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IMMUNOASSAY DETECTION OF DRUGS IN RACING HORSES III. DETECTION OF MORPHINE IN EQUINE BLOOD AND URINE BY A ONE STEP ELISA ASSAY

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SUMMARY

A one step enzyme-linked immunosorbent assay (ELISA) test for morphine was evaluated as part of a panel of pre- and post-race tests for narcotic analgesics in racing horses. This ELISA test is very sensitive to morphine with an I-50 for morphine of about 400 pg/ml. The test is also rapid, and ten samples, a normal pre-race complement, can be analyzed in about thirty minutes. The test can be read with an inexpensive spectrophotometer, or even by eye. The test readily detects the presence of morphine or its metabolites in equine blood for up to six hours after administration of sub-therapeutic doses. The antibody also cross-reacts with hydromorphone, orymorphone, nalorphine, levorphanol, and codeine, and the test either can detect or is likely to detect these drugs in blood or urine shortly after their administration to horses. As such this test is capable of dramatically improving the speed and efficacy of both pre-race and post-race testing for morphine and its congeners in racing horses. On initial introduction into post-race urine screening this test flagged 18 of 166 samples positive for opiates, and 13 of these samples were confirmed positive for opiates by mass spectrometry.

INTRODUCTION

The concept of pre-race testing for drugs in horses goes back to the earliest days of drug testing, when the tests used were biological. These tests involved injection of saliva from suspect horses into frogs and mice and the tests were read and interpreted by inspection of the animals behavior (Tobin, 1983). With the advent of more elaborate chemical tests, these approaches fell into disfavor, and post-race urine testing became the norm in North America for drug testing of racing horses.

The concept of pre-race testing, however, remains attractive from the regulatory point of view. Ideally, horses are sampled and tested for drugs pre-race and any horses found to be improperly medicated are eliminated. In recent years pre-race testing strategies based on gas liquid chromatography and also on thin layer chromatography have been introduced in Ohio, New York, New Jersey, and Pennsylvania (Tobin, 1983). However, the efficacy and cost effectiveness of these programs has been debatable and at least one of these programs has been abandoned. Their regulatory value is debatable because of the limitations of the thin layer chromatographic technology on which these programs are based. Thin layer chromatography (T.L.C.) simply does not have the sensitivity to detect in blood any drugs other than "soft" acidic drugs such as phenylbutazone and furosemide, which drugs are legal in many juridictions. The "hard" or uniformly illegal drugs such as the narcotic analagesics are not detectable in blood by T.L.C., principally because the concentrations of these drugs in blood are too low (Tobin et al., 1979; Tobin, 1981).

To effectively screen pre-race blood samples for high potency drugs simple, rapid, inexpensive and highly sensitive detection techniques are required. The only currently viable solution to this problem is to develop rapid and sensitive immunoassays for these agents. To this end we have been developing a panel of immunoassay based tests, concentrating on particle concentration fluorescence immunoassay (PCFIA) and one step enzyme-linked immunosorbent assay (ELISA) tests. In this report we outline the development of an ELISA test for morphine and we evaluate its application to both pre-race and post-race blood testing for drugs in horses.

MATERIALS AND METHODS

Horses

Mature Thoroughbred, half Thoroughbred and Standbred horses (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 sq M) approximately 12 hours prior to dosing for acclimatization.

Urine and plasma samples from racing horses were collected by the authorities in charge at the individual racecourses after races and delivered to the drug testing laboratory of the racing jurisdiction.

Dosing and Sampling

Authentic morphine standard was obtained from Alltech-Applied Science (State College, PA). Morphine was injected as morphine sulfate injection USP from Lilly Company (Indianapolis, IN). All injections were by rapid IV injection into the jugular vein. All urine samples were collected by bladder catherterization, and were stored frozen until assayed. Urine samples were diluted and filtered through Spin-X® microcentrifuge filters (Costar®, Chantilly, VA) prior to assay.

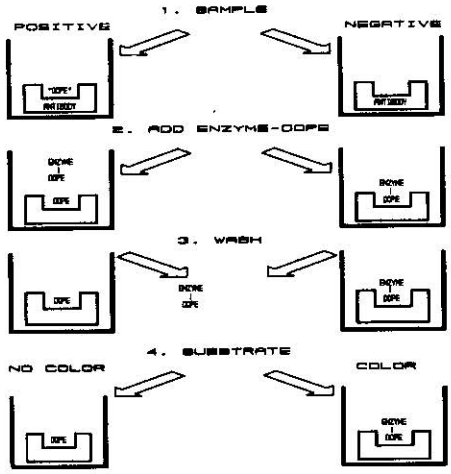
All plasma samples were treated with trichloroacetic acid (TCA) to denature and remove excess plasma proteins and endogeous fluorescent substances. In this step 300 µl of 4% TCA were added to 300 µl of plasma, vortexed and allowed to react at room temperature for 20 minutes. The samples were then centrifuged at 8,800xg for 10 minutes and the supernatant removed. To 50 µl of the supernatant 12 µl of 0.24M sodium carbonate, pH 10 were added, the mixture vortexed and an aliquot taken for assay.

One Step ELISA Test

The one step ELISA tests were performed as described by Voller *et al.* (1976) and Yang *et al.* (1987). Briefly, anti-morphine antibody was linked

to flat bottom Immulon Removawells® (Dynatech, Cantilly, VA) as described by Voller. Similarly, morphine hemisuccinate was linked to horse radish peroxidase (HRP), as described by Wie et al. (1982), to give rise to a covalently linked morphine-HRP complex. All assay reactions were run at room temperature. The assay was started by adding 20 µl of the standard,

Figure 1. Reaction sequence of the one step ELISA test.



Antibody to the drug is bound to the well, and test and control samples are added directly to the well. In control samples those sites remain free and bind the drug enzyme conjugate when this is added. In "positive" sample wells the drug enzyme conjugate cannot bind, because the antibody sites are already occupied. Unbound drug-enzyme is removed by the wash step and substrate added to develop the test. An absence of color, indicating that no drug-enzyme complex bound to the antibody, represents a positive test.

test or control samples to each well, along with 100 µl of the morphine-HRP solution. During this step, the presence of free drug or cross-reacting metabolites competitively prevented the antibody from binding to the

morphine-HRP conjugate. The degree of antibody-morphine HRP binding was therefore inversely proportional to the amont of drug in the sample. After ten minutes of incubation the fluid was removed from the microtiter wells and the wells washed three times with buffer. Substrate (tetramethylbenzamine, Kirkegaard and Perry, Gaithersburg, MD) was then added to all wells and their absorbance read at 560 nm in an International Diagnostic System (St. Joseph, MI) microwell reader at 0, 5, 10, 15, and 60 minutes after addition of substrate. This sequence of events is presented schematically in Fig. 1.

Particle Concentration Fluorescence Immunoassay (PCFIA)

Antibody cross-reaction studies were performed by PCFIA as previously described (Jolley et al., 1984; McDonald et al., 1987; Yang et al., 1987) on a Pandex Fluorescence Concentration Analyzer 15-010-1 (Pandex, Mundelein, IL). Anti-morphine antibody and reagents were supplied by International Diagnostics Systems. The drugs used in the PCFIA cross-reaction studies were supplied by the Illinois Racing Board Laboratory, Elgin, IL.

Mass-Spectroscopy

Gas chromatography/mass-spectroscopy (GC-MS) was performed on a Hewlett-Packard GC model 5890 equipped with a Hewlett-Packard MS-5970 detector and a 12 meter HP-1 (methylsilicone) capillary column (Hewlett-Packard, Palo Alto, CA). The samples were injected directly into the detector in the non-split mode. GC oven temperature was programmed from 70°C to 280°C at 35°C/min. Urine samples were subjected to enzymatic hydrolysis as previously described (Combie et al., 1982). Urine samples (50 ml) were incubated (63°C, 3 hr) with β-glucuronidase from Patella vulgata (Sigma Chemicals, St. Louis, MO) (5,000 units/5ml urine). The samples were made basic (pH 9.5) with ammonium hydroxide and extracted into

dichloromethane (DCM)/isopropanol (6:1) (6 ml solvent/5 ml urine). The samples were then back extracted into 2 ml 0.2N sulfuric acid, made basic (pH 9.5) with 2 ml 0.6N ammonium hydroxide, and re-extracted into DCM/isopropanol (6 ml solvent/5 ml urine). The samples were evaporated to dryness, redissolved in methyl acetate, and purified by preparative thin layer chromatography (E Merck F-254 silica gel-60 plates, Alltech-Applied Science, Deerfield, IL). The solvent system was chloroform/methanol/propionic acid (80:15:5). The plates were scraped and the samples were eluted into 1 ml isopropanol and extracted with 2 ml 0.2N sulfuric acid. The samples were made basic with 2 ml 0.6N ammonium hydroxide (pH 9.5) and extracted into 6 ml DCM. The samples were evaporated to dryness and 30 µl ethylacetate were added to each tube. After thorough mixing, 2 µl of the sample were injected on the GC/MS.

RESULTS

The data of Fig. 2 shows the time course and sensitivity to added morphine of the one step ELISA test. In the absence of added morphine the reaction rapidly runs to completion, with an apparent absorbance value of about 2.0 being attained between 10 and 15 minutes after starting the reaction. The addition of increasing concentrations of morphine acts to inhibit the reaction, with virtually complete inhibition of the reaction occurring after addition of 10 ng/ml of morphine.

The data of Fig. 3 shows that half maximal inhibition occurs at about 400 picograms/ml of morphine, suggesting that the test is likely to be sufficiently sensitive to detect morphine administration in pre-race testing. The dotted line shows the sensitivity of the PCFIA technique previously described when the same anti-morphine antibody and a morphine-β-phycoerythrin complex are used to construct the assay.

Figure 2. Time course of ELISA reaction in the presence of increasing concentrations of morphine.

The symbols show the time course of the ELISA reaction in the presence of the indicated concentration of morphine.

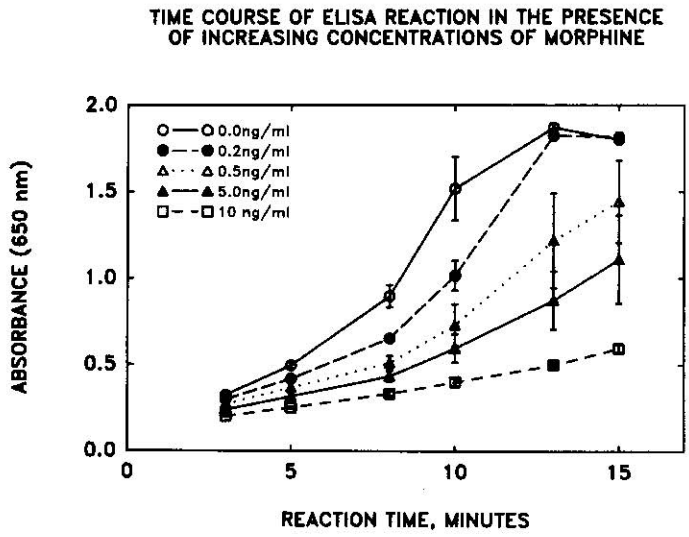
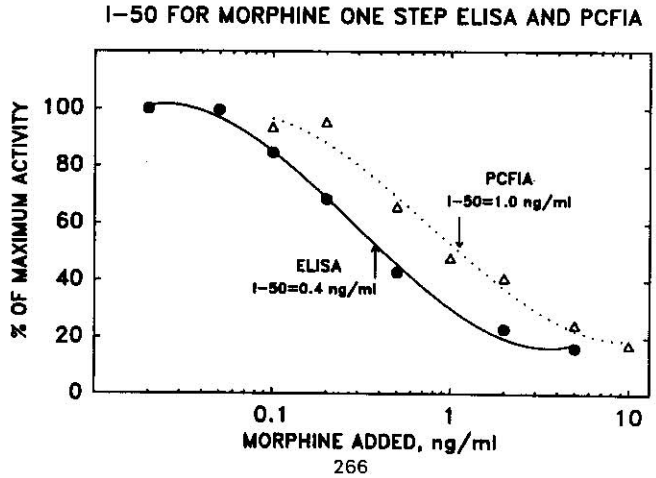


Figure 3. I-50 for morphine in one step ELISA system.

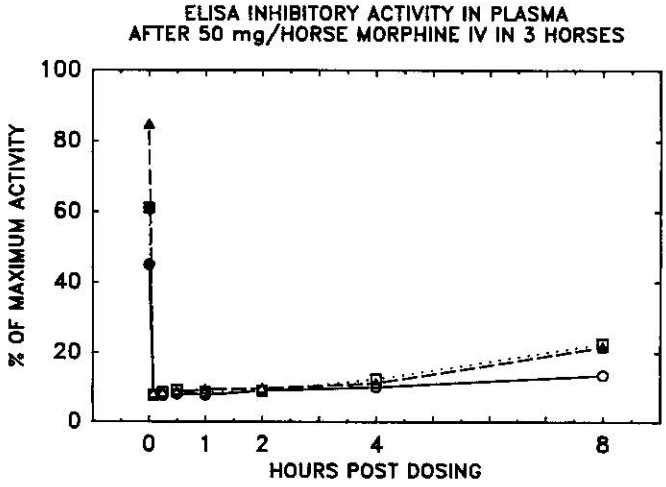
The solid symbols (●-●) show the percentage inhibition of maximal activity observed in the presence of the indicated concentrations of morphine. The dotted line (Δ...Δ) shows the I-50 for the same antibody in the PCFIA system.



The data for Fig. 4 shows that the test readily detects plasma levels of morphine or its metabolites after administration of 50 mg of morphine IV to horses. The ELISA reaction is initially inhibited about 90%, which inhibition is sufficient to be readily visible to the naked eye. In addition, the amount of inhibition observed appears within minutes after administration of the drug, and remains at a high level for the 8 hours over which this inhibition was followed in our horses. The data suggests that the administration of morphine to the horse will be readily detectable in plasma within minutes after administration of morphine and for several hours thereafter.

Figure 4. ELISA inhibitory activity in the plasma of horses after 50 mg/horse of morphine IV.

The symbols show the ability of equine plasma to inhibit ELISA activity before (●, ■, ▲) and after (○, □, △) IV injection of 50 mg/horse morphine into three horses.



To be maximally effective as a pre-race test, a test should not simply detect morphine, but also detect other related medications. We therefore tested our anti-morphine antibody for cross-reactivity with other

medications. As shown in Table I this antibody cross-reacted with morphine, oxymorphone, hydromorphone, and levorphanol, and in fact appears more reactive with hydromorphone than with morphine. Similarly, in other experiments we determined that this antibody also recognized nalorphine and codeine at levels of about 1 ng/ml, likely more than sufficient to allow detection of these agents in blood and urine of dosed horses.

Table I. Cross reactivity of anti-morphine antibody with other narcotic analgesics in PCFIA.

Drug/ Conc	Morphine	Oxymorphone	Hydromorphone	Levorphanol	Nalorphine	Codeine
0	100	100	100	100	100	100
0.1	69%	75	55.0	79%	-	-
0.5	34%	-	28.0	53%	-	-
1.0	28%	68	17.0	46%	68%	69%
10.0	14.6	31	6.6	16%	-	-

The cross reactivity of the anti-morphine antibody was determined by particle concentration fluorescence immunoassay as described by McDonald et al. (1987). Drug concentrations are expressed in ng/ml. All data points are expressed as a percentage of fluorescence in pre-drug samples, which were arbitrarily assigned a value of 100%.

Table II shows that doses of morphine as low as 1 mg/horse, of hydromorphone as low as 2 mg/horse, and oxymorphone as low as 3 mg/horse are easily detectable with PCFIA using this antibody. The inhibition appears within 30 minutes or less, and was still present in all horses tested at 2 or more hours after drug administration. In each case the inhibition is greater than 50%, and as such is sufficient to allow easy identification of samples from dosed horses.

The one step ELISA test described here also readily detected morphine or its metabolites in equine urine. As shown in Fig. 5, the ELISA reaction is essentially completely inhibited for the first 24 hours after administration of morphine at a dose rate of 50mg/kg to three horses. By 48 hours after dosing however, the ability of these urine samples to inhibit the ELISA

In other experiments on the ability of this test to detect morphine congeners in equine urine, we took urine samples provided by the Quality Assurance Program of the National Association of State Racing Commissioners and determined the ability of this test to detect opiates. As shown in Table III the test readily indicated the presence of an opiate in the urines of horses dosed with codeine, hydromorphone, levorphanol and oxymorphone.

Table III. Cross-reactivity of morphine ELISA with morphine congeners in dosed horse urine samples.

Drug administered	Dose	Route	Time Post-dose	ELISA value
Codeine	30mg	IV	2 hr	0.193
Hydromorphone	10mg	IM	2 hr	0.211
Levorphanol	10mg	IV	2 hr	0.226
Oxymorphone	5mg	IM	2 hr	0.262
Post-Race Urines ($\bar{X}$+SEM), n=28				0.562 ± 0.015

The right hand column shows the ELISA readings (measured after color development) of individual urine samples from horses dosed with the indicated drugs as part of the National Association of State Racing Commissioners Quality Assurance Program. All horses were dosed at Cornell University, Ithaca, NY, and the samples were shipped to Industrial Laboratories, Denver, CO, for analysis. All samples were stored at -40°C between dosing and analysis. The control value is the mean of the values obtained in the simultaneous analysis of post-race urine samples in which no narcotic analgesics were detected.

This ELISA test for morphine proved to be particularly effective in field trials. The results of an analysis of a day's complement of post-race urine samples from a track are presented in Fig. 6. As shown in this figure, the samples tested fell into two groups. Most of the samples gave ELISA readings that were greater than the morphine standard, and the color reaction had gone to completion within ten minutes of starting the enzymatic

Table II. Detection of morphine and its analogs in equine plasma by PCFIA.

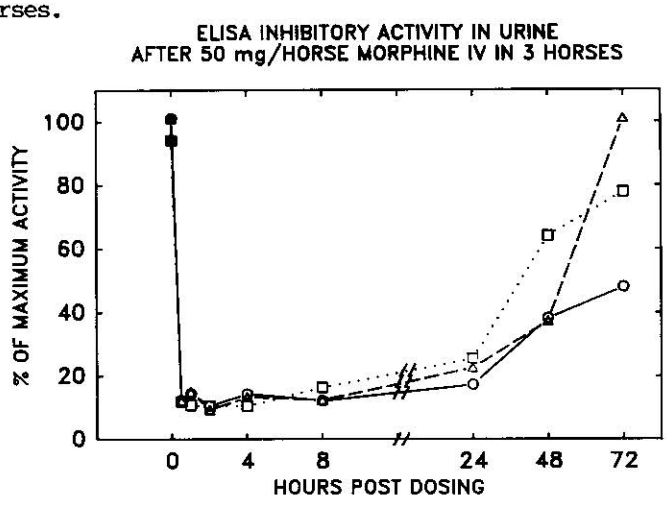
Drug Dose/ Horse	Morphine 10 mg	Morphine 1 mg	Oxymorphone 3 mg	Hydromorphone 8 mg	Hydromorphone 2 mg
CONTROL	100	100	100	100	100
15 min.	-	-	41.7	43.6	41.2
30 min.	20.2	36.7	45.0	33.2	33.6
1 hr.	-	-	41.0	28.1	33.3
2 hrs.	14.6	41.8	37.8	32.0	40.0
4 hrs.	-	-	42.0	-	-
6 hrs.	17.2	43.0	-	-	-

The columns show inhibition of morphine sensitive fluorescence in horse plasma after administration of the indicated doses of morphine or its congeners to horses. All data points are expressed as a percentage of fluorescence in pre-drug samples, which were arbitrarily assigned a value of 100%. The left hand column shows the time post-dosing at which the samples were taken.

reaction was declining and at 72 hours after dosing the ELISA readings had returned to control in one horse, and was only about 50% inhibited in the other two.

Figure 5. ELISA inhibitors activity in horse urine after 50mg/horse morphine IV.

The symbols show ELISA inhibitory activity in the urine of three test horses before (●,■,▲) and after (○,□,△) the injection of 50mg of morphine into these horses.



portion of the assay. For two samples in the assay, however, the ELISA reaction went slowly. One of these was a test urine sample from a morphine dosed horse and another was a track sample. This sample behaved like a typical drug containing sample in that little or no color developed and the sample therefore remained virtually colorless over the entire test period. In addition, because of the easily detectable blue color of the negative ELISA tests, this sample was readily distinguished by eye from the other samples. Mass spectrometric analysis of this sample showed the presence of oxymorphone in this sample (Fig. 7).

Figure 6. One step ELISA reactions in a series of post-race urine samples.

The open triangles (Δ-Δ) show the activity in this one step ELISA test of post-race urine samples. The open squares (□-□) show the effect of 0.5mg/ml of morphine added to this system. The open diamonds (◇-◇) show ELISA activity in a dosed horse urine from Fig. 5, and the solid circles (●-●) show ELISA activity in a sample subsequently determined to contain oxymorphone.

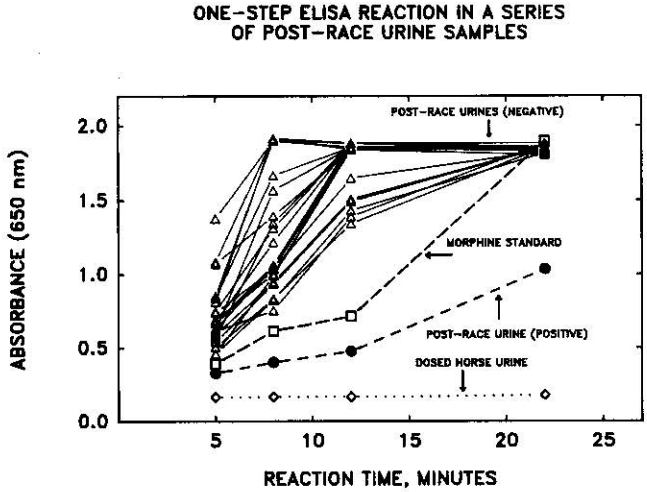
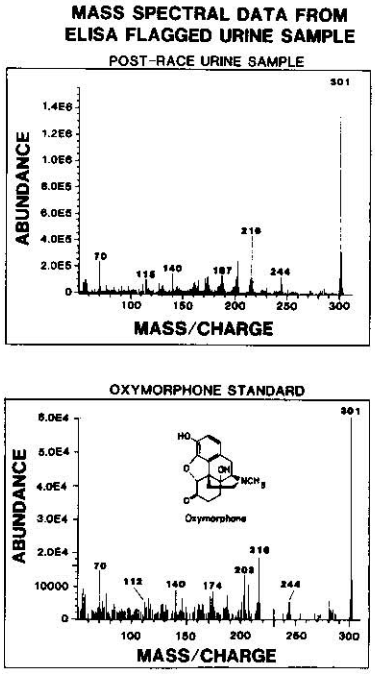


Figure 7. Mass spectrum of material identified in the "positive" sample from Fig. 6.

The lower panel shows the mass spectrum of an oxymorphone standard without derivatization, while the upper panel shows the mass spectrum of a material recovered from the "positive" sample of Fig. 6.



A summary of the results obtained on preliminary introduction of this test into post-race urine analysis is presented in Table IV. The test was applied to nine days of post-race samples from tracks in two southwestern racing jurisdictions. From a total of 162 samples 18 were flagged positive for opiates and with few exceptions all of these samples were confirmed positive for oxymorphone by mass spectral analysis. For some of these samples insufficient urine was available for complete mass spectral analysis. For this reason the confirmation rate of 72% is likely an underestimate of the true number of opiate positives in this population of post-race urines.

Table IV. ELISA screening of post-race urine samples followed by mass spectral analysis.

Sample Date	# Urine Samples	# Flagged by ELISA	# Positive by GC/MS Analysis	Drug Identified
10-3,4-87	34	5	3	Oxymorphone
10-4-87	16	1	1	Oxymorphone
10-11-87	8	1	1	Oxymorphone
10-17-87	36	3	2	Oxymorphone
10-17,18-87	27	3	1	Oxymorphone
10-20-87	21	4	4	Oxymorphone
10-27-87	24	1	1	Hydromorphone
TOTALS/ 9 Days Racing	166	18	13	

Post-race urine samples from two racing jurisdictions were screened for morphine and its analogs by the ELISA test and then subjected to gas chromatography/mass spectroscopy (GC/MS). The dates on which the samples were collected, the number of samples in each analysis batch, and the number of samples flagged "suspicious" by ELISA are presented in the first three columns. The results of GC/MS analysis of the flagged samples are shown in columns four and five. About 72% of the ELISA positives were determined by GC/MS to contain either oxymorphone or hydromorphone. For some of the unconfirmed ELISA positives, insufficient sample was available for complete GC/MS evaluation of their opiate status.

DISCUSSION

Pre-race testing is conceptually the most desirable form of testing. An effective system would allow horses to be tested for drugs pre-race, and those horses that contain illegal medications would be "scratched" or eliminated from the race. Given analytical methodology that can do this, pre-race testing is a very desirable testing strategy, since it prevents any effect of medication on the outcome of the race. Currently, medication testing in racing horses in most jurisdictions is done post-race and the results of the test are generally not available for at least 24 or more hours post-race. The betting payoff, which occurs within minutes of the end

of a race is therefore susceptible to manipulation by the use of illegal medication.

Because of these considerations, a number of jurisdictions have introduced pre-race testing in various forms. Currently, the major practitioner of pre-race testing is the state of New York. All horses racing in New York have blood samples drawn from them within about five hours before racing. These samples are then tested in a trackside pre-race testing laboratory for the presence of illegal medications. If a horse is found to contain illegal medication extra samples are drawn for further analysis and the horse in question is scratched from the race.

The problem with pre-race testing in this form is that it detects only acidic drugs that are found in the plasma in high concentrations. These drugs are, in general, legal medications if present in low concentrations in racing horses, and their detection pre-race is not viewed as a high priority. This is particularly so in view of the cost of pre-race testing, which can amount to several million dollars annually. While effective for acidic drugs, the thin layer chromatography systems now in force have to our knowledge yet to detect a illegal basic medication, such as morphine, in pre-race testing.

The inability to detect basic drugs is a serious deficiency in a pre-race testing system. This is because basic drugs are the medications with the greatest ability to affect the performance of a horse. Basic drugs include the narcotic analgesics, which have been used in racing horses for at least 100 years, and most stimulants, depressants, local anaesthetics, and tranqualizers. Many of these drugs are active in very low concentrations, and the doses are commonly in the area of 10 mg/horse or less, which makes their detection in post-race urine challenging.

Obviously, therefore, their detection in pre-race blood is an even more substantial challenge, and one that thin layer chromatography, in its current configuration, is not likely to attain.

In an alternative approach to pre-race testing we have chosen to utilize immunoassay as our testing mode. Because of the speed requirement, we have limited ourselves to simple, non-radiolabel immunoassays. Among the possible testing modes, we have chosen two, PCFIA and one step ELISA. Work on the utilization of PCFIA in post-race testing has been reported previously (McDonald et al., 1987; Yang et al., 1987). In this communication we examine the role of a simple one step ELISA test in pre- and post-race testing for morphine and related narcotic analgesics.

The test reported here is very rapid and sensitive. To complete a panel of about ten tests, the average number of horses in a race, takes about thirty minutes when this one step ELISA is used. As shown in Fig. 2, the test takes about 15 minutes to set up, and about another 15 minutes to read. Based on these times, the ELISA test is clearly suitable for pre-race testing, where the rapid completion of the analysis is critical.

The speed with which this test determines the opiate status of a sample is very useful in post-race testing. Since this test detects opiates without the necessity of a hydrolysis step, samples likely to contain opiates can be identified within thirty minutes of receipt of the samples in the laboratory. These samples can then be immediately subjected to further appropriate analysis for opiates at a substantial saving in time and effort over the old procedures.

As well as being rapid, this test is also very sensitive to morphine. As shown in Fig. 3, the test is able to detect morphine with a sensitivity of about 400 pg/ml, which is considerably more sensitive than thin layer

chromatographic technology. While this improved sensitivity is not critical in post-race testing, where the concentrations of drug in the system tend to be higher, these differences is critical in pre-race testing, where the concentrations of drug in the blood are low. For morphine itself, sensitivity is clearly more than adequate, since it appears that about 50 mg of morphine have to be administered to an adult horse to produce a pharmacological effect. However, if a more potent narcotic analgesic is being used whose effective dose will be less than that of morphine, and which may not react with this anti-morphine antibody as readily as morphine, then the sensitivity of the test becomes very important.

This test will also detect narcotic analgesics other than morphine. As shown in Tables I and II, PFCIA will detect doses of morphine of as little as 1 mg/horse, and doses of hydromorphone and oxymorphone of approximately similar size. Similarly, there are suggestions that the test may also be able to detect nalorphine and codeine administration. These broad spectrum characteristics of the test are very useful, since none of these drugs has ever been detected in a pre-race test.

This test is similarly effective in post-race urine testing. Because of the concentrating power of the kidney and the fact that most of these drugs are excreted as glucuronide conjugates, these drugs are much easier to detect in urine than in blood. As shown in Fig. 6 morphine was detectable in post-race urine for at least 48 hours and likely longer. This sensitivity raises the possibility that in practice these drugs will detect a relatively wide range of morphine congeners and contribute to control of these agents.

This possibility is further supported by the data of Table III, which shows that the test is capable of detecting administration of codeine,

oxymorphone, hydromorphone and levorphanol. Given this range of sensitivity, this simple one step ELISA assay is likely to be very effective as a post-race test for illegal medication in racing horses.

This likelihood has been borne out in our initial experience with this assay in practice, since this test detected use of oxymorphone and hydromorphone in racehorses in two southwestern racing jurisdictions. On its initial introduction into routine testing this test flagged 18 of 166 post-race urines as possible opiate positives, and 13 of these were confirmed positive for opiates by mass spectral analysis. The test appears to be particularly effective and has contributed substantially to equine medication control in racing horses. Similar assays for fentanyl and other drugs used illegally in racing horses are currently being developed by our research programs and are being incorporated into equine drug testing programs.

In summary, therefore, we have developed a simple one step ELISA for morphine that readily detects morphine in blood or urine after its administration at therapeutic and sub-therapeutic doses. The test also detects administration of small doses of codeine, oxymorphone, hydromorphone and levorphanol in blood or urine after their intravenous administration to horses. Utilization of this test in pre-race and post-race testing programs would appear to offer swift and sensitive testing for morphine and a significant number of its congeners at very reasonable cost.

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