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Atmospheric pressure desorption/ionization on silicon ion trap mass spectrometry applied to the quantitation of midazolam in rat plasma and determination of midazolam 1′-hydroxylation kinetics in human liver microsomes

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The application of atmospheric pressure desorption/ionization on silicon (AP-DIOS) coupled with ion trap mass spectrometry (ITMS) was investigated for the quantification of midazolam in rat plasma, and determination of midazolam 1′-hydroxylation kinetics in pooled human liver microsomes. Results indicate good sensitivity with absolute detection limits for midazolam in rat plasma of approximately 300 femtograms. A linear dynamic range from approximately 10–5000 ng/mL was obtained in rat plasma with analysis times of 1 min per sample. Kinetic constants for midazolam 1′-hydroxylation in human liver microsomes yielded an apparent K_m of 10.0 μM and V_{max} of 6.4 nmol/min/mg. Studies investigating the inhibition of 1′-hydroxymidazolam formation by the cytochrome P450 3A4 model inhibitor ketoconazole yielded an IC_{50} of 0.03 μM. Quantitative precision for replicate analysis of rat plasma and human liver microsomal samples was variable with relative standard deviation (RSD) values ranging from a low of approximately 3% to over 50%, with the highest variability observed in data from human liver microsomal incubations. While preliminary studies investigating the application of AP-DIOS-ITMS suggested feasibility of this technique to typical pharmacokinetic applications, further work is required to understand the underlying causes for the high variability observed in these investigations. Copyright © 2006 John Wiley & Sons, Ltd.

Desorption/ionization on silicon mass spectrometry (DIOS-MS) is an evolving chip-based laser desorption platform that has demonstrated utility in analysis of low molecular weight compounds. Unlike matrix-assisted laser desorption/ionization (MALDI), the DIOS technique relies on deposition of the compound on a variety of porous silica surfaces and subsequent laser desorption to produce protonated or deprotonated molecular ions in the gas phase utilizing a matrix free environment. The absence of typical MALDI matrices eliminates interfering low molecular weight gas-phase ions from the spectral background thereby allowing for analysis of compounds with $m/z < 300$. A variety of low molecular weight biomolecules and drugs have been characterized using DIOS-MS. These compounds include: cimetidine, midazolam, chlorpromazine, dopamine, norepinephrine, pregnenolone, choline, acetylcholine, and a variety of monosaccharides.[1–4] Finally, the chip-based DIOS platform allows for rapid analysis due to fast desorption times (<1 min/sample), and the ability to rapidly raster the laser over multiple samples on a single chip in an automated fashion. Coupled with high-resolution time-of-flight (TOF)-MS (or tandem TOF-MS) and utilizing stable isotope labeled internal standards, DIOS has demonstrated similar quantitative abilities to electrospray ionization (ESI)-MS.[5] Therefore, the technique may have a broader application within the arena of bioanalytical analysis in the pharmaceutical industry.

A desire to provide rapid and precise qualitative pharmacokinetic (PK) data throughout the drug discovery and development process has led to the evaluation of a number of high-throughput MS approaches to maximize use of laboratory resources. The general focus of these efforts is to utilize rapid and simple sample preparation techniques, and detection methods optimized to decrease sample analysis time while maintaining assay precision and robustness.

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In a related application, *in vitro* investigations using model systems are often designed to predict the enzymatic mechanism of drug clearance and drug-drug interactions and are important studies to perform early in drug discovery to predict the *in vivo* characteristics of a drug in the target species. The cytochrome P450 (CYP) enzymes as a family play a critical role in the clearance mechanism of xenobiotics in humans and as such have been studied using a variety of techniques including radiometric,[6] fluorescence,[7] and MS-based[8] analytical approaches. The nature of these analytical approaches are constantly being challenged to improve reliability and analytical capacity as the number of candidates for screening continues to increase in the face of decreasing analytical resources. For example, Cohen *et al.* have demonstrated a lack of correlation between CYP inhibitors *in vitro* when using fluorescent vs. conventional P450 probe substrates, arguing for liquid chromatography (LC)/MS-based approaches.[7] The major disadvantage of LC/MS-based approaches in this case is lower throughput relative to fluorescence-based methods.

In this work we explore the utility of atmospheric pressure desorption/ionization on silicon coupled with ion trap mass spectrometry (AP-DIOS-ITMS) for the quantification of midazolam from rat plasma and the quantification of 1′-hydroxymidazolam metabolites from *in vitro* human liver microsomal incubations. The objective is to challenge this technique with samples prepared from biological matrices typically encountered in the drug discovery and development process in order to determine the feasibility of AP-DIOS-ITMS for routine analytical application in the R&D environment.

EXPERIMENTAL

Materials

Midazolam, 1′-hydroxymidazolam, verapamil, propafenone, ketoconazole and potassium phosphate buffer were purchased from Sigma (St. Louis. MO, USA). The solvents used included acetonitrile and methanol purchased from EM Science (Darmstadt, Germany), and Milli-Q organic-free water (Millipore Corporation, Bedford MA, USA). Blank EDTA rat plasma was obtained from Bioreclamation (Hicksville NY, USA) and pooled human liver microsomes (specific activity of 0.33 nmol P450/mg protein) were purchased from BD Biosciences (Bedford, MA, USA). β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Mass spectrometry

AP-DIOS-ITMS experiments were performed on an Agilent (Palo Alto, CA, USA) MSD 1100 MSD/Trap XCT Plus mass spectrometer equipped with an AP-MALDI source. The MALDI target was modified to accommodate DIOS chips. Data acquisition was automated using Agilent software. The ion trap was operated under multiple reaction monitoring (MRM) using the product ions indicated below. The DIOS plate sample spot was randomly moved at 0.1 mm steps to maximize the signal. The laser was operated at 10 Hz. Analysis of each sample took approximately 1 min.

DIOS chip preparation

Preparation of DIOS chips has been described elsewhere.[3] Briefly, DIOS chips were prepared by etching low resistivity wafers (Silicon Quest) in 25% v/v hydrofluoric acid under white light. Photopatterning was performed to create 36 sample spots on each chip. The DIOS chip was then rinsed with ethanol and dried in a stream of nitrogen to give an H-terminated surface, which was oxidized by exposure to ozone and immersed in 5% HF to regenerate the Si-H terminations. Then the Si–H surface was oxidized again by O_3 and modified with (heptadecafluoro-1,1,2,2-tetrahydrododecyl)dimethylchlorosilane as silylating reagent. This simple silylating procedure generates a modified DIOS chip terminated with a fluoroalkyl group.

Rat plasma standards

Plasma standards (0–5250 ng/mL), plasma blanks, and controls were prepared by fortification of rat plasma with methanolic solutions of midazolam. Plasma samples were then subsequently extracted for analysis by protein precipitation. Briefly, to 50 μL of rat plasma standards, blanks, or controls, 150 μL of acetonitrile containing verapamil as an internal standard were added. Samples were centrifuged for 15 min and the supernatant was transferred into new polypropylene tubes that were frozen at −80°C until analysis. Prior to analysis samples were thawed at room temperature and centrifuged at 16 100 *g* for 10 min at 4°C. In the DIOS experiments, 1 μL of each sample was pipetted onto a DIOS chip then the remaining surface sample was immediately removed by pipettor. This process allows for adsorption of analyte onto the silylated fluoroalkyl surface and partial removal of some potential interfering solvent, matrix, and buffer residues. Precursor ions (*m*/*z*) of 326 (midazolam) and 455 (verapamil) were chosen for fragmentation. MS/MS spectra in the *m*/*z* range of 100–500 were collected and averaged for data analysis. Product ions of *m*/*z* 291 (midazolam) and *m*/*z* 303 (verapamil) were chosen for detection based on acceptable product ion intensity and specificity for the compounds of interest in the tested plasma matrix (data not shown). Proposed sites of product ion formation for midazolam and verapamil are illustrated in Fig. 1.

Microsomal preparation and incubations

Microsomal incubations characterizing midazolam 1′-hydroxylation kinetics were carried out using 0.2 mg/mL human liver microsomal protein in 100 mM potassium phosphate buffer pH 7.4 with a midazolam concentration range of 0–50 μM. Incubation mixtures (150 μL final volume) were pre-incubated at 37°C for 3 min, and then 1 mM NADPH was added to initiate the reaction. After a 5 min incubation, ice-cold acetonitrile (100 μL) was added to quench the reaction, and samples were frozen at −80°C until analysis. Prior to analysis samples were thawed at room temperature and centrifuged at 16 100 *g* at 4°C for 10 min. A volume of 10 μL of each sample was mixed with 10 μL of 1 μM propafenone (MW 341). Samples were dried in a SpeedVac at room temperature and then resuspended in 10 μL of water. Incubation conditions were similar for investigating inhibition of midazolam 1′-hydroxylation by ketoconazole. A

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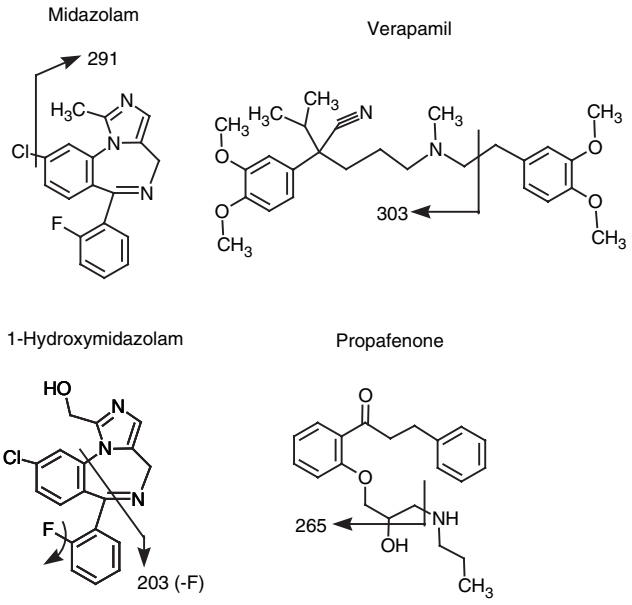

Figure 1. Chemical structures for compounds analyzed in this report (proposed site of product ions used for DIOS analysis are illustrated).

ketoconazole concentration range of 0.002–5 μM was used, with a single concentration of midazolam set at 2.5 μM (apparent K_m from previous studies, data not shown). Formation rates of 1′-hydroxymidazolam were quantified against a standard curve (0–4000 nM) substituting 100 mM potassium phosphate for NADPH. Standard curve samples were incubated and prepared similar to midazolam incubations above.

In the DIOS experiments, 1 μL of each sample was applied to the DIOS chip as detailed above. Samples were then analyzed in MRM mode. The average intensities of the product ions formed for the transitions of *m/z* 342 → 203 (1′-hydroxymidazolam) and 342 → 265 (propafenone) were used for quantitation. Product ions were selected for detection based on acceptable product ion intensity and specificity for the compounds of interest in the tested microsomal matrix (data not shown). Proposed sites of product ions for 1′-hydroxymidazolam and propafenone are illustrated in Fig. 1.

Calculations

Standard curve regression analysis and quantification of midazolam in rat plasma as well as enzyme kinetics and IC_{50} determinations from human liver microsome experiments were performed in GraphPad Prism 4.0 (San Diego, CA, USA). Statistical analysis of the data was performed in Excel 2000. Standard curves were constructed using linear regression forced-through-the-origin. No weighting was employed. Kinetic constants (K_m and V_{max}) were determined by fit of mean data to the Michaelis-Menten equation ($v = V_{max} \times S/(K_m + S)$). Estimation of IC_{50} for ketoconazole was calculated using the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10^{(X - \text{LogEC50})})$.

RESULTS

Quantification of midazolam in rat plasma

In this work we studied the linearity and precision of midazolam quantified in rat plasma in order to evaluate the utility of the AP-DIOS-ITMS technique for providing rapid analysis times with minimal sample preparation. A standard curve was generated by plotting the mean peak intensity ratios of the midazolam product ion peak (*m/z* 291) intensity divided by the verapamil product ion peak (*m/z* 303) intensity averaged over the 1-min sampling interval (Fig. 2). The fortified plasma standard curve samples

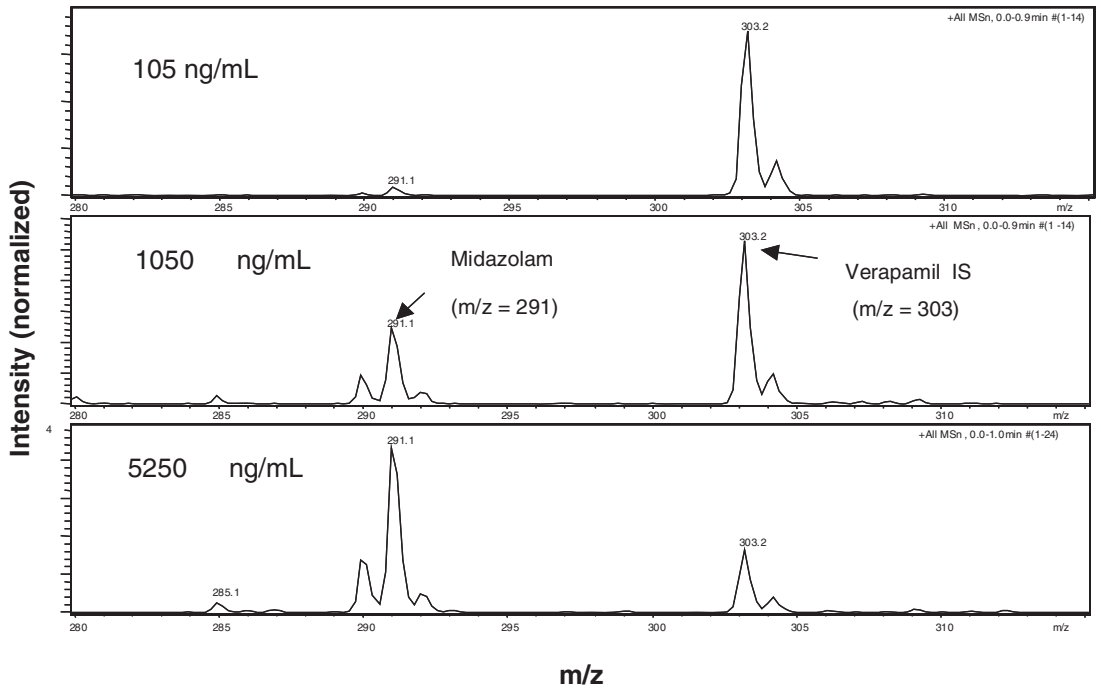

Figure 2. Representative AP-DIOS-ITMS product ion spectra from rat plasma standards showing midazolam (*m/z* 291) and the verapamil internal standard (*m/z* 303).

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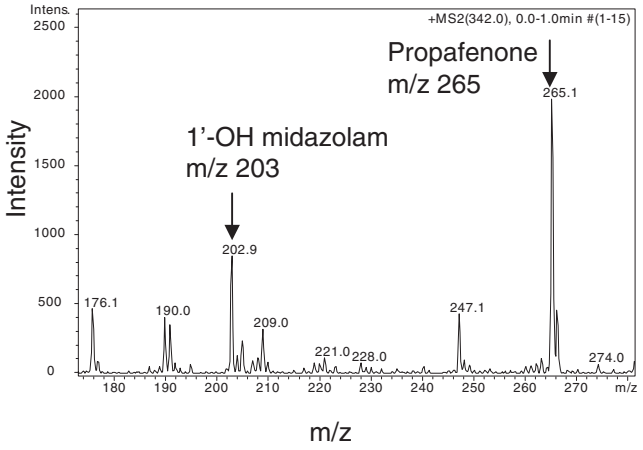

Figure 3. Representative AP-DIOS-ITMS product ion spectra from a representative microsomal standard showing 1′-hydroxymidazolam (*m/z* 203, 490 nM concentration) and the propafenone internal standard (*m/z* 265).

Figure 4. Standard calibration curve for midazolam in rat plasma correlating the intensity ratio of midazolam divided by the internal standard with the concentration of midazolam. Error bars denote standard deviation (SD) of three replicate analyses by AP-DIOS-ITMS.

demonstrated good linearity ($R^2 = 0.9977$) and dynamic range (10–5000 ng/mL), with precision ranging from 3.1–25.2% over the entire range of the curve (Fig. 4, Table 1). Replicate blank samples showed no interfering peaks at the masses monitored for midazolam or the verapamil internal standard. Control sample recovery ranged from 112% of target at the high concentration quality control (QC) level to 179% at the lower limit of quantitation (10 ng/mL) (Table 2).

Midazolam kinetics in human liver microsomal incubations

In vitro investigations into drug disposition often involve the measurement of substrate metabolism in a liver microsomal matrix with the intention of understanding the kinetics of this process. This may be performed as a minimalist screen involving sparse sampling at a few predictive concentrations, or more definitive analysis designed to provide accurate kinetic values. These data can subsequently be scaled to estimate the pharmacokinetic characteristics of a test compound in test species or man. As a second part of this investigation, we explored the utility of AP-DIOS-ITMS for rapid throughput reaction monitoring as applied to the determination of the cytochrome P450 3A4 (CYP3A4)-mediated midazolam 1′-hydroxylation in pooled human liver microsomes. In addition, the utility of DIOS for use in high-throughput drug-drug interaction screening was explored by evaluation of the inhibitory effect of the model CYP3A4 inhibitor ketoconazole on midazolam 1′-hydroxylation kinetics in human liver microsomes.

A product ion of *m/z* 203 was observed for 1′-hydroxymidazolam, consistent with reports showing this to be a unique fragment for this metabolite.[9] Utilizing this fragment ion and a product ion of propafenone (*m/z* 265) as internal standard (Fig. 3), a calibration curve was constructed for determination of 1′-hydroxymidazolam in microsomal incubations (Fig. 5). The resulting Michaelis-Menten plot and associated kinetic constants for 1′-hydroxymidazolam formation in human liver microsomes are shown in Fig. 6. The formation rate of 1′-hydroxymidazolam exhibited typical Michaelis-Menten saturation kinetics with a V_{max} of 6.4 ± 0.49 nmol/min/mg, and an apparent K_m of 10.0 ± 2 μM, which are in agreement with the range of literature values in human liver microsomes.[10,11] Determination of the inhibitory potential of ketoconazole on the formation rate of 1′-hydroxymidazolam is illustrated in Fig. 7. The IC_{50} for

Table 1. Midazolam standard curve data from rat plasma assayed by AP-DIOS-ITMS showing back-calculated concentrations of individual replicates, assay variability (relative standard deviation, RSD) and accuracy

Nominal conc. ng/mL	Determined concentrations ng/mL			Mean ng/mL	RSD %	% Accuracy
	Rep 1	Rep 2	Rep 3			
0.0	0.0	0.0	0.0	0.0	NA	NA
10.5	11.4	19.1	15.9	15.5	25.2	147.3
26.3	24.8	26.3	25.3	25.5	3.1	96.8
52.5	43.0	60.5	56.9	53.5	17.3	101.8
105.0	96.4	111.8	105.6	104.6	7.4	99.6
262.5	320.7	244.6	296.7	287.3	13.5	109.5
525.0	729.1	524.3	740.0	664.4	18.3	126.6
1050.0	750.3	909.2	1030.1	896.5	15.7	85.4
2100.0	2009.7	2333.9	1794.3	2046.0	13.3	97.4
3150.0	3297.0	3069.1	2669.9	3012.0	10.5	95.6
4725.0	4546.6	5067.1	5073.5	4895.7	6.2	103.6
5250.0	4677.9	5185.0	5785.9	5216.3	10.6	99.4
Mean	NA	NA	NA	NA	12.83	105.73

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Table 2. Midazolam rat plasma quality control (QC) sample results showing back-calculated concentrations of individual replicates, assay variability (RSD) and accuracy

QC Nominal conc. ng/mL	Determined conc. ng/mL	% Bias	Mean conc.	SD	RSD	Mean % accuracy
3937.5	4085.5	103.8				
	5230.0	132.8				
	3882.2	98.6	4399.2	726.6	16.5	111.7
262.5	327.6	124.8				
	264.6	100.8				
	340.6	129.8	310.9	40.7	13.1	118.4
31.5	33.4	106.0				
	53.3	169.1				
	69.8	221.7	52.2	18.3	35.0	165.6
10.5	13.3	126.3				
	25.4	242.1				
	17.7	168.4	18.8	6.2	32.8	178.9

ketoconazole was determined to be approximately 0.03 μM, and is in range with reported inhibitory potency for ketoconazole under similar experimental conditions.[12] Calibration curve linearity and variability observed from replicate analysis was high, ranging from 23–54% relative standard deviation (RSD) for the 1′-hydroxymidazolam kinetic determinations.

DISCUSSION

Quantitative applications of DIOS using TOF-MS have been previously described.[4,5,13] In general these studies have demonstrated quantitative utility of the technique in relatively simple sample matrices such as purified enzyme or simple solutions of low molecular weight compounds, and have typically employed stable isotope labeled internal standards and vacuum region MALDI source designs. In this work we extend these investigations to explore the utility of AP-DIOS-ITMS for quantitative analytical applications as applied to a pair of prototypical sample types encountered in pharmaceutical discovery and development investigations.

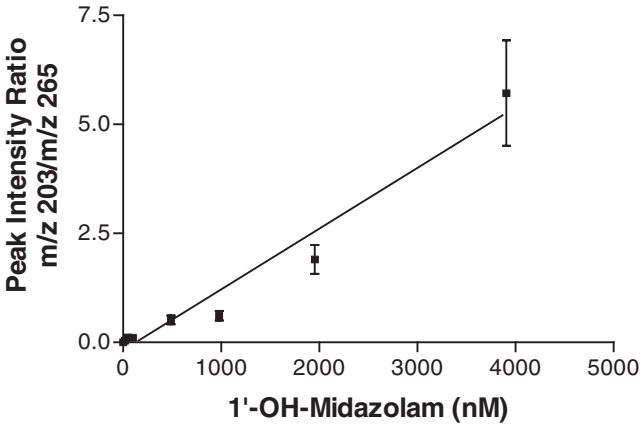
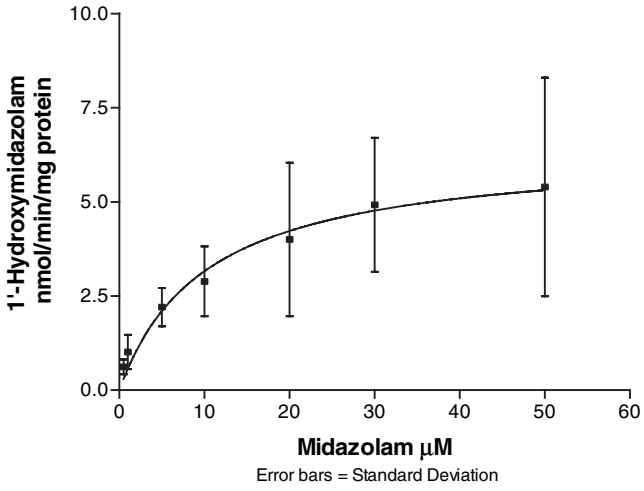


Figure 5. Standard calibration curve for 1′-hydroxymidazolam in microsomal incubations correlating the intensity ratio of 1′-hydroxymidazolam to the internal standard with the concentration of 1′-hydroxymidazolam. Error bars denote SD of three replicate analyses by AP-DIOS-ITMS.



Kinetic Parameter	1'-OH Midazolam ± SE
V_{max} nmole/min/mg	6.40 ± 0.49
K_m (μM)	10 ± 2
r^2	0.9773
n	3

Figure 6. Kinetic plots and kinetic parameters of 1′-hydroxymidazolam formation from midazolam following incubation in human liver microsomes. Error bars denote SD of three replicate analyses by AP-DIOS-ITMS.

Samples prepared from biological matrices such as plasma and liver microsomal isolates often present analytical challenges due to the complex array of constituents present in these samples. Therefore, sample extraction and purification techniques are often required prior to analyte detection in order to minimize the likelihood of interference of other matrix components with the detection technique. In addition, selective methods of detection including a resolving chromatographic separation and detection using tandem mass spectrometry, and analog or stable isotope labeled internal standards, are necessary to further enhance selectivity for the compound in question, but may add time and complexity to the analytical process.

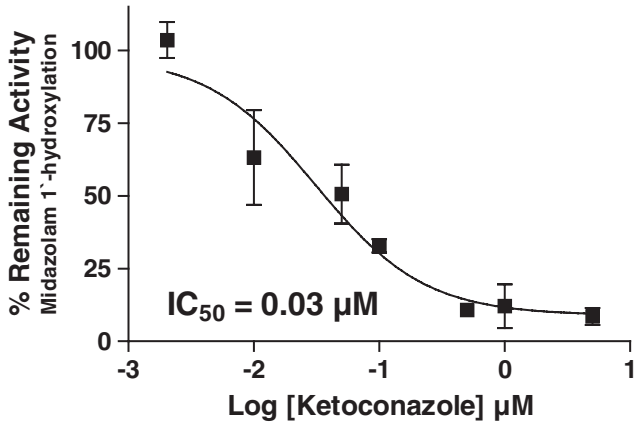


Figure 7. IC_{50} plot of ketoconazole inhibition of 1′-hydroxymidazolam formation in pooled human liver microsomes.

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In this work we used a sample prepared from crude acetonitrile extractions from rat plasma and human liver microsomal incubations of which only 1 μL of sample extract was pipetted on the DIOS plate. The subsequent removal of residual solvent immediately following sample placement provides additional sample cleanup by preferential absorption of analytes to the modified silica surface and removal of excess solvent, buffer, and sample constituents which could interfere with sample analysis. The internal standard compounds (verapamil and propafenone) selected for this work are typical of the types of compounds often used during early discovery efforts where chemically diverse compounds are often screened with a single internal standard in a generic assay design. Use of the AP-MALDI interface facilitated easy loading and unloading of DIOS sample chips, and detection using the Agilent MSD XCT ion trap facilitated detection selective to the analytes of interest with a 1-min analysis time per sample.

Data obtained for rat midazolam quantitation demonstrated acceptable linearity and dynamic range; however, variability of replicate analysis and the analytical precision (especially at low concentrations) were outside of the range typically required for precise pharmacokinetic analysis, and in general do not approach the less stringent precision allowances for other high-throughput screening applications. In the case of the 1′-hydroxylation kinetic determination in human liver microsomes, mean determinations of replicate samples produced kinetic constants consistent with literature values; however, as with the rat plasma quantitative results, improvement in quantitative precision and accuracy would be required for application of this technique to routine analysis in the pharmaceutical environment. The observed high variability for these investigations may be due to a variety of reasons including the limited dynamic range of the ion trap, limitations on ion transfer rates in the AP-MALDI source, variables in deposition of sample and/or the lack of a stable isotope internal standard controlling the analysis. The dynamic range of ion traps has been demonstrated to be acceptable for quantitation over a 3–4 magnitude range (similar to the ranges evaluated in this work) when utilizing ESI.[14,15] However, for this work, an AP-DIOS interface was employed for analysis of rat plasma and human microsomes and as such may represent a seminal report on the utility of the AP-DIOS technique for these types of bioanalytical quantitation challenges. Other potential sources of variability may result from interfering sample constituents via ion inhibition, ion complex formation, or competition reactions, details of which were not pursued in this work. Future investigations into these elements will provide additional insight into the experimental conditions necessary to optimize AP-DIOS-ITMS for improved detection limits and enhanced precision required for quantitative bioanalytical analysis.

CONCLUSIONS

While the application of AP-DIOS-ITMS demonstrated preliminary results suggesting feasibility of this technique to typical pharmacokinetic applications, providing adequate sensitivity, dynamic range and sample throughput for bioanalytical studies, and yielding kinetic constants consistent with reported values for midazolam metabolite production in human liver microsomes, further work is required to understand the underlying causes of high variability observed from replicated samples in these investigations.

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