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A. INTRODUCTION

1. Theory

Trenbolone acetate is an anabolic steroid with strong androgenic activity. The microtiter wells are coated with capture antibodies directed against anti-trenbolone antibodies. Standards or sample solution, trenbolone enzyme conjugate and anti-trenbolone antibodies are added. Free and enzyme conjugated trenbolone compete for the antibody binding sites (competitive enzyme immunoassay (EIA)). At the same time, the anti-trenbolone antibodies are also bound by the immobilized capture antibodies. Any unbound enzyme conjugate is then removed in a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colorless chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm (optional reference wavelength, 600 nm). The absorption is inversely proportional to the trenbolone concentration in the sample.

2. Applicability

This test is applicable for the qualitative analysis of trenbolone in bovine liver at levels ≥ 5 ppb.

B. EQUIPMENT

Note: Equivalent apparatus and instrumentation may be substituted for the following items.

1. Apparatus

   a. RIDASCREEN® Trenbolon Test Kit:

      Each kit contains sufficient materials for 96 measurements (including standard analyses). Each test kit contains:

      i. 1 x Microtiter plate with 96 wells (12 strips with 8 removable wells each) coated with capture antibodies.

      Note: Keep unused wells together with the drying agent and well sealed in the foil bag. Keep stored at 2 - 8 °C.

      ii. 1 x Conjugate (0.7 mL), peroxidase conjugated trenbolone concentrate, red cap.

      Note: The trenbolone enzyme conjugate (bottle with red cap) is provided as a concentrate. Since the diluted enzyme conjugate has a limited stability, only the amount, which actually is needed, should be reconstituted. Before pipetting, the enzyme conjugate should be shaken carefully. For reconstitution, the conjugate concentrate is diluted 1:11 (1+10) in buffer (e. g. 200 µL conjugate concentrate + 2 mL buffer, sufficient for 4 microtiter strips).
iii. 1 x Anti-trenbolone antibody (0.7 mL), concentrate, black cap.

The anti-trenbolone antibody (bottle with black cap) is provided as a concentrate. Since the diluted antibody solution has a limited stability, only the amount, which actually is needed, should be reconstituted. Before pipetting, the antibody concentrate should be shaken carefully. For reconstitution, the antibody concentrate is diluted 1:11 (1+10) in buffer (e.g. 200 µL antibody concentrate + 2 mL buffer, sufficient for 4 microtiter strips).

iv. 1 x Substrate (7 mL), contains urea peroxide, green cap.

v. 1 x Chromogen (7 mL) contains tetramethylbenzidine, blue cap.

The colorless chromogen is light sensitive, therefore, avoid exposure to direct light. Any coloration of the chromogen solution is indicative of deterioration and the reagent should be discarded.

vi. 1 x Stop solution (14 mL) contains 1 N sulfuric acid, yellow cap.

vii. 1 x Buffer (25 mL), Conjugate and antibody dilution buffer.

Note: Store the kit at 2 - 8 °C. Do not freeze. Bring all reagents to room temperature (20 - 25 °C) before use. Return all reagents to 2 - 8 °C immediately after use.

b. Incubator - Labline, model 100.

c. Evaporator - Fisher sample concentrator, model 100.


e. Scintillation vials - VWR, borosilicate glass with screw cap, 66022-004.

f. Micropipettes - 10 -100 µL, P1000, multichannel pipetter 8 spot 50 - 300 µL.


h. Centrifuge tubes - 15 mL, Falcon.

i. Disposable culture tubes - borosilicate glass, 16 x 125 mm, Fisher brand.

2. Instrumentation

Microtiter plate spectrophotometer - 450 nm.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted for the following items.

1. Reagents


b. Tert-butylmethyleneether - Sigma Aldrich, HPLC grade, 293210-2L.
c. NaOH pellets - J.T. Baker, 500g, 3722-11.
d. Glacial Acetic Acid (CH₃COOH) - J.T. Baker, 500 mL, 9507-00.

2. Solutions
   a. 20% acetic acid:
      Add 20 mL of glacial acetic acid to a 100 mL volumetric flask. Dilute to volume with
deionized water.
   b. 500 mM sodium acetate buffer, pH 4.8:
      Add 41 g sodium acetate (CH₃COONa) to a 800 mL beaker add 700 mL of deionzed
      water. Adjust to pH 4.8 with 20% acetic acid. Transfer to a 1000 mL volumetric flask
      and dilute to volume with deionized water.
   c. 20 mM PBS-buffer, pH 7.2:
      Add 0.55 g NaH₂PO₄ ⋅ H₂O + 2.85 g Na₂HPO₄ ⋅ 2 H₂O + 9.0 g NaCl to a 1000 mL
      volumetric flask. Dilute to volume with deionized water.
   d. 80% Methanol:
      Add 800 mL of Methanol to a 1000 mL volumetric flask. Dilute to volume with
      deionized water.
   e. 40% Methanol:
      Add 400 mL of Methanol to a 1000 mL volumetric flask. Dilute to volume with
      deionized water.

D. STANDARDS

1. Source
   17 alpha-Trenbolone
   RIVM laboratory for Residue analysis,
   P.O. Box 1 NL 3720 BA Bilthoven, The Netherlands.

2. Preparation
   a. Trenbolone stock solution (100 μg/mL):
      Note: Allow the ampoules to warm to room temperature before opening. Place them
      in the dark at ambient temperature for at least 30 minutes.
      i. Open the ampoule by carefully breaking the neck. Pipette 1.0 mL of ethanol
      into the ampoule. Close the ampoule carefully with film (to avoid evaporation
during the following steps).

ii. Vortex the ampoule for at least 1 minute.

iii. Place the ampoule in an ultrasonic water bath for at least 5 minutes.

iv. Prepare the standard solution.

b. Trenbolone working solution (500 ng/mL):
   Pipette 50 μL of the stock solution into a 10 mL volumetric flask and dilute to volume
   with methanol.

c. Fortification Solution (50 ng/mL):
   Pipette 1 mL of the working solution into a 10 mL volumetric flask and dilute to volume
   with methanol.

d. Spotting standard #1 (10 ppb):
   Pipette 20 μL of the fortification solution into a 10 mL volumetric flask and dilute to volume
   with methanol.

e. Spotting standard #2 (5 ppb):
   Pipette 5 mL of spotting standard #1 into a 10 mL volumetric flask and dilute to volume
   with methanol.

f. Spotting standard #3 (0 ppb):
   Use 40% methanol for this solution.

3. Storage and Stability
   a. The stock solutions are stable for 6 months if kept in freezer.
   b. The working standard solutions are stable for up to 2 months if kept in freezer.
   c. Spotting standards are stable for up to 2 months if kept in freezer.

E. SAMPLE PREPARATION

Homogenize liver or muscle samples and freeze if samples will not be started the same day
as prepared.

F. ANALYTICAL PROCEDURE

1. Extraction Procedure

   a. Weigh 1 g of ground liver sample into a 15 mL Falcon tube. Weigh 2 blank samples to
      be used as a blank and fortified control. For fortified control, add 100 μL of 50 ng/mL
      fortification solution for a concentration of 5 ppb.

   b. Add 2 mL of 0.5 M sodium acetate buffer, pH 4.8, to each sample and homogenize.
c. Add 8 µL of glucuronidase/arylsulfatase of Helix pomatia.

d. For hydrolysis, cap tube and incubate the solution for 3 h at 37 ± 1 °C, or alternatively over night at room temperature.

e. Add 5 mL of tert-butylmethyl ether to the hydrolyzed product in the 15 mL Falcon tube and shake vigorously for 30 - 60 min.

f. Centrifuge: Approximately 10 min /≈ 3000 g /≈ 10 - 15 °C.

g. Transfer the supernatant to a scintillation vial.

h. Repeat the extraction with another 5 mL of tert-butylmethyl ether.

i. Reduce the combined ether layers to dryness using concentrator and then dissolve with 1 mL of methanol (80%) by shaking on a shaker for 15 min.

j. Dilute the methanolic solution with 2 mL of 20 mM PBS-buffer.

k. Purify the hydrolysis product by means of RIDA® C18 column in the following manner (flow rate: approximately 1 drop per sec):

i. Rinse the column with 3 mL methanol (100%).

ii. Equilibrate the column with 2 mL of 20 mM PBS-buffer, pH 7.2.

iii. Apply sample (volume after hydrolysis or extraction as described).

iv. Rinse the column with 2 mL 40% methanol. Press out residues of rinse-solution, dry column for 3 min by pressing air or N₂ through it.

v. Elute slowly with 1 mL 80% methanol (flow rate: approximately 15 drops per min).

vi. Dilute the eluate 1:2 (1+1) with deionized water.

vii. Use 20 µL per well in the test.

2. Test Procedure

Note: Do not allow microwells to dry between working steps.

a. Insert a sufficient number of wells into the microwell holder for all standards and samples to be run in duplicate. Record the standard and sample positions. The 5 ppb fortified control should be randomly placed into 6 wells throughout the plate.

b. Add 20 µL of each spotting standard solution or prepared sample to separate duplicate wells and add 50 µL of diluted enzyme conjugate to each well.

c. Add 50 µL of the diluted anti-trenbolone antibody solution to each well. Mix gently by rocking the plate manually several times and incubate 2 h at room temperature (20 - 25 °C).

Note: Avoid direct sunlight during all incubations. Covering the microtiter plates is recommended.
d. Pour the liquid out of the wells and tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all wells with 250 µL of deionized water and pour out the liquid again. Repeat two more times.

e. Immediately add 50 µL of substrate and 50 µL of chromogen to each well. Mix gently by rocking the plate manually several times and incubate for 30 min at room temperature (20 - 25 °C) in the dark.

f. Add 100 µL of the stop solution to each well. Mix gently by rocking the plate manually and measure the absorbance at 450 nm against an air blank. Read within 60 minutes after addition of stop solution.

3. Instrumental Settings
Set photometer at 450 nm.

G. CALCULATIONS

1. Evaluate sample results based on absorbance values for fortified control tissue. Trenbolone concentration is inversely related to intensities of yellow color.

2. Calculate the mean and standard deviation (SD) for the absorbance readings of the six 5 ppb control replicates. Use these to determine a decision level (DL) using the formula DL = Mean + 3*SD. Identify the sample as positive if its average absorbance is less than the decision level.

H. SAFETY PRECAUTIONS AND INFORMATION

1. Required Protective Equipment - Lab coat, safety glasses and protective gloves.
2. **Hazards**

<table>
<thead>
<tr>
<th>Procedure Step</th>
<th>Hazard</th>
<th>Recommended Safe Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stop solution</td>
<td>Fumes are corrosive. Spattering may result in serious eye, skin, and respiratory damage.</td>
<td>Prepare solutions in a well-ventilated area such as a fume hood and dispense using repipettors wherever possible. Wear plastic gloves.</td>
</tr>
</tbody>
</table>

3. **Disposal Procedures**

<table>
<thead>
<tr>
<th>Procedure Step</th>
<th>Hazard</th>
<th>Recommended Safe Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stop solution</td>
<td>See above</td>
<td>Neutralize and flush down the sink. Observe all Federal, state and local environmental laws.</td>
</tr>
</tbody>
</table>

I. **QUALITY ASSURANCE PLAN**

1. **Performance Standard**
   a. A plate must meet all of the following criteria:
      i. The external standard curve has absorbances continuously increasing from the 10.0 ng/mL through each lower concentration standard through the 40% methanol blank spotting solution.
      ii. The absorbances increase from the 10.0 ppb to the 0 ppb fortified control tissue wells.
      iii. No false positives for the negative control or false negatives for the positive control.

2. **Critical Control Points and Specifications**
   a. Do not use RIDASCREEN® Trenbolon kit past the expiration date on the kit label. Dilution or adulteration of these reagents may result in loss of sensitivity.
   b. Do not interchange individual reagents between kits of different lot numbers.
   c. Reproducibility in any EIA is largely dependent upon the consistency with which the
microwells are washed. Carefully follow the recommended washing sequence as outlined in the EIA test procedure.

d. Incubations performed at steps 2c and 2e must be carried out at room temperature (20 - 25 °C).

3. Readiness To Perform
   a. Familiarization
      i. Phase I: Standards- Duplicate external standard curves on each of 3 consecutive days, which will include the following:
         (a) 0 ng/mL
         (b) 5.0 ng/mL
         (c) 10.0 ng/mL
      ii. Phase II: Fortified samples - Triplicate analyses at 0, 5, and 10 ppb over a period of 3 different days using bovine liver tissue.
         Note: Phase I and Phase II may be performed concurrently.
      iii. Check samples for analyst accreditation.
         (a) 20 unknown beef liver samples. The sample fortifications including the number of blanks are to be blind to the analyst. 6 - 10 of the 20 samples should be blank and the rest fortified at 5 ppb. All samples should be put in duplicate wells. The samples must be randomized throughout the set. An external curve must be run to help monitor plate acceptability.
         (b) Report analytical findings to the Laboratory Quality Assurance Manager (QAM).
         (c) Letter from QAM is required to commence official analysis.
   
b. Acceptability criteria
      i. No false negatives or positives determined for controls.
      ii. Refer to section I.1 above.

4. Intralaboratory Check Samples
   a. System, minimum contents.
      i. Frequency: 1 weekly per analyst as samples analyzed.
      ii. Records are to be maintained by the analyst
Refer to section I.1 above.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability
   a. Matrix: Beef liver
   b. Sample receipt size: Approximately 200 g.
   c. Sample receipt condition: Frozen
   d. Sample storage:
      i. Sample: 25 weeks if stored frozen at \( \leq -20 \) °C.
      ii. Extract:
          a. Hydrolyzed samples can be stored prior to C18-purification for one or two days at 2 - 8 °C and for two weeks at \( \leq -20 \) °C.
          b. Eluates in 80% or 40% methanol can be stored well-sealed for two months at \( \leq -20 \) °C or two weeks in the refrigerator.

Note: Frequent thawing and freezing should be avoided.

6. Sample Set
   a. Each sample set must contain:
      i. Matrix blank
      ii. Fortified control
      iii. Samples

7. Sensitivity
   a. Lowest detectable level (LDL): Not determined.
   b. Minimum proficiency level (MPL): 5.0 ppb.

J. WORKSHEET
   The worksheet on the following page can be removed for photocopying.
**TRENBOLONE ELISA WORKSHEET**

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<th>Remarks</th>
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<td>tert-Butylmethyl ether</td>
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K. APPENDIX

1. Specificity
   The specificity of the RIDASCREEN® Trenbolon test was determined by analyzing the cross-reactivities to corresponding substances.
   Cross-reactions:
   - 17β-trenbolone: 100%
   - Trendione: 100%
   - 17α-trenbolone: approx. 82%
   - 17β-trenbolone-glucuronide: approx. 82%
   - 17α-trenbolone-glucuronide: approx. 7%
   - 19-nortestosterone: approx. 0.06%
   - Testosterone, estradiol, zeranol, DES and Chloramphenicol: < 0.01%

2. Reference
   RIDASCREEN® Trenbolon, 02-10-28

Approved By:                      Date Approved:

Milo Stubban                        11-22-04
Tom Mallinson                      11-22-04
Terry Dutko                        11-19-04
Sher Ali for Jess Rajan             11-22-04
Charles Pixley                     11-19-04
Phyllis Sparling                   11-19-04

Approvals on file.