Chemical Assay of Drugs and Drug Metabolites

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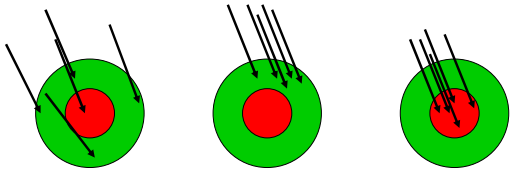
Lecture Outline

- 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

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

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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

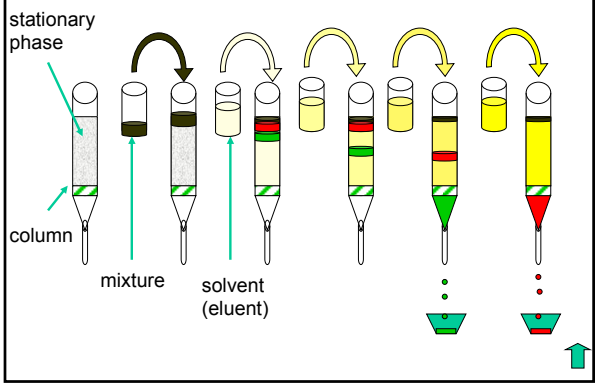
Assay Issues

- 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

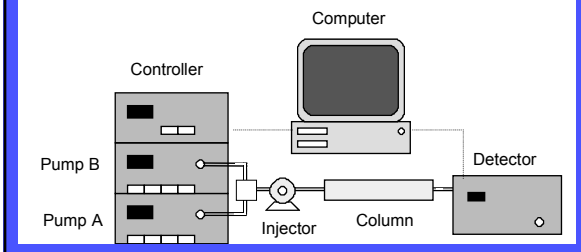
Liquid Chromatography

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

Liquid Chromatography



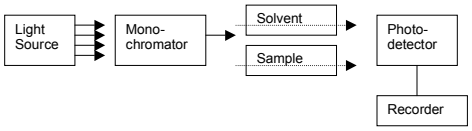
HPLC Apparatus



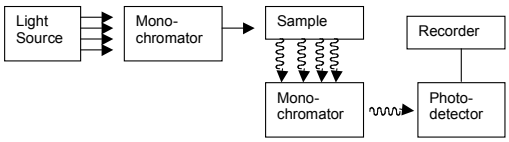
Detection Principles (1)

- 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



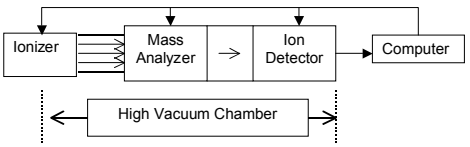
Emission Spectrophotometer



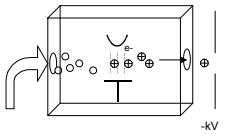
Detection Principles (2)

- 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 monitoring

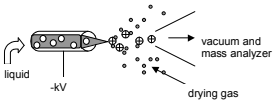
Mass Spectrometer Component Overview



Mass Spectrometer Ionizers

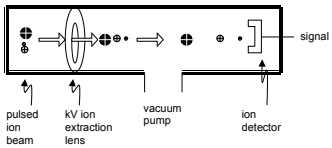


Electron Ionization (in vacuo)

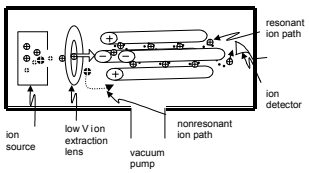


Electrospray Ionization (external)

Mass Analyzers



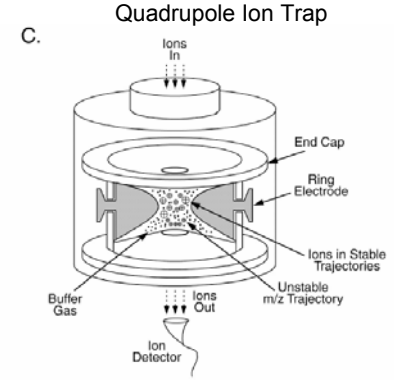
Time-of-flight (TOF)



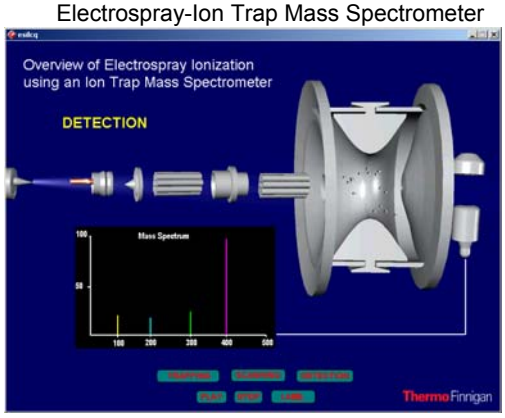
Quadrupole (q)

Quadrupole Ion Trap

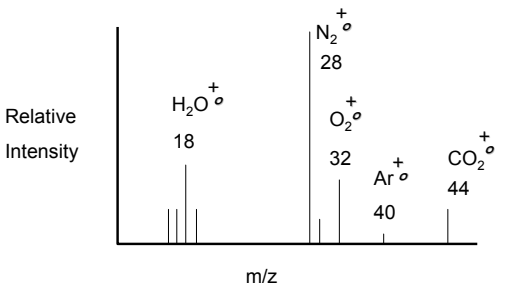
C.



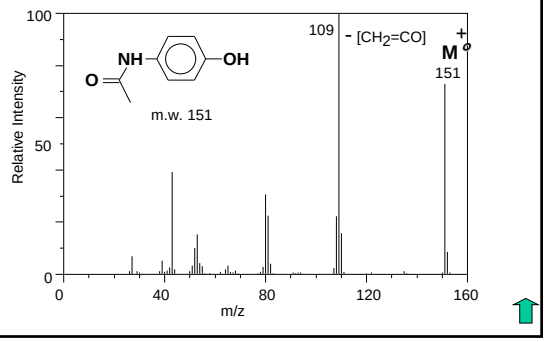
Electrospray-Ion Trap Mass Spectrometer



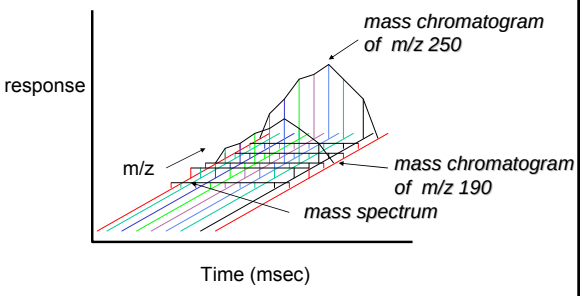
Mass Spectrum of Air



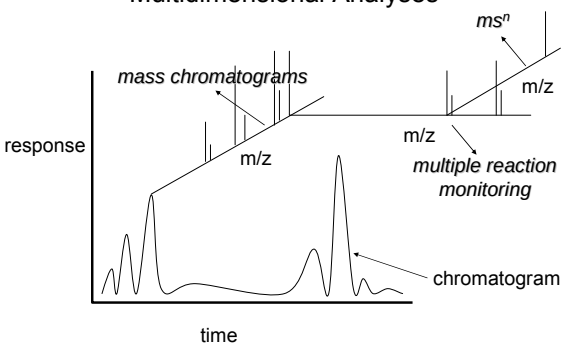
Mass spectrum of acetaminophen (Electron Ionization)



Mass Chromatography



Multidimensional 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 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

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.

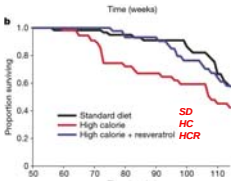
Resveratrol

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

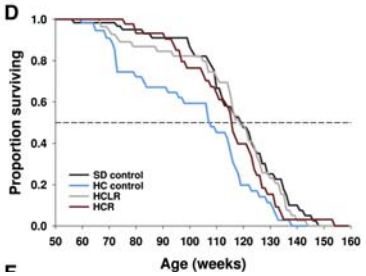


- Resveratrol is a polyphenolic SIRT1 activator
  - ameliorates insulin resistance
  - increases mitochondrial content

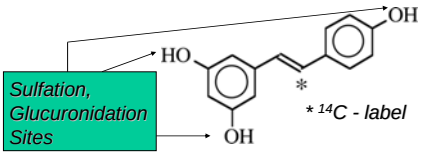
Lagouge, M. et al. Cell 127, 1109–1122 (2006)

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



Resveratrol



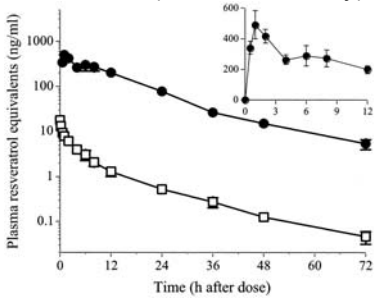
Sulfation, Glucuronidation Sites

** ^{14}C - label*

- 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)

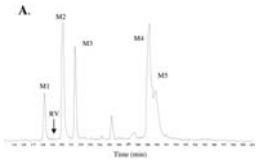


Oral 25 mg ● i.v. 0.2 mg □

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

Resveratrol Urine Metabolites (0-12 hr)

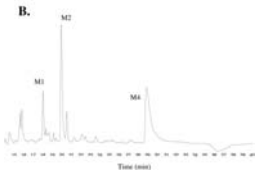
LC/MS
(selected ions)
M1,2=RV-Glu
M3= RV-H_2-Glu
M4= RV-SO4
M5 = RV-H_2-SO4



LC/UV

305 nm

(note absence of M3,M5)



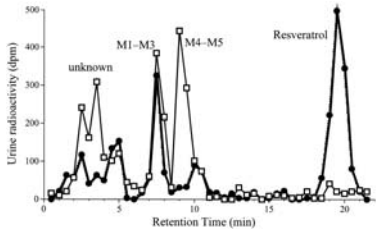
T.Walle et al., Drug Metab Disp 32:1377-1382 (2004)

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



□ *Urine* ● *After aryl sulfatase*

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

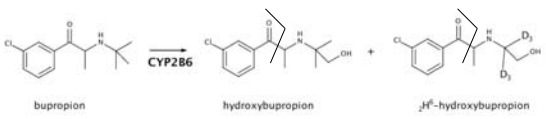
Example 2: LC/MS/MS CYP GLP Assays

- 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

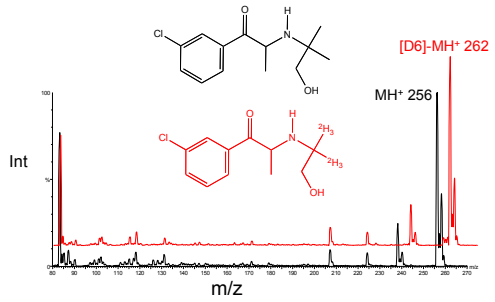


m/z 256 → 139 *262 → 139*

multiple reaction monitoring

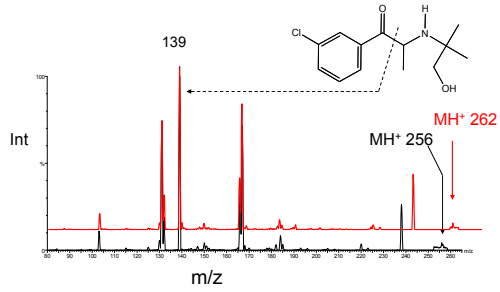
From RL Walsky & RS Obach

Hydroxybupropion - ESI-MS
+ [D6]-hydroxybupropion



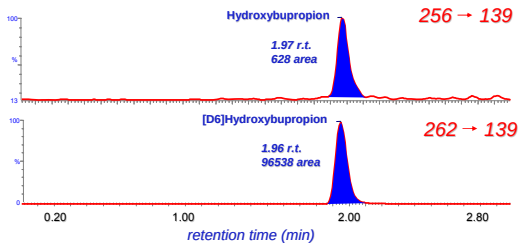
From RL Walsky & RS Obach

Hydroxybupropion - CID of MH+ 256
[D6]-Hydroxybupropion - CID of MH+ 262



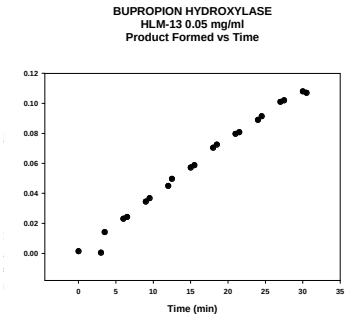
From RL Walsky & RS Obach

Example: CYP2B6 Assay
Bupropion substrate



From RL Walsky & RS Obach

Example: CYP2B6 Results



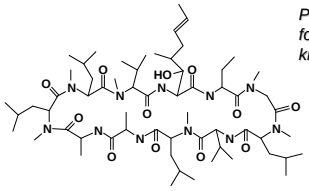
From RL Walsky & RS Obach

Partial Summary of CYP Activities

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

Enzyme	Assay	Inhibitor	IC_{50} (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)



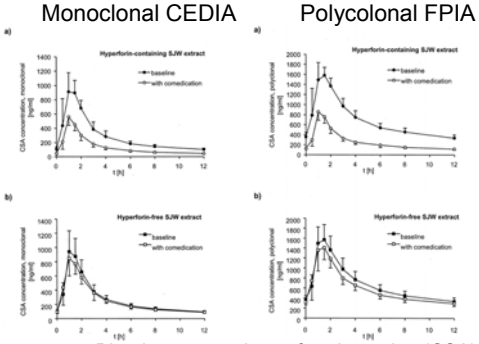
Potent immunosuppresive drug for transplantation; irreversible kidney damage if dose too high

- 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)

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



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