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Influence of pH, buffer species, and storage temperature on physicochemical stability of a humanized monoclonal antibody LA298

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Abstract

The purpose of this work is to study the effect of pH, buffer species, and temperature on the physicochemical stability of a humanized monoclonal antibody LA298. The study was carried out in solution state of the antibody in the presence of different buffer species at different pH values and storage temperature. No significant changes in total protein content were observed for any of the solutions with different buffers at different pH values when stored for 8 weeks at both 5 °C and 25 °C or at 37 °C for 1 week. Known asparagines (Asn55) deamidation of LA298 was found to be dependent on pH, buffer type, and temperature. The estimated rate constant of the double heavy chain Asn55 deamidation in phosphate buffer at pH 6.5 and 7.0 was much higher than that in citrate buffer under the same storage conditions. However, comparable results were obtained for single heavy chain Asn55 deamidation in citrate and phosphate buffer. Aggregation of LA298 was not significant for samples at different pH values, buffers, and temperatures as the monomer of LA298 decreased dramatically over time. Less decrease in monomeric LA298 was observed in citrate buffer, pH 5.0–5.5. In conclusion, to minimize deamidation and loss of LA298 monomer, it is important to optimize its solution pH, buffer species, and storage temperature.

Keywords: Humanized monoclonal antibody; Degradation; Stability; Protein; HPLC; Deamidation; Solution; Preformulation; Protein aggregation

1. Introduction

Monoclonal antibodies (mAbs) have a long history of use as diagnostic and research agents. Recently, mAbs have been developed for therapeutic use in a range of diseases most notably inflammatory and autoimmune diseases as well as cancer therapy (Eccles, 1991; Bright et al., 2003). Some mAb therapy, especially humanized antibodies, has emerged in the marketplace. Examples include Synagis for RSV infection, Zenapax for transplantation rejection, Anakinra and Enbrel for rheumatoid arthritis, and Herceptin for breast cancer (Jiang et al., 2000; Cunnane et al., 2001; Arend and Dayer, 1995). Humanized antibodies have several potential advantages over non-human and chimeric antibodies for use in human therapy, including less antibody response from the human immune system, a longer in vivo half-life in the circulation, and better interaction with the other parts of the human immune system because the effector portion is the same as that of humans.

LA298 is an analog of the humanized monoclonal IgG1 antibody Hu007 expressed from a Chinese hamster ovary (CHO) and intended for human therapy. The transfection was supported with vectors developed at PDL encoding the genomic form of the humanized antibody. LA298 like any other antibody is a large, Y-shaped protein molecule composed of two light chains and two heavy chains connected by disulfide bonds. The C-terminal lysines of the heavy chain are susceptible to cleavage. Many proteins are more complex than typical small molecule pharmaceuticals and have been known to undergo many chemical reactions such as deamidation, aspartate isomerization, oxidation, and peptide bond hydrolysis (Arakawa et al., 2001). Physical instability, such as aggregation and conformational change, is expected to be the important degradation pathway of proteins (Henson et al., 1970). From early development work, it was determined that LA298 was prone to chemical degradation soon after manufacture. One mechanism was identified as asparagine (Asn55) deamidation. Therefore, thorough characterization of the chemical and physical stability of the antibody was essen-

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tial for the design of a stable formulation to achieve the desired efficacy and shelf life. In this study, the primary aim was to investigate the effect of pH, buffer species, and storage temperature on the physicochemical stability of LA298 including Asn55 deamidation, aggregation, and change in protein content.

2. Experimental

2.1. Materials

LA298 was a fully human monoclonal IgG1 antibody provided by Eli Lilly and Company (Indianapolis, IN). The antibody has a molecular weight of approximately 148.9 kDa with a p*I* range of 8.1–8.8. All chemicals including tartaric acid, dibasic sodium phosphate, sodium acetate, succinic acid, sucrose, sodium chloride, and polysorbate 80 were of USP/NF or ACS analytical reagent grade and from commercial sources such as Sigma Chemical Company (St. Louis, MO). USP water for injection from Eli Lilly and Company was used to prepare all solutions.

2.2. Solution stability study

Concentrated bulk solution containing 150 mM NaCl were diluted with different pH and buffer species to yield approximately 0.5 mg/mL antibody concentration with the buffer molarity of 10 mM. The resultant solutions were filtered through a 0.22 μm Millex-GV filter membrane into 1.2 mL corning cryotubes. The solutions were stored at 5 °C, 25 °C, and 37 °C and pulled out for analysis at initial, 24 h, 1 week, 4 weeks, 6 weeks, and 8 weeks, respectively. The analytical assays included total protein content by UV, monomer or aggregation polymer by size-exclusion (SEC)-HPLC, and deamidation impurities by cation-exchange (CEX)-HPLC.

2.3. Analytical characterization

2.3.1. UV–vis spectrophotometer

Protein concentration was determined spectrophotometrically using $A_{280,\,1\,mg/mL,\,1\,cm} = 1.52$ mL/(mg cm). Sufficient water for injection (WFI) was added to the sample to adjust the antibody concentration to a range between 0.3 mg/mL and 1 mg/mL. The light scattering correction was calculated by determining the absorbance at 320 nm. The sample protein content was determined based on light scatter-correct absorbance at the maximum wavelength, the extinction coefficient, and any applicable dilution factor.

2.3.2. SEC-HPLC

Aggregation of LA298 was analyzed by isocratic size-exclusion HPLC with UV detection. The method employed a TSK-GEL G3000SW$_{XL}$ column (7.8 mm × 300 mm, 5 μm particles, TosoHaas). The mobile phase contained 150 mM NaCl in 50 mM phosphate at pH 7.0. The flow rate was 0.5 mL/min and detection was at 214 nm. Mass load of the antibody ranged from 8 μg to 16 μg with an injection volume of 20 μL. The percentage of each individual peak (monomer or polymer) in total protein peak area was calculated and reported. The integrated HPLC peak area of monomer was plotted versus time, and the rate constant (k) for monomer degradation was obtained by linear regression analysis.

2.3.3. CEX-HPLC

Analysis of impurities from Asn deamidation of LA298 was performed by gradient CEX-HPLC with UV detection. The method employed a ProPac WCX column (4.0 mm × 250 mm, 10 μm particles, Dionex). Mobile phase A contained 20 mM phosphate at pH 6.3, and mobile phase B contained 250 mM NaCl in 20 mM phosphate at pH 6.3. The flow rate was 1.0 mL/min and detection was at 214 nm. The sample was prepared into mobile phase A. The column started eluting with 15% of mobile phase B for 5 min. Then, mobile phase B was linearly increased to 32% in 50 min and remained in the composition for 15 min. Lastly, mobile phase B increased to 100% from 32% in 3 min and remained for 5 min followed by reduction of the mobile phase B to 15% in 3 min. The total run time was 80 min. Mass load of the antibody ranged from 8.5 μg to 30 μg with an injection volume of 25 μL. The percentage of each individual peak in total protein peak area was calculated and reported. The rate constant (k) for single or double Asn55 deamidation was obtained by linear regression analysis of the peak growth–time curve.

3. Results and discussion

3.1. Effect of pH and temperatures on total protein content

Total protein content of the samples was determined using a UV method. Usually, a loss of total protein content may result from the formation of insoluble aggregates and/or precipitation. As shown in Fig. 1, no significant changes in the protein content were observed for the solutions in citrate buffer at different pH when stored for 8 weeks at both 5 °C and 25 °C or at 37 °C for 1 week. Similar results were observed for tartrate at pH 4.0 and 4.5, succinate at pH 5.0 and 6.0, and phosphate at pH 6.5 and 7.0. These data indicated that LA298 in solution with different buffer

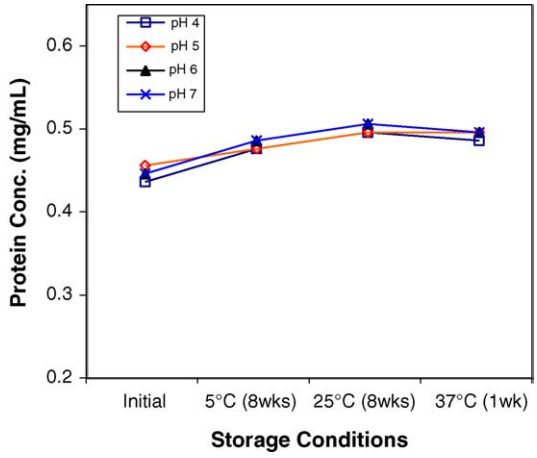

Fig. 1. Effect of citrate buffer on total protein content under different storage conditions.

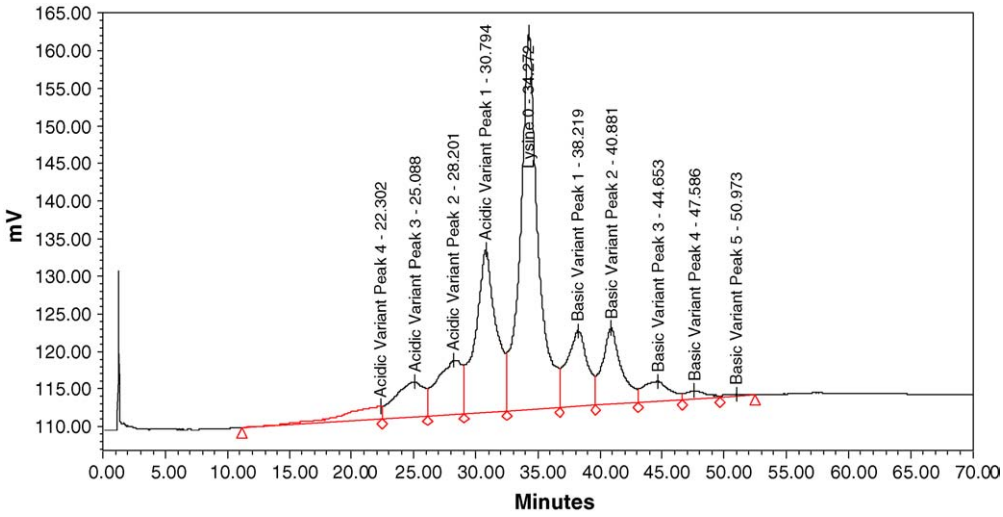

Fig. 2. Elution of a LA298 sample from a cation-exchange HPLC column detected at 214 nm. Acidic variant peak 1: Asn55 single deamidation in single heavy chain; acid variant peak 2: Asn55 double deamidation in both heavy chains.

species at pH 4.0–7.0 should be stable under storage conditions of 5 °C and 25 °C in terms of protein content. Even at 37 °C, no significant loss of protein was observed at 1 week at pH between 4.0 and 7.0. However, it appears that the protein concentration slightly increased after storage. One possibility for this increase may be due to a change in protein conformation; however, using circular dichroism spectral analysis, no change in spectra was observed between initial and aged samples (data not shown). The data suggested no significant changes in the secondary and tertiary structure of the antibody. Nevertheless, it cannot be ignored that a slight change in protein folding could lead to an increase in UV absorbance, and thus, increases in the protein concentration. Overall, the data suggested that a remarkable loss of total protein content due to precipitation and/or formation of insoluble aggregates would not occur during the storage of LA298.

3.2. Effect of pH, buffer, and temperatures on deamidation of LA298

Deamidation appears to be the most common degradation pathway for protein pharmaceuticals. The deamidation of Asn and glutamine (Gln) side chains are among the most widely studied non-enzymatic covalent modifications to proteins and peptides. Deamidation of the Asn55 residue in the heavy chains has been found for LA298 (unpublished data). The deamidation could happen in one heavy chain, i.e., single deamidation, or both heavy chains, i.e., double deamidation. Deamidation of LA298 could be analyzed using a CEX-HPLC method. A typical chromatogram is shown in Fig. 2. Acidic variant peak 2 was the Asn55 deamidation peak of both heavy chains while acidic variant peak 1 was the Asn55 deamidation peak of only one heavy chain.

Formation of deamidated Asn55 antibody in citrate buffer at 25 °C is shown in Fig. 3. In the initial LA298 samples, approximately 19% of relative single deamidation peak area and 7% of double deamidation peak area were determined. During storage, both single and double deamidated peaks went up significantly with increasing pH values from 4 to 7 at 25 °C. Similar deamidation profiles of LA298 were also obtained for citrate buffer at 5 °C and 37 °C. However, the deamidation in 5 °C was much slower than that at 25 °C and 37 °C (data not shown). The deamidation rate constants were calculated with a good approximation and used for assessment of deamidation of LA298 under differ-

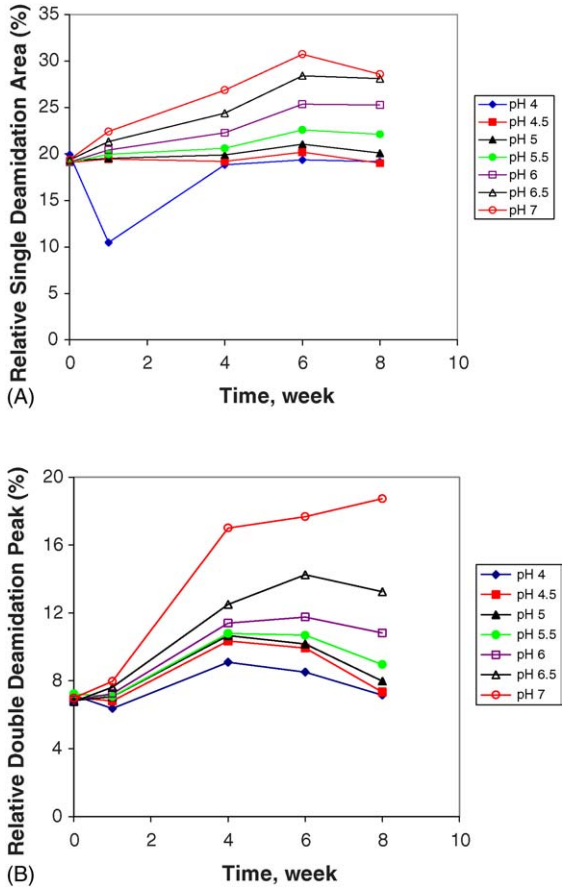

Fig. 3. Asn55 deamidation of LA298 in citrate buffer at 25 °C: (A) single heavy chain deamidation and (B) double heavy chain deamidation.

Table 1
Effect of pH, buffer species, and storage temperature on Asn55 deamidation of LA298

pH	Buffer	Rate constant, k (mg/(mL m))			
		Double deamidation		Single deamidation	
		25 °C	37 °C	25 °C	37 °C
4.0	Citrate	0.56	−8.74	−0.34	−11.66
4.5	Citrate	0.86	−1.97	0.13	−0.99
5.0	Citrate	1.16	−3.21	0.64	0.04
5.5	Citrate	1.54	3.47	1.76	7.63
6.0	Citrate	2.61	9.73	3.47	18.73
6.5	Citrate	4.07	15.13	4.97	26.74
7.0	Citrate	6.90	21.13	5.40	38.61
4.0	Tartrate	0.60	−2.01	0.21	−4.29
4.5	Tartrate	1.24	2.74	0.00	−3.09
5.0	Succinate	1.33	4.11	0.51	−0.17
5.5	Succinate	3.00	6.64	2.14	5.06
6.0	Succinate	2.79	6.94	2.83	10.89
6.5	Phosphate	7.80	31.80	3.47	25.11
7.0	Phosphate	9.26	59.14	−2.57	2.83

ent storage conditions. The deamidation rate of LA298 in citrate buffer at different pH and storage temperatures is summarized in Table 1. In citrate buffer, the single and double deamidation at 25 °C increases as pH increases from 4.0 to 7.0. At pH 7, the calculated rate of the deamidation at 25 °C reached 6.90 mg/(mL m) for double deamidation and 5.40 mg/(mL m) for single deamidation, respectively. The pH dependence of the growth in the degradation peaks also suggested deamidation as the source of the formation of the five-membered ring succinimide intermediate. Subsequent hydrolysis of the intermediate is favored under basic conditions (Geiger and Clarke, 1987; Stephenson and Clarke, 1989). At 37 °C, the rates at lower pH are negative indicating that some other mechanism of degradation is occurring at the higher temperatures. At pH 7, the increase of the deamidation rate constant at 37 °C is remarkable with rates of 21.13 mg/(mL m) for double deamidation and 38.61 mg/(mL m) for single deamidation (Table 1). Hence, Asn55 deamidation of LA298 can be significantly minimized in the citrate buffer at a pH less than 5 and at lower storage temperatures.

In tartrate or succinate buffers, the deamidation rates of LA298 at 25 °C and 37 °C were similar to that in citrate buffer as discussed above (Table 1). However, in phosphate buffer at pH 6.5 and 7.0, the rates of LA298 deamidation at 25 °C and 37 °C were different than in the citrate buffer. At 25 °C, the double deamidation rates at pH 6.5 and 7.0 were 7.80 mg/(mL m) and 9.26 mg/(mL m), respectively, which is two times higher than the rates observed for the citrate buffer. When increasing the storage temperature to 37 °C, the double deamidation rates of LA298 in phosphate buffer were found to be as high as 31.80 mg/(mL m) at pH 6.5 and 59.14 mg/(mL m) at pH 7 (Table 1). It was noted that single deamidation at higher temperatures in phosphate buffer seems different from that in citrate buffer. The rate constant of single deamidation at pH 6.5 in phosphate buffer is comparable to that in citrate buffer at both temperatures of 25 °C and 37 °C. However, the rate of single deamidation at pH 7.0 in phosphate buffer is much slower than that in citrate buffer at higher temperatures. Overall, total deamidation of LA298 in phosphate buffer should be higher than that in citrate buffer. From the available data, it is clear that the deamidation of LA298 in an aqueous environment is dependent on pH, buffer, and temperature. Higher temperature and higher pH in an aqueous solution will accelerate the reaction, and also phosphate ions may catalyze the deamidation of LA298. On the other hand, the data suggests that a solution preparation of the antibody might pose a long-term stability problem in terms of deamidation. Buffer catalysis of deamidation has been observed in some model peptides and proteins (Tyler-Cross and Schirch, 1991; Capasso et al., 1991). It has been suggested that phosphate ion could act on the aqueous solvent to increase basicity of water molecules without forming free hydroxide ions (Brennan and Clarke, 1995). However, direct evidence to support this hypothesis has not yet appeared in the literature.

3.3. Effect of pH, buffer, and temperatures on aggregation of LA298

Protein aggregation is another common and troubling manifestation of protein instability which may be induced by a variety of physical factors, such as temperature, ionic strength, vortexing, surface/interface adsorption, or simply time. Protein molecules may aggregate simply by physical association with one another without any changes in primary structure (physical aggregation) or by formation of a new covalent bond (chemical aggregation), resulting to formation of either soluble or insoluble aggregates (Wang, 2005). Protein aggregates may have no or reduced biological activity, reduced solubility, and altered immunogenicity. As discussed previously, insoluble aggregates of LA298 formed under different storage conditions appear negligible since no significant loss of total protein content was observed (Fig. 1). For soluble aggregates or polymers from protein aggregation, quantitative assessment has been performed using SEC-HPLC. A typical chromatogram of LA298 from SEC-HPLC is shown in Fig. 4. Both polymer and monomer peaks of LA298 were eluted from the column according to their molecular size. There were several unknown peaks following the monomer peak.

The aggregation peak from LA298 bulk solution was low, and the estimated value from SEC-HPLC was approximately 0.72–0.87%. In citrate buffer (pH 4–7), the increase in the aggregation peak over time is negligible under all storage conditions tested (data not shown). Similar results were also observed for tartrate (pH 4.0 and 4.5) and succinate (pH 5.0–6.0) buffers. However, in phosphate buffer, an increase in the polymer peak was observed at 5 °C and 25 °C (Fig. 5). As can been seen, the peak in pH 6.5 phosphate buffer increased from 0.84% to 1.64% at 25 °C over 6 weeks and decreased to 0.69% after 8 weeks. The reason for the decreasing polymer level after 8 weeks is not clear. It is possible that the formed polymer is not stable and subjects to further degradation over time. Overall, there is a tendency of increased LA298 aggregation if a phosphate buffer is used.

From SEC-HPLC, the change in the LA298 monomer level was also monitored. In citrate buffer, changes in monomer level

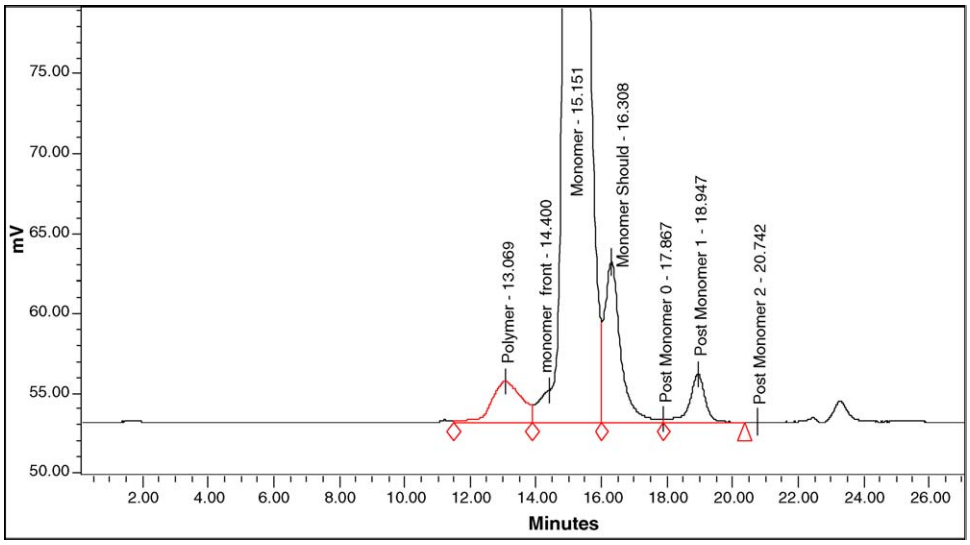

Fig. 4. Elution of a LA298 sample from a size-exclusion HPLC column detected at 214 nm.

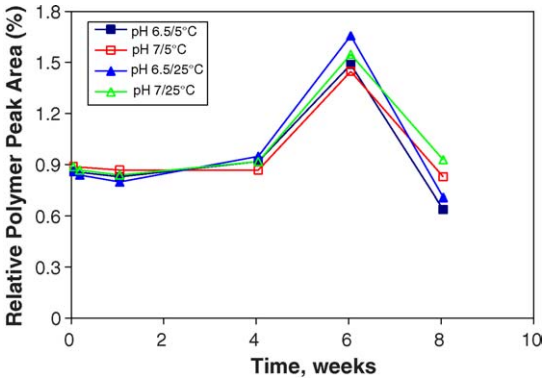

Fig. 5. Formation of soluble aggregates in phosphate buffers at 5 °C and 25 °C.

were negligible if the solution pH was between 4 and 7.0 at 5 °C (data not shown). However, at 25 °C, the amount of monomeric LA298 decreased with time (Fig. 6) especially at pH 4.0 and 7.0. At these pH values, greater than 11% of monomer decreased over 56 days when compared with the initial. The rate constants for degradation of LA298 monomer were 5.87 mg/(mL m) at pH 4.0 and 7.46 mg/(mL m) at pH 7.0, respectively (Table 2).

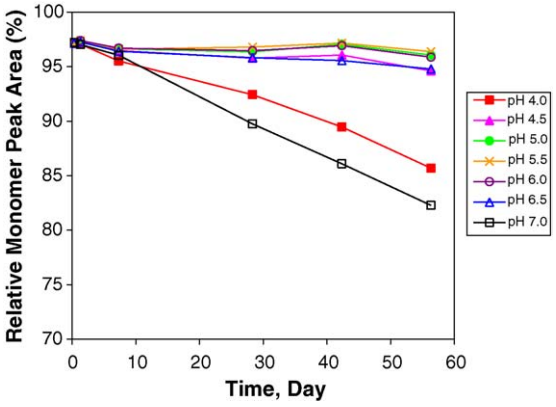

Fig. 6. Decrease of monomeric LA298 in citrate buffers at 25 °C.

It appears that citrate buffer at pH 5.0 and 5.5 displayed the least decrease in monomeric antibody with a rate constant less than 0.21 mg/(mL m). As discussed previously, no increase in polymer was seen under all conditions so that the decrease in monomer is mostly due to fragmentation. The increase in fragments can be seen as an increase in smaller molecular weight species by SEC chromatography.

In tartrate and succinate buffer, the effect of pH on LA298 monomer is comparable to that in citrate buffer (Table 2). However, phosphate buffer showed a significant impact on intact LA298 monomer even when stored at 25 °C. At pH 7, the amount of LA298 monomer in phosphate buffer decreased at a rate of 10.46 mg/(mL m) (Table 2). Therefore, at a given temperature, LA298 monomer decreased with increasing pH of the solution. In the case of buffer species, LA298 monomer decreased much faster in phosphate buffer than in citrate, tartrate, and succinate at 25 °C. These results suggest that the loss of the monomeric

Table 2
Effect of pH, buffer species, and storage temperature on decrease of LA298 monomer

pH	Buffer	Buffer concentration (mM)	Rate constant at 25 °C, k (mg/(mL m))
4.0	Citrate	10	−5.87
4.5	Citrate	10	−1.07
5.0	Citrate	10	−0.21
5.5	Citrate	10	−0.17
6.0	Citrate	10	−0.43
6.5	Citrate	10	−1.11
7.0	Citrate	10	−7.46
4.0	Tartrate	10	−3.56
4.5	Tartrate	10	−0.73
5.0	Succinate	10	−0.51
5.5	Succinate	10	−0.34
6.0	Succinate	10	−1.11
6.5	Phosphate	10	−5.74
7.0	Phosphate	10	−10.46

antibody into smaller fragments in an aqueous environment is associated with pH, temperature, and buffer species. The antibody is more stable when it is in citrate buffer at pH 5.0 and 5.5 and at lower temperatures.

4. Conclusions

The humanized monoclonal antibody LA298 is not chemically stable. It undergoes significant deamidation during storage. The single and double deamidation rates are dependent on the pH, buffer species, temperature, and storage time. The least amount of deamidation was observed in solution pH less than 5.0. Also, the loss of the monomeric antibody is associated with pH, temperature, and buffer species. The antibody is more stable in citrate buffer at pH of 5.0–5.5 and lower temperature. However, LA298 is physically stable. Significant loss of total protein content and formation of aggregates (insoluble and soluble) were not observed over the time of the test period. To improve the chemical stability of the antibody, careful selection of pH, buffer species, and storage temperature should be considered.

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