Cytochrome P450 Reaction Phenotyping by Targeted/Non-targeted Metabolomics Workflow and Accurate Mass and High Resolution LC-QTOF-MS

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Wei Chen[1], Bih Hsu[1], Patrick Lin[1], Xuejun Peng[2], Guillaume Tremintin[2]
[1]Pharmaout Laboratories Inc., Fremont, CA USA;
[2]Bruker Scientific LLC, San Jose, CA USA

Introduction

Reaction phenotyping is the process to identify specific pathways of drug-metabolizing enzymes involved in the clearance of a drug. It is commonly used as an initial strategy during drug discovery or the early drug development stage to provide early insight on evaluation of the potential drug-drug interactions or possible affinities to functionally polymorphic enzymes which can cause inter-subject variability[1]. Cytochrome P450 (CYP) shows polymorphic expression in human populations and contributes to the variable exposure levels of drug metabolism.

Here a combined targeted and non-targeted metabolomics workflow to study CYP reaction phenotyping was established. Based on the incubation of the model analyte diazepam with cDNA expressed P450 enzymes, a simultaneous identification and detection of drug substrate depletion and all metabolites formations by liquid chromatography and ultra-high resolution QTOF MS analysis were conducted.

Methods

cDNA expressed human supersomes isoforms CYP 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 and controls were spiked into a pre-incubated reaction mixture containing 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride, 100 mM potassium phosphate (pH 7.4) and 0.1 mM diazepam, and incubated at 37 ^{0}C for 60min. The reactions were stopped by the addition of cold acetonitrile solvent and centrifuged at 15,000 rpm for 10 min. 100 µL of supernatant was transferred into insert vials and 2 µL sample was injected. Samples were separated by an Elute UHPLC in a reversed phase C18 column (2.2µ, 120A, 2.1x100 mm) gradient elution, and analyzed (n=6) by an ultra-high resolution LC-ESI maXis II QTOF MS (Bruker, Figure 1)

Figure 1. Elute UHPLC and maXis II QTOF MS

Results and Discussion

Metabolic Pathway

Two metabolites from diazepam human supersomes CYP phase I metabolism are nordazepam, the demethylation of diazepam; and temazepam, the hydroxylation of diazepam. Nordazepam and temazepam undergo further demethylation or hydroxylation to form oxazepam. Both temazepam-glucuronide and oxazepam-glucuronide can be formed if phase II metabolism involved.

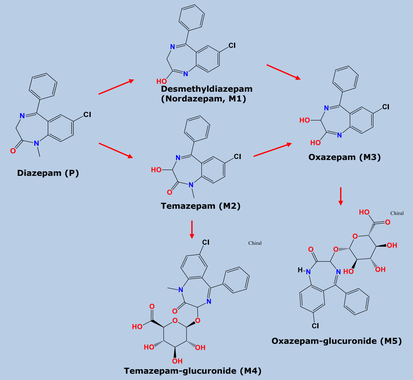

Figure 2. Diazepam metabolic pathway

Separation Chromatogram

No significant difference was observed between samples of each of the individual cDNA expressed human supersomes isoforms CYP incubations (Figure 3).

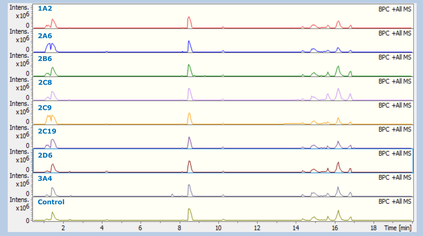

Figure 3. LC-MS BPC chromatogram

Metabolite Normalization

Metabolite **M1** nordazepam was observed in CYP 2A6, 2B6, 2C8, 2C9, 2C19 and 3A4 with the intensity 3A4 (54%) > 2C19 (32.2%) > 2B6 (6.5%) > 2C8 (4.4%) > 2C9 (2.3%) > 2A6 (0.5%), and **M1** was not detected in CYP 1A1 and 2D6; **M2** temazepam and **M3** oxazepam were found only in CYP 2C19 and 3A4 with the major contribution from 3A4 (94.4% for **M2** and 91.6% for **M3**) (Figures 4-5). The results confirm CYP3A4 and CYP2C19 are the major enzymes for diazepam phase I metabolism[2].

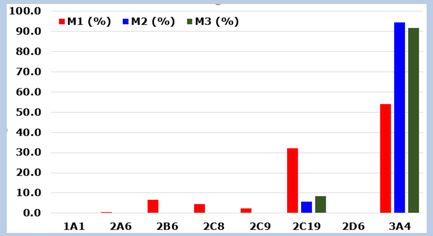

Figure 4. Metabolite Normalization

Data evaluation using MetaboScape

Box plots of major metabolites of **M1** and **M2** are shown in Figure 6 and represent six replicates samples of CYP reaction phenotyping with diazepam.

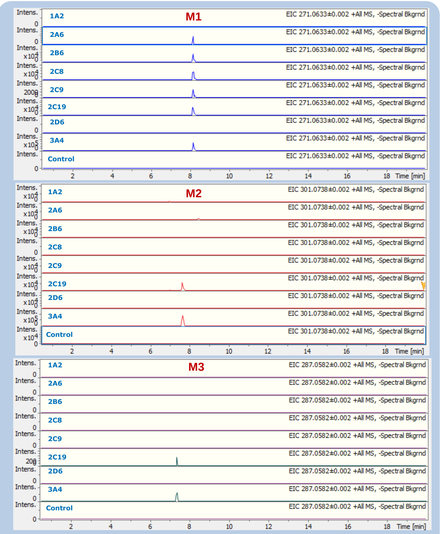

Figure 5. Extracted ion chromatogram

M1 – m/z 271.0633 ± 0.002; Nordazepam
M2 – m/z 301.0738 ± 0.002; Temazepam
M2 – m/z 287.0582 ± 0.002; Oxazepam

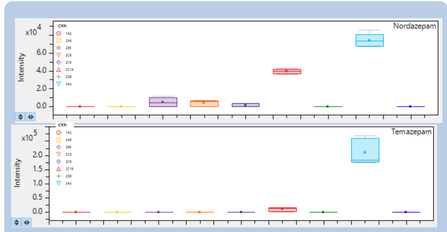

Figure 6. Box Plots of Nordazepam and temazepam (n=6)

Metabolite Characterization

M1 molecular formula and chemical structure were verified based on SmartFormula annotation, database searching and *in-silico* MetFrag confirmation (Figure 7) using MetaboScape.

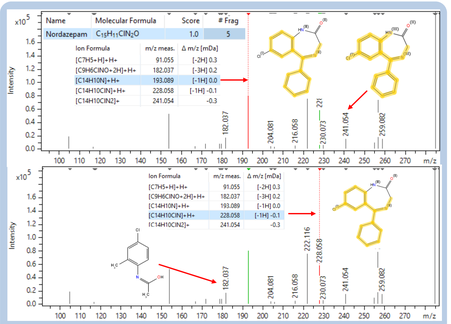

Figure 7. Metabolite Characterization

References

(1) US FDA/CDER, *Guidance for Industry, Drug Interaction Studies – Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendation*. February, 2012.

(2) Samantha Luk et al., *Journal of Analytical Toxicology*, 2014, 38:135-142

Conclusions

- A metabolite reaction phenotyping workflow based on an *in vitro* CYP diazepam metabolism model is presented
- Ultra-high resolution LC-MS QTOF analysis provided reproducible qualitative and quantitative information for reaction phenotyping
- The fully integrated MetaboScape software solution proved to be a powerful tool for statistical data analysis and for enabling to readily identify and verify drug metabolites.

Metabolite Profiling/ Reaction Phenotyping