GSH Conjugate Screening with the New QTRAP® 5500 LC/MS/MS System

Claire Bramwell-German, Hesham Ghobarah, and Elliott Jones
AB SCIEX, Foster City, California and Toronto, Canada

Overview

Screening for reactive metabolites of drug candidates using GSH trapping is an important part of early safety assessment in pharmaceutical discovery and development. Due to the toxicity of reactive metabolites, detection of these species even at trace levels can be relevant in the optimal design of a therapeutic drug. Current low and high-resolution single MS based detection can often be insufficient in terms of sensitivity of detection and confirmation. The QTRAP 5500® system is the first LCMS system which can combine the most powerful modes of GSH metabolite detection into a single injection workflow with sufficient sensitivity to ensure an accurate result. A series of groundbreaking workflows with optimization of scan speed, quadrupole MS/MS sensitivity, rapid positive/negative switching and unprecedented LIT MS/MS confirmation sensitivity will be presented in this application overview.

Introduction

Drug induced idiosyncratic hepatotoxicity is a concern to pharmaceutical companies, especially as these drugs are often missed by pre-clinical safety assessment and clinical trials, probably due to the low frequency of occurrence and low level of reactive metabolite formation.

Reactive metabolites are capable of covalent modification of proteins or nucleic acids through nucleophilic substitution. These types of reactions may contribute to iodisyncratic hepatotoxicity due to the interruption of certain cellular processes. The process and causes of idiosyncratic drug hepatotoxicity is not fully understood. However, the formation of reactive metabolites appears to be associated with various toxicological events.

Reactive/electrophilic metabolites are known to conjugate with the tri-peptide Glutathione (Figure 1). GSH conjugation occurs either spontaneously or through catalysis by GSH-S-transferases (GST's) present in the cytosol or endoplasmic reticulum.

The presence of GSH conjugated metabolites is an indication of the formation of reactive metabolites and is thus critical to identify and monitor in metabolite profiling studies within the drug discovery and development process.

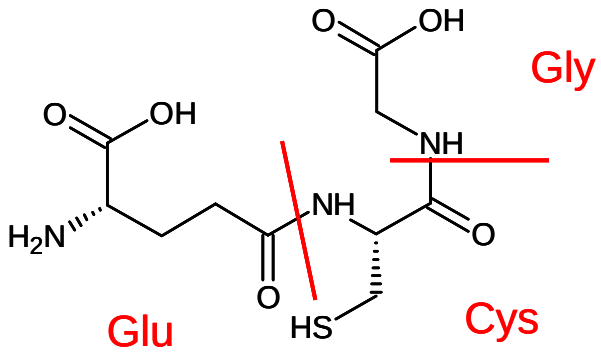

Figure 1. Structure of GSH (glutamylcysteinylglycine)

A number of QTRAP® system specific methods are possible for GSH detection. Historically, a constant neutral loss (NL) scan of 129 Daltons has been proven to be an effective method of detection compared to single MS. This triple quadrupole scan is based on fragmentation which significantly improves selectivity since background ions are excluded from the spectra in favor of GSH formations which produce this unique fragment pathway[1]. In some rare cases, a neutral loss scan of 129 can miss a metabolite based on the nature of GSH conjugation.

More recently a negative precursor ion scan of 272 has also been suggested as a superior method for GSH detection[2]. In general, the negative ionization mode has a lower background since far fewer small molecule compounds ionize in this polarity. Also, GSH variation and bond linkage showed fewer cases in which detection would not be possible using the precursor 272 scan (PI), compared to the positive mode NL 129.

The QTRAP® 5500 system is the first LCMS with the capability to do both negative precursor and positive neutral loss in one fast IDA (Information Dependent Acquisition) scan cycle. The QTRAP® 5500 system has the ability to scan in both precursor and neutral loss mode at up to 2000 Da/sec. Furthermore, the QTRAP 5500® system has the ability to switch between positive and negative ion mode in 50 ms. Since the instrument is also a hybrid Linear Ion Trap (LIT), it is possible to obtain trap-based ultra high sensitivity MS/MS spectra automatically. This can be achieved via IDA based triggering on the notable peaks found by the precursor, pMRM and/or neutral loss scans in real time. The QTRAP 5500® system also has the ability to scan 5X faster

Total Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0.00	250	98.0	2.0
3.00	250	98.0	2.0
30.00	250	10.0	90.0
35.00	250	10.0	90.0
37.00	250	98.0	2.0
47.00	250	98.0	2.0

Table 1. HPLC gradient

(20,000 Da/sec) in LIT mode compared to the original QTRAP® systems. Given these ground-breaking speeds, it is possible to perform a combined PI/NL with polarity switching in IDA, with 4 dependent ion trap MS/MS scans in approximately 2 seconds. This rate of acquisition has significant advantages for the application of 1.8 µm high pressure chromatography with short (5 to 10 minute) run times, which is desirable for high throughput GSH screening in pharmaceutical discovery and development. Furthermore, the ability to run 4 LIT MS/MS scans (at 20,000 Da/sec) ensures that all major GSH formations can be assigned from a single LCMS injection.

A second novel and highly effective method of GSH detection possible on the QTRAP® is pMRM (predictive MRM). This method uses the next generation MRM builder in LightSight® software v2.1 coupled with Excel™ to create a complete set of potential MRM transitions for any GSH biotransformation. In this case, a comprehensive set of greater than 60 phase I transformations can be created in a GSH specific way to produce a positive MRM list based on the neutral loss of 129 and a negative list based on the precursor of 272. This pMRM GSH

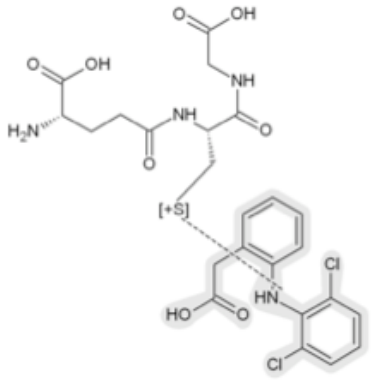

Figure 2. Diclofenac + GSH

method is fundamentally a pos/neg switching IDA experiment with 3 to 5 dependent LIT MS/MS scans. Since the MRM has a superior signal to noise ratio compared to all other survey scan modes, this often returns the greatest number of GSH confirmations in a single injection. Both the pMRM and PI/NL methods are highly effective and generally superior to single MS and GSH labeling strategies, as well as mass defect filter (MDF) high-resolution experiments[3]. In this application note both of these methodologies, as well as the unique abilities of the QTRAP 5500® will be examined for GSH analysis.

Experimental Conditions

Diclofenac was chosen as a reference compound in this application example. It was recently cited in a paper by Ming She Zhu et at. as an example of LC/MS GSH detection[3]. In an effort

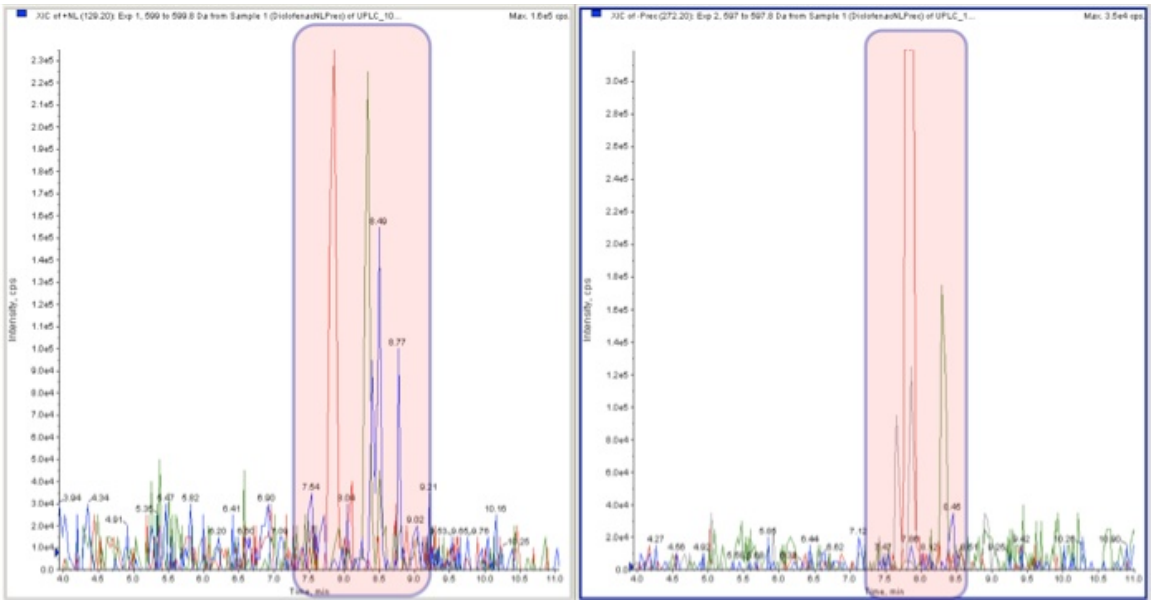

Figure 3. XIC trace of +/- NL/PI IDA Diclofenac GSH analysis, using a short high pressure gradient.

to allow for comparison, identical incubation and LC conditions as well as a faster high-pressure gradient were employed in this analysis. Diclofenac was incubated at 10 µM in human liver microsomes (HLM) with GSH and NADPH co-factor for 30 minutes in potassium phosphate buffer (100 mM, pH 7.4) to give a total volume of 2 mL. After centrifugation, the 2 mL fractions were dried and reconstituted in 200 µL of 5 % acetonitrile. This gave a final concentration of 100 µM for Diclofenac in the T0 control.

The HPLC gradient used is noted in Table 1. A 20 µL volume of control or 30 minute incubation was injected onto a Zorbax™ C-18 (2.1X150 mm) HPLC column. A similar gradient was run in about half the time using a 1.8 µm Agilent XDB 2X50 mm column at a higher flow rate and pressure.

Two IDA methods were run on the QTRAP 5500® system to detect GSH conjugates. The first is the dual survey neutral loss (NL) 129 (+), precursor ion (PI) 272 (-) with 4 dependent EPI (+) scans. Data was acquired at the fastest quadrupole scan rate of 2000 Da/sec for the precursor and neutral loss experiments. The LIT experiments were acquired at a scan rate of 20,000 Da/sec with a fixed fill time of 35 ms and Q0 trapping to increase duty cycle. The second method incorporated a list of pMRM's based on a neutral loss of 129 followed by a negative pMRM list based on the transformed parent m/z for Q1 to a negative fragment of 272 for Q3. The strength of this method is that only two MRMs are needed for each predicted biotransformation, one for positive and one for negative. This keeps the number of MRM's to around 120, based on a theoretical transformation list of ~60 phase I metabolites plus GSH. A dwell time of 5 ms was used for all pMRM's, with a pause of 5 ms. A pos/neg switching time of 50 ms was used for the two requisite polarity changes needed for this experiment. All data was processed in LightSight® software v2.0 and ACD Fragmenter / Specmanager™ to find and assign major metabolites.

Results and Discussion

Figure 2 shows the structure of diclofenac coupled to GSH, in a geometrically unassigned fashion. Two experiments were run to elucidate the possible GSH metabolites, the (+/-) pMRM IDA and the (-) PI 272 / (+) NL 129 IDA. Figure 3 shows the XIC trace for Precursor Ion 272 (-) & Neutral Loss 129 (+) IDA. Figure 4 shows the XIC trace for the (+/-) pMRM GSH specific IDA analysis. In both figures major GSH metabolites are shaded in red.

Figure 5 illustrates a comparative diagram for the total number of GSH metabolites detected by the PI/NL, pMRM and accurate mass (result referenced in paper[3]) workflows. In this case, the combined PI/NL IDA method detected and confirmed a total of 4 GSH conjugates in a single injection. The pMRM IDA method also detected and confirmed 4 GSH conjugates plus two

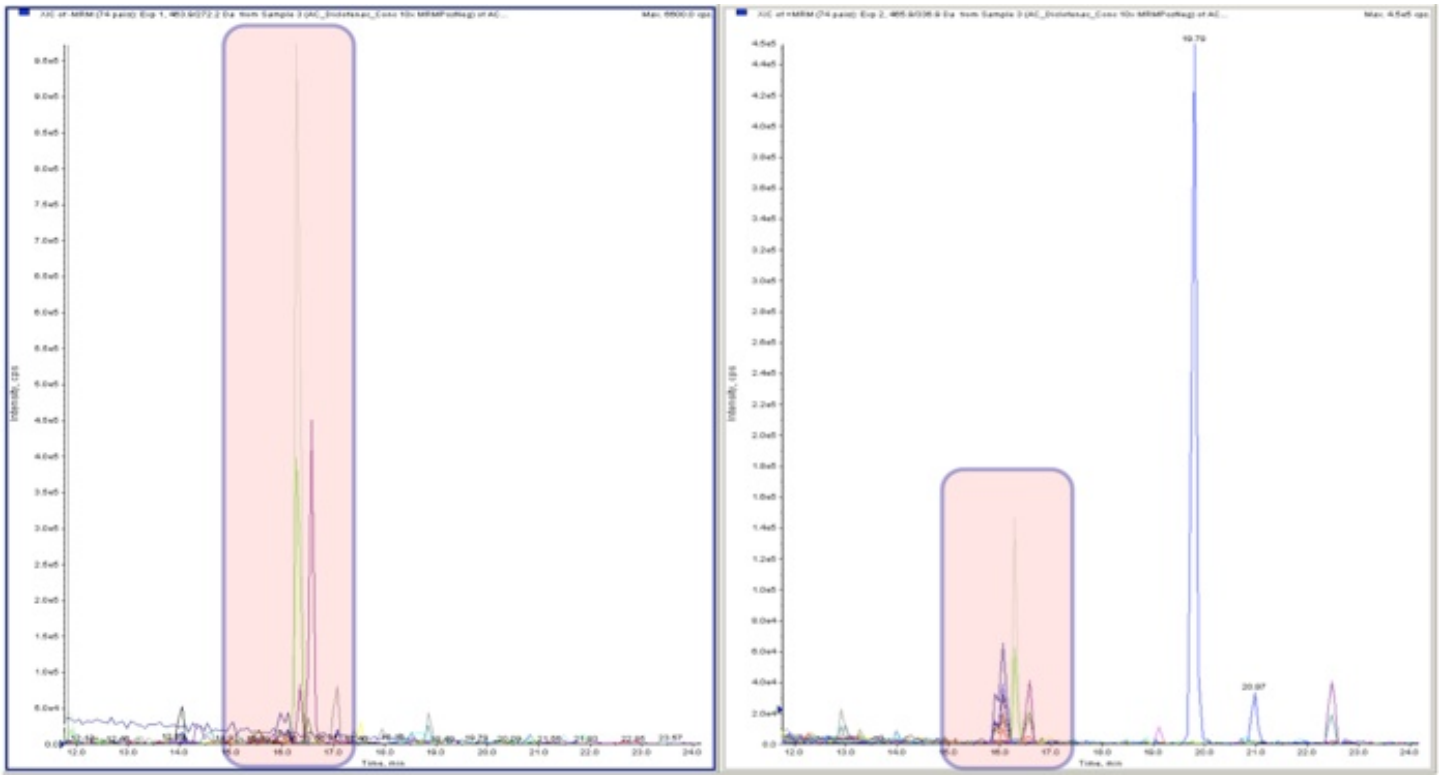

Figure 4. XIC trace of +/- pMRM IDA Diclofenac GSH analysis, using long low-pressure gradient (same as reference).

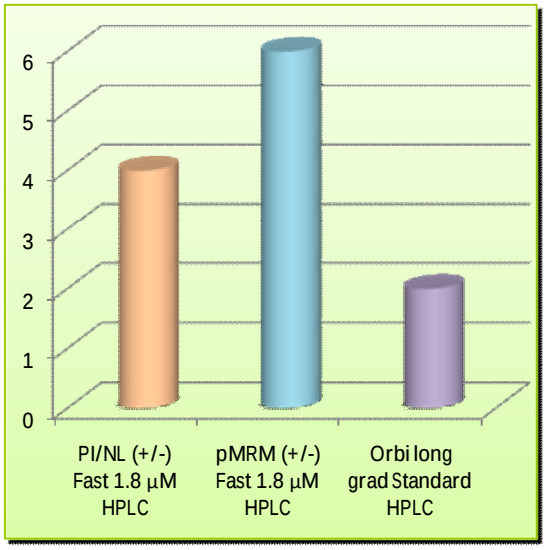

Figure 5. Tabulated total number of GSH metabolites of Diclofenac, high pressure PI/NL and pMRM IDA vs. longer gradient standard HPLC, accurate mass analysis.

additional species that were detected but not confirmed in this preliminary evaluation. In both cases, the QTRAP 5500® system showed a significant improvement in detection and sensitivity of confirmation in a single injection with high throughput workflow, amenable to early discovery level screening. Furthermore, the improved sensitivity in LIT mode on the QTRAP 5500® system provides superior fragmentation pattern and spectrum confirmation that would not be achieved on less sensitive accurate mass instruments. The additional dynamic range of analysis allows for a complete picture of GSH metabolism for a drug of interest.

Figures 6 & 7, show two examples of the fragmentation analysis for notable GSH metabolites with a MW of 581 and 598. The richness of fragmentation provided by the QTRAP 5500® system allows for the detection of 20 diagnostic fragments for the MW 581 & 598 metabolites. A number of the major fragments are noted on the structure for reference.

Summary

In this preliminary study and evaluation of novel workflows, the QTRAP® 5500 system shows a strong ability to detect trace level metabolites not easily seen by standard LCMS workflows. Furthermore, the increased scan rates (4 to 5X quadrupole and 5X trap) allow for advanced IDA dual survey methods (and as many as 4) including polarity switching, thus ensuring complete GSH detection. These improved scan rates are also highly compatible with fast LC high-throughput screening preferred by discovery level GSH detection. The improved sensitivity and coverage of detection applies well to development level toxicology analysis. Further investigation will be pursued to evaluate this ground braking technology for GSH analysis to reinforce this early evaluation.

Highlights

- Improved quadrupole sensitivity (API 5000 level MRM response & S:N, PI & NL)
- Sensitivity improvement in TRAP modes (EPI, 10-100 fold)
- 4 times faster QqQ scanning
- Multiple full scan survey possibilities (eg. Precursor Ion, Neutral Loss)
- Faster Polarity switching (50 ms)
- 5 times faster LIT scanning (20,000 amu/sec)
- Shorter fill time capabilities (50 µs)
- Unique workflows ideal for GSH conjugate detection

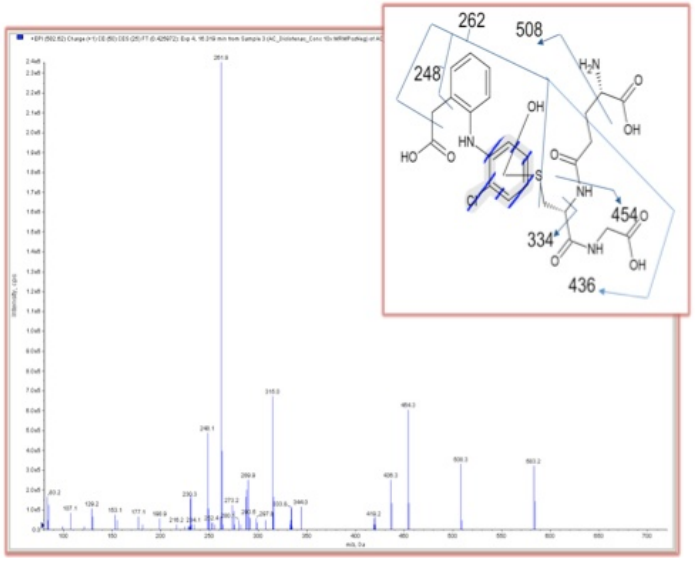

Figure 6. GSH metabolite MW 582, with fragment assignment

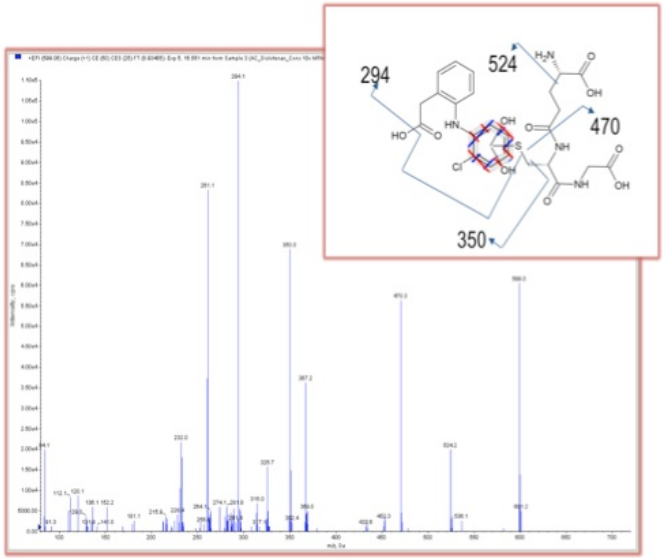

Figure 7. GSH metabolite MW 598, with fragment assignment

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(2) Wen, B.; Ma, L.; Nelson, S. D.; Zhu, M. Anal. Chem. 2008, 80, 1788-1799.

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Headquarters
353 Hatch Drive | Foster City CA 94404 USA
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