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Use of liquid chromatography tandem mass spectrometry (LC-MS/MS) and gas chromatography (GC-MS) in the detection of fluoxymesterone metabolites

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Introduction

The knowledge of the metabolism of anabolic steroids is of utmost importance in order to monitor the abuse of these substances. Different studies have been performed to determine fluoxymesterone (A1) urinary metabolites by GC-MS [1,2]. Kammerer et al. [2] found that fluoxymesterone abuse can be monitored by the detection of two major metabolites: 6-hydroxy-fluoxymesterone (A4) and 9-fluoro-17α-methyl-androst-4-ene-3α,6β,11β,17β-tetrol (A2). Additionally, other metabolites were also detected in that study but at low and/or very variable concentrations [2]. Using GC-MS Schänzer and Donike confirmed the presence of the two major metabolites as well as 9-fluoro-18-nor-17,17-dimethyl-4,13-diene-11-ol-3-one (A3) by comparison of their EI mass spectra of the trimethylsilyl (TMS) derivatives with those obtained from synthesized reference standards [1].

However, the use of the GC-MS approach for the elucidation of metabolites has some limitations. The most important one is the need to perform a derivatization step, normally trimethylsilylation. The derivatization of two fluoxymesterone metabolites, however, required between 5 min and 2 h at 80°C depending on the number and the sterical hindrance of hydroxyl groups present in the molecule [1].

The use of liquid chromatography tandem mass spectrometry (LC-MS/MS) in doping analysis has minimised some problems related to traditional GC-MS methods. Additionally, LC-MS/MS generates complementary data about the analyte structure to that obtained by GC-MS. Therefore, the combination of LC-MS/MS and GC-MS is a powerful tool in the characterization of anabolic steroids metabolites.

The aim of this work is to study the capabilities of LC-MS/MS and GC-MS in the metabolism of fluoxymesterone in relation to doping analysis.

Experimental

Instrumentation

LC-MS/MS analysis was performed on a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo) using the electrospray interface. The LC separation was performed using 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. Sheath gas, ion sweep gas and auxiliary gas (nitrogen) were set at flows of 70, 2 and 5 units, respectively. Spray voltages of 4000V was used in positive ionization mode. The capillary temperature was set to 300ºC and the source CID to 2 units. The collision gas was Argon. A gradient program was used; the percentage of organic solvent was changed linearly as follows: 0 min., 30%; 1.5 min., 30%; 8 min., 55%; 15 min., 55%; 29.5 min., 95%; 30.5 min., 95%; 31 min, 30%, 34 min 30%.
GC/MS analysis was carried out on an Agilent 5973 mass spectrometer (Palo Alto, CA, USA) directly coupled to an Agilent 6870 gas chromatograph equipped with a J&W-Ultra 1 column (J&W, Folsom, USA), length of 17 m, internal diameter of 0.2 mm and a film thickness of 0.11 μm. The GC was operated in constant flow mode at a flow rate of 0.7 ml/min (linear velocity 43 cm/s). The oven temperature was as follows: 120°C (0 min) @ 60°C/min → 183°C (1.05 min) @ 3°C/min → 232°C (0 min) @ 40°C/min → 310°C (3 min). Half a microliter was injected in the splitless mode. The mass spectrometer was operated in the full scan mode between m/z 50 and 650.

Study of the ionization and fragmentation of standards

For the study of the ionization and fragmentation, a standard mixture containing compounds A1, A2 and A3 at 1 μg/ml was injected into the system using the gradient described above. The formation of different adducts depending on the steroid structure was studied. A full scan mass spectrum between 175 and 1000 Da was acquired.
Additionally, the product ion spectra of each adduct formed was acquired in centroid mode at two different collision energies: 25eV and 40eV. A mass range between 30 and 400 Da was selected using a peak width of 0.7 mDa at a speed of 0.5 s/scan.
For GC-MS analysis, the standards were derivatised and analysed. The full scan spectra were acquired between 50 Da and 650 Da at a scan speed of 2.5 scans/s.

Sample preparation

Urine samples obtained after the administration of fluoxymesterone were provided by other doping control laboratories, the WAADS, the IOC and WADA or were samples declared positive in a doping control test at DoCoLab.

For the analysis of the unconjugated fraction by LC-MS, the pH of 5 ml of urine was adjusted to 9.2 by addition of sodium hydrogen carbonate and potassium carbonate. A liquid-liquid extraction was performed with 5 ml of diethyl ether. After centrifugation, the organic layer was separated and evaporated under nitrogen at 40°C. The remaining residue was dissolved into 200µl of mobile phase. 20µL were injected into the system.

For the conjugated fraction, 5 ml of urine were hydrolysed after addition of phosphate buffer (pH=7) and 50 µl of glucuronidase solution. After cooling to room temperature, the previous method was followed.

For GC-MS analysis, the dry residues were derivatised using MSTFA/NH_4I/ethanethiol (320/1/2; v/w/v, 100 µl) at 80°C for 60 min and 0.5µL were injected into the system.

Neutral loss experiments

The same experimental conditions as described above were applied for neutral loss experiments. A neutral loss of 20 corresponding to the loss of HF was selected. The scan speed was set to 0.8 s/scan, the TLV to 100 V and the collision energy at 20 eV. The spectrum of each detected peak was obtaining after background subtraction.

Elucidation of metabolites

The more feasible molecular formula for each metabolite was established based on mass accuracy experiments [3]. Then, both the CID product ion and the EI spectra were studied and compared with the obtained for reference compounds. The occurrence of either common ions or common losses was used in order to propose a feasible structure for each metabolite.

Results and Discussion

Study of the ES ionization and CID fragmentation of standards

The ionization of the three reference standards studied was in agreement with a previous ionization study [4]. Thus, analytes with a conjugated keto in their structure (A1 and A3) presented a predominant $[M+H]^+$ in the MS spectrum due to their higher proton affinity (Figure 1). However in the case of A2, the absence of any keto function avoid its protonation and the formation of different adducts was found to be the only way to ionize this analyte. The $[M+Na]^+$ was the most abundant adduct found for this compound despite the absence of sodium in the mobile phase.

The CID spectra for these analytes (Figure 1) showed also high differences depending on the adduct selected as precursor ion. In this way, the selection of $[M+Na]^+$ as precursor ion only produced an abundant product ion corresponding to the loss of HF. After increasing the collision energy to 40eV, no product ions were observed.

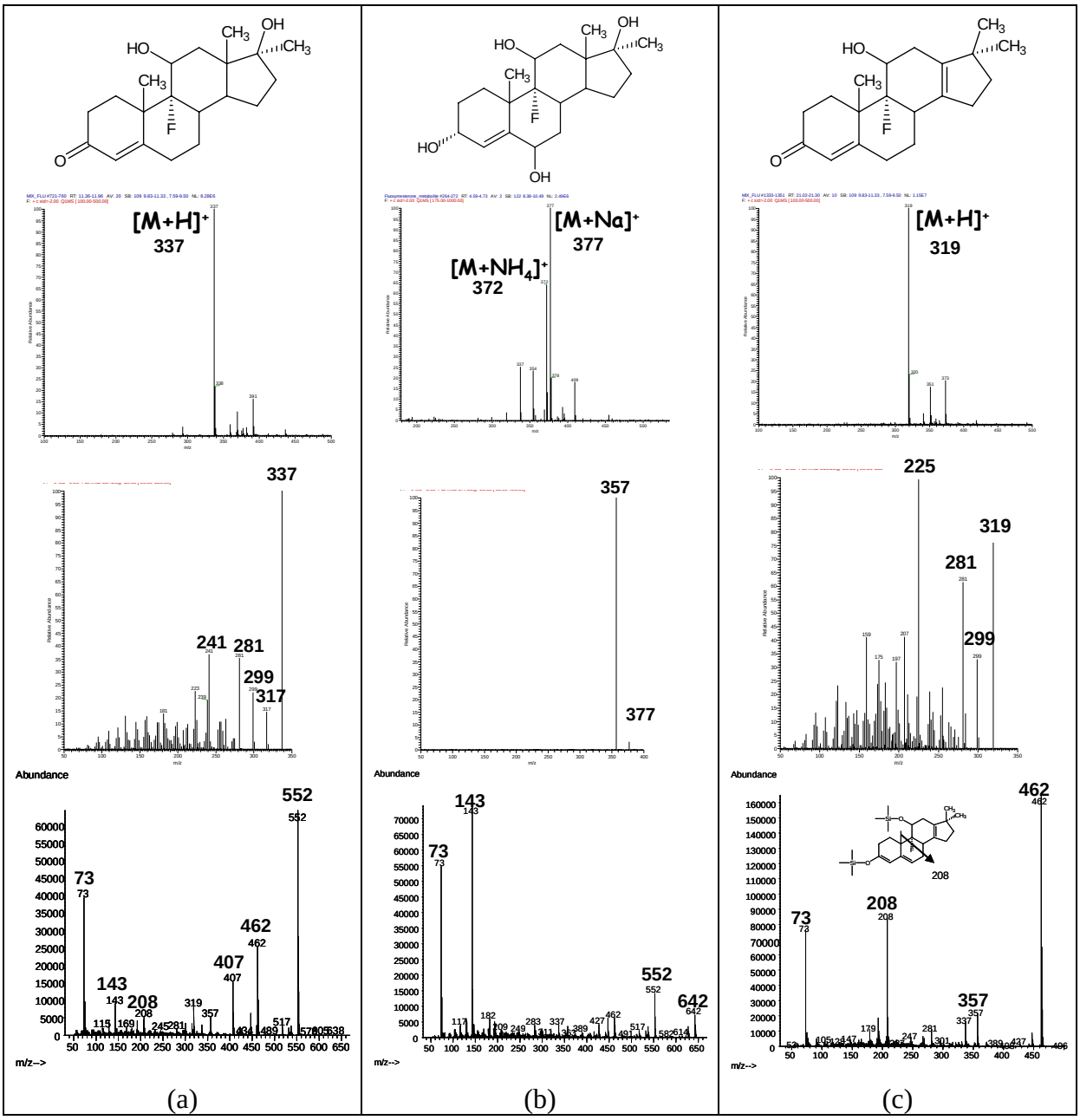


Figure 1. Structures and spectra (top) full scan electrospray spectra (medium) product ion scan and (bottom) GC-EI-MS obtained for available standards (a) A1, (b) A2 and (c) A3;

A different behaviour was observed when analytes with $[M+H]^+$ as predominant ion were fragmented. Both A1 and A3 presented the loss of HF and subsequently several losses of water. The main difference found for their fragmentation was the loss of acetone for A1 (product ion at m/z 241) and the loss of isobutene for A3 (product ion at m/z 225). The fragmentation of these analytes (Table 1) has been confirmed by MS^n experiments [3].

Study of the EI spectra of standards

The EI spectra of the three analytes (Figure 1) showed different losses of 90 corresponding to the loss of TMSOH together with losses of CH_3. These losses are common for derivative

anabolic steroids [5] and in this case are combined with the losses of 20 corresponding to the neutral loss of HF (Table 1).

The ion at m/z 143 was present in the EI spectra of A1 and A2 and was not present in the spectrum of A3. This ion is common for 17-methyl, 17-hydroxy anabolic steroids [6] and therefore it was not present in A3 which has a 17, 17-dimethyl structure.

Finally, the ion at m/z 208 can be assigned to the fragmentation of the B ring in those cases where the A ring contains a conjugated keto function. As expected this ion is present in A1 and A3 but it is not present in A2.

Table 1. LC-MS/MS product ions at 25 eV and GC-EI-MS ions from available standards

	LC-MS/MS			**GC-MS**	
Analyte	Precursor ion (m/z)	Product ions (m/z)		Ions (m/z)	
A1	337	317	-HF	552	M
		299	-HF, -H_2O	462	M-TMSOH
		281	-HF, -2H_2O	447	M-CH_3-TMSOH
		241	-HF, -H_2O-$(CH_3)_2C$=O	407	M-$(CH_3)_2CHCH_2$-TMSOH[a]
				357	M-CH_3-2TMSOH
		223	-HF, -2H_2O-$(CH_3)_2C$=O	208	A ring
				143	D ring
				73	TMS
A2	377	357	-HF	642	M
				552	M-TMSOH
				462	M-2TMSOH
				447	M-CH_3-2TMSOH
				357	M-CH_3-3TMSOH
				143	D ring
				73	TMS
A3	319	299	-HF	462	M
		281	-HF, -H_2O	447	M-CH_3
		225	-HF, -H_2O-$(CH_3)_2C$=CH_2	357	M-CH_3-TMSOH
				337	M-CH_3-HF-TMSOH
		207	-HF, -2H_2O-$(CH_3)_2C$=CH_2	247	M-CH_3-HF-2TMSOH
				208	A ring
				73	TMS

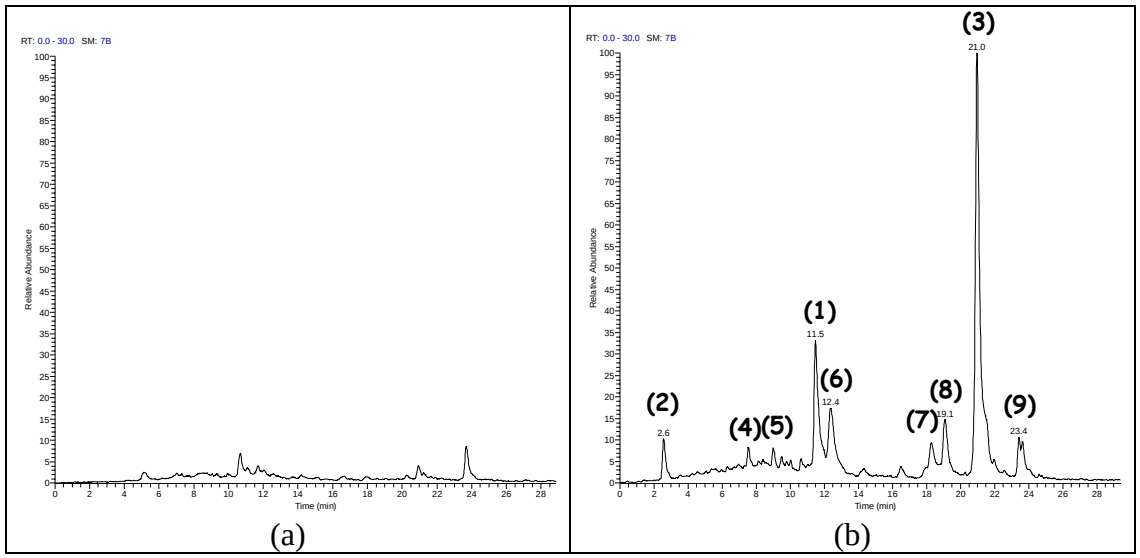
[a] *tentative assignment*

Neutral loss experiments

In order to discriminate the fluoxymesterone metabolites from other endogenous compounds, a neutral loss scan in the LC-MS/MS system was performed. The neutral loss of HF (neutral loss of m/z 20) was selected because it was the only common loss showed by all analytes independently of their ionization. Additionally, the low abundance of fluorine compounds in endogenous urine increased the selectivity of this neutral loss compared with the other possibilities such as losses of water, acetone or isobutene (Figure 2).

The neutral loss chromatogram of the selected excretion urine showed 8 abundant peaks when no hydrolysis was carried out. This number increased up to 9 after enzymatic hydrolysis due to the occurrence under these conditions of peak 1.

The m/z of the precursor ions of obtained for some peaks were consistent with previously reported metabolites. Thus, peaks 2, 3 and 4 presented precursor ions at m/z 377, 353 and 319 respectively which corresponded to the m/z expected for three previously reported metabolites [1]. Therefore, these peaks were assigned to tetrol, dehydration and 6-hydroxylation respectively. Peak 1 only appeared after hydrolysis. The retention time and the m/z of this compound corresponded to the parent drug. No reported structure was found to be coincident with the m/z found for the other 5 metabolites.



(a) (b)

Figure 2. Neutral loss chromatograms for (a) blank urine and (b) excretion urine of fluoxymesterone

GC-MS analysis

The extracted ion chromatograms at the expected m/z of each analyte were recorded. Under these conditions, 7 out of 9 compounds found in neutral loss were also detectable by GC-MS. Only metabolites A7 and A9 did not show any signal. The full scan spectrum of each analyte was obtained. The EI spectra of metabolites A1, A2 and A3 were unequivocally identified using the available standards. The EI spectrum of metabolite 4 was coincident with the previously reported for 6-hydroxy-fluoxymesterone [7]. No information was found for the other 5 metabolites.

Elucidation of metabolites

In order to propose one coherent structure for unreported metabolites, their ES ionization and their fragmentation in both CID and EI spectra were studied. The presence of an abundant

$[M+H]^+$ in the ES full scan spectrum implied the presence of a keto function. On the contrary, the occurrence of a $[M+Na]^+$ was considered as evidence for the absence of this moiety.

The CID fragmentation allowed us to deduce if the metabolite presented a hydroxyl moiety in the position 17 (neutral loss of acetone) or if it contained two methyl groups in this position (neutral loss of isobutene).

In the EI spectra, the occurrence of the ion at m/z 143 was considered as a confirmation of the presence of a hydroxyl group in the position 17. Finally, the ion at m/z 208 was selected as an evidence for the presence of a conjugated keto in the A ring of the molecule.

The combination of both ionization and fragmentation together with previously reported data [1,2] allowed us to establish a tentative structure for each analyte. As an example, the elucidation of A5 is presented. The accurate mass measurements for A5 and the complete data for all metabolites can be found elsewhere [3].

Metabolite A5

The ES ionization of A5 showed an abundant $[M+H]^+$ (Figure 3a) indicative for the presence of a keto function in the molecule. Additionally, this spectrum also presented a peak at m/z 143 corresponding to a 17-methyl, 17-hydroxyl structure (Figure 3c). The absence of the peak at m/z 208 implied a variation in the A-B ring from the fluoxymesterone which can be explained by the presence of a hydroxyl group in C-6.

The CID fragmentation (Figure 3b) of this compound showed a loss of 28 together with losses of HF, water and acetone (confirming the presence of a 17-methyl, 17-hydroxy structure). The loss of 28 was associated with CO due to the oxidation of the 11-hydroxyl group. Therefore, A5 was assigned as 11-keto-6-hydroxy-fluoxymesterone.

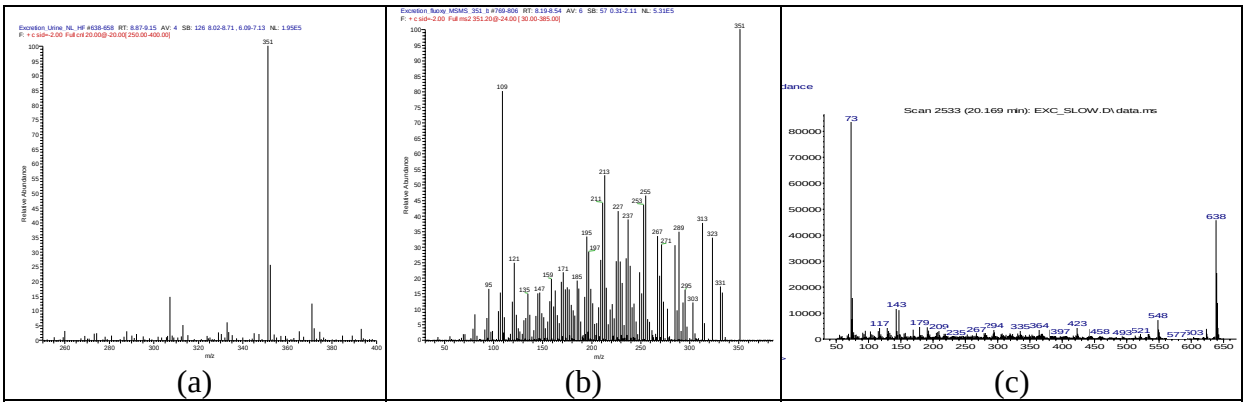


Figure 3. Spectra obtained for metabolite A5: (a) full scan-electrospray-MS (b) product ion electrospray-MS/MS and (c) GC-EI-MS

Table 2. LC-MS/MS and GC-EI-MS ions obtained from metabolite A5

		LC-MS/MS			GC-MS	
Analyte	Molecular formula	Precursor ion (m/z)	Product ion (m/z)		Ions (m/z)	
A5	$C_{20}H_{27}O_4F$	351	333	$-H_2O$	638	M
			323	-CO	623	$M-CH_3$
			313	-HF, $-H_2O$	548	M-TMSOH
			295	-HF, $-2H_2O$	533	$M-CH_3$-TMSOH
			285	-CO, -HF, $-H_2O$	443	$M-CH_3$-2TMSOH
			237	-HF, $-2H_2O$-$(CH_3)_2C=O$	423	$M-CH_3$-HF-2TMSOH
					143	D ring
					73	TMS

Proposal of metabolic pathway

A feasible metabolic pathway for fluoxymesterone has been proposed (Figure 4). Although, as stated before, these structures are in agreement with both ionization and fragmentation data, the synthesis and subsequent analysis of the different standards is necessary in order to corroborate this proposal [1]. The proposed metabolic pathway implies four metabolic reactions which are common for steroids: hydroxylation in allylic position (6-position in this case), reduction either in 3-keto or 4-ene, 11-oxydation and backbone rearrangement at C17 by migration of the C-18 methyl group and subsequent formation of the 13-14 double bond. Additionally, conjugation seems to be the preferred way to excrete the parent compound. Metabolite A10 was not detected in the neutral loss scan. However its occurrence has been hypothesized as an intermediate between metabolite 3 and 6.

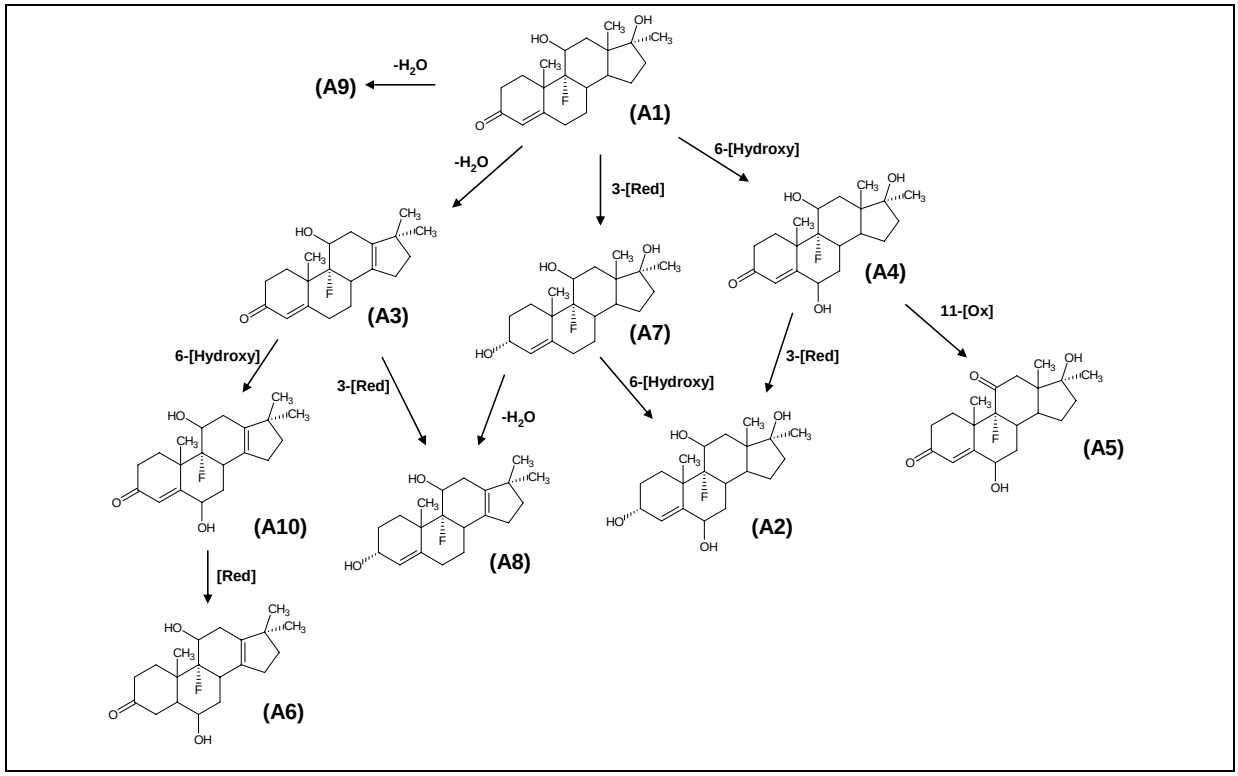


Figure 4. Proposed metabolic pathway for fluoxymesterone

Application to other positive samples

In order to check if the metabolites were commonly present after fluoxymesterone abuse, 6 negative samples and 4 different positive urine samples were analysed. In order to increase the sensitivity of the analysis, a SRM method has been developed for the LC-MS/MS detection and a SIM method for the GC-MS detection (Table 3). The LC-MS/MS method included two transitions for each analyte when available, while at least three ions were selected in the GC-MS method. Two hypothetical LC-MS/MS transitions for A10 were also introduced in the method.

All negative samples did not present any peak at the corresponding retention time in both LC-MS/MS and GC-MS. Positive samples presented abundant peaks for all metabolites in LC-MS/MS (Figure 5) and only A7 and A9 were not detected by GC-MS.

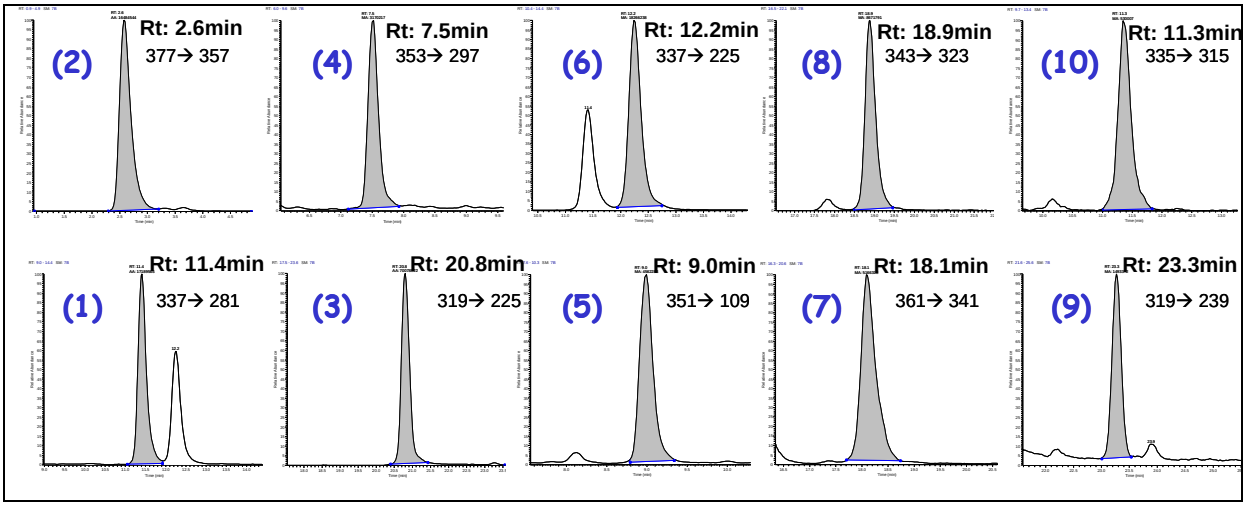


Figure 5. LC-MS/MS chromatograms for a positive urine for fluoxymesterone applying the SRM method developed (number of analytes shown between brackets).

Table 3. Experimental conditions in SRM and SIM methods for the detection of fluoxymesterone metabolites

	LC-MS/MS		GC-MS
Analyte	Precursor ion (m/z)	Product ions (m/z)	Ions (m/z)
A1	337	281 (30[a]), 241 (35)	552, 462, 407
A2	377 372	357 (20) 95 (25)	642, 552, 447
A3	319	225 (25), 159 (40)	462, 208, 357
A4	353	297 (30), 131 (40)	640, 143, 550
A5	351	109 (25), 91 (40)	638, 143, 548
A6	337	225 (30), 159 (35)	552, 462, 407
A7	361	341 (20)	554[a]
A8	343	323 (20)	464, 196, 176
A9	319	239 (25), 131 (35)	462[a]
A10	335	315 (20), 297 (25)	355, 281, 550

[a] *Collision energy (eV)*

Conclusions

LC-MS/MS is a powerful tool in the elucidation of steroid metabolism. The use of neutral losses of HF allowed for the discrimination between fluoxymesterone metabolites and other endogenous components in the urine. By this approach, 9 compounds including the parent drug and 8 metabolites have been detected. Five of them have not been previously reported (A5, A6, A7, A8 and A9). Compounds A1, A2, A3, A4, A5, A6 and A8 have also been confirmed by GC-MS using SIM mode analysis of TMS derivatives. Feasible structures for the detected metabolites and a metabolic pathway for fluoxymesterone have been proposed. These structures are in accordance with four important metabolic pathways: 6-hydroxylation, reduction (either of 3-keto or 4-ene), 11-oxidation and backbone rearrangement with a loss of water. All these analytes have been found in the free fraction except for the parent drug (A1) which was only detectable after hydrolysis. In order to know which metabolite is more adequate for detecting fluoxymesterone abuse, the analysis of urine collected at different times after administration needs to be performed.

Acknowledgements

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