Identification of SIL-GSH Reactive Metabolites Using LC-HRAM and Pattern Matching

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ABSTRACT

New software tool to effectively and quickly find reactive metabolites from SIL-GSH trapping experiment using pattern scoring approach for flagging peaks of unknown compounds.

INTRODUCTION

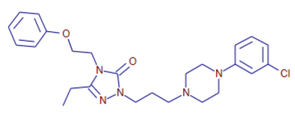
Metabolite profiling including detection of trapped reactive metabolites has become an integral part of the modern drug discovery process. The purpose of this work was to validate application of LC-HRAM with post-acquisition data processing using new software Compound Discoverer. The model compound nefazodone, is used as positive control in metabolite profiling studies at Absorption System. The metabolism of NFZ leads to generation of a number of soft and hard reactive metabolites, conjugates of which were detected using "classical" scanning LC-MS/MS methodologies. Here we show significant improvement in overall coverage of trapped reactive metabolites using LC-HRAM data and new Pattern Scoring node in Compound Discoverer software.

MATERIALS AND METHODS

The LC-HRAM analysis was executed using Dionex XR3000 quaternary solvent HPLC system interfaced with LTQ Orbitrap™ XL mass spectrometer equipped with HESI-II ion source probe.

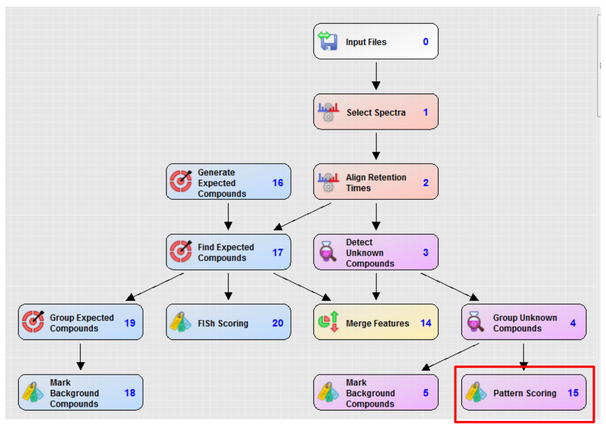
Human liver microsomes (HLM) were purchased from XenoTech (pool of 200 donors). The stable labeled ($^{13}C2,^{15}N$ -Gly) GSH (SIL-GSH) were from Cambridge Isotope Laboratories, all other reagents (analytical or HPLC grad) were from Sigma-Aldrich. The incubation mixture in PBS buffer contained 0.5 mg/ml of HLM , 50 µM of nefazodone (NFZ) and mixtures of GSH/SIL-GSH (1:1; total 5 mM) and KCN/ $K^{13}C^{15}N$ (1:1, total 1mM). The reaction was initiated by addition of NADPH (final c=1 mM). The reaction mixture was then incubated in a shaking water bath at 37°C for 60 minutes. The reaction was terminated by the addition of ice-cold MeCN (1:1, v/v). After centrifugation at 13,000 rpm for 10 minutes, supernatant from all samples were transferred into HPLC vials for analysis.

Parent Compound: Nefazodone:
Formula: C25H32ClN5O2
MW: 469.2244



Thermo Scientific™ Compound Discoverer 2.0 software was used for data processing. The node-assembled workflow from Compound Discoverer included both expected metabolite search and unknown compound detection within the same workflow. The workflow employed the new Pattern Scoring node which flagged peaks of unknown compounds showing mono-isotopic pattern characteristic for the employed mixture GSH/SIL-GSH

Figure 1. Compound Discoverer workflow including both expected and unknown with Pattern Scoring



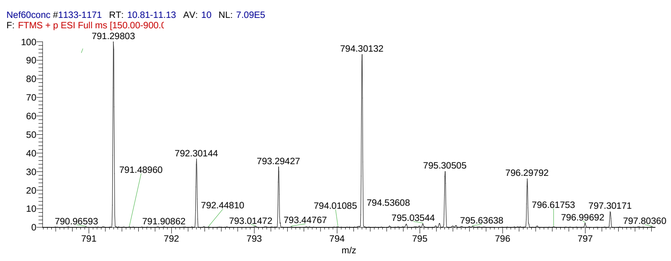
Compounds flagged by Pattern Scoring were examined based on match scores. Artifacts from neutral losses and solvent blank were removed from the final list. Number of GSH conjugate metabolites found were compared with literature [Ref 1].

RESULTS

Defining the Pattern

The pattern used in Pattern Scoring node was based on the raw averaged full ms spectrum for the GSH conjugate compound of Nafazodone with m/z 791.2980. See Figure 2 showing the mass range of interest in Qual Browser.

Figure 2. Averaged Full MS spectrum between RT 10.81-11.13min zoomed in between 790.5 to 797.5 showing the unlabeled and labeled pattern



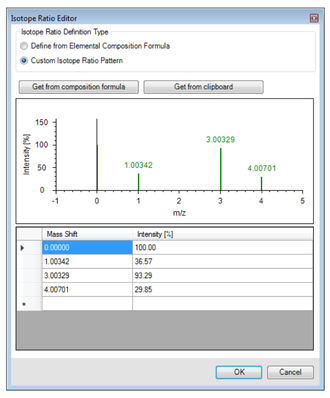
To export the exact masses of the profile peaks in Figure 2, right click and choose View | Spectrum List, then choose Export | Clipboard (Exact Mass). This exports the centroid masses from the displayed spectrum.

Pattern Scoring node

GSH conjugates of trapped reactive metabolites could be present at very low levels (~1E4), resulting in distortion of isotopic patterns. Therefore pattern containing only A0s and A1s of GSH and SIL-GSH was used(Figure 3).

Pattern Scoring node is designed to flag any unknown compound that displays the user defined pattern in full MS with a match status and a match score. The pattern matching algorithm applies mass tolerance (5ppm) and intensity tolerance of (30%) to each mass packets during pattern matching.

Figure 3. Isotope Ratio Editor in Pattern Scoring node.

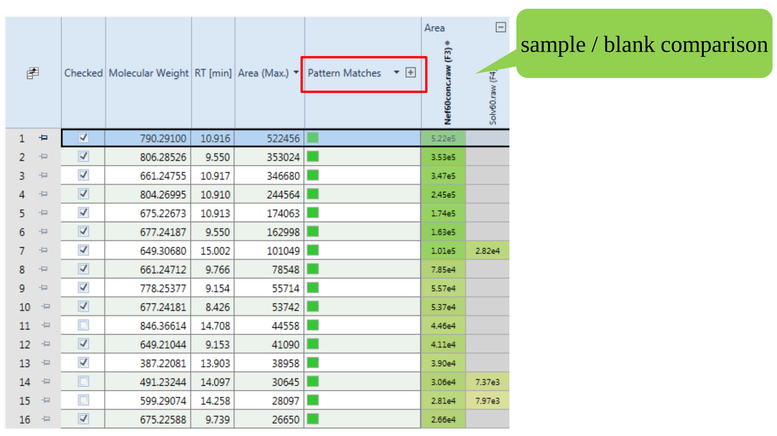


Compounds Flagged by Pattern Scoring

In order to detect metabolites including low level ones, the Min. Peak Intensity in the Detect Unknown Compounds node was set to 500. There were total of 18584 compounds detected excluding compounds in blank. Out of the 18584 compounds, 60 compounds matched pattern.

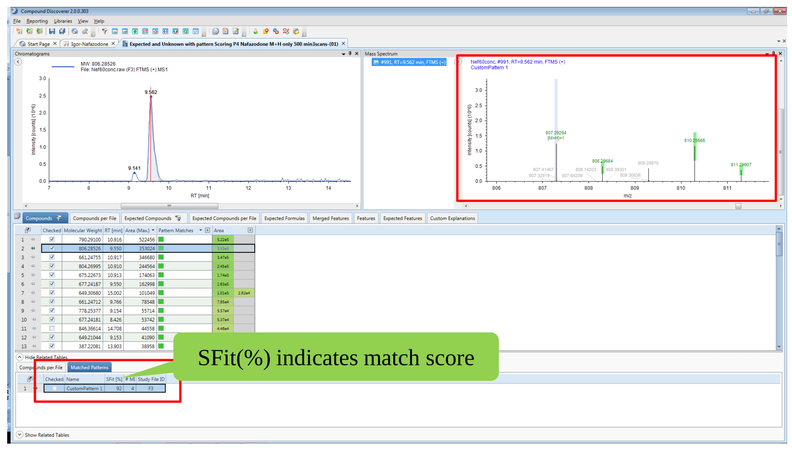
After column sorting on Pattern Matches status and Area (Max), the 60 compounds are listed in order in the Compounds table. Area for sample and blank are displayed next to each other for easy comparison. See Figure 4.

Figure 4. Compounds match pattern after column sorting.



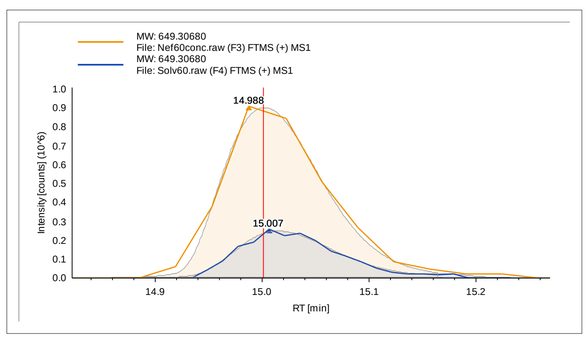
For each of the matched compounds, the matched pattern is displayed in the spectrum view for enhanced confidence of analysis results. See Figure 5.

Figure 5. Data review of pattern matched compounds



Out of the 60 matched compounds, 46 compounds had SFit% of > 60%. From the 46 compounds, we removed false positives by excluding compounds which were neutral losses of 129 and compounds that existed in the blank with lower concentration (see Figure 6).

Figure 6. Comparison between sample and blank helps removal of false positive compounds that match the pattern



An area threshold filter of1e4 was also applied to ignore the tiny metabolites. This resulted in total of 13 GSH conjugate metabolites in the final list (Figure 7)

Figure 7. Final list of GSH conjugate metabolites from Merge Features table.

	Checked	Apex m/z	RT [min]	Max. Area	Detect Unknown Compounds	Find Expected Compounds	Group Areas
1	✓	807.29254	9.550	353024			3.53e5
2	✓	807.29244	9.141	26795			2.68e4
3	✓	805.27722	10.919	244564			2.45e5
4	✓	805.27667	10.346	11771			1.18e4
5	✓	791.29828	10.915	522456			5.22e5
6	✓	791.29822	10.406	22253			2.23e4
7	✓	787.34698	10.204	13739			1.37e4
8	✓	779.26105	9.154	55714			5.57e4
9	✓	757.33594	9.923	18702			1.87e4
10	✓	678.24908	8.426	53742			5.37e4
11	✓	676.23315	9.739	26650			2.66e4
12	✓	662.25439	9.766	78548			7.85e4
13	✓	388.22809	13.903	38958			3.90e4

Why Is This Workflow Valuable?

In this study, we combined both expected and unknown with pattern scoring in the same workflow.

The expected search included comprehensive dealkylation / dearylation predictions combined with combinatorial transformations search. The combinatorial steps was set to 4, which means the node automatically combines single step transformations for up to 4 reaction steps. Dealkylation / dearylation is considered as one step. Phase II transformation was limited to 1 step in the node. See Figure 8.

The unknown workflow with pattern scoring in Compound Discoverer 2.0 found 13 possible GSH reactive metabolites. 6 of them were reported by literature [Ref 1]. The expected search was able to explain 7 of the 13 metabolites including 757 that was previously not reported in literature (see Table 1).

Figure 8. (a) Generate Expected Compounds node settings; (b) single step transformation list used by the node

(a)

Parameters of 'Generate Expected Compounds'	
Show Advanced Parameters	
1. Compound Selection	
Compound	Nafazodone (C25 H32 Cl N5 O2)
2. Dealkylation	
Apply Dealkylation	True
Apply Dearylation	True
Max. # Steps	2
Min. Mass [Da]	150
3. Transformations	
Phase I	Dehydration (H2 O ->); Desaturat
Phase II	Acetylation (H -> C2 H3 O); Argir
Others	
Max. # Phase II	1
Max. # All Steps	4
4. Ionization	
Ions	[M+H]+1

(b)

	Name
	Aa
1	Acetylation
2	Arginine Conjugation
3	Dehydration
4	Desaturation
5	Glucoside Conjugation
6	Glucuronide Conjugation
7	Glutamine Conjugation
8	Glycine Conjugation
9	GSH Conjugation (on Bromine)
10	GSH Conjugation (on Chlorine)
11	GSH Conjugation (on Fluorine)
12	GSH Conjugation 1
13	GSH Conjugation 2
14	Hydration
15	Methylation
16	Nitro Reduction
17	Ornitine Conjugation
18	Oxidation
19	Oxidative Deamination to Alcohol
20	Oxidative Deamination to Ketone
21	Oxidative Debromination
22	Oxidative Dechlorination
23	Oxidative Defluorination
24	Palmitoyl Conjugation
25	Reduction
26	Reductive Debromination
27	Reductive Dechlorination
28	Reductive Defluorination
29	Stearyl Conjugation
30	Sulfation
31	Taurine Conjugation
32	Thiourea to Urea

Table 1. Summary of GSH conjugate metabolites identified by Compound Discoverer

#	Apex m/z	RT [min]	Area	Detected by Unknown	Detected by Expected	Proposed Conjugate Composition	Reported in Literature
1	807.2925	9.55	353024	Y	Y	P+2O+GSH-2H	Y
2	807.2924	9.14	26795	Y	Y	P+2O+GSH-2H	Y
3	805.2772	10.92	244564	Y	Y	P+2O+GSH-2-4H	Y
4	805.2767	10.35	11771	Y	Y	P+2O+GSH-2-4H	Y
5	791.2983	10.92	522456	Y	Y	P+O+GSH-2H	Y
6	791.2982	10.41	22253	Y	Y	P+O+GSH-2H	Y
7	787.3470	10.20	13739	Y	N	Not defined	N
8	779.2611	9.15	55714	Y	N	Not defined	N
9	757.3359	9.92	18702	Y	Y	P-Cl+O+GSH-2H	N
10	678.2491	8.43	53742	Y	N	P+2O+GSH-2H-yGln	N
11	676.2332	9.74	26650	Y	N	P+2O+GSH-4H-yGln	N
12	662.2544	9.77	78548	Y	N	P+O+GSH-2H-yGln	N
13	388.2281	13.90	38958	Y	N	Not defined	N

CONCLUSIONS

- Unknown compound detection and pattern scoring combined with expected combinatorial search workflow in Compound Discoverer 2.0 is a very powerful and effective workflow for profiling of GSH trapped reactive metabolites;
- Combining HRAM and Compound Discoverer 2.0 software not only increased the confidence of metabolite profiling but also dramatically decreased the data processing time.
- This workflow can benefit other unknown and labeling compound studies;
- Future work will focus on structure elucidation of these unreported metabolites.

REFERENCES

1. Jian W[1], Liu HF, Zhao W, Jones E, Zhu M: Simultaneous screening of glutathione and cyanide adducts using precursor ion and neutral loss scans-dependent product ion spectral acquisition and data mining tools. J Am Soc Mass Spectrom. 2012 May; 23(5):964-76.

TRADEMARKS/LICENSING