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STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR QUANTITATIVE ESTIMATION OF PIOGLITAZONE AND VILDAGLIPTIN IN SYNTHETIC MIXTURE

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ABSTRACT

The RP-HPLC was used to develop the method and for validation of Pioglitazone and Vildagliptin from synthetic mixture. For chromatographic condition the mobile phase used was Acetonitrile: Methanol: Water (20:40:40 v/v) on the stationary phase of Hypersil ODS C18 column (250 mm*4.6 mm), the detection observed was at 210nm.Forced degradation study was performed by applying stress condition like Acid hydrolysis, Oxidation, Alkali Hydrolysis and Thermal Degradation. The response of the calibration curve was found to be linear in range by Pioglitazone and Vildagliptin 0.9989 and 0.9975 respectively. The retention time observed is 2.324 min for Pioglitazone and 4.241 min for Vildagliptin at the run time of 15 minutes. The limit of detection for Pioglitazone is 0.42 µg/mL and Vildagliptin is 2.017 µg/mL. However, the limit of quatification for pioglitazone is 1.28 µg/mL and Vildagliptin is 6.11 µg/mL. The accuracy for the pioglitazone is 99.14% and Vildagliptin is 99.26% as per method performed. The limit for precision is RSD<2 therefore, for pioglitazone is 0.83-1.03 and vildagliptin is 0.87-1.20. The experiment was carried out as per International Conference on Harmonization (ICH) guidelines and the statistical analysis of the data is linear, precise, accurate and robust. Therefore, the method can be successfully employed for the synthetic mixture of Pioglitazone and Vildagliptin in pharmaceutical formulation.

KEYWORDS: Pioglitazone, Vildagliptin, RP-HPLC, Forced Degradation

INTRODUCTION

Currently, the combination of both the drugs is under clinical phase-III trial conducted by Synokem Pharmaceuticals at a dose level of 30mg or 15mg of Pioglitazone and 100mg of Vildagliptin. When detailed review of literature was carried out, it was found out that, there is one closest reported RP-HPLC method which determines glimepiride, pioglitazone and vildagliptin from bulk and marketed preparation. But the method is not stability indicating. (Amin MM et al. Optimization and Validation of HPLC Method for Simultaneous Determination of Vildagliptin, Pioglitazone Hydrochloride and Glimepiride in Bulk and Tablets. IOSR Journal of Pharmacy and Biological Sciences. 2017, 12(2): 18-27.) In the reported method, Vildagliptin is being eluted at around 1.1 minutes which can not be termed as separation as it elutes at the column dead volume and method can not be accepted as per standard guideline. Use of chromatographic conditions such as flow rate of 1.5 ml/min and buffer concentration of 0.05M is quite harsh and column life is not sustainable with this condition. With the consideration of all the above mentioned points, it was aimed at "Stability Indicating RP-HPLC method development and validation of Pioglitazone and Vildagliptin in synthetic mixture"

1. Developing and optimizing RP-HPLC method for separation of Vildagliptin and Pioglitazone.
2. To produce forced degradation conditions for the formulation and claim the stability of mentioned components under degradation conditions (Degradation between 10-20%)
3. To demonstrate that the formed degradation products do not interfere with the estimation of Vildagliptin and Pioglitazone (Specificity)

MATERIALS AND METHODS

Make	Shimadzu
Model	LC 2010
Type	Binary Gradient
Detector	UV detector
Software	LC solution
Column	Hypersil ODS C_{18} (250*4.6 mm, 5 Micro-meter)
Pump	High Pressure Gradient (Reciprocating pump)

Table 1 Instrument specification for High Performance LC

Make	Mettler Toledo
Sensitivity	0.1 milligram
Minimum weighing Capacity	1 milligram

Table 2 Instrument specification for weighing balance

Make	Gallenkamp
Design No.	889339

Table 3 Instrument Specification for melting point apparatus

Make	: Shimadzu
Model	: UV 1800
Type	: Double beam spectrophotometer
Detector	: Photodiode
Scanning Range	: 190 – 1100
Output	: %T & Absorbance
Software	: U.V. Probe 2.42

Table 4 Instrument Specification for UV double beam Spectrophotometer

SELECTION OF ANALYTICAL /DETECTION WAVELENGTH

A Working standard of PIO (15 µg.ml^{-1}) and VIL (50 µg.ml^{-1}) using methanol as a solvent, were scanned in UV 200-400 nm region and overlapped.

OPTIMISATION OF CHROMATOGRAPHIC CONDITIONS

Trial 1

Column: Hypersil ODS C18 (250*4.6 mm, 5µm)

Mobile Phase: Methanol: Water (50:50 v/v),

Detection: 210 nm

Flow rate: 1 ml/min

Run Time: 20 minutes

Trial 2

Column: Hypersil ODS C18 (250*4.6 mm, 5µm)

Mobile Phase: Methanol: Water (50:50 v/v),

Detection: 210 nm

Flow rate: 1 ml/min

Run Time: 15 minutes

Trial 3

Column: Hypersil ODS C18 (250*4.6 mm, 5µm)

Mobile Phase: Acetonitrile: Methanol: Water (20:40:40 v/v)

Detection: 210 nm

Flow rate: 1 ml/min

Run Time: 15 minutes

PREPARATION OF STANDARD STOCK SOLUTION

	Preparation of solution
Master Stock Solution:	Accurately weighed 30 mg PIO dissolved in 100 ml methyl alcohol (300 µg.ml $^{-1}$)
Stock Solution:	Withdraw 1.0 ml from Master Stock Solution and make up to 10 ml with methyl alcohol (30 µg.ml^{-1})

Table 5 Preparation of standard stock solution of PIO

	Preparation of solution
Master Stock Solution:	Accurately weighed 100 mg VIL dissolved in 100 ml methyl alcohol (1000 µg.ml $^{-1}$)
Stock Solution:	Withdraw 1.0 ml from Master Stock Solution and make up to 10 ml with methyl alcohol (100 µg.ml^{-1})

Table 6 Preparation of standard stock solution of VIL

	Preparation of solution
Master Stock Solution:	Accurately weighed 100 mg VIL dissolved in 100 ml methyl alcohol (1000 µg.ml $^{-1}$)
Stock Solution:	Withdraw 1.0 ml from Master Stock Solution and make up to 10 ml with methyl alcohol (100 µg.ml^{-1})

Table 7 Preparation of standard stock solution of mixture

SYSTEM SUITABILITY PARAMETERS:

Solution of PIO+VIL (30+100 µg.ml^{-1}) was injected 3 times for determination of System suitability parameters which includes Retention time (R_t), Tailing factor (T_f), Resolution (R_s) and number of theoretical plates. System suitability parameters for selected concentration were determined by C.V.

FORCED DEGRADATION STUDIES:

Acid Hydrolysis studies:

Trials	Condition	Inference	% Degradation	
			PIO	VIL
Trial 1	0.1N HCl, refluxed at 40°C for 30 minutes	No degradation observed	3.12 %	3.07 %
Trial 2	0.2N HCl, refluxed at 40°C for 60 minutes	Minor degradation was observed for both the drugs	6.24 %	5.97 %
Trial 3	0.5N HCl, refluxed at 60°C for 1 hour	Degradation has been observed for both the drugs along with degradants	13.58 %	13.39 %

Base Hydrolysis studies:

Trials	Condition	Inference	% Degradation	
			PIO	VIL
Trial 1	0.1N NaOH, refluxed at 60°C for 30 minutes	Minor degradation was observed for both the drugs	4.81 %	5.22 %
Trial 2	0.2N NaOH, refluxed at 60°C for 1 hour	Degradation has been observed for both the drugs along with degradants	14.40 %	15.08 %

Oxidative stress Trials

Trials	Condition	Inference	% Degradation	
			PIO	VIL
Trial 1	1% v/v H_2O_2, refluxed at 60°C for 1 hour	Minor degradation was observed for both the drugs	1.99 %	2.34 %
Trial 2	3% v/v H_2O_2, refluxed at 60°C for 1 hour	Degradation has been observed for both the drugs along with degradants	10.93 %	10.51 %

Thermal stability Trials

Trials	Condition	Inference	% Degradation	
			PIO	VIL
Trial 1	2 hours at 60ºC	No degradation was observed for both the drugs	2.37 %	3.68 %
Trial 2	3 hours at 70ºC	Degradation has been observed for both the drugs along with degradants	11.73 %	10.24 %

Table-8: Conclusion of Forced degradation studies:

Forced Degradation Condition	Acid Hydrolysis	Base Hydrolysis	Oxidative stress	Thermal stability
Amount of Drug taken (milligram)	15 milligram PIO and 50 milligram VIL			
STEP - I	Transferred in 100 milliliters volumetric flask and add 50 milliliters of 0.5N HCl	Transferred in 100 milliliters volumetric flask and add 50 milliliters of 0.2N NaOH	Transferred in 100 milliliters volumetric flask and add 20 milliliters of 3% Hydrogen peroxide	Transferred into petri dish and kept in hot air oven 70 Degrees for 3 Hrs.
STEP - II	Heat the flask at 60 Degrees on Hot plate for 1 Hr. After the heating cool down the solution and neutralize the contents with 0.5N KOH or NaOH. Make up the volume up to mark with mobile phase if necessary	Heat the flask at 60 Degrees on Hot plate for 1 Hr. After the heating cool down the solution and neutralize the contents with 0.2N HCl. Make up the volume up to mark with mobile phase if necessary	Heat the flask at 60 Degrees on Hot plate for 1 Hr. After the heating cool down the solution and dilute the solution up to the mark with mobile phase if necessary	Rinse the content with mobile phase in 100 milliliters volumetric flask and make up the volume with the same.
STEP - III	From the above solution take 1.0 milliliters and transfer it to a 10-millilitre volumetric flask and dilute the contents up to mark with mobile phase. (PIO+VIL = 15+50 $\mu g.ml^{-1}$).			
STEP - IV	above solution was injected and % degradation was calculated by comparing the obtained area of treated sample with controlled sample (zero-hour sample). whole procedure was repeated in same manner for blank.			

VALIDATION AND DEVELOPED RP-HPLC METHOD FOR ESTIMATION OF PIO AND VIL

Linearity and range

Preparation of Solution for linearity studies:

For the purpose of linearity, accurately weighed amount of PIO (30 mg), and VIL (100 mg) was taken into the volumetric flask (100 ml) and volume of the flask was raised to 100 ml with methyl alcohol to give stock solution containing 300 µg/ml of PIO, and 1000 µg/ml of VIL. Various aliquots from this stock solution were transferred to another 10 ml volumetric flask and volume was raised to the mark with mobile phase to give final solutions containing 15+50, 30+100, 45+150, 60+200 and 75+250 µg/ml of PIO and VIL respectively.

Concentration of Stock solution	Volume taken (ml)	Dilution volume with methanol	Final concentration (PIO+VIL)
300 µg/ml of PIO and 1000 µg/ml of VIL	0.5	Up to 10 ml	15+50
	1.0	Up to 10 ml	30+100
	1.5	Up to 10 ml	45+150
	2.0	Up to 10 ml	60+200
	2.5	Up to 10 ml	75+250

Table 9 Preparation of dilutions for Linearity studies

All above solutions were injected at volume of 20 µL into column by employing optimized chromatographic conditions.

Repeatability:

Prepared standard working solution of mixtures having concentration of PIO (15 to 75 µg/ml) and VIL (50 to 250 µg/ml) were injected at volume of 20 µL into column by employing optimized chromatographic conditions. Each standard mixture was injected 5 time and peak area was monitored. Each concentration was monitored for repeatability by RSD.

Limit of Detection (LOD) and Limit of Quantification (LOQ):

LOD and LOQ were determined by Statistical method by utilization of repeatability data.

$$LOD = 3.3\ X\ (\frac{\sigma}{S})$$

$$LOQ = 10\ X\ (\frac{\sigma}{S})$$

Where, σ = Standard deviation of intercept

S = mean of slope

Accuracy:

Accuracy of the analytical method has been performed by spiking of placebo with the standard. Spiking of the placebo was performed at 50, 100 and 150 % of the target concentration.

Concertation of stock solution	300 µg/ml of PIO and 1000 µg/ml of VIL			
Volume taken from SS	-	0.5 ml	1.0 ml	1.5 ml
Quantity of Placebo added	200 mg	200 mg	200 mg	200 mg
Volume made up with	10 ml	10 ml	10 ml	10 ml
Diluent	Methanol	Methanol	Methanol	Methanol
Identification	Unspiked	50 % Spiked	100 % Spiked	150 % Spiked
Concentration PIO+VIL	-	15+50	30+100	45+150

Each solution was chromatographed for 3 time and area obtained was subjected to statistical analysis to get idea about mean % recovery.

Composition of Placebo: HPMC (4 mg), MCC (190 mg), Magnesium stearate (4 mg), Talc (2 mg). Role of HPLC-Film forming agent, MCC- Directly compressible material, MS, gliding agent, Talk, Lubricating agent.

Intra-day and Inter-day Precision:

- Method precision was determined by performing intraday and interday precision.
- Mixture that represents overall range (PIO+VIL = 15+50, 45+150 and 75+250 µg/ml) were analyzed on same day at different time interval for intraday precision.
- Mixture that represents overall range (PIO+VIL = 15+50, 45+150 and 75+250 µg/ml) were analyzed on different days for interday precision.

Robustness:

Following parameters were altered one by one for determination of robustness of the method and their effect was observed by comparing with the standard preparation.

i) Mobile phase flowrate (± 0.1 mL/min), optimized flowrate was 1.0 mL/min.

ii) Mobile phase composition (± 2 mL), in optimized ratio

Determinations of PIO+VIL = 30+100 µg/mL for each alteration were carried out and RSD was measured.

Assay:

Sample Preparation	
Label Claim: Composition of synthetic mixture: HPMC (4 mg),MCC (190 mg), Magnesium stearate (4 mg), Talc (2 mg), Role of HPLC- Film foaming agent, MCC- Directly compressible material, MS, Gliding agent, Talk, Lubricating agent, PIO (30 mg) and VIL (100 mg) was taken into the volumetric flask (100ml) and volume of the flask was raised to 100 ml with methyl alcohol to give stock solution containing 300 mg/ml of PIO and 1000mg/ml of VIL (Sonicate the solution for 10 mins and filter the same from 0.45Whatman filter paper)	
Test Solution:	Withdraw 1.0 ml from above filtrate in 10mlvolumetric flask; make up the volume with mobile phase, which contain PIO+VIL=30+100mg/ml

RESULT AND DISCUSSION:

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY:

SELECTION OF ANALYTICAL/DETECTION WAVELENGTH

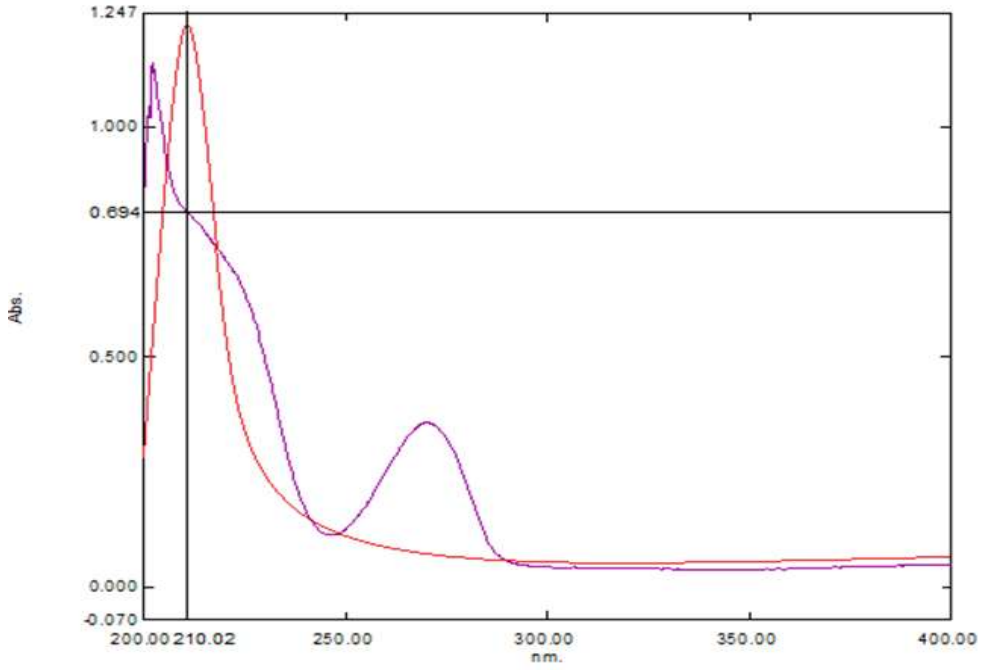

Figure 1 : Selection of Analytical wavelength for HPLC method

Mandatory requirements for selection of analytical wavelength in HPLC with UV detection is that both the drugs should give adequate response at selected wavelength.

It's quite evident from review of literature that, the Vildagliptin (VIL) lacks the chromophore and hence it doesn't possess the UV absorbance in adequate amount, but it does show end absorption at 210 nm, there are several evidence that 210 nm can be selected as analytical wavelength and at this wavelength both the drug possess adequate absorbance and hence it was selected as analytical wavelength.

Optimization of Chromatographic Conditions:

Trial 1

Column: Hypersil ODS C18 (250*4.6 mm, 5µm)

Mobile Phase: Methanol: Water (50:50 v/v),

Detection: 210 nm

Flow rate: 1 ml/min

Run Time: 20 minutes

Observation: Peak detected but with interference.

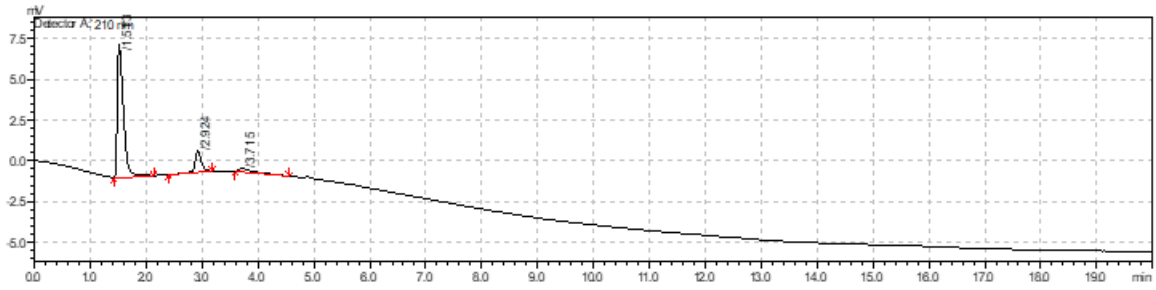

Figure 2: Trial 1: Chromatogram of PIO (10 µg.ml^{-1})

Trial 2

Column: Hypersil ODS C18 (250*4.6 mm, 5µm)

Mobile Phase: Methanol: Water (50:50 v/v),

Detection: 210 nm

Flow rate: 1 ml/min

Run Time: 15 minutes

Observation: No peak detected.

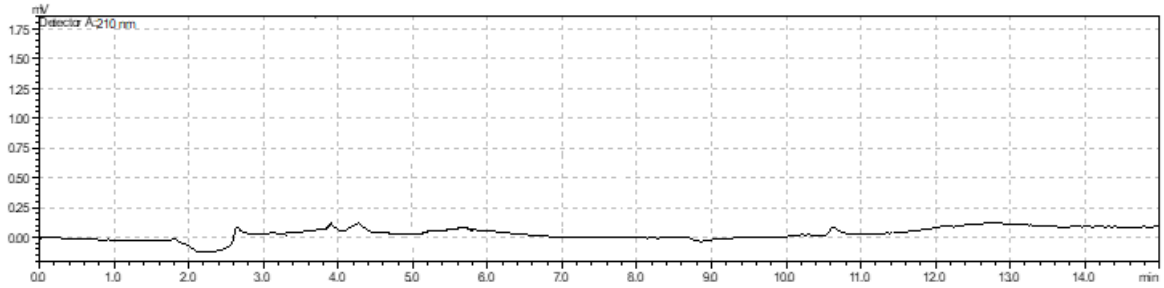

Figure 3: Trial 2: Chromatogram of VIL (10 µg.ml^{-1})

Trial 3

Column: Hypersil ODS C18 (250*4.6 mm, 5µm)

Mobile Phase: Acetonitrile: Methanol: Water (20:40:40 v/v)

Detection: 210 nm

Flow rate: 1 ml/min

Run Time: 15 minutes

Observation: Sharp peaks with adequate separation observed.

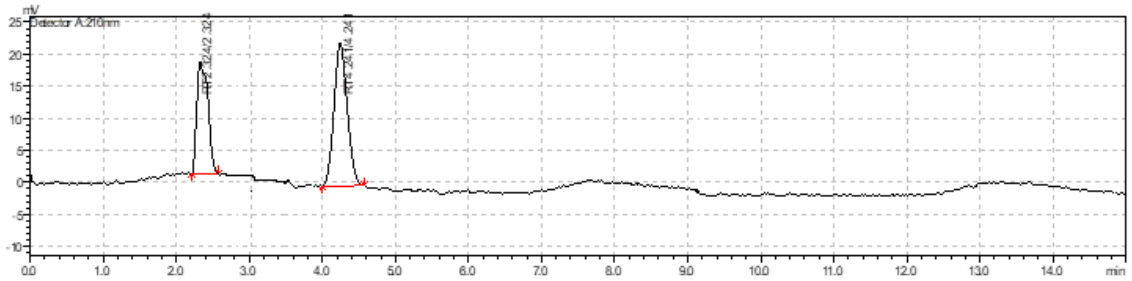

Figure 4: Trial 3: Chromatogram of PIO+VIL (10+10 µg.ml^{-1})

Optimized Chromatographic Condition:

Table 10: Optimized Chromatographic Condition

Stationary Phase	HYPERSIL ODS C18, 250 mm*4.6 mm
Mobile Phase	Acetonitrile: Methanol: Water (20:40:40 v/v)
Detection wavelength	210 nm
Flow rate	1 ml/minute
Run Time	15 minutes
Retention Time	PIO: 2.324 min, VIL: 4.241 min

System Suitability Parameters:

Table 11: System suitability parameter for PIO+VIL (30+100 µg.ml^{-1})

Parameter	PIO			VIL		
	Mean	± SD (n=3)	RSD	Mean	± SD (n=3)	RSD
Retention time (R_t)	2.34	0.01	0.45	4.29	0.06	1.47
Tailing Factor	1.07	0.01	0.93	1.14	0.02	1.34
Number of theoretical plates	21544.33	271.05	1.26	5224.33	84.97	1.63
Resolution (R_s)	3.17	0.05	1.47	3.17	0.05	1.47

Force Degradation Studies:

1) Acid Hydrolysis

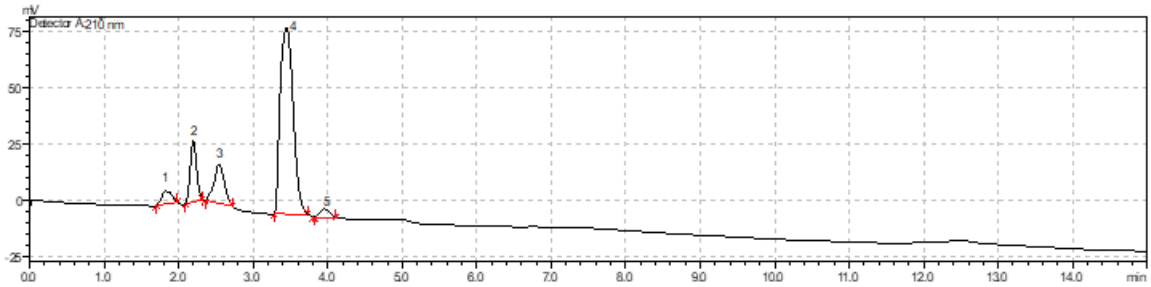

Figure 5 Chromatogram of Acid Hydrolysis (Treated)

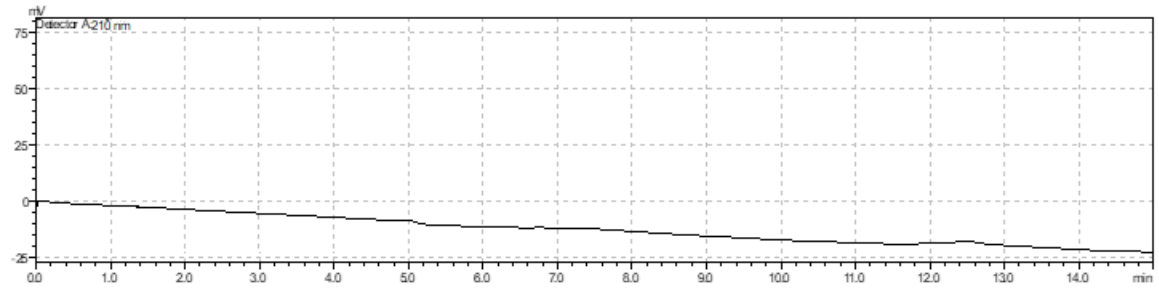

Figure 6 Chromatogram of Acid Hydrolysis (Blank)

2) Base Hydrolysis:

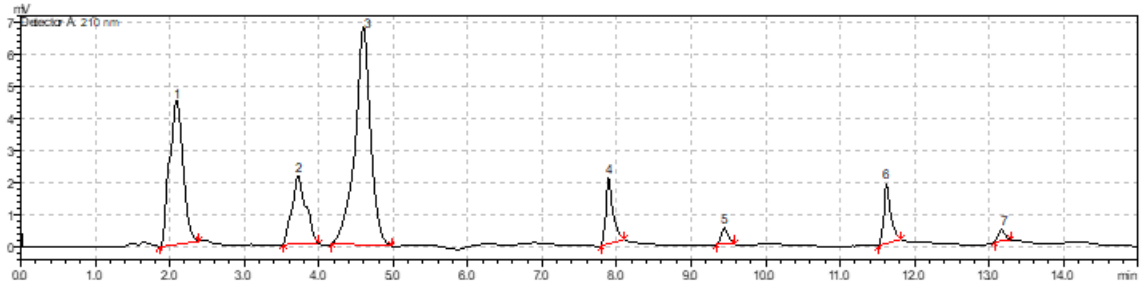

Figure 7 Chromatogram of Base Hydrolysis (Treated)

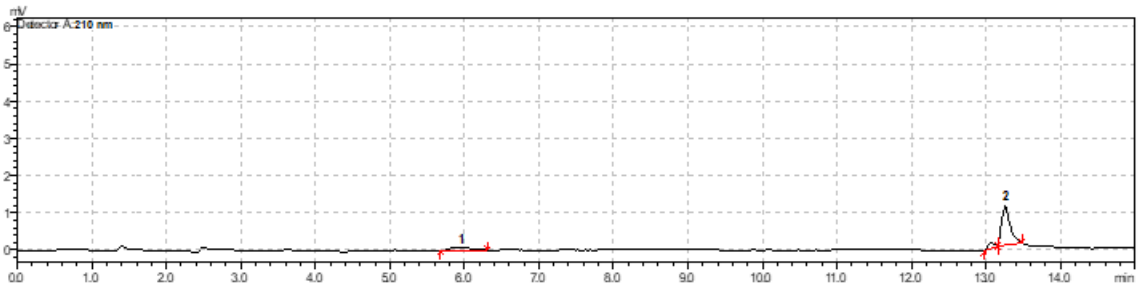

Figure 8 Chromatogram of Base Hydrolysis (Blank)

3) Oxidative Stress:

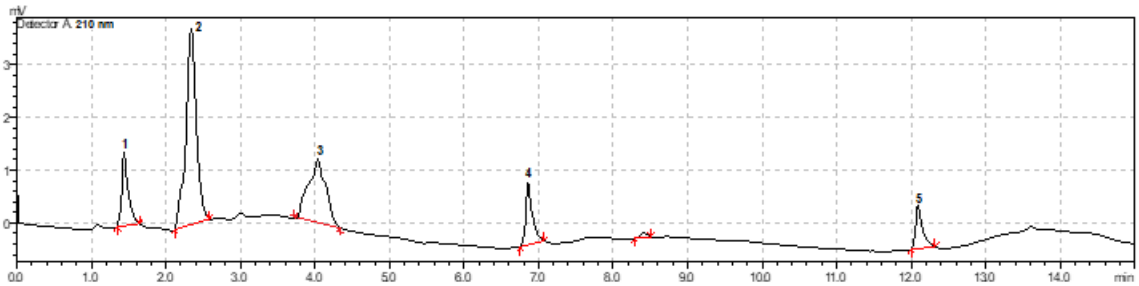

Figure 9 Chromatogram of Oxidative Stress (Treated)

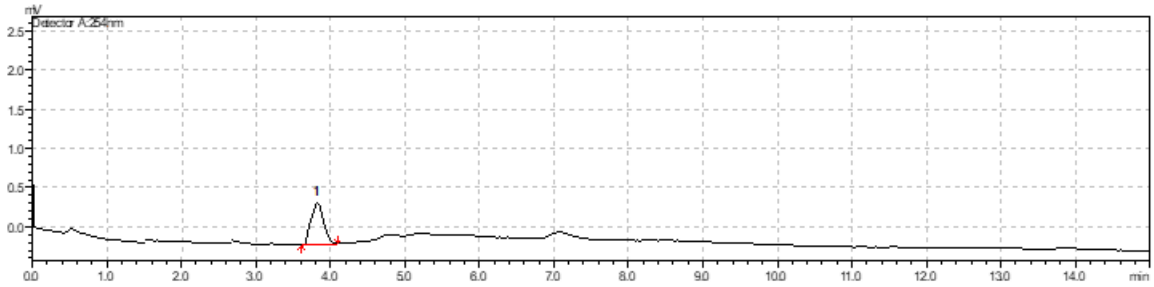

Figure 10 Chromatogram of Oxidative Stress (Blank)

4) Thermal Degradation:

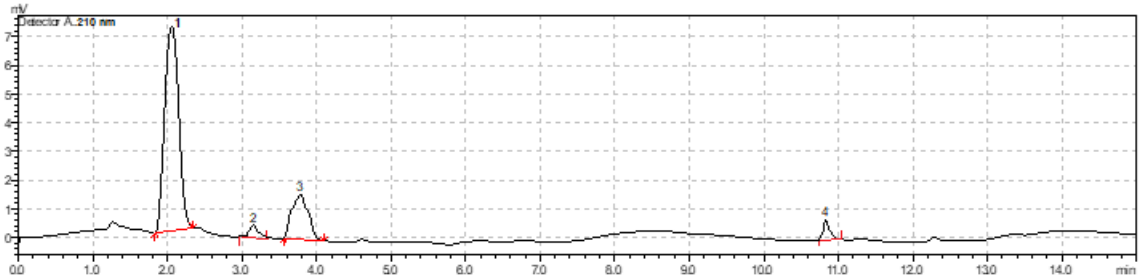

Figure 11 Chromatogram of Thermal Stress (Treated)

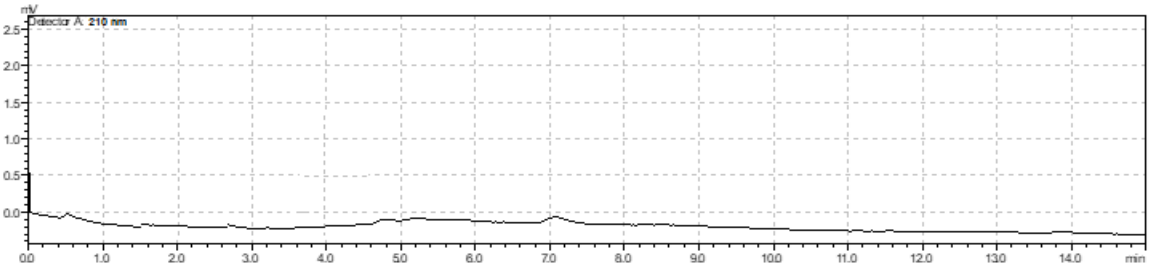

Figure 12 Chromatogram of Thermal Stress (Blank)

Evaluation table of Forced Degradation Studies

Stress Condition	Area	PIO	VIL	% Degradation (PIO)	% Degradation (VIL)
Acid Hydrolysis	Standard Area	51236	247469	13.58 %	13.39 %
	Observed Area	44274	214324		
Base Hydrolysis	Standard Area	51236	247469	14.40 %	15.08 %
	Observed Area	43856	210145		
Oxidative Stress	Standard Area	51236	247469	10.93 %	10.51 %
	Observed Area	45632	221457		
Thermal Degradation	Standard Area	51236	247469	11.73 %	10.24 %
	Observed Area	45223	222125		

Validation of developed RP-HPLC method for estimation of PIO and VIL

Linearity and Range:

Table 12 : Linearity data of PIO

Sr. No.	Concentration (µg/ml)	Mean area (µV. s)	± SD (n=5)	RSD
1	15	51704.4	644.15	1.25
2	30	112554.6	1384.43	1.23
3	45	164303.2	2009.27	1.22
4	60	222796.8	2271.24	1.02
5	75	287571.6	2064.98	0.72

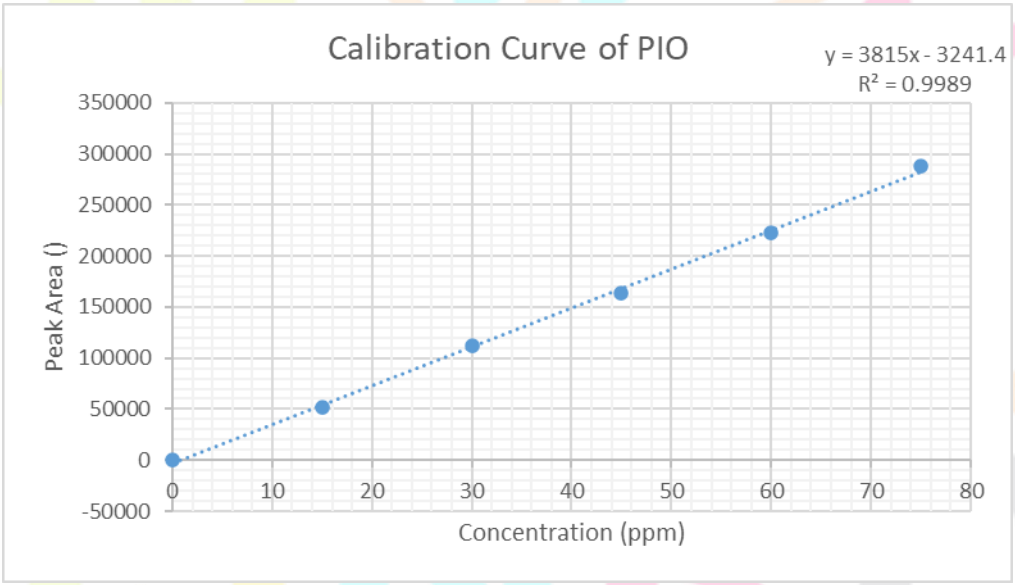

Figure 13: Calibration curve of PIO

Table 13: Linearity data of VIL

Sr. No.	Concentration (µg/ml)	Mean area (µV. s)	± SD (n=5)	RSD
1	50	248212.8	3362.83	1.35
2	100	570790	6644.99	1.16
3	150	937266	10627.55	1.13
4	200	1191154.08	12264.72	1.03
5	250	1536455.96	11150.90	0.73

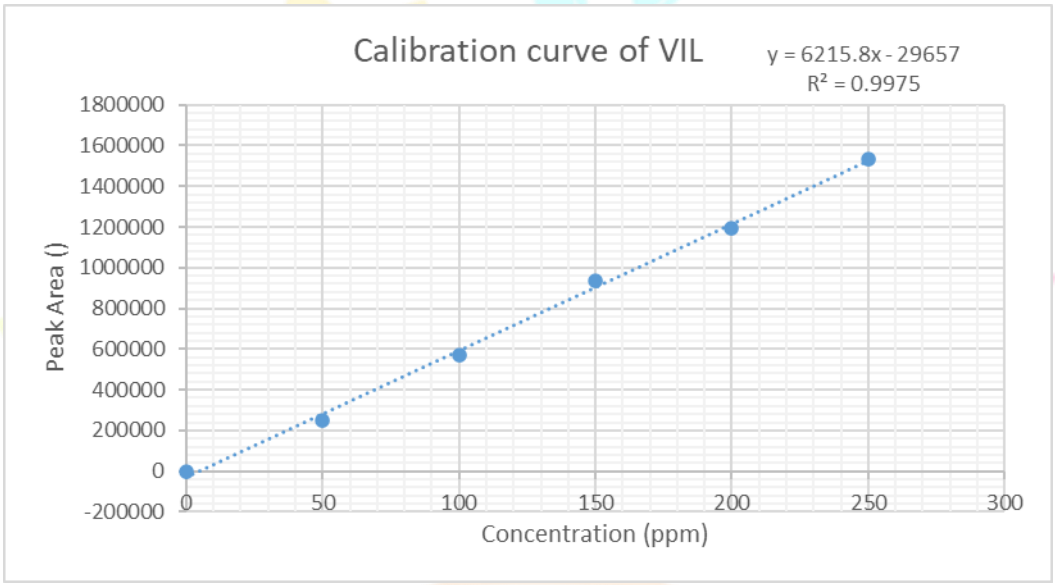

Figure 14: Calibration curve of VIL

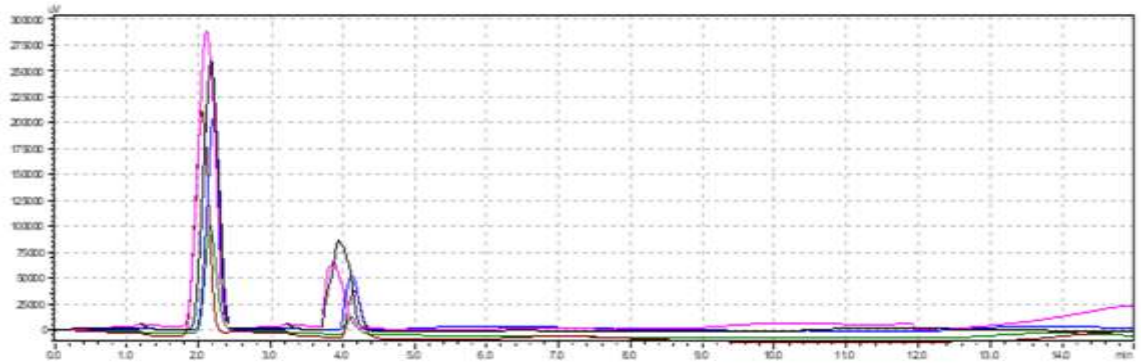

Figure 15: Overlain chromatography for linearity

Conclusion:

As per ICH guidelines the value of R^2 should be greater than 0.995, and observed R^2 for given concentration range for PIO and VIL is 0.9989 and 0.9975 respectively.

Hence, we can say that developed method is linear over the range of 15-75 µg/mL and 50-250 µg/mL for PIO and VIL, respectively.

REPEATABILITY:

Table 14: Repeatability data of PIO

Sr. No.	Concentration (µg/mL)				
	15	30	45	60	75
1.	51236	112458	166758	222367	286314
2.	51765	112974	164247	222968	286019
3.	50945	114278	164124	223864	287235
4.	51989	110432	165146	219314	291145
5.	52587	112631	161241	225471	287145
MEAN	51704.4	112554.6	164303.2	222796.8	287571.6
± SD (n=5)	644.15	1384.43	2009.27	2271.24	2064.98
RSD	1.25	1.23	1.22	1.02	0.72

Table 15: Repeatability data of VIL

Sr. No.	Concentration (µg/mL)				
	50	100	150	200	250
1.	247469	571254	924789	1193475	1543247
2.	246258	568777	936841	1190229.6	1544840
3.	246201	562518	937985	1185391.2	1538273.6
4.	246982	580978	932841	1209961.2	1517159.6
5.	254154	570423	953874	1176713.4	1538759.6
MEAN	248212.8	570790	937266	1191154.08	1536455.96
± SD (n=5)	3362.83	6644.99	10627.55	12264.72	11150.90
RSD	1.35	1.16	1.13	1.03	0.73

Conclusion:

As per ICH guidelines the value of RSD should be less than 2, and observed RSD is less than 2 for all concentrations of PIO and VIL.

Hence, we can say that developed method is repeatable over the range of 15-75 µg/mL and 50-250 µg/mL for PIO and VIL, respectively.

LIMIT OF QUATIFICATION (LOQ) AND LIMIT OF DETECTION (LOD):

Limit of Detection (LOD):

PIO	VIL
LOD = 3.3 x (σ/S) = 3.3 x (488.65/ 3815.02) = 0.422 µg/mL	LOD = 3.3 x (σ/S) = 3.3 x (3799.93/ 6215.76) = 2.0174 µg/mL

Limit of Quantification (LOQ):

PIO	VIL
LOQ = 10 x (σ/S) = 10 x 488.65/ 3815.02) = 1.280 µg/mL	LOQ = 10 x (σ/S) = 10 x 488.65/ 3815.02) = 6.113 µg/mL

ACCURACY:

ACCURACY												
	PIO						**VIL**					
	50%		**100%**		**150 %**		**50 %**		**100 %**		**150%**	
	Amount of drug recovered (mg)	% Recovery	Amount of drug recovered (mg)	% Recovery	Amount of drug recovered (mg)	% Recovery	Amount of drug recovered (mg)	% Recovery	Amount of drug recovered (mg)	% Recovery	Amount of drug recovered (mg)	% Recovery
	14.98	99.87	29.63	98.77	44.84	99.64	49.26	98.52	3.96	99.67	149.25	99.50
	14.84	98.93	29.55	98.50	44.68	99.29	49.36	98.72	3.93	98.22	148.87	99.25
	14.72	98.13	29.89	99.63	44.32	98.49	49.68	99.36	3.97	98.48	148.56	99.04
Mean	14.85	98.98	29.69	98.97	44.61	99.14	49.43	98.87	3.95	98.79	148.89	99.26
± SD	0.13	0.87	0.18	0.59	0.27	0.59	0.22	0.44	0.02	0.77	0.35	0.23

INTRA DAY AND INTER-DAY PRECISION:

METHOD PRECISION													
	Intraday Precision							Inter-day Precision					
	PIO			VIL				PIO			VIL		
	15	**45**	**75**	**50**	**150**	**250**		**15**	**45**	**75**	**50**	**150**	**250**
	52348	165845	287223	248126	921657	1544186		52624	166745	285632	249634	921463	1538964
	51474	166136	282634	251457	923864	1548347		53118	169234	289647	254114	925147	1549234
	52445	163441	284054	245984	936314	1563964		53887	170056	290234	248326	937864	1561382
MEAN	52089.00	165140.67	284637.00	248522.33	927278.33	1552165.67	MEAN	53209.67	168678.33	288504.33	250691.33	928158.00	1549860.00
SD	534.81	1479.13	2349.39	2757.94	7902.54	10427.32	SD	636.47	1724.02	2504.77	3035.41	8605.10	11222.10
RSD	1.03	0.90	0.83	1.11	0.85	0.67	RSD	1.20	1.02	0.87	1.21	0.93	0.72

ROBUSTNESS:

Parameter	**Level of Change**	**Effect on assay volume**			
		PIO		**VIL**	
		Assay ± SD	**RSD**	**Assay ± SD**	**RSD**
Flowrate	0.9 mL/min	99.10 ± 0.73	0.74	99.65 ± 0.39	0.32
	1.1 mL/min	98.56 ± 0.25	0.26	99.57 ± 0.30	0.30
Mobile Phase Composition	22:38 :40	98.26± 0.11	0.11	99.70 ± 0.15	0.15
	20:42 :38	98.58± 0.39	0.40	99.56 ± 0.29	0.29
(n = 3 determinations)					

SUMMARY AND CONCLUSIONS:

Evaluation table of Forced Degradation Studies

Stress Condition	Area	PIO	VIL	% Degradation (PIO)	% Degradation (VIL)
Acid Hydrolysis	Standard Area	51236	247469	13.58 %	13.39 %
	Observed Area	44274	214324		
Base Hydrolysis	Standard Area	51236	247469	14.40 %	15.08 %
	Observed Area	43856	210145		
Oxidative Stress	Standard Area	51236	247469	10.93 %	10.51 %
	Observed Area	45632	221457		
Thermal Degradation	Standard Area	51236	247469	11.73 %	10.24 %

Table 35 Optimized Chromatographic Condition

Stationary Phase	HYPERSIL ODS C18, 250 mm*4.6 mm
Mobile Phase	Acetonitrile: Methanol: Water (20:40:40 v/v)
Detection wavelength	210 nm
Flow rate	1 ml/minute
Run Time	15 minutes

Table 36 Validation parameters

Parameter	Limit	Result		Conclusion
		PIO	VIL	
Linearity and Range	$R^2 > 0.995$	0.9989 (15-75 µg/mL)	0.9975 (50-250 µg/mL)	Method was linear
Repeatability	RSD < 2	0.72-1.25	0.73-1.35	Method was repeatable
LOD	-	0.42 µg/mL	2.01 µg/mL	-
LOQ	-	1.28 µg/mL	6.11 µg/mL	-
Intraday Precision	RSD < 2	0.83-1.03	0.67-1.11	Method was precise
Inter-Day Precision	RSD < 2	0.87-1.20	0.72-1.21	Method was precise
% Recovery	98 - 102 %	98.97-99.14	98.79-99.26	Method was accurate
Robustness	RSD < 2	0.11-0.74	0.15-0.32	Method was robust

Thus, we found that method was comply with all the validation parameters according to ICH Q2R1 guideline.

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