Further refinement and validation of the only ultrasensitive biomarker method for benzo[a]pyrene exposure by urinary metabolite

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Presentation flow

- Overview of biomarkers for cigarette smoking.
  - Modified Risk Tobacco Products: Heat-not-burn

- Polycyclic Aromatic Hydrocarbon Overview

- Initial Method Development and Validation

- Biomarker Qualification – initial assay
  - Lessons learned about biomarker statistics

- Method Development and Re-validation

- Biomarker Qualification – updated assay
  - Bioanalytical impact upon study design

- Conclusion
Examples of Tobacco Biomarkers

- Biomarkers of exposure:
  - Nicotine in plasma
  - Nicotine equivalents in urine
  - Polycyclic Aromatic Hydrocarbons (PAHs) in urine
  - Tobacco specific nitrosamines in urine
  - Aromatic amines in urine
  - Mercapturic acids in urine

- Biomarkers of effect:
  - 11-dehydro-thromboxane B2
  - Isoprostanes like 8-iso-PGF2 (type III)
  - sICAM
  - Biomarkers of oxidative stress
Heat-not-Burn Products

- When a cigarette burns at 600 - 900°C many chemical reactions occur
- Heat-not-burn products burn tobacco at <250°C and produce less chemical reactions

Images from www.pmiscience.com
Polycyclic Aromatic Hydrocarbons (PAH’s)

- PAH’s are tasty!
- Polycyclic aromatic hydrocarbons are formed through the incomplete combustion of organic material
- Tobacco smoke, and diet are primary sources of PAH exposure in humans
- Most tobacco related exposure studies performed today focus on the exposure of pyrene through a measure of total 1-hydroxypyrene in human urine to determine the overall PAH exposure.
- Pyrene exposure, while toxic at high levels, is not considered carcinogenic
- Benzo[a]pyrene is mutagenic and is a considered a primary carcinogen in cigarette smoke. It was the 1st carcinogen identified in cigarette smoke.
What is 3-OH Benzo[a]pyrene (3-OH-B[a]P)?

- Benzo[a]pyrene is a Polycyclic Aromatic Hydrocarbon (PAH) and is a biomarker of cigarette exposure
- Celerion has 27 validated urinary biomarkers of exposure and >5 biomarkers of effect
  - Analyzed >2,000,000 urinary biomarkers for tobacco exposure
- As a general rule, biomarker assays for tobacco exposure are much more complex than a typical bioanalytical drug assay
Benzo[a]pyrene metabolism

J. Carcinog. 2012, 11:1
Initial Assay Parameters for 3-OH-B[a]P

- Linear Range: 50.0 - 2000 fg/mL
- Control matrix: Canine urine
- Assay volume: 3.0 mL
- Sample processing:
  - Hydrolysis of glucuronide
  - Extraction
  - Derivatization
- Chromatography: UPLC with reversed phase retention
- LC-MS/MS Platform: Positive ions measured in MRM mode via SCIEX API 5000 or QTRAP® 5500
- Quality Control Design: 4 QC concentrations in human urine were measured. 2 measured basal level QCs were used for stability and precision.
Chromatograms:
Extracted 3-OH-B[a]P LLOQ & Blank

**Structural Isomers of B[a]P**

- 50 fg/ml LLOQ urine extract
- Control blank urine extract

- Deuterated Internal Standard

Approx. 6:1 S/N
Initial Assay 3-OH-B[a]P Validation Statistics

- Inter-day Precision: 3.6 - 10.9%
- Inter-day Accuracy: -3.6% - 0.2%
- Approximate recovery: 81%
- Intra-day Precision: 1.7 - 12.7%
- Intra-day Accuracy: -1.4 - 7.2%
- Average S/N at LLOQ: 6.7

In Matrix Stabilities
- Short-term Stability: 26 hr at ambient temperature
- Long-term Stability: 98 days at -20°C
- Freeze/thaw Stability: 6 cycles from ambient temp. to -20°C

Multi-lot Matrix Effect testing
- 9 of 10 lots quantitated within 15% of the expected concentration near the LLOQ (basal measurement plus supplement)
- 9 of 10 lots quantitated within 15% of the expected concentration near the high QC (basal measurement plus supplement)
3-OH-B[a]P Biomarker Qualification: Initial Assay Subject Results

- Average conc. from Smoker Urine ~240 fg/mL
  - Smokers used at least 10 conventional cigarettes per day
  - Spot collection of 1st morning void
  - No specific brand identified
  - Diluted samples could not be measured

- Average conc. from Light Smoker Urine ~25 to 50 fg/mL
  - Smokers used less than 10 conventional cigarettes per day
  - Spot collection of urine, not 1st morning void
  - No specific brand identified
  - Most samples could not be measured

- Average conc. from non-Smoker Urine < LLOQ
  - Spot collection of urine, not 1st morning void
  - No non-smoker samples could be measured
Fit-for-Purpose Validation
What is the purpose for each assay?

- **Routine Biomarkers**
- **Clinical Assays**
- **Drug PK Assays**

**Biomarker Assays**
- **Novel & routine Biomarkers**
- **Exposure**
- **Diverse purposes - PD, Efficacy & Safety Surrogacy**
- **Drug Development**

Is the value normal or abnormal?

- Response
  - Initial
  - Treated
3-OH-B[a]P Biomarker Qualification: Conclusion Initial Assay

- Sh!t – The assay isn’t sensitive enough.
  - If the concentration of 3-OH-B[a]P from light smokers < LLOQ then we probably wouldn’t see 3-OH-B[a]P from heat-not-burn product testing.

- What is the value of an LLOQ sample for statistical analysis?
  - Extremely Valuable Data
  - Don’t want to give it a value of “missing”
  - Assign it a value of ½ LLOQ??
  - Conclusion: lower the LLOQ

- The new SCIEX Triple Quad 6500 allows the ability to lower the LLOQ
Updated Assay Parameters for 3-OH-B[a]P

- Linear Range: 25.0 - 600 fg/mL
- Control matrix: Diluted human urine
- Assay volume: 2.7 mL
- Sample processing:
  - Hydrolysis of glucuronide
  - Extraction
  - Derivatization
- Chromatography: UPLC with reversed phase retention
- LC-MS/MS Platform: Positive ions measured in MRM mode via SCIEX Triple Quad 6500
- Quality Control Design: 4 QC concentrations in human urine were measured. 2 measured basal level QCs were used for stability and precision.
Chromatographic Comparison 3-OH-B[a]P: Initial versus Updated Assay

Initial assay: 50 fg/ml

LLOQ urine extract

S/N ~ 6:1

Updated assay: 25 fg/ml

Control blank urine extract

S/N ~ 12:1
Updated Assay 3-OH-B[a]P Validation Statistics

- Inter-day Precision: 4.5 - 15.8%
- Inter-day Accuracy: -2.8 - 1.6%
- Approximate recovery: 95%
- Intra-day Precision: 1.4 - 17.6%
- Intra-day Accuracy: -20.0 - 11.2%
- Average S/N at LLOQ: 12.4

In Matrix Stabilities
- Short-term Stability: 55 hours at ambient temperature
- Long-term Stability: 92 days at -20°C
- Freeze/thaw Stability: 6 cycles from ambient temp. to -20°C

Multi-lot Matrix Effect testing
- 9 of 10 lots quantitated within 15% of the expected concentration near the LLOQ (basal measurement plus supplement)
- 10 of 10 lots quantitated within 15% of the expected concentration near the high QC (basal measurement plus supplement)
3-OH-B[a]P Biomarker Qualification: Updated Assay Subject Results

- **Average conc. from Smoker Urine ~240 fg/mL**
  - Smokers used at least 10 conventional cigarettes per day
  - Spot collection of 1st morning void
  - No specific brand identified
  - All smoker samples could be measured. Most samples were between 5 and 10-fold of the LLOQ

- **Average conc. from Light Smoker Urine ~25 to 50 fg/mL**
  - Smokers used less than 10 conventional cigarettes per day
  - Spot collection of urine, not 1st morning void
  - No specific brand identified
  - All light smoker samples could be measured

- **Average conc. from non-Smoker Urine ~10 to 30 fg/mL**
  - Some non-smoker samples could be measured
Method Validation – Sensitivity
Cost Savings: Able to Dose Fewer Subjects

Number of Subjects required to demonstrate statistical significance

- 100 fewer subjects
- 60 fewer subjects
- 20 fewer subjects
- < LLOQ
- LLOQ

High LLOQ: 20% samples required to be statistical significant.
Appropriate LLOQ: 20 fewer subjects required.
With the improved sensitivity the method is now capable of measuring all smoker and light smoker urine concentrations of total 3-hydroxybenzo[a]pyrene

- Some non-smoker samples could also be measured

The improvement in sensitivity also improved the method reproducibility in the important range of 50 - 250 fg/mL where most clinical samples were measured

The updated assay with improved sensitivity has the ability to reduce the number of subjects in clinical studies
Thank you for your time

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