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
  - 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

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

Accuracy vs. Precision

Graphic illustration of three targets with arrows in them. In the first target, most arrows missed the center (Example of good accuracy, poor precision). In the second target all arrows missed the center and landed in approximately the same area of the outer ring (Example of poor accuracy, good precision). In the third target all arrows hit the middle (Example of 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 (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

Cation & Anion Exchange - charge separation

Smaller particle size, higher pressures - higher performance (UPLC)

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Liquid Chromatography

Graphic illustration of chromatography columns, stationary phase, and solvent (eluent).

High Performance Liquid Chromatography (HPLC)

Graphic illustration 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

Graphic illustration of the flow of the light source into the monochromator, then into a solvent/sample, then into a photo-detector, and lastly into a recorder.

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Emission Spectrophotometer

Graphic illustration of the flow of a light source into a monochromator, then into a sample and from there into a mono-chromator. From that point it the light source goes into a photo-detector and from there into a recorder.

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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

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Mass Spectrometer Component Overview

Graphic illustration showing ionizer, mass analyzer, ion detector, high vacuum chamber, and computer.

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Mass Spectrometer Ionizers

Graphic illustration of electron ionization (in vacuo).

Graphic illustration of electrospray ionization (external)

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Mass Analyzers

Graphic illustration of time-of-flight (TOF)

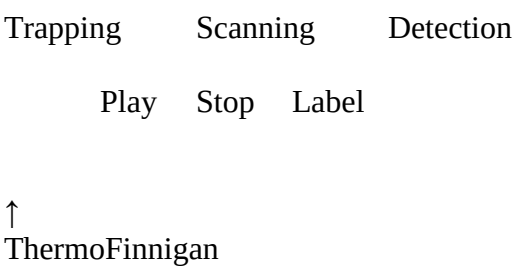
Graphic illustration of Quadrupole (q)

Quadrupole Ion Trap

Graphic illustration showing Ions going in the quadrupole ion trap at the top, then through the end cap into the ring electrode where ions are in stable trajectories until they reach the bottom where there is buffer gas and unstable m/z trajectory. Ions then come out the bottom of the quadrupole ion trap into an ion detector.

Electrospray-Ion Trap Mass Spectrometer

Graphic illustration of an overview of electrospray ionization using an ion trap mass spectrometer



Mass Spectrum of Air

Graphic illustration of the relative peak intensity of mass spectrum of air over m/z

Mass spectrum of acetaminophen
(Electron Ionization)

M.W. 151

Relative intensity from 0 to 100 over m/z

Up arrow

Mass Chromatography

Graphic illustration of response over time (msec). Mass chromatogram of m/z 250 is the highest point in the illustration. Mass chromatogram of m/z 190 is a lower point. Mass spectrum is at an even lower point.

Multidimensional Analyses

Graphic illustration of response over time including multiple reaction monitoring for both mass chromatogram and chromatogram.

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

Chart showing the percent of the method (HPLC, GC/MS, LC/MS/MS, RIA, and Preliminary lead profile time) used from 1990 to 2009.

HPLC use went from 75% to 2%

GC/MS use went from 12% to 0%

LC/MS/MS use went from 3% to 98%

RIA use went from 10% to 0%

Preliminary lead profile time went from 18 m to 0.

Conclusion: requirement for speed (not instrumentation 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

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
4. Acetaminophen – metabolomics, 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

22.4 ± 0.4 mg/kg^{-1}/day^{-1} in food

Resveratrol is a polyphenolic SIRT1 activator
ameliorates insulin resistance
increases mitochondrial content
Lagouge, M. et al. Cell 127, 1109–1122 (2006)

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

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

Line chart showing D proportion surviving from 0.0 to 1.0 over age (weeks) from 50 weeks to 160 weeks. Over time life span decreases for all (SD control, HC control, HCLR and HCR) with HC control apparently having the shortest life span at approximately 138 weeks.

Resveratrol

Chemical structure of resveratrol 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)

Line chart showing Plasma resveratrol equivalents (ng/ml) from 0 to 1000 over Time (h after dose) from 0 to 72. Oral 25 mg shows much lower concentrations-time than i.v. 0.2 mg.

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

Resveratrol
Recovery of Radioactivity

Chart indicating greater recovery in urine.

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

HPLC Radiochromatogram
0-12 hr urine extract

Scatter chart showing Urine radioactivity (dpm) from 0 to 500 over Retention time (min) for M1-M3, M4-M5 and Resveratrol with Resveratrol having the highest retention time.

Glucuronidase shifts M1-M3 to Reservatrol r.t

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

Resveratrol Study
Conclusions

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

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

Small molecule activators of SIRT1 replicate signaling pathways triggered by calorie restriction in vivo.

Smith JJ, et al. BMC Syst Biol. 2009 Mar 10;3:31.

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 structures of bupropion, hydroxybupropion (m/z 256 →139), and $_2H^6$-hydroxybupropion (262→139)

Multiple reaction monitoring

From RL Walsky & RS Obach

Hydroxybupropion - ESI-MS

Chemical structures and mass spectrum

From RL Walsky & RS Obach

Hydroxybupropion - CID of MH^+ 256

[D6]-Hydroxybupropion - CID of MH+ 262

Chart showing Int over m/z (mass spectrum)

Chemical structure

From RL Walsky & RS Obach

Example: CYP2B6 Assay
Bupropion substrate

Two chromatograms showing hydroxybupropion and stable isotope.

From RL Walsky & RS Obach

Example: CYP2B6 Results

BUPROPION HYDROXYLASE
HLM-13 0.05 MG/ML
PRODUCT FORMED VS TIME

Chart showing metabolite formation from 0.00 to 0.12 over time (min) from 0 to 35. There is a linear increase starting at time 4 min.

From RL Walsky & RS Obach

Partial Summary of CYP Activities

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

Chart of CYP isozymes, substrates and inhibitors with IC_{50} values.

Example 3: Cyclosporin A (CsA)

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

Chemical structure

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)

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

There are 4 line charts, 2 for monoclonal CEDIA and 2 for Polycolonal FPIA.

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

Metabolomics

Systematic and comprehensive study of small-molecule metabolite profiles

Preclinical drug development

Monitoring clinical trials

Biomarkers for efficacy and toxicity”

Mouse Metabolomics

Photographs of metabolic cages → test tubes → UPLC-TOFMS

F. Gonzales, NCI

LC-MS-based Metabolomics

High-resolution LC-MS

UPLC (intensity over time) and TOFMS (intensity → time over m/z) TOFMS

1. Align based on m/z

2. Compare
Relative abundance

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Data matrix

↓

F. Gonzales, NCI

Graphic illustration

F. Gonzales, NCI

LC-MS-based Metabolomics for Metabolite Identification

Sample collection→ Classification →Identification
Use of wild-type, knockout, and transgenic mice.

F. Gonzales, NCI

Acetaminophen (APAP)

Chemical structure
C8H9N02, MW 151.16

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

Photograph of two bottles of Tylenol

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

APAP Metabolomics

Graphic illustration

F. Gonzales, NCI

APAP Metabolites

Chemical structures of acetaminophen and 8 metabolites.

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 tutorial 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/course/#chemistry

in-depth course materials for chemistry