Original Article

Liquid chromatographic method for simultaneous estimation of Metformin HCl, Pioglitazone HCl and Glibenclamide in rat plasma

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ABSTRACT

Separation of three analytes, namely metformin (MET), pioglitazone (PIO), and glibenclamide (GLB), was achieved within a single chromatographic run for the first time. Chromatographic method included stationary phase C_{18} Column (100 mm × 4.6 mm, 3.5 µm i.d.) and mobile phase consisting of ion-pair aqueous component and organic component in a gradient mode at 1 mL/min flow rate, and detection was monitored at 230 nm. Mobile-phase compositions and combinations were optimized for type and concentration of ion-pair reagents. Plasma sample preparation was based on protein precipitation by means of cold aqueous solution of 10% (w/v) trichloroacetic acid in combination with organic solvent addition. Ranitidine was used as the internal standard for MET, whereas rosiglitazone and amlodipine played the same role for PIO and GLB, respectively. Calibration curves were plotted from lower limit of quantification to 10,000 ng/mL for MET, PIO, and GLB. Weighing schemes of $1/X$ and $1/X^2$ were applied to observe goodness of fit in calibration curves. Precision was characterized by relative standard deviations below 15%. Stability analysis showed that all analytes are stable for at least 3 months when stored at −20°C. The validated method was applied for determination of MET, PIO, and GLB in pharmacokinetic study samples.

Keywords: Glibenclamide, high-performance liquid chromatography, metformin, pharmacokinetic, pioglitazone, plasma

INTRODUCTION

Metformin (MET; Figure 1) is an antidiabetic drug from the biguanide class. Pioglitazone (PIO; Figure 1) affects abnormal glucose and lipid metabolism associated with insulin resistance by enhancing insulin action on peripheral tissues. Glibenclamide (GLB; Figure 1), also known as glyburide, is an antidiabetic drug belonging to the sulfonylurea class, used in the treatment of type 2 diabetes (Granner and Davis 2001).[1] In most patients, monotherapy by MET is not sufficient in time due to a decline in β-cell function and/or weight gain. MET combined with thiazolidinedione and sulfonylurea is a second-line drug designed for type 2 diabetes mellitus treatment when either drug alone does not improve glycemic control (Kahn e*t al.*, 2007).[2]

Tablet dosage forms containing MET, PIO, and GLP in dosage strength of 500 mg, 15 mg, and 1 mg and 500 mg, 15 mg, and 2 mg, respectively. About 17 brands are available in Indian market. Literature data report several individual methods for the determination of MET, PIO, and GLB in biological samples. MET has been reported to be determined in biological fluid using pre-column derivatization (Lang *et al.*, 2006;Tache *et al.*, 2001)[3,4], normal phase (Koseki *et al.*, 2005).[5], and ion-pair chromatography (IPC) (Aburuz *et al.*, 2005; Tache and Albu, 2007).[6,7] MET (pKa >11.5) (Block and Beale, 2003)[8] remains ionized in chromatographic working pH range, i.e. 3-7 (Snyder *et al.*, 2007).[9] Hence, for optimal retention of MET, the

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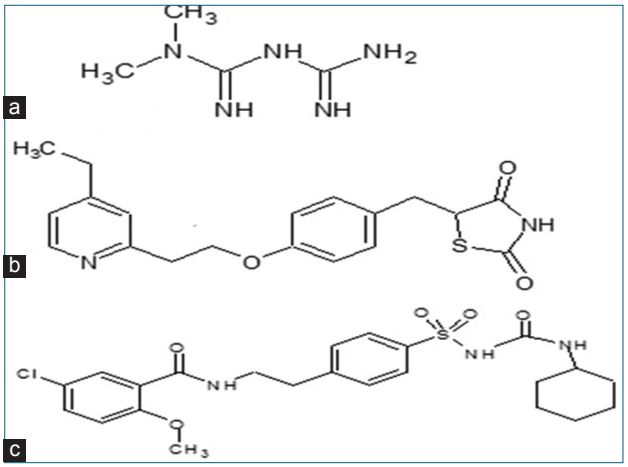

Figure 1: Chemical structure of (a) metformin, (b) pioglitazone, (c) glibenclamide

mode of chromatography should be either hydrophilic interaction liquid chromatography (Rimawi, 2009; Amini *et al.*, 2005)[10,11] or ion-exchange[12] (Gabr *et al.*, 2007) or ion-pair supported (Klaczkow and Anuszewska, 2010; Liu *et al.*, 2009)[13,14] chromatography. Although some reports showing retention of MET in reversed-phase conditions are available in the literature, all were applicable to estimate MET in pharmaceutical preparations (Kar and Chaudhary, 2009; Sultana *et al.*, 2011)[15,16] only. Simultaneous estimation of MET and PIO in laboratory mixture and in biological fluid is done by derivative ultraviolet (UV)-spectroscopy (Goswami *et al.*, 2010)[17,18] and high-performance liquid chromatography (HPLC) (Chaturvedi and Sharma, 2008). Simultaneous estimation of MET and GLB in biological fluid is reported using ion-pair HPLC (Aburuz *et al.*, 2005).[11,19] The HPLC method reported by Chaturvedi and Sharma (2008) showed elution of MET in the column void; hence, the only reported HPLC was not eligible to quantify the selected combination of biological fluids.[14]

To the best of our knowledge, there was no report published in literature regarding separation of MET, PIO, and GLB in pharmaceutical preparation and biological fluids. Therefore, an attempt was made to develop a new, accurate, and sensitive method for the simultaneous determination of MET, PIO, and GLB in the rats and rat plasma. To access the reproducibility and wide applicability of the developed method, it was validated as per the U.S. Food and Drug Administration[17] (Guidance for Industry: Bioanalytical method validation, USFDA, 2013) and European Medicines Agency[16] (Guideline for Industry: Bioanalytical method validation, EMA, 2011) guidelines.

EXPERIMENTAL

Chemicals and reagents

Qualified standards of MET HCl, PIO HCl, and GLB were gifted by Torrent Research Centre (Ahmedabad, India). Analytical/HPLC grade chemicals and solvents used were obtained from Ranbaxy Fine Chemicals Limited (Delhi, India). All buffers and solutions were prepared with purified and deionized water. Unless otherwise specified, all solutions were filtered through a 0.45 µm Ultipor® Nylon-66 membrane filter (Pall Life Sciences, USA) before use.

Chromatography apparatus and initial chromatographic conditions

A high-performance liquid chromatographic system (JASCO, Kyoto, Japan) composed of a PU-2089 plus Quaternary pump solvent delivery module, a manual rheodyne injector with a 20 µL fixed loop and a UV-2075 intelligent UV–Visible detector. For statistical calculations in bioanalytical method validation, GraphPad PRISM® version 5.1 for Windows (GraphPad Software Inc., California, USA) software was used.

MET was freely soluble in water and less soluble in methanol (MeOH). However, PIO and GLB were less soluble in water and freely soluble in selected organic solvents such as MeOH and acetonitrile (ACN). The chromatographic conditions were optimized by different means (using different columns, different buffers, different organic phases, and different ion-pair regents). Early chromatographic work was performed with different brands of C_8 and C_{18} columns as stationary phase and anionic ion-pair agents chain length containing C_6 and C_8 carbon atoms with various combinations of buffered (pH 2.5~4.0) organic phases (ACN and/or MeOH). The flow rate of mobile phase was varied within 1.0~1.5 mL/min. Wavelength for monitoring the eluent was selected by scanning standard solution of drugs within 200–400 nm using a double-beam UV–visible spectrophotometer (JASCO UV V630, Japan).

To achieve an optimum separation, effects of varying concentrations of buffer agent on different peak parameters were evaluated, *viz.*, mobile phase 15 mm dibasic phosphate buffer (pH 3.0) added with variable concentrations of 1-hexane sulfonic acid (HXSA), 1-heptane sulfonic acid (HTSA), and 1-octane sulfonic acid (OSA) in the range of 5-35 mM with MeOH at 1.0 ml/min flow rate in gradient mode.

Moreover, the effects of different level of all these factors were systematically addressed on system suitability parameters such as % relative standard deviation (% RSD) of peak area, retention time, capacity factor, asymmetry, resolution, and peak width.

All noted measurements were performed with an injection volume of 10 µL and UV detection at 230 nm of samples dissolved in a diluent consisting of buffer phase and organic phase in the ratio of 2:3, respectively. During development of bioanalytical method, diluent was changed accordingly.

Preparation of standard and resolution solution

Diluted standard solution of each analyte representing 10 µg/mL concentration was prepared with diluent. Ranitidine (RAN; IS-1), rosiglitazone (ROSI; IS-2), and amlodipine (AMLO; IS-3) were used as the internal standards for MET, PIO, and GLB, respectively. Resolution solution containing MET, PIO, and GLB (10 µg/mL each) and their IS (5.0 µg/mL each) was prepared accordingly.

For optimization purposes, 20 µL of resolution solution was injected into chromatograph, and system suitability parameters, *viz.*, % RSD of peak area for six injections of all analytes, % RSD of retention time for six injections of all analytes, peak asymmetry factor at 10% peak height and resolution, were studied.

Sample preparation and extraction

The protein precipitation was the preferred choice of separation because of the minimized steps in extraction of drug from matrix. The protein precipitation method was attempted using 10% trichloroacetic acid (TCA) and ACN. The mixture of 1 part plasma, 0.1 part drug, and 0.05 part of IS was taken for protein precipitation purposes. Diluted standard solution containing all analytes (15 µL) was added to 150 µL of plasma previously spiked with internal standards in a 1.5 ml capacity microcentrifuge tube. The blend was subjected to vortex for about 3 min. The mixture was allowed to stabilize for 2 min and then 100 µL of cold aqueous 10% (w/v) TCA was added and subjected to vortex for 2 min. PIO and GLB were less soluble in aqueous environment; therefore, about 245 µL of ACN was added and mixture was subjected to vortex for 10 min followed by centrifugation for 10 min at 13,000 rpm. The mixture was allowed to stabilize for 2 min and then 100 µL of supernatant was withdrawn and diluted with 100 µL of buffer phase and 20 µL of resulting solution was injected into HPLC system.

Bioanalytical method validation

The developed HPLC conditions were validated as per the USFDA and EMA guidelines for bioanalytical method validation.

Specificity

To demonstrate the specificity of the method, blank plasma from five different lots, spiked plasma samples, and plasma samples spiked with frequently prescribed medications were analyzed. Selectivity was established by injecting six samples at the lower limit of quantification (LLOQ) level and each of the six blank plasma samples were tested for interference by comparing the mean peak response obtained by injecting blank plasma samples to that of mean peak response of LLOQ (0.05 µg/mL for MET and 0.02 µg/mL for GLB and PIO). Representative chromatograms were generated to show that other components that could be present in the sample matrix are resolved from the parent analytes.[17]

Calibration curve

The standard curve was determined on each day of the 3-day validation; the slope, intercept, and correlation coefficient were determined. Each run consisted of a double control, system suitability sample, blank samples, zero sample, calibration curve consisting of 12 non-zero samples covering the total range (0.05-10 µg/mL for MET and 0.01-10 µg/mL for GLB and PIO) and quality control (QC) samples at three concentrations ($n = 6$, at each concentration). Such runs were generated on 6 consecutive days. Calibration samples were analyzed from low to high at the beginning of each run, and other samples were distributed randomly through the run. For calculation of the standard curve, plots of peak area ratios against concentration were used.

Sensitivity

The sensitivity (LLOQ) was determined by signal-to-noise ratio. The resolution solution was serially diluted and spiked with the rat plasma. Similarly, blank plasma samples were also processed and injected into chromatograph. The LLOQ was expressed for the analyte concentration having response at least 5 times more compared to blank response.[17]

Precision and accuracy

Intraday precision, interday precision, and accuracy were calculated from data obtained during a 6-day validation. Three concentrations were chosen from the high, medium, and low range of the standard curve (LLOQ, 200, 3000, and 8000 ng/ml and upper limit of quantification [ULOQ] for PIO and GLB and LLOQ, 500, 3000, and 8000 ng/ml and ULOQ for MET) as QC samples. Plasma samples spiked at five concentrations, i.e. LLOQ, low QC (LQC), medium QC (MQC), high QC (HQC), and ULOQ, were analyzed at each day of the 6-day validation ($n = 6$ at each concentration). Accuracy was expressed as the mean relative error (RE%). A precision and accuracy value (% CV) ≤15% for all samples was acceptable.

Recovery

Recovery of MET, PIO, and GLBN was evaluated by comparing the mean peak response (peak area ratio of MET, PIO, and GLBN with respect to IS) of processed LLOQ, ULOQ, and three QC samples (low, medium, and high) to mean peak response of unprocessed/without plasma of the same concentration. Recovery of ranitidine, rosiglitazone, and amlodipine (IS) was evaluated by comparing the mean peak areas of processed samples to mean peak areas of unprocessed reference solutions of the same concentration.

Stability studies

The stability experiments were aimed at testing all possible conditions that the samples might experience after collecting and before analysis. The stability of the drug spiked at two QC levels (LQC and HQC) for short-term bench top (at room temperature for 4 h, 8 h, 16 h, and 24 h), freeze thaw (3 cycles; −20°C to room temperature), and long term (30, 60, and 90 days at −20°C) were evaluated. Stability of all analytes in analytical solution was observed at room temperature and in refrigerated conditions for a period of 48 h.

In vivo plasma drug analysis

Two groups of male rats 8–10 weeks of age were used for the study. Each group consisted of six animals. All animals were fasted for 18 h before the administration of the drug.

Access to water was maintained during the experiment, but animals were fasted beginning the night before the experiment and through 4 h of the experiments. Each group of animals received a single oral dose of 50 mg/kg of MET and 10 mg/kg of PIO and GLB. A homogenous suspension of MET, PIO, and GLB was prepared in a vehicle comprising of 0.5% (w/v) carboxymethyl cellulose in water and polyethene glycol 400 (90:10, v/v). About 0.2 ml of blood was withdrawn from the tail vein of a rat into heparinized Eppendorf® tubes at various time points, *viz.,* 0.5 h to pre-dose, at 0.5, 1.0,

1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 8.0, 10.0, 12.0, and 24.0 h after dose administration. Blood samples were centrifuged at 15000 rpm for 10 min and plasma was separated and stored at −20°C until use. The samples were processed as per the protein precipitation protocol. The study adheres to "Principles of Laboratory Animal Care" and is approved by the institutional animal ethics committee/committee for the purpose of control and supervision of experiments on animals.

The pharmacokinetic parameters, namely maximum plasma concentration, time point of maximum plasma concentration, area under the plasma concentration–time curve from 0 to the last measurable concentration, and area under the plasma concentration–time curve from 0 to infinity, were estimated using non-compartmental analysis of Pharmacokinetic add-in provided in Microsoft Excel.

RESULTS AND DISCUSSION

Optimization of the mobile phase

Our preliminary experiments indicated that using different concentrations of ion-pair reagents and with varying amount of organic phase results in controllable retention of MET. Hence, a reversed-phase IPC method was developed for MET determination and the ion-pair reagent which was most suitable for MET was OSA. HXSA was not able to produce optimal retention of MET. The peak asymmetry value was increased with reduction in chain length of ion-pairing agents. The developed mobile phase was 20 mM sodium OSA and 10 mM potassium dihydrogen phosphate in gradient mode. The pH was adjusted at 3.0 using concentrated phosphoric acid to keep all the analytes in ionized state. The UV absorbance for all the analytes was taken at 230 nm wavelength as all analytes showed optimum and homogenate response at the selected wavelength [Figure 2].

PIO and GLB and their I.S. are more lipophilic than MET, having log p-value more than 4.0. The lipophilicity of all the analytes was sufficiently different from each other; therefore, for co-elution of selected analytes in a single run, a gradient run was proposed. In this study, a series of experiments were conducted to optimize the gradient composition of mobile phase for the simultaneous determination of MET, PIO, and GLB with their respective internal standards.

Type and concentration of IP reagent

With the aim of the optimization of type and concentration of IP reagents (HXSA, HTSA, and OSA), the remaining three factors, i.e. mobile-phase composition (15 mM dibasic phosphate buffer: MeOH; in gradient mode), pH of the buffer phase (pH 3.0), and flow rate of 1 ml/min, were kept constant. The ion pairs formed can enhance selectivity because of electrostatic interaction with analytes of opposite charges and also due to hydrogen-bonding capacity. The capacity factor was increasing with the increase in non-polar chain and concentration of IP reagents. The asymmetry of the peak was decreasing with increase in chain length and concentration of IP reagent. The peak $As_{10\%}$ was least for OSA in the concentration in the range of 10–20 mM. The peak asymmetry factor and capacity factor results were poor in the case of HXSA. The resolution between MET and RAN and between AMLO and GLB was optimum at 20 Mm OSA. Resolution was less than the USFDA limit, and the effect was ambiguous at higher concentration of OSA. Looking at the importance of the different chromatographic parameters, 30 mm HTSA and 20 mM OSA were found to be optimum. An overlain chromatogram representing co-elution of all the analytes in the optimized ion-pair HPLC methods is shown in Figure 3.

Mobile phase A was prepared using 10 mM dibasic phosphate buffer with 20 mM OSA (pH 3.0 adjusted with phosphoric acid). Mobile phase B was prepared using 20 mM OSA (previously adjusted to pH 3.0 with phosphoric acid) in a 90:10 mixture of ACN and water. The mobile phase A and mobile phase B were passed through column at 1.0. mL/min in gradient mode given in Table 1.

The results of system-suitability studies in the final and optimized ion-pair HPLC method are given in Table 2.

Development of protein precipitation method

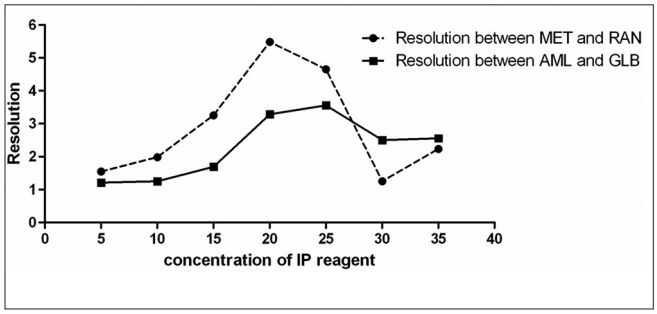

Figure 2: Method development of metformin and ranitidine

Table 1: Gradient mode for simultaneous elution of MET, PIO, and GLB using ion pair-HPLC method

Time (in min)	% Mobile phase A	% Mobile phase B
0.00	75	25
0.00→13.99	38	62
14.00→19.99	38	62
20.00→25.00	75	25

HPLC: High-performance liquid chromatography MET: Metformin, PIO: Pioglitazone, GLB: Glibenclamide

Table 2: System suitability studies for resolution solution of MET, PIO, and GLB (n=6) in the optimized ion pair-HPLC method

System suitability parameter	USP Limit	MET	PIO	GLB
Retention time	-	3.86	14.49	18.32
% RSD of Rt	-	0.56	0.3	0.15
Mean peak area ratio	-	1.9	1.1	0.82
% RSD of peak area ratio	≤2.0	0.65	1.90	1.26
Peak asymmetry factor (at 10%)	≤1.5	1.45	1.01	1.08
Capacity factor (k')	5–20	2.9	13.5	17.3

HPLC: High-performance liquid chromatography MET: Metformin, PIO: Pioglitazone, GLB: Glibenclamide

The protein precipitation was the preferred choice of extraction of drugs from biological fluids because of the minimized steps in extraction and less costly. It may provide maximum matrix effect and therefore make method more robust. The protein precipitation method was attempted using 10% TCA and MeOH. Several trials were taken to get optimum recovery of all the analytes. Recovery of MET was > 85% when precipitation of plasma protein was done with TCA alone, but the recovery of PIO and GLB was <50%. PIO and GLB are less soluble in aqueous environment; therefore, MeOH was taken as an organic partner of TCA as a precipitating agent. The pH of precipitating media was <2.0 due to TCA that help to keep all analytes in ionized state. Overlain chromatogram representing processed plasma blank in different precipitation conditions is shown in Figure 4.

The protein precipitation was carried out by 1 part of plasma, 0.1 part drug, and 0.05 part of I.S. Plasma previously spiked with internal standards was used in precipitation. At the end of precipitation, supernatant was mixed with buffer of mobile phase to make the sample more compatible with the developed method. The % mean recoveries for all the analytes ranged from 90% to 95% in the developed method.

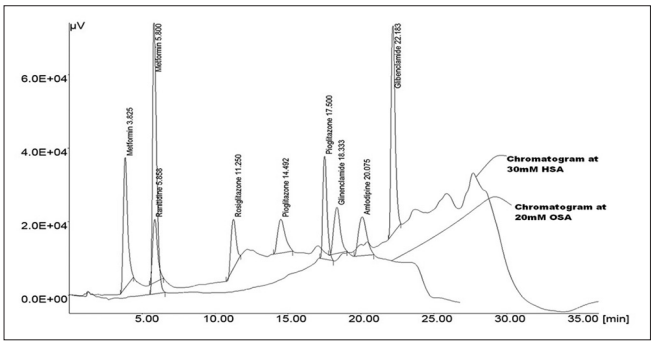

Figure 3: Method development chromatogram

Table no. 3: List of drugs not interfering with retention time of analyte of interest

Name of drugs commonly prescribed in cardiovascular disease		
Gliclazide	Atenolol	Enalapril
Nateglinide	Codeine phosphate	Nifedipine
Glimepiride	Lisnopril	Nevivolol
Amlodipine	Furosemide	Carvedilol
Itraconazole	Candersartan	Losartan
Fluconazole	Valsartan	Captopril
Fenofibrate	Irbesartan	Sidenafil citrate
Metaprolol	Atorvastatin	Paracetamol
Diclofenac	Levofloxacin	Ambroxol
Oseltamivir phosphate	fenofibrate	Terbutaline
Ciprofloxacin	Amphotericin B	
Ranitidine	Domperidone	

Bioanaytical method validation

The developed method was validated for its specificity, sensitivity, working range, recovery, precision, accuracy, and stability as per the bioanalytical method validation guidelines suggested by the USFDA.

Specificity

The specificity of optimized bioanalytical HPLC method was evaluated by observing interference due to matrix and probable comedicated drugs.

There were no endogenous substances in the plasma that interfered with the other peaks of interest as the blank analysis gave no responses at the retention times of the peaks of interest, and the resolutions between all the peaks are more than 2.

Standard curve and linearity

The standard curve was determined on each day of the 6-day validation; the slope, intercept, and correlation coefficient were determined. The goodness of fit was applied for mean ± SD for each analyte for its calibration range. Different weighing schemes, i.e. $1/X$ and $1/X^2$, were applied to calculate the slope and intercept. The results of slope, intercept standard error, and regression coefficient Were shown in Table 3. The % bias in recovery calculation at LLOQ level was least (<5%) for weighted calibration curve when a weight of $1/\times^2$ was taken. By examining the calibration curves and it was concluded that the relationship between area ratio and concentration was weighted linear ($1/\times^2$) within the studied concentration range. The results of unweighted and weighted ($1/\times^2$) calibration curves are shown in Table 4.

The mean % RE was <15% for all the selected drugs.

Accuracy and precision

The values obtained during the 6-day validation for plasma intraday and interday precision and accuracy are summarized in Table 5.

All values of accuracy and precision were within recommended limits. Intraday precision ranged between 1.04% and 7.75% whereas the interday precision was between 1.38% and 14.09%. The intraday mean error was between −1.98% and 9.5% whereas the interday mean % RE was between −2.88% and −10.82%.

Sensitivity

Table 5 details the accuracy and precision at LLOQ, for each of the investigated analytes. Concentrations down to the LLOQ were detected with acceptable accuracy and precision using this method (CV% and RE% <15%). The mean background response of blank

Table 4: Comparison of weighted and unweighted calibration curves for MET, PIO, and GLB

Name of drug	Calibration range (in ng/mL)	Unweighted linearity curve					1/X2 weighted linearity curve			
		m	c	Sy.x	r2	%MRELLOQ	m	c	SEc	%MRELLOQ
MET	100–10000	3.272	−0.029	0.29	0.999	220.6	3.232	0.04209	0.01563	10.25
PIO	50–10000	1.934	0.0409	0.26	0.995	25.65	1.944	0.008322	0.004445	9.11
GLB	50–10000	1.606	−0.0066	0.108	0.999	40.56	1.590	−0.002815	0.003414	12.36

m and c are slope and y-intercept, respectively, for line equation of y=mx+c. SEc is standard error of Y-intercept. And %MRELLOQ is %Mean RE at LLOQ level

was also calculated and found to be <20% of the response at the limit of quantification. The LLOQ level of MET was kept higher due to blank interference at the R_t of MET. The mean background was also calculated and found to be 18.9% (MET), 12.4% (PIO), 17.6% (GLB), 4.25% (RAN), 4.58% (ROSI), and 3.97% (AMLO) in selected conditions. Besides that, the mean background interferences at the retention of all internal standards (% background interference <5%) were within limits.

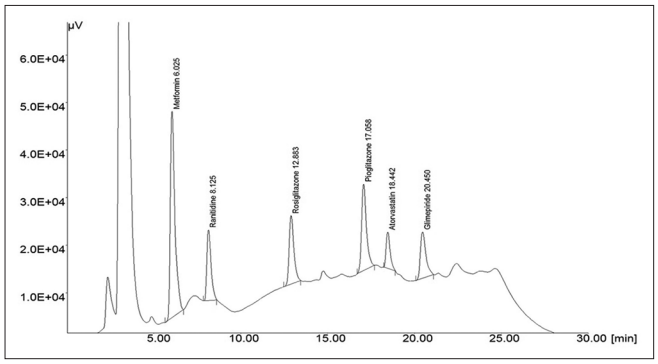

Figure 4: Overlain chromatogram representing processed plasma blank in different precipitation conditions

Table 5: Interday precision and accuracy data for assays of MET, PIO, and GLB in plasma (*n*=6)

Nominal concentration in ng/mL	Precision (% CV)		Accuracy (mean % RE)	
	Intraday	Interday	Intraday	Interday
MET				
20 (LLOQ)	5.60	2.5	−1.98	−5.68
500 (LQC)	1.20	3.43	−2.78	−4.9
3000 (MQC)	0.93	1.38	−3.86	−5.15
8000 (HQC)	1.89	2.96	−3.4	−2.88
10000 (ULOQ)	2.45	3.69	−2.65	−6.64
PIO				
10 (LLOQ)	5.61	11.57	−9.5	−9.28
200 (LQC)	3.03	5.23	−7.01	−7.84
3000 (MQC)	1.04	2.19	−4.39	−8.04
8000 (HQC)	3.1	4.83	−3.17	−5.53
10000 (ULOQ)	2.99	3.65	−2.19	−3.98
GLB				
10 (LLOQ)	7.75	14.09	−5.58	−7.22
200 (LQC)	2.74	3.3	2.72	−10.82
3000 (MQC)	1.25	2.34	−4.82	−4.73
8000 (HQC)	3.34	3.78	3.77	4.34
10000 (ULOQ)	2.98	1.95	2.49	5.65

Table 6: % Mean recoveries for MET, PIO, and GLB in finally optimized method

Concentration	% Mean recovery for MET (n=6)	% Mean recovery for PIO (n=6)	% Mean recovery for GLB (n=6)
LLOQ	96.83±3.35	90.92±8.39	92.22±9.98
LQC	95.52±2.48	91.92±4.25	89.46±2.97
MQC	96.75±1.25	92.99±1.89	95.24±2.12
HQC	94.03±2.09	94.41±3.89	95.26±3.56
ULOQ	90.84±3.15	93.59±5.36	92.08±2.54
Mean±SD	94.8±2.48	93.37±2.21	92.85±2.44

MET: Metformin, PIO: Pioglitazone, GLB: Glibenclamide

Recovery and dilution integrity

The extraction recovery was calculated at LLOQ, QC samples, and ULOQ level (n = 6). The highest recovery of MET was using protein precipitation method with 10% cold aqueous solution of TCA (97.76%), and the lowest recovery was using MeOH (<60.35%) at 10 µg/mL of MET. This was to be expected as the protein-binding capacity of MET is low (<4%) and MET is more soluble in aqueous solution as compared to organic solvent/s. The recoveries of PIO and GLB ranged between 47.7% and 61.9% in TCA solution whereas the recoveries were ranged between 79.85% and 91.26% in MeOH at test concentration level (i.e. 10 µg/mL). This was to be expected as high solubility of PIO and GLB in organic solvents. Therefore, a combination of TCA and MeOH was taken as final precipitating media, and data for mean % recovery in finally optimized protein precipitation method are given in Table 6.

Stability

Stock solution of MET, PIO, and GLB and all IS were stable at room temperature for 24 h and at 2–8°C for 48 h. MET, PIO, and GLB analytes in control rat plasma at room temperature were stable at least for 24 h and for minimum of three freeze and thaw cycles.

Spiked plasma samples, stored at −20°C for long-term stability experiment, were stable for minimum of 90 days. Different stability experiments in plasma with values for precision and percent change are shown in Table 7.

The pharmacokinetic profile of MET, PIO, and GLB in male Wistar rats (n=6). The results of pharmacokinetic parameters are given in Table 8.

Table 7: Stability of MET, PIO, and GLB at LQC and HQC level

Stability	% Mean change		
	MET	PIO	GLB
Benchtop			
Room temperature (24 h)			
LQC	2.0	4.25	0.61
HQC	1.1	−0.18	3.19
Freeze and thaw			
After 3rd cycle at−20°C			
LQC	2.3	6.23	7.49
HQC	1.2	−2.51	5.13
Long-term stability			
90 days at−20°C			
LQC	3.0	5.91	−2.42
HQC	0.4	0.95	−2.01

MET: Metformin, PIO: Pioglitazone, GLB: Glibenclamide

Table 8: Pharmacokinetic profile of MET, PIO, and GLB in male Wistar rat (n=6)

Pharmacokinetic parameter	Metformin	Pioglitazone	Glibenclamide
Dose (mg/kg)	50	10	10
Cmax (µg/mL)	3.48±0.48	13.9±1.8	0.239±0.0291
Tmax (h)	0.5±1.5	1.5±1.0	3.00±0.63
Ke (1/h)	0.63±0.048	0.16±0.012	0.063±0.01
t1/2 (h)	3.5±0.5	5.0±0.5	11.20±1.81
AUC0−t	13.4±1.18	105.3±11.0	1.89±0.34

CONCLUSION

The developed method is a suitable and valid method for the determination of a combination of MET, PIO, and GLB. The use of this method can save time and effort when monitoring a population of diabetic patients who take several diabetes medications; there is no need to have more than one HPLC system or to change the HPLC column to measure plasma from patients on different medication regimes. The validity, LLOQ, and the calibration range of the method make it an acceptable method for preclinical as well as clinical studies in diabetic patients taking these medications. The validated method can also be used in assaying the MET, PIO, and GLB in pharmaceutical formulations.

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