Confident characterization and identification of glucuronide metabolites using diagnostic fragments from orthogonal MS/MS data

Rahul Baghla I Manager Scientific Marketing

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The Power of Precision

Presentation outline

- Challenges with small molecule metabolite identification
- Introduction to instrumentation and electron activated dissociation (EAD) technology
- Diagnostic fragments and confident structure assignment using EAD
- Conclusions
- Acknowledgments

Challenges with identification of small molecule metabolites

Extensive fragmentation coverage for small molecules

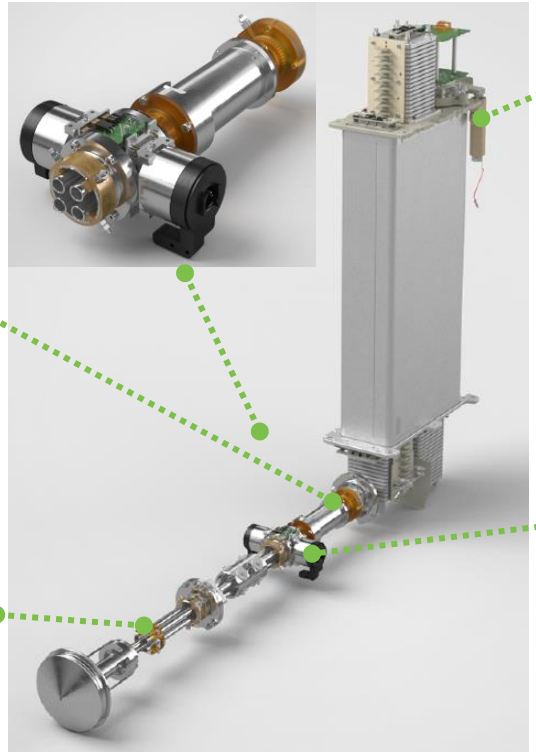
- Low abundance of relevant circulating metabolites
- Ionization efficiency in complex matrices
- Qualitative information and a lack of fragmentation coverage with CID
- Phase II conjugations with non-determinative CID fragments to confirm
  I. Type of conjugation (relies on accurate mass and observed neutral losses)
  II. Glucuronide position (for example, N-, O-, acyl-)

System hardware advancements

New

Zeno trap

- Improved MS/MS duty cycle gain of ≥90%



New

Wide dynamic range

10-bit ADC with 40 GHz TDC timing and a 25 psec detection rate

- 5 GHz
- High-speed pulse counting to maintain resolution and mass accuracy up to 133 Hz and a linear dynamic range (LDR) of over 5 orders of magnitude

New

Complementary fragmentation

- Increased sensitivity using the EAD cell

New

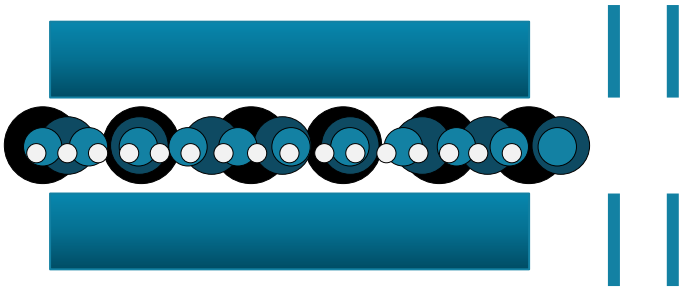
New Q0 design

- Improved ion transmission and maintenance

What is duty cycle?

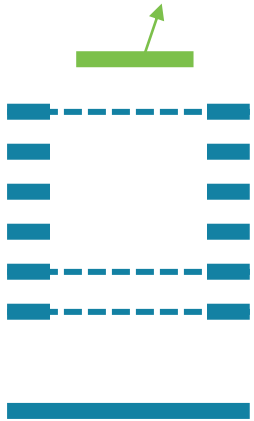
… And why is duty cycle important?

- What is duty cycle?
  - % of ions injected into the TOF
  - Typically, ~5-25%
    - Dependent on
      - Fragment mass
      - Scan range upper limit
- Why is duty cycle not 100%?
  - Ion losses occur when combining:
    - Pulsed measurement technique
      - TOF
    - Continuous ion beam
      - Quadrupole



Quadrupole region

Continuous ion beam



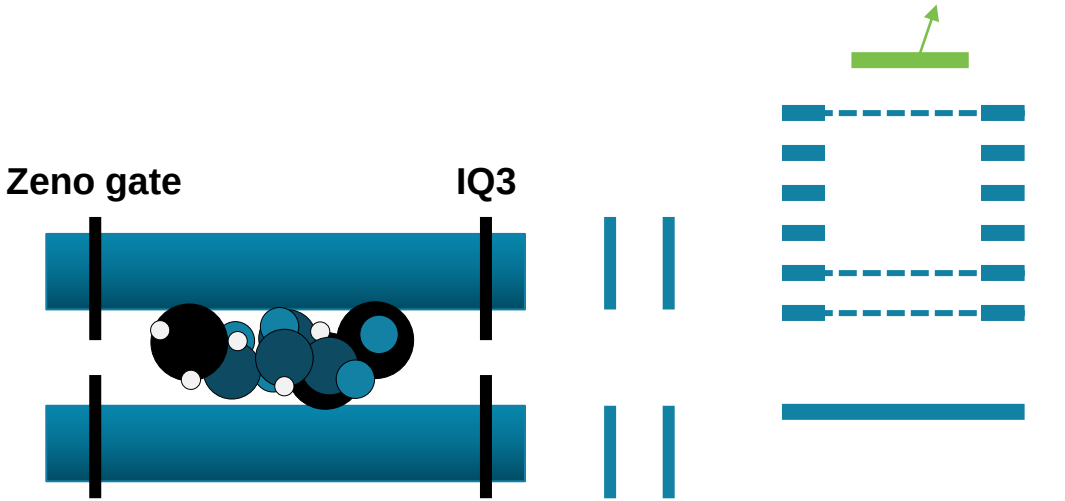
TOF accelerator

Ion pulsing

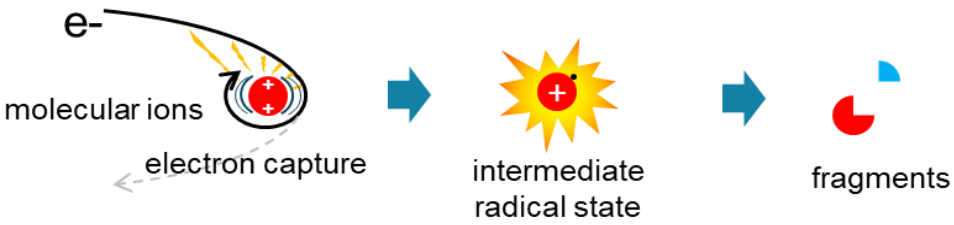
Zeno trap

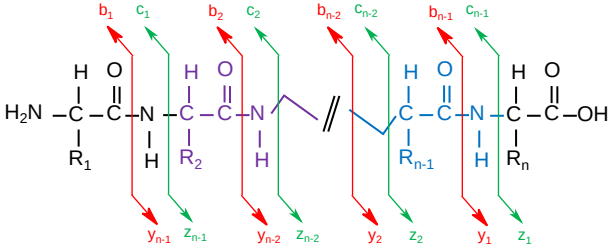
FOR SENSITIVITY GAINS IN MS/MS

- The Zeno trap provides control of the ion beam from the collision cell into the TOF accelerator
- Ions exit the Zeno trap in an ordered release based on potential energy
  - Ions are generally released from a high *m/z* to a low *m/z*
  - All ions now arrive in the TOF accelerator at the same time and location

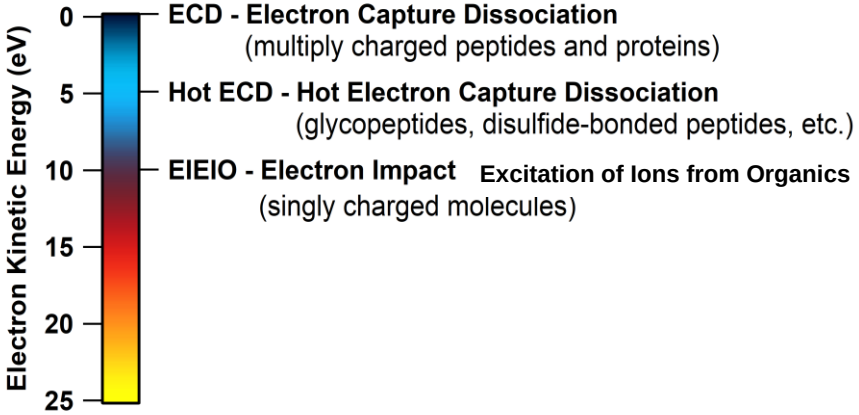


EAD technology





- Free electrons are captured by ions and form a radical state that then fragments
- Electrons introduced with different energies will induce fragmentation in different molecule types
- EAD cell enables you to perform ECD, hot ECD and EIEIO in a single instrument



T. Baba *et al. Anal.Chem*. 2014

EAD leads to more confident structure assignment

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Experimental details

Verapamil, buspirone, darunavir and nefazodone metabolism

- **Sample preparation:**
  - Verapamil, buspirone, darunavir and nefazodone were incubated at 37ºC in rat hepatocytes at a starting concentration of 1µM
- **ExionLC AD system:**
  - Column: Phenomenex Luna Omega Polar C18 column (2.1 x 100 mm, 3 µm, 100 Å)
  - Mobile phase A: 0.1% (v/v) formic acid in water
  - Mobile phase B: 0.1% (v/v) formic acid in acetonitrile
- **ZenoTOF 7600 system:**
  - The samples were analyzed in data dependent acquisition (DDA) mode using Zeno CID DDA and Zeno EAD DDA
  - CID conditions: CE of 35 V with CE spread of 15 V
  - EAD conditions for verapamil, buspirone and nefazodone:
    - Electron kinetic energy: 10 eV
    - Electron beam current: 5000 nA
  - EAD conditions for darunavir:
    - Electron kinetic energy: 11 eV
    - Electron beam current: 8000 nA

Chromatographic gradient.

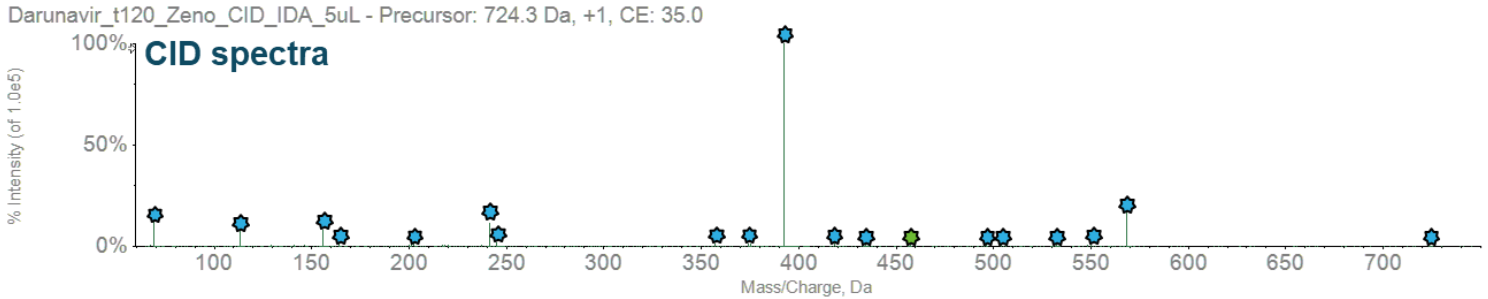
Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.00	*95*	*5*
0.50	*95*	*5*
1.50	*85*	*15*
3.50	*50*	*50*
4.75	*5*	*95*
5.75	*5*	*95*
5.80	*95*	*5*
6.50	*95*	*5*

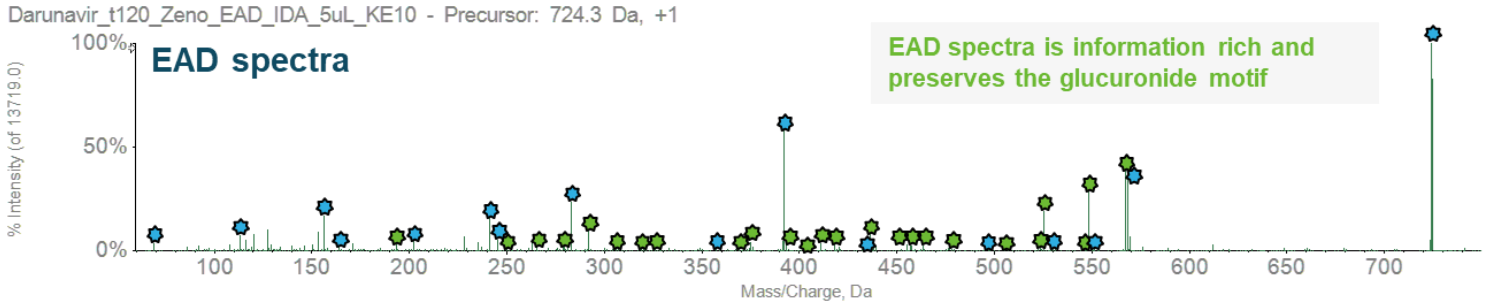
Injection volume: 5 µL

Darunavir-o-glucuronide fragmentation comparison

Alternative fragmentation provides more structural information

Darunavir was incubated at 37ºC in rat hepatocytes at a starting concentration of 1µM





EAD spectra is information rich and preserves the glucuronide motif

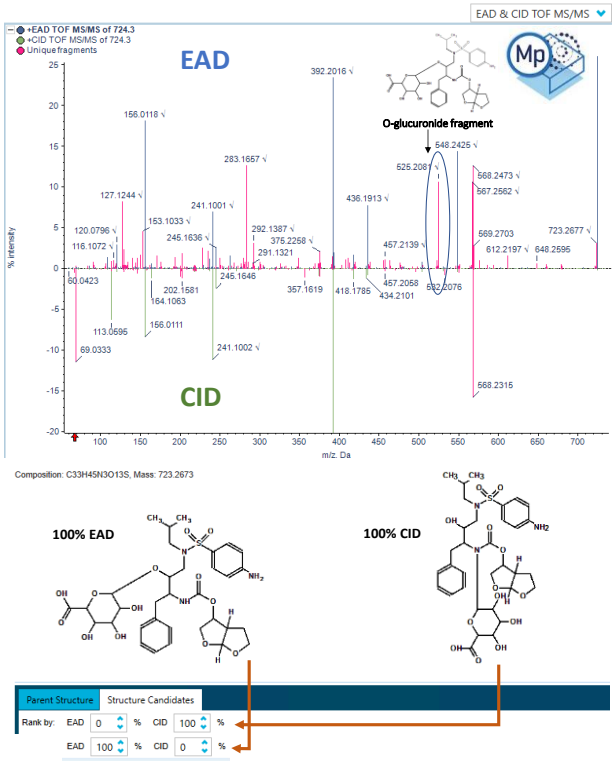
Fragments which **do not** contain glucuronide

Fragments which **do** contain glucuronide

What does EAD offer?

COMPLEMENTARY AND INFORMATION-RICH STRUCTURE ELUCIDATION WITH EAD

- **CID is a soft, thermal fragmentation technique**
  - Often leads to cleavage of most labile sites
  - Results in few diagnostic fragments
  - Insufficient cleavage without protonation sites
- **EAD offers complementary fragmentation information**
  - Radical dissociation mechanism
  - Can maintain labile modifications
  - Potential to result in many diagnostic fragments



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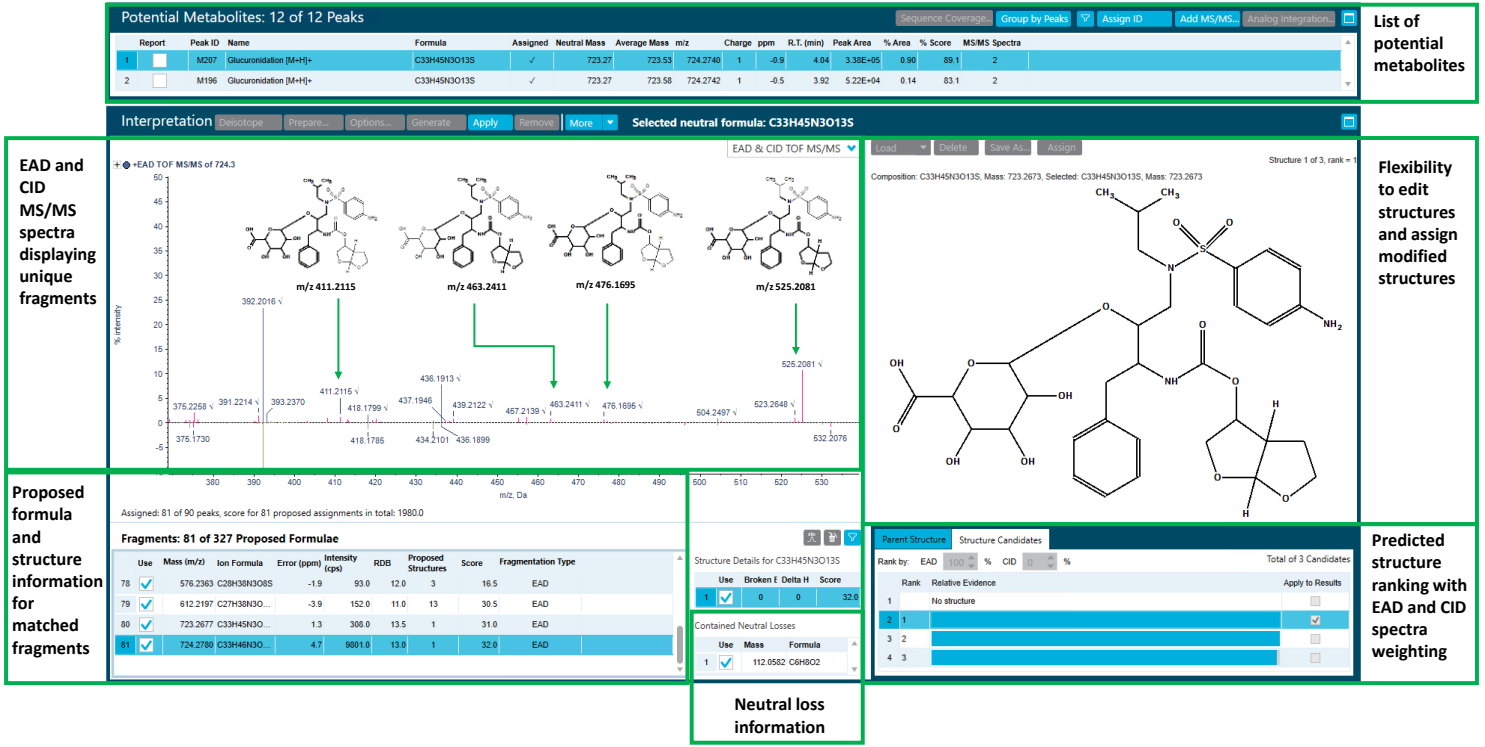
Interpretation pane from Molecule Profiler software

Features of Molecule Profiler software

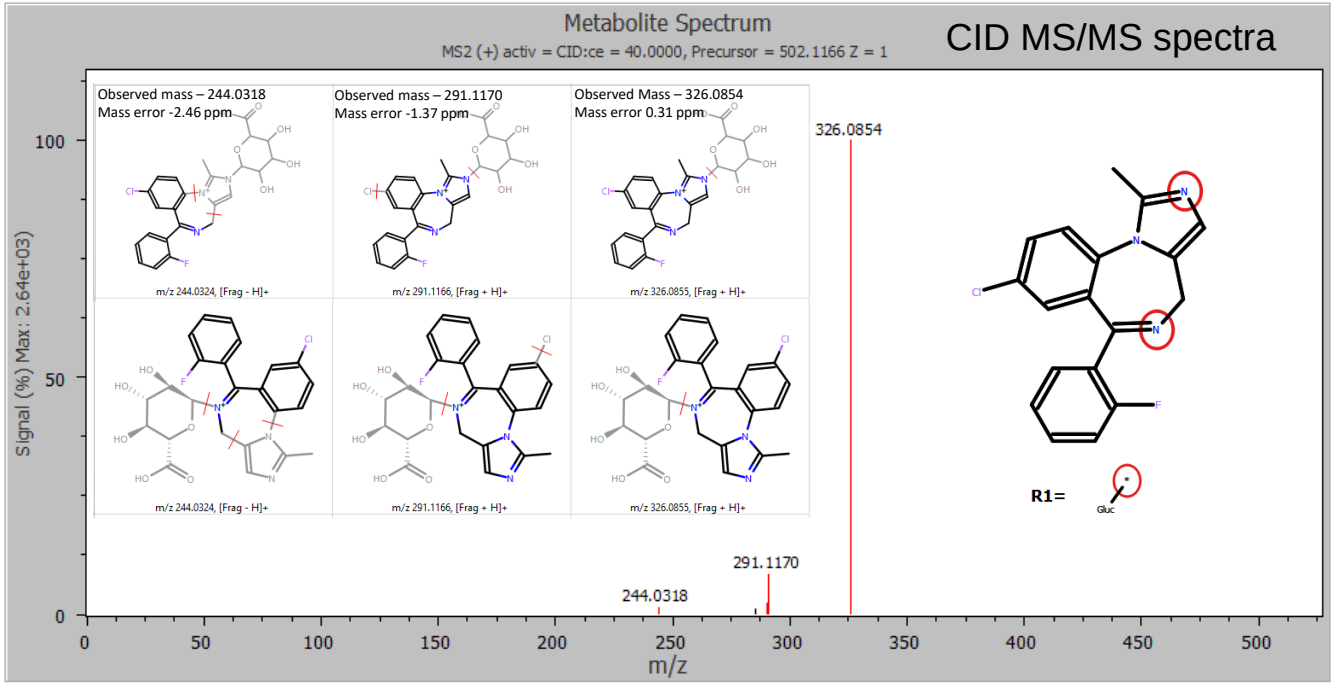


- The interpretation pane displays detailed information on both spectra and unique fragments, along with user options for modifying and reassigning metabolites
- EAD spectra show unique fragments (m/z 476.1695, 463.2411, 411.2115 and 525.2081), supporting the identification of o-glucuronide darunavir metabolite

Identification of midazolam N-glucuronide

CID FRAGMENTS ORIGINATED FROM THE PRIMARY MIDAZOLAM STRUCTURE

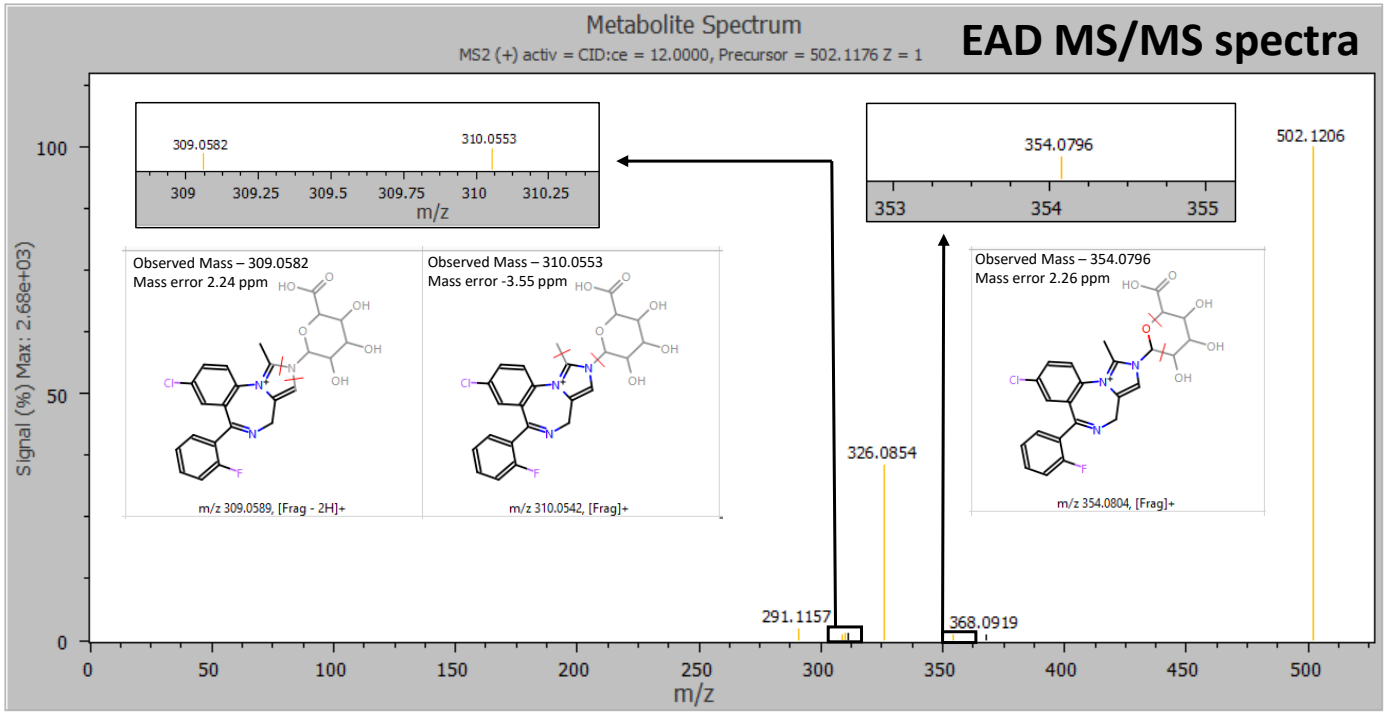
- The 240-min incubation sample showed a significant peak for midazolam N-glucuronide at a retention time of 4.21-min. Zeno CID DDA did not indicate any specific fragments for midazolam N-glucuronide.
- Instead, fragments from CID originated from the primary midazolam structure. The Mass-Metasite software predicted 2 possible sites of metabolism with CID MS/MS spectra.

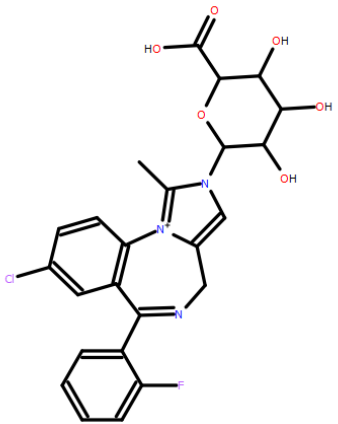


Characterization of N-glucuronide midazolam

UNIQUE FRAGMENTS USING EAD ENABLED CONFIDENT IDENTIFICATION

- EAD showed unique fragments at *m/z* 309.0589 and *m/z* 354.0804 and confirmed N-glucuronide conjugation on the imidazole ring





N-glucuronide midazolam

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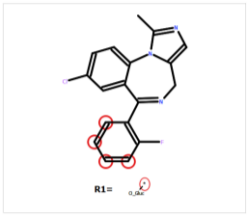
Aromatic/aliphatic hydroxylation and o-glucuronide conjugation

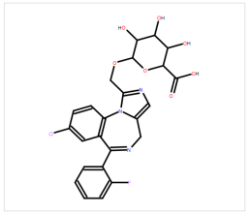
EAD ENABLES SITE-SPECIFIC IDENTIFICATION

- Due to the absence of any glucuronide-specific fragments with CID, 4 possible sites of metabolism on the benzene ring of midazolam were predicted
- Site-specific information from EAD enabled the identification of the peak as 1-hydroxymidazolam o-glucuronide



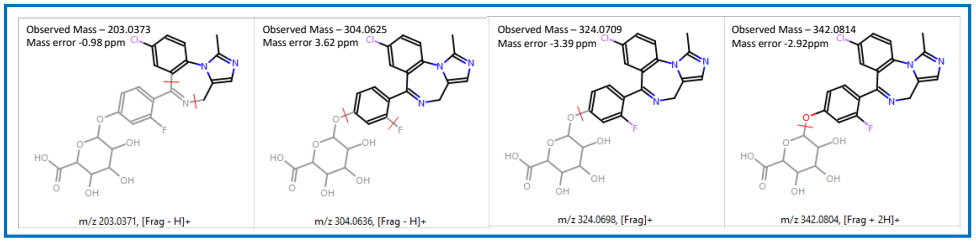
CID

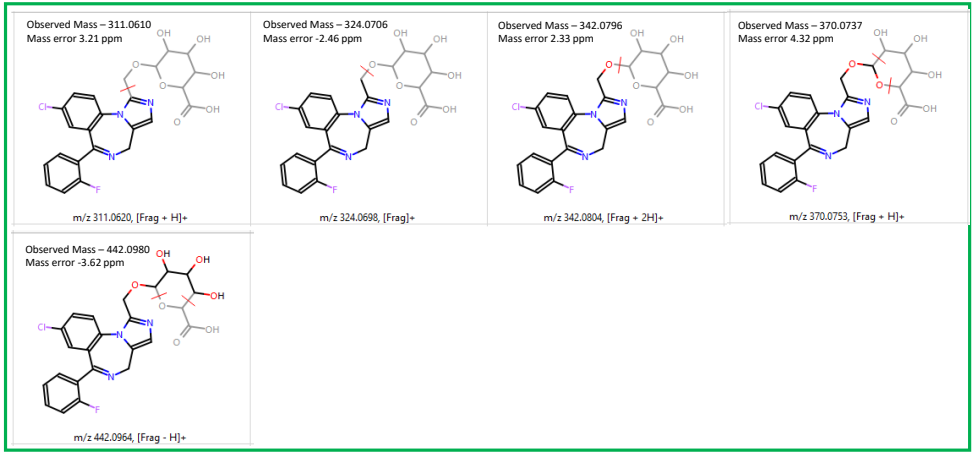
EAD



1-hydroxymidazolam o-glucuronide

Aromatic/Aliphatic Hydroxylation - Glucuronidation

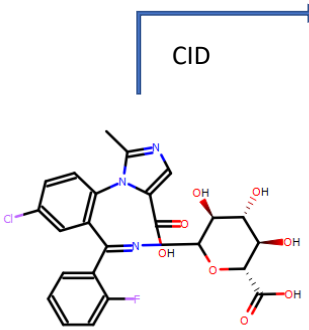




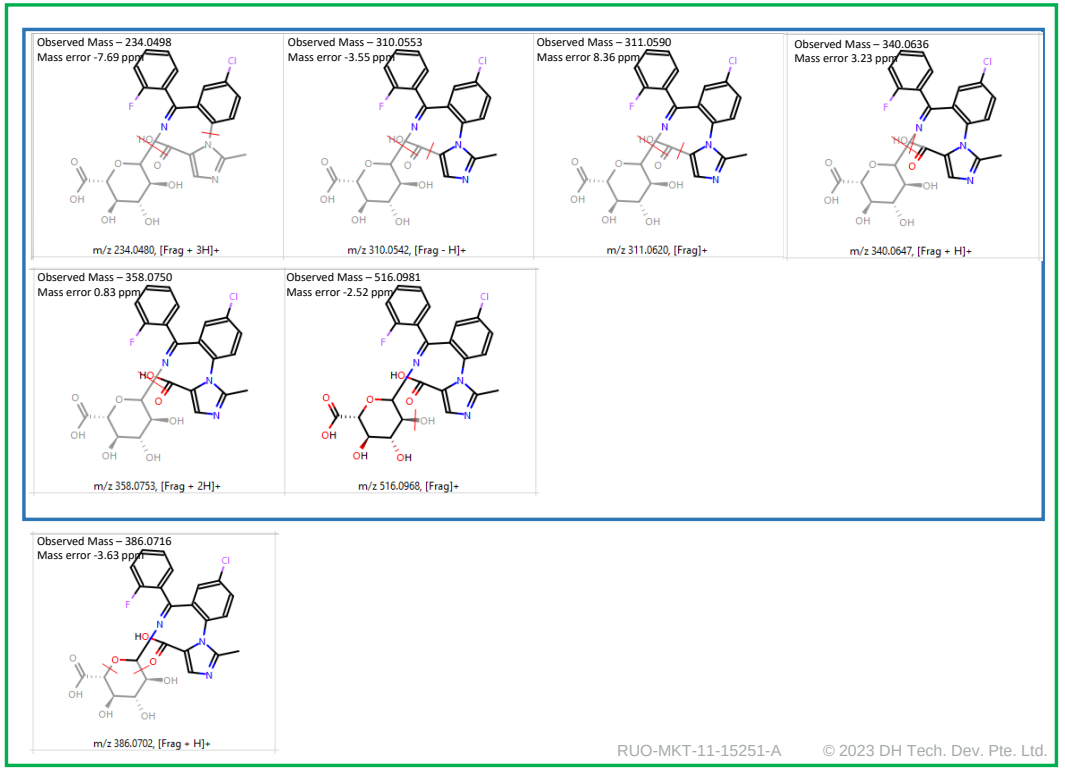
Identification of an N-dealkylated midazolam N-glucuronide metabolite

UNIQUE FRAGMENTS USING EAD ENABLED CONFIDENT IDENTIFICATION

- EAD provided rich MS/MS spectra, enabling the identification of an N-dealkylated midazolam N-glucuronide metabolite
- EAD spectra included all fragments generated using CID along with a glucuronide-specific fragment at *m/z* 386.0702, confirming the site of conjugation



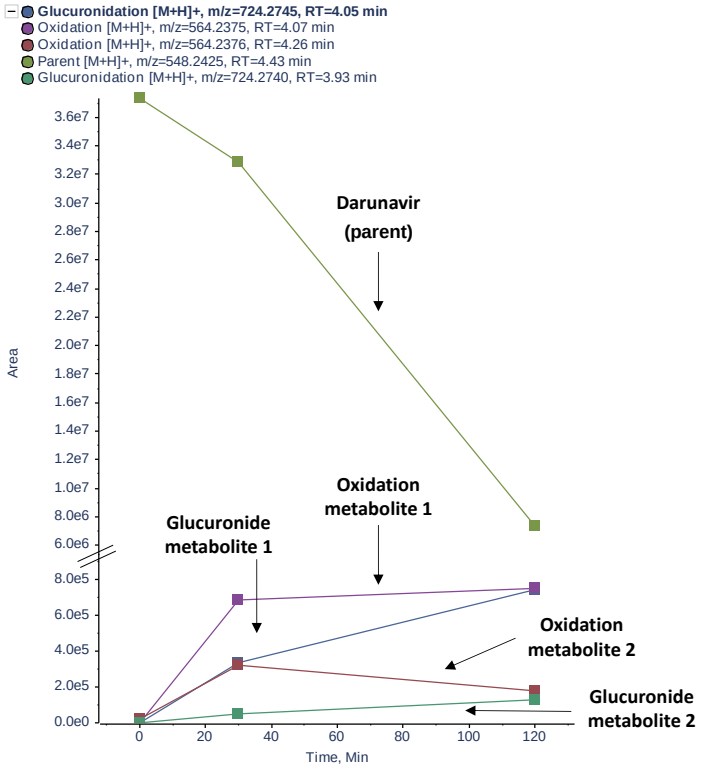
Glucuronidation (tertiary amine) – N Dealkylation



Correlation analysis

Study of drug metabolism over time

- A correlation analysis was performed for drugs and metabolites using peak area based on precursor ion peaks from a TOF MS experiment
- The relative quantitation of drugs and metabolites were correlated and revealed a decrease in drug concentration relative to an increase in different metabolite concentrations over time
- The high mass accuracy enabled the confident prediction of metabolites in an in vitro metabolism study



Metabolite and fragment identification were performed with <10 ppm mass error

Conclusion

- Electron activated dissociation combined with the Zeno trap shows great promise as a new fragmentation technique for delivering information that is complementary to CID
- Using examples such as darunavir and midazolam, n- and o-glucuronide structures along with dealkylation and aromatic/aliphatic hydroxylation glucuronide metabolites were identified with good mass accuracy (MS and MS/MS level)
  - Structures were successfully assigned with unique fragmentation pathways using EAD
- Quick analytical methodology with the software-aided approach for metabolite identification was demonstrated to support high throughput workflows for drug discovery process.

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Thank You