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Better recoveries in confirmation of stanozolol metabolites by LC/MS technique

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Introduction

Stanozolol is a prohibited substance for which the WADA-TDMRPL requires the sensitivity of detection for its metabolite 3'-hydroxy-stanozolol at a concentration level of 2ng/mL in urine [1].

The usual method used by doping control laboratories to prove for 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, a strategy which provides low detection limits [2-5]. For confirmatory analysis instead, this strategy requires time consuming and laborious purification steps, such as immunoaffinity chromatography (IAC) [6].

A modern approach [7] is based on a simple extraction of 4β-, 16β- hydroxylated metabolites of stanozolol and Stanozolol (PC) 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 [7] 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.

Materials and Methods

Sample preparation

For estimation of 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 XAD2 column;
- intermediary spike (Ref interm), in the same time with KOH 5N;
- final spike (Ref fin), before last evaporation to dryness.

Two routine doping control samples were also prepared according to the extraction flow chart presented in figure 1 *right*.

Equipment

All analyses were performed on an Agilent 1200/6410 LC/MS^2 triple quadrupole with ESI source, on a Zorbax 5µm SB-C18 column (50 x 2.1mm i.d., 5µm particle size). The flow rate was 0,3mL/min, the solvents used were A: water with 5mM ammonium formate and 1‰ formic acid and B: acetonitrile with 10% water, 5mM ammonium formate and 1‰ formic acid, the gradient: 30%B→50%B in 1min, 50%B→70%B in 3min, 5min at 70%B and re-equilibration for 5min at 30%B. Ion transitions for 16β-hydroxystanozolol: 345 > 121, 109, 107, 95, 93, 91, 81 and 67 and for 4α- and 4β-hydroxystanozolol: 345>309.

Results and Discussion

In the published method [7] (fig.1 *left*), the final volume resulting 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, we swapped the extractions. Reconstituting the evaporated sample in just 100µL methanol, the sample preparation results in a final volume 4 times more concentrated (fig.1 *right*).

At the acid liquid-liquid extraction step, n-pentane is added to the TBME, as in the published protocol [7], in order to turn the ethereal phase more hydrophobic and force the distribution of 16β- and 4β-hydroxystanozolol in the aqueous phase. After removing the organic phase, the aqueous phase is adjusted to pH 13-14, and then extracted with TBME. The compounds of interest pass in the organic phase; the ethereal phase is evaporated and the sample reconstituted in a small volume of methanol**.** As target metabolite is monitored the 16β-hydroxystanozolol, which has 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.

In figure 2 *left* typical chromatograms generated from a blank urine sample spiked at 5ng/mL of 4β- and 16β-hydroxystanozolol is 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.2 *right,* shows the internal standard, traces of 4β-hydroxystanozolol

and 16β-hydroxystanozolol detectable at approximately 7ng/mL. A good compliance between the relative abundances of the transitions and the retention times of the two real suspicious samples and the 5ng/mL reference was noticed.

The recovery was estimated by direct comparison of response factors of the base transition against the internal standard in the urine samples fortified with stanozolol metabolites at the beginning, after acidic and, respectively, after basic L-L extractions (table 1).

Table 1. Recovery of stanozolol metabolites

	16ß-OH-Stanozolol	4ß-OH-Stanozolol
L-L extraction at pH 1.5-2	46%	44%
L-L extraction at pH 13-14	92%	99%
Total recovery	43%	43%

Conclusions

•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.

Acknowledgements

The authors wish to thank the Romanian National Anti-Doping Agency for support.

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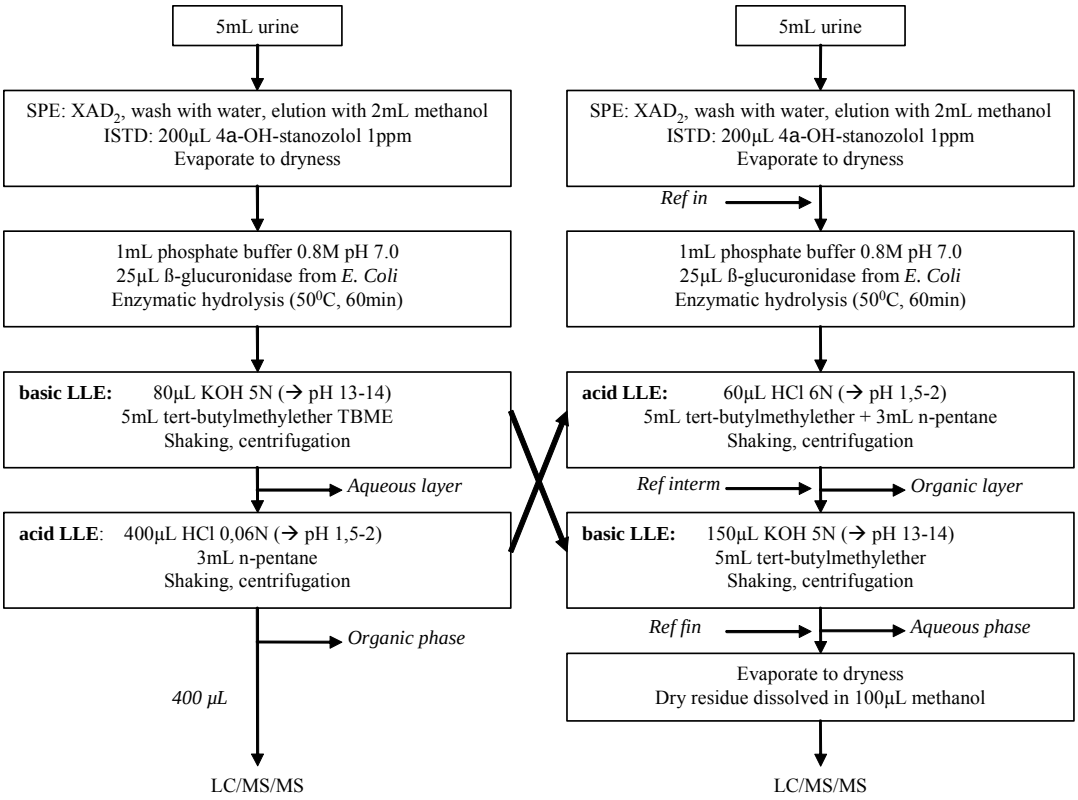

Figure 1. The extraction flow chart: the protocol according to [7] (*left*), alternative method (*right*)

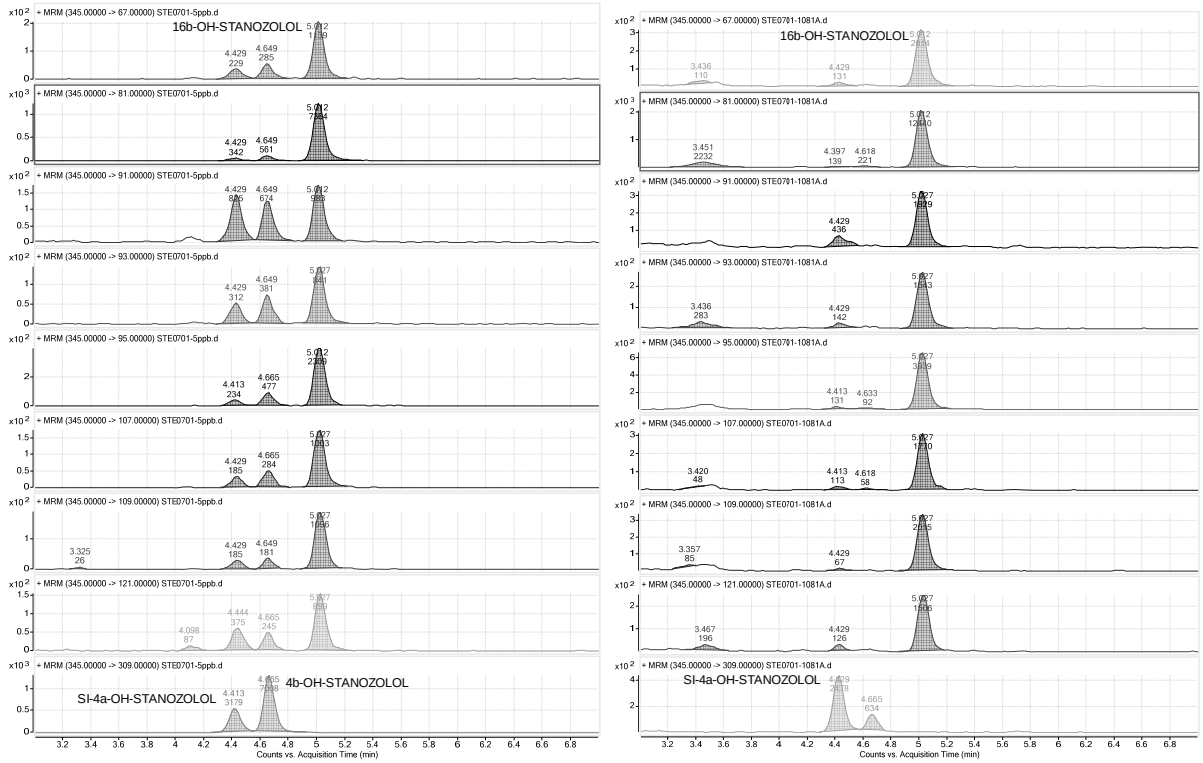

Figure 2. LC/MS/MS chromatograms obtained from a blank urine fortified with 16β-OH-stanozolol and 4β-OH-stanozolol at 5ng/mL each (*left*) and from a real sample suspicious on stanozolol abuse (*right*).