PRE- AND POST-RACE TESTING FOR DRUGS IN RACING HORSES

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SUMMARY

Testing for drugs in horses starts with the taking of the samples. Samples should be split immediately after they are drawn and the referee portion of the sample stored independently of the sample that is to be analyzed. The sample to be analyzed is then shipped in a secure fashion to the laboratory for analysis.

In the analytical process drugs are subjected to liquid/liquid extraction and screened for the presence of illegal medications. The most commonly used screening method is thin layer chromatography. Other screening methods include gas chromatography and high pressure liquid chromatography and, more recently, immunoassay, including non-isotopic immunoassays such as enzyme linked immunosorbent assay (ELISA) and particle concentration fluorescence immunoassay (PCFIA). The immunoassay methods are particularly sensitive and, at least in the ELISA and PCFIA formats, rapid. Due to the sensitivity of immunoassay based screening developed in North America many high potency medications positives have been detected recently.

If a drug is detected in the screening process, its presence in the sample is confirmed by other chromatographic methods, particularly by gas chromatography-mass spectrometry. The qualitative detection of drugs in forensic samples is a developed art, and most drugs can be identified in blood or urine samples with a high degree of accuracy. Drugs can be quantititated in blood or urine with an accuracy of plus or minus 25% or better. These scientific determinations on a sample can be independently verified in the referee samples, and form the scientific basis for the regulatory process of medication control.

INTRODUCTION

Racing has the longest established, most elaborate, and most technically accurate systems for drug testing of any human endeavor (Tobin 1981). The medication of racing

horses was declared illegal by the English Jockey Club about 1903. The first "positive", using frogs as the test animal, and "determining" from their croaks whether the horse was positive, was carried out in Russia in about 1905. The first positive reported by analytical chemistry was called in 1912. Since then, analytical chemistry and drug testing have made major strides and analytical chemistry is now an established discipline. However interpretation of the forensic significance of the findings of an analytical chemist in terms of the types of rules that can be enforced based on the analyst's findings, and, indeed, how these rules should be drafted and interpreted, is a less well-defined area, and the subject of much debate in the industry.

SAMPLE COLLECTION

Both blood and urine should be collected, since the expense of collecting a blood sample is small and blood is the only sample from which drug concentration data can be interpreted with any confidence. Additionally, a decision has to be made as to the nature of the blood sample drawn. While plasma was once the sample of choice for forensic work, the advent of enzyme linked immunosorbent assay (ELISA) and particle concentration fluoroimmunoassay (PCFIA) tests has rendered serum the more satisfactory sample. This is because the presence of proteins in plasma samples can inhibit the ELISA reaction and our experience with these assays suggests that serum is the more satisfactory sample, with less likelihood of non-specific inhibition of the ELISA system. Alternatively, plasma samples can be extracted to avoid the interfering problems with plasma proteins and maintain the efficacy of ELISA testing.

Urine samples need to be drawn into a chemically clean container, and our preference is for a sample which does not contain preservatives. If the urine sample is stored cold and shipped to the laboratory promptly, there should be no significant problems with changes in the urine sample. Additionally, the addition of preservatives interferes with the sample, likely altering its pH, which may be important in the eventual interpretation of the significance of the results of the tests. Beyond this, drugs such as furosemide or other diuretics should not be used to obtain a urine sample since they act to dilute certain drugs and drug metabolites in equine urine, and are therefore likely to interfere with the testing process.

BLOOD VERSUS URINE

The backbone of drug testing in North America today is post-race urine testing, with the utilization of blood testing on the increase (Blake and Tobin 1986). Urine testing is generally superior to blood testing as urine is available in relatively large amounts (200 ml plus), tends to contain higher levels of the parent drug than does the corresponding blood sample, and almost invariably contains much greater concentrations of drug metabolites than a corresponding blood sample. On the other hand, urine is slow and difficult to collect, and because of the lack of correlation between blood and urinary concentrations of drugs and drug metabolites, it is virtually impossible to determine the forensic significance of a given urinary concentration of a drug.

On the other hand, blood samples are easy to collect, and once a drug is identified and quantitated in blood, one can usually estimate its pharmacological effect with a reasonable degree of accuracy. The principal problem with blood testing is that the volume

of sample is small and the concentration of drug available in the sample, and especially the concentration of drug metabolites, tends to be small. This is a major problem with blood testing, and it means that, given the current state-of-the-art, blood testing must always be used in conjunction with post-race urine testing for effective medication control.

PRE-RACE TESTING AND POST-RACE TESTING

PRE-RACE TESTING

Pre-race testing is based entirely on blood sampling in North America, although at one time pre-race testing in Hong Kong was based on urine sampling. The blood sample is drawn at a period of two to four hours before the race and subjected to screening and, if possible, gas chromatography- mass spectrometry (GC/MS) analysis. In theory, pre-race testing allows the chemist to detect a medicated horse before it runs, scratch the horse, and in this way prevent the running of an illegally medicated horse. Pre-race testing is thus seen as the ultimate drug testing strategy, and one that can actually prevent the use of medication to manipulate the betting payoff, which post-race testing cannot do (Tobin *et al* 1979).

The problem with pre-race testing is that, until very recently, the testing technology has not been sensitive enough to detect the use of "hard", or illegal, medications pre-race. Using thin layer chromatographic (TLC) screening systems, acidic drugs such as phenylbutazone and furosemide can be detected but, as a general rule, TLC-based testing does not have sufficient sensitivity to detect the abuse of basic illicit drugs pre-race. This is all the more so because horses are post-race tested for illegal medications and no medication that can be detected readily in urine is likely to be used. This restricts the use of illegal medications to relatively potent drugs that are unlikely to be detected in urine, and if a medication is undetectable in urine it is, in general, highly unlikely to be detectable in blood by TLC. For this reason pre-race testing based on TLC has had a very poor record of detecting hard, or illegal, medications pre-race, and the concept of pre-race testing requires a much more sensitive drug detection technology than TLC-based testing. It was to answer this need for more sensitive pre-race testing that the non-isotopic immunoassay tests of ELISA and PCFIA were initially introduced.

Currently, the state of Kentucky takes post-race blood and urine samples, and testing in Kentucky is based on both of these analyses. Once drawn, the samples are shipped in a secure container to the laboratory at the University of Kentucky, where they arrive the next day. The box is opened in the presence of a witness, the volume and pH (acidity) of each sample noted, and the analytical process begun.

CHEMICAL ANALYSIS OF THE SAMPLE

Classical chemical analysis of a blood or urine sample is a three step procedure. The first step is extraction of the drug from the blood or urine, the second step is the screening of the sample for suspected drugs, and the third step is confirmation of the presence of the drug. The first step in this analysis is the extraction process done by a procedure called liquid-liquid extraction.

LIQUID-LIQUID EXTRACTION

Liquid-liquid extraction is based on the transfer of the drug from blood or urine (aqueous phase) into a solvent that dose not mix with water. Liquid-liquid extraction of drugs follows the extraction rule (Blake and Tobin 1986). By this rule, acidic drugs extract

under acidic conditions, and basic drugs extract under basic conditions. To implement this rule, the analyst takes small portions of the sample (usually about 2 to 3 ml) and makes them either acidic or basic. To make the urine acidic, about 5 ml of an acidic buffer is added which changes its pH value to about 4.0. To make the urine basic, a few drops of ammonium hydroxide are added, which will change the pH of the urine to about 9.0.

To extract the drug, an organic solvent, such as dichloromethane is added, and the sample placed on a mechanical shaker for about 5 minutes or more. For the acidic sample, acidic drugs are contained in the dichloromethane, while for the basic sample, basic drugs are contained in the dichloromethane. The sample is then centrifuged to allow the dichloromethane to separate from the aqueous layer, which is pipetted off. The drugs are now contained in this dichloromethane layer, which is evaporated down to a small volume in order to concentrate the drugs. This small volume will contain any drugs extracted from the urine, and at this point the chemist is ready to submit the extraction to drug screening procedures.

DRUG SCREENING

The screening tests that the chemist uses have been, until recently, almost invariably chromatographic tests. In chromatography, the drug is placed in a mobile phase, which moves past a stationary phase. Depending on the affinity of the drug toward the stationary phases, and thus the amount of time that the drug spends on the stationary phase, the drug may move right along with the mobile phase, may stay stuck to the stationary phase, or may be anywhere in between. Based on this principle, the chromatography may be performed on thin layer plates, or in a gas or liquid chromatographic system. However, by far the most commonly used screening system is TLC (HPTLC for High Performance Thin Layer Chromatography).

THIN LAYER CHROMATOGRAPHY

In thin layer chromatography, the urine extract is spotted onto a thin layer of silica (generally less than 1 mm thick) on a glass plate, alongside with appropriate standards. The plate is then placed in a glass tank and "developed" by allowing a solvent mixture to run up the plate by capillary action. As the solvent (mobile phase) runs up the plate, the different drugs in the sample move along the plate at different rates, characteristic of the drug and dependent on TLC conditions. However, in the last analysis, they still give only one single piece of information about the drug, which is that it chromatographs in the same way as the standard. In contrast, a technique that allows one to actually fragment a drug molecule and see what it is made of is gas chromatography/mass spectrometry.

IMMUNOASSAY BASED TESTING

While TLC based testing is relatively inexpensive, broad in scope, and sufficiently sensitive to allow the detection of many medications, particularly in urine, there are a number of medications which are difficult to detect by TLC in blood or urine. For these drugs the only testing modality with the requisite sensitivity and flexibility has generally been immunoassay, and immunoassay has been suggested to be the most practical approach to the problems of equine medication control (Tobin *et al* 1988). This is especially true in the case of pre-race testing, where the volume of sample available is small and the concentration of drug present in the sample low. For these reasons, the sensitivity of immunoassay techniques renders this a very attractive technology and about

two years ago we began a broad scale approach to the challenge of developing immunoassays for use in equine drug testing. Since it is conceptually and practically the simplest testing format, we will restrict this discussion to ELISA tests, although other non-isotopic test formats are also available.

Performing an ELISA test is relatively simple. As shown in Figure 1, the antibody to the drug is bound to the bottom of the test well. 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-horseradish peroxidase (drug-HRP) solution. During this step, the presence in the sample of free drug or cross-reacting drugs or metabolites competitively prevents the antibody from binding the

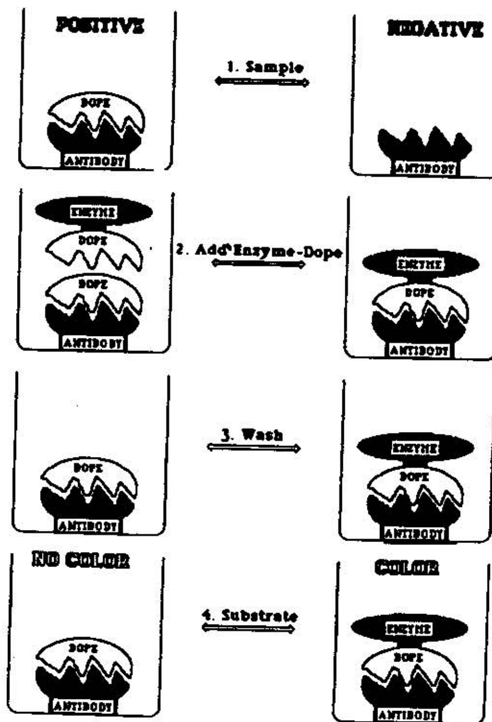

Figure 1:

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.*

drug-HRP conjugate. The degree of antibody:drug-HRP binding is therefore inversely related 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. During this process the antibody and bound drug remain fixed to the bottom of the wells. Substrate (tetramethylbenzidine) is then added to all wells, a color-producing reaction occurs between the substrate and antibody-bound drug-HRP enzyme in the wells, and their absorbance read at 560 nm in an microwell reader. Higher optical absorbance corresponds to lower drug concentration in the sample. A diagram outlining this sequence of events is presented schematically in Figure 1.

These ELISA tests can be particularly potent and effective in drug detection. They can be as sensitive as radioimmunoassays (RIA), can be completed very rapidly, and a good ELISA is comparable to a RIA in terms of accuracy.

A morphine ELISA is paricularly effective in terms of detection of opiates. 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 by GC/MS (McDonald *et al* 1988).

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 analogues by the ELISA test and then subjected to gas chromatography/mass spectrometry (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. Path. Pharmacol.*

Similar patterns of positives were seen across the Western United States whenever these immunoassay 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* 1988). The efficacy of these ELISA tests in racing chemistry was clear and their ability to control the use of high potency medications established.

Establishing the efficacy of PCFIA and ELISA based immunoassays (Table II), exposed deficiencies in TLC as a screening methodology. No TLC method for buprenorphine existed, so use of this drug was complete uncontrolled. Similarly, sufentanil abuse was uncontrolled and even "bragged on" by horsemen until the advent of this technology. 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-detectable 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 tests rendered them highly effective screening tests and far superior to TLC as a screening method for high potency drugs.

TABLE II.
Efficacy of PCFIA and ELISA Tests

Drug	State	TLC Status	Immunoassay Positives
Buprenorphine	New Mexico	No test	Multiple (>50)
Oxymorphone	New Mexico	Low sensitivity	Multiple (>30)
Sufentanil	Oklahoma	No test	10/300*
Mazindol	Western States	Low sensitivity	Multiple (>20)
Cocaine	California	Low 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 asterisk represent the ratio of positives called to total number of samples tested.

**Acepromazine initially detected in pre-race samples.

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DRUG CONFIRMATION

GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Mass spectrometry (MS) has become the standard instrumental method for the confirmation of the presence and identity of a drug in a sample. After a solvent extraction to partially isolate the drug from the sample, the material to be analyzed is further separated by gas chromatography (GC) or, occasionally, by liquid chromatography (LC) and the separated components from the GC or LC are successively introduced into the vacuum chamber of the mass spectrometer. In the "ion source" of the MS, the sample

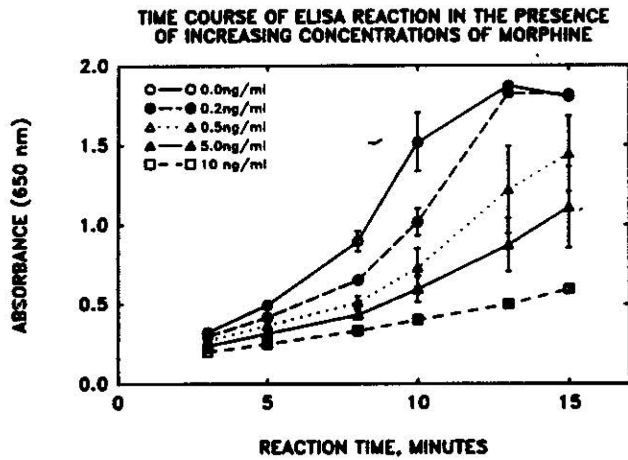

Figure 2:

Time course of ELISA reaction in the presence of inceasing 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.*

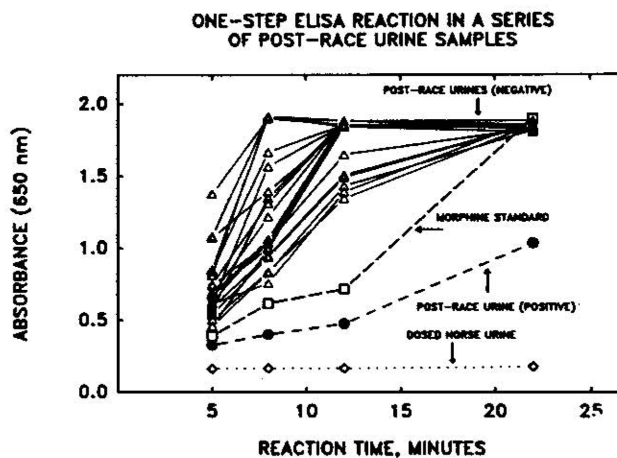

Figure 3:

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

The open triangles (△-△) show the activity in this one step ELISA test of post-race urine samples. The open squares (□-□) show the effect of 0.5 mg/ml of morphine added to this system. The open diamonds (◇-◇) show ELISA activity in a dosed horse urine, and the solid circles (●-●) shows ELISA activity in a sample subsequently determined to contain oxymorphone. Reproduced with permission from *Res. Comm. Chem. Pathol. Pharmacol.*

components are bombarded by a beam of electrons or by a reagent gas such as methane. This charges, or ionizes, the drug molecules and, depending on the severity of impact and stability of the drug molecule, produces either an ion with the molecular mass of the parent drug or molecular fragments. The ions are accelerated through an electromagnetic field, which separates them based on their charge and molecular mass. The ions strike an ion detector and the number of ions at each mass is measured. The graph of ion intensity versus mass is call a mass spectrum.

The mass spectrum is characteristic of the individual particular drug. The pattern produced by the drug and its fragment ions may be visualized as a molecular "fingerprint" and thus the spectrum is routinely accepted as evidence of the drug's identity. The chromatographic characteristics of the drug also add to its confirmation. The mass spectrometer is sensitive down to the level of nanograms (one billionth of a gram) and rapid; it can scan a mass spectrum in a fraction of a second.

A state-of-the-art GC/MS system consists of a GC for sample separation, a mass spectrometer, and a computerized data system to precisely control the instrument and to collect and analyze the chromatographic and mass spectral data. It may also contain a computerized library of reference spectra to aid in the identification of unknown samples.

CALLING A "POSITIVE"

By the time that the chemist has completed the TLC, immunoassay, and GC/MS analyses, sufficient evidence will have been accumulated for the analyst to be persuaded as to the presence of the drug or drug metabolite in the sample. If the medication is a prohibited drug, the analyst is in a position to "call a positive". The act of formally reporting to the authority the presence of a forbidden medication in a sample is to "call a positive." If the analyst does this, the purse is generally not released, and a hearing is scheduled to investigate the positive.

"Calling a positive" occurs when the analyst "positively" identifies the presence of a banned drug in a sample, which is likely to result in substantial penalty to the trainer, and the analyst's findings may be challenged in a formal proceeding. Under these circumstances, the analyst will want to make as good case as possible for the presence of the drug, and the quality of the analytical chemistry should be sufficient to allow unequivocal identification of the drug.

THE REFEREE SAMPLE

As a general rule, the field of analytical chemistry is a rigorous and accurate discipline. If a well-trained chemist with a well-equipped laboratory does the analysis and calls the positive, then the results reported are virtually always repeatable in another qualified lab. However, if the analyst is inexperienced, or not well-trained, or under pressure, then errors can be made, as in any other field of human endeavor.

The most important independent check on the ability and integrity of the chemist is to have available a referee sample, which is an independently sealed and stored sample. If the trainer so desires, this sample can be sent to an analyst of choice, and the analytical work repeated. In this author's experience, work from good laboratories on which positive calls are based is virtually always repeatable. On the other hand, there have been instances where positive calls have not turned out to be repeatable in the hands of an independent chemist, so the precaution of holding a referee sample is important. When the chemist is confident of the quality of the analytical work being done in the laboratory, the analyst should welcome requests for referee samples; these are seen primarily as an opportunity to have the quality of work independently verified.

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