A HTS MEHTODOLOGY FOR REACTIVE METABOLITE DETECTION AND IDENTIFICATION USING AN LC/MS/MS DATA INDEPENDENT STRATETGY

Waters
THE SCIENCE OF WHAT'S POSSIBLE.™

Authors: Jan Claereboudt[1], Mike Van Oosterhout[2], Jose Castro-Perez[3], John Shockcor[3], Kate Yu[3]
Affiliations: [1]Waters Belgium, [2]Waters Chromatography, The Netherlands, [3]Waters Corp, Milford, USA

INTRODUCTION

The identification of metabolites, whether for in vitro or in vivo samples, is an ongoing challenge for drug discovery and development. Metabolite identification typically uses an array of chromatographic and mass spectrometric methods, and therefore may require multiple injections of the same sample. This is to ensure that enough information has been collected to detect all metabolites and have fragmentation patterns available to elucidate structures. We describe a new 'workflow' that enables the collection of both parent and fragment information from a single injection (Figure 1). Data acquisition used two scan functions interleaved such that the first scan function collected information about the intact metabolites and the second scan function collected fragment ions. With this approach, the entire data set is then mined post-acquisition for specific metabolite masses, precursor and product ions, and neutral losses as all the necessary data is collected simultaneously. Selectivity for drug–related material is accomplished through exact mass measurement. A variety of data processing algorithms can be used to extract metabolite information from these data. The data is acquired using high resolution (10K FWHM) and with mass accuracies typically in the sub 3 ppm range. The advantage of this approach is that, since all the data is collected in one run, post-acquisition processing for multiple product ions is possible. Neutral loss chromatograms may also be generated from the data using exact mass differences between the precursor and fragment ions. Since the data are acquired with no preconceptions on the likely routes of metabolism, this approach has the potential to be truly comprehensive and universal in its use for in vitro reactive metabolism screening. From a single injection it is possible to obtain neutral loss information and precursor ion information with exact mass containing diagnostic losses for reactive metabolites for both neutral and precursor ions acquisitions. In turn, these diagnostic neutral losses and precursor ions may be used in conjunction with the low energy data to confirm the presence of a reactive intermediate. We illustrate this approach by using an incubation of human liver microsomes in the presence of GSH for Nefazodone.

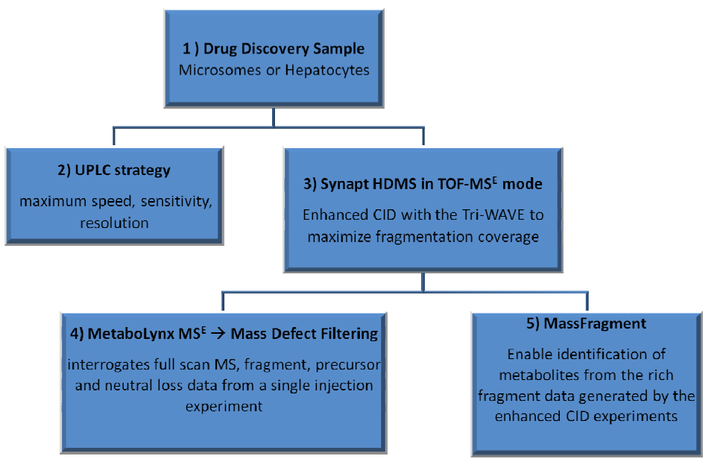


Figure 1. Workflow showing the strategy for reactive metabolite screening

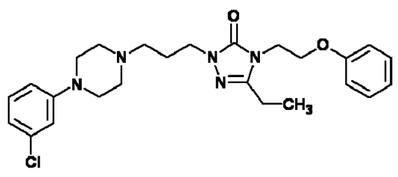
METHODS

Samples

Nefazodone was incubated with human liver microsomes at 10 µM at 37°C, in a solution of 50 mM potassium phosphate adjusted to pH 7.4 containing the appropriate co-factors. GSH was added at a concentration of 10mM to the microsomal incubation. The reaction was terminated after 90 minute with 2 volumes of cold acetonitrile to 1 volume of sample. Then, the sample was centrifuged at 13,000 rpm for 15 minutes and the supernatant was diluted 1/2 with Water +0.1 % formic acid Finally, the samples were centrifuged and the supernatant was injected directly to the UPLC-TOF-MS system for analysis.

Background on Nefazodone

➢ Nefazodone is an antidepressant which was approved in the USA in late 1994



Nefazodone

➢ In spite of its therapeutic effects there has been a number of cases (55 causes of liver failure (20 fatal) and another 39 cases of less severe liver failure) reported showing hepatobiliary dysfunction and cholestasis (ref: *Kalkutkar et al DMD 33:243-253, 2005*)

LC-MS Methodology

LC-conditions:
LC System : Waters Acquity UPLC™
Column: Acquity BEH C18 Column 100x2.1mm id, 1.7µm
Column Temperature: 45 °C
Mobile phase A: 0.1 % formic acid; B: Acetonitrile.
Flow rate: 0.6 mL/min
Gradient: 98%A – 40% A in 8 mins, ramp to 40-0%A in 1.5 min before returning to 100% A for re-equilibration
Injection volume: 5 µL

MS-conditions:
Mass Spectrometer: Synapt HDMS™
MS scan range: 50-900 Da
Mode of Operation: + ion and –ion mode ESI
Lock Mass: Leucine Enkephalin at 200pg/mL

MS^E Methodology:
The Synapt HDMS™ was operated in a parallel data acquisition mode with a wide band RF mode in Q1(Figure 2). Thus, allowing all ions enter into the collision cell without pre-filtering. This resulted in one single injection in which data was collected under one single data file with two functions. Function (1) Low energy acquisition (5eV) which contained the intact compounds and Function (2) High energy or MS^E acquisition (20eV-50eV ramp) which contained all of the fragmented ions

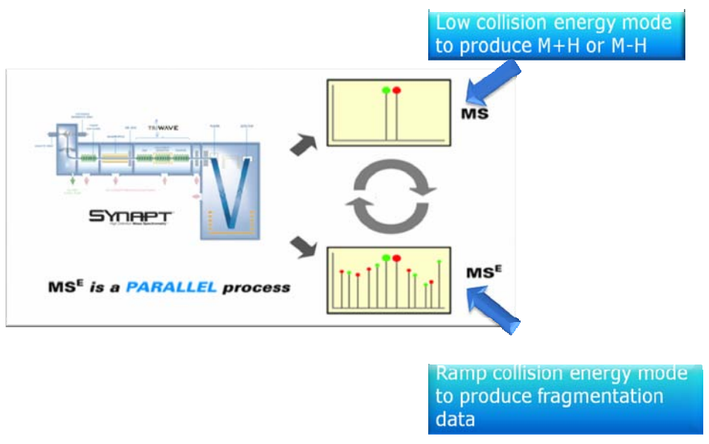


Figure 2. Schematic showing the data independent methodology for reactive metabolite screening

The use of the TriWave device was beneficial as it was possible to conduct fragmentation in two regions (Trap and Transfer) in a parallel fashion (Figure 3). This enhances the fragmentation coverage throughout the mass range acquired.

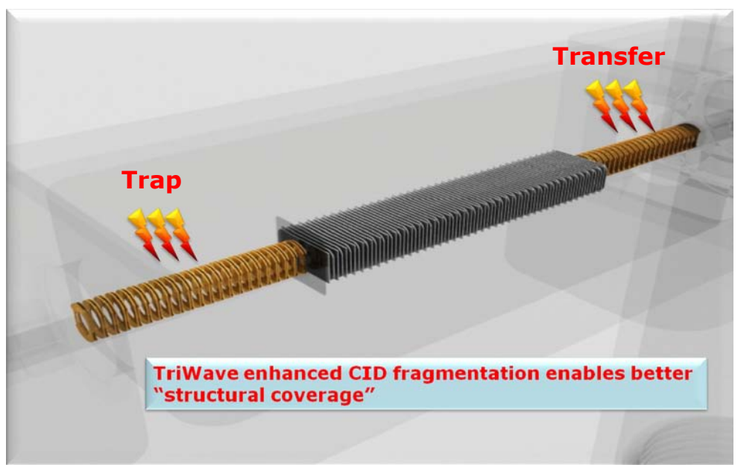


Figure 2. Schematic showing the TriWave fragmentation using the Trap and Transfer region

Processing software
➢ MetaboLynx was used for the MS^E data mining and peak detection of putative metabolites
➢ MassFragment was used for the structure elucidation step

GSH reactive metabolite screening methods by LC-MS/MS

➢ GSH 'Trapping' [1,2]
- Typical in-vitro incubation in microsomes forcing the reaction to form GSH 'adducts'

➢ *For positive ion mode;*
- Monitor the loss of the pyroglutamic acid moiety m/z 129 (Figure 4)
- Monitor the loss of GSH m/z 307 for aliphatic and benzylic thioethers
- Monitor the loss of glutamic acid m/z 147 for thioesters

➢ *For negative ion mode*
- Monitor the precursor ion at m/z 272 arising from the γ glutamyl-dehydroanalyl-glycine. Other GSH fragments may also be used

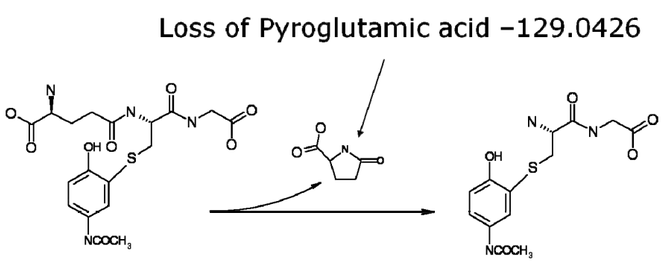


Figure 3. Neutral loss monitoring with the loss of the pyroglutamic acid moiety

RESULTS

- The data obtained by this approach was processed with MetaboLynx and 3 GSH adducts were detected m/z 791 (+O+GSH) , 805 (+O2-H2 +GSH) and 807 (+O2+GSH)
- The 3 GSH adducts were confirmed by in the low energy scan using exact mass and the high energy scan by monitoring the loss of the pyroglutamic acid m/z 129.0426 (Figure 4)

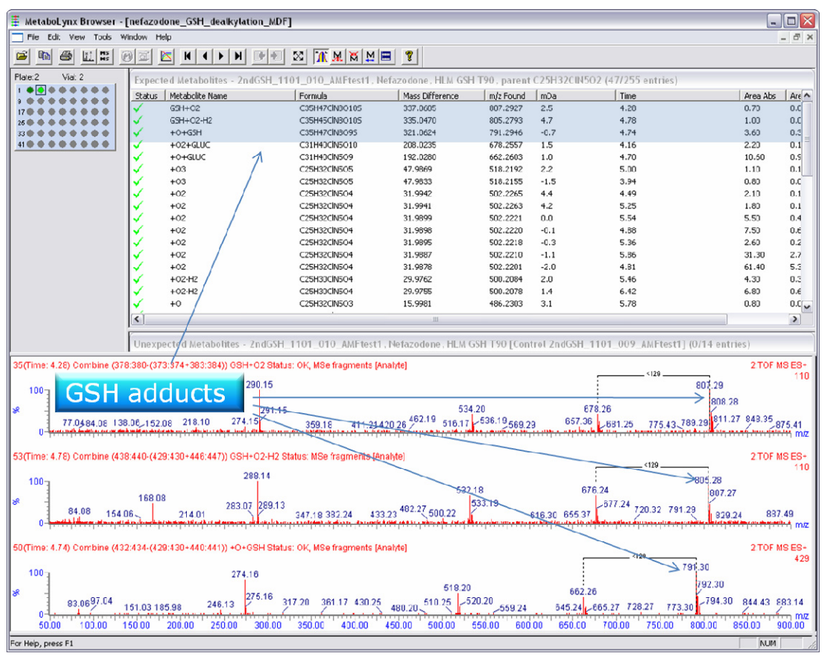


Figure 4. MetaboLynx browser report showing all 3 GSH adducts as well as other biotransformations

- Negative ion MS^E was also carried out and the GSH adducts were confirmed by extracting the precursor ion of m/z 272 (Figure 5). In this example we are showing the fragment data derived from the precursor ion of m/z 272 and then the structure assignment confirmed with exact mass and MassFragment for the GSH adduct at m/z 789 (+O +GSH)

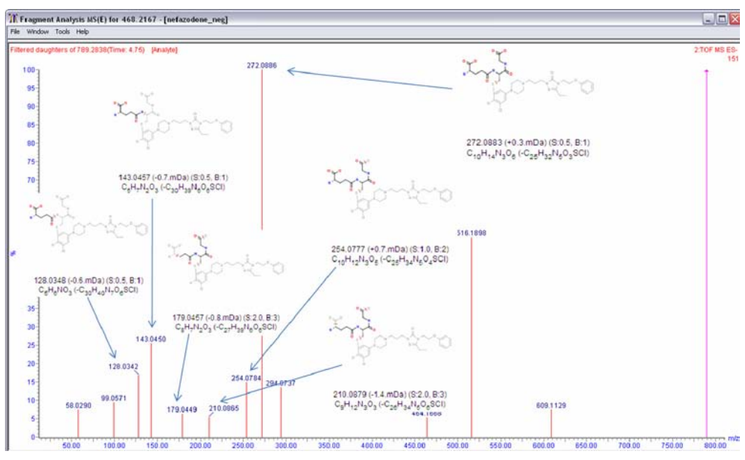


Figure 5. Negative ion fragment information from m/z 789 corresponding to (+O+GSH) adduct of Nefazodone

- Not only GSH information was available with this approach but also other metabolite data
- From this strategy we were able to extract the low and high energy information for all metabolites and utilize the exact mass information from the low energy to remove false positives and confirm metabolites expected and unexpected
- The high energy information was used to obtain fragment ion and precursor ion information for structure elucidation (Figure 6 and 7)

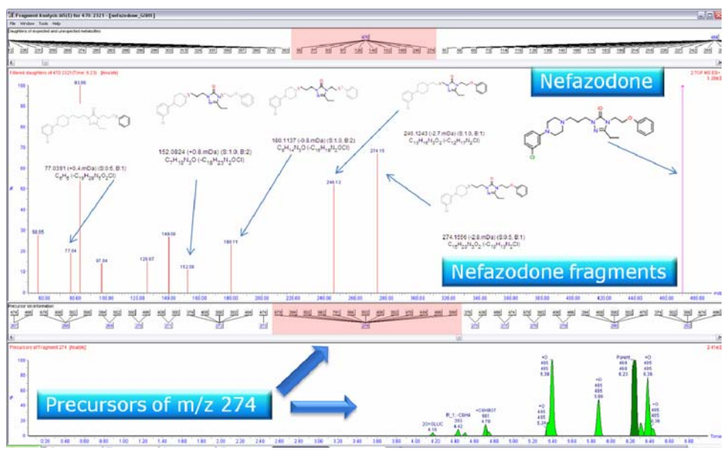


Figure 6. MetaboLynx fragment and precursor ion information for Nefazodone

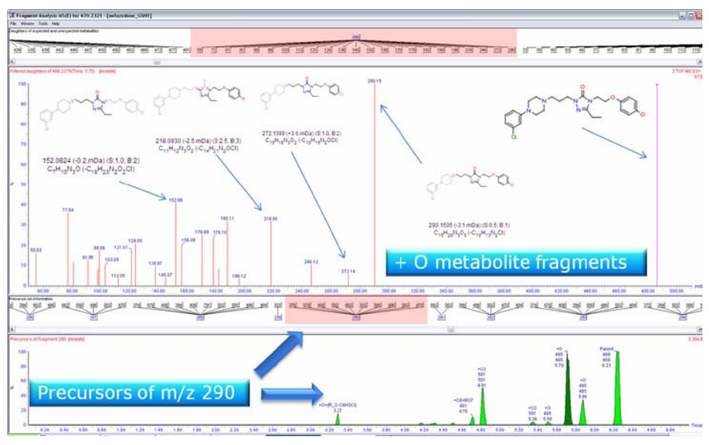


Figure 7. MetaboLynx fragment and precursor ion information for +O metabolite of Nefazodone

CONCLUSION

- Demonstrated a holistic approach to metabolite id and specific search for GSH conjugations
- We can carry out as many NL or precursor ion experiments as required, we have access to 'all the data all the time'
- One single injection provides information equivalent of multiple injections when compared to data dependant experiments and tandem quadrupoles or ion traps
- Software tools and exact mass are very important to data mining and very helpful to rationalize the putative metabolites

References

1. Baillie & Davis Biol Mass Spectrom 1993, 22(6),319-25
2.Dieckhaus et al. Chem Res Toxicol 2005, 18(4), 630-8

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS

720002471EN