High Throughput Workflow for Midazolam and 1-Hydroxymidazolam Analysis in Human Plasma

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OVERVIEW

•Our work illustrates a high throughput work flow for the analysis of a parent drug and metabolite in human plasma.

•This involves 96-well SPE sample extraction using an automated SPE workstation.

•The extracts are then evaporated to dryness, reconstituted, and spotted onto a 96-well plate specific for Laser Diode Thermal Desorption (LDTD) onto a tandem MS/MS.

•Current technology for this work extracting 96 columns and performing an LC/MS/MS analysis would require approximately 10 hours for the clean-up and separation. Our high throughput workflow resulted in an approximately 10 fold reduction in time to 1 hour for the 96 well extraction and LDTD-MS/MS analysis.

•We will present data showing a decrease in sample time while maintaining or improving the reproducibility of the data.

INTRODUCTION

•In pharmaceutical laboratories, the pressure is on to increase sample throughput while decreasing human intervention. In an effort to meet this demand, we developed a high throughput workflow for the analysis of Midazolam, a well-known benzodiazepine derivative and its phase I metabolite, 1-Hydroxymidazolam. This workflow allows the automated solid phase extraction of the compounds and their analysis in tandem mass spectrometry using a Laser Diode Thermal Desorption (LDTD) ionization source known for its speed of operation.

•An automated liquid handler system is used to extract 96 SPE samples at the same time reducing the sample preparation time and human manipulation. On the analytical side, an LDTD-MS/MS system is used as a shotgun approach where no chromatographic separation is involve prior to the MS/MS analysis, reducing the analytical time significantly.

LazWell plate (Figure 1)
– 96-well plate
– Allows a sample volume from 1 to 10 µL
– Standard dimension for liquid handler system
– Metal insert sheet for the thermal desorption

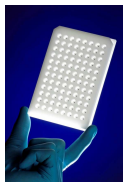

Figure 1 LazWell plate (96-well).

METHOD

Sample Preparation

- 500 µL Human Plasma.
- Internal Standard Diazepam-d5 at 200 ng/mL.
- 500 µL 0.1 M Phosphate Buffer pH 6.0.

Extraction Method

- SPE performed on Caliper Life Science's Zephyr™ SPE Workstation
- SPE Plate: UCT, LLC 96-Well SPE plate Part # WORDAU11

- SPE Method :

Condition Step

900 µL Methanol	Vacuum = 30 sec
900 µL DI Water	Vacuum = 45 sec
900 µL 0.1 M PO_4 Buffer pH 6.0	Vacuum = 60 sec

Load Sample

1000 µL Sample	Vacuum = 100 sec

Wash

1050 µL DI Water	Vacuum = 45 sec
1050 µL 20% ACN/80% PO_4 buffer	Vacuum = 600 sec
1800 µL Methanol	Vacuum = 300 sec

Elution

500 µL Ethyl Acetate with 2% NH_4OH	Vacuum = 20 sec
500 µL Ethyl Acetate with 2% NH_4OH	Vacuum = 20 sec
500 µL Ethyl Acetate with 2% NH_4OH	Vacuum = 45 sec

- Post Extraction :
  The sample is evaporated to dryness in a TurboVap® at 35 °C. The dry plate is reconstituted with 200 µL of 50:50 Methanol:Water and then diluted 1:10 with Ethyl Acetate containing 90 µg/mL of stearic acid.

- Analysis:
  2 µL of the reconstituted extracted samples were deposited in a LazWell™ 96-well plate. The solvent evaporates at room temperature.

LDTD-MS/MS Parameters

LDTD Parameters
– Laser power pattern :
  – Increase laser power to 55 % in 3.0 sec.
  – Hold at 55 % for 1.0 sec.
  – Decrease laser power to 0 %
– Carrier gas flow : 3 L/min (Air)
– Corona voltage value : 4 kV
– Deposited sample volume: 2 µL

MS/MS Parameters
– Scan time : 0.05 s
– Q1 width : 0.70 amu
– Q3 width : 0.70 amu
– Q2 gas pressure (Ar) : 1.5 mTorr
– SRM transition (**Table 1**)

Table 1 SRM transition and collision energy.

Analytes	Q1 (m/z)	Q3 (m/z)	Collision Energy (V)
Diazepam -d_5	290.2	159.1	27
1'-OH-midazolam	342.1	203.1	27
Midazolam	326.0	291.0	27

RESULTS

Manual v. Automated Well Spotting

Comparison of manually spotting to using the Zephyr SPE Workstation to spot the LazWell 96-well plates. One sample plate was prepared with plasma spiked with known concentrations of Midazolam, 1-Hydroxymidazolam and Diazepam-d5 as an internal standard (IS). This plate was extracted following the specified method using the Zephyr SPE Workstation. The plate was evaporated, reconstituted and diluted as specified.

From this plate the Zephyr SPE Workstation was used to deposit 2 µL spots in a LazWell 96-Well plate. From the same plate, LazWell 96-Well plates were spotted manually. The 2 spotted plates were analyzed using the LDTD-MS/MS detection. The raw signal in terms of area count as well as the normalized area ratio (with IS) was compared for precision of the manual versus automated spotting technique.

As shown in **Table 2**, spotting the LazWell plate with 2 µL using a robotic device significantly increases the precision by approximately 4%. The normalized signal give the same precision which indicates that the robotic device adequately corrected for the volume spotted. However, spotting manually takes at least 10-times more time than the robotic device.

Table 2 Relative standard deviation (RSD) on raw and normalized signal for manual and robotic well spotting.

Plate spotting	Analyte	% RSD	
		Raw area count	Normalized against IS
Manual	Diazepam-d_5	14.6	-
	Midazolam	15.9	4.2
	OH-Midazolam	18.7	7.6
Robot	Diazepam-d_5	11.6	-
	Midazolam	11.5	4.3
	OH-Midazolam	12.3	7.5

Increased Throughput Workflow

In our work the increase in throughput work flow primarily comes from 2 areas:
a) the SPE step and b) the analysis step

SPE Extraction- Manual Extraction v. Automated Liquid Handling
- Running 96 samples manually in a vacuum box, and experienced operator can process each sample in 2 minutes, x 96 samples = 192 minutes.
- Using an automated workstation with a 96 well plate the entire plate of 96 samples is completed in ~40 minutes.

Analysis- LC/MS/MS v. LDTD-MS/MS
- Many labs are using LC/MS/MS for analysis, requiring about an average of 5 minutes chromatography time per sample, x 96 samples is 480 minutes.
- The higher throughput option is LTDT-MS/MS completing an entire 96 well plate in 20 minutes.

Increased Throughput Workflow

The proposed method of analysis (Automated SPE followed by LDTD-MS/MS) leads to a ~11X increase in the throughput capability when compared to traditional analysis workflow involving manual SPE followed by LC-MS/MS (**Table 3**).

Table 3 Analytical time relative to the traditional manual SPE LC-MS/MS workflow versus high throughput automated SPE LDTD-MS/MS workflow.

	Workflow	
	Traditional	***High Throughput***
SPE step	96 individual columns on a vacuum box 2 minutes/sample x 96 samples = 192 minutes	96 well SPE plate on an automated workstation 40 minutes /96 well plate
Analysis step	LC-MS/MS 5 minutes/sample x 96 samples = 480 minutes	LDTD-MS/MS 20 minutes /96 well plate
Total workflow	672 minutes	60 minutes

Calibration Curve for Quantitation

Using the automated system, human plasma was spiked in order to evaluate the linearity and the limit of detection for the Midazolam and OH-midazolam quantitation. The calibration curve is shown in **Figure 2**. The r^2 of 0.997 for both analytes are excellent and the LOD was estimated to be 1 ng/mL based on the blank signals.

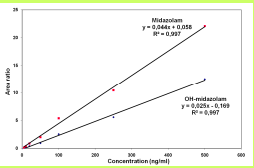

Figure 2 Calibration curve for Midazolam and OH-Midazolam.

CONCLUSIONS

Comparison of the manually spotted to robotically spotted LazWell 96-Well plates shows less variability of the signal area for the robotically spotted plates than the manually spotted plates.

When the same data is normalized against the Diazepam-d5 internal standard, both plates show comparable results indicating good precision and accuracy and limited variations in LDTD/MS/MS sampling volume.