United States Department of Agriculture
Food Safety and Inspection Service, Office of Public Health and Science

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Title: Phenylbutazone Residues in Bovine Kidney by ELISA.		
Revision: .00	Replaces: NA	Effective: 11/8/2002

Contents

Approval **Signatures on file**

A. INTRODUCTION

1. Theory

   This procedure is used to screen bovine kidney samples for Phenylbutazone residues at concentrations of 50 ppb or higher. Cotton swabs are inserted into kidney samples to absorb tissue fluids. The swabs are extracted with a dilute acidic phosphate buffer and analyzed using a commercial Phenylbutazone ELISA kit (PBZ ELISA kit, Neogen Corporation). Samples exhibiting absorbances lower than an estimated minimum for a 50 ppb fortified tissue are identified as positives.

2. Applicability

   The method is applicable to bovine kidney.

B. EQUIPMENT

1. **Apparatus**

Equivalent apparatus may be substituted for the following:

a. Test Kit. PB ELISA kit. - Cat. # 9 104710 (NEOGEN Corporation, ELISA Technologies Division, 628 E. 3rd Street, Lexington, Kentucky 40505). The kit is composed of 12 eight-well strips in a plastic (6x12x1.5 cm) holder. Each strip is removable from the kit. In addition, the strip of wells may be broken so that a partial strip may be used for analysis.

b. Plate Reader. Biotek Autoreader ELx 808. - Equipped with650 nm filter and a printer (ELISA Technologies). The reader is calibrated using an U.S. National Bureau of Standards Microplate Reader Calibration Plate Accuracy Test Filter

c. Centrifuge. - International Equipment Company B-22M Superspeed Refrigerated Centrifuge with Rotor #876, (Refrigeration is not required) International Equipment Company, Clinical Centrifuge (CAT #20671-007, VWR Scientific, San Francisco, CA).

d. Eppendorf pipettors - Variable volume pipettes were obtained from Fisher Scientific: 2-20 µL (Cat. #05-402-46), 10-100µL (Cat #05-402-48), 50-200 µL (Cat. #05-402-49), 100-1000 µL (Cat. #05-402-50) and 500-2500µL (CAT. #05-402-51).

e. pH meter - Orion 601A Calibrated at pH 4 and 7.

f. Transferpette, Multichannel Pipettes. - Brinkmann, 50-300µL #50-08-030-7 (CAT #53512-376 VWR Box 39396 Denver, CO 80239).

g. Glassware - Volumetric glassware includes 10-mL, 25-mL, 50-mL and 100-mL flasks. CLASS A.

h. Pipettes - 0.5-mL, 1.0-mL, 25 mL and 50-mL glass pipettes CLASS A.

i. Test tubes - 16mm x 125mm disposable Borosilicate glass culture tubes, (Kimble).

j. Test tubes.- 50-mL polyallomer tube with polypropylene screw closure. - CAT #3139-0050, (Nalge Company, Rochester NY 14602-0365).

k. Sterile Cotton-tipped swabs - 6" x 1/12" in diameter (two per package) Cat # 10805-154 (VWR Scientific Products).

C. REAGENTS AND SOLUTIONS

1. Reagents

Note: Equivalent reagents may be substituted for the following:

a. Sodium Phosphate, dibasic, anhydrous. Na_2HPO_4, (Aldrich Chemical Company, Inc., Milwaukee WI 53233).

b. Potassium Phosphate, Monobasic crystals. KH_2PO_4. (Mallinckrodt).

2. Solutions

Note: Solutions and solvents may be substituted for the following:

a. Extraction Buffer (0.2M Phosphate). - 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 extraction buffer fresh when it becomes cloudy. The pH should be checked periodically to verify that it is 6.8.

b. Deionized water.

c. Methanol, LC-Grade (Fisher Scientific).

d. 1M HCL (Fisher Scientific).

e. 1M NaOH (Fisher Scientific).

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D. STANDARDS

1. Source

Phenylbutazone standard is available from ICN Biomedical Inc., Cat. #153567, Chillicithe Rd, Aurora, OH 44203. Other sources for the standard material may be acceptable.

2. Preparation of Standards

   a. Stock PBZ Standard Solution 500 µg/mL in methanol. - Accurately weigh 50.0 ± 0.1mg PBZ standard into a 100-mL volumetric flask. Dissolve and bring to volume with methanol. Stable for two months.

   b. Intermediate PBZ Standard Solution. 250 µg/mL in methanol. - Pipet 25 mL PBZ stock standard (a) into a 50 mL volumetric flask and bring to volume with methanol.

   c. Working PBZ Standard Solutions:

      i. 150 ng/mL. - Dilute 30 µL of intermediate standard (b) to 50 mL. with deionized water in a 50-mL volumetric flask.

      ii. 300 ng/mL. - Dilute 60 µL of intermediate standard (b) to 50mL with deionized water in a 50-mL volumetric flask.

      Note: These working standard solutions are used to generate the standard curve and for sample fortification.

3. Storage conditions

Store standards (a) & (b) in a refrigerator at 2-8ºC, and standard (c) at room temperature.

4. Shelf Life Stability

   a. Stock and intermediate standards are stable for 2 months.

   b. Working PBZ solution (D.2.c) must be prepared fresh daily.

E. SAMPLE PREPARATION

After slaughter, intact kidney samples (one/cow) are frozen and then shipped overnight with the cold-frozen gel-packs to the laboratory. The samples are stored in the freezer at -20ºC. Prior to analysis, the samples are brought to room temperature and two swabs are inserted into two separate locations on each kidney sample for 30 minutes to absorb kidney tissue fluids. Swabs are stored at -40ºC if not analyzed immediately.

1. Control tissue

One pair of control kidney tissue swabs is run with each plate. Control is generally a (store-bought bovine kidney) previously analyzed and found to contain no PBZ. Blended control kidney tissue is stored in plastic bag in a freezer at -20ºC.

2. Fortified tissue

Control kidney tissue (blended) is fortified at three levels (0, 50, and 100 ppb) and one set of swabs at each level is run with each day's samples. The levels are pre-determined to be within the linear range of the ELISA plate based on the conditions of the extraction. The tissue portions are prepared as follows:

Fortification	Tissue (g)	Amount of PBZ added
50 ppb	4	2.0 mL of 150 ng/mL (D.2.c.i)
100 ppb	4	2.0 mL of 300 ng/mL (D.2.c.ii)

Each fortification level is based on the concentration of PB added to the tissue divided by the total g of product (4 g of tissue plus 2 g of water or standard solution = 6 g total). Approximately 0.3 mL of tissue fluid is absorbed by each pair of cotton swabs per sample and each pair of swabs is then placed in 1.0 mL of phosphate buffer giving an approximate concentrations of 0 ng/mL for control blank, 15 ng/mL for 50 ppb fortification and 30 ng/mL for 100 ppb fortification. Application of 20 µL of these solutions on each well represents 0 ng, 0.3 ng and 0.6 ng of PBZ, respectively for 0, 50 and 100 ppb fortifications.

F. ANALYTICAL PROCEDURE

1. For Analysis of samples

   a. Place each pair of sample swabs, a pair of control swabs and a pair of 50 ppb control swabs separately in 1.0 mL of 0.2M pH 6.8 phosphate buffer in disposable test tubes (B.1.i) and agitate vigorously for 1 min on a vortex mixer.

   b. Allow the swabs to sit in solution for 5 min with sonication, then agitate again.

   c. After approximately 5 min, squeeze both swabs against the tube sidewall, and discard (2 swabs contain ca. 0.3 mL tissue fluids).

   d. Centrifuge the tubes for 2 min at approximately 1000 RPM.

2. Control and Fortified Kidney Swab Preparation

a. Weigh 4 control kidney blanks (4 g each) in 50-mL polypropylene centrifuge tubes as described in section E.2. above. Use one of the four tubes as control.

b. Add 2.0 mL of 150 ng/mL working standard (D.2.c.i) separately to two tubes and 2.0 mL of 300 ng/mL working standard (D.2.c.ii) to the remaining tube.

c. Add 2 mL of water to the control tube.

d. Agitate tubes vigorously for 1 min using a Vortex mixture and sonicate for 1 min.

e. Centrifuge tubes for 15 min at 15,000 RPM at 4ºC.

f. Decant each supernatant into another set of disposable test tubes.

g. Place two cotton swabs into each supernatant to absorb the kidney/water fluid for approximately 2-3 min.

h. Place each pair of sample swabs in 1.0 mL of 0.2M pH 6.8 phosphate buffer in a disposable test tube (B.1.i) and agitate vigorously for 1 min on a vortex mixer.

i. Allow the swabs to sit in solution for 5 min with sonication, and then agitate again.

j. After approximately 5 min, squeeze both swabs against the tube sidewall, and discard.

k. Centrifuge the tubes for 2 min at approximately 1,000 RPM at 4ºC.

3. Analysis

a. Apply 20 µL extract into individual ELISA plate wells.

Note: One of the 50-ppb fortified control extracts must be distributed randomly into 6 wells in order to facilitate estimation of a decision level (see Section G).

b. Following ELISA kit directions, add 180 µL diluted drug-HRP conjugate solution (HRP) to each well. The HRP solution and EIA buffer are supplied in the test kit and are diluted (1:180 HRP: EIA) or per Kit Instruction. After addition of the HRP: EIA solution to each sample, mix the solutions by gently vibrating the plate on a flat surface.

c. Cover plate to avoid possible dust/dirt contamination. Incubate for one hour at room temperature. Shake gently at least twice during the incubation period.

d. Invert the plate after the incubation period, to remove matrix solutions.

e. Wash the wells 4 times with (300µL/time) with diluted washing buffer (diluted 1:9 with water and supplied with the test kit). Tamp the inverted plate on a paper towel between washings and make sure that the plate is free from liquid and bubbles prior to adding K-Blue substrate.

f. Add 150 µL K-Blue -substrate (another reagent supplied in the test kit) to each well. Allow the reaction to proceed for 15–30 minutes with intermittent gentle shaking of plate, especially before taking an optical density (absorbance) reading.

g. Read results at 650nm on a plate reader. Continue to take readings every five minutes until an optimum absorbency reading (although the absorbency values continue to increase, “optimum" is considered to be between 1.0 and 2.0) is obtained for the control tissue (read against air). Color development may take 20 to 30 minutes.

2. Instrumental Settings —

Read plate at 650 nm.

G. CALCULATIONS

Evaluate sample results based on absorbance values for fortified control tissues. The extent of the color development in each well is inversely proportional to the amount of drug in the sample or control.

Calculate the mean and standard deviation (SD) for the absorbance readings of the six 50-ppb fortified control tissue replicates (see F.3.a.). Use these to calculate a decision level (DL) using the formula DL = Mean + 3*SD. A sample will be identified as positive if its absorbance is less than the decision level.

Since the use of PBZ in food animals is not approved in the United States, marginally positive sample (extract) must be reanalyzed in duplicate wells to make sure that the sample is PBZ positive before sending out for confirmation.

H. HAZARD ANALYSIS

1. Method Title. — Phenylbutazone Residues in Bovine Kidney by ELISA.

2. Required Protective Equipment. — Lab coat, safety glasses and gloves.

3. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Methanol	This solvent is flammable and may produce toxic effects to skin, eyes and the respiratory system.	Use reagents in an efficient fume hood away from all electrical devices and open flames.
Acid and base	Corrosive	Wear gloves and safety glasses.

4. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*

Methanol	See Above	Collect waste in tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.
Acid and base	Can cause burns on skin and eye injury	Neutralize the acid or base for disposal down the drain in accordance with local, state, and Federal regulations.
Used plates	None	Dispose of plates in accordance with local, state, and Federal regulations

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range*	*Acceptable Recovery*	*Acceptable Repeatability (CV)*
PBZ	50 - 100 ppb	N/A	N/A

a. Each sample set must meet all the following criteria before data can be reported:

i. The control tissue absorbance should be between 1.0 and 2.0 absorbance units vs. air.

ii. The standard curve absorbance values continuously increase from the 100 ppb to the 0 ppb concentration in HRP-EIA buffer solution.

iii. The absorbence values continuously increase from the 100 ppb to the 0 ppb fortified control tissue wells.

iv. The CV calculated for the six spiked replicates should be less than or equal to 20%.

b. No false negatives at 50 ppb.

2. Critical Control Points and Specifications

	Record	***Acceptable Control***
a.	E: Swab contact with kidney fluids	At least 30 minutes.
b.	F.1.b-c & f.2.i-j: Swab contact with extraction buffer.	At least 10 minutes.
c.	F.3.e: After washing the ELISA plate make sure that the plate is free of bubbles and liquid prior to proceeding to next step of adding K-blue solution.	Evacuate plate of all liquid or bubbles prior to the addition of the K-blue solution.

3. Readiness To Perform

a. Analyst Training

i. Phase I: Standards- Duplicate external standard curves on each of 3 consecutive days, which will include the following:

(a) 0 ng/mL (0 ppb): buffer solution.

(b) 15 ng/mL (50 ppb): Dilute100 µL of Working Standard (D.2.c.i) to 1 mL with buffer solution.

(c) 30 ng/mL (100 ppb): Dilute 100 µL of Working Standard (D.2.c.ii) to 1mL with buffer solution.

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ii. Phase II: Fortified samples- 3 sets of 10 blank and 10 fortified samples at 50 ppb level 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.) The analyst is to receive 30 ground beef kidney samples. The sample fortifications including the number of blanks are to be blind to the analyst. At least 10 of the 30 samples should be blank and the rest spiked at 50 ppb level. These samples will be prepared and provided by Supervisor or QA Manager (QAM). All samples should be analyzed in duplicate wells. The samples must be randomized throughout the set.

(b.) An external curve at 0 ng/mL, 15 ng/mL and 30 ng/mL (equivalent to 0, 50, and 100 ppb, respectively) must be run to help monitor plate acceptability.

(c) Report analytical findings to the Laboratory Quality Assurance Manager.

(d) A letter from the QAM is required to commence official sample analysis.

b. Acceptability criteria.

i. No false negatives at the 50 ppb level.

ii Refer to section J.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

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

ii. Records of check sample results are to be maintained by the analyst and reviewed by the supervisor and Laboratory QAM.

b. Acceptability criteria.

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: Bovine kidney.

b. Sample receipt size: One intact kidney/cow.

c. Sample receipt condition: Frozen.

d. Sample storage: Kidney at -20ºC and fortified control swabs at -40ºC for one week.

6. Sample Set

a. Each sample set should contain
   i. One control tissue blank,
   ii. 3 fortified tissues: two at 50 ppb and one at 100 ppb levels.
   iii. Samples.

7. Sensitivity
Minimum proficiency level: 50 ppb.

J. WORKSHEET

Examples of worksheets, on the following two pages, may be copied for use.

PBZ (PHENYLBUTAZONE) WORKSHEET

Date: _____ Reviewed by: _____

Analyst: _____

Representative 96-wells ELISA Plate

Row	Col 1	Col 2	Col 3	Col 4	Col 5	Col 6	Col 7	Col 8	Col 9	Col 10	Col 11	Col 12
A												
B												
C												
D												
E												
F												
G												
H												

Calculations of Reagents for ELISA Kit/Set of Samples

Amount of Drug enzyme conjugate needed:

A = No. of wells will be used ___ + 8 = ___ (rounded to nearest 5's).
B = mL of EIA buffer needed = A __ x 180/1000 = ____ mL.
C = (A ___ x 300 x 4)/1000 = ____ (rounded to the nearest 10's).
Amount of DW needed = C ___ - 10% of C __ - (0.1 x ___) = ____ mL.
Amount of Wash buffer needed = 10% of C ___ - 0.1 x ___ = ___ mL.

Instruments and Settings

Plate Reader: _____
Plate Reader Filter: _____
Plate Reader Mode (ABS1): _____
Centrifuge: _____
Centrifuge Speed: _____
Centrifuge Time: _____
Centrifuge Temp: _____
Centrifuge Rotor: _____
Centrifuge: _____
Freezer: _____
Balance: _____
pH Meter: _____
Refrigerator: _____

SPECIFICATIONS

Standard Solutions

PBZ Stock Soln	Conc: 500 µg/mL
PBZ Intermediate Soln	Conc: 250 µg/mL
PBZ Working Std (i)	Conc: 150 ng/mL
PBZ Working Std (ii)	Conc: 300 ng/mL
50 ppb Std Curve/Fortification Soln	Conc: 15 ng/mL
100 ppb Std Curve/Fortification Soln	Conc: 30 ng/mL

Reagents

Phosphate Buffer	0.2M
Wash Buffer	1:2 Diluted with deionized H_2O
Diluted Drug-Enz conjugate	1:180 Diluted with EIA Buffer
Kit #	

Plate Acceptability Criteria

	PBZ Conc Based on 0.3 g Smp	Amount/20 µL /well	Absor-bence	Normalized Value	Passed/ Failed (P/F)
Reagent Blk	0 ng/mL	0 ng		-	
50 ppb Std	15 ng/mL	0.3 ng			
100 ppb Std	30 ng/mL	0.6 ng			
Tissue Blank	0 ng/mL	0 ng		-	
50 ppb Fort	15 ng/mL	0.3 ng (estd)			
100 ppb Fort	30 ng/mL	0.6 ng (estd)			

Estimation of Decision Level (DL)

(Based on 6 replicates of one 50 ppb fortification Sample)

Replicate Numbers	Amount/20 µL/Well	Absorbance	Normalized Value
Tissue Blank	0.0 ng		-
#1	0.3 ng (estimated)		
#2	0.3 ng (estimated)		
#3	0.3 ng (estimated)		
#4	0.3 ng (estimated)		
#5	0.3 ng (estimated)		
#6	0.3 ng (estimated)		
		Avg:	
		SD:	
		DL = SD x 3 =	x 3 =

PBZ SAMPLE RESULTS WORKSHEET

Analyst: ________________

Date Started: ____________

Date Completed: __________

Set Number: ____________

Reviewed By: ____________

DL (Decision Level Absorbance Calculated from PBZ Worksheet) = ______

Note: A Sample is PBZ positive (+) if its absorbance is < DL.

Position	Sample ID Lab No.	Form No	Absorbance	Normalized value	Results (+) or (-)
	Tissue Blk	-----------			

K. REFERENCE

Susan B. Clark, Food and Drug Administration, Denver District Laboratory, Denver Federal Center April 2001**.** Phenylbutazone residues in Bovine Kidney by ELISA,

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Stephen Powell	10/23/2002
Leon Ilnicki	10/23/2002
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