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ANALYTICAL METHOD: URIDINE ASSAY AND RELATED SUBSTANCES DETERMINATION BY UPLC WITH UV DETECTION

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1. PURPOSE:

1.1. To provide Analysts with a procedure for determining assay and related substance quantitation of Uridine by UPLC with UV detection.

2. SCOPE:

2.1. This analytical method applies to the measurement of Uridine assay and related substances on the Waters ACQUITY UPLC.

2.2. The related substances considered in this analytical method are pseudouridine and uracil at the limit described below.

Related Substance	Related Substance Limit (%area)
Uracil	0.5%
Pseudouridine	2%
Unspecified	0.1%
Total Impurities	3.0%

2.3. The Assay specification for uridine is 98.0% - 102.0%

2.4. This uridine assay and related substances method was validated as a category I and category II quantitative test, respectively.

3. RESPONSIBILITIES:

3.1. The Director of Laboratory Services, analysts and/or the Laboratory Technology Manager, if necessary, are responsible for the control, training, implementation and maintenance of this procedure

3.2. The analysts and/or qualified designee are responsible for performing the testing as stated in this procedure.

3.3. The analysts performing this procedure, with help and training from the Laboratory Technology Manager, are responsible for documenting the results obtained from testing.

3.4. Safety: Standard laboratory safety regulations apply. Before working with any chemical, read and understand the Safety Data Sheet (SDS).

4. REFERENCES:

4.1. BSI-RPT-0946, Analytical Method Validation Report: Uridine assay and Related Substances

4.2. BSI-SOP-0098, Balance SOP

4.3. BSI-SOP-0126, Laboratory Notebooks

4.4. BSI-SOP-0134, Pipette SOP

4.5. BSI-SOP-0348, Waters Acquity UPLC H-Class Plus SOP

4.6. BSI-SOP-0422, Empower 3 General Procedure

4.7. BSI-SOP-0436, Analytical Methods Validation Master Plan

4.8. USP-NF Current

4.9. USP <1225> Validation of Compendial Procedures

4.10. USP <1226> Verification of Compendial Procedures

5. MATERIALS AND EQUIPMENT:

- 5.1. Analytical Balance
- 5.2. Micro Balance
- 5.3. Weighing supplies: Weighing boats/funnels and spatulas
- 5.4. Waters ACQUITY UPLC
  - 5.4.1. Manufacturer: Waters
    - 5.4.1.1. Model: H-Class
- 5.5. Reagents
  - 5.5.1. UPLC Grade Water
  - 5.5.2. Potassium Phosphate Monobasic
- 5.6. Supplies
  - 5.6.1. Class A Volumetric Flasks.
  - 5.6.2. Polypropylene transfer funnels or aluminum weighing boats
  - 5.6.3. Analytical Balance
  - 5.6.4. HPLC auto sampler vials and caps
  - 5.6.5. Micropipettes
  - 5.6.6. Micropipette Tips
  - 5.6.7. Transfer pipettes
  - 5.6.8. 10mm Screw Thread Vial Convenience Kit
- 5.7. Reference Standards
  - 5.7.1. Uridine RS
  - 5.7.2. Uracil RS
  - 5.7.3. Pseudouridine RS
- 5.8. Column
  - 5.8.1. Luna Omega 3 µm Polar C18, 100 x 4.6 mm
    - 5.8.1.1. Supplier: Phenomenex
    - 5.8.1.2. Part number: 00D-4760-E0

6. PROCEDURE:

6.1. Solution Preparation:

6.1.1. Note: Solutions may be scaled as needed

6.1.2. Mobile Phase: 0.68% KH_2PO_4 (0.68:100, W/V)

6.1.2.1. Combine 6.80 g (±5%) of potassium phosphate monobasic and 1000 mL of UPLC grade water in a suitable container. Mix until the solution is clear with no visible solids.

6.1.3. Diluent: Mobile Phase

6.1.4. Column Storage Solution: 65% Acetonitrile: 35% Water

6.1.5. Sample Solutions (For finished goods, prepare in duplicate)

6.1.5.1. *Sample Test Solution* (100 µg/mL Uridine): Using a microbalance, accurately weigh and transfer 10.0 mg (± 10%) of uridine sample into a 100 mL volumetric flask, fill ~3/4 full with diluent, and sonicate with occasional swirling until the solution is clear with no visible solids. Allow the solution to equilibrate to RT, dilute to volume with diluent, and mix by inversion.

6.1.5.2. Stability: 11 days when stored stoppered in clear glassware at normal laboratory conditions.

6.1.6. Standard Solutions:

6.1.6.1. *Assay/System Suitability Solution* (100 µg/mL Uridine Reference Standard, prepare in duplicate): Using a micro balance, accurately weigh and transfer 10.0 (± 10%) mg of uridine reference standard into a 100 mL volumetric flask, fill ~3/4 full with diluent and sonicate with occasional swirling until the solution is clear with no visible solids. Allow the solution to equilibrate to RT, dilute to volume with diluent, and mix by inversion.

6.1.6.1.1. **Label SS1 and SS2, respectively.**

6.1.6.1.2. Stability: 11 days when stored in clear glassware at normal laboratory conditions.

6.1.6.2. *LOQ Solution* (0.05 µg/mL Uridine): Transfer 1.0 mL of the *Assay/System Suitability Solution* to a 100 mL volumetric flask, fill to volume with diluent, and mix by inversion (1.0 µg/mL). Transfer 5.0 mL of this solution to a 100 mL volumetric flask, fill to volume with diluent, and mix by inversion.

6.1.6.2.1. **Note: Prepare one replicate using SS1**

6.1.6.3. *Resolution Solution Stock* (50 µg/mL Uracil, 200 µg/mL Pseudouridine,): Using a micro balance, accurately weigh and transfer 5.0 (± 10%) mg of uracil and 20.0 (± 10%) mg of pseudouridine into a 100 mL volumetric flask. Fill ~3/4 full with diluent and sonicate with occasional swirling until the solution is clear with no visible solids. Allow the solution to equilibrate to RT, dilute to volume with diluent, and mix by inversion.

6.1.6.4. *Resolution solution* (100 µg/mL Uridine, 0.50 µg/mL Uracil, 2.0 µg/mL Pseudouridine): Accurately weigh and transfer 10.0 (± 10%) mg of uridine reference standard into a 100 mL volumetric flask and transfer 1.0 mL of the *Resolution Solution Stock* to flask. Fill ~3/4 full with diluent and sonicate with occasional swirling until the solution is clear with no visible solids. Allow the solution to equilibrate to RT, dilute to volume with diluent, and mix by inversion.

6.1.6.4.1. This solution is for qualitative purposes only (system suitability and related substance identification) and is considered stable until uracil and pseudouridine peaks can no longer be reliably identified. It is recommended to inject this solution prior to initiating an analysis to ensure the solution is *acceptable for use*. Store stoppered in clear glassware at normal laboratory conditions.

6.1.6.4.2. Approximate RRT of uracil: **0.45**

6.1.6.4.3. Approximate Impurity Response of uracil: **0.49%**

6.1.6.4.4. Approximate RRT of pseudouridine: **0.48**

6.1.6.4.5. Approximate Impurity Response of pseudouridine: **1.95%**

6.2. Instrument Setup

TABLE 1: INSTRUMENT METHOD PARAMETERS

Parameter	Setting
Flow Type	Isocratic
Diluent	0.68% KH_2PO_4
Mobile Phase	0.68% KH_2PO_4
Needle Wash	100% Purified Water
Flow Rate	1.0 mL/min
Injection Volume	20 µL
Detector	UV- 266 nm
Sample Temperature	Ambient
Column Temperature	30° C
Run Time	15 minutes
Sampling Rate	20 points/sec

TABLE 2: INJECTION SEQUENCE

Sample ID	Number of Injections
System Suitability	
Diluent	≥ 2
LOQ[1]	1
Resolution Solution	1
SS1	5
SS2	2
Samples[2]	
Diluent	1
Samples[3]	≤ 10
SS1	1

[1]LOQ may be omitted if performing assay only.
[2]Repeat the sample injection sequence if additional samples are to be analyzed.
[3]Samples may be substituted with diluent injections.

TABLE 3: SYSTEM SUITABILITY CRITERIA

System Suitability Parameter	Acceptance Criteria
%RSD for the peak area response of Uridine of the first 5 SS1 injections	NMT 0.73%
%RSD for the peak area response of Uridine of all standard SS1 injections	NMT 0.73%
USP Resolution between Pseudouridine and Uracil in the *Resolution Solution*	NLT 1.1
USP Tailing of Uridine in the first SS1 injection	NMT 1.5
USP Plate Count of Uridine in the first SS1 injection	NLT 10000
System Suitability Solution %Agreement between first 5 SS1 injections and SS2 injections	99% - 101%
S/N of Uridine in the LOQ injection	NLT 10

6.3. Calculations:

Related Substance	Relative Response Factor
Uracil	0.6
Pseudouridine	1.2

6.3.1. Assay:

6.3.1.1. Result = $(r_u/r_{SS1}) \times (C_{SS1}/C_u) \times 100$

6.3.1.2. Where:

6.3.1.2.1. r_u= peak area response of uridine from the *Sample Solution*

6.3.1.2.2. r_{SS1}= average peak area response of uridine from all SS1 injections

6.3.1.2.3. C_{SS1}= concentration of uridine in the SS1 solution

6.3.1.2.4. C_u= concentration of uridine in the *Sample Solution*

6.3.2. Adjusted Peak Area Response

6.3.2.1. Result = (r_I x RRF)

6.3.2.1.1. r_I = peak area response for each individual impurity peak in the *Sample Solution*

6.3.2.1.2. RRF = relative response factor

6.3.2.1.3. Assume an RRF of 1.0 for unspecified impurities

6.3.3. Impurity Response (% Area adjusted with the RRF):

6.3.3.1. Result = $(r_I \times RRF)/r_t \times 100$

6.3.3.2. Where:

6.3.3.2.1. r_I = peak area response for each individual impurity peak in the *Sample Solution*

6.3.3.2.2. RRF = relative response factor

6.3.3.2.3. r_t = total Adjusted Peak Area Response in the *Sample Solution*

6.3.3.2.4. Disregard system and diluent peaks.

6.3.3.2.5. Assume an RRF of 1.0 for unspecified impurities

6.3.4. Purity (Main Peak Area %):

6.3.4.1. Result = $(r_u / r_t) \times 100$

6.3.4.2. Where:

6.3.4.2.1. r_u= peak area response of uridine from the *Sample Solution*

6.3.4.2.2. r_t = total Adjusted Peak Area Response in the *Sample Solution*

6.3.4.2.3. Omit system and diluent peak from total peak area

6.3.5. Standard %Agreement:

6.3.5.1. Result = $(r_{SS2}/r_{SS1}) \times (C_{SS1}/C_{SS2}) \times 100$

6.3.5.2. Where:

6.3.5.2.1. r_{SS2} = average peak area response of uridine from the SS2 injections

6.3.5.2.2. r_{SS1} = average peak area response of uridine from the first 5 SS1 injections

6.3.5.2.3. C_{SS1} = concentration of uridine in the SS1solution

6.3.5.2.4. C_{SS2} = concentration of uridine in the SS2 solution

6.4. Reporting

6.4.1. **Assay**: Calculate the % Uridine for both replicates and report the average.

6.4.1.1. If any replicate has a result that is OOS, an OOS checklist will be issued to evaluate further.

6.4.1.2. If the results vary within ±2% of each other, no results will be averaged or reported until evaluated by the QC manager to determine if the results are valid/reportable or if any further action is required.

6.4.2. **Related Substances**: Calculate the percentage of each related substance for both replicates and report the average

6.4.2.1. If any replicate has a result that is OOS, an OOS checklist will be issued to evaluate further.

6.4.2.2. If a reportable related substance or unknown impurity is present in one replicate and not present in the other, an OOS checklist will be issued to evaluate further.

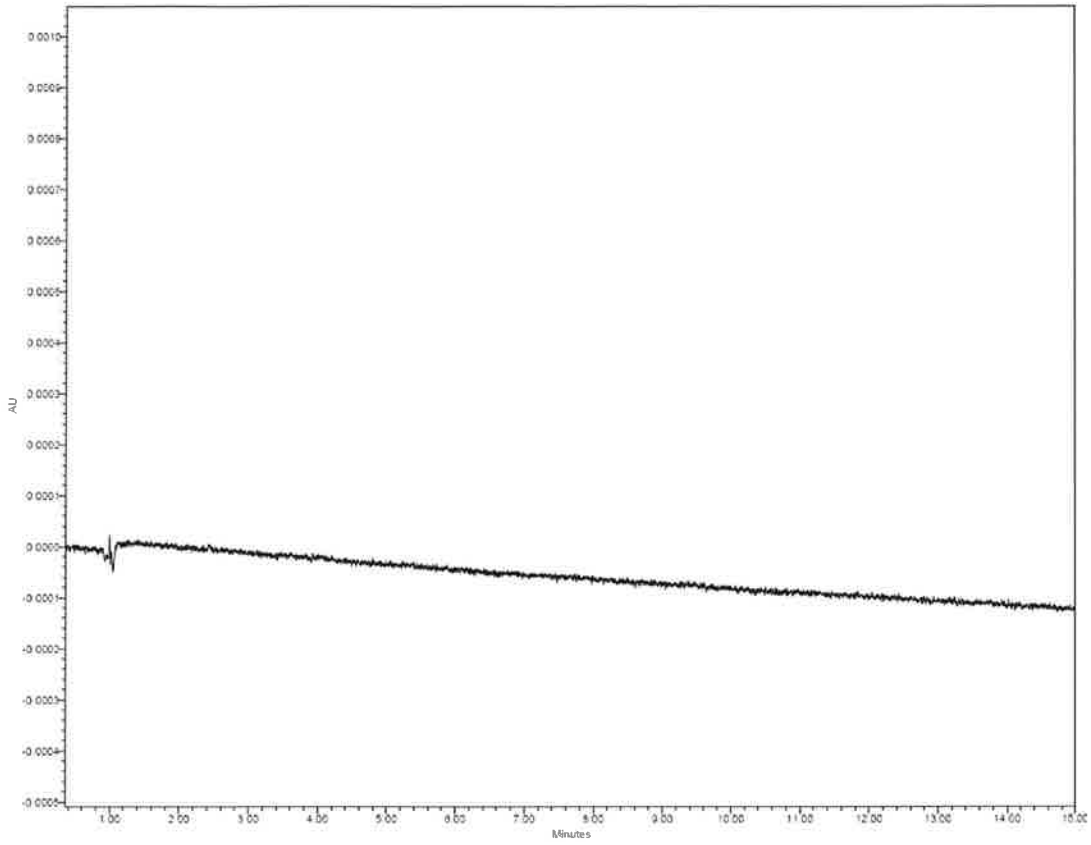
6.4.2.3. If an Impurity is detected in both replicates, and one is below the reporting threshold (0.05%) and one is above, the result above the reporting threshold will be reported.

6.4.3. Report Assay values to 1 (one) decimal place.

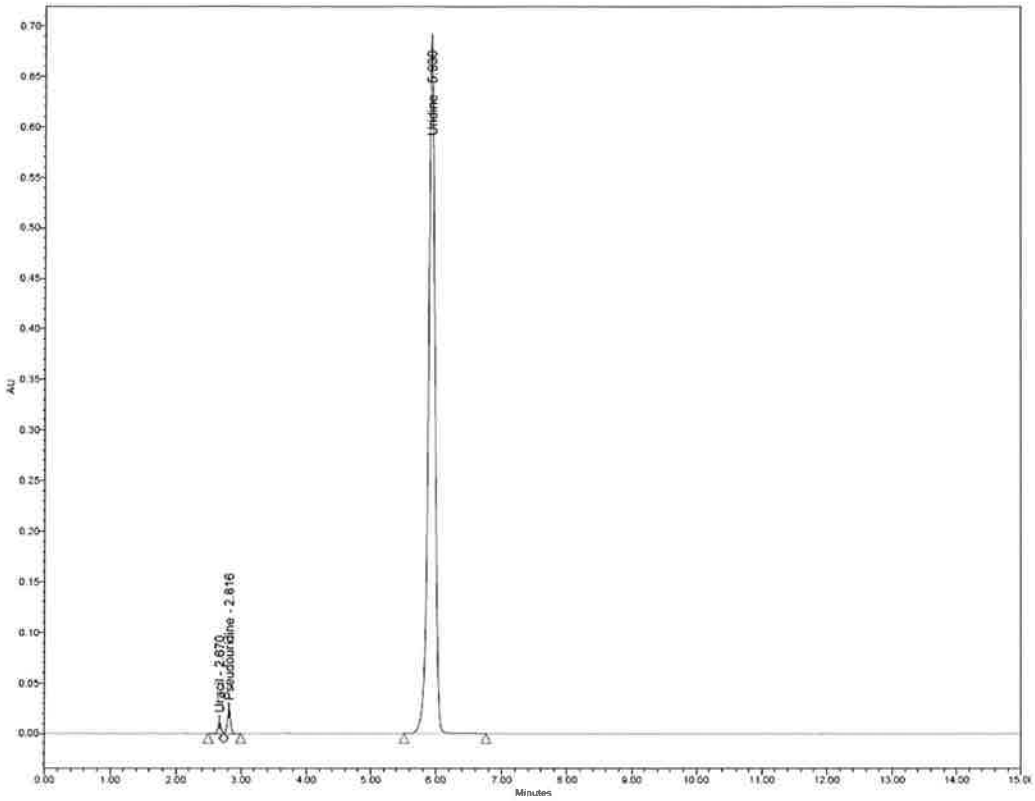
Related Substance Reporting	
Result	**Reporting**
If < 0.05%	Report as < LOQ
If ≥ 0.05% and < 1.0%	Report to two (2) decimal places
If > 1.0 %	Report to one (1) decimal place

6.5. Example Chromatograms and Integrations

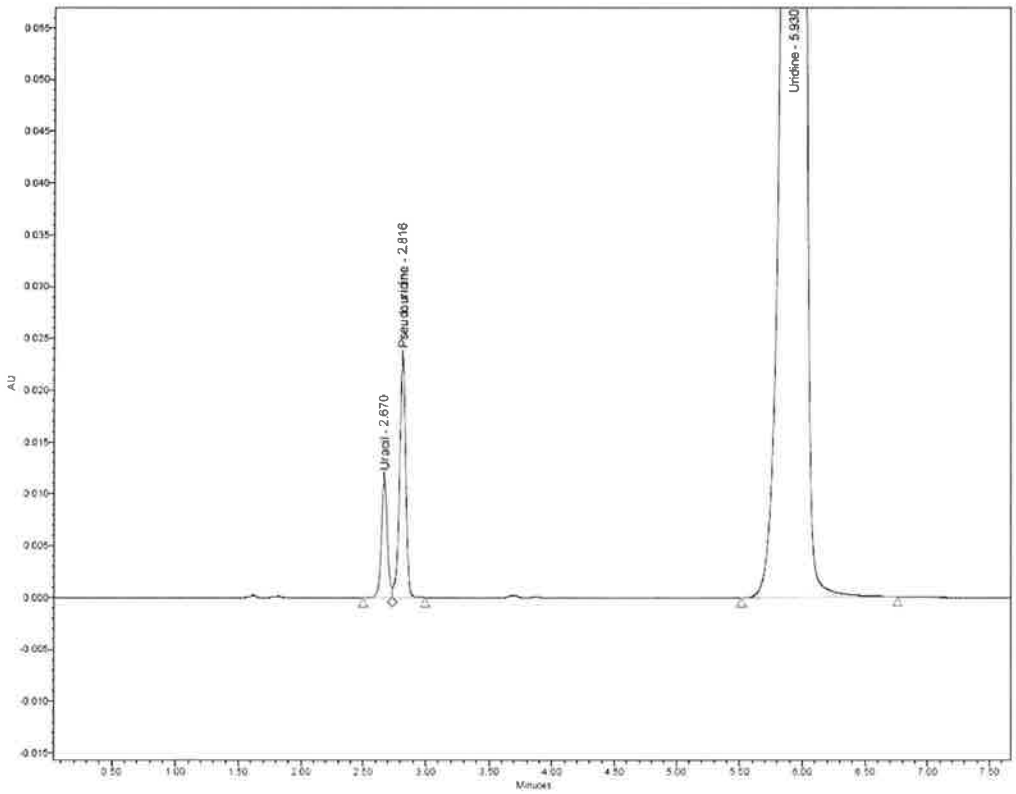
6.5.1. Diluent:



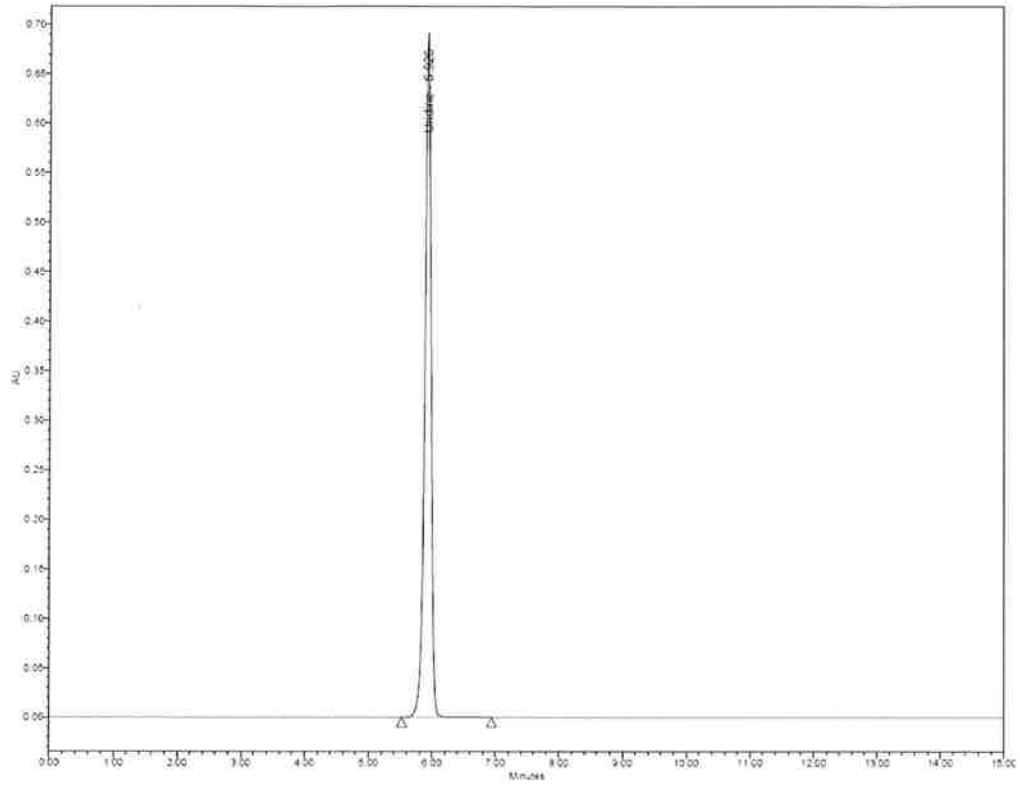
6.5.2. Resolution Solution:



6.5.3. Resolution Solution (Critical Pair Integration)



6.5.4. Assay/System Suitability Standard:



6.5.5. Standard (Main component integration)