Detection of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in Smokers' Urine

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Amendment History

Version	Date	Author/s	Amendment Details
2	25/5/2016	Wenying Lu	Minor amendments to calibration sample preparation.
3	14/09/2017	Gudrun Wells	Reviewed

Purpose:

Aim to determine and quantify tobacco specific nitrosamines – 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) – main metabolite: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in smokers' urine by using ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS).

Principle

The tobacco-specific nitrosamine (TSNA), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), is formed from nicotine and related compounds by a nitrosation reaction during tobacco curing and is classified as a human carcinogen. Its major metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is considered a biomarker of NNK exposure. It is thought that NNK determination could be useful in assessment of harm minimisation with smoking cessation interventions.

Responsibility:

Appropriately trained persons are responsible for analysing urine. Principal Researcher(s) are responsible for ensuring staff have read, understood, and follow the procedure as listed below.

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 NNAL in urine samples by staff in the laboratory that have been trained and are competent undertaking this analysis.

Materials:

- NNAL (Toronto Research Chemicals, 10 mg, CA);
- Deuterated NNAL-d_3 (Toronto Research Chemicals, 1 mg, CA);
- Ethyl acetate (Merck KGaA, 4 L, Germany);
- Ammonia (analytical grade) (Sigma Aldrich, 2 mL, Australia);
- Acetic acid (analytical grade) (Sigma Aldrich, 2.5 mL, Australia);
- Acetonitrile (HPLC 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 C18 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, before immediate re-equilibration to the initial conditions for three minutes. The mass spectrometer is operated in positive electrospray ionisation with multiple reaction monitoring (MRM). The ion source temperature is 130 °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* 210.1→180.1 (NNAL), and *m/z* 213.1→183.1 (NNAL-d_3). The cone voltage is 24 V for all analytes. The dwell time is 78 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:** NNAL, at concentration of 200 µg/mL and NNAL-d_3, at concentration of 100 µg/mL are prepared in methanol and stored at 4 °C.
2. **Internal standard solution:** deuterated internal standards NNAL-d_3, at concentration of 5 ng/mL in water is freshly prepared from the stock solution.
3. **Working standard solution:** NNAL standard solution, at concentration of 1000 ng/mL in water is freshly prepared from the stock solution. NNAL working standard solution is freshly prepared from 1000 ng/mL to 5 ng/mL with Milli-Q water.
4. **Calibration samples:** nine ascending concentrations over a calibration range from 0, 25, 50, 100, 200, 400, 1000, 2000, and 8000 pg/mL of standard NNAL are prepared by serial dilution of working standard solution at concentration 8 ng/mL (prepared from 1000 ng/mL to 8 ng/mL, 125-fold dilution). Each calibration concentration solution (500 µL) and 100 pg of internal standards (20 µL of 5 ng/mL) are made up to 1 mL with Milli-Q water; the volume used for UPLC-MS/MS is 100 µL of each calibration concentration.
5. **Blank urine samples:** blank urine obtained from a non-smoker without any passive exposure to tobacco smoke in the previous 48 hours.

   For NNAL:
   - Blank urine is thawed and centrifuged at 2,000 *g* for 5 minutes;
   - An aliquot blank urine (2 mL) is spiked with 0.08 mL of NNAL working standard solution equivalent to a level of approximately 20 pg/mL of standard NNAL;
   - All the samples are then spiked with 0.04 mL of the internal standard solution equivalent to a level of approximately 100 ng/mL of NNAL-d_3;
   - An aliquot ethyl acetate (2 mL) is added to each sample;
   - Samples are shaken by hand for 1 minute and then vortexed for 3 minutes and kept for 5 minutes to allow the separation of the ethyl acetate and aqueous phase;
   - All the samples are centrifuged at 2,000 *g* for 8 minutes;
   - Transfer the top ethyl acetate phase to autosampler vials;
   - Add another aliquot ethyl acetate (2 mL) into each urine sample;
   - Same extract procedure as above;
   - The ethyl acetate phase is blow to near dryness with nitrogen gas at 25 °C;
   - All the samples are reconstituted with 100 microliters of Milli-Q water and transfer into a glass insert for UPLC- MS/MS determination of NNAL and NNAL-d_3 with injection volume of 20 microliters.

6. **Smokers' Urine samples**:
   - Urine samples from smokers are thawed at room temperature and centrifuged at 2,000 *g* for 5 minutes;
   - An aliquot urine (2 mL) is spiked with 0.04 mL of the internal standard solution equivalent to a level of approximately 100 ng/mL of internal standard;

- An aliquot of ethyl acetate (2 mL) is added to each sample;
- Samples are shaken by hand for 1 minute and then vortexed for 3 minutes and kept for 5 minutes to allow the separation of the ethyl acetate and aqueous phase;
- All the samples are centrifuged at 2,000 *g* for 8 minutes;
- Transfer the ethyl acetate phase to autosampler vials;
- Add another aliquot ethyl acetate (2 mL) into each urine sample;
- Same extract procedure as above;
- Each ethyl acetate phase is blow to near dryness with nitrogen gas at 25 °C;
- All the samples are reconstituted with 100 microliters of Milli-Q water and transfer into a glass insert for UPLC- MS/MS determination of NNAL and NNAL-d_3 with injection volume of 20 microliters.

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 1 to 400 pg/mL of NNAL.
- **Recovery** was calculated based on the NNAL-d_3 concentration in urine samples compared to a NNAL-d_3 standard prepared to a concentration equivalent to 100% theoretical recovery from the urine samples;
- **Intra-day and inter-day accuracy and precision of NNAL** are measured using quality control standards at concentrations of 20, and 100 pg/mL of NNAL standard (n=6). On each day in which samples are analysed, for intra-day accuracy and precision, repeat injection of the same standards (n=3) through the day are required. Inter-day accuracy and precision across several days 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 is greater than 3 months.
- **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 20 pg/mL level for NNAL;
- **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 20 pg/mL level for NNAL;
- **Acceptance criteria** is defined as accuracy and intra-day precision <15% at 20 pg/mL and 100 pg/mL, which considered as low and high levels of NNAL.
- **Freeze/thaw stability,** was determined by freezing (-20°C) and thawing (ambient temperature) standard solutions (20 pg/mL and 100 pg/mL, n=6) for three cycles;
- **Bench top stability,** was assessed over 8 hours by leaving the standard solutions (20 pg/mL and 100 pg/mL, n=6) on the bench top at ambient temperature;
- **Sample storage stability,** was analysed at low (~50 pg/mL), medium (~120 pg/mL), and high levels (~300 pg/mL) (n=6) of subject urine samples after stored in the freezer (-20°C) for 1 month.

References: