# Automated workflow for metabolite identification and microsomal clearance analysis using collision-induced dissociation (CID) and electron-activated dissociation (EAD)

***Automated identification and microsomal clearance analysis using the Molecule Profiler software***

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This technical note describes a software-aided automated workflow to study microsomal clearance and metabolite identification using the ZenoTOF 7600 system paired with Molecule Profiler software. Confident metabolite structure assignments were performed using both CID and EAD data. TOF MS-based relative quantification was used to generate clearance profiles. The more informative MS/MS spectra provided by EAD aided in the software-based identification of drug metabolites to support drug development.

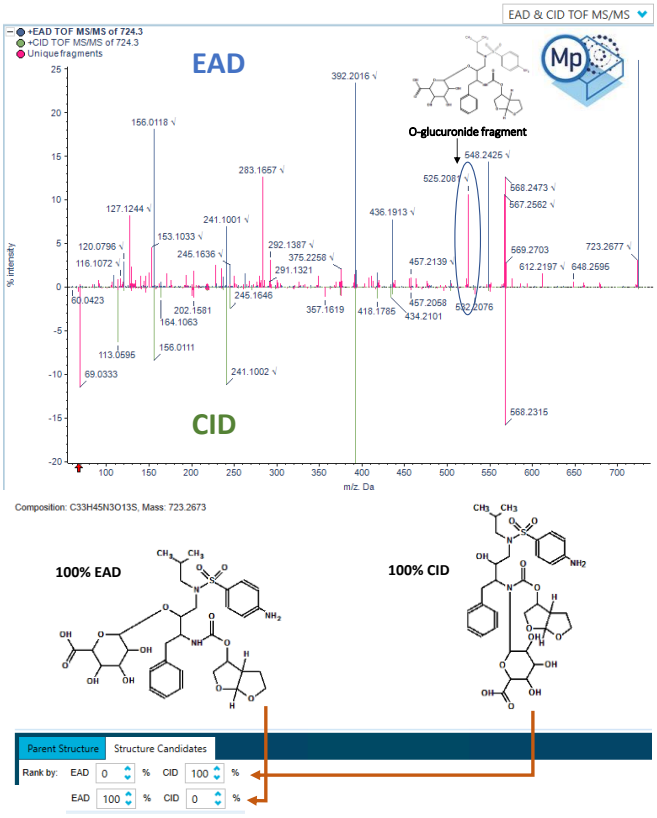


**Figure 1. Panel view of the MS/MS spectra and structure assignments using the Molecule Profiler software.** The software displayed an inverted overlay of the spectra from EAD and CID with unique fragments (red) and assigned possible structures based on spectra weightage. A unique fragment at m/z 525.208 was observed in the EAD spectra, supporting the metabolite identification as an o-glucuronide.

Early drug discovery microsome assays can be used to predict metabolic clearance rates and identify sites of metabolism. LC-MS instruments are commonly used to conduct these measurements as it provides quantitative and qualitative data with sufficient sensitivity, particularly for unknown metabolite identification. Data were generated by employing an advanced metabolite identification workflow using the ZenoTOF 7600 system and Molecule Profiler software. An end-to-end workflow was used to study quantitative clearance profiles and qualitative metabolic soft spots, making it ideal for accelerating the early drug discovery process.

Molecule Profiler software now supports the consolidation and ranking of structures based on EAD and CID data (Figure 1), making it an ideal tool for comparing MS/MS spectra to identify unique fragments in a single results file.

## Key features for the study of metabolites using the ZenoTOF 7600 system with Molecule Profiler software

- **Confident structure assignments:** Analyze information-rich EAD spectra and CID spectra in a single results file to achieve more confident structure assignments for drug metabolites
- **Comprehensive and simultaneous qualitative analysis and relative quantification:** Acquire and process qualitative and quantitative data for analyzing metabolites and microsomal clearance using SCIEX OS software integrated with the ZenoTOF 7600 system
- **Quick metabolite identification and correlations:** Perform quick and efficient software-aided identification and correlation studies of drug metabolites using Molecule Profiler software with the ZenoTOF 7600 system
- **Streamlined data acquisition and processing workflow:** Develop confident structure-metabolic stability relationships for drugs utilizing a quick and easy-to-use workflow from data acquisition to analysis

## Methods

***Sample preparation:*** Darunavir was incubated at 37°C in rat hepatocytes at a starting concentration of 1µM. Samples were removed from incubation and quenched with acetonitrile at 0-, 30- and 120-minute intervals for analysis.

***Chromatography:*** Separation was performed on a Phenomenex Luna Omega Polar C18 column (2.1 x 150 mm, 3 µm, 100 Å) at a temperature of 40°C. Mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was 0.1% (v/v) formic acid in methanol. A 5 µL injection was used for analysis.

The chromatographic gradient conditions used are summarized in Table 1.

**Table 1. Chromatographic gradient.**

| Time (min) | Mobile phase A (%) | Mobile phase B (%) |
|---|---|---|
| *0.0* | *95* | *5* |
| *0.5* | *95* | *5* |
| *1.5* | *85* | *15* |
| *3.5* | *50* | *50* |
| *4.75* | *5* | *95* |
| *5.75* | *5* | *95* |
| *5.8* | *95* | *5* |
| *6.5* | *95* | *5* |

***Mass spectrometry:*** The samples were analyzed using the data-dependent acquisition (DDA) method with Zeno CID DDA and Zeno EAD DDA on the ZenoTOF 7600 system. The source and gas conditions used are summarized in Table 2. The method conditions used are summarized in Table 3.

**Table 2. Source and gas conditions.**

| Parameter | Setting |
|---|---|
| *Curtain gas* | *40 psi* |
| *Ion source gas 1* | *55 psi* |
| *Ion source gas 2* | *65 psi* |
| *CAD gas* | *7* |
| *Ion spray voltage* | *5500 V* |
| *Source temperature* | *500°C* |

**Table 3. Zeno DDA parameters.**

| Parameter | Setting |
|---|---|
| *Method duration* | *6.5 min* |
| *TOF MS start-stop mass* | *100–1000 Da* |
| *Maximum candidate ions* | *8* |
| *Accumulation time (TOF MS)* | *0.05 s* |
| *TOF MS/MS start-stop mass* | *60–1000 Da* |
| *Accumulation time (TOF MS/MS)* | *0.06 s* |
| *Collision energy (CID)* | *35 V* |
| *Collision energy spread (CID)* | *15 V* |
| *Electron kinetic energy (EAD)* | *11 eV* |
| *Electron beam current (EAD)* | *8000 nA* |

***Data processing:*** SCIEX OS software, version 3.0 was used for data acquisition. The Molecule Profiler software was used to predict biotransformation sites using Zeno CID DDA and Zeno EAD DDA data.

## Metabolite identification using Molecule Profiler software

Several phase 1 and phase 2 metabolites were identified and studied using Zeno CID and Zeno EAD data. Molecule Profiler software enabled the processing and analysis of Zeno CID and Zeno EAD data in a single file.

A 30-minute incubation sample of darunavir showed 12 potential phase I and phase II metabolites. The results panel from Molecule Profiler software displays an intuitive and detailed view of the selected metabolite, including its assigned structure, score, extracted ion chromatogram (XIC) and TOF MS and MS/MS spectra information for CID and EAD.

Interpretation of the site of metabolism was enabled by the automated assignment of the structures based on the relative weighting of Zeno EAD and Zeno CID spectra on a scale of 1-100%. The software also provides the flexibility to modify structures in the interpretation pane and the total score for the modified structures. Figure 2 shows the overview of the results panel, where users can view the list of potential metabolites and an overview of assigned structures and scoring information. Data can be presented with TOF MS or MS/MS spectra and XIC views. The software also displays the mass defect and isotope pattern of the metabolites. Figure 3 shows the interpretation pane with more detailed information on product ion matches for both spectra, unique fragments and user flexibility to modify and re-assign the metabolite structures. EAD spectra show unique fragments (m/z 476.1695, 463.2411, 411.2115 and 525.2081) supporting metabolite identification as o-glucuronide darunavir (Figure 3). Figure 4 shows higher scoring and more confident structure assignment for oxidation metabolite with EAD compared to CID data. The software also ranks all possible structures based on the total score for every metabolite structure.[1,2,3]

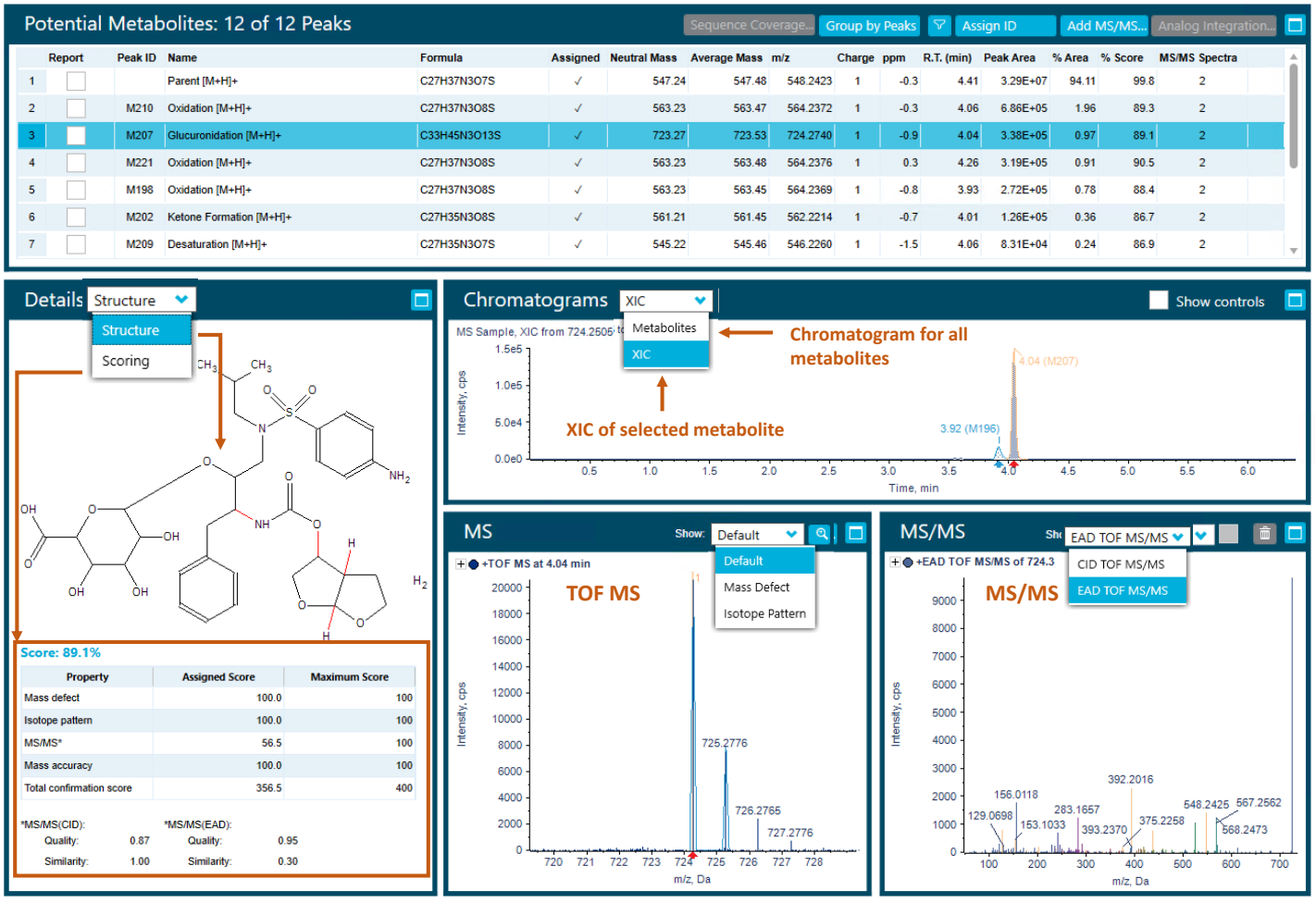


**Figure 2. Results view in Molecule Profiler software.** The software shows the number of potential metabolites with detailed information on the structure and scoring, options to view the XIC of selected metabolites or all metabolites and TOF MS spectra. Molecule Profiler software also displays the mass defect, isotope pattern and CID and EAD spectra of the selected metabolite.

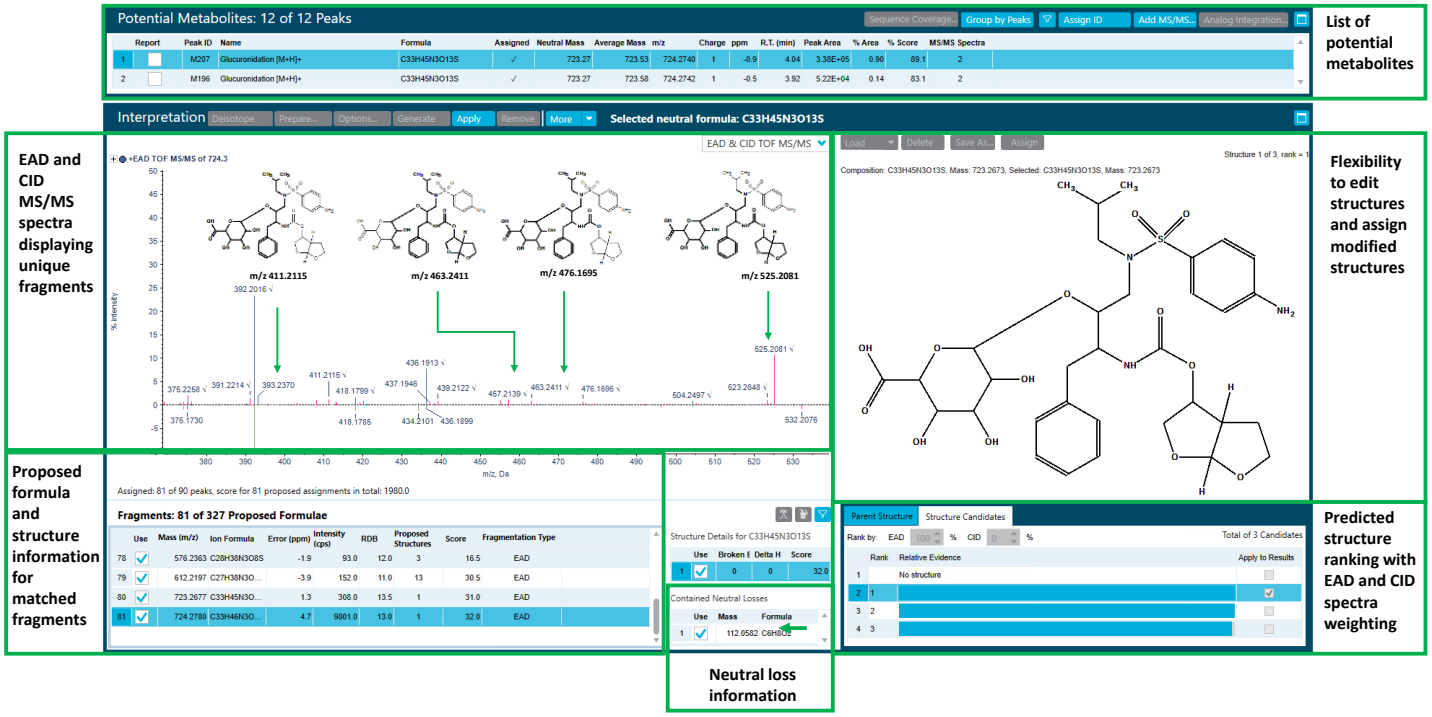


**Figure 3. Interpretation pane of Molecule Profiler software.** The software displays a list of predicted metabolites, CID and EAD spectra for a predicted o-glucuronide metabolite and a predicted structure ranking based on the total score with weighting information. Molecule Profiler software also permits users to edit and assign new structures to the selected peaks.

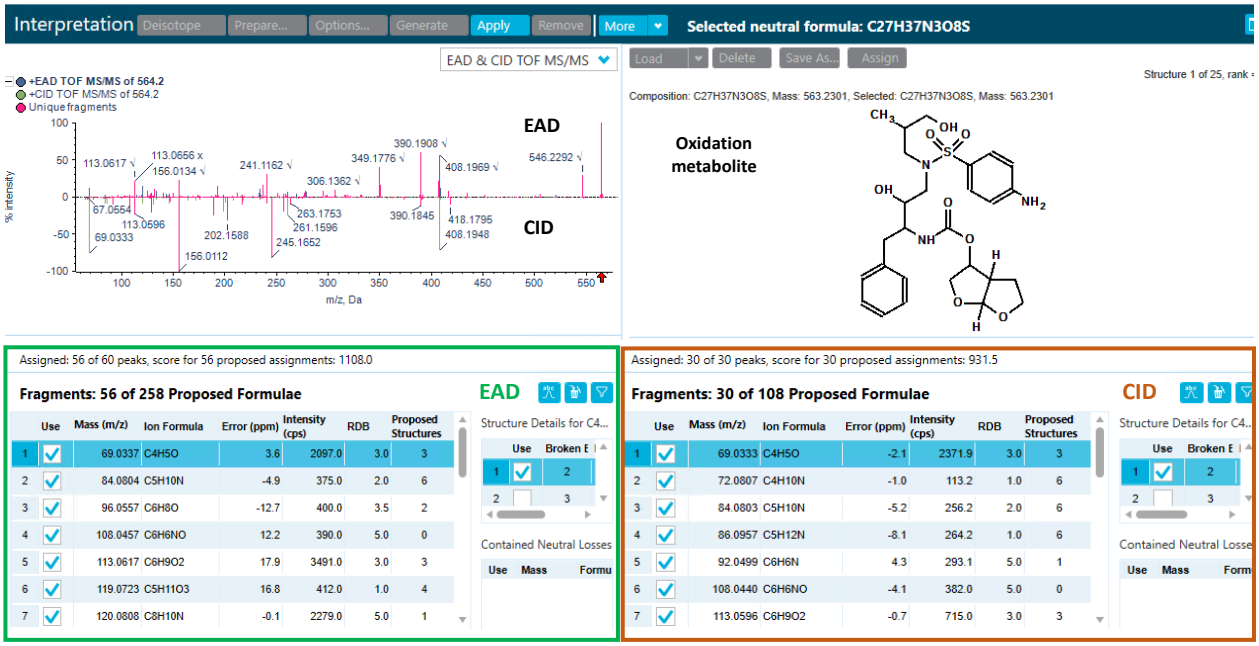


**Figure 4. Interpretation pane for analysis of a darunavir oxidation metabolite.** Molecule Profiler software shows a more confident structure assignment for the oxidation metabolite of darunavir with EAD data, resulting in more fragment ion matches and a higher overall score (green) compared to CID data (orange).

A correlation analysis was performed for drugs and metabolites using peak area based on precursor ion peaks from a TOF MS experiment. The relative quantifications of drugs and metabolites were correlated and revealed a decrease in drug concentration relative to an increase in different metabolite concentrations over time (Figure 5).

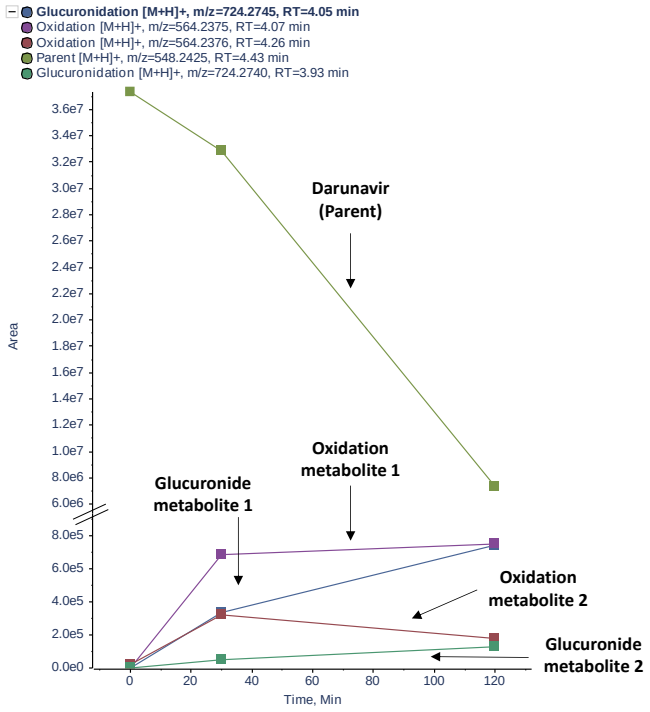


**Figure 5. Correlation analysis using Molecule Profiler software**. Correlation analysis showed increased metabolite concentration with time and decreased parent concentration in incubated samples.

Correlation analysis and DDA acquisition enabled parent and metabolite product ion analysis to identify the most prominent metabolites and provide a visual summary of metabolism. Metabolite and fragment identification were performed with <10 ppm mass error. The high mass accuracy enabled the confident prediction of metabolites in an in vitro metabolism study.

## Conclusions

- An innovative feature from Molecule Profiler software was utilized to identify unique fragments from EAD and CID spectra in a single results file to achieve more accurate structure assignment of metabolites
- Quick and efficient software-aided identification and correlation analysis of drug metabolites was performed with Molecule Profiler software coupled with the ZenoTOF 7600 system
- The enhanced sensitivity provided by the Zeno trap supports confident identification and characterization of low-abundant metabolites
- Data acquisition and processing were streamlined in a single software platform to expedite data reduction and build confident structure-metabolic stability relationships

## References

1. Orthogonal fragmentation mechanism enables new levels of metabolite characterization. SCIEX technical note, RUO-MKT-02-13348-A

2. Comprehensive metabolite characterization using orthogonal MS/MS data. SCIEX poster, RUO-MKT-10-14711-A

3. Confident characterization and identification of glucuronide metabolites using diagnostic fragments from electron activated dissociation (EAD). SCIEX technical note, RUO-MKT-10-14711-A

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