively (Crone *et al.* 1995).

The assay described can be effective for screening for caffeine, theobromine and pentoxifylline. It will meet the sensitivity requirements for the above threshold levels. With proper use, it will indicate high levels of caffeine shortly after administration, and differentiate from trace levels.

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