Guest Viewpoint: Dr. Thomas Tobin discusses use of an ELISA test to detect specific drugs

ENZYME LINKED IMMUNOASSAY DRUG TESTING

BY THOMAS TOBIN

At a recent horsemen's conference in Lexington, I received an unusual thank you, from an Oklahoma horseman for moving him up from second to leading horseman at a recent meet in Oklahoma. I had been able to do this because we had assisted the Oklahoma drug testing laboratory by screening some of their samples for Sufentanil. During this work, we detected about 10 Sufentanil positives in the relatively small number of samples that we screened. Then, when the dust had settled on these positives, the trainer had moved up from second to leading trainer at the meet, and he was understandably pleased with the results of our drug testing.

We also were pleased with the results of our drug testing, since the Sufentanil positives were the third series of positives resulting from research by Dr. J. W. Blake and myself at the University of Kentucky. This research has been carried out in close cooperation with John McDonald of the Illinois Racing Board Laboratory, Chuck Prange of International Diagnostic Systems Corp. of St. Joseph, Mich., and Frank Ozog of Industrial Laboratories of Denver, Colo.

For the last year, our groups have been engaged in intensive research and developmental work, work that we believe has begun to change the way equine drug testing will be performed for the foreseeable future.

The change in equine drug testing that we are working on is the development of sensitive, simple, and inexpensive immunoassays for drugs that are abused in racing horses. Immunoassay is the only effective way to control the use of highly potent drugs and narcotics in racing horses. Until recently, however, immunoassay tests have been difficult to develop and expensive to use. This is because these tests have always involved the use of radioactivity, which is inherently difficult and expensive to work with. The technological breakthrough that we are exploiting is the development of immunoassays that do not require radioactivity. This technology is known in the trade as ELISA, an acronym for Enzyme Linked ImmunoSorbent Assay.

This ELISA technology is now widely used in infectious disease work, and, for example, the test used to screen for AIDS is an ELISA test. To make an ELISA for a drug you must first make an antibody to the drug. Then you bind this antibody to a clear plastic well. What you now have is a clear plastic well that can very specifically bind the drug you are going to test for. This well and the antibody bound to it are presented schematically in Fig. 1.

The second step in ELISA testing is to add the test sample to the well. For simplicity, let us assume that the sample you have added does not contain any drug. Since there is nothing of significance in the sample, nothing binds to the antibody. The antibody is therefore still free to bind to a drug, even if the drug is linked to an enzyme.

The third step in the test is to add just such a drug-enzyme complex. If the antibody sites are vacant (a negative sam-

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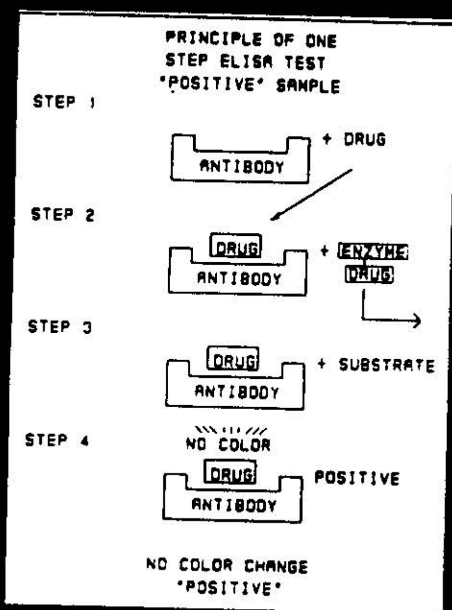

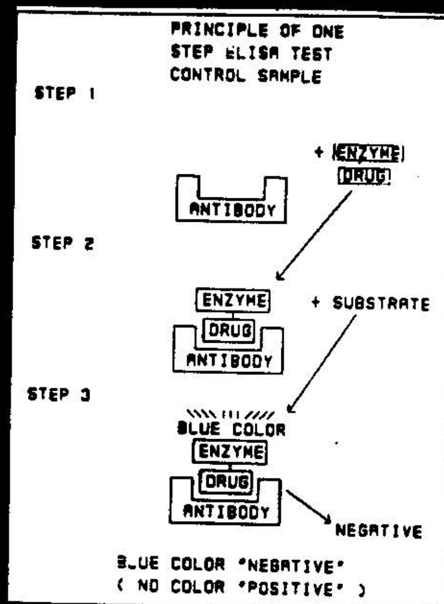

ple) the drug-enzyme complex binds to the sites. You then perform a simple development step, and the presence of the enzyme shows up as blue color. This is a negative test, and a negative test is virtually certainly a true negative.

A positive test (Fig. 2) occurs when there was drug present in the test sample which bound to the antibody before the drug-enzyme complex is added. The drug in the test sample occupies the binding sites and prevents the drug-enzyme complex from binding. Since no enzyme is bound, no color can develop and the sample well remains clear. In the lab, we call these "whiteouts," and they stand out clearly as clear "positives" against a line of blue negative tests (Fig. 3). A positive can be read by eye, or, if necessary, on an inexpensive spectrophotometer (Fig. 4).

If you use a spectrophotometer you can follow the time course of development of the color reaction as shown in Fig. 1. In the negative or control reactions the color develops rapidly, and depending on the particular test, the color reaction can be complete in from five to 15 minutes. By this time the negative samples will have developed an easily visible clear blue color. In contrast, a "strong" positive sample remains clear, and no color change will appear in a strong positive sample over the normal development time of these tests. Samples which contain an intermediate concentration of drug will show a slowly developing blue color, and these concentrations can be used for quantitative purposes. In this way the test can be used as either a screening test to determine the presence or absence of a specific agent, or, alternatively, as a quantitative method to estimate the concentration of a drug in a sample.

As well as being rapid and inexpensive, these tests are simply overwhelmingly more sensitive than the Thin Layer Chromatographic (TLC) tests currently used for routine drug screening by virtually all drug testing programs in the United States. While quantitative comparisons are difficult to make, the data with fentanyl highlights the greatly improved sensitivity of this testing technology. While a Thin Layer Chromatographic method for fentanyl exists, this method is marginally useful, and to this author's knowledge has never given rise to a positive call for fentanyl. Not only can this new technology readily detect fentanyl in urine; it can also detect fentanyl administration in blood, (Fig. 2), thereby giving rise to the possibility of an effective pre-race testing technology based on these rapid immunoassay tests.

These tests are also useful for pre-race testing because they can be done on very small quantities (50 u liters or less than a drop) of plasma in about the same amount of time that it will take to read this article. For post-race testing, they can be done on raw urine, straight out of the horse. In fact, a simple "cup" version of the test can be performed in minutes on a kitchen table and read by eye, at a cost of a dollar or less per test. Fig. 5 shows such a test for fentanyl, known on the street to human abusers as "China White."

These tests have turned out to be remarkably effective when introduced into routine post-race urine testing in horses. As pointed out earlier, their most important characteristic is that they are much more sensitive than the existing Thin Layer Chromatographic (TLC) tests on which equine drug testing is now based. This was made abundantly clear when the first of these tests, a test for buprenorphine, or Buprenex, was introduced into equine drug testing.

Buprenorphine is a narcotic analgesic which is marketed for use in human

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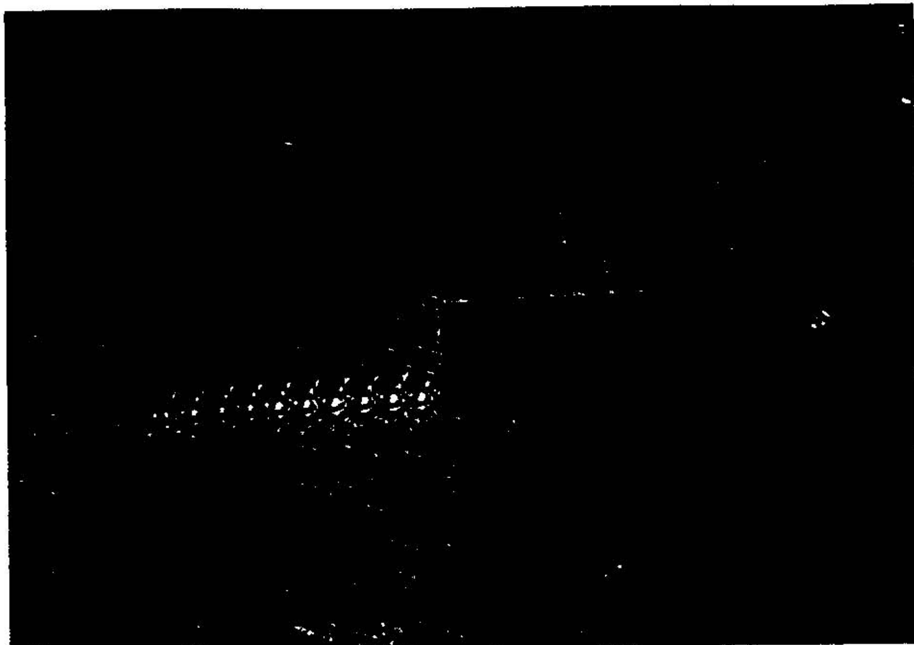

FIGURE 3. A line of wells, with negative samples blue and positive samples clear, are readied for reading in an inexpensive spectophotometer (Fig. 4, below).

medicine. It is chemically related to Etorphine or "Elephant juice," and is about as potent as fentanyl. As a Schedule V drug, it is readily available in human medicine, which means that it is also available for deversion into horse racing. No test for this drug was available before we introduced our ELISA test, which meant that we were virtually certain to obtain dramatic results when we introduced our buprenorphine ELISA into post-race testing.

Our expectations in this regard were not disappointed. When our test for buprenorphine was introduced in several Southwestern states, it immediately showed a positive call rate of about five per cent of the samples tested in some cases.

When these positives were subjected to Mass Spectrometric confirmation, the great bulk of them were confirmed as buprenorphine positives. The first ELISA test for a drug abused in horse racing had been developed, introduced into post-race urine testing, and found to be remarkably effective. A widespread and relatively common pattern of drug abuse was stopped dead in its tracks by a simple, sensitive, and inexpensive test.

The next test that we introduced was an ELISA test for morphine. Although tests for morphine exist and are used throughout the United States, this did not prevent our morphine ELISA from uncovering a pattern of drug abuse based on oxymorphone, a close relative of morphine. This ability of the morphine test to pick up oxymorphone positives points to one of the advantages of immunoassays, which is that they will detect close relatives of a drug of abuse in addition to the drug for which they were originally developed.

All in all, when the oxymorphone test was introduced in the West and Southwest, it flagged about 18 of the first 166 samples positive for opiates. Of these positives 13 were confirmed by Mass Spectrometry for oxymorphone suggesting an abuse rate of more than five per cent in the samples tested. This is a minimum estimate, since some samples could not be confirmed positive either due to either a small sample amount or because the quality of the Mass Spectrum was marginal. At the end of the day, however, the data showed that of the initial group of horses tested at least five per cent and likely more were running on oxymorphone. The morphine ELISA had uncovered a substantial pattern of drug abuse and, again, stopped it dead in its tracks.

Our work with oxymorphone highlighted the practical strengths of these tests. In the first place, a Quality Assurance Program test for oxymorphone already existed and was being used in most laboratories. This test, however, was not sensitive enough to control the use of oxymorphone in racing horses. This is at least partly because the test is laborious, requiring a three-hour hydrolysis step and then Thin Layer Chromatographic (TLC) analysis. Once the TLC plate is developed, it requires a skilled operator to read it, and even then a positive might be missed.

FIGURE 4. Use of a spectophotometer can allow researchers to follow the time course of development of the color reaction as illustrated in Fig. 1 (page 295).

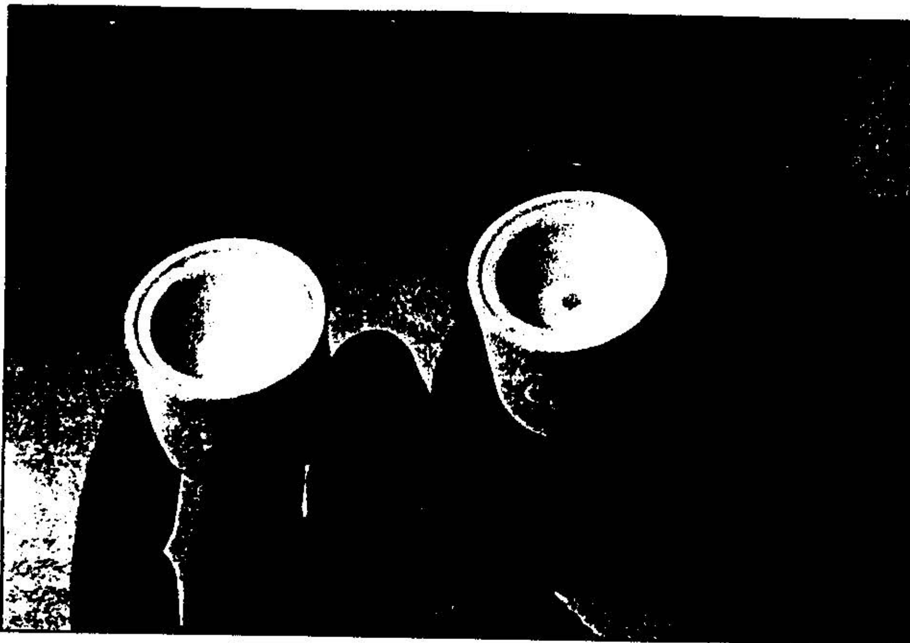

FIGURE 5. "Cup test" for fentanyl or "China White." The entire test can be performed in minutes and read by visual inspection. The cup on the left, with no color, is positive for fentanyl, while the cup on the right is a negative control and shows a blue spot.

With the immunoassay test, no hydrolysis step is required, and the screening test is complete within 30 minutes of receipt of the samples in the laboratory. The results of the test are relatively unambiguous, so samples are flagged for opiates and can go to Mass Spectrometry within an hour of their receipt in the laboratory. In our laboratory in Kentucky, we have replaced an entire sequence of thin layer tests with our morphine-oxymorphone ELISA.

The next step in this sequence of events came from the Oklahoma laboratory, which reported that horsemen were bragging that they were using Sufentanil and it was not being detected. Sufentanil is an analog of fentanyl that is about 10 times more potent than fentanyl and is not detected by the standard fentanyl test. To ensure success in this venture we used both our new ELISA test for fentanyl and a radioimmunoassay test for Sufentanil that we had developed at the University of Kentucky. The samples were screened at both the University of Kentucky and Industrial Laboratories in Denver, Colo., where much of the screening work with buprenorphine and oxymorphone had been carried out.

In the first 200 or so samples screened from Oklahoma we uncovered about 10 Sufentanil positives using our new techniques. Further, as pointed out to me in Lexington, more than one of these positives came from the same stable, which led to a change in the identity of the leading trainer. As before, application of an immunoassay technique led to the identification of a substantial pattern of drug abuse and rapidly ended it.

Based on these very encouraging results, our research group has launched an all-out program to develop and bring into use in routine equine drug testing these simple one step ELISA tests. This is not an easy proposition. In the first place, one needs skilled and experienced synthetic chemists to make the drug derivatives needed to develop the essential antibodies and drug-enzyme conjugates. Then one needs experienced immunoassay scientists to construct the tests themselves. In the third phase, the formats of the tests must be standardized, and they have to be introduced into field testing, then evaluated and verified. While an academic unit can perform the first two of these steps, the third phase requires expertise in the application of ELISA tests to large-scale screening. We have been very fortunate in that all of these skills are represented in our research groups, and our success to date is due to the successful combination of a diverse selection of skills and talents.

The potential impact of this technology extends far beyond post-race testing of drugs in horses. The Illinois Racing Board is actively pursuing the application of these tests to pre-race testing. The unfortunate truth about current pre-race testing is that virtually all that it can detect are Bute and Lasix. However, an immunoassay based pre-race testing system would be vastly more effective than the current systems and would offer the ability to detect many drugs of abuse prior to the race. John McDonald of the Illinois Racing Board laboratory is spearheading research on these applications of immunoassay testing.

This technology can also be used for rapid estimation of the quantities of drugs in blood. Many states currently expend substantial effort quantitating phenylbutazone in the blood of horses to ensure compliance with the 5ug/ml. limit. A simple ELISA test for phenylbutazone would enable these tests to be completed within minutes and at a very competitive cost. A similar quantitative test for Lasix, now being developed in our laboratory, has the potential to eliminate the detention barn and security personnel for Lasix, again giving rise to substantial savings.

Other applications of this technology include human drug-abuse monitoring, especially in circumstances where a rapid presumptive test is useful. Such circumstances include law enforcement applications, such as monitoring parolees. With this technology a parole officer can perform a drug test in front of a parolee and present him with the result on the spot. The immediacy of the test serves to render the officer's control over the parolee much more compelling, and parole is thereby greatly enhanced as a behavior modification tool.

In summary, therefore, we are in the process of applying the new technology of ELISA testing to drug testing in racing horses. Pilot trials with three tests have uncovered major patterns of drug abuse, and led to the calling of at least 50 positives for narcotic analgesics in the second half of 1987. This number of positives is likely greater than the total combined narcotic positives called by the old technology in all of 1987. This technology is rapid, sensitive, and inexpensive and is vastly superior to the old screening technology. It promises to make pre-race testing an effective reality, to make drug quantitation rapid and inexpensive, and it also has applications in many areas outside of horse racing.■