Influence of *Helix pomatia* Enzyme Preparations on the Oxidative Conversion of Some Clostebol Acetate Metabolites in Urine

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Abstract

Clostebol acetate (4-chloro-testosterone acetate) is an anabolic steroid used for fattening purposes in cattle breeding. To safeguard public health, its use has been prohibited by the European Commission since 1986. Screening for its urinary metabolites is therefore an important tool for the control of possible violations. Because those metabolites appear conjugated to glucuronic acid or sulfate, deconjugation prior to analysis is necessary. This work describes the variability in results seen with the use of various commercial preparations of *Helix pomatia* (SHP) for enzymatic hydrolysis of the conjugates. A simultaneous oxidative side reaction was observed, converting metabolites with a 3-OH-4-ene structure into a 3-oxo-4-ene structure. This was not observed when samples were incubated without enzyme or in the presence of heat-inactivated SHE. GC-MS analysis revealed oxidation of some metabolites of clostebol acetate, 4-chloro-4-androsten-3α-ol-17-one and 4-chloro-4-androsten-3α,17β-diol, changing them into other metabolites, 4-chloro-4-androsten-3,17-dione and clostebol (4-chloro-testosterone), respectively. Based on the difference in cross-reactivities of the antibodies for these metabolites, comparative analysis in enzyme immunoassay, following enzymatic hydrolysis, confirmed this transformation. This oxidative conversion phenomenon could be of great importance when considering the choice or target analytes for screening bovine urine.

Introduction

Clostebol acetate is strongly metabolized in cattle (1). An overview of the main metabolites is given in Figure 1. In bovine urine, most steroid metabolites appear as hydrophilic glucuronide or sulfate conjugates that need to be efficiently hydrolyzed in order to recover virtually all the free metabolites prior to further analysis. Deconjugation can be performed by either enzymatic or chemical hydrolysis. The latter method is drastic and sometimes accompanied by additional "modification" of the structure of the analytes, risking a false diagnosis and causing problems in reproducibility, but it is very efficient from the point of view of hydrolysis yield. Another advantage is that there are no differences in the hydrolysis of conjugates in different positions or conformations (α or β).

![Figure 1. Chemical structures of clostebol acetate and its major metabolites.](https://academic.oup.com/jat/article-abstract/26/2/73/739248)
In veterinary residue control, however, enzymatic hydrolysis is the most popular mode of deconjugation and different enzymatic preparations are available to perform this "soft" hydrolysis. Although the method is liable to empiricism, with each laboratory using different brands and incubation conditions of time, temperature, and pH, the Helix pomatia (SHP) preparation, containing both β-glucuronidase and arylsulfatase activity, is used frequently in the study of the metabolism of different anabolic steroids.

Despite its relative specificity, some 17-sulfate steroids are reported to be resistant to the action of arylsulfates (2–6), and its use is often preferred because it ensures milder conditions than acid or alkaline chemical hydrolysis.

In the literature side reactions caused by the digestive juice of SHP have been reported. Messeri et al. (7), Massé et al. (8), and O’Keeffe (9) describe the degradation of some androgens during hydrolysis. After incubation of dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one) solution with an enzymatic preparation of SHP, the steroid recovery was markedly decreased, apparently proportional to the enzyme concentration used. Also, the recovery of 5α-androstane-3β,17β-diol and of 3β-hydroxy-5α-androstan-17-one was lowered after incubation with SHP, but no transformation occurred when 3α-isomers were tested.

The presence of 4-androsten-3,17-dione within the incubation products suggests that the enzymatic preparation contains at least 3-hydroxy-steroid dehydrogenase and 3-oxosteroid-Δ2-Δ4 isomerase. Moreover, the phenomenon appeared to be batch dependent.

In another study, Vanluchene et al. (10) reported the conversion of pregnenolone, 5-pregnen-3β,20α-diol, and 17-hydroxy-pregnenolone to progesterone, 20α-hydroxy-4-pregnen-3-one, and 17-hydroxy-progesterone, respectively. They suggested that enzyme impurities, present in SHP, were responsible for this phenomenon. Steroid analysis carried out after hydrolysis with SHP showed progressively decreasing concentrations of some 3β-OH-5-ene steroids when the volume of the enzyme extract used was increased. Together with this decrease, a concomitant formation of corresponding 4-ene-3-oxo-steroids was observed. Neither incubations in the absence of SHP nor incubations by means of bacterial β-glucuronidase did result in any transformation of the 3β-OH-5-ene steroids. These results provided evidence of the presence of 3β-hydroxy-steroid dehydrogenase and 5→4-ene-steroid-isomerase, both of which are necessary for a 3β-OH-5-ene to a 4-ene-3-oxo-steroid transformation in SHP extract.

Finally, a similar observation has been made by Leyssens et al. (11). In their paper, the isomerization and oxidation of methandriol (17α-methyl-5-androstone-3β,17β-diol) to methyltestosterone (17α-methyl-3-oxoandrost-4-en-17β-ol) during the hydrolysis with SHP was described. The authors showed that SHP had a negative effect on the extraction efficiency of the two steroids from buffer solutions. They found that percentage recoveries decreased from 70% without SHP to 30% in the presence of SHP. There is no doubt that this phenomenon also plays a role in the overall extraction recovery of steroids from urine samples. This conversion was not observed with E. coli, with heat-inactivated SHP, or in the absence of SHP.

Until now, the transformation of a 3-OH-5-ene into a 3-oxo-4-ene configuration during hydrolysis with SHP has been described by several authors. Our study, however, is the first to demonstrate the particular conversion of 3-OH-4-ene structure into a 3-oxo-4-ene structure, leaving the double bond in position 4 unshifted (Figure 2), related to the use of SHP. This was proved by testing different SHP preparations under different conditions for the hydrolysis of urine samples containing clostebol acetate metabolites or structural analogues.

Experimental

Reagents and materials

Organic solvents were all of analytical reagent grade. Ultrapure water was obtained with the Milli-Q System of Millipore (Brussels, Belgium). Clostebol was obtained from Alltech (Applied Science Laboratories, State College, PA). Epiclostebol (4-chloro-epitestosterone) was generously donated by RIVM (Bilthoven, The Netherlands). Dehydroepiandrosterone (DHEA), its 3-sulfate and 3-glucuronide conjugates, testosteron-17-sulfate, N-methyl-N-trimethylsilyltrifluoroacetamide (99% purity, MSTFA), dithioerythritol (DTE), and NH4I were purchased from Sigma-Aldrich (Milano, Italy). 17-Methyltestosterone was acquired from Salars (Como, Italy). Some clostebol metabolites (4-chloro-4-androsten-3-ol-17-one, 4-chloro-4-androsten-3,17-diol) were synthesized in the Department of Organic Chemistry of the University of Firenze, and 4-chloro-4-androsten-3,17-dione (CLAD) was a kind gift of Dr. Leyssens (Dr. L. Willems-Instituut, Diepenbeeck, Belgium). 4-Androsten-3,17-diol and 19-nor-4-androsten-3,17-diol were a kind gift of Dr. L. Dehennin (L.A.B., Fédération Nationale des Courses Françaises, Chatenay-Malabry, France). C18 cartridges were purchased from IST (Stepbio, Bologna, Italy) and Varian (Torino, Italy).

Enzymes

The following Helix pomatia preparations were used and compared.

*Sigma β-glucuronidase type H-2 from SHP (Sigma-Aldrich).*

Two lots of production were used: lot no. 125 H 3381 containing 99,200 Fishman units/mL of β-glucuronidase and 4500 Roy units/mL of arylsulfatase and lot no. 97 H 3386 containing 134,600 Fishman units/mL of β-glucuronidase and 5200 Roy units/mL of arylsulfatase.

Helicase, the lyophilized form of SHP (IBF, Clichy, France).
The solution was reconstituted with distilled water as indicated by the producer and contained about 100,000 Fishman units/mL of β-glucuronidase and about 1,000,000 Roy units/mL of arylsulfatase.

**Experiment 1: enzymatic hydrolysis of standard solutions followed by GC–MS analysis.** Two standard mixtures were prepared, one containing testosterone-17-sulfate, 4-chloro-4-androsten-3-ol-17-one, and 4-chloro-4-androsten-3,17-diol, another containing the 3-sulfate of DHEA, 4-androsten-3,17-diol, and 19-nor-4-androsten-3,17-diol. The two mixtures were separately added to 1 mL of 0.2M phosphate buffer (pH 7.0) or 1 mL of 0.2M acetate buffer (pH 5.2) to obtain a final concentration of 250 ng/mL for each analyte. Subsequently, the solutions were submitted to hydrolysis. After the addition of 17-methyltestosterone as the internal standard, the appropriate amount of SHP was added: approximately 4000–6000 Fishman units/mL of β-glucuronidase and 35,000–40,000 Roy units/mL of sulfatase for the IBF, Merck, and Boehringer Mannheim preparations and approximately 4000 Fishman units/mL and 160–180 Roy units/mL for the Sigma preparation. Three different hydrolysis conditions were used: 37°C for 16 h, 52°C for 2 h, and 52°C for 16 h. Finally, the samples were passed through a C18 cartridge, previously conditioned with methanol and 0.2M acetate buffer (pH 5.2), washed with distilled water, dried under vacuum, and eluted with 5 mL of methanol. The eluates were taken to dryness under N2 flow at 50°C and resuspended in 2 mL of a hexane/diethyl ether (3:1, v/v) mixture. This solution was washed with 1 mL of 1N KOH and 1% NaCl in 1-mL portions until the washing solution reached neutral pH. The organic layer was taken to dryness under N2, dried under vacuum over KOH/P2O5, and derivatized with 70 µL of silylating agent (MSTFA/NH4I/DTE, 1000:4:6, v/w/w) for 30 min at 70°C. A 1–2-µL aliquot was injected into the GC–MS instrument.

**Experiment 2: enzymatic hydrolysis of spiked urine samples followed by GC–MS analysis.** One milliliter of blank bovine urine was spiked with two metabolites of clostebol, 4-chloro-4-androsten-3-ol-17-one and 4-chloro-4-androsten-3,17-diol, each at a concentration of 250 ng/mL. One part of the urine was submitted to hydrolysis after adjusting the pH to 5.2 or 7.0 with 2M acetic acid buffer or 2M phosphate buffer, respectively. Another part was first purified through a C18 cartridge to reduce potential matrix effects prior to hydrolysis. In the latter case, the methanolic eluate was taken to dryness, and the residue was resuspended in an adequate buffer (2 mL of 0.2M acetate, pH 5.2, or 0.2M phosphate buffer, pH 7.0). Subsequently, all samples were hydrolyzed and analyzed as described for experiment 1.

**Experiment 3: chemical hydrolysis of standard solutions followed by GC–MS analysis.** Both the standard mixtures of experiment 1 were submitted to chemical hydrolysis in ethyl acetate/methanol/95% sulfuric acid (900:95:5, v/v/v) for 1 h at 50°C. After addition of 17-methyltestosterone as the internal standard, the solutions were neutralized, extracted with hexane/diethyl ether (3:1, v/v), washed with 1% NaCl, evaporated to dryness under N2, and derivatized with the silylating agent (MSTFA/NH4I/DTE, 1000:4:6, v/w/w). The results obtained from the analysis of this solution were compared with those obtained from the enzymatic hydrolysis procedures.

**Experiment 4: enzymatic hydrolysis of standard solutions and spiked urine samples followed by enzyme immunoassay.** For the approach using an enzyme immunoassay, a procedure based on an experiment described by Leysens et al. (11) was followed. Standard solutions and spiked urine samples were hydrolyzed using SHP from Boehringer Mannheim under different conditions of time, temperature, or pH followed by solid-phase extraction (SPE) and final measurements in ELISA.

Different standard solutions of 4-chloro-4-androsten-3α-ol-17-one (50 ng/mL), clostebol (500 ng/mL), epiclostebol (10 ng/mL), and 4-chloro-4-androsten-3α,17β-diol (5000 ng/mL) were prepared. Two series of solutions with different pH were made for each standard: the pH of the solutions was adjusted to either pH 5.2 by adding 500 µL of acetic acid buffer (2M, pH 5.2) or to pH 7.5 by adding 1 mL of phosphate buffer (0.01M, pH 7.5) and adjusting with HCl (1M) if needed.

For each standard solution at each pH, three different samples were made successively by adding 50 µL of active SHP, inactivated SHP (heat inactivation of SHP was achieved by incubating the enzyme solution for 5 h at 80°C), or no SHP (enzyme blank).

Samples were then hydrolyzed using different times (2 h or 16 h) and temperatures (37°C or 52°C). In a second phase, part of the experiment was repeated using blank bovine urine samples instead of water.

After hydrolysis, the samples were cooled down, and further cleanup was achieved by SPE according to a procedure described by Walsh et al. (12) with some modifications. The sample was applied onto a C18 column previously conditioned with 5 mL of methanol, 2 × 5 mL of ultrapure water, and 5 mL of 0.2M acetate buffer (pH 5.2). The column was washed with 2 × 5 mL of ultrapure water and allowed to dry for 5 min by drawing air through
Hexane (2 mL) was passed through the column, and the column was dried again before elution with 5 mL of ethylacetate/methanol (90:10, v/v). The eluate was successively washed with 2 x 2 mL of 1N KOH and 2 mL of 1% NaCl. After evaporation, the residue was redissolved in buffer and measured by an ELISA according to a procedure developed in our laboratory (1) using monoclonal CLAD antibodies and a CLAD calibration curve.

### Results

**Experiments 1 and 3**

First, experiment 1 was set up to evaluate the ability of arylsulfatase to hydrolyse testosterone-17-sulfate and DHEA-3-sulfate. This was achieved by adding these standard molecules to buffer and submitting them to enzymatic hydrolysis followed by a GC-MS analysis.

For testosterone-17-sulfate, a certain deconjugation was observed under all experimental hydrolysis conditions, although some 17-sulfate steroids are reported to be resistant to the arylsulfatase action. Table I shows the hydrolysis yield of testosterone 17-sulfate in acetate buffer (0.2M, pH 5.2), expressed in percentage of the chemical hydrolysis yield obtained in experiment 3. The yield is proportional to temperature and time, whereas the different amounts of arylsulfatase present in the commercial enzyme preparations seem to have only a limited influence.

A similar evaluation was not possible for DHEA-3-sulfate. When the enzymatic preparations from Boehringer Mannheim, IBF, and Merck were used for sulfate deconjugation, only a weak GC-MS signal for the free DHEA was obtained, and a new signal identified as 4-androsten-3,17-dione appeared. These observations correspond with results reported earlier (7,9). For hydrolysis with the Merck preparation, only a small amount of sulfate had been hydrolyzed after 2 h at 52°C, and an oxidative reaction was evident. This oxidative transformation was complete after 16 h of hydrolysis. The Sigma preparation showed a different behavior: the sulfatase activity was very efficient, and oxidase activity was almost absent. This resulted in a GC-MS signal for DHEA comparable to the one obtained from chemical hydrolysis of its 3-sulfate (experiment 3), and the 4-androsten-3,17-dione signal was only present to a very low extent in samples after 16 h of hydrolysis.

In a second stage, experiment 1 was also used to evaluate the presence of 3-hydroxy-steroid dehydrogenase activity of the different enzymatic preparations. This was achieved by hydrolysis of a buffer solution containing 4-androsten-3,17-diol and 19-nor-4-androsten-3,17-diol. GC-MS analysis revealed that the Boehringer Mannheim, IBF, and Merck preparations transformed 4-androsten-3,17-diol and 19-nor-4-androsten-3,17-diol into their corresponding 3-oxo analogues, testosterone and 19-nortestosterone, respectively. The Sigma product had a lower amount of this non-specific enzymatic activity and caused only very limited oxidation of these.
Two standards in buffer

Conversion with IBF

Conversion with Merck

Conversion with Sigma

Conversion with Boehringer

Figure 4. Conversion of 4-Cl-4-androsten-3α-ol-17-one into CLAD under the influence of various enzyme preparations of SHP (pH 5.2, 16 h, 32°C).
Figure 5. Conversion of 4-Cl-4-androsten-3α-ol-17-one into CLAD: comparison of different hydrolysis conditions with Boehringer SHP. After hydrolysis almost no signal is observed any more for 4-Cl-4-androsten-3α-ol-17-one.
molecules. Figure 3 compares the results of enzymatic hydrolysis of 4-androsten-3,17-diol and 19-nor-4-androsten-3,17-diol for 2 h at 52°C with the Sigma and Boehringer Mannheim preparations.

Identical results were obtained for the buffer solution containing the clostebol metabolites 4-chloro-4-androsten-3-ol-17-one and 4-chloro-4-androsten-3,17-diol. A limited oxidation was observed with the Sigma preparation, whereas the use of the other enzymatic preparations resulted in the formation of oxidized analogues only. The conversion of metabolite 4-chloro-4-androsten-3-ol-17-one into CLAD under different conditions is shown in Figures 4 and 5.

Experiment 2

The second experiment was used to evaluate a potential matrix effect on the hydrolysis and oxidation processes. Blank bovine urines spiked with clostebol metabolites were submitted to enzymatic hydrolysis with or without prepurification of the sample prior to deconjugation. Using a prepurified sample, the GC–MS signal was cleaner compared to the unpurified sample, but no significant differences were noted for the hydrolysis yield. The oxidation process was only slightly reduced when performing the hydrolysis in urine matrix.

Experiment 4

In a previous study (13), CLAD was found to be the main urinary metabolite after intramuscular administration of clostebol acetate. For this reason, CLAD was chosen as the antigen for the production of monoclonal antibodies and for the setup of a competitive ELISA format (1). The antibodies produced were not only specific for CLAD (100%) but showed also cross-reactivity towards the metabolites clostebol (1%), epiclostebol (35%), 4-chloro-4-androsten-3α-ol-17-one (6%), and 4-chloro-4-androsten-3α,17β-diol (0.05%).

Based on this difference in cross-reactivity for the metabolites, the oxidative conversion, revealed by GC–MS, could explain the different results obtained for samples hydrolyzed with active enzyme (SHP) or samples hydrolyzed without (enzyme blank) or with inactivated enzyme (iSHP).

For samples spiked with 4-chloro-4-androsten-3α-ol-17-one and 4-chloro-4-androsten-3α,17β-diol, Table II shows apparently increased concentrations after hydrolysis with active SHP. This corresponds with the oxidative side reaction converting the metabolites into CLAD and clostebol, respectively, compounds for which the antibodies have a higher cross-reactivity.

Samples containing clostebol or epiclostebol showed no change in response after hydrolysis with active SHP or without SHP, indicating that no conversion or structural modifications had occurred.

With active SHP, a conversion could be detected, which did not happen when iSHP or the enzyme blank solution was used. In some cases results found for the enzyme blank solution were a little higher than for the iSHP solution, indicating that the presence of SHP had a negative influence on the recovery. In general, the conversion rate was higher at pH 5.2 than at pH 7.5.

<table>
<thead>
<tr>
<th>Table II. Apparent Concentrations Measured in ELISA after Enzymatic Hydrolysis under Different Experimental Conditions*</th>
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</thead>
<tbody>
<tr>
<td>Spiked conc. (ppb)</td>
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<tr>
<td>Metabolite</td>
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<td>--------------</td>
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<tr>
<td><strong>A. Hydrolysis of standard solutions</strong></td>
</tr>
<tr>
<td>Clostebol</td>
</tr>
<tr>
<td>Epi-clostebol</td>
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<tr>
<td>4-Ch-4-androsten-3α-ol-17-one</td>
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<tr>
<td>16h-52°C·pH 5</td>
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<tr>
<td>16h-37°C·pH 5</td>
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<tr>
<td>2h-52°C·pH 7</td>
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<tr>
<td>16h-52°C·pH 7</td>
</tr>
<tr>
<td>16h-37°C·pH 7</td>
</tr>
</tbody>
</table>
| **B. Hydrolysis of urine samples**
| 4-Ch-4-androsten-3α,17(αβ)-diol | 5000 | 2h-52°C·pH 5 | 5.2 | 1.2 | 1.7 |
| 2h-52°C·pH 5 | 35.2 | 2.8 | 3.0 |
| 16h-52°C·pH 5 | 35.0 | 2.6 | 2.8 |
| 16h-37°C·pH 5 | 25.8 | 2.4 | 2.5 |
| 2h-52°C·pH 7 | 30.3 | 2.5 | 2.3 |
| 16h-52°C·pH 7 | 20.5 | 4.5 |
| 16h-37°C·pH 7 | 25.2 | 4.9 |

* Means of duplicates are given.

Discussion

* Helix pomatia was able to oxidize 4-chloro-4-androsten-3α-ol-17-one to CLAD and 4-chloro-4-androsten-3,17-diol to clostebol and its 17-epimer epiclostebol, depending on the 17 configuration (β or α). During this process, the double bond in position 4-5 was left unchanged. The oxidation is not influenced by the urine matrix or by the presence of a 4-Cl substituent, and the yield was strictly related to hydrolysis conditions.

As a consequence, it is reasonable to conclude that CLAD, earlier (13) identified as the main urinary metabolite of clostebol acetate, could be an artifact produced during hydrolysis with various enzymatic preparations.

The presence of additional non-specified enzyme activity in SHP used for hydrolysis of urinary samples may be at the origin of the different results found in studies of clostebol metabolites in different laboratories. The same cattle's urine samples were analyzed by GC–MS in an Italian and a French laboratory working in this period on clostebol metabolism in cattle.
Conclusions

In conclusion, the impact of this oxidative phenomenon in the field of residue analysis cannot be underestimated. The blind use of enzymatic preparations for hydrolytic steps in the analytical procedure could lead to errors in the study of the metabolism of 4-ene steroids (e.g., clostebol) or to erroneous assay results (false negatives, incorrectly measured levels).

In the case of clostebol acetate, the variability observed in standard assays for its metabolites may relate to the differing abilities of these enzymatic preparations to catalyze the oxidation.

This work aims to highlight the potential of SHP extracts to induce further enzymatic changes, resulting in unexpected assay results, rather than to solve this problem.

Acknowledgments

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Table III. Abundance of the Major Metabolites Identified by GC-MS in Urine Samples after Intramuscular Administration of Clostebol Acetate. Measurements were Done by Two Different Laboratories in France and Italy

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>France</th>
<th>Italy</th>
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<tbody>
<tr>
<td>Clostebol</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Epiclosterol</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>4-Cl-4-androsten-3α-ol-17-one</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>4-Cl-4-androsten-3,17-dione</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>4-Cl-4-androsten-3α,17β-diol</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

*+++ high abundance; ++ intermediate abundance; + low abundance.

References


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