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Experiments on production of "endogenous boldenone"

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Introduction

Microbes can affect doping analysis in many ways e.g. intestinal microbes in the gut can metabolize exogenous and endogenous substances. Microbes can also contaminate urine samples, resulting in complete or partial degradation of endogenous substances or drugs and their metabolites.

It has been earlier shown that in some rare cases bacterial activity in the gut is able to result in formation of "endogenous boldenone" and its metabolites in man [1]. This rare reaction is proposed to be initialized by seldom distributed microbes of human digestive tract converting testosterone (**T**) or androst-4-ene-3,17-dione (**AED**) to androsta-1,4-diene-3,17-dione (**ADD**), that can be absorbed through the gut epithelium and further metabolized by liver to boldenone (**B**) and its metabolites (Figure 1.) [1]. Experimental set-ups to confirm "endogenous boldenone" production by incubating a fecal sample from a boldenone positive athlete with relevant substrates have also been suggested [1,2]. In cases where a very low concentration of **B** is reported the World Anti-Doping Agency's (WADA) Prohibited list guides anti-doping laboratories to confirm exogenous/endogenous origin of **B** either by using isotope ratio mass spectrometry (IRMS) or by studying the longitudinal urinary **B** profile of the athlete of concern [3]. In spite of many proposed hypotheses, the mechanism how and in which circumstances "endogenous boldenone" is formed is not yet clear [4].

In this study we applied human large intestinal simulation model using **T** and **AED** as substrates to study the frequency of **B** producing trait among humans, and, the intestinal microbial fermentation conditions that favor formation of **ADD** – a **B** precursor.

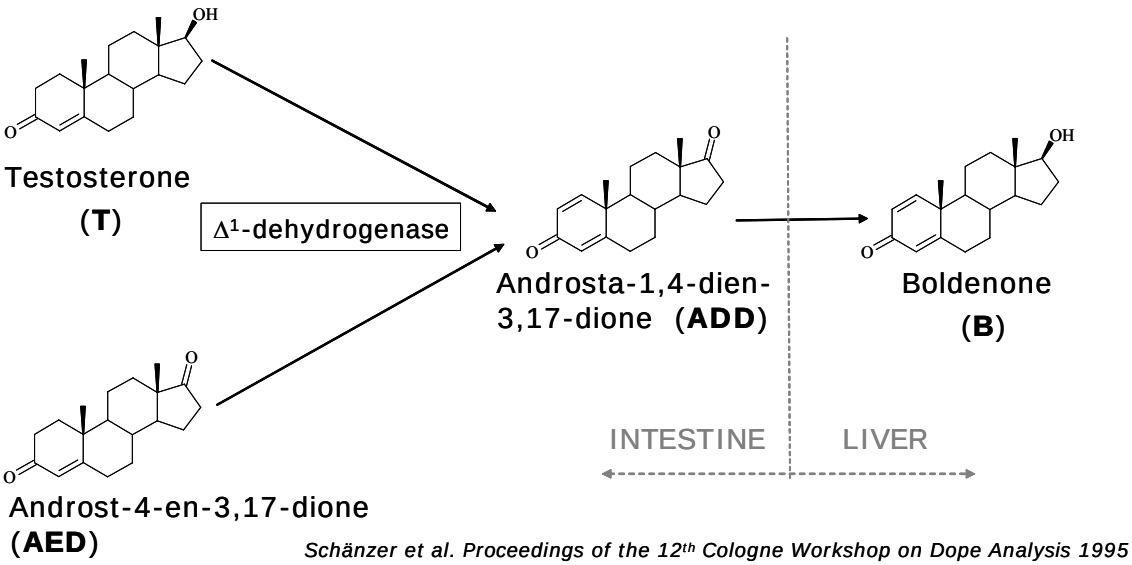

Figure 1. Hypothesis for the explanation of endogenous boldenone production [1].

Experimental

Fecal samples

Fecal samples were collected from over 1000 human subjects (both genders, healthy and diseased subjects, various age groups) from which 100 samples were randomly chosen to colon simulations and pooled to ten simulation groups. In addition, 20 samples were studied individually. All samples were stored frozen.

Steroid substrates

T and **AED** were used as substrates in incubation studies. Concentration of substrates in the incubation mixture was 10 µg/ml. Substrates were incubated either separately or together. In some experiments also **ADD** was used as a substrate. All steroids were purchased from Sigma Aldrich.

Colon simulation procedures

The colon simulations were carried out following the principles discussed more thoroughly by Apajalahti et al. [5]. Colon simulations were performed in 20 ml of specially formulated 30 mM phosphate buffer (pH 6.8) that mimics the chemical composition of colon medium. In simulations which mimic the authentic (normal) large intestinal conditions characterized by

total anaerobiosis (redox potential lower than -300 mV), 47.5 ml of reductant solution containing cysteine and sodium sulfide was added to the buffer. In simulations under extreme conditions, the authentic conditions were manipulated with oxygen addition that increase redox-potential (redox potential higher than -100 mV) to give more thermodynamic space for possible oxidative reactions. In all experiments 200 mg of fecal inoculate was used. Simulation vessels were incubated at 37 °C up to 48 hours. Samples for gas chromatographic/mass spectrometric (GC/MS) analysis were normally collected at 4, 20 and 48 hours after initiation of the simulation.

Sample pretreatment for GC/MS analysis

Extraction of samples was modified from the procedure described by Geyer et al. [6]. A 2-ml aliquot of incubation mixture was spiked with 1000 ng of methyltestosterone (internal standard). Then 2 ml of aqueous potassium carbonate:potassium bicarbonate solution (1:1, 20 %, w/v) was added and the sample was 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 (0.5 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) either in scan (m/z 50 – 700) or selected ion monitoring (SIM) mode. In SIM, 23 different ions with dwell times of 10 ms were monitored for the internal standard, **T** and **AED**, and their expected oxidation and reduction products.

Results and Discussion

In simulations under authentic colon conditions **T** and **AED** yielded only reduced metabolites and there was no indication of **ADD** or **B** or any other oxidized steroids. This means that under normal anaerobic and low redox- potential (less than -300 mV) colon conditions the microbes use steroids as electron acceptors and therefore produce their reduced forms. The

main reduced metabolites formed from **T** were 5β-androstan-17β-ol-3-one (**T+2H**) and 5β-androstan-3α,17β-diol (**T+4H**). Respectively, the main reduced metabolites formed from **AED** were 5β-androstan-3,17-dione (**AED+2H**) and 5β-androstan-3α,17-one (**AED+4H**). Interestingly, some of the sample pools as well as the individual not pooled samples showed remarkable differences in reaction rates, but not in overall metabolic profiles. In conclusion, all trials under authentic colon conditions produce only reduced steroid intermediates from **T** and **AED** but not any **ADD**.

In simulations where the authentic conditions were manipulated with oxygen addition that increased redox-potential to give more thermodynamic space for possible oxidative reactions, in all studied fecal samples **ADD** was formed in large quantities in addition to the above mentioned reduced metabolites at 48 hours time point. It was observed that increased exposure to air enhanced the **ADD** production, and that under reductive conditions **ADD** was rapidly reduced back to initial substrates. The data indicate that the observed Δ^1-dehydrogenase activity is reversible and can be modulated by changing the simulation conditions, but under "authentic" conditions reductive metabolism dominates. An example of the GC/MS data obtained from a simulation favoring formation of **ADD** is presented in Figure 2.

Conclusion

Bacterial Δ^1-dehydrogenase activity which can convert **T** or **AED** into **ADD** is a common feature of all human fecal samples but under authentic colon conditions the formation of **ADD** or **B** is not obvious. Conditions which can "activate" formation of **ADD** in man are still unclear. Because the bacterial Δ^1-dehydrogenase activity exists in the intestinal tract of every human being, verification of the "endogenous boldenone" production in doping control either by using fecal incubation or by longitudinal urinary boldenone profile is questionable and should not be used. Thus carbon isotope ratio mass spectrometry (IRMS) is the only reliable method to verify exogenous and endogenous boldenone.

Furthermore, fecal contamination of urine samples and their storage at room temperature are prone to yield boldenone and its metabolites under aerobic conditions, and – because the enzymatic reaction is reversible in nature – if anaerobic conditions prevail in a sealed sampling vessel, the disappearance of xenobiotic boldenone and its metabolites is also potential.

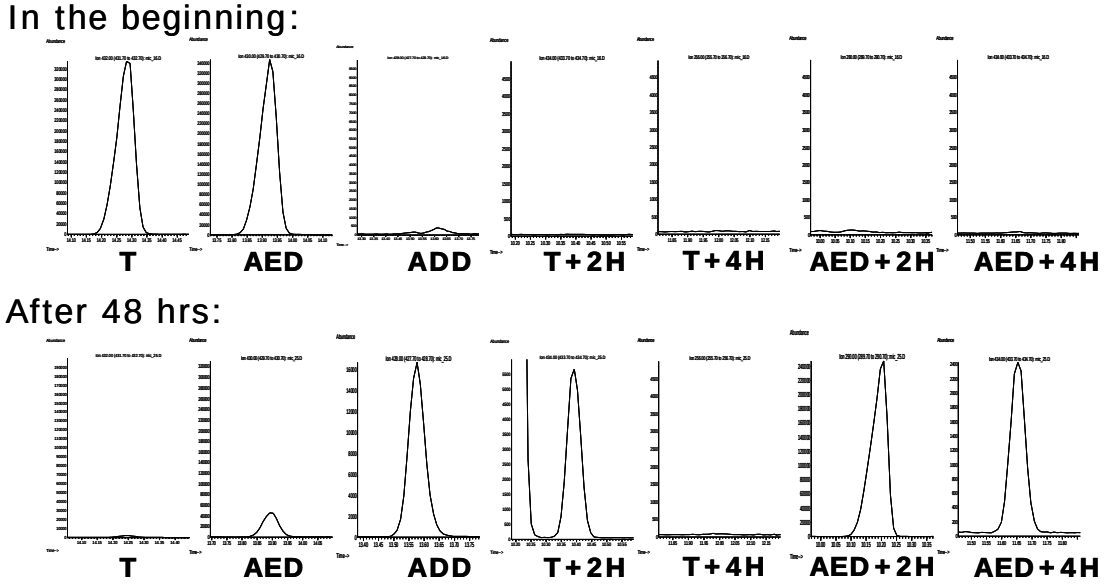

Figure 2. Selected ion chromatograms obtained from GC/MS-SIM analysis of a fecal sample incubated with **T** and **AED** in abnormal colon conditions (aerobic conditions with increased redox-potential).

Acknowledgements

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