Chromatography Focus

FROM SPORTS ARENA TO POSITIVE DRUG TEST - THE LIFE OF AN ANTI DOPING URINE SAMPLE

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The delivery of an effective sports anti-doping program is dependent on several major elements. One of these is an appropriate sample testing strategy combining the analysis of both in and out of competition samples for substances prohibited in the sport.

This article outlines the steps in this process whilst focusing on the application of separation science in the analytical elements.

To illustrate the process, the lifetime of a hypothetical human sample from collection to positive result is described.

Samples may be collected for analysis from competitors at an actual sporting event (termed 'in competition') or through an unannounced visit to the sports person outside of competition (termed 'out of competition' or 'testing in training').

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The collection of the urine sample itself is facilitated and witnessed by the sample collection officer. The voiding of the urine sample is fully observed by the sample collection officer to ensure that the sample is genuine. There have been instances where other urine samples have been substituted at this point. Two bottles are filled. These are termed the A and the B bottles (*Figure 1*). The pH and specific gravity (SG) of the sample are measured and recorded, the paperwork completed and the sample sealed and packaged for dispatch by courier to HFL.

Figure 1. Sample A and B bottles

On arrival at HFL, the sample is signed for and the packaging is inspected for any evidence of tampering. If all is correct, the sample details are logged into the laboratory information management system (LIMS). This system automatically assigns a suite of tests to the sample and generates barcoded labels for each of the tests.

The A bottle sample is opened and sufficient urine to perform the first pass screening analysis is poured into a clean container. At this point, the pH and SG of this fraction of the sample is measured again and compared with the values obtained at sample collection.

Any difference in result may be an indication of sample deterioration. If the pH or SG is abnormal then the question 'has adulteration of the sample occurred' should be addressed. If the sample condition is satisfactory, the analysis of the sample can commence.

SCREENING CATEGORIES

The initial aliquot of urine is further sub-divided to give aliquots for each screening test (5 in total). The general rationale in assembling a suite of tests for a screening analysis is to attain as broad a compound coverage as possible in as few analytical processes as possible with acceptable sensitivity, selectivity, robustness and cost. An appropriate sensitivity is generally in the high picogram to low nanogram per milliliter range. To put these levels into everyday terms, 1ng/ml or 1 part per billion, is the same as 1 second in your life if you were 30 years old! To achieve this for human sports testing, HFL utilizes a combination of Enzyme Linked Immuno Sorbent Assays (ELISA) together with gas chromatography (GC) and high performance liquid chromatography (HPLC) both linked to Mass Spectrometry (MS).

hCG

The first aliquot is submitted for ELISA analysis. The use of ELISA is limited to those compounds such as peptide hormones that are not suited to detection by any of the chromatography screens. Human chorionic gonadotrophin (hCG) for example is detected using ELISA (*Figure 2*).

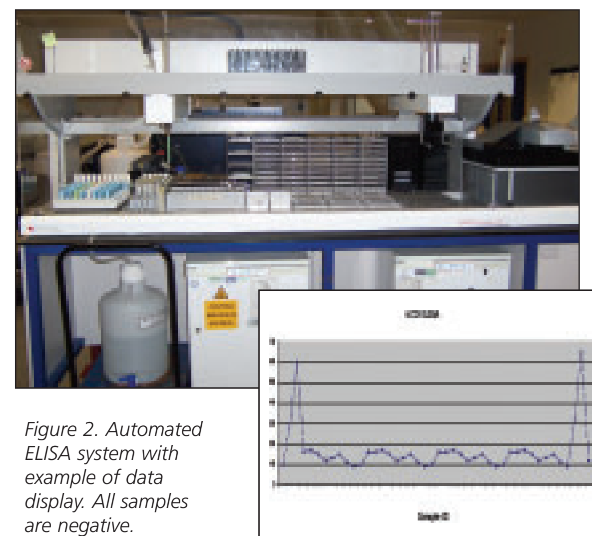


Figure 2. Automated ELISA system with example of data display. All samples are negative.

Conjugated Basic Drugs

The second aliquot is used to prepare a sample for a broad coverage full scan GCMS analysis incorporating a wide range of basic drug compounds including stimulants, narcotics and beta blockers.

The first step of the analysis is to modify the pH of the sample and also to add internal markers that demonstrate that the whole process is functioning correctly. As many of the drug compounds, or metabolites of those compounds, are excreted as glucuronic acid conjugates, an enzymatic hydrolysis using β–glucuronidase is performed.

The pre-treated sample is then cleaned and concentrated for analysis by automated solid phase extraction (SPE) utilizing a mixed mode C8/benzenesulphonic acid sorbent. The resulting eluant is evaporated and reconstituted in a silylating mixture for analysis by full scan electron impact (EI) GCMS.

The acquired data file is submitted to an automated data processing routine using enhanced peak detection, library searching and subsequent data reduction to present a concise report to the analyst for data review.

Due to the nature of this screen, many non-prohibited compounds of exogenous origin are found. Metabolites of quinine for example are common (*Figure 3*)

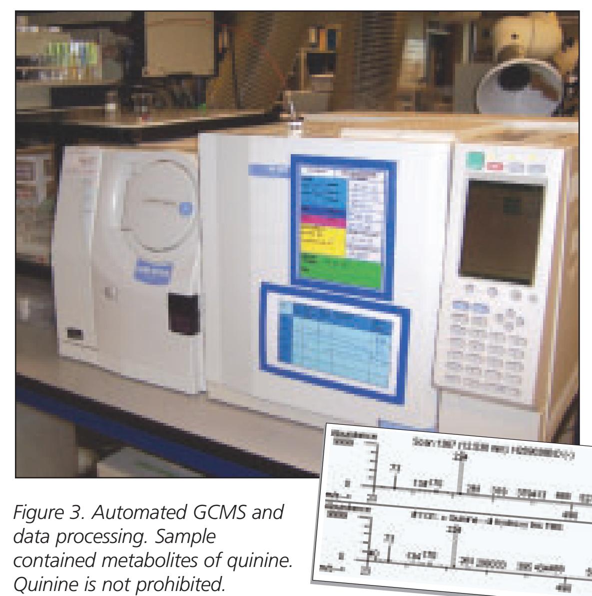


Figure 3. Automated GCMS and data processing. Sample contained metabolites of quinine. Quinine is not prohibited.

Conjugated Anabolic Steroids

The third aliquot of urine is once again enzyme hydrolysed after pH modification and addition of internal markers. It is then extracted using a combination of polymeric SPE cartridges to give a neutral fraction and a basic fraction. The neutral fraction is further prepared for analysis by EI GCMS by forming trimethysilyl (TMS) derivatives of the anabolic steroids in the presence of an enolising reagent. The GCMS analysis is performed in selected ion monitoring (SIM) mode with 30 different acquisition time windows each containing between 10 and 15 ions. The resulting report is generated automatically by the system and contains ion windows for each analyte covered and calculations of approximate levels of endogenous anabolic steroids such as testosterone and epitestosterone (*Figure 4*)

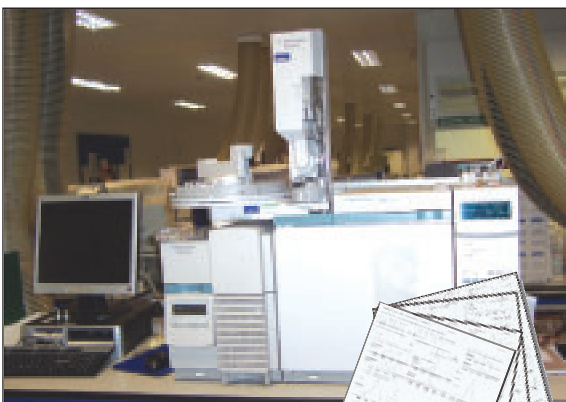


Figure 4. Conjugated anabolic steroid analysis report showing testosterone and epitestosterone (ratio approximately 1:1)

The basic fraction is analysed by LCMSMS looking for compounds not readily detectable in the conjugated basic fraction prepared for GCMS. Included in this screen are compounds such as clenbuterol, stanozolol and the aromatase inhibitors letrozole and anastrozole.

Unconjugated Anabolic Steroids and Diuretics

The fourth aliquot of urine is prepared for analysis by pH adjustment and addition of internal marker. Sample extraction is then performed on a polymeric SPE sorbent to give a fraction containing a wide range of uncojugated analytes. The eluant is then split into two portions. The first portion is analysed as the TMS derivative by GCMS for anabolic steroids and other miscellaneous substances. The second portion is analysed by LCMSMS for a broad range of diuretic compounds including furosemide and hydrochlorthiazide.

Miscellaneous Compound

The final aliquot of urine is used in a LCMSMS screen designed to target problem analytes. The sample is initially enzymatically hydrolysed as in some of the previously described methodologies and is then subjected to a SPE preparation using a polymeric sorbent. The resulting sample is analysed by an ion trap LCMSMS methodology utilizing MS2 and MS3 scan functions. Automated data processing as in all instrumental screens completes the instrumental process. This screen covers compounds such as the designer anabolic steroid tetrahydrogestrinone (THG), basic diuretics and certain neutral stimulants. The sample is also re-analysed on a triple quadrupole LCMSMS instrument for corticosteroids. The sample on this test was suspicious for THG (*Figure 5*).

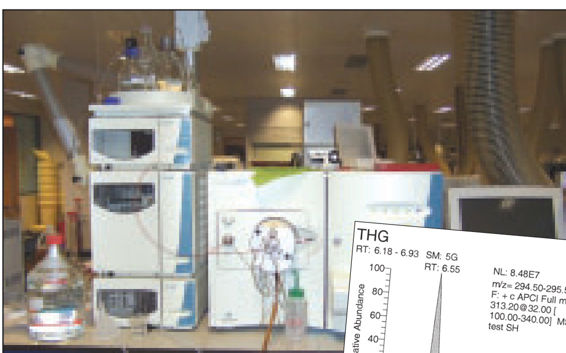


Figure 5. Suspicious peak for THG in screen

CONFIRMATORY ANALYSIS

When a screening test generates a suspicious finding as in *Figure 5*, the sample is re-analysed using a fresh aliquot of urine from the primary 'A' bottle. This analysis is performed with the likelihood that the data will be used in a legal process such as in the Court of Arbitration for Sport.

To this end, the analysis is performed by a single analyst on the one sample, utilizing blank urine samples and certified reference materials to demonstrate the unequivocal presence and identity of the drug substance. In the vast majority of cases, mass spectrometry is used in this process.

The sample analysed through the screen was suspicious for the designer steroid THG and hence requires confirmation. The required confirmatory analysis is assigned to an individual analyst who performs all the steps in the confirmation of the original screening finding.

A new aliquot is taken from the 'A' bottle. In addition, a negative control urine (blank urine), a reagent blank (water or buffer) and a positive control urine (urine augmented with a certified reference standard for the analyte being tested for - in this case THG) are also prepared.

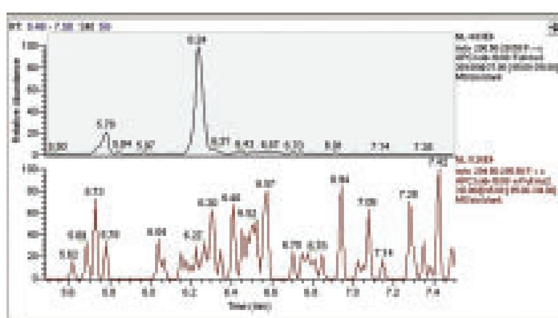
All the samples are extracted using the methodology employed in the screen but this time without the automation. This gives the analyst full control of the process.

The samples, once prepared, are analysed by an optimized full scan ion trap LCMSMS method. The analytical sequence is always the same with the samples being analysed in the following order:-

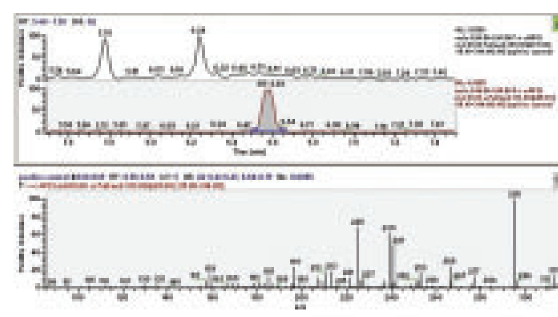
- Negative control
- Reagent blank
- Test sample
- Negative control
- Positive control

This run order demonstrates that the system is clear of drug prior to the analysis of the sample and also demonstrates that there is no carry-over into the positive control sample.

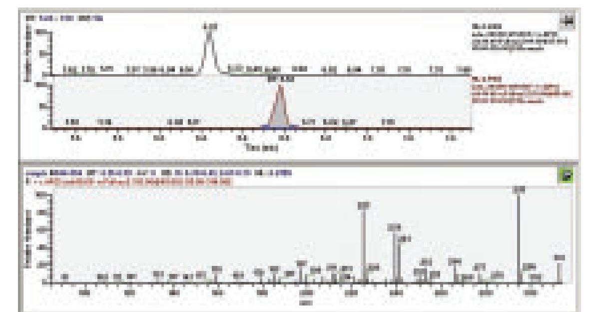
The presence of THG in the sample and the subsequent reporting of a 'failed drug test' or 'adverse analytical finding' is determined by matching the mass spectrum of THG in the sample with the mass spectrum of THG in the positive control sample. In addition, the negative controls and reagent blanks should contain no THG.



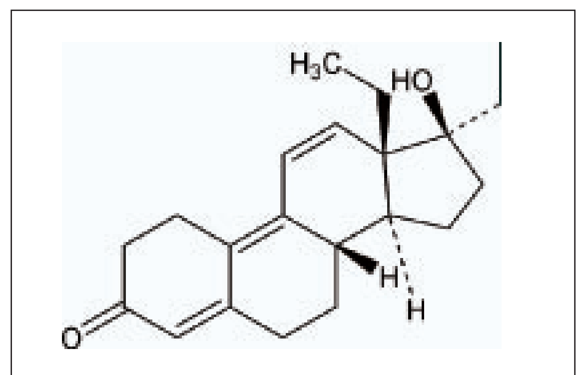
Negative control - The top trace is the internal marker (gestrinone). There is no peak in the bottom trace for THG.



Test sample - The top trace is the internal marker (gestrinone) The shaded peak is THG and the mass spectrum in the bottom window is the full scan MS/MS spectrum of the protonated molecular ion of THG (m/z 313).



Positive control - The top trace is the internal marker (gestrinone) The shaded peak is THG and the mass spectrum in the bottom window is the full scan MS/MS spectrum of the protonated molecular ion of THG (m/z 313)



Tetrahydrogestrinone (THG) MW 312

Figure 6 . Negative control, positive control and test sample in the THG confirmatory analysis. Shaded peaks are THG peaks. The peak at 6.24 minutes is the internal marker, gestrinone

The mass spectra for THG from both sample and positive control are generated. The abundances of a minimum of 3 diagnostic ions are calculated relative to the abundance of the most abundant ion in the mass spectrum (the base peak).

Criteria for comparing relative abundances in the sample and positive control are defined in World Anti Doping Agency (WADA) guidelines. In addition, chromatographic retention time data is taken into account by comparing absolute retention time or relative retention time of the analyte in the sample with the analyte in the positive control. The relative retention time is calculated by dividing the retention time of the analyte by the retention time of an internal marker compound.

Table 1: Mass Spectral reporting criteria

	Abundance (% base peak)		Difference	Difference
Ion m/z	Test sample	Positive Control	% absolute	% relative
295	100	100	0	0
225	68	82	14	17.1
239	61	57	4	7.0
241	47	44	3	6.8
RRT	1.056	1.053	RRT difference = 0.003	

If the criteria are satisfied as above (*Table 1*), the sample is reported to the sport regulator (in this case the national anti doping organisation or NADO) as an adverse analytical finding. This however is not the end of the story.

The witnessed or un-witnessed analysis of the 'B' bottle can now be requested by the competitor through the NADO. Should this prove to also be positive, action may then be taken by the controlling body of the sport involved against the sports person concerned. This may result in a lengthy ban from the sport and a massive reduction in earnings especially if the person is a top performer. Ultimately it may signify the end of a career.

The analysis of samples as part of an anti doping program is a complex process utilising state of the art separation and detection technologies to detect doping substances at very low levels. The confirmatory analysis process after the initial screen protects both the sports person and the laboratory against the reporting of a false positive finding which in turn would have serious consequences for the career of the sports person and possibly also the laboratory.