ISSN : 0974-7419 Volume 16 Issue 4

Analytical CHEMISTRY
An Indian Journal
Review

ACAIJ, 16(4) 2016 [173-181]

Analytical strategies in the detection of stimulants and narcotics in sports drug testing

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ABSTRACT

The desire to win has led some athletes to resort to use of performance enhancing drugs since ancient times. The stimulants and narcotics represent one of the oldest classes of doping agents. The stimulants have a direct stimulating effect on the central nervous system (CNS), by increasing the excitation of brain and spinal cord, cardiac output and rate of metabolism. The four most notorious examples of stimulants that are used in sport are amphetamine, cocaine, ephedrine and caffeine. The narcotics are powerful pain killers like opiates buprenorphine, heroin, hydromorphone, morphine, oxymorphone, oxycodone, pethidine, dextromoramide, fentanyl and its derivatives. A critical overview of the contributions of analytical detection techniques viz. GC-NPD, GC-MSD and LC–MS/MS to testing of stimulants and narcotics in sports is provided, with special emphasis on mass spectrometry. The review also includesprevalence of misuse in sports of stimulants and narcotics.

KEYWORDS

Stimulants;
Narcotics;
Doping control;
GC-NPD;
GC-MS;
LC-MS/MS.

INTRODUCTION

The problem of drug abuse in sports in the modern age was first tackled by the International Olympic Committee (IOC) in the 1960s, with the first definition of doping given in 1964 and the foundation of medical Commission in 1967. Later, the international agency to regulate and harmonize doping controls worldwide was established in 1999 as World Anti Doping Agency (WADA). The misuse of the performance enhancing substances/methods is prohibited by WADA and its prohibited list is updated on regular basis to ensure an environment of drug free sports[1-4].

The substances prohibited at all times (in- and out-of-competition) include Anabolic steroids, Hormones and related substances, β2-agonists, Agents with anti-estrogenic activity, Diuretic and other masking agent. The methods prohibited at all times (in- and out-of-competition) include Enhancement of oxygen transfer,Chemical and physical manipulation and Gene doping. The substances prohibited incompetition includeStimulants, Narcotics, Cannabinoids, Glucocorticosteroids and the substances prohibited in particular sports include alcohol and β-blockers[5, 6].

Review

Drug testing is a highly complex taskthe anti-doping laboratories are one of the most important element in the whole doping control process as they are responsible for producing reliable results. The analytical challenge for the experts working in the field of drug testing in sports is to improve the effectiveness of antidoping tests, which means to reduce as much as possible the percent of false-negative cases, avoiding at the same time the risk of any false-positive result[3, 7, 8]. The ongoing research in various areas to provide measures to combat doping is of equal importance for sporting community[9-11].

Stimulants & narcotics abuse in sportsand their analytical aspects

Stimulants

Stimulants are substances, which have a direct stimulating effect on the central nervoussystem (CNS), because they mimic the adrenaline activity and enhance the cardiac rhythm. They are used therapeutically to increase or maintain alertness, to counteract fatigue, to counteract abnormal states that diminish alertness, consciousness to promote weight loss as well as to enhance the ability to concentrate in people diagnosed with attentional disruption (ADHD) [12, 13].

The class of stimulants prohibited by the WADA[6] contains various agents with different structural features. Many of these compoundsare derived from phenethylamine or phenylpropanolamine core structures and represent drugs such as amphetamine (1), methamphetamine (2), methylenedioxymethamphetamine(MDMA, ecstasy,) (3), or cathine (4), ephedrine (5), and metamfepramone(6). Additional alkaloids with stimulating properties are cocaine (7) andstrychnine (8), which bear entirely different structures based on tropane and indolenuclei. Moreover, alkylamines such as tuaminoheptane (9) or 4-methylhexan-2-amine(10) as well as designer substances such as the hybrid of amphetamine and piracetamreferred to as carphedone (11) were considered relevant for doping controls. For most of the stimulants the minimum required performance limit is 100 ng/ml. In contrast ephedrine, methylephedrine, and cathine which arebanned only when they exceed a urinary threshold level of 10 mg/ml (ephedrineand methylephedrine) or 5 mg/ml (cathine) [14].

The first case of doping offence in sports as per modern regulations was recorded in 18thcentury for abuse of cocaine in race walking competition. During the earlier half of the 19th century with advancements of pharmacognosy, natural product & synthetic chemistry, isolation & production of various other natural origin as well as synthetic stimulants like strychnine, ephedrine was evidentiary[15, 16]. Sincethen, stimulants have been major problems in elite sports and numerous adverse analytical findings (AAFs) have been annually reported by doping control laboratories worldwide.

The WADA statistics of 2003 to 2013is summarizedin TABLE 1, indicating thatin last 11 years 6.4-19% of all AAFs were related to drugs belonging to the classof stimulating agents. In 2003, more than 50% of doping offenses with stimulantswere because of ephedrine and its stereoisomer pseudoephedrine. The latter wasremoved, together with caffeine, from the prohibited list at the end of 2003. Inthe following five years, amphetamine was constantly the most frequently detected stimulant,representing up to 54% of all AAFs resulting from stimulant misuse. However,it must be considered that various drugs categorized as stimulating agents metabolizeto give amphetamine, which might contribute to and explain the prominent occurrenceof amphetamine cases. The year 2010 brought a new substance "methylhexaneamine" hitting the top of AAF% (21.4%)in stimulants which is constantly topping the list of AAFs in this category since then.

Detection methods for stimulants in doping controls

Gas chromatography, mass spectrometry/nitrogen–phosphorus specific detection

In the late 1950s, 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[17-21]. Analyzers such as flame ionization and nitrogen–

TABLE 1 : Prevalence of misuse of stimulants in sports in 11 years (2003-2013)

Year	Total samples tested	Total AAFs	Total AAF stimulants	% AAF of stimulants	Top 5 drugs	% within drug class
					Pseudoephedrine*	36.6
					Ephedrine	9.4
2003	151210	2,716	516	19.0	Cocaine & metabolites	9.3
					Amphetamine	8.3
					Caffeine**	7.6
					Amphetamine	29.3
					Ephedrine	26.7
2004	169187	3,305	382	11.6	Cocaine & metabolites	19.6
					MDMA	3.9
					Phentermine	3.4
					Amphetamine	38.1
					Ephedrine	18.3
2005	183337	4,298	509	11.8	Cocaine &metabolites	16.7
					Methylphenidate	3.3
					Cathine	2.8
					Amphetamine	40.6
					Cocaine &metabolites	17.3
2006	198143	4,332	490	11.3	Ephedrine	13.5
					Methylphenidate	6.5
					Cathine	4.5
					Amphetamine	54.2
					Cocaine &metabolites	12.7
2007	223898	4,850	793	16.4	Ephedrine	6.3
					Methylphenidate	4.8
					Cathine	4.2
					Amphetamine	35.2
					Cocaine & met	16.3
2008	274615	5,523	472	8.5	Ephedrine	11.4
					Methylphenidate	8.5
					Sibutramine	3.6
					Cocaine	18.5
					Ephedrine	13.5
2009	277928	5,084	325	6.4	Methylphenidate	9.5
					Methylhexaneamine	9.5
					Amphetamine	8.3
					Methylhexaneamine	21.4
					Amphetamine	19.5
2010	258,267	5,546	574	10.3	Methylphenidate	12.7
					Cocaine	11.3
					Ephedrine	5.6
					Methylhexaneamine	39.4
					Amphetamine	18.5
2011	243,193	5,600	718	12.8	Methylphenidate	8.2
					Cocaine	5.6
					Ephedrine	4.6
					Methylhexaneamine	45.9
					Cocaine	8.5
2012	285,868	4,500	697	15.5	Amphetamine	8.3
					Methylphenidate	6.7
					Methamphetamine (D-)	4.4
					Methylhexaneamine	31.9
					Methylphenidate	12.5
2013	269,878	5,271	530	10	Cocaine	9.8
					Amphetamine	8.9
					Oxiloferine	4.2

****Removed from WADA prohibited list in 2004 and re-introduced in 2009; *Removed from WADA prohibited list in 2004; Data derived from WADA, Laboratory statistics, 2003-2013. Available at: http://www.wada-ama.orgAcessedon 27thJuly 2014.**

Review

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 toobtain 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.

Trimethylsilylation(e.g., N-methyl-N-trimethylsilyltrifluoroacetamide [MSTFA]; acylation(e.g., acetic or heptafluorobutyric anhydride, bis[acylamide]); alkylation; formation of several Schiff-bases (eg, acetone-, propionaldehyde, benzyl methyl ketone-Schiff-bases); or preparation of mixed derivatives were used[22]. Seminal assays for doping controls were finally based on trimethylsilylationor acylation as established by Donike and coworkers[23-26],

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.

As an example, the proposed dissociation pathway of ephedrine,which gives rise to an EI mass spectrum is depicted in Figure 1 which states that the molecular ion [$M^{+\cdot}$] is hardly or not observed in mass spectra after EI. However, the elimination of a hydrogen atom yields the ion at *m/z* 164 in case of ephedrine. The subsequent loss of water (- 18 Da) gives rise to *m/z* 146. The fragment ions at *m/z* 117 and 115 result from further dissociation of *m/z* 146, that eliminates HCN (- 27 Da) and one or two hydrogen molecules, respectively.

Liquid chromatography–(tandem) mass spectrometry

The considerable proton affinity of amines, has 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[27]. The analytes are commonly ionized by means of electrospray ionization (ESI) that yields a protonated molecule $[M+H]^+$. Subsequent collision-induced

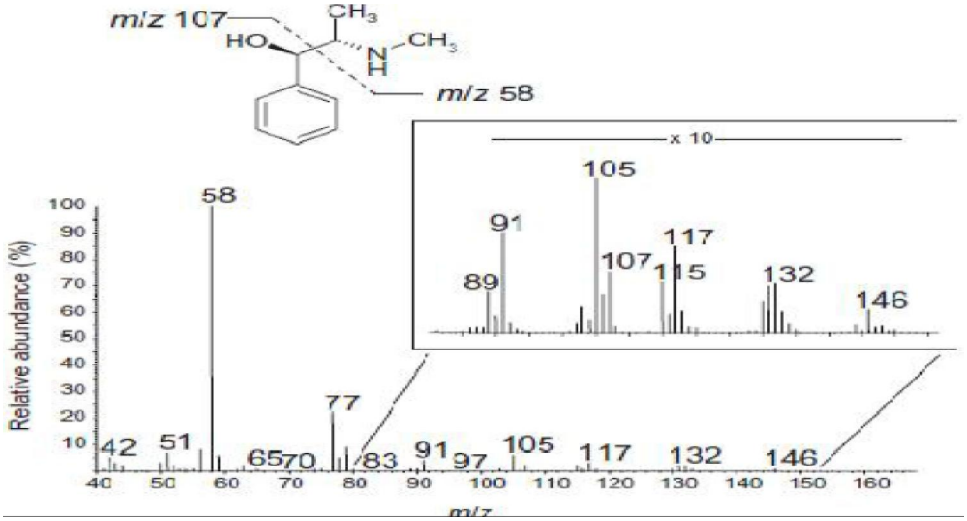

Figure 1 : EI mass spectra of (a) ephedrine (mol wt = 165) with an inset enlarging the region *m/z* 80 – 150

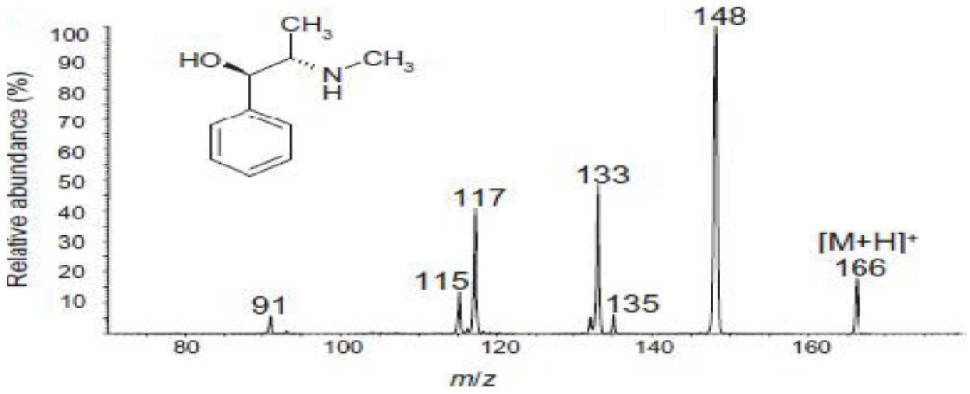

Figure 2 : ESI product ion mass spectra of protonated molecules $[M+H]^+$ of ephedrine (mol.wt. =165)

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 orthermolabile analytes. The positive ESIyielded informative product ion mass spectra for ephidrine as shown in Figure 2.

Narcotics

The narcotic analgesicsare derived from opium, which in turn isderived from the poppy plant (papawersomnifereum). They act on the CNS & surrounding tissues by stimulating opioids receptorsand reduce feelings of pain. Narcotics analgesics have been and are abused in sports, and therefore the IOC medical commission has issued a ban on their use during the Olympic Games in 1967[2]. These drugs are widely used to provide relief from pain, hence their use, misuse and abuse potential in sports may be high because of pressure on the athlete to perform competitively despite varied musculoskeletal injuries. They are listed as banned substances in competition but are permitted when out of competition. There is a strict legal control over most narcotics because of high potential for addiction. The exception to this control is codeine, which is widely available in variety of medications such as cough syrups and cold remedies. Generally, the level of codeine in these medications istoo low to produce the adverse effects.

The WADA statisticsof 2003 to 2013 for narcoticsis summarizedin TABLE 2,indicating that in last 11 years 0.4-1 % of all AAFs were related to drugs belonging to the class of narcotic agents. Since 2003 till date, morphine has topped the list of most abused narcotic.

Detection methods for narcotics in doping controls

Gas chromatography-mass spectrometry detection

The most common method forscreening of these drugs in urine is liquid-liquidextraction (LLE) or solid-phase extraction(SPE) for sample preparation, followed by derivatizationof the polar functional groups andanalysis of the extract by gas chromatographymass spectrometry (GC-MS). Screening methods for the analysis of conjugatednarcotic agents using GC–MS requirederivatising agents to generate the required number of threediagnostic ions characterizing the detected substance. In dopingcontrol most often *N*-methyl-*N*-trimethylsilyltrifluoroacetamide(MSTFA) is used creating *O*-TMS functionalities. Moreover, MSTFA is frequently combined with *N*-methylbistrifluoroacetamide(MBTFA) resulting in*N*-TFAfunctionalities[28, 29]. Although, the properties ofGC and instability of some derivativesmay limit the use of GC-MS for routine analysis of narcotics in doping control,[30, 31] which led to the development of alternative techniques for screening of narcotics in doping control.

The mass spectrometric behavior of morphine shows that the initial ionization of morphine takes place at the nitrogenatom, which induces typical α - cleavages as and concurrent rearrangement and fragmentationpathways yields the most abundant ions

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TABLE 2 : Prevalence of misuse of narcotics in sports in 11 years (2003-2013)

Year	Total samples tested	Total AAFs	Total AAF narcotics	% AAF of narcotics	Top 5 drugs	% within drug class
2003	151,210	2,716	26	1	Morphine Pethidine Hyromorphone Methadone --	84.6 7.7 3.8 3.8 --
2004	169,187	3,305	15	0.5	Morphine Methadone Dextromoramide Hydromorphone Oxycodone	66.7 13.3 6.7 6.7 6.7
2005	183,337	4,298	17	0.4	Morphine Methadone Hydromorphone -- --	88.2 5.9 5.9 -- --
2006	198,143	4,332	16	0.4	Morphine Methadone Hydromorphone Pethidine --	68.8 18.8 6.3 6.3 --
2007	223,898	4,850	21	0.4	Morphine Methadone Hydromorphone -- --	90.5 4.8 4.8 -- --
2008	274,615	5,523	28	0.5	Morphine Methadone Hydromorphone Heroine Oxycodone	75 7.1 7.1 3.6 3.6
2009	277,928	5,084	24	0.5	Morphine Oxycodone Methadone Hydromorphone Buprenorphine	70.8 16.7 4.2 4.2 4.2
2010	258,267	5,546	20	0.4	Morphine Oxycodone Methadone Hydromorphone Buprenorphine	30.5 15 10 10 10
2011	243,193	5,600	20	0.4	Morphine Methadone Oxycodone Hydromorphone --	55 30 10 5 --
2012	285,868	4,500	26	0.6	Morphine Oxycodone Fentanyl & derivatives Methadone --	53.8 23.1 19.2 3.8 --
2013	269,878	5,271	43	0.8	Morphine Oxycodone Methadone Fentanyl & derivatives Buprenorphine	58.1 14 11.6 9.3 4.7

Data derived from WADA, Laboratory statistics, 2003-2013. Available at: http://www.wada-ama.orgAcessed on 27th July 2014

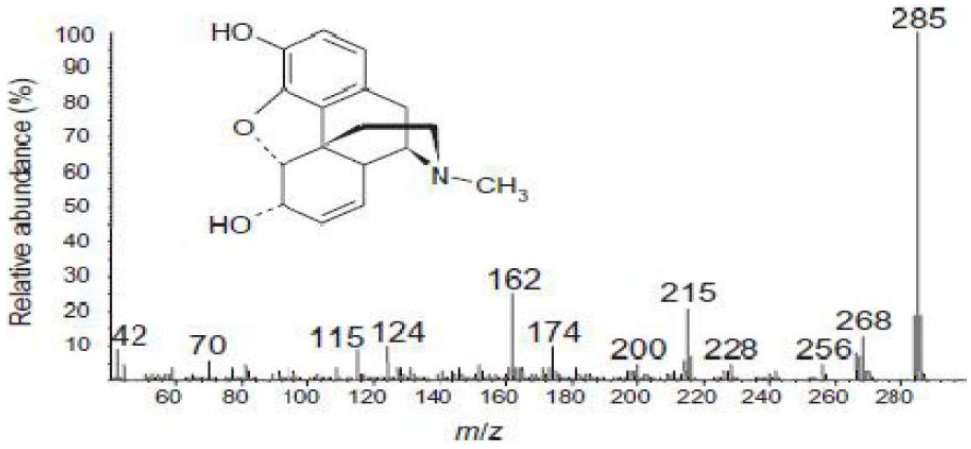

Figure 3 : EI mass spectra of morphine (mol wt = 285)

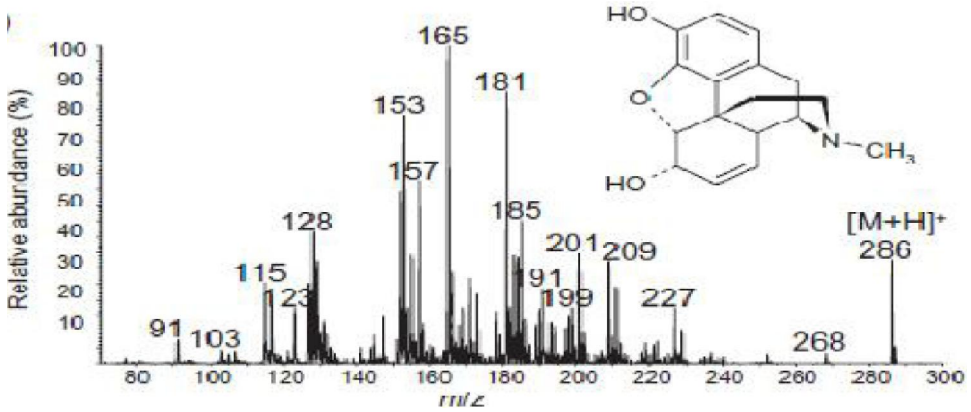

Figure 4 : ESI product ion mass spectra of protonated molecules $[M+H]^+$ of morphine (mol. wt. =285)

as shown in theEI spectrum of morphine (Figure 3) yielding the ions at *m/z* 268, 215, 174, 162, 124, 115, and 70.

Liquid chromatography–(tandem) mass spectrometry

The high proton affinity of most narcotics such as morphine andrelated compounds (e.g., heroin, buprenorphine) or the syntheticopioids pethidine, fentanyl, and its derivatives allows the efficient ionization by means of ESI and, thus sensitive detectionof the active drugs as well as major metabolites in doping controlsamples[32, 33]. The product ion massspectrum of morphine is depicted in Figure 4, which illustrates thefragmentation of the protonated molecule after collisionalactivation.

CONCLUSION

The use of stimulants and narcotics during competition is prohibited in sports since the very beginning and the same is applicable as per 2015 WADA prohibited list. These drugs are known for their medicinal and recreational properties since ancient time. The analysis of stimulants and narcotics most frequently rely on gas chromatography coupled nitrogen–phosphorusdetector (NPD) or mass spectrometric detector (MSD). However, LC–MS/MS has considerably influenced doping control analysis of stimulants and narcotics. This has led to reduced sample preparation efforts and significantly improved detection limits for the determination of stimulants and narcotics in doping control. The future major area of research interest is now focused on metabolic studies of stimulants and narcotics and improvement in their detection techniques employing high resolution mass spectrometry technique.

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