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5-Alpha reductase inhibitors detection in Doping Analyses

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Introduction

5α-reductase inhibitors are used in therapy for the treatment of benign prostatic hyperplasia (BHP) or androgenic alopecia. These pathologies are linked to high 5α-dihydrotestosterone (5α-DHT) levels and then to 5α-reductase activity.

These compounds were included in the WADA 2005 list of prohibited substances, in section *S5. Diuretics and other masking agents* and exemplified with finasteride and dutasteride.

The possible use of finasteride as a masking agent has already been demonstrated [1] with a combined administration of finasteride and nandrolone. As a consequence, the concentrations of norandrosterone are markedly decreased compared with the single nandrolone administration. The changes in steroid profiles that may have influence on the interpretation of longitudinal studies [2], the prevention on the detection of steroids mainly metabolized through 5α-reductase and lowering the time window of detection of some compounds (5α-DHT) are other observations that justified the prohibition of these compounds.

The main objectives of the present work are the description of the effects of the administration of 5 mg of finasteride (Proscar®) and 0.5 mg of dutasteride (Avodart®) on the steroid profile as a first marker of abuse and the detection of the metabolites of the substances administered and their implementation in the existing routine procedures.

Materials and methods

Excretion studies

One tablet of Proscar ® containing 5 mg of finasteride or 0.5 mg of dutasteride (Avodart ®) were administered to two male test subjects. All urine samples were collected during 60 h and then first morning urine spot samples for 12 days on spontaneous voiding.
Samples were frozen at -20ºC until analysis.

Sample analysis

All samples collected were analyzed by the routine steroid screening analysis by GC/MS in order to determine the steroid profile. Testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Et), 5α-DHT and androstandiols (5α-androstan-3α,17β-diol and 5β-androstan-3α,17β-diol) were monitored.

LC/MS/MS analysis of finasteride and dutasteride

For the analysis of both 4-azasteroid inhibitors the routine extraction procedure for corticosteroids was applied. A description of the extraction procedure used is presented in Figure1.
The analysis was performed in a Waters Alliance 2795 HPLC coupled to a Micromass Quattro micro™ mass spectrometer. Chromatographic separation was done in a Waters-XTerra MS C18 150x2.1 mm, 5 μm column. More details on instrumental parameters are presented in Figure 1.

Results and discussion

After the administration of finasteride and dutasteride, and as expected [4,5], a deep inhibition of 5α-reductase is observed through the analysis of the urinary steroid profile. A/Et and 5a/5b-diol ratios are decreased and kept low for at least 3 days for finasteride and 8 days for dutasteride (Figures 2-3). The ratio T/DHT was markedly affected for dutasteride administration until day 5 post-administration but even though DHT excretion was suppressed with finasteride, the ratio T/DHT showed a greater variability.

Effect on the T/E ratio a case study

During an elevated T/E follow-up case of a football player was using finasteride (Propecia) daily for more than one year, we investigated the possibility that this observation could be the result of the finasteride ingestion.

At a single dose administration of both compounds, no effect on the T/E ratio of the volunteers can be observed (CV 12.4% for finasteride, 18.7% for dutasteride) (Figures 2-3) Another parameter that may be used in the evaluation of longitudinal studies of elevated T/E ratios is the A/T ratio. A value below 20 may be an additional indication of testosterone abuse [3]. In both administrations, values of A/T far below the proposed value are reached adding confusing information in this case.

In the case of our football player, he was informed that in year 2005 finasteride was not permitted any more. While samples collected in 2004 showed the presence of finasteride metabolite, in 2005 samples were negatives, The T/E ratio remained stable but the A/Et, 5a-/5b-diol were back to normal values. The elevated T/E ratio was not related to the use of finasteride.

Detection of finasteride and dutasteride by LC/MS

The mass spectra of ω-carboxy-finasteride main finasteride metabolite, dutasteride and the proposed fragmentation pattern are presented in Figure 4.

The detection of ω-carboxyfinasteride after a single dose administration is possible (Figure 5) and acceptable detection times are obtained using the screening procedure for corticosteroids. The confirmation procedure shown in Figure 1 improved the detection of the metabolite. Using the confirmation procedure extraction the metabolite of finasteride was detectable for at least 2 days.

By applying the same analytical approach, the samples of the dutasteride excretion study were analyzed.

Dutasteride was not detected in any of the collected samples. None of the described metabolites [5, 6] (1,2-dihydrodutasteride, 6-OH-dutasteride, 4'OH-dutasteride, 15-OH-dutasteride or 6,4-di-OH-dutasteride) were detected in the urine samples. Some explanations to this may be: 1) the correct metabolite has not been considered or 2) the metabolites are excreted in very low concentrations and the method is not sensitive

enough or 3) the most reasonable is that as expected the main route of elimination is not urine but feces. Less than 0.1-1 % of unchanged dutasteride is excreted in urine [5, 6]. The detection of dutsateride may be possible by LC/MS in plasma samples if available [7] or during a follow-up process.

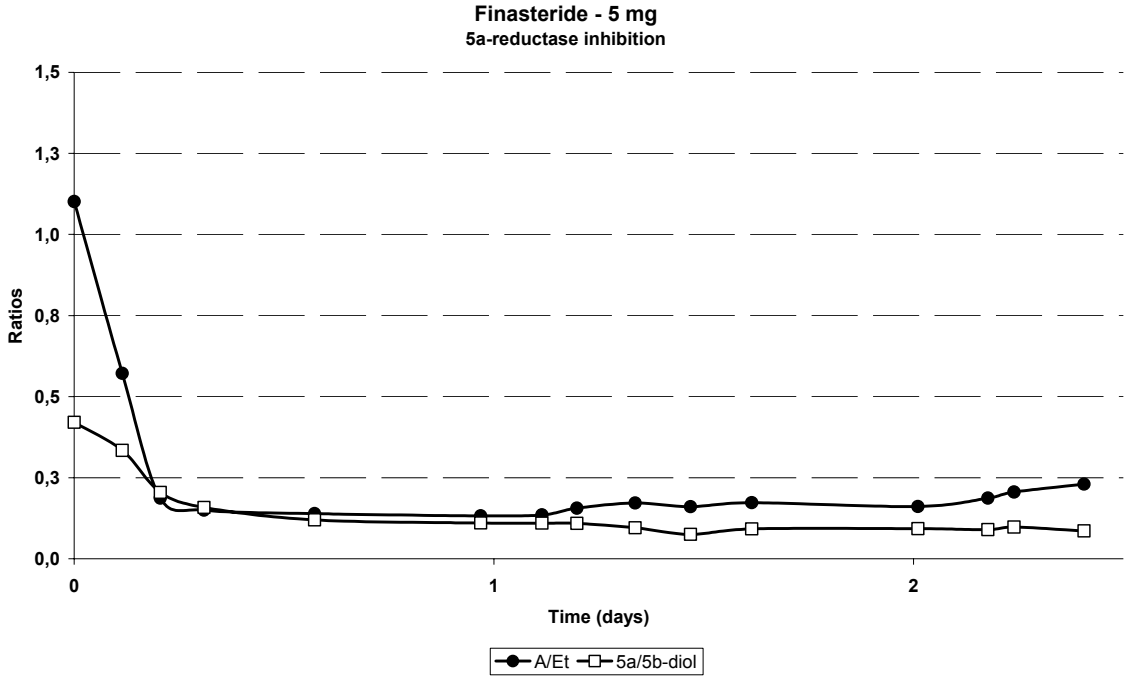
Conclusions

Important changes on the urinary steroid profile are observed after the administration of 5α-reductase inhibitors finasteride or dutasteride. Their intake is easily detected through the determination of A/Et or 5a-/5b-diol ratios. A/T is also reduced to a great extend.
At single doses of the substances studied, no changes of the T/E ratio are observed nor for repeated doses of finasteride.
The detection of finasteride metabolite ω-carboxyfinasteride is possible by LC/MS and has been incorporated in our routine screening procedures along with corticosteroids. Due to its pharmacokinetic properties, neither dutasteride nor the metabolites are detected in urine and other biological samples are needed (feces or plasma).

Acknowledgements
We acknowledge Dr. Douwe de Boer, former Scientific Director of the LAD of Lisbon that started the work on 5α-reductase inhibitors and for his enthusiasm on doping analysis work.

This work is dedicated to Dr. Carmo Manzoni that passed away days before the start of the 23rd Cologne Workshop. The memories that Carmo left among us are unforgettable and present in our daily life and work.

Figure 2. Effect of finasteride (5mg p.o.) on some parameters of the steroid profile



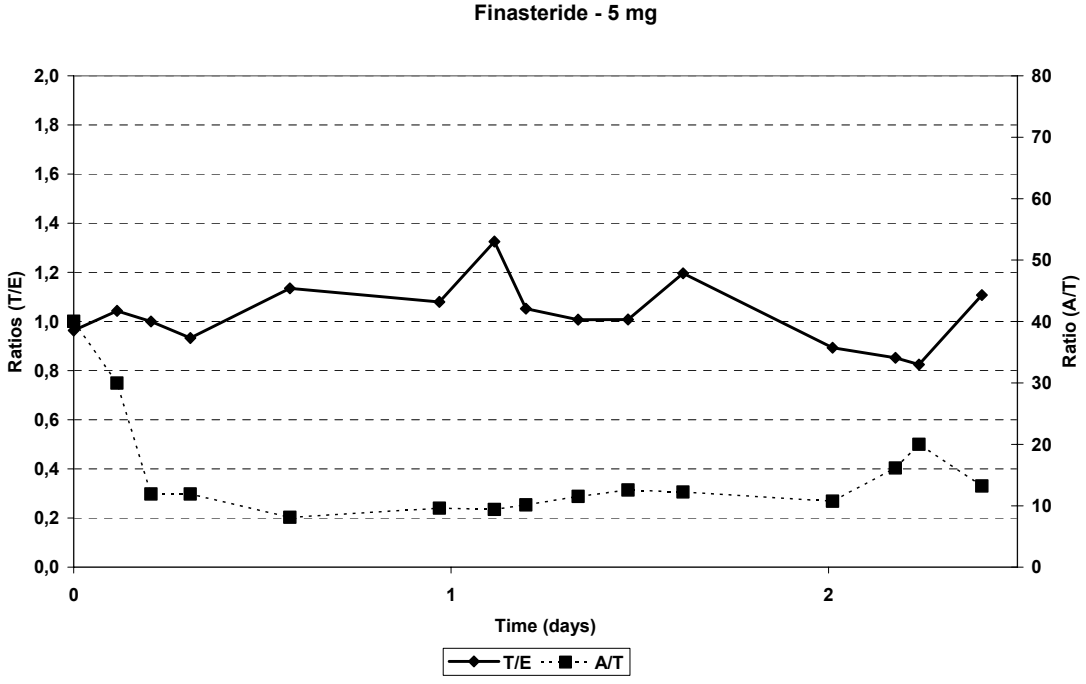
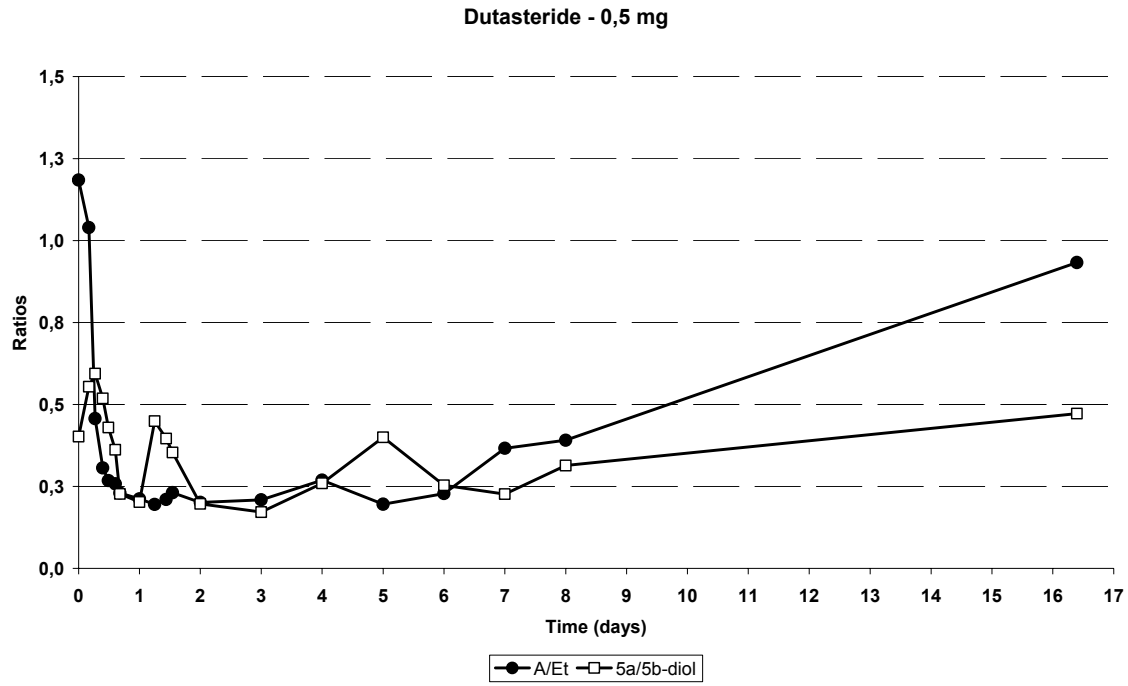


Figure 3 : Effect of dutasteride (0.5mg p.o.) on some parameters of the steroid profile



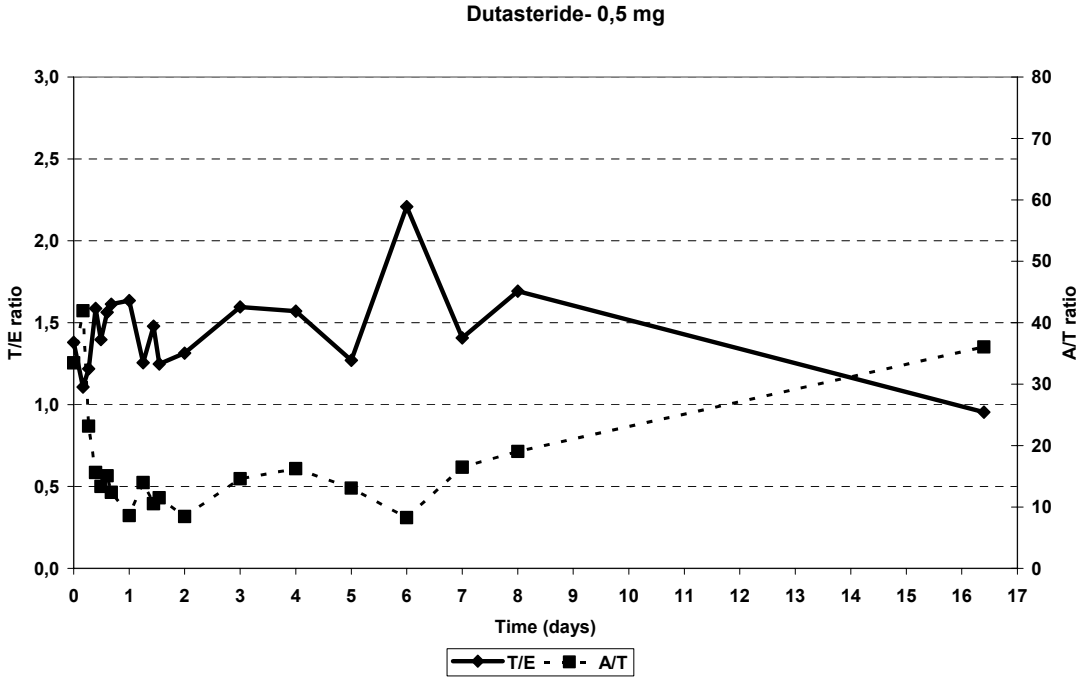


Figure 4. ESI mass spectra of ω-carboxyfinasteride (upper), dutasteride (lower) and proposed fragmentation patterns.

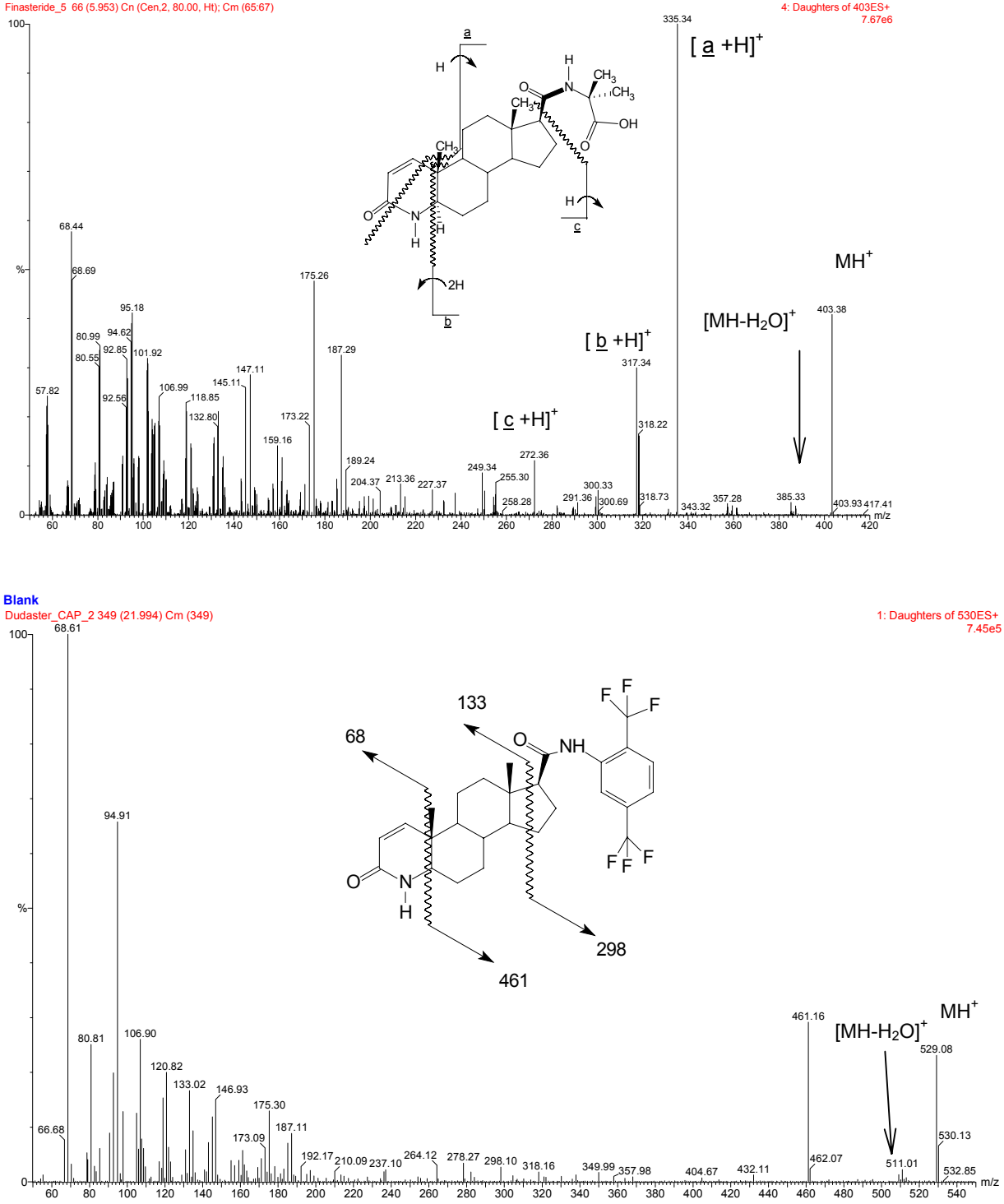


Figure 5. Excretion of ω-carboxyfinasteride in urine determined by LC/MS

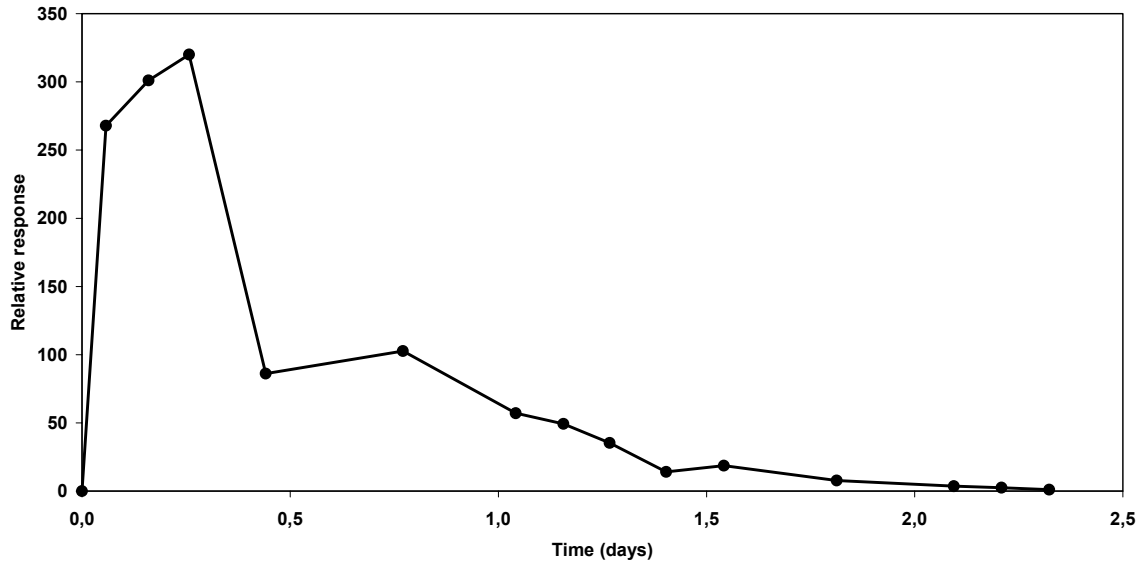


Figure 6. Examples of the chromatographic profile of ω-carboxyfinasteride detection in urine by LC/MS.

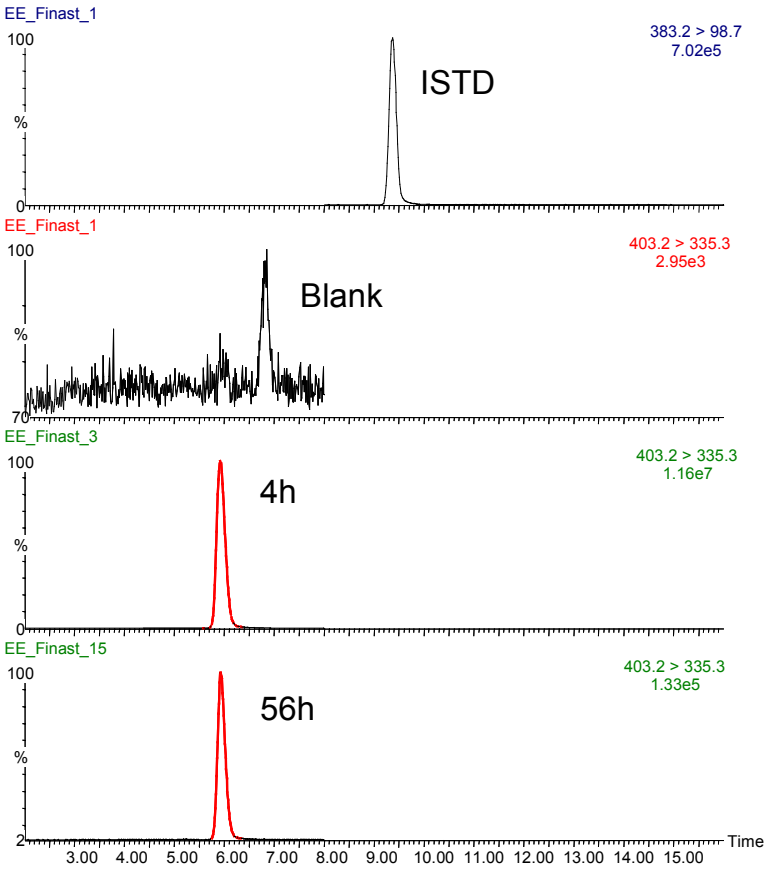


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Figure 1 Scheme of the sample preparation procedure and some analytical parameters

Sample preparation
(screening method- IIIb)

3 mL of urine + IS (methyltestosterone)
↓
Hydrolysis:
sodium acetate buffer pH 5.2 and β-glucuronidase/ arylsulfatase
55°C, 2h
↓
Condition:
Oasis® HLB
methanol and water
↓
Load samples
↓
Wash:
sodium hydroxide 0.02M: methanol (6:4) and water
↓
Dry
↓
Elute:
tert-butylmethyl ether: methanol (9:1)
↓
Evaporate under nitrogen at 45°C.
↓
Reconstitute in mobile phase
↓
Analyse by LC/MS/MS

Sample preparation
(confirmation method)

3 mL of urine + IS (mefruside)
↓
Adjust to pH 2-3 with hydrochloric acid 1N
↓
Extract with methylene chloride
↓
Shake for 20 min and centrifuge
↓
Separate the organic phase and dry
↓
Reconstitute in mobile phase
↓
Analyse by LC/MS/MS

Analytical parameters

Gradient program

solvent A	ACN/HCOOH 0.1% (95:5)
solvent B	HCOOH 0.1%/ACN (95:5)
flow rate	0.3 mL
injection volume	10 µL

Mass spectrometric parameters

acquisition mode		MRM
function 1	time	2-8 min
	(403.2→175.3) cone 50V coll 35eV	
	(403.2→187.3) cone 50V coll 35eV	
	(403.2→335.3) cone 50V coll 35eV	
function 2	time	8-16 min
	(383.2→98.7) cone 20V coll 14eV	
	(383.2→129.1) cone 20V coll 14eV	
…	(383.2→285.1) cone 20V coll 14	