A HTS APPROACH FOR CROSS SPECIES COMPARISON USING UPLC-TOF-MS

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INTRODUCTION

Within the pharmaceutical and biotech industry LC/MS is a very well established technique for drug metabolism studies due to its inherent specificity and sensitivity. Recent development in UPLC has significantly improved the assay throughput without sacrificing sensitivity and sensitivity. A major bottleneck for the LC/MS drug metabolism study is the data processing. In addition, to carry out multiple experiments in parallel and to have their results processed all at once has been an extremely difficult task.

In this poster, we will present a specific and sensitive high throughput assay strategy for cross species metabolism study. By using the UPLC-TOF MS technique with the exact mass measurement. We identified and correlated drug metabolites in three different species (rat, dog, and human) with a short assay turnaround time. The metabolic stability of the parent drug and the metabolites formed with relative concentrations were monitored from a single UPLC injection. Data collection was obtained by full scan MS with the exact mass measurement. An in-house software algorithm was used to process and report the UPLC-MS data. False positive was easily eliminated by using the exact mass filter.

METHODS

Samples

The drug substrate used for this study was buspirone (MW 385). The parent drug was incubated using freshly prepared microsomes at a protein content of 1 mg/mL. Separate incubations were carried out for rat, dog and human microsomes. For each species, samples from four time points were obtained: 0, 15, 30, and 90 minutes. The enzyme reactions were terminated with ice-cold acetonitrile. Samples were centrifigued and their supernatants were kept at -20 °C. Samples were diluted with water/acetonitrile (80/20) in one to four ratio prior to the UPLC-TOF MS analysis.

LC-MS Methodology

Mass Spectrometer: Q-Tof Premier™
MS scan range: 50-800 Da
Mode of Operation: +/-ve ion mode ESI
V-mode, pDRE (dynamic range enhancement)
Lock Mass: Leucine Enkephalin at 200pg/mL

LC-conditions:
Aquity UPLC™
Acquity BEH C18 Column 50x2.1mm id, 1.7µm
Mobile phase A: 0.1 % formic acid
Mobile phase B: acetonitrile
Flow rate: 0.75 mL/min
Gradient: 0-0.25 min 100% A, 0.25-3.25 min 5% A, 5.5 min 100% A
Injection volume: 5 µL

Analysis Strategy

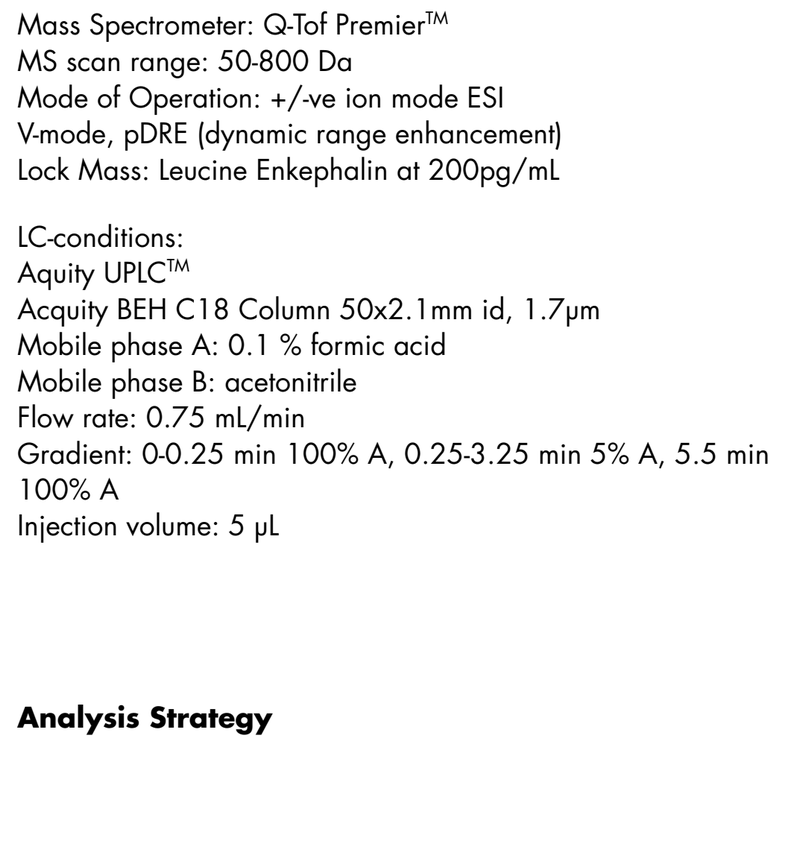

Figure 1.Analytical flow for HTS in cross species comparison in Drug Discovery

RESULTS

UPLC™ Separation

To study the major Buspirone metabolites produced by the CYP3A4 isoform [1], we obtained the extracted ion chromatograms of the single hydroxylated metabolite of Buspirone (m/z 402) in Figure 2 and the double hydroxylated metabolite of Buspirone (m/z 418) in Figure 3.

As shown on these two figures, a complete UPLC separation was obtained in less than 2 minutes. In addition, the levels of the single and double hydroxylated metabolites were different amongst different biological species.

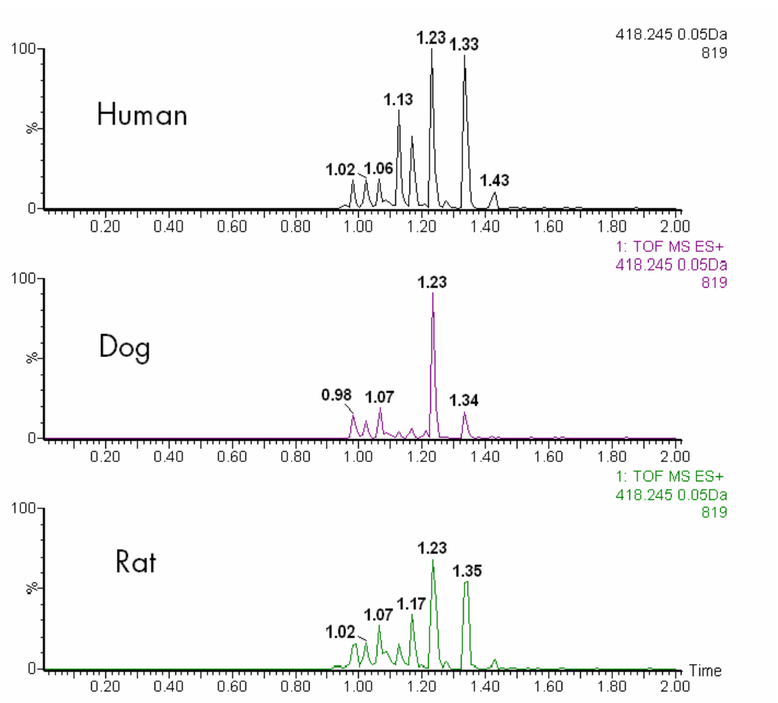

Figure 2.Rapid UPLC-QTof detection for the double hydroxylated metabolites of Buspirone in different biological species

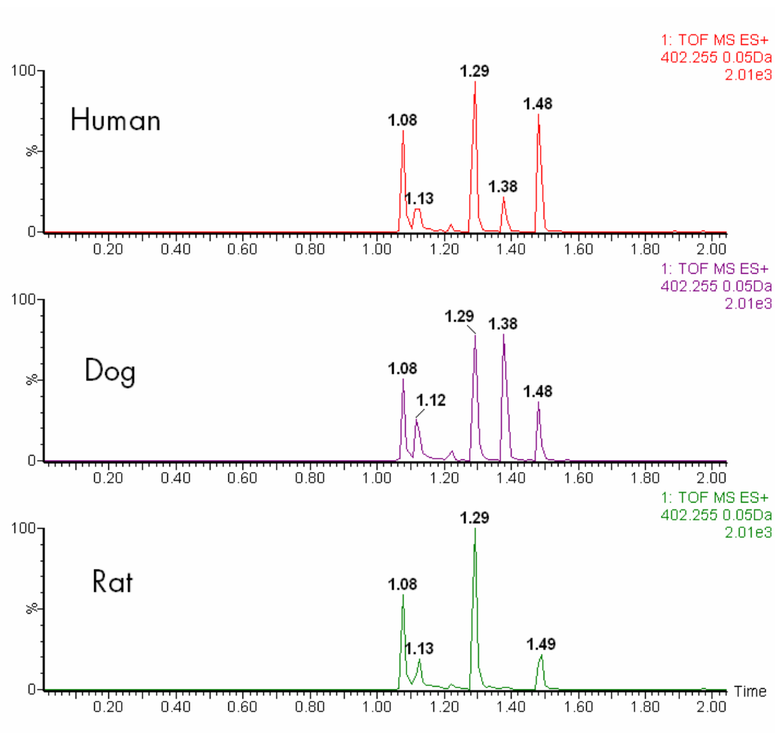

Figure 3.Rapid UPLC-QTof detection for the hydroxylated metabolites of Buspirone in different biological species

Exact Mass Calculations and i-FIT™

Exact mass proved to be a very important tool to confirm all the detected components in the samples. For this study, we used an elemental composition calculator with MetaboLynx. Another parameter, i-FIT™ works on the basis of exact mass and matches the elemental composition suggested by the isotopic pattern from the metabolite of interest (Figure 4). In thi way, confirmation from the exact mass-elemental composition and the isotopic pattern matching algorithms is achieved. Typically, the lower the i-FIT™ value, the most likely that it is the correct answer. The iFIT™ is used together with the exact mass measurement to help eliminate the false positives.

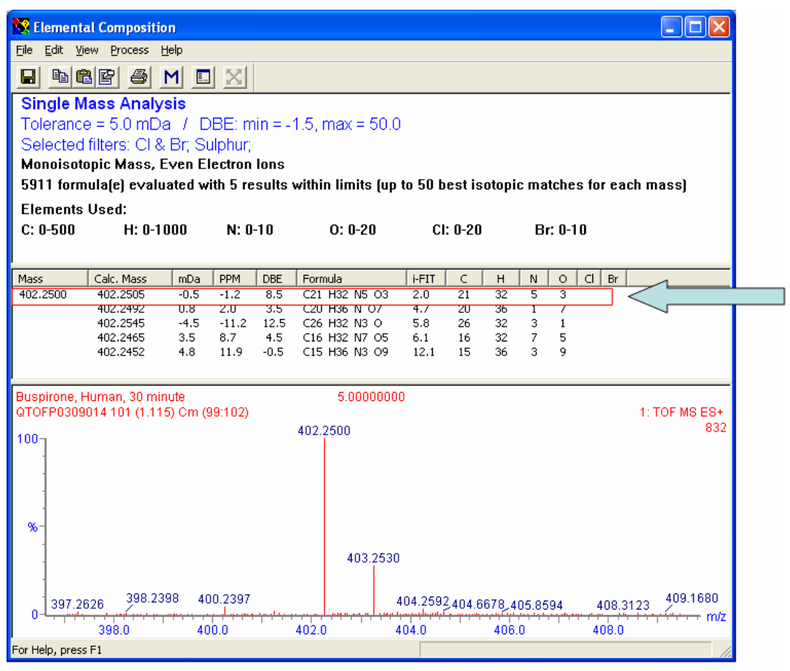

Figure 4.Elemental composition report using i-FIT™ for one of the hydroxylated metabolites of Buspirone

MetaboLynx Data Processing and Exact Mass Filters

The use of an automated software algorithm (MetaboLynx Figure 5) enabled us to data mine the samples in a rapid and semi automated manner.

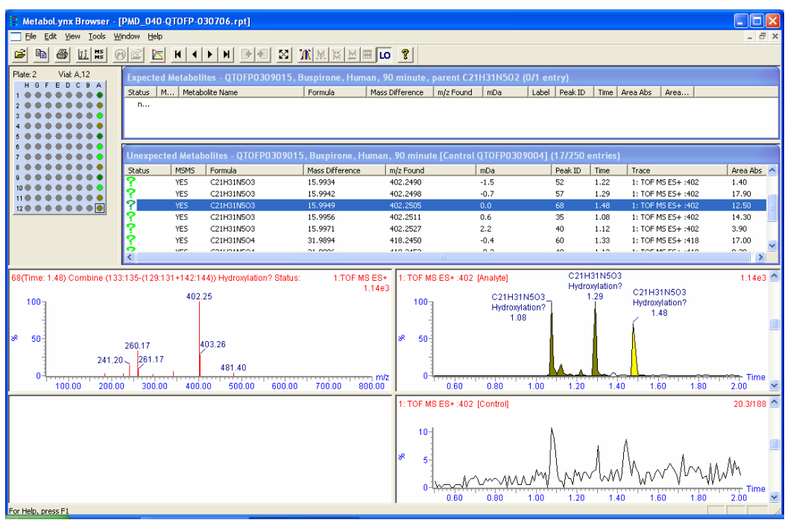

Figure 5. MetaboLynx Browser for Buspirone metabolites in all samples from Human, Dog and Rat microsomes

Exact mass is a crucial part in drug metabolism studies. As shown in Figure 5, there are total of 250 entries for the unexpected metabolites from a single sample injection. To go through each entry manually would be extremely time consuming. The speed for this process can be significantly improved by using an exact mass data filter along with the other filters (retention time and peak area thresholds). To avoid method re-optimization and lowering the risk of loosing important metabolite information, one can set a very low threshold during the MetaboLynx data processing, then apply the filters from the browser page accordingly to remove the false positives (Figure 6). In this example, after applying the filters only '17 true putative metabolites remained'.

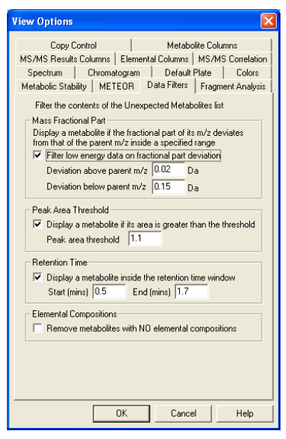

Figure 6. Exact mass data filter used to remove false positives

The cross species correlation for the single and double hydroxylated Buspirone metabolites is demonstrated on the table below. The methodology in this paper allowed for a very fast turnaround from the sample submission to the reporting of the results.

Rat Microsome Incubation			Dog Microsome Incubation		Human Microsome Incubation	
Metabolite Name	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area	Retention Time (min)	Peak Area
Hydroxylation (1)	1.08	10.9	1.08	12.3	1.08	14.1
Hydroxylation (2)	1.13	5.8	1.12	11.7	1.12	3.8
Hydroxylation (3)	1.22	0.4	1.22	2.7	1.22	1.4
Hydroxylation (4)	1.29	16.4	1.29	18.8	1.29	16.7
Hydroxylation (5)	Not Found	Not Found	1.38	16.5	1.38	0.4
Hydroxylation (6)	Not Found	Not Found	1.48	7.7	1.48	12.5
Dihydroxylation (1)	0.99	6.1	0.98	5.8	0.98	4.1
Dihydroxylation (2)	1.07	5.2	1.07	4.4	1.06	5
Dihydroxylation (3)	1.13	2.2	1.13	1.2	1.12	9.4
Dihydroxylation (4)	1.17	6.5	1.17	3.3	1.17	8.8
Dihydroxylation (5)	1.23	13.4	1.23	9.8	1.23	9.8
Dihydroxylation (6)	1.34	2.3	1.34	4	1.33	17
Dihydroxylation (7)	Not Found	Not Found	1.43	0.5	1.43	2

Table 1.Cross-correlation between all the major metabolites of Buspirone in all different species analyzed

CONCLUSION

- **UPLC™ coupled with QTof technology provided a very fast sample turnaround without compromising data quality**
- **Exact mass data acquisition and data processing with i-FIT™ provided unequivocal results for metabolite identification**
- **MetaboLynx enabled a fast turnaround in the interrogation of the data and also provided rapid removal of false positives**
- **With this analytical approach it provided us with information about metabolites formed, cross species comparison and also metabolic stability within one single injection**

References

1. Mingshe Zhu, Weiping Zhao, Humberto Jimenez, Donglu Zhang, Suresh Yeola, Renke Dai, Nimish Vachharanjani, James Mitroka. Cytochrome P450 3A-mediated metabolism of buspirone in human liver moicrosomes. Drug Metabolism and Disposition 33:500-507,2005.

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