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Extended stability of a biosimilar of trastuzumab (CT-P6) after reconstitution in vials, dilution in polyolefin bags and storage at various temperatures

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Objectives: This study assessed the long-term stability of the trastuzumab biosimilar CT-P6 (Herzuma®, Biogaran) in vials under various storage conditions; after reconstitution of the lyophilized powder (21 mg/mL) and after dilution for final concentrations of 0.8 mg/mL and 2.4 mg/mL in polyolefin bags, stored at 4°C and 22°C.
Methods: The physical and chemical stability of the drug was evaluated by several complementary methods.
Results: After 90 days of storage at both 4°C and 22°C, no signs of physical instability, such as the formation of aggregates or oligomers, were observed, regardless of the antibody concentration. After 90 days at 4°C, there was no change in the distribution of the seven ionic variants of the compound. The tertiary structure of the compound was unaltered after storage for 28 days at 4°C.
Discussion: When stored at 22°C for 28 days, tertiary structure was slightly altered and there were signs of hydrolysis, but no observable aggregate formation or significant signs of thermodynamic destabilization. The same conclusions can be made for reconstituted vials at 21 mg/mL.
Conclusion: The trastuzumab biosimilar CT-P6 remained physically and chemically stable for at least 90 days, when stored at 4°C and away from light. These results strongly support the safe use of this biosimilar in several contexts such as preparation in advance, dose banding, supply of prepared but un-administered drug or cold chain rupture. Such usage could improve patient management, lessen nurse and pharmacy workload, and avoid non-justified losses of this expensive drug.

Keywords: Biosimilar, extended stability, in-use stability, trastuzumab

Introduction

Trastuzumab (TTZ) is an IgG1-class recombinant humanized monoclonal antibody (mAb) to human epidermal growth factor receptor 2 (HER2). Several studies show that disease-free survival is shorter in patients with breast cancer whose tumours overexpress HER2 than those who do not [1]. TTZ is used in the management of metastatic gastric cancers and HER2 positive breast cancers [2-6].

The pharmacological properties of TTZ have been reviewed previously [7, 8]. TTZ binds with high affinity and specificity to sub-domain IV, a juxta membrane region of the extracellular domain of HER2. The binding of TTZ to HER2 inhibits ligand-independent activation. Its binding also prevents proteolytic cleavage of its extracellular domain, a mechanism for activating HER2. As a result, TTZ inhibits the proliferation of human tumour cells that overexpress HER2, as shown by *in vitro* and animal studies. In addition, TTZ is a potent mediator of antibody-dependent cellular cytotoxicity (ADCC). *In vitro*, it has been established that TTZ-related ADCC is preferentially exerted on cancer cells overexpressing HER2 compared to cells that do not exhibit this overexpression.

Since expiry of the patent of the TTZ originator (Herceptin®, Roche Laboratories) in numerous countries, especially in Europe, several biosimilars of TTZ have been in the pipeline for registration. At the end of January 2018, the first biosimilar of TTZ was registered in the European Union (EU) (CT-P6; manufactured by Celltrion, South Korea, and marketed in France by Biogaran). Biosimilars marketed in the EU are authorized only after a full comparative exercise using an extensive evaluation of their physicochemical properties including primary, secondary, and higher-order structures; post-translational modifications; glycosylation; charge variants; purity; and biological and clinical properties [9]. However, minor variations in terms of charge and glycosylation or other characteristics can be accepted between the reference drug and its biosimilars if no consequence on biological properties, clinical activity and toxicity is demonstrated.

Drug stability is defined as the ability of the pharmaceutical dosage form to maintain its physical, chemical and microbial characteristics during storage at a level to guarantee sufficient pharmacological activity after administration to the patient. Drug stability studies therefore theoretically permit determination of an expiry date, after which the drug cannot be used due to an unacceptable loss of the active product. However, these studies are often performed in standardized conditions which do not correspond to 'real-world' practices (also described as 'in use stability') [10-12]. Several studies have demonstrated that some widely used mAbs are dramatically more stable after dilution and extended storage periods indicated in their summary of product characteristics (SPCs), if prepared under conditions guaranteeing their sterility [13-16]. In these conditions, stability depends only on the physical and chemical properties of the molecule. However, manufacturers themselves do not perform this type of extended stability study, since regulatory authorities

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do not require it for marketing authorization. Moreover, it has been accepted that injectable drugs must be administered immediately after their preparation, because they are often prepared by nurses under poorly controlled aseptic conditions. Most of the stability data presented in SPCs (generally 24–48 hours at 4°C) are not based on actual physicochemical instabilities but rather on a precautionary principle, considering a risk of bacterial contamination during handling. Thus, this interpretation of the stability of a drug is highly questionable if handling practices are validated from the aseptic point of view, which is the case in pharmacy reconstitution units. Therefore, academic extended stability studies are crucial for pharmacists (and payers) considering the very high cost of these drugs and could also be an efficient response to unwanted events (cold chain rupture) or enable preparation in advance.

For a biosimilar, which is by definition highly comparable to its originator, it makes sense to assume that its stability will also be comparable. However, the required comparability exercises include only short-term stability studies after reconstitution and/or dilution. Thus, it is possible that minor variations between a biosimilar and its originator could induce a different stability profile during extended storage periods. Although recent studies of the extended stability of mAb biosimilars, such as infliximab [17] and rituximab [18], have demonstrated identical profiles as compared to their originator, the behaviour of other mAbs might be different. Therefore, it is crucial to ascertain for each mAb that its biosimilar exhibits the same extended stability.

For the TTZ originator (Herceptin®), reconstituted in vials or diluted in polyolefin bags, stability for up to one month at 4°C [14] has been demonstrated, however, no data are available for its biosimilars. In this study, we studied the extended stability of the TTZ biosimilar CT-P6 (Herzuma®) by several complementary methods, both in reconstituted vials at a concentration of 21 mg/mL and after dilution to bracket concentrations of 0.8 and 2.4 mg/mL in polyolefin bags, after storage at 4°C and 22°C for up to three months.

Materials and methods

All the analytical methods used have been previously validated as indicative of stability during our previous studies on mAbs, such as the originator rituximab, TTZ and ipilimumab [10, 12, 13].

Reagents

Potassium dihydrogen phosphate, disodium hydrogen phosphate and sodium azide were obtained from Merck (Darmstadt, Germany). Acetonitrile for high performance liquid chromatography (HPLC) and MES (2-(N-Morpholino) ethanesulfonic acid) were furnished by Sigma-Aldrich (St Louis, MO, USA). Isotonic saline (batch 13KIS24; expiry date: 09/2018) was provided by Fresenius Kabi (Louviers, France). All other reagents were of analytical grade.

The vials of biosimilar TTZ (150 mg lyophilized powder; CT-P6; Herzuma®), manufactured by Celltrion (Seoul, South Korea), were kindly supplied by Biogaran (France). Three different vial batches were used (batches 17 A4C01; 17 A4C02; 17 A4C03). For the preparation of bags and dilutions, sterile 0.9% NaCl polyolefin bags were used (FreeFlex®, Fresenius Kabi; batch 13 LIF281 expiry date: 09/2019). For several experiments, the analytical samples were diluted in a dilution buffer (20 mM KH_2PO_4, pH 6.0, filter-sterilized using a 0.22 µm pore filter).

Preparation of bags

The lyophilized powders were reconstituted with water for injection (WFI) under validated sterile conditions (vertical laminar flow hood) for a final concentration of 21 mg/mL according to the manufacturer's recommendations. Then, they were diluted to final concentrations of 0.8 mg/mL (C1) or 2.4 mg/mL (C2) in 50 mL 0.9% NaCl. Twelve bags were prepared: three bags at the C1 concentration for the temperature 4°C; three bags at C2 concentration for the temperature 4°C; three bags at C1 concentration for temperature 22°C; and three bags at C2 concentration for temperature 22°C. For the preparation of the 0.8 mg/mL bags, 2.9 mL of the concentrated 21 mg/mL solution was introduced into a 100 mL bag previously completely emptied of its saline content and then the volume made up to 75 mL with 0.9% NaCl. For the 2.4 mg/mL bags, 8.6 mL of the concentrated solution was made up to 75 mL of NaCl according to the preceding protocol. The bags were then weighed.

Storage and sampling for analysis

Immediately after the bags were prepared, a 7 mL syringe sample was taken from each bag in order to make the initial determinations, known as day 0 (T0; reference). The bags or reconstituted vials were then placed under the storage conditions: certified refrigerator (temperature range: +2°C to +5°C or air-conditioned room (22°C ± 2°C), protected from light and under constant temperature monitoring. The bags removed at different storage times (7, 14, 21, 28 days and 3 months; marked as T7, T14, T21, T28 and T90, respectively) were weighed before sampling to determine their initial weight for the calculation of possible water losses during storage. The bags and vials were stored with the sampling device left in place. For the vials reconstituted at 21 mg/mL, a 2 mL sample was taken to carry out all sterility assessments. The necessary vial content was diluted and to be analysed under the same conditions as the bags.

Spectroscopic and turbidimetry determinations

Turbidimetry is extensively used to estimate the amount of micron-size protein aggregates by measuring the optical density at 350 nm. At this wavelength, the intrinsic chromophores of proteins do not absorb. Only suspended aggregates diffract the incident light causing a pseudo-absorbance which permits an estimation of the extent of aggregation.

The second derivative ultraviolet (UV) spectroscopy method makes it possible to evaluate the impact of dilution and conservation on the aromatic amino acids of the molecule: phenyalanine (Phe), tyrosine (Tyr) and tryptophan (Trp). The position of the absorbance peaks of the second derivative spectra is sensitive to the microenvironment of these amino acids and thus provides an overall view of the tertiary structure of the molecule. The spectrum was analysed from 220 to 320 nm. The data were unsmoothed and the derivate spectra were obtained using a nine-point algorithm. Samples were diluted to 0.4 mg/mL using phosphate buffer (20 mM, pH 6.00, filtered at 0.22 µm; prepared in-house). Five major peaks were monitored: 222-Phe, 228-Phe, 275-Tyr, 284-Thy/Trp and 292-Trp, and two minima at 287 and 295 nm.

All analysis was performed using a UV spectrophotometer (Cary 50 Probe, Varian Inc, Palo Alto, USA) and 1 cm quartz micro-cuvettes. Turbidity analyses were performed on pure samples. For scan measurements the samples were diluted to 0.4 mg/mL in dilution buffer. A scan was performed between 220 and 400 nm against the dilution buffer at a rate of 200 nm/min. Absorbances were determined from the spectrum.

Fluorescence spectroscopy

Possible modifications to the drug's tertiary structure during storage were estimated by fluorescence spectrometry through intrinsic fluorescence of the aromatic amino acids: phenylalanine, Tyr and, mostly, Trp, absorbing UV light at 227, 275 and 280 nm, respectively [18]. Samples diluted in the dilution buffer were excited at 279 nm at 22°C and emission spectra recorded between 310 and 410 nm using a Perkin Elmer fluorimeter (Orsay, France).

Ion-exchange chromatography (IC)

The charge variants were analysed by weak cation exchange chromatography under gradient salt mode [12, 19]. A biocompatible Thermo Scientific Ultimate 3000 HPLC system, consisting of an ISO-3100 SD pump, a WPS-3000 automatic injector and an UV multi-wavelength 3000 detector, was used. Samples were injected pure for C1 concentration or diluted to a concentration of 1 mg/mL in dilution buffer for the other sample concentrations. A Propac® WCX-10 column, fitted with a pre-column Propac 10G (Dionex) maintained at 35°C and eluted at a flow rate of 0.8 mL/min (pressure 56 bars), was used with a detection of 280 nm. An excipient blank was used to check for the absence of interference. The mobile phases consisted of a phase A (20 mM MES and 60 mM NaCl; pH 6.0) and a phase B (20 mM MES and 180 mM NaCl; pH 6.0), both in HPLC grade water, filtered at 0.22 µm and degassed by ultrasound. The gradient profile was at T0: phase A 45%/phase B 55%, increasing to 100% phase B in 40 min, then a return to initial values in 10 min and an equilibrium period of 10 min before the next injection.

Size-exclusion chromatography (SEC)

The formation of dimers and high molecular weight species (HMWS) and molecule fragmentation during storage were evaluated by size-exclusion chromatography [19]. After centrifugation (15,000 × G for 5 min) the samples were diluted at the required concentration with dilution buffer. 100 µL of diluted sample was injected onto a Yarra 3000 Phenomenex® (300 × 7.8 mm) column protected with a Phenomenex® security guard GFC3000® (4 × 3 mm) pre-column, maintained at room temperature and eluted at a flow rate of 0.6 mL/min (Biocompatible Dionex chromatographic system). Detection was performed using a Dionex WWD diode-array detector at 280 nm. The mobile phase consisted of 0.2 M K_2HPO_4, 0.22 M KCl and 0.05% NaN_3 in HPLC grade water, adjusted to pH 7.0 with 1 M H_3PO_4 and filtered at 0.22 µm. A reconstituted excipient solution was used as blank.

Dynamic light scattering

Submicron particles estimation

For evaluation of the size (hydrodynamic diameter) of monomers, oligomers, small (50–1,000 nm) and large (1–6 µm) aggregates, dynamic light scattering (DLS) was used. DLS is a sensitive method to measure the Brownian motion of particles, which is linked to their size. A Malvern Zetasizer Nano ZS laser-light scattering system (Worcestershire, UK) and NanoZS® software were used to obtain DLS measurements with a 633 nm laser source. We analysed, in triplicate, 200 µL of each undiluted sample in a quartz micro-cuvette previously rinsed with injection-grade water (0.22 µm filtered). All steps were performed in a laminar flow hood to prevent contamination with external particles. The hydrodynamic diameters of the antibodies and small aggregates were determined, together with their dispersity index (DI), for estimation of dimerization rates.

Thermal aggregation curve (thermodynamic stability)

During stress (thermal, mechanical, chemical, light exposure) or simply during long-term storage, the higher order structures (secondary, tertiary and quaternary) of proteins may change, signalling unfolding of the protein. Due to an increase in the exposure of hydrophobic surfaces, these modifications can induce aggregation. These events can be observed by DLS, because they lead to major changes in protein size. By gradually increasing temperature (1°C/min, 3 min equilibration between steps) from 55°C to 78°C and measuring hydrodynamic diameter at each temperature, we can obtain a thermal aggregation curve. The melting temperature (Tm, at which the aggregated fraction is 0.5) can be determined using a non-linear least squares method to fit diameter dependence on temperature to a sigmoidal model (KaleidaGraph® software). A comparison of the curves obtained for each storage condition can be used to estimate even small alterations of the three-dimensional structure, caused by energy accumulation by the molecule. This accumulation cannot be detected by other methods, which detect only already denatured states [19].

Particle search by microscopy

Microscopy was used to identify particles between 10–50 microns formed in the samples. All materials were rinsed beforehand with WFI (filtered at 0.22 µm) and dried under the laminar flow hood. The samples were prepared in a laminar flow hood to avoid external contamination and then introduced (50 µl) into a Mallassez cell covered with a coverslip and examined by an optical reverse microscope (X 400; Olympus IM; Olympus SA, Rungis, France) equipped with a colour camera XCD-U1000 CR; 1,600 x 1,200 pixels (Sony, Japan). The captured images were analysed using image analysis software (Ellix, Microvision Instruments, Evry, France).

General properties

Several general properties (density, osmolarity, pH) of the biosimilar TTZ solutions were determined as a function of storage conditions.

Density

The density of undiluted samples was measured by the oscillating U-tube method, at 22°C in a densimeter (DMA 35 Anton PAAR portable).

pH

An electronic pH meter with a glass combination microelectrode (Consort P901 pH meter, Bioblock Scientific) was used to measure the pH of the samples. The pH meter was calibrated before each series of measurements, which were precise to 0.01

pH unit. pH values were expressed at 22°C by application of temperature correction.

Osmolarity

The osmolarity of the solutions was measured using the freezing point depression method using 100 µL of sample, with calibration against a 300 mOsmol/L standard (Hermann Roebling automatic micro-osmometer, model: 12/12DR).

Sterility test

Sterility tests were performed on pooled bags for each batch and on remaining material in the vials at T28, since they represent the highest risk of contamination in this study. These tests were performed with Millipore Steritest Symbio Pumps®, based on the membrane filtration technique. Two culture media were used: thioglycolate medium (incubated at 32°C) and casein and soy hydrolysate medium (incubated at room temperature). After 14 days of incubation, microbial growth was assessed by comparing the cloudiness of the medium with that of a negative control.

Presentation of data and statistical analyses

All experiments were performed in triplicate unless otherwise indicated. Results are represented as the mean ± SD. The non-different found values between the conditions tested: batch, temperature, concentration, were pooled to be presented on average ± SD. Characteristic chromatogram plots have been included for visualization of the data.

The results were analysed using the PAST 3.06 software [20] by parametric tests, following verification of the homogeneity of the variances to ascertain their normal distribution by the F test.

Results

After preparation, there was no significant difference in weights between the bags (as demonstrated by an analysis of variance (ANOVA)), indicating excellent homogeneity.

Aggregation estimated by turbidimetry

For each point determined in triplicate, the variation between measurements was very small, typically less than 1 milli Optical Density (mOD) unit. No significant difference in initial turbidance between the three batches for C1 and C2 ($p = 0.4329$, ANOVA) was noticed. This small variability between batches matches our previous studies on the extended stability of biosimilars from the same manufacturer [17, 18].

As expected, the mean turbidity was slightly higher (approximately + 6 mOD) for a TTZ concentration of 2.4 mg/mL vs 0.8 mg/mL (48.2 ± 0.2 mOD vs 42.5 ± 1.7 mOD; $p = 0.003$). As expected, the turbidance was higher in the vials than the bags because of the higher concentration of the solution (74.7 ± 1.8 mOD). A slight difference between batches was found, with a maximum variation of less than 4 mOD (range: 72.50 ± 1.11 mOD for batch 3 at 76.61 ± 0.17 mOD for batch 1) The evolution of turbidance as a function of storage time is presented in Table 1. There was no change in turbidity at 350 nm after 28 days of storage compared to T0 for both concentrations at 4°C. At 22°C, a slight increase in turbidity was noted for both concentrations. On the other hand, after 90 days, turbidity did not increase, see Table 1. For storage in vials at 4°C, as with bags, we identified no significant change in turbidity as a function of the storage time. The slope of the mean values versus time is not statistically different from zero.

Table 1: Evolution of turbidance expressed in mOD at 350 nm versus time expressed in day for the two concentrations (0.8 mg/mL) and (2.4 mg/mL)

		T0	T28	T90
C1	4°C	42.47 ± 1.70	43.57 ± 0.50	46.47 ± 1.55*
	22°C	42.47 ± 1.70	46.07 ± 0.29*	46.40 ± 1.01*
C2	4°C	48.16 ± 0.20	48.68 ± 1.29	50.69 ± 2.16
	22°C	48.16 ± 0.20	52.01 ± 0.31*	49.36 ± 0.25*
Flacons	4°C	74.70 ± 1.81	73.16 ± 1.44	ND
	22°C	74.70 ± 1.81	91.73 ± 0.18*	ND

*p < 0,0001 vs T0, test of Tukey's pairwise.
mOD: milli optical density; ND: not determined.

At 22°C, a slight but significant increase in turbidity was noted for both concentrations in bags (approximately +2.5 to 4 mOD). After 90 days however, turbidity remained stable. Conversely, for storage of vials at 22°C, a significant increase in OD was observed (+17 mOD).

We did not identify light diffracting aggregates during storage for 90 days at + 4°C either in vials or after dilution in bags. However, prolonged storage at room temperature resulted in some aggregation.

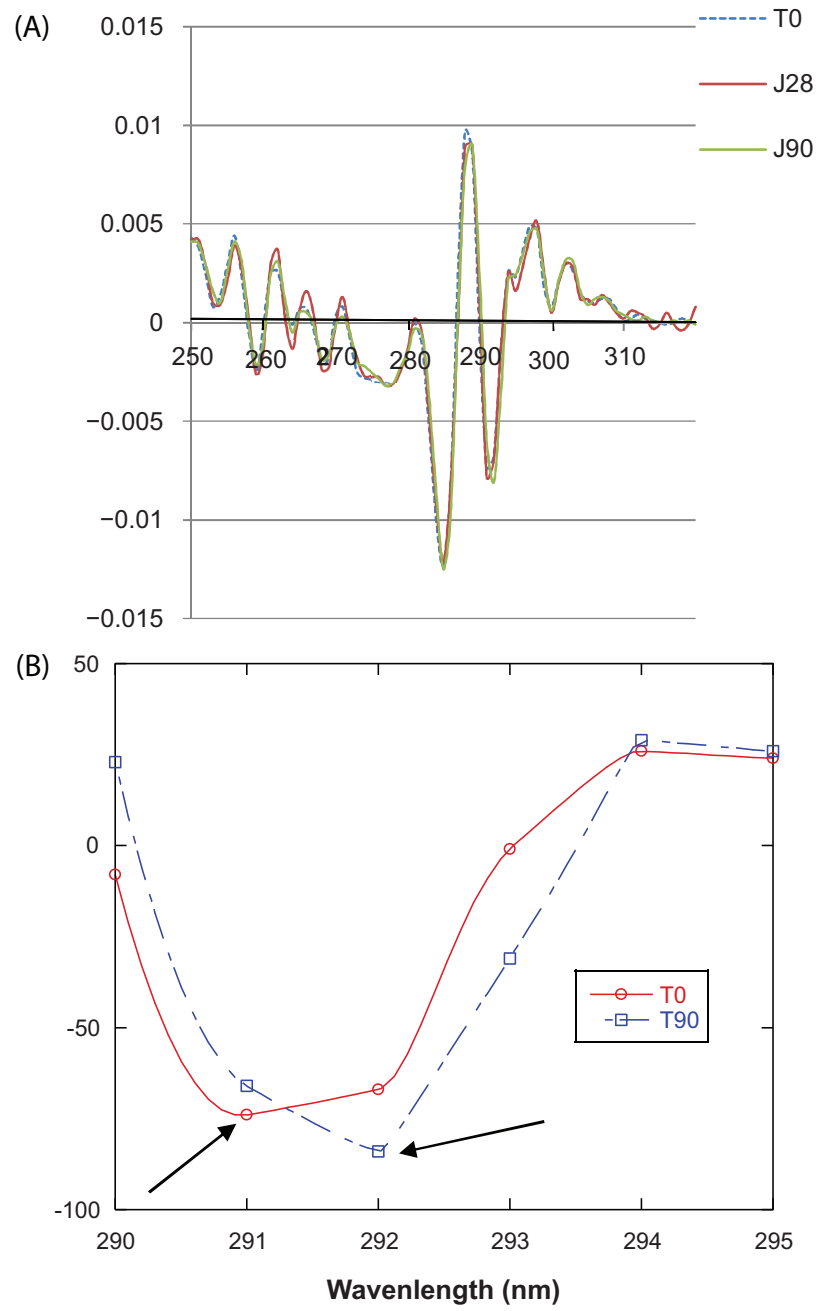
Modification of tertiary structure

Second derivative spectrometric analysis of UV spectra in the aromatic amino acid region (260–300 nm) was performed to estimate changes to the tertiary structure of the antibody. The position of the absorbance peaks of the second derivative are sensitive to the polarity of the microenvironment of these amino acids and thus provide an overview of the tertiary structure of the molecule. Figure 1 shows that TTZ, stored at 4°C and 22°C (0.8 and 2.4 mg/mL) for 28 days, retained its tertiary structure. On the other hand, after storage at 4°C or 22°C for 90 days, discrete offsets were noted, especially for the maxima at 287 and 291 nm which undergo a bathochromic (red shift) effect of 1 nm (288 and 292 nM, respectively).

The absence of modifications in the 250–270 nm region makes it possible to exclude environmental disturbances of phenylalanine which occur by exposure to water during unfolding. In contrast, bathochromic shifts between 290 and 300 nm with a peak shift at 291 nm are consistent with a change in the environment of tyrosine and tryptophan residues [21]. These results are in agreement with those of our previous study on the stability of the originator TTZ (Herceptin®) which showed extended shifts at 269, 296 and 301 nm after six months storage at 40°C [14].

The fluorescence emission spectra show an identical profile with the same emission maxima as a function of storage conditions. However, variations in intensity suggest that the environment of the tryptophan residues have been modified, in agreement with the second derivative UV spectrum (data not shown).

Figure 1: Representative second-order derived UV spectrum of diluted trastuzumab (0.8 mg/mL) stored at 4°C (A); extended region showing the shift (B)



No modification is noted at 28 days. By contrast, after 90 days, a red shift of 1 cm^{-1} is noted in black arrows.

It can thus be concluded that storage of the biosimilar TTZ at 4°C or 22°C up to one month did not alter its tertiary structure. However, 90-day storage (even at 4°C) induces a tertiary structure modification, with exposure of a region possessing tyrosine and tryptophan residues. This behaviour suggests that the alteration of the higher order structure of TTZ is not only dependent on temperature but also on the duration of exposure to an aqueous environment.

Analysis of ionic variants by ionic chromatography

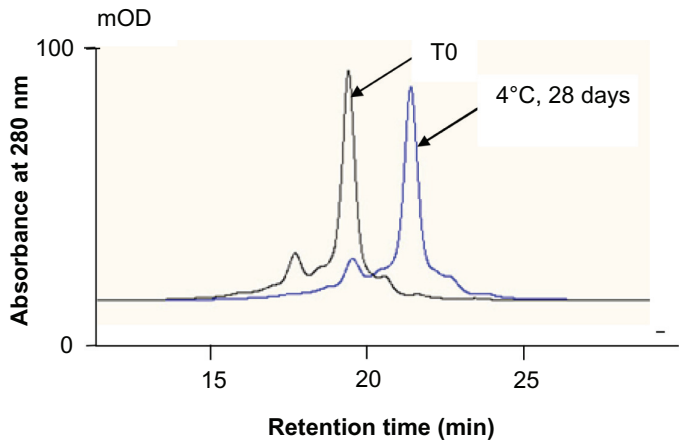
Salt gradient ionic chromatography revealed seven ionic variants of TTZ, Figure 2 shows a representative chromatogram. On the basis of the manuscript by Harris et al. these seven variants have been tentatively identified [22]. By comparison, we found only three acid variants instead of four and three basic variants instead of two. The integration of the peaks allowed us to determine the respective areas under curve (AUC). The percentage of each peak relative to the sum of the AUCs was determined for each condition: concentration, batch, temperature and storage time.

The main ionic variant was peak 2, which represented approximately 87% of the total peaks under our conditions. This peak corresponds to a variant without deamidation of the asparagine (Asn) residues at position 30 of the light chain and at position 55 of the heavy chain, nor isomerization of the aspartate (Asp) residue at position 102 of the heavy chain. There were three peaks of acidic variants (A, B and 1), corresponding to deamidated species (approximately 10.24% in total) and two peaks of basic variants (isomerization and succinimide formation, approximately 1.1%), see Table 2. Unsurprisingly, for the vials, the initial percentages were identical to those of the bags prepared from them (data not shown). By ANOVA and Student-Newman-Keuls Multiple Comparison test analysis, we found no significant difference at T0 in the variant percentages between different batches.

Evolution during conservation

No new peak, either in the acidic or basic part of the chromatograms was observed, suggesting that no new degradation products appeared. There was no significant change in variant percentages after storage for up to 90 days at 4°C at both concentrations, see Table 2 and Figure 2. Similarly, storage at 22°C for 15 days did not significantly affect stability, suggesting that a thermal excursion (cold chain break during the weekend, for example) would not affect the stability of CT-P6 (data not shown). By contrast, after storage at 22°C for 28 days, almost all peaks were significantly altered, see Table 3 and Figure 3. The acidic variants A and B increased respectively from 0.38% to 0.73% and 9.57% to 13.77%. The main variant decreased from 87.30% to 81% ($p < 0.001$; ANOVA). This is indicative of a heat-induced hydrolytic deamidation, especially of the light chain Asn, as has been demonstrated under stress conditions (90 days at 25°C, see Figure 4). There was also a net increase of basic peak n° 3 (1.34% to 2.68%) and peak C (0.37% to 1.06%), with a concomitant decrease of peak n° 4 (0.74% to 0.24%).

Figure 2: Ionic chromatography profiles of biosimilar trastuzumab at T0 and stored at 4°C for 28 days in a diluted 0.8 mg/mL bag



No difference is observed. An offset of 3 minutes between the traces has been made for more readability.
mOD: milli optical density.

Table 2: Percentage of variants by CEX between the 3 batches (mean ± SD, n = 4, determined on bags)

Peak	Batch 1		Batch 2		Batch 3		Mean and SD	
	%	SD	%	SD	%	SD	%	SD
A	0.39	0.0003	0.40	0.0001	0.36	0.0004	0.38	0.0002
B	9.51	0.0036	9.79	0.0034	9.41	0.0044	9.57	0.0020
1	0.34	0.0003	0.26	0.0008	0.27	0.0008	0.29	0.0004
2	87.21	0.0039	87.03	0.0040	87.67	0.0052	87.30	0.0033
3	1.41	0.0092	1.43	0.0090	1.18	0.0078	1.34	0.0013
4	0.78	0.0046	0.74	0.0053	0.72	0.0048	0.74	0.0003
C	0.38	0.0017	0.35	0.0016	0.38	0.0015	0.37	0.0002

No difference between batches (ANOVA).
CEX: ionic chromatography; SD: standard deviation.

Estimation of oligomerization by SEC

SEC chromatography allows visualization of the monomer of biosimilar TTZ and possible oligomers (including dimers, trimers and high molecular weight products [HMWPs]). These indicate the beginnings of aggregation which can lead to the formation of submicron, then micron, aggregates. SEC chromatography can also identify intra-catenary or extra-catenary fragmentation, such as broken disulphide bridges at the level of the hinge. At T0 in both vials and bags, TTZ exhibited two peaks, the monomer at retention time (Rt) of approximately 13 minutes representing 99.81 ± 0.02% of the total area.

There was on average 0.19 ± 0.01% of the second peak, considered as dimer at a Rt of approximately 11 min. There was no significant difference between the concentrations or batches.

Dimer or oligomer formation, as well as potential catenary breaks, were evaluated for up to 90 days for the bags and 28 days for the vials. There was no additional peak occurring at T28 or even at T90 for the 22°C storage condition, demonstrating the absence of oligomers and modifications of the molecule leading to fragmentation, both for the dilute solutions as well as for the vials, see Figure 3.

These results show that, even after dilution and storage at 22°C for more than 90 days, CT-P6 exhibits no significant signs of oligomer formation or chain cleavage. These results are consistent with those obtained by turbidimetry and DLS.

Estimation of aggregation and thermodynamic stability by DLS

DLS was used to address the hydrodynamic parameters of the monomer, diameter and polydispersity index (PDI), to search for the appearance of submicron populations reflecting an intramolecular nucleation phenomenon, and to obtain thermal aggregation curves and Tm (melting point) parameters.

Hydrodynamic diameter and submicron populations

After reconstitution and dilution, only one monomer peak was observed. The average hydrodynamic diameter at T0 for the concentration at 0.8 mg/mL was 11.35 ± 0.19 nm with a PDI of 0.07 ± 0.02. For the concentration of 2.4 mg/mL, the average hydrodynamic diameter was 11.47 ± 0.16 nm with a PDI of 0.06 ± 0.01, see Table 4. There was no significant difference between the batches or concentrations (p = 0.0244, Kolmogorov-Smirnov test).

The very low PDI values strongly suggest that CT-P6 had no dimers or oligomers, consistent with SEC, although it is important to note that this method may break the weak associations between molecules. The measured diameter and PDI were in agreement with those determined in our previous study on the stability of TTZ (range: 11.21–11.39 nm and 0.071–0.074, respectively) [14]. No differences were observed as a function of temperature or batch conditions between T0 and T28 for the two concentrations (Student-Newman-Keuls Multiple Comparison Test: NS (non-significant) for all comparisons). The very low values of the PDI suggest a lack of dimerization, demonstrating excellent agreement with the SEC results.

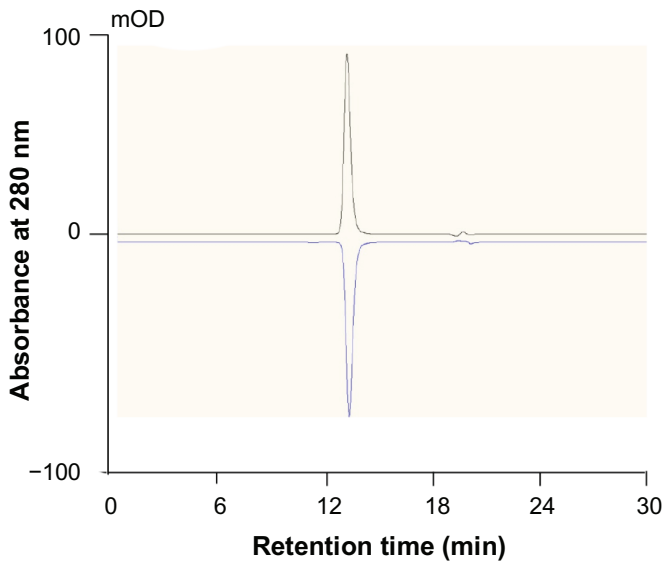
For the 21 mg/mL vials diluted in PPI water, the average hydrodynamic diameters were much lower for the three batches than for the diluted solutions (3.95 ± 0.01 nm, $p < 0.001$; Student-Newman-Keuls Multiple Comparison Test). This difference could be explained by the reconstitution in the WFI of the lyophilizate. In vials, in which the drug is reconstituted in water, there are fewer Na^+ and Cl^- ions compared to bags, in which the solution is diluted in NaCl. This minimizes the solvation layer of the mAb monomer. The diameter of the

Table 3: Evolution of variant percentages after storage at 4°C and 22°C during 28 days and at 4°C during 90 days (mean and SD of three batches in triplicate)

Conc	Peak	Percentages (mean + SD)							
		Initial		28 days				90 days	
				4°C		22°C		4°C	
C1	A	0.38	0.02	0.38	0.03	0.73	0.03	0.38	0.02
	B	9.57	0.20	9.70	0.15	13.77*	0.10	9.61	0.39
	1	0.29	0.04	0.29	0.01	0.44	0.02	0.33	0.05
	2	87.30	0.33	87.48	0.10	81.00*	0.37	87.77	0.37
	3	1.34	0.13	1.07	0.22	2.50*	0.08	0.98	0.05
	4	0.74	0.03	0.66	0.03	0.25*	0.03	0.70	0.02
	C	0.37	0.02	0.33	0.01	1.22*	0.03	0.32	0.02
C2	A	0.38	0.02	0.41	0.03	0.71	0.02	0.43	0.001
	B	9.57	0.20	9.66	0.29	14.20*	0.22	10.16	0.10
	1	0.29	0.04	0.38	0.02	0.44	0.05	0.32	0.04
	2	87.30	0.33	87.96	0.37	80.67*	0.34	87.44	0.14
	3	1.34	0.13	0.71	0.08	2.68*	0.18	0.86	0.01
	4	0.74	0.03	0.56	0.03	0.24*	0.05	0.48	0.04
	C	0.37	0.02	0.33	0.03	1.06*	0.02	0.31	0.02

*= $p < 0.05$ vs initial value.

Figure 3: Typical SEC chromatogram of 0.8 mg/mL solution of the TTZ biosimilar stored at 22°C



Mirror representation; Upper trace: T0; Lower trace: after 90 days of storage. No difference was observed.
mOD: milli optical density; SEC: size-exclusion chromatography; TTZ: trastuzumab.

monomer-solvation layer, displacement of which can be measured by laser beam, is thus smaller than that of the monomer surrounded by a layer of ions and trapped water. The value of approximately 4 nm is therefore closer to the molecular diameter of the monomer.

After 28 days of storage at 4°C and 22°C, diameters were not significantly increased (11.53 ± 0.16 nm, NS vs T0, p (identical) = 0.0047 per Tukey's pairwise test). PDIs were also not modified. However, after 90 days of storage at 4°C and 22°C, hydrodynamic diameters were slightly increased compared to T0 (12.36 ± 0.03 nm and 11.98 ± 0.06 nm, respectively; $p < 0.001$ ANOVA) but were not significantly different between temperatures, corresponding to a pooled value of 12.18 ± 0.06 nm.

This increase in hydrodynamic diameter under stress remained limited, although significant with a difference between the averages [range] of + 0.77 nm [0.64–0.99] ($p < 0.0001$; t test). This increase could be due to the modification of tertiary structure, as shown by second-derivative UV spectroscopy. Unfolding reduces molecular compaction and increases its solvation layer, leading to an increase in hydrodynamic diameter. However, populations of higher hydrodynamic diameter, particularly in the size range between 100 and 1,000 nm, were not observed, indicating the absence of precursors of nucleation-induced aggregates.

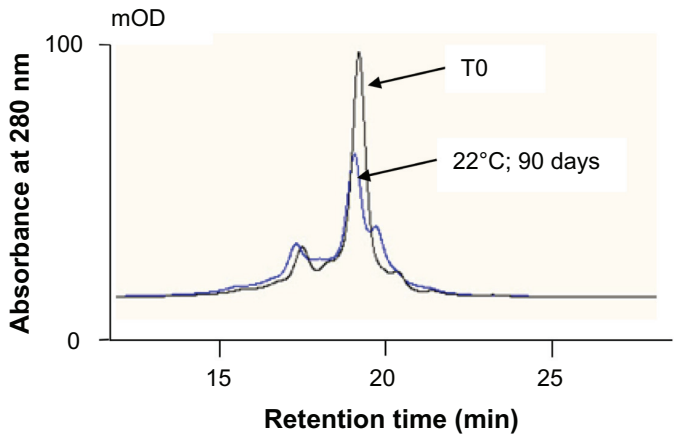
This is consistent with our turbidimetry data and SEC results, which also showed no significant increase in dimer peaks.

It can therefore be concluded that the storage of TTZ for one month at 4°C and 22°C does not influence its hydrodynamic diameter or induce the formation of submicron aggregate populations. After 90 days at 4°C, there is a slight increase of hydrodynamic diameter, but this does not appear to have any consequences for aggregation.

Thermodynamic stability

DLS was used to estimate possible changes in the thermodynamic resistance of TTZ when stored in solution by establishing temperature-dependent aggregation curves to determine characteristic melting temperature (Tms). The initial average Tm value of 77.83°C ± 0.06°C was very similar to that published previously by our team for the TTZ originator (77.33°C ± 0.52°C) [14]. The measured Tm did not depend on concentration, batch, temperature or storage duration (Newman-Keuls Multiple Comparison Test: NS for all comparisons), see Table 4. The absence of modification of the Tm even after storage at 22°C for 90 days seems contradictory with the slight destabilization of the tertiary structure after 28 days at 22°C, as shown by second-derivative UV spectrometry. However, the modification of thermal stability curves requires a substantial energy accumulation by the molecule [23]. Thus, for CT-P6, the energy required to destabilize the tryptophan-rich region responsible of the red shift of the tryptophan peak (291 to 292 nm) would be insufficient to induce modification of the mAb. Moreover, the Tm value for TTZ is high, demonstrating thermal resistance. This suggests large amounts of energy are required to decrease its Tm. Overall therefore, we found no effect of storage duration or temperature on the aggregation state or thermal resistance of CT-P6.

Figure 4: Ionic chromatography profiles of biosimilar TTZ after storage at 22°C for 90 days



Deep changes are visible including a collapse of the main peak 2 as compared to TTZ (trastuzumab).
mOD: milli optical density.

Table 4: Melting temperature (Tm) of the biosimilar TTZ as determined by DLS

Melting temperature	T0	T7	T15	T28	T90
Tm (°C)	77.83	77.48	77.20	77.50	77.34
SD	0.06	0.45	0.42	0.00	0.36

There is no difference between batches and storage temperatures; the individual results were thus pooled for each time. No difference was observed between storage times.
SD: standard deviation; TTZ: trastuzumab.

Particle search

Microscopic examination showed no significant difference to the particles after a storage of 28 days, regardless of concentration or storage temperature.

General parameters: density, pH and osmolarity

Density

The density measured by the oscillating tube method at T0 was 1.0047 ± 0.0003 for the 0.8 mg/mL bags, 1.0075 ± 0.0002 g/mL for the 2.4 mg/mL bags and 1.011 ± 0.0003 g/mL for the 21 mg/mL vials. After 28 days storage, the densities were not significantly modified for any of the conditions tested.

pH

The pH at T0 were the same for all concentrations in the bags: 6.11 ± 0.01 for 0.8 mg/mL and 6.12 ± 0.01 for 2.4 mg/mL, and for the vials (6.11 ± 0.01). After 1 week of storage, there was a significant increase in pH (6.28 ± 0.03: + 0.17 pH units) which then decreased slightly until D28 and increased again after 90 days. Similar behaviour was observed with the vials (a clear increase in pH on Day 7) but afterwards stabilized 6.24 ± 0.05, see Figure 1. These pH variations could be attributed to the introduction of atmospheric CO_2 during dilutions and diffusion through the bag walls, fluctuating with its partial pressure during storage. This would explain the greater variations observed with the bags as compared with the vials, the atmosphere of which was kept more consistent by hermetic closure to gases. However, these pH changes did not affect the stability of TTZ.

Osmolality

For 0.8 mg/mL dilution, the initial osmolality was 274.5 ± 0.8 mOsmol/L and after 28 days of 277.5 ± 1.9 mOsmol/L, with no significant change between T0, T7, T14, T21 and T28 (Kruskall Wallis test, p = 0.0578, NS). There was no significant difference between batches. For 2.4 mg/mL dilution, a higher osmolality was observed than for 0.8 mg/mL: 265, 5 ± 3.14 mOsmol/L at T0, without significant change until T28: 265.7 ± 2.3 mOsmol/L. For the vials, the initial osmolality was 126.3 ± 0.9 mOsmol/L, consistent with the use of water as the solvent. After 28 days, it was 125.3 ± 0.9 mOsmol/ L (NS vs T0) whatever the conditions of storage. After three months of storage at +4°C, for the 0.8 mg/mL concentration, the osmolarity remained stable (277.3 ± 0.3 mOsmol/L) but increased to 283.0 ± 0.0 mOsmol/L at 22°C (+ 2.2%; p = 0.014). This increase is attributed to a slight change in concentration of the solution due to evaporation of water through the bag over the 3-month period (difference in weight loss of 3.5 % between storage at 22°C vs 4°C).

Sterility

No bacterial or fungal contamination was observed in any of the samples tested, even after one month storage at room temperature. This result confirms that well validated aseptic techniques can guarantee the sterility of CT-P6 preparations. Thus, the extended stability of CT-P6 appeared to depend only on its intrinsic physical and chemical properties.

Discussion

The stability of CT-P6, a biosimilar of TTZ marketed by Biogaran (Herzuma®), has been studied in two contexts: in vials after reconstitution (21 mg/mL) and after dilution in polyolefin bags for final concentrations of 0.8 mg/mL and 2.4 mg/mL. Additionally, two storage temperatures were tested: 4°C and 22°C. Stability was evaluated by several complementary methods.

Compared to the freshly prepared bags, after 28 days of storage at 4°C and 22°C, no sign of physical instability was observed, including no formation of submicron, micron or particulate aggregates. Gel exclusion chromatography revealed no oligomer formation or breakdown of the compound's molecular structure. Furthermore, ion chromatography did not show any significant change in the distribution of the ionic variants after 90 days storage at 4°C, indicating no chemical modification of the compound's initial structure, in particular by hydrolysis (asparagine deamidation or isomerization). However, after 28 days storage at 22°C, significant signs of hydrolysis were observed.

Second derivative UV and fluorescence-derived spectral analysis showed no change to the tertiary structure of the antibody when stored at 4°C for 28 days. On the other hand, small changes to the tertiary structure were observed after storage at 22°C for 28 days and 90 days at 4°C. However, thermal denaturation curves were identical between batches and these

storage conditions, suggesting no strong destabilization of the three-dimensional structure even after 90 days storage at 22°C. Identical results were observed for the drug in vials after reconstitution. The consequences of the modification of tertiary structure (possibly the cause of increased hydrodynamic diameter) after 90 days storage at 4°C on pharmacological activity remain unclear. However, because there were no signs of chemical degradation nor aggregation, they are likely to be very limited.

The relative percentages of the peaks we identified differ to those published by Harris et al. for the TTZ originator. The main peak (n° 2) represented 88% for the biosimilar and 73.8% for the originator. The peak of Asn deamidation represented 9.27% for the biosimilar vs 13.6% for Harris et al. The ion chromatography method we used differs slightly from that of Harris et al., however enabled excellent relative integration of the peaks and therefore cannot explain this difference. It should be noted that variants other than the main peak represent degradation products. It is also noted that under stress conditions (storage 90 days at 22°C; see Figure 3), the main peak decreased significantly, with a concomitant increase in acidic and basic peaks, confirming that these peaks are indeed degradation products. It is interesting to note that the major deamidation peak B (denoted IEX-1 by Harris), representing the hydrolysis of Asn in position 30 on the light chain, only represents 70% of the biological activity of the major peak. This would suggest that CT-P6 has fewer degradation products than the originator batch analysed by Harris et al.

It can be concluded that, after dilution under sterile conditions with 0.9% NaCl in polyolefin bags at the concentration range of 0.8 to 2.4 mg/mL, the biosimilar TTZ CT-P6 remained physically and chemically stable for at least 90 days, when stored at 4°C and away from light. When stored at 22°C for 28 days, tertiary structure appeared to be slightly altered and there were signs of hydrolysis, but without any noticeable aggregate formation. The same conclusions can be made for reconstituted vials at 21 mg/mL.

Conclusion

Our results have important implications for hospital use of CT-P6, justifying advanced preparation and implementation of dose-banding strategies. We also suggest an efficient response to unwanted events such as accidental breaks in the cold chain or the presence of un-administered drug, i.e. providing a drug dilution previously prepared but not administered. In particular, the ability to dilute this drug could have major implications for the paediatric use of products previously packaged for adult patients.

These practices could improve patient management, reduce nurse and pharmacy workload and avoid unjustified losses of this expensive drug. Moreover, the extended stability demonstrated here could improve the general acceptability of biosimilars by practitioners and encourage pharmacists to make an objective choice for biosimilar TTZ during tenders.

Considered alongside previously published data on infliximab and rituximab biosimilars [17, 18], these results confirm that a TTZ biosimilar could exhibit the same extensive stability as its originator, and thus justify its use under the same conditions.

Our results also highlight the importance of academic studies on the in-use stability of drugs. As demonstrated by the numerous papers already accessible on the Stabilis database [24], this topic appears to be a major research field for hospital pharmacists and should be encouraged.

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