International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Method Development and Validation Studies of Anti-Diabetic Drug Lobeglitazone from Pharmaceutical Formulation and Human Plasma by Hplc-Dad

Nutan Pustode[1], Dr. Anup Barsagade[2]

[1]Student, Maharashtra Institute of Pharmacy Betala, Bramhapuri, Gondwana University, Gadchiroli
[2]Associate Professor, Maharashtra Institute of Pharmacy Betala,Bramhapuri, Gondwana University, Gadchiroli

ABSTRACT
An optimized isocratic method using HPLC was performed and validation for lobeglitazone tablet dosage form.The chromatographic separation was achieved on a Shimadzu SCL-10AVP in built with binary pump (LC-10ATVP), UV detector (SPD-10AVP),Rheodyne 20ul loop capacity manual injector (P/N 77251) was used throughtout the analysis.
The retention time for Lobeglitazone was determined to be 2.157min. The HPLC method was successfully developed,validated as per ICH guidelines. Theproposed method was simple, precise,sensitive,rapid,robust for the estimation of Lobeglitazone.

KEYWORDS: Lobeglitazone, Method development, Validation, HPLC-DAD

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is characterized by abnormalities of glucose and lipid homeostasis, which exhibit the micro- and macrovascular complications. Clinical evidence indicates that maintaining glycemic control and reducing postprandial glucose excursions can lower the risk of diabetic complications, e.g. reduce the risk of myocardial infarction, renal disease and retinopathy [1,2]. Despite the availability of multiple classes and combinations of antidiabetic agents, the clinical management of T2DM remains challenging, with the majority of patients failing to achieve and maintain target glycemic levels in practice [3,7].

1.1 Lobeglitazone overview

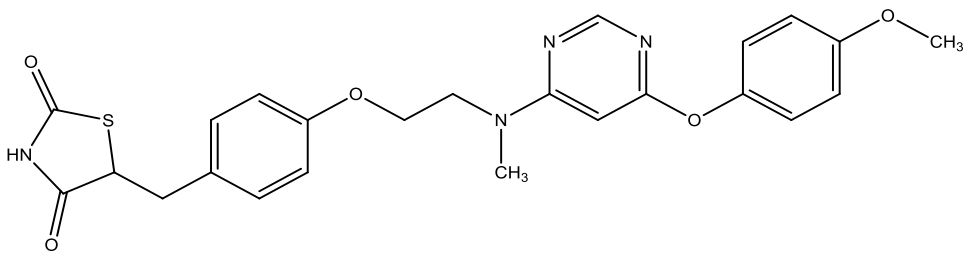

Figure.1.1Molecular structure of lobeglitazone

Chemistry Lobeglitazone is a pharmacophore which has a 2,4-thiazolidinedione group with an ethoxy-benzyl N-methylamino group bound to this as a connecting link. Its structural formula is C24H24N4O5S, and the chemical name is 5-[4-(2-{[6-(4-Methoxy-phenoxy)-pyrimidin-4-yl]-methyl-amino}-ethoxy)- benzyl]-thiazolidine-2,4-dione hydrosulphuric acid. The cocrystal structure of lobeglitazone with PPARγ is shown in Supplementary Fig. 1. Lobeglitazone was based on modification of the rosiglitazone structure to introduce a p-methoxy phenoxy group at the 4-position of the pyrimidine moiety [14].The p-methoxy phenoxy group enables extended interaction with the hydrophobic pocket, and this could also affect the cyclin dependent kinase 5-mediated phosphorylation of PPARγ at Ser245, which changes the expression of genes such as adiponectin and adipsin that are associated with insulin sensitivity without general transcriptional activity of PPARγ [15].

1.2 HPLC Introduction

High Performance Liquid Chromatography (HPLC) was derived from the classical column andchromatography, is one of the most important tools of analytical chemistry today. The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behaviour of different components, elution at different time takes place. High Performance Liquid Chromatography (HPLC) is more versatile than gas chromatography (GC) since, it is not limited to volatile and thermally stable samples, and the choice of mobile and stationary phases is wider [16, 17].

1.3 Method development

Analytical method development and validation studies play an important role in discovery, development and manufacture of pharmaceuticals. These methods used to ensure the identity, purity, potency, & performance of the pharmaceutical drug products. There are many factors to consider when developing methods. The initially collect the information about the analyte's physicochemical properties (pKa, log P, solubility) and determining which mode of detection would be suitable for analysis (i.e., suitable wavelength in case of UV detection) [18]. Steps involve in method development are:

1. Understand the physicochemical properties of drug molecule.
2. Set up HPLC conditions.
3. Preparation of sample solution for method development.
4. Method optimization.
5. Validation of method.

1.4 Buffer selection in HPLC

Choice of buffer is typically governed by the desired pH. The typical pH range for reversed phase on silica-based packing is pH 2 to 8. It is important that the buffer has a pKa close to the desired pH since buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value [21-26].

1.5. Column selection in HPLC

The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Generally, modern reverse phase HPLC columns are made by packing the column housing with spherical silica gel beads which are coated with the hydrophobic stationary phase. The stationary phase is introduced to the matrix by reacted a chloro-silane with the hydroxyl groups present on the silica gel surface. In general, the nature of stationary phase has the greatest effect on capacity factor, selectivity, efficiency and elution. There are several types of

matrices for support of the stationary phase, including silica, polymers, and alumina. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily derivatised, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One shortcoming of a silica solid support is that it will dissolve above pH 7.[27-28]

1.6 Mobile phase selection in HPLC

The mobile phase effects resolution, selectivity and efficiency. In reverse phase chromatography, the mobile phase consists of an aqueous buffer and a non-UV active water miscible organic solvent. The effect of the organic and aqueous phase and the proportions in which they are mixed will affect the analysis of the drug molecule. Selection of the mobile-phase and gradient conditions is dependent on the ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixture respectively. The aqueous buffer serves several purposes. At low pH, the mobile phase protonates free silanols on the column and reduces peak tailing. At sufficiently low pH basic analytes are protonated; when ionized the analyte will elute more quickly but with improved peak shape. Acidic analytes in buffers of sufficiently low pH will remain un-charged, increasing retention [34, 35].

1.7 Changing Gradient in HPLC

Gradient elution is employed for complex multicomponent samples since it may not be possible to get all components eluted between k (retention factor) 1 and 10 using a single solvent strength under isocratic conditions. This leads to the general elution problem where no one set of conditions is effective in eluting all components from a column in a reasonable time period while still attaining resolution of each component [29].

1.8 Preparation of Sample Solutions for Method Development

The drug substance being analyzed should be stable in solution (diluent). During initial method development, preparations of the solutions in amber flasks should be performed until it is determined that the active component is stable at room temperature and does not degrade under normal laboratory conditions.

The sample solution should be filtered; the use of a 0.22 or 0.45 µm pore-size filter is generally recommended for removal of particulates. Filtration is a preventive maintenance tool for HPLC analyses [26]. Sample preparation is a critical step of method development that the analyst must investigate. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants/insoluble components without leaching undesirable artifacts (i.e., extractables) into the filtrate. If any additional peaks are observed in the filtered samples, then the diluent must be filtered to determine if a leachable component is coming from the syringe filter housing/filter.

1.9 Method optimization in HPLC

The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. Stability indicating assay experimental conditions will be achieved through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, Injection volume and diluents solvent type. Validation of method Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures [35]. All

analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH guidelines.

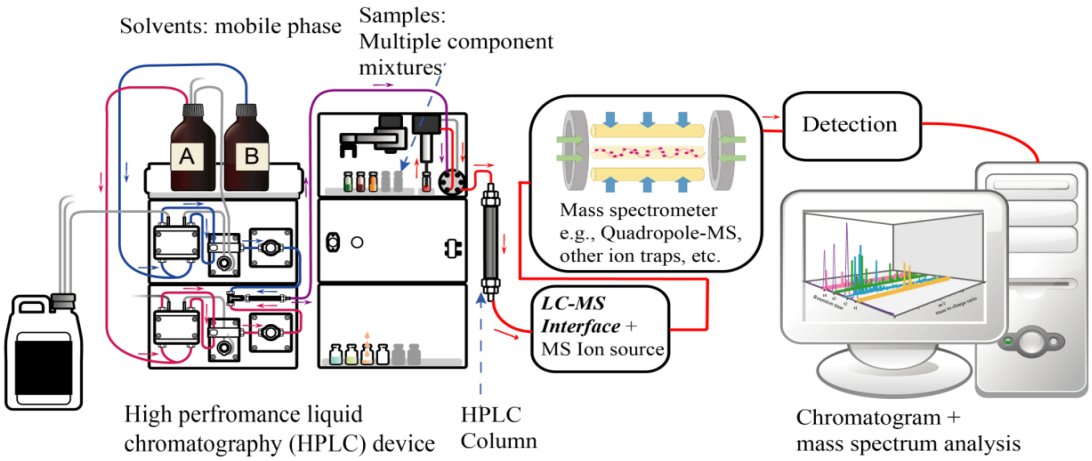

Figure 1.2; Schematic representation of HPLC-MS instrument

2.1 Method Validation studies

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures. All analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH guidelines. Components of method validation studies which have to be evaluated during methods validation are as follows [36, 37, 65, 67].

A. System Suitability test
B. Repeatability
C. Precision studies (Intraday and Interday/intermediate)
D. Linearity/Calibration studies
E. Detection limit (LOD)
F. Quantitation limit (LOQ)
G. Robustness
H. Accuracy/Drug recovery studies
I. Forced degradation/Stability indicating studies
J. Specificity

2.2 Types of HPLC Techniques

Depending on the substrate used i.e. stationary phase used, the HPLC is divided into following types;

1. Normal Phase HPLC- In this method the separation is based on polarity. The stationary phase is polar, mostly silica is used and the non-polar phase used is n-hexane, chloroform, dichloromethane, diethyl ether, ethyl acetate, dioxane, iso-propyl-alcohol (IPA). The polar samples are retained on column. The examples are cyano, amino, diol, silica and hypercarb (100% porous graphite particles) [54,55].
2. Reverse Phase HPLC- It is reverse to normal phase HPLC. The mobile phase is polar and the stationary phase is non-polar or hydrophobic. The more is the non-polar nature the more it will be

retained. For instance, C_{18}, octadecyl silane (ODS), base deactivated silica (BDS), C_8, C_4 etc [56].

3. Size-exclusion HPLC- The column will be incorporating with precisely controlled substrate molecules. Based on the difference in molecular sizes the separation of constituents will occur [57, 58].
4. Ion-exchange HPLC- The stationary phase is having ionic charged surface opposite to the sample charge. The mobile phase used is aqueous buffer which will control pH and ionic strength like Strong cation exchanger (SCX), weak cation exchanger (WCX), strong anion exchanger (SAX) and weak anion exchanger (WAX) [59, 60].
5. Mix mode chromatography (MMC). It is a fast-growing area in recent years, It involved new generation of mixed-mode stationary phases which facilitates multimode drug-stationary phase interactions. MMC has superior applications towards the separation of compounds that are normally not retained or not well resolved by reversed-phase LC techniques, particularly for polar and charged drug molecules. Owing to the multiple retention modes; a MMC column can offer additional dimension towards the separation by adjusting the mobile phase conditions. Mixed-mode media is also an effective way to clean up complex sample matrices for purification purposes or for sensitive detection of trace amounts of analytes. Mixed-mode stationary phases and separation mechanisms have recent advances in pharmaceutical and biopharmaceutical separations including the analysis, isolation purification and characterisation of counterions, small molecule drugs, impurities, formulation excipients, peptides and proteins [61, 64].

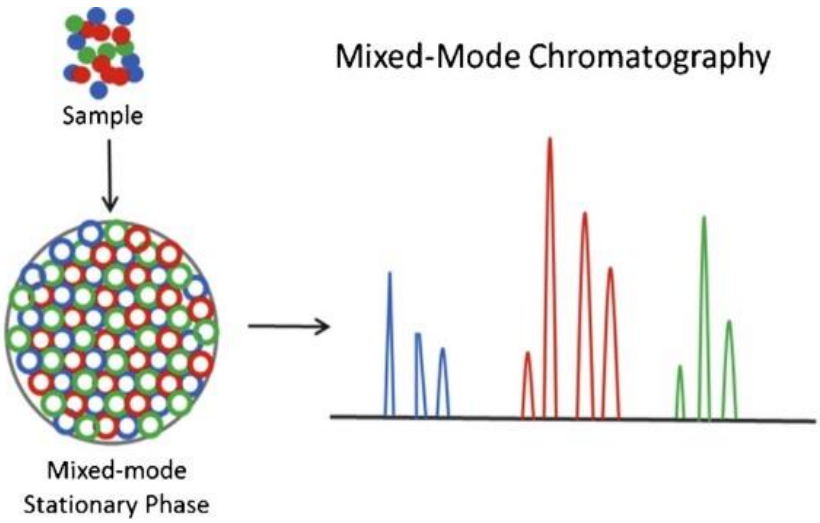

Figure 1.2; Mix mode chromatography (MMC) separation patter

6. Chiral Chromatography (CC). The method for resolving enantiomers we discussed above illustrates the general principle that the key to the separation of enantiomers is the formation of diastereomers. This can be achieved by chiral chromatography, a process in which the column itself contains a chiral ligand. Chiral chromatography depends upon a partition between compounds in the moving phase—the solution of enantiomers passing through the column—and a stationary phase, the chiral column material itself. When a solution of enantiomers passes through the column, the enantiomers bind to the column with different affinities because an (*R*-ligand/*R*-enantiomer) interaction differs from an (*R*-ligand/*S*-enantiomer) interaction (Ali, et al, 2009).

Cyclodextrins bonded to silica gel can also permit the separation of optical isomers, via the formation of inclusion complexes within the cyclodextrins cavity. Importantly, *'d'* and *l* enantiomers of various compounds attributed to inclusion complexes with varying strengths within the cavity and retained to varying degrees, enabling their separation. Enantiomers of compounds like tryptophan, ibuprofen, atenolol and norgestol can be separated with cyclodextrin-functionalized silica stationary phases.

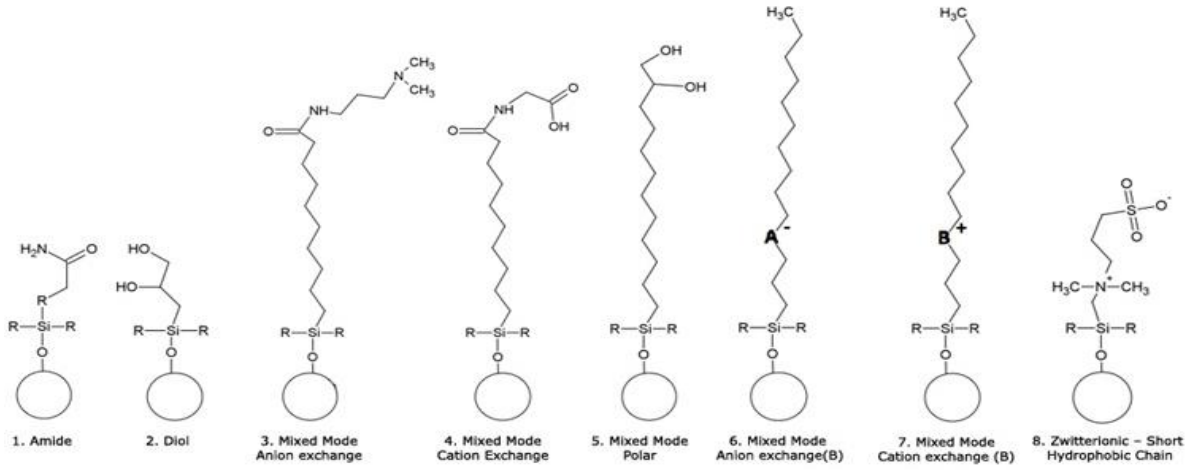

Figure 1.3; various stationary phases for MMC and HILIC chromatography

7. HILIC Chromatography. Typically, HILIC is used when retention times in reversed phase mode are insufficient, and this typically involves more polar or ionisable analytes (i.e. where log P values are below 0). HILIC chromatography can be used as a replacement for Normal Phase chromatography and is particularly useful when using electrospray mass spectrometry as the analyte can be ionised in solution (ensure good ESI efficiency) whilst still showing good retention and the high organic content of the mobile phase ensures efficient droplet desolvation in the API interface of the LCMS (Gama et al, 2012; Nováková et al, 2014; Kahsay, et al, 2014).

The exact mechanism of HILIC chromatography is still subject to a certain amount of speculation, however most experts agree that the bulk mechanism involves polar analyte partitioning into and out of a layer of water which is adsorbed (associated) onto the surface of the polar stationary phase. Although it is also widely understood that as well as this liquid-liquid partitioning behaviour, dipole–dipole and electrostatic interactions are also involved, especially where the eluent pH is adjusted so that the stationary phase surface is charged – predominantly via the anionic surface silanol species at pH less than 5. Mobile phases for HILIC chromatography contain a high degree of organic solvent; specifically, acetonitrile, and a typical gradient in HILIC would involve altering the aqueous composition between 5 and 30%.

8. HILIC Chromatography. Typically, HILIC is used when retention times in reversed phase mode are insufficient, and this typically involves more polar or ionisable analytes (i.e. where log P values are below 0). HILIC chromatography can be used as a replacement for Normal Phase chromatography and is particularly useful when using electrospray mass spectrometry as the analyte can be ionised in solution (ensure good ESI efficiency) whilst still showing good retention and the high organic content of the mobile phase ensures efficient droplet desolvation in the API interface of the LCMS (Gama et al, 2012; Nováková et al, 2014; Kahsay, et al, 2014).

The exact mechanism of HILIC chromatography is still subject to a certain amount of speculation, however most experts agree that the bulk mechanism involves polar analyte partitioning into and out of a layer of water which is adsorbed (associated) onto the surface of the polar stationary phase. Although it is also widely understood that as well as this liquid-liquid partitioning behaviour, dipole–dipole and electrostatic interactions are also involved, especially where the eluent pH is adjusted so that the stationary phase surface is charged – predominantly via the anionic surface silanol species at pH less than 5. Mobile phases for HILIC chromatography contain a high degree of organic solvent; specifically, acetonitrile, and a typical gradient in HILIC would involve altering the aqueous composition between 5 and 30%.

3. Experimental

3.1 Instrumentation

The high-performance liquid chromatography (HPLC) of Shimadzu SCL-10A_{VP} inbuilt with binary pump (LC-10AT_{VP}), UV detector (SPD-10A_{VP}), Rheodyne 20µl loop capacity manual injector (P/N 77251) was used throughout the analysis. The LC-Solution software was used to interpret the HPLC reports. Zodiac-100 C8 (5µm; 150 x 4.6 mm ID.) column was purchased from zodiac life sciences. (Hyderabad, India) was used throughout the analysis. Digital weighing balance (ME-204) purchased from Mettler-Toledo (USA), ultra-sonicator Labman® purchased from UltraChrom Ltd, India. Digital pH meter from Mettler-Toledo was purchased from (Mumbai-India). 50 µ micro-syringe was purchased from Hamilton USA. 0.20µ and 0.45µ nylon membrane filters were purchased from Phenomenex® Mumbai, India.

3.2. Reagents and reference samples

The reference standard; lobeglitazone was obtained as a gift sample from Yarrow chem Ltd. Ammonium acetate was purchased from Merck Ltd. (Mumbai-India) HPLC grade acetonitrile, methanol and HPLC grade water were purchased from Merck (Mumbai, India). 0.20µ and 0.45µ nylon membrane filters were used and purchased from UltraChrom Innovatives Pvt. Ltd. (India). All other chemicals and reagents were used of HPLC grade.

3.3 Selection of solvent and wavelength

Lobeglitazone is soluble in acetonitrile; partially soluble in water and methanol. Hence, standard stock solution of lobeglitazone was prepared in acetonitrile-methanol-water (60:30:10% v/v). Lobeglitazone shows maximum UV absorbance (λ_{max}) at 210 and 250 nm wavelength; hence, both wavelengths were selected throughout the HPLC Analysis.

3.4 Preparation of standard solution

Exactly, 7 mg of lobeglitazone standard was weighed and dissolved in 7 ml of acetonitrile-methanol-water (6:3:1,v/v) to get 1000 ppm (1000 µg/ml) solution. It was sonicated for 2-5 minutes and then as per the need, serial dilutions were made to get the final concentration 100 ppm for the determination of repeatability, precision and robustness.

3.5 Chromatographic conditions

20 µl of freshly prepared stock solution of lobeglitazone was injected into the Zodiac-100 C8 (5µm; 150 x 4.6 mm ID.) column and eluted using the mobile phase as solvent A; 15mM ammonium acetate (AA) and solvent B; acetonitrile-methanol (90:10, v/v) at 1.0 ml/mins flow rate for 10 mins. Separation was carried out at room temperature and monitored at 210 and 250 nm wavelength.

3.6 System suitability studies

Freshly prepared stock solution of lobeglitazone (100 ppm) was injected 6 times to determine the closeness of results achieved for relative standard deviation (RSD) in percentage; The calculated values should always less than 2%. Moreover, other system suitability parameters including, retention time, capacity factor (k'), theoretical plates (N), tailing factor/peak asymmetry (As) and separation factor (α) were tested and evaluated.

3.7. Sample preparation for drug accuracy studies

Exactly 5 tablets of LOBG® 0.5 manufactured by Glenmark pharmaceuticals Ltd. consisting 0.5 mg of lobeglitazone was weighed and the average weight was calculated. They were mixed and crushed to fine powder into the mortar and pestle. An accurately weighed amount of the finely powdered equivalent to 7 mg was dissolved in 7 ml of acetonitrile-methanol-water (6:3:1, v/v). It was then ultrasonicated for 5-10 mins and then filtered through 0.45µ nylon filter. Furthermore, serial dilutions were made in accordance to get the final concentration 100 ppm of lobeglitazone. The solution was then sonicated and analysed as per the chromatographic condition mentioned in section 5.x.

3.8. Sample preparation for Linearity/Calibration studies

1000 ppm (1000 µg/ml) of standard stock solution of lobeglitazone was made. Subsequently, serial dilutions of five different concentrations ranging between 3.12–50 ppm were made, ultrasonicated and then analysed as per the chromatographic condition in section 5.x. Furthermore, the calibration curve (linearity graph) was plotted by calculating the peak area against known concentration to determine regression equation, regression coefficient (R^2), limit of quantification (LOQ) and limit of detection (LOD).

3.9. Precision studies of the proposed method

Freshly prepared stock solution of lobeglitazone (100 ppm) was analyzed thrice within the same day (intraday precision) and three successive days (intermediate precision) were tested and evaluated. Furthermore, their mean, standard deviation and relative standard deviation (RSD) were calculated which should be less than 2% as per the ICH guidelines.

3.10 Robustness for the chromatographic method

The flow rate of the mobile phase was changed by 1.00 ±1 decimal from 1 mL/min to 1.1 mL/min and to 0.9 mL/min to evaluate the effect of the flow rate on separation pattern of lobeglitazone. Similarly, small but deliberate variation of organic modifier as solvent B (50%) was changed by ±2% in its isocratic elution mode to investigate the effects on retention time (t_R), capacity factor (k') and theoretical plates (N). Finally, the effect of wavelength was monitored by making deliberate variation from 250 ±2 nm to 252 and 248 nm and the differences in retention time (t_R), capacity factor (k'), resolution (Rs) and theoretical plates (N) were tested and evaluated. Robustness study was performed as per the procedure mentioned under the experimental section 5.x

3.11 Plasma sample preparation and plasma drug recovery

Approximately, 4 ml of blood from selected non-diabetic healthy volunteers was collected into the EDTA tube. The withdrawn blood was spiked with known concentration of 100 µg/ml of each linagliptin and empagliflozin and then centrifuged at 4000 RPM for 10 mins. Plasma was removed; deproteinized with acetonitrile (100%) and then recentrifuged at 4000 RPM for 10 mins. The supernatant was removed, filtered through 0.20µ nylon filter and injected to HPLC-UV as per the chromatographic condition mentioned in section 5.4.

4. Results and discussion

Application of new proposed RP-HPLC method

After comprehensive literature survey, there are very few HPLC analysis of lobeglitazone have been reported. Moreover, the reported article has used the C18 based RP-HPLC for the quantification of lobeglitazone. However, since the lobeglitazone is highly polar diabetic drug so it hardly retains in C18 based RP-HPLC. Moreover, it is recently approved in the indian market so very few studies have been done on this particular drug.

Therefore, owing to it polar nature, alternative technique like Mix-Mode chromatography have been attempted. Most particularly, Acclaimed mix mode HILIC-1, 5µ column; 150 x 4.6 mm exhibited the good peak symmetry and height. Moreover, it improved peak sensitivity at 230 nm UV detection since as shown in figure 6.1 its λmax value is almost 230 nm; Importantly, along with method development and validation studies its force degradation studies have also been performed.

4.1 UV spectral analysis of lobeglitazone

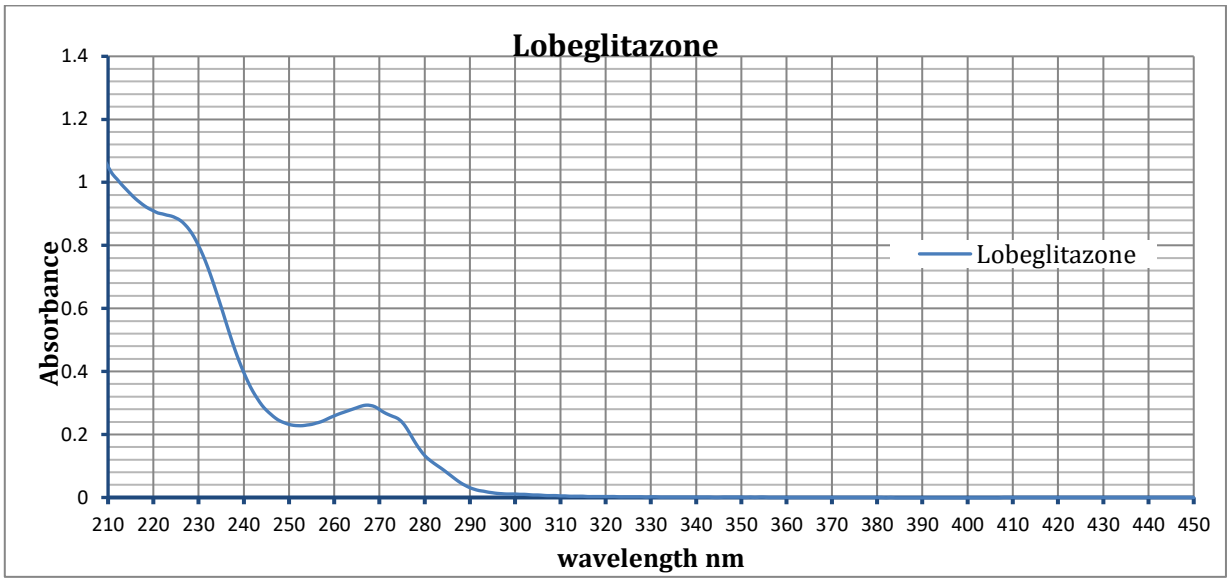

Figure 4.1; UV spectra of Lobeglitazone

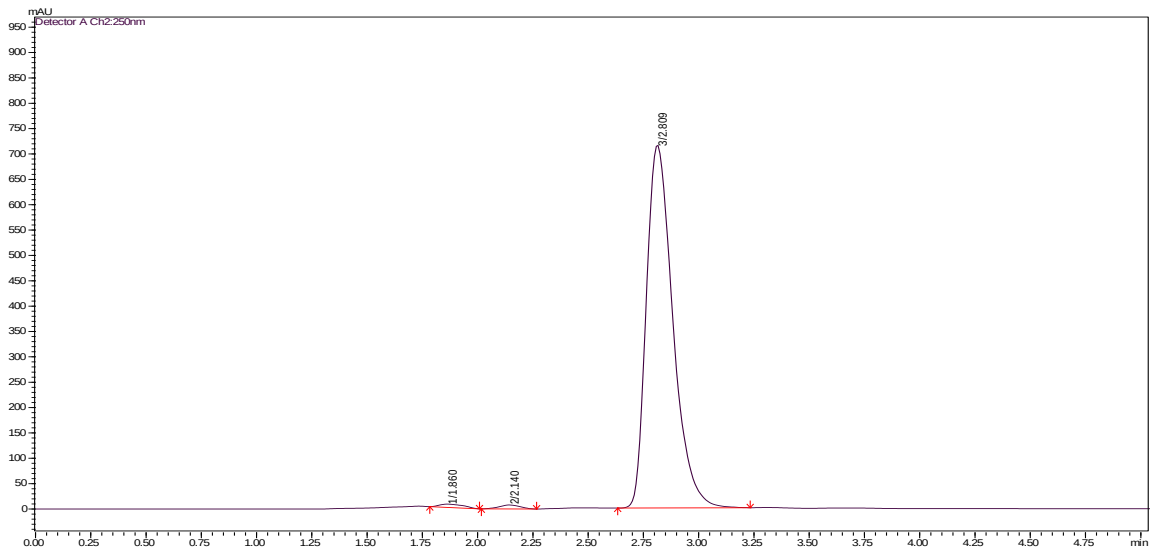

Figure 4.2; HPLC graph of lobeglitazone

Table 1; trial-2 data of lobeglitazone

Peak #	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.86	49830	6452	0.8204	1270.458	--	0	1.494	0
2	2.14	52200	7894	0.8595	2013.27	1.404	0.151	0.998	0
3	2.809	5971474	714882	98.3201	2481.415	3.215	0.511	1.467	3.387

Chromatographic Parameters

1. Analytes: lobeglitazone (100ppm)
2. Column: Zodiac C18; 5µ, 150 X 4.6 mm. ID.
3. Mobile Phase: 15 mM KH_2PO_4- acetonitrile; 10:80 v/v
4. Flow rate: 1 ml/min
5. Elution mode: Isocratic elution mode
6. Wavelength selected: 230 nm
7. Temperature: Room temperature
8. Run time: 20 mins
9. Retention time: lobeglitazone (2.22 min)

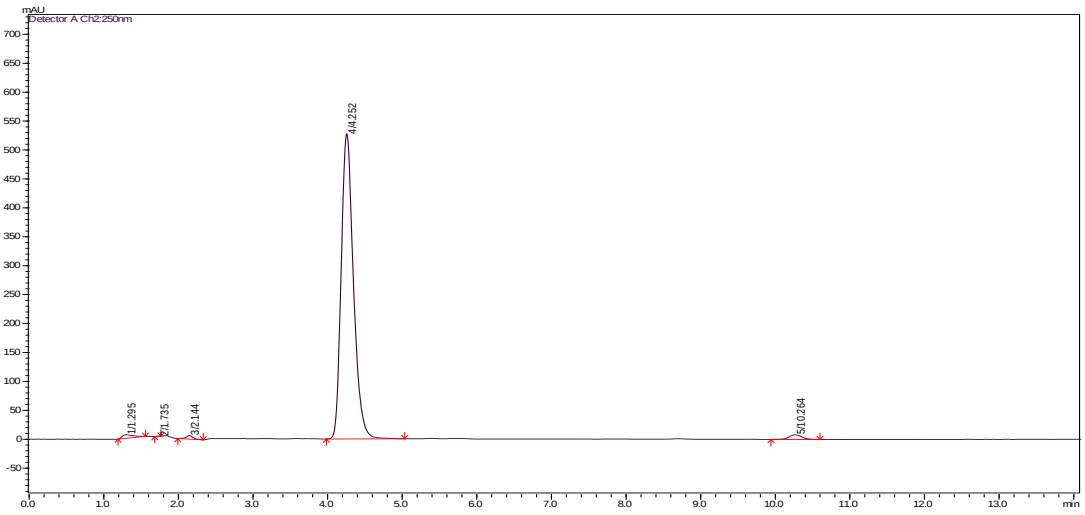

Figure 4.3; developed HPLC method of lobeglitazone.

Table 2; method development data of lobeglitazone

Peak #	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.295	65988	6259	1.0602	312.727	--	0	1.864	0
2	1.735	1097	346	0.0176	7841.496	2.371	0.34	0.858	0
3	2.144	40402	6253	0.6491	2345.37	3.209	0.65	1.041	1.932

					7		6		
4	4.252	6021419	527718	96.7408	3154.818	8.783	2.284	1.302	3.48
5	10.264	95371	7903	1.5322	16528.78	19.326	6.928	1.12	3.033

Chromatographic Parameters

1. Analytes: lobeglitazone (100ppm)
2. Column: Zodiac C18; 5µ, 150 X 4.6 mm. ID.
3. Mobile Phase: 15 mM KH_2PO_4- acetonitrile; 10:80 v/v
4. Flow rate: 1 ml/min
5. Elution mode: Isocratic elution mode
6. Wavelength selected: 230 nm
7. Temperature: Room temperature
8. Run time: 20 mins
9. Retention time: lobeglitazone (2.22 min)

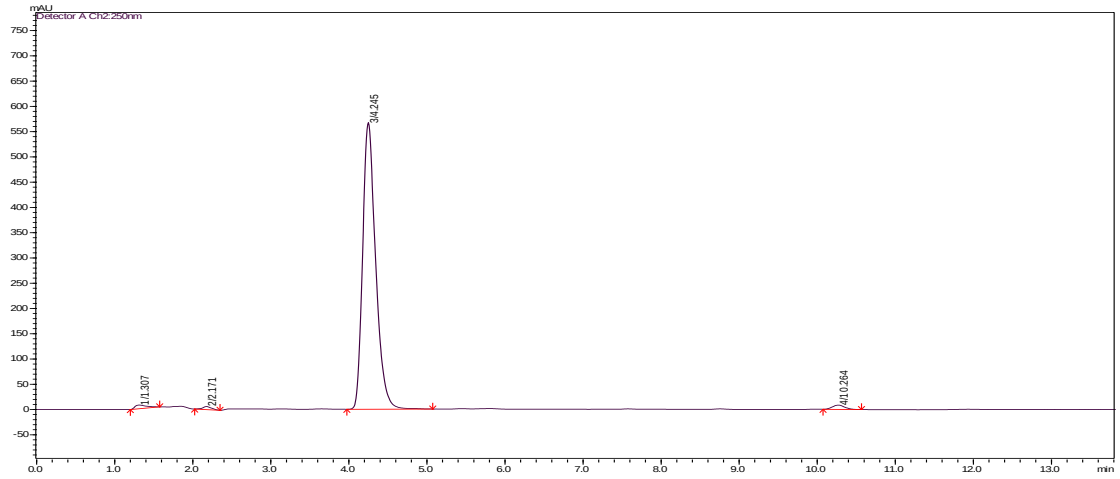

Figure 4.4 Repeatability studies of Lobeglitazone

Table 3; repeatability study of lobeglitazone

Peak #	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.307	76981	7271	1.1341	344.39	--	0	1.873	0
2	2.171	47473	6117	0.6994	1481.977	3.405	0.661	1.083	0
3	4.245	6570087	567075	96.7881	2981.307	7.729	2.247	1.353	3.4
4	10.264	93575	8427	1.3785	18985.32	19.77	6.851	1.202	3.049

Implementing the procedure mentioned under section,the freshly prepared stock solution of lobeglitazone of same concentrations (100 µg/mL), were evaluated for six injections within the same day. The % RSD was calculated and found it is less than 2%.

Table 4; Repeatability data of lobeglitazone

S. No.	**lobeglitazone**
	Peak Area; Conc. 100 ppm
1	8054477
2	8022523
3	8056201
4	8066521
5	8052996
Mean	8050543
STD. DEV.	16537.25
RSD (%)	0.20

4.2 System suitability tests for lobeglitazone

System suitability test included the theoretical plate (N), capacity factor (k'), resolution (R), separation factor (α), tailing factor (*T*), Mean±SD and RSD% which should always less than 2% for 6 repeatetive injections of same concentration. Table No. 6.2; displayed the system suitability studies for lobeglitazone.

Table5;System suitability dataof lobeglitazone

System suitability parameters	lobeglitazone (IMG)	Acceptable Values
Theoretical plates (*N*)	3874	> **2000**
Capacity Factor (*K'*)	2.766	> **1.5 - <10**
Resolution (*R*)	---	≥ **2**
Selectivity/Separation factor (α)	0.00	>***k'***
Asymmetry/Tailing factor (*T*)	1.03	> **2**
Retention time (t_R)	4.903 min.	>***k'***
Wavelength of Detection (nm)	230 nm	> **200 nm**
Repeatability (%RSD)	0.20	< **2**
Intra-Day Precision (%RSD)	0.20 – 0.84	< **2**
Inter-Day Precision (%RSD)	0.07 – 0.88	< **2**
Linearity range	15.65 – 100 µg/ml	**NA**
Regression equation	Y= 35958x – 43049	**NA**
SE of intercept (S_e)	92429.58	**NA**
SD of intercept (S_a)	41335.76	**NA**
Correlation Coefficient (r^2)	0.998	**NA**
LOQ (µg/mL)	3.79 µg/ml	**NA**
LOD (µg/mL)	11.49 µg/ml	**NA**

4.3 Precision studies for lobeglitazone

The precision (intraday and interday/intermediate) of HPLC method reflects its closeness to the agreement among the series of repetitive results, those derived after multiple sampling of the same homogenous mixture of selected drugs under the given conditions. As displayed in after calculating their relative standard deviation (RSD) in percentage, this developed method found to be significantly precise for selected drugs lobeglitazone. Moreover, the peak area of the studied drugs was also correlated with their selected concentration; where the % RSDs for lobeglitazone were <2%. The RSDs were observed well below 2% that reflects an acceptable precision with minimum variations of the proposed method.

4.3.1 Intraday precision study of Lobeglitazone

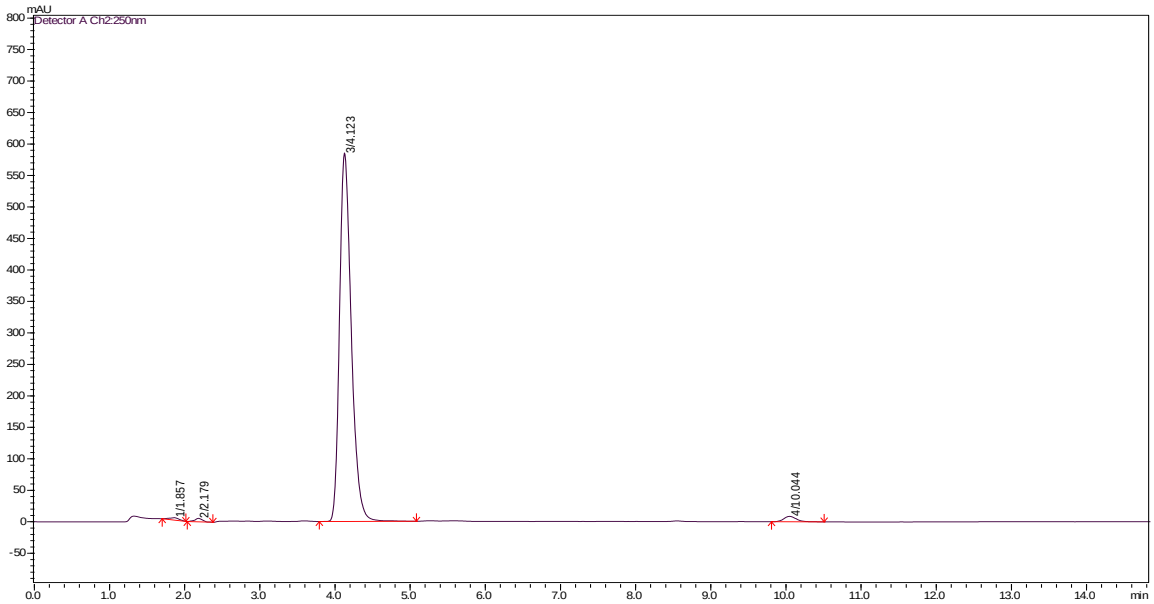

Figure 4.4; intraday precision study of lobeglitazone

Table 6; intraday precision study of lobeglitazone

Peak #	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.857	34389	3711	0.5255	797.719	--	0	0.996	0
2	2.179	38574	5519	0.5895	2008.816	1.408	0.173	0.971	0
3	4.123	6369545	585249	97.3388	3249.158	8.039	1.221	1.35	7.038
4	10.044	101176	8665	1.5462	16550.44	19.684	4.41	1.202	3.612

Implementing the procedure mentioned under section (5.5), the freshly prepared stock solution of LOBEGLITAZONE of three replicates of three same concentrations; 250ppm were tested and evaluated within the same day (intra-day precision). The %RSD was calculated and found less than 2%.

Table 7; Intraday Precision data of lobeglitazone

Drug Name: Lobeglitazone					
S. No.	Concentration (ppm)	Area	Average	Std. Deviation	%RSD
1	50 ppm	16900992	16661958	241832.99	1.45
	50 ppm	16417420			
	50 ppm	16667463			
2	50 ppm	16983506	17064761	70896.43	0.42
	50 ppm	17096753			
	50 ppm	17114023			
3	50 ppm	17296149	17227914	106229.46	0.62
	50 ppm	17105520			
	50 ppm	17282072			
	Range of % RSD				**0.42-1.45**

4.3.2 Interday precision study of lobeglitazone

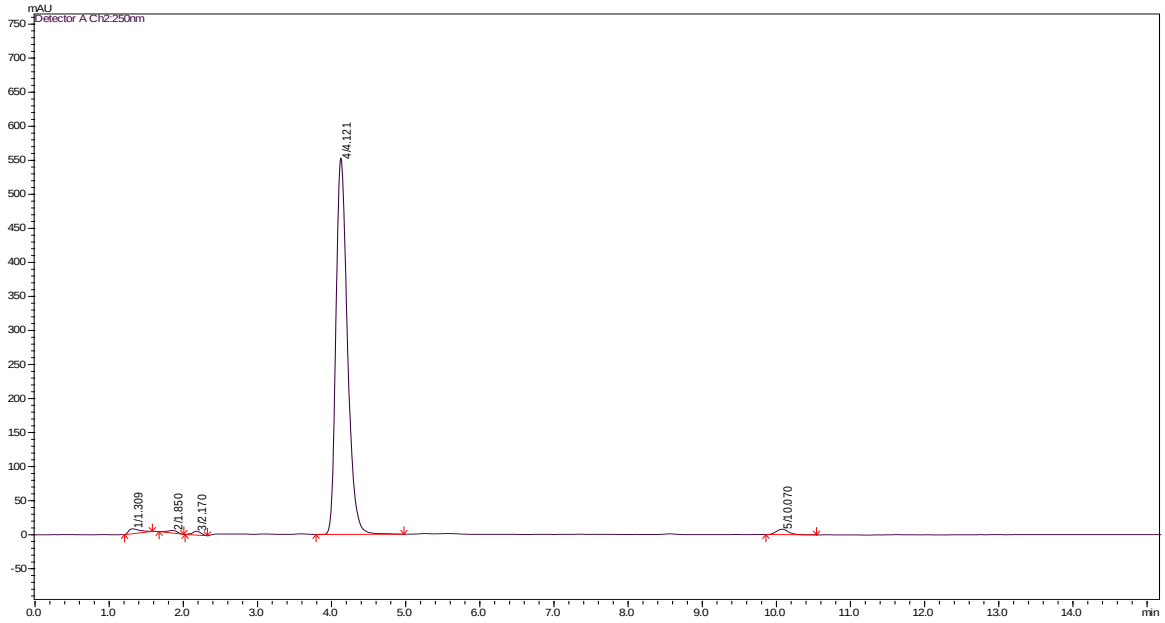

Figure 4.5; interday precision study of lobeglitazone

Table 8; interday precision study of lobeglitazone

Peak #	Ret. Time	Area	Height	Area %	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.309	77046	7251	1.2406	342.479	--	0	1.941	0
2	1.85	35696	3969	0.5748	860.579	2.024	0.414	0.916	0
3	2.17	43673	5627	0.7032	1603.79	1.361	0.65	1.002	1.589

					2		8		
4	4.121	5963531	552631	96.024	3368.208	7.794	2.149	1.369	3.267
5	10.07	90516	7930	1.4575	17785.2	20.301	6.694	1.206	3.115

Implementing the procedure mentioned under experimental section (5.3), the homologous mixture of lobeglitazone of three replicates of selected similar concentrations; were tested and evaluated in three successive days (interday/intermediate precision). The RSDs in percentage were calculated and they are found to be less than 2% for all selected analytes. Results are shown in Table 6.4.

Table 9; Intraday precision data of Lobeglitazone

Drug Name: LOBEGLITAZONE					
S. No.	Concentration (ppm)	Area	Average	Std. Deviation	%RSD
1	100 ppm	8725268	8688447	38641.89	0.44
	100 ppm	8691863			
	100 ppm	8648211			
2	100 ppm	8711173	8689592	32179.56	0.37
	100 ppm	8704998			
	100 ppm	8652606			
3	100 ppm	8684290	8683953	55855.76	0.64
	100 ppm	8739639			
	100 ppm	8627929			
	Range of % RSD				**0.37-0.64**

The above-mentioned concentrations were analyzed on three successive days using, the procedure mentioned under section (2.7).

4.4 Robustness study of lobeglitazone

Robustness of HPLC Method represents its ability to remain unaffected by small but deliberate changes in separation parameters to ascertain its reliability during routine analysis. In this method, robustness was established by making deliberate changes in flow rate (1.0±0.1 ml/mins), organic modifier as solvent B (±2%, v/v), and wavelength (250±2 nm). Robustness studies was determined by small variation in separation parameters like effect of flow rate, organic modifier composition, temperature, pH, wavelength, injection volume on selected separation variables including capacity factor (k'), resolution (Rs), tailing factor (*Tf*), separation factor, theoretical plates (N) and sometimes considered the peak area.

4.4.1 Effect of flow rate on lobeglitazone

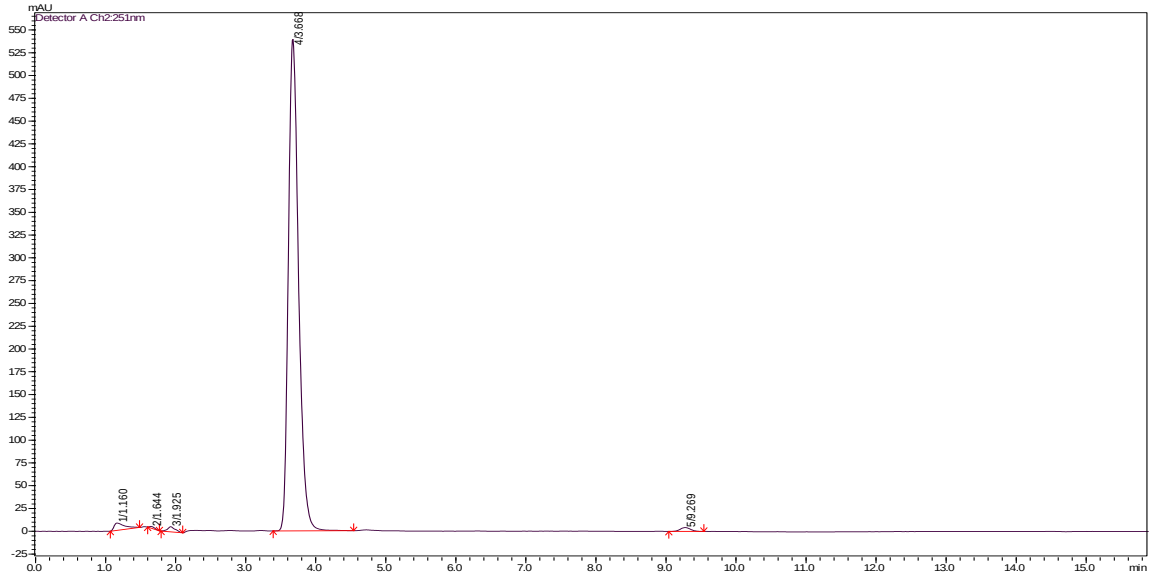

Figure 4.5; effect of flow rate 1.1 ml/min on lobeglitazone

Table 9; effect of flow rate 1.1 ml/min on lobeglitazone

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.16	83800	7995	1.4836	294.346	--	0	2.427	0
2	1.644	8944	1666	0.1583	2083.388	2.335	0.417	1.835	0
3	1.925	42321	5517	0.7493	1212.053	1.539	0.659	1.159	1.581
4	3.668	5469223	539189	96.8287	2991.579	7.121	2.161	1.361	3.278
5	9.269	44064	4254	0.7801	17460.4	20.413	6.99	1.105	3.234

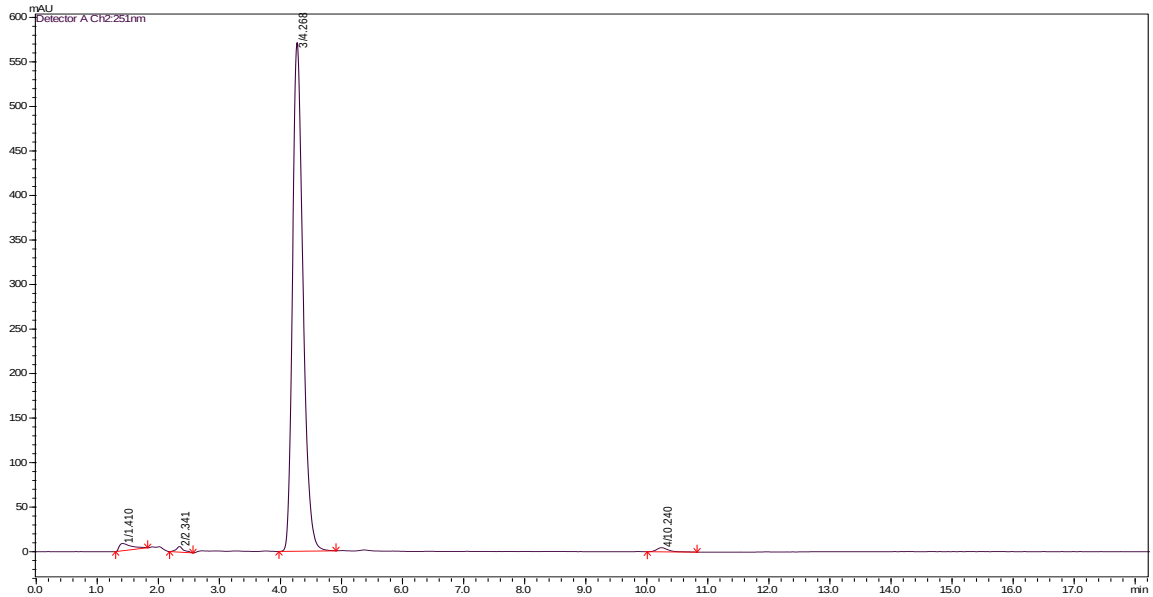

Figure 4.6; effect of flow rate 0.9 ml/min on lobeglitazone

Table 10; effect of flow rate 0.9 ml/min on lobeglitazone

Peak #	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.41	113657	8218	1.6084	277.831	--	0	2.553	0
2	2.341	52065	6229	0.7368	2107.096	3.431	0.66	1.239	0
3	4.268	6837991	571251	96.7675	2864.52	7.369	2.026	1.313	3.07
4	10.24	62697	4670	0.8873	14168.03	18.01	6.26	1.539	3.089

Discussion: Therefore, increased the flow rate by +0.1 ml/minutes, marginally reduced the t_R values of all selected drugs and impurities whereas reducing it, extended slightly the t_R values of same drugs. Similarly, slight variation in organic solvent concentration, considered here as CAN-MeOH (90:10 v/v) 20%±2% for and 70%±2% for have not made any significant changes. Furthermore, small variation in temperature by 28°C ± 2°C has not made any significant changes in the retention pattern of all selected drugs (Fig. 5B).

4.4.2 Effect of solvent composition on lobeglitazone

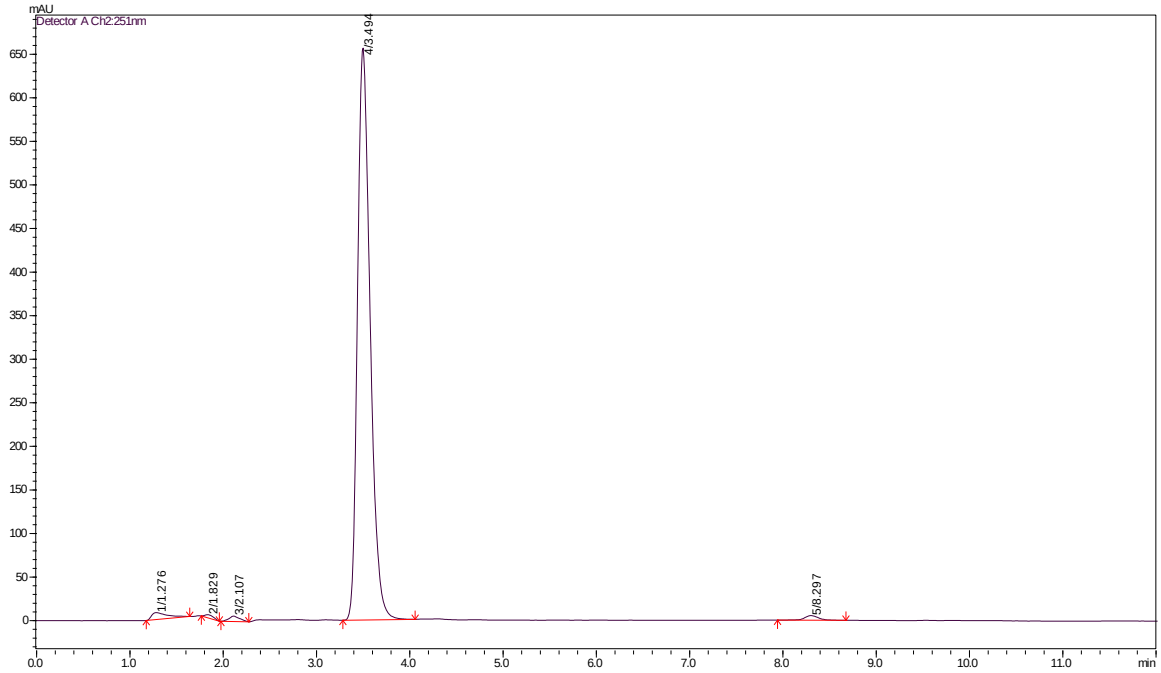

Figure 4.7; effect of organic solvent (B) of 52% on lobeglitazone

Table 11; effect of organic solvent (B) of 52% on lobeglitazone

Peak #	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.276	95994	8137	1.5012	275.803	--	0	2.496	0
2	1.829	22740	3417	0.3556	1647.323	2.271	0.434	1.532	0

3	2.107	50751	6296	0.7937	1228.544	1.322	0.652	1.156	1.502
4	3.494	6167909	656300	96.4582	2982.655	5.585	1.739	1.368	2.667
5	8.297	56989	5318	0.8912	15145.14	18.279	5.504	1.026	3.166

Discussion: Therefore, increased the flow rate by +0.1 ml/minutes, marginally reduced the t_R values of all selected drugs and impurities whereas reducing it, extended slightly the t_R values of same drugs. Similarly, slight variation in organic solvent concentration, considered here as CAN-MeOH (90:10 v/v) 20%±2% for and 70%±2% for have not made any significant changes. Furthermore, small variation in temperature by 28°C ± 2°C has not made any significant changes in the retention pattern of all selected drugs (Fig. 5B).

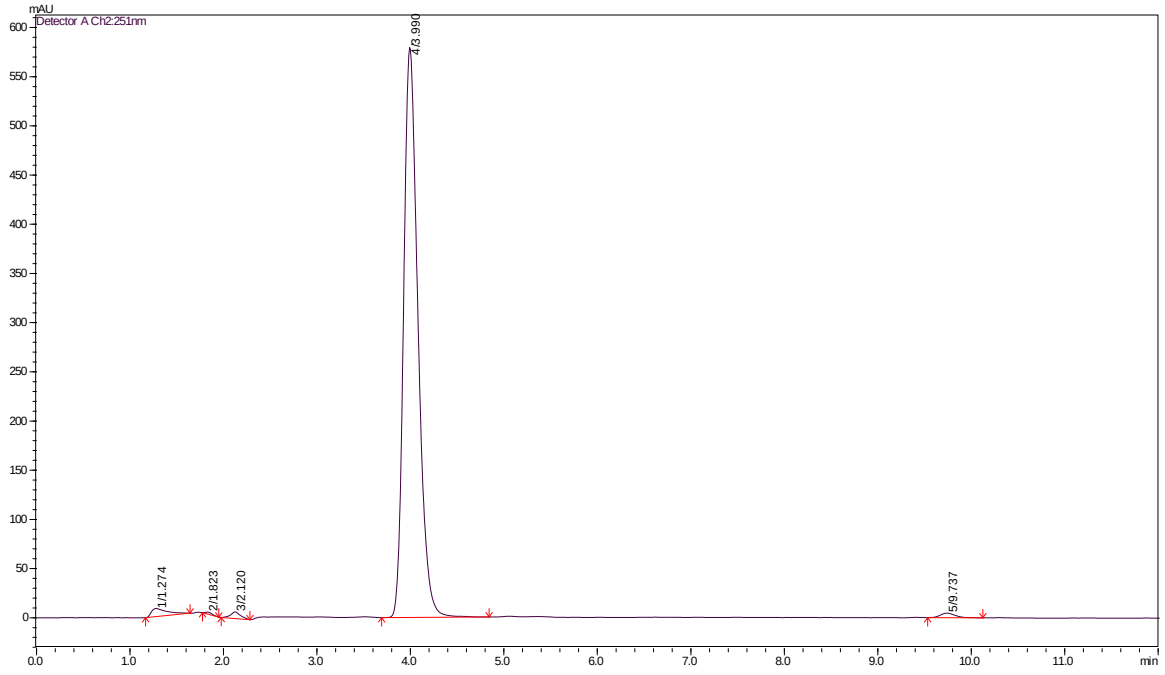

Figure 4.8; effect of organic solvent (B) of 48% on lobeglitazone

Table 12; effect of organic solvent (B) of 48% on lobeglitazone

Peak #	Ret. Time	Area	Height	Area %	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.274	99315	8249	1.557	284.213	--	0	2.426	0
2	1.823	9240	1724	0.1448	2433.407	2.439	0.431	1.767	0
3	2.12	49849	6765	0.7815	1761.963	1.698	0.664	1.068	1.541
4	3.99	6168163	579409	96.698	3095.499	7.65	2.132	1.317	3.21
5	9.737	52224	4655	0.8187	17071.5	19.651	6.64	1.264	3.116

					6		3		

Therefore, increased the flow rate by +0.1 ml/minutes, marginally reduced the t_R values of all selected drugs and impurities whereas reducing it, extended slightly the t_R values of same drugs. Similarly, slight variation in organic solvent concentration, considered here as CAN-MeOH (90:10 v/v) 20%±2% for and 70%±2% for have not made any significant changes. Furthermore, small variation in temperature by 28°C ± 2°C has not made any significant changes in the retention pattern of all selected drugs (Fig. 5B).

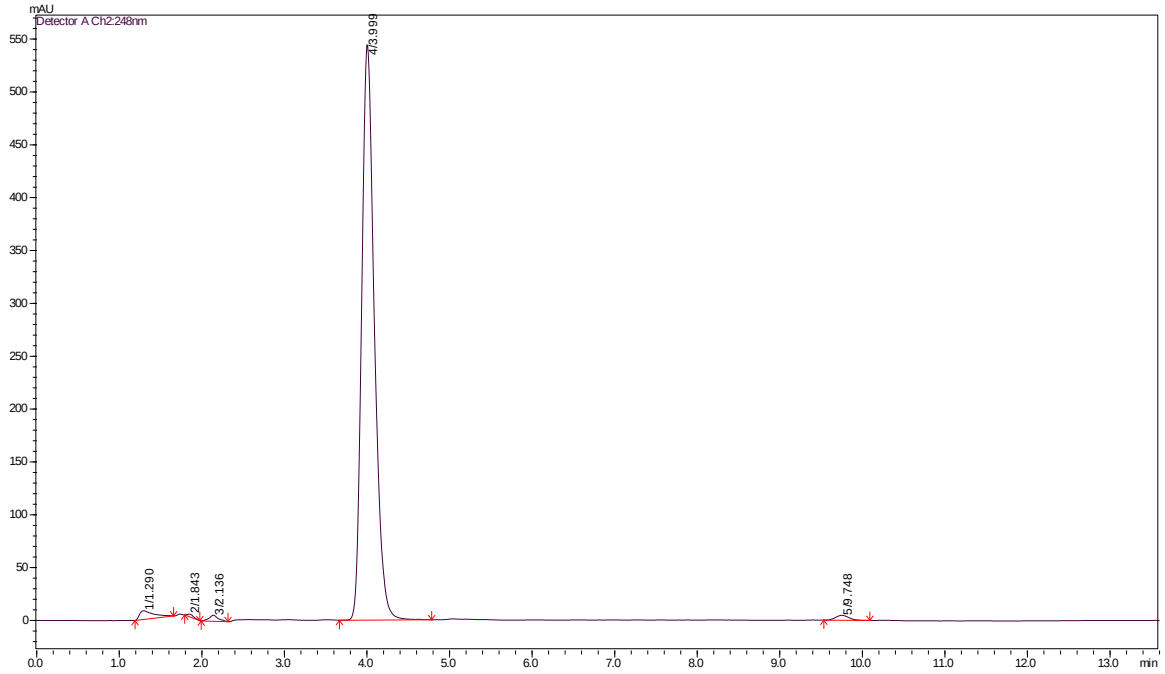

Figure 4.9; effect of 250 nm wavelength on lobeglitazone

Table 13; effect of 248 nm wavelength on lobeglitazone

Peak #	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.29	100014	8368	1.657	295.472	--	0	2.531	0
2	1.843	12144	2199	0.2012	2319.24	2.441	0.429	1.702	0
3	2.136	44466	5729	0.7367	1725.897	1.631	0.656	1.123	1.529
4	3.999	5826634	544234	96.5321	3167.761	7.607	2.1	1.314	3.203
5	9.748	52697	4714	0.873	16121.31	19.443	6.555	1.156	3.122

Discussion: Therefore, increased the flow rate by +0.1 ml/minutes, marginally reduced the t_R values of all selected drugs and impurities whereas reducing it, extended slightly the t_R values of same drugs. Similarly, slight variation in organic solvent concentration, considered here as CAN-MeOH (90:10 v/v) 20%±2% for and 70%±2% for have not made any significant changes. Furthermore, small variation in

temperature by 28°C ± 2°C has not made any significant changes in the retention pattern of all selected drugs (Fig. 5B).

Table No. 14; Robustness data of lobeglitazone

Variables				
	t_R(min)	k'	T_f	N
Flowrate(+0.1mL/min)	3.53	0.77	1.92	2001
Flowrate(-0.1mL/min)	4.29	1.28	0.85	2081
Solvent B (+2%)	3.85	1.28	1.91	2088
Solvent B (-2%)	3.8	1.22	1.53	2019
Wavelength (+2˚C)	3.87	1.28	1.54	2091
Wavelength (-2˚C)	3.87	0.77	1.54	2013
Mean±S.D.	3.87±0.24	1.10±0.26	1.55±0.39	

4.5 Linearity studies of lobeglitazone

The linearity of HPLC method represents its ability to explicit the results that should proportional to the concentration of studied analytes within a selected range. Therefore, as observed from figures (4.10) and tables (15) over the selected concentrations (100, 50, 25, 12.5, 6.25 and 3.12 ppm) of lobeglitazone their relative peak areas were highly proportional since in all studies their regression coefficients (R^2) were almost 0.999 which is closest to 1. It represents that the developed method has a high degree of linearity. Furthermore, based on the standard deviation of the response and the slope of the regression equation their limits of detection (LOD) and limits of quantifications (LOQ) were calculated. As observed, the LOD and LOQ for all selected compounds were well below the 5 µg/ml which signifies the selected wavelength is more sensitive enough to detect the lowestamountofdrugs either from pharmaceutical drugs or biological fluids.

4.5.1 Linearity studies of lobeglitazone

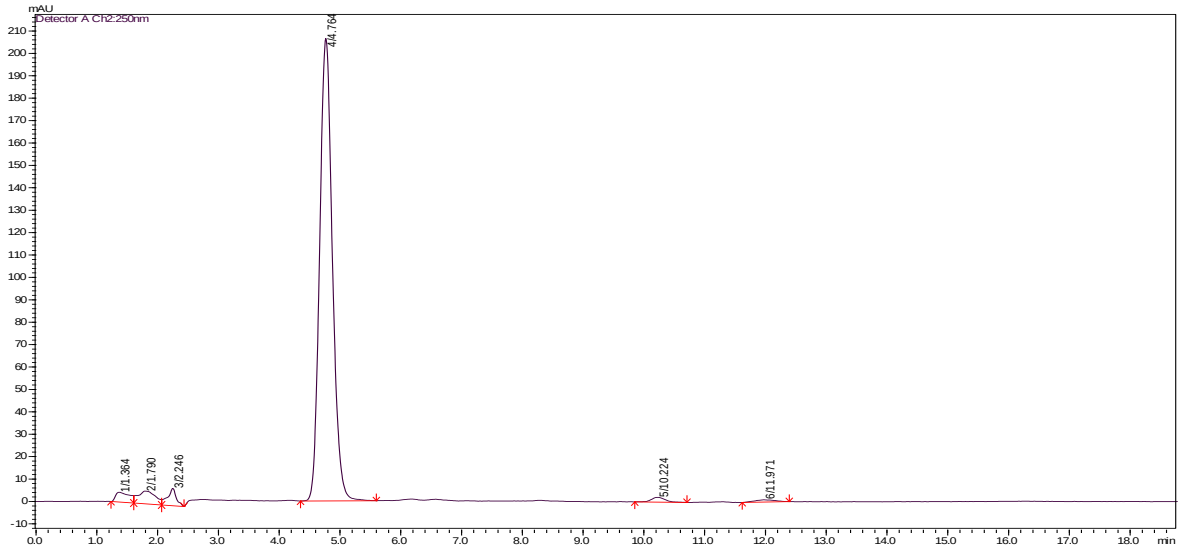

Figure 4.10; HPLC analysis of 50 ppm of lobeglitazone

Table 15; HPLC analysis of 50 ppm of lobeglitazone

Peak #	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.364	71476	4344	2.151	72.809	--	0	--	0
2	1.79	113768	5656	3.4238	14.807	0.34	0.312	--	0
3	2.246	75749	7714	2.2796	1536.59	0.436	0.646	--	2.072
4	4.764	3004238	206397	90.4109	2498.444	8.251	2.491	1.15	3.857
5	10.224	34232	2122	1.0302	9818.783	13.756	6.493	1.103	2.606
6	11.971	23408	1001	0.7044	6080.689	3.404	7.774	1.12	1.197

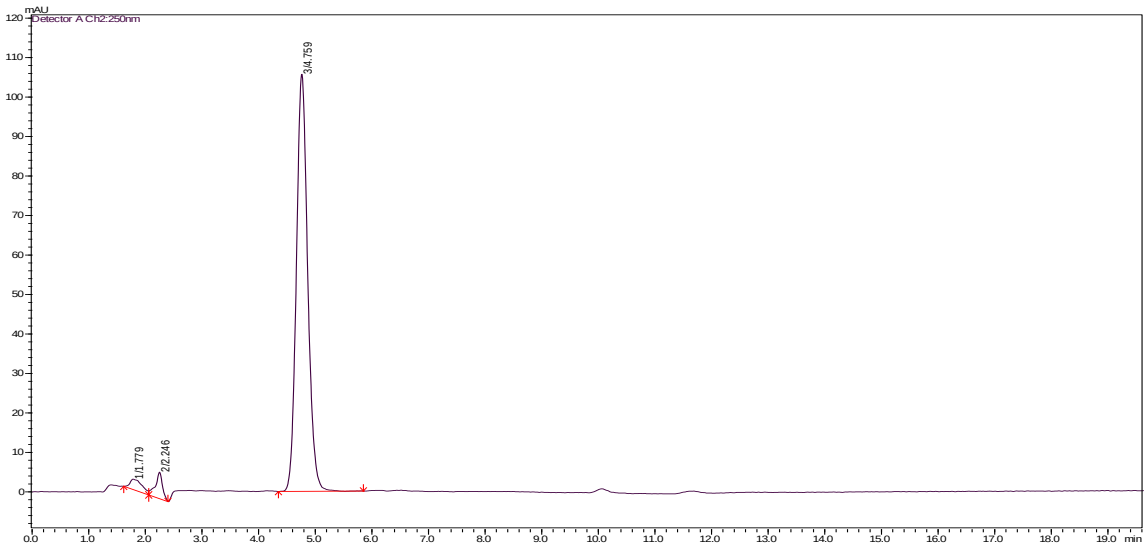

Figure4.11; HPLC analysis of 25 ppm of lobeglitazone

Table 16; HPLC analysis of 25 ppm of lobeglitazone

Peak #	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.779	41825	2606	2.6693	272.807	--	0	--	0
2	2.246	57402	6693	3.6634	1823.222	1.454	0.262	--	0
3	4.759	1467672	105631	93.6673	2752.371	8.768	1.674	1.142	6.388

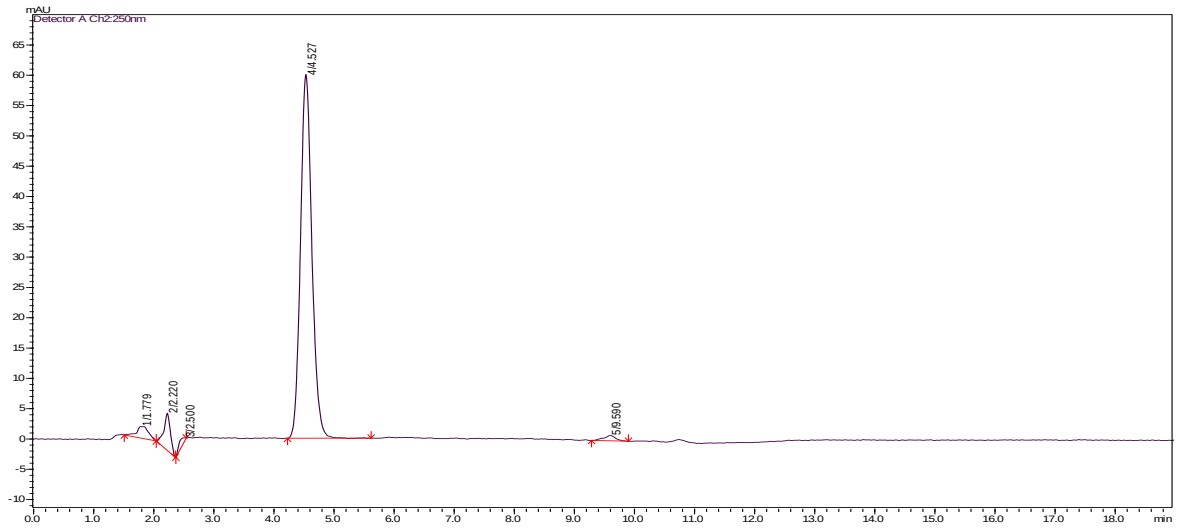

Figure 4.12 HPLC analysis of 12.5 ppm of lobeglitazone

Table 17; HPLC analysis of 12.5 ppm of lobeglitazone

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.779	27567	1903	3.1152	483.425	--	0	0.975	0
2	2.22	45889	6115	5.1857	1951.526	1.684	0.248	0.883	0
3	2.5	9307	768	1.0517	3592.811	1.52	0.406	0.658	1.633
4	4.527	789889	59993	89.2623	2767.643	7.932	1.545	1.167	3.81
5	9.59	12257	872	1.3851	13088.99	14.903	4.392	0.957	2.842

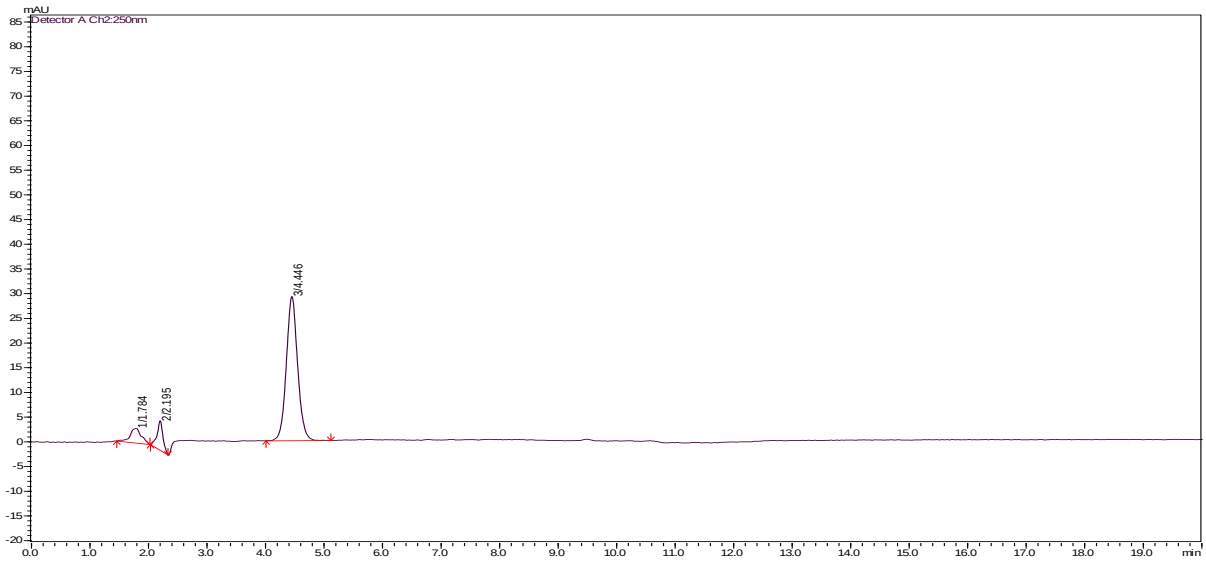

Figure 4.13; HPLC analysis of 6.25 ppm of lobeglitazone

Table 18; HPLC analysis of 6.25 ppm of lobeglitazone

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.784	39715	2964	8.3704	567.287	--	0	0.873	0
2	2.195	41395	5942	8.7245	2291.559	1.701	0.23	0.931	0

3	4.446	393358	29206	82.9051	2445.515	8.292	1.492	1.078	6.482

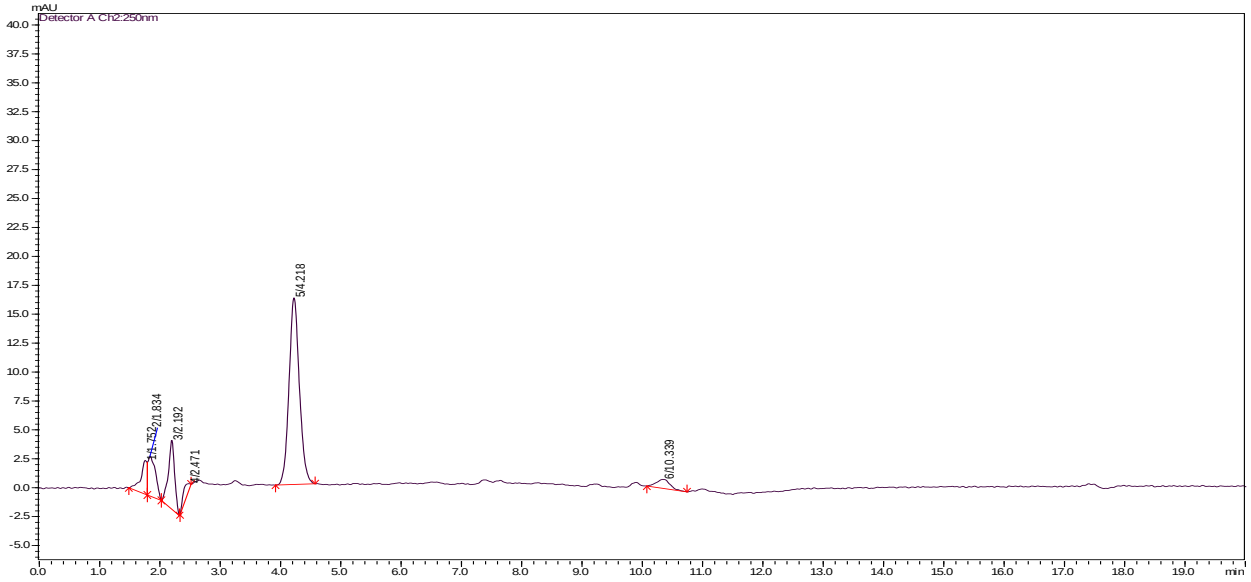

Figure 4.14; HPLC analysis of 3.12 ppm of lobeglitazone

Table 19; HPLC analysis of 3.12 ppm of lobeglitazone

Peak#	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.752	21767	2903	7.0478	50.258	--	0	--	0
2	1.834	29381	3447	9.513	358.51	0.119	0.047	--	0
3	2.192	42929	5926	13.8997	1924.716	1.22	0.251	0.891	5.36
4	2.471	8821	693	2.8561	3067.554	1.475	0.411	0.661	1.634
5	4.218	193573	16142	62.6754	2805.347	7.031	1.408	1.121	3.429
6	10.339	12379	779	4.0081	10180.54	16.804	4.902	1.088	3.481

Table 20; Linearity data of lobeglitazone

Name ofDrug: LOBEGLITAZONE		
S. No.	Concentration (µg/mL)	Area
1	70	11842484
2	35	6007693
3	17.5	3140363
4	8.75	1487561
5	4.375	745567
6	2.1875	362205
Regression Equation		y=169183x + 45048
Correlation coefficient (R^2)		0.9997
Std. error of intercept		47310.73033
Std. Dev. Of intercept		115887.1487

LOQ	6.85 µg/Ml
LOD	2.05 µg/mL

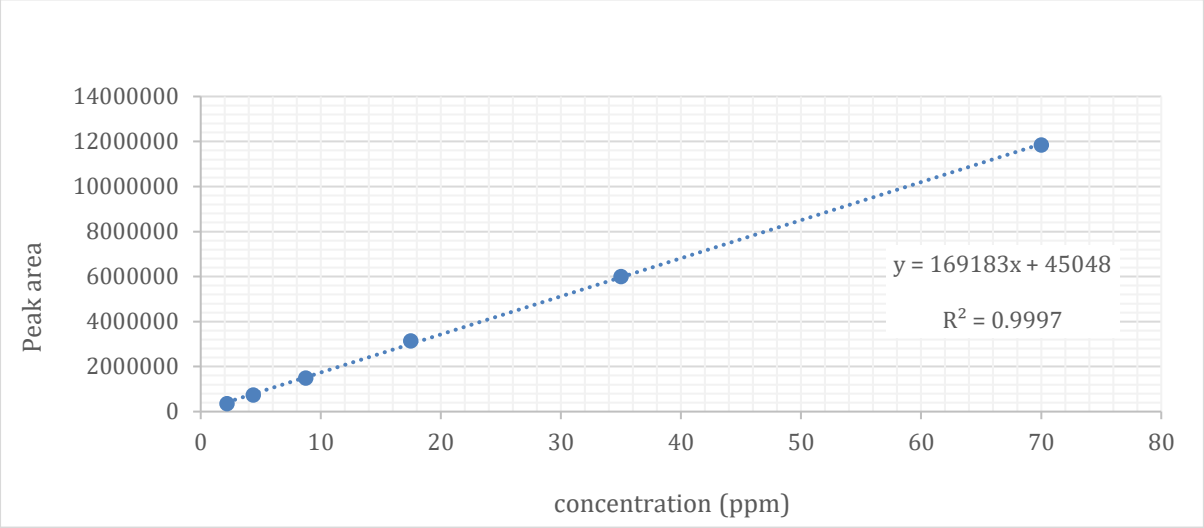

Figure 4.15; Calibration curve of lobeglitazone

4.6 Drug accuracy studies of lobeglitazone

Percentage drug accuracy of three different concentrations; 80%, 100% and 120% (injected thrice) to estimate the and from marketed formulation and results obtained have been reported in Table 7.34. Accuracy can be studied by applying the calibration curve; the Y-intercept and the slope of the graph were used to determine the % drug recovery, attributed to the developed method for the simultaneous quantification of selected drugs or by comparing with similar concentration of reference standard. As resulted, the achieved drug recovery of both and were in the range of 100.4-100.7 and 100-105, respectively. As recommended by international conferences of Harmonization (ICH) guidelines the drug recovery should be within the range of 90-110% and the RSD in percentage should be less than 2%. Hence, the calculated drug recoveries for simultaneous estimation of lobeglitazone represents the drug recovery were in the acceptance limit given by ICH guidelines.

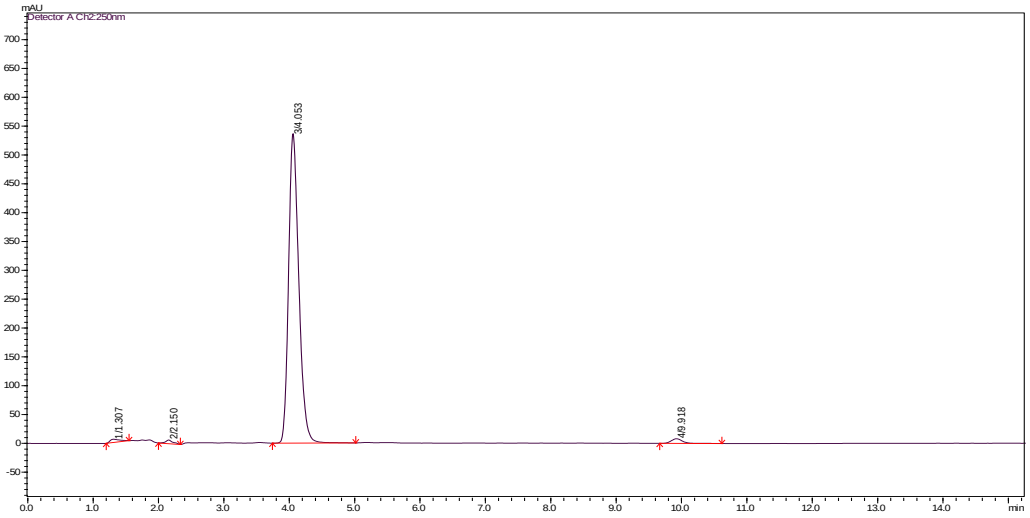

Figure 4.16; HPLC analysis of marketed formulation; LOBG from glenmark

Table 21; HPLC analysis of LOBG tablet from glenmark

Peak #	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.305	14599499	653918	60.4679	41.101	--	0	--	0
2	1.97	1577498	85113	6.5336	173.409	0.942	0.51	--	0
3	4.053	7967225	702761	32.9985	2879.136	4.628	2.107	1.314	4.134

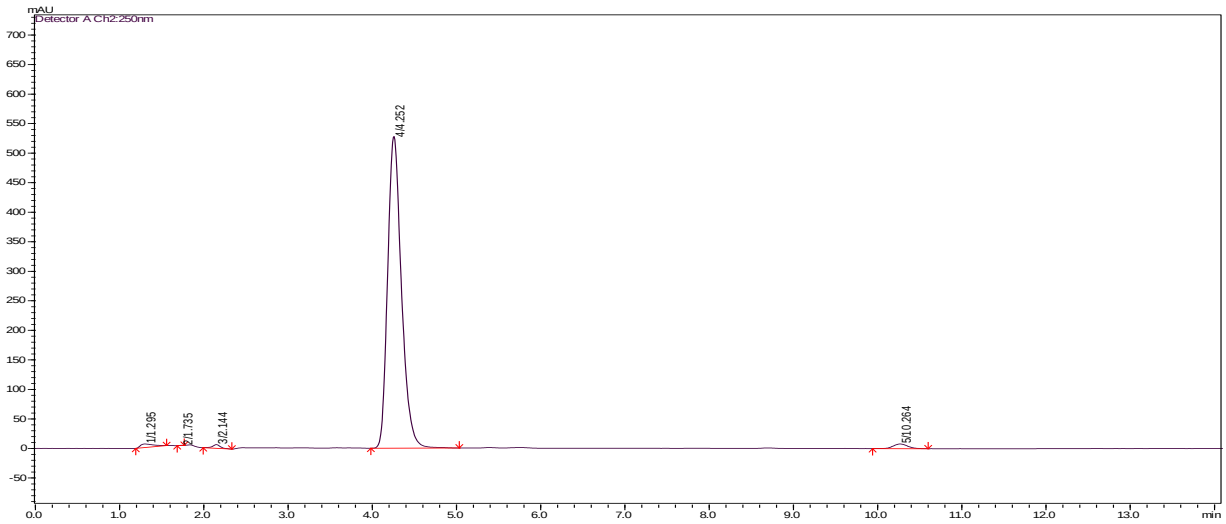

Figure 4.17; HPLC analysis of lobeglitazone API

Table 22; HPLC analysis of lobeglitazone API

Peak #	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.295	65988	6259	1.0602	312.727	--	0	1.864	0
2	1.735	1097	346	0.0176	7841.496	2.371	0.34	0.858	0
3	2.144	40402	6253	0.6491	2345.377	3.209	0.656	1.041	1.932
4	4.252	6021419	527718	96.7408	3154.818	8.783	2.284	1.302	3.48
5	10.264	95371	7903	1.5322	16528.78	19.326	6.928	1.12	3.033

Table 23; Accuracy data of lobeglitazone

Drug Name:LOBEGLITAZONE			Drug content: 500 mg		Marketed formulation: Logs-MF 15 Tablet		
Std. conc. (%)	Std. (ppm)	Peak area	Drug (%)	Drug (ppm)	Peak area	Avg. peak area	Drug Rec. (%)

100%	100 ppm	16878628	80	80	14090409	14135321	104.68
				80	14180233		
			100	100	18542802	18289514.5	108.36
				100	18036227		
			120	120	21047878	21259362.5	104.96
				120	21470847		
			Drug recovery Range (%) as per ICH = 100±10%				104.68 % - 108.36 %

5. Bio-analysis/drug recovery studies from human plasma

Drug recovery studies from Human Plasma

Exact quantification of plasma/urine drug concentrations of selected drug combinations using a proper bioanalytical method is intrinsic for therapeutic drug monitoring to understand their exact pharmacokinetics and dosing optimization. Importantly, for the quantification of lobeglitazone in plasma/urine, very few analytical methods have been reported.

Prior to plasma analysis, two mostly recommended plasma extraction techniques, such as protein precipitation (PPT) with acetone, MeOH, MeCN, and solid phase extraction (SPE) in C18, HLB, SCX cartridges were evaluated. Unfortunately, SPE carried out in C18 and HLB was not much effective for highly polar as it leads to poor recoveries (<40%) with many impurities, causing undesired interferences in HPLC and LC-MS/MS detection.

Protein precipitation with MeOH, MeCN and acetone was attempted for extracting the lobeglitazone from human plasma. However, as observed, in MeOH (with or without 0.1% formic acid), all proteins were not completely precipitated out, resulting in excessive column back pressure and blocking the injection valve, which were the most common problems observed in HPLC and LC-MS/MS. In contrast, extraction carried out with MeCN/MeCN-0.1% formic acid proved more effective for precipitating out of all proteins and peptides by

forming a dense precipitate that was easily removed after centrifugation. Moreover, in both SPE and PPT, the unwanted metabolites were commonly observed and not completely removed. Altogether, protein precipitation carried out in MeCN of lobeglitazone from human plasma was more effective and economical than painstaking SPE with SCX sorbents.

Furthermore, the retention behaviour of simultaneously quantified lobeglitazone from human plasma was estimated in selected SCX, HILIC and mix-mode chromatography. Among them, the differences in drug recovery and reproducibility between pure drugs, spiked plasma and human plasma who received orally a single dose of tablet; Valera-M® (5 mg + HCl 500 mg) were compared. Therefore, from collective efforts towards the quantification of from human plasma (n = 6), the results obtained for all selected techniques have not shown any distinct variabilities with respect to their selectivities and sensitivities, as well as no other interfering peaks from the biological matrix appeared besides the investigated lobeglitazone.

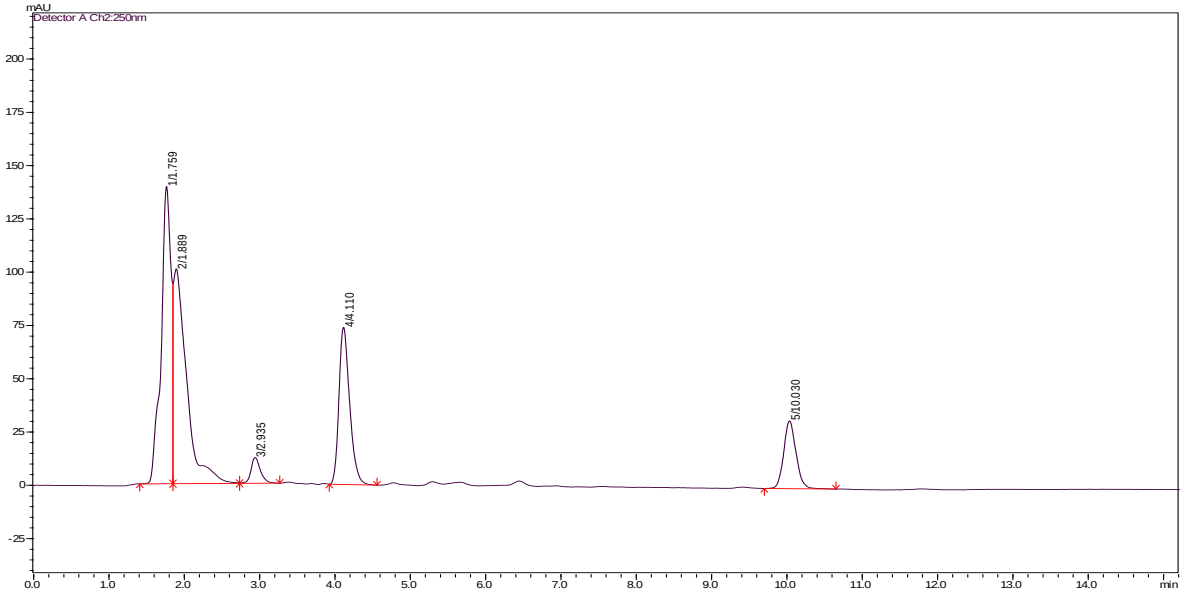

Figure 5.1; bio-analysis studies of lobeglitazone

Table 24; bio-analysis studies of lobeglitazone

Peak #	Ret. Time	Area	Height	Area%	T.Plate#	Resolution	k'	Tailing F.	Separation
1	1.759	1313584	139523	35.2913	471.727	--	0	--	0
2	1.889	1211795	100867	32.5566	97.13	0.238	0.074	--	0
3	2.935	104900	12101	2.8183	2679.415	2.106	0.669	1.263	9.064
4	4.11	730251	73697	19.6192	3791.324	4.759	1.336	1.334	1.999
5	10.03	361593	31806	9.7147	17427.71	20.743	4.703	1.156	3.519

Table-25; Lobeglitazone recovery from human Plasma

	Details of Std.	Recovery before PPT	Recovery after PPT
Peak area	16878682	3348579	2162053
Concentration (ppm)	100	34	34
Injection volume (µL)	20	20	20
Purity of Drug	0.98	NA	NA
Drug content (%)		57.18	36.92

6. Conclusion

From all above results and discussion, it has been concluded that the developed analytical method for the estimation of lobeglitazone in both bulk and tablet formulation has obliged the ICH guidelines. As per the ICH guidelines, the developed method has complied the linearity range (calibration data), accuracy/drug recovery studies (%), repeatability, precision studies (intraday and interday/intermediate), and robustness. Moreover, as per the ICH guidelines, the system suitability test performed for lobeglitazone has achieved all guidelines; including, tailing factor (*T*), separation factors (α), theoretical plates (*N*), capacity factor (*k'*), resolution (*R*) and RSD (%). The validated stress degradation studies under thermal, oxidative, alkali and acid ascertained few degradation products for lobeglitazone. Hence, this developed and validated method for investigation by reverse phase high performance liquid chromatography (RP-HPLC) can be used for routine analysis of estimation of lobeglitazone from marketed formulation.

Reference

1. Holman RR, *et al.* (2008). 10-year follow-up of intensive glucose control in type 2 diabetes. N Engl J Med, 359:1577–1589.
2. UK Prospective Diabetes Study (UKPDS) Group: (1998). Effect of intensive blood glucose control with on complications in overweight patients with type 2 diabetes (UKPDS 34), *Lancet*, 352:854–865.
3. Sami, W. et al, (2017). Effect of diet on type 2 diabetes mellitus: A review. International Journal of Health Science. 11, 65–71.
4. Kohlroser J, Mathai J, Reichheld J, Banner BF, Bonkovsky HL. (2000). Hepatotoxicity due to troglitazone: report of two cases and review of adverse events reported to the United States Food and Drug Administration. *American Journal of Gastroenterology*, 95, 272-6.
5. Chong Kun Dang Pharmaceutical Corp.: Data on file, 2013. Available from: http://www.ckdpharm.com.
6. Kwon MJ, Lee YJ, Jung HS, Shin HM, Kim TN, Lee SH, et al. (2019). The direct effect of lobeglitazone, a new thiazolidinedione, on pancreatic beta cells: a comparison with other thiazolidinediones. Diabetes Research and Clinical Practices, 151, 209-23.
7. Lindholm J. (2004). Development and Validation of HPLC Method for Analytical and Preparative Purpose, Acta Universities Upsaliensis Uppsala, 2004; 13-14.
8. Jeffery GH, Bassett J, Mendham J, Denny RC, Vogel's Textbook of Quantitative Chemical Analysis, fifth edition, Longman scientific & technical.
9. Kaushal C, Srivastava B, A Process of Method Development: A Chromatographic Approach. J Chem Pharm Res, 2010; 2(2): 519-545.
10. Buffers and pH Buffers: available from: www.xtremepapers.com.
11. Chandra M., Buffers: A guide for the preparation and use of buffers in biological system: available from: www.calbiochem.com.
12. How do I Develop an HPLC Method. www.sgc.com.
13. Columns from http://www.waters.com/watersdivision/pdf/ Ic3AC.pdf.
14. Columns from www.agilent.com.
15. Bliesner D.M., Validating Chromatographic Methods, john wliey & sons, Inc. 2006; 88-92.

16. A Guide to Validation in HPLC Based on the Work of G.M. Hearn Perkin Elmer. R.A. van Iterson Drenthe College Emmen Holland for www.standardbase.com.
17. International Conference on Harmonization (ICH); Q2 9(R1): technical requirements for registration of pharmaceuticals for human Use; validation of analytical procedures: text and methodology;
18. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), Geneva, Switzerland, (2005), pp. 1–13.
19. Sinha, S., et al., (2013). Determination of eight isomers and related substance of Aprepitant using normal-phase and reverse-phase HPLC methods with mass spectrophotometric detection, Pharmaceutical Methods, 4, 33-42
20. West, C., et al., (2010). Porous graphitic carbon: A versatile stationary phase for liquid chromatography, Journal of Chromatography A, 1217, 3201-3216
21. Mallik, AK., etal(2018). High molecular-shape-selective stationary phases for reversed-phase liquid chromatography: A review, Trends in Analytical Chemistry, 108, 381-404
22. Richard R. Burgess, A., (2018). A brief practical review of size exclusion chromatography: Rules of thumb, limitations, and troubleshooting, Protein Expression and Purification, 150, 81-85
23. Uliyanchenko, E. (2014). Size-exclusion chromatography—from high-performance to ultra-performance. Analytical and bioanalytical chemistry, 406 (25), 6087-6094.
24. Zatirakha, V., (2016). Preparation and chromatographic performance of polymer-based anion exchangers for ion chromatography: Analytica Chimica Acta, 904, 33-50
25. Fekete, S., et al., (2015). Ion-exchange chromatography for the characterization of biopharmaceuticals, Journal of Pharmaceutical and Biomedical Analysis, 113, 43-55
26. Yang, Y., & Geng, X., (2011). Mixed-mode chromatography and its applications to biopolymers, Journal of Chromatography A, 1218, 8813-25
27. Gama, MR., et al., (2012). Hydrophilic interaction chromatography, Trends in Analytical Chemistry, 48-60
28. Sahu, PK., (2018). An overview of experimental designs in HPLC method development and validation, Journal of Pharmaceutical and Biomedical Analysis. 147, 590-611
29. Rao, RN., & Nagaraju, V., (2003). An overview of the recent trends in development of HPLC methods for determination of impurities in drugs, Journal of Pharmaceutical and Biomedical Analysis, 33, 335-377