Confirmation of Long Term Excreted Metabolites of Stanozolol by Gas Chromatography Coupled with High Resolution Mass Spectrometry (GC/HRMS)

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Upon screening identification of Stanozolol, GC/HRMS confirmation of the suspicious sample is done by reanalysis of the urine specimen, where a specific immunoaffinity purification procedure is used to selectively isolate the long term excreted metabolites of Stanozolol. By meeting the specific identification criteria for more than one metabolite of the same parent compound, additional evidence could be obtained in the decision making process in doping control.

Keywords: Stanozolol, confirmation, GC/HRMS, IAC

Stanozolol -17β-hydroxy-17α-methyl-5α-androst-2-eno[3,2-c]pyrazole – was synthesized in 1959 by the condensation of oxymetholone with hydrazine hydrate leading to a pyrazole ring [1,2]. The 17α-methylated steroids have been ones of the most frequently abused anabolic agents to enhance performance in sport. The use of anabolic androgenic steroids in sport is prohibited by World Anti-Doping Agency's (WADA) requirements [3]. This ban is controlled by the analysis of urine samples collected from athletes, where the excreted anabolic steroids and their metabolites are identified by gas chromatography coupled with mass spectrometry.

The heterocyclic structure, with a pyrazole nucleus fused to the andostan ring system (fig.1, **1**) is the reason for the difficult extraction and isolation from matrix. As many other anabolic steroids, Stanozolol has demonstrated poor chromatographic qualities and elevated background noise. Its detection in urine is difficult due to the rapid metabolization, which leads to low concentration levels of parent compound in urine. The research has been focused thus on identifying its main excreted metabolites which are detected for a much longer period of time in athletes' urine than the parent steroid itself [4,5]. The most abundant metabolites which are detected for the longest time post-administration are 3'-hydroxy-, 4β-hydroxy- and 16β-hydroxystanozolol (fig.1). They are excreted in conjugated forms that can be hydrolyzed with β-Glucuronidase from *E.coli* [6-8] and were selected as target analytes for the long term detection of Stanozolol abuse. Different strategies of extraction, derivatizations and detection by gas chromatography coupled with low or high resolution mass spectrometry are described in the literature [4-7,9,10].

Technical documents elaborated by WADA require the sensitivity of Stanozolol metabolites detection at a concentration level of 2ng/mL of urine [11], limit that might be achieved by application of high resolution mass spectrometry (HRMS) or tandem mass spectrometry (MS/MS) techniques. In lower concentration ranges, the gas chromatographic analysis of Stanozolol hydroxilated metabolites is very difficult as they show high sensitivity to active sites or interferences with matrix compounds. Because of the pyrazole structure the steroid can form bonds with any active sites in the chromatographic system (injector, column head or transfer line) resulting in decreasing intensities of the relevant signals [12].

Lower detection limits and better selectivity could also be achieved with improved sample clean-up strategies in order to eliminate interferences due to the urine matrix, approach generally used especially in confirmatory analysis. The method that provide unequivocal confirmation of Stanozolol abuse long time post-administration, isolates its metabolites by immunoaffinity chromatography (IAC) using an antibody which was prepared for methyltestosterone and showed high cross reactivity to long-lasting metabolites of Stanozolol [8,13].

Following the criteria of identification strictly specified by WADA, a prohibited substance in a suspicious sample is compared with a reference material analyzed in the same analytical batch. According to WADA technical document TD2003IDCR [14] the chromatographic retention time of the analyte shall not differ by more than one percent or ± 0,2 min (whichever is smaller) from that of the same substance in a spiked reference urine. In mass spectrometric detection at least three diagnostic ions must be aquired and the relative intensities of any of these ions shall not differ by more than the amount in table 1 [14] from the relative intensities of the same ions aquired from a spiked urine. The signal-to-noise ratio of the less intense diagnostic ion must be greater than three to one (3:1). The concentration of banned compound should be comparable in the sample and the spiked reference urine.

Relative abundance (% of base peak)	EI-GC/MS	CI-GS/MS; GC/MS^n; LC/MS; LC/MS^n
>50%	±10% (absolute)	±15% (absolute)
25% - 50%	±20% (relative)	±25% (relative)
<25%	±5% (absolute)	±10% (absolute)

Table 1
MAXIMUM TOLERANCE WINDOWS FOR RELATIVE ION INTENSITIES ACCORDING TO WADA DOCUMENTS [14]

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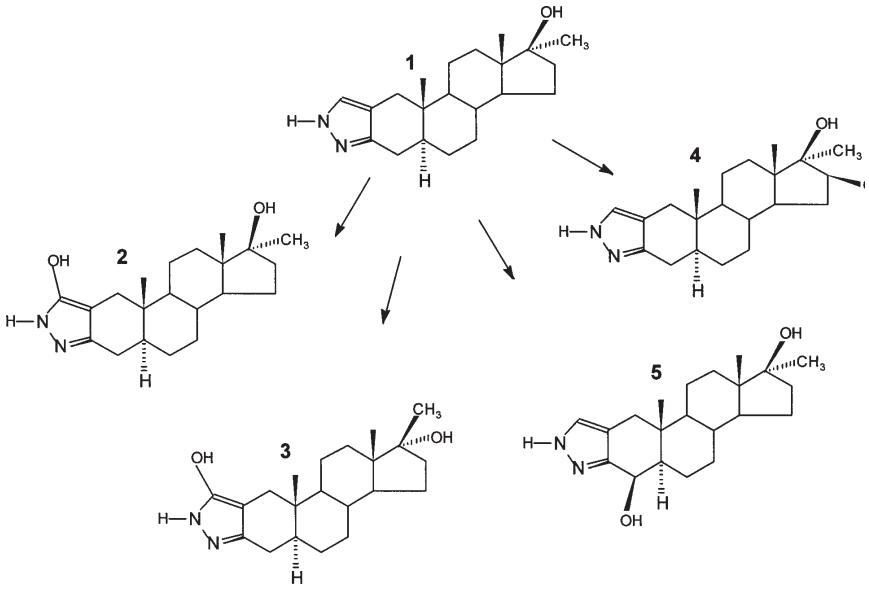
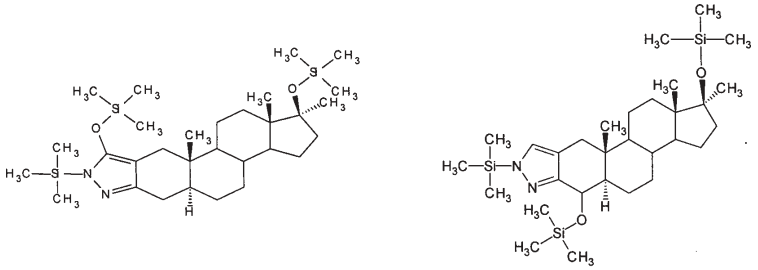


Fig. 1. Metabolism of Stanozolol (1): 3'-hydroxystanozolol (2), 3'-hydroxy-17-epistanozolol (3), 4β-hydroxystanozolol (5) and 16β-hydroxystanozolol (4)



a b

Fig. 2. Trimethylsilyl (TMS) derivatives of Stanozolol metabolites: 3'-hydroxystanozolol tris-TMS (a) and 4β- hydroxystanozolol tris-TMS (b)

Experimental part

Materials

The reference substances for Stanozolol metabolites and internal standard 4α-hydroxystanozolol were purchased from National Analytical Reference Laboratory (NARL, Australia). Derivatization agent N-methyl-N-trimethylsilyltrifluoroacetamide MSTFA (for gas chromatography) was produced by Merck, and the enzyme β-Glucuronidase from *E.coli* was from Roche Diagnostics Manheim. Amberlite XAD_2 resin, was purchased from Supelco, USA, and the immunoaffinity gel from Laboratoire d'Hormonologie, Marloie, Belgium. All the other chemicals and solvents were of analytical and chromatographic grade and were purchased from Sigma and Merck. The PBS buffer (154mmol NaCl, 44mmol Na_2HPO_4 anh., 5.5mmol KH_2PO_4, adjusted to *p*H 7.5 with concentrated HCl) was added 0.05g NaN_3.

To exemplify the confirmation method, a routine doping control sample was used, in compliance with bioethics and identity confidentiality principles. The sample collected on the occasion of a doping testing was declared suspicious on Stanozolol abuse, following the low and high resolution screening analysis.

Isolation of Stanozolol metabolites from urine

The suspicious sample was extracted for the isolation of metabolites 3'-hydroxystanozolol (fig.1, 2) and 4β-hydroxystanozolol (fig. 2, 5) by solid- liquid extraction on Amberlite XAD_2 resin, enzymatic hydrolysis to release the free steroids from conjugated forms, purification by immunoaffinity chromatography (IAC) and for the final detection, gas chromatography coupled with high resolution mass spectrometry technique [8]. In the same analytical batch there were extracted concomitantly a blank urine and a negative urine spiked with pure Stanozolol metabolites solutions (5ng/mL each) as reference sample.

A volume of 8 mL of urine was applied on a glass column conaining about 2 cm of Amberlit XAD_2 resin previously activted. The absorbed steroidic compounds were eluted wit 2 . 1mL of methanol. After evaporation to dryness under reduced pressure of methanol, 1mL phosphate buffer 0.8M, *p*H 7 and 25μL β-Glucuronidase from *E.coli* were added. The mixture was heated 1h to 50°C and the enzymatic hydrolysis was stopped by alkalizing the extract with an aqueous buffer solution of 20% K_2CO_3-$KHCO_3$ (1:1, w/w) *p*H 9. The metabolites of Stanozolol in free form were extracted with 5mL *tert.*–butylmetyl ether and after the organic layer was evaporated t dryness under reduced pressure, the dried residue as further processed by immunoaffinity chromatography.

Selective isolation of Stanozolol metabolites via immunoaffinity chromatography

The urine extract dissolved in 100μL methanol and 5mL PBS buffe (*p*H 7.5) was applied on a glass Econocolumn (Birad), containing 1mL Sepharose CNBr4B, on which the ati-methyltestosterone 3 CMO-BSA anti-body was binded. Afer washing the column with 15% methanol-water solution, the steroids were eluted with 3 mL of a 60% methanol-water solution. Te column was washed again with 5mL of a 60 methanol-water solution, equilibrated with 15mL PBS buffer and stored.

The analytical sequence – blank urine, suspicious sample, reference urine – were applied on the same IAC column. After eluion, 4α-hydroxystanozolol 0.1 ppm was added as internl standard in all the samples (5ng in 50μL). After evaporatio to dryness of methanol-water mixture, the dried extracts were derivatized with 50μL MSTFA/Imidazole mixture (100:2, v:w), by heating at 60°C for 15min.

GC/HRMS analysis

High resolution mass spectrometry analyses were performed with a reverse geometry double focusing mass spectrometer MAT 95XP ThermoFinnigan coupled to an Agilent Technologies 6890N gas chromatograph. On a cross-linked 100% dimethylpolisiloxan capillary column (HP-ULTRA1, J&W Scientific), length 17m, i.d. 0.2mm, film thickness 0.11, 1μL of derivatised extracts was injected in pulsed splitless mode. The carrier gas was helium at a constant flow of 1.6 mL/min. Temperatures of injector and transfer line were set at 300^{0}C. Oven temperature was initially 200^{0}C, ramped by 15^{0}C/min to 310^{0}C and held for 2 min.

The ions were formed by 70eV EI ionization (1mA emission current). The ion source was held at 230^{0}C. High resolution selected ion monitoring was performed by electric field switching and the continuous calibration of the masses, using a fluorocarbon reference compound (fc_5311). The mass resolution has been adjusted to 5000 and the electrons multiplier was set to 1.8 kV. The MS was operated in the multiple ion detection mode (MID) with a single group containing 6 mass fragments characteristic to 3'-hydroxystanozolol and 4β-hydroxystanozolol metabolites, as well as to the internal standard 4α-hydroxystanozolol - m/z 471.3227; 472.3305; 520.3462; 545.3415; 560.3650; 562.3660. The ions were registered with scan cycle time of 0.33sec.

The peaks in the chromatograms presented in this paper are characterized by retention time (RT), height (AH) and signal/noise ratio (SN), parameters selected from Xcalibur software application of MAT 95 XP system. For confirmation, criteria of identification requested by World Anti-Doping Agency [14] were applied, using Excel spreadsheets.

Results and discussions

The monitored metabolites 3'-hydroxy- and 4β-hydroxystanozolol were verified by their chromatographic retention times and mass spectral properties compared to reference compounds. For an improved gas chromatographical behaviour in GC/HRMS analysis, after additional sample clean-up steps, the target steroids were derivatized to yield the trimethylsilyl (TMS) derivatives (figure 2) leading to sharper peaks and lower limits of detection. A reagent mixture MSTFA-Imidazole was used to accomplish the full derivatization of Stanozolol metabolites, steroids without keto groups, and also to obtain a long term stability of their TMS derivatives.

Figure 3 depicts the GC/HRMS screening results of the suspicious sample, without immunoaffinity isolation. The base peak is m/z 545.3415 [M^+-CH_3] for 3'-hydroxy-stanozolol eluting at 6.33 min and m/z 560.3650 [M^+] for 4β-hydroxystanozolol eluting at 6.35 min. It should be noticed the high baseline and the asymmetric shape of the signal. After IAC selective isolation of the steroids and

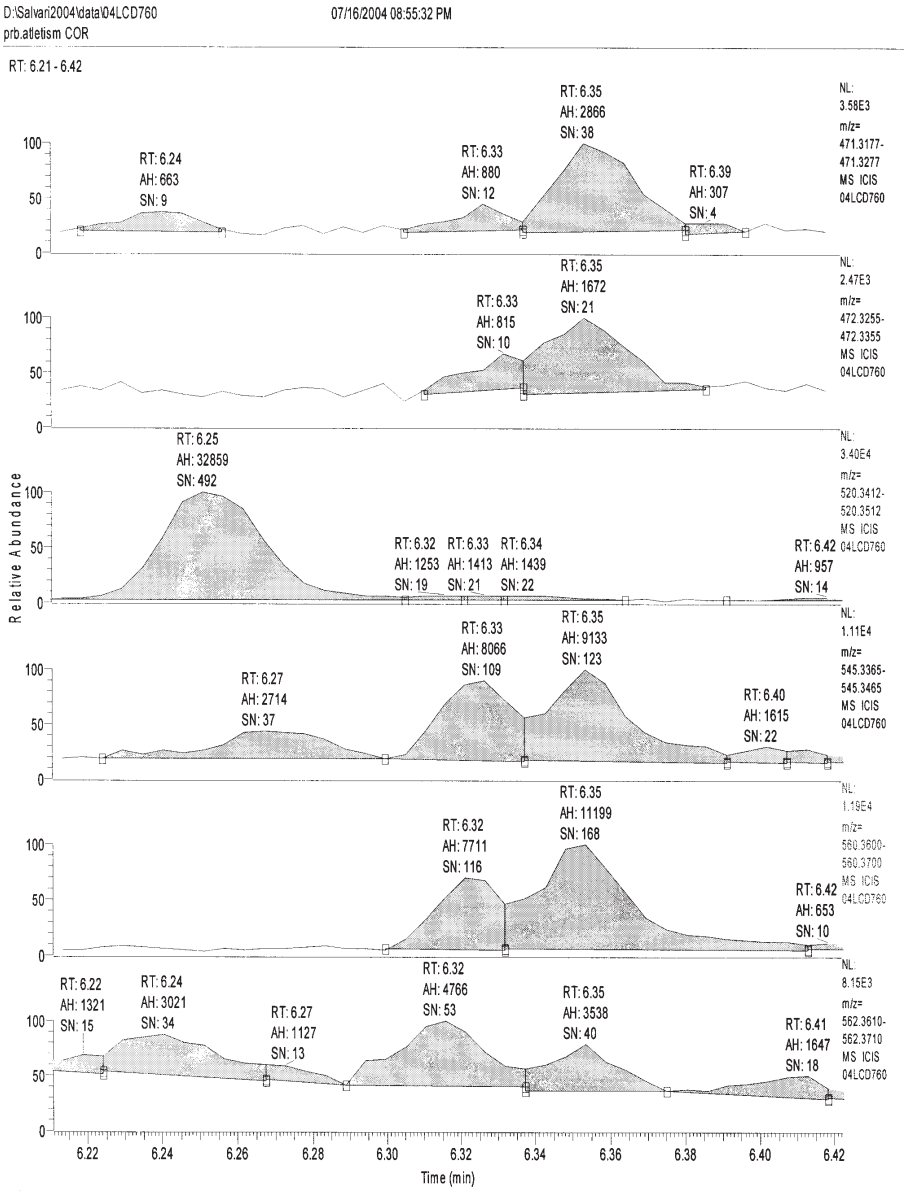


Fig. 3. The chromatogram of the suspicious sample in GC/HRMS screening analysis without IAC purification (3'-hydroxystanozolol RT 6.33min, 4β-hydroxystanozolol RT 6.35min)

Table 2

THE CRITERIA FOR THE IDENTIFICATION OF 3'-HYDROXYSTANOZOLOL METABOLITE IN THE SUSPICIOUS SAMPLE

Reference 3'-hydroxystanozolol (5ng/mL)					Sample		
RT (min)	m/z	Signal's height	Relative abundance (%)	Acceptance range	Relative abundance (%)	Signal's height	RT (min)
8.95	545	679877	100.00		100.00	185642	8.95
RT-IS	520	90148	13.26	8.26-18.26	12.27	22776	RT-IS
9.09	560	557058	81.94	71.94-91.94	85.30	158357	9.09
RRT	562	116872	17.19	12.19-22.19	17.80	33045	RRT
0.985							0.98
							0.00%

IS : 4alfa-hydroxystanozolol

Sample 115207

Reference 3'-hydroxysta 109332 F = 0.949004835

3'-hydroxystanozolol

Sample 185642 * F = 176175.1555

Reference 3'-hydroxysta 679877

==> **1.30 ng/ml**

Table 3

THE CRITERIA FOR THE IDENTIFICATION OF 4β-HYDROXYSTANOZOLOL METABOLITE IN THE SUSPICIOUS SAMPLE

Reference 4beta-hydroxystanozolol (5ng/mL)					Sample		
RT (min)	m/z	Signal's height	Relative abundance (%)	Acceptance range	Relative abundance (%)	Signal's height	RT (min)
9.01	560	475544	100.00		100.00	197187	9.01
RT-IS	471	149169	31.37	25.1-37.64	27.70	54626	RT-IS
9.09	472	57199	12.03	7.03-17.03	11.14	21966	9.09
RRT	545	384642	80.88	70.88-90.88	78.80	155374	RRT
0.99	562	98322	20.68	15.68-25.68	20.97	41348	0.99
							0.00%

IS : 4alfa-hydroxystanozolol

Sample 115207

Reference 4beta-hydroxysta 109332 F = 0.949004835

4beta-hidroxistanozolol

Sample 197187 * F = 187131.4164

Reference 4beta-hydroxysta 475544

==> **1.97 ng/ml**

the 15°C/min ramping GC temperature program a better elution profile was obtained. Clear, symmetrical peaks and very good signal-to-noise ratios for the monitored steroidic compounds are shown in figure 4 (a). The ion traces registered following IAC isolation are those due to the Stanozolol metabolites (3'-hydroxystanozolol RT 8.95min and 4β-hydroxystanozolol RT 9.01min), the biological material which leads to the background ion signals in fig.3 being almost totally removed. The fragment ion m/z 520.3462 is a very specific indicator only for the presence of 3'-hydroxystanozolol, not even for the internal standard of 4α-hydroxystanozolol eluting at 9.09min, synthetic homologous of the analites with a similar EI mass fragmentation. The peaks recorded following IAC isolation, shown in figure 4, have high values for signal-to-noise ratios.

Tables 2 and 3 show the confirmation and concentration estimation data of both monitored metabolites. Retention times (RT) and signals height (AH) in chromatograms of reference urine spiked at 5ng of each metabolite/mL (fig. 4 b) and of the suspicious sample (fig. 4 a) are introduced in Excel spreadsheets. The concentration levels (1,3ng 3'-hydroxystanozolol/mL and 1,9ng 4β-hydroxystanozolol/mL) were estimated by direct comparison of response factors F and *F of the base peaks (m/z 545.3415, respectively 560.3650) against the internal standard IS, in both reference and suspicious sample. For the assesement of the identification criteria additional diagnostic ions have been used. The Excel algorithms calculate the relative abundance of the signals towards base peak's height considered 100% and establish the accepted range according to WADA's requests [14]. It may be noticed that relative abundances of the diagnostic ions in the suspicious sample are comparable to the ones of the reference and are within the acceptance range for all the selected ion fragments.

In the same time the chromatographic retention time criteria are met as RT is not different by more than 1 (one) percent or ±0.2 min compared to reference. Taking into consideration the compliance with the confirmation criteria for both long-lasting Stanozolol metabolites, the sample is declared positive on Stanozolol abuse.

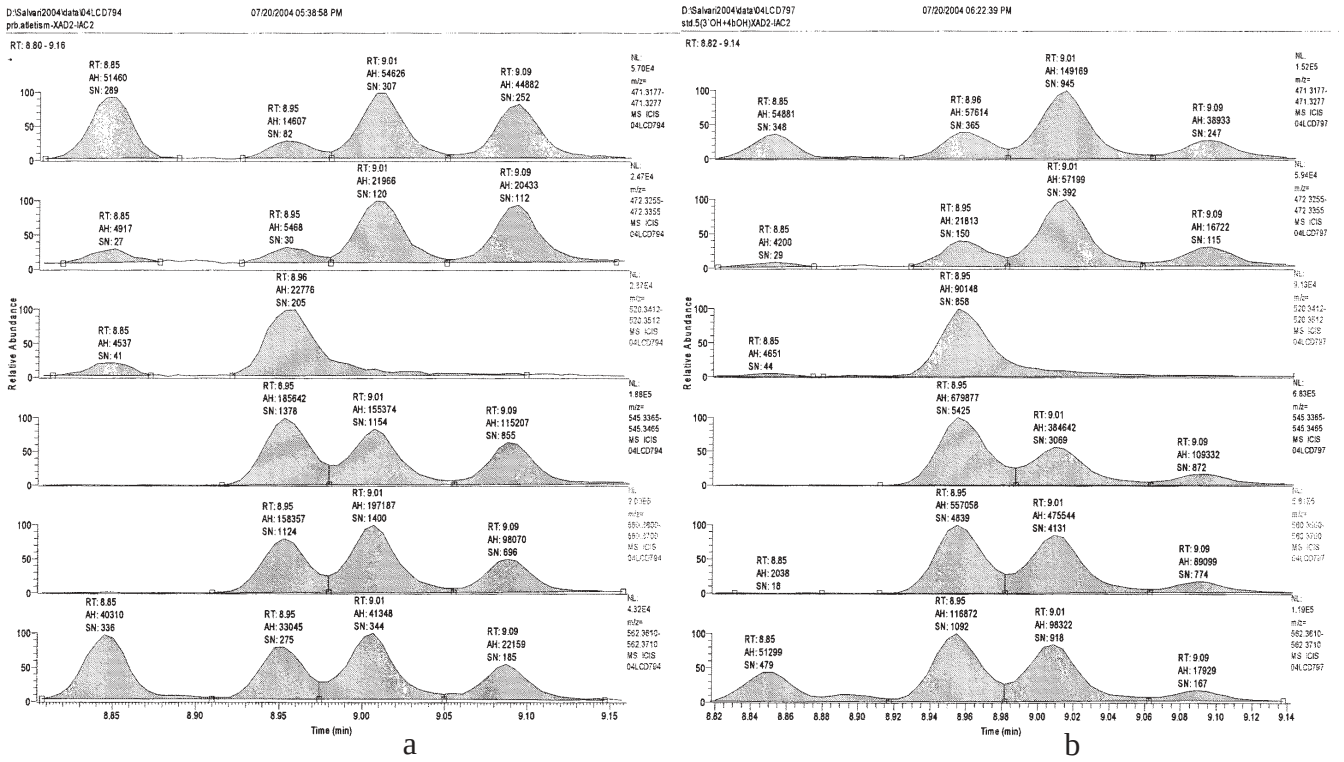


Fig.e 4. The chromatograms of the suspicious sample (a) and reference spiked urine of 5ng/mL (b) in GC/HRMS confirmation analysis after IAC purification (3'-hydroxystanozolol RT 8.95min, 4β-hydroxystanozolol RT 9.01min and IS of 4α-hydroxystanozolol RT 9.09)

Conclusions

Owing to the fact that Stanozolol metabolites are usually very low concentrated when determined in doping control samples and to the coeluting high background noise, complex specific workup and analytical procedures are employed to allow their unambiguous identification. The efficient removal of interferences due to biological matrix by IAC leads to excellent signal-to-noise ratios and to detection limit below 2ng/mL for both long-lasting metabolites. The metabolite 3'-hydroxystanozolol has been in the focus of the determination of Stanozolol abuse by GC/MS approaches due to the long-term retrospective accomplished with it as well as its good gas chromatographic behaviour obtained after derivatization. By HRMS technique it becomes possible to have good confirmation results for a second long-lasting metabolite, 4β-hydroxystanozolol, and meeting the identification criteria for more metabolites of the same parent compound, further proofs could be obtained for a fair decision in doping control.

The immunoaffinity chromatography isolation is a laborious and time-consuming sample preparation step, and thus, an alternative LC/MS/MS approach have been evaluated employing consecutive SPE and LLE with re-extraction into an acidic aqueous layer, with no derivatization step [15]. This strategy allows an improved analysis of hydroxilated metabolites of Stanozolol other than 3'-hydroxystanozolol commonly employed, respectively 4β- and 16β- hydroxystanozolol, which have proven higher abundances and a prolonged traceability in urine specimens.

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Manuscript received: 3.08.2007