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Degradation of doping-relevant Steroids by *Rh. Erythropolis*

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Introduction

Former studies have shown that steroids are potential substrates for microorganisms [1]. As an example, the degradation of testosterone induced by *Rhodococcus erythropolis* was observed. The formation of 4-androstene-3,17-dione, 1,4-androstadiene-3,17-dione (boldione) and 1,4-androstadiene-17β-hydroxy-3-one (boldenone) was confirmed [2]. Consequently a potential endogenous origin of boldenone and its metabolites has to be taken into consideration for the evaluation of routine doping control samples revealing the presence of these substances at low concentration level [3, 4].
This work presents results obtained from further studies related to the microbial conversion of steroid substrates being relevant in doping analysis. For this purpose incubation by *Rh. erythropolis* was applied to examine the influence of structural variations (A/B-ring structure, substitution at position 17, conjugation) on the initial steps of the degradation pathway.

Experimental

Rh. erythropolis culture grown on agar plate "Mueller-Hinton" was utilised. Altogether 16 substrates (see table 1) were examined in this study. The experiments were carried out in a blank urine of a male infant spiked with 1 µg/mL of the selected substrate. Two aliquots of each sample were prepared, one control without addition and one „active" sample with addition of bacteria solution, and incubated at 30°C for 24 hours. Epitestosterone-$19d_3$ was utilised as internal standard. Conjugated substrates were cleaved by enzymatic hydrolysis (*E. coli*) or solvolysis (adapted from [5]), respectively. The solution was adjusted to pH 9 ($NaHCO_3$ / KCO_3) and extracted by n-pentane/ methanol (24:1 v/v) or diethylether (substrates 15 and 16 only). Samples were analysed by GC/MS (substrates 1 to 14, as TMS derivatives) or LC/MS/MS (substrates 15 and 16) to detect and identify the transformation products

formed in the process of microbial degradation. The instrumental conditions for both GC/MS and LC/MS/MS were adapted from procedures commonly used in doping analysis [6, 7].

Results and Discussion

The bacteria strain *Rh. erythropolis* is able to generate several enzymes affecting different sites of the steroid molecule. Taking into consideration the type of the acting enzyme the products found after incubation are listed in table 1. With respect to the structural prerequisites of the substrates the observations can be summarized as follows:

1. Substrates containing a secondary 17β-hydroxy group were first oxidised at this position during incubation with *Rh. erythropolis.* The process of degradation seems to be initiated by this reaction (substrates 1-3 and 9-11). The formed 17-oxo compound then undergoes further transformations via different intermediates depending on the existing A-ring configuration. As a result of these reactions 1,4-androstadiene-3,17-dione (boldione) was detected.
2. However, a secondary 17α-hydroxy group (epitestosterone, substrate 4) remains unchanged in the presence of *Rh. erythropolis*, exhibiting the stereoselectivity of the 17β-OH-DH and the absence of an analogous 17α-OH-DH. Consequently, the substrate is only accessible to dehydrogenases affecting the A-ring (formation of epiboldenone).
3. Attempts to cause a conversion of 17β-hydroxysteroid conjugates failed. Both the glucuronide and the sulphate of testosterone (substrate 5 and 6) are stable in the presence of *Rh. erythropolis*. The oxidation of the 17β-hydroxy group is blocked due to the linkage to the conjugate (ether or ester bond, respectively). Similarly, a conjugated 3α-hydroxy group (substrate 8) is inert against 3-OH-DH. Lacking a 3-oxo function, in both cases no activity in relation to further dehydrogenation reactions in the A-ring could be observed.
4. As expected, an additional substitution at C-17 leading to a tertiary hydroxy group prevents the oxidation at this position (substrates 12 to 16). Methandienone was obtained by incubation of both methyltestosterone (substrate 12) and its main metabolites (substrates 13 and 14) indicating that these substrates are only available for dehydrogenases having an effect on the A-ring structure. A similar experiment with cortisol and cortisone as substrates confirmed this pathway of transformation. The corresponding compounds prednisolone and prednisone were detected at estimated conversion rates of 3% and 16% (after 7 hours) or 23% and 49% (after 24 hours), respectively.

Table 1: Detected transformation products classified by the type of the acting enzyme

no.	substrate	type of the acting enzyme			
		17β-hydroxysteroid-dehydrogenase (17β-OH-DH)	3-hydroxysteroid-dehydrogenase (3OH-DH)	3-oxosteroid-4-dehydrogenase (Δ^4-DH)	3-oxosteroid-1-dehydrogenase (Δ^1-DH)
1	5α-dihydro-testosterone	5α-androstane-3,17-dione	1) 2)	4-androstene-3,17-dione	1,4-androstadiene-3,17-dione
2	5β-dihydro-testosterone	5β-androstane-3,17-dione	1) 2)	4-androstene-3,17-dione	1,4-androstadiene-3,17-dione
3	testosterone	4-androstene-3,17-dione	1) 2)	1) 2)	1,4-androstadiene-3,17-dione
4	epitestosterone	*no reaction at 17α-position*	1) 2)	1) 2)	epiboldenone
5	testosterone glucuronide	3)	1) 2)	1) 2)	*no transformation*
6	testosterone sulphate	3)	1) 2)	1) 2)	*no transformation*
7	etiocholanolone	1) 2)	5β-androstane-3,17-dione	4-androstene-3,17-dione	1,4-androstadiene-3,17-dione
8	etiocholanolone glucuronide	1) 2)	3)	1)	1)
9	5β-androstane-3α,17β-diol	etiocholanolone	5β-androstane-3,17-dione	4-androstene-3,17-dione	1,4-androstadiene-3,17-dione
10	5α-androstane-3α,17β-diol	androsterone	5α-androstane-3,17-dione	4-androstene-3,17-dione	1,4-androstadiene-3,17-dione
11	5α-androstane-3β,17β-diol	epiandrosterone	5α-androstane-3,17-dione	4-androstene-3,17-dione	1,4-androstadiene-3,17-dione
12	methyl-testosterone	1) 4)	1) 2)	1) 2)	methandienone
13	17α-methyl-5α-androstane-3α,17β-diol	1) 4)	17α-methyl-5α-androstanolone	methyltestosterone	methandienone 6)
14	17α-methyl-5β-androstane-3α,17β-diol	1) 4)	17α-methyl-5β-androstanolone	methyltestosterone	methandienone
15	cortisol	1) 5)	1) 2)	1) 2)	prednisolone
16	cortisone	1) 5)	1) 2)	1) 2)	prednisone

notes 1) lack of structural prerequisite
2) target function already existing
3) blocked by conjugation
4) hindered by 17α-methyl group
5) function not existing
6) formation of an additional product, presumably 17α-methyl-5α-androst-1-ene-3-one-17β-diol

Conclusions

The objective of this study was to investigate aspects of the microbial degradation process of steroid substances relevant in doping analysis. The applied bacteria strain *Rh. erythropolis* is able to transform unconjugated steroid substrates yielding defined and stable intermediates.

Depending on the existing A/B-ring configuration and the D-ring substituents, the mechanism of transformation comprises different enzymatic reactions (17β-OH-DH, 3-OH-DH, Δ^4-DH, Δ^1-DH). The microbial formation of methandienone from methyltestosterone and its metabolites in urine serves only as a model and has no practical relevance because the metabolites of methyltestosterone are mainly excreted as glucuronides. In contrast to that, cortisone and cortisol are present in free form at a reasonable concentration level in urine. Therefore a potential conversion of cortisone and cortisol to prednisone and prednisolone caused by microorganisms has to be taken into consideration for the interpretation of corresponding analytical findings.

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