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Physical and Structural Stability of the Monoclonal Antibody, Trastuzumab (Herceptin®), Intravenous Solutions

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Abstract: A major limitation of biological therapeutics is their propensity for degradation particularly in aqueous solutions hence resulting in their short shelf-life. In this study, the stability of trastuzumab (Herceptin®) intravenous (i.v.) solutions, an IgG1 monoclonal antibody (mAb), indicated for the treatment of HER2 positive breast cancer, stored under refrigerated conditions, was evaluated over 28 days. No change in visual appearance or average particle size was observed. The pH values of the trastuzumab i.v. solutions remained stable over time. Interestingly, no change in trastuzumab monomer concentration was observed throughout the 28-day study, as determined by SEC-HPLC. SDS-PAGE showed only a monomer band corresponding to the molecular weight of trastuzumab. Circular dichroism spectra obtained following 28-day storage demonstrated integrity of the secondary structural conformation of trastuzumab. Results from this study show that trastuzumab i.v. solutions remain physically and structurally stable on storage at 2-8°C for 28 days. These findings suggest that trastuzumab in solution may not be as sensitive to degradation as expected for a mAb and therefore may have important implications in extending trastuzumab shelf life for clinical use and reducing associated healthcare cost.

Keywords: Trastuzumab, stability, protein aggregation, SEC, particle size, SDS-PAGE, Circular dichroism.

INTRODUCTION

Biological therapeutics are increasingly utilised in the clinical setting due their specificity to the target site, hence leading to lower adverse events and toxicity. In 2008, 21 monoclonal antibodies (mAbs) were approved in the US with over 200 mAb candidates undergoing clinical study [1]. A major disadvantage of biological therapeutics is their susceptibility to rapid physical, chemical and thermal degradation [2, 3], thereby limiting their shelf-life and adding to the cost of therapy. Most of the therapeutic mAbs are commercially available as lyophilised preparations in multi-dose vials to be aseptically reconstituted before use. Once reconstituted, usually it is further diluted under aseptic conditions, to provide titratable individualised doses for patients. These aqueous dilute solutions of proteins are particularly vulnerable to instability from various types of stressors including shear, thermal, and microbiological and hence caution is recommended during their reconstitution and handling to prevent full or partial denaturation and/or aggregation of these molecules.

Aggregation is a major response of mAbs when subjected to stress of any type and can result in significant loss of biological activity or can result in immunogenic reactions which may be life-threatening [4-6]. Misfolding or denaturation of the protein, leading to the formation of aggregates, may also occur upon storage and is accelerated by a number of factors including heating, agitation and packaging materials [7]. In a clinical setting the stability of the mAb solution, before administration to the patients, is checked by visual inspection for discoloration and presence of sediments, aggregates/particulate matter. Traditionally a visibly clear solution is considered a stable product. However Schellekens *et al.* [8] suggest that smaller aggregates which are invisible to the human eye, may be present and as these may be inactive or immunogenic, they are important to characterise.

Thermal and chemical stressors have been shown to disrupt the secondary structure of mAbs with impact on its physical, chemical stability and hence biological activity and toxicity [9-11]. The concentration of the protein and of formulation excipients such as surfactants in the final solution have been shown to influence the stability of the protein. At high concentration of 1.5 mg/mL (10 μM), proteins such as IgG1 type antibody was reported to exhibit a self-stabilizing ability as a result of its surfactant activity [12].

While the lyophilised mAb products generally have a shelf-life of 18-48 months, the manufacturers recommend a shelf-life of only 24-48 hours for the reconstituted and diluted products, which often leads to wastage and has important cost implications limiting access to such treatment. Recently, studies on various mAbs have demonstrated stability beyond their use date. Reconstituted bevacizumab stored in vials and syringes at 4°C showed 9.6% and 8.8% degradation respectively after 3 months [13]. Chen *et al.* [14] reported less than 10% bevacizumab degradation at 6 months when stored in multi-dose vials at 4°C. Moreover, Ikesue *et al.* [15] reported that aseptically diluted Cetuximab and

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Panitumumab solutions at 2 and 2.5 mg/mL were stable for 14 days at 4°C when stored in PVC bags. Similarly, Kupfer *et al.* [16] reported that alemtuzumab stored in infusion bags was stable for 14 days. We previously reported that exposure of trastuzumab i.v. solutions to various shear stressors such as sonication, spray drying or freeze thawing had no effect on the aggregation or its structural integrity and this was consistent with no significant reduction in the biological activity of trastuzumab [17]. Trastuzumab is a recombinant humanised IgG1 monoclonal antibody indicated for the treatment of HER2 positive breast cancer and more recently for the treatment of metastatic HER2 positive gastric cancer [17, 18].

In the present study, trastuzumab was aseptically reconstituted and diluted to 0.4-4 mg/mL according to the manufacturer's instructions and stored under refrigerated conditions of 2-8°C. The effect of storage time on the physical and structural stability of the i.v. solutions was examined. In addition, the influence of shaking and exposure to increasing temperatures on the trastuzumab i.v. solutions was studied in order to understand the sensitivity of the tratsuzumab i.v. solutions to such potential stressors as may be encountered during their handling and transport in practice.

EXPERIMENTAL

For each test, n=6 trastuzumab samples were used, unless otherwise stated. Changes in colour, cloudiness or presence of particulate matter were examined by visual observation. Particle size analysis was carried out using a Zetasizer NanoZS (Malvern Instruments, Worcestershire, UK) (n=3 samples) and pH was measured using a calibrated CyberScan 510 pH meter. Structural stability was evaluated by size exclusion chromatography (SEC-HPLC), Sodium dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Circular dichroism (CD) analysis as described previously by Pabari *et al.* [17] and Tang *et al.* [19].

Statistics

Data is expressed as mean ± standard deviation (SD), unless otherwise indicated. Statistical analysis was conducted using SPSS (PASW Statistics 18), using Repeated Measures ANOVA, One-Way ANOVA and student's t-tests (unpaired and paired) where appropriate, p values < 0.05 are considered significant.

RESULTS AND DISCUSSION

Visual Appearance and Particle Size Analysis

Trastuzumab i.v. solutions were found to be clear, colourless solutions with no visible particulate material present at any time point tested, irrespective of concentration. Particle size analysis showed no significant change in particle size over the 28-day period regardless of trastuzumab concentrations, confirming absence of aggregates over the study period (Repeated Measures ANOVA, $p>0.05$, n=3) (Table **1**).

pH Stability Profile

The pH of freshly prepared trastuzumab i.v. solutions was in the range of 5.81 to 6.17 which was within the manufacturer's recommended pH range of approximately 6.0. The measured pHs of all solutions remained stable over time and were within the pH range measured for corresponding freshly prepared trastuzumab i.v. solutions over the study period; pH range of 6.13 – 6.26, 6.01 – 6.09 and 5.58 – 5.97 at 4.0, 1.0 and 0.4 mg/mL respectively (Fig. **1**).

SEC-HPLC

Trastuzumab i.v. solutions of 0.4, 1 or 4 mg/mL stored at 2-8ºC showed no significant change in trastuzumab monomer concentration over the 28 days studied (Fig. **2**). Importantly, no new peaks were observed over the time course indicating absence of soluble aggregates or degradants (Fig. **3**). Kupfer *et al.* [16] reported that alemtuzumab in infusion-bags stored over 14 days at 6°C, at room temperature and on a vibrating plate was physically and chemically stable.

SDS-PAGE Analysis

SDS-PAGE analysis confirmed the data observed by SEC-HPLC. A single band at ~150 kDa, corresponding to the molecular weight of intact trastuzumab monomer was obtained for all test solutions (Fig. **4**). Importantly, there was no evidence of reduced trastuzumab or the presence of high molecular weight species (HMWS), signifying the absence of molecule aggregation.

Legends: (a) 4 mg/mL at Day 0. Lanes (1) Molecular marker, (2) Saline, (3 & 4) control, (5-10) sample 1-6; (11 & 12) reduced trastuzumab controls, (13 & 14) reduced samples (1 & 2) (b) 4 mg/mL, (c) 1 mg/mL, (d) 0.4 mg/mL at Day 28. Lanes (1) Molecular marker, (2) Saline, (3) control, (4-9) sample 1-6, (10) reduced trastuzumab control, (11-12) reduced samples (1 & 2).

Table 1. Particle Size (nm) of Trastuzumab i.v. Solutions, Stored at 2-8°C (mean ± SD, n=3).

Trastuzumab	Day 0	Day 2	Day 5	Day 8	Day 15	Day 28
0.4 mg/mL	12.35 ± 0.40	12.16 ± 1.44	11.56 ± 0.51	12.75 ± 1.57	NM*	11.97 ± 0.92
1 mg/mL	11.62 ± 12	11.64 ± 0.62	11.71 ± 0.51	NM*	NM*	11.32 ± 0.47
4 mg/mL	11.24 ± 0.25	11.16 ± 0.07	11.13 ± 0.05	11.14 ± 0.12	11.87 ± 0.75	11.22 ± 0.12

*NM: (Not Measured).

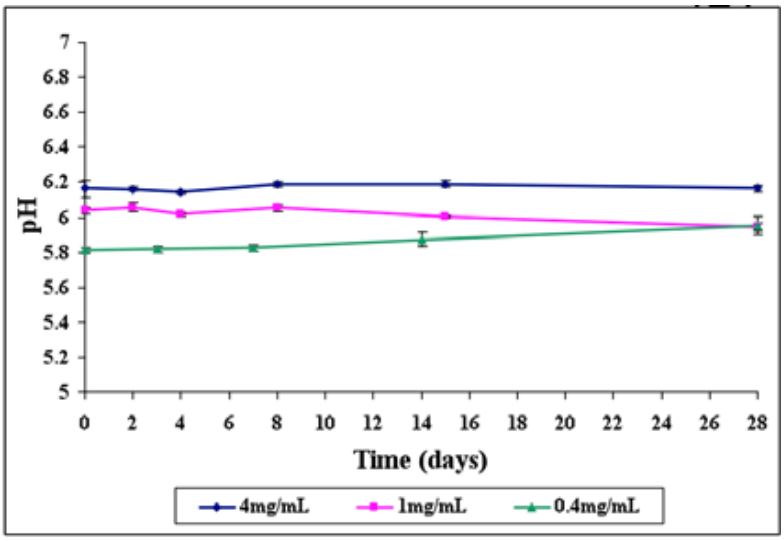

Fig. (1). pH profile of 0.4 – 4 mg/ml trastuzumab i.v. solutions stored at 2-8°C (n = 6).

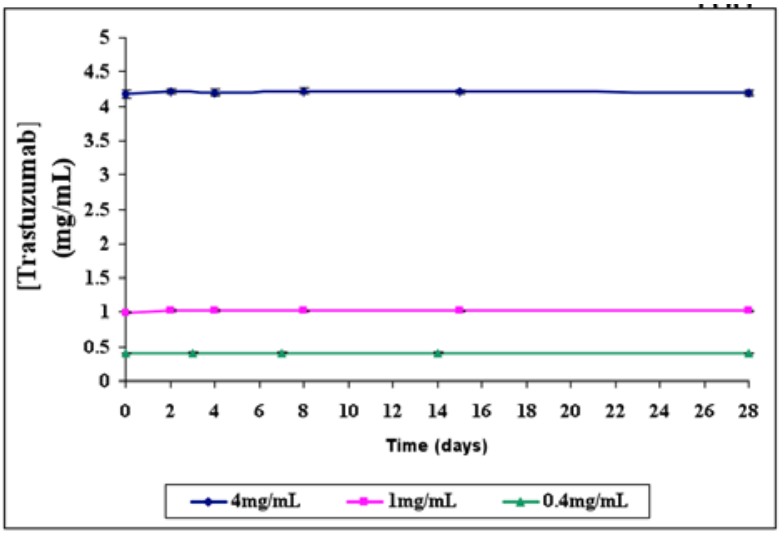

Fig. (2). Concentration-time profile of trastuzumab monomer in i.v. solutions stored at 2-8°C (n = 6).

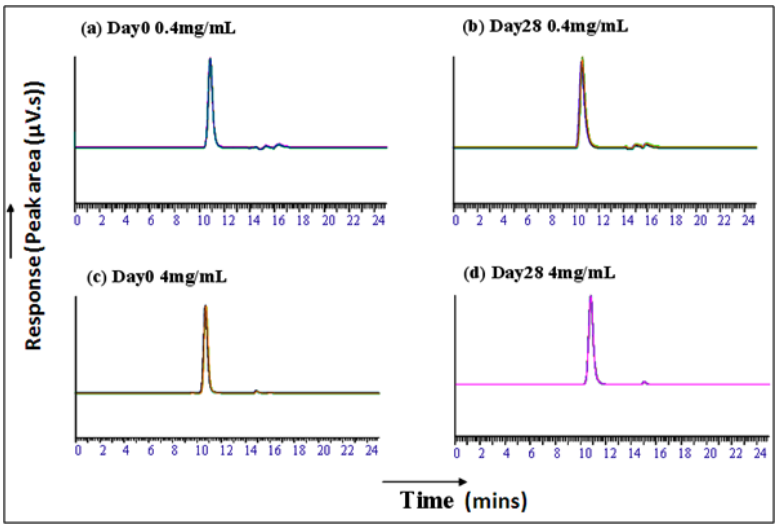

Fig. (3). SEC-HPLC chromatograms of trastuzumab i.v. solutions after storage at 2-8°C over 28 days (n = 6).

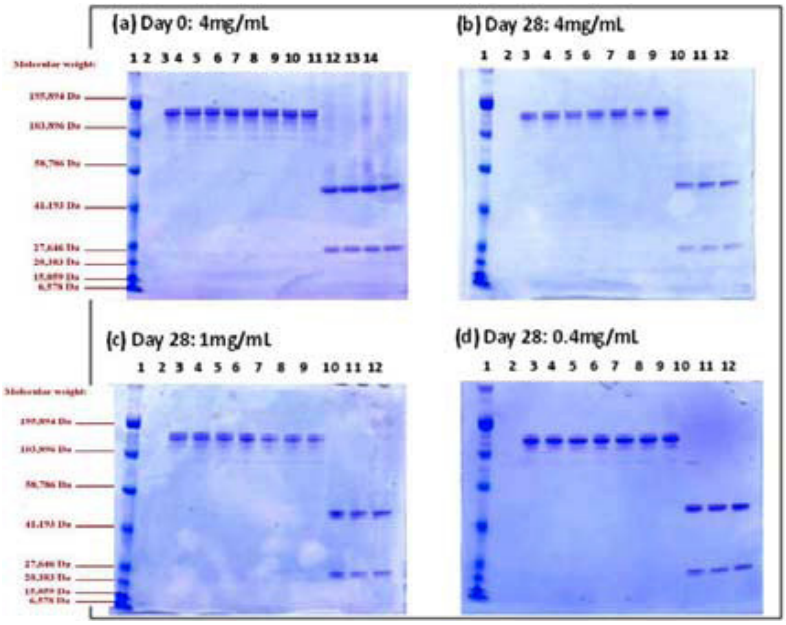

Fig. (4). SDS-PAGE analysis of trastuzumab i.v. solutions stored at 2-8°C over 28 days.

Circular Dichroism

The CD spectrum of the standard trastuzumab i.v. solutions showed a wavelength at zero intensity of 209.1-210.1nm, spectra minima at 217.0-217.2 nm and a broad shoulder at 227.4-228.6 nm (Fig. **5**), similar to that reported for IgG1 molecules and demonstrates a dominance of β-sheet secondary structural conformation [10, 17]. No change in ellipticity or shift in the wavelength was observed over the 28-day stability study period indicating that trastuzumab maintained its native conformation (Fig. **5**).

Influence of Shaking Stress

Shaking resulted in a 2-fold increase in the average particle size of trastuzumab at the lowest concentration of 0.4 mg/mL ($p<0.05$) (Table **2**), probably due to dimerization of trastuzumab. Shaking stress subjects proteins to a high level of air-water interfaces hence exposing the hydrophobic core of the protein to air and resulting in partial unfolding, aggregation and precipitation [9]. Non-ionic surfactants in protein formulations accumulate at the air-liquid interface and therefore protect the protein against interface related stress [9]. Mahler *et al.* [12] found that a polysorbate concentration of 0.005%w/v was sufficient to prevent protein aggregation regardless of the antibody concentrations while Kiese *et al.* [20] detected the presence of soluble aggregates at 0.0025% w/v of polysorbate 20 for a 10 mg/mL of an IgG1 antibody. The trastuzumab i.v. solutions used in our study contained polysorbate 20 at 0.0005 to 0.005%w/v for 0.4 to 4 mg/mL, respectively. At 4 mg/mL trastuzumab concentration, the

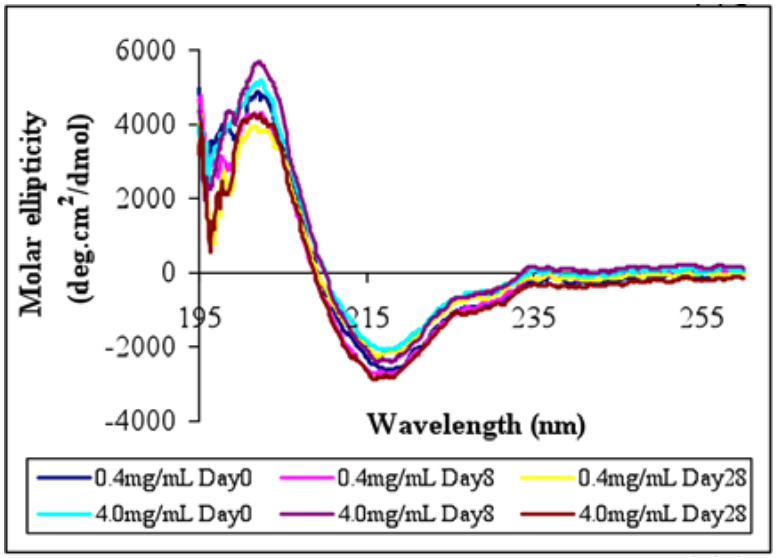

Fig. (5). CD spectra of trastuzumab i.v. solutions on storage at 2-8°C over 28 days.

Table 2. Influence of Physical and Thermal Stressors on the Average Particle Size (nm) of Trastuzumab (Mean± SD).

Trastuzumab	Native (n = 6)	Shaking (n = 6)	35°C (n = 3)	50°C (n = 3)	65°C (n = 3)	75°C (n = 3)
0.4 mg/mL	11.95 ±1.13	24.61 ±8.88	13.48 ±1.20	12.94 ±1.66	12.87 ±1.56	85.16 ±22.57
4 mg/mL	11.22 ±0.10	11.53 ±0.13	11.22 ±0.11	11.32 ±0.13	11.38 ±0.11	93.52 ±27.82

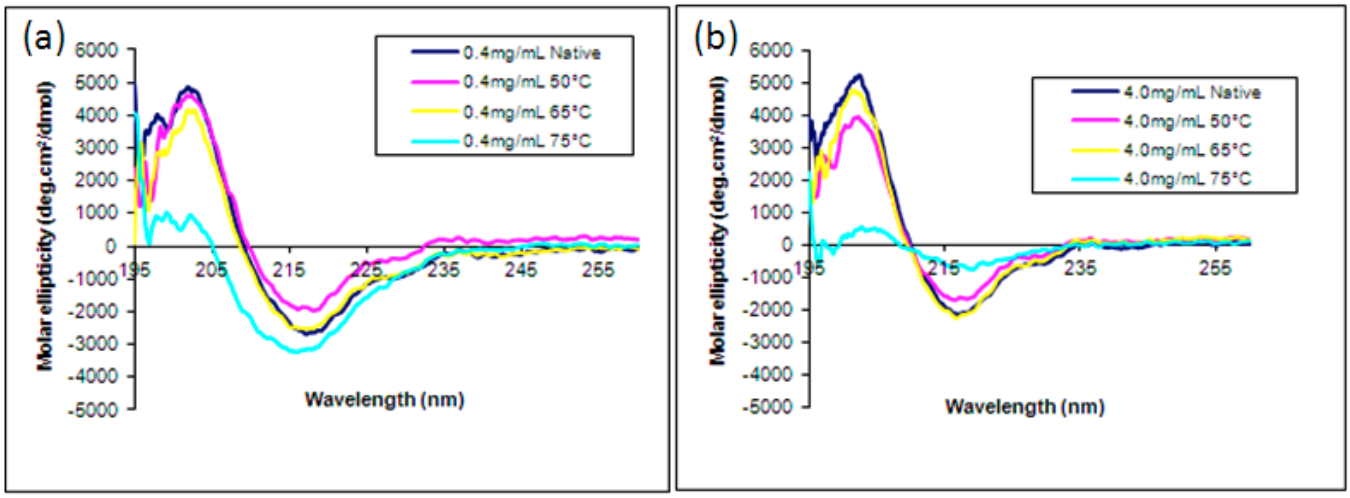

Fig. (6). CD spectra of trastuzumab, (a) 0.4 mg/mL and (b) 4 mg/mL subjected to elevated temperatures.

surfactant concentration was sufficient to prevent aggregation, however at 0.4 mg/mL, trastuzumab aggregation was observed on shaking possibly as a result of the lower concentration of polysorbate 20.

Influence of Elevated Temperatures on Trastuzumab Stability

Trastuzumab solutions showed no increase in particle size up to a temperature of 65°C (Table **2**). At 75°C trastuzumab solutions became turbid, indicating the formation of insoluble aggregates. On centrifugation, particle size analysis showed the presence of soluble aggregates in the clear supernatant (Table **2**).

CD spectrum of trastuzumab remained almost unchanged with increasing temperatures up to 65°C, as was previously reported by Vermeer *et al.* [10] (Fig. **6**). Further elevation of temperature to 75°C resulted in a marked decrease in the wavelength at zero intensity, indicating a loss of ordered structure. In addition, a decrease in intensity of the broad shoulder at ~228 nm reflected an alteration of trastuzumab secondary structural conformation, attributable to a small transition from β-turn conformation to random coil structures.

Trastuzumab i.v. solutions were found to be physically and structurally stable on storage and when subject to shaking and thermal stresses. This stability may be related, in part to the inherent structural stability of trastuzumab molecule and partly through the protective effect of other formulation excipients such as polysorbate 20, L-histidine and α,α-trehalose dehydrate present in Trastuzumab (Herceptin®, Roche) [21].

CONCLUSIONS

The results of this study show that sterile i.v. solutions of trastuzumab remained physically and structurally stable following storage at 2-8°C over 28 days. This is in agreement with previous literature reports of the stability of other mAbs upon storage. Importantly in our study, trastuzumab exhibited resistance to conformational change and physical alteration when subjected to shaking and thermal stressors similar or in excess of those encountered in every day clinical practice. The findings from this study suggest that trastuzumab in solution may not be as sensitive to degradation as expected for a mAb. This may have important implications in extending the shelf life of trastuzumab and other therapeutic monoclonal antibodies in clinical practice and therefore warrants further exploration.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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