IMPROVEMENTS IN THE CONFIRMATION OF STANOZOLOL ABUSE IN DOPING CONTROL

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Timp de mulți ani, metoda folosită de laboratoarele de control doping pentru dovedirea abuzului de stanozolol în sport s-a bazat pe detecția metaboliților săi urinari 3'-hidroxi- şi 4β -hidroxistanozolol, prin cromatografie de gaze cuplată cu spectrometria de masă de joasă sau înaltă rezoluție. Această strategie necesită etape de purificare a probei consumatoare de timp. Lucrări publicate recent prezintă o metodă nouă de identificare a metaboliților stanozololului 4β- şi 16β-hidroxilați, printr-o prelucrare simplă a probei urmată de analiza LC-ESI-MS-MS. Deşi rapidă şi sensibilă, metoda prezintă recuperări între 20 şi 38% pentru metaboliții menționați. Urmând principiul descris, al extracțiilor consecutive în fază solidă şi fază lichidă, dar schimbând ordinea etapelor de extracție la pH acid şi pH bazic, au fost obținute recuperări superioare şi, în consecință, rezultate mult mai bune la confirmare.

The usual method used by doping control laboratories to prove stanozolol abuse in sport has been based for many years on the detection of its urinary metabolites 3'-hydroxy- and 4β-hydroxy-stanozolol by gas chromatography coupled to low or high resolution mass spectrometry. This strategy requires time-consuming sample purification steps. Recently published articles present a new identification method for 4β- and 16β- hydroxylated metabolites of stanozolol by a simple sample preparation followed by liquid chromatography and electrospray ionization tandem mass spectrometry. Although rapid and sensitive, the reported method recovery ranges between 20 and 38% for the above mentioned metabolites. Following the published sample preparation principle, but applying an inverse sequence of the acidic and basic extraction steps, higher recoveries were obtained and, in consequence, better confirmation results.

Keywords: Stanozolol metabolites, LC/ESI/MS/MS

Abbreviations: ESI – electrospray ionization, **GC/MS** – gas chromatography coupled with mass spectrometry, **HRMS** – high resolution mass spectrometry, **IAC** – immunoaffinity chromatography, **ISTD** – internal standard, **LC/MS** – liquid chromatography coupled with mass

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spectrometry, **LLE** – liquid-liquid extraction, **MRM** – multiple reaction monitoring, **MS/MS** – mass spectrometry in tandem, **RRt** – relative retention time, **SPE** – solid phase extraction, **TBME** – tert-butylmethylether, **TMS** – trimethylsilyl, **WADA** – World Anti-Doping Agency

1. Introduction

Stanozolol is one of the most frequently abused anabolic agents to enhance performance in sport. As a consequence, the technical documents elaborated by the World Anti-Doping Agency (WADA) require the sensitivity of detection for stanozolol metabolites at a concentration level of 2ng/mL of urine [1].

Stanozolol presents a rapid metabolization (Fig. 1) leading to low concentration levels of the parent compound in urine (Fig. 1, **1**). It is mainly excreted as hydroxylated metabolites (Fig. 1, **2,4,5**) which are detectable long time post-administration [2,3].

The detection and confirmation of stanozolol abuse in sport is difficult due to the low excretion levels of its urinary metabolites, in combination with elevated biological background and matrix interferences at their chromatographic elution times [4]. The confirmation becomes impossible when signal-to-noise ratio is lower than 3, further purification steps being necessary. Therefore, the doping control analysts focused on an alternative method of sample preparation and detection of stanozolol major metabolites [2,3,5-7]. Doping control laboratories perform different analytical techniques in combination with extraction procedures which are efficient in removing the biological background (table 1).

For many years, the usual method used by doping control laboratories to prove Stanozolol abuse in sport has been based on the detection of its urinary hydroxylated metabolites by gas chromatography coupled to low or high resolution mass spectrometry, a strategy which provides low detection limits.

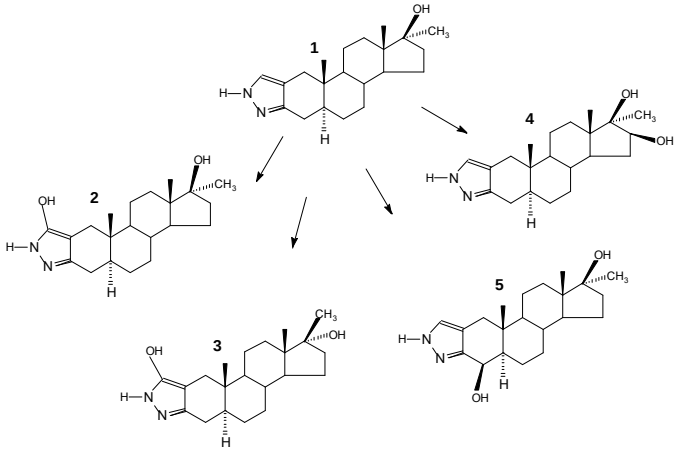

Fig. 1. Stanozolol (**1**) metabolism: 3'-hydroxystanozolol (**2**), 3'-hydroxy-17-epistanozolol (**3**), 4β-hydroxystanozolol (**5**) and 16β-hydroxystanozolol (**4**)

The 3'-hydroxystanozolol metabolite has been employed as target analyte, due to a good gas chromatographical behaviour of its trimethylsilyl (TMS) derivatives. For confirmatory analysis instead, this strategy requires time consuming and laborious purification steps, such as immunoaffinity chromatography (IAC) [8]. A modern approach [9] is based on a simple extraction of two hydroxylated stanozolol metabolites from urine using SPE, LLE and re-extractions at acidic and basic pH, followed by a final detection by liquid chromatography coupled to tandem mass spectrometry with an electrospray ionization interface (LC/ESI/MS/MS). The described assay is rapid and allows for detection limits below 0.5ng/mL with recoveries ranging from 20 to 26% for 4β-hydroxystanozolol and from 27 to 38% for 16β-hydroxystanozolol.

Following the published sample preparation principle [9], but applying an inverse sequence of the acidic and basic extraction steps, higher recoveries were determined for both target metabolites and, in consequence, better confirmation results.

Table 1

Strategies used in doping control for stanozolol abuse detection

Test	Sample preparation	Analytical technique	Target metabolites	Detection limits
Screening	Direct hydrolysis and L-L extraction	GC/MS quadrupol	3'-OH-stanozolol 16β-OH-stanozolol	> 15ng/mL
		GC/HRMS double focusing	3'-OH-stanozolol 4β-OH-stanozolol	> 2ng/mL
		LC (GC)/MS/MS	3'-OH-stanozolol 16β-OH-stanozolol	2-10ng/mL
Confirmation	S-L extraction, hydrolysis, IAC	GC/HRMS double focusing	3'-OH-stanozolol 4β-OH-stanozolol	< 2ng/mL
	S-L and L-L extractions at acidic and basic pH	LC/MS/MS	16β-OH-stanozolol 4β-OH-stanozolol	< 2ng/mL

2. Experimental

Materials

The reference substances for stanozolol metabolites and internal standard 4α-hydroxystanozolol were purchased from National Analytical Reference Laboratory (NARL, Australia). The enzyme β-glucuronidase from *E.coli* was produced by Roche Diagnostics Manheim, and Amberlite XAD_2 resin was purchased from Supelco, USA. All the other chemicals and solvents were of analytical and chromatographic grade and were purchased from Sigma and Merck.

Aiming to exemplify the applicability of the method to real samples, two routine doping control samples were used in compliance with bioethics and identity confidentiality principles. The samples were declared suspicious of stanozolol abuse after the low and high resolution screening analysis, and were

extracted concomitantly with a blank urine and negative urines spiked with pure stanozolol metabolites solutions (1, 2, 5 and 20ng/mL each) as reference samples (Std 1, Std 2, Std 5, Std 20).

In order to estimate the recovery, blank urines were spiked with the target analytes 4β-hydroxy- and 16β-hydroxystanozolol at 5ng/mL each, in different steps of the extraction procedure, as follows:

- initial spike (Ref in), after elution from XAD_2 column;
- intermediary spike (Ref interm), in the same time with KOH 5N;
- final spike (Ref fin), before last evaporation to dryness.

The internal standard of 4α-hydroxystanozolol (4ng/mL) was added in all the samples, before the final evaporation to dryness.

Methods

All the samples were extracted according to the extraction flow chart presented in Fig. 2-*right.* The urine was absorbed on an Amberlite XAD_2 resin column, and the methanolic eluate containing the conjugated stanozolol metabolites was concentrated to dryness, employing a rotary evaporator under reduced pressure. The dry residue was reconstituted in 1mL of sodium phosphate buffer 0.8M (pH 7). 25µl of β-glucuronidase (*E.coli*.) were added. After enzymatic hydrolysis at 50°C for 60 min, hydrochloric acid was added, adjusting the pH to 1.5-2.0, and the sample was extracted in 5mL of TBME and 3mL of n-pentane. The organic layer was discarded, potassium hydroxide (to pH 13-14) and TBME being added to the aqueous phase. After shaking and centrifugation, the aqueous layer was discarded and the dry residue of the organic phase was dissolved in a small volume of methanol which was transferred to a vial for LC/MS/MS analysis. The instrumental analysis was performed using an Agilent 1200/6410 system.

Column: Zorbax 5µm SB-C18 (50 x 2.1mm i.d., 5µm particle size);
Eluents: mobile phase A = 1‰ formic acid + 5mM ammonium formate in water,
mobile phase B = 1‰ formic acid + 5mM ammonium formate in 90% acetonitrile + 10% water;
Flow rate: 0,3mL/min;
Gradient B: 30%→50% in 1min, 50%→70% in 3min, 5min to 70% and re-equilibration for 5min at 10%;
Injection volume: 1µL;
Ionization: positive mode;
Spray voltage: 4000V;
Drying gas: 10L/min N_2 at 350^0C;
Nebulizing gas: 45psi N_2;
Collision gas: nitrogen 5.0;
Acquisition mode: MRM (table 2).

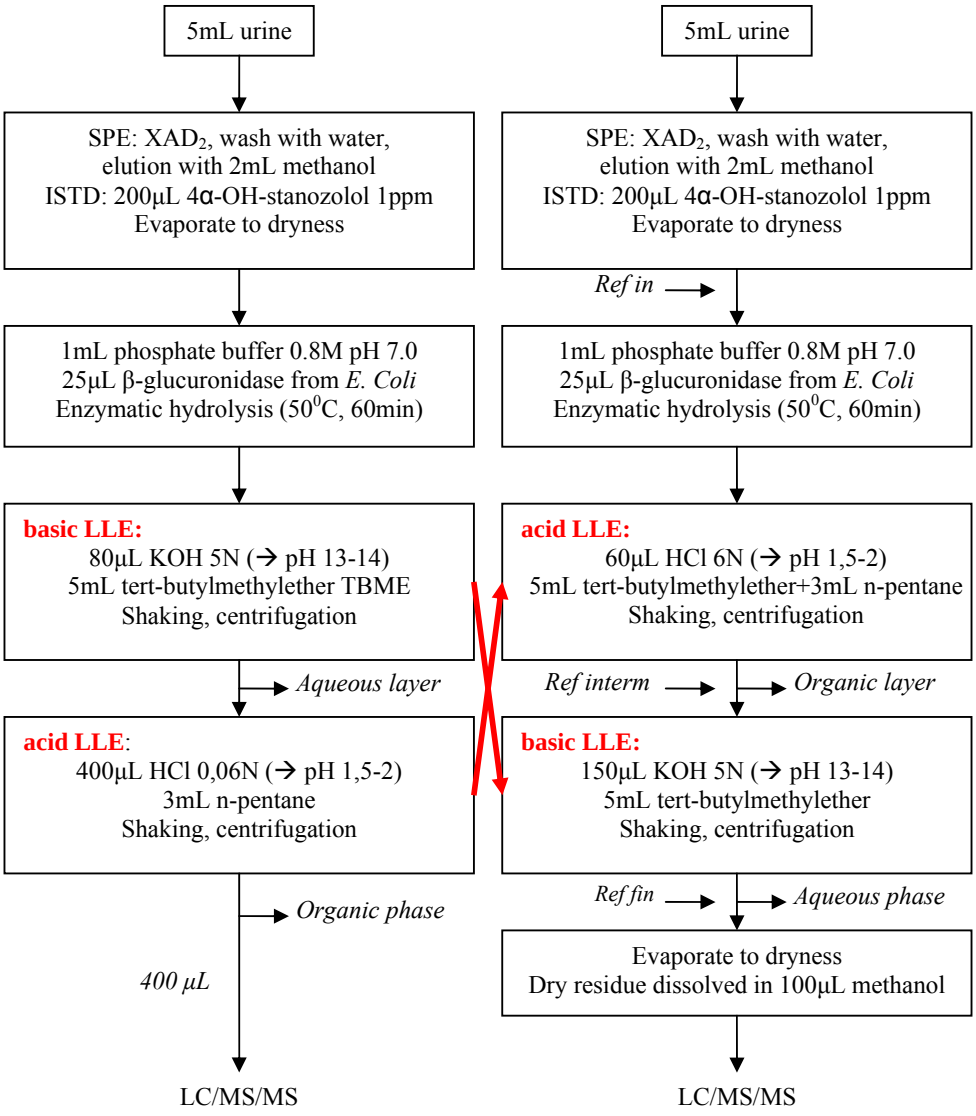

Fig. 2. The extraction flow chart: the literature method (*left*); experimented method (*right*)

3. Results and discussions

Due to the weak basic character determined by the pyrazolic nitrogen, at a basic pH, 16β- and 4β-hydroxystanozolol are in a non-ionized form, more hydrophobic, preferring the ethereal phase, while, at an acidic pH, they are in an ionized form, more hydrophilic, preferring the aqueous phase.

Starting from the published method [9] (Fig. 2-*left*), one noticed that the final volume resulted from the sample preparation is 400μL of an aqueous solution. In order to finish the sample preparation with the compounds of interest in an easily evaporable organic phase, the extractions have been swapped. Reconstituting the evaporated sample in just 100μL methanol, the sample preparation results in a final volume 4 times more concentrated (Fig. 2-*right*). Moreover, the liquid-liquid extraction at the acid pH can be performed with a larger volume of aqueous phase, resulting in an improved distribution ratio of the analyzed compounds in the aqueous phase and, therefore, a better overall recovery.

After the SPE clean-up (Fig.2), the eluted methanolic extract is evaporated and the sample reconstituted in phosphate buffer, for the enzymatic hydrolysis. Afterwards, the pH is adjusted to 1.5-2.0, using a relatively concentrated hydrochloric acid, to compensate the action of the phosphate buffer and of the various acid-basic species from the sample. At the acid liquid-liquid extraction step, n-pentane is added to the TBME in order to turn the ethereal phase more hydrophobic and force the distribution of 16β- and 4β-hydroxystanozolol (which are polar compounds at acid pH) in the aqueous phase, while the organic phase extracts the acid and neutral hydrophobic species. After removing the organic phase, the aqueous phase is adjusted to pH 13-14, using a concentrated solution of potassium hydroxide, to neutralize the previously employed hydrochloric acid, then extracted with TBME. The compounds of interest pass in the organic phase being separated from the neutral hydrophilic species that remain in the aqueous phase; the ethereal phase is evaporated and the sample reconstituted in a small volume of methanol.

16β-hydroxystanozolol is monitored as target metabolite, having a better analytic response in the LC/ESI/MS techniques than 3'-hydroxystanozolol, and a longer excretion period than the 4β-hydroxystanozolol. 4α-hydroxystanozolol is monitored as internal standard; on its MRM transition is also monitored 4β-hydroxystanozolol (table 2).

The recovery was estimated at 5ng/mL concentration by direct comparison of the response factor of the base transition against the internal standard in the urine samples fortified with stanozolol metabolites at the beginning, after acidic and after basic L-L extractions (table 3).

Table 2

MRM transitions monitored in LC/MS/MS technique

Compound	Precursor (fragmentor, V) > Product (collision energy, V)	Dwell time, ms
4α-OH-Stanozolol (ISTD) + 4β-OH-Stanozolol	345 (120) > 309 (10)	100
16β-OH-Stanozolol	345 (120) > 121 (45)	100
16β-OH-Stanozolol	345 (120) > 109 (45)	100
16β-OH-Stanozolol	345 (120) > 107 (50)	100
16β-OH-Stanozolol	345 (120) > 95 (50)	100
16β-OH-Stanozolol	345 (120) > 93 (50)	100
16β-OH-Stanozolol	345 (120) > 91 (75)	100
16β-OH-Stanozolol	345 (120) > 81 (55)	100
16b-OH-Stanozolol	345 (120) > 67 (65)	100

Table 3

Recovery of stanozolol metabolites

	16β-OH-Stanozolol	4β-OH-Stanozolol
L-L extraction at pH 1,5-2,0	46%	44%
L-L extraction at pH 13-14	92%	99%
Total recovery	43%	43%

In Fig. 3-*left*, typical chromatograms generated from a blank urine sample spiked at 5ng/mL of 4β- and 16β-hydroxystanozolol are shown. It should be noticed that the internal standard of 4α-hydroxystanozolol is separated from 4β-hydroxystanozolol, and that the target metabolite 16β-hydroxystanozolol is very well distinguished. The chromatograms of one of the real samples depicted in Fig. 3-*right,* shows the internal standard, traces of 4β-hydroxystanozolol and 16β-hydroxystanozolol detectable at approximately 7ng/mL. Table 4 shows the compliance between the relative abundances of the transitions and the retention times of the two real suspicious samples and the 5ng/mL reference. Fig. 4-*left,* depicts typical chromatograms generated from a blank urine sample, demonstrating no interferences with the interest compounds. The metabolite 16β-hydroxystanozolol is still detectable at retention time 5.012 in a urine sample spiked with 1ng/mL of each of the target metabolites (Fig.4-*right*). Table 5 presents the assessment of the identification criteria according to WADA technical documents [1], for the fortified samples (Std 1, Std 2, Std 5) against the 20ng/mL reference. It may be noticed that the relative abundances for Std 2 and Std 5 samples are comparable to the ones of the reference. For Std 1 sample, the background level affects the abundances of the MRM transitions, but they are still in the range.

Table 4

Assessment of identification criteria for the samples suspicious on stanozolol abuse

	Reference 5ng/mL		Suspicious sample 1	Suspicious sample 2
	Relative abundances	*Acceptance range*	Relative abundances	Relative abundances
345>81	100,00%	-	100,00%	100,00%
345>67	15,47%	*5,47% - 25,47%*	16,19%	14,43%
345>91	13,35%	*3,35% - 23,35%*	15,51%	15,57%
345>93	11,42%	*1,42% - 21,42%*	13,21%	13,03%
345>95	31,36%	*23,52% - 39,20%*	31,66%	30,63%
345>107	13,62%	*3,62% - 23,62%*	14,23%	14,96%
345>109	14,88%	*4,88% - 24,88%*	16,20%	15,10%
345>125	12,21%	*2,21% - 22,21%*	12,11%	11,50%
RRt	1,1357	1,1130 - 1,1584	1,1316	1,1357

Table 5

Assessment of identification criteria for the samples fortified with stanozolol metabolites

	Reference 20ng/mL		Fortified sample Std5 5ng/mL	Fortified sample Std2 2ng/mL	Fortified sample Std1 1ng/mL
	Relative abundances	*Acceptance range*	Relative abundances	Relative abundances	Relative abundances
345>81	100,00%	-	100,00%	100,00%	100,00%
345>67	15,54%	*5,54% - 25,54%*	15,47%	15,71%	18,46%
345>91	14,50%	*4,50% - 24,50%*	13,35%	13,07%	16,51%
345>93	11,91%	*1,91% - 21,91%*	11,42%	11,13%	14,05%
345>95	31,40%	*23,55% - 39,25%*	31,36%	29,92%	39,18%
345>107	13,86%	*3,86% - 23,86%*	13,62%	14,12%	14,36%
345>109	14,98%	*4,98% - 24,98%*	14,88%	16,13%	14,36%
345>125	12,21%	*2,21% - 22,21%*	12,21%	12,21%	11,49%
RRt	1,1357	*1,1130 - 1,1584*	1,1357	1,1357	1,1357

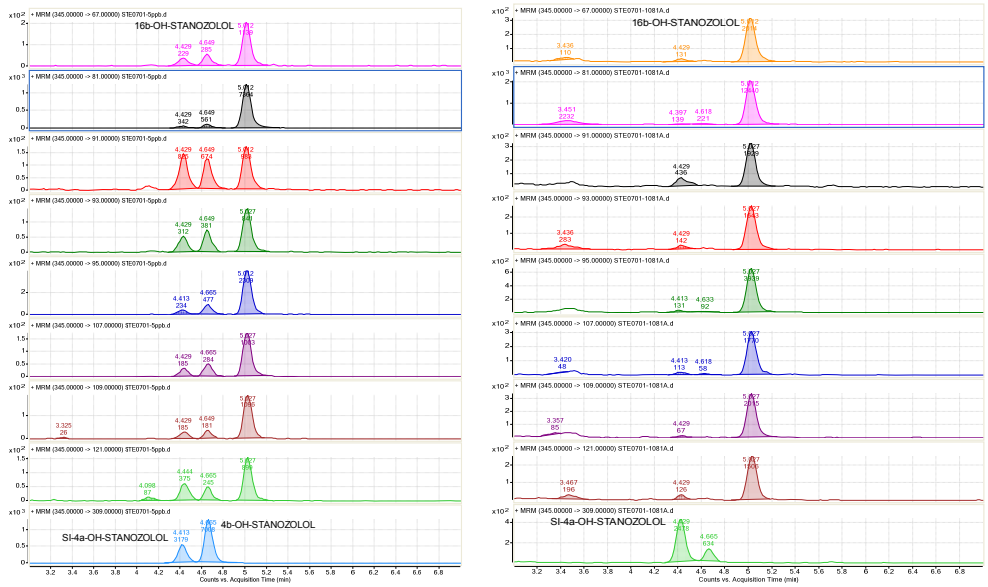

Fig. 3. LC/MS/MS chromatograms obtained from a blank urine fortified with 16β-OH-stanozolol and 4β-OH-stanozolol at 5ng/mL each (*left*) and from a real sample suspicious on stanozolol abuse (*right*)

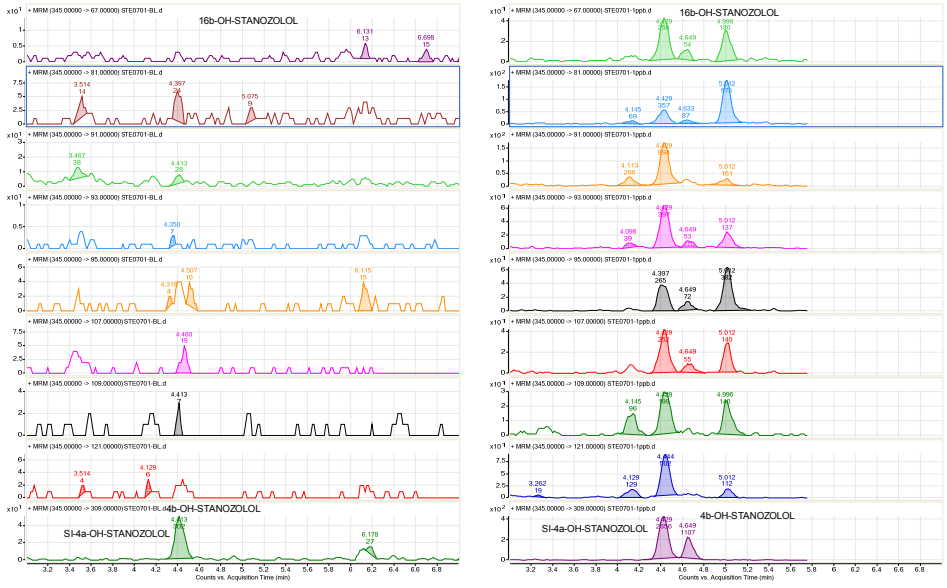

Fig. 4. LC/MS/MS chromatograms obtained from blank urine (*left*) and from a blank fortified with 16β-OH-stanozolol and 4β-OH-stanozolol at 1ng/mL each (*right*)

4. Conclusion

- 16β-hydroxystanozolol metabolite proves to be particularly suitable for long-term detection of stanozolol by LC/MS technique.
- The reversal of the acidic and basic L-L extraction steps leads to an improved recovery of the target metabolites from 30 to 45%.
- The concentrated final solution allows for higher chromatographic signals.

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