# Chemical Assay of Drugs and Drug Metabolites

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# Lecture Outline

- Quantification principles
  - Analytical PK lab tasks
- Chromatography
- Detection - spectroscopies
  - Optical
  - Mass
- Examples
  - Resveratrol
  - Aminoflavone
  - CYP450 Assays
  - Cyclosporin A
- 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
- Limits of quantification (LOQ)
  - 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

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# Accuracy vs. Precision

Picture of “errors and target” diagrams comparing the following:

good accuracy and poor precision,
poor accuracy and good precision and
good accuracy and 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 (5 min LC/MS/MS assay vs. 40 min HPLC)
- Use automated LC/MS/MS methods with high sensitivity and specificity

# 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

- High Performance (HPLC)
  - Reverse Phase - polarity separation
  - Immunoaffinity
  - Cation & Anion Exchange - charge separation
  - Smaller particle size, higher pressures - higher performance

(down arrow)

# Liquid Chromatography

Picture showing various stages of liquid chromatography.

# HPLC Apparatus

Drawing of HPLC apparatus

# 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

# UV Absorption Spectrophotometer

Drawing showing flow from light source to recorder

# Emission Spectrophotometer

Drawing showing light source to photo-detector

(up arrow)

# 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
      - 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 (down arrow) monitoring

# Mass Spectrometer Component Overview

Drawing of mass spectrometer component overview

# Mass Spectrometer Ionizers

Drawing of Electron Ionization (in vacuo)

Drawing of Electospray Ionization (external)

# Mass Analyzers

Drawing of Time-of-flight (TOF)

Drawing of Quadrupole (q)

# Quadrupole Ion Trap

Drawing of Quadrupole Ion Trap

# Electrospray-Ion Trap Mass Spectrometer

Drawing of overview of Electrospray Ionization using an Ion Trap Mass Spectrometer

# Mass Spectrum of Air

Chart showing mass spectrum of air

# Mass spectrum of acetaminophen (Electron Ionization)

Illustration showing electron ionization spectrum for acetaminophen.

# Mass Chromatography

Illustration of mass chromatography

# Multidimensional Analyses

Illustration of multidimentional analyses

Pharmaceutical Industry PK Lab Analytical Assay
Work Load for New Chemical Entities

| **Method** | **1990** | **1998** | **2000** | **2008** |
|---|---|---|---|---|
| HPLC | 75% | 50-60% | 20% | 2% |
| GC/MS | 12% | 3% | 2% | 0 |
| LC/MS/MS | 3% | 40-50% | 60-75% | 98% |
| RIA | 10% | 10% | 10% | 0 |
| Preliminary lead profile time | 18 m | 4 m | 0 | 0 |

CONCLUSION: REQUIREMENT FOR SPEED (NOT INSTRUMENTION COST) DICTATES CHOICE OF ANALYTICAL METHODS

# Popular 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

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# Examples of Analytical Methods Applied in Drug Analyses

- 1. Resveratrol - bioavailability
- 2. CYP450 Assays - LC/MS/MS
- 3. Cyclosporin - FPIA, HPLC/UV, LC/MS/MS

# Example 1 -Where Do Drugs Go?

- Radiochemical tracers ($^{14}C$, $^{3}H$)
  - requires availability of labeled drug
  - useful for bioavailability, kinetics - **Resveratrol**
  - detection of protein adducts/localization (autoradiography)
- Non-radiochemical methods
  - Unique drug elements (fluorine, etc.) or structural property (fluorescence)
  - 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
      - Calcium metabolism

# Resveratrol

Washington Post, November 2, 2006
**A Compound in Red Wine Makes Fat Mice Healthy**
*By* Rob Stein

A substance found in red wine protected mice from the ill effects of obesity and extended their life spans, raising the tantalizing prospect that the compound could do the same for humans and may also help people live longer, healthier lives, researchers reported yesterday…

We've been looking for something like this for the last 100,000 years, and maybe it's right around the corner -- a molecule that could be taken in a single pill to delay the diseases of aging and keep you healthier as you grow old," said David A. Sinclair, a Harvard Medical School molecular biologist who led the study.

# Resveratrol

JA Baur, et al…DA Sinclair
*Nature* **444**, 337-342 (16 November 2006)

Resveratrol improves health and survival of mice on a high-calorie diet

Graph illustrating this.

# Resveratrol Delays Age-Related Deterioration and Mimics Transcriptional Aspects of Dietary Restriction without Extending Life Span

Illustration showing proportion of mice surviving at different ages (weeks)

*Pearson KJ, et al. Cell Metabolism 8, 6 August 2008, 157-168*

# Resveratrol

Chemical structure showing Sulfation, Glucuronidation sites.

- Widely sold at health food stores as antioxidant
- Proposed chemopreventive for cardiac diseases, cancer based on *in vitro* evidence
- Absorption?
- Bioavailability?
- Metabolism?

*HIGH ABSORPTION BUT VERY LOW BIOAVAILABILITY OF ORAL RESVERATROL IN HUMANS, T.Walle et al., Drug Metab Disp 32:1377-1382 (2004)*

# Resveratrol plasma concentration-time curves (total radioactivity)

Graph illustrating this activity.

*T.Walle et al., Drug Metab Disp 32:1377-1382 (2004)*

# Resveratrol Urine Metabolites (0-12 hr)

Scatter charts illustrating Resveratrol urine metabolites 0-12 hours.

*T.Walle et al., Drug Metab Disp 32:1377-1382 (2004)*

## Resveratrol
## Recovery of Radioactivity

| | 25 mg Oral | | 0.2 mg i.v. | |
|---|---|---|---|---|
| | Urine | Feces | Urine | Feces |
| N=6 | 70.5 ± 4.3 | 12.7 ± 6.1 | 64.1 ± 7.7 | 10.4 ± 3.7 |

*T.Walle et al., Drug Metab Disp 32:1377-1382 (2004)*

# HPLC Radiochromatogram
# 0-12 hr urine extract

*Illustration that shows that aryl sulfatase Glucuronidase shifts M1-M3 to Reservatrol r.t.*

*T.Walle et al., Drug Metab Disp 32:1377-1382 (2004)*

# Resveratrol Study
# Conclusions

- Unmetabolized resveratrol not detectable in plasma
- Absorption of resveratrol is at least 70%
- No evidence for further oxidation - only conjugation ± reduction
- Bioavailability of resveratrol limited
  - Highly accumulated in intestinal epithelial cells
  - Target sites of breast and prostate unlikely unless RV-$SO_4$ is active species or reservoir of parent

- Small molecule activators of SIRT1 sought as alternative therapeutics
  - Milne JC et al. Nature 450, 712-716, 2007

# 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

Chemical structure showing multiple reaction monitoring by mass spectroscopy.

From RL Walsky & RS Obach

Hydroxybupropion - ESI-MS
+ [D6]-hydroxybupropion

Chemical structure and mass spectra illustrating this.

From RL Walsky & RS Obach

# Example: CYP2B6 Assay
# Bupropion substrate

Illustration and mass spectra showing this.

From RL Walsky & RS Obach

# Example: CYP2B6 Results

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

Chart of mass spectra illustrating this.

From RL Walsky & RS Obach

Partial Summary of CYP Activities
RL Walsky and RS Obach, Drug Metab Disp 32:647-660, 2004

| Enzyme | Assay | Inhibitor | IC50 (μM) Human | Recomb |
|---|---|---|---|---|
| CYP1A2 | Phenacetin *O*-deethylase | Furafylline | 1.76±0.28 | 1.54±0.16 |
| CYP2A6 | Coumarin 7-hydroxylase | Tranylcyp-romine | 0.449±.073 | 0.895±.262 |
| CYP2B6 | Buproprion hydroxylase | PPP | 7.74±0.47 | 2.02±0.19 |
| CYP2C8 | Amodiaquine *N*-deethylase | Quercetin | 3.06±0.31 | 3.33±0.20 |
| CYP2C9 | Diclofenac 4'-dydroxylase | Sulfaphen-azole | 0.272±.031 | 0.169±.004 |

# Example 3: Cyclosporin A (CsA)

Chemical structure showing potent immunosuppresive 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
  - Keevil BG, Ther Drug Monitor 24: 757-67 (2002)

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# 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

Monoclonal CEDIA Polycolonal FPIA

2 charts 2 charts

Blood concentrations of cyclosporine (CSA)
*Mai I, et al. Clinical Pharmacology & Therapeutics (2004) 76, 330–340*

# WWW Sites (1) HPLC & Drug Metabolism

- Tutorial for HPLC
  - http://kerouac.pharm.uky.edu/asrg/hplc/HPLCMYTRY.HTML
- Prediction Software – pK, structure
  - http://www.acdlabs.com/
- Human Drug Metabolizing Enzymes:
  - Celsis (http://www.celsis.com/index.php)

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