Interpretation of MS/MS Spectra of Small Molecules

Árpád Somogyi

CCIC MSP

OSU Summer Workshop

What do we mean by “small”?

- MW < 1000 Dalton (not well defined)
- Analytes related to
  - Drug metabolism (pharmacokinetics)
  - Environmental science
  - Forensic science/HomeLand security
  - Natural Products
  - Astrobiology model reactions
  - Ion-molecule reactions
- Small enough to produce “strange” results (e.g., involved in ion-molecule reactions)

“Big” biomolecules (enzymes) generate “small” organic molecules that trigger further biochemical/biological processes.
General Questions

• Ionization method?
  – Instrument availability
  – Sensitivity/Quantification
  – Time of analysis

• “In vivo” or sample pre-treatment?
  – Chromatographic/extraction methods: the shorter, the better
    • GC, HPLC, zip-tip, solid phase extraction (SPE), dialysis, etc.
  – Derivatization: the simpler, the better
    • to increase volatility (GC); to study neutral loss; to increase ionization efficiency

Use of isotope labeling and accurate mass/high resolution FT-ICR measurements

negative ion MS/MS spectra

positive ion MS/MS spectra

“Application of mass spectrometry to determination of molecular structure relies on interpretation of fragment ion spectra, however obtained, using a set of rules that are the result of years of experience in extending concepts of classical physical-organic chemistry.”

“It is true that these rules are almost entirely empirical, but their continuing practical success indicates that they must correspond to real phenomena in some sense.”

(Robert Boyd, Árpád Somogyi)
Ion Chemistry and Fragmentation

Richard A. J. O’Hair

University of Melbourne, Victoria, Australia

Progress in mass spectrometry has been so rapid since 1939 that one can hardly predict even the immediate future. . . . Many present difficulties will be overcome when apparatus with better design and more simple operation is generally available, but even before the perfect spectrometer is built, the chemist will find that this tool of the physicist has already earned a place in the chemical laboratory.


Even Electron Mass Spectrometry with Biomolecule Applications
By Bryan M. Ham, John Wiley and Sons, Hoboken, NJ, 2011

“The book is a patch quilt of some correct and good information, some information that is partially correct but twisted, and some information that is completely wrong. Given the latter, this book, when in the hands of someone new to mass spectrometry and wanting to learn the subject, will be more harmful than it will be beneficial.”

“(the book) is not at all bad. For those well versed in the field of mass Spectrometry, some applications will be valuable. The book causes you to Look at some processes differently and more carefully; however, as a first Book in mass spectrometry for a course of self-directed study, it is weak.”

(O. David Sparkman, reviewer, JASMS, 2011, 22, 793-794.)
The Nitrogen Rule

- Compounds* that contain even number of N atoms have even nominal molecular weight
- Compounds* that contain odd number of N atoms have odd nominal molecular weight

But what about singly protonated molecules and accurate molecular weights??

* Common organic compounds

Ion Stabilities

- Even electron ions are more stable than odd electron (radical) ions

How about protonated molecules: even electron or not?
And how about ions formed by electron impact (EI) ionization?
Fragmentation of protonated molecules generated by protonation at lone pairs of heteroatoms.

Fragmentation of protonated molecules generated by protonation at a benzene ring or at an unsaturated bond.
Learning Check on Small Molecules Fragmentation

Screening and identification of unknown contaminants in water with liquid chromatography and quadrupole-orthogonal acceleration-time-of-flight tandem mass spectrometry

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*bMicromass Ltd., Flots Road, Wythenshawe, Manchester M23 9LY, UK
*Micromass Europe, Transvaardstraat 18, 4320 AC Almere, The Netherlands

## Accurate Mass Measurements (Micromass Q-TOF)

<table>
<thead>
<tr>
<th>Compound</th>
<th>[M+H]^+ Elemental Composition</th>
<th>Retention Time (min)</th>
<th>Theoretical Mass</th>
<th>Measured Mass</th>
<th>Δ ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metribuzin</td>
<td>C₈H₁₅N₄OS</td>
<td>25.18</td>
<td>215.0967</td>
<td>215.0969</td>
<td>1.1</td>
</tr>
<tr>
<td>Pirimicarb</td>
<td>C₁₁H₁₉N₄O₂</td>
<td>11.43</td>
<td>239.1508</td>
<td>239.1501</td>
<td>-2.7</td>
</tr>
<tr>
<td>Diuron</td>
<td>C₉H₉Cl₂N₂O</td>
<td>30.20</td>
<td>233.0265</td>
<td>233.0260</td>
<td>-2.3</td>
</tr>
</tbody>
</table>

## Elemental Composition Hits (Search Results)

<table>
<thead>
<tr>
<th>Compound</th>
<th>[M+H]^+ Elemental Composition</th>
<th>Calulated Elecomp Hits</th>
<th>NIST Search Hits</th>
<th>InfoSpec Search Hits</th>
<th>Total Structures Evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metribuzin</td>
<td>C₈H₁₅N₄OS</td>
<td>9</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Pirimicarb</td>
<td>C₁₁H₁₉N₄O₂</td>
<td>9</td>
<td>14</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Diuron</td>
<td>C₉H₉Cl₂N₂O</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Is this enough? NO! MS/MS and accurate masses of fragments needed!

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Nominal Atomic Masses:

- H = 1
- C = 12
- N = 14
- O = 16

Where do you think protonation can occur?
Same chemical formula (exact mass) but

Different Ion Structures!

Are they all *fragmenting* structures????

Predict two main fragments from the protonated structures above!

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**Fig. 2:** Product ions spectra of pirimicarb, obtained at different collision energies (from top to bottom) 15, 20, 25 and 30 eV. For elemental composition of the fragments, see Table 4.
### Accurate Mass Measurements on [M+H]$^+$ and selected fragments

<table>
<thead>
<tr>
<th>[M+H]$^+$ and fragments of Pirimicarb</th>
<th>Elemental Composition</th>
<th>Theoretical Mass</th>
<th>Experimental Mass</th>
<th>Δ ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>C$_3$H$_6$NO</td>
<td>72.0449</td>
<td>72.0465</td>
<td>21.7</td>
</tr>
<tr>
<td>182</td>
<td>C$<em>9$H$</em>{16}$N$_3$O</td>
<td>182.1293</td>
<td>182.1306</td>
<td>6.9</td>
</tr>
<tr>
<td>195</td>
<td>C$<em>{10}$H$</em>{19}$N$_4$</td>
<td>195.1610</td>
<td>195.1627</td>
<td>8.9</td>
</tr>
<tr>
<td>239</td>
<td>C$<em>{11}$H$</em>{19}$N$_4$O$_2$</td>
<td>239.1508</td>
<td>239.1501</td>
<td>-2.9</td>
</tr>
</tbody>
</table>

From which protonated form do you think the fragment at m/z 72 can be generated?
From which structures do you think fragment at m/z 72 can be formed?

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primicarb-I</td>
<td>C_{13}H_{27}N_{2}O_{2}</td>
</tr>
<tr>
<td>Metribuzin-II</td>
<td>C_{9}H_{14}N_{2}O</td>
</tr>
<tr>
<td>Atrazin-IV</td>
<td>C_{8}H_{10}CN_{3}</td>
</tr>
<tr>
<td>Isoxynuron-V</td>
<td>C_{9}H_{12}N_{2}O</td>
</tr>
<tr>
<td>Diuron-VI</td>
<td>C_{13}H_{10}Cl_{2}N_{2}O</td>
</tr>
</tbody>
</table>

m/z 72 \ C_{3}H_{6}NO

Predict structures for fragments at m/z 114 and 83

(hint: use Nitrogen Rule)

\[ [M+H]^+: C_{13}H_{26}N \]
Predict Compound Based on MS/MS spectra

\[ \text{[M+H]}^+: \text{C}_{15}\text{H}_{13}\text{N}_2\text{O} \]

Fig. 4: Product ion mass spectra of an unknown compound with retention time 23.6 min found in surface water extract and the two most plausible structures. Collision energy: 15 eV (A) and 30 eV (B).

Select the Precursor Ion Based on MS/MS Spectra on Next Page

Fig. 6: Possible structural hints for the elemental compositions calculated for the determined exact mass (279.0974 Da) of an unknown compound with retention time 28.3 min, present in surface water extract.
Take Home Messages

• Accurate masses reduce # of possible structures but, generally, not exclusive
• MS/MS is necessary
• Fragmentation rules (e.g., nitrogen rule, odd/even electron ions) apply but MS/MS spectra instrument and collision energy dependent
• Own database is useful
Sedative effects
Facilitate sexual assaults
A sensitive HPLC-MS-MS assay for quantitative determination of midazolam in dog plasma

Department of Pharmacokinetics, Dynamics and Metabolism, Pfizer Central Research Development, Sandwich Kent CT13 9NS, UK

Instrument: API 4000

midazolam (M)

flunitrazepam (F)

Fig. 1. Chemical structures of midazolam (A) and flunitrazepam (B) (internal standard).

Multiple Reaction Monitoring (MRM)

F, 314 → 268
4 ng/ml

M, 326 → 291
10 ng/ml

Dog plasma, after 1 hr

M, 326 → 291
0.1 ng/ml

dog plasma, after 1 hr

Fig. 2. Representative chromatograms of midazolam (m/z 326 → 291) and internal standard (flunitrazepam – m/z 314 → 268) in dog plasma (200 μl). (A) Control plasma blank spiked with 4 ng/ml flunitrazepam. (B) Control plasma spiked with 0.1 ng/ml midazolam and 4 ng/ml flunitrazepam. (C) Control plasma spiked with 10 ng/ml midazolam and 4 ng/ml flunitrazepam. (D) Male dog plasma 1 h after intravenous infusion of midazolam at 0.05 mg/kg.
Mean plasma concentrations of midazolam in male beagle dogs, after single intravenous infusion or oral administration at 0.05 mg/kg.
a. Measure the mass spectra of unchanged drug and its metabolites.
b. Prepare a table comparing (MS)$^n$ fragment ions of a drug with those of its metabolites.
c. Compare the spectral patterns of the drug before and after metabolism.
d. Assign the characteristic peaks of each metabolite in the mass spectrum.
e. Elucidate the structures of the metabolites.

Figure 3. CID mass spectra of the protonated molecules of tiaramide (top) and the single stage mass spectrum before the CID experiment showing the parent ion selected as the dominant peak at m/z 356 (bottom).
Metabolites of tiaramide and their ESI ion trap MS

Metabolism of tiaramide in humans
Integration of Knowledge-Based Metabolic Predictions with Liquid Chromatography Data-Dependent Tandem Mass Spectrometry for Drug Metabolism Studies: Application to Studies on the Biotransformation of Indinavir

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Department of Drug Metabolism, Merck Research Laboratories, WP7SA-201, Summit Hill, PA 18201, United States

**Figure 1.** Metabolite identification strategy based on integration of knowledge-based metabolic predictions with liquid chromatography list-dependent tandem mass spectrometry.
Product ion spectra of protonated indinavir; (A) MS² production ion spectrum of m/z 614 and (B) MS² product ion spectrum of m/z 364.

Knowledge-based metabolic prediction of indinavir
**MS/MS Databases Available?**

- **Databases**
  - EI databases available (NIST, Wiley)
  - MS/MS databases

- **Please remember!**
  - MS/MS more complicated
    - Many instrument types
    - Different ion activation methods/internal energy distributions
    - Different time frame
    - All affect relative ratio of low/high energy processes, i.e., spectra