Metabolite Identification in the Real World
Transforming data into knowledge:
Use of HRMS instrumentation in Drug Discovery and Development

Dr. Axel Pähler
DMPK, Non Clinical Safety, pRED, F. Hoffmann-La Roche Ltd.
**Drug Discovery & Drug Development**

*Quantitative metabolite identification workflows*

**Discovery Research (LI/LO): Find, Quantify? & ID**

Hepatic clearance combined with metabolite ID

Parent disappearance kinetics plus relative metabolite formation rates. Can we move from QqQ to HRMS?

Classical metabolite ID study & peak area ratios (no absolute but relative quantification in tissues/species)

**Drug Development: Find, Quantify & ID**

Compare human metabolites to animal species

In vitro metabolism (relative peak are ratios or 14C)

In vivo systemic metabolite exposure (relative peak area ratios, 14C or authentic standards)
Targeted vs. non Targeted Data Acquisition

MS “all in one” or data dependent “true” MS/MS

- \( \text{MS}^E \) mode: Two acquisition functions are monitored in parallel
  - Principally technology independent QqTOF, (q/trap)-FTMS

- \( \text{MS}^E \) “All in one” DDE
- \( \text{IDA (DBS, MDF)} \)
- All data in one injection
- High data quality

Low energy scan function: precursor ions
High energy scan function: fragment ions
Early Metabolite ID
*Shift towards Qual/Quant workflow*

SoM identification for high Cl compounds → Guide rational Drug Design by Medicinal Chemistry

Soft spot ID (top 4 metabolites) → Structure proposal by MS/MS
Metabolite Identification: The Past

Qual analysis driven by structure & expert knowledge

- Knowledge-based Metabolite prediction
- Structure Input
- Priority list for MS/MS & Intensity threshold
- Substrate/metabolite MS/MS Fragmentation
- Analyte (1 .... n)
- MS experiment (1 .... n) Data dependent MS/MS data
- Dataset (1 .... n)
- Result (1 .... n)
From Prediction to Experiment

Data processing and metabolite fishing

Automated data processing (find expected metabolites)

Search for metabolites based on mass defect filters, isotope clustering and possible de-alkylations: Support detection of possible cleavages and unexpected metabolites

Experienced analysts key for data review and interpretation
Post Acquisition Data Processing
Getting most out of the HRMS data

Mass defect filtering:
Data reduction
Facilitate finding drug related material
On-the-fly MMDF available on 5600+
From Identification to Structure
Data processing in Metabolite ID

- Use MS/MS spectra to localize position of biotransformation in the molecule.
- MS/MS spectra interpretation supported by software (based on general fragmentation rules).
- Definitive structure assignment still manual, often Markush-like.
- Definitive structure by NMR.
Metabolite Identification: The Future

Data driven, untargeted post acquisition analysis
Diversity in Applications

Demand for Versatile LC-MS Instrumentation

*High Sensitivity and Speed:*
Assays conducted at low physiological drug concentrations
Need to detect relevant metabolites and biomarkers
All information, all the time (full scan MS, MS/MS)
Diversity in Applications

Demand for Versatile LC-MS Instrumentation

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*Robust, Reliable & Compatible:*
No significant down time
Proven interface and front-end
Ease of method transfer from existing instrumentation
Diversity in Applications
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All information, all the time (full scan MS, MS/MS)

Robust, Reliable & Compatible:
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Ease of method transfer from existing instrumentation

Ease of Operation:
Automated setup and calibration routine
Shift from instrumentalist to DMPK specialist as main user
Ready to go, still highly customizable
Vendor independent
Waters (*.raw), Thermo (*.RAW): Ion-Trap/Orbitrap, Agilent (*.d), SCIEX (*.wiff)

Automatic peak detection using multiple algorithms
Targeted/unknown analysis, MDF, fragmentation pattern analysis, noise removal, list of mass changes (Phase I and Phase II reactions), background correction (unexpected transformations, des-alkylations)

Fragmentation analysis
Hypothetical fragmentation, comparison parent vs metabolite fragment spectra assignment of score (matches vs mismatches) → structure proposal + scoring

Automatic report system
Customizable excel file format (.xlsx), Database format (.xml format), Markush structure drawing from metabolite structures, Metabolite schemes (.pdf)

Qualitative and quantitative results
Reality Check: P450 Phenotyping

Combined Quant/Qual workflow

**Goal:**
1. Identify P450 enzyme involved in drug clearance (*quant*)
2. Identify metabolites formed by individual enzymes (*qual & quant*)

**Tools:**
1. Matrix samples from 8 time point and 6 enzymes (48 samples)
2. AB Sciex 5600+ QqTOF mass spectrometer & fast gradient HPLC
3. IDA method based on intensity thresholds, DBS active, no inclusion list
4. AB Sciex MetabolitePilot 1.5 software

**Process:**
1. Identify metabolites from individual samples
2. Matching across matrices & time points
3. Flexible reporting
### Case Study: P450 Phenotyping

*MetabolitePilot*

#### Met ID process

1. Peak finding strategy: predicted metabolites (Phase I biotransformation list)
2. Generic parameters (threshold, mass tolerance, peak shape, …)
3. Compound specific parameters (C-heteroatom bond cleavage, isotope pattern, product ion and neutral losses)
4. Formula prediction (include MS/MS)

#### Results: CYP3A4 for drug D

<table>
<thead>
<tr>
<th>Peak ID</th>
<th>Name</th>
<th>Formula</th>
<th>m/z</th>
<th>ppm</th>
<th>R.T. (min)</th>
<th>Peak Area</th>
<th>% Area</th>
<th>% Score</th>
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<tbody>
<tr>
<td>Parent</td>
<td>C18H25NO</td>
<td>272.2005</td>
<td>-1.4</td>
<td>4.26</td>
<td>2.23E+06</td>
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<td>M7</td>
<td>Di-Oxidation</td>
<td>C18H25NO3</td>
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<td>4.10</td>
<td>4.95E+05</td>
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<td>M12</td>
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<td>C17H23NO</td>
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<td>Loss of CH2+Oxidation</td>
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<tr>
<td>M17</td>
<td>Demethylation and Methylene to Ketone</td>
<td>C17H21NO2</td>
<td>272.1636</td>
<td>-3.4</td>
<td>4.45</td>
<td>5.98E+04</td>
<td>0.9</td>
<td>87.4</td>
</tr>
</tbody>
</table>

- Excellent mass accuracy
- Very good match with manual data inspection
- Very few false negative results (threshold setting, MS/MS data was triggered)
Case Study: P450 Phenotyping

MetabolitePilot, flexible reporting options

Drug D: P450 phenotyping

Drug D: Individual Enzyme

parent
M16
M12
M7
In-silico prediction based on P450 binding site and chemical reactivity of drugs towards oxidation

SoM Prediction:
Generally good prediction of soft spot
70-80% success
(experiment within top 3)

Used for prediction of likely SoM in conjunction with experimental MS/MS data and manual data interpretation

*MetaSite* (Molecular Discovery Ltd)
Making full Use of MS/MS Data

Blending computation chemistry and LC-MS/MS Data

Mass MetaSite (Lead Molecular Design)
Finally: What happens to all the data?

Diversity is nice, but…

Some processes are “harmonized”. But need to remember where I parked my car!

Traffic may become terrible…
Cross Platform DMPK Setup
Accommodating various workflows

- Acquisition PC
  - Vendor X 1\textsuperscript{st} gen.
  - Vendor X 2\textsuperscript{nd} gen.
- LC-MS
  - Vendor Y 1\textsuperscript{st} gen.
- PC Data Evaluation
  - Sample Lists
  - Batch Process
  - Metabolite Pilot MassMeta Site
- Web Browser
  - Protocol Instance
  - Protocols
  - Pending Experiments
  - Approved Experiments

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- Web Browser
Conclusions & Outlook

• Emerging technologies (hardware & software) have facilitated workflows in Drug Discovery and Development
  – Unbiased acquisition and post-acquisition data processing
  – “All-in-one” approaches
  – Structure-based data analysis

• Qual/Quant workflows have become routine
  – Combined clearance / Met ID studies
  – Still need for “true” (not relative) quantitative results
  – Benefits of SRM acquisition (linearity, S/N)
  – SWATH & HRMS close to SRM performance

• Structure analysis
  – Computational approaches have emerged
  – Interrogation of LC-MS/MS data most promising
LC-MS/MS applications in biomarker research

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Content

• Introduction
• Experimental set-up
• Results: Method comparison between QTRAP 4000 and TTOF 5600+
• Biological application
• Conclusion
Introduction – Biomarker Research

• Biomarker:
  Endogenous molecules utilized for objective diagnosis or monitoring of physiological, pathophysiological or pathological processes
  – Clinic: Observation or identification of disease state of patients
  – Pre-clinic: Testing of effect / side effect of drug candidates

• Critical characteristics:
  – Reliability (selectivity/sensitivity/robustness)
  – Mode of measurement (invasive vs noninvasive)
Introduction – Biomarker Research

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  – Reliability (selectivity/sensitivity/robustness)
  – Mode of measurement (invasive vs noninvasive)

Detection of severe adverse effect as liver toxicity (“DILI”)
Introduction – Isoprostanes as Biomarkers *in vivo* and *in vitro*

- Generation: Arachidonic acid metabolism

  ![Diagram](https://via.placeholder.com/150)

  - Coronary heart disease
  - Drug induced Oxidant injury
  - Atherosclerosis

  ![Diagram](https://via.placeholder.com/150)

  - Isoprostanes
  - Prostaglandins

- Circulation in plasma, excretion to urine \(\rightarrow\) potential target of investigation

- Generated in cells exposed to oxidative stress i.e. **hepatocytes** when cause is drug \(\rightarrow\) suitable *in vitro* matrix
Experimental –
How to detect and quantify isoprostanes

1. Preparation step: Extraction of prostanoids from matrix
   - Solid phase extraction
   - Liquid liquid extraction
   - Immunoaffinity assays

2. Quantification step: Separation / Focusing of similar analytes + detection
   - LC-MS(/MS)
   - GC-MS
   - Immunoaffinity assays

   - Combination of 1 and 2 favorable to avoid work up steps
     ⇒ column switching LC set up for analyte enrichment
Experimental – Setup for integrated isoprostane measurement: Column switching LC

- Injection volume: 500 µl
- Run time 13.5 min, flow rate 0.4 mL/min
- Flush on trapping column + online dilution
- Backflush of retained analytes to analytical column + gradient elution
- MS/MS detection:
  - 4000 QTRAP (SRM)
  - 5600+ TTOF (FS + PIS → “HR-SRM”)

TC: YMC AQ, 20 x 2.1 mm, 5 µm, YMC Europe
AC: Atlantis T3, 100 x 2.1 mm, 1.8 µm, Waters
Experimental –
MS options for QqQ versus TTOF instruments

• Analytical requirements
  – 10 – 12 data points per peak
  – High selectivity
  – Wide linear range

• Available scan techniques
  – QqQ: Selected Reaction Monitoring (also: MRM)
    best to exclude interferences and achieve linear calibration ranges of
    several orders of magnitude
  – TTOF: 1. Full scan + data dependent scan
    → number of scans too small (many experiments)
  2. Full scan + product ion scan
    → allows to pick matching precursor + product ion
### Optimization of Analytes

- QTRAP: Compound specific SRM transitions; TTOF: no optimization needed

<table>
<thead>
<tr>
<th>Short name (entry)</th>
<th>structure</th>
<th>precursor Q1 m/z</th>
<th>fragment Q3 m/z</th>
<th>DP (V)</th>
<th>CE (V)</th>
<th>CXP (V)</th>
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<tr>
<td>5-iso PF&lt;sub&gt;2α&lt;/sub&gt;-VI</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>353.1</td>
<td>193.0</td>
<td>-65</td>
<td>-34</td>
<td>-13</td>
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<td>(1)</td>
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<td>353.1</td>
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<td><img src="image3.png" alt="Structure" /></td>
<td>351.1</td>
<td>315.0</td>
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<td>351.1</td>
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<td><img src="image4.png" alt="Structure" /></td>
<td>351.1</td>
<td>315.0</td>
<td>-55</td>
<td>-18</td>
<td>-11</td>
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<td>(4)</td>
<td></td>
<td>351.1</td>
<td>271.1</td>
<td>-50</td>
<td>-25</td>
<td>-12</td>
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<td>15(R) PD&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>351.1</td>
<td>315.0</td>
<td>-55</td>
<td>-18</td>
<td>-11</td>
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<tr>
<td>(5)</td>
<td></td>
<td>351.1</td>
<td>271.1</td>
<td>-50</td>
<td>-25</td>
<td>-12</td>
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<td>dihydro-keto (6)</td>
<td><img src="image6.png" alt="Structure" /></td>
<td>351.1</td>
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<td>(8)</td>
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<td>337.0</td>
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<td>-16</td>
<td>-17</td>
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</tbody>
</table>
Results – Overview reference spectra (5 ng/mL)

QTRAP

TTOF

Unit resolution
- 355-319
- 355-275
- 353-309
- 353-193
- 353-115
- 351-333
- 351-315
- 351-271

XIC precursor ± 2.5 mmu
- 351.21770
- 353.23335
- 351.23335
- 355.24470
Results – Overview reference spectra (5 ng/mL)

transition: m/z 353.2 → 115.0

Extracted Product Ion: m/z 115.040 ± 0.0025

(1) – (4): Isobaric F₂α prostaglandins
Results –
Comparison of Calibration data

**QTRAP: 5isoPF$_{2\alpha}$-VI**
- Calibration from 0.02 to 10 ng/mL
- $y=0.0009152x-0.02752$
- $R^2=0.9892$

**TTOF: 5isoPF$_{2\alpha}$-VI**
- Calibration from 0.005 to 10 ng/ml
- $y=0.0001971x-0.01568$
- $R^2=0.9985$
Results –
Comparison of Calibration data

QTRAP: 5isoPF$_{2\alpha}$-VI
- Calibration from 0.02 to 10 ng/mL
- $y=0.0009152x-0.02752$
- $R^2=0.9892$

20 pg/mL:
- SRM 353.2-115.0
- S/N: 3.6

TTOF: 5isoPF$_{2\alpha}$-VI
- Calibration from 0.005 to 10 ng/ml
- $y=0.0001971x-0.01568$
- $R^2=0.9985$

5 pg/mL:
- XIC of precursor
- S/N: 31.8
- XIC of m/z 155.04
- S/N: 9.9
Results –
Comparison of Calibration data

QTRAP: Prostaglandin D₂
• Calibration from 0.02 to 10 ng/mL
• \( y = 0.0009727x - 0.04004 \)
• \( R^2 = 0.9791 \)

TTOF: Prostaglandin D₂
• Calibration from 0.005 to 10 ng/ml
• \( y = 0.0009642x - 0.6372 \)
• \( R^2 = 0.9987 \)
Results –
Comparison of Calibration data

QTRAP: Prostaglandin D$_2$
- Calibration from 0.02 to 10 ng/mL
- $y=0.0009727x-0.04004$
- $R^2=0.9791$

TTOF: Prostaglandin D$_2$
- Calibration from 0.005 to 10 ng/ml
- $y=0.0009642x-0.6372$
- $R^2=0.9987$

20 pg/mL:
SRM 351.2-271.2
S/N: 1.5

5 pg/mL:
XIC of precursor
S/N: 15.3

XIC from 20130109MT.wiff (sample 3) - STD_20 µM _1_3, -MRM (27 transitions): 351.1 /  271.1 Da

SRM 351.2 - 271.2
S/N: 10.4
Results –
Comparison of Calibration data

**QTRAP: Prostaglandin D₂**
- Calibration from 0.02 to 10 ng/mL

**TTOF: Prostaglandin D₂**
- Calibration from 0.005 to 10 ng/ml

Transition:
- m/z 351.2 → 271.2
- S/N 9.5 (➔ 200 pg/mL)

Extracted mass:
- m/z 271.2067 ± 0.0025
- S/N 10.4 (➔ 5 pg/mL)
Biological Application for Oxidative Stress Measurement: Rat hepatocytes

15R PD2 (5)

PD2 (4)

iPF2α-VI (1)

Peak 4

Concentration (pg/ml)

Analyte Peak Ratio
Conclusion

• The TripleTOF 5600+ can be used for quantitative biomarker analysis
• Most suited scan technique: 
  FullScan combined with ProductIonScan (“HR-MRM”)
    – Linear range: Comparable with triple quadrupole type instruments
    – Sensitivity gain in comparison with 4000 QTRAP for presented application
    – Selectivity: Achieved via accurate mass FullScan mode
      Further enhanced by the use of specific product ions
    – Flexibility in post acquisition data evaluation
      • Define processing parameters
      • Most suitable product ion
      • Combined XIC of several selective product ions
## Acknowledgements

<table>
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<tr>
<th>DMPK Roche</th>
<th>Discovery Technologies Roche</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Andreas Brink</td>
<td>• Iris Ruf</td>
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<tr>
<td>• Sandrine Simon</td>
<td>• Martin Binder</td>
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<td>• Ismael Zamora</td>
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<td>• Fabien Fontaine</td>
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<td>• Blanca Serra</td>
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<td>• Esra Nurten Cece</td>
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Thank you for listening!

Questions