A Rapid LC-hrMS Method for Metabolite Identification Simultaneously to Metabolic Stability Assessment on Microsomes at an Early Screening Stage

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ABSTRACT

Introduction: Metabolic stability assessment on liver microsomes is one of the first earlyADME assay in drug discovery. The aim of this assay is to evaluate the intrinsic hepatic clearance of compounds in very short timelines after their synthesis. Structure-Activity Relationship based on metabolic stability has been a powerful tool for medicinal chemists to optimize drug candidates relating to their pharmacokinetic properties. With the leverage of recent chromatography and mass spectrometer technologies, bioanalytical conditions combining a rapid LC method with hrMS detection have been set-up. This will allow the simultaneous study of the metabolic clearance and the metabolite identification for an in-depth drug optimization and for an interspecies comparison.
Method: For the metabolic stability assessment, compounds are incubated at 0.5 µM with human and mouse liver microsomal proteins (0.25 mg/mL) and NADPH-generating system. After each incubation time, the reaction is stopped by adding a quenching solution with internal standard. The samples are injected into a LC-hrMS with a generic method in full scan detection mode. QuickCalc® software is used for quantifying tested compounds based on an automatic search of the molecular ions within the full scan chromatogram. The remaining parent compound is automatically determined. A metabolite identification reprocess with MetWorks® software allows the metabolites data extraction. The present work was focused on Midazolam, Diclofenac and Amitriptyline as examples in human and mouse species.
Results: A first processing with QuickCalc® was used for the microsomal stability assessment of tested compounds. A second reprocessing with MetWorks® software allowed rapid metabolite identification. Midazolam was slightly more metabolized in mouse than in human, but with similar main metabolites: oxidations and di-oxidation. Diclofenac was highly metabolized in human and slightly in mouse. The proportion of oxidized derivatives could explain this interspecies discrepancy. Amitriptyline was more metabolized in mouse than in human. This interspecies discrepancy could be explained by the proportion of desmethylated and oxidized derivatives.
When drug was found unstable, a re-process of the data could be done for identifying which was the main metabolic pathway, helping, by the way, the SAR improvement.
Conclusion: A generic and fast method has been set-up for helping medicinal chemists to optimize the drug candidates based on their microsomal stability and additional metabolic pathway comprehension. The relatively low incubation concentration doesn't allow to precise the correct position of metabolism, but provides helpful information on the metabolite class and potentially on the interspecies metabolism pathway. Specific metabolite identification study will bring structural information.
Novel Aspect: A LC-MS system with high resolution analyzer is a very adapted tool to combine during the first earlyADME studies the parent compound metabolic stability with metabolite identification using a simple, generic and fast analytical method and process.

OBJECTIVE

This assay was set-up to support Structure-Activity Relationship in Hit to Lead and Lead Optimization programs by simultaneously evaluating the intrinsic hepatic clearance of compounds and providing structural information on the generated metabolites within a very short turnaround time.

Sample incubation:

This assay was performed with an automated protocol on robotic platform Sciclone G3 (a Caliper-Perkin Elmer). Compounds were incubated in duplicate at 37 °C at 0.5 µM in a 96-well plate with 0.25 mg/mL of human and mouse liver microsomal proteins and NADPH-generating system (1 mM NADP, 5 mM glucose 6-phosphate, 1 U/ml glucose 6-phosphate dehydrogenase, and 5 mM $MgCl_2$) in 100 mM Tris buffer (pH 7.4). Reactions were initiated by adding tested compound in pre-incubated reaction mixture. After 0, 5, 15 and 30 min, incubations were stopped by adding the quenching solution allowing protein precipitation and containing the Internal Standard. After centrifugation, four 96-well plates (one plate by incubation time) are reformatted in one 384-well plate for the bioanalytical phase.

METHOD

Analytical method:

The samples are injected into a LC-hrMS with a generic method in full scan detection mode.

Equipment:

Instrument	Type	Manufacturer
Mass spectrometer	EXACTIVE PLUS	Thermo Scientific
Autosampler	OAS-3600TXRS	Thermo Scientific
HPLC-pump	HPG-3400RS	Thermo Scientific
Column oven	TCC-3000RS	Thermo Scientific
Analytical column	Accucore RP-MS 30x2.1mm	Thermo Scientific

Gradient:

Time (min)	% Mobile Phase CH_3COO^- NH_4^+ 5mM + HCOOH 0.01%	% Mobile Phase ACN + HCOOH 0.01%	Flow (mL/min)
0.00	98	2	0.7
0.50	0	100	
0.65	0	100	
0.70	98	2	
1.50	98	2	

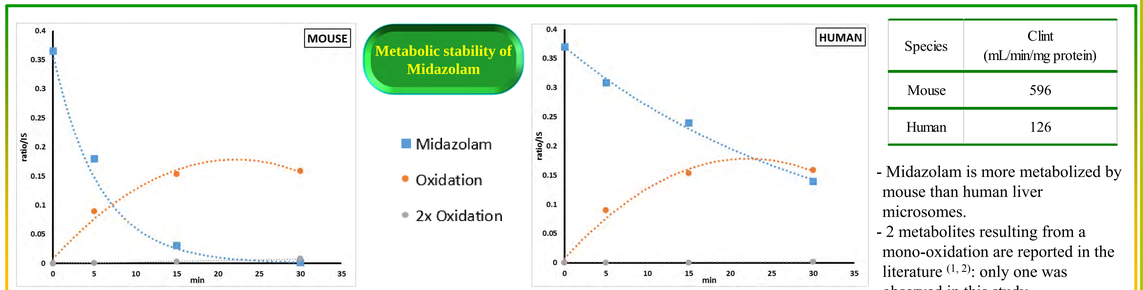
Chromatographic conditions:

Acquisition time (min)	1.5
Oven Temperatue (°C)	45
Autosampler Temperature (°C)	5
Injection volume (µL)	1

Data integration:

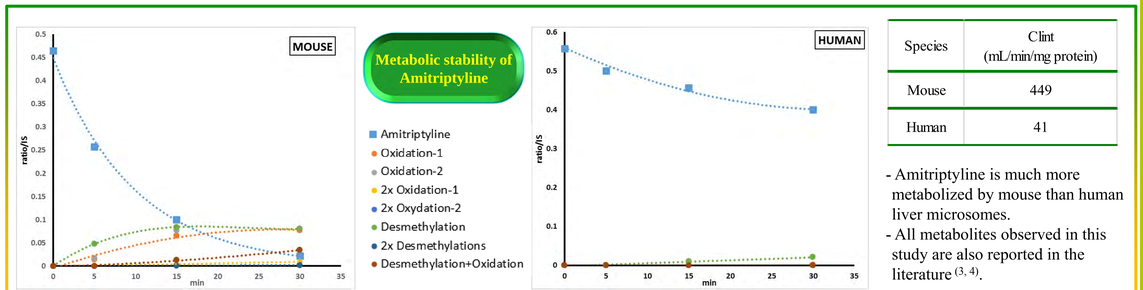
Parent compounds were quantified using QuickCalc® software for the calculation of the intrinsic clearance Clint (mL/min/mg protein). Then, for compound highly cleared, data are reprocessed with MetWorks® software allowing the metabolites data extraction based on a pre-defined list of transformations.

RESULTS



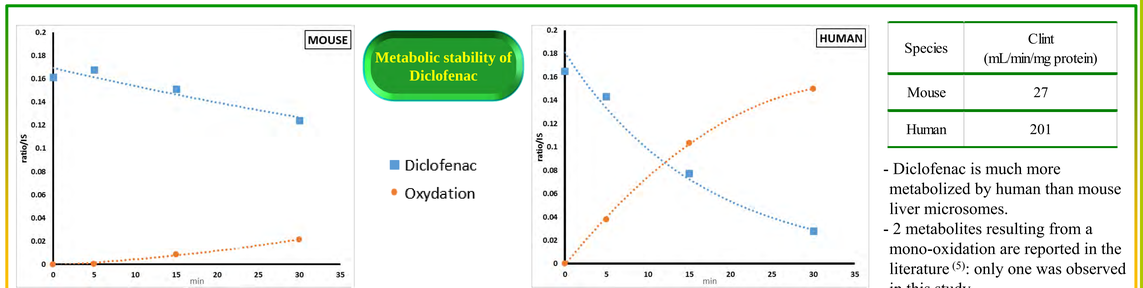
Species	Clint (mL/min/mg protein)
Mouse	596
Human	126

- Midazolam is more metabolized by mouse than human liver microsomes.
- 2 metabolites resulting from a mono-oxidation are reported in the literature [1, 2]: only one was observed in this study.



Species	Clint (mL/min/mg protein)
Mouse	449
Human	41

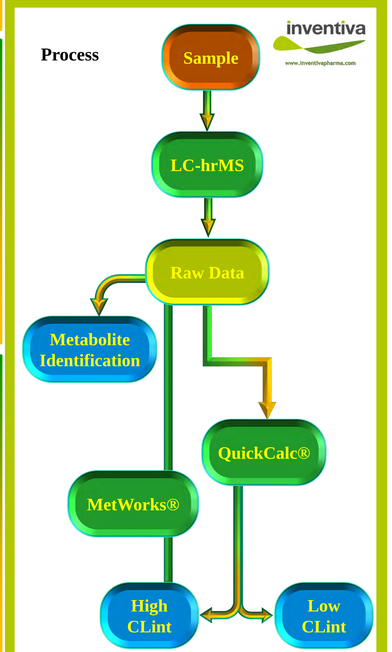
- Amitriptyline is much more metabolized by mouse than human liver microsomes.
- All metabolites observed in this study are also reported in the literature [3, 4].



Species	Clint (mL/min/mg protein)
Mouse	27
Human	201

- Diclofenac is much more metabolized by human than mouse liver microsomes.
- 2 metabolites resulting from a mono-oxidation are reported in the literature [5]: only one was observed in this study.

Both positive and negative detection modes were used in this study and had easily allowed both the clearance calculation of the parent compound and the identification of its metabolic pathway.
The standard deviation was very low for the duplicate samples for the parent compounds as well as for their metabolites. These three compounds are used as positive control references in each metabolic stability run with very low clearance variability (intra- and inter-run).



DISCUSSION

It was demonstrated that this process combining a LC-MS system with high resolution analyzer is a well adapted tool for earlyADME studies supporting Hit to Lead and Lead Optimization programs. It allows to provide important quantitative (intrinsic clearance) and qualitative (metabolite identification) information on the compound metabolic stability using a simple, generic and fast analytical method. Such a powerful tool will help medicinal chemists in their choices optimizing the drug candidates based on their microsomal metabolic stability and pathway comprehension. Moreover, it was shown that inter-species differences can also be evidenced with metabolite hypotheses to explain them.
The limit of such process may be that unexpected metabolites may not be searched automatically, leading to potential unknown metabolic pathways. Moreover, the relatively low incubation concentration, needed for the intrinsic clearance estimation with low risk of CYP inhibition, doesn't allow to precise the exact position of the labile sites, but provides very supportive hypotheses for optimization. Additional specific metabolite identification study further in the process will complete more structural information when needed.

REFERENCES

(1) Hosagrahara VP et al. Drug Metab Dispos. 1999 Dec;27(12):1512-8. (2) Seo KA et al. Drug Metab Dispos. 2010 Nov;38(11):2007-2013. (3) Tang w et al. J Pharmacol Exp Ther. 1999 Dec;291(3):1068-1074. (4) Tang w et al. Drug Metab Dispos. 1999 Mar;27(3):365-372. (5) Linden R et al. J Braz Chem Soc. 2008 Jan;1(1): 35-41