RACEHORSE AND GREYHOUND "DOPE" TESTING

Animals involved in racing are sometimes given drugs to improve their speed, stamina, courage or conduct. It is, however, illegal for an animal to have these compounds in its system on race day. ESR tests urine and blood samples of racing animals to detect such illegal 'doping'.

The testing is done in two stages, and both depend on a knowledge of what type of drugs one is looking for. For this reason it is important that the analysts keep up to date with what compounds are available that could be used illegally in racing.

Step 1 - Extraction
One of two methods can be used. In solid phase extraction the sample is put on a plate coated with a substance that organic compounds (such as drugs) do not adhere to, but to which other compounds in the sample do, and then the organics washed off with a suitable solvent. In liquid-liquid extraction an organic solvent is mixed with the sample, the organic compounds migrate to the organic phase, the two layers are allowed to separate out and finally the aqueous phase is removed.

Step 2 - Separation of components in solution
This is done using chromatography (separating components on the basis of their molecular weight or how well they adhere to a particular solid or liquid) in which the results for the sample are compared with those for known drugs. Quite often the sample first has to be treated to bring the drugs possibly in solution back to their original form, as they are usually changed in a predictable way within the body. After a number of tests have been done it can be stated what, if any, drugs were present in the animal when it was racing.

INTRODUCTION

The term "doping" is associated with the illicit medication of racing animals such as the racehorse and the greyhound. The New Zealand Rules of Racing state that it is an offence to race an animal that has administered to it any substance capable of affecting its speed, stamina, courage or conduct. The rules only apply to substances (e.g. caffeine, stanozolol) present in the animal on race day. Approximately 5000 urine and blood samples per year are collected from racing, harness and greyhound metings and are tested by the Racing Laboratory at ESR (Institute of Environmental Science and Research Ltd.) at Mt. Albert, Auckland. The majority of samples are urine collected from race place-getters. Collection of urine provides a relatively large sample volume (20-300 mL) containing drugs in a concentrated form due to excretion.

The samples are screened in two stages. Firstly a mixture of compounds of interest and other components from the sample matrix is extracted and then various forms of chromatography[1] are used to isolate and identify the drugs.

[1]The term chromatography refers to any separation method in which the components are distributed between a stationary phase and a moving (mobile) phase.

THE SCREENING PROCESS

Step 1 - Extraction

This is carried out using either liquid-liquid or solid phase extraction, and results in a solution that will contain the drugs likely to have been used if such are present.

Liquid-liquid extraction (LLE) is based on the principle of mixing the aqueous sample (urine) with an immiscible organic solvent (e.g. dichloromethane). Drugs present in the aqueous phase should have some solubility in the organic phase, whereas most other compounds will not. If drugs have been administered to the animal this gives an aqueous urine solution with some drugs in it, and an organic solution with the drugs but without many of the other compounds found in urine To selectively extract certain types of drugs the pH of the aqueous phase may be altered (meaning that particular drugs will be relatively more soluble in the organic phase) before extraction. This gives rise to the acidic, basic and neutral classes of drugs. A compound of similar chemical nature to the drugs in question is often added as an internal standard to check on the efficiency of extraction.

Solid phase extraction (SPE) involves the absorption of the aqueous sample onto a solid material. The drugs and other organic soluble components are washed off the solid support selectively by a suitable solvent, giving a solution containing only the organic components. A variation of this is the ion exchange technique (see article) which is used to differentially hold back components of the sample and then release them into the solvent at a specific pH. SPE is suited to automation and usually results in much cleaner extracts than LLE but is more expensive.

Step 2 - Separation of components in solution

Once the sample is extracted the components of the extract must be separated by chromatography. Chromatography is a very important and valuable technique in drug analysis since its application can:

- separate complex mixtures
- detect individual components (selectivity)
- detect low levels of drugs (sensitivity)

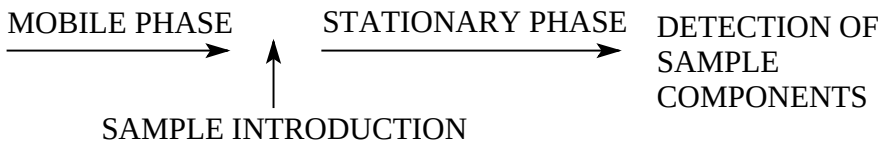

Figure 1 - The basic process of chromatography

Chromatography is based on the principle of having a mobile phase passing through a stationary phase (**Figure 1**). The sample to be analysed is introduced at the beginning of the stationary phase. The components of the sample mixture are separated out on passing through this stationary phase and are individually detected by a specialised detector. Three different types of chromatography are commonly used in racehorse dope testing:

- *Thin-layer chromatography (TLC)*
  Concentrated extracts and control samples each containing one of the drugs likely to be found in the sample are applied close to the edge of a glass plate or paper sheet of solid absorbant. The edge of the plate is placed in a small amount of solvent such that the

solvent does not come as high up the plate as the level at which the extracts were applied. The solvent then runs up the plate, carrying the components of the extract with it. The various components travel at different rates, thus separating the mixture, and the amount by which they have moved can be compared with the known samples to find out what the drug could be. The plate is visualised by UV and/or separated with a dye. Caffeine and clenbuterol are readily detected using this technique.

- *Gas chromatography (GC)*
  The mobile phase in GC is a gas such as nitrogen or helium and the stationary phase is either a liquid or a solid attached to the inside of a column. The sample is injected into the heated column and the components of the sample separate according to how well they adhere to the stationary phase as the sample moves along the length of the heated column. The separated components can be detected by one of a number of detectors positioned at the end of the column including FID (flame-ionisation detector), ECD (electron-capture detector), NPD (nitrogen-phosphorous detector) and MSD (mass selective detector). Many drugs are isolated and detected using GC and one of the above detectors. This technique is very useful for analysing volatile mixtures.

- *High performance liquid chromatography (HPLC)*
  The mobile phase in HPLC is liquid (eluent) and the stationary phace is a solid packed into a column. The liquid sample is injected onto the column and the eluent is pumped through the column. The components (which have been separated according to their adherence to the solid) pass out of the column and enter a detector such as a UV or fluorescence detector.

Derivatisation
Many drugs do not chromatograph well by GC and broad peaks are detected. To enhance chromatographic behaviour and often to give a more distinctive mass spectrum, or fingerprint, for a compound, the extracted drug is chemically derivatised. This is often the case where there are free hydroxyl or phenolic groups in a compound such as morphine.

In animal dope testing, as with human dope testing, the analyst must be aware of how a particular drug is metabolised. A drug will not usually be excreted in an unchanged or parent form and will have probably undergone some chemical modification such as oxidation or the addition of further chemical groups to the basic structure. These groups must often be removed in order to isolate the drug and can be removed either enzymatically or chemically.

Testosterone, for example, is primarily excreted from the horse as the glucuronide (i) and

sulphate (ii) conjugates (**Figure 2**). Both these side chains must be cleaved in order to isolate testosterone which is then derivatised for detection by GCMS. Derivatisation to the Mox TMS (methoxime-trimethylsilyl) derivative of testosterone gives rise to a chromatogram with sharp peaks from which can be obtained a distinctive mass spectrum.

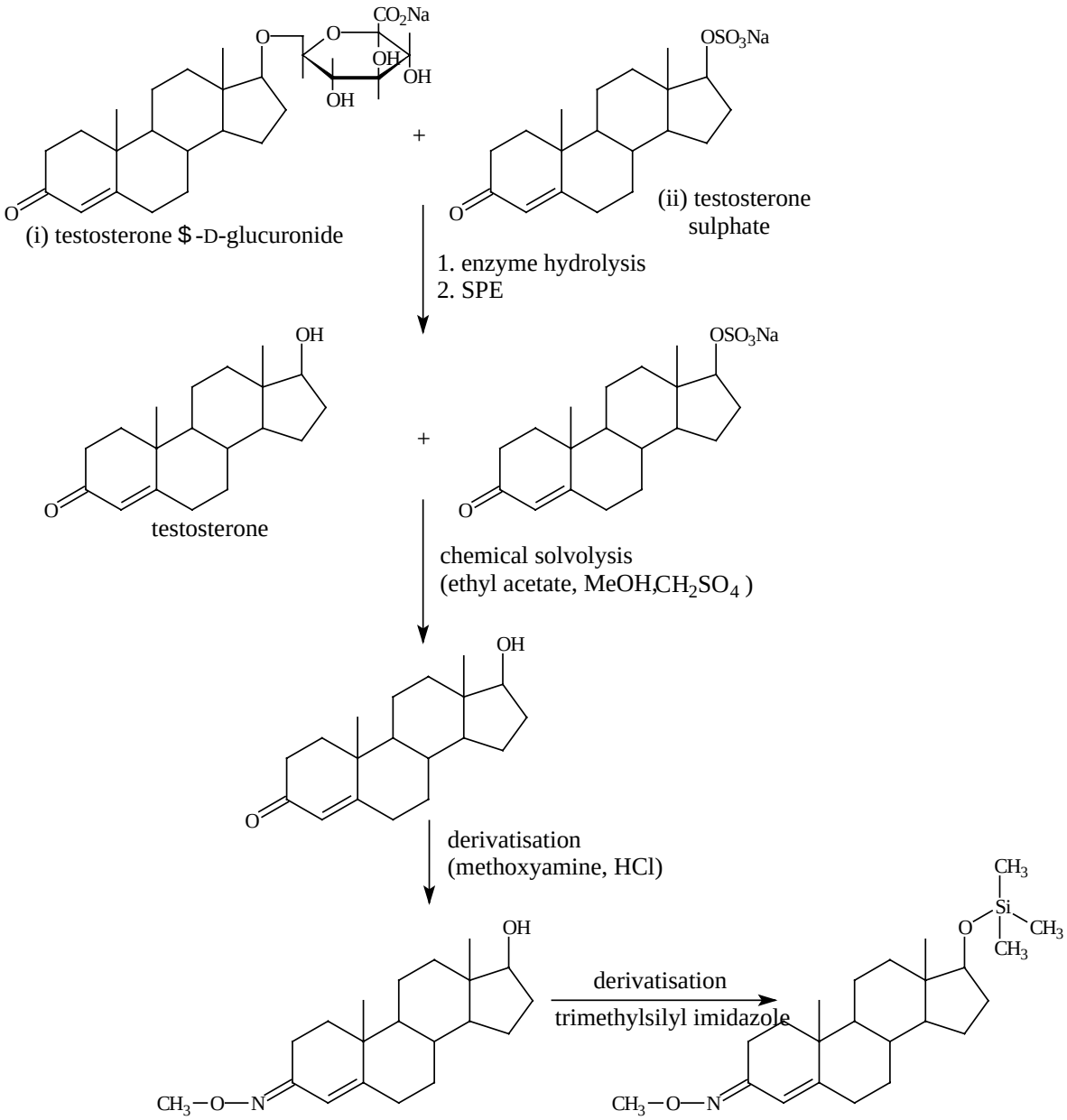

Figure 2 - Treatment of excreted testosterone derivative before chromatography

Detection

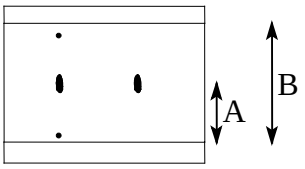

Figure 3

Different chromatographic and detection techniques are utilised according to drug type, e.g. non-steroidal anti-inflammatory drugs are extracted from an acidic medium and are easily screened by HPLC using UV absorbance. Caffeine is extracted from a basic or neutral medium and may be isolated using TLC. The extract is spotted on a TLC plate (**Figure 3**) against a caffeine standard and an extracted urine spiked with caffeine. Caffeine may be visualised under UV at 254nm and the retention factor[2] (Rf) compared with the standard and spiked sample component. When the plate is

[2] Rf = {distance travelled by sample from origin (A)}/{total distance travelled by solvent front (B)}

sprayed with iodine, caffeine appears as a chocolate coloured spot. Caffeine can also be detected using gas chromatography. The extract is injected into a GC in which the components of the mixture are separated as they pass along the column (**Figure 4**). At the end of the column the individual components are detected and recorded as a chromatograph. The retention times of the peaks in the sample are compared to those of a test mixture of drugs and then the identity of the drug is unequivocally confirmed by mass spectrometry (**Figure 5**).

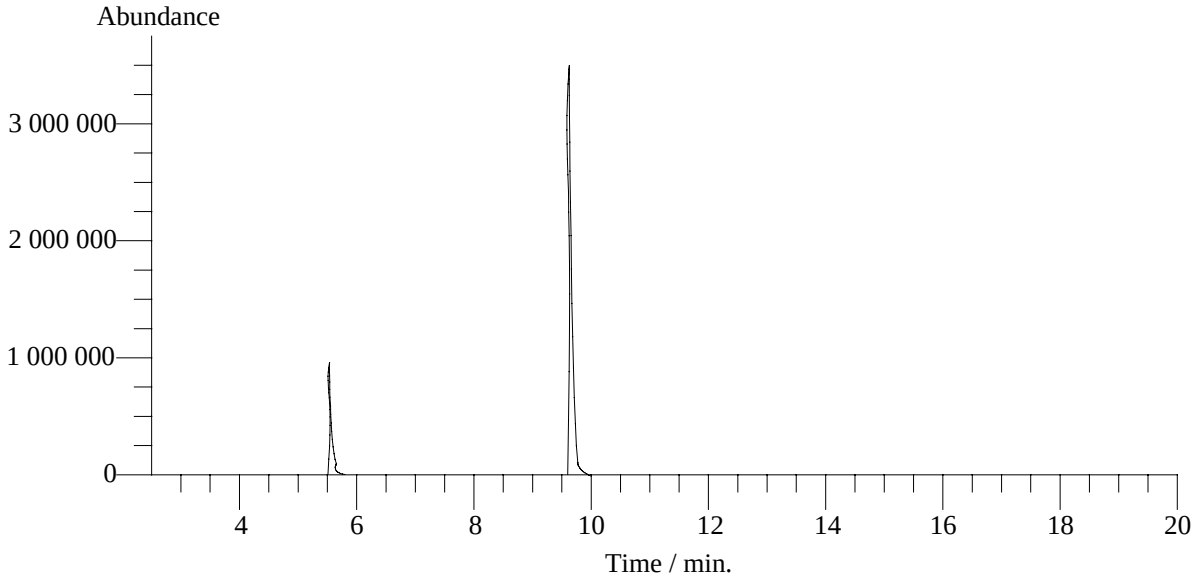

Figure 4 - Ion chromatogram of caffeine

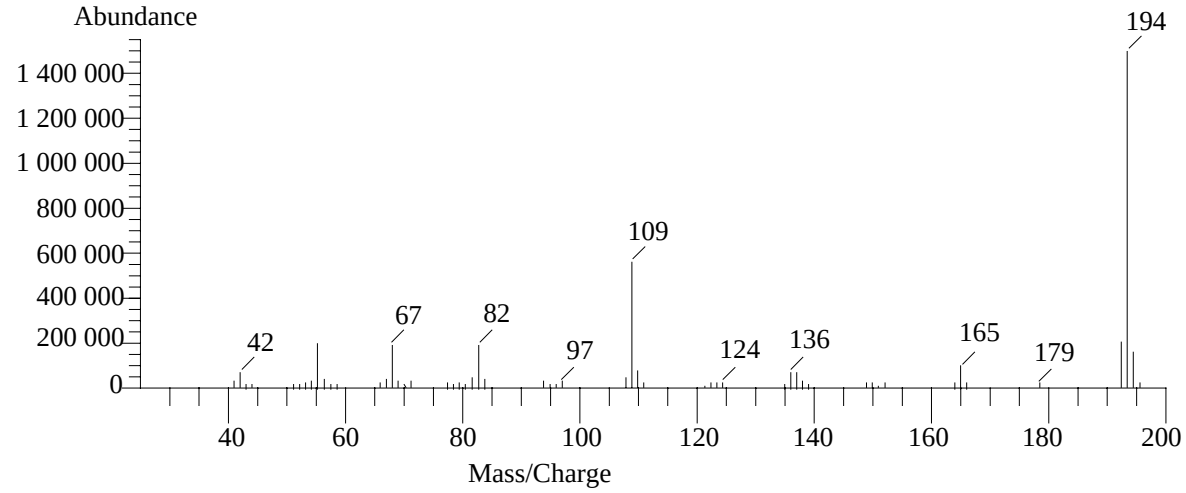

Figure 5 - Mass spectrum of caffeine

Caffeine is best detected by GC using an NPD which has greater sensitivity for nitrogen and phosphorous than for carbon and hydrogen, and thus readily detects drugs such as caffeine which contain nitrogen. The ECD is particulary sensitive to the halogen elements and so is useful for detecting drugs which contain halogens. An MSD gives a useful fingerprint of that compound, and the GC and MSD together form a powerful tool which combines the separation of components of a sample extract with the identification of each component by mass.

In general, dope testing methods must be adapted to processing large numbers of samples, so the most efficient use of instrumentation and extraction technique must be utilised and detection

levels of the drugs of interest determined. As new drugs are constantly being introduced to the market, existing methods must be adapted and new ones developed to incorporate detection of these compounds, and new instrumentation must be evaluated.

ENVIRONMENTAL IMPACT

At present most of the general screening is done using dichloromethane as the extraction solvent. This is collected and redistilled. Following the worldwide trend away from the use of chlorinated hydrocarbons, SPE is being evaluated as a general screening technique as it can be carried out using other solvents.

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