APPENDIX III

IMMUNOASSAY DETECTION OF DRUGS IN RACING HORSES: XIX DETECTION OF BENZOYLECGONINE/COCAINE IN EQUINE URINE BY ELISA

by

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ABSTRACT

We have developed an Enzyme-Linked ImmunoSorbent Assay (ELISA) for benzoylecgonine and cocaine as part of a panel of tests for illegal drugs in racing horses. This ELISA test is very sensitive, with an I-50 for benzoylecgonine of about 0.3 ng/mL. It also cross-reacts with cocaine, having an I-50 for cocaine of about 0.4 ng/mL. It does not cross-react significantly with atropine, procaine, ecgonine, N-norcocaine, or allopseudococaine. This test detects benzoylecgonine and cocaine in equine urine for up to 72 hours after a 40 mg i.v. dose. A frequency distribution of benzoylecgonine/cocaine ELISA background levels in racetrack urine samples showed that with 1:8 dilution this test readily distinguishes negative urines from positive dosed horse samples. This test is more sensitive than thin layer chromatographic tests for cocaine and its metabolites, and is capable of improving the quality of testing for cocaine in race horses. Because of the readily availability of cocaine and the small doses (less than 10 mg/horse) that may alter the performance of racing horses, a simple, inexpensive, and sensitive test for benzoylecgonine/cocaine is essential for effective control of this drug in racing horses.

INTRODUCTION

Cocaine is a stimulant drug that increases endurance in human subjects and has been used in this way for thousands of years (Gilman et al., 1990). As a classic stimulant, cocaine also has a long history of abuse in horse racing, and was a common component in drug mixtures for horse doping that were popular in both America and Europe at the turn of the 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 establishment of the modern racing industry's comprehensive drug testing system for 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 reuptake in noradrenergic synapses, effectively increasing norepinephrine concentration at these sites (Gilman et al., 1990). Cocaine is also a potent local anaesthetic (Pitts and Marwah, 1988). Through its stimulation of adrenergic activity, cocaine increases 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 responding in a variable interval responding paradigms (Wood et al., 1989). With cocaine, as with other stimulant drugs, these effects follow a biphasic response pattern, with low doses producing stimulant effects while higher doses produce inhibition. Experimental investigation of the dose-response threshold doses for

cocaine in the horse shows that doses on the order of 10 mg/horse produce marked stimulatory effects in a variable interval responding paradigm (Tobin, 1981).

Cocaine's low stimulatory threshold poses a challenge for the racing chemist. Small doses of the drug, sufficient to produce stimulatory effects, may therefore go undetected unless sensitive immunoassay-based testing systems are available. This report details the development and evaluation of a highly sensitive ELISA based test for cocaine detection.

MATERIALS AND METHODS

Horses

Six Thoroughbred or Standardbred mares (450-500 kg) were administered cocaine in the in vivo studies. The horses were maintained at pasture until approximately 24 hours before dosing. At that time they were placed in box stalls (17 sq M) and allowed free access to hay and water. All control samples were obtained immediately before dosing.

Dosing and Sampling

Cocaine hydrochloride 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 and stored frozen until assayed.

ELISA test method

The ELISA tests developed here are similar to the ELISA formats used in published report by Stanley et al., 1981. The anti-benzoylecgonine antibody was coated to flat bottom wells as described by Voller, 1976. Benzoylecgonine was linked to horse radish peroxidase (HRP) to give rise to a covalently linked drug-HRP complex by the method described by Rowley et al., 1975. All assays were performed at room temperature. The assay was started by adding 20 µl of the standard, test, or control specimens to each well, along with 180 µl of the drug-HRP solution. During the test, the presence of drug in the specimens competitively prevent the binding of drug-HRP complex to the antibody. Since the HRP enzyme is responsible for the colour-producing reaction in the ELISA test, the concentration of drug in the specimen is inversely related to the optical density of the test well. The optical density (OD_{650}) of the test wells was read at a wavelength of 650 nm with an automated microplate reader approximately 60 minutes after addition of substrate.

Mass Spectrometry (Cocaine)

Gas chromatography-mass spectrometry (GC/MS) was performed on a Hewlett Packard GC Model 5890 equipped with a Hewlett Packard model 5970 Mass Selective Detector (Hewlett Packard, Palo Alto, CA). Urine specimens were adjusted to pH 9 with 2 M sodium hydroxide, extracted with dichloromethane, back-extracted into 0.5 M sulfuric acid, made alkaline with 2 M sodium hydroxide, and re-extracted into petroleum ether. Extracts were then concentrated and injected into the GC in splitless mode. The GC oven temperature was programmed from 90°C to 250°C at 20°C/min. The mass spectrum was operated in the electron-impact ionization mode.

Mass Spectrometry (Benzoylecgonine)

The benzoylecgonine was isolated from a horse urine specimen collected after an intravenous bolus dose of 50 mg of cocaine as cocaine hydrochloride. Gas chromatography-mass spectrometry (GC/MS) was performed on a Hewlett Packard GC Model 5890 equipped with a Hewlett Packard ME-5971A Mass Selective Detector. Extracts were treated with 20 µL of BSTFA at 60°C for 30 minutes, diluted with 20 µL ethyl acetate, and chromatographed on a 15 m x 0.25 mm DB-1 fused silica capillary column. The injector and transfer line temperature were 280°C. The initial oven temperature was set at 100°C and was maintained at that temperature for 1.00 minute after injection. The oven temperature was then increased at a rate of 20°C per minute to a final temperature of 280°C which was maintained for 12.5 minutes. The mass spectrum was operated in the electron-impact ionization mode.

RESULTS

A calibration curve for the benzoylecgonine ELISA test indicates that addition of 100 pg benzoylecgonine/mL to the system produced about 50 percent inhibition (Figure 1). Increasing concentrations of benzoylecgonine increased the inhibition in a sigmoidal manner, with half-maximal inhibition occurring at approximately 0.3 ng benzoylecgonine/mL. The antibody also cross-reacted well with cocaine, (I-50 0.4 ng/mL), while N-norcocaine and allopseudococcaine showed little cross-reactivity. Cross-reactivities for procaine and ecgonine were also evaluated (Figure 2), and this antibody did not have significant affinity for these drugs.

The ability of this ELISA test to detect cocaine or benzoylecgonine in the urine of horses dosed with the drug was also evaluated. Cocaine was administered intravenously at 40 mg/horse to one of three horses. Cocaine equivalents were readily detectable in the urine of these horses (Figure 3). At the 40 mg/horse dose, inhibition of the ELISA 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 80% inhibited with inhibition still detectable at 72 hours post-dose. The inhibition profile for

the 40 mg/horse and 10 mg/horse doses were similar, with the only difference being that inhibition had returned to base line by 72 hours after the 10 mg/horse dose.

When ELISA tests are used for post-race screening in race horse urines, unknown substances in horse urine gives variable levels of background that interfere with the assay. To evaluate this endogenous background activity, we add 20 µL aliquots of about 40 post-race urines to the ELISA system and plot a frequency distribution of the levels of "apparent drug" due to these background materials. We then compared the highest level of "apparent drug" with the I-50 of the test for the drug. If, as was the case for cocaine, the level of apparent cocaine in any of the 40 samples is above the I-50 for cocaine, dilution of the samples prior to assay is recommended. As shown in Figure 4, dilution of horse urines 1:8 prior to assay reduced the highest apparent background in these samples to less than 0.5 ng/mL of apparent cocaine. This level of apparent background is sufficiently below the apparent I-50 of this test that background interference is not a problem. When reading this test using 1:8 diluted urines, therefore, a positive is readily discernable as a clear "whiteout" well against a panel of blue negative tests.

Our source of urine samples for background evaluation is regular post-race urine samples. During our evaluation of natural background in post-race urine samples, one of the forty samples tested showed substantial inhibition of this assay, independent of the level of dilution of the sample. Mass spectral analysis indicated this sample to be positive for benzoylecgonine, at less than 25 ng/mL, confirming the efficacy of this test as a highly sensitive screening method for cocaine or benzoylecgonine in post-race urines. The mass spectrum for the trimethylsilyl derivative of benzoylecgonine is shown in Figure 5. The data of Figure 6 shows a mass spectrum for cocaine isolated from a dosed horse urine specimen along with a control mass spectrum (Figure 7).

DISCUSSION

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

The development of immunoassay based drug screening technology has substantially improved the ability of racing chemists to detect

drugs in racing horses. The ELISA system reported here is very sensitive to cocaine with half-maximal inhibition occurring at about 0.4 ng/mL cocaine. After a dose of cocaine as low as 10 mg/horse, this test can detect the presence of cocaine or its metabolite benzoylecgonine in horse urine for at least 48 hours. This level of sensitivity represents a improvement over TLC methods. Moreover, since this level of detection sensitivity corresponds well with the known stimulatory threshold doses for cocaine in the horse, it seems clear that this test has the ability to detect cocaine when this drug is used at threshold doses for pharmacological effect.

The ELISA technique is also rapid, requiring minimal pre-assay preparation for horse urine specimens. ELISA testing thus satisfies the requirement for speed of analysis, and represents a substantial saving of time and effort over older TLC-based procedures for pre- and post-race testing.

As shown by Shults and coworkers in 1982, doses of as little as 4 mg/horse can produce clearcut stimulant effects in individual stalled horses. It appears likely that racehorses in training will be at least as sensitive to cocaine as stalled animals, and the ability of narcotics and stimulants to potentiate each other's actions suggests that the use of small doses of drugs such as cocaine cannot be discounted.

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

In summary therefore, we have developed a fast and sensitive ELISA test for cocaine and benzoylecgonine. This test is much more sensitive than the previously available TLC test for cocaine. The test readily detected cocaine and benzoylecgonine in urine after dosing with relatively small (10 mg/horse) doses of this drug. When used to screen post-race samples from a major racing state, it detected one low concentration (<25 ng benzoylecgonine/mL) sample "suspect" for cocaine, which sample was later confirmed positive for benzoylecgonine by GC/MS. Based on the ready availability of cocaine, the small doses of this drug that are effective in horses, and the ease with 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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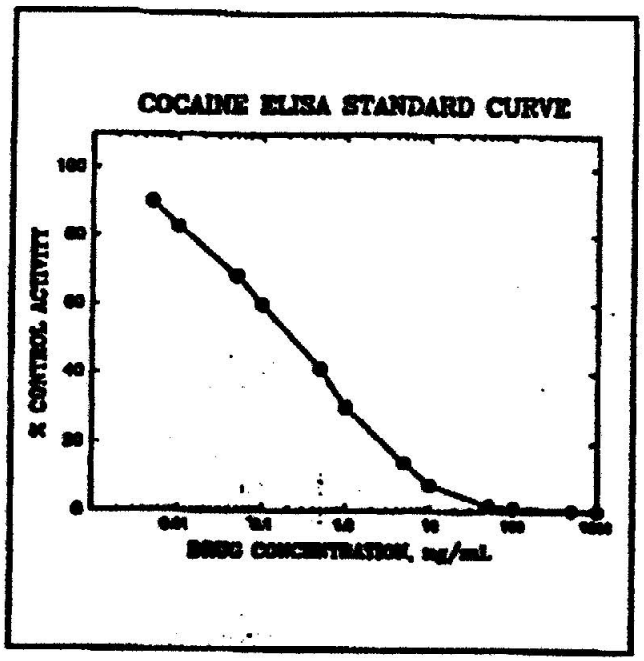

Figure 1. A standard curve for the cocaine ELISA test indicates that the addition of 0.4 ng/ml cocaine to the system produced half-maximal inhibition.

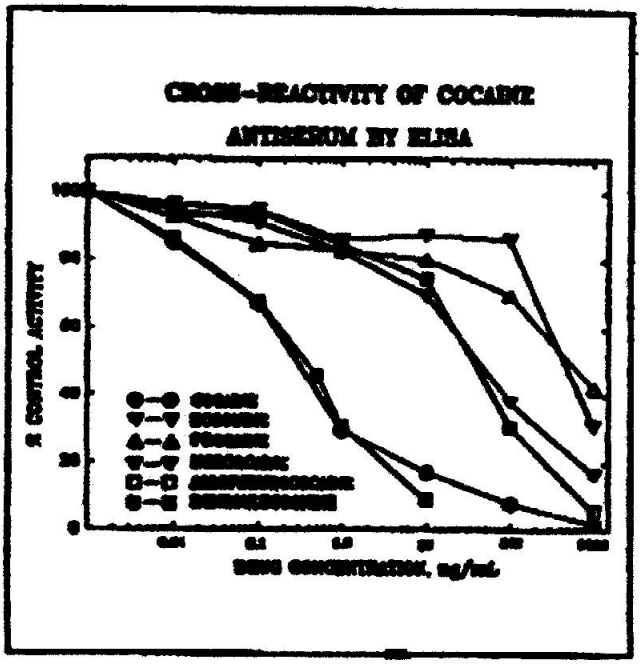

Figure 2. ELISA test activity for the anti-cocaine antibody as a function of added drug is shown.

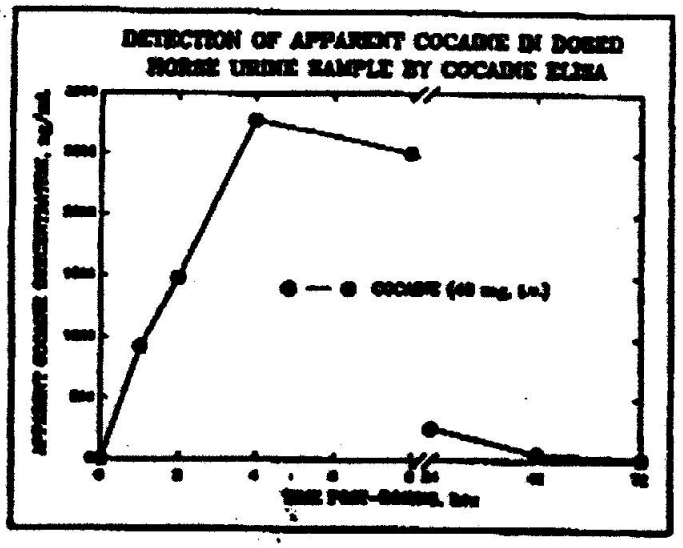

Figure 3. ELISA detection of cocaine in equine urine following a 40 mg IV dose. Control optical density was measured using the predose sample.

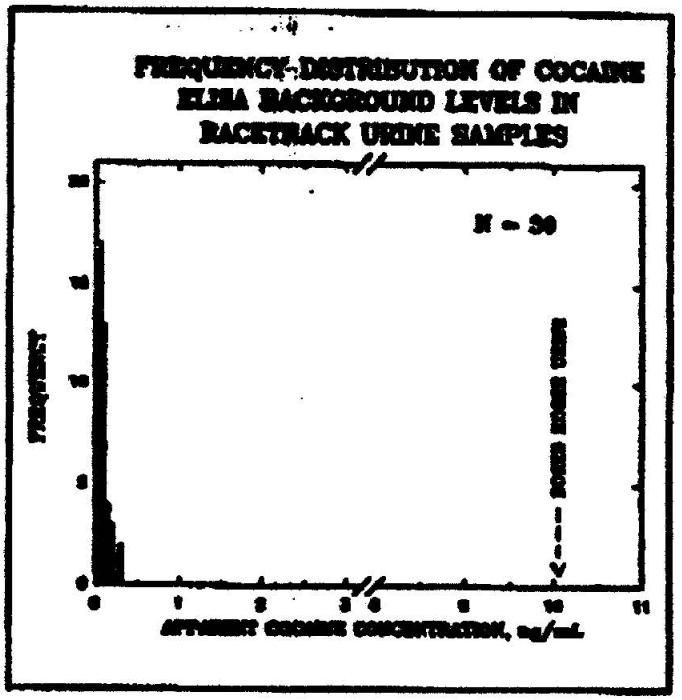

Figure 4. Distribution of "apparent" cocaine values for 39 post-race urine samples.

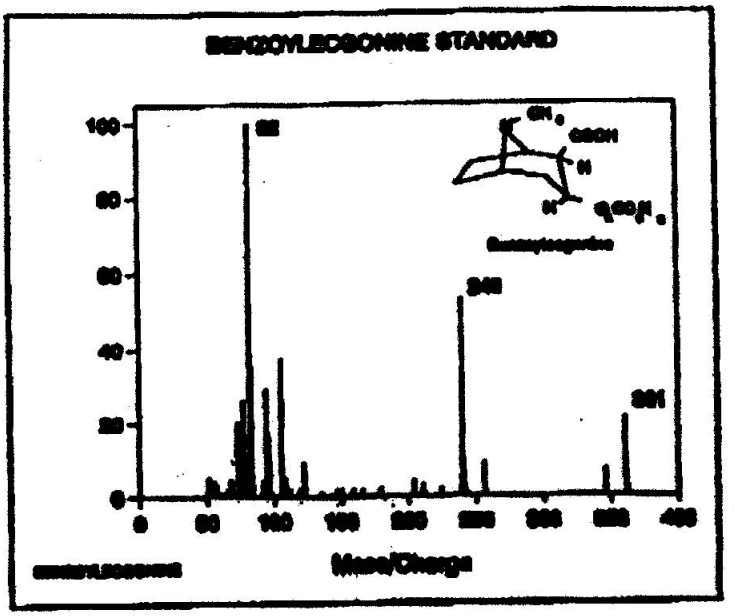

Figure 5. Electron-impact mass spectra of benzoylecgonine standard

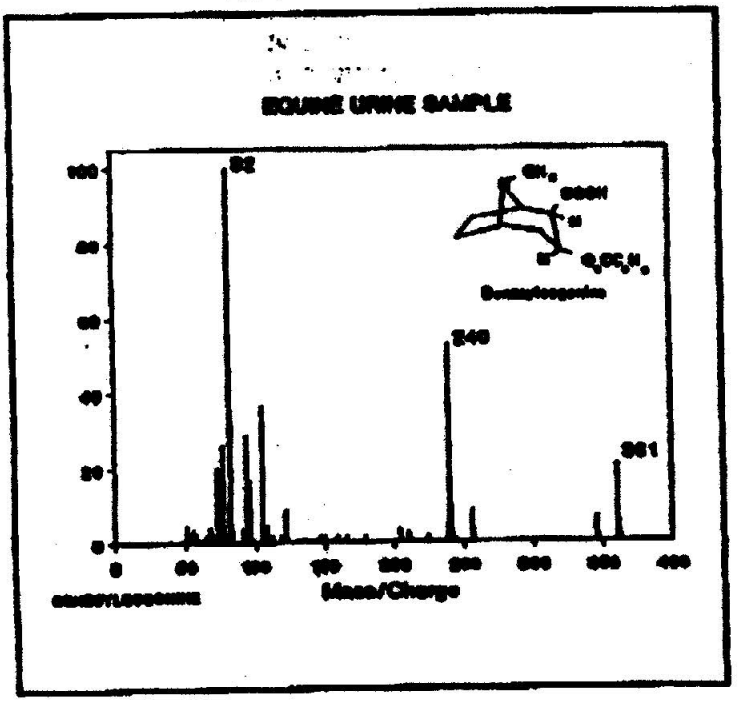

Figure 6. Electron-impact mass spectra of benzoylecgonine isolated from horse urine specimen.

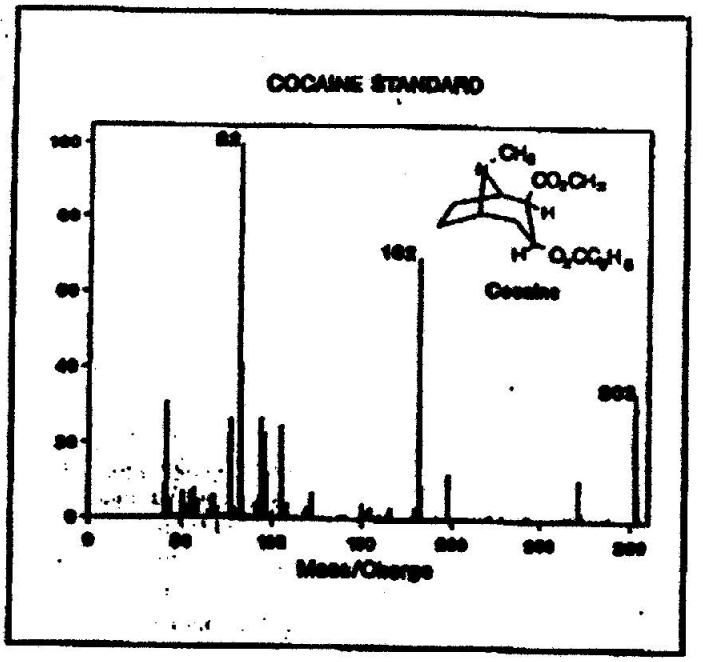

Figure 7. Electron-impact mass spectra of cocaine standard

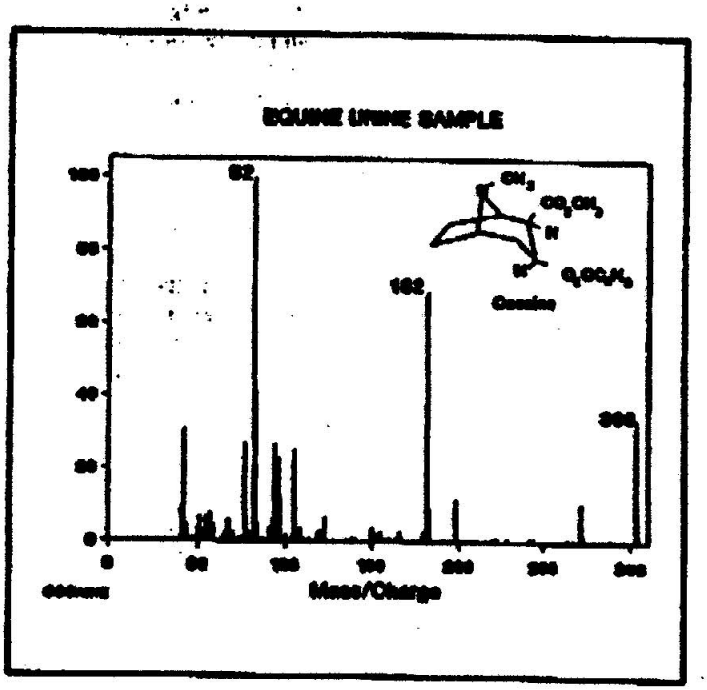

Figure 8. Electron-impact mass spectra of cocaine isolated from horse urine specimen.