Pharmacology

Invited Review*

Testing for Drugs in Horses

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SUMMARY

Drug testing today depends primarily on post-race urine testing, with blood testing and pre-race testing as adjuncts to the testing process. Drugs are extracted from urine by a process called liquid-liquid extraction, and then screened for the presence of illegal agents. Screening is generally done by Thin Layer Chromatography (TLC analysis). If a drug is detected in the screening process, its presence in the sample is confirmed by other chromatographic methods, and most especially by Gas Chromatography-mass Spectrometry (GC-MS.) The qualitative detection of drugs in forensic samples is a well worked-out art, and most drugs can be indentified in 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 more. These scientific determinations on a sample can be independently verified in referee samples, and form the scientific basis of the regulatory process of medication control.

INTRODUCTION

Racing has the longest established, most elaborate, and technically accurate systems for drug testing of any human endeavor. The medication of racing horses was declared illegal by the English Jockey Club about 1903. The first "positive"[a], 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 a well worked out discipline. However, interpretation of the forensic significance of the findings of an analytical chemist in terms of the types of rules that can be based on the analyst's findings, and indeed how these rules should be drafted and interpreted is a controversial area, and the subject of much debate in the industry. In this series of articles, we will first discuss the techniques of testing for drugs in horses, the technical difficulties in regulating this area, the different regulatory strategies in use around the world and, finally, the efficacy and cost of drug testing as it is practiced today.

DRUG TESTING

Blood vs. Urine:

The backbone of drug testing in North America today is post-race urine testing, with the utilization of blood testing on the increase. 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 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 correspondence between blood and urinary concentrations of

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*See Author's Guidelines, page 106.

[a]A positive is the detection of a medication in a horse in violation of a rule, and is likely to result in a substantial penalty

drugs or 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

In this country, pre-race testing is based entirely on blood testing. The blood sample is drawn before the race, usually within 4 to 6 hours of post-time, and analyzed for the presence of drugs in a track-side laboratory. Because of the technical limitations of pre-race testing, the drugs that are detected by pre-race testing are usually acidic drugs of the phenylbutazone-furosemide type. Basic drugs, stimulants, depressants, local anesthetics, narcotic analgesics and tranquilizers, are much more difficult to detect in blood, and are much less likely to show up in pre-race testing. This is a major technical limitation of pre-race testing. However, pre-race testing is a highly visible deterrent for illegal medication use, and it is the only method that can actually prevent the running of an illegally medicated horse. It is, however, an expensive mode of testing and its coverage of the basic or "hard" medications is less than satisfactory.

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

Chemical analysis of a blood or urine sample is a three stage procedure. The first step is extraction of the drug from the 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 process is the extraction process done by a process called liquid-liquid extraction.

Liquid-Liquid Extraction

Liquid-liquid extraction of drugs follows the extraction rule. 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 cc) and makes them either acidic or basic. To make the urine acidic, he will add about

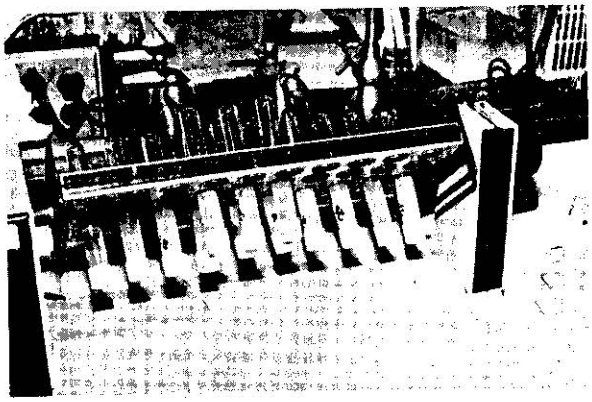

Figure 1. Tubes containing urine, dichloromethane and buffer are being "shaken" on a rotorack.

5 cc of an acidic buffer which changes its pH value to about 4.0. To make the urine basic, he will add a few drops of ammonium hydroxide, which will change the pH of the urine to about 9.0. This done, he proceeds to the extraction step.

To extract the drug, he simply adds an organic solvent, such as dichloromethane, and shakes the sample on a mechanical shaker for about 5 minutes or more (Figure 1). In the acidic sample, acidic drugs wind up in the dichloromethane, while in the basic sample, basic drugs wind up in the dichloromethane. The sample is then centrifuged to allow the dichloromethane to settle out, and pipetted off. The drugs are now contained in this dichloromethane layer, which is evaporated down to a small spot. This small spot will contain any drugs extracted from the urine, and at this point the chemist is ready to submit the extract to his drug screens.

Drug Screening

The screening tests that the chemist uses are almost invariably chromatographic tests. In chromatography, the drug is placed in a mobile phase, which moves past a stationary phase. Depending on 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 on thin layer plates, or in a gas or liquid chromatographic system. However, by far the most commonly used screening system is Thin Layer Chromatography (TLC or 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 on a glass plate, along with appropriate standards (Figure 2). The plate is then placed in a glass tank and "developed" by allowing a solvent mixture to run up the plate by capillary action (Figure 3). As the solvent (mobile phase) runs up the plate, the different drugs in the sample are spaced out along the plate depending on

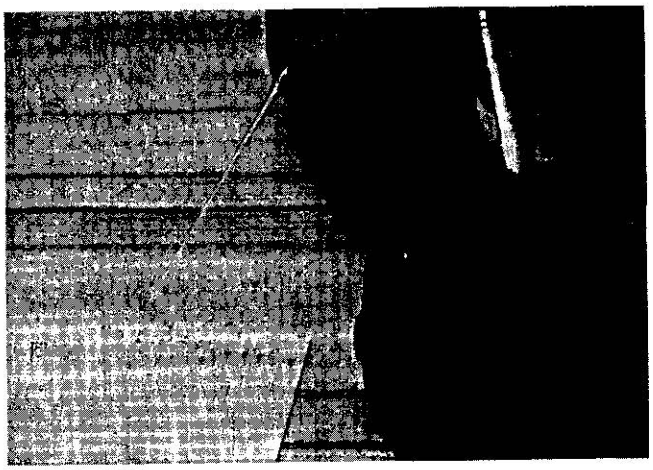

Figure 2. The dichloromethane urine extract is being spotted on a thin layer plate.

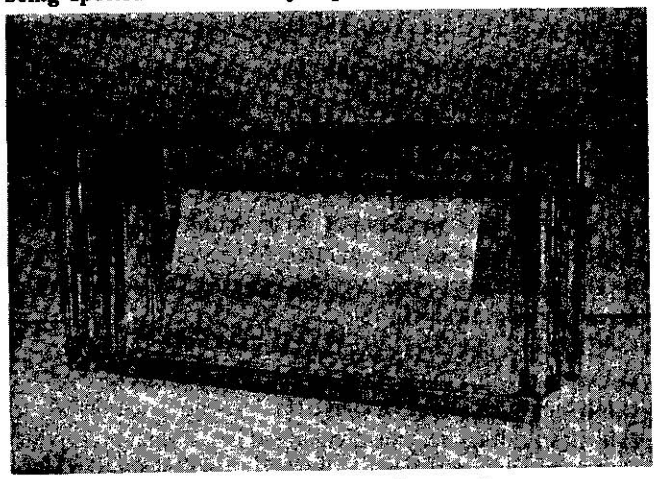

Figure 3. The solvent front is running up the thin layer plate, drawing with it materials from the drug extract.

Figure 4. Oversprays are being applied to the thin layer plate to develop the drug colors.

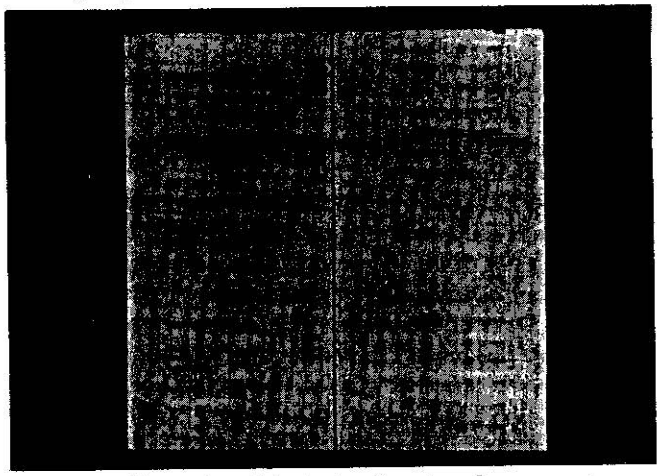

Figure 5. When the plate is developed and oversprayed, normal urine constituents and drug spots show up on the plate.

their affinity for the stationary silica phase. At the end of the process, a Thin Layer Chromatogram (TLC Plate) has been formed. The drugs on the plate are then observed by looking at the plate under UV light or by spraying with oversprays (Figure 4), with and without heat, to bring out the colors of the drug spots (Figure 5). This technique is simple, rapid, and inexpensive. Thin layer chromatography, however, is no more than a screening method, and can only suggest the presence of a drug, and give an approximate idea as to the amount of drug present. For a more definitive determination of the identity of the drug, one has to use more elaborate methods, usually gas chromatography (GC), high performance liquid chromatography (HPLC), and, almost invariably now, gas chromatography-mass spectrometry.

In gas chromatography, the drug must be volatile, and it is chromatographed between a mobile gas phase and a liquid or stationary phase. In liquid chromatography, the mobile phase is liquid and the stationary phase is solid. Both of these methods are better at separating individual drugs than thin layer chromatography. 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.

Gas Chromatography-Mass Spectrometry

Confirmation of the presence of a drug in a sample is nowadays almost invariably done by gas chromatography-mass spectrometry (GC-MS) (Figure 6). In GC-MS the drug is separated from the other blood or urine components on the gas chromatograph. Then the drug "peak" from the gas chromatograph is fed into a vacuum chamber in the mass spectrometer, where it is bombarded with electrons. These electrons charge the drug molecule, and depending on their energy, also fragment it. These fragments are then accelerated through a magnetic or electrical field, which separates them on the basis of their mass and electric charge. At the end of the analysis tube, the impact of these

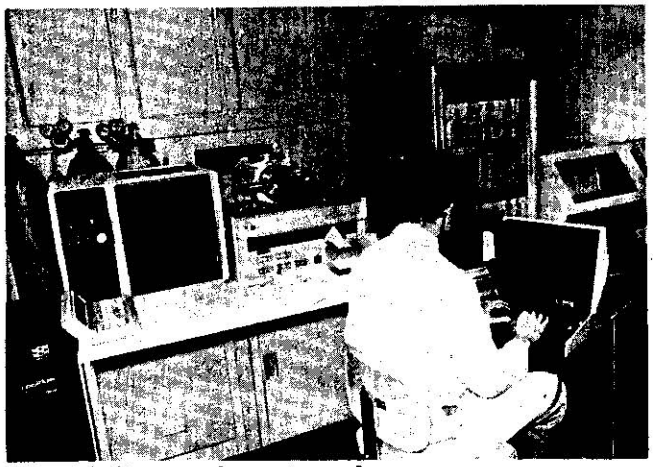

Figure 6. The gas chromatograph-mass spectrometer

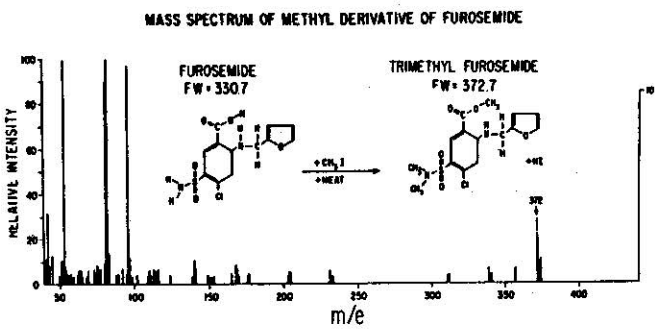

Figure 7. Mass spectrum of trimethlfurosemide. Furosemide (Lasix®) was methylated to convert it into trimethyl furosemide, a form of furosemide that "runs" well on a gas chromatograph. The furosemide derivative was then subject to gas chromatography-mass spectrometry.

fragments is recorded on an ion detector and the number of fragments at each mass is counted. This process is extremely rapid, and in about 1 second a mass spectrum for a drug can be produced.

A mass spectrum yields considerable information about the material contained in the sample. Usually some of the parent drug survives the process intact, and it turns up in the spectrum as a charged fragment with a molecular weight similar to that of the parent drug. When the drug breaks down into fragments, it breaks into specific patterns of fragments of specific masses. When plotted out, these fragmentation patterns are called mass spectra, and the spectra that they yield are specific for individual drugs. They can be thought of as yielding virtual "fingerprints" of the drug in question, and mass spectra are commonly accepted as the best evidence of the identity of a drug (Figure 7). In addition, the mass spectrometer can detect nanogram, or one billionth of a gram, quantities of drugs in fluid. As such, the mass spectrometer is sufficiently sensitive to be useful for drug detection in the body fluids of horses.

A first class mass spectrometer comes equipped with a gas chromatograph (GC), to separate out the drugs, a mass spectrometer (MS) to fragment the drug and generate the Mass Spectrum, and a computerized data system (DS) to generate, collate and compare the mass spectra obtained with known standards. The whole system is therefore a gas chromatograph, mass spectrometer, data system, or GC-MS-DS for short. A good mass spectrum is a very sophisticated product, containing information as to the chromatographic characteristics of the drug, the molecular weight of the drug, and the major fragments that the drug breaks down to. All in all, this is quite a substantial amount of information, and a good mass spectrum is very good evidence indeed that the drug in question is contained in the sample.

Calling a "Positive"

By the time that the chemist has completed his Thin Layer Chromatographic, Gas Chromatographic and GC-Mass-Spec analyses, he will have accumulated sufficient evidence to be persuaded as to the presence of the drug or drug metabolite in the sample. If the medication is an illegal drug, he will then be in a position to call a positive. The act of formally reporting to his authority the presence of a prohibited medication in a sample is to "call a positive." If he does this, the purse is generally not released to the horseman, and a hearing will be scheduled to investigate the positive.

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

The Referee Sample

As a general rule, the field of analytical chemistry is a well-worked discipline. If a well-trained chemist with a well-equipped laboratory does the analysis and calls the positive, then the results he reports are virtually always repeatable in another 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 a referee sample, which is an independently sealed and stored sample, available. If the horseman so desires, this sample can be sent to an analyst of his 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 an Authority of the Chemist is confident of the quality of the analytical work being done in

its laboratory, it should welcome requests for referee samples; these are seen primarily as an opportunity to have their positive calls and the quality of their work independently verified.

Forensic Significance of the Chemist's Findings

For most drugs, all the chemist has to do is to unequivocally detect the drug in the sample, and report its presence. Under some rules, however, the chemist may be required to estimate the time of administration of the drug. In general, while a good chemist can very reliably detect the presence of a drug in a sample, and measure its amount with fair accuracy (± 25% or more), it is much more difficult for a chemist to estimate the time of administration of a drug to a horse. The reason for this is the very great uncertainty as to the blood or urinary concentrations of drugs that are likely to be found after administration of drugs to horses, the subject of the next article in this series.

HYDROGEN IONS, pH AND DRUG EXTRACTION

Hydrogen Ion Concentration or pH

The terms acidic or basic refer to the hydrogen ion concentration of a solution. A solution is acidic (pH 1 to 6) if it has a high concentration of hydrogen ions. A neutral solution has a pH of 7.0, while a basic (alkaline) solution has a pH of greater than 8. A horse's urine can have a pH from 4.5 to 9.

Drugs

Virtually all drugs are either weak acids (phenylbutazone) or weak bases (morphine). An acidic drug loses a hydrogen ion (H+) in basic urine and becomes negatively (-) charged. Once charged, the drug cannot be extracted from urine, except by removing the negative charge

The Extraction Rule

To remove the negative charge, the chemist simply adds excess positive charge. This he does by simply adding H^+ ions (ie, he makes the urine acidic). The H^+ goes back onto the drug molecule, and the drug is no longer charged and is readily extracted. The drug has followed the extraction rule, which holds that acidic drugs extract under acidic conditions, while basic drugs extract under basic conditions.

The Trapping Rule

This is the converse of the extraction rule. Because charged molecules "trap" in urine, acidic drugs will "trap" in basic (alkaline) urine, while basic drugs "trap" will trap or be held in acidic urines.

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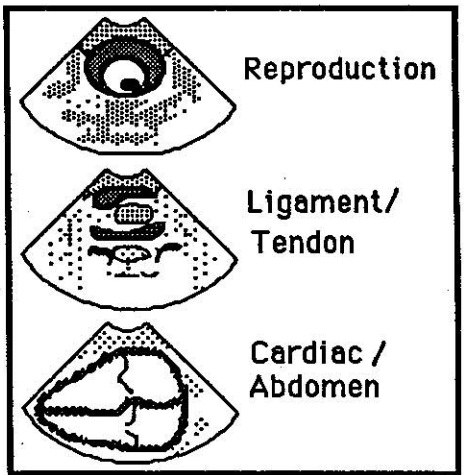

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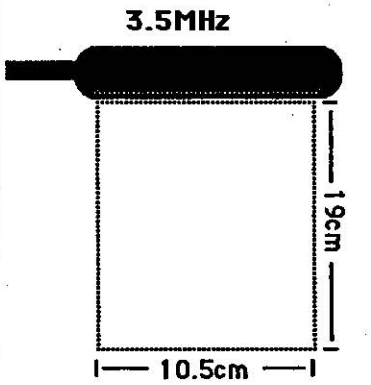

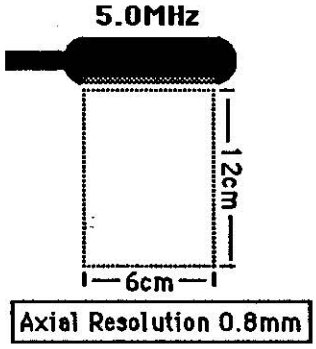

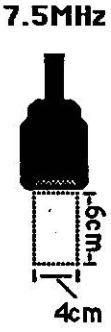

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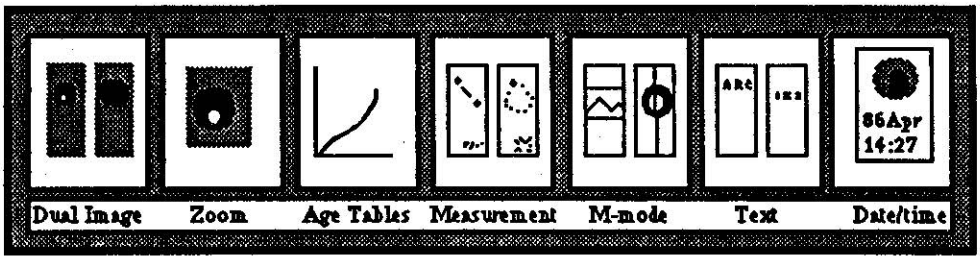

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