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

CLG-PBZ3.03		Page 1 of 11
Title: Screening of Phenylbutazone by ELISA		
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Contents

A. INTRODUCTION

1. Summary of Procedure

This procedure is used to screen bovine kidney samples for phenylbutazone residues at concentrations of 50 ppb or higher. Blended kidney tissues 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 a calculated decision level are identified as positives.

2. Applicability

This method is suitable for the screening of phenylbutazone in bovine kidney and equine muscle at levels ≥ 50 ppb.

Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA.

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

a. Balance, analytical - Model No. MT5, sensitive to 0.0001 g, Mettler.

b. Balance, top loader - Model No. PM2000, sensitive to 0.01g, Mettler.

c. Vortex mixer - Super Mixer Model No.1290, Lab-Line.

d. Shaker, platform (two speed) - Eberhard.

e. Volumetric dispenser, bottle top - Capable of delivering from 1 to 10 mL, Brinkmann.

f. Stirrer/hot plate - Model No PC-4420, Corning.

g. Centrifuge - International Equipment Company B-22M Superspeed Refrigerated Centrifuge with Rotor No. 876, International Equipment Company, Clinical Centrifuge, Cat. No. 20671-007, VWR Scientific.

h. Eppendorf pipettors - Variable volume pipettes, 2-20 µL (Cat. No. 05-402-46), 10-100 µL (Cat. No. 05-402-48), 50-200 µL (Cat. No. 05-402-49), 100-1000 µL (Cat. No. 05-402-50) and 500-5000 µL (Cat. No. 022472151),.

i. epMotion 5075 - Liquid Handling Workstation from Eppendorf (Cat. No. 960020006), TS 50 – 1 to 50 µL dispensing tool (Cat. No. 960001010), TS 1000 – 40 to 1000 µL dispensing tool (Cat. No. 960001036), TM 1000-8 – 40 to 1000 µL 8-channel dispensing tool (Cat. No. 960001061).

j. pH meter - Orion 601A Calibrated at pH 4 and 7, readable to 0.01 pH.

k. Transferpette, Multichannel Pipettes - Brinkmann, 50-300µL #50-08-030-7 (Cat. No. 53512-376, VWR).

l. Glassware - Volumetric glassware includes 50-mL, 100-mL, 1000-mL, and 2000-mL flasks. CLASS A.

m. Pipettes - 25 mL glass pipettes. CLASS A.

n. Test tubes (for use with Liquid Handling Workstation) - 12mm x 75mm disposable Borosilicate glass culture tubes, (Kimble).

o. Centrifuge tubes - 50-mL polyallomer tube with polypropylene screw closure, Cat. No. 3139-0050, Nalge Company.

2. Instrumentation

a. Plate Reader: Biotek Autoreader ELx 808 - Equipped with 650 nm filter and a printer (ELISA Technologies).

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependant on the expiration date of the components used or the listed expiration date, whichever is soonest.

1. Reagents

a. Test kit – Phenylbutazone Enhanced ELISA Kit, Cat. No. 104710-I or 104715-I (bulk kit) (Neogen Corporation, ELISA Technologies Division).

Contents of the bulk kit include the following:

i. EIA Buffer (200 mL).

ii. 10x Wash Buffer Concentrate (100 mL). Dilute before use according to manufacturer's instructions.

iii. K-Blue Substrate (100 mL). Stabilized 3,3',5,5'-Tetramethylbenzidine (TMB) plus hydrogen peroxide in a single bottle. Light sensitive.

iv. Drug-Enzyme Conjugate (1mL). Drug-horseradish peroxidase concentrate. Dilute before use according to manufacturer's instructions.

v. Precoated Plates (5). Each Costar plate consists of 96 wells in strips of 8 breakaway wells coated with anti-drug antisera. The plates are ready for use. Do not wash until the sample/drug-conjugate incubation is complete.

Note: The test kits must be stored in a refrigerator at 2 - 8 °C. Do not use past expiration date.

b. Sodium Phosphate, dibasic, anhydrous. Na_2HPO_4, Cat. No. 3828-01, J.T.Baker.

c. Potassium Phosphate, Monobasic crystals. KH_2PO_4, Cat. No. 3246-01, J.T.Baker.

d. Deionized water.

e. Methanol, LC-Grade, 4L, Cat. No. H488-10, Mallinckrodt.

f. 1N HCl, 1L, Cat No. SA48-1, Fisher Scientific.

g. 1N NaOH, 1L, Cat. No. SS266-1, Fisher Scientific.

h. Buffer solution, pH 4.01, Cat. No. 238217, Hamilton.

i. Buffer solution, pH 7.00, Cat. No. 238218, Hamilton.

2. Solutions

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. Add approximately 950 mL of deionized water to dissolve reagents. Adjust pH to 6.8 ± 0.1 with 1N HCl or 1N NaOH solutions. Dilute to final volume with deionized water. Prepare fresh extraction buffer when it becomes cloudy. The pH should be checked periodically to verify that it is 6.8 ± 0.1.

b. Horseradish peroxidase (HRP) enzyme conjugate (1:180)– EIA buffer dilution (1 + 179). Prepare in a ratio of 1µL HRP conjugate to 179 µL EIA buffer each day of use or per manufacturer's instructions.

c. Wash buffer, dilution – Dilute 1:9 with deionized water or per manufacturer's instructions. This solution is assigned the same expiration date as the concentrated wash buffer and shall be stored at 2 - 8 °C.

D. STANDARD(S)

Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependant on the expiration date of the components used or the listed expiration date, whichever ends sooner.

1. Standard Information

a. Phenylbutazone standard is available from MP Biomedicals. (Cat. No. 153567). Other sources for the standard material may be acceptable.

2. Preparation of Standard Solution(s)

   a. Stock PBZ Standard Solution, 500 µg/mL in methanol:

   Weigh 50.0 ± 0.1 mg PBZ standard into a 100-mL volumetric flask. Dissolve and bring to volume with methanol. This standard solution is stable for 2 months when stored at 2-8 °C unless the note above applies.

   b. Intermediate PBZ Standard Solution, 250 µg/mL in methanol:

   Pipet 25 mL PBZ stock standard (D.2.a.) into a 50-mL volumetric flask and bring to volume with methanol. This standard solution is stable for 2 months when stored at 2-8 °C unless the note above applies.

   c. Working PBZ Standard Solution, 300 ng/mL:

   Dilute 60 µL of intermediate standard (D.2.b.) to 50 mL with extraction buffer (C.2.a.) in a 50-mL volumetric flask. This standard solution must be prepared fresh for each set and kept at room temperature.

   Note: The working standard solution is used to generate the standard curve and for sample fortification.

   d. External Standard Solutions (0, 30, and 60 ng/mL) are prepared daily for each set as follows:

      i. 0 ng/mL (0 ppb): extraction buffer solution (C.2.a).

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

      iii. 60 ng/mL: Dilute 200 µL of Working Standard (D.2.c.) to 1 mL with extraction buffer solution (C.2.a).

E. SAMPLE PREPARATION

1. Samples

Samples collected fresh must be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples must be frozen (<10 °C) prior to grinding if they cannot be prepared on the day of receipt. Once frozen, the sample should be allowed to thaw, while keeping it as cold as possible. Dissect away fat and connective tissue from kidney or muscle. Homogenize kidney tissue in blender and muscle tissue in food processor. Store samples frozen (<-10 °C) prior to analysis.

2. Negative control tissue

Negative control tissues for each bovine kidney and equine muscle are prepared by forming a composite of at least six different bovine kidney samples which were previously found to be screened negative. The tissues are combined and blended to

ensure homogeneity.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls

   a. Preparation of blank samples for establishing the decision level

      i. Bovine Kidney

         (a) Weigh 6 known kidney blanks (4 ± 0.1 g each) into 50 mL polyallomar centrifuge tubes.

         (b) Add 2.0 mL of extraction buffer (C.2.a.) to each of six tubes.

         (c) Continue the process following steps F.2.c. through F.2.e.

      ii. Equine Muscle

         (a) Weigh 6 known kidney blanks (1 ± 0.1 g each) into 50 mL polyallomar centrifuge tubes.

         (b) Add 1.0 mL of extraction buffer (C.2.a.) to each of six tubes.

         (c) Continue the process following steps F.2.c. through F.2.e.

   b. Preparation of 50 ppb control sample and negative control

      i. Bovine Kidney

         (a) Weigh one known kidney blank (4 ± 0.1 g each) into two 50-mL polyallomar centrifuge tubes.

         (b) For the 50 ppb control sample, add 1.0 mL of 300 ng/mL working standard (D.2.c.) plus 1.0 mL of extraction buffer (C.2.a.) to the tube. For the 0 ppb negative control, add 2.0 mL of extraction buffer (C.2.a.) to the tube.

         (c) Continue the process following steps F.2.c. through F.2.e.

         Note: The fortification level is based on the concentration of PBZ added to the tissue divided by the total g of product (4 g of tissue plus 2 g of extraction buffer or standard solution = 6 g total for bovine kidney). Application of 20 µL of these solutions on each well represents 0 ng and 3 ng of PBZ, respectively, for 0 and 50 ppb fortifications.

   Note: The fortification level is based on the concentration of PBZ added to the tissue divided by the total g of product (4 g of tissue plus 2 g of extraction buffer or standard solution = 6 g total for bovine kidney). Application of 20 µL of these solutions on each well represents 0 ng and 3 ng of PBZ, respectively, for 0 and 50 ppb fortifications.

ii. Equine Muscle

(a) Weigh one known muscle blank (1 ± 0.1 g each) into two 50-mL polyallomar centrifuge tubes.

(b) For the 50 ppb control sample, add 0.333 mL of 300 ng/mL working standard (D.2.c.) plus 0.667 mL of extraction buffer (C.2.a.) to the tube. For the 0 ppb negative control, add 1.0 mL of extraction buffer (C.2.a.) to the tube.

(c) Continue the process following steps F.2.c. through F.2.e.

NOTE: Weigh an additional blank for check sample when needed.

2. Extraction Procedure

a. For Bovine Kidney

i. Weigh 4 ± 0.1 g of blended sample into a 50 mL polyallomar centrifuge tube.

ii. Add 2.0 mL of extraction buffer (C.2.a.) to each tube.

iii. Continue the process following steps F.2.c. through F.2.e.

b. For Equine Muscle

i. Weigh 1 ± 0.1 g of blended sample into a 50 mL polyallomar centrifuge tube.

ii. Add 1.0 mL of extraction buffer (C.2.a.) to each tube.

iii. Continue the process following steps F.2.c. through F.2.e.

c. Vortex vigorously for at least 1 minute and sonicate for 5 minutes.

d. Centrifuge tubes for 15 min at ~15,000 RPM at 4 °C.

e. Extracts are ready for plating. Keep extracts refrigerated until beginning step F.3.

3. Instrumental Settings/ELISA

Note: The instrument parameters may be optimized to ensure system suitability.

Note: The following steps are based on kit manufacturer instructions and may be subject to change. If any discrepancies exist, follow the current manufacturer instructions.

a. Apply in duplicate 20 µL extracts of each external standard(s), control(s), and sample(s) into individual ELISA plate wells.

Note: The six blank extracts must be distributed randomly in duplicate into 12 wells covering the area of the plate used in order to facilitate estimation of a decision level (see Section G).

b. Add 180 µL HRP: EIA solution (C.2.b) to each well. Mix the solutions by gently vibrating the plate on a flat surface.

c. Cover plate to avoid possible dust/dirt contamination.

d. Incubate for 45-60 minutes at room temperature. Shake gently at least twice during the incubation period.

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

f. Wash the wells 4 times using 300 µL/well per wash of diluted wash buffer (C.2.c). Tap 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.

g. 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.

h. Evaluate the plate using a plate reader at 650 nm.

Note: Optimum total incubation time will be that where the absorbance reading for the plate blank just begins to plateau. This may be established by recording plate absorbances at 5 minute intervals until the absorbance reading of the 0 external standard no longer shows a significant increase.

4. Sample Set

a. Six different control tissue blanks to establish the decision level.

b. One negative control.

c. Fortified tissue at 50 ppb.

d. Standards at 0, 30, and 60 ng/mL.

e. Samples up 37 per set

G. CALCULATIONS / IDENTIFICATION

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

2. Calculate the mean and standard deviation (SD) for the absorbance readings of the six unfortified control tissue replicates (see F.2.a.). Use these to calculate a decision level (DL) using the formula DL = Mean - 2.78*SD.

3. A sample run using duplicate aliquots is screen positive if it meets one of the following conditions:

a. Both absorbance readings are less than the decision level.

b. One reading is less than the decision level and the average of both readings is less than the decision level and the relative percent difference between readings is less than 10%. Determined as follows:

i. Calculate the absolute difference between the two measurements and divide by two. Multiply this number by 100. Divide this product by the mean of the two measurements (resultant calculation is expressed as percentage).

Note: If one well has an absorbance greater than the decision level and the relative difference between duplicate wells is greater than 10%, the sample must be analyzed again.

H. SAFETY INFORMATION AND PRECAUTIONS

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

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Methanol	Flammable; may produce toxic effects to skin, eyes and 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

3. Disposal Procedures

Follow local, state and federal guidelines for disposal.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range*	*Acceptable Recovery*	*Acceptable Repeatability (CV)*
PBZ	≥ 50 ppb	NA	NA

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

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

ii. The external standard curve absorbance values continuously increase from the 60 ng/mL to the 0 ng/mL concentration.

iii. The day of use 0 ppb standard and 0 ppb fortified control wells must screen negative.

iv. The CV calculated for the six negative control tissue replicates (12 measurements) used to establish the decision level must be ≤ 20%.

b. No false negatives at ≥ 50 ppb fortified tissue.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
a. 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 (F.3.g).	Evacuate plate of all liquid or bubbles prior to the addition of the K-blue solution.

3. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One per week per analyst when samples analyzed.

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Investigate following established procedures.

ii. Take corrective action as warranted.

4. Sample Condition upon receipt

Cool or frozen.

J. APPENDIX

1. References

Susan B. Clark, Sherri B. Turnipseed, Gene J. Nandrea, Mark R. Madson, Emma R. Singleton, Jeffrey A. Hurlbut, John N. Sofos, and Craig E. Shultz. "Identification and

Confirmation of Flunixin Meglumine and Phenylbutazone Residues in Animal Kidney by ELISA Screening and Liquid Chromatography Mass Spectrometry." LIB 4246, Food and Drug Administration, Denver, CO. Vol. 17, No. 5, May 2001.

2. Minimum Level of Applicability (MLA): 50 ppb

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Division.