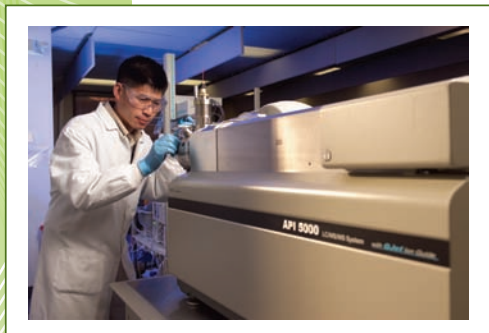


# Evaluation of Alternative HT Intrinsic Clearance Measurement in Rat Liver Microsomes Using FIA/MS/MS, MALDI-TOF MS, and MALDI-TOF MS/MS



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## ABSTRACT

Flow injection analysis (FIA) and matrix-associated laser desorption ionization (MALDI) coupled with tandem mass spectrometry (MS/MS) were evaluated against the conventional LC/MS/MS as alternative high-throughput (HT) analytical methods for measuring intrinsic clearance of ten (10) known compounds in rat liver microsomes. Samples after the microsomal incubation were processed with either protein precipitation or solid-phase extraction for comparisons. Processed samples were analyzed using LC/MS/MS and FIA/MS/MS, MALDI-TOF MS, as well as MALDI-TOF MS/MS. The intrinsic clearance values obtained by the alternative high-throughput methods were compatible with those published in the literature. Moreover, metabolites of compound interests could be simultaneously detected through MALDI-TOF MS and MALDI-TOF MS/MS analyses.

## INTRODUCTION

A LC/MS/MS has been widely used to support absorption, distribution, metabolism, and excretion (ADME) for drug discovery. A fast HPLC chromatography coupled with column switching techniques has been commonly employed as a high-throughput (HT) tool for ADME study. In this report, we evaluated FIA/MS/MS, MALDI-TOF MS, and MALDI-TOF MS/MS as alternative HT analytical tools to study the intrinsic clearance of ten (10) known drug compounds in rat liver microsomes in comparison with the LC/MS/MS method. Protein precipitation and solid-phase extraction were used to process rat liver microsomes incubated with the drugs, followed by LC/MS/MS, FIA/MS/MS, MALDI-TOF MS, and MALDI-TOF MS/MS analyses.

## MATERIALS

The ten compounds: albendazole, alprenolol, acebutolol, desipramine, haloperidol, nicardipine, pyrimethamine, quinine, tolbutamide, and verapamil were purchased from Sigma (St. Louis, MO). Trazodone (internal standard for LC/MS/MS analyses),  $\alpha$ -cyano-4-hydroxycinnamic acid (MALDI matrix), acetonitrile (ACN), formic acid, methanol, isopropanol, and trifluoroacetic acid were obtained from Sigma. The rat liver microsomes were bought from XenoTech (Kansas City, Kansas).

## EXPERIMENTAL

### MICROSOMAL INCUBATION

The microsomal incubation of each compound (final concentration of 2  $\mu$ M) was conducted in a 120  $\mu$ L final volume of 50 mM potassium phosphate buffer (pH 7.4), 3 mM  $MgCl_2$ , 1 mM EDTA containing 0.5 mg/mL of rat liver microsomes, and 2 mM NADPH in a 96-well plate for each time point. No NADPH was added for the 0 time control. The reactions were terminated by adding 12  $\mu$ L of 1 N HCl to each designated well at specific time points (0, 5, 10, 15, 20, and 30 minutes). The time points were adjusted for compounds with very high or low expected intrinsic clearance rates.

### PROTEIN PRECIPITATION

145  $\mu$ L of 90% acetonitrile in water was added to each aliquot of 55  $\mu$ L of the microsomal incubation sample at each time point. All tubes were vortexed and centrifuged at 13,000 rpm for three minutes. An aliquot of 80  $\mu$ L of the supernatant from each sample was transferred into a 96-well plate in duplicates. Each sample was evaporated to dryness using Zymark Turbo Vap® 96 and then reconstituted with 20  $\mu$ L of 0.01  $\mu$ g/mL of trazodone (internal standard) in acetonitrile/0.2% formic acid in water (v/v; 50/50).

### SOLID-PHASE EXTRACTION

Waters Oasis® HLB  $\mu$ Elution Plate (30  $\mu$ m) was used with a vacuum manifold. The plate was conditioned by washing each well with 200  $\mu$ L of methanol, followed by 200  $\mu$ L of Milli-Q water. After mixing 55  $\mu$ L of each microsomal incubation mixture with 45  $\mu$ L of water, the diluted mixture was passed through the solid-phase well plate and washed with 200  $\mu$ L of Milli-Q water. Each sample was eluted onto the collection plate with 120  $\mu$ L of acetonitrile/isopropyl alcohol (v/v; 40/60). Each eluent was transferred to a 96-well plate and was evaporated to dryness using Zymark Turbo Vap® 96 and reconstituted with 50  $\mu$ L of 0.01  $\mu$ g/mL of trazodone in acetonitrile/0.2% formic acid in water (v/v; 50/50).

### LC/MS/MS AND FIA/MS/MS

A four-minute HPLC separation was performed using an Aquasil C18 column (50 x 2.1 mm, 3 micron, Thermo Electron Corporation, Waltham, MA). A linear gradient program from 30% mobile phase B (100%  $H_2O$  with 0.1% formic acid) to 95% mobile phase B (ACN/ $H_2O$  with 0.1% formic acid; v/v; 90/10) in two minutes and kept at 95% mobile phase B for additional one minute was applied to elute the microsomal reaction mixture extract. The total run time including column re-equilibration was four minutes at a flow rate of 400  $\mu$ L/min. A total of ten (10) specific parent/daughter ion pairs (Table 1) were monitored under a multiple reaction monitoring (MRM) mode using an electrospray positive (ES+) ionization source on API 4000. The same MS/MS settings were used for FIA/MS/MS analysis, except that no analytical column was used and an isocratic LC program using 10% mobile phase A and 90% mobile phase B was applied for a two-minute run at a flow rate of 120  $\mu$ L/min. The samples were diluted when necessary to avoid the saturation of the peak signals for FIA/MS/MS analysis. The injection volume was 10  $\mu$ L in all cases.

## MALDI MS AND MALDI MS/MS

The samples were diluted 1 to 10 with  $\alpha$ -cyano-4-hydroxycinnamic acid at 5 mg/mL in 50% ACN/0.1% TFA. 0.5  $\mu$ L of diluted samples were spotted directly to a 192-spot MALDI sample plate and then profiled via MS and MS/MS analyses using an ABI 4700 TOF/TOF (Applied Biosystem, Foster City, CA) instrument in a reflector-positive mode. MS analysis was acquired from a mass range of m/z 150-800. MS/MS analysis was performed using the "Precursor Mass Methods" on a 4700 TOF/TOF.

## DATA HANDLING AND PROCESSING

LC/MS/MS and FIA/MS/MS data were acquired and processed using Analyst 1.4 (Applied Biosystems, Foster City, CA). The results (peak area ratios) were output to Microsoft Excel for further data analysis.

MALDI MS and MS/MS data were collected using a 4700 Explorer 2.0 (Applied Biosystems, Foster City, CA). Data Explorer 4.5 (Applied Biosystems, Foster City, CA) was used to display mass spectra. The MS and MS/MS mass spectra were processed using a stand-alone STAR program, developed in-house, and then the results (peak area ratios for MALDI MS and peak areas for MALDI MS/MS) were placed in Excel for data analysis.

## CALCULATIONS

A set of output peak area ratios (LC/MS/MS, FIA/MS/MS, and MALDI MS) or peak areas (MALDI MS/MS) at different time points against the 0 time was obtained for each compound. A semi-log curve of the peak area ratio or peak area versus incubation time was plotted (Figure 1). The half-life of the drug in the microsomal reaction mixture was determined using the plotted semi-log curve. The intrinsic clearance ( $CL_{int}$ ) rate was then calculated by applying the half-life value in the formula shown as follows:

$$CL_{int} = \frac{0.693}{\text{Half-life}} \times \frac{\text{Vol (Incubation Mixture)}}{\text{mg of Microsomes}} \times \frac{45 \text{ mg}}{\text{(g) Liver}} \times \frac{\text{Wt of liver (g)}}{\text{Body Wt (kg)}}$$

## RESULTS AND DISCUSSION

For these ten (10) test compounds, the results suggested that FIA/MS/MS could be an alternative HT approach for the  $CL_{int}$  measurement in comparison with the LC/MS/MS method if protein precipitation was used to process microsomal samples. The results also showed that the solid-phase extraction was essential for the MALDI analysis of the microsomal extracts (Table 2) to obtain expected  $CL_{int}$  values. Approximately 70% of the test compounds could not be analyzed by MALDI MS or MALDI MS/MS if the samples were processed by protein precipitation.

In terms of throughput, the MALDI MS and MALDI MS/MS was an ultra-fast tool (10 seconds vs. 2 minutes for FIA/MS/MS) when determining the  $CL_{int}$  measurement. FIA/MS/MS could be another alternative HT method used for the  $CL_{int}$  study; however, it would not allow any metabolite information to be obtained. Through the profiles of microsomal samples using MALDI MS and MALDI MS/MS, the metabolites of interests could be observed.

Figure 1 illustrates a representative semi-log intrinsic clearance plot obtained from Albendazole. Figure 2 shows the detection of verapamil metabolites by using MALDI MS and MALDI MS/MS.

**Table 1. A list of parent/daughter ion pairs for 10 test compounds monitored by LC/MS/MS and FIA/MS/MS**

TEST COMPOUNDS	MONOISOTOPIC MW (DALTONS)	PARENT ION (M/Z)	DAUGHTER ION (M/Z)
Albendazole	265.1	266.1	234.1
Alprenolol	249.2	250.0	116.4
Acebutolol	336.2	337.1	116.2
Desipramine	266.2	267.2	71.9
Haloperidol	375.1	375.8	165.0
Nicardipine	479.2	480.2	315.2
Pyrimethamine	248.1	249.0	233.2
Quinine	324.2	325.0	307.4
Tolbutamide	270.1	271.1	155.0
Verapamil	454.3	455.3	165.1

Table 2. A summary of intrinsic clearance measurement in rat liver microsomes

TESTING COMPOUNDS	CL <sub>r</sub> (L/hr/kg)												LITERATURE**
	PROTEIN PRECIPITATION			SOLID-PHASE EXTRACTION						MALDI MS/MS	MALDI MS	MALDI MS/MS	
	LC/MS/MS	FIA/MS/MS	MALDI MS	MALDI MS/MS	LC/MS/MS	FIA/MS/MS	MALDI MS	MALDI MS					
Albendazole	5.9	5.3	6.2	—	9.4	6.5	5.2	4.9	7.9				
Alprenolol	44.2	42.4	—	—	43.1	47.7	—	36.8	55.5				
Acebutolol	2.6	2.0	—	—	2.2	4.5	1.3	2.6	3.1				
Desipramine	8.8	17.6	—	—	7.6	9.5	19.1	9.8	9.0				
Haloperidol	4.7	8.9	—	7.8	5.5	5.6	3.7	6.2	7.5				
Nicardipine	55.8	54.1	—	—	69.2	64.4	—	44.1	45.0				
Pyrimethamine	1.0	0.6	1.8	0.7	0.5	0.9	0.6	0.5	1.2				
Quinine	1.1	0.6	—	—	0.6	0.8	3.9	2.5	1.5				
Tolbutamide*	0.7	—	—	—	0.8	0.5	—	—	0.3				
Verapamil	12.6	12.4	17.9	10.6	13.8	11.9	10.1	10.4	12.7				

\*Tolbutamide could not be ionized using the MALDI source and no signal was observed.

Figure 1. A representative (albendazole) semi-log intrinsic clearance plot

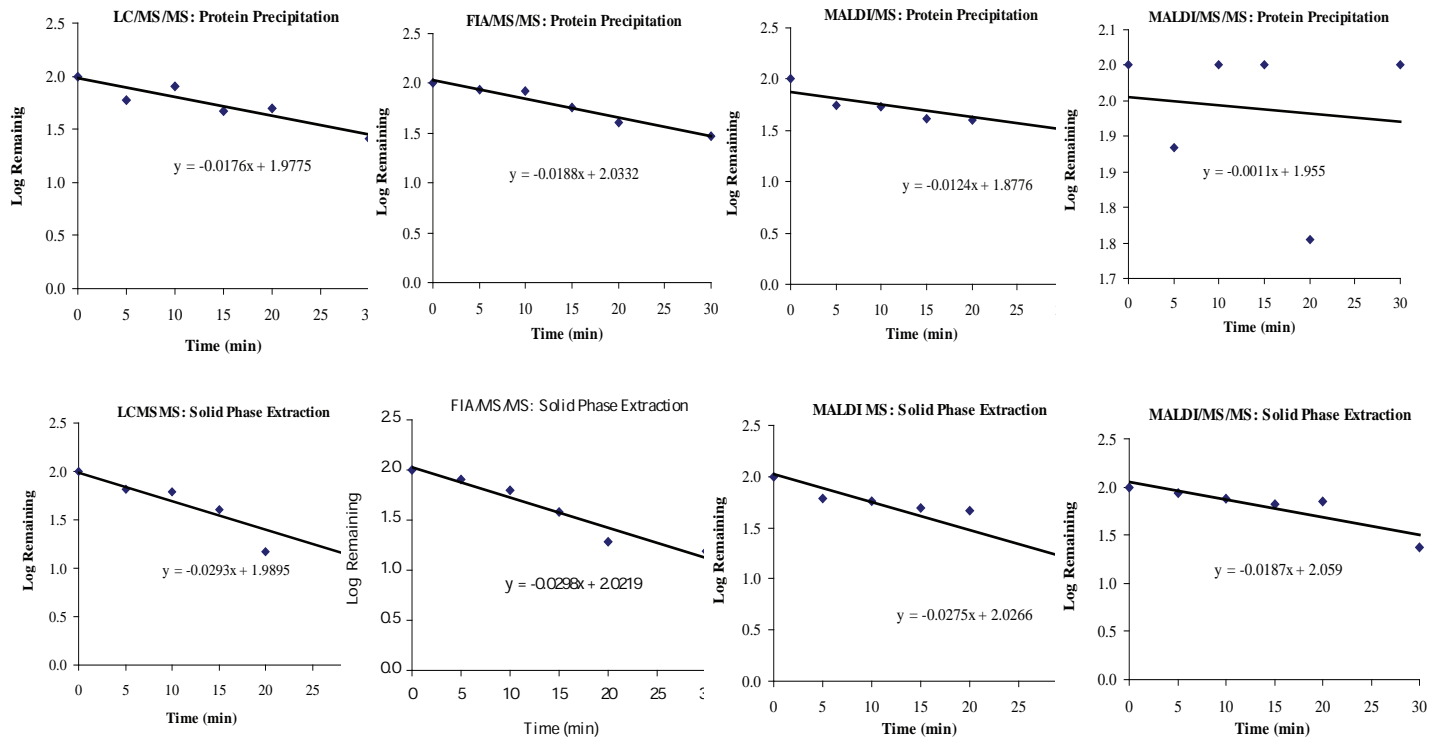


Figure 2A. MALDI-TOF MS spectra of rat liver microsomes incubated with verapamil at 0 and 30 minutes

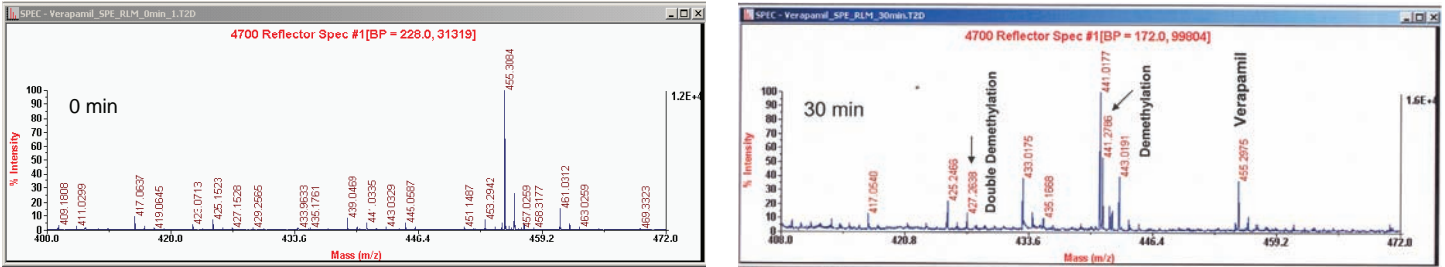


Figure 2B. Time course profiles of rat liver microsomes incubated with verapamil (0, 5, 10, 15, and 30 minutes)

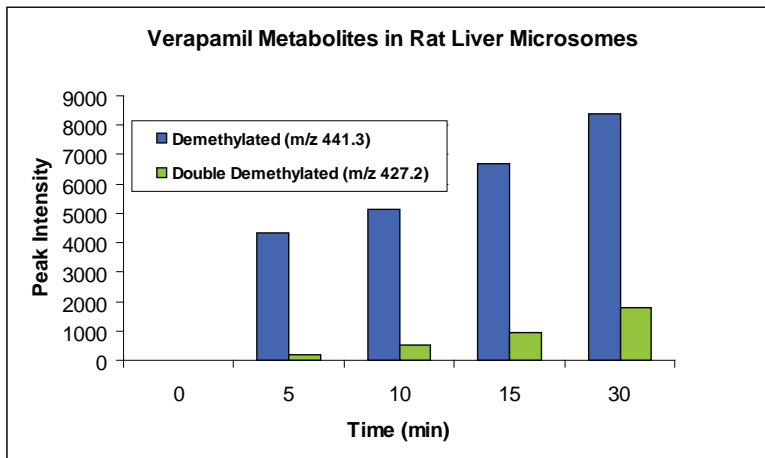
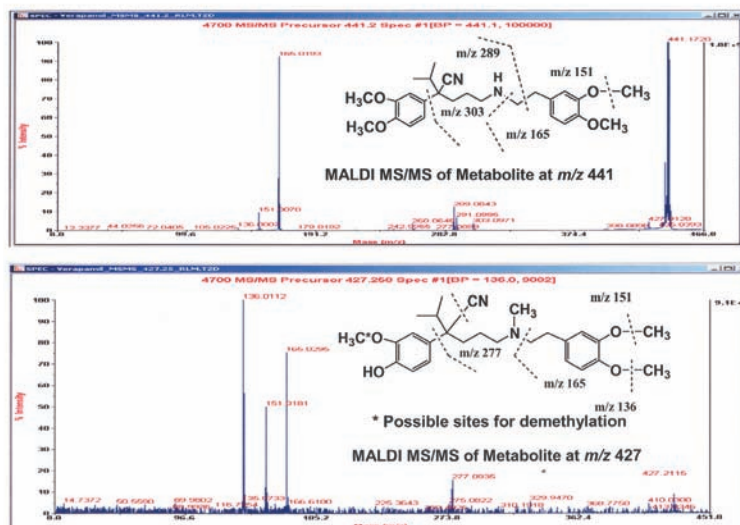


Figure 2C. Examples of identifying verapamil metabolites by MALDI-TOF MS/MS



## CONCLUSIONS

With the solid-phase extraction procedure, both FIA/MS/MS and MALDI MS/MS were viable as alternative HT methods for the measurement of the intrinsic clearance of compounds. MALDI-TOF MS could be used to detect potential metabolites through the profiles of the samples.

MALDI-TOF MS/MS would provide MS/MS fragmentation information to allow elucidation of metabolite structures in addition to  $CL_{int}$  measurement.

## REFERENCE

\*\* Zhixia Yan, Chuang Lu, Jing-Tao Wu, Larry Elvebak, and Adam Brockman. Rapid Commun Mass Spectrom 2005; 19:1191.

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