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Title: Screening for Zeranol by ELISA		
Revision: 02	Replaces: CLG-ZRL.01	Effective: 02/26/2007

Contents

A. INTRODUCTION

1. Theory

Liver or muscle samples are homogenized with water. Homogenized liver samples are incubated with glucuronidase solution to release bound zeranol.

Free zeranol is extracted from the homogenized muscle or liver hydrolysate with diethyl ether, and the ether extract is taken to dryness. Residues are redissolved in chloroform, and zeranol is partitioned into a NaOH solution. After acidification, zeranol in the aqueous extract is partitioned back into ethyl ether. The ether is evaporated and the residue redissolved in an aqueous buffer. An aliquot of this solution is analyzed using a RIDASCREEN® Zeranol competitive enzyme immunoassay (EIA) ELISA test kit.

2. Applicability

This method is applicable for the qualitative analysis of zeranol in liver or muscle, at levels $\geq$ 0.5 ppb.

B. EQUIPMENT

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

1. Apparatus

a. RIDASCREEN® Zeranol Test Kit -

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

i. 1 x Microtiter plate (12 strips with 8 removable wells each) coated with antibodies directed against anti-zeranol antibodies.

Note: Keep unused wells together with the drying agent and well sealed in the foil bag. Store in refrigerator.

ii. 6 x Zeranol standard solutions, 1.3 mL each, 0 ppt (zero standard), 31.25 ppt, 125 ppt, 500 ppt, 2000 ppt, 8000 ppt zeranol in aqueous solution, ready to use.

Note: A value of less than 0.6 absorbance units ($A_{450\ nm} < 0.6$) for the zero standard may indicate deterioration of reagents.

iii. 1 x Conjugate (0.7 mL), peroxidase conjugated zeranol concentrate, red cap.

Note: The zeranol 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).

iv. 1 x Anti-zeranol-antibody (0.7 mL), concentrate, black cap.

The anti-zeranol 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).

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

vi. 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.

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

viii. 1 x Buffer (50 mL), conjugate, antibody and sample dilution buffer.

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

b. Incubator - 37 °C, Model No. 100, Lab-line Instruments.

c. Nitrogen evaporator (N-Evap) - Model 112, Organomation Associates, Inc.

d. Micropipettes - Adjustable 0 µL to 100 µL, (Gilson Pipetman P100) and 500 µL to 1000 µL, (Labnet VE-1000XR).

e. Pipette tips - 1 - 200 µL, Redi-Tip General Purpose, 21-197-8E, and 1000 µL, Redi-Tip, 21-197-8A, Fisherbrand.

f. Balance - top loader, Model No. PC 440, Mettler.

g. Centrifuge - Damon IEC Centra, Model No. GP8R, International Equipment Company, Needham Heights, MA.

h. Adjustable Volumetric delivery devices - 1 - 5 mL, and 1 - 10 mL Dispensett, Cat. No. 13-986-525D, Brinkman.

i. Centrifuge tubes - 50 mL screw cap Number 8422, Pyrex.

j. Borosilicate disposable culture tubes - Cat. No.14-961-27, Fisherbrand.

k. Explosion Proof ISOtemp combination refrigerator/freezer - Model No. 13-986-525D, Fisher Scientific.

l. Mechanical shaker - flat bed, Eberbach Corporation.

m. Vortexer - Thermolyne Maxi MixPlus, Model Number M63215, Barnstead International.

n. Ultra low temperature freezer - Capable of at least -25 °C, Model No. SLT10LS90APP, Harris.

2. Instrumentation

Microtiter plate spectrophotometer - 450 nm. BioTek Instrument EL x 808, Highland Park, Winooski, VT.

C. REAGENTS AND SOLUTIONS

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

1. Reagents

a. Methanol - Cat. No. 9093-03, J.T. Baker.

b. ß-glucuronidase - Cat. No. G-7770, Sigma.

c. Diethyl ether - Cat. No. 17, 926-4, Aldrich.

d. Chloroform - Cat. No. C297-4, Fisher Optima.

e. Deionized water - Cat. No. PL5112, US Filter.

f. Sodium hydroxide (NaOH) pellets - Cat. No. 3728-05, J.T. Baker.

g. Acetic acid (glacial) - Cat. No. 9507-000, J.T. Baker.

2. Solutions

a. 1 M NaOH:

Weigh 20 g NaOH and dissolve with distilled water. Bring to volume with distilled water in a 500 mL volumetric flask.

b. 90% Acetic acid:

Add 50 mL distilled water and 450 mL acetic acid to a 500 mL volumetric flask. Mix.

D. STANDARDS

Note: Equivalent standard and solutions may be substituted for any of the following.

1. Source

a. The external standards used are supplied with the kit at levels of 0, 0.5, and 2.0 ng/mL.

b. Zeranol (α-zearalanol) - Cat. No. Z 0292, Sigma.

2. Preparation

   a. Zeranol stock solution (200 µg/mL):

      Weigh 20 mg of zeranol into a 100 mL volumetric flask. Dilute to volume with methanol.

   b. Zeranol working solution (0.2 ng/µL):

      Dispense 100 µL of zeranol stock solution into a 100 mL volumetric flask. Dilute to volume with methanol.

   c. Fortification solution (0.02 ng/µL):

      Dispense 10 mL of zeranol working solution into a 100 mL volumetric flask. Dilute to volume with methanol.

   Note: Allow standards to equilibrate to room temperature before use.

3. Storage and Stability

   a. The stock solutions are stable for 1 year if kept in freezer.

   b. The working standard solutions are stable for up to 6 months if kept in freezer.

   c. The kit standards have an expiration date set by the manufacturer and should be stored in the refrigerator with the kit.

E. SAMPLE PREPARATION

Homogenize samples and freeze prior to extraction if analysis is not begun on the same day as preparation.

F. ANALYTICAL PROCEDURE

1. Extraction Procedure

   a. Weigh 1 g of homogenized sample into a 50 mL screw cap centrifuge tube. Weigh 2 blank samples to be used as a blank and fortified control. For fortified control, add 25 µL of 0.02 ng/µL fortification solution for a concentration of 0.5 ppb.

   b. Add 2 mL of distilled water.

   c. Shake vigorously for 10 minutes. Proceed to F.1.e for muscle samples.

   d. Add 8 µL of ß-glucuronidase to liver homogenate and incubate for 2 hours at 37 ± 1 °C.

   e. Add 10 mL diethyl ether, cap, and shake vigorously to check for leaks.

   f. Shake for another 10 min and then centrifuge for approximately 10 min / ≅3,000 g / ≅15 °C.

g. Freeze samples at -25 °C for 60 min or at least -60 °C for 30 min or freeze vial on dry ice / acetone. Decant the ether layer to a centrifuge tube.

h. Allow the aqueous phase to thaw, add 5 mL diethyl ether, shake vigorously and repeat extraction as in steps f - g.

i. Evaporate pooled ether extracts to dryness (at 60 ± 3 °C preferably under a gentle stream of nitrogen).

Stopping point: Dried residues obtained from step F.1.i can be stored capped and frozen for up to 1 month.

j. Dissolve dried residue in 1 mL chloroform.

k. Add 3 mL of 1 M sodium hydroxide and shake vigorously for 30 sec.

l. Centrifuge: approximately 10 min / ≅2,000 g / ≅15 °C.

m. Pipet the sodium hydroxide extract (upper aqueous layer) into a vial containing 250 µL of 90% acetic acid.

n. Add 5 mL diethyl ether, and shake vigorously for 30 sec.

o. Centrifuge: approximately 10 min / ≅2,000 g / ≅15 °C.

p. Freeze at - 25 °C for 60 min or at least -60 °C for 30 min or freeze vial on dry ice / acetone and decant the ether phase into a glass vial.

q. Evaporate to dryness (at 60 ± 3 °C preferably under a gentle stream of nitrogen).

Stopping point: Dried residues obtained from step F. 1. q can be stored capped and frozen for up to 1 month.

r. Dissolve the dried extract in 0.5 mL of sample dilution buffer.

Note: A further dilution of the extract, up to 1:10, in sample dilution buffer may be made to decrease matrix effects.

Stopping point: The ready to use extract obtained from step F.1.r can be stored capped at 2 - 8 °C for up to 3 days.

2. Test Procedure

a. Insert a sufficient number of wells into the microwell holder for all external standards and samples to be run in duplicate. Record standard and sample positions. The 0.5 ppb fortified control should be randomly placed into 6 wells throughout the plate.

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

c. Add 50 µL of the diluted anti-zeranol antibody solution to each well. Mix gently by rocking the plate manually 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 distilled water and pour out the liquid again. Repeat two more times.

e. Add 50 µL of substrate and 50 µL of chromogen to each well. Mix gently by rocking the plate manually and incubate for 30 min at room temperature (20 - 25 °C) covered 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. Zeranol concentration is inversely related to intensities of yellow color.

2. Calculate the mean and standard deviation (SD) for the absorbance readings of the six 0.5 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 INFORMATION AND PRECAUTIONS

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

2. Hazards

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

3. Disposal Procedures

Procedure Step	Hazard	Recommended Safe Procedures
Stop solution	See above	Neutralize and flush down the sink. Observe all Federal, state and local environmental laws.

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 2.0 ng/mL through each lower concentration standard through the EIA buffer.
      ii. The absorbances increase from the 0.5 ppb to the 0 ppb fortified control tissue wells.
      iii. No false positives for the negative control.
      iv. The absorbance of the zero standard must be > 0.6 absorbance units or at the level specified in the kit.

2. Critical Control Points and Specifications
   a. Do not use RIDASCREEN® Zeranol 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. Do not allow microwells to dry between working steps.
   d. 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.

3. Readiness to Perform
   a. Familiarization
      i. Phase I: Standards- Duplicate standard curves on each of 3 consecutive days, which will include the following:
         (a) 0 ng/mL
         (b) 0.5 ng/mL
         (c) 2.0 ng/mL

ii. Phase II: Fortified samples - Triplicate analyses at 0, 0.5, and 1.0 ppb over a period of 3 different days.

Note: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 20 unknown 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 0.5 ppb.

(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 determined for controls at the 0.5 ppb level.

ii. Refer to section I.1 above.

4. Intralaboratory Check Samples

a. Frequency: 1 weekly per analyst when samples are analyzed.

b. Acceptability criteria.

Same as I.3.b. 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 and muscle

b. Sample receipt size: Approximately 200 g.

c. Sample receipt condition: Chilled or Frozen.

d. Sample storage:

i. Sample: $\leq$ 6 months if stored frozen.

ii. Extract:

(a) Dried residues obtained from step F.1.h and F.1.p can be stored frozen for up to 1 month.

(b) The ready to use extract obtained from step F.1.q can be stored at 2 - 8 °C for up to 3 days.

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

7. Sensitivity
   a. Minimum proficiency level (MPL): 0.5 ppb.

J. WORKSHEET

The following worksheet is an example.

Weighed _____ *Analyst* _____ *Date* _____

Analysis Started _____

Analysis Completed _____

Reviewed By: _____

Frz__ to Frz__ _____

Frz__ to Frz__ _____

Set Number: _____

Reader Wavelength (nm) _____

Time

Incubator, AM/PM _____

Incubator, AM/PM _____

Centrifuge _____

Freezer 1 _____

Evaporator1 _____

Freezer 2 _____

Evaporator2 _____

Temp

Equipment	ID #
Shaker	
Balance	
Centrifuge	
Evaporator	
Micropettor	
Incubator	
Plate reader	
Freezer	
Refrigerator (for extracts)	

Standards/Reagents Used	ID #
Zeranol Std.	
diethyl ether	
1M Sodium Hydroxide	
β-Glucuronidase	
chloroform	
90% Acetic Acid	
Sample dilution buffer	
DI H2O	
Zeranol Elisa Kit	

Position Number	Lab Number	Form Number	Wt. of sample 1.00 ± 0.10 g	Result (+) or (-)
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				

Position Number	Lab Number	Form Number	Wt. of sample 1.00 ± 0.10 g	Result (+) or (-)
25				
26				
27				
28				
29				
30				
31				
32				
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47				
48				

Remarks:

K. APPENDIX

1. Cross-reactivity:

   Zearalanone.....ca. 2 %
   ß-Zearalanol.....ca. 4 %
   Zearalenone..... < 0.5 %
   α-Zearalenol.....ca. 21 %
   ß-Zearalenol.....ca. 2.5 %
   17ß-Estradiol..... < 0.02 %
   Clenbuterol..... < 0.02 %
   Testosterone ..... < 0.02 %

2. Reference

   RIDASCREEN® Zeranol, 02-06-12.
   Note: If package insert changes, use up to date instruction sheet from the test kit.

L. APPROVALS AND AUTHORITIES

1. Approvals on file.
2. Issuing Authority: Laboratory Quality Assurance Division.