

Note d'application

Reactive Metabolism and New Screening Methodology Using Exact Mass Neutral Loss UPLC-MS/MS

Jose M. Castro-Perez, Michael McCullagh, Alan L. Millar

Waters Corporation



Abstract

This application note demonstrates the use of exact mass neutral loss mode with the Waters Micromass Q-ToF Premier Mass Spectrometer for the UPLC-MS analysis of GSH adducts in *in vitro* samples.

Introduction

Metabolic stability, toxic metabolite production, p450 inhibition and induction are all routinely monitored to prevent 'poor' compounds from progressing forward in the drug discovery and development process.

The challenge is to determine, as early as possible in the discovery process, which compounds will produce toxic reactive metabolites so that drug attrition in late-stage development can be minimized. One such reactive metabolite is glutathione (GSH). GSH is a tripeptide consisting of Glutamine, Cysteine and Glycine and it is present in mammalian systems. The formation of GSH adducts is a manifestation for the body to remove potentially toxic reactive intermediate metabolites. Therefore, assays focusing on detecting and identifying GSH adducts will pick up a significant portion of reactive metabolites.

To do so, there are several methods to monitor reactive metabolites by the use of chemical trapping agents, such as reduced GSH or cyanide trapping, to form stable adducts that allows characterization by liquid chromatography-tandem mass spectrometry. These methods, however, are particularly time-consuming and laborious. In order to screen for these reactive metabolites in a high throughput drug discovery environment, new analytical techniques are required. Atmospheric pressure ionization, triple quadrupole and time-of-flight (ToF) mass spectrometry address this need while affording extra selectivity and sensitivity for both quantitative and qualitative analysis.

Initial experiments carried out using triple quadrupole mass spectrometers operating in neutral loss mode show potential for this application. Here, GSH conjugates are fragmented under collision-induced dissociation (CID) giving a characteristic loss corresponding to the pyroglutamic acid (129 Da) moiety which can be monitored (Figure 1). However, this methodology can result in many false positives due to the nominal mass characteristic of these instruments. To eliminate this false positive potential, the extra selectivity and sensitivity of exact mass neutral loss measurement using a hybrid quadrupole time-of-flight mass spectrometer can be an attractive alternative. Additionally, full scan MS information is retained for further exploration to confirm the presence of the GSH adduct or other metabolites of interest. In this study, we will

demonstrate the use of exact mass neutral loss mode with the Waters Micromass Q-ToF Premier Mass Spectrometer for the UPLC-MS analysis of GSH adducts in *in vitro* samples. The resulting exact mass measurement yielded unequivocal results, confirming all reactive metabolites with errors less than 3 ppm RMS.

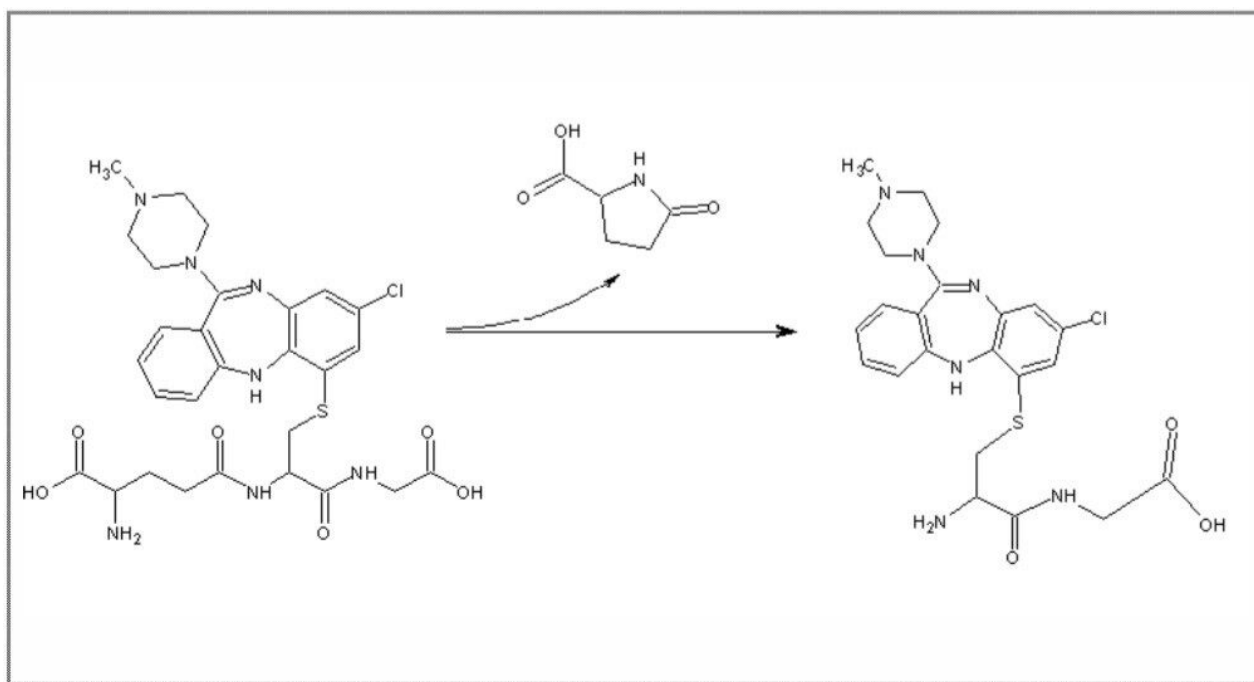


Figure 1. Neutral loss of the pyroglutamic acid moiety for the Clozapine GSH adduct.

Experimental

Exact mass neutral loss detection¹ with the Q-ToF Premier is achieved via sequential low and high energy MS acquisitions (Figure 2). MS/MS is then carried out on the parent mass of interest to confirm the common neutral loss. *In vitro* samples from a microsomal incubation (rat) with Clozapine were analyzed by UltraPerformance LC-MS using the Waters ACQUITY UPLC System and the Q-ToF Premier.

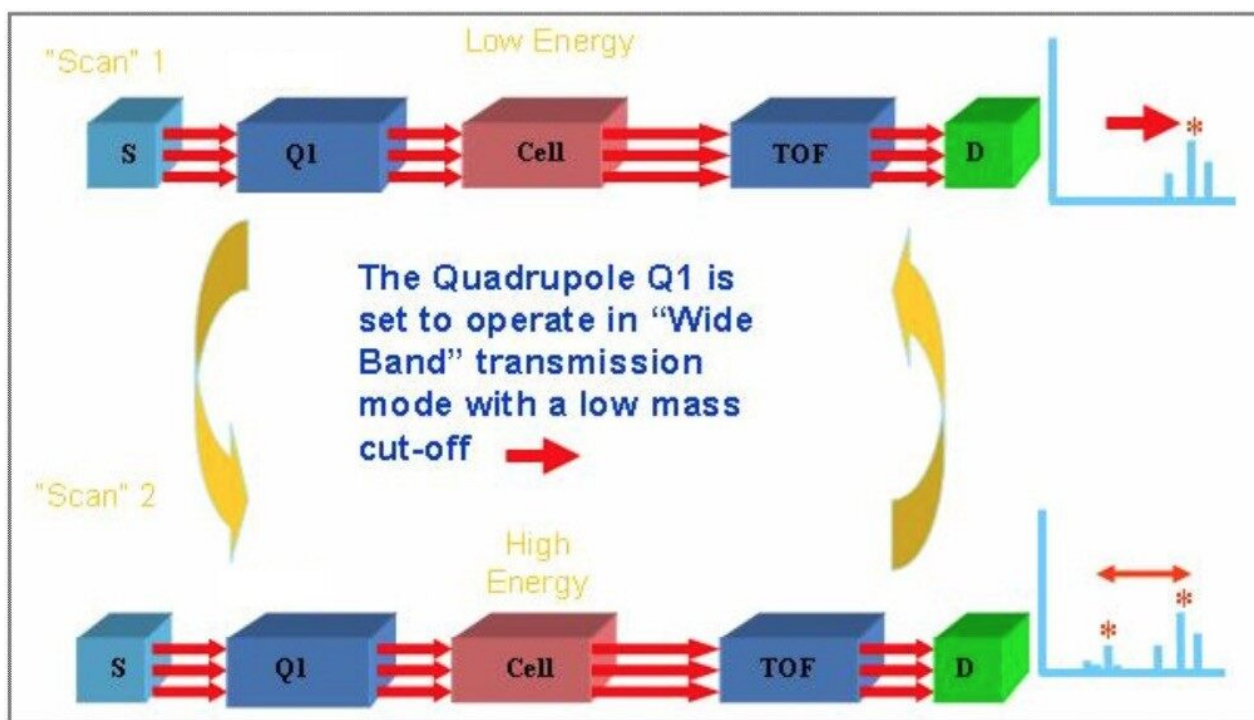


Figure 2. High and low collision energy sequence on the Q-ToF Premier for exact mass neutral loss acquisitions.

Sample Preparation

In vitro metabolism:

The *in vitro* incubations were performed using rat liver microsomes (1 mg/mL protein) in 96-deep well refill tubes, using a 50 mM potassium phosphate buffer (pH 7.4, 500 μ L total volume), at 37 °C on a shaking water bath at 45 rpm for 90 minutes. The test compound, Clozapine, was dissolved in dimethyl sulfoxide (DMSO) in buffer at a concentration of 100 μ M. Glutathione (GSH) was added in buffer for a final concentration of 10 mM GSH. The reaction was initiated by an NADPH-generating system consisting of 0.44 mM NADP⁺, 5.53 mM glucose-6-phosphate and 1.2 units/mL glucose-6-phosphate dehydrogenase together with 3 mM MgCl₂. The incubation was terminated after 90 minutes by the addition of acetonitrile (100 μ L), 6% acetic acid. After quenching, the samples were placed on ice for approximately 10 minutes. The quenched samples were centrifuged at 2,250 g for 20 minutes. The supernatants of the samples were removed and a 5 μ L aliquot was injected directly into the UPLC-MS System.

LC Conditions

LC system:	Waters ACQUITY UPLC
Column:	ACQUITY UPLC BEH C ₁₈ , 1.7 μm, 2.1 mm x 100 mm
Mobile phase A:	10 mM Ammonium Formate, pH 9
Mobile phase B:	Acetonitrile
Gradient:	0 min 98% A, 7 min 2% A, 8 min 2% A, 8.1 min 98% A
Injection volume:	5 μL
Column temp:	40 °C

MS Conditions

MS system:	Waters Micromass Q-ToF Premier
Ionization mode:	Electrospray, positive ion
Capillary voltage:	3 kV
Cone voltage:	45 V
Source temp:	120 °C
Desolvation temp:	320 °C
Acquisition range:	70–900 amu
Lock mass:	Leucine enkephalin, m/z =556.2771
Concentration:	0.2 ng/μL

Results and Discussion

The high sensitivity of the ACQUITY UPLC/Q-ToF Premier facilitated the detection of all of the Clozapine GSH adducts using exact mass neutral loss (Figure 3). A total of three metabolites were detected. We can see that the use of the ACQUITY UPLC System allowed this analysis to be completed rapidly, with an overall run time of less than 9 minutes. Despite this speed, the extra resolution of UPLC maintained the critical separation of these glutathione metabolites (Figure 3). The UPLC peak capacity was greatly improved compared to HPLC, allowing a superior chromatographic separation with little method optimization. The method was directly transferred from the HPLC methodology previously used (9 minutes with UPLC, as compared to the original 18 minutes). This is a significant improvement over the previous methodology reported.¹

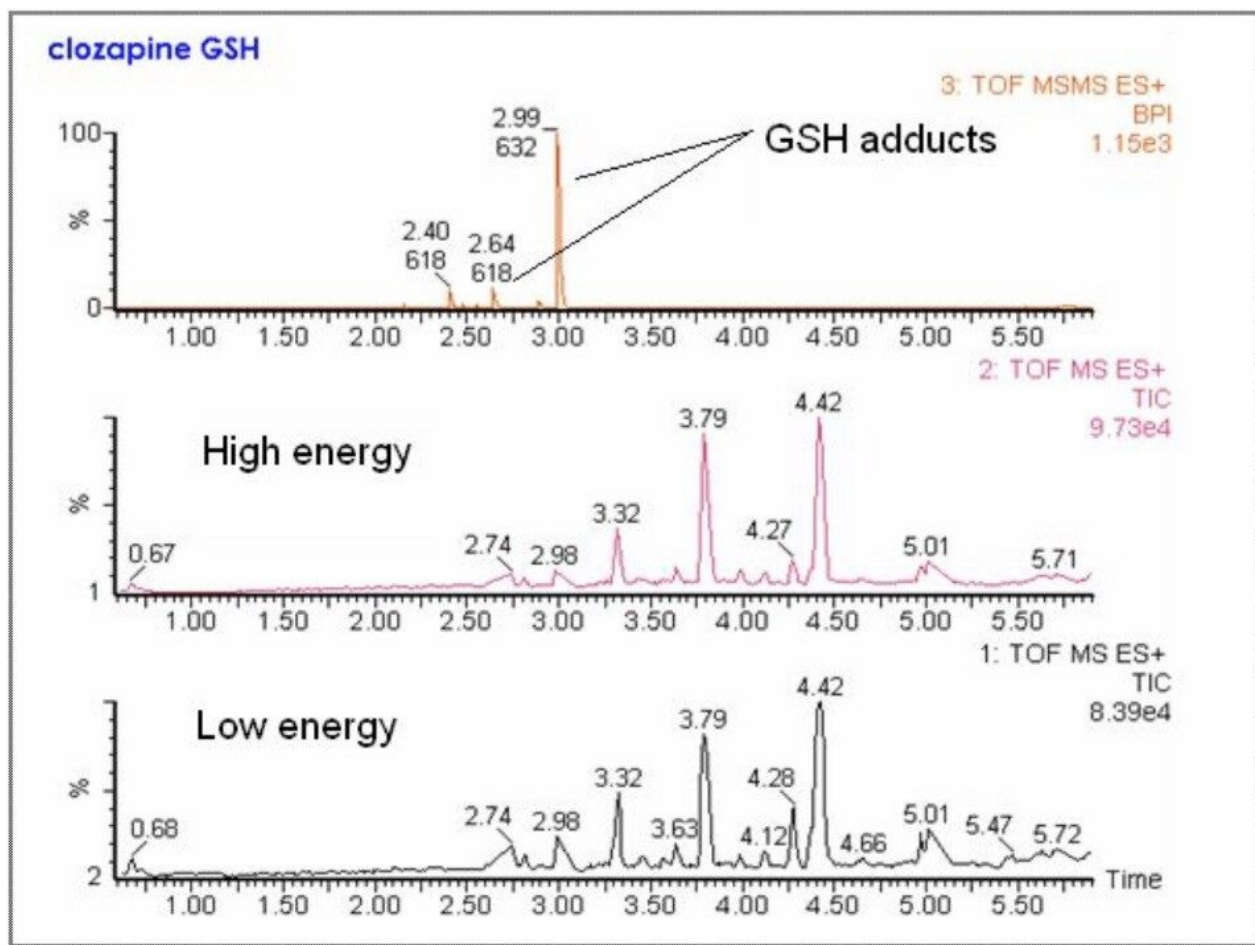


Figure 3. Clozapine sample and results for exact mass neutral loss screening with different acquisition modes, all happening simultaneously from a single injection.

In addition to detecting the potentially toxic GSH metabolites, this mode of operation also provided an information-rich data set in which full scan MS (low energy), pseudo MS/MS (high energy) and MS/MS (MS/MS) are acquired (Figure 4).

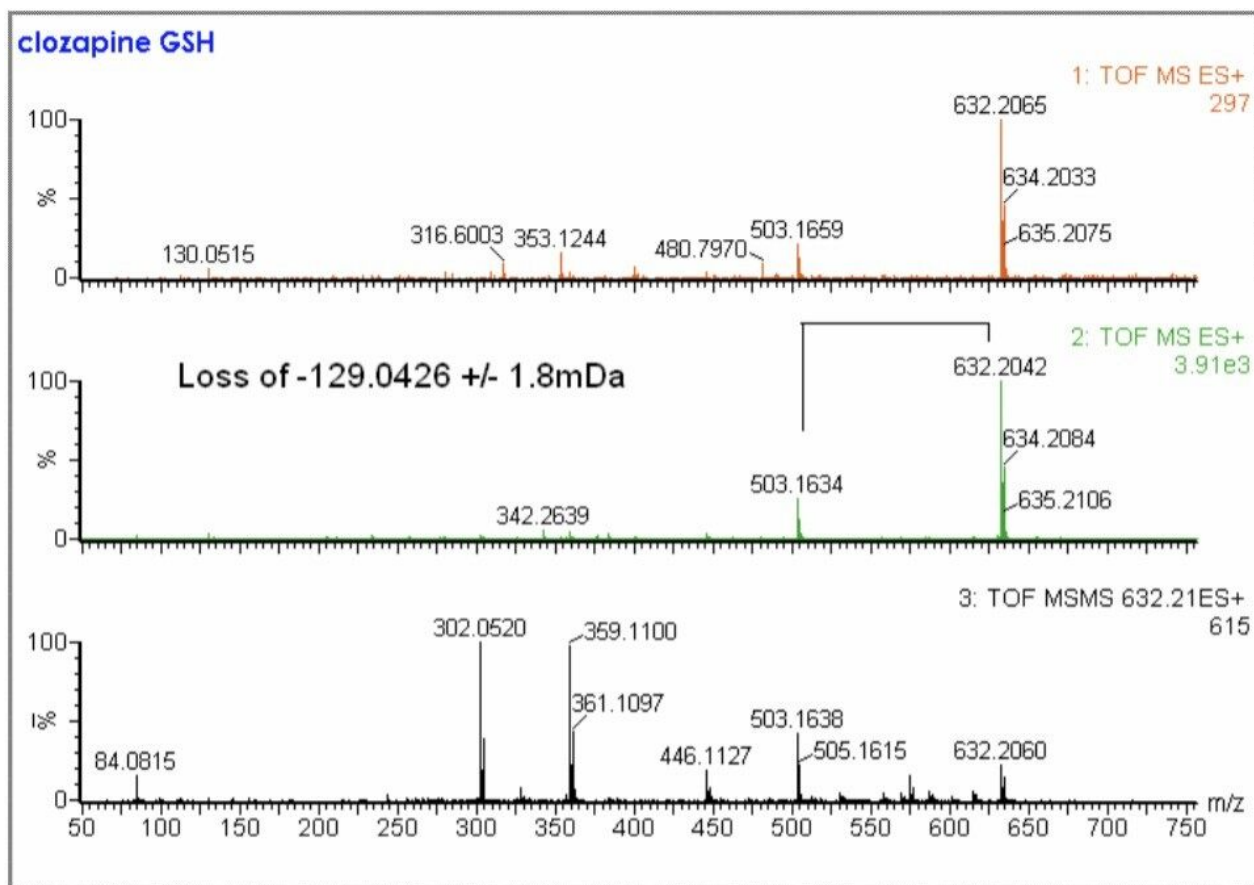


Figure 4. Individual spectra for each one of the acquisition modes in low energy (top), high energy full scan mode (middle) and MS/MS (bottom) to confirm the loss of the pyroglutamic acid moiety.

The application of exact mass analysis using the Q-ToF Premier significantly increased confidence in the identification of metabolites of interest (Figure 5). We can see that one of the GSH adducts was identified by a combination of the high energy scan data with the neutral loss of the pyroglutamic acid.

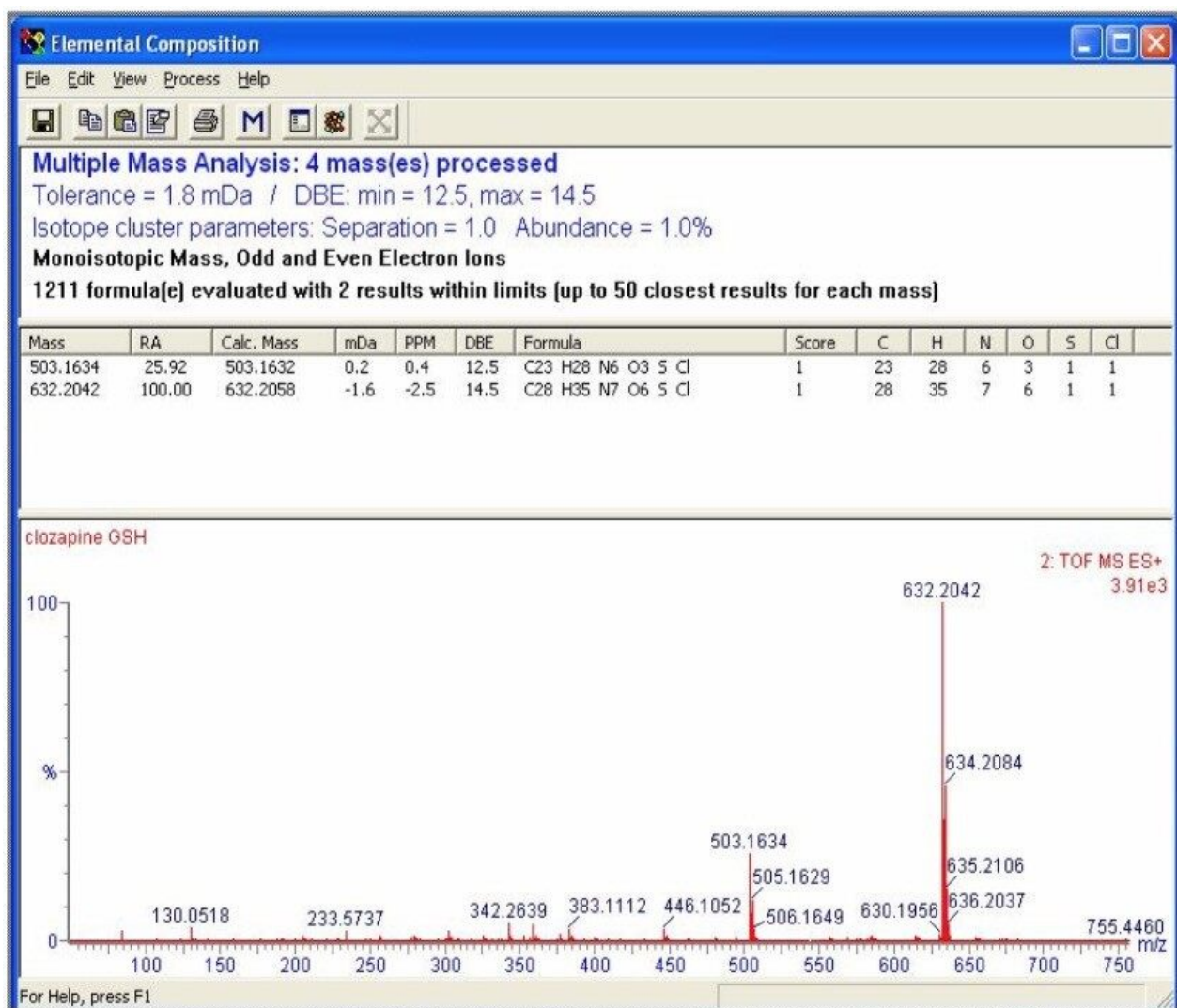


Figure 5. Exact mass obtained for one of the GSH conjugates and the loss of the pyroglutamic acid moiety. Measurements were below 3 ppm.

The low energy acquisition mode full scan MS data was used to detect further Phase I metabolites of Clozapine (Figure 6), illustrating the versatility of this new mode of data acquisition. A further advantage of obtaining the LC-MS information in the low energy mode (Figures 7) is that the data may be subsequently processed and interrogated at a later stage, if the need arises, with specialized application managers such as MetaboLynx for MassLynx Software to screen for other Phase I or Phase II metabolites. This data can be mined in conjunction with exact mass in order to identify putative Phase I metabolites.

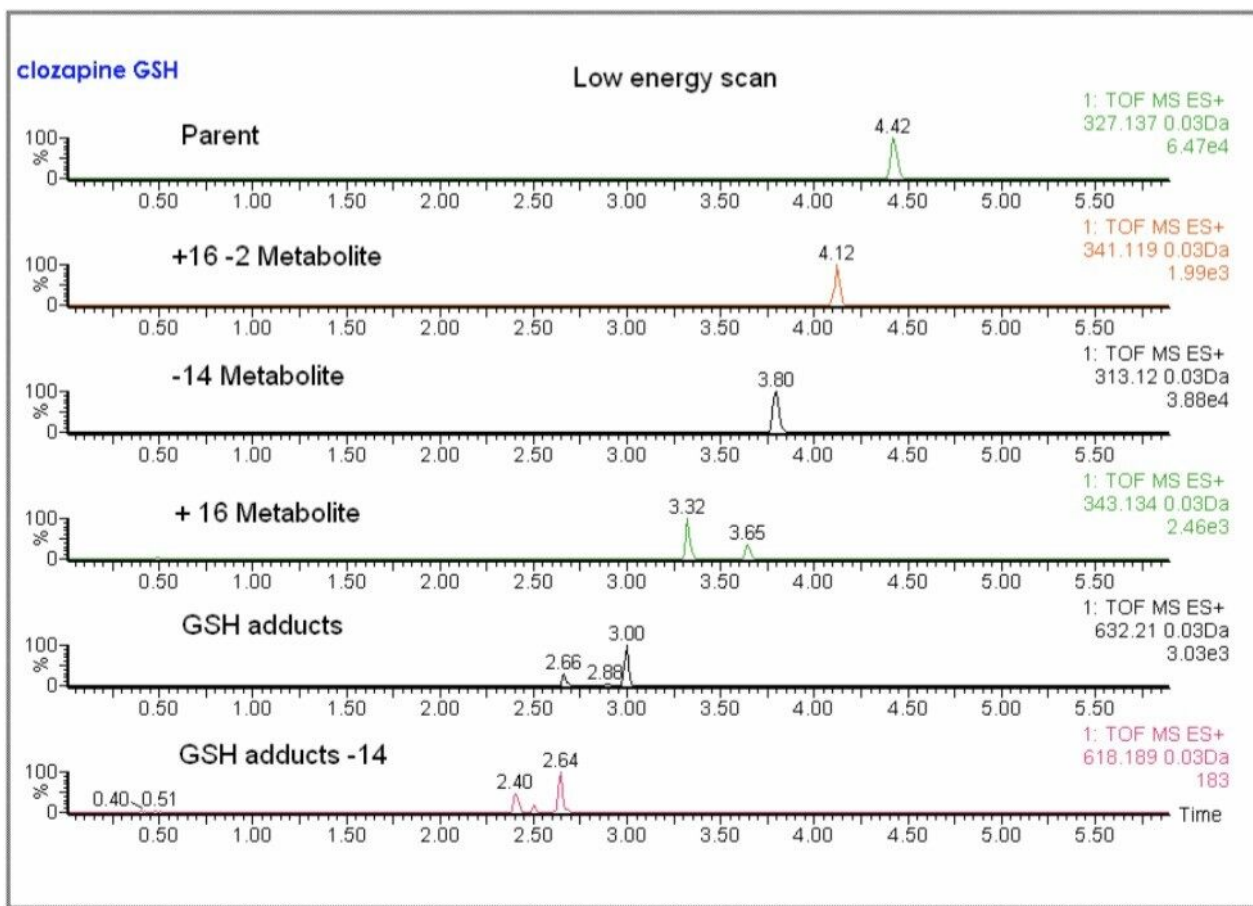


Figure 6. All major metabolites and parent compound from the low energy acquisition mode with exact mass extracted ion chromatogram windows.

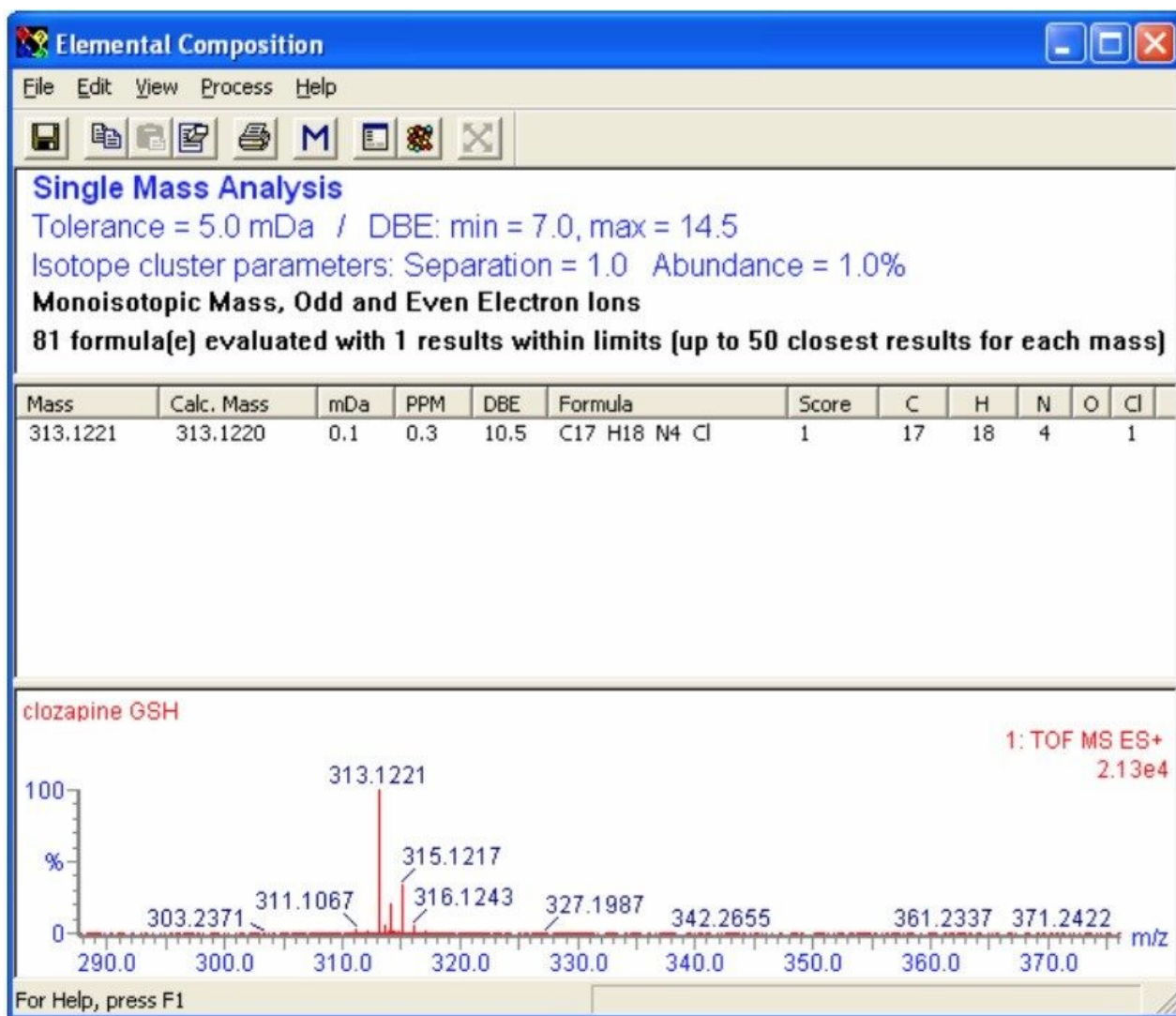


Figure 7. Spectra for N-dealkylated metabolite with exact mass from the low energy acquisition.

Conclusion

The exact mass neutral loss approach demonstrated here using the ACQUITY UPLC System combined with the Q-ToF Premier provided excellent selectivity and generated rich structural full spectrum MS information and targeted MS/MS for glutathione conjugations. Using this new application, fewer false positives were generated in exact mass mode compared with conventional neutral loss on a tandem quadrupole instrument. Additionally, the ability to acquire full scan MS information will allow this data to be interrogated at a later

stage for other putative drug metabolites of interest. The use of ACQUITY UPLC System enabled this analysis to be performed in a high throughput mode with an analysis time of less than 9 minutes - a 2 fold improvement over the previous HPLC methodology.

References

1. Castro-Perez J, Plumb R, Liang L, Yang E. A High-Throughput Liquid Chromatography/Tandem Mass Spectrometry Method for Screening Glutathione Conjugates using Exact Mass Neutral Loss Acquisition. *Rapid Commun Mass Spectrom*. 2005;19(6):798–804.

Acknowledgements

The authors would like to thank Dr. Eric Yang and Dr. Li Liang from GSK, King of Prussia, PA for the kind donation of the samples and guidance for this work.

Featured Products

ACQUITY UPLC System <<https://www.waters.com/514207>>

720001395, October 2005