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Subject-based profiling for the detection of testosterone administration in sport

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The use of reference intervals (previously called 'normal ranges') in clinical chemistry, based on measurements of a healthy population for a multiplicity of substances, has been the accepted model for many years. Occasionally, with routine tests, a measured value of a substance is found to be outside of the reference interval but with unknown cause, investigations revealing no pathological condition in the individual concerned. One possible conclusion is that this individual is presenting as a natural biological outlier. Likewise, in sport, the detection of the administration of the anabolic steroid testosterone (T) has previously been based on a population reference interval of a urinary hormone ratio, often referred to as 'T/E', but natural outliers with raised T/E are known to occur.[1–6] It is also recognised that other athletes who use testosterone can remain within the population interval despite a large within-subject variation in this ratio. This commentary discusses the merits of using the individual as his own reference, a so-called athlete's biological passport. A record for each athlete, in which the results of all doping tests, both urine and blood (where applicable), are collated over a period of time, comprises their passport and results can be compared to that individual's expected profile to determine whether a doping offence may have occurred. The most experience gained in terms of such profiling is with respect to T/E, and it is this ratio that serves as the model example in this commentary. A subsequent commentary will deal with the topic with respect to haematological issues.

Testosterone is noted for its anabolic properties in increasing muscle size and strength and for its androgenic (masculinisation) properties. It is considered to be widely misused in sport.[7] In a comprehensive review of the history of the use of anabolic steroids in sport, Todd[8] noted that, as early as 1945, the science writer Paul de Kreuf commented that 'it would be interesting to watch the productive power of [a] professional group that would try a systematic supercharge with testosterone. . .' De Kreuf's comment was remarkably perspicacious, and over 60 years later testosterone continues to be a problem in sports that participate in anti-doping programmes, the use of such performance enhancers being considered as cheating. Of all the anabolic steroids, testosterone is by far the most common agent reported by World Anti-Doping Agency (WADA) accredited laboratories. Of the 2% of samples that failed the drug test as reported by laboratories in 2007, about a half (1607 findings) were for testosterone. The most likely reason that testosterone is administered is because it is more difficult to prove administration of a substance that is also produced naturally within the body. Moreover testosterone, unlike most xenobiotic anabolic steroids, is available as transdermal, buccal and sublingual formulations. Such formulations, for legitimate daily replacement therapy, can be exploited by the athlete who dopes, as the steroid formulation is rapidly eliminated on stopping administration in anticipation of an anti-doping test.

A test based on determining whether a urine concentration of T exceeds the upper limit of a population reference interval would be insensitive because of the wide variability in excretion associated with a single urine collection. To overcome the problem, Brooks *et al.*[9] introduced the concept of the hormone ratio in 1979, a ratio being considered to be independent of urinary flow rates. The test developed was based on that of the concentration of testosterone to luteinising hormone (LH) but the only viable method for routine measurement of the glycoprotein LH necessitates the use of an immunoassay, a technique that is considered to be evidentially less informative than mass spectrometry. An added complication is the variability in the urinary excretion of LH over the menstrual cycle and also the suppression of LH caused by oral contraceptives and thus the measurement of T/LH is unsuitable for detecting testosterone use by female athletes. Donike *et al.*[10] subsequently proposed the measurement of testosterone to epitestosterone (T/E) by gas chromatography mass spectrometry. Epitestosterone is the 17α-epimer of testosterone, the testis being a major source of this steroid, with a minor contribution from the ovary, and probably by peripheral conversion of steroid precursors secreted by these glands, as well as by the adrenal cortex.[11,12] In 1982, the T/E ratio was adopted by the International Olympic Committee for the detection of administration of testosterone.

Testosterone administration causes an increase in the urinary T/E ratio, the laboratory reporting threshold originally being set at a T/E = 6. The T/E decision limit was derived empirically in the early 1980s from an observed distribution of measurements in urine samples collected from athletes. Subsequent reports of large-scale studies underpinned the fact that the median T/E ratio in male and female athletes approximates unity,[13–15] although a bimodal distribution can be attributed to multi-ethnic origins, with males of Asian origin having a lower ratio than for other ethnic groups. Further, large differences in T excretion between Asian and Caucasian men associated with an UGT2B17 polymorphism may result in discordant T/E values following T administration – with the same dose of T a Caucasian is more likely to exceed the urinary reporting threshold than an Asian.[16–18] It may be that this difference between ethnic groups is the reason why WADA lowered this threshold to a T/E = 4 in 2005. Unfortunately, this has resulted in a larger number of possible cases being reported by laboratories as 'physiological outliers' of Caucasian ethnicity, many being less than T/E = 6.

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The WADA permits the use of approved reliable methods such as gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) to confirm that an administration has taken place following the finding of a T/E $>$ 4 but improvements to this approach are needed. Otherwise, further investigations are required to ascertain whether a doping offence has occurred with emphasis based on intra-individual T/E profiling. This is a limited individual reference study that benefits from measurements from previous samples where available, or from further samples as necessary.[19] In addition, the possibility of a pathological condition, such as a testosterone-secreting tumour, accounting for an augmented ratio in a sports competitor must not be neglected, although there is no such case report described in the scientific literature (possibly because such tumours are likely to be of testicular origin and also to secrete epitestosterone).

The application of T/E intra-individual profiling was first discussed by Donike and co-workers[20] and by Baenziger and Bowers.[21] In a subsequent article,[22] the statistical test proposed by Harris[23,24] was applied for biochemical analyses, to assess the suitability of using such data. This statistical test evaluates whether it is appropriate to use an intra-individual (subject-based) reference interval, as opposed to an inter-individual (population-based) reference interval, for assessing changes in biochemical status. Catlin *et al*.[15] have reviewed the data on intra-individual variability and found that the maximum coefficient of variation (%CV) observed from drug-free males was 55% (variation from the collection of three or more samples of urine taken at monthly or greater intervals). In contrast, they report an example of a case of an athlete with an initial T/E ratio of 8.2 and, after being sampled four times, had a CV of 114%, indicating that T administration had occurred. This pattern was considered to be typical of an individual who discontinues T administration after the initial sample collection. In these authors' experience, most T users who provide three or more urine samples have a CV $>$ 60%. However, those that have a CV $<$ 60% and a T/E ratio between 6 and 10, are tentatively classified as 'naturally increased' (at the time of the publication by Catlin *et al*., the T/E threshold was 6), and Garle *et al*.[6] have reported on a number of such cases that would fall within this classification.

The WADA, in its Technical Document (TD2004EAAS),[19] states:

> *In males, the individual T/E values have been shown to vary from their mean value by less than 30% (screening values). In females, a low concentration of some urinary steroids such as epitestosterone and testosterone, close to the limit of detection using current analytical methods occurs. Normal variation of up to 60% may be expected.*

Furthermore where the suspicious test result, when compared with the basal value using appropriate statistical evaluation, is found to be significantly different, this is evidence that T has been administered. The comparison of screening results and confirmed results is accepted. The WADA document provides a useful bibliography but it is difficult to determine whether the CVs quoted are generally accepted outside of the WADA fraternity in light of limited published data.

Sottas *et al*.[25] discuss the weakness of an approach based solely on a reporting threshold for T/E of 4 together with a CV greater than 30%. Based on the model and parameters previously described,[26] these authors comment in their later publication[25] 'that one over 300 athletes should present naturally with a mean T/E value higher than 4.0 as well as a CV higher than 30%' (as verified by personal communication with Dr Sottas). These investigators therefore propose an alternative: a screening test based on the Bayesian statistical approach, where the population-derived T/E threshold is used for the prior probability that is adapted to an individual according to the sequential measurements of the T/E ratio in that individual and hence the power of the test increases with increasing sample number. This approach appears to be superior in that not only can it reduce the number of presumptive false positives but it should also reduce the number of false negatives, as samples can also be exposed from individuals using testosterone whose T/E ratio does not exceed 4, which could then be subjected to confirmatory analyses. Given the demands of a confirmatory test based on GC-C-IRMS, even with encouraging advances in the application of this complex technique,[27–30] it follows that there is much interest that the Bayesian approach to evidential analysis could also serve to confirm that doping has occurred. This Bayesian approach, where a limit is modified on the basis of previous tests, is already considered to have merit for the detection of blood doping,[31] and also for clinical purposes, such as the early detection of ovarian cancer.[32] Obvious questions include what statistical probability should be chosen for concluding whether an intra-individual variation for T/E is abnormal and what is the required level of analytical certainty?

Skilled personnel are available in WADA-accredited laboratories to evaluate whether an individual's longitudinal T/E profile is abnormal but it is highly desirable that a standardised approach be applied worldwide, with software available to national and international organisations to alert them when an athlete's longitudinal profile appears abnormal. The WADA has assisted in ensuring that its accredited laboratories provide data of a quality fit for inter-laboratory comparison of T/E even from screening data (index cases of elevated T/E being based on confirmed results). Furthermore, WADA's ADAMS athlete data management system has been designed to facilitate these comparisons. Nevertheless, it is not certain that all reviews are currently undertaken against the same criteria. Laboratories also make use of additional data (such as T/LH, the epitestosterone concentration, and the androsterone to testosterone ratio) to assist their decision as to whether the raised T/E could reasonably have come from a 'pathological or physiological' abnormality.

In addition to a high intra-individual variation in T/E, an abnormally large T/LH ratio accompanying a high T/E ratio in a urine sample collected from a male is supplementary evidence of T use,[6] despite the disadvantage of immunoassay of LH being less informative than mass spectrometry. As an adjunct, there is some evidence to support that the T/LH ratio is a more sensitive retrospective marker of chronic administration of T than the T/E ratio.[33] Sample handling is an important consideration and also urinary LH measurement between laboratories is yet to be standardised,[34] although some laboratories have determined their own assay reference interval. Exercise stress appears to attenuate rather than augment the ratio.[35]

With the increasing number of blood samples taken to detect transfusion and human growth hormone administration, due consideration should be also given to the longitudinal profiling of testosterone in serum, not least because this matrix is under strong homeostatic control and hence very much less subject to the problems of variability in dilution that is associated with urinalysis. In the past, this would not have been feasible given that one to two ml of serum would be required for extraction but it has recently been demonstrated that quantification by LC-MS/MS can be based on as little as 50 µL.[36]

In conclusion, intra-individual profiling appears to offer a distinct advantage over doping tests simply based on population statistics, as exemplified with respect to the detection of testosterone administration. With due consideration of the issues discussed above, including adequate research regarding factors that may affect intra-individual variation, this approach should simplify the evaluation of T/E cases and help exonerate the innocent athlete. It ought also to facilitate the detection of testosterone administration in athletes whose basal T/E ratio is small and hence less likely to exceed the reporting threshold. Finally, this approach should help to address the problem of athletes who try to beat the test by periodically titrating themselves with testosterone so that their T/E ratio remains below the reporting threshold.

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