Creating Unique Fragmentation: Small Molecule Structural Elucidation Using UVPD

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ABSTRACT

Purpose: Utilizing ultraviolet photodissociation (UVPD) to increase the fragmentation efficiency resulting in more structurally informative product ions relative to high energy collisional dissociation (HCD).

Methods: Perform comparative LC-MS/MS experiments on a series of standards as well as a set of hepatocyte metabolite incubations using 213 nm laser as compared to beam-type fragmentation.

Results: For all standards evaluated, compounds with more than 3 Pi bonds showed greater fragmentation using UVPD than HCD as well as resulting in many more unique fragments with both even and odd electrons. In addition, UVPD provides greater diagnostic fragmentation to localize the site of conjugation by retaining the entire glucuronide modification.

INTRODUCTION

Small molecule structural elucidation using LC-MS^n presents significant challenges due to the overwhelming structural diversity, lack of mobile protons and/or multiple basic/anionic sites, and biotransformations. Collisional activation/dissociation-based methods results in preferential unimolecular dissociation pathways that may limit the formation of structurally informative product ions. This issue is particularly common for conjugated metabolites or fused-ring structures. We have developed instrument methods designed to leverage the commercially available 213 nm UVPD source on the Thermo Scientific™ Orbitrap Tribrid™ mass spectrometers to increase the structural elucidation capabilities for a series of small molecule standards and a set of isobaric conjugated metabolites from Phase II drug incubations. The LC-MS^n method consisted of standard data dependent acquisition (DDA) and dynamic exclusion (DE) settings changing only the dissociation methods between HCD and UVPD. Evaluation of the number of unique product ions and structural coverage were used to determine the effectiveness of UVPD relative to collisional activation, as well as gauge the limitations of UVPD based on the compound structure.

MATERIALS AND METHODS

Sample Preparation

Two sets of standards were used to initially evaluate UVPD performance and optimal settings. One standard contained an equal molar mixture of a range of compounds with various numbers of aryl rings and double bonds. The second set of standards consisted of base structures and its isomeric metabolites such as Olmesartan and isomeric glucuronides structures. Each compound individually analyzed by HCD and UVPD with a common LC gradient and then mixed in an equal molar mixture. Lastly, a set of Phase II hepatocyte incubations of verapamil was analyzed by the comparative methods.

Test Method(s)

All samples were evaluated on a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer and Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer using a common LC gradient and DDA/DE method. A binary solvent system was used comprised of A) 0.1% formic acid and B) MeCN with 0.1% formic acid and an 8-minute gradient (10-35%) on a Hypersil Gold aQ column with dimensions of 100 x 2.1 and 1.9 µm particles. The DDA/DE method consisted of a full scan MS (m/z 200-800) and a top 5 method with the MS and MS/MS spectra acquired with a resolving power of 60k and 30k, respectively. The maximum ion fill time for all MS/MS spectra was 54 msec. For HCD activation a normalized collision energy (NCE) of 35% was used while the UVPD pulse duration was maintained at 75 msec.

Data Analysis

All data was processed using Thermo Scientific™ FreeStyle™ Software and Thermo Scientific™ Mass Frontier™ 8.0 spectral interpretation software. Chemical structures were created and saved as a library file as well as used predict product ions using the following settings in Mass Frontier 8.0 software: general fragmentation libraries, both even and odd electron considerations, and a maximum of 5 steps for a unimolecular reaction coordinate. A mass measurement error of 2 ppm was used for initial screening to determine product ions.

RESULTS

Leveraging the unique capabilities of the Tribrid mass spectrometer

The goal of the study was to introduce alternative fragmentation mechanisms to generate complimentary unimolecular dissociation pathways relative to the standard beam-type activation (HCD) to enhance small molecule structural characterization and elucidation workflows. The primary method used in this study was UVPD due to the energy imparted per absorbed photon. A representative energy diagram is presented in Figure 1 comparing common activation methods used on commercial mass spectrometers.[1] Clearly the utilization of 213 nm photons imparts the greatest increase of internal energy per unit of time resulting in altering fragmentation methods from primarily kinetically controlled dissociation pathways to thermodynamically controlled pathways resulting in a higher probability of generating unique and structurally definitive product ions being measured. The Tribrid instrument architecture is ideal for implementing UVPD as the ion flight path and optics enables optimal precursor isolation (quadrupole mas filter), collection (ion routing multipole), trapping for irradiation and collection of the resulting unreacted precursors and all product ions (linear ion trap), and high resolution accurate mass (HRAM) measurement of all ions (Ultra-high field Orbitrap mass analyzer) is controlled by on-the-fly decisions (Dynamic Scan Management) enabling LC-MS/MS analysis on UHPLC time scale (6 second wide peaks) for both high and low-abundant precursor ions. The UVPD laser is contained within the footprint of the instrument to minimize required laboratory space and controlled within the standard instrument control software.

Figure 1. Cartoon representation of relative energy deposition resulting from comparative ion activation methods. The reaction coordinate demonstrates the required energy needed to access various unimolecular dissociation channels resulting in various fragments demonstrating the significant increase in energy deposition following the absorption of a single UV photon. Pi bonds (C=C, C=N) have strong absorption bands overlapping with the commercially available UVPD laser to promote fragmentation.

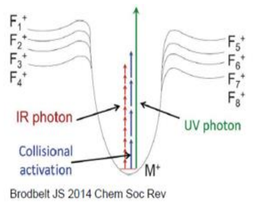

Figure 2. Schematic representation of the Orbitrap Tribrid mass spectrometer with UVPD laser location and introduction into the instrument through the linear ion trap.

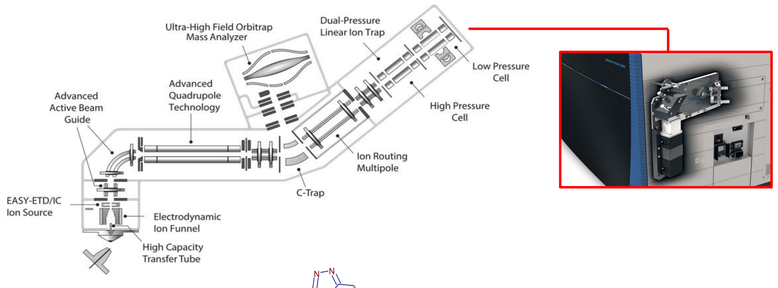

Figure 3. Comparative fragmentation spectra for Alprazolam resulting from HCD and UVPD. Product ions were annotated using Mass Frontier 8.0. Unique product ions are labeled with red font. UVPD generated greater numbers of product ions as well as unique product ions ensuring sequence coverage. The precursor ion activation is also greater using UVPD than HCD. Many of the UVPD product ions have not been labeled but create a series of subtle differences to increase structural annotation.

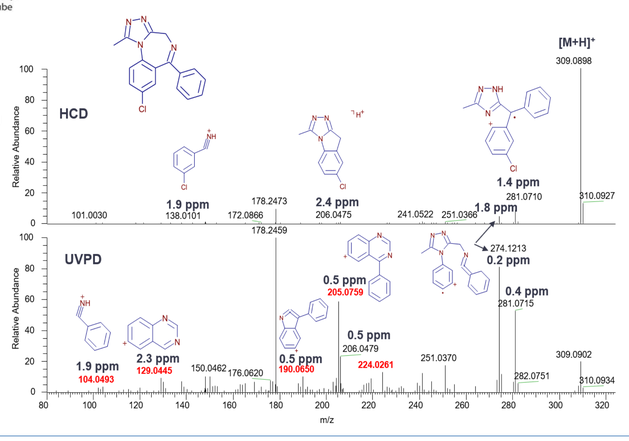

Figure 4. Evaluation of structural elucidation for hydroxy-diclofenac structural isomers using (Fig. 4A) HCD and (Fig. 4B) UVPD. The two isomers shown below demonstrate the challenges using collisional activation of small molecules containing one primary basic site resulting in primarily two fragments that do not enable differentiation. Figure 4B shows the resulting fragmentation following UVPD with much greater fragmentation, and as highlighted, many more unique fragments that are unique per structure that are highlighted in red. Specifically, the highlighted product ion mass range is shown to the right, further demonstrating the importance of high resolution and mass accuracy for detection of key product ions despite it being formed in the 1+ charge state.

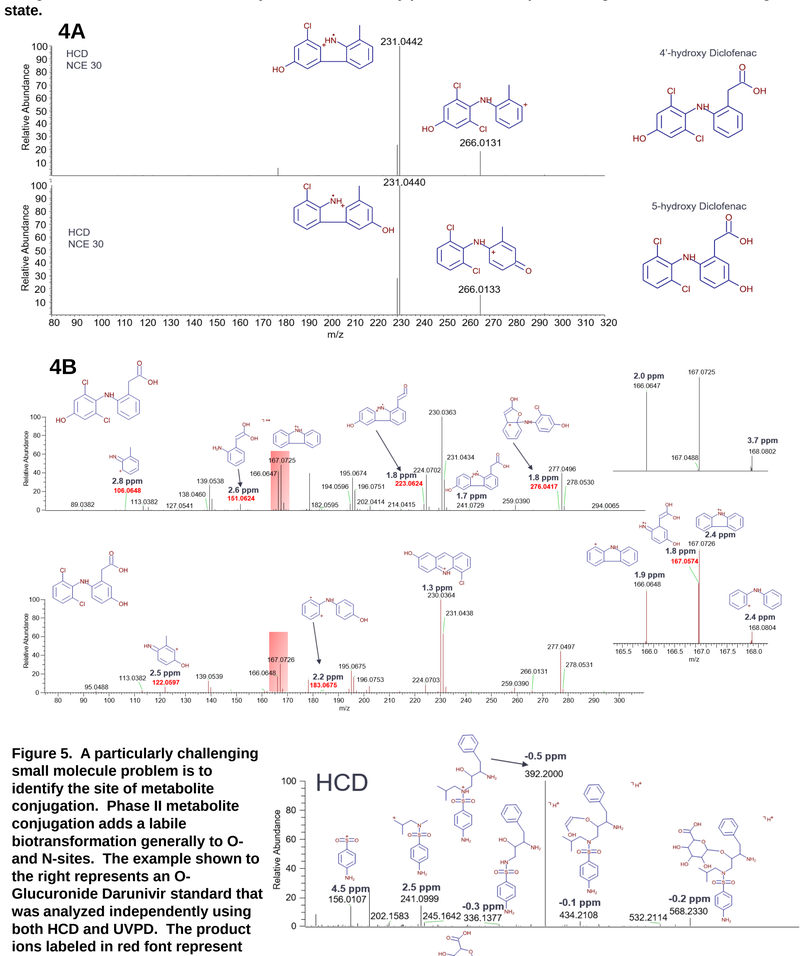

Figure 5. A particularly challenging small molecule problem is to identify the site of metabolite conjugation. Phase II metabolite conjugation adds a labile biotransformation generally to O- and N-sites. The example shown to the right represents an O-Glucuronide Darunivir standard that was analyzed independently using both HCD and UVPD. The product ions labeled in red font represent unique product ions measured following UVPD. Of particular interest is that UVPD creates a number of product ions with the glucuronide modification still attached. Additionally, these key product ions cover the various N- and O-sites helping to determine the specific site of biotransformation.

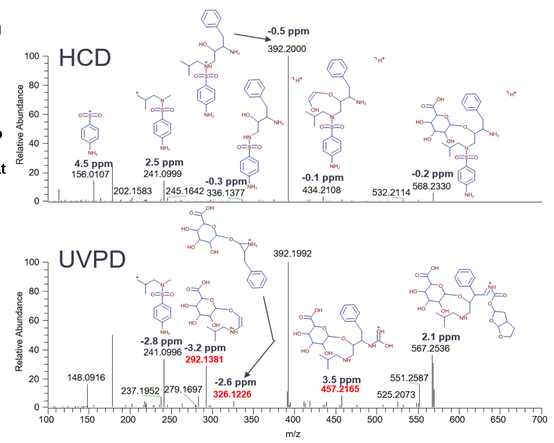

Figure 6. Comparative UVPD MS/MS spectra analyzed independently. The specific fragments were mapped to demonstrate discriminatory capabilities to localize the site of glucuronidation. Note that product ions are being formed with both even and odd electrons despite the molecular ions being formed from protonation [M+H]+.

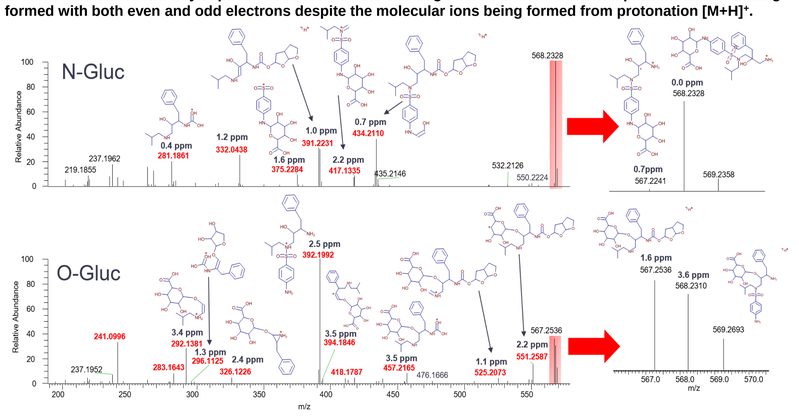

Table 1. List of small molecules evaluated in the two standard sets. The table lists the key attributes per compound that may play a role in the effectiveness of UVPD for structural elucidation. Specifically the number of Pi bonds leading to the number of unique fragment ions between HCD and UVPD. The last column evaluates the ratio of summed product ion intensity to the unreacted precursor for the two activation methods. The larger the number the greater the degree of fragmentation.

Compound Name	Formula	[M+H]+	Number of Aryl Rings	Number of Pi Bonds	Fused Rings	total Pi Bonds	Unique Fragments		Product:Parent	
							HCD	UVPD	HCD	UVPD
Zolmitriptan	C16H21N3O2	288.1707	1	1	2	4	6	17	1.70	0.28
Sulfamethazine	C12H14N4O2S1	279.091	2	0	0	6	7	16	0.71	2.71
Sulfamethoxazole	C10H11N3O3S1	254.0594	2	2	1	5	16	5	27.60	0.94
Bupropion	C12H18Cl1N1O1	240.115	1	0	1	3	14	16	373.14	1.39
Propranolol	C16H21N1O2	260.1645	2	0	5	2	12	18	1.86	82.89
Prednisone	C21H26O6	359.1853	0	2	4	2	22	9	147.25	0.07
Erythromycin degradant	C37H65N1O12	716.4685	0	0	0	0	38	2	458.50	0.02
Alprazolam	C17H13N4Cl1	309.0902	2	3	3	9	6	33	0.16	11.45
Loperamide	C29H33N2O2Cl1	477.2303	3	0	3	9	7	34	21.11	8.58
Terfenadine	C32H41N1O2	472.321	3	0	0	9	3	42	2.99	3.07
Darunavir	C27H37N3O7S1	548.2352*	2	0	2	6	1	6	481.40	0.59
Darunavir Glu	C33H45N3O13S1	724.2746	2	0	2	6	26	16	205.00	0.76
Olmesartan Acid	C24H27N6O3	447.2139	4	0	0	10	6	16	2800.00	42.00
Olmesartan Glu	C30H35N6O9	623.246	4	0	0	10	20	24	1167.00	62.00
Diclofenac	C14H12N1O2Cl2	296.0168	2	0	0	6	0	7	6677.00	1.12
Diclofenac OH	C14H13N1O3Cl2	310.0168	2	0	0	6	1	10	857.83	2.30

Figure 7. Base peak chromatogram of the Verapamil incubation (Fig. 7A) as well as a comparative incubation of a second small drug used as a background control to demonstrate which peaks are more specific to the Verapamil incubation. The inset shows the XIC for the m/z 617 ions representing Verapamil glucuronidation ([M-14+176]+) to demonstrate the narrow chromatographic peak widths less than 3 seconds at the baseline. Two of the three primary glucuronidated isomers are labeled for comparative structural analysis resulting from UVPD and detection.

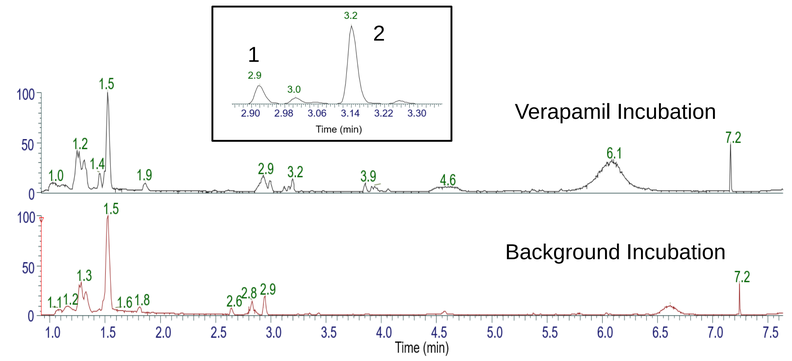

Figure 8. Comparative UVPD MS/MS spectra for the m/z 617 metabolites identified as verapamil glucuronidation. Due to the symmetry of the verapamil molecule, prediction of the site of glucuronidation is challenging when evaluating in a mixture without standards. Two of the three m/z 617 structural isomers were sampled by UVPD as marked in Figure 7. The UVPD MS/MS resulted in fragmentation-rich product ion spectra generating structurally different ions as labeled below. The relative retention times and detection of specific MS/MS product ions align with previous publications.[2]

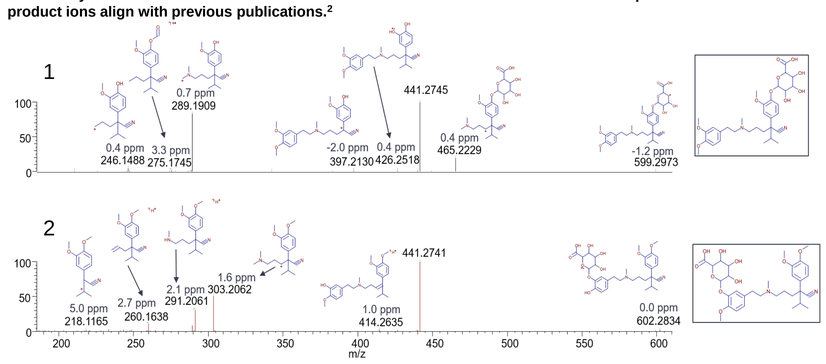

Table 2. List of Phase I and II verapamil metabolites resulting from 30-minute incubation using mouse hepatocytes. The goal of the table is to list which diagnostic fragment ions were formed and detected in the measured UVPD MS/MS spectra. The complementary product ions for Phase I metabolites such as m/z 151:165 and m/z 277:291 and 289:303 as well as high-mass product ion pairs for Phase II conjugate metabolites including m/z 427, 441, 457, and 465 are used to evaluate sites of biotransformation. Note the compression of elution profiles, have narrow chromatographic peak widths, and that most metabolites are low-level relative to the parent drug due to a short incubation time.

Metabolite (m/z)	Retention Time (min)	Integrated Peak Area	Diagnostic Product Ions (m/z)												
			151	165	233	248	260	277	289	291	303	427	441	457	465
455 (parent)	3.95	9.5e6	x	x					x		x				
277.1910	2.78	7.6e5	x												
603.2908	2.90	1.1e6		x	x							x			
617.3070	2.93	1.2e6		x					x				x		x
617.3071	3.03	3.9e5		x		x	x		x	x	x				
633.3023	3.12	6.8e5			x		x			x	x			x	
617.3070	3.15	5.0e6	x			x				x	x				
603.2914	3.17	4.8e6	x				x	x	x			x			
291.2065	3.18	1.6e7	x		x	x	x								
277.1910	3.20	4.8e6	x	x											
441.2745	3.84	1.5e7		x	x	x	x								
457.2697	3.94	9.9e5		x			x		x		x				

CONCLUSIONS

Incorporation of a commercially-available UVPD source for augmented tandem mass spectral analysis of small molecules provides more energetic activation, resulting in formation of unique, structurally relevant product ions increasing structural identification and elucidation. In addition, performing UVPD MS/MS analysis on an Orbitrap Fusion Lumos Tribrid mass spectrometer enables the following:

- Direct implementation of UVPD activation by a drop-down menu option for ease of use
- A 75 msec pulse duration enabled comprehensive metabolite sampling using UHPLC separations
- Mass Frontier 8.0 enabled comparative in silico fragmentation modeling for both even and odd electron unimolecular pathways

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2. Welles, M., et al. "Metabolism of verapamil: 24 new phase I and II metabolites identified in cell cultures of rat hepatocytes by liquid chromatography-tandem mass spectrometry" J. of Chrom. B *798*, **2003**, pp. 265-274

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TRADEMARKS/LICENSING

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