Metabolite Identification with the New QTRAP® 5500 LC/MS/MS System: Sensitivity, Selectivity, Speed & Unique Workflows

Overview

The new QTRAP® 5500 System reduces the challenges of metabolite identification for the drug discovery and development pipeline. The enhanced speed & sensitivity of the QTRAP® 5500 system means we have much more efficient workflow possibilities for metabolite identification; the predictive MRM (pMRM) approach and the use of multiple precursor ion and/or neutral loss survey scans in a single analysis, including polarity switching, means the ability to find and identify lower-level metabolites is better than ever before. When used with LightSight® software, it's a real workhorse for this environment and complements an accurate mass approach.

Introduction

Metabolite identification is central to many of the activities in the discovery and development pipeline. From rapid structural identification during the discovery process to provide an early perspective on the metabolically labile sites, or soft spots of a drug candidate, to a more complete characterization of pharmacokinetic properties to establish dose and toxicity levels, there's an ever increasing demand on throughput. The ability to find, identify and confirm metabolites as fast as possible is critical; not only for the major metabolites, but the very low-level metabolites as well. Being able to do this in a single analysis is a highly sought after goal. The utility of QTRAP® system technology with its unique combination of triple quadrupole and linear ion trap scan capabilities together with automated approaches to metabolite identification has brought this goal closer to reality.

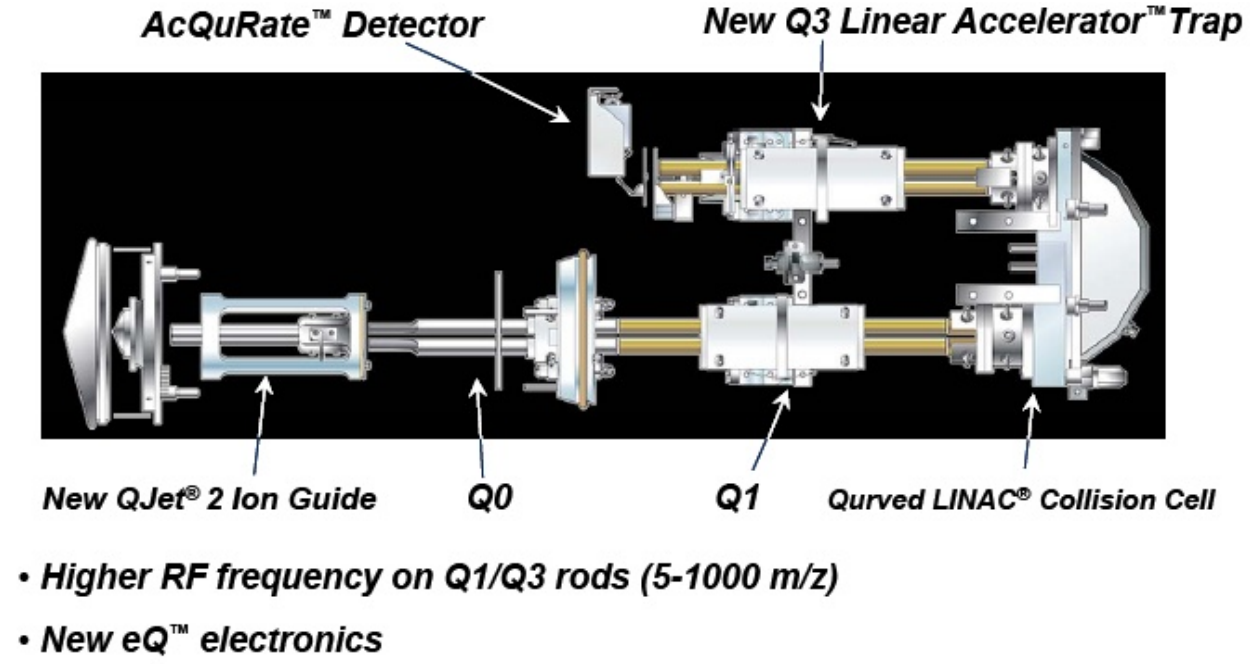

Figure 1. QTRAP® 5500 System Ion Path

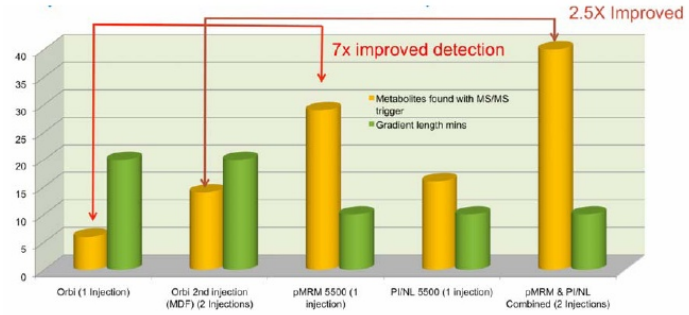

Figure 2. Tabulation of all found metabolites, in either single or double injection workflows, QTRAP® 5500 System shows far greater detection efficiency than high-resolution MDF two injection workflows.

The QTRAP® 5500 System brings a number of key advantages to metabolite identification. The addition of the QJet® 2 Ion Guide and improved Q3 linear ion trap (LIT) shows increased signal response of a factor of 9 in quadrupole mode and 10 to 100 fold in LIT mode. The overall duty cycle is further improved with trap fill times as short at 50 µsec, because of the increase in linear ion trap sensitivity, and polarity switching as low as 50 msec. The system also has enhanced scan rates of up to 20,000 amu/sec in LIT mode and 4 times faster in quadrupole mode. This greater cycle rate allows for compatibility with ultra fast chromatographic analyses as well as improved IDA coverage provided by a greater number of MS/MS dependent scans on relevant peaks.

Here we describe several key strategies to identify metabolites in a single analysis.

QTRAP® 5500 System Overview

The QTRAP® 5500 system is a completely redesigned platform. The ion path (Figure 1) consists of a curved collision cell allowing a reduction in the platforms footprint and various changes have led to improved performance.

The new QJet 2 Ion Guide is 2.5 times longer (125mm) than what was first implemented in the API 5000™ system. The addition of the QJet® ion guide offers improved protection from contamination of the ion optics, but its main function is to efficiently capture and focus ions into the Q0 region. A major advancement in the ion path is the new Linear Accelerator™ Trap which brings LINAC® technology to the Q3 quadrupole. It improves resolution and simplifies the tuning procedure. Aside from this, it has provided improved ion extraction efficiencies for as much as 100 times gain in sensitivity for ion trap scan modes. Additionally, there's been a reduction in ion cooling fragmentation times producing superior MS^3 results. Together with new electronics, all of the above changes have resulted in significant performance enhancements in speed, selectivity and sensitivity.

- Improved quadrupole sensitivity (MRM response & S:N, PI & NL)
- 10-100 times sensitivity improvement in TRAP modes (EPI MS/MS)
- 4 times faster QqQ scanning
- Faster Polarity switching (50 ms)
- 5 times faster LIT scanning
  - 20,000 amu/sec
- Shorter fill time capabilities (50 µs)

The enhanced speed & sensitivity of the QTRAP® 5500 System means we have much more efficient workflow possibilities for metabolite identification; the predictive MRM (pMRM) approach and the use of multiple precursor ion and/or neutral loss survey scans in a single analysis, including polarity switching, means the ability to find and identify lower-level metabolites is better than ever before. Below we discuss very challenging applications that are simplified with the new QTRAP® 5500 System

Metabolite ID Examples

1. In-vivo Analysis
2. Microdosing Studies
3. Metabolic Stability Studies
4. GSH Conjugate Screening (Reactive Metabolites)

In Vivo Analysis

Metabolite detection in in vivo sample matrix at clinically relevant concentrations is a critical challenge for drug discovery and development. A wide range of complex matrices (bile, plasma, urine and fecal extracts) poses a number of critical analytical issues and challenges. One critical limitation is the low level of many circulating metabolites, requiring improved sensitivity to enable detection. Furthermore the suppressive nature of these biofluids can cause a further decrease in sensitivity. Another limiting factor for in vivo analysis is the complexity of background signals, which increases the complexity of MS and MS/MS data analysis. In general the selective nature of MRM, Precursor and Neutral Loss scans provide the highest level of detection comparing to any single MS based methodology.

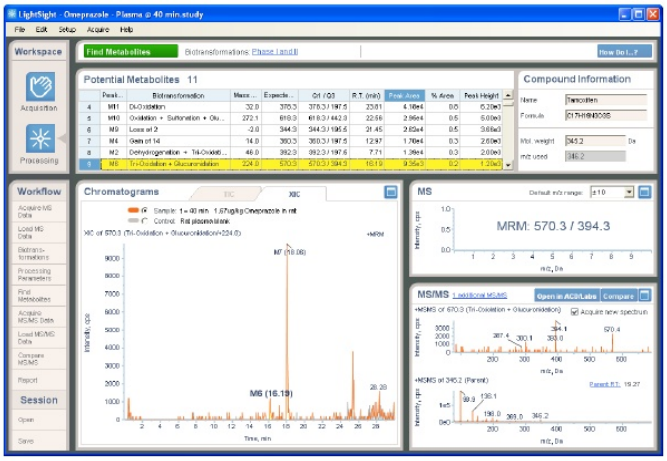

Figure 3. A minor metabolite (tri-oxidation + glucuronidation) of omeprazole detected and confirmed.

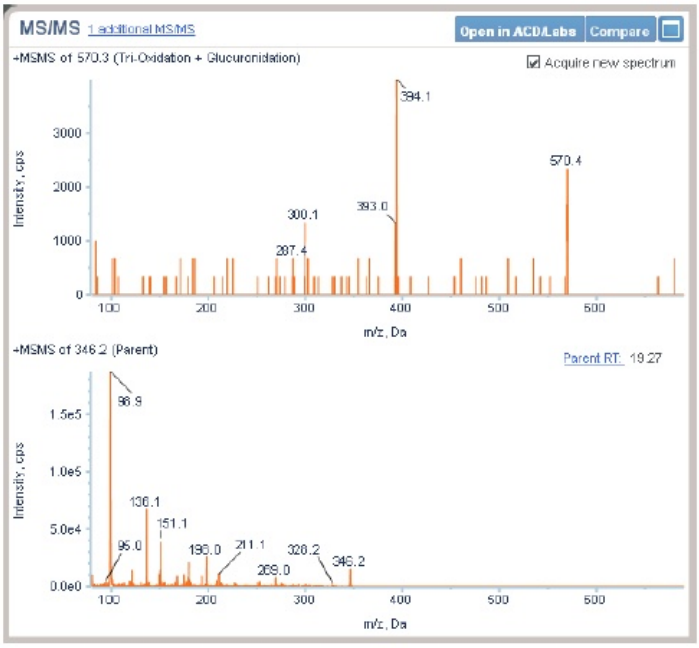

Figure 4. MS/MS of a minor metabolite (tri-oxidation + glucuronidation) of omeprazole; m/z 394 is a diagnostic fragment for this metabolite.

In this work a set of verapamil in vitro hepatocyte metabolites were spiked into bile matrix at a low level (2.5 µM) to mimic the in vivo sample matrix. LightSight® software v2.1 has allowed for a new novel survey mode called pMRM (predictive MRM). This new type of IDA experiment can be created automatically using LightSight® software v2.1 and loaded directly to the QTRAP® system for rapid analysis. The advantage of pMRM is the improved signal to noise ratio, IDA selectivity and efficiency compared to single MS strategies. The QTRAP® 5500 System with pMRM survey mode as well as dual precursor and neutral loss scan mode was evaluated against a two injection mass defect filter (MDF) high-resolution experiment and is discussed in detail in a technical note titled, “Breakthrough in vivo metabolites analysis using the QTRAP® 5500 System and Predictive MRM”.

Using the targeted pMRM approach, 158 transitions were created by LightSight® software 2.1 to search for phase I and II metabolites. A total of 1.5 seconds was the total duty cycle for the method which included automated collection of MS/MS at 20,000 amu/sec. When employing the multiple precursor ion and neutral loss approach, the overall duty cycle was 2.2 seconds. Both workflows had cycle times well suited for high throughput chromatographic analyses.

To summarize, even at higher throughput chromatographic conditions (by a factor of 2), the QTRAP® 5500 System found and identified with confirmatory MS/MS, 7 times using a single injection workflow than the high resolution approach. With the addition of a second injection the QTRAP® 5500 system still managed to top the high resolution experiments by a factor of 2 (Figure 2).

Microdosing Studies

Many new chemical entities fail in Phase I clinical trials due to poor pharmacokinetics. Until Phase I, human pharmacokinetics can only be predicted from animal models. Microdosing is an approach which may make human clinical trials more effective, predictable and quicker. The microdosing strategy allows for early assessment of human pharmacokinetics and bioavailability of a drug candidate. Microdosing studies are designed to evaluate pharmacokinetics or imaging of specific targets without inducing pharmacologic effects. According to the FDA guidance, a microdose is defined as less than 1/100th of the dose of a test substance calculated (based on animal data) to yield a pharmacologic effect of the test substance with a maximum dose of ≤ 100 micrograms[1]. Identifying unique and major human metabolites early in the drug development process is important because it will allow for timely assessment of potential safety issues.

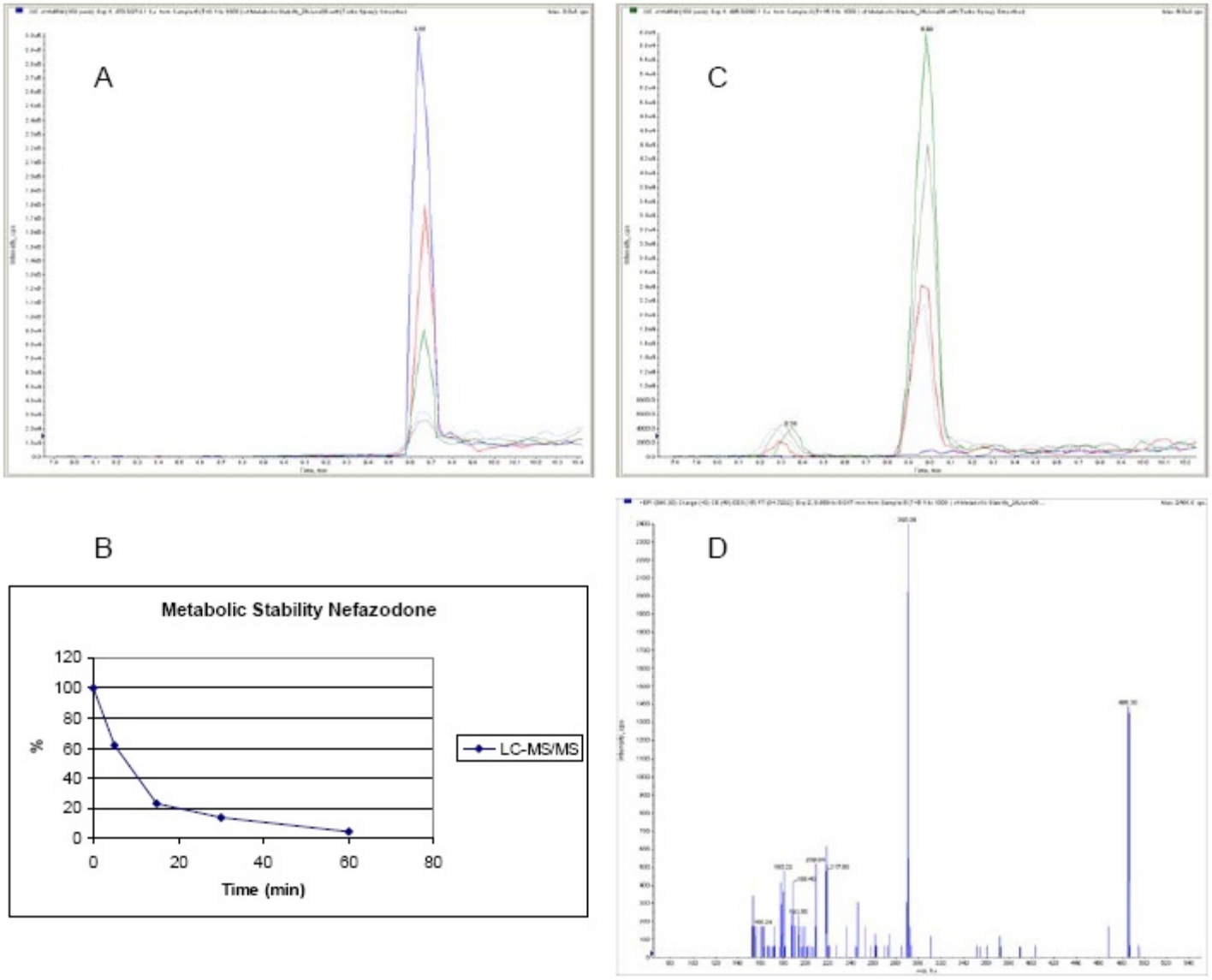

Figure 5. Microsomal incubation in rate liver microsomes was performed on Nefazodone at 0, 5, 15 and 30 minute time points. The half life is determined by monitoring the parent drug, while at the same time major and minor metabolites can be found, ID'd and confirmed all in the same analysis over the time course study. A) Parent drug B) metabolic stability of parent drug, C) oxidative metabolite, D) MSMS of oxidative metabolite

The current technique for evaluating microdosing studies is accelerator mass spectrometry (AMS). AMS is an ultra-sensitive technique where the lower limit of quantitation is in the low fg/mL range. However, this technique has many drawbacks including the cost for routine use because the synthesis of radio-labeled compounds is required. It has been shown[2-4] that conventional liquid chromatography-tandem mass spectrometry (LC/MS/MS) has sufficient sensitivity to support quantification of parent drug in human microdosing studies.

Comprehensive detection and confirmation of metabolites can be achieved at microdosing levels in a single injection using LC/MS/MS. In this work, metabolites of omeprazole were detected and confirmed in rat plasma at microdosing using a QTRAP® 5500 system. In this study, three Jugular vein-cannulated Sprague-Dawley rats were dosed via oral gavages at 1.67 µg/kg of omeprazole. Plasma was taken at 20, 40, 60, 80, and 120 minutes from each rat. A pMRM method was generated with 129 MRM transitions with a pause time of 5 ms and dwell time of 5 ms per transition. MS/MS data was collected automatically via Information Dependent Acquisition (IDA) at a scan rate of 10,000 Da/sec. The overall duty cycle time for this method was approximately 2.6 seconds.

This application is discussed in detail in a technical note titled, "Detection and Confirmation of Metabolites at Microdosing

Levels using the New QTRAP® 5500 LC/MS/MS System". Figures 3 & 4 highlight some results that improved sensitivity of the QTRAP® 5500 System has provided for this sensitivity challenged application area.

To summarize, the sensitivity and speed improvements in the QTRAP® 5500 system have made it the ideal tool for microdosing studies, in particular for finding and identifying metabolites at microdosing levels (1/100th the normal dose that produces a pharmacological effect). This system and workflow has significant advantages over AMS technologies. While AMS has been traditionally associated with microdosing studies because the sensitivity levels achievable, there are some drawbacks to the technology. A comparison of the techniques is reviewed below:

Accelerator Mass Spectrometry (AMS) Drawbacks:

1. Very hardware-intense technology – requires highly qualified staff to keep running
2. Preparation of radio-labeled compounds – time consuming and expensive
3. Not a routine technique, and it can only do a very limited number of analytes at once.
4. The turnaround time from sample preparation to acquisition to results is very slow.

All of these are not an issue with the QTRAP® 5500 System.

QTRAP® 5500 System Advantages

1. Bench-top equipment
2. Made for routine use
3. Easy-to-use
4. Labeling not required
5. High throughput
6. Increased QqQ and LIT sensitivity

Metabolic Stability Studies

The metabolic stability of a drug candidate impacts its bioavailability and half-life in vivo and should be evaluated early in the drug discovery and development process. In vitro methods using liver microsomes or hepatocytes provide valuable insights into metabolic stability and aid in the selection of drug candidates with favorable pharmacokinetic properties. Also important is the identity of metabolites present as the parent drug is metabolized. Being able to collect both qualitative (Metabolite ID) and quantitative (Metabolic Stability, PK, PD) data at the same time can improve the efficiency within the drug discovery and development process and get critical data, faster.

Figure 5 shows an example of monitoring for hundreds of predicted metabolites (using MRM transitions) and monitoring the metabolic stability of the parent drug, also via an MRM transition over a time course study. Here we achieve the

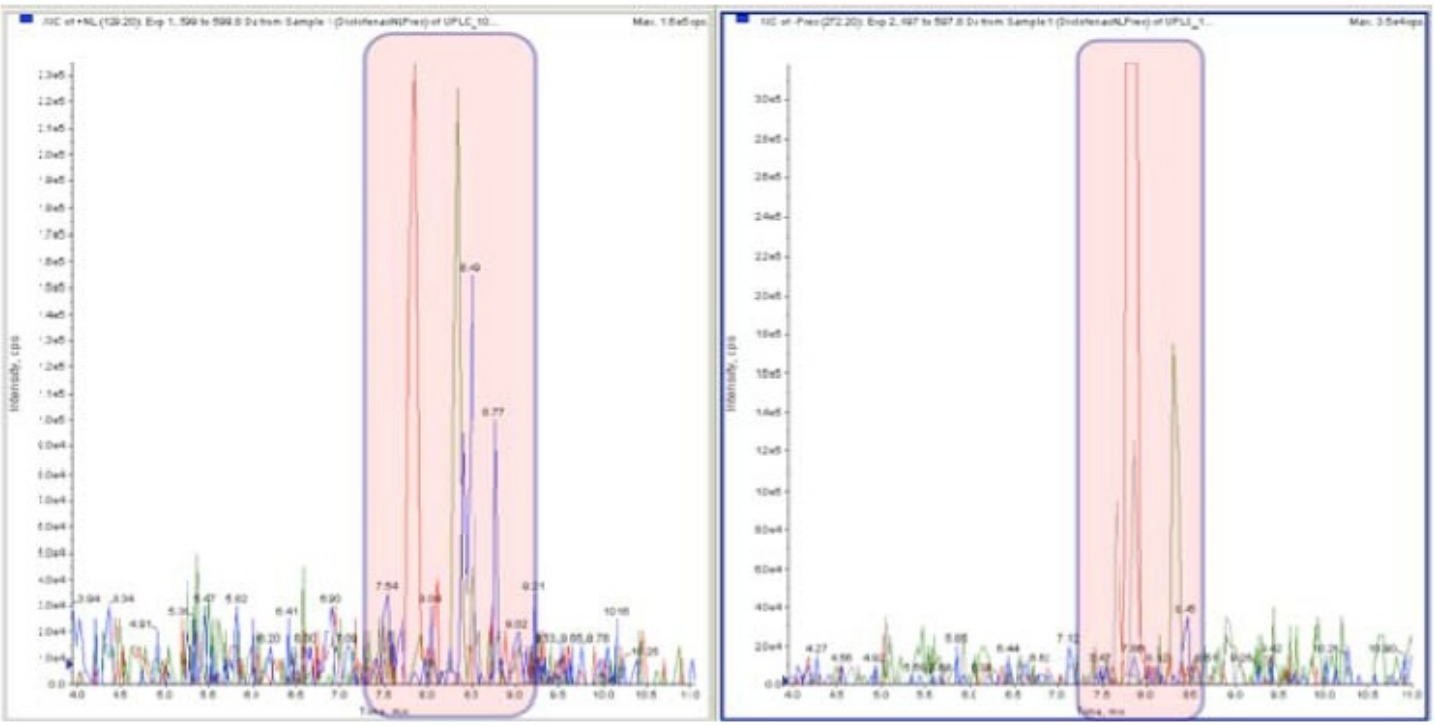

Figure 6. XIC trace of +/- NL/PI IDA Diclofenac GSH analysis, using a short high pressure gradient.

quantitative results required to determine the half life while at the same time monitor the presence of both major and minor metabolites with confirmatory MS/MS all in the same automated experiment.

GSH Conjugate Screening

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.

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 idiosyncratic 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.

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.

Here, 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. 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 v2.1 and ACD Fragmenter / Specmanager™ to find and assign major metabolites. Figure 6 shows the XIC trace for Precursor Ion 272 (-) & Neutral Loss 129 (+) IDA™, the major GSH metabolites are shaded in red. A more detailed discussion can be found in a technical note titled “GSH Conjugate Screening with the New QTRAP® 5500 LC/MS/MS System”

The QTRAP® 5500 System showed a significant improvement in detection and sensitivity of confirmation in a single injection with a high throughput chromatographic separation, 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.

Summary

The QTRAP® 5500 System provides unique workflow possibilities and enhanced speed, sensitivity and selectivity. The hybrid technology allows the ability to combine quadrupole scan functions (to help find potential metabolites) with high sensitivity linear ion trap scans (to identify and confirm metabolites). The pMRM approach allows one to screen for hundreds of potential metabolites and trigger the collection of full scan MS/MS to ID in a single analysis. Multiple precursor ion and/or neutral loss combinations, together with polarity switching, allows significantly better workflow strategies for reactive metabolite identification in a single analysis. With improvements in speed and sensitivity, we achieve much better peak definition at lower levels which leads to better identification, even at the highest of chromatographic throughput. Next generation metabolite in silico prediction via LightSight® software is a perfect complement to the instrument capabilities and allows even novice metabolite investigators to achieve complete results easily.

Highlights

- Improved quadrupole sensitivity (MRM response & S:N, PI & NL)
- Sensitivity improvement in TRAP modes (EPI, 10-100 fold)
- 4 times faster QqQ scanning
- Faster Polarity switching (50 ms)
- 5 times faster LIT scanning (20,000 amu/sec)
- Shorter fill time capabilities (50 µs)
- Unique workflows ideal for metabolite detection
  - pMRM
  - Multiple full scan survey possibilities (precursor ion, neutral loss), including polarity switching
  - Qualitative and Quantitative information together

References

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Publication number: P-1037610-01

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