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Development and validation of a reversed phase HPLC method for simultaneous determination of antidiabetic drugs alogliptin benzoate and pioglitazone HCl

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ABSTRACT

A simple reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been developed and validated for simultaneous determination of alogliptin benzoate and pioglitazone HCl in bulk and tablet dosage form. Chromatographic analysis was performed on an Inertsil® ODS-3 column (250x4.6 mm, 5µm) with a mixture of methanol: phosphate buffer (pH 3) in the ratio 80:20 as mobile phase, at a flow rate of 1.0 mL min^{-1}. UV detection was performed at 269 nm using DAD detector. The method was validated according to ICH guidelines. The retention times of alogliptin benzoate and pioglitazone HCl were 2.31±0.00 and 4.92±0.096 min, res pectively. Calibration plots were linear over the concentration ranges 5-100 µg mL^{-1} for both drugs. The Limits of detection were170 and 215 ng mL^{-1}and the quantification limit were 500 ngmL^{-1} and 650 ng mL^{-1} for alogliptin benzoate and pioglitazone HCl respectively. The accuracy of the proposed method was determined by recovery studies and was found to be 99.56% for alogliptinand101.38% for pioglitazone. The method was transferred to UPLC system using Syncronis®C18 column (50x 2.1 mm, 1.7 µm) and binary mixture was separated with good resolution within less than one minute.

Keywords: Pioglitazone HCl, Alogliptin Benzoate, RP-HPLC, Validation, RP-UPLC

INTRODUCTION

Many patients suffering from type 2 diabetes require treatment with more than one antihyperglycemic drug to achieve optimal glycemic control[1].

Alogliptin is a dipeptidyl peptidase (DPP)-4inhibitor which stimulates glucose dependent insulin release.

Chemically, alogliptin is 2-({6-[(3R)-3-aminopiperidine-1-yl]-3-methyl-2-4-dioxo-3, 4dihydropyrimidin-1(2H)-yl} methyl benzonitrile.

Pioglitazone is a thiazolidinedione derivative, used to reduce insulin resistance by enhancing insulin action on peripheral tissues.

It is chemically 5-({4-[2-(5-ethylpyridin-2yl) ethoxy] phenyl} methyl)-1, 3-thiazolidine-2, 4-Dione. (Figure **1**)

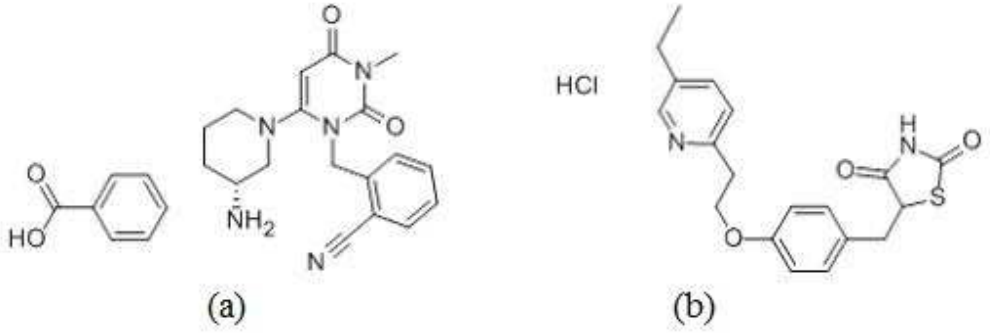

Fig.1: Chemical structure of alogliptin benzoate (a) and pioglitazone hydrochloride (b)

The review of literature revealed a few methods reported for determination of alogliptin benzoate. These include UV spectrophotometric[2], HPTLC [3], HPLC methods[4–7],chiral HPLC method for the enantiomeric purity of alogliptin benzoate[8]. For pioglitazone HCl UV spectrophotometric[9–13], HPLC methods[14–17]. Only two methods for simultaneous determination of alogliptin and pioglitazone have been reported including UV spectrophotometric method[18], Stability Indicating HPLC Method[19].

The present work describes the development and validation of suitable highly precise and accurate analytical method for the estimation of both drugs in their tablet dosage form. Reversed phase high performance liquid chromatography (RP-HPLC) method was developed, then to minimize the elution time the method was transferred to ultra-high performance liquid chromatography (UPLC). So both drugs can be separated and determined within one minute. So it can be effectively applied for routine analysis in research institutions, quality control department in industries, approved testing laboratories, Bio-pharmaceutics and Bio-equivalence studies and in clinical pharmacokinetic studies.[20]

MATERIALS AND METHODS

Standard of pioglitazone HCl (assigned purity 99.76%) and alogliptin benzoate (assigned purity 99.85%) was obtained as a gift sample from Sigma Pharmaceutical Industries, Quesna, Egypt. Methanol HPLC grade (Fischer Scientific, UK) and potassium dihydrogen phosphate analytical grade (Oxford laboratory, Mumbai, India) were used throughout the experiment.

Instrumentation and chromatographic conditions:
HPLC instrument: Dionex UltiMate 3000 RS system (Thermo Scientific™, Dionex™, Sunnyvale, CA, USA), equipped with Quaternary RS pump, RS auto-sampler injector, Thermostated RS column compartment and RS diode array detector (DAD). The data acquisition was performed by Chromeleon® 7.1 software.

Column: Inertsil® ODS-3: (250 mm x 4.6 mm i.d) and particle size: 5µm. The column maintained at ambient temperature and eluent was detected at 269nm.The mobile phase mixture containing phosphate buffer and methanol (20:80) V/V, the flow rate was 1.0 mL min^{-1}.

For UPLC Application:
Column: Syncronis®C18 column (50x 2.1 mm i.d) and particle size: 1.7 µm, the flow rate was 0.4mL min^{-1}.

Preparation of Buffer solution:
Phosphate Buffer pH 3: Dissolve 3.4 gm potassium dihydrogen phosphate in 1000 mL of distilled water, adjust the pH to 3.0 with phosphoric acid. [(Solvent A)], buffer solution was filtered through a 0.22µm membrane filter and degassed by sonication for twenty minutes before use.

Standard solution
Standard stock solutions 1.0 mg mL^{-1} of each pioglitazone and alogliptin were prepared in methanol. The working standard solutions were prepared by diluting different aliquots from stock solutions with methanol to obtain mixture of pioglitazone and alogliptin over the linearity concentration range.

Preparation of working standard solutions:
Accurately measured volumes of stock standard solutions of each pioglitazone and alogliptin were transferred into a series of 10 ml volumetric flasks and diluted appropriately with methanol to obtain working standard solutions with concentration range 5-100 µg mL^{-1} for both drugs.

Construction of calibration curves:
A volume of 20 µl was injected from each working standard solution for each drug under the optimum chromatographic conditions. Then the calibration curve was obtained by plotting the peak area of each drug versus its corresponding concentration. Then the regression equations were calculated for both calibration curves.

Assay of dosage form:
Since the dosage form Oseni® is not available in the local market, a mixture of active ingredients and excipient was prepared by mixing 25 mg of alogliptin benzoate, 30 mg pioglitazone HCl, 20 mg magnesium stearate, 20 mg croscarmellose and 405 mg microcrystalline cellulose. The laboratory prepared mixture was transferred to 100 mL volumetric flask and the volume was made up to the mark using methanol as solvent. The solution was sonicated for 20 minutes. The solution was filtered; First aliquot of filtrate was discarded and 1 mL was diluted to 10 mL with methanol. From resulting solution containing 25 µg mL^{-1} alogliptin and 30 µg mL^{-1} pioglitazone, 20 µL was injected for HPLC analysis and the concentration was calculated from the corresponding regression equation.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions:
As alogliptin used is alogliptin benzoate form excess benzoate peak was found to interfere with alogliptin and pioglitazone peaks, different factors affecting the separation of alogliptin benzoate, Benzoic acid and pioglitazone HCl were thoroughly studied and optimized.

Effect of pH of aqueous solvent in mobilephase:
Different pH (2.5-6) were tried using 0.2% TEA in distilled water and pH adjusted using glacial acetic acid. Low pH 2.5 resulted in overlapped peaks of pioglitazone and benzoic acid, High pH (5-6) resulted in splitted , tailed peak of alogliptin and longer run time, and pH 3 was selected as it gave acceptable resolution between peaks with short run time and symmetric peaks. Figure **2**

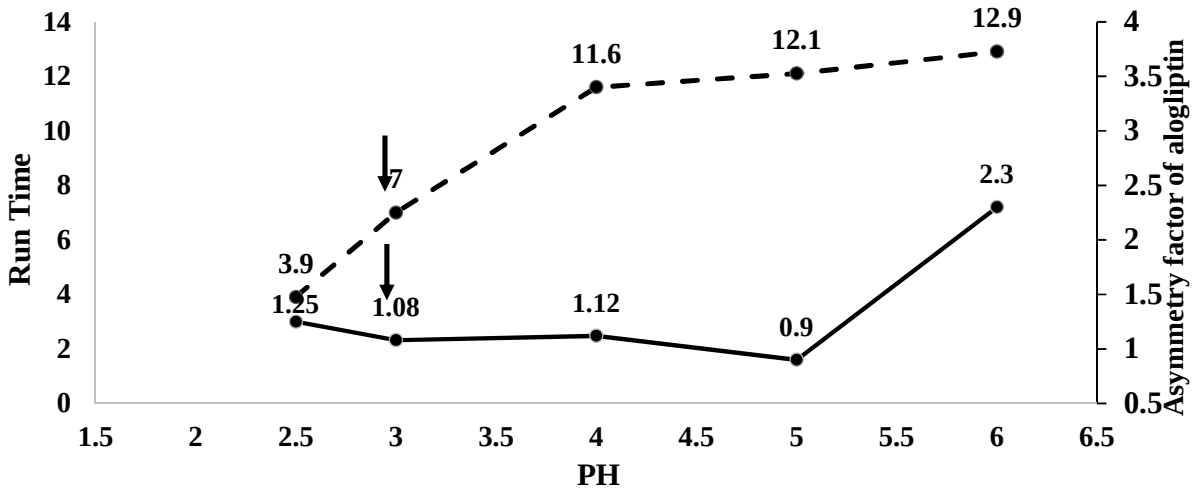

Fig.2: The effect of pHon run time and asymmetry factor of alogliptin peak(0.2% TEA in distilled water different pH: methanol 30:70%)

Effect of mobile phase ratio:
Different mobile phase ratios were tried using 0.2% TEA in distilled water and pH adjusted to 3 using glacial acetic acid. Ratio (20% aqueos-80% methanol) was chosen with respect to runtime. (Figure **3**)

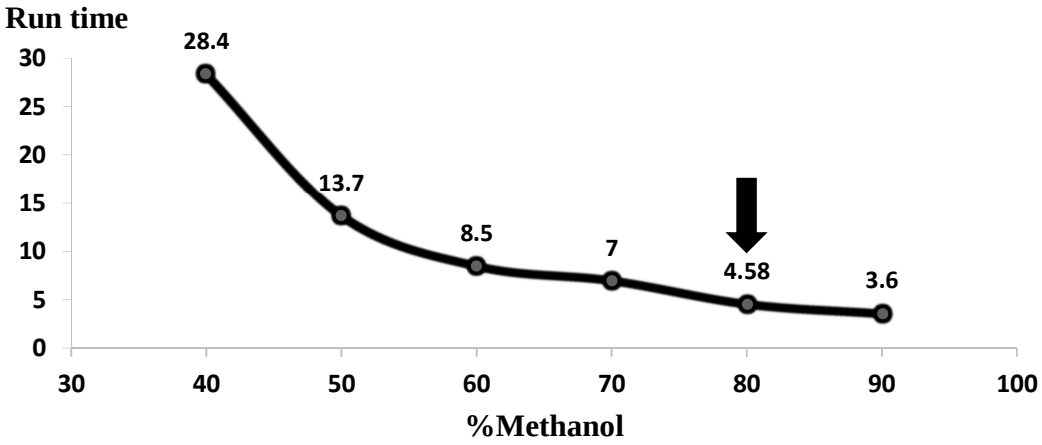

Fig.3: The effect of Methanol ratio on run time (0.2% TEA in distilled water pH 3:Methanol different ratios)

Effect of Buffer type:
Different buffers (pH 3) were tried and compared to 0.2% TEA in distilled water. Acetate buffer pH 3 resulted in poor resolution between benzoic acid and pioglitazone peaks. Phosphate buffer pH 3 was chosen as it gave better symmetric alogliptin peak compared with 0.2% TEA in distilled water.

Effect of flow rate:
It was found that by increasing flow rate, both resolution and run time decreased thus 1.0 mL min^{-1} was accepted as an optimum flow rate with respect to run time and resolution .Flow rates below 1.0 mL min^{-1} resulted in longer run times and tailed peaks, while flow rates above 1.0 mL min^{-1} resulted in low theoretical plates count.

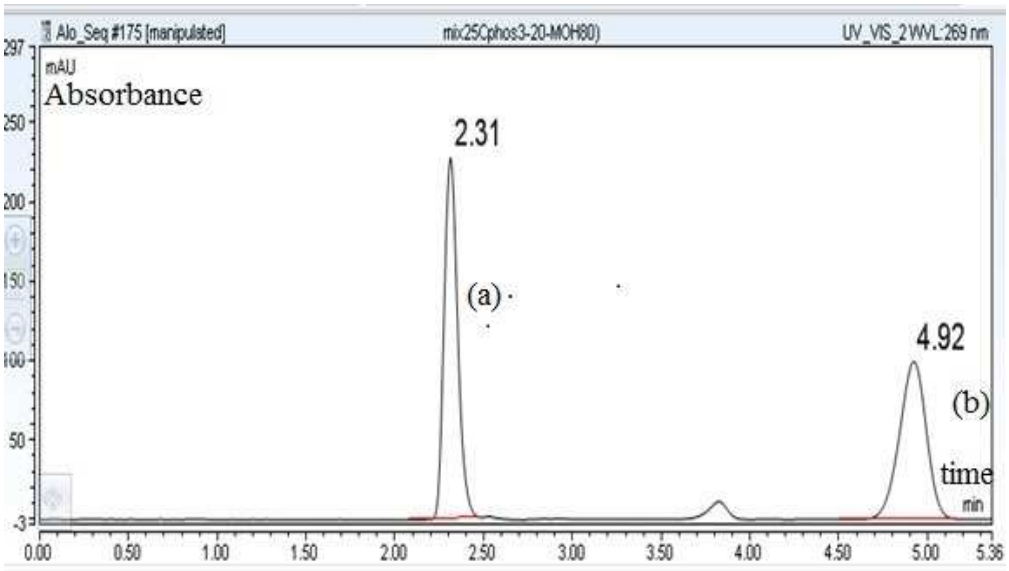

Fig.4: Chromatogram of alogliptin benzoate 50 µg mL^{-1}(a) and pioglitazoneHCl 50 µg mL^{-1}(b) using the proposed HPLC method, mobile phase consisting of methanol: phosphate buffer in ratio of 80:20 (v/v) ; pH= 3 at flow rate = 1 ml/min, temp.= 25°C and inj. vol.= 20 µl

Effect of column temperature:
Variations in the column temperature were also investigated from 20–45^{o}C. Column temp 25^{o}C was chosen as lower temperature resulted in asymmetric peaks, higher temperature resulted in slight decrease in run time, so ambient is more applicable even in absence of column compartment

Finally simultaneous determination of alogliptin benzoate and pioglitazone HCl was carried out using 80% methanol, 20% phosphate buffer pH 3 with a flow rate of 1.00 mL min^{-1}. Under this condition, both drugs were well separated with retention times 2.31 min for alogliptin and 4.92 min for pioglitazone. The above method is suitable for routine pharmaceutical applications involving the analysis of alogliptin benzoate and pioglitazone HCl. As retention time of each analyte was very reproducible with relative standard deviations less than 0.1 and a very good resolution of both drugs in short runtime around six minutes, final chromatogram of binary mixture is shown in Figure **4.**

Transfer of the proposed HPLC method for UPLC determination of Alogliptin and pioglitazone:
The proposed method was modified for UPLC determination of alogliptin benzoate and pioglitazone HCl using RSLC Method Transfer Tool 2.3 software.

Using Syncronis®C18 column (50x 2.1 mm i.d) and particle size: 1.7 µm the flow rate was 0.4 mLmin^{-1} and injection volume was 1 µL, the two drugs were separated with retention time 0.33, 0.76 min and very good resolution Figure 5

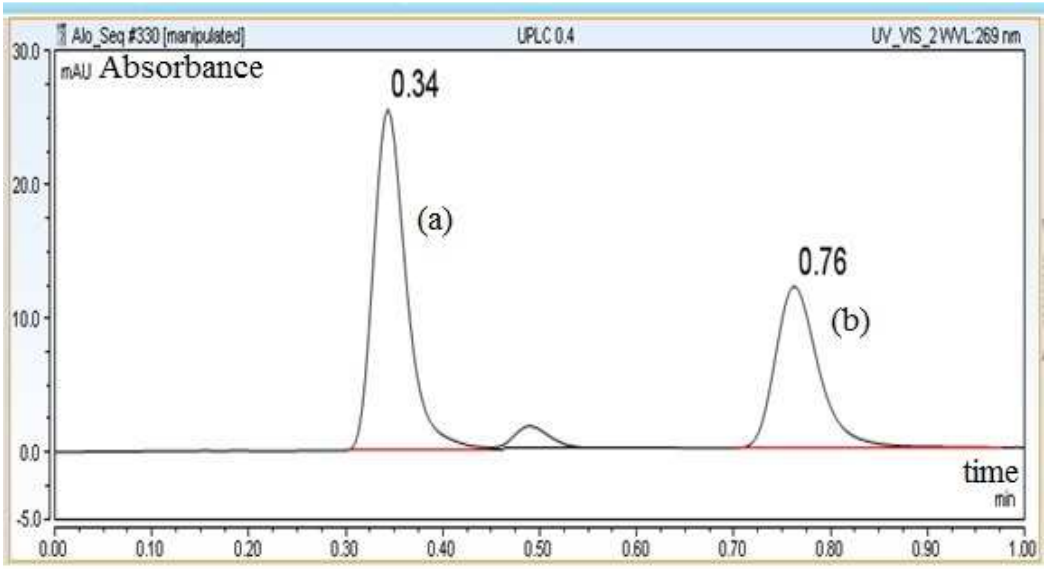

Fig. 5: Chromatogram of alogliptin benzoate 50 µg mL^{-1} (a) and pioglitazone HCl 50 µg mL^{-1} (b) by the proposed UPLC method, mobile phase consisting of methanol: phosphate buffer in ratio of 80:20 (v/v) ; pH= 3 at flow rate = 0.4 ml/min, temp.= 25°C and inj. vol.= 1 µl

Chromatograhic conditions for HPLC and UPLC methods are summarized in (Table 1)

As the drugs were separated within less than one minute, the developed method saves cost due to consumption of small volumes of solvent, so it can be effectively applied for routine analysis in research institutes, quality control department in industries, approved testing laboratories, Bio-pharmaceutics, Bio-equivalence studies and in clinical pharmacokinetic studies.

Table 1: Chromatograhic conditions for HPLC and UPLC methods

Stationary Phase	Inertsil ODS-3® C_{18} 5µm column (4.6 mm i.d.× 250 mm)	Syncronis®C_{18}1.7 µm column (50x 2.1 mm i.d)
Mobile Phase	Methanol : phosphate buffer pH 3 (80:20% v/v)	Methanol : phosphate buffer pH 3 (80:20% v/v)
Flow Rate	1.0 mL min^{-1}	0.4 mL min^{-1}
Detection Wavelength	269 nm	269 nm
Injection Volume	20 µL	1 µL
Column Temperature	25 ± 1 °C	25 ± 1 °C

Method Validation:
The method was validated according to ICH guidelines[21] The following validation characteristics were addressed:

Linearity and range:
A calibration curve was obtained for alogliptin benzoate using eight data points and triplicate injection of standard at each concentration level in the linearity range (5-100 µg mL^{-1}) and for pioglitazone HCl using six data points and triplicate injection of standard at each concentration level in the linearity range (5-100µg mL^{-1})

Mean peak areas were plotted versus the corresponding concentration of alogliptin benzoate and pioglitazone HCl, The results shows an excellent linearity between peak area and concentration for both alogliptin benzoate and pioglitazone HCl. The high values of the correlation coefficients with negligible intercepts indicate the good linearity of calibration graphs.

The statistical parameters for the calibration curves are summarized in Table 2

Table.2: statistical parameters for the calibration curves of alogliptin benzoate and pioglitazone HCl using the proposed HPLC method

Drug / Parameters	Alogliptin benzoate	PioglitazoneHCl
Linearity range	5 – 100 µg mL^{-1}	5 – 100 µg mL^{-1}
slope	0.3474	0.2686
SE of slope	0.0021	0.0019
Intercept	0.2279	-0.0377
SE of Intercept	0.1090	0.105625
Correlation coefficient (r)	0.9998	0.9998
SE of estimation	0.1928	0.162689
LOD	170 ng mL^{-1}	500 ng mL^{-1}
LOQ	215 ng mL^{-1}	650 ng mL^{-1}

Detection and quantitation limits:
Detection (LOD) and quantitation limits (LOQ) were calculated from the following equations:
LOD = 3.3* σ/S
LOQ = 10* σ/S

Where S is the value of the calculated slope and σ is the standard deviation of responses for triplicate blank injections, LOD and LOQ were calculated theoretically and were found to be 170 and 500 ng mL^{-1} respectively for alogliptin, 215 and 650 ng mL^{-1} respectively for pioglitazone.

Accuracy:
The accuracy of the proposed method was evaluated by analyzing three different concentrations of alogliptin benzoate and pioglitazone HCl working standard solutions within the linearity range. Accuracy was expressed as the recovery% for alogliptin benzoate and pioglitazone HCl, (Table 3) illustrates the method accuracy for alogliptin benzoate and pioglitazone HCl with satisfactory recovery%.

Table.3: Recovery data of alogliptin benzoate and pioglitazone HCl using the proposed HPLC method

Alogliptin benzoate			PioglitazoneHCl		
Claimed conc. (µg mL^{-1})	Obtained conc. (µg mL^{-1})	Recovery* (%)	Claimed conc. (µg mL^{-1})	Obtained conc. (µg mL^{-1})	Recovery* (%)
12	11.894	99.124	15	15.2936	101.958
25	24.62	99.498	30	30.4167	101.389
80	79.35	99.498	45	45.35	100.783
Mean % recovery	99.18		101.3766		
SD	0.24		0.48		
RSD%	0.39		0.47		

**mean of three determinations*

Precision:

Repeatability (Intra-day):

The intra-day precision for the determination of alogliptin benzoate and pioglitazone HCl was carried out by analyzing alogliptin benzoate and pioglitazone HCl sample solutions at three concentrations using the proposed method, within the same laboratory, using the same analyst, with the same equipment, in the same day. Repeatability was assessed using three determinations each injected triple. RSD % of the results obtained was calculated. (Table 4)

Intermediate precision (Inter-day):

The inter-day precision for the determination of alogliptin benzoate and pioglitazone HCl was carried out by analyzing alogliptin benzoate and pioglitazone HCl sample solutions at three concentrations using the proposed method, within the same laboratory, using the same analyst, with the same equipment but on three consecutive days, each determined triple (n=9). Precision was expressed as the percentage relative standard deviation (RSD %) for alogliptin benzoate and pioglitazone HCl. Data indicated a good agreement among the individual test results. The criterion for intra-day and interday precisions is a RSD less than 2%. (Table 4)

Table.4: Intraday and inter day precision experiments for determination of alogliptin benzoate and pioglitazone HCl using the proposed HPLC method

Intraday Precision							
Alogliptin benzoate				Pioglitazone HCl			
Claimed conc. (µg mL^{-1})	Conc found (µg mL^{-1})	Mean conc found (µg mL^{-1})	%RSD	Claimed conc. (µg mL^{-1})	Conc found (µg mL^{-1})	Mean conc found (µg mL^{-1})	%RSD
12	11.76	11.82	0.41	15	15.23	15.29	0.31
	11.82				15.33		
	11.88				15.32		
25	24.25	24.62	1.07	30	30.60	30.42	0.64
	24.79				30.50		
	24.83				30.15		
80	79.09	79.35	0.23	45	45.58	45.35	0.4
	79.46				45.35		
	79.49				45.13		
Inter Day Precision							
12	11.82	11.96	1.1	15	15.29	15.303	0.34
	11.92				15.37		
	12.13				15.25		
25	24.91	24.84	0.6	30	30.56	30.665	0.6
	24.98				30.77		
	24.62				31.01		
80	80.27	79.92	0.51	45	47.1	46.22	1.54
	80.13				46.21		
	79.35				45.35		

Robustness:

The robustness of the method was determined by deliberate changes in the method like alteration in pH of the mobile phase by ±0.2, percentage organic content by ±2%, changes in the wavelength by ± 2 n.m and column temperature by ± 5°C.The robustness of the method shows that there is no marked changes in the chromatographic parameters (Table 5)

Table.5: Robustness results for the HPLC method

Parameters	Modification	Alogliptin benzoate		Pioglitazone HCl	
		S.D of retention time (R_t)	S.D of peak area	S.D of retention time (R_t)	S.D of peak area
Mobile phase ratio	82:18	0.03	0.11	0.43	0.06
	80:20				
	78:22				
pH	2.8	0.01	0.18	0.25	0.3
	3				
	3.2				
Wavelength (nm)	267	0.0	0.8	0.0	0.3
	269				
	271				
Temperature (°C)	20	0.02	0.42	0.6	0.21
	25				
	30				

Specificity:

Specificity of the method was shown by quantifying the analyte of interest in the presence of matrix and other components. Commonly used excipients (microcrystalline cellulose, croscarmellose and magnesium stearate) were used in laboratory prepared mixture. Also excess benzoate from alogliptin benzoate was completely separated from peaks of two drugs.

The method specificity was determined by % recovery obtained from analysis of laboratory prepared mixtures. The mean % recovery obtained by the proposed method was found to be 99.75% and 100.23% for alogliptin and pioglitazone, respectively. This indicates that there are no interferences from the excipients.

System Suitability:

A binary solution of 50 µg $mL^{-1}$of pioglitazone and 50 µg mL^{-1} of alogliptin was prepared and was injected using HPLC and UPLC systems, then the system suitability parameters, retention time, asymmetric factor, resolution between alogliptin and pioglitazone peaks, and number of theoretical plates were calculated and compared to that obtained from UPLC system. The results are summarized in (Table 6).

Table.6: Results of system suitability tests for HPLC and UPLC systems

	HPLC system		**UPLC system**	
Parameters	alogliptin benzoate	pioglitazone HCl	alogliptin benzoate	pioglitazone HCl
Retention time (R_t) (min)	2.3	4.9	0.33	0.76
Retention factor K`	1.3	3.9	1.29	4.09
Resolution (R_s)	12.5		6.17	
Theoretical plates (N)	4388	4999	538	1514
Asymmetry factor	1.02	0.92	1.3	1.3

CONCLUSION

A simple, rapid and accurate RP-HPLC method was developed for the simultaneous determination of alogliptin benzoate and pioglitazone HCl in pharmaceutical formulations. The analytical conditions and the solvent system developed provided good resolution for alogliptin benzoate and pioglitazone HCl within a short run time. The HPLC method was validated and demonstrated good linearity, precision, accuracy and specificity. Thus, the developed HPLC method can be utilized for routine analysis.

The method is successfully transferred to UPLC system, the two drugs are well resolved in very short time not exceed one minute which save time and cost**.**

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