Detection of Cotinine and 3-hydroxycotine in Smokers' Urine

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Amendment History

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2	14/09/2017	Gudrun Wells	Reviewed

Purpose:

Aim to determine and quantify nicotine main metabolites—cotinine, 3-hydroxycotine and their corresponding glucuronide conjugate—in smokers' urine by using ultra-high performance liquid chromatography (UPLC).

Principle

3-Hydroxycotine: cotinine ratio, also known as nicotine metabolite ratio, assists as a marker of cytochrome 2A6 phenotype. The 3-hydroxycotinine: cotinine ratio is considered an important determinant of cessation likelihood and responsiveness to smoking cessation pharmacotherapies. In addition, the nicotine metabolite ratio based on total cotinine (free + glucuronide) and total 3-hydroxycotinine (free + glucuronide) is a better measure of cytochrome 2A6 activity than calculating the ratio based on free levels only.

Responsibility:

Appropriately trained persons are responsible for analysing urine. The CI is responsible for delegating and training staff in how to analyse urine (as recorded in the Delegation Log and the Training Log).

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 nicotine metabolites—cotinine and 3-hydroxycotinine in urine samples by staff in the laboratory that have been trained and are competent undertaking this analysis. See Urine Collection SOP for details on how urine is to be collected.

Materials:

- Cotinine (Santa Cruz Biotechnology, 5 mg, CA);
- *trans*-3'-hydroxycotinine (Santa Cruz Biotechnology, 5 mg, CA);
- Deuterated cotinine-d_3 (CDN Isotopes, 100 mg, Canada);
- Deuterated *trans*-3'-hydroxycotinine-d_3 (Toronto Research Chemicals, 1 mg, Canada);
- Cotinine-d_3 *N*-Beta-D-glucuronide (Sapphire Bioscience, 1 mg, Australia);
- *trans*-3'-hydroxycotinine-d_3 *O*-Beta-D-glucuronide (Sapphire Bioscience, 1 mg, Australia);
- Ammonia (analytical grade) (Sigma Aldrich, 2 mL, Australia);
- Acetic acid (analytical grade) (Sigma Aldrich, 2.5 mL, Australia);
- Acetonitrile (ultra-high performance liquid chromatography grade) (Sigma Aldrich, 1 mL, Australia);
- 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 chromatography coupled to a Waters Xevo triple quadrupole mass spectrometer 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 solvent includes 0.4% ammonia (A) and acetonitrile (B). The flow rate is 0.4 mL/min with 100% A for 0.3 minutes, a linear gradient to 30% A at four minutes, followed by re-equilibration to the initial conditions for three minutes. Based on differences in column retention, the phase II glucuronide metabolites are separated using 1% acetic acid as solvent A, 100% A for 1.5 minutes followed by a linear gradient to 30% A, and 70% B at four minutes before immediate re-equilibration to the initial conditions. 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 1000 L/hour. The desolvation temperature is 300 °C and the capillary voltage is 2.8 kV. The MRM transitions for quantitation are *m/z* 177.1→80.05 (cotinine), *m/z* 180.1→80.05 (cotinine-d_3), *m/z* 193.1→80.05 (*trans*-3'-hydroxycotinie), and *m/z* 196.1→80.05 (*trans*-3'-hydroxycotinine-d_3). For the glucuronide metabolites, the MRM transitions for quantitation are *m/z* 353.2→177.1 (cotinine-glucuronide), *m/z* 356.2→180.1 (cotinine-d_3-glucuronide), *m/z* 369.2→193.1 (*trans*-3'-hydroxycotinie-glucuronide), and *m/z* 372.2→196.1 (*trans*-3'-hydroxycotinine-d_3-glucuronide). The cone voltage is 27 V for all analytes, and the collision energy is 21 V and 25 V for cotinine and *trans*-3'-hydroxycotinine transitions, respectively, and their corresponding glucuronides. The dwell time is 25 ms per channel.

Procedure:

Safety precautions

1. Dispose of used urine sample tubes/vials/pipette tips/gloves into biohazard waste bags (double bagged) with each bag tied. Give the biohazard 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% ethanol, and dispose of the clean-up cloths/tissues into the double bagged biohazard waste above (point 1 above).
4. Clean used flasks with laboratory grade detergent.

Calibration standards and sample preparation

1. **Stock solutions:** cotinine, cotinine-d3, cotinine-d3-glucuronide, *trans*-3'-hydroxycotinine, and *trans*-3'-hydroxycotinine-d3 at concentration of 1 mg/mL each; *trans*-3'-Hydroxycotinine-d3-glucuronide at a concentration of 0.25 mg/mL are prepared in water and stored at -20 °C.
2. **Internal standard solution:** containing each of the deuterated internal standards at concentration of 100 ng/mL in water is freshly prepared from the stock solutions.
3. **Calibration samples:** blank urine obtained from a non-smoker without any passive exposure to tobacco smoke in the previous 48 hours.
    a. *For cotinine and trans-3'-hydroxycotinine:*
        i. Blank urine (1 mL) is spiked with ten ascending concentrations over a calibration range from 0, 5, 25, 50, 100, 250, 500,1000, 10000, 20000 ng/mL and centrifuged at 12,000 g for 5 minutes;
        ii. An aliquot (0.2 mL) of the supernatant is then diluted 100-fold with water; a 1 ml aliquot of the diluted sample is then spiked with 0.1 mL of the combined internal standard solution equivalent to a level of approximately 10 ng/mL internal standard;
        iii. Samples are then vortexed for 30 seconds and centrifuge at 12,000 g for 5 minutes;
        iv. An aliquot (1 mL) of the supernatant is then transferred to autosampler vials for UPLC-tandem mass spectrometry (MS/MS) determination of unconjugated (free) levels of cotinine and trans-3'-hydroxycotinine with injection volume of 5 microliters.
    b. *For cotinine-d_3-glucuronide and trans-3'-hydroxycotinine-d_3-glucuronide*:
        i. Eight ascending concentrations of cotinine-d3-glucuronide and trans-3'-hydroxycotinine-d3-glucuronide are spiked into blank urine (1 mL) diluted 5-fold with water over a calibration range from 0, 5, 25, 50, 100, 500, 1000, 2000 ng/mL, and centrifuged;
        ii. An aliquot (0.2 mL) of the supernatant is then diluted 20-fold with water, vortex mixed for 30 seconds, and centrifuged at 12,000 g for 5 minutes;

iii. An aliquot (1 mL) of the supernatant is then transferred to autosampler vials for UPLC-tandem mass spectrometry (MS/MS) determination using external standardisation to demonstrate linearity with an injection volume of 5 microliters.

4. **Urine samples**:
    a. Urine samples from smokers are thawed and centrifuged for 5 minutes;
    b. An aliquot (0.2 mL) of the supernatant of each sample is diluted 100-fold with water and spiked with 100 µl of the mixed deuterated internal standard prepared as previously described;
    c. Samples are analysed using the two separate UPLC solvent programs as described for cotinine, trans-3'-hydroxycotinine, and the respective glucuronide determinations.

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.

1. **The correlation coefficient (r^2)** is used as an estimate of linearity over the range 5 to 20000 ng/mL for cotinine and *trans*-3'-hydroxycotinine, and from 5 to 2000 ng/mL for cotinine-d_3-glucuronide and *trans*-3'-hydroxycotinine-d_3-glucuronide.
2. **Intra-day and inter-day accuracy and precision of cotinine and *trans*-3'-hydroxycotinine** are measured using quality control standards at concentrations of 25, 1000, and 10000 ng/mL of standards. 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.
3. **Intra-day and inter-day accuracy and precision of the cotinine and *trans*-3'-hydroxycotinine glucuronides** are measured using quality control standards at concentration of 25, 1000, 2000 ng/mL of standards, in the same manner as cotinine and *trans*-3'-hydroxycotinine (n=5) above. 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.
4. **Method detection limit (MDL),** defined as a signal-to-noise ratio of three, is determined from the signal-to-noise ratio of replicate determination (n=5) at the 25 ng/mL level for cotinine and trans-3'-hydroxycotinine, and 125 ng/mL for both glucuronides;
5. **Lower limit of quantification (LLoQ),** defined as a signal-to-noise ratio of ten, is determined from the signal-to-noise ratio of replicate determination (n=5) at the 25 ng/mL level for cotinine and trans-3'-hydroxycotinine, and 125 ng/mL for both glucuronides;
6. **Acceptance criteria** is defined as accuracy and intra-day precision <10% at the 1000 ng/mL level for cotinine and trans-3'-hydroxycotinine.

References:

Madhur D. Shastri, Wenying Lu, Stuart G. Ferguson, Glenn A. Jacobson. Determination of cotinine, 3'-hydroxycotinine, and their glucuronides in urine by ultra-high performance liquid chromatography. *Analytical Letters, 2015*, 48:1217-1233. **DOI:** 10.1080/00032719.2014.979363