METHOD DEVELOPMENT AND VALIDATION OF RP-UPLC METHOD FOR THE DETERMINATION OF SEMAGLUTIDE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT *The objective of the study was to develop RP-UPLC method for the determination of purity of semaglutide in bulk and pharmaceutical dosage form. The chromatographic separation was achieved with Acquity BEH C18 (50mm x 1.6 mm) 1.8µm column thermostated at 30°C with mobile phases containing 0.01N potassium dihydrogen phosphate (3.2 pH): acetonitrile in the ratio of 50:50 v/v. The flow rate was maintained at 0.4ml/min and injection volume was found to be 0.50µl. The detection was done at 292 nm using TUV detector. The retention time was found to be 1.026min. Linearity study was carried out between 25% to 150 % concentration levels and R^2 value was found to be as 0.999. The %RSD of repeatability and intermediate precision was found to be 0.5 and 0.6 µg/ml. The LOD and LOQ were found to be 0.086 µg/ml and 0.261 µg/ml, respectively. Forced degradation was carried out under various stress conditions to demonstrate the stability-indicating capability of the developed UPLC method. The proposed method was found to be simple, precise, accurate and validated according to the International Conference on Harmonization guidelines*

Keywords:

INTRODUCTION

Validation is the process of establishing a documentary evidence demonstrating that a procedure, process, or activity carried out in testing and then production maintains the desired level of compliance at all stages [Lavanya et al, 2013]. The validation of the method was based on FDA guidelines and on standard bioanalytical method validation recommendation [Ambadas and prasanna, 2011]. The goal of equipment validation is to produce constant result with minimal variation without compromising the product and performance of the equipment. The system suitability parameters ensures that the UPLC system and procedures are adequate for the analysis performed and validated according to ICH guidelines [Kumar et al, 2011].

The drug 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. It is a large synthetic spacer and modified by the presence of a α-aminobutyric acid in position 8 which gives stability against the dipeptidylpeptidase-4 [Gotfredsen et al, 2014]. It reduces blood glucose through a mechanism where it stimulates insulin secretion and lowers glucagon secretion, both in a glucose dependent manner [Hjerpsted et al., 2018]. It is indicated to improve glycemic control in adults with type 2 diabetes mellitus as an adjunct of diet and exercise [Hjerpsted et al., 2018]. In clinical trials of semaglutide, reduction of glycated hemoglobin (HbA1c) compared to other medications like sitagliptin, exenatide and insulin glargine U100 were noticed. The HbA1c protein is a standard measure of high glucose as in normal conditions the hemoglobin forms 1-deoxyfructose. It also showed that the ability of semaglutide reduces the body weight. The stability of semaglutide by acylation permits high-affinity albumin binding and gives a long plasma half-life which allows the once-daily dosage[Hjerpsted et al., 2018]. The main aim and objective of the work was to develop and validate RP-UPLC method for the determination of semaglutide in bulk and pharmaceutical dosage form. The developed UPLC method was validated with respect to linearity, precision, accuracy, specificity, robustness, limit of detection and limit of quantification.

MATERIALS AND METHODS

Chemicals and Reagents

Sample of semaglutide was supplied by Spectrum pharma research solutions (Hyderabad). Methanol, acetonitrile and potassium dihydrogen phosphate were obtained from Rankem. High purity water was attained by using Millipore Milli Q Plus water purification system.

Instruments

The chromatography analysis was performed using Waters Acquity UPLC separation module (Waters

Corporation, Milford, USA) equipped with an UV/Visible detector, binary solvent manager and auto sampler system. The signal output was checked and processed using Empower 2 software. The pH of the solutions was measured by Mettler-Toledo pH meter (Switzer5land).

Chromatographic conditions

The method was developed by using BEH C18 (50mm x 1.6 mm, 1.8µ) column with a mobile phase comprising a mixture of 0.01N potassium dihydrogen phosphate: acetonitrile in the ratio of 50:50 v/v. The pH was adjusted to 3.2 and the flow rate was maintained at 0.4ml/min. The column temperature was maintained at 30 °C and the eluted compounds were monitored at the wavelength of 292 nm. The sample injection volume was 0.50µl.

Preparation of diluents

The diluent used for the analysis was selected based on the solubility of the drug, so that acetonitrile and potassium dihydrogen phosphate (0.01N) were taken in the ratio of 50:50.

Preparation of 0.01N potassium dihyrogen phosphate

Accurately weighed 1.36gm of potassium dihyrogen phosphate in a 1000ml of volumetric flask. To that about 900ml of Milli-Q water added and sonicated. Finally the volume was made up with water. 1ml of triethylamine was added and then pH was adjusted to 3.2 with dilute orthophosphoric acid solution.

Preparation of mobile phase

A mixture of potassium dihydrogen phosphate and acetonitrile in the ratio of 50:50 (v/v) was prepared and the pH was adjusted to 3.2. The mixture was filtered through 0.045 µm filter under vacuum filtration.

Preparation of standard stock solution

25mg of semaglutide was accurately weighed and transferred into a 50ml of volumetric flask to that 3/4th part of diluent was added and sonicated for 10 minutes. The flask was made up with diluent and labeled as standard stock solution (500µg/ml). From the above solution, 1ml was transferred into 10 ml volumetric flask and the final volume were made up with diluents which was labeled as standard working solution (50µg/ml).

Preparation of sample stock solution

Tablets 5 were accurately 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. Diluent (50 ml) was added and sonicated for 25 min. Further, the volume was made up with diluent and filtered by UPLC filters which was labelled as sample stock solution (100 µg/ml). From the filtered solution, 5ml was transferred into 10ml of volumetric flask and made up the final volume with diluent which was labeled as sample working solution (50µg/ml).

Method development

Initially, reverse phase liquid chromatography separation was tried to develop method by using different combinations. Trial-1 was carried out by taking water : methanol in the ratio of 50:50 using Hibra C18 100mm x 1.8 mm, 2.6µ column. Trial-2 was done by using methanol : potassium dihydrogen phosphate(50:50) with Hibra C18 100mm x 1.8 mm, 2.6µ column. Trial-3 was performed by using water : acetonitrile in 50:50 ratio with Hibra C18 100mm x 1.8 mm, 2.6µ column. Trial-4 accomplished by using acetonitrile : potassium dihydrogen phosphate (50:50) with Hibra C18 100mm x 1.8 mm, 2.6µ column. These trials showed bad peak shape, low plate count and tailing. So 5th trial was carried out by using potassium dihydrogen phosphate : acetonitrile (CHS C18 100mm x 1.8 mm, 1.6µ) in which peak shape was broad and tailing was observed. In addition, USP plate count was very less. So, further trial was carried out. Trial-6 was performed with combination of Potassium dihydrogen phosphate (0.01N) : acetonitrile in the ratio of 50:50 v/v at a flow rate of 0.4 ml/min. BEH C18, 50mm x 1.6 mm, 1.8µ size column (30°c temp) was used as stationary phase. The elution of drug obtained with good peak shape. Further, retention time and tailing factor were also within the limits.

Method validation

The method was validated for linearity, precision, accuracy, robustness and ruggedness, according to ICH guidelines [Sunil et al, 2015].

System suitability

System suitability were evaluated to verify system performance. It was determined by six replicate injections of standard solution. Other suitability parameters like peak area, retention time, relative standard deviation, theoretical plates and tailing factor were also evaluated [Mallikarjuna et al, 2013].

Linearity

Linearity was performed by testing six different concentration of sample solutions (25%, 50%, 75%, 100%, 125% and 150%). Each concentration was performed in triplicate according to optimized chromatographic

conditions and checked over by plotting the graph as peak area verses concentration. Thus the data treated by linear regression analysis.

Precision

Precision was evaluated by repeatability and intermediate precision. Then, the same sample solution was performed on same day and on different days at different time intervals [Parvathi et al, 2014]. Each stage of precision was investigated by six sequential replicates of injections with concentrations of 25%, 50%, 75% , 100 %, 125 % and 150 µg/ml. The precision was expressed by % RSD).

Repeatability

Semaglutide sample solution of 10 µg/ml concentration were spiked for repeatability of the method. The precision was examined by analyzing six replicates of 25%, 50%, 75%, 100%, 125% and 150 µg/ml. The retention time and relative standard deviation (%RSD) were calculated.

Intermediate precision

Working sample solution of 50ppm were injected on next day at six concentration levels of 25%, 50%, 75% , 100 %, 125 % and 150 µg/ml respectively. The % RSD of the analytical responses were calculated.

Accuracy

The accuracy of semaglutide was evaluated in triplicate at three concentration levels, i.e 50%, 100% and 150% of working concentration of sample. The percentage of recoveries for impurities were calculated [Kishore et al, 2012].

LOD and LOQ

The lowest concentration of analyte was detected by limit of detection and The lowest concentration of the substance was quantified by limit of quantification [Paramasivam and Nagappan, 2014]. The LOD and LOQ were determined by using signal to noise ratio approach as defined in ICH guidelines.

Robustness

Robustness was studied by testing the influence of small changes in pH of buffer (± 0.2 units), column temperature (± 5%), organic content of mobile phase (± 2%) and flow rate (± 5%) [Seshukumar et al, 2012].

Degradation studies

As per ICH guideline, The forced degradation studies were performed on drug substance to establish its inherent stability characteristics in order to demonstrate selectivity and stability indicating capability of the proposed method. The standard substances were exposed to forced degradation studies such as oxidation, acid degradation, alkali degradation, dry heat degradation, photo stability and neutral degradation studies.

Oxidation

1ml of 20% hydrogen peroxide (H_2O_2) was added to 1 ml of stock solution (semaglutide). The solutions were kept at 60^0c for 30 min. For UPLC study, the resultant solution was diluted to obtain 50ppm and 0.50 µl was injected into the system. To access the stability of sample, chromatogram was recorded.

Acid degradation

1 ml of 2N hydrochloric acid was added to 1 ml of stock solution (semaglutide) and refluxed for 30mins at 60^0C. The resultant solution was diluted to obtain 50ppm by using injection volume of 0.50 µl. The chromatogram was recorded to attain the stability of sample.

Alkali degradation

1 ml of 2 N sodium hydroxide was added to1 ml of stock solution (semaglutide) and refluxed for 30mins at 60°c. The resultant solution was diluted to obtain 50ppm and 0.50 µl was injected into the system and the chromatogram was recorded to access the stability of sample.

Dry heat degradation

The standard drug solution was placed in an oven at 105^0C for 6 hrs to attain dry heat degradation. For UPLC study, the resultant solution was diluted to 50ppm and 0.5µl was injected into the system and the chromatogram was recorded to gain the stability of the sample**.**

Photo Stability studies

The photochemical stability of the drug was also studied by exposing the (500ppm) solution to UV light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For UPLC study, the resultant solution was diluted to obtain 50ppm and 0.50µl was injected into the system and the chromatogram was 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 UPLC study, the resultant solution was diluted to 50ppm solution and 0.50µl were injected into the system and the chromatogram was recorded to assess the stability of the sample**.**

Assay

Standard solution and sample solution were injected separately into the UPLC system from which peak area response for analytes was measured. The standard was prepared from API and sample was prepared from formulation. Both standard and sample were analyzed by six replications. semaglutide was estimated (formulation) by taking standard as reference.

RESULTS

Chromatograms depicting the method development of semaglutide

Different chromatographic trials were done to achieve the better efficacy of the chromatographic system. The trial-1 chromatogram showed base line disturbances and tailing factor (fig.1). The trial-2 chromatogram demonstrated base line disturbances and low plate count (fig.2). The trial-3 chromatogram exhibited base line disturbances with low plate count and tailing factor (fig.3). The trial-4 chromatogram displayed bad peak shape with low plate count (fig.4). Further, trial was carried out by altering both mobile phase and column. The trial-5 chromatogram obtained was of broad peak with low plate count (fig.5). The trial-6 chromatogram (Semaglutide) eluted with good peak shape. Retention time and tailing were within the limit (fig.6). potassium dihydrogen phosphate (0.01N) : acetonitrile in ratio of 50:50 v/v was fixed as mobile phase. The wavelength of semaglutide showed maximum absorption at 292nm which was selected as detection wavelength for TUV detector.

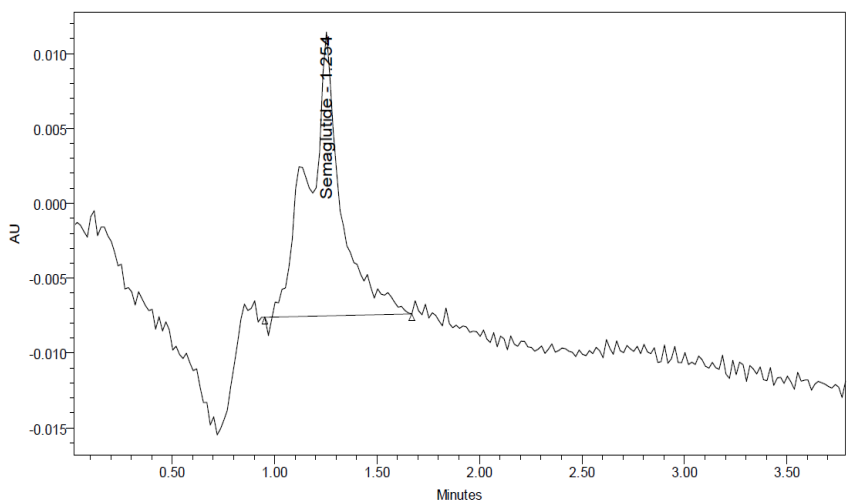

Fig.1: First trial chromatogram run of Semaglutide

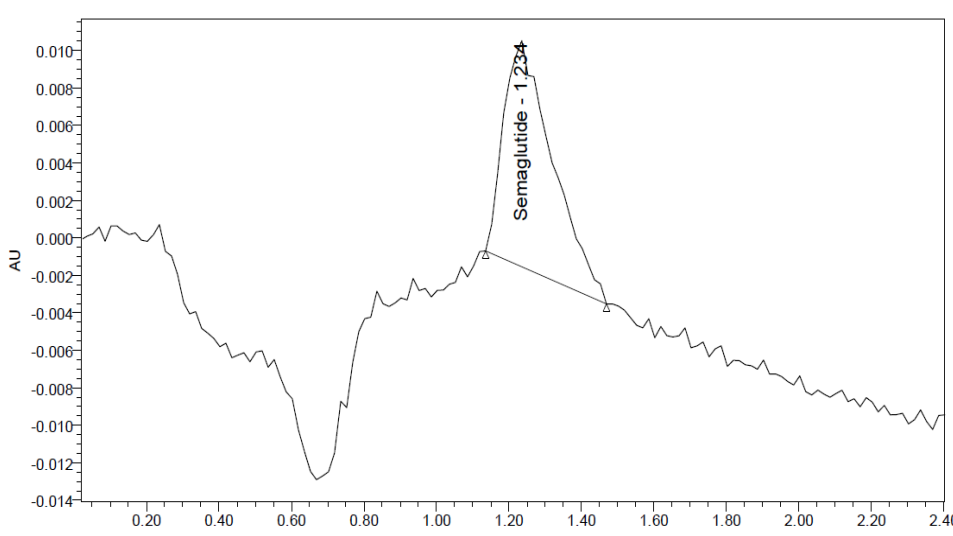

Fig.2: Second trial chromatogram run of Semaglutide

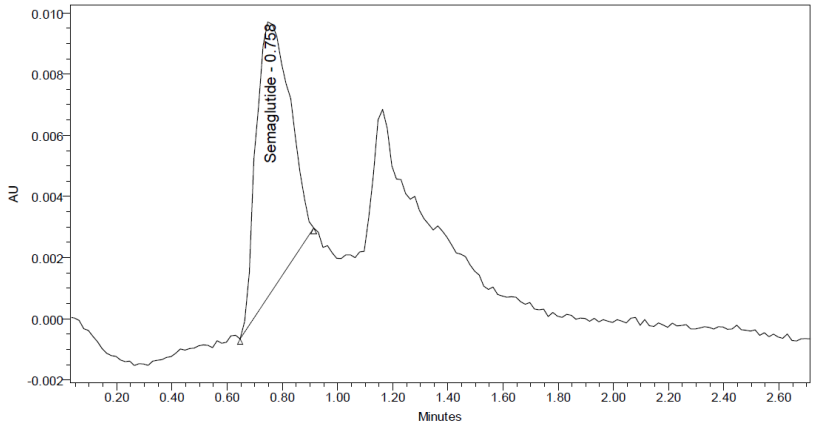

Fig.3: Third trial chromatogram run of Semaglutide

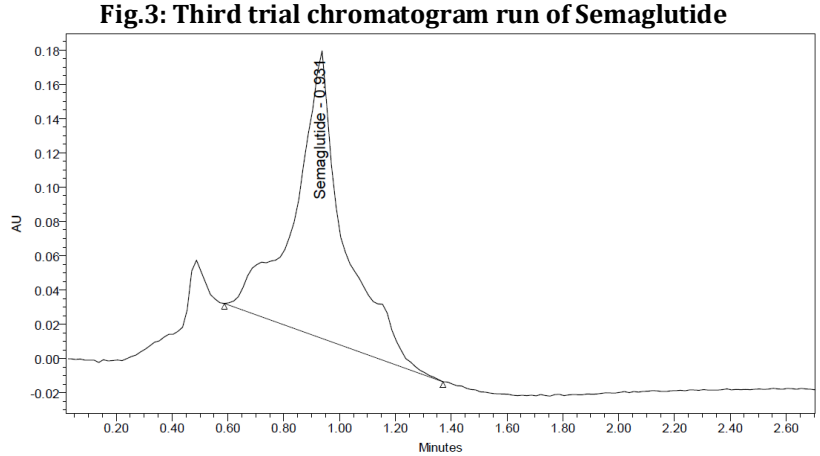

Fig.4: Fourth trial chromatogram run of Semaglutide

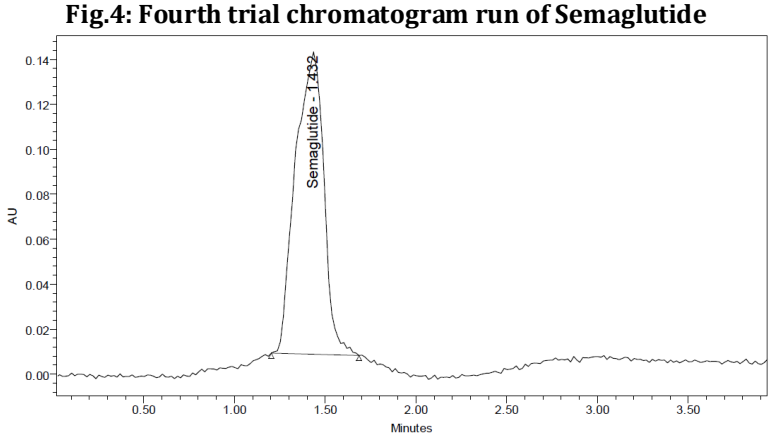

Fig.5: Fifth trial chromatogram run of Semaglutide

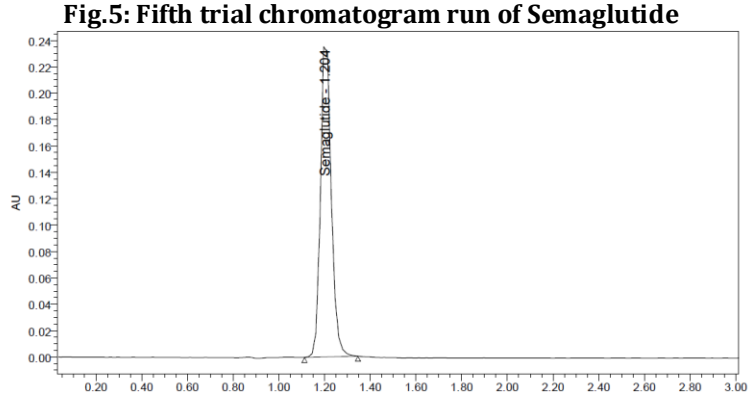

Fig.6: Optimized chromatogram of Semaglutide by proposed method

System suitability

System suitability results were well defined by several representative chromatographic trials. Retention time (1.026), USP plate count (2974), tailing factor (1.13), %RSD (0.3), standard deviation (2894.1) and mean area (853461) were evaluated by six replicate injections of drug (semaglutide). The results were shown in table.1.

Table1: system suitability data of semaglutide

S.NO	Peak Name	Retention Time	Area	% Area	USP Plate Count	USP Tailing
1	Semaglutide	1.019	854675	100.00	2836	1.27
2	Semaglutide	1.019	850385	100.00	2864	1.27
3	Semaglutide	1.021	851818	100.00	2472	1.16
4	Semaglutide	1.022	852153	100.00	2433	1.17
5	Semaglutide	1.026	853136	100.00	2810	1.15
6	Semaglutide	1.026	858602	100.00	2974	1.13
Mean			853461			
Standard deviation			2894.1			
% RSD			0.3			

Linearity: The linearity of the optimized method was determined for six concentrations and the correlation coefficient for semaglutide was found to be 0.9996 (fig.7). The calibration curve was linear for concentration between 12.5 and 75 µg/ml. Linearity was plotted between concentration and peak area. Slope and Y-Intercept was found to be 16726 and 42223. It shows that the linearity was within limit and obeys beer lambert's law. The linearity results were listed in table.2

Precision: The % RSD for the repeatability (intra day) and interday precision were found to be 0.5 and 0.6 which was within the limit (NMT 2.0%). The results of precision were shown in table.3 and table.4

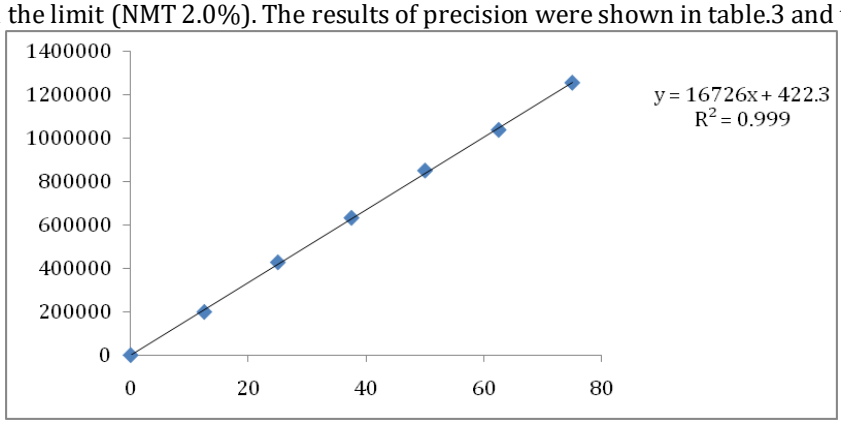

Fig. 7: Linearity plot of Semaglutide

Table 2: Linearity Concentration and Response

Linearity Level (%)	Concentration (ppm)	Area
0	0	0
25	12.50	198581
50	25	426652
75	37.5	631263
100	50	848661
125	62.5	1035740
150	75	1252556

Table 3: Repeatability Data

S. No	Peak Area
1	855072
2	854754
3	846126
4	850619

5	846303
6	850724
Average	850600
Standard deviation	3892.3
%RSD	0.5

Table 4: Intermediate precision data

S. No	Peak Area
1	818162
2	820852
3	826198
4	822022
5	832651
6	825223
Average	824185
Standard deviation	5076.7
%RSD	0.6

Accuracy: Three concentrations of 50%, 100%, 150% were injected in a triplicate manner and the mean% recovery was found to be 100.25% which was in the acceptance limit of 98.0 to 102.0%. The accuracy data was represented in table 5

Table 5: % Recovery data of accuracy

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean % Recovery
50%	25	25.10	100.40	100.25%
	25	25.18	100.74	
	25	25.26	101.03	
100%	50	50.86	101.72	
	50	49.63	99.25	
	50	49.64	99.27	
150%	75	74.95	99.94	
	75	74.64	99.52	
	75	75.30	100.40	

Robustness: The robustness of the developed method was determined by deliberate changes in the experimental conditions. The % RSD of flow minus, flow plus, mobile phase minus, mobile phase plus, temperature minus and temperature plus were found to be 0.3, 0.5, 45:55, 55:45, 25 and 35 respectively. Retention time and tailing factor were shown adherence to the limits. The robustness data was represented in table.6

Limit of Detection and Limit of Quantification: LOD and LOQ were determined by signal to noise ratio of 3:1 and 10:1 respectively. The limit of detection and limit of quantification were found to be 0.086µg/ml and 0.261µg/ml which was within the acceptance range. LOD and LOQ chromatograms were shown in fig.8 and fig.9

Table 6: Robustness Data

Parameter	%RSD
Flow Minus	0.3
Flow Plus	0.5
Mobile phase Minus	45:55
Mobile phase Plus	55:45
Temperature minus	25
Temperature plus	35

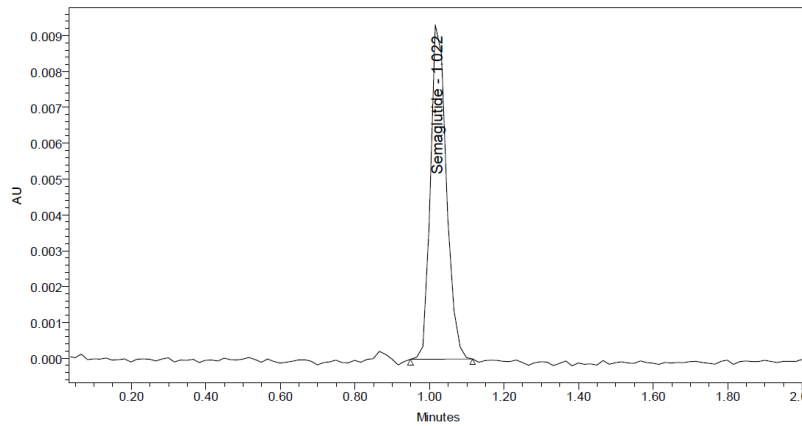

Fig.8: LOD Chromatogram of Semaglutide

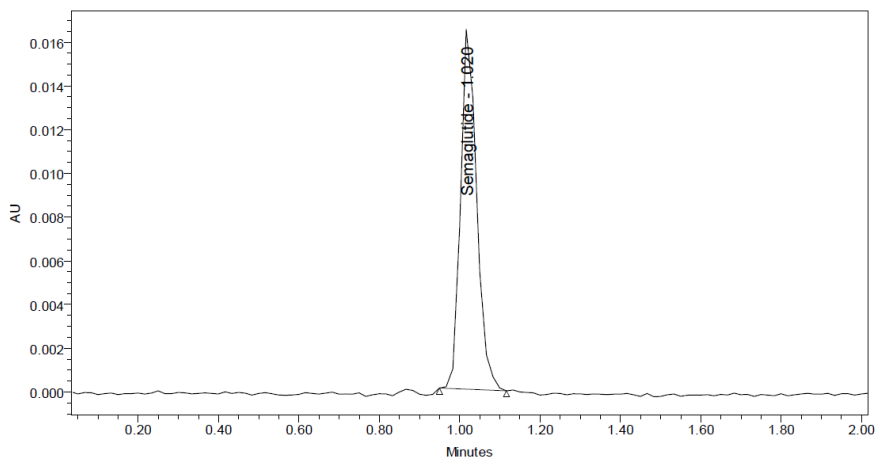

Fig.9: LOQ Chromatogram of Semaglutide

Forced degradation studies

Degradation studies were performed with formulation and degraded samples. The % degradation of the drug for acid, alkali, oxidation, thermal, uv and water were found to be 5.48, 4.34, 4.70, 2.29, 1.41, 0.98 respectively which was within the limit. Degradation data was mentioned in table.7

Table 7: Degradation Data of Semaglutide

S.NO	Degradation Condition	% Drug Degraded
1	Acid	5.48
2	Alkali	4.34
3	Oxidation	4.70
4	Thermal	2.29
5	UV	1.41
6	Water	0.98

Assay of marketed formulation

Standard and sample solution were injected separately into the system. The % RSD of marketed formulation was found to be 0.46. The results of assay (marketed formulation) were demonstrated in table.8

Table 8: Assay of Formulation

Sample No	%Assay
1	100.09
2	100.05
3	99.04
4	99.57
5	99.06

6	99.58
Average	99.57
Standard deviation	0.4556
%RSD	0.46

DISCUSSION

Linearity is the method ability to obtain test results, which are directly proportional to the concentration of analyte in the sample. Semaglutide showed a linearity response between 12.5-75µg/ml. The correlation coefficient was found to be 0.9996. hence it was greater than 0.999 which was within the limit. There is a greater correlation between peak area and concentration of analyte. The slope and Y-Intercept was found to be 16726 and 42223. The method of precision determines the closeness of agreement between a series of measurements of the same sample. The % RSD values were found to be 0.5 and 0.6µg/ml respectively. The values obtained was NMT 2.0% which was within the limit and good precision of assay method was confirmed. To demonstrate the accuracy of the method, standard addition and recovery experiments were conducted. The mean % recovery was found to be 100.25% which was within the acceptance range of 98-102%. The measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions known as robustness. Retention time and tailing factor were shown adherence to the limits. The limit of detection is the lowest concentration of analyte that can be detected. The limit of quantification is the lowest concentration of analyte that can be quantified. The LOD and LOQ determined by signal to noise ratio approach as defined in ICH. The S/N ratio was 3 for LOD and 10 for LOQ. The LOD and LOQ were found to be 0.086µg/ml and 0.261 µg/ml respectively, which was within the acceptance range. The peak purity test results which are derived by UV detector confirmed that semaglutide peaks were pure and homogeneous in all the analyzed stressed conditions. The purity threshold was found to be more than the purity angle which was within the acceptable range. This shows that the method was specific and stability indicating. Validation of the developed method was done as per the ICH guidelines. The method was found to be simple, accurate, precise , specific and stability indicating.

CONCLUSION

The chromatographic separation was achieved by using BEH C18 (50mm×1.6mm)1.8µm column thermostated at 30^{o}C. The mobile phase contains 0.01N potassium dihydrogen phosphate: acetonitrile in the ratio of 50:50 and flow rate was maintained at 0.4ml/min. The wave length detected at 292nm and injection volume was found to be 0.50µm. Suitability parameters were studied by injecting standard six times and the results were well under the acceptance criteria. Linearity study was carried out between 25% to150 % levels, R^2 value was found to be as 0.9996. Precision was found to be 0.5 for repeatability(intraday) and 0.6 for intermediate (inter day) precision. LOD and LOQ are 0.086µg/ml and 0.261µg/ml respectively. By using above method assay of marketed formulation was carried out 99.57% was present. Degradation studies of semaglutide was done in all conditions. The purity threshold was found to be more than purity angle and within the acceptable range. The proposed method was found to be simple, precise, accurate and validated according to ICH guidelines with respect to linearity, precision, accuracy, robustness and specificity studies which remained well within the limit.

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REFERENCES

1. Ambadas R and Prasanna A. (2011). Development and validation of HPTLC method for simultaneous estimation of gatifloxacin and ornidazole in human plasma: Journal of Chromatography Separation Techniques, 2:115.
2. Gotfredsen CF, Molck AM, Thorup I, Nyborg NC, Salanti Z, Knudsen LB, Larsen MO. (2014). The human GLP-1 analogs liraglutide and semaglutide: absence of histopathological effects on the pancreas in nonhuman primates: Diabetes, 63(7), 2486-2489.
3. Hjerpsted JB, Flint A, Brooks A, Axelsen MB, Kvist T, Blundell J. (2018) . Semaglutide improves postprandial glucose and lipid metabolism, and delays first-hour gastric emptying in subjects with obesity: Diabetes, Obesity and Metabolism, 20(3), 610-619.
4. Kanaka Parvathi K, Nagarjan V, Shantha A. (2014). Method development and validation for simultaneous assessment of paracetamol and naproxen in mixed tablet dosage form by RP-UPLC: World Journal of Pharmacy and Pharmaceutical Sciences, 3(3), 1973-1980.
5. Kishore Kumar K, Kameswara Rao C, Vijaya Lakshmi M, Mukkanti K. (2012). A validated stability Indicating RP-UPLC method for atrovastain calcium: American Journal of Analytical Chemistry, 3, 392-399.

6. Kumar N, Sangeetha D, Balakrishna P. (2011). Development and validation of a UPLC method for the determination of duloxetine hydrochloride residues on pharmaceutical manufacturing equipment surfaces: Pharmaceutical Methods, 2(3), 161-6.
7. Lavanya G, Sunil M, Eswarudu MM, Chinna Eswaraiah M, Harisudha K, Spandana B. (2013). Analytical method validation: An updated review: International Journal of Pharmaceutical Science and Research, 4(4), 1280-1286.
8. Mallikarjuna S, Ramalingam P, Sriram P, Garima J and Srinivas SK. (2013). Development and Validation of Stability-Indicating RP-UPLC Method for simultaneous estimation of amlodipine besylate and atorvastatin calcium in pharmaceutical dosage forms: Journal of Chromatography Separation Techniques, 4:187.
9. Paramasivam B and Nagappan K. (2014). Development and validation of stability-indicating RP-UPLC method for simultaneous estimation of thiocolchicoside and aceclofenac in combined dosage form: International Current Pharmaceutical Journal, 3(7), 296-300.
10. Seshukumar D, Abhishek G, Srinivas KS, Ravi Shankar G and Vinod Prasad S. (2012). Development and validation of stability indicating RP-UPLC method for simultaneous determination in fixed dose combination of ezetimibe and simvastatin: Journal of chromatography separation techniques, 3, 131.
11. Sunil Reddy P, Veera Raghava Raju T , Satyanarayana Raju P, Sunil Varma N, Sudhakar Babu K. (2015). Development and validation of a stability-indicating rp-UPLC method for the estimation of impurities in cinacalcet hydrochloride API and its formulation: Scientia Pharmaceutica, 83, 583-598.