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# Investigations of the microbial transformation of cortisol to prednisolone in urine samples

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ABSTRACT

Doping control samples are normally collected under non-sterile conditions and sometimes, storage and transportation are influenced by parameters such as the temperature. Therefore, microbial contamination and subsequent alteration of a sample's composition are possible. Studies regarding sample collection in cattle breeding have already shown enzymatic transformation of endogenous testosterone to boldenone causing false-positive findings. The aim of the present study was to investigate whether positive doping cases with the synthetic corticosteroids prednisolone and prednisone may result from microbial transformation of the endogenous corticosteroids cortisol and cortisone, respectively. A method comprising parameters such as pH values and screening results for synthetic glucocorticosteroids as well as incubation experiments followed by liquid chromatographic and mass spectrometric analysis was employed to test for contaminating germs with $\Delta^1$-dehydrogenase activity. Over 700 urine samples comprising inpatient and doping control specimens were investigated. In none of them, 1,2-dehydrogenating activity was confirmed. These findings are in accordance with other studies. However, the problem of microbial alteration of doping control specimens with special respect to 1,2-dehydrogenation must not be underestimated.

Article from a special issue on steroids and microorganisms.



## 1. Introduction

Urine is the most common biological matrix used for doping control purposes. The worldwide number of samples taken from athletes to control the use and abuse of substances is constantly growing [1]. As a positive doping case might be followed by e.g. a ban for 2 years, it is of paramount importance that the characteristics of each specimen such as its composition stay unchanged from collection till analysis [2]. Beside deliberate alteration of a urine using, e.g. proteases [3], its integrity is challenged by sample collection under non-sterile circumstances or the transport to the accredited laboratories under possibly insufficient transportation conditions. Especially in warm periods of the year the number of urine specimens containing microbial activity increases [4]. The specimens can be contaminated by normal microbial flora (skin, urethra, vagina, intestine), pathogens causing, e.g. urinary tract infections or environmental species [5], whereas women seem to be more susceptible to infections due to their shorter urethra [6]. Enzymes deriving from such micro-organisms (e.g. $\Delta^1$-dehydrogenase, $\Delta^4$-dehydrogenase, 20-keto-reductase, 3-hydroxy-steroid-dehydrogenase, sulfatase, glucuronidase) are able to change a specimen's composition by e.g. altering the ratio of substances already present, oxidation processes yielding new derivatives or changing the conjugation state [5]. Conversion processes may be a possible source of false-positive results.

The literature of the last decades provides a lot of examples dealing with microbial transformation of endogenous androgens [7] such as testosterone. Especially the formation of boldenone from endogenous testosterone was extensively investigated, as boldenone is banned as a growth promoter in cattle breeding, e.g. within the European Union. Since the 1990s, various studies were able to show that faeces of untreated cattle containing boldenone could contaminate urine specimens depending on the kind of sample collection. Deliberate contamination of blank samples with suspicious faeces formed boldenone after incubation [8–10].

A similar problem arose in human samples in the mid nineties, when endogenous formation of boldenone was confirmed [11–13]. The conversion of testosterone to boldenone can be performed by $\Delta^1$-steroid-dehydrogenase ($\Delta^1$-SDH, Fig. 1a).

Just like the anabolic androgenic steroid testosterone the endogenous glucocorticosteroids (GCS) cortisol (also known as hydrocortisone) and cortisone enclose a 3-oxo-4-ene structure (Fig. 1b). This structural element is a possible target of the enzyme $\Delta^1$-SDH for the 1,2-dehydrogenation (Fig. 1) [14,15]. The products resulting from such transformations of cortisol and cortisone

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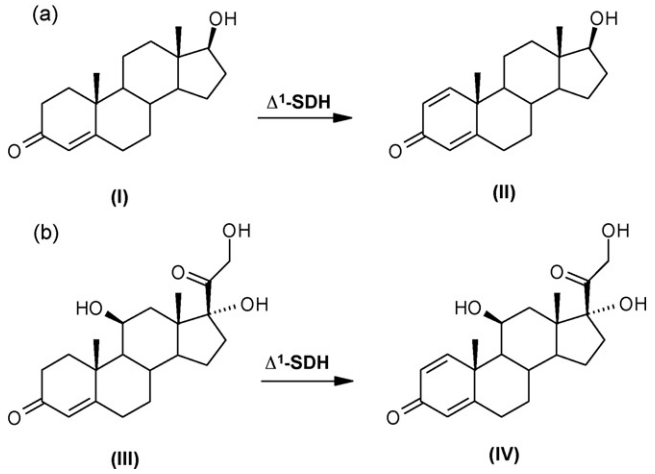


**Fig. 1.** The microbial conversion of steroids by $\Delta^1$-SDH: transformation (a) of testosterone (I) to β-boldenone (II) and (b) of cortisol (III) to prednisolone (IV).

are prednisolone and prednisone, respectively. Both are frequently used anti-inflammatory drugs banned in sports by the World Anti-Doping Agency (WADA) for in-competition use when administered by systemic (e.g. oral), non-systemic and non-topical (e.g. intraarticular, inhalation) routes [16].

There are lots of species containing the enzyme $\Delta^1$-SDH (see [17] and Table 1). *In vitro* experiments with e.g. the common soil bacterium *Rhodococcus erythropolis* showed considerable formation rates of prednisolone and prednisone from cortisol and cortisone after incubation at 30 °C for 24 h [18]. Using *Arthrobacter simplex*, this enzymatic transformation is commonly employed for the industrial production of prednisolone [19–21]. The dehydrogenation can be boosted in the presence of electron acceptors such as riboflavin (vitamin $B_2$) [22–24].

The aim of the present study was to elucidate, whether it would be possible to create false-positive findings due to microbial transformation of endogenous GCS such as cortisol and cortisone.

For this purpose, two studies were carried out. The first one included experiments with selected doping control samples. Specimens with a pH >7.5 and found to contain prednisolone in routine doping screening assays were further investigated. The urine samples were incubated at defined conditions, and possible bacterial species providing $\Delta^1$-SDH activity were searched for. A liquid chromatographic/mass spectrometric method was developed for the detection of cortisol, cortisone, prednisolone and prednisone beside endogenous substances with similar molecular masses and/or retention times such as e.g. 20-dihydrocortisone and 20-dihydrocortisol.

In a second study, 690 inpatient urines were analysed for the presence of contaminating species. Isolates of each species were tested for providing potential of transforming cortisol into prednisolone.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Cortisol (11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione, 98%) as well as prednisolone (11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione, 99%) and prednisone (17α,21-dihydroxy-pregna-1,4-diene-3,11,20-trione) were obtained from Sigma (Taufkirchen, Germany). Cortisone (17α,21-dihydroxy-pregn-4-ene-3,11,20-trione, pure) was purchased from Serva (Heidelberg, Germany). The metabolites 20α/β-dihydrocortisol (11β,17α,20α/β,21-tetrahydroxy-pregn-4-ene-3-one) and 20α/β-dihydrocortisone (17α,20α/β,21-trihydroxy-pregn-4-ene-3,11-dione) were from Steraloids (London, UK). (9,11,12,12)-$D_4$-cortisol was synthesised in our laboratory. Methanol (HPLC gradient grade) was purchased from VWR (Leuven, Belgium). The

**Table 1**
Selected micro-organisms with $\Delta^1$-steroid-dehydrogenase activity found in the literature.

| Name | Alternate names | Main isolation site | References |
|---|---|---|---|
| *Corynebacterium simplex* | *Arthrobacter simplex*<br>*Nocardioides simplex* | Soil | [5,17,19–21,43,44] |
| *Rhodococcus erythropolis* | *Arthrobacter picolinophilus*<br>*Nocardia calcarea* | Soil | [5,18] |
| *Mycobacterium flavum* | *Xanthobacter flavus* | Soil | [36,45] |
| *Mycobacterium smegmatis* | | | [17,46,47] |
| *Rhodococcus rhodochrous* | *Rhodococcus roseus* | Soil<br>Water | [48] |
| *Bacterium hevanicensis* | | | [45] |
| *Bacterium mycoides* | | | [17,45] |
| *Mycobacterium rhodochrous* | *Rhodococcus coprophilus* | Various | [45] |
| *Pseudomonas testosteroni* | *Comamonas testosteroni* | Sewage<br>Soil | [44] |
| *Pseudomonas fluorescens* | | Soil<br>Water | [49] |
| *Nocardia restricta* | *Rhodococcus equi*<br>*Corynebacterium hoagii* | Soil<br>Animals | [17,44] |
| *Bacillus sphaericus* | *Lysinibacillus sphaericus* | Air<br>Animals | [17,44] |
| *Bacillus cyclooxydans* | | | [17,44] |
| *Bacillus aureus* | | | [22] |
| *Phycomyces Blakesleeanus* | | | [50] |
| *Fusarium oxysporum* | | Plants | [51] |
| *Fusarium moniliforme* | *Fusarium proliferatum* | Various | [51] |
| *Fusarium solani* | | Rotting food<br>Man<br>Soil | [51] |
| *Gliocladium simplex* | | | [51] |
| *Helminthosporium speciferum* | | | [51] |
| *Trichoderma hamatum* | | Soil | [52] |