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The "Anatomy" of a Performance-Enhancing Drug Test in Sports

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Supporting Information

ABSTRACT: The components of a performance-enhancing drug (PED) test in sports include sample selection, collection, establishing sample integrity, sample pretreatment, analyte detection, data evaluation, reporting results, and action taken based on the result. Undergraduate curricula generally focus on the detection and evaluation steps of an analytical procedure, but the other steps often determine the quality of the final result. Following the whole analytical process in a PED test can provide a wealth of useful pedagogical examples for the undergraduate analytical curriculum, including practical illustrations of chemical equilibria and a deeper appreciation of analytical protocol. Moreover, actions taken based on the analytical results in PED testing are usually public knowledge because they involve prominent athletes. As a consequence, students get to see that the analytical result is often one of several possible inputs that produce an ultimate decision, which places the analytical result in a "real-world" context.

KEYWORDS: *Upper-Division Undergraduate, Analytical Chemistry, Curriculum, Interdisciplinary/Multidisciplinary, Applications of Chemistry, Drugs/Pharmaceuticals*

The use of examples from the analyses of performance-enhancing drugs (PEDs) in sports affords an excellent opportunity to reinforce analytical pedagogy by exploring the full timeline of an analytical procedure. This includes establishing sampling protocol, sample collection, establishing sample integrity, sample pretreatment, analyte detection, data evaluation, results reporting, and eventual action taken based on the final results. Undergraduate chemistry curricula generally focus on the analyte detection and measurement step and, to a lesser extent, on sampling and data evaluation. But the quality of the final result is often due to the time and effort spent in the other steps of the overall analytical procedure. Moreover, the sample pretreatment step is especially relevant for reinforcing chemical equilibrium topics from the undergraduate curriculum. In addition, the action taken from these PED measurements is generally public knowledge because it often involves possible sanctioning of a prominent athlete. Discussion of the outcomes from PED analyses shows that a positive result for a PED (adverse analytical finding) does not always lead to the athlete being sanctioned due to mitigating factors. As such, this places the analytical result in a "real-world" context: the analytical result is often only one of several inputs upon which an ultimate decision is made.

The "anatomy" of a performance-enhancing drug (PED) test refers to all the procedures involved in the detection of analyte(s) in a given PED sample. A general scheme for the components of this overall process is given in Figure 1.

The rules governing such samples are set by agencies such as the World Anti-Doping Agency (WADA) and the United States Anti-Doping Agency (USADA). WADA was established in 1999 to be a central body in promoting antidoping activities at the national and international level; USADA was established a year later to implement WADA code in the United States. Among the events

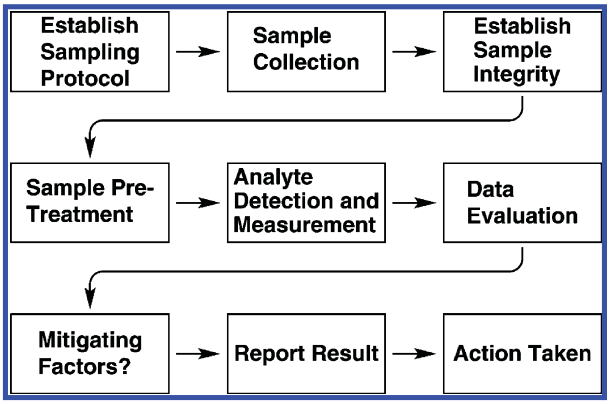

Figure 1. The anatomy of a sports antidoping test.

and organizations that follow WADA antidoping guidelines are the Olympic and Commonwealth Games, World Cup, Tour de France, and international federations for track and field, basketball, hockey, tennis, swimming, and gymnastics. WADA also accredits the laboratories that do PED testing; there are currently 35 WADA-accredited laboratories worldwide. The Web sites of WADA[1] and USADA[2] are major sources for the discussions below.

■ ESTABLISHING SAMPLING PROTOCOL

The most common sample matrices for PED detection are urine and blood. Urine sampling is preferred because it is less invasive than blood sampling. In addition, many PEDs are found at higher levels in urine than in blood, which is also the

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more problematic matrix from an analytical perspective. In-competition (IC) sampling occurs when samples are obtained during a major event, such as the Olympic Games. For such samples, the protocol would determine which athletes would be tested in a given event. Testing might include athletes finishing first and second in an event plus other athletes from the event chosen at random. In addition, target testing can be employed for athletes when, for example, their performance improves dramatically over a short time. Out-of-competition (OC) sampling is usually done unannounced and often in the off-season for a given sport. Most doping experts believe that OC testing, done sufficiently often and anonymously, is more effective than IC testing for curtailment of PED use. Many sports organizations and events follow WADA guidelines for testing, whereas others, such as the NFL and MLB, have their own guidelines for testing.

SAMPLE COLLECTION

The WADA technical document for testing is 91 pages in length, and much of the document deals with the rules and logistics of sample collection.[3] What follows is an overview of the information in this document.

IC sampling is done at doping control stations that are normally run by the antidoping organization running the event, for example, the International Olympic Committee in the case of the Olympic Games. OC sampling is normally done with no advance warning by contacting the athlete at specified locations. Once an athlete is selected for IC or OC sampling, he or she must remain under constant observation by the doping control officer, or a designated chaperone, until the sample is given.

To facilitate OC sampling, WADA has instituted a whereabouts rule for athletes. All WADA-registered athletes must produce a Whereabouts Filing indicating where they will be living, training, and competing over the next three months. In addition, they must designate a 60 min slot during each day of this period in which they would be available for testing. Repeated failure to file and missed OC sampling attempts will result in an antidoping violation for the athlete.

The collected sample is divided into two containers, labeled A and B, in the athlete's presence. If screening and confirmation analysis of the A sample shows the presence of a prohibited PED, the B sample will then be analyzed for confirmation. A positive result for the PED, called an adverse analytical finding (AAF), will only be reported if the A and B tests are in agreement.

The doping control officer and the athlete make sure that the sample is labeled correctly, so that chain-of-custody can be documented during the transfer of the sample to the lab designated to do the analysis and for subsequent sample storage. The doping control officer ensures that the sample is stored properly for transfer to the lab.

ESTABLISH SAMPLE INTEGRITY

The doping control officer will do on-site testing of the urine sample to ensure that the sample volume and specific gravity are adequate for analysis. A low specific gravity could indicate that prohibited methods (e.g., use of diuretics) have been employed to dilute the sample. If the specific gravity is lower than, typically, 1.005, the athlete may be asked to provide an additional sample. The specific gravity result, when compared to the expected value, can also be used to provide a correction for levels of PEDs in the urine sample.

SAMPLE PRETREATMENT

Sample pretreatment is often the most time-consuming and important step in the analytical procedure. Urine and blood are complex matrices containing many hundreds of natural substances. Moreover, PEDs are usually present at very low levels. The major goals of sample pretreatment are to enhance selectivity by eliminating as many potential interferents as possible and to enhance sensitivity by preconcentrating the sample before the measurement step (e.g., 10−20 mL of urine reduced to a few microliters for measurement).

A discussion of the details of sample pretreatment for PED analyses can reinforce chemical concepts and introduce students to the challenge of analyzing complex samples such as those from urine and blood. Some examples of this are as follows:

- Urine samples contain enzymes that convert steroids to glucuronide conjugates to make them more easily excreted in urine. These must be converted back to the free steroid form with β-glucuronidase before analysis.
- Solvent−solvent extraction or the use of solid-phase extraction (SPE) cartridges is required to isolate the desired analytes from potential interferents. The choice of extraction solvents and SPE solid phases provides excellent opportunities to discuss the mechanisms that lead to solvent and SPE surface selectivity.
- Analyte derivatization may be required before the detection step because of the analyte volatility requirement when using GC (e.g., steroid analysis by GC−MS).

A key feature in sample pretreatment is the addition of a suitable internal standard, which is often a stable isotope-labeled form of one of the analytes or a substance that has a chemical structure very similar to the analytes. The internal standard is added to the initial sample aliquots at a fixed and known concentration. Any factor that would affect analyte signal (e.g., chromatographic peak height or area) should have a similar effect on the internal standard signal. As a consequence, measuring the ratio of analyte signal to internal standard signal should obviate this problem. Thus, analyte concentration is related to this ratio rather than to the analyte signal by itself. This corrects for variation in analyte signal due to factors such as loss of sample during pretreatment, variation in sample injection volume, and ion matrix effects with the electrospray ionization in LC−MS.

Details on sample preparation for the analyses of common PEDs, such as steroids and stimulants (e.g., amphetamine), using hyphenated MS methods can be found in the literature.[4−7] A summary is given in the Supporting Information, which includes example reactions for the enzyme-catalyzed conversion of testosterone conjugates to free testosterone and the derivatization of testosterone required for GC−MS.

ANALYTE DETECTION

The detection step depends on the type of target PED. For example, the WADA list of banned substances contains about 60−70 examples of exogenous steroids, which have considerable structural similarities. These substances are likely to be used in training and be present in low concentrations in urine. In this case, screening methods using hyphenated MS methods employing selective ion monitoring and selective reaction monitoring scans are the most sensitive ways to detect these substances. By contrast, other classes of PEDs with structural

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similarities, such as stimulants and diuretics, are most effective when used in competition. Their urine concentrations are expected to be high when tested for during competition, thereby often allowing the less sensitive but better qualitative full-scan MS mode to be employed for detection.

Doping with endogenous steroids, such as testosterone and its precursors, is detected by looking for elevated concentrations of these substances and by using special MS methods. For example, screening for testosterone doping is done by using GC−MS to measure the ratio of testosterone to its epimer, epitestosterone, a nonanabolic steroid that the body normally excretes in about a 1:1 ratio with testosterone.[8] Testosterone/epitestosterone ratios exceeding 4 suggest the use of exogenous (pharmaceutical) testosterone. The use of exogenous testosterone can be confirmed by total combustion of GC components as they elute from a GC followed by isotope ratio MS (IRMS) measurements of the $^{13}CO_2$ and $^{12}CO_2$ produced to determine the ratio of ^{13}C to ^{12}C in the body's total testosterone. Isotopic fractionation occurs when testosterone is produced either from precursor molecules in the body (endogenous testosterone) or by lab synthesis (exogenous testosterone). The depletion of ^{13}C occurs to a greater extent when testosterone is produced in the latter way. As a result, the $^{13}C/^{12}C$ ratio is decreased by a statistically significant and measurable amount when exogenous testosterone is used.[9]

Protein hormone PEDs are most often analyzed by isoelectric focusing and SDS−PAGE (erythropoietin, EPO) or by immunoassays (human growth hormone).

Details on the applications of all of the above methods for PED analysis have recently been published in this journal.[10]

■ DATA EVALUATION

Guidelines

The evaluation of data, which will ultimately lead to a reported result, must conform to strict guidelines set forth by the doping control agency. WADA guidelines for the identification of PEDs from hyphenated MS are described in WADA Technical Document TD2010DCR.[11] A sampling of these guidelines is given below:

- Analyte retention times must agree with those in spiked urine samples or from reference collections by no more than 1% (GC) or 2% (LC).
- When the method relies on chromatographic retention times as part of the identification process, the peak(s) of interest should preferably have capacity factors in the range of 3−10 to optimize the separation factor and detectability.
- If the banned substance is present at a concentration greater than 100 ng/mL, a full-scan mode mass spectrum is preferred for qualitative analysis. The ions used for identification are called diagnostic ions; the intensities of the diagnostic ion peaks (relative abundances) must be within specific tolerance ranges of the diagnostic peak intensities seen in reference data from spiked urine samples.
- If the banned substance is present at a concentration less than 100 ng/mL, selective ion monitoring (SIM mode) or selective reaction monitoring (SRM mode) may be required to detect the substance. In the case of the SIM mode, at least three diagnostic ions are required for detection; all of the ions must be within tolerances from reference spectra intensities and must have a signal-to-noise ratio of at least 3:1. The SRM mode requires tandem MS for detection. In general, two precursor−product ion transitions should be monitored for detection, but one may be sufficient if the transition is unique.
- Concentration determinations require the use of appropriate internal standards, and require comparisons of the ratio of the intensities of a diagnostic ion to that of an internal standard ion in the sample to this ratio in a spiked urine control sample.

Data evaluation for another commonly used PED, synthetic or recombinant erythropoietin (rEPO), involves a pattern recognition approach using the banding patterns resulting from the isoelectric focusing technique. Here again, a WADA technical document provides specific guidelines for differentiating between the isoelectric focusing pattern for normal human erythropoietin (uEPO) and several forms of rEPO.[12] Bands must be present in a specified region of the isoelectric focusing gel and must have defined relative intensities measured by densitomery in order to assign an isoelectric focusing pattern to one of the known rEPO types.

Mitigating Factors

Before a final determination is made on whether an adverse analytical finding (AAF) should be reported, mitigating factors from additional analytical data will be considered. Some examples[13] are listed below:

- Confirmation of elevated steroid levels requires that measured levels be corrected for abnormal sample specific gravity levels.
- The sample must be checked for evidence of microbial contamination. In order to excrete testosterone into urine, the body enzymatically attaches a glucuronide group to the testosterone structure. Microbes present in a nonsterile sample can convert the glucuroconjugates back to free testosterone. To report an AAF from an elevated testosterone/epitestosterone value, testosterone or epitestosterone concentration or any other endogenous steroid parameters, the concentration of free testosterone or epitestosterone in the specimen is not to exceed 5% of the respective glucuroconjugates. In some cases, higher than expected levels of steroid metabolites in the free form may also indicate microbial contamination.

Athletes may have a legitimate medical need for a banned PED substance. In this case, an AAF will not constitute a violation, or lead to a sanction, providing the athlete has obtained a therapeutic use exemption (TUE) for the banned substance. Strict rules govern the granting of TUEs to reduce the possibility of their misuse. The WADA Web site lists the TUE guidelines for athletes in sports governed by WADA rules.[14] Some examples[15] of PEDs that athletes may use with TUEs include oral corticosteroids for severe asthma; diuretics for renal conditions; stimulants for attention hyperactivity disorder (ADHD) and narcolepsy; and insulin for type 1 diabetes.

■ REPORT RESULT

In the case of an AAF from a WADA-accredited lab, the result would be reported to the client (e.g., the International Olympic Committee) that sent the sample to the lab. These reports are extensive and include documentation on sample custody, results from specific analyte tests and from appropriate standards to ensure that the instrumentation was functioning properly and that the methods were correctly employed. Documentation

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provided to WADA by the UCLA Olympic Analytical Laboratory for three positive EPO tests from the 2002 Winter Olympics consisted of a pile of documents and folders about two feet high![16]

■ ACTION TAKEN

When an AAF is reported to the client organization, the organization may decide to sanction the athlete according to guidelines established for the sport. For WADA clients, the sanction for a first offense is normally a two-year ban on competition and possible loss of prize money and championship recognition. If the athlete has been sanctioned previously, subsequent sanctions are much stronger.

An athlete can appeal a sanction, normally by appealing to a panel before a client organization and, if that appeal is denied, the athlete can take his or her case to the Court of Arbitration for Sport (CAS), which is based in Switzerland. The decision of the CAS is final.

A striking example of the process is the case of the cyclist Floyd Landis, who tested positive for external testosterone (based on testosterone/epitestosterone and IRMS data) after stages of the 2006 Tour de France that he won. As an American athlete, Landis had the right to appeal to the American Arbitration Association (AAA). An AAA review panel then considered his appeal, as well as testimony from USADA defending the doping allegation. In September of 2007, the doping allegation was upheld, and the International Cycling Union (UCI) stripped Landis of the Tour title and banned him from the sport for two years. Landis subsequently appealed to the CAS to reverse the sanction, but the appeal was denied in June of 2008. In May of 2010, Landis admitted to the use of PEDs during his pro cycling career.

One example of the type of appeal that the CAS has upheld involves a female gold medal winner in judo at the 2008 Beijing Olympics, whose A and B samples showed evidence for the banned substance clenbuterol. The CAS upheld her subsequent appeal because of a "procedural failure" in the analysis of her B sample. According to the procedure, when her A sample produced a positive test, she or her designated agent should have been invited to view the analysis of the B sample. Because this was not done, the CAS upheld her appeal in a decision that was strongly criticized by the client organization (The International Judo Federation).[17]

"Dog-ate-my-homework" appeals can bedevil sports organizations when athletes contest AAFs on the basis of inadvertent use of a PED. The antidoping sports world works on the principle of the "strict liability rule", whereby athletes are held responsible for what is found in their systems regardless of whether a PED was taken intentionally or unintentionally.[18] This can be especially problematic when athletes use dietary supplements, which, unlike food and drugs, do not require FDA approval before they are marketed. The Dietary Supplement Health and Education Act of 1994 limits the FDA's role in supplement monitoring to a reactive one. It can only ban a supplement shown to be dangerous after it is marketed but not one that is ineffective. Moreover, the FDA is underfunded to do adequate quality controls on many supplement manufacturers. As a result, athletes have tested positive for PEDs from the use of supplements that are contaminated with a PED during production or by intentional addition of a PED to make the supplement "effective".[19]

Although there is some flexibility in individual cases of AAFs due to supplement use, most of these cases result in the athlete being sanctioned. In 2008, six NFL players received four-game suspensions after a positive test for a banned diuretic in weight-loss pills, and a Philadelphia Phillies pitcher got a 50-game suspension for using a steroid-contaminated over-the-counter supplement marketed as a testosterone booster.[17] American swimmer, Jessica Hardy, was able to convince the CAS to reduce her initial two-year suspension for a clenbuterol positive to one year. The CAS considered evidence that clenbuterol was a likely contaminant in a nutritional supplement that she took.[20]

■ USE IN THE CLASSROOM

Much of the material about sports drug testing included herein is appropriate for enrichment of lectures on sampling issues, practical acid–base equilibria, and the illustration of analytical methods, such as chromatography and mass spectrometry. Moreover, the topic is one that can generate debate about the ethics of PED use, as well as the social and economic costs of such use. Although such discussions may seem far afield, my experience is that they enhance student interest and thereby provide incentive for greater mastery of the underlying analytical chemistry. Students also enjoyed the chance to present an end-of-course poster on the analytical detection of an assigned PED.

■ CONCLUSION

Following the "anatomy" of an analytical test can provide students with a deeper appreciation of the full analytical process, from initial sample selection to an ultimate decision based on the measurement result. PED tests embody fundamental chemical equilibrium concepts, must conform to specific analytical criteria, require the use of important instrumental techniques and ultimately contribute to a decision that is public knowledge. As such, these tests can provide relevant examples of chemical principles, as well as a perspective on the role of analytical measurement in the outcome of "real-world" questions.

■ ASSOCIATED CONTENT

Supporting Information

Details on the pretreatment of PED samples for hyphenated MS analysis, including a figure showing the steps in the preparation of a steroid (testosterone) for GC–MS analysis and schemes showing the chemical reactions for the conversion of testosterone glucuronide to free testosterone and the derivitization of testosterone using *N*-methyl-trimethylsilyltrifluoracetamide. The advantages of SPE cartridges over extraction for sample cleanup are listed. An example of a sample preparation procedure for the analysis of stimulants (amphetamine) by LC–MS is also presented. This material is available via the Internet at http://pubs.acs.org.

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Notes

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