

Analysis of Anabolic Steroids for Doping Control Purposes by GC-TOFMS and GCxGC-TOFMS

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1. Introduction

GC-MS analysis after trimethylsilyl (TMS) derivatization is commonly used by doping control laboratories for a large part of doping substances. When using scanning MS detectors, SIM mode (for quadrupoles) or MS-MS modes (for ion traps) has to be applied in order to reach low ppb detectability required for some compounds by the World Anti-Doping Agency (WADA). However, both the above-mentioned approaches are targeted at a given set of analytes only and cannot discover the presence of doping (or other) substances not present on the targeted list. This might be a disadvantage, considering the fact that new substances with physiological effects are being developed and added to the athlete's diet, many times with the intention to make the new chemical structures "invisible" for analytical methods. Contrary to these target approaches, TOFMS provides full mass spectra at low pg detectability, which can bring advantages in terms of better confirmation of identity (full MS instead of 3 ions of SIM) as well as detection of non-targeted compounds.

Recently, GCxGC-TOFMS has been successfully applied for the steroids analysis and validated with respect to WADA criteria.^[1,2]

In this application note, the performance of both one-dimensional GC-TOFMS and GCxGC-TOFMS were evaluated, with special attention to the detectability and the confirmation reliability at low ppb concentration. For this purpose, five anabolic steroids were selected, for which very low detection limits (2 ng/mL) are required by the World Anti-Doping Agency.

2. Experimental Conditions

Samples

Mixed standard of 5 doping compounds: clenbuterol, 19-norandrosterone, epimethendiol, methyltestosterone M2-metabolite and 3'-OH-stanozolol. Methyltestosterone was used as an internal standard.

A QC sample at 2 ng/mL was prepared from negative human urine by extraction and derivatization to trimethylsilyl esters (TMS). Methyltestosterone was added to the sample as an internal standard at a concentration of 50 ng/mL. In order to determine retention times and reference spectra of target analytes, a solvent standard at a concentration of 10 ng/mL was also injected.

Analysis Conditions

GCxGC-TOFMS Analysis:

Pegasus® 4D, LECO Corp., USA

GCxGC Parameters:

Agilent 7890N Gas chromatograph equipped with a consumable-free (CFM) LECO GCxGC thermal modulator and a secondary oven. Medium for cold jets was dry air from a compressor, cooled by immersion cooling. Medium for hot jets was resistively heated air.

Injection:

1 µL splitless, 250°C

Primary Column:

BP-5 30 m x 0.25 mm x 0.25 µm (SGE, Australia)

Secondary Column:

BPX-50 2.5 m x 0.1 mm x 0.1 µm (SGE, Australia)

Carrier Gas:

Helium, 1 mL/min, corrected constant flow

Primary Oven Program:

150°C (1 min), 15°C/min to 270°C, 5°C/min to 320°C, 15°C/min to 350°C (6 min)

Secondary Oven Program:

160°C (1 min), 15°C/min to 280°C, 5°C/min to 330°C, 15°C/min to 360°C (6 min)

Modulator Offset: 50°C
Modulation Time: 3 s
Hot Pulse Time: 0.9 s
Transfer Line Temp.: 240°C
Total Run Time: 27 min

MS Parameters: LECO Pegasus® HT TOFMS
Ionization: EI, -70 eV
Source Temperature: 220°C
Stored Mass Range: 35-600 u
Acquisition Rate: 200 spectra/s

Instrument control and data processing including Automated Peak Find, deconvolution, peak combination and library search was done using LECO ChromaTOF® software v 4.24.

GC-TOFMS Analysis:

The BP-5 30 m x 0.25 mm x 0.25 µm (SGE, Australia) column was used. four spectra/sec were collected by TOFMS. All the other parameters were identical as above.

3. Results and Discussion

A quality control sample (QC) serves to doping laboratories as a tool to ensure the quality of the analytical process. The concentration level of a QC standard should correspond to the required detection limit of a particular compound given by the WADA criteria. For the group of anabolic steroids analyzed here, the required limit is 2 ng/mL, i.e. 2 pg injected onto the column when using a 1 μ l injection volume.

For the purpose of target analyte search, a Reference from a standard at 10 ng/mL was created in ChromaTOF software. This Reference comprised retention times and spectral information for the target analytes. Afterwards the QC sample was automatically processed using this Reference and the analytes were sought automatically.

In Table I and Table II, the results from the analysis of a QC sample at 2 ng/mL by both one-dimensional and GCxGC methods are shown. The tables comprise signal-to-noise ratios and match factors (compared to the reference spectrum) for each analyte. In Figure 1 and Figure 2 the detection of individual peaks at 3-4 characteristic ion traces is shown together with the mass spectra.

By using the 1D GC-TOFMS method, all the analytes, with the exception of epimethendiol, were detected at minimum 3 ion traces and their mass spectra exhibited match factor higher than 670. Signal-to-noise ratio of the main quantification ion ranged from 77 (19-Norandrosterone) to 299 (Clenbuterol). Epimethendiol peak was only detected at 2 characteristic masses and the spectral match was too low.

Using the GCxGC-TOFMS method, we observed up to a tenfold signal-to-noise enhancement compared to the 1D method. Besides that, better analyte-matrix separation has been achieved. All the steroids were detected and confirmed in urine extract at a 2 ng/mL concentration level. The spectral match factors were typically higher than 900, with the exception of epimethendiol, where a match factor of 863 was obtained.

Table I. Results from 1D GC-TOFMS analysis of doping substances, QC sample 2 ng/mL

Analyte	R.T. (s)	Quant Mass	Quant S/N	Match
Clenbuterol, bis-TMS	694.00	335	299	677
19-Norandrosterone, bis-TMS	873.25	405	77	737
Epimethendiol, bis-TMS		n.d.		
Methyltestosterone M2 metabolite, bis-TMS	972.25	435	245	939
Methyltestosterone, bis-TMS (ISTD)	1074.25	446	16976	957
3'-OH-Stanozolol, tris-TMS	1306.75	545	92	736

Table II. Results from GCxGC-TOFMS analysis of doping substances, QC sample 2 ng/mL

Analyte	R.T. (s)	Quant Mass	Quant S/N	Match
Clenbuterol, bis-TMS	692, 2.390	335	2093	940
19-Norandrosterone, bis-TMS	872, 2.830	405	1004	970
Epimethendiol, bis-TMS	884, 2.977	358	252	863
Methyltestosterone M2 metabolite, bis-TMS	974, 0.105	435	460	954
Methyltestosterone, bis-TMS (ISTD)	1076, 0.550	446	21689	923
3'-OH-Stanozolol, tris-TMS	1310, 1.335	545	114	902

4. Conclusions

From the above results several conclusions can be made. One-dimensional GC-TOFMS provides satisfactory results for the purpose of screening anabolic steroids in urine. The signal-to-noise ratios of minimum 3 ion traces were sufficiently high at 2 ng/mL concentration level (except epimethendiol). However, the spectral match factors were lower than 800 for some compounds, and the correct identification of ultra-trace levels of steroids was difficult in some cases due to the high complexity of urine matrix.

Using GCxGC-TOFMS, enhanced analyte-matrix separation has been achieved. Thanks to that, excellent mass spectra of target sterols were obtained at 2 ng/mL level. According to the signal-to-noise values, the estimated LODs are in sub-pg levels. GCxGC-TOFMS is therefore not only a suitable screening method, but can be used for the confirmative purposes as well.

5. Acknowledgement

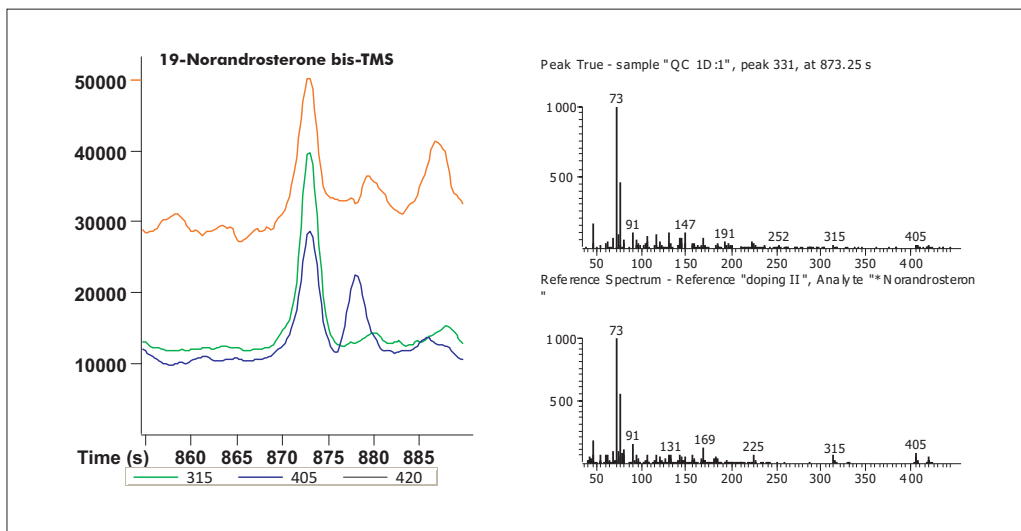
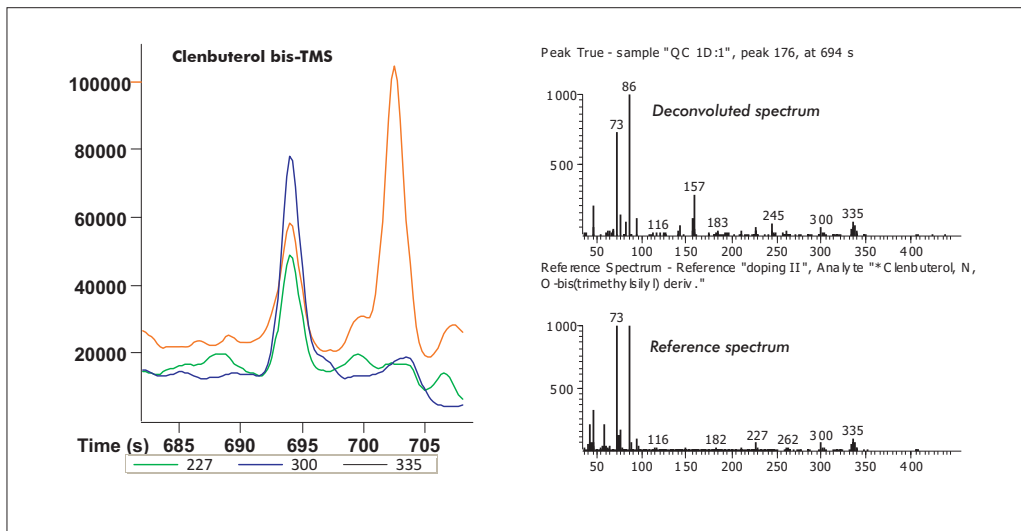
The samples for this study were kindly provided by Zdeněk Chundela, Ph.D, from the Department of Doping Control, University Hospital Prague, Czech Republic.

6. References

- ^[1]Mitrevski et. al.: Application of comprehensive two-dimensional gas chromatography to sterols analysis, J. Chromatogr. A, 1214 (2008) 134-142.
- ^[2]Mitrevski et. al: Evaluation of World Anti-Doping Agency criteria for anabolic agent analysis by using comprehensive two-dimensional gas chromatography-mass spectrometry, Anal. Bioanal. Chem. 396 (2010) 2503-2511.



Figure 1. Detection of doping substances in a quality control sample 2 ng/mL, 1D GC-TOF MS analysis.



Epimethendiol bis-TMS	n.d.
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Figure 1 continued.

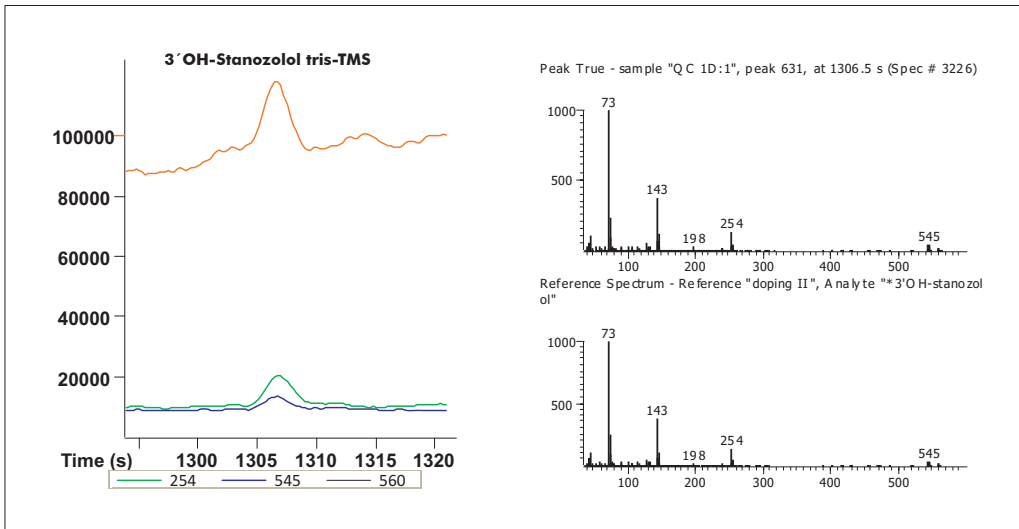
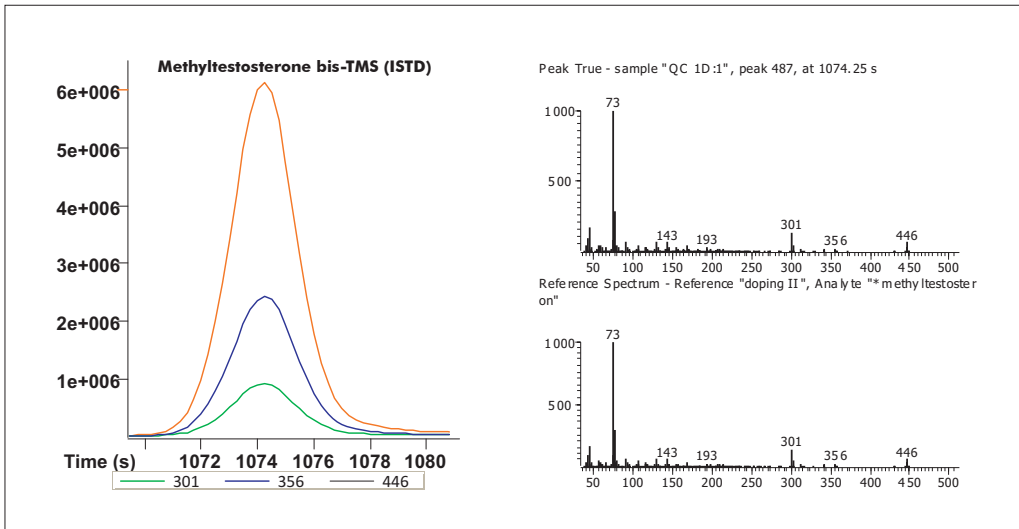
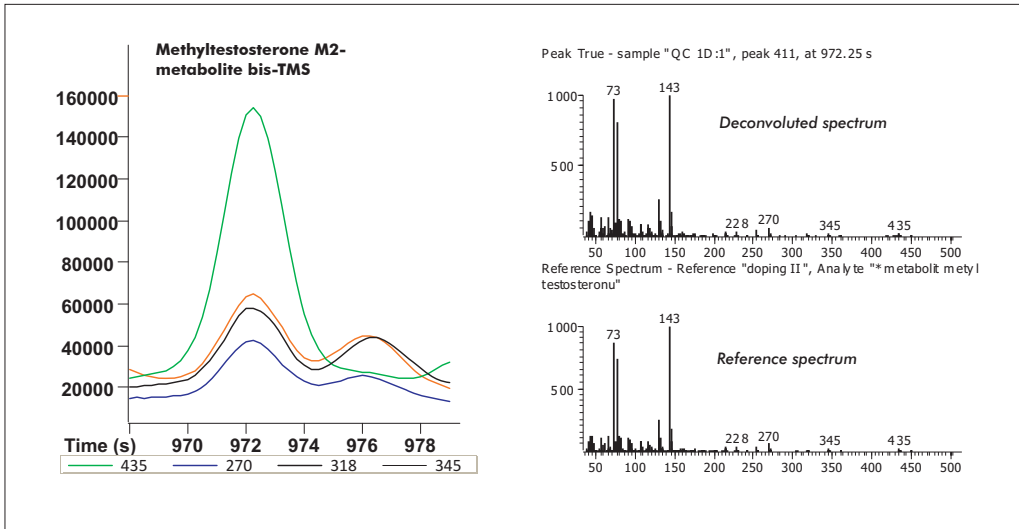


Figure 2. Detection of doping substances in a quality control sample 2 ng/mL, GCxGC-TOF MS analysis

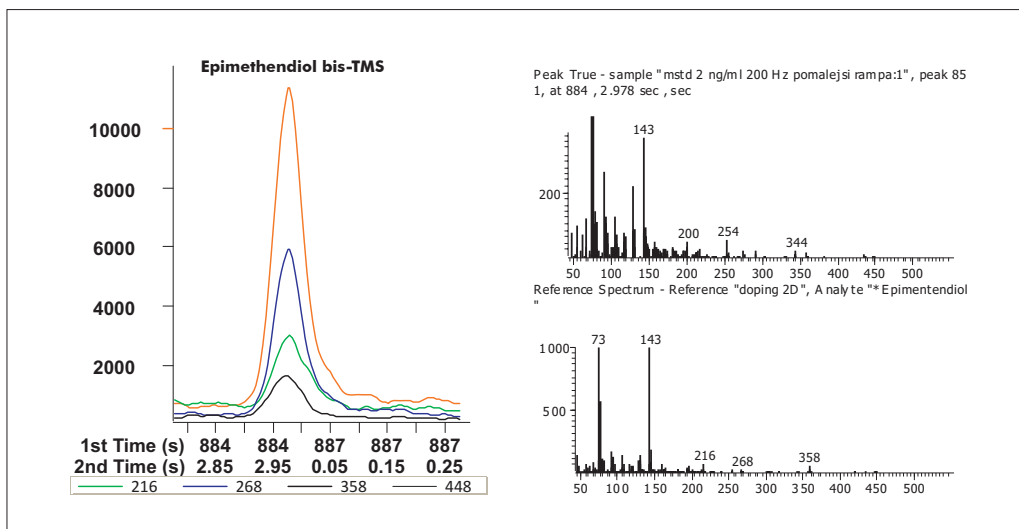
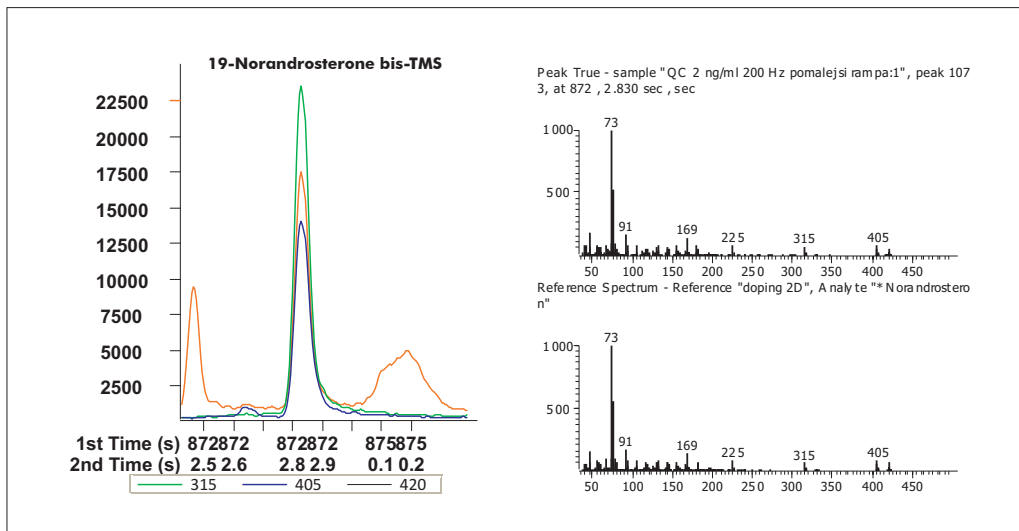
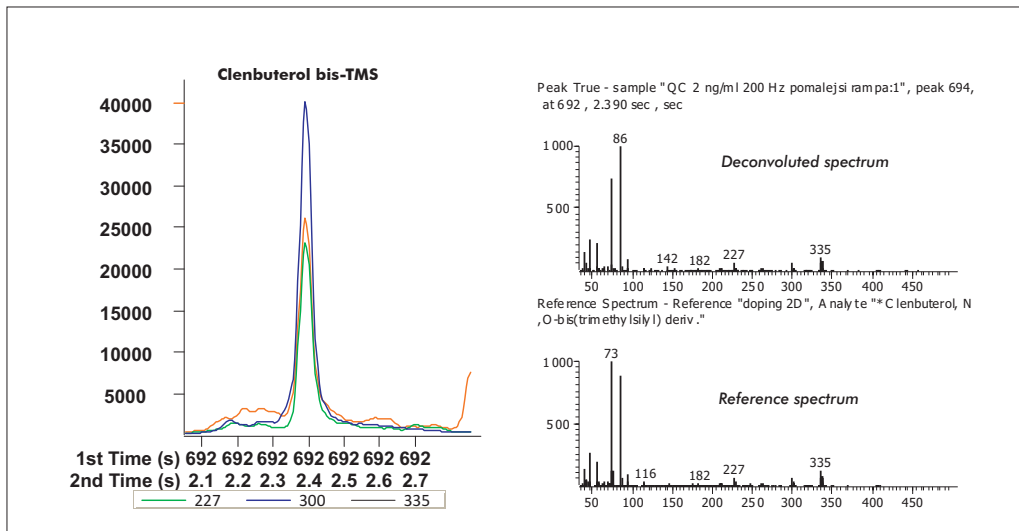


Figure 2 continued.

