DEVELOPMENT AND VALIDATION OF METFORMIN IN HUMAN PLASMA USING DORAVIRINE, AS INTERNAL STANDARD BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract:

The current LC-MS/MS method was developed and validated for the estimation of Metformin in human plasma using Doravirine as an internal standard. Phenomenex Synergi, 4m, 4.675mm was used with an injection volume of 25 L, a run time of 20 minutes, and a mobile phase consisting of 5mM Ammonium Acetate buffer containing 0.1% Formic Acid: Acetonitrile (40:60 v/v) to achieve the best results using positive ion mode (API 4000Q Trap). Electrospray ionisation (ESI) tandem mass spectrometry operating in positive ion mode is used for detection at room temperature and atmospheric pressure. The precursor-to-product ion transitions of m/z452.42>71.32 for Metformin and m/z271.2>228.8 for Doravirine (Internal standard) were utilised for quantification. Doravirine (Internal standard) had a retention time of 1.56 minutes, while Metformin's was 7.04 minutes. Metformin's linearity was determined over a concentration range of 8.0 pg/mL to 160 pg/mL (r=0.999), and the drug's overall percentage recovery was 99.3% (compared to 100.7% for Doravirine, the internal standard). Accuracy and precision of the proposed method were determined to be within 15% CV for Metformin. Metformin stability studies found CV% values of accuracy and precision of 15%, indicating the proposed method is stable. Method specificity, precision, accuracy, linearity, robustness, reproducibility, and results dependability have all been demonstrated through the use of LC-MS in the course of this study's development and validation.

Keywords: Metformin Hcl, Doravirine, LC-MS/MS, Method development, Method Validation,

INTRODUCTION

The analytical chemistry technique known as liquid chromatography-mass spectrometry (LC-MS) combines the physical separation powers of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS)[1].

Coupled chromatography - MS systems are widely used for chemical analysis due to the complementary nature of the various techniques involved[2]. Mass spectrometry provides spectral information that can be used to identify (or confirm the suspected identity of) each component removed from a mixture using liquid chromatography[3].

Metformin, which also goes by the brand name Glucophage, is the first drug of choice for people with type 2 diabetes who are overweight[4]. It is also used in the treatment of polycystic ovary syndrome (PCOS)[5].

"Metformin HCl has a chemical name of 1, 1-Dimethylbiguanide hydrochloride, a molecular formula of C4H11N5 • HCl, and a molecular weight of 165.62 g/mole[6]. Doravirine (MK-1439) is a highly specific HIV-1 nonnucleoside reverse transcriptase inhibitor with IC50s of 4.5 nM, 5.5 nM and 6.1 nM against the wild type and K103N and Y181C reverse transcriptase mutants, respectively"[7].

The objective of the study is to develop and validate LC-MS analytical method for quantifying Metformin with Doravirine as Internal Standard by LC-MS. In these current study validation parameters performed according to ICH guidelines.[8]

Materials and Methods

Instrumentation

"An LC-MS/MS method was performed on a liquid chromatographic system consisting of Mass Lynx 4.1 SCN805, an auto sampler of Shimadzu (SIL-HTC) coupled with an API 4000 Trap triple quadruple mass spectrometer with electrospray ionization (ESI) used for analysis and Mass Lynx 4.1 SCN805Analyst software (version 1.4.2) for processing and data collecting. Phenomenex Synergi, 4µm (4.6×75mm) Column is used as a stationary phase. An ultrasonic bath sonicator(Frontline FS 4, Mumbai, India), semi-micro analytical balance (India) and Whatmann filter paper No.1 is used in the study".

Reagents & Chemicals

Aurobindo Laboratories, in Hyderabad, India, was where we were able to purchase our supply of metformin. The doravine used as the internal standard was purchased from Aurobindo Laboratories in Hyderabad. Chemical Laboratory in Hyderabad supplied the acetonitrile, which was of HPLC quality. The HPLC-grade water came from Ammonium phosphate, and the HPLC-grade orthophosphoric acid was purchased.

Method

Preparation of mobile phase

Five millimolar concentration (mM) Dissolved ammonium acetate and orthophosphoric acid in water at a 0.1% concentration About 0.7708g of ammonium acetate was added to 500ml of water before the volume was brought up to 2000ml. The mixture was stirred thoroughly and sonicated. Two thousand millilitres of formic acid was added to the previous buffer. Keep the solution at room temperature and label it. Details were documented on a Buffer form.

Preparation of Mobile Phase

We sonicated and mixed together 600 ml of acetonitrile and 400 ml of the above buffer. We put the solution in a labelled container and put it in the fridge. Then, they entered the information into the form used to get ready for the mobile phase.

Preparation of standard and working solutions for Metformin

Stock Metformin solution (10000 µg/mL) was generated by dissolving 1000 mg of Metformin in 1% ammonia solution in acetonitrile and bringing the volume up with the same in a 100 mL volumetric flask. The ideal temperature range for this solution was 2-8 degrees Celsius, so it was stored there. Diluent for spiking into plasma was used to dilute the stock solutions to the appropriate concentrations for use in constructing a calibration curve and obtaining quality control samples. The mobile phase was responsible for all other dilutions.

Preparation of stock solution for Doravirine (Internal standard)

A stock solution of Doravirine (Internal standard) was made by dissolving 50 mg of Doravirine in a mixture of HPLC grade acetonitrile and water (60:40, v/v) and filling up a 50 mL volumetric flask with the same mixture to make a 1000g/mL solution. At 2–8 °C, this solution was kept in the fridge. Working IS solutions were made by diluting the above-mentioned stock solution with the right amount of water right before use.

Preparation of plasma samples

Collections of human blood were placed in K2-EDTA-treated polypropylene tubes for further plasma production. The supernatant from each tube was centrifuged for 10 minutes at 8000 rpm and collected. When the plasma proteins had precipitated, the supernatant was treated with 2 mL of acetonitrile and left at room temperature for 15 minutes before being collected.

Preparation of sample solution

"After bulk spiking, aliquots of 100µL for calibration curves and 100 µL for quality controls of spiked plasma samples were pipette out into a pre-labelled polypropylene micro-centrifuge tubes and then all the bulk spiked samples were stored in the deep freezer at -70 °C ± 10 °C, except twelve replicates each of LQC and HQC, which were stored in -20 °C ± 5 °C for generation of stability data. The thawed samples were vortexes to ensure complete mixing of the contents".

Selectivity and Sensitivity[8]

A sensitive bio analytical method development and validation of Metformin in human plasma by LC-ESI-MS/MS with an additional haemolysed group and lipedimic group to test for interference at the retention times of analytes was carried out by analysing human blank plasma samples from six different sources (donors). Comparison of the LLOQ of the analyte with a blank plasma sample was used to determine the level of sensitivity. Peak areas in blank samples must be below the limits of quantification (LOQ) for both Metformin and Doravirine (20% and 5%, respectively).

Precision[8]

Lower limit of quantification (LLOQ), lower quality control (LQC), medium quality control (MQC), high quality control (HQC), and upper limit of quantification (ULOQ) were established through replicate analysis of quality control samples (n = 6). Accuracy should also be within 15%, with the exception of LLOQ, where it should be within 20%, and the CV should be less than 15%.

Matrix effect

By contrasting the absolute response of QC samples following pre-treatment (LLE) with that of reconstitution samples extracted blank plasma samples spiking with the analyte, we were able to assess the ion suppression/enhancement in the signal caused by the matrix effect due to the plasma matrix. Experiments were run in triplicate with six different plasma lots, and the acceptable precision (%CV) was ≤ 15% at MQC levels.

Recovery[8]

Analyzing quality control samples allowed us to calculate the extraction recovery of Analyte and IS from human plasma. By comparing peak areas obtained from the plasma sample and the standard solution spiked with the blank plasma residue, the recovery at three concentrations (10%, 100%, and 150%) was calculated. A 50% or greater rate of recovery was deemed sufficient in order to achieve the necessary level of sensitivity.

Linearity[8]

The linearity of Metformin was assessed at seven concentration levels in the range of 8, 25, 40, 60, 80,120 and 160 µg/mL in plasma samples. The calibration curve was derived by measuring the peak-to-background ratios of each solution against its concentration.

Results and Discussion

Table 1: System Suitability Results

Parameter	**Resolution Doravirine and Metformin**	**% RSD**	**USP Tailing**
Result	6.51	0.3	1.02

Table 2: System Suitability Results

Injection	**Doravirine**		**Metformin**	
	RT	Area	RT	Area
01	1.56	667533	7.04	4445905
02	1.57	667330	7.05	4448379
03	1.58	667759	7.04	4438849
04	1.57	666813	7.06	4427722
05	1.56	667156	7.05	4456545
Mean	667318		4443480	
RSD	0.05		0.24	
Std.Dev	361.2889		10844	

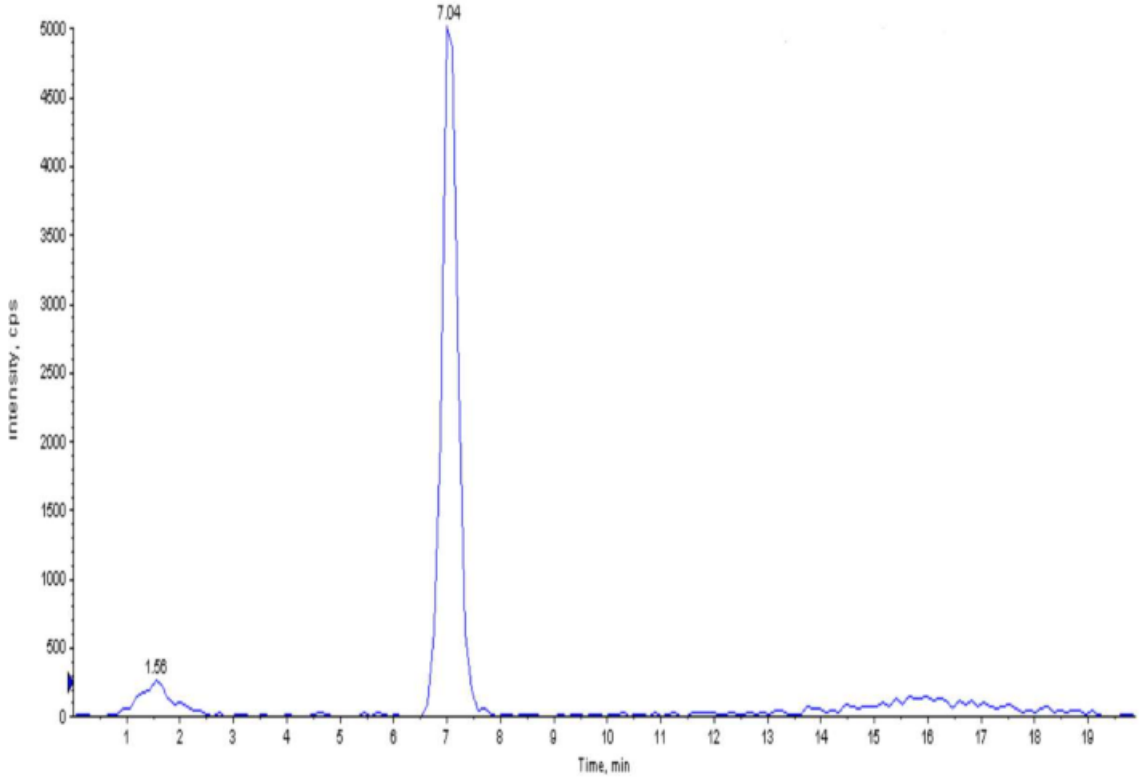

Figure 1: System Suitability Chromatograms for Doravirine & Metformin Hydrochloride

Conclusion

The above results depicted in Table 1-2 & Figure 1 reveal that the system meets the required system suitability.

Precision

System Precision

As per methodology, injected blank and standard solution five times.

Table 3: System Precision Results

Injection	**Doravirine**		**Metformin**	
	RT	Area	RT	Area
01	1.56	667533	7.04	4445905
02	1.57	667330	7.05	4448379
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04	1.57	666813	7.06	4427722
05	1.56	667156	7.05	4456545
Mean	667318		4443480	

RSD	0.05	0.24
Std.Dev	361.2889	10844

Table 4: System Precision Results

Parameter	**Resolution Doravirine and Metformin**	**% RSD**	**USP Tailing**
Result	6.51	0.3	1.02

Conclusion

The above results depicted in Table 3-4 reveal that the system meets the required System Precision.

Table 5: Method Precision Results

Injection	**Doravirine**		**Metformin**		**% Assay Found**	
	RT	Area	RT	Area	Doravirine	Metformin
01	1.57	4421056	7.04	59394054	98.11	98.97
02	1.57	4409060	7.05	58455412	97.85	98.41
03	1.58	4432747	7.04	60124842	98.37	100.19
04	1.57	4525154	7.06	59854126	100.42	99.74
05	1.56	4548584	7.05	58554523	100.94	97.57
06	1.55	4427722	7.05	58341742	98.26	97.22
Mean	4460721		59120783		99	99.19
RSD	1.34		1.31		1.34	1.19
Std.Dev	59975.28		773418		1.33	1.18

Conclusion

The above results depicted in Table 5 reveal that the method is precise.

Linearity

Linearity for Doravirine and Metformin was determined in the concentration range from LOQ (10%) to 200% of concentration levels.

Table 6: Doravirine Linearity Results

Doravirine Linearity							
S.NO	%Level	**Standard Stock**	**Dil**	**Vol Taken**	**Final Volume**	**Final Conc.**	**Area**
1	10	50	50	0.8	100	8	65254
2	25	50	50	2.5	100	25	171358
3	50	50	50	1	25	40	325321
4	75	50	50	1.5	25	60	510215
5	100	50	50	2	25	80	667318
6	150	50	50	3	25	120	1018412
7	200	50	50	4	25	160	1335241
						Intercept	-14180.44
						Slope	8503.82
						Correlation	0.999
						%Y-Intercept	-2.125

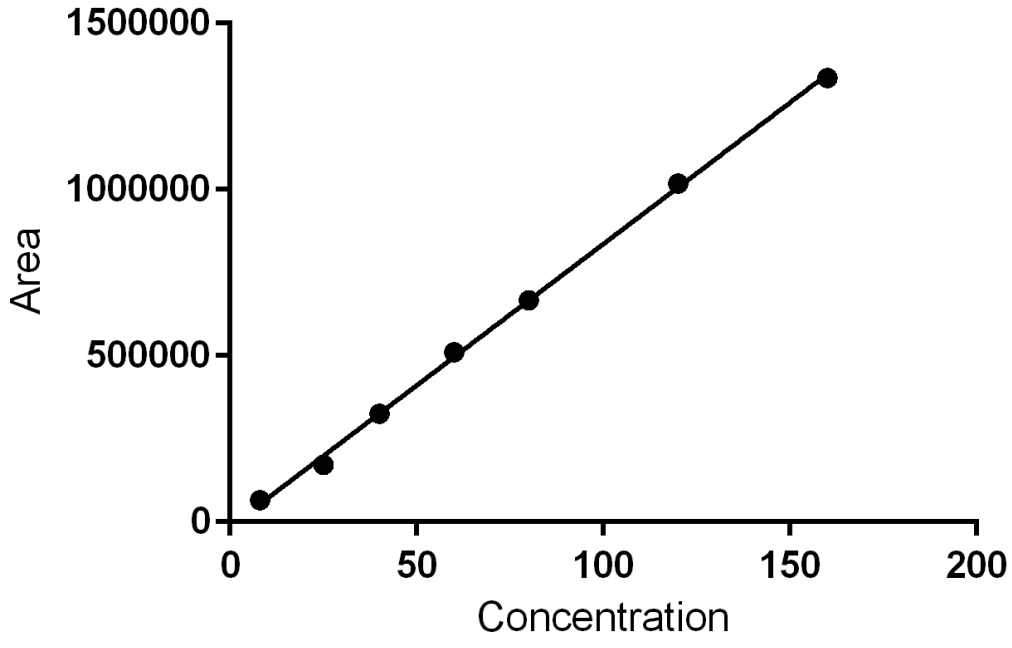

Figure 2: Linearity for Doravirine

Table 7: Metformin Linearity

Metformin Linearity							
S.NO	%Level	Standard Stock (mg)	Dil	Vol Taken	final Volume	Final Conc	Area
1	10	1000	100	0.8	100	80	421251
2	25	1000	100	2.5	100	250	1024157
3	50	1000	100	1	25	400	2321523
4	75	1000	100	1.5	25	600	3412253
5	100	1000	100	2	25	800	4443482
6	150	1000	100	3	25	1200	6214523
7	200	1000	100	4	25	1600	8921541
						Intercept	-68673.53
						Slope	5525.24
						Correlation	0.997
						%Y-Intercept	-1.545

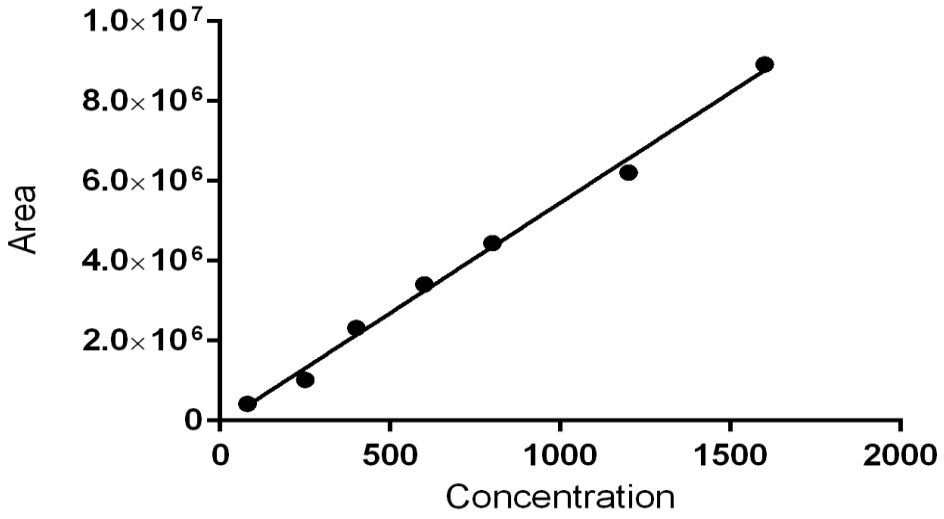

Figure 3: Linearity for Metformin Hydrochloride

Accuracy

As per the method, a blank, 10%, 100%, and 150% of the Assay were injected into the HPLC and the accuracy was shown on a sample. Calculated the parameters for system suitability and the mean recovery.

Table 8: Accuracy of Doravirine

Sample No	Spike level	(%w/w) added	(%w/w) found	'%' Recovery	'%' Mean recovery	%RSD
1	10%	25.15	24.98	101.98	102.1	0.2
2	10%	25.11	24.85	102.29		
3	10%	25.10	24.95	102.09		
1	100%	50.12	49.99	100.73	100.7	0.1
2	100%	50.15	50.10	100.64		
3	100%	50.02	49.85	100.84		
1	150%	75.25	74.82	101.86	101.8	0.1
2	150%	76.02	75.8	101.67		
3	150%	75.83	75.223	101.74		

Table 9: Accuracy of Metformin

Sample No	Spike level	(%w/w) added	(%w/w) found	'%' Recovery	'%' Mean recovery	%RSD
1	10%	500.15	500.0	99.1	99	0.2
2	10%	500.35	500.10	98.9		
3	10%	500.14	500.21	99		
1	100%	998.3	998.1	99.6	99.3	0.5
2	100%	1000.01	999.98	99.3		
3	100%	999.85	999.81	99.0		
1	150%	1500.25	1500.10	98.1	98.46	0.80
2	150%	1500.14	1498.2	98.3		
3	150%	1498.98	1498.10	99.0		

CONCLUSION

Conclusion In accordance with ICH recommendations, we developed and validated the present LC-MS/MS technique for the measurement of Metformin in human plasma using Doravirine as an internal reference. A high degree of sensitivity, selectivity, reproducibility, and excellent recovery, stability, and minimal matrix effects were demonstrated by the designed and validated procedures. The criteria for recognising the chromatographic assay as a reliable and practical approach were met. There is a high degree of specificity, accuracy, robustness, and speed with which a large number of samples can be analysed. So, this technique can be utilised for everyday analysis.

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