

trends

Doping control of athletes

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History

The origin of the word doping is not certain. It first appeared in the English dictionaries at the end of the nineteenth century. The first reference to the use of doping by athletes was in 1864 during swimming competitions that were held in the canals of Amsterdam. The use of what is known as a 'speedball', a mixture of heroine and cocaine, by cyclists was described in 1869. In 1904 the Russian chemist Bukowski was able to identify some alkaloids in the saliva of horses¹.

Australia was the first country to take measures against doping in sports in 1962, and England followed with the drugs bill in 1964. During the Olympic Games of Mexico (1968) the first doping control tests were performed but it took another 10 years before the use of anabolic steroids could also be detected and tested for.

Doping is a topic that attracts a lot of publicity from the media, e.g. the cases of the athlete Sandra Gasser (1987), the skater Goeljajev (1988) and Ben Johnson (during the Olympic games in Seoul). From the publications in the media it is obvious that doping is used in all kinds of sports including body building, power lifting, cycling, athletics, skating, tennis and even billiards. Athletes such as Steve Cram and Carl Lewis have openly attacked the current procedures for doping control, asking for an even more stringent but fair testing procedure. It should be impossible for sportsmen to make arrangements with the organization committees about doping control. Every athlete should be tested during a competition.

What is doping?

It is very difficult to find a satisfactory definition for doping. Many experts and committees have spent valuable time trying to answer this question without success. When does a cup of coffee become a forbidden drug instead of a socially accepted stimulant?

The definition of the Council of Europe for doping is²:

'The administration to or the use by a healthy individual in any way of compounds which are not natural to the organism or the use of physiological compounds in abnormal doses or in an abnormal way with the only purpose to artificially and dishonestly influence the performance of this person during competition.'

Another possible definition is:

'The use of compounds which are not synthesized by the human body or the use of endogenous compounds in excessive doses with the aim to improve performance in sport competitions.'

A simpler definition is:

'The use of forbidden drugs.'

The last definition can only be used when we have a clear and unambiguous list of forbidden drugs. The Medical Commission of the International Olympic Committee (IOC) has decided which compounds should be listed as 'forbidden' and fortunately most international and national sport federations comply to the IOC rules. However, there are still some non-olympic sports with their own regulations, which can lead to confusion.

Why doping control?

The IOC started its doping control program for several reasons. First of all, all competitors should have an equal chance and the use of doping should therefore be forbidden. Secondly, the use of certain drugs can result in a decrease of motoric control which can endanger the other competitors, e.g. cyclists riding close together in a pack. Last but not least is the possible risk of side-effects such as addiction to amphetamines or narcotics, or possible liver damage and cancer from anabolic steroids. Of course, it is always difficult to prove that certain side-effects result from the use of doping. Double-blind cross-over studies with athletes using anabolics are very difficult and to measure possible damage the follow-up should continue for years.

Forbidden compounds and their use

The current IOC list^{3,4} consists of five groups of doping classes with forbidden drugs.

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(I) Stimulants

According to their action, several groups of stimulants can be discriminated although the boundaries between them are not clear and the classification therefore debatable⁵. The most well known group of stimulants is formed by the *psychomotoric stimulants* such as amphetamine. Amphetamine and its derivatives reduce the appetite, and feelings of fatigue. They can raise morale and self-confidence while improving alertness, initiative and physical activity. The known side-effects are headache, dizziness, hallucinations, but especially dangerous for athletes is the disturbance of body temperature regulation. The characteristic reduced self-criticism also leads to unnecessary risks (also for other competitors). Examples are amfepramone, benzphetamine, fencamfamin and propylhexedrine. Also the anorexic drugs such as fenfluramine and the central nervous system stimulants cocaine and pemoline belong to this group.

Ephedrine and other phenylethylamines belong to the *sympathomimetics*, a group of drugs which, like adrenaline stimulate the sympathetic nervous system. Compounds that activate the central nervous system are called *analeptics* and they are used to stimulate respiratory functions (pentetrazol, beme-gride) or the heart (nikethamide, mefentermine, strychnine). Caffeine is also regarded an analeptic drug. This socially accepted stimulant has been used for centuries and millions of people need their daily dosis of caffeine; without it they get headaches or become moody. It is also known that caffeine will increase the combustion of fat thereby saving the glycogen in the muscles which results in increased stamina.

(II) Narcotics

All narcotic analgesics are forbidden in sports. Because of their euphoric and analgesic effect these drugs are used to lengthen performance time. Besides morphine, heroin, pethidine and dextropropoxyphene, codeine also belongs to this group which often leads to problems because it is present in many commonly used cough and cold preparations.

(III) Anabolic steroids

The use of hormones in sports is probably the most well-known form of doping. The use of the male sex-hormone testosterone increases protein synthesis and therefore creates more muscle: the anabolic effect. Androgenic effects also occur. Virilization effects are particularly obvious in women users. The pharmaceutical industry has therefore synthesized testosterone derivatives with less androgenic effects, but with high anabolic action: these are called ana-

bolic steroids. There are many medical indications for the use of such anabolic steroids. In sports anabolics are used not only for increasing muscle mass but also to improve recuperation of the muscles after heavy physical strain. Also, they help injuries to heal quicker. The potential severe side-effects — liver damage, impotency or cancer — are reduced by using lower doses of short-acting anabolics under the control of a physician. However, high doses of several anabolics are still being used in sports such as powerlifting and bodybuilding.

(IV) Beta-blocking agents

In January 1987 the use of beta-blockers was forbidden. They were found to be used by ski-jumpers to calm their nerves, as well as in sports such as pentathlons, shooting and billiards. These drugs block the beta-receptors in the heart resulting in a decreased cardiac rhythm. This can be useful in sports where aiming is important.

(V) Diuretics

The last group added to the list is the group of masking agents, the diuretics. These drugs increase the urine volume thereby diluting the concentration of the possible forbidden doping agents. In the analytical analysis some compounds may well fall under the detection limit by this dilution. Diuretics are also used to lose weight quickly for placement in lower weight categories in certain sports, e.g. judo, taekwondo, or weightlifting. During the Olympic games in Seoul four athletes were found positive on the diuretic furosemide.

Another compound that attracted a lot of attention in 1988 was probenecid. This compound reduces the excretion of antibiotics and steroids. It may well reduce the concentration of anabolic steroids in urine to 1–10%, thus making analysis impossible⁶. This compound has therefore been banned and is easily detected in the screening procedures for stimulants and anabolic steroids (own results).

Doping control regulations

The procedures for doping control vary for different sports, depending on the regulations of the international sport federations such as the IAAF (athletics) and UCI (cycling). The legal situation is also different in each country. Five European countries take legal action against doping: Belgium (1965), France (1965), Italy (1971), Turkey (1971) and Greece (1976). In Switzerland, Germany and the Scandinavian countries the national sport federations have strict regulations against doping. In the Netherlands there is no law against doping as the government is

TABLE I. Examples of forbidden drugs according to the IOC regulations

<i>Stimulants</i>				
Amfepramone	Clorprenaline	Etilamfetamine	Methylphenidate	Pipradrol
Amfetaminil	Cocaine	Fencamfamin	Morazone	Prolintane
Amiphenazole	Cropropamide ^a	Fenetylline	Nikethamide	Propylhexedrine
Amphetamine	Crotetamide ^a	Fenproporex	Pemoline	Pyrovalerone
Benzphetamine	Dimetamfetamine	Furfenorex	Pentetrazol	Strychnine
Caffeine	Ephedrine	Mefenorex	Phendimetrazine	
Cathine	Etafedrine	Methamphetamine	Phenmetrazine	And chemically or
Chlorphentermine	Etamivan	Methoxyphenamine	Phentermine	pharmacologically
Clobenzorex		Methylephedrine	Phenylpropanolamine	related compounds
<i>Narcotic analgesics</i>				
Alphaprodine	Dextropropoxyphen	Ethylmorphine	Pentazocine	And chemically and
Anileridine	Diamorphine (heroin)	Levorphanol	Pethidine	pharmacologically
Buprenorphine	Dihydrocodeine	Methadone	Phenazocine	related compounds
Codeine	Dipipanone	Morphine	Trimeperidine	
Dextromoramide	Ethoheptazine	Nalbuphine		
<i>Anabolic steroids</i>				
Bolasterone	Fluoxymesterone	Methyltestosterone	Oxymesterone	And chemically and
Boldenone	Mesterolone	Nandrolone	Oxymetholone	pharmacologically
Clostebol	Metandienone	Norethandrolone	Stanozolol	related compounds
Dehydrochlormethyltestosterone	Metenolone	Oxandrolone	Testosterone	
<i>Beta-blockers</i>				
Acebutolol	Labetalol	Oxprenolol	And chemically and	
Alprenolol	Metoprolol	Propranolol	pharmacologically	
Atenolol	Nadolol	Sotalol	related compounds	
<i>Diuretics</i>				
Acetazolamide	Bumetanide	Diclofenamide	Mersalyl	And chemically and
Amiloride	Canrenone	Ethacrynic acid	Spirolactone	pharmacologically
Bendroflumethiazide	Chlormerodrin	Furosemide	Triamterene	related compounds
Benzthiazide	Chlortalidone	Hydrochlorothiazide		

^a Compound of Micoren®.

not interested in this problem. In Norway the sport authorities are allowed to take urine samples from athletes during training. The IAAF has also adapted its regulations in this direction, because many drugs tend to be taken during training and their administration stopped just in time for the athlete concerned to be clean for a major sport event. During World or European championships, samples are screened for all the drugs on the IOC-list. However, at national sport events, local organizations or sport federations in certain countries are at liberty to test only for one group of drugs. Also, often only the first three places are checked: a proper, stringent doping control should always be performed for all forbidden drugs and the samples should be taken at random.

Analytical procedures

As can be seen from the IOC list (Table I) the analytical laboratory is faced with five groups of com-

pounds each consisting of a large number of compounds with totally different structures and chemical properties. It is therefore impossible for all compounds to be isolated and detected by one procedure. Of course the analytical laboratories will try to keep the number of procedures as small as possible. Although there are differences in the approaches taken by the doping control laboratories, the general outline given below is the same. All procedures are based on those recommended by the IOC and published by Donike *et al.*⁷.

Stimulant screening

For the stimulants and some narcotics two screening procedures are used^{8,9}. The first procedure, which is suitable for most amphetamines, is a simple ether extraction after alkalization of the urine. Another extraction at pH 10 is performed after an enzymatic hydrolysis. In this way we can also analyze the

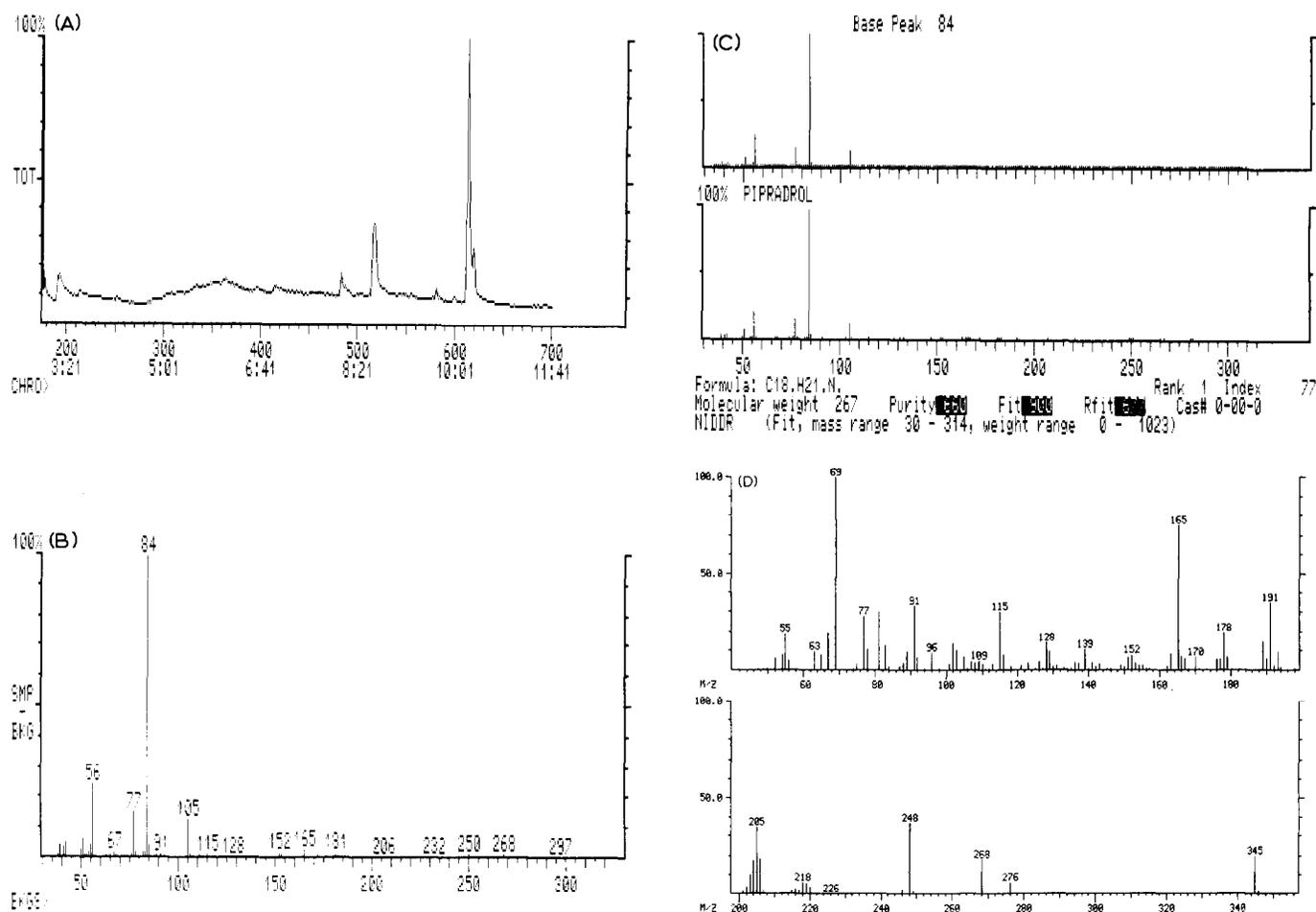


Fig. 1. (A) Total ion chromatogram of a positive urine sample measured with the Finnigan MAT ITD. (B) The background subtracted spectrum of the suspected peak at 10:14 min. (C) Although the file search result gives a 90% fit for pipradol the spectrum with basepeak m/z 84 is not characteristic. (D) The EI spectrum of the TFA-derivative of the suspected positive urine results in a far more informative spectrum.

conjugated stimulants and narcotics. Both extracts are injected in a gas chromatograph with nitrogen-phosphorus (NP) detection using a capillary fused-silica column. An internal standard is added to all urines before the extraction so the relative retention times of suspected peaks can be compared with our library of peaks of forbidden drugs.

Confirmation of stimulants

When a urine sample is suspect, a second aliquot of the same urine is reanalyzed together with a reference urine of the suspected drug and a blank urine. The extracts are usually converted to their N-trifluoroacetyl-O-trimethylsilyl (N-TFA-O-TMS) derivatives according to the procedures published by Donike *et al.*¹⁰. In this way the chromatographic properties improve considerably. When the relative retention times of the suspected peak and the reference urine match, the presence of this compound will be confirmed by mass spectrometry. Only mass spectrometry can give the necessary proof for a positive urine sample and is therefore always required by the

IOC. We use a Finnigan MAT Ion Trap Detector 800 (ITD 800) for confirmation of our findings in the screening procedures. The ITD has proven to be very sensitive in the full scan mode which is favourable for this kind of screening. However, most amphetamines give electron impact (EI) spectra with only one or a few non-characteristic peaks. We therefore always run a chemical ionization (CI) spectrum as well on our Finnigan MAT TSQ-45, which provides us with the extra (molecular) information (Figs. 1 and 2). Nowadays there is also a CI option available on the ITD 800, which is very easy to operate, making the CI information available just by pressing a key.

Narcotics screening

Although a number of narcotics will be detected while screening for stimulants, we have developed a special narcotics screening for all known narcotic analgesics. As most narcotics are conjugated with sulphate or glucuronic acid we perform an enzymatic hydrolysis overnight on 2 ml of the urine sample.

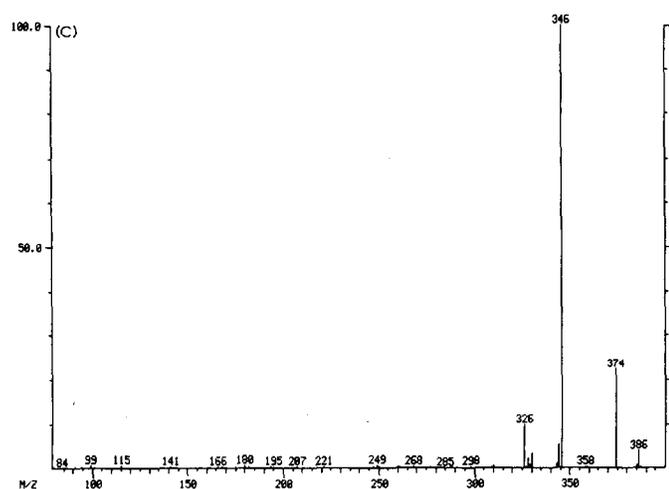
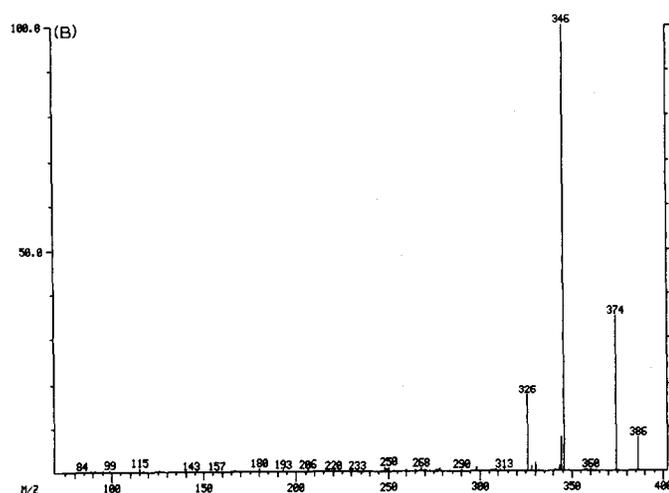
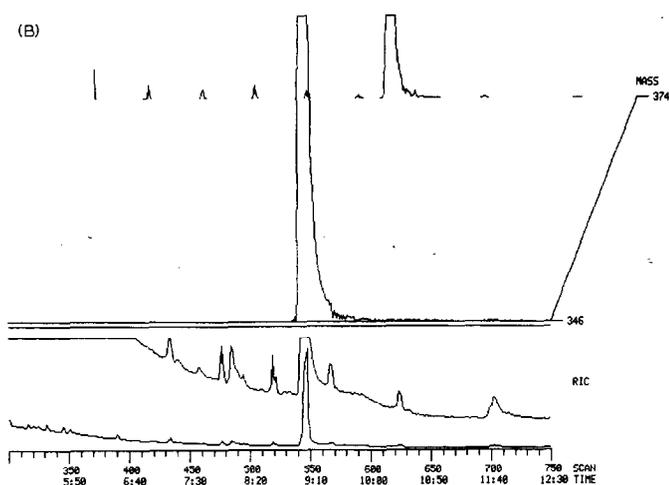


Fig. 2. (A) Highly illustrative plot of the chromatogram of a reference sample of the TFA derivative of pipradol together with the mass chromatograms for two characteristic masses (m/z 346 and 374). Comparison of the CI reference spectrum (B) with the suspected peak (C) clearly confirms the presence of pipradol.

Using Bond-Elut C_{18} columns we do a solid phase extraction at pH 9 and after washing the columns we elute them with 500 μ l methanol. Clean extracts are obtained which can then be converted to trifluoroacetyl (TFA) derivatives. Extracts are screened and confirmed directly by CI mass spectrometry using ammonia as the reagent gas. As the solid phase extraction technique has a lot of advantages over the conventional liquid-liquid extraction as used by most doping control laboratories, we are currently investigating the use of solid phase extraction techniques in stimulant screening as well.

Pemoline screening

Unfortunately, pemoline is a stimulant which cannot be extracted and detected by the described methods. Therefore one can either use a liquid chromatographic technique or oxidate the pemoline as we do. After extraction of the urine with diethyl ether at pH 9.6 the extract is hydrolysed with 1 M hydrochloric acid. The 5-phenyl-oxazolidin-2,4 dione formed is then extracted with diethyl ether and silylated with N-methyl-N-trimethylsilyltrifluoroacetamide-trimethylsilyl chloride (MSTFA-TMSCl) (100:2). Using the selected ion monitoring mode we can determine the trimethylsilyl (TMS)-derivative of the hydrolysis product by gas chromatography-mass spectrometry (GC-MS).

Beta-blockers screening

Beta-blockers consist of two groups of structurally related drugs which can be screened by the same procedures that are used for the stimulants after derivatization. The technique commonly used is selective derivatization with MSTFA and N-methyl-bis-trifluoroacetamide, resulting in a N-TFA-O-TMS derivative of the beta-blocker¹¹.

Screening by GC-MS is then easily carried out by measuring the two base peaks of the two types of beta-blockers. Confirmation with CI mass spectra is always necessary.

Diuretics screening

The diuretics group consists of several different groups of compounds which are extracted from the urine with diethyl ether or ethyl acetate and screened on a liquid chromatograph with gradient elution. Confirmation is by GC-MS after methylation with methyl iodide or by direct insertion of the underivatized high-performance liquid chromatographic (HPLC) fraction via the probe-inlet (off-line)¹². Some efforts have been made to analyze diuretics directly by thermospray LC-MS, but the information contained in such spectra is limited¹³.

Anabolic steroid screening

Great efforts have been made by Donike *et al.*^{14,15} to develop screening procedures for the routine detection of anabolic steroids. The determination of anabolic steroids is very time-consuming. Most steroids are conjugated so after isolation of the steroids from the urine by a XAD-2 column the methanolic eluate is evaporated to dryness and the free steroids separated from the conjugated steroids by extraction with diethyl ether. The conjugated fraction is hydrolyzed overnight at 37 °C with β -glucuronidase. When pressed for time a shorter hydrolysis in 1–3 h at higher temperature can be used.

The two fractions have to be trimethylsilylated individually according to different methods. Both the free and conjugated fraction can then be analyzed by GC-MS. However, because of the very low concentrations of steroids in urine, the selected ion monitoring (SIM) mode has to be used. For this purpose we employ the Hewlett-Packard MSD 5970B, which is capable of measuring different sets of characteristic ions automatically switched in time. We have therefore written an automatic program that will screen for the base peaks of all the anabolics on the IOC list.

Up to now we have not mentioned the metabolites of the forbidden compounds although they are of course very important. In the case of stimulants and narcotics, the concentration of the free drug is usually above the detection limit. However, when a urine sample tests positive, we always will look for the known metabolites of the drugs to confirm its presence. With the anabolic steroids there are often only metabolites present in the urine. Some pharmacological understanding of steroid metabolism is essential in order to be able to interpret the GC-MS results.

Confirmation of anabolic steroids

When a base peak of an anabolic steroid is present in the screening at the right retention time the whole work-up procedure is repeated. A reference urine containing the suspected steroid, a blank urine and the positive urine are all measured again in duplicate. The derivatization step is often adjusted to the specific structure of the suspected drug to get optimal results. The GC-MS analysis is again performed in the SIM mode but this time at least four characteristic masses for that particular steroid are detected (Fig. 3). If possible, more than one metabolite is analyzed to confirm the positive case. The presence and concentration level of the metabolites of testosterone, *e.g.* etiocholanolone and *cis*-androsterone, are important. Low levels indicate that the urine has either been diluted by diuretics or the use of anabolic

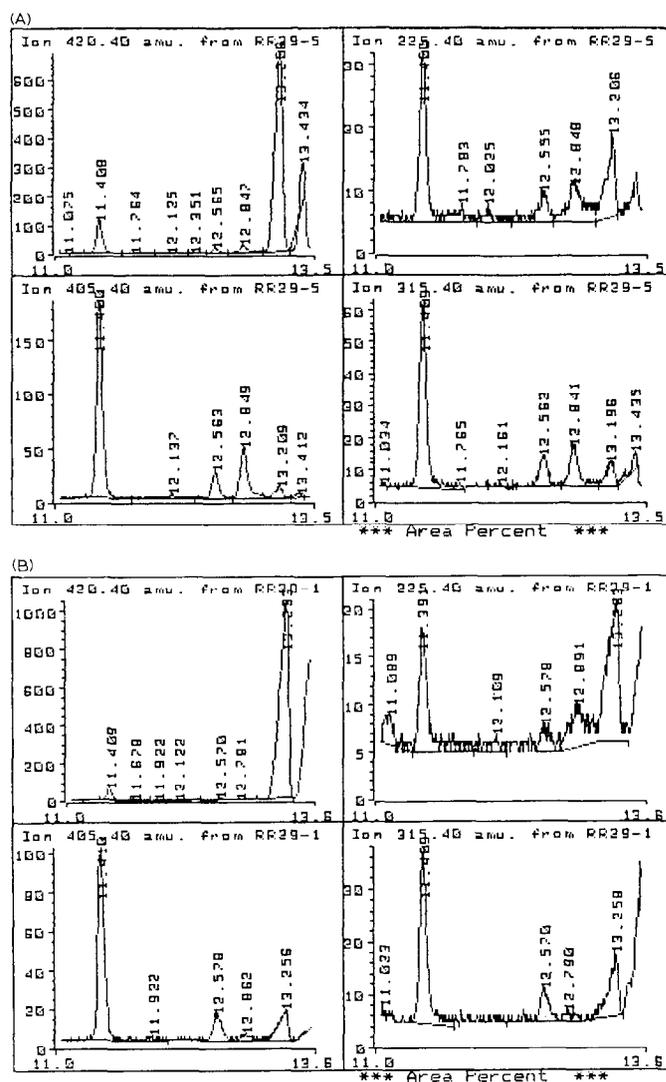


Fig. 3. Anabolic steroids are detected by SIM of four masses as in this example showing nortestosterone. A plot of the four ions 420, 405, 315 and 225 shows nortestosterone at 11.4 min in the reference urine (A). The same ions should be present in the positive urine (B) with the same relative abundances.

steroids has suppressed the endogenous hormones. The use of probenecid will also result in low levels of these metabolites.

Testosterone and hCG

As testosterone is always found in urine, its abuse cannot be indicated just by its presence. Therefore the IOC has implemented a rule based on the presence of the two isomers, testosterone and epitestosterone. In a normal male or female the ratio between those two isomers (T/E) is 0.5–1.5. Any externally administered testosterone will increase this ratio as the level of epitestosterone will remain the same. The IOC has defined a T/E ratio of 6.0 or higher as a positive indication of testosterone abuse¹⁶.

The human chorionic gonadotropin (hCG) is now

banned because it acts on the hypophysis to increase testosterone secretion and hence provokes an indirect anabolic effect.

Quantitative analysis

Quantitative analysis is also performed in doping control. Caffeine is a forbidden drug but only at levels above 12 $\mu\text{g/ml}$. It is very difficult to get above this limit by just drinking coffee or tea. So when high levels of caffeine are found during the stimulant screening we perform a quantitative analysis by HPLC in order to be able to analyze its metabolite theophylline as well.

Only very recently has the IOC commented on a quantitative analysis for ephedrine and codeine¹⁷. Because these compounds are present in many over-the-counter cold prescriptions they always create problems with doping controls. Now the IOC has suggested that only concentrations above 1 $\mu\text{g/ml}$ should be reported and has advised the international sport federations to enforce only a minor sanction for concentrations of 1–10 $\mu\text{g/ml}$.

Quality control and accreditation

All of the listed and structurally related compounds are forbidden according to the IOC. However, as no quantitative levels are acceptable to the IOC the detection limit of a particular instrument and/or technique used often will determine whether or not a urine sample will test positive. Especially when analysing for anabolic steroids the traces in the urine can be very low and the four ions measured in the SIM mode give the only reliable information. Ion abundances in EI spectra always vary but how much variance between the positive urine and the reference urine can we allow and still confirm the sample as positive? No general rules apply. On the basis of experience with a large number of positive nortestosterone cases we have set up a protocol in which relative ion ratios should be within a 15% boundary to identify a sample as positive.

It is essential in such complicated analysis as doping tests, with such far-reaching consequences for the athletes concerned, that the quality control of the doping control laboratories is efficient. Therefore the medical commission of the IOC has set up an accreditation procedure. Of course, the laboratory should work according to Good Laboratory Practice (GLP) regulations and should have the required equipment and qualified personnel. When all this has been checked by the IOC, a test program is performed that involves positive urine samples from all forbidden classes of drugs. This (re)accreditation test should be performed every two years. In this way the quality of the analyses of the now 21 accred-

ited laboratories throughout the world can be controlled.

At the sixth Cologne workshop held in May 1988, new proposals were announced concerning an even more stringent quality control program based on the GLP rules of the National Institute for Drugs of Abuse (NIDA, U.S.A.). If these proposals are accepted by the medical commission of the IOC a proficiency testing program will start in early 1989 with tests every four months¹⁸.

In 1987 the accredited laboratories performed 37 882 analyses, of which 854 (*i.e.* 2.25%) gave positive results. More than 500 cases involved anabolic steroids, mostly nortestosterone, testosterone and metenolone. In 300 cases forbidden stimulants were found, of which 100 involved pseudoephedrine. In 24 cases the masking agent probenecid was found and more than 50 cases of narcotics were identified (mainly codeine and dextropropoxyphene)¹⁹.

MS-MS in doping control

Although the IOC does not yet require the use of GC-MS-MS, we use it to confirm positive samples²⁰. In a normal urine extract there will always be chemical background limiting the detection level of GC-MS. A better sample preparation could improve this, but will probably lead to a low recovery and is time-consuming. With MS-MS the first quadrupole acts as a (very expensive) filter to select the compound of interest. In a collision chamber (also a quadrupole) we generate collisions with argon and use the third quadrupole to measure a mass spectrum. However the mass spectrum from the third quadrupole can only come from the selected ion and not from any background. These so-called CAD (collisionally activated dissociation) spectra are therefore highly specific but at the same time offer better detection limits. Although MS-MS is rather expensive the developments in instrument ion trapping techniques look very hopeful for a cheap MS-MS in the future.

New forms of doping

The task of the doping control laboratory is becoming increasingly difficult, not only because more substances have been added to the list of forbidden compounds but also because more and more endogenous hormones are being used. We have already mentioned the use of hCG which now has been banned and can be tested for using the commercially available pregnancy tests based on monoclonal antibodies. However the interpretation of those results is not easy as cross reactions with luteinizing hormone or other hormones occur and not enough information on 'normal' values is available.

Other hormones such as growth hormone and adrenocorticotrophic hormone are being used as well. When more and more hormones (e.g. certain releasing factors) become available it will be virtually impossible for the laboratories to discriminate between this kind of doping and the natural endogenous hormones.

Blood doping has also been banned by the IOC because of the ethics and the risks involved. However, no reliable testing procedure is available to date, making it impossible to detect this doping method.

Conclusions

The quality control of the analytical procedures to determine the use of forbidden drugs is in principle efficient. However, the communication between the accredited laboratories needs to be improved because the exchange of data and reference material is not optimal.

The use of techniques such as MS-MS and LC-MS is likely to become increasingly important in the next five years. Also GC-Fourier transform IR has been improved considerably over the last couple of years and could well supply the extra structural information on isomeric structures that is often lacking with mass spectrometry.

The use of immunoassay techniques will have to be implemented more and more in screening procedures of the IOC-accredited labs as the use of endogenous hormones and releasing factors becomes more widespread. However, the confirmation of positives will be difficult. Perhaps HPLC-MS can help in certain cases, but this will certainly require more sophisticated and expensive mass spectrometers with higher mass ranges and introduction systems like continuous-flow fast-atom bombardment.

Of course, one can argue about the usefulness of doping control as more than 20 years of doping control has not solved the problem. We believe that supplying information, especially to the young athletes, should have a high priority but that the controls should continue. In a recent enquiry²¹ nearly 50% of the athletes interviewed responded that they would take a drug that could make them an Olympic champion even if it had lethal side-effects within five years. With this attitude it is obvious that controlling the abuse of drugs is still necessary.

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