Metabolite Identification and Characterization

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Outlines

• Introduction
• Metabolism Reactions
• LC-MS strategies for metabolite identification
  ▪ Triple Stage Quadrupole (TSQ) LC/MS/MS
  ▪ Ion Traps (LCQ and LTQ)
  ▪ QTOF
• Analytical techniques combined with mass spectrometry for characterization of metabolites
  ▪ Derivatization
  ▪ H/D exchange
• LC-NMR
Fate of Drugs in Living Organisms

Drug → Receptor → Pharmacological Activity

Affinity

Biotransformation

Phase I

Metabolites

Phase II

Bioactivation

Excreted

Reactive intermediates

Conjugated metabolites

Excreted → Detoxification

Toxicity/Adverse drug reaction

Adducts
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.
- Pharmaceutical industries are mandated by regulatory agencies to identify metabolites of NCE.
- Metabolites may provide new leads.
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
Phase I Metabolism

- Hydroxylation - aliphatic, aromatic
- Epoxidation - aliphatic, aromatic
- O-, N-, S- Dealkylation
- Oxidative Deamination
- N-, S-, P- Oxidation
- Reduction
- Hydrolysis
- Aromatization
Phase II Metabolism

- Glycoside Conjugation
  - Glucuronide
  - other sugars
- Sulfate Conjugation
- Methylation (O-, S-, N-)
- Acylation
- Amino acid Conjugation
- Glutathione Conjugation
Identifying Metabolites—Prerequisite

- Knowledge of *Basic Organic Chemistry*
- Knowledge of *Drug Metabolism and Basic Metabolic Reactions*
- Knowledge of Concepts of Mass Spectrometry
- *Interpretation of Mass Spectra* for Structural Elucidation
- *Interpretation of NMR Spectra* for Structural Elucidation
Identifying Metabolites-Basic Needs

- Need the molecular weight
- Need product ions to get structure information
- Need product ion spectrum of the parent drug and metabolites
- Use the nitrogen rule
Techniques for the identification of metabolites

- **LC-API MS/MS**
  - Single Stage Quadrupole (SSQ) LC/MS
  - Triple Stage Quadrupole (TSQ) LC/MS/MS
  - Ion Traps (LCQ and LTQ)
  - QTOF

- **Analytical Techniques combined with MS**
  - Derivatization
  - Enzymatic hydrolysis
  - H/D exchange

- **LC/NMR**
LC/MS

- LC
- Ion Source
- Mass Analyzer

- Atmosospheric Pressure Chemical Ionization (APCI)
- Electrospray (ESI)
- Single Quadrupole
- Triple Quadrupole
- Ion Trap (LCQ)
- Q-TOF
General Rules for Choosing Polarity of Ion Detection and pH

- Positive ion Detection
  - Basic samples
  - Decrease pH →
    - Acetic acid pH (3-5)
    - Formic acid pH (2-3)
    - TFA pH (1-2)
  - pH at least 2 units below pKa of samples
General Rules for Choosing Polarity of Ion Detection and pH

• Negative ion Detection
  – Acidic samples
  – Increase pH
    • Ammonium hydroxide
  – pH at least 2 units above pKa of samples
Quadrupole

Single stage quadrupole (SSQ)

Ion Source

Q0 → Q1 → Detector

Triple stage quadrupole (TSQ)

Ion Source

Q0 → Q1 → Q2 → Ar → Q3 → Detector
Advantages of a TSQ MS

• Renders selectivity due to mass separation at two stages.
• Helps to rapidly identify metabolites in matrices without purification.
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 $\Rightarrow$ Molecular mass
  – Fragment ions $\Rightarrow$ Structure information
  – Background ions $\Rightarrow$ from non-analyte species
## Natural Isotopic Abundance of Common Elements

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<thead>
<tr>
<th>Element</th>
<th>Isotope Mass</th>
<th>%</th>
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<td>Oxygen</td>
<td>$^{16}$O</td>
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<td>Nitrogen</td>
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<td>Sulfur</td>
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# Mass

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<th>Element</th>
<th>Nominal Mass</th>
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<th>Exact Mass</th>
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<tr>
<td>S</td>
<td>32</td>
<td>32.06</td>
<td>31.972</td>
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</tbody>
</table>
Average vs. Exact Mass

• Average mass results from occurrence of isotopes. (See below)
  – 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)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Integer</th>
<th>Avg. Mass</th>
<th>Exact Mass</th>
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</thead>
<tbody>
<tr>
<td>Caffeine</td>
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<td>C8H10N4O2</td>
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<tr>
<td>Xanomeline</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C14H23N3OS</td>
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<td></td>
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<tr>
<td>Ziprasidone</td>
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</tr>
<tr>
<td>C21H21N4OSCl</td>
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<td>Xanomeline</td>
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<tr>
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<td>412</td>
<td>412.9197</td>
<td>412.1120</td>
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<tr>
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# Nitrogen Rule

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<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Even number of nitrogens (0, 2, 4)</td>
<td>Even</td>
<td>odd</td>
<td>Even</td>
</tr>
<tr>
<td>Odd number of nitrogens (1, 3, 5)</td>
<td>odd</td>
<td>Even</td>
<td>odd</td>
</tr>
</tbody>
</table>

Mass Changes Associated with Phase I Metabolism

- Hydroxylation- aliphatic, aromatic
  - Lose H & add OH = (M+16)
- Epoxidation- aliphatic, aromatic
  - Lose H & add OH = (M+16)
- O-, N-, S- Dealkylation
  - Add H & lose alkyl (R) = (M+1-R)
- N-, S- Oxidation
  - Add O = (M+16)
- Reduction
  - Add 2H = (M+2)
Mass Changes Associated with Phase II Metabolism

- **Glucuronide Conjugation**
  - Lose H & add glucuronic acid \((M-1+176) = (M+176)\)

- **Sulfate Conjugation**
  - Lose H & add \(\text{HSO}_3^-\) \((M-1+81) = (M+80)\)

- **Methylation (O-, S-, N-)**
  - Lose H & add \(\text{CH}_3^-\) \((M-1+15) = (M+14)\)

- **N-acetylation**
  - Lose H & add \(\text{CH}_3\text{CO}^-\) \((M-1+43) = (M+42)\)

- **Amino acid Conjugation**
  - Lose \(\text{H}_2\text{O}\) & add amino acid \((M-18+R)\)

- **Glutathione Conjugation**
  - Various addition of SG-amino acid \((M+306-X; X=\text{leaving group})\)
LC/MS/MS Techniques for the Identification of Metabolites
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

HLM Control

HLM + Substrate

Background subtracted
Problem Set:
Full Scan MS of Metabolites of Compound X (MW 394)
Determine possible additions of functionality of metabolites
Product Ion Spectrum

One ion selected (parent ion) → OR → Q1 → Q2 → Q3 → All ions scanned (product ions)

Fragmentation

Rel. intensity (%)

m/z

50 162 375 421

A product ion spectrum

From column

N₂
CID Product Ion Spectrum of Compound Y
**Precursor Ion Scan**

- From column
- OR
- Q1: Scanned over a mass range
- Q2: Fragmentation (all ions fragmented)
- Q3: Ions with specific product ions traced
- Precursor ion scan
- m/z
- M1, M2, M3

**Diagram Description:**
- 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.
Interpreting Product Ion MS/MS Spectrum

Full Scan MS

Product ion MS/MS

MW=212

M+H = 213
Systematic Approach for the Identification of Metabolites by LC/MS/MS

- GET A Q1 SCAN OF THE COMPOUND IN QUESTION
- OBTAIN A PRODUCT ION SPECTRUM OF THE COMPOUND: INTERPRET THE SPECTRUM
- IDENTIFY MAJOR FRAGMENT ION AND NEUTRAL LOSS
- IDENTIFY MAJOR FRAGMENT ION AND NEUTRAL LOSS
- RUN PRODUCT ION SCANS FOR ALL POSSIBLE METABOLITES IDENTIFIED FROM STEP 4 PLUS EXPECTED METABOLITES
- INTERPRET THE SPECTRA AND ASSIGN STRUCTURES OF METABOLITES
Example
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

485-A
Ret. Time 29.9 min

485-B
Ret. Time 36.6 min
CID Product Ion Spectra of Metabolites 455-A and 455-B

455-A
Ret. Time 28.1 min

455-B
Ret. Time 37.7 min
ION TRAP MS
ION TRAP MS

• Sensitivity
  – Ion accumulation
    (10-1000 times better sensitivity than quadrupole MS)

• Specificity
  – Multistage MS capabilities (MSn)

• Speed
  – Can complete an entire scan in 100 ms

• Data Dependent Acquisition
  – Acquire MW information and MSn spectra in the same run

• High value/Cost Ratio
Triple Quad vs LCQ (MS/MS)
Schematic of data-dependent analysis using LCQ
MS, MS$^2$, MS$^3$ and MS$^4$ spectra of trocade on a ion trap
Proposed fragmentation pathways of trocade for the major fragments
Fig. 2. MS" behavior of zolpidem.
Fig. 4. MS" behaviour of 7-aminoflunitrazepam.

Adapted from Smyth et. al., Analytica Chimica Acta 506(2004) 203-214
Q-TOF
Why use a Q-TOF?

**Sensitivity**
- detection of low level metabolites in complex matrices in vivo

**Exact mass (high resolution mass measurement)**
- added confidence in confirming expected metabolites (confirm elemental composition for metabolites with the same nominal mass)

**LC/MS/MS**
- confirmation of metabolites (compare MS/MS spectra)
- data dependent MS $\rightarrow$ MS/MS (time saving, High throughput)
Operating principle of the Q-TOF mass analyzer

MS operation: Quadrupole MS transmits – TOF detects all ions transmitted: full scan mass spectrum

MS/MS operation: Precursor ion selection in quadrupole, collision induced dissociation (CID) in hexapole collision cell – product ion detection in TOF: MS/MS spectrum
Instrument Capabilities

• **High Resolution MS and MSMS**
  - 20,000 resolution
  - Peak Width of 0.025 at 500 amu

• **High Resolution = High Selectivity**
  - Able to easily separate masses that differ in 0.1 amu easily

• **TOF allows fast scan speeds without sacrificing sensitivity or scan ranges in MS or MS/MS modes**
## Mass

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</table>
Biotransformation of Ziprasidone

Question: Can we differentiate the structures of these metabolites 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

<table>
<thead>
<tr>
<th>Metab</th>
<th>Cal. Mass</th>
<th>Obs. Mass</th>
<th>Mass +/-mDa</th>
<th>+/-ppm</th>
<th>Mol. Formula</th>
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<tbody>
<tr>
<td>M9</td>
<td>429.1516</td>
<td>429.1520</td>
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<td>0.9</td>
<td>C22H26N4OSCl</td>
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<tr>
<td>M10</td>
<td>429.1152</td>
<td>429.1151</td>
<td>-0.1</td>
<td>-0.3</td>
<td>C21H22N4O2SCl</td>
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<tr>
<td>Parent</td>
<td>413.1203</td>
<td>413.1205</td>
<td>0.2</td>
<td>0.4</td>
<td>C21H22N4O2SCl</td>
</tr>
</tbody>
</table>
MS/MS Spectra of Metabolites M9 and M10

- MS/MS Spectra of Metabolites M9 and M10 are depicted with molecular structures and their corresponding m/z values.
- The spectra show the fragmentation patterns of metabolites M9 and M10, highlighting the differences in their molecular structures and masses.
- The m/z values are accompanied by their respective ppm deviations from the expected masses, indicating the precision of the measurements.

For example, the m/z 196.0369 with a deviation of 0.9 ppm compared to the expected mass. Similarly, m/z 429.1516 with a deviation of 0.3 ppm from the expected mass.

The diagrams also include molecular structures with highlighted chemical bonds and functional groups, providing a visual representation of the metabolites' chemical compositions.
Two Isobaric metabolites of Compound X

![Chemical structures showing two isobaric metabolites of Compound X.](image-url)
TOF MS Spectrum of Structure A

- Mass corrected and centroid
- Exact mass: 242.0793
- $\Delta$ -1.0 mda; ppm error= -3.9
- Formula= C12H11NOF3
TOF MS Spectrum of Structure B

- Mass corrected and centroid
- Exact mass: 242.0429
- $\Delta$ 0.3 mda; ppm error = 1.2
- Formula = C11H7NO2F3
Analytical techniques combined with mass spectrometry for characterization of metabolites
When Derivatization is Useful

- Metabolite is unstable
- Metabolite is very polar
- Metabolite is volatile
- To characterize the functional group
- To prove MS fragmentation
- To improve sensitivity when metabolite is available in only trace amounts
Derivatizations of Hydroxyl Groups

**-Acetylation**

ROH or ArOH + CH₃COCl → ROCOCH₃ or ArOCOCH₃

**-Silylation**

ROH or ArOH + MTBSTFA → ROBDMS or ArOBDMS

**-Methylation**

ArOH + CH₂N₂ → ArOCH₃

**-Dansylation**

ArOH + MTBSTFA; Tert-butyldimethylsilyl-N-methyltrifluoroacetamide → SO₂Cl

MTBSTFA; Tert-butyldimethylsilyl-N-methyltrifluoroacetamide
Derivatizations of Amines

**-Acetylation**

\[
\text{RNH}_2 + \text{CH}_3\text{COCl} \rightarrow \text{RNHCOCH}_3
\]

\[
\text{RNH}_2 + (\text{CH}_3\text{CO})_2\text{O} \rightarrow \text{RNHCOCH}_3
\]

**-Dansylation**

\[
\text{RNH}_2 + \text{SO}_2\text{Cl} \rightarrow \text{RNHCOCH}_3
\]

MBTFA; N-methylbis(trifluoroacetamide)
Derivatization of Carboxyl Group

-Methylation

\[
\text{RCOOH} + \text{CH}_{3}\text{OH}/\text{HCl} \rightarrow \text{RCOOCCH}_{3}
\]

-Triphenylphosphonium Derivative

\[
\text{RCOOH} + \text{H}_{2}\text{NCH}_{2}\text{CH}_{2}\text{P(Ph)}_{3} \rightarrow \text{RCO HNCH}_{2}\text{CH}_{2}\text{P(Ph)}_{3}^{+}
\]

-Reduction

\[
\text{RCOOH} + \text{LiAlH}_{4} \rightarrow \text{RCH}_{2}\text{OH}
\]
Functional group-specific chemistry

\[ R_3N \rightarrow O \quad \text{TiCl}_3 \rightarrow R_3N \]

\[ R_2S=O \quad \text{TiCl}_3 \rightarrow R_2S \]

\[ R \overset{\text{NH}}{\equiv} \overset{\text{NH}}{\equiv} \quad \text{HFAA} \quad \text{Hexafluoroacetylacetone} \rightarrow \quad \text{R} \]

\[ \text{Hexafluoroacetylacetone} \]
CID product ion spectra of glucuronide conjugates

![CID spectra diagrams](image-url)
CID product ion spectrum of glucuronide conjugates after methylation
CID Product Ion Spectrum of Sunepitron

CID product ion spectrum of M18
Mass Spectra of M18 Before and After Deuterium Exchange
CID product ion spectrum of M18 after treatment with HFAA

- Reduced polarity
- Diagnostic mass increase
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
What information does NMR provide

• Each proton (or carbon atom) in a molecule typically has a different resonant frequency (chemical shift)
  – Thus, NMR spectrum is a fingerprint of a molecule
  – Chemical shifts are governed by nuclear environment, e.g. CH₃O, CH₃CN, CH₃NH₂
• Adjacent NMR-active nuclei 9-4 bonds apart) in a molecule may couple to one another
  – Coupling constants can be identified by inspection of 1D spectra
Activation of Acetaminophen by Cytochrome P450 to N-acetyl-p-benzoquinonimine (NAPQI) and subsequent conjugation with Glutathione
Proposed Reaction Product of N-acetyl-p benzoquinone imine (NAPQI) with Glutathione

NAPQI + GSH → IPSO ADDUCT
CID Product Ion Mass Spectra of Reaction Mixture
$^1$H NMR Data Obtained on The Reaction Mixture
Characterization of Metabolites of Compound X

![Chemical Structure](image)
HPLC Radiochromatograms of Compound X Metabolites
CID Product Ion Spectrum of compound X
$^1$H NMR of Compound X

Proton double presat 278K d1=10sec CP-533,536
LC peak at 57.41 minutes (m/z=471) injection # 636

Expansion of Aromatic Region

3 Methyl groups

-NCH$_2$
-OCH$_2$
CID mass and NMR spectra of M4

Proton double presat 283K CP-533,536 metabolite
LC peak at 18.27 minutes (m/z=488) injection 605

MH⁺=485
MD⁺=488

ACN-d3

<---HOD

2 Methyl groups
CID mass and NMR spectra of M24

Proton double presat 278K CP-533,536 metabolite
LC peak at 45.30 minutes (m/z=485) injection # 622

MH⁺ = 483
MD⁺ = 485

2 Methyl groups

aldehyde resonance

- NCH₂

- OCH₂

impurities

MeOD

ACN-d3

<--- HOD
Conclusions

• Combination of LC/MS/MS with other analytical approaches is a powerful tool for solving difficult problems encountered in the analysis of drug metabolites.
SOME REFERENCES

• Biochemistry of reactions by Bernard Testa.
• Biotransformation of Xenobiotics - Andrew Parkinson - in Casarett and Doull’s Toxicology, 5th edition.
• Drug Metabolism - Bernard Testa - in Burgers Medicinal Chemistry, 5th edition.
• Metabolism of Heterocycles by L. A. Damani in Comprehensive Heterocyclic Chemistry