BIOSPECTRA

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UREA ASSAY VIA HPLC

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1. PURPOSE:

1.1. To provide the Quality Control (QC) Analysts with a procedure for Urea Assay determination and for operating the Perkin Elmer Flexar HPLC.

2. SCOPE:

2.1. Applies to USP Urea Assay on the Perkin Elmer Flexar HPLC.

3. RESPONSIBILITIES:

3.1. The QC Manager, or other qualified designated individual, is responsible for the control, implementation, training and maintenance of this procedure.
3.2. The QC Analysts are responsible for complying with the requirements of this procedure.
3.3. If any abnormalities are determined during routine use of the HPLC or during calibration, the QC Manager shall be promptly notified. If necessary, the HPLC will be serviced and recalibrated by Perkin Elmer before being approved for use.

4. REFERENCE:

4.1. BSI-SOP-0098, Balance SOP
4.2. *Urea Assay HPLC Training and Troubleshooting Checklist*
4.3. *USP Urea*
4.4. *USP <621> Chromatography*

5. MATERIALS AND EQUIPMENT:

5.1. Instrumentation
  5.1.1. Analytical Balance
    5.1.1.1 Secura 124-1S S/N 29212172
  5.1.2. HPLC Flexar
    5.1.2.1. Flexar Autosampler S/N 293H32080804A
    5.1.2.2. Flexar LC pump S/N 291S133111109F
    5.1.2.3. Flexar Oven S/N OVHF130915868
    5.1.2.4. Flexar UV/Vis S/N 292S14031703F
5.2. Reagents
  5.2.1. HPLC Grade Acetonitrile
  5.2.2. HPLC Grade Water
  5.2.3. HPLC Grade 0.1% Formic Acid in Water
5.3. Supplies
  5.3.1. Micropipettes
  5.3.2. Micropipette Tips
  5.3.3. Transfer pipettes
  5.3.4. Screw Top Vials, 2mL 10 mm x 32 mm
  5.3.5. Screw Top with Slit Vials
5.4. Reference Standards
  5.4.1. USP Traceable Related Compound A Reference Standard
  5.4.2. USP Traceable Urea Reference Standard
5.5. HPLC Column
  5.5.1. Ascentis Express OH5 15cm x 4.6 mm. 2.7 um
    5.5.1.1. Part number: 53778-U

6. PROCEDURE:

6.1. If previous instrument use utilized any other mobile phases than 0.1% formic acid in water and acetonitrile, flush the system without a column before use to ensure that the system is primed with the appropriate and compatible mobile phases.

6.2. Ensure correct column is installed. If column is not installed, install the column by putting it in the HPLC oven and screwing both ends in to the respective flow lines. Do not over tighten fittings. Reference column documentation for column information if required.

6.2.1. Fill the mobile phase reservoir labeled "Solution A" with 0.1% formic acid in water.

6.2.2. Fill the mobile phase reservoir labeled "Solution B" with HPLC grade acetonitrile

6.2.3. Fill the vessel containing the sample line with HPLC grade acetonitrile

6.3. Standard Preparation: Ensure all USP traceable standards are traceable to the "current" lot by checking the Reference Standard offerings on USP-NF.

6.3.1. HPLC Diluent

6.3.1.1. Refer to "Laboratory Chemicals" for preparation.

6.3.2. Urea Standard (5mg/mL Urea)

6.3.2.1. Dissolve USP primary or secondary standard in HPLC diluent to a final concentration of 5.00mg/mL in an appropriately sized volumetric flask.

6.3.3. Organic Impurity Standard (0.01 mg/mL RCA, 0.01 mg/mL Urea)

6.3.3.1. Refer to "Laboratory Chemicals" for preparation.

6.3.4. System Suitability (0.01 mg/mL RCA, 10 mg/mL Urea)

6.3.4.1. Refer to "Laboratory Chemicals" for preparation.

6.4. Sample preparation

6.4.1. **Note - Possible sources of error in this analysis include but are not limited to: glassware/glove/weigh boat contamination, crystals on the analytical balance, improper transfer of sample and incorrect transfer of sample to vials. To avoid these errors; inspect weigh boats for contaminants prior to use, gloves should be checked for crystals after weighing samples, and clean the balance with an anti-static brush before each preparation.**

6.4.2. Assay

6.4.2.1. Accurately weigh 0.1250g of sample and transfer analytically into a 25mL volumetric flask. Dilute to line with HPLC diluent for a final concentration of 5mg/mL.

6.4.3. Organic Impurity

6.4.3.1. Accurately weigh 0.1000 grams of sample. Transfer to a 10mL volumetric flask. Dilute to line with HPLC diluent for a final concentration of 10mg/mL.

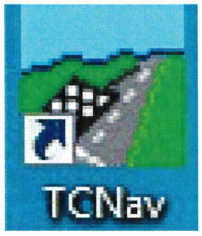
6.4.3.2. Equivalent sample preparation can be performed as long as the final concentration is equivalent to the concentration prepared in step 6.3.3.1.

6.5. Setting up the instrument:

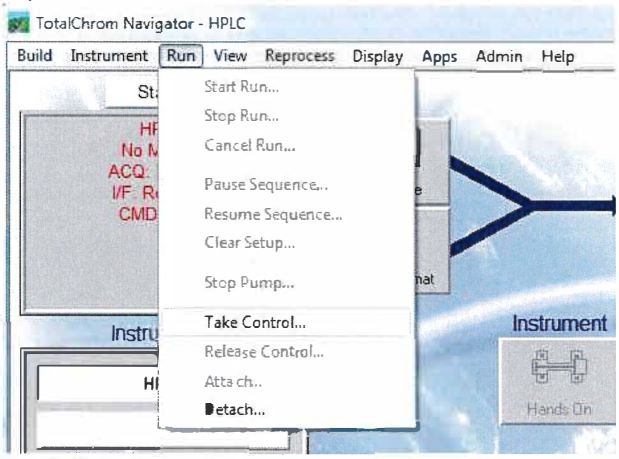
6.5.1. If the instrument is off, hold the power button on the Flexar Auto Sampler, Binary Pump, Column Oven, and the UV/VIS detector.

6.5.2. Open the TotalChrom Workstation and sign in.

6.5.2.1. Double click "TCNAV" icon on the desktop.



6.5.3. Under the "Run" tab, click "Take Control"



6.5.4. This will allow the software to gain control of the instrument.

6.5.5. Setting column temperature

6.5.5.1. Double click the oven icon on the desktop

6.5.5.2. The temperature of the column should be set at 30.0 degrees Celsius

6.5.6. Setting UV detector

6.5.6.1. Double click the UV/Vis icon

6.5.6.2. Set wavelength to 195nm.

6.5.6.3. Click "enter".

6.5.6.4. Ensure the detector is set to 195nm, urea does not exhibit high absorptivity andwill not be measurable at low concentrations when using another wavelength, if peak intensities are not as expected, check this parameter.

6.5.7. Starting the pump

6.5.7.1. On the main control panel click "Hands On"

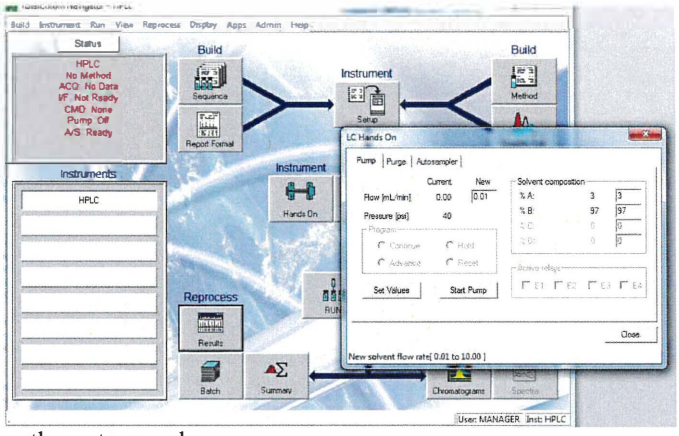
6.5.7.2. In the text box below "New", set the value of the pump at "1" to allow the pump to pump solution at a rate of 1 mL/min

6.5.7.3. Click "Set Value"

6.5.7.4. Allow the pressure to equilibrate (~1 hour)

6.5.7.4.1. The pressure may vary from one column to another.

6.5.7.4.2. The pump pressure should be monitored. Base level pressure may differ from column to column (900-1200 psi @ 1mL/min). A drastic change (±100 psi) of pressure may indicate column wear and/or an air bubble in the line.



6.5.8. Flushing the autosampler

6.5.8.1. Before beginning any runs, the autosampler should be flushed 3 times

6.5.8.1.1. Under "Hands On", click on the "Autosampler" tab

6.5.8.1.2. Click "Flush" to begin the process

6.6. Condition the column with 5-10mg/mL of urea using the column condition sequence. System suitability solution is an appropriate choice for column conditioning. Allow to run until qualitative equilibrium is achieved and desired resolution is demonstrated; the baseline should be stable and peaks should be separated and distinct. Conditioning can usually be obtained in less than 3 cycles. A simple check is to compare urea area counts of each condition to each other; they should not vary by more than 1% RSD. If the %RSD is unstable, consider filtering the solution (0.2micron), purchasing a column guard, remaking of solutions, back flushing the column, preparing in fresh glassware, reseating of the column, flush the column with more polar mobile phase, back flush the column with the most polar mobile phase, back flush the column with the most nonpolar mobile phase at high flow to remove any insoluble contaminants in the column frit, inspect tubing for bubbles, bleed system, check for kinks in tubing or any instrument faults.

6.6.1. A System Suitability run will precede the standard/sample run to ensure the method conditions are suitable for analysis.

6.6.1.1. The RSD can be NMT 1.0% for Urea

6.6.1.2. Calculate resolution utilizing the following equations:

6.6.1.2.1. (retention time 2 – retention time 1)/[0.5*(Width 1+ Width 2)]

6.6.1.2.2. Width=Area/Height

6.6.1.2.3. The resolution should be NLT 1.5

6.6.1.2.4. If system suitability requirements are not met, trouble shoot the system using step 6.6 or refer to DCN 18-001980 for troubleshooting as well as the user manual or Perkin Elmer service.

6.6.2. Standards will be run after System Suitability

6.6.2.1. The RSD must be NMT 1.0% for Urea in order to consider data acceptable.

6.6.2.2. The average area of the three or more consecutive standard runs will be used for the final sample assay result calculation.

6.6.3. A system suitability run will follow sample runs (at the end of the daily run and/or every5 samples) as a QC check.

6.6.3.1. System suitability may be run more frequently, every 5 samples is the minimum amount of times it may be run.

6.6.4. Setting up the sequence

6.6.4.1. Before running samples, a sequence containing sample name, vial number,method file, and data file needs to be set.

6.6.4.1.1. Any number of sequences may be used to complete the analysis.

6.6.4.2. Under the "Build" panel, click "Sequence"

6.6.4.3. Choose the "Urea Assay" sequence.

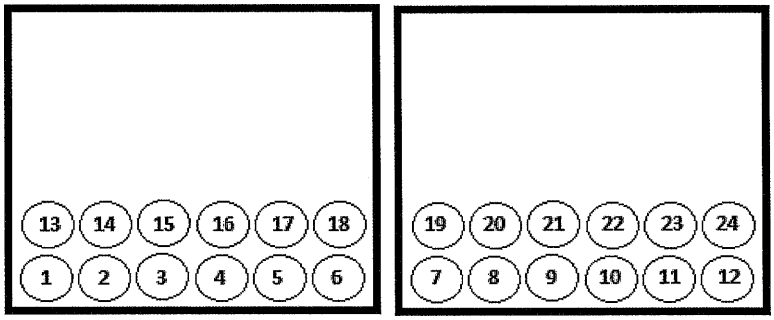
6.6.4.3.1. The file will contain rows for each sample that will be run.

6.6.4.3.2. Verify that the correct method file is selected under the "Method" column.

6.6.4.3.3. Under the "Name" column header, enter the sample names.

6.6.4.3.4. System suitability should be run prior to any standard and sample runs.

6.6.4.3.5. Enter the vial number that corresponds to where the sample was placed in the autosampler, as shown below.



6.6.4.3.6. A new Data save file will need to be created under the "Data" column header for each run.

6.6.4.3.6.1. Double click the Data cell for the first row, a window will open.

6.6.4.3.6.2. Double Click on "Urea"

6.6.4.3.6.3. Create a New Folder with the current date and initials of the analyst running the samples

6.6.4.3.6.4. Inside this folder, enter your sample name as your file name. Click "Select" to confirm the location that the data will go and to return to the Sequence Editor

6.6.4.3.6.5. For replicate runs (such as suitability) the software can create separate data files

6.6.4.3.6.5.1. After one data folder is created, drag the cell of the designated file in the sequence editor down to the last similar sample. Right click and select"smart fill"

6.6.4.3.6.5.2. This is to make sure that each run gets its own file; failure to follow this step will cause all the runs to overwrite.

6.6.4.4. Place the samples in the vial order set up in the sequence.

6.6.4.5. Sequence example:

Sample

Row	Type	Study name	Name	Note	Number	Vial
1	Sample	Urea assay	System Suitability 1		1	1
2	Sample	Urea assay	System Suitability 2		2	1
3	Sample	Urea assay	System Suitability 3		3	1
4	Sample	Urea assay	System Suitability 4		4	1
5	Sample	Urea assay	System Suitability 5		5	1
6	Sample	Urea assay	USP Urea Standard 1		6	2
7	Sample	Urea assay	USP Urea Standard 2		7	2
8	Sample	Urea assay	USP Urea Standard 3		8	2
9	Sample	Urea assay	Organic Impurity Standard 1		9	3
10	Sample	Urea assay	Organic Impurity Standard 2		0	3
11	Sample	Urea assay	Organic Impurity Standard 3		1	3
12	Sample	Urea assay	SAMPLE ASSAY		2	4
13	Sample	Urea assay	SAMMPLE OI		3	5
14	Sample	Urea assay	QC System Suitability		4	6

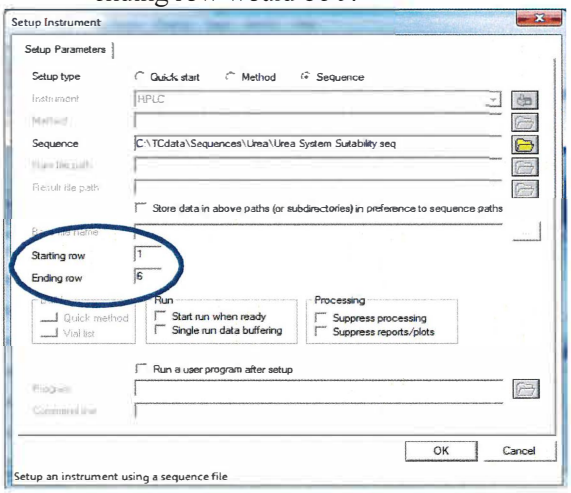
6.6.4.6. In the Sequence Editor window, click the "Actions" tab, and select "Setup".

6.6.4.6.1. The Setup window will appear with the selected sequence.

6.6.4.6.2. Ensure that the starting row is set at "1" and the ending row matches the number of samples that will be run in the sequence.

6.6.4.6.3. Click "OK" to finish the setup.

6.6.4.6.3.1. In the sequence below, starting row 1 indicates the first line of the sequence setup and ending row 6 indicates the last line of the sequence. If there were 9 samples to run, the ending row would be 9.



6.6.4.7. Allow the status of the HPLC to turn green.

6.6.4.7.1. Once ready, click "RUN" and then "Start Run"

6.6.4.7.2. The analysis can be viewed using the "Real-Time Plot" window.

6.6.4.8. Once the run has completed, the setup must be cleared in order to initiate the sample sequence.

6.6.4.8.1. Click "RUN" and select "Clear setup". This will cause the status of the HPLC to turn red.

6.7. Result Reporting

6.8. A qualitative inspection of chromatograms utilized in system suitability, standard data acquisitions and sample analyses will be performed to ensure integration areas are consistent and data is acceptable for reporting. If chromatograms do not align qualitatively, the difference will be documented and the system will be troubleshot.

6.9. In addition to a qualitative inspection of chromatographic quality, a quantitative evaluation should be made of integration area for Urea to assess drift between the system suitability solutions (QC Checks). Drift will be monitored in the same manner as the system suitability runs, with a 1%RSD max acceptance criteria.

6.9.1. The assay result will be calculated using the following equation:

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

6.9.1.1.1. R_u = peak response from Sample solution

6.9.1.1.2. R_s = peak response from Standard solution

6.9.1.1.3. C_s = concentration of Urea in the Standard solution (mg/mL)

6.9.1.1.4. C_u = concentration of Urea in the Sample solution (mg/mL)

6.9.2. The organic impurity result will be calculated using the following equation:

6.9.2.1. Result = $(r_U/r_S) \times (C_S/C_U) \times 100$

6.9.2.1.1. R_u = peak response of Urea Related Compound A from Sample solution

6.9.2.1.2. R_s = peak response of Urea Related Compound A from Standard solution

6.9.2.1.3. C_s = concentration of Urea in the Standard solution (mg/mL)

6.9.2.1.4. C_u = concentration of Urea in the Sample solution (mg/mL)

6.9.3. The unspecified impurity result will be calculated using the following equation:

6.9.3.1. Result = $(r_U/r_S) \times (C_S/C_U) \times 100$

6.9.3.1.1. R_u = peak response of any impurity from Sample solution

6.9.3.1.2. R_s = peak response of Urea from Standard solution

6.9.3.1.3. C_s = concentration of Urea in the Standard solution (mg/mL)

6.9.3.1.4. C_u = concentration of Urea in the Sample solution (mg/mL)

6.9.3.1.5. Disregard any individual impurity below 0.05% and report as “Not Reportable”.

6.10. Shutting Down the Instrument

6.10.1. Once the run has completed, click “Hands On” and turn the pump off.

6.10.2. Under the “Run” tab, click “Release Control”

6.10.3. Power down each component of the HPLC unit.

6.10.4. Remove and store column according to manufacturer literature for long periods of shutdown. (Greater than two weeks).