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Title: Screen for Furazolidone and Furaltadone Metabolites by ELISA		
Revision .00	Replaces: NA	Effective: 03/07/2005

Contents

A. INTRODUCTION

Furazolidone and Furaltadone rapidly degrade to metabolites 3-amino-2-oxazolidinone (AOZ) and 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ) respectively, which readily bind to cellular proteins. This procedure screens for those metabolites.

1. Theory

Blended liver is heated overnight in a dilute acid solution containing 2-nitrobenzaldehyde to hydrolyze, extract and derivatize AOZ and AMOZ residues to NPAOZ and NPAMOZ, respectively. The hydrolysate is then made alkaline and the derivatives extracted into ethyl acetate. An aliquot of the ethyl acetate extract is evaporated and the residue is redissolved into hexane. The derivatives are then partitioned into aqueous sample buffer. Aliquots of the buffer are then analyzed using commercial Nitrofuran ELISA kits.

2. Applicability

This procedure will detect AOZ in bovine, porcine and avian (poultry) liver at ≥ 0.5 ppb, and AMOZ in bovine and porcine liver at ≥ 0.5 ppb and avian liver at ≥1 ppb.

B. EQUIPMENT

Equivalent equipment may be substituted for the following:

1. Apparatus

a. Centrifuge - Model No. PR 7000, rotor # 269, test tube holder (CAT. No. 32A, test tube adapter (CAT. No. 1106), Damon IEC.

b. Vortex mixer - Vortex Genie 2 (CAT. No. 12-812), Fisher Scientific.

c. Shaker - Eberbach Brand (CAT. No. 57007-000), VWR.

d. Test tube rack - CAT. No.5930-0020, Nagle Nunc International.

e. N-evap - Model No. 112, Organomation.

f. Magnetic stirrer.

g. Incubator - Model No. 120-923, Lab-Line Instruments, Inc.

h. Pasteur pipettes - Fisher brand (CAT No. 13-678-20B), Fisher Scientific.

i. Eppendorf pipettors - Variable volumes: single channel, 50 -200 µL, CAT. No. 022470256, 500-5000 µL, CAT. No. 022472151; Multi-channel, 30 -300 µL, CAT. No. 02452045, Brinkman instruments.

j. Balance - Top loading, model number PM300, Mettler.

k. Polypropylene conical tubes - Blue Max 50 mL, 30 x 115 mm, CAT. No. 352098, and 15 mL, 17 x 120 mm. CAT. No. 352096, Becton Dickinson Lab ware.

l. Glassware - Fisher brand, Class A 1 L volumetric flask or graduated Cylinder, Fisher Scientific.

m. Dispensette - 5-25 mL, CAT. No. 22-22-030-6, 0.5-5 mL, CAT. No. 4701-130, Brinkman instruments.

2. Instrumentation

a. Plate reader - BioTek Automated Microplate reader, model number ELx 808, with printer. Equipped with 450 nm filter. Bio-Tek instruments Inc.

C. REAGENTS AND SOLUTION

1. Reagents

a. ELISA Test kits - RIDASCREEN Nitrofuran AOZ and Nitrofuran AMOZ, CAT. No. R3701, R7311, R-Biopharm, Inc.
Note: Store at 2 - 8 °C.

Equivalent reagents and solutions may be substituted for the following:

b. N-Hexane (or n-Heptane) - EM Omnisolv, CAT. No. EM-HX0295-6 (hexane) or EM-HX0078-6 (heptane), VWR.

c. Ethyl acetate - Fisher brand (CAT. No. E195-4), Fisher Scientific.

d. Dimethylsulfoxide (DMSO) - CAT. No. 27685-5, Sigma Aldrich.

e. 2-Nitrobenzaldehyde - CAT. No. N1080-2, Sigma Aldrich.

f. 1 N HCL- Fisher brand (CAT. No. SA48-1), Fisher Scientific.

g. 1 N NaOH - Fisher brand (CAT. No. SS266-1), Fisher Scientific.

h. K_2HPO_4 - Potassium phosphate dibasic anhydrous, Fisher brand (CAT. No. P288-500), Fisher Scientific.

i. Deionized water.

2. Solutions

a. 10 mM 2-Nitrobenzaldehyde in Dimethylsulfoxide:

Add 7.6 mg ± 0.2 mg of 2-Nitrobenzaldehyde into 5 mL of DMSO. Prepare freshly each day of use.

b. 0.1 M K_2HPO_4 Solution:

Weigh 17.41 g of K_2HPO_4 into a 1 L volumetric flask or graduated cylinder. Dilute to volume with deionized water.

c. Kit buffer (sample and washing buffer):

Empty one pouch of buffer salt into a 1 L volumetric flask or graduated cylinder. Dilute to volume with deionized water. Refrigerate between 2 - 8 °C. Stable for one month. Bring to room temperature before use.

D. STANDARDS

1. Source

Ridascreen Nitrofuran AOZ and AMOZ test kits contain multiple calibration standard solutions. The AOZ kit provides NPAOZ standards equivalent to 0, 50, 150, 450, 1350, and 4050 ppt. AOZ The AMOZ kit provides NPAMOZ standards equivalent to 0, 100, 300, 900, 2700, and 8100 ppt AMOZ.

AOZ (CAT. No. 33347) and AMOZ (CAT. No. 33349) standards may be obtained from Sigma Chemical Co., St Louis MO.

2. Preparation of Fortification Standards

Note: Different solution concentrations may be prepared as long as fortification volumes are adjusted accordingly.

a. AOZ and AMOZ Stock standard solutions (25 µg/mL):

Accurately weight 2.5 ± 0.1 mg AOZ and AMOZ into separate 100 mL amber volumetric flasks. Dissolve and bring to volume with methanol.

b. AOZ, AMOZ Intermediate standard solutions (250 ng/mL):

Pipet 1.0 mL of each stock standard into a separate100 mL amber volumetric flask and bring to volume with methanol.

c. AOZ, AMOZ Combined Working Standard solution (10 ng/mL):

Pipet 2.0 mL of each Intermediate standard solution into a 50 mL amber volumetric flask and bring to volume with methanol.

3. Storage and Stability

a. Refer to manufacturer's insert for storage and stability information regarding kit standards.

b. Store all fortification standards in a freezer. Stock, intermediate, and working standards are stable for at least 5 months.

E. SAMPLE PREPARATION

Blend partially thawed sample, then store in a freezer.

F. ANALYTICAL PROCEDURE

1. Sample Extraction:

   a. Weigh 1.0 ± 0.1 g of blended bovine, porcine, or poultry liver into a 50 mL conical centrifuge tube.

      Prepare tissue controls at this time. A total of 6 different tissue blanks and at least one fortified control must be included with every sample batch. Blanks used should match the species of samples being analyzed. Prepare a 1 ppb fortified control by adding 100 µL of 10 ng/mL Combined Working Standard to a tissue blank.

   b. Add 4 mL of deionized water, 0.5 mL of 1 N HCL, and 100 µL 10 mM 2-nitrobenzaldehyde in DMSO to each tube. Mix by shaking or vortexing.

   c. Cap tube and Incubate over night (approx. 16 h) at 35-39 °C.

   d. Add 5 mL 0.1 M K_2HPO_4, 0.4 mL of 1 N NaOH, and 5 mL of ethyl acetate. Shake vigorously for 30 seconds.

   e. Centrifuge approximately 10 min at 3000 g to separate layers.

   f. Transfer 2.5 mL of the ethyl acetate layer into a new vessel and reduce to dryness on an N-evap at about 60 ºC. (This is not critical; the samples can be dried under nitrogen or dry air without a waterbath.).

   g. Dissolve the residue in 1 mL n-hexane/heptane and mix with 1 mL of sample buffer (supplied with kit).

   h. Centrifuge 10 minutes at 3000 g.

   i. Remove the top organic phase.

   j. Remaining buffer contains AOZ and AMOZ derivatives.

2. ELISA

   The following instructions apply to both the AOZ and AMOZ test kits.

   a. Prepare reagents supplied with the test kit according to manufacturer's instructions.

   b. Insert a sufficient number of wells into the microwell holder to accommodate all standards, controls and samples (Standards require 4 wells).

   c. Pipet 50 µL of each of the following kit standards into one well of the test kit: (AOZ - 0, 150, 450, 900 ppt; AMOZ - 0, 300, 900, 2700 ppt). Pipet 50 µL aliquots

for each control and sample analyzed into duplicate sample wells. Add 50 µL of the enzyme conjugate to each well.

d. Add 50 µL of the antibody to each well. Mix gently, either by rocking the plate manually every fifteen or twenty minutes, or placing on a vortex mixer equipped with microwell plate insert and mixing for 60 min. at lowest speed setting. This mixing/incubation is done at room temperature (20 - 25 °C).

e. Pour the liquid out of the wells, then tap the microwell holder upside down vigorously (3 times) against absorbent paper to effect complete removal of liquid.

f. Fill all wells with 250 - 300 µL washing buffer, and pour out liquid as described in step e. Repeat wash step three times, for a total of 4 washes.

g. Add 100 µL of substrate/chromogen to each well and mix gently. Incubate for 15 min. at room temperature in the dark.

h. Add 100 µL of stop solution to each well and mix gently. Measure the absorbance of all wells at 450 nm against an air blank within 60 minutes of addition of stop solution.

G. CALCULATIONS

a. Check suitability of ELISA.

i. Reagent stability: The absorbance of the 0 standard must be >0.6.

ii. Sensitivity: Divide the absorbance of the mid-level standard by the absorbance of the 0 level standard. The resulting fraction should typically be below 0.6.

iii. Linearity: Subtract the absorbance of the mid-level standard from that of the low standard, and subtract the absorbance of the high standard from that of the mid-level standard. Divide the larger difference by the smaller difference. This ratio must be <2.

Deviations from any of the above performance requirements indicate a likely problem related to reagent quality or test execution. Rerun ELISA analysis if the 0 standard absorbance is < 0.6 or the analysis fails to meet both sensitivity and linearity requirements.

b. Calculate the mean and standard deviation (SD) for the averaged absorbance readings of the six blanks included in the sample set.

c. Use this mean and SD to determine a cutoff value to be used to decide if a reading is screen positive as follows:

i. For all species-tissue combinations except AMOZ in poultry, calculate the cutoff absorbance as:

Cutoff = Mean - 3 x SD.

ii. For AMOZ in poultry, calculate the cutoff absorbance as::

Cutoff = Mean - 2 x SD.

d. Compare both readings taken for the positive control and all samples against the cutoff.

e. A sample is screen positive if it meets one of the following conditions

i. Both absorbance readings are < cutoff value.

ii. The average of both readings is < cutoff value, and the relative difference between readings [100(b-a)/a], where b>a] is less than 10%.

Note: In all other instances where only 1 well shows a reading < cutoff value, re-analyze the sample extract.

f. Screening results may be reported if the following conditions are met.

i. The ELISA appears to be working properly.

ii. The positive control is screen positive.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment — Safety glasses, laboratory coat.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Acids and bases: HCl, NaOH	Can cause burns on skin and eye injury. Corrosive.	Wear gloves and protective clothing when handling.
Organic solvents: n-hexane, ethyl acetate, DMSO	Flammable liquid and vapor, can cause eye, nose, and upper respiratory tract irritation. DMSO may be harmful if absorbed through the skin	Use in a fume hood, away from all open flames and electrical devices. Wear gloves when handling.
2-Nitrobenzaldehyde	Harmful by inhalation or skin contact. Irritant to eyes, respiratory tract, and skin	Use in fume hood. Wear gloves when handling.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Acids and bases: HCl, NaOH	See Above	Neutralize the acid or base for disposal down the drain in accordance With local, state, and federal regulations.
Organic solvents: n-hexane, ethyl acetate, DMSO, 2-Nitrobenzaldehyde	See above	Collect waste and store in a tightly sealed container away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	Species	Detection Limits	Acceptance criteria (set)
AOZ	Bovine, Porcine & Avian	≥ 1 ppb	ELISA QC is acceptable, positive control is screen positive
AMOZ	Bovine, Porcine & Avian	≥ 1 ppb	

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Absorbance of 0 External Std.	>0.6

3. Readiness To Perform (FSIS Training Plan)

a. Familiarization

Note: All analyses must be done using both AOZ and AMOZ test kits.

i. Phase I: Standards: Analyze duplicate standard curves using all standards supplied by Kit manufacturer on three different days.

ii. Phase II: Fortified blanks: Analyze a minimum of 6 blanks and 6 recoveries fortified at 1 ppb using beef, pork, and poultry tissues. Analyze a different species each day.

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

iii. Phase III: Check samples for analyst accreditation.

(a) Using full method, analyze a minimum of 6 samples that are blind to the analyst. At least one, but no more than two of the samples may be blanks. The rest are to be fortified at approximately 1 ppb. Any tissue may be used for this phase.

(b) Report analytical findings to QAM.

(c) Authorization from QAM is required to commence official analysis.

b. Acceptability criteria.

Refer to I.1.

4. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One per week when analyses are being conducted.

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I.1.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Condition or receipt: Frozen.

b. Sample storage: Store frozen when not in use.

6. Sample Set

Sample Set will consist of:

a. Six blank tissues.

b. Recovery fortified with AOZ, AMOZ at 1 ppb.

c. Samples to be analyzed.

7. Sensitivity

a. Lowest detectable level (LDL): Typically <0.5 ppb AOZ, AMOZ in most tissues (1 ppb AMOZ in poultry).

Minimum Proficiency Level[1] (MPL): 1 ppb AOZ, AMOZ.

[1]Minumum analyte concentration analyst must consistently detect.

J. WORKSHEET

Reserved.

K. APPENDIX

1. References

RIDASCREEN Nitrofuran (AOZ)
Enzyme Immunoassay for the quantitative analysis of AOZ
Art. No.: R3701
R-Biopharm AG, Darmstadt, Germany

RIDASCREEN Nitrofuran (AMOZ)
Enzyme Immunoassay for the quantitative analysis of AMOZ
Art. No.: R3711
R-Biopharm AG, Darmstadt, Germany

The above references are published as kit inserts.

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