A PRECISE AND ROBUST METHOD FOR QUANTIFYING METFORMIN IN HUMAN PLASMA USING DORAVIRINE, AS INTERNAL STANDARD BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract:

The present LC-MS/MS method for the estimation of Metformin in human plasma by using Doravirine as an internal standard was established and validated as per ICH guidelines. The best response was obtained with Phenomenex Synergi, 4µm, 4.6×75mm, and a mobile phase containing a mixture of 5mM Ammonium Acetate buffer with 0.1% Formic Acid: Acetonitrile (40:60 v/v) was delivered at a flow rate of 1.0 mL/min by positive ion mode (API 4000Q Trap) with an injection volume of 25 µL and a run time of 20min. Detection is performed by atmospheric pressure electrospray ionization (ESI) tandem mass spectrometry in positive ion mode. The precursor to product ion transitions is m/z452.42>71.32 for Metformin and m/z 271.2>228.8 for Doravirine (Internal standard) were used for quantization. The retention time of Doravirine (Internal standard) and Metformin was found to be 1.56 min and 7.04 min respectively. This method development Stable, Robust, Reproducible and Capable of producing reliable results.

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

INTRODUCTION

Liquid chromatography–mass spectrometry (LC–MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS)[1].

Coupled chromatography - MS systems are popular in chemical analysis because the individual capabilities of each technique are enhanced synergistically[2]. While liquid chromatography separates mixtures with multiple components, mass spectrometry provides spectral information that may help to identify (or confirm the

suspected identity of) each separated component[3].

Metformin, sold under the brand name Glucophage, among others, is the main first-line medication for the treatment of type 2 diabetes, particularly in people who are overweight[4]. It is also used in the treatment of polycystic ovary syndrome[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 IC_{50}s of 4.5 nM, 5.5 nM and 6.1 nM against the wild type and K103N and Y181C reverse transcriptase mutants, respectively[7].

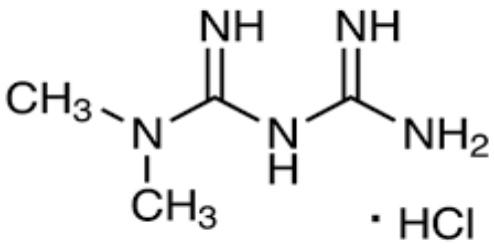

Figure 1: Metformin Hydrochloride.

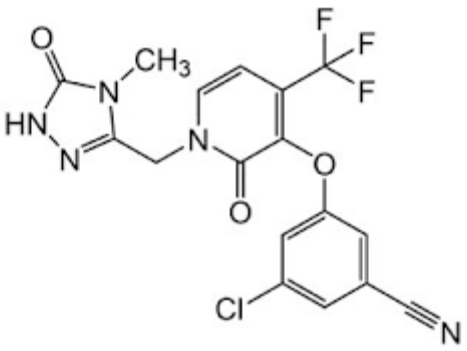

Figure 2: Doravirine

The objective of the study is to develop and validate following parameters like Precision, linearity, Accuracy LC-MS analytical method for quantifying Metformin with Doravirine as Internal Standard was published and reported [8, 9] In continuation with the literature reported[9], following validation parameters like System suitability and Robustness has performed as per ICH guidelines[8].

Materials and Methods

Apparatus & Equipment

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

Metformin was procured from Aurobindo Laboratories, Hyderabad. Doravirine (Internal Standard) was procured from Aurobindo Laboratories Hyderabad. Acetonitrile of HPLC grade was procured from Chemical Laboratory Hyderabad. The water of HPLC grade was obtained from Ammonium phosphate and orthophosphoric acid of HPLC grade was procured.

Preparation of mobile phase

Preparation of 5mM Ammonium acetate with 0.1% orthophosphoric acid in water To approximately 500mL of water added 0.7708g of ammonium acetate, made up the volume to 2000mL with water, mixed well, and sonicated. To the above buffer added 2.000mL of Formic Acid. Labelled and stored the solution at ambient temperature. Recorded the details in Buffer preparation form.

Preparation of Mobile Phase

To 400mL of above buffer added 600mL of Acetonitrile, mixed well, and sonicated. Labelled and stored the solution at ambient temperature. Then recorded the details in the Mobile phase preparation form.

Preparation of standard and working solutions for Metformin

The Metformin stock solution was prepared by dissolving 1000 mg of Metformin in 1% ammonia solution in acetonitrile and made up the volume with the same in a 100 mL volumetric flask to produce a solution of 10000µg/mL. This solution was kept in a refrigerator at 2-8 °C. The stock solutions were diluted to suitable concentrations using diluent for spiking into plasma to obtain calibration curve standards and quality control samples for further use. All other dilutions were made in the mobile phase.

Preparation of stock solution for Doravirine (Internal standard)

A stock solution of **Doravirine (Internal standard)** was prepared by dissolving 50 mg of Doravirine in diluents (mixture of HPLC grade acetonitrile and water in a ratio (60:40, v/v) and made up the volume with the same in a 50 mL volumetric flask to produce a solution of 1000µg/mL. This solution was kept in the refrigerator at 2-8 °C. Working IS solutions were prepared by suitably diluting the above-mentioned stock solution fresh before use.

Preparation of plasma samples

For the preparation of plasma samples, human blood samples were collected into polypropylene tubes containing K2-EDTA. Each tube was centrifuged for 10min at 8000 rpm and the supernatant was collected in another tube. To the

supernatant 2 mL of acetonitrile was added and kept for 15 min for the plasma proteins to precipitate and then the supernatant was collected for further use.

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]

Selectivity was performed by analysing the human blank plasma samples from six different sources (donors) 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. The sensitivity was compared with the LLOQ of the analyte with its blank plasma sample. The peak area of blank samples should not be more than 20% of the mean peak area of LOQ of Metformin and 5% of the mean peak area of Doravirine.

Precision[8]

It was determined by replicate analysis of quality control samples (n = 6) at LLOQ (lower limit of quantification), LQC (low quality control), MQC (Medium quality control), HQC (high quality control) and ULOQ (upper limit of quantification) levels. The % CV should be less than 15%, and accuracy should be within 15% except LLOQ where it should be within 20%.

Matrix effect

The matrix effect due to the plasma matrix was used to evaluate the ion suppression/enhancement in a signal when comparing the absolute response of QC samples after pre-treatment (LLE) with the reconstitution samples extracted blank plasma sample spiking with the analyte. Experiments were performed at MQC levels in triplicate with six different plasma lots with the acceptable precision (%CV) of ≤ 15%.

Results and Discussion:

System suitability

Injected Doravirine and Metformin Standard solution into the Chromatographic system. Five Replicate standards injected into the chromatographic system and recorded the %RSD and the resolution between Doravirine and Metformin.

System suitability:

1. Resolution between Doravirine and Metformin should be not less than 2.0.
2. % RSD for peak area of Doravirine and Metformin from replicate injections of reference standard solution should be not more than 5.0.
3. USP Tailing should be not more than 2.0.
4. USP Platecount should be not less than 2000.

Table 1: System Suitability Results

Parameter	Resolution Doravirine and Metformin	% RSD	USP Tailing	USP Plate count
Result	6.51	0.3	1.02	3561

Table 2: System Suitability Results

Injection	Doravirine		Metformin		Resolution
	RT	Area	RT	Area	
01	1.56	667533	7.04	4445905	7.51
02	1.57	667330	7.05	4448379	7.56
03	1.58	667759	7.04	4438849	7.54
04	1.57	666813	7.06	4427722	7.55
05	1.56	667156	7.05	4456545	7.53
Mean	667318		4443480		
RSD	0.05		0.24		
Std.Dev	361.2889		10844		

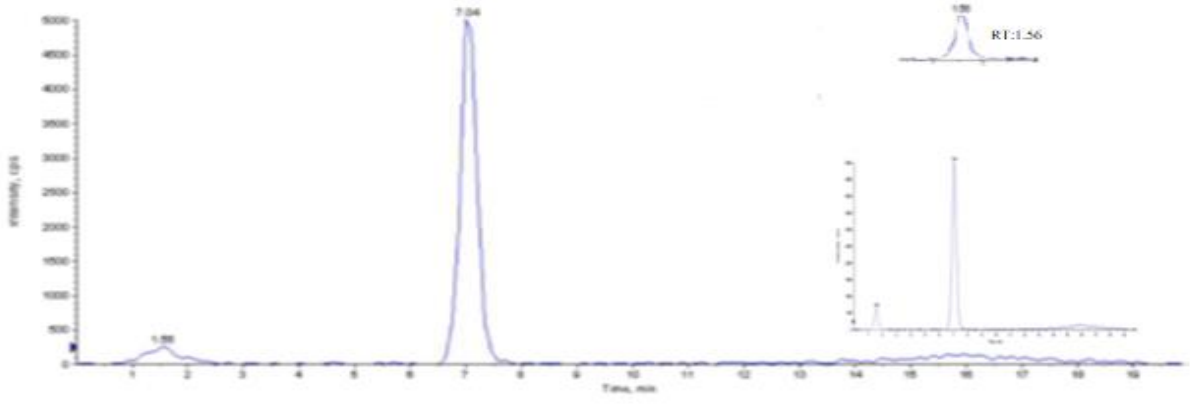

Figure 3: System Suitability Chromatogram for Metformin Hydrochloride & Doravarine

Conclusion

The above results reveal that the system meets the required system suitability.

Stability of standard & test preparation[8]:

This guide distinguishes between the analyte stability in calibration and stock solutions and stability in sample matrix and stresses the influence of storage conditions, matrix and container system on stability, besides the intrinsic properties of the analyte itself. The intended compounds are determined by calculating their separation or resolution (Rs) from other compounds. Stability of the analyte is evaluated using both low- and high-level QC samples. The investigation of stability should cover short-term stability at room temperature or sample processing temperature.

Table 3: Stability of standard at 2-8°C at 6 Hours

Injection	**Doravirine**		**Metformin**		**Resolution**
	RT	**Area**	**RT**	**Area**	
01	1.55	667543	7.03	4425905	7.55
02	1.56	667231	7.02	4348379	7.54
03	1.57	667641	7.04	4538849	7.56
04	1.58	656513	7.04	4527722	7.51
05	1.57	677556	7.05	4446545	7.55
Mean	667297		4457480		
%RSD	1.12		1.76		

Injection	**Doravirine**		**Metformin**		**Resolution**
	RT	Area	RT	Area	
01	1.52	657541	7.01	4424915	7.55
02	1.54	654132	6.99	4448376	7.49
03	1.55	657341	7.02	4448849	7.56
04	1.56	656523	7.05	4437222	7.55
05	1.59	655546	7.03	4449852	7.51
Mean	656217		4441843		

%RSD	0.21	0.24	

Table 4: Stability of standard at -70 °C ± 10 °C at 6 hours

Robustness[8]:

The term robustness refer to the ability of an analytical method to remain unaffected by small variations in method parameters (mobile phase composition, flow rate and column oven temperature, etc.) and influential environmental factors (room temperature, air humidity, etc.) and characterize its reliability during normal usage. The notion of remaining unaffected by varying a method parameter has two possible interpretations – it can be interpreted as: (a) no change of the detected amount of the analyte in a certain sample in spite of the variation of the method parameter or (b) no change of the critical performance characteristics (e.g. limit of quantitation) by the variation of the method parameter. In experimental evaluation of robustness either one of these interpretations can be used.

Effect of Variation in buffer [8]:

Design:

Analyzed system suitability preparations as per the methodology at high (45%) & low buffer (35%).

Table 5: System Suitability Comparisons Variation in Buffer % Change

Mobile Phase Composition ACN:Buffer(%)	Doravirine RT	Metformin RT	Resolution
65:35	1.35	6.12	7.22
55:45	1.95	8.14	8.01

Conclusion: The above results reveal that the method is robust at change of buffer %.

Effect of Variation in Flow rate[8]:

Design:

Analyzed system suitability preparations as per the methodology at low column flow (0.8 mL/min) and high flow (1.2 mL/min).

Table 6: System suitability comparison data of flow rate variation

Flow rate	Doravirine RT	Metformin RT	Resolution
0.8mL/min	1.88	8.02	7.65

1.2mL/min	1.41	6.78	7.14

Conclusion:

The above results reveal that the method is robust at flow between 0.8 mL/min and 1.2 mL/min.

Effect of Variation in Column Oven Temperature[8]:

Design:

Analyzed system suitability preparations as per the methodology at low column oven temperature (20°C) and high column temperature (30°C).

Table 7: System suitability comparison data of temperature variation

Column Oven tempearture	Doravirine RT	Metformin RT	Resolution
20°c	1.62	7.21	7.12
30°c	1.41	6.74	6.51

Conclusion: The above results reveal that the method is robust at column oven temperature between 20°C to 30°C variations.

CONCLUSION

The present LC-MS/MS method for the estimation of Metformin in human plasma by using Doravirine as internal standard was established and validated as per ICH guidelines. The developed and validated method shows a high degree of sensitivity, selectivity, reproducibility and high recovery, stability with less matrix effects. The chromatographic method fulfilled all the requirements to be recognized as a reliable and feasible method. It is highly specific, precise accurate, rugged and robust analytical procedure and allows the analysis of large number of samples in a short period of time. So, this method can be used for routine analysis.

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