Chemical Assay of Drugs and Drug Metabolites

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NIMH

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Lecture Outline

• Quantification principles
  – Analytical PK lab tasks
• Chromatography
• Detection - spectroscopies
  – Optical
  – Mass
• Examples
  – Resveratrol
  – CYP450 Assays
  – Cyclosporin A
  – Metabolomics - APAP
• References
Definition of Analytical Terms

• Limits of detection (LOD)
  – Sensitivity is the minimum detectable concentration change that can be observed at a specified concentration
  – LOD is the minimum mass or concentration of analyte that can be detected at an acceptable signal to noise (S/N) ratio

• Lower limits of quantification (LLOQ)
  – Analyte mass or concentration required to give an acceptable level of confidence in the measured analyte quantity
  – Always greater (usually 3x) than the minimum LOD

• Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample
Accuracy vs. Precision

- Good accuracy
  Poor precision
- Poor accuracy
  Good precision
- Good accuracy
  Good precision
Pharmaceutical Industry PK Lab
Analytical Assays (1)

• Parent drug usually the target analyte for Phase 1 dose response and safety determinations
• Scale of runs: 30-50 samples/patient, plus 10-15 standards, procedural blanks, plus 10-15 QC pools or previously analyzed samples
• Several patients per run - effort to optimize patient/(standards + QC) ratio. Result is >100 samples/run
• Analytical runs require automation & rugged instrumentation, continuous operation for assay cycle time X number of samples
• Develop assays on 96 well or 384 well devices
Pharmaceutical Industry PK Lab
Analytical Assays (2)

• Speed of assay development principal determinant of methodology choice
  – Avoid derivatization chemistry
  – Use solid phase extraction or simple methanol/acetonitrile protein precipitation

• Time is money (3 min UHPLC/MS/MS assay vs. 40 min HPLC)

• First option: use automated UHPLC/MS/MS methods with high sensitivity and selectivity
Assay Issues

• **What to assay (what is important?)**
  – **Species** -
    • man, non-human primate, rat, mouse (transgenic)
  – **Tissue/Fluid**
    • liver, target organ, plasma, excreta
  – **Isolated organ/tissue fluids**
    • liver slices, human liver microsomes, CYPs, other enzymes
Assay Issues

• Commercial Aides
  – Drug metabolizing preparations
    • Human liver tissue or hepatocytes – all enzymes present in fresh (not frozen) tissue – single use only
    • Microsomes from frozen liver; easily stored
    • Recombinant CYPs and other enzymes - widely available (yeast, baculovirus, bacteria) and some mammalian cells with NADPH CYP reductase
    • CYP substrates, antibodies, inhibitors, inducers
  – Computer software - predict metabolites, pKa, pLogD, logP
  – Contract Research Organizations
Liquid Chromatography

• Most pharmaceuticals small molecules (<1000 Da) with some lipid solubility, adsorb to silica particles coated with stable organic hydrocarbon films

• A single analytical system can be used for many types of analyses, tailored to each by changing the solvents and gradients

• High Performance or High Pressure - HPLC
  – 3-5 µm surface coated, hard particles
    • 500-1000 psi pressures common
    • 1-5 mm diameter, 10-15 cm length columns
  – Reverse Phase - polarity separation
  – Cation & Anion Exchange - charge separation
    • Ultra High Pressure LC - higher performance
  – 1-2 µm particle size
    • 10,000-30,000 psi (thousands of atmospheres)
Liquid Chromatography – simple principles

stationary phase

column

mixture

solvent (eluent)
Liquid Chromatography – typical instrumentation

Pump A

Pump B

controller

column

detector

chromatogram

computer
Detection Principles (1)

- **Ultraviolet** or **Fluorescence Spectroscopy**
  - chromophore in drug or derivatized drug
  - most useful for known target analytes

- **Nuclear Magnetic Resonance Spectrometry**
  - most useful for totally unknown chemical structure characterization
  - least sensitive
Absorption Spectrophotometer

- Light source
- Lens
- Grating
- Monochromator
- Flow cell
- Sample detector
- Reference detector
- Absorption spectrum

Diagram of an absorption spectrophotometer showing the flow of light through the system.
Emission Spectrophotometer

Light Source

Monochromator

Sample

Monochromator

Recorder

Photo-detector
Detection Principles (2)

- **Mass Spectrometry**
  - versatile ionization modes for liquids and gases
    - electron, chemical, *electrospray*, desorption
  - versatile **mass analyzers** with varying capabilities
    - magnetic, ion trap, quadrupole, time-of-flight
    - combination analyzers in series
      - triple quadrupole
      - quadrupole-time-of-flight
      - linear trap-orbitrap, etc, etc
  - very sensitive and structurally informative
    - example: *air, acetaminophen*
  - added specificity through mass chromatography
    - tandem mass chromatography = multiple reaction monitoring
Mass Spectrometer Ionizers - Electron Ionization

-Filament
-Repeller
-Neutral molecule
-Trap
-Electron beam
-Odd electron positive ion
-

High vacuum chamber

Acceleration lens

Mass analyzer

kV
Mass Spectrometer Ionizers - Electrospray Ionization

HPLC
+kv capillary
electrosprayed ions
drying gas

vacuum interface
mass analyzer (high vacuum)
Mass Spectrometer Ionizers - Matrix Assisted Laser Desorption Ionization (MALDI)
Mass Analyzers: Time-of-Flight (TOF)

ion inlet
acceleration lenses
heavy ions
light ions
vacuum chamber
ion detector
Mass Analyzers: Quadrupole (q)
Mass Analyzers: Linear trap quadrupole (LTQ)

- Ion beam entrance
- Resonant trapped ion cloud
- Quad guide 1
- Quad guide 3
- Quadrupole 2 - trap and analyzer
- Detector
- Detector
Electrospray-Ion Trap Mass Spectrometer

Overview of Electrospray ionization using an Ion Trap Mass Spectrometer

DETECTION

Mass Spectrum

Thermo Finnigan
Mass Spectrum of Air

- $\text{H}_2\text{O}^+$ at m/z 18
- $\text{O}_2^+$ at m/z 32
- $\text{Ar}^+$ at m/z 40
- $\text{N}_2^+$ at m/z 28
- $\text{CO}_2^+$ at m/z 44
Mass spectrum of acetaminophen (Electron Ionization)
Multi-dimensional analyses
Pharmaceutical Industry PK Lab Analytical Assay
Work Load for New Chemical Entities

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<tr>
<td>HPLC</td>
<td>75%</td>
<td>50-60%</td>
<td>20%</td>
<td>2%</td>
</tr>
<tr>
<td>GC/MS</td>
<td>12%</td>
<td>3%</td>
<td>2%</td>
<td>0</td>
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<tr>
<td>LC/MS/MS</td>
<td>3%</td>
<td>40-50%</td>
<td>60-75%</td>
<td>98%</td>
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<tr>
<td>RIA</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>0</td>
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<tr>
<td>Preliminary lead profile time</td>
<td>18 m</td>
<td>4 m</td>
<td>0</td>
<td>0</td>
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</table>

Conclusion: requirement for speed (not instrumentation cost) dictates choice of analytical methods
Methods for Qualitative & Quantitative Assays in Clinical Pharmacology

• LC/MS/MS
  – High speed, reduced requirement for sample preparation

• HPLC/UV or Fluorescence
  – Very robust, routine assay technology

• Enzyme Linked Immunoassay (ELISA)
  – Many 96 well formatted colorimetric or radiometric commercial assay kits for specific compounds

• Florescence polarization immunoassay (FPIA)
  – Measures difference in florescence between bound and free antigen
  – Important in therapeutic drug monitoring – CsA

• Accelerator Mass Spectrometry - microdosing
Examples of Analytical Methods Applied in Drug Analyses

• 1. Vorapaxar – UHPLC/MS/MS
• Antiviral nucleoside - microdosing
• 2. CYP450 Assays - LC/MS/MS
• 3. Cyclosporin - FPIA, HPLC/UV, LC/MS/MS
  – Dried Blood Spot technology
• 4. Moxifloxacin – imaging mass spectrometry
UHPLC-MS/MS Assays of New Chemical Entities

vorapaxar

MS/MS spectrum of 493

Reproducible analyses at LLOQ

$^{13}$C$_6$- vorapaxar  
m/z 499 -> 463  

Black and blue traces are runs #1 and #279

vorapaxar  
m/z 493 -> 447
Example 1 - Where Do Drugs Go?

- Radiochemical tracers ($^{14}$C, $^3$H)
  - requires availability of labeled drug
    - useful for bioavailability, kinetics
    - detection of protein adducts/localization (autoradiography)
  - Specific atom or isotope detectors
    - Accelerator mass spectrometry (AMS) - detection of 14C at near natural background levels for drug pharmacokinetics
      - Ideal for human studies of toxic mechanisms - DNA

- Non-radiochemical methods
  - Unique drug elements (fluorine, etc.) or structural property (fluorescence)
Accelerator Mass Spectrometry - AMS

• Equipment is special – usually at national lab resource, or central shared industrial facility

• Samples converted to elemental carbon- first to CO₂, then by reduction to carbon in sealed tubes (10 µL plasma, 400 µg carbon required) ~1000 atoms $^{14}$C
  – LLOD: 30 aCi/mg Carbon; 3 amoles agent/sample with 5% precision

• Precise isotope ratio measured $^{12}$C:$^{13}$C:$^{14}$C for quantification


Compound A antiviral

$^{14}$C label
AMS measurement

Conventional dose
1.0 mg/kg P.O.

Microdose
0.02 mg/kg P.O.

Total $^{14}$C measured by AMS
Parent levels measured by LC/MS/MS

AMS - Microdosing

• Can microdose studies in humans provide an early measure of the pK properties of a NDE?
• Pharmacokinetics in the dog linear over a 50-fold dose range for compound A
• European Microdosing AMS Partnership Programme (EUMAPP) tested 7 drugs (2006-8)
  – Drugs selected were problematic for pK predictive models (eg in vitro and animal species)
  – Conclusion: ‘Intravenous microdose data predicted $t_{1/2}$ (half-life), CL (clearance) and V (volume of distribution) very well. Oral dose data did not scale as well as the IV dose but in general, the data obtained would have been useful in the selection of drug candidates for further development (or dropped from the development pipeline).

cyclosporine
tacrolimus
tissue slide → matrix application

laser ablation/ionization tandem mass spectrometry

array of MS1/MS2 spatial data

MS2 compound specific histological images
Example 2: LC/MS/MS CYP GLP Assays

- 12 Semi-automated assays for 10 human CYP450 enzymes described
- Microsomes pooled from 54 human livers
- Microsomes, NADPH, substrate in 96 well plate; stable isotope internal standards added with quenching solvent
- Recombinant CYP450 enzymes (Sf9 cells) from PanVera run in parallel; reference values published
- High speed LC/MS/MS conditions established for each analyte and internal standard (2 min/assay)
- Interassay precision of reaction velocity <10%

Validated Assays for Human Cytochrome P450 Activities, RL Walsky and RS Obach, *Drug Metab Disp* 32:647-660, 2004
CYP 450 Validated Assay
Bupropion and hydroxy metabolite

bupropion \xrightarrow{\text{CYP286}} \text{hydroxybupropion} + \text{\textsubscript{2}}^{\text{H}}\text{hydroxybupropion}

\[
\begin{align*}
m/z 256 & \rightarrow 139 \\
262 & \rightarrow 139
\end{align*}
\]

*multiple reaction monitoring*

From RL Walsky & RS Obach
Hydroxybupropion - ESI-MS

+ [D6]-hydroxybupropion

From RL Walsky & RS Obach
Hydroxybupropion - CID of MH$^+$ 256

[D6]-Hydroxybupropion - CID of MH$^+$ 262

From RL Walsky & RS Obach
Example: CYP2B6 Assay
Bupropion substrate

From RL Walsky & RS Obach
Example: CYP2B6 Results

BUPROPION HYDROXYLASE
HLM-13 0.05 mg/ml
Product Formed vs Time
## Partial Summary of CYP Activities

RL Walsky and RS Obach, Drug Metab Disp 32:647-660, 2004

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay</th>
<th>Inhibitor</th>
<th>1C50 (µM)</th>
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<tr>
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<td></td>
<td></td>
<td>Human</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Recomb</td>
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<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethylase</td>
<td>Furafylline</td>
<td>1.76±0.28</td>
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<td></td>
<td></td>
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<td>1.54±0.16</td>
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<tr>
<td>CYP2A6</td>
<td>Coumarin 7-hydroxylase</td>
<td>Tranylcypromine</td>
<td>0.449±0.073</td>
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<tr>
<td></td>
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<td>0.895±0.262</td>
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<tr>
<td>CYP2B6</td>
<td>Bupropion hydroxylase</td>
<td>PPP</td>
<td>7.74±0.47</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2.02±0.19</td>
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<tr>
<td>CYP2C8</td>
<td>Amodiaquine N-deethylase</td>
<td>Quercetin</td>
<td>3.06±0.31</td>
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<tr>
<td></td>
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<td></td>
<td>3.33±0.20</td>
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<tr>
<td>CYP2C9</td>
<td>Diclofenac 4’-hydroxylase</td>
<td>Sulfaphenazole</td>
<td>0.272±0.031</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>0.169±0.004</td>
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Example 3: Cyclosporin A (CsA)

Potent immunosuppressive drug for transplantation; irreversible kidney damage if dose too high

• HPLC - UV (210 nm) method first used for clinical analyses
  – LOQ - 20-45 µg/L (therapeutic range 80-300 µg/L)

• LC/MS/MS method for fingerprick samples
  – 25 µL; LOQ 10 µg/L
Cyclosporin Immuno Assays

- **Florescence polarization immunoassay (FPIA)**
  - Homogeneous immunoassay
  - Fluorescein tagged drug competes with patient drug for monoclonal Ab
  - Polarized light excites Ab-tagged drug complex most efficiently
  - LOQ 25 µg/L; analysis of 20 samples in 19 min
- **Enzyme monitored Immunoassay Technique (EMIT) and Cloned Enzyme Donor Immunoassay (CEDIA)**
  - Competitive: enzyme labeled antigen competes with sample antigen; enzyme labeled antigen-Ab complex changes rate
- **Multiple cyclosporin metabolites exhibit cross-reactivity in immunoassays**
Blood concentrations of cyclosporine (CSA)

Metabolomics

*Systematic and comprehensive study of small-molecule metabolite profiles*

- Preclinical drug development
- Monitoring clinical trials
- Biomarkers for efficacy and toxicity
Mouse Metabolomics

Metabolic cages

UPLC-TOFMS

F. Gonzales, NCI
1. Align based on m/z

2. Compare

Data matrix

<table>
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<tr>
<th>Sample_1</th>
<th>Ion_1 (RT₁, m/z₁)</th>
<th>……</th>
<th>Ion_n (RTₙ, m/zₙ)</th>
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</thead>
<tbody>
<tr>
<td>Sample_n</td>
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</table>

Relative abundance

F. Gonzales, NCI
Correlation

MD

A

Samples plot

Ions plot

Correlation

Metabolite Identification

Structural elucidation

F. Gonzales, NCI
LC-MS-based Metabolomics for Metabolite Identification

Sample collection

Vehicle

Xenobiotic

Xenobiotic

Isotope-labeled xenobiotic

Vehicle/Xenobiotic

Wild-type

Knockout

Transgenic

Classification

Identification

F. Gonzales, NCI
Acetaminophen (APAP)

Over-the-counter drug;
relieving pain,
reducing fever,
relieving the symptoms of
allergies, cold, cough, and flu.

Co-administration:
Sedative
Antihistamine
Vasoconstrictants
Expectorants
Antitussive
Analgesics

*Tylenol*
*(Top seller, controlling 35% of the pain killer market in North America)*

C₈H₉NO₂, MW 151.16

F. Gonzales, NCI
APAP Metabolomics

Xenobiotic

Isotope-labeled xenobiotic

→ LC-MS

→ MDA

→ Metabolite Identification

F. Gonzales, NCI
### APAP Metabolites

<table>
<thead>
<tr>
<th>Ion</th>
<th>Identity</th>
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<tbody>
<tr>
<td>I</td>
<td>APAP</td>
</tr>
<tr>
<td>II</td>
<td>Cys-APAP</td>
</tr>
<tr>
<td>III</td>
<td>NAC-APAP</td>
</tr>
<tr>
<td>IV</td>
<td>APAP glucuronide</td>
</tr>
<tr>
<td>V</td>
<td>APAP sulfate</td>
</tr>
<tr>
<td>VI</td>
<td>SAMP</td>
</tr>
<tr>
<td>VII</td>
<td>3-methoxy-APAP-G</td>
</tr>
<tr>
<td>VIII</td>
<td>3,3’-biacetaminophen</td>
</tr>
<tr>
<td>IX</td>
<td>benothiazine compound</td>
</tr>
</tbody>
</table>

*F. Gonzales, NCI*
Useful Reference Web Sites

• Prediction software – pK, structure
  – http://www.acdlabs.com/

• Human drug metabolizing enzymes:
  – Celsis (http://www.celsis.com)

• http://ull.chemistry.uakron.edu/classroom.html
  – Excellent introductory tutorials in analytical methods including chromatography and mass spectrometry

• http://ionsource.com/
  – Site with very useful links for mass spectrometry including tutorials, freeware

• http://ocw.mit.edu/courses/#chemistry
  – In-depth course materials for chemistry
Web Sites (2): Mass Spectrometry Information Education

• http://ull.chemistry.uakron.edu/classroom.html
  – Excellent introductory tutorials in analytical methods including chromatography and mass spectrometry

• http://svmsl.chem.cmu.edu/vmsl/default.htm
  – Virtual mass spectrometry laboratory with tutorial and excellent case study examples