Sweating the small stuff---the influence of metabolite extraction and separation on metabolomic studies

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Excellent References

• Mass spectrometry-based metabolomics

• Xenobiotic metabolomics: major impact on the metabolome

• The intestinal metabolome: an intersection between microbiota and host
Metabolomics

• Metabolomics is the systematic analysis of the unique chemical fingerprints left behind by specific cellular processes
  • These small molecule metabolite profiles provide insight into cellular status.

• All “-omics” based scientific disciplines aim at the collective characterization and measurement of their particular constituent molecules
  • A comprehensive approach to study complete pools of biological molecules
  • Defines the structure, function and dynamics of an organism.

• Vast chemical diversity among small molecule metabolites has made extended coverage of the metabolome challenging
  • Size (50 – 1500 Da)
  • Concentration ( pM – mM)
  • Physicochemical properties (diverse log P values)
  • Stereochemistry (distinct biological activity)

Metabolite Extraction

• Currently no analytical technique exists that is capable of in-situ measurement of all classes of cellular metabolites

• Metabolite extraction therefore becomes a crucial step in any type of metabolomics study
  • Critical to both targeted and global based profiling strategies.

• Optimized extraction methodology should fulfill several criteria:
  • Extract the largest number of metabolites
  • Unbiased and non-selective - physical or chemical properties of a molecule
  • Non-destructive - no modification of metabolites
Separation of Metabolites

- Mass spectrometry usually requires some form of chromatographic separation
  - Most systems use either liquid or gas chromatography
  - CE-MS gaining popularity

- Fractionation of sample components simplifies the resulting mass spectra while ensuring more accurate compound identification
  - Capacity factor (k) is critical to optimizing resolution
  - Increased resolution allows longer MS dwell times resulting in better signal/noise ratios

- Inadequate chromatographic separation of metabolites results in:
  - signal suppression – ion suppression
  - compromised metabolite quantification
  - reduced metabolite coverage

Hypothesis

Extraction and separation of metabolites may influence metabolomic studies as much as the disease process being investigated

Rationale

Developing optimized protocols for extraction efficiency and chromatographic resolution based on metabolite class and/or characteristics will dramatically improve accuracy and reproducibility of metabolomic data sets.
Definitions

- **Isomer** – same chemical formula, different chemical structure

- **Stereoisomer** – same chemical formula, same order/sequence of bonded atoms, different 3-dimensional orientation

- **Isobar** – same mass, but different chemical formula
Resolution of Bile Acid Metabolites by RPLC using Waters BEH C18

1. TCA
2. GCA
3. TCDCA
4. CA
5. UDCA
6. GCDCA
7. CDCA
8. DCA

Resolution of Taurine Conjugated MCA Isomers by RPLC on WATERS BEH C18

1) Tauro-ω-MCA
2) Tauro-α-MCA
3) Tauro-β-MCA
Resolution of Taurine Conjugated MCA Isomers by RPLC on
Restek Rapture Biphenyl

1) Tauro-α-MCA
2) Tauro-β-MCA
3) Tauro-ω-MCA

Resolution of Taurine Conjugated MCA Isomers by RPLC on
Restek Ultra AQ C18

1. Tauro-ω-MCA
2. Tauro-α-MCA
3. Tauro-β-MCA
Why should you care about chromatography?

OUTLINE

- Extraction ---- Acetaminophen-Induced Hepatotoxicity
- Chromatography --- Search for Lung Cancer Biomarkers
METABOLIC PROFILING FOR UNDERSTANDING DRUG TOXICITY---ACETAMINOPHEN

ACETAMINOPHEN-INDUCED HEPATOCYTE NECROSIS
I. ROLE OF DRUG METABOLISM
J. B. MITCHELL, D. J. JOLLOW, W. E. POTTER
D. C. DAVIS, J. R. GILLETTE AND B. R. BRODIE
Laboratory of Chemical Pharmacology, National Heart and Lung Institute, National Institute of Health, Bethesda, Maryland

ACETAMINOPHEN-INDUCED HEPATIC NECROSIS
II. ROLE OF COVALENT BINDING IN VIVO
D. J. JOLLOW, J. R. MITCHELL, W. E. POTTER
D. C. DAVIS, J. R. GILLETTE AND B. R. BRODIE
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ACETAMINOPHEN-INDUCED HEPATOCYTE NECROSIS
III. CYTOCHROME P-450-MEDIATED COVALENT BINDING IN VITRO
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ACETAMINOPHEN-INDUCED HEPATOCYTE NECROSIS
IV. PROTECTIVE ROLE OF GLUTATHIONE
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APAP → NAPQI → Toxicity

N-acetyl-p-benzoquinone imine
NORMAL MOUSE LIVER  
NECROTIC MOUSE LIVER  
(400 mg/kg APAP 6 HOURS)

- Contained in 100s of products
- One of the most common pharmaceuticals associated with accidental and intentional poisoning (>7 g per adult per day)
- APAP overdose serves as a model for drug-induced liver toxicity
- Excess NAPQI (with reduced glutathione levels) leads to oxidative damage and inflammation leading to hepatocellular death/necrosis
Acetaminophen Metabolomics

Vehicle Wild-type

APAP Wild-type

LC-MS

Data Analysis

Metabolite Identification

Unlabeled

Ring - labeled

Acetyl - labeled

Mouse Urinary Proteins (MUPs)

• Dilute equal volume of mouse urine with an equal volume of 50% methanol
Mouse Urinary Proteins

- Dilute equal volume of mouse urine with an equal volume of 100% methanol

TOTAL REMOVAL OF MUPS = IMPROVED SIGNAL, REDUCED ION SUPPRESSION

APAP Metabolism Study #4 Score Scatter Plot
PCA model
APAP Metabolism Loading Scatter Plot

PCA Model

- Drug Metabolites
- Depleted with APAP Treatment
- Enriched with APAP Treatment

$R^2 \times 1 = 0.247$, $R^2 \times 2 = 0.159$

X-variable Trend Plot for L-Carnitine (m/z=162.114+)

- Control
- Acetaminophen
- Depleted with APAP Treatment

Obs ID (Obs. Sec. ID: 1)
**X-Variable Trend Plot for Propionylcarnitine (m/z=218.14+)**

- **Control**
- **Acetaminophen**
- **Depleted with APAP Treatment**

**X-Variable Trend Plot for Acetylcarnitine (m/z=204.124+)**

- **Control**
- **Acetaminophen**
- **Enriched with APAP Treatment**
X-Variable Trend Plot for Decanoylcarnitine (m/z=316.247+)

Control

Acetaminophen

Enriched with APAP Treatment

STRUCTURAL ELUCIDATION USING INFORMATION DEPENDENT ACQUISITION
**Typical Metabolomics Workflow**

1. **LC-MS Data files**
2. **Import, align, normalize**
3. **Assign group IDs (optional)**
4. **Analyze data (PCA, OPLS)**
5. **Interpret results**
6. **Metabolite ID**
7. **Generate features (Ret Time & m/z pairs)**
8. **Exclude variables**

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**Metabolite ID is Time Consuming**

1. Populate MS/MS product ion acquisition parameter list with m/z and RT values
2. Prepare new samples
3. Acquire MS/MS product ion spectra
4. Optimize collision energy and reacquire spectra
5. Align new chromatograms with the previously obtained ones
6. Compare product ion spectra with reference spectra
IDA Metabolite ID Workflow

1. Conventional LC-MS survey scan used to trigger LC-MS/MS product ion acquisition
2. Up to 50 MS/MS product ions for each survey scan, optimal number 5-20
3. Optimal values are dependent on chromatographic peak widths and sample complexity
4. Compromise between missing a compound and acquiring many noisy unusable spectra
5. Dynamic background subtract
6. Collision energy spread

IDA Metabolite ID Workflow

• Autonomous acquisition of product ion spectra
• No product ion m/z list generation, sample re-preparation or chromatographic realignment required
• Ideally high quality MS/MS product ion spectra acquired for all components present in mixture
• If subsequent data analysis reveals previously unidentified features, have archived MS/MS product ion spectra
Lipid Extract IDA Workflow

- Bovine liver total lipid extract 0.5 mg/ml (Avanti)
- Gradient elution aqueous isopropanol/acetonitrile w/ 10mM ammonium formate 0.1% formic acid
- Waters CSH® 2.1 x 100 mm C18 column
- Up to 20 IDA scans (100 ms)
- 2.1s duty cycle
- Exclude for 10 s after 1 occurrence
- Product ion m/z 150-1150
- Total 3408 product ion mass spectra
Lipid Extract IDA Workflow

- Limit m/z range to 50-200 Da increments
- Up to 10 IDA scans (200 ms)
- 2.1s duty cycle
- Exclude for 10 s after 2 occurrences
- Product ion m/z 800-850
- Total 1564 product ion mass spectra
TARGETED m/z 800-850
1564 MS/MS

PC 22:6, 18:1
Liver Toxicity 

APAP → NAPQI → Serum Acylcarnitines 

PPARα 

MITOCHONDRIAL DYSFUNCTION

β-oxidation

PPARα
INFLUENCE OF EXTRACTION PROTOCOL

Influence of pH on Metabolite Extraction from Mouse Liver
Influence of pH on Metabolite Extraction from Mouse Liver

Influence of pH on Metabolite Extraction from Mouse Liver
Extraction Efficiency of L-Carnitine from Mouse Liver

![Graph showing extraction efficiency of L-carnitine from mouse liver.](image)

Resolution of Acyl Carnitine Standards by RPLC on Waters BEH C18

![Graph showing resolution of acyl carnitine standards.](image)

1. L-carnitine
2. Propionylcarnitine
3. Hexanoylcarnitine
4. Octanoylcarnitine
5. Decanoylcarnitine
6. Lauroylcarnitine
7. Myristoylcarnitine
8. Palmitoylcarnitine
Resolution of Coenzyme A (CoA) Thioester Metabolites by RPLC using Waters BEH C18

1. Coenzyme A
2. Acetyl CoA
3. 3-dephospho CoA
4. Propionyl CoA
5. 2-butenyl CoA
6. Isobutyryl CoA
7. Butyryl CoA
8. Phenylacetyl CoA
9. Hexanoyl CoA
10. Octanoyl CoA
11. Decanoyl CoA
12. Lauroyl CoA
13. Myristoyl CoA
14. Lineoleoyl CoA

Extraction Efficiency of Stearoyl Carnitine from Mouse Liver

- n-hexanol: 16.6%
- acidified IPA: 16.7%
- basic IPA: 50.1%
- MeOH: water (pH 4.0): 41.4%
- MeOH: water (pH 10.0): 51.4%
Acylcarnitine Extraction in Acidified IPA

AcylCoa Extraction via Modified Bligh/Dyer
AcylCoA Extraction in Acidified IPA

Coenzyme A
3-dephospho acetylpropionylcrotonyl
2-butenoylbutyrylisobutyrylmalonylhexitanoyl
myristoylpalmitoleoylpalmitoyllinoleoyloleoylstearoyl

β-oxidation

PPARα
Long Chain CoA Carnitine

METABOLIC FOR CASE CONTROL STUDIES – LUNG CANCER BIOMARKERS
**Lung Cancer Biomarkers**

- Most common cause of cancer deaths worldwide
- **Training Set**
  - 536 population controls, 469 lung cancer patients (pre-treatment)
- **Validation Set**
  - 78 population controls, 80 lung cancer patients
- **Quantitation Set**
  - 106 population controls, 92 lung cancer patients
- **Tumor Tissue Set**
  - 48 non-tumor, 48 tumor
Quality Control

ESI+

ESI-

Pearson's Correlation Coefficients

Frequency

-0.5 0.0 0.5 1.0

0 50 100 150 200

Random Pairs (N=2500)

Duplicate Pairs (N=254)

ESI+

ESI-

Pearson’s Correlation Coefficients

Frequency

-0.2 0.0 0.2 0.4 0.6 0.8 1.0

0 50 100 150 200

Random Pairs (N=2500)

Duplicate Pairs (N=254)
Creatine Riboside (non IUPAC)

Maillard Reaction (makes grilled stuff tasty)

Creatine + D-ribose + heat = creatine riboside
Reverse Phase
Waters BEH C18
Poor retention of small, polar metabolites

HILIC
Waters BEH Amide
Effect of Column Bore Diameter on Sensitivity

SAICAR std (10.0 μM)

1.0x150mm Acquity BEH amide 1.7μM

10X

2.1x100mm Acquity BEH amide 1.7μM
Histamine is metabolized to a similar riboside (Williams)
  - Similar mechanism for creatine?

Creatine and ribose can form a mutagen similar to PhIP (Kaddurah-Daouk)

Synthesis of creatine riboside with SRI as part of the NIH Common Fund

Summary

- Demonstrated the value of optimized metabolite extraction
- Emphasized study design (particularly for human studies) and the value of good chromatography
- Provided an improved means to simultaneously collect high quality MSMS spectra for later metabolite structural elucidation
- Annual meeting present findings from interlab comparisons
Conclusions

• Extraction protocols can impact metabolomic data sets considerably

• Solvent system composition and pH exhibit the most dramatic effects on metabolite recovery
  • The magnitude of these effects depend on metabolite class
  • Some classes of metabolites

• The number of extraction repetitions also plays a role in enhancing metabolite recovery
  • Tradeoff - longer sample prep time
  • Larger sample volumes to process (evaporate)

Conclusions

• Traditional RPLC methods can provide efficient separation of acyl-carnitine, bile acid and CoA thioester mixtures.
  • Advancements in hybrid particle technologies
  • Allowing for extremes in mobile phase pH and temperature – manipulate selectivity
  • Complex ligand stationary phase interactions

• HILIC methods are superior at separating highly polar metabolites.
  • Nucleotides and derivatives
  • Small polar metabolites – sugars, organic acids, amino acids, hydrophilic vitamins

• Advanced column chemistries (amide, aminopropyl, biphenyl, graphite, phenyl-hexyl) and alternative chromatographic methodologies (HILIC) can provide enhanced coverage of the metabolome.
Future Plans

• There’s no one “perfect” extraction or LC method available capable of efficiently resolving all components or features in the metabolome

• Therefore, our goal is to continue to develop optimized extraction and chromatography protocols for various classes of liver metabolites

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