Using Multiple Mass Defect Filters and Higher Energy Collisional Dissociation on an LTQ Orbitrap for Fast, Sensitive and Accurate Metabolite ID

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Overview

Purpose: To evaluate the use of Multiple Mass Defect Filters (MMDF) with LTQ Orbitrap data for metabolite identification; to investigate the use of Higher energy Collisional Dissociation (HCD) for structural elucidation in metabolite identification experiments.

Methods: Rat hepatocyte incubation samples of Irinotecan were analyzed using an LTQ Orbitrap with HCD functionality. Both Collision Induced Dissociation (CID) MS/MS as well as HCD MS/MS were acquired for the potential metabolites. MMDF were then used to process the acquired raw file.

Results: MMDF were able to filter out the vast majority of the background ions in the full scan spectra, while preserving those related to the parent drug. Comparison with the results using only a single Mass Defect Filter (MDF), results from MMDF are more distinct and more specific, allowing users to do faster, more sensitive and more accurate analyses. HCD provides complementary fragmentation pathways in addition to the Collision Induced Dissociation (CID) available in the ion trap, and produce low mass diagnostic ions in MS/MS spectra that are very useful for metabolite structural elucidation.

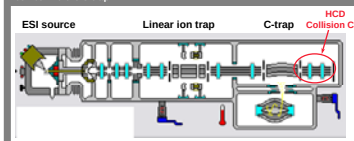
Introduction

An integral part of drug discovery and development is the identification of drug metabolites formed through phase I and phase II metabolic reactions. These metabolites may have intrinsic pharmacological activity or display specific toxicity. LC-MS has become the cornerstone in drug metabolite identification because of its sensitivity and ability to analyze complex mixtures. Particularly, LC-MS^n employing linear ion trap (LIT) technology has become widely used because of its speed, sensitivity and robustness in generating rich structure information. However, challenges still remain in detecting and identifying metabolites in the presence of highly complex biological matrices.

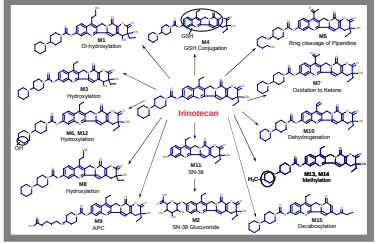
Coupling an Orbitrap mass analyzer to the LIT has greatly facilitated the task of metabolite identification, because it not only enables parallel data acquisition with high mass accuracy and resolution, but also provides post LIT ion manipulations. High resolution and accurate mass help to resolve and identify metabolite peaks from background matrix ions, and also allow the use of post-acquisition data processing tool like Mass Defect Filter (MDF) to reduce the number of false positives by removing the vast majority of matrix-related background ions. Higher energy Collisional Dissociation (HCD) was recently introduced on the Orbitrap as an alternative dissociation method by adding a new collision cell behind the C-trap region (Figure 1.). HCD can be used to generate low mass diagnostic ions in MS/MS mode, and can be used in combination with CID in the LIT for MS^n experiments.

Irinotecan (CPT-11; 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin) is a water-soluble carbamate prodrug of camptothecin and is activated in vivo to SN-38, a potent topoisomerase I inhibitor.[1,2] Currently, Irinotecan in combination with 5-fluorouracil and leucovorin is approved by the U.S. Food and Drug Administration as first-line therapy in the treatment of metastatic carcinoma of colon or rectum.[2] In this study, we will utilize MMDF and HCD on an LTQ Orbitrap to study the biotransformations of Irinotecan in hepatocyte incubation and identify its metabolites.

Figure 1. Scheme diagram of the LTQ Orbitrap, highlighting the HCD collision cell behind the C-trap.



Scheme 1. Proposed structures and biotransformation pathways of Irinotecan metabolites.



Materials & Methods

Samples: Incubation was carried out using rat hepatocytes pooled from 1 male and 1 female with a cell density of 0.5million/mL and 10uM of Irinotecan in the final 1mL incubation solution. The solution was shaked overnight and quenched by cooling down on dry ice, followed by the addition of 200uL chilled AcN. The solution was then vortexed and centrifuged. The supernatant (~1mL) was taken out and 10uL from such solution was directly injected for each LC-MS/MS run.

HPLC: Accela™, Thermo Fisher Scientific

Column: Hypersil gold, 1 x 100mm, 1.9μM particle size, Thermo Fisher Scientific

Gradient:

Time	A%	B%	μL/min
0	98	2	150
2	98	2	150
2.5	85	15	150
14	75	25	150
14.9	20	80	150
15	98	2	150
20	98	2	150

Mass Spectrometer: LTQ Orbitrap XL™ with HCD collision cell

Table 1. Summary of the putative metabolites identified from 10mM Irinotecan rat hepatocyte incubation. Those highlighted in different colors were filtered by different Mass Defect Filters.

MDF Template	#	R.T.	Metabolite Identity	Formula Change	Integrated Peak Area	% of Parent	Theoretical MH+ m/z	Measured MH+ m/z	Ppm	MW (Da) from Irinotecan	ΔMD from Irinotecan	ΔMD from Template
Irinotecan MH+ = 587.2864	M1	7.19	Di-hydroxylation	+O2	4.06E+6	0.027	619.2762	619.2755	-1.1	31.9898	+ 0.0102	+ 0.0102
	M3	8.46	Hydroxylation	+O	1.66E+6	0.107	603.2813	603.2805	+1.3	15.9949	0.0051	+ 0.0051
	M4	8.46	GSH conjugation	+C10H15N3O5S	8.76E+5	0.006	892.3046	892.3051	0.6	305.0682	+ 0.0682	+ 0.0682
	M5	8.84	Ring cleavage of Piperidine	+H2O	9.86E+5	0.653	605.2970	605.2965	-0.8	18.0106	+ 0.0106	+ 0.0106
	M6	9.92	Hydroxylation	+O	1.36E+6	0.867	603.2813	603.2800	-2.2	15.9949	-0.0051	-0.0051
	M7	10.34	Oxidation to Ketone	+O-O	9.70E+4	0.065	601.2657	601.2650	+1.2	13.9792	-0.0208	-0.0208
	M8	10.64	Hydroxylation	+O	8.66E+4	0.059	603.2813	603.2805	+1.3	15.9949	-0.0051	-0.0051
	M9	11.11	APC	+O2	1.75E+5	0.113	619.2762	619.2756	-0.6	31.9898	+ 0.0102	+ 0.0102
	M10	11.55	Dehydrogenation	-H2	3.75E+5	0.247	585.2757	585.2699	-1.4	-2.0157	-0.0157	-0.0157
	P	11.68	Irinotecan	N.A.	1.55E+8	100	587.2864	587.2860	-0.7	0	0	0
	M12	12.68	Hydroxylation	+O	1.20E+5	0.080	603.2813	603.2803	+1.7	15.9949	-0.0051	-0.0051
	M13	12.72	Methylation	+CH2	1.10E+6	0.007	601.3021	601.3013	+1.3	14.0157	+ 0.0157	+ 0.0157
	M14	13.67	Methylation	+CH2	1.56E+6	0.010	601.3021	601.3022	0.2	14.0157	+ 0.0157	+ 0.0157
	M15	15.77	Decarboxylation	-CO2	3.06E+5	0.213	543.2966	543.2965	+1.1	+43.9898	+ 0.0102	+ 0.0102
SN-38 MH+ = 393.1445	M2	7.44	SN38 Glucuronide	+C6H10O6	4.06E+5	0.267	569.1766	569.1754	-2.1	176.0321	-0.1106	+ 0.0321
	M11	11.90	SN-38	N.A.	6.16E+5	0.407	393.1445	393.1440	-1.3	0	-0.1445	0

Results & Discussions

Multiple Mass Defect Filters (MMDF) is a new feature in the latest version of Thermo Scientific Metworks™ software that combines the results from up to 6 different MDFs. Through the use of MMDF and the combination of HCD and CID MS/MS, fifteen Irinotecan metabolites were identified from the incubation sample using LTQ Orbitrap XL™ with less than 3ppm mass accuracy. Table 1 summarizes the 15 metabolites identified, while Scheme 1 displays their corresponding structures.

As shown in Table 1, all 15 metabolites were found with peak areas less than 1% of that of the parent. Figure 2 shows how the base peak chromatogram from the same LC-MS/MS run changed after a single MDF, and after MMDF processing. Due to the low abundance of the metabolites, in all these chromatograms, the intensity (y axis) for retention time 5.7-11.2 minute and 12.5-16.1 minute was expanded by 50 times to better illustrate the effectiveness of MMDF. While all the peaks from the Irinotecan metabolites were well buried in the original chromatogram (Figure 2a), the most abundant metabolite peaks (e.g. M2 @ 7.44 minute, M5 @ 8.84 minute, M7 @ 9.92 minute) starts to show up after applying a single MDF (Figure 2b). However, even with a single MDF, peaks from background matrix ions that are unrelated to the metabolite were still prominent (e.g. peaks at 6.55 minute, 7.85 minute, and 11.1 minute). This is due to the fact that in order to use only one single MDF to capture all the phase I and II metabolites, including those from the hydrolysis product SN-38, a relatively wide mass defect range needs to be used (-150 mmu, +70mmu). Therefore, a portion of the background ions still remains after the mass defect filtering. When MMDF were applied to the original data (Figure 2c), four different mass defect filters were used, whose corresponding metabolites identified were highlighted using different colors in Table 1: phase I metabolites of Irinotecan are shown with white background; phase II of

FIGURE 2. Base peak chromatograms of 10mM Irinotecan rat hepatocyte incubation: (a) Original; (b) After single Mass Defect Filter; (c) After Multiple Mass Defect Filters (MMDF).

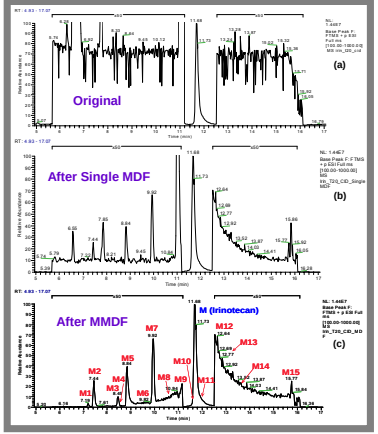
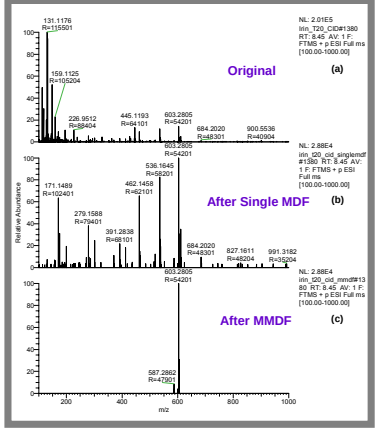


FIGURE 3. Full MS spectrum at 8.45 minute: (a) original, (b) after single Mass Defect Filter, (c) after Multiple Mass Defect Filter (MMDF).

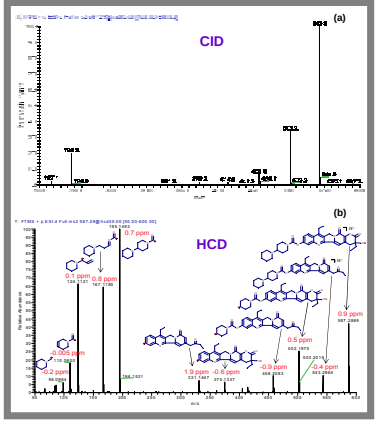


Irinotecan are shown in light blue; phase I of SN-38 are shown in yellow; phase II of SN-38 are shown in pink. Comparing to the results using a single MDF (Figure 2b), results from MMDF (Figure 2c) are cleaner and more specific to the metabolites related to Irinotecan, therefore easier to interpret.

Figure 3 further illustrates the power of MMDF by giving an example of how the full MS spectrum changed after the same single MDF, and MMDF were applied. The peak at 603.2805 m/z is a hydroxylation metabolite M3 that elutes at 8.45 minute. The original full scan MS is dominated by background ions and M3 peak only has less15% relative abundance. After a single MDF was applied, the M3 peak becomes the base peak, however, there are still a lot of background peaks remaining in the spectrum. After MMDF were applied, only the M3 peak and trace of the parent remains in the spectrum while all the background ions are gone.

After MMDF processing, HCD and CID MS/MS spectra of Irinotecan and its potential metabolites were analyzed and their corresponding structures were elucidated. Figure 4a shows the CID MS/MS of Irinotecan from LTQ and Figure 4b shows the HCD MS/MS from the Orbitrap. While all the major fragment ions in the CID spectrum were also observed in the HCD spectrum, HCD spectrum contains additional fragment ions and has no low mass cut off. A portion of the parent ions still remains in the HCD spectrum. These are characteristics similar to those from a quadruple collision cell. Less than 2ppm mass accuracy and high resolution were obtained on the fragment ions in the HCD MS/MS spectrum because it was acquired in the orbitrap. Mass Frontier™ software with its accurate mass capability was used to assist spectrum interpretation. Structures of the fragment ions were assigned accordingly. The fact that HCD spectra display rich fragment ions, especially in the low mass region, as well as high mass accuracy on these product ions greatly facilitates the MS/MS interpretation.

FIGURE 4. MS/MS spectra of Irinotecan: (a) CID MS/MS acquired in the LTQ. (b) HCD MS/MS acquired in the Orbitrap.



Conclusions

With the help of MMDF and the combination of HCD and CID MS/MS, 15 Irinotecan metabolites whose peak areas are less than 1% of that of the parent were identified on an LTQ Orbitrap XL™ coupled to an Accela™ LC.

This reports demonstrates that MMDF is more effective than a single MDF to uncover phase I and II metabolites specifically and concurrently. It also allows the detection of metabolites from hydrolysis or N-dealkylation, even when the products from such processes have mass defects that are significantly different from the parent. MMDF allows users to use low threshold values during data processing so that metabolites at very low level can be easily identified. The resulting chromatogram from MMDF is very accurate and specific because it is based on exact mass and mass deficiencies, which are highly specific to the parent drug compound. It provides speed, sensitivity and accuracy to facilitate the identification of drug metabolites in drug discovery and development.

HCD provides an alternative fragmentation method on the LTQ Orbitrap in addition to the CID in the linear ion trap. HCD spectra display characteristics similar to those from a quadruple collision cell: rich in product ions, has no low mass cut off, typically a portion of the parent ions still remains. The fragment ions in HCD spectra have high mass accuracy and resolution. These characteristics of HCD spectra compliments the power of ion trap MS^n and allow easy spectrum interpretation and high confidence in structural elucidation .

References

(1) Haaz *et al*., Drug Metabolism and Disposition, **1998**, 26 (8), 769-774.

(2) Sanghani *et al*., Drug Metabolism and Disposition, **2004**, 32 (5), 505-511.

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