

Identification of GSH Conjugates Using Accurate Mass Data and MetWorks Software

Amber Kohl, Thermo Fisher Scientific, West Palm Beach, FL, USA

Heng-Keang Lim, Johnson and Johnson Pharmaceutical Research Institute, Raritan, NJ, USA

Yingying Huang, Thermo Fisher Scientific, San Jose, CA, USA

Key Words

- LTQ Orbitrap Hybrid MS
- MetWorks Metabolite Identification Software
- Surveyor Plus™ HPLC
- Accurate Mass
- GSH Conjugates
- Isotope Search
- Mass Defect Filter

Introduction

Incorporating metabolite profiling for ADME properties early in discovery across the pharmaceutical industry has resulted in an increase in drug success in development by decreasing failure due to poor pharmacokinetic properties.¹ However, since it is difficult to predict how toxicity screens compare to human toxicity *in vivo*, there has been a shift to drug attrition during development due to preclinical or clinical safety issues. This is due to the fact that there are no animal models that adequately predict drug toxicity involving the cardiovascular and hepatic systems in humans.^{1,2} In order to avoid drug toxicity in development, the screening for formation of electrophilic reactive intermediates has been widely embraced by the pharmaceutical industry as part of drug design.³

Neutral loss scanning of 129 Da using a triple-stage quadrupole mass spectrometer has been the key analytical method of choice in the detection and characterization of glutathione conjugates.⁴ The neutral mass of 129 Da results from the loss of pyrroglutamic acid from glutathione conjugates. This method of detection can lack selectivity due to false positives from compounds with the same neutral mass loss of 129 Da which are unrelated to pyrroglutamic acid. The selectivity of the neutral loss method has been improved first by trapping of the compound with an equimolar ratio of glutathione: [¹³C₂,¹⁴N-Gly] glutathione and removal of false positives without the correct isotope pattern⁵ and also by exact neutral mass loss detection of pyrroglutamic acid.⁶ In this report, a cofactor-fortified human liver microsomal incubation of 10 μM of nefazodone was analyzed using the LTQ Orbitrap™ which also incorporated use of isotopic pattern triggered data-dependent high-resolution accurate mass analysis. A selective method was developed to identify glutathione conjugates using mass defect filtering followed by isotopic pattern recognition techniques in MetWorks™. Structural elucidation of detected glutathione conjugates was aided by generating chemical formulas from accurate masses of the precursors and their product ions.

Materials and Methods

Reagents

β-Nicotinamide adenine dinucleotide phosphate (monosodium salt), D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase, ethylenediaminetetraacetic acid (disodium salt, 0.5 M) [EDTA], magnesium chloride (1.0 M) [MgCl₂], glutathione and nefazodone were purchased from Sigma-Aldrich (St. Louis, MO).

Human Liver Microsomal Incubation

All human liver microsomal incubations (1 mL) consisted of 2 mM EDTA, 5 mM MgCl₂, 1 mg/mL microsomes, 10 μM nefazodone, 5 mM glutathione and 1 mM NADPH. All reagents were prepared in 0.1 M phosphate buffer (pH 7.4), except for the drug, which was spiked as 10 μL of 1 mM acetonitrile:H₂O (1:1) stock solution to keep the final acetonitrile content to less than 0.1%. After incubation for one hour at 37 °C in an open tube, the incubation was terminated by the addition of 3 mL of ice-cold acetonitrile, and the tube was vortexed prior to centrifugation at 1,203 g for 10 minutes at 5 °C. The supernatant was then transferred to a clean tube and dried at room temperature under a gentle stream of nitrogen. The residue was reconstituted in 200 μL of water:acetonitrile (9:1), hand-vortexed and then passed through a 0.45 μm nylon filter using centrifugation at 7,200 g for 10 minutes at room temperature. The filtrate was analyzed by LC/MS and MS/MS using the methods described below:

HPLC

LC Instrument: Surveyor Plus HPLC System
(Thermo Scientific)

Column: HyPURITY Aquastar™ column
(100 x 2.1 mm ID, 3 μm) (Thermo Scientific)

Mobile Phase: A: Water with 0.2% Formic acid

B: Acetonitrile with 0.2% Formic acid

Flow Rate: 400 μL/min.

Injection Volume: 20 μL

Gradient: t (min.)	A%	B%
0	98	2
0.5	98	2
2	96	4
4	93	7
6	91	9
8	86	14
10	81	19
12	76	24
15	62	38
17	42	58
19	5	95
22	5	95
23	98	2
28	98	2

Mass Spectrometer

The LTQ Orbitrap mass spectrometer was operated in positive electrospray ionization mode with an electrospray voltage of 5 kV. The sample was sprayed into the mass spectrometer with the sheath, auxiliary and sweep gas set at 70, 20, and 5 arbitrary units, respectively, and desolvation was further aided by an ion transfer tube temperature of 300 °C. An isolation width of 2 amu was used with either 30% or 35% normalized collision energy (CID) for MSⁿ experiments. Isotopic data dependence was enabled in order to get MSⁿ data of compounds containing a chlorine isotope pattern by using an accurate mass difference of 1.99705 Da and an expected isotopic abundance ratio of 0.32. All scan events were detected in the FT detector to give accurate mass data for precursor ions and for MSⁿ ions using internal mass calibration mode.

Results and Discussion

The LTQ Orbitrap workflow used to trigger MSⁿ scans for compounds containing a chlorine isotope pattern is illustrated in Figure 1. In each full scan MS, the most intense peak

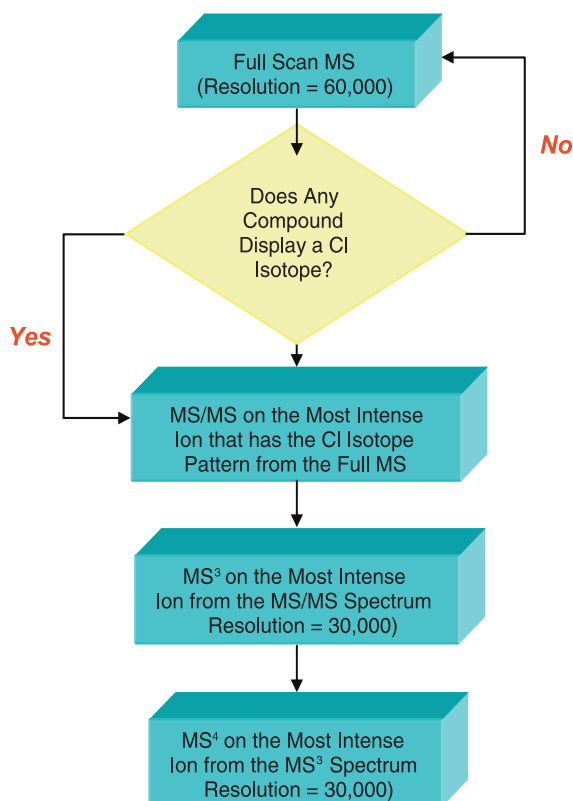


Figure 1: Schematic of LTQ Orbitrap method setup using Isotopic Data Dependence to trigger MSⁿ on compounds containing a chlorine isotope pattern

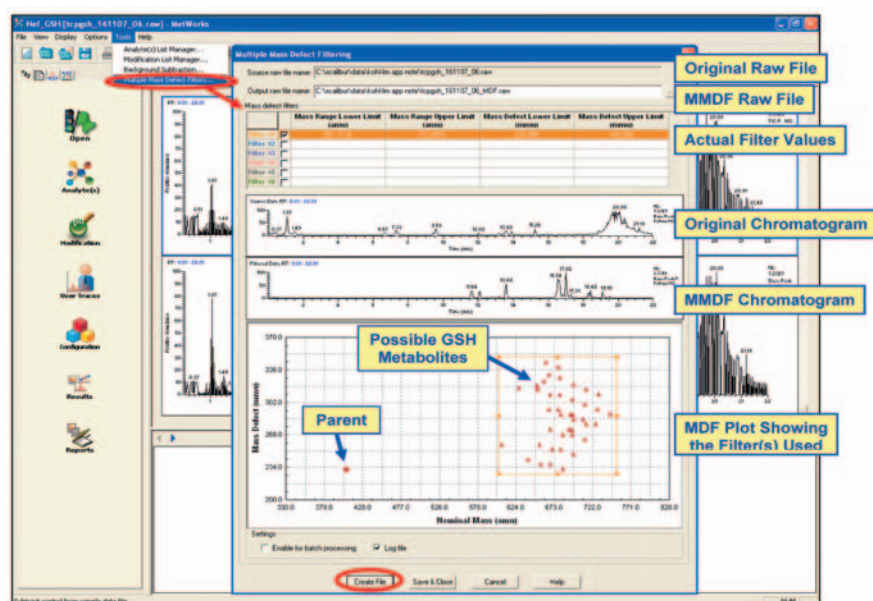


Figure 2: Multiple Mass Defect Filter dialogue box in MetWorks 1.2. This dialogue box can be accessed by clicking on "Tools" → "Multiple Mass Defect Filters...".

that displayed the specified chlorine isotope pattern was identified, and its MSⁿ data was acquired. If there were no compounds above a set threshold that contained the specified chlorine isotope pattern, no MSⁿ data were acquired for any ions in that scan. This workflow increased the likelihood that MSⁿ data acquisition be triggered from metabolites of interest containing the specific chlorine isotope pattern.

The high resolution accurate mass data from the analysis of human liver microsomal incubation of nefazodone was processed using MetWorks software which automates the Data DependentTM LC/MSⁿ analyses of drugs and their metabolites. For full scan accurate MS data, the Multiple Mass Defect Filters (M MDFTM) option in MetWorks software (see Figure 2) can be utilized to filter out many background ions that are unrelated to the parent drug and improve the capabilities of finding unexpected modifications of an analyte.

Figure 2 shows the Multiple Mass Defect Filter dialogue box in MetWorks 1.2. To take full advantage of the M MDF function, the formula of nefazodone was specified in the Analyte(s) Manager, and a list of potential GSH conjugates was built in the Modifications Manager. Once this information is supplied, a mass defect filtered (MDF) plot displaying the parent compound and the possible GSH metabolites is shown at the bottom of the M MDF dialogue box. Users can move the cursor over each metabolite, and the corresponding modification information will be shown. A box around the metabolites of interest can then be drawn by clicking and dragging the cursor. This box represents the mass defect filter and actual values are automatically translated in the M MDF table at the top of the dialogue box.

Mass defect filters can also be specified by typing values directly in the table. Up to six different mass defect filters are allowed. Once the box containing all the GSH conjugates is drawn, a filtered base peak chromatogram appears on top of the MDF plot. An enlarged plot of base peak chromatograms before and after M MDF processing

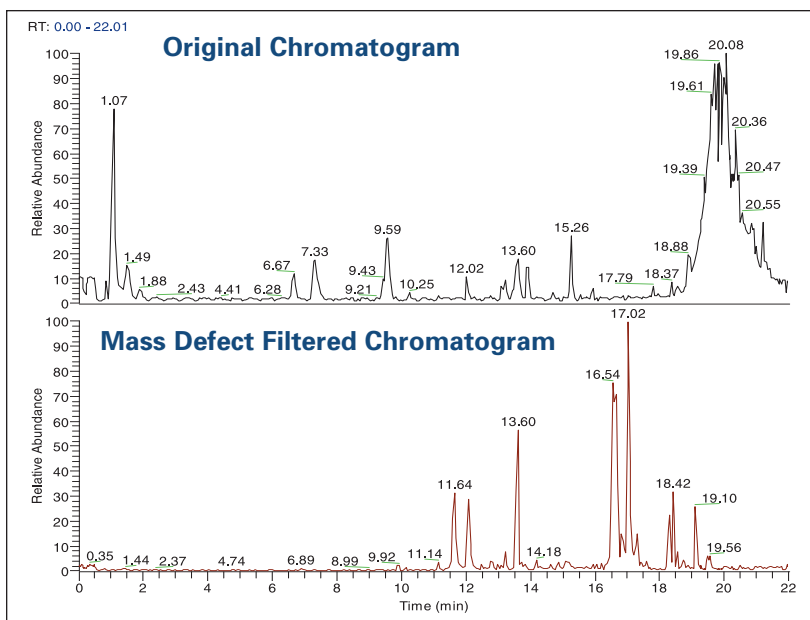


Figure 3: Base peak chromatograms of the full scan data comparing the unfiltered chromatogram to the mass defect filtered chromatogram

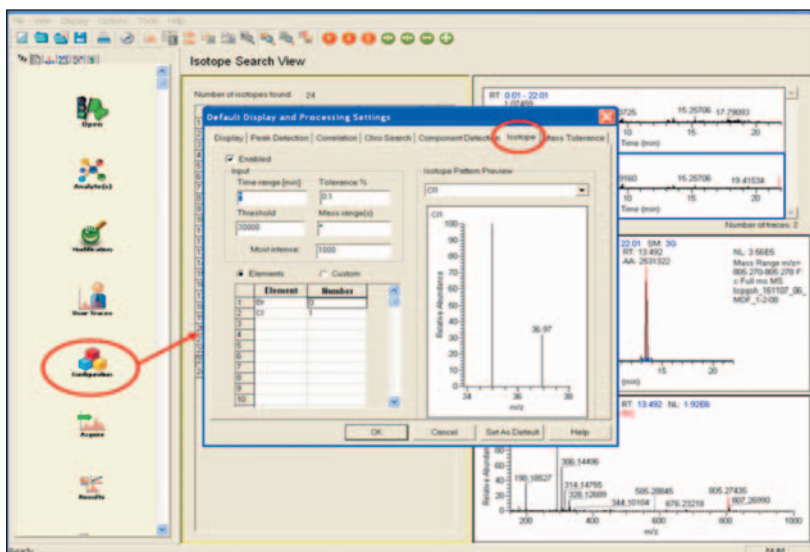


Figure 4: Parameters for configuration of the isotope search

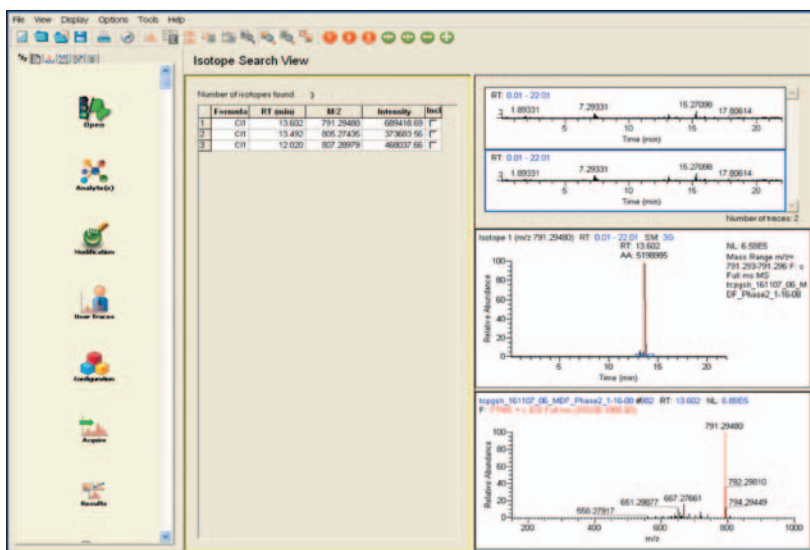


Figure 5: Isotope search results showing glutathione conjugates containing a chlorine isotope

is shown in Figure 3. Users can make adjustments to the boxes drawn, and the filtered chromatogram will be refreshed accordingly. Once the MMDF parameters are set, a new raw file is created by clicking the “Create File” button on the bottom of the dialogue box and all ions that fall outside the filter box (i.e. ions whose mass defects significantly differ from the expected GSH conjugates) are removed from the full scan MS data. The MMDF parameters can be saved in the MetWorks method file (*.mbx file) for future access or batch processing.

After applying the mass defect filter to the data, many of the ions unrelated to nefazodone were filtered out from the full scan MS chromatogram (Figure 3). The glutathione conjugates of interest at retention times of 12.02, 13.49, and 13.60 were much more apparent in the mass defect-filtered chromatogram. In addition, most of the components eluting at a high concentration of organic mobile phase from approximately 18.5 to 22 minutes were filtered out of the chromatogram. Clearly there were still several peaks due to unrelated compounds that were not filtered out using the mass defect filter, so the use of the isotope search was explored to further refine the data.

The next step in the data analysis was to identify which of the nefazodone metabolites in the filtered chromatogram contained a chlorine isotope pattern. In order to do this, the isotope search in MetWorks was utilized. An isotope search allows searching for compounds with specific isotope ratios. The search can include multiple labels for a single atom (for example, multiple Cl atoms), multiple labels for different atoms (for example, multiple Cl and Br atoms), or any customized isotope pattern. Using MetWorks, the isotope search can take advantage of full scan accurate MS data. An important point when configuring the isotope search parameters is to accurately determine the noise threshold below which no compounds will be identified. To do this, an average spectrum was obtained before one minute in the

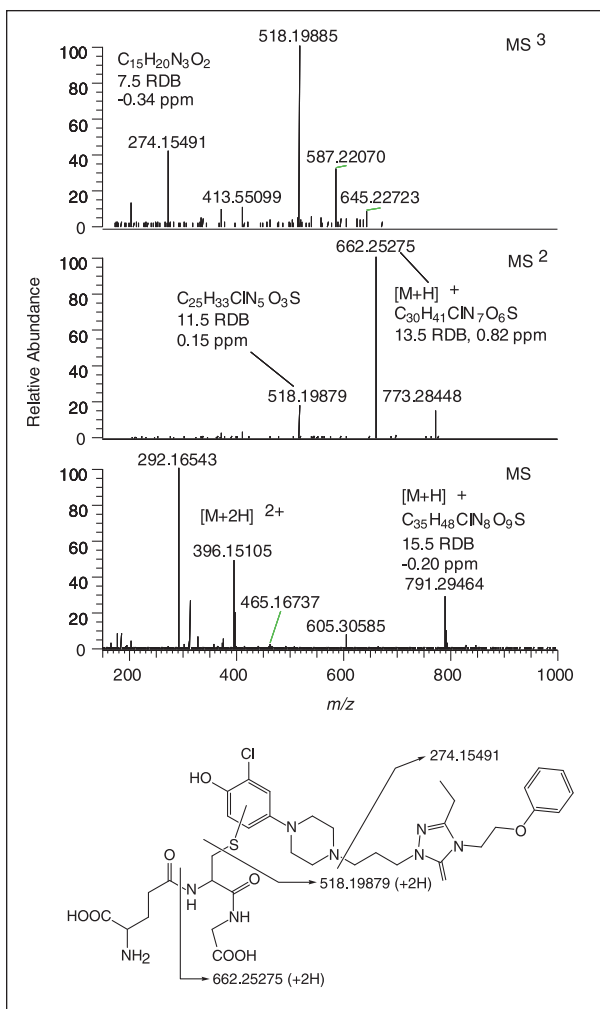


Figure 6: Full scan and product ion mass spectra of the glutathione conjugate at 13.60 minutes

chromatogram where no compounds were eluting, and the noise level was determined by examining the spectrum. A threshold of $3e^4$ was observed and specified for the isotope pattern search (Figure 4). Additionally, the last tab in the configuration parameters is where the mass tolerance is defined. To analyze this data, a mass tolerance of 2 ppm was used.

Following the isotope search, three metabolites from the mass defect filtered chromatogram were found at m/z 791, 805, and 807 (Figure 5). The mass defect filter followed by the isotope search provided a simple method to identify these GSH metabolites in the data. Identification of these conjugates was confirmed from the product ion mass

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spectra generated from data-dependent scans of m/z 791 as shown in Figure 6. The presence of the product ion in the MS² data at m/z 662.2528 from loss of pyrroglutamic acid from CID of the protonated molecule (m/z 791.29480) confirmed that this was a GSH conjugate. The ion in the MS³ spectrum at m/z 274.1549 suggests GSH addition to the 2-chloro-4-piperazin-1-yl-phenol moiety. This GSH conjugate is consistent with that previously reported.⁷

Accurate mass measurements of the glutathione conjugates at 12.02 and 13.49 minutes gave chemical formulae of $C_{35}H_{48}ClN_8O_{10}S$ and $C_{35}H_{46}ClN_8O_{10}S$, which are consistent with glutathione composition of nefazodone + 2O + GSH - 2H and nefazodone + 2O + GSH - 4H, respectively. These glutathione conjugates are reported here for the first time and were readily detected after using the isotope pattern search.

Conclusions

An approach for identifying GSH conjugates from incubations of compounds has been developed using the LTQ Orbitrap and MetWorks. The Isotopic Data Dependence in the method setup allows for selective triggering of MSⁿ data for metabolites of interest. Compared to other methods for post-processing of data, multiple mass defect filters allow the user to identify known as well as unknown or unexpected metabolites of interest. Followed by the accurate mass isotope search, this combination is well-suited for taking advantage of accurate mass data for selectively identifying GSH conjugates that have distinct isotope signature patterns. The availability of both accurate mass full scan and product ion mass spectra in the data allows structure elucidation of the conjugates for a single LC/MSⁿ experiment. Additional examples employing this workflow and more in-depth discussions can be found in reference 8.

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