TITLE

A novel study demonstrating practical shelf life extension (Beyond Use Dating) of Trastuzumab (Herceptin®, Roche) when stored within a Closed System Transfer Device (CSTD) Tevadaptor® (marketed as OnGuard™ by B.Braun in the United States of America) as a container system for preservative free single use drug vials for up to 28 days when stored 2-8^{o}C protected from light (PFL).

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Abstract

Objective: Evaluation of the stability of a monoclonal antibody Trastuzumab (Herceptin, Roche) when stored within a closed system transfer device (CSTD) Tevadaptor® for up to 28 days at 2-8^{o}C protected from light (PFL), a novel beyond use dating BUD study that is compliant with current USP and NHS guidelines.

Design: A study compliant with USP chapter <797>(1), NHS pharmaceutical quality assurance committee guidelines for deriving and assessment of stability of biopharmaceuticals(5), and with the methods of the USP summary validation report for Trastuzumab(6).

Methods: Trastuzumab was freshly prepared in drug vials fitted with Tevadaptor® CSTD by reconstitution with sterile water for injection (WFI) within an ISO class 5 environment on Day 0. Sampling was performed from test Trastuzumab drug vials (n=4) fitted with Tevadaptor® on days: 0, 7, 14, 21 and day 28. On each day of test control Trastuzumab vials were freshly prepared by reconstitution using standard needle and syringe. Both test (n=4) and control (n=2) Trastuzumab samples were subjected to a range of physicochemical and biological tests for a full assessment of stability. On day 28 samples were withdrawn from the test Trastuzumab vials and used to directly inoculate tryptic soya broth (TSB) (n=2) and fluid thioglycollate media (FTM) (n=2). Sterility testing was performed as per the USP<71> incubating TSB for 14 days at 20-25^{o}C for set one (n=2) and 30-35^{o}C for a second set of TSB media fill vials (n=2). FTM media fills (n=2) were incubated at 30-35^{o}C for 14 days. The vials were inspected on each day for the first 7 days and then on the 8th and 14th day for signs of microbial growth.

Setting: All hospitals in the United States of America, the United Kingdom and Europe within hospital pharmacy compounding unit for aseptic preparation of parenteral products and in the home healthcare setting.

Results: The results show that when Trastuzumab is prepared and then stored at 2-8^{o}C protected from light within the CSTD Tevadaptor® the drug product remains stable for up to

28 days allowing beyond use dating for the first time for a monoclonal antibody product using a CSTD. A range of physicochemical and biological analytical techniques were applied to the study to make a full assessment of stability for Trastuzumab in accordance with current NHS guidance and USP summary validation report documents. In addition sterility testing was performed within an ISO class 5 environment and demonstrated that Tevadaptor® is able to maintain a sterile barrier for up to 28 days extended storage of Trastuzumab.

Conclusions: This study represents a first for establishing extended stability of up to 28 days of a monoclonal antibody drug Trastuzumab when prepared and stored in a CSTD Tevadaptor®. Stability was confirmed using a range of physicochemical and biological analytical methods including a cellular assay and sterility assessment was made on Day 28 of the study by direct inoculation in to TSB and FTM media fills and incubation for 14 days. All media fills showed no sign of microbiological growth confirming the ability of Tevadaptor® to maintain a sterile barrier for up to 28 days for a monoclonal drug Trastuzumab when prepared and manipulated under ISO class 5 conditions.

Introduction

Trastuzumab (TZM) is the INN name for the recombinant humanised monoclonal antibody IgG_1 antibody product sold under the brandname Herceptin® (Roche). In Europe Herceptin is supplied in type I glass vials containing 150mg TZM as a pale yellow lyophilized powder for re-constitution. On addition of 7.2mL sterile water for injection (SWI) this provides a solution at 21mg/m at pH 6.0. This is diluted in 0.9% sodium chloride to the required concentration based on the patient's body mass for infusion from an IV bag. Trastuzumab is indicated for the treatment of adult patients with Human Epidermal Growth Factor Receptor 2 (HER2+) positive metastatic breast cancer (MBC) or HER2+ gastric cancer. Controlled cell proliferation occurs in healthy cells when human epidermal growth factor protein (HER2) binds to its receptor. In some cancers, notably certain types of breast cancer, HER2 is over-expressed, and causes the cells to reproduce uncontrollably. The mode of action of Trastuzumab is believed to be to bind to the surface cell receptor HER2 on cancerous cells and prevent homo and hetrodimer formation from binding with other EGFR proteins. This leads to suppression of intracellular tyrosine kinase, attenuation of cell signaling, and hence suppression of tumor proliferation. Down regulation of HER2 production and inhibition of angiogenesis leads to cell death. There are two approved uses for Trastuzumab in treatment of metastatic breast cancer: (1) as adjuvant therapy with chemotherapy drug Paclitaxel for the first line treatment of Human Epidermal growth factor Receptor 2-positive (HER2+) metastatic breast cancer and (2) for the treatment of HER2-positive breast cancer in patients who have received one or more chemotherapy courses for metastatic disease as a single treatment. Dosing for all indications is according to body mass in the therapeutic range 0.8 to 2.4 mg/Kg. Trastuzumab is also available in 5mL vial at 600mg as a solution for subcutaneous injection. The study reported here only refers to the form supplied for IV infusion. According to the manufacturer's instructions Trastuzumab is only stable for 48 hours following reconstitution with SWI in preservative free single use

drug vials. When Trastuzumab is reconstituted using bacteriostatic water in multidose drug vials it can be safely stored for up to one month however, this is limited to 48 hours if sterile water for injection is used for reconstitution. This suggests that stability assessment is based only on microbiological sterility rather than physicochemical drug stability grounds. There is therefore an opportunity for extending the practical shelf life (beyond use dating) following first puncture by performing a robust assessment of stability of Trastuzumab in Tevadaptor® as a container system when manipulated under ISO class 5 conditions. This should be performed in accordance with USP<797>. Tevadaptor® offers the operator and healthworker an additional engineering control to limit occupational exposure from potential liquid, aerosol and vapour release. In addition to this Tevadaptor® can also provide a sterile barrier to the product allowing beyond use dating to be used. This is only possible if a supporting body of scientific evidence by way of physicochemical, biological stability data and microbiological sterility data is provided to cover the practical "in use" period of 28 days storage within pharmacy. This can lead to a significant reduction in drug wastage at a time when increasing financial pressure is being placed on already stretched healthcare budgets. The study described here provides supporting data for the pharmacist to safely extend the in use period of Trastuzumab when stored in Tevadaptor® closed system for up to 28 days at 2-8oC protected from light.

An independent stability assessment study performed on diluted Trastuzumab in an infusion bag was previously conducted by Kaiser and Krämer in 2011(2). In this study, one month stability following storage at 2-8◦C was found. However the study only partially addressed physico-chemical stability of Trastuzumab and made no attempt to assess biological activity over the storage period. A follow up study was performed by Paul et al in 2013 (3) in an attempt to fully assess the physical and chemical stability of diluted Trastuzumab at clinically relevant concentrations (0.8 and 2.4 mg ml−1) in polyolefin IV bags upon storage for up to six months

at 2-8◦C. Again in this second study no attempt was made to address the biological aspects of Trastuzumab and assess biological stability and function. A more recent publication by Young et al in 2015 makes an evaluation of the physicochemical and functional stability of diluted Reminsa, a biosimilar of infliximab (Remicade, Merck, Sharp and Dohme), upon extended storage(4). This is a study that was designed to be compliant with the current NHS (UK) guidance on assessment of stability of biopharmaceuticals published by the pharmaceutical quality assurance committee of the NHS and includes both physicochemical and biological assessment of drug stability(5). There is a requirement within UK hospitals that all extended stability studies of aseptic parenteral drug products need to be compliant with the NHS guidance documents for acceptance within NHS hospital pharmacy. The study described here meets that standard as well as incorporating the methods and techniques of the USP summary validation report for Trastuzumab published in 2013(6).

More recently there has been increasing concern among health professionals in the safe handling and administration of monoclonal antibody (mAb) and biopharmaceutical drug products. Monoclonal antibodies (mAbs) do not pose as high a risk as some hazardous drugs (HDs) such as antineoplastic agents (unless they are coupled to a cytotoxic payload) and are not included on the current NIOSH list of hazardous drugs(7), however there is a paucity of data on their potential to cause harm to the health worker(8)(9)(10). The only consensus guidelines published on the safe handling of mAbs comes from the Australian healthservice who published a guidance document on the safe handling and administration of MAbs in 2014(11). The use of Tevadaptor® offers not only the possibility of providing a container system to support extension of shelf life of monoclonal antibody and biopharmaceutical drug products within a hospital pharmacy setting but also to safe guard both the healthworker against accidental exposure to potentially hazardous materials and provide a sterile barrier to microbiological ingress. The study described aims to address all of these areas and provide

robust scientific data to support the assessment of stability of Trastuzumab within Tevadaptor®, representing a first for a stability study of a mAb within a closed system transfer device (CSTD). According to the NHS pharmaceutical quality assurance committee data obtained using one container system cannot be extrapolated to another container system, as such the stability data generated with Tevadaptor® and presented in this paper cannot be used to extend the shelf life of Trastuzumab when manipulated by other closed system transfer devices, only when Tevadaptor® closed systems technology is employed.

Study design

There is a paucity of guidance documentation to support beyond use dating and extension of practical “in use” stability for monoclonal antibody products to support practical pharmacy. In the UK, the National Health Service (NHS) have published a guidance document for deriving and assessment of stability for aseptic preparations of biopharmaceuticals as part of the pharmaceutical quality assurance (PQA) committee series of standard protocols, these are locally referred to as the yellow cover documents (YCDs) (5).

The above guidance document was used to help define acceptance criteria and as a template for the current study design. This in combination with the published USP summary validation report for Trastuzumab and the USP<797> were the main guiding documents for performing this beyond use dating study for Trastuzumab when stored in the original container system fitted with Tevadaptor® CSTD for up to 28 days. Table 1 below summarises the requirements of a robust scientific stability study as defined by the NHS guidance alongside the parameters selected for this study.

Table 1. NHS PQA guidance requirements taken from yellow cover document (YCD) for design of a robust stability study compared with parameters employed in this study showing compliance.

Study parameter	**Guidance requirement**	**Parameter employed in this study**
Diluent	Water for injection (WFI)	Water for injection (WFI)
Containers	Type I glass drug vial as supplied.	Type I glass drug vial as supplied for control drug vials. Type I glass drug vial fitted with Tevadaptor® CSTD system for test drug vials.
Storage	Refrigerated 2-8^{o}C, protected from light (PFL) following reconstitution.	Refrigerated 2-8^{o}C, protected from light (PFL) following reconstitution.
Concentrations	Low and high clinically significant.	Tested at clinically relevant concentration for reconstituted product within original container at 21 mg/mL.
Storage period	Normally 6 hours from first puncture when prepared in ISO class 5 conditions according to USP<797>. SPC states 48 hours if stored 2-8^{o}C and if reconstituted under validated aseptic conditions.	28 day storage at 2-8^{o}C protected from light (PFL) in pharmacy refrigerator. Stored when not in use in uncontrolled environment.
Sampling strategy	For studies <6 months, at least 4 sampling points in addition to the baseline (T=0) data.	Sampling performed at day = 0, 7, 14, 21 and 28 at 21mg/mL concentration.

Sampling number	Three independent batches. Three replicates per batch	Three independent batches at the same concentration. Three replicates per batch.

Study parameters

All Trastuzumab drug vials used in the study were reconstituted using sterile water for injection (WFI) in accordance with the manufacturer's instructions for use (IFU) and summary of product characteristics (SPC).(12) The NHS guidance document requires that study concentrations be selected matching both clinically low and clinically high values to allow for interpolation. However in this study as we were only concerned with the extended storage of reconstituted Trastuzumab prior to dilution, thus only one concentration is relevant for the study which is 21mg/mL.

The stability data, in accordance with the NHS guidance document refers only to clinical practice where the Tevadaptor® CSTD system is used to protect the operator and drug product and forms part of the final container system and this data should not be extended to other CSTDs. A sampling strategy of four sampling points in addition to the baseline (day 0) data provides the requisite number of study data points for a 28 day stability study and is compliant with the NHS requirements. Finally three batches of product were tested in triplicate on each test day. A summary of all test methods employed in this study is provided below in Table 2. The selection of test methods was made using both the NHS guidance document and the USP summary validation report for Trastuzumab. Acceptance criteria were taken from the USP document unless absent in which case the criteria defined in the NHS document was applied for acceptability.

Table 2. Summary of physicochemical, biological and microbiological sterility assessment assay methodologies employed in this study taken either from the NHS PQA guidance (YCD) or the Trastuzumab USP summary validation report 2013 and USP<71> with appropriate acceptance criteria adopted.

Assay	Test Days	Acceptance Criteria
Size Exclusion Chromatography (SEC)	0, 7, 14, 21, 28	No interference from the matrix; the quantitative main peak height of the Test injections are within ± 10% of those obtained with Control injections and the qualitative chromatogram of the Test injection is concordant with that of the Control injection; %RSD for % area of the impurity peak (dimer) should be not more than (NMT) 2.0%.
C18 Reverse Phase High Performance Liquid Chromatography (RP-HPLC)	0 & 28	The matrix profile should not show any peak responses within the integration window; the profile of peaks of the standard injection should show both heavy and light chain CDR regions 1, 2 & 3; Test injections should show heavy and light chain CDR regions 1, 2&3 and be comparable with the relative heights of those obtained with Control injections and the qualitative chromatogram of the Test injection is concordant with that of the Control injection.
Visual Appearance	0, 7, 14, 21, 28	Injection remains clear, colourless and free from visible particles
pH	0, 7, 14, 21, 28	Test injections remain within 0.5 pH unit of control injections
Cation Exchange Chromotography	0, 7, 14, 21 & 28	The matrix profile should not show any peak responses within the integration window. Resolution between the main peak of Trastuzumab (K0) and the pre-peak should be NLT 1.3 for

		standard and test solutions. The trastuzumab main peak in test solutions are within ± 10% of those obtained with control solutions and no new acidic or basic variant peaks appear in the test drug solutions that are not present in the control drug solutions.
Sodium Dodecyl Sulphate Polyacryalamide Gel Electrophoresis (SDS-PAGE)	0, 7, 14, 21 & 28	The intensity of principle bands of the test injections are within ± 10% of those obtained with Control injections and no new bands appear in the test drug solutions that are not present in the control drug solutions. Performed under both reducing and non reducing conditions.
Biological Potency - Enzyme linked Immunosorbent Assay (ELISA)	0, 7, 14, 21, 28	Mean biological activity of test injections are within ± 10% of those obtained with Control injections
Biological Activity- Cellular Proliferation Assay	0 & 28	Mean biological activity of test injections is comparable to that of corresponding control injection samples which will be determined using a statistical t- test with n=8 replicates for each test/control within a 96 microwell plate format. Individual potencies should be within the range 80-120%. Placebo (formulation buffer) should not show a dose response. Standard solution should show dose response curve with a fold anti-proliferation of ≥1.3.

Sterility test	28	All test Trastuzumab drug vials should be free of any microbiological growth following the 14 day incubation period. Tested by direct inoculation in to tryptic soya broth (TSB) and fluid thioglycollate media (FTM) and incubation for 14 days.

Materials and methods

Reagents

Water for injection was purchased from Baxter Ltd (Thetford, Cambridgeshire, UK). Sodium phosphate monobasic, sodium phosphate dibasic dehydrate and sodium chloride, NIST pH reference standards, HPLC grade Methanol and Acetonitrile and all other reagents were purchased either from Fisher or Scientific Laboratory Supplies (SLS) in the UK. Water for preparing buffer solutions and HPLC mobile phase was prepared using a MilliQ system and used without further purification. Heat inactivated foetal bovine serum (FBS), Penicillin/Streptomycin, phosphate buffered saline (PBS), Hybri-Care (ATTC-46-X) were all purchased from LGC (ATCC) in the UK. Alamar Blue (Resazurin) (PN 13494309) and 1-step Ultra-TMB substrate (10647894) were purchase from Thermo Scientific Pierce and trypsin (59428C), tween 20 (CHE3852) from SLS, trypsin for protein digestion and Carboxypeptidase B were both purchased from Sigma Aldrich. Bovine serum albumin (BSA) (A9647) was purchased from Sigma Aldrich.

Materials

Trastuzumab 150mg vials (Herceptin, Roche Pharma AG) was supplied by Andacon N.V. in Belgium (Batch: H4429B03, expiry date: 30/April/2018). Protein 230 assay kits (5067-1517) for performing capillary electrophoresis on chip were supplied by Agilent in the UK and were used as supplied. Human anti-Trastuzumab (HCA166), human anti-Trastuzumab clone 18018 (HCA168), Lynx rapid HRP kit (LNK001P) and HRP stabilising diluent (BUF052A) were all

purchased from BioRad in the UK. Tevadaptor® (marketed as OnGuard™, B. Braun, in the United States of America) CSTD systems were all supplied direct from Teva Medical in Israel. Tevadaptor®/ OnGuard™, syringe adaptor (PN MG245567) and Tevadaptor®/ OnGuard™, 20mm cap size vial adaptor (PN MG245548). Two part lubricant free luer-lock inject syringes (2mL and 5mL) from B. Braun were used for reconstitution of Trastuzumab. HTB-20 (BT474, ATCC) (Lot 61136823) cell line was purchase from LGC standards. 96 well solid black flat bottom TC-treated microplates (3916) were purchased from SLS. Corning T75 tissue culture treated flasks, stripettes (5, 10, 25 and 50mL) and 0.22 micron media filter flasks were purchased from Fisher Scientific. RP HPLC columns: Acclaim^{TM} C18 reverse phase column with dimensions 100 mm × 2.1 mm and USP packing material L1 of particle size 2.2 microns and 120 Ångstrom pore size was supplied by Thermo Scientific, UK. Tosoh Biosciences G3000SWXL size exclusion column dimensions 7.8mm x 30cm, 250 Angstrom pore size, 5 micron packing (L59) (PN Y00495) and Tosoh TSK gel CM-STAT column with dimensions of 4.6 mm ×100 mm (PN 821966) and packing material particle size 7µm USP L53 (Sigma Aldrich, UK). A micro pH combination glass electrode (part 10655091) for measurement of pH in small volume and PD-10 Sephadex G-25 columns were purchased from Thermo Fisher, UK.

Methods

Trastuzumab products used in this study were all prepared in accordance with the instructions outlined within the summary of product characteristics for Herceptin (150mg, Roche). All manipulations were performed under ISO class 5 aseptic conditions within a LAF and using validated pharmacy processes in line with good manufacturing practice (GMP). Both test Trastuzumab vials and control vials were manipulated and re-constituted under identical conditions except that the reference control drug vials were re-constituted using a standard needle and syringe approach. All Trastuzumab drug vials were reconstituted with 7.2 mL of

sterile water for injection (WFI) using two additions, one from a 5mL two part lubricant free luer lock syringe the second from a 2mL two part lubricant free luer lock syringe for greater accuracy, furnishing a drug solution at a concentration of 21 mg/mL.

On day 0 of the study Trastuzumab drug vials (n=4) were fitted with the Tevadaptor® CSTD system following the manufacturer's instructions for use (IFU). Two additional control Trastuzumab drug vials (n=2) were also freshly reconstituted using water for injection (WFI) using a standard needle and syringe approach. All handling of the drug vials was performed in an ISO Class 5 laminar air flow hood sited in an uncontrolled environment.

Samples were taken and analysed on the day of production of the Trastuzumab test product day 0 and then on days: 7, 14, 21 and 28. At each time point an aliquot (0.5 mL) was withdrawn from each of the test drug vials (n=4) using the closed system and this was subjected to analysis using the suite of physico-chemical, biological and microbiological techniques to assess the physical, chemical, biological function and sterility profiles of Trastuzumab at each time point.

On each day of test two replicate Trastuzumab (n=2) drug vials were freshly reconstituted according to the instructions outlined in the summary of product characteristics and using a standard needle and syringe approach. These product vials were used as the reference control samples for baseline measurements and used to compare with the test Trastuzumab samples.

Forced degradation

The stability indicating nature of each of the test methods was established by performing forced degradation studies (data not presented). Although less well understood than their small molecule counterparts, monoclonal antibody molecules undergo degradation through exposure to a number of stress conditions. Each of the stress conditions: low pH (10mM HCl), high pH (10mM NaOH), oxidation (hydrogen peroxide) and exposure to ultraviolet light at short wavelength (365nm) were used to assess different potential degradation pathways for Trastuzumab. For example the exposure of Tratsuzumab to ultraviolet light is known to induce dimer formation. As part of the system suitability tests, the dimer product should be synthesised and used to demonstrate that the technique of size exclusion chromatography (see method section 3.8.1 below) is stability indicating by resolving the dimer from the monomer. The ability to indicate stability was successfully tested with this forced degradation approach and applied to most test methods. For the liquid chromatographic methods similarity of the chromatograms using diode array as the detection method of choice was established based on peak purity analysis and a match score analysis available within the Chromeleon software (v6.8) operating under Windows 7 control.

Visual characteristics

Visual inspection of duplicate test samples were performed to evaluate the solution for clarity, color and the presence of particulates. The visual inspection was performed against white and black backgrounds (matt) using a white fluorescent light as per the British Pharmacopoeia(13). The acceptance criteria for stability, was that the solution remain clear and free from visible particles when viewed under the different lighting and background conditions. The Trastuzumab drug solution should either remain colorless or should maintain the same color as that at the start of the study and reference control vials freshly reconstituted on the day of test

and in accordance with what is stated in the summary of product characteristics from the manufacturer.

pH

Drug solution was transferred to a mini Eppendorf tube to facilitate pH measurement using a small volume micro glass pH electrode. The pH was measured on duplicate test or control samples using a calibrated pH meter (Orion model 420A) in conjunction with a micro standard single junction glass pH electrode. The pH meter was calibrated on each day of use prior to measurement of samples (using NIST traceable standard solutions at pH 4.0; pH 7.0 and pH 10.0 as supplied by Fisher UK). Linearity checks were performed on the pH measurement system prior to use. Triplicate measurements were taken and the mean average values presented with the standard deviation (SD). The acceptance criteria for indicating drug stability was for all test solution samples to remain within ±0.5 pH unit of the appropriate control sample solution. Results are presented as the average of triplicate measurements recorded.

Sub-visible particle counting

Particle counting was performed using an industry standard Hiach Royco 9703 (SN E10658, Beckman Coulter) light obscuration instrument. The instrument was fitted with a HRLD400 (SN E01006) and was recently performance qualified (PQ) using NIST standard microspheres (Thermo Scientific). Signal to noise was established as 3.01 milliVolts at 2 microns with resolution of 1.83% at 10 microns. The sensor demonstrated excellent linearity from 2 to 100 microns. Prior to use on each day of test the instrument was subjected to system performance checks using NIST microsphere counting standards (3000 particles/mL at 15 microns) and NIST size standards (10 and 25 microns). Sampling was performed using 20mL of sample solution rather than 25mL as required by the USP to reduce product burden. The mean average of three particle count runs was determined and used as a final measurement. Particle free water counts per mL at 10 and 25 micron cumulative counts were not deducted as the particle counts

for the water were close to zero. Data was analysed using the supplied PharmSpec software version 2.0 and further processed offline using a validated Microsoft excel spreadsheet (Microsoft office 2013). Testing of Trastuzumab samples stored in vials closed with Tevadaptor® and accessed using the Tevadaptor® system was performed at the following time points: day 0, day 7 and day 30 during an extended "in use" period of 30 days. Due to the product volume requirement of the assay separate drug vials were used for this aspect of the testing.

The Trastuzumab test samples were analysed as a low volume parenteral product <100mL and product was co-collected from more than one test vial (n=5) and combined in a clean container to prepare samples for analysis. Test Trastuzumab samples were removed from the Tevadaptor® closed system using a 5mL lubricant free two part B. Braun injekt syringe and Tevadaptor® syringe adaptor on each day of test.

The acceptance criteria was taken from USP<788> (14) for parenteral injections of low volume <100 mL which states that particle counts should not exceed 6000 particles per container at 10 microns or greater and should not exceed more than 600 particles per container at 25 microns or greater. The stability indicating nature of this method was not confirmed.

Physicochemical analysis

Size Exclusion – High Performance Liquid Chromatography (SEC-HPLC)

Size exclusion HPLC (SEC-HPLC) was performed using an Ultimate 3000 UHPLC system (Thermo Scientific, UK) according to the size exclusion method provided in the USP validation report for Trastuzumab. The equipment consisted of the following modules: LPG-3400SD Gradient Pump unit, a SR-3000 solvent rack, a WPS-3000TSL analytical autosampler, a TCC-3000SD Column Thermostat, and a DAD-3000 Diode Array Detector. Trastuzumab samples were eluted using a TSKgel® G3000SWXL size exclusion column (Tosoh Biosciences,

Stuttgart, Germany) with dimensions 7.8mm x 300mm and 5 micron particle size with internal pores 250 Angstrom, as per the USP packing L59 (PN Y00495). The mobile phase used was 0.2 Molar potassium phosphate with 0.25 Molar potassium chloride solution prepared using MilliQ water. The pH was adjusted to 6.2 using sodium hydroxide solution. The injection volume was 20 micro Litres with an isocratic flow rate of 0.5 mL/minute over 35 minutes and detection was made using a diode array detector set to 280nm wavelength. Data was acquired and analysed using Chromeleon version 6.8 running under the windows 7 operating system. Trastuzumab drug concentrations were obtained by calculating the area under the peak in combination with a linear regression response curve obtained using Trastuzumab RS reference standards versus absorption at 280nm. A full validation of the method was performed to test for system suitability, demonstration of accuracy, precision, and linearity over the concentration measurement range. Verification was performed that the limits of detection (LOD) and lower limits of quantitation (LLOQ) were adequate for robust evaluation of Trastuzumab at clinically relevant concentration used in this study. Forced degradation studies were also conducted as per the USP guidance document to demonstrate that the method was stability indicating. Specifically the method was assessed for its ability to separate excipients from Trastuzumab drug substance (DS) including both the monomeric active pharmaceutical ingredient (API) and the high molecular weight impurity dimer. The method was demonstrated to be capable of separating all species and to be able to separate the Trastuzumab monomer from dimer with the required resolution according to the USP criteria of not less than (NLT) 2.0 minutes. Forced degradation of Trastuzumab RS at 10mg/ml was performed to generate an enhancement in dimer contribution as a control. To this end, the Trastuzumab 10 mg/ml solution was exposed to UV light (354nm) for 2 hours. Following exposure to UV light the percentage of high molecular weight impurity dimer increased from around 0.2-0.3% to 1%. No further attempt to degrade the Trastuzumab drug substance was made.

C18 Reverse Phase – High Performance Liquid Chromatography (RP-HPLC)

The procedure was performed only on test days Day 0 and Day 28. An Ultimate 3000 UHPLC system (Thermo Scientific, UK) according to the size exclusion method provided in the USP validation report for Trastuzumab. The equipment consisted of the following modules: LPG-3400SD Gradient Pump unit, a SR-3000 solvent rack, a WPS-3000TSL analytical autosampler, a TCC-3000SD Column Thermostat, and a DAD-3000 Diode Array Detector. Trastuzumab samples were eluted using an Acclaim^{TM} C18 reverse phase column with dimensions 100 mm × 2.1 mm and USP packing material L1 of particle size 2.2 microns 120 Ångstrom pore size (Thermo Scientific, UK) as per the USP packing L1.

The mobile phase gradient flow used started with 98% water containing 0.1% Trifluoroacetic acid (TFA) and 2% Acetonitrile containing 0.1% Trifluoroacetic acid (TFA) for five minutes before increasing to 45% Acetonitrile containing 0.1% Trifluoroacetic acid (TFA) over 115 minutes before changing to 100% Acetonitrile containing 0.1% Trifluoroacetic acid (TFA) at 125 minutes and this being maintained until the end of the run at 130 minutes flowing at 0.3 mL/minute and detection set to 214nm. The injection volume was 10 micro Litres. Data was acquired and analysed using Chromeleon version 6.8 running under the windows 7 operating system. Trastuzumab complimentary binding domain regions (CDRs) were identified using mass spectrometry (data not shown) and then assigned to the chromatographic profile obtained from using the UHPLC-Diode Array. This instrument was then used for all study time point anlaysis. Drug concentrations were not calculated for this method but relative retention times based for each peptide CDR fragment was assessed based on an internal reference peak with retention time of 89 minutes. Each of the heavy and light fragment peak CDRs 1,2 and 3 were assigned for all study time points and used to assess the stability profile of stored Trastuzumab.

Test and control Trastuzumab samples were prepared in the following way: a 48 micro Litre Trastuzumab RS sample at 21mg/mL in water was mixed with 452 micro Litres of 6 Molar Guanidine hydrochloride containing 1mM EDTA in 0.25 Molar tris buffer (pH 7.5). To this was added 10 micro Litres of 0.5 Molar Dithiothreitol (DTT) in water. The mixed solution was incubated at 37^{o}C for 30 minutes before adding 24 micro Litres of 0.5 Molar Iodoacetamide and incubating for a further 30 minutes in the dark at room temperature. Finally 10 micro litres of 0.5 Molar Dithiothreitol solution was added to provide stock solution 1. A PD-Sephadex G-25 column was then washed with 20 mL of water, equilibrated with 35mL of 2 Molar urea in 0.1 Molar tris buffer (pH 7.8). The column was then loaded with the stock solution 1 and this eluted using 2 Molar urea in 0.1 Molar tris buffer pH 7.8. Fractions were collected in 750 micro Litre volumes and each measured using UV absorbance at 280nm. Only the fraction providing an absorbance of between 1.3 and 2.0 was used in the next step, this was standard stock solution 2. 50 micro Litres of standard stock solution 2 was then mixed with 2 micro Litres of 1mg/mL trypsin and incubated for 18 hours at 37^{o}C. To this was added 2 micro Litres of Trifluoroacetic acid (TFA) and the standard solution stored at -20^{o}C until analysed. test Trastuzumab samples were prepared similar to that of the standard solution above. All control standard solutions and test Trastuzumab solutions were prepared at the same time using the same reagents.

The purpose of this test procedure was to identify any chemical change within the Trastuzumab peptide chains by performing enzymatic digest with trypsin followed by analysis using reverse phase chromatographic separation of all peptide cleavage products. Any changes in relative intensity or the absence of specific peptide domains within the test Trastuzumab samples would indicate potentially harmful changes upon extended storage. The USP method described visually comparing peptide profiles obtained from the test Trastuzumab samples with those from the freshly prepared reference Trastuzumab RS samples.

Sodium Dodecyl Sulphate Capillary Gel Electrophoresis under reducing conditions (SDS-CE)

The cited CE method in the USP validation report for Trastuzumab was taken and applied using an Agilent BioAnalyzer which performs capillary electrophoresis (CE) on a glass microchip. This was used with the proprietary Agilent Protein 230 assay kit for assessment of impurities from non-glycosylated heavy chain (NGHC) fragments within the Trastuzumab samples. Analysis was performed under denaturing Sodium dodecyl sulphate (SDS) and reducing conditions as per the instructions provided in the Protein 230 kit (1). The reference control non-glycosylated heavy chain (NGHC) samples of Trastuzumab were generated to test system suitability and prepared as follows: 25 micro litres of Trastuzumab RS (4mg/mL) in WFI was mixed with 70 micro litres of 0.1M Tris HCl at pH 9.0 with 1% SDS (as sample buffer) and 5 micro litres of β-Mercaptoethanol. The solution was heated to 70^{o}C for 10 minutes and cooled. 0.38 micro litres of PNGase F enzyme (Fisher) was then added to the cooled solution and this was incubated at 37^{o}C for 15 hours. This was then used as the control Trastuzumab for non-glycosylated heavy chain product to test system suitability and the ability to resolve the glycosylated form from the non-glycosylated heavy chain fragment. System suitability as defined in the USP report for CE was performed for the above instrumental technique (data not shown) and the Protein 230 kit demonstrated equivalent resolution, accuracy, ruggedness, repeatability and linearity to the USP method. Analysis of the Trastuzumab test samples following storage in the Tevadaptor® CSTD container over 28 days was performed and compared with Trastuzumab RS control samples freshly prepared on the day of test and with the baseline Day 0 measurements. The amount of non-glycosylated heavy chain (NGHC) fragment was calculated for both test and control Trastuzumab RS samples as a percentage of the total product and the values reported in the results tables below. Acceptability was taken

from the NHS guidance document for assessment of stability of biopharmaceuticals because of the absence of an acceptance limit for NGHC within the USP validation report. Acceptability was selected for the glycosylated heavy chain at 95-105% of starting concentration.

Sodium Dodecyl Sulphate Capillary Gel Electrophoresis under non-reducing conditions (SDS-CE)

The cited CE method in the USP validation report for Trastuzumab was taken and applied using an Agilent BioAnalyzer which performs capillary electrophoresis (CE) on a glass microchip. This was used with the proprietary Agilent Protein 230 assay kit for assessment of impurities from the loss of a light chain (LC) fragment from the intact Trastuzumab samples which contain two heavy and two light peptide chains and hence designated HHLL. The impurity peak is designated HHL based on the loss of one light chain fragment. Analysis was performed under denaturing Sodium dodecyl sulphate (SDS) and non-reducing conditions as per the instructions provided in the Protein 230 kit (1). The reference control HHL samples of Trastuzumab RS were generated to test system suitability and prepared as follows: 25 micro litres of Trastuzumab RS (4mg/mL) in WFI was mixed with 70 micro litres of 0.1M Tris HCl at pH 9.0 with 1% SDS (as sample buffer) and 5 micro litres of a 0.25M Iodoacetamide (Fisher) solution. The solution was heated to 65^{o}C for 4 minutes and cooled. This was then used as the control Trastuzumab HHL solution for checking system suitability and the ability to resolve the HHL form of Trastuzumab from the intact HHLL form of Trastuzumab under the conditions of the assay. System suitability as defined in the USP report for CE was performed for the above instrumental technique (data not shown) and the Protein 230 kit demonstrated equivalent resolution, accuracy, ruggedness, repeatability and linearity to the USP method. Analysis of the Trastuzumab test samples following storage in the Tevadaptor® CSTD container over 28 days was performed and compared with Trastuzumab RS control samples freshly prepared on the day of test and with the baseline Day 0 measurements. The amount of Trastuzumab HHL

impurity within the Trastuzumab test and control samples was calculated as a percentage of the total protein product and the values reported in table X below. Acceptability was taken from the NHS guidance document for assessment of stability of biopharmaceuticals because of the absence of an acceptance limit for the Trastuzumab HHL impurity within the USP validation report, this was set at the amount of intact Trastuzumab HHLL remaining at the end of the study being between 95-105% of control samples freshly prepared on the day of test.

Cation Exchange Liquid Chromatography (CEX-HPLC)

Cation Exchange HPLC (CEX-HPLC) was performed using an Ultimate 3000 UHPLC system (Thermo Scientific, UK) according to the size exclusion method provided in the USP validation report for Trastuzumab. The equipment consisted of the following modules: LPG-3400SD Gradient Pump unit, a SR-3000 solvent rack, a WPS-3000TSL analytical autosampler, a TCC-3000SD Column Thermostat, and a DAD-3000 Diode Array Detector. Trastuzumab samples were eluted using a Tosoh TSK gel CM-STAT column with dimensions of 4.6 mm ×100 mm (PN 821966) and packing material particle size 7µm USP L53 (Sigma Aldrich, UK). The column temperature was set to 45^{o}C and injection volume was 80 micro Litres.

The mobile phase used was a gradient starting at 100% 10 mM sodium phosphate buffer solution (pH 7.5) for five minutes changing to 70% 10 mM sodium phosphate buffer (pH 7.5): 30% 10 mM sodium phosphate buffer with 100 mM sodium chloride solution (pH 7.5) at 43 minutes and then changing to 0% 10 mM sodium phosphate buffer (pH 7.5): 100% 10 mM sodium phosphate buffer with 100 mM sodium chloride solution (pH 7.5) at 44 minutes. At 46.01 minutes the percentage of 10 mM sodium phosphate buffer solution (pH 7.5) was increased to 100% and this was then maintained until 55 minutes and the end of the run at a constant flow rate of 0.8 mL/minute. Detection was made using a diode array detector set to 280nm wavelength.

Carboxypeptidase B Enzyme stock solution was prepared by dissolving 1.9 mg Carboxypeptidase B Enzyme (lyophilized) in 380 µL Milli-Q water. This was then stored in a pharmacy refrigerator at 2-8°C, for a period up to three months. Standard Trastuzumab RS control solution was prepared by adding 2 micro litres of the above Carboxypeptidase B Enzyme stock solution to 200 micro Litres of a 1mg/mL USP Trastuzumab RS sample and this incubated for 2 hours at 37ºC. Test Trastuzumab solutions were prepared in an analogous manner to the control Trastuzumab samples and these were prepared at the same time using the same reagents. It was decided not to continue with preparing the resolution solution which contains an inhibitor for the enzyme resulting in zero cleavage of terminal L-lysine residues as it was discovered that the batch of Trastuzumab used in the study had no charge variation and no intact L-Lysine modifications to undergo cleavage by this process.

Data was acquired and analysed using Chromeleon version 6.8 running under the windows 7 operating system. Trastuzumab drug concentrations were obtained by calculating the area under the peak in combination with a linear regression response curve obtained using Trastuzumab RS reference standards versus absorption at 280nm. A full validation of the method was performed to test for system suitability, demonstration of accuracy, precision, and linearity over the concentration measurement range. Verification was performed that the limits of detection (LOD) and lower limits of quantitation (LLOQ) were adequate for robust evaluation of Trastuzumab at clinically relevant concentration used in this study.

The acceptance criteria for resolution of the method taken from the USP could not be assessed due to the lack of basic charge variants in the reference Trastuzumab RS solution. The USP does not state an acceptable limit for the amount of Trastuzumab (K0) following treatment with caboxypeptidase in test samples compared with control samples. Therefore we applied the

criteria that all test Trastuzumab samples should be within 95-105% of the starting concentration for the Trastuzumab (K0) peak compared to control Trastuzumab RS samples freshly prepared using standard needle and syringe approach. This meets the UK NHS guidance requirements for assessment of stability of biopharmaceuticals. The data supports the data obtained from the orthogonal technique of size exclusion chromatography.

Biological activity assay

Determination of the biological activity of Trastuzumab samples was assessed by their ability to interfere with cell proliferation using the adherent human carcinoma derived cell line HTB-20 supplied by ATCC. The method used was taken directly from the USP validation report and used a Thermo Scientific Ascent Fluoroskan microplate reader to report the fluorescence readout from the pro-fluorescent substrate Alamar Blue (Resazurin). The microplate reader was set up for excitation at 530nm and emission at 590nm using a filter set supplied by Thermo scientific. A black 96 well plate that was Nunc coated for tissue culture was chosen to reduce extraneous fluorescence from the microplate substrate and eliminate stray background light.

In addition to the USP method a second in house method was developed and validated based on an enzyme linked immune sorbent assay (ELISA) for Trastuzumab. This was performed in addition to the cellular assay because of the lower inherent variability of the ELISA for reporting biological activity values so as to comply with the NHS guidance document criteria for assessment of stability of biopharmaceuticals

Enzyme Linked Immuno Sorbent Assay (ELISA)

Trastuzumab samples were analyzed according to a modified protocol supplied by AbD Serotec (now Biorad, UK) (1) who manufactured and supplied the HCA-166 Human Anti-Trastuzumab (Fab antibody, V5 and StrepX-StrepX-tagged) as well as the HCA168 Human anti-Trastuzumab (Fab FLAG and HIS-6-tag) antibodies used in this test method. A LYNX rapid Horse Radish peroxidase (HRP) conjugation kit supplied by AbD Serotec was used as

supplied to conjugate HRP to the detection antibody HCA168. HCA166 (0.5mg/ml) was used as supplied following dilution down to a working concentration of 1 microgram/ml by dilution in phosphate buffered saline (PBS). A standard control Trastuzumab RS sample was prepared on each day of test by reconstitution from lyophilised drug in 7.1 mL water for injection, after reconstitution this was stored at 5±3°C and used for analysis on the day of test. Four vials of HCA166 as received were mixed making 800µL HCA166 at 0.5 mg/mL. To this was added 80 µL LYNX Modifier reagent from the LNK003P LYNX Rapid Conjugation kit®. This mixture was then pipetted directly onto lyophilized LYNX mix from the LNK003P LYNX Rapid Conjugation kit® and pipette up and down twice to re-suspend. The solution was then incubated for 3 hours at room temperature. Finally 80µL of the LYNX Quencher reagent was added from the LNK003P LYNX Rapid Conjugation kit®. This was left the for 30 min room at temperature. This furnished a 1mL ready-to-use conjugated HRP-HCA166 antibody conjugate at 0.5 mg/mL The conjugate was stored in a pharmacy refrigerator for up to 2 weeks at 5±3°C.

Coating of 96 well plates with the capture chemistry was achieved by first preparing the Anti Trastuzumab antibody solution (2 µg/mL). 5 mL was needed for coating a 96 well plate. 20 µL anti Trastuzumab antibody stock solution (0.5mg/mL) was diluted to 5 mL with PBS. This was used the same day.

The anti Trastuzumab antibody (4 µg/mL) detection solution was prepared in the following way: 80 µL HRP conjugated anti-Trastuzumab antibody stock solution (0.25mg/mL) was diluted to 5 mL with PBS + 1% BSA. Plate wash solution was prepared by adding 0.5 mL Tween®-20 to 1 Litre of PBS.

Plate blocker solution was prepared in the following way: 10.0 g BSA was dissolved in approximately 80 mL PBST and the volume adjusted to 100mL with PBST. This solution was also used for sample dilution if required.

The detection antibody was diluted where required using the solution prepared by: 0.5 g BSA was dissolved in approximately 40 mL PBS and the volume was adjusted to 50 mL with PBS. A working stock of Trastuzumab was created by diluting 4 µL stock Trastuzumab solution (21 mg/mL) with 836 µL ultrapure water to give 840 µL working stock at 100 µg/mL. This was then used to create a set of 9 reference standard Trastuzumab stocks by serial dilution in the range of 0-500 ng/mL using PBS as diluent.

The format of for the ELISA was a standard sandwich ELISA using anti-Trastuzumab capture antibody onto which Trastuzumab from the test or control samples binds. Detection was obtained through a HRP conjugated anti-Trastuzumab detection antibody which bound selectively to Trastuzumab. 50 µL of anti-trastuzumab antibody coating solution (2 µg/mL) was added to each well on the 96 well plate, this was covered with plate sealer and incubated overnight (20 hours) at 5±3°C. The plate washed with 300 µL washing solution, four times. The plate was then blocked with 150 µL blocking solution (PBST 10% BSA buffer), and incubated for 60 minutes (37°C) in an incubator. Following washing of the plate with 300 µL wash solution four times, 50 µL of the diluted samples were added along with Trastuzumab calibration standards in triplicates to the plate. The plate was incubated for 60 minutes at RT on a plate shaker. The plate was then washed with 300 µL washing solution five times on plate washer. 50 µL HRP conjugated anti trastuzumab antibody detection solution (4 µg/mL) was then added to the plate. The plate then incubated with the HRP conjugate for 60 minutes at RT on plate shaker. Washing the plate was performed with 300 µL washing solution, and 50 µL Ultra TMB substrate diluted 1:1 with ultrapure water was added and incubated for 5 minutes. Finally a 50 µL stop solution aliquot was added to the plate and the OD measured within 30

minutes on a Biotek Epoch Microplate Reader at 450 nm with a 620 nm reference filter. Data collection was performed using the proprietary Gen5 software supplied with the Biotek instrument. The analysis software used a 4 parameter fit algorithm to fit all of the experimental data to the curve. This was assessed as the best fit for the data and the recommended standard fitting algorithm for interpretation of ELISA binding data.

The methods section in the USP summary validation document did not contain any reference to the use of an ELISA for determining biological potency and so the acceptance criteria of 80-120% was selected for the assay based on the principles of the UK NHS guidance.

Cellular Potency Assay (Anti-proliferation assay)

The USP summary validation document for the assessment of stability of Trastuzumab using a cell based anti-proliferation assay was followed to assess biological potency of test Trastuzumab following preparation and storage with Tevadaptor® systems over 28 days. The test samples were compared with control Trastuzumab RS samples freshly prepared on each day of test for Day 0 and Day 28 only. The approach used a human breast carcinoma cell line that over expresses the surface cell receptor HER2, which is the target for Trastuzumab. Automated cell counting equipment and automated liquid cell suspension handling systems were employed in the USP validation work which resulted in high repeatability in dispensing and seeding cells in to 96 well plates. The study described here used manual cell loading and preparation of 96 well tissue culture plates and hence has a higher variability. The acceptance criteria for system suitability based on repeatability is a dose response curve with a fold anti-proliferation of ≥1.3, whereas placebo should show no dose response according to the USP guidance document.

HTB-20 (LGC, UK) Cells were sub cultured in T75 flasks (Nuncleon) using Hybricare media (LGC, UK) supplemented with 10% FBS and incubated at 37^{o}C in 5% CO_2 and high relative humidity (RH). Cell suspensions were harvested from T75 flasks at 70% confluence and

prepared at a concentration of 0.9-1.0 x 10^5 cells per mL in Hybricare media with 2% FBS. To the test and control wells on a 96 well plate was added 100 micro litres of cell suspension. Media control wells contained 100 microlitres of Hybricare+2% FBS media. The plate was shaken to mix and incubated for 3 hours at 37^{o}C and high RH. Each test and control well on the 96 microwell plate had an appropriate serially diluted Trastuzumab drug solution added (100 microlitres) and the plate was shaken to mix. In total ten control drug dilution solutions were prepared using a stock Trastuzumab (RS) at 2.5 micrograms/mL and diluting 1:1 each time down to the lowest concentration of 0.005 micrograms/mL. The same was done for all test Trastuzumab samples giving ten separate test Trastuzumab samples in the range of concentrations from 2.5 microgram/mL down to 0.005 microgram/mL. Each concentration was added in triplicate wells to the plate. Negative controls (Hybricare + 2% FBS media) and positive controls using freshly prepared Trastuzumab at 2.5 micrograms/mL were added to every plate (n=3). The plate was mixed and incubated for 90 ± 3 hours at 37^{o}C in a humidified CO_2 incuabtor. Then the plate was removed, 30 microlitres of Alamar Blue was added to each well, swirled and then incubated at 37^{o}C or an additional 7 ± 1 hours. The plate was removed, cooled and placed on plate shaker for 30 minutes. The potency was then determined for each well by recording the relative fluorescent units (RFU) on a Thermo Scientific Ascent Fluoroskan plate reader operating under Thermo Scientific Ascent software and using the 530nm, 590nm excitation and emission filter set. Dose response curves were calcuated for both control Trastuzumab RS and test Trastuzumab solutions (n=3). All control Trastuzumab samples were prepared using a standard needle and syringe approach and appropriate system controls were run using negative and positive samples. On each day of test a number of wells containing Alamar blue were added to a new plate and autoclaved at 120^{o}C according to the manufacturer's instructions for use. This generated the fully reduced Alamar blue product for calculating dose responses (100% fluorescence value). All

manipulations of the cell line were done using good aseptic technique and in accordance with standard tissue culture procedures in a LAF.

The potency assay for test Trastuzumab compliments the Trastuzumab ELISA biological binding assay in terms of assessment of biological function providing additional information as to the functionality of the test Trastuzumab product following preparation and storage in Tevadaptor® systems. The challenge with all cellular assays is repeatability and variance in the data. Figure 1 below shows a characteristic raw fluorescence response curve for control Trastuzumab (RS) versus test Trastuzumab solutions following preparation and storage in Tevadaptor® systems.

Microbiological Sterility Testing

Validated 20-25°C (+/- 1°C) and 30-35°C (+/- 1°C) incubators with continuous temperature monitoring using a validated Comark temperature monitoring system were used for the study. Laminar air flow hood (LAF) Certified to EUGMP Class A according to BSEN14644; EU GMP; COSHH 2002; Regulation 9 was sited inside a grade B (ISO class 7) environment. This was used to prepare the growth media vials for use in the sterility study. The tryptic soya broth (TSB) and fluid thioglycollate (FTM) media vials were produced in a single batch and incubated at 30-35°C for 14 days as part of the quality control and only media vials showing no growth were released for use in the study. Media growth vials from the same batch were subjected to growth promotion testing as per the USP<71> method and was shown to support the growth of all ATCC organisms defined in the USP pharmacopeial method. A second LAF certified EU GMP Class A according to BSEN14644; EUGMP; COSHH 2002; Regulation 9 was used during sampling and manipulation of Trastuzumab test vials and this was sited in an uncontrolled environment. The three aliquots (1mL each) for sterility testing were withdrawn by manipulating the closed system vials (n=2) after the final withdraws had been made for

physicochemical and biological testing on the 28th day of the study. The aliquots were placed directly in to the TSB (n=2) and FTM (n=2) growth media. Acceptance criteria was that following direct inoculation of test and control Trastuzumab aliquots (1mL) in to TSB and FTM growth media and incubation, the vials were inspected on each day for the first 7 days and then on the 8th and 14th day for signs of microbial growth.

Direct inoculation in to tryptic soya broth (TSB) media (n=2) and incubation at 20-25°C (set 1) and 30-35°C (set 2) for 14 days.

On day 28, the OnGuard™ vial adaptor caps (n=2) were removed from TEVADAPTOR® vial adaptors (n=2) attached to two reconstituted Trastuzumab test drug vials (n=2), the elastomers were swabbed with sterile IPA 70% preparation pad and left to dry for 2 minutes. Two TEVADAPTOR® syringe adaptors (n=2) were aseptically removed from their packaging and each was attached to a 2 ml syringe. One TEVADAPTOR® syringe adaptor and syringe was connected to the TEVADAPTOR® vial adaptor attached to each test drug vial of Trastuzumab and 2.0 ml drug was withdrawn from the test drug vial. The protective cap was removed from four vials of Tryptic Soy Broth growth medium (TSB), the rubber stoppers were swabbed with sterile IPA 70% preparation pad and left to dry for 2 minutes.

An TEVADAPTOR® vial adaptor was aseptically removed from its packaging and attached to each of the four TSB vials leaving the TEVADAPTOR® vial adaptor cap in place. One TEVADAPTOR® syringe adaptor and syringe containing the 2.0 ml drug sample aliquot was attached to the TEVADAPTOR® vial adaptor on the first of the two duplicate TSB vials for the first Trastuzumab drug sampled from test vial 1. One mL (1.0mL) of the contents of the syringe were transferred into the first TSB vial. The TEVADAPTOR® syringe adaptor, with the syringe attached, was disconnected from the TEVADAPTOR® vial adaptor. The same

TEVADAPTOR® syringe adaptor and syringe containing the remaining 1.0 mL drug sample aliquot within the syringe was then re-attached to the TEVADAPTOR® vial adaptor on the second of the two duplicate TSB vials for the first Trastuzumab drug sampled from test vial 1. The contents of the syringe (1.0 mL) were transferred into the second TSB vial. The Tevadaptor® syringe adaptor, with the syringe attached, was disconnected from the TEVADAPTOR® vial adaptor attached to the TSB vial and discarded. The above procedure was then repeated for the second Trastuzumab test drug vial.

One set of duplicate TSB vials inoculated with 1.0 ml Trastuzumab were incubated for 14 days at 20-25°C. The other set of duplicate TSB vials inoculated with 1.0 ml of Trastuzumab were incubated for 14 days at 30-35°C. The vials were inspected on each day for the first 7 days and then on the 8^{th} and 14^{th} day for signs of microbial growth.

Direct inoculation in to fluid thioglycollate (FTM) growth media and incubation at 30-35°C for 14 days.

On day 28, the TEVADAPTOR® vial adaptor caps (n=2) were removed from TEVADAPTOR® vial adaptors (n=2) attached to the two reconstituted Trastuzumab test drug vials (n=2), the elastomers were swabbed with sterile IPA 70% preparation pad and left to dry for 2 minutes. Two TEVADAPTOR® syringe adaptors were aseptically removed from their packaging and each was attached to a 2 ml syringe. One TEVADAPTOR® syringe adaptor and 2 ml syringe was connected to the TEVADAPTOR® vial adaptor attached to each test drug vial (test 1 and test 2) of Trastuzumab and 1.0 ml of the drug was withdrawn from the test drug vial. The protective cap was removed from two vials of Fluid Thioglycollate growth medium (FTM), the rubber stoppers were swabbed with sterile IPA 70% preparation pad and left to dry for 2 minutes. A TEVADAPTOR® vial adaptor was aseptically removed from its packaging and attached to each of the FTM vials leaving the TEVADAPTOR® vial adaptor cap in place. One TEVADAPTOR® syringe adaptor and syringe containing the 1.0 ml drug

sample aliquot was attached to the TEVADAPTOR® vial adaptor on each of the FTM vials. The contents of each syringe were transferred into the FTM vials. The TEVADAPTOR® syringe adaptor, with the syringe attached, was disconnected from the TEVADAPTOR® vial adaptor attached to the FTM vial and discarded.

The FTM vials inoculated with 1.0 ml Trastuzumab were incubated for 14 days at 30-35°C. The vials were inspected on each day for the first 7 days and then on the 8^{th} and 14^{th} day for signs of microbial growth.

Results

Visual appearance

All Trastuzumab test samples (n=2) closed with the Tevadaptor® system remained clear over the extended storage period of 28 days when stored at 2-8^{o}C protected from light. There was no evidence of any particulate matter in the visible length scale (>100 microns) as viewed with the unaided eye viewed under the different lighting and background conditions of the test method employed. Throughout the study period there was also no evidence for any change in colour or turbidity and the test solutions all remained clear and transparent after the in use period of 28 days.

pH

No significant change in pH was observed for the pH of test Trastuzumab solutions at 21mg/mL stored within the Tevadaptor® system over the extended storage period of 28 days 2-8^{o}C protected from light. A small variation in pH values was observed over the five time points studied but the pH remained within the limits of acceptance throughout the 28 day storage period as can be seen from Table 3 below. None of the test Trastuzumab samples gave a pH reading that was significantly different from the control day 0 values or that stated in the summary of product characteristics for Trastuzumab (Herceptin, Roche) which gives a pH of 6.20.

Table 3. Table shows variation in pH measurements for Trastuzumab test drug vials fitted with Tevadaptor® system and stored over 28 days 2-8^{o}C protected from light versus control drug vials freshly prepared on the day of test for time points: day 0, 7, 14, 21 and day 28.

	Day				
pH measurement	**0**	**7**	**14**	**21**	**28**
pH (test 1)	6.17	6.05	6.15	6.13	6.02
pH (test 2)	6.20	6.13	6.15	6.11	6.06
pH (average) *	**6.19**	**6.09**	**6.15**	**6.12**	**6.04**
pH (control 1)	6.20	6.11	6.16	6.15	6.06
pH (control 2)	6.10	6.06	6.15	6.19	6.01
pH (average) *	**6.15**	**6.09**	**6.16**	**6.17**	**6.04**

All Trastuzumab test drug vials fitted with Tevadaptor® system were within the accepted criteria, of ± 0.5 pH units, of the Trastuzumab control drug vials freshly prepared on the day of test.

Sub-visible particle counting

Sub-visible particles were counted and quantified for all of the Trastuzumab test solutions studied over the extended storage period of 28 days at 2-8^{o}C protected from light when accessed using the Tevadaptor® closed system. Measurements were recorded from 2 microns to 100 microns however only the particle counts per mL at >10 microns and >25 microns are reported in accordance with the USP<788) test method for low volume parenteral injections. At all of the time points studied the particle counts at the >10 micron size bin were larger than those recorded for the >25 micron size bin. The average mean particle count values per mL were found to have a large variation between different containers tested (n=5) although the measurement from each of the time points were below the required acceptability levels

for low volume parenteral products (<100mL dosage) of <6000 10-25 micron particles/container and <600 particles/dosage greater than 25 microns. The data for the study is presented below in Figure 1.

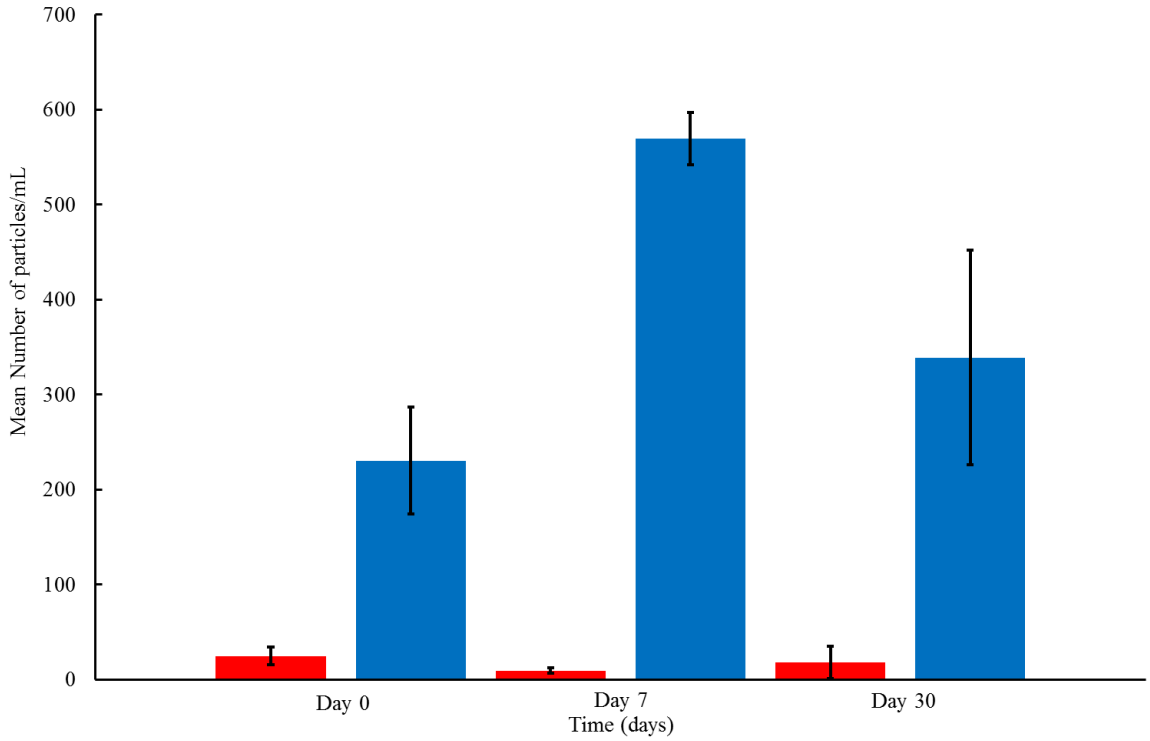


Figure 1. Figure showing the mean particle number in counts per mL from testing of the Trastuzumab test solutions at 21mg/mL and contained using Tevadaptor® systems over 28 days stored 2-8^{o}C protected from light. Particles >10 microns are shown in blue and particles >25 microns are shown in red. Error bars represent ±1 SD.

All test Trastuzumab solutions were shown to have less than the allowed number of particles per container at both >10 micron and >25 micron size bins as stated in the USP<788> for low volume parenteral injections. As such all samples stored using Tevadaptor® CSTD systems were found to meet the acceptance criteria.

Physicochemical analysis

Size Exclusion – High Performance Liquid Chromatography (SEC-HPLC)

A typical chromatogram for Trastuzumab (TZM) RS freshly prepared solution is shown below in Figure 2 with Trastuzumab monomer eluting at a retention time of 16.4 minutes and the high molecular weight dimer impurity eluting at a retention time of 14 minutes. This characterised all SEC-HPLC chromatograms obtained for test and control Trastuzumab samples obtained during the course of the study.

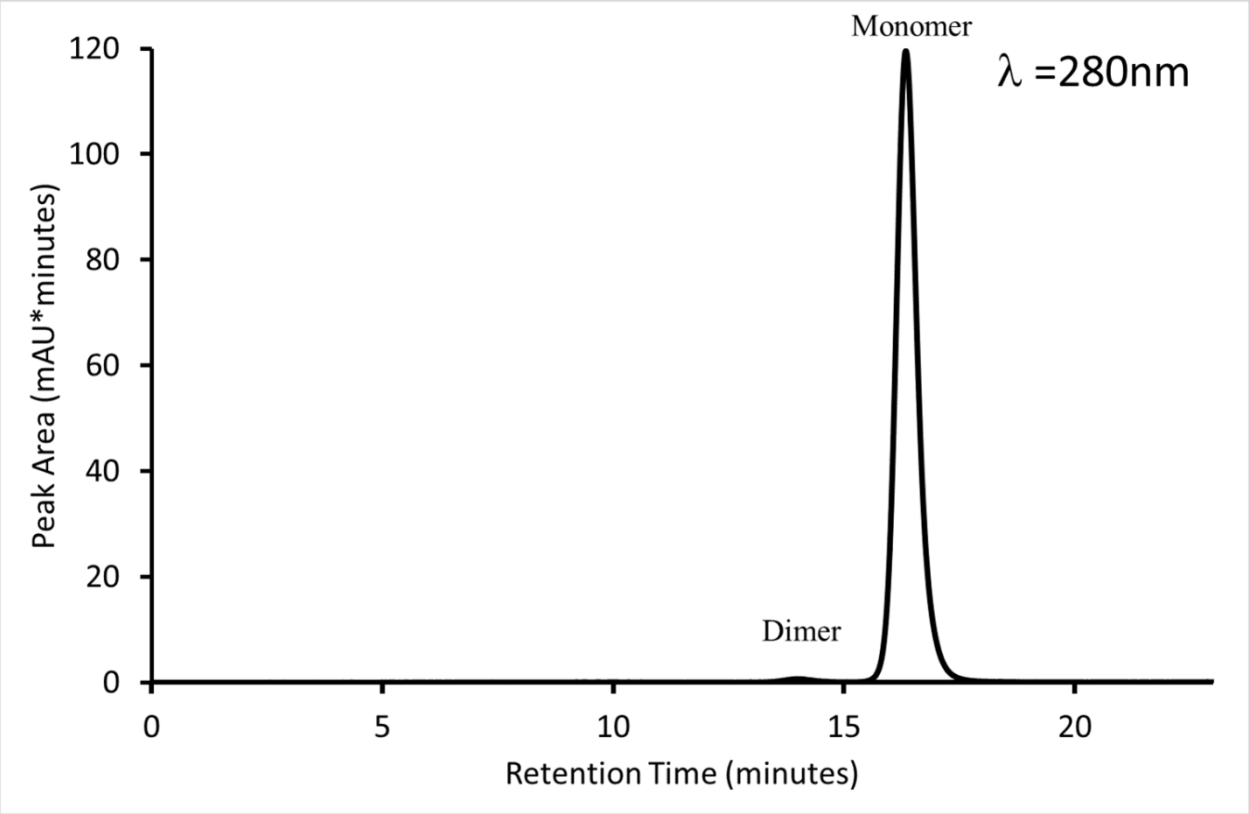


Figure 2. Figure showing a representative chromatographic profile for freshly prepared Trastuzumab RS (10mg/mL) at a detection wavelength of 280nm using the validated SEC-HPLC method.

The resolution between the dimer and the intact monomer peak representing the active pharmaceutical ingredient (API) Trastuzumab was 2.37 minutes well within the acceptance

criteria of not less than (NLT) 2.0 minutes for the USP method. Analyses were performed at two detection wavelengths 214 nm and 280 nm. Data presented in Table 4 and Table 5 below demonstrate the system resolution between the dimer and Trastuzumab monomer peaks. The retention time of the dimer species was consistent with the molecular weight of 300 kDa according to the calibration curve of proteins of varying molecular weight versus retention time (data not shown).

Table 4. Table showing the level of high molecular weight Trastuzumab (TZM) impurity (dimer) present in the 10mg/mL Trastuzumab RS sample freshly prepared and prior to exposure by UV light. Data recorded at 214nm.

UV stability run: Area (mAU*min), 10mg /mL						Average	Std Dev.	%CV
Dimer	5.390	5.398	5.331	5.435	5.439	5.399	0.044	0.8
TZM_{intact}	621.915	621.615	620.906	625.475	625.952	623.173	2.354	0.4
% dimer	1%	1%	1%	1%	1%	0.009	0.000	0.5

Table 5. Table showing the level of high molecular weight Trastuzumab (TZM) impurity (dimer) present in the 10mg/mL Trastuzumab RS sample freshly prepared after exposure to UV light at 365nm for 2 hours. Data recorded at 214nm.

UV stability run: RT (min), 10mg/mL						Average	Std Dev.	%CV
Dimer	14.01	13.99	13.98	13.99	13.99	13.99	0.01	0.1
TZM_{intact}	16.36	16.36	16.36	16.36	16.36	16.36	0.00	0.0
RT diff	2.35	2.37	2.39	2.38	2.37	2.37	0.01	0.6

The Trastuzumab monomer content in all test Trastuzumab solutions stored in vials fitted with Tevadaptor® remained within 95-105% of the starting concentration and within 95-105% of that of freshly prepared reference Trastuzumab RS samples that were reconstituted using a standard needle and syringe approach. The data is presented below in Table 6.

Table 6. Table showing the SEC-HPLC analysis for test Trastuzumab (TZM) monomer given as the mean AUC (mAu min) recorded at 280nm at each time point, when stored 2-8ºC protected from light at a concentration of 21 mg/mL using Tevadaptor® systems (n=2).

	Sampling Date				
Sample ID	**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Test TZM	98.48 ± 3.37	100.22 ± 1.81	101.47 ± 1.81	103.42 ± 6.79	100.07 ± 4.42
Control TZM	100.00 ± 3.57	100.00 ± 3.57	100.00 ± 1.73	100.00 ± 2.22	100.00 ± 1.33
Difference: Control - Test	1.52 ± 3.47	0.22 ± 2.69	1.47 ± 1.77	3.42 ± 4.51	0.07 ± 2.88

No significant change in Trastuzumab concentration was observed throughout the duration of the 28 day study period indicating that concentration levels of the active pharmaceutical ingredient were constant and remained within acceptable limits after storage in Tevadaptor® for 28 days. Furthermore analysis of the percentage dimer contribution for the same test Trastuzumab solutions shows percentage of Trastuzumab dimer (high molecular weight impurity) within Trastuzumab samples prepared using Tevadaptor® systems and then stored for up to 28 days remains identical to that of control Trastuzumab solutions freshly prepared

on the day of test using a standard needle and syringe approach. Data was obtained at 280nm and is presented below in Table 7.

Table 7. Table showing the SEC-HPLC analysis of test Trastuzumab (TZM) dimer given as a percentage of the aggregated AUC (mAu min) for combined dimer and monomer recorded at 280nm for each of the time points when stored at 2-8^{o}C protected from light at a concentration of 21 mg/mL and prepared and stored using Tevadaptor® systems (n=2).

	Sampling Date				
Sample ID	**Day 0**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
% Dimer in Test	0.28 ± 0.04	0.26 ± 0.04	0.20 ± 0.04	0.26 ± 0.05	0.26 ± 0.05
% Dimer in Control	0.26 ± 0.06	0.27 ± 0.03	0.20 ± 0.05	0.29 ± 0.06	0.19 ± 0.04
Difference: Control - Test	0.02 ± 0.1	0.01 ± 0.07	0.00 ± 0.09	0.03 ± 0.11	0.07 ± 0.09

All data support the ability of Tevadaptor® to be used for reconstitution and extended storage of Trastuzumab solutions at 21 mg/mL in the original container for up to 28 days.

C18 Reverse Phase – High Performance Liquid Chromatography (RP-HPLC)

The acceptance criteria states that there should be no peak response in the integration window when matrix only is analysed, also that the profile from the standard Trastuzumab RS samples should give all CDR regions 1,2 and 3 for both heavy and light chains. Following digestion with trypsin according to the above method the test Trastuzumab samples were analysed and compared with the digest from freshly prepared Trastuzumab samples manufactured on the day of test using a standard needle and syringe approach. In all

characteristic chromatograms for Trastuzumab all six CDR regions were first identified and assigned as belonging to either heavy or light chain fragments and a direct comparison made between Trastuzumab profiles for product stored in vials fitted with Tevadaptor® and product that was freshly reconstituted on the day of test. A typical result for test and control Trastuzumab after 28 days storage (test) is shown in Figure 3 below.

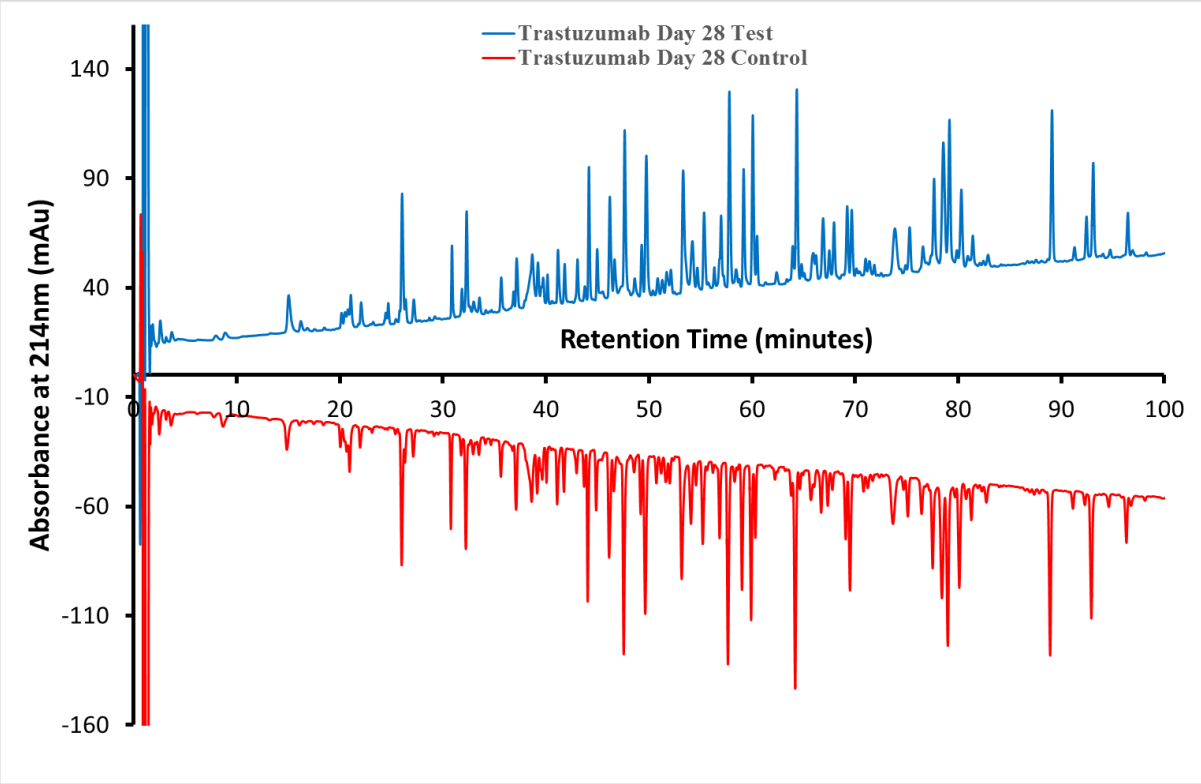


Figure 3. Figure showing the Day 28 C18 Reverse Phase High performance Liquid Chromatography (RP-HPLC) analysis of test Trastuzumab samples following 28 day storage at 2-8^{o}C protected from light within the Tevadaptor® closed system versus control samples freshly prepared on the day of test. Data collected at 214nm.

As can be seen from the above figure the test and control Trastuzumab samples appear to be identical in terms of the peptide profiles. No significant difference is seen between these two samples showing no change in the stored product. Furthermore all CDR region peptides were

identified and assigned in all samples and the full data for both test days, Day 0 and Day 28 following extended storage of Trastuzumab in Tevadaptor® systems based on retention of heavy and light CDR peaks are provided in Table 8 and Table 9 below.

Table 8. Table showing Day 0 peptide mapping profiles performed on both test Trastuzumab samples prepared and stored using Tevadaptor® system (n=2) and Trastuzumab RS freshly reconstituted in a glass vial on the day of analysis as control (n=2). All devices were sampled in duplicate and analysed in triplicate. Detection at 214nm.

Relative Retention Time (RRT) w.r.t reference peak at 89 minutes.

Calculated value as ratio RT peak/RT Ref (214 nm)

Peptide ID	Ret time (min)	Control	Test	Acceptance Criteria	Difference Observed
HC-CDR1	49.60	0.56 ± 0.0	0.56 ± 0.0	0.50 ± 0.03	0.00
HC-CDR2	32.20	0.36 ± 0.0	0.36 ± 0.0	0.30 ± 0.03	0.00
HC-CDR3	78.40	0.88 ± 0.0	0.88 ± 0.0	0.81 ± 0.03	0.00
LC-CDR1	46.10	0.52 ± 0.0	0.52 ± 0.0	0.46 ± 0.03	0.00
LC-CDR2	77.50	0.87 ± 0.0	0.87 ± 0.0	0.79 ± 0.03	0.00
LC-CDR3	80.10	0.90 ± 0.0	0.90 ± 0.0	0.84 ± 0.03	0.00
Reference	89.00	1.0 ± 0.0	1.0 ± 0.0	not applicable	not applicable

Table 9. Table showing Day 28 peptide mapping profiles performed on both test Trastuzumab samples prepared and stored using Tevadaptor® system (n=2) and Trastuzumab RS freshly reconstituted in a glass vial on the day of analysis as control (n=2). All devices were sampled in duplicate and analysed in triplicate. Detection at 214nm.

Relative Retention Time (RRT) w.r.t reference peak at 89 minutes.

Calculated value as ratio RT peak/RT Ref (214 nm)

Peptide ID	Ret time (min)	Control	Test	Acceptance criteria	Difference Observed
HC-CDR1	49.60	0.56 ± 0.0	0.56 ± 0.0	0.50 ± 0.03	0.00
HC-CDR2	32.20	0.36 ± 0.0	0.36 ± 0.0	0.30 ± 0.03	0.00
HC-CDR3	78.40	0.88 ± 0.0	0.88 ± 0.0	0.81 ± 0.03	0.00
LC-CDR1	46.10	0.52 ± 0.0	0.52 ± 0.0	0.46 ± 0.03	0.00
LC-CDR2	77.50	0.87 ± 0.0	0.87 ± 0.0	0.79 ± 0.03	0.00
LC-CDR3	80.10	0.90 ± 0.0	0.90 ± 0.0	0.84 ± 0.03	0.00
Reference	89.00	1.0 ± 0.0	1.0 ± 0.0	not applicable	not applicable

As can be seen from the above retention time values and CDR peptide fragment assignments there is no significant different between the peptide profiles for test Trastuzumab samples stored for up to 28 days, 2-8^{o}C protected from light having been prepared and stored in Tevadaptor® systems to that from control Trastuzumab RS samples freshly prepared on the day of test.

Sodium Dodecyl Sulphate Capillary Gel Electrophoresis under reducing conditions (SDS-CE-PAGE)

Protein separation of the denatured and reduced Trastuzumab RS control samples at all concentrations tested were characterised by an electropherogram with a heavy chain fragment peak at an apparent molecular weight of 58 kDa and a light chain fragment peak at an apparent molecular weight of 28 kDa which is consistent with the known structure of Trastuzumab. There was also a much smaller peak at an apparent molecular weight of 53.5 kDa which was assigned to the non-glycosylated (NG) form of the heavy chain (HC) or low molecular weight impurity. This was assigned by spiking of the RS control sample with known amounts of the purified non-glycosylated heavy chain reference sample (synthesis described above) at the 1-4% level as per the USP guidance. This NGHC fragment was naturally present as impurity at the 0.5% level in all control Trastuzumab RS samples (data not shown). Electropherograms were also recorded for the sample matrix and for another biopharmaceutical product to demonstrate system suitability and show system peaks (data not shown). A typical electropherogram for the electrophoretic separation of a control Trastuzumab RS solution at 21mg/mL under denaturing and reducing conditions is shown in Figure 4 below.

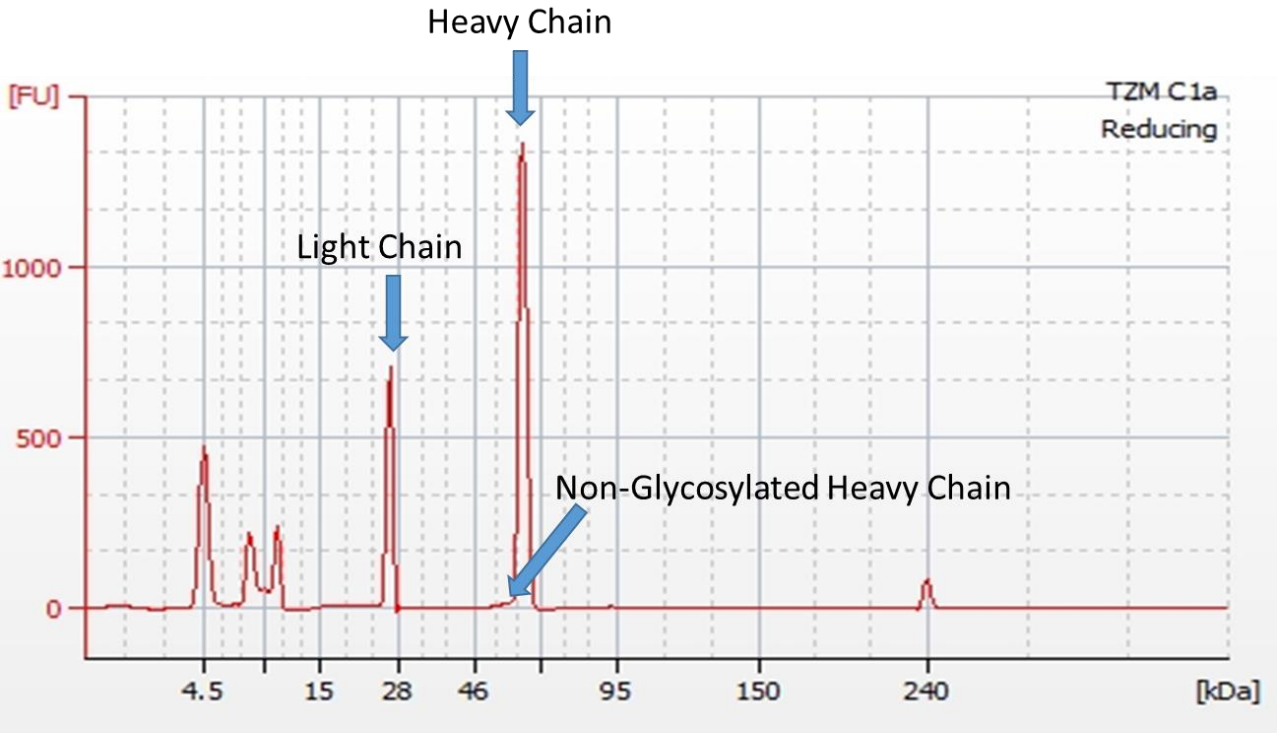


Figure 4. Figure showing a typical CE electropherogram for control Trastuzumab RS sample under denaturing and reducing conditions.

Using the applied NHS guidance criteria for assessment of stability of biopharmaceuticals all replicate batches of Trastuzumab test samples following storage in Tevadaptor® CSTD were analysed at each study time point Day 0, 7, 14, 21 and 28 and the results presented in Table 10 below. Values for the percentage of non-glycosylated heavy chain are also presented for the reference control Trastuzumab RS samples that has been freshly prepared on each day of test.

Table 10. Table showing average mean assessment of Trastuzumab test samples (n=3) following storage in Tevadaptor® CSTD for a period of 28 days 2-8^{o}C protected from light versus freshly reconstituted control Trastuzumab RS samples prepared on the day of test (n=3). All samples prepared under denaturing and reducing conditions.

Assignment of Trastuzumab Peptide Fragments: LC[a]; NGHC[b] & HC[c] kDa	**Test Day (Percentages of total protein)**				
	0	**7**	**14**	**21**	**28**
Fragment Light Chain 26.4 kDa (Av Test LC)	31.6	31.7	32.3	31.7	31.6
Fragment Light Chain 26.4 kDa (Av Control LC)	31.6	31.6	31.8	31.5	31.5
Difference |Control-Test| (Percentage, %)	**0.0**	**0.2**	**0.5**	**0.3**	**0.2**
Non-Glycosylated Heavy Chain 53.5 kDa (Av Test NGHC)	0.6	0.8	0.7	0.8	0.7
Non-Glycosylated Heavy Chain 53.5 kDa (Av Control NGHC)	0.5	0.9	0.6	0.6	0.7
Difference |Control-Test| (Percentage, %)	**0.1**	**-0.1**	**0.1**	**0.1**	**0.0**
Glycosylated Heavy Chain 58.1 kDa (Av Test HC)	67.5	67.7	66.4	67.4	67.1
Glycosylated Heavy Chain 58.1 kDa (Av Control HC)	67.8	66.8	67.5	67.1	66.9
Difference |Control-Test| (Percentage, %)	**-0.3**	**0.9**	**-1.1**	**0.3**	**0.2**

[a]LC = Light chain fragment, [b]NGHC = Non-Gycosylated Heavy chain impurity, [c]HC = heavy chain fragment,

Even after 28 days storage in the closed system there is no significant difference between the levels of low molecular weight non-glycosylated heavy chain impurity (NGHC) in the test samples as compared with the reference control samples freshly prepared on the day of test. This provides support to the hypothesis that Trastuzumab at 12mg/mL is stable when stored in Tevadaptor® CSTD for up to 28 days 2-8^{o}C protected from light.

Sodium Dodecyl Sulphate Capillary Gel Electrophoresis under non-reducing conditions (SDS-CE-PAGE)

Protein separation of the denatured and non-reduced Trastuzumab RS control samples at all concentrations tested were characterised by an electropherogram which shows a single major peak corresponding to the intact Trastuzumab molecule containing two heavy chain peptides and two light chain peptide fragments (HHLL) at the appropriate indicated molecular weight of circa 157 kDa, see Figure 1 below. The assignment of molecular weight was based on the internal molecular weight markers present within each experimental run (14-230 kDa). In addition to the main intact Trastuzumab peak for the HHLL species there is also a smaller impurity peak at lower molecular weight indicated at around 141 kDa which represents the loss of a light chain peptide molecule. This is the high molecular weight impurity HHL and is the subject of this stability test. The assignment for HHL was achieved by synthesis of a control reference sample from Trastuzumab RS by heating in the presence of Iodoacetamide (details provided above).

Electropherograms were also recorded for the sample matrix and for another biopharmaceutical product to demonstrate system suitability and show resolution from system peaks (data not shown). A typical electropherogram for the electrophoretic separation of a control Trastuzumab RS solution at 21mg/mL under denaturing and non-reducing conditions is shown in Figure 5 below which also shows the slight shoulder peak due to the presence of the HHL impurity.

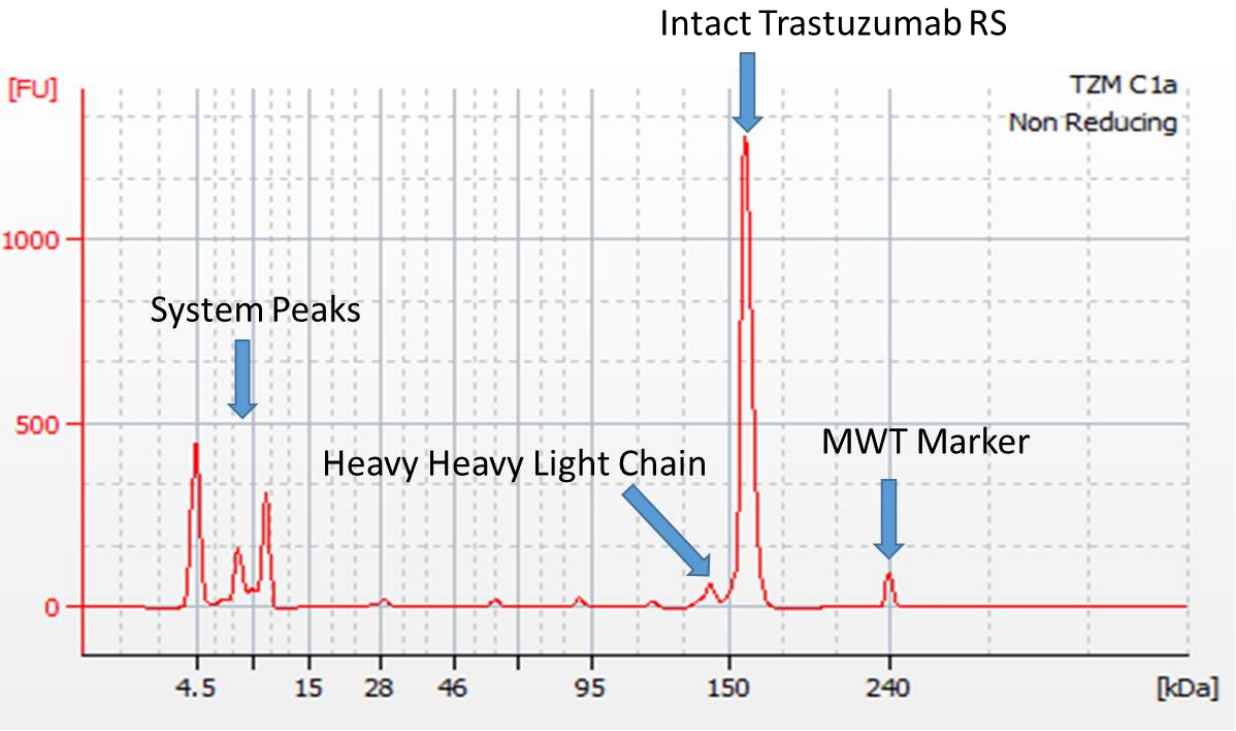


Figure 5. Figure showing a typical CE electropherogram for control Trastuzumab RS sample under denaturing and non-reducing conditions.

Using the applied NHS guidance criteria for assessment of stability of biopharmaceuticals all replicate batches of Trastuzumab test samples following storage in Tevadaptor® CSTD were analysed at each study time point Day 0, 7, 14, 21 and 28 and the results presented in Table 11 below. Values for the percentage of the high molecular weight impurity HHL fragment for Trastuzumab test samples are presented alongside those for the reference control Trastuzumab RS samples that had been freshly prepared on each day of test.

Table 11. Table showing average mean values from assessment of Trastuzumab test samples (n=3) following storage in Tevadaptor® CSTD for a period of 28 days 2-8^{o}C protected from light versus freshly reconstituted control Trastuzumab RS samples (n=3) prepared on the day of test. All samples prepared under denaturing and reducing conditions.

Assigned Trastuzumab species & Size of Molecular Species (kDa)	**Test Day (Percentages of total protein)**				
	0	**7**	**14**	**21**	**28**
Trastuzumab HHL 141 kDa (Av Test)	5.1	5.8	5.8	4.9	5.8
Trastuzumab HHL 141 kDa (Av Control)	5.1	5.6	6.1	4.8	6.1
Difference |Control-Test| (Percentage, %)	**0.0**	**0.1**	**-0.4**	**0.1**	**-0.3**
Trastuzumab HHLL intact 157 kDa (Av Test)	85.6	88.6	88.9	90.5	88.5
Trastuzumab HHLL intact 157 kDa (Av Control)	88.9	87.5	87.8	90.4	89.0
Difference |Control-Test| (Percentage, %)	**-3.3**	**1.0**	**1.0**	**0.0**	**-0.5**

Test =samples stored in Tevadaptor® CSTD system 2-8^{o}C protected from light. Control = aseptically prepared Trastuzumab RS using standard needle and syringe approach. HHLL = Heavy Heavy Light Light intact Trastuzumab RS; HHL = Heavy Heavy Light Chain high molecular weight impurity.

All test Trastuzumab presentations prepared and stored using Tevadaptor® systems for up to 28 days demonstrated acceptability within the criteria set 95-105% of control samples freshly prepared on the day of test.

Cation Exchange Liquid Chromatography (CEX-HPLC)

Treatment of Trastuzumab resolution solution (RS) with Carobxypeptidase B according to the methods used should cleave any terminal L-lysine residues from the polypeptide chains (four in total) of Trastuzumab producing a single Trastuzumab (K0) peak in the characteristic chromatogram, see Figure 6 below. In the batch of Trastuzumab used in the study all four L-

lysine residues from each peptide chain had already been removed by exogeneous carboxypeptidases from the manufacturing process of the drug substance. As such the analysis of Trastuzumab resolution solution (RS) pre-Carboxypeptidase B treatment and following treatment with this enzyme showed no difference in their chromatographic profiles. The peak corresponding to Trastuzumab K0 was consistently the single largest peak and there was no significant presence of any basic charge variant species relating to K1, K2, K3 and K4 (where K represents an L-Lysine amino acid residue) with retention times greater than the Trastuzumab K0 species as separated under the chromatographic conditions used.

A typical chromatogram for Trastuzumab RS sample freshly prepared following treatment with Carboxypeptidase B solution is shown below in Figure 6. The Trastuzumab K0 charge variant peak is shown eluting at a retention time of 23.4 minutes, there are no other charge variant peaks present following enzyme digestion and therefore no other peaks appearing at longer retention time to the main K0 peak.

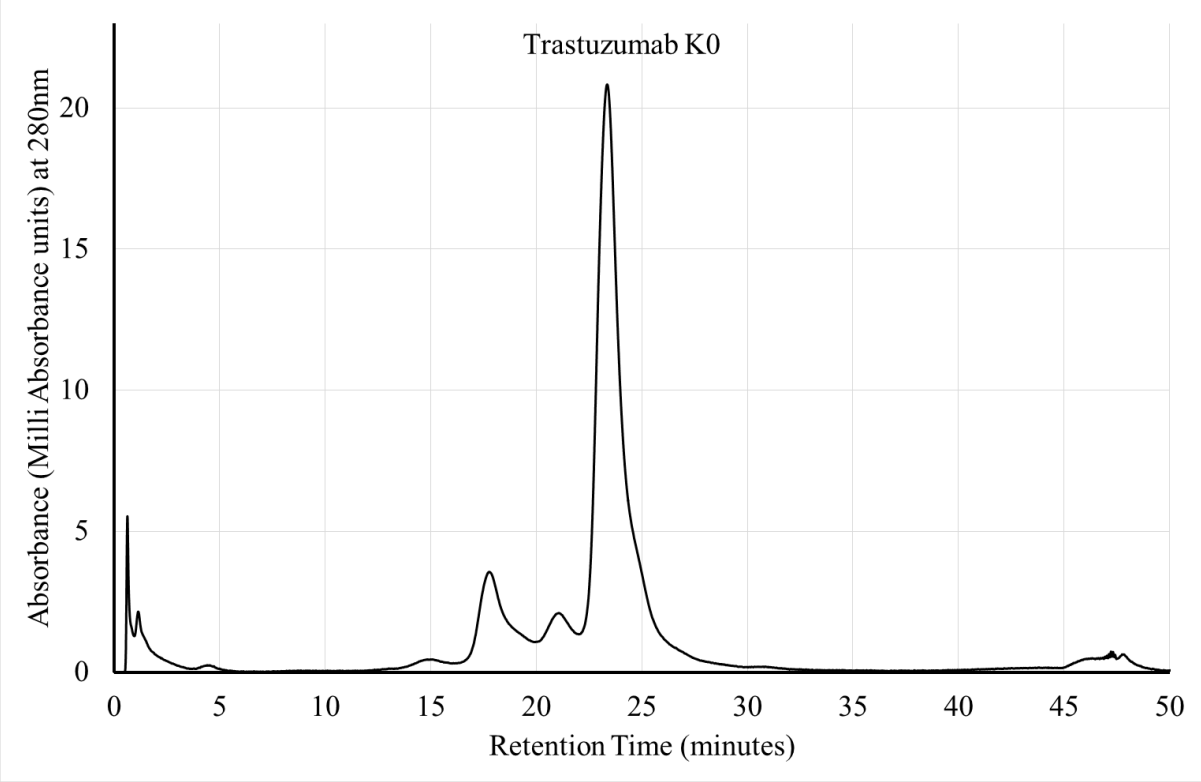


Figure 6. Figure showing a representative cation exchange chromatographic profile for freshly prepared Trastuzumab RS (1.0mg/mL) following treatment by Carboxypeptidase B enzyme at a detection wavelength of 280nm using the validated SEC-HPLC method.

It should be noted that the chromatographic profile of the same Trastuzumab RS sample at 1.0 mg/mL without Carboxypeptidase B treatment is identical to that shown above in Figure 6.

Table 12 below shows the percentage recovery of Trastuzumab (K0) charge variant in test Trastuzumab samples stored in Tevadaptor® system for up to 28 days as compared with control Trastuzumab samples freshly on the day of test using a standard needle and syringe approach. There is higher variability in the day 0 baseline test Trastuzumab K0 sample values however no significant difference is seen between test and control samples over the 28 day period using the 95-105% acceptance criteria adopted in this study.

Table 12. Analysis of basic charge variants by cation exchange high performance liquid chromatography (HPLC-CEX) with detection at a wavelength of 280nm at test days 0, 7, 14, 21 and 28. The data was obtained from duplicate test and control Trastuzumab samples prepared with duplicate sampling and triplicate injections.

Sample ID	**Sampling Date**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Test Trastuzumab K0	94.5 ± 7.2	102.8 ± 4.5	99.6 ± 2.7	99.6 ± 6.1	101.5 ± 4.8
Control Trastuzumab K0	100.0 ± 4.3	100.00 ± 6.8	100.00 ± 2.0	100.00 ± 3.9	100.00 ± 5.6
Difference: Control - Test	5.5 ± 7.2	2.8 ± 6.8	0.4 ± 2.7	0.4 ± 4.51	1.5 ± 5.6

Analysis by cation exchange chromatography (CEX-HPLC) demonstrates that Trastuzumab solutions remain stable for up to 28 days when prepared using Tevadaptor® systems and stored 2-8^{o}C protected from light. No significant difference in the amount of Trastuzumab charge variant K0 was observed between test Trastuzumab solutions (n=2) prepared using Tevadaptor® systems compared with control Trastuzumab RS samples freshly prepared on the day of test using a standard needle and syringe approach. It is clear from the above chromatographic profile in Figure 6 that all L-Lysines have already been cleaved during manufacture of this batch of Trastuzumab. Only charge variant K0 is present in the profiles obtained. All test Trastuzumab solution K0 peak area values remained within 95-105% of the starting concentration values on day 0 and so the data shows no significant change when Tevadaptor® is used to prepare and store Trastuzumab under the test conditions.

Biological activity assay

Enzyme Linked Immuno Sorbent Assay (ELISA)

Analysis using the modified AbD Serotec Trastuzumab ELISA binding assay was used to quantify the amount of active Trasuzumab within the test Trastuzumab (n=2) presentations prepared and stored using Tevadaptor® systems and presentations of Trastuzumab RS freshly reconstituted using the standard needle and syringe approach on the day of test (n=2). The binding curves obtained using reference Trastuzumab RS from validation runs performed on different days throughout the 28 day study period showed excellent agreement and inter repeatability with the method used. Representative binding curves for Trastuzumab resolution solution are shown in Figure 7 and Figure 8 below for Day 0 and Day 28 of the study respectively. Both response curves demonstrate good binding characteristics across the range of Trastuzumab concentrations tested within the assay from 0 to 500ng/ml and show a linear instrument response for Trastuzumab in the range 100 to 220 ng/ml.

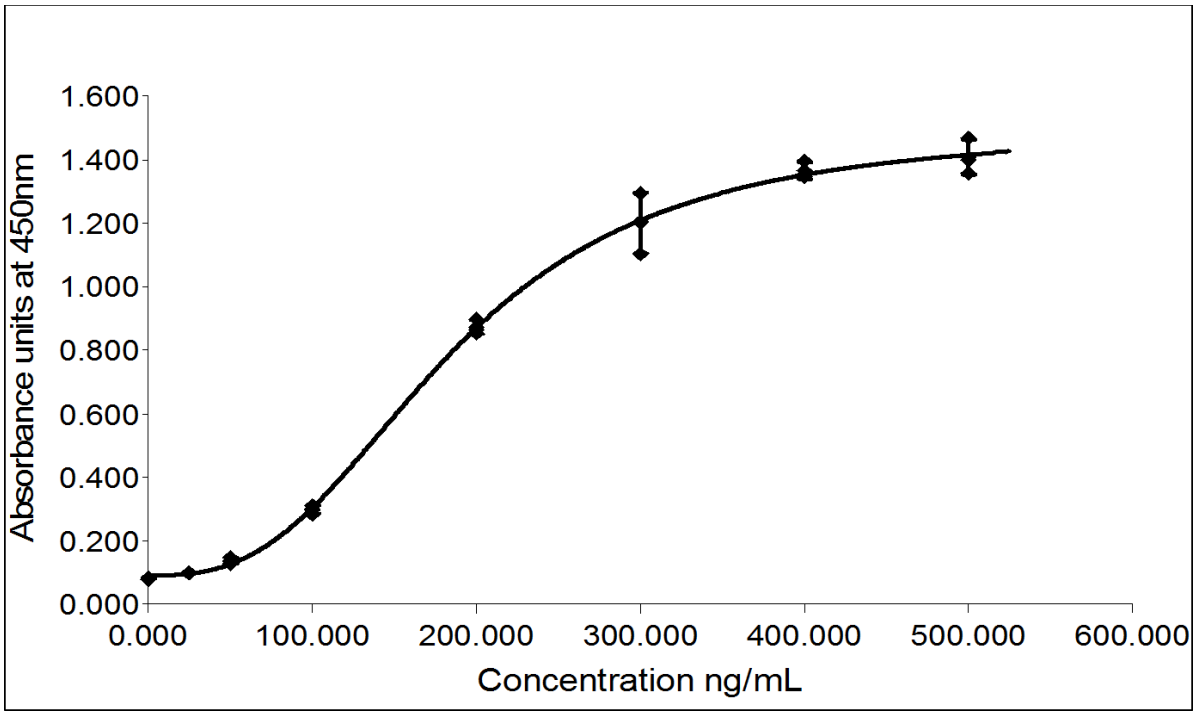


Figure 7. A characteristic binding curve for Trastuzumab RS freshly prepared at concentrations between 0 and 500ng/mL on Day 0 of the study. Detection was made at 450nm wavelength.

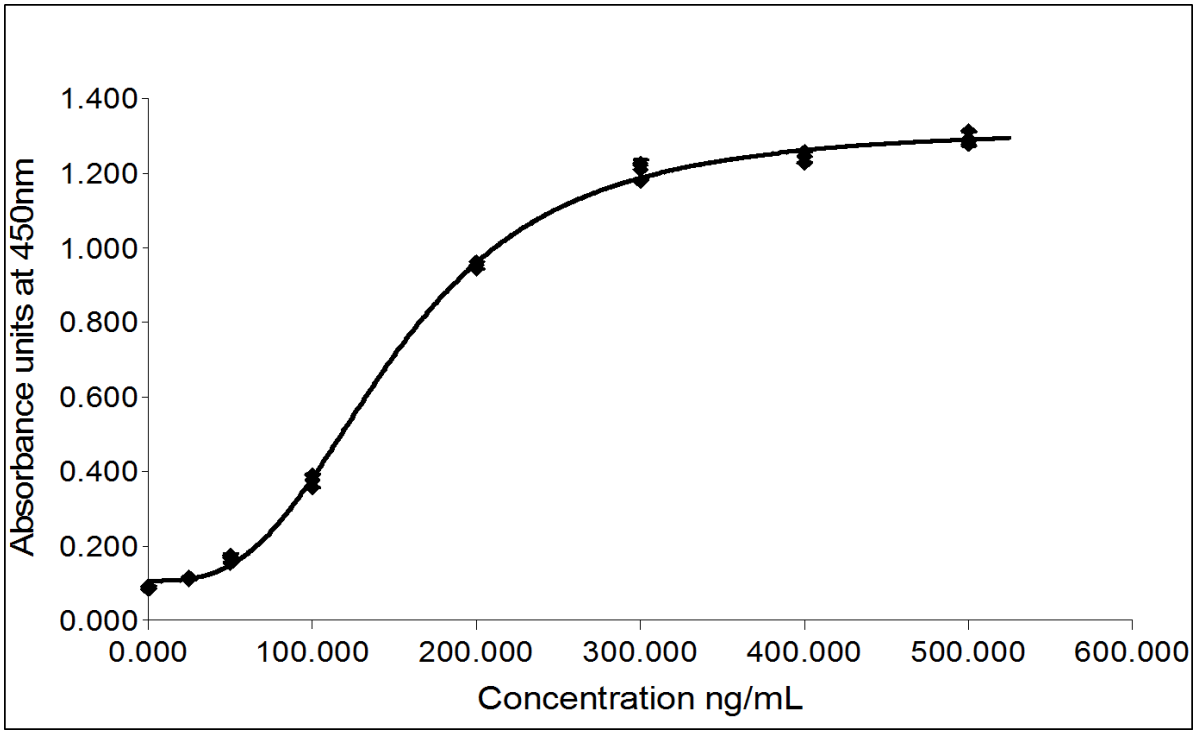


Figure 8. A characteristic binding curve for Trastuzumab RS freshly prepared at concentrations between 0 and 500ng/mL on Day 28 of the study. Detection was made at 450nm wavelength.

Figure 7 and Figure 8 above demonstrate the reproducibility of the ELISA method for Trastuzumab as used in the study. The binding assay provides almost identical response curves for Trastuzumab across the range of drug concentrations within the assay (0 to 500ng/ml) which covers around 75% of the dynamic response of the instrument at 450nm detection wavelength. Table 1 below shows the percentage active Trastuzumab from test Trastuzumab presentations prepared and then stored in Tevadaptor® systems for up to 28 days versus, 2-8ºC protected from light. Control Trastuzumab RS freshly prepared using a standard needle and syringe approach on the day of test is used for reference. Data is presented as % recovery based

on absorbance readings at 450nm for the Horse Radish Peroxidase substrate Ultra-TMB. The data presented in Table 13 was obtained from duplicate test (n=2) and control devices (n=2) with duplicate sampling and triplicate injections (n=3).

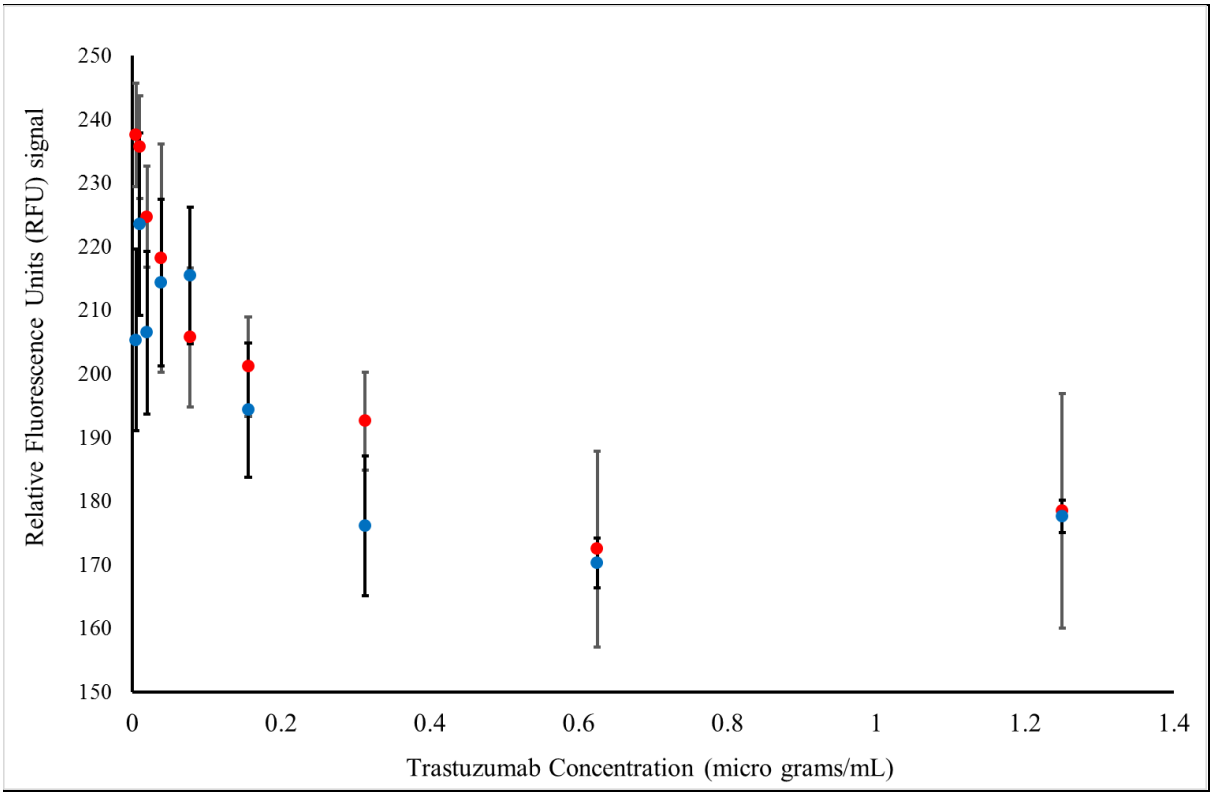
Table 13. Biological activity of Trastuzumab test and control samples (n=2) assessed by an anti-Trastuzumab antibody binding assay (ELISA) with readout at 450nm from Ultra-TMB.

Sample ID	**Sampling Date**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 28**
Test Trastuzumab	82.8 ± 16.6	108.0 ± 9.0	105.8 ± 9.8	106.8 ± 9.4	101.7 ± 10.4
Control Trastuzumab	100.0 ± 11.1	100.00 ± 13.9	100.00 ± 9.5	100.10 ± 4.4	100.00 ± 7.6
% Diff.: Control - Test	17.2 ± 16.6	8.0 ± 13.9	5.8 ± 9.8	6.7 ± 9.4	1.7 ± 10.4

All test Trastuzumab solutions remained within 80-120% of the starting control Trastuzumab values as determined using reference standards of Trastuzumab RS freshly prepared on the day of test. The data for Day 0 in Tevadaptor® systems shows a wider variance than the other time points and this may a reflection of the effect of sample dilution on day 0. The activity is then recovered on subsequent test days. An unpaired t-test was performed to test for significance between control and test data and this returned a p-value of 0.841 ($P>0.05$). The test solutions prepared using Tevadaptor® systems do not therefore appear to be significantly different to those prepared using the standard syringe and needle approach on Day 0 (control performed on each day of test) and for days 7 onwards all values appear to be within 90-110% of starting values.

Cellular Potency Assay (Anti-proliferation assay)

The results from performing the cell anti-proliferation assay for Day 28 versus freshly prepared control Trastuzumab are shown below in Figure 9 . Due to the known inherent variability of the cellular assay compared with ELISA which was also performed as part of this technique was only applied at two test time points.



Test Trastuzumab stored for 28 days in Tevadaptor® (n=2) blue circles and control Trastuzumab RS (n=2) freshly prepared on day of test (Day 0) red circles.

Figure 9. Figure showing the raw fluorescence data and characteristic drug dose response curve for control Trastuzumab RS (n=2) versus a characteristic dose response curve for test Trastuzumab prepared and stored using Tevadaptor® over 28 days. Readout was measured as emission of fluorescent light at 590nm.

Table 14. Table showing biological potency of test Trastuzumab using a cellular anti-proliferation assay (HTB-20) for samples prepared using Tevadaptor® systems and stored for 28 days 2-8^{o}C protected from light versus control Trastuzumab freshly prepared using a standard needle and syringe approach on the day of test.

Sample ID	**Sampling Date**		**Acceptance**
	Day 0	Day 28	**Criteria**
Trastuzumab potency (%) Test samples (n=2)	91.8 ± 5.4	96.6 ± 7.6	(90-110%)
Trastuzumab potency (%) for Control samples (n=2)	100.0 ± 4.7	100.0 ± 9.1	(90-110%)
% Difference Test - Control	8.2 ± 5.4	3.4 ± 9.1	0-20%

The data obtained above for test and control samples of Trastuzumab assessed on Days 0 and 28 demonstrate that there is no significant loss of activity and biological action of test Trastuzumab following storage in Tevadaptor® systems for up to 28 days 2-8^{o}C protected from light. There was no significant difference between test and control Trastuzumab RS freshly prepared on the day of test using a standard needle and syringe approach. The dose response curve for Trastuzumab met the acceptance criteria and showed no dose response for a placebo (data no presented).

Microbiological Sterility Testing

Results from sterility testing of test Trastuzumab (n=2) drug vials, samples taken directly into both TSB and TFM growth media are presented below.

Table 15. Summary of the results for the method suitability test required to establish the validity of the sterility test method using standard ATCC reference organisms as per the requirements of USP<71>.

Growth Medium	Microbial Strain	Growth Promotion Method Suitability Test Result†
TSB	*Staphylococcus aureus NCTC 10788*	Meets Requirements
TSB	*Bacillus subtilis NCTC 10400*	Meets Requirements
TSB	*Pseudomonas aeruginosa NCTC 12924*	Meets Requirements
TSB	*Candida albicans NCPF 3179*	Meets Requirements
TSB	*Aspergillus brasiliensis NCPF 2275*	Meets Requirements
FTM	*Staphylococcus aureus NCTC 10788*	Meets Requirements
FTM	*Bacillus subtilis NCTC 10400*	Meets Requirements
FTM	*Pseudomonas aeruginosa NCTC 12924*	Meets Requirements
FTM	*Candida albicans NCPF 3179*	Meets Requirements
FTM	*Aspergillus brasiliensis NCPF 2275*	Meets Requirements
FTM	*Clostridium sporogenes ATCC 11437*	Meets Requirements

†Growth observed within 5 days following inoculation with <100 Colony Forming Units

The system suitability shows that the TSB and TFM growth media prepared as part of this study supported growth across all ATCC organisms when subjected to direct inoculation (<100 CFU) and incubated. The results from the sterility testing of the duplicate Trastuzumab (n=2) drug vials following 28 days storage in Tevadaptor® systems at 2-8ºC protected from light are presented Table 16 below.

Table 16. Table showing the results from the direct inoculation sterility testing of the test Trastuzumab vials prepared and stored using Tevadaptor® systems for up to 28 days 2-8ºC protected from light.

Sample Day	**Trastuzumab Vial**	**Growth Media**	**Test Result**	**Incubation Temperature & Time**
Day 28	1	TSB	Meets Requirement for Sterility	20-25°C; 14 Days
	1	TSB	Meets Requirement for Sterility	30-35°C 14 Days
	1	FTM	Meets Requirement for Sterility	30-35°C 14 Days
Day 28	2	TSB	Meets Requirement for Sterility	20-25°C; 14 Days
	2	TSB	Meets Requirement for Sterility	30-35°C 14 Days
	2	FTM	Meets Requirement for Sterility	30-35°C 14 Days

From the above sterility tests it can be seen that sterility was maintained in the single dose preservative free Trastuzumab drug vials when Tevadaptor® systems are used to prepare and

store the vials for up to 28 days at 2-8ºC (accessed 5 times over the 28 days for physicochemical sampling and an additional 3 times for sterility testing). his demonstrates the ability of Tevadaptor® to maintain a sterile barrier to the Trastuzumab drug substance following first puncture of the drug vial and this barrier is then maintained up to 28 days when accessed on multiple days over a 28 day "in use" period.

Discussion

This is the first example of a robust scientific study on the use of a closed system transfer device (Tevadaptor®, marketed by B.Braun in USA) that is able to maintain sterility and support the extended practical shelf life of a monoclonal antibody Trastuzumab for up to 28 days when stored 2-8ºC and protected from light. The study has taken both study design and testing methodologies that are compliant with the NHS guidance document requirements for assessment of stability for biopharmaceuticals and applied where present, acceptance criteria and methodologies from a USP validation summary report for Trastuzumab.

We have applied a number of relevant analytical and biological techniques to make a full assessment of physicochemical and biological stability as well as microbiological sterility for Trastuzumab stored in Tevadaptor® CSTD systems. Overall no physical or chemical changes were observed and the stored Trastuzumab was found to retain all of its biological activity as determined by both ELISA and cellular assay. This goes beyond the current standard for biological assessment of stability of biopharmaceuticals. The technique of cation exchange chromatography was able to confirm the amount of Trastuzumab monomer assessed by the orthogonal technique of size exclusion chromatography and also that the batch of drug tested in the study had no heterogeneity from charge variants. This was most likely due to exogeneous carboxypeptidase removal of all four C-terminal L-Lysine residues during the manufacturing process. This study applied a significant number of robust scientific

techniques and presents a compelling body of evidence to support the use of Tevadaptor® with Trastuzumab as an example of a monoclonal antibody drug that can be safely stored within a closed system for up to 28 days at 2-8^{o}C PFL.

Conclusions

Previous stability assessment attempts have been made to extend the "in use" period for Trastuzumab following first puncture of the drug vial beyond that stated in the summary of product characteristics(2,3). However all of the studies performed to date did not comply with current NHS guidance and often did not consider the assessment of biological activity of Trastuzumab following reconstitution and storage in a different container system. This study demonstrates compliance with the current NHS guidance on assessment of stability of biopharmaceuticals(5) and also utilises methodologies taken from the USP summary validation report published in 2013(6). In this present study we have demonstrated the stability of preservative free Trastuzumab at 21mg/mL following reconstitution and storage in Tevadaptor® systems as part of the container system when stored at 2-8^{o}C protected from light for up to 28 days. In addition to the physicochemical and biological assessment we have also demonstrated the ability of Tevadaptor® to maintain the sterile barrier over 28 days in use period. This data will help the pharmacist reduce drug wastage as part of beyond use dating at a time when costs in healthcare are becoming unsustainable, placing an increased need to seek new ways of working to reduce drug wastage of high cost drugs used in oncology.

Conflict of Interest

None

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