LC-NMR in Drug Discovery

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Personal Background

- Undergraduate in Biochemistry and Biophysics in 1997
- Ph.D. in Medicinal Chemistry in 2001
  - Pharmacognosy -- Marine Natural Products
- Wyeth Pharmaceuticals (PA) -- NMR Spectroscopist in Discovery
- Amgen Inc. (CA) -- NMR Spectroscopist in Discovery/PKDM/Process Chemistry
- Pfizer Inc. (CT) -- NMR Group Leader in Pharmaceutical Sciences (Development)
What we do in the Pharm. Sci. NMR group

- NMR need throughout all of drug development. This includes but is not limited to:
  - Filing characterizations
  - Lot confirmations
  - Solution confirmations
  - New technology development and integration
  - STRUCTURE ELUCIDATION
    - Impurities (e.g. process related)
    - Degradants (e.g. process related or forced degradation)
Intent of this Lecture

- What is LC-NMR – No NMR theory, just application oriented.
- Limitations
- Configurations/Options
- Practical Considerations – Overlying Theme
  - When/When not to use it
LC-NMR: Simplistic Concept

- HPLC is plumbed in line with a “flow” NMR system
- Sample components are physically separated by HPLC
- Each component flows, in turn, from the LC column and UV detector to the NMR sample cell
  - Multiple different configurations for this
- NMR is performed on each desired fraction / peak
  - Always the rate limiting step
- Continuous or stopped flow mode
  - Additional methods are also available – Peak “parking” and “trapping”.
Modes of Operation

- **Continuous Flow**
  - Eluent sampled in “real-time” as flowing through NMR Detection Coil
- **Time Slices**
  - Regions, or “time-slices” of interest are analyzed
- **Stopped Flow**
  - Pump is stopped at desired location and data acquired
- **Peak Parking**
  - Peaks of interest are “parked” in off-line sample loops
- **Peak Trapping**
  - Solid Phase Extraction cartridges are used to “re-concentrate” samples.
General Schematic for an LC-NMR
General Cartoon of Loop Collection

LC Pump → DAD Detector → Loop Collection

Direct Stop Flow

36 Loop Cassette

NMR Spectrometer
LC-NMR Hardware Configuration

- Binary or Quaternary LC Pump
- Degasser
- UV Detector
- Manual Injection
- RF Gradients
- Temperature Control Unit
- LC Column Temperature Control
- Loop Collection
- 600 MHz NMR Console
LC-NMR Probe Schematic

NMR detection coil built directly onto flow cell (4mm OD)

From LC

To Waste
Traditional Probe Configurations

- Most common configuration – inverse probes – best proton sensitivity

- Flow Cells – Active Volumes
  - 3mm – 60µL
  - 4mm – 120µL
  - 5mm – 240 µL
  - Others “non-traditional” will be covered later

- Typically probes are outfitted with z-gradients
  - For gradient experiments and shimming
1. Structural determination of metabolites:

~50 µg of metabolites
From microsomal incubations

4.6 mm or 6mm Reverse-phase HPLC
Mobile phase: CH$_3$CN/D$_2$O+(0.1%TFA or DCOOD), solvent gradient < 2%/min

2. Structural determination of impurities:

Large volume injections in high % of aqueous phase
More then 1 mg

4.6 mm or 6 mm reverse-phase HPLC
Mobile phase: CH$_3$CN/D$_2$O+(0.1%TFA or DCOOD), solvent gradient < 2%/min
1D $^1\text{H}$ and homonuclear NMR experiments are the most sensitive and accessible experiments for LC-NMR.

1D $^{13}\text{C}$ and heteronuclear NMR experiments are very insensitive and are typically inaccessible to LC-NMR applications (in most cases).
When to use LC-NMR

- Fairly resolved peaks.
- Relative abundance of entities similar.
- Known stability issues.
- A significant amount of information is known about the compound
Impurity Analysis: Low Volume (20µL) Injection of Sample

20µL injected

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<thead>
<tr>
<th>t</th>
<th>%B</th>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td>48</td>
<td>100</td>
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<td>50</td>
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<tr>
<td>50.5</td>
<td>10</td>
</tr>
<tr>
<td>51</td>
<td>10</td>
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1.5mL/min
4.6x150mm Luna 5u C8(2)
A: 0.1% TFA/D_2O
B: 0.1% TFA/MeCN

~0.3-0.5% Impurities (215nm)

In order to achieve sufficient NMR sensitivity it was necessary to overload the HPLC column without sacrificing peak resolution. This goal was achieved by maximizing sample concentration in the highest content of aqueous phase followed by large volume column injections.
Impurity Analysis: Large Volume (500µL) Injection of Sample

500µL injected

t | %B
0 | 10
48 | 100
50 | 100
50.5 | 10
51 | 10

1mL/min
4.6x150mm Luna 5u C8(2)
A: 0.1% TFA/D$_2$O
B: 0.1% TFA/MeCN

---

UV (215.0nm)
Pressure
Impurity Analysis: Solved Structures
100µL of ~4mg/mL injected

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<tr>
<td>0</td>
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<td>37</td>
<td>95</td>
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<td>38</td>
<td>35</td>
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<tr>
<td>40</td>
<td>35</td>
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</tbody>
</table>

1.0mL/min
4.6x250mm YMC-AQ 5u 120A
A: 0.1% d-FA/D$_2$O
B: 0.1% d-FA/MeCN

Metabolite Analysis:
Metabolite Analysis: Solved Structures
Metabolite Analysis:

500 µL injected

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<td>90</td>
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<tr>
<td>46</td>
<td>20</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
</tr>
</tbody>
</table>

1.0 mL/min
6x150mm YMC-AQ 3u 12n
A: 0.1% d-AcOH/D_2O
B: 0.1% d-AcOH/MeCN

DAD (254)
When not to use LC-NMR
Regiochemistry

- Sample submitted for determination of the regiochemistry of the primary amine moiety

Structure A

Structure B
\[ ^1H \text{ Assignment} \]

Very little help using Chemical Shift Arguments

\[ \text{N} \quad \text{O} \]

\[ \text{NH}_2 \]

A B C D E

A B C D E

\[ \text{E} \]

\[ \text{A} \quad \text{B} \quad \text{C} \quad \text{D} \quad \text{E} \]

\[ \text{E} \]

\[ \text{A} \quad \text{B} \quad \text{C} \quad \text{D} \quad \text{E} \]

\[ \text{E} \]

\[ \text{A} \quad \text{B} \quad \text{C} \quad \text{D} \quad \text{E} \]
1H-15N HMBC Data

Background -- Simplified

• Pulse sequence allows one to detect 1H’s long-range coupled (~8 Hz) to 15N
  – Depending on the experiment used one can choose to omit or retain the 1J_{NH}

\[ ^nJ_{NH} (n = 2,3) \] ? Will result in a correlation centered at the 1H and 15N chemical shift

\[ ^1J_{NH} \] ? Will result in a correlation centered at the 15N chemical shift and a split signal centered on 1H
$^{1}H^{15}N$ HMBC Data Allows Assignment

> Primary Amine

> Tertiary Amide

$^n J_{NH}$ (n = 2,3)

$^1 J_{NH}$

70 Hz
Other Options Available

- **LC-NMR/MS**
  - Allows on-line MS and NMR evaluation of samples

- **Peak Trapping (Column Trapping)**
  - Potentially allows multiple LC peaks to be “trapped” and concentrated prior to NMR data acquisition.

- **Microcoil Probes**
  - Has potential to allow microscale separation mechanisms (e.g. CapLC).

- **CryoProbe Technology**
  - Significantly lowers “noise floor” through cryo-cooling RF electronics in the probe.
LC-NMR/MS

- LC & MS Control System
- NMR Control System
- LC Pump
- Injector
- Column/Column Heater
- Loop Cassette
- OR
- 95%
- 5%
- UV
- Splitter
- MS Component
- 600MHz NMR Spectrometer
- Waste Or Fraction Collector
Hardware Setup for LC-NMR-MS (without magnet)

- HPLC
- LC-NMR-MS Interface
- BNMI
- UV detector
- BPSU-36/
- BSFU-O
- MS-Rough pump
- NMR Spectrometer electronics
- Esquire Ion Trap Mass-Spectrometer

Graphic Borrowed from Bruker-Biospin
Actual System

- 36 Loop Cassette
- 3 Port Switching Valves
- Column Heater/Injector
- Agilent 1100
- Esquire 3000+ Ion Trap MS
- BNMI
- MS Rough Pump
- NMR Electronics
- DAD Detector
Other Options

LC-SPE-MS-NMR

Chromatographic System → Spark Prospekt 2 → Spark Prospekt 2 → NMR Spectrometer

Peak Trapping

Drying
Closer Look at the SPE

Spark Holland Prospekt SPE
Robot gripper for SPE cartridges

2 flow lines where trap cartridges are inserted

SPARK HOLLAND SPE-UNIT

Graphic Borrowed from Bruker-Biospin

Trap cartridge size
- 10mm * 2mm (ID)
- 10mm * 1mm (ID)
- 10mm * 3mm (ID)

~ 2 $ per cartridge

Various commercial packings available

Bruker provides a set of 4 different solid phase types to start
Major New Developments

- Miniaturization – Microcoil Probes
- Integration of new (to commercial NMR) MS
- Cryogenic NMR Flow Probes
MicroCoil Probe

- Horizontal copper RF solenoid Coil
- Vertical (Z) pulse field gradient (PFG) coil
- Flow cell is surrounded by CF-43 fluorocarbon for susceptibility matched to copper coil
- 1.5 µL active volume with a 5 µL total volume
- 7 µL total volume from inlet to outlet (3 µL transfer from injection assembly)
- Lock power > 45 db to prevent saturation
- $\pi/2$ pulse width of 8.4 µs at 18 db power level
- Low power needed for 90% H$_2$O/ 10% D$_2$O (75 db) saturation
Advantages vs. Disadvantages

**Advantages**
- Extremely mass sensitive
- Capillary-scale fluidics allow transport of µL volume samples over distances of 5-10 meters with virtually no degradation in analyte peak volume.
- Diffusion and mixing effects at the capillary scale are very limited so that peaks can be parked overnight with negligible loss of S/N.
- Residual protonated solvents are significantly reduced – need for multiple solvent suppression avoided in most cases.
- Acquisition of data in fully protonated solvents is reasonable.

**Disadvantages**
- Manipulation of 5µL aliquots can tedious.
- Availability of HT platform poor
- Samples of poor solubility
Capillary Probe Data

One Hour acquisition time per sample

Sample in 5µL cell (conc. mg/mL)

<table>
<thead>
<tr>
<th>Concentration (mg/mL)</th>
<th>Sample S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µg (20 mg/mL)</td>
<td>845</td>
</tr>
<tr>
<td>50 µg (10 mg/mL)</td>
<td>440</td>
</tr>
<tr>
<td>25 µg (5 mg/mL)</td>
<td>229</td>
</tr>
<tr>
<td>12.5 µg (2.5 mg/mL)</td>
<td>116</td>
</tr>
<tr>
<td>6.3 µg (1.3 mg/mL)</td>
<td>62</td>
</tr>
<tr>
<td>3.1 µg (0.63 mg/mL)</td>
<td>36</td>
</tr>
<tr>
<td>1.5 µg (0.31 mg/mL)</td>
<td>25</td>
</tr>
<tr>
<td>780 ng (0.16 mg/mL)</td>
<td>18</td>
</tr>
<tr>
<td>390 ng (0.08 mg/mL)</td>
<td>16</td>
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</tbody>
</table>

HOD Saturation
• The microTOF-LC can provide the exact mass of the analyzed sample and therefore access to the sum formula.
• microTOF-LC allows HyStar™ to trigger collection of chromatographic peaks into loops (LC-NMR), or SPE cartridges (LC-SPE™ NMR) based on mass chromatograms
• However, no additional structural information is provided (e.g. fragmentation). This information is sometimes more relevant than the molecular formula.
CryoProbes

Installation of a 600MHz triple resonance $^1\text{H}$/$^{13}\text{C}$/$^{15}\text{N}$ CryoProbe™ system.

Picture taken from presentation made by Dr. Kim Colson at Bruker Biospin
Overall Conclusions

- LC-NMR is an extremely useful tool in very specific instances.
- Additional “hyphenation”, in some cases, provides an enormous amount of pertinent structural information.
- Decreasing the noise floor (cryoprobes) is allowing NMR to routinely analyze samples that were previously impossible by NMR.
Acknowledgements

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