Orthogonal fragmentation mechanism enables new levels of metabolite characterization

Electron activated dissociation on the ZenoTOF 7600 system enables thorough characterization of difficult to classify metabolites

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Thorough characterization of drug candidate metabolism is a requisite part of early to late stage drug discovery. Early ADME (absorption, distribution, metabolism and excretion) studies are used to determine if a drug candidate is prone to metabolic breakdown or oxidation/conjugation. This is typically done by incubation of a compound in liver microsomes, followed by LC-MS analysis (on a triple quadrupole or high resolution mass spectrometer) of the compound to assess a drop in concentration over time. In many cases, the products of these early assays are not extensively characterized and generally do not represent all of the potential metabolic activity a drug could be exposed to *in vivo*.

As drug candidates progress through research, more comprehensive metabolic characterization is performed. These studies could involve incubation in hepatocytes, or even analysis of *in vivo* samples. Samples are generally analyzed on a high resolution mass spectrometer (such as TOF MS) to identify metabolites, but MS1 provides no information about the site of metabolism, which is crucial for assessment of potential toxicity and for providing information to chemists working to improve chemical structures. Fragmentation is required to provide structural information about metabolites. The classic mode of analysis is collisional induced dissociation (CID), which fragments ions by accelerating them in the presence of a neutral gas (N_2), yielding consistent and efficient fragmentation. But, in some cases, truly diagnostic fragment ions are not generated by this process (Figure 1).

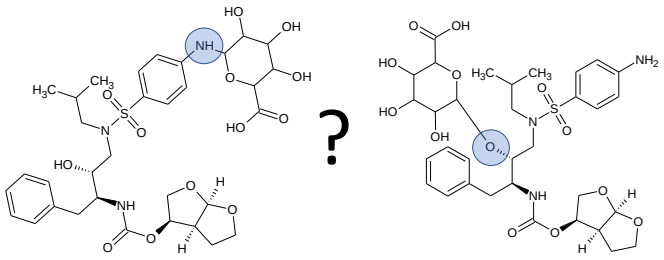

Figure 1. Possible glucuronide conjugates of darunavir. Site of conjugation can be difficult to identify using conventional fragmentation methods.

Glucuronide conjugation can be difficult to thoroughly characterize by MS/MS alone, as the glucuronic acid bond is often very labile both in the ionization source and the collision cell of most mass spectrometers. Electron activated dissociation (EAD) on the ZenoTOF 7600 system is an orthogonal fragmentation technique that is able to generate fragments of glucuronide conjugates that retain the glucuronic acid group, which allows for confident assessment of the site of conjugation.

The drug darunavir, a currently prescribed protease inhibitor, was incubated at 1 µM in rat liver hepatocytes and timepoints were analyzed by LC-MS/MS using the ZenoTOF 7600 system to investigate the *in vitro* metabolism. Two glucuronide conjugates were identified, one of which could be confidently characterized using both CID and EAD, while the other could only be characterized using the fragments generated by EAD.

Key features of the ZenoTOF 7600 system for metabolite identification

- EAD is a reagent free, tunable technique that is applicable to a broad range of molecule types available on the ZenoTOF 7600 system
- EAD provides orthogonal, complimentary fragmentation to CID, allowing more thorough, confident metabolic structure elucidation than with CID alone

- The Zeno trap provides enhanced sensitivity in MS/MS modes, allowing characterization of low level metabolites.

Methods

Sample preparation: Darunavir was incubated in rat hepatocytes (1 million cells/mL) at a starting concentration of 1 µM. In addition to time 0, 30 min and 120 min timepoints were pulled from the incubation and quenched with 1 volume of CH_3CN. Samples were vortexed, then centrifuged, and then the supernatant was transferred to an HPLC vial, before delivering the samples for analysis.

Chromatography: An ExionLC AD HPLC system was used for separation on a Phenomenex Luna Omega Polar C18, 3µm, 150 x 2.1 mm (P/N 00F-4760-AN) column using 5 µL injection volume. Table 1 below shows LC gradient parameters.

Table 1. HPLC gradient.

Time (min)	Flow (mL/min)	%B
0.0	*0.5*	*5*
0.5	*0.5*	*5*
1.5	*0.5*	*15*
3.5	*0.5*	*50*
4.75	*0.5*	*95*
5.75	*0.5*	*95*
5.8	*0.5*	*5*
6.5	*0.5*	*5*

Mobile phase A was water with 0.1% formic acid
Mobile phase B was CH_3CN with 0.1% formic acid

Mass spectrometry: The samples were analyzed twice, once using Zeno IDA with CID method and once with a Zeno EAD IDA method. Method parameters are shown in Table 2. Data was acquired using SCIEX OS software.

Data processing: All data was processed in MetabolitePilot software v2.0.4. The default list of phase I and phase II metabolites was used for searching, as well as compound specific fragmentations that were based on reference spectra for darunavir. The data processing parameters for both CID and EAD sets were the same for both fragmentation approaches, except for the assignment of the parent reference spectra. CID and EAD spectra were used for each respective set of data.

Table 2. MS method parameters.

Parameter	TOF MS (Survey)	CID MS/MS	EAD MS/MS
Start Mass	*100*	*60*	*60*
Stop Mass	*1000*	*1000*	*1000*
Accumulation time (sec)	*0.05*	*0.06*	*0.06*
Maximum IDA Candidates	*8*		
CE (volts)	*5*	*35*	*10*
*eV**			*11*
*nA**			*8000*

*EAD only values

Identification of metabolites with CID

Several metabolites were observed for darunavir using both CID and EAD fragmentation, and those shown in Figure 2 were able to be interpreted consistently using both fragmentation approaches.

In addition to the metabolites shown in Figure 2, there were also two distinct peaks corresponding to glucuronide conjugates, shown in Figure 3.

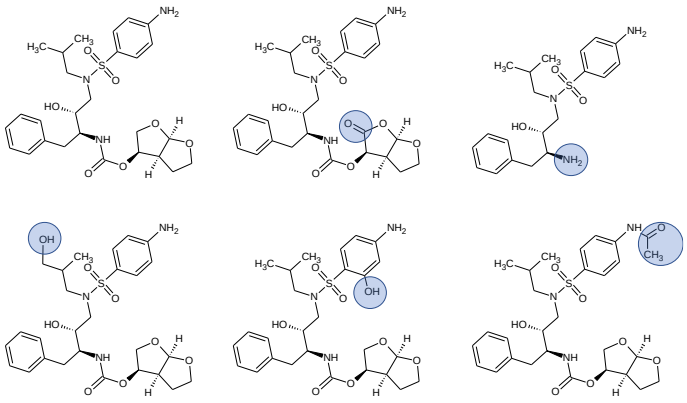

Figure 2. Darunavir and metabolites described by both CID and EAD. Top, left: darunavir; top, center: oxidation (ketone formation) of *bis*-THF; top, right: hydrolysis of carbamate bond, loss of *bis*-THF. Bottom, left: oxidation of isobutyl group; bottom, center: oxidation of aniline ring; bottom, right: acetylation of aniline group.

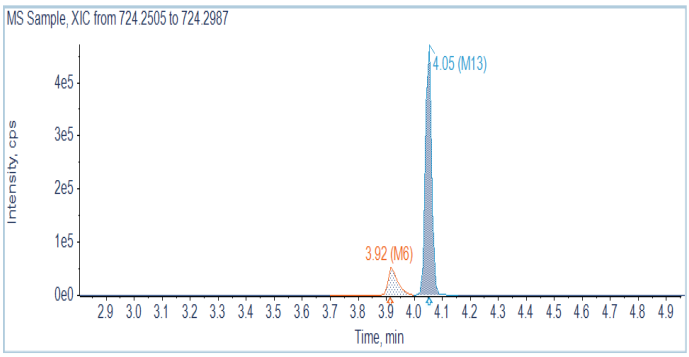

Figure 3. XIC of m/z 724.2739, corresponding to the glucuronide conjugates of darunavir. The presence of two peaks suggest the presence of two different glucuronide conjugates.

The extracted ion chromatograms for the parent m/z of darunavir also showed peaks at retention times matching those of the glucuronide conjugates, indicating the typical in-source fragmentation of these labile conjugates back to the parent. The peak at 3.92 min contained enough diagnostic fragments in both CID and EAD to make a confident assignment of the site of conjugation. Figure 4 shows a mirror plot comparison of the MS/MS spectra of the two fragmentation mechanisms. This case is an uncommon example of CID fragmentation that contains multiple fragments where the glucuronide conjugate is conserved, allowing a confident assignment of the site of conjugation.

Confirmation of isomeric structures with EAD

The peak at 4.05 mins was difficult to characterize with CID spectra alone. The mirror plot comparison of EAD and CID spectra for the peak at 4.05 min is shown in Figure 5. The CID spectra did not show fragments that allowed an assignment of the position of the glucuronide conjugate. The much richer EAD spectra had three peaks of high abundance containing the glucuronide, and as many as 33 total fragments identified by the software, which allowed for a confident assignment of the site of conjugation.

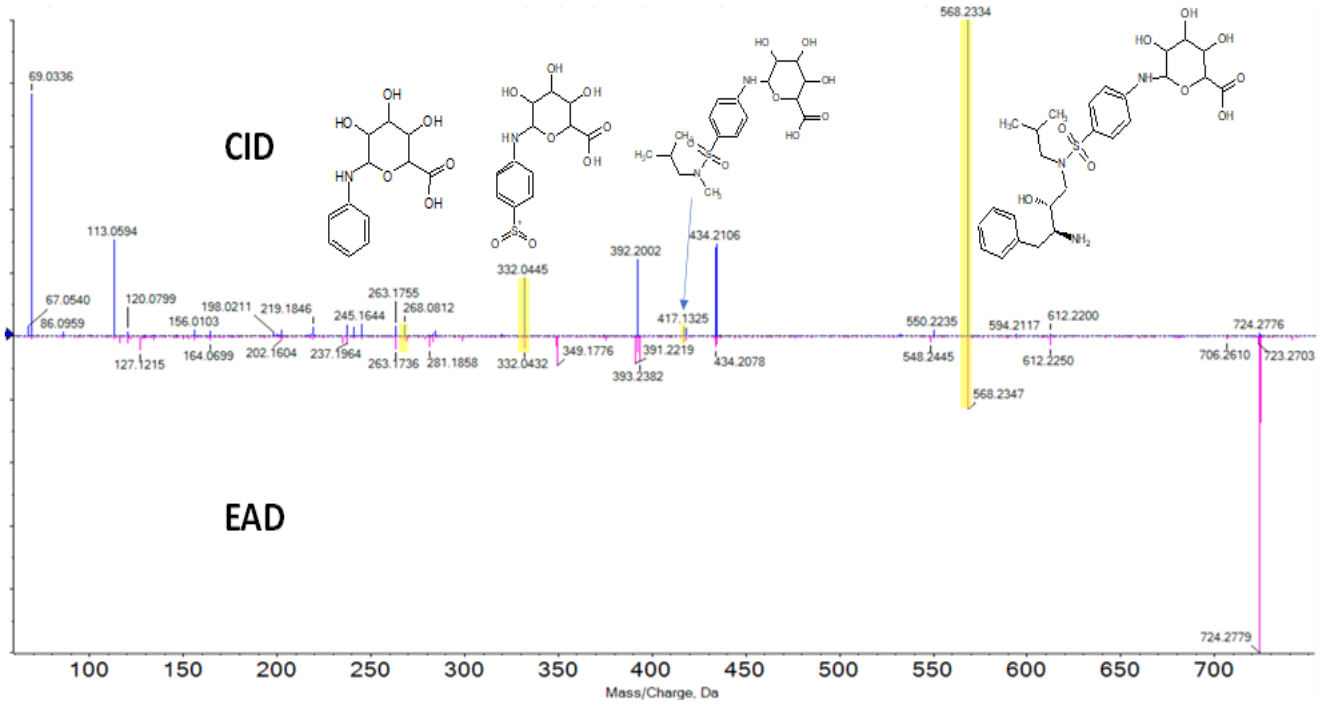

Figure 4. MS/MS spectra comparison of CID and EAD for the glucuronide conjugate at 3.92 min. Highlighted peaks in both spectra correspond to intact glucuronide fragments, allowing confident assignment of the site of conjugation. Note that the EAD spectrum contains significant amounts of the parent m/z, which is typical of this technique.

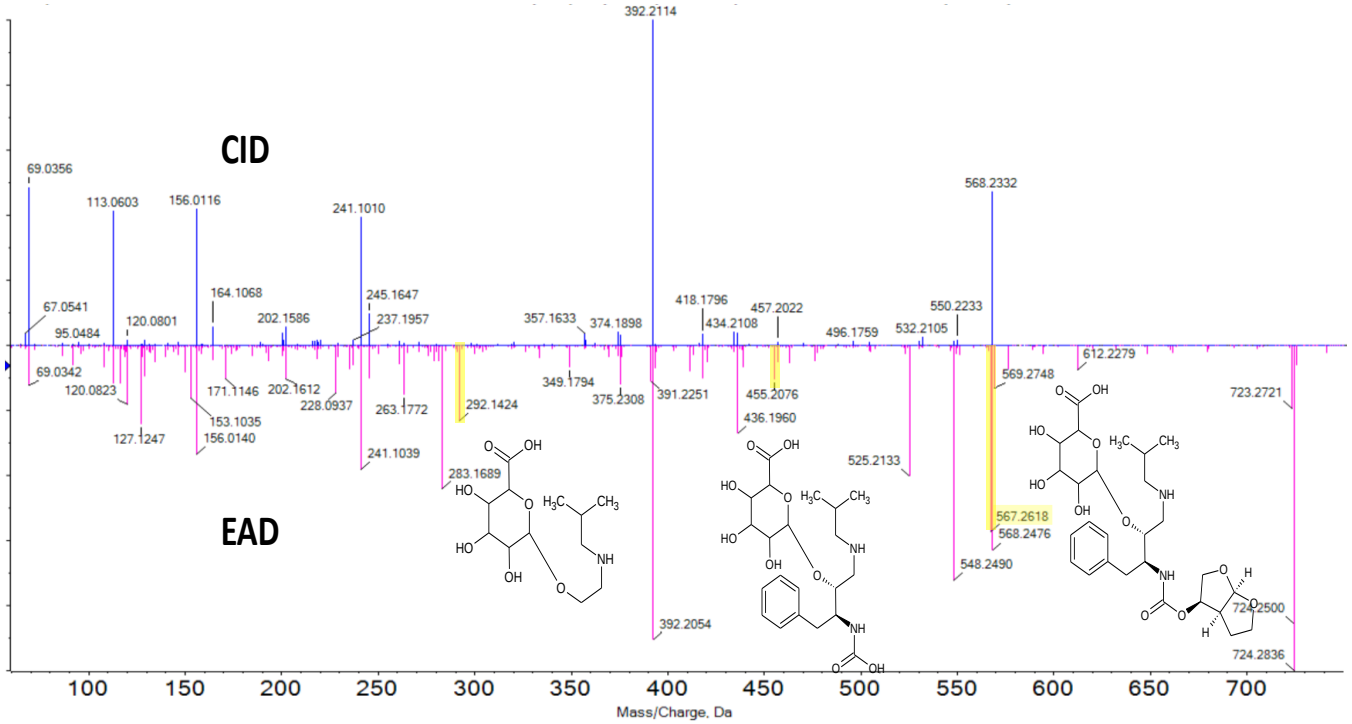

Figure 5. MS/MS spectra comparison of CID and EAD for the glucuronide conjugate at 4.05 min. Highlighted peaks in the EAD spectrum correspond to major intact glucuronide fragments, while there are no fragments in the CID spectrum with the glucuronide conjugation conserved**.** There were 33 unique fragments in the EAD spectrum confirming the site of conjugation.

Conclusions

The ability of the ZenoTOF 7600 system to perform both EAD and CID fragmentation brings a new level of interpretive power to the routine, but often challenging, process of metabolite identification. The high energy electron-based mechanism of EAD produces a richer fragmentation pattern than CID, and can generate diagnostic spectral peaks for labile fragments that would otherwise be lost. In the example illustrated here, EAD allows confident assignment and differentiation of O- and N-glucuronide conjugates. This differentiation is important because each can exhibit different toxicities, which means the improved confidence in understanding the site of conjugation is very important, and is something that might not otherwise be possible with MS based techniques.

References

1. Qualitative flexibility combined with quantitative power. SCIEX technical note, RUO-MKT-02-13053-A.
2. Electron activated dissociation: A new paradigm for tandem mass spectrometry. SCIEX publication, RUO-MKT-19-13372.
3. Zeno trap: Defining new levels of sensitivity. SCIEX publication, RUO-MKT-19-13373.

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