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Screening method for selected doping substances by gas chromatography – triple quadrupole mass spectrometry: Reaching minimum required performance limits

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Abstract

The minimum performance limits for many forbidden substances have been modified in the latest version of the World Anti-Doping Agency (WADA) technical document (TD2013MRPL). As a consequence, antidoping laboratories have to re-evaluate their routine screening methods in order to prove that they are capable of reaching these limits. This is not always feasible with the commonly employed GC-MS and LC-MS/MS methodologies. Thus, laboratories may take advantage of recently introduced instruments for some compounds. In particular, the use of gas chromatography coupled to triple quadrupole instruments (GC-QqQ) is becoming an essential tool. In the Barcelona antidoping laboratory, the screening strategy has introduced GC-QqQ in order to cope with the new MRPL for selected analytes which show limitations either when analyzed by GC-MS or LC-MS/MS.
The aim of this work was to develop and validate a GC-QqQ method able of reaching the new MRPL for some doping agents. The list of analytes includes anabolic steroids and other doping agents.The final selected reaction monitoring (SRM) GC-QqQ method contains two specific transitions per compound. The method was tested by extracting ten blank urine samples, six samples fortified at low concentration, six at high concentration, and six samples spiked after extraction procedure for recovery calculations. Without modifying the work-up procedure employed for the screening of anabolic steroids by GC-MS, 27 compounds were successfully validated at the new MRPL with adequate intra-assay precisions. No interferences were detected confirming the specificity of the method.

Introduction

The minimum required performance limits which need to be reached for each compound have been reviewed in a recent technical document [1]. For this reason, antidoping laboratories have to re-evaluate their routine screening methods in order to prove that they are able of reaching the new limits. The use GC-QqQ is becoming an essential tool for anabolic androgenic steroids (AAS) [2,3]. This is particularly important in the analysis of some AAS which show limitations when being analyzed by GC-MS or LC-MS/MS. The screening strategy for AAS in Barcelona antidoping laboratory has been modified by analyzing those substances that show these limitations by GC-QqQ without modifying the work-up procedure commonly employed for the screening of anabolic agents.

Experimental

Sample preparation

Screening of anabolic androgenic steroids and their metabolites includes the analyses of glucuronoconjugates and unconjugated excreted steroids, the so-called total fraction. Briefly, 25 µL of internal standard solution, 1 mL of 1M phosphate buffer at pH 7.0 and 30 µL of ß-glucuronidase from E. coli were added to 2.5 mL of urine. The mixture was hydrolyzed at 55 °C for 1 hour in a water bath. The hydrolyzate was left to cool at room temperature and 200 mg of Na_2CO_3/ $NaHCO_3$ (2:1 w/w) were added in order to increase the pH to 9. Sample was extracted with 6 mL of tert-butylmethyl ether.

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After centrifugation (5 min at 1400 *g*), the organic phase was separated and evaporated to dryness under nitrogen stream in a water bath. The extract was reconstituted and derivatised with 50 µL of MSTFA: NH4I: 2-mercaptoethanol (1000:2:6 v/w/v) for 20 min at 60 °C and 2 µL were injected into the system.

GC-MS/MS instrument and conditions

A GC 7890A gas chromatograph equipped with a 7693 autosampler and a 7000A Series Triple Quadrupole (Agilent Technologies, USA) was used to carry out all experiments. HP-Ultra 1 capillary column was used; length 16.5 m, I.D. 0.2 mm, film thickness 0.11µm. Helium gas at constant flow was used as a carrier gas. The system operated in split mode (1:10). The GC initial temperature was 185°C, increased at 25°C/min to 230°C, 10°C/min to 290°C and 70°C/min to 310°C with 2.5 min of hold time (total run time 10.586 min). The injector, transfer line, ion source and quadrupole temperatures were 280°C, 280°C, 230°C and 150°C, respectively. The SRM transitions are listed in Table 1. Nitrogen at 1.5 mL/min and helium 2.25 mL/min were used as a collision gas and quenching gas, respectively.

Compound	Transitions	CE	Compound	Transitions	CE
Clenbuterol	335.3 → 300.3	15	5α-Androstan-2α,17α-dimethyl-	449.4 → 269.2	19
(CLE)	335.3 → 227.2	15	3a,17β-diol (METm)	449.4 → 213.2	19
Anastrazole	293.2 → 209.2	20	Stenbolone	446.4 → 208.2	19
(ANA)	209.2 → 141.1	21	(STE)	431.4 → 193.1	24
Zilpaterol	405.4 → 308.3	6	Boldenone	430.4 → 206.2	18
(ZIL)	308.3 → 218.2	15	(BOD)	206.2 → 196.1	10
Desoxymethyltestosterone	345.3 → 201.1	25	7β,17α-dimethyl-5β -androstane-	284.2 → 227.2	15
(MAD)	345.3 → 105.1	25	3α,17β-diol (CALm)	284.2 → 213.2	15
5β-androst-1-ene-17β-ol-3-one	432.4 → 206.1	14	17α -ethyl-5β-estrane-	421.4 → 331.3	11
(BODm)	432.4 → 194.1	14	3α,17β-diol (NOREm1)	421.4 → 241.2	11
NorAndrosterone	420.4 → 405.4	13	7α,17α -dimethyl-5β-androstane-	284.2 → 269.2	15
(NorA)	420.4 → 315.3	13	3α,17β-diol (BOLm)	284.2 → 227.2	15
Epimethendiol	358.3 → 301.3	12	Methenolone	195.1 → 179.1	16
(EMD)	358.3 → 196.1	12	(METE)	195.1 → 105.1	16
NorEtiocholanolone	420.4 → 405.4	13	Oxabolone	506.4 → 195.1	34
(NorEt)	405.4 → 315.3	11	(4OHN)	506.4 → 147.1	34
2α-methyl-5α-androstan-3α-ol-17-one	448.4 → 433.4	13	α-Zearalanol	538.4 → 433.4	7
(DROm)	448.4 → 343.3	13	(aZER)	433.4 → 295.2	22
1-methylen-5α-androstan-3α-ol-17-one	446.4 → 431.4	15	β-Zearalanol	538.4 → 433.4	6
(METEm)	446.4 → 341.3	15	(bZER)	433.4 → 295.2	25
9-fluoro-18-nor-17,17-dimethyl-	462.4 → 208.2	20	Fluoxymesterone	552.4 → 462.4	20
4,13-diene,11β-ol-3-one (FLUm)	208.2 → 193.1	13	(FLU)	552.4 → 319.3	20
1α-methyl-5α-androstan-3α-ol-17-one	448.4 → 433.4	13	16β-hydroxyfurazabol	490.4 → 231.2	15
(MESm)	448.4 → 343.3	13	(FURm)	490.4 → 143.1	15
17α-methyl-5β-androstanediol	270.2 → 213.2	15	3'hydroxystanozolol	545.4 → 455.4	40
(MEm2)	270.2 → 199.1	15	(STAm)	545.4 → 147.1	40
17α-methyl-5α-androstanediol	255.2 → 159.1	25			
(MEm1)	255.2 → 145.1	25			

Table 1: Transitions, collision energy (CE) and acronim for each compound included in the GC-QqQ screening as its enol-TMS derivative.

Results and Discussion

GC conditions were chosen to minimize time of analysis distributing all analytes in different acquisition groups to ensure that an adequate number of data points were collected in order to fully define the chromatographic peaks.

Detection conditions (precursor ion, collision energy, and product ion) were optimized for each analyte. Candidates for precursor and product ion were chosen by studying pure standards. Then, final conditions were selected by analyzing extracts from urine samples spiked at 5 times the minimum performance limit required. From the conditions studied, those showing the maximum signal to noise ratio (S/N) were preferred. Table 1 lists all the compounds included in the method together with their assigned abbreviation and their MS parameters.
After optimizing the MS conditions, a validation protocol was applied to the new method in order to evaluate its sensitivity, repeatability, reproducibility and accuracy. Table 2 summarizes the limits of detection (LOD), repeatability and accuracy results found for each analyte at two different concentration levels. The LOD were defined as the lowest concentration tested (half of the MRPL established by WADA). As a result, some compounds could be detected at even lower concentrations.
Regarding reproducibility, Table 3 lists the estimated values for representative analytes. The specificity and selectivity of the method were also evaluated by analyzing ten urine samples from different healthy individuals. No interferences were found at the retention times of the analytes. As an example, Figure 1 shows the chromatograms corresponding to the 27 compounds in a negative sample and in a sample spiked at the LOD.

Compound	RT (min)	LOD*	Repeatability				Recovery (%)	
			ng/mL	RSD (%)	ng/mL	RSD (%)	ng/mL	mean ± sd
CLE	2.98	0.1	0.1	21.3	1	1.9	1	83.4 ± 1.9
ANA	3.67	10	10	4.8	10	6.8	10	100.0 ± 6.8
ZIL	3.91	2	2	0.7	20	3.0	20	56.4 ± 3.0
MAD	4.46	2	2	1.0	20	0.3	20	74.8 ± 0.3
BODm	4.62	2	2	14.1	20	5.3	20	53.1 ± 5.3
NorA	4.64	1	1	6.0	10	2.7	10	66.9 ± 2.7
EMD	4.72	1	1	1.2	10	0.5	10	69.7 ± 0.5
NorEt	4.90	2	2	2.0	20	1.3	20	86.5 ± 1.3
DROm	5.28	2	2	4.6	20	2.4	20	74.9 ± 2.4
METEm	5.47	2	2	0.7	20	3.0	20	92.8 ± 3.0
FLUm	5.56	2	2	1.7	20	3.2	20	90.5 ± 3.2
MESm	5.62	2	2	1.0	20	4.6	20	90.9 ± 4.6
MEm2	5.64	1	1	2.3	10	0.4	10	84.6 ± 0.4
MEm1	5.67	2	2	2.4	20	1.0	20	83.4 ± 1.0
METm	5.85	2	2	8.5	20	2.3	20	78.2 ± 2.3
STE	5.86	2	2	5.8	20	6.7	20	50.6 ± 6.7
BOD	5.87	2	2	2.5	20	2.0	20	89.2 ± 2.0
CALm	5.89	2	2	2.3	20	2.0	20	75.8 ± 2.0
NOREm1	6.08	2	2	3.0	20	2.4	20	100.8 ± 2.4
BOLm	6.10	2	2	5.2	20	1.6	20	79.7± 1.6
METE	6.15	2	2	1.2	20	3.7	20	94.0 ± 3.7
4OHN	6.71	2	2	1.7	20	3.8	20	90.0 ± 3.8
aZER	6.81	2	2	1.9	20	3.6	20	77.3 ± 3.6
bZER	6.90	2	2	3.5	20	1.1	20	73.6 ± 1.1
FLU	7.60	2	2	20.6	20	2.3	20	72.6 ± 2.3
FURm	8.87	2	2	12.1	20	2.0	20	68.5 ± 2.0
STAm	8.89	1	1	7.6	10	2.0	10	48.1 ± 2.0

Table 2: Retention time (RT), repeatability tested at two concentrations (n=6, each concentration), extraction recovery (n= 4) and limit of detection (LOD). The repeatability was measured as the relative standard deviation (RSD) of the areas ratio between the transition of the compound and the transition of the ISTD obtained in different replicates analyzed the same day. The LOD was the lowest concentration tested, with S/N > 3 and RSD or repeatability lower than 25%.

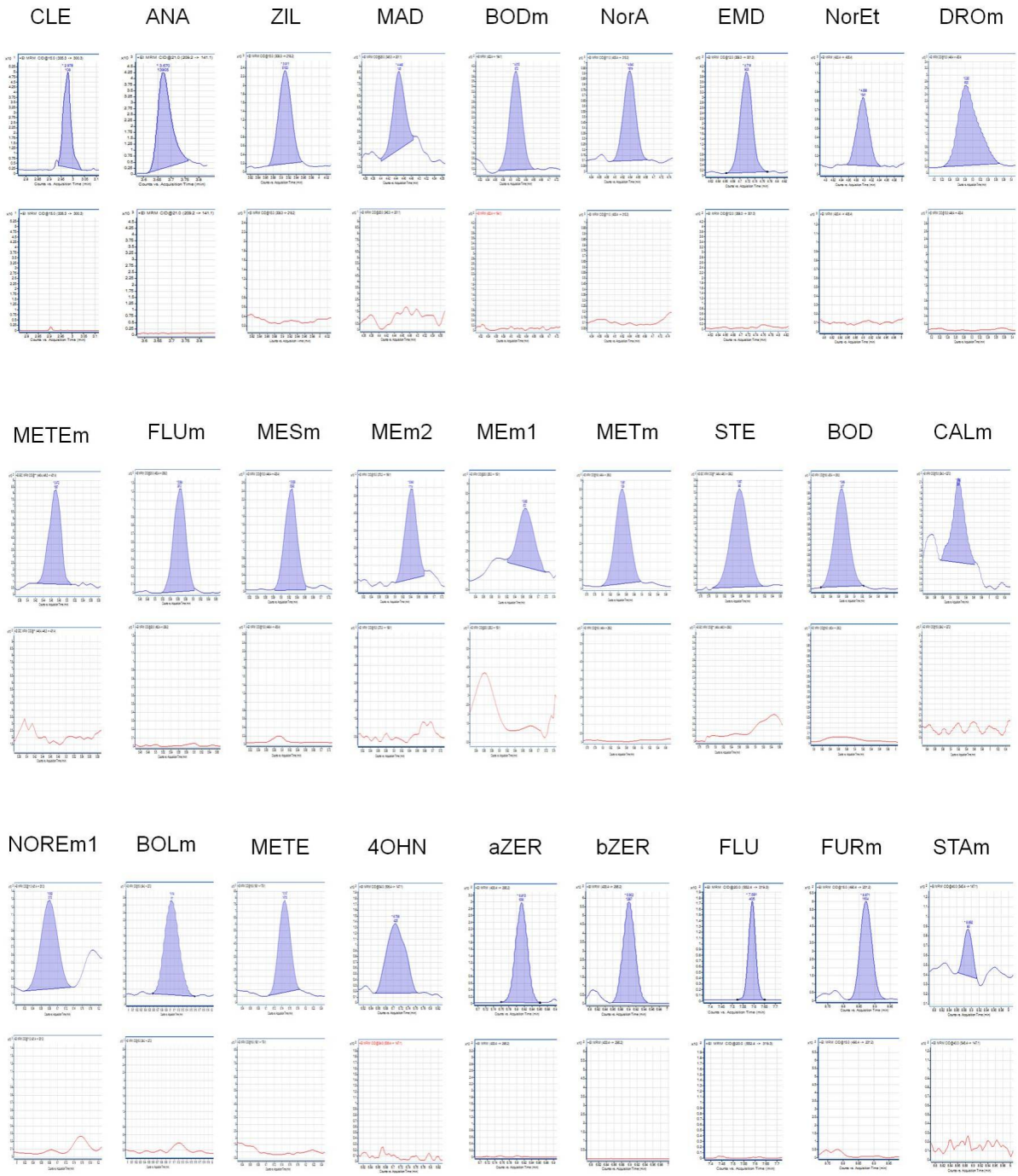

Figure 1: Chromatograms of the characteristic transitions of the enol-TMS derivatives of each compound: upper trace, sample spiked at the LOD indicated in Table 2; lower trace, blank urine.

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Compound	Reproducibility	
	ng/mL	RSD (%)
CLE	2	24.2
BODm	10	5.2
NorA	2	8.9
EMD	2	9.6
NorEt	2	7.1
MEm2	2	6.7
BOD	10	8.4
CALm	10	9.8
BOLm	10	8.3
bZER	10	8.7
STAm	2	22.9

Table 3: Reproducibility for some compounds in 15 analyses performed in different days. The reproducibility was measured as the relative standard deviation (RSD) of the areas ratio between the transition of the compound and the transition of the ISTD obtained in different days.

Conclusions

A new method has been developed to detect 27 compounds at the new MRPL established by WADA. All analytes were successfully validated with adequate intra-assay precisions and recoveries. The specificity and selectivity of the method were confirmed by comparison with ten negative samples from different volunteers.

References

[1] WADA Technical Document TD2013MRPL: Minimum required performance levels for detection and identification of non-threshold substances.http://wada-ama.org/Documents/World Anti-Doping Program/
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