APPLICATIONS

A Fast Approach for the Determination of Metformin in Human Plasma Using Phree™ Phospholipid Removal Plate and LC-MS/MS

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Introduction

Metformin is a generic medication widely used as a first-line treatment for Type 2 diabetes, particularly in overweight patients, and has also shown some promise in helping to prevent the cardiovascular disease and cancer complications associated with diabetes. Metformin decreases high blood sugar primarily by suppressing liver glucose production. With the increasing diagnosis of Type 2 diabetes worldwide, it is expected that treatment with metformin will continue to increase. As its importance increases, quality control testing of metformin oral dose drug products takes on increased importance, and analytical testing such as dissolution and related bioequivalence (BE) studies need to be more robust and efficient.

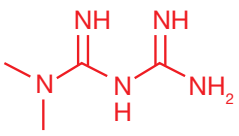
Metformin is a highly polar biguanide molecule, which can present chromatographic challenges in a complex matrix such as plasma. Consistent with common bioequivalence studies used by generic pharmaceutical drug companies, we will demonstrate a fast analytical method including both sample preparation and LC-MS/MS analysis of metformin in human plasma. Sample preparation was done using protein precipitation in conjunction with the elimination of phospholipids using the Phree phospholipid removal plate. This sample preparation technique is a very effective means for the removal of endogenous plasma proteins and phospholipids, which can have a deleterious effect on analytical column lifetime and ion suppression in MS detection. The method was evaluated for sensitivity, linearity, precision, accuracy, and recovery of the extraction method using Phree phospholipid removal plates for fast sample preparation to minimize potential matrix interferences. A core-shell Kinetex® 5 µm C18 50 x 4.6 mm analytical column was utilized for rapid LC-MS/MS analysis (<2 min), which results in time savings for the modern analytical laboratory.

Materials

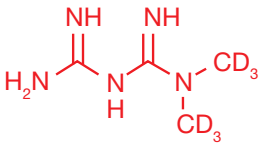
Standards and all other reagents and chemicals were obtained from Sigma-Aldrich®.

Human plasma was purchased from BioIVT® (Westbury, NY).

Structures



Metformin



Metformin-d_6

Experimental Conditions

An eight-point standard curve (n=2) was generated by preparing standards, in duplicate, at 1, 2, 10, 50, 100, 500, 1000, 2000 ng/mL in human plasma. Three levels of QC samples (n = 6) were prepared in human plasma at 8, 800, 1600 ng/mL (QCL, QCM, QCH, respectively). These standards and QC samples were then extracted with the Phree phospholipid removal 96-well plate (Phenomenex Part Number 8E-S133-TGB) as outlined in the sample preparation procedure below.

For the assay recovery experiment, six replicates of pre-extraction spiked samples, equivalent to QCM (800 ng/mL metformin) were prepared by spiking human plasma sample matrix solution containing 20 µL of working internal standard (IS) solution. These samples were processed per the sample extraction procedure above.

Post-extraction samples (Recovery samples, n = 4) consisted of human plasma blank samples containing 20 µL of working internal standard (IS) solution that were spiked with an equivalent of 800 ng/mL ibuprofen **after** extraction with Phree and **before** sample dry down.

Sample Preparation Procedure:

1. Aliquot 50 µL of human plasma sample into 13 x 100 mm glass tube, add 20 µL working internal standard (IS) solution (500 ng/mL of Metformin-d6 in 50:50 Acetonitrile / Water)
2. Add 500 µL of acetonitrile to the sample tubes, mix for at least 30 seconds
3. Load the sample into an unused well on the Phree 96-well plate accordingly, and apply vacuum at 5 mm Hg
4. Using a 2 mL 96 deep well plate (Phenomenex Part Number AH0-7194) collect the eluted sample
5. Take to dryness at 40 °C under N_2
6. Reconstitute with 400 µL of 2 mM ammonium acetate in water
7. Inject 3 µL

LC-MS Conditions:

Column: Kinetex® 5 µm C18
Dimensions: 50 x 4.6 mm
Part No.: 00B-4601-E0
Sample Preparation: Phree™ Phospholipid Removal 96-well plate
Part No.: 8E-S133-TGB
Mobile Phase: A: 2 mM Ammonium Acetate in Water (unadjusted pH)
B: Methanol
Isocratic A/B (95:5)
Flow Rate: 750 µL/min
Injection Volume: 3 µL
Temperature: 40 °C
Detection: MS/MS (SCIEX® 4500 Triple Quad™) ESI Positive
HPLC System: Agilent 1260 Infinity (Agilent Technologies®, Santa Clara, CA, USA)
Run time: 2 minutes
Pressure: ~60 bar

Table 1.
MRM Transitions and MS Settings

ID	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	DP	CE
Metformin 1	130	60	100	45	18
Metformin 2	130	71	100	45	16
Metformin-d_6 1	136.1	60.1	100	80	18
Metformin-d_6 2	136.1	77	100	80	16

Table 2.
Accuracy and Precision

Sample ID	QCL	QCM	QCH
Nominal Concentration (ng/mL)	8.00	800	1600
1	8.50	778	1620
2	7.68	736	1590
3	7.40	781	1590
4	7.17	806	1580
5	7.76	827	1600
6	6.96	786	1600
Mean	7.58	786	1597
S.D.	0.54	30.6	11.5
% CV	7.16	3.89	0.72
% Theoretical	94.7	98.2	99.8
N	6	6	6

Table 3.
Extraction Recovery

Sample ID	Area Ratio (Metformin / Metformin-d3)	Mean	Std. Dev.	CV (%)	Extraction Recovery (%)
QCM 1	1.48E+01				
QCM 2	1.40E+01				
QCM 3	1.49E+01				
QCM 4	1.54E+01				
QCM 5	1.58E+01				
QCM 6	1.50E+01	14.98	0.61	4.06	67.6
Recovery 1	2.20E+01				
Recovery 2	2.26E+01				
Recovery 3	2.33E+01				
Recovery 4	2.07E+01	22.15	1.10	4.98	

Results and Discussion

Table 1 shows the mass transitions used (ESI, positive mode) and the mass spec settings for data acquisition on the SCIEX 4500. **Table 2** shows the mini assay evaluation run results to demonstrate the accuracy and precision of the assay. Three QC levels (QCL, QCM and QCH) were used in the run, with accuracy and precision across all QC samples ranging from 94.7 – 99.8 % with CV from 0.72 -7.16 %, respectively. **Table 3** shows the sample extraction recovery results for the mid-level QC sample (QCM) of 67.6 % with CV of 4.06 % (n=6) using Phree phospholipid removal 96-well plate. A representative chromatogram for the LLOQ at 1 ng/mL, and for the ULOQ at 2000 ng/mL in matrix are shown in **Figures 1** and **2**, respectively. The linear dynamic range of this method was tested with eight calibrators, in duplicate, from 1-2000 ng/mL with acceptable linearity (r = 0.9999) and the calibration curve is shown in **Figure 3**.

Sample preparation using the Phree 96-well plate format for protein precipitation and phospholipid removal allows for the processing of multiple samples simultaneously, thereby reducing the overall time usually devoted to sample preparation. To further minimize sample analysis time, a short (50 x 4.6 mm) Kinetex C18 column was utilized to resolve the metformin analyte from any residual sample matrix interferences which may not be completely removed during sample preparation.

Conclusions

For a new generic drug product, several time points across multiple patient samples would be required for analysis to demonstrate bio-equivalence. These experiments are a significant undertaking for any company. In order to be effective, an efficient method is highly preferred. In this technical note, we have demonstrated an assay with a total run time of only 2 minutes on an LC-MS/MS system using Kinetex C18 5 µm, 50 x 4.6 mm column. In addition, sample preparation with the Phree 96-well plate is an automation friendly format. The Phree phospholipid removal plate gave very clean extraction results, which minimized the possible matrix interference peaks from endogenous phospholipids and proteins.

The fast analysis time demonstrated for the assay of metformin in human plasma would be ideally suited for a bio-equivalence study. A fast analytical method as demonstrated here is ideal for reducing costs in high-throughput analysis in research and production environment, without compromising analytical results.

Figure 1.
Representative chromatogram at LLOQ (1 ng/mL) in human plasma

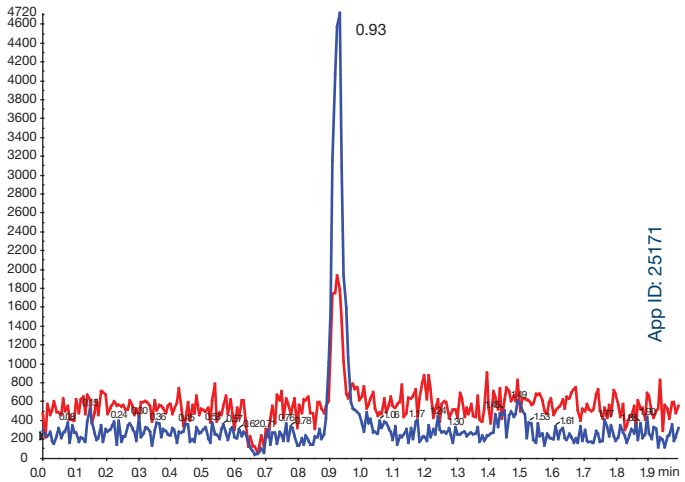


Figure 2.
Representative chromatogram at ULOQ (2000 ng/mL) in human plasma

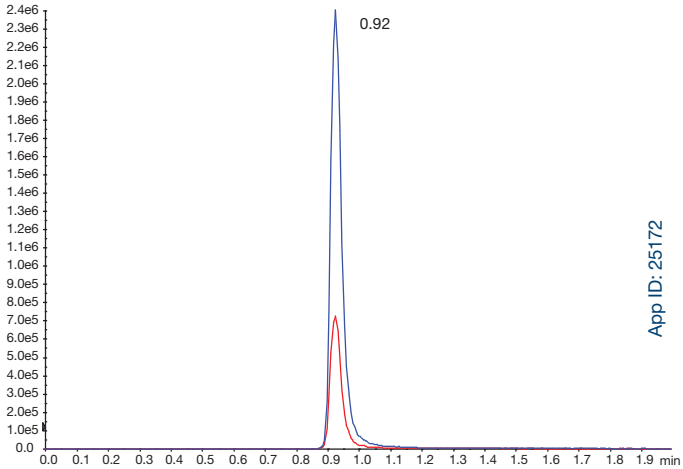
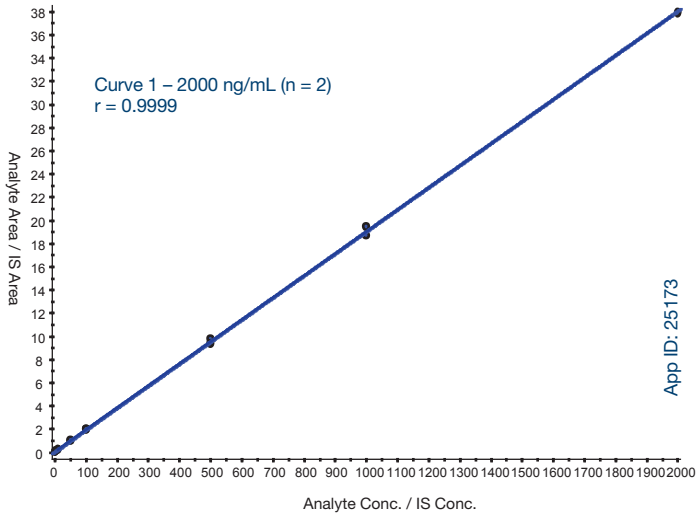


Figure 3.
Representative calibration curve



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Ordering Information

Kinetex® Core-Shell Columns

s5 µm Analytical Columns (mm)					SecurityGuard ULTRA Cartridges‡
Phase	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
C18	00B-4601-E0	00D-4601-E0	00F-4601-E0	00G-4601-E0	AJ0-8768
					for 4.6 mm ID

Phree™ Phospholipid Removal Products

Part No.	Description	Unit
8B-S133-TAK	Phree Phospholipid Removal Tabbed 1 mL Tubes	100/pk
8E-S133-TGB	Phree Phospholipid Removal 96-Well Plates	2/pk

‡SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

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