Examples of Doping Control Analysis by Liquid Chromatography–Tandem Mass Spectrometry: Ephedrines, β-Receptor Blocking Agents, Diuretics, Sympathomimetics, and Cross-Linked Hemoglobins

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Abstract

The application of modern and powerful analytical instruments consisting of liquid chromatographs (LCs), sophisticated atmospheric pressure ion sources, and sensitive mass analyzers has improved quality as well as speed of doping control analyses markedly during the last 5 years. Numerous compounds such as β-receptor blocking agents or diuretics require derivatization prior to gas chromatographic (GC) and mass spectrometric (MS) measurement, which is the reason for extended sample preparation periods. In addition, several substances demonstrate poor GC–MS properties even after chemical modification, and peptide hormones such as cross-linked hemoglobins cannot be analyzed at all by means of GC–MS. With the availability of electrospray ionization and robust tandem MSs (e.g., triple-stage quadrupole or ion trap instruments) many new or complementary screening and confirmation assays have been developed, providing detailed qualitative and quantitative information on prohibited drugs. With selected categories of compounds (ephedrines, β-blockers, β_2-agonists, diuretics, and bovine hemoglobin-based oxygen therapeutics) that are banned according to the rules of the World Anti-Doping Agency and International Olympic Committee, the advantages of LC–MS–MS procedures over conventional GC–MS assays are demonstrated, such as enhanced separation of analytes, shorter sample pretreatment, and identification of substances that are not identified by GC–MS.

Introduction

The desire to artificially enhance physical performance in order to increase the chances to win in sports competition is supposedly as old as mankind itself. Already during the ancient Olympic Games, in approximately 200 B.C., athletes used concoctions of mushrooms and plant seeds, as well as special diets including bovine and canine testicles, as reported by Philostratos and Galen (1,2). The attempt to improve the power and strength of racing animals also has a long history, as it has been reported that the ancient Romans fed their horses so-called "Hydromel", a mixture of honey and water, in order to increase endurance (3,4).

The manipulation of the capacities of an athlete or animal has been referred to as "doping" since 1889, when this term, originating from a dialect spoken in the south-eastern region of Africa, was included in a British dictionary. With the athlete Linton, the first authentic doping-related death occurred in 1886 during a cycle race (Paris–Bordeaux), caused by an overdose of caffeine (2). Nevertheless, sports history that followed has shown numerous cases of doping or attempts to medicinally improve performance; for example, in 1963, Venerando (5) described results of investigations concerning drug abuse in certain sport categories (mainly soccer and cycling) demonstrating "positive" cases ranging from 14% to 46% of the analyzed samples. These numbers were even more unintelligible because of the frightening records of a series of severe intoxications and deaths related to drug abuse during sporting events. Based on these facts, doping control analyses have been performed systematically since 1968 according to the list of prohibited substances and methods of doping (6), established by the Medical Commission of the International Olympic Committee (IOC). With technical innovations, improvements and availability of analytical tools, procedures have been developed based on gas chromatography (GC) interfaced to different kinds of analyzers such as a flame ionization detector, nitrogen phosphorous detector (NPD), or mass spectrometer (MS) (7–22). High-performance liquid chromatography (HPLC) coupled to UV detectors (23–27), as well as immunochemical assays, have been used frequently (28–30). The connection between HPLC and MS has been a challenging task, and several interfaces such as "particle beam" and "thermospray", have demonstrated drawbacks such as insufficient sensitivity for most of the necessary applications relevant for doping control analysis. With

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the introduction of electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photo ionization, new possibilities have arisen that may be employed in various established procedures for sensitive, selective, and fast determination of prohibited compounds in doping control samples. This paper covers several modern and commonly used applications based on LC–MS (MS–MS) that have been recently developed to cover prohibited classes of compounds that are difficult or even impossible to detect or quantitate by other conventional chromatographic/MS techniques.

Ephedrines

In 1967, a global basis of doping control analysis was developed by the detection of stimulants such as amphetamines and ephedrines in human urine (31). At that time, doping controls were performed primarily by means of gas–liquid chromatography (GLC) as well as thin-layer chromatography (TLC) (31,32), and in selected cases, supportive information regarding positive samples was obtained by derivatization, further GLC, TLC, and MS or micro-IR spectroscopy. These techniques were based on a multitude of analytical procedures primarily developed for the investigation of metabolism, pharmacokinetics, and toxicology (33–39). The classical experiments employing colorimetric determinations of amphetamines (40–42) suffered from interferences in biological matrices, until a first, commonly-accepted procedure was established based on liquid extraction, paper chromatography, and visualization (43). More comprehensive and sensitive procedures were established based on GC that was interfaced to NPD (44,45), and with the use of MS, the developed screening and confirmation methods gained even more specificity. For stimulants such as ephedrine, phenylpropanolamine, and their stereoisomers pseudoephedrine and cathine, respectively, the list of prohibited substances and methods of doping provides different regulations. Thus, for suspicious urine samples, a quantitative analysis of the detected compounds is necessary, which includes the chromatographic separation of stereoisomers as an ephedrine threshold has been established at 10 µg/mL, and pseudoephedrine has not been considered a banned substance since January 2004. The GC properties of ephedrines regarding separation of isomers has proven to be a challenging task. Without chemical treatment of the analytes, a differentiation of ephedrine and pseudoephedrine is barely possible, but by means of selective derivatization of hydroxyl and amine functions with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (46) and *N*-methyl-*N*-bistrifluoroacetamide (47,48), a GC isolation of the derivatives was accomplished. Because of the time-consuming effort of sample preparation, a relatively high concentration of ephedrines in the urine samples, and adequate LC behavior of ephedrines, an alternative approach to qualitatively and quantitatively determine these compounds in doping control samples was developed utilizing LC–MS–MS instrumentation. The suspicious urine samples are fortified with the triply-deuterated analogue of ephedrine, and without any further sample preparation, aliquots of a volume of 2 µL are injected into the LC–MS–MS instrument. Based on multiple reaction monitoring, qualitative evidence for the identity of the analyte, as well as quantitation, is accomplished (49). Utilizing an isocratic flow of 1.25 mL of a 5mM ammonium acetate buffer (containing 0.1% acetic acid) and acetonitrile (97:3), a

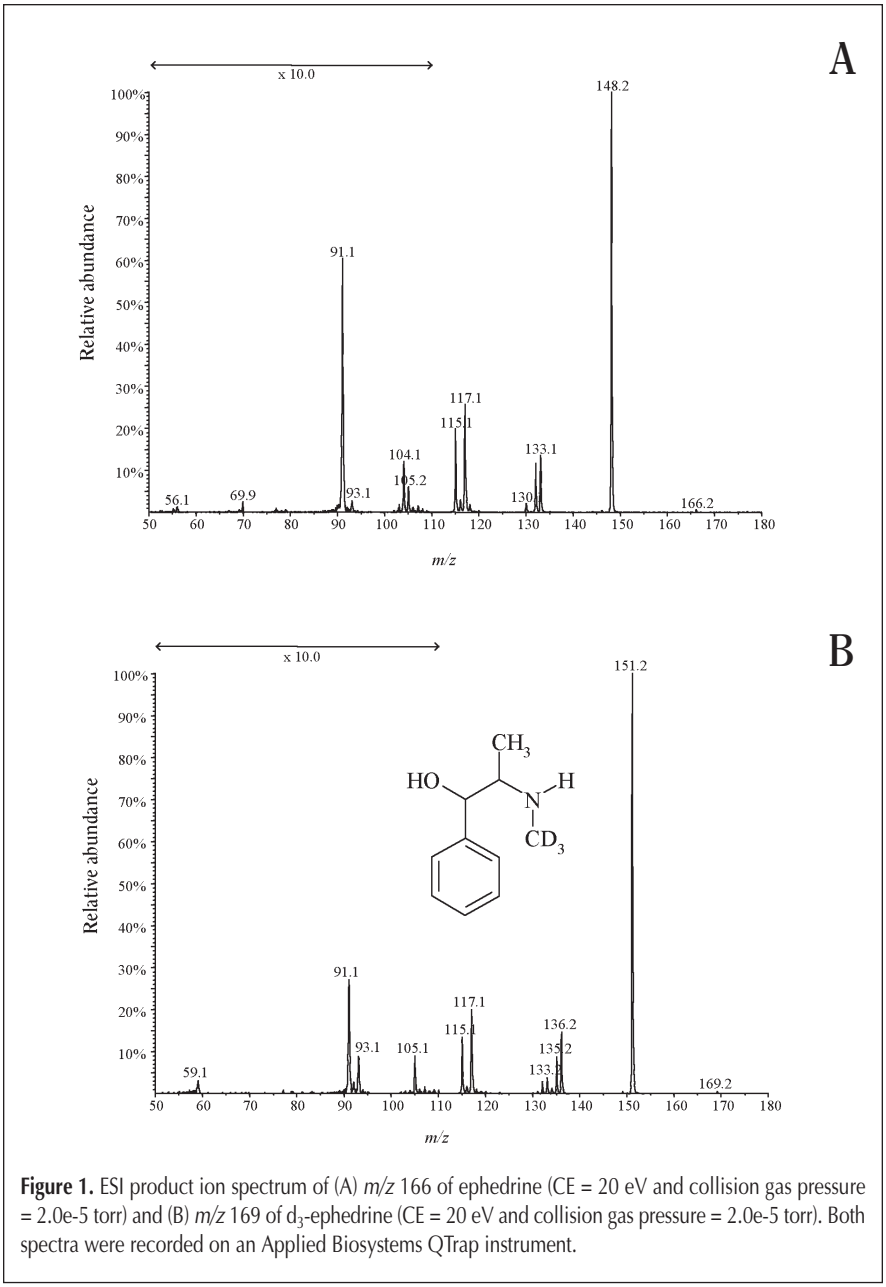

Figure 1. ESI product ion spectrum of (A) *m/z* 166 of ephedrine (CE = 20 eV and collision gas pressure = 2.0e-5 torr) and (B) *m/z* 169 of d_3-ephedrine (CE = 20 eV and collision gas pressure = 2.0e-5 torr). Both spectra were recorded on an Applied Biosystems QTrap instrument.

single analysis is performed within 5 min on a reversed-phase C18 column (4.6 x 55 mm). In Figure 1, the product ion spectra of *m/z* 166 of ephedrine and *m/z* 169 of the internal standard (d_3-ephedrine) are shown. Although the ion transitions *m/z* 166–148 and 169–151 provide signals for quantitation purposes, additional transitions such as *m/z* 166–117, 115, and 91 enable the unambiguous identification of ephedrine or its stereoisomer in combination with their chromatographic separation. A typical analytical result with extracted ion traces *m/z* 166–148 and 169–151 is demonstrated in Figure 2. A comparable approach has been recently published by Truillo and Sorenson in 2003 (50).

Beta-receptor blocking agents

Since 1988, the class of β-receptor blocking agents (β-blockers) is prohibited according to the rules of the IOC during competition for selected sports (6), such as ski jumping, shooting, curling, etc. β-Blockers are commonly administered for the treatment of angina pectoris, control of hypertension, and relief of intraocular pressure (51–53); but presumably because of their effect of heart rate reduction, they have been detected in several urine specimens of doping control samples. In general, most of the β-blockers are either composed by a substituted phenolic ring structure bearing an oxypropanolamine side chain terminating in an isopropyl- or tert-butyl residue (e.g., propranolol and talinolol), or by derivatives of phenylethanolamines (e.g., sotalol and nifenalol). Some exemptions from this generic structure were also developed (e.g., nebivolol and carvedilol). Depending on the physicochemical properties of the drugs, β-blockers are metabolized to a certain extent. Although less lipophilic compounds such as atenolol are renally excreted mainly unchanged, more lipophilic substances such as propranolol are widely oxidized, hydroxylated or dealkylated, and also conjugated, giving rise to glucuronides or sulfates. Therefore, comprehensive screening procedures include sample preparation steps entailing the hydrolysis of conjugates before extraction, derivatization, and subsequent GC–MS analysis. Different approaches were demonstrated in the literature to increase the volatility of β-blockers in order to improve the GC properties (54–56), and adequate procedures are available to doping control laboratories. However, the use of LC–MS–MS instruments also overcomes some drawbacks of GC–MS (e.g., a time-consuming derivatization). Also, for numerous compounds belonging to the class of β-blockers, improved detection limits were demonstrated as meeting the demands and requirements of comprehensive, fast, and robust doping control procedures (18,57). Nowadays, more than 30 substances are analyzed in a single LC–MS–MS run within 5 min, and limits of detection vary between 10 and 100 ng/mL. Therefore, 2-mL aliquots of urine samples are fortified with 500 ng of the internal standard bupranolol. This is followed by an enzymatic hydrolysis employing β-glucuronidase–aryl sulphatase from *Helix pomatia*, liquid–liquid-extraction (LLE) of analytes at pH 9.6 into tert butyl methyl ether, concentration, and subsequent LC–ESI-MS–MS analysis on a C18 reversed-phase HPLC column (4.6 × 55 mm) utilizing the solvents (A) 5mM ammonium acetate (containing 0.1% acetic acid) and (B) acetonitrile. Figure 3 shows part of a typical test urine sample containing 50 ng/mL of talinolol, carvedilol, and propranolol. The extracted ion traces correspond to individual and abundant ion transitions of the selected compounds. In Figure 4A, the product ion spectrum of the representative β-blocker propranolol is shown. Because of the closely related molecular structures of a series of β-blockers, common fragmentation routes yielding identical or related fragment ions (or both) are observed, such as $(M+H)^+$ 77 and *m/z* 116. A proposed origin of these ions is presented in Figure 4B. The knowledge about fragmentation and

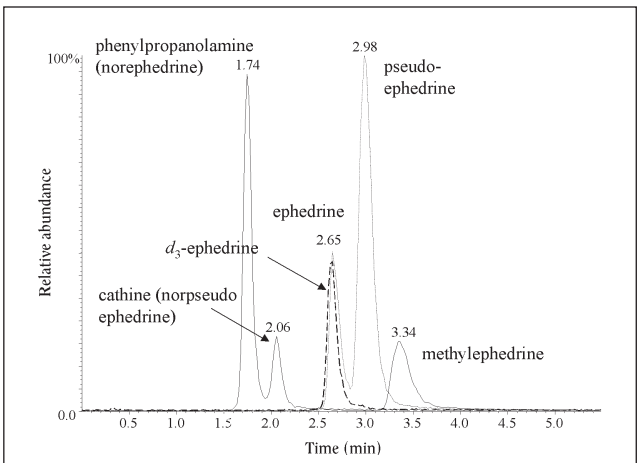

Figure 2. Extracted ion chromatogram of a urine sample containing phenylpropanolamine (*m/z* 152–134), cathine (*m/z* 152–134), ephedrine (*m/z* 180–162), pseudoephedrine (*m/z* 166–148), d_3-ephedrine (*m/z* 169–151) and methylephedrine (*m/z* 181–163).

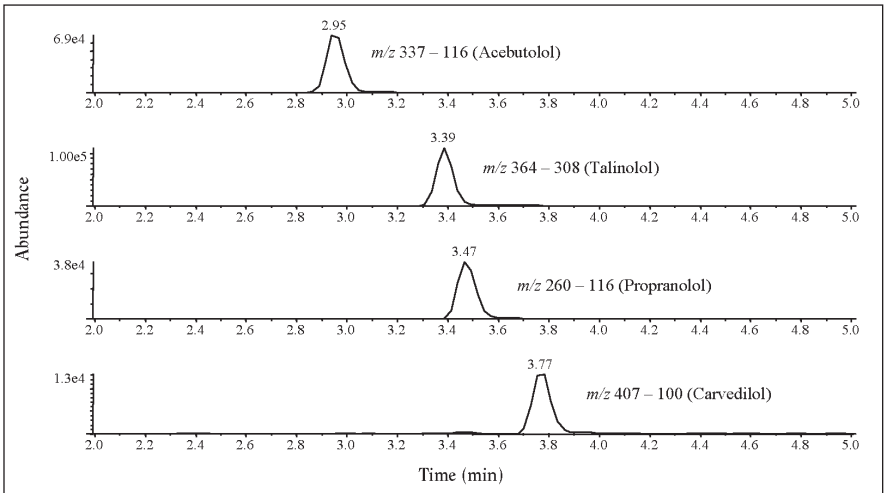

Figure 3. Typical extracted ion chromatogram of a test urine sample containing 50 ng/mL of acebutolol, talinolol, propranolol, and carvedilol recorded on an Agilent (Palo Alto, CA) 1100 Series LC interfaced by APCI to an Applied Biosystems API2000 triple quadrupole MS. The extracted ion traces correspond to specific and abundant ion transitions of the selected compounds.

origin or generation of characteristic fragment ions in CAD spectra supports and substantiates the identification of prohibited compounds that are known, new, and related to doping-relevant substances.

Diuretics

In contrast to β-receptor blocking agents, diuretics are prohibited in every sport discipline in, as well as out, of competition. Thus, all urine samples taken from athletes selected for doping controls are screened for diuretic agents. On the one hand, the need to test for diuretics is based on its weight-reducing effect caused by increased renal elimination of water, the importance of which is apparent in sports that are organized in weight classes. On the other hand, an increased urine flow can lower the concentration of renally excreted compounds that are prohibited when exceeding an established cut-off limit (e.g., the nandrolone metabolite norandrosterone, caffeine, or ephedrine). Therefore, the possibility of manipulation of doping control samples by administration of diuretic agents exists with all specimens requiring a comprehensive doping analysis. The wide variety of diuretics and their heterogeneous chemical structures and physicochemical properties have proven to complicate the development of broad and capacious screening procedures for doping control purposes. Several representatives of this category bear acidic functions (e.g., bumetanide, piretanide, furosemide, and ethacrynic acid), but others are basic compounds such as amiloride and triamterene. In addition, numerous benzothiadiazines (e.g., ethiazide, epithiazide, and althiazide) demonstrate poor GC behavior even after derivatization, and osmotic diuretics such as mannitol are hardly compatible with most of the sample preparation and extraction procedures. Several approaches based on GC–MS, HPLC–UV, and HPLC–plasmaspray were developed in the past (27,58–60) to cope with the complex class of diuretic agents by means of procedures including extractive alkylation, solid-phase extraction (SPE), multiple liquid–liquid extractions under different pH conditions, and derivatization. Employing LC–ESI–MS–MS or LC–APCI–MS–MS, most of the analytes belonging to the class of diuretics are identified in human urine at reasonable detection limits with high specificity as demonstrated in several studies (61–63). Exceptions are generally the osmotic diuretics (such as mannitol), which are neither isolated from urine by commonly accepted sample preparation procedures, nor are these compounds efficiently ionized by ESI or APCI under the given conditions. Different strategies here have been evaluated in order to identify mannitol and its stereoisomers in human urine (64). Because of the heterogeneity of diuretics regarding acidity and basicity, the ionization mode (positive or negative) is also a crucial step of analysis. Although the majority of diuretics with thiazides (e.g., ethiazide, epithiazide, and althiazide), benzoic acid derivatives (e.g., bumetanide, piretanide, and furosemide), benzamide derivatives (e.g., indapamide), and benzanilide derivatives (e.g., xipamide and clopamide) are ionizable by deprotonation (65,66), several drugs such as triamterene, amiloride, and canrenone are only ionized in the positive mode. Hence, analytical procedures have to cover both polarities, either by switching ionization modes scan by scan, or by establishing chromatographic groups of compounds that are ionizable by protonation, deprotonation, or both (63). LC–MS–MS assays utilized in routine doping control analyses cover up to 32 diuretics and masking agents within a single analysis (61), and a typical extracted ion chromatogram of a urine sample containing furosemide, bumetanide, althiazide, and xipamide at 100 ng/mL is presented in Figure 5. Here, a 2-mL aliquot of urine is fortified with 200 ng of mefruside followed by SPE on PAD-1, concentration, and subsequent LC–MS–MS analysis on a reversed-phase C18 column (4.6 × 55 mm) using the solvents: (A) 5mM ammonium acetate (containing 0.1% acetic acid)

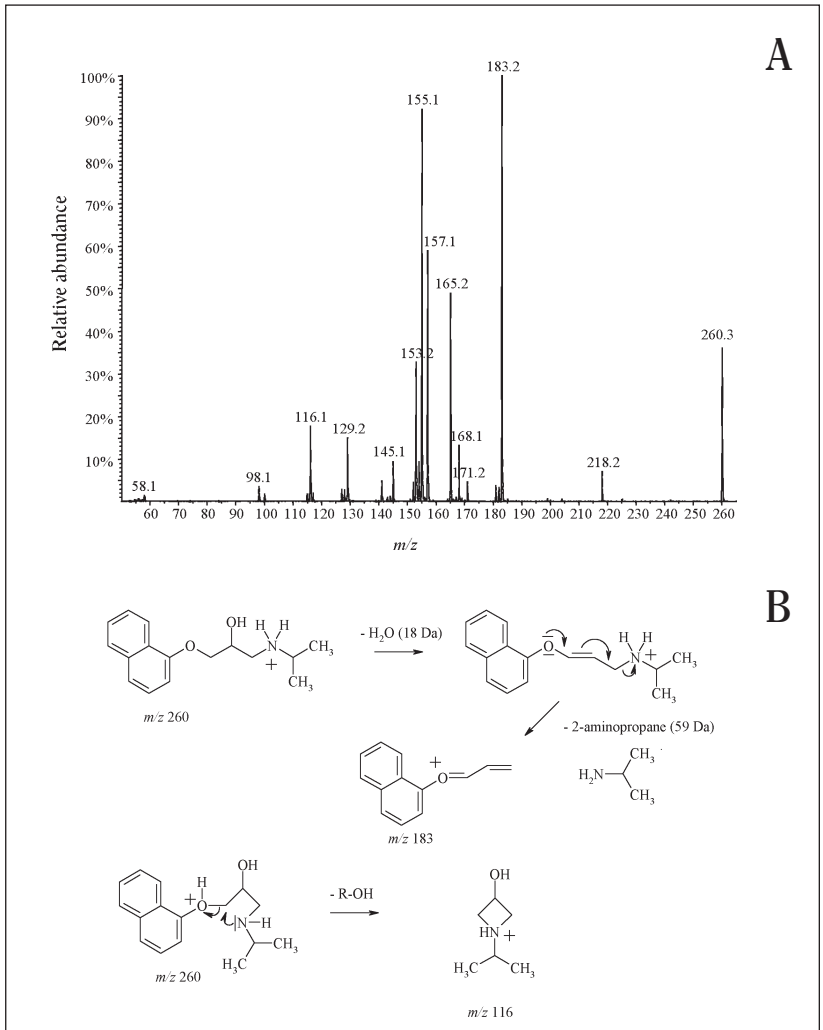

Figure 4. (A) ESI product ion spectrum of *m/z* 260 of propranolol (CE = 20 eV and collision gas pressure = 2.0e-5 torr) recorded on an Applied Biosystems QTrap instrument and (B) proposed fragmentation route of $(M+H)^+$ to common fragment ions $(M+H)^+$ 77 (*m/z* 183) and *m/z* 116.

and (B) acetonitrile at a flow rate of 800 µL/min. A single run is completed within 8 min.

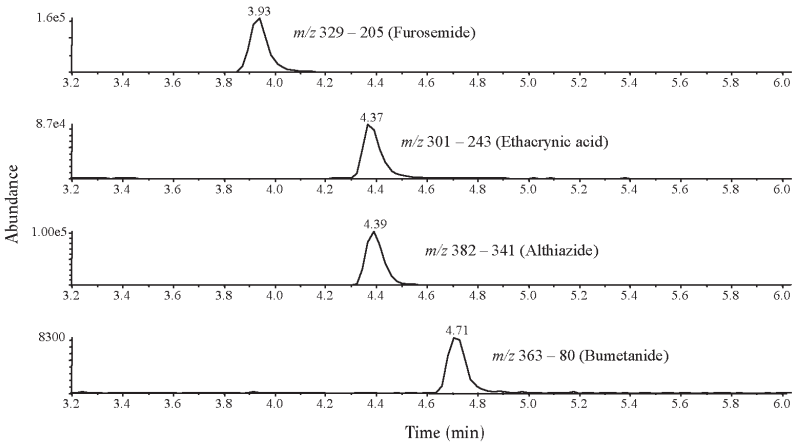

Figure 5. Extracted ion chromatogram of a urine sample fortified with 100 ng/mL of furosemide, ethacrynic acid, althiazide, and bumetanide, analyzed on an Agilent 1100 Series LC interfaced by APCI to an Applied Biosystems API2000 triple quadrupole MS. Ionization was performed by deprotonation (negative mode).

Beta-2-Agonists

Sympathomimetic agents (β_2-agonists) such as clenbuterol, fenoterol, formoterol, and many others are commonly used for treatment of pulmonary diseases such as bronchial asthma or bronchial hyperreactivity (for instance exercise-induced asthma) because of their bronchodilator activity (67). The routes of administration are either orally or via inhalation by means of pressurized metered-dose aerosols. Significant differences in the amounts applied in a single dose are observed within this category of drugs. In the literature, stimulation as well as anabolic effects are described as correlated with the intake of β_2-agonists at levels higher than therapeutically indicated. This includes an increase in heart rate and force of myocardial contraction, incremental gain of muscle protein, and decrease in fat deposition (68–71). Sympathomimetics have been prohibited since 1992 according to the IOC list of prohibited substances and methods of doping (6). They are classified as stimulants as well as anabolic agents with specific regulations in terms of competition and out-of-competition testing. In competition, all β_2-agonists are considered as stimulants (except for salbutamol, salmeterol, terbutaline, and formoterol via inhalation), but only clenbuterol and salbutamol are referred to as anabolic agents. In addition, a limit of 1 µg/mL has been established for free urinary salbutamol in out-of-competition controls. Common screening and confirmation methods for sympathomimetic agents in human urine samples are based on GC–MS (72–74) but usually require complex derivatization of analytes. For several years, qualitative and quantitative analyses of β_2-agonists in different fields of analytical chemistry are also performed employing LC–MS and LC–MS–MS techniques (75–80), demonstrating excellent ionization properties, specificities, and detection limits of modern ion sources interfacing LC to mass analyzers. Sympathomimetics in particular are efficiently protonated and introduced into single- or multistage MSs by ESI, and they give rise to informative and characteristic product ion spectra (as demonstrated in Figure 6, with mabuterol at two different collision energies). The common nucleus of β_2-agonists consists of a singly- or multiply-substituted phenyl residue bearing an ethanolamine side chain that terminates in either a tert butyl residue, an isopropyl group, or in few functionalities different from these common structures. This common nucleus is the basis of sample preparation strategies enabling the detection of numerous sympathomimetics and also for interpretation of their product ion spectra. Some common dissociation behaviors and elimination reactions of β_2-agonists after ESI and CAD are presented in Figure 7 (80), including loss of water, release of a propene or isobutene residue, and generation of side-chain-specific fragment ions as

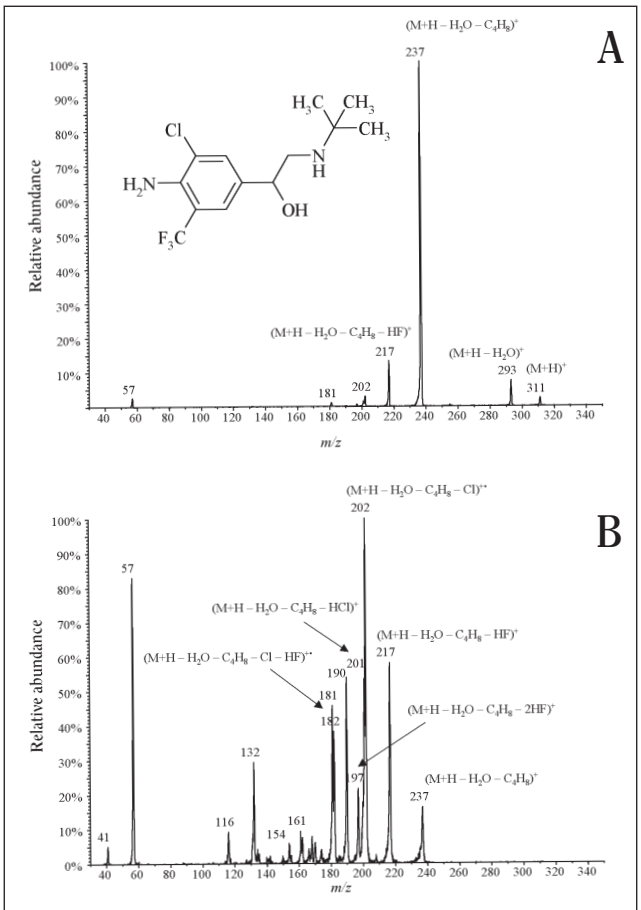

Figure 6. ESI product ion spectrum of (A) *m/z* 311 of mabuterol (CE = 25 eV and collision gas pressure = 2.0e-5 torr) recorded on an Applied Biosystems API2000 instrument and (B) *m/z* 311 of mabuterol (CE = 50 eV and collision gas pressure = 2.0e-5 torr). Common fragmentations of β_2-agonists included an initial loss of water (–18 Da) and a subsequent elimination of isobutene (–56 Da) whenever an ethylamine side chain was present, terminating in an isobutyl residue. With mabuterol in particular, further neutral losses were observed by removal of HF (–20 Da) and HCl (–36 Da), as well as the loss of a chlorine radical (–35 Da).

demonstrated with the compounds cimaterol and fenoterol. The β_2-agonists fenoterol, reproterol, and terbutaline are known to incorporate a methylene unit in the presence of formaldehyde under acidic conditions according to the Pictet–Spengler reaction (81). This reaction yields tetrahydroisoquinoline derivatives, and the corresponding artifacts of fenoterol and terbutaline are observed in excretion study specimens (presumably generated during sample preparation), which are also utilized as target compounds in screening and confirmation procedures (80). In addition, the drug formoterol leads to an artifact produced by deformylation that entails the absence of a parent compound in fortified urine samples after acidic hydrolysis. Here, identification of the prohibited substance is based on the detection of the degradation product.

Except for the derivatization of analytes (in order to increase volatility of β_2-agonists), sample pretreatment can be transferred from established GC–MS (and tandem MS) methods directly to newly developed LC–MS–MS procedures, providing appropriate tools for doping control analysis with reduced time efforts and improved detection capabilities (e.g., with salmeterol and deformylated formoterol). Samples are fortified with 250 ng of the internal standard clenbuterol-d_6, hydrolyzed under acidic conditions, purified by LLE, and the analytes are extracted into tert butyl methyl ether at pH 9.6, followed by concentration and subsequent LC–MS–MS analysis on a C18 reversed-phase HPLC column (4.6 × 55 mm). The solvents (*a*) 5mM ammonium acetate (containing 0.1% acetic acid) and (*b*) acetonitrile were used at a flow rate of 800 µL/min. In Figure 8, an LC–MS–MS chromatogram of a fortified urine sample containing 5 ng/mL of clenbuterol and 50 ng/mL of fenoterol–mabuterol–salmeterol is presented as an example.

Bovine hemoglobin-based oxygen therapeutics

The quest for a perfect artificial oxygen carrier to substitute for stored blood has been of eminent interest ever since shortages of blood arose, and contagious diseases require time-consuming blood screenings before transfusion in addition to tests regarding suitability in terms of blood type and rhesus factor (82). Moreover, blood samples tested and stored at 4°C until infusion are outdated after a period of 3–8 weeks, depending on additives such as stabilizers. Thus, the development of biocompatible, more conveniently handled blood substitutes has been pursued for years. Oxygen therapeutics based on synthetic perfluorocarbons as well as various hemoglobin (Hb) derivatives from different species have been investigated intensively with diverse results (83–85). Intra- or intermolecular crosslinking (or both) of Hb has proven to maintain the stability of hemoglobin molecules and to improve oxygen off-loading by a decreased oxygen affinity, but cell-free Hb lacks the capability of reversible oxygen binding and release (owing to the absence of its polyanionic effector 2,3-diphosphoglycerate) (87). Moreover, its tetrameric structure dissociates into α,β-dimers without crosslinking, giving rise to nephrotoxicity (88). Different preparations have been investigated using either bovine (89) or human hemoglobin crosslinked by various molecules such as *o*-raffinose (e.g., Hemolink), diaspirin (e.g., HemAssist), or glutaraldehyde (e.g., PolyHeme and Hemopure), some of which have limited approval for animal or human application (85) and others that have been withdrawn from clinical studies because of side effects (83,84).

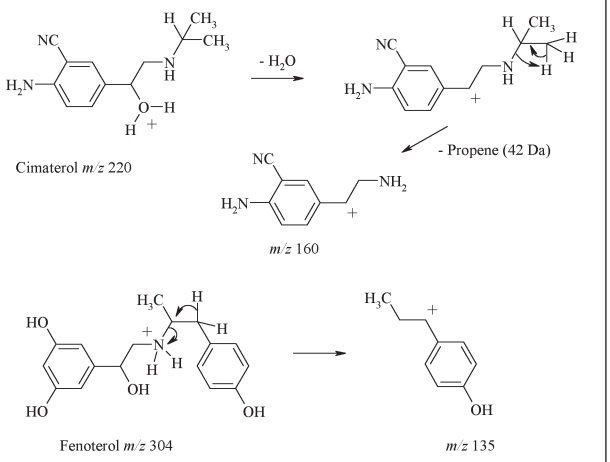

Figure 7. Common fragmentation behavior of β_2-agonists after ESI and CAD demonstrated with cimaterol and fenoterol. The loss of water followed by elimination of propene or isobutene of compounds bearing a isopropyl or *tert.*-butyl group, respectively, was observed frequently. Substances such as fenoterol comprising a larger residue, including a phenolic structure, also generated side-chain-specific fragment ions.

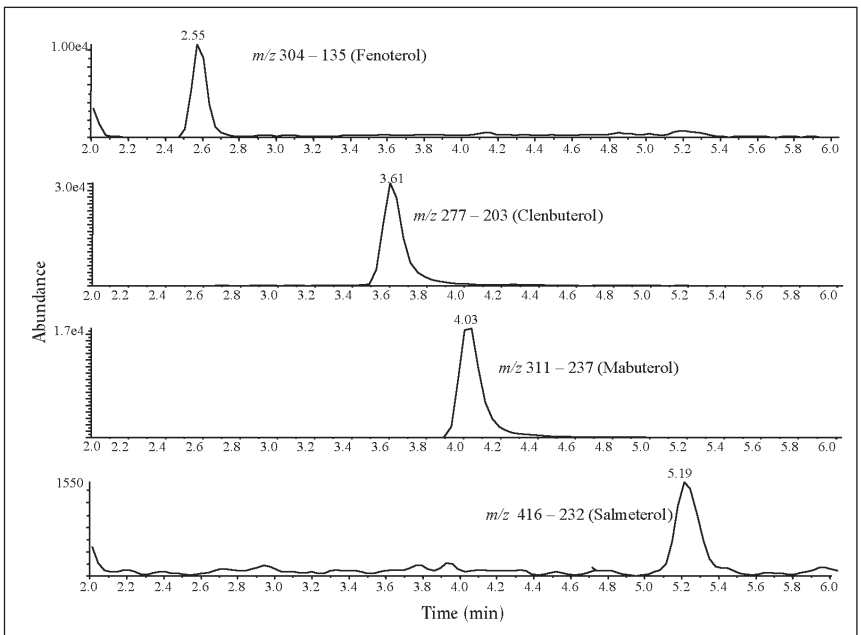

Figure 8. Extracted ion chromatogram of a urine sample fortified with 5 ng/mL of clenbuterol, and 50 ng/mL of fenoterol, mabuterol, and salmeterol, analyzed on an Agilent 1100 Series LC interfaced by APCI to an Applied Biosystems API2000 triple quadrupole MS. Ionization was performed in the positive mode by ESI.

Regarding doping control analysis, artificial oxygen carriers are of interest because of their capabilities to enhance oxygen transport during exercise. Especially, elite athletes performing an endurance sport may try to improve their maximum oxygen uptake and transport by supportive drugs (90). According to this notion, artificial oxygen carriers have been added to the list of prohibited substances of the IOC in January of 2000 (6). One of the therapeutics approved in several countries for animal and human application is Hemopure, which is based on bovine hemoglobin (91) that is intra- and intermolecular crosslinked by glutaraldehyde units at the amino acid residue Lys_{99} of the α-chain, as demonstrated recently (92). Hemoglobin (including numerous natural variants) and also several Hb-based crosslinked oxygen therapeutics have been investigated extensively using the appropriate techniques such as ESI-MS–MS (93–97) or matrix-assisted laser desorption ionization-MS (87,98–100). Moreover, species determination was performed successfully (100). Based on these results, a method for doping control purposes was established that employs LC–ESI-MS–MS analysis of bovine hemoglobin-specific peptides, obtained by enzymatic hydrolysis (101). Here, 50 µL of plasma is diluted to 500 µL with deionized water, filtered through a membrane with a molecular weight cutoff of 100 kDa, and 20 µL of the retentate is fortified with 100 µg of bovine carbonic anhydrase as the internal standard. To the mixture, 1 µg of trypsin (dissolved in 50 µL of 50mM ammonium bicarbonate) and 30 µL of acetonitrile are added, and samples are incubated at 37°C for at least 4 h. A volume of 5 µL of glacial acetic acid is then added (terminating trypsin activity) and the solution is applied to a 10 kDa filter unit. The filtrates are diluted by an equal amount of deionized water, transferred to HPLC vials, and 5 µL is injected into the LC–ESI-MS–MS system employing a reversed phase C18 column (2.1 × 50 mm, pore size 300Å) and the solvents of (A) 0.1% acetic acid (containing 0.01% trifluoroacetic acid) and (B) acetonitrile–water (80:20, v/v, containing 0.01% trifluoroacetic acid) at a flow rate of 200 µL/min. By means of data banks such as SwissProt (102), predicted peptide sequences and masses are calculated for tryptic digestions of human and bovine hemoglobin as well as carbonic anhydrase. Owing to a sequence homology of 85% of human and bovine Hb, LC–ESI-MS–MS measurements are primarily focused on individual peptides generated from artificial oxygen carriers (i.e., cross-linked bovine hemoglobin). For instance, the peptide originating from the α-chain comprising the amino acid residues 69–90 (AVEHLDDLPGALSELSDLHAHK, 2367.2 Da) is not produced by an enzymatic hydrolysis of human Hb and gives rise to a product ion spectrum presented in Figure 9A, obtained by CAD of the triply charged molecule at *m/z* 790. Another example of a bovine hemoglobin-related peptide is composed by the amino acid residues 40–58 of the β-chain (FFESFGDLSTADAVMNNPK, 2089.9 Da), the product ion spectrum of which is shown in Figure 9B, recorded after CAD of the doubly charged molecule at *m/z* 1046.0. Besides several others, those peptides are detectable in human plasma and enable the sensitive and specific determination of bovine hemoglobin. This procedure does

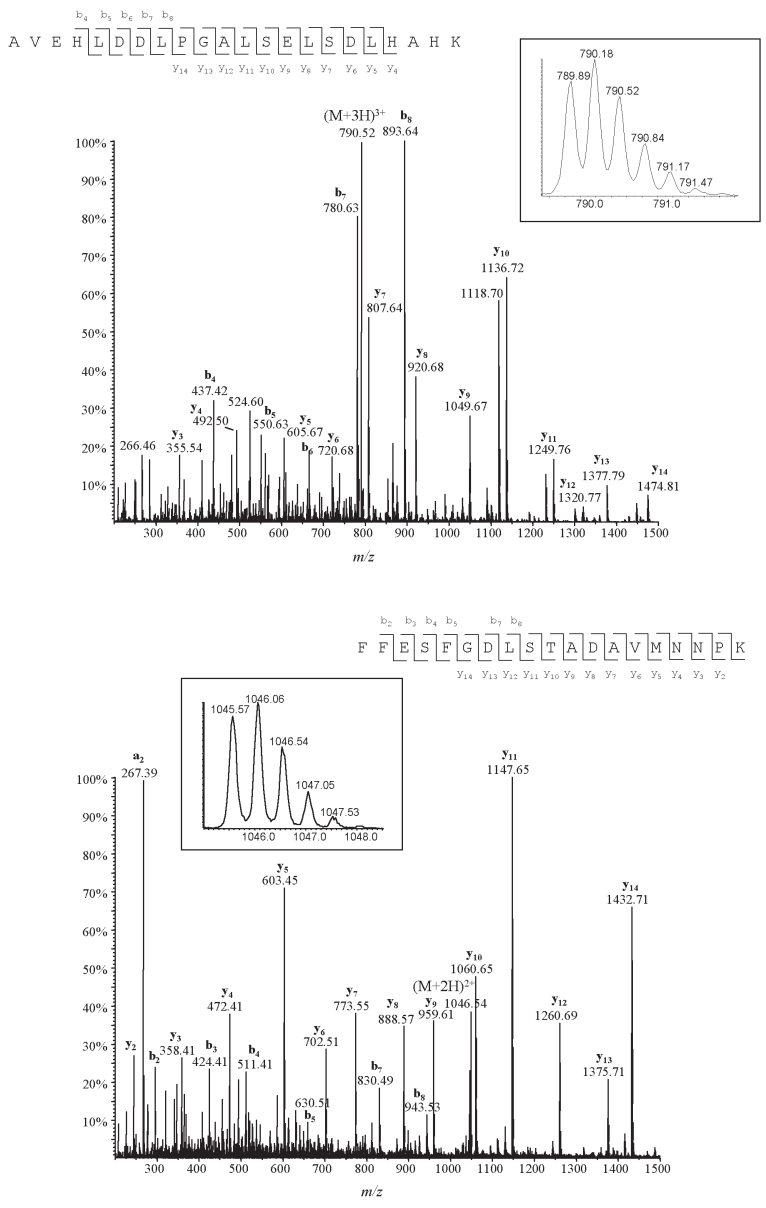

Figure 9. ESI product ion spectrum (A) triply charged molecule at *m/z* 790.4, and (B) doubly charged molecule at *m/z* 1046.2 of bovine hemoglobin specific peptides (CE = 50 eV and collision gas pressure = 4.0e-5 torr). Both spectra were recorded on an Applied Biosystems QTrap instrument and provided comprehensive amino acid sequence tags for protein identification purposes.

not provide information on the cross-linker because the analysis is not focused on connected peptides, but the mere presence of bovine Hb in the plasma specimens of elite athletes is evidence of the administration of oxygen carriers.

Conclusion

With the availability of LC interfaced to MS by sophisticated ionization techniques such as ESI and APCI, doping control analysis has many powerful options for specific, sensitive, fast, and robust procedures. Those provide, in combination with GC–MS assays, a comprehensive screening platform to analyze hundreds of prohibited compounds in urine samples of elite athletes. From this basis, and also new tasks and challenges, will innovative drugs putatively abused in sports be handled.

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