Introduction of the new analytical approaches to the doping control on the XIV Winter Olympic Games

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Summary

In this paper we present introduction and development of some new analytical methods for identification of anabolic steroids, their metabolites and certain hormones, especially determination of exogenous testosterone by means of gas chromatography-mass spectrometry. Identification of central nervous stimulants and corticosteroides has been performed by high performance liquid chromatography.
In desire to achieve better results, to increase strength and endurance, to sharpen reflexes and to reduce stress and anxiety athletes as well as other people use different pharmacological substances, hormones or even illicit drugs. Use of these substances without medical supervision can lead to adverse effects to one's health or even cause a death. At the same time, use of such substances means a kind of cheat that could not be accepted. This is why International Olympic Committee started at 1968 with official doping control that is permanently carried out and continuously increasing number of banned substances. Doping control demands for discover and development of new sensitive and specific methods for detection of banned substances and their metabolites in urine and blood.

Doping control consists of two parts: sample collection and analytical treatment of sample. Collecting procedure varies depending on types of sports; it is different during Olympic games and different on other competitions. Sample collection procedure consists of system for athlete's selection, their approach to the field station for sampling, sample transport to the laboratory and documentation that trace chain-of-custody for each sample. Sampling procedure is described in details since there are the most possibilities for sample manipulation and generally this part of doping control has the most of athletes complain.
Laboratory for doping control has its own characteristics. It is similar to the laboratory for clinical biochemistry qualified for pharmacokinetic studies of drugs metabolism, identification of metabolites in body fluids, especially endogenous and exogenous hormones using specific sophisticated analytical equipment. Specific demands for such laboratory are capability for identification of large number of drug classes and large number of chemically similar substances in each class. Ultimate precision is absolutely necessary since consequences of positive result reach individual competitor, his sport federation and country as well. Laboratories are rigorously reviewed and evaluated through the International Olympic Committee (IOC) reaccreditations and proficiency programme. Drugs and metabolites are identified using the most sensitive and specific methods and every result is accompanied by complete documentation obtained during doping control.
When the first large scale test were officially instituted at the X Olympic Winter Games in Grenobl and the Games of the XIX Olympiad in Mexico City in 1968 equality of the treatment among athletes was easily established. As there had previously been no rules, the International Federations easily accepted those prepared by the IOC containing the procedures and the list of banned substances. From that time up to now following prohibited classes substances and prohibits methods are subject for doping control: stimulants, narcotics, anabolic agents including anabolic androgenic steroids, diuretics, peptide hormones, mimetic and analogues, chorionic gonadotrophin (hCG), pituitari and synthetic gonadotropines (LH), corticosteroides (ACTH), growth hormone (hGH), insulin-like growth factor (IGF-1), erythropoietin (EPO) and insulin. Prohibited methods include following procedures: blood doping, administering of artificial oxygen carriers or plasma expanders, pharmacological, chemical and physical manipulation. In certain circumstances, following substances are prohibited: alcohol, cannabinoids, local anaesthetics, glucocorticosteroides and -blockers.
Problems associated with abuse of anabolic steroids and testosterone started during Olympic games in Moscow in 1980. At that time identification of these substances was carried out by means of radio immunoassay (RIA). RIA as the analytical method lacks enough specificity for these classes of substances because it is not possible to distinguish between or substituted cyclopentanoperhydrophenanthrenes and doping control demands for unequivocal identification of substance and its metabolite. So, Medical Commission of IOC decided that for next Olympic games in Sarajevo and Los Angeles new methods for anabolic steroid analysis should be devel-

oped and introduced in routine. Quantification of exogenic testosterone and caffeine in urine should be done as well, together with identification of corticosteroides. This conclusion of IOC introduced quantitative analysis in doping control for the first time. That caused another problem for laboratory staff. Joint project of Sarajevo and Los Angeles laboratories was to simultaneously investigate determination of testosterone an anabolic steroids using RIA and hyphenated system gas chromatography-mass spectrometry (GC-MS). These investigations demanded for modern equipment and Organizing Committee of XIV Winter Olympic Games purchased adequate high-resolution gas chromatography-mass spectrometry system.

Quantification of exogenous testosterone was a specific problem. Presence of exogenous testosterone significantly influences normal hormonal profile of androgenic hormones and their metabolites. Detection of these changes was solved by tracing ratio of testosterone and its metabolites androsterone and etiocholanolone, as well as ratio of testosterone and other androgenic hormones: epitestosterone, dehydroepiandrosterone, androstendione, 5β-androstandione, 11β-hydroxyandrosterone and 11β-hydroxyetiocholanolone (1-8). Significant indicator of the presence of exogenous testosterone is ratio of testosterone and epitestosterone in urine. If this ratio exceeds 6 that means obvious presence of exogenous testosterone. In this case, quantification was performed by Single Ion Recording (SIR) technique that enables simultaneous tracing of ions in correlation with their chromatographic retention times, while other ions are not registered. Single Ion Recording technique gains sensitivity by approximately 3 orders of magnitude and at the same time avoids registration of impurities from biological matrix.

In table 1 monitored androgenic hormones are listed with molecular ions of their trimethylsylil derivatives and retention times relative to deuterated testosterone. Figure 1 shows fragmentograms of androgenic hormones in urine sample oh healthy male.

During Olympic games in Sarajevo and Los Angeles simultaneous analysis of steroids by RIA and GC-MS were performed. After completed investigation, Medical Commission of IOC accepted GC-MS as the official method for identification of anabolic steroids and quantification of exogenous testosterone (9-17), while RIA was accepted as a screening technique.

High performance liquid chromatography (HPLC) was introduced for quantification of caffeine in urine (18). The definition of positive results is a concentration in urine greater than 12 μg/mL. HPLC was also used for analysis of pemoline (19,24,26) and other stimulants (9, 20, 23, 25).

Identification of -blockers and their metabolites was also subject of our collaboration with Institute for Pharmacology, UCLA, Los Angeles (27-30).

Systemic use of glucocorticoswteroides is prohibits when administered orally, rectaly or by intravenous or intramuscular injection. Some contributions in this field we gave with investigations presented in different journals (31-33).

Particular problem for laboratory for doping control is accreditation by IOC. Accreditation is performed by analysis of ten samples in exactly 48 hours, under supervision of IOC expert. Every result has to be absolutely correct followed by complete documentation including methodology and results interpretation.

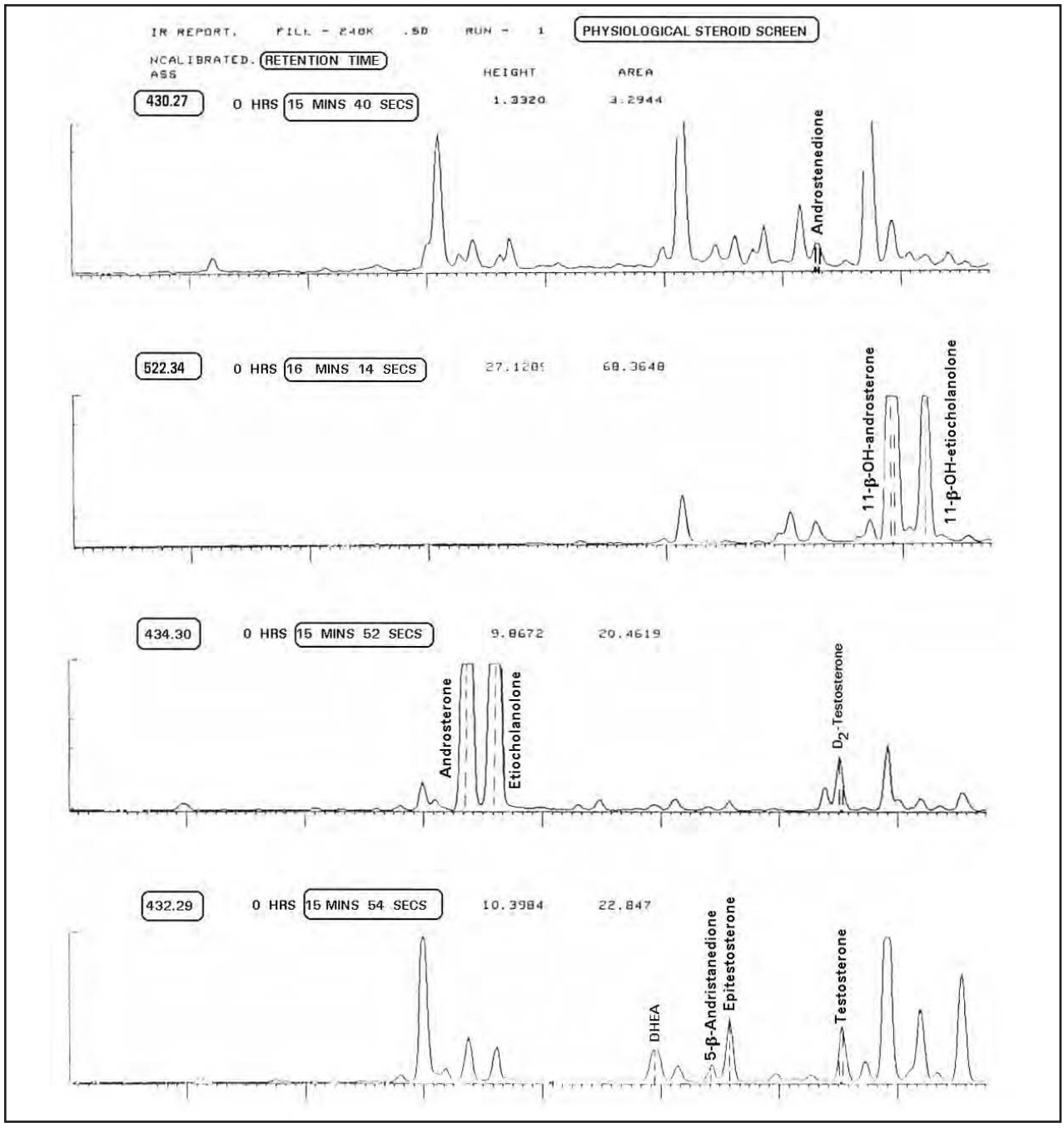
Analysis in a doping control can be interfered by some masking agents that could be intentionally added to the samples. The best-known agents of such kind are bromantane, probenecide and diuretics. There are some

Table 1: Androgenic hormones monitored ions and their relative retention times

Steroide hormones	Monitored molecular ion (M^+)	Relative retention time*
Androsterone	**434**	**0.819**
Etiocholanolone	**434**	**0.836**
Dehydroepiandrosterone (DHEA)	**432**	**0.912**
5-ß-androstandione	**432**	**0.939**
Epitestosterone	**432**	**0.948**
Testosterone	**432**	**1.002**
D_2-Testosterone	**434**	**1.000**
Androstenedione	**430**	**0.987**
5-ß-hydroxy-androsterone	**522**	**1.023**
5-ß-hydroxy-etiocholanolone	**522**	**1.039**

*Relative retention time expressed in relation to D_2-Testosterone

Figure 1: Mass fargmentogram of endogenous steroide hormones and their metabolites of a healthy man who did not use exogenous testosterone



other possibilities for manipulation with sample, like dilution by simultaneous use of diuretics, which put them on the list of banned substances as well. Use of substances that change urine pH value making it acidic or alkaline might slow down excretion of banned substances and their metabolites.

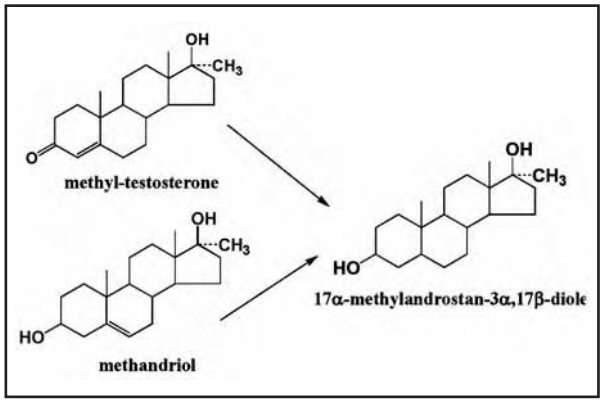
Great problem in analysis is presence of the substances with similar chemical structure. These substances undergo similar metabolic changes and some of them give the same metabolites. This is the case with methyl testosterone and methandriol. Metabolic pathway of these substances is shown in figure 2.

Reliable identification of these substances is possible only by identification of their metabolites and elucidation of their pharmacokinetics. Presence of substances like methyl testosterone and methandriol might lead to false results and they are often used by IOC for doping laboratory testing during competitions.

Laboratory for doping control must have adequate equipment, highly trained multidisciplinary staff, pure test sub-

stances, specific software, bank of spectra and different analytical data including fragmentation pathways, molecular masses and so on. Specific for doping control laboratory is collection of so-called "physiological urines" that are collected in different time course and that serve for metabolite tracking.

Figure 2. Metabolic pathway of methyl testosterone and methandriol



A team of 20 sub specialists carried out accreditation of Sarajevo laboratory: chemists, pharmacists, biologists, physicians and computer experts. Intensive preparations were carried out for a one year and during Olympic games 60 persons worked in laboratory (12 doctors of science, 3 masters of science, chemists, pharmacists, physicians, technicians).

During preparation of laboratory we made intensive collaboration with prof. dr A. Becket and prof. dr D. Cowen, Chelsea College of Science, London; prof. dr Manfred A. Donicke, Bundes Institut für Sportwisenschaft - Institut für Biochemie, Köln; prof. dr V. Semjonov, head of Doping control laboratory in Moscow and prof. dr Catlin, Institute for Pharmacology, UCLA.

Collaborators from our laboratory were on education in Institute Jožef Štefan, Ljubljana (at prof. dr J. Marsel and prof. dr B. Kralj); in Manchester, VG Analytical Education Center (now Micromass) and in Köln attending "Workshop in Dope Analysis of Anabolic Steroids". We are taking the advantage of this opportunity to thank them for their collaboration and help.

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