BIOMARKERS OF EXPOSURE

Selection, Method Development and Validation, and Acceptance

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BIOMARKER DEFINITIONS

- Chemicals, metabolites of chemicals, enzymes, and other biochemical substances found in tissues and body fluids
- Measurements provide data linking ***exposure*** with internal dose and outcome; relevant to process of risk assessment

Biomarkers and Risk Assessment: Concepts and Principles, World Health Organization, Geneva, 1993

EXPOSURE DEFINITIONS

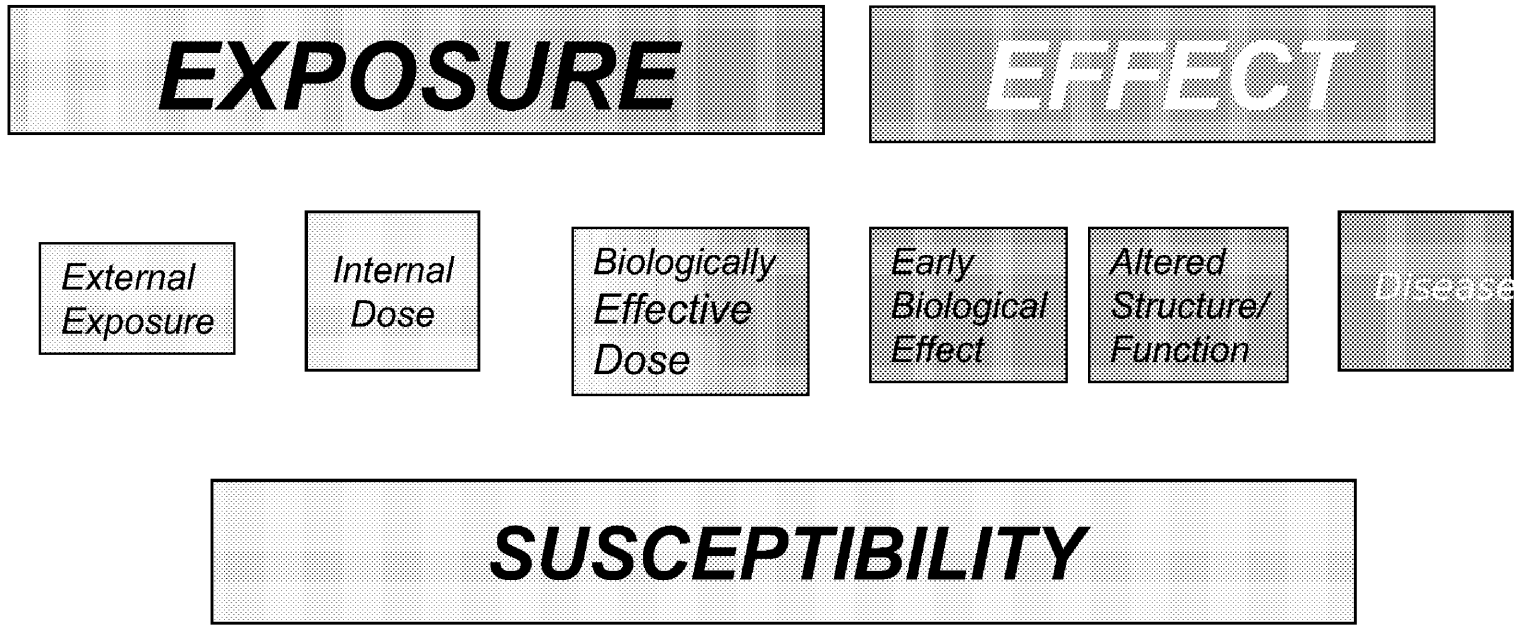
- Exposure is the contact over time and space between a person and one or more biological, chemical or physical agents (US NRC, 1991).
- Exposure is concentration of a substance in the human body over time, (c x t).
- Exposure is the area under the concentration-time-curve, (AUC).

TYPES OF BIOMARKERS

Exposure or Biomarker Assessment	Definition
External Exposure Marker	A tobacco constituent or product that may reach or is at the portal of entry to the body
Biomarker of Exposure	A tobacco constituent or metabolite that is measured in a biological fluid or tissue that has the potential to interact with a biological macromolecule; sometimes considered a measure of internal dose
Biologically Effective Dose (BED)	The amount that a tobacco constituent or metabolite binds to or alters a macromolecule; estimates of the BED might be performed in surrogate tissues
Biomarker of Potential Harm	A measurement of an effect due to exposure; these include early biological effects, alterations in morphology, structure, or function, and clinical symptoms consistent with harm; also includes "preclinical changes"

From Clearing the Smoke: Assessing the Science Base for Tobacco Harm Reduction

The Paradigm: Biological Marker Components in Sequential Progression Between Exposure and Disease



SELECTION OF BIOMARKERS OF EXPOSURE

- Convened panel of PM experts and consultants with significant experience in smoke composition and/or exposure assessment
- Utilized biomarker selection considerations from NRC and N. Benowitz
- Identified candidate smoke constituents
- Chose biomarkers of exposure representative of both smoke phases

SELECTION CRITERIA FOR TES BIOMARKERS OF EXPOSURE

- "Unique or nearly unique" to tobacco smoke, "so that other sources are minor in comparison"
- Reliable analytical methods available ("easily detectable")

Benowitz, N.L. [illegible] *Environmental Health Perspectives*, 1999, 107(Suppl. 2), 349 – 355

- Representative of either particulate or gas-vapor phase
- Representative of health-relevant constituents
- Sampling to acquire material for analysis only minimally invasive
- Constituent metabolism understood
- Increased in smokers as compared to non-smokers

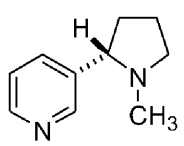
BIOMARKERS OF EXPOSURE FOR THE TES

BIOMARKER	SAMPLE MATERIAL	SMOKE CONSTITUENT	SMOKE PHASE*
Acetonitrile	*Exhalate; blood*	*Acetonitrile*	*GVP*
Carbon monoxide	*Exhalate*	*Carbon monoxide*	
GVP			
Carboxyhemoglobin	Blood	Carbon monoxide	GVP
Hb adducts of 3- and 4-aminobiphenyl	Blood	3- and 4- Aminobiphenyl	PP
Nicotine and nicotine metabolites**	24-hr urine	Nicotine	PP
NNAL and NNAL-glucuronide	24-hr urine	NNK	PP

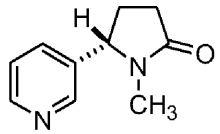
*GVP: gas-vapor phase; PP: particulate phase

**cotinine, 3-hydroxycotinine, nicotine-*N*-glucuronide, cotinine-*N*-glucuronide, and *trans-3'*-hydroxycotinine-*O*-glucuronide,

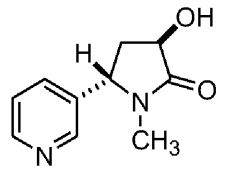
NICOTINE METABOLISM



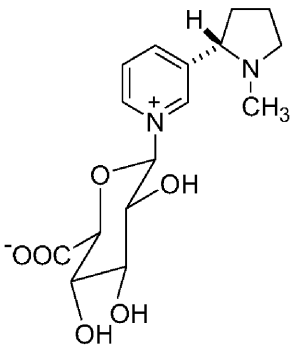
Nicotine



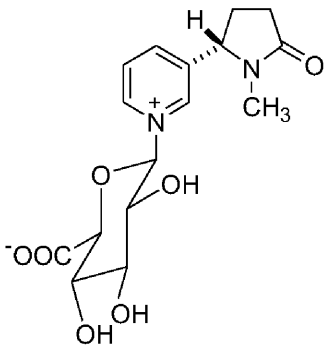
Cotinine



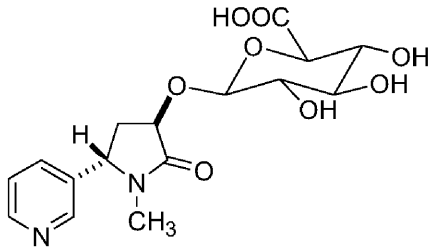
***trans*-3'-hydroxycotinine**



Nicotine-*N*-Glucuronide

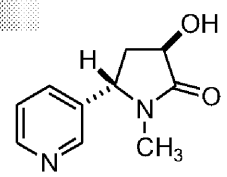


Cotinine-*N*-Glucuronide

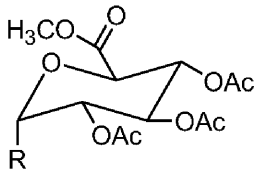


***trans*-3'-Hydroxycotinine-*O*-Glucuronide**

THE QUEST FOR AUTHENTIC REFERENCE STANDARDS

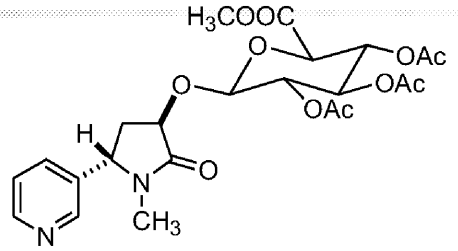


trans-3'-Hydroxycotinine



R = Br, OAc, etc.

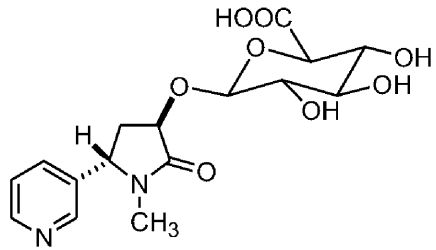
various conditions



Protected Intermediate

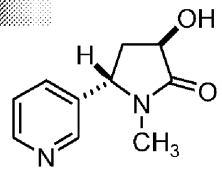
Conditions

1) CH_2Cl_2, reflux, 2d
2) 1.0 eq NaH, 23°C, 2h
3) Ag_2CO_3, 4A M.S., C_6H_6, 23°C, 24h
4) Cs_2CO_3, 4A M.S., C_6H_6, 23°C, 24h
5) CF_3COOAg, Ag_2CO_3, CH_2Cl_2, 23°C, 24h
6) BF_3OEt_2, CH_2Cl_2, reflux, 3d
7) 2.0 eq $SnCl_4$, CH_2Cl_2, 23°C
8) 2.0 eq $SnCl_4$, CH_2Cl_2, reflux
9) 2.0 eq TMSOTf, 4A M.S., CH_2Cl_2, reflux
10) HgI_2, CH_3CN, 23°C, 24h

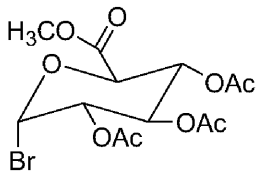


trans-3'-Hydroxycotinine-*O*-Glucuronide

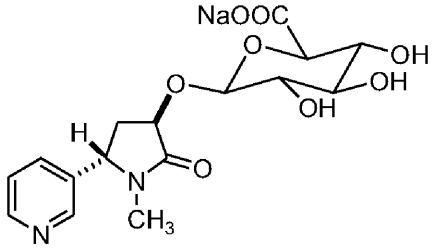
SUCCESSFUL SYNTHESIS OF *trans*-3′-HYDROXYCOTININE-*O*-GLUCURONIDE



(±)-*trans*-3'-Hydroxycotinine



Multiple Steps

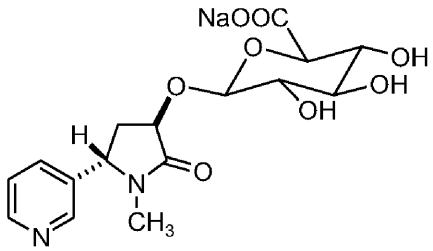


1:1 Mixture of 3'R, 5'S-β-D- and 3'S, 5'R-β-D-Diastereomers

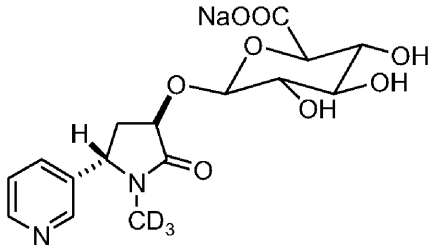
Structural and Purity Proof:

^{1}H-NMR
^{13}C-NMR
2D-NMR (COSY, NOESY, and HETCOR)
MS
MS/MS
Karl Fisher
Residual Solvents
HPLC (UV-PDA, Anion and Cation Scan)
TLC

REGIO-SPECIFIC AND STEREO-SPECIFIC SYNTHESIS OF *trans*-3′-HYDROXYCOTININE-*O*-GLUCURONIDE

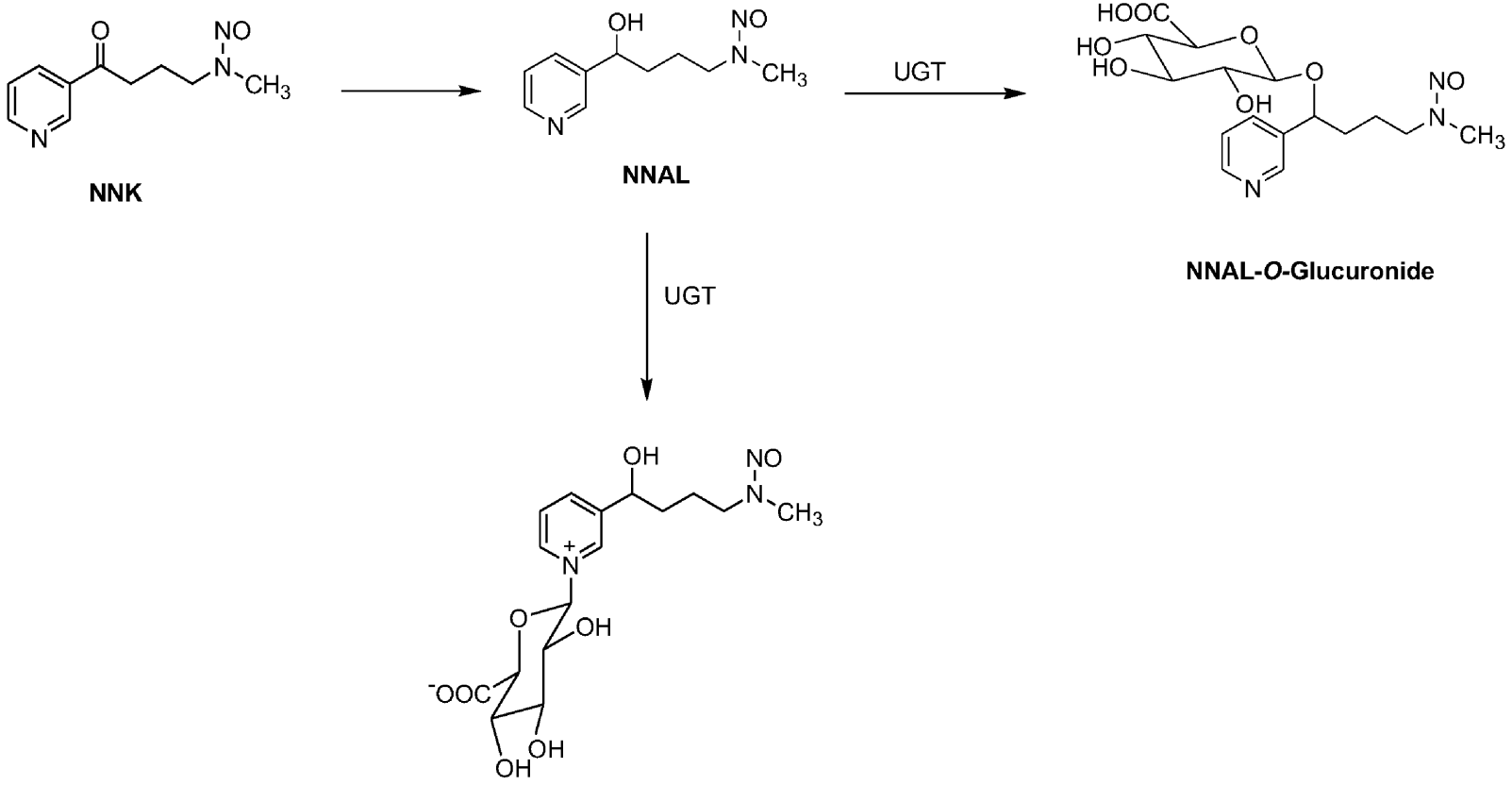


***trans*-3'-Hydroxycotinine-*O*-Glucuronide**
(Single 3'R, 5'S-β-D-Diastereomer)



[$^2H_3$]Methyl-Analog

NNK METABOLISM



Additional Biomarkers for JLI

- Cotinine in blood
- 3-HPMA (3-hydroxypropylmercapturic acid, an acrolein metabolite)
- *Urine mutagenicity (measured by the Ames Salmonella microsome assay)*

ANALYTICAL METHODS

- CO-Oximeter: Carboxyhemoglobin
- LC/MS/MS
  - Nicotine and 5 metabolites
  - NNK metabolites
  - 3-HPMA
- *Urinary mutagens: Ames assay*

ANALYTICAL METHODS

- Nicotine and 5 metabolites
  - Free nicotine, cotinine, and *trans*-3'-hydroxycotinine
  - Acidify 2mL aliquot of a 24-hour urine
  - SPE clean-up
  - LC/MS/MS: Phenomenex 3 μm Phenyl-Hexyl column at 50°C; mobile phase NH_4OAc/ Methanol/Et_3N (65:35:0.002 v/v/v) at 0.2 mL/min; Sciex 3000 tandem mass spectrometer, multiple ion recording mode.

Covance

ANALYTICAL METHODS

- Nicotine and 5 metabolites
  - Free nicotine, cotinine, and *trans*-3′-hydroxycotinine and glucuronides of each (total nicotine, total cotinine, and total *trans*-3′-hydroxycotinine)
  - Treat a second acidified 2mL aliquot of the 24-hour urine collection with a β-glucuronidase (*Helix pomatia)* for 18-22 hr
  - Proceed as for free alkaloids/metabolites
  - Glucuronides determined by difference

Covance

ANALYTICAL METHODS

- Nicotine and 5 metabolites
  - Free nicotine, cotinine, *trans*-3'-hydroxycotinine, and nicotine-*N*-glucuronide
  - Basify 1mL aliquot of a 24-hour urine
  - SPE clean-up
  - LC/MS/MS: MetaChem Inertsil 5 μm Silica column; mobile phase acetonitrile/ water/trifluoroacetic acid (90:10:0.05 v/v/v); Sciex 3000 tandem mass spectrometer, multiple ion recording mode

MDS

ANALYTICAL METHODS

- Nicotine and 5 metabolites
  - Total cotinine, and total *trans*-3'-hydroxycotinine)
  - Treat a second 0.2mL aliquot of the 24-hour urine collection with a β-glucuronidase (*Helix pomatia)* for at least 20 hr
  - Basify and proceed as for free alkaloids/metabolites
  - Glucuronides determined by difference

MDS

ANALYTICAL METHODS

- NNK metabolites
  - Free NNAL
  - Acidify 1.0 mL aliquot of 24-h urine
  - Clean-up with mixed-mode SPE
  - LC/MS/MS
    - Keystone Hypersil silica column with a guard column; mobile phase ACN/H_2O/HCOOH (50:50:1 (v:v:v)); Sciex 3000 MS/MS; multiple ion recording mode

MDS

ANALYTICAL METHODS

- NNK metabolites
  - Total NNAL (free NNAL and NNAL glucuronide)
  - Acidify 1.0 mL aliquot of 24-h urine, and incubate with β-glucuronidase (type H-1 from *Helix pomatia)*
  - Proceed as for free NNAL
  - Glucuronides determined by difference

MDS

ANALYTICAL METHODS

- 3-HPMA
  - 0.200 mL sample of acidified urine
  - Clean-up with solid phase mixed mode extraction column
  - LC/MS/MS
    - Hypersil BioBasic column with a guard column; mobile phase is 80:20 acetonitrile:50 mM ammonium acetate, pH 4.5; Sciex API 4000 MS/MS; multiple ion recording mode

ANALYTICAL METHODS

- Urine Mutageneticity by the Ames Assay
  - Preincubation method with metabolic activation (5% S9)
  - Urine samples concentrated by 250-fold on XAD-2 resin
  - Diluted with dimethyl sulfoxide (DMSO) to yield different dose levels
  - Incubated at 37°C with S9 and YG1024 (tester strain with a high sensitivity for detecting mutagenicity of aromatic amine compounds in human urine)

Biomarker Selection +

Method Development +

Method Validation +

Sample Analysis ⟶

QUALITY DATA

VALIDATION

- "Validation and development of biomarkers will provide a stronger foundation by which to make scientific evaluations and regulatory decisions regarding PREPs."

(Clearing the Smoke Assessing the Science Base for Tobacco Harm Reduction, Advance Copy, Executive Summary, p. 9, 2001.)

- "All of the procedures that demonstrate that a particular method used for quantitative measurement of analytes. . .is reliable and reproducible for the intended use."

(FDA Guidance for Industry Bioanalytical Method Validation, May 2000)

Two Types of Validation

- Analytical validation
  - More commonly discussed
  - More extensively defined
- Biological validation
  - Less guidance available
  - Opportunity for leadership in scientific community

Components of Analytical Validation

- Measures of system suitability and ruggedness
- Statement of data collection and processing instrumentation to be utilized
- Outline of necessary calculations
- Standard curve
- Accuracy
- Precision

Components of Analytical Validation

- SensitivitySpecificity/selectivity
- Stability
- Pre-defined acceptance criteria
- Assessment of carryover and recovery
- Appropriate reporting

FDA GUIDANCE ON VALIDATION

- Precision: <15%; <20% for LLOQ
- Accuracy: ±15%; ±20% for LLOQ
- Standard curve: 75%, or a minimum of six standards, when back-calculated (including ULOQ) should fall within ±15%, except for LLOQ, when it should be ±20% of the nominal value
- Short term stability: includes at least 3 freeze-thaw cycles; tests samples and reagents
- Reproducible recovery

ANALYTICAL VALIDATION

- Carboxyhemoglobin

Performance Parameter	Carboxyhemoglobin
Limit of Quantification	0.3%
Linear Range	0.3% -- 64.7%
Between-Run Precision (% CV)	
Low QC	4.7%
Mid QC	0.7%
Hi QC	0.8%
Between-Run Accuracy (% Error)	
Low QC	-0.6
Mid QC	-0.9
Hi QC	+0.1
Vhi QC	-0.4

ANALYTICAL VALIDATION

- Nicotine, Cotinine, and *trans*-3'-Hydroxycotinine

	Nicotine	**Cotinine**	**trans-3'- Hydroxy cotinine**
Limit of Quantification	1.00 ng/mL (6.17 nmol/L)	1.00 ng/mL (5.64 nmol/L)	1.00 ng/mL (5.18 nmol/L)
Linear Range	1 – 1000 ng/mL	1 – 1000 ng/mL	1 – 1000 ng/mL
Between-Batch Precision (%RSD)			
Low QC (2ng/mL)	10.8 (n=18)	10.9 (n=18)	11.7 (n=18)
Med QC (400 ng/mL)	5.5 (n=18)	4.6 (n=18)	5.8 (n=18)
High QC (750 ng/mL)	3.3 (n=18)	2.3 (n=18)	2.7 (n=18)
Between-Batch Accuracy (%DMT)			
Low QC	1.3 (n=18)	3.5 (n=18)	6.5 (n=18)
Med QC	-9.7 (n=18)	-7.6 (n=18)	-7.4 (n=18)
High QC	-11.2 (n=18)	-10.2 (n=18)	-10.2 (n=18)

ANALYTICAL VALIDATION

- Nicotine, Cotinine, *trans*-3'-Hydroxycotinine, and Nicotine Glucuronide

	Nicotine	**Cotinine**	***trans*-3'-Hydroxy cotinine**	**Nicotine-*N*-glucuronide**
Limit of Quantification	10 ng/mL	10 ng/mL	50 ng/mL	5 ng/mL
Linear Range	10 – 2000 ng/mL	10 – 2000 ng/mL	50 – 2000 ng/mL	5 – 2000 ng/mL
Between-Batch Precision (%RSD)				
Low QC (30ng/mL)	6.7 (n=36)	9.1 (n=36)	7.0 (n=36)	6.7 (n=35)
Med QC (300 ng/mL)	2.6 (n=36)	4.3 (n=36)	6.5 (n=36)	6.3 (n=36)
High QC (1500 ng/mL)	4.2 (n=36)	2.9 (n=36)	4.9 (n=36)	3.9 (n=36)
Between-Batch Accuracy (%DMT)				
Low QC	+2.7 (n=36)	+2.7 (n=36)	+2.0 (n=36)	-0.7 (n=36)
Med QC	+9.0 (n=36)	+0.7 (n=36)	-1.3 (n=36)	+0.3 (n=36)
High QC	-2.5 (n=36)	-0.1 (n=36)	+1.9 (n=36)	+0.4 (n=36)

ANALYTICAL VALIDATION

- Nicotine, Cotinine, and *trans*-3'-Hydroxycotinine* (Total Nicotine + Metabolites)

	Nicotine	Cotinine
Limit of Quantification	1.00 ng/mL (6.17 nmol/L)	1.00 ng/mL (5.64 nmol/L)
Linear Range	1 – 1000 ng/mL	1 – 1000 ng/mL
Between-Batch Precision (%RSD)		
Low QC (2ng/mL)	12.7	14.7
Med QC (400 ng/mL)	5.1	4.4
High QC (750 ng/mL)	4.4	3.5
Between-Batch Accuracy (%DMT)		
Low QC (2ng/mL)	-8.0	-3.5
Med QC (400 ng/mL)	-13.0	-7.0
High QC (750 ng/mL)	-11.9	-7.8

*No data for *trans*-3'-Hydroxycotinine due to unavailability of standard

ANALYTICAL VALIDATION

- Cotinine and *trans*-3'-Hydroxycotinine* (Total Nicotine Metabolites)

	Total Cotinine		Total *trans*-3'-Hydroxycotinine
Limit of Quantification	100 ng/mL		100 ng/mL
Linear Range	100 – 4000 ng/mL		100 – 6002 ng/mL
Between-Batch Precision (%RSD)		Between-Batch Precision (%RSD)	
Low QC (300ng/mL)	4.5 (n=30)	Low QC (300ng/mL)	6.5 (n=30)
Med QC (750 ng/mL)	4.8 (n=30)	Med QC (750 ng/mL)	8.0 (n=30)
High QC (2080 ng/mL)	6.7 (n=30)	High QC (3486 ng/mL)	9.0 (n=30)
Uhigh QC (3000 ng/mL)	3.2 (n=30)	Uhigh QC (4501 ng/mL)	5.6 (n=30)
Between-Batch Accuracy (%DMT)		Between-Batch Accuracy (%DMT)	
Low QC (300ng/mL)	-5.7 (n=30)	Low QC (300ng/mL)	-2.0 (n=30)
Med QC (750 ng/mL)	-2.7 (n=30)	Med QC (750 ng/mL)	+1.3 (n=30)
High QC (2080 ng/mL)	0.0 (n=30)	High QC (3486 ng/mL)	0.0 (n=30)
UHigh QC (3000 ng/mL)	-3.0 (n=30)	Uhigh QC (4501 ng/mL)	+0.7 (n=30)

**trans*-3'-Hydroxycotinine data for QC = 3486 from incurred sample

MDS

ANALYTICAL VALIDATION

- NNK Metabolite (Free NNAL)

Performance Parameter	NNAL
Lower Limit of Quantitation	50.0 pg/mL
Linear Range	50.0-2000 pg/mL
Between-Run Precision (% CV)	
150 pg/mL (Low QC)	8.2 (n=30)
500 pg/mL (Mid QC)	5.5 (n=30)
1500 pg/mL (Hi QC)	5.6 (n=30)
Between-Run Accuracy (% Error)	
150 pg/mL (Low QC)	4.0 (n=30)
500 pg/mL (Mid QC)	1.4 (n=30)
1500 pg/mL (Hi QC)	4.1 (n=30)

MDS

ANALYTICAL VALIDATION

- NNK metabolites (Total NNAL)

Performance Parameter	NNAL
Lower Limit of Quantitation	50.0 pg/mL
Linear Range	50.0-2000 pg/mL
Between-Run Precision (% CV)	
150 pg/mL (Low QC)	7.0 (n=30)
500 pg/mL (Mid QC)	4.5 (n=30)
773 pg/mL (Incurred QC)	7.5 (n=30)
1500 pg/mL (Hi QC)	5.2 (n=30)
Between-Run Accuracy (% Error)	
150 pg/mL (Low QC)	2.7 (n=30)
500 pg/mL (Mid QC)	4.0 (n=30)
773 pg/mL (Incurred QC)	0.0 (n=30)
1500 pg/mL (Hi QC)	4.8 (n=30)

MDS

ANALYTICAL VALIDATION

- 3-HPMA

Performance Parameter	3-HPMA
Lower Limit of Quantitation	35.0 ng/mL
Linear Range	35.0 -- 5000 ng/mL
Between-Run Precision (% CV)	
187 ng/mL (Low QC)	7.5 (n=34)
687 ng/mL (Mid QC)	6,0 (n=34)
3837 ng/mL (High QC))	5.3 (n=34)
Between-Run Accuracy (% Error)	
187 ng/mL (Low QC)	4.3 (n=34)
687 ng/mL(Mid QC)	2.6 (n=34)
3837 ng/mL(Hi QC)	2.1 (n=34)

WHO RECOMMENDATIONS FOR BIOLOGICAL VALIDATION

- Determination of specificity
- Exposure-related toxicokinetics and toxicodynamics
- Dose-response relationship
- Biological variation associated with the marker
  - Intra-individual
  - Inter-individuals
  - Group differences

WHO RECOMMENDATIONS FOR BIOLOGICAL VALIDATION

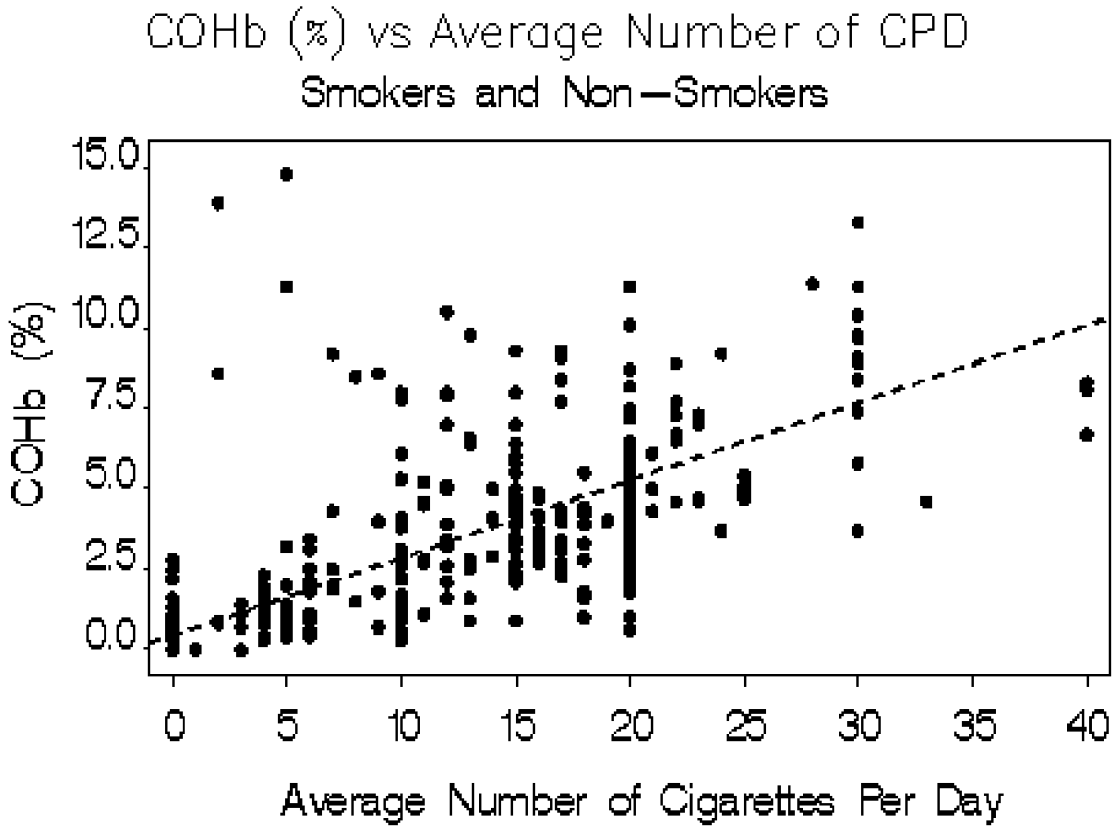
- Route of exposure
- Type of health effect
- Behavioral factors influencing exposure
- Generation of baseline or normative data

(Human Exposure Assessment, Environmental Health Criteria 214, World Health Organization: Geneva, 2000.)

BIOLOGICAL VALIDATION: TES EXPOSURE BIOMARKERS

- Determination of specificity: ***There was a statistically significant difference between carboxyhemoglobin levels in smokers and non-smokers, and the same was observed for nicotine equivalents levels.***
- Dose-response relationship: ***Estimating dose as cigarettes/day***

CARBOXYHEMOGLOBIN DOSE RESPONSE



R. D. Kinser, B. L. Nelson, et al. "Assessment of Human Exposure to Cigarette Smoke Constituents: Pilot Study Results for Carbon Monoxide" Society of Toxicology, March, 2002

BIOLOGICAL VALIDATION: TES EXPOSURE BIOMARKERS

- Determination of specificity:*There was a statistically significant difference between carboxyhemoglobin levels in smokers and non-smokers, and the same was observed for nicotine equivalents levels.*
- Dose-response relationship: *Using dose as cigarettes/day*
- Biological variation associated with the marker: *Assessed by collecting biological specimens on multiple occasions, at differing times of the day, and on work days and leisure days*

VARIABILITY IN THE PILOT TES

BIOMARKER	INTRA-	INTER-
Nicotine Equivalents	18.4%(S) 229.4% (NS)	64.5% (S) 279.5% (NS)
COHb	25.1% (S) 61.3% (NS)	70.0% (S) 75.0% (NS)

BIOLOGICAL VALIDATION: TES EXPOSURE BIOMARKERS

- Inter-individual differences (demographics, genetic differences, metabolites, anthropometrics, behavior, biological/circadian rhythms, previous exposure, lifestyle factors, dietary habits): ***Most of these factors will be addressed by a questionnaire.***
- Route of exposure: ***Addressed by a questionnaire***

BIOLOGICAL VALIDATION: TES EXPOSURE BIOMARKERS

- Toxicokinetics: ***Considered half-life information as a biomarker selection criterion***

HALF-LIFE DATA FOR TES BIOMARKERS

BIOMARKER	$t_{1/2}$(elim)
COHb	3.1 h (awake) and 6.9 h (sleeping)
Nicotine	2 h
Cotinine	19 h
trans-3'-OHCot	6.4 h
Nicotine-gluc; Cotinine-gluc	Not available
trans-3'-OHCot-gluc	7.2 h
NNAL	45 days

BIOLOGICAL VALIDATION: TES EXPOSURE BIOMARKERS

- Toxicokinetics: *Considered half-life information as a biomarker selection criterion*
- Generation of baseline or normative data: ***A U.S. adult smokers population study is underway***

BIOLOGICAL VALIDATION: JLI BIOMARKERS

- 3-HPMA
  - Literature indicates differentiation between smokers and non-smokers
    - Mascher, D. G. et al., J. Chrom B, **750** (2001) 163 – 169

      Smokers (n=27) 2809±385 μg/24 hr

      Non-smokers (n=41) 812±123 μg/24 hr
  - Biomarker of exposure to allylic moiety
    - Acrolein, allylic alcohol

CHALLENGES

- Authentic standards
- Analyte-free matrix for QC samples
- Alternate methods
- Certified standards (standard reference materials)

1 ❒ BIOMARKERS OF EXPOSURE

Selection, Method Development and Validation, and Acceptance

Robin Kinser
Senior Principal Scientist
Clinical Evaluation, Worldwide Scientific Affairs

2 ❒ BIOMARKER DEFINITIONS

- Chemicals, metabolites of chemicals, enzymes, and other biochemical substances found in tissues and body fluids
- Measurements provide data linking ***exposure*** with internal dose and outcome; relevant to process of risk assessment

Biomarkers and Risk Assessment: Concepts and Principles, World Health Organization, Geneva, 1993

3 ❒ EXPOSURE DEFINITIONS

- Exposure is the contact over time and space between a person and one or more biological, chemical or physical agents (US NRC, 1991).
- Exposure is concentration of a substance in the human body over time, (c x t).
- Exposure is the area under the concentration-time-curve, (AUC).

4 ❒ TYPES OF BIOMARKERS

5 ❒ The Paradigm: Biological Marker Components in Sequential Progression Between Exposure and Disease

EXPOSURE

6 ❒ SELECTION OF BIOMARKERS OF EXPOSURE

- Convened panel of PM experts and consultants with significant experience in smoke composition and/or exposure assessment
- Utilized biomarker selection considerations from NRC and N. Benowitz
- Identified candidate smoke constituents
- Chose biomarkers of exposure representative of both smoke phases

7 ❒ SELECTION CRITERIA FOR TES BIOMARKERS OF EXPOSURE

- "Unique or nearly unique" to tobacco smoke, "so that other sources are minor in comparison"
- Reliable analytical methods available ("easily detectable")

Benowitz, N.L. [illegible], 1999, 107(Suppl. 2), 349 – 355

- Representative of either particulate or gas-vapor phase
- Representative of health-relevant constituents
- Sampling to acquire material for analysis only minimally invasive
- Constituent metabolism understood
- Increased in smokers as compared to non-smokers

8 ❒ BIOMARKERS OF EXPOSURE FOR THE TES

BIOMARKER	SAMPLE MATERIAL	SMOKE CONSTITUENT	SMOKE PHASE*

Acetonitrile	*Exhalate; blood*	*Acetonitrile*	*GVP*
Carbon monoxide	*Exhalate*	*Carbon monoxide*	*GVP*
Carboxyhemoglobin	Blood	Carbon monoxide	GVP
Hb adducts of *3-* and 4-aminobiphenyl	Blood	3- and 4- Aminobiphenyl	PP
Nicotine and nicotine metabolites**	24-hr urine	Nicotine	PP
NNAL and NNAL-glucuronide	24-hr urine	NNK	PP

*GVP: gas-vapor phase; PP: particulate phase
**cotinine, 3-hydroxycotinine, nicotine-*N*-glucuronide, cotinine-*N*-glucuronide, and *trans*-3'-hydroxycotinine-*O*-glucuronide,

9 ☐ NICOTINE METABOLISM

10 ☐ THE QUEST FOR AUTHENTIC REFERENCE STANDARDS

11 ☐ SUCCESSFUL SYNTHESIS OF *trans*-3'-HYDROXYCOTININE-*O*-GLUCURONIDE

12 ☐ REGIO-SPECIFIC AND STEREO-SPECIFIC SYNTHESIS OF *trans*-3'-HYDROXYCOTININE-*O*-GLUCURONIDE

13 ☐ NNK METABOLISM

14 ☐ Additional Biomarkers for JLI

- Cotinine in blood
- 3-HPMA (3-hydroxypropylmercapturic acid, an acrolein metabolite)
- *Urine mutagenicity (measured by the Ames Salmonella microsome assay)*

15 ☐ ANALYTICAL METHODS

- CO-Oximeter: Carboxyhemoglobin
- LC/MS/MS
  - Nicotine and 5 metabolites
  - NNK metabolites
  - 3-HPMA
- *Urinary mutagens: Ames assay*

16 ☐ ANALYTICAL METHODS

- Nicotine and 5 metabolites
  - Free nicotine, cotinine, and *trans*-3'-hydroxycotinine
  - Acidify 2mL aliquot of a 24-hour urine
  - SPE clean-up
  - LC/MS/MS: Phenomenex 3 μm Phenyl-Hexyl column at 50°C; mobile phase NH_4OAc/ Methanol/Et_3N (65:35:0.002 v/v/v) at 0.2 mL/min; Sciex 3000 tandem mass spectrometer, multiple ion recording mode.

Covance

17 ☐ ANALYTICAL METHODS

- Nicotine and 5 metabolites
  - Free nicotine, cotinine, and *trans*-3'-hydroxycotinine and glucuronides of each (total nicotine, total cotinine, and total *trans*-3'-hydroxycotinine)
  - Treat a second acidified 2mL aliquot of the 24-hour urine collection with a β-glucuronidase (*Helix pomatia)* for 18-22 hr
  - Proceed as for free alkaloids/metabolites

- Glucuronides determined by difference

Covance

18 ANALYTICAL METHODS

- Nicotine and 5 metabolites
  - Free nicotine, cotinine, *trans*-3′-hydroxycotinine, and nicotine-*N*-glucuronide
  - Basify 1mL aliquot of a 24-hour urine
  - SPE clean-up
  - LC/MS/MS: MetaChem Inertsil 5 μm Silica column; mobile phase acetonitrile/ water/trifluoroacetic acid (90:10:0.05 v/v/v); Sciex 3000 tandem mass spectrometer, multiple ion recording mode

MDS

19 ANALYTICAL METHODS

- Nicotine and 5 metabolites
  - Total cotinine, and total *trans*-3′-hydroxycotinine)
  - Treat a second 0.2mL aliquot of the 24-hour urine collection with a β-glucuronidase (*Helix pomatia)* for at least 20 hr
  - Basify and proceed as for free alkaloids/metabolites
  - Glucuronides determined by difference

MDS

20 ANALYTICAL METHODS

- NNK metabolites
  - Free NNAL
  - Acidify 1.0 mL aliquot of 24-h urine
  - Clean-up with mixed-mode SPE
  - LC/MS/MS
    - Keystone Hypersil silica column with a guard column; mobile phase ACN/H_2O/HCOOH (50:50:1 (v:v:v)); Sciex 3000 MS/MS; multiple ion recording mode

MDS

21 ANALYTICAL METHODS

- NNK metabolites
  - Total NNAL (free NNAL and NNAL glucuronide)
  - Acidify 1.0 mL aliquot of 24-h urine, and incubate with β-glucuronidase (type H-1 from *Helix pomatia)*
  - Proceed as for free NNAL
  - Glucuronides determined by difference

MDS

22 ANALYTICAL METHODS

- 3-HPMA
  - 0.200 mL sample of acidified urine
  - Clean-up with solid phase mixed mode extraction column
  - LC/MS/MS
    - Hypersil BioBasic column with a guard column; mobile phase is 80:20 acetonitrile:50 mM ammonium

acetate, pH 4.5; Sciex API 4000 MS/MS; multiple ion recording mode

23 ANALYTICAL METHODS

- Urine Mutageneticity by the Ames Assay
  - Preincubation method with metabolic activation (5% S9)
  - Urine samples concentrated by 250-fold on XAD-2 resin
  - Diluted with dimethyl sulfoxide (DMSO) to yield different dose levels
  - Incubated at 37°C with S9 and YG1024 (tester strain with a high sensitivity for detecting mutagenicity of aromatic amine compounds in human urine)

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25 VALIDATION

- "Validation and development of biomarkers will provide a stronger foundation by which to make scientific evaluations and regulatory decisions regarding PREPs."

(Clearing the Smoke Assessing the Science Base for Tobacco Harm Reduction, Advance Copy, Executive Summary, p. 9, 2001.)

- "All of the procedures that demonstrate that a particular method used for quantitative measurement of analytes. . .is reliable and reproducible for the intended use."

(FDA Guidance for Industry Bioanalytical Method Validation, May 2000)

26 Two Types of Validation

- Analytical validation
  - More commonly discussed
  - More extensively defined
- Biological validation
  - Less guidance available
  - Opportunity for leadership in scientific community

27 Components of Analytical Validation

- Measures of system suitability and ruggedness
- Statement of data collection and processing instrumentation to be utilized
- Outline of necessary calculations
- Standard curve
- Accuracy
- Precision

28 Components of Analytical Validation

- SensitivitySpecificity/selectivity
- Stability
- Pre-defined acceptance criteria
- Assessment of carryover and recovery
- Appropriate reporting

29 FDA GUIDANCE ON VALIDATION

- Precision: <15%; <20% for LLOQ
- Accuracy: ±15%; ±20% for LLOQ

- Standard curve: 75%, or a minimum of six standards, when back-calculated (including ULOQ) should fall within ±15%, except for LLOQ, when it should be ±20% of the nominal value
- Short term stability: includes at least 3 freeze-thaw cycles; tests samples and reagents
- Reproducible recovery

30 ANALYTICAL VALIDATION

- Carboxyhemoglobin

31 ANALYTICAL VALIDATION

- Nicotine, Cotinine, and *trans*-3'-Hydroxycotinine

32

33 ANALYTICAL VALIDATION

34

35 ANALYTICAL VALIDATION

36 ANALYTICAL VALIDATION

37 ANALYTICAL VALIDATION

38 WHO RECOMMENDATIONS FOR BIOLOGICAL VALIDATION

- Determination of specificity
- Exposure-related toxicokinetics and toxicodynamics
- Dose-response relationship
- Biological variation associated with the marker
  - Intra-individual
  - Inter-individuals
  - Group differences

39 WHO RECOMMENDATIONS FOR BIOLOGICAL VALIDATION

- Route of exposure
- Type of health effect
- Behavioral factors influencing exposure
- Generation of baseline or normative data

(Human Exposure Assessment, Environmental Health Criteria 214, World Health Organization: Geneva, 2000.)

40 BIOLOGICAL VALIDATION: TES EXPOSURE BIOMARKERS

- Determination of specificity: ***There was a statistically significant difference between carboxyhemoglobin levels in smokers and non-smokers, and the same was observed for nicotine equivalents levels.***
- Dose-response relationship: ***Estimating dose as cigarettes/day***

41 ❒ CARBOXYHEMOGLOBIN DOSE RESPONSE

42 ❒ BIOLOGICAL VALIDATION: TES EXPOSURE BIOMARKERS

- Determination of specificity:*There was a statistically significant difference between carboxyhemoglobin levels in smokers and non-smokers, and the same was observed for nicotine equivalents levels.*
- Dose-response relationship: *Using dose as cigarettes/day*
- Biological variation associated with the marker: *Assessed by collecting biological specimens on multiple occasions, at differing times of the day, and on work days and leisure days*

43 ❒ VARIABILITY IN THE PILOT TES

44 ❒ BIOLOGICAL VALIDATION: TES EXPOSURE BIOMARKERS

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- Route of exposure: ***Addressed by a questionnaire***

45 ❒ BIOLOGICAL VALIDATION: TES EXPOSURE BIOMARKERS

- Toxicokinetics: ***Considered half-life information as a biomarker selection criterion***

46 ❒ HALF-LIFE DATA FOR TES BIOMARKERS

47 ❒ BIOLOGICAL VALIDATION: TES EXPOSURE BIOMARKERS

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- Generation of baseline or normative data: ***A U.S. adult smokers population study is underway***

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  - Biomarker of exposure to allylic moiety
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- Analyte-free matrix for QC samples
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- Certified standards (standard reference materials)