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Validation of a rapid and economical RP-HPLC method for simultaneous determination of metformin hydrochloride and sitagliptin phosphate monohydrate: Greenness evaluation using AGREE score

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Abstract: Reported high performance liquid chromatographic (HPLC) methods for estimating metformin hydrochloride (MET) and sitagliptin phosphate monohydrate (SIT) are either laborious or contain higher proportions of organic solvents in mobile phase, thus presenting exorbitant procedures. So, a rapid, significantly more economical and eco-friendly HPLC method for synchronized analysis of both drugs was aimed to develop and validate in current study. Analytical evaluation was executed on Shimadzou® C_{18} column (250mm × 4.6mm, 5µm) using acidified water and methanol 60:40 (v/v) as mobile phase at a flow of 1mL/min; while peaks were detected at 260nm at 25°C. Resultant values of accuracy, precision, linearity, limit of detection (LOD), limit of quantification (LOQ), robustness and specificity depicted that the method was validated in accordance with the ICH Guidelines. The approximate retention time for MET and SIT were 1.96 and 3.70 min, correspondingly. The greenness score of the developed method was evaluated using AGREE software and was found better (0.81) as compared with the methods reported (<0.8). Conclusively, the developed method was time saving, economical, rapid, robust, rugged, precise, accurate and found to be applicable for simultaneous determination of MET and SIT in commercial tablets.

Keywords: ICH guidelines, method development and validation, metformin, RP-HPLC, sitagliptin.

INTRODUCTION

In fixed dose combination, metformin hydrochloride (MET) and sitagliptin phosphate monohydrate (SIT) have proven as an effective therapy in achieving glycemic control in type 2 diabetic (DM) patients. Two fixed dose combinations have been approved by the Food and Drug Administration authority (Zhang *et al.*, 2021). Currently, immediate and extended release tablets of MET and SIT are available worldwide (Maina, 2018). Metformin is the first line of oral medication in type 2 DM patients according to NICE and ADA guidelines. MET can be used either as single drug or combined with other agents in its class (van Dalem *et al.*, 2021). Chemically, MET is an N, N-diethyl imido dicarbonimidic diamide hydrochloride and comes under the biguanides class of anti-diabetic drugs. The mechanism of action includes improvement in the peripheral sensitivity to insulin by up regulation of glucose in skeletal cells and by inhibiting intestinal glucose uptake as well as hepatic gluconeogenesis from non-sugar precursors (Al Bratty *et al.*, 2017). The drug has a reported half-life of about 5 to 6 hours and excreted unchanged in the urine with the meagre amounts of protein binding. Elimination occurs through kidney occur via active tubular secretion (Bahrambeigi *et al.*, 2019). MET is nearly insoluble in acetone, ether and chloroform, easily soluble in water and faintly soluble in 95% v/v ethanol (Sirigiri *et al.*, 2018). It is reported that less than 50% of patients could not achieved adequate glycemic control with metformin alone, therefore, advised to progress with add on therapy. However, sitagliptin can effectively be used with metformin as a dual therapy in order to necessarily reduce the progression of disease (Sharma *et al.*, 2017). Whereas, Sitagliptin phosphate monohydrate (SIT), is related to class dipeptidyl peptidase-4. The chemical name of Sitagliptin is -[(3R)-3-amino-1-oxo-4-(2,4,5-trifluorophenyl) butyl]-5,6,7,8tetrahydro-3-(trifluoromethyl) -1,2,4-triazolo[4,3-a]pyrazine phosphate. It acts by prolonging the anti-diabetic action of incretin hormones, including glucagon-like peptide and glucose-dependent insulinotropic polypeptide, thus by inhibiting their breakdown (Ahmed *et al.*, 2018). Sitagliptin is absorbed well upon oral administration having bioavailability of 87%. It is majorly removed unchanged from the body through kidneys (Scott, 2017). It is insoluble in isopropanol, very slightly dissolved in acetonitrile and ethanol; whereas sparingly soluble in methanol but soluble in water (Stofella *et al.*, 2019).

Multiple analytical techniques of simultaneous estimation for MET and SIT are evident in literature but these are either time consuming to conduct quantitative analysis (Peraman *et al.*, 2013) or expensive due to high feeding of

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organic solvents (Malleswararao *et al.*, 2012, Shyamala *et al.*, 2011). Thus, a simple, fast, ecological and cost-effective method development is the need of present time. To improve in existing techniques, the current study aimed to develop a robust quick as well as an inexpensive technique for simultaneous estimation of MET and SIT by reverse phase HPLC. Then, it was validated according to the guidelines of International Council for Harmonization (ICH) of Technical Requirements for Pharmaceuticals for Human Use (Syed *et al.*, 2021). To accomplish linearity, system suitability, precision, accuracy, robustness, limit of detection (LOD), limit of quantification (LOQ) and specificity for quantitative evaluation of drugs in marketed dosage form. Finally, the

MATERIALS AND METHODS

Metformin hydrochloride, sitagliptin phosphate monohydrate were obtained from Consolidated Chemical Laboratories®, Pakistan on kind basis. Double distilled water was used throughout the experiment unless otherwise specified. Analytical chemicals/ solvents/ reagents were of research grade and used as received.

Instrumental conditions

The analysis was performed on Shimadzu® (LC20) HPLC gradient system with manual sampler injector (20µL). The temperature of C_{18} column (250mm × 4.6 mm, 5µm) was maintained at 25°C from which the analytical substance was flowing and eluted for detection at 260 nm. The temperature of the column was maintained at 25°C while flowing through the column at 1mL/min.

Preparation of mobile phase (MP) and standard solutions

For the preparation of acidified water, 5.5mL of concentrated sulphuric acid was diluted to 1L with distilled water. Then 1mL of dilute sulfuric acid was further diluted in one liter of distilled water to make acidified water. This acidified water was mixed with methanol in a respective ratio of 60:40 volumetrically to constitute the mobile phase. The final pH was then adjusted to 3.0. Standard solution was prepared by dissolving 500 and 50mg of MET and SIT in 100mL of distilled water to form standard solution 'A'. Then further concentrations were prepared from 'A' by diluting in order to achieve linearity of both drugs.

Validation parameters

The system suitability was evaluated by finding the resolution, tailing factor, retention time (min), and plate count before establishing accuracy, precision, robustness, LOD and LOQ (Hanif *et al.*, 2021). The resolution (R_s) of both peaks of drugs in the chromatogram was found using the Equation 1.

$R_s = (t_{R2}-t_{R1})/ 0.5\ (t_{W1} + t_{W2})$ ……. Eq. 1.

Where 't_{R1}' and 't_{R2}' are the retention time of MET and SIT, respectively. While 't_{W1}' and 't_{W2}' are the corresponding chromatographic peak width (Swartz and Krull, 2018). Then tailing factor (T_f) or coefficient of peak symmetry as reported in United States Pharmacopeia (USP) was calculated (Equation 2) as reported (Syed *et al.*, 2021).

$Tf = (a+b)/2a$ ……. Eq. 2.

Where 'a' is the distance from the front half of the peak to the peak midpoint (upright from the peak highest point) that is evaluated at 5% of peak altitude and b is the distance from the peak center (perpendicular from the peak maximum point) to the trailing edge of the peak estimated at 5% of peak height (Mullick *et al.*, 2021). For linearity, different dilutions from standard solution 'A' were prepared and 6 injections of each dilution were injected in sequence run on HPLC and peak area were determined respectively. Accuracy was determined by evaluating the percentage recoveries of standard solution at three different levels of drug concentrations i.e. 80, 100 and 120% (Syed *et al.*, 2021). The precision was identified by analyzing samples at different days on different machines by two independent analysts. However, concentrations similar to prepared for accuracy experiment were used in precision determination. The robustness of developed procedure was evaluated at slight above and lower used conditions of pH, flow rate and temperature for 100% concentrations of both drugs. While LOQ is the procedure where, minor quantity of a substance is quantitatively determined with suitable accuracy and precision. The parameters LOD (Equation 3) and LOQ (Equation 4) were evaluated as follows.

$LOD = (\sigma/m) \times 3.3$ ………. Eq. 3.
$LOQ = (\sigma/m) \times 10$ …………Eq. 4.

The 'm' indicates slope of line and 'σ' indicates the standard deviation of the lowest concentration of analyte prepared in the study. In specificity, the peaks of drugs in the dosage form was compared with peaks in standard solution and the blank injection of dissolution media (Le *et al.*, 2019). Then peaks were evaluated if any change in the shape or retention time were observed for both MET and SIT.

Assay of pharmaceutical dosage form

After the validation of analytical method, the conditions were applied to calculate the concentration of drugs from commercial dosage form. For this, sample of SITA-MET® (manufactured in 2020) was purchased from the local market of Lahore (Pakistan) for analysis. Initially, 20 tablets containing both MET and SIT were taken and ground to fine powder. Then the weight equivalent to the weight of a single tablet was taken and dissolved in the MP and made solution in mobile phase according to the prescribed analytical procedure (Alquadeib, 2019). Moreover, the conditions were tested on a mucoadhesive buccal sustained release formulation (Hanif *et al.*, 2017) using dissolution conditions as reported by the researchers (Hussain *et al.*, 2016, Razzaq *et al.*, 2018).

STATISTICAL ANALYSIS

The statistical analysis of different validation parameters were evaluated using IBM SPSS v.20 software package and standard deviation along with percentage determination of the concentration of analyte were calculated (Razzaq *et al.*, 2021).

Greenness evaluation

The agreement of the established analytical procedure for greenness was calculated using the AGREE software v0.5 beta developed by the Universida de Vigo as reported. To accomplish, different parameters related to the extent to toxic substances, waste materials and use of resources were evaluated with a predefined criterion and penalty points on twelve different evaluation parameters (Pena-Pereira *et al.*, 2020). The score earned by the method after deducting penalty points were types at the center on a circular pictogram. The criteria where the developed method ideally agreed to the greenness conditions was colored green while the extent to total disagreement was marked with red. For response in between the two extremities, it was graded between both contours (fig. 2).

RESULTS

Method Development and Optimization

Method was optimized by altering the column conditions, pH as well as concentration of mobile phase and it was set with the ratio of 60:40 with acidified water and methanol respectively. Two sharp peaks were observed in the chromatogram (fig. 1), representing MET and SIT.

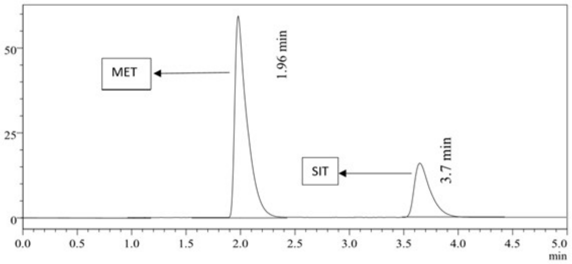

Fig. 1: Typical chromatogram of metformin hydrochloride (MET) and sitagliptin phosphate monohydrate (SIT)

(Bhende *et al.*, 2012)
a

(Sirigiri *et al.*, 2018b)
b

Current Study
c

Fig. 2: Comparison of the AGREE score between the developed HPLC method and some methods, reported in the literature.

Method Validation

System suitability

Results showed that relative standard deviation of peak areas was less than 2% and retention peak time observed for MET and SIT was 1.96 and 3.70 min, respectively. The theoretical plates numbers (N) was at least 4500 per each peak and tailing factor was less than 1.5 for both MET and SIT as summarized in table 1.

Linearity

In current conditions, the linearity for both drugs were found satisfactory with good coefficient linear of regression values (table 1). The respective coefficient of regression (r^2) values for MET and SIT were found to be 0.9998 and 0.9993. The linearity range for MET and SIT calculated in the current findings were 100-300 and 10-35 ppm, respectively.

Accuracy

Accuracy was estimated by performing recovery studies at three varied concentrations of both drugs i.e. 80, 100 and 120% prepared from the standard solution. The respective values of all the concentrations were with the value of 2%, suggesting that the developed method was accurate.

Precision

Precision was performed by testing the analytical procedure at inter day by two different analysts using two different analytical machines. Outcome revealed that the standard deviation was less than 2% which suggests that the conditions for the estimation of MET and SIT were precise.

Robustness

For robustness, the analytical conditions were slightly changed to check whether the impact of minute variations affects the chromatographic outcome or not. It was evaluated by varying conditions like change in the pH of mobile phase i.e. ±0.5, flow (0.85 and 1.15mL/min) and temperature from 20 to 30°C (table 5). Results revealed that the developed method was stable and no significant change in the peak was observed relative to each drug. The values of RSD were also with limit of less than 2% (Syed *et al.*, 2021) that shows the ruggedness of the method.

Specificity

Specificity is degree of interference of added ingredients in the peak of analytical drug which was observed when the drugs were formulated in the medicinal product. It was compared with standard and blank solution of SIT and MET. Outcome showed no remarkable interference of placebo or ingredients present in the formulation on retention time.

Table 1: System suitability and linearity of the proposed method for MET and SIT

Parameters	MET	SIT
System suitability		
Retention time (min)	1.96	3.70
Tailing factor	0.95	0.79
Plate count (USP)	3103	7158
Peak area RSD	0.437	1.021
Resolution	-	5.19
Linearity		
Linear function	$y = 0.2803x -3.6276$	$y = 1.3133x + 0.1167$
Coefficient of linear regression (r^2)	0.9998	0.9993
Linearity range (ppm)	100-300	10-35

Table 2: Accuracy data for metformin hydrochloride

Concentration level (%)	Theoretical (µg/mL)	Mean area	Content recovered (µg/mL)	Recoveries (%)
80	200.6	58.23	201.41	100.40 ± 0.88
100	250.75	72.26	249.94	99.68 ± 0.76
120	300.9	87.90	304.01	101.03 ± 0.11

Table 3: Accuracy data for Sitagliptin phosphate monohydrate

Concentration level (%)	Theoretical (µg/mL)	Mean area	Content recovered (µg/mL)	Recoveries (%)
80	19.99	24.74	19.96	99.87 ± 1.30
100	24.98	31.31	24.98	100.0 ± 1.19
120	29.98	37.11	29.82	99.46 ± 1.87

Table 4: Response of MET and SIT in terms of precision

Interday precision		
Concentration level (%)	MET (% ± RSD)	SIT (%±RSD)
80	99.37±1.67	99.54±0.87
100	100.87±0.73	100.26±1.82
120	100.51±1.18	100.80±1.28
Intraday precision		
80	100.38±1.42	99.21±0.83
100	101.13±0.91	98.49±1.73
120	100.65±1.67	100.17±1.25

Table 5: Robustness of the proposed method for MET and SIT at 100% concentration

Parameters	MET (% ± RSD)	SIT (% ± RSD)
Optimized conditions		
pH 3.0, 25°C, 1.00mL/min	99.83±1.62	100.53±1.02
Flow rate		
0.85mL/min	100.02±0.55	98.89±0.69
1.15mL/min	98.92±0.87	99.12±1.39
Temperature change		
20°C	99.30±1.58	99.20±0.98
30°C	100.76±0.91	99.37±0.86
Mobile phase (pH)		
pH 2.5	99.51±1.25	98.86±0.84
pH 3.5	99.73±0.53	100.23±0.64

Table 6: LOQ and LOD of metformin hydrochloride and sitagliptin phosphate monohydrate

Parameters	MET	SIT
LOQ (µg/mL)	59.99	3.96
LOD (µg/mL)	19.77	1.30

Table 7: Assay of commercial tablets according to the developed method

Active	Label claimed (mg)	Amount found (%)	USP interpretation
SIT	50	97.2	Pass
MET	500	99.6	Pass

Table 8: Comparison between current HPLC method and some reported methods in the literature

Parameter	Method I		Method II		Current	
Reference	(Bhende *et al.,* 2012)		(Sirigiri *et al.*, 2018)		Current study	
Drug	MET	SIT	MET	SIT	MET	SIT
Range (ppm)	50-150	50-150	277.7-833.3	27.7-83.3	100-300	10-35
Retention time (min)	2.42	3.05	2.39	17.11	1.96	3.70
Flow rate (mL/min)	0.6		1		1	
Mobile phase (v/v)	Phosphate buffer solution, acetonitrile and methanol (35:45:20)		Ammonium acetate buffer and methanol (60:40)		Acidified water: Methanol (60:40)	
Column	C_8		C_{18}		C_{18}	
Wavelength (nm)	260		225, 265		260	
Pros	Retention ≤4 min		Using methanol in place of acetonitrile		Retention ≤4 min, less organic mobile phase	
Cons	More organic mobile phase, less resolution		Fumigated mobile phase, retention up to 17 min, range too high		Retention time 3.70 min	
AGREE Score	0.76		0.73		0.81	

LOD and LOQ

The LOD and LOQ of developed conditions were calculated by relative standard deviation technique (Habib *et al.*, 2020), which indicated that the method can be applied for quantification and detection of MET and SIT for such lower concentrations of MET and SIT (table 6).

Assay of Marketed tablets

The marketed tablets were also evaluated as a sample for the estimation of MET and SIT according to the validated HPLC conditions. The observed peaks were undisturbed which also confirmed that no interference from the ingredients were observed in the developed analytical method (table 7). While, no interference was observed in the peaks of SIT and MET from mucoadhesive sustained releasing dosage form and the peak time was uninterrupted when compared with the standard values.

Greenness evaluation

Scoring greenness of the generated method on the AGREE software revealed that the cumulative penalty on twelve different assessing parameters was 0.19 which showed that the score of current study was found to be 0.81 (fig. 2c). The circular pictogram of the current study revealed that the score of parameters were close to greenness, as indicated by contour, except for point 11 which corresponded for the presence of toxic solvent used. In current study, 40% methanol was used volumetrically in the mobile phase. All the charts, scoring and list of evaluation parameters are available in literature (Pena-Pereira *et al.*, 2020).

DISCUSSION

The current was designed to improve the analysis time of metformin hydrochloride and sitagliptin phosphate monohydrate. Preliminarily, ultraviolet spectroscopic scans (Razzaq *et al.*, 2018) of both drugs were investigated to find the wavelength where reasonable absorbance of both drugs can be studied. Then, chromatographic conditions were optimized to study the impact of solvent concentration in the mobile phase and changing pH. In our study, we introduced a mobile phase composition that was volumetric solvent combination of acidified water and methanol. It has distinct advantages regarding analysis time, availability and cost as compared with those of the previously reported methods (Peraman *et al.*, 2013). Method was validated according to ICH guidelines. The results obtained were of good accuracy and precision (Bashir *et al.*, 2017).

The percentage recoveries were found to be satisfied as shown in results of accuracy (table 2-4). The method suitability was ensured by finding tailing factor, number of theoretical plates and peak area. Results shown that theoretical plates numbers (N) was at least 4500 per each peak, tailing factor was less than 1.5 and RSD values for peak areas were less than 2% for both MET and SIT (Swartz and Krull, 2018). Robustness of suggested method indicated that small changes of chromatographic conditions have did not put significant impact on the percentage drugs recovered and their respective RSD values (table 5), indicating that the method was rugged.

Specificity did not show any additional or interfering peak in the chromatographic pattern of drugs (Iqbal *et al.*, 2018). Hence, the suggested developed method can be applied for the quality control analysis of the MET and SIT in combined dosage form. To compare the developed parameters of current study, the data of previously published studies have been represented (table 8). In the first study, it was observed that the organic volume in mobile phase was more (Bhende *et al.*, 2012) than the current study while in the second column, the retention time was more (Sirigiri *et al.*, 2018) as compared with current findings. While, pros and cons of developed methods have been presented in table 8. The greenness of the current method was also compared with some previously reported methods in literature (Bhende *et al.*, 2012, Sirigiri *et al.*, 2018). The outcome of greenness has been presented in Fig. 2 (b and c). The final score of reported methods after penalties were found to be 0.76 and 0.73 for method I and method II, respectively. If we compare the greenness score of the current method with previous one, it was found that the number of run for Method II were just 3 due to increased retention time. This score is being shown by the orange contour in AGREE response of method II. It has been improved in our current study and the greenness score was incremented. While, the points were also gained under evaluation parameter where the solvents used in the current study were bio-based i.e. the precursors are obtained from the natural source. The AGREE score of the current study was improved and in close agreement with greenness compared with reported literature. It was mainly because the percent of toxic solvent (methanol) according to AGREE tool was reduced. Moreover, the sampling time was also decreased.

CONCLUSION

In the current study, we proposed a simple, economic as well as ecologically effective technique for concurrent analysis of metformin hydrochloride and sitagliptin phosphate monohydrate through reverse phase HPLC technique in combined dosage form. The corresponding retention time for metformin and sitagliptin were 1.96 and 3.70 min. Validation testing for developed technique were found linear, precise, accurate, robust and specific. Moreover, the proposed method could be practically applicable to quantify metformin hydrochloride and sitagliptin phosphate monohydrate in a combined dosage form. The AGREE tool analysis on the developed method depicted a value of 0.81, which was comparatively improved as compared with the stated methods. The suggested method has distinct benefits as compared to previous methods regarding time and cost. Furthermore, the devised method is also ecologically effective due to less consumption of organic phase.

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