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

Kate Comstock; Tim Stratton; Hongxia (Jessica) Wang; Yingying Huang
Thermo Fisher Scientific, San Jose, CA USA

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.

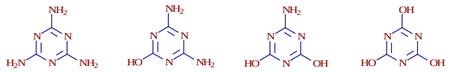
Results: Simultaneous detection and quantification of melamine and its analogues in Albumin and Guar gum were achieved with a high degree of confidence.

Introduction

Potential drug contamination by melamine and its analogues remains a major concern of the FDA. [1] 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.[1]

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.[1] 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



Melamine $C_3H_6N_6$ **(M+H)⁺ 127.0727** | **Ammeline** $C_3H_5N_5O$ **(M+H)⁺ 128.0567** | **Ammelide** $C_3H_4N_4O_2$ **(M+H)⁺ 129.0407** | **Cyanuric Acid** $C_3H_3N_3O_3$ **(M-H)⁻ 128.0102**

Methods

Material and Reagents

Melamine CAS # 108-78-1, Ammelide CAS# 645-93-2, Ammeline CAS# 645-92-1, and Cyanuric acid CAS#108-80-5, purchased from ChromaDex Inc. California.
Albumin CAS# 9048-46-8, Sigma-Aldrich, p/n A7906.
Guar CSA# 9000-30-0, Sigma-Aldrich, p/n G4129.
Acetonitrile and Water, Fisher.
Ammonium Acetate, Sigma-Aldrich, p/n 73594-25G-F.
Acetic Acid, Sigma-Aldrich, p/n A6283-1L.

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.

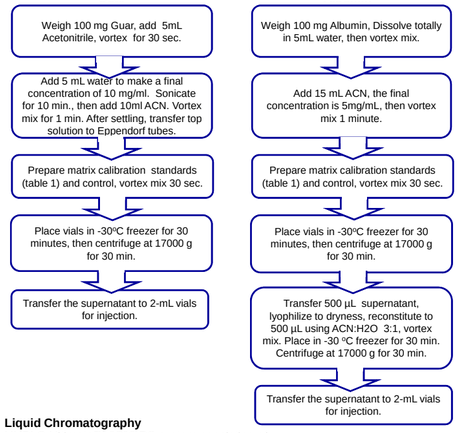
Matrix effects and percent recoveries were determined by spiking neat solutions into 5 mg/mL Albumin and Guar solutions.

The sample preparation flowchart is shown in Figure 2.

Table 1. Matrix Calibration Standard Preparation

Standard mix	Level	100ng/mL	100ng/mL	1ug/mL	1ug/mL	1ug/mL	10 ug/mL
	Volume	2.5ul	12.5 ul	2.5 uL	5 uL	12.5 uL	2.5 uL
Matrix 5mg/ml		997.5 uL	987.5 uL	997.5 uL	997.5 uL	997.5 uL	997.5 uL
Finial concentration		0.05ppm	0.25 ppm	0.5 ppm	1 ppm	2.5 ppm	5 ppm

FIGURE 2. Sample Preparation Flowchart



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 ºC
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 N_2
Auxiliary gas flow rate: 10 units N_2
Spray voltage (KV):+3.5 for positive, -3.4 for negative
Capillary temp (ºC): 320
S-lens RF level: 50.0
Heater temp (ºC): 400

Q Exactive Method Parameters

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

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

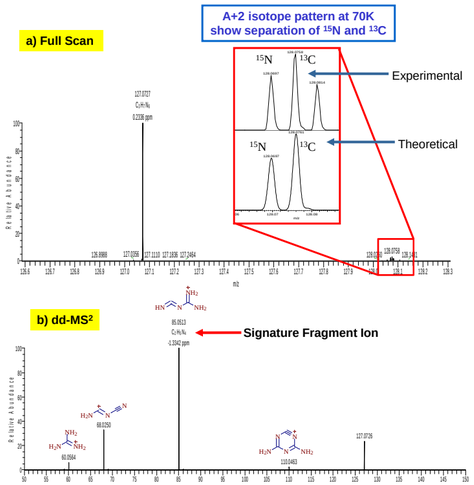


FIGURE 4. Extracted Ion Chromatogram of Albumin Fortified with Melamine and Related Compounds at 0.25 µg/g level

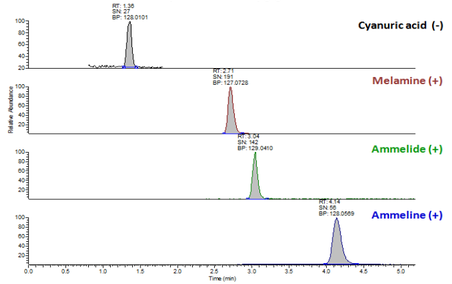


FIGURE 5. Calibration Curves of Neat Mix Standards (0.05 ppm to 5 ppm, linear fit)

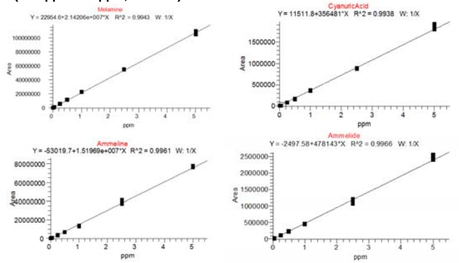


FIGURE 6. Calibration Curves of Albumin Fortified with Melamine and Related Compounds from 0.05 ppm to 5 ppm (linear fit)

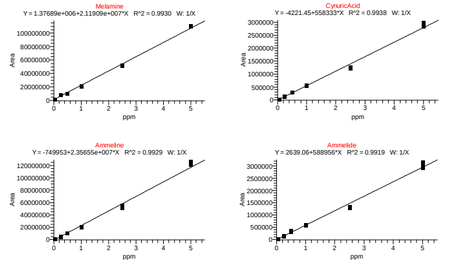
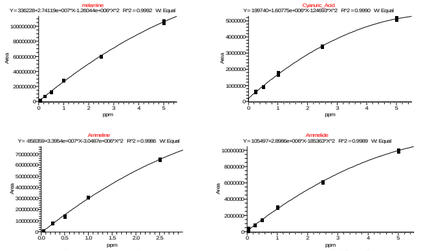


FIGURE 7. Calibration Curve of Guar Gum Fortified with Melamine and Related Compounds, Quadratic fit *



* 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 5ppm and 0.05 to 2.5 ppm, respectively.

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 ^{13}C and ^{15}N 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 (r^2>0.99), (see figure 6).

For the analyses of guar gum fortified with melamine and analogues, melamine and ammelide have acceptable quadratic fit for six-point calibration curves over the range from 50 ppb to 5 ppm with regression coefficients (r^2>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 (r^2>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 quantification of melamine, ammeline and ammelide 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