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A. INTRODUCTION

1. Theory

This qualitative method for the rapid screening of clenbuterol, salbutamol, and cimaterol in animal retinal tissue uses a simple one-step extraction procedure. The choroid/pigmented retinal epithelium (choroid/PRE) is excised, mixed with a phosphate buffer and mashed using a glass rod. The tissue extract is centrifuged at high speed to produce a clear supernatant. The supernatant is then analyzed using the enzyme linked immuno-assay (EIA) kit, which determines analyte concentration as a function of color intensity at 650 nm.

2. Applicability

This ELISA method is applicable to bovine, ovine, porcine, and caprine retinal tissue for clenbuterol at levels ≥ 3 ppb and applicable to bovine and porcine retinal tissue for salbutamol at levels ≥ 3 ppb and cimaterol at levels ≥ 6 ppb.

B. EQUIPMENT

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

1. Apparatus

a. Test Kit ELISA Generic Bronchodilator Kit - Neogen Corporation, ELISA Technologies Division, 628 E. 3rd Street, Lexington, Kentucky, CAT #100310, ELISA Technologies Div.

b. Plate Reader - Biotek Autoreader EL 311, with printer (650 nm filter used) ELISA Technologies.


d. pH meter - Orion 601A, calibrated at pH 4 and 7.

e. Eppendorf pipettors - Variable volume, 5 µL to 1000 µL, Brinkmann Instruments, Inc., Westbury, NY.

f. Transferpette - Multichannel Pipettes, Brinkmann, 50 - 200 µL #50-08-030-7, CAT #53512-376 VWR, Denver, CO.

g. Volumetric Glassware - 100 mL and 10 mL amber flasks Class A; 1.0 mL and 0.5 mL glass pipettes Class A.
h. Glassware - 50 mL polyallomer tube with polypropylene screw closure (CAT #3139-0050, Nalge Company, Rochester NY). Glass rods, 10 mm in diameter by 25 cm, fired and rounded at both ends. Disposable culture tubes, Borex, 12 x 75 mm.


j. Petri dish - disposable polystyrene, Optilux 100 x 20 mm style, CAT #1005, Becton Dickinson Labware, Lincoln Park, NJ.


C. REAGENTS AND SOLUTIONS

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

1. Reagents
   a. Sodium Phosphate, dibasic, anhydrous (Na₂HPO₄) - Aldrich Chemical Company, Inc., Milwaukee WI.
   b. Potassium Phosphate, Monobasic crystals (KH₂PO₄) - Mallinckrodt.
   c. Deionized water.
   e. 1M HCL - Fisher Scientific.
   f. 1M NaOH - Fisher Scientific.

2. Solutions
   Extraction Buffer:
   Weigh 13.6 g of potassium phosphate monobasic and 14.2 g of sodium phosphate dibasic into a 1 L class A volumetric flask or graduated cylinder. Dilute to volume with deionized water. Adjust pH to 6.8 with 1M HCl or 1M NaOH solutions. Prepare fresh extraction buffer when solution becomes cloudy. The pH should be checked periodically to verify that it is 6.8.

D. STANDARDS

Note: Equivalent standards may be substituted for the following items.

1. Source
   a. Clenbuterol HCl (CLEN) - Sigma Chemical Co., St. Louis MO, CAT#C-5423, approximately 95% pure.
b. Cimaterol (CIM) - MP Biochemicals Inc., Aurora, OH, CAT# 159757.

c. Salbutamol (SAL) - Sigma Chemical Co., St. Louis MO, CAT# S-8260, approximately 95% pure.

2. Preparation of Standards
   a. Clenbuterol standards
   
      i. Stock CLEN standard solution (25 µg/mL):
         Accurately weigh 2.5 ± 0.1 mg clenbuterol standard into a 100 mL amber volumetric flask. Dissolve and bring to volume with methanol.

      ii. Intermediate CLEN standard solution (250 ng/mL):
         Pipet 1.00 mL ± 0.01 mL CLEN stock standard into a 100 mL amber volumetric flask and bring to volume with water.

      iii. Working CLEN standard solution (12.5 ng/mL):
         Prepare daily. Pipet 0.10 mL ± 0.01 mL CLEN Intermediate Standard Solution into a 10 mL volumetric flask and bring to volume with Extraction Buffer.

         Plate standard series: Into individual 12 x 75 mm test tubes containing 0.4 mL, pipet 100 µL, 50 µL, 25 µL, 10 µL, and 5 µL of Working CLEN Standard Solution. Vortex to mix.

         The standard series: 2.5 ng/mL, 1.4 ng/mL, 0.7 ng/mL, 0.3 ng/mL, 0.2 ng/mL CLEN - EIA buffer (found in the test kit) are applied (0.02 mL) to the ELISA plate.

      iv. CLEN fortification solution (0.75 ng/mL):
         Pipet 0.075 mL of the intermediate CLEN standard solution into a 25.0 mL volumetric flask. Dilute to volume with Extraction Buffer.

   b. Salbutamol standards
   
      i. Stock SAL standard solution (25 µg/mL):
         Accurately weigh 2.5 ± 0.1 mg SAL standard into a 100 mL amber volumetric flask. Dissolve and bring to volume with methanol.

      ii. Intermediate SAL standard solution (250 ng/mL):
         Pipet 1.00 mL ± 0.01 mL SAL stock standard into a 100 mL amber volumetric flask and bring to volume with water.

      iii. SAL fortification solution (0.75 ng/mL):
         Pipet 0.075 mL of the intermediate SAL standard solution into a 25.0 mL volumetric flask. Dilute to volume with Extraction Buffer.
c. Cimaterol standards

i. Stock CIM solution (25 µg/mL):
   Accurately weigh 2.5 ± 0.1 mg CIM standard into a 100 mL amber volumetric flask. Dissolve and bring to volume with methanol.

ii. Intermediate CIM standard solution (250 ng/ml):
   Pipet 1.00 mL ± 0.01 mL CIM stock standard into a 100 mL amber volumetric flask and bring to volume with water.

iii. CIM fortification solution (1.5 ng/ml):
   Pipet 0.15 mL of the intermediate CIM standard solution into a 25.0 mL volumetric flask. Dilute to volume with Extraction Buffer.

3. Storage and Stability

a. Stock and intermediate standards are stable for 2 months if stored in the refrigerator.

b. Working CLEN, SAL, and CIM solutions must be prepared fresh daily and should be kept at room temperature until use.

E. SAMPLE PREPARATION

1. Eyeballs should be thawed at room temperature just enough so that the outside tissues can be manipulated but the aqueous and vitreous humors are still frozen. This takes about 45 to 60 minutes. The eyeball will not deform any noticeable amount when squeezed.

2. With a razor blade, incise the eyeball by slowly cutting across the cornea horizontally and vertically. The length of the incisions should include the full width and length of the cornea, and extend into the sclera to allow the eyeball to be evened. Force the semi-frozen contents (aqueous and vitreous humors and lens) out of the eyeball and retain in a separate whirl-pak bag. (Eye contents may be discarded if retinal results are negative for clenbuterol.)

3. Evert the eyeball, and scrape the choroid/PRE layer (distinctive in bovine, ovine, and caprine due to bluish-green coloration on black) and neural retina (black filmy tissue emanating from the optic nerve area) into a petri dish. With a new razor blade, mince the tissue into fine pieces prior to weighing.

Note: The retina is composed of ten layers. The innermost nine layers are called the neural retina, which is unpigmented. The tenth layer of the retina is the highly pigmented epithelium and is intimately connected to the choroid, which is a pigmented, vascular coat of the eye. “Detached retina” refers to a condition in which the neural-retina (inner
nine layers) is separated from the pigmented epithelium and, upon dissecting the eye, is found as a dull, gray, fold of tissue, approximately 30 - 50 mm in length, attached only at the optic disc (depressed area where optic nerve attaches to the back of the eye). The everted eyeball is smooth and darkly pigmented with an iridescent metallic sheen (tapetum lucidum). This layer is the PRE with the adjacent choroid.

Everted porcine eyes do not display the greenish-blue metallic sheen. Typical choroid/PRE yields for bovine, ovine, caprine, and porcine are: 1.5 g, 0.8 g, 0.8 g, and 0.2 g respectively.

F. ANALYTICAL PROCEDURE

1. Weigh 0.1 g minced bovine, ovine, caprine, or porcine retinal tissue into a 50 mL polyallomer centrifuge tube. (When retinal tissue from a porcine eye is limited, weigh 0.05 g retinal tissue into a 50 mL centrifuge and add 0.2 mL Extraction buffer.)

Weigh six different blank retinal tissues to use for calculating the Decision Level (DL).

2. Weigh four 0.1 g retinal control tissues. Use one as the control blank and fortify the other three as follows:
   a. Recovery 1: 400 μL of 0.75 ng/mL CLEN fortification solution (approx. 3 ppb),
   b. Recovery 2: 400 μL of 0.75 ng/mL SAL fortification solution (approx. 3 ppb), and
   c. Recovery 3: 400 μL of 1.5 ng/mL CIM fortification solution (approx. 6 ppb).

   Distribute fortifications randomly into at least 2 wells.

3. Add 0.40 mL ± 0.01 mL Extraction Buffer to centrifuge tube.

4. Vigorously mash retinal tissue with glass rod for one minute.

5. Cap and centrifuge tube at 15,000 RPM for 15 minutes.

6. Pour supernatant into a 12 x 75 mm test tube.

7. Aliquot 0.020 mL supernatant into duplicate wells of the Generic Bronchodilator ELISA plate.

8. Following ELISA kit directions, add 180 μL diluted Terbutalene-HRP Conjugate Solution (HRP) to each well. The HRP solution and EIA buffers are supplied in the test kit and are diluted 1:180 HRP:EIA. After addition of the HRP:EIA solution to each sample, mix the solutions by gently vibrating the plate on a flat surface.

9. Cover plate to avoid possible dust/dirt contamination. Incubate for one hour at room temperature. Shake gently at least twice during the incubation period.
10. Invert the plate after the incubation period, to remove matrix solutions.

11. Wash the wells four times with 300 µL each of diluted washing buffer (diluted according to kit instructions supplied in the test kit). Tamp the inverted plate on a paper towel.

12. Add 150 µL of the K-Blue substrate supplied in the test kit to each well. Allow the reaction to proceed for 15 minutes with intermittent gentle shaking of plate, especially before taking an absorbance reading.

13. Set the plate reader at 650 nm. Read the plate when the absorbance of the control tissue blank reads between 1 and 2 (typically 30 minutes).

G. CALCULATIONS

Note: Since the ELISA antibody integrates its response over the entire spectrum of compounds that it recognizes, it is not possible to determine the identity or actual concentrations of the beta-agonists present in a positive sample. All positives are identified as ppb Clenbuterol.

Evaluate sample results based on absorbance values for blank control tissues. With this ELISA, beta agonist concentration is inversely related to color intensity. (The highest concentration of beta agonist results in a very pale blue-whereas the reagent blank is a deep blue color).

Calculate the mean and standard deviation (SD) for the mean absorbance readings of the duplicate wells for the six blanks (6 values total). Use these to calculate a decision level using the formula: DL = Mean - 3*SD. Average the duplicate wells for each sample. A sample will be identified as positive if its absorbance is ≤ the DL.

H. SAFETY PRECAUTIONS AND INFORMATION

1. Required Protective Equipment - Lab coat, safety glasses. Gloves should be worn when working with the eyeballs.

2. Hazards

<table>
<thead>
<tr>
<th>Procedure Step</th>
<th>Hazard</th>
<th>Recommended Safe Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>This solvent may be flammable and may produce toxic effects to skin, eyes and the respiratory system.</td>
<td>Use reagents in an efficient fume hood away from all electrical devices and open flames.</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>Methanol</td>
<td>See Above</td>
<td>Collect waste in tightly sealed container and store away from non-compatibles in a cool, well</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state,</td>
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<td>and Federal regulations.</td>
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</tbody>
</table>

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. A plate should meet all the following criteria:
      i. The control tissue absorbance should be between 1.0 and 2.0 absorbance units vs. air,
      ii. The standard curve has absorbencies continuously increasing from the 2.5 ng/mL through each lower concentration standard through the EIA buffer,
      iii. The absorbencies continuously increase from the 3 ppb to the 0 ppb fortified control tissue wells, and,
      iv. A variability of less than ± 25% between duplicate sample wells is obtained. Determine acceptable variability by using the following:
         \[
         \frac{\text{Larger Absorbance Value}}{\text{Smaller Absorbance Value}} \leq 1.25
         \]
      v. The control blank must fall within the Mean ± 3 SD range.
      vi. The CV for the six duplicate blank replicates must be ≤ 20%.
2. Critical Control Points and Specifications

<table>
<thead>
<tr>
<th>Record</th>
<th>Acceptable Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Variability between</td>
<td>± 25%</td>
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<tr>
<td>duplicate wells.</td>
<td></td>
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<tr>
<td>b. Time of retinal tissue</td>
<td>At least 10 minutes.</td>
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<td>contact with extraction</td>
<td></td>
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<tr>
<td>solution.</td>
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<tr>
<td>c. After applying aliquots to the ELISA plate the plate is devoid of</td>
<td>Evacuate plate of all liquid or bubbles prior to the addition of the</td>
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<td>liquid prior to the</td>
<td>K-blue solution.</td>
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<tr>
<td>addition of the K-blue</td>
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<tr>
<td>solution.</td>
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</table>

3. Readiness To Perform  (FSIS Training Plan)

a. Analyst Training
   i. Phase I: Standards- Duplicate external standard curves for Clenbuterol on each of 3 consecutive days, which will include the following:
      (a) Blank
      (b) 0.2 ng/mL
      (c) 0.3 ng/mL
      (d) 0.7 ng/mL
      (e) 1.4 ng/mL
      (f) 2.5 ng/mL
   
   ii. Phase II: Analyst fortified samples
      (a) 3 replicates at 0, 3.0, and 6.0 ppb for Clenbuterol over a period of 3 different days.
      (b) 3 replicates at 0, 3.0, and 6.0 ppb for Salbutamol over a period of 3 different days.
      (c) 3 replicates at 0, 6.0, and 12.0 ppb for Cimaterol over a period of 3 different days.
iii. Phase III: Check samples for analyst accreditation.

   (a) 30 unknown beef or swine eyeball samples. The sample fortifications including the number of blanks are to be blind to the analyst. 10 -15 of the 30 should be blank and the rest spiked at 3 ppb for Clenbuterol and Salbutamol and 6 ppb for Cimaterol. 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 at the 3 ppb level for Clenbuterol and Salbutamol and 6 ppb for Cimaterol.

   ii. Refer to section I.1 above.

4. Intralaboratory Check Samples

   a. System, minimum contents.

      i. Frequency: At least 1 weekly per analyst if samples analyzed.

      ii. Records are to be maintained by the analyst and reviewed by the supervisor and Laboratory QAM for positive or negative results for QA samples.

   b. Acceptability criteria.

      i. No false negatives at the 3 ppb level for Clenbuterol and Salbutamol and 6 ppb for Cimaterol.

      ii. Refer to section I.1 above.

   c. 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: Retinal tissue

   b. Sample receipt size: Eyeball
c. Sample receipt condition: Frozen
d. Sample storage:
   Time: Eyeballs should be stored at -20 °C or lower. They can be kept for one year. Extracted retinal tissue may be stored for one week at -40 °C or lower (longer tends to dry the tissue).

6. Sample Set
   a. Each sample set must contain:
      i. 6 different blanks
      ii. Blank control
      iii. 3 Fortified controls. (Two at 3 ppb Clenbuterol and Salbutamol, and one at 6 ppb Cimaterol).
      iv. Samples
      v. Standards at 2.5, 1.4, 0.7, 0.3, and 1.2 ng/mL Clenbuterol.

7. Sensitivity

J. WORKSHEET

An example of a worksheet on the following page can be removed for photocopying.
**United States Department of Agriculture**  
**Food Safety and Inspection Service, Office of Public Health Science**

**SOP No: CLG-CLN3.01**  
**Page 12 of 13**  
**Title: ELISA Screening for β-Agonist Residues in Animal Retinal Tissue**  
**Revision: .01**  
**Replaces: .00**  
**Effective: 7-19-04**

### BETA-AGONIST WORKSHEET

**DATE:**  
**ANALYST:**  
**SUPERVISOR REVIEWED BY:**  
**REVIEWED BY:**

<table>
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</tbody>
</table>

**INSTRUMENTS AND SETTING**

- **PLATE READER:**
- **PLATE READER FILTER:**
- **PLATE READER MODE: ABS1**
- **CENTRIFUGE:**
- **CENTRIFUGE SPEED:**
- **CENTRIFUGE TIME:**
- **CENTRIFUGE TEMP:**
- **CENTRIFUGE ROTOR:**
- **PIPETTE:**
- **FREEZER:**
- **BALANCE:**
- **pH METER:**
- **REFRIGERATOR:**

### STANDARDS AND REAGENTS

<table>
<thead>
<tr>
<th></th>
<th>CLEN</th>
<th>SAL</th>
<th>CIM</th>
<th>STANDARDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>BETA-AGONIST STOCK:</td>
<td></td>
<td></td>
<td></td>
<td>0.2 ng/mL</td>
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<tr>
<td>BETA-AGONIST INTERMEDIATE:</td>
<td></td>
<td></td>
<td></td>
<td>0.3 ng/mL</td>
</tr>
<tr>
<td>BETA-AGONIST WORKING SOL:</td>
<td></td>
<td></td>
<td></td>
<td>0.7 ng/mL</td>
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<tr>
<td>BETA-AGONIST SPiking SOL: 0.75 ng/mL</td>
<td></td>
<td></td>
<td></td>
<td>1.4 ng/mL</td>
</tr>
<tr>
<td>BETA-AGONIST SPiking SOL: 1.5 ng/mL</td>
<td></td>
<td></td>
<td></td>
<td>2.5 ng/mL</td>
</tr>
<tr>
<td>BETA-AGONIST SPiking SOL: 3 ng/mL</td>
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</tr>
</tbody>
</table>

**Calculations:**
- Amount of drug enzyme conjugate needed. Calculation A
  - A = No. of wells used ___ + 8 = ___(round to the nearest 5's)

**PHOSPHATE BUFFER:**  
Sample size = 0.10 gram

**WASH BUFFER:**  
Weighing Date: _____  
Weighing Analyst: _____

**DILUTED DRUG ENZYME CONJUGATE:**

**KIT #:**

**COMMENTS :**

- Amount of deionized water needed: C - 10%C (0.1 x ___) = _____
- Amount of wash buffer needed: 10% of C 0.1 x ____ = _____
K. REFERENCE

Susan B. Clark, W. Douglas Rowe, and Jeffrey A. Hurlbut, FDA Laboratory Information Bulletin #43, July 1996.

Approved by:

Stephen Powell
Leon Ilnicki
David Martin
Jess Rajan
Charles Pixley
Phyllis Sparling

Approvals on file.