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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING ASSAY METHOD FOR ESTIMATION OF SEMAGLUTIDE BULK AND PHARMACEUTICAL DOSAGE FORM BY RP-HPLC

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ABSTRACT

Background: A simple, Précised, Accurate method was developed for the estimation of Semaglutide by RP-HPLC technique. Chromatographic conditions used are stationary phase Agilent C18 250mm x 4.6 mm, 5µ ,Mobile phase Water : Acetonitrile in the ratio of 60:40 and flow rate was maintained at 1.0ml/min, detection wave length was 292nm, column temperature was set to 30°C and diluent was mobile phase Conditions were finalized as optimized method. System suitability parameters were studied by injecting the standard six times and results were well under the acceptance criteria. **Results:** Linearity study was carried out between 25% to150 % levels, R^2 value was found to be as 0.999. Precision was found to be 0.4 for repeatability and 1.2 for intermediate precision.LOD and LOQ are 0.21µg/ml and 0.62µg/ml respectively. By using above method assay of marketed formulation was carried out 99.46% was present. Degradation studies of Semaglutide were done, in all conditions purity threshold was more than purity angle and within the acceptable range.
Conclusions: Degradation studies of Semaglutide were done, in all conditions purity threshold was more than purity angle and within the acceptable range. Full length method was not performed; if it is done this method can be used for routine analysis of Semaglutide.

INTRODUCTION

Background: Semaglutide is a once-daily glucagon-like peptide-1 analog that differs to others by the presence of an acyl group with a steric diacid at Lys26 and a large synthetic spacer and modified by the presence of a α-aminobutyric acid in position 8 which gives stability against the dipeptidylpeptidase-4.[1] The stability of semaglutide by the acylation permits a high-affinity albumin binding and gives it a long plasma half-life which allows the once-daily dosage. It was developed by the Danish pharmaceutical company Novo Nordisk and FDA approved on December 5, 2017[2]. Drugs like semaglutide affect the glucose control through several different mechanisms like the increase of insulin secretion, slow of gastric emptying, and reduction of postprandial glucagon and food intake. Glucogon like peptide-1 has a major role in glucose management and semaglutide presents an analog structure which allows it to perform all the activities of GLP-1[3].The innovative section of semaglutide is the presence of structural modifications (amino acid substitution at position 8) that generate

superior stability against dipeptidyl peptidase-4 which is an enzyme that degrades incretins like Glucagon like peptide-1.

MATERIALS AND METHODS:

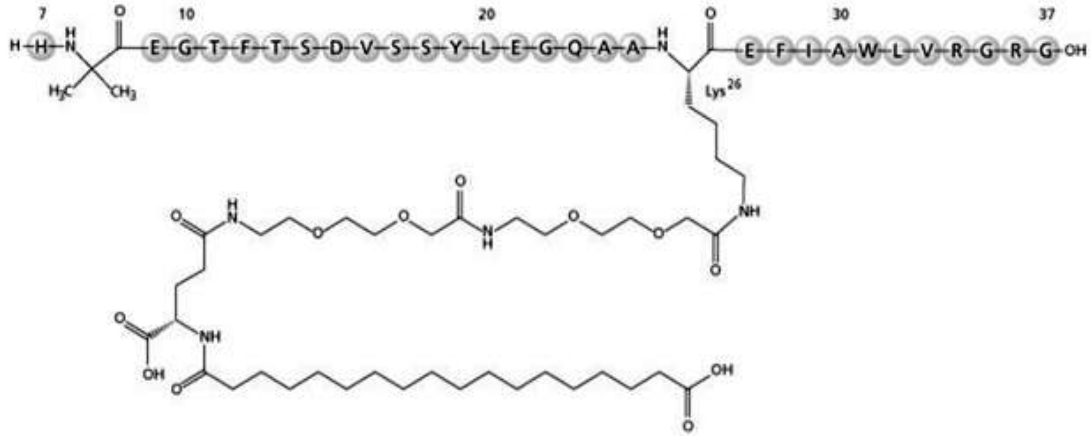

Fig: 1- Chemical Structure of Semaglutide

Chemicals and reagents: HPLC Water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem.

Apparatus: WATERS HPLC 2965 SYSTEM Auto Injector and PDA Detector. Sonicator (Ultrasonic sonicator) P^H meter (Thermo scientific), Micro balance (Sartorius), Vacuum filter pump

Optimised method:

Column: Agilent C18 250mm x 4.6 mm, 5m.
Mobile phase: Water: Acetonitrile (50:50)
Flow rate : 1.0 ml/min
Detector : PDA 292nm
Temperature : 30^0C
Injection Volume : 3.00µL

Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and Water taken in the ratio of 50:50

Preparation of Standard stock solutions:

Accurately weighed 5mg of Semaglutide transferred 50ml and volumetric flasks, 3/4 Th of diluents was added and sonicated for 10 minutes. Flasks were made up with diluents and labeled as Standard stock solution (100µg/ml of Semaglutide).

Preparation of Standard working solutions (100% solution): 1ml of Semaglutide from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (10µg/ml of Semaglutide).

Preparation of Sample stock solutions:5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 10 ml volumetric flask, 50ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (100 µg/ml of Semaglutide).

Preparation of Sample working solutions (100% solution): 1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (10µg/ml of Semaglutide)

Preparation of buffer: 0.1%OPA Buffer: 1ml of Perchloric acid was diluted to 1000ml with HPLC grade water.

Buffer: 0.01N Potassium dihyrogen ortho phosphate: Accurately weighed 1.36gm of Potassium dihyrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then added 1ml of Triethylamine then PH adjusted to 3.0 with dil. Ortho phosphoric acid solution.

Standard drug :

Semaglutide of purity 99% produced from Spectrum laboratory

Validation:

System suitability parameters: The system suitability parameters were determined by preparing standard solutions of Semaglutide (10ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%.

Specificity: Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at

retention times of these drugs in this method. So this method was said to be specific.

Precision:

Preparation of Sample stock solutions: 5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 10 ml volumetric flask, 5ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters.(100 µg/ml of Semaglutide)

Preparation of Sample working solutions (100% solution): 1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (10µg/ml of Semaglutide)

Linearity: Preparation of Standard stock solutions: Accurately weighed 5mg of Semaglutide transferred 50ml and volumetric flasks, 3/4 to diluents was added and sonicated for 10 minutes. Flasks were made up with diluents and labeled as Standard stock solution (100µg/ml of Semaglutide).

25% Standard solution: 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (2.5µg/ml of Semaglutide)

50% Standard solution: 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (5.0µg/ml of Semaglutide)

75% Standard solution: 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (7.5µg/ml of Semaglutide)

100% Standard solution: 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (10µg/ml of Semaglutide)

125% Standard solution: 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (12.5µg/ml of Semaglutide)

150% Standard solution: 1.5ml each from two standard stock solutions was pipettete out and made up to 10ml. (15µg/ml of Semaglutide)

Accuracy: Preparation of Standard stock solutions: 5 tablets were weighed and the average weight of each tablet was calculated, then the weight equivalent to 1 tablet was transferred into a 10 ml volumetric flask, 5ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters.(100 µg/ml of Semaglutide).

Preparation of 50% Spiked Solution: 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 100% Spiked Solution: 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Preparation of 150% Spiked Solution: 1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

Robustness: robustness of the study method was Evaluated by assaying test solutions Slight but delebrate changes in the analytical conditions like Flow minus (1.1ml/min), Flow plus (1.3ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected The in duplicate manner.

LOD sample Preparation: 0.25ml of Standard stock solution was pipette out and transferred to 10ml volumetric flasks and made up with diluents. From the above solution 0.1ml Semaglutide, were transferred to 10ml volumetric flasks and made up with the same diluents.

LOQ sample Preparation: 0.25ml of Standard stock solution was pipetted out and transferred to 10ml volumetric flasks and made up with diluents. From the above solution 0.3ml Semaglutide, were transferred to 10ml volumetric flasks and made up with the same diluents.

Degradation procedure: Acid Degradation studies: To 1 ml of s tock solution Semaglutide 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c .The resultant solution was diluted to obtain (10ppm) solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation studies: To 1ml of stock

solution Semaglutide 1 ml of 2 N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain (10ppm) solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Oxidation: To 1 ml of stock solution of Semaglutide 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60^0c. For HPLC study, the resultant solution was diluted to obtain (10ppm) solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies: The standard drug solution was placed in oven at 105^0c for 6 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to (10ppm) solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Photo Stability studies: The photochemical stability of the drug was also studied by exposing the (100ppm) solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain (10ppm) solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies: Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60ºc. For HPLC study, the resultant solution was diluted to (10ppm) solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

Results: System Suitability: The system suitability parameters were evaluated from standard Chromatograms obtained by calculating the % RSD of retention time, tailing factor, theoretical plates and peak areas from five replicate injections are within range and Results obtained were satisfactory and in accordance with guide lines. System suitability Results were shown in Table 1.

Precision:

Repeatability: Six working sample solutions of 10ppm are injected and the % Amount found was calculated and %RSD was found to be with in the limits (<2%). Repeatability Results were shown in Table 2.

Intermediate precision: Five working sample solutions of 10ppm are injected on the next day of the preparation of samples and the % Amount found was calculated and %RSD was found to be With in the limits.the results of Intermediate Precision were shown in Table 3.

Linearity: The linearity of Semaglutide was found to be 2.5 ppm to 15 ppm of Semaglutide. Plot a graph to concentration versus peak area. Slope obtained was 41859 Y-Intercept was 6064 and Correlation Co-efficient was found to be 0.999, the data was shown in Table 4 and Linearity plot was shown in Fig .2.

Accuracy: Three Concentrations of 50%, 100%, 150% are Injected in a triplicate manner and %Recovery was calculated as 99.81, And Shown in Table 5

LOD: Detection limit of the Semaglutide in this method was found to be 0.21µg/ml as shown in Fig:3

LOQ: Quantification limit of the Semaglutide in this method was found to be 0.62µg/ml, as shown in Fig 4.

Robustness: Small Deliberate change in the method is made like Flow minus, flow plus, Mobile phase minus, Mobile phase plus, Temperature minus, Temperature Plus. %RSD of the above conditions are calculated. The results of Robustness study was shown in Table 6.

Assay: The marketed formulation was analysed by the proposed method . In Accordance with ICH guidelines .The results were found in with in the limits as shown in Table 7, and Chromatogram was shown in Fig 5

Degradation Studies: Degradation studies related to Acid, Alkali, Peroxide, Dry Heat, Photo Stability, Neutral degradation studies were represented in Table 8, The Chromatograms are shown in Fig 6, Fig 7, Fig 8, Fig 9, Fig 10, Fig 11 Respectively.

Table 1: System suitability data

SN0.	Peak Name	RT	Area	USP Plate count	USP Tailing
1	Semaglutide	2.277	286181	5774	1.03
2	Semaglutide	2.278	281143	7175	1.01
3	Semaglutide	2.278	289455	7187	1.00
4	Semaglutide	2.281	288499	8373	0.98
5	Semaglutide	2.281	288411	8374	0.98
6	Semaglutide	2.282	287562	8697	0.97
Mean			**286875**		
Std. Dev.			**3015.4**		
% RSD			**1.1**		

Table 2: Repeatability data

S.No	Peak Area
1	286338
2	286252
3	285592
4	288997
5	285663
6	286024
AVG	286478
STDEV	1270.5
%RSD	0.4

Table 3: Intermediate precision data

S.No	Peak Area
1	271468
2	270668
3	276199
4	279023
5	277425
6	274859
AVG	274940
STDEV	3308.9
%RSD	1.2

Table 4: Linearity Concentration and Response

Linearity Level (%)	Concentration (ppm)	Area
0	0	0
25	2.5	115503
50	5	215291
75	7.5	330889
100	10	416635
125	12.5	525408
150	15	636325

Table 5: Accuracy data for Semaglutide

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	5	4.98	99.53	99.81%
	5	5.01	100.16	
	5	4.97	99.42	
100%	10	9.92	99.18	
	10	9.91	99.13	
	10	10.06	100.61	
150%	15	14.92	99.46	
	15	15.06	100.39	
	15	15.06	100.40	

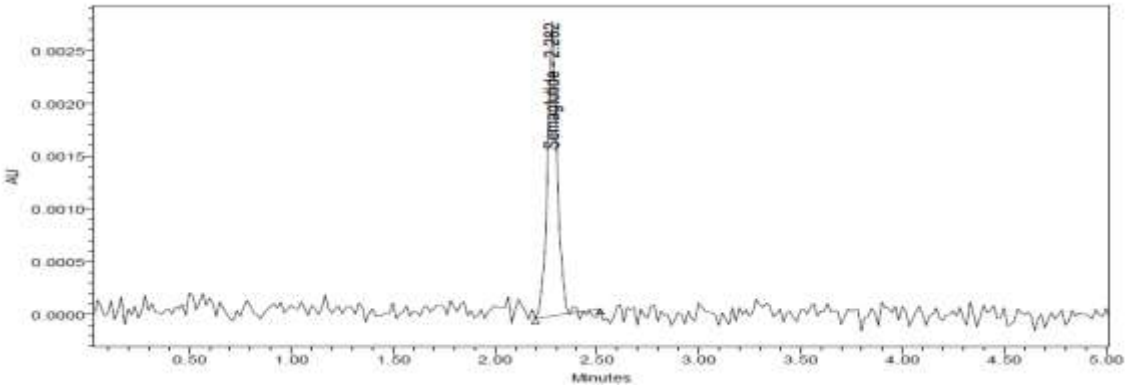

Fig 3: LOD Chromatogram of Semaglutide

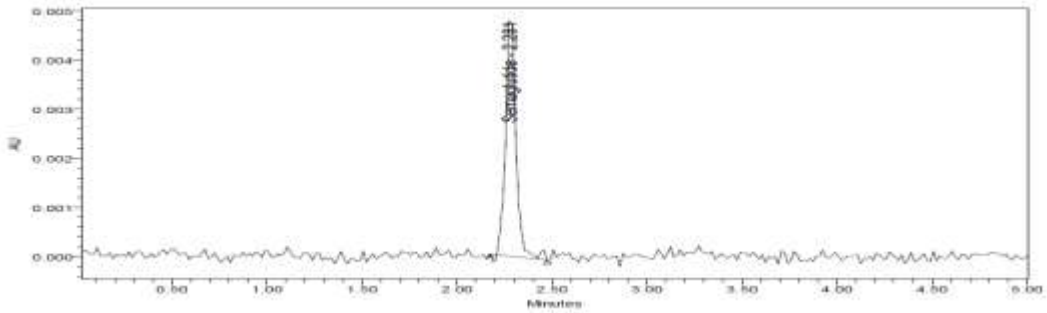

Fig :4 LOQ Chromatogram of Semaglutide

Table: 6 Robustness Data

Parameter	%RSD
Flow Minus	0.8
Flow Plus	0.3
Mobile phase Minus	0.2
Mobile phase Plus	0.6
Temperature minus	0.5
Temperature plus	0.4

Table 7: Assay of Formulation

Sample No	% Assay
1	99.41
2	99.38
3.	99.15
4.	100.34
5.	99.18
6.	99.30
AVG	99.46
STDEV	0.44

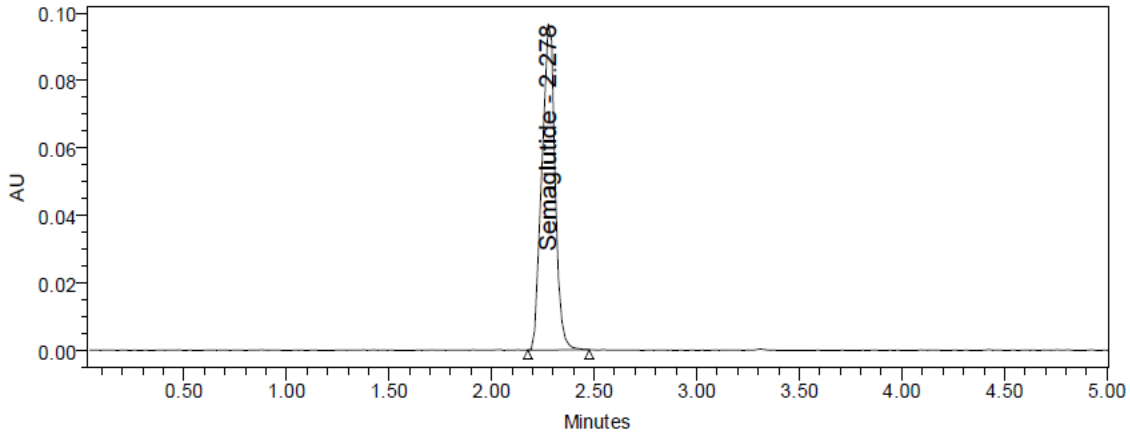

Fig 5: Assay Chromatogram

Table 8: Degradation Data of Semaglutide

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold

1	Acid	6.61	0.165	0.289
2	Alkali	4.20	0.189	0.288
3	Oxidation	4.97	0.271	0.302
4	Thermal	1.93	0.266	0.303
5	UV	1.15	0.459	0.602
6	Water	0.72	0.302	0.313

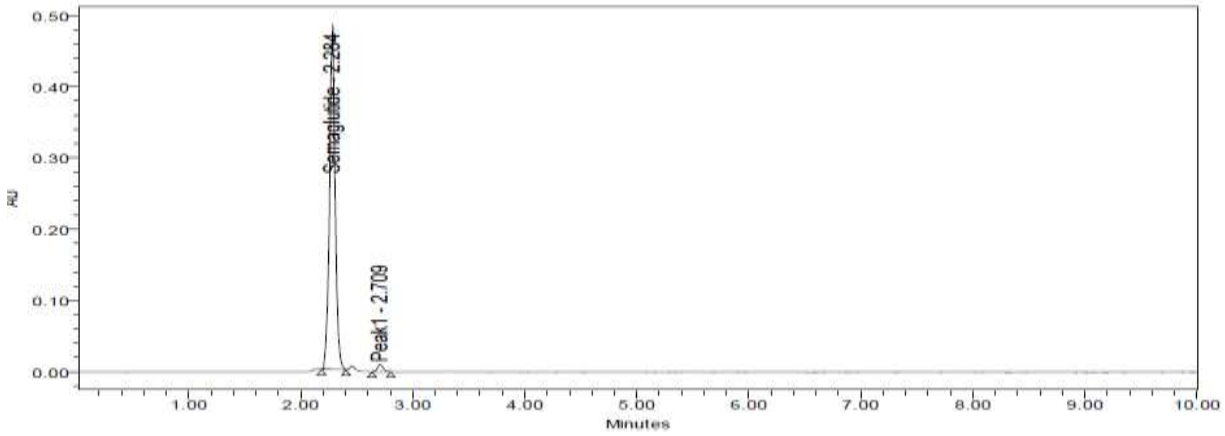

Fig 6: Acid degradation chromatogram

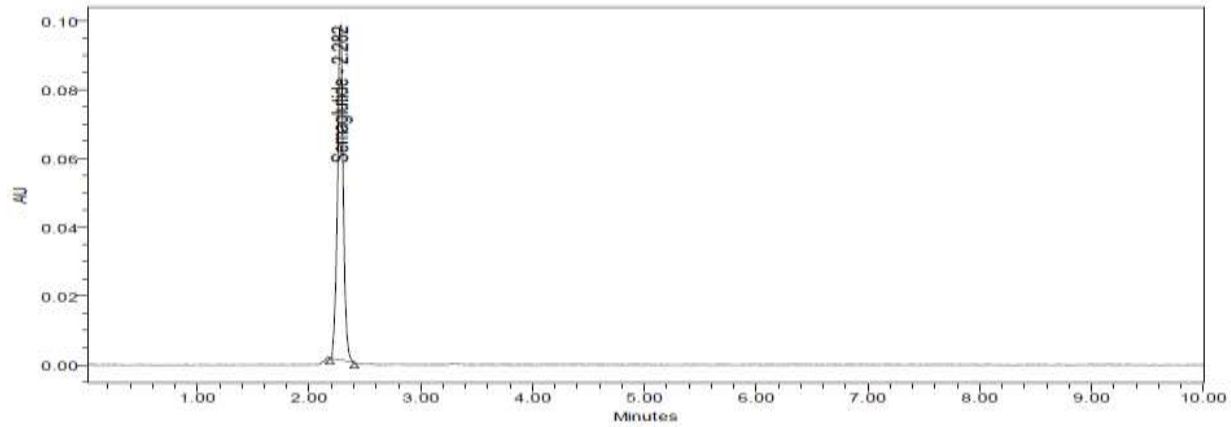

Fig 7: Base degradation chromatogram

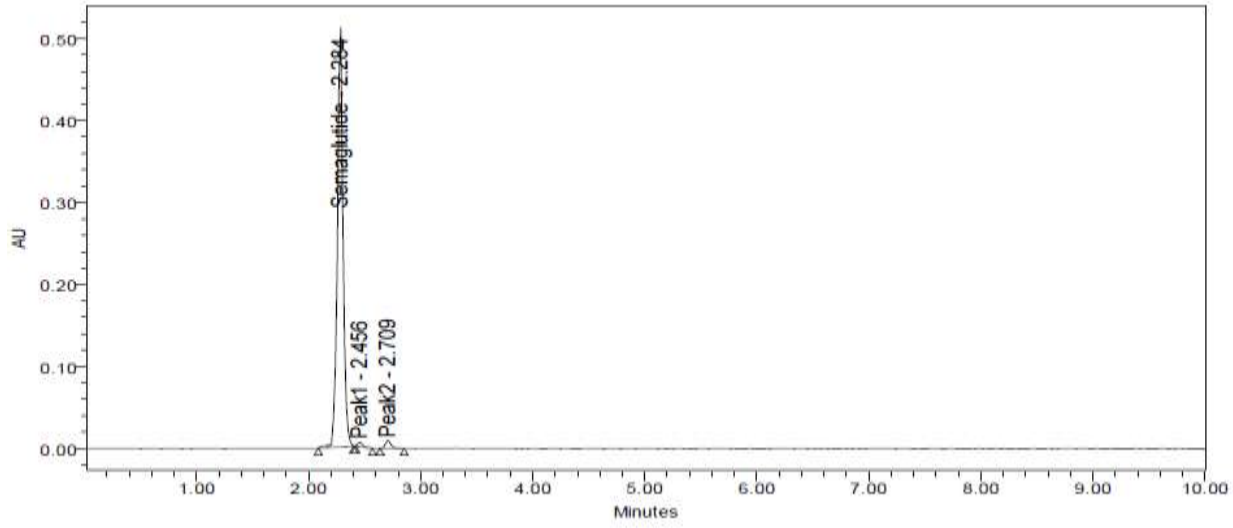

Fig 8: Peroxide degradation chromatogram

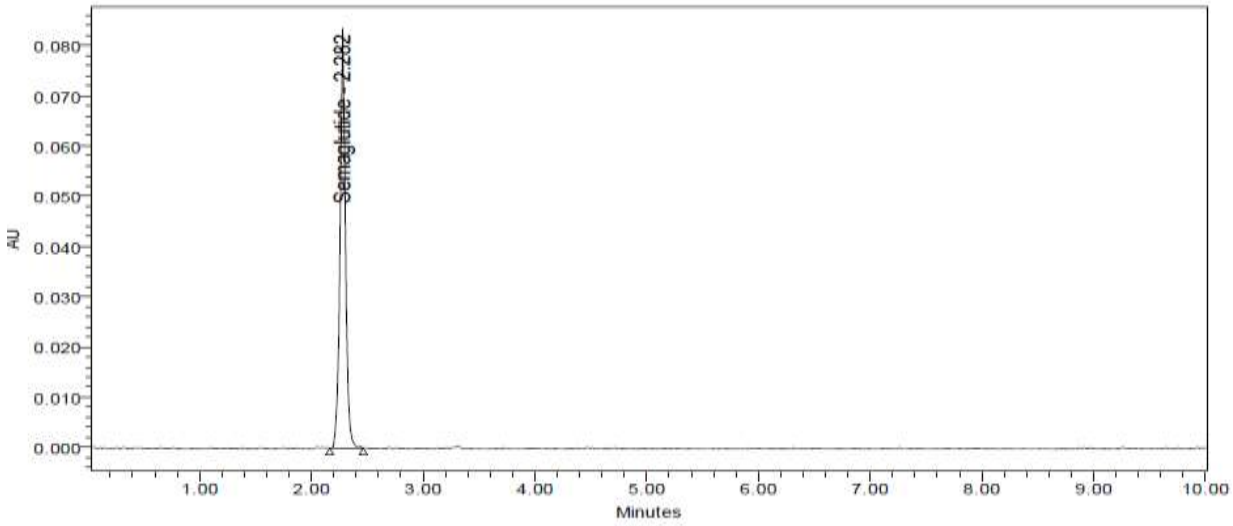

Fig 9: Thermal degradation chromatogram

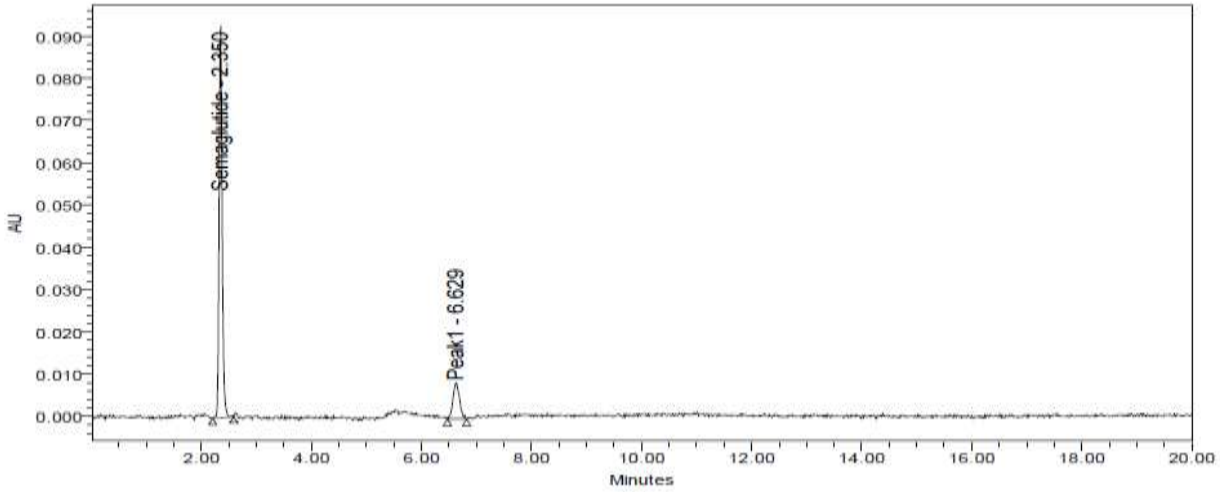

Fig 10: UV degradation chromatogram

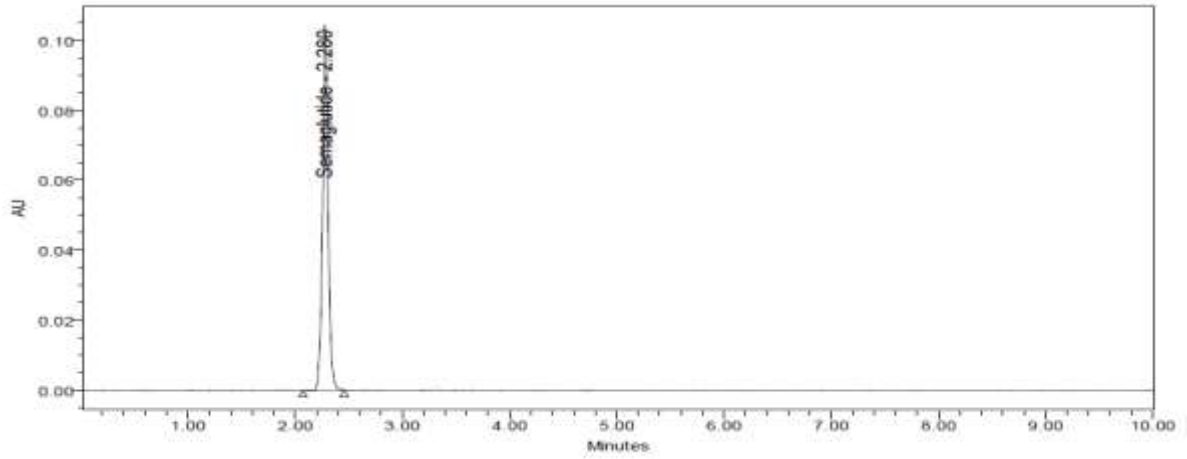

Fig 11: Water degradation chromatogram

Discussion: Regarding the pH adjustment in mobile phase for the acid and base degradation studies have movement in retention time of drugs. But due to neutralized acid sample with 2N Base solution and base sample with 2N Acid solution there will be no change in retention time.

Summary Table

Parameters		**Semaglutide**	**LIMIT**
Linearity : Range (µg/ml)		2.5-15 µg/ml	R< 1
Regression coefficient		0.999	
Slope(m)		41859	
Intercept(c)		6064	
Regression equation (Y=mx+c)		y = 41859x + 6064	
Assay(% mean assay)		99.46%	90-110%
Specificity		Specific	No interference of any peak
System precision %RSD		1.1	NMT 2.0%
Method precision %RSD		0.4	NMT 2.0%
Accuracy %recovery		99.81%	98-102%
LOD		0.21	NMT 3
LOQ		0.62	NMT 10
Robustness	**FM**	0.8	%RSD NMT 2.0
	FP	0.3	
	MM	0.2	
	MP	0.6	
	TM	0.5	
	TP	0.4	

SUMMARY AND CONCLUSION

Chromatographic conditions used are stationary phase Agilent C18 250mm x 4.6 mm, 5µ ,Mobile phase Water: Acetonitrile in the ratio of 60:40 and flow rate was maintained at 1.0ml/min, detection wave length was 292nm, column temperature was set to 30^{o}C and diluent was mobile phase Conditions were finalized as optimized method. System suitability parameters were studied by injecting the standard six times and results were well under the acceptance criteria. Linearity study was carried out between 25% to150 % levels, R^2 value was found to be as 0.999. Precision was found to be 0.4 for repeatability and 1.2 for intermediate precision.LOD and LOQ are 0.21µg/ml and 0.62µg/ml respectively. By using above method assay of marketed formulation was carried out 99.46% was present. Degradation studies of Semaglutide were done, in all conditions purity threshold was more than purity angle and within the acceptable range. Full length method was not performed; if it is done this method can be used for routine analysis of Semaglutide.

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Conflict of Interest: The authors declared no conflict of interest.

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