An Ultra High Throughput Method for Simultaneous Determination of Rates and Routes of Metabolism with a Single Injection using UPLC-QTof

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INTRODUCTION

Over the years we have seen many developments on the hardware and software for the mass spectrometers that has allowed the users to benefit from lower levels of detection and ease-of use. One area in which development pace has been slower is in HPLC. Improving the chromatographic resolution will allow us to obtain superior separations with high peak capacity, thus reducing co-elution of metabolites and also enhance the sensitivity in the MS system but reducing ion suppression. Having better chromatographic separations will not only help to detect more metabolites which were co-eluting before but also to reduce 'ion suppression'. To investigate this we have employed an Ultra Performance Liquid Chromatography system (UPLC™). By the use of this technology, it will allow us to run faster and separations with excellent peak capacities for very complicated biological matrices. This instrument has been coupled to a hybrid quadrupole-TOF mass spectrometer (QTof Premier ™). In order to show the potential of this particular development, we will show the results from the analysis of a number of marker compounds Midazolam, Phenacetin, Diclofenac, Diazepam and all drugs mentioned incubated as a cocktail. The substrates were incubated at 2uM using rat hepatocytes.
The full scan TOF-MS sensitivity allowed very good levels of detection for all samples analyzed with exact mass and excellent semi-quantitative properties. The run times for each sample were 5 minutes in comparison with a typical run time of 20 minutes when the same experiments were carried out using HPLC. No difference in the CL_{int} of parent compounds and their metabolites formed was observed when incubated individually or as a cocktail.

METHODS

Samples

Fresh hepatocytes were prepared from male Sprague-Dawley rats. Cryopreserved human hepatocytes were purchased from In Vitro Technologies (Baltimore, US).
A drug cocktail stock solution (20 µM) was made of phenacetin, diclofenac, diazepam, bufuralol, midazolam and 7-hydroxycoumarin in water and 1% DMSO.
Hepatocytes were incubated with 2 µM of all cocktail compounds in Krebs-Henseleit buffer at a cell density of 1x106 cells/ml in a final volume of 100 µl. The reactions were carried out in duplicate at 37°C for 0, 5,15, 30 and 60 minutes (rat) or 0, 15, 30, 60 and 90 minutes (human), gently shaken under an atmosphere of 5% CO2/95% air. The reactions were terminated with ice-cold acetonitrile. The well plates were centrifuged and the supernatants obtained were analyzed. Analysis of the disappearance of parent compounds and metabolites formed was followed using full scan UPLC-TOF mass spectrometry. The predicted bioavailability over the liver, F*H*, was calculated as previously described [1].

LC-MS Methodology

Mass Spectrometer: Q-Tof Premier™
MS scan range: 70-900 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 100x2.1mm id, 1.7µm
Mobile phase A: 0.1 % formic acid
Mobile phase B: acetonitrile
Flow rate: 0.6 mL/min
Gradient: 0-0.5 min 100% A, 0.5-4 min 10% A, 4.1-5 min 100% A
Injection volume: 10 µL

RESULTS

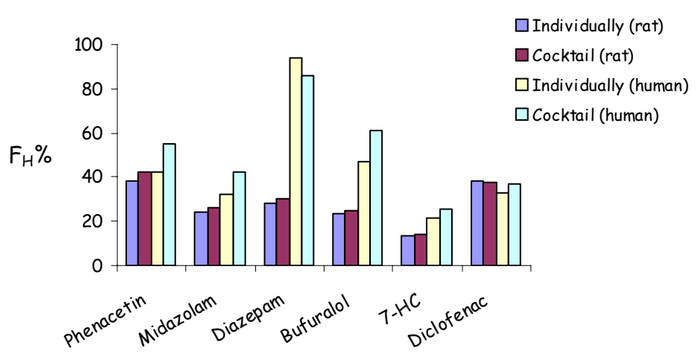

Figure 1.Inter-substrate interaction check. Predicted F_H data for compounds after incubation of each compound individually and in the cocktail with rat and human

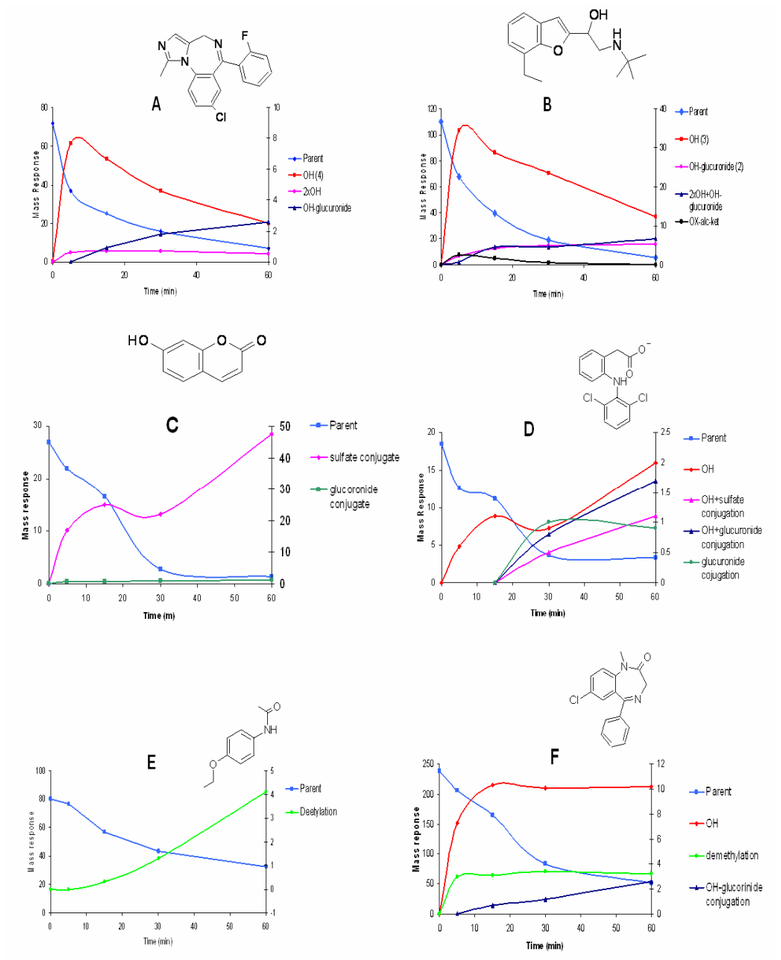

Figure 2. Metabolic profile for the 6 cocktail compounds in rat hepatocytes, A: midazolam, B: bufuralol, C: 7-hydroxycoumarin, D: diclofenac , E: phenacetin, F: diazepam. Mass response for the parent compounds is shown on the left y-axis and for the metabolites on the right axis. The number of the same products found are indicated in the brackets

UPLC™ Separation

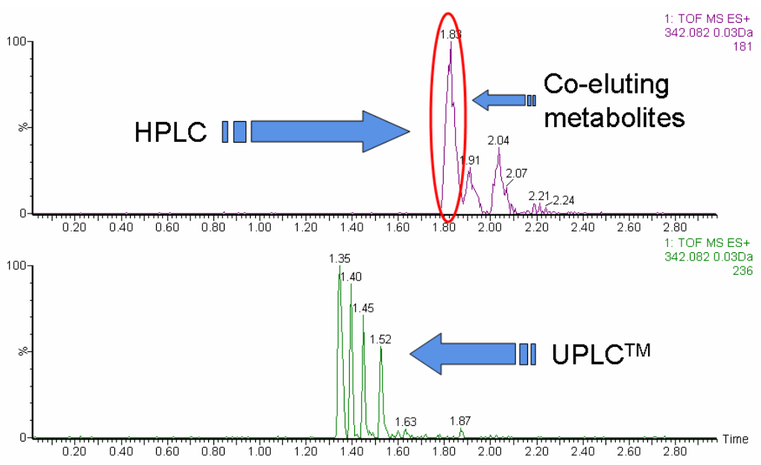

Figure 3.Chromatogram of 4 hydroxylated metabolites of midazolam. With the conventional HPLC method, only 3 possible metabolites were detected whilst with UPLC 4 metabolites were detected

MS/MS was carried out using all peaks of interest to prove that they were four OH- metabolites of midazolam (Figure 4).

MS/MS Data

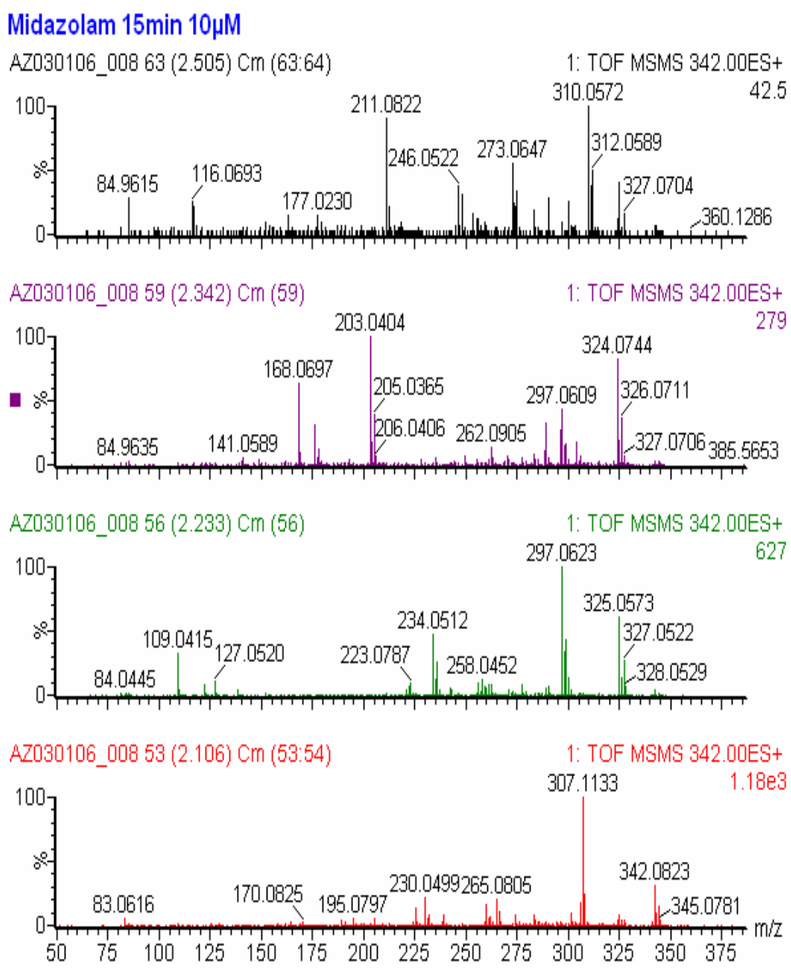

Figure 4. MS/MS spectra for 4 hydroxylated metabolites of Midazolam.

Typically MS/MS provides very important information. But sometimes the metabolites of interest are of very low concentration. To be able to run MS/MS there is a need to increase the signal/noise ratio in the mass detector. Here, another mode of acquisition is used, unique to this instrument EDC (Enhanced duty cycle) in which the T-WAVE collision cell shapes the ion beam into packets of ions. The ion packets are then released from the collision cell at certain intervals with the pusher of the oa-Tof, synchronised to operate as the ion of interest enters the extraction region. By increasing the duty cycle to 100 % this can be used in MS and MS/MS mode. For this work it can be observed that when using this mode of operation (Figure 5) a 10-fold higher signal was obtained, especially for the peak at 2.5 minutes which could clearly be detected.

Enhanced Sensitivity and EDC

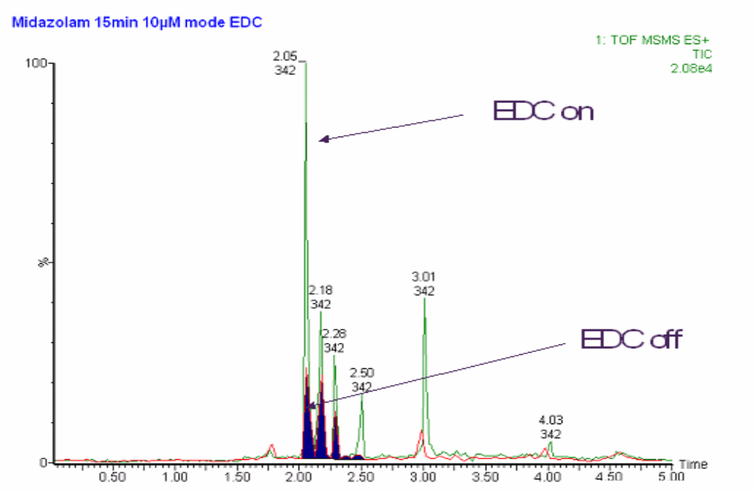

Figure 5. EDC example for hydroxylated metabolites of Midazolam

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 we used an elemental composition calculator, i-FIT™ which works on the basis of exact mass and matching the elemental composition suggested with the isotopic pattern for the metabolite of interest (Figure 6). In this way confirmation from the exact mass-elemental composition and the isotopic pattern matching algorithms is achieved. Typically, the lowest i-FIT™ value the most likely is the correct answer, this is used together with the exact mass measurement obtained. The metabolites of midazolam found by using UPLC-TOF (Figure 4) were verified for isotopic matching and exact mass with i-FIT™ . All four products gave a positive match.

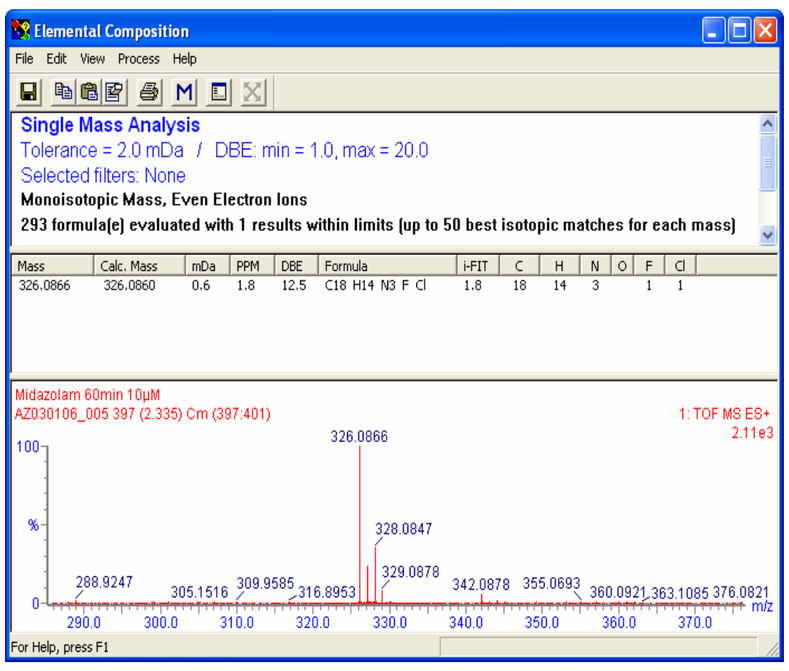

Figure 6. Elemental composition calculator with i-FIT™ for Midazolam

CONCLUSION

- The metabolic fate of all probe substrates was determined in this study.
- Both Phase I and Phase II metabolites were readily detected.
- Incubations of each compound individually demonstrated that none of the cocktail compounds were affected by inter-substrate interactions.
- EDC enabled us to obtain very good quality data for MS/MS especially when looking at low level metabolites.
- The software, Metabolynx 4.1, is user-friendly and performs an excellent job in extracting metabolites with exact mass from the *in vitro* incubations.

References

1. Floby E, Briem S, Terelius, Y, Sohlenius-Sternbeck A-K. Use of a cocktail of probe substrates for drug-metabolising enzymes for the assessment of the metabolic capacity of hepatocyte preparations. Xenobiotica 2004; 34:949-959.

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