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Characterization of chemically modified steroids for doping control purposes by electrospray ionization tandem mass spectrometry

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The discovery of the designer steroid tetrahydrogestrinone (THG) in elite athletes' doping control samples in 2003 demonstrated the availability of steroid derivatives prepared solely for doping purposes. Modern mass spectrometers utilizing electrospray ionization and collisionally activated dissociation (CAD) of analytes allow the structural characterization of steroids and their derivatization sites by the elucidation of fragmentation behaviors. A total of 21 steroids comprising either a 4,9,11-triene, a 3-keto-4-ene or a 3-keto-1-ene nucleus were investigated regarding their dissociation pathways, deuterated analogues were synthesized and fragmentation routes were postulated, permitting the identification of steroidal structures and modifications. Compounds based on a 4,9,11-triene steroid with an ethyl residue at C-13 (gestrinone analogues) generate abundant fragment ions at *m/z* 241 and 199, whereas the substitution of the C-13 ethyl group by a methyl residue (trenbolone analogues) results in a shift of *m/z* 241 to 227. Substances related to testosterone with a 3-keto-4-ene structure give rise to abundant fragment ions at *m/z* 109 and 97 whereas steroids with a 3-keto-1-ene nucleus eliminate the A-ring including the carbons C-1–C-4, in addition to C-19 that is proposed to migrate from C-10 to C-1 under CAD conditions. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: designer steroids; doping; electrospray ionization; mass spectrometry; fragmentation

INTRODUCTION

Ever since anabolic androgenic steroids were prohibited by international sports federations such as the International Olympic Committee (IOC)[1] and the World Anti-Doping Agency (WADA), representatives of this class of drugs have frequently been detected in urine specimens from elite athletes. Doping offenses with remedies developed to treat emaciation or debilitating diseases[2] were revealed by the determination of the administered drug, its metabolites and/or their carbon isotope ratios, in particular by means of mass spectrometry (MS) following chromatographic separation by gas chromatography (GC) or liquid chromatography (LC). In 2002, an anabolic agent termed norbolethone (13-ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-3-one, Scheme 1, **18**) was detected in a doping control urine sample[3] although this compound was never marketed by pharmaceutical companies, indicating the willingness of some athletes to employ chemicals without medical approval in order to promote muscle growth and strength. In October 2003, the discovery of another steroid named tetrahydrogestrinone (THG, 13-ethyl-17-hydroxy-18,19-dinor-17α-pregn-4,9,11-trien-3-one, Scheme 1, **3**) triggered an even greater avalanche in the world of sport[4–7] as several elite athletes were convicted of its abuse and sanctioned by WADA. THG was presumably prepared from gestrinone (Scheme 1, **1**), a drug utilized for the treatment of endometriosis,[2] by catalytic hydrogenation of the 17-ethinyl function, and its effects on human or animal physiology have never been clinically tested. As a consequence of gestrinone hydrogenation, conventional screening procedures of doping control laboratories did not register the presence of the resulting prohibited compound, because most analytical assays are based on target analysis, i.e. the determination of known compounds or their metabolites.[8–12] Mass spectrometry has proven manifold to be a powerful tool for clinical, forensic and doping control purposes, and it has been employed routinely for decades for the detection and identification of drugs in various biological matrices. In order to understand common and individual mass spectrometric properties of analytes of interest, numerous studies on many classes of substances have been performed in the past, in particular on steroids after electron ionization by Djerassi and co-workers.[13–17] To improve the comprehensiveness of doping controls, the enormous potential of modern mass spectrometric instruments have to be exploited, in particular of those analyzers

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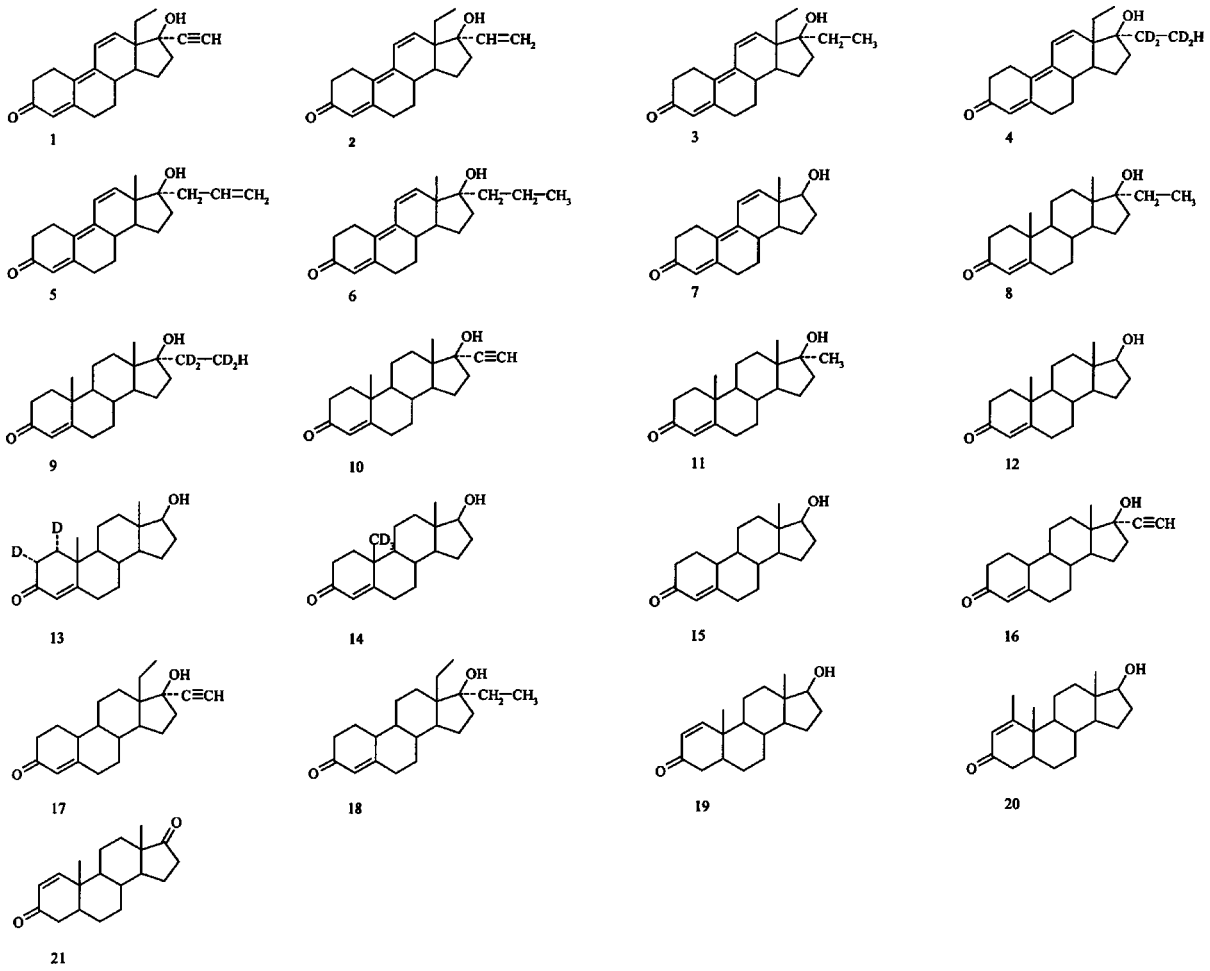


Scheme 1. Structure formulae of steroids investigated by means of ESI and CAD: gestrinone (**1**), dihydrogestrinone (DHG, **2**), tetrahydrogestrinone (THG, **3**), d_4-THG (**4**), altrenogest (**5**), propyltrenbolone (**6**), trenbolone (**7**), 17-ethyltestosterone (**8**), d_4-17-ethyltestosterone (**9**), ethisterone (**10**), methyltestosterone (**11**), testosterone (**12**), 1α,2α-dideuterotestosterone (**13**), 19-trideuterotestosterone (**14**), 19-nortestosterone (**15**), norethisterone (**16**), norgestrel (**17**), norbolethone (**18**), 5α-androst-1-en-17β-ol-3-one (1-testosterone, **19**), metenolone (**20**) and 5α-androst-1-en-3,17-dione (1-androstenedione, **21**).

equipped with atmospheric pressure interfaces allowing the detection and characterization of modified or derivatized steroidal agents. Hence a variety of known steroids, stable labeled counterparts and synthesized analogues (Scheme 1) were investigated in the present study. Common and individual fragmentation routes resulting from collisionally activated dissociation (CAD) after electrospray ionization (ESI) were elucidated for steroids with common nucleic structures, providing information necessary for the identification of alterations and their influences on the mass spectrometric behaviors of analytes relevant for doping control analyses.

EXPERIMENTAL

Chemicals

Platinum(IV) oxide hydrate was obtained from Fluka (Buchs, Switzerland). Palladium on activated carbon (10% Pd) and ethylene glycol (99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrogen and deuterium were bought from Linde (Cologne, Germany). Toluene-4-sulfonic acid (p.a.), glacial acetic acid (p.a.), *n*-pentane (p.a.), and silica gel 60 (70–230 mesh) were purchased from Merck (Darmstadt, Germany). Benzene (dried) was obtained from Aldrich (Deisendorf, Germany). *tert*-Butyl methyl ether (TBME), (distilled before use) and ethyl acetate were bought from Kraemer & Martin (St. Augustin, Germany).

Steroids

5α-Androst-1-en-17β-ol-3-one, gestrinone and altrenogest were obtained from Thinker Chemical (Hangzhou, China). Trenbolone, norgestrel, ethisterone, norethisterone, testosterone, nortestosterone, metenolone and methyltestosterone were purchased from Sigma-Aldrich. 19-d_3-Testosterone was a generous gift from Dr R. Kazlauskas of the Australian Drug and Sport Testing Laboratory (ADSTL). 5α-Androst-1-en-3,17-dione was obtained from Steraloids (Newport, RI, USA). Ethyltestosterone, d_4-ethyltestosterone, norbolethone, dihydrogestrinone, tetrahydrogestrinone, d_4-tetrahydrogestrinone, propyltrenbolone and 1α,2α-dideuterotestosterone were prepared in our laboratory.

Selective hydrogenation and H/D exchange of steroids

Various derivatives of commercially available compounds were prepared by commonly employed derivatization and

hydrogenation procedures,[18] which are described in brief in the following.

*Hydrogenation of gestrinone (**1**)—preparation of DHG (**2**), THG (**3**) and d_4-THG (**4**)*

In order to prepare THG (**3**) or d_4-THG (**4**), 500 mg of **1** (1.6 mmol) were dissolved in 250 ml of methanol in a round-bottomed flask, 10 mg of PtO_2 were added and hydrogen or deuterium, respectively, was flushed into the reaction mixture by means of a glass capillary. After 25 min, the reaction mixture was allowed to stand at room temperature for 30 min for sedimentation of the catalyst, the methanolic layer was transferred to a new flask, evaporated to dryness, and the resulting product was purified from residues of PtO_2 by the addition of 100 ml of deionized water and liquid–liquid extraction into TBME. After evaporation of TBME, pure THG and its d_4-labeled analogues were obtained at purities of >97% and yields of 78 and 81% of theory, respectively.

Compound **2** was prepared from **1** in analogous manner, except that PtO_2 was replaced with palladium on charcoal and 300 µl of pyridine were added to the reaction mixture prior to hydrogenation in order to limit reduction potency.[18] Here, the synthesis yielded 75% of **2** that was further purified by HPLC fractionation. By means of a Merck (Darmstadt, Germany) semi-preparative reversed-phase column (LiChrospher, 250 × 10 mm i.d., particle size 10 µm), **2** was separated from **3** utilizing a gradient composed of (A) deionized water and (B) acetonitrile (97% A to 0% A in 11 min, 0% A until 18 min, re-equilibration at 97% A for 5 min), and the respective fractions were collected and evaporated to dryness, yielding **2** with a purity of >97%.

*Hydrogenation of altrenogest (**5**)—preparation of propyltrenbolone (**6**)*

Propyltrenbolone was obtained by hydrogenation of **5** in accordance to the protocol described above at a purity of >95% and yield of 81%.

*Synthesis of ethyltestosterone (**8**) and d_4-ethyltestosterone (**9**)*

The testosterone analogues **8** and **9** were synthesized from ethisterone (**10**). An amount of 400 mg (1.28 mmol) of **10** was dissolved in 60 ml of benzene containing 30 mg of toluene-4-sulfonic acid and 5 ml of ethylene glycol. The reaction mixture was refluxed with water separation for 16 h, 100 ml of deionized water were added and ethisterone-3-ethylene ketal was recovered by liquid–liquid extraction into 300 ml of TBME. The organic layer was evaporated to dryness and the crude product was stored in a desiccator over phosphorus pentoxide for 12 h. Hydrogenation or deuteration of ethisterone-3-ethylene ketal was performed according to the procedure described above, and subsequently the ketal function was removed by acidic hydrolysis. For this purpose, the intermediate products were dissolved in 50 ml of acetic acid (2 M) and refluxed for 1 h. A volume of 50 ml of deionized water was added, and the desired compounds were extracted into 300 ml of TBME. The organic layer was washed twice with 100 ml of aqueous potassium hydroxide (1 M) and evaporated to dryness. The resulting products were purified by flash chromatography on silica gel (*n*-pentane–ethyl acetate (4 : 1, v/v)), yielding 24 mg (12% of theory) of **8** (purity >95%) and 30 mg (15% of theory) of **9** (purity >95%).

*Hydrogenation of norgestrel (**17**)—preparation of norbolethone (**18**)*

Norbolethone (**18**) was obtained by hydrogenation of 200 mg (0.65 mmol) of **17** (dissolved in methanol) utilizing 20 mg of (1,4-bis(diphenylphosphino)butane)(1,5-cyclooctadiene)rhodium(I)BF_4 as catalyst. Here, PtO_2 or Pd on activated carbon were not an option as their selectivity was not sufficient, resulting in additional hydrogenation of the steroidal A-ring. After complete reduction of the 17-ethinyl residue of **17**, the methanolic solution was evaporated to dryness, 100 ml of deionized water were added and the target compound was extracted into 300 ml of TBME, yielding 173 mg (83% of theory) of **18** (purity >97%).

*H/D exchange of 5α-androst-1-en-17β-ol-3-one (**19**) and metenolone (**20**)*

The exchange of labile hydrogens by deuterium atoms was accomplished by incubation of 5 mg of each steroid in 5 ml of a mixture of CH_3OD, D_2O and 40% NaOD in D_2O (4 : 0.95 : 0.05, v/v/v) for 12 h at 60 °C. The hydrogens located at C-1 and C-4 or C-2, C-4 and C-20 were substituted in **19** or **20**, respectively. The efficiency of deuterium exchange was tested by ESI-MS, and the results were as follows: **19** $d_1 = 4\%$, $d_2 = 100\%$, $d_3 = 88\%$; **20** $d_5 = 18\%$, $d_6 = 100\%$, $d_7 = 64\%$.

Purity

The purity of the synthesized material was determined by HPLC/UV analysis performed on a Hewlett-Packard (Waldbronn, Germany) 1090 Series II HPLC system, equipped with a Nucleosil 120-5 C_{18} column (Macherey–Nagel, Düren, Germany) of 120 × 4 mm i.d. The solvents used were (A) distilled water and (B) acetonitrile. A gradient was employed starting with 65% A decreasing to 30% A in 14 min at a flow-rate of 1 ml min^{-1}. Wavelengths of 208, 246 and 350 nm and also full UV spectra were utilized for the detection of target compounds and contaminants.

NMR spectroscopy

Structure confirmation of the synthesized products was obtained by ^1H NMR spectroscopy performed on a Bruker (Bremen, Germany) Avance DPX 200 instrument. Amounts of 5 mg of each compound were dissolved in d_6-acetone and spectra were recorded at room temperature.

Mass spectrometry

Commercially obtained and synthesized steroids were dissolved in a mixture of 0.1% acetic acid and acetonitrile (1 : 1, v/v) at concentrations of 5 µg ml^{-1} and analyzed by ESI and CAD on an Applied Biosystems (Darmstadt, Germany) QTrap instrument at room temperature. Nitrogen was employed as curtain and collision gas (5.33×10^{-3} Pa) delivered from a Whatman K75-72 nitrogen generator, and samples were introduced into the mass spectrometer by means of a syringe pump at a flow-rate of 5 µl min^{-1}. Declustering potentials were optimized for respective protonated molecules, and collision energies (CE) were adjusted to allow

efficient fragmentation of analytes retaining the precursor ion at a relative abundance of ~10% in product ion spectra. Unit resolution was employed for mass selection in Q1, and the linear ion trap was operated at a scan speed of 1000 u s^{-1}.

With MS^3 experiments, the first precursor ion was isolated in Q1, dissociated in Q2 and the resulting fragment ion serving as second precursor ion was isolated in the linear ion trap of the mass spectrometer and dissociated with different excitation energies providing respective product ion spectra.

Aliquots from H/D exchange experiments were dissolved only in acetonitrile and introduced into the mass spectrometer according to the conditions described above.

RESULTS AND DISCUSSION

NMR spectroscopy

The structural modifications of all synthesized compounds (**2**–**4**, **6**, **8**, **9**, **18**) were confirmed by ^{1}H NMR spectroscopy and correspond to data for the structurally related reference compound **1** and also published data.[7,19]

Mass spectrometry

ESI product ion experiments were performed on all analytes, and in Fig. 1 the resulting tandem mass spectra of **3, 8** and **19** are shown exemplarily. All major common and individual fragment ions of the compounds analyzed are listed in Table 1.

Steroids with 4,9,11-triene nucleus

This class of steroids includes compounds **1**–**7** in Scheme 1. The principal fragmentation behavior of representatives will be discussed by means of the product ion spectrum of substance **3** (Fig. 1(a)). The protonated molecule $[M + H]^+$ at m/z 313 gives rise to a variety of abundant fragment ions upon CAD. The neutral loss of water (−18 u), presumably originating from C-17, generates the product ion at m/z 295. Here, no hydrogen from the ethyl residue linked to C-17 is involved in the elimination process as the deuterated analogue **4** also releases 18 u, proving the origin of the hydrogen necessary for the neutral loss of water from a position remote of the ethyl group at C-17. Several mass spectrometric studies demonstrated that eliminations of water upon electron ionization (EI) from both steroidal and other cyclic organic molecules include hydrogens originating from various positions with particular regard to stereochemical properties. Primarily 1,3- and 1,4-eliminations were observed, and losses of hydrogens adjacent to the hydroxyl function were excluded.[16,20–22]

The fragment at m/z 295 (Fig. 1(a)) subsequently releases an ethyl radical, generating the ion at m/z 266. This is an exception to the commonly accepted 'even-electron rule',[23] several of which have been reported in the literature recently.[24–27] All gestrinone analogues (compounds **1**–**4**) expel 29 u from the fragment ion obtained from the respective protonated molecules after elimination of water (Table 1). Hence the primary origin of the ethyl radical is proposed to be C-13 that is located in an allylic position with respect to a large conjugated 8-π-electron system, explaining the

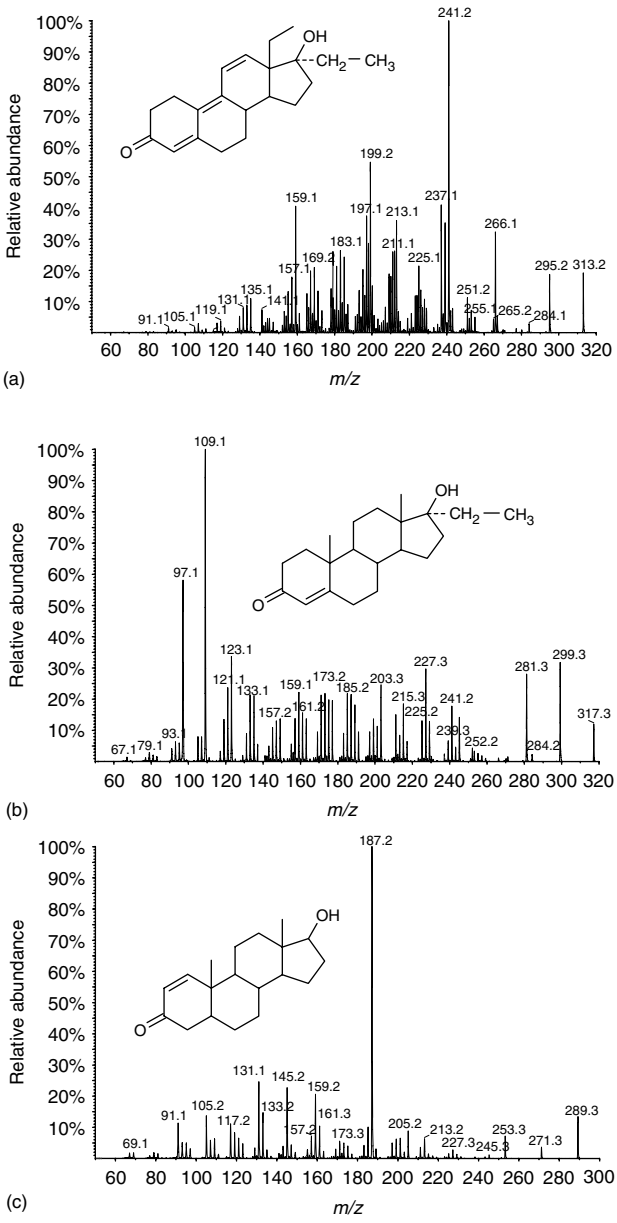


Figure 1. ESI product ion spectra of (a) tetrahydrogestrinone (M_r = 313 u, CE = 40 eV), (b) 17-ethyltestosterone (M_r = 317 u, CE = 40 eV) and (c) 5α-androst-1-en-17β-ol-3-one (M_r = 289, CE = 40 eV).

stable character of the generated cation. Compounds **3** and **4** contain additional ethyl side-chains at C-17 amenable for elimination, and the product ion spectrum of **4** (Table 1) provides information that proves the generation of m/z 266 by release of an ethyl residue originating either from C-13 or C-17. The introduction of four deuterium atoms into the C-17 side-chain allows their distinction as both 29 and 33 u are expelled from the ion at m/z 299 giving rise to the product ions at m/z 270 and 266, respectively, in a ratio of ~2.5 : 1.

In contrast to the fragments generated by the losses of water and ethyl residues, ions at m/z 241 and 199 are present in all gestrinone analogues (compounds **1**–**4**, Table 1) requiring a structure lacking the C-17 substituents. Hence a neutral loss of C-16 and C-17 including their substituents is postulated, giving rise to m/z 241 as demonstrated in

Table 1. Relative abundances (%) of selected fragment ions of steroids studied by ESI and CAD

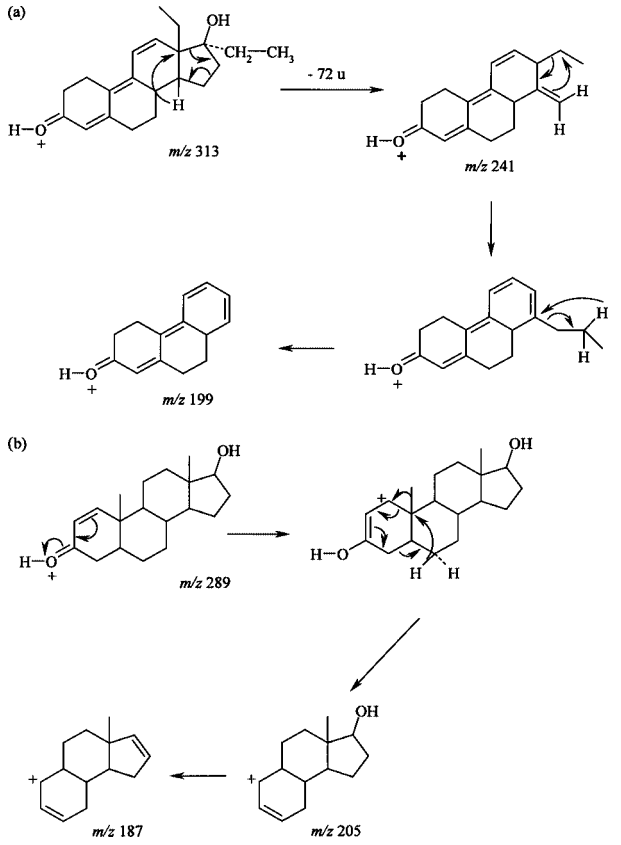
Compound	Precursor ion (m/z)	CE (eV)	Common fragment										Individual fragment ions: m/z (relative abundance, %)[a]	
			$[M+H]^+ - 18$	$[M+H]^+ - 18 - 29$	m/z 245	m/z 241	m/z 231	m/z 227	m/z 199	m/z 187	m/z 185	m/z 109	m/z 97		
1	309	40	291 (14)	262 (38)	—	(75)	—	—	(100)	—	(14)	—	—	228 (29)	179 (22)
2	311	40	293 (10)	264 (25)	—	(100)	—	—	(59)	—	(24)	—	—	213 (34)	159 (40)
3	313	40	295 (17)	266 (33)	—	(100)	—	—	(56)	(8)	(22)	—	—	213 (35)	159 (45)
4	317	45	299 (11)	270 (24)/266 (10)[b]	—	(100)	—	—	(80)	(9)	(26)	—	—	213 (48)	159 (39)
5	311	40	293 (27)	—	—	—	—	(100)	(30)	—	(13)	—	—	269 (34)	251 (45)
6	313	43	295 (9)	—	—	—	—	(100)	(43)	—	(24)	—	—	252 (10)	237 (11)
7	271	40	253 (76)	—	—	—	—	(40)	(100)	(7)	(23)	—	—	238 (23)	214 (34)
8	317	40	299 (36)	—	(17)	(22)	—	(35)	(16)	(25)	(26)	(100)	(69)	159 (22)	123 (34)
9	321	40	303 (41)	—	(34)	—	—	(31)	(7)	(16)	(19)	(100)	(66)	159 (16)	123 (27)
10	313	40	295 (6)	—	(7)	—	—	(12)	(11)	(10)	(17)	(100)	(61)	237 (7)	171 (15)
11	303	40	285 (12)	—	(7)	—	—	(26)	—	(12)	(14)	(100)	(89)	159 (18)	123 (24)
12	289	40	271 (10)	—	—	—	—	—	—	(14)	(9)	(100)	(62)	213 (9)	123 (23)
13	291	40	273 (8)	—	—	—	—	—	—	—	—	111[c] (100)	99[c] (62)	214 (9)	125 (21)
14	292	40	274 (10)	—	—	—	—	—	—	(13)	(6)	(100)	100[d] (72)	216 (11)	126 (7)
15	275	40	257 (11)	—	—	—	(6)	—	(13)	(16)	(13)	(100)	—	105 (35)	91 (37)
16	299	40	281 (23)	—	—	—	(100)	—	(18)	(26)	(51)	(75)	—	213 (70)	171 (87)
17	313	40	295 (12)	266 (11)	(100)	—	—	(57)	(32)	(40)	(69)	(46)	—	227 (55)	185 (68)
18	317	40	299 (64)	—	(100)	(36)	—	(95)	(38)	(91)	(66)	(73)	—	281 (44)	187 (88)
19	289	40	271 (6)	—	—	—	—	—	(7)	(100)	(11)	(6)	—	159 (18)	145 (21)
20	303	40	285 (5)	—	—	—	—	—	—	(100)	(12)	—	—	159 (21)	145 (45)
21	287	40	269 (6)	—	—	—	—	(5)	(9)	(9)	(100)	—	(16)	159 (23)	143 (32)

[a] –Relative abundance <5%.

[b] Loss of 33 u (CD_2CD_2H).

[c] Incremented by 2 u.

[d] Incremented by 3 u.



Scheme 2. (a) Proposed fragmentation pathway of tetrahydrogestrinone (**3** in Scheme 1) generating fragment ions at *m/z* 241 and 199; (b) proposed fragmentation pathway of 5α-androst-1-en-17β-ol-3-one generating the fragment ion at *m/z* 187.

Scheme 2(a). The ion at m/z 241 is generated directly from the protonated molecules as MS^3 experiments, e.g. from $[M + H]^+ - H_2O$ (i.e. m/z 291), did not yield the respective fragment. The ion at m/z 199 results from a neutral loss of 42 u from m/z 241, also confirmed by MS^3 experiments, where the protonated molecule (m/z 309) was dissociated, and the resulting fragment ion at m/z 241 was isolated and fragmented in the linear ion trap. Here, an elimination of propene is suggested, including C-15, C-18 and C-19, which requires a migration of the ethyl residue as shown in Scheme 2(a). The linkage between C-13 and C-18 is proposed to generate a double bond between C-13 and C-14, while C-18 establishes a new bond to C-15. A neutral loss of propene (−42 u) accompanied by a hydrogen shift from the leaving group (C-15, C-18 and C-19) is postulated, giving rise to m/z 199. This fragment in particular is observed in all product ion spectra of steroids with a 4,9,11-triene nucleus investigated in this study, whereas compounds bearing a methyl residue at C-13 instead of an ethyl group give rise to a fragment at m/z 227 instead of m/z 241. These phenomena are in accordance with the fragmentation pathway proposed for the CAD of gestrinone analogues, where m/z 241 includes the C-13-linked alkyl residue, and m/z 199 is generated by removal of this particular part of the molecule. Exchanging the ethyl function of gestrinone by a methyl group results in a decrement of m/z 241 by 14 u, yielding m/z 227, as detected in the product ion spectra of the **5**–**7** (Table 1). The ion at m/z 227 eliminates 28 u (ethene) upon CAD in a comparable way as suggested for the propene elimination from m/z 241. Hence a distinction between substances related to either trenbolone or gestrinone (i.e. with a methyl or ethyl residue at C-13) can be accomplished by the determination of m/z 227 or 241, a valuable parameter for the identification of unknown designer steroids or their metabolites. An example is given, for instance, with THG (**3**) and propyltrenbolone (**6**), both of which consist of $C_{21}H_{28}O_2$ and generate a protonated molecule at m/z 313. Product ion spectra of both compounds contain signals at $[M + H]^+ - 18$ and m/z 199, but **3** gives rise to m/z 241 whereas **6** generates a base peak at m/z 227.

Steroids with 3-keto-4-ene nucleus

Testosterone and 19-nortestosterone analogues comprise 3-keto-4-ene nuclei and demonstrate a distinct difference in dissociation behavior compared with the class of 4,9,11-triene steroids. Product ion spectra of compounds related to testosterone (**8**–**12**) contain abundant fragments at m/z 97 and 109 (Table 1), the proposed generation of which was described for testosterone by Williams *et al*.[28] This particular dissociation pathway is valid also for 17-substituted testosterone analogues such as **8**–**11**, which yield abundant fragments originating from A- and B-ring cleavages. However, to a smaller extent, also fragment ions generated by D-ring dissociation are found in product ion spectra of **8**–**11**. Ethisterone (**10**) bears an ethinyl residue at C-17, and its protonated molecule eliminates 68 u to give m/z 245 in accordance with the dissociation of gestrinone (**1**, Table 1 and Scheme 2(a)). Owing to four additional hydrogens in the B- and C-rings, the fragment ion at m/z 241, as found in the product ion spectra of **1**–**4**, is shifted to m/z 245. The hydrogenation of the 17-ethinyl residue of **10** gives rise to **8**, which demonstrates comparable fragmentation routes upon CAD. Here, the protonated molecule expels 72 u by means of D-ring dissociation, giving rise to m/z 245, and an additional fragment ion is detected at m/z 241, which must comprise the 17-alkyl residue as with the quadruply deuterated counterpart **9** this particular fragment is incremented by 4 u, yielding an ion at m/z 245 obtained from two different fragmentation pathways. Moreover, **10** produces a fragment at m/z 237 substantiating the proposal of a fragmentation remote from the D-ring generating m/z 241 in the case of **8**. In order to determine the part of molecule released to generate m/z 241 of **8**, testosterone (**12**), 1α,2α-dideuterotestosterone (**13**) and 19-trideuterotestosterone (**14**) were investigated regarding analogous fragment ions. Compound **12** generates m/z 213 corresponding to m/z 241 of **8**. The ion at m/z 213 of **12** is incremented to m/z 214 in the case of **13**, and m/z 216 is observed in the product ion spectrum of **14**, demonstrating the presence of one and three deuterium atoms, respectively, in the fragment ion of interest. Hence an elimination of water from C-17 followed by the release of carbons C-2, C-3 and C-4 as acetone (−58 u) is postulated, giving rise to m/z 213 and 241 in the case of **12** and **8**, respectively. The origins of the hydrogens that migrate to the leaving group allowing the loss of acetone from $[M + H]^+ - 18$ were not assigned,

as various hydrogen transfer reactions during fragmentation processes are reported in the literature, which are to be elucidated by specific deuterium labeling.

Product ion spectra of compounds structurally related to 19-nortestosterone (**15**) such as **16, 17** and **18** demonstrate dissociation behaviors that are in accordance with both gestrinone and testosterone analogues. The protonated molecule of **17**, which can also be considered tetrahydrogenated gestrinone (in the B- and C-rings), eliminates 18 u (H_2O) and subsequently 29 u (ethyl radical), giving rise to m/z 266 as detected also in the product ion spectrum of **3** (Table 1). In contrast to **3**, **17** does not generate a base peak at m/z 241 but at m/z 245 owing to four additional hydrogens in the B- and C-rings and an expulsion of C-16 and C-17 of the D-ring as described for **1–7**. Hence the resulting product ion composed of intact A-, B- and C-rings is incremented by 4 u, allowing enabling the differentiation of the principle steroidal structures, i.e. the 3-keto-4-ene and 4,9,11-triene nucleus, of these closely related substances. The product ion spectrum of **16** contains fragment ions corresponding to those observed with **17**. Here, the protonated molecule at m/z 299 is decremented by 14 u compared with **17** owing to a methyl residue at C-13 instead of an ethyl group. Hence fragments resulting from neutral losses of water or D-ring dissociation such as m/z 231 are also reduced by 14 u as the steroid nucleus including the C-ring and its substituent at C-13 are still present.

A characteristic A- and B-ring dissociation of 19-nortestosterone analogues is observed with the fragment ion at m/z 109. This ion corresponds to m/z 123 of testosterone analogues generated from A- and B-ring cleavages[28] and is detected in all spectra of the investigated compounds related to 19-nortestosterone (**16**–**18**) regardless of C-13 or C-17 substitution.

Steroids with 3-keto-1-ene nucleus

Steroids such as 5α-androst-1-en-17β-ol-3-one (**19**), metenolone (**20**) and 5α-androst-1-en-3,17-dione (**21**) demonstrate a different preference regarding their fragmentation behavior upon CAD compared with those comprising a 3-keto-4-ene structure. In product ion spectra of the respective protonated molecules, fragment ions at m/z 187 (in the case of **19** and **20**) and m/z 185 (in the case of **21**) are generated (Table 1), which are proposed to result from an elimination of the carbons C-1–C-4 and C-19, followed by a loss of water, as depicted in Scheme 2(b). Indications for the suggested fragmentation route were obtained by the detection of m/z 187 in the product ion spectra of **19** and **20**, proving the release of C-1 (and its substituent) from the steroids. In addition, H/D exchange of **20** at C-2, C-4 and C-20 did not influence the fragment at m/z 187 (data not shown), demonstrating the elimination of the entire steroidal A-ring during the fragmentation process. The postulated generation of m/z 187 requires the migration of C-19 from C-10 to C-1 of the leaving group (Scheme 2(b)). The basis of this proposal was MS^3 experiments proving the generation of m/z 205 from 289 of **19** (−84 u) and the subsequent elimination of 18 u giving rise to m/z 187. In accordance with this, m/z 185 was observed in MS^3 experiments with **21**, where the protonated molecule at m/z 287 was dissociated to a fragment at m/z 203 that subsequently expelled 18 u to give m/z 185. The migration of methyl residues of steroids in solution under various conditions is a well-known phenomenon,[29–32] in particular for 17-methyl steroids such as methyltestosterone and methandienone,[33] but it has never been reported for C-19 of 5α-androst-1-en-17β-ol-3-one and analogues in the gas phase. This migration can be explained according to a Wagner–Meerwein rearrangement starting with a positive charge at C-1 that triggers the shift of C-19 from C-10 to C-1 followed by the release of the A-ring. Complementary observations in acidified solutions have been described in the literature,[30] where the migration of C-19 to C-5 was reported, initiated by the elimination of water including a hydroxyl function at C-5, causing a positive charge at this particular carbon. NMR studies confirmed the shift of C-19 to C-5 and the corresponding final structure of the steroid.

Doping control analysis

The knowledge of dissociation pathways of selected steroid nuclei upon ESI and CAD allows the creation of screening procedures complementary to established methods in doping control analysis. The possibility of utilizing precursor ion scanning or neutral loss scanning allows the determination of classes of steroids and does not limit the analytical assay to selected drugs, hence permitting also the detection of artificially modified drugs such as THG or norbolethone. Precursor ion scans of m/z 97 and 109 indicate steroids with a 3-keto-4-ene structure (e.g. testosterone (**12**), ethyltestosterone (**8**)), while the presence of a signal at m/z 109 in combination with the absence of m/z 97 can represent 19-norsteroids with a 3-keto function such as nortestosterone (**15**) and analogues. Precursor ion scans of m/z 241 indicate structures comparable to gestrinone (**1**), signals generated by m/z 227 represent trenbolone (**7**) nuclei and fragment ions at m/z 187 represent the A/B-ring structure of an androst-3-keto-1-ene steroid (**19**). With sufficient sensitivities of modern triple-quadrupole mass spectrometers, doping control urine samples can be analyzed in addition to commonly accepted procedures by means of LC/ESI-MS/MS in precursor ion scanning modes. In the case of suspicious signals in the resulting urinary steroid profiles, subsequent experiments are required either to substantiate or to negate the suspicion regarding drugs of abuse and their putative metabolites.

CONCLUSION

ESI and CAD-MS/MS of steroids with a 4,9,11-triene or a 3-keto-4-ene nucleus provide distinct information about the principle structure of the analyte. The detection of abundant product ions at m/z 241 and 199 or m/z 227 and 199 indicate the presence of a 4,9,11-triene steroid with a C-13-linked ethyl or methyl group, respectively. In addition, the neutral loss of 68 u from the protonated molecule is generally observed in the case of a D-ring bearing a 17-ethinyl and 17-hydroxyl function. In contrast, in product ion spectra of testosterone analogues comprising a 3-keto-4-ene structure, fragments generated by A- and B-ring cleavages are predominant, in particular the ions at m/z 97 and

109. With removal of the angular 19-methyl group giving rise to molecules related to 19-nortestosterone, product ion spectra are obtained that are not dominated by A- and B-ring fragment ions but contain an ion at m/z 109 (corresponding to m/z 123 of testosterone analogues) and fragments generated by commonly observed D-ring fragmentation releasing C-16 and C-17 including their substituents. 5α-Androst-1-en-17β-ol-3-one and its analogue metenolone eliminate primarily the carbons C-1–C-4 including all substituents, in addition to C-19 that migrates to the leaving group under CAD conditions generating an abundant fragment at m/z 187. The removal of the A-ring is charge-driven, presumably according to a Wagner–Meerwein rearrangement, and was only observed with testosterone analogues bearing a double bond between C-1 and C-2. Based on this information, unknown substances with steroidal structure can be classified and elucidated regarding the principal structure and possible modifications. Moreover, more comprehensive screening procedures can be established focusing on common fragmentation pathways. Here, features of modern triple-stage mass spectrometers such as neutral loss or precursor ion scanning as employed routinely in metabolism studies can provide a broader view in urine analysis, supporting an improved analytical assay at required sensitivities. Compounds with fragmentation routes comparable to known steroids but with different molecular masses or different chromatographic behavior can be detected and probably identified by MS^n analysis, chemical derivatization, synthesis of the putative compound, etc. Hence substances that do not belong to regular urinary steroid profiles may be discovered and give indications for the abuse of unknown drugs.

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