Chemical Assay of Drugs and Drug Metabolites

Sanford P. Markey
Laboratory of Neurotoxicology
NIMH

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Lecture Outline

• Quantification principles (1-8)
  – Analytical PK lab tasks
• Chromatography (10-11)
• Detection - spectroscopies
  – Optical (12-14)
  – Mass (15-27)
• Examples
  – Vorapaxar – UHPLC/MS/MS (28-31)
  – CYP450 Assays – HPLC/MS/MS (32-38)
  – Cyclosporin A – FPIA, HPLC/UV (39-43)
    • Dried Blood Spot – UHPLC/MS/MS
  – Antiviral nucleoside – microdosing, AMS (44-47)
  – Moxifloxacin, Olanzapine – Imaging MS (48-50)
• References (51)
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
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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
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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
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
- sample detector
- flow cell
- reference detector
- absorption spectrum

Sample text: 13
Emission Spectrophotometer

Light Source → Mono-chromator → Sample → Monochromator

→ Photo-detector

Recorder
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

- High vacuum chamber
- Filament
- Electron beam
- Acceleration lens
- Mass analyzer
- Repeller
- Neutral molecule
- Trap
- Odd electron positive ion
- Mass spectrometer ionizers
- Electron ionization
- kV
- High vacuum chamber
- Electron beam
- Mass analyzer
- Electron ionization
- kV
Mass Spectrometer Ionizers - Electrospray Ionization

- HPLC
- +kV capillary
- vacuum interface
- mass analyzer (high vacuum)
- drying gas
- electrosprayed ions
Mass Spectrometer Ionizers - Matrix Assisted Laser Desorption Ionization (MALDI)
Mass Analyzers: Quadrupole (q)

- Ion source
- Detector
- Resonant ion
- Non-resonant ion
Mass Analyzers: Linear trap quadrupole (LTQ)
Electrospray-Ion Trap Mass Spectrometer

Overview of Electrospray ionization using an Ion Trap Mass Spectrometer

DETECTION
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
- $\text{CO}_2^+$ at m/z 44
- $\text{N}_2^+$ at m/z 28
Mass spectrum of acetaminophen (Electron Ionization)
MS1 mass chromatograms

Total ion chromatogram

Signal intensity

Time

Multi-dimensional analyses
Pharmaceutical Industry PK Lab Analytical Assay
Work Load for New Chemical Entities

<table>
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<tr>
<th></th>
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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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<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
Examples of Analytical Methods Applied in Drug Analyses

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

Vorapaxar (mol. wt. 492)

MS/MS spectrum of 493

Sample preparation for UHPLC-MS/MS

- Protein removal
  - dilute 100 µL plasma with 500 µL acetonitrile:acetone (95:5, v:v)
  - vortex; chill to precipitate proteins, centrifuge
  - transfer 200 µL to a fresh well
- Robotic system
  - scale procedures to automated 96-well plates
- 10 µL aliquot injected into UHPLC-MS/MS
- Validated over a dynamic range of 1.00–1000 ng/mL.
- Caution: copious quantities of potentially interfering phospholipids in crude plasma extract
  - Phospholipids eluted at end of each run
  - cycle time of 3 minutes/injection
  - programming the UHPLC mobile phase to elute vorapaxar at 1.5 min, then ramp to 98% acetonitrile:methanol:acetic acid (70:30:0.1, v:v:v) and holding for 1 minute to remove contaminants.
Consistency and reproducibility of UHPLC-MS/MS analyses at LLOQ

Blue/black traces are overlays of runs 270 injections apart

Method used for >13,000 samples to support clinical pK studies

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{H_2O-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

- Hydroxybupropion
  - 1.97 r.t.
  - 628 area

- [D6]Hydroxybupropion
  - 1.96 r.t.
  - 96538 area

From RL Walsky & RS Obach
Example: CYP2B6 Results

BUPROPION HYDROXYLASE
HLM-13 0.05 mg/ml
Product Formed vs Time

From RL Walsky & RS Obach
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay</th>
<th>Inhibitor</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Human</th>
<th>Recomb</th>
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<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethylase</td>
<td>Furafylline</td>
<td>1.76±0.28</td>
<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>
<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>
<td>2.02±0.19</td>
<td></td>
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<tr>
<td>CYP2C8</td>
<td>Amodiaquine N-deethylase</td>
<td>Quercetin</td>
<td>3.06±0.31</td>
<td>3.33±0.20</td>
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</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac 4’-dydroxylase</td>
<td>Sulfaphen-azole</td>
<td>0.272±0.031</td>
<td>0.169±0.004</td>
<td></td>
</tr>
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</table>
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
- Dried Blood Spot – UHPLC-MS/MS
  - LOD, LOQ 8.5 µg/L for CsA, and 0.5 and 2.3 µg/L tacrolimus
  - Hinchliffe, J Chrom B, in press, 2011
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)

Dried Blood Spot vs Whole Blood Assay for CsA assay by UHPLC-MS/MS

Dried Blood Spots for pK and other measurements

- For CsA, 6 mm discs punched using a stationery paper hole punch, placed into 96-well plates, and ultrasonically extracted using hot methanol in the presence of added internal standards
- Photodegradation of light sensitive compounds less than solution storage - Bowen, Bioanalysis 2:1823-8 (2010)
- Multiple commercial collection devices (paper, coated surfaces) marketed
Example 4 - 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 $^{14}$C, $^3$H 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 CO$_2$, 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

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 New Chemical Entity?
- 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 t1/2 (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).

Example 5: Imaging Mass Spectrometry Workflow

tissue slide

matrix application

laser ablation/ionization tandem mass spectrometry

array of MS1/MS2 spatial data

MS2 compound specific histological images
Imaging Mass Spectrometry – Drug Metabolism

Olanzapine (8 mg/kg, oral) Distribution after 2 hr from MS/MS images

Imaging Mass Spectrometry – kinetics of moxifloxacin distribution in lung

Fluoroquinone anti-TB agent

Useful Reference Web Sites

• 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