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How safe are our internal procedures? Some preliminary experimental evidence on the problem of "lab-oriented" potential masking agents

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INTRODUCTION

A masking agent is generally defined as a substance capable of altering the pharmacokinetic profile of a drug/class of drugs. Some of the main effects of the use of masking agents are therefore aimed to obtain a reduced concentration of a forbidden drug/metabolite in urine, caused by either a delayed excretion (and/or a reabsorption) or an altered metabolism. So far, no information is available on the potential use by the athletes of substances capable of interfering not (only) with biometabolic processes "in vivo", but (also) with the analytical procedures carried out in the anti-doping laboratories. This problem is becoming of increasing interest since a noteworthy amount of information concerning the internal procedures of the WADA accredited laboratories is to be included in the Laboratory Documentation Package [1], i.e. the document transmitted to the testing authority, upon request, following the report of an Adverse Analytical Finding.

In a previous study, we have showed that the oral administration of propyphenazone can affect the urinary concentration of some natural steroids (see Figure 1) while it does not affect the testosterone and epistestosterone urinary profile [2]. In this work we have tried to clarify the mechanism leading to the suppression of the measured urinary concentration of those androgenic steroids, observed following oral administration of propyphenazone; in particular, we have studied the possible effect of propyphenazone and its main metabolites on: i) the biotransformation and excretion of steroids; ii) the steroid sample preparation steps (hydrolysis, liquid/liquid execration and derivatization).

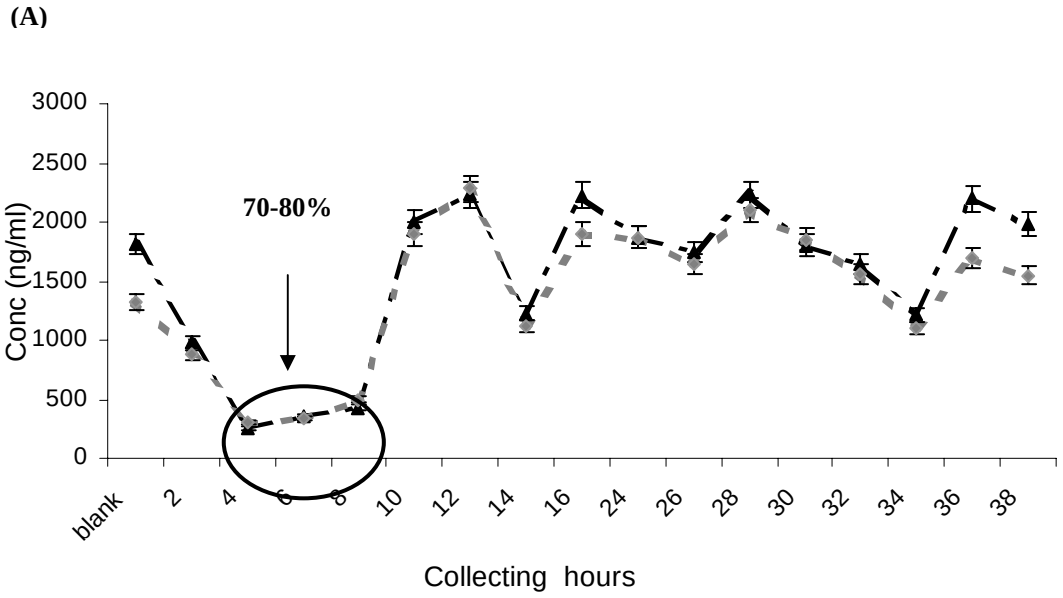

Figure 1A. Circadian variability of the urinary concentration of testosterone metabolites (▲=androsterone; ◆= etiocholanolone) after propyphenazone administration.

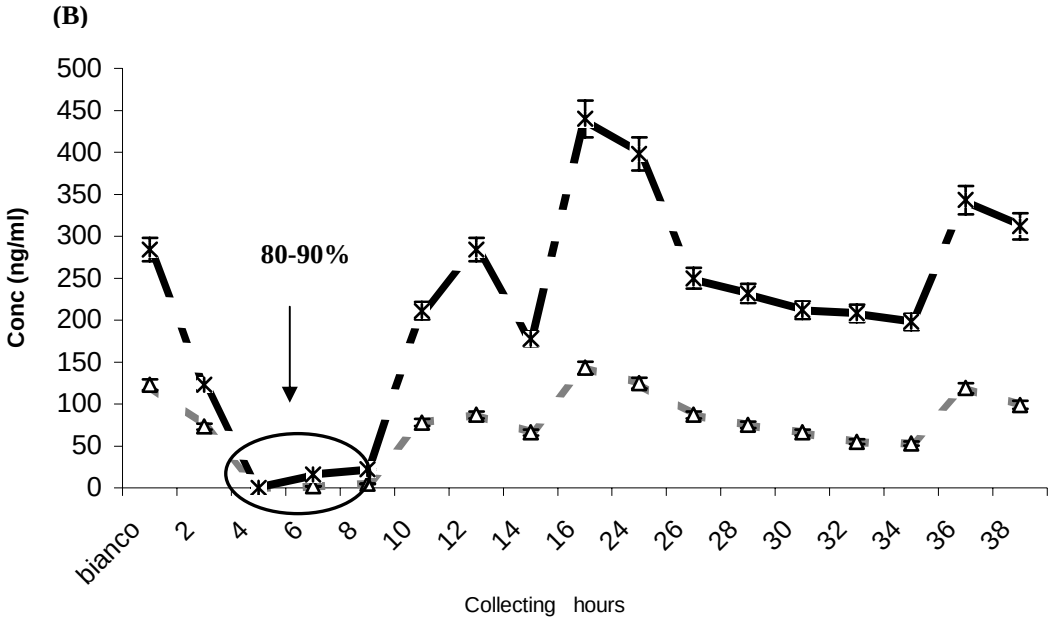

Figure 1B. Circadian variability of the urinary concentration of testosterone metabolites (×=11β-OH-androsterone; △= 11β-OH-etiocholanolone) after propyphenazone administration.

EXPERIMENTAL SECTION

Propyphenazone excretion study

The experiments have been carried out on patients (two males and two females, age 35±5 years), engaged in normal physical activity, undergoing treatment with propyphenazone. Circadian variability of the endogenous androgens profile was assessed for 24 hours after treatment with a single dose of 400 mg (each tablet) of propyphenazone by collecting urine samples every two hours. The following endogenous hormones (glucuronate+free fraction) were considered: testosterone, epitestosterone, androsterone, etiocholanolone, 11β-OH-androsterone, 11β-OH-etiocholanolone. The samples were stored on ice packs after collection to prevent changes in the concentrations of steroids caused by bacterial contamination or thermal degradation [3-6]. All concentration values were adjusted to a specific gravity of 1.020 g/L; samples with pH>7 were not considered for the study.

GC/MS parameters

All gas-chromatographic-mass spectrometric (GC-MS) systems, with auto sampler, were from Agilent Technologies (GC 5890/MS 5973A), equipped with HP1 columns (fused silica capillary cross linked methylsilicone 17 m × 0.2 mm id, 0.11 μm film thickness). The thermal ramp was as follows: 180 °C for 4.5 min, 3 °C/min to 230 °C, 20 °C to 290 °C, held for 2 min, 30 °C/min to 320 °C, held for 0.80 min; the temperature of the injection port and the detector was set at 280 °C and 300 °C respectively; injection was carried out in split mode (1:10), the mass selective detector was operated in the electron impact (EI) mode at 70 eV. Acquisition was carried out in selected ion monitoring (SIM)

LC/MS parameters

All LC/MS-MS experiments were performed using an Agilent 1100 Series liquid chromatograph equipped with a Supelco Discovery C18 column (2.1 × 150 mm), interfaced by an ESI to an Applied Biosystems API4000 triple quadrupole mass spectrometer. Chromatography was accomplished using 0.1 % acetic acid (eluent A) and acetonitrile with 0.1 % acetic acid (eluent B). A gradient was employed starting at 15 % B and increasing to 60 % B in 7 min and then, after 6 min, to 100 % B in 1 min. The column was flushed for 1 min at 100 % B and finally re-equilibrated at 15 % B for four minutes. The flow rate was set at 0.25 mL/min. The ion source was operated in the positive mode at 550 °C, and multiple reaction monitoring (MRM) experiments were performed employing collision-induced dissociation

(CID) using nitrogen as collision gas at 5.8 E^{-3} Pa, obtained from a dedicated nitrogen generator system (Parker-Balston model 75-A74). The collision energy was set at 30 eV for all experiments, while MRM was used as acquisition mode.

Sample preparation

To 3 mL of urine 50 µL of internal standard (200 ng/mL of 17α-methyltestosterone), 1 mL of phosphate buffer (pH 7.4) and 30 µL of beta-glucuronidase from E. coli were added and hydrolysis was performed for 1 h at 50 °C. After hydrolysis the buffered solution was alkalinised with 1 mL of carbonate buffer (pH 9) and the steroids were extracted with 10 mL of tert-butylmethyl ether on a mechanical shaker for 5 minutes. After centrifugation, the ethereal layer was transferred and evaporated to dryness under vacuum; the residue i) was derivatized by 50 µL of MSTFA:NH_4I:Dithioerythrytol (1000:2:4 v/w/w) and 1 µL of the derivatized extract was injected directly into the injection port for the GC/MS screening procedure, ii) was reconstituted in 50 µL of tert-butylmethyl ether and 1 µL was injected directly into the injection port for the GC/MS hydrolysis inhibition study, iii) was reconstituted in 50 µL of mobile phase and 15 µL were injected for LC-MS/MS experiments.

RESULTS

Evaluation of the effect of propyphenazone on testosterone and epitestosterone metabolism

The potential effect of propyphenazone administration on the metabolism of testosterone and epitestosterone was studied on two males and two females volunteers.

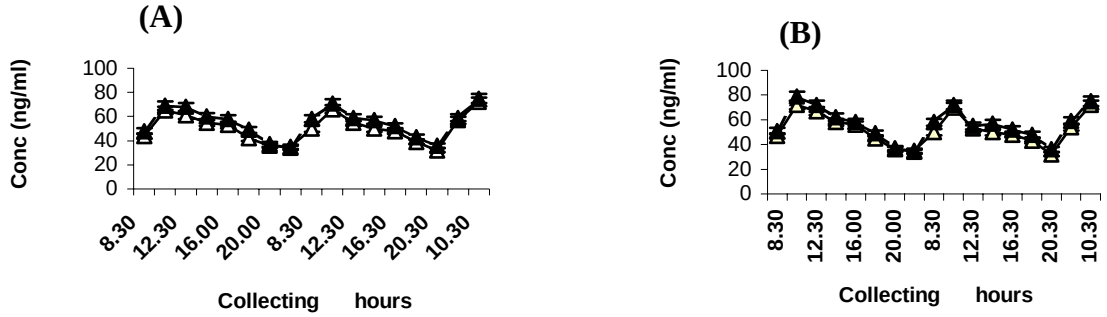

Figure 2. Circadian variability of the urinary concentration of androgens (▲=epitestosterone △=testosterone) before **(A)** and after **(B)** propyphenazone administration.

Figure 2 shows the mean excretion values of the males volunteers testosterone and

epitestosterone. The circadian variability of the urinary concentration of testosterone indicated that, before and after propyphenazone administration, no significant variation is recorded. These data is in agreement with the conclusion of a previous study focused on the influence of propyphenazone on the urinary profiling of testosterone and epitestosterone [7].

Evaluation of the effect of propyphenazone on the urinary excretion of testosterone main metabolites

The effect of propyphenazone administration was also evaluated with respect to the urinary excretion of the main testosterone metabolites (androsterone, etiocholanolone, 11β-OH-androsterone and 11β-OH-etiocholanolone). Figure 3 shows the mean excretion values of androsterone glucuronide, etiocholanolone glucuronide 11β-OH-androsterone glucuronide and 11β-OH-etiocholanolone glucuronide. The endogenous glucuronide urinary concentration profile indicated that, before and after propyphenazone administration, there is not any significant variation on both male and female volunteers.

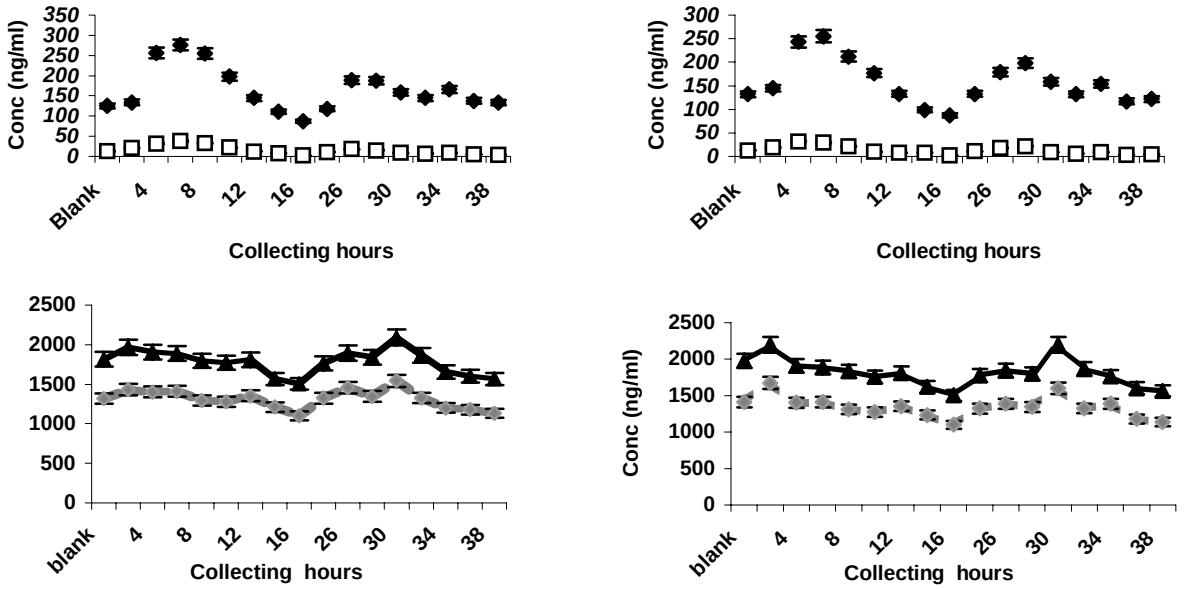

Figure 3. Circadian variability of the urinary concentration of androgen glucuronides (above: ♦=11β-OH-androsterone; □=11β-OH-etiocholanolone; below: ▲=androsterone; ●=-etiocholanolone) before (left) and after (right) propyphenazone administration.

Evaluation of the effect of propyphenazone administration on the pre-treatment procedure

Hydrolysis

The samples were prepared following the screening procedure for anabolic steroids, but without the derivatization step. Figure 4 shows the hydrolysis recovery of androsterone, etiocholanolone, 11β-OH-androsterone and 11β-OH-etiocholanolone. Data show that, before and after propyphenazone administration, there is not any significant variation in the amount of the steroids monitored.

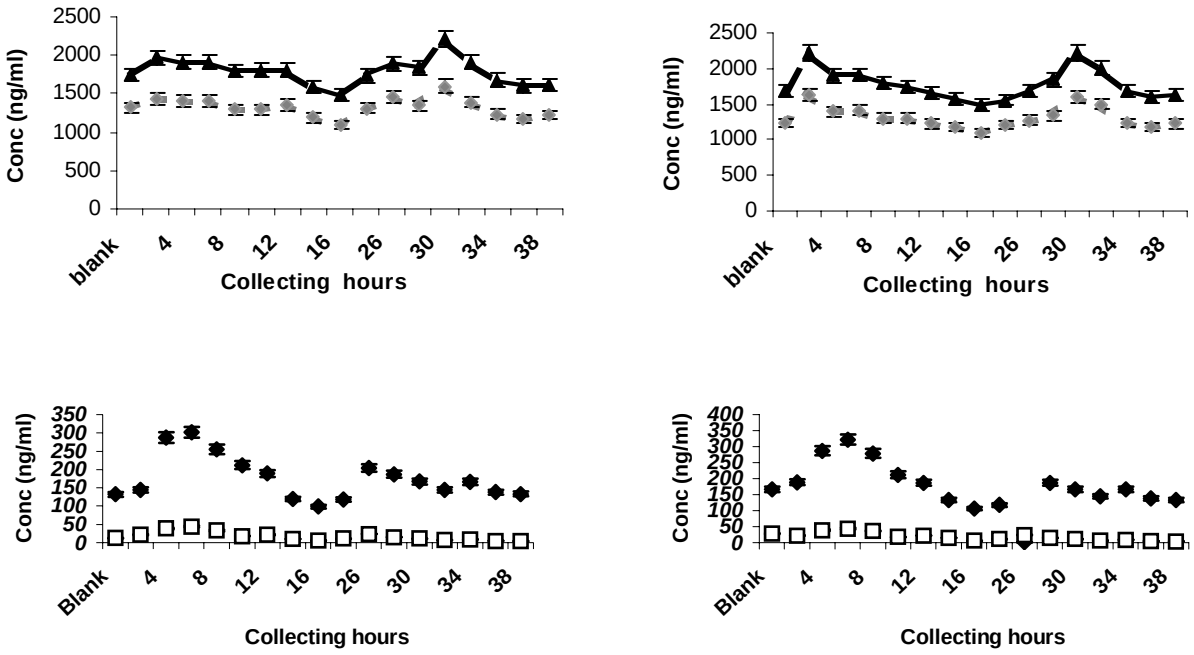

Figure 4. Circadian variability of the urinary concentration of androgens (above: ▲=androsterone; ●=-etiocholanolone; below: ♦=11β-OH-androsterone; □=11β-OH-etiocholanolone) before (left) and after (right) propyphenazone administration.

Derivatization

The effect of propyphenazone administration was finally evaluated also on the derivatization step, using the complete procedure of sample pre-treatment for the steroid screening analysis. Figure 5 and Figure 6 show androsterone, etiocholanolone, 11β-OH-androsterone and 11β-OH-etiocholanolone profile after propyphenazone administration. The

endogenous profile indicated that, before and after propyphenazone administration there is a significant variation on the endogenous urinary concentration of testosterone metabolites, if assayed by GC/MS of the corresponding TMS-derivatives.

Propyphenazone excretion study

The excretion study indicates that the urinary concentration of propyphenazone reaches the maximum during the first hour, to quickly decrease afterwards; while its metabolites increase in the first two hours and reach a maximum in 2-8 hours, to slowly decrease until their minimum.

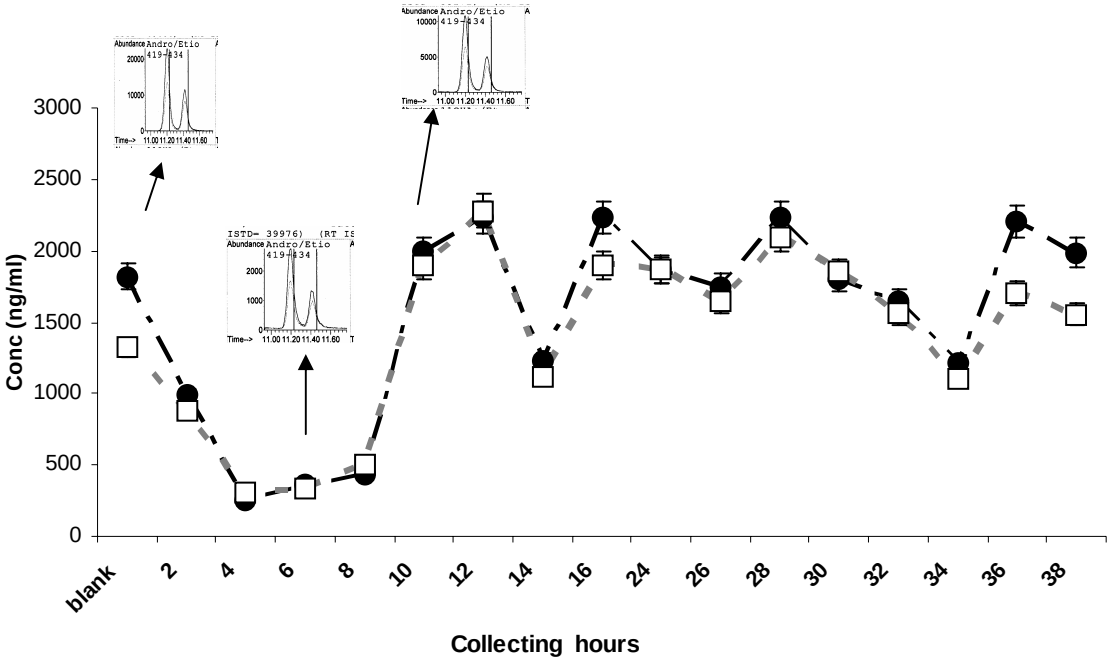

Figure 5. Circadian variability of the urinary concentration of androsterone and etiocholanolone after propyphenazone administration (☐=androsterone; ●= etiocholanolone).

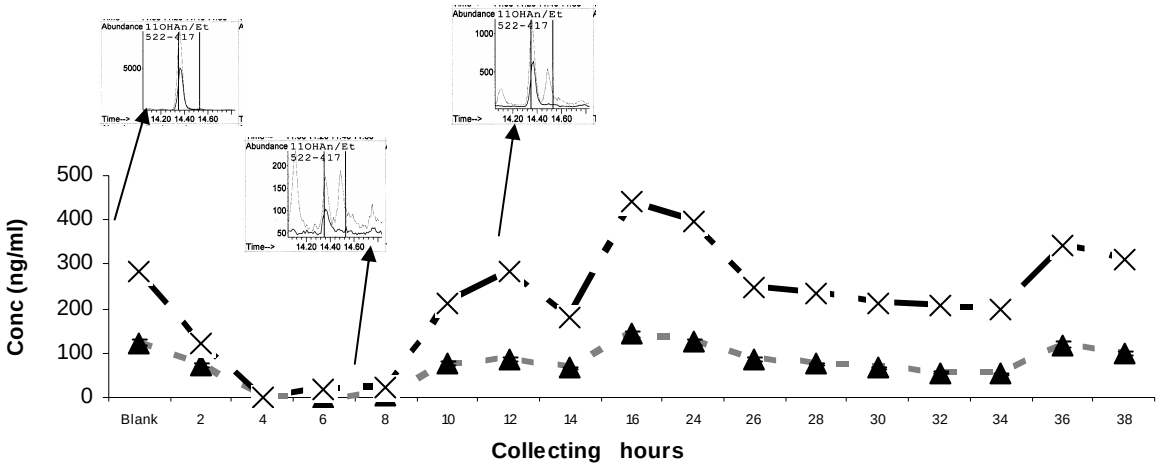

Figure 6. Circadian variability of the urinary concentration of 11beta-hydroxy-androsterone and 11beta-hydroxy-etiocholanolone after propyphenazone administration (× =11β-OH-androsterone; ▲= 11β-OH-etiocholanolone).

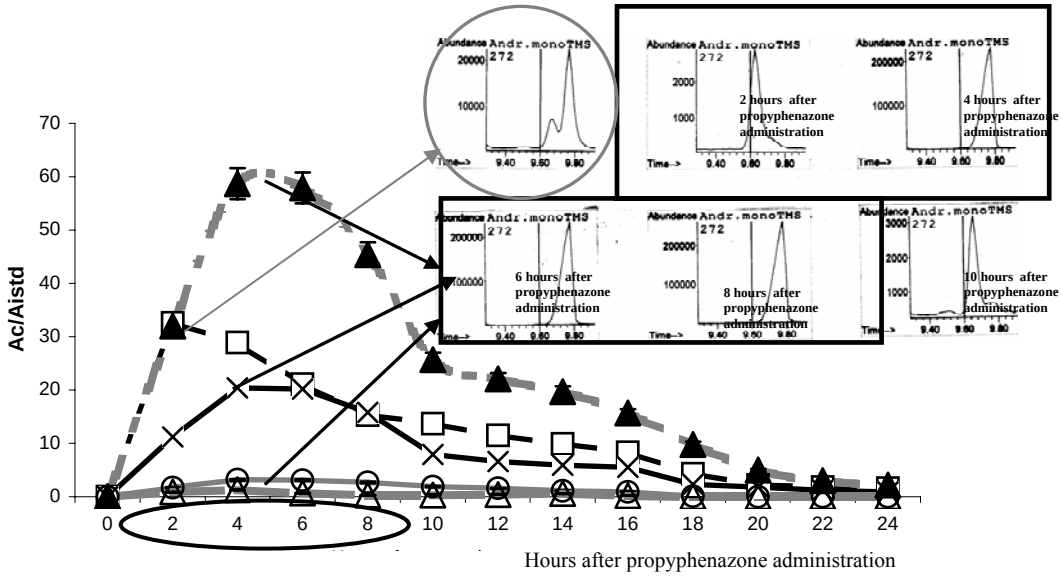

Figure 7. Propyphenazone and its metabolites urinary profile (☐ propyphenazone; ▲ methylhydroxypropyphenazone; x propylhydroxypropyphenazone △ nor-propyphenazone; ○ dihydroxypropyphenazone) and their effect on derivatization step.

Figure 7 shows that the maximum value of the urinary concentration of hydroxypropyphenazone metabolites overlaps, in all subjects studied, with the observed

suppression of 11β-OH-androsterone, 11β-OH-etiocholanolone, androsterone, etiocholanolone and with the maximum of mono-TMS-androsterone.

DISCUSSION

The results obtained show that: i) the observed effects are due to the presence of the main propyphenazone metabolites, namely hydroxypropyphenazone; ii) the effect is due to a reduced yield of the derivatization step; iii) the effect seems to be specific of this drug, since the cross examination of the urinary steroid profile of our routine samples (in the period September 2006 – January 2007) confirmed that no suppression of the steroid urinary concentration was observed in the presence of other non-steroidal anti-inflammatory drugs (i.e. aspirin, diclofenac, ibuprophen, paracetamol).

Our finding suggest to monitor the presence of propyphenazone metabolites in the steroid screening procedure (also indirectly, by monitor the ratio mono- to bis-TMS androsterone or etiocholanolone), and, in a broader context, to impose the lab to carefully check, in all batches, for the effectiveness of all stages of urine sample pretreatment. The evidence that some substances are capable of interfering with the procedures followed by the labs for the pre-treatment of samples forces us to reconsider the definition of "masking agent". For indeed, compounds capable of interfering with the normal lab routine procedures can in principle be selected (if not even designed) by studying the lab analytical procedures as they are described in the Laboratory Documentation Packages; in some cases, a double check, carried out by a parallel analytical procedure (eg LC/MS-MS to complement GC/MS data) should be carried out in the case of doubtful or abnormal findings.

Although reduced on an absolute scale, the effects may become significant in the case of the combined use of multiple non-forbidden compounds, which can produce synergistic effects (eg propyphenazone + ascorbic acid, an inhibitor of beta-glucuronidase [8], an apparently mild and common therapy for an athlete, can bring the concentration of 19-NA below the threshold). The short-medium-long term implication are terrible (think of the proposal of routinely measuring the concentration of some key endogenous steroids, in view of the future implementation of the "athlete passport"…).

ACKNOWLEDGEMENT

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