Automated workflow to study microsomal clearance and analysis of metabolites using collision-induced dissociation and electron activated dissociation MS/MS data

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ABSTRACT

Studies of in vitro metabolism of drugs in human and animal tissues help to predict the metabolic clearance rate of compounds and identify major metabolism pathways ("soft spots"). For such studies, metabolite identification is critical, and it is often software-aided to ensure proper metrics are used for confident identification and prediction of the metabolism site. A software-aided methodology was developed to quantitatively study microsomal clearance and qualitatively identify the soft spots for metabolism, aiding in the acceleration of the early drug discovery process. Datasets from collision-induced dissociation (CID) and electron activated dissociation (EAD) were applied to predict the sites of metabolism.

INTRODUCTION

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 because they provide quantitative and qualitative data with sufficient sensitivity, particularly for unknown metabolite identification.

Here, data were generated and analyzed with 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, making it an ideal tool for comparing MS/MS spectra to identify unique fragments in a single results file (Figure 1).

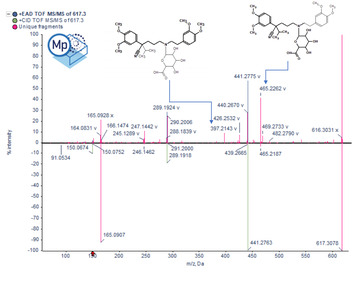

Figure 1. Panel view of the MS/MS spectra and structure assignments in Molecule Profiler software. The software displayed an inverted overlay of the EAD and CID spectra, highlighting unique fragments (pink) and putative structure assignments based on spectra weightage. Diagnostic fragments such as m/z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

MATERIALS AND METHODS

Sample preparation:
Verapamil, buspirone, darunavir 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
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. Optimised source and gas conditions are summarized in Table 2. The Zeno DDA parameters are shown 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**

**For darunavir, electron kinetic energy was 11 eV and electron beam current was 8000 nA*

Data processing:
- SCIEX OS software was used for data acquisition
- Molecule Profiler software was used to predict biotransformation sites based on the Zeno CID DDA and Zeno EAD DDA data

RESULTS

- Metabolites from the incubation of verapamil, buspirone, darunavir 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.

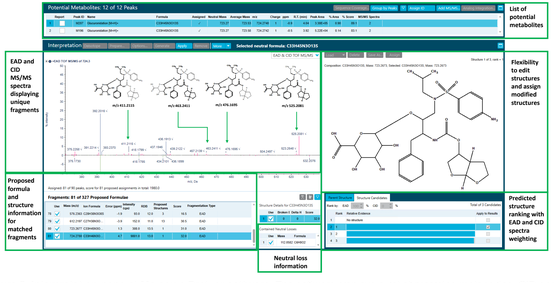

Figure 2. Interpretation pane of Molecule Profiler software. The software displays a list of predicted metabolites, CID and EAD spectra for a predicted o-glucuronide metabolite and a predicted structure ranking based on the total score with weighting information. Molecule Profiler software also permits users to edit and assign new structures to the selected peaks.

- Figure 2 shows the interpretation pane with more details on product ion matches for both spectra, information on unique fragments and user flexibility to modify and reassign 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 2).

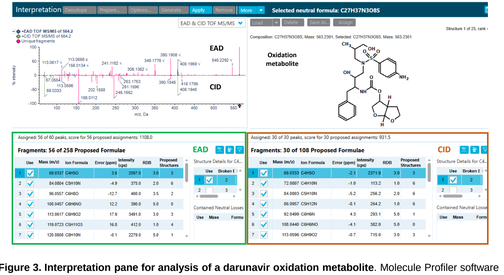

Figure 3. Interpretation pane for analysis of a darunavir oxidation metabolite. Molecule Profiler software shows a more confident structure assignment for the oxidation metabolite of darunavir with EAD data, resulting in more fragment ion matches and a higher overall score (green) compared to CID data (orange).

- Figure 3 shows higher scoring and a more confident structure assignment for oxidation metabolites with EAD compared to CID data.
- 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 (Figure 4).
- The software also ranks all possible structures based on the total score for every metabolite structure (Figure 5).

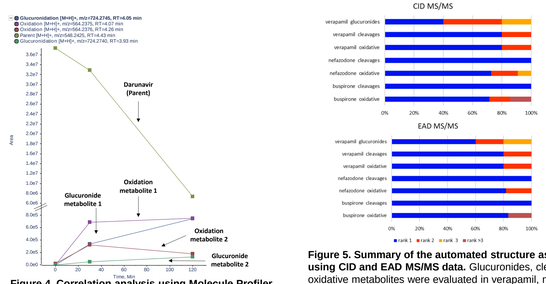

Figure 4. Correlation analysis using Molecule Profiler software. Correlation analysis showed an increased metabolite concentration with time and decreased parent concentration in incubated samples.

Figure 5. 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

- 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

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