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Development and Validation of RP-HPLC Method for Metformin HCl and Sildenafil Citrate in Rat Plasma-Application to Pharmacokinetic Studies

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ABSTRACT

Metformin hydrochloride (MET) used in the treatment of non-insulin-dependent diabetes mellitus not responding to dietary modification Sildenafil citrate (SIL) is used in the treatment of erectile dysfunction and other chronic disorders. For the pharmacokinetic investigation of MET, SIL and CET (IS). we developed a simple and sensitive method for the estimation of MET and SIL in rat plasma by reverse phase high-performance liquid chromatography (RP-HPLC). The drug samples were extracted by Solid phase extraction with stationary phase strata-X Cartridges with 1ml mixture of mobile phase. Chromatographic separation was achieved on C18 column using (0.3) Triethyl anime buffer: methanol: Acetonitrile (70:05:25 v/v) as mobile phase at a flow rate of 1 ml/min and UV detection at 224 nm. The retention time of MET, SIL and CET was found to be 2.923, 3.877 and 10.091 min having a separation Run time 15 min. The developed method was validated for accuracy, precision, linearity and recovery. Linearity studies were found to be acceptable over the range of 0.4–6.4 ug/ml. The method was successfully applied for the analysis of rat plasma sample for the application in pharmacokinetic study, drug interaction, bioavailability and bioequivalence.

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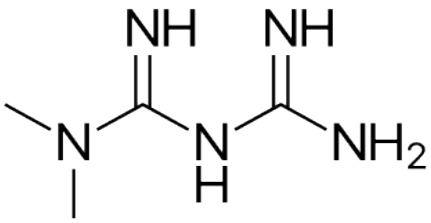
1 INTRODUCTION

Metformin hydrochloride (MET) is a biguanide is the first-line of oral therapy in patients with type 2 diabetes mellitus (T2DM) used in the treatment of non-insulin-dependent diabetes mellitus not responding to dietary modification. Metformin chemically is (3-(diaminomethyli dene)-1, (dimethylguanidine; hydro-chloride) decreases blood glucose levels by decreasing hepatic glucose production, decreasing intestinal absorption of glucose and improving insulin sensitivity by increasing peripheral glucose uptake and utilization.[1][2].

Sildenafil citrate (SIL), an oral therapy for erectile dysfunction, is the citrate salt of sildenafil, a selective inhibitor of cyclic guanosine monophosphate (cGMP) – specific phosphodiestrase type 5 (PDES). [3] Sildenafil citrate (1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo-[4,3-d]pyrimidin-5-yl) phenylsulphonyl]-4-methylpiperazine) is widely prescribed for the treatment of impotence and male erectile dysfunction [1]. Sildenafil is rapidly absorbed after oral administration, with absolute bioavailability of about 40%. It is rapidly and extensively metabolized in the liver to the active N-desmethyl sildenafil metabolite. [4]

A careful literature survey reveals that there are many chromatographic methods reported for the determination of SDF in biological fluids, including HPLC coupled with UV detection, liquid Chromatography-mass spectrometry (LC/MS) and liquid chromatography-tandem mass spectrometry (LC/MS/MS). [5] Some of these methods used gradient elution to separate the tested analytes. Literature also reveals the use of ion pairing technique and micellar liquid chromatography to develop a successful HPLC method for the determination of Sildenafil Citrate (SIL) and Metformin (MET). [6]

The present study is aiming to develop and validate a simple, sensitive, rapid, economic and isocratic HPLC method for the determination of both Metformin [8] and Sildenafil Citrate [7] on the same chromatographic run without the need for derivatization or precolumn treatment.



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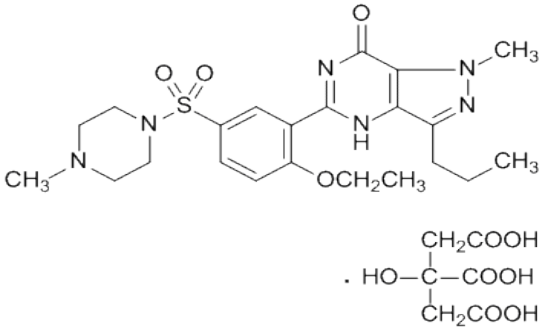

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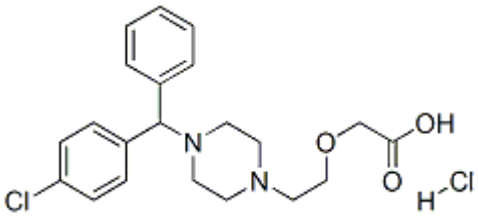


(c)

FIGURE NO. 1: STRUCTURE OF (A) MET, (B) SIL AND (C) CET (IS)

TABLE NO. 1: RESULTS OF ACCURACY STUDIES

Sr. No.	Nominal Comcentrations (Ug/ml)	Recovered amount (ug/ml)		Accuracy (%)	
		MET	SIL	MET	SIL
1	0.4	0.38	0.23	95	57.5
2	1.6	1.38	1.43	86.25	89.37
3	6.4	6.09	5.9	95.15	92.18

2. EXPERIMENTAL

2.1. INSTRUMENTATION:

A double beam UV–vis spectrophotometer, model UV-1600 PC (Thermo) with 10 mm matched quartz cell was used.

The HPLC instrument consisted of thermo separation product quaternary gradient equipped with a Water's 600 having an inline membrane degasser, detector was a PDA detector

belonging to spectra system 515 Autosampler injector with 20 ul loop. All the data were processed using Empower software. Separation was achieved using a Phenomenex C-18 Evo stationary phase (150 · 4.6 mm i.d. 5 um particle size) and the analytical column was protected by a Phenomenex C18 guard column (4 · 2.0 mm, i.d.).

2.2. MATERIALS AND REAGENTS:

Metformin HCl and Sildenafil citrate was donated by Yarrowchem products, Pvt. Ltd. All the reagents and chemicals used were of AR analytical and HPLC grade. Methanol (Spectrochemie), Acetonitrile (spectrochemie), Tri-ethyl anime (Loba chemic) and water (Lobachemic) used were of HPLC grade.

2.3. CHROMATOGRAPHIC CONDITIONS:

All determinations were carried out at room temperature. The isocratic separation of compounds was carried out by using mobile phase consisting of (0.3ml) Triethylamine buffer:methanol: Acetonitrile (70:05:25 v/v). The flow rate was maintained at 1 ml/min. The volume of injection was 20ul. The mobile phase was filtered through 0.45 um membrane filter and degassed by ultrasonification.

2.4. PREPARATION OF STANDARD SOLUTIONS

2.4.1. Metformin HCl and Sildenafil citrate stock and working solutions

The stock solution of MET and SIL was prepared by dissolving 1mg in 100 ml of 0.3ml of Triethyl anime buffer and further dilutions were prepared in 0.3ml Triethyl anime buffer to obtain the working solution of MET and SIL in the range of 0.4–6.4 ug/ml.

2.5. PREPARATION OF SAMPLE

Plasma samples were stored at 20^0 C and allowed to thaw at room temperature before processing. In brief, to 600 ul of plasma, 500 ul aliquot of working standard solution of MET and SIL was added in Strata-X Cartridges and then were added 300 ul of methanol and washed with 1000ul water then add plasma samples and remove plasma samples with suction vacuum then add 1000 ul of mobile phase. Resultant samples were injected in developed chromatographic conditions.

2.6. APPLICATION OF THE ASSAY

The above method was successfully applied for the pharmacokinetic studies of MET HCl and SIL citrate in rats. Sprague–Dawley rats (200–250 g) were housed with free access to food and water. The rats were fasted overnight with free access to water before administration of drugs. After a single oral administration of 200 mg/kg of MET [9] and 2.5 mg/kg of SIL [10], 0.5 ml of blood samples were collected from the retro orbital plexus sinus at 0, 1, 2, 4, 6, 12 and 24 h timepoints. Plasma was separated by centrifugation and stored at 20˚ C until analysis. Aliquots of 0.1 ml serum samples were processed and analyzed for MET and SIL concentrations[10]. The pharmacokinetic parameters were calculated with a non-compartmental model using Kinetica TM Soft-ware (version 4.4.1 Thermo Electron Corporation, USA). Each value is expressed as mean ± SD.

3. RESULTS AND DISCUSSION

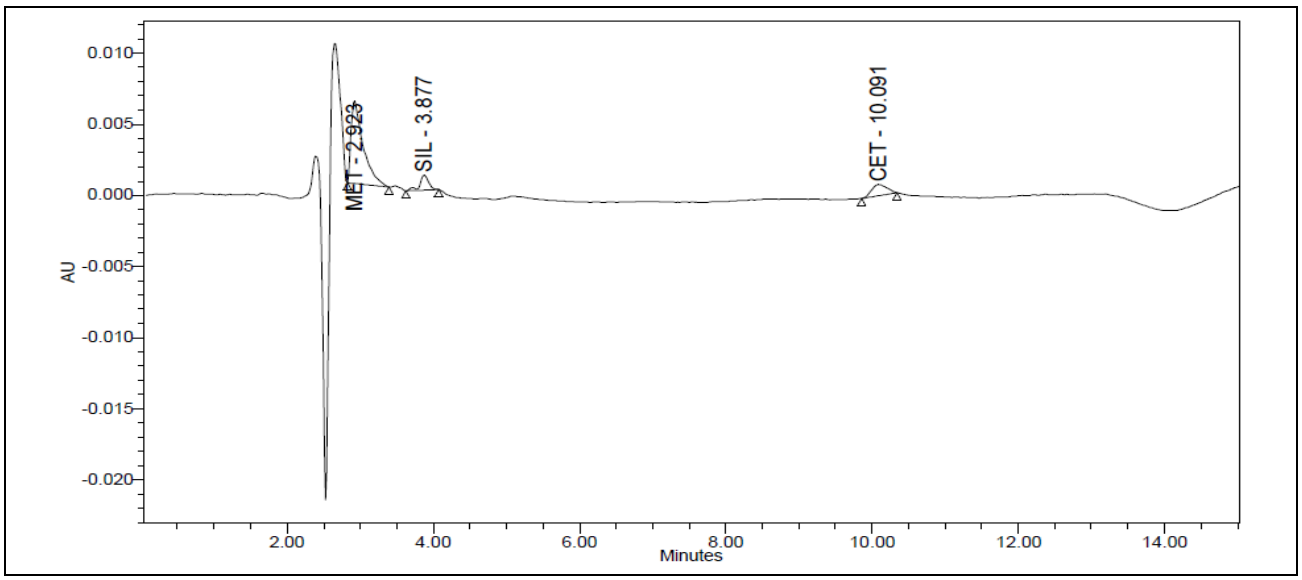


FIGURE NO. 2: CHROMATOGRAM QUALITY CONTROL TEST

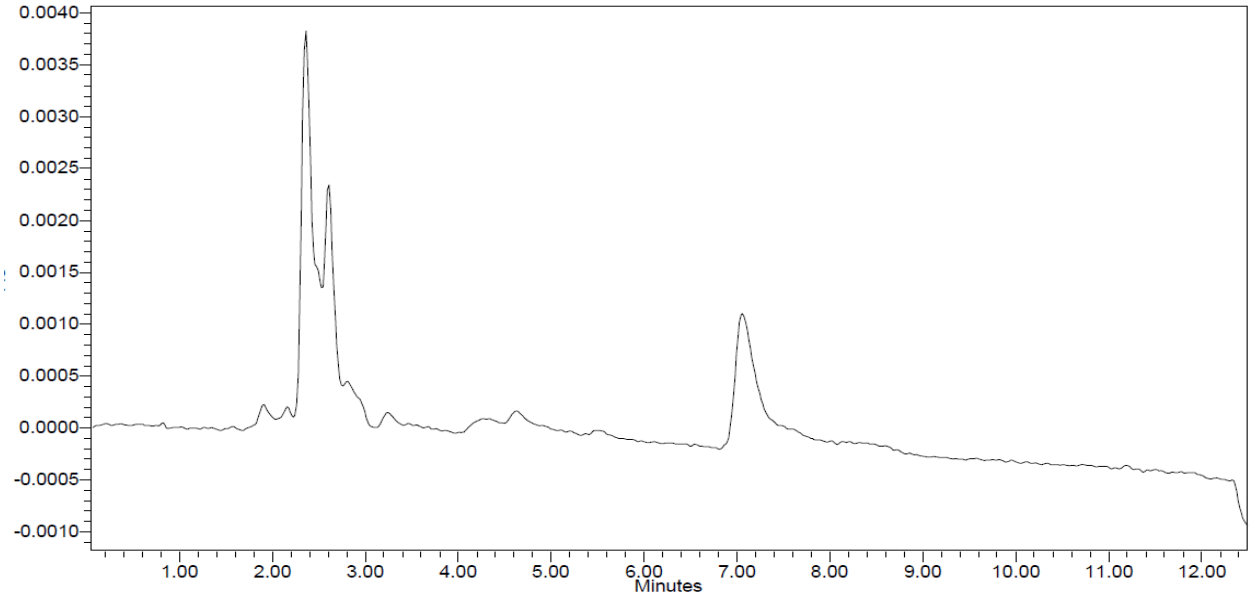


FIGURE NO. 3: BLANK PLASMA SAMPLE

3.1. METHOD VALIDATION

3.1.1. Selectivity and specificity

Blank plasma was studied for endogenous interference. A representative chromatogram of the plasma blank is shown in figure 3.

3.1.2. Linearity and limit of quantitation

Linear calibration curves with correlation coefficients greater than 0.9999 were obtained over the concentration range of 0.4–6.4 ug/ml for MET HCl and SIL in plasma. The co-efficient of regression i.e., r^2 = 0.895 for MET and 0.932 for SIL. The results had shown that within the concentration range indicated there was an excellent correlation between peak area ratio and each concentration of MET HCl and SIL.

The limit of quantitation, defined as the lowest concentration was analyzed with an accuracy of ±15% and a coefficient of variation of<15%, 0.38 ug/ml and 0.23 ug/ml for MET and SIL in plasma.

3.1.3. Accuracy

Accuracy studies were performed for MET and SIL in terms of recovery studies. For this 0.4, 1.6 and 6.4ug/ml of both drugs in plasma were injected and % recovery and % RSD were calculated. (See, Table 1).

3.1.4. Precision

Inter-day and intra-day precision studies were done by injecting three serial dilutions in developed chromatographic conditions (n = 6). For precision studies 0.4, 0.8 and 1.6 ug/ml were injected (n = 6). Peak areas were calculated for % RSD values, results for inter-day and intra-day precision are shown in Table 2.

TABLE NO. 2: RESULT OF PRECISION STUDIES (INTER DAY AND INTRA DAY)

Sr. No.	Quality Control Samples (ug/ml)	Inter-day Precision			
		Peak Area MET	% RSD	Peak Area SIL	% RSD
1	0.4	10042	±0. 86	11496	±2.55
2	1.6	11949	±1.22	17464	±1.60
03	6.4	37881	±0.63	33198	±1.50

Sr. No	Quality Control Sample (ug/ml)	Intra-day Precision			
		Peak Area MET	% RSD	Peak Area SIL	%RSD
1	0.4	10015	±0.96	11426	±2.34
2	1.6	11915	±1.70	17430	±1.99
3	6.4	37715	±0. 70	33131	±1. 84

3.1.5. Extraction recovery

Extraction recovery of MET and SIL was determined by comparing peak areas obtained from extracted plasma samples with those found by extracting blank matrices through the extraction procedure and spiking with a known amount of MET and SIL. The results showed that the mean extraction recoveries of SIL were >85% at concentrations of 0.4, 1.6 and 6.4 ug/ml, respectively (Table 3). Different organic extraction solvents were evaluated in the experiment, including methanol, acetonitrile, and 0.3ml triethyl amine buffer. Methanol, acetonitrile, and 0.3ml triethyl amine buffer combination proved to be the most efficient in

extracting MET and SIL from plasma and had a small variation in extraction recoveries over the concentration range.

Averaged for six measurements at each concentration level (n = 6).

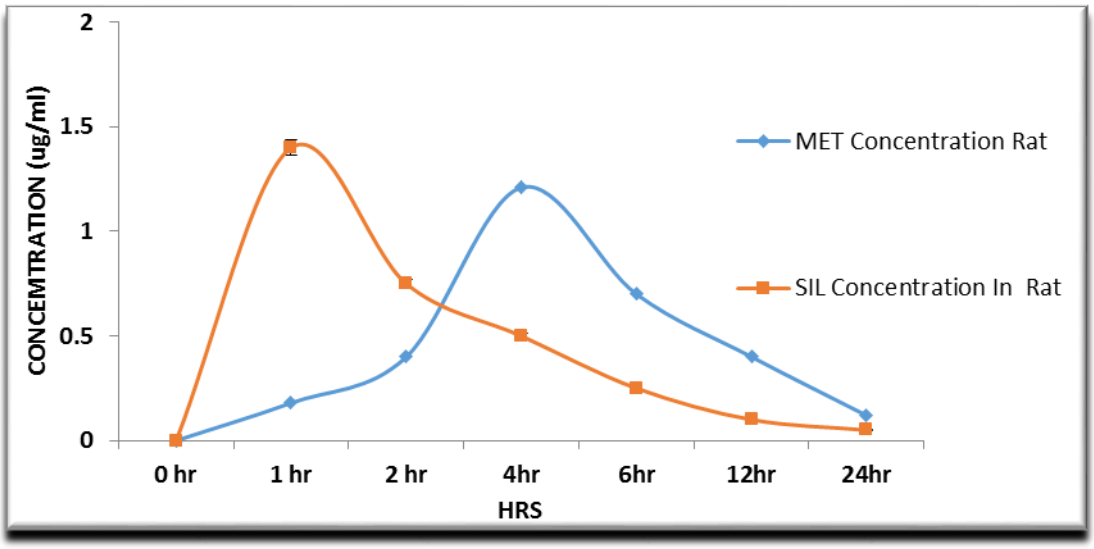
% recovery = (response of extracted spike) (response of post-extracted spike)- 1× 100

Sr. No.	Nominal concentration (ug/ml)	Mean % Recovery	
		MET	SIL
1	0.4	12.55 %	80.23 %
2	1.6	7.58 %	83.35 %
3	6.4	8.77 %	82.76 %

3.2. Application of the analytical method in pharmacokinetic studies

The described method was applied to a pharmacokinetic study in rats. After a single oral administration of MET(200 mg/kg) and SIL (2.5 mg/kg) to rats, plasma concentrations were determined over a period of 24 h after administration. The mean serum concentration–time curve after an oral dose of MET (200 mg/kg) [9] and SIL (2.5 mg/kg) [10] is shown in Fig. 3 and the main pharmacokinetic parameters are summarized in Table 4. The Cmax of MET and SIL detected in the rats was 15 ug/ml and 28.56 ug/ml, and the Tmax was 4 h in both drug.

Sr. No.	Group	AUC_{0-t} (ug.h/ml)	$AUC_{0-\infty}$ (ug.h/ml	C max (ug/ml)	Tmax (hr)	Kel	C- last (ug/ml)	$T_{1/2}$ (hr)
1	Metformin HCl	59.50 ± 1.2	13.5 ± 2.2	15 ± 0.5	4 hr	0.013 hr^{-1}	0.7	7.5hr
2	Sildenafil Citrate	98.60 ± 4.1	14.93 ± 2.2	28.56 ± 0.5	4 hr	0.211 hr^{-1}	0.05	6.2hr



3.3 DISCUSSION

Different trial had performed for the development of the method on HPLC. HPLC method had validated by system suitability, linearity, precision, LOD and LOQ. Extraction method was developed on the spiked human plasma. The developed HPLC method was applied for spiked plasma and even linearity was performed and R^2 value is 0.999 and 0.998 for Metformin HCl and Sildenafil Citrate respectively. For precision percent RSD values of peak areas were below 5%.

After the administration of drugs, 0.5ml of blood was withdrawn at an interval of 0 min, 1hr , 2 hr, 4 hr, 6 hr, 12 hr, 24 hr. there was significant (**P<0.0001) increase in the concentration of Sildenafil citrate in plasma.

4. CONCLUSION

In the present study a simple, accurate, and precise method was developed for the estimation of MET and SIL by RP-HPLC. The developed method was simple employing water and buffer as component of mobile phase. The developed method was elution of MET and SIL Run time was 15 min and specific with no interferences of blank matrix interfering with the quantification of MET and SIL. The developed method was applied successfully for pharmacokinetic studies of MET and SIL in rats. The applicability of method suggests its further application for bioequivalence, bioavailability and drug interaction studies.

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