Contents lists available at UGC-CARE

International Journal of Pharmaceutical Sciences and Drug Research

[ISSN: 0975-248X; CODEN (USA): IJPSPP]

journal home page : http://ijpsdr.com/index.php/ijpsdr

Research Article

Method Development and Validation for the Determination of Higher Molecular Weight Species of Calcitonin Salmon in Calcitonin Salmon Injection by High-Performance Size Exclusion Chromatography

Akhilesh K Kuril*, K Saravanan

Bhagwant University, Ajmer, Rajasthan, India

ARTICLE INFO

Article history:

Received: 30 January, 2024

Revised: 15 February, 2024

Accepted: 22 February, 2024

Published: 30 March, 2024

Keywords:

Aggregation, Oligomer, Higher molecular weight impurities, Multimer, Calcitonin salmon.

DOI:

10.25004/IJPSDR.2024.160216

ABSTRACT

Protein aggregation is the process by which misfolded proteins self-assemble into soluble oligomers and insoluble aggregates. An isocratic HP-SEC method was developed in order to determine the protein aggregation in the calcitonin salmon injection. Since the label claim of the calcitonin salmon in calcitonin salmon is 33.33 µg/mL at this level of test concentration, no aggregate was observed even with 500 µL injection volume of as such test preparation. Therefore, attempts were made to detect aggregates or high molecular weight impurities to increase the test concentration of calcitonin salmon injection by evaporating the test product under a nitrogen stream and subsequently reconstituting it with a diluent. An isocratic HP-SEC method was developed in order to determine the protein aggregation in the calcitonin salmon injection. The chromatographic separation was achieved at SEC column, i.e., insulin, HMWP, 300 x 7.8 mm with 0.1%TFA in a mixture of Water: Acetonitrile (70:30) with a 0.5 mL/min flow rate. About 100 µL of samples were injected at 40°C column temperature and UV detection occurred at 220 nm. The developed method is highly specific and oligomers are completely separated from the principal peak with USP resolution of 1.5. The developed method was found to be accurate, linear, and precise in the range of 0.092 to 300 µg/mL. The method also examined stress factors such as temperature, light, agitation, freezing, and thawing to identify and study the factors that induce calcitonin salmon aggregation in calcitonin salmon drug product.

Introduction

The biological activity of proteins or peptides strongly depends on the molecule's molecular confirmation/ three-dimensional structure. Proteins also participate in non-covalent self-association (oligomerization) reactions. Desired association events include the creation of aggregates and common, frequently complicated degradation pathways. Unwanted association reactions include the possibility of a dimeric native functional state.[1] Simply put, protein aggregation results from unwanted assemblages of peptide or protein molecules, such as monomers.[2] A variety of aggregates can form, differing in size (from nm to mm in diameter), shape (from spherical to elongated), bonding type (covalent versus non-covalent), and irreversible versus reversible aggregation nature.[3,4] Peptides and proteins naturally gravitate toward greater molecular weight species or aggregates from their initial functional form. The terms and definitions for protein aggregation used in the literature vary, encompassing aggregates, oligomers, multimers, and higher molecular weight protein species.[1,4,5] Protein aggregation is frequently the result of structural alterations in proteins. Physical protein breakdown and decreased immunogenicity are linked to protein aggregation.[6] Therefore, protein aggregates can adversely affect therapeutic drugs and cause unwanted drug-resistance reactions.[7,8] Process of formation of oligomers and matured fibrils in the nucleation and elongation phase mentioned in Fig. 1a.

***Corresponding Author:** Mr. Akhilesh K Kuril

Address: Bhagwant University, Ajmer, Rajasthan, India

Email ✉: gentleakhilesh@gmail.com

Tel.: +91-76759 63234

Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

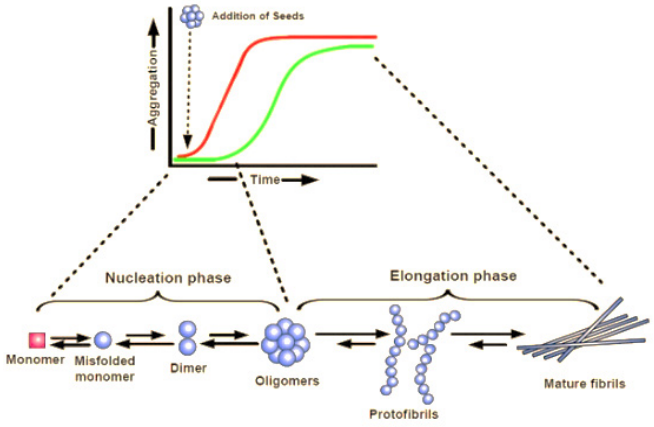

Fig. 1a: Process of formation of oligomers

The following categories are used to group aggregates: size, confirmations, reversibility or solubility, intermolecular bonding, and so on.[4] Non-covalent aggregates by held together by weak electrostatic forces and covalent aggregates like disulfide linkages.[4,9,10] Reversibility: reversible versus irreversible aggregates.[7,8,11,12] The size of aggregates/oligomers ranges from 1 to 25 mm. [13-15] Aggregates of protein products are effective triggers of immune responses to therapeutic protein products. Protein aggregation can be induced by several factors such as temperature, freeze and thawing, agitation stress, concentration of protein, and solvent effect.[4,16-21]

According to regulatory guidelines, accurate quantification of aggregates is required to meet pharmaceutical specifications and understand the nature of protein structure and protein aggregation conditions. Different orthogonal methods are required to assess the protein aggregation in drug substances (rDNA origin or Synthetic) and drug products from DS. Common methods used for studying the formation and structural characteristics of aggregates are listed in Fig. 1b.[22]

Sedimentation velocity analytical ultracentrifugation (SV-AUC) and asymmetric-flow field-flow fractionation (AF4) are widely utilized techniques that regulatory bodies request as an orthogonal method in addition to the HP-SEC method. In this work, the HP-SEC method for the determination of aggregation/oligomer has been developed and validated, also, the method is tested against the above-mentioned stress conditions to determine the effect of heat, freezing, agitation, and light exposure.

No methodology for the evaluation of aggregates in the synthetic forms of calcitonin salmon DS and calcitonin salmon DP was discovered throughout the literature study. Thus, considering the therapeutic importance of calcitonin salmon, high-performance size exclusion chromatography was used to design and validate the peptide aggregation method. Since oligomers and aggregates might elicit an immune response, it is imperative to track both the qualitative and quantitative aggregation of medicinal products from release through stability testing.

	Monomer	Oligomer	Protofibril	Fibril	Amyloid (in vivo)
Atomic structure	Solution-state NMR			Solid-state NMR	
				X-Ray crystallography	
	X-Ray absorption				
Secondary structure	Circular dichroism spectroscopy				
	Fourier transform infrared spectroscopy				
			X-Ray fiber diffraction		
			Neutron scattering		
			Congo red binding / Thioflavin S fluorescence		
				Birefringence	
			Thioflavin T fluorescence		PIB/FDDNP/SB13/BF227
Morphology		Transmission electron microscopy			
		Scanning transmission electron microscopy			
		Scanning tunneling microscopy			
		Atomic force microscopy			
Tertiary/quaternary structure	Electron spin resonance				
	Hydrogen–Deuterium exchange				
	Limited proteolysis				
	Fluorescence methodologies				
Assembly size/size distribution	Gel electrophoresis				
	PICUP				
	Size-exclusion chromatography				
	Analytical ultracentrifugation				
	Dynamic light scattering				
	IMS–MS				

Fig. 1b: Analytical methods used to study amyloid protein structure, folding, and assembly

Material And Methods

The active ingredient of calcitonin (Salmon) injection is a synthetic calcitonin consisting of 32 amino acids in the same linear sequence as that found in salmon calcitonin. The amino acid sequence of calcitonin is mentioned in Fig. 2.

Calcitonin Salmon Injection

Calcitonin salmon drug substance is used to determine the oligomeric impurity of calcitonin Salmon in calcitonin salmon injection. Trifluoroacetic acid (HPLC grade), acetonitrile (HPLC grade), and HPLC grade water were used for the mobile phase preparation.

Method Development

SEC-HPLC is the most suitable and reproducible technique for determining the peptides and proteins' oligomer or high molecular weight species (HMWs). Different SEC columns from different makes were tried, but the best suitable results in terms of peak shape, resolution, and reproducibility were observed with the insulin HMWP column. The major obstacle to determining the oligomer in calcitonin salmon observed was the sample concentration, i.e., 33.33 µg/mL. As such, a sample (undiluted sample) was used to determine the oligomer but no peak of the oligomer was observed at such low concentration hence the sample concentration was increased by evaporating the sample in the nitrogen evaporator to 200 µg/mL. In order to attain the recovery (90–110%) from LoQ level to 150%, different proportions of TFA were used in the Water and Acetonitrile mixture. TFA was optimized at 5% with water: Acetonitrile present at a ratio of 70:30. The optimized chromatographic conditions are given below.

Chromatographic Conditions

Chromatographic separation and quantification of oligomeric impurity, HMW species, or aggregates was achieved using a size exclusion column viz. Insulin, HMWP, 300 x 7.8 mm (Part No. WAT201549, Waters, Ireland) on HPLC (Make; Waters, Model; Alliance) equipped with UV visible detector. An isocratic elution strategy was implemented, employing mobile phase as 0.1% TFA in a mixture of Water: Acetonitrile (70:30) filtered through a 0.45-µm nylon membrane filter (Millipore) and degassed by sonication for 10 minutes before use. The flow rate was optimized at 0.5 mL/min. The injection volume was adjusted to 100 µL, and UV detection occurred at 220 nm. Column and sample cooler temperature was maintained at 40 and 5°C, respectively. The final run time of injection was 35 minutes.

Diluent

Prepared a mixture of Water:Acetonitrile:Trifluoroacetic acid (TFA) in the ratio of 70:30:5 v/v/v. Mixed it well and sonicated it to degas.

Fig. 2: Amino acid sequence of calcitonin salmon

Resolution solution

Since the oligomers were not present in the calcitonin DS at a significant level, the oligomers were generated by heating the calcitonin salmon DS at 60°C in a hot air oven for 24 hours, followed by cool at room temperature and the solution was prepared at the concentration of 200 µg/mL in the diluent stated above and resulting solution will be taken as the resolution solution to demonstrate the resolution between the oligomer and monomer as a part of system suitability.

Test preparation

About 1.5 mL of sample was transferred into a 15 mL centrifuge tube and the centrifuge tube in a nitrogen evaporator for complete drying of solution. After drying, the dried residue was reconstituted with 250 µL of diluent and vortexed for 2 minutes to mix the contents uniformly (Test concentration about 200 µg/mL).

The method was validated against the method precision, linearity, accuracy, solution stability, specificity, and accelerated aggregation study.

Method Validation

System precision

About 0.5 µg/mL of calcitonin salmon was prepared in diluent as a standard solution and six replicates injection into the chromatograph to determine the system precision. The results are mentioned in Table 1a.

Method precision

Approximately 1.5 mL of sample was transferred into a 15 mL centrifuge tube, and the centrifuge tube was kept in a nitrogen evaporator for complete drying of solution. After drying, the dried residue was reconstituted with 250 µL of diluent and vortexed for 2 minutes to mix the contents uniformly. (Test concentration about 200 µg/mL). The remaining five preparations were prepared similarly to the above-mentioned procedure.

Results are discussed under Table 1b in the results and discussion section.

Limit of detection and limit of quantification

LoD and LoQ concentration were determined by the visual method. LoQ was established by performing the LoQ precision and recovery in diluent. The LoQ was established at 0.046% of the test concentration, i.e., 0.092 µg/mL and the LoD of the method was optimized at 0.012% (0.023 µg/mL) of the test concentration.

Linearity of the method

Linearity of the method was performed in diluent from LoQ to 150% of the test concentration (200 µg/mL) of calcitonin salmon. The method was found linear with a

Table 1a: Results of system precision (From Standard solution)

S no	*Sample name*	*Area*
1	Standard solution 1	298959
2	Standard solution 2	299221
3	Standard solution 3	298904
4	Standard solution 4	298789
5	Standard solution 5	296876
6	Standard solution 6	298345
Mean area		298516
Standard deviation		852.9
%RSD		0.29

Table 1b: Results of Method Precision Study (From Test Preparations)

S. No.	*Sample name*	*Area of monomer (Calcitonin)*	*Total area of oligomer peaks*	*% Oligomer*
1	Method precision sample 1	27967675	36098	0.129
2	Method precision sample 2	27607657	34597	0.125
3	Method precision sample 3	28027793	36677	0.131
4	Method precision sample 4	28071368	36589	0.130
5	Method precision sample 5	27876696	35977	0.129
6	Method precision sample 6	27728591	34676	0.125
Mean area		27879963	35769	0.128
Standard deviation		180697.3664	-	0.002
%RSD		0.65	-	1.9

correlation coefficient (R) of 0.9999, and the Y-intercept at 100% was observed to be 0.43.

The results of linearity are mentioned in Table 2, and the linearity curve is depicted in Fig. 3.

Accuracy

The accuracy of the method was determined at LoQ, 50, 100, and 150% of the test concentration (200 ppm). Accuracy was performed by spiking the known concentration of calcitonin salmon at LoQ, 50, 100, and 150% in the placebo. Accuracy was performed in triplicate for each level. The accuracy results are mentioned in Table 3 under the results and discussion. Fig. 4 depicts the linearity curve obtained from accuracy results.

Specificity

Specificity was demonstrated by injecting blank, placebo, and test solution. No interference was observed at the retention time of oligomer and calcitonin salmon.

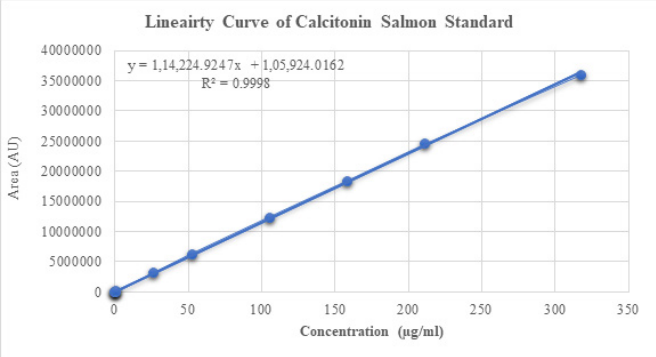

Fig. 3: Linearity curve of calcitonin salmon (LoQ to 150%)

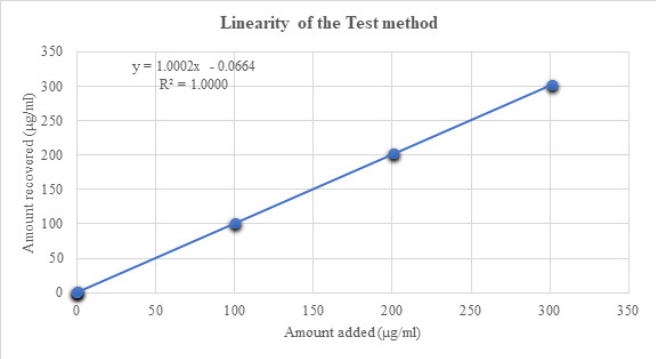

Fig. 4: Linearity curve of calcitonin salmon from accuracy

Table 2: Linearity results of calcitonin salmon from LoQ to 150%

S. No.	*%Level of calcitonin salmon w.r.t test concentration*	*Concentration of calcitonin salmon (µg/mL)*	*Area*
1	0.046	0.092	11242
2	0.115	0.23	27824
3	0.23	0.46	57544
4	0.46	0.92	115372
5	13	26.443	3124032
6	25	52.886	6303388
7	50	105.771	12317652
8	80	158.657	18388592
9	100	211.542	24600895
10	150	317.313	35981150
Correlation coefficient (R)			0.9999
Slope			114224.9247
Intercept			105924.0162
Y- intercept OR % bias			0.43

Accelerated aggregation study

The acceleration aggregation study was performed using physical methods of aggregation such as heating at 80°C and freezing at -20°C, high-speed centrifugation, and UV light exposure.

Table 3: Accuracy results of calcitonin salmon injection

Recovery level	*Preparation no.*	*Amount added (µg/mL)*	*Amount recovered (µg/mL)*	*%Recovery*	*%RSD*
LoQ	1	0.095	0.0881	92.74	
LoQ	2	0.095	0.0885	93.16	0.51
LoQ	3	0.095	0.0876	92.21	
0.50%	1	0.95	0.922	97.05	
0.50%	2	0.95	0.919	96.74	0.44
0.50%	3	0.95	0.914	96.21	
50%	1	100.5	100.25	99.75	
50%	2	100.5	100.34	99.84	0.05
50%	3	100.5	100.28	99.78	
100%	1	201	201.2	100.10	
100%	2	201	200.92	99.96	0.08
100%	3	201	200.91	99.96	
150%	1	301.5	301.61	100.04	
150%	2	301.5	301.45	99.98	0.03
150%	3	301.5	301.48	99.99	
Mean Recovery				97.83	
Overall % RSD				3.04	
Correlation coefficient				1.0000	

Aggregation under UV light

About 1.5 mL of sample was transferred into 15 mL of centrifuge tube and kept in a UV chamber for 48 hours. After 48 hours the sample was removed from the UV chamber and kept in a nitrogen evaporator for complete drying, after drying reconstitute it with 250 µL of diluent and mixed well.

Aggregation under heat (Pyrolysis)

About 1.5 mL of sample was transferred into 15 mL of volumetric flask and kept in hot air oven for 48 hours. at 80°C. After 48 hours. sample was removed from oven and allowed the sample to cool at room temperature and kept in nitrogen evaporator for complete drying, after drying reconstitute it with 250 µL of diluent and mixed well.

Aggregation under agitation

About 1.5 mL of sample was transferred into 15 mL of centrifuge tube and centrifuged at 12000 rpm for 15 hours. at 5°C. After 15 hours. sample was removed from the centrifuge apparatus allowed the sample to attain room temperature and kept in a nitrogen evaporator for complete drying, after drying reconstitute it with 250 µL of diluent and mixed well.

Aggregation under freezing and thawing

About 1.5 mL of sample was transferred into 15 mL of centrifuge tube and kept in freezer at (-20°C) for 48 hours. After 48 hours. sample was removed from -20°C allowed the sample to attain the room temperature and kept in nitrogen evaporator for complete drying, after drying reconstitute it with 1-mL of diluent and mix well.

The results of the accelerated aggregation study are mentioned in Table 4.

Table 4: Results observed from the forced aggregation study

S. No.	*Degradation type*	*%Of aggregation (by Area normalization)*
1	As such sample (Control sample)	0.1
2	Photolytic degradation	0.1
3	Pyrolytic degradation	9.2
4	Agitation stress	0.41
5	Freezing and thawing	0.70

Results And Discussion

Data acquisition, analysis, and reporting were performed by Empower 3 software, and %oligomer was calculated by using by the area normalization method.

The method is based on size exclusion chromatography, in which the compounds with high molecular weight will elute first, followed by the compounds with low molecular weight. As our objective is to quantitively evaluate the oligomer/HMWs the peaks were not integrated after the main peak, i.e., calcitonin salmon because after the calcitonin salmon peak, the phenol peak elutes at about 24 minutes confirmed from the placebo. Hence, all the peaks eluted after the main peak were disregarded. All the peaks eluted before the main peak will be considered as the oligomeric impurities of calcitonin salmon, and the same were reported using the area normalization method. Since the concentration of the calcitonin salmon in its drug product is very low i.e. 33.33 µg/mL. It is very difficult to analyze the oligomer or higher molecular weight (HMW) species of calcitonin if it is present even with a 500 µL injection volume. Hence, using a nitrogen evaporator, strategy was employed to increase the sample concentration. Using the nitrogen evaporator, the sample 1.5 mL of the sample was evaporated and reconstituted with diluent and analysed in SEC-HPLC at 200 µg/mL of the test concentration. While the oligomer or HMWs are not available in their physical form (individual impurity), oligomers or HMWs are the unspecified impurity of calcitonin salmon and the validation study has been performed on the calcitonin salmon in calcitonin salmon injection. System suitability of the method was determined by injecting the resolution solution in which the oligomer was generated by heating the calcitonin salmon DS at 60°C and diluted with diluent to achieve the concentration of 200 µg/mL. The retention time of calcitonin salmon and oligomer/HMWs was observed at about 18 minutes and 16 minutes, respectively.

The USP resolution between the oligomer and calcitonin salmon was observed at 1.54 (NLT 1.0) For a representative chromatogram, refer to Fig. 7.

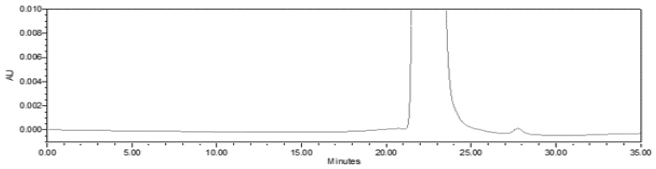

Fig. 5: Representative chromatogram of blank (Diluent)

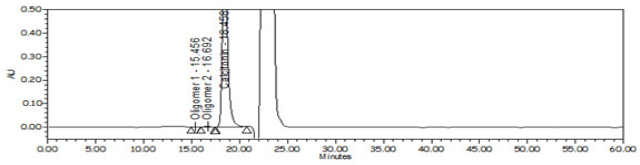

Fig. 7: Representative chromatogram of system suitability solution

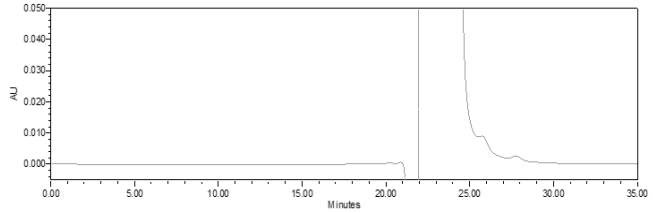

Fig. 6: Representative chromatogram of placebo

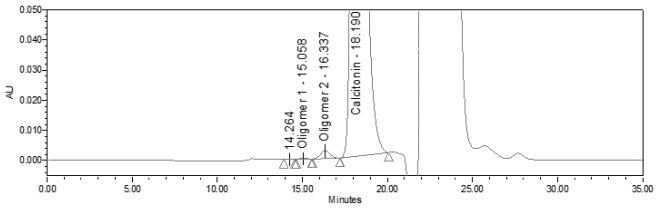

Fig. 8: Representative chromatogram of test solution

System Precision

The %RSD of six standards was observed at 0.29% (NMT 5.0%); hence the system was found precise (Refer Table 1a).

Method precision

It was observed from the results mentioned in Table 1b that the %RSD of oligomer from the six preparations was observed at 1.6% (NMT 10.0%) calculated based on %area normalization and for the calcitonin salmon peak area was observed at 0.6%. Hence, the method was considered precise. For a representative chromatogram refer to Fig. 8.

LoD and LoQ

The sensitivity of the method was performed by establishing LoQ at 0.05% of the test concentration (200 µg/mL) i.e., 0.092 µg/mL, and the LoD of the method was optimized at 0.012% (0.023 µg/mL) of the test concentration.

Linearity

A graph showing peak mean area versus concentration was plotted in order to create the calibration curve. Using linear regression analysis, the calibration curve's linearity was assessed. The regression equation was as follows: Y = 105,924.0162 + 114,224.9247x. The correlation coefficient of 0.9999 satisfies the acceptance criteria for analytical method validation (NLT 0.99). Y-intercept or % bias was observed at 0.38%. Thus, the method's linearity over the concentration was demonstrated in the range of 0.05 to 150% of the test concentration (Refer to Table 2).

Accuracy

The method's accuracy was performed at LoQ to 150% of the test concentration; the results are mentioned in Table 3. The %recovery was observed in between 90 to 110% at each level and the mean recovery was observed at 97.83% with 3.1 %RSD.

The test method's linearity was performed by plotting a graph of the amount added (µg/mL) versus the amount recovered (µg/mL). The linearity of the calibration curve was evaluated using linear regression analysis. A regression equation was y = 1.0002x - 0.0664. The correlation coefficient is 1.000 which meets the analytical method validation acceptance criteria (NLT 0.999).

Range

The range was established by performing the linearity and accuracy from LoQ to 150% of the test concentration. The method was found to be linear and accurate in the range of about 0.092 to 300 µg/mL.

Specificity

No blank and placebo peak was observed at the retention time of calcitonin salmon and oligomer; hence the method is said to be specific. Refer to Fig. (5-8) for representative chromatograms of blank and placebo.

In blank, a large peak was observed at RT about 22 minutes, belonging to trifluoroacetic acid (TFA) (Refer to Fig. 5).

In the placebo, a large and broad peak was observed at RT for about 22 minutes, which belongs to TFA + Phenol (Refer to Fig. 6).

Forced aggregation study

A forced aggregation study was performed to determine the stress condition that can accelerate the aggregation process in the drug product. Hence different stress conditions were applied to the calcitonin drug product and the results were mentioned in Table 4. From the results, maximum aggregation was observed in the pyrolysis (Thermal degradation) which was 9.2%. Meanwhile, 0.7 and 0.4% were observed in freezing, followed by thawing at RT and agitation stress, respectively. However, no aggregation or similarity to that of the control sample was observed in photolytic degradation.

Stability of analytical solution

Stability of the standard solution and test solution was performed for 48 hours at 5°C and found stable for 48 hours.

Conclusion

A simple isocratic HP-SEC method with UV detection was developed and validated to identify and quantify oligomer or high molecular weight impurities of calcitonin salmon in calcitonin salmon injection. The method was successfully validated and statistical data confirmed precision, linearity, sensitivity, and accuracy of the proposed method. The method can serve as an important tool in determining the molecular weight of the oligomers by using HPLC with an MALS detector or MS detector, as the method is mass-compatible. Since the method has the advantage of short HPLC run time and reproducibility and sensitivity, it can be adopted in quality control for routine analysis and can be used for the stability study of calcitonin salmon injection.

References

1. Philo JS. Characterizing the aggregation and conformation of protein therapeutics. American biotechnology laboratory. 2003 Oct;21(11):22-9.
2. Den Engelsman J, Garidel P, Smulders R, Koll H, Smith B, Bassarab S, Seidl A, Hainzl O, Jiskoot W. Strategies for the assessment of protein aggregates in pharmaceutical biotech product development. Pharmaceutical research. 2011 Apr;28:920-33.
3. Chi EY, Krishnan S, Randolph TW, Carpenter JF. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. Pharmaceutical research. 2003 Sep;20:1325-36.
4. Mahler HC, Friess W, Grauschopf U, Kiese S. Protein aggregation: pathways, induction factors and analysis. Journal of pharmaceutical sciences. 2009 Sep 1;98(9):2909-34.
5. Cromwell ME, Hilario E, Jacobson F. Protein aggregation and bioprocessing. The AAPS journal. 2006 Sep;8:E572-9.
6. Valerio M, Colosimo A, Conti F, Giuliani A, Grottesi A, Manetti C, Zbilut JP. Early events in protein aggregation: molecular flexibility and hydrophobicity/charge interaction in amyloid peptides as studied by molecular dynamics simulations. Proteins: Structure, Function, and Bioinformatics. 2005 Jan 1;58(1):110-8.
7. Arakawa T, Timasheff S. The stabilization of proteins by osmolytes. Biophysical journal. 1985 Mar 1;47(3):411-4.
8. Arakawa T, Bhat R, Timasheff SN. Preferential interactions determine protein solubility in three-component solutions: the magnesium chloride system. Biochemistry. 1990 Feb 1;29(7):1914-23.
9. Dobson CM. Protein folding and disease: a view from the first Horizon Symposium. Nature Reviews Drug Discovery. 2003 Feb 1;2(2):154-60.
10. Andya JD, Hsu CC, Shire SJ. Mechanisms of aggregate formation and carbohydrate excipient stabilization of lyophilized humanized monoclonal antibody formulations. Aaps Pharmsci. 2003 Jun;5:21-31.
11. Kendrick BS, Cleland JL, Lam X, Nguyen T, Randolph TW, Manning MC, Carpenter JF. Aggregation of recombinant human interferon gamma: kinetics and structural transitions. Journal of pharmaceutical sciences. 1998 Sep 1;87(9):1069-76.
12. Arakawa T, Prestrelski SJ, Kenney WC, Carpenter JF. Factors affecting short-term and long-term stabilities of proteins. Advanced drug delivery reviews. 2001 Mar 1;46(1-3):307-26.
13. Carpenter JF, Kendrick BS, Chang BS, Manning MC, Randolph TW. [16] Inhibition of stress-induced aggregation of protein therapeutics. In Methods in enzymology 1999 Jan 1 (Vol. 309, pp. 236-255). Academic Press.
14. Mahler HC, Müller R, Frieβ W, Delille A, Matheus S. Induction and analysis of aggregates in a liquid IgG1-antibody formulation. European journal of pharmaceutics and biopharmaceutics. 2005 Apr 1;59(3):407-17.
15. Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. International journal of pharmaceutics. 1999 Aug 20;185(2):129-88.
16. Kelly JW. The alternative conformations of amyloidogenic proteins and their multi-step assembly pathways. Current opinion in structural biology. 1998 Feb 1;8(1):101-6.
17. Marcon G, Plakoutsi G, Chiti F. Protein Aggregation Starting from The Native Globular State1. Methods in enzymology. 2006 Jan 1;413:75-91.
18. Holm NK, Jespersen SK, Thomassen LV, Wolff TY, Sehgal P, Thomsen LA, Christiansen G, Andersen CB, Knudsen AD, Otzen DE. Aggregation and fibrillation of bovine serum albumin. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2007 Sep 1;1774(9):1128-38.
19. Makin OS, Serpell LC. Structures for amyloid fibrils, Febs j. 2005 Nov 5; 272(23):5950-61.
20. Rosenberg AS. Effects of protein aggregates: an immunologic perspective. The AAPS journal. 2006 Sep;8:E501-7.
21. Zapadka KL, Becher FJ, Gomes dos Santos AL, Jackson SE. Factors affecting the physical stability (aggregation) of peptide therapeutics. Interface focus. 2017 Dec 6;7(6):20170030.
22. Li H, Rahimi F, Sinha S, Maiti P, Bitan G, Murakami K. Amyloids and protein aggregation–analytical methods. Encyclopedia of analytical chemistry. 2009 Sep 15;10(9780470027318):a9038.

HOW TO CITE THIS ARTICLE: Kuril AK, Saravanan K. Method Development and Validation for the Determination of Higher Molecular Weight Species of Calcitonin Salmon in Calcitonin Salmon Injection by High-Performance Size Exclusion Chromatography. Int. J. Pharm. Sci. Drug Res. 2024;16(2):260-266. **DOI:** 10.25004/IJPSDR.2024.160216