

## Direct Detection of Glutathione Conjugates in Human Liver Microsomes Using ACQUITY UPLC I-Class, Xevo G2-S QTof, and UNIFI

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### APPLICATION BENEFITS

- MS<sup>E</sup> enabled positive electrospray ionization allows simultaneous detection of parent, phase I metabolites, and phase II metabolites.
- Automatic charge state deconvolution and detection allows GSH adducts/charge states to be automatically grouped together, facilitating easy interpretation.
- Trend plots in UNIFI software allow visual confirmation of detected metabolites across time and/or experimental conditions.

### WATERS SOLUTIONS

Metabolite Identification Application  
Solution with UNIFI

ACQUITY UPLC® I-Class System

ACQUITY UPLC BEH Column

Xevo® G2-S QTof

UNIFI® Scientific Information System

### KEY WORDS

Clozapine, metabolite, reactive metabolite, GSH trapping, glutathione, microsome, UPLC, HRMS, MS<sup>E</sup>, structural elucidation

### INTRODUCTION

Screening for reactive metabolites is commonly carried out in pharmaceutical drug discovery/development as part of the risk assessment of a compound, or compound class. The reactive metabolite hypothesis implicates bioactivation of drug compounds and subsequent haptization or inactivation of proteins with clinical toxicity. To that end, significant effort has been made towards reducing the propensity of candidate drugs to undergo metabolic activation.

One way that reactive metabolites can be assessed is through the use of *in vitro* incubations fortified with trapping reagents such as glutathione (GSH). The formation of reactive intermediates can be followed by detection and characterization of “trapped” glutathione adducts using a variety of LC/MS approaches. Popular methods for detecting GSH conjugates include monitoring the ESI<sup>+</sup> signature GSH neutral loss of 129,<sup>1</sup> ESI<sup>+</sup> precursor ion scanning for the 272 diagnostic product ion,<sup>2</sup> or using 1:1 stable isotope labeled GSH and detection based on a signature isotope difference with similar peak intensity under ESI<sup>-</sup> conditions.<sup>3</sup>

In addition to understanding the rate and pathways of formation for glutathione-containing metabolites, information on parent compound is often desired to estimate conversion rate of the compound in the study. Another complication of glutathione adducts is that they often exist as multiple-charged species with detection based on positive electrospray ionization, the widely used ionization technique for the majority of parent compounds. A failure to detect metabolites at low levels, and low and/or variable response for precursor ions and their product ions, can result in false negatives.

High-resolution mass spectrometry (HRMS) approaches are becoming more popular as gathering detailed precursor and product ion information is possible. Software tools can then comprehensively interrogate full scan data for expected and difficult-to-predict reactive intermediates.

Since Waters introduced MS<sup>E</sup> based data acquisition, which provides simultaneous acquisition of full scan precursor and product ion data, it has been possible to perform a generic data acquisition and produce fragment ion information in a non-selective manner, thus simplifying data acquisition.<sup>4</sup>

## EXPERIMENTAL

## LC conditions

LC system:	ACQUITY UPLC I-Class
Column:	ACQUITY UPLC BEH C <sub>18</sub> 1.7 μm, 2.1 x 100 mm
Column Temp.	60 °C
Sample Temp.	10 °C
Injection Volume:	10 μL
Flow Rate:	0.6 mL/min
Mobile Phase A:	H <sub>2</sub> O with 0.1% formic acid
Mobile Phase B:	Acetonitrile with 0.1 % formic acid
Gradient:	0-60% B in 6 min, 60-90% B in 2 min, held at 90% B for 1 min before returning to the initial condition. The total run time was 10 min.

## MS conditions

MS system:	Xevo G2-S QTof
Ionization mode:	ESI <sup>+</sup> , resolution
Experiment:	MS <sup>E</sup>
MS <sup>E</sup> settings:	Low CE 2.0 eV, high CE Ramp 10-30 eV
Acquisition range:	50-1200 <i>m/z</i>
Capillary voltage:	1 V
Cone voltage:	30 kV
Source temp.:	120 °C
Desolvation gas temp.:	550 °C
Cone gas flow:	20L/h
Desolvation gas flow:	1000L/h
Scan time:	0.1 s

## Data acquisition, processing, and reporting

UNIFI Scientific Information System

This application note demonstrates the use of MS<sup>E</sup> data acquisition for detecting reactive metabolites using clozapine as a model compound. Through integrated charge state deconvolution and detection using the Metabolite Identification Application Solution in UNIFI, all metabolites, including parent, phase I metabolites, and phase II GSH conjugates are detected and tracked across the dataset automatically.

## Sample description

Clozapine with final concentration of 10 μM was incubated in human liver microsomes (1 mg/mL final protein concentration, 150 donor pool, BD Gentest) in the absence or presence of GSH (5 mM) and/or cytosol (1 mg/mL). After starting the reaction by NADPH addition, the reaction was followed at 37 °C for 2 hours with 100-μL aliquots withdrawn at 15-min intervals. For each withdrawn sample, 2 volumes of cold acetonitrile were added to quench the reaction. The quenched solution was then centrifuged for 20 min at 15,000 RPM and 10 °C to precipitate proteins. Finally, the supernatant was transferred to a 2-mL analytical vial and diluted with 1 volume of H<sub>2</sub>O.

Experiments were performed on an ACQUITY UPLC I-Class System and a Xevo G2-S QTof high resolution mass spectrometer, using the UNIFI Scientific Information System.

## RESULTS AND DISCUSSION

Clozapine, an antipsychotic agent, carries a black box warning for agranulocytosis, among other side effects. While clozapine alone does not exert any direct cytotoxicity in *in vitro* tests, it has been postulated that the nitrenium reactive intermediate formed either from clozapine or its phase I metabolites may play a possible role (Figure 1).<sup>5</sup> Clozapine metabolites from both phase I and phase II biotransformation pathways have been extensively characterized by LC/MS and NMR<sup>6</sup> through the use of trapping studies.

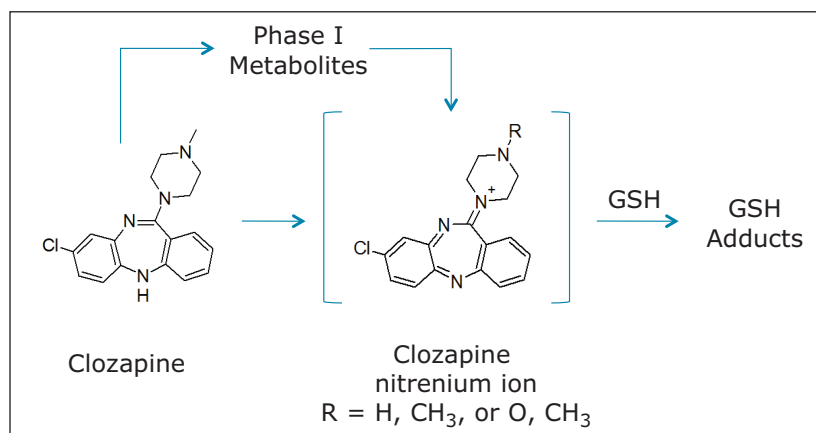


Figure 1. The proposed biotransformation pathways for clozapine phase I and phase II metabolism.

In the study, clozapine was incubated in human liver microsomes. Three conditions were tested: -GSH; +GSH; and +GSH +cytosol. Time points for each condition were collected at 15 min intervals over two hours. These samples were analyzed by LC/MS using MS<sup>E</sup> acquisition with positive electrospray ionization on the Xevo G2-S QToF MS platform under generic conditions. Data was processed using a generic set of processing conditions which searches for common phase 1 biotransformation pathways as summarized in Table 1.

Identification of GSH adducts was enabled by checking the “Specify a trapping reagent” box in UNIFI Software and choosing GSH. For charge state deconvolution, 2 was chosen for the “Maximum allowed absolute charge for adduct combination” in the “Adduct” panel in data processing (Figure 2).

In UNIFI’s data review mode, a summarized XIC (extracted ion chromatogram) of all identified components is displayed for each sample/injection. Figure 2 shows data for the 90-min incubation containing GSH. Four major components are visible: clozapine, a +O metabolite, the -CH<sub>2</sub> metabolite, and one +GSH-2H adduct at RT 2.1 min (Figure 3, top). Each component can be visualized either by choosing a specific XIC either by clicking on the peak in the summarized XIC, or by selecting a row in the component table where all identified metabolites are listed. For example, by choosing the +GSH-2H adduct peak at RT 2.1 min, a chromatogram is displayed showing, in addition to the major peak, two additional minor GSH adducts having the same mass (Figure 3, middle). Similarly, by choosing +O+GSH-2H metabolite in the component table, an extracted chromatogram is displayed showing one major and one minor metabolites with the same mass (Figure 3, bottom).

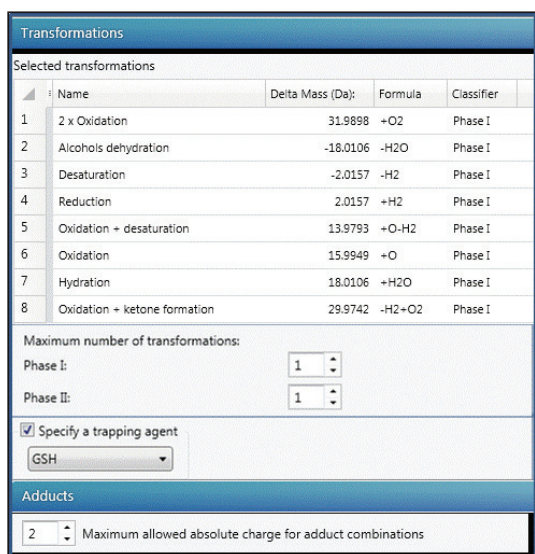


Figure 2. Summary of biotransformation acquisition parameters.

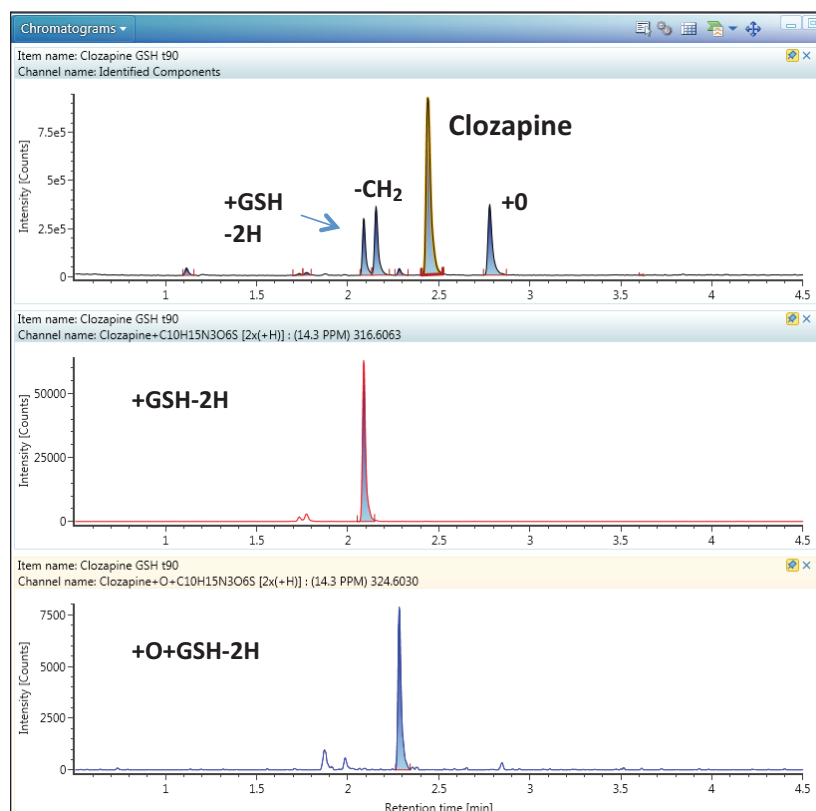


Figure 3. Extracted ion chromatograms for the 90-min sample in the presence of GSH. Top, all identified; middle, +GSH-2H metabolites; bottom, +O+GSH-2H metabolites.



Component Name	Phase	label	Formula	m/z	Observed RT (min)	Mass error (mDa)	Mass error (ppm)	Response	Percentage of Parent Response (%)	Adducts
Clozapine		P	C <sub>18</sub> H <sub>19</sub> ClN <sub>4</sub>	327.1372	2.44	0.1	0.2	148572		+H, 2x(+H)
Clozapine+O	I	M1	C <sub>18</sub> H <sub>9</sub> ClN <sub>4</sub> O	343.1312	2.78	-0.8	-2.25	28509	19	+H, 2x(+H)
Clozapine+O	I	M2	C <sub>18</sub> H <sub>9</sub> ClN <sub>4</sub> O	343.1307	1.12	-1.3	-3.91	3158	2.1	+H
Clozapine+O <sub>2</sub>	I	M3	C <sub>18</sub> H <sub>9</sub> ClN <sub>4</sub> O <sub>2</sub>	359.1266	1.44	-0.3	-0.93	181	0.12	+H
Clozapine-C <sub>2</sub> H <sub>2</sub>	I	M4	C <sub>16</sub> H <sub>17</sub> ClN <sub>4</sub>	301.1204	1.21	-1.0	-3.44	921	0.62	+H
Clozapine-CH <sub>2</sub>	I	M5	C <sub>17</sub> H <sub>17</sub> ClN <sub>4</sub>	313.1213	2.16	-0.1	-0.44	50537	34	+H, 2x(+H)
Clozapine-CH <sub>2</sub> +O	I	M6	C <sub>17</sub> H <sub>17</sub> ClN <sub>4</sub> O	329.1153	0.90	-1.1	-3.24	590	0.37	+H
Clozapine+C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S (+GSH-2H)	II	G1	C <sub>28</sub> H <sub>34</sub> ClN <sub>7</sub> O <sub>6</sub> S	316.6063	2.09	0.1	0.10	10717	7.2	2x(+H), +H
Clozapine+C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S (+GSH-2H)	II	G2	C <sub>28</sub> H <sub>34</sub> ClN <sub>7</sub> O <sub>6</sub> S	316.6062	1.77	-0.2	-0.28	694	0.47	2x(+H)
Clozapine+C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S (+GSH-2H)	II	G3	C <sub>28</sub> H <sub>34</sub> ClN <sub>7</sub> O <sub>6</sub> S	316.6067	1.73	0.8	1.23	383	0.26	2x(+H)
Clozapine+O+C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S (+GSH-2H)	II	G4	C <sub>28</sub> H <sub>34</sub> ClN <sub>7</sub> O <sub>7</sub> S	324.6030	2.29	-1.4	-2.22	2344	1.6	2x(+H)
Clozapine+O+C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S (+GSH-2H)	II	G5	C <sub>28</sub> H <sub>34</sub> ClN <sub>7</sub> O <sub>7</sub> S	324.6030	1.88	-1.5	-2.36	353	0.24	2x(+H)
Clozapine-CH <sub>2</sub> +C <sub>10</sub> H <sub>15</sub> N <sub>3</sub> O <sub>6</sub> S (+GSH-2H)	II	G6	C <sub>27</sub> H <sub>32</sub> ClN <sub>7</sub> O <sub>6</sub> S	309.5972	1.98	-2.5	-4.01	372	0.23*	2x(+H)

Table 1. Metabolite summary of clozapine incubation in human liver microsome at 90 min in the presence of GSH.

\*Observed in the +GSH sample at 60 min incubation.

Table 1 summarizes data for the 12 metabolites observed in this study, including six phase I metabolites and six GSH adducts. The summary table also contains an “Adducts” column, where the observed charge state is displayed and listed in order of detected abundance. The column shows that for phase I metabolites, all compounds exist predominantly in a single-charge stage. For phase II metabolites, on the other hands, all the detected adducts are present exclusively in the doubly-charged state.

This typical behaviour is shown in Figure 4 in which clozapine is compared to the GSH adduct at RT 2.09 min, G1, as an example. In the spectrum of clozapine, the compound exists primarily as a singly charged species with an observed  $[M+H]^+ = 327$  (Figure 4, top), while the GSH adduct, G1, exists mostly as the doubly charged species  $[M+2H]^{2+} = 316.6$  with only minor levels of the singly charged  $[M+H]^+ = 632.2$  species (Figure 4, bottom). Less abundant GSH adducts were not observed as singly charged ions. Automatic deconvolution and grouping of all detected charge states (prior to data interpretation) is therefore essential for detecting GSH adduct under ESI<sup>+</sup> conditions.

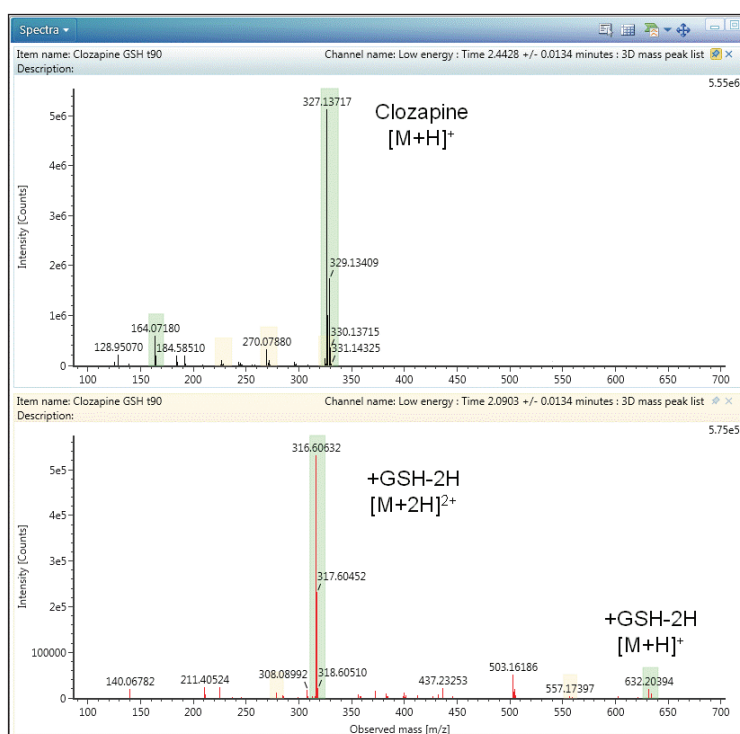


Figure 4. ESI<sup>+</sup> low energy spectra obtained from MS<sup>E</sup> experiment, (top) clozapine, (bottom) clozapine+GSH-2H adduct.

Finally, a complete picture of the study can be obtained by plotting observed components across all samples. Figure 5 is a summary plot for the response of clozapine and its major metabolites. The plot shows a decrease in clozapine concentration over time, with a corresponding increase in the concentrations of both phase I and phase II metabolites. Approximately 40% clozapine was metabolized at 90 minutes. The plot provides additional visual confirmation and supports positive identification of these metabolites.

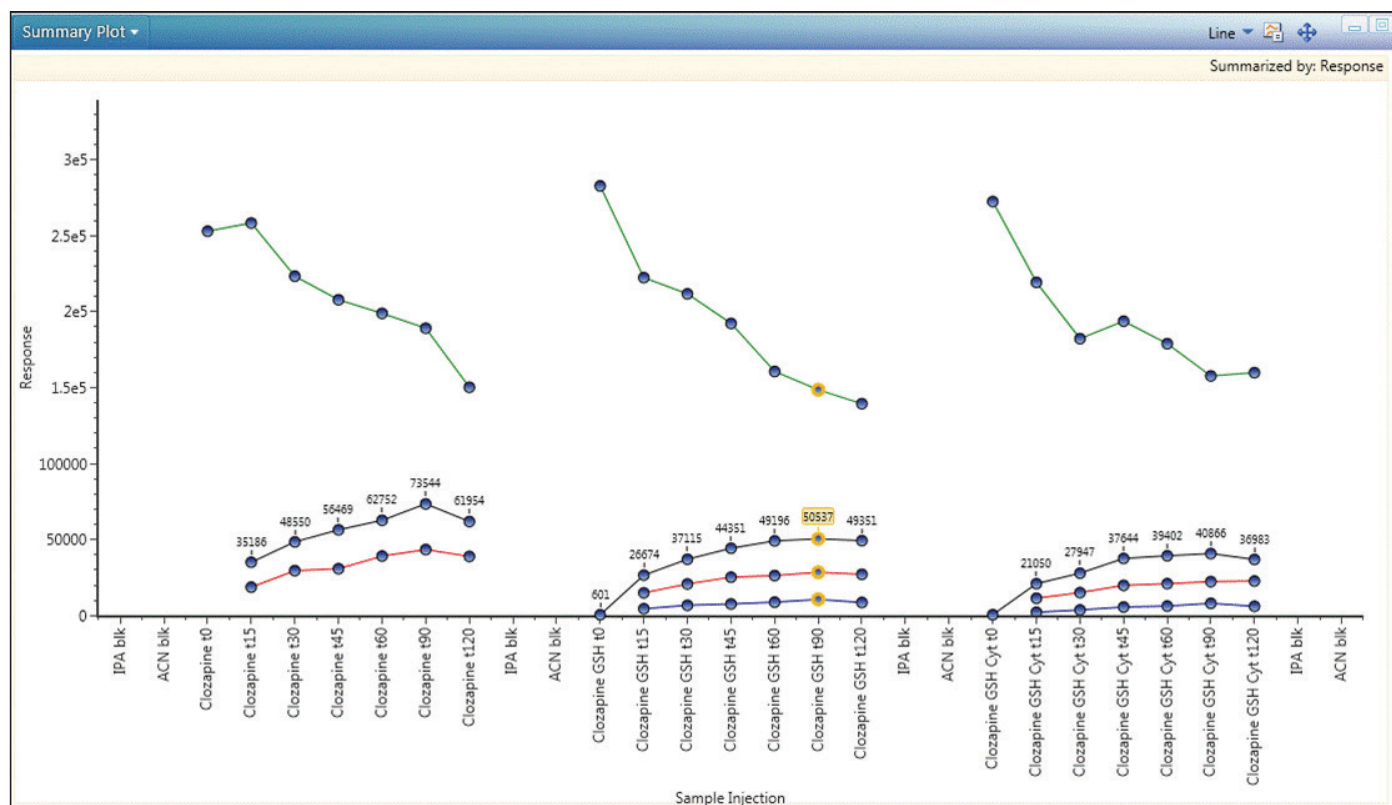


Figure 5. Summary plot of observed components across samples. (green line) clozapine, (dark blue): -CH2 metabolite (M5), (red): +O metabolite (M1), (light blue): +GSH-2H metabolite (G1).

## CONCLUSIONS

A simple and generic method has been described for the simultaneous detection and quantitation of parent compound, phase I metabolites, and GSH adducts in a microsomal incubation, using MS<sup>E</sup> generic acquisition under positive electrospray ionization conditions. Under positive electrospray ionization conditions, glutathione adducts are typically present as multiple-charged species, resulting in no detection or low sensitivity based on singly charged precursor ion detection. The automatic data deconvolution and charge state summarization of UNIFI Software has provided the key functionality necessary for their detection under ESI<sup>+</sup> conditions.

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