Comprehensive metabolite identification with electron-activated dissociation (EAD) and collision-induced dissociation (CID)

Automated metabolite identification on the ZenoTOF 7600 system using Molecule Profiler software

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This technical note describes a streamlined, software-aided automated workflow to study metabolite identification using the ZenoTOF 7600 system paired with Molecule Profiler software. Confident metabolite structure assignments were performed using both CID and EAD data. The more informative MS/MS spectra provided by EAD lent higher confidence to the software-based identification of drug metabolites to support drug development.

The qualitative capabilities of accurate mass spectrometry, such as automated LC-MS/MS workflows using CID, have been important for investigating the metabolism of candidate modalities in the early stages of pharmaceutical drug development.

Recent accurate mass spectrometry advancements, including improvements in the duty cycle, enabled the application of EAD on LC time scales and the integration of EAD into LC-MS/MS workflows, providing more confident characterizations of compounds of interest.[1-3]

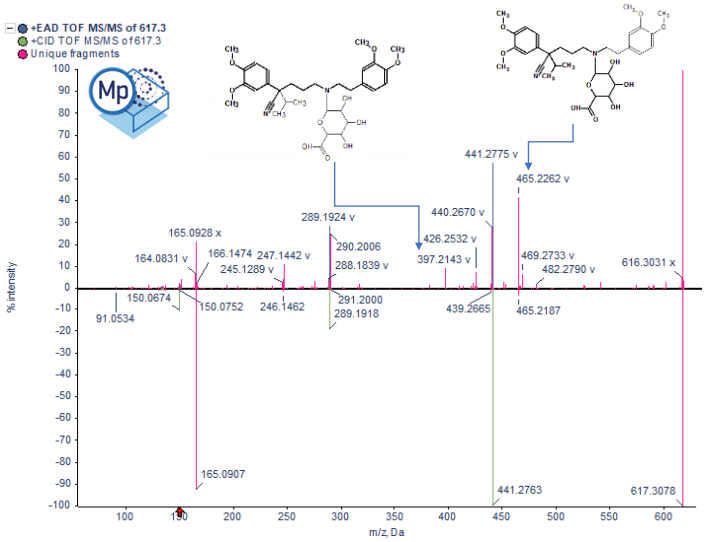


Figure 1. Panel view of the MS/MS spectra and structure assignments in Molecule Profiler software. The software displayed an inverted overlay of the EAD and CID spectra, highlighting unique fragments (pink) and putative structure assignments based on spectra weightage. Diagnostic fragments such as m/z 397 and 465 support the N-glucuronide conjugation of verapamil. EAD TOF MS/MS fragment mass accuracy was within 5 ppm, enabling confident metabolite confirmation and identification.

Here, data were generated and analyzed using a single SCIEX OS software platform to test metabolite identifications. The data were acquired using an advanced metabolite identification workflow using the ZenoTOF 7600 system and processed with Molecule Profiler software. 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 metabolite identification using the ZenoTOF 7600 system and Molecule Profiler software

- **Confident structure assignments:** Analyze EAD and CID spectra from a single results file to achieve more confident structure assignments
- **Enhanced structure assignment:** EAD preserves fragile modifications to easily localize phase II conjugates. EAD also provides information-rich MS/MS spectra that enable more confident identification of phase I metabolites than CID.
- **Efficient metabolite identification:** Perform fast and efficient software-aided identification of drug metabolites using Molecule Profiler software with the ZenoTOF 7600 system
- **Streamlined data acquisition and processing workflow:** Utilize a quick and easy-to-use workflow from data acquisition to analysis in SCIEX OS software with the integration of Molecule Profiler software

Methods

Sample preparation: Verapamil, buspirone and nefazodone were incubated in rat hepatocytes at a 1µM starting concentration. Samples were removed from incubation and quenched with acetonitrile at 0-, 30- and 120-minute time points.

Chromatography: Separation was performed on a Phenomenex Luna Omega Polar C18 (2.1 x 150 mm, 3 µm, 100 Å) column at 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 acetonitrile. An injection of 5 µL 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	*35 psi*
Ion source gas 1	*55 psi*
Ion source gas 2	*55 psi*
CAD gas	*7*
Ion spray voltage	*5500 V*
Source temperature	*550°C*

Table 3. Zeno DDA parameters.

Parameter	Setting	
	Zeno CID DDA	**Zeno EAD DDA**
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	*40–1000 Da*	*60–1000 Da*
Accumulation time (TOF MS/MS)	*0.06 s*	*0.09 s*
Collision energy (CID)	*35 V*	
Collision energy spread (CID)	*15 V*	
Electron kinetic energy (EAD)		*10 eV*
Electron beam current (EAD)		*5000 nA*

Data processing: SCIEX OS software was used for data acquisition. Molecule Profiler software was used to predict biotransformation sites using Zeno CID DDA and Zeno EAD DDA data.[4]

Metabolite identification using Molecule Profiler software

Metabolites from the incubation of verapamil, buspirone and nefazodone in rat hepatocytes were analyzed using Zeno CID and Zeno EAD. Molecule Profiler software enabled the processing and analysis of Zeno CID and Zeno EAD data in a single results file.

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% to 100%. The interpretation panel in the software allows users to modify structures and the total score for the modified structures. Figure 2 shows the overview of the results panel, in which users can view the list of potential metabolites, assigned structures and scoring information. A potential metabolite is scored based on the mass defect, isotope pattern, MS/MS data and mass accuracy. The data can be viewed using TOF MS or MS/MS spectra and XICs. The software also displays the mass defects and isotope patterns of the metabolites.

Using Zeno CID and Zeno EAD MS/MS data can reduce the ambiguity in identifying positional isomers (Figure 1). For glucuronide conjugates, the EAD data provided more specific and unique MS/MS fragments than CID, aiding in the correct assignment of the site of metabolism. Figure 3 shows an example of verapamil metabolites identified in rat hepatocytes (t = 30 minutes). Based on the EAD data, 15 metabolites and cleavages were identified. The Molecule Profiler software enables rapid metabolite identification from automated data processing.

Metabolites were assigned rank values of 1, 2, 3 or >3 based on the probability of occurrence (Figure 4). Metabolites assigned rank 1 have the highest probability of occurrence based on the total score derived from the mass defects, isotope patterns, MS/MS data and mass accuracy. Based on the datasets acquired from verapamil, nefazodone and buspirone, EAD provided more informative MS/MS spectra that

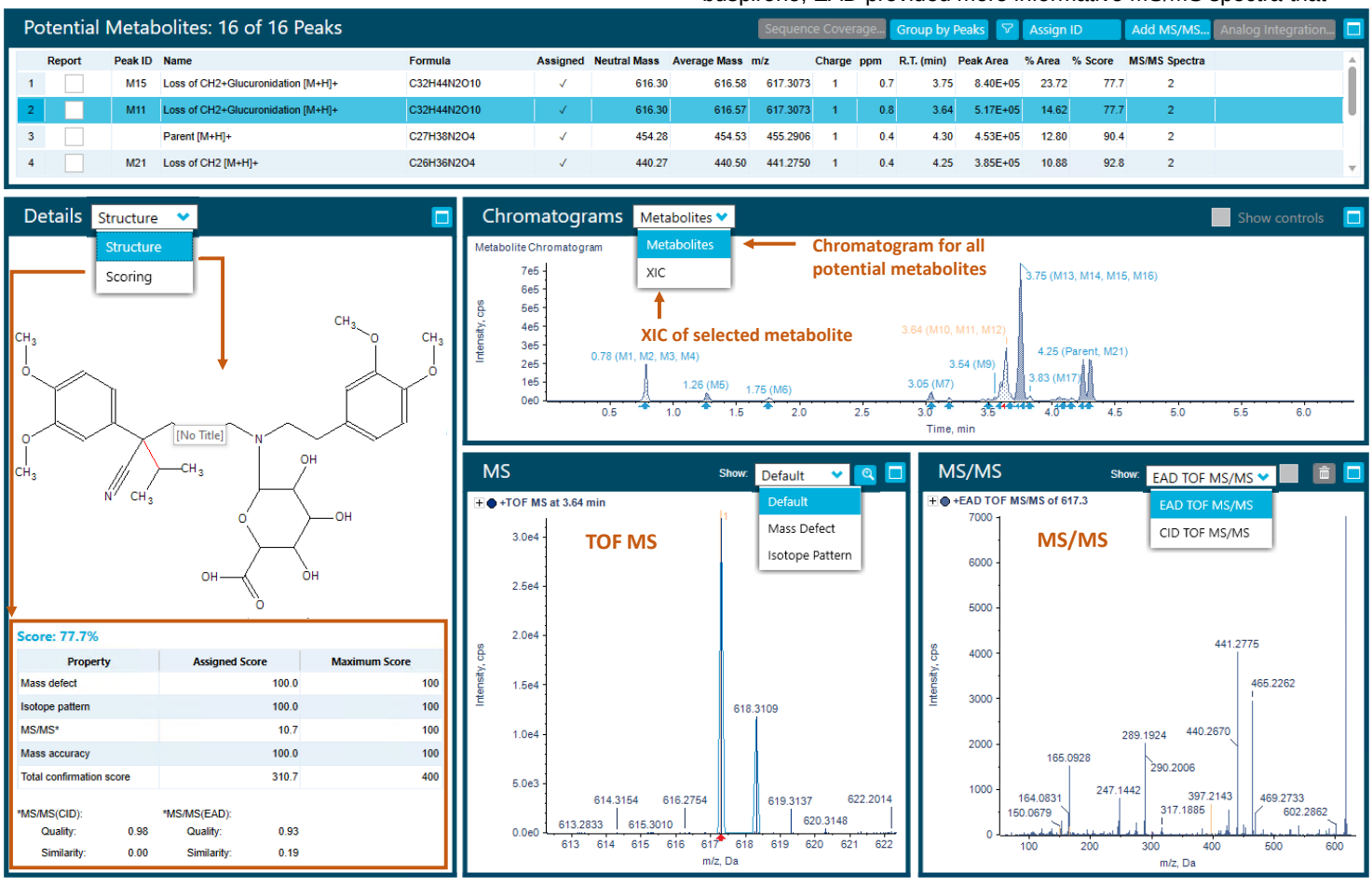
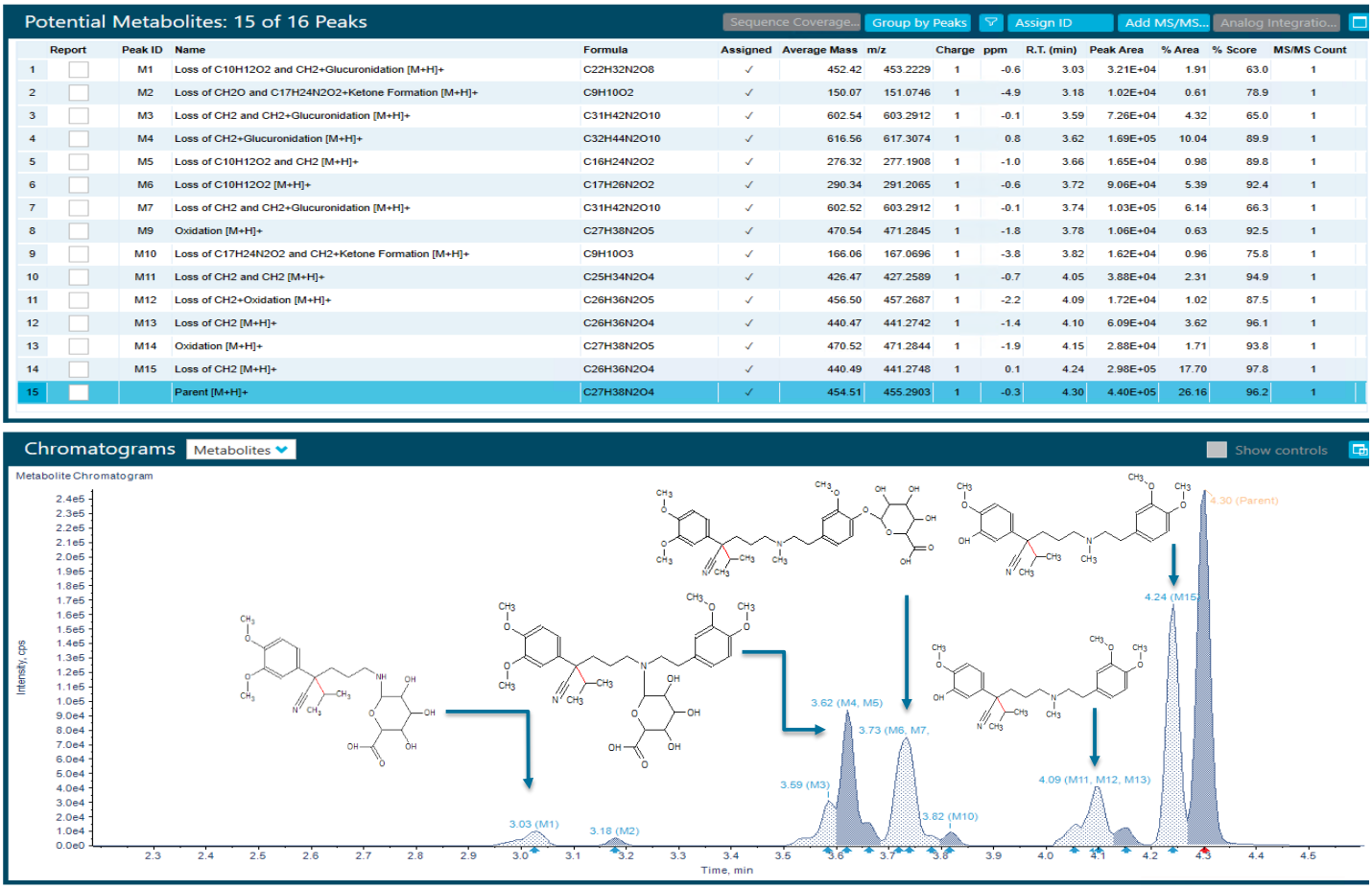


Figure 2. Results view in Molecule Profiler software. The software shows the number of potential metabolites with detailed information about the structure and scoring, options to view the XICs of selected or all metabolites and TOF MS spectra. Molecule Profiler software also displays the mass defect,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

provided greater confidence in metabolite identification. For example, 40% of glucuronide metabolites from verapamil were ranked 1 based on the CID data, whereas 60% of the glucuronide metabolites were ranked 1 based on the EAD data (Figure 4). Similarly, in the buspirone dataset, 70% of oxidative metabolites were ranked 1 based on the CID data, while the remaining metabolites were ranked 2 or 3. Based on the EAD data, however, >80% of the oxidative metabolites were ranked 1 in the buspirone dataset. Metabolite confirmations were further supported by CID data. In the verapamil dataset, cleavage and oxidative metabolites were equally confirmed by CID and EAD data, with 80% of metabolites placed as rank 1 (Figure 4).



	Report	Peak ID	Name	Formula	Assigned	Average Mass	m/z	Charge	ppm	R.T. (min)	Peak Area	% Area	% Score	MS/MS Count
1		M1	Loss of C10H12O2 and CH2+Glucuronidation [M+H]+	C22H32N2O8	✓	452.42	453.2229	1	-0.6	3.03	3.21E+04	1.91	63.0	1
2		M2	Loss of CH2O and C17H24N2O2+Ketone Formation [M+H]+	C9H10O2	✓	150.07	151.0746	1	-4.9	3.18	1.02E+04	0.61	78.9	1
3		M3	Loss of CH2 and CH2+Glucuronidation [M+H]+	C31H42N2O10	✓	602.54	603.2912	1	-0.1	3.59	7.26E+04	4.32	65.0	1
4		M4	Loss of CH2+Glucuronidation [M+H]+	C32H44N2O10	✓	616.56	617.3074	1	0.8	3.62	1.69E+05	10.04	89.9	1
5		M5	Loss of C10H12O2 and CH2 [M+H]+	C16H24N2O2	✓	276.32	277.1908	1	-1.0	3.66	1.65E+04	0.98	89.8	1
6		M6	Loss of C10H12O2 [M+H]+	C17H26N2O2	✓	290.34	291.2065	1	-0.6	3.72	9.06E+04	5.39	92.4	1
7		M7	Loss of CH2 and CH2+Glucuronidation [M+H]+	C31H42N2O10	✓	602.52	603.2912	1	-0.1	3.74	1.03E+05	6.14	66.3	1
8		M9	Oxidation [M+H]+	C27H38N2O5	✓	470.54	471.2845	1	-1.8	3.78	1.06E+04	0.63	92.5	1
9		M10	Loss of C17H24N2O2 and CH2+Ketone Formation [M+H]+	C9H10O3	✓	166.06	167.0696	1	-3.8	3.82	1.62E+04	0.96	75.8	1
10		M11	Loss of CH2 and CH2 [M+H]+	C25H34N2O4	✓	426.47	427.2589	1	-0.7	4.05	3.88E+04	2.31	94.9	1
11		M12	Loss of CH2+Oxidation [M+H]+	C26H36N2O5	✓	456.50	457.2687	1	-2.2	4.09	1.72E+04	1.02	87.5	1
12		M13	Loss of CH2 [M+H]+	C26H36N2O4	✓	440.47	441.2742	1	-1.4	4.10	6.09E+04	3.62	96.1	1
13		M14	Oxidation [M+H]+	C27H38N2O5	✓	470.52	471.2844	1	-1.9	4.15	2.88E+04	1.71	93.8	1
14		M15	Loss of CH2 [M+H]+	C26H36N2O4	✓	440.49	441.2748	1	0.1	4.24	2.98E+05	17.70	97.8	1
15			Parent [M+H]+	C27H38N2O4	✓	454.51	455.2903	1	-0.3	4.30	4.40E+05	26.16	96.2	1

Figure 3. Major verapamil metabolites in rat hepatocytes (t = 30 minutes). Fifteen significant metabolites were assigned putative structures during the automated processing with Molecule Profiler software.

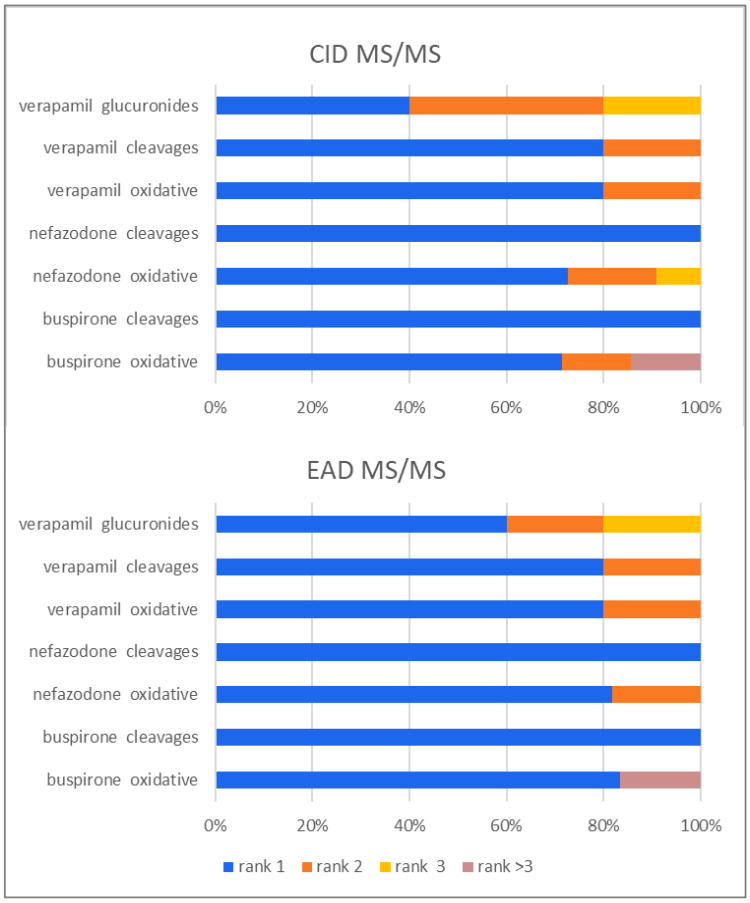


Figure 4. Summary of the automated structure assignment using CID and EAD MS/MS data. Glucuronides, cleavages and oxidative metabolites were evaluated in verapamil, nefazodone and buspirone. Each assignment was based on software rankings of 1, 2, 3 and >3. Metabolite structures with the highest total scores were assigned as rank 1.

Conclusions

- Accurate and comprehensive CID and EAD MS/MS data were generated on the ZenoTOF 7600 system using a fast LC gradient workflow
- An innovative feature in Molecule Profiler software was used to identify unique fragments from EAD and CID spectra to achieve more accurate metabolite structure assignments
- The enhanced sensitivity provided by the Zeno trap supported confident identification and characterization of low-abundant metabolites
- Data acquisition and processing were streamlined in a single software platform to expedite data management and analysis for drug metabolism studies

References

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