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Review

Chromatographic techniques—the basis of doping control☆

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Abstract

The principal definition of doping, the groups of banned compounds and the basic analytical problems and strategy of doping analysis are outlined, and the position of chromatography in doping analysis is explained. Examples of the application of GC–MS, especially high-resolution MS, and of LC–thermospray MS to doping problems are given. A practical case is presented briefly, showing the post-analytical problem of evaluating even unequivocal results.

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1. Introduction

The complexity of doping control, which relies heavily on the use of chromatographic techniques, stems from the following: definition of doping; choice of analytical strategies; control management; and interpretation and consequences of the results.

The pre- and post-analytical problems are sometimes far more crucial than the analytical problems (e.g., ignorance and controversies about the meaning of definitions of doping; uniformity or discrepancies among sports associations of different levels, legal aspects, public opinions). Therefore, some general aspects will first be outlined.

Even the generally valid, very simple doping definition of the International Olympic Committee (IOC) seems to be controversial: "Doping is the incorporation of substances out of the banned groups of agents or the application of forbidden methods". This general definition is detailed by the categories and groups listed in Table 1.

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Table 1
Doping definition of the Medical Commission of the IOC 1994 list of banned substances and methods

I. Doping classes
A. Stimulants
B. Narcotics
C. Anabolic agents
D. Diuretics
E. Peptide and glycoprotein hormones and analogues

II. Doping methods
A. Blood doping
B. Pharmacological, chemical and physical manipulation

III. Classes of drugs subject to certain restrictions
A. Alcohol
B. Marijuana
C. Local anaesthetics
D. Corticosteroids
E. Beta-blockers

Examples of the agents are listed for each group of banned substances, followed each time by the addition "... and related compounds". However, since even the meaning of the term "related" has been disputed, the IOC Medical Commission (and, e.g., also the German Sports League) additionally defined in 1993: " 'related' means related by chemical structure or by the intended action". The so-called "complete doping list" has not been defined in order to avoid "grey zones" of newly introduced agents (as experienced in the legislative banning of controlled narcotic drugs of addiction). This means, *vice versa*, that the absence of an agent among those examples of doping groups does not necessarily exempt it from being banned.

While some decades ago the so-called "classical doping agents", stimulants (group 1A of the IOC definition; see Table 2) and narcotics (group 1B), prevailed, their leading role has now been taken over by the anabolic agents (group 1C; Table 3), which are intended to produce effects similar to the male sexual hormone testosterone e.g., (muscle production, enhancement of competitiveness and aggressiveness).

In addition to those substances, their numerous metabolites and interfering compounds in the biological sample become relevant, thus considerably enlarging the number of potential analytes to be detected and identified. This determines the generally analytical strategy: a predomoninantly two-step qualitative analysis involving first a "screening" for the large number

Table 2
Group 1A: examples of stimulants

Amfepramone	Fenproporex
Amphetaminil	Furfenorex
Amineptine	Mefenorex
Amiphenazole	Mesocarb
Amphetamine	Methamphetamine
Benzphetamine	Methoxyphenamine
Caffeine[a]	Methylephedrine
Cathine	Methylphenidate
Chlorphentermine	Morazone
Clobenzorex	Nikethamide
Clorprenaline	Pemoline
Cocaine	Pentetrazol
Cropropamide	Phendimetrazine
Crotethamide	Phenmetrazine
Dimethamfetamine	Phentermine
Ephedrine	Phenylpropanolamine
Etafedrine	Pipradol
Ethamivan	Prolintane
Ethylamfetamine	Propylhexedrine
Fencamfamine	Pyrovalerone
Fenethylline	Strychnine
	and related compounds

[a] For caffeine the definition of a positive result depends on the concentration of caffeine in the urine. The concentration in urine may not exceed 12 μg/ml.

Table 3
Group 1C (part 1): examples of anabolic androgenic steroids; Group 1C (part 2): examples of other anabolic agents

Group 1C (part 1)	
Bolasterone	Methyltestosterone
Boldenone	Nandrolone
Clostebol	Norethandrolone
Dehydrochloromethyl-testosterone	Oxandrolone
Fluoxymesterone	Oxymesterone
Mesterolone	Oxymetholone
Methandienone	Stanozolol
Methenolone	Testosterone[a]
	and related compounds
Group 1C (part 2)	
Beta-2-agonists, e.g., clenbuterol and related compounds	

of relevant compounds and second a scrutinous conformation of any positives.

Quantitative determinations (although done on the basis of signal intensities and comparison with control samples, internal standards, biological matrix) are demanded for only a few compounds (e.g., caffeine, ephedrine, physiological compounds such as testosterone).

The requirements of the IOC for the accreditation of doping laboratories already reflect this philosophy (see also below).

The analysis itself has to rely on an adequate sample collection. The doping checks during competitions, which are the responsibility of the organizing national or international sports associations, imply the analysis of all banned groups of substances.

Because anabolic agents, owing to their long-term effects, are misused only during training phases, so-called "out-of-competition tests" were introduced some years ago. These samples are analysed only for anabolic agents (plus diuretics in the case of low urine densities).

Strong efforts have been and still are directed towards the increase in the number of samples analysed, equal inclusion of all sports and countries and the strict obeyance of scrutinized rules for sample withdrawal, intended to eliminate any possibilities for manipulation.

Similar harmonizations are necessary for the consequences of positive doping analyses, to diminish discrepancies and uncertainties between nations and international and national sports associations on the ground of internal regulations, legal aspects and political and public pressure.

Numerous compounds of the banned groups cover a wide range of chemical structures. They are almost exclusively pharmaceuticals with reasonable medical indications, which are misused for doping. It is not true that there is a growing number of new agents especially synthesized for doping purposes, a process suggested to be always ahead of the analytical methodology. Even considering the immense grey market of doping preparations, this would probably never be economically efficient.

The wide range of chemical structures requires the use of a complex analytical strategy.

2. Analytical strategy

The analytical strategy is determined by the chemical structures of the substances, biochemical aspects, such as dosages, metabolism and excretion behaviour, and various administrative demands.

Chemical structures are slightly correlated with the intended pharmacological activity, hence a subdivision of relevant substances into categories such as stimulants and narcotics, β-blockers, anabolic steroids and diuretics is useful also from an analytical viewpoint. Additionally, a differentiation between substances excreted unchanged or conjugated is necessary, especially in the case of anabolic steroids. The hydrolysis of conjugates is essential for steroids excreted as glucuronides, but should be avoided otherwise, since disturbing matrix components may be formed. Finally, different classes of substances in competition or out of competition testing are covered.

Although mainly restricted to anabolic steroids and related compounds, the out-of-competition testing requires also the detection of uricosuric agents (probenecid), which can impede renal excretion and therefore mask the presence of doping agents, and which are consequently included in the screening procedure. Therefore, analytical flexibility is required, and the screening procedures may comprise different species regardless of their divergent chromatographic properties, concentration ranges or pharmaceutical actions.

The first step of the doping analysis consists of the screening procedures:

Screening I	Volatile N compounds
Screening IIa	Slightly volatile conjugated compounds
Screening IIb	β-Blockers
Screening III	Pemoline, caffeine
Screening IVa	Anabolic steroids free fraction
Screening IVb	Anabolic steroids conjugated fraction
Screening V	Diuretics

They are intended to detect all banned compounds and their metabolites (and possibly further agents for the observed of upcoming cus-

toms). This resembles the concept of systematic toxicological analysis (STA), although the number of doping agents is much smaller and well defined compared with potentially toxic compounds in "general unknown" suspected poisoning cases.

After the initial check of density and pH, liquid–liquid and solid-phase extractions are used for sample preparation.

Gas chromatography with nitrogen-sensitive flame ionization detection (GC–NFID) and with mass spectrometric detection (GC–MS), high-performance liquid chromatography (HPLC) and its combination with mass spectrometry (HPLC–MS) are used in various configurations. Immunoassays are applied to the determination of peptide hormones, which cannot yet be detected by the more informative combination of chromatographic and spectroscopic principles.

Sophisticated instrumentation is demanded by the IOC Requirements for Accreditation. The introduction of high-resolution and tandem MS, and also combined HPLC–MS, will certainly become more and more important in the near future. Only a few examples can be given to illustrate the practical application of the outlined analytical (mainly chromatographic) strategies.

The GC–NPD screening procedure for the so-called "classical" doping agents such as amphetamines or ephedrines was introduced more than 20 years ago. This example illustrates the fact that the information obtained from one analysis may considerably increase with improvements in the chromatographic techniques applied. Only packed columns were available at the beginning, and the nitrogen/carbon selectivity of the detectors used at that time was lower than today. Time-consuming and partly sophisticated methods were necessary to deactivate the chromatographic system and to tune the detector

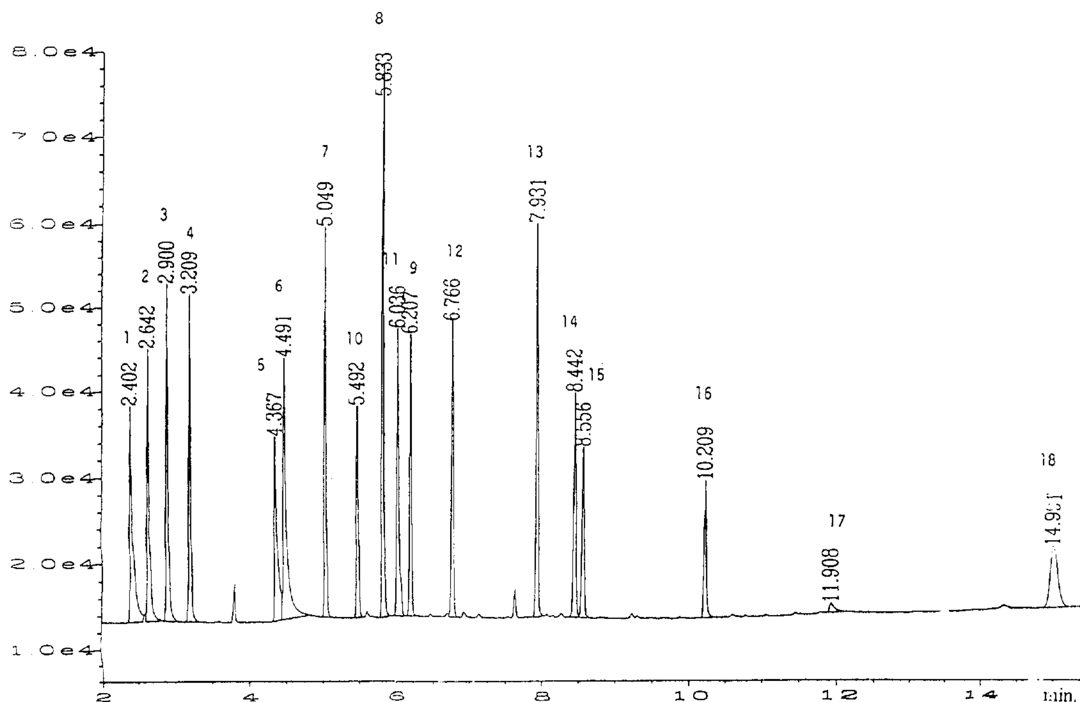


Fig. 1. GC–NFID of a mixture of reference compounds (the strychnine peak represents 40 pg). 12.5 m column OV-17; 0.32 mm I.D.; 0.26 μm d_f. Peaks: 1 = heptaminol, 2 = amphetamine, 3 = methamphetamine, 4 = dimethamphetamine, 5 = norephedrine, 6 = ephedrine, 7 = phenmetrazine, 8 = nikethamide, 9 = diphenylamine (I.S.), 10 = prolintane, 11 = fencamphamine, 12 = methylphenidate, 13 = caffeine, 14 = normethadone, 15 = methadone, 16 = codeine, 17 = quinine, 18 = strychnine.

selectivity. The lower overall chromatographic performance led to a higher degree of uncertainty in peak allocation and hence to a higher load of analytical work. The tremendous advances in commercially available chromatographic equipment during the past two decades, in connection with easy-to-use data handling systems now give us the opportunity to do this work more efficiently. During injection, the ethereal extract from a urine sample is split between two fused-silica capillary columns with different stationary phases (HP-5 and HP-17), yielding simultaneously two characteristic retention parameters per analyte. This enhanced chromatographic information combined with the high selectivity and sensitivity of NPD (see Fig. 1; the strychnine peak represents about 40 pg) provide an effective tool for screening of volatile nitrogen-containing compounds.

Screening for anabolic steroids is an example of the application of GC in combination with highly informative and sensitive mass selective detection. The strategy for the detection of anabolic steroids in urine samples has to take into consideration the following aspects: steroids may undergo extensive metabolism; metabolites are excreted either in the free form or as conjugates (in humans predominantly as glucuronides); GC analysis of the usually polar compounds requires derivatization, mainly to trimethylsilyl (TMS) derivatives; and low detection limits are necessary.

To achieve detection limits at the low ppb level, it is necessary to operate the quadrupole MS detector at the highest sensitivity in the selected-ion monitoring (SIM) mode (Fig. 2). The windowed print-out of every sample (Fig. 3) with two diagnostic ions per analyte gives a comprehensive overview of the presence of the target analytes. All monitored diagnostic ions of a distinct analyte can be reconstructed from the raw data file for a more detailed data evaluation (Fig. 4).

The work-up procedure for anabolic steroids (agents) includes the following steps: XAD-2 extraction of the urine sample to isolate steroids and steroid conjugates; separation of free steroid fraction (screening 4a) and total steroid fraction (screening 4b); enzymatic hydrolysis to cleave the steroid glucuronides (screening 4b only); liquid–liquid extraction with diethyl ether to recover the steroids from the aqueous phase; evaporation to

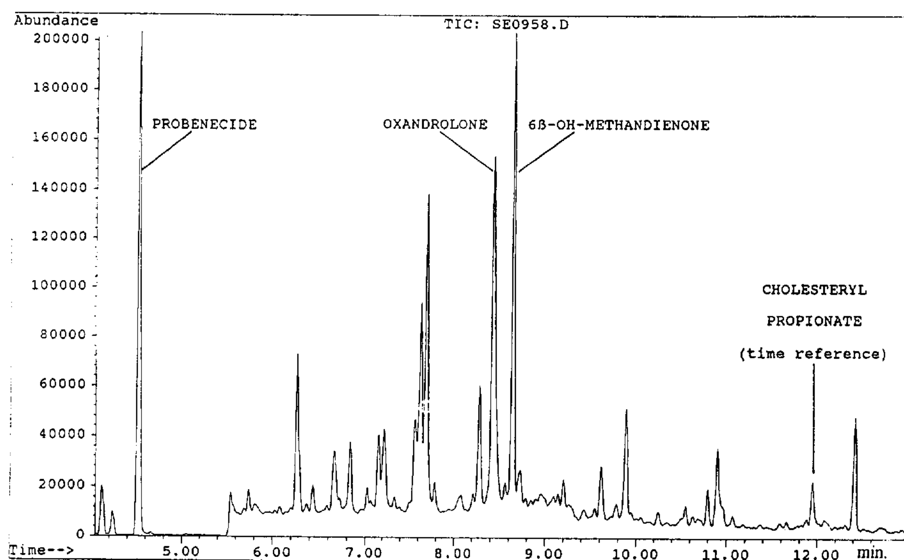


Fig. 2. GC–MS in SIM mode (pg range).

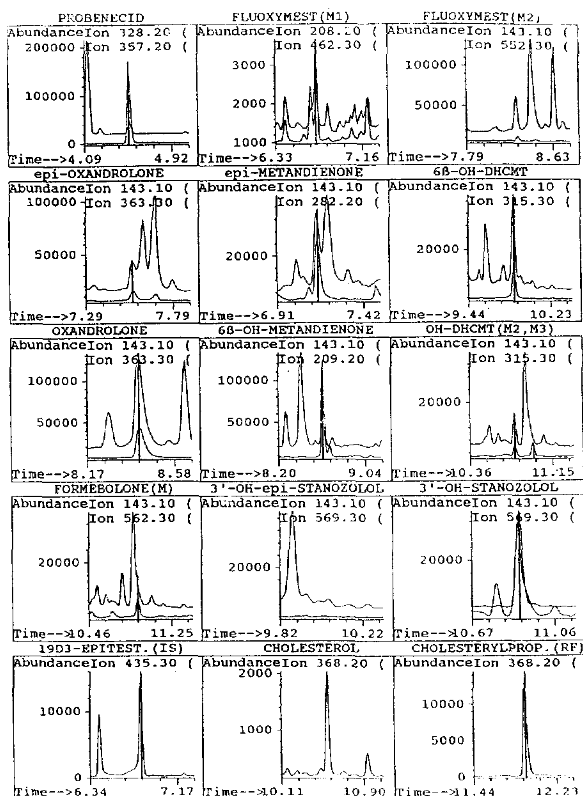


Fig. 3. Windowed printout for a single sample (two diagnostic ions per analyte). Time in min.

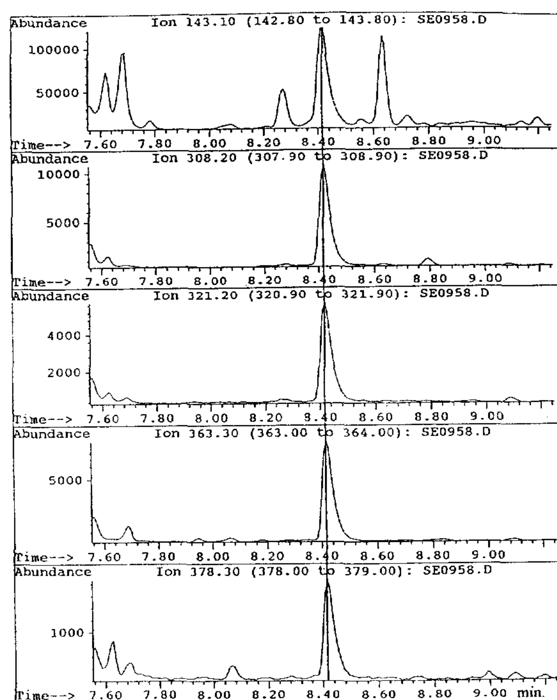


Fig. 4. Reconstructed chromatograms (diagnostic ion traces).

dryness; and derivatization of the extract to convert the hydroxyl and keto groups into TMS-ethers and TMS-enol ethers, respectively.

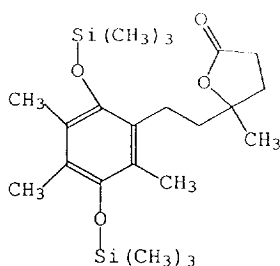
The problem with the determination of steroids in urine is the need to detect comparatively low concentrations in a complex matrix. Principle possibilities to solve these problems are to improve either the chromatographic or the detection conditions. Even a very high chromatographic performance is not sufficient to separate all possible substances of a urine matrix, owing to the variety of existing endogenous substances, including their metabolites and chromatographic artifacts. Therefore, additional optimization of the detection methods is required. There is no general approach to optimize the chromatographic detection; NPD seems to be appropriate for a screening for nitrogen-containing substances, whereas the structural similarity of illicit steroids with endogenous substances is a serious problem for the detection of steroids. Interfering substances may have a manifold higher concentration, identical elemental composition, similar metabolism and identical derivatization properties and fragmentation pattern.

In the case of nandrolone metabolites there is co-elution of norandrosterone (TMS derivative) with a minor fragment of a comparatively highly concentrated vitamin E metabolite (Fig. 5).

Application of a quadrupole mass spectrometer in the SIM recording mode is not sufficient for the problem, as the unit mass resolution is not capable of differentiating between the fragments of the co-eluting substances. Both masses 420.215 of the vitamin E metabolite and 420.288 of the molecular ion peak of norandrosterone are of the same average mass. To differentiate between them, a resolution R of

$$R = \frac{m}{m_{\mathrm{Norand.}} - m_{\mathrm{Vit.}EMb.}} \approx \frac{420}{0.07} = 6000$$

is required. The corresponding metabolite can be detected without vitamin E interferences at any



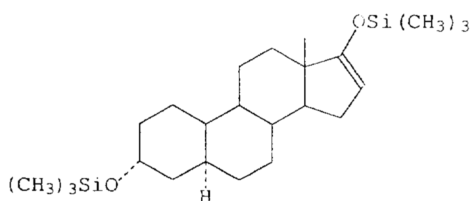


Fig. 5. Co-eluted norandrosterone TMS derivative (M_r = 420) and vitamin E metabolite (M_r = 422).

resolution higher than this threshold. In Fig. 6, the measurement of a presumed nandrolone-positive sample is shown, compared with the corresponding control urine. This comparison proves the presence of both metabolites in the suspected sample, and moreover confirms the fact that the signals at a mass of 420.288, specific for nandrolone metabolites, are not influenced by vitamin E or other impurities.

In spite of optimization of the chromatographic separation and detection parameters, some analytical problems in doping analysis cannot be solved adequately by gas chromatography. The identification of the stimulant mesocarb by GC–MS failed owing to its considerable thermal instability. Only mass spectra of defined pyrolysis products may be determined.

HPLC proved to be a useful separation technique for these problems. The application of an LC–MS detector becomes necessary, because MS is a mandatory identification criterion in doping analysis, while UV detection is preferred for HPLC screening procedures.

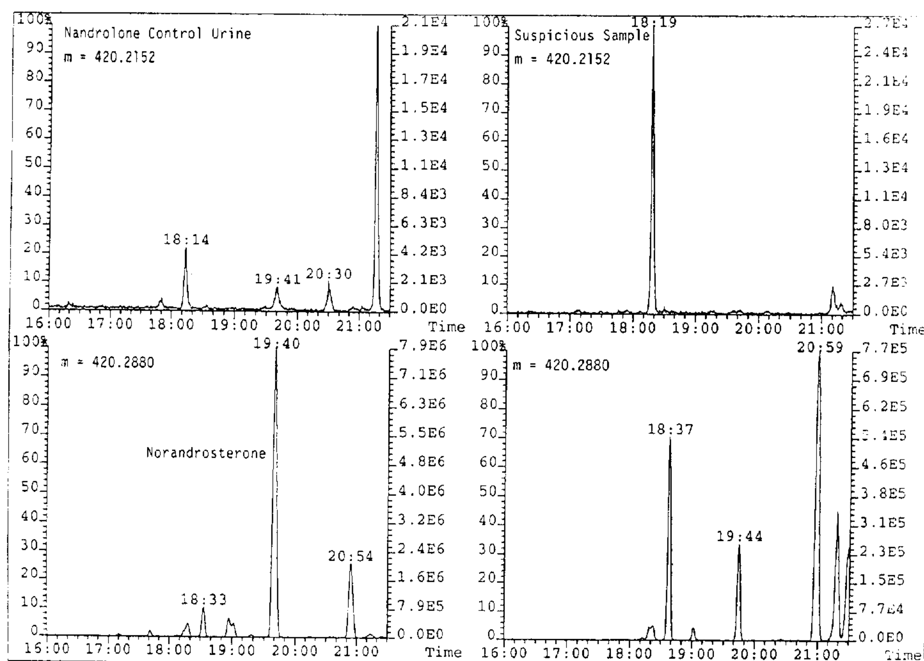


Fig. 6. Comparison of a suspicious sample (right) with a nandrolone control urine (left). The upper windows exhibit the traces of m/z 420.2152 to identify the vitamin E fragment. Below the molar mass, signals of the nandrolone metabolites (norandrosterone at a retention time of 19:42 min, noretiocholanolone at 20:56 min) are demonstrated.

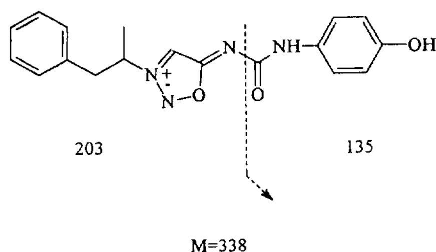


Fig. 7. Structure of mesocarb and of its main fragment.

By application of a reversed-phase HPLC separation with an ammonium acetate buffer–acetonitrile gradient as mobile phase, identification of the parent compound and the metabolite was possible. Moreover, the confirmation of the uncertain composition of a conjugate was possible by identification of the unchanged sulphate by thermospray MS (Figs. 7 and 8).

Compared with other typical thermospray spectra, the thermospray mass spectra of mesocarb (parent compound, metabolite and conjugate) exhibit considerable fragmentation. Therefore, the significance of a full-scan mass spectrum is sufficient for a positive identification, and a screening procedure based on SIM of five key fragments may be effective (Fig. 9).

3. Evaluation of results

An unusual practical case emphasizes the analytical scope and the problems in evaluating the results. During the screening of six urine samples from an international competition, an

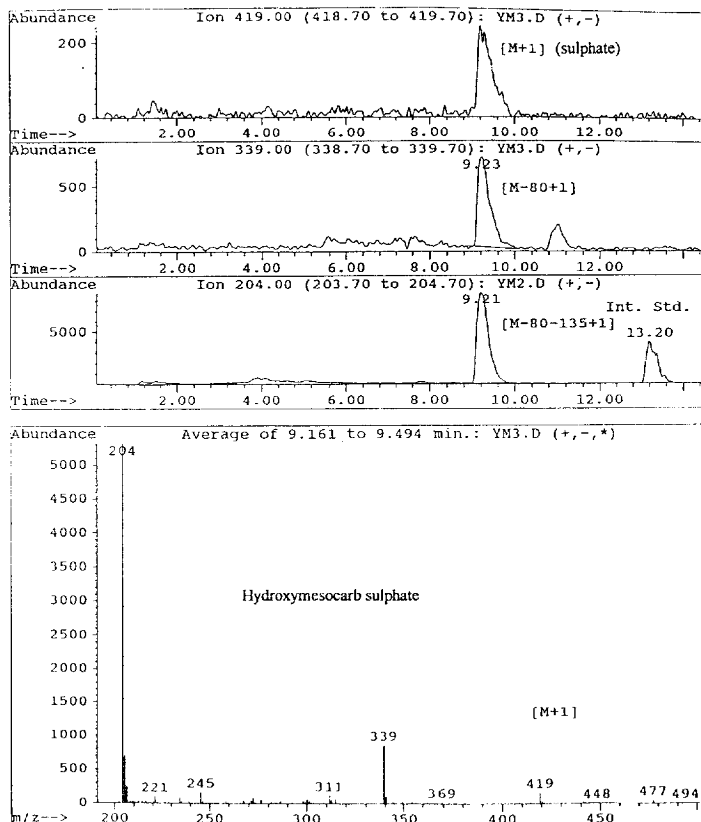


Fig. 8. Full-scan MS of a mesocarb control urine. The extract ion plot shows the three most intense ions. Thermospray mass spectrum of conjugated hydroxymesocarb in the range m/z 200–500. Time in min.

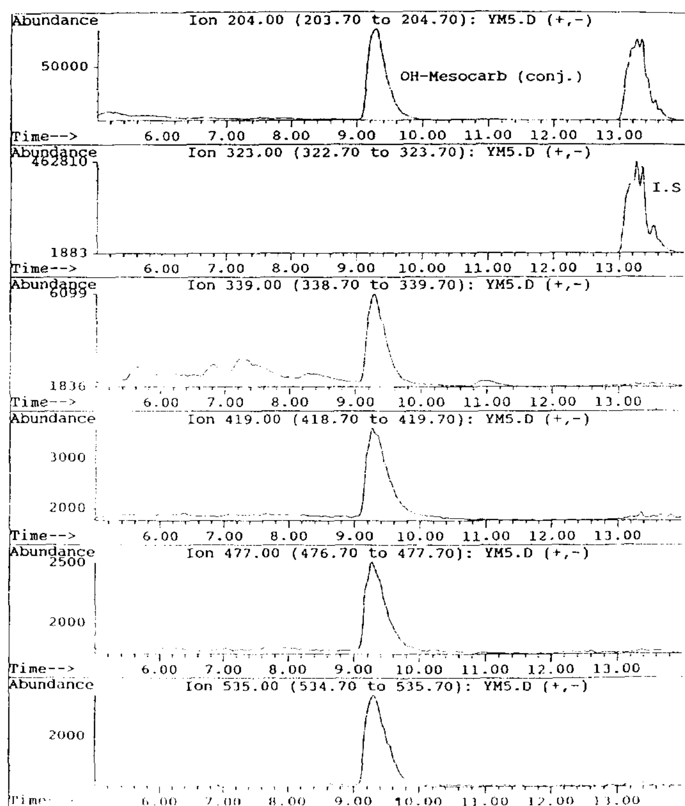


Fig. 9. SIM recording of a mesocarb control urine as a screening method. Time in min.

immense GC peak appeared in the extraction residues of procedure 1 in two of the samples (for one example, see Fig. 10). GC–MS led to the suggestion of the drug piracetam (Fig. 11). Repeated chromatograms after derivatization (trifluoroacetyl and trimethylsilyl derivatives) confirmed this assumption both on the basis of retention parameters and comparison of mass spectra (Fig. 12).

The identification of the nootropic agent piracetam was a surprise, because this pharmaceutical agent, present in urine samples from two athletes in the same team, was not declared on the sampling protocols, whereas the intake of ascorbic acid was confessed: the nootropic agent piracetam is not explicitly included in the list of examples of doping class I A "Stimulants", although its support of cerebral metabolism, normally used in geriatry, might have been the reason for its use; a reasonable medical indication could not be recognized, and it had become the more improbable when administered to two people simultaneously; and the concentration in urine was extremely high.

Quantification and a comparison with a control trial (intake of the usual daily dose of 3 × 800 mg, which yielded much lower concentrations showed that the drug must have been taken in extraordinary overdoses. This led to our conclusion to state positive *analytical* results of the two doping control samples (remaining negative according to the IOC doping definition) together with the recommendation to the sports association involved, to react adequately towards the people responsible and further to observe a possible new tendency of misuse in sports.

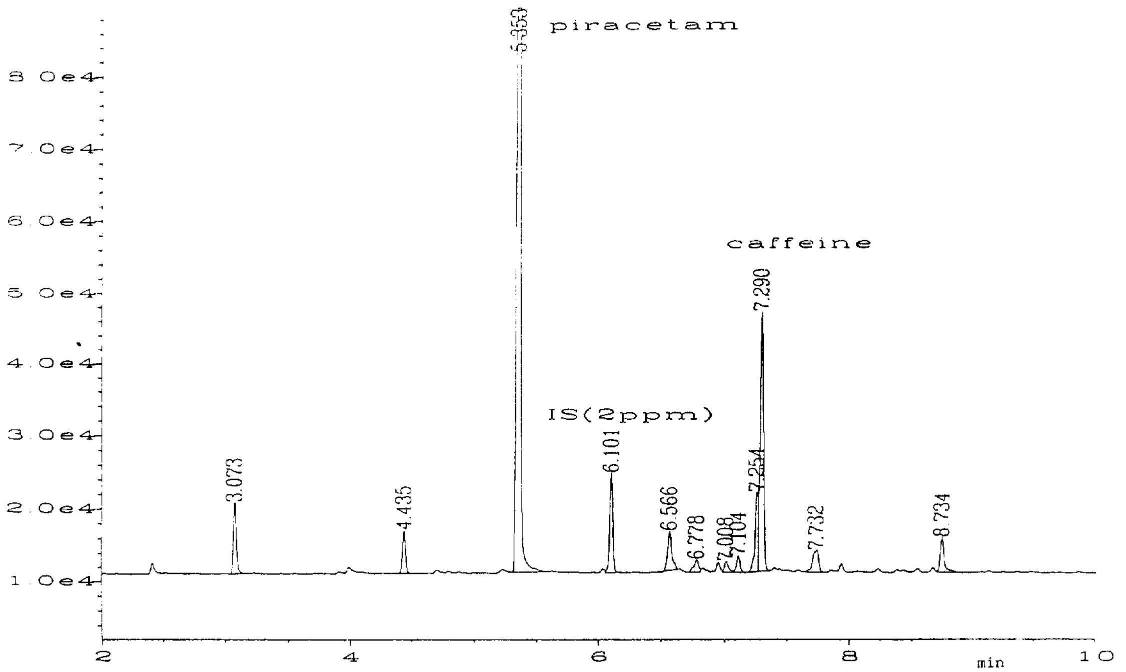


Fig. 10. Gas chromatogram with unknown (piracetam) peak.

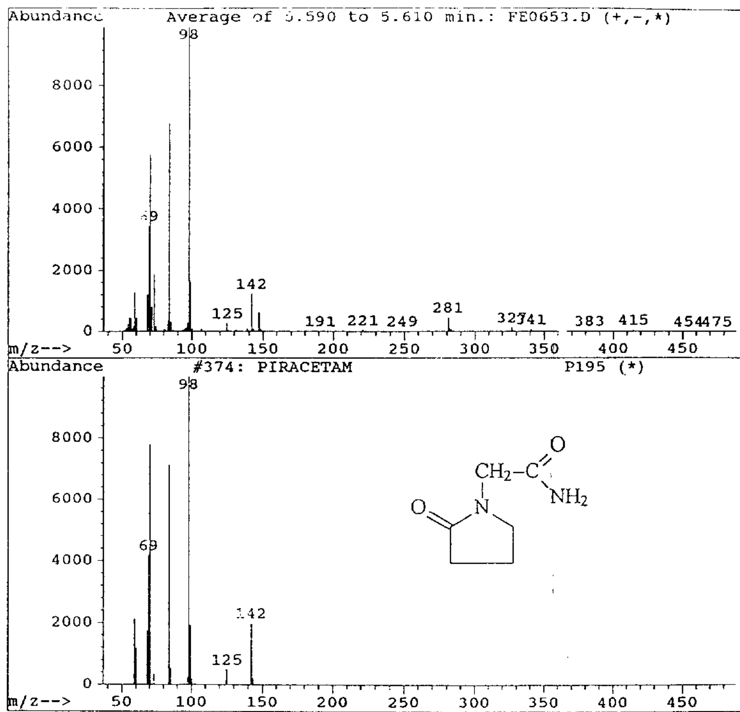


Fig. 11. Mass spectrum of piracetam.

Fig. 12. Result of MS library search: comparison of sample and library spectra.

4. Conclusions

Although doping control can rely on an advanced analytical strategy including several chromatographic techniques, several aspects have to be kept in mind for further progress towards fair, drug-free sport: the continuous observation of upcoming tendencies of misuse; the subsequent amendment of the lists of banned substances; the development of analytical techniques for substances which are either new or which cannot yet be detected or identified with sufficient certainty or sensitivity; and harmonization of the final evaluation and of the consequences of positive and of "grey zone" results of doping analyses.

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