

# Morphine and Etorphine: XIV. Detection by ELISA in Equine Urine\*

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## Abstract

We have raised antibodies to morphine and etorphine and developed one-step enzyme-linked immunosorbent assays (ELISA) for these drugs as part of a panel of post race tests for drugs in racing horses. These tests are simple, can be completed in 2 h, and can be read by visual inspection. The morphine ELISA has an  $I_{50}$  for morphine of about 1.5 ng/mL, while the etorphine ELISA has an  $I_{50}$  for etorphine of 250 pg/mL. Cross-reactivity studies show that the antimorphine antibody cross-reacts well with levorphanol, hydromorphone, and oxycodone, while the anti-etorphine antibody showed no cross-reactivity with buprenorphine, diprenorphine, oxymorphone, morphine, or thebaine. The morphine test readily detected parent morphine or its metabolites in equine urine for at least 8 h after administration of 50 mg/horse, while a 0.1  $\mu$ g/kg dose of etorphine was detectable for up to 48 h post dosing. For each test the background activity in post-race urines was equal to or less than the  $I_{50}$  for the standard curves, making them useful equine forensic tests. Each of these tests has detected "positives" in post race urine samples and as such these tests are capable of substantially improving the speed and efficacy of both pre-race and post-race testing for morphine, etorphine, and their congeners in racing horses.

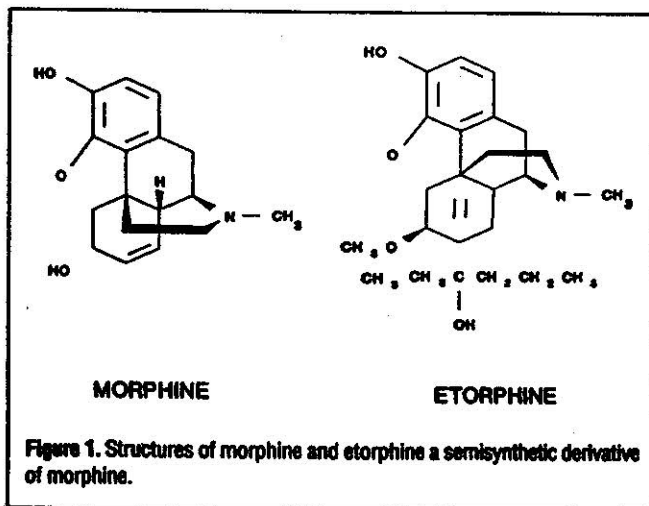
## Introduction

Morphine (7,8-didehydro-4,5-epoxy-17-methyl-morphinan-3,6-diol), Figure 1, is obtained from opium, derived from the milky exudate of the incised unripe seed capsules of the poppy plant, *Papaver somniferum*. Opiate alkaloids are divided up into two main chemical classes, phenanthrenes and benzylisoquinolines. The principal phenanthrenes are morphine (10% of opium), codeine (0.5%), and thebaine (0.2%)(1).

Morphine is a classic mu receptor agonist and it may be used in the horse as analgesic for the relief of pain (particularly to relieve the acute pain of spasmodic colic) or as preanesthetic medication. The analgesic dose of morphine for the horse is considered to be about 0.12 mg/kg (2).

Etorphine (4,5-epoxy-3-hydroxy-6-methoxy- $\alpha$ ,17-dimethyl- $\alpha$ -propyl-6,14-ethenomorphinan-7-methanol), Figure 1, is a semisynthetic opiate derivative. It is synthesized from thebaine, a pharmacologically inactive opium alkaloid, and is structurally related to morphine. Thebaine differs from morphine only in that both OH groups are methylated and that there are two double bonds in the ring ( $\Delta 6,7$  and  $\Delta 8,14$ ). Etorphine is administered as the hydrochloride salt (M99<sup>®</sup>, oripavine), commonly in combination with a phenothiazine tranquilizer (Immobilin<sup>®</sup>-Large Animal Immobilin<sup>®</sup>; 2.45 mg etorphine HCl/mL with acepromazine; Small Animal Immobilin<sup>®</sup>; 0.074 mg etorphine HCl/mL with methotrimeprazine) (3). Because etorphine has been widely used in the capture of wild animals (such as elephants), it is sometimes referred to as "elephant juice". Under the name "elephant juice" it is widely known in racing circles as a potent and difficult to detect analgesic and stimulant.

Etorphine is a typical opiate narcotic analgesic characterized by extremely high potency (at least 10,000 times that of morphine), rapid onset, and short duration of action. The pharma-



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cological actions of etorphine in humans are similar to those of morphine, including the subjective effects and euphoria, but show rapid onset and short duration (4). In the horse, etorphine in combination with acepromazine results in analgesia and sedation. Pharmacological responses to etorphine in the horse include tachycardia, depression of respiration rate, spastic rigidity of the limbs and muscular tremors (5). In the horse, etorphine produces the classic locomotor stimulant response of the narcotic analgesics (6).

Because morphine and etorphine have analgesic and stimulant effects in racing horses (6,7) they have long been used illegally in racing. To control abuse of this group of drugs we are developing a panel of ELISA-based immunoassay tests for drugs in racing horses. In this communication we report on the development and application of such ELISA tests for morphine and etorphine.

## Materials and Methods

### Horses

Thoroughbred or standardbred mares (450–500 kg) were administered etorphine or morphine in the *in vivo* studies. The horses were maintained at pasture until approximately 24 h before dosing. At that time, they were placed in box stalls (17 m<sup>2</sup>) and allowed free access to hay and water. All control samples were obtained on the day of the dosings.

### Dosing and sampling

Injectable etorphine hydrochloride (M99, oripavine, D-M Pharmaceuticals, Inc.) and morphine sulfate injection (Eli Lilly Co.) were used. All drugs were administered by rapid IV bolus injection into the jugular vein. All urine samples were collected by bladder catheterization and stored frozen until assayed. Blood samples were collected in serum vacuum tubes (Becton-Dickinson). The blood samples were centrifuged and the serum fraction aliquoted and frozen at –20°C until assayed.

### ELISA test method

The ELISA tests (WTT ELISA Tests) developed here are similar to the ELISA formats used in published reports by

Kwiatkowski et al. (9) and Tobin et al. (10). The anti-morphine antibody or anti-etorphine antibody was coated to flat bottom wells (Costar®) as described by Voller (11). Morphine and etorphine were linked to horse radish peroxidase (HRP) to give rise to a covalently linked drug-HRP complex by the method described by Rowley et al. (12). All assays were performed at room temperature. The assay was started by adding 20 µL of the standard, test, or control samples to each well, along with 180 µL of the drug-HRP solution. During the test, the presence of drug in the sample competitively prevents the binding of drug-HRP complex to the antibody. Because the HRP enzyme is responsible for the color-producing reaction in the ELISA, the concentration of drug in the sample is inversely related to the optical density of the test well. The optical density (OD<sub>650</sub>) of the test wells was read at a wavelength of 650 nm with an automated microplate reader (Biotek Instruments) approximately 60 min after addition of substrate.

Backgrounds due to extraneous material in horse urine are a considerable problem when ELISA testing is applied to equine drug testing. To measure the extent of this problem for individual tests they are run against 35–40 randomly selected post-race horse urines and the inhibitory effect of horse urine evaluated. The inhibitory effect is expressed in terms of parent drug equivalents estimated from the standard curve for parent drug. This method allows direct comparison of the sensitivity of the test with apparent drug levels due to background.

### Mass spectral confirmation of morphine

Gas chromatography/mass spectrometry (GC/MS) was performed on a Hewlett-Packard GC Model 5890 equipped with a Hewlett-Packard MS-5870 detector and a 12-m HP-1 methyl-silicone capillary column (Hewlett-Packard). The samples were injected directly into the detector in the non-split mode. GC oven temperature was programmed from 70 to 280°C at 35°C/min. Urine samples were subjected to enzymatic hydrolysis as previously described (13). Urine samples (50 mL) were incubated (63°C, 3 h) with beta-glucuronidase from *Patella vulgata* (Sigma Chemical) (5,000 units/5 mL 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

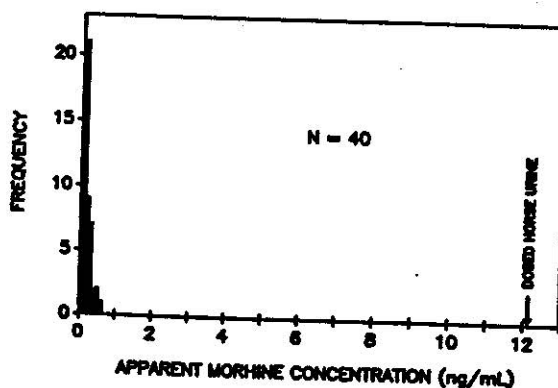


Figure 2. ELISA test activity for the antimorphine antibody as a function of added drug is shown. Half-maximal inhibition occurs at around 1.5 ng/mL for morphine. Hydromorphone, levorphanol, and oxycodone also showed cross-reactivity to the antimorphine antibody.

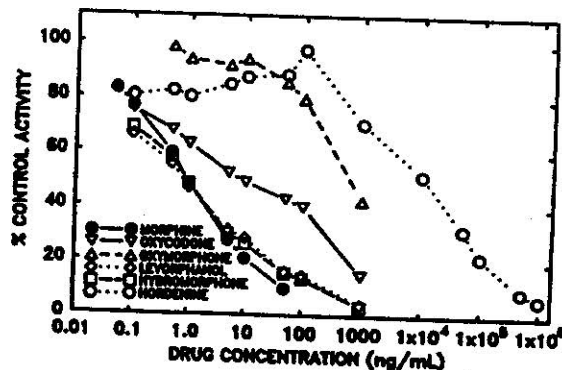
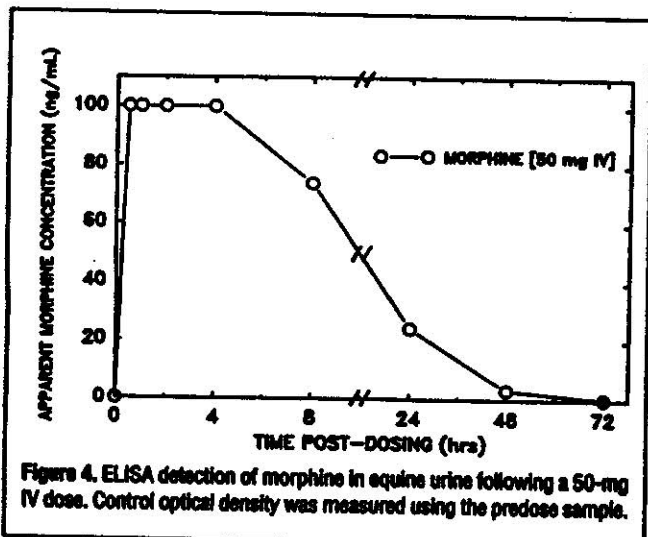


Figure 3. Distribution of "apparent" morphine values for 33 post-race urine samples.

(6 mL solvent/5 mL urine). The samples were then evaporated to dryness, redissolved in methylacetate, and purified by preparative thin-layer chromatography (E Merck F-254 silica gel-60 plates, Alltech-Applied Science). 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 0.6 ammonium hydroxide (pH 9.5) and extracted into 6 mL DCM. The samples were evaporated to dryness and 30  $\mu$ L ethylacetate was added to each tube. After thorough mixing, 2  $\mu$ L of the sample was injected on the GC/MS.

#### Mass spectral confirmation of etorphine

The presence of etorphine<sup>1</sup> was assayed by the ELISA and confirmed by GC/MS. A 0.10-g solution of beta-glucuronidase was added to a 50-mL sample of urine (adjusted to pH 4.0-4.2 with acetic acid). The urine was incubated overnight at 37°C. Following the incubation period, an additional 0.050 g of the beta-glucuronidase enzyme solution was added and the urine incubated at 65°C for 3 h. The solution was then cooled, made basic (pH 9.0) with ammonia + ammonium chloride buffer, and extracted in 7- to 9-mL portions with approximately 60 mL DCM in a 125-mL separatory funnel. The DCM extract was dried with a small amount of sodium sulfate. The DCM portion was extracted with two portions of 1% sulfuric acid (one of 4 mL and the other of 2 mL). The acid extracts were combined and made basic (pH 9.0) with the ammonium chloride + ammonia buffer. The aqueous solution was then extracted with 3  $\times$  12-mL portions of petroleum ether. The petroleum ether extracts were combined and evaporated just to dryness at 65°C. The resulting residue was prepared for GC/MS analysis for the formation of the TMS derivative by a method proposed by Jindal and coworkers (14). Briefly, the residue was taken in a Reacti-Vial, to this 50  $\mu$ L of pyridine and 150  $\mu$ L of BSTFA were added. The sample was heated at 120°C for 2 h. Following this the reagents were evaporated with N<sub>2</sub> gas; the residue was reconstituted in 5  $\mu$ L of benzene and a suitable aliquot injected into the GC/MS. The samples were analyzed on an HP 5890 GC equipped with an HP-1 methyl-silicone capillary column and a Model 5970 MSD (Hewlett-Packard).

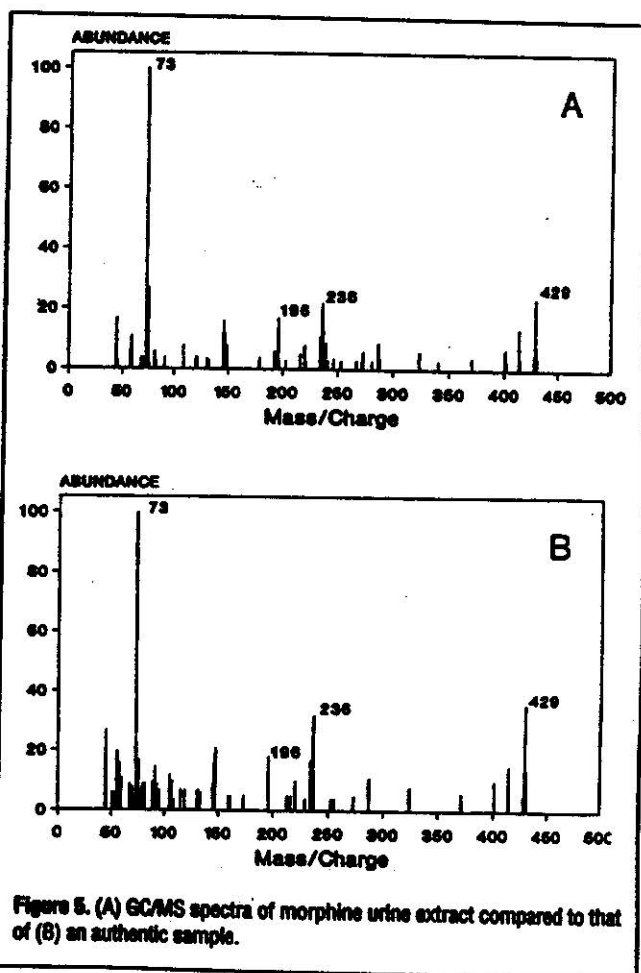


## Results and Discussion

A critical problem in the development of ELISA tests for use in racing horses is the background levels seen in equine urines. Horse urine contains large quantities of proteins, mucus, phosphates, sulfates, and other poorly identified materials at least one of which can produce substantial background levels in ELISA tests on equine urine. These spontaneous urine background levels vary between ELISA tests and can cause serious problems in the application of ELISA testing in racing horses. As shown in Figure 2, the background levels of apparent morphine equivalents seen in 40 equine urine samples were all less than the I<sub>50</sub> value for morphine in this assay. Therefore, this test can be used in the large-scale screening of horse urine samples with reasonable assurance that substantial inhibition (>75%) of this ELISA test is likely to be caused by an opiate or closely related drug ("true positive") and not by extraneous background materials (false positive).

The data of Figure 3 shows the sensitivity of the morphine ELISA test to morphine and its cross-reactivity with other opiates. Morphine inhibited this ELISA test with an I<sub>50</sub> of about 1.5 ng/mL and this test also cross-reacted very well with hydromorphone and levorphanol. This broad spectrum reactivity and the relatively good reaction with oxycodone suggests that this test is likely to be useful in routine screening for opiates in racing horses.

<sup>1</sup>The material assayed by ELISA and confirmed by GC/MS consisted of syringes and needles, which were confiscated in a backstreet search.



This test readily detected morphine or its metabolites in horse urine. As shown in Figure 4, administration of 50 mg of morphine to a 1000-lb. horse resulted in significant inhibition of this ELISA test for about 24 h. Because 50 mg of morphine per horse is a subtherapeutic dose of morphine it appears likely that this ELISA test will readily detect abuse of morphine or closely related opiates in racing horses.

On introduction into routine screening of post-race urine samples in Canada, California, and Kentucky, this test readily detected opiate drug positives, with a low incidence, estimated at one in 200 or less, of false positives. Figure 5A shows the mass spectrum of a morphine positive from one of these jurisdictions along with a control mass spectrum (Figure 5B). This test is currently in use in routine post-race testing in the United States with satisfactory forensic results as outlined above.

The sensitivity and cross-reactivity of our etorphine ELISA to parent etorphine and related drugs is presented in Figure 6. Based on an "early bleed" of this antibody the ELISA test showed an  $I_{50}$  for etorphine of about 250 picograms/mL and little cross-reactivity with a variety of closely related narcotic analgesics. Therefore, this ELISA test appears to be highly sensitive to and relatively specific for etorphine.

Because of the high potency of etorphine and the correspondingly small doses (100  $\mu$ g/horse) administered to horses, it was

important that this test be both sensitive to etorphine and have low background values in horse urine. As shown in Figure 7 the background values observed in horse urine are very low, running at less than 250 pg/mL in 40 post-race urine samples. Based on this high sensitivity to etorphine and the low background values seen in equine urine samples, it appears likely that this ELISA would be a very effective test for etorphine in post-race urine samples. In good agreement with this information are the data of Figure 8, which show that this etorphine ELISA test readily detected etorphine administration to horses at doses of 100  $\mu$ g/horse even though these etorphine samples had been stored frozen for several years. Similarly, recent work at Ohio State University with this ELISA test has demonstrated that it can detect administration of 50  $\mu$ g of etorphine/horse for up to 48 hours in fresh unfrozen samples (7).

This ELISA test for etorphine has been made available for use in racing jurisdictions in the United States and Canada, and has performed well with a very low incidence of false positives. One of the most potent narcotic analgesics commercially available, etorphine is strictly regulated and its availability is restricted. Positives for etorphine are rare but sporadic outbursts of etorphine positives have been reported (7). Shown in Figure 9A is the mass spectrum of etorphine obtained from an etorphine-containing syringe discovered in a barn search. This syringe was screened for etorphine using this ELISA test. Figure 9B shows a control mass spectrum from an etorphine reference sample.

More recently, the Illinois Racing Laboratory reported the results of screening about 2,000 post-race urines with this ELISA. In the 2,000 samples screened, this ELISA flagged just two samples positive, and one of these was confirmed positive for etorphine by mass spectrometry.

Narcotic analgesics that have mu agonist activity modify three very useful actions in racing horses (8). Firstly, these drugs are directly stimulant to horses, producing a clear-cut and easily quantitated locomotor stimulation (8). Secondly, these drugs are analgesic, suppressing the perception of pain by the horse, and thus enabling a superior performance by a less-than-sound horse (6). Finally, these drugs appear to have the ability to extend the time to exhaustion of horses, apparently by reducing the performance-limiting effects of blood lactate (15). All of these pharmacological effects are particularly useful in racing horses and likely account for the fact that narcotic analgesics have been abused in racing horses for more than 100 years (6).

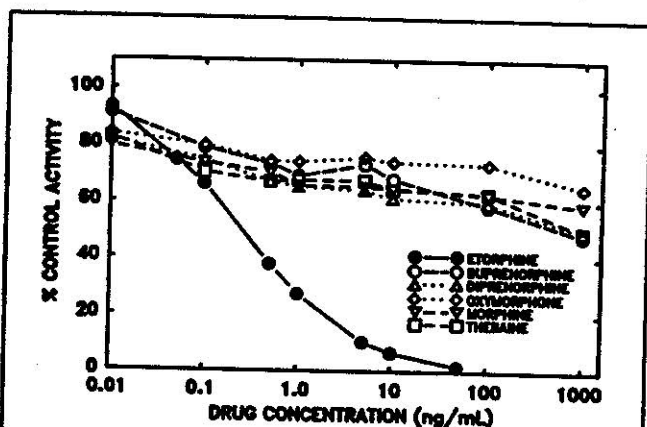


Figure 6. ELISA test activity for the anti-etorphine antibody as a function of added drug is shown. Half-maximal inhibition occurs at around 250 pg/mL for etorphine. None of the other drugs tested show significant cross-reactivity.

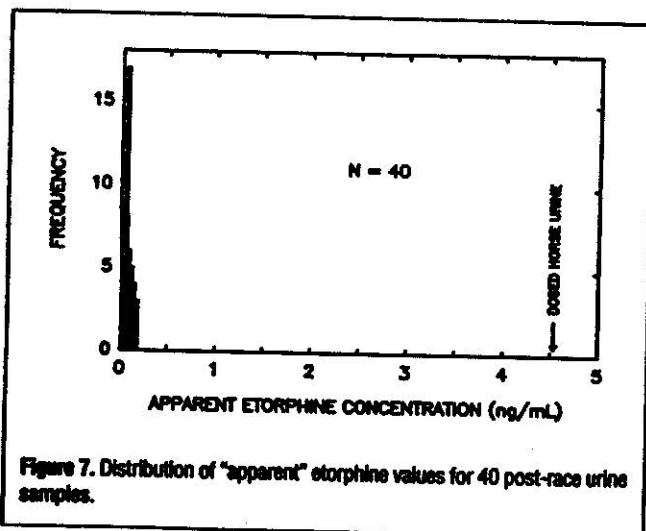


Figure 7. Distribution of "apparent" etorphine values for 40 post-race urine samples.

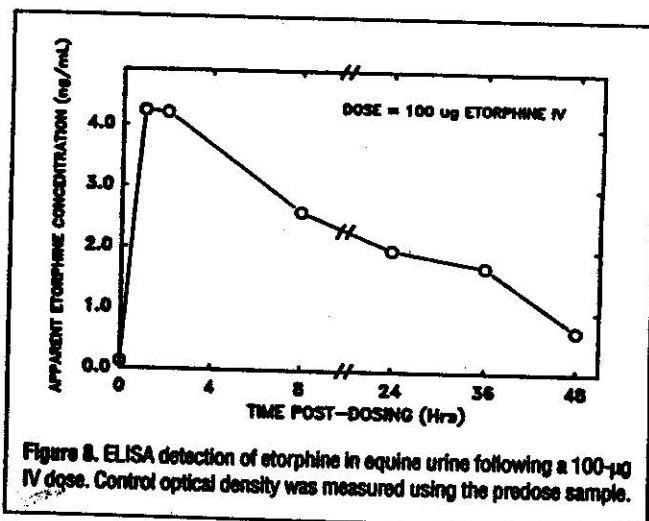


Figure 8. ELISA detection of etorphine in equine urine following a 100- $\mu$ g IV dose. Control optical density was measured using the predose sample.



For the greater part of this 100-year period chemists have been attempting to control the use of these drugs. More recently, however, with the development of highly potent opiate and opiate-like drugs, the advantage has appeared to lie with those willing to abuse these drugs in racing horses. Drugs such as etorphine, fentanyl, and carfentanil are effective at doses of much less than 5 mg/horse, which are very difficult to detect by traditional thin-layer chromatographic (TLC) techniques (8). For this reason, we have developed radioimmunoassay (RIA) and more recently, ELISA tests to detect abuse of these drugs in racing horses. ELISA tests offer considerable advantages over RIA and other immunoassay testing techniques for drugs in racing horses.

With a test time of less than two hours, the ELISA method is inexpensive and rapid. No elaborate equipment is required for these tests—they can be performed on a tabletop and read by eye. Additionally, these tests are a clear improvement over RIA because no isotope is required, and they are markedly superior to TLC, which is generally unable to detect high potency drugs at the doses typically administered to racing horses. These points were dramatically borne out when ELISA testing was first introduced into racetrack testing several years ago, when multiple patterns of positives for high potency drugs were uncovered (10).

Full utilization of the scope of ELISA testing in racing horses requires that a complete and readily extended panel of equine drug tests be available. To this end, the Kentucky Equine Drug Testing and Research programs have made a commitment to the

development of a panel of ELISA tests for high potency drugs in racing horses. We have selected the ELISA format over other nonisotopic formats because of its proven efficacy and its minimal instrumental requirements. This technology is readily adaptable to post-race testing and is also adaptable to pre-race testing and to testing in non-laboratory surroundings such as at horse show grounds and other event sites.

The present data shows that these tests are sufficiently sensitive to detect drug or drug metabolite residues in post-race urines of horses dosed with etorphine, the most potent narcotic analgesic tested for in racing horses. Because etorphine was detectable for about 48 h after a 100- $\mu$ g/horse dose and for a similar period after a 50- $\mu$ g/horse dose in experiments carried out at Ohio State University, it appears that, in principle, at least, this technology is sufficiently sensitive to detect most high potency drugs administered to racing horses. This holds greater validity in light of the fact that these samples were tested as 20  $\mu$ L of raw urine added directly to the ELISA test system. Because immunoassay samples can presumably be extracted and concentrated in the same way as is now done in thin-layer chromatographic analysis, the sensitivity of ELISA-based testing can theoretically be increased several hundred fold to render testing extremely sensitive indeed. It seems reasonable therefore to conclude that immunoassay-based testing is likely to be sufficiently sensitive to detect virtually any known drug in horse urine, although the ability of these tests to detect very low levels of drug in equine blood in pre-race testing situations remains to be explored.

When used on raw urine, ELISA tests are very fast and effective tests. If the incidence of positives is high, as it may well be when a new test is first introduced, the use of ELISA tests can be very dramatic, with large numbers of positives being detected and sent for mass spectral confirmation. However, if the incidence of positives is low or nonexistent then the bulk of what the ELISA test reports is the background activity or "false positive" activity in horse urine.

With the more widespread use of ELISA testing it has become apparent that there are materials in horse urine that give rise to high background or false positives in horse urine levels and these substances are found in high levels in certain urines. It further appears that some ELISA tests are very sensitive to these materials while others are quite resistant. The quality of an ELISA test for use in equine drug testing depends to a large extent on the  $I_{50}$  of the test for the drugs of interest, and the apparent natural background levels in raw urine, which should be less than the apparent  $I_{50}$  of the test for the drug against which the ELISA was raised.

As shown in Figures 3 and 7, the background levels in raw urine for these ELISA tests are each less than the  $I_{50}$  for the drug against which the antibody was raised. For the morphine ELISA, analysis of the background levels in 40 post-race urines shows that no background levels greater than 1.2 ng/mL were observed, while the  $I_{50}$  for morphine in this test was about 1.5 ng/mL. As a practical matter, this means that inhibition of this ELISA is more likely caused by an opiate positive than to extraneous background in equine urine, suggesting that the morphine test is likely to be a useful and practical field test.

The etorphine ELISA turned out to be an extremely sensitive and effective ELISA test. As shown in Figure 6, this test has an  $I_{50}$  for etorphine of about 250 picograms/mL, an extremely sensitive ELISA test. The test is relatively specific for etorphine, also a useful characteristic. Most important, however, is the fact

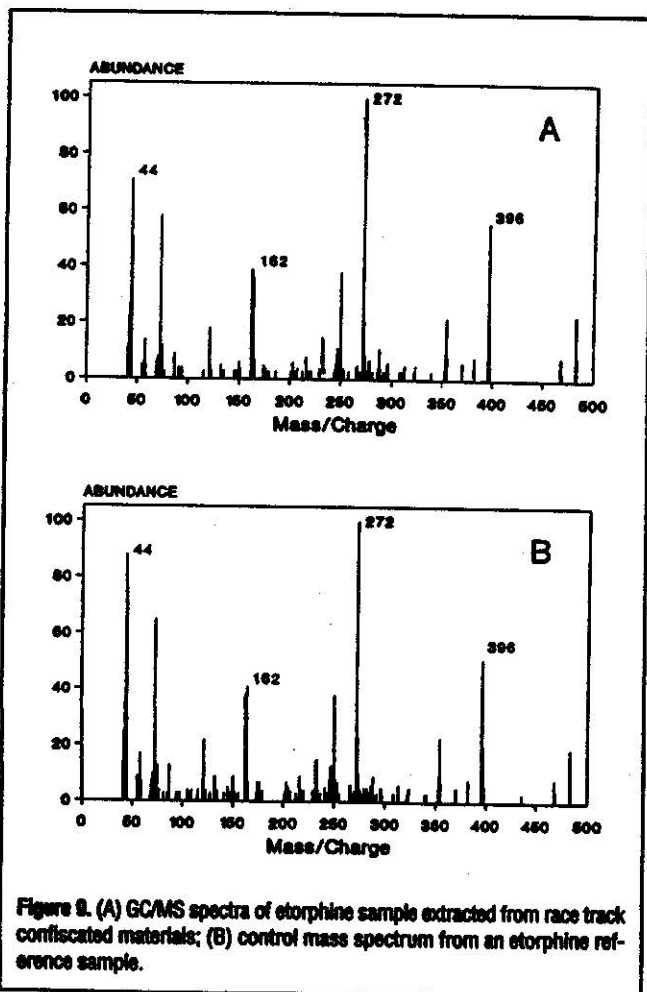


Figure 8. (A) GC/MS spectra of etorphine sample extracted from race track confiscated materials; (B) control mass spectrum from an etorphine reference sample.

that this test is relatively insensitive to the endogenous background material in horse urine. As shown in Figure 7, the backgrounds seen with this assay are all less than 250 picograms/mL, which means they are virtually undetectable to the eye. This ELISA test is a rare example of an ELISA test that has essentially no background levels in equine urine.

This test readily detects etorphine or its major metabolites in equine urine after administration of small doses of this drug to horses. As shown in Figure 8, administration of a dose of 100 µg/horse gave rise to detectable inhibition of the etorphine ELISA for up to 48 h after administration of this drug.

Because the pharmacological actions of etorphine are unlikely to last more than about five hours after its administration, it appears clear that this test is sufficiently sensitive to control the use of this very potent drug in racing horses. This conclusion has been borne out by recent results reported from Illinois, where 2,000 screened samples yielded one confirmed positive and one apparent false positive.

The speed and ease of use of these tests is a major advantage with regard to their forensic effectiveness. For example, with a radioimmunoassay for etorphine it takes a laboratory technician one day to analyze one day's quota of samples in a lab handling about 30 samples per day or 10,000 samples per year. In addition to the expense of labor there is the expense of scintillation counters and scintillation cocktails, and the problem of disposition of radioactive wastes. In contrast, approximately three to four ELISA tests can be carried out in the same time and with an equivalent amount of labor as is required for one RIA. In general, therefore, it appears that ELISA tests are forensically and economically superior to RIA assays and this is evidenced by the popularity and effectiveness of ELISA testing for illegal drugs in racing horses.

In summary, we have developed and deployed ELISA tests for morphine and etorphine in racing horses. These tests are sufficiently sensitive, economical, and free of background problems to be very effective forensic tests for use in racing horses. The morphine test detects morphine, hydromorphone, and levorphanol with an  $I_{50}$  of about 1.5 ng/mL. It is currently in widespread use in North American racing jurisdictions and has given rise to the calling of several opiate positives in racing horses. The etorphine ELISA has an  $I_{50}$  for etorphine of 250 picograms/mL and equivalently low background values. The etorphine ELISA is unique in that no comparable nonisotopic immunoassay for this drug is available. It is also currently in use in several racing jurisdictions in North America and has recently yielded a "true positive" for etorphine in a post-race sample.

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