VOL. 62, NO. 3
DECEMBER 1988

Research Communications in
Chemical Pathology and Pharmacology

NON-ISOTOPIC IMMUNOASSAY DRUG TESTS IN RACING HORSES: A REVIEW OF THEIR APPLICATION TO PRE- AND POST-RACE TESTING, DRUG QUANTITATION, AND HUMAN DRUG TESTING

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Published as Kentucky Agricultural Experiment Station Article No. 88-4-241 with the approval of the Dean and Director, College of Agriculture and Kentucky Agricultural Experiment Station.

Publication No. 156 from the Kentucky Equine Drug Testing and Research Programs, Department of Veterinary Science and the Graduate Center for Toxicology, University of Kentucky.

Supported by a grant entitled "Immunoassay Tests for High Potency Narcotic Analgesics in Racing Horses" from the Kentucky Equine Drug Research Council and the Kentucky State Racing and Harness Racing Commissions. Additionally, this research was supported by grants for research on detomidine from the American Horse Shows Association; research funds from the Illinois Racing Board to support their pre-race testing programs; research funds from the Oklahoma Horse Racing Commission and the Oklahoma Horsemen's Association for work on Lasix and research support to the University of Kentucky from International Diagnostic Systems Corporation.

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Contents

1. Summary

We have introduced large scale non-isotopic immunoassay testing into pre- and post-race drug testing in racehorses. The technologies utilized are Particle Concentration Fluorescence Immuno Assay (PCFIA) and the one-step Enzyme Linked ImmunoSorbent Assay (ELISA). These technologies are rapid, inexpensive, and highly effective. On introduction into post-race testing in the Western United States, these ELISA tests exposed several previously undetected patterns of drug abuse. The drugs detected were buprenorphine, oxymorphone, mazindol, sufentanil and cocaine. This led to the suspension of a large number of trainers and exposed the high false negative rate of thin layer chromatography (TLC) based testing.

More recently, we have introduced both PCFIA and ELISA assays into pre- and post-race testing in Illinois. Within days, our pre-race PCFIA tests detected signs of acepromazine abuse. Directed searches of post-race urines from these horses showed evidence for acepromazine metabolites in the urine of these horses. Examination of frozen samples from associated horses yielded about 70 ELISA "positives" for acepromazine. To date, about 25 of these ELISA "positives" have been confirmed by mass spectrometry.

We have also raised antibodies to phenylbutazone and furosemide to enable rapid and inexpensive quantitation of these permitted medications. Furosemide is a particular

problem since its use requires a pre-race detention barn. For furosemide, we have developed a regulatory schedule based on our immunoassay test that allows elimination of the detention barn. For phenylbutazone, we have developed a similar immunoassay that allows rapid and inexpensive quantitation of this drug in blood.

To enable racing authorities to test jockeys and other racetrack personnel, we have adapted PCFIA technology to human drug testing, and a full range of very sensitive tests for human drugs of abuse is available.

These immunoassays are sufficiently sensitive to control abuse of the most potent drugs available to horsemen. In principle, an immunoassay can be raised to any drug within about six months, and made available worldwide at competitive rates. It appears clear that these non-isotopic immunoassays provide racing with the only technological basis that is sufficiently sensitive to detect the most potent abused drugs pre- and post-race, and has the flexibility to be readily adaptable to different drugs.

Because of the high false negative rate generated by TLC, credible pre- and post-race testing programs cannot be based on TLC alone. Rather, such programs must be spearheaded by vigorous, sensitive, broad scope, and flexible immunoassay testing for the high potency drugs abused in racing horses.

2. *Background*

Horse racing is the biggest spectator sport in North America, with an estimated audience of about 85 million, and a betting handle of about twelve billion dollars per annum*. As such, racing is sensitive to suggestions that the process is tainted. A consistent problem in racing has been the use by horsemen of illegal medications to influence the outcome of races. More recently, however, the industry has made dramatic progress in this area. In this review, we will outline the industry's progress with the introduction of simple, rapid, and inexpensive immunoassay tests into racing. We will show that these tests are much more effective than the old technology, are revolutionizing drug testing and, properly applied, should free racing from the problem of undetectable medications.

3. *The Old Technology: Thin Layer Chromatography*

Until very recently, control of the use of illegal medication in horses in North America depended on thin layer chromatography (TLC) (Tobin, 1981). Post-race urine samples taken from racing horses were shipped to post-race laboratories, extracted by liquid/liquid extraction, and subjected to TLC. Samples showing evidence for the presence of drugs were subjected to further testing, including gas chromatography/mass spectroscopy (GC/MS) confirmation. With minor exceptions, medication control in racing horses in North America has depended on the TLC technology outlined above. This technology

*Personal communication. T. Chamblin. Association of Racing Commissioners International. Lexington, KY (1988).

was the basic technology of the old National Association of State Racing Commissioners and was encoded as such by laboratories at Cornell and Ohio State Universities.

These TLC tests are in general use on post-race urine samples throughout the country. Beyond this, this same technology is used for pre-race blood testing in New York, New Jersey, and until recently, Pennsylvania (Tobin *et al.*, 1979). While the efficacy of TLC testing for post-race urines is marginal, there is no question whatsoever concerning its efficacy in pre-race testing. Because of the small volume of blood drawn in pre-race testing, and the high potency of the drugs used as illegal medications, TLC based pre-race testing cannot and does not detect "hard"* illegal medications pre-race. This is despite the expenditure of substantial funds on both development of this technology and its day to day application by various official testing laboratories. In a nutshell, pre-race testing systems based on TLC are of very limited value since they are unable to pick up evidence for the use of high potency drugs pre-race. Horsemen have long recognized these facts about TLC based pre- and post-race testing, and freely use the many illegal medications that are undetectable by TLC.

4. Immunoassay Based Testing: The Illinois Initiative

In 1986, the Illinois Legislature mandated pre-race testing for the State of Illinois as part of a package of changes in Illinois racing. One of us (John McDonald) was charged with determining the nature of the scientific approach that would be taken to pre-race testing. In essence, the choice was two-fold. Illinois could install a TLC based pre-race system that would be forensically worthless, or Illinois could develop a more effective technology. The choice was made to develop a more effective immunoassay based pre-race testing technology.

Previous immunoassay experience in racing involved radioimmunoassay (Tai *et al.*, 1988; Wood *et al.*, 1988; Woods *et al.*, 1986). Radioimmunoassays, however, are too slow to be applicable to pre-race testing, which must be completed within two hours. It was, therefore, clear from the outset that the new testing system would have to be fast and non-isotopic. Additionally, the testing system would have to be flexible. Unlike human drug testing, where the six major drugs of abuse show up again and again, horse racing shows rapidly changing patterns of drug abuse. In horse racing, the drugs abused are very potent, and are changed as soon as it is evident that they can be detected. Any new testing system would have to be sufficiently flexible to keep pace with the rapidly changing pattern of equine drug abuse.

*"Hard" illegal medications are the stimulants, depressants, local anesthetics, narcotic analgesics and tranquilizers that are illegal in all racing jurisdictions. "Soft" medications are drugs such as phenylbutazone and furosemide that are legal in most racing states.

5. Changing Patterns of Drug Abuse: The Need for Flexibility

A classic example of the ease with which drug abuse patterns change in horse racing occurred in the late seventies and the early eighties. In the late seventies, fentanyl* was the illegal drug of choice in racing horses, and it was widely known to be undetectable (Tobin, 1981). When an immunoassay for fentanyl was developed (Woods *et al.*, 1986), its use stopped, and its place was taken by etorphine**. More recently, an immunoassay for etorphine has been developed (Tai *et al.*, 1988), and the pattern of abuse shifted to other drugs, such as oxymorphone, sufentanil and buprenorphine. The discovery and control of these other patterns of drug abuse is in large part the substance of this review.

In approaching the problem of immunoassay-based pre-race testing, it was apparent that a completely new technology would have to be developed. The assays developed would have to be sensitive, since the drugs must be detected in blood. The assays would have to be fast, and they would have to be non-isotopic, for ease of use. Beyond this, the system would have to be able to develop and bring on line sensitive new tests at a significant rate, since once drug "X" was "called," drug "Y" would be substituted. Given the number of drugs abused in horse racing and the ability of horsemen to identify new drugs, this could be the most challenging part of the program. The first technology that McDonald evaluated was particle concentration fluoroimmunoassay (PCFIA).

6. Particle Concentration Fluorescence Immunoassay (PCFIA)

Particle concentration fluorescence immunoassay (PCFIA) was selected as the primary test vehicle because this technique is fast, sensitive, non-isotopic and readily adaptable to automation (Jolley *et al.*, 1984; Prange *et al.*, 1988b). In this approach, the blood or urine sample is allowed to react with antibody and the drug-fluorophore complex in a microtiter well (Figure 1). After an equilibration time of five to ten min, second antibody-coated latex particles are added to the system (1) and the complex concentrated by vacuum at 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 was sufficiently fast and sensitive to be adaptable to pre- and post-race drug screening in horses.

The only problem with PCFIA was that there were no suitable drug detection kits available, not even for human drugs of abuse. If we were to develop a pre-race testing program based on PCFIA, we would have to develop the immunoassays ourselves. To help McDonald with test development, the manufacturers of the PCFIA system (Bax-

*Fentanyl, Sublimaze®, a potent short acting narcotic analgesic.

**Etorphine, a short acting narcotic analgesic 100-fold more potent than fentanyl.

ter/Pandex, Mundelein, IL), introduced him to Charles Prange of International Diagnostic Systems Corporation*.

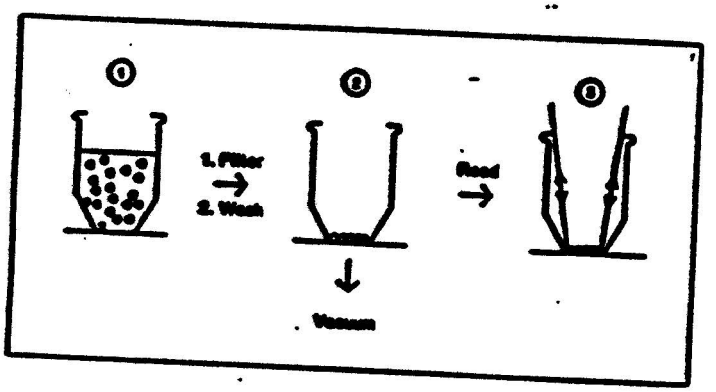

Figure 1. Principle of Particle Concentration Fluoroimmunoassay

A drug is allowed to displace drug-R-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).

Prange was experienced in non-isotopic immunoassay development, and he also visited with those racing researchers who had developed immunoassays. In this way, the University of Kentucky provided him with antibodies to fentanyl and etorphine, while other sources provided other reagents. Soon the Illinois group had the beginnings of a pre-race testing panel, and they began to work up a pilot pre-race testing system. Among the first kits that were developed were tests for buprenorphine (Ozog *et al.*, 1988), fentanyl (McDonald *et al.*, 1987), morphine and mazindol. Since then, at least twenty other tests, including a full line of human drugs of abuse assays have been developed (Kwiatkowski *et al.*, 1988a, 1988b; Prange *et al.*, 1988b, 1989; Woods *et al.*, 1988).

7. Enzyme-Linked Immunosorbent Assays (ELISA) and Post-Race Testing

While we were working on these pre-race tests, a request came from the State of New Mexico for help with their post-race testing system. The chairman of the New Mexico State Racing Commission, Harris Hartz, was unhappy with their drug testing and sent some samples to International Diagnostic Systems Corp. for further screening. These samples immediately began to show positives for buprenorphine, and also for opiates. On confirmation, the opiate positives turned out to be due to oxymorphone (McDonald *et al.*,

*IDS Corporation, St. Joseph, MI.

1988). Because the State of New Mexico was drawing from stored frozen samples, the positives continued to pile up. When the sequence of tests was complete, approximately 49 trainers had been suspended for medication violations.

Working with the New Mexico samples, we soon became convinced that the enzyme-linked immunosorbent assay (ELISA) test we had developed was the most satisfactory format for urine testing. The ELISA tests could be performed on raw urine, while the FCFIA tests required an extraction step. Beyond this, the ELISA tests could be read by eye, which made them highly portable, while the FCFIA tests required a fluorescence reader. Based on our experiences with the New Mexico samples, we introduced the ELISA format of our tests for post-race urine screening.

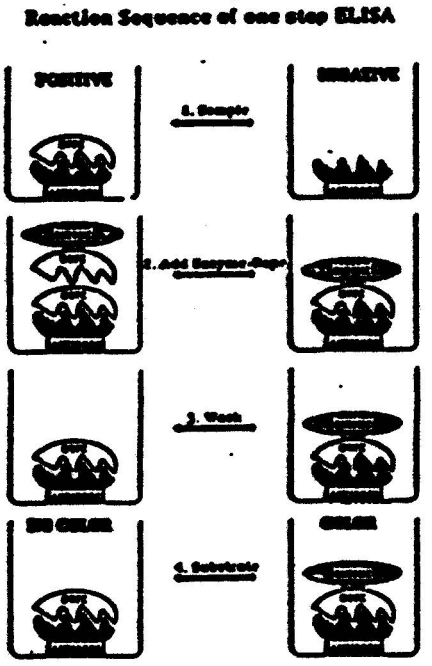

Figure 2. Reaction sequence of the one step ELISA test.

Antibody to the drug is bound to the well, and test and control samples are added directly to the well. In control samples these 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.

The ELISA tests are based on those described by Voller *et al.* (1976) and Yang *et al.* (1987). Briefly, the anti-drug antibody is linked to flat bottom Immulon Removawells® (Dynatech, Chantilly, VA) as described by Voller. Similarly, drug-hemisuccinate is linked to horse radish peroxidase (HRP), as described by Pradelles *et al.* (1985), to give rise to a covalently linked drug-HRP complex. All assay reactions are run at room temperature. The assay is started by adding 20 µl of the standard, test or control samples to each well, along with 100 µl of the drug-HRP solution. During this step, the presence of free drug or cross-reacting drugs or metabolites competitively prevents the antibody from binding the drug-HRP conjugate. The degree of antibody:drug-HRP binding is, therefore, inversely proportional to the amount of drug in the sample. After ten minutes of incubation, the fluid is removed from the microtiter wells and the wells washed three times. Substrate (tetramethylbenzidine, Kirkegaard and Perry, Gaithersburg, MD) is then added to all wells and their absorbance read at 560 nm in an International Diagnostic System microwell reader. A diagram outlining this sequence of events is presented schematically in Figure 2.

The ELISA tests outlined above are particularly effective. For example, Figures 3 and 4 show, respectively, the time course and sensitivity of the morphine ELISA, a typical "run" on a series of track samples and, in Table I, the results of the introduction of this test into routine post-race testing. As shown in Table I, of 166 samples screened in the Western United States, 18 were "flagged" by ELISA and of these, 13 confirmed positive on GC/MS (McDonald *et al.*, 1988).

Similar patterns of positives were seen across the Western United States wherever these tests were introduced. In general, about 1% to 5% of the early samples tested were positive for a narcotic analgesic. Similarly, when the mazindol test was introduced in early 1988, about two to five percent of the early samples were positive when confirmed by GC/MS (Prunge *et al.*, 1988a). The efficacy of these ELISA tests in racing chemistry had been dramatically established and a major false negative problem with TLC based screening had been exposed.

8. Comparative Efficacy of TLC and Immunoassay Screening

Establishing the efficacy of FCFIA and ELISA based immunoassays (Table II), exposed major deficiencies in TLC as a screening methodology. No TLC method for buprenorphine existed, so use of this drug was completely uncontrolled. Similarly, sufentanil abuse was uncontrolled and even "bragged on" by horsemen until the advent of this technology (Tobin *et al.*, 1988). While TLC methods for cocaine, oxymorphone and mazindol existed, these methods were unable to detect the small doses of these drugs being used in horses. This was especially so for mazindol, where the TLC dose was about 400 mg/horse*, while the dose used on the track was about 4 mg horse (Prunge *et al.*, 1988a). Overall, the great sensitivity and speed of the ELISA tests rendered them highly effective screening tests and far superior to the old TLC screening methods. The ELISA tests established the

*NASRC Quality Assurance samples shipped to participating laboratories.

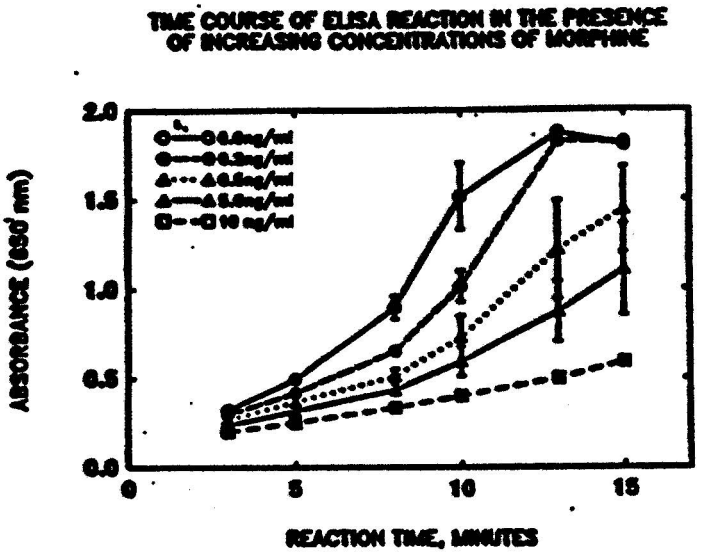

Figure 3. Time course of ELISA reaction in the presence of increasing concentrations of morphine.

The symbols show the time course of the ELISA reaction in the presence of the indicated concentration of morphine. Reproduced with permission from Res. Comm. Chem. Path. Pharmacol.

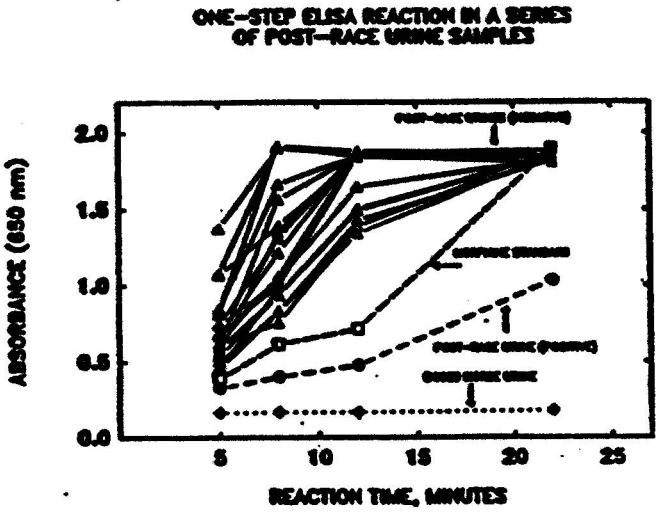

Figure 4. One step ELISA reactions in a series of post-race urine samples.

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 0.5ng/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. Reproduced with permission from Res. Comm. Chem. Path. Pharmacol.

TABLE I

ELISA Screening of Post-Race Urine Samples Followed by GC/MS Analysis

Post-race urine samples from two racing jurisdictions were screened for morphine and its analogs by 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. Above 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. Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.

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	

efficacy of this technology in post-race testing, and similar events soon took place in pre-race testing.

9. Development of an Acepromazine Test

The next application of this technology was in pre-race testing. While our ELISA assays were being evaluated in post-race testing in the Western United States, work at the Illinois Racing Board Laboratory on the application of this technology to pre-race

TABLE II

Efficacy of PCFIA and ELISA Tests

Drug	State	TLC Status	Immunoassay Positives
Buprenorphine	New Mexico	No Test	Multiple (>50)
Oxymorphone	New Mexico	Poor Sensitivity	Multiple (>20)
Sufentanil	Oklahoma	No Test	20 /500*
Mazindol	Western States	Very poor sensitivity	Multiple (>20)
Cocaine	California	Poor sensitivity	265*
Acepromazine	Illinois	Fair sensitivity	Multiple** (>25)

*The table compares the TLC and immunoassay status of 6 drugs for which immunoassay tests have been introduced since August 1987. Figures marked by an asterisk represent the ratio of positives called to total number of samples tested.
**Acepromazine initially detected in pre-race samples.

testing continued. One of the industry's greatest needs is for antibodies to speciality veterinary drugs abused in racing. To this end, we specifically targeted a number of veterinary drugs for antibody development. Among the targeted drugs was the veterinary tranquilizer acepromazine, which is readily available and widely used in racing horses (Kwiatkowski *et al.*, 1988b).

Tranquilizers are used in racing horses to make excitable horses more manageable prior to post time and also during the race itself. A "washy" horse is one that runs his race "in the paddock," and a judicious dose of a tranquilizer calms a "washy" horse and helps him run his race on the track. Similarly, an excitable horse will tend to fight his jockey and be difficult to control during a race. Again, a carefully selected dose of a tranquilizer such as acepromazine will prevent an excitable horse from fighting his jockey and allows him to run the best race possible. For these reasons, very small doses of tranquilizers such as acepromazine* are widely used in racing horses to ensure that excitable horses turn in their best possible performance (Tobin, 1981).

In September of 1987, we targeted acepromazine for antibody development. Within one month, we had acepromazine linked to bovine serum albumin (BSA) and in rabbits. Three months later, we had good antibodies to acepromazine and were working on test development. By early April, the pre-race PCFIA format of the acepromazine test was introduced into pre-race testing in Illinois. Within days, this test began to detect evidence of acepromazine abuse in Illinois racing.

*Other tranquilizers are also abused in this way, and tests for other tranquilizers are under development.

10. Application of the Acepromazine Test in Pre-Race Testing

Because a secondary test for acepromazine was not available, the initial response was to "special" the horses in question for post-race testing. When urines from these horses were examined with our post-race acepromazine ELISA, they gave strong reactions for acepromazine. However, when these urines were sent to GC/MS, evidence for the presence of acepromazine could not be developed, which initially slowed the pace of the investigation. However, when the urine samples in question were hydrolyzed, subjected to TLC, and the plates scraped and each individual scrape subjected to ELISA analysis, it became clear that the reactive materials in the urine samples were metabolites of acepromazine*. When these metabolites were identified, confirmation of acepromazine positives in the ELISA "flagged"** urine samples became routine (Kwiatkowski *et al.*, 1988b).

Since Illinois stores urine samples for up to three years, the next step was a directed search of these stored samples for evidence of acepromazine abuse. The search was directed because it encompassed other horses trained by individuals whose horses had been identified as acepromazine positive. Additionally, samples from other horses treated by a veterinarian involved were tested. This search developed about 70 ELISA "hits" for acepromazine, and of these "hits" about 25 have to date been confirmed by GC/MS.

The overwhelming power of this new immunoassay based testing is now apparent. Prior to development of the immunoassay technology, it was impossible to detect drugs such a morphine, mazindol or acepromazine in blood samples pre-race. With the pre-race system, all horses can be screened for these and other drugs pre-race. While at this time, the most likely action taken is simply to "special" the horse for further tests post-race. This simple event led to the identification of what is apparently the largest numbers of pre- and post-race positives for any single drug in any jurisdiction to date.

11. Quantitation of Permitted Medications by Immunoassay

Under the rules of racing in most jurisdictions, the use of non-steroidal anti-inflammatory drugs such as phenylbutazone and the diuretic furosemide are permitted (Tobin, 1981). Use of phenylbutazone is usually regulated by the use of quantitative levels, such as the 5 μg/ml level recommended by the Association of Racing Commissioners International (ARCI). Furosemide is permitted under more complex regulations which stipulate both the dose of drug permitted (250 mg/horse), and the time prior to post at which it must be administered (4 hours) (Clay *et al.*, 1983). Because of the complexity of the furosemide rule, it is often enforced by use of a detention barn in which the horses are sequestered and supervised for four hours prior to the race (Woods *et al.*, 1988).

*This technology represents a marriage of the separating power of TLC and the detection sensitivity of ELISA and is called ELISA-fingerprinting.

**The terms "flagged" or "hit" are used interchangeably to indicate that a sample has shown up as suspicious for the presence of a drug family in our immunoassay tests.

It is vitally important that the rules with regard to furosemide be strictly enforced. This is because the unregulated use of furosemide can facilitate illegal medication. Unpublished work from our laboratories has shown that for at least the first 90 min after administration of furosemide, a recent administration of buprenorphine cannot be detected. This is despite the fact that our buprenorphine ELISA is the most sensitive test available for this drug. Since detention barns are only as good as the quality of supervision that a horse receives, the possibility of illegal administration of a second dose of furosemide always exists. Based on recent work from our groups, we have, therefore, developed a quantitative method for furosemide to monitor compliance with furosemide regulations which we believe to be superior to, and more economical than, the detention barn system.

12. Furosemide Quantitation

Detention barns are an administrative problem for racetracks. They are cumbersome to manage, expensive to use, disliked by horsemen, and unless meticulously supervised, of doubtful efficacy. While the appropriate dose of furosemide is given under supervision shortly after the horse enters the barn, it is quite possible for a horse to receive a second dose of furosemide at any time prior to post. This second dose obviates the function of the test barn, which is to ensure that such second doses of furosemide are not used to "cover up" administration of illegal drugs.

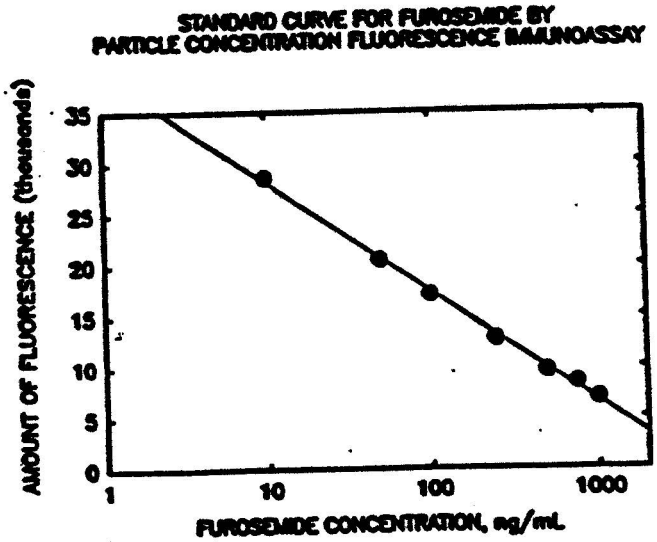

Figure 5. The standard curve for the inhibition of furosemide-BPE fluorescence by the addition of the indicated concentrations of furosemide was constructed. Furosemide (10-1000 ng/ml) was added to normal race track serums which were diluted 1:10 for assay. No extractions were conducted. Reproduced with permission from Res. Comm. Chem. Path. Pharmacol.

Supported by the Oklahoma Horse Racing Commission and the Oklahoma Horsemen's Association, we have investigated the use of quantitative testing to substitute for the furosemide detention barn. We have raised an antibody to furosemide and developed a rapid and sensitive PCFIA test for furosemide (Figure 5). Using this test, we can rapidly and sensitively determine the plasma levels of furosemide in a sample (Woods *et al.*, 1988). Based on recent previous work, we can then determine whether or not this level of furosemide is in excess of the regulatory level of furosemide permitted by the Oklahoma rule (Chay *et al.*, 1983). The Oklahoma State Racing Commission has chosen to set this level at 50 ng/ml of furosemide, since the probability of a horse dosed with the specified amount of furosemide exceeding this level is less than one in one million.

In the event that a horse exceeds this regulatory level in the immunoassay screen, the remainder of the plasma sample will be subjected to high performance liquid chromatography (HPLC) or GC/MS to definitively establish the level of furosemide in the sample. The final quantitative analysis will then be reported to the Commission for regulatory purposes.

Because of the characteristic plasma kinetics of furosemide (Chay *et al.*, 1983), this regulatory method will be highly effective and should easily detect administration of a second dose of furosemide close to post-time. This is in contrast with the detention barn and honor systems, which leave the possibility of such second doses open.

This basic approach can easily be modified to accomodate different regulatory strategies. For example, in Illinois a blood sample is drawn as soon as the horse enters the pre-race detention barn at two hours prior to post, and these samples are screened for furosemide. Such early samples are much closer the time of drug administration and greatly increase the power of the test to detect improper administrations. Using this strategy of a first test at two hours prior to post-time and the second test as close to post-race as possible, greatly increases the ability of the immunoassay test to detect improper administrations of furosemide.

13. Phenylbutazone Quantitation

A similar quantitative PCFIA has also been developed for phenylbutazone (Kwiatkowski *et al.*, 1988a). As shown in Figures 6A and 6B, our phenylbutazone assay readily detects levels of phenylbutazone in the low μg/ml level. Since PCFIA technology is highly automated, this immunoassay can be used as an inexpensive screening test for phenylbutazone. As with furosemide, test samples clearly in excess of the regulatory standard would be assayed by HPLC to unequivocally establish the serum level of phenylbutazone in the sample for regulatory purposes. These tests are under field trial in Illinois, Kentucky and New Mexico, and preliminary results are very encouraging.

These quantitative immunoassay tests for phenylbutazone and furosemide offer substantial advantages over the old extraction-quantitation technologies. The immunoassay methods are fast, flexible, accurate and readily automated. This means that the

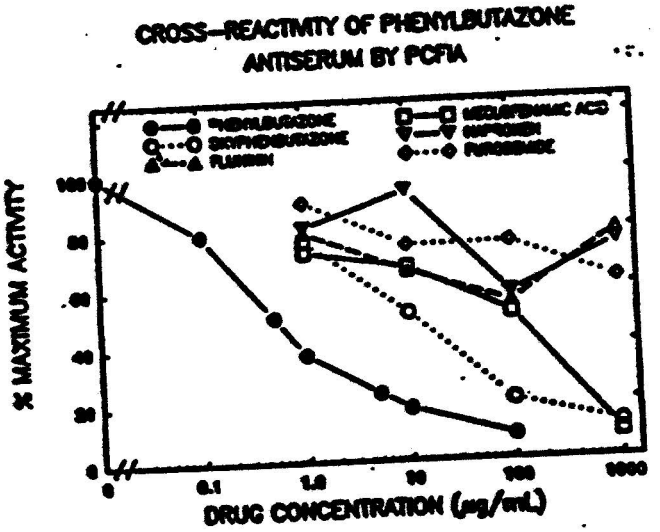

Figure 6A. Cross-reactivity of anti-phenylbutazone antibody.

The solid circles (●-●) in this panel show the displacement of phenylbutazone-HRP from our anti-phenylbutazone antibody. The open circles (○-○) show displacement by oxyphenbutazone and the other symbols show displacement by furosemide and other non-steroidal anti-inflammatory drugs.

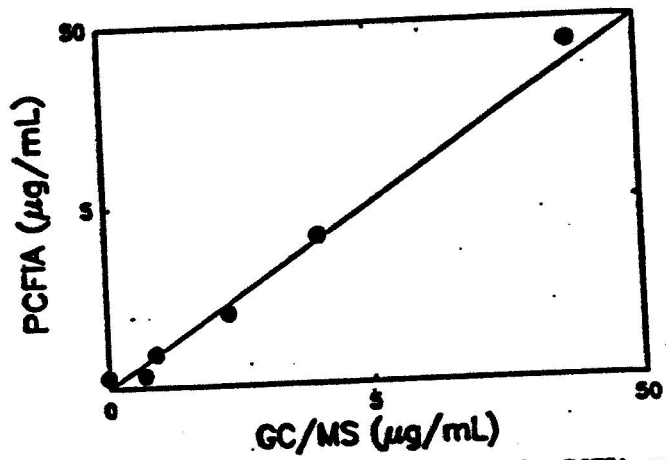

Figure 6B. Serum quantitation of phenylbutazone by PCFIA and GC/MS.

The relationship between PCFIA and GC/MS values for spiked serum quantitations are shown. 0, 0.5, 1.0, 2.0, 5 and 48 µg/ml of phenylbutazone were spiked into equine serum and quantitated by both PCFIA and GC/MS. The solid symbols represent the individual values obtained in each quantitation, while the solid line represents the actual values.

tests can be readily applied and the results communicated to horsemen. For example, with the phenylbutazone test it should be readily possible to give horsemen a printout of their horses' phenylbutazone readings. This information would be of great value to horsemen since it would guide them in their dosing schedules, and, thus, assist them in obeying the rules of racing. Similarly, the furosemide test can be applied at any time to horses in order to monitor very stringently their compliance with furosemide medication rules if this is deemed necessary.

14. Screening for Drugs Abused by Humans

Because racing laboratories may be asked to test samples generated by the human participants in racing, we have also developed a full line of assays for drugs abused by humans. These include tests for opiates, cocaine, tetrahydrocannabinol, amphetamines, barbiturates, and phencyclidine. As with the equine PCFIA tests, these assays are highly sensitive and rapid (Frange *et al.*, 1988b).

The I_{50} values for the major drugs of abuse in each assay were determined. The cocaine assay was half-maximally inhibited by about 50 ng/ml benzoylecgonine, and was much more sensitive to parent cocaine. The amphetamine assay was half-maximally inhibited by 10 ng/ml of *d,l*-amphetamine, the phencyclidine assay by 1 ng/ml of phencyclidine, the tetrahydrocannabinol assay by 350 pg/ml of 11-nor-delta-8-tetrahydrocannabinol-9-carboxylic acid, the opiate assay by less than 1 ng/ml of morphine, and the barbiturate assay by less than 1 ng/ml of secobarbital. The sensitivity of the assays was such that human test urines were diluted ten-fold prior to testing.

No false positives were observed in a series of 50 control urines, and very few false negatives were observed. In cross-reactivity studies, the specificity and sensitivity of our panel of PCFIA tests compared favorably with that of the Abbott TDx® system. In our hands, the PCFIA system was more sensitive than the TDx and correlated better with the GC/MS status of some of these samples. Beyond this, the potential for automation and sample throughput with the Baxter/Pandex Screen Machine and PCFIA technology is currently unmatched by any other immunoassay technology.

15. Development of New Tests

To be effective in the control of medication abuse in racing horses, new tests must be developed and brought on line at a significant rate. As pointed out above, a broad range of tests must be available and a mechanism in place to develop, evaluate and bring on line such tests. We believe, based on our experience in this field, that tests such as those that we have developed (Table III), described in the literature (see references), have under market evaluation (Table IV), and are in the process of developing (Table V), demonstrate both the feasibility and practicality of using non-isotopic immunoassays to control the abuse of high potency drugs in racing horses.

TABLE III

Assays Currently Available[*]

Drug	ELISA Format	FCFIA Format
Buprenorphine	X	X
Etorphine		X
Morphine and related opiates (oxy- and hydromorphone, levorphanol, etc.)	X	X
Tricyclic anti-depressants	X	X
Promazine tranquilizers (acepromazine, propiopromazine, etc.)	X	X
Fentanyl (Sublimaze®) family	X	X
Sufentanil Specific	X	X
Carfentanil Specific	X	X
Meperidine		X
Phencyclidine		X
Mazindol (Sanorex®)	X	X
Amphetamines	X	X
Cocaine		X
Lidocaine and related compounds		X
Methadone	X	X
THC		X
Barbiturate family(Surital® etc.)	X	X
Benzodiazepines (Valium®, Librium® etc.)	X	X
Ibuprofen		X
Reserpine and Yohimbin		X
Ethacrynic Acid	X	X
Bumetanide	X	X
Furosemide (Lasix®) (Quantitative Blood Test)		X
Detomidine		X
Phenylbutazone (Quantitative Blood Test)		X

[*]All assays/tests listed above are either available from or under development by International Diagnostic Systems Corp., St. Joseph, Michigan.

16. Costs of Test Development

While it is clear that the cost of developing an immunoassay test is greater than the minimal cost of developing a TLC test, these costs must be measured against the investment that the racing industry makes in each "hard" drug "positive" that it calls. The national average rate for "hard" drug calls is about one per one thousand samples tested, and each test costs on average about $38.00 (Tobin, 1986; Tobin *et al.*, 1985). Each "hard drug positive," therefore, represents an approximately $38,000 investment by the industry. Viewed in this perspective, the $30,000 to $60,000 investment to create an immunoassay test is much less prohibitive. This is especially so when one considers that the costs

TABLE IV

*Assays in Market Evaluation**

Drug	ELISA Format	FCFIA Format
Glucocorticosteroids (Dexamethasone/Triamcinolone)	X	X
Anabolic Steroids:		
Testosterone	X	X
Boldenone		X
Nandrolone		X
Nortestosterone	X	X
Haloperidol		X
Theophylline		X
Digoxin/Digitoxin		X
Hydromorphone Specific		X
Albuterol Specific		X

*All assays/tests listed above are either available from or under development by International Diagnostic Systems Corp., St. Joseph, Michigan.

TABLE V

*Assays Under Development**

Drug	ELISA Format	FCFIA Format
Butorphanol		X
Oxymorphone Specific		X
Nalbuphine		X
Flunixin		X
Ivermectine		X
Methylphenidate (Ritalin®)		X
Methamphetamine		X
Meteraminol		X
Dextromethorphan		X
Clenbuterol		X
Mephenteramine		X
Ephedrine		X
Phenylpropanolamine		X
Terbutaline		X
Dextromoramide (Palfium®)		X
Hydrochlorothiazide		X
Naproxen		X
Bupivacaine		X

*All assays/tests listed above are either available from or under development by International Diagnostic Systems Corp., St. Joseph, Michigan.

of developing these tests continue to drop as experience is gained in their development. Beyond this, development costs can be amortized against a large number of positives worldwide over a period of up to ten or more years.

If one is considering these immunoassays for use in pre-race testing, the cost considerations are favorable to the point of being compelling. Based on the fragmentary evidence available, we estimate the cost of calling a single pre-race "hard" drug positive with TLC technology as being in excess of $200,000 per positive. Since this is about five times the cost of developing a single immunoassay test, it makes no sense whatsoever to attempt pre-race testing without exploring this technology.

17. Technical Challenges

While the application of ELISA and FCFIA testing to racetrack testing is only about one year old, the general pattern of development of these tests is clear. When a new test for a drug is developed and introduced into an unsuspecting population, the initial positive call rate is likely to be high. While this will vary with the patterns of drug abuse, which show regional and national variations, it is not unusual for one to five percent or more of samples to be positive if a drug is being widely abused. This was our experience with these tests when they were introduced in the Western United States, and a broadly similar pattern was found in England when anabolic steroid tests were introduced (Moss and Haywood, 1984). Thus, one may expect a short run of positives, initially at about the five percent rate, which then drops rapidly to zero. If frozen samples are available, as was the case in New Mexico and Illinois, then the tests can be extended backwards in time to detect past violators, and to very dramatically and unequivocally establish patterns of abuse.

After initial introduction of the test, the real technical challenge begins. The users of illegal drugs may choose to substitute new drugs, to reduce the doses of drugs, to use structurally related agents, or to use furosemide to dilute out the sample. Under these conditions, immunoassay "hits" become rarer, background noise more difficult to distinguish, and immunoassay testing becomes hard work.

We have long found it useful to "marry" immunoassay to other techniques. With our iodinated radioimmunoassays for etorphine and fentanyl, we routinely subjected our immunoassay "hits" to hydrolysis and a second immunoassay (Tai *et al.*, 1988; Woods *et al.*, 1986). If the "hit" survived this second screen, it was considered a candidate for GC/MS. With ELISA assays, it is very productive to marry ELISA and TLC to obtain further information about an immunoassay "hit." In evaluating the acepromazine positives, we found that by combining TLC and ELISA we could locate the metabolites of acepromazine that were giving the ELISA positives, and with this information guide our GC/MS confirmation. We are currently investigating this marriage of TLC and ELISA for work with other drugs, and have chosen to call it "ELISA fingerprinting."

ELISA fingerprinting is carried out in the same way as regular TLC, except that "visualization" is by means of ELISA. In standard TLC, drugs are visualized by oversprays

which react chemically with the drug or drug metabolite. This approach is useful only if the amount of drug on the TLC plate is large. It is not useful if high potency drugs have been administered to the horse and the amount of drug on the plate is small. A logical solution to this problem is to use the detecting power of ELISA to localize the drug or drug metabolite on the TLC plate. To do this, the TLC plate is divided into ten zones and each zone scraped and the drug or drug metabolite eluted from the gel with solvent. The TLC pattern of fingerprint obtained is then compared with that of an authentic drug administration. If the TLC fingerprint from the test horse matches the fingerprint from the control horse, this is strong presumptive evidence that the material in the urine is related to the drug for which the sample has been flagged.

Carfentanil and sufentanil are drugs suspected of being abused in racing horses and for which we have developed an ELISA test. Table VI shows an ELISA fingerprint for a

TABLE VI

ELISA Fingerprinting of a Fentanyl Family Flagged Sample

A post-race sample "flagged" positive for fentanyl family was hydrolyzed and subjected to the analysis along with carfentanil, sufentanil and negative control urines. The numbers are the optical density at 650nm. The presence of ELISA inhibiting materials in scrapes 8, 9 and 10 is consistent with the track sample containing fentanyl related materials.

TLC Fraction	Negative Control Horse Urine	50 µg IV Carfentanil Horse Urine	100 µg IV Sufentanil Horse Urine	Post-race Sample
1	.658	.569	.545	.551
2	.652	.653	.655	.628
3	.632	.645	.632	.651
4	.638	.629	.635	.642
5	.691	.630	.652	.635
6	.651	.642	.631	.615
7	.662	.631	.625	.619
8	.637	.459	.248	.298
9	.672	.237	.125	.219
10	.639	.388	.240	.300

racetrack sample which tested positive for a carfentanil/sufentanil-like drug. However, although this sample is indistinguishable from a carfentanil dosed horse urine sample and is, therefore, strongly suspect for carfentanil or a related substance, no confirmation method for carfentanil in horse urine exists. At this time, therefore, the presence or absence of carfentanil in a post-race sample cannot be confirmed for lack of an effective confirmation technique. The second half of the technical challenge, developing confirmation methods for high potency drugs such as carfentanil, remains to be completed for this and for many other high potency drugs abused in racing horses.

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The pressing need for work on GC/MS confirmation methodologies was brought home by a recent sequence of events in Kentucky. Recently, we detected and confirmed a low concentration (1 ng/ml) morphine positive and sent it to another laboratory for referee analysis. This laboratory reported the sample negative, and had to be directed through a confirmation procedure that was sufficiently sensitive to confirm the presence of morphine. Clearly, the industry's GC/MS confirmation methods need to be both expanded and increased in sensitivity to keep up with the advances under way in drug screenin. Two technologies that offer particular promise in this area are the use of XAD resins and the development of affinity columns for specific drugs.

18. False Negatives, Sample Pooling, "False Positives"

If the advent of ELISA testing has shown one thing, it is that the medication control problem faced by racing is the problem of false negatives. Re-analysis of the frozen samples in New Mexico showed large numbers of false negatives, based on TLC testing. Retesting of frozen samples from other states has shown similar results. The overwhelming lesson of drug testing is that if a drug is not detectable, then the drug will be widely used. This puts honest horsemen in an unfortunate position. They have the choice of obeying the rules and being disadvantaged, or they can use illicit medications. As shown elsewhere, up to ten percent of samples can test positive for certain drugs when a new test is introduced without warning, suggesting that substantial abuse of undetectable medications does occur.

In attempts to extend the range and reduce the costs of immunoassay testing, some laboratories "pool" samples. Pooling of samples occurs when several samples are combined and the resulting pool screened. Then if the pool turns up positive, the individual samples are re-screened to identify the positive sample. The objective is to extend the scope and reduce the costs of immunoassay testing. We believe that pooling during routine screening is false economy, since the pooling of samples invariably reduces the scope of immunoassay testing and increases the numbers of false negatives obtained.

The only circumstance under which pooling is acceptable is when samples are being screened for a specific drug, with a known sensitivity cutoff. If, under these circumstances, the sensitivity of the test is sufficient to allow pooling, then the technique will be effective. A classic example of such a circumstance is the large directed searches for fentanyl-suspects conducted by one of us (John McDonald) on stored frozen samples. On the other hand, the ^{3}H-etorphine assay could not be used in this way, because the assay was not sensitive enough to allow pooling of samples.

The problem with pooling of samples in routine screening is that one is not using the full scope of the immunoassay. For example, when one of us (T.T.) used an iodinated assay for fentanyl, he found that this very sensitive test readily allowed the pooling of a day's racing samples. In this way, a week's worth of samples could be screened for fentanyl in one day, leading to large economies of labor (Woods *et al.*, 1986). In retrospect,

however, pooling firmly eliminated the possibility of this assay picking up a sufentanil positive, which drug would have "gone through" the pooled screen, and, thus, yielded false negatives for this drug.

When our opiate ELISA was introduced, a similar false negative situation would have occurred for oxymorphone if we had chosen to pool samples. The antibody in this test was raised to morphine, and oxymorphone is a cross-reacting substance with substantially less reactivity in the test. If we had pooled samples submitted to our opiate screen, we would not have detected the oxymorphone positives, and, thus, reported false negatives. The rule in screening, therefore, is that *One Must Always Use the Test in its Most Potent Configuration* (i.e., on undiluted samples*), and follow up all ELISA "hits" vigorously, for they may well be reactions to structurally related agents which one would otherwise report as false negatives. The relatively low cost of ELISA tests and the ease and speed with which these tests can be performed eliminates the major argument for pooling, which was the labor and expense of radioimmunoassay. With multiple ELISA tests now available, it is far better strategy to screen each individual sample, and to change the screens at regular intervals if nothing of interest shows up.

It is to be expected that these highly sensitive ELISA tests that cross-react with structurally related drugs will yield ELISA "hits" that are difficult to confirm. In the first place, as our experience with the morphine positives made clear, not all laboratories operate at a sufficient level of sensitivity to routinely confirm ELISA positives. Confirmation methodologies developed to confirm TLC positives are clearly insufficiently sensitive to confirm ELISA positives, which are up to 1000 times more sensitive than TLC. In the second place, since ELISA tests cross-react with other drugs†, the assays will yield "hits" in response to members of drug families which are unidentified and for which confirmation methods have never been developed. Complicating the picture further is the fact that these drugs will be metabolized by unknown pathways, and these unknown metabolites will yield responses in the ELISA and FCFIA assays. For these reasons, samples cannot be declared drug free until they have been ELISA tested and shown to be clean. If samples yield ELISA "hits" that are difficult to confirm, then these samples become the subject of further investigation. To label them "false positives" is completely misleading, since their drug status is unknown. They are simply unconfirmed ELISA "hits," and may be due to cross-reactivity with a non-drug substance or, more likely, to a hitherto undetected pattern of drug abuse, or metabolism.

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*When a chemist pools samples, he dilutes them, performing gratis for the horseman the exercise that furosemide detention barns are built to prevent.

†Although an immunoassay is raised to a specific drug, most antibodies cross-react well with related drugs, and the tests should be thought of as reacting with drug families. For example, our acepromazine antibody has a higher affinity for chlorpromazine and propiopromazine than acepromazine.

References

Clay, S., Woods, W.E. Rowse, K. Nugent, T.E., Blake, J.W. and Tobin, T. The pharmacology of furosemide in the horse. V. Pharmacokinetics and blood levels of furosemide after intravenous administration. Drug Metab. Disposition *11*: 226-231 (1983).

Jolley, M.E.: Principles of particle concentration fluorescence immunoassay. Pandex Res. Rep. No. 1. pp. 1-2. July (1983).

Jolley, M.E., Wang, C.-H., Ehrenberg, S.J., Zudin, M.W. and Kaiso, D.M.: Particle concentration fluorescence immunoassay (PCFIA): a new, rapid immunoassay technique with high sensitivity. J. Immunol. Method *67*: 21-35 (1984).

Kwiatkowski, S., Goodman, J.P., Stanley, S.D., Yang, J.-M., Wood, T., Sturma, L., Woods, W.E., Watt, D.S., Wang, C.-J., Tai, H.-H., Weckman, T.J., Chang, S.-L., Blake, J.W., Tobin, T., Wie, S., Chang, R.A., Brecht, J.M., Conner, J.C., Dahl, P.A., Lewis, E.L., Prange, C.A., McDonald, J., Kalita, S., Bass, V.D., Wenge, P. and DeLeon, B.: Immunoassay detection of drugs in racing horses. X. Detection of phenylbutazone in equine blood and urine by particle concentration fluoroimmunoassay. In draft (1988a).

Kwiatkowski, S., Sturma, L., Dai, M.R., Tai, H.-H., Watt, D.S., Tai, C.L., Woods, W.E., Weckman, T.J., Yang, J.-M., Wood, T., Chang, S.-L., Blake, J.W., Tobin, T., Prange, C.A., Brockus, C., Stobert, D., Wie, S., Chang, R.A., McDonald, J., Bass, V.D., Merchant, S. and Artemenko, M.: Immunoassay detection of drugs in racing horses. VII. Detection of acepromazine in equine urine and blood by ELISA and PCFIA. Res. Commun. Chem. Pathol. Pharmacol. *61*: 391-412 (1988b).

McDonald, J., Gall, R., Wiedenbach, P., Bass, V.D., DeLeon, B., Brockus, C., Stobert, D., Wie, S., Prange, C.A., Ozog, F.J., Green, M.T., Woods, W.E., Tai, C.L., Weckman, T.J., Tai, H.-H., Yang, J.-M., Chang, S.-L., Blake, J.W. and Tobin, T.: Immunoassay detection of drugs in racing horses. III. Detection of morphine in equine blood and urine by a one step ELISA assay. Res. Commun. Chem. Pathol. Pharmacol. *59*: 259-278 (1988).

McDonald, J., Gall, R., Wiedenbach, P., Bass, V.D., DeLeon, B., Brockus, C., Stobert, D., Wie, S., Prange, C.A., Yang, J.-M., Tai, C.L., Weckman, T.J., Woods, W.E., Tai, H.-H., Blake, J.W. and Tobin, T.: Immunoassay detection of drugs in horses. I. Particle concentration fluoroimmunoassay detection of fentanyl and its congeners. Res. Commun. Chem. Pathol. Pharmacol. *57*: 389-407 (1987).

Moss, M.S. and Haywood, P.E.: Survey of positive results from racecourse antidoping samples received at Racecourse Security Services' Laboratory. Equine Vet. J. *16*: 39-42 (1984).

Ozog, F.J., Green, M.T., McDonald, J., Gall, R., Wiedenbach, P., Bass, V.D., DeLeon, B., Brockus, C., Stobert, D., Wie, S., Prange, C.A., Yang, J.-M., Tai, C.L., Dai, M.-R., Woods, W.E., Weckman, T.J., Wood, T., Tai, H.-H., Blake, J.W. and Tobin, T.: Immunoassay detection of drugs in racing horses. XI. Detection of etorphine and buprenorphine in equine blood and urine by radioimmunoassay, particle concentration fluoroimmunoassay and enzyme-linked immunosorbent assay. In draft (1988).

Pradelles, P., Grassi, J. and Maclouf, J.: Enzyme immunoassays of eicosanoids using acetylcholine esterase as label: an alternative to RIA. Anal. Chem. *57*: 1170-1173 (1985).

Prange, C.A., Brockus, C., Stobert, D., Wie, S., McDonald, J., Gall, R., Wiedenbach, P., Bass, V.D., DeLeon, B., Ozog, F.J., Green, M.T., Woods, W.E., Tai, C.L., Dai, M.R., Weckman, T.J., Tai, H.-H., Yang, J.-M., Chang, S.-L., Blake, J.W. and Tobin, T.: Immunoassay detection of drugs in racing horses. V. Detection of mazindol in equine blood and urine by a one step ELISA assay. Res. Commun. Subst. Abuse *9*: 13-30 (1988a).

Prange, C.A., Brockus, C., Wie, S., Chang, R.A., Brecht, J.M., Conner, J.C., Dahl, P.A., Lewis, E.L., McDonald, J., Kalita, S., Bass, V.D., Wegge, P., DeLeon, B., Kwiatkowski, S., Goodman, J.P., Sturma, L., Stanley, S.D., Yang, J.-M., Woods, T., Henry, P., Weckman, T.J., Woods, W.E., Watt, D.S., Tai, H.-H., Chang, S.-L., Blake, J.W. and Tobin, T.: Immunoassay detection of drugs in racing horses. VIII. Detection of cocaine in equine blood and urine by particle concentration fluorescence immunoassay. Res. Commun. Subst. Abuse. *10*: 1-14 (1989).

Prange, C.A., Wie, S., Brockus, C.L., Dahl, P.A., Lewis, E.L., Brecht, J.M., Conner, J.C., Chang, R.A., McDonald, J., Bass, V.D., Merchant, S., Kwiatkowski, S., Woods, W.E., Watt, D.S., Tai, H.-H., Blake, J.W., Chang, S.-L. and Tobin, T.: Screening for human drugs of abuse by particle concentration fluorescence immunoassay (PCFIA). Res. Commun. Subst. Abuse *9*: 129-156 (1988b).

Tai, C.L., Wang, C.-J., Weckman, T.J., Popot, M.A., Woods, W.E., Yang, J.-M., Tai, H.-H., Blake, J.W. and Tobin, T.: Radioimmunoassay for etorphine in horses with a ^{125}I analog of etorphine. Amer. J. Vet. Res. *49*: 622-628 (1988).

Tobin, T.: *Drugs and the Performance Horse*. Charles C. Thomas. Springfield, IL. pp. 190-193 (1981).

Tobin, T.: Medication control: the efficacy and cost of drug testing. J. Equine Vet. Sci. *6*: 333-336 (1986).

Tobin, T., Maylin, G.A., Henion, J., Woodward, C., Ray, R., Johnston, J. and Blake, J.W.: An evaluation of pre- and post-race testing and blood and urine testing in horses. J. Equine Med. Surg. *3*: 85-90 (1979).

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Tobin, T., Tai, H.-H., Tai, C.L., Houtz, P.K., Dai, M.R., Woods, W.E., Yang, J.-M., Weckman, T.J., Chang, S.-L., Blake, J.W., McDonald, J., Gall, R., Wiedenbach, P., Bass, V.D., Ozog, F.J., Green, M., Brockus, C., Stobert, D., Wie, S. and Prange, C.A.: Immunoassay detection of drugs in racing horses. IV. Detection of fentanyl and its congeners in equine blood and urine by a one step ELISA assay. Res. Commun. Chem. Pathol. Pharmacol. *60*: 97-116 (1988).

Tobin, T., Woods, W.E. and Blake, J.W.: Efficacy and cost of testing for illegal medication in horses. In: *Proceedings of the 6th International Conference of Racing Analysts and Veterinarians*. Edited by Crone, D.L., Hong Kong. pp. 113 - 121 (1985).

Voller, A., Bidwell, D.E. and Bartlett, A.: Enzyme linked immunosorbent assay (ELISA). Bull. WHO *53*: 55-56 (1976).

Wood, T., Tai, C.L., Taylor, D.G., Woods, W.E., Wang, C.-J., Houtz, P.K., Tai, H.-H., Weckman, T.J., Yang, J.-M., Sturma, L., Chang, S.-L., Blake J.W., Tobin, T., Wie, S., Chung, R.A., Brecht, J.M., Conner, J.C., Dahl, P.A., Lewis, E.L., Prange, C.A., McDonald, J., Kalita, S., Bass, V.D., Weege, P. and DeLeon, B.: Immunoassay detection of drugs in racing horses. IX. Detection of detomidine in equine blood and urine by radioimmunoassay. In draft (1988).

Woods, W.E., Tai, H.-H., Tai, C., Weckman, T., Wood, T., Barios, H., Blake, J.W. and Tobin, T.: High-sensitivity radioimmunoassay screening method for fentanyl. Amer. J. Vet. Res. *47*: 2180-2183 (1986).

Woods, W.E., Wang, C.-J., Houtz, P.K., Tai, H.-H., Wood, T., Weckman, T.J., Yang, J.-M., Chang, S.-L., Blake, J.W., Tobin, T., McDonald, J., Kalita, S., Bass, V.D., Weege, P., DeLeon, B., Brockus, C., Wie, S., Chung, R.A., Brecht, J., Conner, J., Dahl, P., Lewis, E., Prange, C.A., Ozog, F.J. and Green, M.: Immunoassay detection of drugs in racing horses. VI. Detection of furosemide (Lasix®) in equine blood and urine by a one step ELISA. Res. Commun. Chem. Pathol. Pharmacol. *61*: 111-128 (1988).

Yang, J.-M., Tai, C.L., Weckman, T.J., Woods, W.E., Tai, H.-H., Blake, J.W., Tobin, T., McDonald, J., Gall, R., Wiedenbach, P., Bass, V.D., DeLeon, B., Brockus, C., Stobert, D., Wie, S. and Prange, C.A.: Immunoassay detection of drugs in racing horses. II. Detection of carfentanil in equine urine by RIA, FCFIA and ELISA. Res. Commun. Subst. Abuse *8*: 59-75 (1987).