ELISA BASED IMMUNOASSAY TESTS IN POST-RACE TESTING*

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SUMMARY

We have developed and applied enzyme linked immunoassay testing to post-race testing in race horses in North America. These tests are rapid, inexpensive, and highly sensitive. They can be read with inexpensive spectrophotometer or by eye. At this time we are developing a panel of these tests for use in pre- and post-race testing, in which these tests have turned out to be particularly effective.

When these tests were introduced into post-race testing in the western United States they immediately began to detect the presence of illegal medications that were not being detected by the generally used thin layer chromatographic (TLC) screening systems. Multiple positives for buprenorphine, oxymorphone, sufentanil, mazindol, cocaine and acepromazine were detected and their presence confirmed. In one major race four of ten starters were medicated with oxymorphone. This level of drug use in racing horses in the western United States has led to a reevaluation of the effectiveness of TLC and to a renewed research effort in the area of non-isotopic immunoassay.

Current work in this area is directed at expanding the range and scope of the immunoassays available. We propose to develop immunoassay tests for any high potency illegal drug that is abused in racing horses, and also to develop quantitative immunoassays for drugs such as phenylbutazone and furosemide for which certain residue levels are legal.

INTRODUCTION

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 thin layer chromatographic (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; Woods et al., 1986; Wood et al., 1988). 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.

Initially this immunoassay based testing system was directed towards improving pre-race testing but as events turned out its first major impact was to be in post-race testing.

ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA) AND POST-RACE TESTING

While we were working on the pre-race tests described previously by Mr. McDonald, 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, Mr. 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., 1988). Because the State of New Mexico was drawing from stored frozen samples, the positives

continued to pile up, and 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 persuaded that the enzyme-linked immunosorbent assay (ELISA) test we had developed (Tobin et al., 1988a) was the most satisfactory format for urine testing. The ELISA tests could be performed on raw urine, while the PCFIA tests required an extraction step. Beyond this, the ELISA tests could be read by eye, which made them highly portable, while the particle concentration fluoroimmunoassay (PCFIA) tests of Dr. McDonald 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.

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^{R} (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 ul 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. Following 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 1.

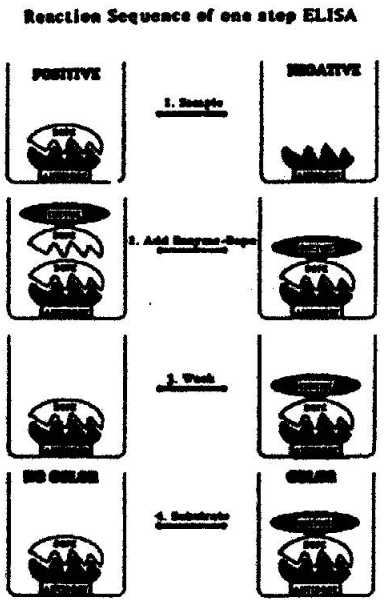

Figure 1. Reaction sequence of the one step ELISA test.

Antibody to the drug is bound to the well, and test and control samples are added directly to the well. In control samples those 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. Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.

The ELISA tests outlined above are particularly effective. For example, Figures 2 and 3 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 gas chromatography/mass spectroscopy (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 (Prange 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.

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., 1988b). 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 (Prange et al., 1988). Overall, the great sensitivity and speed of the ELISA test rendered them highly effective screening tests and far superior to the old TLC screening methods.

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

*National Association of State Racing Commissioners Quality Assurance samples shipped to participating laboratories.

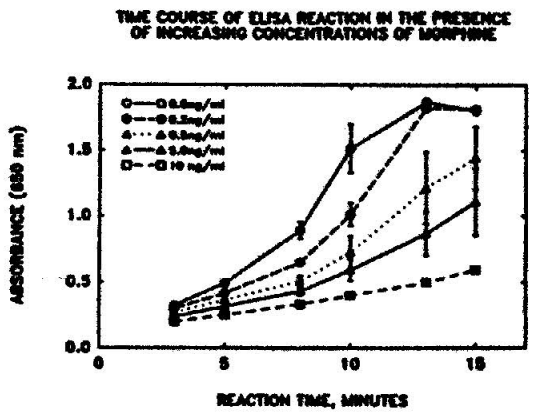

Figure 2. Time course of ELISA reaction in the presence of increasing concentrations of morphine.

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. Pathol. Pharmacol.

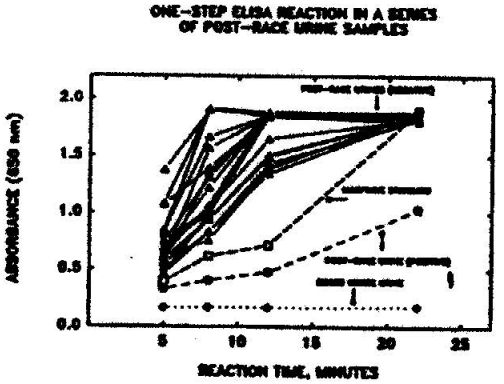

Figure 3. One step ELISA reactions in a series of post-race urine samples.

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 and the solid circles (●-●) show ELISA activity in a sample subsequently determined to contain oxymorphone. Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.

Table I. ELISA screening of post-race urine samples followed by GC/MS analysis.

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	

Post-race urine samples from two racing jurisdictions were screened for morphine and its analogs by the 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. About 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.

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 (>30)
Sufentanil	Oklahoma	No test	10/300*
Mazindol	Western States	Very poor sensitivity	Multiple (>20)
Cocaine	California	Poor sensitivity	2/83*
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 asterick represent the ratio of positives called to total number of samples tested. Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.

**Acepromazine initially detected in pre-race samples.

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) (Chay 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.

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 ninety minutes 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.

PHENYLBUTAZONE QUANTITATION

A similar quantitative ELISA has been developed for phenylbutazone (Kwiatkowski et al., 1988). As shown in Figure 4 and Figure 5, our phenylbutazone assay readily detects levels of phenylbutazone in the low ug/ml level. Since ELISA technology can be automated, this immunoassay can be used as an inexpensive screening test for phenylbutazone. Test samples clearly in excess of the regulatory standard would be assayed by high performance liquid chromatography (HPLC) to unequivocally establish the

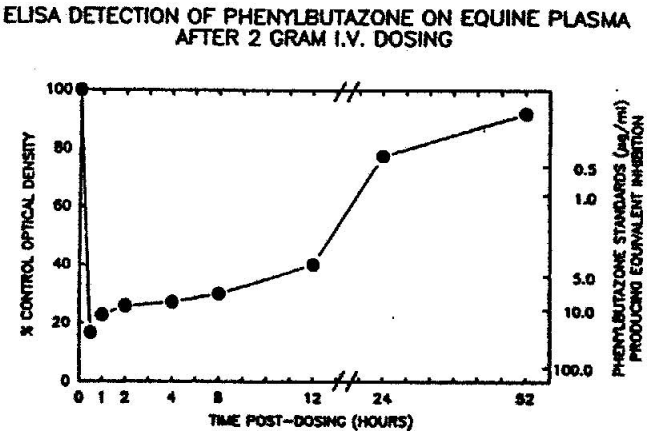

Figure 4. ELISA detection of phenylbutazone on equine plasma after 2 gram IV dosing.

The plasma phenylbutazone equivalents of a horse dosed with 2 grams phenylbutazone IV as measured by ELISA. "Control" optical density was the reading when no drug was added. Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.

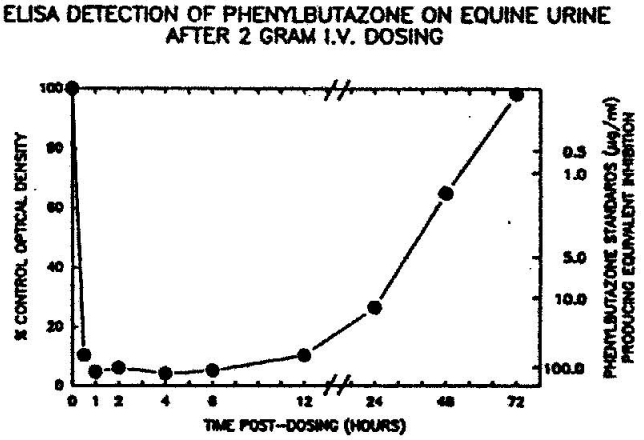

Figure 5. ELISA detection of phenylbutazone on equine urine after 2 gram IV dosing.

The urine phenylbutazone equivalents of a horse dosed with 2 grams phenylbutazone IV as measured by ELISA. "Control" optical density was the reading when no drug was added. Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.

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. In other work we are developing a similar quantitative test for lasix to allow us to eliminate the lasix detention barn.

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 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 to very stringently monitor their compliance with furosemide medication rules if this is deemed necessary.

SUFENTANIL DETECTION

The data of Figure 6 shows the time course and sensitivity to added sufentanil in our ELISA test (Tobin *et al.*, 1988c). In the absence of added sufentanil the reaction rapidly runs to completion, with an apparent absorbance value of about 1.6 being attained between 50 and 60 minutes after starting the reaction. The addition of increasing concentrations of sufentanil up to 2.5 ng/ml acted to increasingly inhibit the reaction.

Because these assays are ususally run on serum and urine samples added directly to the sample wells, we studied the effect of addition of human and equine serum samples to our ELISA system. A small inhibition of the reaction rate was seen after addition of equine urine (Figure 7), and there was little or no inhibition after addition of human urine or equine or human serum.

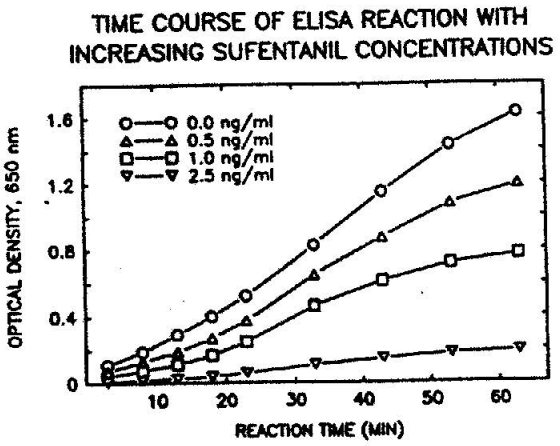

Figure 6. Time course of ELISA reaction with increasing sufentanil concentrations.

The ELISA reaction was inhibited by the addition of increasing concentrations of sufentanil citrate. The sample was 20 µl of phosphate buffer, pH 7.4. Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.

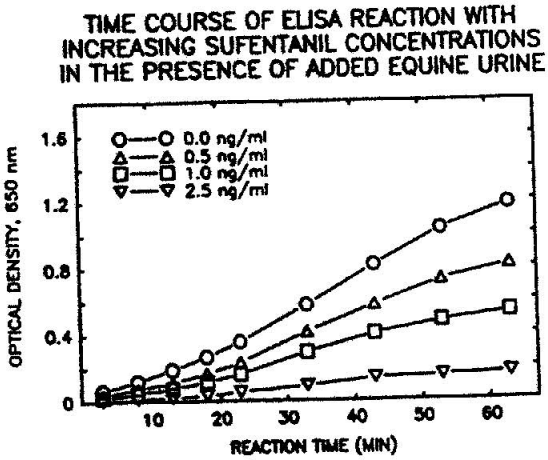

Figure 7. Time course of ELISA reaction with increasing sufentanil concentrations in the presence of added equine urine.

The addition of 20 µl of blank equine urien slowed the ELISA reaction, but the added urine did not reduce the inhbition by the increasing concentrations of sufentanil. Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.

This ELISA tests is sufficiently sensitive to detect sufentanil in equine plasma after administration of small doses of this drug to horses (Figure 8). The ELISA test was almost completely inhibited at five minutes after drug administration but declined rapidly thereafter. By sixty minutes after drug administration inhibition was no longer apparent, consistent with the expected pharmacokinetic pattern of a highly lipid soluble drug.

Urinary detection of sufentanil (Figure 9) followed a broadly similar pattern. For the first two hours after dosing with sufentanil citrate virtually complete inhibition of the ELISA test was observed, with the inhibition declining thereafter at a rate dependent on the dose. When a 0.05 mg dose was administered, the inhibition had returned to control by between 8 and 24 hours after administration. If the dose of sufentanil was larger, the urine contained significant quantites of sufentanil equivalents, producing about 40% inhibition of the ELISA test at 24 hours after dosing (Tobin et al., 1988c).

NEW TEST DEVELOPMENT

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) demonstrates both the feasibility and practicality of using non-isotopic immunoassays to control the abuse of high potency drugs in racing horses.

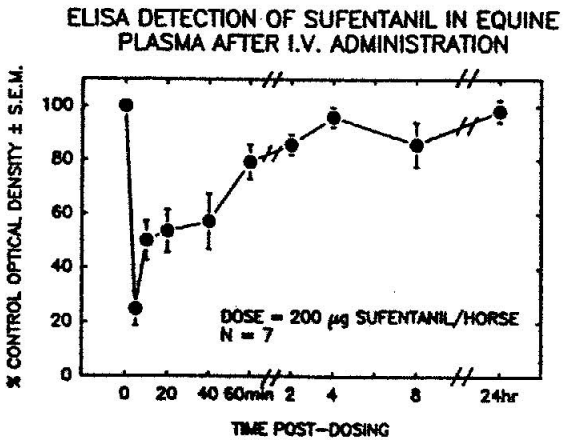

Figure 8. ELISA detection of sufentanil in equine plasma after IV administration.

The plasma sufentanil equivalents of a horse dose with 200 μg sufentanil IV as measured by ELISA. Each sample was assayed 7 times and the interassay standard error of the mean (SEM) was calculated. "Control" optical density was the reading when no drug was added. Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.

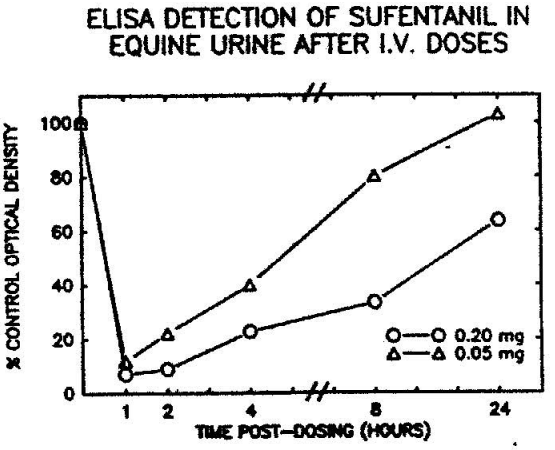

Figure 9. ELISA detection of sufentanil in equine urine after IV doses.

The urine sufentanil equivalents concentrations of 2 horses dosed IV with 200 μg (O-O) and 50 μg/horse (Δ-Δ) sufentanil was determined by ELISA. "Control" optical density was the reading when no drug was added. Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.

Table III. Assays currently available.*

	ELISA Format	PCFIA Format
Buprenorphine	X	X
Eterphine		X
Morphine and related opiates (oxy- and hydromorphone, levorphanol, etc.)	X	X
Tricyclic anti-depressants	X	X
Promazine tranquilizers (acepromazine, propriopromazine, 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

Table IV. Assays in market evaluation.*

	ELISA Format	PCFIA Format
Glucocorticosteroids (Dexamethazone/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
Alfentanil Specific		X

*All assays/tests listed above are either available from or under development by International Diagnostic Systems Corp., St. Joseph, Michigan. Reproduced with permission from Res. Comm. Chem. Pathol. Pharmacol.

Table V. Assays under development.*

	ELISA Format	PCFIA Format
Butorphanol		X
Oxymorphone Specific		X
Nalbuphine		X
Flunixin		X
Isoxsuprine		X
Methylphenidate (Ritalin^{R})		X
Methamphetamine		X
Metaraminol		X
Dextromethorphan		X
Clenbuterol		X
Mephentermine		X
Ephedrine		X
Phenylpropanolamine		X
Terbutaline		X
Dextromoramide (Palfium^{R})		X
Hydrochlorothiazide		X
Naproxen		X
Bupivacaine		X

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 et al., 1985; Tobin, 1986). 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 of developing these tests continues 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.

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

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.

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, and re-testing 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 medication. 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 occurs.

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 rescreened 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 likely 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

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

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 positive 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. Further complicating the picture is that fact that these drugs will be metabolized by unknown pathways and these unknown metabolites will yield responses in the ELISA and PCFIA assays. For these reasons samples cannot be declared drug free until they have been ELISA tested and shown to be clean. If samples yield difficult to confirm ELISA "hits", then these samples become the subject of further research. To label them "false positives" is completely misleading, since their drug status is

*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 propriopromazine than acepromazine.

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.

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