Review of Equine Drug Testing

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Forensic equine drug testing has made comprehensive changes in the past few decades, resulting in analytical sensitivity of parts per trillion for many analytes. This highly sensitive testing has provided a strong deterrent to and increased the integrity of the racing industry. The coinciding detection of equally small amounts of therapeutic drugs, however, has resulted in numerous violations that previously would have been undetected. Authors’ address: Equine Analytical Chemistry Laboratory, California Veterinary Diagnostic Laboratory System, University of California, Davis, P.O. Box 1770, Davis, CA 95617-1770. © 1997 AAEP.

1. Introduction
In the past several decades the field of equine analytical chemistry has changed substantially. Increased public consciousness of the dangers of drug abuse has prompted regulatory agencies to initiate programs to eliminate use of unauthorized medications. Toward that end, these agencies have asked analytical laboratories to employ state-of-the-art technology. The choice of analytical methodologies by a laboratory depends on many factors, including sample workload, required sensitivity, reliability, instrument capability, turnaround time, and cost. A suitable analytical protocol for the detection of unauthorized medication in equine urine samples should consist of two phases. In the first phase, a sensitive screening method is used to identify suspect samples for further testing and to eliminate samples with negative results. In the second phase, samples considered suspicious in the first phase undergo a highly specific confirmatory analysis that is at least as sensitive as the screening test. The test method used for the second phase of analysis should be based on a different chemical principle than the screening test and is used to confirm the identity of the substance detected in the first phase. Both of these phases of equine drug testing have undergone numerous changes. For example, nearly every equine drug testing laboratory in the world now uses immunoassay technology to screen samples. The most widely used method is enzyme-linked immunosorbent assay, or ELISA. When these assays were introduced in the 1980’s, their use resulted in the detection of highly potent narcotics at concentrations that had been previously undetectable. The early ELISA’s were developed exclusively for high-potency drugs of abuse; however, regulators concerned about the unauthorized use of therapeutic medications prompted assay manufacturers to develop ELISA’s for numerous therapeutic drugs. These ELISA’s, when applied to routine testing, dramatically alter the withdrawal time necessary for clearance of these therapeutic medications. A good example is acepromazine. When used at a therapeutic dose, acepromazine can be detected by thin-layer chromatography (TLC) for approximately 72 h. The use of an ELISA for acepromazine, however, will allow detection for roughly 168 h after administr-
tion. Technological advances have also resulted in improved detection limits for instrumental analysis. Improvements in instrumental analysis have been made for the following equipment: high-pressure liquid chromatography (HPLC), mass spectrometry (MS), gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS) and tandem mass spectrometry (MS–MS). Because instrumental testing methods are characterized as more sensitive than ELISA, their use for acepromazine should result in detection of the drug for longer than 168 h after administration.

Practitioners and regulatory veterinarians need to be familiar with the current methods employed by equine analytical laboratories for the detection of unauthorized medications. These modern methods of chemical analysis have significantly affected the detection of therapeutic medications, and therefore they could alter how veterinarians can use them in current practice.

2. Traditional Screening Analysis

Traditional screening methods, primary TLC complemented with ELISA, are time consuming, labor intensive, and highly dependent on technologist interpretation.

A. Thin-Layer Chromatography

The use of TLC in racing chemistry dates back to the early 1960's, when Maynard and Smith presented their research on this technique in the Bulletin of the Association of Official Racing Chemists.¹ The following year Maynard and Wong² described TLC as an "extremely valuable confirmatory test." At present, thin-layer chromatography is commonly used as a screening test in equine analytical laboratories. To summarize the technique, it is a method of chromatography in which a mobile phase moves by capillary action across a uniform thin layer of finely divided stationary phase bonded onto a plate. A sample is applied to this plate and developed with the mobile phase. Any drugs present in the sample will move across the plate at different rates, depending on their individual chemical characteristics (i.e., solubility, pKₐ value, and hydrogen-bonding capabilities).³ As most drugs are colorless, various reagent sprays are applied to the plates to make them visible. TLC methods provide good drug resolution and permit the simultaneous identification of a wide range of substances in a single analysis. A primary drawback of this methodology is that it's very laborious, and therefore results in long turn-around times. In addition, the sensitivity and resolving powers are inferior to instrumental methods. Finally, several years of training may be required for a chromatographer to discriminate the drug spot from the spots of other naturally occurring compounds. It has retained favor as an analytical method essentially because it is simple, dependable, and low cost, and because it allows for the detection of a wide range of drug substances.

B. Immunoassay

Enzyme-linked immunosorbent assays are securely established in routine equine drug testing. Their popularity has been secured by their simplicity, sensitivity, and specificity. The majority of laboratories in the U.S., however, still use ELISA to complement their TLC testing backbone.

The principal components in enzyme immunoassays are a drug labeled with a specific enzyme, an antibody specific to that drug, a substrate capable of producing a measured optical signal when initiated by the enzyme, and the test sample.⁴ In an ELISA, the drug labeled with an enzyme competes with the drug in the test sample for a limited number of antibody binding sites. Generally, the one-step ELISA is started by adding urine (20 µl) to the wells of a microtiter plate previously coated with antibody. Next the drug–enzyme conjugate is added to each well and the plates is incubated. If there is any drug present in the sample, specific to the ELISA employed, it will bind to the antibody already present. After the incubation period the fluid is removed and the plate washed with buffer. The final step is to add peroxidase substrate solution to each well. The wells will rapidly change color from clear to blue when no drug is present. If a sample does contain the drug specific to that ELISA, no color change will occur. When appropriate standards and calibration samples are used, the ELISA can provide an estimation of the amount of the drug in the sample.⁵

The principle advantages of ELISA tests are their extreme sensitivity and specificity. Limits of detection of 1.0 ng/ml for many substances are typical. In contrast, limits of detection in the range 25–2000 ng/ml are typical for many substances in TLC screening methods. The specificity of ELISA's is high because the antibody will only react to one drug or a small group of structurally related drugs.

Recently, several laboratories have instituted testing schemes based exclusively on ELISA tests. This presents a limitation because of the expense of ELISA tests and the narrow range of drug assays available to laboratories. A common misconception is that equine testing laboratories have an ELISA for every drug.

3. Instrumental Analysis

Although some have advocated comprehensive instrumental drug screening programs, their impact on the industry has so far been limited. In addition, many laboratories are hesitant to commit to the inherent expense of equipment. With the availability of lower-cost instruments and automated sample preparation, GC–MS has become the gold standard for equine drug screening. Instrumental methods provide a wide range of substance screening and minimize the limitations found in traditional equine drug testing. With continued technological advances and declining prices, the use of instrumental methods in drug screening will definitely accelerate.
A. High-Pressure Liquid Chromatography

In high-pressure liquid chromatography, a liquid mobile phase is pumped through a narrow-diameter tube packed with small particles of silica gel, alumina, or other material. Drugs are separated from each other and from other substances in the sample extract as a result of different degrees of interaction with functional groups on the surfaces or pores of the particles. Drug molecules are carried through the mobile phase as it is pumped through the column. The mobile phase containing the separated drugs flows through one or more detectors as it exits the column. The two detectors most commonly used are ultraviolet and fluorescence. They measure the drugs' UV wavelength absorption or fluorescence capabilities.

This analytical method has traditionally been used for quantification of authorized medications such as nonsteroidal anti-inflammatory drugs (such as phenylbutazone) and furosemide. However, the introduction of the on-line photodiode array detector (DAD) and the development of HPLC methods for broad-spectrum drug screening have attracted the interest of equine analytical laboratories. The HPLC-DAD coupling fulfills the requirement for confirming the identity of unknowns by obtaining secondary chemical information (i.e., retention time and ultraviolet spectrum). This combination, as a single system for confirmation value, is second only to GC-MS.

The efficiency of HPLC as commonly used is between that of gas chromatography and thin-layer chromatography. Data generated by HPLC are presented so as to be easily identified by matching the retention times and ultraviolet spectra with reference compounds in the library.

B. Mass Spectrometry

The mass spectrometer provides specificity of identification and increased sensitivity over routine drug screening. Mass spectrometry operates on the principle that a charged particle moving through a magnetic or an electric field can be separated from other charged particles by producing charged molecules and molecule fragments, and by measuring the mass (or mass-to-charge ratio) of each. MS works by the strategy divide and conquer, chemically breaking down the molecules to smaller, more easily identifiable pieces. The processes that occur in MS are (a) ionization, (b) ion separation, and (c) ion detection. The types of ionization used fall into two classes. Hard ionization produces a substantial proportion of ionized molecules with such high internal energies that they fragment before leaving the ion source. The masses generated are basic structures that provide the information used in interpretation. Soft ionization, in contrast, minimizes fragmentation, thereby leaving the molecule intact. A record of the ions formed and the relative abundance of each is used as a fingerprint to determine the structural identification of unknowns.

This technique is used with an inlet system, which facilitates introduction of the sample into the mass spectrometer. The most common combination of interfacing chromatography with MS is gas chromatography. More recently, liquid chromatography has permitted many compounds to be studied that are not suitable for GC analysis.

C. Gas Chromatography–Mass Spectrometry

Gas chromatography is widely used to separate complex mixtures in a gas or vapor phase. In the late 1960’s the most significant development with respect to racing chemistry was the introduction of gas chromatography–mass spectrometry. This link allows compounds already in the vapor phase to enter the mass spectrometer. In the 1970’s, improvements in computer operating systems and the evolution of lower-priced bench-top instruments resulted in almost every analytical chemistry laboratory having this capability. The primary use of GC-MS in racing chemistry continues to be the confirmation testing of samples screened by TLC or immunoassay. Mass spectrometry has become mandatory for many racing regulatory authorities and a requirement for laboratory accreditation. GC–MS confirmation allows drug identification to stand up to ever-increasing scrutiny by the judicial court system. For example, in California nearly every analytical finding for the California Horse Racing Board (CHRB) of drugs in Classes 1–3 has been challenged and has required an administrative hearing to resolve.

D. Liquid Chromatography–Mass Spectrometry

The use of LC methodology is increasing rapidly as interfaces are improved (i.e., atmospheric pressure chemical ionization (APCI) and electrospray ionization (EI)). The single most important piece of information that may be obtained from the mass spectrum is the molecular weight. The development of soft ionization techniques, such as APCI and EI, that maximize the molecular ions have resulted in increased identification specificity. These new techniques present the opportunity for developing on-line LC–MS methods capable of identifying the intact parent drug, bypassing laborious and time-consuming chemical derivatization procedures. Because of this soft ionization approach, thermally labile compounds such as conjugated metabolites, which were traditionally hydrolyzed for analysis, can be characterized unaltered. The value of this technique has already been demonstrated with methods developed for steroids and butorphanol, compounds difficult to detect with other methods.

Relatively little use has been made of this technology in equine drug testing, although wider use of LC–MS is anticipated as more difficult to detect drugs are developed and the identification of metabolites becomes more important.

E. Tandem Mass Spectrometry

There are several driving forces behind the rapid acceptance of MS–MS: (a) the need for an increased...
speed of analysis, (b) the need for decreased cost per sample, and (c) the need for improved sensitivity. \textsuperscript{7} Tandem mass spectral techniques are having a major impact on the future of equine analytical chemistry laboratories. These instruments can reduce sample preparation, improve accuracy of identification, and tremendously improve detection limits. Compared with conventional MS methods, MS–MS generally lowers detection limits by at least 1 order of magnitude. \textsuperscript{17}

The principle of MS–MS is simple and comparable with GC–MS. An extract is introduced by an inlet system where sample ionization occurs. The ions produced have characteristics of the individual drug compounds, called the parent ion. Once identified, the parent ion is targeted for further fragmentation into secondary ions, called daughter ions. The daughter ions resulting from the fragmentation of the parent ion are mass analyzed in the second mass spectrometer stage. The resulting analysis of the daughter ion provides an unparalleled and highly specific identification of the targeted parent ion. \textsuperscript{17}

The technology still has some drawbacks in that it is high priced and requires the use of a highly skilled operator.

4. Discussion

Instrumental analytical techniques are gradually replacing traditional TLC and ELISA testing programs in equine analytical chemistry laboratories. Because a laboratory’s accreditation status, reputation, and economic well-being depend on consistently accurate results, it is important to use optimal analytical procedures to maximize reliability and precision. Mass spectrometry clearly has a central and crucial role in the screening analysis of unauthorized medications today and in the future. The direction toward more sensitive methods will continue, and with it the need for regulatory officials to address the consequences and importance of the analytical findings will continue as well.

In the future, formidable challenges may arise with regard to substances of environmental contamination, dietary contamination, and recombinant hormones. These are issues to be addressed collectively by the analysts, veterinarians, and other industry representatives.

References