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REPORT

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Restoring the biological activity of crizanlizumab at physiological conditions through a pH-dependent aspartic acid isomerization reaction

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ABSTRACT

In this study, we report the isomerization of an aspartic acid residue in the complementarity-determining region (CDR) of crizanlizumab as a major degradation pathway. The succinimide intermediate and iso-aspartic acid degradation products were successfully isolated by ion exchange chromatography for characterization. The isomerization site was identified at a DG motif in the CDR by peptide mapping. The biological characterization of the isolated variants showed that the succinimide variant exhibited a loss in target binding and biological activity compared to the aspartic acid and iso-aspartic acid variants of the molecule. The influence of pH on this isomerization reaction was investigated using capillary zone electrophoresis. Below pH 6.3, the succinimide formation was predominant, whereas at pH values above 6.3, iso-aspartic acid was formed and the initial amounts of succinimide dropped to levels even lower than those observed in the starting material. Importantly, while the succinimide accumulated at long-term storage conditions of 2 to 8°C at pH values below 6.3, a complete hydrolysis of succinimide was observed at physiological conditions (pH 7.4, 37°C), resulting in full recovery of the biological activity. In this study, we demonstrate that the critical quality attribute succinimide with reduced potency has little or no impact on the efficacy of crizanlizumab due to the full recovery of the biological activity within a few hours under physiological conditions.

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Introduction

Crizanlizumab (Adakveo®) is an intravenously administered monoclonal IgG2 antibody developed by Novartis Pharmaceuticals for the prevention of vaso-occlusive crises in patients with sickle cell disease. Crizanlizumab binds to P-selectin, thereby blocking its interaction with P-selectin glycoprotein ligand-1 (PSGL-1).[1,2]

Degradation during manufacturing and storage of biopharmaceuticals such as crizanlizumab is a common phenomenon that may result in structural and functional changes. Main degradation mechanisms are therefore characterized during technical development and the critical quality attributes affecting the safety or efficacy are controlled during release and stability testing of drug substances and drug products.

Several monoclonal antibody (mAb)-related degradation products can be detected as charge variants using methods such as capillary isoelectric focusing (cIEF), ion exchange chromatography (IEX) or capillary zone electrophoresis (CZE). Common charge variants of mAbs can be caused by sialic acids, C-terminal lysine, N-terminal glutamine, unprocessed leader sequence, as well as degradation products such as clippings, deamidation, oxidation, glycation, or isomerization of aspartic acid to iso-aspartic acid through the cyclic imide intermediate succinimide. Other sequence, chemical, or post-translational modifications may lead to further antibody microheterogeneity.[3]

The chemical isomerization reaction is favored in the presence of glycine at the C-terminus of the isomerization site, namely at a DG motif, and has been studied in detail using model peptides.[4–9] Additional influencing factors of the isomerization reaction are the protein structure itself,[10] as well as environmental conditions. During aspartic acid isomerization, the rate-limiting[10] accumulation of succinimide is favored at mildly acidic conditions[8] and under elevated temperatures.[11] The following succinimide hydrolysis to iso-aspartic acid and aspartic acid is preferred at neutral and basic pH with a ratio of approximately 3:1.[4,12–15] MAbs are often stored under mildly acidic or neutral conditions to ensure their stability,[16] which promotes the formation of succinimide and its subsequent hydrolysis in this class of biopharmaceuticals.

Biologics technical development integrates developability assessment as a major project selection milestone to minimize late-stage development issues, building on *in silico* scoring tools for the prediction of degradation hotspots, such as aspartic acid in the DG motif prone to isomerization. If chemical modifications exist in the antibody's complementarity-determining regions (CDR), an effect on the binding activity is possible. The influence on the binding activity by the presence

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of succinimide in the CDR has already been reported.[17–20] Isoaspartic acid as isomerization variant has also been shown to result in a decreased binding activity, if present in the CDR.[18,21,22] Degradation hotspots, such as the DG isomerization site, are scored negatively during the developability assessment using *in silico* prediction tools.

While optimized formulation conditions minimize degradation of biologics during storage, the environment after the administration in patients, such as the blood stream, potentially triggers uncontrolled modifications. Physico-chemical properties such as pH, redox potential, temperature of human serum and human plasma may lead, for example, to deamidation, oxidation, or aggregation.[23] The impact of physiological pH and temperature can easily be controlled in a buffer system to study the dynamics of these chemical modifications. For example, it has been reported, that succinimide hydrolyses at pH 7.6 and 37°C.[17] Also, the serum matrix and its components are potentially additional influencing factors. The study of degradation pathways in such a complex matrix cannot be performed by standard physicochemical methods like CZE or IEX. However, mass spectrometry (MS) is able to analyze site-specific degradation products, such as deamidation, within such complex matrices.[24]

In this study, we investigated the isomerization reaction of an aspartic acid located in an isomerization hotspot of the CDR of crizanlizumab and the effect on its biological activity. We demonstrated that a succinimide variant in the CDR of crizanlizumab, initially considered as a critical quality attribute due to the loss of activity, has little or no effect on the efficacy of the molecule due to the full recovery of the biological activity within a few hours under physiological conditions. Our study highlights the importance of *in vitro* experiments to assess the impact of a degradation product and its criticality as a quality attribute in regard to safety and efficacy.

Results

Characterization of charge variants

CZE was used to monitor the charge variants of crizanlizumab during release and stability testing. The only relevant changes of the charge profile, detected during real-time storage conditions or during forced degradation studies, are changes of the peaks BP3, BP1, main, AP1, and AP2 (Figure 1). The peaks were enriched using anion exchange chromatography (AEX) fractionation from stressed (40°C for 6 weeks at pH 5.7) and unstressed (stored frozen at pH 5.7) crizanlizumab (Figure 1). AEX fractions were analyzed by peptide mapping for the identification of the different charge variants. Differences between the AEX fractions were only observed for a light chain peptide named L2-3 including one of the CDR of the molecule (Figure 2). The relative abundance of the native peptide L2-3 was decreased in the basic and acidic fractions compared to the "main" fraction (Figure 2).

In both acidic fractions, an early-eluting isomer of L2-3 was increased. To characterize the early-eluting isomer, online electron transfer dissociation (ETD) was used to produce diagnostic ions,[25–27] more specifically c + 57 and z – 57 for isoaspartic acid. This was also applied to the unmodified CDR peptide. Figure 3(a-b) shows zoomed views of the resulting

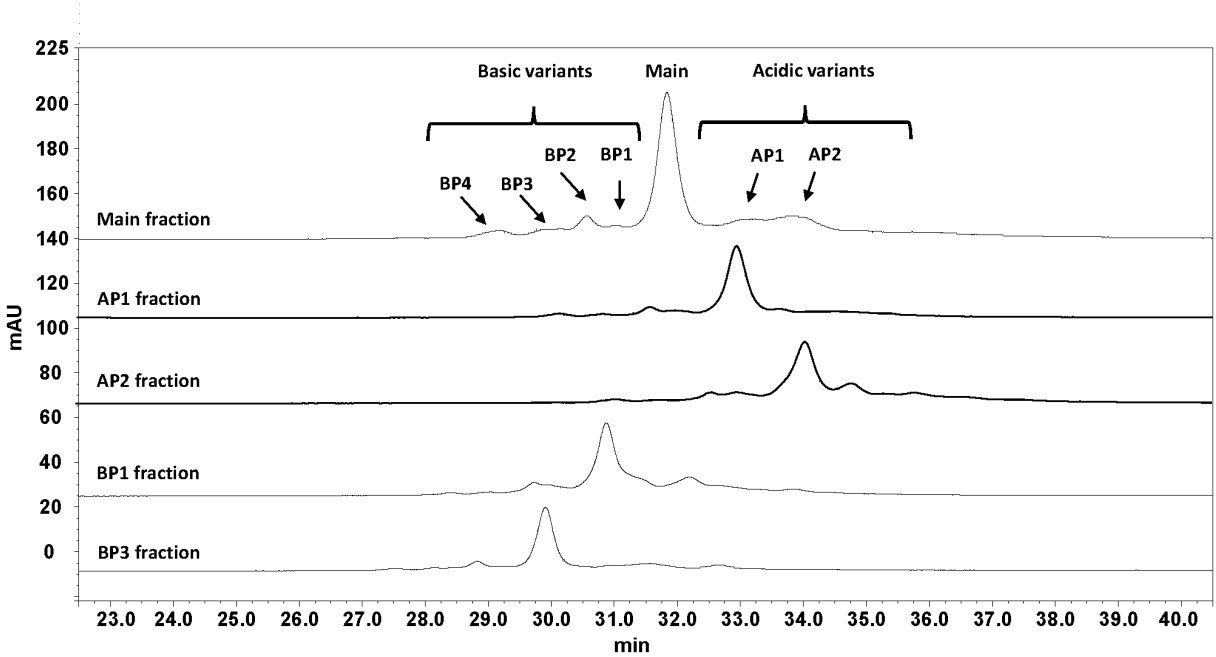


Figure 1. CZE electropherograms of the isolated crizanlizumab variants. Crizanlizumab isoforms were enriched using anion exchange chromatography (AEX) fractionation from stressed (40°C for 6 weeks at pH 5.7) and unstressed material (stored frozen at pH 5.7). The fractionation was done with an Agilent HPLC system 1100 equipped with a cooled fraction collection unit and a weak anion exchange column (Dionex ProPac WAX-10 BioLC, 4 mm x 250 mm). Separation of the isoform was achieved using a NaCl salt gradient at pH 7.9. Fractions were diluted (50%: 50%) in citrate buffer (5 mM, pH 2.8) in order to preserve the stability of the basic variants. The collected fractions were concentrated in centrifugal filter devices (15 ml capacity). The final protein content determination was performed by UV spectroscopy (using Nanodrop). The CZE analysis of the AEX fractions was performed using a Beckman Coulter PA800 Plus capillary electrophoresis instrument equipped with an uncoated fused-silica capillary. Electrophoresis was performed in running buffer (400 mM 6-aminocaproic acid/acetic acid pH 5.7 with 2 mM TETA and 0.03% Tween 20) at a constant voltage of 20 kV in positive to negative (normal) polarity mode for 45 or 30 minutes. AP: acidic peak; BP: basic peak.

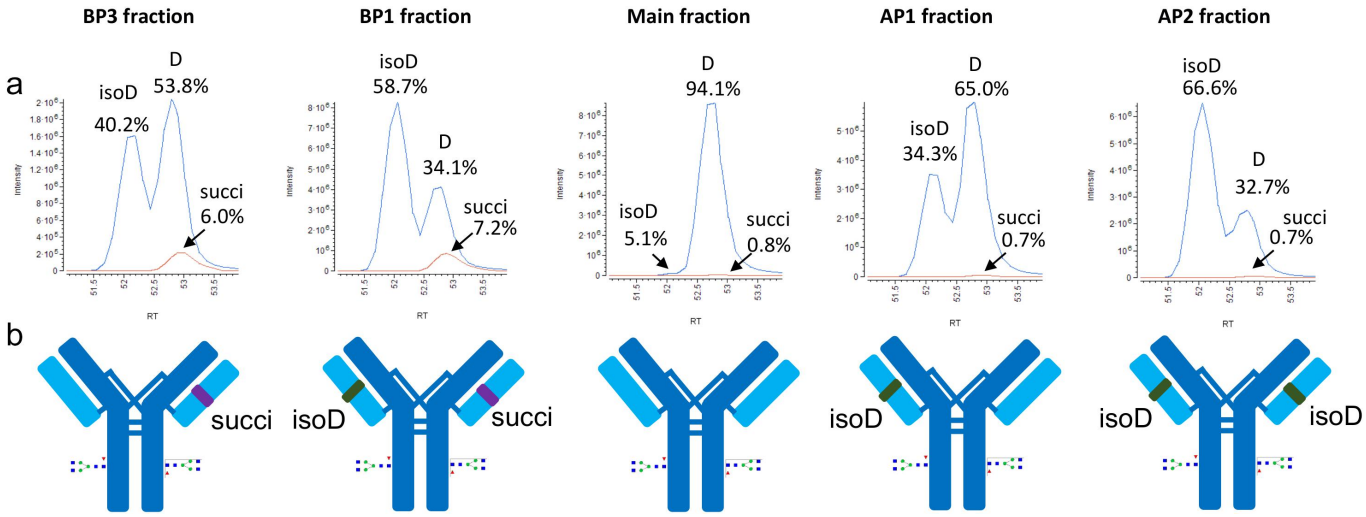


Figure 2. Analysis of isolated charge variants of crizanlizumab by Lys-C peptide mapping at pH 7.5. A: Extracted ion chromatograms of the iso-aspartic acid and aspartic acid precursor (863.72 m/z) in blue and of the succinimide precursor (857.72 m/z) in Orange of the L2-3 peptide in each fraction acquired with the Orbitrap Elite after peptide separation on the Vydac C18 column. The iso-aspartic acid, aspartic acid and succinimide charge variants are eluting at 52.1, 52.8 and 53.0 min, respectively. AP: acidic peak; BP: basic peak. D: aspartic acid; isoD: iso-aspartic acid; succi: succinimide. B: Depictions of crizanlizumab containing the basic succinimide or the acidic iso-aspartic acid charge variants of the light chain enriched in each fraction. isoD: iso-aspartic acid; succi: succinimide.

ETD MS/MS spectra for both peaks. Figure 3(a) reveals the diagnostic ions $c'_7{}^{1+}$ + 57 and $z''_{15}{}^{1+}$ – 57 at the DG motif of the peptide identifying the presence of iso-aspartic acid in the early-eluting peptide. Since iso-aspartic acid has been reported to have a lower pKa value than D,[28] the presence of this variant in the acidic fraction is expected. For acidic AEX fractions AP1 and AP2, ~ 34% and ~ 67% of iso-aspartic acid were enriched, respectively. The difference in the iso-aspartic acid level between the two fractions correspond to an enrichment factor of 2 in AP2. This strongly suggests that AP1 contains mainly crizanlizumab with one light chain exhibiting one iso-aspartic acid variant and AP2 the two light chains exhibiting two iso-aspartic acid variants. The differences between observed and theoretical levels (34% vs 50% for AP1 and 67% vs 100% for AP2) may either result from the co-enrichment of other molecules or from conversion during fraction collection and digestion at pH 7.5.

In basic fractions, the iso-aspartic acid isomer of L2-3 was increased as well, reaching higher levels in fraction BP1 than in fraction BP3. The basic fractions also contained a variant bearing a mass shift of −18 Da co-eluted with the unmodified CDR peptide L2-3 (Figure 2). Interpretation of the MS/MS spectra of the variant acquired with collision-induced dissociation (CID) and higher energy collisional dissociation (HCD) fragmentation types confirmed the loss of 18 Da at the expected aspartic acid residue. This localization at a DG motif suggests the enrichment of a succinimide in the CDR peptide in basic fractions (Figure 3(c)). The succinimide formation from an aspartic acid is a direct loss of a carboxylic acid group, resulting in an apparent basic variant, from a negatively charged to a neutral species at mildly acidic conditions (pH > 5). Succinimide has been reported as a basic variant by others as well.[29] The reason for the relatively low amounts of succinimide enriched in the basic fractions can be explained by the fast hydrolysis of succinimide intermediate to aspartic acid and iso-aspartic acid during peptide mapping sample preparation at pH 7.5, as shown by others.[4,12,13]

Taking into consideration this conversion mechanism and the observed levels for each variant, we concluded that the AEX fraction of BP3 is enriched with crizanlizumab molecules containing one succinimide in one light chain (Figure 2(b)). The AEX fraction of BP1 may consist mainly of antibody molecules with the presence of one succinimide in one light chain and the concomitant presence of one acidic iso-aspartic acid in the second light chain (Figure 2(b)).

Further identification of succinimide or iso-aspartic acid by more advanced technologies, which includes the labeling of succinimide with 18O, was not deemed necessary for this study.[12,15,30]

All other basic CZE peaks have been identified as typical basic modifications of mAbs (e.g., N-terminal glutamine, C-terminal prolinamide, C terminal lysine), which are not related to the isomerization reaction, and hence are not further discussed here.

Impact of the different charge variants on biological activity

The potency of the AEX fractions was determined by ELISA and cell-based assay (CBA) (Table 1). The potency of each fraction is reported in percent because it is the relative potency in comparison to the crizanlizumab reference standard. Fractions BP3 and BP1 contain succinimide as a major variant and show a decreased potency in both potency assays. The potency decrease was more pronounced in the functional CBA compared to the binding ELISA. The main peak AEX fraction containing the aspartic acid variant, as well as the acidic fractions AP1 and AP2 with iso-aspartic acid as a major form, did not show reduced potency compared to the crizanlizumab reference sample. The results reveal that only the presence of the cyclic imide in the CDR on the light chain

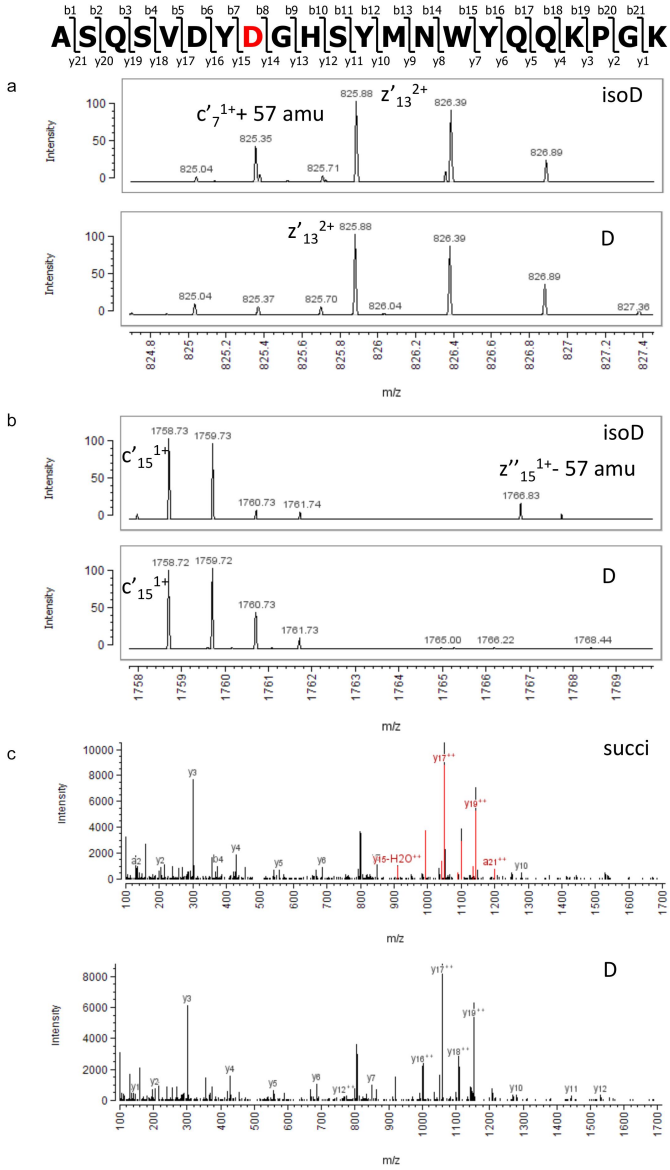


Figure 3. Identification of iso-aspartic acid at position 32 in the CDR region of the light chain of crizanlizumab. Sequence of the peptide L2-3 is shown on top. Zoomed views of ETD MS/MS spectra onto diagnostic ions c + 57 (a) and z – 57 (b) confirming iso-aspartic acid at the DG motif in the peak eluting at 52.1 min (top panel) compared to the peak eluting at 52.8 min (bottom panel) using a Vydac C18 column. C: Identification of the succinimide variant at position 32 in the CDR region of the light chain of crizanlizumab eluting at 53.0 min. Consolidated CID/HCD MS/MS spectra of the unmodified peptide (bottom panel) and the peptide with succinimide at DG motif (top panel). Fragment ions highlighted in red exhibit a loss of 18 Da at the aspartic acid residue corresponding to the succinimide.

(BP3 and BP1) has a negative impact on the potency. Furthermore, none of the other basic charge variants (BP2 and BP4), related to typical IgG basic modifications mentioned above, had a significant impact on the potency.

Influence of pH and temperature on charge variants

The pH and temperature are known to be the main contributors, influencing the isomerization reaction of aspartic acid.[8,14] The influence of the pH on the aspartic acid isomerization reaction in the CDR of crizanlizumab was investigated. Crizanlizumab exhibits a different degradation pathway, dependent on the pH conditions. At pH 5.0, the formation of basic variants is favored, particularly BP3, the major succinimide containing variant. New CZE peaks appeared, which are even more basic compared to BP3 (Figure 1). These are likely caused by crizanlizumab variants containing succinimide on two light chains and other combinations of succinimide containing crizanlizumab with other basic variants present (e.g., N-terminal Q, C-terminal prolinamide). At pH 7.0 the formation of the acidic variants is favored, which is related to the formation of an iso-aspartic acid residue at one or both light chains of crizanlizumab (AP1 and AP2, respectively).

To investigate the influence of pH and temperature on the isomerization reaction in more detail, samples formulated at pH 5.0, 5.5, 5.7, 6.0, 6.3, 6.5, and 7.0 were incubated at 5, 25 and 40°C. CZE analysis was used to monitor succinimide and iso-aspartic acid containing variants for this investigation. The sum of acidic variants was used to monitor changes to the iso-aspartic acid levels, as only iso-aspartic acid variants were identified in fractions AP1 and AP2 (Figure 1 and 2). The sum of basic variants was used to monitor changes to the amount of succinimide, as the levels of other basic variants (e.g., N-terminal Q, C-terminal prolinamide) and their variations under these conditions remained low. Major changes during the study were only observed for BP3 and to a lesser extent to BP1 (Figures 1 and 2 for peak assignment), allowing a direct correlation between the changes of basic variants by CZE and the succinimide levels in the sample. Figure 4 illustrates the dynamic behavior of the basic variants as a function of time, temperature, and pH, for pH 5.7 (Figure 4(a)), pH 6.0 (Figure 4(b)), and pH 6.3 (Figure 4(c)) at 5, 25 and 40°C. At pH 5.7 and pH 6.0 the basic variants initially increased (corresponding to succinimide formation), reaching a maximum, followed by a decrease (corresponding to succinimide hydrolysis) over time at 25°C and 40°C. While the maximum amount of basic variants for temperatures of 25°C and 40°C was reached after a short period of time (2 weeks and 4 weeks, respectively), this value steadily increased over a period of 12 months at 5°C. At pH 6.3, no increase of the basic variants (succinimide formation) was observed for any storage temperatures or time points, but only a decrease of the basic variants (succinimide hydrolysis) was observed. The fastest decrease at pH 6.3 was observed at 40°C followed by 25°C, while the slowest decrease was at 5°C. The decrease of the basic variants (succinimide hydrolysis) correlated well with an increase of the acidic variants (iso-aspartic acid generation from succinimide hydrolysis; data not shown)

In Figure 5 the relative amounts of basic variants as a function of pH before and after storage for 1 month at 25°C is illustrated. At mildly acidic conditions (pH below 6.3) the basic variants increase due to succinimide formation revealed by a distinct increase of the BP3 peak, while the succinimide hydrolysis and its conversion to aspartic acid and iso-aspartic acid (AP1 and AP2 peaks) resulted in a decrease of basic variants at pH conditions above 6.3.

The maximum relative amount of basic variants that are correlated to the maximum amount of succinimide is

Table 1. Potency of crizanlizumab charge variants. Crizanlizumab charge variants (isoforms) were enriched using anion exchange chromatography (AEX). For peak assignment of the enriched isoforms see Figure 1 and for the identification of the isoforms see Figure 2. The enriched charge variants were analyzed by ELISA and CBA for potency determination.

CZE peak	Potency ELISA [%]	Potency cell-based assay [%]
BP3	78	45
BP1	63	33
Main	90	102
AP1	105	125
AP2	119	141

influenced by the pH (36.9% for pH 5.7; 29.8% for pH 6.0; no maximum for pH 6.3), whereas the temperature affects the duration until the maximum is reached. The higher the temperature, the faster the reaction.

Finally, an additional experiment was performed to confirm the reversibility of the isomerization mechanism. A crizanlizumab sample was stored for a longer time period at neutral pH conditions to increase the iso-aspartate variants and to decrease both basic and main variants. The sample was later incubated at pH 5.0, which led to increased basic variants (succinimide) and decreased acidic variants (iso-aspartic acid variant), thus confirming the conversion of iso-aspartic acid to succinimide and aspartic acid (data not shown).

Impact of the succinimide presence on the biological activity

Previous experiments showed that the increased level of basic variants observed during the incubation at mildly acidic conditions was related to the formation of the succinimide variant and that the succinimide variant is less active compared to the main antibody variant containing aspartic acid in the CDR and the acidic variants containing iso-aspartic acid in the CDR. To assess the criticality of the succinimide variant, which is the major variant of the basic CZE peaks, we further investigated the impact of succinimide formation in the CDR on the biological activity under physiological conditions. A stressed crizanlizumab sample (pH 5.0 incubated for 4 weeks at 25°C; Figure 6), containing 52% basic variants with a reduced potency of 67% by CBA was incubated at physiological conditions (pH 7.4 and 37°C) for up to 24 h (Figure 6(a)). After 24 h, the amounts of basic variants decreased from 52% to 14%. To a similar extent, the relative amounts of acidic variants changed from 24% to 59%. As a result, the main peak was almost steady (24% before and 26% after incubation; Figure 6(a)). The change of basic and acidic variants is mainly caused by a decrease of BP3 and of those peaks, which are more basic than BP4, leading to increased AP1 and AP2 peaks. Strikingly, the relatively low potency at the beginning (67% by CBA) increased after 24 h

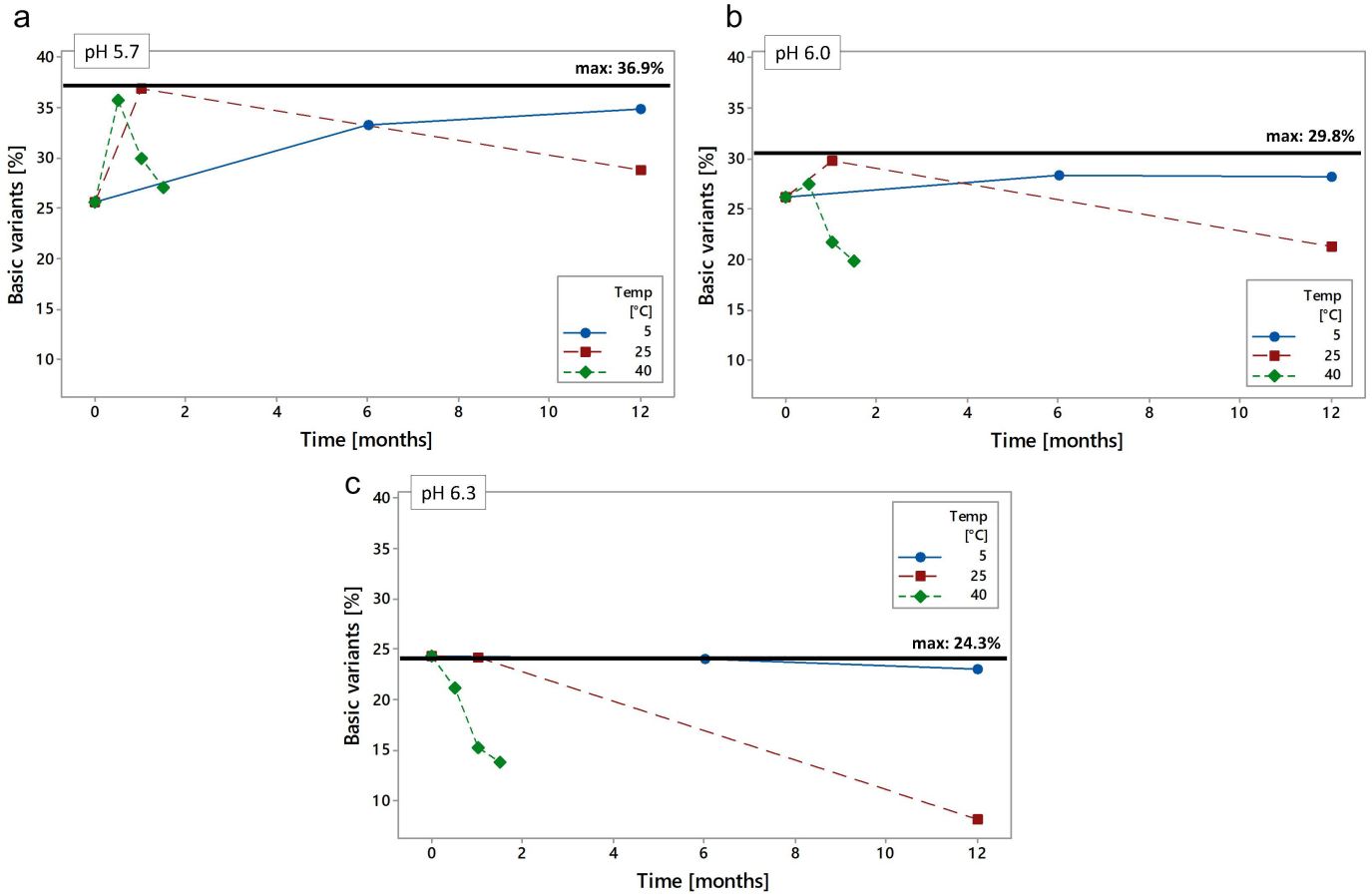


Figure 4. Basic variants of crizanlizumab by CZE as a function of time. 10 mg/mL crizanlizumab were compounded at pH 5.0, 5.5, 5.7, 6.0, 6.3, 6.5, 7.0 in parallel and stored at different temperature conditions (5°C, 25°C and, 40°C). Samples were analyzed by CZE at different times to determine the levels of charge variants. A: incubation at 5, 25 and 40°C at pH 5.7, B: incubation at 5, 25 and 40°C at pH 6.0, C: incubation at 5, 25 and 40°C at pH 6.3.

incubation under physiological conditions to 121% by CBA. These results show that the formation of iso-aspartic acid does not result in physiologically relevant decreased potency, since the potency is restored with the conversion of succinimide to iso-aspartic acid occurring at physiological pH conditions. The succinimide hydrolysis has been identified as a relatively fast first-order reaction. The half-life of 3.9 h was calculated after curve fitting (first-order reaction) of the basic variants as a function of time during incubation at pH 7.4 and 37°C (Figure 6(b)).

Reversibility reaction in human serum

In addition to the buffer experiments, the fate and impact of succinimide on the biological activity in human serum matrix was investigated. As in the buffer experiment above, stressed crizanlizumab incubated at pH 5.0 for 4 weeks at 25°C enriched in succinimide with a reduced potency (79% by CBA) was spiked into human serum at a relevant concentration of 150 µg/mL and incubated at 37°C for different periods of time. The CZE method could not be used to analyze the samples and to monitor succinimide due to the low concentration of crizanlizumab in serum. Therefore, we developed a liquid chromatography coupled to an MS method in order to analyze the samples after enzymatic digestion at pH 6.0. We used parallel reaction monitoring (PRM) to specifically detect the precursors and specific fragment ions of the peptides bearing aspartic acid, succinimide, and iso-aspartic acid of crizanlizumab in human serum. To increase sensitivity, formic acid was used in the mobile phase instead of trifluoroacetic acid (TFA), which is a known ion suppressor. A C18 CSH column with low-level positively charged particles was used to obtain sharp peaks with formic acid. Under these chromatographic conditions, the succinimide species is eluting earlier than the aspartic acid and iso-aspartic acid species.

Figure 7(a) shows the workflow used for PRM scheduled for the elution order of succinimide, aspartic acid, and iso-aspartic acid, confirming the charge/hydrophobicity of each variant. Figure 7(b) shows the extracted ion chromatograms for the specific fragments used to monitor the conversion of the succinimide variant into aspartic acid and its isomer in human serum as a factor of time. In Figure 8 the kinetics of the succinimide hydrolysis at 37°C in human serum is shown. A similar kinetics of the succinimide hydrolysis was observed in serum (half-life of 3.1 h) as compared to the buffer experiments (half-life of 3.9 h). The minor differences in the kinetics might be within the variability of the experiments due to minor differences in the pH, temperature, or other parameters, like the different analytical detection methods used (CZE vs MS). However, we cannot exclude that the serum composition might affect the succinimide hydrolysis reaction, in addition to the pH and the temperature.

Finally, the biological activity was also restored in serum, as confirmed by CBA. After 24 h incubation at 37°C, the relative potency was increased (92% CBA) compared to the starting conditions (79% CBA).

Discussion

In this study, we investigated the isomerization reaction of an aspartic acid located in an isomerization hotspot in the CDR of crizanlizumab and the effect on its biological activity. The kinetics of the aspartic acid isomerization and its relationship with pH and temperature have been extensively described.[4,8, 10–14]

The influence of pH on the aspartic acid isomerization

Under acidic conditions (below pH 6.0) the formation of succinimide is the dominant degradation product of the isomerization reaction of aspartic acid 32 located in the CDR region of crizanlizumab. The reversibility of the isomerization was confirmed in this study, where the formation of succinimide can result from the isomerization of both aspartic and iso-aspartic acid residues, detectable by a decrease of the CZE main peak (the aspartic acid variant), and the decrease of the CZE acidic peaks (the iso-aspartic acid variants), respectively.

At pH 6.0, basic variants (mainly the BP3 variant) and acidic variants increased, showing that succinimide is formed, which partly hydrolyzes to iso-aspartic acid (AP1 and AP2) and aspartic acid (main). Since the main peak decreases and the acidic peaks 1 and 2 increase, the formation of iso-aspartic acid is finally predominant. This is based on the succinimide hydrolysis to iso-aspartic acid and aspartic acid with a ratio of 3:1.[4,12,13] Accumulation of succinimide is known to be accelerated under those mildly acidic conditions[8] combined with increased temperatures.[11] Independent of the tested pH, the formation of succinimide is not unlimited: although the cyclization reaction is favored at pH conditions below pH 6.3, succinimide also hydrolyzes at those conditions, reflected by an increase of iso-aspartic acid (acidic variants). Even at pH 5.0 the basic variants decrease again after reaching a maximum (data not shown). The equilibrium between the succinimide formation and its hydrolysis is pH dependent, so the maxima that basic variants can reach differ. The lower the pH, the more the equilibrium favors succinimide formation. Higher succinimide maxima are present at lower pH values compared to neutral pH conditions. This behavior is also in agreement with reported rate constants for the succinimide formation, which increase by lowering the pH from 6 to 4. It has been reported that rate constants of the succinimide hydrolysis were increased by raising pH conditions from 6 to 8.5.[11]

At a pH of 6.3 and higher, both the main and basic variants of crizanlizumab decrease over time. The aspartic acid main variant (main peak in CZE) first needs to form a succinimide variant as the rate limiting step[10] prior to undergoing hydrolysis to aspartic and iso-aspartic acid. Since the basic variants are decreasing, the succinimide variant is not stable at pH 6.3 and above, leading to increased iso-aspartic acid variants, as already reported for similar pH conditions.[17] This confirms the predominant succinimide hydrolysis at neutral or alkaline pH.[14] Zhou and colleagues have successfully detected and quantified succinimide in intact protein via hydrazine trapping and chemical derivatization.[31] Although this elegant methodology enables the trapping of labile succinimide species in a stable hydrazide and subsequent analysis, the present study describes the conditions (pH, temperature) leading to the formation and hydrolysis of succinimide in its

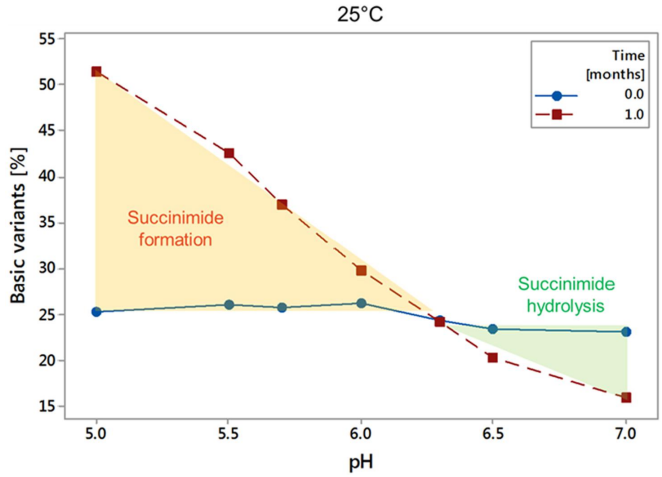


Figure 5. Basic variants of crizanlizumab by CZE as a function of pH. 10 mg/mL crizanlizumab were compounded at pH 5.0, 5.5, 5.7, 6.0, 6.3, 6.5, 7.0 in parallel and stored at 25°C for 1 month. Samples were analyzed by CZE to determine the levels of charge variants.

native form by CZE to demonstrate the reversible behavior of this mechanism in crizanlizumab. Recent work from other groups confirmed that succinimide formation and racemization result in four isomer products, namely L-aspartic acid, L-iso-aspartic acid, D-aspartic acid, and D-iso-aspartic acid.[32–34] The L- and D-stereoisomers of aspartic acid and iso-aspartic acid can be differentiated by reversed phase liquid chromatography using standards containing L- and D-amino acids, or using proteases specific to chiral amino acids. Radical directed dissociation coupled with MS is also a method sensitive to structural differences that can be applied to differentiate stereoisomers in synthetic peptides using a photocleavable radical precursor. Those techniques were not used in the present study because D-stereoisomers are expected to remain within a small fraction (~10%) of all isomerization products.[34]

Impact of isomerization on potency and its behavior as a function of pH

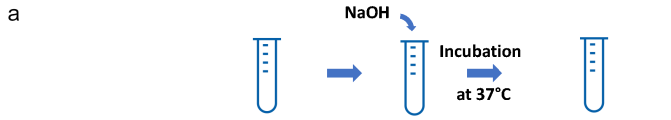
Aspartic acid isomerization in the CDR can affect the target-binding behavior. For crizanlizumab, potency was decreased by the presence of increased succinimide levels. The impact of succinimide on the potency is also reported for other molecules[17,18,20,35] and can be explained by a change in the binding affinity, potentially caused by the cyclization.[20] The potency of crizanlizumab was recovered after 24 h at physiological temperature and pH conditions, due to the succinimide hydrolysis resulting in the formation of iso-aspartic acid-containing variants. This reveals that iso-aspartic acid does not lead to a decreased biological activity under physiologic conditions. It furthermore shows that the potency is comparable to the aspartic acid variant. The increased potency (above 100%) of the sample incubated at pH 7.4 compared to an unstressed crizanlizumab reference sample (used for the assay) with a defined potency of 100% can be explained by the presence of less succinimide in the pH 7.4 sample, and is not due to the iso-aspartic acid itself. This assumption is also supported by the potency for the AEX fractions main, AP1 and AP2. The potency obtained by CBA increases from main with 102% to 141% for AP2 (see Table 1). Since AEX fractions are mixtures, more succinimide in the main fraction is expected compared to AP1 or AP2, based on the separation behavior. Hence, the potency is increased with decreasing succinimide amounts from main to AP1 and to AP2.

From a structural point of view, iso-aspartic acid introduces an additional atom into the polypeptide chain compared to aspartic acid.[10] This may result in structural changes, potentially influencing the target-binding behavior. An example is the isomerization reaction in the heavy chain CDR of an IgG1.[17] In a study by Yan *et al.*, where CEX fractions containing mainly succinimide show a loss in potency of 70%.[17] Hydrolyses of the succinimide to iso-aspartic acid and aspartic acid at pH 7.6 and 37°C for 20 h resulted in an additional decrease of potency in that fraction. In another study, the isomerization of aspartic acid in the CDR of a mAb is reported with a loss in affinity for both the succinimide and iso-aspartic acid variant.[18] Furthermore, a loss in target binding has been reported, if iso-aspartic acid is present, due to deamidation and/or isomerization.[21,22] There are also concerns with regard to immunogenicity for iso-aspartic acid variants.[36,37]

In our study, we showed that the isomerization to iso-aspartic acid for crizanlizumab is not critical in regard to potency. Formation of the succinimide variant results in a decreased potency, but iso-aspartic acid, which is the dominantly formed variant under physiologic conditions, does not. Therefore, the structural change induced by the iso-aspartic acid is not relevant to the antigen binding. This may suggest a stabilizing salt bridge at the aspartic acid, which may also be accommodated in the presence of iso-aspartic acid.

Considering the testing principle in general, we note that, depending on how the analytical method is performed, the obtained results might differ, since the isomerization reaction is pH and temperature sensitive. For instance, if a sample is incubated for an increased time at neutral/physiological conditions within the test procedure, hydrolysis of succinimide must be expected, resulting in unchanged potency.[38] If iso-aspartic acid as the predominant reaction product would show no decreased potency, as in our case, but succinimide would, decreased potency might not have been detected during potency

a



Charge variants			
Basic variants	52%	46%	14%
Acidic variants	24%	29%	59%
Main variant	24%	25%	26%
Potency (CBA)	67 %	73%	121%
Potency (ELISA)	95 %	103%	112%

b

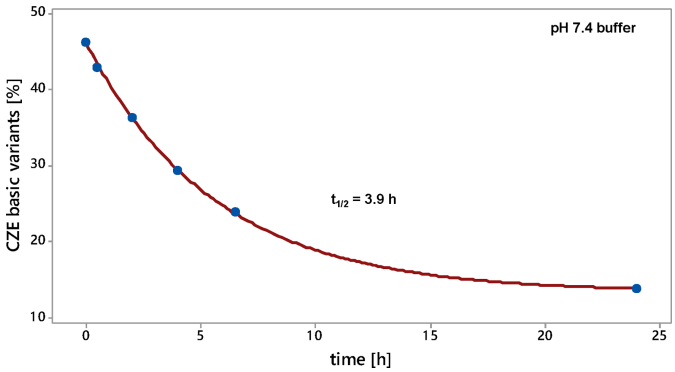


Figure 6. Restoring the biological activity of a stressed crizanlizumab under physiological conditions. The pH of a crizanlizumab sample stressed for 1 month at 25°C, pH 5 was increased to pH 7.4 using NaOH and incubated for 24 hours at 37C. Samples were taken at 0, 0.5, 2, 4, 6.5, and 24 h. Samples were analyzed by CZE, CBA and ELISA to determine the charge variants and biological activity. A: Sample preparation workflow and results from CZE, CBE, and ELISA. B: basic charge variants by CZE over time at pH 7.4 and 37°C. The data was fitted using MiniTab® first-order reaction equation to calculate the half-life.

testing due to the fast hydrolysis of succinimide. Since we showed for the first time a reduced potency for succinimide, but not for iso-aspartic acid in the CDR, other cases might also exist, which may have not been detected, due to the fast succinimide hydrolysis at the testing conditions.[17] This scenario is also supported by the relatively short half-life of the hydrolysis: For a succinimide in the CDR of an IgG1 at pH 7.6, a half-life of around 3 hours is reported.[17] This is in agreement with our results by CZE. Sample incubation times under physiological conditions of more than 3 h or even up to several days are not uncommon for bioassays and hence the potential impact on the results for certain test methods is obvious.

Succinimide hydrolysis in human serum

To our knowledge, the present study reports for the first time the potency recovery of an inactive succinimide variant under physiological conditions.

The succinimide variant of crizanlizumab can be considered as transient species since a physiological pH of 7.4 results in succinimide hydrolysis (Figure 7). Ouellette et al. reported as well that, in blood from cynomolgus monkeys, the succinimide intermediate was not stable during asparagine deamidation.[20] We confirmed the hydrolysis of the succinimide variant in human serum with a comparable half-life compared to the buffer experiment. The minor difference in the hydrolysis rates (3.9 h in buffer vs. 3.1 h in serum) is most likely due to the different experiment setup, but increased succinimide hydrolysis due to serum components can also not be excluded. Importantly, peptide mapping at pH 6 was used to minimize the formation and hydrolysis of the succinimide during sample preparation of the serum sample. These conditions also minimized the formation of known artifacts of sample preparation, such as deamidation, although deamidation was not relevant to the peptide of interest.[39,40]

The recovery of the potency in serum was less pronounced compared to the buffer experiment at pH 7.4. This may also be due to differences in the experimental setup, such as different concentrations of crizanlizumab used and the different sample matrix (containing serum), which in itself might have an impact on the bioassay. The samples were only diluted about 15 times before analysis, potentially allowing for some matrix interferences on the assay compared to the usual 100-fold dilution for a typical drug product sample. Experiments at physiological conditions in buffer or in serum, already reported for other molecules,[24,38,41] are valuable tools to gain information on the behavior of biopharmaceuticals under the conditions after administration.

The specificity and sensitivity offered by modern MS techniques offers the opportunity to monitor the fate of critical quality attributes in biological matrices. Degradation hotspots, such as the DG isomerization site, are scored negatively during the developability assessment using *in silico* prediction tools. Here, we demonstrate that a mAb degrading into a non-active succinimide variant even under optimized formulation conditions should not automatically be excluded from clinical development, as the full biological activity could potentially be restored upon its rapid hydrolysis under physiological conditions as reported here.

Materials and methods

Materials

Crizanlizumab, a recombinant monoclonal IgG2 antibody, was produced and purified at Novartis. Endoproteinase Lys-C was purchased from Wako pure chemical (Osaka, Japan). All other chemicals were at analytical grade and ordered from ThermoScientific or Sigma Aldrich.

Methods

Anion exchange chromatography fractionation

The fractionation was done with an Agilent HPLC system 1100 or higher, 214 nm UV-detection and equipped with a cooled fraction collection unit and a weak anion exchange column (Dionex ProPac WAX-10 BioLC, 4 mm × 250 mm). Crizanlizumab was diluted to a concentration of 4.0 mg/mL with mobile phase A (15.8 mM Na-phosphate, pH 7.9) prior to the injection of 8 µg on the column. Mobile phase A (15.8 mM Na-phosphate, pH 7.9) and mobile phase B (500 mM NaCl, 15.8 mM Na-phosphate, pH 7.9) were used for applying a salt gradient (time [min]/mobile phase B [%]: 0/20, 1.0/20, 11.0/42, 13.0/42, 13.5/44, 15.5/44, 16.0/46, 18.0/46, 25.0/80, 30.0/100, 30.1/20). A flow rate of 1.0 mL/min and column temperature of 40°C was set. Fractions were diluted (50%: 50%) in citrate

buffer (5 mM, pH 2.8) in order to preserve the stability of the basic variants. The collected fractions were concentrated in centrifugal filter devices (15 ml capacity). The final protein content determination was performed by UV spectroscopy (using Nanodrop).

Different material was used for the fractionation: Temperature stressed crizanlizumab material (6 weeks at 40° C) was used for the identification by MS, bioassay (CBA, ELISA) and CZE, except the analysis of the main and P7 by MS and the main by bioassay was analyzed using unstressed crizanlizumab.

Identification of isolated charge variants by Lys-C peptide mapping

For the characterization of AEX fractions, crizanlizumab samples were prepared using Lys-C at pH 7.5 as previously described.[42] The impact of digestion pH on succinimide hydrolysis was confirmed using Lys-C at pH 6.0. Briefly, crizanlizumab (200 µg) was first denatured in 50 µL of denaturing buffer (6 M guanidine hydrochloride, 5 mM EDTA, 20 mM His-HCl pH 6.0), and afterward reduced with 1.5 µL of 1 M dithiothreitol (DTT) at 37°C for 1 hour and alkylated with 3 µL of 1 M iodoacetamide at room temperature in the

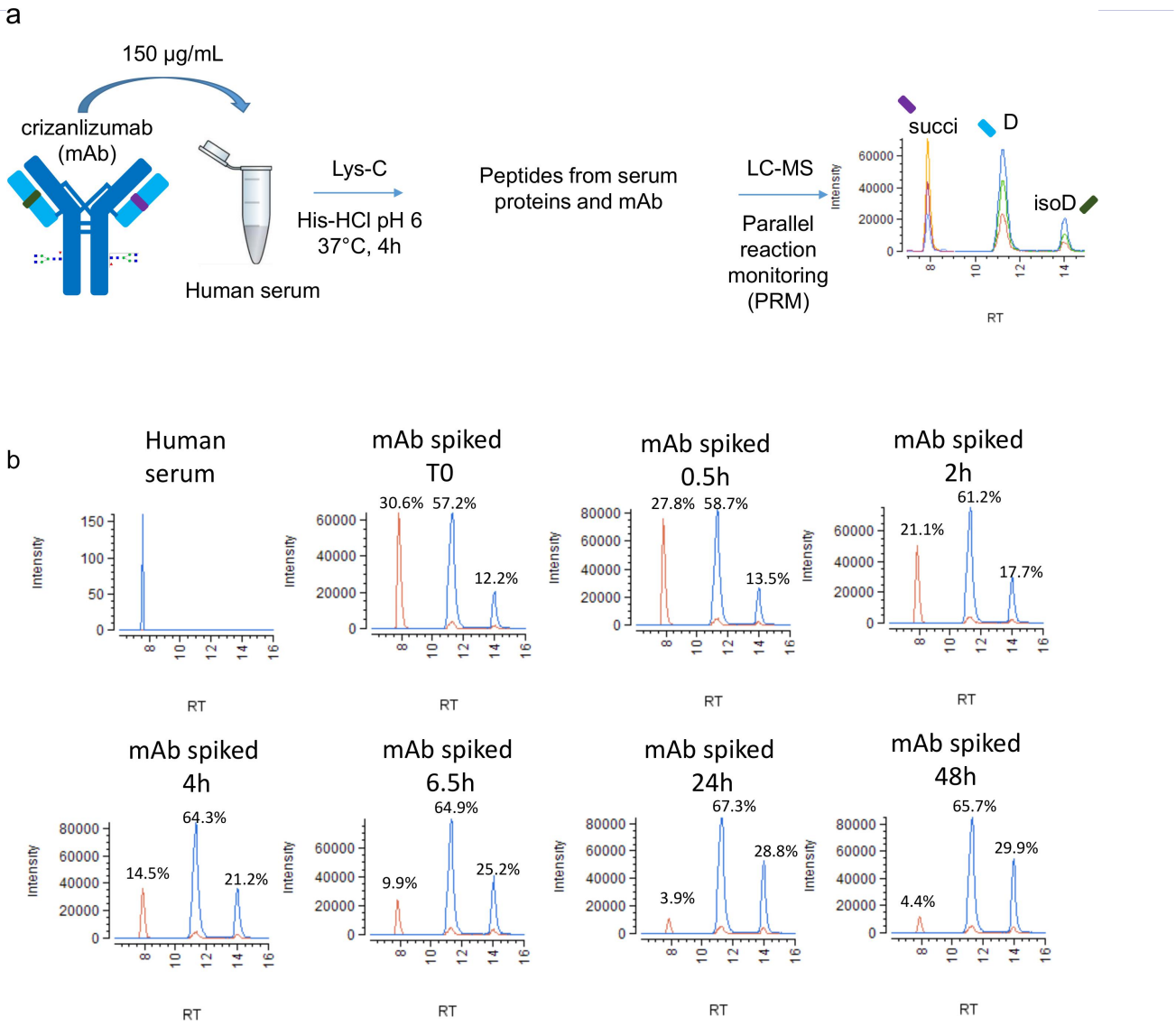


Figure 7. Parallel-reaction monitoring (PRM) of crizanlizumab succinimide conversion in human serum. A: Workflow of the PRM methodology to monitor the succinimide conversion into aspartic acid and iso-aspartic acid over a 24 h time course in human serum. Crizanlizumab stressed for 1 month at 25°C, pH 5 was spiked in human serum at a final concentration of 150 µg/mL and incubated for 0.5, 2, 4, 6.5, 24, and 48 h. Peptide mapping at pH 6 was used to keep the succinimide intact during sample preparation. Targeted L2-3 peptide detection was performed using an Orbitrap Fusion Lumos mass spectrometer and PRM methodology, by acquiring full MS/MS spectra for the succinimide L2-3 precursor at 857.72 m/z aspartic acid and iso-aspartic acid L2-3 precursor at 863.72 m/z in a scheduled method to enhance sensitivity. Multiple fragment ions were monitored for each species to increase the specificity of L2-3 detection in human serum. An example of three specific extracted fragment ion chromatograms is shown for succinimide, aspartic acid and iso-aspartic acid species. succi: succinimide; D: aspartic acid; isoD: iso-aspartic acid. B: Overlay of extracted fragment ion chromatograms for y'172+ for aspartic acid and iso-aspartic acid (blue) and y'172+ - 18 for succinimide (orange) for different time points following the spiking of crizanlizumab in human serum. In-source loss of water for y'172+is observed for aspartic acid and isoaspartic acid species.

dark. The alkylation was quenched by addition 1 µL of 1 M DTT. The reduced and alkylated samples were diluted with 750 µL of 20 mM His-HCl, pH 6.0 followed by digestion with endoproteinase Lys-C (1:25, w:w) at 37°C for a total of 4 hours. The enzymatic reaction was stopped by the addition of 5 µL of TFA. Peptides (22 µg) were then separated at a flow rate of 0.2 mL/min on a Vydac C18 column (2.1 × 150 mm, 5 µm, 300 Å) operated at 40°C by an Agilent 1200 (Agilent), with a mixture of 0.1% TFA in water as mobile phase A and 0.09% TFA in 90% acetonitrile as mobile phase B. Online detection was performed with an Orbitrap Elite analyzer (Thermo Fisher Scientific). For the CID and HCD fragmentation of the unmodified CDR peptide and its succinimide variant, acquisition was performed in the Orbitrap at 30 K resolution with each full scan between 300 m/z and 2000 m/z followed by 2 MS/MS CID fragmentation events with 35 normalized collision energy and 2 MS/MS HCD fragmentation events with 32 normalized collision energy on each precursor. For the ETD fragmentation of the unmodified CDR peptide and its isomerization variant, acquisition was performed with a full scan acquired at 30 K between 300 m/z and 2000 m/z, and a targeted MS/MS on the CDR peptide precursor m/z with isolation width of 3 m/z, activation time of 50 ms. Reagent ion source temperature was set at 160°C, reagent ion source emission current at 50 uA, reagent vial 1 injection time of 100 ms and reagent vial 1 AGC target of 300000, with 25 supplemental activation energy. FTMS MSn AGC target was set at 100000. Ion trap and FTMS MSn maximum injection time of 250 ms and 1000 ms, respectively. Targeted MS/MS acquisition was performed between 120 m/z and 2000 m/z at 120 K resolution. Data analysis was performed with Genedata Refiner MS software. Mass accuracy for the CDR peptide was below 5 ppm.

Parallel reaction monitoring of the CDR peptide in buffers and human serum

For kinetics samples in human serum, the Lys-C peptide mapping in reducing conditions was performed at pH 6.0 as described above. Peptides (22 µg) were then separated at a flow rate of 0.2 mL/min on a CSH C18 column (2.1 × 150 mm, 2.1 µm, 300 Å) operated at 40°C by an Agilent 1200 (Agilent), with a mixture of 0.1% formic acid (FA) in water as mobile phase A and 0.1% FA in 90% acetonitrile as mobile phase B. Online detection was performed with an Orbitrap Fusion Lumos analyzer (Thermo Fisher Scientific). The H-ESI source was used with a static spray voltage at 3800 V, ion transfer tube temperature at 275°C, vaporizer temperature at 45°C, sheath gas, and auxiliary gas parameters at 35 and 10, respectively. The instrument was scanned in the Orbitrap during the first 5 min of acquisition to enable PRM data analysis in Genedata Refiner MS software as described below. Then, targeted MS/MS was scheduled to enhance sensitivity on the precursor m/z of the succinimide between 5 and 9 min and aspartic acid and iso-aspartic acid between 9 and 15 min. Precursors were isolated by quadrupole with an isolation window of 1.6 m/z then CID fragmentation was performed with a normalized collision energy of 30 for 10 ms and Activation Q of 0.25. Detection was performed in the orbitrap between 400 m/z and 2000 m/z at a resolution of 30 K. RF lens was 60%, AGC target was 2.0e5 with a maximum injection time of 2000 ms. Internal calibration with EASY-IC was selected. Data analysis was performed with Genedata Refiner MS software. Mass accuracy for the fragments of interest was below 1 ppm.

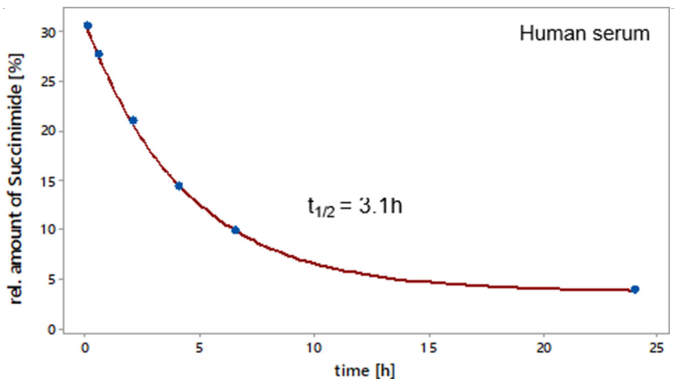


Figure 8. Relative amounts of succinimide in the CDR of crizanlizumab over time in human serum at 37°C. Crizanlizumab stressed for 1 month at 25°C, pH 5 was spiked in human serum at a final concentration of 150 µg/mL and incubated for 0.5, 2, 4, 6.5, 24, and 48 h at 37°C. The relative amount of succinimide in the CDR of crizanlizumab was determined by parallel-reaction monitoring (PRM) as shown in Figure 7. The data up to the 24 h time point was fitted using MiniTab® first-order reaction equation to calculate the half-life.

Identity and charge heterogeneity by CZE

The CZE analysis was performed as described in the following. The capillary electrophoresis instrument (Beckman Coulter PA800 Plus) equipped with an uncoated fused-silica capillary (inner diameter: 50 µm; total length: 50 cm; capillary length from inlet to detector/separation length: 40 cm). The separation of charge variants was monitored by UV at 214 nm. Crizanlizumab was diluted to 3.0 mg/mL with sample buffer (5 mM phosphate pH 7.2–7.4) and injected by applying a pressure of 0.5 psi for 8 seconds. The capillary temperature was fixed at 25°C for the separation and the auto-sampler temperature was 15°C. Electrophoresis was performed in running buffer (400 mM 6-aminocaproic acid/acetic acid pH 5.7 with 2 mM TETA and 0.03% Tween 20) at a constant voltage of 20 kV in positive to negative (normal) polarity mode for 45 or 30 minutes. The charge variants were quantified by relative time corrected peak area determination.

Binding of crizanlizumab to P-selectin by ELISA

In the ELISA, potency of crizanlizumab samples was measured based on their ability to bind P-selectin. ELISA plates were coated with recombinant human P-selectin (R&D Systems #ADP3), and graded amounts of crizanlizumab were added. Bound crizanlizumab was quantified using an anti-human IgG antibody coupled to horseradish peroxidase (Pierce #31413) followed by the addition of a colorimetric substrate. Results of the colorimetric reaction were measured by light absorption.

The potency of a crizanlizumab test sample was quantified by comparing its ability to bind to its target protein to that of a crizanlizumab reference standard. The samples and the standard are normalized on the basis of protein content. Relative potency is calculated using a parallel line assay according to the European Pharmacopoeia. The final result is expressed as

relative potency/bioactivity (in percent) of a sample compared to the reference standard.

Inhibition of adhesion of P-selectin expressing cells with PSGL-1

Potency (CBA) testing was performed as follows. V-bottom microtiter plates were coated with PSGL-1 and then graded amounts of crizanlizumab were added. Cells, presenting P-selectin on their surface, were fluorescently labeled and also added to the plate. After short incubation, the plate was centrifuged and non-adherent cells accumulate at the bottom of the V-shaped wells, where they were measured using a fluorescence reader.

The potency of a crizanlizumab test sample was quantified by comparing its ability to inhibit the adhesion of P-selectin-expressing cells to its ligand PSGL-1, to that of crizanlizumab reference standard. The samples and the standard were normalized on the basis of protein content. Relative potency was calculated using a parallel line assay according to the European Pharmacopoeia. The final result is expressed as relative potency/bioactivity (in percent) of a sample compared to the reference standard.

pH incubation experiments

10 mg/mL crizanlizumab at pH 5.0, 5.5, 5.7, 6.0, 6.3, 6.5, 7.0 were compounded in parallel and stored at different temperature conditions (5°C, 25°C, and 40°C).

Buffer incubation experiment

The pH of a pre-incubated crizanlizumab sample (approximately 9–10 mg/mL) stored at pH 5 for 4 weeks (1 months) at 25°C was increased to pH 7.4 using NaOH.

Serum incubation experiment

A crizanlizumab sample pre-incubated at pH 5 for 4 weeks at 25°C was spiked with a final concentration of 150 µg/mL into human serum (Innovative Research, MI, USA) and incubated for different timepoints (t0, t = 30 min, t = 2 h, t = 4 h, t = 6.5 h, t = 24 h, t = 48 h) in a humidified incubator (37°C at 5% CO2). At each designated timepoint, the respective aliquot was taken out of the incubator, immediately sub-aliquoted for the required analysis, shock frozen on dry ice and stored at <-60° C until analysis. The t0 sample was not placed in the incubator but treated immediately as described.

Abbreviations

AEX: anion exchange chromatography; CDR: complementarity-determining regions; CZE: capillary zone electrophoresis; ELISA: enzyme-linked immunosorbent assay; ETD: electron transfer dissociation; mAb: monoclonal antibody; PSGL-1: P-selectin glycoprotein ligand-1

Disclosure statement

All authors were employed directly by Novartis Pharma or indirectly supporting the work by have been providing services to Novartis Pharma AG.

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