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Anabolic Steroid Detection Poses Analytical Challenges

By Amy B. Cadwallader, Melinda K. Shelby, Ryan B. Paulsen, Dennis J. Crouch, and David L. Black

Anabolic androgenic steroids (AAS) and other anabolic agents are widely abused as performance-enhancing drugs by athletes of all abilities—from elite competitors to participants in amateur sports to high-school students. AAS and other anabolic compounds are banned by most amateur sports anti-doping programs, the World Anti-Doping Agency (WADA), and many professional sports organizations (1). In 2010, 60% of all adverse analytical and atypical findings reported by WADA were for AAS or related substances (2).

Although anecdotal evidence suggested AAS use was prevalent among athletes in the early 1970s, inadequate testing methods limited efforts to detect their use. A breakthrough came in the mid-1970s with the introduction of AAS testing using radioimmunoassay (RIA) (3,4). Not long thereafter, AAS testing by gas chromatography-mass spectrometry (GC-MS) began (5).

By 1975, the International Olympic Committee (IOC) banned the use of AAS. The first official AAS testing occurred during the 1976 Olympic Games in Montreal, where RIA was used to screen urine specimens and GC-MS was used for confirmation (6). GC-MS quickly became the gold standard for steroid testing and has been used for both screening and confirmatory analyses. Recently, however, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been used increasingly for the detection of analytically challenging AAS. In modern anti-doping laboratories both GC-MS(/MS) and LC-MS/MS are used to detect and confirm AAS use.

Currently, anti-doping scientists are faced with two major challenges regarding the detection of AAS use. The first challenge is to differentiate administration of endogenous steroids from those naturally produced; the second is to detect the use of synthetic "designer steroids."

Unequivocal proof of synthetic AAS administration can be demonstrated by using MS coupled with separation by GC or LC to identify the steroid, its metabolites, or both in urine. However, none of these techniques can readily distinguish exogenous use of natural steroids, such as testosterone and its precursors, from endogenous steroids. Similarly, the use of synthetic designer compounds cannot be determined easily using routine MS screening and confirmation methods unless the compound has been previously identified and the method has been programmed to detect it.

The doping agents and techniques used by athletes are constantly evolving, requiring creative approaches for detection and presenting ongoing challenges for anti-doping scientists.

Endogenous steroids

Although many synthetic AAS were prohibited by the IOC in 1975, testosterone was not banned until 1982. To enforce the ban, laboratories needed to develop methods capable of distinguishing exogenously administered testosterone from that naturally produced by the athlete. The MS techniques used at the time could not unequivocally determine the origin of the athlete's urinary testosterone, so alternate approaches such as "steroid profiling" were explored (7). Steroid profiling is based on the concept that exogenous use of a compound such as testosterone pro-

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New Tools Emerge to Test Newborns for Drug Exposure

By Joseph Jones

In utero drug and alcohol exposure is associated with a range of negative health outcomes, including intrauterine growth retardation, premature labor, placental abruption, convulsions, and even death (1). Over the past decade, neonatal abstinence syndrome has become more common as more babies are born to mothers who abuse prescription medications, such as pain-killing narcotics (2). The most recent National Survey on Drug Use and Health indicated that 5.1% of pregnant women self-report illicit drug use and almost 1% report heavy drinking (defined as five or more binges in the previous month), rates that have not changed significantly since 2002 (3).

Neonatal-care professionals need reliable tools to identify newborns who have been exposed to drugs and alcohol in utero.

Newborn urine

Drug and alcohol monitoring throughout the entire pregnancy is impractical. Typically, no specimen is obtained for the purpose of a drug or alcohol screen until near the time of birth. Historically, the most common specimen used has been newborn urine because urine analysis is relatively inexpensive and most hospital laboratories can provide an extended profile of drug classes.

Newborn urine as a specimen has a number of limitations in this application (4,5). First, collection is cumbersome and time-consuming. Second, the best specimen is the first void after birth, which can be easily missed without anyone noticing it. Third, sample volume is so limited that a sufficient volume for confirmation testing may not be available. Last, newborn urine has a very short window for detecting exposure, usually only a day or two. The lack of a confirmation test and the short detection window drastically reduce the value of this specimen.

Meconium

The epidemic of "crack babies" in the 1980s spurred interest in newborn toxicology. A number of specimen types were evaluated, including hair, vernix, placenta, and amniotic fluid. The specimen type that proved most useful was meconium, the newborn's first fecal material.

In most cases, there is a sufficient amount of specimen for testing and the detection window covers the last half of the pregnancy, which is a vast improvement over newborn urine. Dose-response studies for illicit drug detection in meconium have not been performed because of ethical concerns; however, meconium begins to form near mid-term, which establishes an upper limit for the detection window. Meconium analysis for drugs of abuse has been available commercially since 1991, and today extended profiles of illicit drugs, prescription drugs, and alcohol biomarkers are available from a number of hospital and reference laboratories.

As with newborn urine, meconium presents significant problems (6,7). The first issue is availability. Kwong and Ryan reported that 22% of ordered specimens are not available for a variety of reasons, including that it was passed in utero, it was passed before the order was made, the quantity was not sufficient, and it was disposed of inadvertently (5).

Second, meconium is heterogeneous; the best specimen contains all the meconium that has passed thoroughly mixed together. This characteristic creates a dilemma when an adequate weight has been collected but the newborn is still passing meconium. Third, the sickest infants may not produce enough meconium for analysis or may take five to seven days to pass a sufficient amount. Last, meconium collection is typically performed by multiple collectors over multiple shifts, which is an expensive use of valuable personnel and complicates the chain-of-custody documentation.

Umbilical cord

Recently, the use of a six- to eight-inch segment of umbilical cord as a specimen has been gaining attention (8–15). Montgomery et al. demonstrated that the positivity rates for matched pairs of meconium and umbilical cord are very similar, with a concordance of greater than 90% (8,9).

As with meconium, dose-response studies for illicit drugs have not been performed because of ethical concerns; however, the close agreement of positive rates between umbilical cord and meconium indicates that the detection windows are similar. In the case of buprenorphine, Concheiro et al. demonstrated that umbilical cord analysis could be used to monitor in utero exposure, although the measured concentrations did not correlate well with dose (12,13). On the other hand, Marin et al. reported that the measured concentrations of nicotine and metabolites found in umbilical cord correlated nicely with tobacco self-report (15).

There are a number of advantages to using umbilical cord as a specimen. First, in contrast to the problems with obtaining adequate meconium, a sufficient quantity is available for each birth. In our laboratory, we have had only one specimen rejected for "quantity not sufficient" in more than 37,000 cases. Second, the collection procedure can be performed

immediately after birth, saving up to several days of turnaround time. Third, the collection procedure is a single event with a single collector, as opposed to multiple collections by multiple collectors over multiple shifts added to a single container. The simple collection procedure improves the integrity of the chain of custody and dramatically reduces the cost of collection.

Conclusion

A newborn toxicology test may result in serious clinical and legal consequences. The specimen is extremely precious because it can be gathered only once in a lifetime. There is no opportunity to recollect. As clinical laboratorians, we have an opportunity to make a positive impact on the life of our smallest patients.

As with any drug-monitoring program, each specimen type has its advantages and disadvantages, including collection invasiveness, specimen availability, technical capability, cost, and overall value. Therefore, it is crucial that each organization establish thoughtful, thorough protocols and procedures to ensure high-quality and forensically defensible newborn toxicology results.

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Joseph Jones, MS, NRCC-TC, is vice president of laboratory operations at United States Drug Testing Laboratories in Des Plaines, Illinois.

Electronic Suggestion Box

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Lactate Dehydrogenase Can Cause False-Positive Ethanols

By Donald L. Frederick and Gregory S. King

A study of laboratories that participate in a College of American Pathologists (CAP) proficiency testing program shows that a high level of lactate and lactate dehydrogenase (LDH) can cause false-positive ethanol results in commonly used assays (1).

Almost 3,000 laboratories participate in the CAP Serum Alcohol/Ethylene Glycol/Volatiles (AL2) Proficiency Survey, and some 98% of them perform serum ethanol tests using a clinical chemistry analyzer. The serum ethanol assay in most analyzers uses added nicotinamide adenine dinucleotide (NAD^+) and alcohol dehydrogenase (ADH) to form acetaldehyde and NADH monitored at 340 nm. In uncommon situations, such as severe trauma in which the body releases high levels of lactate and LDH, the NAD^+ in the ethanol assay can oxidize the lactate to pyruvate and NADH. This interference can cause some ethanol assays to measure lactate as ethanol, and thus lead them to report a false-positive result (2–6).

The CAP Toxicology Resource Committee sponsored a pilot study in late 2010 of the potential for lactate and LDH to cause false-positive ethanol results.

Case study

The survey material that came with the test material included the case of a 17-year-old male who was brought to the emergency department by emergency transport. The history received from friends indicated the male left a graduation party driving his new pickup truck, with his girlfriend as a passenger. By all accounts, he had had no alcohol to drink. The truck struck a concrete culvert. The driver and passenger sustained severe trauma to head, chest, and abdomen.

On arrival at a local hospital, the driver was in shock. Lab results were Na, 140 mmol/L; K, 4.0 mmol/L; Cl, 90 mmol/L; HCO_3, 14 mmol/L; Ca, 9.1 mg/dL; creatinine, 1.2 mg/dL; BUN, 12 mg/dL; glucose, 89 mg/dL; and ethanol, 90 mg/dL. The driver died within a few hours. No autopsy was performed by the county coroner and no further toxicology testing was performed. The case illustrates how the release of enzymes from trauma can cause a false-positive ethanol result.

Source of interference

In most clinical settings, the levels of lactate and LDH are too low to generate significant positive interference with the ethanol assays on the market. However, lactate can accumulate as a result of tissue hypoperfusion. High levels of LDH are also seen in liver disease, acute liver failure, liver contusion and laceration, leukemia, and lymphoma. Lactate and LDH can be significantly elevated in major trauma such as in vehicular accidents in which hemodynamic shock from blood loss and tissue injury lead to lactate elevations that coexist with hepatic injury from contusion or laceration.

The analysis

The study included 50 laboratories that volunteered to perform serum ethanol analysis. They received test aliquots containing 120 mM sodium chloride, 25 mM sodium lactate, and 5000 U/L human LDH (mixed isoenzymes). The level of lactate/LDH interference varied greatly by instrument and assay manufacturer.

The results can be seen in Table 1. Abbott Aeroset, Microgenics, and Beckman platforms demonstrated the largest false-positive ethanol interference. The Roche assay demonstrated intermediate interference. The Siemens, Ortho, and Abbott AxSYM tests had little, if any, interference. This group of instrument and assay manufacturers represents more than 90% of all lab results reported in the CAP AL2 Survey.

Table 1. Ethanol Values Reported for Survey (mg/dL)

	Average
Beckman LX and DxC 51, 75, 73, 77, 83, 75, 72, 62, 80, 74, 74, 70, 87, 72	73.21
Siemens 1, 10, <10, 0, 13, 1, <3, <3, <3, 0.2	4.20
Microgenics Dimension 105, 107	106.00
Abbott Aeroset 131, 127, 82, 117, 75, 83	102.50
Abbott AxSYM <13, 9	9.00
Ortho Vitros <10, 10.2, <10, <10, 9, 10, 10	9.80
Roche Various 26, 17, 38, 19, 53, 16, 36	29.29

The laboratories were instructed to report any level of ethanol detected, but some did not quantitate below their limit of detection. This result is indicated by the < symbol.

Countering interference

What should a laboratory that uses one of the platforms showing a high level of interference do to prevent reporting false-positive ethanol results?

• Determine the anion gap. An elevated anion gap is an effective screen for the presence of elevated lactate on ethanol-positive samples. The anion gap calculation could be made part of a middleware rule that would lead to automatic validation of the ethanol result.

• Measure LDH and/or lactate on ethanol-positive samples. Because these tests are not part of any common laboratory test panel, this approach would incur additional cost.

• A laboratory that uses an open platform chemistry analyzer could switch to an assay that does not demonstrate the lactate/LDH interference.

Assay protocols that can reduce the lactate interference include starting the assay by adding NAD^+ only, measuring a basal rate of NADH production (which indicates the presence of lactate interference), then adding the ADH and measuring the rate again. The difference in the two rates is a measure of ethanol.

Hopefully, the results of this study will prompt manufacturers to alter their assays to eliminate this interference, which was first described almost 20 years ago.

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Donald Frederick, PhD, DABFT, is with the Peoria Tazewell Pathology Group, Peoria, Illinois. Gregory S. King, MD, FCAP, is with Christian Hospital in St. Louis.

Use of On-Site Drug-Testing Devices Continues to Expand

By Robert E. Willette

On-site drug testing has come a long way since the 1970s, when laboratory-based immunoassays were first introduced for opiates and other abused drugs. The first major workplace drug-testing program was launched by the U.S. Navy in the 1980s. It used portable benchtop immunoassay instruments at military bases and on board most large Navy ships. The portable instruments made it possible to test for a wide variety of drugs wherever there was an electrical outlet, but researchers continued to look for ways to cut the cord.

The first hand-held, "cord-free" devices for drugs were used to test for ethanol in saliva and urine. The technology advanced with the development of lateral flow strips that had antibody-coated particles at the start of the strip with drug conjugates impregnated in discrete zones. When a specimen is applied to the end of the strip, the urine or saliva migrates up, with the presence of a drug indicated by color development. For example, any morphine present binds to the opiate antibody, preventing the antibody conjugate from binding in the opiate zone, thus preventing a color from developing in that zone, which indicates a positive result. Some devices are configured the opposite way and produce a colored line for positives.

These devices also include a control zone at the terminal end of the strip to show that the sample migrated sufficiently to complete the test. Some are also available with instrumented readers.

There was a virtual explosion of such devices starting in the early 1990s, with dozens of publications in various journals. A book, *On-Site Drug Testing*, was published in 2002 (1).

FDA approval

As with all in-vitro diagnostic tests, manufacturers of drugs-of-abuse screening tests must submit data and labeling information to the Food and Drug Administration (FDA) as part of a 510(k) submission

for marketing approval. The devices fall under different classifications, depending on factors such as where they will be used (for example, in the laboratory; in the workplace and other "repetitive testing sites" outside of laboratories; or at home). Home-use devices, typically sold in drug stores, require special labeling information. Before a manufacturer can market a device, it must be cleared by the FDA unless it is labeled for forensic use only.

The test systems known as point-of-collection test (POCT) and non-instrumented drug test (NIDT) devices are used most widely in criminal justice and workplace testing programs. Federal, state, and local criminal justice programs use these devices as part of their arrest procedures and for monitoring violators as part of pretrial release, while they are on probation, and while they are incarcerated.

The use of these devices in workplace drug testing is limited to programs that are not part of the various federal drug-testing programs, such as those regulated by the Department of Transportation and others. Federally mandated workplace drug-testing programs must follow the "Mandatory Guidelines for Federal Workplace Drug Testing Programs" of the Substance Abuse and Mental Health Services Administration (SAMHSA).

Although SAMHSA has periodically considered permitting the use of NIDT devices in the programs it regulates over the past 26 years, the guidelines still do not allow them, apparently because of continuing concern about the lack of confirmation of on-site positive results (2).

Nonregulated testing

Nevertheless, these devices are widely used for drug testing of employees in nonregulated workforces worldwide. In most of these programs, positive specimens are sent to a laboratory for confirmation testing.

Their use has also expanded significantly in physician offices and pain management programs. The pain clinics created a problem by charging separately for each drug assay included in a test cup, when they were really doing only one test for multiple drugs. Federal regulators put an end to the practice by instituting a billing category for multi-assay test cups.

In addition to devices using urine as a specimen, there has been considerable interest in devices for testing oral fluid. The major challenge for these devices is the low concentrations of analytes in oral fluid. The greatest interest is in using them for roadside testing. Several studies evaluating the devices have been conducted by European organizations, including the Roadside Testing Assessment (www.rosita.org) and Driving Under the Influence of Drugs, Alcohol and Medicines (www.druid-project.eu). The proceedings of several meetings and conferences are also available on these websites.

How good are on-site drug tests?

More than 100 published studies have evaluated these devices. One of the earliest evaluated an immunoassay-based test strip for morphine in 1983 (3). Since then, a stream of reports has ranged from studies of a single new device to comparisons of multiple devices.

Two of the first large-scale studies involving multiple devices were conducted by Duo Research for the U.S. courts in 1986 and, two years later, as part of SAMHSA's ongoing evaluation of NIDT devices for inclusion in the federal workplace guidelines (1).

The two studies evaluated 15 different devices using real specimens that had been previously screened by immunoassay and, if positive, analyzed by GC/MS analysis. The studies found that the devices had a range of favorable and unfavorable characteristics. Some devices were better in design and performance than others, with the better ones considered good enough to be used in criminal justice and workplace testing programs.

In an ongoing effort to evaluate the NIDT devices currently available, Duo Research periodically sends kits of proficiency testing samples to probation offices to be tested along with real specimens. The specimens include some drug-free samples and some that contain drugs below cutoff levels. The results are graded and provided to the offices. A consistent observation is that most devices are "aggressive" in that they detect some drugs below the stated cutoffs.

Recent studies

A recent study on the use of NIDT devices to monitor patients with mental illnesses concluded that they "may be a helpful tool for measuring clinical and research outcomes" (4). In an evaluation of several clinical POCT devices and one POCT drugs-of-abuse device in a number of hospitals in the United Kingdom, the drugs-of-abuse device proved to be the least accurate (5).

Investigators in Italy recently evaluated a device in a variety of workplace settings, in compliance with Italian workplace regulations (6). This study concluded that the device did not provide satisfactory results compared with laboratory testing and that it performed poorly around the cutoff. However, some of the spiked samples contained a combination of parent drug and metabolites totaling the cutoff concentration. Duo Research's experience with a wide variety

of devices has been that they detect drugs below the stated cutoffs.

For drug treatment programs, this characteristic may be beneficial. Obviously, it can create discord with GC/MS confirmation, with some device-positive specimens failing to confirm at conventional confirmation cutoffs. However, when such specimens are tested at confirmation levels of detection, almost all the positives have the drug in question present.

Because these devices are used for a wide variety of medical testing, the American Association for Clinical Chemistry formed the Critical and Point-of-Care Testing Division, which addresses bedside clinical testing and offers point-of-care specialist training.

The path ahead

Clearly, the use of POCT and NIDT devices will expand to more areas and their performance will improve in the areas currently using them. For the testing arenas that use these devices the most, such as law enforcement, drug-treatment programs, and various clinical settings, we may still be in the early phase of development and innovation.

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Robert E. Willette, PhD, is president of Duo Research Inc., in Eagle, Colorado.

Anabolic Steroid Detection

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duces discernible changes in the athlete's excretion pattern of naturally occurring steroids and their metabolites. By monitoring an athlete's urinary steroid profile, samples indicating endogenous steroid administration may be identified. At a minimum, a profile consists of the relative and absolute concentrations of steroids such as testosterone; epitestosterone; dehydroepiandrosterone (DHEA); androsterone; etiocholanolone; 5α-androstan-3α,17β-diol; and 5β-androstan-3α,17β-diol.

The T/E ratio

To identify "abnormal" profiles, studies were performed to determine "normal" reference ranges and to establish population-based thresholds for steroids that comprise the urinary endogenous steroid profile. The first population-based endogenous steroid threshold to be introduced was the ratio of testosterone to epitestosterone (T/E ratio), which is an indicator of exogenous testosterone use. Testosterone administration increases urinary testosterone concentrations, which increases the T/E ratio. Chronic testosterone administration can also decrease the normal production of epitestosterone, further increasing the T/E ratio. Of the changes that occur in the urinary steroid profile following use of testosterone, an increase in the T/E ratio is considered the most diagnostic.

Based on empirical data published by Manfred Donike and colleagues, in 1982 the IOC adopted a T/E ratio of greater than 6 as the threshold for indicating exogenous testosterone administration (7–9). WADA later reduced this ratio to 4:1. Although sports governing bodies use different T/E ratio thresholds (6:1 vs. 4:1), the T/E ratio remains the most widely used indicator of testosterone administration today. However, after the implementation of the T/E ratio as a threshold, it was discovered that a T/E ratio greater than 4 or even 6 does not necessarily prove exogenous use of testosterone because some individuals have naturally elevated T/E ratios (10). Thus, a method to distinguish exogenous from endogenous testosterone was needed to supplement the T/E ratio.

Subject-based reference ranges

In the mid-1990s, Donike and colleagues introduced the concept of "subject-based" reference ranges derived from longitudinal studies of the athlete's endogenous urinary steroid profile. They dem-

onstrated that subject-based thresholds were superior to population-based thresholds in discerning testosterone and epitestosterone administration (9).

Continued refinement of this approach has led to development of the athlete biological passport. The biological passport program uses longitudinal monitoring of markers of prohibited substances, including hematological parameters and the urinary steroid profile. A longitudinal profile is used to establish ranges for markers that are specific to the athlete. The concept of the program is that variation from an athlete's established values (subject-based ranges) may indicate doping more readily than the use of population-based thresholds. In addition, subject-based thresholds accommodate the influence of factors such as sex and age that may complicate population-based thresholds. One reason for this is that subject-based thresholds accommodate the influence of factors such as sex and age that may complicate population-based thresholds.

More recent refinement in this approach includes the application of a Bayesian statistical model to longitudinal data to improve the identification of doping (11).

Because subject-based testing is not always feasible, researchers have also been working to better establish reference ranges for markers, from which population-based thresholds can be developed or refined. In addition to this, researchers have been working to identify new screening markers to improve detection of doping. These recent studies demonstrate the continued importance of improving screening methods and identification of "abnormal" samples requiring follow-up or confirmation testing (12,13).

GC-C-IRMS

Even with improved screening identification of abnormal samples using steroid profiling, longitudinal evaluation, and subject-based testing, there remains the need for unequivocal proof of administration of naturally occurring steroids. GC-combustion-isotope ratio mass spectrometry (GC-C-IRMS) was introduced in the mid-1990s as a confirmation technique with the means to distinguish pharmaceutical from endogenous testosterone and provide such proof (14).

In this application, GC-C-IRMS analysis combines GC separation of targeted steroids, combustion of the selected steroids into carbon dioxide, and the precise measurement of the relative abundance of the ^{13}C and ^{12}C isotopes of the steroids.

The relative abundance of these isotopes is revealing because pharmaceutical preparations of steroids such as testosterone have $^{13}C/^{12}C$ ratios that differ from their endogenous counterparts (15–17). This is because synthetic testosterone is synthesized from plants. Plants that rely on the C_3 carbon fixation process (including wheat, rice, and soybean) incorporate CO_2 differently than C_4 plants (corn and sugar cane), resulting in different carbon isotopic compositions. C_4 plants are more permissive to ^{13}C and thus are considered enriched in ^{13}C compared with C_3 plants. Pharmaceutical testosterone is synthesized from C_3 soy plants, whereas steroids produced within the body reflect the diet of the individual, typically a mixture of C_3 and C_4 plants (18).

To determine whether or not the steroid in the sample is present from natural production or exogenous use, the $^{13}C/^{12}C$ ratios of endogenous reference compounds (ERCs) are measured in addition to the steroids of interest. ERCs are typically compounds upstream in the biosynthetic pathway of steroids but not involved in the androgen metabolic pathway. The reference compounds have $^{13}C/^{12}C$ ratios that are not affected by the administered AAS use and can therefore be used to establish the individual's normal $^{13}C/^{12}C$ ratio. The difference between the $^{13}C/^{12}C$ ratio of the ERC and the steroid and/or metabolites of interest indicates whether or not the steroid is consistent with endogenous production or exogenous use.

Because hydrogen is also abundant in steroid molecules, investigators have started to examine hydrogen isotope ratios, although these methods require some 10 times more material because the natural abundance of ^{2}H is much less than that of ^{13}C (19,20). GC-C-IRMS is an evolving and promising technique for detecting the administration of endogenous steroids.

Designer steroids

To deter the use of steroids, in 1990 the federal government added AAS to its schedule of controlled substances. In 1994, Congress passed the Dietary Supplement Health and Education Act, which allows for the sale of steroid precursors as "food supplements." Some steroid precursors are included in lists of controlled substances, but many are not, so supplement manufacturers can continue to sell them.

More stringent regulation came with the approval of the Anabolic Steroid Control Act of 2004. The intent of this law was to discourage the use of AAS, but it had the unintended consequence of encouraging the clandestine synthesis of designer steroids (21). These compounds are designed to be structurally unique to avoid the definitions in the law, which also makes them difficult for anti-doping laboratories to detect. Minor structural modifications of a steroid can produce a new compound that is undetectable by conventional testing protocols but can have

increased anabolic potency or be resistant to metabolism. Safety is of particular concern with designer steroids because few are evaluated for toxicity or adverse effects (21,22).

A 2004 scandal brought the problem of designer steroids to the attention of anti-doping scientists when a previously undetectable steroid, later identified as tetrahydrogestrinone, was discovered to be in widespread use (21,23). The number of designer steroids in current use is unknown because routine detection methods often miss them.

Research aimed at the detection of new designer anabolic agents focuses on more sophisticated use of MS technology, including full-scan data acquisition/electrospray ionization orthogonal acceleration time-of-flight mass spectrometry (23), full-scan liquid chromatography/time-of-flight mass spectrometry (24), comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (25), and precursor ion scanning after liquid chromatography-electrospray-tandem mass spectrometry (26). However, even these sophisticated technologies may not detect anabolic agents with unknown structures.

Anti-doping developments

Progress is being made in applying "effect-based" strategies to anti-doping testing. Biological and receptor-based assays are being developed that not only detect the presence of illicit compounds, but can also assess their relative biological activity. In addition, the fields of transcriptomics, proteomics, and metabonomics are being applied to better understand the physiological signatures of AAS use, which could be detectable even when the anabolic agent and its metabolites are not.

Biological assays

There are many assays that can determine the presence of AAS and related compounds based on their biological effects elicited via the androgen receptor. There are also bioassays in development based on other steroid receptors (glucocorticoid, estrogen, and progesterone). A recent review details the benefits and pitfalls of several bioassays (21).

Assays that detect the biological effects of a prohibited substance do not require knowledge of the steroid's structure because they detect its biological activity as opposed to the steroid molecule itself. Bioassays, therefore, detect all compounds that interact with a chosen endpoint regardless of their structure.

Bioassays use living cells to mimic the in vivo drug actions of receptor activation, compound uptake into cells, and eventual protein production, biological response, or biological endpoint. One bioassay uses the human androgen receptor transfected into COS-7 cells. Transfected plasmids use the cell's machinery to produce luciferase when the androgen receptor is activated. Luciferase can then be measured as an indicator of bioactivity.

Other applications of bioassays, beyond their ability to simply detect the presence of banned substances, include their use in conjunction with mass spectrometry techniques to determine the structure of new ligands, their ability to determine the relative biological activity of steroid receptor ligands, and their ability to be coupled with other molecular biology techniques such as microsomal metabolism for pharmacological and toxicological studies of newly identified compounds. With all their benefits, the use of bioassays in the fight against anti-doping is increasing.

Transcriptomic/proteomic/metabolomic strategies

Transcriptomics is the study of the entirety of a cell's RNA transcripts (called the transcriptome), typically through DNA microarray technology. Proteomics is the study of expressed proteins (the totality of which is called the proteome). Metabolomics is the study of all the small organic molecules associated with metabolic function (the metalobome). Metabonomics focuses on metabolome dynamics.

Methodologies developed in these fields are being used to expand our understanding of the physiological signatures of steroid use and to identify RNA transcripts, proteins, and metabolites that are highly responsive to AAS as potential markers of AAS doping (27). These markers could be incorporated into longitudinal monitoring schemes that track athletes over time. Changes and patterns in these markers can potentially signal AAS use even when no steroids or metabolites are directly detectable.

The physiological effects of AAS are mediated by quantifiable changes in gene expression and metabolism. For instance, prostate tissue is highly sensitive to androgens and prone to display a strong steroid use signature in its gene expression pattern (28).

Potential new markers

For practical reasons, anti-doping programs are largely limited to blood/serum and urine as specimens. A recent proteomic study profiled peripheral blood lymphocytes treated with supraphysiological doses of dihydrotestosterone (DHT). It demonstrated that 30 proteins were differentially expressed and could potentially serve as markers for AAS abuse (29). An analogous study examined the steroid metabolites created in response to oral administration of testosterone undecanoate in an attempt to uncover ad-

ditional steroid use markers in urine. This proof-of-principle study found a number of markers that are potentially more sensitive to AAS use than the list of seven currently monitored by WADA (30).

A fundamental challenge with "–omics" approaches (and indeed with any indirect longitudinal monitoring scheme) is establishing a "normal" metabolome or proteome profile to which a "doping" metabolome or proteome profile can be compared. These approaches study complex biosystems that are affected by myriad factors such as genetics, sex, age, diet, environmental exposure, injury, and physical activity. The factors that affect individual and population variability over time are poorly understood.

To address this need, WADA commissioned a pilot study in 2006 to study metabonomic signatures in bicycle athletes. The study employs nuclear magnetic resonance spectroscopy and mass spectrometry in conjunction with multidimensional statistical techniques to detect deviations from the athlete's normal metabonomic profiles over multiple years, during training and competition. The study will provide insight into the feasibility of longitudinal metabolome or blood proteome monitoring and help characterize the types and levels of population and individual variation (31).

In addition to the challenges of variability, the complexity of the data presents significant obstacles to the discovery of new markers. The causal relationships between steroid use and changes in proposed doping markers are not always clear. Many technical challenges in the still-young "-omics" fields limit the completeness of the physiological picture they can provide. While challenges remain, it seems probable that the discovery of new markers through "-omics" studies will refine future longitudinal monitoring for AAS doping.

Conclusion

AAS testing has advanced significantly since the development of the first RIAs in the 1970s. Anti-doping scientists have worked to keep up with developments in the pharmaceutical industry as well as black market synthesis of designer compounds. Although anti-doping programs can for the most part identify known steroids and anabolic compounds, the direct methods of GC-MS(/MS), LC-MS/MS, and IRMS are limited and likely inadequate for future needs. Subject-based longitudinal testing using athletes' biological passports is gaining momentum because of its ability to detect subtle alterations in an individual's natural biomarker profile. This approach will continue to be refined and improved by the contributions of proteomic and metabonomic investigations. In addition, research is moving toward effect-based testing with receptor assays complementing the current direct detection methods.

As long as athletic competition exists, the desire to gain an advantage through performance-enhancing drugs with anabolic effects will continue. And so will the need to improve detection methods.

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Amy B. Cadwallader, PhD, is senior scientist for life sciences; Melinda K. Shelby, PhD, is senior scientist for sports services; Ryan B. Paulsen, PhD, is laboratory director of healthcare services; Dennis J. Crouch, MBA, FTS-ABFT, is a consulting scientist; and David L. Black, PhD, DABFT, FAIC, is president and CEO, at Aegis Sciences Corporation in Nashville, Tenn.

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- Discuss relevant regulations, such as analytical performance requirements, or the legality of new drugs of abuse.
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