

# **Advances in Equine Nutrition**

## **Volume I**

Edited by

**J.D. Pagan**



## TESTING FOR DRUGS, MEDICATIONS AND OTHER SUBSTANCES IN RACING HORSES

THOMAS TOBIN,<sup>1</sup> J.D. HARKINS,<sup>1</sup> W.E. WOODS<sup>1</sup>  
N. C. HALL,<sup>1</sup> ANTONIO QUEIROZ-NETO,<sup>2</sup> SCOTT D. STANLEY,<sup>3</sup>  
GEORGE D. MUNDY<sup>4</sup>

<sup>1</sup>*The Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, Kentucky, USA,* <sup>2</sup>*Departamento de Morfologia e Fisiologia, Animal Faculdade de Ciencias Agrarias e Veterinarias de Jaboticabal, Unesp. Sao Paulo, Brazil,* <sup>3</sup>*Truesdail Laboratories, Inc., Tustin, California,* <sup>4</sup>*The Kentucky Racing Commission, Lexington, Kentucky, USA*

### Summary

Testing for drugs in horses starts with the taking of samples. Samples, usually blood and urine, should be split immediately after they are drawn and the referee portion of the sample stored independently of the sample 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 enzyme linked immunosorbent assay (ELISA). ELISA screening is particularly sensitive and rapid. Due to the sensitivity of immunoassay-based screening, most high-potency medications are first detected on ELISA screening.

If an agent is detected in the screening process, its presence in the sample is confirmed by other methods, but most especially by gas chromatography-mass spectrometry. The qualitative detection of drugs in forensic samples is a well developed art, and most drugs can be identified 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 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.

These techniques detect much more than medications administered to horses, and one of the challenges of equine drug testing is to fairly and equitably distinguish between natural substances in horse urine and medicinal substances. In this review we examine the techniques used or that may be used to make this distinction for a number of dietary, environmental and endogenous substances found in horse urine.

## **Introduction**

Racing has the longest established, most elaborate, broadly based and technically accurate systems for drug testing of any human endeavor (Tobin 1981). The medication of racing horses was formally declared illegal by the English Jockey Club about 1903. The first reported medication violation, using frogs as the test animal, and “determining” from their croaks whether the drug was present, was reported in Russia in about 1905. The first medication violation reported by analytical chemistry was reported 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 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 industry.

## **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 enzyme linked immunosorbent assay (ELISA) 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 more satisfactory, 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. Beyond this, drugs such as furosemide or other diuretics should generally 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. Urine testing is generally superior to blood testing since urine is available in relatively large amounts (200 ml plus), tends to contain higher concentrations of the parent drug than does the corresponding blood sample, and almost invariably contains much greater (50 fold 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 difficult to determine the forensic significance of a given urinary concentration of a drug.

In contrast, 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 sample volume is small and 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, the state of Kentucky takes post-race urine samples only, and testing of Kentucky samples is carried out by one of us (SDS) at Truesdail Laboratories Inc. in Tustin, California. Following collection at Kentucky tracks, the samples are shipped in a secure container to Truesdail, where they arrive the next day. 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 and post-race testing**

### **PRE-RACE TESTING**

Pre-race testing, which is no longer performed on a significant basis in North America, was based entirely on blood sampling, although at one time pre-race testing in Hong Kong was based on urine 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, pre-race testing allowed the chemist to detect a medicated horse before it ran, and then to scratch the horse, and in this way prevent the running of an illegally medicated horse. Pre-race testing was thus seen as the ultimate drug testing strategy, the one that could 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 the testing technology has never been sensitive enough to detect the use of high-potency, 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 high-potency, 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 readily detected in urine is likely to be used. This restricts the illegal use of 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. For this reason pre-race testing based on

TLC had an extremely poor record of detecting high potency illegal medications pre-race, and the concept of pre-race testing required a much more sensitive drug detection technology than TLC-based testing. 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 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, 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 (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 is placed on a mechanical shaker for about 5 minutes or more. For the acidic sample, acidic drugs move into the dichloromethane, while for the basic sample, basic drugs move into the dichloromethane. The sample is centrifuged to allow the dichloromethane to separate from the aqueous layer, which is pipetted off. 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**

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 immobile on 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 or HPTLC (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, along 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, the test yields only one single piece of information about the drug, which is that it chromatographs 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 the detection of many medications, particularly in urine, there are many medications that 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 is low. For these reasons, the sensitivity of immunoassay techniques renders this a very attractive technology, and, about nine years ago, we began a broad scale approach to 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  $\mu$ l of the standard, test or control samples to each well, along with 100  $\mu$ l 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 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 are

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 a microwell reader. Higher optical absorbance corresponds to lower drug concentration in the sample.

These ELISA tests can be particularly potent and effective in drug detection. They can be as sensitive as radioimmunoassays (RIA), the test can be completed within about one hour and a good ELISA is comparable to a RIA in terms of accuracy.

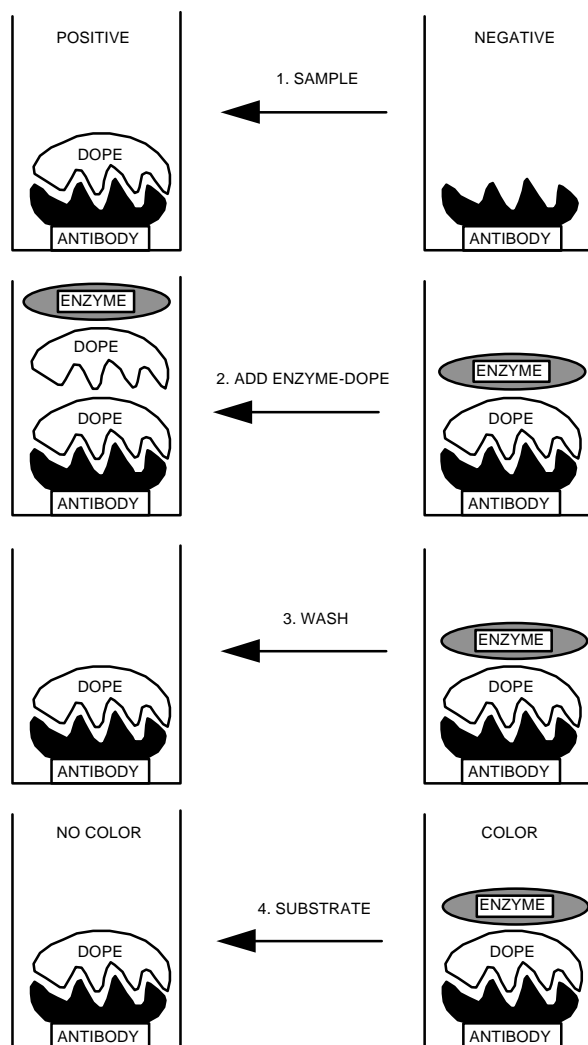
For example, a simple morphine ELISA was particularly effective in terms of detection of opiates. Figures 2 and 3 show, respectively, the time course and sensitivity of the morphine ELISA, a typical "run" on a series of track samples, and in Table I, the results of the introduction of this test into 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 to contain a narcotic substance by GC/MS (McDonald *et al.*, 1988).

**TABLE 1.** ELISA SCREENING OF POST-RACE URINE SAMPLES FOLLOWED BY GC/MS ANALYSIS

<i>Sample Date</i>	<i>No. Urine Samples</i>	<i>No. Flagged by ELISA</i>	<i>No. Positive by GC/MS Analysis</i>	<i>Drug Identified</i>
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

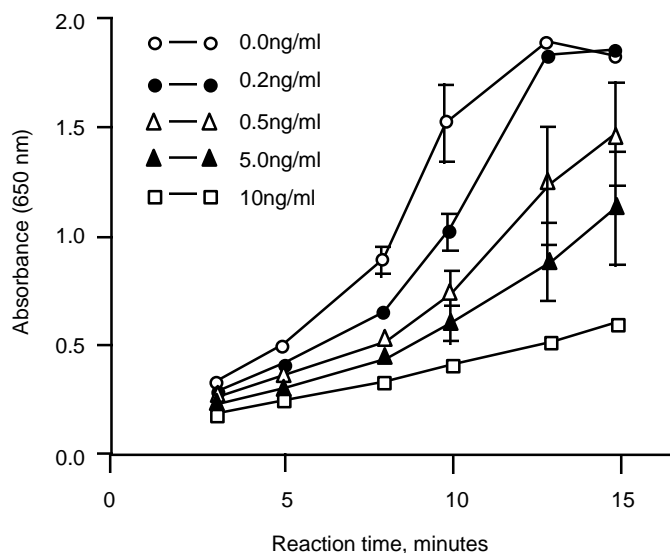
\*9 Days Racing

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



**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 the substrate is 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.*

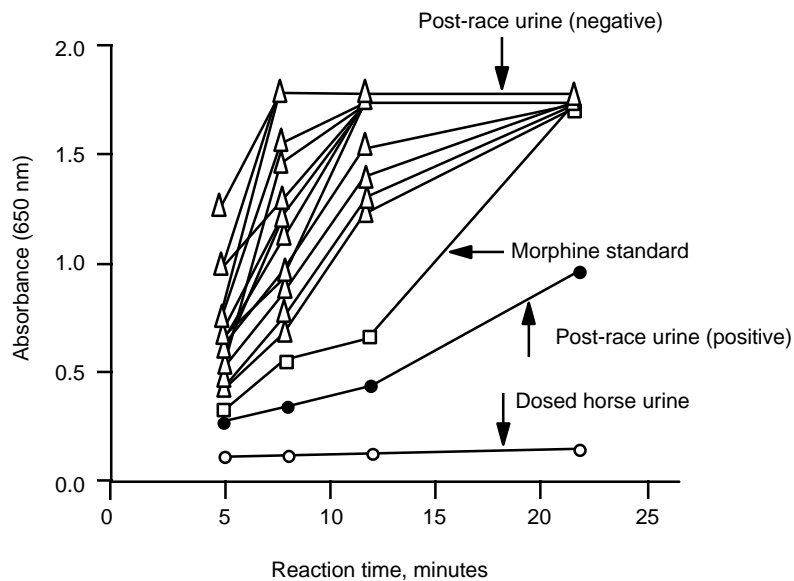
hydromorphone. For some of the unconfirmed ELISA identifications, insufficient sample was available for complete GC/MS evaluation of their opiate status.



**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.*

Similar patterns of medication violations 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 found to contain a narcotic analgesic. Similarly, when the mazindol test was introduced in early 1988, about 2 to 5 % of the early samples were 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 was established.

Establishing the 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 completely 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.



**Figure 3.** One-step ELISA reaction in a series of post-race urine samples. The open triangles (Δ) 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 (●) show ELISA activity in a sample subsequently determined to contain oxymorphone.

It is, however, worth noting that once an effective test became available abuse of these medications stopped at once, and did not resume. This was clearly demonstrated when business disputes and marketing strategies among competing ELISA companies left the western United States without access to ELISA tests for a period of at least one year through 1989. Then, beginning in 1990, ELISA tests again became commercially available to western testing laboratories. When these tests were re-introduced into the western United States there was no evidence of a return to the frequent pattern of abuse of narcotic analgesics characteristic of this region prior to 1987.

**Table 2.** EFFICACY OF PCFIA AND ELISA TESTS

<i>Drug</i>	<i>State</i>	<i>TLC Status</i>	<i>Immunoassay Positives</i>
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.

Reproduced with permission from *Res. Comm. Chem. Pathol. Pharmacol.*

**Table 3.** PARTIAL LIST OF COMMERCIALY AVAILABLE ELISA TESTS

Alfentanil	Dexamethasone	Meperidine
Amphetamine	Diprenorphine	Mepivacaine
Anileridine	Doxapram	Methylprednisolone
Azaperone	Droperidol	Nalbuphine
Barbiturate Group	Ethacrynic Acid	Nandrolone
Benzodiazepine Group	Etorphine	Opiate Group
Boldenone	Fentanil Group	Oxymorphone/Oxycodone
Bronchodilator Group	Fentanyl	Pentazocine
Bumetanide	Fluphenazine	Phenylbutazone (Blood Only)
Buprenorphine	Furosemide (Blood Only)	Procaine
Butorphanol	Glycopyrrolate	Promazine Group
Caffeine/Pentoxifylline	Haloperidol	Pyrilamine
Carfentanil	Haloperidol Metabolites	Reserpine
Clenbuterol	Isoxsuprine	Sufentanil
Cocaine/Benzoylecgonine	Ketorolac	Sulfamethazine
Corticosteroid Group	Levallorphan	THC Metabolites
Cromoglycate	Lofentanil	Theophylline
Dantrolene	Mazindol	Triamcinolone Acetonide
Detomnine	Mazindol Metabolites	Tricyclics Group

## **Drug confirmation**

### **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 as little as picogram quantities of analyte can be measured as long as the material can exist in the gaseous state at the temperature and pressure of the ion source. The mass of the analyte can typically range from 10-800 Atomic Mass Units. The major advantages of MS are its initial sensitivity over most other analytical techniques and its specificity in confirming the presence of suspected compounds.

Mass spectrometry provides both qualitative and quantitative information about the atomic and molecular composition of organic as well as inorganic materials. After 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 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 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 at each mass. The resulting mass spectrum is a plot of counted ions versus mass of the ions.

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 sub-nanograms (trillionths of a gram) levels and is very rapid; it can develop a full 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.

### **UNEQUIVOCAL IDENTIFICATION OF SUBSTANCE**

By the time the chemist has completed his 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 issue a chemical finding. This act of formally

reporting to the authority the presence of a chemical substance initiates a sequence of administrative events that may end in significant disciplinary action against certain individuals, and is only undertaken when the analyst is absolutely certain that he is able to unequivocally identify the material present in the sample.

Since issuing an analytical report may eventually result in substantial penalty to the trainer, 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 material in question, and the quality of the analytical chemistry should be sufficient to allow unequivocal chemical identification of the drug.

### THE SPLIT (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 performs the analysis and reports an analytical finding, then the results reported are virtually always repeatable in a similarly equipped and staffed laboratory. 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 split or referee sample, which is an independently sealed and stored sample. If the trainer so desires, this sample can be sent to an analyst of his (or her) choice, and the analytical work repeated. In this author's experience, 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 turned out to be repeatable in the hands of an independent chemist, so the precaution of holding a split or referee sample is important. When the analyst is confident of the quality of the analytical work being done in the laboratory, the analyst should welcome requests for split or referee samples; these should be seen primarily as an opportunity to have the quality of the analytical work independently verified.

### VETERINARY REVIEW/THRESHOLDS

With the increase in scope and sensitivity of medication testing, the bulk of the chemical findings reported by analysts are not illegal medications improperly used, but rather are trace residues of legal and appropriate therapeutic medications. The frequency of these findings in recent years has created somewhat of a dilemma for the industry. Under the old rules, any finding of a foreign substance led to confiscation of the purse and suspension of the trainer. Under the new and highly sophisticated techniques available to analytical chemists, however, routine and rigorous application of this approach would close down racing.

The solution to this problem is two-fold. In the first place, 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. California, which has just appointed an Equine Medical Director, is the first major racing state to take this approach to the problem. One of us (GDM) serves in a similar capacity in the state of Kentucky.

No veterinarian, no matter how well trained or experienced, can have at his fingertips answers to all of the problems that can arise from the application of sophisticated analytical chemistry to 10-40,000 post-race urines/year. For this reason both the California and Kentucky Boards / Commissions function in close conjunction with university based research programs to enable them to respond appropriately when questions concerning findings of drugs, therapeutic medications, and environmental and dietary substances in racing horses arise.

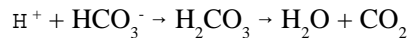
The second portion of the solution is formal thresholds or cutoffs for therapeutic medications in racing horses. Again, California has been the first state to implement this solution, with the recent establishment of thresholds for eight therapeutic medications as set forth in table III, and one of us (TT) has an active research program in the area of thresholds for therapeutic medications in racing horses.

**Table 4.** APPROVED AND PROPOSED "THRESHOLDS" FOR MEDICATIONS IN RACING HORSES MARCH 30, 1995

Phenylbutazone	5 µg/ml	Plasma	North America (ARCI)
Oxyphenbutazone	5 µg/ml	Plasma	North America (ARCI)
Furosemide	50 ppb	Plasma	Oklahoma
Flunixin	1 µg/ml	Plasma	California
Flunixin	0.1 µg/ml	Plasma	Pennsylvania
Naproxen	1 µg/ml	Plasma	California
Procaine	25 ppb	Plasma	Canada
Procaine	10 ppb	Urine	California
Acepromazine	25 ng/ml	Urine	California
Mepivacaine	10 ng/ml	Urine	California
Promazine	25 ng/ml	Urine	California
Albuterol	1 ng/ml	Urine	California
Atropine	10 ng/ml	Urine	California
Benzocaine	50 ng/ml	Urine	California
Theobromine	2 µg/ml	Urine	International
Arsenic	0.3 µg/ml	Urine	International
Salicylate	6 µg/ml	Blood	International
Salicylate	750 µg/ml	Urine	International, California
DMSO	15 µg/ml	Urine	International
DMSO	1 µg/ml	Plasma	International
Hydrocortisone	1 µg/ml	Urine	International

## Bicarbonate

In recent years, there has been considerable interest in the use of  $\text{NaHCO}_3$  in athletes to counteract the acidosis associated with intense exercise. The accumulation of hydrogen ions ( $\text{H}^+$ ) during intense, short-duration exercise suppresses glycolysis through the inhibition of the enzyme phosphofructokinase (Hood *et al.*, 1988; Wilkie 1986). During intense exercise the  $\text{H}^+$  associated with lactate formation are buffered by  $\text{HCO}_3^-$  in the reaction:



Carbon dioxide is eliminated through respiration, and  $\text{HCO}_3^-$  is depleted. As blood  $[\text{HCO}_3^-]$  decreases,  $\text{H}^+$  accumulate causing blood pH to decrease. The goal of  $\text{NaHCO}_3$  administration is to augment the blood bicarbonate buffering system thereby delaying the accumulation of  $\text{H}^+$ .

Studies using human athletes have delayed the onset of fatigue with  $\text{NaHCO}_3$  during moderately heavy but not supramaximal exercise. For  $\text{NaHCO}_3$  to be beneficial to performance, work bouts must last at least 2 minutes in duration (Costill *et al.*, 1984). Sodium bicarbonate provides no beneficial effect during long term aerobic exercise since lactic acid buildup is not a problem in that type of work. In fact, endurance exercise produces alkalosis.

In studies using equine athletes, the fatigue-delaying effects of  $\text{NaHCO}_3$  are equivocal; however, anecdotal evidence and the perception that  $\text{NaHCO}_3$  can improve performance, particularly in Standardbreds, is substantial. Since a majority of Standardbred races last longer than 2 minutes and Standardbred trainers use multiple pre-race warm-up heats, pre-race treatment with  $\text{NaHCO}_3$  may supply an ergogenic benefit to Standardbred racing horses. Mixtures of  $\text{NaHCO}_3$ , water and confectionery sugar, known as “milkshakes,” administered by nasogastric tube have been used to a considerable extent at Standardbred race tracks. It is estimated that as many as 90% of trainers at some Standardbred tracks have used these concoctions (Bergstein 1989).

Following  $\text{NaHCO}_3$  administration, renal and respiratory compensatory mechanisms will seek to return systemic acid-base balance to normal. Renal mechanisms respond to acid-base changes by altering the excretion of electrolytes,  $\text{H}^+$ , and  $\text{HCO}_3^-$ . Horses fed a grain mixture containing 2.0%  $\text{NaHCO}_3$  (about 0.4 g/kg) had a urine pH of 8.20 compared to 7.46 for horses not consuming  $\text{NaHCO}_3$  (Robb and Kronfeld 1986). The urine pH of human athletes following an 800 meter run was 6.17 during recovery following a placebo but was increased to 7.61 when athletes were given  $\text{NaHCO}_3$  (0.3 g/kg BW) before the run (McKenzie 1988).

Several racing organizations have ruled that concoctions containing  $\text{NaHCO}_3$  are not permitted. The Illinois Racing Board regards the tubing of a horse on race day as a violation of its rules. Furthermore, an excess of  $\text{NaHCO}_3$  is a rules violation because it is a foreign substance that changes the normal physiological state of the horse (Milbert

1991). In Illinois, any horse not being treated with furosemide for exercise-induced pulmonary hemorrhage that has a pre-race plasma  $[\text{HCO}_3^-]$  of 38 units or higher, blood pH above 7.43, and a  $[\text{Na}]$  over 147 mEq/L, is promptly retested. If the second test shows similarly high levels, the stewards are requested to scratch the horse (Bergstein 1989).

## **Dietary, environmental and endogenous substances in racing horses**

With the increasing sensitivity of equine drug testing, the incidence of detection of dietary, environmental and endogenous substances in post-race blood and urine samples has increased. We have categorized these substances under three headings: dietary substances, environmental substances and endogenous substances.

### **DIETARY SUBSTANCES**

#### ***Definition***

A dietary substance is any substance that is part of the normal and ordinary feeding of horses. In this section we are concerned with substances that yield materials in post-race samples that trigger administrative actions. With dietary substances there are generally clear-cut geographic, seasonal and food source influences on the appearance of these materials in post-race samples.

#### ***Salicylate***

Salicylic acid (Salicylate), the prototype Non Steroidal Anti-Inflammatory Drug (NSAID), is found in the post-race urine of all horses and has long been recognized as “normal” in horse urine. Based on a number of studies, thresholds of 6.0 g/ml and 750 g/ml of salicylate in plasma and urine respectively were established. These thresholds are internationally recognized, and the 750 g/ml threshold is being reviewed for adoption as the urinary threshold for this agent in California. Salicylate is an ARCI Class 4 agent (Moss *et al.*, 1985).

#### ***Hordenine***

Hordenine is a plant alkaloid closely related structurally and pharmacologically to epinephrine. It gets its name from *Hordeum vulgare* or barley, a common source of hordenine. Other common sources include Reed Canary grass, brewers grains and sprouting barley. Like salicylate, hordenine is likely to be found in a large number of post-race urine samples if they are examined at high enough sensitivity. There are,

however, regional reports of unusually high concentrations of hordenine being found in the post-race urines of horses racing in Minnesota and also in Queensland. It seems likely that these geographically related high concentrations of hordenine are also seasonally related (Frank *et al.*, 1990). There is no formal threshold for hordenine. Hordenine is not classified by ARCI. Some European laboratories report hordenine to their authorities.

#### ***Dimethyl sulfoxide (DMSO)***

DMSO is found in all horse urines and is thought to be entirely of dietary origin. DMSO and its metabolite, dimethylsulfone (MSM) therefore occur normally in horse urine. In horses on a diet of Lucerne hay, urinary concentrations as high as 5 µg/ml of DMSO in urine have been reported. DMSO is a Class 5 substance in the ARCI classification system. DMSO is often readily identifiable in post-race urine samples and some US labs report DMSO to their authorities. The international thresholds for DMSO are 1 µg/ml in plasma and 15 µg/ml in urine (Crone 1995).

#### ***Morphine***

Morphine is found in significant quantities in hay grown in certain parts of Australia and worldwide in poppy seed used in baked products such as bagels and muffins. Occasional low concentrations of morphine or its metabolites in post-race horse urine in eastern Australia have been traced to horses eating feed contaminated with poppy capsules. Another possible source of morphine is codeine, which is metabolized to morphine in man and presumably also in the horse; the pharmacological activity of codeine in man may be due to its metabolism to morphine. Findings of morphine in post-race urine samples may therefore be associated with contaminated hay in certain geographic areas, inadvertent feeding of poppy seed bagels, accidental contamination from prescription codeine or with morphine from other sources. Because poppies grow wild in Australia, there are clear seasonal and geographic influences on the incidence of morphine identifications in this country. No published or unpublished thresholds for morphine have been reported; however, Australian researchers use chemical-ionization GC/MS procedures to identify equine urine samples where morphine is derived from *P. setigerum* contamination of cereal crops (Batty 1995). Morphine is an ARCI Class 1 agent.

#### ***Scopolamine***

Scopolamine is an alkaloid closely related to atropine that is available as a pharmaceutical agent and also from various plant sources. The most common plant source of scopolamine in the US is “jamestown” or jimson weed, which grows wild across much of the southern United States. Scopolamine identifications are rarely

reported in racing horses, and unequivocally distinguishing between pharmaceutical scopolamine and scopolamine from plant sources is far from trivial. However, a seasonal finding of jimson weed in close association with horses and an associated finding of scopolamine in post-race urines clearly raises the possibility of environmental contamination (Feenaghty 1982). Within the last two years, a number of scopolamine identifications have been made in the US and elsewhere. Scopolamine is an ARCI Class 3 agent. No published or unpublished thresholds for scopolamine have been reported.

### ***Bufotenine***

Bufotenine or NN-dimethylserotonin is an indole alkaloid found in the leaves and seeds of *Piptadenia* and also from *Amanita*. Bufotenine is also hallucinogenic, and materials from frog and toad skin are sometimes ingested for their hallucinogenic effects. At least one identification of bufotenine in a post-race urine sample has been reported in the US, and a number of identifications have been made outside the US. Although no penalty was assessed, no formal threshold for this agent in post-race urine exists. Bufotenine is not classified by ARCI. No seasonal or geographic associations for bufotenine identifications have so far been reported.

### ***Arsenic***

Arsenic is ubiquitous in nature and is found in all horse urines. However, it can be used as a tonic in small amounts and as a “stopper” in large amounts. A threshold was therefore needed to distinguish between normal arsenic and unusually high concentrations of arsenic in post-race urines. Crone and his co-workers analyzed 4,000 post-race samples in Hong Kong between 1983 and 1988. The international threshold for arsenic is now 0.3 µg/ml of arsenic in urine (Crone 1995). While it is highly likely that there are geographic influences on arsenic concentrations in post-race urine, these are not described. Arsenic is not classified by ARCI.

## **ENVIRONMENTAL CONTAMINANTS**

### ***Definition***

Environmental contaminants are substances brought into the environment of the horse by man and are unlikely to be found in horses not closely associated with man. Horses may be exposed to these materials pre-race, in which case metabolites of the materials will be found in the post-race samples. Identification of parent contaminant alone in

the absence of metabolites is presumptive evidence of post-collection contamination.

### ***Caffeine***

Caffeine is the most widely used psychoactive agent in the world. It is consumed daily by humans in considerable (125 mg) amounts. Caffeine is extensively metabolized in the horse, with only about 2-3% of a dose being excreted in the urine as unchanged caffeine. A finding of caffeine in a urine sample with associated metabolites generally means that the caffeine went through a horse. Finding of caffeine without associated metabolites generally means that the caffeine did not go through a horse, with the implication that the caffeine resulted from post-collection contamination. Because of the widespread environmental presence of caffeine, Hong Kong has an unpublished threshold of 0.01 µg/ml in plasma and 0.03 µg/ml in urine. Malaysia also has an in-house threshold of 0.01 µg/ml in plasma (Cheng 1988). Caffeine is an ARCI Class 2 agent.

### ***Theobromine***

Theobromine is 3,7-dimethylxanthine, and for two decades theobromine from cocoa husk was the most commonly identified material in horse urine in England. It proved very difficult to remove cocoa husk from the feed, so the approach was taken of developing a threshold. Studies were carried out at the Horse Racing Forensic Laboratory in England, and 2 µg/ml in urine was established as the regulatory threshold (Greene 1983). Theobromine is an ARCI Class 4 agent.

### ***Nicotine***

Nicotine is ubiquitous in the human environment, and is occasionally identified in post-race urine samples from horses. The metabolism of nicotine in the horse has not been described; however in man cotinine and trans-3-hydroxycotinine are its major urinary metabolites. Based on what is known of the metabolism of nicotine in humans, the likelihood of free nicotine entering horse urine by any route other than contamination is small. In the absence of cotinine or other nicotine metabolites, a nicotine identification is presumptive evidence of post-collection contamination (Stanley 1993). Nicotine is not currently classified by ARCI.

### ***Cotinine/trans-3-hydroxycotinine***

These agents are the major urinary metabolites of nicotine in man. Their identification in horse urine in significant concentrations is presumptive evidence that the horse was exposed to nicotine, such as bedding on tobacco stalks. They are not classified by

ARCI (Stanley 1993).

### ***Cocaine***

Cocaine is ubiquitous in certain human environments, and cocaine and/or its metabolites have been found in tongue ties, in saliva samples from horses entering races and in post-race urine samples from horses. Most of these identifications have been at relatively low concentrations, and their pharmacological and forensic significance is often unclear. In Illinois, control of the use of cocaine on tongue ties has been implemented by use of a pre-race cocaine ELISA. Application of this test allows pre-race detection of cocaine contamination; the trainer is then invited to withdraw his horse, and most elect to do so. This approach avoids the problem of determining the source, pharmacological effect and forensic significance of trace detections of cocaine or its metabolites in post-race urine. Determining the significance of traces of cocaine or its metabolites in post-race samples is made more difficult by the fact that cocaine spontaneously hydrolyses to breakdown products difficult to distinguish from authentic metabolites (Jensen 1995). Cocaine is classified as an ARCI Class 1 agent.

## **ENDOGENOUS SUBSTANCES**

### ***Definition***

Endogenous substances are substances that are specifically synthesized within the horse and are independent of dietary or other sources.

### ***Hydrocortisone***

Hydrocortisone is an endogenous corticosteroid hormone produced by the adrenal gland and essential to normal life. It is also available as an injectable pharmaceutical, and its release in the horse can be specifically stimulated by administration of ACTH. To control its use in racing horses, a urinary threshold of 1 g/ml has been established. Hydrocortisone is an ARCI Class 4 agent.

### ***Testosterone***

Testosterone is normal in the plasma and urine of geldings and fillies but at very low concentrations. Testosterone can also be used for its anabolic actions in fillies and geldings. To control this use of testosterone, a threshold for this agent is required. The Australian authorities use a threshold of 100 ng/ml of testosterone, although by what method this threshold was devised is not quite clear (Batty 1995). Testosterone is an ARCI Class 4 agent.

## Acknowledgments

The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published as a Kentucky Agricultural Experiment Station Article with approval of the Dean and Director, College of Agriculture and Kentucky Agricultural Experiment Station.

Publication #201 from the Kentucky Equine Drug Testing and Research Programs, Department of Veterinary Science and the Graduate Center for Toxicology, University of Kentucky.

Supported by grants entitled "Development of a Test for Procaine in Horses" and "Thresholds and Clearance Times for Therapeutic Medications in Horses" funded by the Equine Drug Council and the Kentucky Racing Commission, Lexington, Kentucky, and the National Horsemen's Benevolent and Protective Association, New Orleans, Los Angeles and the Conselho Nacional de Pesquisa (CNPq), Brazil.

## References

- Batty D. Morphine and the Australian analytical experience, in *Proceedings*. Workshop on "Testing for Therapeutic Medications, Environmental and Dietary Substances in Racing Horses," Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky 1995;93-97.
- Bergstein S. "Milkshakes": A bad idea the sport can do without. *Harness Horse* 1989; 54:6.
- Blake JW, Tobin T. Testing for drugs in horses. *J Equine Vet Sci* 1986; 6:93-97.
- Cheng AS. Threshold levels of prohibited substances in horse body fluids and their relevance to the rules of racing — with particular reference to caffeine, in *Proceedings*. 7th International Conference of Racing Analysis and Veterinarians 1988;97-99.
- Costill DL, Verstappen F, Kuipers H, *et al.* Acid-base balance during repeated bouts of exercise: Influence of  $\text{HCO}_3^-$ . *Int J Sports Med* 1984; 5:228-231.
- Crone DL. Arsenic and DMSO thresholds, in *Proceedings*. Workshop on "Testing for Therapeutic Medications, Environmental and Dietary Substances in Racing Horses," Maxwell H. Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky 1995;63-69.
- Feenaghty DA. Atropine poisoning: Jimsonweed. *J Emergency Nursing* 1982; 8:139-141.
- Frank M, Weckman TJ, Wood T, *et al.* Hordenine: pharmacology, pharmacokinetics and behavioral effects in the horse. *Equine Vet J* 1990; 22:437-441.

- Greene EW, Woods WE, Tobin T. Pharmacology, pharmacokinetics, and behavioral effects of caffeine in horses. *Am J Vet Res* 1983; 44:57-63.
- Hood VL, Schubert C, Keller U, et al. Effect of systemic pH on pHi and lactic acid generation in exhaustive forearm exercise. *Am J Physiol* 1988; 255:F479-F485.
- Jensen R. Personal communication. 1995.
- McDonald J, Gall R, Wiedenbach P, et al. Immunoassay detection of drugs in racing horses. III. Detection of morphine in equine blood and urine by a one step ELISA assay. *Res Comm Chem Pathol Pharmacol* 1988; 59:259-278.
- McKenzie DC. Changes in urinary pH following bicarbonate loading. *Can J Sport Sci* 1988; 13:254-256.
- Milbert N. Illinois horsemen say "No thanks" to milkshakes. *Hoofbeats* 1991; 59:27-32.
- Moss MC, Blay P, Houghton E, et al. Normal and post-administration concentrations of salicylic acid in thoroughbred horses, in *Proceedings*. 6th International Conference of Racing Analysts & Veterinarians, Hong Kong 1985;97.
- Prange CA, Brockus C, Stobert D, et al. Immunoassay detection of drugs in racing horses. V. Detection of mazindol in equine blood and urine by a one step ELISA assay. *Res Comm Subst Abuse* 1988; 9, 13-30.
- Robb E and Kronfeld D. Dietary sodium bicarbonate as a treatment of exertional rhabdomyolysis in a horse. *J Amer Vet Med Assoc* 1986; 188:602-605.
- Stanley SD, Gairola CG, Diana J, et al. Development and characterization of an ELISA for cotinine in biological fluids. *Inhalation Toxicology* 1993; 5:403-413.
- Tobin, T. *Drugs and the Performance Horse*. CC Thomas, Springfield, IL, 1981;85-110.
- Tobin T, Maylin GA, Henion J, et al. An evaluation of pre- and post-race testing and blood and urine testing in horses. *J Equine Med Surg* 1979; 3:85-90.
- Tobin T, Watt DS, Kwiatkowski S, et al. Non-isotopic immunoassay drug tests in racing horses: a review of their application to pre- and post-race testing, drug quantitation, and human drug testing. *Res Comm Chem Pathol Pharmacol* 1988; 62, 371-396.
- Wilkie DR. Muscular fatigue: effects of hydrogen ions and inorganic phosphate. *Fed Proc* 1986; 45:2921-2923.

