ELISA Detection of Acepromazine in the Horse

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Abstract

We have developed and evaluated a one step enzyme-linked immunosorbent assay (ELISA) test for acepromazine as a part of a panel of pre- and post-race tests for illegal medications in racing horses. This ELISA test detects acepromazine with an I-50 of about 150 pg/ml. The test is economical and can be read with an inexpensive spectrophotometer, or even by eye. The test is rapid and ten urine samples can be analyzed in twenty minutes. The test readily detects the presence of acepromazine or its metabolites in equine blood and urine from 8 to 72 hours or longer, respectively, after administration of sub-therapeutic doses. In vitro, the test cross-reacts with analogues of acepromazine, suggesting that it will also detect the use of other phenothiazine tranquilizers. As such this test is capable of both improving the quality and reducing the cost of testing for acepromazine in racing and show horses.

Introduction

Acepromazine (acetylpromazine or 1-[10-[3-(dimethylamino)propyl]-10H-phenothiazin-2-yl]ethanone) is an acetylated derivative of the phenothiazine tranquilizer promazine. Acepromazine is commonly used for sedation in equine medicine with a recommended dose of between 0.5 mg/kg and 0.1 mg/kg.[1] The pharmacological properties of acepromazine include sedation, reduction of respiratory rate penile protrusion, reduction of hematocrit, and hypothermia.[2]

Acepromazine and related phenothiazine tranquilizers have been used to illegally affect the performance of both racing and show horses. Treating nervous horses with a small amount of tranquilizer prior to a race or show event is believed to "take the edge off" such horses and allow them to settle sufficiently for a concentrated performance effort. The phenothiazine derivatives, particularly acepromazine and proprio-promazine, are commonly used in this regard. They are readily available in veterinary circles and as extremely potent drugs are difficult to detect with currently available analytical methodology.[3,4]

Regulation of the use of the phenothiazine tranquilizers is also complicated by the fact that these drugs have legitimate veterinary uses.[5,6,7] They may be used in "breaking" young horses, to calm horses in loading and transporting them, and also in association with minor surgical and other interferences with the horse. For this reason traces of these tranquilizers may show up in horse urine after a legitimate medical use of these agents, posing a problem for horsemen and regulators.

One approach to the problem of the illegal use of these drugs is to develop a more sensitive test for these agents. To this end we have raised a series of antibodies to acepromazine and incorporated these antibodies into a one step enzyme-linked immunosorbent assay (ELISA) test for acepromazine. In this report we detail the development and evaluation of this ELISA test

for acepromazine.

Materials and Methods

Horses

Mature Thoroughbred, half Thoroughbred and Standardbred mares (400-600 kg) were used throughout. The animals 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

Acepromazine maleate was obtained as the authentic drug standard and as an injectable preparation.[a] The drug (either 5, 1, 0.1 or 0.05 mg/horse) was administered by rapid IV injection into the jugular vein.

All urine samples were collected by bladder catheterization. Blood samples were collected by venipuncture into vacuum blood tubes[b] containing potassium oxalate and sodium fluoride (for plasma) or into serum separation vacuum tubes and centrifuged. All samples were stored frozen until assayed.

Urine samples were analyzed directly. Blood samples were diluted 1:3 with PBS buffer before assay.

One Step ELISA

The one step ELISA tests were performed as previously described.[8,9,10] Briefly, anti-acepromazine antibody was linked[11] to flat bottom wells.[c] An acepromazine oxime derivative was linked to horseradish peroxidase (HRP) to give rise to a covalently bound acepromazine-HRP complex.[12]

The assay was started by adding 20 ul of the standard, test, or control samples to each well, along with 100 ul of the acepromazine-HRP solution. Acepromazine standards were prepared in PBS buffer. During this step, the presence of free drug or cross reacting metabolites competitively prevented the antibody from binding to the acepromazine-HRP conjugate. The degree of antibody-acepromazine-HRP binding was therefore inversely proportional to the amount of drug in the sample. After 25 minutes of incubation the fluid was removed from the microtiter wells and the wells washed three times with buffer. Substrate solutions[d] were then added to all wells and their optical density read at 650 nm in microwell reader[e] at least 15 minutes after addition of substrate.

Results

The time course and sensitivity to added acepromazine of our one step ELISA test are shown in Figure 1. The standards were added in PBS buffer. In the absence of added acepromazine the reaction reached an apparent optical density value of about 0.75 after 60 minutes of reaction time. The addition of increasing concentrations of acepromazine acted to inhibit the reaction, with virtually complete inhibition of the reaction occurring after the addition of 20 ul of 100 ng/ml acepromazine standard.

Data of Figure 2 show standard curves of the acepromazine ELISA test run in PBS buffer with increasing reaction reading times. After 15 minutes there appeared to be no significant improvement of apparent sensitivity of the assay. Therefore ELISA tests in buffer or urine were read after at least 15 minutes of reaction time. The apparent I-50 for acepromazine in this assay was about 150 pg/ml.

The ability of the anti-acepromazine antibody to detect several phenothiazine congeners was evaluated *in vitro*. Tests showed strong cross-reactivity of the antibody to the promazine family of phenothiazines. In fact, the antibody was even more sensitive to the tested promazine analogs than to acepromazine (Figure 3). The antibody was less reactive with some other phenothiazine congeners (Figure 4). These results suggest that an ELISA assay based on an anti-acepromazine antibody should be able to detect phenothiazine congeners in horses dosed with these drugs.

The ability of the ELISA test to detect acepromazine and its metabolites in the blood and urine of horses dosed intravenously with the drug was investigated. Acepromazine was administered at dose levels ranging from 5 to 0.05 mg/horse to either of two horses.

Acepromazine equivalents were readily detectable in urine at these dose levels. Inhibition of the ELISA reaction was essentially total from 30 minutes to 8 hours at dose levels from 5 mg/horse to 0.1 mg/horse.

Inhibition was still evident at 24 hours at all dose levels, and at 72 hours for 5 mg/horse and 1 mg/horse doses (Figure 5).

In blood, acepromazine was assayed at the 5 mg/horse and 1 mg/horse dose levels. The ELISA reaction readily detected the drug at both dose levels in serum for up to 4 hours post-dose and at the 5 mg/horse level up to 8 hours (Figure 6). In plasma the ELISA reaction detected acepromazine in samples for up to 8 hours at the 5 mg/horse dose level (Figure 7).

Discussion

The one step ELISA test for acepromazine described in this report is the most sensitive test for acepromazine developed to date. *In vitro* it can detect levels of acepromazine of less than 0.1 ng/ml. As such this ELISA will easily detect clinically effective doses of acepromazine in horses, and will also likely detect sub-therapeutic doses of acepromazine of the kind used in performance horses. This test also offers hope of detecting phenothiazine tranquilizers in the blood stream of horses after clinical-level doses, which to date has not been possible with any testing technology.

In its current configuration this test is able to detect the administration of less than 100 ug of acepromazine/horse. This is a very small dose of acepromazine indeed, and raises the question of whether or not such a sensitive test is necessary for a drug such as acepromazine, particularly in view of the legitimate uses of this drug in veterinary medicine. The answer to this question depends at least in part on the minimum dose of acepromazine that will produce a pharmacological effect, and on how this dose relates to any other testing methods that may be available for acepromazine.

The currently used testing methodology for acepromazine is based on thin layer chromatography. Based on the samples used in interlaboratory trials, the minimum dose of acepromazine that standard TLC methodology will detect is about 2.5 mg/horse. Since our ELISA test is able to detect doses of 100 ug/horse and possibly less, our ELISA test is at least twenty-five times more sensitive than the currently used thin layer chromatographic technology and approximately equivalent to the limit of HPTLC technology (100 ug/horse) under optimal conditions.[13]

There is good evidence that acepromazine can produce subtle pharmacological effects in horses at doses of less than 2.5 mg/horse. Studying the effects of acepromazine on penile protrusion we found that this relatively crude indicator of a tranquilizing effect of acepromazine was half maximally affected after a dose of about 5 mg/horse, and that the threshold for this effect was about 2.5 mg/horse.[2] However, when we examined the effects of acepromazine on hematocrit, a much more sensitive indicator of an adrenergic blocking effect, we found that doses of 1 mg/horse produced good pharmacological effects. Since at that time (1982) we had no method of detecting such low doses of acepromazine, we did not pursue this effect to determine its threshold. However, it appears clear to us that the threshold dose for a pharmacological effect of acepromazine in the horse is less than 500 ug/horse, and likely close to the ultimate sensitivity of this test. There appears, therefore, to be a need for a test with the sensitivity of our ELISA test in the regulation of the use of acepromazine in performance horses.

The analysis presented here assumes the patterns of phenothiazine tranquilizer use is limited to the administration of one phenothiazine at a time only. It would be a relatively simple matter, however, for an individual who wished to circumvent the current technology to simply combine one or more phenothiazine tranquilizers. Since TLC detection technology depends on separating these drugs prior to their identification, administration of three different phenothiazine tranquilizers would give rise to a three fold pharmacological effect without proportionally increasing the risk of detection. With the immunoassay technology described in this report, however, there is a good probability that the different drugs will each cross-react with our anti-phenothiazine antibody and thereby increase the probability of detection of the medication offense.

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Footnotes

[a]PromAce®, Aveco Co., Inc., Fort Dodge, IA.
[b]Becton Dickinson Vacutainer Systems, Rutherford, NJ.
[c]Immulon Removeawells, Dynatech, Chantilly, VA
[d]TMB Microwell Peroxidase, Kirkegaard & Perry, Gaithersburg, MD
[e]Ultrascan, International Diagnostic Systems Corp., St. Joseph, MI

Figure 1. *Time course of the ELISA reaction in the presence of increasing concentrations of acepromazine.*

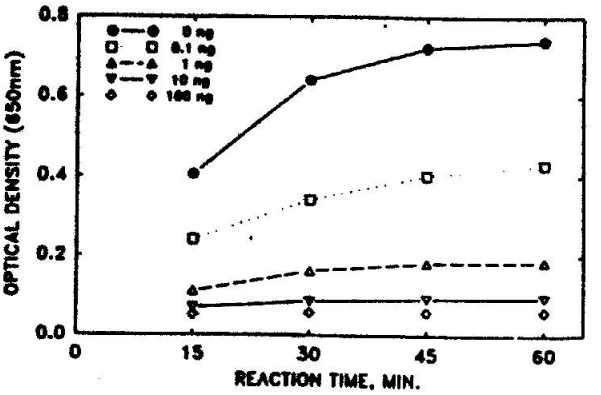


Figure 2. *I-50 for acepromazine in the one-step ELISA system.*

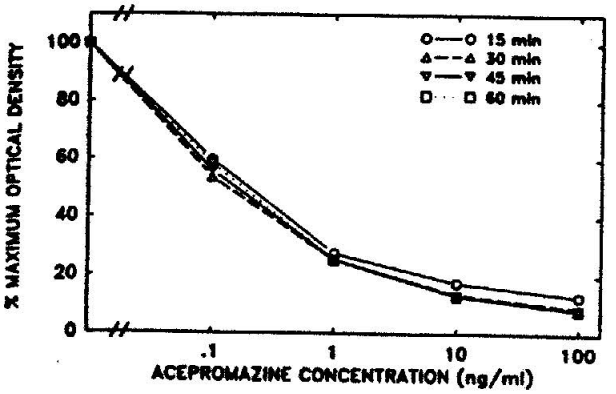


Figure 3. *Inhibition of the ELISA reaction by promazine congeners.*

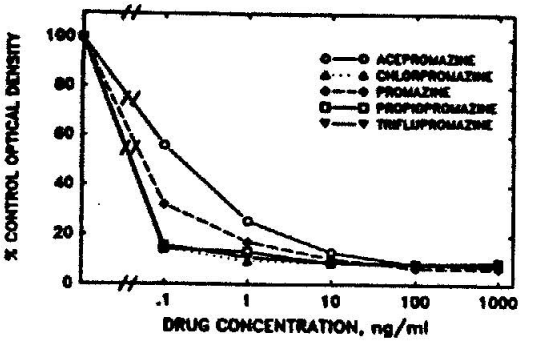


Figure 4. *Inhibition of the ELISA reaction by phenothiazine congeners.*

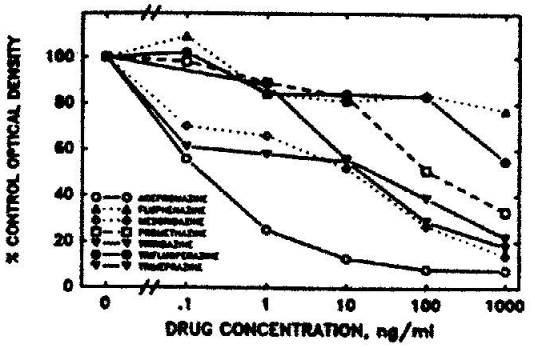


Figure 5. *ELISA inhibitory activity in equine urine after IV administration of acepromazine.*

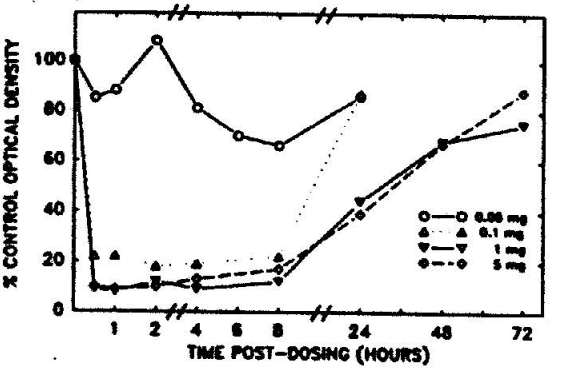


Figure 6. *ELISA inhibitory activity in equine serum after IV administration of acepromazine.*

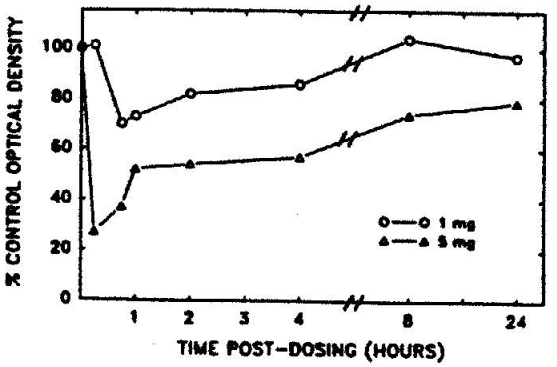
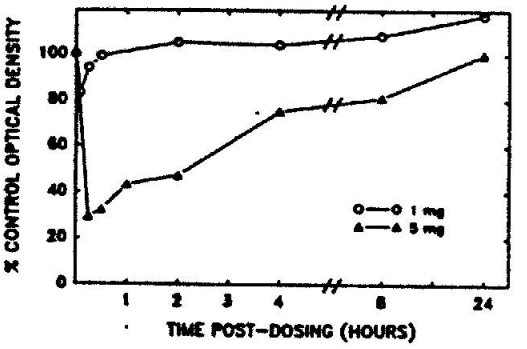


Figure 7. *ELISA inhibitory activity in equine plasma after IV administration of acepromazine.*



Discussion

RON JENSEN Did you look at acepromazine at therapeutic levels longer than 24 hours? And if so, how long were you able to see it?

JMY Do you mean in the incubation curve for the high dose level?

RJ You said you could detect it up to 24 hours, and I am wondering if you looked at longer than 24 hours at therapeutic levels; 30 ml, 25 ml.

JMY Right now, we don't have the data for those high levels. We have some levels from a paper done by another graduate student of Dr. Tobin's a few years ago, that those levels run like 30-50 and it was done by gas chromatography method. Right now, we don't have the data for those high dose levels.

PIERRE BEAUMIER This paper disturbs me, in that on the other ELISA techniques we were looking for narcotic analgesics where we needed sensitivity. Now, if I read one of your slides correctly, you are still getting reaction at 72 hours. If someone is shipping a horse, it may be within 72 hours of race time that he is using acepromazine. I think this is a different situation over someone administering acepromazine right before a race to change the performance of a horse. We may be getting into a situation of quantitation of acepromazine with this technique. Can you comment on this?

JMY I would like to say, if someone doses a horse at 72 hours before the shipping?

PB Shipping the horse days before the race, using acepromazine, we will still be detecting it and presumably able to confirm it for positives. There is a difference with that and trying to change the horse right before the race.

JMY Even with the high dose level of up to 50 ml per horse, usually the horse will recover to a control level, after 4 hours. So actually there is no pharmacological effect after....

PB That is my question. Basically, if you administer acepromazine to ship a horse two days before a race, he may end up positive with this ELISA assay, the drug not having an effect on the horse at the time of the race. Will we need regulatory levels on acepromazine?

JMY I would recommend to check on the level plasma in the serum. If the presence of acepromazine is metabolized still very high in the plasma you will probably still have some pharmacological effect on the horse.

PB Basically, we will need a blood sample with every urine sample.

JMY Yes.

ROBERT LANTZ Have you checked this for cross-reactivity with phenothiazine, itself?

JMY No.

TT The answer to Pierre's questions is the one that Jaime pointed out. This is an extremely sensitive test and we only used 5 mg doses in our studies in Kentucky. If we gave a 30 mg dose it would be detected in the urine for quite a while and we are going to investigate the use of the test on blood levels to come up with the regulatory level. It is quite clear from Dr. Smith's comments this morning about morphine and the abilities of this assay for the derivatives that the business of trace levels is now extending into the illegal drug area as well as what we call in the US, the soft drugs.
