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A. INTRODUCTION

1. Theory

Carbadox metabolite derivative methyl quinoxaline-2-carboxylate (QME) is confirmed in extracts from the determinative method CLG-CBX1 by Gas Chromatography/Electron Ionization Ion-Trap MS (GC/EI IT-MS) in Selective Ion Monitoring (SIM) mode. Confirmation is based on comparison of sample GC retention time and relative ion abundance ratios against those obtained for a reference standard.

2. Applicability

This method is applicable to quinoxaline-2-carboxylate in swine liver at ≥ 30 ppb.

3. Structure

![Chemical Structure Diagram]

B. EQUIPMENT

1. Apparatus

Note: Equivalent apparatus may be substituted for the following:

a. 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 - 2500 µL, Cat No. 05-402-51, Fisher Scientific.

b. Teflon® membrane Syringeless Filter Device, 0.2 µm Pore Size, Cat. No. UN203NPENYL, Whatman.

2. Instrumentation

Note: An equivalent can be used for any instrumentation listed below.


b. Gas Chromatograph - Varian Chrompack CP3800 GC equipped with Varian
C. REAGENTS AND SOLUTIONS

1. Reagents
   Note: Equivalent reagents or solutions may be substituted for the following.
   a. Iso octane - Cat. No. 362-4, Burdick & Jackson.
   b. Toluene - Cat. No. AH 347-4, Burdick & Jackson.

D. STANDARDS

GC/MS External Standard – 0.15 µg/mL methyl quinoxaline-2-carboxylate (QME):
Pipet 10 µL Stock Solution 1 from CLG-CBX1 D.2.b.i into GC vial. Add 990 µL Toluene and mix.

E. SAMPLE PREPARATION

See CLG-CBX1 section E.

F. ANALYTICAL PROCEDURE

1. Weigh, extract, and clean up the sample as described in Determinative Method CLG-CBX1, sections F.1 and F.2.
   Note: Confirmatory sample sets require a tissue blank and a fortified recovery spiked at a concentration necessary to yield an extract of approximately the same concentration as the sample to be confirmed.

2. Filter the undiluted toluene extract, from step F.2.e.iv of the determinative method, through a Teflon® membrane. The extract is ready for GC-MS analysis using the Ion Trap MS.

3. Set Instrument Operating Conditions:
   Note: The instrument parameters listed here may be optimized, if necessary, to accommodate differences between individual instruments.
   a. Gas Chromatograph Parameters:
      
      Carrier Gas      Helium
      Column Flow Rate 1.0 mL/min
Injector Temperature  220 °C
Injection Volume  2 µL
Injection Mode  Pulse injection, splitless at 30 psi for 1 min.

Temperature Program:
- Initial temp: 100 °C
- Initial hold time: 1 min
- Program rate up to 180 °C: 20 °C/min
- Hold time at 180 °C: 2 min
- Program rate up to 270 °C: 25 °C/min
- Program rate up to 270 °C: 5 min
- Run time: 15.6 min

b. Mass Spectrometer Parameters:
- EM voltage  Autotune to autotune +300 as needed.
- Electron energy  70 eV
- Emission current  25 µA
- Detector temperature  150 °C
- Manifold temperature  80 °C
- Transferline temperature  220 °C

c. Data Acquisition Selected Ion monitoring mode:
- Ion 76, scan width 5d, ionization time factor 100 %
- Ion 102, scan width 5d, ionization time factor 100 %
- Ion 130, scan width 5d, ionization time factor 50 %
- Ion 158, scan width 5d, ionization time factor 100 %

4. Optimize MS
   a. Tune the instrument on the day of the analyses.
   b. Before analyzing a set of samples, verify system suitability by injecting a 0.15 µg/mL QME external standard.

5. Analyze sample set
Recommended injection sequence for analysis set:

a. Standard
b. Solvent blank (if necessary)
c. Blank (Negative tissue control)
d. Sample
e. Solvent blank (if necessary)
f. Recovery
g. Solvent blank (if necessary)
h. Standard

Note: Carry-over may be observed when too high a concentration of QME sample is injected. It is always prudent to inject the solvent blank after a high concentration of QME sample is analyzed. Multiple sample and/or reference injections may be used to provide averaged response values.

G. CONFIRMATION

1. For each chromatogram showing a positive analyte response, record the retention time and ion abundance for each ion peak detected. Calculate ion abundance ratios, relative to the ion showing the highest abundance in the external standard.

2. Compare response for each sample against that obtained for the nearest reference. Normally the external standard should be used for this purpose. However, if sample response shows evidence of matrix effects, the recovery may be substituted. Compute a % difference for the retention times and for each ion abundance ratio, where % Difference = 100 X (Sample value – Reference value)/(Reference Value).

3. Confirmation of QME presence in a sample extract requires that the following criteria be met:
   a. The retention time of the QME peak in the sample chromatogram is within ± 2 % of that determined for the reference.
   b. All monitored ions (76, 102, 130, and 158) are present in the sample and reference.
   c. At least 2 fragment ion ratios calculated for the sample match those of the reference within a relative difference of ± 20 % for ratios between 20 - 100 %, and within ± 50 % for ratios <20 %.
   d. The negative control extract does not contain QME.

H. SAFETY INFORMATION AND PRECAUTIONS
1. Required Protective Equipment - Safety glasses, disposable gloves, lab coats.

2. Hazards

<table>
<thead>
<tr>
<th>Reagents / Solutions</th>
<th>Hazard</th>
<th>Recommended Safe Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso octane, Toluene</td>
<td>Flammable</td>
<td>Wear gloves and work in the hood. Use protective eyewear.</td>
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3. Disposal Procedures

<table>
<thead>
<tr>
<th>Reagents / solutions</th>
<th>Hazard</th>
<th>Recommended Safe Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso octane, Toluene</td>
<td>See above</td>
<td>Store waste 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.</td>
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I. QUALITY ASSURANCE PLAN

1. Performance Standards
   a. No false positives from blank tissues.
   b. No false negatives at ≥ 30 ppb.

2. Readiness To Perform (FSIS Training Plan)
   a. Familiarization
      i. Phase I, Standard(s):
         Analyze a 0.15 μg/mL QME injection standard solution for fragment ions, 76, 102, 130 and 158 using GC/ EI-IT-MS in SIM mode. Repeat this analysis on three different days to verify parameters.
      ii. Phase II, Fortified samples:
         Analyze 3 replicates at 0 and 30 ppb 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) Analyze a minimum of 8 check samples provided by the Supervisor/Quality Assurance Manager (QAM). At least one check sample should be negative. At least 4 samples should be fortified at ≥30 ppb level.
   (b) Report analytical findings to Supervisor/QAM.
   (c) Notification from QAM is required to commence official sample analysis.

3. Intralaboratory check samples
   a. System, minimum contents.
      i. Frequency: A minimum of four per year.
      ii. Records are maintained.
   b. Acceptability criteria: Refer to sections I.1 above.
      If unacceptable results are obtained, then:
      i. Stop the sample analysis.
      ii. Take corrective action.

4. Sample set must include
   a. Positive control (recovery).
   b. Tissue blank.
   c. Sample extract(s).

5. Sensitivity

J. WORKSHEET
# QME CONFIRMATION WORKSHEET

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<td>Date Completed</td>
<td>Tissue Code</td>
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<tr>
<td>Instrument</td>
<td>Dilution or Concentration Factor</td>
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<td>Injection Volume</td>
<td>Result Confirmed (yes or no)</td>
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## CONFIRMATION

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%Diff RT |
%Diff RT

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</table>
K. APPENDIX

1. Chromatograms and Spectra

Figure 1. GC/EI IT mass spectrum of QME

QME Structure and Ions

Molecular weight of QME: 188 g/mol
Quasi molecular ion: [MH]+ 189

Structurally significant fragment ions:
- [MH - OCH₃]+ 138
- [MH - COCH₂]+ 139
- [MH - H - COOH]+ 129
- [MH - N=COCH₂]+ 103
- [MH - H - N=COOH]+ 102
- [MH - N=C=H]COCH₂]+ 75
- [MH - H - N=C=H]COCH₂]+ 75
Figure 2. TIC of External Standard Equivalent to 30 ppb QME

Figure 3. TIC of Tissue Blank
2. Reference:

Title: Confirmation of Carbadox Metabolite in Swine Liver by GC/EI Ion-trap MS

Revision .00
Replaces: CBX part II, 1991
Effective: 8/22/05

Approved by:       Date

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In Suk Kim         8/02/05
Jess Rajan         8/03/05
Charles Pixley     8/03/05
Phyllis Sparling   8/04/05

Approval records are on file.