Beyond Sports-Doping Headlines:

The Science of Laboratory Tests for Performance-Enhancing Drugs

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INTRODUCTION

Young athletes breaking into the elite level can look forward to giving interviews, autographs… and urine samples. This article shows what happens when the latter are tested for prohibited doping agents at the laboratory—how roomfuls of regulations and teams of specialized professionals ensure that the laboratory work is conducted accurately, and that the test results are handled properly. Drug testing, along with drug education, research, and results management, is how an antidoping program enforces the rules, protects fair play, and defends the clean athletes' freedom to compete without drugs.

REGULATORY FRAMEWORK

The fight against drug abuse in sports has grown and improved ever since doping control began in the 1960s. Worldwide antidoping efforts are better organized, harmonized, and structured than ever. This is true not only of the rules, prohibited substances and methods, sanctions, and appeals, but also of laboratory accreditation and reporting criteria. A positive test result, or laboratory report that a prohibited drug was found in a sample, is referred to in antidoping jargon as an adverse analytical finding. It is the antidoping organization that determines whether the case is positive.

The World Anti-Doping Agency (WADA, see Box 1. for common acronyms) has the support and participation of World Anti-Doping Code signatories, such as governments and private entities, to work with the International Olympic Committee (IOC), National Anti-Doping Organizations (NADOs), sports federations, and athletes to control doping in sport. NADOs testing programs, such as that of the US Anti-Doping Agency (USADA), fall under WADA regulations, but those of United States professional sports, the NCAA, and United States high schools do not.

Box 1. List of acronyms

CIR: carbon isotope ratio (same as carbon IRMS)
DAD: diode array detector
EPO: erythropoietin
ERC: endogenous reference compound
GC: gas chromatography
GC-C-IRMS: gas chromatography-combustion-isotope ratio mass spectrometry
GC-MS: gas chromatography-mass spectrometry

Table 1. List of acronyms (continued)

GC-MS-MS: gas chromatography-tandem mass spectrometry
GH: growth hormone
HBOC: hemoglobin-based oxygen carrier
hCG: human chorionic gonadotropin
HES: hydroxyethylstarch
HPLC: high-performance liquid chromatography
IA: immunoassay
IEC: International ElectrochemicalCommission
IEF: isoelectric focusing
IOC: International Olympic Committee
IRMS: isotope ratio mass spectrometry (when applied to carbon, same as CIR)
ISL: International Standard for Laboratories (WADA)
ISO: the symbolic name of the International Organization for Standardization
LC-MS: liquid chromatography-mass spectrometry
LC-MS-MS: liquid chromatography-tandem mass spectrometry
LH: luteinizing hormone
MRM: multiple reaction monitoring
MS: mass spectrometry
NADO: national antidoping organization
NCAA: National Collegiate Athletic Association
RSR-13: a pharmaceutical hemoglobin modifier
SIM: selected ion monitoring
SOP: standard operating procedure
SRM: selected reaction monitoring
TD: technical document (WADA)
THG: tetrahydrogestrinone
TMS: trimethylsilyl
TUE: Therapeutic Use Exemption
USADA: United States Anti-Doping Agency
WADA: World Anti-Doping Agency

The WADA 2007 List of Prohibited Substances and Methods [1] includes prohibitions effective at all times or only in-competition. Among them are anabolic agents (eg, anabolic steroids), hormones

(eg, erythropoietin [EPO]), diuretics and other masking agents, chemical and physical manipulation, stimulants, and more. The list gives examples in each class and includes "other substances with a similar chemical structure or similar biological effect(s)." Therefore, athletes cannot claim innocence merely because the drug they used was not listed by name. Only nine of several hundred prohibited drugs have a cut-off [2]; for all the others, any detectable amount constitutes an adverse analytical finding. In the United States, professional sports, the NCAA [3], and high schools have their own prohibited lists, which overlap with WADA's list.

National antidoping agencies, such as USADA, must use WADA-accredited laboratories to test the samples that they collect. A prerequisite to WADA accreditation is ISO accreditation (ie, the laboratory must meet the requirements of ISO/IEC 17025) [4]. These include a quality assurance program; standard operating procedure (SOP) for assays, instrument operation and maintenance, personnel qualifications, restricted access to premises, computers, and electronic records; internal audit trails; and traceability of results to reference standards.

The WADA requirements for laboratories reflect those of ISO and are stated in the International Standard for Laboratories (ISL) [4] and WADA Technical Documents. WADA generally does not require laboratories to follow prescribed SOPs; instead, laboratories are required to meet performance criteria. For example, laboratories must be able to detect 2 ng/mL of clenbuterol in urine [2]; the sample preparation procedure and analytical techniques are up to each laboratory. WADA requires laboratories to have research activities to optimize tests performance and to keep up with cheaters. WADA accreditation is up for renewal annually.

SAMPLE IDENTITY AND INTEGRITY

The first step in a doping-control urine test is getting an authentic urine sample from the correct person and getting it sealed and documented for shipment to the laboratory. Thus, the first crucial link in the doping-control process is the Doping Control Officer or Sports Drug Testing Collector and his or her staff.

Athletes who enter sports competitions agree to follow the rules—including antidoping rules, from being subjected to doping-control tests to accepting the consequences of a positive test. Athletes selected for a test identify themselves before they urinate in a cup, under direct observation by an official of the same gender. Next, the urine is poured into a pair of bottles, A and B, labeled only with numbers (eg, 963852A and 963852B) and the bottles are sealed. Only the sport organization—not the laboratory—knows which number corresponds to which athlete. Chain of custody paperwork documents who has custody of the

samples or where they are locked up, from the moment the bottles are sealed, to their receipt at the lab, to the day when they are finally discarded.

Blood is rarely collected in the major United States sports drug testing programs. At the Olympics, blood is collected, but not as often as urine. For example, at the 2004 Athens Olympics, the laboratory received 2926 urine samples and 691 blood samples [5]. Some federations, such as Union Cycliste Internationale, collect blood before races for health tests (not doping-control tests), and athletes with atypical values deemed medically unsafe (eg, high hematocrit) are not allowed to compete [6].

Testing urine is better than testing blood for most prohibited substances (small molecules, molecular weight less than ~800 atomic mass units). Urine collection is noninvasive and yields a large volume of sample, with higher drug concentrations than in blood and with far fewer cells and proteins to complicate extraction.

Is it possible to tamper with the sample containers? Not without leaving evidence of it. Sample integrity is checked and documented upon receipt at the laboratory, by technicians who inspect containers and tamper-evident seals visually, then record whether the chain of custody was intact. The bottles used at the Olympics are sealed with a thick plastic cap over the stopper, and the only way to access the sample is to destroy the cap.

To deter urine substitution, urination is observed. This led to the discovery at the Athens Olympics of a contraption consisting of a bag of clean urine up the rectum and plastic tubing running along the underside of the penis [7].

High school drug-testing programs may test only for street drugs or only for anabolic steroids. In some programs, samples are not split and adverse analytical findings are not reported by individual bottle number, but only in an aggregate fashion, reporting the finding of a drug with no further detail than “in one sample in the batch.”

A DOPING-CONTROL LABORATORY TEST CONSISTS OF MORE THAN ONE TEST

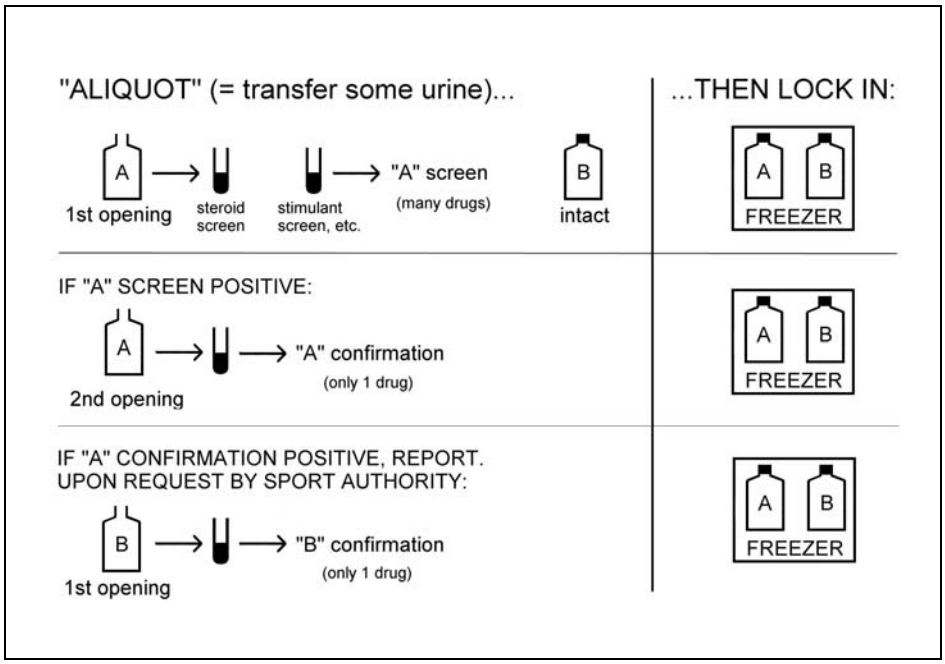

Fig. 1. Typical antidoping laboratory procedure

At the laboratory (Fig. 1), all “A” samples (a portion or “aliquot” of each) undergo screening for all the drugs on the relevant (in or out-of-competition) list (menu). The goal of screening is to rapidly sort samples into two categories: certainly negative and maybe containing a target compound. A well-designed screen is quick, detects a broad variety of substances, and provides a mere indication, not full proof, that the compound is present. Because drugs tend to be chemically similar within each class (eg, stimulants, steroids), but chemically different between classes, the best conditions for extraction and detection tend to be the same within a class, but different between classes. Typically, laboratories conduct one stimulant screen, one or two steroid screens, one diuretic screen, and so forth on each sample in a batch of test tubes; each test tube represents one athlete. The batch also includes quality-control test tubes.

If the screening data contain any indication that a drug might be present, a fresh portion (aliquot) of the “A” sample undergoes a confirmation attempt. Although a screen collects a little bit of data on each of numerous target compounds, to see if any might be present, a confirmation collects a lot of data on only the suspected compound.

Typically, the time elapsed between receiving the samples at the laboratory and reporting results on the "A" samples (turn-around time) is one to two weeks for year-round testing, and it can get as short as 24 hours for negative "A" results during major events. "A" confirmations take longer. Results are needed as soon as possible when the world is watching and athletes compete more than once, because if they used drugs, they should be removed from competition.

If the "A" sample analysis confirms the presence of a drug, the laboratory reports to the antidoping program its finding in the sample, identified only by code number—the only identification known to the laboratory. The athlete is notified and has the right to come to the laboratory or send a representative of his or her choice to witness the "B" confirmation. After verification of identity, the witness examines the "B" sample exactly as it was last seen by the athlete when it originally was sealed for shipment to the laboratory; paperwork is filled out and signed. The witness may then observe the "B" confirmation, which takes two to three days, depending on the drug. Some witnesses choose to watch the process; others choose to leave before the laboratory work begins. After completion and conclusion, the laboratory reports the result of the "B" confirmation to the antidoping program. What remains of positive samples is securely frozen for the length of time that meets applicable regulatory and contractual requirements. Typically, negative samples are disposed of sooner. Disposal is the last entry for each sample's chain of custody documentation.

MAIN ANALYTICAL TECHNIQUES

The goal of this article is to cover key points without being exhaustive and to focus on urine analysis and data interpretation. The laboratories' job is to detect hundreds of substances. Table 1 shows common technologies. The choice of technology is determined primarily by chemical characteristics (eg, solubility in water or organic solvents, volatility, thermolability, polarity) and secondarily by logistics (eg, resident expertise, staff or instrumentation, capacity and throughput in different laboratory sections). Many substances are routinely detected by more than one approach [5,8].

Table 1. Screening technologies for classes of prohibited substances and methods

FROM WADA 2007 PROHIBITED LIST	URINE SCREENING TECHNOLOGY					
SUBSTANCES AND METHODS PROHIBITED AT ALL TIMES (IN AND OUT OF COMPETITION)	IA	GC	GC-MS	LC-MS or LC-MS-MS	Miscellaneous	IEF
PROHIBITED SUBSTANCES						
Anabolic agents (anabolic steroids)			X	X		
Hormones	hCG, LH					EPO
Beta-2-agonists			X			
Antiestrogens			X			
Diuretics and other masking agents			X	X	HES dextran by biochemical analyzer	
PROHIBITED METHODS						
Enhancement of oxygen transfer			HES, RSR-13			HBOCs
Chemical and physical manipulation						
Gene doping	No urine test					
SUBSTANCES AND METHODS PROHIBITED IN COMPETITION						
Stimulants		X	X			
Narcotics		X	X			
Cannabinoids	X		X			
Glucocorticosteroids				X		
SUBSTANCES PROHIBITED IN PARTICULAR SPORTS						
Alcohol					Dipstick or GC	
Beta-blockers			X	X		

SAMPLE PREPARATION

The work-up ranges from one hour to one day, depending on the screen. Because metabolism attaches sugars (conjugates) to some drugs (eg, anabolic steroids), the sugars need to be cleaved (deconjugated) using an enzyme (eg, β-glucuronidase) or an acid for some incubation time. The freed drugs that are still too polar and involatile to be vaporized for analysis need to be derivatized, or reacted with chemicals that will "cap" their polar functional groups (eg, to convert hydroxyl groups into trimethylsilyl ether or OTMS groups in the case of anabolic steroid screening).

ANALYSIS

Chromatography

Chromatography is an analytical chemistry technique used to separate (resolve) the chemical compounds in a mixture. Gas chromatography (GC) is done in the gas phase. A gas chromatograph (GC) has three parts: a sample introduction system (injector), an oven containing a chromatography column to achieve separation, and a detector. Typically, a microliter of liquid urine extract is automatically injected into the injector, a chamber at a high temperature. The sample is vaporized and swept along a hair-thin glass tube (capillary column, many meters long, flexible enough to be rolled up in a coil) by a carrier gas (mobile phase), such as helium. Different compounds travel at different speeds because of the differences in boiling point, polarity, and relative solubility in the carrier gas versus the coating of the inner wall of the column (stationary phase). The compounds emerge from the column outlet at different times after injection (the chromatographic retention time)—separated from each other. Under identical operating conditions, the retention time is characteristic of each chemical compound. If two compounds have the same retention time, they may be identical (eg, testosterone). If two compounds have different retention times, they certainly are different (eg, testosterone and methyltestosterone). Matching retention times between an unknown and a reference standard is one element of identification.

A graph of the amount of substance as a function of the retention time is a chromatogram (Fig. 2A) [9]. Two common GC detectors in antidoping labs are the nitrogen-phosphorus detector (NPD) and the mass spectrometer (MS). The NPD detector is ideal for detecting nitrogen-containing compounds such as stimulants.

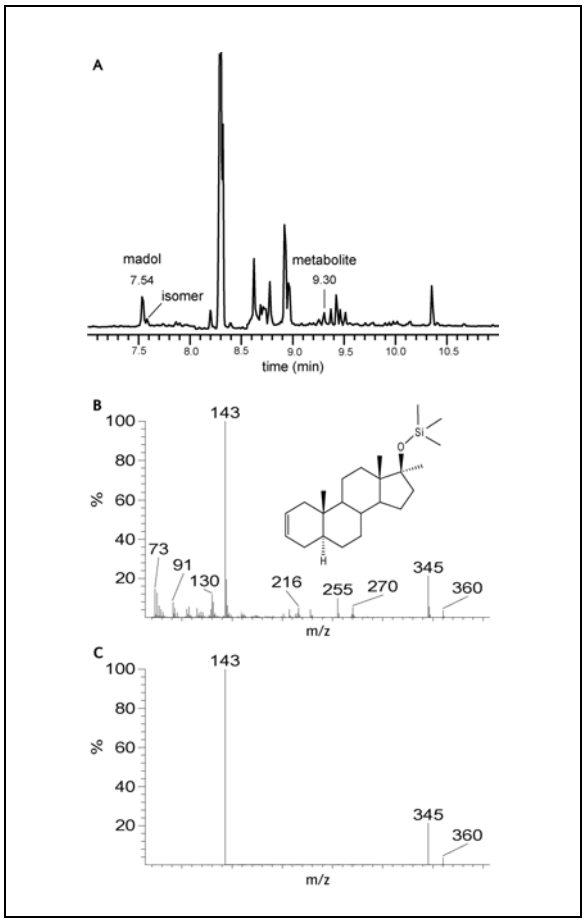

Fig. 2. GC-MS data for designer steroid madol.

(A) chromatogram; the isomer differs only by the position of the double bond.

(B) full scan.

(C) selected ion monitoring (SIM) scan.

Mass spectrometry

Mass spectrometry (MS) is an analytical chemistry technique used for structure elucidation of unknowns or identification of known compounds. A mass spectrometer has three parts: an ion source where the compound is ionized to form a molecular ion and fragmented into smaller ions; a mass filter that separates ions by mass-to-charge ratio (m/z); and a detector. The graph of ion abundance as a function of m/z is a mass spectrum. In Figure 2B, the molecular ion is 360 and significant ions are 345 and 143 (largest = base peak = 100%). The fragmentation pattern is determined by weak bonds and other physicochemical characteristics; therefore, fragmentation is reproducible and characteristic of the

molecular structure, and the mass spectrum is like a fingerprint of the compound. Matching mass spectra between an unknown and a reference standard is another element of identification. Significant ions are so characteristic that matching only three ions (eg, 143, 345, 360) and their percent abundance relative to the most intense of the three (eg, 143) has long been widely accepted as proof of chemical identification.

Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is the technique that is used most widely in antidoping laboratories. The GC effluent enters the mass spectrometer continuously, and the mass spectrometer continuously records roughly one mass spectrum (scan) per second. There are two main modes of MS operation: the full-scan mode and the selected ion monitoring (SIM) mode (see Fig. 2). In the full-scan mode, the mass spectrometer records the whole mass spectrum (from m/z 70 to 400), monitoring hundreds of ions. In the SIM mode, only selected ions are monitored (eg, 143, 345, 360); therefore, a longer time is spent recording each ion. In physics, signal strength (signal-to-noise ratio) increases with the time spent collecting data. Therefore, on the same instrument SIM is more sensitive than full scan: it can detect smaller amounts of drug. Other types of MS that are more sensitive include high-resolution MS, tandem MS and ion traps. High-resolution MS is designed to measure m/z not only to the nearest unit or decimal, but out to several more decimals. This makes it possible to mathematically deduce the molecular formula (how many carbon, hydrogen, oxygen and other atoms it contains); the more decimals, the fewer combinations of atoms fit, the narrower the possibilities. High-resolution MS instruments happen to be inherently more sensitive. Tandem MS instruments have two mass spectrometers back to back. The first one can be used to select only one ion, the precursor ion, which can be the molecular ion. The second mass spectrometer monitors only one (or at most a few) characteristic fragmentations (transitions to product ions). This is called the Multiple Reaction Monitoring (MRM) or Selected Reaction Monitoring (SRM) mode. (Alternatively, the first mass spectrometer can be used to select only the molecular ion and the second mass spectrometer can be used to record a full scan.) Tandem MS is more sensitive because it is blind to interferences. Unlike all the above MS types, which let all ions formed continually escape from the ion source, ion traps trap all ions until they are released, one m/z at a time, to determine their abundance.

Liquid chromatography

Whereas GC is done in the gas phase, liquid chromatography (LC) is done in the liquid phase. This is a crucial difference because it works for thermolabile compounds (destroyed by GC) and polar compounds (cannot be vaporized). The separation principles are the same. A typical high-pressure or high-performance LC (HPLC) column is a steel tube the size of a fat marker pen, packed with microbeads on

the surface of which is the stationary phase. The mobile phase is a liquid solvent, often a mixture whose composition is programmed to change during the run (gradient elution).

Two common HPLC detectors are the diode-array detector (DAD) and the mass spectrometer. The DAD monitors UV absorption over a range of wavelengths or at selected wavelengths; it detects only those compounds that absorb UV light. When the HPLC is connected to an MS, the instrument is called LC-MS. The most advanced type of LC-MS can do tandem MS by one of several choices of conceptual and hardware approaches. It is called LC-MS-MS or LC-tandem MS.

For a given class of drugs, such as diuretics (Fig. 3), the LC-tandem MS screen is far superior to the GC-MS screen. Sample preparation time can be well less than an hour, down from a full day's work, because unlike GC, LC does not require removal of water or salts, deconjugation or derivatization. Typically, the instrumental analysis run-time is two to three times shorter, well under 10 minutes per sample, because LC-MS-MS is blind to interferences; therefore, chromatographic resolution is not required, and LC run times can be shortened.

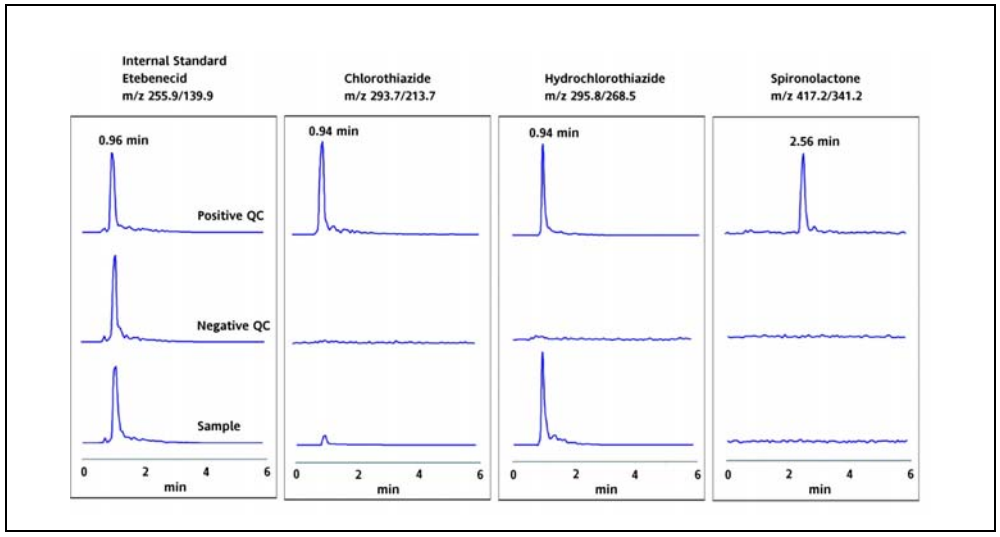

Fig. 3. Example of diuretic screen LC-MS-MS data.
Top row: positive quality control (QC) urine spiked with diuretics chlorothiazide, hydrochlorothiazide, and spironolactone. Middle row: negative quality control urine. Bottom row: unknown urine sample. The internal standard (etebenecid) is added to each sample and control during work-up; detecting it shows that the assay performed as expected. Detecting the diuretics spiked into the positive control confirms this. The sample screens positive for hydrochlorothiazide.

Drug identification

Except for proteins, such as EPO, most prohibited drugs are identified by GC-MS, the workhorse of doping-control laboratories. LC-MS is used increasingly for diuretics, some anabolic steroids, and corticosteroids. Doping-control scientists identify a substance, in the laboratory and in court, by matching chromatographic retention time and mass spectra between unknown and standard. They need an authentic reference standard—a sample of the substance, certified to be correct. The standard may be a white powder or an excretion urine from a volunteer who took the drug. Chromatography coupled with mass spectrometry makes it possible to identify not just drug classes, but specific chemicals, with absolute certainty.

Pharmaceuticals include some synthetic compounds that do not occur naturally (eg, the anabolic steroid stanozolol) and some that do (eg, testosterone). Unfortunately, GC-MS and LC-MS cannot distinguish natural, endogenous testosterone from pharmaceutical, exogenous testosterone; however, normal human urine samples contain a testosterone isomer with no known function, epitestosterone. The urinary ratio of testosterone to epitestosterone (T/E ratio) is roughly 1:1 in most normal men, and it increases upon testosterone administration. Since the 1984 Olympics, the T/E ratio has been used to screen for testosterone use. Adverse analytical findings are defined by a T/E cutoff, which currently is 4. The two problems with any cutoff are that rare, drug-free individuals might have a naturally elevated T/E and that T/E may never exceed the cutoff in some users, either because their T/E is not responsive to administration or because they use small doses and titrate themselves. To distinguish users from nonusers, longitudinal profiling consists of plotting T/E and other urinary androgen parameters over time, expecting stability for nonusers and a spike for users. In the 1990s a new approach was introduced: isotope ratio mass spectrometry (IRMS) [10].

Isotope ratio mass spectrometry or carbon isotope ratio

It so happens that there is a measurable difference in carbon-13 content between endogenous and pharmaceutical testosterone. Most carbon atoms in nature are carbon-12, with a nucleus containing six protons and six neutrons. Radiocarbon dating relies on the rare carbon-14, an unstable, radioactive isotope, with a nucleus containing six protons and eight neutrons, which decays over time. Between the two is carbon-13, a stable isotope with six protons and seven neutrons. Roughly 1.1% of carbon in nature is carbon-13. Pharmaceutical testosterone contains a few parts per thousand less carbon-13 than does natural testosterone. This is because they arise from biosynthetic pathways that are sufficiently different. Humans make endogenous testosterone from cholesterol, itself made from acetate or coming from the diet. Pharmaceutical companies make testosterone by semisynthesis from plant sterols. All

carbon in living beings is ultimately derived from atmospheric carbon dioxide (CO_2), fixed in plants by photosynthesis. Different plants make the first multicarbon intermediates and downstream biosynthetic compounds differently. Animals eat plants, humans eat plants and animals, and we are what we eat. At every biosynthetic step, carbon-13 is left behind. This is because of the isotopic effect: chemical reactions go faster with lighter compounds; the molecule with a carbon-12 reacts sooner than the molecule with a carbon-13 instead. Because the pathways from atmospheric CO_2 to endogenous or pharmaceutical testosterone are different enough, carbon-13 is depleted to different extents; the difference happens to be measurable.

The technique used to make the measurement is gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS, also referred to as IRMS or carbon isotope ratio, CIR). Before application to doping control, it had long been used to detect fraudulent substitution of synthetic compounds in place of natural compounds in the food, flavor, and fragrance industries. The anabolic steroids are extracted from urine and separated by GC. The separated testosterone enters the pencil-size combustion oven where it is pyrolyzed. Every carbon atom in the molecule is converted to CO_2, and every hydrogen atom is converted to water (H_2O). The water is scrubbed out and only the CO_2 enters the IRMS. This type of mass spectrometer measures only three m/z: 44 for $^{12}C^{16}O_2$, and 45 and 46 for variants containing carbon-13, oxygen-17, or oxygen-18. From the relative abundances, the instrument software calculates the $\delta^{13}C$ (delta) value. It reflects the $^{13}C/^{12}C$ ratio, but it is actually the difference between the $^{13}C/^{12}C$ ratio of the sample and that of an international standard. The units are ‰ (per mil). By definition, the delta value of the international standard is 0‰. Examples of values are –24‰ for natural testosterone and –29‰ for pharmaceutical testosterone. The values are negative because both compounds contain less carbon-13 than the international standard: 29 fewer parts per thousand for the pharmaceutical testosterone.

After exogenous testosterone administration, the delta values of urinary testosterone metabolites become more negative (Fig. 4). In contrast, the delta values of testosterone precursors, or of endogenous steroids not involved in testosterone metabolism, remain unchanged; therefore, they can be used as endogenous reference compounds (ERCs). A gap in delta value between testosterone or its metabolites and an endogenous reference compound indicates the use of testosterone or of any steroid in its metabolism. If the difference between the delta values of one metabolite and the endogenous reference compound is three delta units or more, the WADA requirement for reporting an adverse analytical finding has been met [11]. The power of this approach is that it can detect the use of not only testosterone itself, but also of any one of many testosterone precursors and metabolites. The second advantage is that it is not affected by factors that might influence baseline delta values. For example, diet influences the carbon-13 content of endogenous steroids—all of them to a similar extent. Although interpreting vastly different delta values

from one individual to the next might be difficult, a difference in delta values between a testosterone metabolite and an endogenous reference compound clearly reveals drug use. In short, the approach compensates for individual variability. The third advantage is that it does not require identifying or even knowing what exact compound was taken.

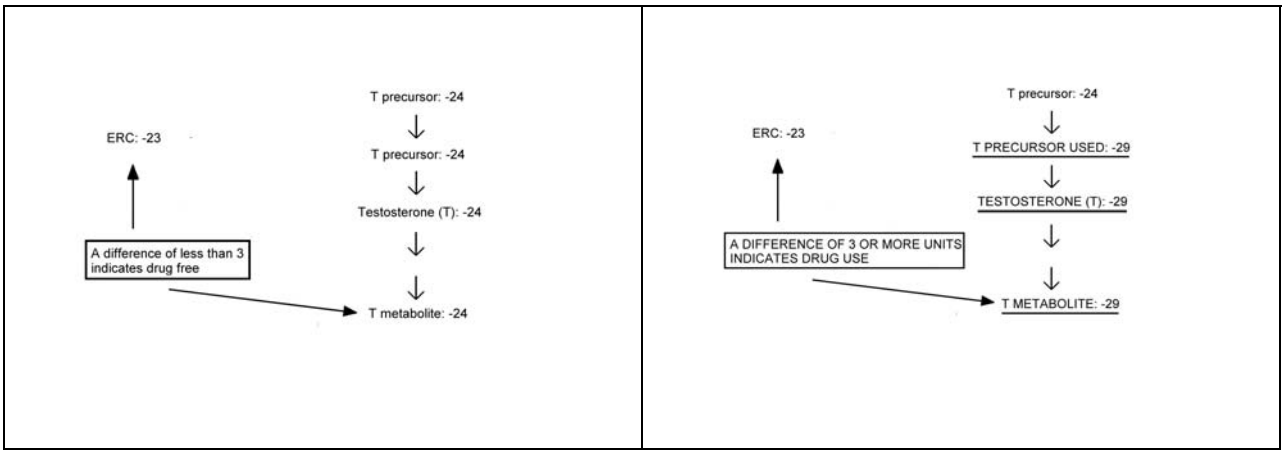

Fig. 4. How an IRMS test detects doping

A testosterone (T) precursor is metabolized to another T precursor, which is metabolized to T, which undergoes one or more metabolism steps to a T metabolite. The endogenous reference compound (ERC) is a steroid not involved in T metabolism; therefore, it remains unaffected by administration of pharmaceutical, exogenous T, or of its precursors.

IRMS testing has been applied to various T precursors, T metabolites, and endogenous reference compounds. It is currently done for samples with T/E greater than 4 or on request by the sports authority.

Isoelectric focusing

Isoelectric focusing (IEF) is used to detect recombinant EPO in the urine EPO test [12-14]. Historically, the EPO test at the Olympics (2000 to 2006) was done on paired blood and urine samples collected simultaneously. The blood test is an indirect test because it does not detect the presence of recombinant EPO. Instead, it measures multiple parameters (eg, hemoglobin, hematocrit, percentage of reticulocytes) and calculates a score which indicates whether the individual is on or recently off recombinant EPO [15]. Since 2002, EPO tests done by United States sports authorities have included only the urine test, a direct test that identifies recombinant EPO. EPO tests are done on only some of all of the urine samples, upon request by the sports authority.

Endogenous human EPO is a glycoprotein with a known amino acid sequence and glycosylation pattern. More precisely, it consists of a family of isoforms (molecules that differ only by their degrees of glycosylation). As a result, the pH at which each isoform bears as many negative as positive charges (isoelectric point or PI) is different.

Recombinant human EPO differs from endogenous human EPO only by its overall glycosylation pattern (ie, it consists of a different family of isoforms). The difference in overall pattern of isoforms allows differentiation between recombinant and endogenous human EPO.

The urine EPO test, also known as the French test or the IEF test, consists of four steps: sample preparation, IEF, double blotting, and visualization. Sample preparation concentrates EPO by multiple ultrafiltrations that leave the proteins of desired molecular weight in the filtration "retentate."

Next, the retentate is deposited on a gel with an embedded pH gradient and a current is applied to achieve electrophoretic separation of the isoforms (IEF). Unknown samples, reference standards, and known positive and negative quality controls are normally run on each gel. Each sample, standard, or control spreads out in its own "lane." Each isoform is charged, therefore it migrates in the electrical field until it reaches the distance on the gel at which the pH is equal to its PI. There the isoform is electrically neutral so it stops migrating. Its position or distance up the gel is the key, and the goal of the remaining steps is to visualize it.

The first blotting step transfers all proteins (erythropoietic and other) to a first membrane. The membrane is incubated with antibodies specific to erythropoietic proteins. The second blot transfers only these specific antibodies to the second membrane, thus transferring the isoform pattern, but leaving behind all proteins, including some that otherwise would obscure the final image.

Visualization is based on chemiluminescence; it involves incubation with a second antibody that binds to the first antibody and a chemical reaction that emits light. The image (electropherogram) is captured with a special digital camera. All steps use commonplace molecular biology techniques. The electropherogram contains one lane per sample, standard, or quality control sample (Fig. 5). In each lane, the isoform pattern consists of bands. The pattern (number of bands, positions, relative intensities) allows identification.

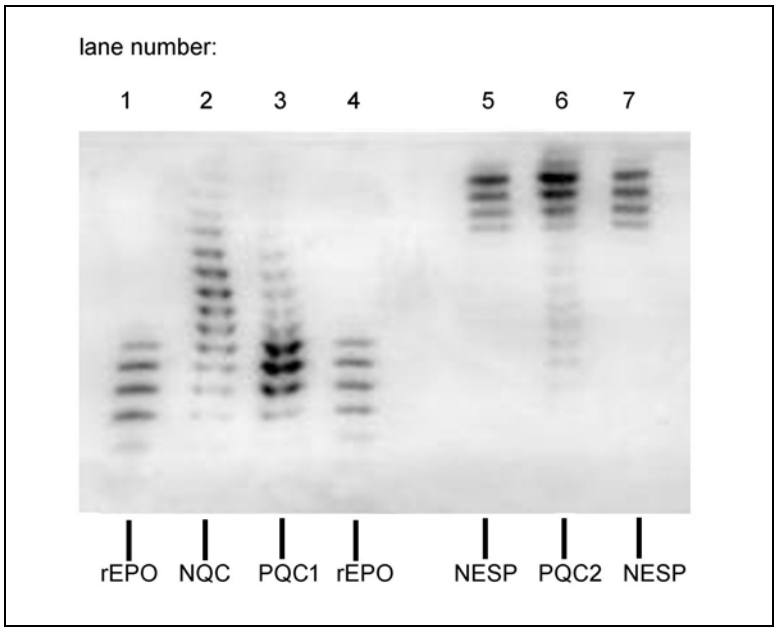

Fig. 5. EPO test result

Lane number and content:

1 & 4: rEPO = recombinant EPO (rEPO), pure standard

2: NQC = negative quality control = research subject urine before rEPO administration

3: PQC1 = positive quality control 1 = research subject urine after rEPO administration

5 & 7: NESP = darbepoetin = long-lasting rEPO, pure standard

6: PQC2 = positive quality control 2 = urine from different research subject after NESP administration

In common language, a negative EPO test often is discussed as if it reflects the absence of EPO, but of course what it means is that there was no recombinant erythropoietic protein in the urine sample, which normally would (hopefully!) contain natural, endogenous EPO.

Blood tests

Blood screening [5] is done at the Olympics, but not in the main United States sports drug-testing programs. At the 2004 Athens Olympics, whole blood was tested by cytometry to detect blood transfusions. Serum was tested by LC-MS-MS to detect hemoglobin-based oxygen carriers (HBOCs) and by immunoassay to detect recombinant human growth hormone (GH). Natural GH is a family of isoforms, including a major one of 22 kDa (22,000 atomic mass units) and some non-22-kDa isoforms, whereas recombinant GH is 100% 22kd isoform. Administration of recombinant GH suppresses endogenous GH production. The current approach to recombinant GH detection in serum is based on estimating the ratio

of the amounts of the 22-kd isoform to non-22-kd isoforms by immunoassay; it can detect administration for three hours after the last dose [16]. The test was conducted at the 2006 Winter Games in Torino as well. No adverse analytical findings were reported. This test can be implemented more widely as soon as reagents can be manufactured in sufficient quantities [17].

LABORATORY REPORT INTERPRETATION

The laboratory urine drug test can determine what substance is present in the urine sample, not the brand, formulation, route of administration, dose, or how long before urine collection the drug was taken. Reasons why a urine drug test is negative include: the drug is not prohibited by this program; the drug was never used; the drug was used long enough ago to have been completely eliminated; the drug is present below the cut-off; the drug is present below the limit of detection of the test; the drug is a prohibited (designer) drug that the laboratory doesn't look for; the sample was manipulated; and the sample wasn't real urine. The latter can be revealed by steroid screen data devoid of natural steroids in cases that would be missed by commercial adulteration tests and dipsticks.

Many factors determine test retrospectivity (Table 2), or how long after the end of administration the test can detect the drug in urine: among them are the dose, body burden, elimination pharmacokinetics, and test sensitivity. Anabolic steroids can be detected for as little as only a few days or as long as many months after the user stops taking them, depending on type used (eg, short-acting pill or long-acting oily injection), how much was used, and for how long. In addition, some steroids are easier to detect than others because of chemical differences. Individuals who have been in a drug-testing program for some time are less likely to use long-acting, easy-to-detect steroids.

Table 3. Urine drug test retrospectivity

Prohibited drugs	**Period of detectability after last dose**
Stimulants	a few hours to a few days
Anabolic steroids	from a few days… (short-acting, water soluble, small doses)
	…to many months (long-acting oily injection, large doses for long time)
Diuretics	a few hours to a few days
Marijuana	some weeks
EPO	a few days

The test result on a follow-up sample collected some time after an initial, positive sample needs to be interpreted in light of the above. If the follow-up test is positive for the same drug, it may be because the drug was not completely eliminated yet or because the athlete used the drug again in the meantime. Comparing the laboratory data from both tests may or may not provide an indication of which is the case. The follow-up test is expected to be negative if the drug was eliminated completely. This is why a negative follow-up test is not relevant to determining the accuracy or inaccuracy of a positive result on a sample collected previously. Conversely, a negative follow-up test is a valid check that the athlete has stopped using the drug.

Drug users who expect to be tested at events try to time their discontinuation to pass the test; this is why no-notice, out-of-competition testing was implemented in the 1980s. In the early 2000s, United States track and field athlete Kelli White passed 17 drug tests while on steroids (tetrahydrogestrinone [THG], testosterone), stimulants (modafinil), and EPO before she was caught on modafinil, then confessed to having used the whole regimen [18]. THG was not found in her samples because laboratories were still blind to this designer steroid (used only to beat the test). Testosterone use was not detected because she masked it by taking epitestosterone as well; because her T/E never exceeded the cutoff, it never triggered IRMS analysis, which would have detected exogenous testosterone. Modafinil was first targeted and found by the French WADA-accredited lab; her EPO use was not detected because sprinters' samples were not tested for EPO yet.

It is said that the test is blind to designer steroids because the test is targeted and finds only what it looks for. Typically, WADA-accredited labs screen for most anabolic steroids by GC-MS in the more sensitive SIM mode, monitoring only a few ions per target compound (eg, an ion of 415 atomic mass units). A designer steroid could differ from a known one by only two extra hydrogens, give an ion of 417 atomic mass units upon fragmentation, and escape detection because the test monitors 415, not 417. Or the designer steroid could happen to fragment to ions that happen to be monitored, in which case data readers would see suspicious signals and investigate further. The first reported designer steroid (norbolethone) [19] was a pharmaceutical abandoned decades before, during clinical trials. It resurfaced upon further investigation of an athlete's urine sample devoid of normal endogenous androgens, a telltale sign of endocrine suppression, which is expected after androgen administration because of negative feedback. The second designer steroid (THG) [20] was discovered because a coach turned in a used syringe. THG simply is not detected in the standard steroid screen, probably because its chemical properties are such that it disintegrates along the way. Different modifications of the screen now allow its detection.

Are the tests accurate? What are the risks of "false positive" or "false negative"? Both phrases can have widely different meanings in common language compared to antidoping jargon. In common language, a "false positive" might be any adverse analytical finding that does not result in a sanction, perhaps because the athlete had a therapeutic use exemption, or because a courier's signature was missing on a shipping document, or because the prohibited drug was a supplement contaminant. Supplements are not regulated by the US Food and Drug Administration (FDA); athletes should not only use them at their own risk, but question whether they need them to win [21]. A case in which on appeal, an arbitrators' panel had purely legal reasons to exonerate the athlete, might casually be called a "false positive." But for the laboratory, a false positive is only the case where the laboratory reports the presence of a drug and it is later proven scientifically that the drug was not present.

As for a "false negative," in common language that might be a case where the athlete used a drug but passed the test. This could be because the metabolite was accurately detected just below the cutoff—a perfectly accurate negative result. Other possible explanations for negative results following drug use were listed above.

HANDLING OF RESULTS BY THE SPORTS AUTHORITY

Based on the laboratory "B" report, the sports authority decides whether to charge the athlete with a doping offense. For USADA, the process includes automatic consideration by a Review Board and the opportunity for the athlete to request an arbitration hearing to contest the sanction [22]. The NCAA protocol includes an appeal process [23]. The laboratory remains involved when additional documentation (administrative or technical), scientific support for the legal team, or testimony is requested.

When doping cases lead to legal activities, the laboratories' decision criteria are reviewed. The WADA criteria for drug identification are not novel; they have long been widely used. GC-MS, LC-MS, and GC-C-IRMS technology were not invented for doping control; instead doping control is merely one of many fields of application.

The days of genuine or alleged inadvertent use being excused are over now that strict liability is enforced. It has room for refinement (lesser penalties) when authorities believe that the athlete made an honest mistake. Sanctions for the athlete's entourage (eg, team physicians) are meted out in more cases.

Antidoping program administrators and attorneys, such as those of USADA, are the third key link in the chain that began with the sample collection team and continued with the laboratory team. They take the

drug test result to its final conclusion, carrying the baton over the finish line. They have also gone beyond drug testing by charging athletes in cases involving “non-analytical positives” (ie, evidence of doping that doesn’t include a positive test result).

Testing statistics are available online. In 2000, WADA took over the IOC’s annual collection of statistics from worldwide accredited laboratories, including the total number of samples tested per laboratory, number of adverse analytical findings (total per laboratory, per drug worldwide, and per sport worldwide) [24]. USADA posts annual testing statistics and the test history of all United States athletes tested by USADA since inception in 2000 [25]. The NCAA results since 2001 are online [26].

CURRENT TRENDS

Will antidoping science ever get ahead of the cheats? (Not that we can ever catch every last one.) The pace of medical progress makes doping control an endless escalade in complication and expense. If society wants no performance-enhancing drugs in sports, the prospects for a technological fix for values gone out of line might be dim. This is, in part, because crooked scientists can market new designer drugs overnight with no concern for FDA approval, and some athletes pay good money to be the ones to discover safety and efficacy… or the lack thereof. Meanwhile, antidoping scientists need months or years to develop and validate new tests.

Yet major, recent improvements include the increased commitment of government entities in the United States and in Europe to the fight against doping, the speed at which sports authorities will add a drug to the prohibited list, and the expansion of profiling as a means to detect drug use.

Physicians have long monitored patient biomarkers (eg, blood cholesterol) for preventive purposes. Drug use is expected to affect common clinical test results and additional ones selected for their responsiveness to doping agents. Two examples are how longitudinal T/E profiling helps spot users and how the absence of endogenous urinary steroids led to the discovery of designer steroid norbolethone. Extending the review of steroid profiles to all athletes undergoing doping-control tests has not been done yet—although it has long been possible technically because urinary steroid profiles are archived at laboratories and sports authorities know which bottle numbers represent each athlete. Now that blood collection is more common in sports, more parameters could be added. Several programs in different countries are gearing up to formally and prospectively collect athlete urine and blood profile data. Some of those programs are voluntary, and although they all look similar at first glance, only one of them is all carrots and no sticks: envisioned by Don Catlin [27], it is designed to help clean athletes show the world that they are drug-free, so that when they win they do not have to suffer suspicion of drug use. Beyond

public recognition, the program might offer free medical care, and nutrition and fitness advice. Deviations from normal variability would be discussed with a trusted health care team. If a deviation had no explanation other than drug use, the athlete merely would be dropped from the program. No athlete would be sanctioned or suspended from competition or kicked off teams or contracts. With no sanctions, the lower risk for legal activities would lower the cost of the program. The central question would be: "What if an anti-doping program rewarded drug-free athletes instead of punishing drug-using athletes?" Could it trigger a shift in culture or is that just a wild hope for a crazy idea?

At the heart of any program is a trusting relationship with health care professionals, something that patients can experience at a young age with their pediatricians, who could be among the most influential people in turning around the culture of drug use in sports.

SUMMARY

Pediatricians or their patients may have to deal with sports doping-control tests and positive results. A substantial international regulatory framework is in place to harmonize sports rules and drug-testing laboratories. In major programs, urine and blood samples are split into an "A" and a "B" sample, and urination is observed directly. Chain of custody paperwork documents who has custody of the samples or where they are locked up, from the moment the bottles are sealed, to their receipt at the lab, to the day when they are finally discarded. If an "A" sample screens positive, the finding is confirmed twice before sanctions are considered: by reanalyzing the "A" sample and then analyzing the "B" sample. The main analytical chemistry technologies in doping-control laboratories are GC-MS, LC-MS-MS, IRMS detection of exogenous testosterone use, and IEF detection of recombinant EPO use. The approaches, technologies, and drug identification criteria are not novel; they have long been widely used in other fields. Although every medical advance has a potential for abuse by athletes, overnight and underground, antidoping scientists, who work above board, are slowed by the requirements of testing research, development, and validation. To try to leap ahead of the curve, the newest trend in doping control is the expansion of all elite athletes' profiling by monitoring biomarkers and watching for deviations that may be indicative of drug use.

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SYNOPSIS

This primer on urine analysis in sports doping control is an overview with an emphasis on the main analytical chemistry technologies in use: GC-MS, LC-MS-MS, IRMS detection of exogenous testosterone use, and IEF detection of recombinant EPO use. Included are graphic examples of GC-MS SIM and full scan, LC-MS-MS, and EPO test electropherogram data; a list of common acronyms; and answers to questions frequently asked about tampering and test accuracy.