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High Throughput LDTD-MS/MS IC_{50} Determination of CYP Inhibition in HLM

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Overview

- High throughput analysis of CYP 1A2 / 2C9 / 2D6 / 3A4 inhibition assays ;
- Probe : Phenacetin, Diclofenac, Tolbutamide, S-mephenytoin, Bufurolol, Dextromethorphan, Midazolam, Testosterone and Nifedipine ;
- Inhibitors : Furafylline (1A2), Sulfaphenazole (2C9), Ticlopidine (2C19), Quinidine (2D6) and Ketoconazole (3A4) ;
- ***Acetaminophene (1A2) analyzed in negative APCI***
- IC_{50} comparable with LC-MS/MS and LDTD-MS/MS methods ;

Instrumentation

- LDTD ion source, T-960 (Phytronix Technologies) interface on a TSQ Vantage™ triple quadrupole (Thermo Fisher Scientific) ;

- Laser power pattern :
  Increase laser power to 40 % in 2.0 s
  Hold at 40 % for 2.0 s
  Decrease laser power to 0 %

Introduction

In early drug discovery, identifying potential drug candidates is a common practice. One important step in this discovery process is to identify drug-drug interactions. The most widespread procedure for this is to perform cytochrome P450 (CYP) inhibition assays using human liver microsomes (HLM). The most commonly used method for analyzing CYP inhibition assay samples is LC-MS/MS. However, this method is time-consuming and represents the bottleneck in this type of assay. To increase the throughput, we propose to use the LDTD as ionization source.

The LDTD is a shotgun approach where the sample is introduced into the mass spectrometer without chromatographic step, the separation being achieved by operating the mass spectrometer in MS/MS mode. The specificity of the method is therefore achieved by selecting specific MS/MS transitions for each target analyte. Eliminating the chromatographic step, increase dramatically the analytical speed.

Samples Preparation

Table 1 shows the incubation conditions of the CYP probes and inhibitors used in this study. The protein concentration was 0.25 mg/mL of HLM prepared in a pH 7.4 phosphate buffer. Reactions were initiated by adding NADPH and all samples were incubated in 300-µL round bottom 96-well plates at 37 ^{0}C for 10 min. The incubations were quenched by adding equal volume of cold acetonitrile.

LC-MS/MS : After vortexing and centrifuging, the supernatant were transferred to a 96-well injection plate. The resulting samples were analyzed by the Roche Palo Alto in house LC-MS/MS (Sciex API 4000, instrumentation conditions not listed). Bucetin was used as internal standard.

LDTD-MS/MS : After vortexing and centrifuging, the supernatant were dissolved with four (4) volume of acetonitrile and 2µL were transferred to a 96-well LazWell plate. The solvent was evaporated to dryness at room temperature. This dilution step was necessary to reduce the unvolatile content into the final dry samples. The resulting dry samples were analyzed in LDTD-MS/MS. Labelled internal standard were used for OH-midazolam and OH-diclofenac only.

Table 1 Incubation conditions of the CYP assay.

Isozyme / Probe substrate	Probe substrate conc. (µM)	Inhibitor / Highest conc. (µM)	Monitored metabolites
CYP 1A2 / Phenacetin	10	Furafylline / 10	Acetaminophen
CYP 2C9 /Diclofenac	10	Sulfaphenazole / 10	4'-OH-diclofenac
CYP 2C9 / Tolbutamide	100		4'-OH-tolbutamide
CYP 2C19 / S-Mephenytoin	50	Ticlopidine / 10	4'-OH-mephenytoin
CYP 2D6 / Bufurolol	5	Quinidine / 5	1'-OH-bufurolol
CYP 2D6 / Dextromethorphan	5		Dextrorphen
CYP 3A4 / Midazolam	2	Ketoconazole / 5	1'-OH-midazolam
CYP 3A4 / Testosterone	50		6β-OH-testosterone
CYP 3A4 / Nifedipine	10		OH-nifedipine

Table 2 SRM transition and APCI mode.

Compound	APCI Mode	Q1 (m/z)	Q3 (m/z)
Acetamoniphene	-	150.0	107,16
4'-OH-diclofenac	+	310.0	265.98
4'-OH-tolbutamide	+	287.0	88.9
4'-OH-mephenytoin	+	235.0	150.08
1'-OH-bufurolol	+	278.0	186.05
Dextrorphen	+	258.1	133.07
1'-OH-midazolam	+	326.0	291.01
6β-OH-testosterone	+	289.3	109.2
OH-nifedipine	+	344.98	284.3

Results and Discussion

Method specificity

Operating without chromatographic separation in APCI without liquid mobile phase, the LDTD in-source fragmentation needs to be evaluated. Each probe were analyzed in matrix at the working concentration (2 to 100 µM, see **Table 2**) and the corresponding metabolites were monitored.

In (+)APCI a signal of Acetaminophen (MW 151.2 g mol^{-1}) SRM was observed when a blank containing 50 µM of Phenacetin (MW 179.2 g mol^{-1}) was analyzed. The signal intensity correspond to a Phenacetin conversion into Acetaminophen of 1.5 % affecting the IC_{50} determination. The same analysis has performed in (-)APCI and under these conditions the observed conversion was 10-times lower. The IC_{50} was calculated and the value obtained was comparable to the LC-MS/MS method. ***Therefore, in LDTD-MS/MS, the Acetaminophen should be monitored in (-)APCI to get accurate IC_{50} determination.***

IC_{50} determination and accuracy

Data were fitted using Xlfitting and representative inhibition curves obtained in LDTD-MS/MS are presented in **Figure 1**. Calculated IC_{50} values are reported in **Table 3**. The calculated IC50 values as well as inhibition curves were comparable to the results obtained in LC-MS/MS, suggesting acceptable performance from the LDTD-MS/MS system. Using an internal standard (CYP 2C9 for 4'-OH-diclofenac and CYP 3A4 for 1'-OH-midazolam) allows to get better accuracy with the LDTD. On the other hand, We observe more discrepancy results between LDTD and LC-MS/MS IC_{50} values when the probe substrate concentration is higher then 50µM (Tolbutamide, S-Mephenytoin and Testosterone). This could from a mass-effect into the well which seems to affect the thermal desorption. Diluting the final extract should lower this effect leading to more accurate results.

Table 3 Summary of IC_{50} values obtained.

Compound	CYP Isoform	IC_{50} (M) LC-MS/MS	IC_{50} (M) LDTD-MS/MS
Acetamoniphene	1A2	2.427	3.145
4'-OH-diclofenac	*2C9*	*0.585*	*0.555*
4'-OH-tolbutamide	2C9	0.515	0.357
4'-OH-mephenytoin	2C19	2.99	1.852
1'-OH-bufurolol	2D6	0.041	0.017
Dextrorphen	2D6	0.025	0.031
1'-OH-midazolam	*3A4*	*0.093*	*0.095*
6β-OH-testosterone	3A4	0.090	0.062
OH-nifedipine	3A4	0.199	0.159

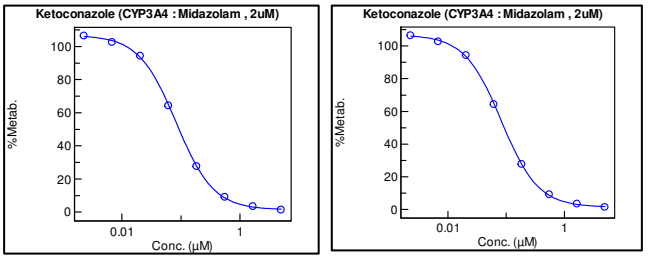

Figure 1 Representative inhibition curves for the CYP isoforms

High throughput advantage of LDTD-MS/MS over LC-MS/MS

The reproducibility has been evaluated in LDTD-MS/MS by running 4 to 6 replicates of the same sample. The reproducibility has been calculated from the % RSD. and being operated without using an internal standard, The reproducibility is comparable to the one obtained in LC-MS/MS, however it should be improved by using an internal standard.

The analytical speed of the LDTD which allows 4 seconds per sample is 63-times faster then traditional LC-MS/MS method (252 seconds / sample). The analytical speed provided by the LDTD allow to increase the CYP inhibition assays throughput without compromising the IC_{50} determination according to the obtained results.

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