Simultaneous, Fast Analysis of Melamine and Analogues in Pharmaceutical Components Using Q Exactive - Benchtop Orbitrap LC-MS/MS

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Overview

Purpose: Demonstrate a simple workflow for the simultaneous analysis of melamine and analogues in pharmaceutical components using a Q Exactive benchtop Orbitrap mass spectrometer.

Method: A sensitive, simple and robust method employing high resolution mass spectrometry coupled with UHPLC and HILIC mode chromatographic separation.

Results: Simultaneous detection and quantification of melamine and its analogues in Albumin and Guar gum were achieved with a high degree of confidence.

Introduction

Potential drug contamination by melamine and its analogues remains a major concern of the FDA. Currently, the FDA requires that the method used should be suitable for detecting melamine contamination in at-risk components down to at least 2.5 parts per million to give a high degree of assurance that they are not contaminated.

We present a workflow for simultaneous detection, confirmation, and quantification of melamine and its analogues: ammeline, ammelide, and cyanuric acid (shown in Figure 1) in at-risk pharmaceutical components Albumin and Guar gum. A Thermo Scientific Q Exactive benchtop Orbitrap™ mass spectrometer coupled with Thermo Scientific Dionex Ultimate 3000 RS UHPLC system were employed in this study.

Figure 1. Structures of Melamine and Analogue

![Image](image_url)

Melamine $\text{C}_6\text{H}_6\text{N}_6\text{(M+H)$^+ 127.0727$}

Ammeline $\text{C}_6\text{H}_8\text{N}_6\text{O}\text{(M+H)$^+ 129.0567$}

Ammelide $\text{C}_6\text{H}_8\text{N}_6\text{O}_2\text{(M+H)$^+ 129.0407$}

Cyanuric Acid $\text{C}_3\text{H}_2\text{N}_2\text{O}_3\text{(M-H)$^+ 128.0102$}

Methods

Material and Reagents


Sample Preparation

Individual stock solutions of melamine, cyanuric acid, ammeline and ammelide were prepared at 1.0 mg/mL. Melamine and cyanuric acid were dissolved in water. Ammeline and ammelide were dissolved in 2 N ammonium hydroxide. Standard mixture of 100ng/mL, 1µg/mL and 10 µg/mL were used to prepare neat and matrix calibration standards.

Neat standard calibration

Neat standard mixtures were prepared by serial dilution of 10 µg/mL standard mixture using 3:1 acetonitrile/water to final concentration of 0.25, 1.25, 2.5, 5, 12.5 and 25 ppb, which are equivalent to 0.05, 0.25, 0.5, 1.0, 2.5 to 5 µg/g of standard mixture in 5 mg/mL matrix solution.

Extracted matrix calibration standards preparation

Extracted matrix calibration standards were prepared at 0.05, 0.25, 0.5, 1.0, 2.5, and 5 ppm level by adding standard mixtures to 5 mg/mL Albumin and Guar solutions as shown in Table 1. Matrix effects and percent recoveries were determined by spiking neat solutions into 5 mg/mL Albumin and Guar solutions. The sample preparation flowchart is shown in Figure 2.
**Table 1. Matrix Calibration Standard Preparation**

<table>
<thead>
<tr>
<th>Standard mix</th>
<th>Level 1</th>
<th>100ng/mL</th>
<th>100ng/mL</th>
<th>1ng/mL</th>
<th>1ng/mL</th>
<th>1ng/mL</th>
<th>10 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>2.5µl</td>
<td>12.5 µL</td>
<td>2.5 µL</td>
<td>5 µL</td>
<td>12.5 µL</td>
<td>2.5 µL</td>
<td></td>
</tr>
<tr>
<td>Matrix: 5mg/ml</td>
<td>997.5 µL</td>
<td>997.5 µL</td>
<td>997.5 µL</td>
<td>997.5 µL</td>
<td>997.5 µL</td>
<td>997.5 µL</td>
<td></td>
</tr>
<tr>
<td>Final Concentration</td>
<td>0.05ppm</td>
<td>0.25 ppm</td>
<td>0.5 ppm</td>
<td>1 ppm</td>
<td>2.5 ppm</td>
<td>5 ppm</td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 2. Sample Preparation Flowchart**

**Guar**
- Weigh 100 mg Guar, add 5mL Acetonitrile, vortex for 30 sec.
- Add 5 mL water to make a final concentration of 10 mg/mL. Sonicate for 10 min., then add 10mL ACN. Vortex mix for 1 min. After settling, transfer top solution to Eppendorf tubes.
- Prepare matrix calibration standards (table 1) and control, vortex mix 30 sec.
- Place vials in -30°C freezer for 30 minutes, then centrifuge at 17000 g for 30 min.
- Transfer the supernatant to 2-mL vials for injection.

**Albumin**
- Weigh 100 mg Albumin, Dissolve totally in 5mL water, then vortex mix.
- Add 15 mL ACN, the final concentration is 5mg/mL, then vortex mix 1 minute.
- Prepare matrix calibration standards (table 1) and control, vortex mix 30 sec.
- Place vials in -30°C freezer for 30 minutes, then centrifuge at 17000 g for 30 min.
- Transfer 500 µL supernatant, lyophilize to dryness, reconstitute to 500 µL using ACN/H2O 3:1, vortex mix. Place in -30°C freezer for 30 min. Centrifuge at 17000 g for 30 min.
- Transfer the supernatant to 2-mL vials for injection.

**Liquid Chromatography**
UHPLC Ultimate™ 3000 RS system consisting of:
- DGP-3000RS pump
- WPS-3000RS sampler
- TCC-3000RS column compartment

**LC Conditions:**
- Thermo Scientific Accucore HILIC Column 100x3 particle (µ) 2.6 Thermo Scientific p/n 17526-103030
- Guard cartridge: Accucore ™ HILIC 10x3.0 mm 2.6 µm Thermo Scientific p/n 17526-013005
- Mobile Phase A: Water
- Mobile Phase B: Acetonitrile
- Mobile Phase C: 100 mM Ammonium Acetate, adjusted to pH 5 using Acetic acid
- LC program: Isocratic 90% B 10% C for 5.5 minutes
- Flow rate: 0.5 mL/min.
- Column Temp: 35 °C
- Injection Volume: 5 µL

**Mass Spectrometry**
- Q Exactive high resolution benchtop Orbitrap MS
- Ion source conditions:
  - Ionization mode: alternating positive/negative ESI
  - Ion source: HESI-II
  - Sheath gas flow rate: 45 units N₂
  - Auxiliary gas flow rate: 10 units N₂
  - Spray voltage (KV):+3.5 for positive, -3.4 for negative
  - Capillary temp (°C): 320
  - S-lens RF level: 50.0
  - Heater temp (°C): 400

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**References**

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Overview

Weigh 100 mg Guar, add 5 mL 500 µL using ACN:H2O 3:1, vortex mix 1 minute. Place vials in -30°C freezer for 30 minutes, then centrifuge at 17000 g for 30 min. Transfer 500 µL supernatant, mix 1 minute. Add 5 mL ACN to vials and vortex mix for 10 min., then add 10 mL ACN. Vortex mix 1 minute, then centrifuge at 17000 g for 30 min. Weigh 100 mg Albumin with sub 2 ppm Error. Prepare matrix calibration standards at 0.05, 0.25, 0.5, 1.0, 2.5, and 5 ppm. Melamine and cyanuric acid were dissolved in water. Ammeline and ammelide were dissolved in 2 N ammonium hydroxide. Standard calibration curves were prepared in the ranges of 0.25 to 5 ppm and 0.05 ppm to 5 ppm, respectively. The analyses of albumin fortified with melamine and analogues have good linearity with six-point calibration curves over the range from 50 ppb to 5 ppm. The signal to noise ratio at 70,000 resolution (FWHM) were utilized for confident detection and quantification of melamine and its analogues. Albumin and Guar gum were fortified with sub 2 ppm Error.

Q Exactive Method Parameters
Full MS / dd-MS² with polarity switching
Resolution: full scan 70,000 FWHM; dd-MS² 35,000 FWHM (at m/z 200)
AGC target: 1e6
Scan Range (Full MS): 123 to 133 amu

Results: Simultaneous detection and quantification of melamine and its analogues in at-risk pharmaceutical components Albumin and Guar gum were fortified with sub 2 ppm Error. The analyses of albumin fortified with melamine and analogues have good linearity with six-point calibration curves over the range from 50 ppb to 5 ppm. The signal to noise ratio at 70,000 resolution (FWHM) were utilized for confident detection and quantification of melamine and its analogues. Albumin and Guar gum were fortified with sub 2 ppm Error. The analyses of albumin fortified with melamine and analogues have good linearity with six-point calibration curves over the range from 50 ppb to 5 ppm. The signal to noise ratio at 70,000 resolution (FWHM) were utilized for confident detection and quantification of melamine and its analogues. Albumin and Guar gum were fortified with sub 2 ppm Error.

FIGURE 3. Full Scan and MS/MS Spectra of 0.25 ppm Melamine in 5 mg/mL Albumin with sub 2 ppm Error.

a) Full Scan

b) dd-MS²

Signature Fragment Ion

FIGURE 4. Extracted Ion Chromatogram of Albumin Fortified with Melamine and Related Compounds at 0.25 µg/g level
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**Experimental**

Weigh 100 mg Albumin, Dissolve totally in 5mL water, then vortex mix. Place vials in -30°C freezer for 30 min. Weigh 500 mg Guar Gum, Dissolve totally in 5mL water, then vortex mix. Place vials in -30°C freezer for 30 min. Prepare matrix calibration standards:

- Melamine
- Cyanuric acid
- Ammeline
- Ammelide

Each stock solution was prepared at 1.0 mg/mL. Melamine and cyanuric acid were dissolved in water. Ammeline and ammelide were dissolved in 2 N ammonium hydroxide. Standard solutions as shown in Table 1.

**Results**

The sample preparation flowchart is shown in Figure 2. The signal to noise ratio at 250 ppb level was 27 for cyanuric acid and 191 for melamine which exceed FDA specification (S/N > 5:1 for ions of quantification) (see figure 4).

**Discussion**

**Introduction**

Melamine and its analogues: ammeline, ammelide, and cyanuric acid (shown in Figure 1. Structures of Melamine and Analogue Scientific Dionex Ultimate 3000 RS UHPLC system were employed in this study.

**Material and Reagents**

- Albumin CAS# 9048-46-8, Sigma-Aldrich, p/n A7906.
- Cyanuric acid CAS#108-80-5, purchased from ChromaDex Inc. California.

**Sample Preparation**

Ammeline and ammelide were dissolved in 2 N ammonium hydroxide. Standard solutions as shown in Table 1.

**Liquid Chromatography**

LC Conditions:
- LC program: Isocratic 90% B 10% C for 5.5 minutes
- Mobile Phase A: Water
- Mobile Phase B: Acetonitrile
- Mobile Phase C: 100 mM Ammonium Acetate, adjusted to pH 5 using Acetic acid

**Mass Spectrometry**

- Sheath gas flow rate: 45 units N2
- Ion source: HESI-II
- Ionization mode: alternating positive/negative ESI
- Desolvation gas flow rate: 10 units N2
- Heater temp (°C): 400
- Quadrupole temperature (°C): 175
- Number of cycles: 50
- Number of dwell time: 1
- Auxiliary gas flow rate: 10 units N2

**Matrix Effects**

Matrix effects and percent recoveries were determined by spiking neat solutions of melamine, cyanuric acid, ammeline and ammelide to 5 mg/mL Albumin and Guar Gum. Extracted matrix calibration standards were prepared at 0.05, 0.25, 0.5, 1.0, 2.5, and 5 ppm level by adding standard mixtures to 5 mg/mL Albumin and Guar Gum.

**Calibration Curves**

Melamine and Ammelide have 6 point calibration curves in the ranges of 0.05 to 5 ppm. Cyanuric acid and Ammeline have 5 point calibration curves in the ranges of 0.25 to 5 ppm and 0.05 to 2.5 ppm, respectively.

**References**

Results

Confident detection and quantification of melamine and its analogues were achieved using HR/AM full scan and ms/ms in a data-dependent fashion with polarity switching. HR/AM full scan data at 70,000 resolution (FWHM) were utilized for quantitation, the high quality full scan spectra have sub-2 ppm mass accuracy with external calibration and fine isotopic pattern of A+2 isotope ion on 15C and 16N for positive identification (see figure 3 a), which ensure accurate detection and quantification of ions of interest in the presence of complicated matrices. High resolution accurate mass measurement of signature product ions were utilized for confirmation of melamine and its analogues (see figure 3 b).

Isocratic LC separation was conducted using an Accucore HILIC column. Reproducible baseline separations were obtained within 5 minutes. Excellent sensitivity and selectivity were obtained by the HR/AM. The signal to noise ratio at 250 ppb level was 27 for cyanuric acid and 191 for melamine which exceed FDA specification (S/N > 5:1 for ions of quantification) (see figure 4).

The analyses of albumin fortified with melamine and analogues have good linearity with six-point calibration curves over the range from 50 ppb to 5 ppm with excellent linear regression coefficients (r²>0.99), (see figure 6).

For the analyses of guar gum fortified with melamine and analogues, melamine and ammeline have acceptable quadratic fit for six-point calibration curves over the range from 50 ppb to 5 ppm with regression coefficients (r²>0.99), and for cyanuric acid and ammeline, five-point quadratic fit calibration curves were obtained over the range from 0.25 to 5 ppm (cyanuric acid) and 0.05 to 2.5 ppm (ammeline), both with good regression coefficients (r²>0.99), (see figure 7).

Compared to commonly used triple quadrupole MS methods, this workflow is simple to set up and allows added post-analysis capability. It avoids the upfront selection of specific compound masses required for SRM methods. This method provides simultaneous detection and quantitation of melamine, ammeline and ammeline at 50 ppb level, and 250 ppb level for cyanuric acid, which is well below the FDA current requirement (2.5 ppm).

Conclusion

Simultaneous determination, confirmation, and quantitation of melamine and its analogues in at-risk pharmaceutical components Albumin and Guar gum were achieved. This was accomplished by using a complete Thermo Scientific solution, consisting of a high resolution Q Exactive benchtop Orbitrap mass spectrometer, Ultimate 3000RS UHPLC system, and Accucore HILIC column. This UHPLC-HRMS MS/MS method is simple, fast, and robust. The combination of HR/AM narrow range full scan and dd-MS² provides rapid, confident identification and quantification.

Further experiments will be carried out to investigate the potential for screening of melamine and its analogues in other at-risk pharmaceutical components using this workflow.

In addition, with appropriate sample preparation in place, this HR/AM full scan and dd-MS² method can be utilized for trace amount material identification, confirmation, and quantitation in other applications.

References

1. US FDA Guidance for industry: Pharmaceutical Components at Risk for Melamine Contamination
2. US FDA LIB 4421 Melamine and Cyanuric Acid Residues in Infant Formula October 2008

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