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Use of ion trap gas chromatography–multiple mass spectrometry for the detection and confirmation of 3′hydroxystanozolol at trace levels in urine for doping control

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Abstract

Stanozolol, a synthetic anabolic androgenic steroid, is often abused in sports to enhance performance. Consequently, the anti-doping laboratories daily screen for its metabolites (3′hydroxystanozolol and 4β hydroxystanozolol) in all urines, mainly by GC–MS, after enzymatic hydrolysis and TMS derivatization. A sensitive and specific method by GC–MS^3 has been developed for the identification in urine of 3′hydroxystanozolol at trace levels. Full mass spectra and diagnostic ions are presented and a case report is commented. In this case, it was possible to attest the presence of a low concentration of stanozolol metabolite in a sample obtained from a competition test. This would have not been possible with other analytical techniques used in the laboratory. Through this case report, it was also possible to demonstrate the importance of sampling and the difficulties that has to face the laboratory when the pre-analytical step is not correctly performed.

Keywords: Stanozolol; Anabolic steroids; Doping; Urine; $GC–MS^n$

1. Introduction

The use of anabolic androgenic steroids (AAS) in sports has been banned since 1974 by the International Olympic Committee (IOC), National and International Sport federations and more recently, by the World Anti-Doping Agency (WADA). The use of anabolic steroids increased during the 1980s and more particularly, stanozolol was often misused in sport by athletes during these last 20 years, not only during the competition events, but also during the training periods [1,2]. Stanozolol, 17α-methyl-17β-hydroxy-5α-androstano-(3,2-C)-pyrazole (Fig. 1), was initially synthesized in 1959 [3,4] and clinically used in cases of deficiency in protein synthesis and osteoporosis [5]. Rapidly, it has been one of the most abused anabolic steroids in numerous sports as well as in horse-races to enhance performance. For example, stanozolol was among the anabolic steroid-positive tests reported at the 1988 Olympic Games in Seoul [6]. Long-term effects on liver such as peliosis hepatis, cholestasis or hepatic tumors as well as cardiovascular diseases and neurologic disorders have been reported after steroid abuse, particularly in young steroid abusers [7–12].

Stanozolol and its main metabolites (3′hydroxystanozolol and 4β hydroxystanozolol) are structurally different from most anabolic steroids and are particularly difficult to detect in urine: these compounds have a poor gas chromatographic behavior and the measured concentrations are generally very low due to their slow excretion rate. Indeed, only 16% of stanozolol metabolites are excreted in urine during the first day, while 40–60% are excreted in the feces [13]. Moreover, anabolic steroids are frequently taken for periods ranging from 4 to 18 weeks, alternating with drug-free periods of 1 month to 1 year [14]. As a matter of fact, AAS are mainly misused during training periods and athletes discontinue their use at sometime prior to competition. After administration, stanozolol is rapidly metabolized and the metabolites can be detected in urine until 6 days, depending on the dose

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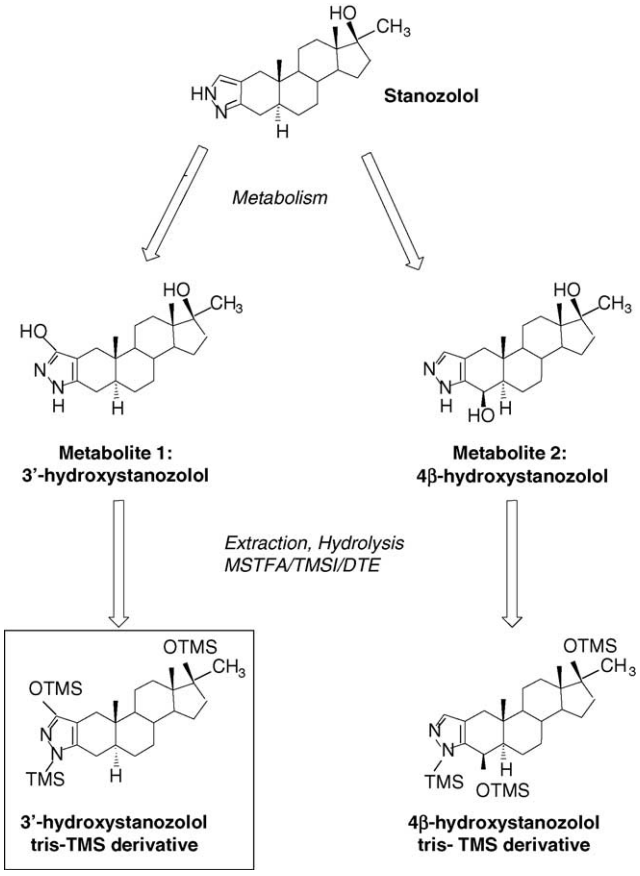

Fig. 1. Structure of investigated compounds: metabolism of stanozolol and derivatized products obtained after extraction and hydrolysis.

administered and the individual metabolism [15]. Furthermore, stanozolol metabolites are mainly excreted in urine as conjugated forms and in order to achieve exact identification of low concentrations in complex matrices, both the analytical and the extraction techniques must provide good recovery, selectivity and specificity. Consequently, all these aspects decrease the chance that AAS and their metabolites can be detected in the urine of the athlete when controlled at a sporting event. Then, a sensitive and specific analytical method is needed for the screening of anabolic steroids and particularly for stanozolol metabolites.

Analytical methods reported for the detection of anabolic steroids include radio-immunoassay [16], high performance liquid chromatography [17–19] and gas chromatography-mass spectrometry (GC–MS) [20–26]. Currently, the most reliable, sensitive and specific analytical methods for anabolic steroids screening are GC–MS in SIM mode with electron impact (EI) ionization, GC–MS^n and high resolution mass spectrometry (HRMS) [27–34]. Even if a few studies deal with the detection of stanozolol in hair [35–36], urine remains the favorite matrix for the analysis of this anabolic steroid and its metabolites [15,37–39]. Regarding stanozolol, the IOC accredited laboratories mainly focus on two main metabolites, 3′hydroxystanozolol and 4β-hydroxystanozolol, after enzymatic hydrolysis and TMS derivatisation of the urinary extract (Fig. 1).

The minimum required performance limit according the WADA code for the detection of stanozolol is 2 ng/ml in urine [40]. For stanozolol, this limit is particularly difficult to achieve by classical analytical techniques as GC–MS in SIM mode, usually used by the accredited laboratories for the screening of anabolic steroids. Actually, only HRMS and GC–MS^n analytical equipments are able to respect the WADA requirements for this substance.

Numerous applications in the literature attest of the sensitivity and specificity that can be achieved by using ion trap GC–MS^n systems [41–44] for the analysis of complex matrices as biological samples. In this study, a sensitive and specific method by GC–MS^3 was developed for the detection of 3′hydroxystanozolol. This method is now routinely applied at the laboratory for the screening and confirmation of this anabolic steroid and a positive case is presented in this article. In this particular case, the analysis of B sample did not allow to confirm the presence 3′hydroxystanozolol metabolite that was found in A sample.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical grade: *tert*-butyl methyl ether (TBME) was purchased by Acros (Geel, Belgium). Sodium carbonate (Na_2CO_3), potassium dihydrogen phosphate (KH_2PO_4) and di-sodium hydrogen phosphate (Na_2HPO_4) were obtained from Merck (Darmstadt, Germany), whereas sodium hydrogen carbonate ($NaHCO_3$) was form Acros (Geel, Belgium). Sodium sulphate (Na_2SO_4) was purchased by BDH Laboratory Supplies (Poole. England). *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) was provided by Macherey-Nagel (Düren, Germany), trimethyliodosilane (TMSI) from Sigma (St. Louis, MO, USA) and dithioerythritol (DTE) from Acros (Geel, Belgium). *E. Coli* β-glucuronidase (200 U/ml specific activity) was purchased by Roche Diagnostics (Mannheim, Germany). 3′Hydroxystanozolol was obtained from Promochem (Molsheim, France), whereas methyltestosterone was provided by Sigma (St. Louis, MO, USA). Methanol (MeOH) from Biosolve (Valkenswaard, The Netherlands) and bi-distilled water were used for the Sep-Pak C_{18} cartridge conditioning (J.T. Baker, NJ, USA).

2.2. Urine sample preparation

2.5 ml of urine was added with 20 ng/ml methyltestosterone, used as internal standard, and applied over a Sep-Pak C_{18} cartridge (previously washed with 5 ml of methanol and 5 ml of water). The cartridge was then washed with 5 ml of water to eliminate most of the water soluble urinary constituents, which had not been adsorbed on the

solid support. The steroids (free and conjugated) were then eluted with 3 ml × 1 ml of methanol. The entire effluent was evaporated to dryness under a nitrogen stream at 40 °C and the residue was dissolved with 1 ml phosphate buffer (KH_2PO_4–Na_2HPO_4, 0.2 M, pH 7.0). Then, hydrolysed was performed during 1 h at 50 °C with 50 μl β-glucuronidase from *E. Coli* (200 U/ml specific activity). After addition of approximately 200 mg of solid carbonate buffer (Na_2CO_3–$NaHCO_3$, 1:10), the sample was extracted with 5 ml TBME by shaking during 10 min. After centrifugation (2500 × *g* for 5 min), the organic phase was collected, dried with Na_2SO_4 and the residue was derivatized with 50 μl MSTFA–TMSI–DTE (1000:5:5, v/v/w) during 30 min at 60 °C. This method was initially optimized and validated by the Cologne anti-doping laboratory (Germany) several years ago and since, has been slightly modified and is commonly used by the anti-doping community for the extraction of anabolic steroids from urine [15,37–39].

2.3. GC/MS^n parameters

The gas chromatograph was a TRACE GC 2000 series (Thermo Quest, Italy), equipped with an A200S autosampler from Fisons Instruments (from Finnigan, USA). The GC system was interfaced to a Finnigan GCQ^{TM} Polaris ion trap mass spectrometer (USA). Chromatographic separation was performed by using a capillary column (DB-XLB; column length 15 m × 0.25 mm with a 0.25 μm film thickness) from J&W Scientific (Agilent Technologies, USA). The GC temperature program was as follows: the initial temperature was 150 °C for 1 min, then increased with a temperature program of 25 °C/min to a temperature of 300 °C which was held for 4 min. Samples (2 μl) were injected in the splitless mode. The injector temperature was set at 270 °C. Helium was used as carrier gas at a flow rate of 1 ml/min. The transfer line temperature was 280 °C and the ion source temperature was 230 °C. The MS instrument was operated in the electron impact ionization mode at 70 eV and product ion scan was used as detection mode. For collision induced dissociation in MS^2 and MS^3, helium was used as collision gas. Other MS^2 and MS^3 instrumental conditions for the detection of 3′hydroxystanozolol and the internal standard (methyltestosterone) are indicated in Table 1. With these analytical conditions, both compounds are well separated as the retention times are 7.2 min and 9.3 min for methyl-testosterone and 3′hydroxystanozolol, respectively. In addition, as it can be seen in Table 1, the corresponding product ions attest of the selectivity of the method.

Table 1
Main MS^2 and MS^3 parameters for 3′hydroxystanozolol and methyltestosterone (internal standard)

MS^n parameters	Compound		
	3′Hydroxystanozolol		Methyl-testosterone
	MS^2	MS^3	MS^2
Precursor ion	545	455	446
Isolation time (ms)	8	8	8
Excitation voltage (V)	1.75	1.60	1.10
Excitation time (ms)	15	15	15
q-Value	0.30	0.225	0.225
Product ions	455	439, 425, 347, 277[a], 239	356, 341[a], 301, 251

[a] Ion used as mass trace.

3. Results and discussion

3.1. Gas chromatography–tandem mass spectrometry detection

Fragmentation of the precursor ion is performed by collision-induced dissociation (CID) with helium molecules, the carrier gas which fills the ion trap. The main parameters determining the fragmentation behavior of an ion are: isolation time, excitation time, excitation voltage, maximum excitation energy (low: $q = 0.225$, medium: $q = 0.30$ and high: $q = 0.45$). The last two parameters, as well as the final selection of the mass trace, are the ones which have to be carefully optimized to ensure optimum performance of the technique.

In the case of the TMS derivative of 3′hydroxystanozolol, the criterion followed for the election of the parent ion were those of selectivity and intensity of the ion. Indeed, the parent ion chosen was not present in the background and did not commonly interfere. Furthermore, another important criterion for selecting the parent ion was that the ion chosen should have an intense ionic current to permit greater sensitivity and ideally, a correct and sufficient fragmentation for identification. Fig. 2A shows the electronic impact mass spectrum of 3′hydroxystanozolol. In this case, the *m*/*z* 545 was chosen as the precursor ion for further fragmentation in the trap. By using helium as a collision gas, the fragmentation of the parent ion was carried out to produce the daughter ions. In this particular case, the ion *m*/*z* 545 which is stable at $q = 0.30$, shows a very poor fragmentation even at high CID voltages and the unique daughter ion was *m*/*z* 455 (Fig. 2B). As a matter of fact, at very high CID voltage, the ion *m*/*z* 455 is unstable, explodes and leads to a dramatic loss in sensitivity. Consequently, with a CID voltage at 1.75 V, it was possible to achieve a 100% recovery of ion *m*/*z* 455 from precursor ion *m*/*z* 545, before explosion. Indeed, unlike to ion *m*/*z* 545, ion *m*/*z* 455 was quite unstable for fragmentation at $q = 0.30$ and consequently, was stocked in the trap at $q = 0.225$ (low excitation energy) for the next fragmentation. By this way, it was possible to achieve a nice fragmentation of ion *m*/*z* 455 without a dramatic loss in sensitivity (Fig. 2C). The experimental conditions used for the first and second fragmentation with the ion trap spectrometer are reported in Table 1. In the case of 3′hydroxystanozolol, the CID voltage selected for the second fragmentation in the trap was that which made it possible to obtain a spectrum with a highly abundant base peak, which remains *m*/*z* 455, a minimal initial ionic current loss, and the presence of enough representative fragments ions *m*/*z*

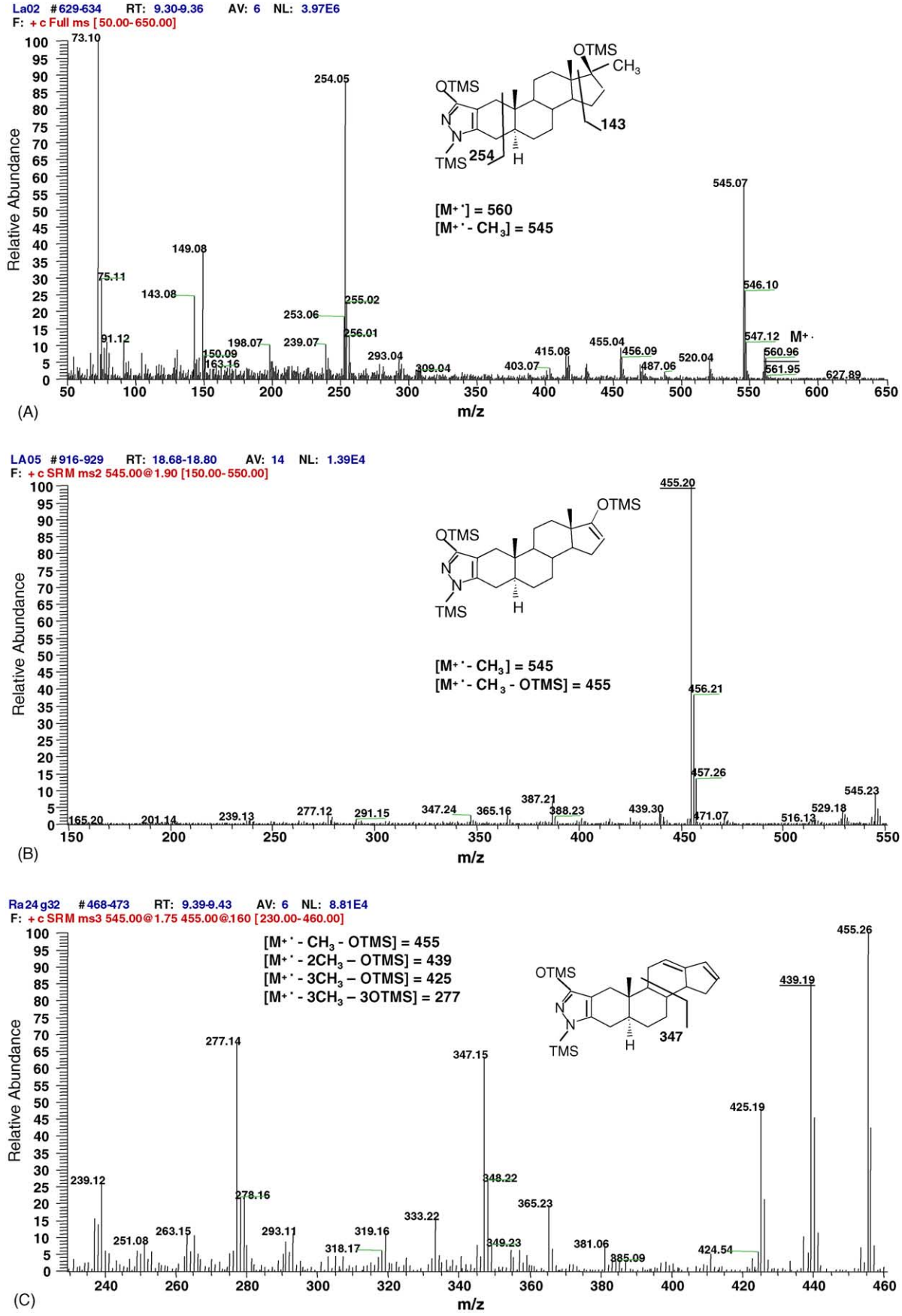

Fig. 2. Mass spectra and proposed fragmentation pattern of 3′hydroxystanozolol by (A) GC–MS, (B) GC–MS^2 and (C) GC–MS^3.

Table 2
Intra- and inter-day precision data

	3′Hydroxystanozolol spiked urines (concentration level)			
	2 ng/ml	5 ng/ml	10 ng/ml	20 ng/ml
Intra-day precision				
Retention time	0.01%	0.05%	0.03%	0.07%
Peak area ratio[a]	17.1%	18.2%	17.7%	20.8%
Inter-day precision				
Retention time	0.02%	0.04%	0.07%	0.09%
Peak area ratio[a]	18.2%	20.6%	23.1%	24.9%

[a] The peak area ratio is defined as the analyte peak area divided by the internal standard (methyltestosterone) peak area.

Table 3
Maximum tolerance windows for relative ion intensities (from WADA code [40])

Relative abundance (% of base peak)	GC–MS	GC–MS^n
>50	±10% (absolute[a])	±15% (absolute[a])
25–50	±20% (relative[b])	±25% (relative[b])
<25	±5% (absolute[a])	±10% (absolute[a])

[a] The absolute difference is calculated by subtracting the stated percentage from the relative abundance obtained for the studied ion from the positive control urine.
[b] The relative difference is calculated by multiplying the stated percentage by the relative abundance obtained for the studied ion from the positive control urine.

439, 425, 347, 277 and 239. In doping analysis field, when GC–MS^n is used, a minimum three transition ions have to be monitored (intensity > 5%) from the mass spectrum, and at least the ion ratios must correspond to that of the standard analyte at the same concentration between a permitted tolerance of ±25%. Consequently, the *m*/*z* 455, 439, 425, 347, 277 and 239 ions were selected for identification of 3′hydroxystanozolol in the product scan mode. In particular, the ion *m*/*z* 277 was selected as mass trace because of its intensity and very high specificity when analyzing complex urinary matrices. By this way, it was possible to identify 3′hydroxystanozolol in urine even at very low concentration, as it can be seen in Fig. 3A and B. Initially, the analytical method was developed for the two metabolites of stanozolol, simultaneously. The optimized conditions of the trap were not the same for both metabolites. Consequently, in order to achieve better sensitivity, only the experimental conditions for 3′hydroxystanozolol were kept in the method, as it is the metabolite which is excreted in the urine for the longest time period. The developed GC–MS^3 method offers the advantage to issue a specific mass spectrum with almost six characteristic ions which fulfill the identification criteria requested by WADA [40].

Samples for intra- and inter-day assays were prepared at four different concentrations, 2, 5, 10 and 20 ng/ml. Each sample was analyzed six times a day for 3 days. The relative standard deviations (R.S.D.) of the intra-day precision ($n = 6$) ranged between 17% and 21%, whereas the inter-day precision ($n = 18$) ranged between 18% and 25%, depending on the investigated solute concentration (Table 2). The detection limit (LOD) was estimated by preparing and analyzing several spiked urines with 3′hydroxystanozolol ranging between 0.1 and 2 ng/ml. The ion chromatograms were established using the mass trace *m*/*z* 277 and corresponding product ion spectra were evaluated. The LOD, is generally defined as the lowest value that differs from the blank (signal to noise ≥ 3). In the case of stanozolol metabolite, sensitivity was evaluated in terms of confirmation limits and expressed as the concentration of the anabolic compound needed to permit a matching of less than 25% of the ions ratios, which is in accordance with the WADA requirements (Tables 3 and 4) and was estimated to be 0.5 ng/ml. Finally, the overall extraction recovery of the procedure was 73% for 3′hydroxystanozolol ($n = 20$, R.S.D. = 28%). In this last case, the total recovery include both the extraction of the compound from the urinary matrix and the hydrolysis step. This was achieved by comparing the signal obtained with a 3′hydroxystanozolol glucuronide spiked urine with

Table 4
Identification criteria for 3′hydroxystanozolol (ion ratios)

Ions	Std 3′OH-stano 2 ng/ml[a]		US 3′OH-stano 0.5 ng/ml[b]		Difference	
	Abundance	Relative abundance (%)	Abundance	Relative abundance (%)	Absolute (%)	Relative (%)
455	10315	100.00	2276	100.00	0.0	0.0
439	6888	66.78	1730	76.01	9.2	12.1
425	3871	37.53	1055	46.35	8.8	19.0
347	5726	55.51	1415	62.17	6.7	10.7
277	4823	46.76	1168	51.32	4.6	8.9

[a] Standard of 3′hydroxystanozolol in methanol at 2 ng/ml (WADA required performance limit).
[b] Spiked urine with 3′hydroxystanozolol at 0.5 ng/ml (LOD).

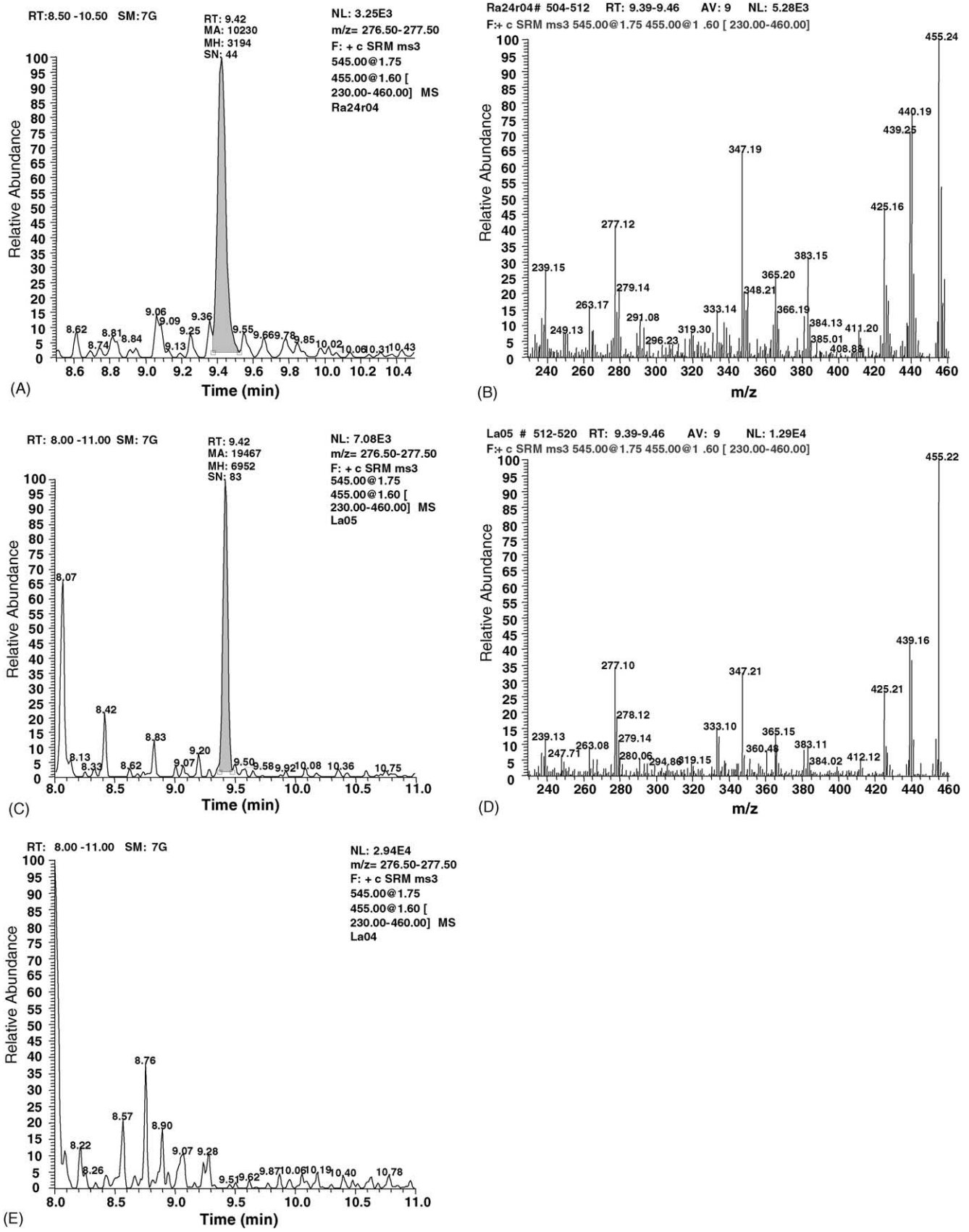

Fig. 3. Data from the case report: ion chromatograms of mass trace *m*/*z* 277 and corresponding product ion spectra after liquid–liquid extraction and TMS derivatization. (A–B) 3′Hydroxystanozolol spiked urine at 2 ng/ml. (C–D) A sample. (E) B sample.

the free substance only derivatized. The developed method has been tested in the laboratory during 1 year, in parallel with GC–MS screening analysis in SIM mode, on numerous blank urines and hundreds of samples from males and females. The GC–MS^3 method proved to be much more selective and specific than the screening method and allowed to differentiate without doubt between negative and positive samples. The good results obtained with proficiency and inter-laboratory tests, also attest of the performance of the developed method.

The analytical performance of the method is in agreement with the WADA code regarding stanozolol, as the minimum required performance limit is 2 ng/ml in urine. This very low limit is difficult to achieve with other analytical methods, particularly with GC–MS methods in SIM mode, which is the common way used by the laboratories to screen the anabolic steroids in urine. The 3′hydoxystanozolol mass spectrum with six specific ions obtained by GC–MS^3 analysis attests of the substance and is more characteristic than analytical results that can be obtained with HRMS.

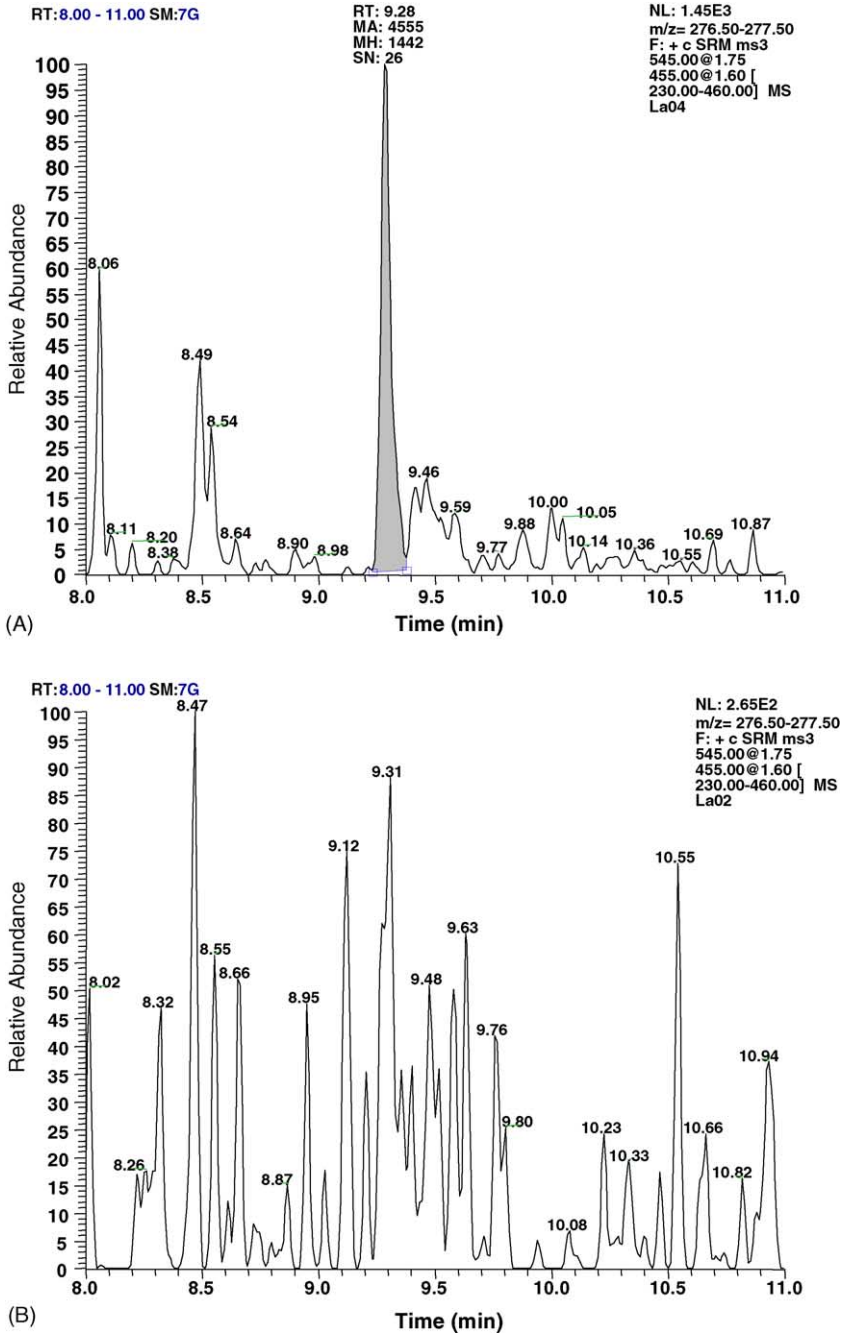

Fig. 4. GC–MS^3 analysis (ion chromatogram of mass trace *m*/*z* 277) of an excretion study from stanozolol after liquid–liquid extraction and TMS derivatization. (A) Urinary lower phase. (B) Urinary upper phase.

3.2. Analysis of a positive sample: a case report

The data shown in Fig. 3 correspond to a positive A sample with 3′hydroxystanozolol obtained from an athlete, with the MS^3 technique. As it can be seen in Fig. 3C and D, the presence of the diagnostic ions *m*/*z* 439, 425, 347, 277 and 239 attested of the presence of 3′hydroxystanozolol at an estimated concentration of 4 ng/ml. For comparison, Fig. 3A and B shows the mass spectrometric signals for a spiked urine with stanozolol at 2 ng/ml. The analysis was performed three times in the laboratory before the analytical report sending. Surprisingly, the presence of 3′hydroxystanozolol was not confirmed in the B sample (Fig. 3E). Consequently, several investigations were performed at the laboratory in order to explain this unexpected result. It was rapidly admitted that no possible contamination of the bottle A with stanozolol metabolite happened in the laboratory. Indeed, sterile and disposable equipment is used to take urine aliquots from the bottle for the measure of pH and specific gravity, or for the screening purpose. In particular, the aliquot used for the pH measurement is thrown away. The second hypothesis was the possible degradation of the substance in the bottle B, even if the sample was kept frozen. No references in the literature attest of this possible degradation and no degradation was observed in the excretion urines generated by the laboratory many years ago and kept frozen, even in the case of very low stanozolol metabolite concentrations.

The third hypothesis was the possibility that the urine in bottle B was not exactly the same as in bottle A. As a matter of fact, in some cases the urine aliquots in bottles A and B are visually not at all the same, if the sampling is not correctly done, even if both samples are from the same athlete. Indeed, if the athlete gives on two times urine in order to reach the volume needed for each bottle and if the aliquots are not mixed before the partitioning between both bottles, urine can be not the same in bottles A and B. But in this particular case, no difference on the pH of bottles A and B was observed, no real difference on the specific gravity and color of the urines, and no difference on the steroid profiles. The only remark noted down on the data base when registering the samples concerned the sedimentation which was present in sample A and not in sample B.

In order to verify this hypothesis, an excretion urine containing a similar 3′hydroxystanozolol concentration was defrosted, allowed to settle and the upper and lower phases were extracted and analyzed separately. Surprisingly, the presence of stanozolol metabolite was identified in the lower phase and nothing in the upper phase (Fig. 4A and B). In this particular case, 3′hydroxystanozolol is linked to sedimentation in urine and the urinary sample taken from the athlete probably settles during all the administrative step and the upper layer was put in bottle B and the rest in bottle A, as often performed. This hypothesis might explain the contradictory results obtained by the laboratory when performing this urinary sample. To our knowledge, this phenomenon has never been described for other anabolic steroids or substances.

4. Conclusion

The use of GC–MS^n ion trap system is effective in the analysis of some compounds in complex matrices such as urine and hence is a powerful tool for the detection and confirmation of anabolic substances, for example, at low concentrations. The GC–MS^3 analytical method used for 3′hydroxystanotolol is highly sensitive, reliable and specific in confirming positive results. Investigations are in progress in the laboratory for the negative chemical ionization analysis of stanozolol metabolite with the ion trap system.

Through the case report, it seems important to heighten sport federations awareness of the importance of sampling and the difficulties that has to face the laboratory when dealing with such a case.

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