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(RESEARCH ARTICLE)

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Development and validation of HPLC method for determination of amino acid mole ratio in Semaglutide

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Abstract

This study describes the development and validation of HPLC method for determination of amino acids mole ratio in Semaglutide. System suitability, specificity, Linearity and Range, Precision at specification level and Solution stability, Accuracy were studied to ensure amino acid mole ratio. The analytical method used for determination of amino acid mole ratio. System Suitability, Specificity, Linearity and range, Precision at specification level, and Accuracy. Hence, the method stands validated. The method can be used routinely for determination of amino acid mole ratio in Semaglutide in the quality control department.

Keywords: Semaglutide; HPLC; Manufacturing equipment; Quality control department

1. Introduction

Semaglutide, a synthetic 34 amino acid peptide. Semaglutide belongs to peptide family. Semaglutide is an agonist of glucagon-like peptide-1 receptors (GLP-1 AR) and used for the treatment of type 2 diabetes. [1-2] The purpose of this study is to develop and validate analytical method for determination of amino acid content in Semaglutide meets the requirements for its intended analytical application. [3-4]

2. Material and methods

Semaglutide was available at Precise Biopharma Pvt Ltd. Sodium Acetate Trihydrate, Acetonitrile, Glacial Acetic Acid, n-Hexane, Hydrochloric acid and solvents of analytical grade/IP/BP/USP equivalent grade available in the laboratory.

2.1. Method of Analysis by HPLC Procedure

2.1.1. Chromatographic Condition Buffer Preparation

Weigh and transfer about 13.6 g sodium acetate trihydrate, in 1000ml water and mix well to dissolve, adjust pH to 6.50 with diluted Glacial Acetic Acid or Sodium Hydroxide solution.

- **Mobile Phase A:** Mix 930 ml sodium acetate trihydrate solution (Buffer) and 70ml acetonitrile, mix well and Filter through 0.22µm filter. [5-6]
- **Mobile Phase B:** Mix Acetonitrile: Water (80:20) (%v/v), Mix well and sonicate to avoid baseline disturbances.
- **Diluent:** Water
- **Column:** Ultsil Amino Acid,5µm,4.6 x 250mm
- **Flow Rate:** 1.0ml/min **Injection Volume:** 10µL **Column Temperature:** 40ºC

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Autosampler Temperature: Ambient

- **Detector:** PDA/UV
- **Wavelength:** Semaglutide: 254nm
- **Run time:** 35 minutes
- **Retention Time of amino acids are as mentioned below:** (For Information only)

Asp about 4.59 mins, Glu about 5.22 mins, Ser about 9.78 mins, Gly about 10.57 mins, His about 10.98 mins, Arg about 12.63 mins, Thr about 13.76 mins, Ala about 14.49 mins,*Pro about 15.04 mins, Tyr about 20.50 mins, Val about 21.90 mins, *Met about 22.85 mins, *Cys about 24.44 min, Ile about 25.63 mins, Leu about 26.07 mins, he about 28.22 mins, Lys about 30.64 mins. [7-8]

- **Note:** *Pro, *Met and *Cys these peaks are not part of system suitability solution and calculation of amino acid, as these amino acids are not part of Semaglutide molecule.
- **Elution Mode:** Gradient, Mobile phase B: 0 mins-0%, 11 mins-7%, 13.9 mins-12%, 14.0 mins-15%, 29.0 mins-34%, 32.0 mins-70%, 35.0 mins-100%, 42 mins-100%,45 mins-0% and 60 mins-0%.
- **Blank:** Diluent

2.2. Preparation of Solution

2.2.1. Preparation of 6M Hydrochloric acid solution

Transfer 5 ml of Conc. Hydrochloric acid into 10.0 ml of volumetric flask. Dilute up to the mark with water.

2.2.2. Preparation of Derivatizing Solution

There are 2 derivatizing reagent i.e. A and B.

Preparation of derivatizing reagent A

Take 1ml of Welch Derivatizing reagent A add 4 ml of acetonitrile into it.

Preparation of derivatizing reagent B

Take 1ml of Welch Derivatizing reagent B add 4 ml of acetonitrile into it.

2.3. Preparation of solutions

2.3.1. Solution-1: Preparation of Standard solution

Transfer 80 µl standard solution in a test tube, add 80µl water add 100µl each diluted reagent A and B, mix well and react at ambient temperature for 60 min; add 400 µl n-hexane solution and shake for 5-10 second, stand at room temperature for separation of two layers.

Dilute 200 µl lower (aqueous solution) solution of test tube and with 800 µl water (consider this as solution-A).

Dilute 200 µl of above solution (Solution-A) with 800 µl water, mix well. (Note: If solution found hazy then filter through 0.22 µm membrane).

2.3.2. Solution-2: Preparation of Hydrolyze Sample Solution

Weigh 3 mg of sample into a headspace vial add 1.0 ml of 6 M hydrochloric acid solution to dissolve, place aluminum cap with septa on headspace vial and crimp the Headspace vial. Hydrolyze the sample at 110°C for 16 hours in heating block, after 16 hrs. cool down the headspace vial at room temperature and evaporate the solution by keeping the solution in water bath at 75°C and apply vacuum in HS Vial. [9-11]

OR

Keep solution in oven at 75°C under vacuum.

After evaporation add 1 ml of water into it.

2.3.3. Solution-3: Preparation of Sample solution

Transfer 80 µl above sample solution (Solution-2) in a test tube, add 80µl water add 100µl each diluted reagent A and B, mix well and react at ambient temperature for 60 min; add 400 µl n-hexane solution and shake for 5-10 second, stand at room temperature for separation of two layers. [12-13]

Dilute 200 µl lower (aqueous solution) solution of test tube and with 800 µl water (consider this as solution-A).

Dilute 200 µl of above solution (Solution-A)

with 800 µl water mix well. (Note: If solution found hazy then filter through 0.22 µm membrane).

(Prepare in Duplicate)

2.4. Validation parameters

2.4.1. System Suitability

Preparation of Standard Solution

Transfer 4ml of the Semaglutide standard stock solution into a 200ml volumetric flask. Dilute up to the mark with diluent and mix well. (Semaglutide: 10ppm)

- **Acceptance criteria:** %RSD of area of each amino acid peak obtained from three replicate standard solution should be not more than 5.0

2.5. Specificity[14]

2.5.1. Preparation of Standard Solution

Solution-1: Preparation of Standard solution

Transfer 80 µl standard solution in a test tube, add 80µl water add 100µl each diluted reagent A and B, mix well and react at ambient temperature for 60 min; add 400 µl n-hexane solution and shake for 5-10 second, stand at room temperature for separation of two layers.

Dilute 200 µl lower (aqueous solution) solution of test tube and with 800 µl water (consider this as solution-A).

Dilute 200 µl of above solution (Solution-A) with 800 µl water. (Note: If Solution found hazy then filter through 0.22 µm membrane.

Solution-2: Preparation of Hydrolyze Sample Solution

Weigh 3 mg of sample into a headspace vial add 1.0 ml of 6 M hydrochloric acid solution to dissolve, place aluminum cap with septa on headspace vial and crimp the Headspace vial. Hydrolyze the sample at 110°C for 16 hours in heating block, after 16 hrs. cool down the headspace vial at room temperature and evaporate the solution by keeping the solution in water bath at 75°C and apply vacuum in HS Vial.

OR

Keep solution in oven at 75°C under vacuum.

After evaporation add 1 ml of water into it.

Solution-3: Preparation of Sample solution

Transfer 80 µl above sample solution (Solution-2) in a test tube, add 80µl water add 100µl each diluted reagent A and B, mix well and react at ambient temperature for 60 min; add 400 µl n-hexane solution and shake for 5-10 second, stand at room temperature for separation of two layers.

Dilute 200 µl lower (aqueous solution) solution of test tube and with 800 µl water (consider this as solution-A).

Dilute 200 µl of above solution (Solution-A) with 800 µl water, mix well. (Note: If solution found hazy then filter through 0.22 µm membrane).

(Prepare in Duplicate).

2.6. Acceptance criteria

- All amino acid peaks obtained from standard solution should be well resolve from each other.
- All amino acid peaks obtained from standard solution should pass peak purity test.

2.7. Linearity and Range

Inject Amino acid standard solutions in triplicate ranging from 12.5% to 200% of the specification level by covering at least five points.

Perform the regression analysis and report the linearity range as the range for determining Amino acid mole ratio of Semaglutide.

2.7.1. Preparation of Standard Solution

Transfer 240 µl standard solution in a test tube, add 240µl water add 300µl each diluted reagent A and B, mix well and react at ambient temperature for 60 min; add 1200 µl n-hexane solution and shake for 5-10 second, stand at room temperature for separation of two layers.

Dilute 600 µl lower (aqueous solution) solution of test tube and with 2400 µl water (consider this as solution-A).

Dilute 200 µl of above solution (Solution-A) with 800 µl water, mix well (Note: if solution found hazy filter through 0.22 µm membrane).

2.7.2. Procedure

Inject each level in triplicate and plot the graph of peak area response versus concentration and check correlation co-efficient of average peak response.

Report y-intercept (at 100%), slope of regression line and residual sum of squares

2.7.3. Calculation

$$\text{Y} - \text{intercept value at 100\% level} = \frac{\text{Y Intercept}}{\text{Area of 100\% level}} \times 100$$

2.8. Acceptance criteria

- The plot of concentration versus peak area for each component should be linear with a correlation coefficient (R) not less than 0.99.
- Report the slope and intercept values. % Y intercept should be not more than ±10.0 % of response of 100% standard solution.

Table 1 Preparation linearity level

Level	% of Linearity Level	volume of Standard Solution in ml to be added (µl)	Diluted to volume (µl) with Diluent	Cone. of amino acids (%)
1	12.5	25	975	12.5
2	25	50	950	25
3	50	100	900	50
4	100	200	800	100
5	200	400	600	200

Table 2 Injection Sequences

S.No	Solution Name	No. of Injections
1	Blank (Diluent)	1
2	Linearity Level (12.5%)	3
3	Linearity Level (25%)	3
4	Linearity Level (50%)	3
5	Linearity Level (100%)	3
6	Linearity Level (200%)	3

*Three individual preparations of each levels were prepared and injected separately.

2.9. Precision

Method precision should be performed by preparing six different sample preparations of Semaglutide test sample and analyzing as per the method.

2.9.1. Preparation of Standard Solution

Transfer 80 µl standard solution in a test tube, add 80µl water add 100µl each diluted reagent A and B, mix well and react at ambient temperature for 60 min; add 400 µl n-hexane solution and shake for 5-10 second, stand at room temperature for separation of two layers.

Dilute 200 µl lower (aqueous solution) solution of test tube and with 800 µl water (consider this as solution-A).

Dilute 200 µl of above solution (Solution-A) with 800 µl water, mix well and filter through 0.22 µm membrane.

2.9.2. Preparation of Hydrolyze Sample Solution

Weigh 3 mg of sample into a headspace vial add 1.0 ml of 6 M hydrochloric acid solution to dissolve, place aluminum cap with septa on headspace vial and crimp the Headspace vial. Hydrolyze the sample at 110°C for 16 hours in heating block, after 16 hrs. cool down the headspace vial at room temperature and evaporate the solution by keeping the solution in water bath at 75°C and apply vacuum in HS Vial.

OR

Keep solution in oven at 75°C under vacuum.

After evaporation add 1 ml of water into it.

2.9.3. Preparation of Sample solution

Transfer 80 µl above sample solution (Solution-2) in a test tube, add 80µl water add 100µl each diluted reagent A and B, mix well and react at ambient temperature for 60 min; add 400 µl n-hexane solution and shake for 5-10 second, stand at room temperature for separation of two layers. (15-17)

Dilute 200 µl lower (aqueous solution) solution of test tube and with 800 µl water (consider this as solution-A).

Dilute 200 µl of above solution (Solution-A) with 800 µl water, mix well. (Note: If solution found hazy then filter through 0.22 µm membrane). (Prepare Six preparations).

2.10. Acceptance criteria

% RSD of each amino acid mole ratio in six test preparations should be not more than 5.0.

2.11. Intermediate Precision (Ruggedness)

The ruggedness should be performed by preparing six different sample preparations of Semaglutide test sample and analyzing as per the method by considering different analyst, different day, different column and different instrument.

2.11.1. Preparation of Standard Solution

Transfer 80 µl standard solution in a test tube, add 80µl water add 100µl each diluted reagent A and B, mix well and react at ambient temperature for 60 min; add 400 µl n-hexane solution and shake for 5-10 second, stand at room temperature for separation of two layers.

Dilute 200 µl lower (aqueous solution) solution of test tube and with 800 µl water (consider this as solution-A).

Dilute 200 µl of above solution (Solution-A) with 800 µl water, mix well and filter through 0.22 µm membrane.

2.11.2. Preparation of Hydrolyze Sample Solution

Weigh 3 mg of sample into a headspace vial add 1.0 ml of 6 M hydrochloric acid solution to dissolve, place aluminum cap with septa on headspace vial and crimp the Headspace vial. Hydrolyze the sample at 110°C for 16 hours in heating block, after 16 hrs. cool down the headspace vial at room temperature and evaporate the solution by keeping the solution in water bath at 75°C and apply vacuum in HS Vial.

OR

Keep solution in oven at 75°C under vacuum.

After evaporation add 1 ml of water into it.

2.11.3. Preparation of Sample solution

Transfer 80 µl above sample solution (Solution-2) in a test tube, add 80µl water add 100µl each diluted reagent A and B, mix well and react at ambient temperature for 60 min; add 400 µl n-hexane solution and shake for 5-10 second, stand at room temperature for separation of two layers.

Dilute 200 µl lower (aqueous solution) solution of test tube and with 800 µl water (consider this as solution-A).

Dilute 200 µl of above solution (Solution-A) with 800 µl water, mix well. (Note: If solution found hazy then filter through 0.22 µm membrane). (Prepare Six preparations)

2.12. Acceptance criteria

1) % RSD of each amino acid mole ratio in six test preparations should be not more than 5.0.

2) Cumulative % RSD of each amino acid mole ratio from twelve determinations (From method precision and intermediate precision) should be not more than 5.0

2.12.1. Accuracy

Accuracy will be determined by injecting Semaglutide standard solution in triplicate at 80 %, 100%, and 120 % levels and analyzing as per method.

Table 3 Sample Preparation

S.No	Accuracy Level	Weight of Semaglutide standard in mg (A)	From Solution A transfer (µl)	Diluted to volume (µl) with Diluent	Cone. of amino acids (%)
1	80%	2.4	200	800	80
2	100%	3.0	200	800	100
3	120%	3.6	200	800	120

Table 4 Injection Sequences

S.No	Solution Name	No. of Injections
1	Blank (Diluent)	1
2	Standard Solution	3
3	Accuracy Level (80%)	3
4	Accuracy Level (100%)	3
5	Accuracy Level (120%)	3

Table 5 The retention time and Specification is as follows form Amino acid

Sr No.	Component	Retention Time (mins)	RRT	Specification Limit
1	Asp	4.59	1.00	0.8 to 1.2
2	Glu	5.22	1.14	4.0 to 6.0
3	Ser	9.78	2.13	2.4 to 3.6
4	Gly	10.57	2.30	3.2 to 4.8
5	His	10.98	2.39	0.8 to 1.2
6	Arg	12.63	2.75	1.6 to 2.4
7	Thr	13.76	3.00	1.6 to 2.4
8	Ala	14.49	3.16	2.4 to 3.6
9	*Pro	15.04	3.28	0.7 to 1.2
10	Tyr	20.50	4.47	1.6 to 2.4
11	Val	21.90	4.77	0.8 to 1.2
12	*Met	22.85	4.98	1.6 to 2.4
13	*Cys	24.44	5.32	1.6 to 2.4
14	Ile	25.63	5.59	0.7 to 1.2
15	Leu	26.07	5.68	0.8 to 1.2
16	Phe	28.22	6.15	4.0 to 6.0
17	Lys	30.64	6.68	2.4 to 3.6

Note : *Pro, *Met and *Cys these peaks are not part of system suitability solution and calculation of amino acid ratio, as these amino acids are not part of Semaglutide molecule.

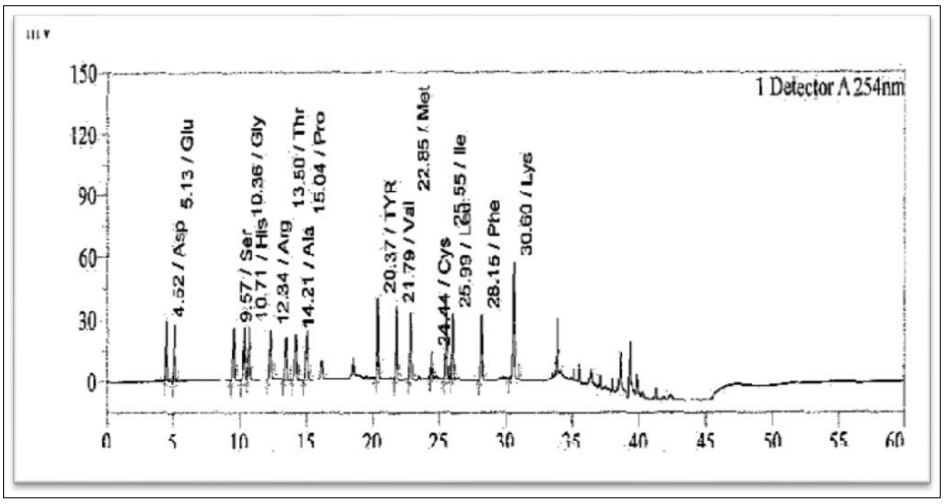

Figure 1 Standard chromatogram

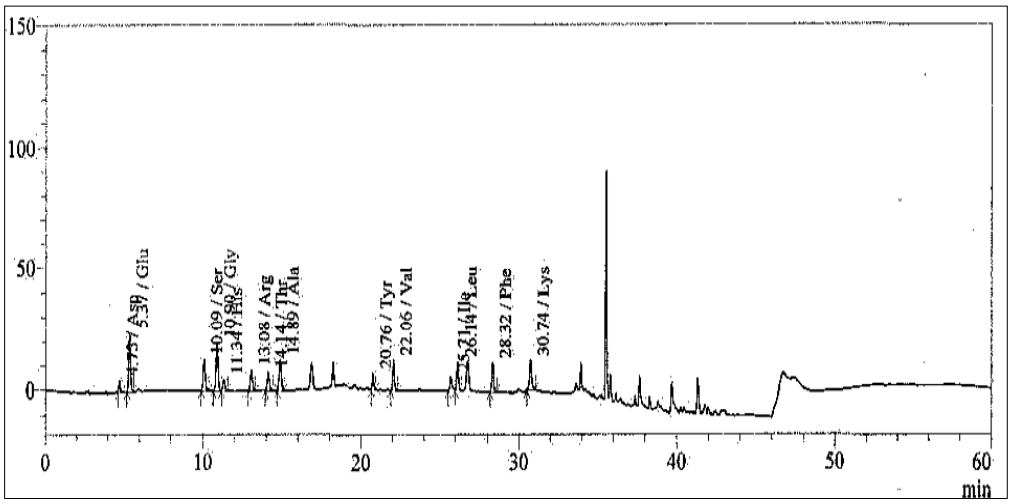

Figure 2 Test sample chromatogram

3. Results and discussion

%RSD of area of each amino acid peak obtained from three replicate standard solution is found to be less than 5.0.

It was concluded system was suitable for analysis of Amino acid mole ratio.

In specificity all amino acid peaks obtained from standard solution are well resolve from each other.

In linearity of the plot of concentration versus peak area for each component was linear with a correlation coefficient (R) not less than 0.99 for all amino acid.

In Accuracy The mole ratio values and % RSD of mole ratio at each level from 80% to 120% obtained was within the acceptance criteria. Hence, accuracy was established.

Samples analysed found complies with the specification for amino acid as per above table

4. Conclusion

From the results obtained by applying the suggested procedures, it is obvious that the proposed methods are accurate, precise, simple, sensitive, rugged, robust and rapid and can be applied successfully in routine analysis for the estimation of amino acid mole ratio in Semaglutide.

Compliance with ethical standards

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Disclosure of conflict of interest

I declare that I have no conflict of interest.

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