Confirmation of In Vitro Nefazodone Metabolites using the Superior Fragmentation of the QTRAP® 5500 LC/MS/MS System

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Overview

Recently, nefazodone (anti-depressant) has been the target compound for metabolite studies due to the quantity and diversity of biotransformations than can occur in the CYP450 enzymes. Several scientific publications have evaluated different workflows and mass spectrometry platforms for this specific application.[1-3] Herein, we report an effective and sensitive method to identify Nefazodone metabolites using the new QTRAP® 5500 LC/MS/MS System. The system's significant performance improvement for Quadrupole and Linear Ion Trap sensitivity and scan speed, allow for highly effective detection and confirmation of in vitro and in vivo metabolites. The faster scan rates are a perfect match for small particle, high-pressure LC workflows for better separation and throughput. Using a pMRM approach and a rapid 10 minute chromatographic analysis, a significant number of metabolites were found and confirmed in a single injection (more than any other MS technologies used in previous literature references). This level of identification in a rapid chromatographic analysis demonstrates the abilities of the QTRAP® 5500 System for high throughput and a detailed metabolite analysis, with a depth of detection not possible on other MS technologies. Furthermore, the improved sensitivity in LIT mode allows for fine structural confirmation, since a greater number of diagnostic low level fragments are detectable compared to less sensitive accurate mass systems.

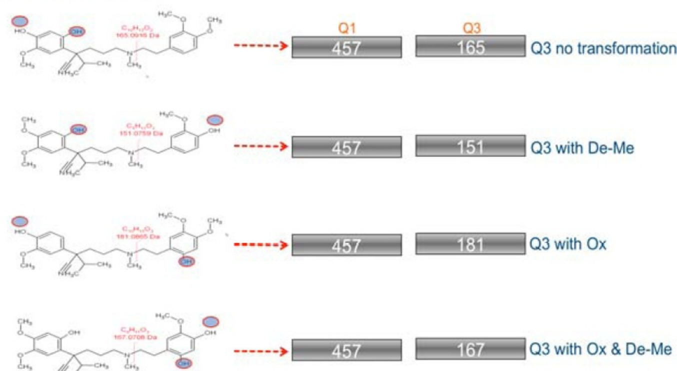

Figure 1. Example of pMRM transition prediction for a Oxidation and Demethylation transformation. In this case LightSight® software v2.1 creates four MRMs which will cover any fragment / transformation possibility. Since MRM has better duty cycle than any other MS method, sensitivity and selectivity are very important in this mode.

Introduction

Metabolite profiling plays a critical role in the discovery and developmental level of analysis for lead drug candidates. Many aspects of drug activity are affected by metabolism (pharmacokinetics, bioavailability, drug distribution, toxicity and adverse drug reactions). The enhanced scan rate (4X Quadrupole mode, 5X LIT) and sensitivity (8-10X response in Quadrupole mode and 10 to 100X in LIT mode) allow for a number of unique experiments with unprecedented sensitivity.

The most effective scan mode for first pass metabolic screening on the QTRAP 5500 system is pMRM (predictive MRM). Figure 1 shows an example of this method of MRM detection in which a comprehensive list of possible biotransformations along with parent drug fragment pattern are used to create a range of theoretically derived MRMs to cover all possibilities. The only major area in which pMRM might miss a metabolite is N-dealkylation and other exotic breaks in the drug core structure. In this case a second injection using a dual precursor and neutral

QTRAP® 5500 4000 QTRAP®

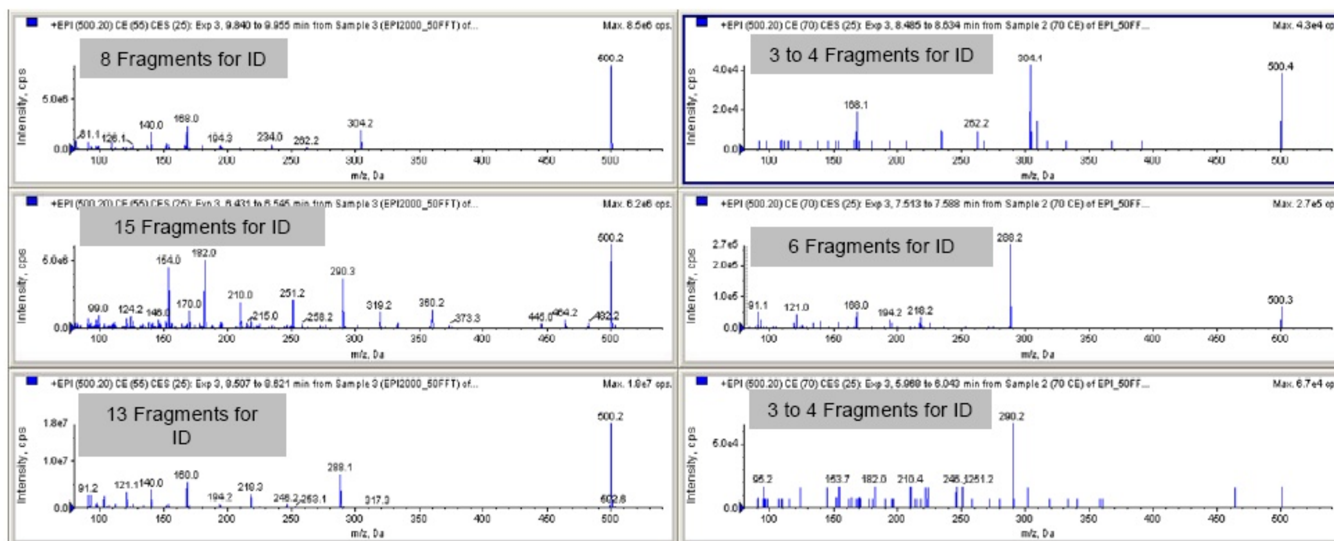

Figure 2. Three metabolites with corresponding LIT MS/MS spectra on the QTRAP® 5500 and 4000 QTRAP Systems. In each case a significant increase in interpretable fragments is exhibited on the QTRAP 5500 System.

loss survey based on the parent drugs fragmentation can be employed to pick up any missed metabolites. This second

injection would not be required if the molecule is unlikely to undergo an N-dealkylation transformation. The strength of the QTRAP® 5500 System is that a cycle of ~300 pMRMs or a dual precursor and neutral loss survey with 4 dependent Ion Trap MS/MS can be done approximately 4X faster than current 3200 and 4000 QTRAP® system experiments. For example, a dual scan experiment with both a precursor and neutral loss on a standard QTRAP system, with four EPIs running at the fastest scan rate possible on the 4000 QTRAP system could take ~7.5 seconds. Since this cycle rate is too slow for most chromatographic peaks, only a single survey and one or two EPIs could be done on an LCMS time frame. However, as the QTRAP 5500 System can scan 4 to 5X faster, this same cycle would be reduced to around ~2 seconds. A two second cycle rate will allow for 3 points over even a very sharp 6 seconds chromatographic peak. The combined precursor and neutral loss is also particularly effective since few metabolic changes would affect both the neutral and charged parts of the molecule, allowing for broad metabolite detection. In the case of many metabolic forms co-eluting, a greater number of dependent EPIs is possible based on the 5X faster LIT scan rate, which will also allow for more MS/MS spectra in a single injection. Thus the dual scan survey with four EPIs can allow for greater metabolite detection without compromising scan rate. (Dynamic Background Subtraction (DBS) is another way to ensure an efficient ion selection process is made during an automated experiment and doesn’t require more than one dependant EPI scan; significantly reducing the cycle time). This optimal mode is only possible on a QTRAP 5500 System. As with dual survey mode, pMRM also benefits by a 4X reduction in scan rate compared to the 4000 QTRAP System, in much the same way.

Another differential feature is the LIT MS/MS sensitivity. Compared to the 4000 QTRAP System, an improvement of 10 to 100X in intensity is apparent. This has two main benefits, the first being the detection of metabolites at an unprecedented low level. Often in in vivo PK work, MRMs track metabolites at very low levels. For the first time, the QTRAP 5500 System can now allow qualitative identification at much the same levels. The second benefit is the greater number of fragments for detailed structural identification on the QTRAP 5500 System. The presence of more fragments aids in greater refinement of interpretation. Figure 2 shows an example of the QTRAP 5500 and 4000 QTRAP System in terms of the number of interpretable fragments for the same metabolites.

To illustrate these points, a Nefazodone incubation was analyzed on the QTRAP 5500 System and two survey modes were used: 1) pMRM IDA and 2) dual scan precursor and neutral loss IDA. A number of highly refined MS/MS confirmations will be presented to demonstrate the level of refinement possible using the high sensitivity LIT MS/MS of the new QTRAP 5500 system.

Experimental Conditions

Incubations

Solutions

50 mM Phosphate Buffer (pH 7.4), 5.0 mM of NADPH in phosphate buffer solution, (A) 2.0 mg/mL of rat microsome in phosphate buffer, (B) 20 µM of nefazodone solution in 5.0 mM of NADPH solution (C) Acetronitrile solvent.

Control (t = 0 min)

- In a 1.8 mL Eppendorf tube the following solutions were added: 200 µL (B), 400 µL (C) and 200 µL (A)
- 1.0 min. vortex, left a few minutes in ice, centrifuged for 2.0 min at 10.000 rpm
- Final volume 800 µL
- Final substrate concentration 5.0 µM + 1.25 mM NADPH + 25 mM phosphate buffer

Incubation (t = 60 min)

- In a 1.8 mL Eppendorf tube the following solutions were added: 200 µL (B), 200 µL (A)
- Incubation for 60 min (37 °C – Eppendorf apparatus, 350 rpm agitation)
- Quench with 400 µL of (C)
- 1.0 min. vortex, left a few minutes in ice, centrifuged for 2.0 min at 10,000 rpm
- Final volume 800 µL* 20 µL of the control and 60 min incubation solutions were diluted with 200 µL 95% H_2O (5.0 mM ammonium formate)/5% ACN (v/v) solution and injected
- After dilution in mobile phase A, the final concentration of pre-incubations solution was 2.5 µM

Chromatography (LC)

- System: Shimadzu Prominence (Shimadzu Corp, Kyoto, Japan)
- Column: Stable Bond C18 (4.6 mm ID x 50 mm) 1.8 µm (Agilent, Palo Alto, CA)
- Mobile Phase: A) H_2O + 0.1 % Formic Acid and B) Acetonitrile + 0.1 % Formic Acid
- Column oven temperature: 45°C
- Flow Rate: 1.00 mL/min
- Injection Volume: 10 µL
- Gradient Runtime: 10.0 min (see table 1)

Step	Total Time(min)	Flow Rate(µl/min)	A (%)	B (%)
0	0	1000	95	5
1	1	1000	95	5
2	2	1000	70	30
3	10	1000	60	40
4	10.1	1000	5	95
5	10.5	1000	5	95
6	10.6	1000	95	5
7	15	1000	95	5

Table 1. HPLC gradient

Mass Spectrometry

The new QTRAP® 5500 System and LightSight® software v2.1 were used for all LCMS experiments. A single injection pMRM approach was employed, triggering the collection of confirmatory MS/MS in an automated experiment (IDA). A second injection using dual scan parent Precursor/Neutral Loss IDA was also used, although no major metabolites were missed in the first injection. In both cases the 20,000 amu/sec scan rate was used with four dependent EPI's (enhanced product ion scan). All methods were generated automatically using the LightSight® software v2.1 automatic method builder.

Results

A number of novel metabolites will be presented that show the identification abilities of the ultra high sensitivity LIT mode on the

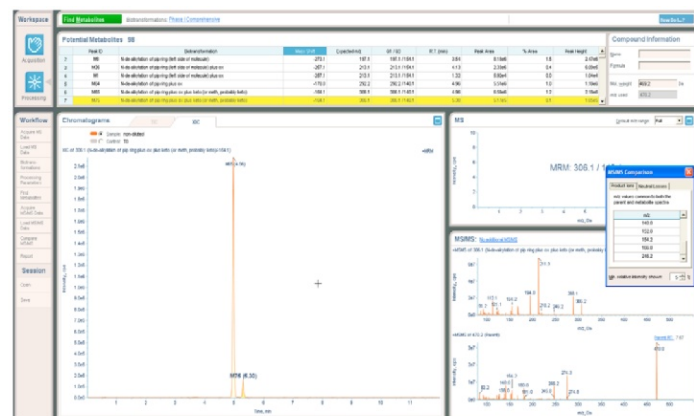

Figure 3. The results of a sample control comparison in LightSight software v2.1. In this example a low level N-dealkylation, oxidation and di-dehydrogenation is observed. The MS/MS comparison list in the middle right of the figure allows for quick comparison of metabolite vs. parent fragmentation. The QTRAP® 5500 Systems LIT MS/MS sensitivity is excellent for this 0.1% level metabolite based on the MRM TIC.

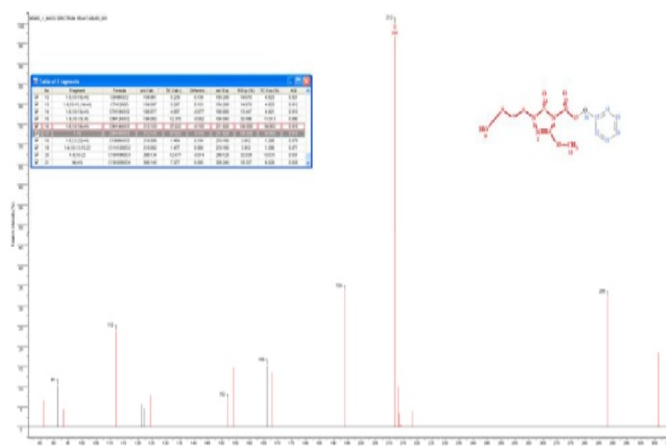

Figure 4. The fragment analysis of the same N-dealkylation, oxidation and di-dehydrogenation observed in LightSight software v2.1. ACD Fragmentor™ overlays structural fragment assignments on top of the MS spectra. The user can then scroll through the list deselecting any incorrect predictions.

QTRAP® 5500 System. Initial screening of the metabolites was accomplished by sample and control comparison using LightSight® software. Figure 3 shows the XIC trace, MS/MS spectrum and other relevant information in the LightSight® software interface for an N-dealkylation, oxidation and di-dehydrogenation metabolite. This metabolite represents 0.1% of the total TIC area and represents a good example of a low level species. Even for this trace level metabolite, 18 high quality fragments are apparent in the spectrum. LightSight software v2.1 allows for the direct import of the spectra into ACD Spectrum Manager and Fragmentor™, for MS/MS automated analysis. Figure 4 shows the fragment assignment accomplished using ACD Fragmentor™, in this case 80% of the fragments were assigned using the automated workflow. The metabolically created carbonyl group is likely to be on the 6, 7, 8 or 12 carbon atom.

One of the other interesting metabolites, an N-dealkylations plus oxidation and dehydrogenation is found at a retention time of 4.13 minutes. Figure 5 shows the XIC and MS/MS data found by LightSight software. Examination of the MS/MS spectra reveals 19 fragments of high quality. ACD Fragmentor was able to assign 70% of these to the predicted structure with minimal manual review (Figure 6). The metabolically created ketone group is likely to be at positions 21, 20, 11 and 10 on the backbone of the structure.

Two novel oxidative de-chlorinations were detected by the pMRM IDA. Figure 7 shows an example of the oxidative de-chlorinations found at 4.25 minutes in the LC run. The LIT MS/MS spectrum shows 14 diagnostic fragments at excellent sensitivity. Figures 8 and 9 contain the ACD Fragmentor assignments for this metabolite.

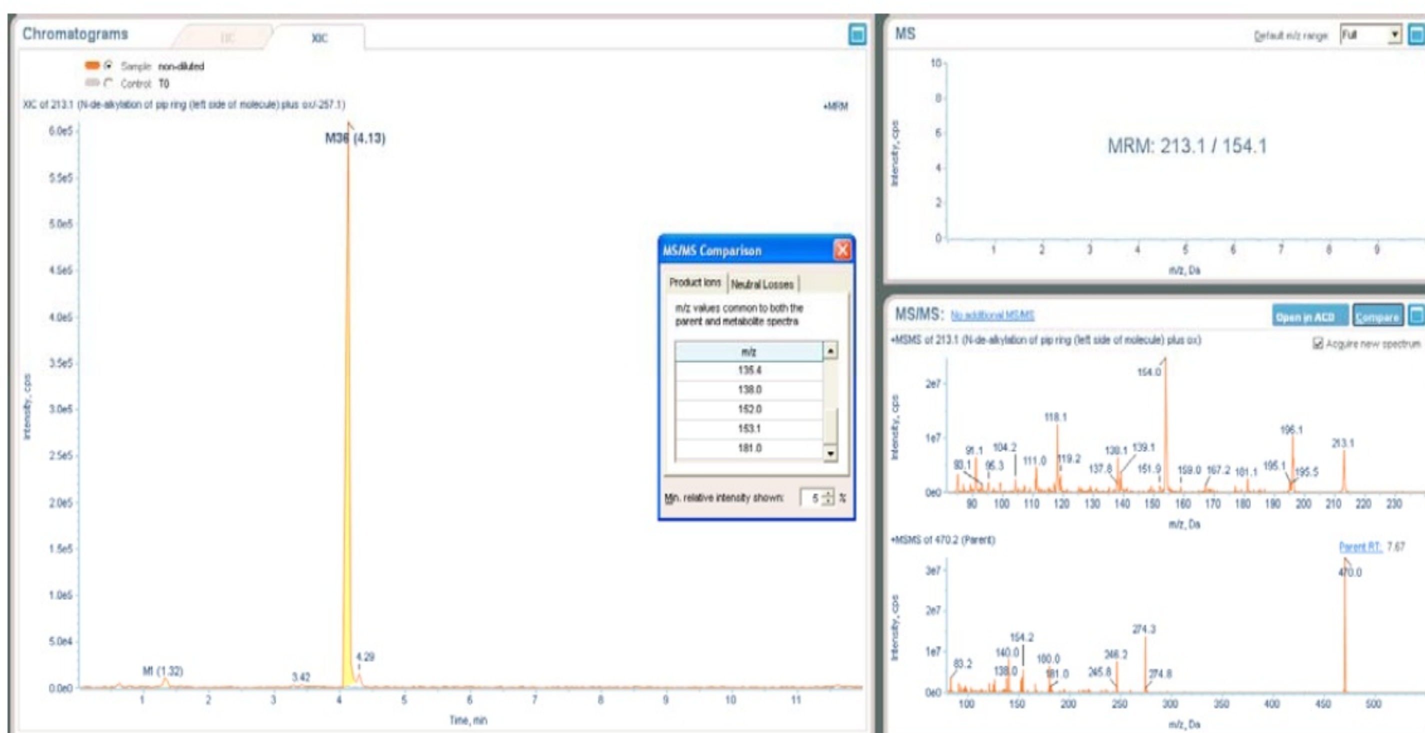

Figure 5. N-dealkylation of the chlorine containing ring on the left half of the molecule plus addition of an oxidation and dehydrogenation. The QTRAP® 5500 System LIT sensitivity provides superior fragmentation detail.

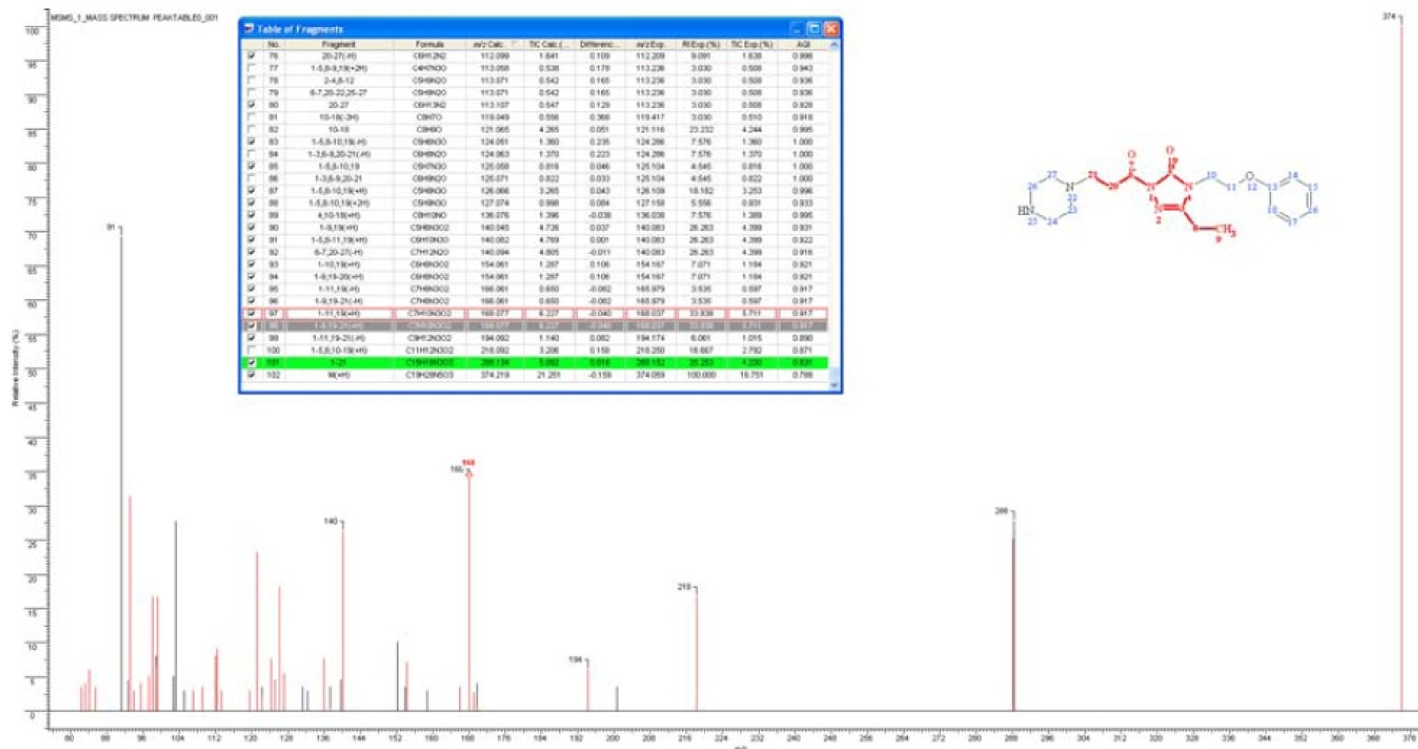

Figure 6. Fragmentation of an N-dealkylation of the chlorine containing ring on the left half of the molecule plus addition of an oxidation and dehydrogenation. ACD Fragmentor confirmed around 70 percent of the major fragments automatically. The ketone group matches well to positions 21, 20, 10 and 11 on the aliphatic backbone of the molecule.

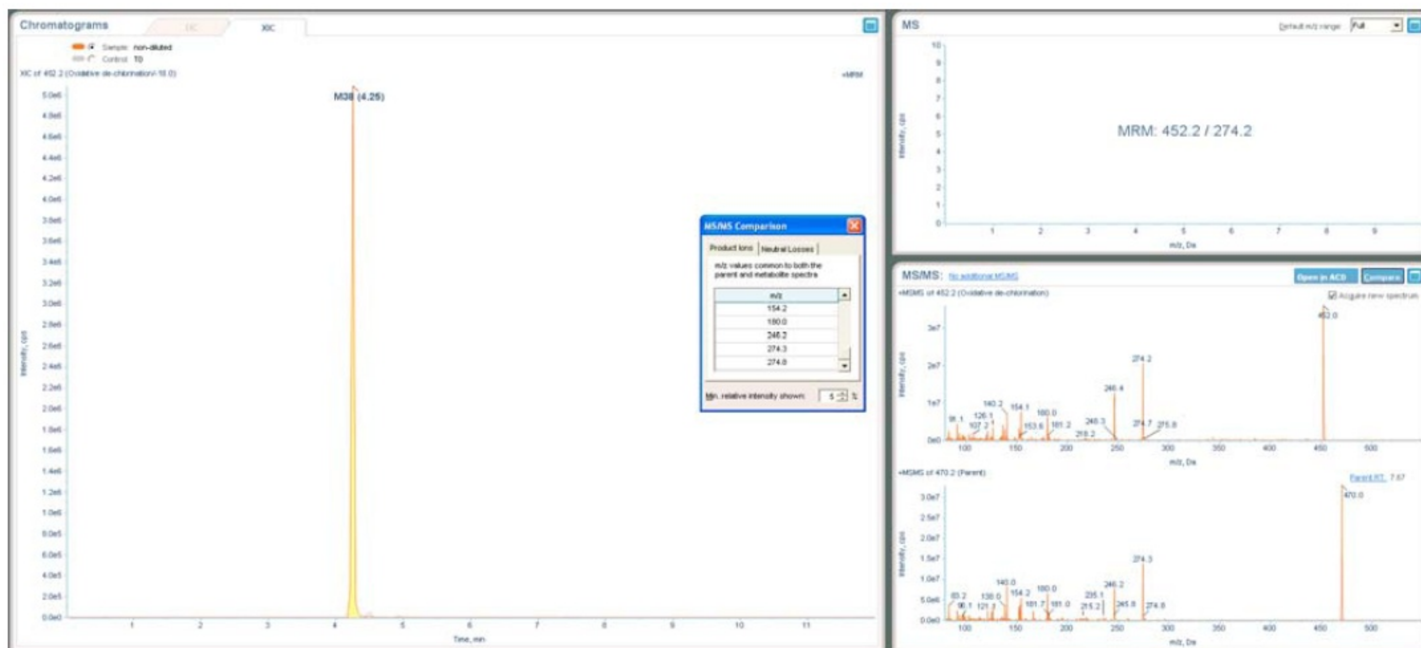

Figure 7. LightSight® software v2.1 XIC and MS/MS of oxidative de-chlorination.

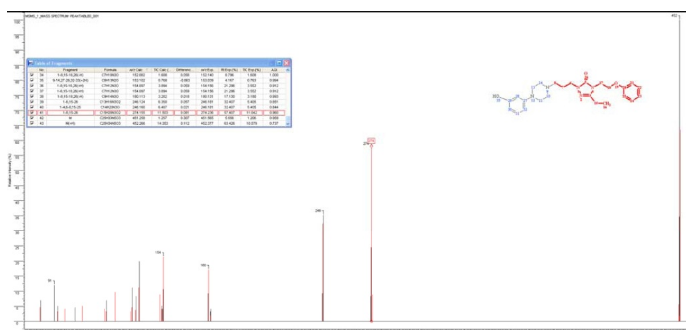

Figure 8. ACD Fragmenter assigns the m/z 274 fragment to the right half the molecule. This is consistent with oxidative de-chlorination taking place on the left ring. Position 29, 30 or 31 are most likely sites for the oxidation.

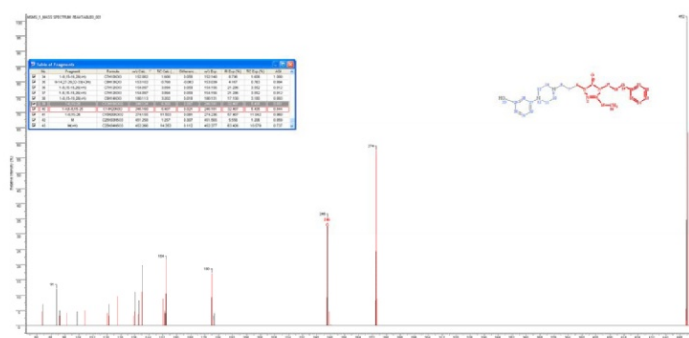

Figure 9. ACD Fragmenter assigns the m/z 246 fragment to the right half the molecule. This is consistent with oxidative de-chlorination taking place on the left ring. Position 29, 30 or 31 are most likely sites for the oxidation.

Conclusions

- The QTRAP® 5500 System has excellent sensitivity for both detection and confirmation of metabolites
- The pMRM as well as dual scan Precursor and Neutral Loss IDA methods provide comprehensive detection of in vitro metabolites in a single injection. In most cases, a single injection using pMRM alone is more then adequate for a complete analysis.
- The 10 to 100X LIT MS/MS sensitivity improvement allows for superior fragmentation not possible on less sensitive Trap or TOF instruments. This greater degree of fragmentation allows for a high level of structural assignment.
- The improved scan rate 4 or 5X over previous QTRAP® Systems allows for greater IDA coverage, even with sharp peaks delivered by the most progressive small particle high-pressure chromatography methods.

References

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