# Effects of pharmaceutical excipients on the chemical stability of therapeutic proteins in a parenteral formulation

**Emmanuel Gulangayan Deniola**

Degree Project in Pharmaceutical Technology, 2022
Department of Food Technology, Engineering and Nutrition

Lund University
Sweden

# Effects of pharmaceutical excipients on the chemical stability of therapeutic proteins in a parenteral formulation

Emmanuel Gulangayan Deniola

Degree Project in Pharmaceutical Technology, 2022
Department of Food Technology, Engineering and Nutrition
Lund University
Sweden

30 ECTS credits


LUND UNIVERSITY

**Supervisors:** Ingrid Ramm and Lars Nilsson

**Examiner:** Marie Wahlgren

## POPULAR SCIENCE SUMMARY

Proteins and peptides are biomolecules that have a wide range of functions in the human body. As such, they are widely used in the treatment and management of various illnesses such as cancer, diabetes, and cardiovascular diseases. This increased the demand for protein-based products such as vaccines, enzymes, and many other drugs. Nowadays, concerted efforts lead to many protein-based drugs in the market and a lot of which are undergoing clinical trials for approval. During the production of the drug, the protein may be affected by various factors such as temperature, humidity, pharmaceutical excipients, and many other stressors. These excipients are the other substances aside from the protein present in a formulation and they typically comprise the majority of the final product. Their function is to ensure that the protein retains its potency and stability until its administration. The loss of stability of a protein is sometimes coupled with the formation of degradation products. This may result in a decrease in the efficacy and shelf-life of the drug and increases the risk to consumers, as they may be toxic.

In this study, various substances were tested for their effects on the stability of two therapeutic proteins, GA-Z, and somatropin. Stability was measured in terms the of chemical degradation of the proteins. Chemical degradation is characterized by a change in the chemical properties of the protein, which leads to changes in its structure and function. For GA-Z, substances such as sucrose, polysorbate 80, and polyethylene glycol 600 in varying concentrations were tested to see if they have stabilizing properties. On the other hand, glycerol was tested for its effect on the chemical stability of somatropin. In the stability studies, both proteins were stored in an incubator at 37°C, with and without excipients, for up to 30 days. After the incubation period, a technique called liquid chromatography was used to determine the amount of the chemically degraded protein. The results showed that sucrose and polysorbate 80 protected GA-Z from chemical breakdown. However, this was not observed in the case of polyethylene glycol.

Unlike GA-Z, a method had to be first created for somatropin prior to testing. In this process, equipment settings were tested out to see how they improved the separation and detection of various components present in the somatropin samples. The use of this developed liquid chromatography method showed that glycerol helped protect somatropin from chemical destruction. The identities of the degradation products from both proteins were then determined using another technique.

Based on the results, the protective abilities of these substances should be further examined. The use of other advanced methods will also provide information on how these substances affect the structure of the protein. The results may then be used as new formulation strategies for the increased stability of protein-based drugs.

## ABSTRACT

Proteins and peptides are widely used for the prevention, management, and cure of various illnesses. This vast therapeutic potential represents a significant portion of the pharmaceutical industry in the form of vaccines, antibodies, enzymes, and other protein-based therapeutics. During the drug development process, these proteins/peptides are affected by various environmental factors such as temperature and humidity, as well as the presence of pharmaceutical excipients. These affect the physical and chemical stability of the proteins, which may compromise the safety, efficacy, and shelf-life of the product. With this, stability testing is performed to provide data on how such factors affect the degradation of proteins. In this study, various pharmaceutical excipients were tested for their effect on the chemical stability of two therapeutic proteins, GA-Z, and somatropin over a 30-day incubation period at 37°C. The effects of sucrose (40-120 mg/mL), polysorbate 80 (0.05-1 mg/mL), and polyethylene glycol 600 (20-40%) on the chemical stability of GA-Z were tested. Moreover, glycerol concentrations from 10% to 50% were tested for their effect on somatropin. The results of HPLC-UV analysis of both protein samples provide information on how much native protein remained throughout the incubation period. Sucrose and polysorbate 80 demonstrated an increase in GA-Z stability as their concentrations were increased. Unlike GA-Z, there was no existing LC-UV method for the analysis of somatropin and its degradation products. With this, method development and optimization were performed using a BioResolve reversed-phase column. This process focused on optimizing the flow rate, gradient profile, injection volume, and column oven temperature to improve chromatographic resolution and analyte sensitivity. With the use of this developed method, it was shown that increased glycerol concentrations improved the stability of the somatropin. In conjunction with the LC-UV analysis, further experiments show that the decrease in the degradation of GA-Z is not linked with decreased water activity. However, this decreased chemical degradation in somatropin may be coupled with water activity. Mass spectrometric analyses provided confirmation that the degradation peaks are results of hydrolysis, deamidation, and isomerization. Future work with Asymmetrical Flow Field-Flow Fractionation and Small Angle Neutron Scattering is necessary to provide information on how the excipients affect the tertiary and quaternary structure of the proteins and how these excipients change the protein conformation.



**Keywords:**

Chemical stability, protein degradation, GA-Z, somatropin, pharmaceutical excipients, liquid chromatography

## ACKNOWLEDGEMENT

Firstly, I would like to thank Marie Wahlgren for presenting the availability of this project opportunity. I would like to express my deepest gratitude and appreciation to Ingrid Ramm for serving as my supervisor during this endeavor. I have learned a lot of invaluable knowledge and practical laboratory skills that enhanced my competence. Thank you very much for your never-ending patience and tutelage. I would also like to extend the same courtesy to my co-supervisor, Lars Nilsson. This undertaking will not be possible without the resources provided by the NextBioForm consortium, the Department of Food Technology, Engineering and Nutrition, and the Department of Chemical Engineering. Moreover, it is with great pleasure that I am crediting the Swedish Institute for awarding me a fully-funded scholarship for the entire duration of my studies. My heart goes to my family, friends, schoolmates, and teachers for all the love and support.

# TABLE OF CONTENTS

# PROJECT AIM

## General objective:

This study aims to evaluate the effects of various chemical excipients on the chemical stability of two therapeutic proteins, namely: GA-Z and Somatropin within a thirty-day incubation period at an ambient temperature of 37°C.

## Specific objectives:

- Assess the effects of 0.05-1 mg/mL polysorbate 80, 20-40% v/v polyethylene glycol 600, and 40-120 mg/mL sucrose on the chemical stability of GA-Z
- Assess the effect of 10-50% v/v glycerol on the chemical stability of somatropin
- Perform LC-UV analysis to provide quantitative data on the degradation of the therapeutic proteins over the duration of thirty days
- Conduct LC-MS analysis to provide data regarding the identity of degradation products of the therapeutic proteins
- Perform LC-UV method development for the analysis of somatropin and its degradation products

# INTRODUCTION

## Therapeutic proteins and peptides

Proteins and peptides have the ability to cure various illnesses, making them integral to the pharmaceutical sector. Such agents include enzymes, antibodies, vaccines, interferons, and many others. In recent years, significant progress in the field of pharmaceutical biotechnology and engineering paved for the increased value and attention for protein-based therapeutics (Ramm et al., 2021). Nowadays, many protein and peptide-based therapeutics are approved in the market and a lot of which are undergoing clinical trials. These are used for various purposes including disease diagnosis, management, prophylaxis, and cure. Therapeutic proteins gain increased attention due to their potency in curing many illnesses such as cancer, diabetes, and heart problems (Akash et al., 2015). The foremost advantage of proteins and peptides as therapeutic agents is that they are highly specific to their targets, thereby resulting in much higher efficacy and less adverse side effects. It is estimated that the market value of peptide and protein-based drugs is 40 billion USD, which translates to approximately 10% share of the pharmaceutical market. With the recent research and interest in protein-based therapies and recombinant proteins, the market share is steadily increasing (Craik et al., 2012).

A class of proteins called affibody has gained attention in recent years due to its therapeutic and diagnostic potential. These are small non-immunoglobulin proteins developed through robust protein engineering efforts. They are of significant therapeutic interest because of the wide array of biotechnological applications in cancer research, receptor signal blocking, and inflammatory diseases, among others (Ståhl et al., 2017). One example of such protein is GA-Z, a novel therapeutic affibody containing both a Z-domain and an albumin-binding domain connected via a linker (Ramm et a., 2021). Aside from GA-Z, somatropin, also known as the human growth hormone (rhGH) is also considered a highly valuable protein as it is vital for various physiological processes such as growth, cell regeneration, cell reproduction, and metabolic processes. Over the past years, the demand for rHGH has increased due to its health and therapeutic benefits. With the increasing prevalence of diseases that can be treated with rhGH or directly associated with growth hormone deficiency, there is an increased market demand (Ranke and Wit, 2018).

## Protein Stability

There are many factors that influence the stability of therapeutic proteins, which include pH, temperature, pharmaceutical excipients (sugars, surfactants, etc), ionic strength, as well manufacturing-related stress. These affect the chemical and physical stability of proteins and peptides, which in turn compromises shelf-life, safety, and the efficacy of the drug product (Manning et al., 2010). In parenteral formulations, proteins and peptides may undergo chemical degradation through routes such as hydrolysis, deamidation, isomerization, and oxidation. These result in changes in the covalent linkages within the protein, thereby altering its chemical structure and possibly its function (Shirwaikar et al., 2006; Manning et al., 2010). On the other hand,

physical degradation is characterized by aggregation, protein unfolding, and adsorption to surfaces. Moreover, physical degradation can also be a result of chemical degradation reactions such as deamidation and isomerization (Chi et al., 2003; Krause and Sahin, 2019; Ramm et al., 2021).

The post-translational modification (PTM) of proteins is a ubiquitous process that occurs in vivo and in vitro. Some examples of such reactions are oxidation, glycosylation, phosphorylation, and deamidation. These reactions may present deleterious effects such as loss of biological activity, as in the case of oxidation or deamidation (Gervais, 2015). One of the most common routes of chemical degradation in proteins and peptides is through asparagine deamidation. Some of the earliest examples of this modification were observed in proteins such as insulin, human growth hormone, and hemoglobin (Manning et al., 2010). Deamidation is a highly undesirable PTM in manufacturing proteins as it may lead to the alteration of the secondary, tertiary, and quaternary structure of the protein, leading to the loss of activity or promoting aggregation of proteins. Other notable physico-chemical effects include lowering of pI, change in the charge, and change in hydrophobicity (Van der Walle, 2011; Gervais, 2015). It is thus important to perform chemical stability studies on therapeutic proteins such as GA-Z and somatropin, considering that they both contain a number of asparagine and aspartic acid residues.

Asparagine deamidation is initiated through the nucleophilic attack by the C-terminus residue's backbone nitrogen atom to the asparagine's side chain amide group carbon atom (Jia and Sun, 2017). This forms the succinimide intermediate, which will result in the formation of two degradation products, aspartic acid and iso-aspartic acid residues as exemplified in Figure 1 (Krause and Sahin, 2019). These degradation products may form both stereoisomers of the succinimide intermediate, thus forming a racemic mixture, though the L-form proved to be the more common. During deamidation, the neutral amide in the protein is replaced by the negatively-charged carboxylic acid thereby resulting in a charged variant. It is worth noting that some amino acid sequences, particularly Asn-Gly or Asn-Ser are more prone to deamidation since the small size of the flanking residue increases the conformation flexibility of the protein (Phillips et al., 2017).

As shown in Figure 1, the other reaction of interest is the isomerization of aspartic acid to form iso-aspartic acid. This reaction is also considered to be a ubiquitous post-translational modification in proteins, usually through the cyclic succinimide intermediate. The formation of iso-aspartic acid adds an extra methylene group into the protein backbone, alongside the shortening of the Asp side chain. As a result, the protein structure is altered, resulting in the reduction/loss of protein activity (Eakin et al., 2014).

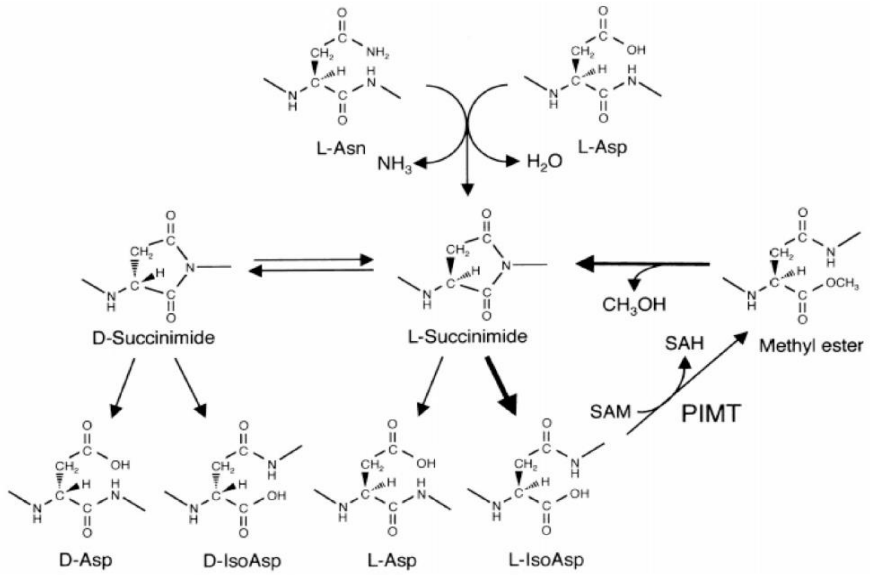



**Figure 1**. Routes of asparagine deamidation and isomerization of aspartic acid. This Figure has been taken with no further modifications from the publication of Yang and Zubarev (2010).

In regards to the detection of deamidation and isomerization in proteins, mass spectrometric (MS) analysis is the most commonly used method. MS identification of the deamidated protein/peptide is relatively easy since this reaction adds approximately +0.984 Da (the mass difference between -OH and -NH2 groups) to the mass of the original molecule (Yang and Zubarev, 2010). On the other hand, isomerization is challenging to detect since the molecular weights of Asp and iso-Asp are the same, and there are no charge differences (Krause and Sahin, 2019). However, isomerization affects the structure of the protein, thereby changing the retention time of the peptide in reversed-phase liquid chromatography mass spectrometry (RP-LCMS). It is worth noting that differentiating between the isomers still presents a hefty challenge. Other notable methods of detection include studies using bioluminescent assay (Hsiao et al., 2017) and another type of MS configuration using Fourier-Transform Ion Cyclotron Resonance Mass Spectrometry (Sargaeva, 2009).

Protein integrity and function are highly influenced by thermodynamic parameters such as time and temperature. It is also important to consider factors such as formulation, manufacturing process, and the packaging for the protein to remain effective and safe within the specified shelf-life and storage conditions. While protein and peptide-based drugs shouldn't be assumed to remain in these optimal specifications, it is important to consider how other aspects of operation such as manufacturing, inspection, transport, and device assembly may affect protein stability.

## Stability Testing

Stability is a key attribute of pharmaceuticals as this plays a critical role in the entire drug development process. This testing procedure is conducted to provide objective evidence on how the drug is influenced over time by environmental factors such as humidity, temperature, and light, as well as product-related factors including pharmaceutical excipients, physico-chemical properties of the API, and packaging materials (WHO, 2012). With this, stability testing can give insights into how various factors affect the expiration date of the pharmaceuticals, and both their chemical and physical stability.

In regard to the industry, its application can be further funneled in various stages of the drug development process such as pre-clinical formulation, process development, packaging, and post-marketing process (Huynh-Ba, 2009). The loss of stability of the drug directly affects the efficacy, purity, and safety of the final drug product. If these drug properties are significantly modified during testing, the established data on the safety and efficacy of the product will not be applicable. One major risk is the formation of toxic degradation products which may endanger consumer safety. Moreover, these substandard products may incur additional costs for manufacturers and put the company in a bad light with regulatory agencies such as FDA, should they be released into the market. Aside from establishing a retest date for the drugs, estimating the shelf-life of the product, and recommending optimal storage conditions, stability studies provide data in support of clinical trials, registration submission, and product marketing (Huynh-Ba, 2009).

## Pharmaceutical Excipients

Pharmaceutical excipients are necessary to stabilize the protein from its processing until and during administration. These substances are crucial in ensuring that the active drug achieves the intended potency and stability. Moreover, they help in preserving the sterility of products, providing isotonicity, and helping reconstitute lyophilized products (Gervasi et al., 2018). In a typical drug product, the pharmaceutical excipients represent the bulk of the formulation while the active component is only a small percentage. As such, it is important to select appropriate excipients during formulation to avoid or reduce negative effects including loss of activity, stability, and solubility. Excipients such as polysorbate, polyethylene glycol, glycerol, and sucrose are commonly used in parenteral formulations to preserve and stabilize the active pharmaceutical ingredient (API). Polysorbates are surfactants that can act as emulsifying or solubilizing agents. However, its major drawback is its ability to undergo hydrolysis and oxidation, leading to the formation of hydrogen peroxide and formic acid. Polyethylene glycol (PEG) is a polymer that is widely used in various pharmaceutical formulations to aid drug delivery, improve biological activity, and increase solubility (Rowe et al., 2015; Strickley and Lambert, 2021; Pramanick et al., 2015). Glycerol and sucrose are added to protein formulations due to their stabilizing properties. They are known to prevent irreversible aggregation, reduce the loss of activity, and increase the protein’s thermal transition temperature (Lee and Timasheff, 1981; Ramm et al., 2021).

# METHODS

## Materials and Reagents

The proteins, excipients, and other chemicals used in this project were provided through the efforts of the NextBioForm consortium. The GA-Z affibody (11.9 kDa) was provided at a stock concentration of 90 mg/mL. This formulation contains 125 mM NaCl (VWR, PA, USA) and 25 mM sodium phosphate buffer (Merck Millipore, MA, USA), at pH 7.0. Prior to analysis, the protein was stored at −80 °C. An intermediate solution of 50 mg/mL somatropin was prepared from the powdered somatropin (99%), which was initially stored at -20°C. It was also ensured that other chemicals are of sufficient purity and grade: acetonitrile (HPLC grade, 99.9%), sodium chloride (NaCl, 99.5%), formic acid, glycerol (98%), sucrose (> 99.5%), PEG600 (density: 1.064 g/mL), polysorbate 80 (density: 1.064 g/mL), trifluoroacetic acid (HPLC graded, > 99%), Sodium phosphate dibasic dihydrate ($Na_2HPO_4$•$2H_2O$, 99–102%), sodium phosphate dibasic monohydrate ($NaH_2PO_4$•$H_2O$, 98.5–100.5%), sodium azide ($NaN_3$, 99.5%). LC-MS grade chemicals such as acetonitrile (99.9%) and formic acid (99%) were also used. The water used for mobile phases, samples, and reagent preparation was Milli-Q grade.

## Sample Preparation

The GA-Z samples for the chemical stability evaluated three excipients, namely: PEG600, sucrose, and polysorbate 80. The samples contained 20-40 v/v% PEG600, 40-120 mg/mL sucrose, and 0.05-1 mg/mL polysorbate 80, while maintaining a GA-Z concentration of 9 mg/mL, 25 mM sodium phosphate buffer, and 125 mM of NaCl. All three concentrations of polysorbate 80 are above the critical micelle concentration (CMC) of the surfactant (Zhou et al., 2021), which is estimated to be around the concentration range of 0.014-0.019 mg/mL (Bide et al., 2021, Patist et al., 2000). These excipient concentrations were based on common formulations of commercially available antibodies/proteins (Gervasi et al., 2018; Pramanick et al., 2013; Stickley and Lambert, 2021). The GA-Z stock already contains 25 mM sodium phosphate buffer and 125 mM of NaCl. With this, the number of moles of NaCl and phosphate present in the GA-Z stock was taken into account when the buffer-salt solution was prepared to make sure that the resulting concentrations are correct. The sodium phosphate buffer was prepared by mixing equal moles of sodium phosphate dibasic dihydrate and sodium phosphate dibasic monohydrate, totaling to 25 mM. The pH was then adjusted to 7.0 to resemble physiological conditions. For the sucrose, an intermediate stock solution at 300 mg/mL was prepared. The same was done for polysorbate 80, wherein a 5 mg/mL intermediate stock solution was prepared, before further diluting it to 0.05, 0.1, and 1 mg/mL. Both polysorbate 80 and PEG600 are very viscous, thus making it difficult to prepare via pipetting. In the case of PEG600, direct preparations were done wherein the PEG was weighed into polypropylene tubes and was added with the necessary volume of buffer, salt, and diluent (Milli-Q water) to make 20% and 40% v/v PEG600. Approximately 9 mL of each PEG concentration was made to constitute for 5 trials. This method of preparing separate buffer solutions in polypropylene tubes containing the excipient, salt, and diluent was also done for

sucrose (40 mg/mL and 120 mg/mL) and Polysorbate 80 (0.05, 0.1, and 1 mg/mL). It is worth noting that in the case of sucrose, a salt concentration of 137.5 mM was used. As such, the blank used for these samples contains the same salt concentration. For the somatropin samples, glycerol was weighed directly into the polypropylene tubes, the same way as PEG600. An appropriate amount of buffer, salt, and diluent was also added to give a concentration of 25 mM phosphate buffer and 125 mM salt for each glycerol concentration of 10%, 20%, and 50%.

These solutions are then autoclaved to ensure that everything is sterile prior to the addition of the protein and incubation. Once the autoclaved solutions have cooled down, they are pipetted into a 2 mL screw cap vial. The GA-Z (200 uL) was then pipetted into the different vials, corresponding to various days of incubation. The same is done with somatropin, wherein 200 uL of the somatropin 90 mg/mL intermediate stock solution was pipetted into the vials, to give a formulation with 9 mg/mL somatropin. For each formulation, replicates are prepared corresponding to specific time intervals (0, 10, 20, 30 days). This subsequent sample preparation was done inside a biosafety cabinet to ensure that sterile conditions are observed. In this way, the possibility of bacterial contamination is significantly reduced. Moreover, gloves and filtered pipette tips were used to avoid contamination. After gently mixing the samples, they were stored in an incubator at a temperature of 37 °C. The samples were taken out and stored in a freezer at -80°C after the specified incubation period, before LC-UV and LC-MS analysis

**Water Activity**

The AquaLab system was used to measure the water activity of the samples at 20 °C. Before the analysis, the equipment was calibrated with pure water ($a_w$ = 1) and with an AquaLab reference sample of water activity 0.50. For the 9 mg/mL GA-Z samples, formulations containing 20-40% PEG600, 40-120 mg/mL sucrose, and 0.05-1 mg/mL polysorbate 80 was analyzed. The same protocol was performed for the 9 mg/mL somatropin containing 10-50% glycerol. All of the measurements were performed in triplicates.

**Liquid Chromatography**

The samples from the stability study were analyzed using the Shimadzu LC-20AD system equipped with a DAD UV detector, pump, autosampler, and a BioResolve reversed-phase column (Polyphenyl, 450 Å, 2.7 µm, 3 × 150 mm, Waters, MA, USA). For the analysis of GA-Z samples, an established method (Ramm et al., 2021) was used to quantify the chemical degradation over the incubation period. This method utilized a gradient mode with mobile phases A (Milli-Q water with 0.08% formic acid and 0.02% trifluoroacetic acid) and mobile phase B (LC grade acetonitrile with 0.08% formic acid and 0.02% trifluoroacetic acid). The mobile phase B increased 10-31% for the initial 15 minutes, 31-40% during 15-45 minutes, and 40-95% in the 45–50-minute period. The method utilized a flow rate of 1 mL/min, a column oven temperature of 60 °C, and an injection volume of 5 µL. Before the injection, samples were diluted with a solution containing 10% acetonitrile. After the dilution, the concentration of the protein was 0.33 mg/mL.

On the other hand, LC method development/optimization was first performed prior to somatropin analysis. This was conducted using the Shimadzu LC-20AD system equipped with a DAD UV detector, pump, autosampler, and a BioResolve reversed-phase column (Polyphenyl, 450 Å, 2.7 μm, 3 × 150 mm, Waters, MA, USA). The method optimization process focused on varying chromatographic parameters that improve chromatographic separation of the native protein with its degradation products. The parameters that were evaluated were the flow rate, gradient profile, injection volume, and column oven temperature. Firstly, the gradient profile using water and acetonitrile was optimized to create a method that can separate somatropin and its degradation products within a reasonable analysis time. It is worth noting that both mobile phases contain 0.08% formic acid and 0.02% trifluoroacetic acid as additives. After which, various column oven temperatures of 50, 60, 70, and 80 °C were tested to see their effects on resolution. The setting which leads to better resolution and analyte sensitivity was chosen before proceeding to the next parameter. Various flow rates (0.2, 0.4, 0.6, 0.8, and 1.0 mL/min) and injection volumes (3, 6, 12, 24 uL) were also tested. Overall, the chosen parameter settings are the ones that provided the best resolution and highest sensitivity. For the developed method, the mobile phase B increased from 10-43% for the initial 23 minutes, 43-52% during 23-41 minutes, and 52-95% in the 41–45-minute period. The method utilized a flow rate of 0.8 mL/min, a column oven temperature of 60 °C, and an injection volume of 12 μL.

For both proteins, two wavelengths were used for signal measurement: 220 nm and 280 nm. These wavelengths are particularly useful for protein analysis as they respectively relate to the UV absorption of the protein backbone and absorption maxima of residues containing aromatic rings. Although, the 280 nm wavelength was primarily used for peak integration, as it provides better sensitivity, thus sharper peaks. To avoid potential degradation of the protein, the sampler rack cooler was operated at 4 °C. The peak integration of LC chromatograms was performed using the Shimadzu LabSolutions software.

**Liquid Chromatography-Mass Spectrometry**

Samples containing 9 mg/mL GA-Z and 20-40% PEG600, 40-120 mg/mL sucrose, and 0.05-1 mg/mL polysorbate 80 were analyzed along with samples containing 9 mg/mL somatropin containing 10-50% glycerol with LC-MS to identify the various degradation products. In this experiment, the Agilent 1260 II infinity system connected to a 6545 Q-TOF LC/MS was utilized. The Agilent 1260 II infinity system was equipped with an Agilent 1260 II infinity autosampler and an Agilent 1260 II infinity pump. The LC method for GA-Z and somatropin were the same as described under the Liquid Chromatography section with the exception of the use of LC-MS grade acetonitrile. For the GA-Z analysis, the LC flow was connected to the MS source during the 3–50-minute period. On the other hand, this was 3-45 minutes for the somatropin. The MS was run in the positive mode in the mass range of 100–3000 m/z, and an acquisition rate of 5 spectra $s^{-1}$. The ESI parameters consist of a drying flow rate of 12 L/min, nebulizer pressure of 55 psi, sheath gas temperature of 400 °C, drying gas temperature of 350 °C, and sheath gas flow of 12 /min. Other

MS parameter settings utilized a skimmer voltage of 65 V, fragmentor voltage of 175 V, nozzle voltage of 2000 V, octupole RF voltage of 750 V, and capillary voltage of 4500 V. The Agilent MassHunter Qualitative Navigator B.08.00 software was used for peak integration and extraction of MS spectrum.

## RESULTS AND DISCUSSION

A previously developed LC method (Ramm et al., 2021) was utilized to analyze the degradation of the proteins GA-Z. The method was able to identify the GA-Z native peak as well as multiple degradation products. The chromatograms presented in Figure 2, show the effect of various incubation periods of GA-Z samples without the tested excipients. It is observed that as the incubation period progressed, degradation also increased. This can be noted in terms of the increased number of degradation peaks and decreased signal intensity (peak area) of the native GA-Z. Since GA-Z and somatropin are known to chemically degrade, these results were expected as per the prior experimental data (Ramm et al., 2021), considering the prolonged exposure to an ambient temperature of 37°C and lack of stabilizing excipients. The same trend was noticed for samples containing somatropin as well, as shown in Figure 3.

a.

b.

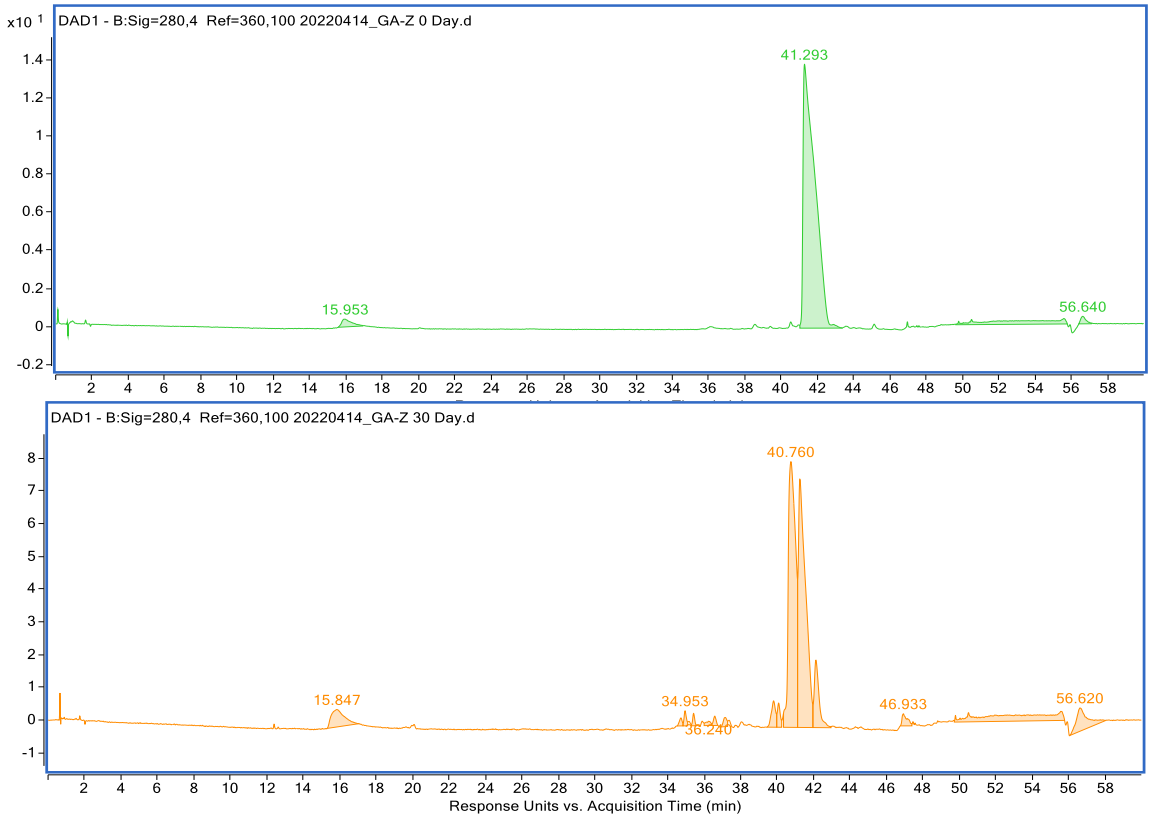



**Figure 2**. Degradation of GA-Z samples (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for (**a**) 0 day and (**b**) 30 days, as detected using LC-UV-MS at 280 nm.

a.

b.

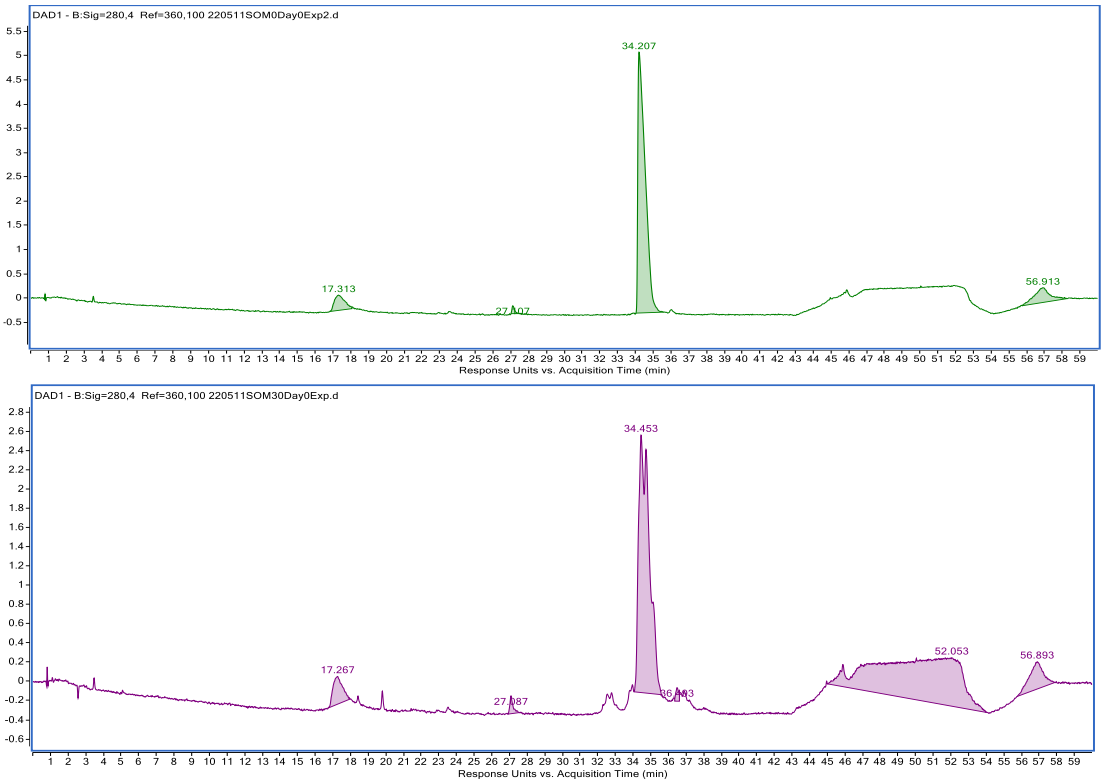



**Figure 3**. Degradation of somatropin samples (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for (**a**) 0 day and (**b**) 30 days, as detected using LC-UV-MS at 280 nm.

For a more detailed analysis, charts were illustrated to establish how various excipient concentrations affect the degradation of the therapeutic proteins. Figures 4, 5, and 6 show the trend in GA-Z degradation by varying the sucrose, polysorbate 80, and polyethylene glycol 600 concentrations respectively. Meanwhile, Figure 7 illustrates the relationship of glycerol concentration with somatropin degradation. The degradation rate for both proteins was measured as the percentage of the native peak, relative to the other peaks in the chromatogram.

As observed in Figure 4, the addition of sucrose at 40 mg/mL and 120 mg/mL proved to be efficacious in lowering the degradation rate of GA-Z in the formulation, with the latter producing better results. At each incubation time, the percentage of the native GA-Z is higher in formulations containing sucrose, with the exception of the 20-day incubation for the 40 mg/mL formulation. The stabilizing effects of polyols and sugars on biological macromolecules such as proteins were established in the nineties. The addition of these excipients is known to prevent irreversible aggregation, reduce the loss of activity, and increase the protein's thermal transition temperature (Lee and Timasheff, 1981). Further investigations posit that such excipients do not bind to the protein. Instead, they affect the water tension surrounding the protein as described by the phenomenon called preferential exclusion. This results in an increased chemical potential of the protein, which is directly proportional to the surface area of the protein in contact with the solvent.

By the action of Le Chatelier's Law, the system will thus favor the condition leading to the smallest surface area to reduce the effects of preferential exclusion. In a thermodynamic sense, the addition of sucrose leads to an increase in the protein's Gibb's free energy and stability (Kendrick et al., 1997; Ruan et al., 2003).

Chemical degradation such as asparagine deamidation and aspartic acid isomerization is strongly linked with the physical properties of the solution and the amino acid residues (Ramm et al. 2021). As mentioned previously, these degradation reactions are initiated with the nucleophilic attack from the nitrogen of the peptide backbone to the asparagine and aspartic acid side chains. Protein conformations that lead to the decreased distance between these reaction sites increase the rates of chemical degradation. Since the structure affects the protein's intra-molecular mobility and distance, it is a key factor in determining the rate of deamidation and degradation. In regards to the stabilizing effect of sucrose at 120 mg/mL, it is likely that preferential exclusion was minimized leading to improved stability of GA-Z through the reduction of its surface area. However, this needs to be confirmed with further analysis to determine whether this was influenced by changes in the structural conformation of the protein.

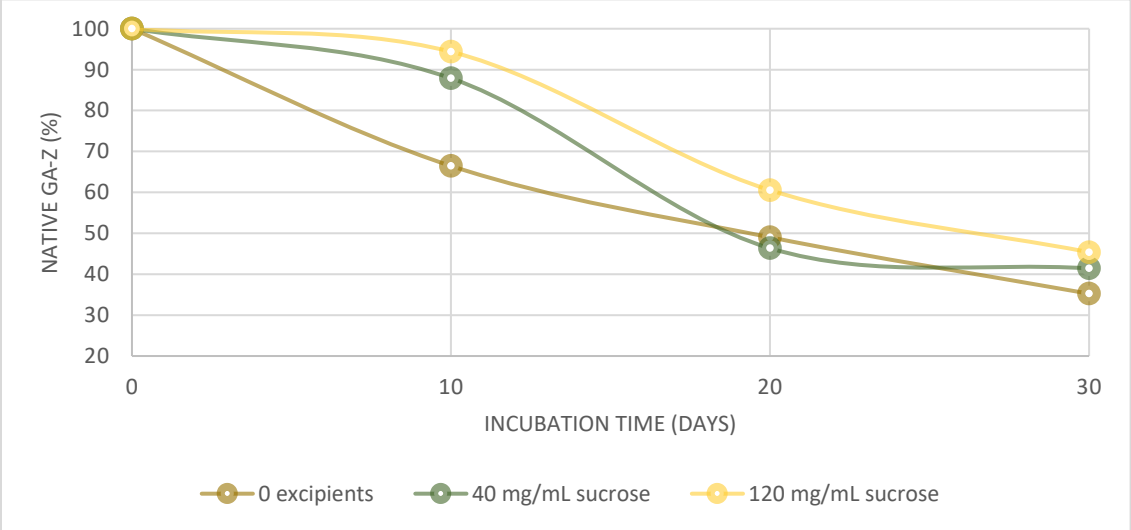



**Figure 4.** Degradation of GA-Z with sucrose (0, 40 mg/mL, 120 mg/mL) after incubation at 37°C, as determined by LC-UV analysis at 280 nm.

The decreased protein degradation is also more pronounced with the addition of polysorbate 80, as shown in Figure 5. It is observed that increasing the polysorbate concentration lessens the degradation rate, with more pronounced effects at the highest concentration, 1 mg/ml. It was also noted that at this concentration, the rate of degradation of GA-Z is significantly lowered from an incubation time of 10 days to 30 days, as compared to the two lower concentrations of polysorbate 80. These data points correspond to a decrease in the GA-Z protein from 82.69% to 76.35%. Polysorbate 80, a non-ionic surfactant, has long been used as to inhibit protein aggregation due to

its low toxicity, efficacy at low concentrations, and its capacity to prevent protein surface adsorption (Agarkhed et al., 2012). It is thought that protein adsorption is prevented or reduced through the competitive binding of these compounds to the surface. Non-ionic surfactants typically bind to interfaces stronger as compared to proteins/peptides or protein-based complexes. As such, polysorbate 80 prevents GA-Z from reaching various interfaces and protects it from denaturation, aggregation, and adsorption (Mahler et al., 2010). Even though surfactants are widely used as a stabilizer in protein formulations, the mechanisms of how they provide stability are not fully understood. Non-ionic surfactants were shown to protect from protein degradation by increasing the free energy of protein unfolding, by decreasing molecular interactions by binding to the hydrophobic parts of the protein, and by acting as a molecular chaperones in facilitating protein folding (Agarkhed et al., 2012). It is also possible that chemical degradation was decreased since polysorbate 80 lowered the conformational flexibility of the protein.

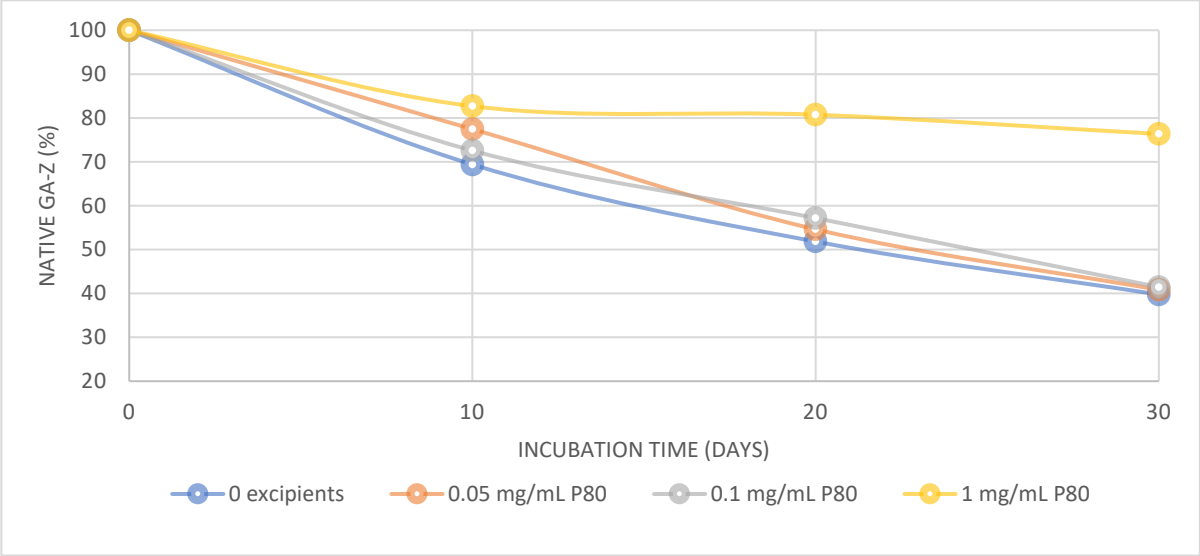



**Figure 5.** Degradation of GA-Z with Polysorbate 80 (0, 0.05 mg/mL, 0.1 mg/mL, 1 mg/mL) after incubation at 37°C, as determined by LC-UV at 280 nm

On the other hand, the use of polyethylene glycol as an excipient did not provide the same effect as with the other previously mentioned excipients. As shown in Figure 6, the addition of PEG 600 to GA-Z increased the degradation rate instead. Polyethylene glycol is a hydrophilic, non-toxic, and non-ionic polymer that is widely used in various pharmaceutical formulations to aid drug delivery, improve biological activity, and increase solubility (Rowe et al., 2015). In a previous study (Ramm et al., 2021), the chemical degradation of GA-Z was lowered with the addition of glycerol. It is thought that the compaction of the protein domains reduces the mobility of the peptide backbone, which may lower the degradation rate of the protein. Moreover, the stiffness of the backbone may have decreased the mobility of the GA-Z linker and water accessibility. These

factors contributed to the change in structural conformation of GA-Z, which lead to decreased degradation.

The destabilizing effect of PEG600 may be attributed to the presence of impurities in the sample. Previous studies (Wu et al., 2011; Waterman et al., 2008) have linked drug instability in formulations to trace amounts of aldehydes, organic acids, and peroxides. Peroxides, in particular, can lead to free radical-initiated oxidation reactions, which contribute to the formation of other reactive substances such as organic acids and low molecular weight aldehydes in PEG (Hemenway et al., 2012). Such substances can lead to N-methylation and N-formylation of drug substances containing an amine moiety, as in the case of proteins and peptides (Robnik et al., 2020). The presence and formation of peroxides in the samples containing PEG could explain the increased GA-Z degradation over time as PEG600 concentration was increased. However, it was not confirmed if this decreased stability was indeed due to the presence of peroxides in the sample, as it was not possible to perform LC-MS analysis for samples containing PEG.

Moreover, PEG is discouraged as a crowding agent in formulations since it has the potential to interact with the parts of the protein (Zhou et al., 2008). Even though macromolecular crowding leads to excluded-volume effects, non-specific interactions between crowders and proteins may overcome its stabilization effects, leading to protein degradation. It is inferred that GA-Z destabilization is due to the intermolecular interactions between PEG and the hydrophobic/non-polar side chains of the protein. In a study, such mechanism was exhibited by PEG2000 in the destabilization of the structural conformation of a protein, leading to decreased thermal stability (Zhang et al., 2012). However, further research must be conducted if such results will be replicated in the case of PEG600 or with other polymers.

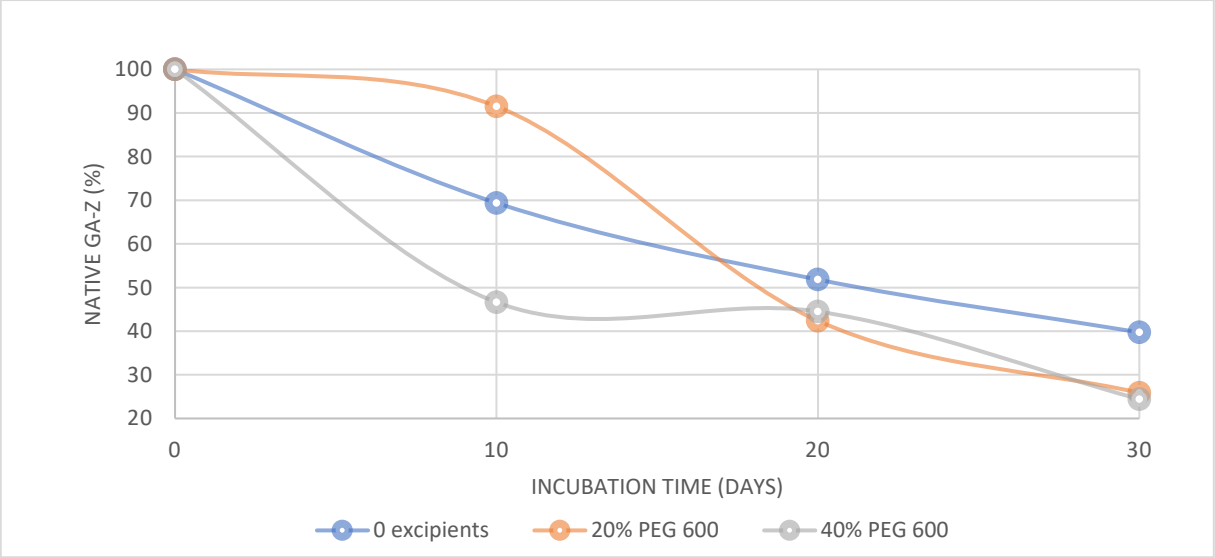



**Figure 6.** Degradation of GA-Z with Polyethylene glycol (0%, 20%, 40%) after incubation at 37°C, as determined by LC-UV at 280 nm.

The stabilizing effect of glycerol is shown in Figure 7, wherein 30 and 50% glycerol concentrations led to the decreased degradation of somatropin. This effect of glycerol was demonstrated in a previous study (Ramm et al., 2021), albeit using the GA-Z protein. The mechanism involved in formulations with 50% glycerol or less stems from a decrease in conformational flexibility of the protein. This led to the reduction of the overall mobility of the peptide backbone and potentially water accessibility. Such mechanism may be plausible in regards to its stabilizing effect on somatropin, but more analysis should be performed to conclude this.

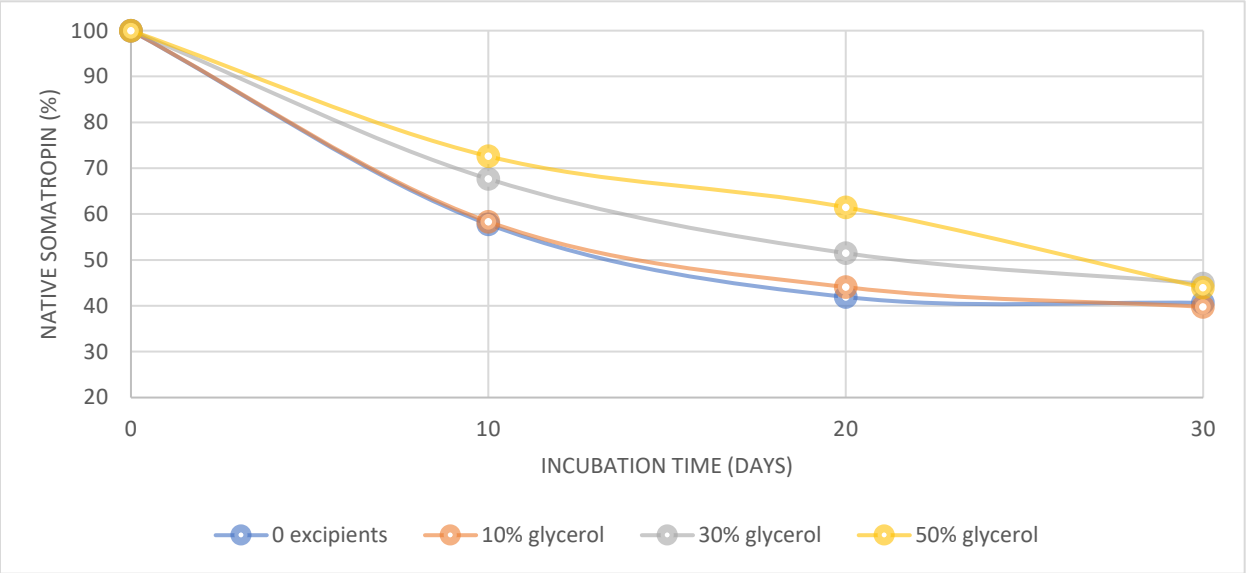



**Figure 7.** Degradation of Somatropin with glycerol (0%, 10%, 30%, 50%) after incubation at 37°C, as determined by LC-UV at 280 nm.

The relationship between water activity, protein degradation, and excipient concentration were also assessed for both GA-Z (Figures 8, 9, 10) and somatropin (Figure 11). These graphs show the percentage of the degraded protein after 30 days of incubation at a temperature of 37°C. It can be observed from Figure 8, Figure 9, and Figure 10 that the decrease in the degraded GA-Z is not attributed to the decrease in the water activity since they were still high ($a_w$>0.9). On the other hand, the decrease in degraded somatropin with increasing glycerol concentration in Figure 11 may be coupled with the decrease in water activity. It is important to assess whether water activity produces a significant effect, considering that the main chemical degradation pathways are dependent on water availability (Ramm et al., 2021). It can be inferred that lowered chemical degradation rate in somatropin is due to the reduced amount of available water in the formulation, which decreased as glycerol concentration is increased. This decreased water accessibility influenced by glycerol-induced compaction is a key factor in decreasing deamidation rates (Yan et al., 2018). Such structural changes in the protein may be a result of the preferential exclusion of glycerol, which leads to a decrease in the exposed area of the protein available for water.

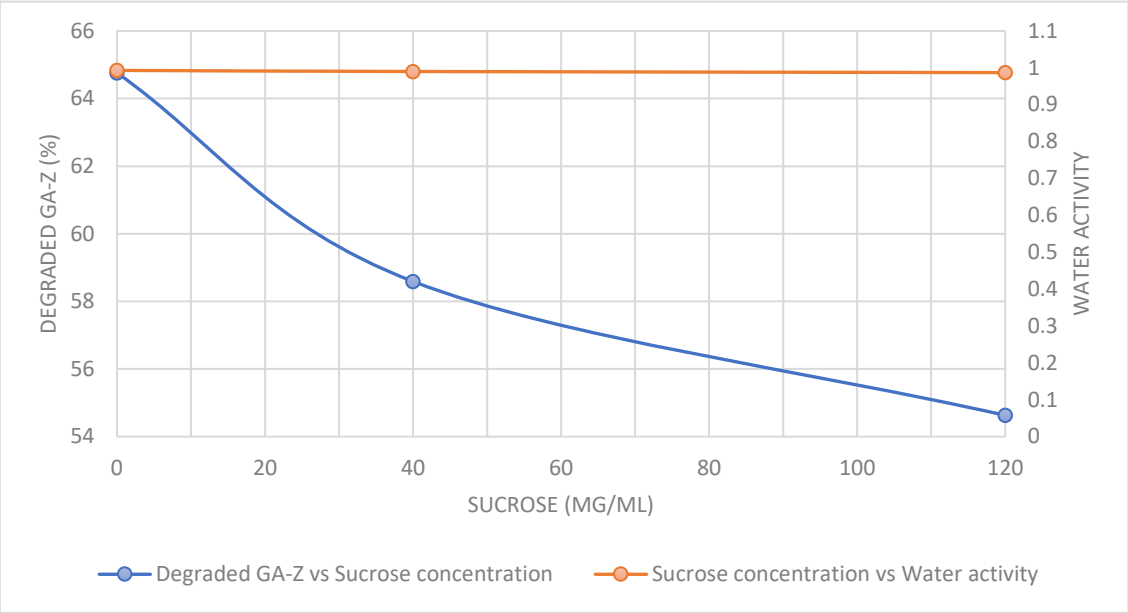



**Figure 8.** Relative amount of GA-Z and water activity vs sucrose concentration after 30 days incubation at 37°C.

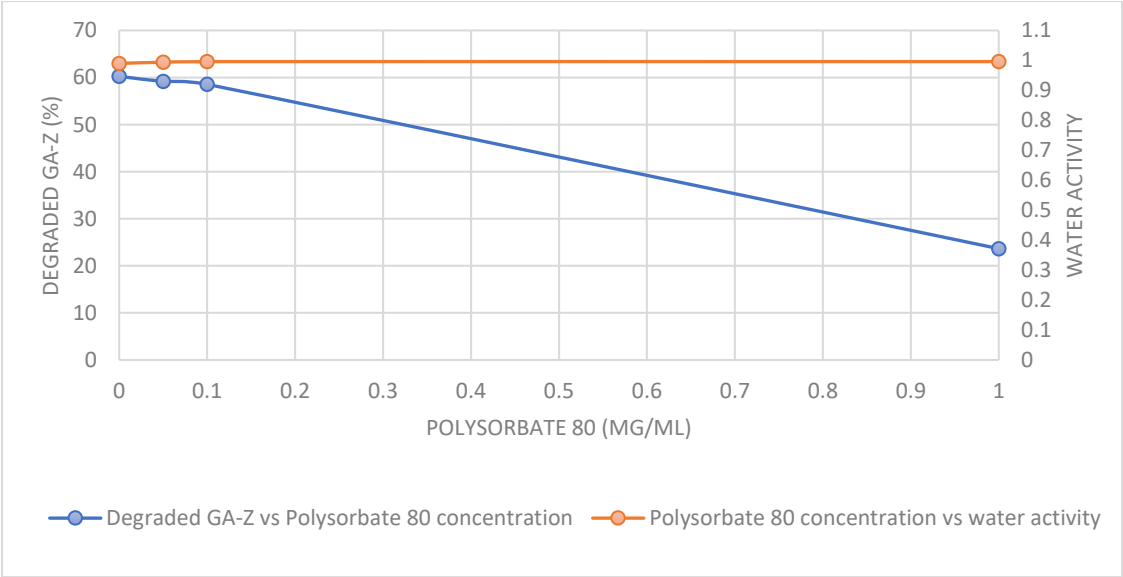



**Figure 9.** Relative amount of GA-Z and water activity vs Polysorbate 80 concentration after 30 days incubation at 37°C

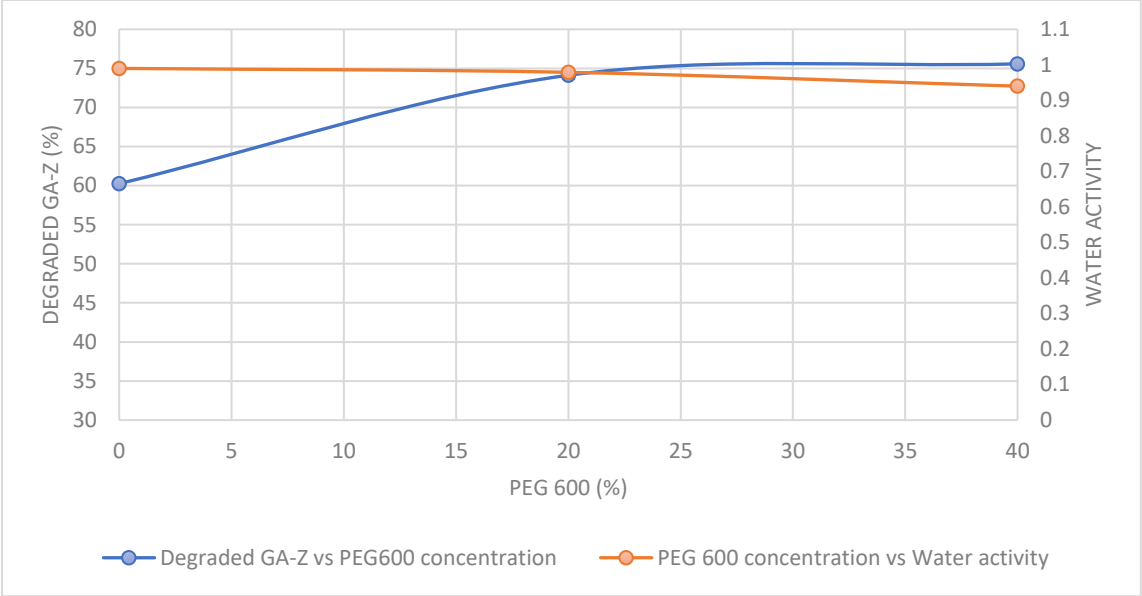



**Figure 10.** Relative amount of GA-Z and water activity vs PEG 600 concentration after 30 days incubation at 37°C.

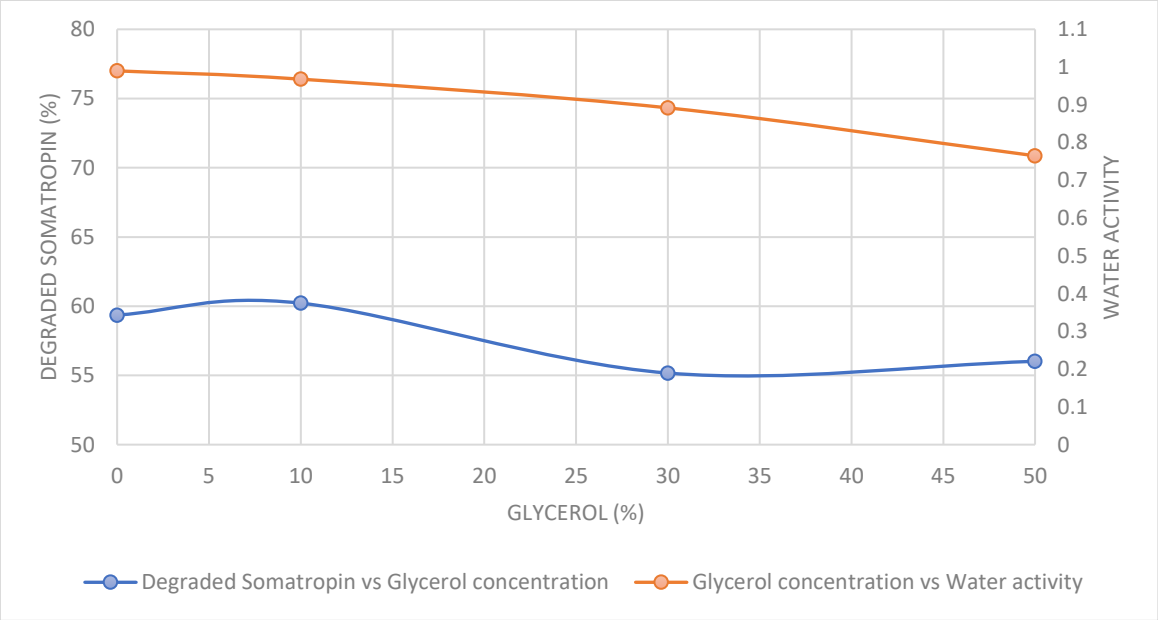



**Figure 11.** Relative amount of Somatropin and water activity vs glycerol concentration after 30 days incubation at 37°C.

Unlike GA-Z, there was no developed method for the analysis of somatropin. With this, method development was first conducted to produce a fast and reliable method capable of separating and identifying somatropin and its degradation products using the Shimadzu LC-20AD system. The parameters that were optimized were the gradient profile, flow rate, injection volume, and column

oven temperature. During the gradient optimization, the method started from a low acetonitrile concentration for a long period to establish a baseline and gather information regarding the initial degradation profile. From this, the portions of the chromatogram with poor resolution were modified to have a slower gradient to promote better separation. Various flow rates were also assessed in terms of their effect on analyte sensitivity and retentivity. The injection volume directly relates to the amount of analyte loaded into the HPLC column; as such, it is important to determine the optimal volume to maximize analyte sensitivity without compromising the peak symmetry. Lastly, the column oven temperature was optimized to determine its effects on the retentivity and sensitivity of the analytes. The kinetic properties of the analyte and the mobile phase are directly affected by temperature, leading to increased solute diffusivity and faster mass transfer. The method used a diode-array detector, using the wavelengths 220 nm and 280 nm, although the latter was solely used during integration as it produced sharper peaks. The final method utilized a flow rate of 0.8 mL/min, a column oven temperature of 60 °C, and an injection volume of 12 µL, and a gradient profile of 45 minutes.

Mass spectrometric analysis of both GA-Z and somatropin samples were also performed to identify the degradation peaks, shown in Figure 12 and 13, respectively. As per the chromatogram shown in Figure 12, the native protein was identified along with various degradation products, namely hydrolysis (A), hydrolysis/deamidation/isomerization (B), isomerization (C), and deamidation (D). This is in agreement with the previously established data (Ramm et al., 2021). The identification of degradation products was also done for somatropin, as indicated in Figure 13. The degradation peaks were identified to be products of hydrolysis (A) and isomerization/deamidation (B). Deamidation is shown by an increase in mass of 1 Da and isomerization is shown by a change in retention time but no difference in mass.

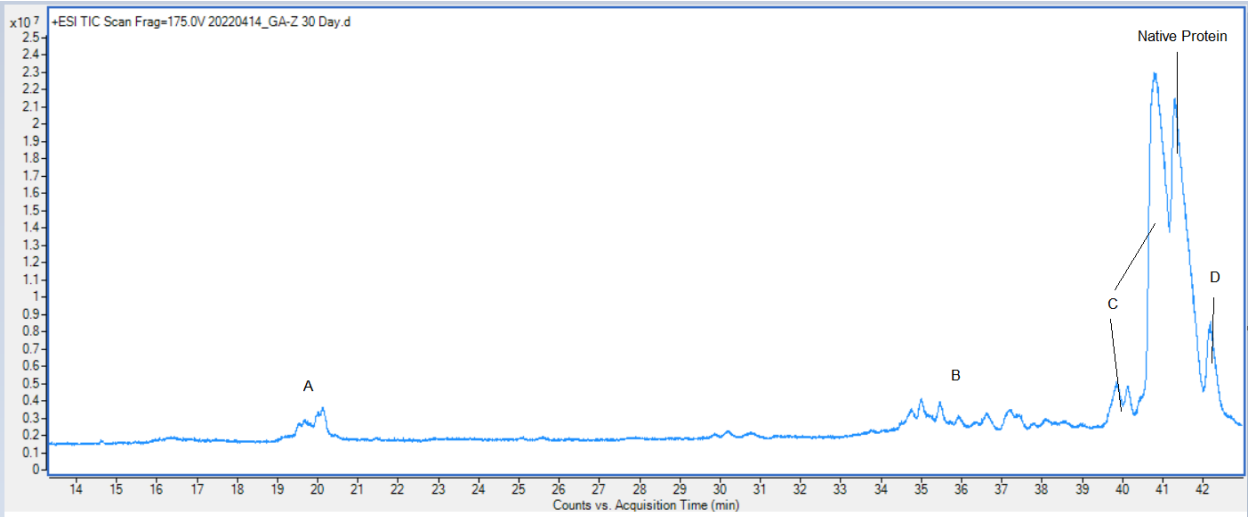



**Figure 12.** Total Ion Chromatogram of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 30 days, as determined by LC-MS analysis.

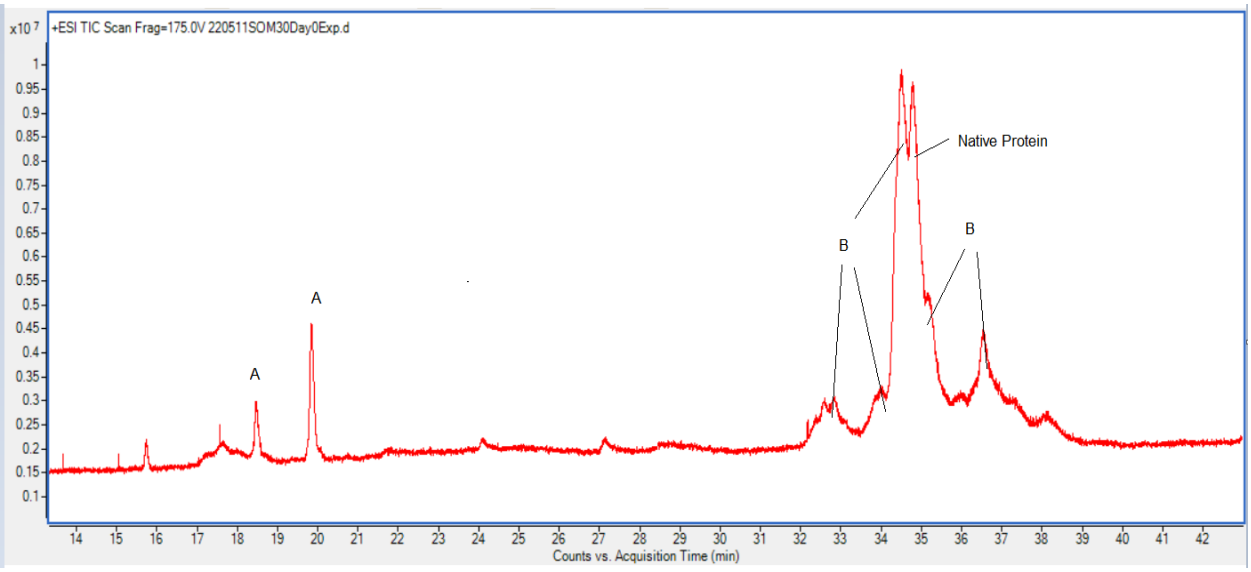
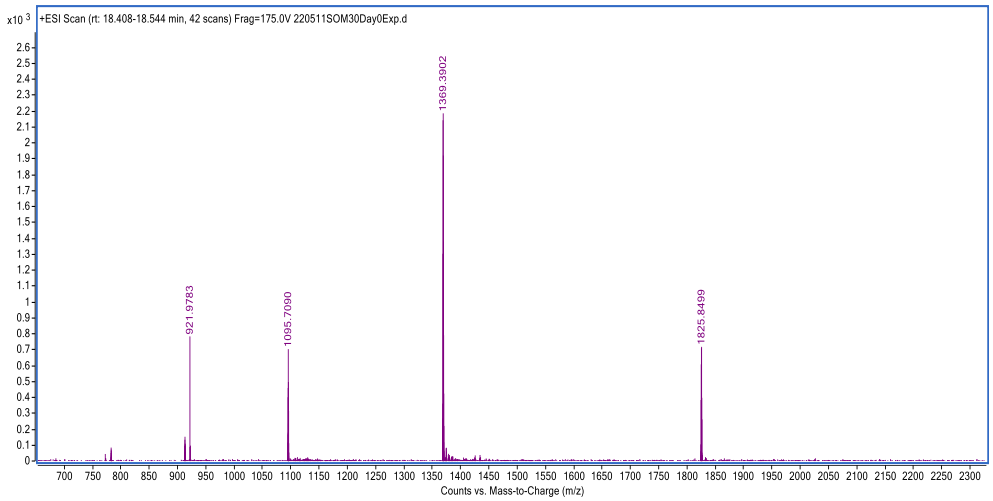



**Figure 13.** Total Ion Chromatogram of Somatropin sample (9 mg/mL Somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 30 days, as determined by LC-MS analysis.

As an example of how the data was analyzed, the mass spectrum of a hydrolysis product of somatropin is given in Figure 14 below. This spectrum was extracted from 18.408 minutes to 18.544 minutes from the given chromatogram in Figure 13. Upon closer inspection of the mass spectrum, the monoisotopic mass of the hydrolysis product was calculated to be 5470.5509 m/z [(1824.5223*3)-(3*1.008)]. The 1825.5223 m/z peak and the charge state +3 was extracted from the ion distribution shown in Figure 14b. It is possible to discern the identity of a degradation product by cross-referencing the calculated monoisotopic mass from the MS to a somatropin fragment ion table. This fragment ion table represents various monoisotopic masses of the fragmented somatropin as per its amino acid sequence at a particular charged state. The calculated monoisotopic mass is close to the monoisotopic mass of the fragment K145-F191, Y(+1), which is 5473.62 Da. There is however a difference in mass of 2 Da. This monoisotopic mass from the fragment ion table represents the cysteine residues existing in its S-H form. Thus, the difference in mass of 2 Da can be attributed to the possible formation of disulfide bonds between cysteine residues in the fragment. Before the hydrolysis, the protein formed disulfide bridges between C53-C165 and C182-C189. Having the residue C165 in S-H form and the residues C182 and C189 linked in S-form would generate a fragment with the monoisotopic mass seen in Figure 14.

a.




b.

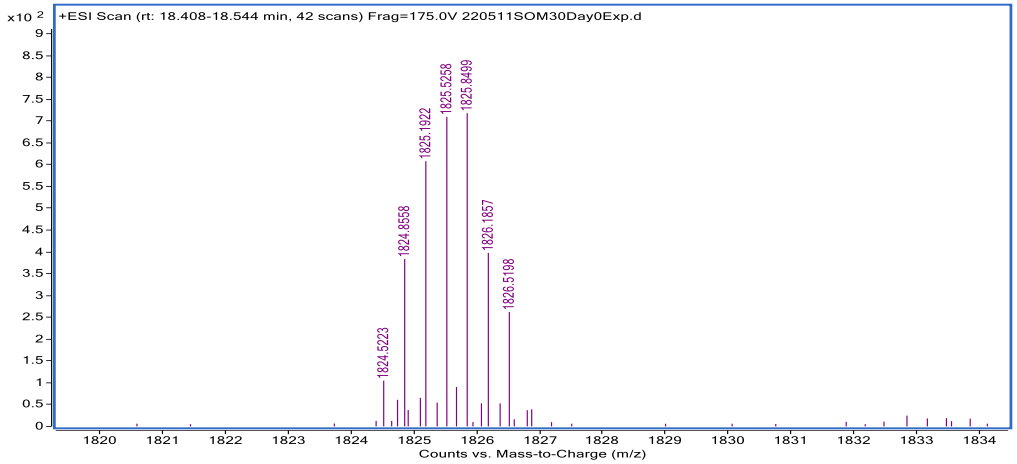



**Figure 14.** Sample mass spectrum (**a**) for Somatropin sample (9 mg/mL Somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 30 days, as determined by Q-TOF LC/MS. Further examination (**b**) of the m/z 1825.5223 fragment is also provided.

## CONCLUSIONS

The effects of sucrose, polysorbate 80, and PEG 600 on the chemical degradation of GA-Z were determined, along with the effect of glycerol on somatropin. Overall, the chemical degradation of GA-Z was lowered with increasing sucrose and polysorbate 80 concentration. However, PEG 600 did not lower the degradation rate of the protein. On another note, the stability of somatropin increased with increasing glycerol concentrations. Mass spectrometric analysis confirms that the degradation products from both proteins are the result of deamidation, isomerization, and hydrolysis reactions. Moreover, an LC-UV method was successfully developed for the analysis of somatropin and its degradation products. These preliminary results may be used to develop formulation strategies for the increased stability of protein-based drugs.

## FUTURE DIRECTIONS

As this study serves as an initial screening for pharmaceutical excipients with stabilizing effects in protein parenteral formulations, it is recommended that further exploration be done on the effects of sucrose and polysorbate 80 on GA-Z and glycerol on somatropin. This study is limited only to the separation and identification of degradation products for both proteins, with the use of LC-UV and LC-MS analysis. Thus, future work involving Asymmetrical Flow Field-Flow Fractionation (AF4) will give insights into how the various excipients affect the tertiary and quaternary structure of the proteins. Moreover, Small Angle Neutron Scattering (SANS) can examine how the excipients change the protein conformation. Both analyses are integral in forming more conclusive explanations as to how these excipients affect the stability of the protein. One other recommendation is to expand the concentration range and to provide more concentration points to thoroughly evaluate the trend at which the excipients provide stability to the protein. With this, it will be fairly easy to discern at which concentration will an excipient be the most effective or at which point will it not provide further stability. It is also highly recommended to test the effects of other possible excipients on the chemical degradation of the tested proteins In regards to the developed LC-UV method for somatropin, further optimization can be explored for its improvement. This includes the use of other mobile phases and the use of an experimental design for optimization. It is also possible to conduct LC-UV method validation for both GA-Z and somatropin to provide objective evidence that the method complies with necessary standards and may be adapted for routine work in the industry.

**REFERENCES**


- Agarkhed, M., O'Dell, C., Hsieh, M., Zhang, J., Goldstein, J., & Srivastava, A. (2012). Effect of Polysorbate 80 Concentration on Thermal and Photostability of a Monoclonal Antibody. AAPS Pharmscitech, 14(1), 1-9. doi: 10.1208/s12249-012-9878-0
- Akash, M., Rehman, K., Tariq, M., & Chen, S. (2015). Development of therapeutic proteins: advances and challenges. Turkish Journal of Biology, 39, 343-358. doi: 10.3906/biy-1411-8
- Bam, N., Cleland, J., & Randolph, T. (1996). Molten Globule Intermediate of Recombinant Human Growth Hormone: Stabilization with Surfactants. Biotechnology Progress, 12(6), 801-809. doi: 10.1021/bp960068b
- Bide, Y., Fashapoyeh, M., & Shokrollahzadeh, S. (2021). Structural investigation and application of Tween 80-choline chloride self-assemblies as osmotic agent for water desalination. Scientific Reports, 11(1). doi: 10.1038/s41598-021-96199-6
- Chi, E., Krishnan, S., Randolph, T., & Carpenter, J. (2003). Pharmaceutical Research, 20(9), 1325-1336. doi: 10.1023/a:1025771421906
- Craik, D., Fairlie, D., Liras, S., & Price, D. (2012). The Future of Peptide-based Drugs. Chemical Biology &Amp; Drug Design, 81(1), 136-147. doi: 10.1111/cbdd.12055
- Eakin, C., Miller, A., Kerr, J., Kung, J., & Wallace, A. (2014). Assessing analytical methods to monitor isoAsp formation in monoclonal antibodies. Frontiers In Pharmacology, 5. doi: 10.3389/fphar.2014.00087
- Gervais, D. (2015). Protein deamidation in biopharmaceutical manufacture: understanding, control and impact. Journal Of Chemical Technology &Amp; Biotechnology, 91(3), 569-575. doi: 10.1002/jctb.4850
- Gervasi, V., Dall Agnol, R., Cullen, S., McCoy, T., Vucen, S., & Crean, A. (2018). Parenteral protein formulations: An overview of approved products within the European Union. European Journal Of Pharmaceutics And Biopharmaceutics, 131, 8-24. doi: 10.1016/j.ejpb.2018.07.011
- Hemenway, J., Carvalho, T., Rao, V., Wu, Y., Levons, J., & Narang, A. et al. (2012). Formation of Reactive Impurities in Aqueous and Neat Polyethylene Glycol 400 and Effects of Antioxidants and Oxidation Inducers. Journal Of Pharmaceutical Sciences, 101(9), 3305-3318. doi: 10.1002/jps.23198
- Hsiao, K., Alves, J., Patel, R., Adams, M., Nashine, V., & Goueli, S. (2017). A High-Throughput Bioluminescent Assay to Monitor the Deamidation of Asparagine and Isomerization of Aspartate Residues in Therapeutic Proteins and Antibodies. Journal Of Pharmaceutical Sciences, 106(6), 1528-1537. doi: 10.1016/j.xphs.2017.02.022
- Huynh-Ba, K. (2009). Handbook of Stability Testing in Pharmaceutical Development. doi: 10.1007/978-0-387-85627-8
- Jia, L., & Sun, Y. (2017). Protein asparagine deamidation prediction based on structures with machine learning methods. PLOS ONE, 12(7), e0181347. doi: 10.1371/journal.pone.0181347

- Kendrick, B., Chang, B., Arakawa, T., Peterson, B., Randolph, T., Manning, M., & Carpenter, J. (1997). Preferential exclusion of sucrose from recombinant interleukin-1 receptor antagonist: Role in restricted conformational mobility and compaction of native state. Proceedings Of The National Academy Of Sciences, 94(22), 11917-11922. doi: 10.1073/pnas.94.22.11917
- Krause, M., & Sahin, E. (2019). Chemical and physical instabilities in manufacturing and storage of therapeutic proteins. Current Opinion In Biotechnology, 60, 159-167. doi: 10.1016/j.copbio.2019.01.014
- Kumar, V., & Kalonia, D. (2006). Removal of peroxides in polyethylene glycols by vacuum drying: Implications in the stability of biotech and pharmaceutical formulations. AAPS Pharmscitech, 7(3), E47-E53. doi: 10.1208/pt070362
- Lee, J., & Timasheff, S. (1981). The stabilization of proteins by sucrose. Journal Of Biological Chemistry, 256(14), 7193-7201. doi: 10.1016/s0021-9258(19)68947-7
- Mahler, H., Huber, F., Kishore, R., Reindl, J., Rückert, P., & Müller, R. (2010). Adsorption Behavior of a Surfactant and a Monoclonal Antibody to Sterilizing-Grade Filters. Journal Of Pharmaceutical Sciences, 99(6), 2620-2627. doi: 10.1002/jps.22045
- Manning, M., Chou, D., Murphy, B., Payne, R., & Katayama, D. (2010). Stability of Protein Pharmaceuticals: An Update. Pharmaceutical Research, 27(4), 544-575. doi: 10.1007/s11095-009-0045-6
- Patist, A., Bhagwat, S., Penfield, K., Aikens, P., & Shah, D. (2000). On the measurement of critical micelle concentrations of pure and technical-grade nonionic surfactants. Journal Of Surfactants And Detergents, 3(1), 53-58. doi: 10.1007/s11743-000-0113-4
- Phillips, J., Buchanan, A., Andrews, J., Chodorge, M., Sridharan, S., & Mitchell, L. et al. (2017). Rate of Asparagine Deamidation in a Monoclonal Antibody Correlating with Hydrogen Exchange Rate at Adjacent Downstream Residues. Analytical Chemistry, 89(4), 2361-2368. doi: 10.1021/acs.analchem.6b04158
- Pramanick, S., Chandel, V. & Singodia, D. (2013). Excipient Selection In Parenteral Formulation Development. Pharma Times. 45. 65 - 77.
- Ramm, I., Sanchez-Fernandez, A., Choi, J., Lang, C., Schagerlöf, H., Wahlgren, M., & Nilsson, L. (2021). The Impact of Glycerol on an Affibody Conformation and Its Correlation to Chemical Degradation. Pharmaceutics, 13(11), 1853. doi: 10.3390/pharmaceutics13111853
- Ranke, M., & Wit, J. (2018). Growth hormone — past, present and future. Nature Reviews Endocrinology, 14(5), 285-300. doi: 10.1038/nrendo.2018.22
- Robnik, B., Naumoska, K., & Časar, Z. (2020). A Novel Testing Approach for Oxidative Degradation Dependent Incompatibility of Amine Moiety Containing Drugs with PEGs in Solid-State. Pharmaceutics, 12(1), 37. doi: 10.3390/pharmaceutics12010037
- Rowe, R., Sheskey, P., & Quinn, M. (2015). Handbook of pharmaceutical excipients. [S.l.]: Pharmaceutical Press.

- Ruan, K., Xu, C., Li, T., Li, J., Lange, R., & Balny, C. (2003). The thermodynamic analysis of protein stabilization by sucrose and glycerol against pressure-induced unfolding. The typical example of the 33-kDa protein from spinach photosystem II. European Journal Of Biochemistry, 270(8), 1654-1661. doi: 10.1046/j.1432-1033.2003.03485.x
- Sargaeva, N., Lin, C., & O'Connor, P. (2009). Identification of Aspartic and Isoaspartic Acid Residues in Amyloid β Peptides, Including Aβ1−42, Using Electron−Ion Reactions. Analytical Chemistry, 81(23), 9778-9786. doi: 10.1021/ac901677t
- Ståhl, S., Gräslund, T., Eriksson Karlström, A., Frejd, F., Nygren, P., & Löfblom, J. (2017). Affibody Molecules in Biotechnological and Medical Applications. Trends In Biotechnology, 35(8), 691-712. doi: 10.1016/j.tibtech.2017.04.007
- Strickley, R., & Lambert, W. (2021). A review of Formulations of Commercially Available Antibodies. Journal Of Pharmaceutical Sciences, 110(7), 2590-2608.e56. doi: 10.1016/j.xphs.2021.03.017
- Van Der Walle, C. (2011). Peptide and Protein Delivery. doi: 10.1016/c2010-0-64887-5
- Waterman, K., Arikpo, W., Fergione, M., Graul, T., Johnson, B., & MacDonald, B. et al. (2008). N-Methylation and N-Formylation of a Secondary Amine Drug (Varenicline) in an Osmotic Tablet. Journal Of Pharmaceutical Sciences, 97(4), 1499-1507. doi: 10.1002/jps.21119
- WHO guidelines on stability testing of active pharmaceutical ingredients and finished pharmaceutical products. (2022). Retrieved 19 May 2022, from https://www.who.int/publications/m/item/who-guidelines-on-stability-testing-of-active-pharmaceutical-ingredients-and-finished-pharmaceutical-products
- Wu, Y., Levons, J., Narang, A., Raghavan, K., & Rao, V. (2011). Reactive Impurities in Excipients: Profiling, Identification and Mitigation of Drug–Excipient Incompatibility. AAPS Pharmscitech, 12(4), 1248-1263. doi: 10.1208/s12249-011-9677-z
- Yan, Q., Huang, M., Lewis, M., & Hu, P. (2018). Structure Based Prediction of Asparagine Deamidation Propensity in Monoclonal Antibodies. Mabs, 10(6), 901-912. doi: 10.1080/19420862.2018.1478646
- Yang, H., & Zubarev, R. (2010). Mass spectrometric analysis of asparagine deamidation and aspartate isomerization in polypeptides. ELECTROPHORESIS, 31(11), 1764-1772. doi: 10.1002/elps.201000027
- Zhang, D., Wu, L., Chen, J., & Liang, Y. (2012). Effects of macromolecular crowding on the structural stability of human α-lactalbumin. Acta Biochimica Et Biophysica Sinica, 44(8), 703-711. doi: 10.1093/abbs/gms052
- Zhou, H., Rivas, G., & Minton, A. (2008). Macromolecular Crowding and Confinement: Biochemical, Biophysical, and Potential Physiological Consequences. Annual Review Of Biophysics, 37(1), 375-397. doi: 10.1146/annurev.biophys.37.032807.125817
- Zhou, X., Galparsoro, D., Madsen, A., Vetri, V., van de Weert, M., Nielsen, H., & Foderà, V. (2021). Polysorbate 80 Controls Morphology, Structure and Stability of Human Insulin Amyloid-Like Spherulites. doi: 10.1101/2021.08.10.455855

**APPENDICES**

A. HPLC chromatograms

1. GA-Z blank (for Sucrose)

a. GA-Z blank: 0 day

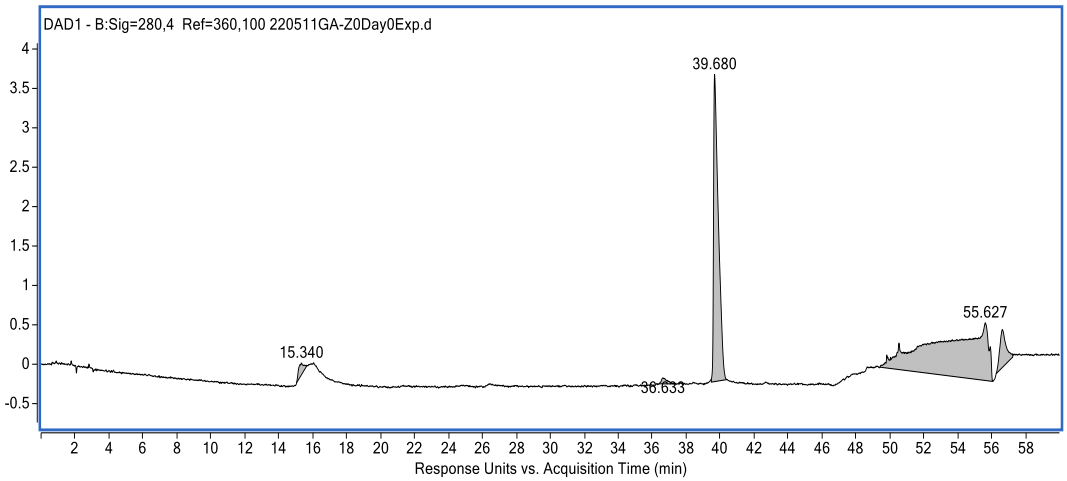



**Figure A1**. Degradation of GA-Z sample (9 mg/mL GA-Z, 137.5 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

b. GA-Z blank: 10 days

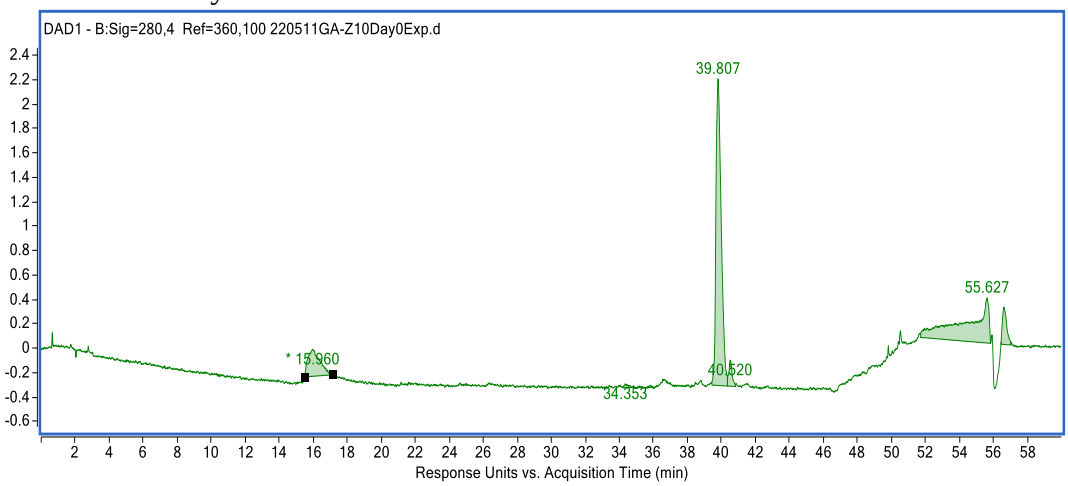



**Figure A2**. Degradation of GA-Z sample (9 mg/mL GA-Z, 137.5 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

c. GA-Z blank: 20 days

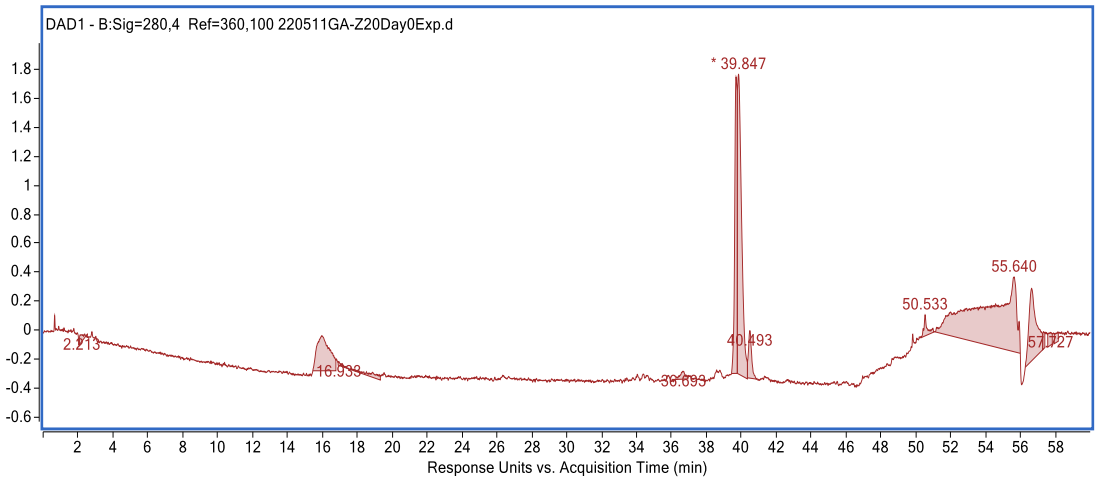



**Figure A3**. Degradation of GA-Z sample (9 mg/mL GA-Z, 137.5 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

d. GA-Z blank: 30 days

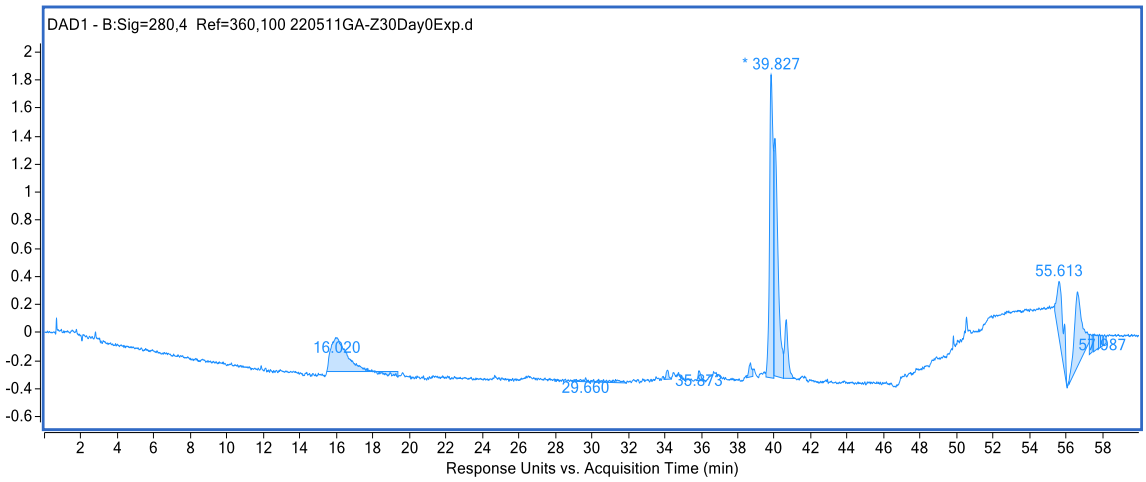



**Figure A4**. Degradation of GA-Z sample (9 mg/mL GA-Z, 137.5 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.

2. GA-Z blank(for Polysorbate and PEG)
   a. GA-Z blank: 0 day

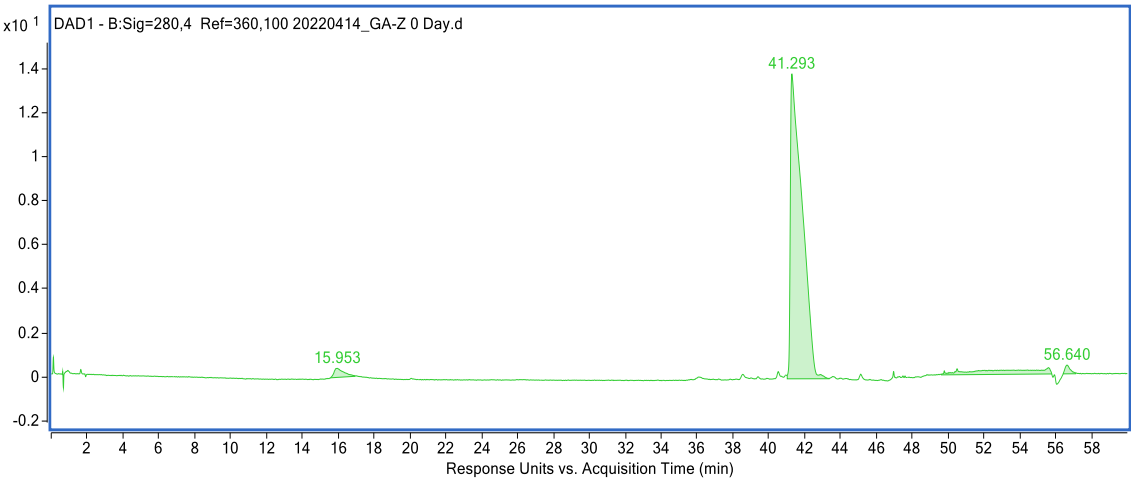



**Figure B1**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

   b. GA-Z blank: 10 days

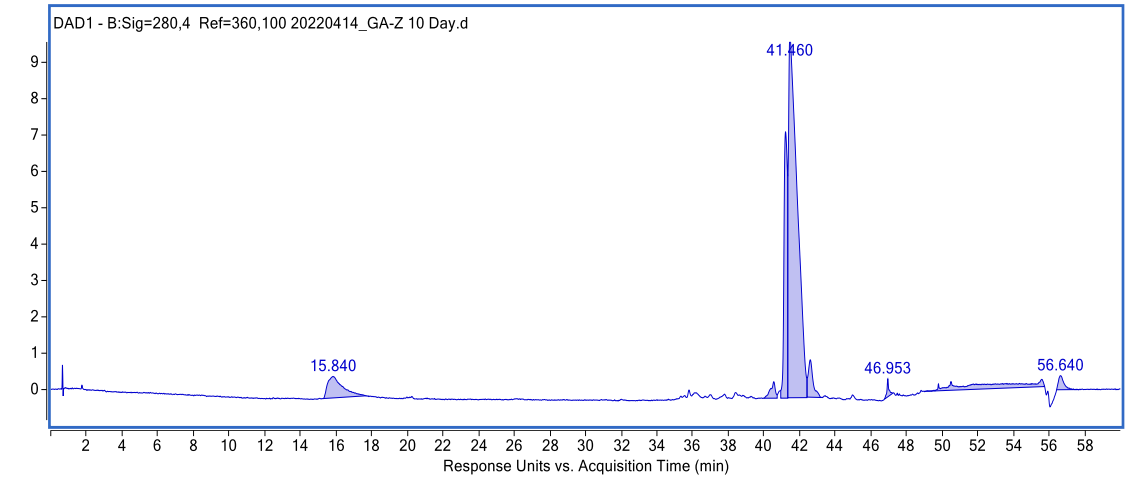



**Figure B2**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

c. GA-Z blank: 20 days

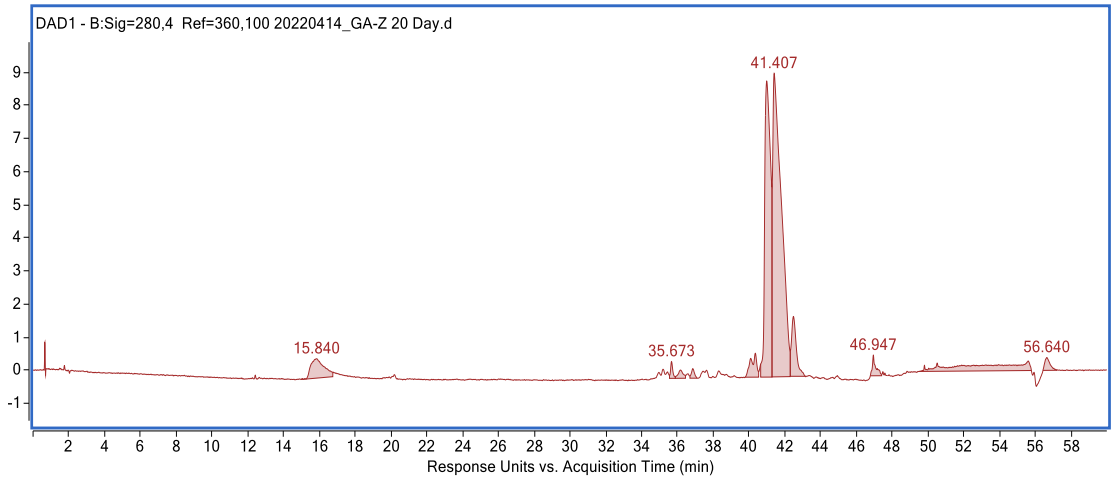



**Figure B3**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

d. GA-Z blank: 30 days

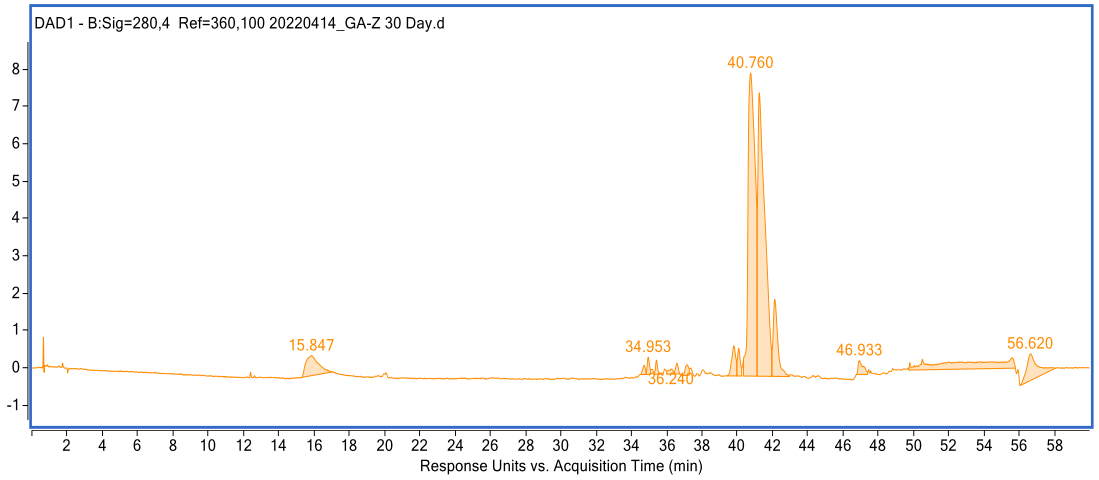



**Figure B4**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.

3. GA-Z with Sucrose

a. GA-Z with Sucrose (40 mg/mL): 0 day

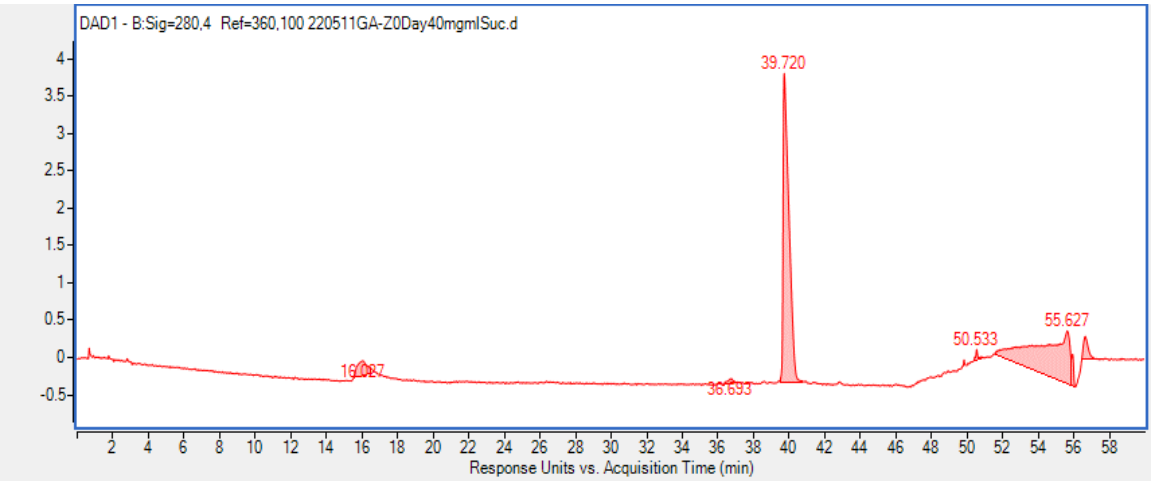



**Figure C1**. Degradation of GA-Z sample (9 mg/mL GA-Z, 137.5 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 40 mg/mL sucrose, after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

b. GA-Z with Sucrose (40 mg/mL): 10 days

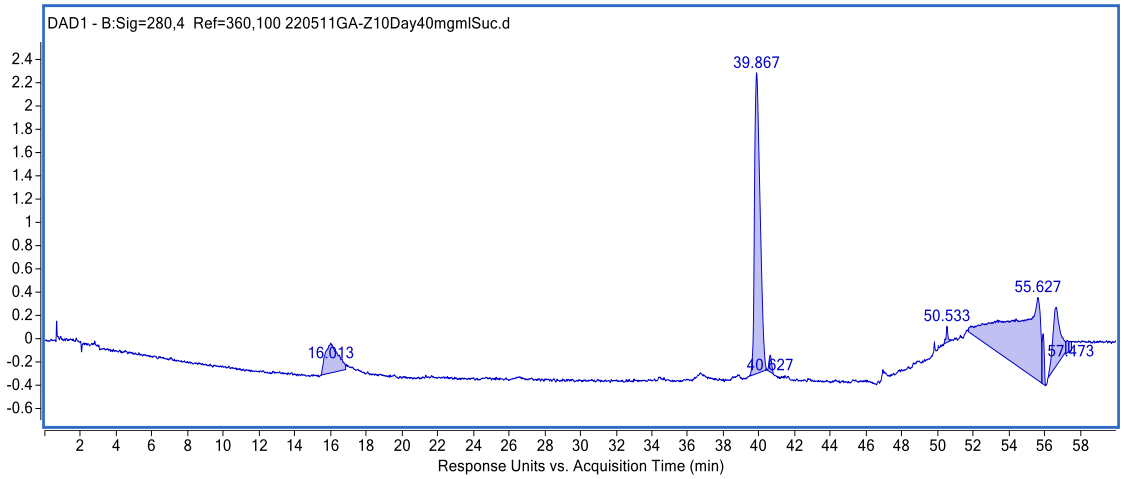



**Figure C2**. Degradation of GA-Z sample (9 mg/mL GA-Z, 137.5 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 40 mg/mL sucrose, after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

c. GA-Z with Sucrose (40 mg/mL): 20 days

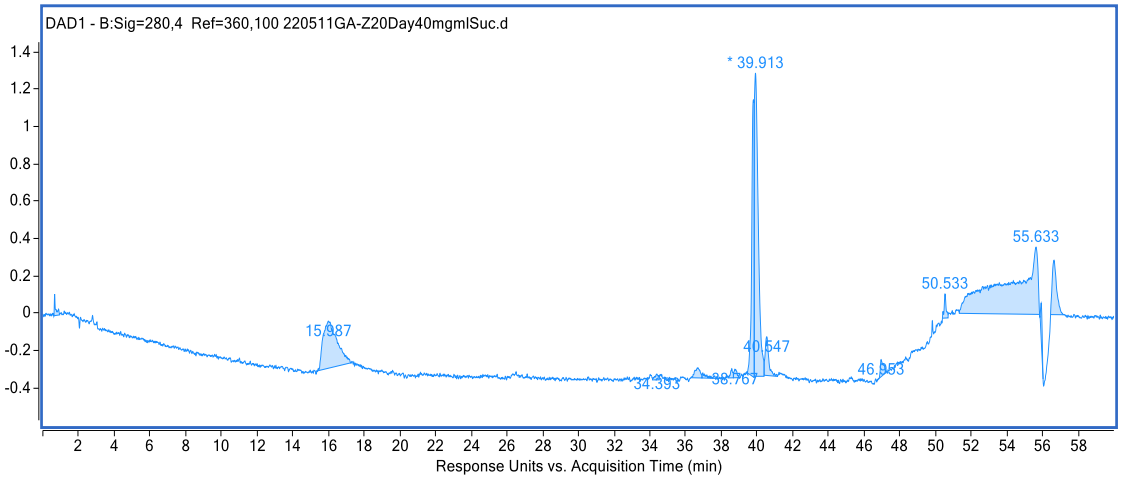



**Figure C3**. Degradation of GA-Z sample (9 mg/mL GA-Z, 137.5 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 40 mg/mL sucrose, after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

d. GA-Z with Sucrose (40 mg/mL): 30 days

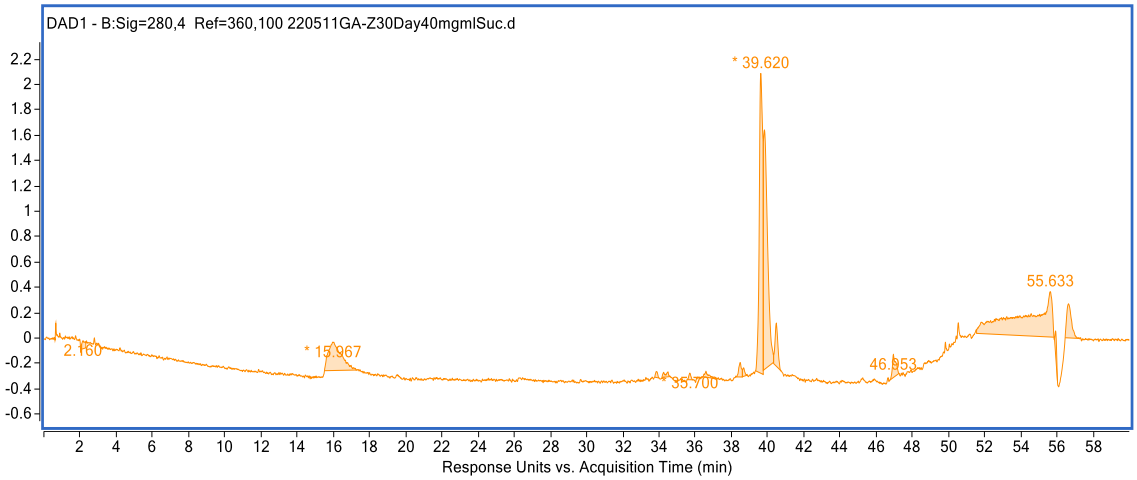



**Figure C4**. Degradation of GA-Z sample (9 mg/mL GA-Z, 137.5 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 40 mg/mL sucrose, after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.

e. GA-Z with Sucrose (120 mg/mL): 0 days

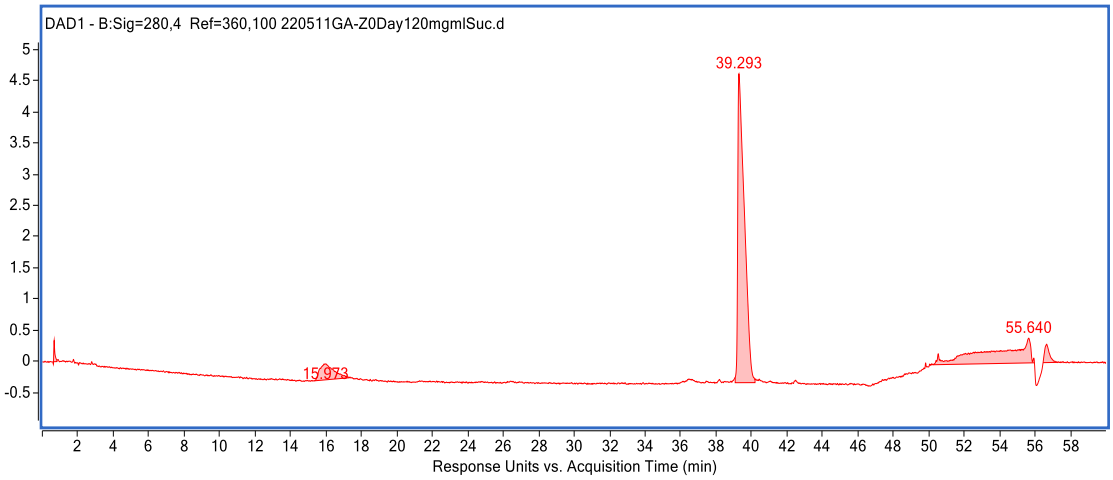



**Figure C5**. Degradation of GA-Z sample (9 mg/mL GA-Z, 137.5 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 120 mg/mL sucrose, after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

f. GA-Z with Sucrose (120 mg/mL): 10 days

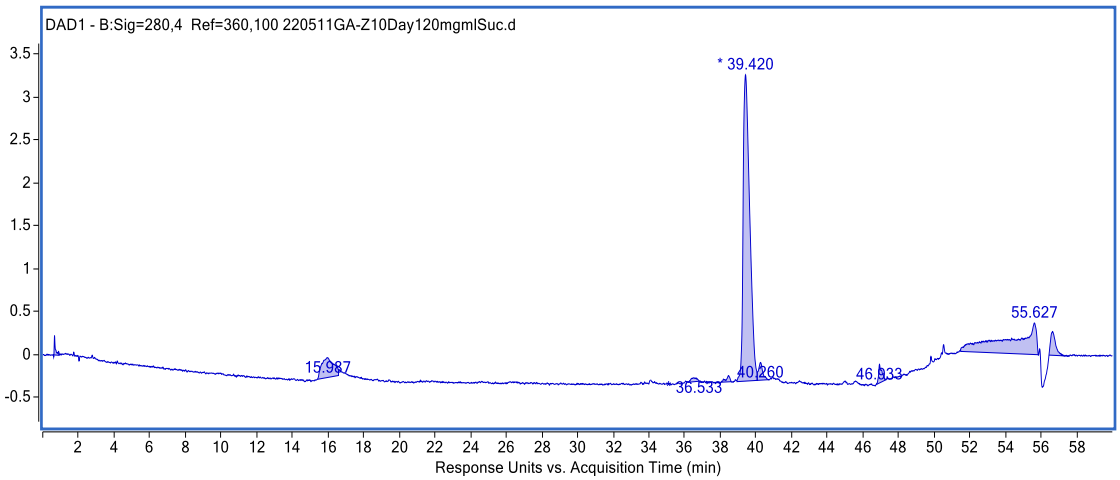



**Figure C6**. Degradation of GA-Z sample (9 mg/mL GA-Z, 137.5 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 120 mg/mL sucrose, after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

g. GA-Z with Sucrose (120 mg/mL): 20 days

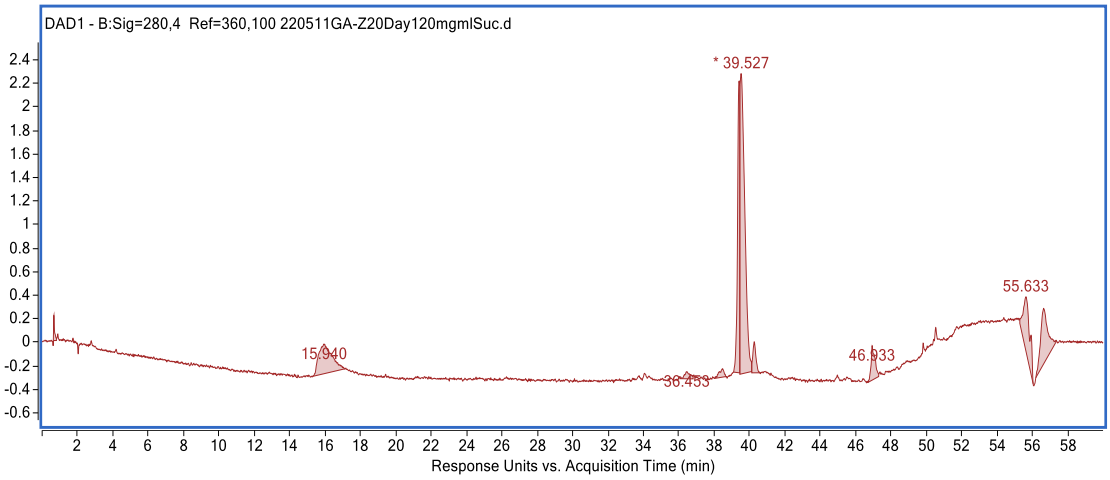



**Figure C7**. Degradation of GA-Z sample (9 mg/mL GA-Z, 137.5 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 120 mg/mL sucrose, after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

h. GA-Z with Sucrose (120 mg/mL): 30 days

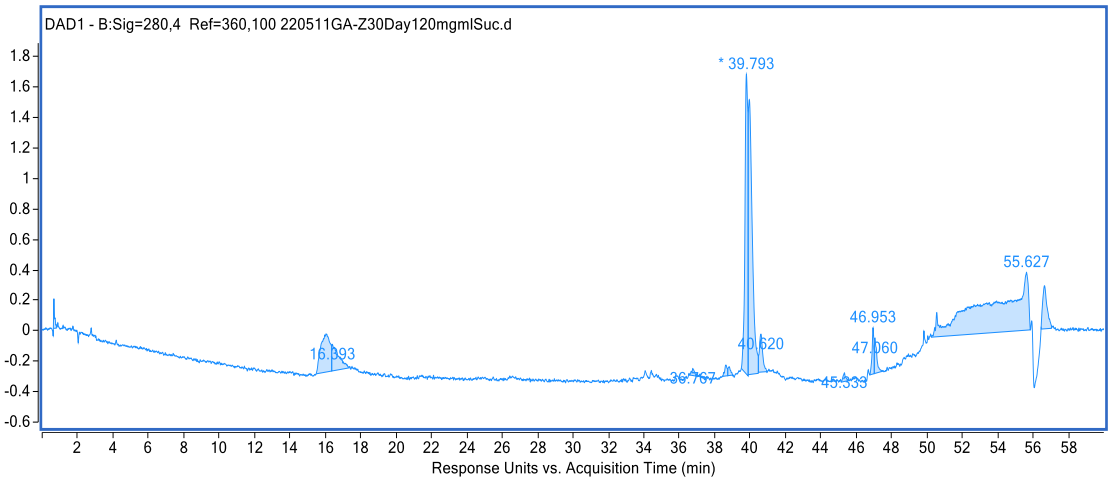



**Figure C8**. Degradation of GA-Z sample (9 mg/mL GA-Z, 137.5 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 120 mg/mL sucrose, after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.

4. GA-Z with Polysorbate 80

a. GA-Z with Polysorbate 80 (0.05 mg/mL): 0 day

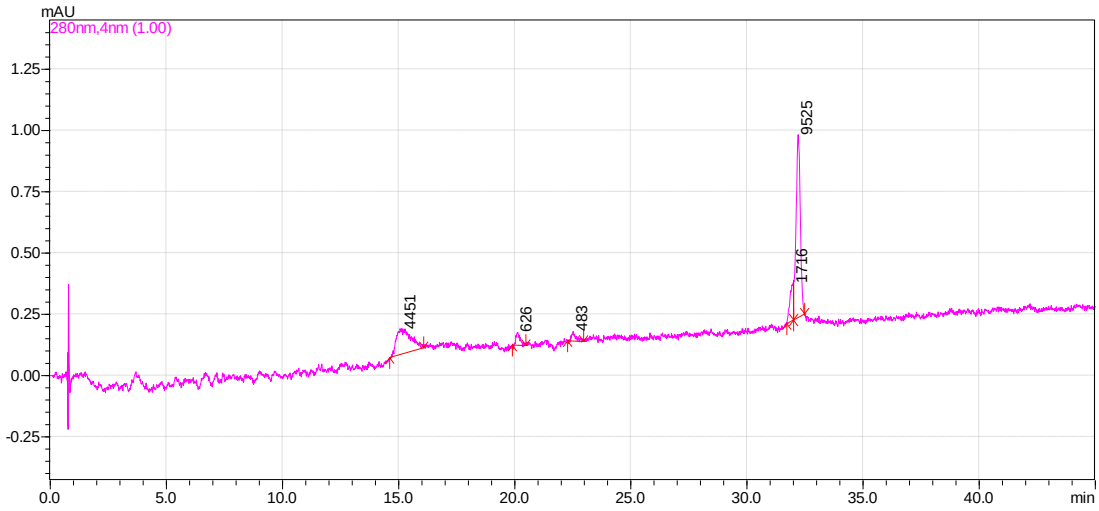



**Figure D1**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 0.05 mg/mL Polysorbate 80, after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

b. GA-Z with Polysorbate 80 (0.05 mg/mL): 10 days

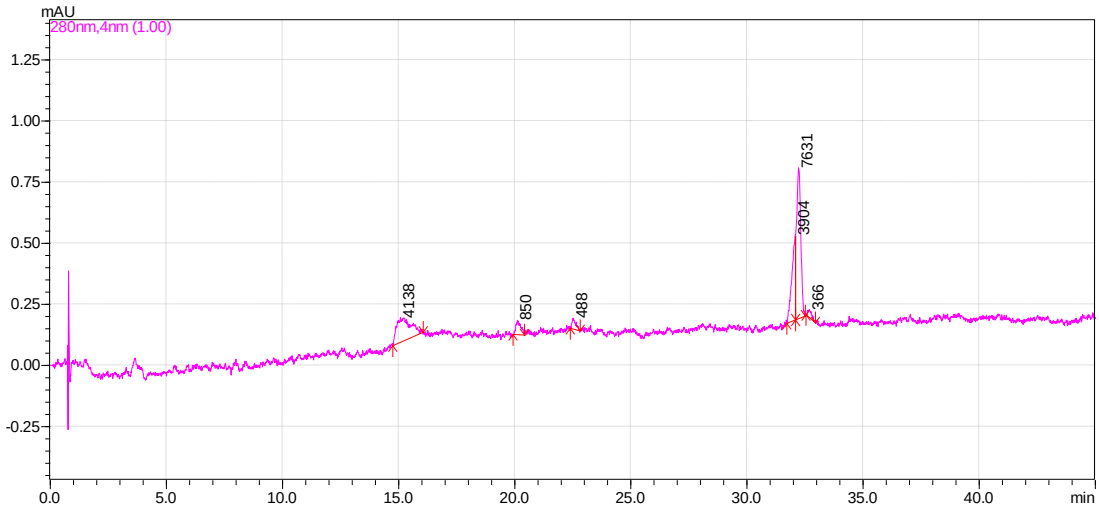



**Figure D2**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 0.05 mg/mL Polysorbate 80, after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

c. GA-Z with Polysorbate 80 (0.05 mg/mL): 20 days

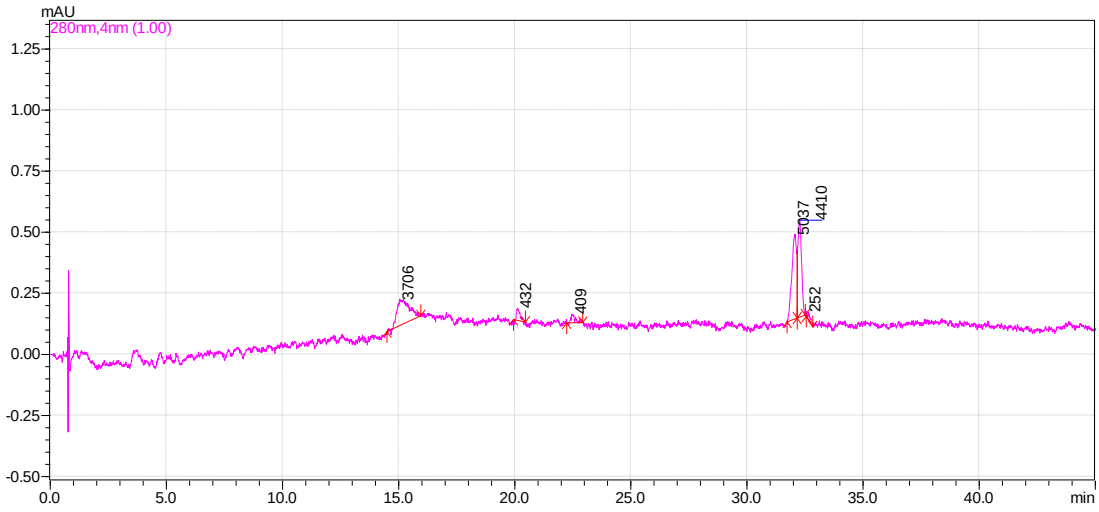



**Figure D3**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 0.05 mg/mL Polysorbate 80, after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

d. GA-Z with Polysorbate 80 (0.05 mg/mL): 30 days

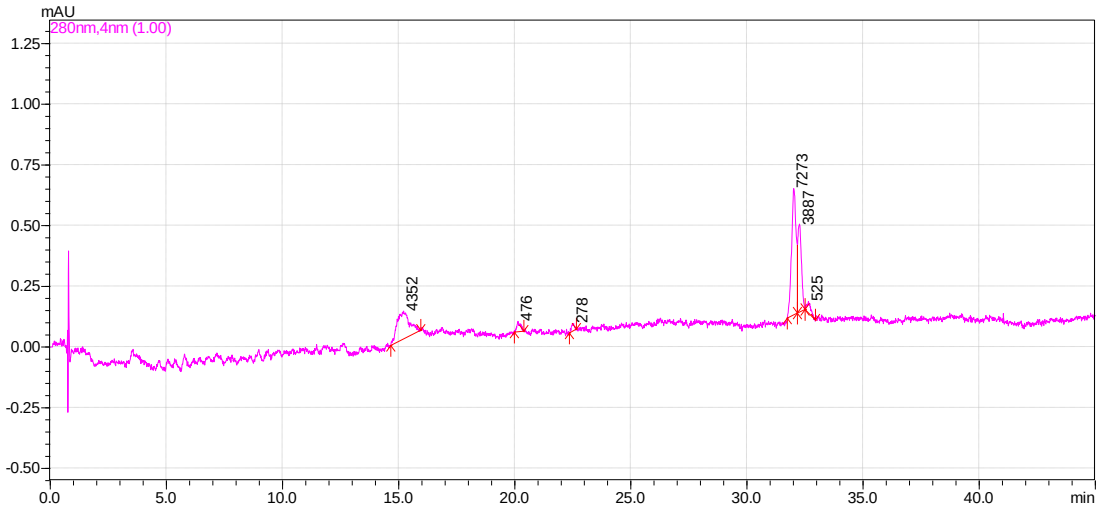



**Figure D4**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 0.05 mg/mL Polysorbate 80, after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.

e. GA-Z with Polysorbate 80 (0.1 mg/mL): 0 day

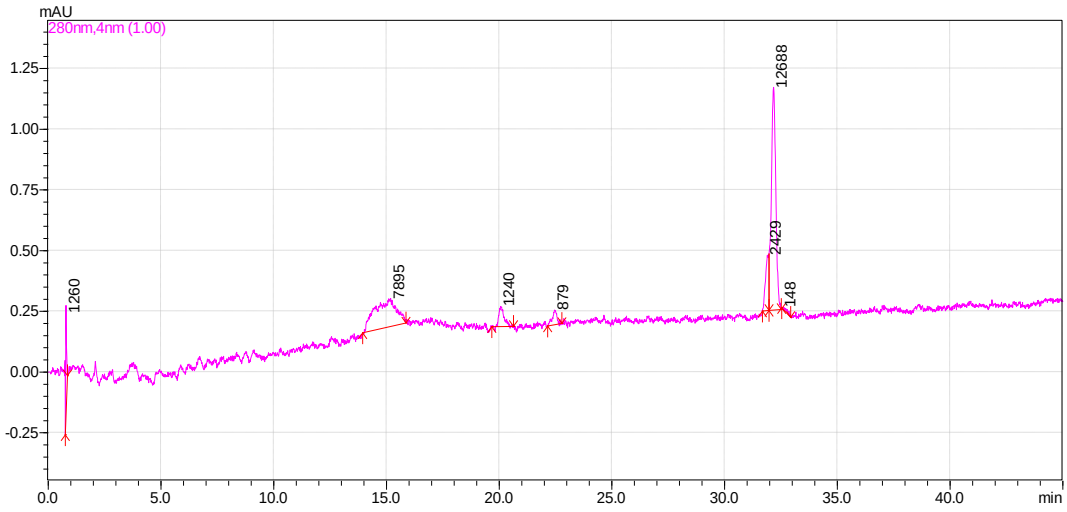



**Figure D5**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 0.1 mg/mL Polysorbate 80, after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

f. GA-Z with Polysorbate 80 (0.1 mg/mL): 10 days

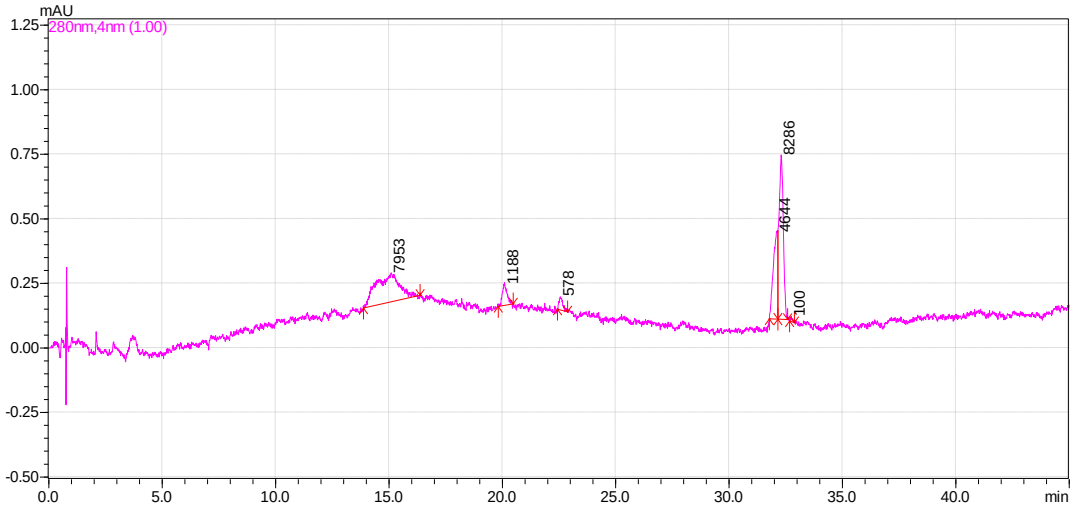



**Figure D6**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 0.1 mg/mL Polysorbate 80, after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

g. GA-Z with Polysorbate 80 (0.1 mg/mL): 20 days

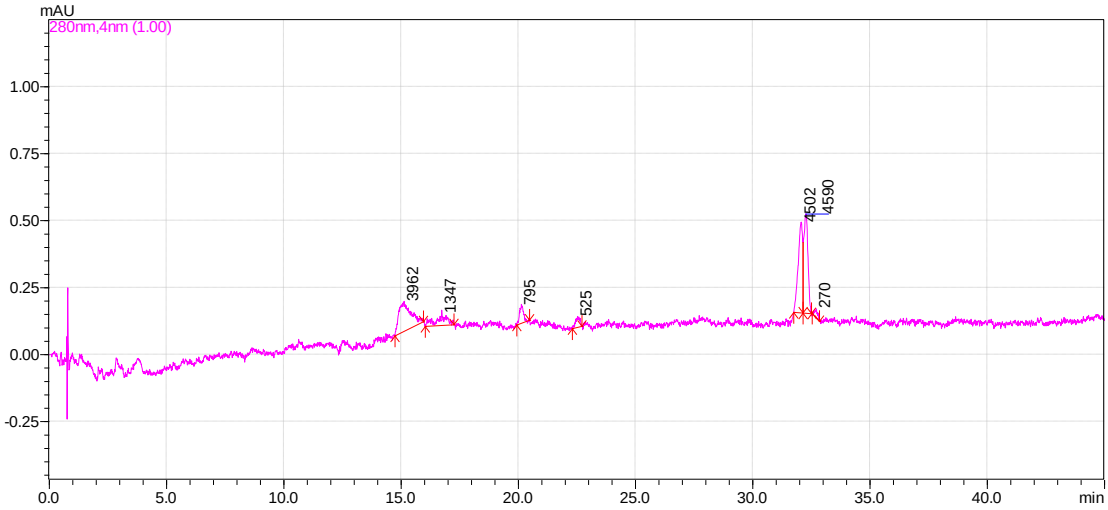



**Figure D7**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 0.1 mg/mL Polysorbate 80, after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

h. GA-Z with Polysorbate 80 (0.1 mg/mL): 30 days

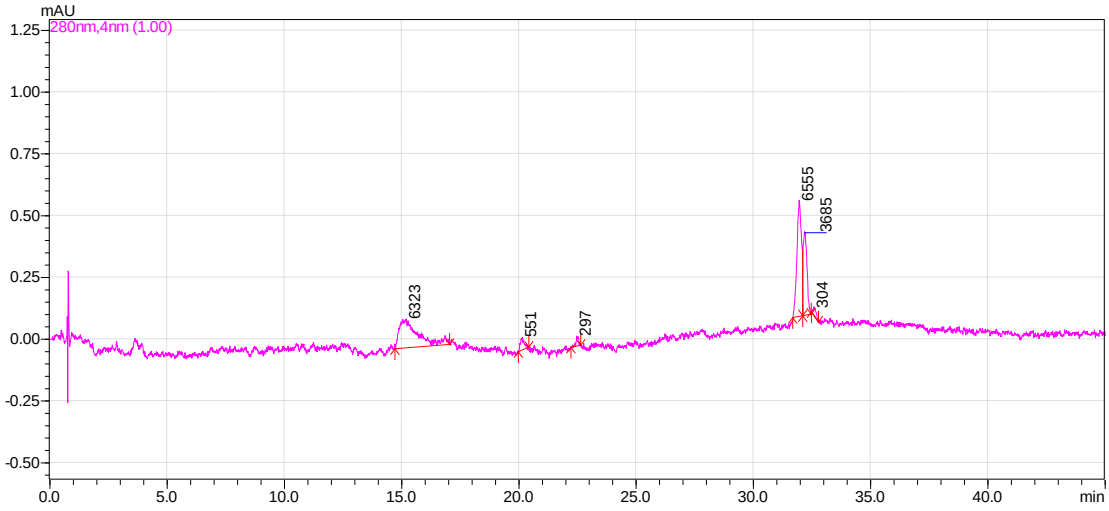



**Figure D8**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 0.1 mg/mL Polysorbate 80, after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.

i. GA-Z with Polysorbate 80 (1 mg/mL): 0 day

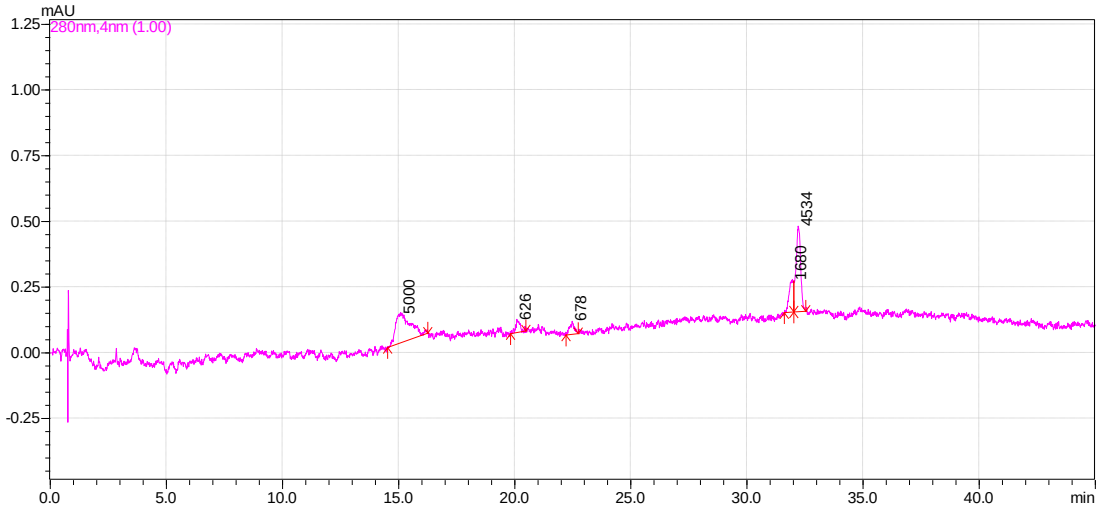



**Figure D9**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 1 mg/mL Polysorbate 80, after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

j. GA-Z with Polysorbate 80 (1 mg/mL): 10 days

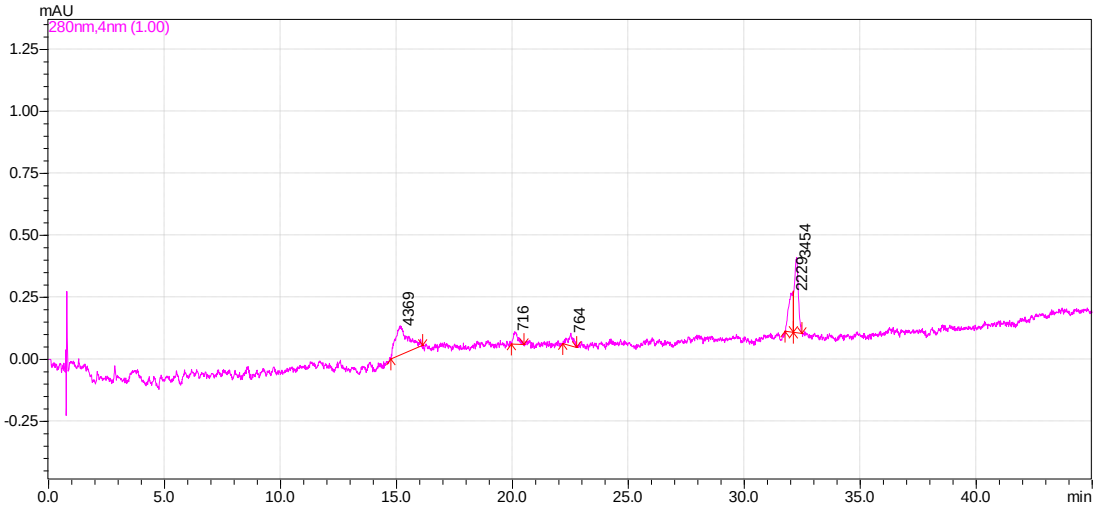



**Figure D10**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 1 mg/mL Polysorbate 80, after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

k. GA-Z with Polysorbate 80 (1 mg/mL): 20 days

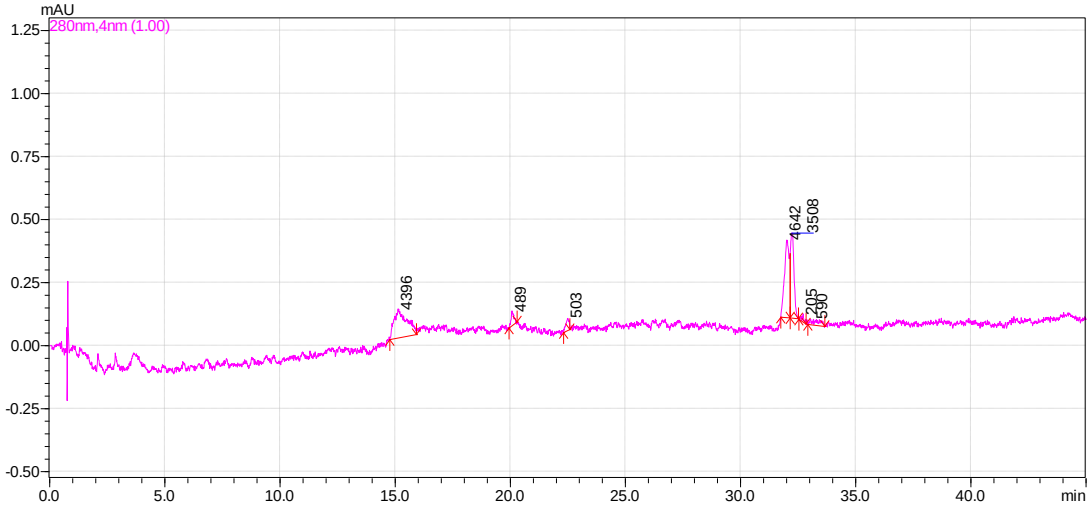



**Figure D11**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 1 mg/mL Polysorbate 80, after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

l. GA-Z with Polysorbate 80 (1 mg/mL): 30 days

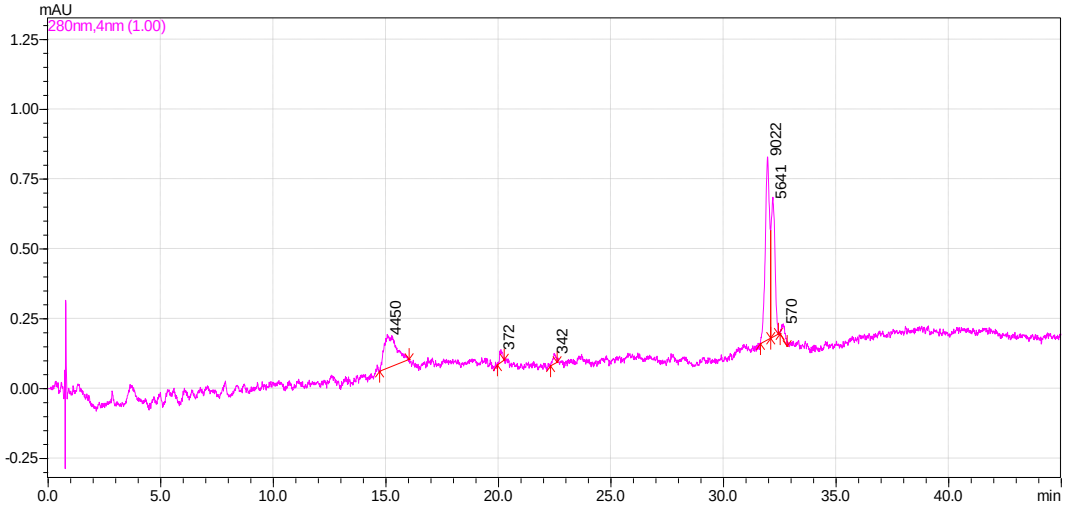



**Figure D12**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 1 mg/mL Polysorbate 80, after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.

5. GA-Z with PEG600
   a. GA-Z with PEG600 (20%): 0 day

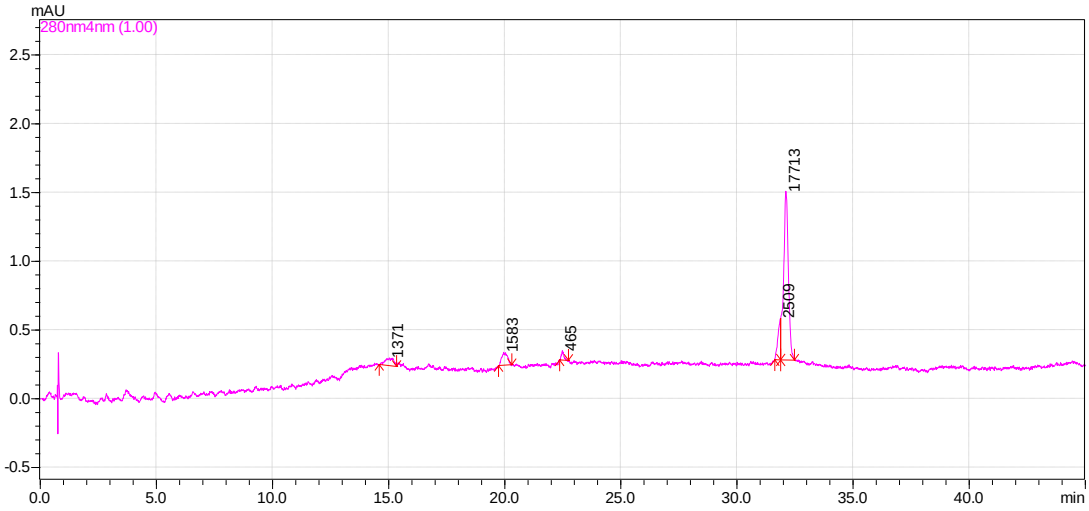



**Figure E1**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 20% PEG600, after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

   b. GA-Z with PEG600 (20%): 10 days

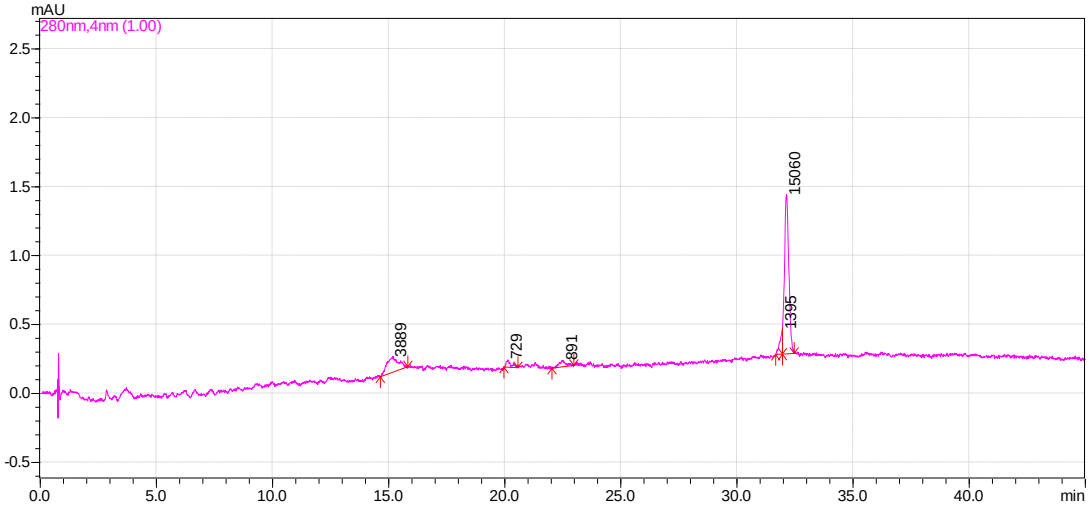



**Figure E2**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 20% PEG600, after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

c. GA-Z with PEG600 (20%): 20 days

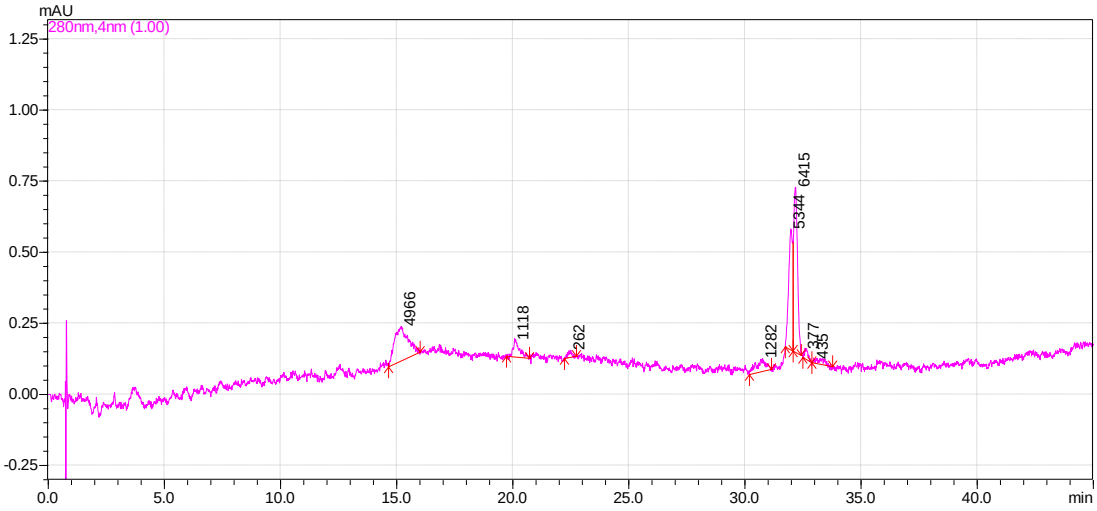



**Figure E3**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 20% PEG600, after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

d. GA-Z with PEG600 (20%): 30 days

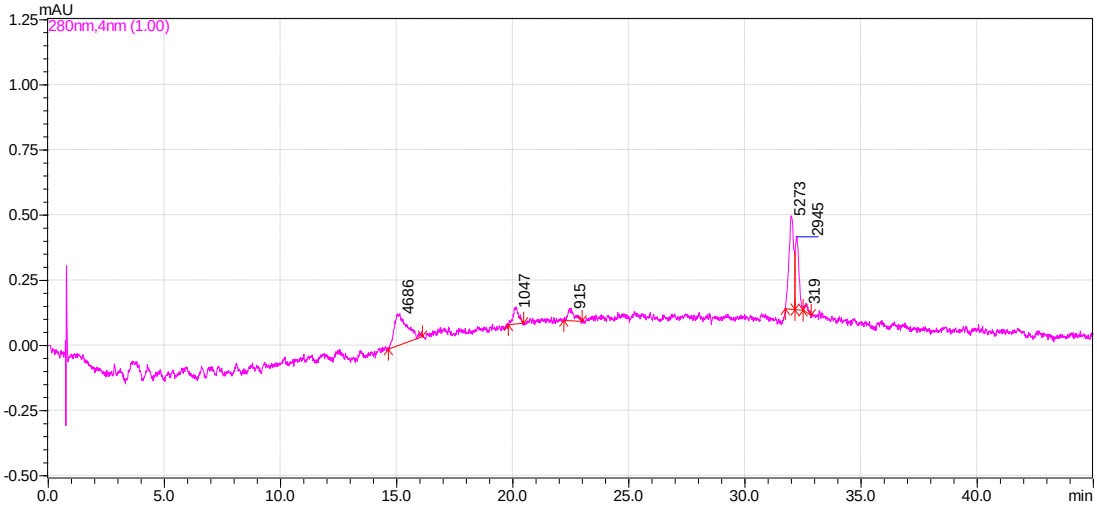



**Figure E4**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 20% PEG600, after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.

e. GA-Z with PEG600 (40%): 0 day

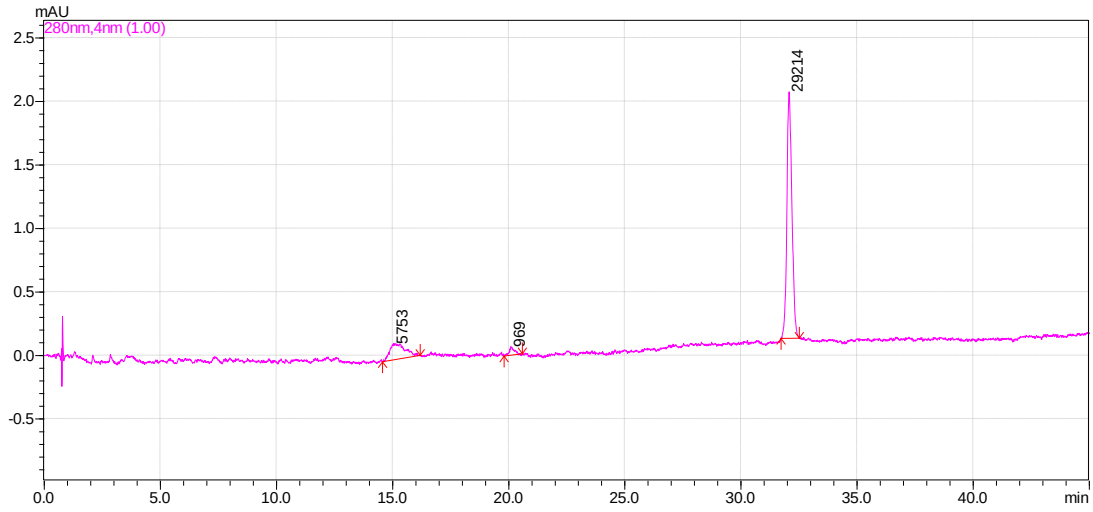



**Figure E5**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 40% PEG600, after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

f. GA-Z with PEG600 (40%): 10 days

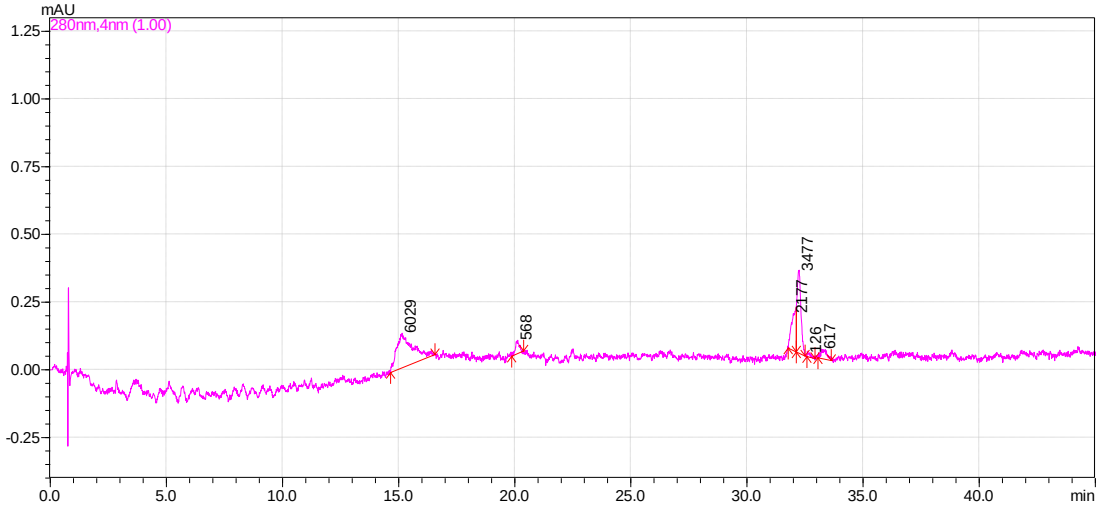



**Figure E6**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 40% PEG600, after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

g. GA-Z with PEG600 (40%): 20 days

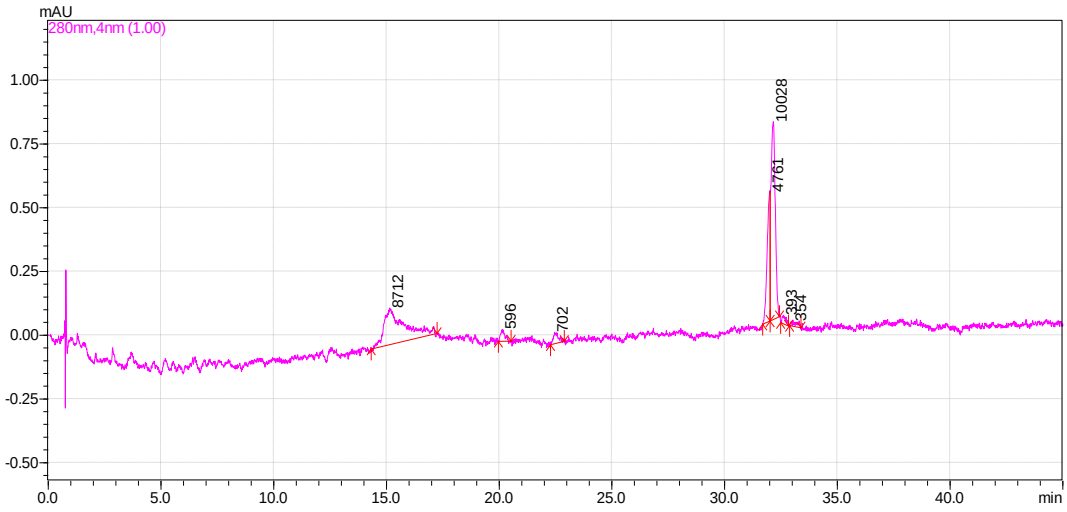



**Figure E7**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 40% PEG600, after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

h. GA-Z with PEG600 (40%): 30 days

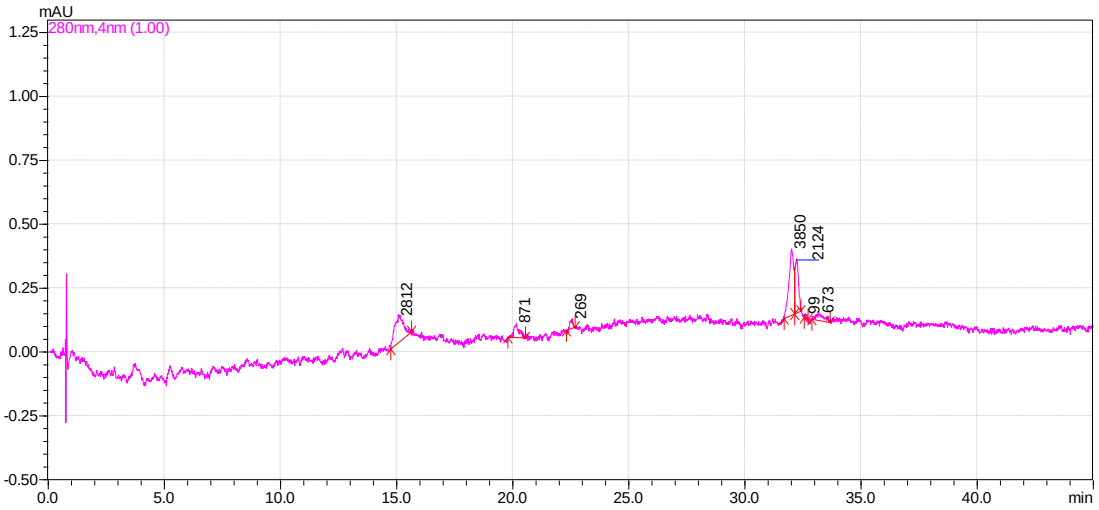



**Figure E8**. Degradation of GA-Z sample (9 mg/mL GA-Z, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 40% PEG600, after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.

6. Somatropin blank

a. Somatropin blank: 0 days

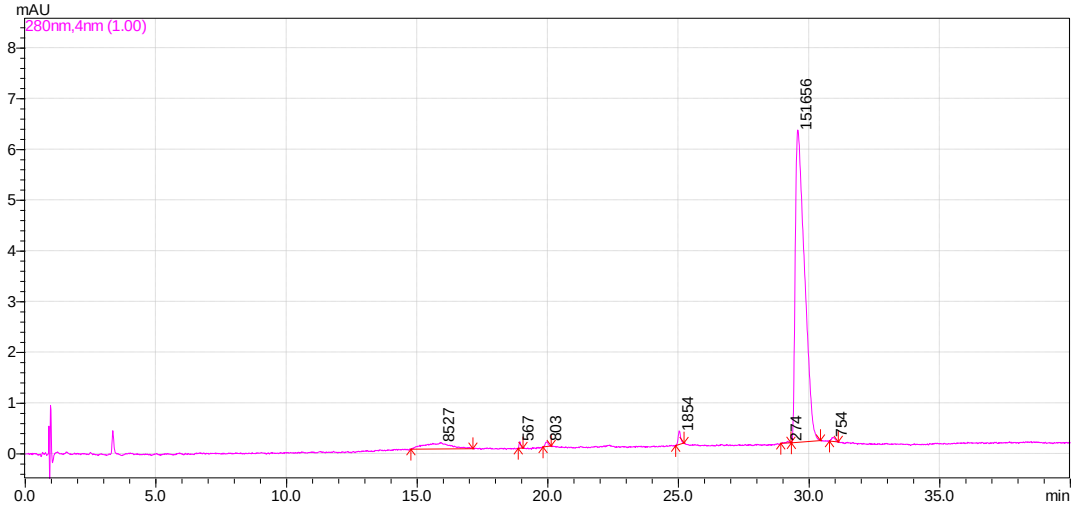



**Figure F1**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

b. Somatropin blank: 10 days

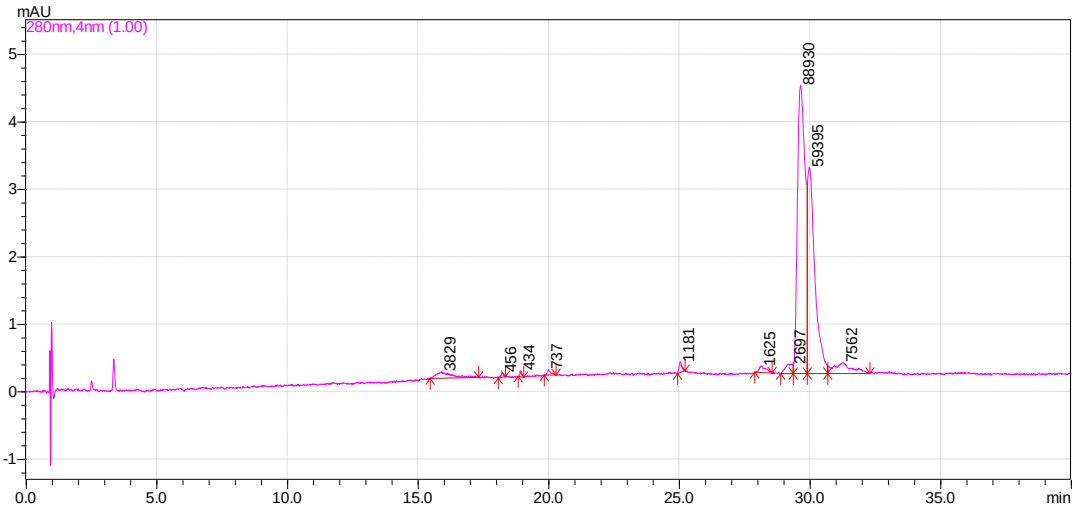



**Figure F2**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

c. Somatropin blank: 20 days

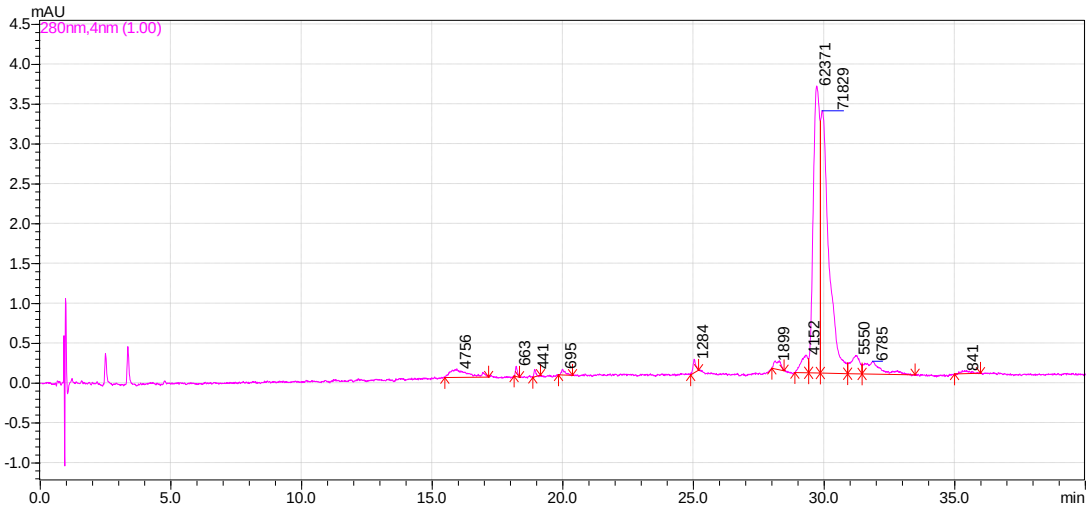



**Figure F3**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

d. Somatropin blank: 30 days

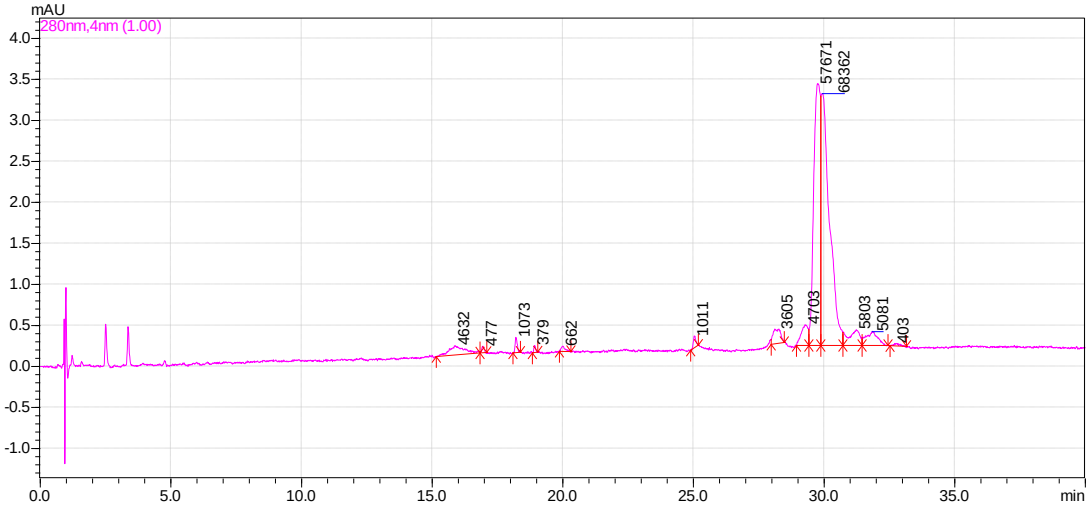



**Figure F4**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0), after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.

7. Somatropin with Glycerol

a. Somatropin with Glycerol (10%): 0 day

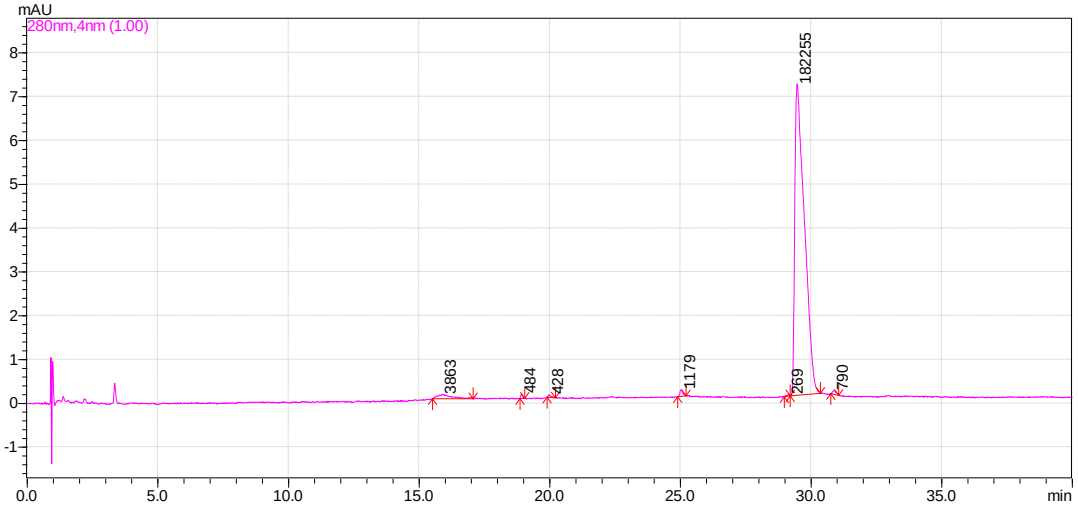



**Figure G1**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 10% glycerol, after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

b. Somatropin with Glycerol (10%): 10 days

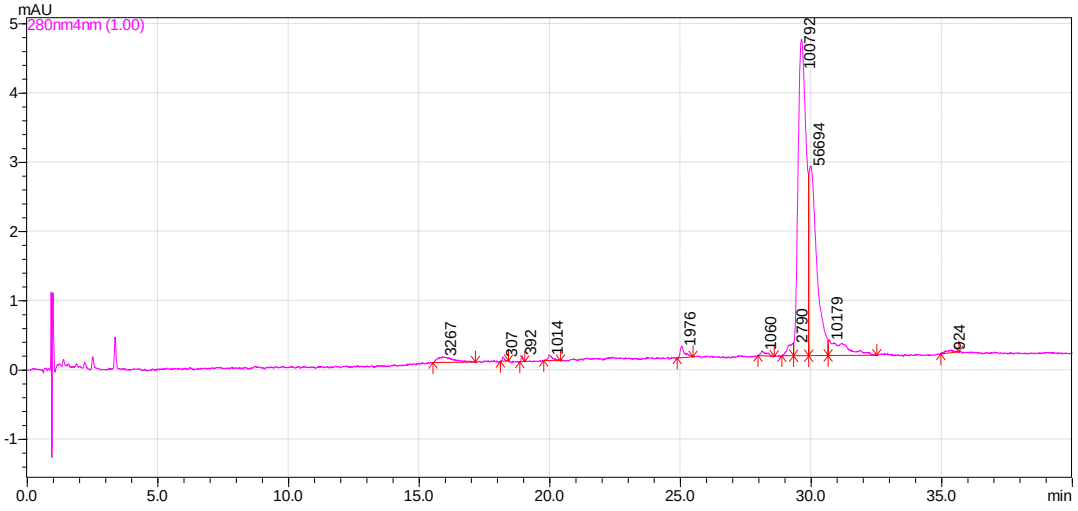



**Figure G2**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 10% glycerol, after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

c. Somatropin with Glycerol (10%): 20 days

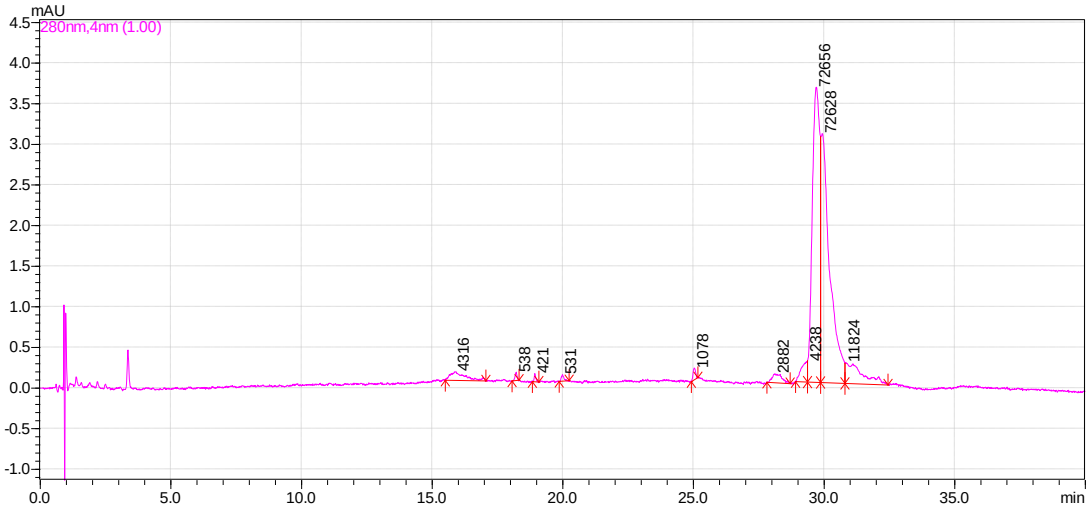



**Figure G3**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 10% glycerol, after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

d. Somatropin with Glycerol (10%): 30 days

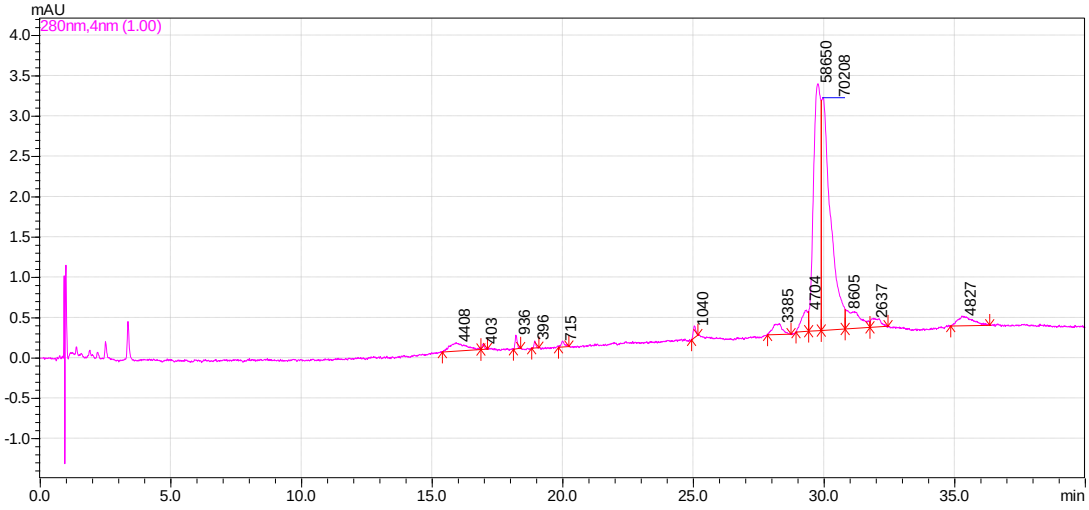



**Figure G4**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 10% glycerol, after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.

e. Somatropin with Glycerol (30%): 0 day

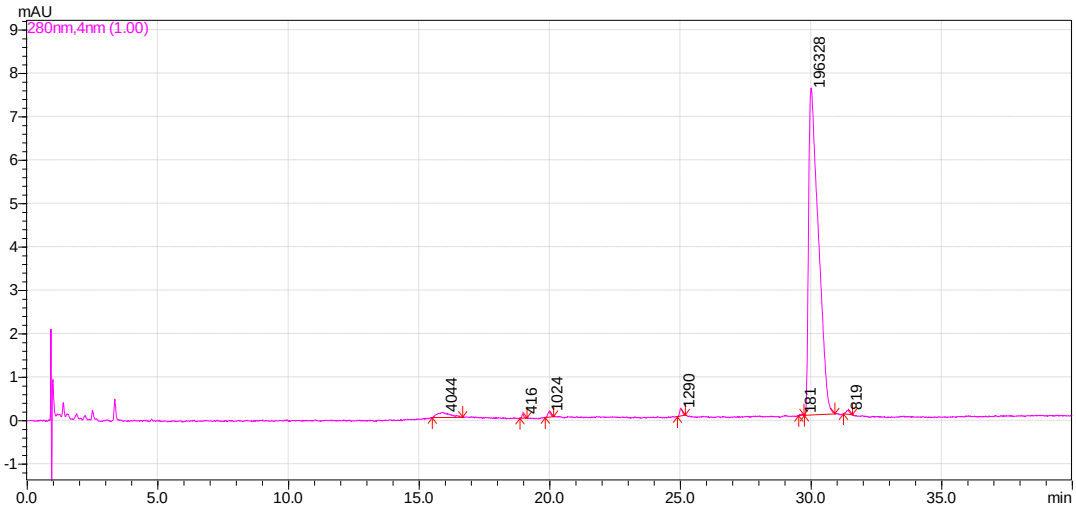



**Figure G5**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 30% glycerol, after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

f. Somatropin with Glycerol (30%): 10 days

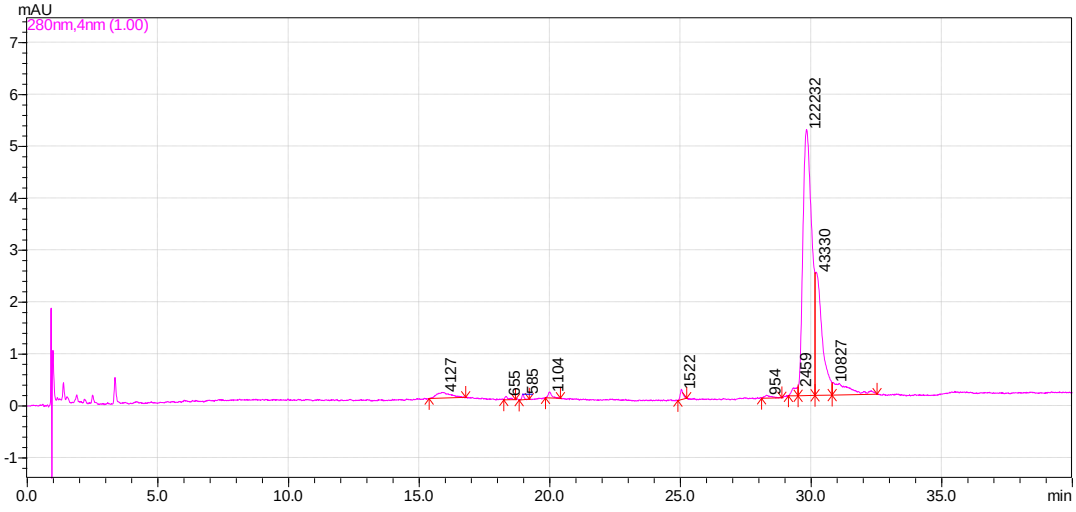



**Figure G6**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 30% glycerol, after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

g. Somatropin with Glycerol (30%): 20 days

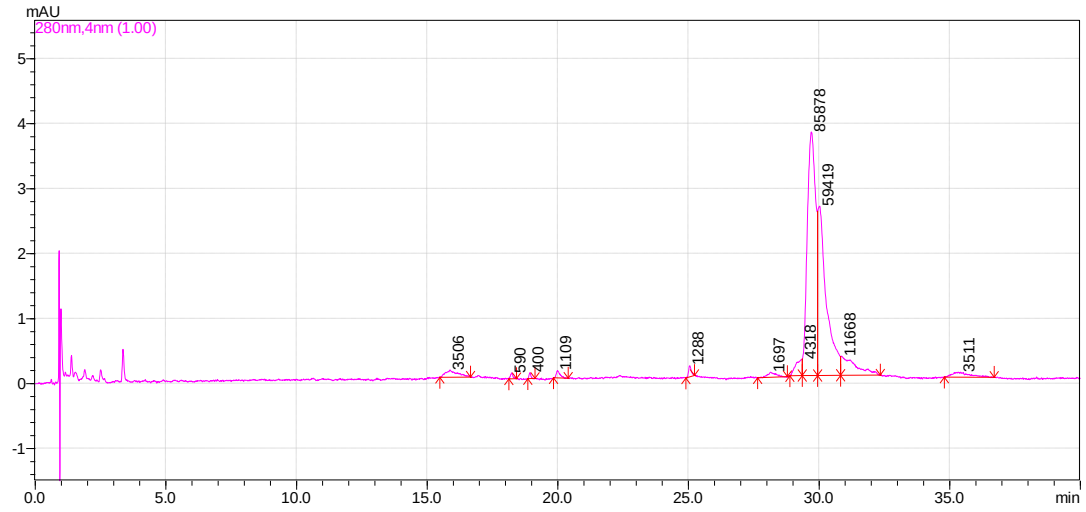



**Figure G7**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 30% glycerol, after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

h. Somatropin with Glycerol (30%): 30 days

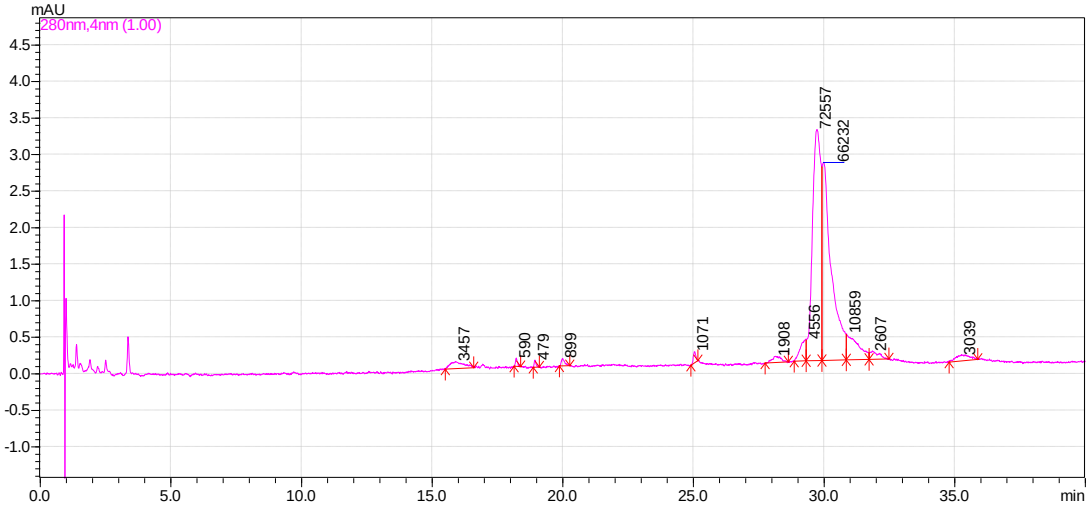



**Figure G8**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 30% glycerol, after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.

i. Somatropin with Glycerol (50%): 0 day

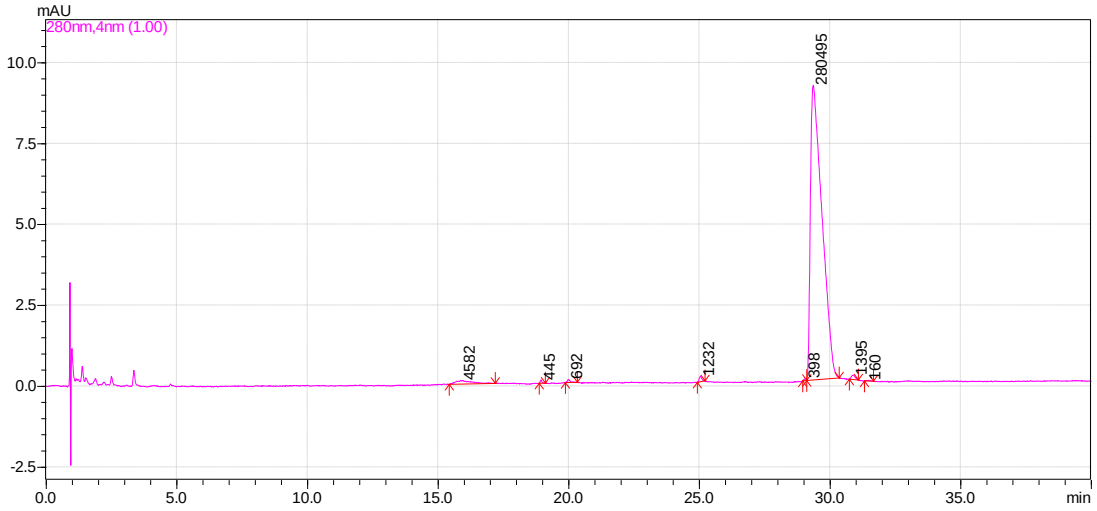



**Figure G9**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 50% glycerol, after incubation at 37°C for 0 days, as detected using LC-UV at 280 nm.

j. Somatropin with Glycerol (50%): 10 days

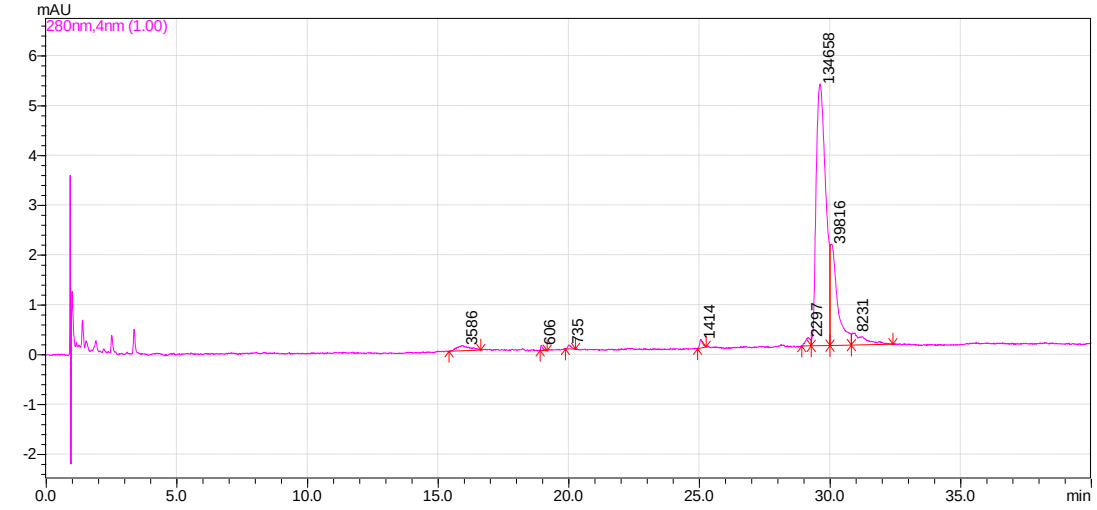



**Figure G10**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 50% glycerol, after incubation at 37°C for 10 days, as detected using LC-UV at 280 nm.

k. Somatropin with Glycerol (50%): 20 days

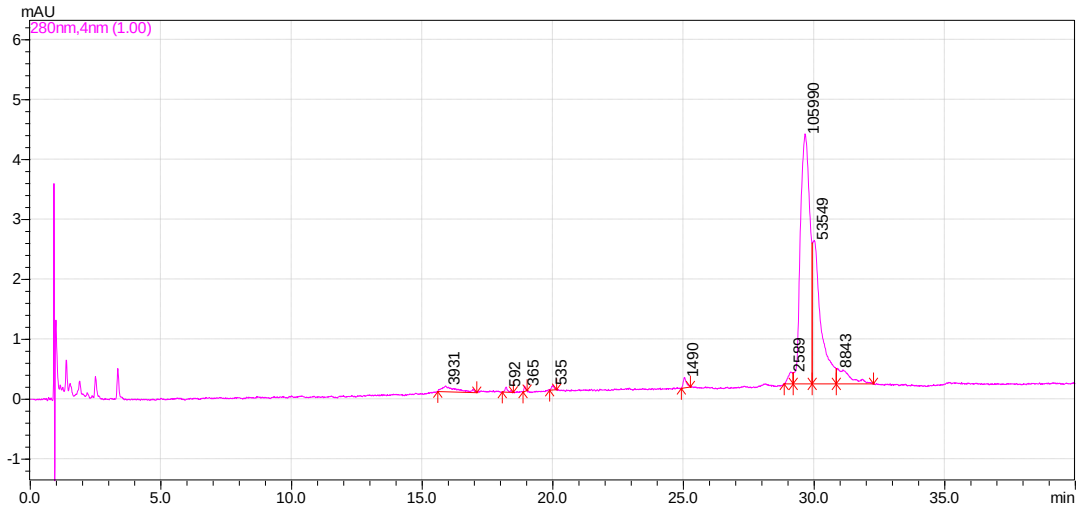



**Figure G11**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 50% glycerol, after incubation at 37°C for 20 days, as detected using LC-UV at 280 nm.

l. Somatropin with Glycerol (50%): 30 days

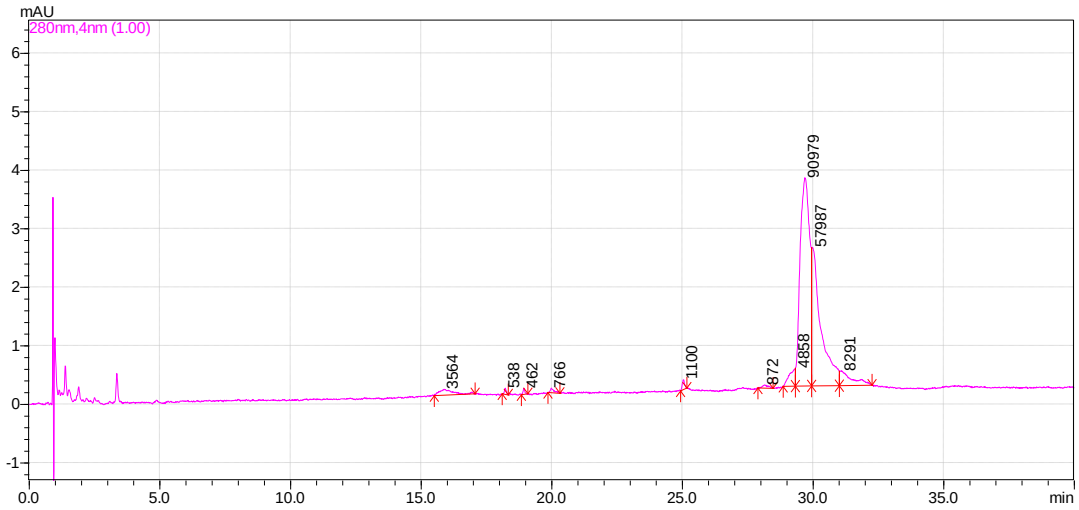



**Figure G12**. Degradation of Somatropin sample (9 mg/mL somatropin, 125 mM NaCl, 25 mM phosphate buffer, pH 7.0) and 50% glycerol, after incubation at 37°C for 30 days, as detected using LC-UV at 280 nm.