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Title: Screen of Carbadox Metabolite QCA by HPLC-MS-MS		
Revision: .02	Replaces: CLG-CBX4.01	Effective: 03/31/2016

Contents

A. INTRODUCTION

1. Background

   Carbadox is an anti-microbial drug that is used as a growth promoter and to prevent swine dysentery and bacterial enteritis.

2. Summary of Procedure

   Homogenized liver tissue is digested with 3M NaOH for 30 minutes in an oil bath. The tissue is acidified, extracted and centrifuged. The extract is then purified by passing through an SCX cartridge. After washing the cartridge with different solvents, 2-quinoxalinecarboxylic acid (QCA), is eluted with 70:30 0.1 M sodium hydroxide: methanol. The eluate is extracted with ethyl acetate, evaporated to dryness, reconstituted with methanol/water, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS-MS).

   Note: 2-Quinoxalinecarboxylic acid is often referred to as quinoxaline-2-carboxylic acid.

3. Applicability

   This method is suitable for the *screening* of QCA in swine liver at levels ≥ 15ppb.

   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. Top- Load Balance - sensitive to 0.01 g, Model PM 360, Mettler

   b. Analytical Balance – sensitive to 0.0001 g, Model AG204, Mettler.

   c. Oil Bath – High Temperature Bath, Fisher Scientific.

   d. Shaker – Eberbach.

   e. Centrifuge – Sorvall RC 4 Centrifuge, Thermo Electron Corporation.

   f. Nitrogen Evaporator – Model 112 Organomation.

   g. Vortex – Genie-2 Cat. No. 12-812, Fisher Scientific.

   h. Centrifuge tubes – 15 mL glass disposable, with Teflon-lined screw caps, conical bottom, Fisher Scientific.

   i. Glass centrifuge tubes – 50 mL glass, Heavy Duty, with screw cap, 25 X 150 mm, Cat. No. 9826-25, Corning.

j. Micropipettes – Adjustable, 10-100 µL, 100-1000 µL, 500-5000 µL, plus tips, Eppendorf.

k. Vacuum manifold – SPE 20 port, Waters.

l. SPE column – Benzenesulfonic acid cation exchange (SCX) 500 mg/3 mL, Cat. No. 2323, Applied Separations.

m. Liquid dispensers – Adjustable 1-10 mL, 5-50 mL, Brinkmann.

n. Disposable glass culture tubes – 15 mL, 16 X 100 mm, Cat. No. 73500 16100, Kimble.

o. pH meter – Ultrabasic pH meter, UB-5, Denver Instruments.

2. Instrumentation

a. Waters HPLC/MS/MS – Waters Quattro Micromass.

b. Alliance Column – Pursuit XRs C18, 3 µm, 100 x 2 mm

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependent on the expiration dates of the compounds used. The maximum length of time that a working reagent shall be used is 1 year unless the laboratory has produced extension data.

1. Reagents

a. Sodium hydroxide - Pellets, Cat. No. SX0590-14, EMD.

b. 3N Sodium Hydroxide - Cat. No. BDH 3472-1, BDH.

c. 1N Sodium Hydroxide - Cat. No. 7450-32, Ricca Chem Company.

d. Hydrochloric acid – Reagent grade, Cat. No. 9535-33, J.T Baker.

e. 1N Hydrochloric acid - Cat. No. BDH 3201-2, BDH.

f. Ethyl Acetate - Reagent grade, Cat. No. 100-4, Burdick and Jackson.

g. Sodium phosphate monobasic - Cat. No. S8282, Sigma Aldrich.

h. Sodium phosphate dibasic – Cat. No. S7907,Sigma Aldrich.

i. Methanol – High Purity Solvent, Cat. No. 230-4, Burdick and Jackson.

j. Nitrogen – Airgas.

k. Argon – Airgas.

l. Formic Acid - Cat. No 94318, Fluka Analytical.

2. Solutions

a. 3 M NaOH:

Add 120 g of NaOH pellets and 1000 mL of Milli Q water and mix.

b. 0.2 M sodium phosphate monobasic:

Weigh out 13.93 g of sodium phosphate monobasic. Then add 500 mL of Milli Q water.

c. 0.2 M sodium phosphate dibasic:

Weigh out 53.65 g of sodium phosphate dibasic. Add 500 mL of Milli Q water.

d. 0.1 M sodium phosphate, pH 8-8.5:

Add 15.9 mL of 0.2 M sodium phosphate monobasic and 285 mL of 0.2 M sodium phosphate dibasic. Add 600 mL of Milli Q water. Measure pH about 8-8.5.

e. 0.1 M NaOH:

Add 100 mL of 1 N NaOH and bring to volume in a 1000 mL class A volumetric flask using Milli Q water.

f. 0.1 M NaOH:MeOH (70:30):

Add 700 mL of 0.1 M NaOH to 300 mL of methanol.

g. 0.1 M HCl:

Add 100 mL of 1 N HCl and bring to volume in a 1000 mL class A volumetric flask using Milli Q water.

h. MeOH: H_2O (10:90):

Add 10 mL of methanol to 90 mL of Milli Q water and mix.

i. 0.3 % formic acid:

Dilute 3 mL of formic acid to 1000 mL with Milli Q water in a 1 L volumetric flask

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 dependent on the expiration date of the components used. In-house prepared standards shall be assigned an expiration date that is no later than the expiration date of the earliest expiring component or no later than the stability stated in the method, whichever ends soonest. The maximum length of time that an in-house prepared standard shall be used is 1 year unless the laboratory has produced extension data.

1. Standard Information

   a. 2-Quinoxalinecarboxylic acid (15 µg/mL in methanol) (QCA) – CAS # 879-65-2, Cat. No. 91819 Absolute Standards, Aldrich Chemicals.

2. Preparation of Standard Solution

   a. QCA solution (0.15 µg/mL):

      Add 250 µL of 15 µg/mL QCA into a 25 mL class A volumetric flask. Dilute to volume using methanol. Mix thoroughly and store at 2 - 4 °C. Prepare every six months.

E. SAMPLE PREPARATION

Homogenize liver samples using a blender or food processor.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls and Samples

   a. Weigh 5.00 ± 0.10 g into a 50 mL glass tube for controls and samples. Weigh out three blank samples. One will be used as a blank, another as a positive control and the third as a check sample.

   b. Fortify the positive control with 500 µL of 0.15 µg/mL QCA and vortex.

   c. Let stand for 10 min.

2. Extraction Procedure

   a. Add 10 mL of 3 M NaOH to each tube and vortex.

   b. Place tubes in an oil bath that has been heated to 95- 100 °C. Leave tubes in the oil bath for 30 minutes. After 30 minutes remove the tubes from the oil bath and cool them to room temperature.

   Stopping point: The samples may be stored in the refrigerator for 24 hours.

   c. Once the tubes are cooled to room temperature add 4 mL of concentrated HCl. Cap and vortex for 30 seconds.

   Caution: Reaction is highly exothermic. Take care to work in a hood and wear eye and hand protection.

   d. Add 6 mL of ethyl acetate and vortex for 15-30 seconds. Centrifuge at 1500 g at 4 °C for 10 minutes.

   e. Transfer the upper layer to a 50 mL polypropylene tube.

   f. Repeat step d. and combine the extracts.

g. Add 8 mL of 0.1 M sodium phosphate solution (C.2.d.) and shake for 1 minute on a shaker. Centrifuge at 2000 g at 4 °C for 10 minutes.

h. Aspirate the upper organic layer.

i. Take a 4 mL aliquot and add it to a 10 mL glass tube containing 1 mL of concentrated, HCl.

j. For solid phase extraction, prime the SCX SPE cartridge with 5 mL of methanol, followed by 5 mL 0.1 M HCl.

k. Add 2 mL of the sample.

l. Wash the cartridge with 5 mL of 0.1 M HCl.

m. Elute with 3 mL of 0.1M NaOH:MeOH (70:30) into a 15 mL centrifuge tube.

n. Add 300 µL of concentrated HCl to each sample.

o. Extract with 2 mL of ethyl acetate. Vortex for 15 seconds. Centrifuge at 1500 g at 4 °C for 10 minutes. Collect the upper organic layer into a 10 mL test tube.

p. Repeat step o. two more times and combine the extracts.

q. Evaporate the combined ethyl acetate extracts at 50-60 °C to dryness.

r. Add 100 µL of MeOH:H_2O (10:90) and vortex for 15 seconds.

s. Transfer to a vial containing an insert.

Stopping point: Samples may be left for up to 48 hours at room temperature.

t. Analyze on the HPLC/MS/MS.

3. Instrumental Settings

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

a. Set HPLC Parameters:

Column Temperature: 30°C

Injection Volume: 15 µL

Total column flow: 0.35 mL/min

Pump gradient: (Total run time of 11.01 min)

Mobile Phase A: Acetonitrile

Mobile Phase B: 0.3 % formic acid

Mobile Phase C: Methanol

Gradient set of Mobile phases as follows:

Time	**A%**	**B%**	**C%**
0.00	2	90	8
10.00	10	50	40
10.01	16	20	64
11.01	2	90	8

b. Set Mass Spectrometric Parameters. System may be adjusted to ensure optimum performance.

Capillary Voltage 3.00
Cone Voltage 20.00
Extractor Voltage 3.00
RF Lens Voltage 0.3
Source Temperature 125 °C
Desolvation Temperature 450 °C

c. Measure the detector response (peak area) for the product ion transitions

175 → 129, Collision energy 30 eV, Dwell time 0.3
175 → 102, Collision energy 18 eV, Dwell time 0.3
175 → 131, Collision energy 14 eV, Dwell time 0.3

4. Injection sequence (if applicable)/Sample Set
   a. External Standard
   b. Negative Control (blank tissue)
   c. Positive Control (fortified tissue)
   d. Intra-laboratory check sample (if needed)
   e. Samples up to a maximum of 17
   f. Positive Control (fortified tissue)

G. CALCULATIONS / IDENTIFICATION

1. Screening Analysis for QCA
   a. A sample will be considered positive if:
      i. The retention time of the analyte in the sample matches the retention time of either the external standard or the positive control run under the same experimental conditions to within ± 5%.
      ii. All product ion transitions listed in Section F.3.c. are present.
      iii. Peaks for all transitions have a signal-to-noise (s/n) ratio ≥ 3.
      iv. The most abundant product ion area is > 25% of the positive control.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment — Safety glasses, disposable gloves, lab coat.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Methanol, Ethyl Acetate	Flammable, poisonous, inhalation will cause headaches, fatigue, nausea.	Wear gloves and work in the hood. Use protective eyewear. Avoid contact with skin, eyes.
Hydrochloric acid, Formic acid	Corrosive	Wear PPE, and avoid contact with skin.
QCA	Carbadox has been shown to cause cancer in laboratory animals but when fed to swine, is metabolized or transformed over a relatively short period of time. May cause photosensitization. This substance is possibly carcinogenic to humans.	Wear PPE, avoid skin contact. Exercise appropriate precautions to minimize direct contact with skin or eyes and prevent inhalation.
Sodium Hydroxide	Corrosive	Wear PPE, and avoid skin contact.
Sodium Phosphate	Slight reactivity rating. Irritant due to its slight acidic nature. Chronic exposure may result in calcium phosphate deposits in the kidneys.	Wear gloves and work in the hood. Use protective eyewear. Avoid contact with skin and eyes.

3. Disposal Procedures

   Follow federal, state and local regulations

I. QUALITY ASSURANCE PLAN

1. Performance Standard

   a. The external standard and the positive control must be positive according to the criteria listed in G.1.

   b. The area response of the negative control must be ≤ 5% of the positive control

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Fortify weighed control sample	Let the sample stand for 10 minutes.
b.	Sample weight	5.00 ± 0.10 g

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

   Cold

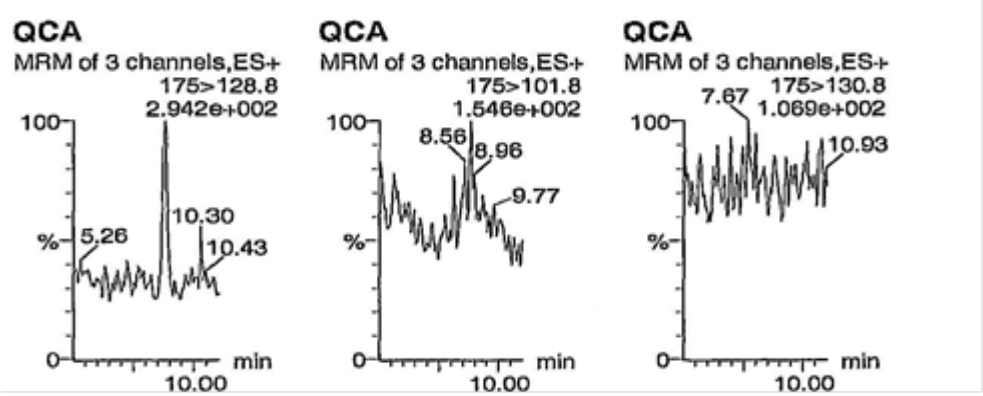
J. APPENDIX

1. References

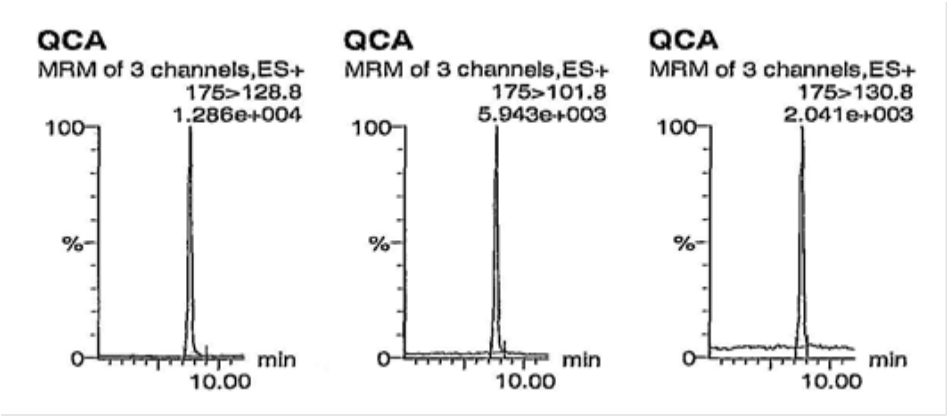
Hutchinson, M.J., Young, P.Y., Hewitt, S.A., Faulkner, D.,Kennedy, D.G.," Development and validation of an improved method of the carbadox metabolite, quinoxaline- 2-carboxylic acid, in porcine liver using LC-electrospray MS-MS according to revised EU criteria for veterinary drug residue analysis" , The Analyst 2002, 127, 342-346.

2. Chromatograms

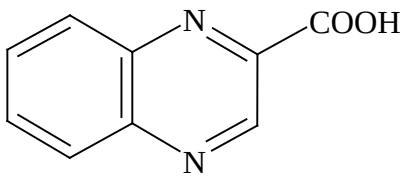
a. Negative Control



b. Positive Control



3. Structure



Quinoxaline-2-Carboxyllic Acid (QCA)

4. Method Information
   a. Screening: Minimum Level of Applicability (MLA): 15 ppb

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Staff