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Studies with steroids and fecal contamination of urine samples

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Introduction

It has been earlier shown by our group that bacterial Δ^1-dehydrogenase activity which can convert testosterone and androst-4-en-3,17-dione into androsta-1,4-dien-3,17-dione (a precursor of boldenone) is a common feature of human digestive tract. However, under authentic colon conditions, characterized by total anaerobiosis, these reactions do not occur, but testosterone or androst-4-en-3,17-dione are converted into their reduced metabolites instead [1].

The aim of this study was to investigate if fecal contamination of urine sample could also cause similar conversion of steroid structures. In the study, artificial urine samples spiked with testosterone, boldenone and methyltestosterone were contaminated with feces and incubated in different conditions.

Experimental

Incubation experiments

The study was carried out completely in artificial phosphate buffer, because the urine may contain substances that inhibit or interfere with microbial growth and metabolism. The incubations were carried out following the principles presented more thoroughly by Apajalahti et al. [2]. Half of the simulation vessels were kept open to the atmospheric oxygen that increase their redox-potential (redox +50 mV) and provide oxidative conditions to the metabolic reactions. The other half was sealed under oxygen free nitrogen gas and supplemented with reductant (redox potential -300 mV), providing thereby highly reductive anaerobic conditions to the simulation vessels. All vessels (volume of 10 ml) were inoculated with 200 µl of 10 % pooled human fecal mass and thereafter supplemented by 100 µg of

testosterone, boldenone and methyltestosterone. All samples were incubated for 48 hours at 37°C temperature and thereafter analyzed for steroid metabolites.

Sample pretreatment for GC/MS analysis

Extraction of samples was modified from the procedure described by Geyer et al. [3]. A 2-ml aliquot of incubation mixture was mixed with 2 ml of aqueous potassium carbonate:potassium bicarbonate solution (1:1, 20 %, w/v) and extracted with 5 ml of diethyl ether. After centrifugation, the organic layer was separated and evaporated to dryness. Finally, the residue was derivatized with 50 µl of MSTFA/ammonium iodide/dithioerythritol (1000:2:4, v/w/w) for 15 min at 60 °C.

GC/MS analysis

GC/MS analysis was performed on an Agilent 6890 gas chromatograph and an Agilent 5973N mass selective detector. Compounds were separated on an Agilent HP-1 fused silica capillary column (16 m, 0.2 mm i.d., film thickness 0.11 µm). Injection of 2 µl was done in split mode (1:20) at 280 °C. Carrier gas was helium (1 ml/min, constant flow). The oven was ramped first from 180 to 230 °C at 3 °C/min and then up to 310 °C at 30 °C/min and held at the final temperature for 3 min. MS was operated in electron ionization mode (70 eV). In selected ion monitoring (SIM) mode, twenty different ions with dwell times of 10 ms were monitored for trimethylsilylated testosterone, boldenone, methyltestosterone and their expected oxidation and reduction products.

Results and Discussion

Conversions of steroids observed in the incubation experiments are presented in Figure 1. Urine sample which was contaminated by feces behaved similarly to the colon simulations [1]. Under oxidative conditions, i.e. microbes have not consumed all oxygen from the test vessel, testosterone was oxidized to androsta-1,4-dien-3,17-dione, the precursor of boldenone. Boldenone itself was also converted almost completely to its precursor. Under reductive conditions, i.e. microbes have consumed all oxygen from the test vessel, testosterone showed reductive metabolites (5β-androstan-17β-ol-3-one and 5β-androstan-3α,17β-diol) and boldenone was reduced to 5β-androst-1-en-17β-ol-3-one. This shows that fecal microbial contamination has the potential to produce boldenone precursors from endogenous

testosterone, or the contamination can remove/reduce boldenone or its metabolites from urine by either oxidative or reductive processes, which all may lead to false analytical findings.

The completely new observation was that microbial contaminants were able to metabolize methyltestosterone. Under aerobic conditions, methyltestosterone was converted to methandienone, and under anaerobic and reduced conditions methyltestosterone was metabolized to 17α-methyl-5β-androstan-3α,17β-diol. Thus, microbial contamination and internal standard also show potential to cause false analytical findings. As it is a standard procedure to add methyltestosterone as an internal standard to urine samples and thereafter incubate it at 50°C together with β-glucuronidase enzyme [3], the risk is considerable.

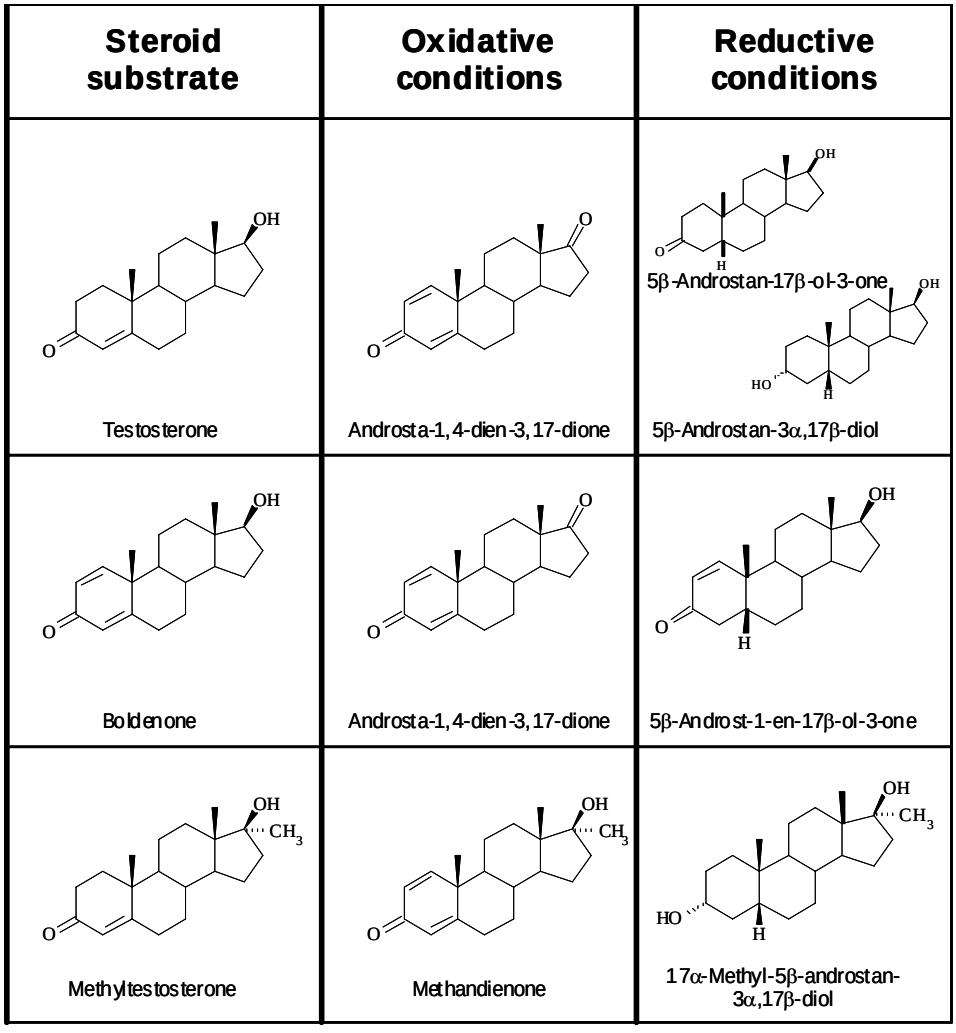

Figure1. Conversions of steroids observed in fecal contaminated artificial urine after incubations in conditions favoring either oxidative or reductive reactions.

The study describes only the maximum contamination potential and should be considered only as a theoretical possibility for highly contaminated authentic urine samples. The experiments were carried out in extreme incubation conditions with significant microbial challenge.

Conclusions

Fecal contamination of urine has a possibility to cause adverse reactions for endogenous and exogenous steroids for instance during analytical sample pretreatment process. Therefore, it is strongly suggested that direct enzymatic hydrolysis of urine sample should never be used in confirmation analysis without destroying or removing possible microbial contaminants first.

Acknowledgements

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References

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