INTRODUCTION
Finding and identifying metabolites in cold in vivo studies by LC-MS is extremely challenging, as metabolites are often present at such low levels that chromatographically they are indistinguishable from the background endogenous materials. To address this problem, two scans with different collision energies were performed, which are referred to as the high energy or MSE acquisition (25eV-40eV ramp) and the low energy or low energy acquisition (5eV), respectively. The high energy scan function combined all of the fragmented ions (25eV - 40eV) collected information about the intact (5 eV) metabolites and the second scan function used a collision energy ramp to collect fragment ions (25eV-40eV). This approach provided a great deal of information, such as metabolite masses, precursor, product ions, and neutral losses. It allows for a true comprehensive and universal in its use for metabolite identification.

METHODS

Sample Preparation
Buspirone was incubated with rat liver microsomes at 100 μM at 37° C, in a solution of 50 mM potassium phosphate adjusted to pH 7.4 containing the appropriate cofactors. The reaction was initiated by the addition of the microsomes and was allowed to equilibrate for 15 minutes at 37° C. Samples were injected directly to the UPLC-TOF-MS system for analysis.

LC-MS Methodology

Mass Spectrometer: Q-Tof Premier™
Mode of Operation: + ion mode ESI V-mode, QDIE (dynamic range enhancement)
Lock Mass: Leucine fragnaphtin at 200μg/ml

MSE Methodology:
The Q-Tof Premier was operated in a parallel data acquisition mode with a wide band RF mode in Q1 (Figure 2). Thus, allowing all ions in the collision cell. This resulted in one single injection in which data was collected under one single data file with two functions. These were:
• Function 1: Low energy acquisition on (5eV) which contained the unfragmented compounds
• Function 2: High energy or MS2 acquisition (25eV-40eV ramp) which contained all of the fragmented ions

LC-conditions:
Acquity UPLC™
Acquity BEH C18 Column 100μm 2.1mm id, 1.7μm
Mobile phase A: 0.1 % formic acid
Mobile phase B: Acetonitrile
Flow rate: 0.5 mL/min
Gradient: 0 min 99% A, 0.4-7 min 30%A, 4.7-5 min 10% A, 5.1-8 min 98% A
Injection volume: 5 μL

RESULTS

Description of software algorithm for data processing - Metabolynx M$^2$ - How does it work?
Metabolynx is a software application manager, which automatically detects putative metabolites and presents results in a data base format. It operates by comparing and contrasting each metabolised sample with a control sample—although unexpected metabolite searching may still be performed in the absence of a suitable control. Samples from in vitro incubations or in vivo dosing experiments can be quickly analysed by LC/MS, followed by a multi-dimensional search which correlates retention time, m/z value, intensity and components from alternative detection technologies (e.g. diode array UV or radiochemical monitoring). Comparison of analyte data with the control sample allows filtering of matrix-related peaks, which would otherwise produce an unmanageable list of false metabolite peaks.

M$^2$ data may also be filtered using exact mass information (Figure 6). In this case the mass of each metabolite is recorded and then filtered accordingly with a moving mass filter set up by the user which is applicable to the mass range of interest.

• M$^2$ data can be filtered using exact mass (Figure 5).
• M$^2$ data may also be filtered using exact mass (Figure 6).

• From this analytical strategy, fragment ion, precursor ion and neutral data was generated in the M$^2$ function

• The approach we were able to determine all the metabolites expected and unexpected from the low energy acquisition.

• Moreover, we were able to obtain important common product and precursor ion information from the high energy scan which allowed spectral correlation between drug and its metabolites. Neutral loss chromatograms were also generated from the data using exact mass differences between the precursor and fragment ions. Since the data was acquired with no preconceptions on the likely routes of metabolism (Figure 1).

• The QTof Premier was operated in a parallel data acquisition mode with a wide band RF mode in Q1 (Figure 2). Thus, allowing all ions in the collision cell. This resulted in one single injection in which data was collected under one single data file with two functions. These were:
• Function 1: Low energy acquisition on (5eV) which contained the unfragmented compounds
• Function 2: High energy or MS2 acquisition (25eV-40eV ramp) which contained all of the fragmented ions

• By this approach we were able to acquire vast amounts of information during the time scale of UPLC experiments.

• Exact mass data filtering provided us with a very powerful strategy to data mine the samples with great accuracy.

• The use of exact mass data filtering also allowed us to remove false positives in a much faster manner without the need of re-injection.

• Methodology easy to set-up without any prior knowledge of metabolites

• Generation of fragment, precursor and neutral loss information from a single injection

• This approach provides a quick and accurate 'snapshot' for fragment ion information

• It will also help to decide what further MS/MS experiments to carry out

DISCUSSION

CONCLUSION

References

Figure 6. Comparison of M$^2$ spectra for OH-Buspirone incubated sample spiked into rat bile without and with mass defect filtering.

Figure 7. M$^2$ fragment analysis for the metabolites of Buspirone spiked in rat bile

Figure 3. M$^2$ data acquisition and processing schematics with Metabolynx

Figure 4. Example for the use of mass defect filtering and Metabolynx M$^2$ - How does it work?

• The use of exact mass data filtering also allowed us to remove false positives in a much faster manner without the need of re-injection

• Methodology easy to set-up without any prior knowledge of metabolites

• Generation of fragment, precursor and neutral loss information from a single injection

• This approach provides a quick and accurate "snapshot" for fragment ion information

• It will also help to decide what further MS/MS experiments to carry out

• M$^2$ data may also be filtered using exact mass (Figure 6).

• In this case the mass of each metabolite is recorded and then filtered accordingly with a moving mass filter set up by the user which is applicable to the mass range of interest

• From this analytical strategy, fragment ion, precursor ion and neutral data was generated in the M$^2$ function

• Here an example is shown where precursor ion information (Figure 7) is used for the confirmation of metabolites found corresponding to the hydroxylated metabolites m/z 420 and giving rise to a common precursor ion with the parent drug at m/z 222