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Comparative detection of Stanozolol metabolites in excretion urines

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INTRODUCTION

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 [1]. Stanozolol metabolism indicates a quick production of mono- and bis-hydroxy- derivatives, excreted mainly in glucuronide form. The most abundant metabolites identified in the human urine are 16β-hydroxystanozolol, 3'-hydroxystanozolol and 4β-hydroxystanozolol [2,3].

The use of the gas chromatography coupled with the double focusing high resolution mass spectrometry (GC/HRMS) in combination with the purification by immunoaffinity chromatography (IAC) increases the sensitivity and specificity of the confirmation analysis of the 3'-hydroxystanozolol and 4β-hydroxystanozolol [4]. Another modern approach is based on the use of the liquid chromatography coupled to tandem mass spectrometry with an electrospray ionization interface (LC/ESI/MS/MS) in combination with a simple extraction of 16β-hydroxystanozolol and 4β-hydroxystanozolol from urine using SPE and LLE extraction/re-extraction at basic and acidic pH [5, 6].

The aim of the presented excretion study was to compare the retrospective detection of Stanozolol and to show how far out the specific long-term excreted metabolites could be confirmed, depending on the administration way and on the detection technique used.

MATERIALS AND METHODS

Drug administration: A male subject (age 30, 62kg) has been orally administered 40mg Stanozolol/day, for 14days, during a cure for a bones disease and his urine samples were collected daily between the 3rd and 43rd day post-application. Another male subject (age 22, 60kg) has been injected 150mg Stanozolol (3 ampoules x 50mg intramuscular) and only the urine samples on 46th, 50th and 52nd day post-administration were collected.

GC/HRMS analysis: All the samples were extracted according to the extraction flow chart presented in Figure 1 *left*. Immunoaffinity chromatography (IAC) was performed on glass Econocolumn (Biorad), containing 1mL Sepharose CNBr4B on which the anti-

methyltestosterone 3 CMO-BSA antibody was binded. The instrumental analysis 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 0.11µm, 1µL of derivatised extracts was injected in pulsed splitless mode. The carrier gas was helium at a constant flow of 1.6mL/min. Oven temperature was initially 200^{0}C, ramped by 15^{0}C/min to 310^{0}C and held for 2min. The mass resolution has been adjusted to 5000 and the electrons multiplier was set to 1.8kV. The MS was operated in the multiple ion detection mode (MID): *m/z* 471.3227, 472.3305, 520.3462, 545.3415, 560.3650, 562.3660.

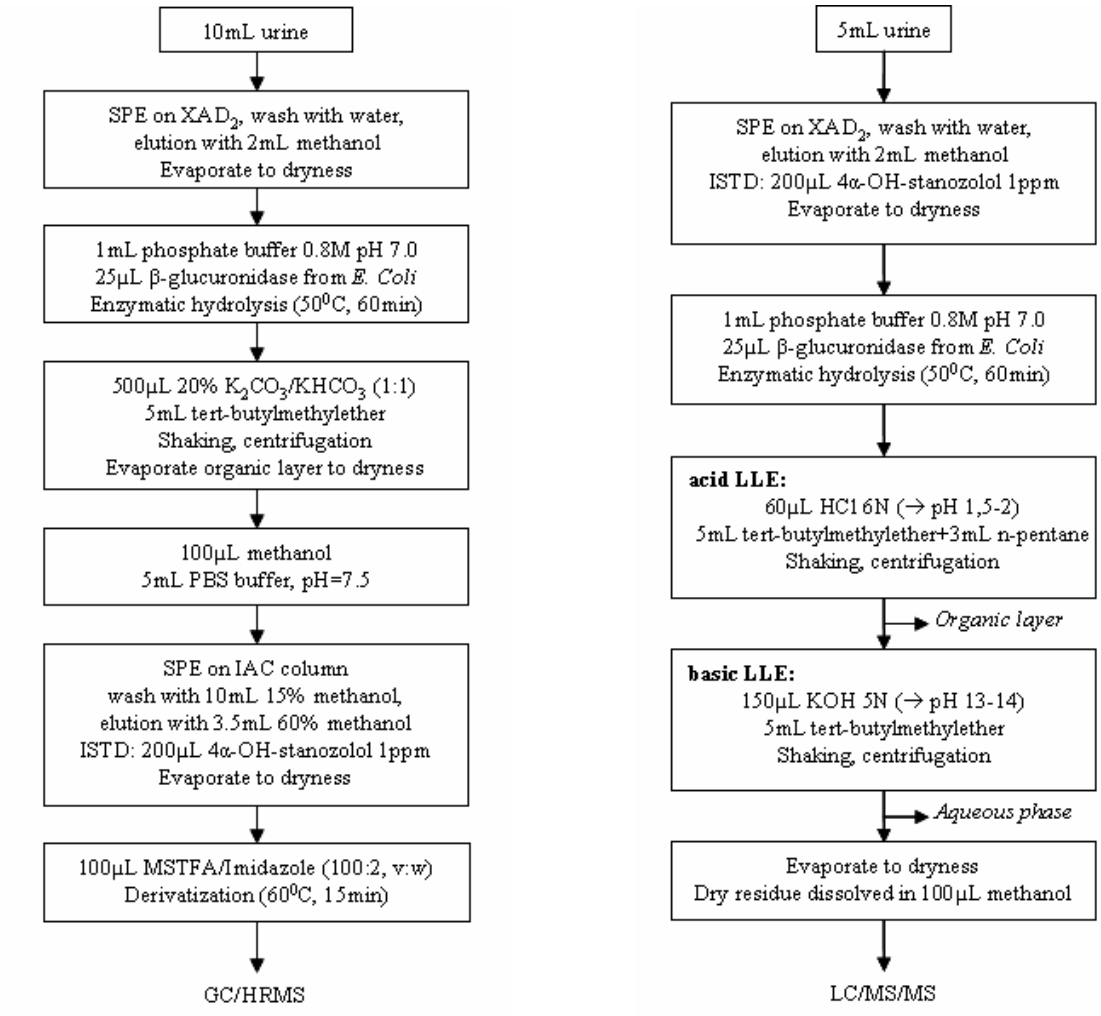

Figure 1. The extraction flow chart for GC/HRMS analysis (*left*) and LC/MS/MS analysis (*right*)

LC/MS/MS analysis: All the samples were extracted according to the extraction flow chart presented in Figure 1 *right*. The instrumental analysis were performed using a triple quadrupole mass spectrometer Agilent 6410 with an electrospray interface coupled to an Agilent 1200 liquid chromatography system. 1µL of the prepared samples were injected on a

Zorbax 5µm SB-C18 column, length 50mm, i.d. 2.1mm, particle size 5µm. The mobile phase consisted in a mixture of solvent A (1‰ formic acid + 5mM ammonium formate in water) and solvent B (1‰ formic acid + 5mM ammonium formate in 90% acetonitrile + 10% water) passing through the column at a flow rate of 0.3mL/min. The B gradient employed: 30%→50% in 1min, 50%→70% in 3min, 5min to 70% and re-equilibration for 5min at 30%. The ions were formed by positive electrospray ionization. The drying gas was held at 10L/min N_2 and 350^{0}C and the nebulizing gas at 45psi N_2. The triple quadrupole was operated in the multiple reaction monitoring mode (MRM): *m/z* 345>67, 81, 91, 93, 95, 107, 109, 121, 309.

RESULTS AND DISCUSSIONS

The monitored metabolites are 3'-hydroxystanozolol and 4β-hydroxystanozolol for the GC/HRMS technique and 16β-hydroxystanozolol for the LC/MS/MS technique. The concentration levels were estimated by direct comparison of response factors of the base peak (*m/z* 545.3415 in GC/HRMS, *m/z* 345>81 in LC/MS/MS) against the internal standard, 4α-hydroxystanozolol, in both reference and suspicious samples. The relative abundances of the diagnostic ions towards base peak's height were calculated for each sample and reference and evaluated according to WADA's criteria [7].

Oral administration. **GC/HRMS:** The day 7 post-oral administration sample was confirmed in compliance with the acceptance criteria for both metabolites 4β-hydroxystanozolol and 3'-hydroxystanozolol and the day 6 and 8 samples were confirmed only for 3'-hydroxystanozolol. The day 9 sample was out of the range for both metabolites. For the days 10, 11 and 12, the acceptance criteria were met for both metabolites, in the same time with an increase of their concentration. Beginning with day 13 the samples could not be confirmed anymore because of the reduced signals and after the 16th day no Stanozolol metabolites were detected. **LC/MS/MS:** The acceptance criteria were met for metabolite 16β-hydroxystanozolol up to day 15. After day 15 the samples could not be confirmed anymore, but the metabolite could be detected up to day 40. The evolution of the metabolites 3'-hydroxy-, 4β-hydroxy- and 16β-hydroxystanozolol concentrations are shown in figure 2.

Intramuscular administration. **GC/HRMS:** The day 46 and 52 post-IM administration samples were confirmed in compliance with the acceptance criteria only for metabolite 3'-hydroxystanozolol. For the day 50 sample the acceptance criteria were not met for any metabolite. The concentration of 3'-hydroxystanozolol dropped from 1.9ng/mL in day 46 to 0.24ng/mL in days 50 and regained 0.50ng/mL in day 52. **LC/MS/MS:** The metabolite 16β-hydroxystanozolol was detected and confirmed in all 3 samples. The concentration of 16β-hydroxystanozolol dropped from 1ng/mL in day 46 to 0.15ng/mL in days 50 and 52.

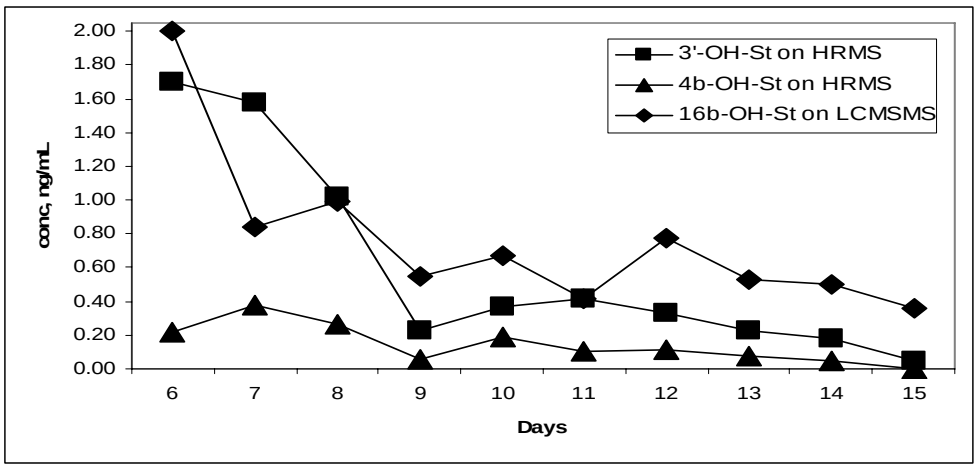

Figure 2. The plot of Stanozolol metabolites concentrations estimated in GC/HRMS and LC/MS/MS confirmatory analysis, between days 6 and 15 after oral application of Stanozolol

CONCLUSIONS

The period in which the stanozolol abuse can be confirmed in compliance with the acceptance criteria depends on the target metabolite, the administration path and the analytic technique: the metabolite 16β-hydroxystanozolol is excreted in general in slightly higher concentrations than the metabolite 3'-hydroxystanozolol and both of them are in significantly higher concentrations than the metabolite 4β-hydroxystanozolol, the samples collected post-intramuscular injection could be confirmed much longer than the samples collected post-oral administration and the LC/MS/MS method showed better results than the GC/HRMS method, while employing an easier sample preparation.

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