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Ned Tijdschr Klin Chem Labgeneesk 2007; 32: 173-175

Detection of drugs-of-abuse in athletes

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Drugs-of-abuse (DOA) in sports is as old as modern sports itself and started at the end of the 19th century. In those days developments in science lead to the isolation and identification of several alkaloids; naturally occurring amines produced by plants, which are pharmacologically active. Consequently, the first kind of drugs applied in sports were the alkaloids such as morphine, heroine (synthetic variant of morphine), caffeine, cocaine and strychnine. Alkaloids remained the drugs of interest until the 2nd World War (Figure 1). Their application was not considered to be an abuse in human sports and therefore detection of alkaloids as DOA was not an issue for athletes. However, at the beginning of the 20th century the administration of alkaloids in horse racing was regarded as game and gambling fraud and because of that testing for horses was initiated as early as 1910.

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In the late twenties of the last century detection of DOA in horse racing consisted of screening for alkaloids in saliva by colorimetry (1). Saliva was chosen, as urine collection was considered to be dangerous and impracticable. Alcohol was applied in order to stimulate sufficient amounts of saliva. For detection different analytical chemical approaches were already available. The STASS-DRAGENDORF procedure for example included distillation to remove ethanol, liquid/liquid extraction and precipitation to isolate the alkaloids. Finally, using general and specific colour reactions it was possible to screen for alkaloids in general and to confirm certain alkaloids specifically.

The 2nd World War induced significant changes as certain drugs were applied to improve the performances of soldiers. Amphetamine was used for example to deprive them from exhaustion (2) and anabolic androgenic steroids (AAS) were supposed to make them more aggressive (3). After the war the returning soldiers are assumed to have introduced these drugs into the society and sports. However, testing of athletes remained a non-issue during the first two decades after the war. Detection of DOA

was only considered for human sports, when in the sixties some athletes died because of heat shock caused by the combination of amphetamine abuse and high atmospheric temperature (4). Because of that testing for psychostimulants (amphetamines, ephedrines, methylphenidate, etc.) was implemented first in cycling and later also in other sports.
In contrast to in horse racing, urine was used to detect psychostimulants in human sports. Applying gas chromatography (GC) combined with Nitrogen Phosphorous Detection (NPD), identification was first purely based on retention times (5). However, due to a lack of specificity substances were misidentified and some athletes were falsely accused of a DOA offence. These findings forced the sport authorities to implement mass spectrometry (MS) for a more adequate identification. Nevertheless, GC/NPD remained to be useful for screening purposes. Nowadays, MS is the overall method of choice for confirmation of prohibited substances, metabolite(s) of prohibited substance or marker(s) of the use of a prohibited substance or method (6). Although urine is still essential as a biological specimen in sport DOA testing, blood is gaining significance, especially for those substances or markers, which are not or poorly excreted in urine.
The development of DOA in sports correlates with the introduction of new analytical technologies (Figure 1). The implementation of GC/NPD improved the screening for psychostimulants in the seventies and pushed the attention to AAS (stanozolol, methandienone, mesterolone, oxandrolone, etc.). Subsequently, the low resolution bench top GC/MS equipment made it possible in the late eighties to detect routinely the abuse of AAS and as a result, peptides and proteins became of interest in the nineties. The latest development is the introduction of liquid chromatography/tandem MS (LC/MS/MS) in order to detect non-volatile and thermo-labile substances including peptides and proteins (insulin, human chroniogonadotropin, tetracosactide ect.) (7). Accordingly, it can be stated that sport DOA testing is actually a battle between sport authorities and athletes. The drive of athletes to enhance their performance is causing a continuing search for new substances or methods to improve their performance. As such sport DOA testing is shifting the problem instead of solving it.
Despite the shift towards peptides and proteins, the abuse of AAS occasionally is reviving because of alternative ways of application (veterinary AAS (boldenone, trenbolone), oral administration and lower dosages). High resolution and tandem MS (HR-MS and MS/MS) increased the possibilities to detect these new applications (6), but the latest progress in sport DOA testing is the use of Combustion/Isotope Ratio MS (C/IRMS) in combination with GC to detect the abuse of pharmaceutically applied endogenous AAS (testosterone, nandrolone, androstenedione, dehydroepiandrosterone, etc.) (8). The GC/C/IRMS method is a powerful supplementation to the classical steroid profiling, which amongst others is based on the ratio between testosterone and epitestosterone. The profiling method has been for decades the method of choice for the detection of abuse of endogenous testosterone in particular (9) and of endogenous steroids in general (10) and combined with GC/C/IRMS it will remain to be essential in DOA testing. However, once more the continuous improvement of detection methods does not prevent abuse and nowadays the synthesis of designer AAS (tetrathydrogestrinone, prostanozol) in order to avoid detection is the next development to provoke sport authorities (11).
The detection of the abuse of recombinant human erythropoietin (rhEPO: α- and β-epoetin, darbepoetin) and growth hormone (rhGH) still suffers from analytical problems. Haematological and endocrinological parameters in blood are merely useful for screening of the abuse of rhEPO (12) and rhGH (13). At this moment the detection of the abuse of rhEPO is based on isoform pattern analysis by isoelectric focussling (IEF) combined with Western blotting and chemiluminescence detection (Figure 1) (14). Current discrepancies observed in sport DOA testing of urinary rhEPO are caused by the lack of a MS based method. IEF/MS is in that respect a promising method, despite some analytical problems, which still have to be solved (15). The future analytical step to deal with the abuse of rhEPO might be LC/C/IRMS as new variants of rhEPO are identical to endogenous human erythropoietin (δ-epoetin). For rhGH even no adequate method exists, as the developed immunoassay is not robust for routine application. Whereas the detection of the abuse of peptides and proteins is a short-term challenge for sport authorities, an important long-term challenge is the detection of genetic engineering purely to improve sport performance. The fact that genetic engineering of primates by implementing cDNA of hEPO in muscle tissue still could be detected by the current analytical technology also proves that probably always an analytical answer to these challenges can be found (16).

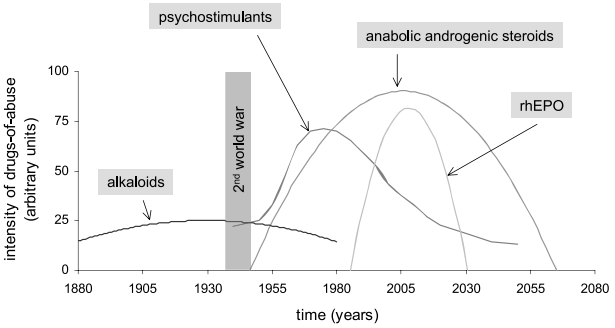

Figure 1. Simulation of some trends in Drugs-of-Abuse testing of sports *versus* the application of new analytical technologies: Alkaloids were substances used in sports before the 2nd World War, while the abuse of psychostimulants and anabolic androgenic steroids exponentially increased after the war. Application of GC/NPD and MS amongst others realized a decrease in psychostimulant abuse, while hyphenated mass spectrometric technologies (GC and LC combined with HR-MS, MS/MS or C/IRMS) currently are realizing a decrease in the abuse of anabolic androgenic steroids. At present the abuse of recombinant erythropoietin (rhEPO) is being tackled by IEF combined with Western blotting and chemiluminescence detection. Promising technologies are IEF/MS and LC/MS/MS.

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Ned Tijdschr Klin Chem Labgeneesk 2007; 32: 175-178

Do NIST SRM 2921 and recombinant cTnI-based serum pools have potential to harmonize cTnI results?

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The history of cardiac troponin assays starts from the late 1980s. Today cardiac troponins (cTn) are "popular" cardiac markers which have largely replaced CK and CK-MB. Dozens of commercial cTnI assays are available nowadays while there is only one cTnT assay because of an existing patent (Roche Diagnostics). For qualitative or quantitative detection of different cTn antigens in body fluids, immunoassays are used in clinical practice. As the sought parameter is the cTn antigen, companies have developed strategies of antibody selection based on the knowledge of the antigen's structure and properties. Notwithstanding well-considered antibody selection, extreme microheterogeneity of the cTnI antigen and lack of a complete cTnI reference system (figure 1) complicate assay standardization, resulting in huge between-method variation for existing cTnI assays (1).

An important prerequisite for guaranteeing comparability of results among different cTnI methods is the availability of suitable reference materials. Nowadays, well characterized Standard Reference Material (SRM) 2921 and recombinant cTnI (rec cTnI) are available to manufacturers for cTnI harmonization and/or standardization (2, 3). From an EQAS viewpoint, the Chemistry Section of the Dutch EQAS (SKML) aims to monitor the effect of ongoing cTnI standardization efforts by in-vitro diagnostic industries. Consequently, we wanted to develop stable, well characterized, matrix-based cTnI pools. Secondly, we aimed to investigate the cTn harmonization potential of these matrix-based cTnI pools.

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