Detection of Creatinine in Smokers

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Version number:	2
Effective date:	14/09/2015
Review due:	1/09/2019 (reviewed every two years)
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Amendment History

Version	Date	Author/s	Amendment Details
2	14/09/2017	Gudrun Wells	Reviewed.

Purpose:

To outline the assay to determine creatinine levels in smokers' urine using ultra high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).

Principle

There are variations in water intake or renal function between individuals, which may influence the levels of nicotine and its main metabolites – cotinine and 3'-hydroxycotinine in urine. The urinary creatinine excretion is important to indicate the level of glomerular filtration.

Responsibility:

Chief Investigators(s) are responsible for delegating and training staff to analyse urine for creatinine (as recorded in the Delegation and Training Logs). All persons analysing urine must be experienced and trained in the procedure, and be observed by senior staff and deemed as proficient prior to undertaking the procedure independently.

Scope:

This Standard Operating Procedure applies to the determination of creatinine in urine samples by staff in the laboratory that have been trained and are competent in the performance of this analysis.

Materials:

- Deuterated creatinine-d_3 (Santa Cruz Biotechnology, 2.5 mg, CA);
- Ultrapure (type I) water (from Milli-Q purifier) (Millipore Corporation, MA).

- All chemicals should follow the conditions for safety storage.

Instrumentation

A Waters Acquity H-class ultra-high performance liquid chromatograph coupled to a Waters Xevo triple quadrupole mass spectrometer (MS/MS) using MassLynx software is used to measure analytes. The separation is performed using a Waters Acquity BEH C_{18} column (2.1 × 100 mm × 1.7 µm particles size). The liquid chromatography solvents include 0.1% (v/v) formic acid (Mobile phase A) and acetonitrile (Mobile phase B). The flow rate is 0.30 mL/min with isocratic elution using 98% A, 2% B over three minutes. An injection volume of 10 µL is recommended for samples and standards. The mass spectrometer is operated using positive electrospray ionisation with multiple reaction monitoring (MRM). The ion source temperature is 150 °C and the desolvation gas is nitrogen at 950 L/hour. The desolvation temperature is 450 °C and the capillary voltage is 2.8 kV. The MRM transitions for quantitation are *m/z* 114.1→44.0 (creatinine), *m/z* 117.1→47.0 (creatinine-d_3), and for qualification are *m/z* 114.1→86.0 (creatinine), *m/z* 117.1→89.0 (creatinine-d_3). The cone voltage is 26 V for all analytes, and the collision energy is 14 V and 10 V for quantitation and qualification transitions, respectively. The dwell time is 78 ms per channel.

Procedure:

Safety precautions

1. Dispose of the assessed urine sample tubes/vials/gloves and used pipette tips in two biohazard waste bags (double bagged) with each bag tied. Give the waste disposal bags to lab manager.
2. Residual chemicals will be disposed as general chemical waste.
3. Clean-up the biosafety cabinet and fume cupboard benches with 70% (w/v) ethanol, and the cleaned-up cloths/tissues will be disposed of in the biohazard bag.
4. Clean used flasks with detergent.

Standards and sample preparation

1. **Stock solutions:** creatinine-d_3 Stock Solution at concentration of 10 µg/mL is prepared in water and stored at -20 °C. An Intermediate Standard is then prepared at 1.0 µg/mL by 10-fold dilution of the Stock Standard in water. A Spiking Standard is made at a concentration of 0.5 µg/mL by 2-fold dilution of the Intermediate Standard.
2. **Linearity standards:** Utilise blank urine obtained from a non-smoker without any passive exposure to tobacco smoke in the previous 48 hours.
    a. Blank urine is diluted 1000-fold with water and centrifuged at 12,000 *g* for 5 minutes;
    b. An aliquot of the diluted urine is then spiked with the Intermediate Standard to produce ascending concentrations over a calibration range from 0, 5, 20, 40, 80, 100 ng/mL of creatinine-d_3, as described in Table 1;

Table 1. Linearity concentration range of creatinine-d_3

Linearity Concentration (ng/mL)	Volume Intermediate Standard (µL)	Volume Diluted Urine (µL)
0	0	1000
5	5	995
20	20	980
40	40	960
80	80	920
100	100	900

c. Standards are analysed using the UPLC-MS/MS instrument as described above.

3. **Urine samples**:
   a. Urine samples from smokers are thawed and centrifuged for 5 minutes (12,000 *g*);
   b. An aliquot of the supernatant of each sample is diluted 1000-fold with water. 990 µL of the diluted urine is then spiked with 10 µL of creatinine-d_3 Spiking Standard (0.5 µg/mL) to yield a concentration of 5 ng/mL in the sample;
   c. Samples are analysed using the UPLC-MS/MS instrument as described above.

Assay performance

The following assay performance measures are undertaken and reported in order to demonstrate the assay meets the acceptance criteria for a given application.

- **The correlation coefficient (r^2)** is used as an estimate of linearity over the range 0 to 100 ng/mL for creatinine-d_3 in urine. The r^2 value of linearity should be greater than 0.995.
- **Intra-day and inter-day accuracy and precision of the creatinine-d_3** are measured using quality control standards at concentration of 5, 40, 100 ng/mL of creatinine-d_3. On each day/batch in which samples are analysed, for intra-day accuracy and precision, repeat injection of the same standards (n=5) through the day are required. Inter-day accuracy and precision across several days/batches will be determined using the results from intra-day samples (no extra samples need to be prepared), however, new calibration samples should be prepared if the duration between batches is greater than 3 months.
- **Method detection limit (MDL),** defined as a mimimum signal-to-noise ratio of three, is determined from the signal-to-noise ratio of replicate determination (n=5) at the 5 ng/mL level for creatinine-d_3 in diluted urine;
- **Lower limit of quantification (LLoQ),** defined as a minimum signal-to-noise ratio of ten, is determined from the signal-to-noise ratio of replicate determination (n=5) at the 5 ng/mL level for creatinine-d_3 in diluted;

- **Acceptance criteria** is defined as accuracy and intra-day precision <10% at the 100 ng/mL level for creatinine-d_3.

References:

Hou, H.W., Xiong, W., Zhang, X.T., Song, D.K., Tang, G.L. and Hu, Q.Y. (2012) LC-MS-MS measurements of urinary creatinine and the application of creatinine normalisation technique on cotinine in smokers' 24 hour urine. *Journal of Analytical Methods in Chemistry*, 2012, Article ID 245415, 8 pages.

Waterval, W.A.H., Scheijen, J., Bakker, J.A. and Bierau, J. (2008) Simultaneous determination of creatine, creatinine and guanidinoacetate in plasma and urine by stable-isotope dilution UPLC-MS/MS. *Journal of Inherited Metabolic Diseases*, 31, 72-72.