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Software

Detection and identification of isotope-labeled glutathione-trapped reactive drug metabolites

A streamlined solution using the Orbitrap Exploris 240 MS and Compound Discoverer software

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Keywords

Drug metabolism, metabolite identification (MetID), high-resolution accurate mass (HRAM), Orbitrap Exploris 240 MS, Compound Discoverer software, labeled metabolite profiling, glutathione screen, GSH neutral loss screen, stable isotope labeled glutathione, structure elucidation, structural characterization

Application benefits

- Accomplish high-throughput metabolites detection and identification through high quality HRAM MS and MS/MS data acquired with high resolution, high mass accuracy, and internal calibration.
- Achieve a comprehensive approach to labeled small molecule profiling and structural characterization with greater confidence using intelligent data processing software.
- Increase overall productivity through high-quality intelligent data acquisition and advanced data processing software.

Goal

To demonstrate the integrated approach for high throughput identification and structural characterization of glutathione (GSH) trapped metabolites utilizing liquid chromatography-high resolution mass spectrometry and efficient data processing in Compound Discoverer software.

Introduction

The investigation of a drug's metabolic fate is an important component of the drug development process, to ensure its efficacy and safety. In particular, formation of metabolites that are capable of reacting with macromolecules (*reactive metabolites*) could contribute to drug-induced toxicity.[1] For this reason, GSH trapping is a widely used assay for detection of these reactive metabolites during the lead optimization stage in the pharmaceutical industry, and the trapped GSH conjugates can be characterized by liquid chromatography mass spectrometry (LC-MS).[2] One of the classic LC-MS methods to detect GSH trapped metabolites relies on monitoring the constant neutral loss of 129 Da using triple quadruple mass spectrometers in the positive ion mode, as

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GSH conjugates undergo pyroglutamic acid moiety cleavage during fragmentation.[2] However, the main limitation of this approach is its low selectivity and sensitivity. In order to improve the selectivity, a mixture of non-labeled and stable isotope-labeled GSH is commonly employed as the trapping agent, allowing for unambiguous identification of GSH conjugates by detecting the characteristic doublet with a distinct mass difference in the mass spectral data.[3,4] Subsequent structural characterization of the GSH trapped reactive metabolite to identify the sites of bioactivation allows for further structure optimization in the drug discovery process.[3,4]

This work demonstrates a solution for streamlined detection and comprehensive structure elucidation of the isotope-labeled GSH trapped reactive metabolites in complex biological matrixes using the high-resolution accurate mass Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer and the Thermo Scientific™ Compound Discoverer™ software.

To showcase the data acquisition and processing, clozapine, an antipsychotic agent, was chosen as a reference compound for the study. Clozapine GSH conjugates were formed in rat liver microsomal incubations supplemented with a mixture of non-labeled GSH and stable isotope-labeled GSH ([$^{13}C_2$, ^{15}N]) at a 1:1 ratio. Samples were analysed using reversed phase chromatography on a Thermo Scientific™ Vanquish™ Flex UHPLC system coupled to a Orbitrap Exploris 240 MS.

Data was collected in positive ionization mode using a FullScan data-dependent MS^2 ($ddMS^2$) method. Data processing was performed using Compound Discoverer 3.3 SP3 software.

Briefly, the results show that the Orbitrap Exploris 240 mass spectrometer generated high-resolution accurate mass (HRAM) Full Scan and HCD-MS^2 data with sub-ppm mass accuracy, enabling confident metabolite detection and structural characterization. Unbiased identification of GSH trapped reactive intermediates were achieved via Compound Discoverer software using a single processing workflow. The workflow employed nodes for both expected and unknown compounds detection. To filter the unknown compound results, the use of the *Pattern Scoring* node allowed the user to flag compounds with the distinct isotopic pattern corresponding to the incorporation of isotope-labeled GSH and the use of the *Search Neutral Losses* node allowed for the detection of compounds with the characteristic GSH neutral loss, increasing confidence in the identification. Finally, the *FISh Scoring* node provided *in silico* fragmentation predication and automatically annotated fragmentation spectra with predicted fragment structures to facilitate structure elucidation of the detected metabolites. Using this data processing workflow, a total of nine GSH-trapped reactive metabolites of clozapine could be detected and identified, as detailed hereafter.

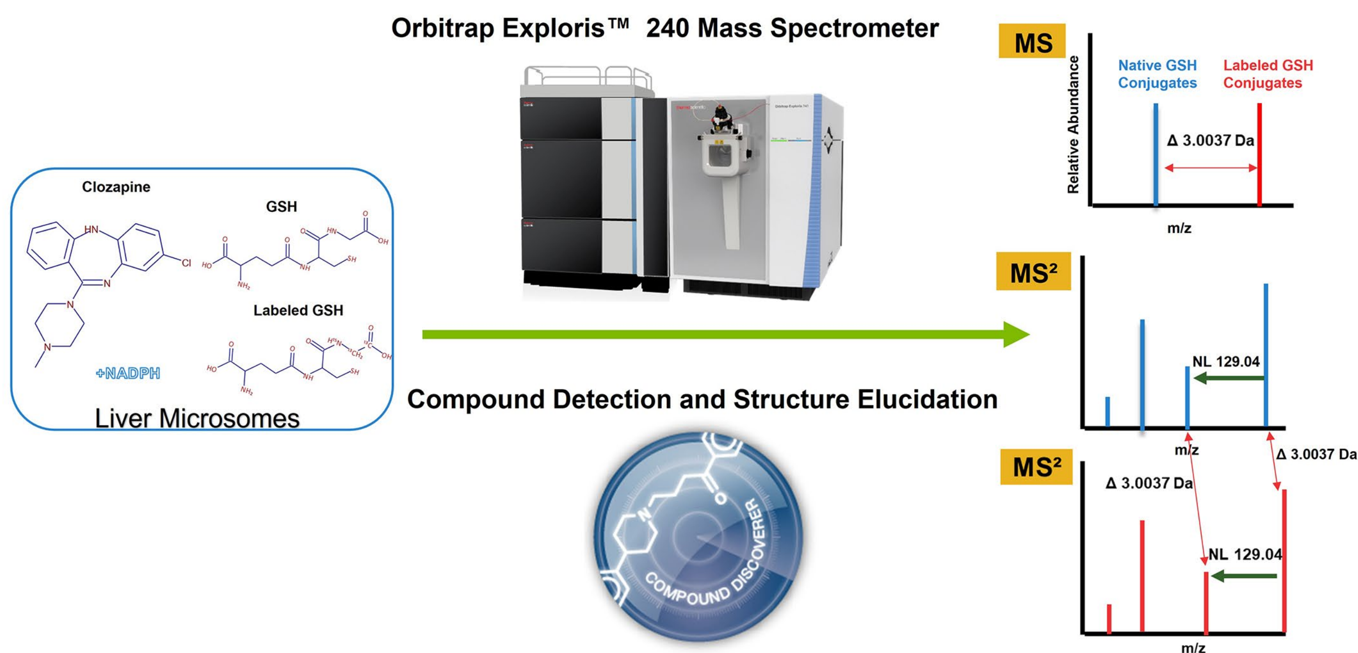


Figure 1. Workflow overview for detection and identification of labeled glutathione-trapped reactive metabolites using the Orbitrap Exploris 240 MS and Compound Discoverer software.

Experimental details

Sample preparation

Clozapine (10 µM) was incubated with rat liver microsomes (1 mg/mL) in the presence of NADPH (1 mM) and a mixture of GSH and [$^{13}C_2$,^{15}N]-GSH (1:1 ratio, total 5 mM). A negative control was prepared with incubations in the absence of drug substrate. The enzymatic reaction was stopped by the addition of one volume of cold acetonitrile containing 0.1% of formic acid at 0 and 60 minutes, followed by centrifugation for protein precipitation. The supernatants were concentrated under a stream of nitrogen and reconstituted to 100 µL with H_2O and ACN (1:4 v:v).

Liquid chromatography and mass spectrometry

Chromatographic separation was performed with a Vanquish Flex Binary system using a reversed phase method. A C18 stationary phase (2.5 µm particle size) in a 100 × 2.1 mm column format was used. Samples of 8 µL each were injected, and the mobile phase was eluted with a constant flow rate of 300 µL/min. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. The gradient condition is shown in Table 1. Mass spectrometric analysis was performed on an Orbitrap Exploris 240 mass spectrometer operated in Full MS data-dependent MS^2 mode. Analysis was performed in positive ionization mode. Full MS scan range of *m/z* 100–1000 enabled unbiased compound detection. Run Start EASY-IC™ was activated for internal mass calibration. Resolution for the Full MS scan was set at 120,000 and at 30,000 for the $ddMS^2$ scans. HCD was performed with normalized stepped collision energies of 20, 30 and 50%. Ion source parameter and scan settings are listed in Table 2 and Table 3 respectively. The *Deep Scan* workflow of the Thermo Scientific™ AcquireX™ data acquisition strategy was subsequently used to improve the fragmentation data coverage for relevant metabolites.

Table 1. LC gradient for sample analysis

Time (min)	B%	Flow rate (mL/min)
0	5	0.3
1.5	5	0.3
9	55	0.3
12	100	0.3
14	100	0.3
14.3	5	0.3
15	5	0.3

Table 2. Ion source properties

Parameter	Value
Ion source type	HESI
Source voltage	+ 3500 V
Sheath gas (Arb)	50
Aux gas (Arb)	10
Sweep gas (Arb)	1
Vaporizer temp (°C)	350
Ion transfer tube temp (°C)	325

Table 3. Scan settings: full scan properties and data dependent MS^2 settings

Parameter	Value
Full scan properties	
Orbitrap resolution	120,000 @ *m/z* 200
Scan range (*m/z*)	100-1000
RF lens (%)	70
AGC target	Standard
Maximum injection time mode	Auto
Data-dependent MS^2 scan properties	
Isolation window (*m/z*)	1.5
HCD collision energies	Normalized, 20, 30, 50%
Orbitrap resolution	30,000 @ *m/z* 200
Scan range mode	Auto
AGC target	Standard
Maximum injection time (ms)	100

Compound Discoverer workflow

Samples were analyzed using Compound Discoverer 3.3 SP3 software. Compound Discoverer software is a powerful data mining tool for confident metabolite identification, which offers a fully customizable node-based processing workflow to process HRAM full scan MS and MS^n data. The MetID-specific processing workflow used for concurrently detecting expected and unexpected metabolites is shown in Figure 2.

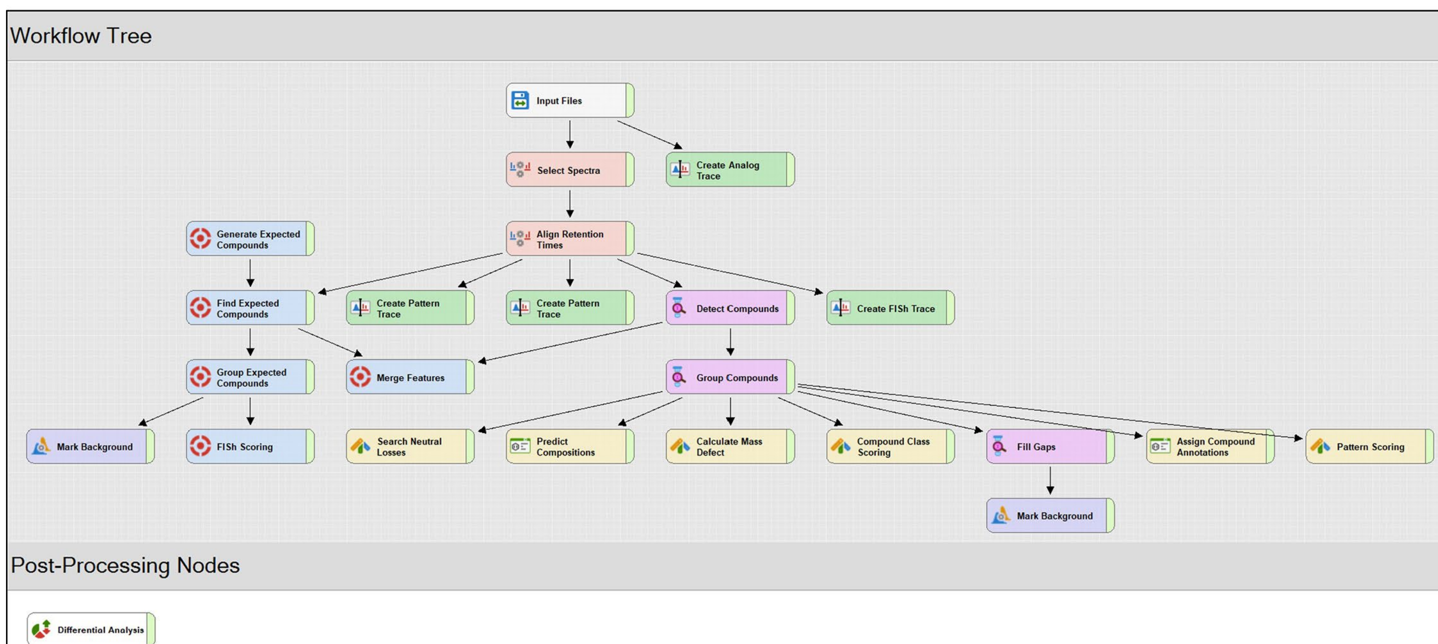


Figure 2. Compound Discoverer workflow tree for this study. This workflow template was customized based on a combination of nodes from the *Compound Discoverer MetID w Stats Expected* and the *Unknown w Background Removal Workflow* trees to create a combined workflow.

The combined workflow uses targeted and untargeted approaches to capture the expected and unknown metabolites.[5] The targeted approach detects the metabolites from the expected metabolite list which was generated based on the intelligent dealkylation/dearylation predictions and list of common metabolic transformations. Importantly, the list of transformations was customized to include the isotope-labeled GSH modification described in this work (Figure 3A).

- The ***Generate Expected Compounds*** node generates a list of expected *m/z* values for the ionized potential clozapine metabolite compounds. This list was curated based on the structure of the clozapine parent compound, the user-specified transformation lists and number of combinatory steps, and the user-specified ionic species (Figure 3B). Glutathione conjugates often exist as multiple-charged species under positive electrospray ionization, so both singly charged and double charged state were chosen in the selection of the adduct ions.

- Subsequently, the ***FISh Scoring*** node (FISh = fragment ion search) was used to annotate the MS^2 scans with matching fragments structures and explain the fragments of the detected expected compounds for structure elucidation.[6] On the other hand, the "unknown workflow" detects untargeted compounds unbiasedly.

- The ***Pattern Scoring*** node flags compounds that have user-defined isotopic patterns, enabling the profiling of isotope-labeled compounds.

- The ***Search Neutral Losses*** node marks compounds that undergo certain neutral losses.

- The ***Compound Class Scoring*** node can annotate compounds that share matching MS^2 fragments with the parent and other known metabolites.

- The ***Differential Analysis*** node calculates the statistics between different sample groups such as the ratios of the compound peak areas in the experimental samples over the negative controls, which helps streamline the data analysis and decrease the number of false positive hits from the biological matrix background.

A

Transformations

New... Edit... Delete Import... Export All...

	Name	Leaving Group	Arriving Group	Leaving Modification	Arriving Modification	ΔM [Da]	Phase	Max Occurrence
1	13C215NGSH Conjugation 1		C8 [13]C2 H15 N2 [15]N O6 S		C8 [13]C2 H15 N2 [15]N O6 S	308.07190	Phase2	1
2	13C215NGSH Conjugation 2		C8 [13]C2 H17 N2 [15]N O6 S		C8 [13]C2 H17 N2 [15]N O6 S	310.08755	Phase2	1
3	13C215NGSH Conjugation on Cl	Cl	C8 [13]C2 H16 N2 [15]N O6 S	Cl	C8 [13]C2 H16 N2 [15]N O6 S	274.11087	Phase2	1

B

Generate Expected Compounds

Parameters of 'Generate Expected Compounds'

Parameter	Value
1. Compound Selection	
Compounds	Clozapine (C18 H19 Cl N4)
2. Dealkylation	
Apply Dealkylation	True
Apply Dearylation	True
Max. # Steps	3
Min. Mass [Da]	150
3. Transformations	
Phase I	Cyanide Adduct (H -> C N); Dehydration (H2 O ->); Desaturation
Phase II	13C215NGSH Conjugation 1 (-> C8 [13]C2 H15 N2 [15]N O6 S); 1
Others	
Max. # Phase II	1
Max. # All Steps	3
4. Ionization	
Ions	[M+2H]+2; [M+H]+1

Show Checked Only (18/22)

Filter...

- 13C215NGSH Conjugation 1 (-> C8 [13]C2 H15 N2 [15]N O6 S)
- 13C215NGSH Conjugation 2 (-> C8 [13]C2 H17 N2 [15]N O6 S)
- 13C215NGSH Conjugation on Cl (Cl -> C8 [13]C2 H16 N2 [15]N O6 S)
- Acetylation (H -> C2 H3 O)
- Arginine Conjugation (H O -> C6 H13 N4 O2)
- Cysteine Conjugation 1 (H -> C3 H6 N O2 S)
- Cysteine Conjugation 2 (-> C3 H7 N O2 S)
- Glucoside Conjugation (H -> C6 H11 O5)
- Glucuronide Conjugation (H -> C6 H9 O6)
- Glutamine Conjugation (H O -> C5 H9 N2 O3)
- Glycine Conjugation (H O -> C2 H4 N O2)
- GSH Conjugation (on Chlorine) (Cl -> C10 H16 N3 O6 S)
- GSH Conjugation 1 (-> C10 H15 N3 O6 S)
- GSH Conjugation 2 (-> C10 H17 N3 O6 S)
- Methylation (H -> C H3)
- Stearyl Conjugation (H -> C18 H35 O)
- Sulfation (H -> H O3 S)
- Taurine Conjugation (H O -> C2 H6 N O3 S)

Figure 3. (A) View of the Transformations list in Compound Discoverer software, showing the custom added GSH conjugations with the [$^{13}C_2$,^{15}N] label included; (B) overview of the Generate Expected Compounds node settings, where "Apply Dealkylation" and "Apply Dearylation" were set to True, Phase I and Phase II including the customized labeled GSH transformations were selected, and the number of combinatory steps and adduct ion species were specified as shown to generate the list of expected clozapine metabolite compounds.

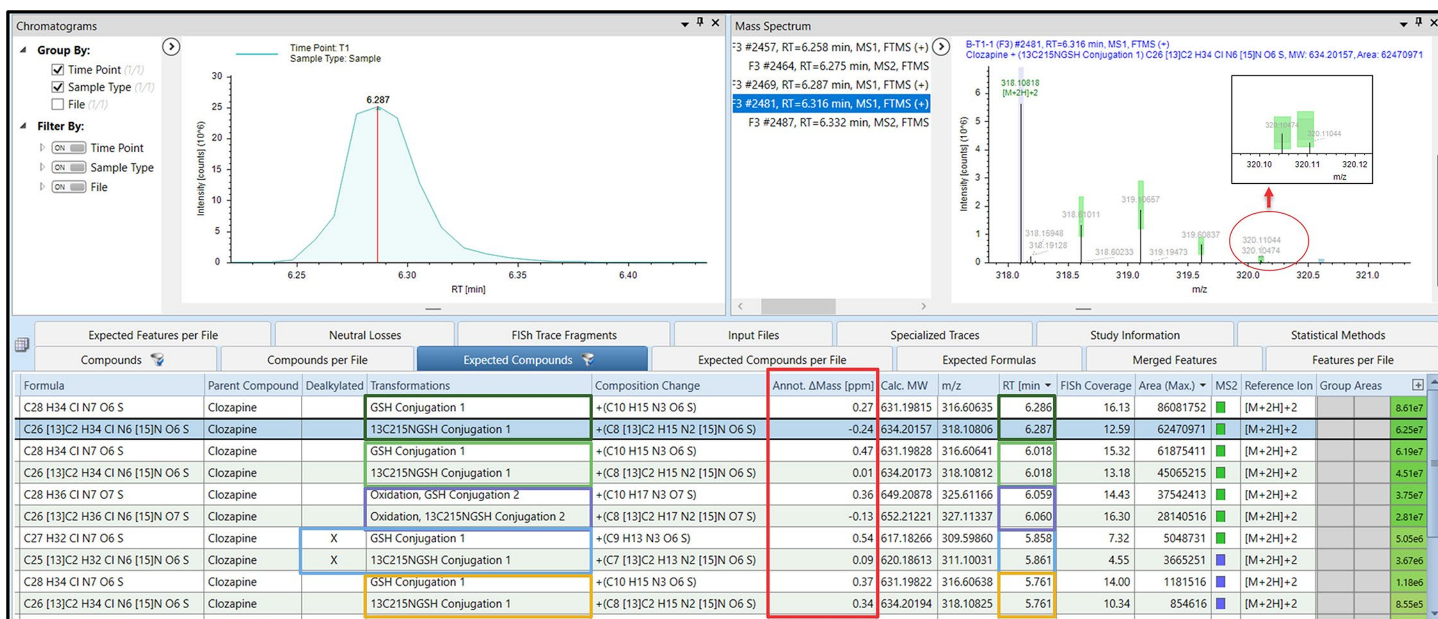
Results and discussion

Targeted compounds table to identify expected GSH conjugates

Once processed by the Generate Expected Compounds node, the annotated results can be reviewed in the results view demonstrated by Figure 4. The Expected Compounds tab shows several examples of both the non-labeled and labeled expected clozapine GSH metabolites detected using the *Find Expected Compounds* node, which predicts possible metabolites of the parent compound based on the intelligent dealkylation/dearylation predictions and the common metabolic pathways lists that were curated to include the customized labeled GSH transformations. The Chromatogram view is populated with the XIC of the selected compound (second row, Formula $C_{26}{}^{13}C_2H_{34}ClN_6{}^{15}NO_6S$, RT 6.28 min, highlighted in blue) in the Expected Compounds table. The Mass Spectrum view displays the MS^1 scans and MS^2 scans of the preferred ion. The MS^1 spectrum shows the isotopic pattern of the selected compound, and the green rectangles highlight mass spectrum peaks that match the theoretical isotopic pattern. Fine isotope pattern visualization confirms the presence of Sulfur, and the sub-ppm mass accuracy obtained with the Orbitrap Exploris 240 mass spectrometer provides increased confidence in the compound identification (column *Annot. Delta Mass [ppm]* in Figure 4). In addition, both non-labeled and stable isotope-labeled GSH trapped clozapine metabolites with the same type of transformations show up at the same retention time confirming the detection of GSH trapped metabolites, as indicated by the paired entries in the colored frames.

Structure annotation via *FISh Scoring* node

Structure annotation of the expected compounds is supported by the *FISh Scoring* node in the expected workflow. MS^2 fragment spectra of detected expected compounds were automatically annotated by the *FISh Scoring* node, which uses Thermo Scientific™ Mass Frontier™ Fragmentation Libraries to predict *in silico* fragments based on the structure of the parent compounds or dealkylation/dearylation products. Direct match fragments are color-coded in green and transformation-shifted fragments in blue. Figure 5 displays the auto annotation and mirror plot of MS^2 fragments of the labeled GSH conjugate (Top, *m/z* 635.209, RT=6.28 min) and non-labeled GSH conjugate (Bottom, *m/z* 632.205, RT=6.28 min) in the MS Spectrum view. The circled pair of fragments have a mass difference of 3.0037 Da, indicating the retention of the labelled part of the glutathione moiety.



Formula	Parent Compound	Dealkylated	Transformations	Composition Change	Annot. ΔMass [ppm]	Calc. MW	m/z	RT [min]	FISh Coverage	Area (Max.)	MS2	Reference Ion	Group Areas
C28 H34 Cl N7 O6 S	Clozapine		GSH Conjugation 1	+(C10 H15 N3 O6 S)	0.27	631.19815	316.60635	6.286	16.13	86081752		[M+2H]+2	8.61e7
C26 [13]C2 H34 Cl N6 [15]N O6 S	Clozapine		13C215NGSH Conjugation 1	+(C8 [13]C2 H15 N2 [15]N O6 S)	-0.24	634.20157	318.10806	6.287	12.59	62470971		[M+2H]+2	6.25e7
C28 H34 Cl N7 O6 S	Clozapine		GSH Conjugation 1	+(C10 H15 N3 O6 S)	0.47	631.19828	316.60641	6.018	15.32	61875411		[M+2H]+2	6.19e7
C26 [13]C2 H34 Cl N6 [15]N O6 S	Clozapine		13C215NGSH Conjugation 1	+(C8 [13]C2 H15 N2 [15]N O6 S)	0.01	634.20173	318.10812	6.018	13.18	45065215		[M+2H]+2	4.51e7
C28 H36 Cl N7 O7 S	Clozapine		Oxidation, GSH Conjugation 2	+(C10 H17 N3 O7 S)	0.36	649.20878	325.61166	6.059	14.43	37542413		[M+2H]+2	3.75e7
C26 [13]C2 H36 Cl N6 [15]N O7 S	Clozapine		Oxidation, 13C215NGSH Conjugation 2	+(C8 [13]C2 H17 N2 [15]N O7 S)	-0.13	652.21221	327.11337	6.060	16.30	28140516		[M+2H]+2	2.81e7
C27 H32 Cl N7 O6 S	Clozapine	X	GSH Conjugation 1	+(C9 H13 N3 O6 S)	0.54	617.18266	309.59860	5.858	7.32	5048731		[M+2H]+2	5.05e6
C25 [13]C2 H32 Cl N6 [15]N O6 S	Clozapine	X	13C215NGSH Conjugation 1	+(C7 [13]C2 H13 N2 [15]N O6 S)	0.09	620.18613	311.10031	5.861	4.55	3665251		[M+2H]+2	3.67e6
C28 H34 Cl N7 O6 S	Clozapine		GSH Conjugation 1	+(C10 H15 N3 O6 S)	0.37	631.19822	316.60638	5.761	14.00	1181516		[M+2H]+2	1.18e6
C26 [13]C2 H34 Cl N6 [15]N O6 S	Clozapine		13C215NGSH Conjugation 1	+(C8 [13]C2 H15 N2 [15]N O6 S)	0.34	634.20194	318.10825	5.761	10.34	854616		[M+2H]+2	8.55e5

Figure 4. Result Review of the Expected Compounds table showing multiple pairs of non-labeled and stable isotope-labeled clozapine metabolites with the same type of transformations at the same retention time (highlighted in pairs with different color), confirming the detection of GSH trapped metabolites. This can be quickly used to help rule out false positive hits missing either the labelled or non-labelled compound.

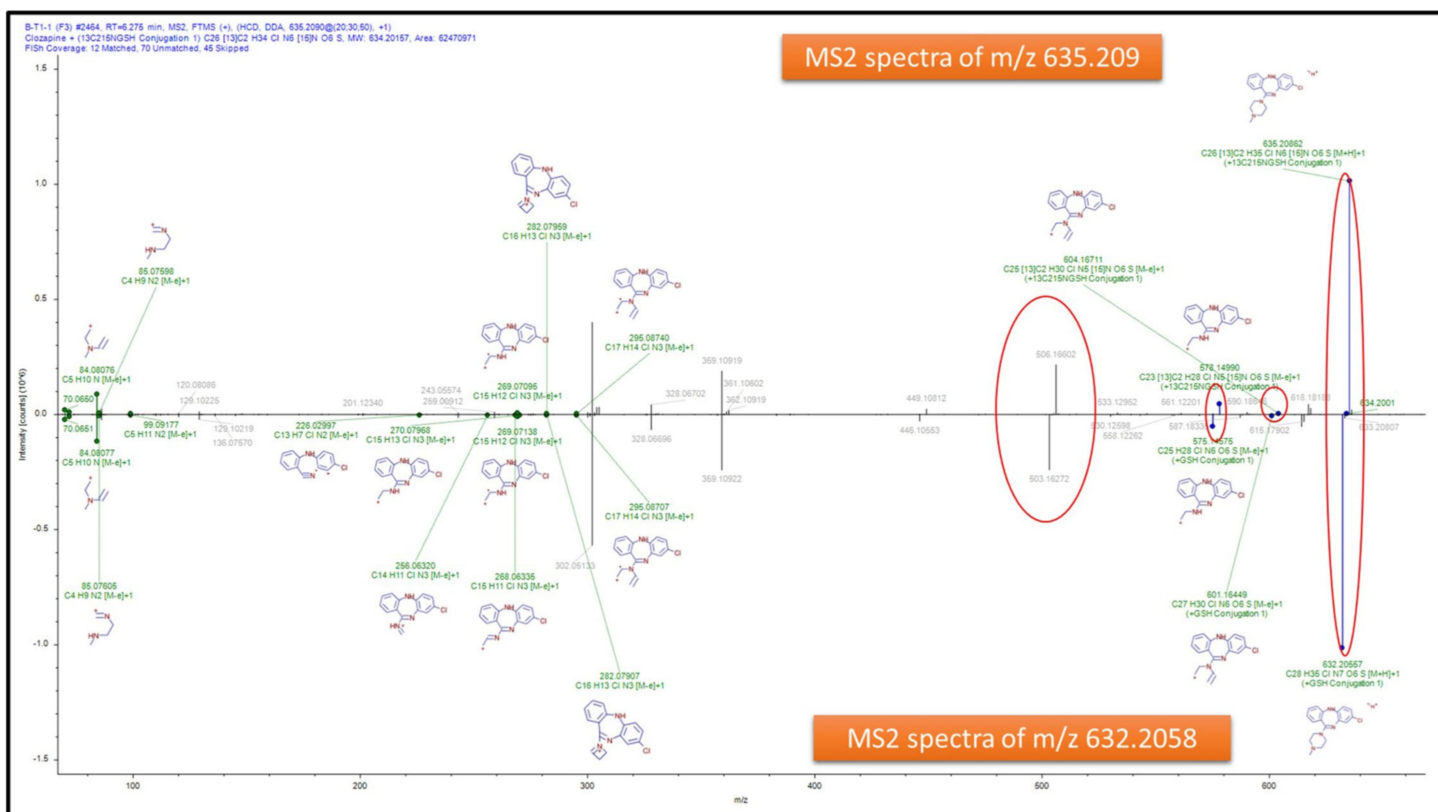


Figure 5. Auto annotation and mirror plot of MS² facilitate structure elucidation. Circled pairs of fragments have a mass difference of 3.0037 Da, which corresponds to the difference between the stable isotope-labeled and non-labeled GSH conjugates.

Unknown Compounds table for labeled GSH conjugates profiling

A customized "unknown workflow" was utilized to annotate the untargeted metabolites detected in the samples, and the results are displayed in the Compounds table (Figure 6).

- The result table contains comprehensive information on extracted compounds and multiple search results from the "unknown workflow."
- The *Compound Class Scoring* node adds the Class Coverage column to the Compounds table which displays the percentage of matched MS^2 fragments with clozapine and its known metabolites in the user curated clozapine compound class library.
- The Mass Defect column shows the calculated mass defect value for each compound.
- The Neutral Losses column annotates compounds with the specified pyroglutamic acid neutral loss in their fragmentation spectra.
- The Pattern Matches column indicates compounds matching the user defined isotope pattern.

The combination of these annotations can be used in an integrated approach enabling the detection of unexpected clozapine GSH conjugates and related compounds, as discussed below.

Results filters for data reduction

A low peak intensity threshold is often applied in the initial workflow to avoid the loss of low abundant metabolites; however, this may result in a large number of potentially false positive entries in the Compounds table, making manual data interpretation challenging. Data reduction could be achieved by using the results filter to extract the compounds related to clozapine GSH conjugates. Figure 7 describes an example of filtering schema leveraging the intrinsic properties of the stable isotope-labeled GSH trapped metabolites capturing compounds with the pyroglutamic acid neutral loss, or specified isotope pattern match, which will be discussed hereafter. This filter also accounts for negative controls, and it narrowed the compounds entries in a complex biological matrix from 3728 to less than 120, enabling further data mining to identify the GSH trapped metabolites that could be missed by the expected workflow.

Figure 6. Results view of the Compounds table. The Compounds table organizes information with columns pertinent to the analysis such as Mass Defect, Class Coverage, Neutral Losses, and Pattern Matches (highlighted in red), and it is interactive with Chromatograms and Mass Spectrum views.

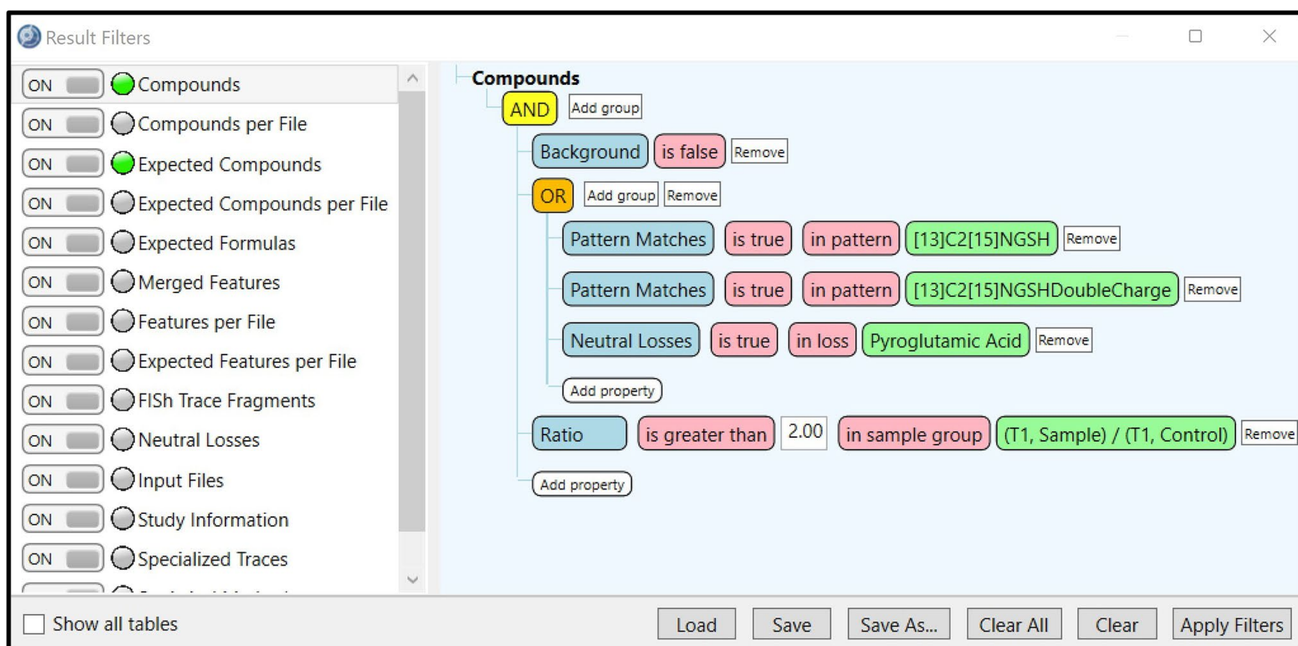


Figure 7. Result Filters. Data reduction is achieved through Results Filters to extract the potential GSH trapped clozapine metabolites.

Pattern Scoring node - setting up the pattern

The *Pattern Scoring* node in the "unknown workflow" takes a user-defined pattern, which can be used to flag compounds bearing unique isotopic patterns. In this study, the trapping agent is a mixture of non-labeled and stable isotope labeled glutathione (GSH) at 1:1 ratio. GSH was labeled to give an overall increase of 3.0037 Da over the naturally occurring substance. GSH trapped reactive metabolites produced a characteristic pattern of "twin ions" separately by 3.0037 Da in mass spectral data. Using the experimental pattern instead of theoretical pattern helps to achieve better pattern match results, which accounts for the purity of the stable isotope-labeled GSH trapping agent.[6] The Isotope Ratio Editor in the *Pattern Scoring* node to set up the customer isotopic pattern is shown in Figure 8A, and experimental pattern is displayed in Figure 8B.

Pattern matched compounds

In the Compounds table, the unknown compounds that match the specified custom pattern are marked. For each compound that matches the pattern, the matched pattern is displayed in the Mass Spectrum window. Thus, the compounds with a characteristic pattern of a "twin peak" with a mass difference of 3.0037 Da can be identified to detect the non-labeled and labeled GSH conjugates, as Figure 9 shows.

Neutral loss screen

The GSH conjugates commonly undergo a neutral loss of pyroglutamic acid in a MS/MS scan in the positive ion mode, which leads to a characteristic loss of *m/z* 129.0425. The Search Neutral Losses node in the "unknown workflow" screens MS^2 spectra and flags compounds with pyroglutamic acid losses in the Compounds table. The Neutral Losses Column in the Compounds table shows the unknown compounds with detected Neutral Losses. For each compound with neutral loss flag, the fragmentation spectrum is annotated with neutral loss (Figure 10). The identification of neutral loss *m/z* 129.0425 fragments in the fragmentation spectra for an unknown compound adds confidence to the identification of the GSH trapped reactive metabolites.

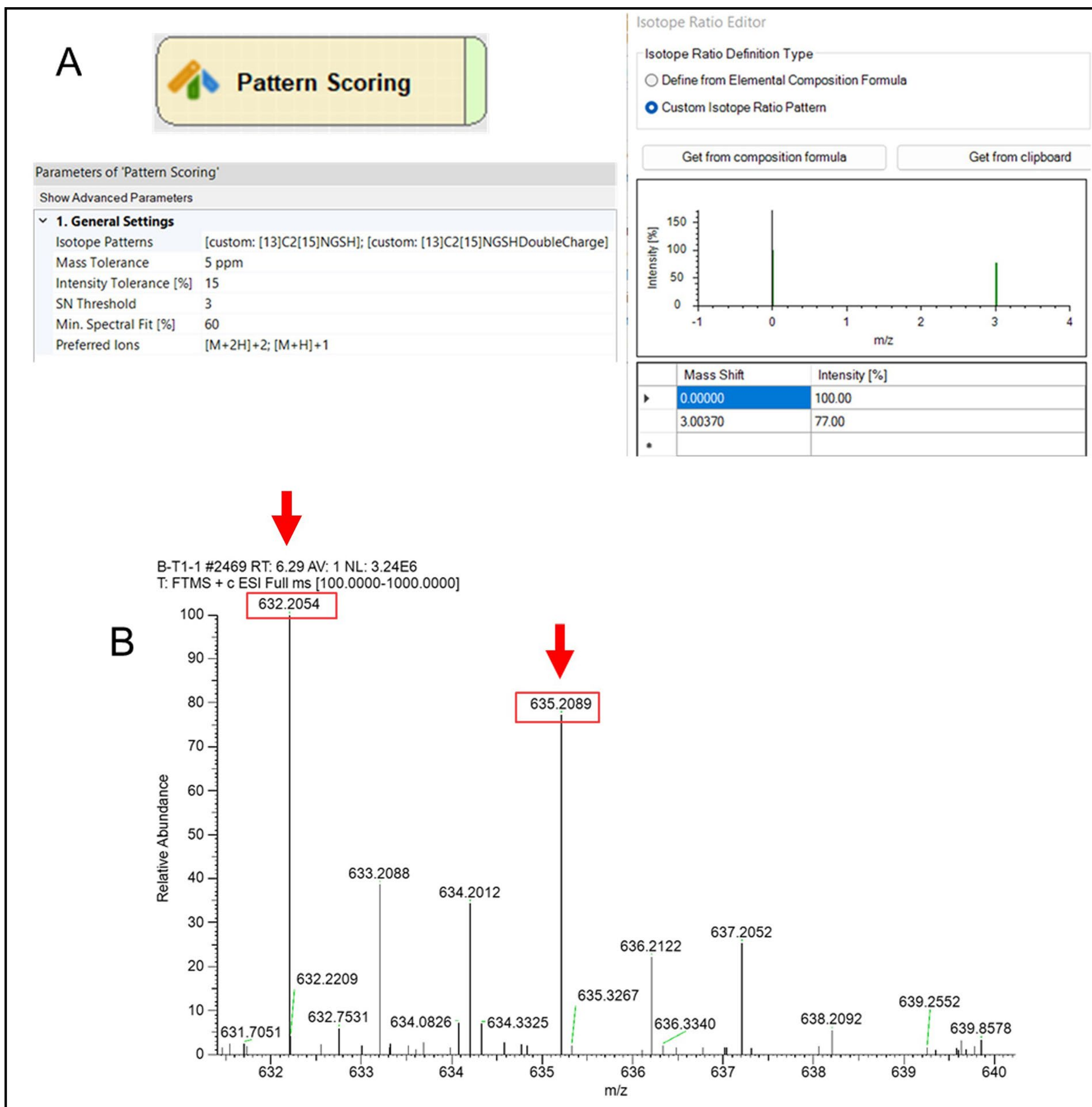


Figure 8. (A) Isotope Ratio Editor in the *Pattern Scoring* node. The isotopic ratio was manually curated to include a mass shift of 3.0037 Da with 77% relative intensity (15% tolerance) to the monoisotopic compound based on the experimental pattern (shown in B). The parameters in the *Pattern Scoring* node such as Mass Tolerance, Intensity Tolerance, SN Threshold, and Min. Spectral Fit threshold facilitate reducing the false positive hit. (B) Raw Full MS spectrum of the glutathione conjugation product of clozapine at 6.29 min, showing the characteristic experimental pattern at a 1:0.77 intensity ratio.

Figure 9. *Pattern Scoring* node flags unknown compounds matching user specified artificial isotopic pattern(s) and it adds the related Matched Patterns table to the result file (highlighted in red).

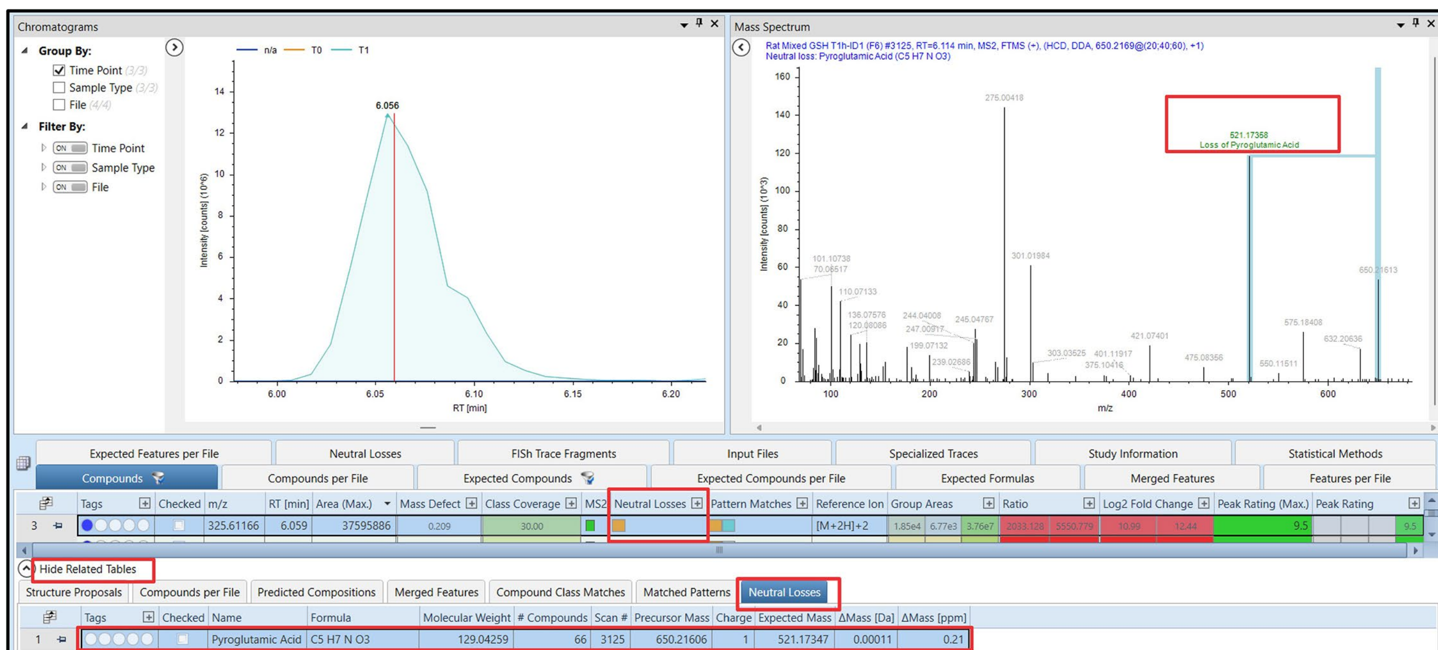


Figure 10. ***Search Neutral Losses* node reports unknown compounds with specified neutral loss fragment.** Related Neutral Losses table provides more detailed information.

In silico fragmentation prediction for structure proposal

Applying FISh Scoring on the user proposed structure of the putative clozapine GSH conjugates automatically allows explanation of the fragmentation spectra with predicted fragment structures. FISh algorithm post processing helps to elucidate the structure of detected metabolites based on experimental MS^2 spectra. In the Expected Compound table, compound with *m/z* 650.2148 at RT 6.06 min was proposed to be GSH trapped reactive metabolite produced through oxidation and GSH conjugation, and the position of oxidation could be further refined via FISh proposals. Figure 11 depicts the *in silico* fragmentation of this GSH-trapped oxidized clozapine and the annotation of the fragments with the predicted structures using general rules and the Thermo Scientific™ HighChem Fragmentation Library™. The FISh is very useful in this example to distinguish the positional isomeric compounds and identify the sites of bioactivation via structural assignment.

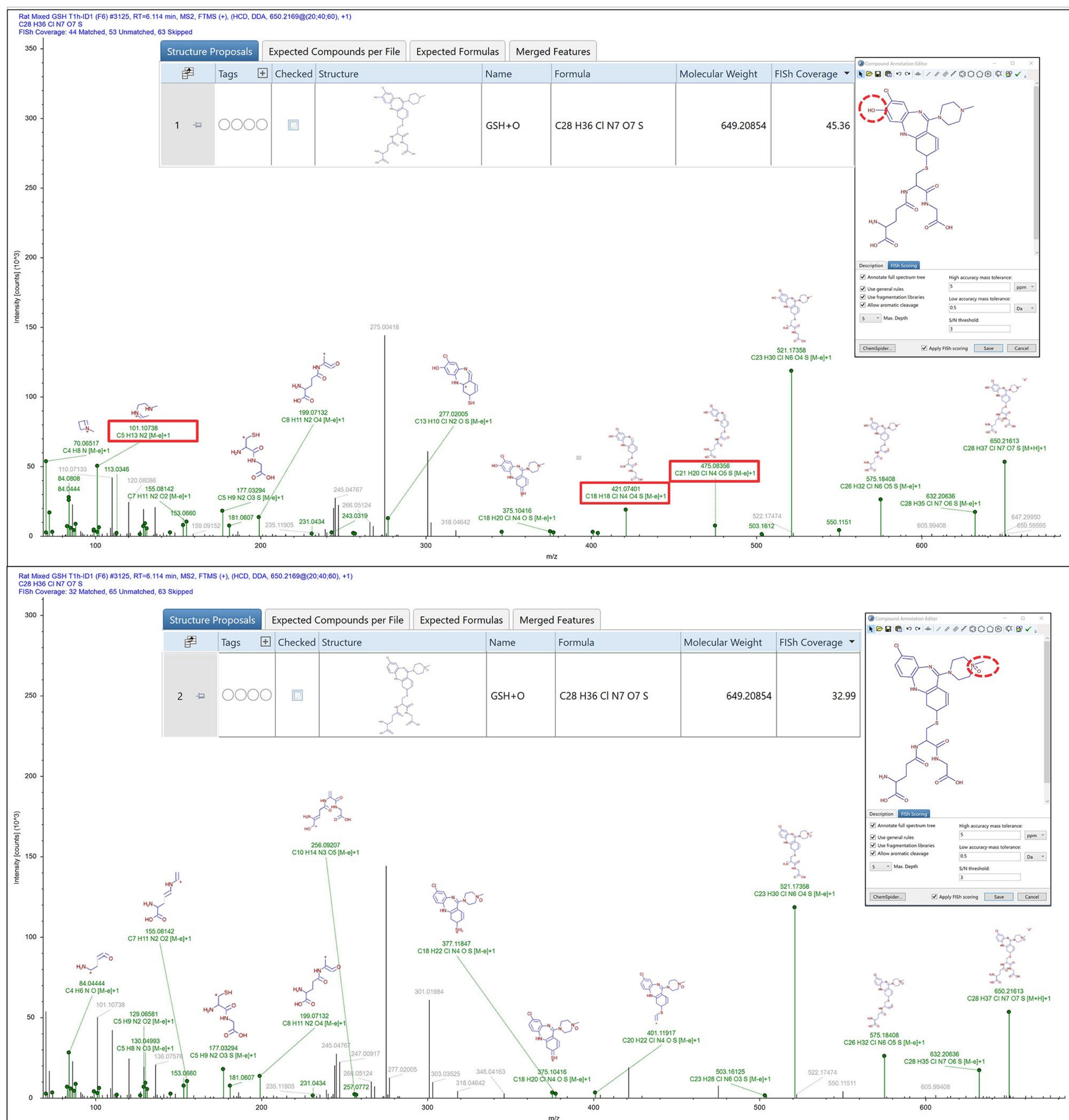


Figure 11. *In silico* fragmentation prediction with user proposed structure. Applying FISh Scoring on the proposed structure (inserted box) of the clozapine GSH conjugate for fragment ion matching and structure annotation. Due to the difference in the oxidation position, the MS^2 spectral interpretation confirmed the fragment ions *m/z* 475.08356, *m/z* 421.07401, and *m/z* 101.10738 are matching with the structure proposal that hydroxylation on the chloro benzene ring and not with N-oxide metabolite.

Summary

Eight expected non-labeled and isotope-labeled GSH trapped reactive metabolites of clozapine were readily identified by the expected workflow. One unexpected clozapine GSH conjugate (*m/z* 534.1209, RT 8.18 min) missed by the expected workflow was putatively identified through the "unknown workflow." Its fragmentation spectra could be explained by the user proposed structure via FISh scoring, as illustrated in Figure 12. A summary of the identified GSH-trapped reactive metabolites of clozapine in the study is listed in Table 4.

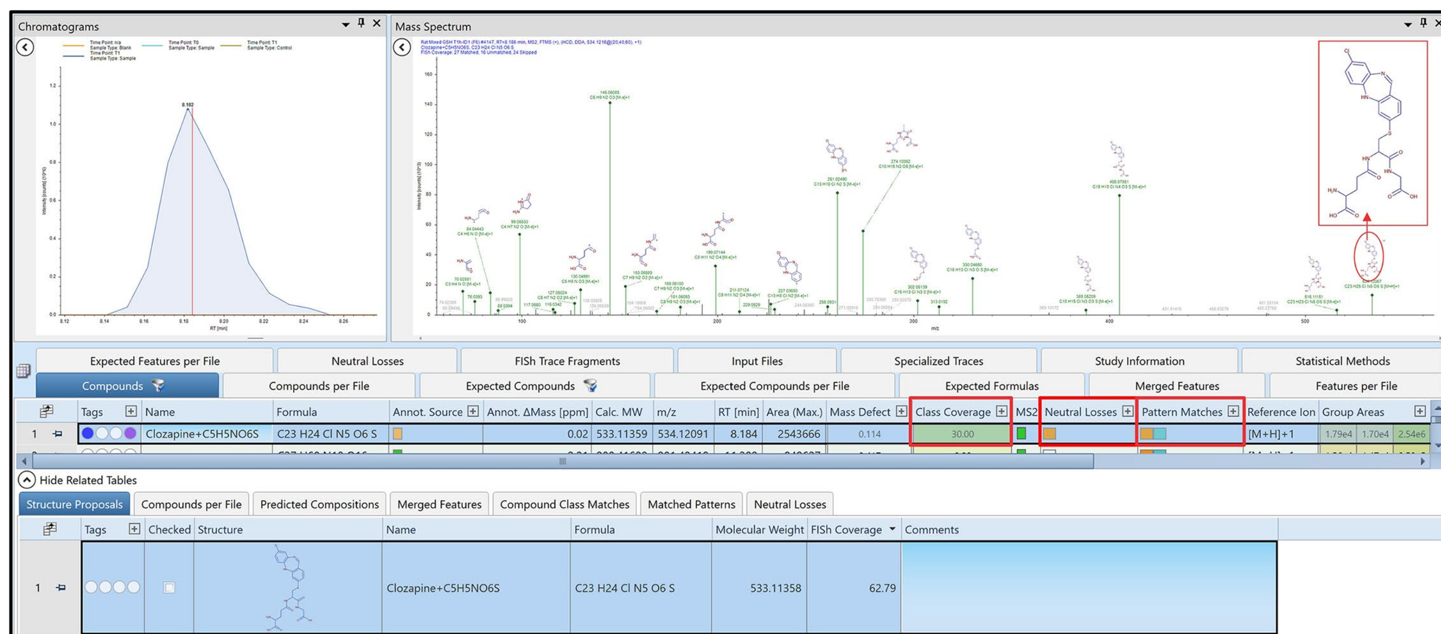


Figure 12. Untargeted GSH clozapine conjugate was putatively identified by the "unknown workflow". The flags in the Pattern Matches, Neutral losses and Class Coverage columns indicate that is a potential GSH clozapine conjugates. The proposed structure (highlighted in red) was given to explain the fragmentation spectra via FISh scoring.

Table 4. Summary of identified GSH-trapped reactive metabolites of clozapine by Compound Discoverer software

RT [min]	Formula	Calc. MW	Proposed GSH Conjugates	Composition Change	Area	Expected Workflow	Unknown Workflow
5.76	C28 H34 Cl N7 O6 S	631.1982	GSH-2H	+(C10 H15 N3 O6 S)	1181516	X	X
5.86	C27 H32 Cl N7 O6 S	617.1827	GSH-CH2-2H	+(C9 H13 N3 O6 S)	5048731	X	X
6.02	C28 H34 Cl N7 O6 S	631.1983	GSH-2H	+(C10 H15 N3 O6 S)	61875411	X	X
6.06	C28 H36 Cl N7 O7 S	649.2088	GSH+O	+(C10 H17 N3 O7 S)	37542413	X	X
6.13	C28 H36 Cl N7 O8 S	665.2039	GSH+O+O	+(C10 H17 N3 O8 S)	868431	X	X
6.29	C28 H34 Cl N7 O6 S	631.1982	GSH-2H	+(C10 H15 N3 O6 S)	86081752	X	X
7.06	C28 H34 Cl N7 O6 S	631.1979	GSH-2H	+(C10 H15 N3 O6 S)	451074	X	X
7.25	C23 H24 Cl N5 O7 S	549.1088	GSH-C5H10N2+O-2H	+(C5 H5 N O7 S)	399717	X	X
8.18	C23 H24 Cl N5 O6 S	533.1136	GSH-C5H10N2-2H	+(C5 H5 N O6 S)	2543666		X

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Conclusion

In this study, we have demonstrated a comprehensive workflow for streamlined identification and structural characterization of drug metabolites from a labeled GSH trapping assay, utilizing data from an Orbitrap Exploris 240 mass spectrometer and Compound Discoverer software. This approach can be routinely used to detect and characterize GSH trapped reactive metabolites at early stages of drug discovery, and the structural information obtained by this method allows for optimization of lead candidates.

- The full scan and MS/MS data generated by the Orbitrap Exploris 240 mass spectrometer with high resolution and sub ppm mass accuracy enables high confident compound identification and structural elucidation of drug metabolites.
- Compound Discoverer software provides an integrated approach for labeled metabolite profiling and structure elucidation with greater confidence and efficiency using customizable node-based workflows and a combined targeted and untargeted compound detection approach.
- The *Pattern Scoring* node and the *Search Neutral Losses* node in the "unknown workflow" enable high throughput GSH conjugate screening with enhanced selectivity and sensitivity.
- The described workflow can also be applied to other small molecule identification and structure elucidation from experiments utilizing similar labeling techniques.

References

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