Simultaneous Analysis of Uric Acid and Creatinine in Plasma and Urine Using Flow Injection Tandem Mass Spectrometry

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Abstract

The levels of uric acid and creatinine in serum and urine have important clinical implications. In this protocol, we developed a simple, rapid and reliable method to simultaneously determine uric acid and creatinine using flow-injection analysis tandem mass spectrometry (FIA–MS/MS) with polarity-switching electrospray ionization. The plasma and urine samples were extracted using the extraction buffer (acetonitrile: methanol (50:50) +5 mM NH4OAC and 1 mM EDTA) prewarmed to 70 ºC. Stable isotope internal standards 15N2-uric acid and creatinine-13C were added into the samples prior to the extraction. Ten µl of extract was injected into MS/MS. Uric acid and its isotope standard were analyzed in negative mode using MRM transitions 167.11—124.00 (Q1-Q3) and 169.10-125.00, respectively. Creatinine and its isotope standard were analyzed in positive mode using 114.12-86.10 and 115.10-87.10, respectively. The dwell time was 180 ms and the polarity switch time was 50 ms. The lower limit of detection (LLOD) was 0.02 µM for uric acid and 0.03 µM for creatinine. The analysis time only takes about 3 min.

Keywords Uric acid, creatinine, flow-injection analysis, MS/MS
Background

Uric acid is the final oxidation product of purine metabolism in human (Figure 1a). Creatinine is a breakdown product of creatine phosphate in muscle (Figure 1b). Creatinine is transported to kidney by blood plasma, whereupon it is eliminated from the body by glomerular filtration and partial tubular excretion. Uric acid and creatinine have important clinical implications. The examination of uric acid and creatinine is the commonly used test for the evaluation of renal function. In addition, the elevated serum uric acid level in hypertensive pregnant women is a useful prognostic indicator of severe hypertension; hemolysis, elevated liver enzymes, low platelets (HELLP) syndrome; and preeclampsia. High levels of creatinine is also reported to be associated with metabolic syndrome. In clinical labs, creatinine concentration is frequently measured using the Jaffe reaction between creatinine and picrate, which generates an orange chromogen. Uric acid concentration is commonly determined by enzymatic methods such as the uricase and phosphotungstate methods. However, the colorimetric methods are not specific and influenced by many other compounds in plasma and urine. Chromatographic assays are not affected by endogenous interferences and are more selective. To date, several chromatographic methods have been reported for analysis of uric acid and creatinine. These include high performance liquid chromatography (HPLC) with UV detection, gas chromatography–mass spectrometry (GC-MS), capillary electrophoresis, and liquid chromatography–tandem mass spectrometry (LC–MS/MS). However, the above methods required column separation which are time-consuming. So far, no analytical method has been published for simultaneous determination of uric acid and creatinine by FIA-MS/MS.

This protocol described a fast, sensitive, specific and reliable method for quantification of uric acid and creatinine by FIA-MS/MS. The analysis time was only 3 min. The lower limit of detection (LOD) was as low as 20 nM for uric acid and 30 nM for creatinine.

Reagents and Equipment

1. ABSCIEX 5500 QTRAP LC-MS/MS
2. Leap CTC Autosampler (Part number:252984) Cooling stack set at 4°C
3. Autosampler Syringe Cleaning Solvent (Hamilton, #18311)
4. Shimadzu CTO-20AC column oven (Set at 70 °C)
5. Dry block heater (Set at 70 °C)
6. 2mM ^15N$_2$-Uric Acid MW 170.11 (Catalog TA0832V, Sigma)
7. 10mM ^13C-Creatinine MW114.12 (Catalog number EB1523V, Sigma)
8. HPLC Grade Methanol (Catalog Number SHBC2652V, Sigma)
9. HPLC Grade Isopropanol (Catalog Number 060771, Sigma)
10. HPLC Grade Acetone (Catalog Number 650501, Sigma)
11. Trace Grade 30% Ammonium Hydroxide (Spectrum chemical)
12. Wash Solvent (1:1:1:1 Water, Methanol, Isopropanol, Acetone; 0.1% [17.4 mM] 30% NH$_4$OH)
13. Mobile Phase (50%:50% Water: Methanol)
14. 80% Methanol Diluent
15. 0.3M NaOH
16. Hot extraction buffer (Prewarm at 70°C): Acetonitrile: Methanol (50:50) with 5mM NH₄OAC and 1mM EDTA

Standard stock solution preparation and storage

2mM $^{15}$N₂-Uric Acid in 30 mM NaOH and miliQ H₂O

1. Tare a 2 ml Eppendorf tube to the nearest microgram.
2. Carefully weigh about 6.80 mg (about 40 µmol) of $^{15}$N₂-uric acid into the tube and note the total mass.
3. Calculate the exact number of µmol to 3 significant digits.
4. Add 2 ml of 0.3M NaOH.
5. Vortex it and make sure UA dissolve completely. It usually only need 1min.
6. Transfer to a 50 ml conical, screw-cap tube.
7. Add about 18 ml of water and mix well.
   a. The exact final volume in ml is the number of µmol, eg 40 µmol, divided 2 µmol/ml.
   b. If the actual mass was 6.8 mg (6.8 mg/170.11 mg/µmol = 0.04 mmol = 40 µmol), then the total volume is 40/2
      = 20 ml.
8. Divide into 30, 1.5 ml aliquots, with Lot and Date Numbers.
9. Store in the dark (eg in a labeled cardboard freezer box) at -20°C.

10mM $^{13}$C-Creatinine in water

1. Tare a 1.6 ml Eppendorf tube to the nearest microgram.
2. Carefully weigh about 22.80 mg (about 200 µmol) of $^{13}$C-Creatinine MW 114.12 into the tube and note the total mass.
3. Calculate the exact number of µmol to 3 significant digits.
4. Add 1.5 ml of water and mix to dissolve.
5. Transfer to a 50 ml conical, screw-cap tube (Corning or Falcon).
6. Add about 18 ml of water and mix well.
   a. The exact final volume in ml is the number of µmol, eg 200 µmol, divided 10 µmol/ml.
   b. If the actual mass was 22.8 mg (22.8 mg/114.12 mg/µmol = 0.2 mmol = 200 µmol), then the total volume is
      200/10 = 20 ml.
7. Divide into 30, 1.5 ml aliquots, with Lot and Date Numbers.
8. Store in the dark (eg in a labeled cardboard freezer box) at -20°C.

Instrument setup

MRMs of the analytes

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Polarity Mode</th>
<th>Exact Parent Mass</th>
<th>MRM (Q1-Q3)</th>
<th>Dwell time (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uric Acid</td>
<td>Negative</td>
<td>[M-H]- = 167.11</td>
<td>167.11--124.00</td>
<td>180</td>
</tr>
<tr>
<td>Uric Acid-$^{15}$N₂</td>
<td>Negative</td>
<td>[M-H]- = 169.10</td>
<td>169.10--125.00</td>
<td>180</td>
</tr>
<tr>
<td>Creatinine</td>
<td>Positive</td>
<td>[M+H]+ = 114.12</td>
<td>114.10--86.10</td>
<td>160</td>
</tr>
<tr>
<td>Creatinine-13C</td>
<td>Positive</td>
<td>[M+H]+ = 115.10</td>
<td>115.10--87.10</td>
<td>160</td>
</tr>
</tbody>
</table>
### MS/MS settings

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Temperature (°C)</th>
<th>Gas1</th>
<th>Gas2</th>
<th>Curtain gas</th>
<th>Collision gas (CAD)</th>
<th>DP</th>
<th>EP</th>
<th>CE</th>
<th>CXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uric Acid</td>
<td>500</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>High</td>
<td>-95</td>
<td>-10</td>
<td>-20</td>
<td>-17</td>
</tr>
<tr>
<td>Creatinine</td>
<td>500</td>
<td>40</td>
<td>30</td>
<td>30</td>
<td>11</td>
<td>31</td>
<td>9</td>
<td>15</td>
<td>4</td>
</tr>
</tbody>
</table>

### Autosampler and LC settings

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Autosampler Cooling Stack T°C</th>
<th>Autosampler Injection Speed (µl/S)</th>
<th>Mobile Phase Composition</th>
<th>Mobile Phase Flow Rate (µl/ml)</th>
<th>Wash Solvent Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uric acid</td>
<td>4°C</td>
<td>10</td>
<td>MEOH:H₂O (50:50)</td>
<td>0.2</td>
<td>Wash solution</td>
</tr>
<tr>
<td>Creatinine</td>
<td>4°C</td>
<td>10</td>
<td>MEOH:H₂O (50:50)</td>
<td>0.2</td>
<td>Wash solution</td>
</tr>
</tbody>
</table>

### Extraction of uric acid and creatinine from urine and plasma

1. Pipette 10 µl urine sample into a 1.5 ml Eppendorf tube on ice and add 990 µl H₂O (100-fold dilution before extraction).
2. Pipette 50 µl plasma or 100-fold diluted urine sample into a new 1.5ml Eppendorf tube on ice.
3. Spike 5 µl of isotope internal standard mixture (15N₂-Uric Acid 1mM+13C-Creatinine 0.5mM) into the plasma or urine and vortex the tube to mix.
4. Add 195 µl of hot extraction buffer in to the tube, vortex it thoroughly and incubate at 70 °C dry block for 5min.
5. Centrifuge at 16,000g for 5 min.
6. Carefully transfer about 200 µl of supernatant to a clean 1.5ml Eppendorf tube without disturbing the pellet. Discard the pellet.
7. Transfer 50 µl of supernatant to another 1.5ml Eppendorf tube and store the rest of supernatant at -20 °C
8. Dilute 50 µl supernatant to 1ml (final dilution = 100-fold; 5x then 20x) by adding 950 µl of 80% methanol and mix it.

### FIA-MS/MS Analysis

1. Open Analyst 1.6.1 software and check the hardware configuration and make sure LC-MS is activated.
2. Create a folder from Analyst top panel and build a new "acquisition method" from Analyst left panel.
3. Click “Build acquisition batch”. Define the set name, number of samples, Rack code, Rack position, plate code, vial position, analysis method, and data folder. Add blank in the beginning and at the end of the whole batch. Add blanks every two injections.

4. Click “submit” to send the samples to queue. Click “View Queue” on the top left panel of analyst and then click “equilibrate” icon.

5. Equilibrate the method and then click “start” button to start the analysis once the instrument is ready.

**Validation of the assay**

<table>
<thead>
<tr>
<th>Plasma</th>
<th>LLOD (µM)</th>
<th>LLOQ (µM)</th>
<th>ULOQ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uric Acid</td>
<td>0.02</td>
<td>0.05</td>
<td>5</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.03</td>
<td>0.05</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Urine</th>
<th>LLOD (µM)</th>
<th>LLOQ (µM)</th>
<th>ULOQ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uric Acid</td>
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<td>Creatinine</td>
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<td>0.05</td>
<td>2</td>
</tr>
</tbody>
</table>
Tips and Notes

1. Uric acid adsorbs non-covalently to the internal surfaces of PEEK HPLC tubing.
2. Wash the PEEK tubing every 10 to 15 samples using the Wash Solvent (1:1:1:1 Water, Methanol, Isopropanol, Acetone; 0.1% [17.4 mM] 30% NH₄OH).
3. Disconnect LC from Mass Spec and activate Qtrap 5500 alone.
4. Click manual tuning on the left panel of Analyst 1.61. Choose a Q1 scan. Set the source temperature to 600˚C, Gas 1 and Gas 2 to 50.
5. Click “start” button on the top panel of Analyst and hit stop immediately. The mass spec will keep the settings for 1h.
6. Turn on the LC-pump manually and wash the tubing at 0.2ml/min for about 2 hours. Keep an eye on mass spec. Hit “start” and “stop” again after 30 min and 1h. Otherwise, the mass spec will be automatically in “standby” mode.

Acknowledgement

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References