A multi-analyte method using liquid chromatography with high resolution accurate mass spectrometry for the determination of aerosol constituents in 3D tissue cultures

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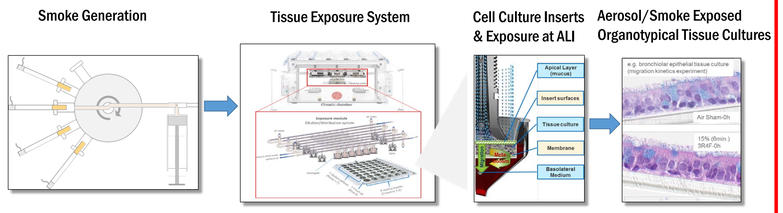
Introduction and Objectives

Measurement of nicotine and its primary metabolites in 3D organotypical tissue cultures following exposure to tobacco derived aerosols is important for understanding the deposition/absorption and metabolic characteristics of such tissue exposure models. Other aerosol constituents or their metabolites (e.g. glutathione adducts) can also serve as markers for exposure normalization for 3D tissue cultures. Therefore, analysis of both compound classes (nicotine metabolites and glutathione adducts) would provide more information regarding metabolism and pharmacokinetics for toxicological assessment using such tissue exposure models.

Analysis of glutathione adducts is challenging using classical reverse phase chromatography, since adducted species are generally too polar to be adequately retained by this technique. Therefore, a novel liquid chromatographic separation with high resolution accurate mass spectrometry (LC-HRAM-MS) in full scan positive electrospray ionization mode (QExactive™, Thermo Fisher) was developed. This was achieved using anion exchange (Biobasic AX®) and pentafluorophenyl (Kinetex® PFP) columns in series, successfully separating nicotine and five metabolites (nicotine-N-oxide, cotinine-N-oxide, cotinine, 3-hydroxycotinine, nicotine-N-glucuronide) plus the glutathione adducts of selected electrophilic aerosol constituents (acrolein, crotonaldehyde, benzoquinone). Quantification was performed using stable isotope labeled standard compounds, which were added prior to sample preparation of tissues and basolateral media.

Methods

Aerosol Generation and Exposure of Organotypical Tissue Cultures at the Air-Liquid Interface (ALI)



Smoking machine: SM2000, Philip Morris International
Puff profile: Health Canada regimen (2 puffs/min. of 55 mL and 2 sec. aspiration and 8 sec. exhaust)

Exposure system: VITROCELL® 24/48 simultaneous exposure of 48 cell culture inserts with up to 7 different smoke dilutions with humidified air and exposing 6 inserts per dilution.

Organotypical Tissue Cultures:
- Bronchiolar, Nasal, Buccal (human)

Surrogate Matrix:
- Phosphate Buffered Saline (PBS)

Figure 1: Aerosol generation and exposure of organotypical tissue cultures at the air-liquid interface.

Analytical Methods

Original method:
- LC-pump: Thermo Accela 1250
- Column: Hypersil GOLD™ aQ
- (150x2.1 mm, 1.9µ)

Time [min]	A[%]	B[%]
0.0	100	0
3.0	100	0
18.0	80	20
23.0	0	100
26.0	0	100
27.0	100	0
30.0	100	0

- [A]: 0.1% formic acid (pH2.7)
- [B]: 0.1% formic acid in acetonitrile
- Flow: 400µL/min, 40°C, Inj.: 5µL

Mass Spectrometry (HRAM-MS): Thermo QExactive™, HESI(+/-)
- Full scan: 80 – 800 Da, res.: 70000

New multi-compound method:
- LC-pump: Thermo Accela 1250
- Columns: 1) Biobasic SAX (50x2.1 mm, 5µ)
  2) Kinetex PFP (150x2.1mm, 2.6µ)

Time [min]	A[%]	B[%]
0.0	100	0
3.0	100	0
11.0	70	30
15.0	5	95
17.0	5	95
19.0	100	0
25.0	100	0

- [A]: 10mM ammonium formate (pH3.5)
- [B]: methanol/isopropanol, 80/20, v/v
- Flow: 200µL/min, 40°C, Inj.: 2µL

Mass Spectrometry (HRAM-MS): Thermo QExactive™, HESI(+/-)
- Full scan: 145 – 650 Da, res.: 70000

Results

Analytical Separation and Detection

Methodologies for the separation and quantification of nicotine and its metabolites plus additional glutathione adducts

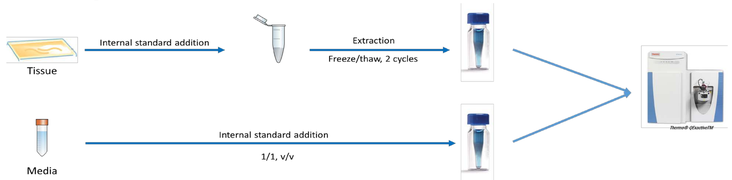


Figure 3: Sample preparation method for determining compounds of interest in aerosol exposed organotypical tissues

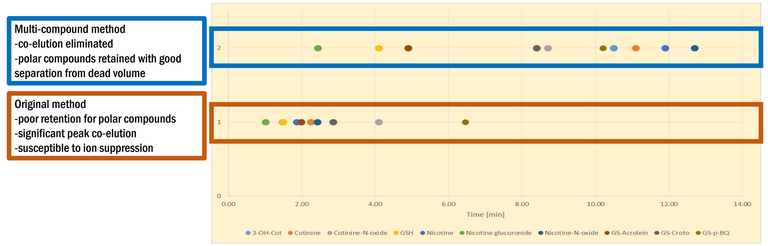


Figure 4: Separation approaches for nicotine and its metabolites (nicotine-N-oxide, nicotine glucuronide, cotinine, cotinine-N-oxide, 3-hydroxycotinine), glutathione (GSH) and GSH adducts of acrolein, crotonaldehyde and benzoquinone

Metabolism

- Human organotypical tissues exposed to whole smoke from the conventional reference cigarette 3R4F
  - Metabolism of nicotine and migration from tissues to basolateral media
  - Metabolites are mainly found in the basal media due to migration during exposure
  - Use as markers for exposure normalization of 3D tissue cultures to smoke exposure

Figure 5: Migration kinetics for nicotine in bronchiolar tissue, exposed to 15% 3R4F for 6 min.

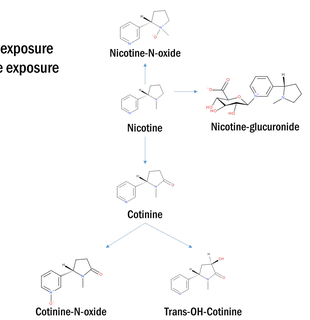


Figure 6: Nicotine and primary metabolites identified after tissue exposure

- Nucleophilic reactions with glutathione result in the formation of glutathione adducts
  - Chemical reaction by GSH with reactive compounds
  - GS-acrolein converts to its reduced form (GS-hydroxypropyl) over time
  - Acrolein adducts and its metabolically reduced product represent the majority of GSH-adducts identified

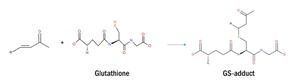


Figure 7: Nucleophile adduct formation by glutathione reaction with compounds of interest

Exposure Characterization

Exposure of small airway bronchial tissues to aerosol from the reference cigarette 3R4F

- Nicotine was more abundant in media compare with tissues due to migration effects
- Nicotine metabolites were predominantly found in media
- GSH-adducts were found in tissues
- Additional GSH-adducts were identified: glutathione adducts of methylisocyanate (GS-Mic) and ethylvinylketone (GS-EVK)

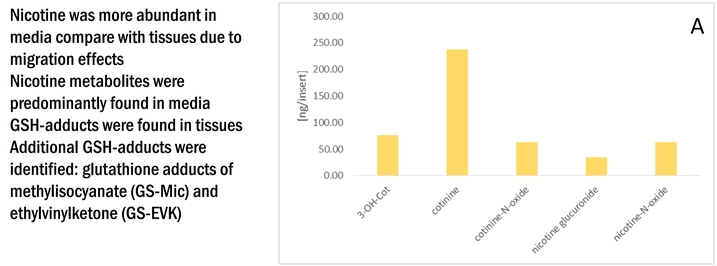


Figure 8: Nicotine deposited after 3R4F exposure (13%) for 28min on bronchial tissues and in the basolateral media (N=3)

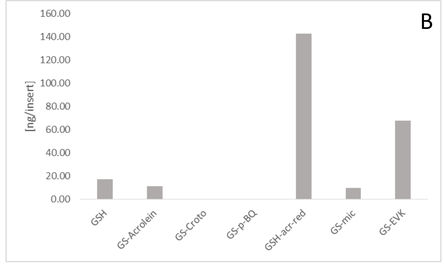


Figure 9: Characterization of bronchial tissues exposed to 3R4F (13%) for 28min (N=3), A) nicotine metabolites after exposure in basolateral media, B) glutathione adduct level in tissues after exposure

Conclusions

- The multi compound method allows full separation of nicotine and its metabolites as well as predominant GSH-adducts formed by nucleophile reaction with reactive compound such as carbonyls within one single run
- The analytical methodology was used to characterize the exposure of 3D organotypical tissues to aerosol from the conventional reference cigarette 3R4F
- Nicotine and the glutathione adducts of reactive carbonyls can be determined to describe the dose in exposed tissues
- Next challenge: the multiplicity of glutathione adducts formed, which are also subject to subsequent metabolic reduction

References

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