

A Validated High Performance Liquid Chromatography Method for the Determination of Metformin in Human Plasma and its Application to Pharmacokinetic Study

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ARTICLE INFO

Received Date: May 13, 2019

Accepted Date: May 31, 2019

Published Date: June 11, 2019

KEYWORDS

Tuberculosis
Anti-tuberculosis treatment
Diabetes mellitus
Metformin
Pharmacokinetics

ABSTRACT

High performance liquid chromatographic method for determination of metformin in human plasma was developed. The method involved deproteinisation of the sample with methanol and analysis of the supernatant using Zorbax 300 – SCX, 4.6 X 150mm ID and UV detection at a wavelength of 233 nm. The assay was specific for metformin and linear from 0.0625 to 2.5µg/ml. The relative standard deviation of intra- and inter-day assays was lower than 10%. The average recovery of metformin from plasma was 106%. Due to its simplicity, the assay can be used for pharmacokinetic studies of metformin.

INTRODUCTION

Diabetes Mellitus (DM) is a disease of metabolic dysregulation, characterized by chronic hyperglycemia due to insufficient insulin action [1,2]. Diabetes is associated with various complications, such as diabetic foot neuropathy, diabetic nephropathy, diabetic retinopathy, cataracts, and glaucoma [3-5]. Metformin HCl (N,N-dimethyl imido dicarbonimidic diamide hydrochloride), (MET), an oral anti hyperglycemic agent, is extensively used as standard first – line pharmacotherapy in the treatment of type 2 diabetes patients. Metformin reduces gluconeogenesis in the liver by activating AMP-Kinase Protein Kinase (AMPK) Via Liver Kinase B1 (LKB1) [6]. It also increases glucose utilization and insulin sensitivity in peripheral tissues including muscles and fat. As a result, MET lowers plasma glucose level, in the fasting condition [7-10]. According to the American Diabetes Association guideline, metformin is suggested as the preferred initial agent, and, if MET monotherapy fails to reduce or maintain blood glucose, other therapeutic agents should be added to MET [11]. Analysis of MET in plasma is important in the clinical management of diabetic patients and also drug monitoring studies in these patients. Literature survey revealed several High Performance Liquid Chromatography (HPLC) and liquid chromatography-tandem mass spectrometry methods for the estimation of plasma MET alone [12-17] or in combination with other drugs [18-20]. Method development for determination of metformin hydrochloride in formulations by RP-HPLC was also reported [21]. The HPLC methods reported were based on derivatisation technique and require special columns, large volume of samples and low sensitivity. Several LCMS/MS methods for the estimation of MET have been reported. However, these expensive techniques are not affordable in developing countries and for resource

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Citation for this article: Mary Rebecca Y, SudhaV and Hemanth Kumar AK. A Validated High Performance Liquid Chromatography Method for the Determination of Metformin in Human Plasma and Its Application to Pharmacokinetic Study. Chromatography And Separation Techniques Journal. 2019; 2(1):119

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poor settings. Hence we developed and validated a simple, sensitive and high throughput analysis using an HPLC for the determination of MET in plasma.

MATERIALS AND METHODS

Pure Metformin Hydrochloride was a kind gift from M/s Aarthi Drugs Ltd. Methanol (99.80% purity), Acetonitrile (99.9% purity), Potassium dihydrogen phosphate (99.5% purity), Di potassium hydrogen orthophosphate (99% purity) were purchased from M/s Qualigens (India) Limited. Deionized water was processed through a water purification system (Siemens, Germany). Pooled plasma was obtained from a Blood Bank, Chennai, India.

CHROMATOGRAPHIC SYSTEM

HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of two pumps (LC-20AD), photodiode array detector (SPD-M20A) and auto sampler (SIL20AC-HT) with built in system controller. Lab solutions software was used for data collection and acquisition. The analytical column used was Zorbax 300 – SCX, 4.6 X 150mm ID, 5 μ m particle size (Agilent, USA) protected by a compatible guard column. An isocratic mobile phase consisted of a mixture of 10 mM phosphate buffer (1.625 gm of KH_2PO_4 & 0.3 gm of K_2HPO_4 in 1000ml of MQ Water, pH 4.8) and acetonitrile in the ratio of 55: 45 (v/v), was used to separate the analyte from the endogenous components. Prior to preparation of the mobile phase, the solvents were degassed separately using a Millipore vacuum pump. The PDA detector was set a wavelength of 233 nm. The chromatogram was run for 10 minutes at a flow rate of 1.3 ml/min. The column temperature was set at 30 $^{\circ}$ C. Unknown concentrations were derived from linear regression analysis vs concentration curve. The linearity was verified using estimates of correlation coefficient (r).

PREPARATION OF STANDARD SOLUTION

A stock solution (1 mg/ml) was prepared by dissolving MET HCL in methanol. The working standards of MET in concentrations ranging from 0.0625 μ g/ml to 2.5 μ g/ml were prepared in pooled plasma.

SAMPLE PREPARATION

To 200 μ l of calibration standards and test samples, 450 μ l of methanol was added and the contents were vortexed vigorously for 3 minutes and centrifuged at 10,000 RPM for

10 mins. 500 μ l of clear supernatant was transferred and evaporated to dryness using nitrogen evaporator for approximately 30 minutes. The dried residue was reconstituted in 150 μ l of mobile phase and 75 μ l was injected into the HPLC column.

METHOD VALIDATION

Accuracy and linearity

The accuracy and linearity of MET standards were evaluated by analysing a set of standards ranging from 2.5 to 0.0625 μ g/ml. The intra- and inter-day variations were determined by processing each standard concentration in duplicate for six consecutive days.

Precision

In order to evaluate the precision of the method, plasma samples containing varying concentrations of MET were analysed in duplicate on three consecutive days.

Recovery

Known concentrations of MET (0.25, 1.0 and 2.5 μ g/ml) were prepared in pooled human plasma and were spiked with lower and higher concentrations of standards. The percentage of drug recovery from plasma samples was calculated by dividing the difference in MET concentrations by the added concentration. Recovery experiments were carried out on three different occasions.

Specificity

Interference from endogenous compounds was investigated by analysing blank plasma samples. Interference from certain anti-tuberculosis drugs - rifampicin, isoniazid, ethambutol, pyrazinamide, levofloxacin, cycloserine, ethionamide, rifapentine, anti-retroviral drugs – nevirapine, efavirenz, lamivudine, stavudine, and zidovudine and sulphonyl ureas – glibenclamide at a concentration of 10.0 μ g/ml was also evaluated.

Limit of detection (LOD) and quantitation (LOQ)

These values were estimated mathematically from the standard curve equations. LOD was calculated using the formula $3.3 \times o/S$, where o is the standard deviation of Y – axis intercepts and S is the slope of the calibration curve. LOQ was calculated using the formula $10.0 \times o/S$, where o is the standard deviation of Y – axis intercepts and S is the slope of the calibration curve.

RESULTS

Under the chromatographic conditions described above, MET was well separated and seen as a discrete peak in the representative chromatograms of extracted MET plasma standards 0.0625 and 2.5 µg/ml and plasma sample from diabetic patient (Figure 1a-c) with the retention time of 6.8 minutes and the run time being 10 minutes. Blank sample did not give any peak at the retention time of MET (Figure 1d).

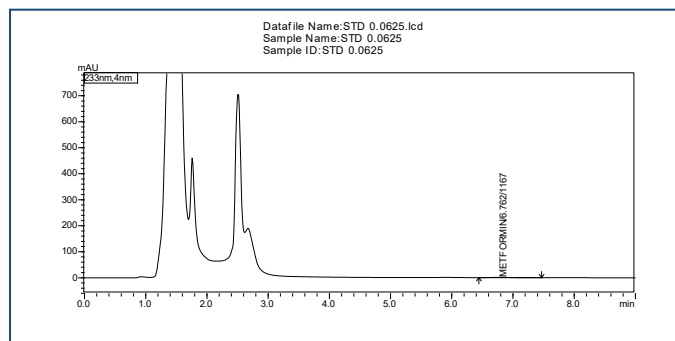


Figure 1a: Standard 0.0625 µg/ml.

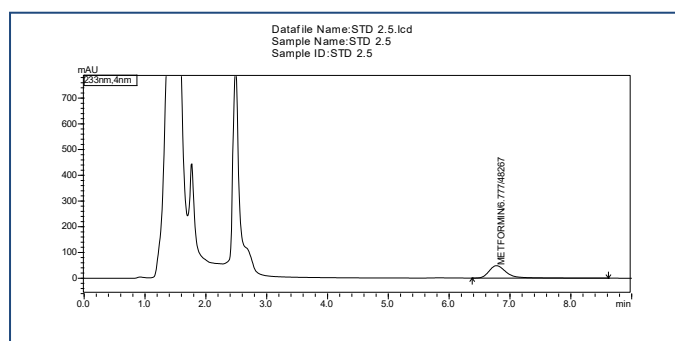


Figure 1b: Standard 2.5 µg/ml.

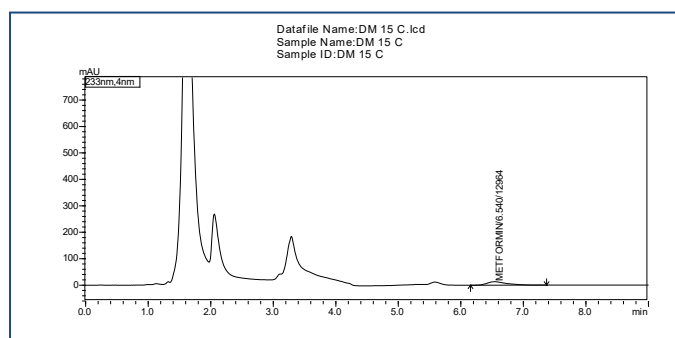


Figure 1c: Patient Sample.

The primary oral antidiabetic drugs for type 2 diabetes mellitus are MET alone or in combination with the second generation sulphonyl ureas like Glibenclamide and also along with other drugs for co-morbidities. It is therefore becomes

necessary to rule out the interferences of other anticipated co administered drugs in diabetic patients with tuberculosis or HIV in the assay of MET and establish the specificity of the method.

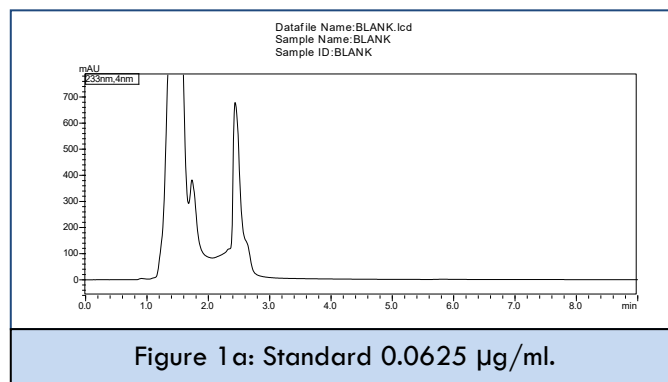


Figure 1a: Standard 0.0625 µg/ml.

No endogenous substances, first line anti-tuberculosis drugs such as rifampicin, isoniazid, ethambutol, pyrazinamide or anti-retroviral drugs like nevirapine, efavirenz, lamivudine, stavudine, zidovudine or anti-diabetic drug like glibenclamide interfered in the MET chromatogram. In the present method, MET concentrations ranging from 0.0625 – 2.5 µg/ml were checked for linearity. These concentrations span the range of clinical interest. The calibration curve parameters of MET from six individual experiments for standard concentrations ranging from 0.0625 – 2.5 µg/ml showed a linear relationship (Figure 2).

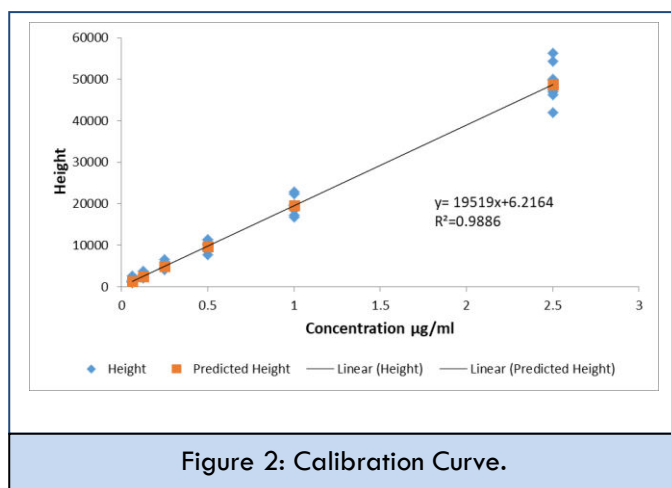


Figure 2: Calibration Curve.

The mean correlation coefficients (r), coefficient of determinants (R²), slope and intercept values were 0.994, 0.989, 19518 and 6.216 respectively. The linearity and reproducibility of the various standards used for constructing calibration graphs for plasma MET are given in (Table 1). The intra- and inter-day

Relative Standard Deviation (RSD) for standards containing 0.0625 – 2.5 µg/ml ranged from 2.1% to 5.8 % and 4.9 % to 7.2% respectively. The accuracy of plasma MET concentrations ranged from 96 % to 108 %. The precision of the method was further evaluated by analysing three plasma samples containing different concentrations of MET (Table 2).

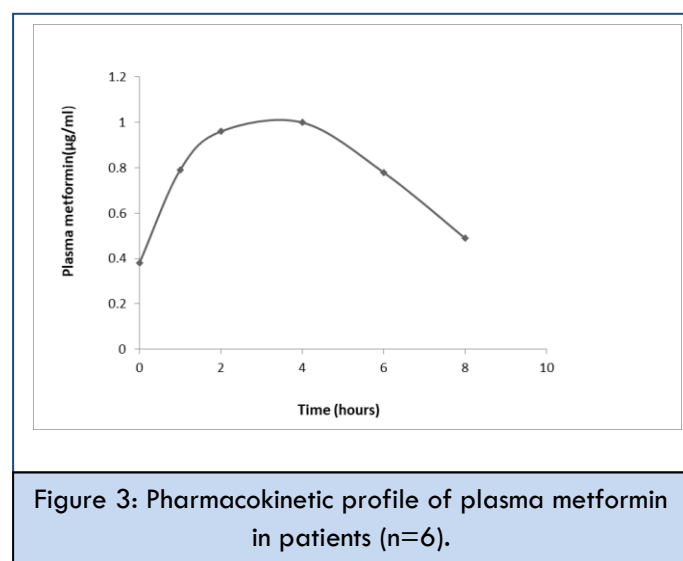
Table 1: Linearity and reproducibility.

Standard concentration (µg/ml)	Mean peak height ratio $\pm$ SD (%RSD)	
	Within day (n=6)	Between day (n=6)
0.0625	1196 $\pm$ 69(5.8%)	1259 $\pm$ 84(6.5%)
0.125	2373 $\pm$ 116(4.9%)	2437 $\pm$ 120(4.9%)
0.25	4931 $\pm$ 249(5.1%)	4868 $\pm$ 352(7.2%)
0.5	10471 $\pm$ 547(5.2%)	10519 $\pm$ 615(5.8%)
1	20343 $\pm$ 809(4.0%)	20359 $\pm$ 1357(6.7%)
2.5	50360 $\pm$ 1048(2.1%)	49824 $\pm$ 3214(6.5%)

Table 2: Precision.

Actual concentration (µg/ml)	Found concentration (µg/ml) mean $\pm$ SD	% RSD
0.0625	0.061 $\pm$ 0.001	98
0.5	0.513 $\pm$ 0.018	103
2.5	2.467 $\pm$ 0.012	99

The RSD for these samples ranged from 98 % to 103 % respectively. The LOD and LOQ estimated mathematically from the standard curve equation were 0.031 and 0.0625 µg/ml respectively. The method reliably eliminated interfering materials from plasma, yielding a recovery of MET that ranged from 105% to 109 % (Table 3).



The mean MET concentrations measure on day 1 and 30, in two plasma samples were 1.01- and 0.93µg/ml respectively. No

degradation (<10%) of MET in human plasma occurred up to 30 days when stored at – 20°C. The method described was applied to the determination of MET concentration in plasma from 6 patients with diabetes who are treated with dose of 500 mg MET (Figure 3). Serial blood samples were collected at predose and at different time points after drug administration. The mean plasma peak concentration of MET was 1.0 µg/ml and the mean time to attain the peak concentration was 4 hours by visual inspection.

Table 3: Recovery.

Base (µg/ml)	Added (µg/ml)	Actual (µg/ml)	Obtained (µg/ml)	Recovery (%)
2.5	1	1.75	1.86	106
1	0.5	0.75	0.79	105
0.25	0.125	0.188	0.204	109

DISCUSSION

Several methods have been described to measure MET concentrations in drug formulations. Only few methods have been developed to determine MET concentrations in plasma for therapeutic drug monitoring and pharmacokinetic studies. A review of these methods revealed that some of these methods involved derivatisation technique, larger sample volume and low sensitivity. Although LCMS/MS methods are available for the quantification of MET in plasma, these expensive techniques are out of reach for laboratories in resource poor settings. Moreover, none of the HPLC or LCMS/MS methods included anti-tuberculosis and anti-retroviral drugs as well as sulphonyl ureas like Glibenclamide in their specificity experiments. Treatment of type 2 diabetes mellitus may be either with MET alone or in combination with sulphonyl ureas. Tuberculosis and HIV infected diabetes patients will receive treatment for these diseases along with anti-diabetic drugs. In order to achieve the objectives of simple sample preparation, sensitive, and economic, HPLC method was developed and validated as per Bio analytical method validation guidelines. The method has utilized simple mobile phase with one step extraction procedure which is economic and efficient. The total run time was 10 minutes and could be proved as better alternative for LCMS/MS method.

CONCLUSION

We have described a method for estimation of MET in plasma, that has the sensitivity to quantitate MET from 0.0625 µg/ml which is sufficient enough for pharmacokinetic studies and therapeutic drug monitoring. This method is reproducible and specific for the determination of MET in human plasma, yielding satisfactory recovery from human plasma. Stability experiments showed that MET was stable up to 30 days when stored at -20°C. This method could be used for pharmacokinetic studies and therapeutic drug monitoring of MET.

ACKNOWLEDGEMENT

The technical assistance rendered by Mr. Vijayakumar and the secretarial assistance rendered by Mr. Sasikumar.

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