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Confirmation of Endogenous Boldenone Production: A Procedure for an *in vitro* Experiment

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Introduction

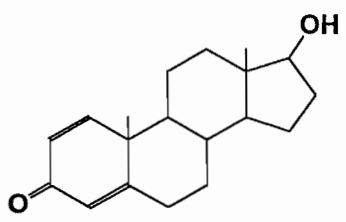
In 1994 a case of endogenous boldenone production has been reported[1]. In this study a hypothesis was presented that boldenone production resulted from bacterial activity in the gut, which converted testosterone or androst-4-ene-3,17-dione to androsta-1,4-diene-3,17-dione, refered to as metabolite VIII. Transport of this metabolite to the liver via the enterohepatic circulation, could result in metabolic conversion to boldenone and metabolites[2].

A possible similar case was suspected for a boldenone positive sample in our laboratory. In several samples taken in time, boldenone and its main metabolite were detected in very low concentrations. The athlete strongly denied boldenone abuse. It was decided to try to collect data on possible endogenous boldenone production with the cooperation of the athlete.

This experiment is the first step in the confirmation procedure followed. An experimental set-up is presented for an *in vitro* experiment, in which faeces of the athlete were incubated in non-specific media with testosterone or androst-4-ene-3,17-dione as metabolic precursors. The test was whether the possible Δ^1-dehydrogenase activity could be used to produce metabolite VIII *in vitro*. As reported by Yang *et al*[3], phenazine methosulfate (PMS) was added as accelerator of Δ^1-dehydrogenase activity. The possible acceleration is based on its capacity as electron acceptor.

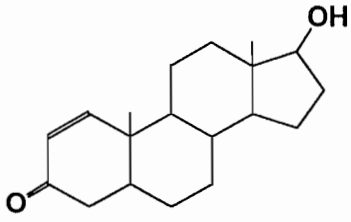
Experimental

A timed urine and faeces sample were collected from the subject and a control subject and stored at -20° C until analysis was performed. The urine sample was analyzed for boldenone and its main metabolite II. The faeces sample was divided and incubated in several media after adding testosterone or androst-4-ene-3,17-dione as precursor.



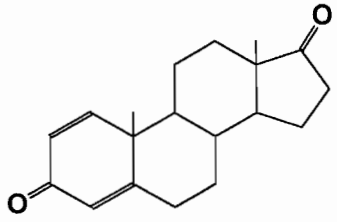
androsta-1,4-dien-17β-ol-3-one

boldenone



5β-androst-1-en-17β-ol-3-one

metabolite II



androsta-1,4,diene-3,17-dione

metabolite VIII

Urine sample analysis

Sample (10 mL) clean-up was performed applying C_{18}-solid phase extraction, selective enzymatic β-glucuronidase hydrolysis and alkaline liquid-liquid extraction with diethyl ether. Further sample clean-up was performed by HPLC fractionation on a C_{18}-column. Prior to analysis derivatization to TMS-enolTMS derivatives by MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v) was performed, followed GC/MS analysis by split (1:10) injection in a HP5890 gas chromatograph equiped with a HP Ultra-1 column (18 m, i.d. 0.20 mm, film thickness 0.11 μm) and a HP 5972 MSD. Methyltestosterone was used as internal standard.

Incubation of faeces samples

Small fractions of the faeces sample were mixed with 30 ml of medium. The following media were used (see appendix 1):

- Cysteine-Pepton-Bouillon
- Cysteine-Pepton-Bouillon with PMS (0.005 mM)
- Brain-Heart XV
- Brain-Heart XV with PMS (0.005 mM)

Testosterone or androst-4-ene-3,17-dione (200 mg) were added as precursor. Incubation was performed at 35° C during 33 days. An overview of the experimental set-up is shown in figure 1.

Incubation media sample analysis

Samples of 4 mL were taken from the incubation mixture at days indicated in figure 1 and centrifuged (10 min, 3000 rpm). The supernatant was sterilized by filtration through a millipore

filter (0.45 µm). Sample clean-up was performed by applying C_{18}-solid phase extraction, selective enzymatic β-glucuronidase hydrolysis and alkaline liquid-liquid extraction with diethyl ether. Prior to analysis derivatization to TMS-enolTMS derivatives by MSTFA/NH_4I/ethanethiol (1000:2:3; v/w/v) was performed, followed by GC/MS analysis. A control sample (faeces incubation at 0 days) and a faeces sample from a control subject were included in the experiment.

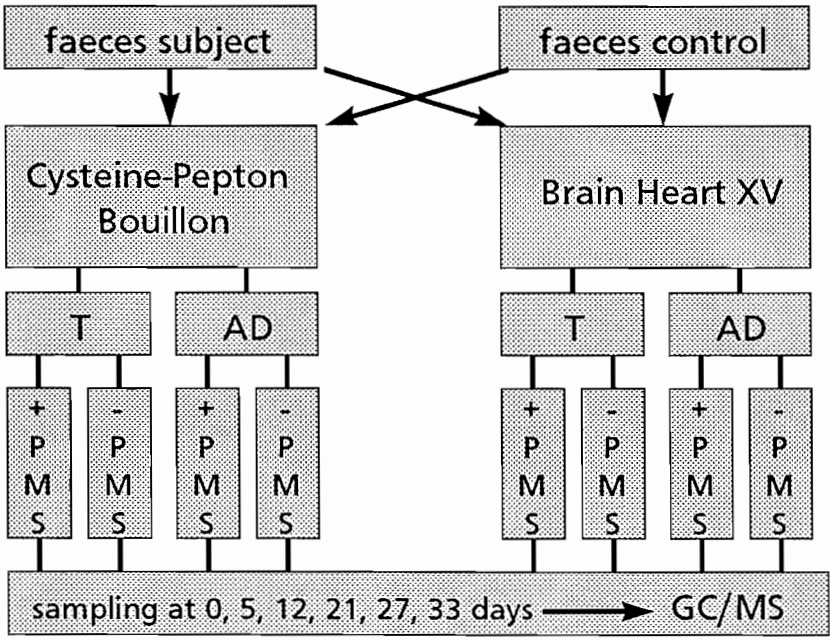


Figure 1: Overview of the incubation experiment. Two different media were used; testosterone (T) or androst-4-ene-3,17-dione (AD) were added as precursor. Phenazine methosulfate (PMS) was used as a proton acceptor. Sampling was timed at indicated days.

Results

Urine analysis for boldenone confirmation

Minor traces of boldenone and its main metabolite (II) were detected. Concentration was too low to be confirmed according the IOC criteria with Low Resolution MS under given conditions (see figure 1a and 1b). This was in agreement with earlier routine analyses regarding this subject, as mentioned before.

Incubation mixture analysis

The Brain Heart XV incubation mixtures were all negative for boldenone or metabolites on all days. Metabolite VIII was detected in low concentrations in the Cysteine-Pepton-Bouillon

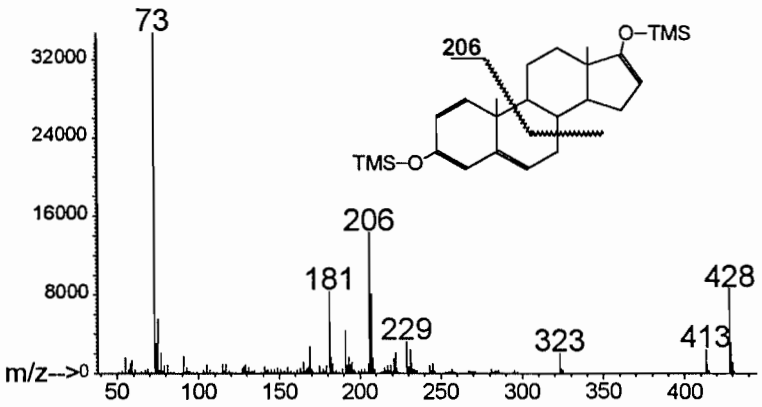


Figure 2: Reference spectrum of metabolite VIII

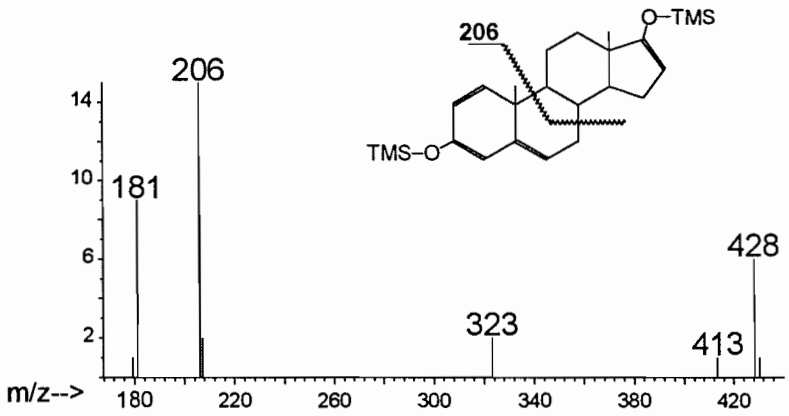


Figure 3: Spectrum of metabolite VIII as detected (SIM)

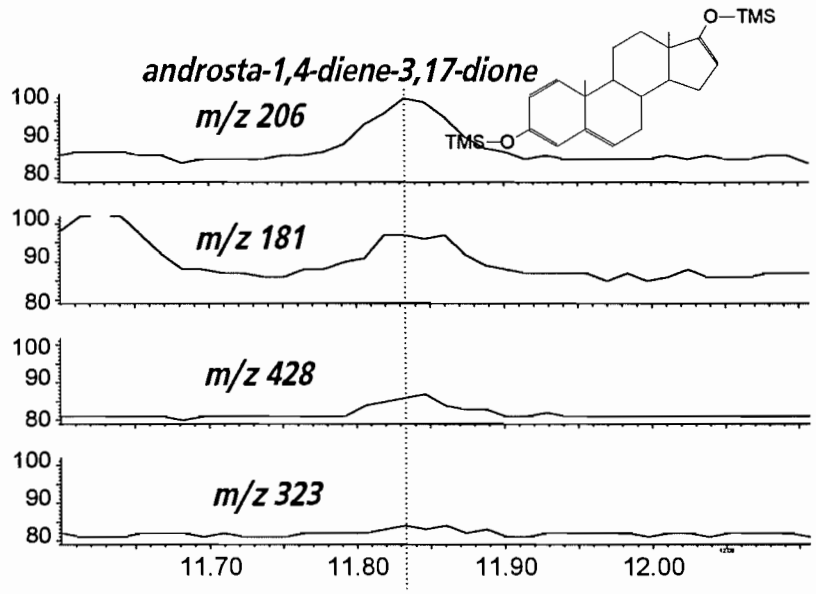


Figure 4: Extracted ion chromatogram of metabolite VIII as detected after 33 days

after 33 days when no PMS was added and testosterone was used as precursor (see figures 2-4). All control and blank samples were negative for the same substances.

Discussion

Schänzer *et al*[1] suggested that the endogenous boldenone production could be explained as is shown in figure 5. According to this idea testosterone glucuronide as a metabolite of testosterone is secreted in bile and transported to the gut. In the intestine around 400, mainly obligate anaerobe, species of microorganisms are present with a total weight of around 1 kg. Several enzymatic activities within the gut have been identified, due to the present microflora[4,5,6]. Some of the activities which can alter steroid structures are glucuronidase, sulfatase, (de)hydrogenase and aromatase activities. However most are reductive pathways, also *in vitro* Δ^1-dehydrogenase activity on 3-oxo-5β-steroids has been reported[7]. Therefore, due to microorganisms testosterone glucuronide can be hydrolyzed to testosterone and could be converted to androsta-1,4,diene-3,17-dione (metabolite VIII). Metabolite VIII could be transported back to the liver and metabolized to boldenone and metabolites. This process consisting of transport by the enterohepatic circulation and chemical conversion by gut bacteria has been reported before for other endogenous compounds[6,8,9]. As a result of this phenonomen the intestine can be regarded as an endocrine organ.

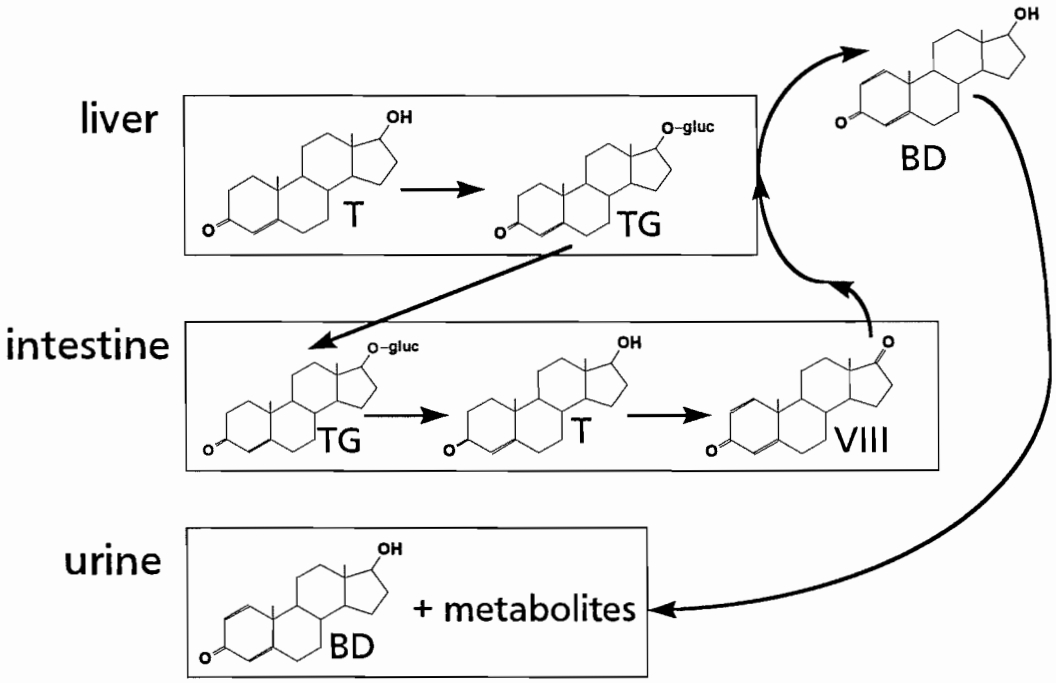


Figure 5: Hypothesis for the explaination of endogenous boldenone production, due to enterohepatic circulation and bacterial enzyme activities in the intestine, with testosterone (TG) and boldenone (BD).

This *in vitro* incubation experiment is the first step in the confirmation of endogenous boldenone production. This experiment has shown the presence of metabolite VIII production in incubated faeces where faeces of the control subject did not show any production at optimal incubation conditions and high precursor concentration. The choice of medium for the incubation appeared to be a critical factor. Adding PMS to the incubation mixture did not appear to accelerate the Δ^1-dehydrogenase activity in this experiment. This should however be confirmed in an incubation experiment with higher recovery. A method is presented to perform this *in vitro* experiment. To continue this project the experimental set-up should be used to perform higher conversion rates applying longer incubation times. In the next step a stable isotope testosterone will be used as a precursor, to prove the specific conversion to metabolite VIII.

Literature

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Appendix 1: Content of the media

Cysteine-Pepton-Bouillon

The content of the mixture was: 40 g yeast extract, 8 g pepton (Difco), 68 g NaCl p.a., 4 g L-cysteine HCl, 80 mL haemine solution in 8 L demineralized water. Haemine solution was prepared by adding 2 mL concentrated NH_4OH solution and 0.1 g haemine to 500 ml sterile demineralized water and sterilized afterwards. After filtration over a 0.45 μm Millipore filter, pH was adjusted to 7.0 and sterilized again for (20 min, 120° C). Under sterile conditions 2 mL of nicotinamide adenine dinucleatide solution in water (2 mg/mL) were added. The total solution was mixed and stored at -20° C until use.

Brain-Heart XV

Haemine solution (6 mL) and iso vitalex (20 mL) were added to sterilized (20 minutes, 120° C) dissolved Brainheart Infusion (38 g from Difco in 1 L demineralized water). Haemine solution was prepared by adding at 100° C, 1 g haemine and 0.8 g NaOH to 100 mL demineralized water.