DCN: 20-003559 v. 1.0

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Effective Date:	20-Nov-2020		: Date of Next Review
Initiated By:	Uhlig, Mark W	N/A	: Supersedes
Reason for Print:	MasterControl		

Approval:

Approvers

Date	Time	Group	Name
20-Nov-2020	11:03:53 AM	SNR MGMT	Uhlig, Mark W
20-Nov-2020	11:11:30 AM	EDITOR	Struss, Adam
20-Nov-2020	11:13:56 AM	QUALITY	Bertsch, Amy M

ANALYTICAL METHOD VERIFICATION REPORT: UREA UPLC ASSAY

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1. PURPOSE:

1.1. The purpose of this Report is to:

1.1.1. Provide performance data that the determination of the Urea Assay procedure used on the Waters ACQUITY UPLC is adequately evaluated and verified.

1.1.2. Provide Verification that the procedure for determining the assay of Urea meets all requirements for accuracy, precision, linearity, range, ruggedness, and specificity.

2. SCOPE:

2.1. This Analytical Method Verification Report applies to the Assay Determination of Urea purity by assay using BioSpectra's Waters ACQUITY UPLC.

3. RESPONSIBILITIES:

3.1. The Quality Control Laboratory supervisor and Quality Control Analysts are responsible for completing the Method Verification Report using conclusions made from the results obtained from testing.

4. REFERENCE:

4.1. *USP Urea*
4.2. *USP <621> Chromatography*
4.3. *USP <1225> Validation of Compendial Procedures*
4.4. *USP <1226> Verification of Compendial Procedures*
4.5. Analytical Methods Validation Master Plan
4.6. Balance SOP
4.7. *Waters Acquity UPLC H-Class Plus SOP*

5. PRE-VERIFICATION REQUIREMENTS:

5.1. Equipment

5.1.1. All equipment to be used in this Verification is in proper working order and with current calibrations.

5.2. Personnel

5.2.1. All personnel who perform this Verification will be properly trained in accordance with the Analytical Method Validation Master Plan.

5.3. Supplies

5.3.1. All supplies used in the Verification will be clean and appropriate for their intended use.

5.4. Reagents

5.4.1. All reagents will be current, meet required specifications, and suitable for their intended use.

5.5. Reference Standards

5.5.1. All standards that will be used in this Verification are listed in the Materials and Equipment section.

5.6. Method

5.6.1. The method on the UPLC follows USP parameters and is set as follows:

5.6.2. Method Parameters:

Parameter	Setting
Flow Type	Gradient Elution
Mobile Phase	See 5.6.3
Flow Rate	1.0mL/min
Injection Volume	3µL
Detector	UV 195nm
Column Temperature	30°C
Run Time	15 min

5.6.3. Gradient:

Table 1

Time (min)	Solution A (%)	Solution B (%)
0.0	2.5	97.5
7.0	10.0	90.0
7.01	2.5	97.5
15.0	2.5	97.5

6. MATERIALS AND EQUIPMENT:

6.1. All materials and equipment utilized in this Verification are outlined in this section.

6.1.1. Analytical Balance

6.1.1.1. Manufacturer: Secura

6.1.1.2. Model: 124-1S

6.1.1.3. Serial Number: 29212172

6.1.1.4. Last Serviced: 10/20

6.1.1.5. Next Service: 4/21

6.1.2. Waters ACQUITY UPLC

6.1.2.1. Manufacturer: Waters

6.1.2.1.1. H-Class Unit

6.1.2.1.1.1. SN: K18CHA186G

6.1.2.1.1.2. Last Preventative Maintenance: 2/20

6.1.2.1.1.3. Next Preventative Maintenance Due: 02/21

6.1.2.1.2. UV Detector

6.1.2.1.2.1. SN: J18TUV016A

6.1.2.1.2.2. Last Preventative Maintenance: 2/20

6.1.2.1.2.3. Next Preventative Maintenance Due: 02/21

6.1.2.1.3. Solvent Manager

6.1.2.1.3.1. SN: K18QSP106A

6.1.2.1.3.2. Last Preventative Maintenance: 2/20

6.1.2.1.3.3. Next Preventative Maintenance Due: 02/21

6.1.2.1.4. Sample Manager

6.1.2.1.4.1. SN: K18FTP166G

6.1.2.1.4.2. Last Preventative Maintenance: 2/20

6.1.2.1.4.3. Next Preventative Maintenance Due: 02/21

6.2. Reagents
- 6.2.1. UPLC Grade Acetonitrile
  - 6.2.1.1. Supplier: Fisher
    - 6.2.1.1.1. Catalog Number: A998-4
      - 6.2.1.1.1.1. Lot: 198791
      - 6.2.1.1.1.2. Expiry Date: 2/28/25
      - 6.2.1.1.1.3. Open Date (If applicable): 8/20/20
- 6.2.2. UPLC Grade Water
  - 6.2.2.1. Supplier: In-House (Millipore)
- 6.2.3. UPLC Grade Water/Formic Acid
  - 6.2.3.1. Supplier: Fisher
    - 6.2.3.1.1. Catalog Number: HB523-4
      - 6.2.3.1.1.1. Lot: 196383
      - 6.2.3.1.1.2. Expiry Date:9/30/24
      - 6.2.3.1.1.3. Open Date (If applicable): 3/17/2020

6.3. Supplies
- 6.3.1. Micropipettes
  - 6.3.1.1. Supplier: Eppendorf
    - 6.3.1.1.1. Model: 100µL – 1000µL Pipette
      - 6.3.1.1.1.1. SN: 039512B
      - 6.3.1.1.1.2. Due: 12/31/20
- 6.3.2. Micropipette Tips
  - 6.3.2.1. Supplier: Eppendorf
    - 6.3.2.1.1. Part Number: 0030071581
- 6.3.3. Transfer pipettes
  - 6.3.3.1. Supplier: Fisher
    - 6.3.3.1.1. Part Number: 13-711-9AM
- 6.3.4. 10mm Screw Thread Vial Convenience Kit
  - 6.3.4.1. Supplier: Fisher
    - 6.3.4.1.1. Part Number: 03-391-18

6.4. Reference Standards
- 6.4.1. USP Traceable Related Compound A Reference Standard
  - 6.4.1.1. Supplier: USP
    - 6.4.1.1.1. Catalog Number:
      - 6.4.1.1.1.1. Lot: R085X0
      - 6.4.1.1.1.2. Expiry Date: Current Lot
      - 6.4.1.1.1.3. Open Date (If applicable): 6/8/20
- 6.4.2. USP Traceable Urea Reference Standard
  - 6.4.2.1. Supplier: Sigma-Aldrich
    - 6.4.2.1.1. Catalog Number: 1706698
      - 6.4.2.1.1.1. Lot: LRAC0302
      - 6.4.2.1.1.2. Expiry Date: 12/23
      - 6.4.2.1.1.3. Open Date (If applicable): 6/7/18

6.5. UPLC Column
- 6.5.1. Ascentis Express OH5 15cm x 4.6 mm. 2.7 um
  - 6.5.1.1. Supplier: Sigma-Aldrich

6.5.1.1.1. Part number: 53778-U

6.5.1.1.1.1. Serial Number: USZL002007

7. PROCEDURE:

7.1. Solution preparation

7.1.1. Diluent

7.1.1.1. Prepared 90:10 Acetonitrile: Water (UPLC Grade).

7.1.2. Mobile phase

7.1.2.1. Mobile Phase A (0.1% Formic acid in water)

7.1.2.1.1. Added 1mL of formic acid to 1L UPLC grade water

7.1.2.2. Mobile Phase B (Acetonitrile)

7.1.2.3. Wash Solvent

7.1.2.3.1. Used diluent listed above

7.1.3. RCA Stock Solution (0.5 mg/mL Biuret)

7.1.3.1. Weighed 4.4mg biuret into a 10mL volumetric flask.

7.1.3.2. Dissolved and brought to volume with diluent.

7.1.4. System Suitability Solution (5mg/mL Urea, 0.01mg/mL RCA)

7.1.4.1. Weighed 125.0mg urea reference standard into a 25mL volumetric flask

7.1.4.2. Pipetted 568.2µL of RCA stock solution into the common flask.

7.1.4.3. Dissolved and brought to volume with diluent

7.1.5. Standard Solution for Urea Assay

7.1.5.1. Refer to system suitability solution.

7.1.6. 80-120% Sample Solutions

7.1.6.1. Prepared solutions in class A volumetric flasks according to the table below using Urea reference material.

Assay Target Range	Urea Weight (mg)	Final Volume (mL)	Final Concentration (C_s)
80%	100.0	25.0	4.0mg/mL
90%	112.5	25.0	4.5mg/mL
100% (Prepare n=6)	125.0	25.0	5.0mg/mL
110%	137.5	25.0	5.5mg/mL
140%	175.0	25.0	7.0mg/mL

7.1.6.2. Dissolved and diluted to volume with diluent.

7.2. Setting up the instrument:

7.2.1. Refer to DCN 19-002766 Waters ACQUITY UPLC H-Class Plus SOP

7.2.1.1. A System Suitability run preceded the standard/sample run to ensure the method conditions are suitable for analysis.

7.2.1.1.1. The RSD can be NMT 1.0% for Urea

7.2.1.1.2. The resolution between Urea RCA and Urea should be NLT 1.5

7.2.1.2. The result will be calculated using the following equation:

7.2.1.2.1. $(R_u/R_s) \times (C_s/C_u) \times 100$

7.2.1.2.1.1. R_u = peak response from Sample solution
7.2.1.2.1.2. R_s = peak response from Standard solution
7.2.1.2.1.3. C_s = concentration of Urea in the Standard solution (mg/mL)
7.2.1.2.1.4. C_u = concentration of Urea in the Sample solution (mg/mL)

8. PERFORMANCE PARAMETERS:

8.1. System Suitability:
8.1.1. Injected the assay system suitability solution 5 times
8.1.2. Acceptance Criteria:
8.1.2.1. NLT 1.0% Urea RSD
8.1.2.2. Resolution NLT 1.5 between Urea and RCA
8.1.2.3. Result: Pass

Replicate	Area Count Urea	Relative Retention Time	Resolution (NLT 1.5)	Average Area Count Urea	% RSD (NMT 1.0%)
1	4569849	0.93	2.5	4584288.5	0.3
2	4575035	0.93	2.5		
3	4598522	0.93	2.5		
4	4574627	0.93	2.5		
5	4603409	0.93	2.5		

8.2. Specificity:
8.2.1. Specificity Solution 1: Three blank solutions were prepared and analyzed to determine whether the actual reagents in the method will interfere with the analysis.
8.2.2. Specificity Solution 2: Prepared a 0.01mg/mL solution of Urea RCA and analyzed in triplicate.
8.2.3. Specificity Solution 3: Prepared a 1.0mg/mL solution of Urea standard and analyze in triplicate.
8.2.4. Acceptance Criteria:
8.2.4.1. Reagents should not interfere with peaks of interest and Urea and RCA are full resolved in the system suitability solution injections. Use Specificity Solution 2 and 3 to identify Urea RCA and Urea retention times, respectively.
8.2.4.2. Result: Pass

Sample	Retention Time RCA (min)	Area Count	Retention Time Urea (min)	Area Count
Blank 1	None Detected	None Detected	None Detected	None Detected
Blank 2	None Detected	None Detected	None Detected	None Detected
Blank 3	None Detected	None Detected	None Detected	None Detected
Urea RCA 1	3.884	96176	None Detected	None Detected
Urea RCA 2	3.887	101049	None Detected	None Detected
Urea RCA 3	3.890	88680	None Detected	None Detected
Urea 1	None Detected	None Detected	4.212	1007708

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Urea 2	None Detected	None Detected	4.212	1000937
Urea 3	None Detected	None Detected	4.210	996631

8.3. Accuracy:

8.3.1. Refer to table 7.1.6.1 for sample preparation table.

8.3.2. Three replicate samples were analyzed at each concentration below and above 100% (mg/mL) level.

8.3.3. Six replicates will be prepared and analyzed at the 100% concentration.

8.3.4. Acceptance Criteria:

8.3.4.1. % RSD: NMT 1.0% At each level

8.3.4.2. % Recovery: 98.0-102.0%

8.3.4.3. % Recovery = (Assay Result % / CoA Urea Accepted Value %) * 100

8.3.4.3.1. Urea CoA Reference Value: 99.3%

8.3.4.4. Result: Pass, 80-110% Levels, Fail 140% Level.

Sample ID	Area Count	Assay Result (% Urea)	%RSD (NMT 1.0%)	% Recovery (98.0-102.0%)	Performance Result
4.0mg/mL Urea 1	3729554	100.9	0.3	101.7	Pass
4.0mg/mL Urea 2	3733035	101.0		101.8	Pass
4.0mg/mL Urea 3	3747244	101.4		102.2	Fail
4.5mg/mL Urea 1	4150032	99.8	0.2	100.6	Pass
4.5mg/mL Urea 2	4154405	99.9		100.7	Pass
4.5mg/mL Urea 3	4139087	99.5		100.3	Pass
5.0mg/mL Urea 1	4580656	99.1	0.2	99.9	Pass
5.0mg/mL Urea 2	4583709	99.2		100.0	Pass
5.0mg/mL Urea 3	4582889	99.2		100.0	Pass
5.0mg/mL Urea 4	4595726	99.4		100.2	Pass
5.0mg/mL Urea 5	4589189	99.3		100.1	Pass
5.0mg/mL Urea 6	4602765	99.6		100.4	Pass
5.5mg/mL Urea 1	5014321	98.6	0.2	99.4	Pass
5.5mg/mL Urea	4994294	98.2		99.0	Pass

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2					
5.5mg/mL Urea 3	4994091	98.2		99.0	Pass
7.0mg/mL Urea 1	6260960	96.8	0.3	97.6	Fail
7.0mg/mL Urea 2	6237438	96.4		97.2	Fail
7.0mg/mL Urea 3	6273500	97.0		97.7	Fail

8.4. Precision / Intermediate Precision:

8.4.1. Six replicate samples of Sample Solution 100% will be prepared. The samples will be prepared and analyzed by two separate analysts on two different days.

8.4.2. Analyst I will perform system suitability and standardizations separate from Analyst II.

8.4.2.1. Acceptance Criteria:

8.4.2.1.1. Relative Standard Deviation (RSD)

8.4.2.1.1.1. RSD = (standard deviation/average) x 100

8.4.2.1.1.2. NMT 1.0%

8.4.2.1.1.3. Precision Result: Pass

Analyst	Sample ID	Assay Result	% RSD (NMT 1.0%)	Performance Result
I	5.0mg/mL Urea 1	99.1	0.2	Pass
	5.0mg/mL Urea 2	99.2		
	5.0mg/mL Urea 3	99.2		
	5.0mg/mL Urea 4	99.4		
	5.0mg/mL Urea 5	99.3		
	5.0mg/mL Urea 6	99.6		

8.4.2.1.2. Combined RSD

8.4.2.1.2.1. Combined RSD between both analyst I and II is:

8.4.2.1.2.2. NMT 2.0

8.4.2.1.2.3. Result: Pass

Analyst	Sample ID	Assay Result	Combined % RSD (NMT 2.0%)	Performance Result
I	5.0mg/mL Urea 1	99.1	0.4	Pass
	5.0mg/mL Urea 2	99.2		
	5.0mg/mL Urea 3	99.2		

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	5.0mg/mL Urea 4	99.4		
	5.0mg/mL Urea 5	99.3		
	5.0mg/mL Urea 6	99.6		
II	5.0mg/mL Urea 1	98.8		
	5.0mg/mL Urea 2	98.6		
	5.0mg/mL Urea 3	98.9		
	5.0mg/mL Urea 4	98.4		
	5.0mg/mL Urea 5	98.9		
	5.0mg/mL Urea 6	99.3		

8.5. Linearity and Range:

8.5.1. The average response from the analysis five concentrations were prepared and analyzed in Section 7.1.6 of this analysis were used to graph instrument response to urea spike level.

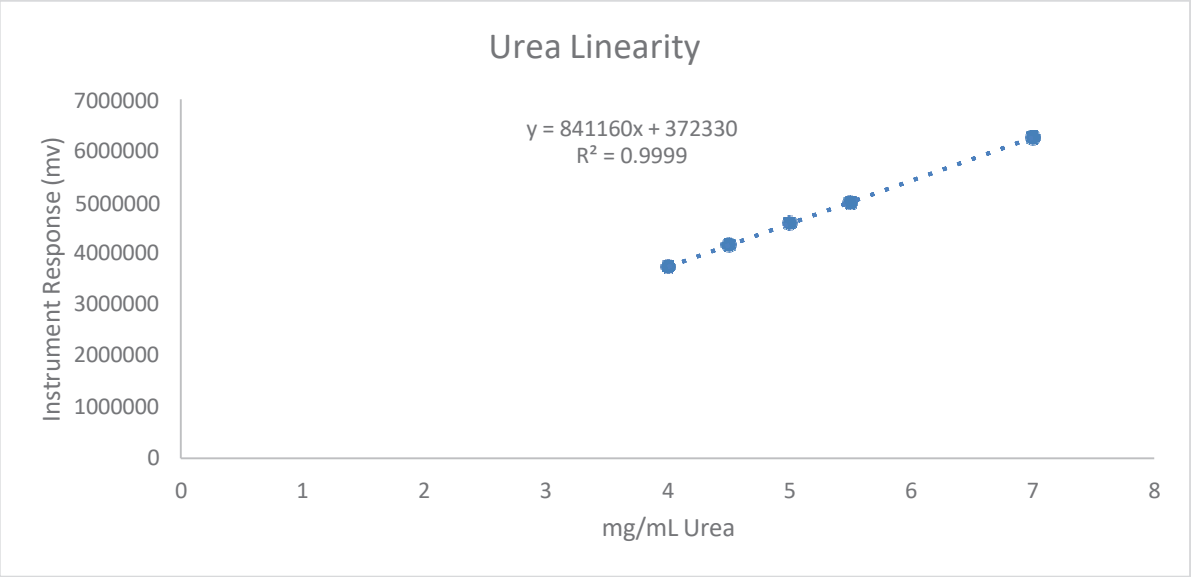
8.5.2. A linear regression line was used to determine the equation of the line and the correlation coefficient.

8.5.2.1. Acceptance Criteria:

8.5.2.1.1. A correlation Coefficient of NLT 0.99 is considered acceptable.

8.5.2.1.2. Result: Pass

Level	Urea Concentration	Average Instrument Response	r^2 (NLT 0.99)	Result
1	4.0mg/mL	3736611	1.00	Pass
2	4.5mg/mL	4147841		
3	5.0mg/mL	4589156		
4	5.5mg/mL	5000902		
5	7.0mg/mL	6257299		



9. VALIDATION STATUS:

9.1. The method of analysis for urea assay determination via UPLC is considered a verified method of analysis as it was found meet all acceptance criteria at the 4.5mg-5.5mg/mL levels and is an approved method of analysis at the BioSpectra Bangor, PA facility.

9.1.1. System Suitability: Pass

9.1.2. Specificity: Pass

9.1.3. Accuracy (98.0-102.0% Recovery): Pass

9.1.4. Precision (NMT 1.0% RSD): Pass

9.1.5. Linearity (r^2 of NLT 0.99): Pass

9.1.6. Range (Report): 4.5-5.5mg/mL Urea

9.1.7. Ruggedness/Intermediate Precision (NMT 2.0%): Pass

9.2. Critical Changes or Failure:

9.2.1. The “120% Level” was prepared at an actual concentration of 140% (7.0mg/mL) and % Recovery did not meet specification. The lower range of analysis investigated also did not meet % recovery criteria at the 80% level. The range of analysis is 90-110% of 5mg/mL urea for assay. Sample solutions lower in concentration should be appropriately diluted to fall within the linear working range of the instrument: 4.5-5.5mg/mL.

9.3. Laboratory Notebook References:

9.3.1. MV6, P97-99

9.3.2. MV7, P13-14

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