GUEST VIEWPOINT

ELISA TESTING

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In January of this year, we reported on the introduction of ELISA tests into equine drug testing and the impact of this technology on post-race testing. At that time we suggested that ELISA (Enzyme Linked ImmunoSorbent Assay) technology showed great promise in pre-race testing and indicated that we had redirected our immunoassay research program to implement this strategy. Six months have passed since our original report on ELISA testing, and we now report on progress in this rapidly developing field.

Since January, the most dramtic developments have been in pre-race testing. In April of this year, the Illinois Racing Board pre-race testing program introduced a fluorescence immunoassay test for acepromazine. This test had been developed specifically for use in pre-race testing, and soon after its introduction it began to "flag"* pre-race blood samples for acepromazine. This pre-race "flagging" of a small number of samples triggered a sequence of events that resulted in confirmation of at least 25 positives for acepromazine in horses racing in Illinois, and in the control of what was clearly a widespread pattern of acepromazine abuse.

Acepromazine and other tranquilizers are improperly used shortly before post-time to improve control of nervous horses. Its best known use is in the treatment of a "washy horse," that is a horse that tends to get overly excited and "run its race in the paddock." Similarly, in classic distance races, the ability of the jockey to "pace" his horse and control its speed is very important. In each case, administration of a very small dose of a tranquilizer calms the horse in the paddock and makes him easier to rate during the race. Horsemen have long recognized these uses of tranquilizers, which have been used to advantage by unscrupulous horsemen.

To develop an effective post-race (urine) test for acepromazine is a substantial technical challenge, since doses of 1 mg. or less have clear pharmacological effects on racing horses. To develop an effective pre-race (blood) test is much more challenging, since the amounts of acepromazine in plasma after 1 mg. doses are so minute that they had never been detected. However, based on our experience with immunoassays in post-race testing, we

*Immunoassays merely draw attention to a sample, indicating that a certain drug, drug metabolite, or a structurally related agent might be present. We use the term "flag" to indicate this marking of a sample or, in our somewhat more intense laboratory jargon, an immunoassay "hit."

targeted acepromazine for immunoassay development, with the tests to be structured as both pre- and post-race tests.

The development, deployment, and forensic effectiveness of the acepromazine test is a classic example of how well immunoassay testing works when everything goes smoothly. Our work with acepromazine started in September of 1987, and soon the drug was derivatized and injected in rabbits. By about Christmas, we knew that we were going to get good antibodies, and the early part of 1988 was spent developing the acepromazine tests.

By early April, the time of the International Conference of Racing Analysts and Veterinarians in Louisville, Ky., we had developed preliminary data and papers on the test. Additionally, the pre-race format of these tests, the particle concentration fluorescence immunoassay (PCFIA) test, had already been introduced into pre-race testing in Illinois, and during the conference we got reports on the first signs of illegal use of acepromazine from these pre-race tests.

As soon as these pre-race samples were "flagged" for acepromazine, the horses involved were "special sampled" for post-race testing and their urines carefully analyzed for acepromazine. This sequence of events points up a major strength of the Illinois pre-race program, which is that *all* horses are tested pre-race. Since normal post-race testing only screens 10 per cent of horses, the scope of the new pre-race screen in Illinois increased the probability of detecting the illegal use of acepromazine at least ten-fold.

When these horses were "specialed" for post-race testing, they also yielded strong ELISA "hits" for acepromazine. Initially, substantial difficulty was experienced in confirming these acepromazine "hits" as acepromazine positives. After several failed attempts to identify parent acepromazine, these samples were hydrolysed and subjected to thin layer chromatography. The thin layer plates were then scraped and each individual scrape subjected to ELISA testing. (This marriage of the detection sensitivity of ELISA tests and the separating power of TLC is called "ELISA fingerprinting.") This technique showed that the material in the urine of these horses was not acepromazine, but two different metabolites of acepromazine. Once the metabolites that were reactive in the ELISA tests had been identified, confirmation of the ELISA "hits" became relatively straightforward.

This experience points up one of the strengths of ELISA tests. These immunoassay tests react with parent drug, major metabolites, and structurally related agents, and this must be taken into account in evaluating the performance of ELISA tests. As shown by our experience with the acepromazine assay, a good test will react well not only with metabolites of the drug in question, but also with unknown metabolites and closely related agents. Our acepromazine test, for example, reacts even more strongly with propriopromazine and chlorpromazine than with acepromazine.

As a practical matter, what this means is that dosing horses with either acepromazine, propriopromazine, or chlorpromazine can give rise to ELISA reacting materials in horse urine that would yield ELISA "hits" that would be difficult to confirm. The "hits" would be difficult to confirm for the simple reason that the metabolites giving rise to the ELISA positives would be unknown and therefore challenging to confirm by mass spectrometry until their structures had been determined.

In general, we expect this pattern to be followed for any immunoassay test. Initially, when the test is introduced, there is likely to be a large number of positives, relatively easily confirmed. This is especially likely if frozen samples extending back in time are being examined. Later ELISA positives, however, are more likely to be due to very low doses of drugs, or to structurally related drugs. These new patterns of drug abuse are likely to be much more difficult to confirm and require the utilization of other screening techniques in combination with ELISA, along with substantial drug metabolite and mass spectral research.

In Illinois, however, these confirmation problems were solved relatively easily, and as soon as the metabolite pattern had been determined, mass spectral confirmation of the ELISA "flagged" samples became routine. Confirmation of the presence of acepromazine and metabolites in the urine samples of these "specialed" horses then set in motion a standard Illinois Racing Board directed search and reanalysis of the samples in the board's refrigerators.

The Illinois Racing Board holds refrigerated urine samples for up to three years as a precautionary measure. When a sample is analysed in Illinois, the report that releases the purse does not explicitly "clear" the sample. While this report releases the purse, it does not preclude further work on the sample. An event such as the detection of a new pattern of drug abuse or the development of a new test can trigger a directed search of the stored samples, usually with new analytical techniques. Such a directed search was an immediate outcome of the acepromazine pre-race "hits."

Based on the detection of acepromazine in the pre-race samples, and the subsequent confirmation of these "hits" in post-race samples, a large number of stored urines were selected for analysis by the new technology. These included previous samples from horses trained by the implicated trainers and samples from other horses treated by the veterinarian who worked

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with the "positive" horses. These samples were pulled from storage and subjected to the newly developed acepromazine ELISA test.

At this point the speed and sensitivity of the ELISA test came into play. Within weeks about 80 ELISA "hits" for acepromazine had been identified and work on their confirmation begun. As of this writing, about 25 of these samples have been confirmed by mass spectrometry. Since ELISA testing a batch of samples takes 30 minutes, while a mass spectral confirmation is one to two days work, ELISA "hits" can be developed far faster than any mass spectrometrist can evaluate or confirm them.

Our experience in developing and implementing this test shows how effective immunoassay-based screening can be in racing chemistry. For many high-potency drugs (those effective at less than 5 mg./horse), immunoassay is the only viable testing method, either pre- or post-race. Development of effective thin layer chromatographic tests for high-potency drugs in urine is very difficult and essentially impossible in blood. On the other hand, immunoassay offers a way in which tests can be tailor-made for racing with a relatively modest investment of research funds and time.

The description "tailor-made" best describes the labor-intensive development of these tests. Currently many groups have presented us with lists of drugs being used in North American racing for which no effective tests exist. From among these drugs we select the most likely candidates and initiate derivative synthesis. Depending on the molecular structure of the drug, derivative synthesis can vary from straightforward to exceedingly difficult. However, if all goes well, the drug is derivatized and the derivative is linked to a protein molecule and injected into rabbits within a period of weeks. We then settle down and wait for a period of months until the antibodies develop.

Development of the antibodies is watched closely, since early antibodies are an excellent sign. As soon as antibodies are ready, they are incorporated into a preliminary test format and evaluated. This is another critical step, for at this time we discover whether or not the antibody is going to be sensitive enough to detect the drug in the blood, and also whether or not the antibody is going to interact with the major metabolites found in the urine. If the antibody passes all three of these tests, then you have a winner on your hands. If not, you go back to square one, chemical synthesis, and start the process again.

The amount of work involved in the development of these tests means that they are not inexpensive. Nevertheless, their costs are well within the reach of the racing industry. While it can cost up to $50,000 to develop a single immunoassay, it appears clear that this cost will decline as experience in the field is gained. This capital cost can be amortized against use of the test, perhaps for 10 years, most likely worldwide. When one remembers that using current technology costs about $35,000 to call a single hard drug-positive,* the cost of developing an immunoassay appears less prohibitive.

If one considers what it costs to call a pre-race hard drug positive (about $200,000 per positive in one jurisdiction), the cost of developing an immunoassay becomes a fraction of the cost of a single pre-race positive. In point of fact, to "run" a pre-race testing program without immunoassay technology is to run a program whose regulatory value is negligible.

The major value of a pre-race testing program based on immunoassay is that all horses are tested by a very sensitive technology. In this way patterns of drug abuse are identified much more rapidly than if random post-race urine testing is used. If the drug is discovered in the blood of a horse which is just about to race, it is virtually certain that the drug has been placed there in an attempt to influence the performance of the horse. Pre-race testing thus avoids a major problem with urine testing, which is determining whether or not the trace of drug in urine is an innocent residue or evidence of an attempt to influence performance. As testing methods become more sophisticated, this problem becomes more acute, and it is a problem that can be addressed only by blood testing. Additionally, if the jurisdiction uses the stored sample system, as do Illinois, California, and Florida, patterns of drug abuse can readily be screened for and/or confirmed.

This sequence of events with acepromazine points up the regulatory strength of both the scientific and administrative structures of the Illinois Racing Board. Scientifically, the new immunoassay technology gives Illinois the most sensitive tests available to control medication in racing horses. These tests are unique in that they can detect evidence of hard drug abuse prerace. Since up to 100 per cent of all horses are screened, all horses can be subjected to these tests.

Horses which show positive in these tests are immediately "specialed" for post-race testing. In this way, the efficacy of the post-race testing system is greatly increased because the samples coming in to it are already targeted for certain drugs. Once evidence of drug abuse is confirmed in the post-race urines, directed searches of the last three years' urine samples can be conducted for further evidence of abuse of the drug. In this way, all the technologies available for medication control are utilized to maximum effect, and the power of the combined pre- and post-race systems is much greater than random post-race testing.

In summary, therefore, within a six-month period we targeted acepromazine for immunoassay development, raised antibodies to it, developed fluorescence and ELISA tests, and deployed them in pre- and post-race testing in Illinois. The pre-race tests immediately picked up evidence of acepromazine shortly before post time in certain horses. These horses were "specialed" for post-race urine testing, and stored samples were "pulled" for re-analysis using the new tests. These tests have to date "flagged" about 70 samples for acepromazine or related agents or metabolites, and of these about 25 have been confirmed positive by mass spectrometry.

These events show the power of immunoassay testing. Immunoassay testing can detect, in blood, evidence of the administration of very potent drugs close to post time, which has hitherto not been possible. Since all horses are screened, the scope of the screening is 100 per cent, as compared with about 10 per cent on post-race testing.

The pre-race data can be used to pinpoint samples with high probabilities of abuse of certain drugs. When suspects are identified, the speed and sensitivity of the post-race ELISA version of this test make it possible to screen large numbers of stored samples rapidly. Using immunoassay technology, therefore, control for the abuse of high potency drugs that are escaping detection by current thin layer chromatographic methods can be rapidly, reliably, and economically developed. ■

*The national average cost per urine test is about $35, and roughly one test in a thousand is positive for a hard drug. Each hard drug positive costs, therefore, about $35,000 to call.