# Development of an ELISA for Procaine in Racing Horses and Dogs

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## Abstract

Local anesthetics are commonly used in veterinary practice for minor surgery. Because procaine possesses both local anesthetic and central stimulant actions, it may be used to either mask lameness or stimulate performance in racing animals. For these reasons its presence in the blood or urine of racing horses is prohibited by most racing authorities. Another problem is posed by the presence of procaine in horse blood or urine samples after administration of procaine penicillin. An enzyme-linked immunosorbent assay was developed with an $I_{50}$ for procaine of 3 to 5 ng/ml. This test is very specific for procaine, showing only slight cross-reactivity with tetracaine and no significant cross-reactivity with other local anesthetics such as mepivicaine and lidocaine. A background distribution analysis from 40 post-race horse urine samples shows that the average level of background is well below the $I_{50}$ of this assay for procaine and the procaine concentrations found in dosed horse urine samples. This assay can readily detect procaine in horse urine up to 7 days after administration of therapeutic doses of procaine and procaine penicillin, respectively.

## Introduction

Local anesthetics are used to reversibly decrease sensory activity in restricted areas of the body by blocking the transmission of nerve impulses (1). As such, these agents can be used to produce local sensory blockade in the diagnosis of lameness and in association with minor surgery. Local anesthetics are therefore widely used in equine medicine and surgery, and are a legitimate part of the practice of veterinary medicine (2).

they can also be used to reduce pain in injured areas and allow an injured horse to run sound. This use of these drugs, however, is considered to increase the risk of serious injury to horses including the possibility of breakdown and death of horses and/or jockeys. For these reasons medication with local anesthetics close to post time is illegal in all racing jurisdictions. To enforce this rule a need exists for highly sensitive tests for local anesthetics.

Procaine (Novocaine®) is a distinctive local anesthetic which possesses both local anesthetic and central stimulant properties. Procaine may be administered subcutaneously or intramuscularly for its local anesthetic activity in minor surgery. High levels of procaine are central stimulant and may affect the performance of racing horses (3). The use of procaine may cause marked excitement in horses under some circumstances (4).

The presence of procaine in the blood or urine of racing horses is prohibited by most racing authorities, and positives for procaine are also not uncommon in dog racing (5). One source of these positives in dog racing is thought to be the presence of procaine from procaine penicillin in meat fed to these dogs prior to racing. For several reasons therefore, a need exists for a sensitive test for procaine and we now report the development of a sensitive and specific enzyme-linked immunosorbent assay (ELISA) for this drug.

## Materials and Methods

### *Horse Urine Samples*

Two mature Thoroughbred mares were used to determine the elimination profile of procaine in equine urine. One mare was administered 100 mg of procaine subcutaneously, while the other was administered $6x10^6$ units of procaine penicillin-G salt intramuscularly. All urine samples were collected by bladder catheterization at +1, +2, +3, +4, +5, +6, +7, +8, +120 and +168 hr post-dose, and were stored frozen at -20°C until assay.

Post-race urine samples of race horses used to study the background levels of apparent procaine were collected by the authorities at racetracks as part of routine post-race drug testing. These samples were [illegible] race testing methods.

### *ELISA Procedures*

Drug derivatization, rabbit immunization, antibody raising and ELISA development were performed as previously described (6). The carboxylic derivative of procaine was first synthesized and then conjugated to bovine serum albumin (BSA) for immunization and to horseradish peroxidase (HRP) for drug-enzyme conjugation. Anti-procaine antibody was coated on polystyrene microtitre plates (EIA/RIA 8-well strip, Costar Inc., Cambridge, MA) at 4°C overnight. The remaining binding sites on the wells were blocked with BSA. All reactions were carried out at room temperature.

To perform the ELISA assay sequential additions of 20 µl of the standard, control or test samples were added to each well along with 180 µl of procaine-HRP conjugate and followed by 60 min of incubation. The amount of drug-enzyme conjugate bound to anti-procaine antibody is inversely proportional to the procaine concentration in the sample. The unbound procaine-HRP was removed by three washes of the wells and followed by addition of tetramethyl benzidine (TMB)/$H_2O_2$ substrate (Kirkegaard and Perry, Gaithersburg, MD). The absorbance at 650 nm was monitored with a microplate reader (Microplate Autoreader, Bio-Tek Inc., Winooski, VT) for 60 min. The absorbance or color is inversely proportional to the concentration of procaine in the tested samples. Standard curves and procaine concentrations were calculated by logit-log transformation.

### *Gas-Chromatography/Mass-Spectroscopy (GC/MS)*

The urine sample (5 ml) was adjusted to pH 10.0 with ammonium hydroxide and extracted with 10% isopropanol in dichloromethane. The organic layer was recovered and evaporated under a stream of nitrogen. The residue was reconstituted in 40 µl methanol and injected into a Hewlett-Packard GC/MS with electron impact ionization (Hewlett-Packard, Palo Alto, CA).

## Results

The sensitivity and cross-reactivity of the procaine ELISA was determined against a panel of selected local anesthetics. The structures of procaine and two of its local anesthetic analogs are shown in Figure 1. Standard inhibition and cross-reactivity curves were developed as previously described (6) and are presented in Figure 2. The procaine concentration necessary for 50% displacement of procaine-HRP from the anti-procaine antibody binding sites ($I_{50}$) was about 5 ng/ml. All local anesthetics tested except tetracaine showed negligible cross-reactivity, with tetracaine showing an $I_{50}$ in this assay of about 30 ng/ml.

The background levels of apparent procaine in this ELISA were determined for 40 confirmed drug free post-race horse urine samples[1] (Fig. 3). None of the assayed samples had an apparent procaine concentration above 5 ng/ml or about the $I_{50}$ of this test for procaine. Therefore no dilution of urine samples is necessary for procaine detection in racing horses.

This procaine ELISA was next used to assay urine samples collected from horses dosed with subcutaneously with procaine hydrochloride and intramuscularly with procaine penicillin[1]. For urine samples from a horse dosed with 100 mg procaine subcutaneously (Fig. 4), it was necessary to dilute several of the samples with assay buffer due to high levels of apparent procaine concentration. The mean concentration of apparent procaine from 40 post-race urine samples was used as control ("0"). The apparent procaine concentration peaked at about 2 µg/ml in the 5 hr sample, and procaine was easily detectable up to 8 hr post-dosing.

A similar elimination profile was seen in urine samples collected from a horse dosed with $6x10^6$ units of procaine penicillin intramuscularly (Fig. 5). In this horse the apparent procaine concentration peaked at about 40 µg/ml in the 7 hr post-dosing sample and procaine was readily detectable at least through 8 hr. Procaine was detectable at up to 7 days post-dosing although "white-out" inhibition was not obtained a this time.

More recently a Western United States horse racing forensic testing laboratory has used this ELISA test as part of their post-race equine and canine urine screening panels and have reported detection and confirmation of several procaine positive from post-race horse and dog urine samples[2]. A typical GC/MS spectrum of procaine from an equine sample positive for procaine is presented in Figure 6.

## Discussion

Procaine is one of the most commonly detected positives worldwide in both canine and equine post-race urine testing and has held this position ever since the introduction of procaine penicillin into veterinary medicine thirty or more years ago. The procaine in procaine penicillin is present to delay the absorption of penicillin and thus extend its plasma half-life. Procaine penicillin is a very effective combination in this regard, but one effect of this combination is to similarly extend the plasma and urinary half-life of procaine.

Procaine may also be administered as procaine hydrochloride for its local anesthetic effects. While once the principal local anesthetic in human and veterinary medical practice, procaine is now rarely used, having been supplanted by more effective agents. Nevertheless, the possibility of its use as a local anesthetic remains and positives for procaine from procaine penicillin cannot generally be distinguished from positives for procaine hydrochloride by the methods available to analytical chemists.

As well as having local anesthetic actions, procaine also has central stimulant actions, although these actions appear only at high or toxic doses of this drug and are not the kind of central stimulant effects that would likely help a racing horse. Taken together, however, these various actions and effects of procaine means that there is considerable regulatory interest in this drug and also some discussion as to what constitutes an appropriate regulatory strategy for this drug.

The ELISA test described in this paper, with an $I_{50}$ for procaine of about 5 ng/ml, is sufficiently sensitive to regulate use of this drug. If procaine is administered as procaine hydrochloride, in an attempt to block a nerve or joint, this test is sufficiently sensitive to detect the administration of

procaine in this way for at least 8 hr post-dosing more than the duration of the pharmacological effect of this drug. In point of fact a more recent reformulation of this test, with an $I_{50}$ for procaine of about 200 pg/ml, should readily detect use of even smaller local anesthetic doses of this agent.

The sensitivity of this test also allows detection of procaine administered as procaine penicillin for at least five days and likely longer. In fact, with the older and less sensitive GC methods, detection of procaine had been reported for up to 13 and 18 days after administration of procaine penicillin. With this test similarly long detection times seem possible and the problem of distinguishing positives caused by procaine penicillin from those due to procaine remains.

Another potential application of this ELISA is to screen meat fed to racing dogs for the presence of procaine. Screening of such meat samples prior to their feeding to dogs close to post time would be very likely to detect the presence of procaine in these samples. Similarly a field kit adaption of this test could also be used by equine practitioners to detect traces of procaine in equine urine samples before entry of their horses, thereby enabling them to avoid the commonest positive of all, a procaine positive for procaine penicillin.

[1]Urine specimens were provided by Analytical Toxicology Laboratory of the Ohio State University, Columbus, OH.
[2]Harris Laboratories, Phoenix, AZ.

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**Figure 1**
*tetracaine*

**Figure 2**
*is illustra*
*procaine*
*cross-reac*

**Figure 1** *Procaine and Analog Structures. The molecular structures of procaine and the local anesthetic analogs tetracaine and procainamide are illustrated.*

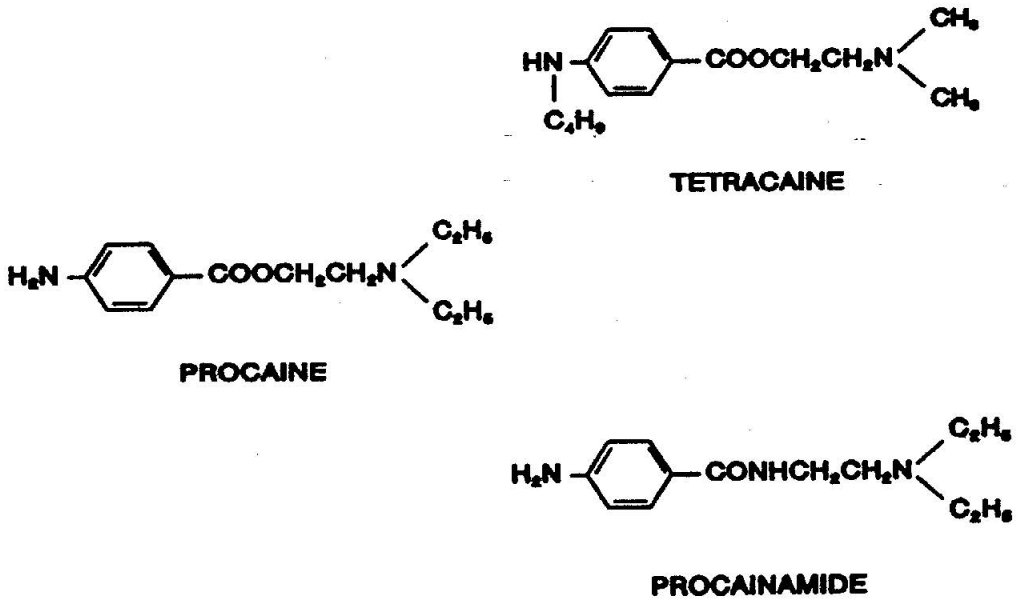



**Figure 2** *Cross-Reactivity Plot. The reactivity of the anti-procaine antibody with procaine and other local anesthetics is illustrated with standard curves in assay buffer. A 50% inhibition was produced by the addition of a 5 ng/ml procaine solution. Tetracaine produced a 50% inhibition at about 30 ng/ml. No other drug exhibited significant cross-reactivity.*

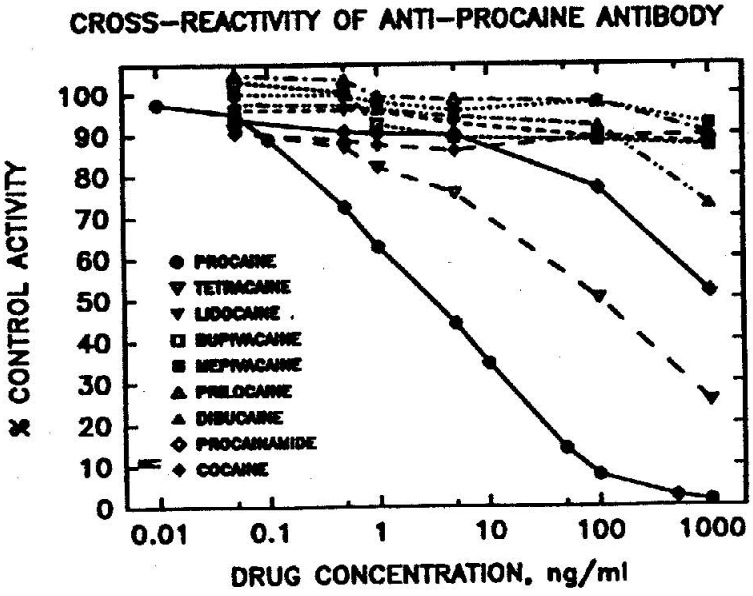


**Figure 3** *Backgrounds Plot. The distribution of "apparent" procaine concentrations for 40 known negative post-race urine samples indicated no interference from horse urine.*

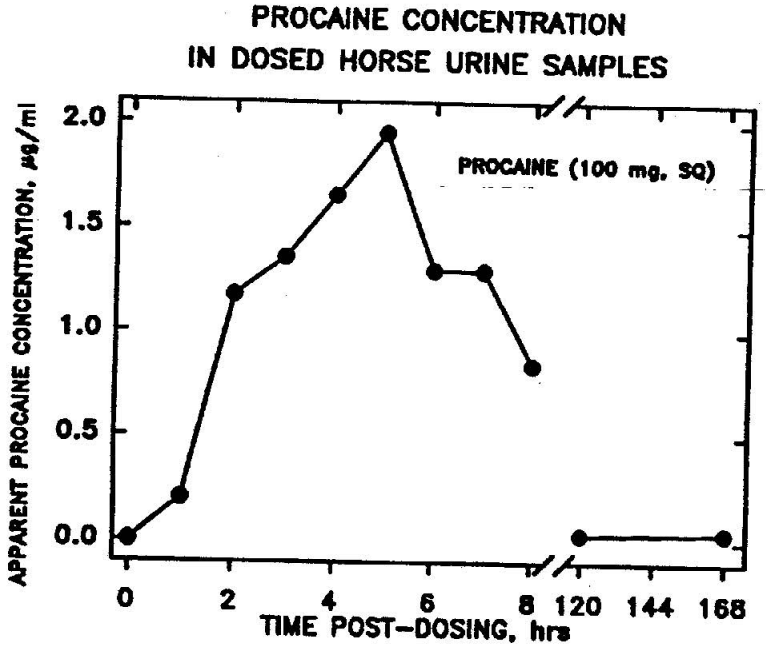



**Figure 4** *Procaine Dosed-Horse Urine Plot. Procaine or its metabolites (procaine equivalents) were detectable for up to 8 hr post-dose in urine collected from a horse dosed with 100 mg procaine subcutaneously. the samples were diluted with assay buffer as necessary to keep the concentrations odserved within the linear range of the standard curve.*

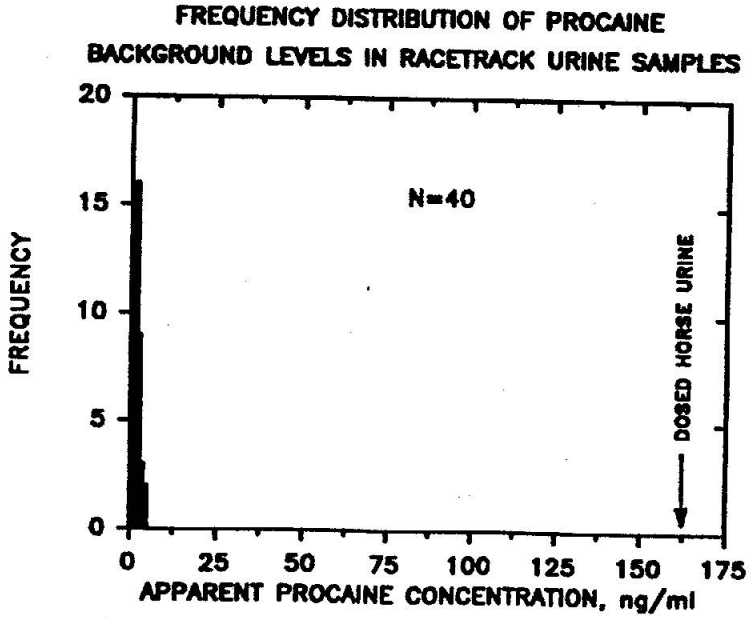


**Figure 5** *Procaine Penicillin Dosed-Horse Urine Plot. Procaine equivalents were detectable up to 7 days post-dose in urine collected from a horse dosed with 6x10⁶ units procaine penicillin intramuscularly. Although not clear from this plot, samples taken at 120 hr and 168 hr post-dose actually are ELISA "positive", with concentrations far enough above the mean post-race urine sample control to nearly "white out" the assay.*

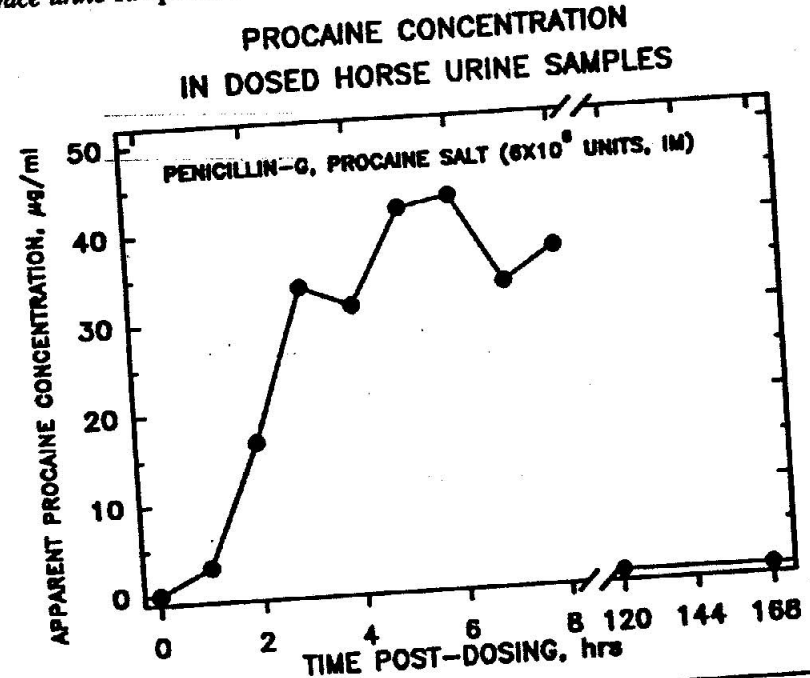
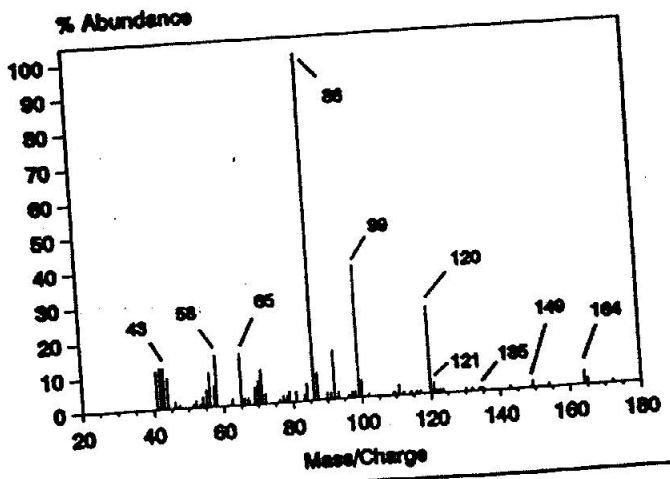



**Figure 6** *Gas Chromatography/Mass Spectroscopy Confirmation. A typical GC/MS procaine confirmation spectrum is illustrated. The procaine ELISA was used to screen this equine urine sample for GC/MS confirmation. (Data provided by Harris Laoratories, Phoenix, AZ).*



## Discussion

*RYAN* Did you try analyzing any plasma samples with your ELISA kit?

*YANG* No, we haven't analyzed plasma samples.

*RYAN* Thank you.

*DALGLISH* Is there any way you can distinguish between the procaine from procaine penicillin and the procaine that's used as a local anesthetic?

*YANG* It's very hard to tell if you just analyze it from the urine. The concentration of the drug in the urine does not tell you anything about administration or when the drug was administered or how long the drug was already in the body. So you cannot really tell. But at least it will give you an indication. Procaine has legitimate use in veterinary medicine, but in some of the jurisdictions, procaine is prohibited. So this will give the veterinarians an idea of the withdrawal time. They should inject the horse probably three or four weeks prior to the race to avoid problems with procaine penicillin.

*DALGLISH* Okay. Thank you.