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Bioanalytical stability indicating method development and validation for the estimation of Lobeglitazone Sulphate in human plasma by using RP-HPLC Method.

Article History:

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Abstract: A simple, rapid, reliable, precise, accurate, sensitive and selective analytical method for the estimation of lobeglitazone. In human plasma and using as an internal standard (IS). Lobeglitazone is a novel thiazolidinedione (TZDs) based peroxisome proliferator-activated receptor (PPAR) agonist, used for the management of type-2 diabetes. The chromatographic separation was achieved on a Rubitas C18 Column (250 mm 4.6mm, 5 µm particle size) and the mobile phase was a combination of Acetonitrile: Methanol (60:40 v/v) .The flow rate was set at 1.0 mL/min, and detection of the effluents occurred at 248 nm. Results: The retention time for Lobeglitazone was determined to be 5.945±0.164 min. The drug exhibited linearity within the concentration range of 0.1-5 µg/mL, the correlation coefficient was established to be 0.9924. The LQC, MQC, HQC were found to be 0.5 µg/ml, 2µg/ml, 4µg/ml. The accuracy of the method was considered satisfactory and the mean recovery percentage is found to be in the acceptable range of 93.87-97.07%. The HPLC method was successfully developed, validated as per ICH guidelines. The proposed method was simple, precise, sensitive, rapid, robust for the estimation of Lobeglitazone Sulphate.

Keywords: Lobeglitazone, Method development, Validation, RP-HPLC.

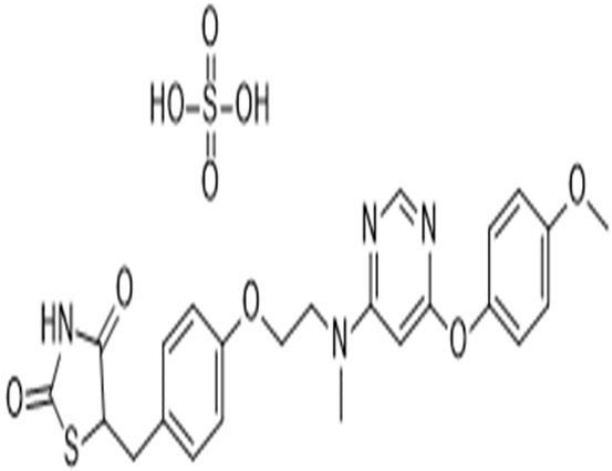
INTRODUCTION

In pharmaceutical industry, there is a need for the invention of suitable novel analytical methods from time to time for testing the quality of bulk drugs, excipients and formulations. Method development and validation is an integral part of drug discovery and drug development. UV-visible spectroscopy and HPLC are the most popular techniques used for the identification and estimation of drugs with good accuracy and precision. Simultaneous method development is useful for analysis of combination of drugs. Bioanalytical method development is used for the estimation of drugs and their metabolites in

various biological fluids like blood, urine, plasma, serum, saliva and cerebrospinal fluid. Worldwide, around 463 millions people are suffering from type-2 diabetes mellitus [International diabetes federation,2019] Anti-diabetic drugs are used for treating type-2 diabetes mellitus. There will be always a need for the development and validation of novel analytical methods for the estimation of drugs in bulk and dosage forms in order to deliver good quality and affordable medicines to patients Degradation studies of the drug substance include both appropriate solution and solid-state stress conditions (e.g., acid/base hydrolysis, oxidation, heat, humidity, and light exposure in accordance with ICH guidelines). Applied stress conditions should result in approximately 10–20 % degradation of the drug substance or represent a reasonable maximum condition achievable for the drug substance. In case, if no degradation is observed under the specified stress conditions, it is recommended to stop stress testing. A better knowledge of therapeutics and physicochemical and toxicological behaviour of drug and its pharmaceutical formulation can be gained from degradation study. This study allows a formulator in formulating more stable drug preparation by using a suitable solvent or vehicle or ingredients, which will prevent or retard the degradation. Further, it helps in deciding the storage conditions and routes of administration of various pharmaceutical dosage forms.

DRUG PROFILE:

Lobeglitazone Sulphate



Molecular Formula: C24H24N4O5S

Molecular weight: 578.6 g/mol
IUPACName:5-[[4-[2-[[6-(4-methoxyphenoxy)pyrimidin-4-yl]-methylamino]ethoxy]phenyl]methyl]-1,3-thiazolidine-2,4-dione;sulfuric acid

Category: thiazolidinedione (TZDs) based peroxisome proliferator-activated receptor (PPAR) agonist, used for the management of type-2 diabetes.

Mechanism of Action:
Lobeglitazone acts as an insulin sensitizer by binding and activating Peroxisome Proliferator-Activated Receptors (PPAR) gamma within fat cells. By promoting the binding of insulin at fat cells, lobeglitazone has been shown to reduce blood sugar levels, lower hemoglobain A1C (HbA1C) levels, and improve lipid and liver profiles 1. Unlike Pioglitazone, which is a dual PPAR agonist at PPAR-alpha and PPAR-gamma, Lobeglitazone is a pure PPAR-alpha agonist.

Uses:
Lobeglitazone sulphate belongs to a class of medicines called antidiabetic drugs. It contains Lobeglitazone sulphate, an insulin sensitiser that works by binding and activating PPAR (peroxisome proliferator-activated receptors) gamma within fat cells. Thus, it promotes insulin binding at fat cells, reduces blood sugar levels, lowers haemoglobin A1C (hba1c) levels, and improves lipid and liver profiles. It reduces blood sugar levels by increasing insulin utilization by the body. Controlling blood sugar levels can help reduce the risk of complications of diabetes like eye damage, kidney damage, nerve problems., etc.

Material and Instrument:

➢ Reagents and chemicals:
- Methanol (HPLC Grade)
- HPLC grade water
- Acetonitrile (HPLC Grade)

Methanol and Acetonitrile HPLC grade was purchased from Merck limited, Mumbai. HPLC grade water used generated using LABLINK water purification system. Pooled plasma was obtained as gift sample from Akshay Blood Bank, Pune.

➢ Instruments:

1. HPLC (UV):
- Borwin- software (version 1.50)
- Model PU 2080 Plus Intelligent HPLC pump
- Rheodyne sample injection port with 20 µl loop
- Rubitas C18 Column (250 mm □ 4.6mm, 5 µm particle size)
- Jasco UV 2075 plus detector
- UV-Visible Double beam spectrophotometer (Shimadzu Model 1780)

2. Shimadzu (model ATX-224) Electronic weighing balance
3. Sonicator: Prama solutions for laboratory
4. LABLINK–XTRAPURE water purification system. Conductivity below 0.055 µS/cm
5. Electronic pH meter
6. Cold Centrifuge (BL 165)
7. Deep Freezer (Make: Classic)

❖ Bioanalytical Method Development

❖ Preparation of standard stock solution of Lobeglitazone Sulfate

For standard stock solution accurately weighed 10 mg of Lobeglitazone Sulfate transferred to 10 ml volumetric flask and the volume was made up to 10 ml with methanol, to get standard stock solution of Lobeglitazone Sulfate (1000 µg/ml). Further dilutions were made with methanol to produce the stock solutions of 1, 2, 5, 10, 20, 30, 40, 50 µg/ml.

❖ **Selection of Analytical Wavelength**

A solution of 10 µg/ml was prepared from standard stock solution of Lobeglitazone Sulfate (1000 µg/ml) and scanned over 200-400 nm in UV Spectrophotometer. The maximum absorbance was shown at 248 nm. Hence it was selected as analytical wavelength; UV spectrum is given in Fig. 1

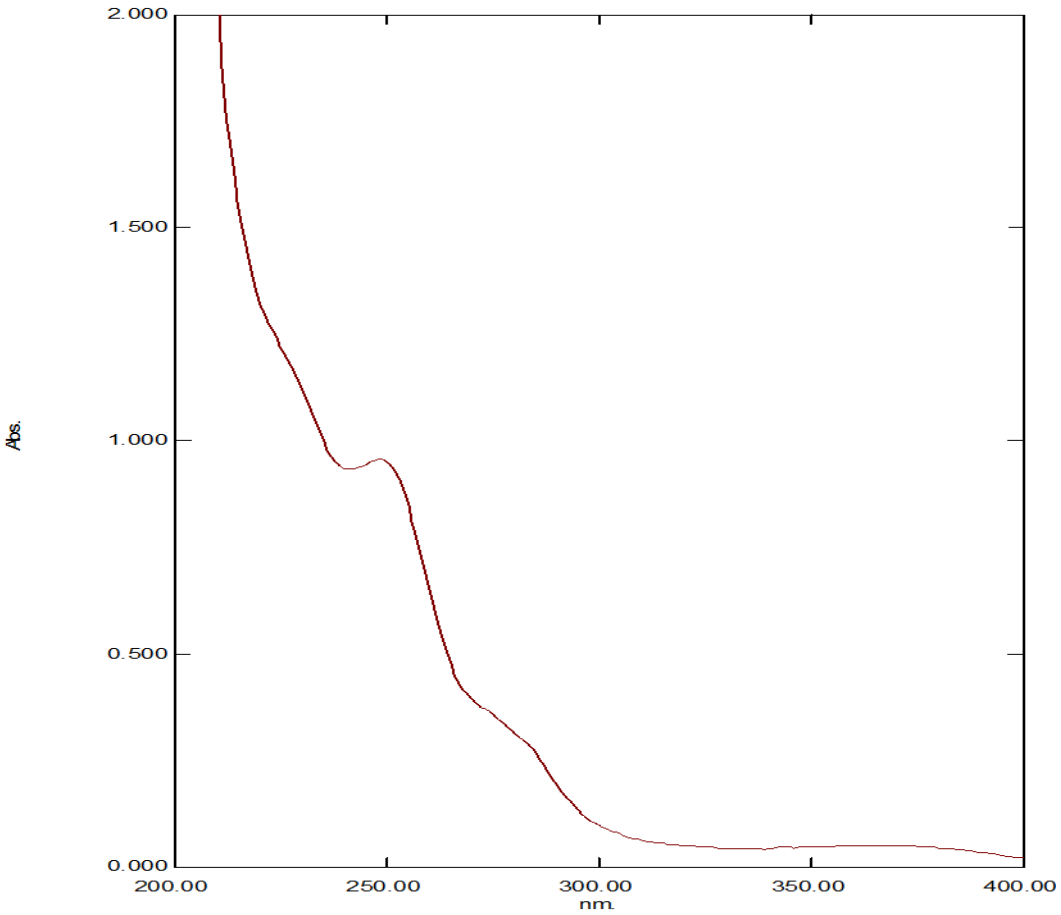


Fig 1: UV-Spectrum of Lobeglitazone Sulfate in methanol (10 µg/ml)

❖**Mobile Phase Optimization**

To achieve optimum chromatographic condition few mobile phases were checked using column Rubitas C18 Column (250 mm □ 4.6mm, 5 µm particle size). The: Acetonitrile: Methanol (20:80 v/v) system was initially tried but did not get a considerable number of theoretical plates as well as peak shape. The ratio changed (60:40 v/v) has resulted in considerable improvement of theoretical plates and appropriate peak shape with appropriate system suitability parameters. The system suitability parameters are given in the Table 1.

Table 1: System Suitability Parameters

Parameter	Obtained values
RT (min)	5.945 ± 0.164
Asymmetry	1.32
Plates (N)	3482

❖ Bioanalytical Method Validation

1. Selectivity/Specificity

Selectivity of analytical method is ability of method to differentiate and quantify the drug sample in presence of other interfering substance. The specificity of method is demonstrated by analysing blank (Mobile Phase), blank plasma, API and spiked plasma (with API). There was no any interfering peak at the same RT of Lobeglitazone Sulfate.

Fig. 2(a): Chromatogram of Blank (MP)

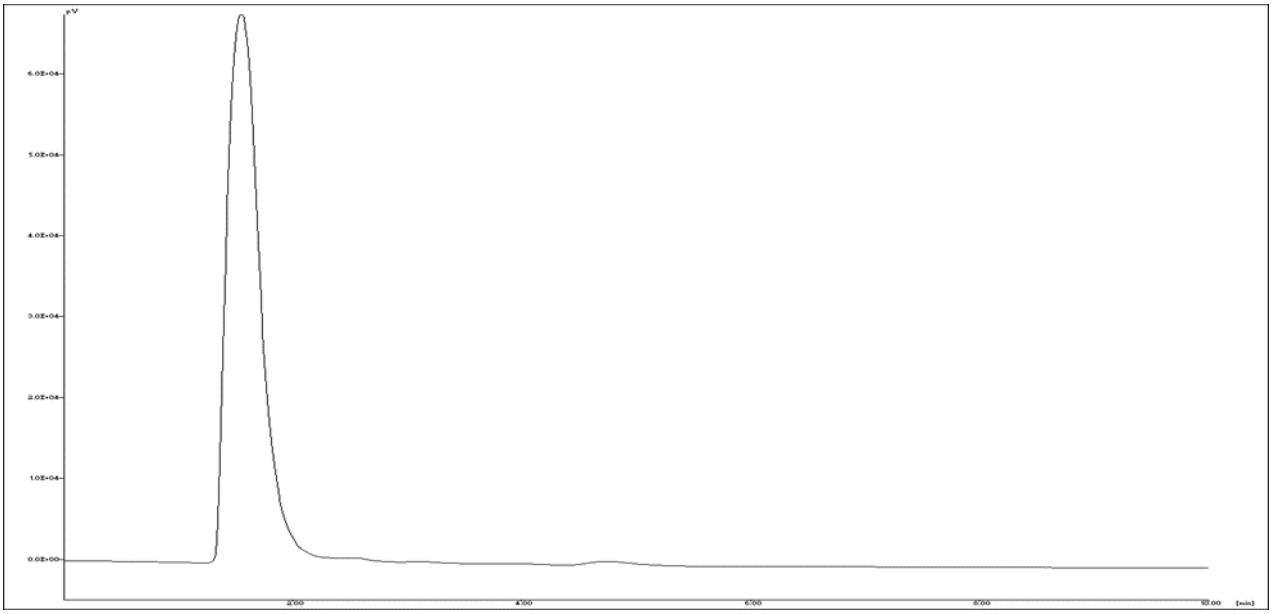


Fig. 2(b): Chromatogram of Blank Plasma

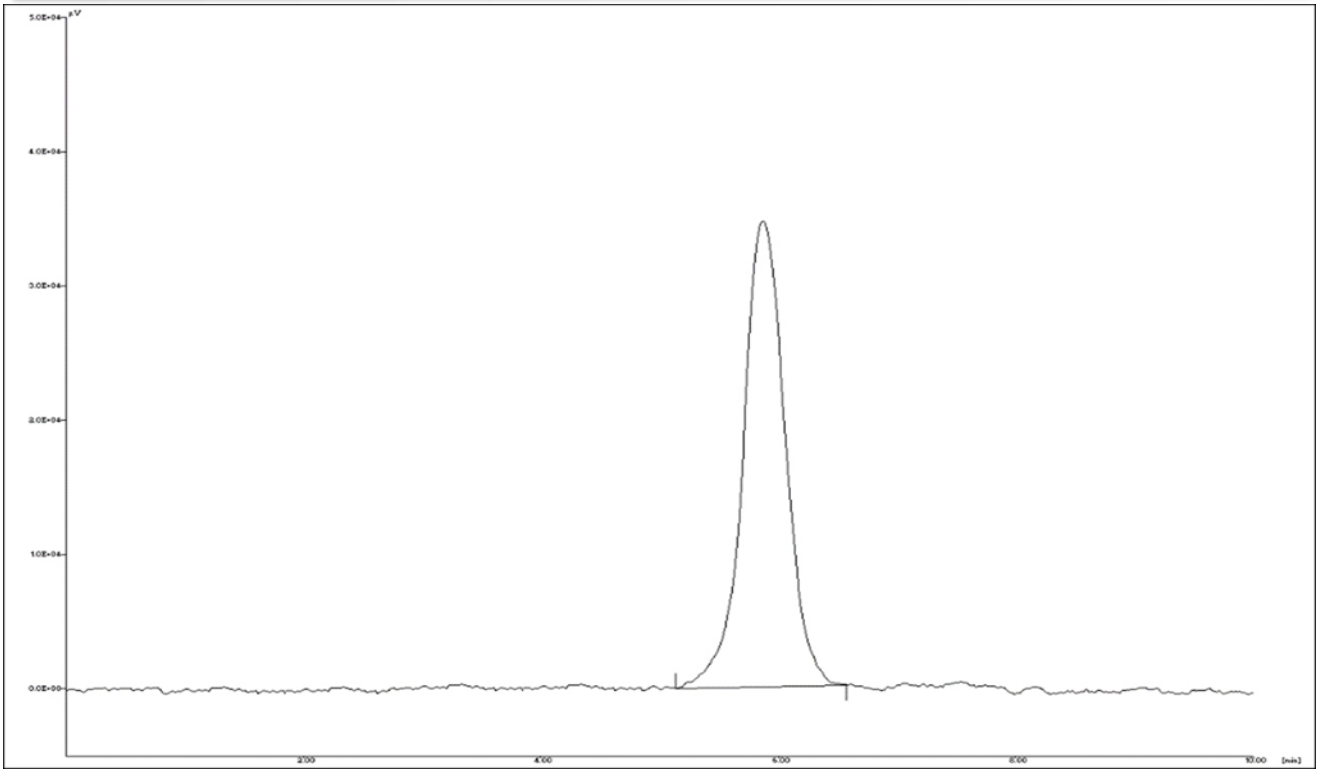


Fig. 2(c): Chromatogram of API - Lobeglitazone Sulfate

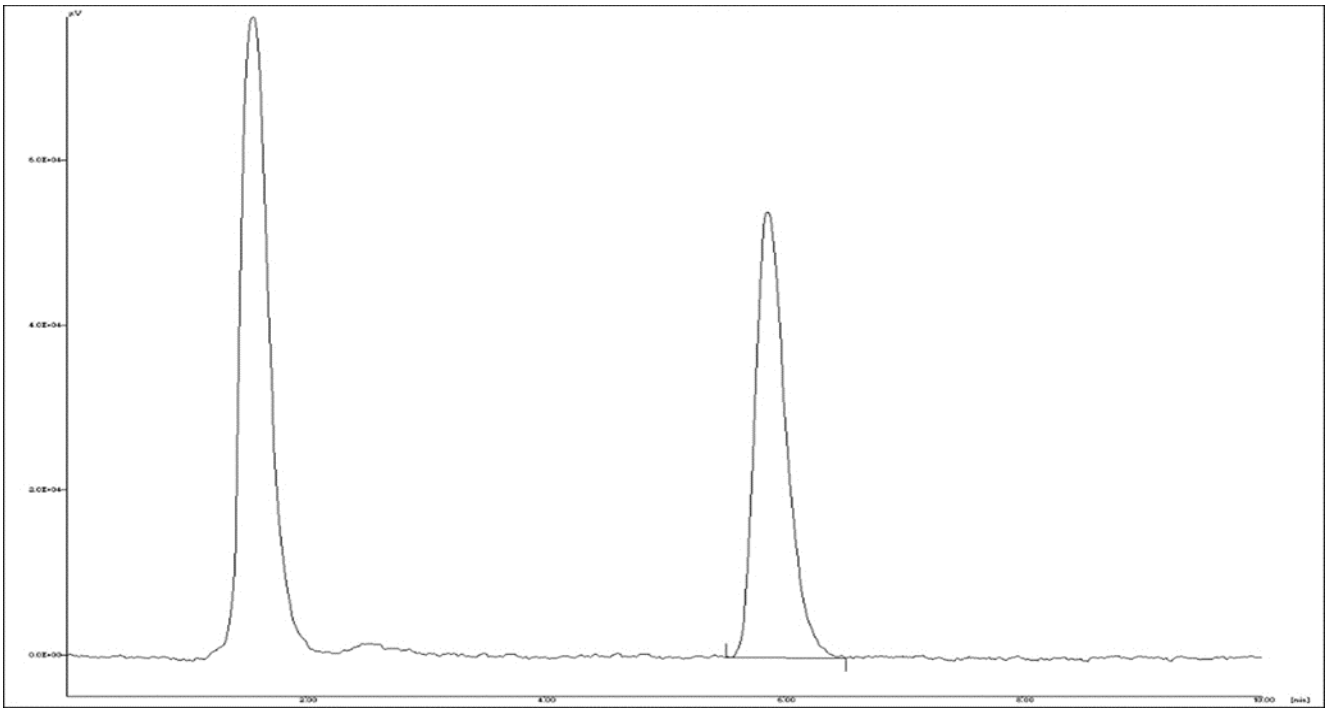


Fig. 2(d): Chromatogram of Spiked Plasma (with API - Lobeglitazone Sulfate)

2. Calibration curve / Linearity

Calibration curve or linearity of method exhibit direct proportionality between detector response and concentration of analyte of interest. Linearity was tested for the range set in concentration of 0.1-5 µg/ml. 6 replicates of QC samples were analysed and peak areas were recorded. The correlation between the known concentration and response was evaluated through a regression analysis of calibration curve constructed using an eight-point (0.1, 0.2, 0.5, 1, 2, 3, 4 and 5 µg/mL) standard calibration curve. Calibration curve was constructed with drug response on Y-axis and concentration on X-axis. The correlation coefficient (R^2) values were calculated.

Table 2: Linearity of Lobeglitazone Sulfate Standard

Conc. (µg/ml).	Area 1	Area 2	Area 3	Area 4	Area 5	Mean	SD	%CV
0.1	52208.33	44064.02	38863.37	41439.15	40978.72	43510.72	5202.28	11.96
0.2	55732.95	59025.33	63300.51	58815.12	68164.66	61007.71	4822.84	7.91
0.5	131676.55	130054.93	124023.9	157189.08	154605.02	139509.90	15256.07	10.94
1	199158.05	208453.7	231631.4	241265.12	242415.98	224584.85	19701.34	8.77
2	399936.81	390785.4	356104.84	446882.35	442743.41	407290.56	37983.19	9.33
3	578507.74	602053.73	570494.47	600813.4	584363.77	587246.62	13862.01	2.36
4	916949.22	835717.16	826287.28	939502.2	862858.56	876262.88	49939.42	5.70
5	981864.52	985779.8	985201.49	1122407.2	1118208.91	1038692.38	74534.34	7.18

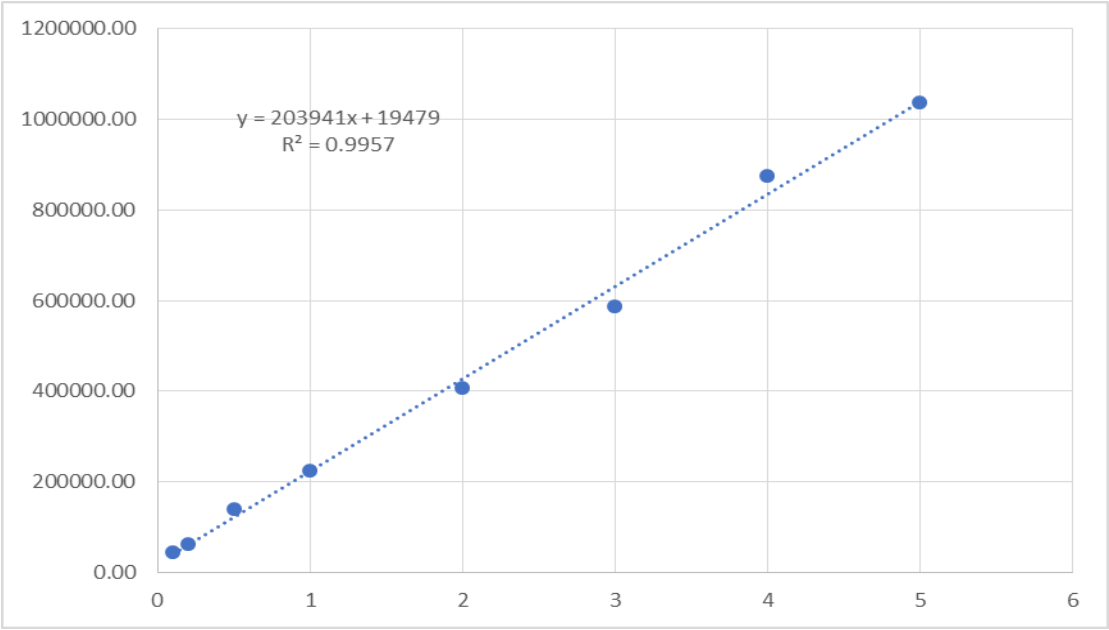


Fig. 3: Calibration curve for Lobeglitazone Sulfate Standard

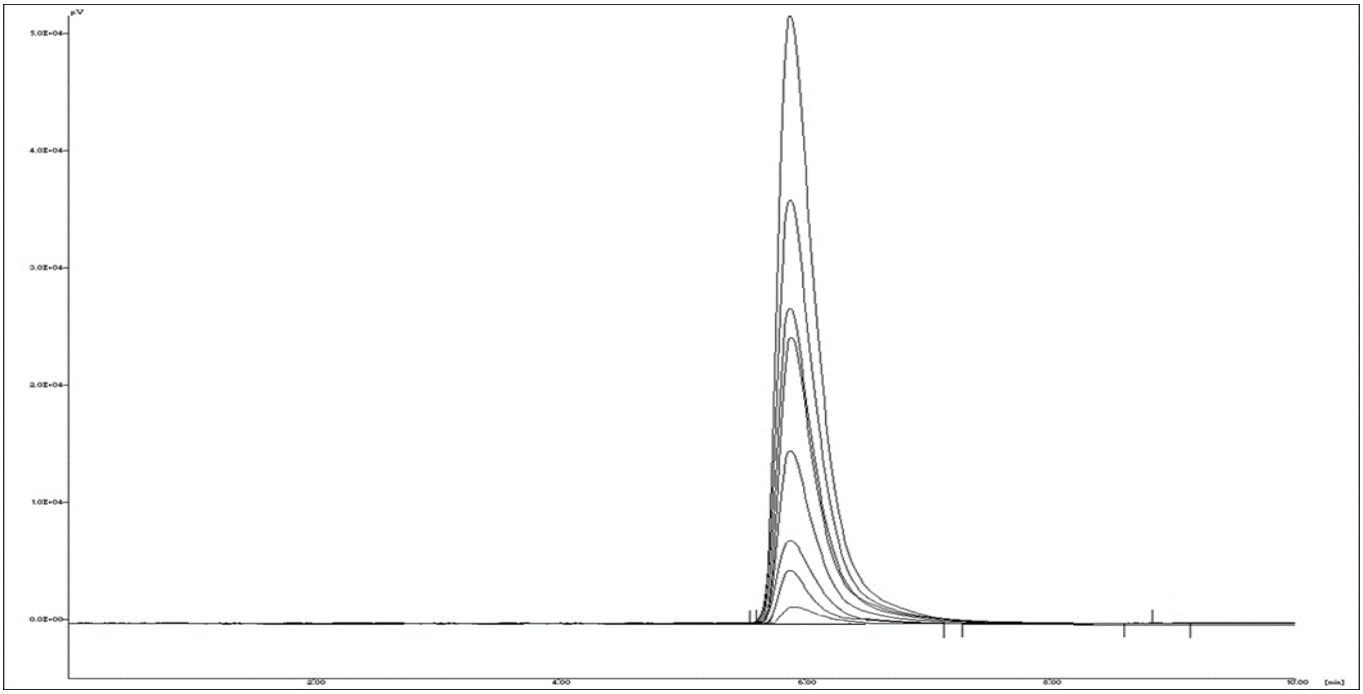


Fig. 4: Overlay Chromatographs of Linearity for Lobeglitazone Sulfate (0.1 to 5 µg/ml)

❖ **Linearity of Lobeglitazone Sulfate in spiked plasma**

Sample preparation consisted of the addition of 0.1 ml of plasma sample in 10 ml test tubes, then 0.1 ml of Standard stock solutions was added, then 0.8 ml of methanol was added as precipitating agent to produce the final conc. of 0.1, 0.2, 0.5, 1, 2, 3, 4, 5 µg/ml, then vortex for 10 minutes, centrifugation for 20 minutes at 3000 r.p.m., then the supernatant was injected into HPLC system. The protein precipitation was the preferred choice of separation because of the minimized steps in extraction of drug from matrix.

Table 3: Linearity of Lobeglitazone Sulfate in spiked plasma

Conc. (µg/ml).	Area 1	Area 2	Area 3	Area 4	Area 5	Mean	SD	%CV
0.1	41186.57	36316.5	32030.25	34532.63	34532.63	35719.72	3415.89	9.56
0.2	55120.5	58376.7	62604.9	58168.8	66632.87	60180.75	4483.72	7.45
0.5	130229.55	128625.75	122661	150279.67	149393.61	136237.92	12734.02	9.35
1	229797.75	240523.5	267267	278382.83	275101.28	258214.47	21762.10	8.43
2	395541.9	386491.05	352191.6	417417.58	422844.83	394897.39	28209.42	7.14
3	591222.2	615285.68	583032.81	614018.09	630324.96	606776.75	19268.34	3.18
4	906872.85	826533.45	817207.2	908529.6	872553.6	866339.34	43185.87	4.98
5	917126.2	920783.33	920243.15	1048402.33	1017695.75	964850.15	63211.69	6.55

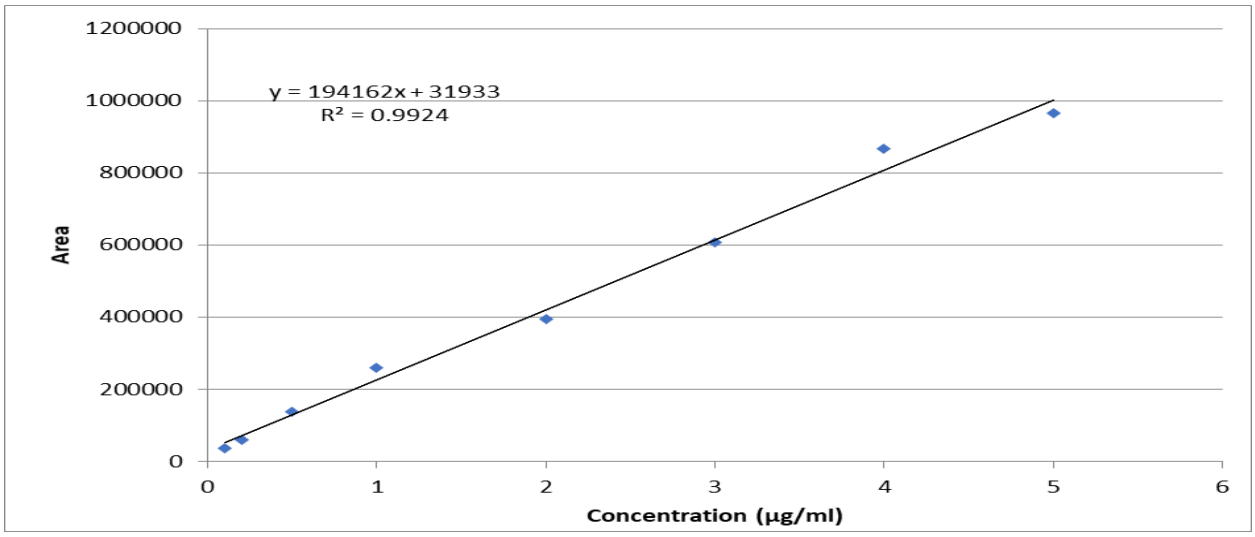


Fig. 4: Calibration curve for Lobeglitazone Sulfate in spiked plasma

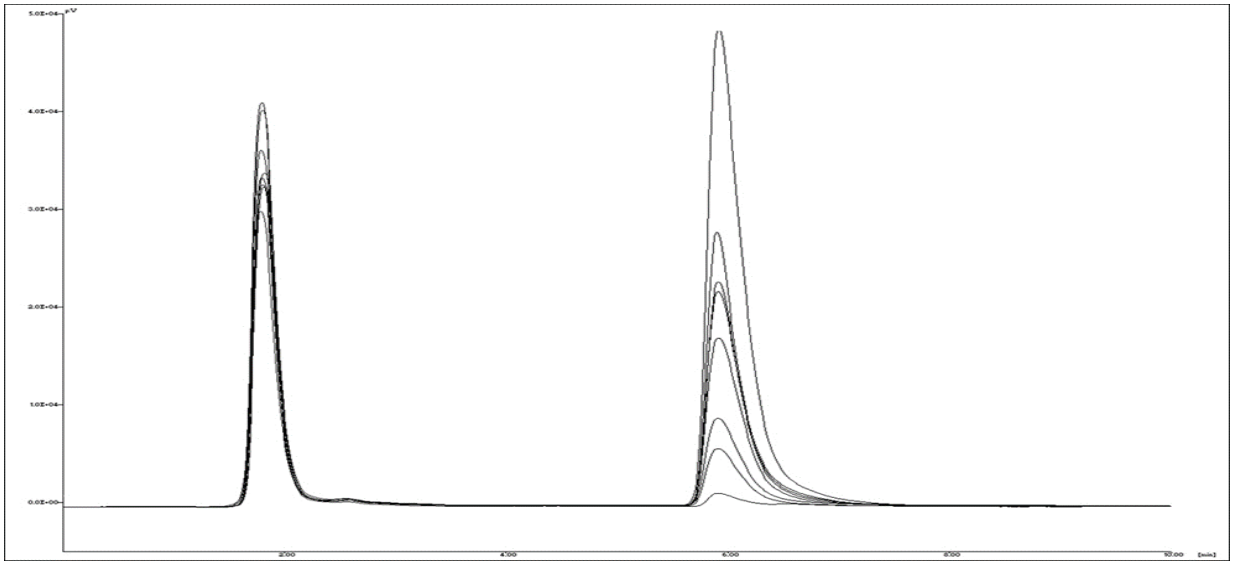


Fig. 5: Overlay Chromatographs of Linearity for Lobeglitazone Sulfate in spiked plasma (0.1 to 5 µg/ml)

1. Accuracy

Accuracy was estimated by using minimum 5 replicates of 3 concentrations i.e., at LQC (0.5 µg/ml, MQC (2µg/ml), HQC(4µg/ml). The % mean accuracy was determined for all QC samples. Drug area was substituted in regression equation (y=mx+c) to get the concentration of the given sample. The deviation of the average from the theoretical value served as the estimation of accuracy. The accuracy at each concentration level should be within ± 15% of the nominal concentration.

Table 4: Results of Accuracy Studies

Replicates	LQC (0.5 µg/ml)		MQC (2 µg/ml)		HQC (4 µg/ml)	
	Area	Calcu. Conc	Area	Calcu. Conc	Area	Calcu. Conc
1	124340.68	0.476	393500.9	1.862	809223.19	4.003
2	126277.33	0.486	387869.54	1.833	766882.73	3.785
3	126021.48	0.485	389763.02	1.843	771879.44	3.811
4	122329.99	0.466	403626.1	1.914	773481.35	3.819
5	123532.36	0.472	392129.38	1.855	792971.31	3.920
Mean Area	124500.37		393377.79		782887.60	
SD	1669.17		6123.91		17755.69	
%CV	1.34		1.56		2.27	
%Accuracy	**95.35**		**93.08**		**96.69**	

4. Precision

Closeness of the individual measured value of the drug analyte among all aliquots of same volume of the plasma was assessed by injecting six replicates at, LQC, MQC and HQC levels. The precision of the method performed on HPLC-UV was evaluated by determining the % CV of the repeated injections. Intraday precision was evaluated by determining % CV of the response of the repeated injections injected on the same day. On the contrary, Interday precision was calculated after comparison of the measured values of the samples injected on three different days. According to the ICH M10 guideline, the precision (% CV) of the concentrations determined at each level should not exceed ±15%.

Table 5: Intraday Precision Studies

Concentration Level	Morning	Afternoon	Evening	Mean	SD	% CV
LQC (0.5 µg/ml)	138332.3	144148.33	139272.95	140584.53	3121.97	2.22
MQC (2 µg/ml)	405516.46	387359.63	418439.75	403771.95	15613.33	3.87
HQC (4 µg/ml)	864526.31	868349.47	849982.22	860952.67	9691.09	1.13

Table 6: Inter-day Precision Studies

Concentration Level	Day 1	Day 2	Day 3	Mean	SD	% CV
LQC (0.5 µg/ml)	141644.45	142683.98	127684.37	137337.60	8376.08	6.10
MQC (2 µg/ml)	413351.27	393856.47	406787.88	404665.21	9919.23	2.45
HQC (4 µg/ml)	887537.69	893780.06	839156.18	873491.31	29898.46	3.42

5. Recovery

Recovery studies were performed by comparing the chromatographic response for samples after extraction at LQC, MQC and HQC with standard samples in three replicates. Recovery need not be 100 percent, but the extent of the recovery of an analyte should be consistent and reproducible.

Table 7: Results of Recovery Studies

Conc level	Area		%Recovery	%Mean Recovery
	Standard	Spiked plasma		
LQC (0.5µg/ml)	148003.55	135657.36	91.66	
	147452.64	138999.03	94.27	**93.87**
	147404.25	141031.47	95.68	
MQC (2µg/ml)	406394.04	397596.68	97.84	
	419137.73	405321.7	96.70	**96.81**
	408765.54	391930.78	95.88	
HQC (4µg/ml)	903857.27	894640.37	98.98	
	894191.63	844313.08	94.42	**97.07**
	901842.21	882045.13	97.80	

6. Carry Over:

Carryover is the impact of the previous injection to the next injection of the analyte. It was determined by injecting blank samples after HQC injection of 4 µg/ml. According to the guidelines, response of samples should be below the LLOQ. Chromatograms obtained are shown below.

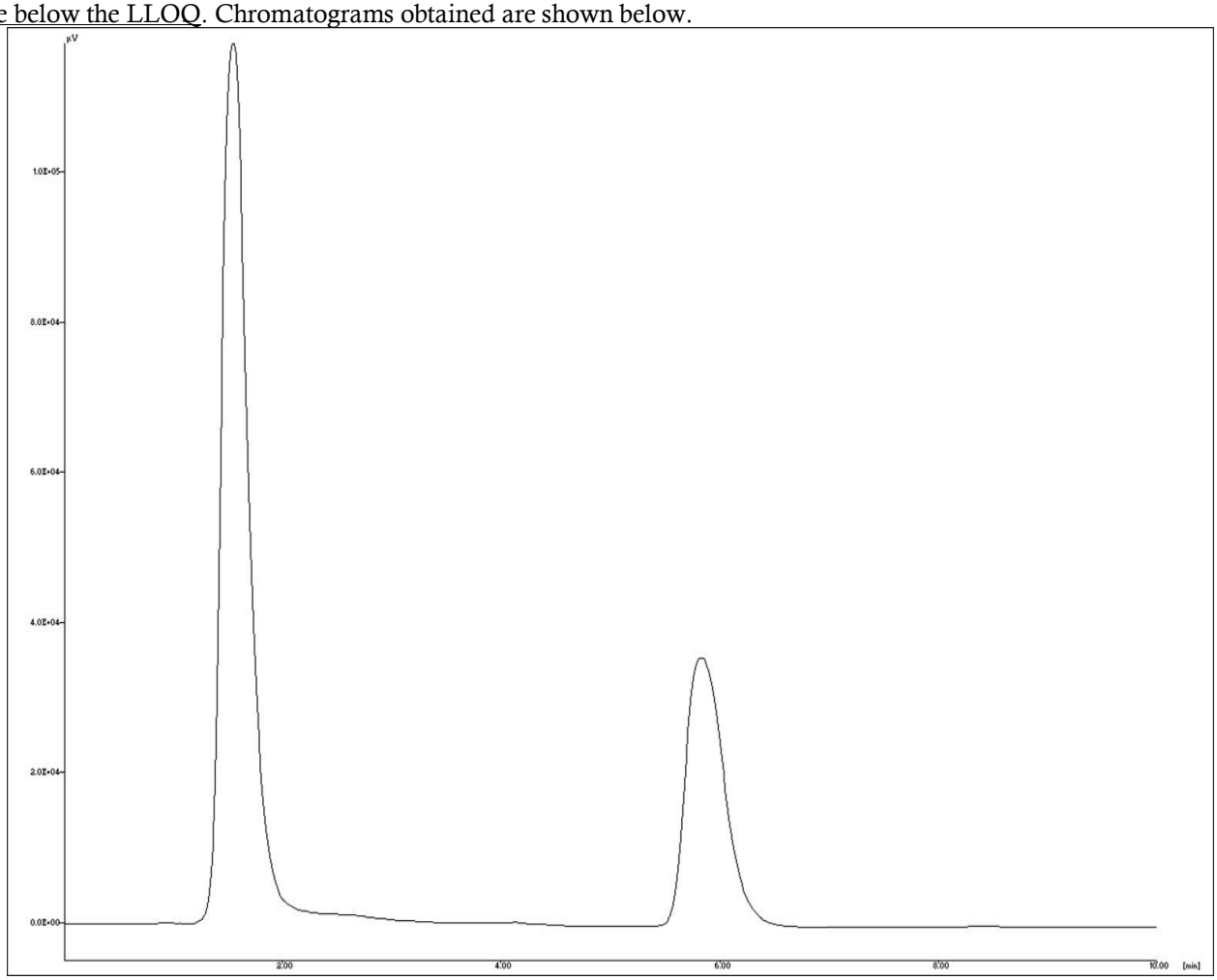


Fig. 6 (a): Chromatogram at HQC (4 µg/ml)

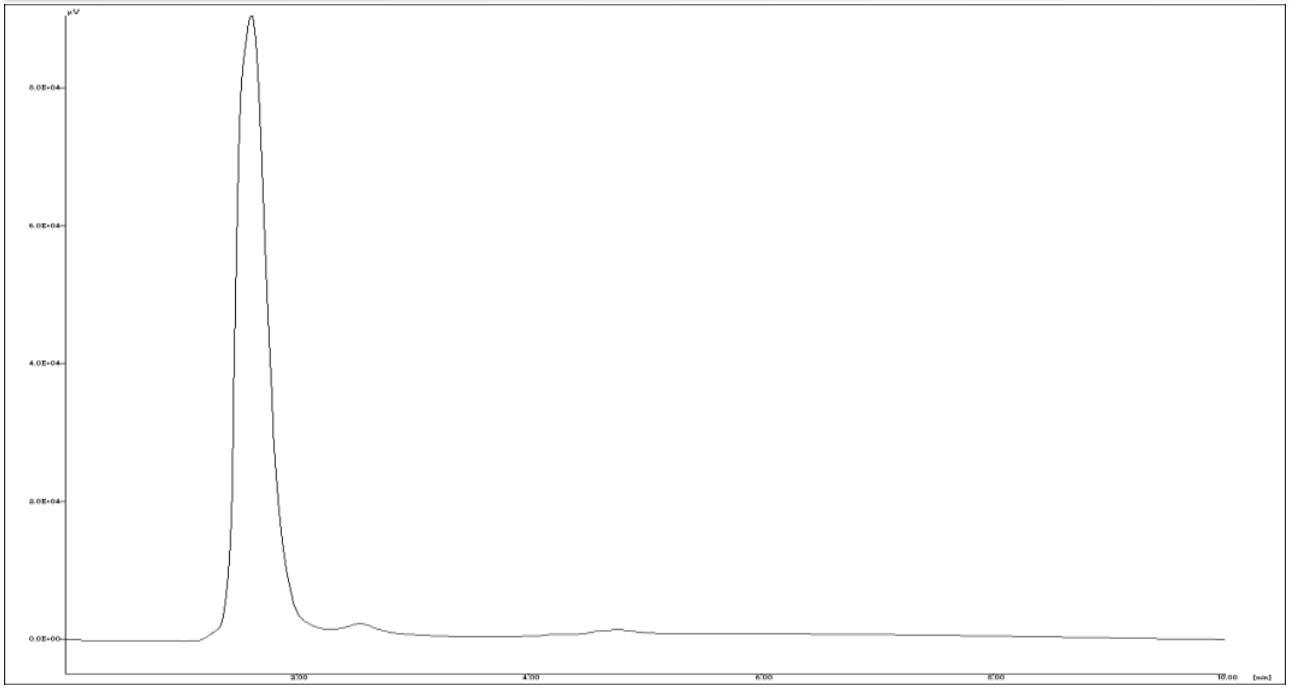


Fig. 6 (b): Chromatogram Blank

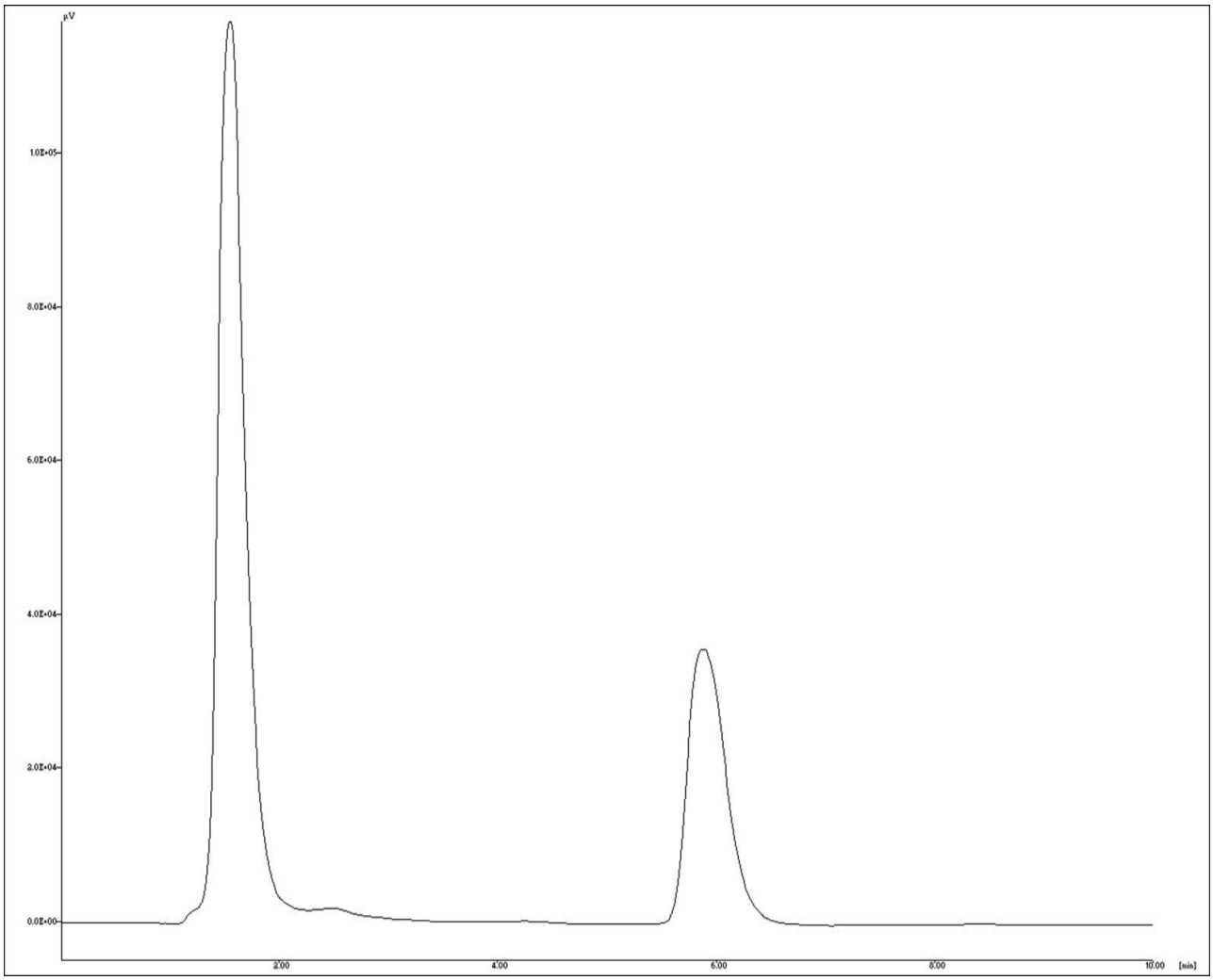


Fig. 6 (c): Chromatogram at HQC (4 µg/ml)

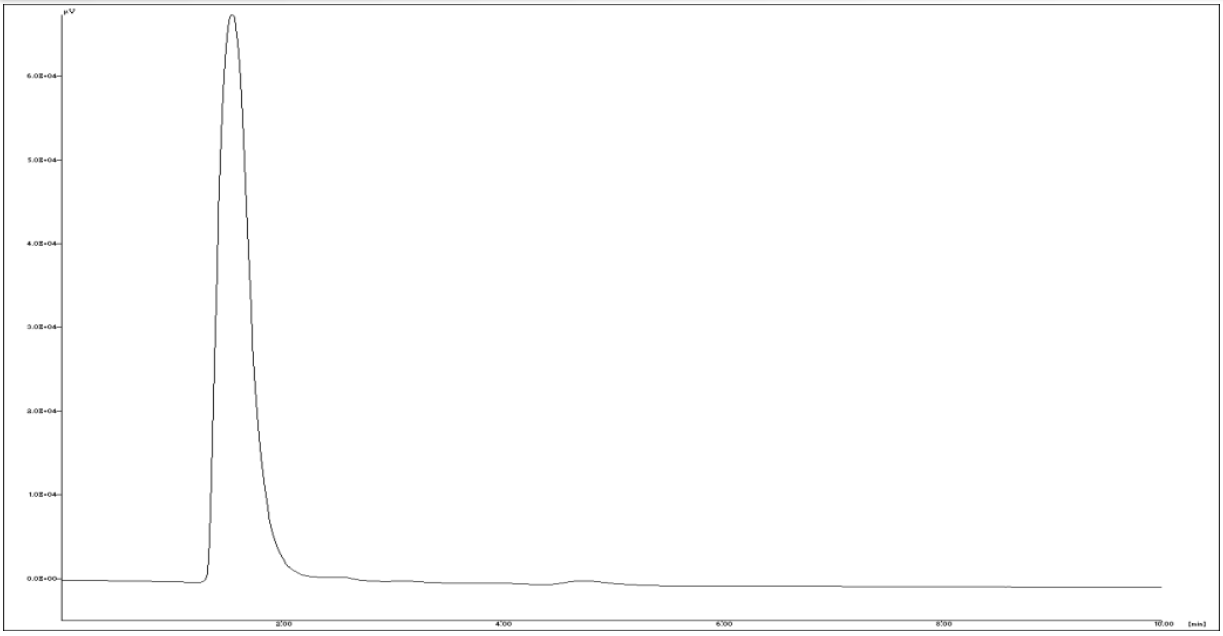


Fig. 6 (d): Chromatogram Blank

7. Stability

The purpose of determining stability is to detect any degradation of analyte occurred during entire process of sample collection, storage, extraction, and analysis. It is recommended to determine stability during short term storage, long term storage as well as during freeze thaw cycles. Stability samples should be compared with freshly prepared QC samples. The acceptance criteria for % mean stability is **85-115%.**

Lobeglitazone Sulfate stability was evaluated using two concentration levels i.e., at LQC and HQC.

For each sample to be tested mean of 3 samples was taken that were stressed, stored, and analyzed.

Following types of stability studies were performed:

- **Short term (Bench Top) stability:** LQCs and HQCs were kept at room temperature for 4 hours and checked for its stability.
- **Long term stability:** LQCs and HQCs were kept in deep freezer at -70 ^{0}C for 7 days, brought to room temperature and then checked for its stability.
- **Freeze thaw stability:** The stability of low- and high-quality concentration samples was determined after three freeze thaw cycles stored at -20 ^{0}C till it freezes, brought to room temperature, and then checked for its stability.
- **Stock solution stability:** Stock solution stability of the drug was determined for 2 hrs at room temperature. Comparing them against the freshly weighed stock solution assessed for stability.

Table 8: Results of Stability Studies

Stability	Conc. (µg/ml)	Area	Avg. Area	SD	%CV	%Mean Stability
Freeze thaw stability (three cycles)	LQC	130892.99	133253.30	2716.57	2.04	96.78
		132644.19				
		136222.71				
	HQC	829627.29	837535.12	37139.82	4.43	95.56
		804986.08				
		877991.99				
Short term stability (for 4h at RT)	LQC	136596.65	134115.72	2426.97	1.81	97.41
		131746.58				
		134003.92				
	HQC	887025.14	867605.11	19104.70	2.20	98.99
		866958.02				
		848832.18				

Long term stability (for 7 days at -20^{0}C)	**LQC**	131214.87	133480.24	1969.43	1.48	96.95
		134440.5				
		134785.34				
	HQC	835248.53	855776.36	24005.52	2.81	97.64
		882171.64				
		849908.90				
Stock solution stability (for 2 hrs)	**LQC**	138002.52	135839.29	3553.50	2.62	98.66
		131738.13				
		137777.23				
	HQC	910522.53	873352.93	43496.28	4.98	99.64
		884021.21				
		825515.06				

8. Matrix Effect

A matrix effect is defined as an alteration of the analyte response due to interfering and often unidentified component(s) in the sample matrix. During method validation the matrix effect between different independent sources/lots should be evaluated. No matrix interference was observed.

Summary of Validation Parameters is shown in Table 8.

Table 9: Summary of Bioanalytical Validation Parameters

Sr. No.	Validation Parameter	Results	
1.	**Linearity**	y = 194162x + 31933 R^2 = 0.9924	
2.	**Range**	0.1-5 µg/ml	
3.	**Precision**	**Conc**	**% CV**
	A) Intraday precision	LQC	2.22
		MQC	3.87
		HQC	1.13
	B) Interday precision	LQC	6.10
		MQC	2.45
		HQC	3.42
4.	**Accuracy**	**% Mean ± % CV**	
	LQC	95.35 ± 1.34	
	MQC	93.08± 1.56	
	HQC	96.69 ± 2.27	
5.	**Recovery**	**% Mean**	
	LQC	93.87	
	MQC	96.81	
	HQC	97.07	
6.	**Stability**	**% Stability**	
	Freeze thaw stability	LQC	96.78
		HQC	95.56
	Short term (Bench Top) stability	LQC	97.41
		HQC	98.99
	Long term stability	LQC	96.95
		HQC	97.64
	Stock solution stability	LQC	98.66
		HQC	99.64
7.	**Specificity**	Specific	

CONCLUSION

A Bioanalytical method was developed for the estimation of lobeglitazone in Human Plasma by HPLC method and was validated. Lobeglitazone is an antidiabetic drug from the class called thiazolidinedione. The method was developed using Acetonitrile: Methanol (60:40 v/v). The peaks obtained for the drug of interest by the present method

was symmetrical in nature with acceptable tailing factor and from the plasma endogenous proteins by Protein precipitation Extraction. The retention time of lobeglitazone was shorter and proves that the method is rapid. All the analytical validation parameters were determined and found in the limit as per ICH guidelines, which indicates the validity of the method. The method was validated with respect to accuracy, precision, linearity and robustness. The results of linearity, intraday and interday precision study and capability of the extraction method were within the limits of Bioanalytical method development. The method was linear with a correlation coefficient of acceptable agreement, which is suitable for the estimation of lobeglitazone human plasma. The method developed can be used in therapeutic drug monitoring units, bioequivalence and bioavailability studies, pharmacokinetic and toxicology studies of lobeglitazone.

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