

A NEW APPROACH FOR THE SIMULTANEOUS DETERMINATION OF RATES AND ROUTES OF METABOLISM USING UPLC-QTOF AND AUTOMATED SOFTWARE ALGORITHMS

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INTRODUCTION

Typically the rate of disappearance for novel compounds has been carried out by triple quadrupole mass spectrometers in multiple reaction monitoring, followed by full scan MS with different mass analysers to detect metabolites and find out major routes of metabolism for drug discovery experiments. There is always a need to improve the throughput of samples and especially in drug discovery where time is of the essence to find new leads and optimise drugs to achieve the desired pharmacokinetic effect. Recently, with new chromatographic strategies running at higher 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. 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 (UPLCTM). This instrument has been coupled to a hybrid quadrupole-TOF mass spectrometer (QToF PremierTM) which has the required sensitivity in full scan mode to detect and obtain a relative amount of parent drug disappearance. 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, Bufuralol and 7-OH Coumarin. The substrates were incubated at 2μM using rat hepatocytes individually and in a cocktail.

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 $C_{L_{int}}$ of parent compounds and their metabolites formed was observed when incubated individually or as a cocktail. This proved that the chromatographic strategy used in this example was adequate enough to prevent ion suppression from co-eluting species.

Moreover, with a single injection both rate of disappearance of the parent compound and metabolites formed were reported simultaneously by the use of an in-house software algorithm (MetabolynxTM) which data mined the raw data. This software algorithm used an exact mass data filter which allowed the removal of false positives.

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, FH, was calculated as previously described [1].

LC-MS Methodology

Mass Spectrometer: Q-ToF PremierTM

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 UPLCTM

Acuity 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

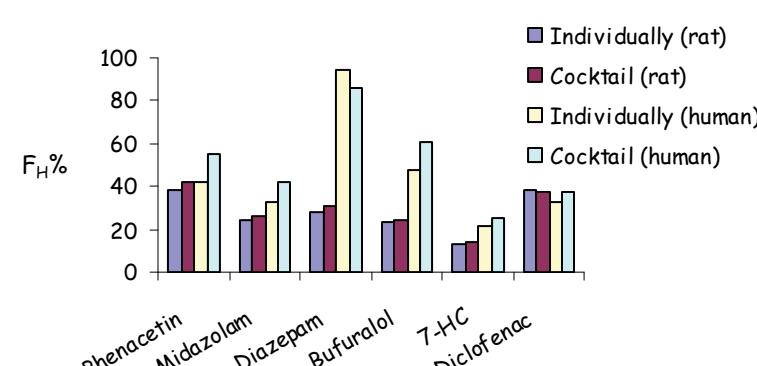


Figure 1. Inter-substrate interaction check. Predicted F_H data for compounds after incubation of each compound individually and in the cocktail with rat and human hepatocyte

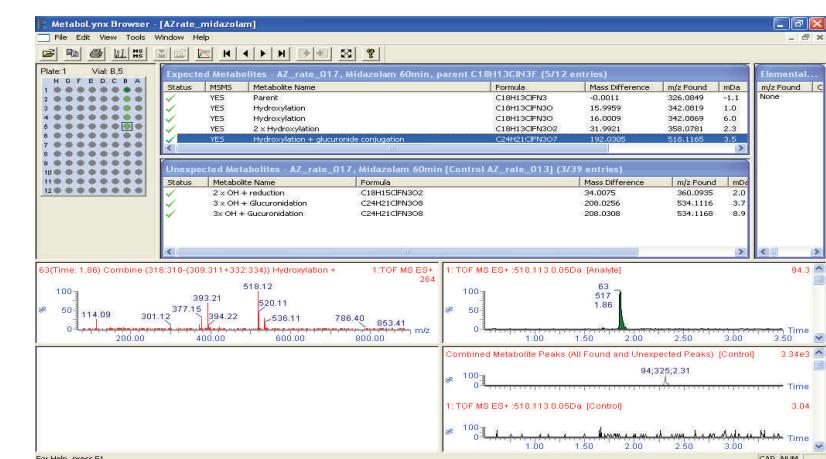


Figure 2. Metabolynx browser report for all expected and unexpected metabolites of Midazolam

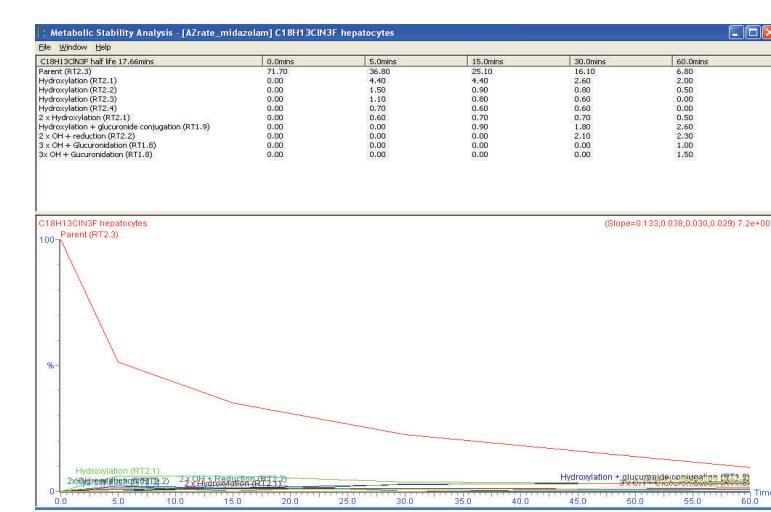


Figure 3. Metabolic stability report for all found metabolites of Midazolam

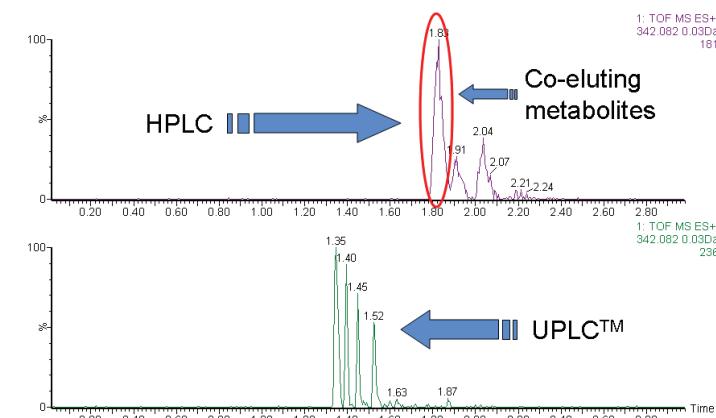


Figure 4. Chromatogram of 4 hydroxylated metabolites of midazolam. With the conventional HPLC method, only 3 possible metabolites were detected whilst with UPLC 4 metabolites were detected

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-FITTM which works on the basis of exact mass and matching the elemental composition suggested with the isotopic pattern for the metabolite of interest (Figure 5). In this way confirmation from the exact mass-elemental composition and the isotopic pattern matching algorithms is achieved. Typically, the lowest i-FITTM 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-FITTM. All four products gave a positive match.

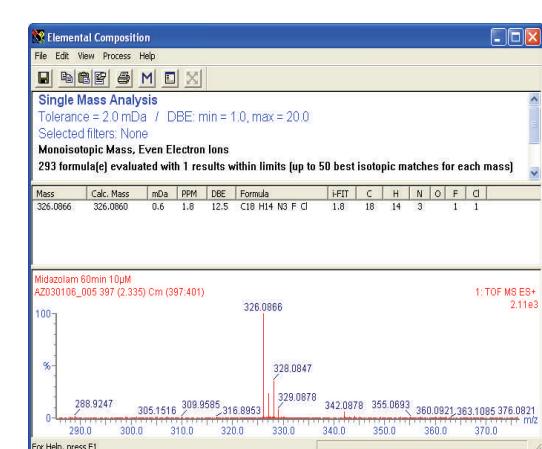


Figure 5. Elemental composition calculator with i-FITTM for Midazolam

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.
- The software, Metabolynx 4.1, is user-friendly and performs an excellent role in extracting metabolites out of the exact mass data from the *in vitro* incubations.
- Within one single injection metabolic stability and identification information was achieved
- UPLCTM provided a rapid sample turnaround, 5 min between injections, and excellent chromatographic resolution.

References

- 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.