ELECTROCHEMICAL SENSORS AND BIOSENSORS FOR THE DETECTION OF DOPING SUBSTANCES AND METHODS

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Abstract: This communication presents the possible use of electrochemical sensors and biosensors for the screening analysis of some doping substances and methods.
An outline of presently studied methods is presented, focusing on those classes of doping substances (primarily ?2?agonists and corticosteroids) missing a quick and reliable screening procedure in doping control analysis, as well as on specific compounds (e.g. some diuretics) whose preliminary screening in urine samples by traditional GC-MS and/or HPLC techniques can be affected by various experimental artifacts.
Depending on the specific class of compounds to be detected, the extent of the pre-purification process, the nature of the electrode and of the applied electrochemical technique, the lowest detection limit varies from 100-200 ng/ml down to few ng/ml, thus theoretically matching the sensitivity needed by an antidoping assay.
The possibility of employing some newly developed electrochemical methods for the analysis of biological fluids different from urine (especially salive), and/or for the "in vivo" monitoring of biophysiological parameters strictly related to the athletic performance, is also discussed.

Keywords: electrochemical sensors and biosensors, doping analysis, ?2?agonists, corticosteroids, diuretics, plasma volume expanders.

INTRODUCTION

According to the International Olympic Committee definition, "doping contravenes the ethics of both sport and medical science. Doping consists of: (1) the administration of substances belonging to prohibited classes of pharmacological agents, and/or (2) the use of various prohibited methods" [1].

The list of banned substances and methods (last update: January 1999) is reported in Table 1 [2].

Table I

List of banned doping substances and methods

I. Prohibited Classes of Substances

A. Stimulants
B. Narcotics
C. Anabolic Agents
   1. Anabolic Androgenic Steroids
   2. Beta-2 Agonists
D. Diuretics
E. Peptide Hormones, Mimetics and Analogues
   1. Chorionic Gonadotrophin (hCG)
   2. Pituitary and Synthetic Gonadotrophins (LH)
   3. Corticotrophins (ACTH, tetracosactide)
   4. Growth Hormone (hGH)
   5. Insulin-like Growth Factor (IGF-1)
   … and all the respective releasing factors and their analogues (.. -RH)
   6. Erythropoietin (EPO)
   7. Insulin

II. Prohibited Methods

A. Blood Doping
B. Pharmacological, Chemical and Physical Manipulation.

III. Classes of Drugs Subject to Certain Restrictions

A. Alcohol
B. Cannabinoids
C. Local Anaesthetics
D. Corticosteroids
E. Beta-blockers

The detection of doping agents and of their metabolites in the athletes urine is generally performed by chromatographic-spectrometric techniques, primarily by gas chromatography–mass spectrometry (GC-MS). These methods (see refs. [3-6] for reviews), although extremely powerful, require an extensive pretreatment of the urine (reviewed in [7]), including an extraction step (solid-liquid or liquid-liquid), enzymatic or chemical hydrolysis (when needed), preconcentration, and derivatization. The last step is often an unavoidable requirement for GC-MS analysis, and many derivatization methods have been developed in the last years (reviewed in [8]), following the pioneristic

work of the late Prof. Manfred Donike, who firstly developed the most common derivatization reagents use in the antidoping laboratory, namely N-methyl-N-(trimethylsilyl)trifluoro-acetamide (MSTFA), and N-methyl-bis-trifluoroacetamide (MBTFA) [9].

While for the confirmation analysis chromatographic techniques with mass spectrometry detection still represent the unique analytical option (also from a merely normative point of view), other analytical methods are being evaluated for the development of alternative screening protocols, mainly because of the increasing number of drugs/metabolites to be searched for and of the correspondingly increasing costs to be sustained by an antidoping laboratory.

At present, several classes of substances, primarily peptide hormones, some drugs of abuse (cocaine, opioids, cannabinoids, and amphetamines), corticosteroids, and even beta adrenergic agonists and antagonists, are preliminarily searched by immunological methods (ELISA, competitive binding assays with fluorescence or chemiluminescence detection): these techniques ensure a very rapid and effective screening of huge populations of samples, but still with a high percentage (in some instances greater than 10%) of false positive results.

Electrochemical sensors and biosensors could represent a faster, simpler and more economical alternative for the preliminary screening analysis of selected classes of doping substances and methods. The use of these devices would indeed allow to drastically reduce the pretreatment and purification of urine samples, which is imposed by the use of chromatographic-spectrometric techniques, and to avoid the chemical derivatization of the urine extracts.

ELECTROCHEMICAL SENSORS AND BIOSENSORS IN THE ANALYSIS OF DRUGS AND METABOLITES IN BIOLOGICAL FLUIDS

Analytical methods involving the use of electrodes and bioelectrodes for the detection of pharmaceuticals and/or their metabolites in biological fluids can be divided into three main classes:

a) combined chromatographic-electrochemical techniques, in which the electrochemical sensor or biosensor, assembled into a flow-through cell, constitutes the sensing element of the chromatographic detection unit;
b) stand-alone electrochemical or bioelectrochemical cells, where the detection unit is employed for batch measurements on a pre-purified fraction of the biological fluid (urine) to be assayed;
c) electrochemical immunosensors, where the immunological interaction between the sensor and the sample gives rise to a detectable change of a defined electrochemical parameter.

While the amount of studies carried out on biosensors belonging to class (c) is still too limited to draw an even preliminary picture of the real potentiality of the relevant methods, sensors included in classes (a) and (b) have already been evaluated on real samples. More precisely, class (a) refers to HPLC methods with amperometric detection, whose advantage with respect to traditional HPLC-UV and also to GC-MS methods is given by a drastically simplified pretreatment procedure. Class (b) includes a wide variety of methods based on polarographic and voltammetric techniques, mainly adsorptive cathodic stripping voltammetry, cyclic voltammetry and differential pulse voltammetry.

Some of the above mentioned applications, and their potential

application in an antidoping laboratory, are outlined below.

Analysis of diuretics by HPLC with electrochemical detection

Diuretics can be illicitly used to reduce the body weight (resulting in a conclusive advantage in those discipline where athletes are divided in weight categories), as well as to mask the administration of other doping agents.

The analysis of diuretics and/or their metabolites in human urine is a challenging task in an antidoping laboratory, due to the many pharmacological and chemical differences among the many compounds belonging to this class. Apart from direct osmotic agents, diuretics exert their pharmacological action at different cellular and subcellular sites, and they also markedly differ in many basic pharmacokinetics parameters [10-11]. It follows that a unique, general method of screening always represents a compromise in terms of sensitivity, selectivity and specificity.

Screening of diuretics is carried out in an antidoping laboratory either by GC-MS or by HPLC [12]. Both of these methods are in some extent integrated by other screening procedure (e.g. the screening for the androgenic anabolic steroids), in order to ensure the complete covering of the whole class of diuretics (more than 30 compounds, not considering the corresponding metabolites)

The combination of liquid chromatography with electrochemical detection could allow the preliminary screening of a broad variety of diuretics with one single chromatographic run.

HPLC techniques with electrochemical (amperometric) detection have been succesfully used for the determination of diuretics like

diuretics like pretanide, furosemide [13], and hydrochlorothiazide [14], matching the sensitivity limits required by an antidoping assay. Any suspicious sample would subsequently be confirmed following the specific GC-MS protocol.

Analysis of ?-2 agonists and corticosteroids by voltammetric techniques

A satisfactory number of drugs is nowaday available for the pharmacological management of asthma, the most common being sodium cromoglycate (cromolyn sodium), H1-antagonists, belladonna alkaloids, methyl xanthines, glucocorticoids and ?-2 adrenoceptor stimulants (?-2 agonists) [15]. Drugs belonging to the last two classes are presently allowed by the IOC provided either they are not administered sistemically (corticosteroids), or used under medical prescription by inhalation only (?-2 agonists like salbutamol, salmeterol and terbutaline).

The challenge for an antidoping laboratory is therefore to set up a reliable method to distinguish the authorized from the prohibited (oral, systemic) administration of drugs belonging to these classes.

A proposed analytical strategy for the analysis of ?-2 agonists, and especially of salbutamol, comprises a conventional, screening procedure (ELISA), carried out to select any suspicious sample, and the subsequent determination of the concentration ratio of non-conjugated enantiomers by enantioselective HPLC [16, 17].

According to data presented in the literature, a more effective pre-test of samples could be carried out by either cyclic voltammetry or differential pulse voltammetry following electrochemical pretreatment of

electrode surface, not only for salbutamol [18], but also for clenbuterol [19] and other ?-2 agonists.

This approach would allow the drastic reduction of the pretreatment step and, at the same time, a more rapid and general screening of urine samples for ?-2 agonists.

An analogous approach could in principle be followed also for the screening of corticosteroids, whose confirmation (electively by LC-MS-MS) is not yet performed on a routine basis by the IOC accredited antidoping laboratories.

Additional possible applications: Preliminary screening for plasma volume expanders (PVE) by enzymatic electrodes

The abuse of the synthetic glycoproteic hormone erythropoietin, (EPO), not detectable yet by the traditional antidoping analysis since it is virtually identical to the endougenous hormone, imposed the study of additional parameters that can be traced to the use of this doping agents. For this reason the control of hematocrit (HCT) is carried out prior to the start of the competition in some disciplines, and a value higher than a threshold limit (usually >50% in male and >48% in female athletes) leads to the suspension of the athlete “for health reasons”.

This situation caused an abuse of plasma volume expanders, i.e. of “masking” agents infused to dilute the blood and to consequently reduce the HCT value.

The most common PVE are made by aqueous solutions of polysaccharides, and primarily by hydroxyethylstarch (HES), widely used in clinical medicine and in surgery for the treatment of hypovolemic shock and of disturbances in capillary blood circulation.

An analytical method for the analysis of HES in urine has recently been proposed [20], and it is presently under evaluation by several IOC accredited antidoping laboratories. The methods is constituted by the GC-MS analysis of several low molecular weight residues produced at the end of an extremely complex procedure. The complete analytical protocol comprises the preliminary storage of the urine sample to be assayed in desiccator, with the residue resolved in DMSO; the permethylation of polysaccharides by NaOH/DMSO suspension and methyliodide; the extraction of products with chloroform and the subsequent evaporation to dryness; the cleavage of carbohydrates by heating with 3 M HCl; the reduction with $NaBH_4$ in methanol/aqueous NaOH; and the final acetylation: a number of products are generated that constitute the “fingerprint” of HES and that are analyzed by GC-MS. It is clear that such a procedure cannot be applied for the analysis of all samples received by an antidoping laboratory.

We are presently evaluating a preliminary screening of urine sample by a pre-test, involving the most common electrochemical biosensor, i.e. the glucose electrode. The sample is added with ?–amilase and maltase to obtain the cleavage of HES (if any) to ?–D–glucose and hydroxyethylated derivatives and then analyzed by a common glucose bioelectrode. An unusually high level of glucose represents a reliable index of HES assumption and the sample is therefore subjected to the “complete” treatment described above.

The system can also be made more elegant by immobilizing two or more enzymes on the same electrode and by checking directly for the presence of HES, thus reducing the overall time and costs of operation.

DISCUSSION AND CONCLUSIONS

The recent developments in bioelectronics, micromachining and mass production of screen printed electrodes suggest that the application of electrochemical sensors and biosensors in the field of pharmaceutical and biomedical analysis will markedly grow in the near future.

Apart from their potential use as alternative methods for the screening, in human urine, of doping agents and methods, electrochemical sensors and biosensors could be applied also for the biomedical study of selected parameters correlated to the sport performance. In these cases electrodes and bioelectrodes can be used both on urine and on whole blood, possibly as the sensing element of a flow-through cell placed on an extracorporeal loop by a microdialysis probe.

Further application of electrochemical sensors and biosensors in sport medicine and doping analysis could come by the analysis of biological fluids different from urine.

Around 1910, the Russian chemist Bukowski developed a method to detect alkaloids in saliva of horses. Two years later this method was used for drug testing in horse racing. At present, saliva is no longer collected (neither in horses nor in humans), since the only biological specimen for the determination of drugs in doping control is urine. Nonetheless, even if only a non-invasively obtained sample is acceptable for routine collection in antidoping analyses, the

acceptability of a urine sample is currently being disputed, due to the potential invasion of privacy, especially if a directly observed collection is advisable to prevent adulteration or substitution of the sample [21].

Another major disadvantage of urine is the variability in the renal clearance of drugs and their metabolites, which is largely due to fluctuations in the flow rate and pH of urine. Moreover, not all drugs are excreted in the urine, and especially the lipid-soluble drugs: for instance, ß-blockers tend to be rapidly eliminated by various metabolism systems in the liver [22].

Finally, the present “mission” of an antidoping laboratory is exclusively to supply the “body of evidence” for the subsequent sport trial, i.e. to give all the analytical information requested in order to prosecute or to release the athlete. In this light the pinpoint analysis of a single sample, even if coming from just one single sampling operation and referring to one single biological fluid, is perfectly suitable for the task. Should the laboratory be called to draw pharmacokinetic profiles on the occasion of a positive case, especially whenever it would be necessary to distinguish between allowed and illicit administration of a drug (as it is, for instance, for corticosteroids, whose use is admitted only by topical administration) it is more than evident that the analysis of a single urine sample is completely useless.

For a wide class of drugs, a direct help would come by the analysis of saliva samples, especially if the screening procedure can be quickly carried out, possibly by highly automated devices. Unlike a urine sample, saliva can be obtained under supervision without direct observation of private functions. Although a qualitative doping control mainly depends on the sensitivity of the assay, the usefulness of electrochemical sensors and biosensors for the analysis of doping

agents in saliva needs, in our opinion, to be further and more extensively explored.

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