# Metabolite Identification and Characterization

Chandra Prakash, Ph. D.
Biogenidec, Cambridge, MA

## Outlines

- Background
- Metabolism Reactions
- LC-MS strategies for metabolite identification
  - Triple Stage Quadrupole (TSQ) LC/MS/MS
  - 3 dimensional and linear ion traps
  - various hybrids: Q-TOF, Triple TOF, trap-orbitrap
- Analytical techniques combined with mass spectrometry for characterization of metabolites
  - Derivatization
  - H/D exchange
  - LC-NMR
- Future Trend

## Why Identify Metabolites?

- Most of the drugs are eliminated from the body by metabolism: Detoxification process-This is good.
- The metabolites modulate the efficacy of drugs in the treatment of disease.
- The metabolites may possess pharmacological activity.
- The metabolites may be toxic: Bioactivation- bad.
- The metabolites can be involved in drug-drug interactions
- For proper safety assessment of a drug for human use, it must be shown that the animal species used for safety evaluation are exposed to the same metabolites as humans

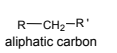
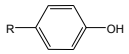
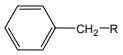
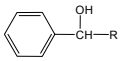
- Identify metabolic liabilities
  - synthesize compounds that are more metabolically stable
- Pharmaceutical industries are mandated by regulatory agencies to identify metabolites of NCE.

## Metabolism Reactions

## Xenobiotic Metabolism

- Phase I (Activation/Detoxification)
  - Polar reactive groups introduced
  - products most often more polar and less lipophilic
  - more water soluble
- Phase II (Detoxification)
  - Covalent "conjugation" to endogenous substances
  - reactions most often abolish biological activity and add to polarity
  - very water soluble

## Oxidation of C-H Centers by CYP

| Functional Group | Product | Shift in m/z |
|---|---|---|
|  aliphatic carbon |  alcohol | +16 |
|  aromatic carbon |  aromatic hydroxylation | +16 |
|  benzylic carbon |  benzylic hydroxylation | +16 |
|  alkene |  epoxide | +16 |

## Oxidation of Heteroatoms (dealkylation) &Reductions

| Functional Group | Product | Shift in m/z |
|---|---|---|
| R—X—$CH_2$—R<br>X = N, O, S, halogen | R—X H + HC(=O)—R'<br>dealkylated product | -R+H |
| R—X—R'<br>X = NR", S | R—X(→O)—R'<br>N or S oxide | +16 |
| R—$NO_2$<br>nitro group | R—NH O H<br>hydroxylamine | -14 |
| R—NH O H<br>hydroxylamine | R—$NH_2$<br>amine | -14 |
| $R_3N$→O<br>N-oxide | $R_3N$<br>amine | -16 |

## Phase II Metabolism: Glucuronidation

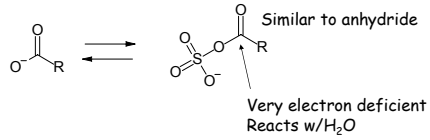
The sites of glucuronidation are electron-rich nucleophilic heteroatoms

The functional groups are:
Aliphatic alcohols R-OH
Phenols Ar-OH
Carboxylic acids R-$COO^-$
Aromatic Amines Ar-$NH_2$ or Ar-NH-R
Free sulfhydryl groups R-SH
Sometimes tertiary amines $R_3$-N

C-glucuronidation is also known - the carbon is sufficiently nucleophilic

## Phase II Metabolism: Sulfonation

The functional groups for sulfonation are:
Aliphatic alcohols R-OH
Phenols Ar-OH
Aromatic Amines Ar-$NH_2$ and
N-hydroxy compounds R-NH-OH
Why not carboxylic acids COOH




Compared to glucuronidation, sulfation is less common
PAPS cellular concentration is considerably lower (75 µM) than UDPGA (350 µM). Hence the capacity of sulfation is low

## Glutathione Conjugation

- GSTs catalyze reaction of reduced glutathione with an electrophile
  - GSH is reactive on its own; the enzyme holds the substrate in place for an increased reaction rate
- Examples of electrophiles that are substrates for GSTs
  - Arene oxides
  - Epoxides
  - α,β-Unsaturated Compounds
  - Alkyl halides
  - Nitroaromatics
  - Quinones, quinoneimines, and quinonemethides

## Tools for Metabolite Identification

## Tools

- LC-MS/MS is extensively and routinely used for metabolite identification
  - Sensitive, selective and quick
- Structural confirmation frequently requires additional tools such as
  - NMR
  - synthesis of authentic standards
  - the lost art of chemical derivatization

## MASS SPECTRUM

- Mass Spectrometers Do Not Measure Mass. It is plot of the mass-to-charge ratios (m/z) vs. the % relative intensities of the ions, where base peak is the most abundant ion in the spectrum
- If single charge, z=1 and m/z = m
- Three types of ions in a mass spectrum;
  - Intact molecule± one or more charges⇒Molecular mass
  - Fragment ions⇒Structure information
  - Background ions⇒from non-analyte species

## Natural Isotopic Abundance of Common Elements

| Element | Isotope Mass | % |
|---|---|---|
| Carbon | $^{12}C$ | 98.9 |
| | $^{13}C$ | 1.1 |
| Hydrogen | $^{1}H$ | 99.98 |
| | $^{2}H$ | 0.02 |
| Oxygen | $^{16}O$ | 99.8 |
| | $^{18}O$ | 0.2 |
| Nitrogen | $^{14}N$ | 99.6 |
| | $^{15}N$ | 0.4 |
| Chlorine | $^{35}Cl$ | 75.8 |
| | $^{37}Cl$ | 24.2 |
| Sulfur | $^{32}S$ | 95.3 |
| | $^{33}S$ | 0.76 |
| | $^{34}S$ | 4.20 |

## Mass

| Element | Nominal Mass | Average Mass | Exact Mass |
|---|---|---|---|
| C | 12 | 12.011 | 12.0000 |
| H | 1 | 1.00797 | 1.0078 |
| O | 16 | 15.9994 | 15.9949 |
| N | 14 | 14.003 | 14.0031 |
| Cl | 35 | 35.45 | 34.9689 |
| S | 32 | 32.06 | 31.972 |

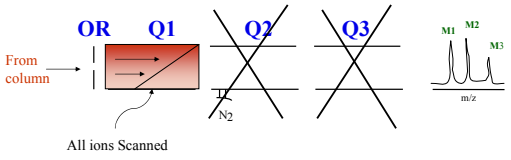
## Average vs. Exact Mass

- Average mass results from occurrence of isotopes.
  - This is what we weigh
- Exact mass results from non-integer masses of sub-atomic particles.
  - This is what the Mass Spec sees
  - Deviation of exact from nominal is the "Mass Defect"

## Examples (C,H,O,N compounds)

| Compound | Integer | Avg. Mass | Exact Mass |
|---|---|---|---|
| Caffeine $C_8H_{10}N_4O_2$ | 194 | 194.1785 | 194.0802 |
| Xanomeline $C_{14}H_{23}N_3OS$ | 281 | 281.4057 | 281.1556 |
| Ziprasidone $C_{21}H_{21}N_4OSCl$ | 412 | 412.9197 | 412.1120 |

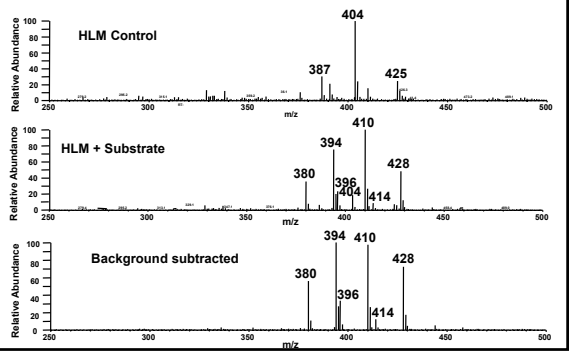
## Q1 or Full Scan



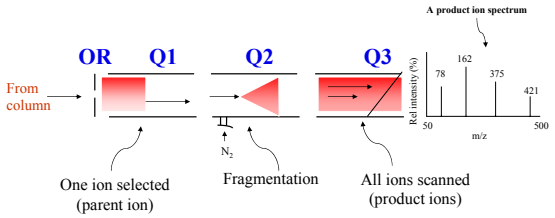

Only Q1 operational (LC/MS mode)

*Similar to an LC/MS total ion chromatogram.*

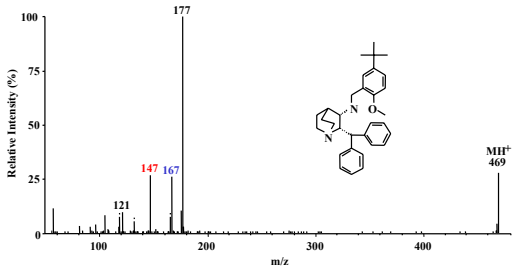
# Full Scan MS of Microsomal Incubation of Compound X



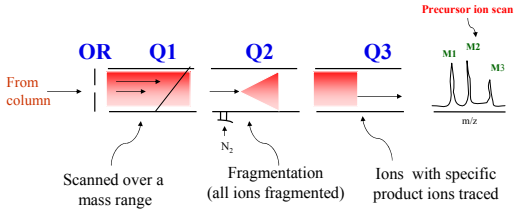

# Product Ion Spectrum




# CID Product Ion Spectrum of Compound Y

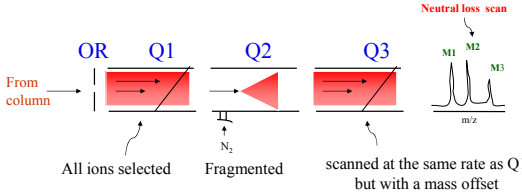



# Precursor Ion Scan




***Precursor ion experiment yields a spectrum of all parent ions which have the same product ion in their spectrum***

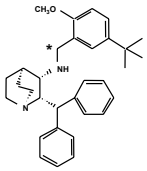
# Neutral Loss Scan



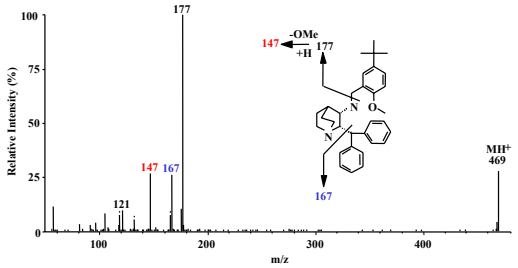

***Mass offset corresponds to the mass of neutral fragment loss during fragmentation***

***Neutral loss experiment yields a spectrum of all parent ions which lose a selected neutral loss fragment***

# Example



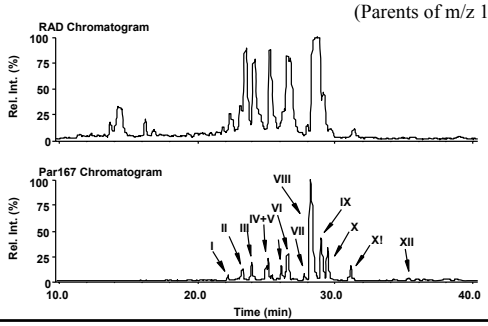
## CID Product Ion Spectrum of a Parent Drug



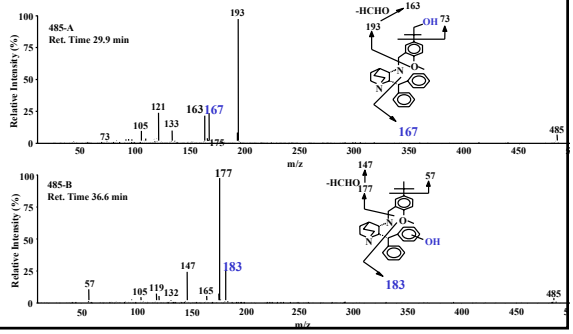

Identify parent scan ions?

## HPLC-RAD and TIC Chromatograms for Biliary Metabolites of CJ-11,972

(Parents of m/z 167)



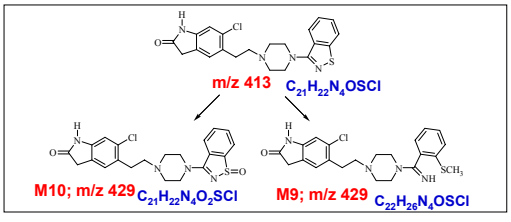

## CID Product Ion Spectra of Metabolites 485-A and 485-B




## LTQ/Orbitrap Mass Spectrometer

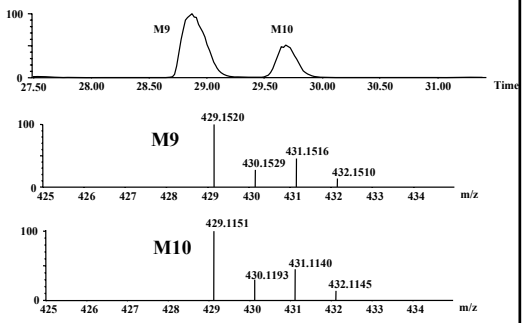
- What it is
  - Hybrid instrument: Linear ion trap (LTQ) / Orbital ion trap (Orbitrap).
  - LTQ can be used as a stand-alone MS, or in tandem with the Orbitrap as an ion preparation/isolation device.
- Features of LTQ
  - Very fast scan rate, ~ 11,000 u/s at unit mass resolution.
  - Capable of $MS^n$, SRM, CRM, "pseudo" precursor-ion and NL scans.
- Features of Orbitrap
  - High resolution – increments from 7,500 to 100,000 (at *m/z* 400).
  - Data-dependent $MS^n$. Capable of concurrent high-resolution scans in Orbitrap and unit mass resolution scans in Linear trap.
  - Can also perform high resolution data-dependent $MS^n$ scans.
- What it is not
  - It is not a time-of-flight (TOF) or ion cyclotron resonance mass spectrometer – there is no magnet or need for cryogens

## Biotransformation of Ziprasidone




**Question: Can we differentiate the structures of these metabolite with m/z 429 by TOF?**
**Previous assignment: S-oxides or S-methyl (+ 16) .**

## Selected Ion Chromatogram and Full Scan MS of M9 and M10

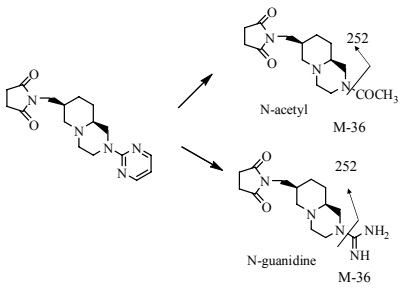


## Mass Measurements of M9 and M10

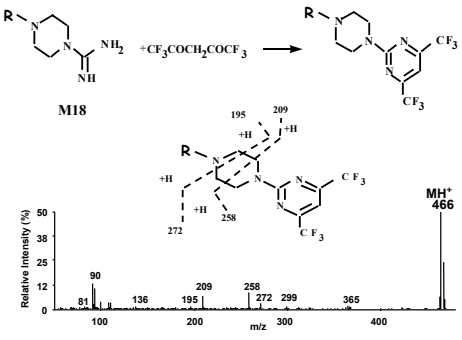
| Metab | Cal. Mass | Obs. Mass | +/-mDa | +/-ppm | Mol. Formula |
|---|---|---|---|---|---|
| M9 | 429.1516 | 429.1520 | 0.4 | 0.9 | C22H26N4OSCl |
| M10 | 429.1152 | 429.1151 | -0.1 | -0.3 | C21H22N4O2SCl |
| Parent | 413.1203 | 413.1205 | 0.2 | 0.4 | C21H22N4OSCl |

## Loss Art of Derivatization for Characterization of Metabolites

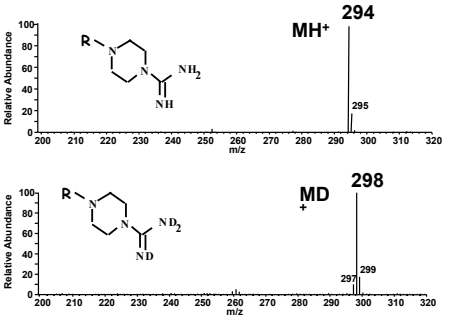
## Metabolites of a Pyrimidinylpieprazine




## $MS^2$ Spectrum After Treatment With HFAA




## Mass Spectra with H/D Exchange




## Identification of Drug Metabolites LC-NMR

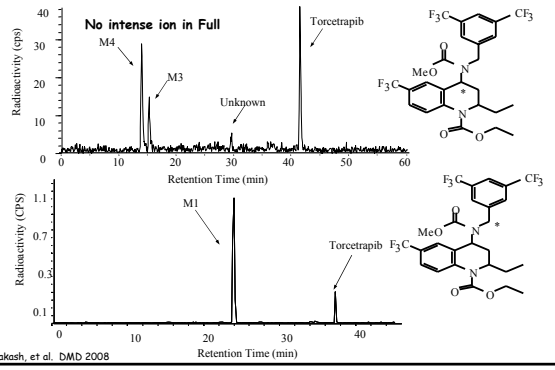
## ADVANTAGES

- LC-NMR (Continuous flow or stopped flow)
- Fast
- Reportedly sensitive (50 - 200 ng)
- Amenable to automation
- Negate the need for isolation
- Sample Stability
- Cleaner Spectra

## Disadvantages and Limitation of LC-NMR

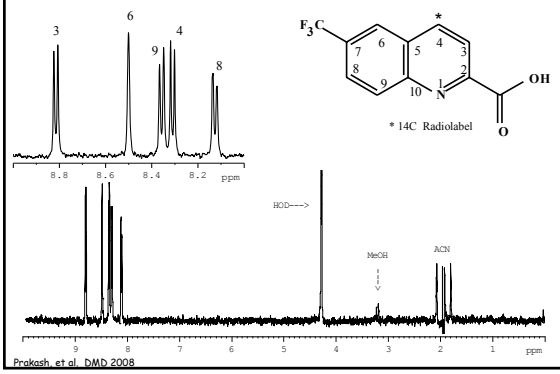
- Sensitivity
  - Nearly eliminates quantitative application
- The Chromatograph
- Solvent Suppression
- Expensive deuterated mobile phase and buffers
- Shimming problems introduced by LC-gradient methods

## Radioprofiles of Torcetrapib Metabolites




Prakash, et al. DMD 2008

## 1H NMR Spectrum of M4




Prakash, et al. DMD 2008

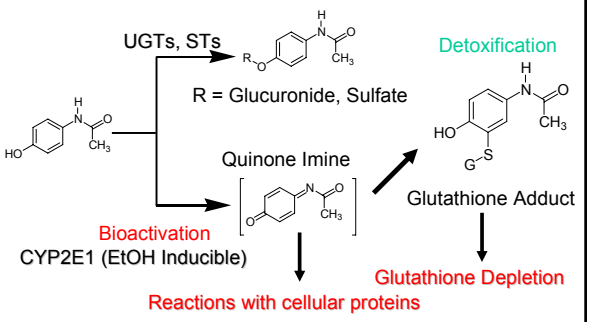
## Bioactivation/Adverse Drug Reactions

## Bioactivation of Drugs

- Attrition due to (pre)clinical toxicity is too high
- In some cases reactive metabolites have been implicated in toxicity, but the link between reactive metabolites and toxicity is complex.
  - hepatotoxicity
  - idiosyncratic reactions
- "It is now assumed that most idiosyncratic drug reactions are due to reactive metabolites, and yet most drugs form reactive metabolites to some degree, and we can not predict with any degree of certainty which drugs will be associated with a high incidence of idiosyncratic reactions."

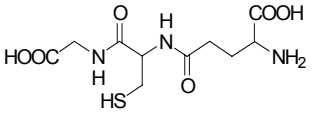
Utrecht, *Current Drug Metab.* **2002**, 3, no. 4, i-i(1).

## Acetaminophen Bioactivation




## Detoxification of Electrophilic Intermediates

- Glutathione is an excellent detoxifying agent
  - Cellular concentration ~ 10 mM
  - Serves as an endogenous nucleophile and reducing agent (free SH group) – protects cells from oxidative damage
  - Two families of enzymes help to catalyze these reactions
    - Glutathione transferase – in the nucleophilic attacks
    - Glutathione peroxidase – helps reduce reactive oxygen species or organic peroxides – reduces oxidative stress



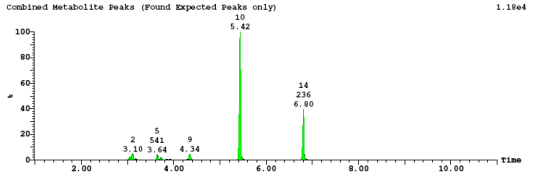
## Results

Sample 11 Bottle 2:A,6 ID File LX-0722_Rxn_HLM_MDF_50 Date 02-Jul-2009 Time 20:56:14 File Text LX-0722_Rxn_HLM

Expected Metabolites:

| Time | Metabolite Name | Formula | Mass Difference | m/z Found | mDa | Area Abs | Area % |
|---|---|---|---|---|---|---|---|
| 6.80 | True Parent | C11H9FN2OS | -0.0004 | 237.0493 | -0.5 | 177.00 | 24.25 (22.48) |
| 4.34 | Reduction? | C11H11FN2OS | 2.0161 | 239.0658 | 0.4 | 21.50 | 2.95 (2.73) |
| 5.42 | Hydroxylation? | C11H9FN2O2S | 15.9933 | 253.0431 | -1.6 | 458.40 | 62.81 (58.23) |
| 3.04 | Hydration? | C11H11FN2O2S | 18.0106 | 255.0603 | -0.0 | 11.40 | 1.56 (1.45) |
| 3.10 | 2 x Hydroxylation? | C11H9FN2O3S | 31.9893 | 269.0391 | -0.5 | 27.10 | 3.71 (3.44) |
| 3.18 | iH + Oxidative Defluorination | C21H25N5O8S2 | 303.0702 | 540.1199 | -2.4 | 3.70 | 0.51 (0.47) |
| 3.64 | GSH + Parent | C21H24FN5O7S2 | 305.0675 | 542.1172 | -0.7 | 7.40 | 1.01 (0.94) |
| 3.63 | GSH + Hydration | C21H26FN5O8S2 | 323.0793 | 560.1291 | 0.6 | 10.60 | 1.45 (1.35) |
| 3.73 | GSH + Hydration | C21H26FN5O8S2 | 323.0815 | 560.1313 | 2.8 | 7.20 | 0.99 (0.91) |
| 3.84 | GSH + Hydration | C21H26FN5O8S2 | 323.0800 | 560.1298 | 1.3 | 3.20 | 0.44 (0.41) |
| 3.92 | GSH + Hydration | C21H26FN5O8S2 | 323.0781 | 560.1279 | -0.6 | 2.30 | 0.32 (0.29) |

Total metabolites: 11




## Future Trends

- Metabolite identification by LC-MS:
  - Provide quantitative information
  - Automated interpretation of MS/MS spectra
  - Direct link with potency assays
- Drug Metabolism:
  - better *in vitro/in vivo* correlations
  - computational models which predict extent and site of metabolism (*in silico*)
  - systems biology approach

## SOME REFERENCES


- Biochemistry of Reactions by Bernard Testa
- Biotransformation of Xenobiotics - Andrew Parkinson - in Casarett and Doull's Toxicology, 5th edition.
- Drug Biotransformations - Neal Castagnoli - in Burgers Medicinal Chemistry 4th edition.
- Drug Metabolism -Bernard Testa- in Burgers Medicinal Chemistry, 5th edition.
- Strategies for Identifying Metabolites – Prakash, Shaffer and Nedderman. Mass Spectrometry Reviews 2007.
- HPLC/API/MS/MS in Drug Metabolism and Toxicology Studies- Kamel and Prakash Curr. Drug Metab. 2006


## Questions?