In: W Schänzer, H Geyer, A Gotzmann, U Mareck (eds.) Recent Advances In Doping Analysis (16). Sport und Buch Strauß - Köln 2008

O.J. Pozo[1)], P. Van Eenoo[1)], K. Deventer[1)], L. Lootens[1)], S. Grimalt[2)], J.V. Sancho[2)], F. Hernandez[2)], F.T. Delbeke[1)]

General guidelines for the CID fragmentation of 3-keto-anabolic steroids

[1)] DoCoLab, UGent, Department of clinical chemistry, microbiology and immunology, Technologiepark 30, B-9052 Zwijnaarde, Belgium

[2)] Research Institute for Pesticides and Water, University Jaume I, E-12071, Castellón, Spain

Abstract

The CID fragmentation of 38 anabolic steroids has been studied in order to extract general guidelines. Eight guidelines have been established for this fragmentation. From these guidelines a general approach for the discovery, elucidation and subsequent detection of unknown anabolic steroids has been proposed. This approach has been applied for the discovery of unreported metabolites for methyltestosterone and for the proposal of a feasible structure for these metabolites.

Introduction

The detection of some anabolic steroids using GC-MS is difficult due to problems in the derivatization step. The use of liquid chromatography tandem mass spectrometry (LC-MS/MS) is becoming more important in the analysis of anabolic steroids and metabolites because it does not require derivatization and it allows for the detection of some steroid metabolites that remained undetected by GC-MS [1,2]. Recently, a few methods based on precursor ion scanning have also been found useful for the detection of unknown (designer) steroids [3,4].

In order to obtain reliable information on the structure of an unknown steroid, detected by LC-MS/MS, detailed studies about the collision induced dissociation (CID) fragmentation pathway of known steroids are needed. The CID fragmentation of several steroid families has been reported [5-8]. Most of them reported that small differences in chemical structure of the steroids, such as a double bond or a methyl group, resulted in significantly different

fragmentation pathways. Therefore, general guidelines in the CID fragmentation of anabolic steroids are needed to facilitate the identification of unknown steroids and metabolites.

The objective of this work is to study the CID fragmentation of a large number of anabolic steroids belonging to different structural groups in order to establish general fragmentation guidelines which are required to facilitate the characterization of unknown steroids and metabolites.

Materials and Methods

<u>Chemicals and reagents</u>

Stanozolol, 16β-hydroxy-stanozolol, 3'-hydroxy-stanozolol, 4β-hydroxy-stanozolol, tetrahydrogestrinone (THG), 4-chloro-17α-methyl-androsta-1,4-diene-6β,17β-diol-3-one, 6β-hydroxymetandienone, 2-hydroxymethyl-17α-methyl-androsta-1,4-diene-11α,17β-diol-3-one (formebolone metabolite), 1-testosterone, 5β-androst-1-ene-17β-ol-3-one (boldenone metabolite), 1(5α)-androstene-3,17-dione (1-androstenedione), metandienone and 9-fluoro-18-nor-17,17-dimethyl-4,13-diene-11-ol-3-one (fluoxymesterone metabolite) were purchased from NMI (Pymble, Australia). 6α-hydroxy-androst-4-ene-3,17-dione (6α-hydroxyandrostenedione), mibolerone, norclostebol, 17-methyl-1-testosterone, 1,4,6-androstatriene-17β-ol-3-one, 4,6-androstadiene-17β-ol-3-one, 4,9(11)-androstadiene-17β-ol-3-one, 4,9(11)-androstadiene-17β-ol-17α-methyl-3-one, 1,4-androstene-3,17-dione, 19-hydroxyandrostenedione, 6β-hydroxytestosterone, 19-nortestosterone and 4-hydroxytestosterone were purchased from Steraloids (Newport, RI, USA). Testosterone, epitestosterone and dihydrotestosterone (DHT) were obtained from Sigma (St. Louis, MO, USA). Gestrinone, boldenone and oxymesterone were obtained from the Institut für Biochemie (DSHS, Cologne, Germany). 17α-trenbolone, methyltestosterone and fluoxymesterone were purchased from RIVM (Netherlands), Organon (Oss, the Netherlands) and Pfizer (Puurs, Belgium), respectively. Danazol, ethisterone, mesterolone, metenolone, metandienone metabolite and oxandrolone were kind gifts from Winthrop, Laboratório de Análises e Dopagem (Instituto do Desporto, Lisbon, Portugal), Schering ACo (Berlin, Germany), King's College London (London, UK), Center for Preventive Doping Research (German Sport University, Cologne, Germany) and Searle & Co (Chicago, Ill, USA), respectively.

HPLC grade methanol and HPLC grade water were purchased from Acros (Geel, Belgium)

and Fischer Scientific (Loughborough, UK), respectively. Ammonium acetate and ammonium formate were obtained from Sigma (St. Louis, MO, USA).

Instrumentation

A HPLC Finnigan Surveyor MS pump plus (Thermo, San Jose, USA) was interfaced to a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo) using the electrospray interface. Twenty µL of the analyte (concentrations between 1 µg/ml and 10 µg/ml) were injected into the system using a Finnigan surveyor autosampler plus (Thermo). Nitrogen was used as sheath gas, ion sweep gas and auxiliary gas, which were set at flows of 50, 2 and 20 units, respectively. Spray voltage of 4000V was used in positive ionization mode. The tube lens voltage (TLV) was set to 100 V. The capillary temperature was set at 350ºC and the source CID at 2 units.

The LC separation was performed on a Varian Omnispher C_{18} column (100 x 2 mm i.d., 3µm) (Varian, Sint-Katelijne-Waver, Belgium), at a flow rate of 300 µL/min using a ChromSep guard column (10 x 2 mm i. d., 5µm) from Varian. Aqueous ammonium acetate (1mM) and methanolic ammonium acetate (1mM) were selected as mobile phase solvents. A gradient program was used; the percentage of organic solvent was changed linearly as follows: 0 min., 20%; 0.5 min., 20%; 2 min., 55%; 10.5 min., 55%; 17 min., 85%; 17.5 min., 85%; 18 min, 20%, 20 min 20%.

Accurate mass experiments were carried out using a hybrid quadrupole time-of-flight (QTOF Premier) mass spectrometer provided with an orthogonal Z-spray-electrospray interface (ESI) (Waters, Milford, MA, USA) interfaced to an UPLC system, Acquity (Waters) for the chromatographic separation. A cone voltage of 40 V and a capillary voltage of 3.0 kV were used in positive ionization mode. The nitrogen desolvation temperature was set to 350 ºC and the source temperature to 120 ºC. TOF MS resolution was approximately 10000 (FWHM) at m/z 556. MS and MS/MS spectra were acquired over a m/z range of 50 to 1000. For automated accurate mass measurement, the lockspray probe was used, with leucine enkephaline 2 µg/mL in acetonitrile/water (50:50) used as lockmass solution.

LC-MS/MS

For the fragmentation study, individual solutions of selected analytes at 10 µg/ml were injected in both QqQ and QTOF instruments. Product ion spectra of the protonated ions were acquired in centroid mode at two different collision energies: 20eV and 30eV. For QqQ

experiments, a m/z range between 30 and 400 was selected using a peak width of 0.7 Da at 0.5 s/scan. In QTOF experiments, the m/z range selected was between 20 and 1000 and the scan time was selected at 0.2 s/scan. MS^3 experiments were carried out by both QqQ and QTOF analysers by increasing the source voltages (TLV to 150 V in the case of QqQ and the cone voltage to 55 V for QTOF) and selecting the adequate precursor ion. The molecular formula of each product ion was obtained from the accurate mass measurements by QTOF.

Results and Discussion

Study of the fragmentation of anabolic steroids

The CID fragmentation of 38 steroids at two collision energy was studied. The complete data set can be seen elsewhere [9]. From these results several guidelines can be extracted:

1. *Isomers have the same CID spectra but with different intensities*

The CID fragmentation of 5 pairs of steroisomers was studied. For each pair, the same product ions were obtained but with different relative abundances. As an example, the product ion spectra for 1-testosterone and boldenone metabolite (Figure 1) exhibited different abundances for ions at m/z 205 and 271.

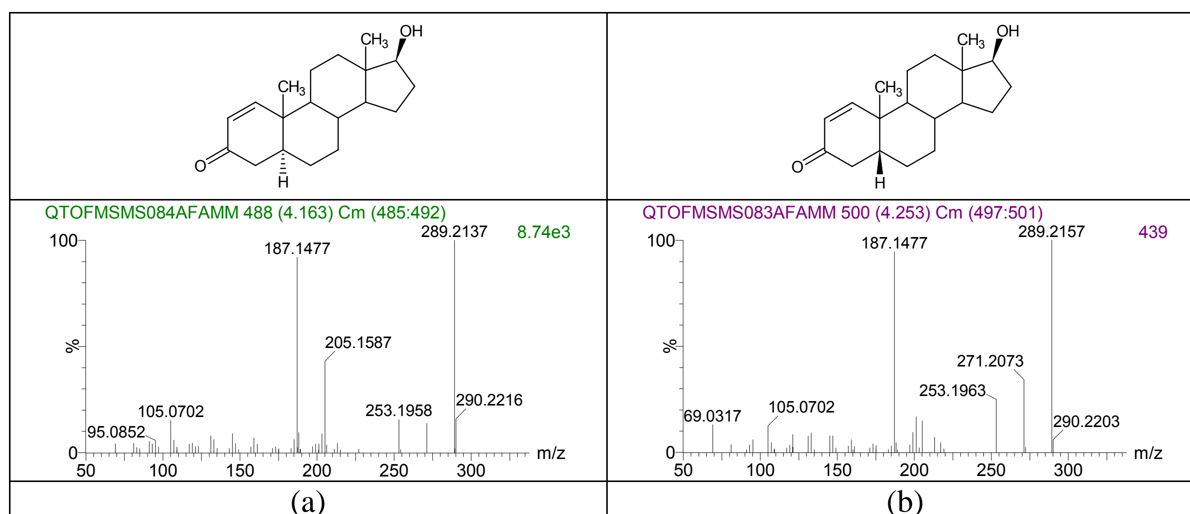

Figure 1. Product ion spectra obtained by QTOF instrument for (a) 1-testosterone and (b) boldenone metabolite

2. *The number of losses of water is related to the number of oxygen atoms and the conjugation of the keto group*

Due to the presence of hydroxyl and keto groups, all anabolic steroids tested showed different losses of water at low collision energy. Although this loss is unspecific, a correlation between the number of water molecules lost and the structure could be obtained.

The number of losses of water is normally equal to the number of oxygen atoms in the steroid excepting for steroids with a highly conjugated 3-keto function where the oxygen of this keto was not lost as a water molecule. Hence, the product ion spectrum of 6-hydroxy-testosterone showed 3 losses of water due to the presence of three oxygens and low conjugation of the keto function (Figure 2a). However, for THG only one loss of water was observed despite the presence of two oxygen atoms due to the high conjugation in the steroid (Figure 2b).

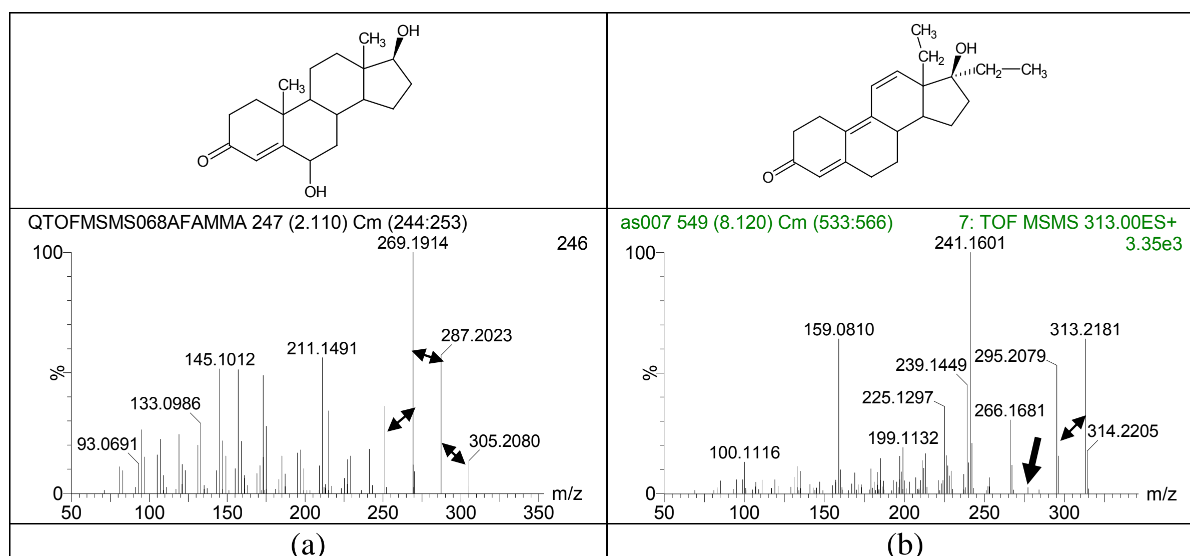

Figure 2. Product ion spectra at 20 eV for (a) 6-hydroxy-testosterone and (b) THG.

3. *The occurrence of OE^+ is related to the presence of double bond in the C ring*

The product ion spectra of anabolic steroids exhibited a large number of ions which can reach more than 30 with intensity higher than 15%. In most of the studied compounds all these ions showed odd m/z corresponding to even electron ions (EE^+). Odd electron ions (OE^+) were formed after the loss of the alkyl group in C13 and found exclusively in those steroids with a double bond in the C ring. This can be due to the stability obtained by the additional conjugation between the radical formed after the alkyl loss and the double bond in the C ring. As an example, the product ion spectrum for 6-hydroxy-testosterone does not contain any OE^+ ion (Figure 2a) while for THG the OE^+ at m/z 266 was one of the abundant product ions obtained (Figure 2b).

4. *The occurrence of common losses can be related to the anabolic structure*

Several common losses were found in the study of the CID fragmentation of steroids. These neutral losses depend on the steroid structure and therefore their occurrence can provide structural information for unknown steroids. Most common neutral losses and the structural

information obtained are shown in Table 1 or elsewhere [4-9].

Table 1. Common losses depending on the steroid structure

Ion	Explanation	Type of steroids	Ion	Explanation	Type of steroids
18	$-H_2O$	unspecific	58	$-(CH_3)_2C{=}O$	androstan-3-keto steroid
20	-HF	fluorinated steroids			17-methyl-3-keto steroids
30	$-H_2CO$	hydroxymethyl-3-keto steroids	84	-A ring	1-ene-3-keto steroids
56	$-(CH_3)_2C{=}CH_2$	17,17-dimethyl-3-keto steroids	176	-Gluc	glucuronidated steroid (unspecific)
		17-methyl-3-keto steroids			

5. *The occurrence of common ions can be related to the anabolic structure*

In the same way, several common ions were found in the CID spectrum of steroids depending on their structure. A list of the most common ions and the structural information is shown in Table 2 or elsewhere [4-9].

Table 2. Common ions depending on the steroid structure

Type of steroids	Ion	Explanation	Type of steroids	Ion	Explanation
4,9,11-triene-3-keto steroids	241/227	A-B-C ring	1-ene-3-keto steroids	187/185	B-C-D ring
	199	A-B-C ring		145	
				159	
1,4-diene-3-keto steroids	135	C-D ring	hydroxyl stanozolol analogues	145	N-ring
	121	A-ring		97	N-ring
4,9-diene-3-keto steroids	147	A-B	stanozolol analogues	95	N-ring
	145/159	C-D		81	N-ring
4-ene-3-keto steroids	109	A ring	steroidal core	105	methyl tropylium
				91	tropylium
19-methyl-4-ene-3-keto steroids	97	A ring		77	phenyl

6. *The position of double bonds guides the fragmentation*

At medium collision energy (30eV) most anabolic steroids only showed between 1 and 3 abundant ions (>60% or abundance). The m/z of these ions was found to be dependent of the number and position of the double bonds. Hence, those steroids with double bonds only in the A ring showed abundant ions containing only one ring (Figure 3a). On the other hand, those with double bonds in rings A and B showed ions corresponding to a two rings structure

(Figure 3b). The same behaviour was observed for those steroids with double bonds in ring A and C. Finally, abundant ions containing three rings were observed for those steroids with double bonds in rings A, B and C. Therefore, the number of rings in the abundant ions at medium collision energy can provide structural information about the steroid.

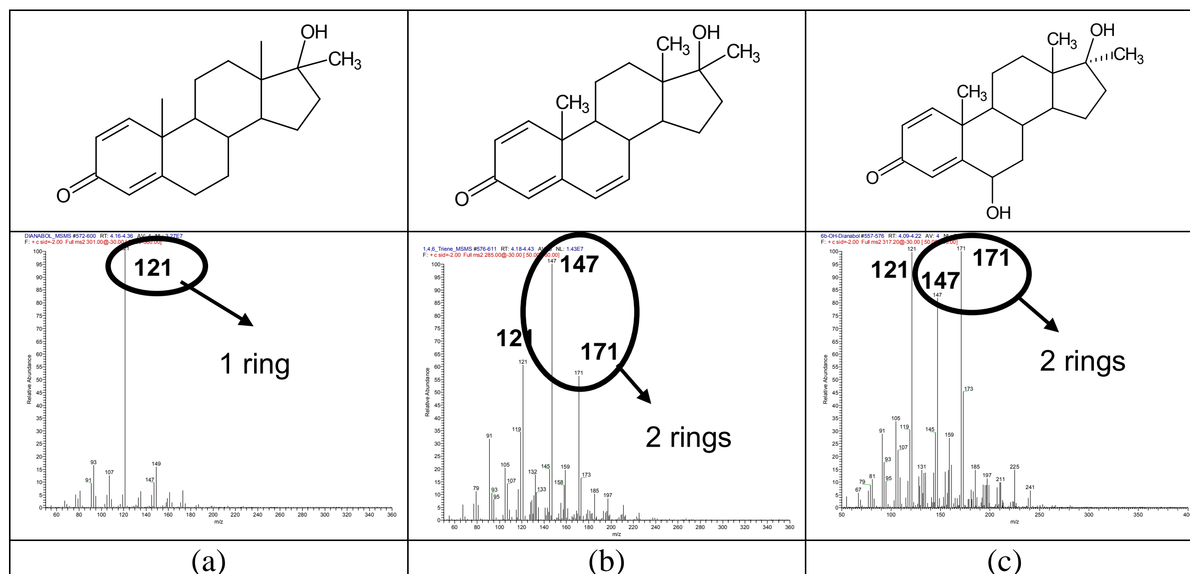

Figure 3. Product ion spectra at 30 eV for (a) metandienone, (b) 6-ene-metandienone and (c) 6-hydroxymetandienone.

7. *The presence of a hydroxyl group easily produces an additional double bond which also guides the fragmentation*

As stated in the guideline 2, several losses of water are commonly observed in the fragmentation of steroids. The loss of these molecules generated a new double bond. This double bond has to be taken into account when applying guideline 6. Hence, steroids with a hydroxyl group in C6 first generates a double bond in B ring due to the loss of water and then, at 30 eV of collision energy, produce ions containing two rings. As an example, the product ion spectrum of 6-hydroxy-metandienone (Figure 3c) exhibited the same abundant ions as 6-ene-metandienone (Figure 3b).

8. *At high collision energy steroids present 3 common ions: m/z 77, 91 and 105*

The fragmentation at low and medium collision energy depends on the steroid structure (guidelines 1-7). However, after increasing the collision energy to 50 eV all the steroids showed a similar behaviour. At this high collision energy all steroids exhibited three main ions at m/z 77, 91 and 105. These ions can be assigned to phenyl, tropylium and methyltropylium respectively and can be explained by the fragmentation of the steroid skeleton. These ions can be useful for the non-target detection of anabolic steroids [4].

General approach

According to the guidelines established before, the fragmentation of steroids at low and medium collision energy depends on their structure while at high collision energy all steroids follow a similar behavior. A general approach was proposed in order to discover, to propose a structure and to detect unknown steroids and metabolites (Figure 4). In a first step, either precursor ion scan or neutral loss scan methods can be applied for those steroids with a partially known structure (unknown metabolites). For the detection of unknown steroids the precursor ion scan of the ions m/z 77, 91 and 105 is the only alternative. Once the steroid has been detected, a feasible structure has to be proposed. For this purpose, full scan using accurate mass measurements is the mode of choice in order to obtain the molecular formula. The product ion scans at low and medium collision energy provide information about the steroid structure helping in the proposal of a structure. Finally, in order to detect the steroid, a SRM method is the most sensitive approach.

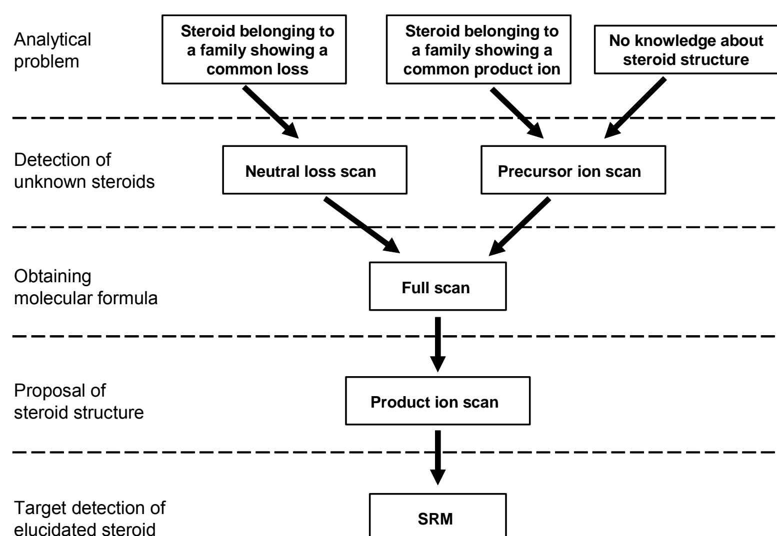

Figure 4. Proposed general approach for the discovery, elucidation and subsequent target detection of unknown steroids and metabolites.

Application to methyltestosterone metabolism

The described approach was applied to post-administration urines of methyltestosterone. Due to the absence of either a common ion or a common loss for this compound, the general methodology for the detection of unknown steroids was applied. In the precursor ion scan chromatogram for the ions m/z 77, 91 and 105 two different peaks were observed (Figure 5a). The metabolite A4 showed a m/z of 303 (an isomer of methyltestosterone) while A5 exhibited a m/z 301 corresponding with 2 hydrogens less than the parent compound (Figure

5b). The metabolite A4 was confirmed as epimethyltestosterone by the acquisition of full scan and product ion spectra at different collision energies. The full scan of A5 with accurate mass measurements showed a peak at m/z 301.2167 corresponding to $C_{20}H_{29}O_2$ for $[M+H]^+$. Therefore, A5 presented a loss of 2 hydrogens from methyltestosterone. Metabolite A5 was characterized by GC-EI-MS (Figure 5c) and ESI-MS/MS (Figure 5d). According to these data 11-dehydro-epimethyltestosterone was suggested as feasible structure for A5.

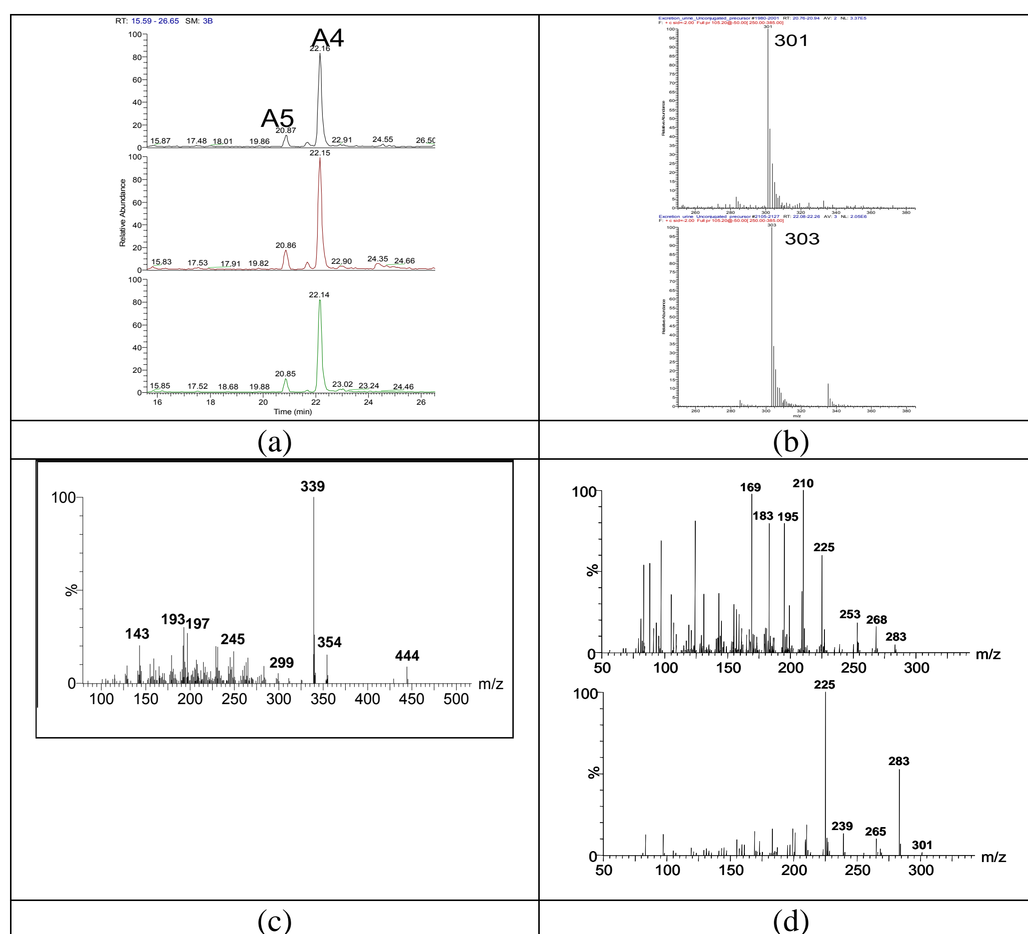

Figure 5. Detection and characterization of metabolite A5. (a) Precursor ion scan chromatogram showing two metabolites, (b) Spectra for both metabolites (A5 top and A4 bottom), (c) GC-EI-MS spectrum for A5 and (d) LC-ESI-MS/MS spectra at two collision energies (30eV top and 20 eV bottom)

Conclusions

The CID behaviour of anabolic steroids has been studied and summarized in 8 general guidelines. The fragmentation of anabolic steroids at low and medium collision energy depends on the number and position of double bonds and hydroxyl groups. At high collision energy a similar behavior is observed and all of them exhibited three ions (phenyl, tropylium and methyltropylium). These three ions can be used as markers for the presence of unknown steroids. A general strategy is suggested for the detection and characterization of unknown

steroids and metabolites. This strategy has been applied to the methyltestosterone metabolism and an unreported metabolite has been characterized.

Acknowledgements

Postdoctoral grants by the Spanish Ministerio de Educacion y Ciencia (O.J.P.) and the Flemish Ministry of Culture, Youth, Sports and Brussels (P.V.E., W.V.T., K.D.) are gratefully acknowledged. WADA is thanked for partially funding this work.

References

[1] Schänzer W, Geyer H, Fuβhöller G, Halatcheva N, Kohler M, Parr MK, Guddat S, Thomas A, Thevis M. (2006) Mass spectrometric identification and characterization of a new long-term metabolite of metandienone. *Rapid Commun. Mass Spectrom.* 20, 2252-2258.
[2] Pozo OJ, Van Thuyne W, Deventer K, Van Eenoo P, Delbeke FT. (2008) Elucidation of urinary metabolites of fluoxymesterone by liquid chromatography-tandem mass spectrometry and gas chromatography-mass spectrometry. *J. Mass Spectrom.* 43, 394-408.
[3] Thevis M, Geyer H, Mareck U, Schänzer W. (2005) Screening for unknown synthetic steroids in human urine by liquid chromatography-tandem mass spectrometry. *J. Mass Spectrom.* 40, 955-962.
[4] Pozo OJ, Deventer K, Van Eenoo P, Delbeke FT. (2008) Efficient approach for the comprehensive detection of unknown anabolic steroids and metabolites in human urine by liquid chromatography-tandem mass spectrometry. *Anal. Chem.*; **80:** 1709-1720.
[5] Thevis M, Makarov AA, Horning S, Schänzer W. (2005) Mass spectrometry of stanozolol and its analogues using electrospray ionization and collision-induced dissociation with quadrupole-linear ion trap and linear ion trap-orbitrap hybrid mass analyzers. *Rapid Commun. Mass Spectrom.* 19, 3369-3378.
[6] Thevis M, Schänzer W. (2005) Mass spectrometric analysis of androstan-17 beta-ol-3-one and androstadiene-17 beta-ol-3-one isomers. *J. Am. Soc. Mass Spectrom.* 16, 1660-1669.
[7] Thevis M, Bommerich U, Opfermann G, Schänzer W. (2005) Characterization of chemically modified steroids for doping control purposes by electrospray ionization tandem mass spectrometry. *J. Mass Spectrom.* 40, 494-502.
[8] Guan F, Soma LR, Luo Y, Uboh CE, Peterman S. (2006) Collision-induced dissociation pathways of anabolic steroids by electrospray ionization tandem mass spectrometry. *J. Am. Soc. Mass Spectrom.* 17, 477-489.
[9] Pozo OJ, Deventer K, Van Eenoo P, Grimalt S, Sancho JV, Hernández F, Delbeke FT, General guidelines for the collision induced dissociation of 3-keto-anabolic steroids and related compounds after electrospray ionization. *Rapid Commun. Mass Spectrom.* submitted