IMMUNOASSAY BASED PRE-RACE TESTING: AN EFFECTIVE PRE-RACE TESTING SYSTEM

By

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SUMMARY

We have developed a panel of immunoassay based pre-race tests that are highly sensitive and effective tools for use in the control of illegal medication. The basic immunoassay technique is particle concentration fluorescence immunoassay, or PCFIA. We have developed a panel of tests for about 30 drug families and rotated these tests through the pre-race testing system in Illinois. Tests for about 20 other drug families are under development.

We developed an acepromazine test for use in this panel of tests based on information that this drug was being abused. When our acepromazine PCFIA test was introduced into pre-race testing in Illinois, it immediately began to detect signs of acepromazine abuse in several horses. These horses were "specialed" and post-race samples from these horses also showed strongly for acepromazine in our post-race enzyme linked immunosorbent assay (ELISA) tests.

These samples were confirmed by mass spectrometry to contain metabolites of acepromazine, and a directed search of frozen urine samples from the trainers and veterinarians associated with these "positives" was begun. At this time about 70 of these samples have been shown to be "positive" by ELISA testing and more than 20 of these positives have been confirmed by mass spectrometry.

This new technology is far superior to thin layer chromatography (TLC) in pre-race testing. This is because TLC based blood testing simply does not have the sensitivity to detect high potency drugs such as narcotic analgesics and tranquilizers pre-race, and is therefore of very limited forensic value.

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, 1981). 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 (GC) and also on thin layer chromatography (TLC) have been introduced in Ohio, New York, New Jersey, and Pennsylvania (Tobin et al., 1979). However, the efficacy and cost effectiveness of these programs have been debatable and at least one of these programs has been abandoned. Their regulatory value is debatable because of the limitations of the TLC technology on which these programs are based. TLC simply does not have the sensitivity to detect in blood any drugs other than "soft" acidic drugs such as phenylbutazone and furosemide, which are legal in many juridictions. The "hard" or uniformly illegal drugs, such as the narcotic analgesics, are not detectable in blood by TLC, 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 a series of PCFIA tests and we evaluate their application to both pre-race and post-race blood testing for drugs in horses.

PARTICLE CONCENTRATION FLUORESCENCE IMMUNOASSAY

We have elected to base our immunoassay based pre-race testing system on the PCFIA technique developed by the Pandex Corporation (Jolley et al., 1984; Jolley, 1983). In this approach the blood and urine sample is allowed to react with the antibody and the drug-fluorophore complex in a microtiter well (Fig. 1). After an equilibration time of 5 to 10 minutes, second antibody coated latex particles are added to the system (1) and the complex concentration by vacuum to the bottom of the microtiter well (2). The particles are then washed to remove unbound fluorescent material and the fluorescence response read (3). The intensity of the fluorescence is inversely related to the amount of free drug in the sample. Preliminary experiments suggested that this system is sufficiently fast and sensitive to be readily adaptable to pre- and post-race drug screening in horses.

The basic functional unit in the Pandex PCFIA is a 96 well plate with a filter base in each plate. To each well is added 20 µl of drug--phycoerythrin (drug-BPE), 40 µl of anti-drug antibody, and 40 µl of blank, standard, or test sample. The system is allowed to equilibrate for about 10 minutes when a second antibody system is added. The second antibody consists of goat anti-rabbit antibody bound to latex beads. The system is allowed to react for another 10 minutes and then the fluid is drawn out of the system through the filter membrane. The reaction system is then washed with about 80 µl of phosphate buffer to resuspend the particles, and the system again drawn down with the vacuum. The filtration step has the effect of concentrating the latex beads 1000-fold, thereby increasing the sensitivity of the method. After the wash step, the fluorescence of the particles at 545 and 575 nm is measured. The mean response from control urines is usually about 25,000-30,000 arbitrary fluorescence units/well.

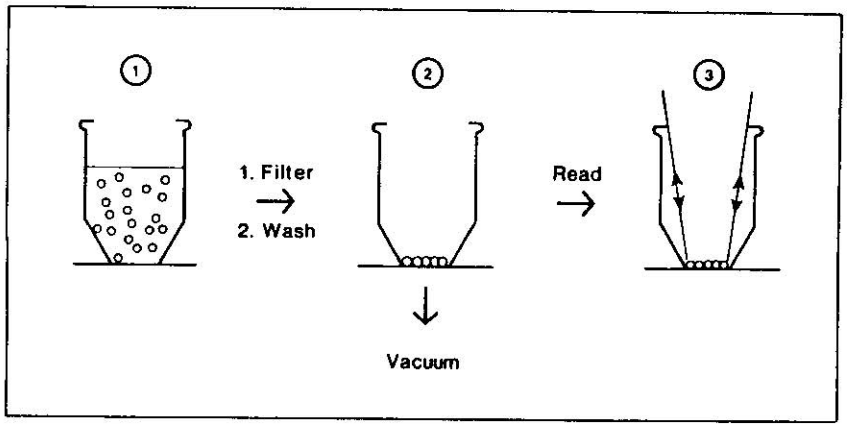

Figure 1. Principle of Particle Concentration Fluoroimmunoassay

A drug is allowed to displace drug-B-phycoerythrin from the anti-drug plasma (1). Second antibody coated latex particles are added to the system and the particles drawn down by application of a vacuum (2). The fluorescence of the resulting layer of particles is then measured and the loss of fluorescence estimated (3). (Adapted from Jolley, 1983).

The advantages of the PCIFA are that the machine is readily adaptable to automation and has a high sample throughput. Using this apparatus, therefore, sensitive and effective screening or pre-race samples can be obtained within 45 minutes of drawing of the blood sample.

TIME AND SAMPLE VOLUME CONSTRAINTS IN PRE-RACE TESTING

In Illinois the time and sample volume constraints in pre-race testing are stringent. The horses are detained for only 2 hours in the pre-race paddock prior to the race and the volume of serum available to assay is about 5 ml. Given the low concentrations of high potency drugs that are likely to be found in such a sample, the small volume of sample available and the short time available for the analysis, immunoassay appears at this time to be the only viable approach. Administratively at this time the immunoassay system is used primarily as a screening system with samples

showing up as a suspect in the pre-race test leading to "specialing" of the horse for post-race urine tests. To illustrate this process in action, I will recount the sequence of events with the acepromazine positives in Illinois.

ACEPROMAZINE

Acepromazine and other tranquilizers are improperly used shortly before post-time to improve the control of nervous horses. Its best known use is in the treatment of "washy horses," that is, horses which tend to get overly excited and "run their races 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.

The development of 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 have never been detected. However, based on our experience with immunoassays in post-race testing, we targeted acepromazine for immunoassay development, with the tests to be structured as both pre- and post-race tests (Kwiatkowski et al., 1988).

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.

DEPLOYMENT OF THE SCREENING TEST

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 PCFIA test (Fig. 2), had already been introduced into pre-race testing in Illinois, and during the conference we had reports on the first signs of illegal use of acepromazine from these pre-race tests.

Table I shows the PCFIA readouts from such a positive pre-race test (Kwiatkowski et al., 1988). A panel of pre-race tests is involved, and the two vertical left hand columns (#1 and #2) represent the tests for acepromazine. The first figure (A) represents a known negative serum sample, the second (B) a known positive sample and the subsequent (C, D, etc.) figures represent live pre-race samples. Inspection of the left hand column shows that the fifth pre-race (G) sample (*) clearly resembles the positive control rather than the negative control, and is thereby flagged for acepromazine.

As soon as the pre-race samples were "flagged" for acepromazine, the horses 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 tests 10% of horses, the scope of the new pre-race screen in Illinois increased the probability of detecting the illegal use of acepromazine at least 10-fold.

When these horses were "specialed" for post-race testing, they also yielded strong ELISA "hits" for acepromazine post-race. Initially, substantial difficulty was experienced in confirming these acepromazine "hits" as acepromazine positives. After several failed attempts to identify

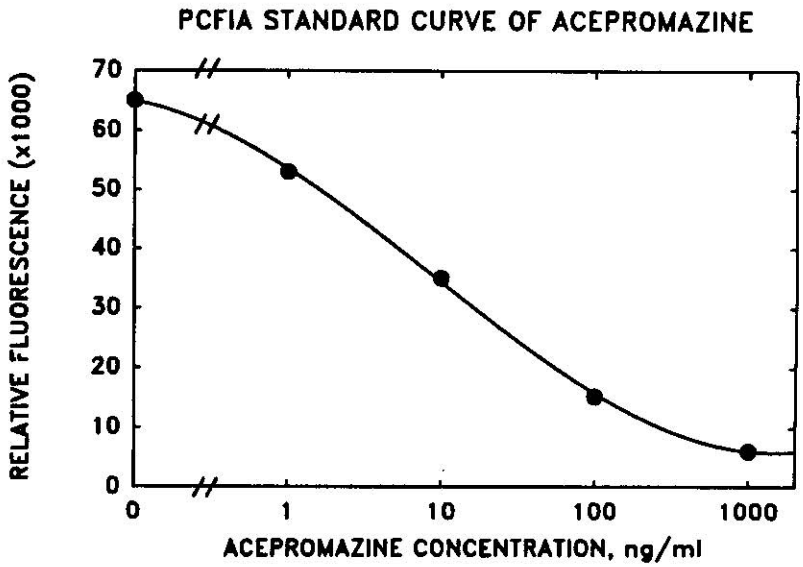

Figure 2. PCFIA standard curve of acepromazine.

The symbols show the inhibition of PCFIA fluorescence in assay buffer observed in the presence of the indicated concentrations of acepromazine. I-50 for acepromazine in this PCFIA system is approximately 10 ng/ml. Control fluorescence value is for buffer with no acepromazine added. (Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.)

Table I. PCFIA readouts for a panel of pre-race drug tests.

Channel Report: 545/575 Gain Setting: 25 Read Time: 100 ms
Plate ID# 00001423

	1	2	3	4	5	6	7	8	9	10	11	12
A	40758	38376	0	23884	22812	0	17032	9878	0	16754	15732	0
B	14944	36316	0	10538	21146	0	5560	7326	0	4380	13132	0
C	41990	33200	0	23484	20220	0	14110	8604	0	16572	13656	0
D	40234	40784	0	23708	22044	0	11520	11916	0	15882	14666	0
E	40162	0	8	21012	0	0	9048	0	0	12358	0	0
F	38228	0	6	22884	0	0	10674	0	0	12378	0	0
G	*19112	0	0	23210	0	0	10396	0	0	13820	0	0
H	39342	18	0	23114	0	0	8582	0	0	13784	0	0

The table represents PCFIA readouts (in relative fluorescence units) for a panel of pre-race drug tests on serum samples. The two left hand columns show data for acepromazine. The first figure is for a known negative control sample, the second for a known positive control. Subsequent figures are for pre-race serum samples. The sample for the fifth horse (*) in this race is "flagged" for acepromazine. Data in other columns are for three other drugs which were simultanously screened. (Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.)

parent acepromazine, these samples were hydrolysed and subjected to TLC. The TLC 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 2 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 (Fig. 3, Table II).

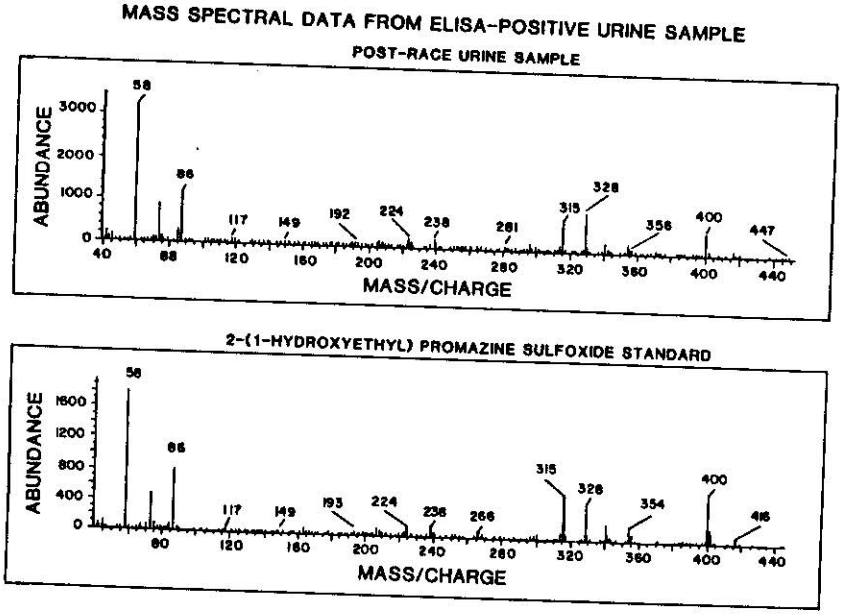

Figure 3. Mass spectral data from ELISA-positive urine sample.

The upper panel shows the mass spectrum of a material recovered from a track urine sample flagged positive for acepromazine by ELISA. The lower panel shows the mass spectrum of the BSTFA derivative of the reduced sulfoxide of acepromazine as a standard. (Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.)

Table II. Acepromazine ELISA results of urine samples from a racetrack screening assay.

Sample #	Race Date	ELISA Optical Density	Confirmation GC/MS (Pos./Neg.)
1	3/17/88	0.168	Pos.
2	3/12/88	0.224	Pos.
3	3/18/88	0.193	Pos.
4	3/26/88	0.132	Pos.
5	3/30/88	0.158	Pos.
6	3/17/88	0.115	Pos.
7	3/2/88	0.267	Pos.
8	3/16/88	0.163	Pos.
9	3/26/88	0.164	Pos.
10	2/26/88	0.303	Pos.
11	3/22/88	0.210	Pos.
12	2/25/88	0.224	Pos.
13	3/7/88	0.177	Pos.
14	3/7/88	0.177	Pos.
15	12/13/87	0.121	Pos.
16	12/11/87	0.291	Pos.
17	3/24/88	0.274	Pos.
18	11/14/87	0.379	Pos.
19	4/1/88	0.135	Pos.
Control dosed 3 mg IV acepromazine (2-4 hr. sample)			
	(x + SD)	0.120 $\pm$ 0.020	n = 10

Acepromazine ELISA optical densities are shown for 19 post-race urine samples obtained from two racetracks in Illinois. These 19 samples were considered to be suspect with a relative optical density reading approximating that of a positive control. The positive control is an equine sample taken from 2-4 hrs after IV administration of 3 mg acepromazine. The x $\pm$ SD represent 10 different ELISA assays. All 19 ELISA positive samples were confirmed by GC/MS to contain either 2-(1-hydroxyethyl) promazine or 2-(1-hydroxyethyl) promazine sulfoxide. (Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.)

PATTERN DEVELOPMENT

The Illinois Racing Board holds frozen urine samples for up to 3 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 that worked 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 in ELISA testing a batch 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 evalute or confirm them.

DISCUSSION

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 TLC 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 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 for perhaps 10 years and most likely worldwide. When one remembers that using current technology it costs $35,000 to call a single hard drug positive, the cost of developing an immunoassay appears less prohibitive (Tobin et al., 1988).

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 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 that 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 will become more

acute, and it is a problem that can be addressed only by blood testing. Additionally, if the jurisdiction uses the frozen sample system, as do Illinois, California, and Florida, patterns of drug abuse can readily be screened for and/or confirmed.

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