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ORIGINAL PAPER

Evaluation of World Anti-Doping Agency criteria for anabolic agent analysis by using comprehensive two-dimensional gas chromatography–mass spectrometry

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Abstract This work presents the validation study of the comprehensive two-dimensional gas chromatography (GC×GC)–time-of-flight mass spectrometry method performance in the analysis of the key World Anti-Doping Agency (WADA) anabolic agents in doping control. The relative abundance ratio, retention time, identification and other method performance criteria have been tested in the GC×GC format to confirm that they comply with those set by WADA. Furthermore, tens of other components were identified with an average similarity of >920 (on the 0–999 scale), including 10 other endogenous sterols, and full mass spectra of 5,000+ compounds were retained. The testosterone/epitestosterone ratio was obtained from the same run. A new dimension in doping analysis has been implemented by addressing separation improvement. Instead of increasing the method sensitivity, which is accompanied by making the detector increasingly "blind" to the matrix (as represented by selected ion monitoring mode, high-resolution mass spectrometry (MS) and tandem MS), the method capabilities have been improved by adding a new "separation" dimension while retaining full mass spectral scan information. Apart from the requirement for the mass spectral domain that a minimum of three diagnostic ions with relative abundance of 5% or higher in the MS spectra, all other WADA criteria are satisfied by GC×GC operation. The minimum of three diagnostic ions arises from the need to add some degree of specificity to the acquired mass spectrometry data; however, under the proposed full MS scan method, the high MS similarity to the reference compounds offers more than the required three diagnostic ions for an unambiguous identification. This should be viewed as an extension of the present criteria to a full-scan MS method.

Keywords WADA criteria · GC×GC · Relative abundance ratio · Tolerance window · Deconvolution · Anabolic-androgenic steroids (AAS)

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Introduction

Anabolic-androgenic steroids (AAS) and β_2 agonists with anabolic effect are the pharmacological substances most frequently abused in doping control. The International Olympic Committee (IOC) and other organisations responsible for anti-doping control (i.e. World Anti-Doping Agency (WADA)) have banned these substances because of their effect on athlete's performance and consequently on the results of competitions.

The biological fluid of choice in current doping control methods is urine. Urine is a complex matrix containing many components in different concentrations and with similar structures, many of which are unknown. Anabolic agents are extracted from this matrix by solid phase extraction [1–3] or liquid/liquid extraction [4, 5], conjugates are hydrolysed, and the free-agents are extracted again and derivatised into corresponding trimethylsilyl (TMS) derivatives. Beside this, many endogenous components are co-extracted to some extent and introduced in the

chromatographic system. Gas chromatography–mass spectrometry (GC-MS) is the preferred technique for analysis, which offers high sensitivity and selectivity. However, the coelution of anabolic agents with endogenous components and the high background noise give rise to false signals in mass spectra and therefore to possible erroneous interpretation [6]. High-resolution MS (HRMS) [2, 7–9] and tandem mass spectrometry (MS/MS) [6, 10] have been proposed to solve this problem and to achieve ever lower limits of detection. Several approaches are proposed for increasing the sensitivity and selectivity of the methods by using an extra step in purification of the samples. In this manner, immunoaffinity chromatography [11] and high-performance liquid chromatography fractioning [12] are employed. Recently a new approach for separation improvement has been proposed by using comprehensive two-dimensional gas chromatography (GC×GC) in sterol [13–16] and drug analysis [17]. GC×GC is a relatively new technique, which exhibits improved separation power compared to traditional one-dimensional gas chromatography (1D GC). The principles of GC×GC have been reported elsewhere [18, 19].

The applicability of GC×GC to doping analysis have been demonstrated previously [15, 16] through its improved separation and identification power when combined with time-of-flight mass spectrometry (TOFMS) as a detector. However, no evaluation of the method performance, as pertaining to assessment against specific WADA criteria, has been presented so far. Thus herein, a systematic and coherent evaluation of analysis of AAS against WADA criteria [20], a point-by-point assessment of GC×GC-TOFMS related to each criterion, is presented. The five key anabolic agents (clenbuterol, 19-norandrosterone, epimethendiol, methyltestosterone M2-metabolite and 3′OH-stanozolol) that the WADA had listed at the low ng mL^{-1} level [21] were used as model components.

Experimental

Reagents and chemicals

Clenbuterol, 19-norandrosterone, epimethendiol (EMD), 17α-methyl-5β-androstane-3α,17β-diol (methyltestosterone M2-metabolite), 3′OH-stanozolol and methyltestosterone were purchased from National Measurement Institute (NMI, Pymble, Australia). *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) was purchased from Sigma Aldrich and β-glucuronidase (from *Escherichia coli*, K12) was supplied from Roche (Mannheim, Germany). All other reagents and chemicals were of analytical grade or higher. Water used in the experiments was of Milli-Q (Millipore) grade.

Sample preparation

Spiked urine samples with anabolic agents were prepared in the concentration range from 0.5–20 ng mL^{-1} by adding an appropriate volume of the standard solutions and 25 μL of the internal standard solution (5 μg mL^{-1}) to the blank urine extracts. The extracts were prepared according to the previously published sample preparation procedure [4, 22]. Prior to analysis, the residue was derivatised by dissolving in 50 μL of derivatisation mixture (MSTFA-NH_4I-ethanethiol, 1000:2:6) with heating at 80 °C for 30 min. Standard solutions of anabolic agents were prepared at a concentration of 0.5 μg mL^{-1} for the purpose of acquiring reference TOFMS spectra. Solutions were stored at 4 °C when not in use.

Instrumentation

A Pegasus III time-of-flight mass spectrometer (LECO Corp., St. Joseph, MI, USA) connected to an Agilent 6890 gas chromatograph fitted with a Longitudinal Modulation Cryogenic System (LMCS) (Chromatographic Concepts Pty Ltd, VIC, Australia) was used in GC×GC-TOFMS experiments. The detector was operated at 1,600 V, and applied electron ionisation voltage was 70 eV. Data acquisition rate was 100 Hz over the mass range from 45 to 600 amu. Data acquisition and processing were performed by ChromaTOF software (LECO Corp.). A separate GC×GC-TOFMS-based in-house library for improved identification was generated using standard solutions of anabolic agents at a concentration of 0.5 μg mL^{-1}. The National Institute of Standards and Technology (NIST) algorithm was used for mass spectra searching.

The column configuration applied was non-polar/polar consisting of 30 m BPX5 (0.25 mm I.D.; 0.25 μm d_f) as a first dimension column (^1D) and 1 m BPX50 (0.1 mm I.D.; 0.2 μm d_f) as a second dimension column (^2D). The oven temperature program was from 120 °C (hold for 1 min) to 320 °C (hold for 5 min) at 4 °C min^{-1}, at a flow rate (He) of 1.3 mL min^{-1}. One-microlitre samples were injected in splitless mode (2.5 min purge time).

Results and discussion

Retention time tolerance criteria

Present WADA criterion:

– The retention time of the analyte shall not differ by more than 1% or ±0.2 minutes (whichever is smaller) from that of the same substance in a spiked urine sample or reference material analysed contemporaneously [20].

Table 1 $^{1}t_R$ and $^{2}t_R$ retention times reproducibility and the relative error of the reference compounds

	Clenbuterol-2TMS		19-Norandrosterone-2TMS		EMD-2TMS		M2-2TMS metabolite		3′OH-stanozolol-3TMS	
	$^{1}t_R$ (s)	$^{2}t_R$ (s)	$^{1}t_R$ (s)	$^{2}t_R$ (s)	$^{1}t_R$ (s)	$^{2}t_R$ (s)	$^{1}t_R$ (s)	$^{2}t_R$ (s)	$^{1}t_R$ (s)	$^{2}t_R$ (s)
AAS average (n=13)	1,670	2.498	2,125	2.577	2,170	2.627	2,343	3.071	2,918	3.075
RSD (%)	0.00	0.65	0.00	0.75	0.18	0.83	0.10	0.64	0.09	0.57
Ref. average (n=2)	1,670	2.480	2,125	2.565	2,165	2.605	2,340	3.045	2,920	3.055
Rel. error (%)	0.00	0.72	0.00	0.47	0.23	0.84	0.13	0.85	0.07	0.65

The relative standard deviation (RSD) of the first dimension ($^{1}t_R$) and second dimension ($^{2}t_R$) retention times of the proposed method has been calculated from a series of spiked urine samples with anabolic agents at different levels of concentration, and the results are given in Table 1. A representative 2D plot of urine extract spiked with sterols at a concentration of 10 ng mL^{-1} is given in Fig. 1. The reproducibility (%RSD) of $^{1}t_R$ has been found to range from 0% for clenbuterol-2TMS and 19-norandrosterone-2TMS to 0.18% for EMD-2TMS, which agrees with the previous findings for GC×GC reproducibility [23]. The relative error of the retention times of the reference compounds are well within the tolerance window of 1% [20]. The lowest deviation (Table 1) has been obtained for clenbuterol-2TMS and 19-norandrosterone-2TMS (0%) and the highest for EMD-2TMS (0.23%). The 0% RSD values for clenbuterol-2TMS and 19-norandrosterone-2TMS are due to the "quantised" values of the $^{1}t_R$ values derived from the modulation process and data processing software. Specifically, ChromaTOF assigns the same $^{1}t_R$ for all components in the same modulation period, regardless of their $^{2}t_R$ values. In addition, since each component generates more than one modulation peak, the $^{1}t_R$ of the component is assigned the retention time of the highest modulated peak. Obviously, the highest modulated peak for clenbuterol-2TMS has been found in the same modulation event at 1670s in each run and likewise for the 19-norandrosterone-2TMS peak at 2125s to give an "arbitrary" $^{1}t_R$ variation evaluated to be 0%. Thus it is not necessarily true that each replicate of each of these compounds has exactly the same $^{1}t_R$, but rather is an artefact of the data presentation. This group has proposed an algorithm for exact $^{1}t_R$ determination in GC×GC [24].

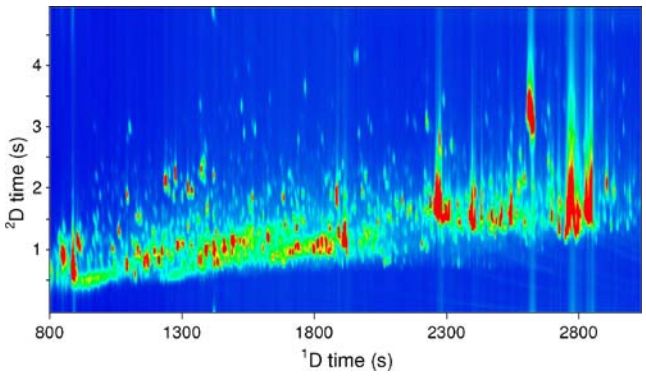

Fig. 1 Two-dimensional plot of urine extract spiked with anabolic agents at a concentration of 10 ng mL^{-1}. Note that the $^{2}t_R$ is shifted vertically by −1 s to provide a better presentation format

The $^{2}t_R$ relative error of the reference compounds, even though not defined as part of the established WADA criteria, fall within the tolerance window of 1%. The reproducibility is in agreement with the previous results [23], and the relative error is from 0.47% (for 19-norandrosterone-2TMS) to 0.85% (for M2-2TMS metabolite). Reproducibility in $^{2}t_R$ values is found to depend on maintaining constant CO_2 cryogen supply, since the temperature of the modulator can influence the $^{2}t_R$; clearly, the values found here, being less than 1% RSD, are acceptable for purposes of this criterion. The high reproducibility of $^{1}t_R$ produces very reproducible elution temperature for all anabolic agents, which in turn means constant and stable $^{2}t_R$. If the modulation process is properly conducted, the $^{2}t_R$ is very robust so the same criteria (1% relative error) can be applied also for this additional parameter. We stress that the retention time reproducibility was carried out on spiked urine samples at different concentration levels, over the range from the limit of detection (2 ng mL^{-1}) to the highest tested concentration level (20 ng mL^{-1}), which demonstrates a rugged reproducibility assessment of $^{1}t_R$. In the event that WADA adopts GC×GC technology, then a new criterion specific for $^{2}t_R$ will need to be considered.

Relative abundance tolerance window criteria

Present WADA criteria [20]:

- All diagnostic ions with relative abundance greater than 10% in the reference spectrum must be present in the spectrum of the unknown peak, and
  - The relative abundance of three diagnostic ions shall not differ by more than the amount shown in Table 2 from the relative intensities of the same ions from that of a spiked urine or reference material.

Table 2 Maximum tolerance windows for relative ion intensities to ensure appropriate uncertainty in identification [20]

Rel. abundance (% of base peak)	EI-GC/MS
>50%	±10% (absolute)
25% to 50%	±20% (relative)
<25%	±5% (absolute)

The mass spectrometric detection and identification in selected ion monitoring (SIM) mode is usually based on monitoring of one quantification ion (for estimation of concentration) and two or more qualification ions (qualifiers, for identification criteria). The relative abundance of these diagnostic ions in unknown samples compared to the relative abundances of the same ions from the reference compounds is used as criteria for positive identification. The graphical view of the maximum tolerance window of relative difference (MTWRD) against the relative abundance of the ions, based on WADA criteria [20], is given in Fig. 2.

Several diagnostic ions and ion ratios for each anabolic agent have been tested against their compliance with the WADA relative abundance criteria. The results are summarised in Table 3.

The results in Table 3 demonstrate that all ions and ion combinations for clenbuterol-2TMS, except for 86 *m/z* alone, comply with the WADA criteria. The low reproducibility of the relative abundance of 86 *m/z* is due to two main factors:

1. The low uniqueness of the base ion 73 *m/z* in TOFMS spectra. This ion is present in all TMS derivatised compounds—endogenous or exogenous—since it is a fragment pertaining to derivatizing agent. In contrast, the base ion in quadrupole based MS spectra (NIST05) is 86 *m/z*, which is more characteristic than 73 *m/z*.
2. The narrower permitted tolerance window of the relative difference for ions with higher relative abundance (see Fig. 2).

However 335 *m/z*, 337 *m/z* and their combinations, including the combinations with 86 *m/z*, have complied with the criteria. The relative abundance ratio has shown higher reproducibility than the relative abundances itself. All ion combinations fell well within the tolerance window.

The relative abundance of the diagnostic ions and their combinations for EMD-2TMS fell within the tolerance window except the 143 *m/z* ion. Again, the main reasons for this are the same as for clenbuterol-2TMS: the low uniqueness of 73 *m/z* as a base ion in TOFMS spectra of EMD-2TMS and the narrower tolerance window for ions with higher abundances. Any tested combination, except 143 *m/z* alone, can be used as a qualifier.

Methyltestosterone metabolite M2-2TMS gives similar results to EMD-2TMS; 143 *m/z* alone cannot be used as a diagnostic ion since it shows low reproducibility and does not comply with the established identification criteria. Another combination which cannot be used is the relative ratio of the 255 *m/z* and 435 *m/z* relative abundance; because they are both of low abundance (less than 3% of the base ion), their ratio can therefore vary greatly. All other ions and ion combinations (255 *m/z*, 435 *m/z*, 255/143 *m/z* and 435/143 *m/z*) can be used as qualifiers.

The 143 *m/z* ion alone, as well as 254 *m/z* alone, did not pass the test as qualifiers for 3′OH-stanozolol-3TMS. However, 545 *m/z*, 545/143 *m/z* and 254/143 *m/z* gave results within the tolerance window; 560 *m/z* cannot be used as a diagnostic ion in samples with a concentration of 3′OH-stanozolol-3TMS lower than 4 ng mL^{-1}, because of the observed bias of TOFMS towards higher masses (Mitrevski et al. [25]). The comparison of TOFMS and quadrupole MS spectra of 3′OH-stanozolol-3TMS is given in Fig. 3. The relative abundance of 560 *m/z* in TOFMS spectrum of 3′OH-stanozolol-3TMS acquired at 0.5 μg mL^{-1} concentration in standard solution (Fig. 3a) shows approximately 20 times lower relative abundance when compared to the quadrupole based MS spectra (Fig. 3b) [9, 26]. However, the TOF mass spectra similarity of 3′OH-stanozolol-3TMS to the in-house created TOFMS library ensures positive identification of this sterol even at the lowest level of concentration spiked in urine extracts.

Each of the diagnostic ions alone for 19-norandrosterone-2TMS (405 *m/z*, 420 *m/z* and 315 *m/z*) have failed the criteria despite the good similarity of TOFMS spectra to the entries in the in-house library. One of the reasons could be the coelution of 19-norandrosterone-2TMS with the endogenous component which shares some of the diagnostic ions (Mitrevski et al. [25]), but most probably the reasons are the same as for the other anabolic agents. However, their combinations (with few exceptions at the lower levels of concentration) gave results within the tolerance window (Table 2). Furthermore, the spectral similarity even at the lowest level of concentration is high enough to ensure unambiguous identification. The similarity

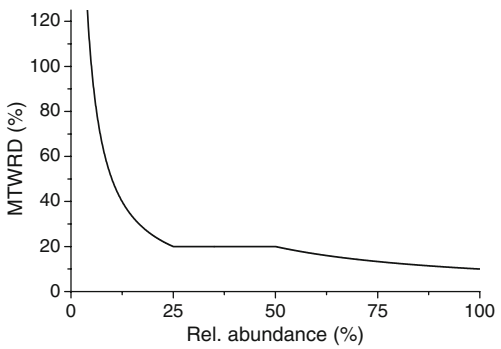

Fig. 2 Graphical view of the MTWRD against the *m/z* abundance, according to the WADA criteria [20]

Table 3 Relative abundance of diagnostic ions and their combinations

Clenbuterol-2TMS (n=12)	Ion (m/z)	86	335	337	335/86	337/86	337/335
	Aver. (%)	65.7	10.2	8.2	12.2	9.0	74.6
	STD	27.1	3.1	2.3	0.6	0.6	2.6
	Out of range[a]	8	–	–	–	–	–
19-norandrosterone-2TMS (n=11)	Ion (m/z)	405	420	315	420/405	315/405	315/420
	Aver. (%)	35.4	14.9	13.4	41.9	36.6	81.0
	STD	20.4	9.7	8.7	3.5	5.1	13.5
	Out of range[a]	8	6	4	–	1	2
EMD-2TMS (n=12)	Ion (m/z)	358	143	216	358/143	216/143	358/216
	Aver. (%)	1.9	67.1	3.4	2.9	5.0	57.6
	STD	0.3	9.0	0.7	0.4	0.7	9.0
	Out of range[a]	–	4	–	–	–	1
M2-2TMS metabolite (n=10)	Ion (m/z)	255	143	435	255/143	435/143	255/435
	Aver. (%)	2.7	86.9	2.8	3.3	3.5	94.7
	STD	0.4	19.0	1.3	0.4	1.3	29
	Out of range[a]	–	4	–	–	–	3
3′OH-stanozolol-3TMS (n=9)	Ion (m/z)	254	143	545	254/143	545/143	545/254
	Aver. (%)	15.1	51.6	6.1	29.5	12.7	44.6
	STD	6.5	23.3	2.8	3.1	3.3	12.9
	Out of range[a]	1	6	1	1	–	1

[a] Number of spiked urine samples (out of the total number of analysed samples), which do not comply with the WADA relative abundance criteria

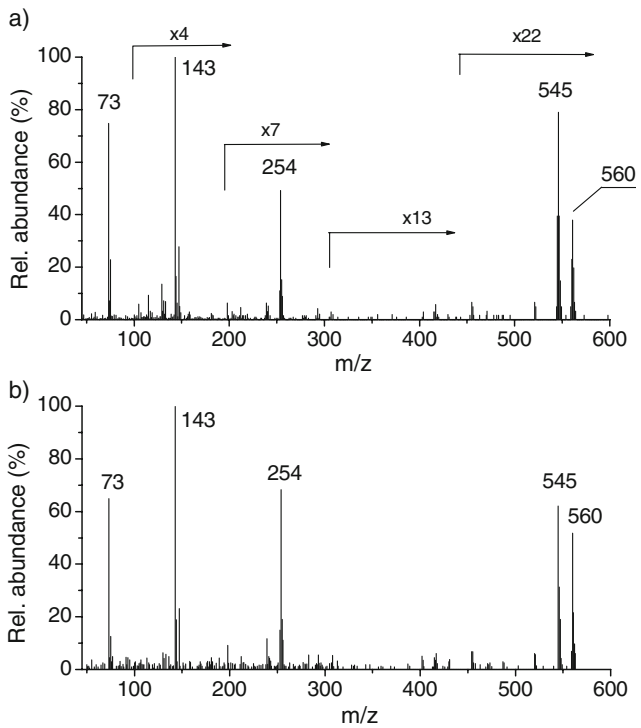

Fig. 3 Comparison of **a** TOFMS spectra of 3′OH-stanozolol-3TMS acquired at 0.5 μg mL^{-1} in standard solutions and **b** its entry from the NIST05 MS database. Note that the abundance of several consecutive mass intervals in TOFMS spectrum is enhanced by factors given above the intervals

of 19-norandrosterone-2TMS spiked in urine extract at different concentrations is given in Table 4. The general trend of increased similarity for higher concentrations has been observed, even though the variation of similarity has been found to be quite large (within ±10%).

The lower relative abundance of higher masses observed in TOFMS spectra of anabolic agents can be seen as an advantage over GC-qMS identification since the maximum tolerance window of the relative difference for lower abundance diagnostic ions is wider (see Fig. 2), and the reproducibility of TOFMS spectra even at the lowest levels of concentration is good. On the other hand, the diagnostic ion ratio, which is not implemented in the WADA criteria, gave reproducible and consistent results, allowing this to be used as a criterion in anabolic agent identification.

Table 4 TOFMS similarity of 19-norandrosterone-2TMS spiked in urine extracts at different levels of concentration against the in-house TOFMS library

Conc. level (ng mL^{-1})	Similarity (n=2)
500 (std sol.)	945
20	926
10	867
8	891
6	888
4	799
2	784

Table 5 Experimental and calculated isotope ratio patterns for clenbuterol-2TMS

	Ion abundance ratio		Area ratio	
	337/335 *m/z*	339/335 *m/z*	337/335 *m/z*	339/335 *m/z*
Average (%) (n=12)	74.6	17.8	76.5	21.1
RSD (%)	3.4	11.7	2.6	11.8
Calculated (%)	73.7	16.7	–	–

Other criteria

Presence of diagnostic ions

Present WADA criteria:

– Diagnostic ion with relative abundance of less than 5% in the reference must be present in the unknown.

All the diagnostic ions with abundance above 10%, which were present in the reference spectra of the anabolic agents, have been detected also in the spiked urine samples. When ions with abundance lower than 5% were chosen as the qualifier, these ions were also detected in spiked samples, even at the lowest concentration tested. As stated previously, the commonly used 560 *m/z* ion for 3′OH-stanozolol-3TMS was not chosen as a qualifier because of the decreased sensitivity of TOFMS at higher masses. Figure 3 shows the comparison of TOFMS spectra of 3′ OH-stanozolol-3TMS and its entry in NIST05 MS database (qMS based spectrum). For the same reason, the ion 448 *m/z* is not considered in EMD-2TMS identification criteria. This creates the biggest challenge in strictly applying the WADA criteria in GC×GC-TOFMS: the lack of three diagnostic ions with abundance above 5%. As can be seen from Table 2, the abundances of 358 *m/z* and 216 *m/z* for EMD-2TMS, and 255 *m/z* and 435 *m/z* for M2-2TMS metabolite, are below 5%. As a comparison, the abundance of these ions in NIST05 library entries of the same sterols is between 10% (216 *m/z*) and 30% (435 *m/z*). However, the high similarity of TOFMS spectra from spiked urine samples to the TOFMS spectra of the reference compounds permits unambiguous identification based on all ions in the spectrum. This has a range of ramifications for establishing a new criterion based on adequacy of library matches to sterol spectra as opposed to simply comparing ion ratios in SIM spectra [15].

Isotope ratio criteria

Present WADA criteria:

– A second ion belonging to the same isotopic cluster may also be used as diagnostic only when the peculiarity of the atomic composition of the fragment so justifies (e.g. presence of Cl, Br, or other elements with abundant isotopic ions).

The clenbuterol molecule contains two chlorine atoms, and its isotope pattern can be another useful criterion in identification of this anabolic agent. The ion abundance ratios of 337/335 *m/z* and 339/335 *m/z* have been compared against the theoretical values calculated by the isotope calculator from the NIST MS Search software package (ver. 2.0). The average ion abundance ratios were calculated from 12 spiked urine samples with anabolic agents over the concentration range from 2 to 20 ng mL^{-1}. The results are given in Table 5.

Good agreement has been obtained between the experimental and the calculated values for both ion ratios: 337/335 *m/z* and 339/335 *m/z* (Table 5). The 337/335 *m/z* and 339/335 *m/z* ratios, when the peak area was used instead of ion abundances, were slightly higher. However, the reproducibility of the 337/335 *m/z* ion abundance and area ratio was better than the 339/335 *m/z* ratios.

The good agreement between the experimental results obtained from clenbuterol-2TMS spiked at different concentration in urine extract to the theoretical values is a result of the deconvolution capabilities of the ChromaTOF software. The high acquisition rate (100 Hz) contributes to the correct deconvolution. A series of deconvoluted MS spectra of clenbuterol-2TMS spiked in urine extract at different concentrations, showing the chlorine isotope pattern, are given in Fig. 4.

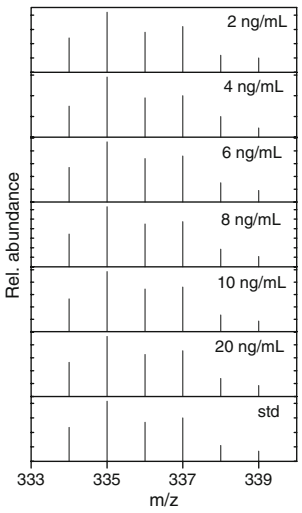

Fig. 4 Deconvoluted TOFMS spectra of clenbuterol-2TMS spiked in urine extracts at different concentrations, expanded over the 334–339 *m/z* cluster and normalised to 335 *m/z* as the base ion

Furthermore, the appearance of 334 *m/z* in the experimental results (but not in the calculated isotope pattern) and the differences arising in the isotope pattern at 336 *m/z* and 338 *m/z* between experimental and calculated results (Fig. 5) may be explained by presuming the formation of a species with A-1 *m/z* (that is, the 334 *m/z* ion). The isotope pattern of the dominant fragment ion at *m/z* 335 is shown in Fig 5. The experimental spectrum in this region extends from 334 to 341 *m/z*. Note that ion 334 *m/z* will not arise from fragment mass loss from the parent ion, but arises from an A-1 ion species. Taking the predicted isotope pattern for the 335 *m/z* ion, including the Cl isotopes, gives the pattern labelled as "calculated without A-1 ion". This is a very poor match with the experimental pattern. In order to calculate the isotope pattern correctly, we invoke the presence of the A-1 ion, and since it contains 2× Cl atoms, it will have a strong isotope contribution at 336 *m/z*. This leads to the large discrepancy between the experimental pattern and the calculated pattern in the absence of the A-1 species. Once the A-1 species is included as an overlapping ion pattern, very good agreement between experimental and calculated patterns arises.

Linearity

The linearity of the proposed method has been calculated from spiked urine extracts with anabolic agents at different concentrations from 0.5 to 20 ng mL^{-1}. The correlation coefficients and the linearity curve equations are given in Table 6. The highest correlation was obtained for EMD-2TMS and 19-norandrosterone-2TMS (0.996) and the lowest for M2-2TMS metabolite (0.992). Please note that the AA's quantification ions are detected down to the lowest tested concentration (except for EMD-2TMS at 0.5 ng mL^{-1}), and unambiguous identification is obtained only at 1 ng mL^{-1} and above for clenbuterol-2TMS, 19-norandrosterone and M2-2TMS and at 2 ng mL^{-1} and above for EMD-2TMS and 3′OH-stanozolol-3TMS (Mitrevski et al. [25]). The good correlation coefficients are the result of the temporal deconvolution of the anabolic agents' mass spectra from the background components, and this is a direct result of the separation of the components in the 2D space. The high acquisition rate of the TOFMS detector (100 Hz) further facilitates the deconvolution in the mass spectral domain. As we have previously shown (Mitrevski et al. [25]), the poorer separation of the components on the 0.1 μm d_f ^2D column gave lower similarity, which is based on the poorer deconvolution. Furthermore, the low acquisition rate of the TOFMS detector (20 Hz) in 1D GC experiments and the separation based on classical 1D GC gave even lower mass spectra similarity against TOFMS entries in the in-house library.

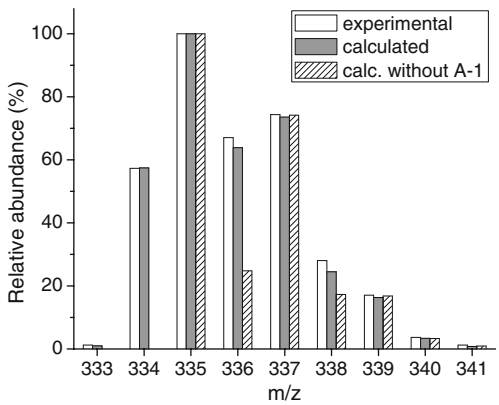

Fig. 5 Cluster isotope pattern of clenbuterol-2TMS in standard solution at 0.5 μg mL^{-1} for experimental data, calculated data by assuming the A-1 fragment has been formed in a ratio 1:2 against A and calculated data without taking the A-1 fragment formation into account

Table 6 Linearity parameters for the anabolic agents spiked in urine extracts at different concentrations from 0.5 to 20 ng mL^{-1}

Anabolic agent	Quant. ion	Linearity equation[a]	Correlation coefficient
Epimethendiol-2TMS	358 *m/z*	$Y=0.0081x+0.0074$	0.996
19-Norandrosterone-2TMS	405 *m/z*	$Y=0.0061x+0.0094$	0.996
M2-2TMS metabolite	255 *m/z*	$Y=0.0144x+0.0070$	0.992
Clenbuterol-2TMS	335 *m/z*	$Y=0.0143x+0.0050$	0.994
3′OH-stanozolol-3TMS	254 *m/z*	$Y=0.0043x+0.0003$	0.994

[a] Y=area (AAS)/area (IS); x=concentration of AAS (ng mL^{-1})

Testosterone/epitestosterone (T/E) ratio

Testosterone/epitestosterone ratio (*T*/*E*) values can be calculated from the same run with unambiguous identification of both sterols. The average similarity of testosterone and epitestosterone TOFMS spectra against the in-house library, across the concentration range of AAS from 0.5 to 20 ng mL^{-1} in urine samples, was 915 and 820, respectively.

Signal-to-noise (S/N) ratio

Present WADA criteria:

– The signal-to-noise ratio of the least intense diagnostic ion must be greater than 3:1.

The final criterion, which must be complied with, is the signal-to-noise (S/N) ratio for the least intense diagnostic ions. The lowest S/N permitted in WADA criteria is 3:1. S/N ratio for quantification ions obtained at 0.1 μg mL^{-1} in

standard solution (corresponding to 2 ng mL^{-1} in spiked urine samples at a concentration factor of 50) has been found to be above 18 (Mitrevski et al. [25]). The lowest S/N (4) has been obtained for EMD-2TMS (358 *m*/*z*) spiked in urine extracts at 2 ng mL^{-1}. This is again due to the bias of TOFMS towards the higher masses, combined with the low relative abundance of 358 *m*/*z*. The lack of potential diagnostic ions in the TOFMS spectra of EMD-2TMS limits the choice.

19-Norandrosterone-2TMS and 3′OH-stanozolol-3TMS are the only anabolic agents, which completely comply with defined WADA criteria, since all others do not contain at least three diagnostic ions with abundance higher than 5% relative to the base ion. However, WADA permits a laboratory to establish its own identification criteria, which can be based on minimum MS similarity against the reference compounds. A minimum acceptable match (MAM) has been defined [15] as a criterion for identification of minor components in complex matrices such as urine, where a library match is required to be above a certain match quality in order to provide satisfactory identification of the component. The MAM in doping control depends on the reproducibility of the MS similarity and probably is best to be set at the average MS similarity of anabolic agents at the positive urine control level, corrected for the standard deviation of the reproducibility. We have found that for unambiguous identification, the MAM can be set at 800 when analysing free sterols in non-hydrolyzed urine samples [15] and to 700 in hydrolysed urine samples (Mitrevski et al. [25]). The main reason is the more complex urine extract from hydrolysed samples. Obviously, MS similarity is just a confirmation of the other identification parameters, such as relative abundance ratio and retention time tolerance windows. In this case, the MAM serves the purpose of providing a minimum value for the quality of full mass spectral similarity matching against, here, the in-house library for sterols. By all measures, this should represent a sound basis for confirmation (subject to the metric by which library searches are performed). The compounds can still be quantified based on a selected single quantification ion if so desired.

Other benefits of the GC×GC-TOFMS method

Amongst *T* and *E*, another eight sterols, which were investigated in our previous work [15], were detected in the urine extract with an average similarity above 900. Once full MS data are available, the presence of sterols can be confirmed in post-run processing, allowing retro-searching for newly designed sterols, should the analyst become aware of such sterols or any other illicit compounds in the future.

Another advantage of having access to the full mass spectral information is characterisation of the interfering components and the matrix. Just as the deconvolution works for the target anabolic sterols, so it works also for matrix components, and their spectra are likewise deconvoluted. This is a simple and inexpensive way to characterise trace components in urine matrix by their full deconvoluted mass spectra and not just a limited suite of sterols defined by selected SIM ions and retention windows.

Finally, as previously stated, several tens of other non-target components were identified with high similarity. Most of them are low molecular mass components, because lower mass components have higher similarity against the NIST05 database than high mass counterparts. At present we have only chosen sterols to be in our custom in-house library. A dedicated comprehensive TOFMS library is needed for proper identification of higher molecular mass components because of the previously stated bias of TOFMS against the higher masses and consequent larger differences between qMS (used for most library entries) and TOFMS spectra.

Conclusions

The results presented here confirm that GC×GC-TOFMS of anabolic agents largely complies with the established WADA identification criteria. This technique has been shown to be a powerful tool for detection and unambiguous identification of trace amounts of anabolic agents in complex matrix as urine, so proved to be a promising choice for doping control in sport competition.

Retention time reproducibility on both columns has been found to be below 0.83%, allowing relative error of the reference components to be within the WADA tolerance window of 1%. The second dimension retention time relative error also complies with these criteria, although it is not explicitly required in the WADA criteria.

The relative abundance tolerance criteria have been complied with by most of the diagnostic ions selected, except for the high abundance ions or the ratio between two low abundance ions. Spectral deconvolution has been demonstrated in the case of clenbuterol-2TMS, where very good agreement has been obtained between experimental and theoretical calculations for the isotope cluster from 334 to 339 *m*/*z*.

Other general criteria complied with include the linearity and minimum spectral match quality for positive identification. The deconvoluted full mass spectral information of the non-target components and the matrix are other benefits from this method. The least-complied criterion of the proposed method is the presence of at least three diagnostic ions in the TOFMS spectra with abundance above 5% of

the base peak. This appears to be due to the predominance of the 73 *m*/*z* in sterol spectra, and if scans were to exclude the 73 *m*/*z* ion by presenting data at >73 *m*/*z*, then the WADA criteria may be complied with. Because of the observed bias towards the higher masses, only 19-norandrosterone-2TMS and 3′OH-stanozolol-3TMS comply with the criteria. However, the high similarity based on the full mass spectra offers an unambiguous identification even at the lowest tested concentrations. This latter benefit of using full-scan TOFMS data is not available for most classical methods used in doping control that rely on SIM analysis.

The present work suggests that the GC×GC-TOFMS method can play an important role in doping control and drug testing in the future. Separation of components in 2D space relaxes the eventually coelutions with the matrix components and deconvoluted full mass TOFMS spectra offers unambiguous identification. This makes the method a good alternative for anti-doping screening, and if sensitivity is improved and confirmed when recovery is taken into consideration, the method can be a promising option for AAS confirmation.

References

1. Schänzer W, Donike M (1993) Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. Anal Chim Acta 275:23–48
2. Kokkonen J, Leinonen A, Tuominen J, Seppala T (1999) Comparison of sensitivity between gas chromatography-low-resolution mass spectrometry and gas chromatography-high-resolution mass spectrometry for determining metandienone metabolites in urine. J Chromatogr B 734:179–189
3. Buiarelli F, Cartoni GP, Amendola L, Botre F (2001) Screening and confirmation analysis of anabolic agents in human urine by gas chromatography hybrid mass spectrometry (high resolution time of flight). Anal Chim Acta 447:75–88
4. Geyer H, Schanzer W, Mareck-Engelke U, Nolteernsting E, Opfermann G (1998) Screening procedure for anabolic steroids—the control of the hydrolysis with deuterated androsterone glucuronide and studies with direct hydrolysis. In: Schanzer W (ed) Recent advances in doping analysis (5). Sport and Buch Strauß, Köln, pp 99–101
5. Ayotte C, Goudreault D, Charlebois A (1996) Testing for natural and synthetic anabolic agents in human urine. J Chromatogr B 687:3–25
6. Munoz-Guerra J, Carreras D, Soriano C, Rodriguez C, Rodriguez AF (1997) Use of ion trap gas chromatography tandem mass spectrometry for detection and confirmation of anabolic substances at trace levels in doping analysis. J Chromatogr B 704:129–141
7. Thieme D, Grosse J, Mueller RK (1997) Observations on new metabolites of clostebol by high-resolution-MS. In: Schanzer W (ed) Recent advances in doping analysis (4). Sport and Buch Strauß, Köln, pp 43–58
8. Horning S, Schanzer W (1997) Steroid screening using GC/HRMS. In: Schanzer W (ed) Recent advances in doping analysis (4). Sport and Buch Strauß, Köln, pp 261–270
9. Schanzer W, Delahaut P, Geyer H, Machnik M, Horning S (1996) Long-term detection and identification of metandienone and stanozolol abuse in athletes by gas chromatography high-resolution mass spectrometry. J Chromatogr B 687:93–108
10. Bowers LD, Borts DJ (1996) Separation and confirmation of anabolic steroids with quadrupole ion trap tandem mass spectrometry. J Chromatogr B 687:69–78
11. Machnik M, Delahaut P, Horning S, Schanzer W (1997) Purification and concentration of anabolic steroids by immuno affinity chromatography. In: Schanzer W (ed) Recent advances in doping analysis (4). Sport and Buch Strauß, Köln, pp 223–237
12. Gotzmann A, Geyer H, Schanzer W (1997) HPLC clean-up for urine samples with disturbing background for confirmation of 17β-methyl-5β-androst-1-ene-3α, 17α-diol (Epimetendiol). In: Schanzer W (ed) Recent advances in doping analysis (4). Sport and Buch Strauß, Köln, pp 239–246
13. Mitrevski B, Brenna JT, Zhang Y, Marriott PJ (2007) A new paradigm in drugs analysis: comprehensive two-dimensional gas chromatography for steroid analysis. Chem Aust 74:3–5
14. Silva AI, Pereira HMG, Casilli A, Aquino Neto FR (2007) Analytical challenges in doping control: GC×GC: is it an option? In: Schanzer W, Geyer H, Goltzmann A, Mareck U (eds) Recent advances in doping analysis (15). Sportverlag Strauß, Köln, pp 217–226
15. Mitrevski BS, Brenna JT, Zhang Y, Marriott PJ (2008) Application of comprehensive two-dimensional gas chromatography to sterols analysis. J Chromatogr A 1214:134–142
16. Silva AI, Pereira HMG, Casilli A, Conceicao FC, Neto FRA (2009) Analytical challenges in doping control: comprehensive two-dimensional gas chromatography with time of flight mass spectrometry, a promising option. J Chromatogr A 1216:2913–2922
17. Kueh AJ, Marriott PJ, Wynne PM, Vine JH (2003) Application of comprehensive two-dimensional gas chromatography to drugs analysis in doping control. J Chromatogr A 1000:109–124
18. Adahchour M, Beens J, Vreuls RJJ, Brinkman UAT (2006) Recent developments in comprehensive two-dimensional gas chromatography (GC×GC): I. Introduction and instrumental set-up. TrAC. Trends Anal Chem 25:438–454
19. Adahchour M, Beens J, Vreuls RJJ, Brinkman UAT (2006) Recent developments in comprehensive two-dimensional gas chromatography (GC×GC): II. Modulation and detection. TrAC. Trends Anal Chem 25:540–553
20. Technical Document TD2003IDCR (2003) Identification criteria for qualitative assays incorporating chromatography and mass spectrometry. World Anti-Doping Agency, Montreal, Canada. www.wada-ama.org
21. Technical Document TD2004MRPL (2004) Minimum required performance limits for detection of prohibited substances. World Anti-Doping Agency, Montreal, Canada. www.wada-ama.org
22. Saudan C, Kamber M, Barbati G, Robinson N, Desmarchelier A, Mangin P, Saugy M (2006) Longitudinal profiling of urinary steroids by gas chromatography/combustion/isotope ratio mass spectrometry: diet change may result in carbon isotopic variations. J Chromatogr B 831:324–327
23. Shellie RA, Xie L-L, Marriott PJ (2002) Retention time reproducibility in comprehensive two-dimensional gas chromatography using cryogenic modulation: an intralaboratory study. J Chromatogr A 968:161–170
24. Adcock JL, Adams M, Mitrevski BS, Marriott PJ (2009) Peak modeling approach to accurate assignment of first-dimension retention times in comprehensive two-dimensional chromatography. Anal Chem 81:6797–6804
25. Mitrevski BS, Wilairat P, Marriott PJ (2010) Comprehensive two-dimensional gas chromatography improves separation and identification of anabolic agents in doping control. J Chromatogr A 1217:127–135
26. Huenerbein A, Marques MAS, Pereira AD, Neto FRD (2003) Improvement in steroid screening for doping control with special emphasis on stanozolol. J Chromatogr A 985:375–386