Anti-doping: Two Scientists' Points of View

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As we get close to the start of the Olympic and Paralympic Games taking place next summer in Tokyo, Japan, athletes are preparing for selection by their host nation. This preparation, like most activities, is governed by the rules of sport. One of the most important rules relates to doping, a practice that is prohibited.

Olympic sports follow the anti-doping rules of the World Anti-Doping Agency (WADA) who publish a Prohibited List annually [1]. The list for the Games has just been approved by the WADA Executive and came into effect on 1 January 2020. This year's Prohibited List is very similar to that of 2019, although interestingly the use of argon, thought to stimulate red cell production, is no longer prohibited. The format of the Prohibited List is to differentiate substances that are banned at any time, such as the anabolic steroids, from those that are banned only at competition time such as stimulants. Interestingly, the WADA Code [2] defines competition time for the purposes of the rules as "in-competition," which is "the period commencing twelve hours before a *Competition* in which the *Athlete* is scheduled to participate through the end of such *Competition* and the *Sample* collection process related to such *Competition*."

Although it is, of course, the administration of particular chemicals or drugs that is banned, the rules classify these according to pharmacological groups, that is, according to the effect of the drug on the human body. These classes include: "anabolic agents," i.e. anabolic/androgenic steroids as well as certain other substances with anabolic activity such as clenbuterol and selective androgen receptor modulators (SARMs); the class called "peptide hormones, growth factors, related substances, and mimetics," comprises substances such as erythropoietin (EPO) and other agents that affect erythropoiesis (red blood cell formation); the next class is "beta-2 agonists" which are used medically to treat exercise-induced bronchoconstriction and asthma, but there are some exceptions to this prohibition dealt with later; "hormone and metabolic modulators" including insulins and meldonium, the drug that Maria Sharapova was accused of misusing; and "diuretics and masking agents," that is, substances taken to reduce the appearance of a banned substance in urine thereby

evading their detection during routine drug testing. All substances in the classes just described are banned at all times, i.e. both in- and out-of-competition.

Generally, it is the mere presence of a Prohibited Substance that provides *prima facie* evidence of a contravention of the rules. For some substances, however, there may be a threshold above which their concentrations in urine must be exceeded before the WADA accredited laboratories that test the samples will report a so-called "Adverse Analytical Finding" (AAF). For example, the beta-2 agonist salbutamol is prohibited if the concentration in urine exceeds 1000 ng/mL. The laboratory will apply a WADA specified measurement uncertainty of 200 ng/mL and will not report an AAF for salbutamol unless the concentration exceeds 1200 ng/mL. The laboratory will ensure that its analytical method has a measurement uncertainty that is less than the WADA specified value, which is considered to be the maximum tolerated value. The laboratory performance, assuring that the laboratory meets the WADA uncertainty requirements [3], is evaluated via external quality assurance schemes (EQAS) organised at least three times a year by WADA. In addition, WADA submits double-blind samples through national anti-doping organisations and sample collection agencies. Even false negative results will render the laboratory liable to an inspection by WADA and possible suspension or even revocation of its accreditation. Even before gaining WADA accreditation, the laboratory is required to be accredited to ISO 17025 as a testing laboratory, generally with a flexible scope of accreditation, covering all the classes of prohibited substances required by WADA from time to time. Getting a flexible scope of accreditation is particularly difficult for a new laboratory since most accrediting bodies, such as the United Kingdom Accreditation Service (UKAS), will require the laboratory first to show its competence in dealing with named substances. Only after satisfactory performance

of getting extensions to scope to include additional named substances, often taking several years, is flexible scope likely to be granted. The need for flexible scope is in order to be able to deal with new “designer” drugs which are banned by WADA under its classification category S0 of “Non-Approved Substances,” which is defined as “Any pharmacological substance which is not addressed by any of the subsequent sections of the List and with no current approval by any governmental regulatory health authority for human therapeutic use (e.g. drugs under pre-clinical or clinical development or discontinued, designer drugs, substances approved only for veterinary use) is prohibited at all times”.

The next group of categories comprise Prohibited Methods. These include: manipulation of blood and blood components (this category includes blood transfusion including one’s own blood); chemical and physical manipulation; gene and cell doping.

As mentioned earlier, stimulants are prohibited in-competition only, as are narcotics (but only specified narcotics such as morphine), cannabinoids (but not cannabidiol), glucocorticoids and beta-blockers in some sports only. Interestingly, morphine is banned but codeine is a permitted substance. This means that since morphine is a metabolite of codeine as well as a drug in its own right, laboratories have to attempt to distinguish the presence of morphine arising from morphine administration from that coming from codeine. To assist this process, WADA includes in one of its Technical

Documents [3] a set of criteria based on the concentrations of the two analytes adjusted for the specific gravity of the sample, if greater than 1.018, and the ratio of the two, to be considered before reporting an AAF.

With the exception of the narcotics, the Prohibited List, despite its name, is simply a list of examples of substances that are controlled. Under many of the headers, the words “including but not limited to” or sometimes “and other substances with similar chemical structure or similar biological effect(s), are prohibited.” The idea is to limit the possibility of designer drug circumventing the regulations.

By now the reader might appreciate that the analytical laboratory needs to be able to deal with a wide range of drugs from relatively small volatile basic compounds like methylhexanamine, also known as DMAA, and amphetamine, more polar compounds like testosterone glucuronide, through to large molecules like human growth hormone and erythropoietin (EPO) as well as erythrocytes and reticulocytes. Of course, with such a wide range of chemistries, no one analytical method is suitable. Furthermore, the concentration of the analytes that need to be monitored varies widely. An EPO isoform may be present at less than 100 attomolar on an isoelectric focussing gel, procollagen III amino terminal propeptide (P-III-NP, which is a biomarker used to evidence growth hormone administration) at around 25 pM, right up to pseudoephedrine that has a reporting threshold of 150 µg/mL or around 1 mM.

Graph illustrating the many factors that need be considered by the anti-doping laboratory

Thus, the concentration range encountered in human sports doping analysis is around 10^{13} orders of magnitude. WADA sets minimum required performance limits (MRPL) [4] that means that laboratories need to be able to detect the different prohibited substances at, and generally have to show that their limits of detection are less than 50 % of, the relevant MRPL in the relevant matrix of urine and/or blood by spiking a suitable number of blank matrix samples. Furthermore, they need to implement confirmation procedures that meet the WADA standards with similar sensitivities to that of the initial testing (or screening) procedure.

The analytical techniques used include a number of specific measurements such as simple pH measurement with a glass electrode, specific gravity inferred from a refractometer and human chorionic gonadotrophin (the hormone often used as a pregnancy test for females but used by males to stimulate testosterone production) by immunoassay.

Gas chromatography- (GC-) coupled mass spectrometry (MS) has been the cornerstone of screening samples and especially effective for the anabolic steroids. In more recent years, the use of tandem MS typically with triple quadrupole instruments has replaced the single quadrupole in anti-doping laboratories. Liquid chromatography (LC) coupled MS/MS or LC-high resolution accurate mass MS (HRMS) is now being used widely and is probably the major analytical technique in use for a variety of analytes covering wide concentration and mass ranges. Dilute and inject techniques are also gaining in popularity particularly as manufacturers improve the sensitivity of their instruments. Electrospray ionisation (ESI) is the main ionisation technique for LC-MS and nano- or preferably micro-spray, because of its greater reliability and ease of use, are becoming more routine. The latest research is looking into the routine use of supercritical fluid chromatography mass spectrometry giving a good degree of orthogonality in terms of retention time compared with reversed-phase LC. For peptide/protein hormones, such as insulin and insulin-like growth factor-I (IGF-I), LC-MS/MS is employed making use of the multiple charges obtained in ESI for the intact hormone. Trypsin digestion of IGF-I and also P-III-NP is being used for the quantification of these hormones too, gradually replacing commercial immunoassays that may change from time to time because of manufacturers changing their products, which affects the traceability of the measurement needed by the WADA accredited laboratory.

Although many of the quantitative assays are for foreign substances with thresholds set by WADA, some of the pseudo-endogenous substances, that is

substances that are chemically identical or virtually identical to the endogenous hormone that are administered against the rules, are only poorly measured against the population reference intervals. In order to improve the sensitivity of detection of these pseudo-endogenous compounds, WADA requires the use of carbon-isotope ratio mass spectrometry (C-IRMS) or the athlete biological passport (ABP) depending on the compound. Although C-IRMS is useful to prove a different isotope signature of the administered steroid, e.g. testosterone, from that produced endogenously, sources of testosterone are available with isotope signatures very similar to that of most athletes. The ABP is now widely used to monitor whole blood using cell cytometers to measure haematocrit, haemoglobin, reticulocytes and several other blood variables, which are then entered onto a WADA database known as the Anti-Doping Administration and Management System or simply ADAMS, which is also used to record athlete whereabouts to facilitate nil-notice sample collection. Using the ABP system, the athlete is measured against themselves rather than against the population, making any change to the athlete's profile more readily seen. Then an expert group of three haematologists independently review the data and if all three conclude that there has been a doping violation rather than a medical disorder or physiological abnormality, this is put forward for possible disciplinary action.

Although an AAF is evidence that an offence has been committed, because the WADA Code allows mitigation if the athlete can prove no intent, many cases focus on claims that a drink had been spiked or a contaminated supplement had been taken. This requires the toxicologists involved in case review to consider sometimes very limited data in order to assist the disciplinary panel hearing the case. Sometimes the defence claim is rather predictable such as the AAF for the cannabis metabolite carboxy-tetrahydrocannabinol. The athlete claims that he was in Amsterdam, where cannabis may be widely available, went out drinking and came back to where he was staying, felt hungry and saw some cakes on the table that he then ate. He discovered the next day that these cakes contained cannabis; this having occurred two weeks before the urine sample that gave the AAF was collected. Or the cocaine defence where the athlete claimed that he was counting bank notes and licking his fingers and bites his nails and this was the source of the cocaine that resulted in an AAF for the cocaine metabolite benzoylecgonine.

Another possible complication is the Therapeutic Use Exemption (TUE) certification allowed by WADA. This allows the athlete needing medication that would

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otherwise contravene the rules to get approval for the use of one or more medications. Thus, although the laboratory may report an AAF, a TUE may be present meaning that there may be no case to answer. However, the laboratory or toxicologist may be asked whether the laboratory findings are consistent with the terms of the TUE or whether the athlete has taken more than they should have or taken additional substances that are prohibited.

The last decade has seen the emergence of a new recreational drug phenomenon whereby hundreds of new psychoactive substances (NPS) has been synthesized and widely distributed either on the dark web, or, depending on the individual country legislation, even in head-shops or petrol station (thus also referred to as "legal highs") [5]. More than 730 new chemical analogues have been reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) by the end of 2018 [6]. These small molecule drugs may be classified according to several clusters, the most reliable one being probably their pharmacological action, such as (sub-) receptor binding and main pharmaco-toxicological effects commonly observed amongst users. The main NPS subclasses include stimulants, synthetic cannabinoids, designer hallucinogens, designer benzodiazepines and synthetic opioids. A large number of fatal or non-fatal intoxications are attributed to these substances every year, posing a major challenge for clinical and forensic toxicologists, policy makers, and public authorities alike. The link between such NPS and drugs in sports may seem tenuous, however drug testing laboratories should implement screening and confirmatory assays to cover NPS, many of which are covered by WADA regulations.

A parallel and recent wave of new synthetic substances with a high potential for misuse in sport has however appeared recently that involves a number of synthetic peptide hormones, such that the established acronym NPS typically used for small molecules could be translated for such new peptide-based substances to NSP (New Synthetic Peptides). These compounds belong to various analogues of endogenously produced peptide hormones, such as IGF-I, insulin, and growth-hormone releasing hormones (GHRHs). The chemical design is centred on structural modifications such that a) the bioavailability is increased via e.g. half-life extension due to the introduction of d-amino acid residues and/or chemically modified amino acids to reduce the rate of metabolism; b) their analytical detection (e.g. via LC-MS/MS) may be overlooked in routine screening tests because of different mass to charge ratios and fragmentation patterns. Little or no information is known about the pharmacology and toxicity

of these NSPs and developing and validating robust analytical procedures for their detection and identification represent an ongoing challenge for the forensic laboratories. People may think that the scientists lag behind the dopers. In fact, WADA has an early warning system regarding the potential misuse of pharmaceuticals even before they reach the market. It has agreements since 2011 with many of the major pharmaceutical companies including GlaxoSmithKline, Pfizer and Roche, as well as the International Federation of Pharmaceutical Manufacturers and Associations and, in September this year, the Japanese Pharmaceutical Company Kyowa Kirin too.

The future is exciting with many new challenges facing the scientist working in this area but, as history has shown, they will continue to make an impact in helping to deter drug misuse in sport. 🏆

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