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Journal of Chromatography B, 687 (1996) 221–238

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Stimulants, narcotics and β-blockers: 25 years of development in analytical techniques for doping control

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Abstract

More than 25 years of developing doping control methods have led to comprehensive screening and confirmation procedures for stimulants, narcotics and β-blockers. Much of this work has been initiated and/or improved by the late Prof. Dr. Manfred Donike. The methodological approach covered in this overview was applied to doping control procedures during the XXV Summer Olympics in Barcelona, Spain, in 1992 and the XVII Winter Olympics in Lillehammer, Norway, in 1994. Urine samples are screened through a combination of two analytical methods that are complementary: (a) gas chromatographic analysis of the parent compound and unconjugated metabolites, following single-step sample extraction and detection by a nitrogen-specific detector based on a retention index identification system and (b) gas chromatographic analysis including also conjugated drugs and metabolites after hydrolysis, solid-phase extraction, derivatisation and mass spectrometric detection. Confirmation and identification is always performed by gas chromatographic separation and full scan mass spectrometric detection. These methods facilitate the rapid screening and confirmation of more than 100 stimulants, narcotic analgesics and β-blockers in urine for at least 24 h after the intake of a pharmaceutical dose. Application of the methods ensures high quality standards for the unequivocal identification of doping agents as well as a rapid turnaround time for sample analyses.

Keywords: Stimulants; β-Blockers; Narcotics

Contents

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PII S0378-4347(96)00276-9

1. Introduction

In the sense of using performance-enhancers based on pharmacological substances (drugs, plant extracts), doping [1] may be as old as competitive sports itself. Doping analyses [2] have been used for doping control in a variety of sports for some 25 years now. During the sixties stimulants of the amphetamine type were the favorite drugs used to increase sports performance. The dramatic increase in the use of amphetamines can be explained by the fact that, during World War II, the amphetamines were administered to soldiers to increase alertness and performance. Thus, the 'benefits' of amphetamines became known to a larger population. In this way, amphetamines were introduced into sports. Sports organisations became concerned for ethical and medical reasons. Not only amphetamines, but also caffeine, respiratory stimulants like nicethamide and crotetamide, cropropamid (Micoren) and narcotic analgesics like morphine and pethidine were used in the form of 'cocktails' [3]. Several sudden deaths occurred during competitions due to amphetamine use [4,5].

The International Olympic Committee's (IOC) Medical Commission [4] was established in 1967 and subsequently stimulants and narcotic analgesics were prohibited throughout most of the international sports federations. The IOC's definition of doping stating that "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," [6] (see Table 1) is today widely accepted. The IOC list of banned substances and methods has been modified and enlarged several times. Anabolic steroids (1976), blood doping (1988), masking agents (1988), β-blockers (1988) (later changed to a class subject to certain restrictions), diuretics (1988), protein hormones (1992) and other anabolic agents (1993) were subsequently also prohibited.

There had been some attempts made by the mid-sixties to develop analytical procedures for the routine detection of stimulants by combining gas chromatography (GC–FID), thin-layer chromatography (TLC) and gas chromatography coupled to mass spectrometry (GC–MS) [7,8]. Nevertheless, the first doping control which really earns the designation 'comprehensive' was performed in 1972 at the XX Summer Olympics in Munich with Manfred Donike as head of the laboratory. Due to the development in analytical techniques and especially the introduction of a long-term stable nitrogen specific detector (N–FID), it was possible to detect

Table 1
IOC Medical Commission: 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. β-Blockers

the amphetamine and other nitrogen-containing drugs effectively. In addition, the application of novel derivatisation reagents such as N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and N-methyl-bis-trifluoroacetamide (MBTFA) [9] for GC and GC–MS analysis developed by Manfred Donike contributed to the success of this analytical approach.

The analytical challenge facing laboratories is to prove the intake of forbidden substances by testing samples of body fluids. To some extent, the pharmacological behavior and metabolism of substances reflect similarities in their chemical structures. Accordingly, the differences between various classes of compounds have formed the historical basis for their classification from an analytical point of view. This explains why stimulants, narcotics and β-blockers (class of drugs subject to certain restrictions) were grouped together in this overview. Nevertheless, a unified screening method has always been one of the chemists' primary objectives [10].

The annually collected statistics for all IOC-accredited laboratories shows for 1994 that 24% of all identified prohibited substances belonged to the class stimulants, while only 2.9% and 1.0% belonged to narcotics and β-blockers (only screened for in certain sports), respectively. The decreasing percentage of positive samples with stimulants reflects the increased efficiency of doping control, especially because of the stimulants' pharmacological characteristics (i.e. short plasma half-life and the need to be administered very close to the competition).

The present overview will be focused on the doping control of stimulants, narcotics, and β-blockers. These substances share several characteristics: (a) they can be excreted into urine as free drug or conjugated as glucuronides or sulphates; (b) they are basic (pK_a 7–10), mainly because they are nitrogen containing compounds, and volatile (molar mass <350 g/mol and amenable to gas chromatography); (c) if there is a poor chromatographic performance, almost all these substances have functional groups that can be derivatised to enhance their gas chromatographic properties; (d) their pharmacological effects are produced by relatively high doses of the drug. The observation of these similarities has suggested the possibility of screening all of these substances simultaneously in a combined analytical procedure.

2. General considerations

Several general doping control rules must be taken into account to gain a fuller understanding of the design of the analytical methods. As of today, urine is the main biological fluid available for doping control. This may be justifiable for ethical reasons as its collection is less invasive than the taking of blood samples. Many other aspects will have to be discussed before blood sample taking might be included [11]. Moreover, urine volumes are sufficiently high to accommodate the menu of substances to be controlled. Additionally, the concentration of most xenobiotics and their metabolites is greater in urine than in blood. In other words, as long as the analytical work aims at proofing the intake of prohibited exogenous substances, urine is an appropriate choice.

However, the lack of a clear relationship between pharmacological effects and concentrations in urine restricts the possibilities for the interpretation of results from a therapeutic perspective. When reporting positive cases, laboratories should provide as much analytical and pharmacological (mainly metabolic) information as possible.

From an analytical perspective, the use of chromatography (mainly gas chromatography and liquid chromatography) coupled with mass spectrometry for the final identification of suspected compounds is mandatory. Any additional analytical information helpful in results interpretation is always welcome (i.e. chiral separation of enantiomers).

As some screening methods compromise by trading off different analytical conditions in order to detect as many compounds as possible, the optimal detection of a specific compound is not always possible.

Several appropriate analytical methods have been developed to verify results by alternative reactions [12,13]. Information from immunological tests designed specifically for the group of substances in question (stimulants, narcotics and β-blockers) is also of interest [14,15].

From a pharmacological perspective, whenever possible, the parent compound and metabolite(s) should be identified to avoid possible sample tampering discussions (e.g., allegations about spiking samples with controlled substances). The identification of

metabolites demonstrates drug biotransformation in the body. From that perspective, the analytical approach described below (one method for unaltered drugs and another for phase I and phase II metabolites) provides complementary information about many controlled substances.

2.1. Qualitative versus quantitative analyses

Substances prohibited by the IOC's Medical Commission must not be present in tested urines. This is the main criteria underlying doping control. Due to the lack of cut-off concentrations for reporting positive results, laboratories report the presence of banned substances on a qualitative basis. As doping controls must routinely cope with several dozen substances and report results within 24 h, analytical procedures do not usually include quantification of the screened substances. The length of the list of controlled substances and the time pressures involved in reporting the results are the main differences that distinguish doping control from related fields such as the forensic sciences or drug abuse testing, both of which report quantitative results but deal with shorter menus, definite quantitative limits and/or more lengthy delays in reporting results. Quantitative guidelines have been developed for reporting results on some stimulants (caffeine (12 μg/ml), pseudoephedrine and phenylpropanolamine (10 μg/ml), cathine and ephedrine (5 μg/ml) and the morphine derivatives but it is not always easy to make a distinction [16] between social or therapeutic use and the misuse or abuse of substances. Quantitative analyses of the morphine derivatives are necessary in order to differentiate between the intake of the prohibited compounds such as morphine and ethylmorphine [17], and the accepted therapeutic use of codeine [18] or pholcodine.

2.2. Chirality

Doping control is occasionally concerned with the interpretation of results associated with the chirality of the substances detected. Most substances are administered/misused as racemates, and the list of prohibited substances does not differentiate among different enantiomers. Nevertheless, in the case of methamphetamine, in which the *R*-enantiomer is present in some over-the-counter cold medications with little activity at the CNS level or can be produced as a metabolite from the anti-Parkinsonian medication, selegiline [19], a report of a positive case should include a differentiation between the enantiomers. This subject has been extensively covered in the reference literature and does not represent a major analytical problem [20–23]. Gas chromatographic separations are mainly based on derivatisation with chiralic reagents such as (*S*)-N-(trifluoroacetyl)prolylchloride, producing diastereomers [24], or they depend on the use of chiral columns [23].

Another example is dextromethorphan, an antitussive agent which is not prohibited, whereas levorphanol, the enantiomer of the dextromethorphan metabolite dextrorphan [25], is banned as a narcotic analgesic. In this case, enantiomeric separation might be required. This could also be performed by capillary zone electrophoresis [26].

2.3. Pharmacology and its relevance for doping analysis

Controlling for stimulants, narcotics and β-blockers means checking for substances assumed to be taken in connection with a competition. To show physiological effects of a performance-enhancing nature in connection with stimulants or β-blockers, or an analgesic effect in the case of narcotic substances, the serum concentration of the drug in question has to be considerable, i.e., it must be in the 'therapeutic' range at the time of the competition. When samples are taken from the athletes within a couple of hours of the competition, we can expect the compounds and/or metabolites to show an excretory pattern equivalent to that demonstrated 4–12 h after drug administration. Based on a normal urine flow and pH, this would result in a urine concentration well above the detection limit, in most cases in excess of 500 ng/ml (1–2 mmol/l) [27]. As a general rule and in the qualitative analytical context of doping control, laboratories validate their analytical procedures analysing control urine samples (collection period 24–72 h) collected from healthy volunteers who have been given a single therapeutic dose of one of the substances on the IOC list of banned substances in the stimulants, narcotics and β-blockers doping classes.

As stated above, most of the banned parent compounds classified as stimulants, narcotics and β-blockers show chemical similarities which make them suitable for a combined analytical approach: They contain nitrogen (in the amine function) as a heteroatom, which imbues them with similar basic properties. This makes extraction under basic conditions and selective nitrogen detection useful. Table 2 shows the structure of some sample compounds, accompanied by their pK_a values.

The pharmacokinetics of stimulants [28], narcotics and β-blockers [29] show extensive phase 1 and phase 2 metabolism for most of the substances (see Table 3 for some typical examples). Additionally, considerable amounts of unconjugated drug are usually excreted in the urine. Nevertheless, recovery of the parent drug may vary as it is urinary flow- and pH-dependent [30–32].

Attention should be also devoted to interpreting metabolic data. Drugs such as the anti-Parkinsonian, seligiline [33] or the analgesic, famprofazone [34] metabolize into methamphetamine and amphetamine. Amphetamine has been reported in rats as metabolite of mesocarb (not yet found in humans) [35]. Mephentermine can be produced by chemical hydrolysis or the metabolism of local anaesthetic, oxethazaine [36]. Several esters and amphetamine derivatives, such as mefenorex, fenproporex or fenetylline, metabolize into amphetamine [37]). Analytical conditions can sometimes artifactually generate metabolites (e.g., cocaine metabolic profile of urines after cocaine ingestion [38]) or modify their normal concentrations (e.g. morazone and its metabolite phenmetrazine [39]).

As regards narcotics, codeine, pholcodine or ethylmorphine metabolize into morphine. The same finding are shown after the ingestion of poppy seeds or antidiarrheal medications containing natural opiates, i.e., the interpretation of metabolic data is of relevance [40,41].

The analytical approach favoured today takes phase I and phase II metabolic reactions into account by applying two different methods: one for the lipophilic parent compounds and one for more polar phase 1 and phase 2 metabolites. Conjugation, as well as differences in basic properties and gas

Table 2
Chemical structure and pK_a values of examples of stimulants, narcotics and β-blockers

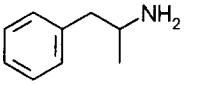
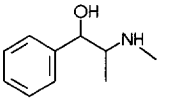
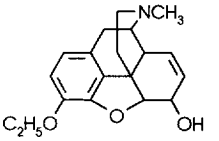
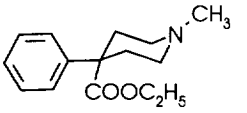
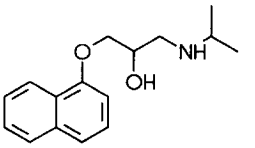
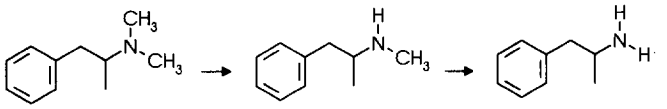
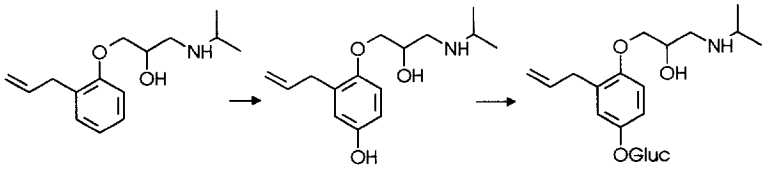
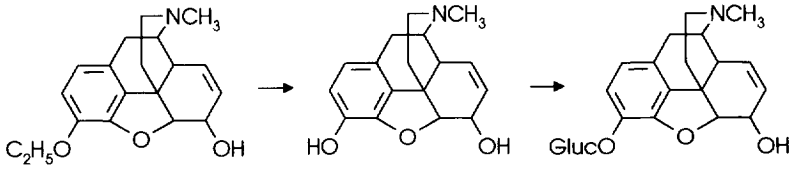
Compound	Structure	pK_a
Amfetamine		9.9
Ephedrine		9.6
Ethylmorphine		8.2
Pethidine		8.7
Propranolol		9.5

Table 3
General metabolic pathways of stimulants, narcotics and β-blockers

Compound	Phase I and phase II metabolism
Dimetamfetamine	 N-Dealkylation
Alprenolol	 Hydroxylation Glucuronidation
Ethylmorphine	 O-Dealkylation Glucuronidation

chromatographic behaviour, led to the development of doping analyses for these kinds of compounds in respect of hydrolysis, extraction and derivatisation.

3. Analysis of unconjugated parent compounds and metabolites in urine

The analytical method (described below) used to screen for unconjugated, nitrogen-containing compounds during the XVII Olympic Winter Games in Lillehammer 1994, was largely based on principles laid down more than 25 years ago by M. Donike [42,43] and A.H. Beckett et al. [7]. The method incorporated minor modifications in sample preparation and was adapted to employ state-of-the-art chromatographic separation and identification techniques.

In the 1960s [44,45] advances in gas chromatography clearly enhanced the potential usefulness of this technique in connection with comprehensive screening for basic drugs. Following the introduction of a nitrogen specific detector [46] for this purpose, the first comprehensive screening of large numbers of samples was undertaken during the XX Olympic Summer Games in Munich in 1972 [47]. The method essentially involved (1) single step extraction, and (2) temperature-programmed gas chromatography, nitrogen-specific detection and the use of an internal standard [48].

The method was further developed and improved [49], then used at major international championships [50] and other Olympic Games [51,52]. Descriptions of several similar gas chromatographic methods have been published for clinical and forensic purposes [53–57]. Although this overview mainly concentrates on gas chromatography, several other chromatographic techniques such as high-performance liquid chromatography (HPLC) [58–61], supercritical fluid chromatography (SFC) [62], capillary electrophoresis (CE) [63–66] and micellar electrokinetic chromatography [67,68] have been used to detect basic drugs in body fluids.

Using gas chromatography to screen for banned substances enables us to detect many compounds of interest in a single test run. It also allows us to identify illegal substances by mass spectrometric detection [69], either in the same run or after further concentration. Comprehensive mass spectra libraries have been published [70] for underivatised drugs,

facilitating drug identification thanks to their availability as commercial software packages [71] integrated into computerized data evaluations [72]. The reference literature also provides comprehensive pharmacological and chromatographic information [73].

3.1. Experimental procedure

3.1.1. Reagents

tert.-Butyl methyl ether (HPLC-grade), anhydrous sodium sulphate (p.a.) and potassium hydroxide (*p.a.*) were purchased from Merck, Darmstadt, Germany. The mixture of seventeen 1-(N,N-diisopropylamino) *n*-alkanes (DIPA) (*n*=4,5,6,7,8,9,10, 12,13,14,15,16,17,18,20,22,24) was a generous gift from Prof. Dr. M. Donike and E. Nolteernsting of the Deutsche Sporthochschule in Cologne, Germany. Diphenylamine was purchased from Sigma (St. Louis, MO, USA) and 7-ethyltheophylline (7-ethyl-1,3-dimethyl-3,7-dihydropurine-2,6-dione) was synthesized from theophylline [74,75]. All reference substances were purchased from Norsk Medisinaldepot (Oslo, Norway) and quality controlled.

3.1.2. Extraction

A 20-μl volume of the internal standard solution (diphenylamine and 7-ethyltheophylline, 0.5 mg/ml in methanol) was added to 5 ml of urine. Then 0.5 ml potassium hydroxide solution (5 mol/l), 2 ml *tert.*-butylmethyl ether and approximately 3 g sodium sulphate were added and the mixture was briefly vortex-mixed. After 20 min of mechanical shaking and centrifugation for 10 min at approximately 300 *g*, the ether layer was transferred to an injection vial and 1 μl was injected onto the GC column.

3.1.3. Gas chromatographic analysis

The gas chromatography was performed for screening purposes on a Hewlett-Packard gas chromatograph 5890, equipped with a nitrogen-specific detector (NPD), and for confirmation on a Hewlett-Packard gas chromatograph 5890 connected to a Hewlett-Packard 5972 mass selective detector (MSD). The separation was carried out on an Ultra-2, cross-linked 5% phenylmethyl silicone gum capillary column (12.5 m×0.2 mm I.D., 0.11 μm film thickness).

GC–NPD: The injection was done in the split mode (1:15), at a temperature of 280°C and in a constant helium flow mode (p=6.0 MPa at 70°C). The oven temperature was programmed from 75°C to 320°C at 25°C/min and a final time of 2 min. The detector temperature was set to 300°C.

GC–MSD: The MSD was run in a scan mode (m/z 40–350) with electron impact ionization (70 eV). The GC parameters were the same as in the GC–NPD experiments. The inlet pressure might be slightly reduced in order to keep the retention times constant.

Alternatively, the injection volume (3 μl) could be split into two columns connected to an NPD and an MSD, respectively [76]. Under circumstances where mass spectrometric identification is not mandatory, identification could be made on the basis of retention data by injecting the sample into two different columns, both connected to a nitrogen-specific detector [77–81].

3.1.4. Retention indices (RI)

The RI values of the analytes were calculated against 1-(N,N-diisopropylamino) alkanes (DIPA) [82] according to the equation:

$$RI = 100n + 100\,(t_x - t_n)/(t_{(n+1)} - t_n)$$

where

n = number of carbons in the n-alkyl chain of the DIPA eluting before the analyte,
t_x = retention time of analyte,
t_n = retention time of DIPA with n carbons,
$t_{(n+1)}$ = retention time of DIPA with $n+1$ carbons.

3.1.5. Confirmation

The urine extract used for the screening procedure described above was concentrated at room temperature using a slight flow of nitrogen. The best results for underivatised drugs (preventing the loss of volatile compounds) were obtained when solvent evaporation is discontinued at a sample volume of about 100 μl. This concentrated extract was transferred to an injection vial, then 2 μl were injected onto the GC–MS. Alternatively, the urine extract

may be derivatised as described below (see Section 5.1.3.).

3.1.6. Standard operating procedure

Each batch of samples contained the following quality control samples: a caffeine calibration urine containing 5 μg/ml caffeine, a caffeine control urine (approximately 10 μg/ml), a first control urine (blank urine fortified with 2 μg/ml of heptaminol, amphetamine, phentermine, methamphetamine, dimetamphetamine, phenylpropanolamine, ephedrine, methylephedrine, phendimetrazine, pentetrazole, fencamfamine, methylphenidate and 1 μg/ml nicotine), a second control urine (blank urine fortified with 5 μg/ml pseudoephedrine, 2 μg/ml methylphenidate, pethidine, oxprenolol, metoprolol, codeine, ethylmorphine and 0.2 μg/ml strychnine), a blank urine and a reagent blank.

The caffeine concentration in each sample was calculated by an internal standard method against 7-ethyltheophylline. The retention indices of all integrated peaks were calculated automatically using a post-run program [83]. The calibration table for this calculation was established by analysing a methanolic solution of the DIPA-mixture (10 μg/ml).

4. Results and discussion

The development of this simple but effective analytical method from 1970 to 1995 has been mainly characterized by technical and instrumental improvements. One of the most important improvements has been the introduction of fused-silica capillary columns [84].

Fig. 1 illustrates the changes in gas chromatographic separation characteristics over the past 25 years, including reductions of retention times and peak widths by factors of 3 and 10, respectively.

Numerous substances belonging to the doping class of stimulants, narcotic analgesics and a few β-blockers can be detected in urine as parent compounds or metabolites, underivatised after a single step extraction and for at least 12–24 h after intake. Table 4 shows the retention index compared with a mixture of 17 homologous 1-(N,N-diisopropylamino) alkanes (DIPA). These indices are fairly robust towards a change in temperature programming, column length, film thickness and inlet pressures, its repeatability during the XVII Olympic Winter Games on different instruments is shown in Table 5.

As retention behaviour on modern fused-silica capillary columns is highly reproducible, retention

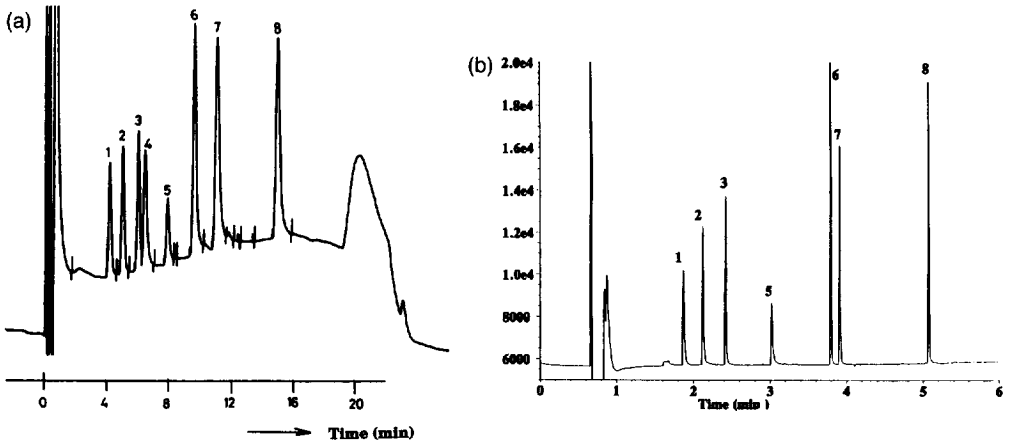


Fig. 1. Improvement of gas chromatographic separation of stimulants. Mixture of amfetamin (1), metamfetamin (2), dimetamfetamin (3), ephedrine (5), nikethamide (6), pentetrazol (7) and caffeine (8); (a) at a concentration of 10 μg/ml on a 2% Igepal and 10% Apiezon on Chromosorb P glass capillary column (1.5 m×2.4 mm I.D.) in 1972 [56]; (b) at a concentration of 2 μg/ml on a fused-silica cross-linked 5% phenyl methyl silicone capillary column (12.5 m×0.2 mm I.D.) in 1994.

Table 4
Retention indices of doping agents and their metabolites/artefacts excreted unconjugated into urine

Compound	Metabolite/artefact	Retention index	Drug/Metabolite	Metabolite/artefact	Retention index
Heptaminol		530	Nikethamide		970
Amfetamine	Amfetaminil A/M		Phenylpropanolamine	Amfepramone-M	
	Benzfetamine M,		(N-diethyl)		994
	Dimetamfetamine-M,		Pentetrazol		999
	Etilamfetamine-M		Benzocaine		1009
	Fenetylline-M		Methylendioxyethyl-		
	Fenproporex-M		amfetamine (MDEA)		1011
	Mefenorex-M,		Mefenorex		1018
	Metamfetamine-M	550	Clorprenaline		1020
Phentermine		589	Fenproporex		1040
Propylhexedrine		593	Diphenylamine (ISTD)		1050
Metamfetamine	Dimetamfetamine-M	610	Fencamfamine (Desethyl)	Fencamfamine M	1051
Tranylcypromine		649	2-Methylamino-1-(3,4)		
Fenfluramin		664	methylendioxyphenyl-		
Etilamfetamine		666	butane (MBDB)		1056
Dimetamfetamine		675	Prolintane		1061
Mephentermine		691	Furfenorex		1117
Cathine	Pseudoephedrine-M	752	Fencamfamine		1118
Phenylpropanolamine	Amferpramone-M		Crotetamide		1123
	Amfetamine-M,		Cotinine	Nicotine-M	
	Ephedrine-M,		Cropropamide		1173
	Fenetylline-M	754	Methylphenidate		1179
Methoxyphenamine	Methoxyphenamine M		Pethidine		1194
(N-desmethyl)		764	Pethidine (Nor-)	Pethidine-M	1223
Nicotine		775	Lidocaine (Desethyl-)	Lidocaine-M	1255
Chlorphentermine		789	Caffeine		1271
Ephedrine	Methylephedrine-M	804	Benzfetamine		1282
Pseudoephedrine		806	Ethyltheophylline (ISTD)		1298
	Mefenorex A	808	Ethoheptazine		1307
OH-Propylhexedrine	Propylhexedrine-M	812	Pyrovalerone		1318
Methoxyphenamine		813	Lidocaine		1328
OH-Propylhexedrine	Propylhexedrine-M	818	Oxprenolol		1361
OH-Propylhexedrine	Propylhexedrine-M		Clobenzorex		1410
	Propylhexedrine-M		Amiphenazole		1467
Amfepramone			Metoprolol		1488
(-N-desethyl)	Amfepramone M	833	EDDP[a]	Methadone-M	1496
	Propylhexedrine-M	838	Dextromethorphan		1598
Methylephedrine		846	Methadone		1603
Phenylpropanolamine	Amfepramone-M		Pipradrol		1609
(-N-ethyl)		863	Dextropropoxyphen		1647
Phenmetrazine	Morazone-A,		Cocaine		1660
	Phendimetrazine-M	879	Levorphanol, dextrorphan	Dextromethorphan-M	
N-Ethylnicotineamide	Nikethamide-M	898		(dextrorphan)	1680
Methoxyphenamine	Methoxyphenamine M			Amiphenazole A	1705
(-O-desmethyl)		899	Pentazocine		1735
Phendimetrazine		900	Codeine		1851
Methylendioxy-			Ethylmorphine		1890
amfetamine (MDA)		908	Dextropropoxyphen (Nor)	Dextropropoxyphen-M	2028
Methylecgonine	Cocaine-M	917		Amineptine-M	2054
Etafedrine		921	Fenetylline		2301
Amfepramone		934	Folcodine		>2400
	Nikethamide-M	939	Strychnine		>2400
Methylendioxymethyl-					
amfetamine (MDMA)		963			

[a] 2-Ethyliden-1,5-dimethyl-3,3-diphenylpyrrolidine.

Table 5
Repeatability of the retention index determination during the XVII Olympic Winter Games in Lillehammer on two different instruments

Compound	Retention index	Coefficient of variation (%) $n=10$	Retention index standard deviation
Amphetamine	553.0	0.33	1.8
Caffeine	1275.7	0.15	1.9
Dimetamfetamine	675.4	0.21	1.4
Diphenylamine (ISTD)	1051.7	0.15	1.5
Ephedrine	803.4	0.17	1.4
Fencamfamine	1120.1	0.15	1.7
Heptaminol	532.1	0.33	1.8
Metamfetamine	610.5	0.26	1.6
Methylephedrine	845.7	0.17	1.4
Methylphenidate	1180.7	0.14	1.7
Nicotine	777.1	0.20	1.5
Pentetrazole	1002.6	0.20	2.0
Phendimetrazine	899.9	0.17	1.5
Phentermine	589.1	0.30	1.7
Phenylpropanolamine	754.1	0.18	1.3

indices give a strong indication of the identity of a drug. Alternative indices have been published [85] for liquid chromatography.

As an extraction solvent, *tert.*-butylmethyl ether provides sufficiently high recovery of a broad range of substances (Table 6), and its boiling point of 55°C allows easy direct injection of the extract onto the GC column.

Table 6
Recovery of stimulants in urine after liquid–liquid extraction with *tert.*-butyl methyl ether at pH 13 ($n=6$)

Compound	Recovery (%)	Standard deviation (%)
Ampetamine	95.9	3.9
Dimetamfetamine	101.4	3.3
Ephedrine	101.2	8.7
Fencamfamine	104.4	5.7
Heptaminol	89.4	11.0
Metamfetamine	99.1	5.3
Methylephedrine	101.8	6.0
Methylphenidate	74.6	13.1[a]
Nicotine	99.9	3.0
Pentetrazole	52.3	3.0
Phendimetrazine	104.7	2.6
Phentermine	101.7	4.1
Phenylpropanolamine	80.8	6.5
Pseudoephedrine	93.9	4.4

[a] The variation of the methylphenidate recovery is partly due to basic hydrolysis of the ester at these high pH.

Due to extraction at a high pH level and the use of a nitrogen-specific detector the normal biological background is very clean. Fig. 2 shows the chromatogram of a blank urine sample from a coffee-drinking smoker and Table 7 shows the retention index output.

In addition to detecting banned substances, this type of detector will reveal numerous other nitrogen-containing drugs, provided they are excreted as a parent compound, unconjugated metabolite or lipophilic, and provided they are basic. Relevant exam-

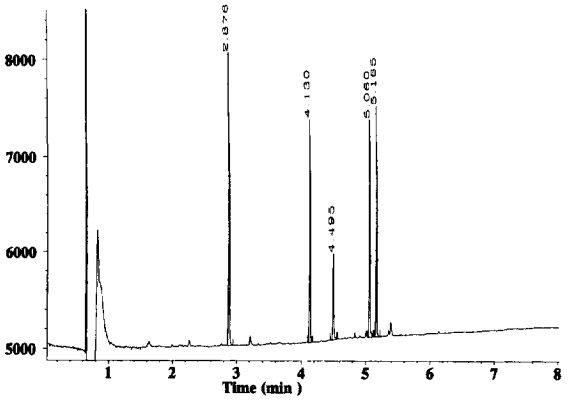


Fig. 2. Gas chromatographic screening procedure with NPD-detection of a blank urine sample containing nicotine, diphenylamine (ISTD), cotinine, caffeine and ethyltheophylline (ISTD), see Table 7.

Table 7
Integration results and determination of retention indices of the gas chromatographic screening procedure with NPD-detection of a blank urine

RT	Area %	Index	Compound
2.875	32.5417	775.898	Nikotin - 775.3
4.310	19.2479	1050.875	Difenylamin (ISTD) - 1049.7
4.495	7.8677	1136.056	Kotinin Nikotin-M - 1135.6
5.060	19.6159	1272.388	Koffein - 1271.4
5.166	20.7269	1298.756	Ethylteofyllin (ISTD) - 1298.0

The urine was collected from a laboratory staff member normally smoking and used to consume coffee (see Fig. 2).

ples include local anaesthetics, anti-histamines and benzodiazepines.

5. Analysis of stimulants, narcotics and adrenergic drugs and metabolites including those excreted in the conjugated fraction in urine

Traditionally, methods for the analysis of nitrogen basic drugs have been based on liquid–liquid extraction [86] and have usually been designed for the extraction and detection of each group of substances separately [52,87]. Even being successful in designing a unified method using liquid–liquid extraction, when analysing a large number of samples, the high amounts of organic solvent residues generated in the extraction procedure are a health and safety issue with regards to the workers and the environment. In addition, such methods are difficult to automate. Within the last few years, the great development of solid-phase extraction procedures (SPE) [88–90] has allowed the building of alternative methods to liquid–liquid procedures obtaining cleaner extracts and optimum recoveries. SPE methods, in addition, save a substantial amount of organic solvent per sample and are more amenable to automation. Some analytical methods for β-blockers [91–94], stimulants [95–98] and narcotic analgesics [99–102] have been already published.

The present method [103] describes a unified analytical procedure for the simultaneous isolation of stimulants, narcotics, β-blockers and many of their metabolites using a SPE extraction procedure. Gas chromatography coupled to mass spectrometry was used for separation and detection. This method was used for screening and confirmatory purposes in the analysis of about 2000 athlete's urine samples during the 25th Olympic Games, July 1992 in Barcelona, Spain.

5.1. Experimental procedure

5.1.1. Chemicals and reagents

Reference standards for all β-blockers, stimulants and narcotics were provided by Sigma.

Stock standard solutions of drugs (1 mg/ml, free base form) were prepared in methanol. Working solutions of 100 μg/ml were prepared by dilution of stock solutions. These solutions were checked by UV spectrophotometry and stored at −20°C.

The internal standards solutions of codeine-D_3 and 3,4-methylene- dioxymethamphetamine-D_5 (MDMA-D_5) (1 mg/ml) were obtained from Radian Corporation (Austin, USA). A mixture solution containing 100 μg/ml of codeine-D_3 and 100 μg/ml of MDMA-D_5 in methanol was used as internal standards working solution.

β-Glucuronidase-aryl sulphatase from *Helix pomatia* was purchased from Sigma. Methanol HPLC grade, chloroform, glacial acetic acid and acetone analytical grade were purchased from Merck. 2-Propanol and ammonium hydroxide 25% reagent grade were supplied by Scharlau (Barcelona, Spain). Deionised water was obtained by Milli-Q system (Millipore, Mulheim, France). N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), N-methyl-bis-trifluoroacetamide (MBTFA) and trimethylchlorosilane (TMCS) were obtained from Macherey–Nagel (Düren, Germany). Bond-Elut Certify™ columns were provided by Analytichem International (Harbor City, PA, USA) and the Visiprep™ vacuum manifold was obtained from Supelco (Bellefonte, PA, USA). Organic phases were evaporated to dryness with a Turbo-Vap from Zymark.

5.1.2. Extraction procedure

Urine samples (2.5 ml) were added with 25 μl of the internal standards working solution to obtain a concentration of 1 μg/ml of codeine-D_3 and 1 μg/ml of MDMA-D_5. Then, 1 ml of 1.1 *M* acetate buffer to reach pH 5 and 50 μl of β-glucuronidase-

aryl sulphatase (about 2600 units of β-glucuronidase per millilitre of urine) were added. The samples were vortex-mixed and heated to 55°C for 2 h on a water bath and later cooled to room temperature. Then, sample pH was adjusted to 8–9 with 1 *M* KOH and the mixture was centrifuged at 160 *g* for 5 min.

Bond Elut Certify™ columns were inserted into a vacuum manifold and conditioned by washing once with 2 ml of methanol and 2 ml of deionised water. The columns were prevented from drying out before applying specimens. Samples were poured into each column reservoir and drawn slowly through the column. The columns were washed with 2 ml of deionised water, 1 ml of 0.1 *M* acetate buffer pH 4 and 2 ml of methanol. Elution of analytes was performed with 2 ml of a mixture of chloroform–2-propanol (80:20, v/v) containing 2% of ammonium hydroxide. The eluates were added to 20 μl of MBTFA, vortex-mixed and then evaporated to dryness under a stream of nitrogen in a 50°C water bath. Samples were kept in a desiccator for 30 min. It has been observed after trying different alternatives (i.e. TMSCl, 20% HCl in methanol) that the best approach, in order to avoid losses of volatile substances (i.e. amphetamine) when evaporating the organic phase under nitrogen, was the addition of MBTFA.

5.1.3. Derivatisation procedure

MSTFA/MBTFA derivatives

A 100-μl volume of MSTFA was added to the dried residue, vortex-mixed and kept at 60°C for 5 min to obtain the trimethylsilyl (TMS) derivatives of hydroxyl, carboxylic and phenolic groups.

After cooling at room temperature, 20 μl of MBTFA were added and the mixture was vortexed and incubated for 10 min at 60°C to obtain trifluoroacetamide (TFA) derivatives of primary and secondary amines [104]. In the first step partially silylated primary and secondary amines are also transformed to the acylated products.

5.1.4. Instrumental analysis

The gas chromatography was performed on a Hewlett–Packard Model 5890 coupled to a mass selective detector Model 5970.

Separation of analytes was carried out using a cross-linked capillary column 12.5 m×0.2 mm I.D., 5% phenylmethyl silicone gum (0.33 μm film thickness; Hewlett-Packard). Injector (split mode, ratio 1:10) and detector temperatures were 280°C. Oven temperature was programmed from 100°C to 290°C at 20°C/min (final time 4 min) and the solvent delay was 2 min. Helium flow was 0.8 ml/min and the sample injection volume was 2 μl.

The mass spectrometer was operated by electron impact ionisation (EI, 70 eV) in the scan acquisition mode (50–600 amu). Data acquisition was done locally by HP Chemstation 59 940 (HP UNIX series).

5.1.5. Results and discussion

The present analytical method is based on a solid-phase extraction on Bond-Elut Certify™ columns. This kind of solid phase contains a proprietary bonded silica sorbent, which exhibits a unique hydrophobic ion-exchange extraction mechanism and will retain basic, neutral and acidic drugs under the proper extraction conditions [92]. In this procedure all the extracted substances have a primary or secondary amine function able to interact with the anionic groups of the extraction column.

Enzymatic hydrolysis was used as it has demonstrated to be more adequate than chemical hydrolysis (acidic or alkaline) to avoid breaking off some β-blockers (like atenolol, pindolol, timolol) and other substances (i.e. 6-monoacetylmorphine).

The GC–MS system working in scan mode is specific and sensitive enough to detect and identify approximately 100 compounds and metabolites in urine for at least 24 h after the administration of doses typically encountered in therapeutics. Table 8, Table 9 and Table 10 summarise chromatographic and mass spectrometric data of several compounds included in the stimulants, narcotics and β-blockers doping classes respectively.

This method is able to quantify many different substances under the optimal chromatographic conditions. Nevertheless for the quantitative analysis of drugs excreted as conjugates (like morphine and codeine) we propose additionally a previous sample clean-up step with XAD-2 or C_{18} columns before the enzymatic hydrolysis in order to have higher and more reproducible recoveries of substances.

Table 8
Gas chromatographic and mass spectrometric data of stimulants and their metabolites after selective derivatisation with MSTFA/MBTFA

Stimulants and their metabolites	t_{rr}[a]	Diagnostic ions		
Derivative		Base peak	M[b]	Others
Amfepramone (diethylpropion)	0.42	100	205	190, 105
Amineptine-N-TFA-O-TMS	1.23	193	505	487, 300
Amineptine-C5-N-TFA-O-TMS (metabolite)	1.12	193	477	459, 178
Amphetamine-N-TFA	0.28	140	231	118, 91
Benzphetamine	0.65	91	239	224, 148
Cathine-N-TFA-O-TMS	0.39	179	319	304, 191
Cathine-bis-N,O-TMS-O-TFA [c]	0.45	179	391	376, 212
Chlorphentermine-N-TFA	0.42	154	279	125, 59
Clobenxorex-N-TFA	0.79	125	355	264, 91
Dimethamphetamine	0.26	72	163	118, 91
Ephedrine-N-TFA-O TMS	0.45	179	333	318, 154
Etafedrine-O-TMS	0.40	86	265	250, 58
Ethylamphetamine-N-TFA	0.38	168	259	140, 91
Ethylefrine-bis-O-TMS-N-TFA	0.67	267	421	406
Fencamfamine-N-TFA	0.67	170	311	142, 91
Fenetylline-N-TFA	1.20	346	437	207, 91
Fenfluramine-N-TFA	0.37	168	327	308, 140
Fenproporex-N-TFA	0.57	193	284	140, 118
Furfenorex	0.54	81	229	138, 91
Heptaminol-N-TFA-O-TMS	0.34	131	313	186, 40
Hydroxyclobenzorex-N-TFA-O-TMS	0.95	125	443	264, 179
Hydroxyephedrine-N-TFA-bis-O-TMS	0.65	267	421	179, 154
Hydroxyetafedrine-N-TFA-bis-O-TMS	0.66	267	353	338, 86
Hydroxyfenproporex-N-TFA-O-TMS	0.76	179	372	206, 140
Hydroxymefenorex-N-TFA-O-TMS	0.77	179	395	216, 140
Hydroxymethoxyphenamine-N-TFA-O-TMS	0.56	236	363	209, 154
MDMA-N-TFA	0.58	154	289	162, 135
MDMA-D_5-N-TFA	0.58	158	294	163, 136
Metenorex-N-TFA	0.58	216	307	140, 91
Methoxyphenamine-N-TFA	0.47	154	275	140, 91
Methylamphetamine-N-TFA	0.36	154	245	110, 91
Methylendioxyamphetamine-N-TFA	0.53	135	275	162, 140
Methylendioxyamphetamine-N-TFA-N-TMS [c]	0.55	212	347	163, 135
Methylephedrine-O-TMS	0.36	72	251	236, 163
Methylphenidate-N-TFA	0.69	180	329	298, 91
N-Ethylnorephedrine-N-TFA-O-TMS	0.47	179	347	332, 140
N,N-Diethylnorephedrine-N-TFA-O-TMS	0.45	100	279	193, 179
Nordiethylpropion-N-TFA	0.44	168	273	140, 105
Phendimetrazine	0.40	57	191	176, 85
Phenmetrazine-N-TFA	0.48	167	273	98, 70
Phentermine-N-TFA	0.29	154	245	230, 91
Phenylpropanolamine-N-TFA-O-TMS	0.39	179	391	304, 191
Phenylpropanolamine-bis-N,O-TMS-N-TFA [c]	0.45	179	391	376, 212
Pholedrine-N-TFA-O-TMS	0.59	179	333	206, 154
Propylhexedrine-N-TFA	0.35	154	251	236, 182
Pseudoephedrine-O-TMS-N-TFA	0.45	179	333	318, 154
Ritalinic acid-N-TFA-O-TMS	0.72	180	387	372, 118
Tyramine-N-TFA-O-TMS	0.51	179	305	206, 192

[a] Relative retention time (t_{rr}) to codeine-D_3.
[b] Molar mass.
[c] Minor derivative.

Table 9
Gas chromatographic and mass spectrometric data of narcotics and their metabolites after selective derivatisation with MSTFA/MBTFA

Narcotics and their metabolites	t_{rr}[a]	Diagnostic ions		
Derivative		Base peak	M[b]	Others
Alphaprodine	0.63	172	261	187, 84
Anileridine-N-TFA	1.24	246	448	447, 375
Butorfanol-bis-O-TMS	1.23	416	471	456
Codeine-O-TMS	1.00	371	371	234, 178
Codeine-D_3-O-TMS	1.00	374	374	237, 181
Dextrometorphan	0.84	271	271	214, 59
Dextrorphan-O-TMS	0.87	59	329	272, 150
Dihydrocodeine-O-TMS	0.97	373	373	315, 236
Dihydromorphine-bis-O-TMS	0.98	431	431	236, 146
Dipipanone	1.01	112	349	334, 165
Ethoheptazine	0.67	57	261	188, 246
Ethylmorphine-O-TMS	1.02	385	385	357, 327
Hydrocodone-O-TFA	0.91	395	395	380, 338
Hydrocodone-O-TMS (c)	1.00	371	371	356, 313
Hydromorphone-O-TFA-O-TMS	0.95	453	453	438, 381
Hydromorphone-bis-O-TMS [c]	1.02	429	429	414, 357
Levallorphan-O-TMS	0.93	355	355	272, 176
Levorphanol-O-TMS	0.87	59	329	272, 150
Methadone	0.84	72	309	294, 91
Monoacetylmorphine-O-TMS	1.06	399	399	340, 287
Morphine-bis-O-TMS	1.03	429	429	414, 401
Nalbuphine-tris-O-TMS	1.28	573	573	518, 428
Norcodeine-N-TFA-O-TMS	1.04	453	453	313, 282
Nordihydrocodeine-N-TFA-O-TMS	1.02	315	455	255, 225
Nordihydromorphine-N-TFA-bis-O-TMS	1.03	373	513	455, 315
Normorphine-N-TFA-bis-O-TMS	1.06	511	511	496, 281
Norpethidine-N-TFA	0.72	241	329	256, 143
Oxycodone-bis-O-TMS	1.02	459	459	370, 312
Oxymorphone-bis-O-TMS	1.08	445	445	430, 287
Pentazocine-O-TMS	0.90	289	357	342, 245
Pethidine	0.60	71	247	218, 172
Phenazocine-O-TMS	1.10	302	393	378, 229
Pholcodine-O-TMS	1.47	114	470	356, 100

[a] Relative retention time (t_{rr}) to codeine-D_3.
[b] Molar mass.
[c] Minor derivative.

6. Conclusions

Doping control for the misuse of stimulants, narcotic analgesics and β-blocking agents is today achieved by the combination of two analytical methods complementing each other:
(1) Chromatographic analysis of the parent compound and unconjugated metabolites, following a single step sample extraction and detection by a nitrogen-specific detector and/or mass spectrometer (with or without derivatisation).
(2) Chromatographic analysis of conjugated metabolites after hydrolysis, extraction, derivatisation and detection through mass spectrometry.

These methods presented allow the screening and confirmation of more than 100 stimulants, narcotic analgesics and β-blocking agents in urine for at least 24 h after the intake of a therapeutic dose. A few

Table 10
Gas chromatographic and mass spectrometric data of β-blockers and their metabolites after selective derivatisation with MSTFA/MBTFA

β-Blockers and their metabolites	t_{rr}[a]	Diagnostic ions		
Derivative		Base peak	M[b]	Others
Acebutolol-N-TFA-O-TMS	1.25	284	504	242, 129
Acebutolol-N-TFA-O-TMS[c]	0.99	284	576	561, 129
Alprenolol-N-TFA-O-TMS	0.76	284	417	402, 129
Atenolol-N-TFA-bis-O-TMS[d]	0.90	284	559	544, 129
Atenolol-nitril-N-TFA-O-TMS[c,e]	1.02	284	417	402, 129
Atenolol-N-TFA-O-TMS[c]	1.03	284	434	242, 129
Bisoprolol-N-TFA-O-TMS	1.02	284	493	478, 129
Carteolol-O-TMS	1.03	86	364	349, 163
Carteolol-bis-O-TMS[c]	0.97	235	436	421, 86
Celiprolol-O-TMS	0.91	86	378	363, 234
Celiprolol-bis-O-TMS[c]	0.93	86	450	435, 200
Diacetolol-N-TFA-O-TMS	0.95	284	548	533, 129
Hydroxyalprenolol-N-TFA-bis-O-TMS	0.90	284	505	242, 129
Hydroxylabetalol-N-TFA-tris-O-TMS	1.39	292	638	623, 179
Hydroxymetropolol-N-TFA-bis-O-TMS	0.96	284	523	478, 129
Hydroxyoxprenolol-N-TFA-bis-O-TMS	0.93	284	521	506, 129
Hydroxypenbutolol-N-TFA-bis-O-TMS	0.88	86	451	436, 365
4-Hydroxypropranolol-N-TFA-O-TMS	1.05	284	515	242, 129
3-Hydroxypropranolol-N-TFA-O-TMS	1.07	284	515	242, 129
Labetalol-nitril-N-TFA-bis-O-TMS isomer 1[c]	1.13	292	550	535, 91
Labetalol-nitril-N-TFA-bis-O-TMS isomer 2[c]	1.14	292	550	535, 91
Levobunolol-O-TMS	0.95	86	363	348
Methoxyhydroxypropranolol-N-TFA-bis-O-CMS	1.12	284	545	242, 129
Metoprolol-N-TFA-O-TMS	0.88	284	435	420, 129
Mepindolol-bis-N,N-TFA-O-TMS	0.94	284	526	242, 129
Nadolol-tris-O-TMS	0.99	86	525	510, 409
Oxprenolol-N-TFA-O-TMS	0.80	284	433	418, 129
Penbutolol-O-TMS	0.82	86	363	348, 57
Pindolol-N-TFA-bis-N,O-TMS	1.02	284	488	242, 129
Pindolol-bis-N,N-TFA-O-TMS[c]	0.91	284	512	497, 129
Practolol-N-TFA-bis-N,O-TMS	1.03	284	506	491, 129
Practolol-N-TFA-O-TMS[c]	1.04	284	434	242, 129
Propranolol-N-TFA-O-TMS	0.93	284	427	419, 129
Sotalol-N-TFA-bis-O-fMS	0.96	344	512	497, 126
Sotalol-N-TFA-O-TMS[c]	0.98	272	440	193, 126
Timolol-O-TMS	0.90	86	388	373, 130

[a] Relative retention time (t_{rr}) to codeine-D_3.
[b] Molar mass.
[c] Minor derivative.
[d] Possible structure; an amide function is supposed to be lost.
[e] An amide function is dehydrated.

compounds which are difficult to detect in the described manner have been integrated into other existing analytical methods for doping control. Examples are pemoline [105] or buprenorphine, which can easily be integrated into the analysis of anabolic steroids, and mesocarb, which can be analysed for in the method for diuretics [106,107]. By applying the presented methods the high quality standards for an unequivocal identification of doping agents as well as a short total time of analysis are achieved.

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