Determination of Chloride, Sulfate, Trifluoroacetic acid, Phosphate, Sodium and Ammonium from Semaglutide by Ion Chromatography

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Abstract: *A developed and validated method on Ion Chromatography for the determination of chloride, sulfate, trifluoroacetic acid, phosphate, sodium and ammonium from Semaglutide active pharmaceutical ingredient (API). It is new, simple, accurate, precise and reproducible method. This Ion Chromatography method requires high capacity columns for best resolution and peak shapes. Chloride, sulfate, trifluoroacetic acid and phosphate were separated using IonPac AS11-HC column whereas sodium and ammonium were separated using IonPac CS16 column. These anions and cations had been analysed by suppressed conductivity detection in two different channels with same IC system. This method is linear from 1.0mg/L to 100.0mg/L of chloride, sulfate, trifluroracetic acid and phosphate, whereas it is linear for 10.0mg/L to 200.0mg/L of Sodium and 0.1mg/L to 5.0mg/L of Ammonium. The limits of detection and quantification have also been established for these anions and cations. This method had been validated and shows good sensitivity, reproducibility, linerarity, specificity and robustness. Therefore, this method can be applicable for routine and regular analysis of anionic and cationic process impurities from semaglutide.*

Keywords: Semaglutide, Anti-Diabetic drug, Peptide, Ion Chromatography, Anions, Cations, Validation, Chromeleon

1. Introduction

India is country where one in every six diabetics globally reside. The country is ranked eighth among the top ten countries for diabetics [1, 8]. Diabetes medications, in combination with a nutritious diet and regular exercise, enable persons with type 2 diabetes and gestational diabetes to maintain stable blood glucose levels. There are several different diabetic medications available, each of which serves a different purpose. Most of patients requires multi-drug therapy to treat diabetes which may include combination of two or more drugs.

A side effect is an unintentional consequence of taking a medicine. Unfortunately, certain diabetes medications have typical side effects include nausea or stomach trouble. In the case of diabetes medications, such as metformin, side effects are frequent but temporary. Different types of Anti-diabetes drugs have various side effects. For example, Sulfonylureas class of drugs will cause low blood sugar, skin rash or itching, weight gain. Biguanides (e.g. Metformin) class of drugs will cause kidney complications, tiredness or dizziness. Alpha glucosidase inhibitors class of drugs will cause gas, bloating and diarrhea. Thizolidinediones class of drugs will cause weight gain, risk of liver disease, anemia risk, swelling of legs or ankles. Meglitinides class of drugs will cause weight gain and low blood sugar [6].

Semaglutide is an anti-diabetic medication which is used for chronic weight management and treatment of type 2 diabetes. It is in a class of medications called incretin mimetics. When blood sugar levels are high, it supports pancreas in liberating the suitable quantity of insulin. Insulin is a hormone that aids in the movement of sugar from the bloodstream to other bodily tissues where it is used for energy. Semaglutide also works by slowing the movement of food through the stomach and may decrease appetite and cause weight loss [2, 3, 4].

Semaglutide Assay and related substances analysis are mostly done by HPLC and UV detection. And if sample matrix is complex, it is analysed by LC-MS methodologies [1]. There should be limit for anions like chloride, sulfate, trifluoroacetic acid and phosphate and cations like sodium and potassium as presence of it might hinder Semaglutide efficacy or its stability. There are several processes during manufacturing through which these anions and cations can be added into Semaglutide and they need to be monitored in final active pharmaceutical drug.

Therefore, Ion Chromatography method had been developed to keep a check on anions and cations during process manufacturing and final active pharmaceutical product. The method described in the present article displays advantages in its simplicity and accuracy of results. Both anions and cations can be precisely analysed using methods illustrated herein the article, with ease and efficiently.

2. Experimental

2.1 Reagents and Chemicals

Semaglutide API was provided by Pharmaceutical firm to develop this method**.** Analytical Reagent (AR) grade reagents and chemicals were used for preparation of reagents, standards and mobile phase. Deionized (DI) water (18.2 MΩ cm & TOC <10ppb) was used as diluent and for preparation of eluent. Sodium Chloride (Merck), Potassium Sulfate (Merck), Sodium dihydrogen Phosphate monohydrate (Merck), Trifluoroacetic acid (Merck) and Ammonium Chloride (Merck) were used for preparation of standards, RFIC-MSA (Reagent Free Ion Chromatography

Methanesulfonic acid) was used for electrolytic generation of Methanesulfonic acid eluent for cations estimation and RFIC-KOH (Reagent Free Ion Chromatography Potassium Hydroxide) was used for electrolytic generation of Potassium Hydroxide eluent for anions estimation.

2.2 Apparatus

The equipment used was Thermo Fisher Dionex Ion Chromatography system **ICS 6000** with dual analysis configuration having AS-AP Autosampler with a 20µL loop (for anions analysis) and 10 µL loop (for cations analysis), IonPac AS11-HC, 4mm analytical and IonPac AG11-HC, 4mm guard column along with ADRS600, 4mm was used for anions analysis and IonPac CS16, 4mm and IonPac CG16, 4mm guard column along with CDRS600, 4mm was used for cations analysis. The experiment was conducted using a pre-degassed DI water for preparation of eluent, standards and samples.

For anions analysis, 30mM Potassium Hydroxide (KOH) was used as eluent at a flow rate of 1.0ml/min with ADRS600, 4mm in constant voltage mode with suppressed conductivity detection technique with run time of 15 minutes

For cations analysis, 30mM Methanesulfonic Acid (MSA) was used as eluent at a flow rate of 1.0mL/min with CDRS600, 4mm in constant voltage mode with Suppressed conductivity detection technique. Column oven temperature used is 40°C. Run time for this experiment was 30 minutes.

Dual Channel ICS6000 Ion Chromatography system was used for simultaneous anions and cations analysis and Software used for data acquisition was Thermo Fisher Scientific Dionex Chromeleon (version: 7.2.10). Chromatograms were monitored simultaneously during analysis and results were interpreted.

2.3 Procedure

Preparation of Diluent: Deionized Water (DI Water) is used as diluent.

For Chloride, Sulfate, Trifluoroacetic acid (TFA) and Phosphate analysis

Preparation of Potassium Hydroxide eluent: DI water is connected to pump and required concentration (30 mM of KOH) was generated electrolytically by Reagent Free Ion Chromatography Potassium Hydroxide (RFIC-KOH) module.

Preparation of standard solutions: Certified Sodium Chloride, Potassium Sulfate, Trifluoroacetic acid (TFA) and Sodium dihydrogen Phosphate monohydrate reagents were procured from Merck. From these, 1000mg/L Chloride and 1000mg/L Sulfate, 1000mg/L TFA and 1000mg/L Phosphate were prepared using diluent. Standards used for validation study were prepared according to Table 1 from their respective stock of 1000mg/L.

Table 1: Concentration of Chloride, Sulfate, TFA and Phosphate used for validation study

Parameter	Chloride mg/L	Sulfate mg/L	TFA mg/L	Phosphate mg/L
Specificity	10.0	10.0	10.0	10.0
Precision	50.0	50.0	50.0	50.0
Linearity	1.0, 5.0, 10.0, 25.0, 50.0 and 100.0	1.0, 5.0, 10.0, 25.0, 50.0 and 100.0	1.0, 5.0, 10.0, 25.0, 50.0 and 100.0	1.0, 5.0, 10.0, 25.0, 50.0 and 100.0
Limit of Detection (LOD)	0.03	0.10	0.10	0.10
Limit of Quantification (LOQ)	0.10	0.50	0.50	0.50

Sample preparation: Weighed accurately 0.10g of Semaglutide sample in 10mL of volumetric flask, added 6mL of diluent and sonicated for 5 minutes to dissolve and finally made up to the mark with diluent. Samples were passed through OnGuard II RP (1.0cc cartridge, Thermo Fisher Scientific Dionex PN 057083) and filtered through 0.2µ nylon membrane filter. This was collected in autosampler vial. This procedure was repeated for each sample along with recovery samples and diluent

For Sodium and Ammonium analysis

Preparation of Methanesulfonic Acid eluent: DI water is connected to pump and required concentration (30 mM of MSA) was generated electrolytically by Reagent Free Ion Chromatography Methanesulfonic acid (RFIC-MSA) module.

Preparation of standard solutions: Certified Sodium Chloride and Ammonium Chloride were procured from Merck. From these, 1000mg/L Sodium and 1000mg/L Ammonium were prepared in diluent. Standards used for validation study were prepared according to Table 2 from their respective stock of 1000mg/L.

Table 2: Concentration of Sodium and Ammonium used for validation study

Parameter	Sodium (mg/L)	Ammonium (mg/L)
Specificity	25.0	1.0
Precision	50.0	1.0
Linearity	10.0, 25.0, 50.0, 100.0 and 200.0	0.10, 0.50, 1.0, 2.50 and 5.00
Limit of Detection (LOD)	0.02	0.03
Limit of Quantification (LOQ)	0.06	0.08

Sample preparation: Weighed accurately 0.10g of Semaglutide sample in 10mL of volumetric flask, added 6mL of diluent and sonicated for 5 minutes to dissolve and finally made up to the mark with diluent. Samples were passed through OnGuard II RP (1.0cc cartridge, Thermo Fisher Scientific Dionex PN 057083) and filtered through 0.2µ nylon membrane filter. This was collected in autosampler vial. This procedure was repeated for each sample along with recovery samples and diluent.

An Autosampler (Dionex AS-AP in sequential injection mode) was used to inject standard solution containing anions or cations into the ion chromatography system.

Subsequently, the standard solution in the sample loop was transferred onto the separator column, on which anions or cations were separated. After separation on the column, these ions were detected by Suppressed Conductivity detector. A sequence containing the blank, standards, samples and recovery samples were run, and results were then interpreted.

3. Results and Discussions

For Chloride, Sulfate, TFA and Phosphate analysis

Limit of Detection (LOD) for Chloride, Sulfate, TFA and Phosphate was 0.03mg/L, 0.10mg/L, 0.10mg/L and 0.10mg/L respectively and injected (n) six times and average signal to noise ratio (S/N) observed was 4.1 for Chloride, 3.2 for Sulfate, 3.4 for TFA and 3.1 for Phosphate. Limit of Quantification (LOQ) for Chloride, Sulfate, TFA and Phosphate was 0.10mg/L, 0.50mg/L, 0.50mg/L and 0.50mg/L respectively, it was injected (n) six times and observed signal to noise ratio (S/N) was 30.1 for Chloride, 21.3 for Sulfate, 20.1 for TFA and 18.5 for Phosphate. It's percent relative standard deviation (RSD) for peak area was 1.54% for Chloride, 2.26% for Sulfate, 0.73% for TFA and 2.34% for Phosphate. Table 3 shows results for LOD and LOQ of Chloride, Sulfate, TFA and Phosphate.

Table 3: LOD and LOQ data for Chloride, Sulfate, TFA and Phosphate

Chloride			
Parameter	Amount, mg/L	S/N	% RSD (n=6)
LOD	0.03	4.1	Not Applicable
LOQ	0.10	30.1	1.54%
Sulfate			
Parameter	Amount, mg/L	S/N	% RSD (n=6)
LOD	0.10	3.2	Not Applicable
LOQ	0.50	21.3	1.81%
TFA			
Parameter	Amount, mg/L	S/N	% RSD (n=6)
LOD	0.10	3.4	Not Applicable
LOQ	0.50	20.1	0.73%
Phosphate			
Parameter	Amount, mg/L	S/N	% RSD (n=6)
LOD	0.10	3.1	Not Applicable
LOQ	0.50	18.5	2.34%

The response of the analytes was linear over the range of 1.0 to 100.0mg/L for each Chloride, Sulfate, TFA and Phosphate. Calibration curve fits well and that is significantly linear having correlation coefficient of 0.9998, slope 0.2700 and offset -0.0779 (figure 1) for Chloride, correlation coefficient of 0.9999, slope 0.1841 and offset -0.0932 (figure 1) for Sulfate, correlation coefficient of 0.9998, slope 0.1037 and offset -0.1084 (figure 1) for TFA and correlation coefficient of 0.9997, slope 0.0809 and offset -0.1379 (figure 1) for Phosphate. This linearity study was performed for the concentration range of 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 mg/L of Chloride, Sulfate, TFA and Phosphate each. Each standard injection was repeated thrice. Therefore, number of calibration points (n) for linearity study was 18. Its data had been shown in table 4.
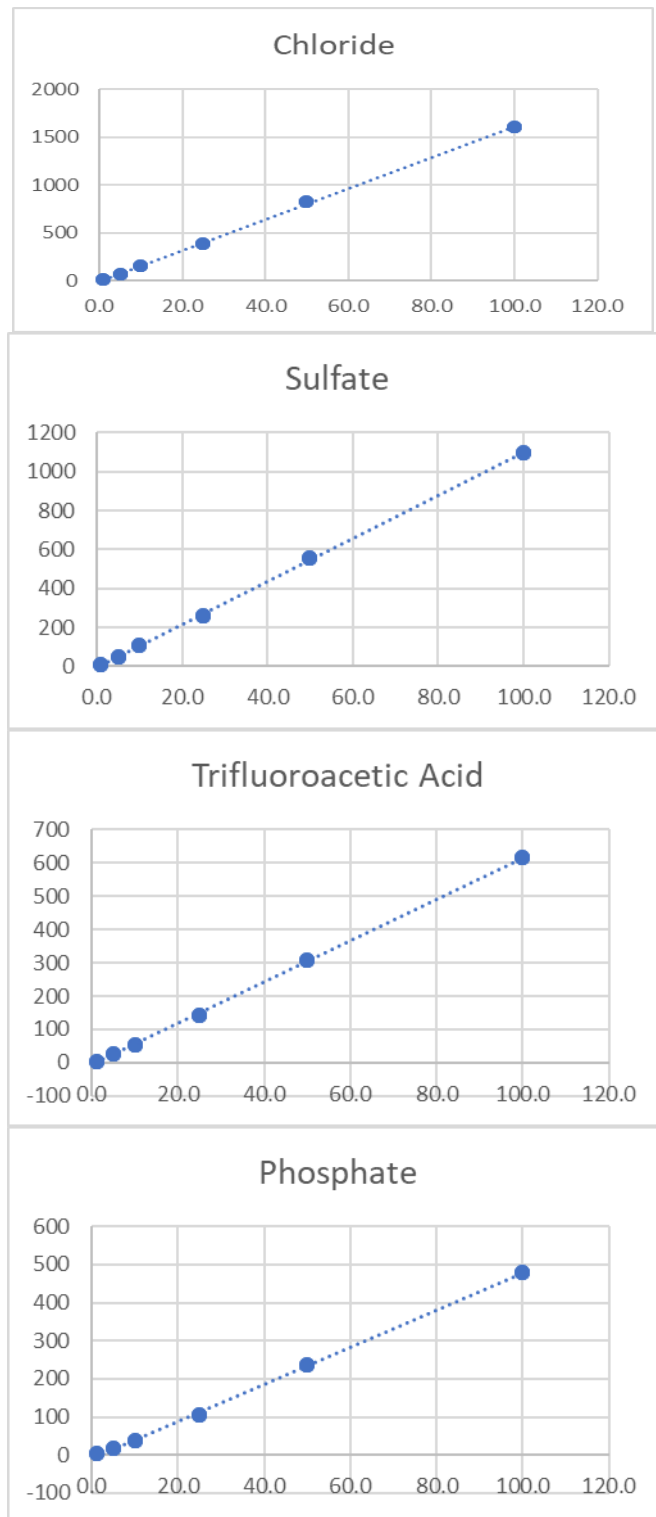

Figure 1: Linearity plot for Chloride, Sulfate, TFA and Phosphate

Table 4: Linearity data for Chloride, Sulfate, TFA and Phosphate

Analyte	Points	Corr. Coeff.	Offset	Slope
Chloride	18	0.9998	-0.0779	0.2700
Sulfate	18	0.9999	-0.0932	0.1841
TFA	18	0.9998	-0.1084	0.1037
Phosphate	18	0.9997	-0.1379	0.0809

Replicate injections mixture of Chloride, Sulfate, TFA and Phosphate standards was done and their percentage relative

standard deviation for peak area was 0.95% for Chloride, 1.23% for Sulfate, 1.32% for TFA and 1.14% for Phosphate. Table 5 shows results for their precision study.

Table 5: Precision data for Chloride, Sulfate, TFA and Phosphate

Analyte	Amount, mg/L	% RSD (n=10)	Resolution (USP)
Chloride	50.0	0.95	4.86
Sulfate	50.0	1.23	3.68
TFA	50.0	1.32	6.54
Phosphate	50.0	1.14	Not Applicable

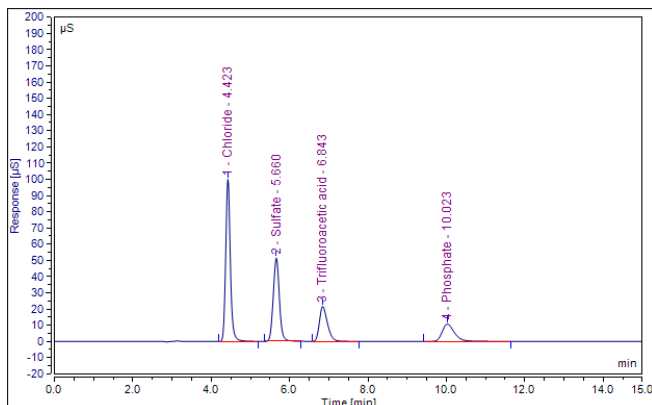Chromatogram for Chloride, Sulfate, TFA and Phosphate standard mixture is shown in figure 2.

Figure 2: Standard chromatogram for Chloride (50.0mg/L), Sulfate (50.0mg/L), TFA (50.0mg/L) and Phosphate (50.0mg/L)

Sample results: Samples were analysed using the linearity calibration method. Replicate injections of same sample was also done. Its results and routine analysis sample results were shown in table 6 and table 7. Sample chromatogram was shown in figure 3.

Table 6: Sample precision

Analyte	Sl. No..	Number of injections	% RSD
Chloride	MCP-01-40	10.0	0.93
Sulfate	MCP-01-40	10.0	1.36
TFA	MCP-01-40	10.0	1.80
Phosphate	MCP-01-40	10.0	Not Detected

Table 7: Routine sample analysis results

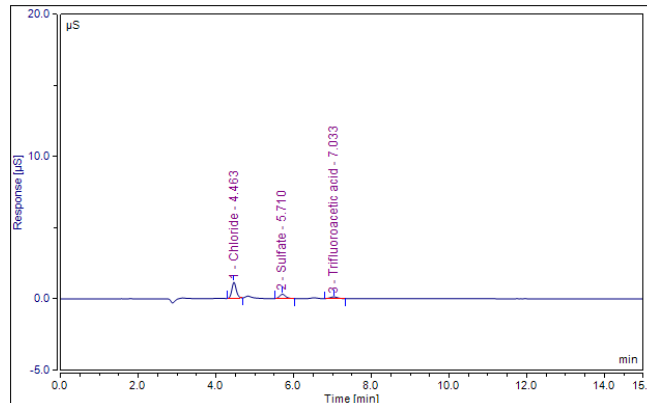| S. No.	Chloride %	Sulfate %	TFA %	Phosphate %
MCP-01-40	0.008	0.008	0.013	Not Detected
MCP-01-41	0.020	0.016	0.011	Not Detected
MCP-01-42	0.018	0.010	0.009	Not Detected
MCP-02-01	0.060	0.031	0.091	0.003
MCP-02-03	0.009	0.015	0.012	Not Detected

Figure 3: Sample chromatogram for Chloride, Sulfate, TFA and Phosphate estimation (Sl. No. MCP-01-40)

Recovery: The sample used for recovery study was Semaglutide Sl. No. MCP-01-40 (average concentration was taken for calculation). Recovery test solutions were injected in triplicate. Also for recovery study, known concentrations of amount was added to sample at three different levels as shown in table 8.

Table 8: Recovery study (Chloride, Sulfate, TFA and Phosphate) for sample Semaglutide (Sl. No. MCP-01-40) (n = 3)

Analyte	Target Concentration	Amount Added, mg/L	Amount Recovered, mg/L	% Recovery
Chloride	50%	25.0	25.5	102.0
	100%	50.0	50.2	100.4
	150%	75.0	75.4	100.5

Analyte	Target Concentration	Amount Added, mg/L	Amount Recovered, mg/L	% Recovery
Sulfate	50%	25.0	25.3	101.2
	100%	50.0	49.2	98.4
	150%	75.0	74.6	99.5

Analyte	Target Concentration	Amount Added, mg/L	Amount Recovered, mg/L	% Recovery
TFA	50%	25.0	24.6	98.4
	100%	50.0	49.1	98.2
	150%	75.0	74.2	98.9

Analyte	Target Concentration	Amount Added, mg/L	Amount Recovered, mg/L	% Recovery
Phosphate	50%	25.0	25.1	100.4
	100%	50.0	49.3	98.6
	150%	75.0	75.3	100.4

Method Ruggedness: Method was tested with different flow rate like 1.2 and 0.8. Also, it was tested using different eluent concentrations and IC system. Method was proven to be rugged as it does not show any changes in sample results due to changes in flow rate, eluent concentration and IC system.

For Sodium and Ammonium analysis
Limit of Detection (LOD) for Sodium and Ammonium was 0.02mg/L and 0.03mg/L respectively and injected (n) six times and observed average signal to noise ratio (S/N) was 4.2 for Sodium and 3.8 for Ammonium. Limit of

Quantification (LOQ) for Sodium and Ammonium was 0.06mg/L and 0.08mg/L respectively, it was injected (n) six times and observed signal to noise ratio (S/N) was 15.6 for Sodium and 12.1 for Ammonium. It's percent relative standard deviation for peak area was 2.12% for Sodium, and 1.54% for Ammonium. Table 9 shows results for LOD and LOQ of Sodium and Ammonium

Table 9: LOD and LOQ data for Sodium and Ammonium

Sodium			
Parameter	*Amount, mg/L*	*S/N*	*% RSD (n=6)*
LOD	0.02	4.2	Not Applicable
LOQ	0.06	15.6	2.12%
Ammonium			
Parameter	*Amount, mg/L*	*S/N*	*% RSD (n=6)*
LOD	0.03	3.8	Not Applicable
LOQ	0.08	12.1	1.54%

The response of the analytes was linear over the range of 10.0 to 200.0mg/L for Sodium and 0.1 to 5.0mg/L for Ammonium. Calibration curve fits well and that is significantly linear having correlation coefficient of 0.99998, slope 0.1060 and offset -0.0227 (figure 4) for Sodium and correlation coefficient of 0.9992, slope 0.0972 and offset 0.00 (figure 4) for Ammonium. This linearity study was performed for the concentration range of 10.0, 25.0, 50.0, 100.0 and 200.0mg/L of Sodium and 0.10, 0.50, 1.00, 2.50 and 5.00mg/L of Ammonium. Each standard injection was repeated thrice. Therefore, number of calibration points (n) for linearity study was 15. Its data had been shown in table 10.
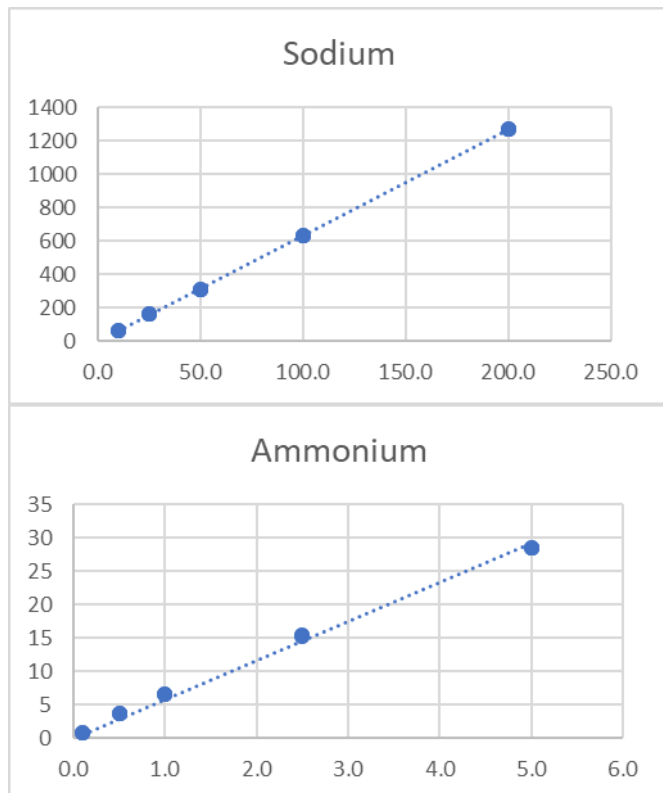

Figure 4: Linearity plot for Sodium and Ammonium.

Table 10: Linearity data for Sodium and Ammonium

Analyte	*Points*	*Corr. Coeff.*	*Offset*	*Slope*
Sodium	15	0.99998	-0.0227	0.1060
Ammonium	15	0.99922	0	0.0972

Replicate injections mixture of Sodium and Ammonium standards was done and their percentage relative standard deviation for peak area was 0.61% for Sodium and 1.52% for Ammonium. Table 11 shows results for their precision study.

Table 11: Precision data for Sodium and Ammonium

Analyte	*Amount, mg/L*	*% RSD (n=10)*	*Resolution (USP)*
Sodium	50.0	0.61	5.23
Ammonium	1.00	1.51	Not Applicable

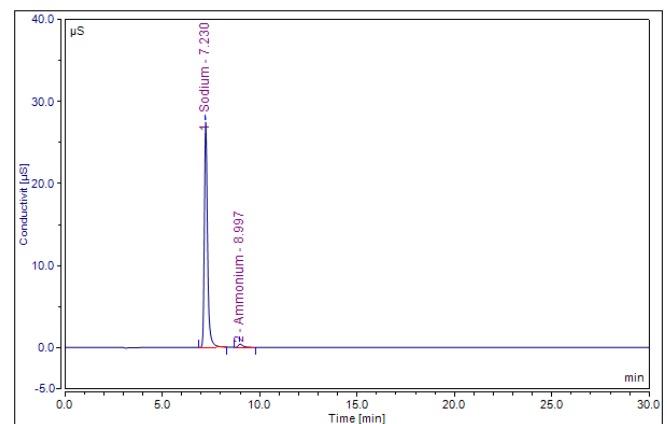Chromatogram for Sodium and Ammonium standard mixture was shown in figure 5.

Figure 5: Standard chromatogram for Sodium (50.0mg/L), and Ammonium (1.0mg/L)

Sample results: Samples were analysed using the linearity calibration method. Replicate injections of same sample was also done. Its results and routine analysis sample results were shown in table 12 and table 13. Sample chromatogram was shown in figure 6.

Table 12: Sample precision

Analyte	*S. No.*	*Number of injections*	*% RSD*
Sodium	MCP-01-40	10.0	1.71
Ammonium	MCP-01-40	10.0	2.85

Table 13: Routine sample analysis results

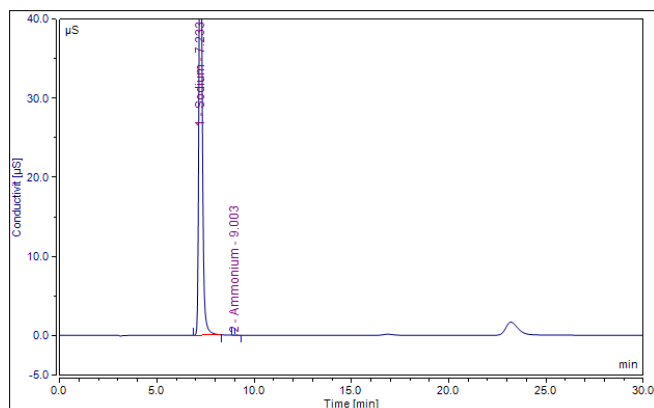| *B. No.*	*Sodium, %*	*Ammonium, %*
MCP-01-40	1.12	0.0001
MCP-01-41	1.36	Not Detected
MCP-01-42	1.28	Not Detected
MCP-02-01	1.42	0.0004
MCP-02-03	1.17	Not Detected

Figure 6: Sample chromatogram for Sodium and Ammonium estimation (Sl. No. MCP-01-40)

Recovery: The sample used for recovery study was Semaglutide Sl. No. MCP-01-40 (average concentration was taken for calculation). Recovery test solutions were injected in triplicate. Also for recovery study, known concentrations of amount was added to sample at three different levels as shown in table 14.

Table 14: Recovery study (Sodium and Ammonium) for sample (Sl. No. MCP-01-40) (n = 3)

Analyte	Target Concentration	Amount Added mg/L	Amount Recovered, mg/L	% Recovery
Sodium	50%	25.0	24.8	99.2
	100%	50.0	50.9	101.8
	150%	75.0	75.3	100.4

Analyte	Target Concentration	Amount Added, mg/L	Amount Recovered, mg/L	% Recovery
Ammonium	50%	0.50	0.511	102.2
	100%	1.00	1.019	101.9
	150%	1.50	1.513	100.9

Method Ruggedness: Method was tested with different flow rate like 0.8 and 1.2. Also, it was tested using different eluent concentrations and IC systems. Method was proven to be efficient as it does not show any changes in sample results due to changes in flow rate, eluent concentration and IC system.

4. Conclusion

In the present research, a fast, simple, accurate, precise and linear Ion Chromatography (IC) method has been developed and validated for quantification of Chloride, Sulfate, Trifluoroacetic acid, Phosphate, Sodium and Ammonium in Semaglutide Active Pharmaceutical Ingredient (API). Hence, it can be employed for routine reaction monitoring, purification procedure and quality control analysis. These Ion Chromatography methods provides good resolution among cited anions and cations. This dual IC setup provides ease of use and convenience for determination of ionic impurities in single ICS6000 system. Sample preparation using OnGuard II RP helps to trap Semaglutide API and excluding ions, which was then subsequently injected into IC system. This technique is proven to be efficient and cost effective with respect to analysis required for keeping check on its limit of anions and cations in Semaglutide by using reagent free ion chromatography. This IC method can be further utilized for estimation of ionic impurities from other peptide-based drugs.

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Author Profile

Chetan Chavan had completed B. Sc (Chemistry). and M. Sc (Chemistry) from Mumbai University and completed his Ph. D in year, 2018., He had joined Dionex India Pvt. Limited, which is now Thermo Fisher Scientific Pvt. Ltd., as Applications Manger for Ion Chromatography (IC) and HPLC and currently working as Sr. Product Manager – IC/SP. His role is to provide technical support coordination with factory for India team requirements, provide guidance to sales, application and service team for IC.

Chanakya Thaker received the B. Sc (Chemistry). and M. Sc (Analytical Chemistry) degrees from Mumbai University in 2006 and 2008, respectively. He had awarded Honorary Doctorate in Life Sciences from CCU/USA in 2019. He had completed MBA-Marketing in 2019 from ITM Vocational University. From 2009, he had joined Dionex India Pvt. Limited, which is now Thermo Fisher Scientific Pvt. Ltd., as Applications Specialist for Ion Chromatography (IC) and HPLC. Currently working as Applications Manager – IC/SP. His role is to manage application team, provide customer training, sample analysis, technical presentations, method development and troubleshooting on IC systems.

Mr. Chetan Chaudhari received the B. Pharm. and M. Pharm. degrees from Mumbai University in 2008 and 2010, respectively. From 2015, he had joined Fisher Scientific Pvt. Ltd., as Applications Specialist for Ion Chromatography (IC) His role is to provide customer training, sample analysis, method development and troubleshooting on IC systems.