Efficacy

Toxicity

Primary Molecules

Secondary Molecules

Filtration

Dilution

Concentration

Resorption

Adapted from D. Robertson, Pfizer Global Research and Development
Metabolomics

Comprehensive Definition:
The quantitative measurement of the time-related multiparametric metabolic response of living systems to pathophysiological exogenous or endogenous stimuli or genetic modification

Operational Definition:
The systematic exploration of biofluid composition using NMR/pattern recognition technology in order to associate target organ toxicity with NMR spectral patterns and identify novel surrogate markers of toxicity.

Adapted from D. Robertson, Pfizer Global Research and Development
**Metabolomics:**
The study of the total metabolite pool (metabolome), metabolic regulation and fluxes in individual cells or cell types. Can be achieved through a wide spectrum of technologic methods including LC-MS, GC-MS, and nuclear magnetic resonance (NMR)

**Metabonomics:**
The study of the systemic biochemical profiles and regulation of function in whole organisms by analyzing a metabolite pool (metabolome) in biofluids and tissues. Usually implies that the study is done specifically through nuclear magnetic resonance profiling
**Metabolome:**
The quantitative complement of all the low molecular weight molecules present in cells in a particular physiological or developmental state

**Biofluid:**
A fluid sample obtained from a living system. The donor might typically be a human or an animal. Fluids can be excreted (such as urine, sweat), expressed or secreted (such as milk, bile), obtained by intervention (such as blood plasma, serum or cerebrospinal fluid), develop as a result of a pathological process (such as blister or cyst fluid), or be applied and collected (such as dialysis fluid)
Advantages of Metabolomics

- Identification of target organ, severity, onset, duration and reversal of the effects (time-course)
- Classify sample as “normal” vs. “abnormal”
- Determine mechanisms of action within the organ
- Potential for identifying novel biomarkers of toxic effect
- Non-invasive
- No *a priori* decisions about samples need be made
- No sample processing necessary other than cold collection
- Complete time course data can readily be obtained
- Minimization of compound requirements
- Relatively fast analysis (200-300 samples/day)
- Useful tool for modeling physiological variation and exposure conditions in animals and humans

Adapted from D. Robertson, Pfizer Global Research and Development
NMR spectroscopy

Spectroscopy deals with the interactions between electromagnetic radiation and matter. Spectroscopy is used to derive the properties of matter at the molecular level. Nuclear magnetic resonance (NMR) exploits the magnetic properties of atomic nuclei.

The method functions as follows: A substance is placed in a magnetic field. Some atomic nuclei (e.g. protons, nuclei of hydrogen atoms) then behave like microscopic compass needles, called nuclear spins. Each nuclear spin orientation corresponds to a different energy level. The spins may jump between the levels when the sample is exposed to radio waves whose frequency exactly matches the energy spacing. This is called resonance. One way of measuring the energy is to change the irradiation frequency. At resonance, the spins flip causing an electric signal. The strength of the signal is plotted as a function of frequency in a diagram, the NMR spectrum.

In metabolomics, it is the patterns that occur when many different biochemical entities are detected simultaneously in a mixture using $^1$H NMR that are interpreted.

From: www.nobel.se
NMR in Metabolomics:

Pro:
• Non-destructive
• Applicable to intact biomaterials
• More information rich in complex-mixture analyses
• No extraction/derivatization is necessary

Con:
• Less sensitive than MS

History:
• NMR has been used to study metabolites in biofluids for over a decade
• Metabolomics technology as it is known today (600 MHz $^1$H NMR) was pioneered by Jeremy Nicholson, Elaine Holmes and John Lindon of Imperial College in London
• Only recently have advances in flow-through NMR hardware and pattern recognition software made the possibility of “high”-throughput in vivo toxicity assessment a practical possibility
NMR Acquisition and Gilson 215 Control System

Varian Inova 600 Shielded magnet 120 ul flow probe

Refrigerated Metabolism Cage (0°C)

Biomek® Robot

Deuterated Buffer TSP

Frozen Storage

+ NaN₃

Data Processing

Adapted from D. Robertson, Pfizer Global Research and Development
Normal Metabolic Profiles

Day 1
Day 2
Day 3
Day 4
Day 5

Adapted from D. Robertson, Pfizer Global Research and Development
“Biomarker Windows”

<table>
<thead>
<tr>
<th>Chemical Agent</th>
<th>Target Organ/Toxicity Type</th>
<th>Associated Biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adriamycin</td>
<td>Heart and kidney (glomerulus)</td>
<td>↑creatinine, taurine, ↓citrate, ↓αKG</td>
</tr>
<tr>
<td>Allyl alcohol</td>
<td>Liver (periportal)</td>
<td>↑creatinine, lactate, phenylacetylglucine, N-methyl nicotinamide, taurine, ↓citrate, αKG, ↓αKG, ↑phenylacetylglucine, DMG</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>Phospholipidosis (lung)</td>
<td>↑phenylacetylglucine, DMG</td>
</tr>
<tr>
<td>α-Naphthylisothiocyanate (ANIT)</td>
<td>Liver (cholestatics)</td>
<td>↑acetate, bile acids, glucose</td>
</tr>
<tr>
<td>2-Bromoethanamine</td>
<td>Kidney (papilla) and mitochondrial dysfunction</td>
<td>↑acetate, bile acids, glucose, ↓citrate, hippurate, αKG, succinate</td>
</tr>
<tr>
<td>Butylated hydroxytoluene</td>
<td>Liver</td>
<td>↑glucose, taurine</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>Testicular</td>
<td>↑glucose, creatine, ↓citrate, αKG, succinate</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>Liver</td>
<td>↑taurine, creatine</td>
</tr>
<tr>
<td>2-Chloroethanamine</td>
<td>Kidney (papilla) and mitochondrial dysfunction</td>
<td>↑taurine, creatine, ↓citrate, αKG, succinate, ↑αKG</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>Phospholipidosis and liver necrosis</td>
<td>↑taurine, creatine, hippurate, αKG, succinate, ↑αKG</td>
</tr>
<tr>
<td>S-(1,2-dichlorovinyl)-L-cysteine (DCVC) z4</td>
<td>Kidney (S2/3 proximal tubular)</td>
<td>↑citrate, succinate, ↑taurine, creatine, hippurate, αKG, succinate</td>
</tr>
<tr>
<td>S-(1,2-dichlorovinyl)-L-homocysteine (DCVHC)</td>
<td>Kidney (S2/3 proximal tubular)</td>
<td>↑taurine, creatine, hippurate, αKG, succinate, ↑αKG</td>
</tr>
<tr>
<td>Ethionine</td>
<td>Liver</td>
<td>↑glucose, taurine</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>Liver (hepatitis-like lesion)</td>
<td>↑acetate, betaine, bile acids, creatine, organic acids, taurine, urocanic acid</td>
</tr>
<tr>
<td>Haxachlorobutadiene</td>
<td>Kidney (S3 proximal tubular)</td>
<td>↑acetate, amino acids, glucose, organic acids</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>Liver (steatosis)</td>
<td>↑2-amino adipate, β-alanine, creatine, N-acetyl-citrulline</td>
</tr>
<tr>
<td>Lanthanum nitrate</td>
<td></td>
<td>↑creatinine, fumarate, hippurate, TMAO</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑acetocarnitine, alanine, aromatic amino acids, DMA, ethanol, glucose, hippurate, αKG, lactate, succinate, taurine, TMAO</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑allantoin, citrate, creatine, glucose, urea</td>
</tr>
</tbody>
</table>

*Toxicogenomics, Hamadeh & Afshari (eds.) Wiley-Liss, 2004*
A systems approach to the problem is important:

- Each step affects the other steps in the workflow.
- Separation is key to success.
- Targeted versus comprehensive analysis:
  - Discovery or
  - Validation – analysis cost issue
- Metabolite identification strategy.
Mass Spectrometry-based Metabolomic Analysis

Sample Analysis – GC/MS or LC/MS

GC/MS

Advantages

• High resolution separation
• High sensitivity
• Identification by EI searchable libraries if they exist
• No ionization suppression
• Most cost effective solution

Disadvantages

• Analyte must be volatile
  – Sample derivatization required
• Molecular ion often missing
Mass Spectrometry-based Metabolomic Analysis

Sample Analysis – LC/MS or GC/MS

LC/MS

Advantages
- Broader sample applicability
  - No sample derivatization
- High sensitivity
- Pseudo-molecular ion observed

Disadvantages
- Moderate resolution separation
- Ionization suppression
  - ESI or APCI
- No spectral libraries for identification
- More expensive solution
Mass Spectrometry-based Metabolomic Analysis

Data Reduction

Convert total ion chromatogram to individual components (metabolites)

- Remove background noise
- Remove unrelated ions
- Create reconstructed spectra
- Create a feature list

Metabolite tracking is by retention time and mass (spectrum)

Process multiple reduced data files

- Normalize the signal intensities and retention times
- Align features

How well this works is very dependant on the separation!!!!
Human plasma (100-500 Da range)
Data Processing Steps in Metabolomic Analysis

Acquisition software
- GC/MS ChemStation
- LC/MS MassHunter Workstation

Data reduction – feature finding
- AMDIS – GC/MS
- Molecular feature extractor - LC/MS

Metabolite identification
- NIST Library

Statistical Analysis - GeneSpring MS
- GC/MS
- LC/MS

Pathway Mapping
Data Analysis in Metabolomics

- NMR Spectra
- Primary Data Processing
- Unsupervised mapping of data in 3D space
- Supervised classification and calculation of confidence intervals

Pattern Recognition (PR) Methods:
PR and related multivariate statistical approaches can be used to discern significant patterns in complex data sets and are particularly appropriate in situations where there are more variables than samples in the data set. The general aim of PR is to classify objects (in this case $^1$H NMR spectra) or to predict the origin of objects based on identification of inherent patterns in a set of indirect measurements. PR methods can reduce the dimensionality of complex data sets via 2 or 3D mapping procedures, thereby facilitating the visualization of inherent patterns in the data.

Principal Components Analysis (PCA):
This is a data dimension reduction method that involves a mathematical procedure that transforms a number of (possibly) correlated variables into a (smaller) number of uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. Use of PCA enables the "best" representation, in terms of biochemical variation in the data set to be displayed in two or three dimensions.
Principal components analysis

(a) Stylized data matrix consisting of \( N \) observations (spectra, \( N=4 \)) and \( K \) variables (spectral regions, \( K=3 \)).
(b) Representation of the three variables placed in a 3D Cartesian coordinate system.
(c) All observations in the data matrix are placed in 3D space, and the computed principal components are shown as vector arrows.

Key: Obs, Observation; Var, Variable.

Adapted from D. Robertson, Pfizer Global Research and Development
**ALT (IU/L)**

- **Control**: ALT = 33-94 (Normal Reference Range)
- **24 hr ALT**: 1228 ±1061
- **48 hr ALT**: 994 ±884
- **96 hr ALT**: 161 ±108

Adapted from D. Robertson, Pfizer Global Research and Development
PCA analysis of vehicle effect on rat urine NMR spectra

Adapted from D. Robertson, Pfizer Global Research and Development
$\alpha$-naphthylisothiocyanite (ANIT)

$p$-Aminophenol (PAP)

Control

Adapted from D. Robertson, Pfizer Global Research and Development
ANIT 100 mg/kg

Adapted from D. Robertson, Pfizer Global Research and Development
Number in parentheses = mean serum total bilirubin (mg/dL)

Adapted from D. Robertson, Pfizer Global Research and Development
ANIT (50 mg/kg) in Mouse

Adapted from D. Robertson, Pfizer Global Research and Development
Metabolomic Detection of Liver Toxicity

Metabolomic Detection of Kidney Toxicity

Adapted from D. Robertson, Pfizer Global Research and Development
Limitations of Metabolomics

• Specialized equipment is required
• Extensive expertise is required
• Information is limited to time- and dose-points taken
• High risk of false positive data:
  a compound causes significant metabolism changes without associated toxicity
• Difficulty in separation of physiological (adaptive) and toxicological (adverse) effects
• Sensitivity of the assay
• Certain pathological states have negligible effects on biofluids:
  liver fibrosis may go undetected until damage is severe
• Availability of biofluids for certain organ toxicity: CNS vs. urine
• Distinguishing effects of multi-organ toxicants:
  biomarkers in different biofluids are different and in one biofluid are inter-mixed
Future Directions in Metabolomics

- Develop comprehensive metabonomic database
- Expand metabonomics applications to many species
- Evaluate cryoprobe technology for increased sensitivity or increased throughput
- Expand technology to novel targets:
  - Cardiac toxicity
  - Adrenal toxicity
- “Grand Unification” of Genomic/Proteomic and Metabonomics technologies
Metabolomics of drugs and drug metabolites

ANIT trajectory represents the aggregate biochemical effects of the metabolic flux (ANIT to ANIC to ANA). The first metabolic direction of the ANA-dosed animals is in the third principal component (PC3). For ANIC, the second direction is in PC3, as it takes time for ANA to be produced. For ANIT, the PC3 direction change takes longer, as ANA is formed after ANIC. This approach, termed ‘metabolic trajectory deconvolution’, allows effects of drugs and their metabolites to be separated for mechanistic purposes. Endogenous metabolic changes that are caused by the effects of successive drug metabolites must also mirror complex time-related changes in the transcriptomic and proteomic patterns, thus limiting the value of single time-point measurements of genes and proteins.