CHAPTER - II

RP-HPLC ESTIMATION OF ALOGLIPTIN AND PIOGLITAZONE SIMULTANEOUSLY IN COMBINED TABLET DOSAGE FORMS

2.1. PHYSICAL, CHEMICAL AND PHARMACOLOGICAL PROPERTIES OF THE DRUGS

2.1.1. Alogliptin

IUPAC name : 2-({6-[(3R)-3-aminopiperidin-1-yl]-3-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-1-yl}methyl)benzonitrile

Molecular formula : $C_{18}H_{21}N_5O_2$

Molecular weight : 339.39 g/mol

Appearance : White to off white crystalline powder

Solubility : Soluble in dimethylsulfoxide; sparingly soluble in water and methanol; slightly soluble in ethanol; very slightly soluble in octanol and isopropyl acetate.

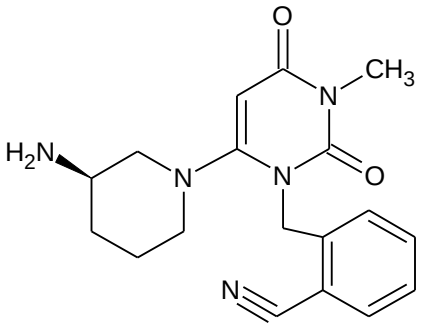

Figure 2.1: Chemical structure of alogliptin

Alogliptin is an oral antihyperglycemic of dipeptidyl peptidase-4 (DPP-4) inhibitor class used in the treatment of type II diabetes milletus [1,2]. Usually, dipeptidyl peptidase 4 degrades the glucagon like peptide 1 and incretins glucose-dependent insulinotropic polypeptide. The glucagon like peptide 1 and incretins glucose-dependent insulinotropic polypeptide stimulate glucose dependent secretion of insulin, repress glucose dependent glucagon secretion, reducing food intake and

gastric emptying. The inhibition of dipeptidyl peptidase 4 by alogliptin increases the quantity of active plasma incretins and glucagon like peptide 1 that helps in glycemic control [3,4]. This enzyme-inhibiting drug is used in combination with metformin for treatment of patients with diabetes that cannot adequately be controlled with metformin alone [5].

2.1.2. Pioglitazone

IUPAC name	**:**	5-({4-[2-(5-ethylpyridin-2-yl)ethoxy]phenyl}methyl)-1,3-thiazolidine-2,4-dione
Molecular formula	**:**	$C_{19}H_{20}N_2O_3S$
Molecular weight	**:**	356.44 g/mol
Appearance	**:**	white to off white solid powder
Solubility	**:**	Soluble in dimethylsulfoxide and dimethyl formamide; insoluble in water and ether; slightly soluble in acetonitrile, alcohol, and acetone.

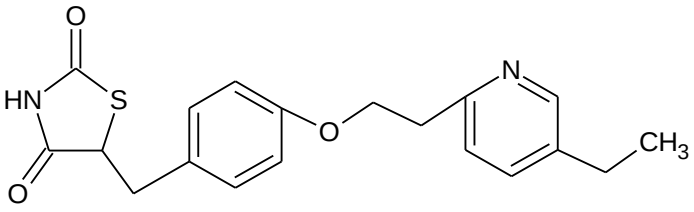

Figure 2.2: Chemical structure of pioglitazone

Pioglitazone is an anti-diabetic drug and belongs to thiazolidinedione class of drugs. Pioglitazone is prescribed to improve control of blood glucose level in adults with type - II diabetes mellitus [6]. Pioglitazone enhances tissue sensitivity to insulin

by acting as a potent and selective agonist at peroxisome proliferator activated gamma receptor in adipose tissue, skeletal muscle and liver [7].

2.2. REVIEW OF LITERATURE

2.2.1. Alogliptin

A few analytical methods have been proposed for the quantification of alogliptin. El-Bagary *et al.,* [8] and Yadav *et al.,* [9] reported reverse phase high performance liquid chromatography methods for the determination of alogliptin concentration in bulk and pharmaceutical dosage forms. Srinivasa rao *et al.,* [10] reported a chiral liquid chromatography method for the determination of enantiomeric purity of alogliptin. A stability RP-HPLC method was developed by Zhou *et al.,* [11] for the separation and quantification of the process related impurities and stress degradants of alogliptin in bulk. Kun *et al.,* [12] have reported a gradient reverse phase high performance liquid chromatography method for the determination of related substances in alogliptin bulk drug and tablets.

In the existing literature, there are few reports regarding the use of spectrophotometry for the quantification of alogliptin in bulk and tablet dosage forms. Keyur *et al.,* [13] described UV spectrophotometric method for the quantification of AGN using water and methanol in the ratio of 50:50 (*v/v*) as solvent. First-order spectrophotometric determination of AGN is proposed by Yadav *et al*., Kumar *et al.,* [15,16] and Raval *et al.,* [17] proposed spectrophotometric methods for the quantification of AGN in formulations.

2.2.2. Pioglitazone

Pioglitazone is described in European Pharmacopoeia and an HPLC method is proposed for its determination [18]. Techniques like spectrophotometry, capillary electrophoresis, high performance liquid chromatography, liquid chromatography–mass spectrometry, potentiometry and high performance thin layer chromatography have been used for the analysis of pioglitazone in biological samples and pharmaceutical formulations.

Mostafa *et al.,* [19] constructed a new composite and classical potentiometric sensor for the direct determination of pioglitazone in pharmaceutical preparations and have been used as indicator electrodes for potentiometric titration. El-Ghobashy *et al.*, [20] proposed membrane selective electrodes for the determination of pioglitazone in tablets and plasma. Badawy *et al.,* [21] quantified piogitazone in Briton–Robinson buffer solution using cyclic voltammetry and differential pulse voltammetry. Al-Arfaj *et al.,* [22] used square-wave adsorptive cathodic stripping voltammetry to determine pioglitazone in pharmaceutical formulations and biological samples.

In the literature few methods were reported for the estimation of pioglitazone in bulk, pharmaceutical dosage forms and biological samples using UV spectrophotometry [23-28] and visible spectrophotometry [29-31]. Micellar electrokinetic chromatographic and HPLC methods were developed by Radhakrishna *et al.,* [32] for the determination of pioglitazone and its unsaturated impurity. Calixto *et al.,* [33] proposed an electrophoretic method for pioglitazone and its main metabolites quantification in a rat liver microsomal fraction. Various analytical

methods were found in the literature for the determination of pioglitazone in pharmaceutical dosages forms, biological samples like plasma, serum, and urine using HPLC [34-43].

LC/MS/MS method was developed by Xue *et al.,* [44], Lin *et al.,* [45], Chinnalalaiah *et al.,* [46] for determination of pioglitazone in human serum and plasma. Dhirender Singh *et al.,* [47] developed an HPTLC method for pioglitazone in bulk and tablet dosage forms.

2.2.4. Alogliptin and pioglitazone combination

With proper diet and exercise, alogliptin and pioglitazone combination is used in the management of high blood sugar levels caused by type II diabetes [48,49]. This combination was approved by FDA in 2013 [50]. The combination of the selected drugs is not official in any pharmacopoeia. Therefore, it is essential to develop an effective analytical method for the simultaneous determination of alogliptin and pioglitazone.

UV spectrophotometric methods like first order derivative, dual wavelength, second order derivative and area under curve methods were described by Raval and Srinivasa [51] and Anusha *et al.,* [52] for the simultaneous estimation of alogliptin and pioglitazone in bulk and pharmaceutical dosage forms. First order derivative and dual wavelength methods developed by Raval and Srinivasa [51] and uses methanol solvent. In the first order derivative method, zero crossing point of alogliptin and pioglitazone were found at 275.60 nm and 268.20 nm, respectively. In dual wavelength method, two wavelengths 270.20 nm and 265 nm were selected as

λ1 and λ2, respectively for the estimation of alogliptin. Wavelengths 280 nm and 271 nm were selected as λ3 and λ4, respectively for the estimation of pioglitazone. Anusha *et al.,* [52] employed second order derivative and area under curve methods. The first method involves measurement of second order derivative spectrum absorbance at 276 nm for alogliptin and 226 nm for pioglitazone. The second method involves measurement of peak area in the wavelength range 221-231 nm and 271-281 nm for pioglitazone and alogliptin, respectively.

High performance thin layer chromatographic method was reported by Komal and Amrita for the simultaneous assay of alogliptin and pioglitazone in combined dosage forms [53]. In HPTLC method, the separation was achieved on Merck HPTLC aluminum sheets coated with silica gel 60F254, with acetonitrile: 1 % ammonium acetate in methanol (4.5:5.5 *v/v*) as mobile phase and densitometric analysis was performed at 254 nm. Though the UV spectrophotometric methods reported by Raval and Srinivasa [51] and Anusha *et al.,* [52] are simple, they are less selective since they involve measurements in the UV range where there is a possibility of absorbance by the tablet excipients. One of the important validation parameter, method robustness, is not reported in the UV spectrophotometric methods. The HPTLC method described by Komal and Amrita [53] requires costly, sophisticated instrumentation and expertise personnel to operate. Moreover, the HPTLC instrument is not commonly available in the developing and under developed countries.

RP-HPLC methods were also applied to the determination of the selected drug combination in bulk and pharmaceutical dosage forms by Raval and Srinivasa [54],

Neelima *et al.,* [55], Manzoor *et al.,* [56] and Mokhtar *et al.,* [57]. In Raval and Srinivasa method, the separation was carried out on an BDS hypersil C18 (250 mm × 4.6 mm, 5 µm) analytical column using buffer with pH 3.5 and methanol (70:30, *v/v*) as mobile phase at a flow rate of 1.0 mL/min with UV detection at 271 nm [54]. Using a Hypersil BDS C18, (250 x 4.6 mm, 5 µm) column as the stationary phase and a phosphate buffer of pH 4.8-acetonitrile (45:55, *v/v*) as mobile phase, the selected drugs combination in pharmaceutical formulations was determined by Neelima *et al.,* [55]. The detection wavelength was set at 215 nm. In Manzoor *et al.,* method [56], alogliptin and pioglitazone was chromatographed on Enable C18 (250 mm × 4.6 mm, 5 µm) column with a mobile phase consisting of phosphate buffer with pH 3.6-acetonitrile (35:65, *v/v*) pumped at a flow rate of 1.0 mL/min with UV detection at 268 nm. Alogliptin and pioglitazone in tablets was assayed by Mokhtar *et al.,* [57] by carrying out chromatography on an Inertsil ODS-3 (250 mm × 4.6 mm, 5 µm) column using a mixture of methanol and phosphate buffer with pH 3.0 (80:20, *v/v*) as mobile phase at a flow rate of 1 mL/min with UV detection at 269 nm.

In the present study, a new RP-HPLC method with photodiode array detector was developed for simultaneous determination of alogliptin and pioglitazone in bulk and combined tablet dosage form. The method validation has been carried out according to the International Conference on Harmonization guidelines [58]. The developed and validated RP-HPLC method was successfully applied to combined tablet dosage form.

2.3. MATERIALS AND METHODS

2.3.1. Reference standard drugs and tablet dosage forms

Alogliptin and pioglitazone reference standard drugs were provided by Lara Drugs Private Limited (Telangana, India) as gift samples. They are used as received. The tablet dosage form, Oseni tablets (strength 25 mg alogliptin and 45 mg pioglitazone), manufactured by Takeda pharmaceuticals America Inc., Deerfield was purchased from the local pharmacy.

2.3.2. Chemicals and solvents

The HPLC grade methanol was obtained from Merck India Ltd., Mumbai, India. Analytical reagent ammonium acetate was obtained from Sd. Fine Chemicals Ltd., Mumbai, India. Water was obtained using a Milli-Q system.

2.3.3. Apparatus and HPLC conditions

The Waters Alliance 2695 Module equipped with a 2998 PDA detector with Empower 2 software was used in the current analysis. The Zorbax C8 column (150 mm x 4.6 mm internal diameter, 5 μm particle size) was used. Isocratic mobile phase was composed of 0.1 M ammonium acetate and methanol (50:50, *v/v*) with pH 3.5 (adjusted with orthophosphoric acid: Sd. Fine Chemicals Ltd., Mumbai). The 0.1 M ammonium acetate solution was prepared by dissolving 7.7 g ammonium acetate (Sd. Fine Chemicals Ltd., Mumbai) in 1000 mL water. Electronic balance ELB 300 was used for weighing the materials. Digisun pH meter was used for all pH measurements. The same mobile phase was used as a diluent for the preparation of standard solutions of alogliptin and pioglitazone. A flow rate of 1.0 mL/min was

maintained. The eluted compounds were monitored at 248 nm. The column temperature was maintained at 30±1 ^{o}C. An injection volume of 10 µL was used.

2.3.4. Standard solutions

A stock standard solution (alogliptin - 250 µg/mL and pioglitazone – 450 µg/mL) was prepared in a 100 mL volumetric flask by dissolving 25 mg of alogliptin and 45 mg of pioglitazone in a final volume of 100 mL mobile phase. Working standard solutions (6.25-18.75 µg/mL for alogliptin and 11.25-33.75 µg/mL for pioglitazone) were prepared from the above stock solution by appropriate dilution with mobile phase.

2.3.5. Tablet sample solution

Average weight of ten tablets was determined, transferred to a clean dry mortar and grinded into fine powder. Tablet powder equivalent to 25 mg of alogliptin and 45 mg of pioglitazone was then transferred to a 100 mL volumetric flask, 30 mL of mobile phase was added and the flask was sonicated for 10 min to dissolve the drugs completely. The mixture was diluted up to volume with the mobile phase to give a solution containing 250 µg/mL and 450 µg/mL of alogliptin and pioglitazone, respectively. This solution was filtered through 0.45 µm pore size membrane filter. Appropriate dilution (12.50 µg/mL of alogliptin and 22.50 µg/mL of pioglitazone) was prepared in mobile phase for analysis.

2.3.6. Calibration graph

Working standard solutions equivalent to 6.25-18.75 µg/mL alogliptin and 11.25-33.75 µg/mL pioglitazone were prepared by appropriate dilution of the stock

standard solution with the mobile phase. 10 µL aliquot of each solution was injected automatically into the column in triplicate and the chromatograms were recorded. The peak areas of the drugs were determined. Calibration graph was constructed by plotting the mean peak area against drug concentration. The concentration of the unknown was calculated from the calibration graph or from the regression equation derived from the mean peak area-concentration data.

2.3.7. Estimation of alogliptin and pioglitzone in combined tablet dosage form

10 µL of the tablet sample solution was injected into the HPLC system in triplicate. The chromatograms were recorded. The peak areas were determined. The concentrations of alogliptin and pioglitazone in the combined tablet dosage form were calculated from the corresponding calibration curves or corresponding regression equations.

2.3.8. Forced degradation

To assess the stability indicating properties of the proposed HPLC method, forced degradation studies were performed. The tablet sample was subjected to acid, alkali, oxidation, thermal and photo degradation.

2.3.8.1. Acid and alkali hydrolysis

Tablet powder equivalent to 25 mg of alogliptin and 45 mg of pioglitazone was transferred to a 100 mL volumetric flask. The powder was mixed with 10 mL of 0.1 N hydrochloric acid (for acid hydrolysis) or 10 mL of 0.1 N sodium hydroxide (for alkali hydrolysis). The solutions were subjected to sonication for 30 min. The samples were neutralized with an amount of acid (for alkali hydrolysis) or base (for

acid hydrolysis) equivalent to that of the previously added. The flask was made up to the volume with mobile phase.

2.3.8.2. Oxidative degradation

Tablet powder equivalent to 25 mg of alogliptin and 45 mg of pioglitazone was transferred to a 100 mL volumetric flask. The contents were mixed with 10 mL of 30 % hydrogen peroxide solution. The reaction mixture was allowed to sonication for 30 min and then the volume of the flask was made up to 100 mL with mobile phase.

2.3.8.3. Thermal and photo degradation

Tablet sample powder (alogliptin- 25 mg and pioglitazone-45 mg) was exposed to 105 ^{o}C for 30 min in oven (for thermal degradation) or subjected to direct sun light for up to 24 hr (for photo degradation). After the specified time, the tablet powder was cooled and dissolved in 30 mL of mobile phase in a 100 mL volumetric flask. The solution thus prepared was diluted to volume with the mobile phase.

The degraded sample solutions were appropriately diluted with mobile phase to obtain a concentration of 12.50 µg/mL (alogliptin) and 22.50 µg/mL (pioglitazone). The solutions were filtered through 0.45 µm pore size membrane filter. A volume of 10 µL was injected into the HPLC system and the chromatograms were recorded.

2.4. RESULTS AND DISCUSSION

2.4.1. Method development

The present study was aimed to establish a sensitive, robust and reliable RP-HPLC method for the simultaneous determination of alogliptin and pioglitazone in combined tablet dosage form. During method development, two different columns like the Hypersil BDS C18 column (250 mm x 4.6 mm internal diameter, 5 µm particle size) and Zorbax C8 column (150 mm x 4.6 mm internal diameter, 5 µm particle size) were tried. Better results (good symmetrical sharp peak, acceptable tailing factor and resolution) were obtained with Zorbax C8 column (150 mm x 4.6 mm internal diameter, 5 µm particle size) maintained at a temperature of 30±1 ^{o}C. Hence, the same column and temperature were chosen for analysis.

Various mobile phases (0.1 % Orthophosphoric aicd**:** Methanol, 0.1 M NaH_2PO_4**:** Methanol, 0.1 M KH_2PO_4**:** Methanol and 0.1 M ammonium acetate**:** Methanol) with different ratios, flow rate, and pH were tried and the responses were recorded. After a series of experiments, highly symmetrical and sharp peaks of alogliptin and pioglitazone with better resolution were obtained at pH 3.5 by using 0.1 M ammonium acetate and methanol (50:50 *v/v*) as mobile phase at a flow rate of 1.0 mL/min. The alogliptin and pioglitazone in the selected mobile phase have sufficient absorption at 248 nm, which was therefore chosen for the analysis. Figure 2.3 shows a typical HPLC chromatogram of alogliptin and pioglitazone using the optimized chromatographic conditions (Table 2.1).

Table 2.1: Optimized chromatographic conditions

S.No	Parameter	Value
1	Column	Zorbax C8 column (150 mm x 4.6 mm internal diameter, 5 µm particle size)
2	Mobile phase	0.1 M ammonium acetate and methanol (50:50 *v/v*): pH -3.5
3	Flow rate	1.0 mL/min
4	Diluent	Mobile phase
5	Column temperature	30±1 ^{o}C
6	Runtime	7 min
7	Retention time	Alogliptin – 2.883 min and pioglitazone – 4.329 min.
8	Volume of injection	10 µL
9	Detection wavelength	248 nm

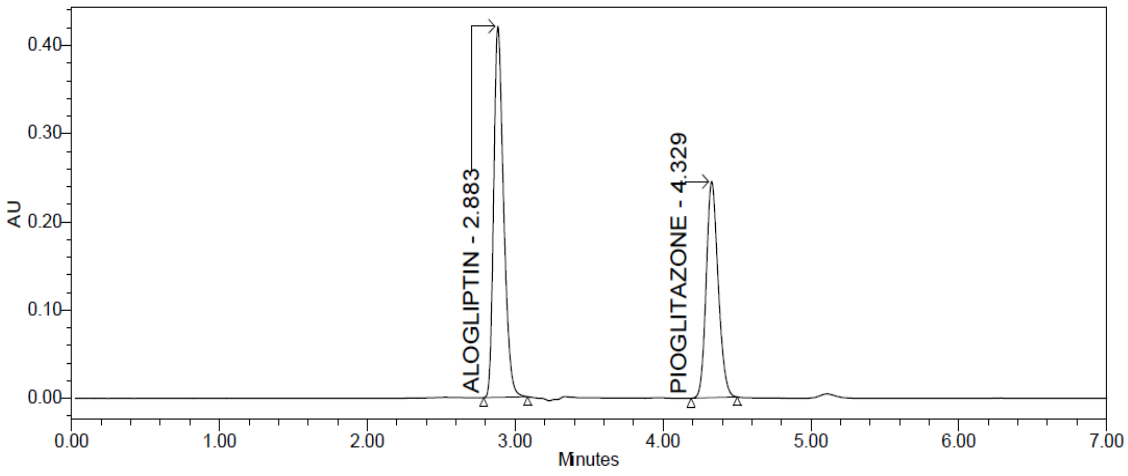

Figure 2.3: Typical HPLC chromatogram of alogliptin and pioglitazone

2.4.2. Method validation

The developed RP-HPLC method was validated using ICH guidelines [58].

2.4.2.1. System suitability test

In order to determine the satisfactory resolution and reproducibility of the method, suitability parameters, including % RSD of retention time, % RSD of peak area, USP plate count and USP tailing factor, were investigated. In order to test the system suitability, standard solution (alogliptin - 12.50 µg/mL and pioglitazone - 22.50 µg/mL) was injected five times into the HPLC system. The results (Table 2.2) demonstrate the method suitability.

Table 2.2: System suitability parameters of the proposed HPLC method

Parameters	Alogliptin		Pioglitazone		Recommended limit
	Value*	RSD (%)	Value*	RSD (%)	
Retention time	2.906	0.761	4.361	0.754	RSD ≤2
Peak area	1976065	0.474	1386415	0.864	RSD ≤2
USP resolution	-	-	10.41	0.686	> 1.5
USP plate count	8997	0.365	13896	0.524	> 2000
USP tailing factor	1.322	0.632	1.186	0.461	≤ 2

**Average of five values*

2.4.2.2. Selectivity

Selectivity was evaluated by comparing the chromatograms of mobile phase blank, placebo blank, working standard solution (alogliptin 12.50 µg/mL and pioglitazone 22.50 µg/mL) and tablet sample solution (alogliptin 12.50 µg/mL and pioglitazone 22.50 µg/mL). For this purpose, solutions of placebo blank, mobile phase blank, working standard and tablet sample was injected into the HPLC system.

The resulting chromatograms are shown in Figures 2.4, 2.5, 2.6 and 2.7. The chromatograms of placebo blank, mobile phase blank, working standard and tablet sample did not show any peaks other than that of alogliptin and pioglitazone. This confirmed the selectivity of the method.

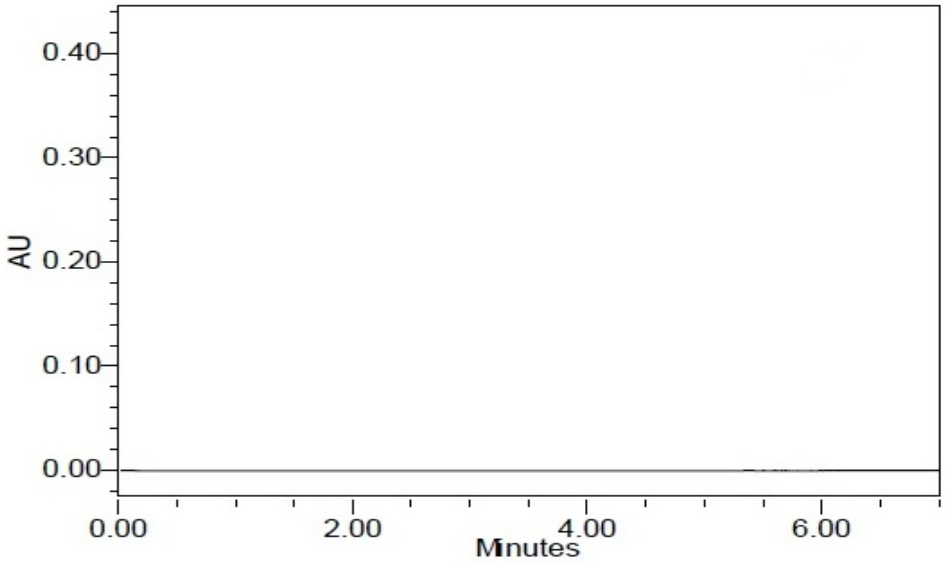

Figure 2.4: Chromatogram of placebo blank solution

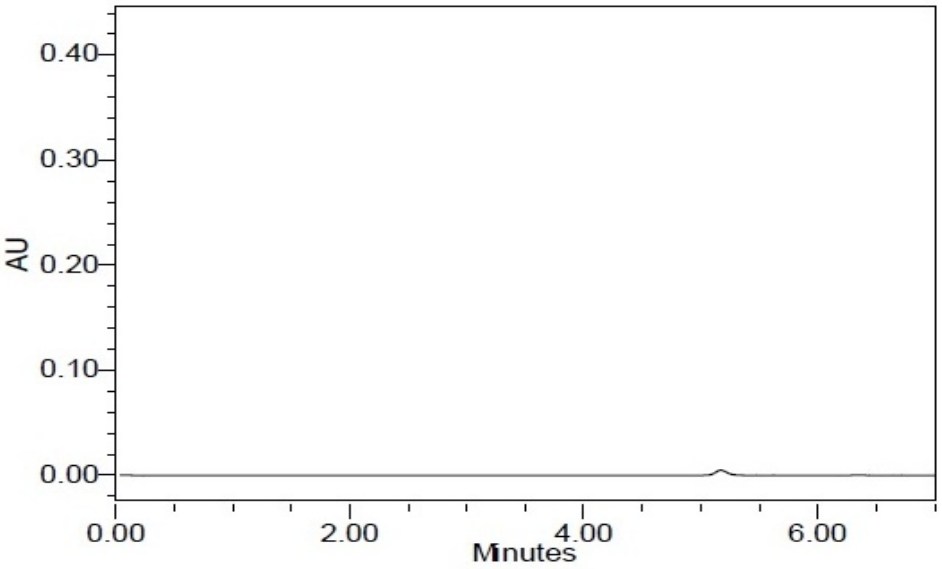

Figure 2.5: Chromatogram of mobile phase blank solution

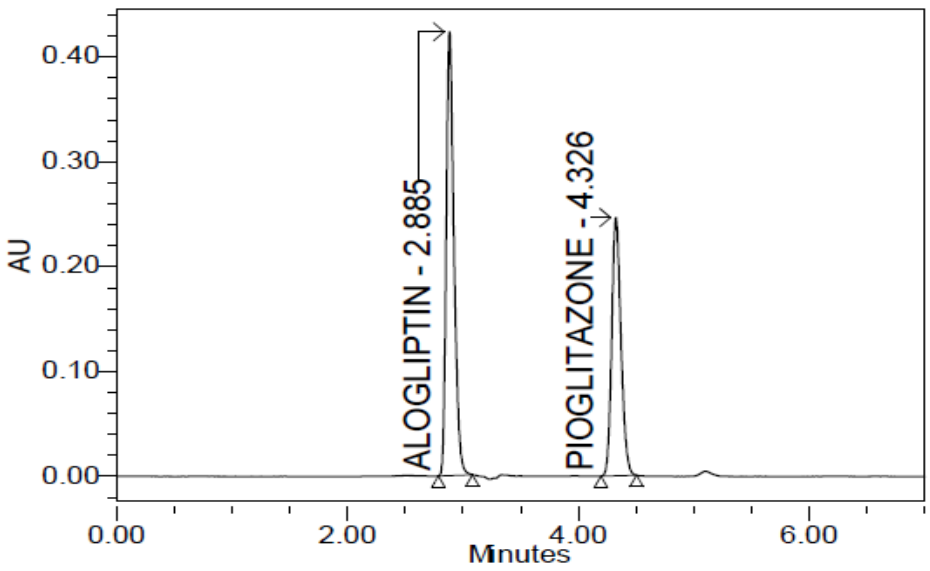

Figure 2.6: Chromatogram of alogliptin and pioglitazone standard solution

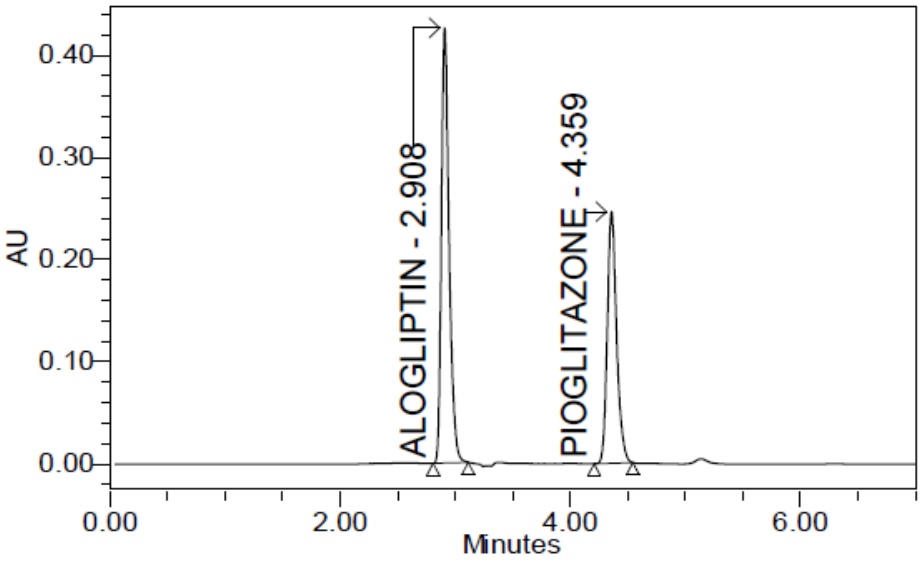

Figure 2.7: Chromatogram of alogliptin and pioglitazone tablet sample solution

2.4.2.3. Linearity

Plot (Table 2.3) of the mean peak area against concentration gave the linear relationship over the concentration range 6.25-18.75 µg/mL for alogliptin (Figure 2.8) and 11.25-33.75 µg/mL for pioglitazone (Figure 2.9). Using the regression analysis, the linear equation obtained was*:* y = 15825 x - 554 (R^2 = 0.9999) for alogliptin; y = 61574 x - 471.2 (R^2 = 0.9998) for pioglitazone where y is the mean peak area, x is concentration in µg/mL and R^2 is the regression correlation.

Table 2.3: Linearity studies for alogliptin and pioglitazone

Linearity of alogliptin		**Linearity of pioglitazone**	
Concentration (µg/mL)	**Peak area**	**Concentration (µg/mL)**	**Peak area**
6.25	988012	11.25	693449
9.38	1482104	16.88	1034328
12.5	1976665	22.5	1386985
15.625	2478118	28.125	1732302
18.75	2963436	33.75	2077170
Regression equation: y = 15825 x - 554 (R^2 = 0.9999)		Regression equation: y = 61574 x - 471.2 (R^2 = 0.9998)	

Where y = peak area and x = concentration of the drug in µg/mL

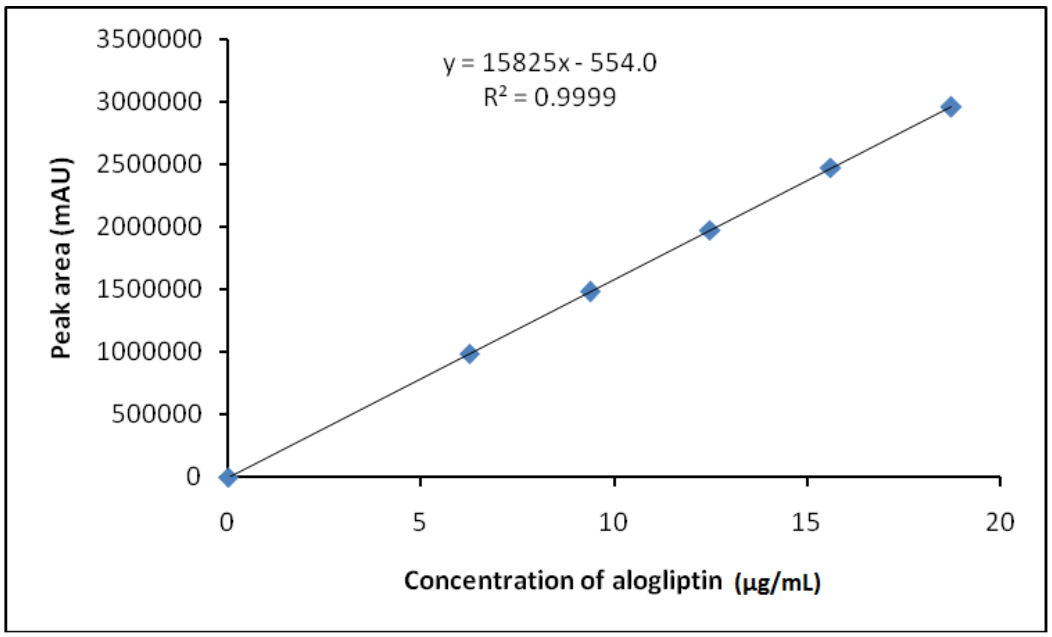

Figure 2.8: Linearity curve for alogliptin

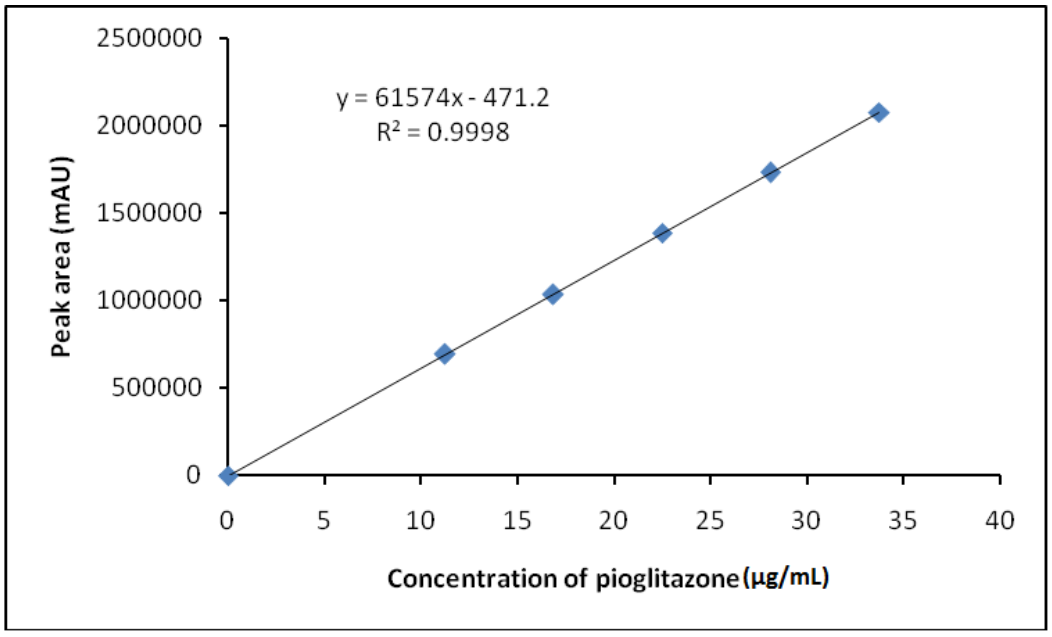

Figure 2.9: Linearity curve for pioglitazone

2.4.2.4. Limit of detection (LOD) and limit of quantification (LOQ)

The limits of detection and quantification were calculated according to ICH guidelines. The LOD for alogliptin and pioglitazone was 0.047 and 0.085 µg/mL, respectively, while LOQ was 0.157 and 0.284 µg/mL, respectively.

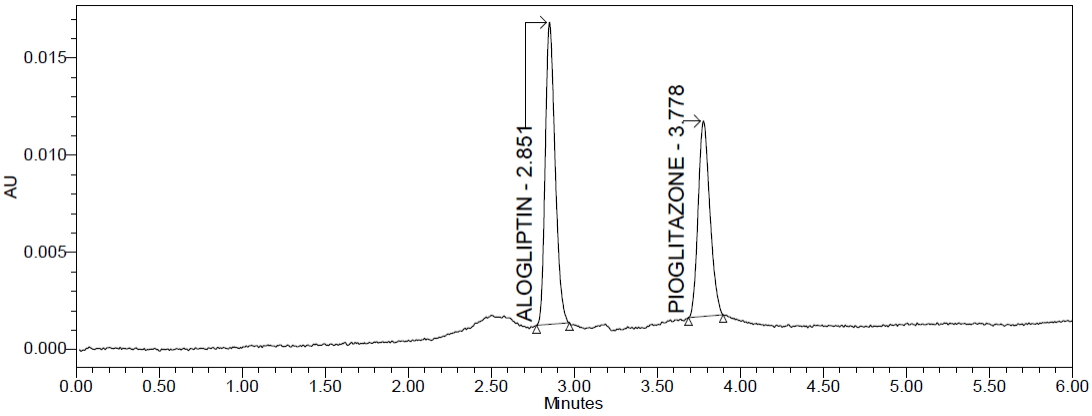

Figure 2.10: Chromatogram of alogliptin and pioglitazone at LOD level

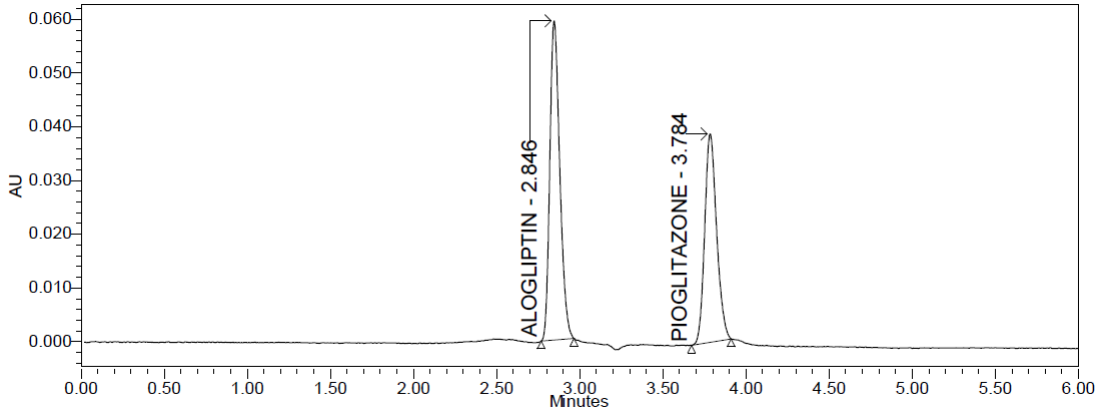

Figure 2.11: Chromatogram of alogliptin and pioglitazone at LOQ level

2.4.2.5. Precision and accuracy

The precision and accuracy of the method were tested by applying the proposed HPLC method for the determination of working standard solution of

alogliptin and pioglitazone at a concentration of 12.50 µg/mL and 22.50 µg/mL, respectively. The working standard solution was assayed six times on the same day. The precision and accuracy were expressed as % relative standard deviation and % recovery, respectively. The results are shown in Tables 2.4. The low values of % RSD and good % recovery values confirm the satisfactory precision and accuracy of the present HPLC method.

Table 2.4: Precision and accuracy data of the proposed HPLC method

Alogliptin		**Pioglitazone**	
Peak area	**Recovery (%)**	**Peak area**	**Recovery (%)**
1976730	98.53	1381221	98.83
1972402	98.32	1388479	99.35
1972552	98.32	1380884	98.80
1974805	98.44	1382787	98.94
1979467	98.67	1387533	99.28
1971548	98.27	1386181	99.18
Average	**98.43**	**Average**	**99.06**
RSD (%)	**0.152**	**RSD (%)**	**0.239**

2.4.2.6. Recovery study

The accuracy of the proposed HPLC method was further checked by the standard addition method. For this, the pre-analyzed sample solution was spiked with known concentration of alogliptin and pioglitazone at three different concentration levels (50 %, 100 %, and 150 %) the percentage recovery data (Table 2.5; Figures 2.12-2.14) show that the proposed method was accurate. Common excipients in tablets did not interfere with the assay of alogliptin and pioglitazone indicating the selectivity of the method.

Table 2.5: Recovery data of the proposed HPLC method

Spiked level (%)	Concentration of alogliptin (µg/mL)		Recovery (%)	Mean (%)	Concentration of pioglitazone (µg/mL)		Recovery (%)	Mean (%)
	Added	Found			Added	Found		
50 %	6.178	6.160	99.709	99.692	11.120	11.163	100.385	100.422
	6.178	6.156	99.652		11.120	11.168	100.433	
	6.178	6.160	99.714		11.120	11.170	100.448	
100 %	12.375	12.314	99.509	99.450	22.275	22.308	100.150	100.184
	12.375	12.282	99.246		22.275	22.313	100.169	
	12.375	12.325	99.596		22.275	22.327	100.232	
150 %	18.534	18.483	99.729	99.626	33.360	33.399	100.116	100.221
	18.534	18.450	99.547		33.360	33.445	100.255	
	18.534	18.460	99.601		33.360	33.458	100.291	

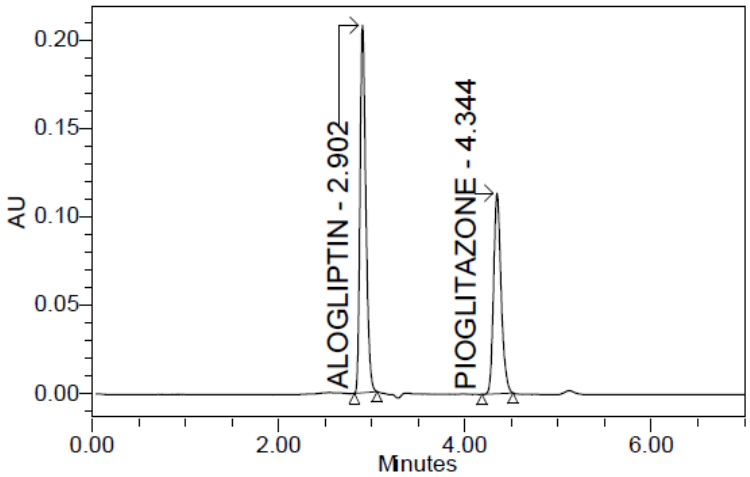

Figure 2.12: Chromatogram of alogliptin and pioglitazone at 50 % level

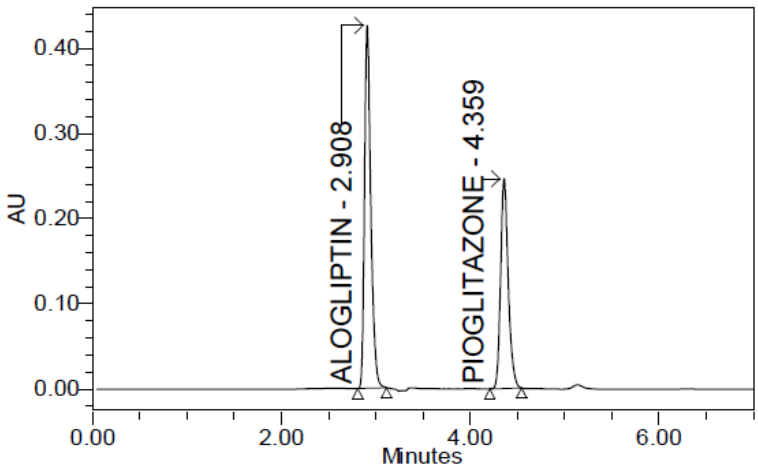

Figure 2.13: Chromatogram of alogliptin and pioglitazone at 100 % level

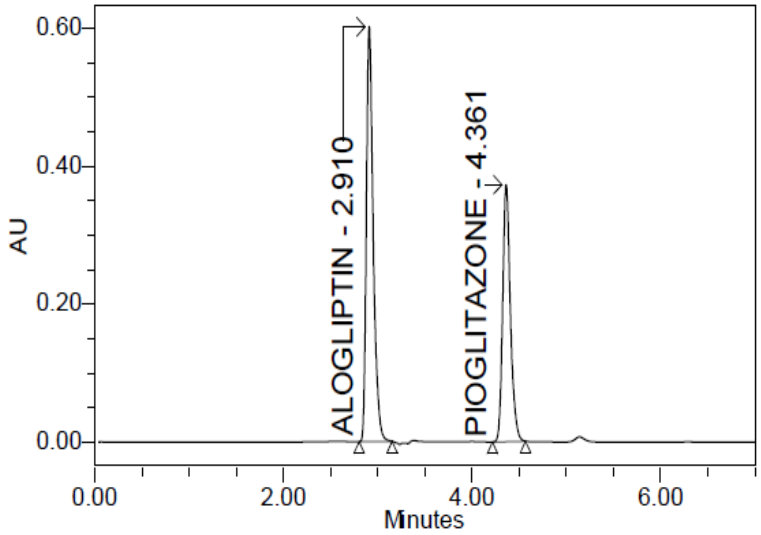

Figure 2.14: Chromatogram of alogliptin and pioglitazone at 150 % level

2.4.2.7. Robustness

The method robustness was performed by evaluating the influence of small and deliberate changes in HPLC conditions on the system suitability parameters of the proposed HPLC method. The selected conditions are flow rate (±0.1 mL/min) and temperature (±2 ^{o}C). Robustness was determined by the working standard solution of alogliptin and pioglitazone at a concentration of 12.50 µg/mL and 22.50 µg/mL, respectively. The results are summarized in Table 2.6. In all cases, good separations of both alogliptin and pioglitazone were achieved and the system suitability parameters are well within the acceptable limits, indicating that the proposed HPLC method remained robust under the optimized conditions.

Table 2.6: Robustness data of the proposed HPLC method

Parameter	Alogliptin			Pioglitazone		
	USP Tailing	USP plate count	USP resolution	USP Tailing	USP plate count	USP resolution
Flow rate 1.0 + 0.1 mL/min	1.36	9687	-	1.25	15120	10.71
Flow rate 1.0 – 0.1 mL/min	1.40	8369	-	1.28	13455	10.17
Temperature 30 + 5 ^{o}C	1.37	9714	-	1.27	15015	10.70
Temperature 30 - 5 ^{o}C	1.42	8508	-	1.29	13532	10.23

2.4.2.8. Stability studies

Forced degradation studies were carried out to elucidate the inherent stability characteristics of the alogliptin and pioglitazone. An ideal stability-indicating HPLC method is one that measures the analytes and also resolves its degradation products. Different stress conditions were applied: acid and base hydrolysis, oxidative, thermal and photo degradation.

Alogliptin and pioglitazone were found to degrade under all the stress conditions employed. Alogliptin was found to be more degraded by thermal degradation and pioglitazone in oxidative degradation condition applied. Less degradation of both the drugs was observed in photolytic degradation. The results of forced degradation studies are shown in Table 2.7. Chromatograms obtained under different stress conditions are shown in Figures 2.15-2.19. The developed HPLC method could effectively resolve the drugs from their degradation products. This confirms the stability indicating the power of the developed HPLC method.

The chromatographic peak purity tool was applied to verify the purity of alogliptin and pioglitazone peaks in all cases. This was done by calculating purity angle and purity threshold for alogliptin and pioglitazone peaks. In all cases, alogliptin and pioglitazone peaks were pure since purity angle was less than purity threshold. This showed that alogliptin and pioglitazone peaks had no detectable impurity peaks and free of co-eluting degradation products.

Table 2.7: Results of degradation studies

Stress condition	Drug	Peak area	Degradation (%)	Assay (%)	Purity angle	Purity threshold	Retention time of degradants
Acid (0.1 N HCl)	Alo	1782654	9.82	90.18	0.554	0.659	5.141
	Pio	1888497	4.46	95.54	0.528	0.667	
Base (0.1 N NaOH)	Alo	1790579	9.41	90.59	0.494	0.658	5.143
	Pio	1287248	7.19	92.81	0.299	0.437	
Oxidation (30 % H_2O_2)	Alo	1791485	9.37	90.63	0.516	0.659	2.540 and 5.131
	Pio	1283480	7.46	92.54	0.305	0.437	
Dry heat (105 ^oC)	Alo	1705211	13.73	86.27	0.565	0.661	3.995 and 5.125
	Pio	1284260	7.41	92.59	0.306	0.439	
Photolytic (sun light 24 hr)	Alo	1888497	4.46	95.54	0.528	0.667	2.532 and 5.134
	Pio	1290513	6.96	93.04	0.315	0.444	

Alo- Alogliptin; Pio-Pioglitazone

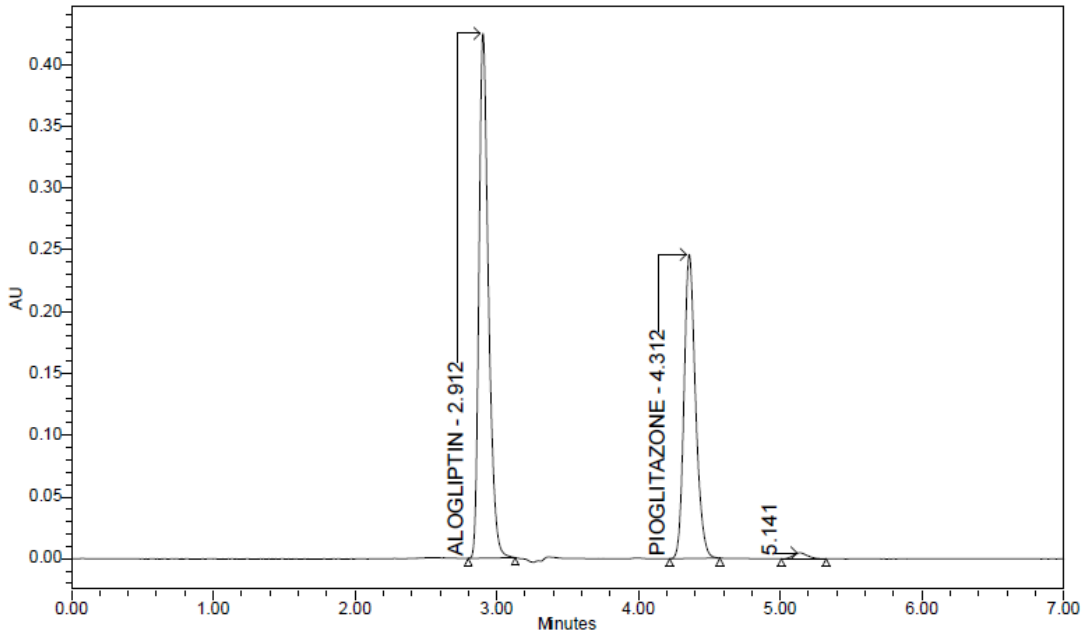

Figure 2.15: Chromatogram of tablet sample treated with 0.1 N HCl

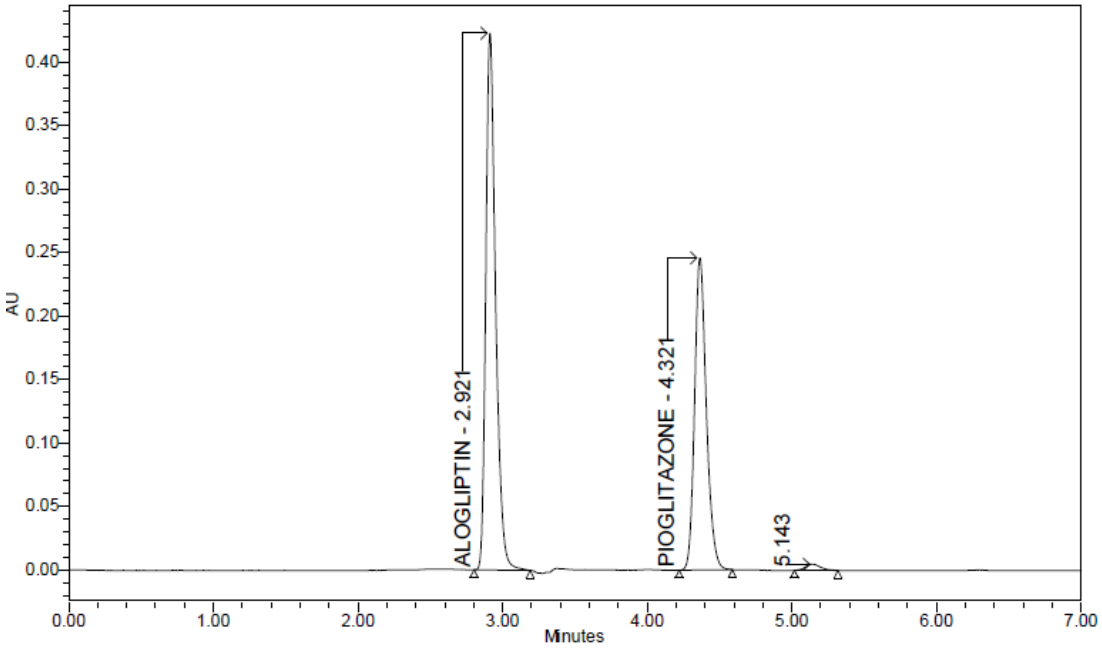

Figure 2.16: Chromatogram of tablet sample treated with 0.1 N NaOH

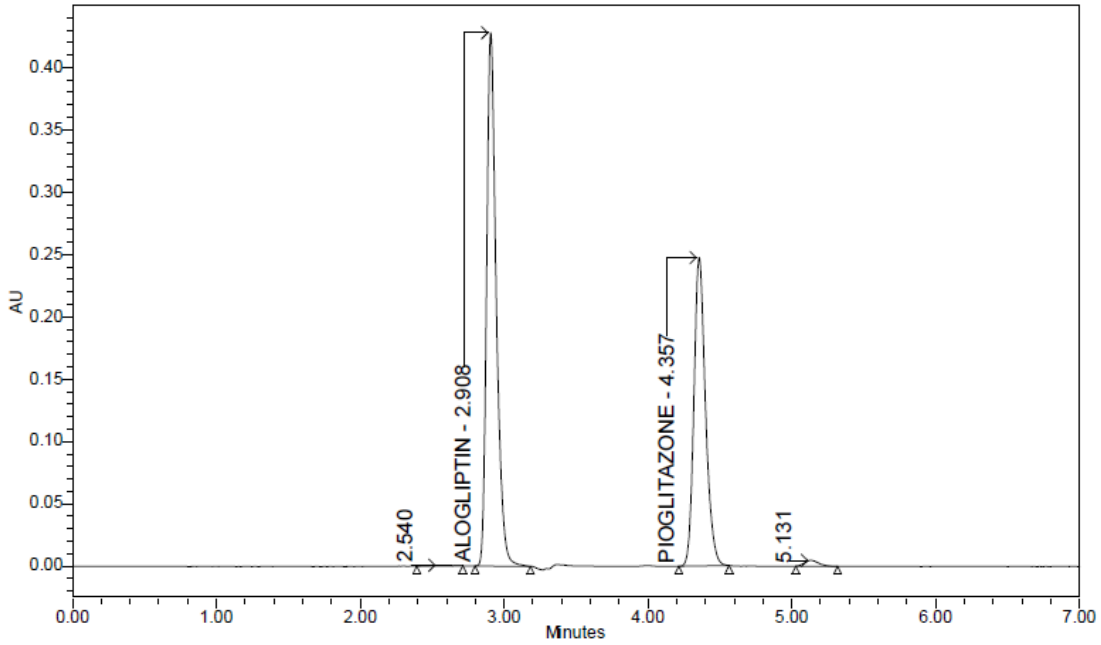

Figure 2.17: Chromatogram of tablet sample treated with 30 % hydrogen peroxide

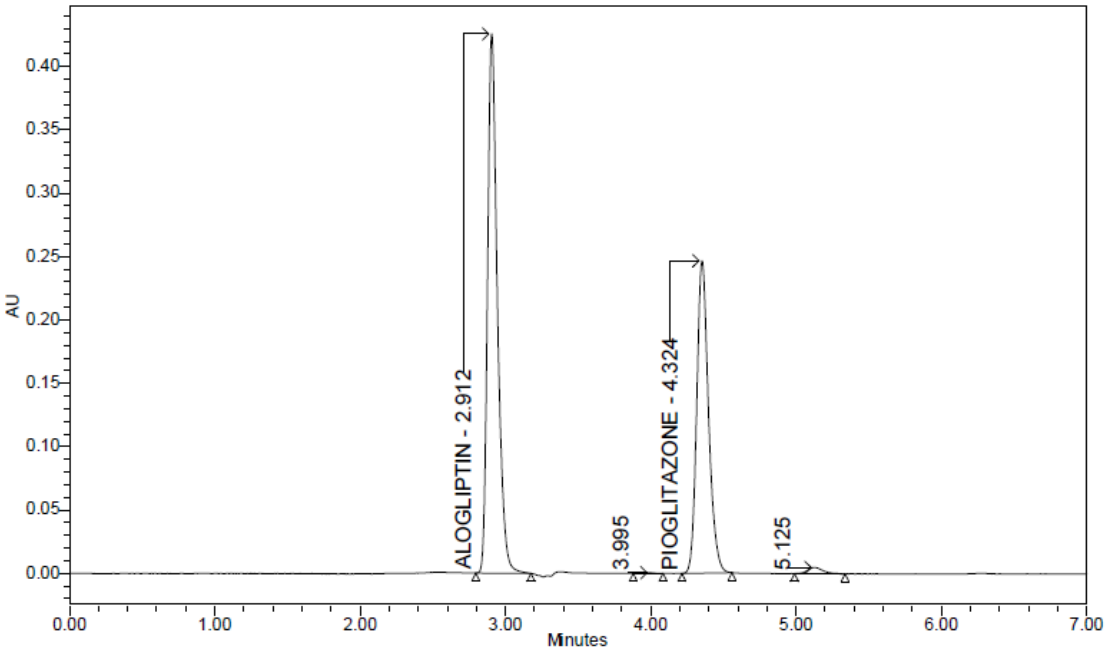

Figure 2.18: Chromatogram of tablet sample exposed to 105 ^{o}C

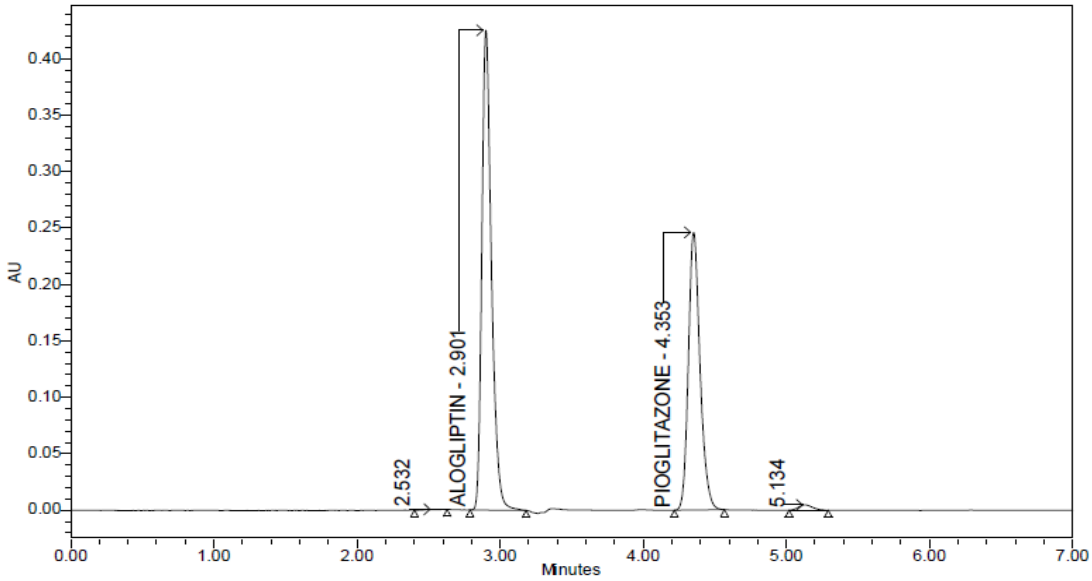

Figure 2.19: Chromatogram of tablet sample exposed to sun light

2.5. ADVANTAGES OF THE PROPOSED METHOD

The methods reported in the sections 2.2.1 and 2.2.2 for the quantification of alogliptin [8-17] and pioglitazone [18-47] were applied only for the individual estimation of the drug. The estimation of combination of these three drugs is not official in any pharmacopoeia.

Four UV spectrophotometric methods [51,52] and one HPTLC method [53] were reported in the literature. Though the UV spectrophotometric methods are simple, they are less selective since they involve measurements in the UV range where there is a possibility of absorbance by the tablet excipients. One of the important validation parameter, method robustness, is not reported in the UV spectrophotometric methods. The HPTLC method requires costly, sophisticated instrumentation and expertise personnel to operate. Moreover the HPTLC instrument is not commonly available in the developing and under developed countries.

Few RP-HPLC methods are found in the literature for the simultaneous assay of alogliptin and pioglitazone. The details of the reported RP-HPLC methods are summarized in Table 2.8. The proposed RP-HPLC method has the advantages of being more sensitive [54-57], more precise [54-57] and more accurate [54-57] than the reported HPLC methods. The total run time of the proposed method was less when compared with the reported HPLC methods [54-56]. The less run time may decrease the utilization of solvents, time and cost of analysis. The proposed method has wider range of linearity than the Manzoor *et al.,* [56] method. The validation parameters like system suitability [55] and specificity [54-56] are not reported in some of the reported HPLC methods. The volume of sample for analysis in the proposed method (10 µL) is lesser than the methods (20 µL) of Manzoor *et al.,* [56] and Mokhtar *et al.,* [57].

Table 2.8: Summary of proposed and reported RP-HPLC methods

Drug	Run time (min)	Linearity (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	RSD (%)	Recovery (%)	Reference
Alo	10	6.25-31.25	0.555	1.680	0.404-1.069	101.01-101.07	Raval and Srinivasa [54]
Pio		3.75-18.75	0.139	0.423	0.553-1.124	99.84-100.77	
Alo	10	31-187	0.339	1.210	0.31	99.87-100.56	Neelima et al., [55]
Pio		75-450	0.516	1.565	0.32	99.62-100.61	
Alo	8	2.5-15	0.034	0.012	0.257	98.44-100.40	Manzoor et al., [56]
Pio		3-18	0.034	0.105	0.230	99.16-100.55	
Alo	6	5-100	0.170	0.500	0.23-1.10	99.12-99.48	Mokhtar et al., [57]
Pio		5-100	0.215	0.650	0.31-1.54	101.38-101.95	
Alo	7	6.25-18.75	0.047	0.157	0.152	99.45-99.69	Proposed
Pio		11.25-33.75	0.085	0.284	0.236	100.18-100.42	

Alo – **A**logliptin: Pio – Pioglitazone

2.6. SUMMARY AND CONCLUSION

The overall results obtained for the proposed method validation were tabulated in Table 2.9.

Table 2.9: Summary of the proposed method

Parameter	Alogliptin	Pioglitazone
Linearity (µg/mL)	6.25-18.75	11.25-33.75
Regression equation	*y* = 15825 x- 554	*y* = 61574 x - 471.2
Regression coefficient (R^2)	0.9999	0.9998
LOD (µg/mL)	0.047	0.085
LOQ (µg/mL)	0.157	0.284
Precision (% RSD)	0.152	0.239
Accuracy (% Recovery)	99.450-99.692	100.184-100.422

In the present study, an attempt was made to develop an accurate, precise, selective and sensitive RP-HPLC method for the simultaneous analysis of alogliptin and pioglitazone in bulk and combined tablet dosage forms. The method was validated in accordance with ICH guidelines. The main features of the developed method are economical, low run time, selective, robust, sensitive and satisfactory precision and accuracy. Therefore, the suggested RP-HPLC method can be used for the simultaneous quantification of alogliptin and pioglitazone in quality control laboratories or industry.

REFERENCES

1. Jarvis CI, Cabrera A, Charron D. Alogliptin: a new dipeptidyl peptidase-4 inhibitor for type 2 diabetes mellitus. *Annals of Pharmacotherapy*, 2013, 47 (11), 1532-1539.

2. Marino AB, Cole SW. Alogliptin: safety, efficacy, and clinical implications. *The Journal of Pharmacy Practice*, 2015, 28 (1), 99-106.

3. Covington P, Christopher R, Davenport M, Fleck P, Mekki QA, Wann ER, Karim A. Pharmacokinetic, pharmacodynamic, and tolerability profiles of the dipeptidyl peptidase-4 inhibitor alogliptin: A randomized, double-blind, placebo-controlled, multiple-dose study in adult patients with type -II diabetes. *Clinical Therapeutics*, 2008, 30 (3), 499-512.

4. Golightly LK, Drayna CC, McDermott MT. Comparative clinical pharmacokinetics of dipeptidyl peptidase-4 inhibitors. *Clinical Pharmacokinetics*, 2012, 51 (8), 501-514.

5. Keating GM. Alogliptin: a review of its use in patients with type -II diabetes mellitus. *Drugs*, 2015, 75 (7), 777-796.

6. Cyrus VD, Vijay S. Pioglitazone in the treatment of type -II diabetes: Safety and efficacy review. *Clinical Medicine Insights Endocrinology and Diabetes*, 2010, 3, 43–51.

7. Luigi B, Julie K. Management of type -II diabetes mellitus in adults. Focus on individualizing non-insulin therapies. *Pharmacy and Therapeutics,* 2012, 37(12), 687–696.

8. El-Bagary RI, Elkady EF, Ayoub BM. Liquid chromatographic determination of alogliptin in bulk and in its pharmaceutical preparation. *International Journal of Biomedical Sciences,* 2012, 8 (3), 215-218.

9. Yadav PJ, Kadam VN, Mohite SK. Development and validation of RP-HPLC method for alogliptin benzoate in bulk drug and dosage form. *International Journal of Pharmacy and Pharmaceutical Research,* 2014, 1 (2), 1-9.

10. Srinivasa rao G, Mallesh K, Kumar GV, Surekha Ch, Rao BV. A validated chiral HPLC method for the enantiomeric purity of alogliptin benzoate. *Der Pharma Chemica,* 2014, 6 (3), 234-239.

11. Zhou Y, Zhou W, Sun L, Zou Q, Wei P, OuYang P. Characterization of process-related impurities including forced degradation products of alogliptin benzoate and the development of the corresponding reverse-phase high-performance liquid chromatography method. *Journal of Separation Science*, 2014, 37 (11), 1248–1255.

12. Kun Z, Panqin M, Wenna J, Xiangrong Z. A developed HPLC method for the determination of Alogliptin Benzoate and its potential impurities in bulk drug and tablets. *Asian Journal of Pharmaceutical Sciences,* 2015, 10 (2), 152-158.

13. Keyur BA, Pooja JM, Namrata BS, Aakanksha AG. Development and validation of a simple UV spectrophotometric method for the determination of alogliptin benzoate in bulk and in pharmaceutical dosage formulation. *Inventi Rapid - Pharm Analysis & Quality Assurance,* 2014, Article ID: Inventi:ppaqa/1502/14.

14. Yadav PJ, Kadam VN, Mohite SK. Development and validation of UV spectrophotometric method for alogliptin benzoate in bulk drug and tablet formulation. Jou*rnal of Current Pharma Research*, 2014, 4 (4), 1286-1290.

15. Kumar AVVNKS, Reddy TV, Sekharan CB. Utility of picric acid and 2,4 dinitrophenol as chromogenic reagents for visible spectrophotometric quantification of alogliptin. *Bulletin of Faculty of Pharmacy, Cairo University,* 2017, 55 (1), 177-184.

16. Kumar AVVNKS, Reddy TV, Sekharan CB. Spectrophotometric determination of alogliptin in bulk and tablet dosage form using bromate-bromide mixture as brominating agent. *Karbala University Journal of Modern Sciences,* 2017, 3 (1), 8-17.

17. Raval K, Srinivasa U. Development and validation of new colorimetric method for the estimation of alogliptin & loteprednol. *International Journal of Advanced Pharmaceutics*, 2015, 5 (2), 56-59.

18. European Pharmacopoeia, Council of Europe, France. 8th Edition, 2014.

19. Mostafa GA, Al-Majed A. Characteristics of new composite and classical potentiometric sensors for the determination of pioglitazone in some pharmaceutical formulations. *Journal of Pharmaceutical and Biomedical Analysis*, 2008, 48 (1), 57–61.

20. El-Ghobashy M, Yehia A, Mostafa A. Application of membrane-selective electrodes for the determination of pioglitazone HCl in the presence of its acid degradant or metformin HCl in tablets and plasma. *Analytical Letters*, 2009, 42(1), 123–140.

21. Badawy WA, El-Ries MA, Mahdi IM. Carbon paste and PVC membrane electrodes as sensitive sensors for the determination of antidiabetic drugs for type -II diabetic patients. *Analytical Science*, 2009, 25(12), 1431–1436.

22. Al-Arfaj NA, Al-Abdulkareem EA, Aly FA. Flow-injection chemiluminometric determination of pioglitazone HCl by its sensitizing effect on the cerium sulfite reaction. *Analytical Science*, 2009, 25(3), 401–406.

23. Mahadik SP, Senthilkumar GP. Method development & validation of pioglitazone in bulk and pharmaceutical dosage forms by using spectrophotometric method. *Asian Journal of Biochemical and Pharmaceutical Research*, 2012, 2 (1), 159–165.

24. Younus MA, Swamy PV, Borgaonkar P. UV spectrophotometric determination of pioglitazone in pharmaceutical dosage forms. *International Journal of Chemical Sciences*, 2008, 6 (4), 2062–2065.

25. Santosh K, Sunayana M, Mali M, Suresh G, Sanjana G, Sayyad SH, Abdul RJ. Area under curve UV spectrophotometric method for the determination of pioglitazone in tablet formulations. *International Journal of Current Medical and Pharmaceutical Research*, 2017, 3 (1), 1483-1487.

26. Mohd S, Kulkarni AP, Zaheer Z. Spectroscopic estimation of pioglitazone hydrochloride. *International Journal of Pharmaceutical Frontier Research*, 2012, 2(2012), 87–94.

27. Shakya P, Singh K. Determination of pioglitazone hydrochloride in bulk and pharmaceutical formulations by UV spectrophotometric method. International Journal of Pharmaceutical Sciences and Research, 2010, 1(11), 153–157.

28. Patil S, Dwivedi S, Bagade S. Development of spectrophotometric method for the estimation of pioglitazone HCl from two different marketed brands. *American Journal of PharmTech Research*, 2011, 1(4), 264–275.

29. Pawan KB, Prabhat KS, Deepti J. Spectrophotometric estimation of pioglitazone hydrochloride in tablet dosage form. *Asian Journal of Pharmaceutics*, 2008, 2 (4), 225-227.

30. Nawal AA, Sawsan AAR, Aziza AAH. Spectrophotometric determination of pioglitazone hydrochloride in tablets and urine. *Asian Journal of Chemistry*, 2011, 23 (9), 4104-4108.

31. Amanlou M, Zarei-Ghobadi M, Rofouei MK. Shahrooz S, Abbas K. Extractive Spectrophotometric method for determination of pioglitazone HCl in raw material and tablets using ion-pair formation. *E-Journal of Chemistry*, 2010, 7 (3), 915–921.

32. Radhakrishna T, Rao DS, Reddy GO. Determination of pioglitazone hydrochloride in bulk and pharmaceutical formulations by HPLC and MEKC methods. *Journal of Pharmaceutical and Biomedical Analysis*, 2002, 29 (4), 593–607.

33. Calixto LA, Bonato PS. Combination of hollow-fiber liquid-phase microextraction and capillary electrophoresis for pioglitazone and its main metabolites determination in rat liver microsomal fraction. *Electrophoresis*, 2013, 34 (6), 862–869.

34. Sripalakit P, Neamhom P, Saraphanchotiwitthaya A. High performance liquid chromatographic method for the determination of pioglitazone in human plasma using ultraviolet detection and its application to a pharmacokinetic study. *Journal of Chromatography B. Analytical Technologies in Biomedical and Life Sciences*, 2006, 843 (2), 164–169.

35. Tahmasebi E, Yamini Y, Saleh A. Extraction of trace amounts of pioglitazone as an anti-diabetic drug with hollow fiber liquid phase microextraction and determination by high-performance liquid chromatography-ultraviolet detection in biological fluids. *Journal of Chromatography B. Analytical Technologies in Biomedical and Life Sciences,* 2009, 877 (20-21), 1923–1929.

36. Ravikanth CH, Kumar AA, Kiran VU, Prashanth S, Madhu B, Reddy YN. Sensitive and rapid HPLC method for the determination of pioglitazone in rat serum. *International Journal of Pharmaceutical Sciences and Drug Research*, 2011, 3 (1), 38–41.

37. Islambulchilar Z, Valizadeh H, Milani PZ. Rapid HPLC determination of pioglitazone in human plasma by protein precipitation and its application to pharmacokinetic studies. *Journal of AOAC International,* 2010, 93 (3), 876–881.

38. Reddy GRK, Rao VSN. Development and validation of stability indicating assay method for pioglitazone drug substance by reverse phase HPLC. *Journal of Global Trends in Pharmaceutical Sciences*, 2012, 3 (2), 584–596.

39. Sharma S, Sharma MC, Chaturvedi SC. Study of stressed degradation behavior of pioglitazone hydrochloride in bulk and pharmaceutical formulation by HPLC assay method. *Journal of Optoelectronic and Biomedical Materials*, 2010, 1 (1), 17–24.

40. Saber AMRL. Determination of pioglitazone hydrochloride in tablets by high-performance liquid chromatography. *Pakistan Journal of Analytical and Environmental Chemistry*, 2008, 9 (2), 118–121.

41. Jedlicka A, Klimes J, Grafnetterova T. Reverse-phase HPLC methods for purity test and assay of pioglitazone hydrochloride in tablets. *Pharmazie*, 2004, 59 (3), 178–182.

42. Wanjari DB, Gaikwad NJ. Stability indicating RP-HPLC method for determination of pioglitazone from tablets. *Indian Journal of Pharmaceutical Sciences*, 2005, 67 (2), 256–258.

43. Madhukar A, Naresh K, Kumar CN, Sandhya N, Prasanna P. Rapid and sensitive RP-HPLC analytical method development and validation of pioglitazone hydrochloride. *Der Pharma Chemica*, 2011, 3 (3), 128–132.

44. Xue YJ, Turner KC, Meeker JB, JaniceP, Mark A, Steve U. Quantitative determination of pioglitazone in human serum by direct-injection high performance liquid chromatography mass spectrometry and its application to a bioequivalence study. *Journal of Chromatography B. Analytical Technologies in Biomedical and Life Sciences*, 2003, 795 (2), 215–226.

45. Lin ZJ, Ji W, Krieger DD, Linyee S. Simultaneous determination of pioglitazone and its two active metabolites in human plasma by LC–MS/MS. *Journal of Pharmaceutical and Biomedical Analysis*, 2003, 33 (1), 101–108.

46. Chinnalalaiah R, Pigili R, Avanapu SR. Liquid chromatography and tandem mass spectrometry method for quantitative determination of pioglitazone and its metabolite 5-hydroxy pioglitazone in human plasma. *Annales Pharmaceutiques Françaises*, 2016, 75 (2), 105-111.

47. Singh SCDD, Ashok K. Development and validation of a HPTLC method for estimation of pioglitazone in bulk and tablet dosage form. *Journal of Pharmacy Research*, 2011, 4 (11), 3919–3921.

48. Raalte VDH, van Genugten RE, Eliasson B, Möller-Goede DL, Mari A, Tura A, Wilson C, Fleck P, Taskinen MR, Smith U, Diamant M. The effect of alogliptin and pioglitazone combination therapy on various aspects of β-cell function in patients with recent-onset type -II diabetes. *European Journal of Endocrinology*, 2014, 170 (4), 565-574.

49. Julio R, Silvio EI, Jochen S, Penny RF, Craig AW, Qais M. Initial combination therapy with alogliptin and pioglitazone in drug-naïve patients with type -II diabetes. *Diabetes Care*, 2010, 33(11), 2406-2408.

50. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm336942.htm

51. Raval K, Srinivasa U. First order derivative and dual wavelength spectrophotometry methods development and validation for simultaneous estimation. *International Journal of Pharmacy and Pharmaceutical Sciences*, 2014, 6 (2), 730-738.

52. Anusha M, Manzoor Ahmed, Satishkumar Shetty A, Vijaya Krishna.C. Aradhya. Simultaneous estimation of alogliptin and piogliazone in bulk and combined tablet dosage form by UV-spectrophotometric methods. *International Journal of Universal Pharmacy and Bio Sciences*, 2014, 3(6), 363-376.

53. Komal S, Amrita P. Development and validation of HPTLC method for simultaneous estimation of alogliptin benzoate and pioglitazone hydrochloride in bulk drugs and combined dosage forms. *International Journal of Pharma Research and Review*, 2015, 4 (11), 35-42.

54. Raval K, Srinivasa U. Development and validation of HPLC method for the simultaneous estimation of pioglitazone and alogliptin in bulk and dosage form. *International Journal of Current Research*, 2014, 6(11), 10201-10207.

55. Neelima B, Kumar PR, Bindu VH, Prasad YR. A validated stability indicating RP-HPLC method for simultaneous determination of alogliptin and pioglitazone in bulk and pharmaceutical formulations. *International Journal of Pharmacy*, 2014, 4 (1), 458-464.

56. Manzoor A, Anusha M, Satishkumar SA, Kuppast IJ, Siddalingaswamy MS, Ravi MC. RP-HPLC method development and validation for simultaneous estimation of alogliptin and pioglitazone in combined tablet dosage form. *World Journal of Pharmacy and Pharmaceutical Sciences*, 2015, 4 (1), 863-874.

57. Mokhtar MM, Sherin FH, Fotouh RM, Mona MA. Development and validation of a reverse phase HPLC method for simultaneous determination of antidiabetic drugs alogliptin benzoate and pioglitazone HCl. *Der Pharmacia Sinica*, 2016, 7(2), 32-40.

58. International conference on harmonization, ICH Guidelines, Validation of analytical procedures technical requirements for registration of pharmaceuticals for human use: Text and methodology Q 2 (R1), International Conference on Harmonization, Geneva, Switzerland, November 2005.