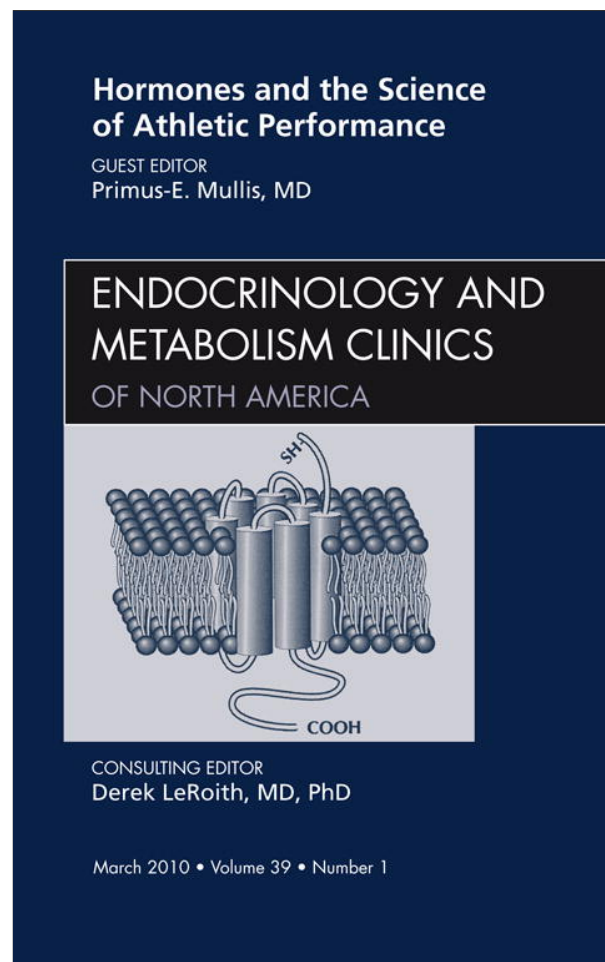


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Stimulants and Doping in Sport

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KEYWORDS

- Doping • Sport • Amphetamine • Ephedrine
- 4-methylhexan-2-amine • Mass spectrometry

Stimulants represent one of the oldest classes of doping agents and have been used to increase performance, endurance, and stamina for centuries. According to reports on the habits of the indigenous people in the Peruvian Andes, the consumption of coca leaves (and thus cocaine) was widespread and mostly uncontrolled in the pre-Inca period.¹ It became also part of religious Inca rituals, which were hardly related to athletic challenges; however, cocaine's potential to reduce pain and hunger as well as to enhance or prolong physical work was recognized, and it was used during long and energy-demanding marches under hypoxic conditions.²

The first documents demonstrating a doping offense with cocaine according to modern regulations were found in racewalking competitions in the eighteenth century. Racewalking, a British invention, potentially arose from the job of "footmen" accompanying wealthy travelers in the sixteenth and seventeenth centuries and later became a competitive sporting event with the goal to complete 100 miles (or more) in 24 hours without breaking into a run. In the late nineteenth century, various astonishing achievements in racewalking were reported and the use of cocaine was frequently mentioned, which further outlined the performance-enhancing properties of the stimulating and fatigue-reducing drug.³

The continuous research on active ingredients of plants in the nineteenth century; the constantly improving possibilities to isolate, purify, and characterize substances from complex mixtures; and the options to chemically modify these compounds have led to the detection, production, and use of various additional stimulating agents for clinical and nonclinical purposes (eg, strychnine,⁴ ephedrine,^{5,6} and related synthetic derivatives).⁷⁻⁹ The efficiency of stimulating drugs such as strychnine, cocaine, ephedrines, and amphetamines on performance was hardly systematically evaluated; only a few studies allowed an estimation of performance enhancement in selected sport disciplines, which ranged from 0.6% to 4% for amphetamines.¹⁰⁻¹⁹

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Although these numbers appear comparably low, such small improvements might represent the competitive edge in elite sport. A great variety of stimulating drugs have been detected in human routine doping control samples since systematic sports drug-testing programs were installed in 1967, and stimulants in general have been among the most frequently found prohibited compounds since.

In the following sections, a selection of stimulants, their prevalence in sports, particular challenges, and detection strategies are described. It is noteworthy that sports drug testing, especially with regard to alkaloid-based stimulants, was first introduced in animal doping controls in the early twentieth century, and much effort was necessary to establish and continuously improve detection methods that sensitively and selectively measure banned compounds in bodily specimens.

CHEMISTRY AND PHARMACOLOGY OF STIMULANTS

Categories of Stimulants

The class of stimulants prohibited by the World Anti-Doping Agency (WADA)²⁰ contains various agents with different structural features. Many of these compounds are derived from phenethylamine or phenylpropanolamine core structures (**Fig. 1**) and represent drugs such as amphetamine (**1**), methamphetamine (**2**), methylenedioxymethamphetamine (MDMA, ecstasy, **3**), or cathine (**4**), ephedrine (**5**), and metamfetramone (**6**). Additional alkaloids with stimulating properties are cocaine (**7**) and strychnine (**8**), which bear entirely different structures based on tropane and indole nuclei. Moreover, alkylamines such as tuaminoheptane (**9**) or 4-methylhexan-2-amine (**10**) as well as designer substances such as the hybrid of amphetamine and piracetam referred to as carphedone (**11**), were considered relevant for doping controls. In contrast to most prohibited stimulants, ephedrine, methylephedrine, and cathine are currently banned only when they exceed a urinary threshold level of 10 µg/mL (ephedrine and methylephedrine) or 5 µg/mL (cathine).

Mechanisms of Action

Detailed studies on the mechanisms of action of selected central nervous system (CNS) stimulants have been conducted for more than 3 decades, and at least 3 major modes of influencing the process of neurotransmission at the nerve terminal were elucidated. The modes include (1) an elevated release of neurotransmitters (eg, dopamine, noradrenaline, and serotonin) into the synaptic cleft, (2) the direct stimulation of postsynaptic receptors, and (3) the inhibition of neurotransmitter reuptake.^{21–24}

One of the most widely studied topics in stimulants is amphetamine (see **Fig. 1, 1**) and its effect on dopaminergic neurons, although numerous articles on the influence of amphetamine on noradrenergic and serotonergic systems have been published also. In the case of dopamine, amphetamine was shown to exert mechanism (1), ie, causing an increased secretion of the neurotransmitter, through manipulation of the Na⁺/Cl[−] dependent dopamine transporter (DAT). The function of the DAT to clear extracellular dopamine from the synaptic cleft is reversed in the presence of extracellular amphetamine, and bursts of dopamine are released into the synaptic cleft in a channel-like mode,²⁵ which intensifies the dopaminergic neurotransmission significantly. Moreover, the inhibitory effect of amphetamine on monoamine oxidase (MAO) was reported, which further interferes with the metabolism and thus elimination of dopamine. Direct interaction of amphetamine with the neurotransmitter receptors and its potential to counteract reuptake were also hypothesized.²¹ In contrast to amphetamine and related drugs, the CNS stimulant cocaine (**7**) (see **Fig. 1**) does not increase the release of dopamine from nerve terminals but elevates concentrations of dopaminergic and

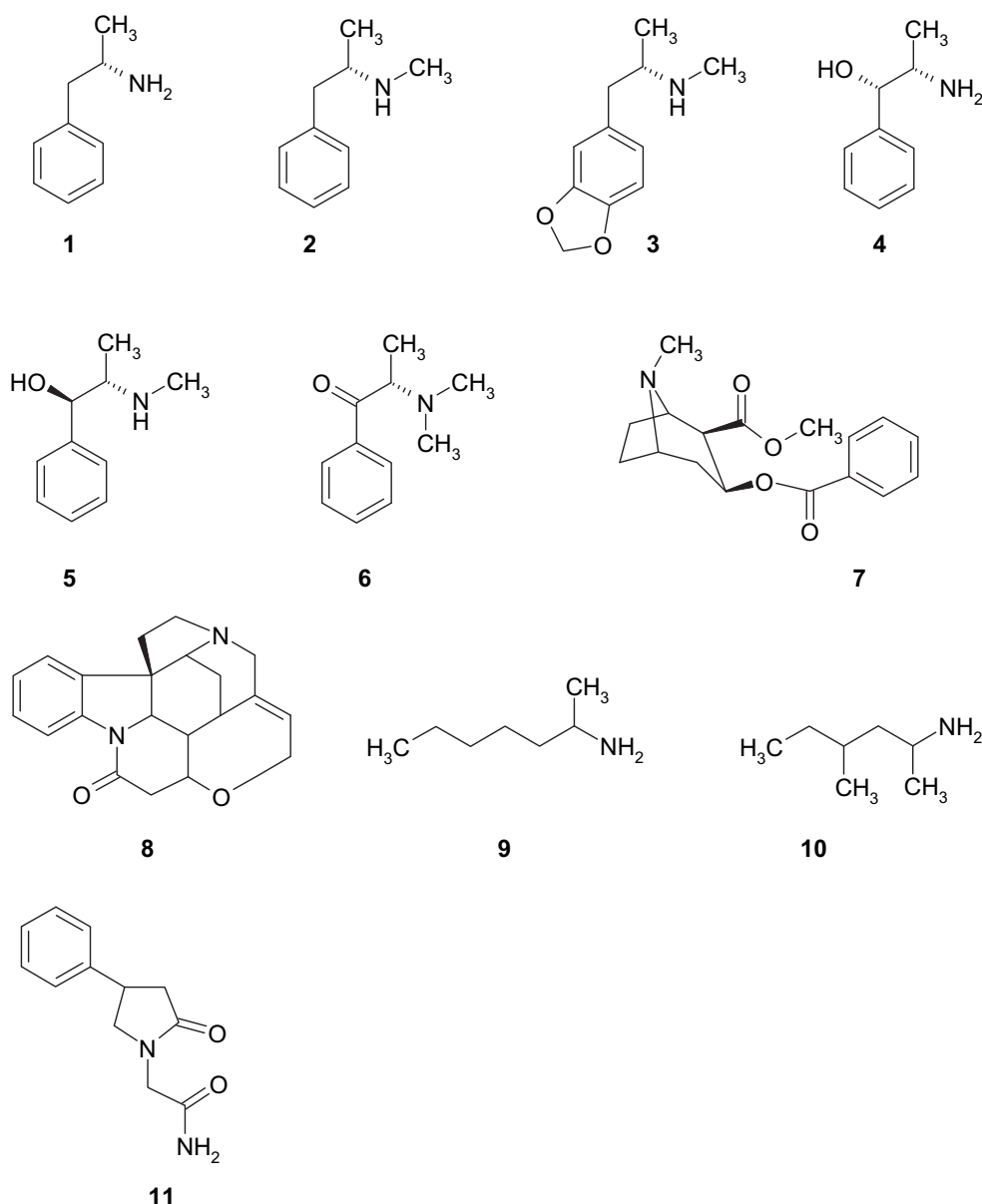


Fig. 1. Structures of selected stimulants: amphetamine (**1**, molecular weight [mol wt] = 135), methamphetamine (**2**, mol wt = 149), MDMA/ecstasy (**3**, mol wt = 193), cathine (**4**, mol wt = 151), ephedrine (**5**, mol wt = 165), metamfepramone (**6**, mol wt = 177), cocaine (**7**, 303), strychnine (**8**, mol wt = 334), tuaminoheptane (**9**, mol wt = 115) or 4-methylhexan-2-amine (**10**, mol wt = 115), and carphedone (**11**, mol wt = 218).

noradrenergic transmitters at the neuronal synapse, predominantly by binding to the DAT and efficiently blocking dopamine reuptake.² In a similar fashion, the sympathomimetic compounds tuaminoheptane (**9**) and 4-methylhexan-2-amine (**10**) (see **Fig. 1**) were shown to hamper the noradrenaline reuptake.²⁶ The mechanism underlying the stimulating activity of ephedrine (**5**) (see **Fig. 1**) is reported to be based on the displacement of neurotransmitters from respective storage sites. Extracellular ephedrine is transferred into the presynaptic neuron and stored in vesicles, where it exhibits considerable resistance against MAOs because of the methylated amino function and further prolongs the effect of released neurotransmitters. In addition to its indirect mechanism of action, ephedrine was also shown to possess weak direct effects on alpha- and beta-adrenergic receptors. It affects primarily the adrenergic receptor system that is a part of the sympathetic nervous system rather than the CNS due to its reduced lipophilicity, which results from the β -positioned hydroxyl function.

The peripheral effects of the sympathomimetic amines commonly include an elevated blood pressure, increased pulse rate, and bronchodilatation, which is complemented by diminished fatigue and improved alertness. These beneficial effects are considered the major reasons for athletes to abuse stimulants in sports, and numerous doping rule violations have been recorded ever since systematic doping controls have been conducted.

PREVALENCE OF STIMULANTS IN SPORT

Stimulants have been a major problem in elite sports and numerous adverse analytical findings (AAFs) have been annually reported by doping control laboratories worldwide. In **Table 1**, the WADA statistics of 2003 to 2007 are summarized,²⁷ indicating that constantly more than 10% of all AAFs were related to drugs belonging to the class of stimulating agents. In 2003, more than 50% of doping offenses with stimulants were because of ephedrine and its stereoisomer pseudoephedrine. The latter was removed, together with caffeine, from the prohibited list at the end of 2003 and was no longer a subject of sanctions when detected in doping control urine samples. In the following 4 years, amphetamine was constantly the most frequently detected stimulant, representing up to 54% of all AAFs resulting from stimulant misuse, complemented predominantly by findings of cocaine and ephedrine applications; however, it must be considered that various drugs categorized as stimulating agents metabolize to give amphetamine,²² which might contribute to and explain the prominent occurrence of amphetamine cases.

Pseudoephedrine—Prevalence Before and After Lifting the Ban

Until the end of 2003, pseudoephedrine, the stereoisomer of ephedrine (**Fig. 2**), was prohibited in sports when a threshold value of 25 $\mu\text{g}/\text{mL}$ of urine was exceeded. The threshold level for pseudoephedrine was initially set to 10 $\mu\text{g}/\text{mL}$, then increased to 25 $\mu\text{g}/\text{mL}$ in 2000, and since January 2004 the presence of this drug in doping control urine samples and its use in sports were no longer sanctioned. Hence, pseudoephedrine represents an interesting object to outline a possible effect of lifting a ban for a drug, the ergogenic properties of which are controversially discussed.²⁴

Data generated and recorded in the doping control laboratory of Cologne between 1996 and 2003 that included a total of 52,347 in-competition analyses yielded 33 and 93 AAFs for pseudoephedrine and ephedrine, respectively, which accounts for an average of 4.1 (0.06%) and 11.6 (0.18%) positive controls per year. In 2007 and 2008, that is, 3 years after pseudoephedrine was removed from the prohibited list, the prevalence of pseudoephedrine and ephedrine was determined in 16,335 in-competition doping control samples. The analyses resulted in 102 (0.62%) and 9 (0.06%) cases of pseudoephedrine and ephedrine use or misuse, respectively, representing a considerable increase of findings for pseudoephedrine at concentrations higher than the formerly valid threshold. One of the major contributors to these samples were cyclists, who provided 53 positive test results in 1343 specimens, including 44 (3.28%) and 9 (0.67%) cases of pseudoephedrine and ephedrine, respectively. This is particularly interesting because only 10 findings were reported in cycling in the period between 1996 and 2003 (4 samples containing pseudoephedrine and 6 samples containing ephedrine exceeding their respective threshold levels). These observations are confirmed by data of the Belgian sports drug-testing laboratory, which reported a significant increase in urine samples containing pseudoephedrine in concentrations higher than 25 $\mu\text{g}/\text{mL}$.²⁸ Over a 3-year period, only 0.2% of all urine specimens were tested positive for pseudoephedrine, which increased to 1.4% AAFs

Table 1 Prevalence of stimulants in elite sports from 2003 to 2007					
Year	Total Number of A-Samples	Total Number of AAF with Stimulants	Percent of Total Number of AAFs Worldwide	Top 5 of The Detected Compounds	Percent of Total Number of AAFs with Stimulants
2003	151,210	516	19.0	Pseudoephedrine Ephedrine Cocaine & metabolites Amphetamine Caffeine	36.6 ^a 19.4 9.3 8.3 7.6 ^a
2004	169,187	382	11.6	Amphetamine Ephedrine Cocaine & metabolites MDMA Phentermine	29.3 26.7 19.6 3.9 3.4
2005	183,337	509	11.8	Amphetamine Ephedrine Cocaine & metabolites Methylphenidate Cathine	38.1 18.3 16.7 3.3 2.8
2006	198,143	490	11.3	Amphetamine Cocaine & metabolites Ephedrine Methylphenidate Cathine	40.6 17.3 13.5 6.5 4.5
2007	223,898	793	16.4	Amphetamine Cocaine & metabolites Ephedrine Methylphenidate Cathine	54.2 12.7 6.3 4.8 4.2

^a Removed from prohibited list in 2004.

Data from WADA. Laboratory statistics. 2008. Available at: <http://www.wada-ama.org/en/dynamic.ch2?pageCategory.id=594>. Accessed February 15, 2008.

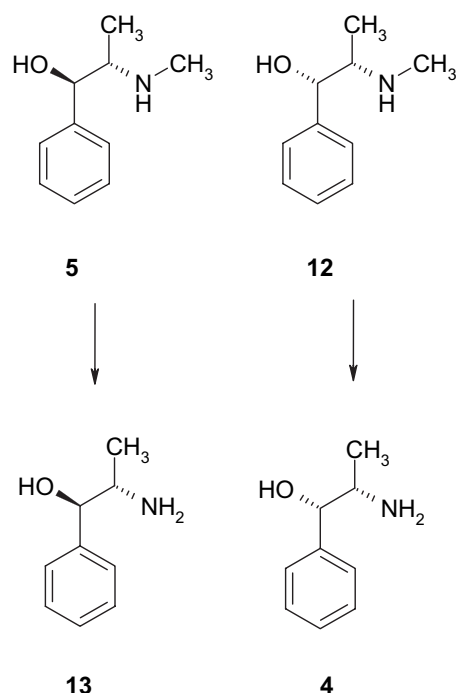


Fig. 2. Structures of ephedrine (**5**, mol wt = 165), pseudoephedrine (**12**, mol wt = 165), and their major metabolites phenylpropanolamine (**13**, mol wt = 151) and cathine (**4**, mol wt = 151).

in 2007/2008. Also here, the major reason was the considerably elevated numbers of pseudoephedrine findings in samples collected during cycling events. While only 0.2% of specimens measured between 2001 and 2003 yielded pseudoephedrine levels greater than 25 µg/mL, 3.9% of all samples analyzed in 2007/2008 were found to contain more than the formerly existing threshold value.

These data suggest that the misuse of the stimulating agent pseudoephedrine was rather limited as long as the substance was prohibited and that lifting the ban resulted in a much more frequent use aiming for performance enhancement. Consequently, it has been suggested to reconsider the implementation of a threshold value to control the nontherapeutic ingestion of the drug, especially since the major metabolite of pseudoephedrine, cathine (**4**) (see Fig. 1), has remained prohibited when exceeding a urinary threshold level of 5 µg/mL despite decontrolling pseudoephedrine. An athlete might thus produce an AAF with cathine, although no banned substance was administered. Amongst others, these facts have led to the installation of a new threshold value for urinary pseudoephedrine of 150 µg/mL becoming effective in January 2010.

DETECTION METHODS FOR STIMULANTS IN DOPING CONTROLS

Gas Chromatography, Mass Spectrometry/Nitrogen–Phosphorus Specific Detection

Stimulants and alkaloids in general were among the first analytes to be tested in systematic doping controls. In the late 1950s, based on chemistry that provided characteristic and more or less quantitative data by means of color reactions, the capability of gas chromatography (GC) to separate compounds relevant for doping controls was recognized and introduced into sports drug testing to measure various classes of analytes, predominantly sympathomimetic amines.^{29–33} Analyzers such as flame ionization and nitrogen–phosphorus detectors (FID and NPD, respectively) as well as ionization β-ray (strontium 90) or electron capture detectors were used, and sample extraction and concentration methodologies were mostly adapted from earlier purely “chemical” procedures. The need to improve GC properties of target analytes and to

obtain supporting information that would provide additional confidence in analytical results led to the development of various derivatization strategies, which improved chromatographic peak shapes and yielded additional data characterizing a substance. A strategy to identify a compound by its retention times obtained from the native and derivatized analyte or 2 different derivatives was termed the “peak-shift technique”³⁴ and was used as a common standard in confirmatory analyses. Trimethylsilylation (using, for example, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide [MSTFA]³⁵); acylation (using, for example, acetic or heptafluorobutyric anhydride, bis[acylamide]); alkylation; formation of several Schiff-bases (eg, acetone-, propionaldehyde-, benzyl methyl ketone-Schiff-bases)³⁶; or preparation of mixed derivatives were used to modify the physicochemical nature of substances and thus enhance their traceability in sports drug testing. Seminal assays for doping controls were finally based on trimethylsilylation or acylation as established by Donike and coworkers,^{37–41} and they were used for the comprehensive doping control program undertaken at the 1972 Olympic Games in Munich and at the subsequently conducted great sporting events.^{42–44} The enormous complexity of biologic matrices and the continuously increasing number of therapeutics have, however, necessitated more specific and unequivocal analyzers than for instance NPD and FID alone. This resulted in the frequent use of GC equipped with NPD plus mass spectrometry (MS), a combination that allows the exploitation of advantages provided by both analytical techniques simultaneously. MS is commonly operated using electron ionization (EI), which frequently results in comprehensive fragmentation of analytes and thus hardly yields information on the molecular weight; however, the obtained EI mass spectra contain diagnostic ions and provide detailed information that enables the characterization and identification of target compounds. Moreover, various derivatives of stimulants have been shown to produce stable molecular ions also under EI conditions. An example of a recent AAF (4-methylhexan-2-amine) as detected by means of GC-EI/MS is presented in Case Vignette 1.

Liquid Chromatography–(Tandem) MS

The considerable proton affinity of amines, which constitute an important structural feature of most stimulants, has also enabled the use of robust and sensitive instruments composed of liquid chromatography (LC) combined with (tandem) mass spectrometers (LC-MS/MS) to detect and quantify stimulants in doping controls.⁴⁵ The analytes are commonly ionized by means of electrospray ionization (ESI) or atmospheric pressure chemical ionization that yields a protonated molecule $[M+H]^+$. Subsequent collision-induced dissociation (CID) of $[M+H]^+$ gives rise to product ion mass spectra that allow the sensitive and specific analysis of numerous stimulants with the advantages that the intact molecular ion is recorded in addition to diagnostic product ions and that no derivatization is required even in case of heavy volatile or thermolabile analytes (eg, phase I or phase II metabolites). The specificity of ion transitions (ie, the direct correlation of precursor and product ions) has been used to establish fast and sensitive detection assays that complement GC-MS/NPD-based procedures, and case vignettes of the finding of 4-methylhexan-2-amine and methoxyphenamine abuse as proved by LC-MS/MS are described in the following sections.

Case Vignette 1—4-methylhexan-2-amine

4-Methylhexan-2-amine (geranamine, **10**) (see **Fig. 1**) is a natural product produced to a minor extent in *Pelargonium graveolens* (also referred to as geranium or Pelargonium), a plant that is indigenous particularly to South Africa. *Pelargonium* is largely cultivated because of the great interest in its foliage that is used for the preparation of various different scents, which are derived from an oily distillate that contains

approximately 0.7% of 4-methylhexan-2-amine. The oil has been approved as a food additive, and “geranium extracts” are frequently declared as ingredients of nutritional supplements and so-called party pills. In addition to its natural occurrence, 4-methylhexan-2-amine is synthetically obtained by the reaction of 4-methylhexan-2-one and hydroxylamine followed by reduction using, for example, hydrogen in the presence of Raney nickel catalyst.⁴⁶ As such, a pharmaceutical product was prepared and patented in 1944, which was marketed as a nasal decongestant (Forthane sulfate [Eli Lilly, Indianapolis, IN]) and as a therapeutic agent for the treatment of hypertrophied gums. Although it was supposed to be less stimulating than drugs such as amphetamine or ephedrine typical sympathomimetic effects, tremor, excitement, or insomnia were reported. Its advantages over amphetamine and ephedrine were greater volatility and reduced toxicity, and its efficacy was greater than that of heptylamine derivatives such as tuaminoheptane (**9**) (see **Fig. 1**).⁴⁷

In January 2007, WADA expanded the list of prohibited substances by adding tuaminoheptane to the list, which has since been monitored in all in-competition samples. In 2009, doping control urine specimens were found suspicious for a drug closely related to tuaminoheptane according to GC-MS and LC-MS/MS data, but retention times did not match the reference of tuaminoheptane. Further studies led to the identification of 4-methylhexan-2-amine using authentic reference material, and detection assays based on GC-MS and LC-MS/MS were established.

The approach based on LC-MS/MS consisted of an alkaline liquid-liquid extraction of the target analyte, followed by concentration and subsequent measurement using diagnostic product ions obtained from the protonated molecule at m/z 116 (mass-to-charge ratio) after CID.⁴⁸ By means of this assay, a detection limit of approximately 50 ng/mL was accomplished, and an AAF with 4-methylhexan-2-amine was reported for an in-competition doping control urine sample that contained about 15 $\mu\text{g/mL}$ of the banned substance, indicating an application shortly before the contest. The test result was further confirmed by GC-MS after derivatization of 4-methylhexan-2-amine to the corresponding Schiff-base using cyclohexanone.

Almost simultaneously, 4-methylhexan-2-amine was found in a doping control sample in Germany using an entirely GC-MS-based procedure, which was initially established for the detection and confirmation of tuaminoheptane (**9**) (see **Fig. 1**).⁴⁹ In brief, the analytes of interest are extracted from urine into methyl tert-butyl ether under alkaline conditions and subsequently derivatized to Schiff-bases by adding a methanolic solution of benzaldehyde. After 30 minutes without further treatment, the samples are injected into a GC-EI/MS/NPD system equipped with a HP-5MS analytical column (Agilent Technologies, Inc, Santa Clara, CA, USA). The extracted ion chromatograms of a mixture containing 4-methylhexan-2-amine and tuaminoheptane are illustrated in **Fig. 3A** along with respective EI mass spectra. Notably, tuaminoheptane (retention time of 4.49 min) yielded 1 signal, and 4-methylhexan-2-amine gave rise to 2 peaks at 4.27 and 4.32 min, indicating the presence of diastereomers as also reported earlier.⁴⁸ The blank urine sample did not yield any signal (see **Fig. 3B**), whereas the AAF in a doping control sample included 2 baseline-resolved peaks of 4-methylhexan-2-amine isomers (see **Fig. 3C**). In the same context, a nutritional supplement (NOX PUMP [Ultralife, Aylesbury, UK]) declaring to contain “geranium root extract” was analyzed and found to contain 4-methylhexan-2-amine at approximately 1 mg/g. A single administration of the product according to the recommended dosage (1 portion of 17 g/d) resulted in the presence of the banned substance in urine samples with nearly identical appearance of diastereoisomers (see **Fig. 3D**) as observed with the reference compound and the doping control urine sample.

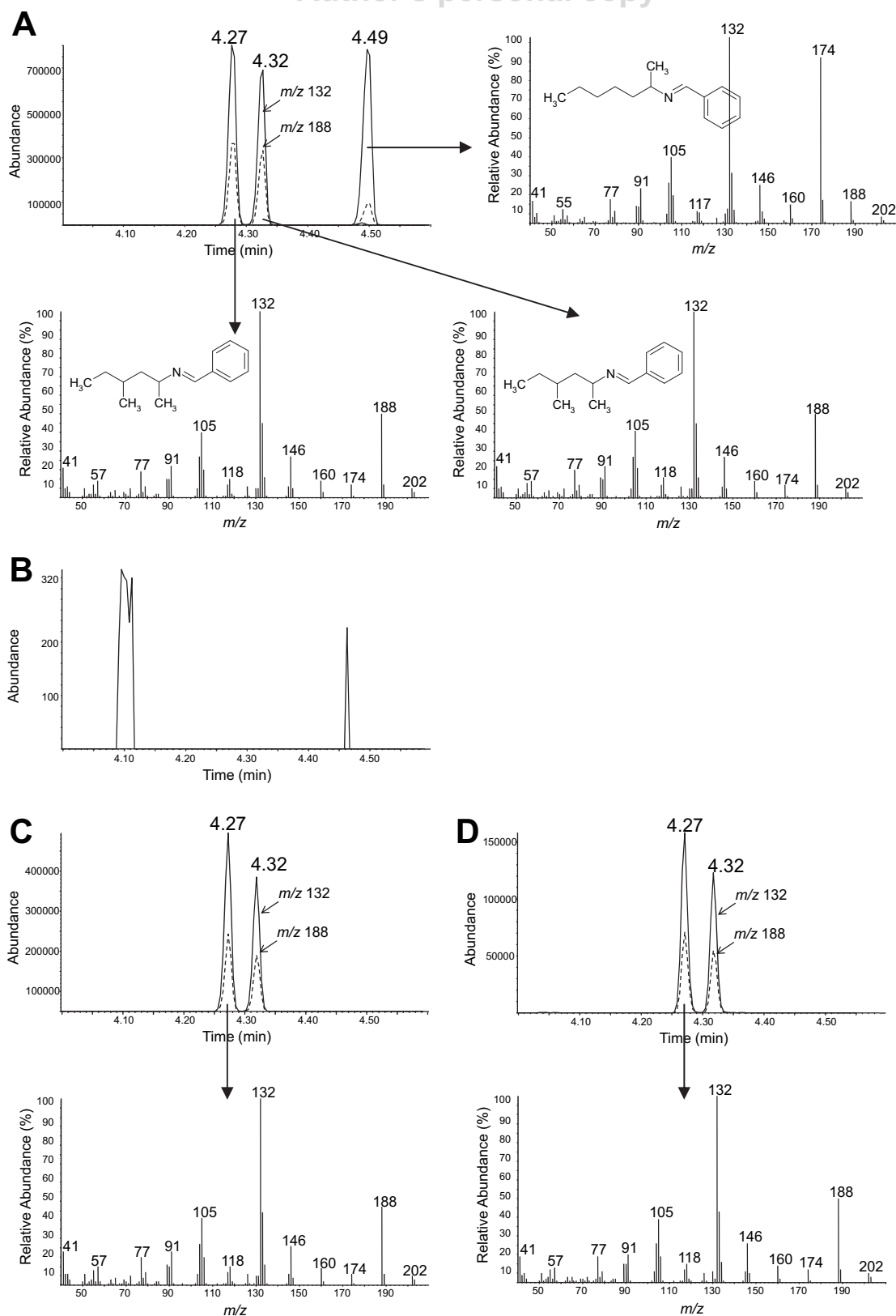


Fig. 3. (A) GC-MS analysis of a mixture of 4-methylhexan-2-amine (10) and tuaminoheptane (9) derivatized with benzaldehyde yielding respective Schiff-bases with a molecular weight of 203 Da; 2 baseline-separated stereoisomers of 10 are observed at 4.27 and 4.32 min, which yield identical EI-MS spectra (*bottom left and right*). The constitutional isomer 9 differs in retention time (4.49 min) and relative abundances of diagnostic fragment ions such as m/z 188 and 174 (*top right*). (B) GC-MS analysis of a blank urine sample for compounds 9 and 10. No signals are detected. (C) GC-MS analysis of a doping control urine sample tested positive for 4-methylhexan-2-amine; appearance of 2 diastereomers and EI mass spectrum are identical with those measured from reference substances. (D) GC-MS analysis of a urine sample collected after administration of a nutritional supplement labeled to contain "geranium root extract." Also here, 4-methylhexan-2-amine was detected with diastereomers.

The mass spectrometric fragmentation of 4-methylhexan-2-amine as benzaldehyde derivative is in accordance with earlier reported dissociation pathways.⁴⁹ Imines such as the derivatized target analytes are likely ionized by EI at the nitrogen atom because of the electron-donating nature of the amino function.⁵⁰ The resulting radical cations can subsequently undergo isomerization triggered by intramolecular hydrogen abstraction and formation of so-called distonic ions.⁵¹ Hence, besides the commonly observed and characteristic α -cleavage products,⁵² complex cascades of rearrangements were described to precede the dissociation of amines, which allowed for the explanation of frequently detected additional fragment ions in EI mass spectra of aliphatic amines. The molecular ion of the condensation product of 4-methylhexan-2-amine at m/z 203 is hardly visible, which is a well-known issue of EI-MS-based assays for primary and secondary amines. However, abundant diagnostic ions originating from α -cleavages are found at m/z 188 and 132 (Fig. 4), which are suggested to represent the cations of benzylidene-(3-methyl-pentyl)-amine and benzylideneethylamine, respectively. These are complemented by losses of alkyl radicals such as ethyl-, propyl- and butyl-residues from the molecular ion to yield the fragments at m/z 174, 160, and 146, which support the MS-based identification of 4-methylhexan-2-amine (see Fig. 3C, D).

Case Vignette 2—methoxyphenamine

Methoxyphenamine (**14**) (*o*-methoxy-*N*, α -dimethylphenethylamine, Fig. 5) was synthesized and clinically evaluated for the treatment of asthma bronchiale in the 1940s^{53–55} and it demonstrated promising bronchodilator activity after oral administration with reduced influence on blood pressure and the CNS as compared with ephedrine.^{56–58} It is metabolized to 3 major products that are derived from *N*- or *O*-demethylation and ring hydroxylation at position 5 (see Fig. 5, 15–17) with introduced hydroxyl functions further conjugated to glucuronic acid. Because of the structural analogy of methoxyphenamine to stimulants such as amphetamine (**1**) (see Fig. 1) and its β_2 -agonistic properties, it has been prohibited in sports according to the rules established by the WADA.⁵⁹ For clinical and forensic purposes, several methods were established allowing the detection of methoxyphenamine, its metabolites, and its designer analogues in plasma and urine using GC or GC-MS, and sports drug-testing procedures have commonly used the previously described GC-MS/NPD methods and, more recently, the methods that use LC-MS/MS.^{45,60–63} Although stimulants

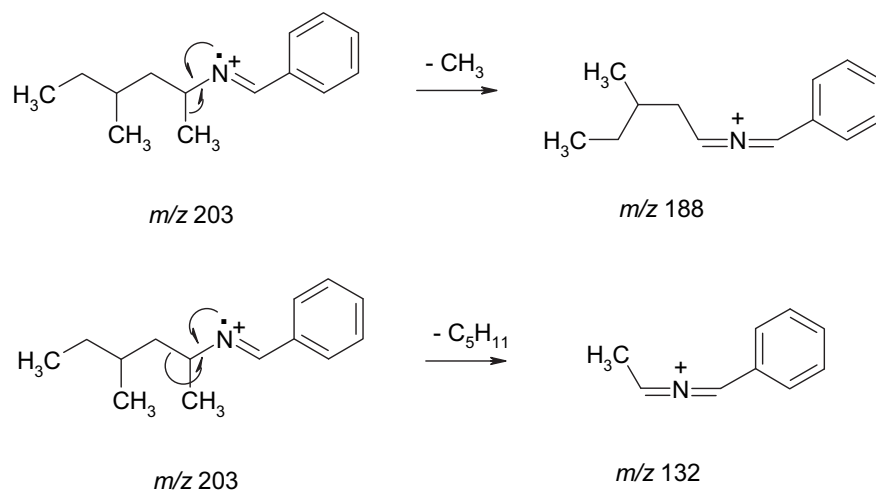


Fig. 4. Proposed fragmentation pathway of the Schiff-base benzaldehyde derivative of 4-methylhexan-2-amine (**10**) under EI conditions.

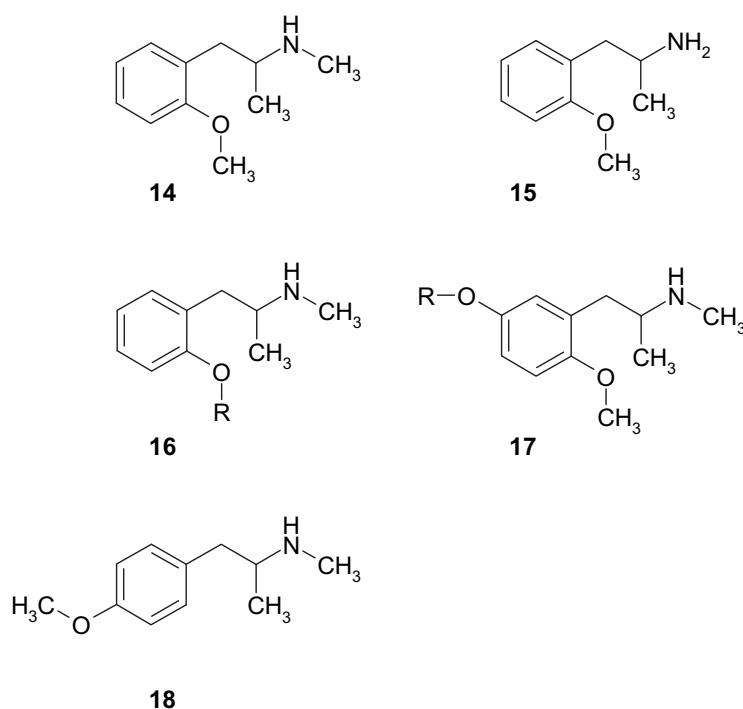


Fig. 5. Structures of methoxyphenamine (**14**, mol wt = 179), *N*-demethyl methoxyphenamine (**15**, R = H: mol wt = 165, R = glucuronic acid: mol wt = 341), *O*-demethyl methoxyphenamine (**16**, R = H: mol wt = 165, R = glucuronic acid: mol wt = 341), 5-hydroxy-methoxyphenamine (**17**, R = H: mol wt = 195, R = glucuronic acid: mol wt = 371), and PMMA (**18**, mol wt = 179).

represent one of the most frequently detected classes of compounds in sports drug-testing samples, the prevalence of methoxyphenamine has been very low during the last 4 years with only 2 findings in doping controls.⁶⁴

In early 2008, another doping control specimen yielded an AAF for methoxyphenamine and because mono-methoxy positional ring isomers are possible,⁶⁵ an LC-MS/MS procedure was used to confirm the presence of the banned substance. The chromatographic separation of the active drug from isomeric compounds such as the designer drug *p*-methoxymethamphetamine (PMMA, **18**) (see **Fig. 5**) was of particular interest. Product ion mass spectra of methoxyphenamine (**14**) and PMMA (**18**) were obtained from protonated molecules (**Fig. 6A, B**, respectively) using identical collision energies of 25 eV. Major product ions derived from the precursor at *m/z* 180 were found at *m/z* 149, 121, 93, and 91, and slightly different relative abundances were observed in particular for the ion at *m/z* 149. The proposed origin of the product ions is illustrated in **Fig. 7**, and supporting information for the suggested dissociation pathway was obtained from MS³ experiments. The protonated molecule at *m/z* 180 yielded the ion at *m/z* 149 by the elimination of methylamine (31 Da), which subsequently released ethylene (28 Da) to yield the product ion at *m/z* 121. Subsequently, *m/z* 121 liberated formaldehyde (30 Da) from the methoxy residue to yield the ion at *m/z* 91.

Besides methoxyphenamine, further analytes were found in the doping control sample and they are attributed to *N*-demethyl methoxyphenamine (**15**), *O*-demethyl methoxyphenamine (**16**) and its glucuronic acid conjugate, and 5-hydroxy-methoxyphenamine (**17**) glucuronide (see **Fig. 5**).⁶⁶ The *N*-demethylation was characterized by the presence of a primary amine (**15**), which eliminated ammonia (17 Da) under ESI/CID conditions giving rise to *m/z* 149 (see **Fig. 6C**), which subsequently released ethylene (28 Da) to *m/z* 121 as observed also with methoxyphenamine (**14**). In contrast,

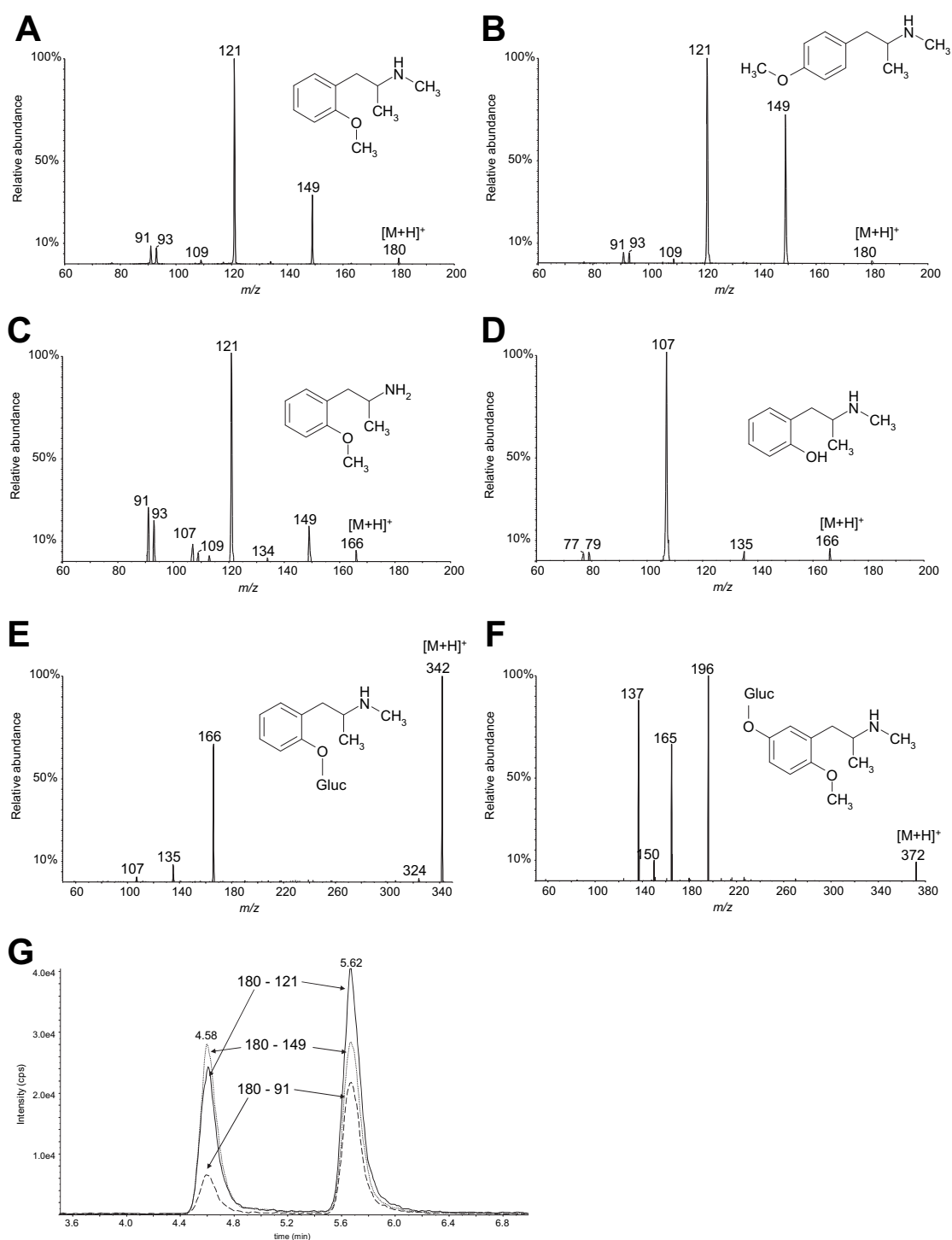


Fig. 6. ESI-MS/MS spectra obtained from protonated molecules of methoxyphenamine (A), *N*-demethyl methoxyphenamine (B), *O*-demethyl methoxyphenamine (C), 5-hydroxy-methoxyphenamine (D), glucuronide of *O*-demethyl methoxyphenamine (E), and glucuronide of 5-hydroxy-methoxyphenamine (F). Extracted ion chromatograms of a mixture containing methoxyphenamine and PMMA further demonstrate the chromatographic and mass spectrometric differentiation of both compounds (G).

the *O*-demethylated analogue (**16**) generated a product ion at m/z 135 instead of 149, indicating the loss of methylamine (31 Da) and the presence of an intact and methylated secondary amine (see Fig. 6D). Consequently, the following elimination of ethylene yielded a product ion at m/z 107, which corresponded to m/z 121 in case of **15** and **17**. Further to the phase I metabolism, the *O*-demethylated metabolite (**16**) and the 5-hydroxylated analogue to methoxyphenamine (**17**) are conjugated to

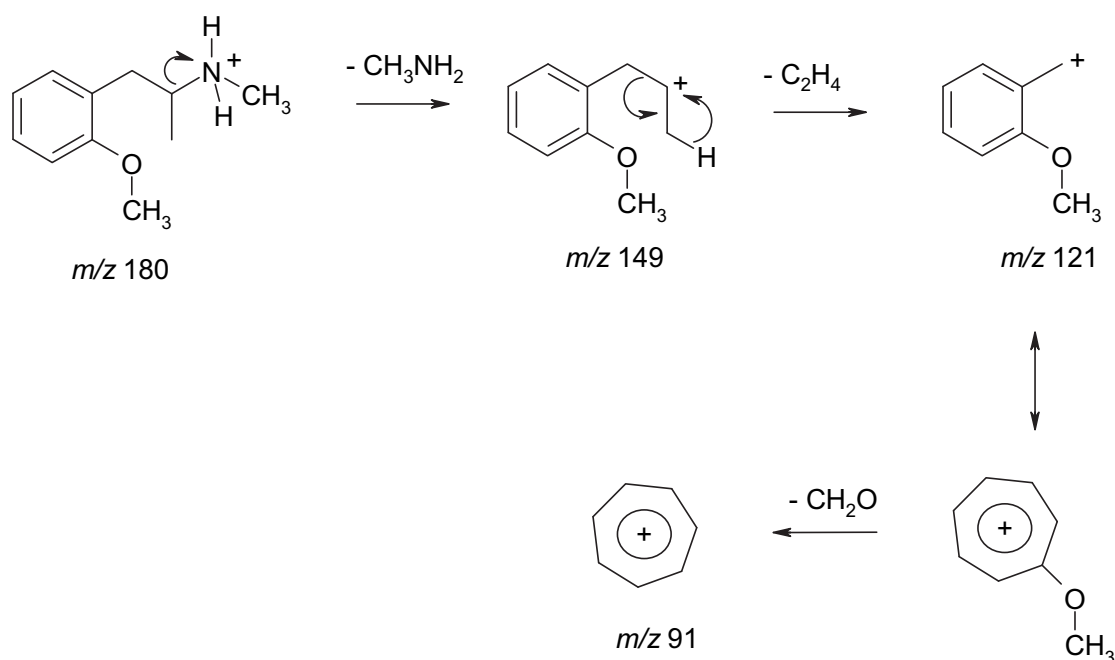


Fig. 7. Proposed dissociation pathway of the protonated molecule of methoxyphenamine under CID conditions.

glucuronic acid in phase II metabolic reactions. Respective product ion spectra were obtained from the doping control urine specimen containing methoxyphenamine as shown in **Fig. 6E, F**. The protonated molecule of *O*-demethylated and glucuronidated methoxyphenamine was observed at *m/z* 342 and yielded product ions at *m/z* 166, 135, and 107 (see **Fig. 6E**). These were attributed to the consecutive losses of the glucuronic acid moiety (176 Da) and the previously reported eliminations of methylamine (31 Da) and ethylene (28 Da), respectively. Accordingly, the precursor ion of 5-hydroxy-methoxyphenamine was detected at *m/z* 372, which gave rise to abundant product ions at *m/z* 196, 165, and 137 (see **Fig. 6F**).

Besides the mass spectrometric identification, chromatographic separation of methoxyphenamine (**14**) and PMMA (**18**) was aimed and accomplished using LC with isocratic elution of the analytes (see **Fig. 6G**). The *para*-substituted amphetamine derivative (**18**) eluted at 4.58 min, whereas methoxyphenamine (**14**) demonstrated a considerably longer retention, eluting at 5.62 min. In addition, the selected ion transitions (180–149, 180–121, and 180–91) yielded different relative abundances, which further supported the unambiguous differentiation of both compounds.⁶⁷

SUMMARY

Stimulants play an important role in sports drug-testing programs. The great variety of compounds belonging to this class of prohibited substances represents a challenge for doping control laboratories, but the sensitive and selective nature of analytical instruments and detection assays has enabled comprehensive screening procedures that not only reveal the misuse but also the presumably unintended intake of banned compounds. Several instances of natural products illegally enriched with synthetic compounds were reported (eg, sibutramine in an herbal tea),⁶⁸ and the addition of synthetically produced natural stimulants (such as 4-methylhexan-2-amine) to nutritional supplements is conceivable.

Studies concerning the prevalence of the stimulating agent pseudoephedrine have highlighted its great misuse potential, that is, lifting the ban for pseudoephedrine in

2004 resulted in a significant increase of findings in doping control urine samples subjected to a monitoring program. Consequently, pseudoephedrine has been added to the list of prohibited compounds being valid from January 2010.

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