VOL. 10, NO. 1
1989

Research Communications in
Substances of Abuse

IMMUNOASSAY DETECTION OF DRUGS IN RACING HORSES VIII. DETECTION OF COCAINE IN EQUINE BLOOD AND URINE BY PARTICLE CONCENTRATION FLUORESCENCE IMMUNOASSAY

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Published as Kentucky Agricultural Experiment Station Article #88-4-223 with the approval of the Dean and Director, College of Agriculture and Kentucky Agricultural Experiment Station.

Publication #152 from the Kentucky Equine Drug Testing and Research Programs, Department of Veterinary Science and the Graduate Center for Toxicology, University of Kentucky.

Supported by a grant entitled "Immunoassay Tests for High Potency Narcotic Analgesics in Racing Horses" from the Kentucky Equine Drug Research Council and the Kentucky State Racing and Harness Racing Commissions.

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SUMMARY

We have developed a Particle Concentration Fluorescence Immunoassay (PCFIA) for cocaine as part of a panel of pre- and post-race tests for illegal drugs in racing horses. This PCFIA test is very sensitive, with an I-50 for cocaine of about 2.5 ng/mL. It does not cross-react significantly with scopolamine, atropine, procaine, or ecgonine. Benzoylecgonine cross-reactivity is less than 10%. The test is rapid, and a normal complement of pre-race tests can be analyzed within one hour. This test detects the presence of cocaine or its metabolites in equine urine for up to 48 hours after a dose of 10 mg/horse, and for at least 72 hours if the dose is 80 mg or larger. In serum, the test detects cocaine for at least 8 hours and likely longer at the 10 mg/horse dosing level. The test is many times more sensitive than thin layer chromatographic tests for cocaine, and is capable of improving the quality of both pre- and post-race testing for cocaine in horses. On initial introduction into post-race urine screening the test flagged 2 of 83 samples "suspect" for cocaine, which samples subsequently confirmed positive for cocaine by mass spectrometry. Because of the ready availability of cocaine and the small doses (less than 10 mg/horse) that are effective in racing horses, a simple, inexpensive, and sensitive test for cocaine is essential for control of abuse of this drug in racing horses.

INTRODUCTION

Cocaine is a stimulant drug that has the ability to increase endurance in human subjects and has been used for this purpose for thousands of years. As a classic stimulant, cocaine also has a long history of abuse in horse racing. It was an ingredient in "homemade recipes" for horse doping preparations popular in both America and Europe at the turn of the twentieth century. In fact, the blatant practice of administering cocaine and other

stimulant drugs to racing horses in the early nineteen hundreds played a significant role in prompting the establishment of the modern racing industry's system of regulatory control of equine medication (Tobin, 1981).

Cocaine, 3-(benzoyloxy)-8-methyl-8-azabicylo-[3.2.1]-octane-2-carboxylic acid methyl ester, acts as a central nervous system stimulant by blocking norepinephrine uptake in sympathetic receptors, effectively increasing norepinephrine concentration at these sites (Gilman et al., 1985). It is also a potent local anaesthetic (Pitts and Marwah, 1988). Through its stimulation of adrenergic activity, cocaine has the ability to increase glycogenolysis in muscle tissue, which is a factor in athletic performance (Giammarco, 1987). In the horse, cocaine's stimulatory effect is manifested by increased respiratory and locomotor rates, and by an increased rate of operant behavioral response (Tobin, 1981). With cocaine as with other stimulant drugs, these effects follow a biphasic response pattern, with low doses producing stimulant effects while higher doses are inhibitory. Experimental investigation of the response-threshold levels for cocaine in the horse shows that doses on the order of 10 mg/horse can produce marked stimulatory effects (Tobin, 1981).

Cocaine's low stimulatory threshold poses a challenge for the racing chemist. The lower limits of cocaine detection of the thin layer chromatography (TLC) methodology currently used as the standard Association of Racing Commissioners International (ARCI) Quality Assurance test for drugs in racing horses is 200 mg/horse. Small doses of the drug, sufficient to produce stimulatory effects, may therefore go undetected with current TLC technology. We have approached the problem of detectability by developing a Particle Concentration Fluorescence Immunoassay (PCFIA) for cocaine. This report details the development and evaluation of this cocaine detection method.

MATERIALS AND METHODS

Horses

Mature Thoroughbred, half Thoroughbred and Standardbred mares (450-550 kg) were used throughout. The horses were kept at pasture and allowed free access to food and water. The horses were placed in standard box stalls (17 m^2) approximately 12 hours prior to dosing for acclimatization.

Dosing and Sampling

Cocaine pure drug standard was obtained from Mallinckrodt, Inc. (St. Louis, MO). The drug was prepared for injection by dissolution in 0.9% sodium chloride USP solution from LyphoMed, Inc. (Rosemont, IL). Cocaine (80, 40, or 10 mg/horse) was then administered by rapid IV injection into the jugular vein.

All urine samples were collected by bladder catheterization. Blood samples were collected by venipuncture into evacuated serum separation tubes (Becton Dickinson Vacutainer systems, Rutherford, NJ). The blood tubes were centrifuged and the serum was collected and frozen until assayed.

Urine samples were filtered through Spin-X^{TM} microcentrifuge filters (Costar^{R}, Cambridge, MA) and diluted (1:5) with 0.01 M phosphate buffered saline, pH 7.4 (PBS buffer) prior to assay. Serum samples were extracted according to the following method before assay: One mL aliquots of serum were made alkaline with approximately 0.15 mL 0.1N sodium bicarbonate, pH 12.5, and then extracted by vortexing briefly with 0.4 mL chloroform: isopropanol (1:3). After centrifugation (8,800 x g, 30 sec) the organic layer was removed and evaporated at 40°C under nitrogen. The residue was taken up in 0.025 mL methanol followed by 0.15 mL PBS buffer. The resultant solution was assayed.

Particle Concentration Fluorescence Immunoassay (PCFIA)

PCFIA studies were performed as previously described (Jolley et al., 1984; McDonald et al., 1987; Yang et al., 1987) on a Pandex Fluorescence

Concentration Analyzer (Pandex Corp., Mundelein, IL). The basic functional unit in the Pandex PCFIA is a 96 well plate with a filter base in each plate. To each well is added 20 µL of cocaine-B-phycoerythrin (cocaine-BPE), 20 µL of anti-cocaine antibody, and 20 µL of blank, standard, or test sample. The system is allowed to equilibrate for 10 minutes after which a second antibody system is added. The second antibody consists of goat anti-rabbit antibody bound to latex beads. The system is allowed to react for another 10 minutes and then the fluid is drawn out of the system through the filter membrane. The reaction system is washed with 80 µL of PBS buffer to resuspend the latex particles, and the system again drawn down with the vacuum. The filtration step has the effect of concentrating the latex beads 1000-fold, thereby increasing the sensitivity of the method. After the wash step, the fluorescence of the particles at 545 and 575 nm is measured. The mean response from control horse urines is usually about 25,000-30,000 arbitrary fluorescence units/well.

Anti-cocaine antibody and reagents were supplied by International Diagnostics Systems Corporation (St. Joseph, MI).

Mass Spectrometry

Gas chromatography-mass spectrometry (GC/MS) was performed on a Hewlett Packard GC Model 5890 equipped with a Hewlett Packard MS-5970 Mass Selective Detector (Hewlett Packard, Palo Alto, CA). Urine samples were adjusted to pH 9, extracted with dichloromethane, back-extracted into sulfuric acid, made alkaline with sodium hydroxide, and re-extracted into petroleum ether. Extracts were then concentrated and injected into the GC in splitless mode. GC oven temperature was programmed from 90°C, after a 1 min. hold, to 250°C at 20°C/min.

RESULTS

A standard curve for the cocaine PCFIA test indicates that addition of 0.1 ng/mL cocaine to the system produced about a ten percent inhibition of fluorescence counts (Figure 1). Increasing concentrations of cocaine increased the inhibition in a log-linear manner, with half-maximal inhibition occurring at approximately 2.5 ng/mL cocaine. Figure 2 compares the sensitivity of the anti-cocaine antibody to detect cocaine with that for two major cocaine metabolites. Cross-reactivity for benzoylecgonine is less

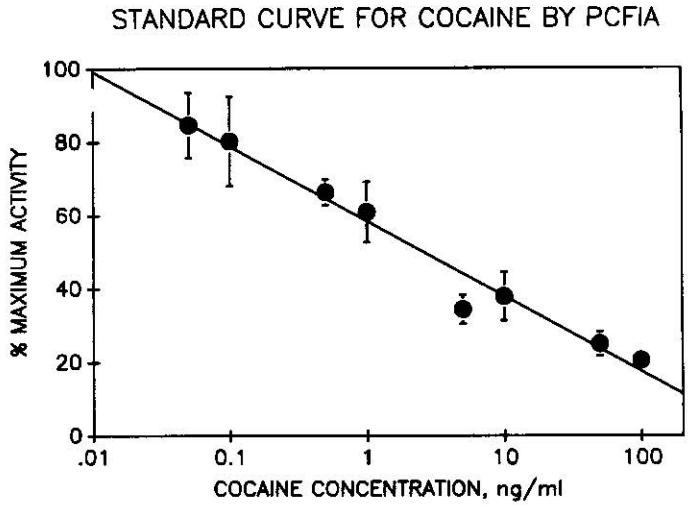

Figure 1. The percentage of PCFIA activity was observed in the presence of the indicated concentrations of cocaine in PBS buffer. I-50 for cocaine in this PCFIA system is approximately 2.5 ng/mL. All data points are expressed as a percentage of fluorescence with no cocaine added to the system, which is assigned a value of 100%. Error bars represent the s.e.m. of 4 determinations.

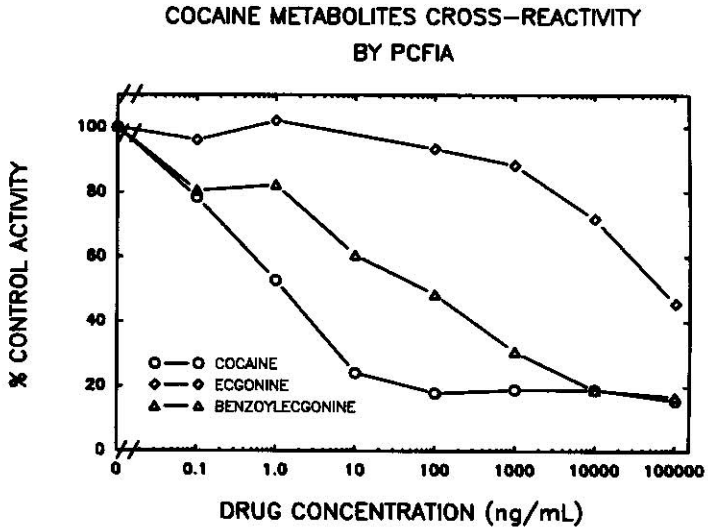

Figure 2. The percentage of PCFIA activity was observed in the presence of the indicated concentrations of cocaine or the cocaine metabolites ecgonine and benzoylecgonine. Fluorescence with no cocaine added to the system represents 100% activity.

than 10%, while ecgonine shows little reactivity at concentrations less than 10 μg/mL. Cross-reactivities for atropine, procaine, and scopolamine were also evaluated (Figure 3). Our anti-cocaine antibody does not have significant affinity for these drugs.

The ability of the PCFIA test to detect cocaine in the blood and urine of mares dosed with the drug was evaluated. Cocaine was administered intravenously at 10, 40, or 80 mg/horse to one of three mares. In serum, inhibition of the PCFIA reaction was essentially complete at the 80 mg/horse dose from 5 minutes through 8 hours post-dose. Inhibition was also strong at both the 40 mg/horse and 10 mg/horse doses through this time period (Figure 4). Cocaine equivalents were readily detectable in the urine of these mares (Figure 5). At the 80 mg/horse dose, inhibition of the PCFIA reaction in urine was essentially complete from 1 hour through 24 hours post-dose. By 48 hours after dosing however, the reaction was only about 70% inhibited. Inhibition continued through 72 hours post-dose. The

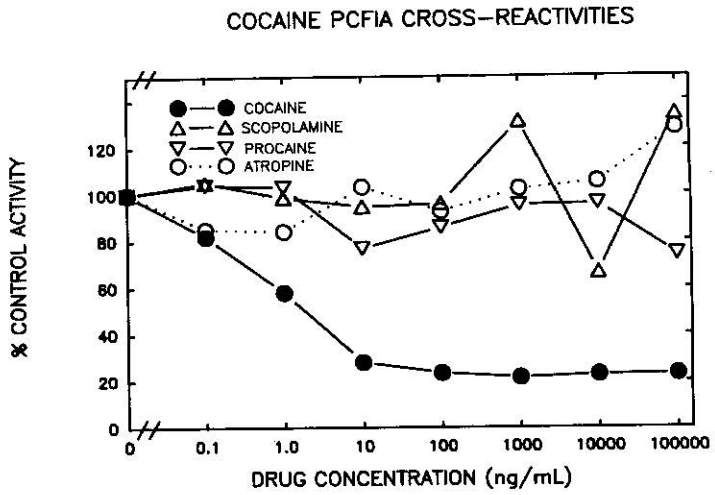

Figure 3. Cross-reactivities of the anti-cocaine antibody toward atropine, procaine, or scopolamine are not significant at concentrations less than 10 µg/mL. Control activity is the fluorescence with no cocaine added to the system.

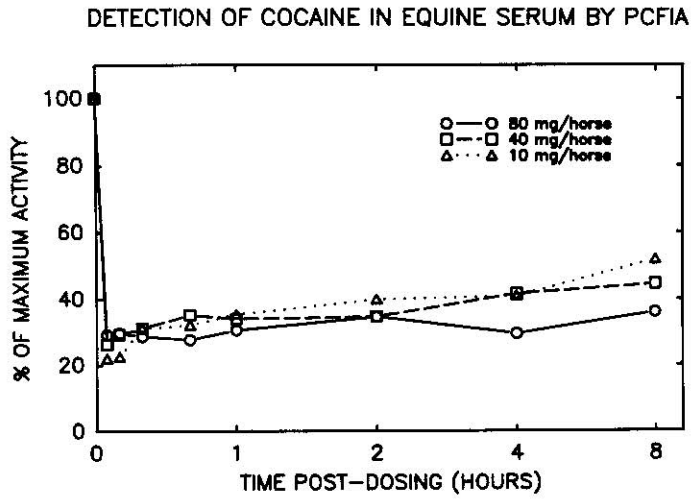

Figure 4. The inhibition of the PCFIA reaction by serum was measured after administration of the indicated amounts of cocaine. Inhibition was evident at the 10, 40, and 80 mg/horse dosing levels throughout the 8 hour sampling period. Maximal activity is the reading for the pre-dose serum sample for each dose level.

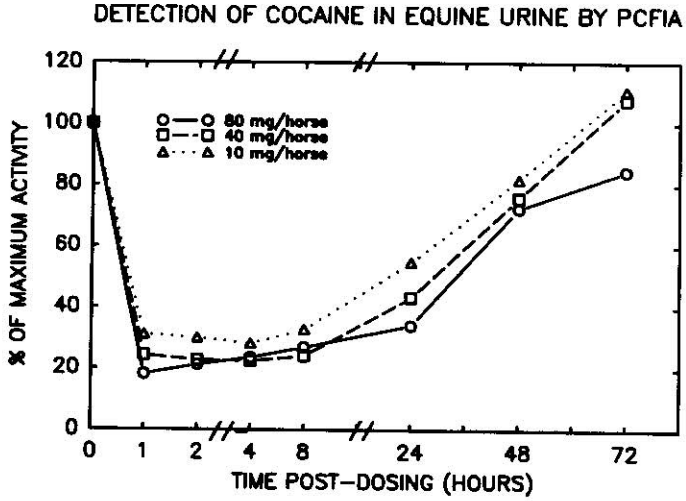

Figure 5. The inhibition of the PCFIA reaction by urine was measured after administration of the indicated amounts of cocaine. Inhibition was evident at the 10, 40, and 80 mg/horse dosing levels for up to 24 hours post-dose, and at the 80 mg/horse dose for up to 72 hours post-dose. Maximum activity is the reading for the pre-dose urine sample for each dose level.

inhibition profiles for the 40 mg/horse and 10 mg/horse doses were similar, with the exception that inhibition had returned to control levels by 72 hours after dosing.

DISCUSSION

The PCFIA test for cocaine reported here is both rapid and sensitive, and as such it represents a marked improvement over the TLC method currently used in pre- and post-race testing of racing horses. Sensitivity is a crucial factor in a screening method. In this regard, regulatory control of equine medication by the racing industry faces problems similar to those in clinical and forensic screening for drugs of abuse in humans (Joern, 1987; Mule and Casella, 1988; Prange et al., 1988). A fundamental difficulty of all such testing is that insufficiently sensitive screening methods yield false negatives by failing to detect drugs which are actually present in test samples.

Until recently the detection of basic drugs in racing horses has been limited by the the sensitivity of the TLC technology used for screening track samples. This technique detects potent basic drugs in racing horse urine with difficulty and virtually not at all in plasma. Such basic drugs of abuse include depressants, local anaesthetics, tranquilizers, narcotic analgesics, and stimulants, cocaine among them. Not only are these drugs the ones most likely to affect a horse's racing performance but they are extremely potent, producing effects after doses of 10 mg/horse or less. With reference to cocaine, the industry standard for TLC detection of 200 mg/horse is clearly capable of "passing" a high percentage of cocaine-containing samples as negative, simply because of the sensitivity limits of the TLC-based detection technology. In a nutshell, TLC-based methods for cocaine detection are likely to yield numerous false negatives if the dose of cocaine used is less than 200 mg/horse.

The development of an immunoassay-based technology has the potential to improve vastly the ability of the racing chemist to detect basic drugs in racing horses. The PCFIA system reported here is very sensitive to cocaine, with half-maximal inhibition of fluorescence occurring at about 2.5 ng/mL cocaine. After a dose of cocaine as low as 10 mg/horse, this test can detect the presence of cocaine or its metabolites in equine serum for at least 8 hours, and in equine urine for at least 48 hours. This level of sensitivity represents an improvement of an order of magnitude over TLC methods. Moreover, since the level of detection sensitivity offered by the PCFIA system corresponds well to known stimulatory threshold levels for cocaine in the horse, it seems clear that this test has the ability to detect cocaine when this drug is used at pharmacologically effective levels.

The PCFIA technique is also rapid, requiring minimal pre-assay workups for equine blood and urine samples and eliminating the need for a

preliminary hydrolysis step to detect cocaine metabolites in urine. PCFIA thus satisfies the requirement for speed of analysis, which is a critical factor in pre-race serum testing, and represents a substantial saving of time and effort over old procedures for post-race urine testing.

It is highly likely that the dose of cocaine used in racing horses will be less than the 200 mg/horse that is the current TLC minimum detectable dose. As shown by Shults and coworkers (Shults et al., 1982), doses of as little as 4 mg/horse can produce a peak stimulant effect in stalled broodmares. It appears likely that racehorses in training will be at least as sensitive to cocaine as stalled broodmares, and the ability of narcotics and stimulants to potentiate each other's action suggests that the use of small doses of drugs such as cocaine cannot be discounted.

A second factor which facilitates abuse of cocaine is its ready availability through the illicit market. This factor, along with its marked stimulant effects means that a certain level of cocaine use is likely in racing horses. Its availability also leads to a third mechanism for its presence in post-race urine samples, namely contamination of the urine sample during collection and handling.

Based on these considerations it was not surprising that when this PCFIA cocaine assay was introducted for testing of a small number of post-race samples in a western state, several were flagged for cocaine. This is because the immunoassay has not been used routinely for cocaine screening in post-race samples and this test is also apparently more sensitive to cocaine than at least one commercially available immunoassay.

Of the samples flagged for cocaine, two were analyzed by GC/MS and were confirmed positive for cocaine (Figure 6). Both the GC retention time and mass spectrum of the plasma sample match that of the cocaine standard.

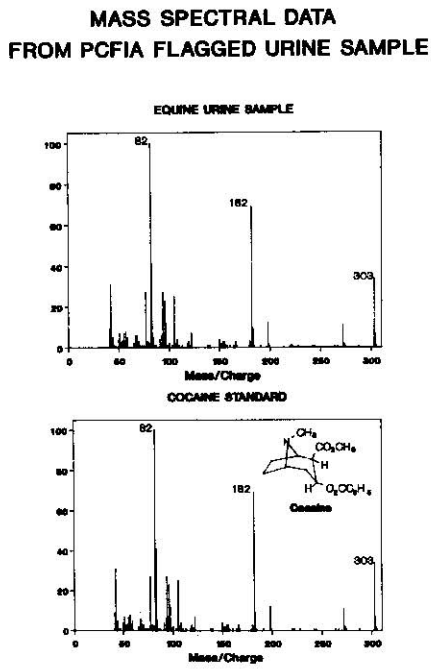

Figure 6. Mass spectrum of urine extract of a sample flagged by PCFIA matches that of a cocaine standard.

However, what was unusual about these samples was that no metabolites of cocaine were detected in these samples. One interpretation for these events is that contamination occurred after the urine sample was drawn from the horse and that the cocaine present in these samples was due to sample contamination.

Other instances of detection of parent cocaine without associated metabolites in post-event urine have occurred and have also been difficult to interpret. Based on data generated by the Quality Assurance Program it appears that the detection of metabolites of cocaine in post-race urines is to be expected. However, one must bear in mind that no studies have been performed on the disposition of cocaine in the urine of horses after small

doses of this drug. Given this circumstance, it remains possible that the ratios of parent drug to metabolites found in experiments using high doses of cocaine may be very different from the ratios of metabolites found after much smaller doses of cocaine. Additionally, since most laboratory experiments are performed using stalled horses, there may be differences between the metabolite patterns in stalled horses and in horses immediately post-exercise. For example, while the presence of drug metabolites may be expected after higher doses of cocaine in stalled horses, the proportions of parent drug to metabolites which may be found in exercised horses after lower doses of cocaine is unknown at this time.

In summary therefore, we have developed a fast and sensitive PCFIA for cocaine. This test is much more sensitive than the currently used TLC test for cocaine, and also appears more sensitive than at least one commercially available immunoassay for cocaine. The test readily detects cocaine or its metabolites in plasma and/or urine after dosing with relatively small (10 mg/horse) doses of this drug. When used to screen 83 post-race samples from a major western racing state, our PCFIA flagged several samples "suspect" for cocaine, and two were confirmed positive for cocaine by GC/MS. Based on the ready availability of cocaine, the small doses of this drug that are effective in horses, and the ease which which this drug can be administered to horses, a simple, inexpensive and sensitive test for cocaine such as the one described here is required for effective control of cocaine abuse in racing horses.

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