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Review

Introduction to the application of capillary gas chromatography of performance-enhancing drugs in doping control

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Abstract

Performance-enhancing drugs banned by antidoping rules are detected in doping control preferably by hyphenated chromatographic techniques, capillary gas chromatography in particular. Based on the prohibited classes of substances and on the general aspects of sample collection and preparation, a survey is given about the usual procedures of screening, identification and confirmation of the most important doping agents: stimulants, narcotics, anabolics, diuretics, β-blockers. In addition to gas chromatography itself, the application of various MS techniques doping is outlined.

Keywords: Doping control; Reviews

Contents

1. Introduction

While performance enhancement is the main aim of doping, the misuse of drugs in sports extends farther to other drug classes with indirect influence onto the ability to compete in sports. As soon as substances are banned by the according rules, doping analysis has to be able to detect them in the control samples taken from athletes.

Performance-enhancing drugs in a more narrow sense – central stimulants like amphetamine and cocaine – are the most classical doping agents, which have been used and have caused health damages and fatalities decades earlier than the ‘doping classes of today’ – anabolics and peptide hormones.

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When doping became forbidden and doping substances have been banned after the midst of the 20th century, central stimulants have been the first target. Attempts to control them started with thin-layer chromatography, soon followed by (packed column) gas chromatography.

The extent of banned doping classes and compounds (present state see Table 1 [1,2]) as well as the analytical possibilities and requirements made fast progresses, and the instrumentation increased considerably in parallel.

Doping analysis on an international level is confined to a relatively small number of laboratories (at present 26) accredited by the International Olympic Committee (I.O.C.), which are in permanent close connection with regard to the analytical strategies, methods, quality assurance and new developments. The present paper is therefore intended to give an introductory survey to the application of capillary gas chromatography rather than an exhaustive literature review. Comprehensive papers on several aspects of GC application in doping analysis could provide more detailed information [3–7].

The official doping definition of the I.O.C., adopted by most international and national sports associations, defines only *classes* of prohibited substances. The classes include synthetic as well as natural compounds, some of the latter being physiological body constituents (creating additional problems according to the evaluation of analytical results). The lists of examples to each prohibited group are by definition incomplete, always followed by the term '...and related substances', so that the analytical strategy has to include many more compounds than the listed ones. This the more, because the biotransformation products have to be reliably detected for the majority of doping agents due to two facts: at first, most relevant compounds are excreted in urine preferably or even only in metabolised form, and at second the presence of metabolites excludes the possibility that the presence of an unchanged agent in the sample could have been caused by sample manipulation.

Table 1
Banned doping classes and compounds

(I) Prohibited classes of substances:
A Stimulants
B Narcotics
C Anabolic agents
D Diuretics
E Peptide hormones
(II) Prohibited methods: Blood doping, manipulation of samples
(III) Classes of drugs subject to certain restrictions: Alcohol, cannabinoids, local anesthetics, corticosteroids, β-blockers

Doping analysis has to rely (almost) exclusively on urine samples, which can be collected non-invasive in sufficient amounts and provide an even longer detectability of many substances than blood. However, from a pharmacological point of view, urine concentrations are less conclusive than blood with regard to concentrations. Blood sampling by venous puncture is not generally applicable due to legal impediments (compared to blood samples for medical reasons or e.g. for blood alcohol determinations). The urine sample is divided to two aliquots – 'A' and 'B' samples – to be transported in sealed, anonymously coded containers to the laboratory.

2. General procedure

The analysis for performance enhancing drugs starts with a pre-analysis procedure to check the integrity, code, seal and visual appearance (colour, turbidity, sediment) of a sample. Density and pH are determined, followed by registration (chain of custody) and aliquotation for the screening procedures.

In principle, doping analyses are performed in two steps: (i) screening by standard operation procedures and (ii) confirmation of positive screening results. Examples of common screening procedures are outlined below (see Table 2).

The confirmation is generally done by MS hyphenated methods (GC or HPLC coupled with MS). The peptide hormones such as hCG (human chorion-gonadotropin), erythropoietin, human growth hormone are analysed by immunometric methods.

3. Sample preparation

In general, gas chromatographic methods imply several steps: sample preparation, sample introduction (injection), chromatographic separation, and

Table 2
Screening procedure

(a) Screening for volatile nitrogen containing compounds (stimulants and narcotics excreted free)
Liquid–liquid extraction
cGC–NPD
(b) Screening for slightly volatile compounds and conjugates (stimulants, narcotics, β-blockers)
Acidic and/or enzymatic hydrolysis
Liquid–liquid extraction
Derivatisation (silylation)
cGC–MS (SIM mode) or cGC–NPD
(c) (Screening HPLC for thermolabile compounds, not accessible for GC separation even after derivatisation)
(d) Screening for anabolic agents (free and conjugated fraction)
SPE (XAD-2, C_{18} etc.)
Enzymatic hydrolysis (β-glucuronidase and/or aryl sulfatase)
Liquid–liquid extraction
Derivatisation (silylation)
cGC–MS (low resolution-SIM mode)
cGC–high-resolution MS (resolution 10000-SIR mode) or cGC–MS–MS
(e) Screening for diuretics
Liquid–liquid extraction
Derivatisation (methylation)
cGC–MS (SIM mode)
(f) (Screening for peptide hormones by immunoassays)

detection. The first step is a time consuming part of the analysis prone to failures or disturbances. The quality of the results depend to a large extend on this part of the procedure.

The isolation or concentration, respectively, for the screening procedures of doping analysis are carried out by liquid–liquid extraction and/or solid-phase extraction (SPE). More recently developed techniques, such as solid-phase microextraction (SPME), are not yet included in the routine procedures, even though they were tested for some typical doping agents [8–10].

On the other hand, immunoaffinity chromatography (IAC) was tested for sample preparation in doping analysis to determine steroids [11,12] and β-agonists [13–15]. Moreover, automated sample preparation and analysis – e.g. by means of a Hewlett-Packard PrepStation [16] – was described.

Conjugated metabolites have to be hydrolysed prior to gas chromatography or prior to derivatisation [17].

Derivatisation is a prerequisite for GC separation of the majority of doping agents. Silylation and methylation are the most important techniques for the according substances [5].

4. Gas chromatographic analysis of doping agents

Gas chromatography – especially capillary gas chromatography (cGC), which has replaced the packed-column alternative completely also in doping laboratories – soon became a leading principle of doping analysis. It is used as a stand-alone method as well as coupled with mass spectrometry. Combined instruments use several mass spectrometric techniques: quadrupol low resolution full-scan and selected ion monitoring (SIM), ion-trap, magnetic high-resolution and tandem MS with electron impact and chemical ionisation.

4.1. Anabolics

Anabolic agents, the anabolic androgenic steroids and β-agonists (antiasthmatic drugs with anabolic and stimulation side-effects) are included in the list of banned substances and still leading the statistics of positive doping cases.

Testosterone – the male sexual hormone – as the physiological model of the synthetic anabolic steroids requires special precaution. It is an always present physiological compound, varying considerably in its concentrations in urine (and also in blood), with generally higher levels in men, lower ones in women. To recognize potential administrations, the varying concentrations are normalized by determining the concentration ratio between testosterone and its epimer epitestosterone (T/E). Being the natural accompanyon of the testosterone biosynthesis, epitestosterone is physiologically inactive [18,19]. According to the I.O.C. rules, a T/E ratio above 6 is considered suspicious, leading to further investigation of the cause of this elevated value (normal range around 1). Therefore, the quantitation of both T and E in low concentrations is essential in doping analysis (Fig. 1).

The inclusion of further compounds (precursors, accompanying compounds, metabolites, 'steroid profiling') besides testosterone is an additional part of the analytical strategy in doping control for the detection of testosterone misuse [20–24].

Recently, the use of isotope ratio mass spectrometry (IRMS) after chromatographic separation was proposed to distinguish external sources of testosterone from endogenously increased concentrations or T/E ratios by the $^{12}C/^{13}C$ ratio.

The isolation of free and conjugated compounds of this class is usually carried out using SPE and liquid–liquid extraction with or without hydrolysis of the conjugates (glucuroneconjugated and sulfoconjugated metabolites) [25–31]. Liquid–liquid extraction after direct hydrolysis in the urine sample for anabolic steroids is described by Ref. [32]. SPE using ion-exchange functional groups for β-agonists is proposed by Refs. [33–35]. Anabolic agents and their metabolites are usually transformed to trimethylsilyl (TMS) derivatives – *O*-TMS or in some instances, *N*-TMS derivatives – before the gas chromatographic separation [3,36–40]. The aims are improvement of their chromatographic behaviour and more characteristic mass fragments in higher mass ranges with lower biological background for MS detection.

N - Methyl - *N* - trimethylsilylfluoroacetamide (MSTFA) is the most important reagent, applied in combination with catalysts such as potassium acetate [41], or trimethylsilylimidazole (TSMIm) [42], trimethylsilyl iodide (TMSI) [43] or NH_4I with reduction agent [25,27,28,44]. The formation of cyclic derivatives was suggested for β-agonists [33,45].

MS detection in the SIM mode [3] is the preferred technique in analysis for anabolic agents, especially for the anabolic compounds, because it provides better detection limits.

However, with respect to the required very low detection limits ($\leq$ 1 ng/ml urine) and the ubiquitary presence of very similar background compounds, detection principles like high resolution MS or tandem MS are increasingly important prerequisites in doping analysis. (This applies the more for special cases – in particular in connection with autopsies after death of high-performance athletes and for hair analyses with the expectation to detect long-term use of doping agents in this matrix [46]).

Fig. 2 demonstrates that the identification of 3′OH-stanozolol-tris-TMS ($<$5 ng/ml in a urine extract) by low-resolution MS failed due to the high amount of background. If the specificity of detection is increased by high-resolution MS (R=10 000) and tandem MS, the detection limit is much improved.

On the other hand, quantitation – e.g. for the metabolites of testosterone androsterone and etiocholanolone – was described using GC-flame ionisation detection (FID) systems equipped with comparatively short columns. Because of the instability of some steroids, it is indispensable to use sufficiently silanised inserts, which have to be regularly changed in order to minimise the influence of active sites [3,47,48].

4.2. Stimulants, narcotics and β-blockers

From the analytical point of view stimulants, narcotics and β-blockers can be grouped together because of similar analytical behaviour. Most of these substances are nitrogen containing bases, excreted in urine as free or conjugated compounds

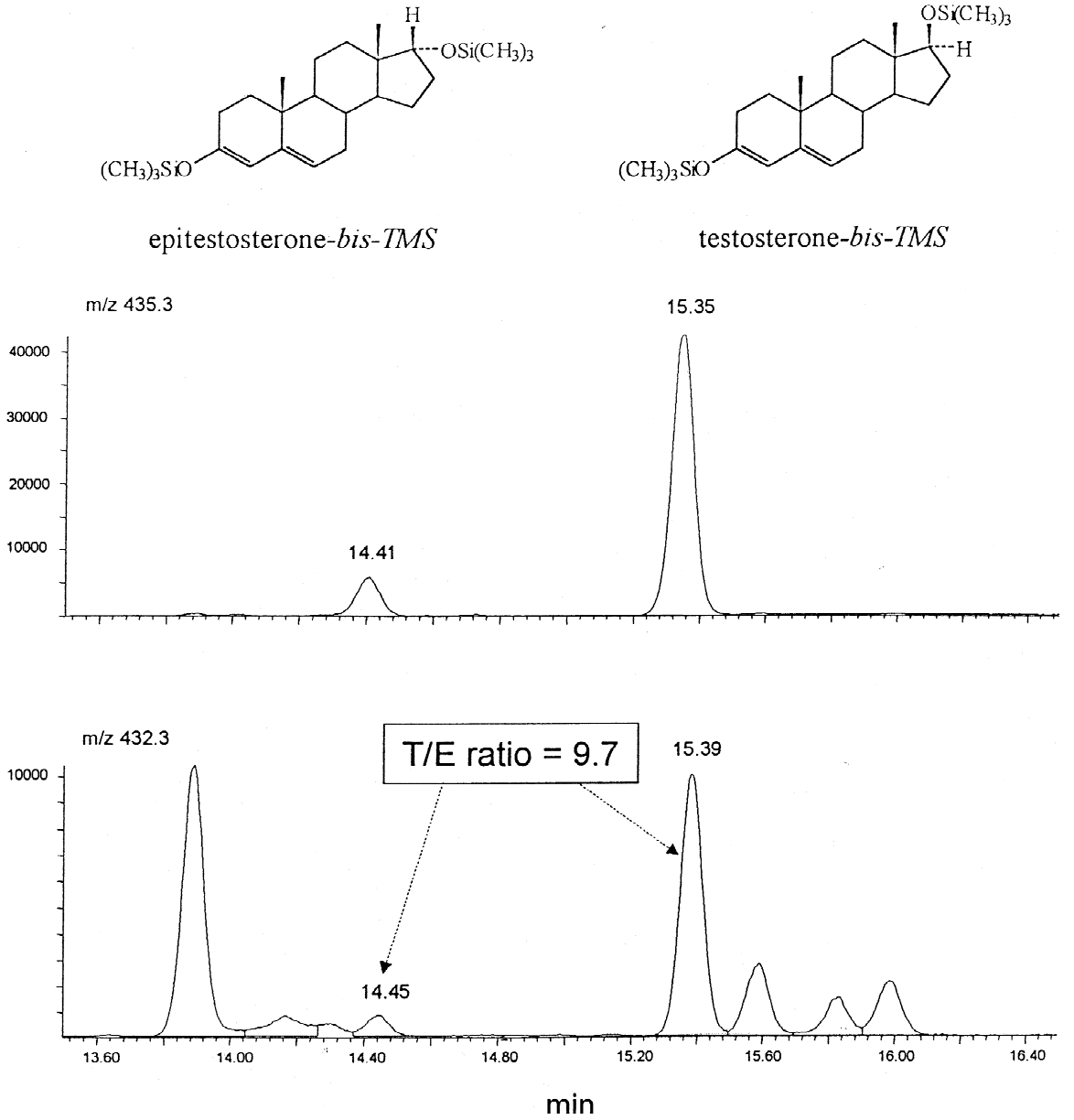

Fig. 1. Determination of the T/E ratio (after enzymatic hydrolysis of conjugates and derivatisation) by GC–MS. The chromatogram of the molecular ion m/z 432.2 shows the signals of testosterone-bis-TMS (t_R 15.39 min) and epitestosterone-bis-TMS (t_R 14.45 min) representing concentrations of 11.6 and 1.2 ng/ml, respectively. The corresponding threefold deuterated internal standards are monitored at m/z 435.3 [($^2H_3$]testosterone at t_R 15.35 min and [$^2H_3$]epitestosterone at t_R 14.41 min).

simultaneously with their metabolites. While for the majority of doping agents their mere presence in a urine sample constitutes an offence according to the I.O.C. doping definition, the quantitative determination of several compounds is necessary with respect to conventionally set thresholds, up to them those agents are not prohibited. In these cases, it is considered necessary to distinguish between prohi-

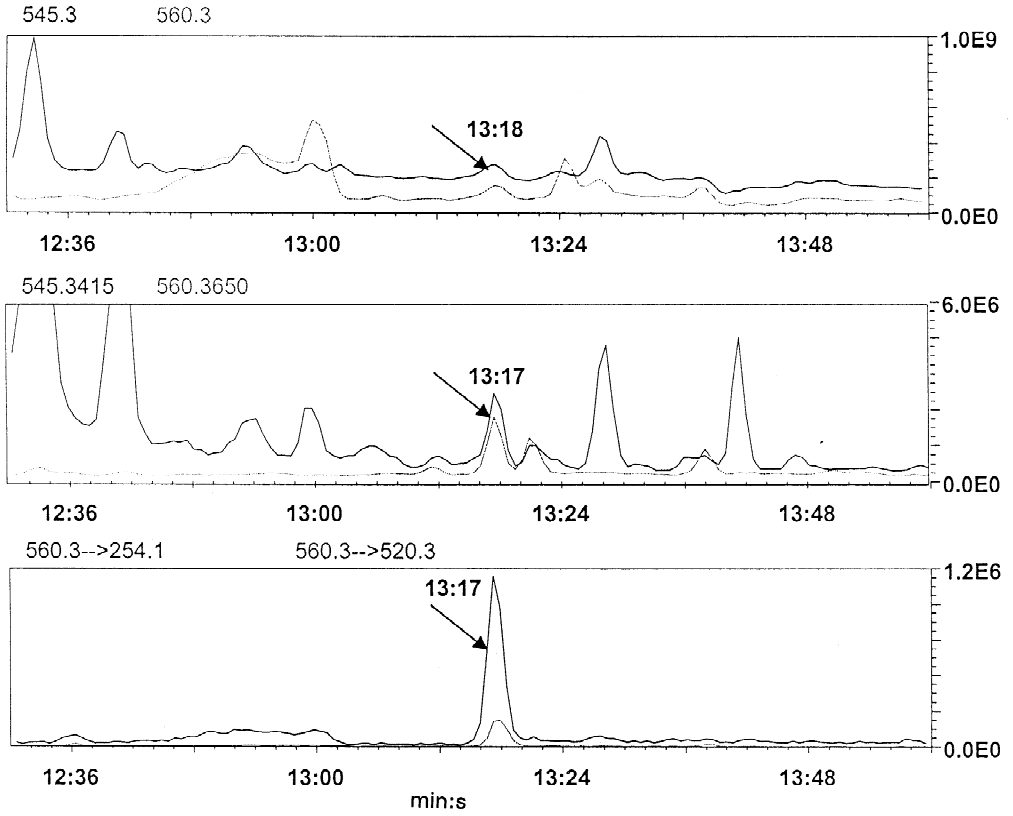

Fig. 2. Identification of 3′OH-stanozolol-tris-TMS (<5 ng/ml in a urine extract). Low-resolution MS (*above*): interference of background; high-resolution R=10 000 (*middle*): increased signal–noise ratio; tandem MS (*below*): considerably improved detection. All other conditions (extraction, GC separation) remained unchanged.

bited intake and therapeutic or normal use, respectively. One of the most important examples is the almost ubiquitary caffeine (permitted threshold 12 mg/l urine), others are ephedrine and cathine(5 mg/l) pseudoephedrine and phenylpropanolamine (10 μg/ml) and morphine (1 μg/ml). Furthermore, a few compounds – such as methamphetamine or levorphanol – require the determination of enantiomers for a clear evaluation of the results [49–52].

The usual procedure for the determination of stimulants, narcotics and β-blockers is a combination of two analytical methods, as already shown in Table 2. The unconjugated substances and the free metabolites are isolated by liquid–liquid extraction and analysed usually without derivatisation [53–56]. Some papers suggest slightly changed variations [57–59]. Identification and quantification of the nitrogen containing compounds can be carried out properly by cGC with nitrogen–phosphorus detection (NPD) – even by injecting the sample into two capillary columns [60–62], but MS detection is also frequently used.

The determination of conjugated compounds usually starts with enzymatic or acidic hydrolysis followed by liquid–liquid extraction, derivatisation (silylation) and GC–MS [59,63,64]. Moreover, SPE-based extraction procedures [65–67] are described for narcotics [68–71], stimulants [72–74] and β-blockers [33,75–77] as well as comprehensive procedures for the whole group. Ionic-exchange SPE materials can be used due to the properties of the hydrolysed analytes [78].

As already mentioned, some of (closely related) compounds coelute and remain insufficiently separated until they have been adequately derivatised [5,79–81]. This is demonstrated by Fig. 3 for the

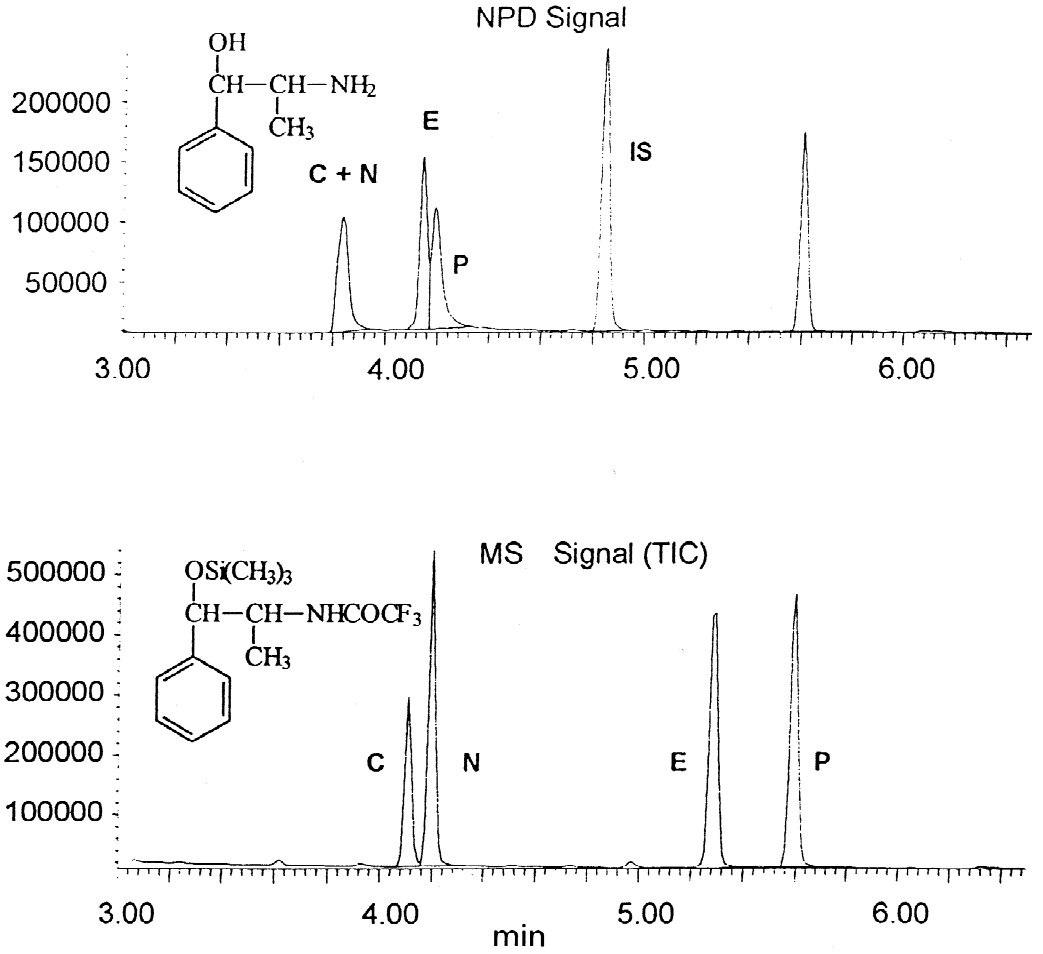

Fig. 3. GC separation of the diastereomers cathine (C) and norephedrine (N) and ephedrine (E) and pseudoephedrine (P). (*Top*) Insufficient separation of unchanged compounds, (*bottom*) improved separation after selective derivatisation with MSTFA-MBTFA.

diastereomeric stimulants cathine and norephedrine (derivatisation by MSTFA-MBTFA *N*-methyl-bis-trifluoroacetamide) [82].

4.3. Diuretics

The group of diuretics – prohibited with respect of possible forced excretion of other doping agents with shortened time of detectability and due to the possibility of quick mass reduction in sports with weight classification – comprises numerous substances, differing in their chemical and physicochemical properties. The vast majority of these compounds were excreted unchanged in urine and the analytical methods are, as a rule, focused onto the parent compounds. Normally, diuretics cannot be analysed by cGC without derivatisation procedures, because most of the substances contain polar functional groups. LC-based methods were therefore frequently described and – compared to the other doping agents – used more in the analysis of diuretics.

The isolation of these group pf analytes can be carried out using liquid–liquid extraction, but different pH values are required to extract basic, acidic and neutral diuretics with good recoveries [83–87]. Extraction at pH 9.5 was suggested as an acceptable

compromise, supported by the salting-out effect [88]. Moreover, SPE procedures were reported by means of C_{18} [87,89] and XAD-2 cartridges [84,89].

In the case of cGC–MS-based methods methylation [3,7] is the favourite derivatisation technique, because the silyl derivatives – especially of sulfonamides – are quite unstable [84]. There are several kinds of methylation procedures using methyl iodide, either after extractive methylation [90–93] with phase-transfer reagents (i.e. tetrahexylammonium salts) or by reaction in dry acetone [84,87,94,95]. In addition, pyrolytic methylation [96,97] in the hot inlet of the GC with quaternary ammonium hydroxides – i.e. trimethylphenylammonium hydroxide or tetramethylammonium hydroxide – is used.

A furosemide positive urine result obtained by cGC–MS after methylation is given as an example in Fig. 4. The total ion current of the MS detector shows no significant signal for trismethyl furosemide, only the extracted ion profiles of characteristic ions are able to show the presence of the diuretic furosemide.

5. Conclusions

cGC is the most important technique of doping

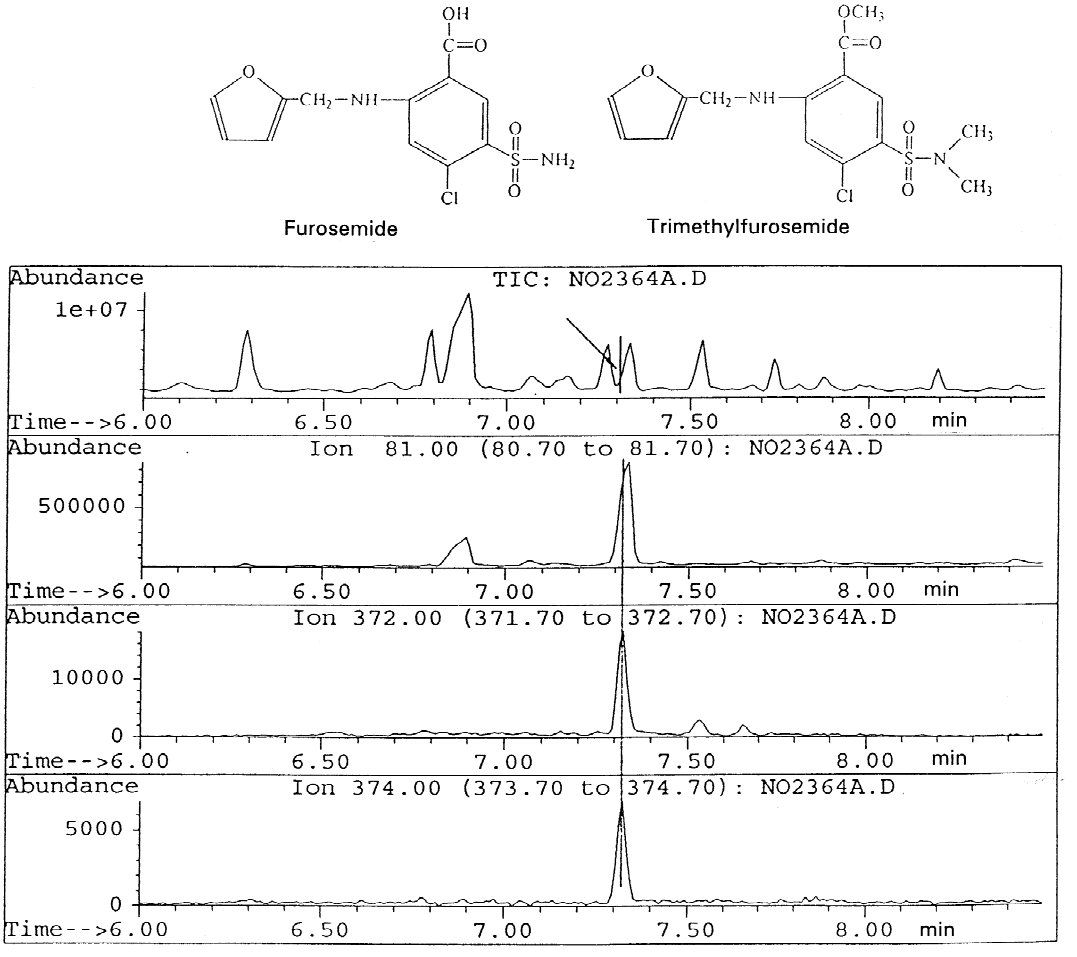

Fig. 4. Detection of furosemide in urine by GC. Total ion current (TIC) (*above*): no signal of the unchanged compound, trismethyl furosemide not detectable in the total ion current chromatogram; selected ion chromatograms 81, 372, 374 (*below*): detection of the characteristic masses of trimethylfurosemide.

analysis. The majority of doping substances – especially the anabolic steroids and their metabolites – require the combination cGC–MS, but many of the more 'classic' doping substances (stimulants, also narcotics) are analysed by GC–NPD. Supplemented by HPLC and additional principles like immunoanalysis, those methods are now constituting the compulsory fundamental equipment of (I.O.C. accredited) antidoping laboratories.

Nevertheless, cGC remains valuable not only in the screening for the mentioned compounds, but also by its potential to primarily detect even substances outside the scope of known doping agents. During the recent years, a number of hitherto unknown compounds could be detected at first by GC as unusual peaks, subsequently identified by the 'whole analytic arsenal' of GC–MS combinations, e.g. Piracetam [98,99] Bromantan, Carphedon [100].

Obviously, some trends in cGC, such as fast GC using narrow bore capillaries or sample introduction techniques like large volume injection [101] are not yet routinely applied in doping analysis so far. In the future, isotope ratio MS in combination with chromatographic separations is expected to become an additional instrumental technique, aiming to the recognition of extracorporal sources of physiological compounds like testosterone and its accompanyons (see above) [102–105]. Similarly, the application of LC–MS will provide additional identification power also for thermolabile and hydrophilic compounds.

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