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MEDICATION REFERENCE GUIDE

National Horsemen's Benevolent & Protective Association

"Horsemen Helping Horsemen"

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EQUINE DRUG TESTING
HOW IT WORKS

EDITOR'S NOTE: The following section was adapted with permission from Thomas Tobin, et al., authors of *Testing for Drugs, Medications and other Substances in Racing Horses.*

INTRODUCTION

Racing has the longest established, most elaborate, broadly-based, and technically accurate systems for drug testing of any sport. The medication of racing horses was formally declared illegal by the English Jockey Club in 1903. By 1905, racing officials in Russia hired an "expert" who used frogs as test animals. He determined from their croaking whether or not drugs were present in samples taken from the saliva of race horses. The first medication violation reported using the techniques of analytical chemistry was reported in 1912. Since then, analytical chemistry and drug testing have made major strides. Analytical chemistry is now an established discipline. However, interpretation of the forensic significance of the analytical findings regarding the types of rules that can be enforced and how these rules should be defined, drafted and interpreted, is currently the subject of much debate within the horse-racing industry.

Testing for drugs in horses starts with the collection of samples. Samples, usually of blood and urine, should be split immediately after they are drawn. The sample to be analyzed is then shipped in a secure fashion to the laboratory for analysis. The referee sample, the second portion of the whole sample, should be carefully stored independently of the sample to be analyzed.

In the analytical process, the samples are subjected to a liquid-to-liquid extraction process and are then screened for the presence of illegal substances. The most commonly used screening method is thin layer chromatography (TLC). Other screening methods include gas chromatography (GC), high pressure liquid chromatography (HPLC) , and more recently, immunoassay including enzyme linked immunosorbent assay (ELISA). ELISA screening is particularly sensitive and can be done quickly. Due to the sensitivity of immunoassay-based screening, most high-potency medications are first detected with ELISA screening.

If an agent is detected in the screening process, its presence in the sample is confirmed by other methods, most often by gas chromatography-mass spectrometry (GC/MS). The qualitative detection of drugs in forensic samples is a well developed art. Most drugs can be identified in equine blood or urine samples with a high degree of accuracy. Drugs can be quantitated in blood or urine with an accuracy of plus or minus 25% or better under optimal conditions. 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.

These techniques detect much more than medications administered to horses, and one of the challenges of modern-day equine drug testing is to fairly and equitably distinguish between natural substances in horse urine and medicinal substances.

SAMPLE COLLECTION

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, both blood and urine should be collected. Additionally, a decision must be made regarding the nature of the blood sample drawn. While plasma was once the sample of choice for forensic work, the advent of the enzyme linked immunosorbent assay (ELISA) has made serum the more satisfactory sample. In plasma, the presence

of proteins can inhibit the ELISA reaction. Therefore a test on a serum sample is more accurate 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 to maintain the efficacy of ELISA testing.

Urine samples should be drawn into a chemically clean container. 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.

BLOOD VERSUS URINE

In the United States and Canada, most of the drug testing done on race horses is post-race urine testing. Urine testing is generally superior to blood testing since urine is available in relatively large amounts (200 ml or more per sample). It tends to contain higher concentrations of the parent drug than a corresponding blood sample, and almost invariably, it contains a much greater (50 fold greater) concentration of drug metabolites than a corresponding blood sample. On the other hand, urine is difficult to collect and because of the lack of correlation between blood and urinary concentrations of drugs and drug metabolites, it is sometimes difficult to determine the forensic significance of a given urinary concentration of a drug.

In contrast, blood samples are easier to collect. Once a drug has been 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 sample volume is small. The concentration of drug available in the sample, 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 is always used in conjunction with post-race urine testing for effective medication control.

Currently, for example, the State of Kentucky takes only post-race urine samples and the testing of Kentucky samples is carried out by Truesdail Laboratories, Inc. of Tustin, California. Following the collection of samples at Kentucky tracks, the samples are packed in a secure container and shipped overnight to Truesdail. The box is opened in the presence of a witness, the volume and pH (acidity) of each sample is noted, and the analytical process begins.

PRE-RACE TESTING

Pre-race testing is no longer performed at most tracks in North America. In past years, such testing was based entirely on blood sampling. In classical American pre-race testing, the blood sample was drawn two to four hours before the race and subjected to screening and, if possible, gas chromatography-mass spectrometry (GC/MS) analysis. In theory, the pre-race testing allowed the chemist to detect a medicated horse before it ran, in order for the stewards to have time to scratch the horse and in this way prevent the running of an illegally-medicated horse. In those days, pre-race testing was seen as the ultimate drug testing strategy, one that could actually prevent the use of medication to manipulate the betting payoff, which post-race testing could not do.

The problem with pre-race testing is that the testing technology was never sensitive enough to detect the use of high-potency, illegal medications. Using thin layer chromatographic (TLC) screening for pre-race testing, acidic drugs such as phenylbutazone and furosemide can be detected but, as a general rule, the TLC-based testing did not have sufficient sensitivity to detect the abuse of

high-potency, illicit drugs. Today, race horses are post-race tested for illegal medications so no medication that can be readily detected in urine is likely to be used. The winners of races are tested along with a randomly selected group of the other runners. This restricts the illegal use of medications to relatively potent, quick-acting drugs that are unlikely to be detected in urine.

When racing officials determined that testing using TLC had an extremely poor record of detecting high-potency, illegal medications pre-race, and that the concept of pre-race testing required a much more sensitive drug detection technology than the existing TLC-based testing, a search for better testing methods began. It was largely to answer this need for more sensitive pre-race testing that ELISA tests were initially introduced into chemical analysis.

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 screening 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, which is performed 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 does not mix with water. Liquid-liquid extraction of drugs follows the extraction rule. By this rule, acidic drugs are extracted under acidic conditions and basic drugs are extracted under basic conditions. To implement this procedure, the chemist 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. It changes the pH value of the sample to about 4.0. To make the urine basic, sodium tetraborate is added. It will change the pH of the urine to about 9.0.

To extract the drug, an organic solvent, such as dichloromethane is added to the sample, then it is placed in a mechanical shaker for about five minutes. In an acidic sample, acidic drugs move into the dichloromethane, but in a basic sample, basic drugs move into the dichloromethane. The sample is spun in a centrifuge to allow the dichloromethane to separate from the aqueous layer, which is siphoned off in a pipette. The drugs are now contained in the dichloromethane layer, which is evaporated down to a small volume in order to concentrate the drugs. This small volume will contain all the drugs, therapeutic medications, environmental substances and natural products extracted from the urine, and at this point the chemist is ready to submit the extract to screening procedures.

DRUG SCREENING

Until recently, the screening tests that the chemists use have been almost entirely chromatographic tests. In chromatography, a portion of the extract from the sample is placed in a mobile phase, which moves past stationary phases. Depending on the affinity of a drug toward the stationary phases, and thus the amount of time that the drug spends on a stationary phase, the drug may move right along with the mobile phase, or it may stay immobile on the stationary phase, or may be anywhere in between. The chromatography may be performed on thin layer plates, in a gas, or in

a liquid chromatographic system. The most commonly-used screening systems are thin layer chromatography (TLC) or high performance thin layer chromatography (HPTLC).

THIN LAYER CHROMATOGRAPHY

In thin layer chromatography, the urine extract is spotted onto a thin layer of silica (generally less than one millimeter thick) on a glass plate, alongside a set of standards to compare it to. 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, if there are drugs in the sample they will move along the plate at different rates, depending on the characteristics of the drug and dependent on TLC conditions. However, in the last analysis the test yields only one single piece of information about the drug, which is that it chromatographs and reacts in the same way as the standard.

IMMUNOASSAY-BASED TESTING

While TLC-based testing is relatively inexpensive, broad in scope, and sufficiently sensitive to allow for the detection of many medications, particularly in urine samples, there are many medications that are difficult to detect by TLC in blood or urine. For such drugs, the only testing modality with the requisite sensitivity and flexibility has generally been immunoassay. It has been suggested that using immunoassay is the most practical approach to the problems of equine medication control. This is certainly true in the case of pre-race testing, where the volume of the sample available is small and the concentration of drug present in the sample is low. For these reasons, the sensitivity of immunoassay techniques renders it a very attractive technology. About 12 years ago, a broad scale approach to developing immunoassays for use in equine drug testing was developed.

OR ELISA TESTS

PERFORMING AN ELISA TEST

Performing an ELISA test is relatively simple. If the urine sample contains the drug or metabolite that you are testing for, they will bind tightly to the anti-drug antibody in the ELISA test well. The indicator contains the same drug bound to an enzyme (drug-enzyme complex). After the mixture sits in the well for a few minutes, the plate is gently washed. The wells containing drug-free horse urine will bind to the drug-enzyme complex, and these wells will turn blue when the enzyme substrate is added. The blue color indicates a "negative" test, one that contained no apparent drug bound to the antibody.

On the other hand, the wells that were filled with samples that contained a drug, will not turn blue because the drug-enzyme-complex will not bind to the antibody. When the substrate is added such samples stay clear. In the laboratory jargon, they are called "whiteouts."

A "whiteout" means that the sample contained a material that bound to the antibody and prevented the indicators from binding. Such samples are ELISA "positive", and as such, they are candidates for more rigorous testing.

These reaction steps are set forth in Table 1, which is reproduced with permission from Thomas Tobin, et al., the authors of *Research Communications in Chemical Pathology and Pharmacology*.

TABLE 1

Reaction Sequence of One Step ELISA

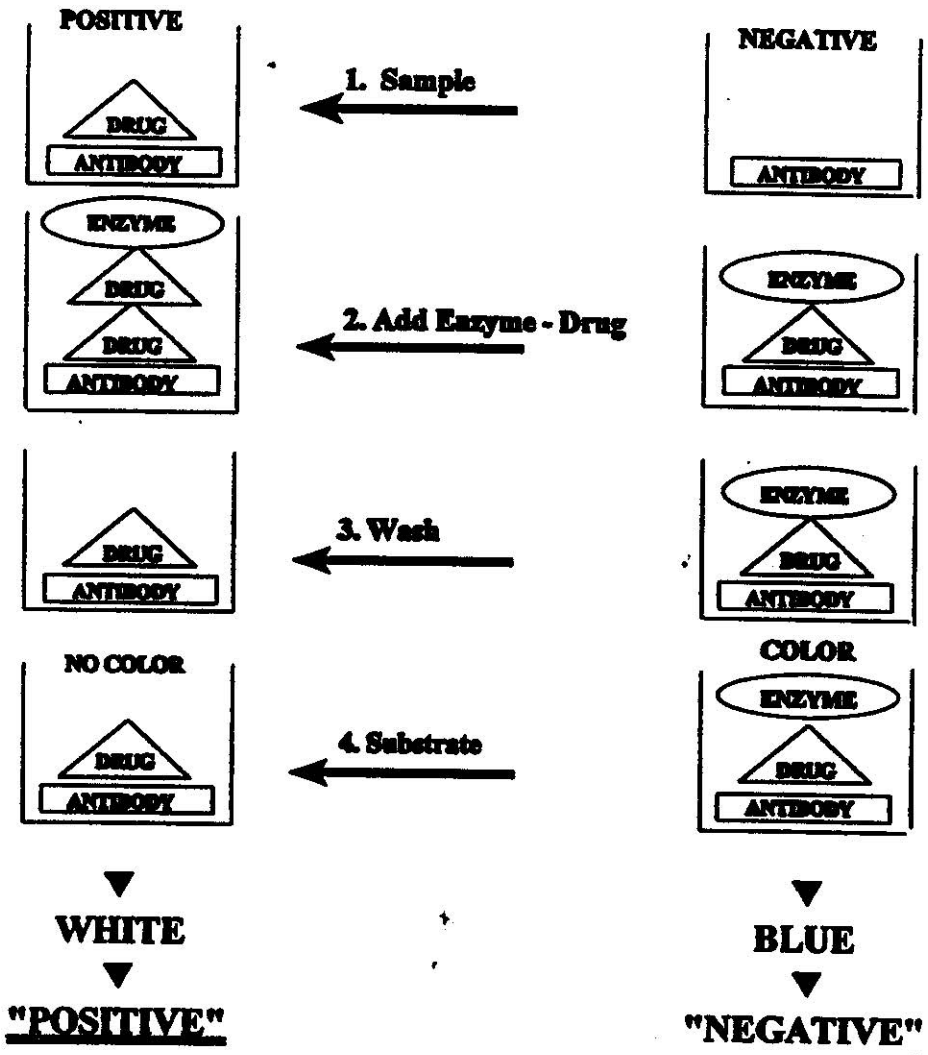

TABLE 2

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	Oxymorphone
TOTALS*	166	18	13	Hydromorphone

* 9 Days Racing

In Table 2 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 identifications were determined by GC/MS to contain either oxymorphone or hydromorphone. For some of the unconfirmed ELISA identifications, insufficient sample was available for complete GC/MS evaluation of their opiate status. Reproduced with permission from Research Communications in Chemical Pathology and Pharmacology. Vol. 59, No.2 (273) Feb. 1988.

Numerous medication violations were reported in post-race tests at several tracks in the Western United States when the immunoassay tests (ELISA) were first introduced. In general about one to five percent of the samples tested with ELISA were found to contain a prohibited substance. The efficacy of the ELISA tests in racing chemistry was clear and their ability to identify the use of high potency medications was established. Overall, the great sensitivity and speed of the ELISA tests have made them highly effective screening tests, far superior to TLC as a screening method for high potency drugs.

However, it is worth noting that once an effective test became available, abuse of these medications stopped at once and did not resume. This was clearly demonstrated in 1989, when business disputes and marketing strategies among competing ELISA companies left the racing commissions in the western United States without access to ELISA tests for a year. The ELISA tests again became commercially available to Western testing laboratories in 1990. When the tests were re-introduced into the post-racing testing procedures in the western United States there was no evidence of return to the pattern of abuse of prohibited substances characteristic of this region prior to 1987.

DRUG CONFIRMATION BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Mass spectrometry (MS) has become the standard instrumental method for confirmation of the presence and identity of a drug in a sample. Samples containing quantities as small as a picogram (part per trillion) quantities of an analyte can be measured as long as the substance can exist in the gaseous state at the temperature and pressure of the ion source. The mass of the substance detected can typically range from 10 to 800 Atomic Mass Units. The major advantages of mass spectrometry testing include its initial sensitivity ~~over most other analytical techniques~~ and its very great specificity in confirming the presence of foreign substances in equine samples.

Mass spectrometry provides both qualitative and quantitative information about the atomic and molecular composition of organic as well as inorganic substances. Preparing a sample for MS is a multi-step process. After solvent extraction partially isolates the foreign substance (drug) from the rest of the sample, the substance 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 chamber of the MS, the sample's components are bombarded by a beam of electrons or by a reagent gas such as methane. The collision of the electron beam and sample molecules produces molecular ions of the parent compound and its fragments. The resulting positive ions are then accelerated through an electromagnetic field, which separates them based on their mass/charge ratio. Once separated, the ions strike a detector, which analyses the number of ions in each mass. The resulting output of the mass spectrum is a graph, or plot of counted ions versus mass of the ions.

Comparisons between the plot of the sample to the reference set of plots of known drugs give the test results. If the mass spectrum "signature" shown in the plot is characteristic of a particular drug, the pattern produced by the drug and its fragment ions may be visualized as a molecular "fingerprint," a match of the sample against the standard, which is routinely accepted as evidence of the drug's identity. The chromatographic characteristics of the drug also add to its conformation. The mass spectrometer is sensitive down to ~~particles the size of sub-nanograms~~ (or levels measured in trillionths of a gram) levels. The MS test is very rapid. It can develop a full mass spectrum in a fraction of a second.

BILLIONTHS

TO NANO GRAM LEVELS

A state-of-the-art GC/MS system consists of a gas chromatograph 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.

UNEQUIVOCAL IDENTIFICATION OF A SUBSTANCE

By the time the chemist has completed a TLC, immunoassay, and GS/MS analysis, sufficient evidence will have been accumulated for the analyst to report on the presence of a drug or drug metabolite in the sample. If the medication is a prohibited agent, the analyst will issue a chemical finding. This act of formally reporting the test results to the racing authorities initiates a sequence of administrative events that may end in significant disciplinary actions against certain individuals. Such a report should only be undertaken when the analyst is absolutely certain that the tests unequivocally identified the foreign substance present in the sample.

THE SPLIT (REFEREE) SAMPLE

As a rule, the field of analytical chemistry is a rigorous and accurate discipline. If a well-trained chemist with a well-equipped laboratory performs the analysis and reports an analytical finding, then the results reported are almost always repeatable in a similarly-equipped and staffed laboratory. However, if the analyst is inexperienced, or not well-trained, or if he or she is under pressure, or other unforeseen circumstances occur, then errors can be made, just as in any other field of human endeavor.

The most important independent check on the ability and integrity of the chemist and lab is to run a second test in a different laboratory on a split, or referee sample, which has been sealed, then safely and securely stored. In a dispute over a positive test result, if the trainer so desires, the referee sample will be sent to an analyst of his (or her) choice, and the analytical work repeated and, if necessary, intended to include quantification and identification of metabolites. Analytical reports from high caliber laboratories on which administrative actions are based are virtually always repeatable. On the other hand, there have been instances where identifications have not been repeatable when the referee samples were tested by independent chemists. So the precautionary measure of holding a split, or referee, sample is important. Even when the Racing commission has confidence in the quality of the analytical work being done in the state-designated laboratory, the regulators should welcome requests for the testing of referee samples. The independent testing should be seen primarily as an opportunity to have the quality of the original analytical work independently verified.

VETERINARY REVIEW/DECISION LEVELS

With the increase in scope and sensitivity of medication testing, the bulk of the chemical findings reported by analysts do not show improperly used, illegal medications, but the tests often identify "trace" residues of legal and appropriate therapeutic medications. The frequency of these findings in recent years has created somewhat of a dilemma for the racing regulators. Under the old rules, any finding of a foreign substance found in a race horse led to confiscation of the purse and suspension of the trainer.

With the advent of the new and highly sophisticated testing techniques available today, the rigorous application of the old racing rule would close down racing.

The solution to this problem is two-fold. First, all analytical reports should be formally reviewed in writing by a veterinarian experienced in the field of racing administration and pharmacology to determine the regulatory significance of the finding. In California, an appointed Equine Medical Director, has been given this task; California is the first major racing state to take this approach to the problem.

No matter how well trained or experienced, however, no single veterinarian can know the answers to all of the problems that can arise from the application of sophisticated analytical chemistry techniques to 10,00 to 40,000 post-race urine tests run each year. For this reason many racing commissions function in close conjunction with university-based research programs. This approach enables the regulators to respond appropriately when questions arise concerning findings of drugs, therapeutic medications, and environmental and dietary substances in racing horses.

The second part of the solution is to establish decision levels, or cut-off levels, for therapeutic medications in race horses. Again, California has been the first state to implement this kind of

solution. Racing regulators there recently established decision levels for eight therapeutic medications. In racing jurisdictions, research programs are underway to determine decision levels for many other therapeutic medications used in race horses.

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