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PRE-PURCHASE AND DRUG TESTING OF HORSES: A REVIEW

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The value of a clinically based pre-purchase evaluation of an animal can be significantly limited if the drug or medication status of the animal is not known. This limitation can be circumvented if blood and/or urine or other samples can be obtained for analysis. Forensically, urine is vastly superior to blood for detection of evidence of prior medication. Blood is superior when it comes to interpreting the forensic significance of an analytical finding. The capability and experience of the analytical laboratory is a critical part of the pre-purchase testing process.

I would suggest that an integral part of the pre-purchase examination is a formal inquiry as to the medication status of the animal. It is important to specifically question the vendor about the medication history of the horse and have the vendor 'sign-off' on a form concerning the same. The medication history should cover at least the preceding three months.

Samples
At least five different samples can be collected: blood, urine, hair saliva and sweat.

Blood
Blood is easily sampled and is the minimum of what should be collected. Generally, blood samples can be stored refrigerated for short periods or frozen for longer periods until analysed. Follow the directions of the laboratory. My preference is to collect in green top tubes, centrifuge or let the sample sediment, and draw off the plasma. For longer-term storage, it is best to freeze plasma or serum rather than whole blood. Drugs containing ester groups, classically procaine, are not stable in equine blood for any significant period of time unless the plasma esterases are specifically inhibited.

Advantages of blood testing:
The form of drug found in blood is the parent drug itself and this generally makes it easier to quantify medications or drugs in blood than in urine. For forensically useful data it is important to be able to quantify the drug.

Disadvantages:
The volume of the sample is small and historically this has been a major problem with blood testing. The concentrations of drugs in blood are generally considerably lower than in urine – not uncommonly, fifty to several hundred fold lesser concentrations are found in blood compared to urine.

Urine
Urine samples are more difficult to collect. Special collection facilities and equipment may be required and the animal may have to be held and observed for a period of up to one hour or more. In females, the sample can be collected post-spontaneous voiding or, as we usually do in the lab, by catheter. I have never collected from a male animal other than by waiting until he volunteered a sample.

Medications are commonly found in urine as metabolites conjugated to water-soluble molecules, typically glucuronides. This makes the accurate quantification of drug metabolites in equine urine typically much more difficult than quantification from blood.
The volume and specific gravity of the urine sample can vary substantially and thus affect the concentration of drug in the sample. The use of furosemide for example, in order to collect the sample, can substantially increase the volume and dilute the sample and thereby significantly reduce drug concentration.

Urine pH has a significant effect on drug concentration. Acidic drugs or metabolites concentrate in basic urine. The phenylbutazone metabolite oxyphenbutazone is acidic and can have a 200 fold greater concentration in alkaline urine than in acidic urine. Parent lidocaine, which is a basic drug, can have a 1000 fold greater concentration in acid urine. It can therefore be very difficult to interpret the pharmacological significance of a urinary finding. In racing, the forensic significance of the finding is defined by the rules of racing.

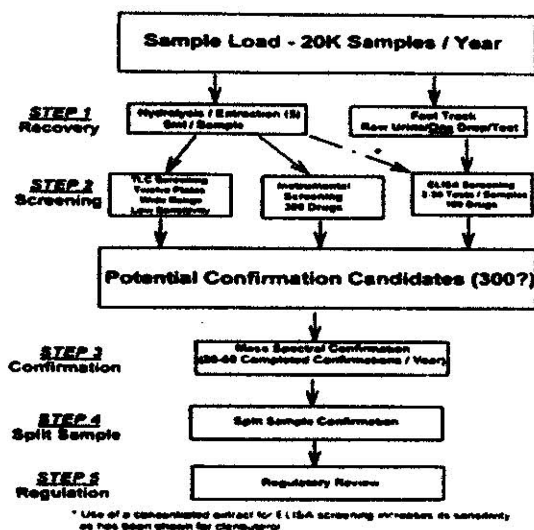
Note:
The way in which urinary findings are reported is often technically inaccurate. For example, a glucuronide metabolite of a drug will be treated (hydrolysed) to recover the parent drug. The material reported to be present in the urine will often be inaccurately presented as the parent drug, when in fact the material present in the urine is not the drug but the conjugated metabolite.

Equine Drug Testing

Equine drug testing consists of a high throughput 'screening' process followed by a lower throughput 'confirmation' process. Screening methods include Enzyme Linked Immunosorbent Assay (ELISA) and Thin Layer Chromatography (TLC). The ideal screening method is sufficiently sensitive and has a high throughput. A screening positive result simply suggests the presence of a drug; unequivocal demonstration of its presence requires mass spectral or other confirmation.

TLC and ELISA tests are relatively fast and inexpensive, but they are not definitive. They will tell you that an agent may be there, but they will not tell you that it is there for certain. If you want to be able to act on a report, it had better be based on a full-scale mass spectral analysis, of the same calibre as would be presented in a racing jurisdiction matter. It would actually be most unfair to all involved in the sale to act on the results of a screening test alone. Mass spectral confirmation can specifically determine the molecular weight of the drug and break it into a fragmentation pattern. Submitting a 'split' sample to a referee laboratory provides independent, or 'split-sample,' confirmation of mass spectral findings. Once the presence of a drug is confirmed, the matter proceeds to a regulatory review.

Flow Chart of Equine Drug Testing



Sequence of Testing and Regulatory Events

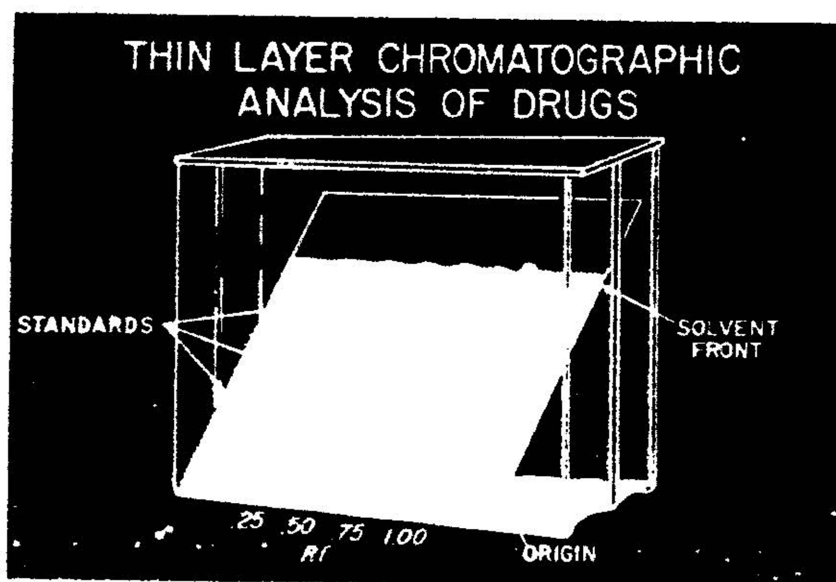
Step 1: Extraction recovery/isolation of drug or metabolite from sample
Step 2: Screening of samples for evidence of the presence of prohibited substances
Step 3: Suspect samples go from screening to confirmation analysis. A high quality mass spectrum usually constitutes definitive evidence of the presence of the drug/metabolite
Step 4: Referee sample confirmation: repeat analysis of a split or referee sample at second laboratory. Once confirmed by a second laboratory, results are unlikely to be contested
Step 5: Regulatory review: ARCI classification residues of therapeutic medications/standardised testing/mitigating factors penalty

Step 1: Drug Recovery

For most analytical techniques drugs or metabolites must be:
1. Hydrolysed from the glucuronide or sulphate moieties that many drugs are linked to for excretion in horses
2. Extracted or recovered via solid phase or liquid-liquid extraction. This yields a concentrated extract of the drug or metabolite.
3. The concentrated extract goes to preliminary screening.
4. Derivatisation: for mass spectral confirmation, the drug/metabolite may need to be chemically modified (derivatised).

Step 2: Screening

Thin Layer Chromatography
Thin layer chromatography (TLC) screening utilises postcard sized glass plates covered with a thin layer (1/32") of silica gel. The analyst applies ('spots') a small volume of concentrated extract of urine to the TLC plate – one plate will run 10 to 20 samples. The plate is placed in a tank containing a shallow level of solvent. This solvent then migrates up the plate by capillary forces. The plate is then dried and developed by over spraying or dipping it in colour inducing solutions (chromatography). The plate is then examined, sometimes under UV light, for suspicious spots.



The throughput with TLC can be high. Multiple batched TLC systems can be used, along with several over sprays. Estimated number of tests at five to ten times sample load, or 100-200,000 tests on 20,000 per annum sample load.

Advantages:
TLC is economical as the components are inexpensive, although the overall process is labour intensive and not readily automatable. The process is rapid: it takes 4 to 8 hours and the throughput is high because samples can be batched. TLC is broad spectrum, covering a wide range of drugs, and new 'spots' may be identified. The system is simple and easy to modify and adapt.

Disadvantages:
TLC is not a particularly sensitive technique – it is generally not effective below 100ng ml. Its use requires experienced and dedicated operators and there are possible health risks from solvents

and sprays. The labour costs are much higher than for ELISA. There are also costs of solvent purchase and disposal.

ELISA
Enzyme Linked Immunosorbent Assay

These are antibody-based tests. which are highly sensitive. A routine sensitivity of about 10ng/ml (parts per billion), may be increased to 0.10ng.ml (100 parts per trillion) if specific drugs or analytes are extracted and concentrated first (eg. Clenbuterol).

ELISA tests have low cross reactivity – an ELISA will detect one drug, plus possibly a few structurally related substances. ELISA tests to cover approximately 100 to 150 drugs are currently available and many identifications of ARCI class 1, 2 and 3 drugs are based on ELISA screening. ELISA allows high throughput. with between 5 and 30 tests possible per sample and up to 100 to 600,000 individual tests per year.

The future aim for ELISA is to develop more tests for specific drugs. These tests must be created on a drug-by-drug basis and each test costs 20 to 50 thousand dollars to develop. A good test is a significant commercial property (Neogen. approaching 100 tests which screen for up to 300 drugs).

Advantages:
ELISA is fast. resulting in high throughput. It has high sensitivity and specificity, is automatable, semi quantitative and good tests are very cost effective.
Disadvantages:
ELISA is drug specific and results in a targeted analysis. In global terms, a limited number of useful tests are available

Screening by Instrumental Analysis

Major laboratory instruments that may be used for drug screening include High Performance Liquid Chromatography (HPLC). Gas Chromatograph Mass Spectrophotometry (GC-MS) and Liquid Chromatograph Mass Spectrophotometry (LC-MS).

In theory, any drug should be detectable by these techniques with a relatively short test development time (perhaps less than 1 month). In practice. the initial costs are greater, but throughput can be high and processes are readily automated. The costs of the instruments have declined steadily and the number of potential drugs detected is theoretically unlimited.

Advantages:
Use of these instruments has the potential to allow identification of a wide range of drugs and analytes. They are suitable for automation, the tests have high sensitivity and specificity and can be used for quantification of drugs. Mass spectral methods may give molecular weight information, which may identify 'unknown' new drugs.
Disadvantages:
Instrumental screening equipment is complex. requiring trained analysts for maintenance and operation. There are high capital costs (S200 to 300.000), high supply and maintenance costs and high workforce costs.