



The in-use stability of the rituximab biosimilar Rixathon[®]/Riximyo[®] upon preparation for intravenous infusion

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Abstract

Purpose: The purpose of this study was to evaluate the in-use physicochemical and biological stability of the Sandoz rituximab biosimilar, marketed under the trade names Rixathon[®] and Riximyo[®] in the European Union, upon preparation for intravenous infusion.

Methods: Three batches of Rixathon[®]/Riximyo[®] in the final month of their 36 month shelf life were exposed to room temperature and light for 14 days to recapitulate a major temperature excursion. Samples were diluted to the lowest allowable concentration of 1 mg/mL in 0.9% NaCl solution in either polypropylene or polyethylene infusion bags and stored for 14 or 30 days at 5 ± 3°C followed by an additional 24 h at room temperature to simulate product handling. Samples stored in infusion bags were analyzed using SEC, CEX, non-reducing CE-SDS, peptide mapping and CDC to assess physicochemical and biological stability.

Results: Analysis of Rixathon[®]/Riximyo[®] diluted to the lowest allowable concentration in 0.9% sodium chloride in either polypropylene or polyethylene infusion bags revealed no change in molecular weight variants, charge variants, deamidation, oxidation, overall composition or potency over a 31-day period.

Conclusion: Physicochemical and biological analyses demonstrate that Rixathon[®]/Riximyo[®] stability is not impacted by dilution and formulation conditions required for intravenous infusion, even under worst case conditions with regard to product shelf life, temperature excursion, light exposure, dilution factor and infusion bag storage time over a 31-day period.

Keywords

Biosimilar, rituximab, stability, sandoz, rixathon

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Introduction

Rituximab is a monoclonal antibody capable of recognizing the CD20 antigen present on mature B-cells and elicits their subsequent elimination through various modes of action including complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and direct induction of cellular apoptosis.^{1–3} Rituximab is administered in oncology for the treatment of non-Hodgkin's lymphoma and chronic lymphocytic leukemia (CLL) and is

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additionally indicated for the treatment of autoimmune diseases including rheumatoid arthritis, granulomatosis with polyangiitis and microscopic polyangiitis.^{4,5} Rituximab is provided in single use vials containing either 100 mg or 500 mg of active pharmaceutical ingredient at a concentration of 10 mg/mL. Prior to intravenous administration, the necessary amount of rituximab must be withdrawn from the commercial vial under aseptic conditions and diluted to a final concentration of 1 to 4 mg/mL in an infusion bag containing either 0.9% sodium chloride or 5% dextrose in water,^{4,5} whereby sodium chloride infusions are preferred due to product stability and clinical safety concerns associated with dextrose.⁶

Prior to regulatory approval, national health authorities require extensive stability testing for biopharmaceuticals such as rituximab to demonstrate that product quality is not compromised by standard transport and storage conditions and that product specifications are met throughout the entirety of the declared shelf life.^{7,8} However, for products intended for immediate use, such as rituximab, these stability testing requirements do not generally include extended in-use conditions such as longer storage of the diluted and reformulated product prior to intravenous infusion, since such conditions are within the responsibility of the compounding health care professional.^{9,10} Thus, reports describing product stability under extended in-use conditions are valuable to health care professionals to provide assurance that pharmaceutical safety and efficacy can be maintained under reformulation and handling conditions not covered by standard stability evaluation.

The extended in-use stability of rituximab upon dilution into 0.9% sodium chloride in infusion bags has been previously reported for both EU approved MabThera[®] and US licensed Rituxan[®].^{11,12} Recently, the Sandoz rituximab biosimilar registered under the trade names Rixathon[®] and Riximyo[®] was endorsed by the Committee for Medicinal Products for Human use (CHMP) at the European Medicines Agency and received a formal marketing authorization from the European Commission.^{13,14} Biosimilars and their corresponding reference medicine must contain a matching active ingredient and must exhibit equivalence upon extensive physicochemical and biological evaluation and comparative phase I and phase III clinical studies.¹⁵⁻¹⁷ Although comparative stability evaluation is an integral part of the biosimilarity assessment, formulation may differ and biosimilar manufacturing and shelf life specifications are established independently based on product specific process capability and stability data, and may not be identical to those of the reference product.^{8,18,19} Thus, although previous studies have evaluated the extended in-use stability of the

Table 1. Determination of protein concentration at different time points following in-use preparation for intravenous infusion.

Batch (bag type)	Time (days)	Protein concentration (mg/mL)
Batch A (PP)	0	1.01
	15	1.02
	31	1.02
Batch A (PE)	0	1.03
	15	1.04
	31	1.02
	RSD	0.01%
Batch B (PP)	0	1.02
	15	1.03
	31	1.02
Batch B (PE)	0	1.01
	15	1.02
	31	1.03
	RSD	0.01%
Batch C (PP)	0	1.02
	15	1.02
	31	1.01
Batch C (PE)	0	1.02
	15	1.02
	31	1.02
	RSD	0%

Note: Assessment of protein concentration using affinity liquid chromatography (ALC) in three Rixathon[®]/Riximyo[®] batches (arbitrarily labeled A, B, and C) diluted to 1 mg/mL in saline solution infusion bags and stored for 0, 15 and 31 days. Samples were stored at 5 ± 3°C for 14 or 30 days followed by an additional 24h at room temperature. Stability was assessed in either polypropylene (PP) or polyethylene (PE) infusion bags. The relative standard deviation (RSD) is calculated for each batch. PP: polypropylene; PE: polyethylene; RSD: relative standard deviation.

rituximab reference product,^{11,12} product specific in-use stability assessments of rituximab biosimilar products will be important to ensure that established formulation and handling practices do not compromise product safety or efficacy. The aim of this study was thus to evaluate the stability of the rituximab biosimilar Rixathon[®]/Riximyo[®] under worst case conditions potentially encountered upon dilution and reformulation of the product for intravenous administration.

Methods

Affinity liquid chromatography

Affinity liquid chromatography (ALC) was used to determine protein concentration under in-use conditions. Chromatographic separation was achieved

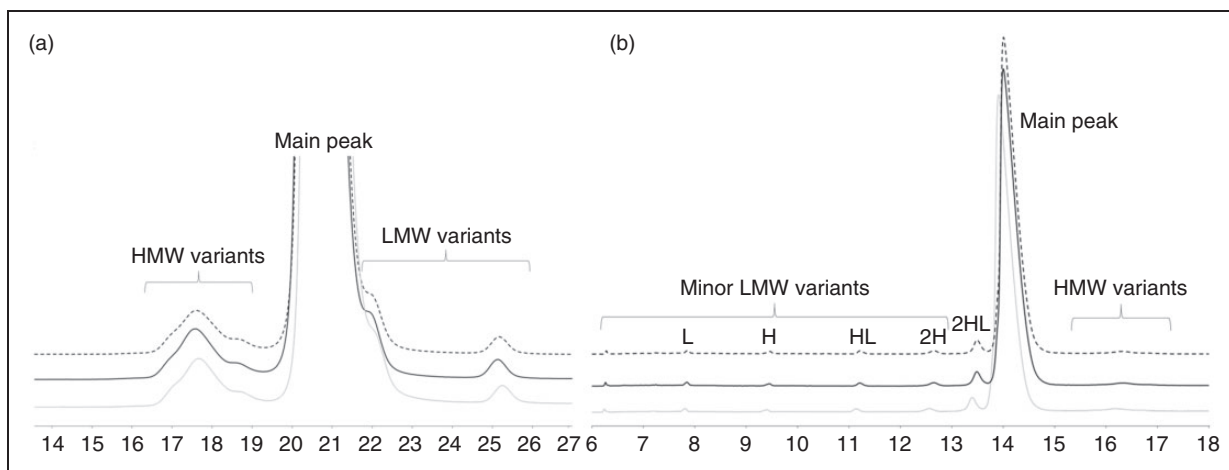


Figure 1. In-use preparation of Rixathon®/Riximyo® for intravenous infusion does not impact the abundance of molecular weight variants. (a) Representative overlay of size exclusion chromatograms (SEC) of Rixathon®/Riximyo® samples diluted to 1 mg/mL in saline solution infusion bags and stored for 0 (grey), 15 (black) and 31 (black dashed) days. Samples were stored at $5 \pm 3^\circ\text{C}$ for 14 or 30 days followed by an additional 24 h at room temperature. (b) Representative overlay of non-reducing SDS gel capillary electrophoresis (CE-SDS) chromatograms of Rixathon®/Riximyo® diluted to 1 mg/mL in saline solution infusion bags and stored for 0 (grey), 15 (black) and 31 (black dashed) days. Samples were stored at $5 \pm 3^\circ\text{C}$ followed by an additional 24 h at room temperature. The main peak, the 2HL fragment (two heavy chains and one light chain) and additional minor low-molecular weight (LMW) variants and high-molecular weight (HMW) variants are indicated. Elution times are shown on the x axis in minutes.

using a Poros PA Immuno DetectionTM column covalently modified with Protein A. Sample was bound to the column at pH 7.5 and subsequently eluted by shifting the gradient to a pH of 2.0. The UV adsorption of the eluting sample peak was measured at 280 nm and compared to a known standard for quantification.

Size exclusion chromatography

Size exclusion chromatography (SEC) was used to assess the formation of high and low-molecular weight variants. Chromatographic separation was achieved using a TSK-Gel G3000SWXL column (Tosoh Bioscience) and UV detection was carried out at 210 nm.

Non reducing SDS capillary electrophoresis

SDS capillary electrophoresis (CE-SDS) under non-reducing conditions was used to assess the formation of fragments and low-molecular weight variants. Analysis was performed on a Beckman PA800 Enhanced (Beckman Coulter, Brea, CA, USA). Samples were denatured, and any free sulfhydryl group from the cysteine side chain was alkylated with iodoacetamide (IAM) to prevent disulfide shuffling before analysis. Samples were pressure-injected, and the separation was performed with -15 kV and UV detection was carried out at 214 nm.

Cation exchange chromatography

Cation exchange chromatography (CEX) separates protein variants based on differences in charge, separate rituximab main compound from acidic and basic variants. Samples were treated with carboxypeptidase B to remove C-terminal lysine residues (CPB; Roche, Mannheim, Germany), and buffer exchanged into 25 mM sodium phosphate pH 7.0. CEX separation was performed using a weak CEX resin (ProPac WCX-10, $4 \times 250\text{ mm}$, Dionex, Germering, Germany), using a linear sodium chloride gradient. UV detection was carried out at 280 nm.

Reducing peptide map analysis

Peptide mapping with UV and mass spectrometry detection was performed to verify the primary sequence and detect modifications such as deamidation and oxidation. Protein samples were reduced and alkylated using dithiothreitol (DTT) and IAM, respectively. Samples were subsequently digested using endoproteinase Lys-C (Wako, Osaka, Japan). After digestion, the peptides were separated on a C18 reversed-phase column (KinetexTM 2.6 μm C18 $50 \times 2.1\text{ mm}$ column, Phenomenex, Torrance, CA, USA). UV detection was carried out at 214 nm. The identities of peptides and modifications were verified using mass spectrometry with electrospray ionization (Orbitrap, Thermo

Table 2. Size exclusion chromatography analysis of Rixathon[®]/Riximyo[®] batches following in-use preparation for intravenous infusion.

Batch (bag type)	Time (days)	Main peak (%)	HMW variants (%)	LMW variants (%)
Batch A (PP)	0	97.1	1.5	1.3
	15	97.2	1.6	1.1
	31	97.4	1.5	1.1
Batch A (PE)	0	97.1	1.6	1.3
	14	97.4	1.6	1.0
	31	97.3	1.6	1.0
	RSD	0.1%	0.1%	0.1%
Batch B (PP)	0	97.8	1.0	1.2
	15	98.0	1.0	1.0
	31	98.0	1.1	1.0
Batch B (PE)	0	97.9	1.0	1.1
	15	98.0	1.0	1.0
	31	97.9	1.1	1.0
	RSD	0.1%	0.1%	0.1%
Batch C (PP)	0	97.9	1.0	1.1
	15	98.1	1.0	0.9
	31	98.0	1.1	1.0
Batch C (PE)	0	97.7	1.0	1.3
	15	98.0	1.0	1.0
	31	97.8	1.2	1.0
	RSD	0.1%	0.1%	0.1%

Note: Assessment of molecular weight variants using size exclusion chromatography (SEC) in three Rixathon[®]/Riximyo[®] batches (arbitrarily labeled A, B, and C) diluted to 1 mg/mL in saline solution infusion bags and stored for 0, 15 and 31 days. Samples were stored at 5 ± 3°C for 14 or 30 days followed by an additional 24 h at room temperature. Stability was assessed in either polypropylene (PP) or polyethylene (PE) infusion bags. The relative abundance of high-molecular weight variants (HMW), low-molecular weight variants (LMW) and purity (main peak) are given. The relative standard deviation (RSD) is calculated for each batch.

PP: polypropylene; PE: polyethylene; RSD: relative standard deviation; LMW: low-molecular weight; HMW: high-molecular weight.

Scientific). Relative quantitation using mass spectrometry was achieved by comparing the extracted ion chromatograms of native and variant peptides.

Potency

To determine the CDC activity of Rixathon[®]/Riximyo[®], Raji B cells expressing CD20 on their surface are incubated with Rixathon[®]/Riximyo[®] and a fixed concentration of rabbit complement. Thereafter, the degree of cell death was determined by a cell viability assay. Cell viability was assessed by

Table 3. Non reducing SDS capillary electrophoresis analysis of Rixathon[®]/Riximyo[®] batches following in-use preparation for intravenous infusion.

Batch (bag type)	Time (days)	Main peak (%)	2HL variant (%)	Sum of minor LMW variants (%)
Batch A (PP)	0	94.8	2.3	2.0
	15	94.9	2.3	2.0
	31	94.9	2.2	2.0
Batch A (PE)	0	94.6	2.4	1.6
	15	94.9	2.3	2.0
	31	95.2	2.2	1.7
	RSD	0.2%	0.1%	0.2%
Batch B (PP)	0	95.3	2.2	1.9
	15	95.6	2.1	1.9
	31	95.5	2.1	1.8
Batch B (PE)	0	95.4	2.2	1.7
	15	95.5	2.2	1.9
	31	96.1	2.1	1.3
	RSD	0.3%	0.1%	0.2%
Batch C (PP)	0	96.1	2.1	1.3
	15	95.5	2.1	1.8
	31	95.6	2.0	1.8
Batch C (PE)	0	95.9	2.1	1.5
	15	95.5	2.0	1.9
	31	95.8	2.1	1.5
	RSD	0.2%	0.1%	0.2%

Note: Assessment of molecular weight variants using non reducing SDS capillary electrophoresis (CE-SDS) in three Rixathon[®]/Riximyo[®] batches (arbitrarily labeled A, B, and C) diluted to 1 mg/mL in saline solution infusion bags and stored for 0, 15 and 31 days. Samples were stored at 5 ± 3°C for 14 or 30 days followed by an additional 24 h at room temperature. Stability was assessed in either polypropylene (PP) or polyethylene (PE) infusion bags. The relative abundance of the 2HL incomplete fragment missing one light chain, the sum of subsequent minor low-molecular weight variants (LMW) and purity (main peak) are given. The relative standard deviation (RSD) is calculated for each batch.

PP: polypropylene; PE: polyethylene; RSD: relative standard deviation; LMW: low-molecular weight

measuring ATP concentration via the luciferin-luciferase system.

Results

The purpose of this study was to evaluate the extended in-use stability of the Sandoz rituximab biosimilar product, marketed under the trade names Rixathon[®] and Riximyo[®] in the European Union, during preparation for intravenous infusion. To best evaluate the stability of this biosimilar product, worst case conditions with regard to product shelf life, temperature excursion, light exposure, dilution factor and infusion bag storage

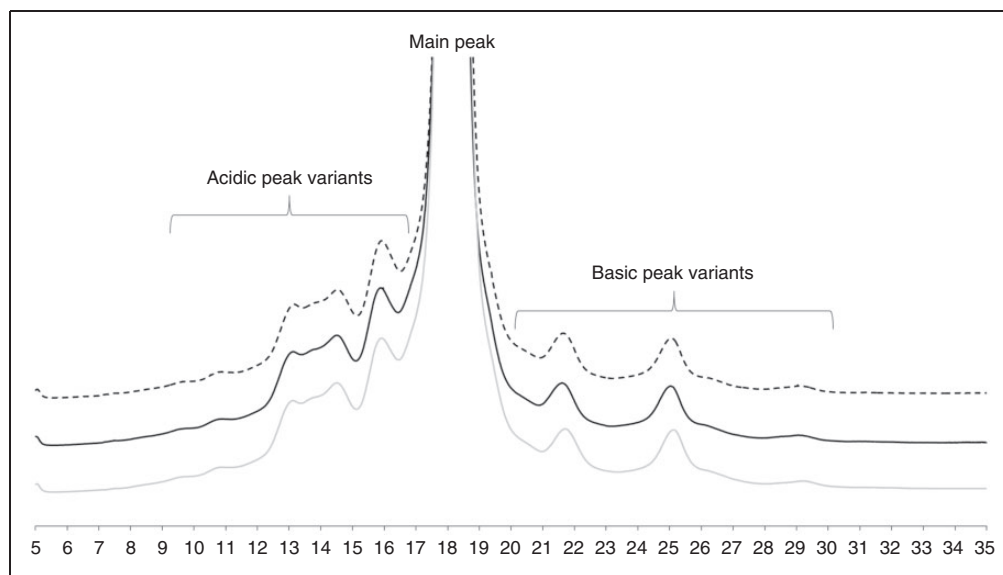


Figure 2. In-use preparation of Rixathon[®]/Riximyo[®] for intravenous infusion does not impact the abundance of charge variants. Representative overlay of cation exchange (CEX) chromatograms of Rixathon[®]/Riximyo[®] samples diluted to 1 mg/mL in saline solution infusion bags and stored for 0 (grey), 15 (black) and 31 (black dashed) days. Samples were stored at $5 \pm 3^{\circ}\text{C}$ for 14 or 30 days followed by an additional 24 h at room temperature. Elution times are shown on the x axis in minutes.

times were tested. State of the art analytics were used to assess the impact of conditions and infusion bag storage times on molecular weight variants, charge variants, oxidation, deamidation, overall composition and potency. As shown in Table 1, no change in protein concentration was observed over a 31-day period, demonstrating that protein adsorption or insoluble particle formation is negligible under in-use conditions. Analytical and functional results from this study demonstrate that physicochemical and biological attributes of Rixathon[®]/Riximyo[®] remain unchanged over a 31-day period in saline infusion bags, under worst case conditions. Although the artificial temperature and light excursions used to mimic production hold times and handling far exceed those that would be acceptable under normal manufacturing conditions, all product acceptance criteria and release specifications were still met at the end of the 31-day test period, indicating that neither the described stress conditions nor subsequent in-use dilution and reformulation significantly impact product quality.

Molecular weight variants

SEC was used to assess the formation of molecular weight variants in Rixathon[®]/Riximyo[®] under in-use conditions for drug infusion. As shown in Figure 1(a) and Table 2, SEC analysis revealed no change in high- or low-molecular weight variants. In addition, non-reducing CE-SDS was employed as a canonical method due to its ability to more completely resolve incomplete

antibody fragments and low-molecular weight variants compared to SEC (see Figure 1(b)). As shown in Table 3, the abundance of the 2HL fragment, as well as the sum of the other minor incomplete low-molecular weight antibody variants remained unchanged during the course of the in-use stability study. Overall, SEC and non-reducing CE-SDS demonstrate no change in Rixathon[®]/Riximyo[®] molecular weight variants over a 31-day period in either PP or PE saline infusion bags.

Charge variants, deamidation and oxidation

CEX was used to resolve variants resulting in increased overall negative charge (acidic variants) and those resulting in increased overall positive charge (basic variants). As shown in Figure 2 and Table 4, the abundance of acidic and basic variants in Rixathon[®]/Riximyo[®] was not impacted by in-use conditions required for infusion. In addition, the degree of deamidation and oxidation was specifically monitored at sites in the molecule most sensitive to these modifications by peptide map analysis at 0 and 31 days. Due to the low abundance of deamidation and oxidation variants, relative quantitation was achieved using mass spectrometry by comparing the extracted ion chromatograms (EIC) of the modified and native peptides. As shown in Figure 3(a) and (b) and Table 5, the abundance of the deamidation on peptide L28H and the degree of oxidation on peptide L17H remained unchanged under in-use conditions. Further, comparison of the peptide map

Table 4. Cation exchange chromatography analysis of Rixathon[®]/Riximyo[®] batches following in-use preparation for intravenous infusion.

Batch (bag type)	Time (days)	Main peak (%)	Acidic variants (%)	Basic variants (%)
Batch A (PP)	0	66.6	21.3	12.1
	15	66.6	22.0	11.4
	31	66.3	21.6	12.1
Batch A (PE)	0	66.3	21.3	12.4
	15	66.9	21.7	11.4
	31	66.4	21.5	12.1
	RSD	0.2%	0.3%	0.4%
Batch B (PP)	0	70.1	17.6	12.3
	15	70.4	17.6	12.1
	31	69.9	18.0	12.1
Batch B (PE)	0	70.2	17.6	12.2
	15	70.2	17.6	12.2
	31	69.7	17.9	12.4
	RSD	0.2%	0.2%	0.1%
Batch C (PP)	0	70.2	17.7	12.2
	15	69.7	17.9	12.4
	31	69.8	18.0	12.2
Batch C (PE)	0	70.4	17.5	12.1
	15	70.1	17.6	12.2
	31	69.9	17.8	12.3
	RSD	0.3%	0.2%	0.1%

Note: Assessment of charge variants using cation exchange chromatography (CEX) in three Rixathon[®]/Riximyo[®] batches (arbitrarily labeled A, B, and C) diluted to 1 mg/mL in saline solution infusion bags and stored for 0, 15 and 31 days. Samples were stored at 5 ± 3°C for 14 or 30 days followed by an additional 24 h at room temperature. Stability was assessed in either polypropylene (PP) or polyethylene (PE) infusion bags. The relative abundance of acidic variants, basic variants and purity (main peak) are given. Mean values and relative standard deviation (RSD) are calculated for each batch.

PP: polypropylene; PE: polyethylene; RSD: relative standard deviation.

UV traces shown in Figure 4 revealed no observable difference in overall protein composition. These results demonstrate that charge variants, deamidation, oxidation and other minor protein variants remain stable over a 31-day period in either PP or PE saline infusion bags.

Potency

The potency of Rixathon[®]/Riximyo[®] was monitored by CDC analysis. Because CDC analysis is a cell-based assay, potency results exhibit greater variability than those observed for other analytical methods, resulting in greater relative standard deviation values.

As shown in Table 6, CDC analysis demonstrates that product potency remains stable over a 31-day period in either PP or PE saline infusion bags.

Discussion

Changes in the concentration, formulation, and temperature, such as those required for the preparation of rituximab for infusion, have the potential to impact the stability of the molecule which in turn can compromise the drug safety and efficacy. Although biosimilar products such as Rixathon[®]/Riximyo[®] must demonstrate physicochemical and clinical equivalence to the reference product prior to regulatory approval,¹⁵⁻¹⁷ the product stability evaluations of single use biologics, such as rituximab, do not generally include an assessment of extended in-use conditions.¹⁰ Thus, health care providers may be reluctant to employ biologics, such as biosimilar versions of rituximab, for indications requiring additional dilution or formulation, due to a paucity of relevant in-use stability data. The purpose of this manuscript was to evaluate the stability of the rituximab biosimilar Rixathon[®]/Riximyo[®] encountered upon dilution and reformulation of the product for intravenous administration.

To ensure that the in-use stability assessment described in this manuscript reflects stability under worst case conditions, Rixathon[®]/Riximyo[®] batches were selected which were in the final month of their 36-month shelf life and were subsequently exposed to 14 days of room temperature and light to recapitulate a major temperature excursion. Samples were diluted to the lowest allowable concentration of 1 mg/mL and stored in infusion bags for 14 or 30 days at 5 ± 3°C followed in each case by an additional 24 h at room temperature to simulate product handling. Although 5% dextrose formulation is also permitted by the rituximab label, this study focused exclusively on stability using 0.9% sodium chloride formulation since dextrose is known to result in product glycation and subsequent degradation over longer periods of storage.⁶ Further, dextrose formulations are often avoided where possible due to clinical risks associated with the provision of dextrose formulation and diabetes.

Although the rituximab label dictates infusion bag dilution to a final concentration anywhere between 1 and 4 mg/mL,^{4,5} samples in this study were diluted to the lowest allowable concentration of 1 mg/ml to evaluate conditions under which samples are most susceptible to adsorption and associated degradation. At lower concentrations, the percent adsorption is highest and can most dramatically compromise the intended soluble dose.²⁰ In addition to impacting protein concentration, protein adsorption to solid surfaces has been associated with unfolding, propagating

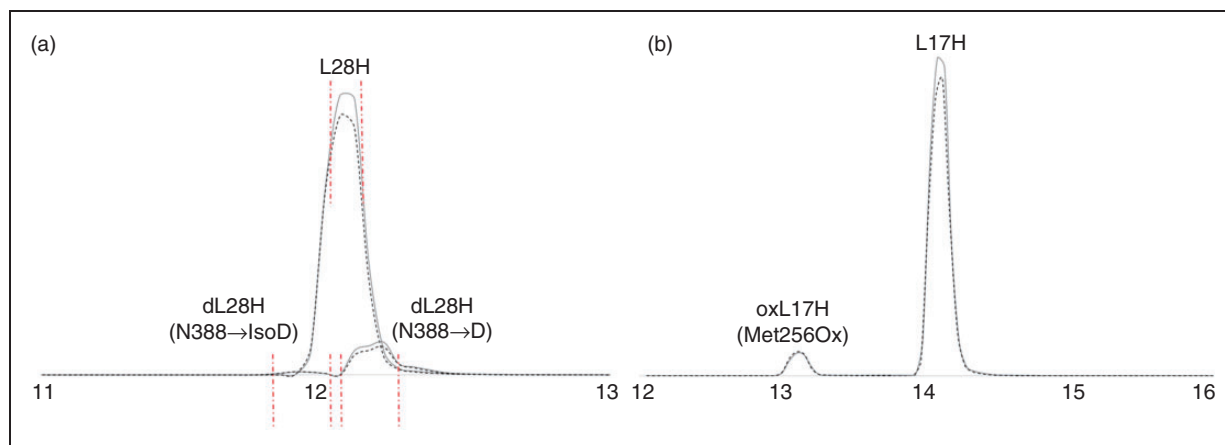


Figure 3. In-use preparation of Rixathon[®]/Riximyo[®] for intravenous infusion does not impact the abundance of deamination or oxidation variants. (a) Representative overlay of mass spectrometer extracted ion chromatograms (EIC) of the native L28H peptide and the deamidated peptide dL28H from Rixathon[®]/Riximyo[®] samples diluted to 1 mg/mL in saline solution infusion bags and stored for 0 (grey) and 31 (black dashed) days. Samples were stored at $5 \pm 3^{\circ}\text{C}$ for 30 days followed by an additional 24 h at room temperature. Both the isoaspartate (N388 to IsoD) and aspartate (N388 to D) forms of the deamidated peptide are indicated, the sum of which is considered total deamidation. Because the deamidated variants elute close to main variant and are low abundant, EIC peaks are defined by red dashed lines for clarity (b) Representative overlay of mass spectrometer extracted ion chromatograms of the native L17H peptide and the oxidized peptide oxL17H from Rixathon[®]/Riximyo[®] samples diluted to 1 mg/mL in saline solution infusion bags and stored for 0 (grey) and 31 (black dashed) days. Samples were stored at $5 \pm 3^{\circ}\text{C}$ for 30 days followed by an additional 24 h at room temperature. Elution times are shown on the x axis in minutes.

Table 5. Reducing peptide map – MS analysis of Rixathon[®]/Riximyo[®] batches following in-use preparation for intravenous infusion.

Batch (bag type)	Time (days)	dL28H (%)	oxL17H (%)
Batch A (PP)	0	3.95	7.86
	31	4.06	7.43
Batch A (PE)	0	6.11	7.07
	31	6.75	7.08
	RSD	1.42%	0.37%
Batch B (PP)	0	4.69	4.80
	31	5.05	4.71
Batch B (PE)	0	4.83	4.56
	31	4.66	4.91
	RSD	0.18%	0.15%
Batch C (PP)	0	3.90	4.78
	31	4.88	4.88
Batch C (PE)	0	3.98	4.44
	31	5.29	4.87
	RSD	0.68%	0.21%

Note: Assessment of deamidation and oxidation variants using peptide mapping coupled with mass spectrometry in three Rixathon[®]/Riximyo[®] batches (arbitrarily labeled A, B, and C) diluted to 1 mg/mL in saline solution infusion bags and stored for 0, 15 and 31 days. Samples were stored at $5 \pm 3^{\circ}\text{C}$ for 14 or 30 days followed by an additional 24 h at room temperature. Stability was assessed in either polypropylene (PP) or polyethylene (PE) infusion bags. The abundance of deamidated and oxidized peptides was assessed by comparison of variant dL28H and oxL17H peptide extracted ion chromatograms, relative to that of the native unmodified peptides, L28H and L17H. Mean values and relative standard deviation (RSD) are calculated for each batch.

PP: polypropylene; PE: polyethylene; RSD: relative standard deviation.

further qualitative differences such as degradation or aggregation.^{21–23} As shown in Table 1, assessment of sample concentration at 0, 15 and 31 days revealed no change in protein concentration, demonstrating that no measurable adsorption of Rixathon[®]/Riximyo[®] occurs under in-use conditions necessary for infusion.

Rixathon[®]/Riximyo[®] stability was evaluated using an array of state of the art methodologies to allow complete assessment of all degradation products and variants which may impact product safety and efficacy. Molecular weight variants were assessed in this study due to their notable sensitivity to changes in formulation and storage conditions and their potential impact on product immunogenicity and potency.^{24,25} While SEC analysis allowed for assessment of both high and low-molecular weight variants, non-reducing CE-SDS was additionally employed as an orthogonal method due to its ability to achieve higher resolution separation of low-molecular weight antibody degradation products and fragments.^{24,26,27} As shown in Figure 1(a) and (b) and Tables 2 and 3, both SEC and non-reducing CE-SDS demonstrate no change in molecular weight variants over the course of the in-use stability study.

Another class of variants which may be influenced by changes in formulation and storage are charge variants. Charge variants represent a broad class of protein modifications influencing the protein isoelectric point and can range from clinically inert variants, such as C-terminal lysine residues, to variants impacting

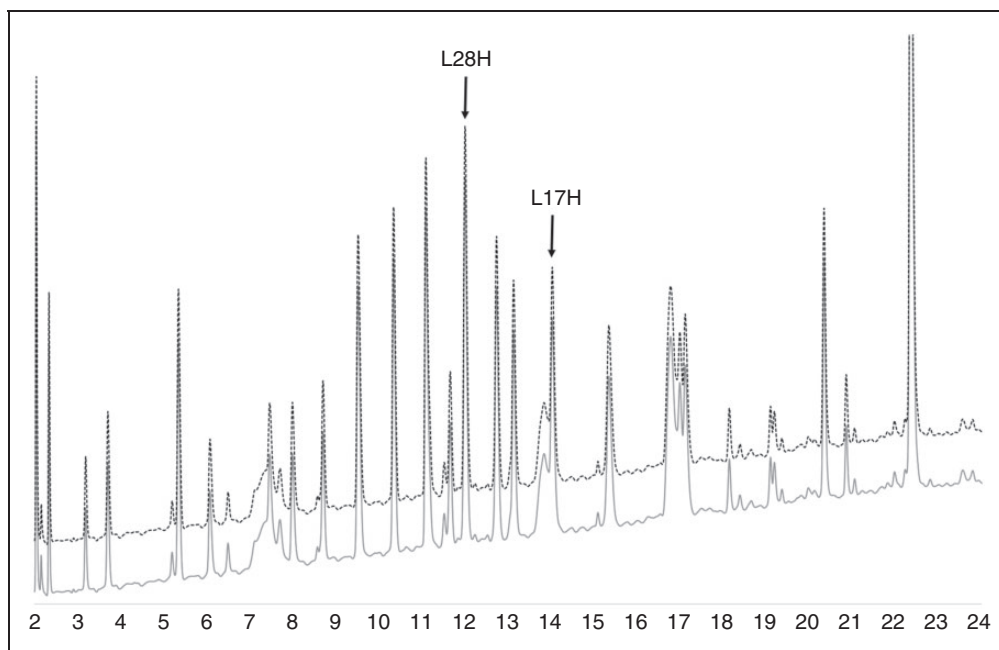


Figure 4. In-use preparation of Rixathon[®]/Riximyo[®] for intravenous infusion does not impact peptide map composition. Representative overlay of reducing peptide map UV chromatograms of Rixathon[®]/Riximyo[®] samples diluted to 1 mg/mL in saline infusion bags and stored for 0 (grey), 15 (black) and 31 (black dashed) days. Samples were stored at $5 \pm 3^{\circ}\text{C}$ for 14 or 30 days followed by an additional 24 h at room temperature. Peptides L28H and L17H, used respectively for assessment of deamidation and oxidation using mass spectrometry, are indicated for clarity. Elution times are shown on the x axis in minutes.

protein structure or function such as deamidations at critical asparagine residues.^{28,29} CEX analysis allowed for a general assessment of variants resulting in an acidic or basic shift in overall charge and, as shown in Figure 2 and Table 4, revealed no effect of in-use dilution and formulation conditions on charge variants.

To evaluate potentially critical minor variants, such as deamidation and oxidation, peptide mapping coupled with mass spectrometry was employed due to the ability of this approach to sensitively detect and characterize molecular variants at specific positions within a protein therapeutic.^{28,30} Although rituximab contains multiple asparagine residues which are potentially susceptible to deamidation, this modification most readily occurs within the SNGQPENNYK consensus sequence found in the CH3 constant domain of the antibody heavy chain.³¹ Similarly, although rituximab contains multiple methionine residues which are potentially susceptible to oxidation, methionine 256 has been shown to be most readily oxidized under endogenous and chemical stress conditions.^{27,30–32} Thus, the L28H peptide containing the deamidation consensus sequence and the L17H peptide, which contains the methionine 256 oxidation hot spot, were used as indicators for changes in the degree of deamidation or oxidation, respectively. As shown in Figure 3(a) and (b) and Table 5, no change in deamidation or oxidation was detected over the course of the in-use stability

study. Further, the high similarity of the peptide map UV chromatograms shown in Figure 4 underlines the equivalence of overall sample consistency over the course of in-use stability study, suggesting that in addition to deamidation and oxidation, the abundance of other minor variants was also not affected.

Taken together, broad physicochemical analyses demonstrate that Rixathon[®]/Riximyo[®] stability is not impacted by dilution and formulation conditions required for intravenous infusion. Assessment of protein concentration using ALC established that no significant protein adsorption or insoluble particle formation occurs under the defined in-use conditions in this study. Further, analysis using SEC, non-reducing CE-SDS, CEX and peptide mapping convincingly verifies that molecular weight and charge variants are unaffected by the dilution and formulation changes required for drug administration. To verify that the samples remain functionally stable under these conditions, CDC was used to compare the potency of Rixathon[®]/Riximyo[®] samples at different stability time points. While rituximab potency relies on multiple modes of action in addition to CDC, CDC activity is ideal for functional comparison as it requires both Fab and Fc integrity and is independent of additional variables such as effector cell activity.²⁷ As shown in Table 6, CDC analysis demonstrates no change in potency over the course of the in-use stability study.

Table 6. Potency of Rixathon[®]/Riximyo[®] batches following in-use preparation for intravenous infusion.

Batch (bag type)	Time (days)	CDC Potency (%)
Batch A (PP)	0	87
	15	81
	31	82
Batch A (PE)	0	89
	15	84
	31	83
	RSD	3%
Batch B (PP)	0	82
	15	83
	31	84
Batch B (PE)	0	81
	15	80
	31	83
	RSD	1%
Batch C (PP)	0	84
	15	89
	31	84
Batch C (PE)	0	89
	15	82
	31	83
	RSD	3%

Note: Assessment of potency using complement-dependent cytotoxicity (CDC) in three Rixathon[®]/Riximyo[®] batches (arbitrarily labeled A, B, and C) diluted to 1 mg/mL in saline solution infusion bags and stored for 0, 15 and 31 days. Samples were stored at $5 \pm 3^{\circ}\text{C}$ for 14 or 30 days followed by an additional 24 h at room temperature. Stability was assessed in either polypropylene (PP) or polyethylene (PE) infusion bags. Mean values and relative standard deviation (RSD) are calculated for each batch. PP: polypropylene; PE: polyethylene; RSD: relative standard deviation; CDC: complement-dependent cytotoxicity.

Although this study employs a broad spectrum of analytical tools to assess in-use stability, quality attributes associated with highly concentrated protein samples, such as sub-visible or visible particles, were not directly assessed as part of this study. However, thorough assessment of parameters such as pH, clarity and particles are required prior to Rixathon[®]/Riximyo[®] release and must meet defined safety standards at the final drug product concentration of 10 mg/mL.³³ Consistent data from this study demonstrating stable protein concentration and unchanged molecular weight distribution, charge characteristics and biological function strongly indicate that pH, clarity and particles are additionally unaffected at lower concentrations of 1–4 mg/mL used for intravenous infusion.

Conclusion

Taken together, physicochemical and biological analyses demonstrate that Rixathon[®]/Riximyo[®] quality is not impacted by dilution and formulation conditions required for intravenous infusion, even under worst case conditions with regard to product shelf life, temperature excursion, light exposure, dilution factor and infusion bag storage times. Thus, provided aseptic working conditions, the stability of the rituximab biosimilar Rixathon[®]/Riximyo[®] is maintained after dilution in saline solution infusion bags over a 31-day period.

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Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Rixathon[®] and Riximyo[®] are registered trademarks of Novartis AG and have been registered across different countries. At the time of manuscript submission, marketing authorization for the Sandoz rituximab biosimilar registered under the trade names Rixathon[®] and Riximyo[®] was formally approved by the European Commission. The authors are employees of the Novartis group of companies, which are developing, manufacturing and marketing biopharmaceuticals, including biosimilar products. MabThera[®] is a trademark registered by F. Hoffmann-La Roche AG. Rituxan[®] is a trademark registered by Biogen MA Inc.

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References

- Smith MR. Rituximab (monoclonal anti-CD20 antibody): mechanisms of action and resistance. *Oncogene* 2003; 22: 7359–7368.
- Weiner GJ. Rituximab: mechanism of action. *Semin Hematol* 2010; 47: 115–123.
- Boross P and Leusen JH. Mechanisms of action of CD20 antibodies. *Am J Cancer Res* 2012; 2: 676–690.
- Food and Drug Administration. Rituxan Label. *Online Referencing*, www.accessdata.fda.gov/drugsatfda_docs/label/2014/103705s54321bl.pdf (2014, accessed 16 May 2017).
- European Medicines Agency. MabThera summary of product characteristics. *Online Referencing*, www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000165/WC500025821.pdf (2016, accessed 16 May 2017).
- Leblanc Y, Bihoreau N, Jube M, et al. Glycation of polyclonal IgGs: effect of sugar excipients during stability studies. *Eur J Pharm Biopharm* 2016; 102: 185–190.

7. International Conference on Harmonization Guideline. Stability Testing of New Drug Substances and Products Q1A (R2). *Online referencing*, www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step4/Q1A_R2_Guideline.pdf (2003, accessed 16 May 2017).
8. International Conference on Harmonization Guideline. Stability testing of new drug substances and products Q5C. *Online Referencing*, www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5C/Step4/Q5C_Guideline.pdf (1995, accessed 16 May 2017).
9. European Medicines Agency. Note for guidance on in-use stability testing of human medicinal products. CPMP/QWP/2934/99. *Online Referencing*, www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003475.pdf (2001, accessed 16 May 2017).
10. European Medicines Agency. Note for Guidance on maximum shelf life for sterile products for human use after first opening or following reconstitution. CPMP/QWP/159/96 corr. *Online Referencing*, www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003476.pdf (1998, accessed 16 May 2017).
11. Paul M, Vieillard V, Jaccoulet E, et al. Long-term stability of diluted solutions of the monoclonal antibody rituximab. *Int J Pharm* 2012; 436: 282–290.
12. Zhang Y, Vermeulen LC and Kolesar JM. Stability of stock and diluted rituximab. *Am J Health Syst Pharm* 2013; 70: 436–438.
13. European Medicines Agency. Rixathon summary of opinion. EMA/CHMP/76832/2017 *Online Referencing*, www.ema.europa.eu/docs/en_GB/document_library/Summary_of_opinion_-_Initial_authorisation/human/003903/WC500226219.pdf (2017, accessed 16 May 2017).
14. European Medicines Agency. Riximyo summary of opinion. EMA/CHMP/76832/2017 *Online Referencing*, www.ema.europa.eu/docs/en_GB/document_library/Summary_of_opinion_-_Initial_authorisation/human/004729/WC500226218.pdf (2017, accessed 16 May 2017).
15. World Health Organization. Guidelines on evaluation of similar biotherapeutic products. *Online Referencing*, www.who.int/biologicals/areas/biological_therapeutics/BIOTHERAPEUTICS_FOR_WEB_22APRIL2010.pdf (2009, accessed 16 May 2017).
16. European Medicines Agency. Guideline on similar biological medicinal products. CHMP/437/04 Rev1. *Online Referencing*, www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/10/WC500176768.pdf (2014, accessed 16 May 2017).
17. Food and Drug Administration. Scientific consideration in demonstration biosimilarity to a reference product. *Online Referencing*, www.fda.gov/downloads/drugsguidancecomplianceregulatoryinformation/guidances/ucm291128.pdf (2015, accessed 16 May 2017).
18. European Medicines Agency. Guideline on similar biological medicinal products containing biotechnology – derived proteins as active substance: quality issues (revision 1). EMA/CHMP/BWP/247713/2012. *Online Referencing*, www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/06/WC500167838.pdf (2012, accessed 16 May 2017).
19. Food and Drug Administration. Quality considerations in demonstrating biosimilarity of a therapeutic protein product to a reference product. *Online Referencing*, www.fda.gov/downloads/drugs/guidances/ucm291134.pdf (2015, accessed 16 May 2017).
20. Herrmann JE and Collins MF. Quantitation of immunoglobulin adsorption to plastics. *J Immunol Methods* 1976; 10: 363–366.
21. Vermeer AW, Bremer MG and Norde W. Structural changes of IgG induced by heat treatment and by adsorption onto a hydrophobic Teflon surface studied by circular dichroism spectroscopy. *Biochim Biophys Acta* 1998; 1425: 1–12.
22. Pinholt C, Hartvig RA, Medicott NJ, et al. The importance of interfaces in protein drug delivery – why is protein adsorption of interest in pharmaceutical formulations? *Expert Opin Drug Deliv* 2011; 8: 949–964.
23. Rabe M, Verdes D and Seeger S. Understanding protein adsorption phenomena at solid surfaces. *Adv Colloid Interface Sci* 2011; 162: 87–106.
24. Vlasak J and Ionescu R. Fragmentation of monoclonal antibodies. *MAbs* 2011; 3: 253–263.
25. Ratanji KD, Derrick JP, Dearman RJ, et al. Immunogenicity of therapeutic proteins: influence of aggregation. *J Immunotoxicol* 2014; 11: 99–109.
26. Salas-Solano O, Tomlinson B, Du S, et al. Optimization and validation of a quantitative capillary electrophoresis sodium dodecyl sulfate method for quality control and stability monitoring of monoclonal antibodies. *Anal Chem* 2006; 78: 6583–6594.
27. Visser J, Feuerstein I, Stangler T, et al. Physicochemical and functional comparability between the proposed biosimilar rituximab GP2013 and originator rituximab. *BioDrugs* 2013; 27: 495–507.
28. Vlasak J and Ionescu R. Heterogeneity of monoclonal antibodies revealed by charge-sensitive methods. *Curr Pharm Biotechnol* 2008; 9: 468–481.
29. Khawli LA, Goswami S, Hutchinson R, et al. Charge variants in IgG1: isolation, characterization, in vitro binding properties and pharmacokinetics in rats. *MAbs* 2010; 2: 613–624.
30. Srebalus Barnes CA and Lim A. Applications of mass spectrometry for the structural characterization of recombinant protein pharmaceuticals. *Mass Spectrom Rev* 2007; 26: 370–388.
31. Du Y, Walsh A, Ehrick R, et al. Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies. *MAbs* 2012; 4: 578–585.
32. Chumsae C, Gaza-Bulsecu G, Sun J, et al. Comparison of methionine oxidation in thermal stability and chemically stressed samples of a fully human monoclonal antibody. *J Chromatogr B* 2007; 850: 285–294.
33. International Conference on Harmonization Guideline. Specifications: test procedures and acceptance criteria for biotechnological/biological products Q6B. *Online Referencing*, www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf (1999, accessed 7 August 2017).