LC-MS Quantitative Screening Method for 18 Anabolic Steroids in Oral Fluid Using MS2 Spectra Data Collected with Q Exactive Orbitrap Mass Spectrometer

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Overview

Purpose: To develop a sensitive method for quantitative screening of 18 anabolic steroids in oral fluid.

Methods: Samples were processed with LLE, analyzed with a 15 min. LC gradient, and compounds were identified with ion ratio calculated for fragments in MS2 spectrum

Results: The LLOQ was 1ng/mL for all analytes except for 6β-Hydroxyfluoxymesterone (6 ng/mL). The UPLQ was between 60-1500 ng/mL, and it was lower for compounds producing high signal in mass spectrometer detector. Matrix effects were not observed: percent recovery in spiked blank oral fluid and analyzed with calibration standards prepared in solvent was in range 78.5-118%.

Introduction

Androgenic-anabolic steroids (AAS) are drugs which mimic effects of testosterone and dihydrotestosterone in the human body. They increase protein synthesis within cells which results in buildup of cellular tissue, especially in muscles. Use of anabolic steroids by athletes to increase body weight is referred to as doping and is banned by major sporting bodies.

In this work we implemented Thermo Scientific™ Q Exactive™ ultra high resolution mass spectrometer to ensure high method specificity and sensitivity.

Methods

Sample Preparation - LLE

1. To 200 µL of oral fluid (in preservation buffer), add 40 µL of internal standard solution (10 µg/mL Testosterone $^{13}C_3$ in MeOH) and 1 mL MTBE
2. Vortex, let samples rest for 5 min. at room temperature
3. Store samples for 30 min. at -20 °C
4. Transfer solvent upper layer to glass tube
5. Evaporate at 37 °C
6. Reconstitute in 50% MeOH
7. Inject 30 µL of the sample onto LC-MS

Liquid Chromatography

Column: Thermo Acucore C18, 100x3 mm, 2.6 µm

Mobile phase:

A: 0.2% Formic Acid in DIW
B: 0.1% Formic Acid in MeOH
C: ACN/IPA/Acetone=45/45/10 v/v/v

LC gradient:

	Time	A%	B%	C%	D%	µl/min
0	0.00	95.0	5.0	0.0	0.0	1000.0
1	0.49	95.0	5.0	0.0	0.0	1000.0
2	0.50	95.0	5.0	0.0	0.0	500.0
3	2.00	50.0	50.0	0.0	0.0	500.0
4	10.00	0.0	100.0	0.0	0.0	500.0
5	10.01	0.0	100.0	0.0	0.0	1000.0
6	11.00	0.0	100.0	0.0	0.0	1000.0
7	11.01	0.0	0.0	100.0	0.0	1000.0
8	12.00	0.0	0.0	100.0	0.0	1000.0
9	12.01	95.0	5.0	0.0	0.0	700.0
10	13.00	95.0	5.0	0.0	0.0	1000.0
11	15.00	95.0	5.0	0.0	0.0	1000.0

Mass Spectrometer

Ionization source: APCI
Resolution: 35K
Isolations width: 2 mu
AGC target: 2e5
Maximum IT = 250 ms
Acquisition mode: t-MS2

MS2 spectra are collected with optimized collision energies specified in method inclusion list (Figure 1) together with acquisition time windows.

Figure 1. MS method inclusion list

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	Mass [m/z]	Polarity	Start [min]	End [min]	nCE	CS [z]	Comment
1	259.07630	Positive	2.75	3.75	40 %		Clenbuterol
2	259.07630	Positive	7.25	8.25	40 %		19-Norandosterone
3	275.20060	Positive	6.00	7.00	50 %		Nandrolone
4	283.20560	Positive	6.12	7.12	40 %		Methandrosterone
5	285.18490	Positive	3.70	4.70	40 %		6b-Hydroxyboldenone
6	287.20060	Positive	5.70	6.70	35 %		Boldenone
7	287.20060	Positive	6.70	7.70	50 %		DHEA
8	289.21620	Positive	5.90	7.90	40 %		Oxandrolone/Testosterone/Epitestosterone
9	303.19550	Positive	3.70	4.70	45 %		Formestane
10	311.24820	Positive	7.10	8.10	90 %		Stanozolol
11	313.21620	Positive	7.44	8.44	50 %		THG
12	319.22680	Positive	6.70	8.70	50 %		Oxymesterone
13	323.17720	Positive	7.00	8.00	40 %		Clostebol
14	292.22630	Positive	6.40	7.40	40 %		Testosterone_3C13
15	337.21740	Positive	5.90	6.90	50 %		Fluoxymesterone
16	345.25360	Positive	5.80	6.80	80 %		3-Hydroxystanozolol
17	353.21230	Positive	4.20	5.20	45 %		6b-Hydroxyfluoxymesterone
18							

Data Processing

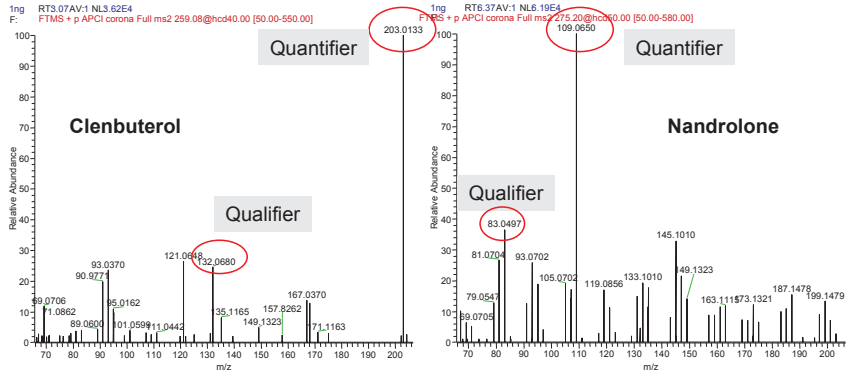
Two most abundant fragments (Table 1) in MS2 spectra (Figure 2) were selected for quantification and confirmation. Ion ratio was calculated and EU guidelines[1] for maximum permitted tolerance were applied.

Table 1. List of analytes, m/z values for parent ion and fragments in MS2 spectrum

Analyte	Formula	m/z	m/z in MS source	Ret Time (min)	Fragment 1	Fragment 2
Clenbuterol	$C_{12}H_{18}Cl_2N_2O$	277.0869	259.0763	3.2	203.0129	132.0679
19-Norandrosterone	$C_{18}H_{28}O_2$	277.2162	259.2056	7.7	241.1942	145.1007
Nandrolone	$C_{18}H_{26}O_2$	275.2006	275.2006	6.5	109.0647	83.0494
Methandrosterone	$C_{20}H_{28}O_2$	301.2161	283.2056	6.6	173.0956	147.0800
6β-Hydroxyboldenone	$C_{29}H_{26}O_3$	303.1955	285.1849	4.3	121.0645	147.0798
Boldenone	$C_{19}H_{26}O_2$	287.2006	287.2006	6.2	121.0648	135.1166
DHEA	$C_{19}H_{28}O_2$	289.2162	287.2006	7.2	97.0653	109.0651
Oxandrolone	$C_{19}H_{30}O_3$	307.2268	289.2162	6.4	135.1165	121.1012
Testosterone	$C_{19}H_{28}O_2$	289.2162	289.2162	6.9	97.0651	109.0650
Epitestosterone	$C_{19}H_{28}O_2$	289.2162	289.2162	7.4	97.0651	109.0650
Formestane	$C_{19}H_{26}O_3$	303.1955	303.1955	4.2	121.0649	171.0802
Stanozolol	$C_{21}H_{32}N_2O$	329.2587	311.2482	7.6	81.0542	107.0857
THG	$C_{21}H_{28}O_2$	313.2162	313.2162	7.9	241.1576	159.0798
Oxymesterone	$C_{20}H_{30}O_3$	319.2268	319.2268	7.2	113.0595	125.0593
Clostebol	$C_{19}H_{27}ClO_2$	323.1772	323.1772	7.5	143.0254	131.0254
Fluoxymesterone	$C_{20}H_{29}FO_3$	337.2173	337.2173	6.4	241.1576	131.0851
3-Hydroxystanozolol	$C_{21}H_{32}N_2O_2$	345.2536	345.2536	6.3	97.0400	107.0855
6β-Hydroxyfluoxymesterone	$C_{20}H_{29}FO_4$	353.2122	353.2123	4.7	95.0857	239.1419
Testosterone-$^{13}C_3$	$C_{16}{}^{13}C_3H_{28}O_2$	292.2263	292.2263	6.9	100.0753	112.0751

Results

FIGURE 2. MS2 spectra for selected analytes collected for 1 ng/mL calibration standard



Linearity Range, LOQ, LOD

Figure 3. Chromatographic peaks reconstructed with m/z accuracy of 5 ppm at LOQ of 1 ng/mL (*3 ng/mL, **6 ng/mL)

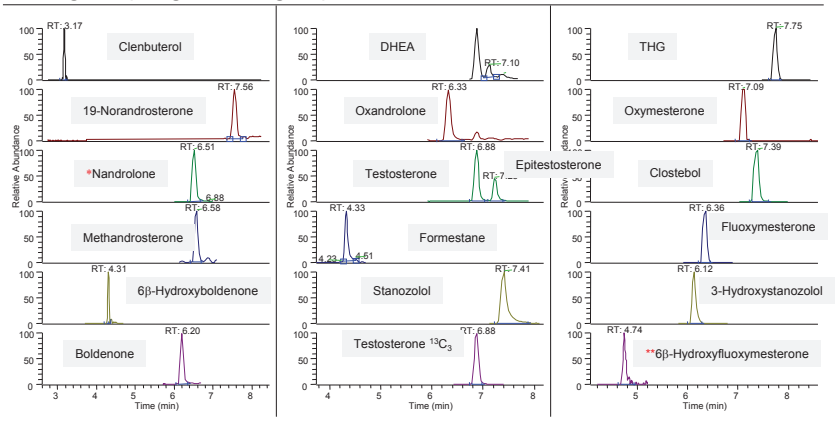
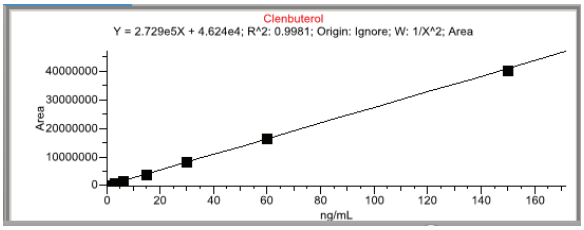
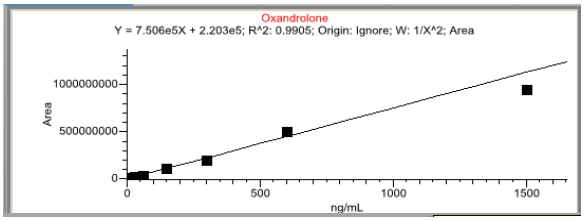


Figure 4. Calibration curves for selected analytes



Theo Amt	Calc Amt	Excluded	% Diff
1.000	0.986		-1.40
3.000	3.119		3.97
6.000	6.187		3.12
15.000	13.899		-7.34
30.000	30.735		2.45
60.000	60.561		0.94
150.000	147.492		-1.67



Theo Amt	Calc Amt	Excluded	% Diff
1.000	0.974		-2.60
3.000	3.058		1.93
6.000	6.557		9.28
15.000	15.531		3.54
30.000	32.688		8.96
60.000	57.033		-4.95
150.000	147.968		-1.35
300.000	267.250		-10.92
600.000	672.905		12.15
1500.000	1259.275		-16.05

Table 2. Linearity ranges, LOQ, LOD

Analyte	Linearity range	R^2	LOQ	LOD
Clenbuterol	1-150 ng/mL	0.9981	1 ng/mL	<1 ng/mL
19-Norandrosterone	1-1500 ng/mL	0.9937	1 ng/mL	< 1 ng/mL
Nandrolone	3-150 ng/mL	0.9926	3 ng/mL	<1 ng/mL
Methandrosterone	1-600 ng/mL	0.9931	1 ng/mL	<1 ng/mL
6β-Hydroxyboldenone	1-600 ng/mL	0.9852	1 ng/mL	<1 ng/mL
Boldenone	1-600 ng/mL	0.9939	1 ng/mL	<1 ng/mL
DHEA	1-600 ng/mL	0.9898	1 ng/mL	<1 ng/mL
Oxandrolone	1-1500 ng/mL	0.9905	1 ng/mL	<1 ng/mL
Testosterone	1-300 ng/mL	0.9896	1 ng/mL	<1 ng/mL
Epitestosterone	1-600 ng/mL	0.9889	1 ng/mL	<1 ng/mL
Formestane	1-600 ng/mL	0.9882	1 ng/mL	<1 ng/mL
Stanozolol	1-300 ng/mL	0.9911	1 ng/mL	<1 ng/mL
THG	1-600 ng/mL	0.9914	1 ng/mL	<1 ng/mL
Oxymesterone	1-300 ng/mL	0.9923	1 ng/mL	<1ng/mL
Clostebol	1-150 ng/mL	0.9961	1 ng/mL	<1 ng/mL
Fluoxymesterone	1-300 ng/mL	0.9916	1 ng/mL	<1 ng/mL
3-Hydroxystanozolol	1-60 ng/mL	0.9952	1 ng/mL	<1 ng/mL
6β-Hydroxyfluoxymesterone	6-150 ng/mL	0.9838	6 ng/mL	3 ng/mL

Method Precision
QC samples with concentrations across calibration range (2 ng/mL, 15 ng/mL, 90 ng/mL, 450 ng/mL) were prepared in blank oral fluid. QC samples were analyzed in 5 replicates in 3 separate batches to obtain intra- and inter- assay precision (Table 3).

Table 3. Intra-assay and inter-assay results

Analyte	Intra assay				Inter assay			
	2 ng/mL	15 ng/mL	90 ng/mL	450 ng/mL	2 ng/mL	15 ng/mL	90 ng/mL	450 ng/mL
Clenbuterol	<10.5	<3.3	<6.2	<15.1	12.6	8.4	5.8	11.0
19-Norandrosterone	<12.4	<11.6	<12.5	17.9	16.3	9.4	12.0	14.1
Nandrolone	NA	<14.2	<12.3	<13.0	NA	12.7	10.0	10.4
Methandrosterone	<13.1	<11.9	<13.9	<18.3	11.5	12.7	13.9	17.3
6β-Hydroxyboldenone	<7.9	<13.3	<11.5	<20.0	14.5	10.8	9.5	13.6
Boldenone	<15.1	<9.4	<11.1	<12.9	12.6	11.3	16.1	18.2
DHEA	<16.6	<13.4	<10.5	<9.7	14.2	10.2	10.2	8.9
Oxandrolone	<11.0	<14.4	<12.9	<19.9	10.6	10.4	10.2	13.7
Testosterone	<14.6	<9.0	<11.9	<19.1	11.5	7.6	9.5	16.7
Epitestosterone	<16.4	<14.4	<10.8	<13.2	14.3	9.8	7.7	8.3
Formestane	<10.4	<10.6	<10.0	<18.1	18.7	13.5	14.3	19.9
Stanozolol	<20.9	<10.9	<10.5	<15.2	19.9	10.9	8.2	13.1
THG	<19.5	<10.1	<11.0	<16.9	16.3	11.1	7.5	13.4
Oxymesterone	<25.0	<12.3	<6.0	<15.0	24.5	9.0	4.9	12.6
Clostebol	<14.8	<12.4	<10.3	<12.8	14.1	11.0	6.6	9.7
Fluoxymesterone	<18.0	<9.6	<11.6	<19.2	24.0	9.0	7.5	14.0
3-Hydroxystanozolol	<15.1	<5.0	<5.3	<12.5	24.8	8.0	5.8	11.0
6β-Hydroxyfluoxymesterone	NA	<12.8	<6.5	<13.7	NA	9.1	9.4	14.2

Matrix Effect
Matrix effects (Table 4) were evaluated by spiking blank oral fluid with all analytes at concentrations of 2 ng/mL, 10 ng/mL, 100 ng/mL and analyzing these samples with calibration standards prepared in solvent.

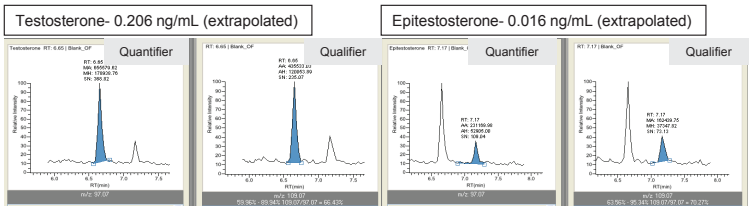
Table 4. Percent recovery in spiked blank oral fluid

Analyte	2 ng/mL	10 ng/mL	100 ng/mL
Clenbuterol	121	131	107
19-Norandrosterone	101	123	101
Nandrolone	ND	97.7	93.5
Methandrosterone	95.0	104	103
6β-Hydroxyboldenone	102	92.4	94.3
Boldenone	101	103	99.6
DHEA	100	127	115
Oxandrolone	93.5	124	109
Testosterone	90.5	105	96.8
Epitestosterone	78.5	99.8	102
Formestane	90.5	92.6	95.3
Stanozolol	80.0	81.5	92.8
THG	94.0	100	95.9
Oxymesterone	89.0	109	113
Clostebol	99.7	110	118
Fluoxymesterone	96.5	101	104
3-Hydroxystanozolol	93.5	92.0	105
6β-Hydroxufluoxymesterone	80.5*	102	104

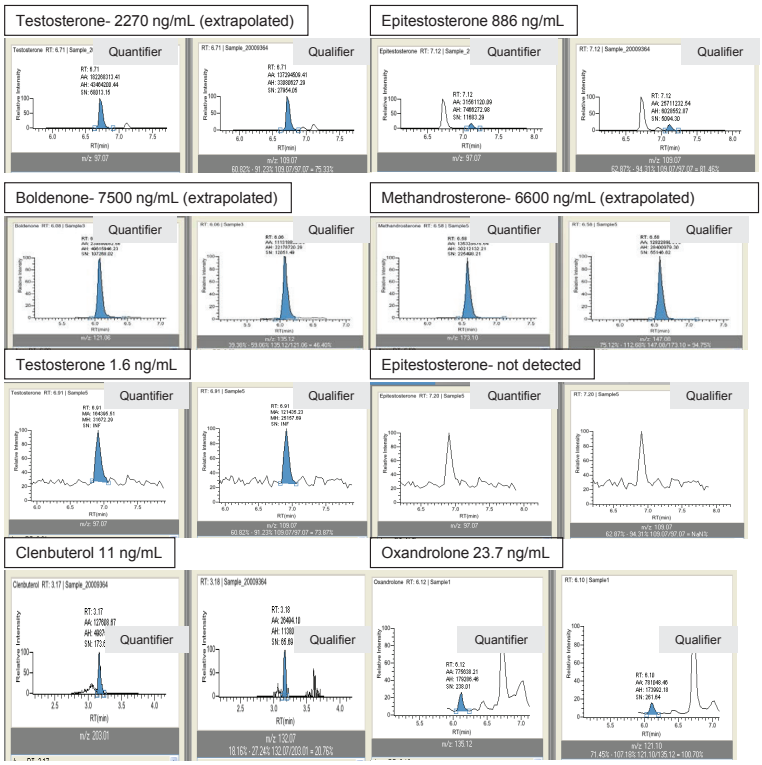
ND: not detected; *concentration (1.61 ng/mL) below LOQ

Donor Samples

Testosterone and Epitestosterone in negative tested oral fluid processed with LLE.



Compounds detected in selected positive tested samples prepared in collaborator lab with protein precipitation method.



Conclusion

We developed sensitive and robust quantitative screening method to analyze anabolic steroids in human oral fluid.

- Implementation of the ultra high resolution Q Exactive mass spectrometer to collect MS2 spectra and ion ratio confirmation results in high confidence in compound identification.
- Method was validated using LLE for sample preparation, but we also detected all analytes in positive tested samples processed with protein precipitation and provided by collaborator laboratory.

Acknowledgement

We would like to thank Erica Guice, Research Director, Western Slope Laboratory, for scientific advice and for providing samples for method testing.

References

1. Draft SANCO 1805/2000 Rev.1 [Revised Commission Decision 93/256 of 14 April 1993] laying down performance criteria for analytical methods to be used for certain substances and residues there of in live animals and animals products according to Council Directive 96/23/EC

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