# Bioactivation of Furans by Human Lung and Liver Microsomes and S9

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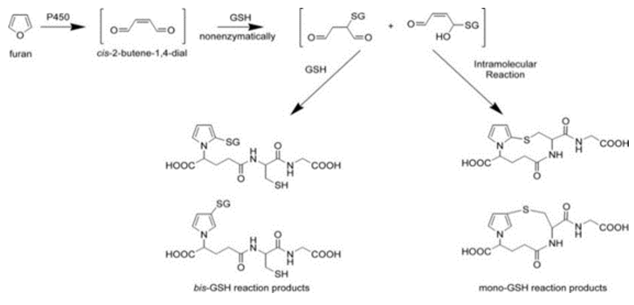
## Introduction

### Background

Direct exposure to inhaled mainstream cigarette smoke is known to cause damage in the human lung. Although the lung has a significant capability for biotransforming many of the harmful or potentially harmful compounds (HPHCs) present in cigarette smoke, thereby reducing acute toxicity, in some instances biotransformation can result in the generation of more reactive, and thus more toxic, metabolites. This is the case for furan and simple alkylfurans[1].

- Furans are naturally present in tobacco leaves and are also formed by thermal degradation of carbohydrate precursors (between 300-400°C)[2] during the burning or heating of tobacco
- Biotransformation of simple furans in the body can be described by two major pathways, either by the initial generation of reactive intermediates (cis-2-butene-1,4-dial and derivatives) by CYP2E1 metabolism[3] or, if substituted with a side chain (e.g., furfuryl alcohol), by oxidation to the corresponding aldehydes and acids prior to Phase II conjugation and excretion from the body

*General scheme for furan bioactivation by CYP2E1 and subsequent conjugation with glutathione*




### Aim

To evaluate the capability of the human lung (by using subcellular fractions as a surrogate model) to generate reactive intermediate metabolites (RIMs) from the biotransformation of simple furans by Phase I oxidation, and Phase II conjugation with activated sulfate, generating potentially toxic sulfate conjugates, with a view to establish a link between exposure to such compounds and disease-relevant mechanisms (e.g., oxidative stress, inflammation, apoptosis).

To compare the kinetic parameters of human lung (smoker and non-smoker) versus human liver subcellular fractions for the metabolism of simple furans.

### Strategy

- Establish a robust and easy to use *in vitro* model to characterize the metabolic fate of furans in human liver and lung subcellular fractions (microsomes as well as S9 for studying Phase I or Phase I+II metabolism)
- Evaluate kinetic parameters for parent compound elimination and metabolite formation for a simple mixture of furans and for cigarette smoke (from 3R4F reference cigarette[4] bubbled through phosphate buffered saline (wsPBS)) containing furans as part of a complex mixture
- Analyze reactive intermediate metabolites by determination of their glutathione adducts and reactive sulfate conjugates using liquid chromatography coupled with high resolution mass spectrometry (LC-HR-MS)

## Materials and Methods

### Test/Reference Material and Application Levels

| System: | Human Microsomes (Phase I) | | | Human S9 (Phase I + II) | | | |
|---|---|---|---|---|---|---|---|
| Organ: | Liver | | Lung | Liver | Lung | Liver | Lung |
| Test item: | Furan-Mix | | | Ref. Cig. 3R4F | | Ref. Cig. 3R4F | |
| Smoke Generation: | - | | | Health Canada (HC) | | Health Canada (HC) | |
| Application level: | 1µM | 10µM | 10µM | 0.08 cig./incub. | | 0.08 cig./incub. | |
| Protein/incubation: | 0.5mg | 0.5mg | 2mg | 1mg | 2mg | 2mg | 2mg |
| Furan | 2.8 | 27.5 | 6.9 | 6.8 | 3.4 | 3.4 | 3.4 |
| 2-Methylfuran | 2.2 | 22.2 | 5.5 | 10.3 | 5.2 | 5.2 | 5.2 |
| 2,5- & 2,4-Dimethylfuran | 3.8 | 37.6 | 9.4 | 10.4 | 5.2 | 5.2 | 5.2 |
| 2-Acetylfuran | 2.0 | 20.0 | 5.0 | 2.3 | 1.2 | 1.2 | 1.2 |
| Furfural | 2.4 | 24.1 | 6.0 | 15.2 | 7.6 | 7.6 | 7.6 |
| 5-Methylfurfural | 2.0 | 20.1 | 5.0 | 4.7 | 2.4 | 2.4 | 2.4 |
| 2-Furanmethanol | 2.3 | 23.0 | 5.8 | 0.6 | 0.3 | 0.3 | 0.3 |
| 5-Methyl-2-furanmethanol | 1.9 | 19.1 | 4.8 | 0.07 | 0.04 | 0.04 | 0.04 |
| 5-Hydroxymethyl-furfural | 1.9 | 19.1 | 4.8 | 15.9 | 8.0 | 8.0 | 8.0 |

[nmol/mg protein]

- *Furan mixes were freshly prepared before each experiment by adding pure compounds to water within the limits of solubility*
- *Applied doses of cigarette smoke to the subcellular fractions were calculated based on semiquantitative levels of furans determined by two-dimensional gas chromatography with time-of-flight mass spectrometry (GCxGC-TOF-MS); compound peak areas were related to internal standards of known concentration without calibration*

### Microsomal and S9 Incubations

- Metabolites were generated *in vitro* using an enzymatic system containing human liver microsomes and S9 fractions combined with a NADPH Regenerating System (NRS) containing NADP, G6P and G6PDH as well as alamethicin and co-factors required for Phase II conjugation (e.g. PAPS, UDPGA…)
- Aqueous smoke samples were pre-incubated with glutathione (GSH) for 30 minutes in order to prevent an irreversible inhibition of CYPs by smoke electrophiles (e.g. acrolein, crotonaldehyde…)
- Reactive intermediate metabolites were trapped using GSH as a soft nucleophile (selected experiments used a mixture of stable isotope labeled (glycine-$^{13}C_2$,$^{15}N$) and non-labeled GSH)
- Individual vials for each incubation time point were used to avoid any loss of volatile compounds
- To avoid adsorption effects at material surfaces, silanized glass was used
- Corresponding negative controls followed the same procedure in the absence of the NRS
- Enzymatic activity was stopped by the addition of methanol, containing a mixture of internal standards, followed by crash freezing using dry ice, thawing and centrifugation
- Supernatants were directly injected onto GCxGC-TOF-MS (cool-on-column) and LC-HR-MS systems

### LC-HR-MS analysis

**LC system:**
**Thermo Fisher Scientific Accela 1250™ LC**
Injection volume: 5 µL
Eluent A: 0.1% formic acid in water
Eluent B : 0.1% formic acid in acetonitrile
Flow rate: 400 µL/min
Column: Thermo Hypersil Gold aQ (150 x 2.1 mm, 1.9 µm)
HPLC gradient:
0-3min (0%B) ->15min (20%B) ->16-19min (100%B)

**MS system:**
**Thermo Fisher Scientific Q Exactive™**
Ionisation mode: ESI (+) / (-)
Scanning mode: full scan
Scan range: 80 – 800 m/z
Resolution: 70000

### GCxGC-TOF-MS analysis

**GCxGC system:**
**Agilent 6890A with ZOEX Modulator**
Injection: cool-on-column, 0.5µL
1D column: SLB-IL59 (30m × 0.25mm × 0.20µm)
2D column: SLB-IL76 (2m × 0.25mm ID × 0.20µm)
T-program 1D: 30°C (2min) – 5°C/min – 250°C (15min)
T-program 2D: 35°C (2min) – 4°C/min – 43°C – 5.5°C/min – 270°C (15min)
Modulation period: 6sec (1sec hot pulse)

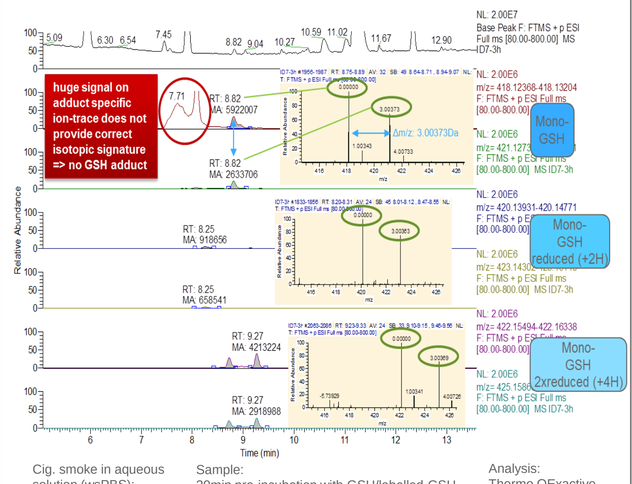
**MS system:**
**LECO Pegasus® *4D***
Ionization mode: EI (70eV)
Scan range: 35 – 500 m/z,
Acquisition rate: 200Hz
Resolution: Unit

## Results

### Glutathione Conjugates for Reactive Intermediate Metabolites of Furans by Phase I Metabolism – the Downstream Modification Challenge

Conjugation of RIMs derived from furans with GSH form a multiplicity of initial adducts (1,4-Michael-addition adducts, intramolecular rearrangement products and bis-GSH-adducts), which are prone to subsequent metabolic processes (e.g. reduction, already known for acrolein-glutathione adducts). The presence of downstream reduced GSH-adducts from furans was demonstrated using a mixture of stable isotope labeled and non-labeled GSH as a trapping system. The presence of carbonyl reductase activity within the subcellular fractions could be confirmed by the reduction of 3-oxopropyl-GSH (initial reaction product of acrolein and GSH) to its reduced form, 3-hydroxypropyl-GSH.

**Downstream reduction of initial RIM glutathione adducts**
Example: RIMs of 2,5- & 2,4-dimethylfuran conjugated to stable isotope labeled/non-labeled GSH provides a characteristic isotopic signature of Δm/z: 3.00374Da

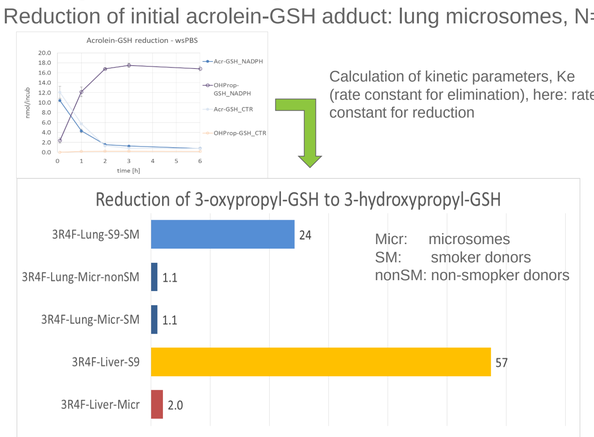



Cig. smoke in aqueous solution (wsPBS): human liver microsomes

Sample: 30min pre-incubation with GSH/labelled-GSH, 3h microsomal incubation + NADPH regenerating system

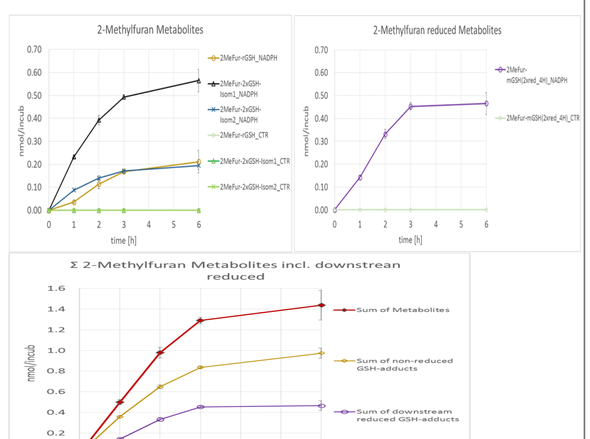
Analysis: Thermo QExactive LC-HR-MS, ESI(+)

**Carbonyl reductase activity for subcellular fractions**
Reduction of initial acrolein-GSH adduct: lung microsomes, N=3

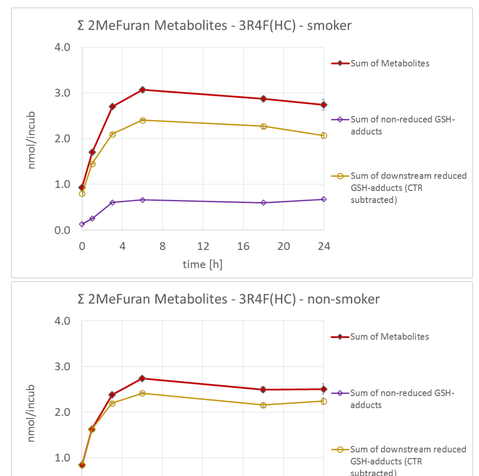



- All tested subcellular fractions showed reductase activity
- Consequently all metabolic experiments included screening for the presence of reduced RIM glutathione adducts

**Furans RIM glutathione adducts, examples**
For the evaluation of metabolite formation rates, all currently known types of RIM adducts were considered.
Examples below illustrate the diversity of adducts formed.
2-Methylfuran in furan-mix (1µM), liver microsomes, N=3

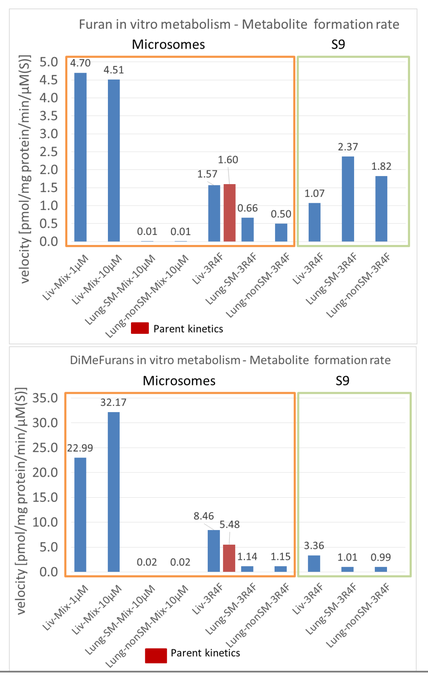


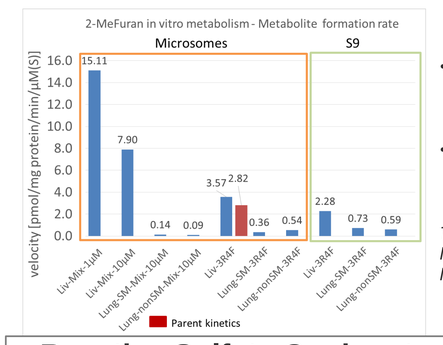

2-Methylfuran - wsPBS-3R4F (6cig./36mL, PBS)




### Formation rate of Reactive Intermediate Metabolites by Human Lung (Smoker versus Non-Smoker) and Human Liver Microsomal Subcellular Fractions

The rate of metabolite formation was evaluated using log transformed averaged kinetics data (N=3) for the sum of all glutathione adduct species for each RIM. Values for velocity of metabolite formation are expressed as pmol metabolite formed per mg protein per minute, normalized for 1µM substrate concentration.




- *All experiments performed in triplicate, rate constants calculated using averaged metabolite kinetics*
- *For each kinetic experiment a minimum of 5 time points were evaluated*

*Typical time-points:*
*liver: 0h, 1h, 2h, 3h, 6h*
*lung: 0h, 1h, 3h, 6h, 18h, 24h*

**Reactive Sulfate Conjugates by Human Lung (Smoker versus Non-Smoker) and Liver S9**

| System: | human S9 | | |
|---|---|---|---|
| Organ: | Liver | Lung smoker | Lung non-smoker |
| Test item: | 3R4F(HC) | 3R4F(HC) | 3R4F(HC) |
| 2-Furanmethanol (reactive sulfate) | not detected | | |
| 5-Methyl-2-furanmethanol (reactive sulfate) | 0.714 | 0.062 | 0.014 |
| 5-Hydroxymethyl-furfural (reactive sulfate) | 0.023 | 0.0033 | 0.0016 |

velocity [pmol/mg protein/min/µM(S)]

- Reactive sulfate conjugates found for 5-methyl-2-furanmethanol and 5-hydroxy-methylfurfural
- Human S9 activity: liver >> lung, smoker (lung) > non-smoker (lung)

## Conclusions

- Human lung subcellular fractions are able to toxify furan and simple alkylfurans by CYP-related Phase I metabolism by generating reactive electrophilic intermediates
- Conjugation of RIMs derived from furans with GSH forms a multiplicity of initial adducts, which are also subject to subsequent metabolic reduction, greatly complicating the evaluation process
- The formation rate for furans reactive metabolites was significantly lower for the tested cigarette smoke fraction compared to a simple furans mixture, indicative for the presence of inhibitory effects in a complex mixture such as cigarette smoke
- The formation rate for furans reactive metabolites for lung subcellular fractions is significantly lower compared to liver, except for furan using S9 fraction
- Human liver and lung S9 fractions generate reactive sulfate conjugates for 5-methyl-2-furanmethanol and 5-hydroxy-methylfurfural, with activity for lung S9 fraction more than 10 times lower compared to liver S9

## References

1) *Peterson L. A. Reactive metabolites in the biotransformation of molecules containing a furan ring. Chem. Res. Toxicol. 26:6 (2013).*
2) *Zhouhong Wang et al. Effect of cellulose crystallinity on the formation of a liquid intermediate and on product distribution during pyrolysis. Journal of Analytical and Applied Pyrolysis. 100: 56-66 (2013)*
3) *Takakusa H. et al. Markers of Electrophilic Stress Caused by Chemically Reactive Metabolites in Human Hepatocytes . Drug Metabolism and Disposition. Vol. 36, No. 5 (2008).*
4) *University of Kentucky (www.3r4f.com), (http://www2.ca.uky.edu/refcig/3R4F%20Preliminary%20Analysis.pdf)*

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