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Research Article

DEVELOPMENT AND VALIDATION OF A STABILITY–INDICATING HPLC-UV METHOD FOR THE DETERMINATION OF PIOGLITAZONE HYDROCHLORIDE AND METFORMIN HYDROCHLORIDE IN BULK DRUG AND COMBINED DOSAGE FORM

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ABSTRACT

A simple, selective and stability indicating high performance liquid chromatographic method was developed and validated for the determination of pioglitazone hydrochloride and metformin hydrochloride in bulk drug and pharmaceutical dosage form. Separation and quantification were achieved on a Kromasil C_{18} 4.6 x 250 mm, 5 µm 100 Å column. The mobile phase was (50:50) methanol: phosphate buffer, pH 6.5 containing 0.01 M sodium dodecyl sulphate, v/v at a flow rate of 1.5 ml/min. Detection was carried out at a wavelength of 270 nm. The method was validated for precision, accuracy, ruggedness and recovery. Pioglitazone and metformin were exposed to acidic, basic and oxidative stress conditions and the stressed samples were analyzed by the proposed method. Good linear relationship in the concentration range of 50-150% of target concentration with correlation coefficient of 0.995 was obtained. Intra- and inter-day precision were less than 2.5% for both analytes. The stressed sample chromatograms demonstrate the specificity of the proposed method for the determination of target analytes in presence of degradants.

Keywords: Pioglitazone/ Metformin/ Stability indicating/ HPLC-UV

INTRODUCTION

Pioglitazone hydrochloride, (±)-5-{[[4-2-(5-ethyl-2-pyridinyl) ethoxy] phenyl]methyl}-2,4-thiazolidinedione monohydrochloride (PIO) , is an oral anti-hyperglycemic agent belongs to the thiazolidinedione class which acts by binding to peroxisome proliferator-activated receptors gamma, thus increasing the receptor sensitivity to insulin in muscle and adipose tissues and inhibits hepatic gluconeogenesis. PIO is used either as a monotherapy or in combination with other hypoglycemic agents in the treatment of type-II diabetes (non-insulin-dependent diabetes mellitus). After administration, PIO decreases insulin resistance in the periphery and liver resulting in increased insulin dependent glucose disposal and decreased hepatic glucose output [1-3]. Metformin HCl (MET), (1,1-Dimethyl biguanide hydrochloride) is a biguanide hypoglycemic agent commonly used for the treatment of type II diabetes mellitus. It acts by increasing glucose transport across the cell membrane in the skeletal muscle and it is recommended in case of overweight patients [4-6]. Although MET was used decades ago it is wildly prescribed for the treatment of diabetes either as a monotherapy or in combination with other compounds. [5]. Monotherapy with an oral anti-diabetic agent is not adequate for many type II diabetes patients; multiple drugs may be necessary to achieve sufficient blood sugar control. A combination of MET and the second generation sulphonylureas (glipizide, gliclazide, glibenclamide or glimepiride) is commonly prescribed for type II diabetes [7]. Liquid chromatography (LC) methods have been reported for the determination of pioglitazone and its metabolites in biological fluids [8-10] and for analysis of PIO in bulk drug and in pharmaceutical formulations [1]. A UPLC method has been developed for the simultaneous determination of PIO with another six anti-diabetic drugs in a single run; the method has been applied for determination of these compounds in pharmaceutical formulations using UV detection [2]. A liquid chromatography tandem mass spectrometry (LC-MS/MS) method has been developed and validated for the simultaneous determination of PIO and its two metabolites M-III (keto-derivative) and M-IV (hydroxyderivative) in human plasma. [11]. LC-MS/MS method has been reported for the simultaneous determination of PIO and candesartan in human plasma for human pharmacokinetic and bioequivalence studies [3]. Solid phase extraction [9], and hollow fiber liquid phase micro-extraction (HF-LPME) [10]. Procedures have been applied for extraction and pre-concentration of PIO from biological fluids before quantitative determination by high-performance liquid chromatography (HPLC). A simple HPLC with UV detection has been developed and validated for the simultaneous determination of MET and rosiglitazone in human plasma [14].method A stability indicating capillary electrophoresis method has been reported for the analysis of MET in tablet formulation. [5]. HPLC method has been reported for the determination of MET in human plasma and urine using small sample volume and Octadecyl silane column [15]. High performance thin layer chromatographic (HPTLC) method has been used for simultaneous determination of MET and glyburide in tablets [16]. Determination of MET and glyburide in an anti-hyperglycemic binary mixture has been studied using HPLC-UV and spectrometric methods. [17]. A HPLC–UV method has been developed and validated for the determination of MET in tablets containing croscarmellose sodium as an additive [18]. Different spectrophotometric procedures have been reported for determination of MET such as interaction with ninhydrin in alkaline medium [19] and oxidation with hydrogen peroxide [20]. Simultaneous spectrophotometric determination of MET and repaglinide in a synthetic mixture has been also reported [21]. Gas chromatography [22], NMR spectrometry [23], capillary electrophoresis, [24], potentiomretry and spectroflourometry [25, 26] methods have been reported for determination of MET. Spectrophotometric and chemometric methods have been applied for determination of MET and PIO in binary mixture and in their ternary mixture with pioglitazone acid degradate [27]. HPLC and spectrophotometric methods have been developed and validated for determination of MET and PIO in a combined pharmaceutical-dosage form [4]. To our knowledge, no stability-indicating method for the determination of PIO and MET in combined dosage form has been published. Degradation profiles of PIO and MET in combined dosage form did not investigated before, the aim of this work is to develop a simple stability indicating method for determination of MET and PIO in bulk and combined dosage form. Chemical structures of PIO and MET are shown in Figure 1.

EXPERIMENTAL

Reagents and chemicals

Pioglitazone Hydrochloride (PIO) (USP R.S) and Metformin Hydrochloride (Met) were obtained from Dr. Reddy's Laboratories

Limited, (Hyderabad, India). Compectat® capsules were purchased from the local market. Methanol (HPLC grade), phosphoric acid, hydrochloric acid and hydrogen peroxide 30% were purchased from Scharlau (Barcelona, Spain). Sodium dodecyl sulfate was obtained from REDA Industrial Division (Saudi Arabia). Disodium hydrogen phosphate was obtained from Technopharmchem (India). Sodium hydroxide was purchased from Panreac (Barcelona, Spain). Potassium hydroxide was purchased from lobacheme (Mumbai, India). Ultra pure water (Milli-Q) (Millipore Corporation, Billerica, MA, USA) was used.

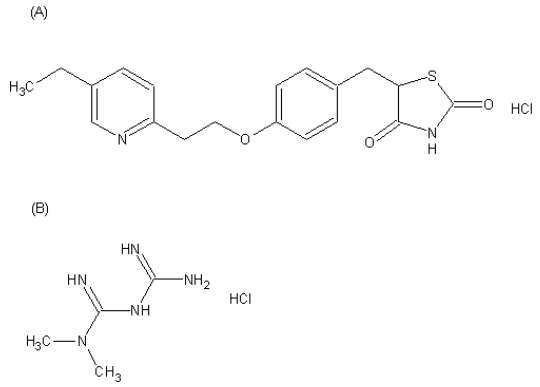

Figure 1: It shows chemical structures of (A) Pioglitazone hydrochloride and (B) Metformin hydrochloride.

Instrumentation and chromatographic conditions

The HPLC system (Waters, USA) was equipped with Autosampler, Binary HPLC Pumps, Dual lamb Absorbance Detector and In-Line Degasser ISA Card. Data acquisition was performed on Empower software. The detector was set at 270 nm. The HPLC separation and quantitation were achieved on Kromasil® C_{18} 4.6 x 250 mm, 5 µm 100 Å analytical column (Phenomenex, USA). All determinations were performed at 40 ºC. The mobile phase was (50:50) methanol: phosphate buffer, pH 6.5, containing 0.01 M sodium dodecyl sulphate, v/v, which was run Isocratic. Flow rate was 1.5 ml/min and injection volume was 20 µl.

Preparation of standard solutions

A combined standard solution of PIO and MET was prepared by dissolving 15 mg of PIO and 850 mg of MET in 100 ml 0.1N HCl. Appropriate dilutions were made in mobile phase to obtain working solutions. 100% standard solution was prepared by diluting 5 ml of stock standard solution to 50 ml with mobile phase.

Preparation of sample solutions

The content of ten capsules of Compectat® were accurately weighed and well mixed. A portion of the powder equivalent to one capsule was accurately transferred to a 100 mL volumetric flask and dissolved in 100 ml 0.1 N HCl in an ultrasonic bath for 10 min and then filtered through 0.45 µm membrane filters (Millipore, Milford, MA, USA). 100% sample solution was prepared by diluting 5 ml of stock sample solution to 50 ml with mobile phase.

Forced degradation conditions

Acid degradation: A 6 ml of stock solution was transferred into 50 ml volumetric flask and 5 ml of 5N HCl was added, the mixture was shaken for 5 minutes and left in the dark at room temperature for 1 hour, then neutralized by 5 ml of 5N NaOH, the volume was completed to 50 ml with the mobile phase. Another experiment was performed by heating the acidic solution for 15 minute at 80 ˚C followed by cooling, neutralization and dilution.

Alkaline degradation: A 6 ml of stock solution was transferred into 50 ml volumetric flask and 5 ml of 5N NaOH was added, the mixture was shaken for 5 minutes and left in the dark at room temperature for 1 hour, then neutralized with 5ml of 5N HCl, the volume was completed to 50 ml with the mobile phase. Another experiment was performed by heating the acidic solution for 15 minute at 80 ˚C followed by cooling, neutralization and dilution.

Oxidative degradation: A 6 ml of stock solution was transferred into 50 ml volumetric flask and 5 ml of 30 % H_2O_2 was added, the mixture was shaken for 5 minutes and left in the dark at room temperature for 1 hour, then the volume was completed to 50 ml with the mobile phase. Another experiment was performed by heating the solution for 15 minute at 80 ˚C followed by cooling and dilution.

Validation procedure

The method was validated in accordance with the ICH requirements [28], which involved accuracy, precision, linearity, selectivity, limit of detection and limit of quantitation

System suitability

The system suitability parameters resolution (Rs), area repeatability and asymmetry factor (As) were calculated as previously reported [29, 30].

Specificity

Specificity is the ability of the analytical method to discriminate between target analyte and other components that may be present. To assess the method selectivity the excipients used for Compectat® without PIO and MET were used. For HPLC analysis the solution was prepared using the same procedure of analytical sample, specificity of the developed method was also assessed by performing forced degradation studies. Moreover PIO and MET were injected separately.

Robustness and Ruggedness

Robustness is the ability of the analytical method to remain unchanged by small, but deliberate changes in method parameters. To determine the robustness of the proposed method, the experimental conditions were deliberately changed; variation of the mobile phase flow rate by ± 0.1 ml/min, column temperature by ± 3.0 ºC and organic strength of the mobile phase by ± 2.0 % were studied. Ruggedness is the degree of reproducibility of test results under normal operational conditions such as laboratory to laboratory and analyst to analyst. The ruggedness of the assay was studied by analysis of the same sample in triplicate under a variety of test conditions such as different days, analysts, and instruments.

Linearity, LOD and LOQ

The linearity of an analytical method is the ability of this method, within a given range, to obtain test results which are directly, or through a mathematical transformation, proportional to the concentration of analyte. Linearity of the method was evaluated at five concentration levels by diluting the standard solutions to give solutions over the ranges 50–150% of the target concentration for PIO and MET, calibration curves were constructed by plotting the peak areas against concentrations. Lower limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. LOD was calculated as 3.3 (SD/S); where (SD) is the standard deviation of intercept of the regression line and (S) is the slope of the calibration curve. Lower limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the proposed conditions. LOQ was calculated as 10 (SD/S); where (SD) is the standard deviation of intercept of the regression line and (S) is the slope of the calibration curve.

Precision

Precision is the degree of agreement of test results when the analytical method is applied to multiple samples. Precision was evaluated in terms of intra-day repeatability and inter-day reproducibility. The intra-day repeatability was investigated using six separate sample solutions prepared, as reported above at 100 % of the target level. The peak areas obtained were used to calculate means and RSD % values. The inter-day reproducibility was checked on three different days at concentration of 80%, 100% and 120% of the target concentration, the means and RSD% values were calculated from peak areas.

Accuracy

Accuracy is the closeness of test results obtained by the analytical method to the nominal value. To assess accuracy, sample solutions of Compectat® capsules at concentrations of 80, 100 and 120% of the target concentration were analyzed. Each solution was injected in triplicate and the peak areas were used to calculate means and RSD% and % recovery.

RESULTS AND DISCUSSIONS

Method development

All determinations were performed at 40˚C. The mobile phase was (50:50) methanol: phosphate buffer, pH 6.5, v/v, which was run Isocratic. Flow rate was 1.5 ml/min A Kromasil® C_{18} 4.6 x 250 mm, 5 µm 100 Å analytical column (Phenomenex, USA), maintained at (40 ˚C) was used for the separation of PIO, MET and their related degradation products. The method was validated for the determination of PIO and MET in Compectat® capsules. Inertsil® C_{18} 4.6 x 250mm, 5 µm 100 Å column was initially used for separation of PIO and MET, however, a significant tailing was observed for PIO. Kromasil® is a spherical, totally porous silica particle; it has a high loading capacity, narrow pore size distribution and excellent chemical and mechanical stability. Kromasil® is stable at high pH levels up to pH 9.0 and provides excellent peak shape. The composition, pH and the flow rate of the mobile phase were changed to optimize peak shape, elution time for MET and PIO and also to optimize the separation using stressed samples of the two compounds of interest. MET was initially eluted at ~ 2.0 minutes using different combinations of mobile phases. A mobile phase consisting of phosphate buffer pH 6.5 – methanol (50:50, v/v) set at a flow rate of 1.5 ml/min was selected for method validation after several preliminary investigatory chromatographic runs; addition of ion pairing agent was investigated to improve peak shape and also to avoid elution of MET in the column void volume. Addition of different ion pairing agents such as triethyamine, octane sulfonic acid sodium salt and tetrabutylammonium hydrogen sulphate were studied. The addition of 0.01 M sodium dodecyl sulphate into the hydro-methanolic mobile phase was found to be an excellent tool to improve MET and PIO retention, peak shape and symmetry (Figure 2). Under the optimized chromatographic conditions, all peaks were well resolved and free from tailing. The effects of small deliberate changes in the mobile phase composition, pH and flow rate were evaluated as a part of testing for method robustness.

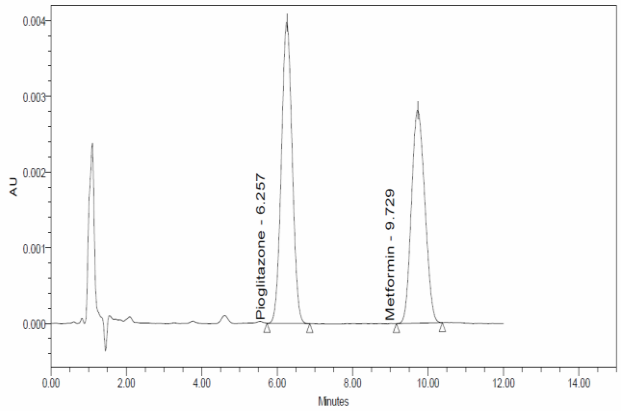

Figure 2: It shows HPLC-UV chromatogram of Compectat® capsules at concentration of 100% of the target concentration for PIO and MET.

Forced degradation studies

Forced degradation studies were established by subjecting samples of PIO and MET standard solutions to degradation in NaOH, HCl and H_2O_2. The degradation samples were analysed using the proposed method. Minor degradations of PIO (~18%) and MET (~10%) were observed under acidic conditions at room temperature and (80 ˚C). Small degradation product peaks were observed as shown in Figure 3. All acidic degradation products were chromatographically resolved from target analytes.

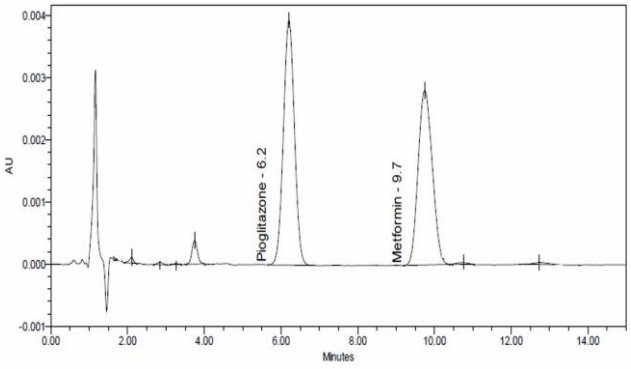

Figure 3: It shows HPLC-UV chromatogram of PIO and MET degradation in 5N HCl (80˚C).

PIO and MET peaks showed approximately 25% and 15% degradation under alkaline condition at both investigated temperature, respectively. The elution profiles of degradation products at 80 ˚C are shown in Figure 4. The main alkaline degradation products at 80 ºC were eluted at 2.0 -3.7 min and at 12.7 minutes, resolution between each two successive peaks was greater than 2. Figure 4

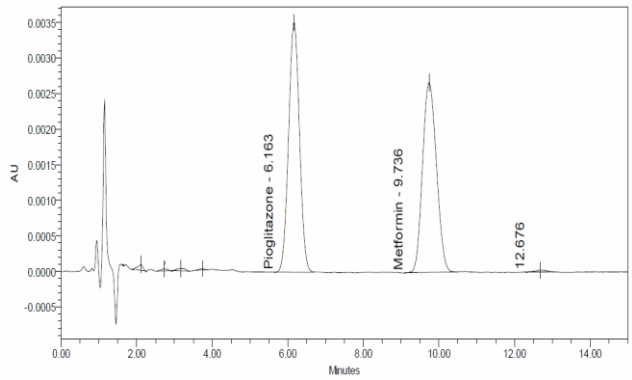

Figure 4: It shows HPLC-UV chromatogram of PIO and MET degradation in 5N NaOH at room temperature.

Minor degradations of PIO (~ 5%) and MET (~ 8%) were also observed under oxidative conditions at room temperature, elevated temperature showed more degradation for PIO (~ 28%) The elution profiles of degradation products at 80 ˚C are shown in Figure 5. The main degradant peak was eluted at 1.92 min. All degradation products peaks under different stress conditions were chromatographically resolved from target analytes peaks.

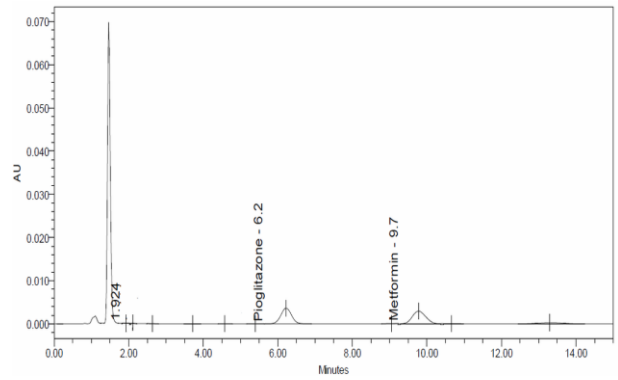

Figure 5: It shows HPLC-UV chromatogram of PIO and MET degradation in 30% H_2O_2 (80˚C).

METHOD VALIDATION

The developed method was validated according to the ICH guidelines [28], for the following parameters: system suitability, specificity, linearity, precision, accuracy and LOD/LOQ.

System suitability

As system suitability test is an integral part of chromatographic method development and it is used to verify that the system is satisfactory for the analysis to be performed, system suitability parameters for PIO and MET are reported in Table 1.

Table 1: System suitability parameters

Parameters	PIO	MET	Degradants	Acceptance criteria
Asymmetry	≤ 1.03	≤ 1.1	≤ 1.1	≤ 2
Resolution		≥ 5.4	> 2	> 2
Theoretical plates	>2000	> 3000	> 2000	> 2000

Selectivity

Selectivity is the ability of an analytical method to assess unequivocally the analytes in the presence of components that are present in the sample matrix. The analysis of the placebo solution constituted by excipient blend showed no peak interfering with analytes; moreover all degradation products were chromatographically resolved from target analytes. Overall, these data demonstrated that the excipients and the degradation products did not interfere with the PIO and MET peaks, indicating selectivity of the method.

Linearity

Five concentration levels within 50–150% of the target concentration range for PIO and MET were used to study the method linearity. The results of the regression statistics obtained for PIO and MET are presented in Table 2. The square of the correlation coefficient (r^2 > 0.995) demonstrated a significant correlation between the concentration of analytes and detector response. LOD was 0.01 and 0.05 µg/mL for PIO and MET, respectively. LOQ was 0.03 and 0.16 µg/mL for PIO and MET, respectively.

Table 2: Five level calibration graphs for PIO and MET

Analyte	Range (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	Slope ± SD	Intercept ± SD	r^2 ± SD
PIO	7.5-22.5	0.01	0.03	79952.3 ± 189.1	- 2629 ± 261.8	0.994 ± 0.002
MET	425-1275	0.05	0.16	55254.7 ± 1068.2	12456 ± 893.7	0.996 ± 0.001

PRECISION

The intra-day repeatability (intra-day precision) refers to the use of analytical procedure within a laboratory over a short period of time using the same operator with the same equipment. Inter-day reproducibility (inter-day precision) involves estimation of variations in analysis when a method is used within a laboratory on different days. The results obtained are shown in Table 3. In all cases the % RSD values were within 2.2 % and 1.6 % for PIO and MET, respectively.

Table 3: Inter and intra-day precision (%RSD) data for PIO and MET

Analyte	Intra-day precision		Inter-day precision	
	100%	80%	100%	120%
PIO	1.48	2.20	2.00	0.20
MET	1.24	0.7	1.6	0.20

Accuracy

The accuracy of the method has been determined by application of the analytical procedure to recovery studies, where sample solutions of Compectat® capsules were analyzed using the proposed method. The results of accuracy studies were shown in Table 4; recovery values demonstrated that the method was accurate within the proposed range. The representative chromatogram PIO and MET in Compectat® capsules (Figure 2) showed no interfering peaks from excipient components.

Table 4: Accuracy (% recovery) data for PIO and MET

% of targeting concentration	PIO % recovery ± SD	MET % recovery ± SD
80%	100.39 ± 0.33	99.71 ± 0.79
100%	100.60 ± 0.46	100.55 ± 1.00
120%	101.07 ± 0.07	101.46 ± 0.17

CONCLUSIONS

In this work, a sensitive, specific, accurate and stability-indicating HPLC-UV method for the determination of PIO and MET in the presence of degradation products was developed and validated. The stability of PIO and MET under various stress conditions were investigated using a forced degradation study. All of the degradation products were well resolved from the target analytes demonstrates the stability-indicating power of the method. The information presented in this study could be used for quality control studies of active pharmaceutical ingredients in their dosage forms and to monitor drug quality during stability studies.

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