

High Throughput Laser Diode Thermal Desorption Mass Spectrometry for the Determination of Time-Dependent Cytochrome P450 Inhibition in Human Liver Microsomes

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OVERVIEW

The objective of this study was to compare the two analytical techniques, namely, LC-MS/MS and laser diode thermal desorption (LDTD)-MS/MS for the estimation of time-dependent inhibition (TDI) of cytochrome P450 in human liver microsomes (HLM) using automated screening assay. LDTD-MS/MS was found to be a higher through-put technique with results comparable to LC-MS/MS.

INTRODUCTION

TDI of cytochrome P450 can cause clinically relevant drug-drug interactions¹. The greater demand to screen new chemical entities (NCEs) for TDI of human cytochrome P450 in drug discovery has resulted in the need for higher throughput assay options. Currently, this has been partially met by automation of the screening assays. However, time consuming analysis of the samples remains as a bottleneck in the screening process. Therefore, establishment of higher throughput analytical methods for screening of NCEs is highly desirable. Since the analytical support for screening assays is most widely provided by LC-MS/MS, the current study was undertaken to compare LC-MS/MS and a higher throughput analytical technology using LDTD-MS/MS for the analysis of TDI assay samples.

METHODS

Primary incubations were performed using selected reference and AstraZeneca (AZ) compounds added to human liver microsomes (HLM at 1 mg/mL). The incubation was initialized upon the addition of NADPH or buffer. Following this primary incubation period (30 minutes), aliquots were taken and transferred into a secondary incubation (refer to Scheme 1 for details) containing a cocktail of specific probes for major human P450s² (Table 1). This mixture was incubated in the presence of NADPH for 15 minutes. The reactions were stopped by addition of one volume of acetonitrile containing stable isotopes (used as internal standards) corresponding to each measured metabolites (Table 1). Aliquots of samples were further diluted with methanol/water containing clomiphen citrate (used as consistency control) prior to analysis using the LDTD-MS/MS. For both methods, LC and LDTD, Agilent triple quad 6410 was used as MS/MS. For LDTD-MS/MS analysis, samples were dried onto the bottom of a well from a standard 96-well plate with a metal sheet insertion. Thermal desorption was induced by a laser at 980 nm (no photon-sample interactions) (Scheme 2). TDI assay for CYP2C19 was conducted using a different batch of microsomes because of low CYP2C19 activity in the batch used for assay of other CYPs.

Table 1: Specific probe substrates and internal standards

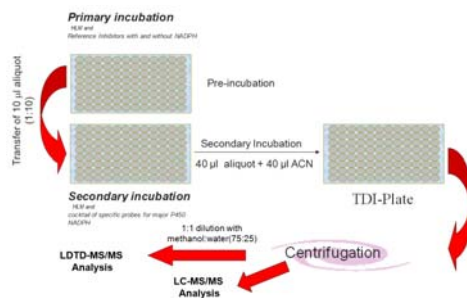
CYPs	Probe Substrate (substrate reaction)	Internal Standard
1A2	Phenacetin (150 µM) (Phenacetin O-dealkylation)	13C ₂ , 15N-acetaminophen
2C9	Diclofenac (10 µM) (Diclofenac 4'-hydroxylation)	4'-hydroxydiclofenac-[13C ₆]
2C19	S-Mephenytoin (100 µM) (Mephenytoin 4'-hydroxylation)	Rac-4-hydroxy mephenytoin-d ₃
2D6	Dextromethorphan (10 µM) (Dextromethorphan O-demethylation)	Dextrophan-d ₃
3A4	Midazolam (10 µM) (Midazolam 1'-hydroxylation)	Hydroxymidazolam-[13C ₃]

The percentage of TDI was determined by comparing the peak areas of formed metabolites in samples and the DMSO control (acetaminophen, 4-hydroxydiclofenac, 4-hydroxymephenytoin, dextrophan and 1-hydroxymidazolam) using both LC-MS/MS and LDTD-MS/MS systems.

TDI (%) was calculated using the following equation:

$$\%TDI = \left(1 - \frac{\% \text{ control activity with NADPH}}{\% \text{ control activity No NADPH}} \right) \times 100$$

Scheme 1: Incubation method for Time-Dependent Inhibition Assay



RESULTS

Table1: TDI values of selected reference compounds estimated using LC-MS/MS and LDTD-MS/MS.

Reference Compound (Concentration)	CYP	TDI % (Mean ± SD)	
		LC-MS/MS	LDTD-MS/MS
Torleandomycin (2 µM)	CYP3A4	63.54 ± 2.09	59.99 ± 1.54
Tielineic Acid (4 µM)	CYP2C9	65.66 ± 3.37	77.75 ± 7.54
Paroxetine (2 µM)	CYP2D6	82.38 ± 0.74	75.03 ± 2.72
Verapamil (10 µM)	CYP3A4	68.77 ± 1.93	64.21 ± 4.07
Ticlopidine (5µM)	CYP2C19	57.66 ± 6.09	44.51 ± 15.49

Data are mean ±SD (n=3).

Figure1: Correlation between TDI values for all CYPs estimated using LC-MS/MS and LDTD-MS/MS for various compounds. (No statistical bias was observed between the methods; p<0.05)

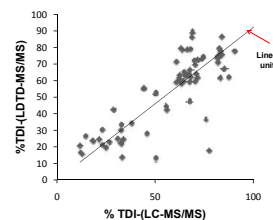
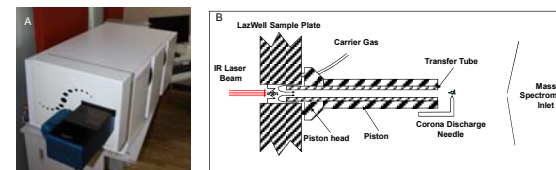


Table 2: Comparison of TDI of selected AZ compounds (at 10 µM) estimated using LC-MS/MS and LDTD-MS/MS. Different set of compounds were used for CYP2C19 TDI assay. Data are mean ±SD (n= 2 or 3).

Compound	TDI % (Mean ± SD)							
	CYP2C9		CYP2D6		CYP3A4		CYP2C19	
	LC-MS/MS	LDTD-MS/MS	LC-MS/MS	LDTD-MS/MS	LC-MS/MS	LDTD-MS/MS	LC-MS/MS	LDTD-MS/MS
1	<10	<10	<10	<10	<10	<10	83.08 ± 8.53	78.32 ± 12.39
2	<10	<10	16.82 ± 7.33	24.56 ± 6.28	<10	<10	32.84 ± 5.05	33.36 ± 3.65
3	<10	<10	<10	<10	28.58 ± 5.39	25.45 ± 6.54	28.98 ± 6.82	27.32 ± 7.80
4	<10	<10	<10	<10	14.93 ± 15.75	15.75 ± 9.37	20.35 ± 13.25	19.13 ± 5.54
5	11 ± 1.67	15 ± 0.65	<10	<10	24.98 ± 1.75	28.27 ± 7.11	30.60 ± 15.27	28.21 ± 4.03
6	<10	<10	<10	<10	<10	<10	71.14 ± 10.55	62.37 ± 16.13
7	<10	<10	19.79 ± 2.77	25.19 ± 4.16	10.01 ± 5.55	10.55 ± 13.36	67.89 ± 10.88	54.79 ± 25.69
8	<10	<10	<10	<10	<10	<10	39.33 ± 12.92	28.87 ± 22.72
9	<10	<10	<10	<10	<10	<10	41.21 ± 35.87	22.69 ± 7.89
10	<10	<10	<10	<10	36.19 ± 8.84	38.39 ± 12.02	56.21 ± 11.07	48.77 ± 14.67
11	<10	<10	<10	<10	70.45 ± 5.02	68.13 ± 6.87	39.46 ± 25.00	13.48 ± 44.06

<10 = TDI values below 10%. The TDI values below 10% are not reported because TDI below 10% is considered to be of low risk for drug-drug interactions



Scheme 2. (a) Photograph of LDTD ion source coupled to Agilent Triple Quad. 6410; (b) Schematic representation of LDTD ionization source.

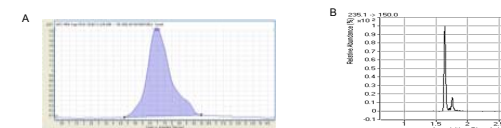


Figure 2. Representative peak of 4-Hydroxy Mephenytoin in (A) LDTD-MS/MS (B) LC-MS/MS indicating differences in retention time and hence sample run time.

DISCUSSION

- In general, the % TDI values estimated by LC-MS/MS were similar to those estimated by LDTD-MS/MS (Tables 1 and 2). More importantly, no false positive or negative TDI values were observed by LDTD-MS/MS.
- With the exception of a few outliers, the percent TDI values for CYP2C9, CYP2D6, CYP2C19 and CYP3A4 for selected reference and AZ compounds showed excellent agreement between LC-MS/MS and LDTD-MS/MS methods (Figure 1; no statistical bias was observed between the methods; p<0.05).
- LDTD-MS/MS could not be used for estimation of TDI for CYP1A2, because of in-source degradation of phenacetin (probe substrate). Therefore, another specific CYP1A2 probe will have to be evaluated for sample analysis using the LDTD.
- Analysis of 96-well plate samples by LDTD-MS/MS reduced the sample run time (Figure 2) by more than ten-fold when compared with the LC-MS/MS method (1.2 hour vs 13 hours, respectively). As a result, all samples could be analyzed within a day.

CONCLUSIONS

Our data suggests that LDTD-MS/MS is a faster analytical technique with TDI results comparable to the LC-MS/MS. Thus, it can be considered as an alternative higher throughput analytical technique for TDI assay.

REFERENCES

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- A. Atkinson, J.R. Kenny, K. Grime, *Drug Metab Dispos* 2005, 33: 1637-1647.