Force Degradation Comparative Study on Biosimilar Adalimumab and Humira

Estudio comparativo de la degradación de la fuerza en biosimilar Adalimumab y Humira

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Resumen

CinnoRA® has been developed by CinnaGen Co., as a biosimilar adalimumab of originatHUMIRA®. Biosimilars are defined as "similar" or "highly similar" to the reference medicinal products (originator products) which are large molecules with an inherent physicochemical complexity. Such complex molecules with a variety of functional groups are susceptible to instability through different degradation pathways, it is a matter of great concern as it affects the quality, safety and efficacy of the drug product. So force degradation or stress stability studies are expected to be part of the demonstration of similarity of a biosimilar to reference product as well as orthogonal analytical techniques to characterize the physicochemical and functional properties of biosimilar in comparison with originator. A force degradation protocol designed to expose sample to oxidative, acid and base hydrolysis, thermal, photolysis and mechanical stress conditions. Samples were tested in defined time points by validated analytical method to detect aggregated forms by size exclusion chromatography, acidic and basic charged variants by ion exchange chromatography and TNF-α neutralizing activity of adalimumab by invitro bioassay. The results were analyzed by Minitab statistical software and the results demonstrate the comparable degradation pathway between biosimilar and reference product in all conditions.

Keywords: degradation product(s), biosimilar(s), monoclonal antibody(s), stability, protein, protein aggregation.

Abstract

CinnoRA® ha sido desarrollado por CinnaGen Co., como un adalimumab biosimilar del originador HUMIRA®. Los biosimilares se definen como "similares" o "muy similares" a los productos medicinales de referencia (productos originadores) que son moléculas grandes con una complejidad fisicoquímica inherente. Tales moléculas complejas con una variedad de grupos funcionales son susceptibles a la inestabilidad a través de diferentes vías de degradación, es un motivo de gran preocupación ya que afecta la calidad, la seguridad y la eficacia del producto farmacéutico. Por lo tanto, se espera que los estudios de degradación de la fuerza o de estabilidad del estrés formen parte de la demostración de la similitud de un producto biosimilar al de referencia, así como de las técnicas analíticas ortogonales para caracterizar las propiedades fisicoquímicas y funcionales del biosimilar en comparación con el originador. Un protocolo de degradación de fuerza diseñado para exponer la muestra a condiciones de estrés por oxidación, ácido y base de hidrólisis, térmica, fotólisis y estrés mecánico. Las muestras se analizaron en puntos de tiempo definidos mediante un método analítico validado para detectar formas agregadas mediante cromatografía de exclusión por tamaño, variantes cargadas ácidas y básicas mediante cromatografía de intercambio iónico y actividad neutralizadora de TNF-α de adalimumab mediante bioensayo de invitro. Los resultados fueron analizados por el software estadístico Minitab y los resultados demuestran la vía de degradación comparable entre biosimilares y productos de referencia en todas las condiciones.

Palabras clave: producto (s) de degradación, biosimilar (es), anticuerpo (s) monoclonal (es), estabilidad, proteína, agregación de proteínas.

Introduction

Abbreviation: International Conference on Harmonization (ICH), United States Food and Drug Administration U.S FDA, European Medicines Agency (EMA), World Health Organization (WHO)., 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), N-methyl dibenzopyrazine Methyl Sulfate (PMS).

Introduction: ICH the international conference on harmonization, U.S FDA United States food and drug administration, EMA European medicines agency, WHO, The World Health Organization. HOS high order structure, XTT 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, PMS N-methyl dibenzopyrazine methyl sulfate,

Introduction: Adalimumab is a mono clonal antibody which blocks tumor necrosis factor (TNF)-alpha and is indicated in a number of inflammatory conditions for treatment of Rheumatoid Arthritis (RA), Psoriatic Arthritis, Ankylosing Spondylitis, Crohn's Disease and Plaque Psoriasis[1,2]. With the increasing use of monoclonal antibodies, especially in the areas of cancer and autoimmune disease, there has been a great demand for the development of biosimilar therapeutic monoclonal antibodies. Biosimilars having a similar level of quality, efficacy and safety compared to that of the originator products provide additional advantages to patients in terms of affordability and low cost of therapy, while expanding patient access to therapies. Force degradation or stress stability studies are expected to be part of the demonstration of similarity to establish degradation profiles and this study provides direct comparison of the proposed biosimilar product (CinnoRA® manufactured by CinnaGen Co.) with the reference product (HUMIRA® manufactured by Abbvie)[3,4]. On the other hand, these experiments play an important role in the drug development process, drug formulation design, selection of storage conditions and packaging, better understanding of potential liabilities of the drug olecule chemistry, and solving of stability-related problems[5]. Also the need of force degradation studies is described in various international guidelines such as ICH6-11, EMA12-14, U.S FDA15 and WHO guidelines[16-18].

To provide an orthogonal assessment of degradation trends, typically the exposure of drug substance or drug product to the relevant stress conditions of light, heat, humidity, acid/base hydrolysis, and oxidation is involved. The chemical and physical instabilities could disturb tertiary and frequently secondary structure of proteins; formation and/or breakage of covalent bonds in the first order structure of biopharmaceuticals, this conformational and chemical alteration could impair biological activity of biopharmaceuticals and could induce irreversible protein aggregation, oxidation, hydrolysis adsorption, deamidation, unfolding, denaturation, elimination, racemization and disulfide exchange[19-21,36,37].

Oxidation is one of the major degradation routes for proteins, potentially all 20 natural amino acids can be oxidized, however, methionine (Met), cysteine (Cys), histidine (His), phenylalanine (Phe), tryptophan (Trp) and tyrosine (Tyr) are generally most prone to oxidation, due to the high reactivity of sulfur atoms and aromatic rings towards various reactive oxygen species[22-25]. Oxidation of Met residues, has been proposed as a marker for protein degradation by cellular machinery[26,34]. There are three major oxidative pathways important to consider for drug degradation: (i) radical- initiated oxidation (also known as autoxidation); (ii) peroxide-mediated oxidation; (iii) electron transfer mediated oxidation[27,39]. H_2O_2 is an excellent starting point, generally mainly yielding methionine sulfoxide[28]. Oxidative modifications can lead to HOS changes with aggregate formation resulting in potentially reduced biological activity[22,23,42]. Baertschi et al. employed hydrogen peroxide in force degradation at strengths from 0.3% to 30%[27]; moreover, Bhagyashree et al. used H_2O_2 30% to reach the sufficient degradation of mefenamic acid[47]. In addition, some studies were conducted at elevated temperatures by Alsante et al. and Srivastava for oxidative stressing with 3% Hydrogen Peroxides in up to 60°C for 5 days to result 5-20% degradation of sample[32,33], and Timm et al. reached to the methionine residue oxidation of rat/mouse hybrid monoclonal antibody by H_2O_2 0.05% at 37°C[48].

Hydrolysis is a common degradation problem hydrolysis reactions are typically acid or base catalyzed. Acidic and basic conditions should therefore be employed in order to induce all potential hydrolytic reactions[27]. For acid and base degradation recommended condition by Baertschi et al. and Huynh-Ba is 1-13 pH range during a 7-day study[27,5]. More so, Iram recommends pH 2,4,6,8 in 40 and 60°C in 5 days to achieve degradation in active pharmaceutical ingredients[37]. Hoitinc evaluated the stability of gonadotropin releasing hormone in the pH range of 5–6 resulted in hydrolysis at the N-terminal side of the serine residue[49]. Hydrochloric acid or sulfuric acids (0.1–1 M) for acid hydrolysis whereas sodium hydroxide or potassium hydroxide (0.1–1M) for base hydrolysis are suggested as suitable reagents for hydrolysis[30-32,37,29].

Photostability testing is accepted as an integral part of stress testing, especially when the drug substance or drug product are photosensitive. It needs to be confirmed that when a drug product or substance experiences an exposure to light this does not result in a not acceptable change[18]. The method that has been used in different literature is according to Q1B[8].

Thermal analysis can help in development of degradation profile of proteins which are sensitive to temperature, liquid drug substance and drug products can be exposed to heat without humidity.30 In thermal degradation studies the temperature should be equal or greater than accelerated stability study are normally conducted at 40˚C to 80˚C[18], Sharma recommends 60°C and 80°C in 5 days to reach 5-20% degradation in drug substance and drug product[38], for proteins Tamizi and her colleague indicate 40°C to result degradation in rhGH and mAbs[42].

Mechanical stress condition (agitation) is the most important factor in influencing the particle size and Shaking-

stressed protein aggregates were made by shaking samples side to side.

In force degradation study it should be carefully considered that conditions selected on a case-by-case basis the conditions have varied greatly from compound to compound and from investigator to investigator[7].

All oxidation, pH, heat and agitation could lead aggregated forms in proteins, aggregation is the formation of complexes between macromolecules. They can be dimers, trimers, and heavier multimers. The complexes may be covalently bonded or just associated through hydrophobic interactions. The formation of aggregates can cause changes in protein binding and activity (potency), and have been implicated in immunogenic reactions in the patient. Control of aggregate formation during process and formulation development is, therefore, very important5, the aggregated forms were analyzed by Liu et al. by SE-HPLC system which was equipped with an LS detector and AUC sedimentation velocity (AUC-SV)[40].

Charged isoforms in drug product samples of adalimumab were separated on a CEX-HPLC

Pro Pac_ WCX-10 and assessing biological activity were indicated by TNFa-mediated apoptosis inhibition by Liu et al. and Moo-Young Song et al.[40,41].

The main the present work was to design a complete force degradation study which is included all stress to conditions to illustrate degradation profile of biosimilar adalimumab in comparative way with reference product which has not studied before.

Materials and methods

All reagents were of analytical grade, all aqueous solutions were prepared using purified water from milli-q water purification system merck [Germany], 0.2 M phosphate buffer (pH 6.2±0.1) containing 27.2g di-potassium hydrogen phosphate merck [Germany] and 18.65g potassium chloride merck [Germany] made up to 1000ml with milli-q water, 20 mM MES (morpholinoethane sulfonic acid) himedia buffer [India] (pH 6.0±0.1) containing 8.52g MES made up to volume 2L with milli-q water and pH was adjusted with sodium hydroxide 10N merck [Germany].

Agilent HPLCsSystem 1290 [Santa Clara, CA, USA] - LC column: weak cation exchange, dionex pro pac WCX-I0 thermo fisher scientific [Waltham, MA, USA] 4mm ID x 250mm L. for Ion exchange chromatography, the system equipped with UV detector and refrigerated auto sampler.

LC Column: Tosoh [Tokyo, Japan] 7.8mm ID x 30cm L, 5µl, 250A˚ was used for Size exclusion chromatography. Murine L929 cells ATCC (American Type Culture Collection), [Manassas, VA, USA], RPMI-1640 medium [ATCC, Manasas, VA, USA], XTT tetrazolium salt [ATCC, Manassas, VA, USA], PMS [ATCC, Manassas, VA, USA]

Methods

Photolysis study is performed same as Q1B[8]. 3 batches of cinnora® Drug product in industrial scale were entered in the study in comparison with 3 batches of HUMIRA®. The force degradation study designed (table 1) according to physicochemical and structural properties of adalimumab by considering mostly used conditions in the different scientific literature[29,33-38].

Table 1: Force degradation condition for drug product

Stress type	Conditions	Time (day)	Tests
Oxidative	3.0% H_2O_2/25±2°C DP Control (without H_2O_2)	1, 3, 7	
Acid hydrolysis	HCL 0.1M /25±2°C/ pH=2 DP Control (without acid)	1, 3, 7	IEC
Base hydrolysis	NaOH 0.1M /25±2°C/ pH=8 DP Control (without base)	1, 3, 7	SEC
Thermal hydrolysis	40±2°C	1, 3, 7	Biological activity
Photolytic	1.2 million lux hours /25±2°C DP wrapped in aluminum foil as control	1, 3, 8	
Mechanical study	512 rpm/ 25±2°C	2,4	

In order to determine aggregated forms, the sample was diluted in the SEC running buffer (0.2 M phosphate buffer, pH:6.2±0.1) to obtain the concentration of 1mg/ml. 20µl of the prepared sample was injected into a SE column operated with a flow rate of 0.5 ml/min for 35 min and aggregated forms were detected by UV spectrophotometer at 280 nm.

To detect various charged species variants of adalimumab Agilent HPLC system 1290 was used Prior to injection the sample to HPLC, dionex pro pac WCX-I0 column was saturated with 100% V/V 20 mM MES buffer (pH 6.0 ± 0.1) with the flow rate of 0.7mL/minute. 1mg/ml Concentration of adalimumab incubated Differently charged species variants of adalimumab were separated out of the column in 115 minutes and detected by UV spectrophotometer at 280 nm.

To assess the TNF-αneutralizing activity of adalimumab. L929 cells were cultured on T-flask. Prior to addition to the cell plate, a series of diluted originator humira® and cinnora® samples were prepared by RPMI 1640 and added

to 96 well cell plate in the presence of optimized amounts of TNF-α to allow neutralization. Cell plates were further incubated at 37°C with 5% CO2 and 95-98% humidity for 46±2 hours. At the end of incubation, TNF-neutralizing activity was estimated by Adding a mixture of XTT and PMS to well plates, incubation at 37°C with 5% CO2 and 95-98% humidity for 2h±30 min and reduction assay. Results obtained as a percent of cell viability (absorbance at 570 nm) were plotted against different concentrations (ng/mL) of samples applied in a four-parameter sigmoidal dose-response curve by using graphpad prism software. The TNF-α neutralizing activity of cinnora® was expressed as a relative potency with respect to the originator Humira® PLA of TNF neutralizing assay on L929 cells. All result obtained in the study were statistically analyzed by Minitab 18 in ANOVA interval plot to check comparability of results between biosimilar and reference product statistically.

Results

Photolytic:

The results of detected parameters in photolytic condition as representative data shown in (table 2). The results analyzed by minitab ANOVA interval plot in Figure1,2 and 3 which illustrate no significant difference of bioassay, aggregated forms and acidic/basic charged variants between Cinnora® and Humira® in all 1,3 and 8 days.

Table 2: The results of bioassay, total aggregated forms and acidic/basic charged variants in the photolytic stress testing are presented. The results are given in each time point (day 1,3,8) beside control samples of cinnora® and Humira®.

Conditions	Parameters	Samples	Control	Day 1	Control	Day 3	Control	Day 8
Photolytic	Bio assay	CinnoRA® 09	96	97	100	94	94	85
		CinnoRA® 10	90	99	97	90	87	90
		CinnoRA® 11	93	96	101	96	82	85
		HUMIRA® 01	95	101	94	89	90	88
		HUMIRA® 02	100	97	98	93	100	86
		HUMIRA® 03	98	95	99	90	98	84
	Total aggregated forms	CinnoRA® 09	1	3	2	3	2	3
		CinnoRA® 10	2	3	2	3	2	3
		CinnoRA® 11	1	2	1	2	1	2
		HUMIRA® 01	1	2	1	2	1	2
		HUMIRA® 02	2	2	2	2	2	3
		HUMIRA® 03	1	1	1	2	1	2
	Acidic charged variants	CinnoRA® 09	17	16	15	16	15	15
		CinnoRA® 10	18	18	18	17	15	16
		CinnoRA® 11	16	16	17	17	16	17
		HUMIRA® 01	18	16	17	17	18	17
		HUMIRA® 02	16	17	16	17	16	19
		HUMIRA® 03	18	18	17	18	17	18
	Basic charged variants	CinnoRA® 09	11	10	9	11	9	10
		CinnoRA® 10	9	11	9	12	10	9
		CinnoRA® 11	12	12	13	15	15	14
		HUMIRA® 01	13	13	14	12	14	12
		HUMIRA® 02	11	14	12	15	12	15
		HUMIRA® 03	10	12	10	13	12	13

Figure 1: Comparison of acidic and basic charged variants by Ion exchange chromatography in photolysis condition (day 1) between Humira® and Cinnora®. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira® (01,02,03). This degradation pattern is followed in both day 3 and day 7.

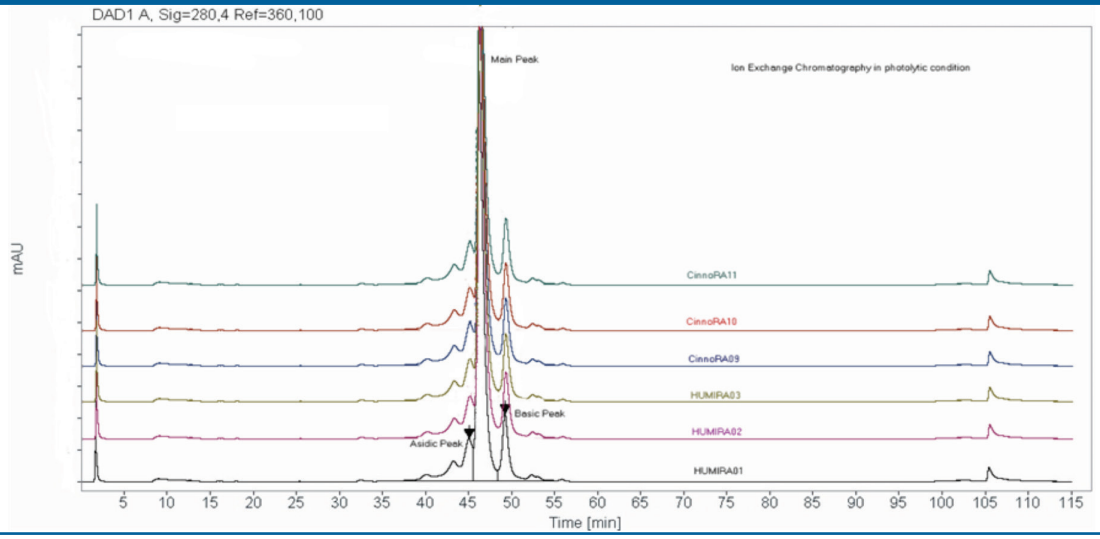


Figure 2: Comparison of total aggregated forms by size exclusion chromatography in photolytic condition (day 1) between Humira® and Cinnora®. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira® (01,02,03). This degradation pattern is followed in both day 3 and day 7.

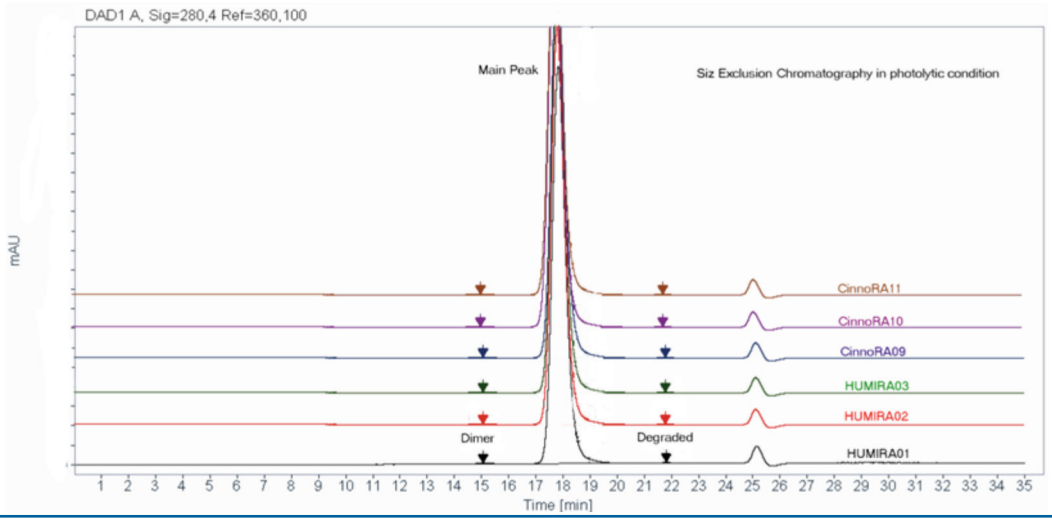
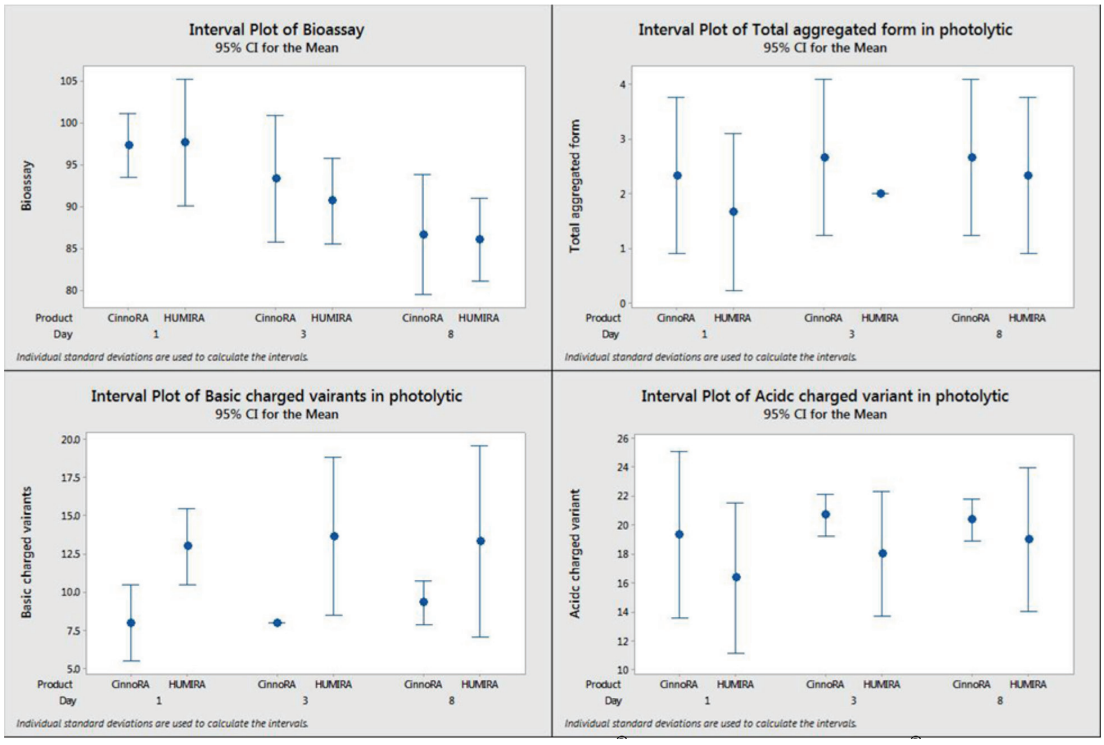


Figure 3: Interval plot of the photolytic condition of Cinnora® in comparison with Humira®



Basic hydrolysis:

Basic pH used for degradation of Humira® and Cinnora® batches the results of aggregated forms, acidic and basic charged variants and bioassay are presented (table 3).

Based on ANOVA statistical analysis in figure 4,5 and 6 the degradation profile of biosimilar and the reference product is similar. In addition, a significant difference is observed between control and degraded sample (table 3).

Table 3: The results of bioassay, total aggregated forms and acidic/basic charged variants in basic, acidic and oxidative stress testing are presented. The results are given in each time point (day 1,3,8) beside control samples of Cinnora® and Humira®.

			Day 1				Day 3				Day 7		
Parameters	Samples	Control	Basic Hydrolysis	Acidic Hydrolysis	Oxidative	Control	Basic Hydrolysis	Acidic Hydrolysis	Oxidative	Control	Basic Hydrolysis	Acidic Hydrolysis	Oxidative
Bio assay	CinnoRA® 09	93	60	7	66	94	57	0	59	97	48	0	56
	CinnoRA® 10	101	57	4	60	97	48	0	55	95	25	0	47
	CinnoRA® 11	102	63	2	59	98	54	0	48	92	46	0	43
	HUMIRA® 01 HUMIRA® 02 HUMIRA® 03	100 95 91	57 50 63	7 5 5	56 61 60	88 87 92	46 45 40	0 0 0	54 58 58	90 89 90	30 42 38	0 0 0	48 55 50
Total aggregated forms	CinnoRA® 09	2	6	70	5	2	6	70	5	2	7	86	5
	CinnoRA® 10	2	5	72	5	2	7	79	6	2	7	87	6
	CinnoRA® 11	2	6	78	2	2	7	83	5	2	7	85	6
	HUMIRA® 01 HUMIRA® 02 HUMIRA® 03	1 2 1	3 4 3	71 88 75	5 4 4	1 2 1	3 5 3	83 89 85	6 5 6	1 2 1	4 5 4	86 87 87	8 6 8
Acidic charged variants	CinnoRA® 09	15	17	9	11	15	20	8	9	15	26	15	9
	CinnoRA® 10	15	16	8	12	15	20	9	8	15	26	15	13
	CinnoRA® 11	16	17	9	13	16	20	11	8	16	27	14	9
	HUMIRA® 01 HUMIRA® 02 HUMIRA® 03	15 15 16	16 17 17	10 9 8	11 12 10	15 16 16	19 20 21	13 13 14	8 8 8	15 16 16	25 26 25	16 15 16	10 12 5
Basic charged variants	CinnoRA® 09	8	8	31	66	9	8	42	72	9	8	52	75
	CinnoRA® 10	10	7	33	65	10	8	42	68	10	8	51	75
	CinnoRA® 11	11	8	30	64	10	7	41	64	11	7	51	75
	HUMIRA® 01 HUMIRA® 02 HUMIRA® 03	5 5 6	4 4 7	40 41 40	66 65 70	5 5 7	5 3 7	43 44 43	72 68 68	5 5 7	2 2 5	47 50 47	75 75 59

Figure 4: Comparison of acidic and basic charged variants by Ion exchange chromatography in basic hydrolysis condition (day 1) between Humira® and Cinnora®. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira® (01,02,03). This degradation pattern is followed for day 3 and 7. *mAU:milli area under curve

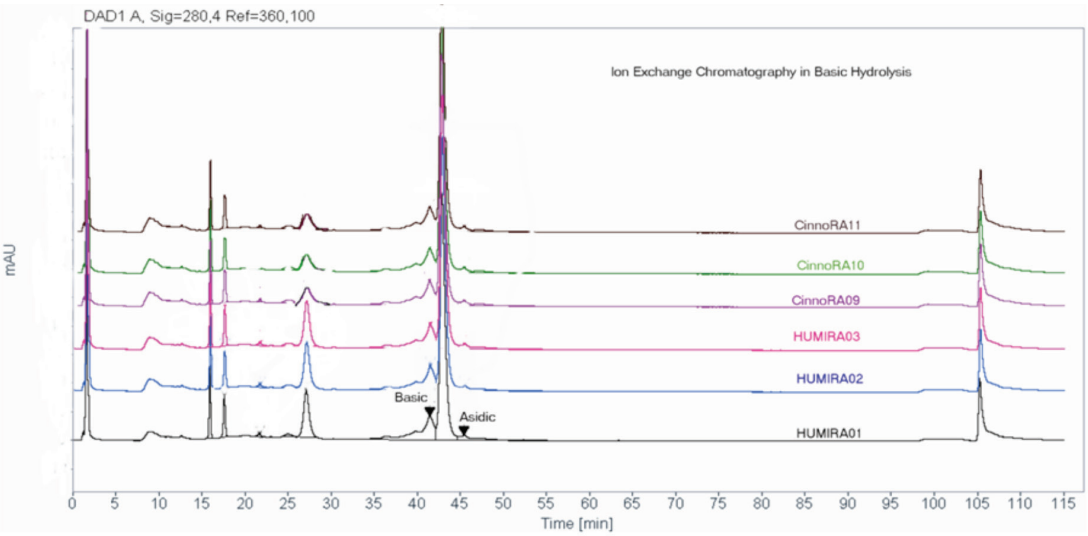


Figure 5: Comparison of total aggregated forms by size exclusion chromatography in basic hydrolysis condition (day 1) between Humira® and Cinnora® are given. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira® (01,02,03). This degradation pattern is followed for day 3 and 7.

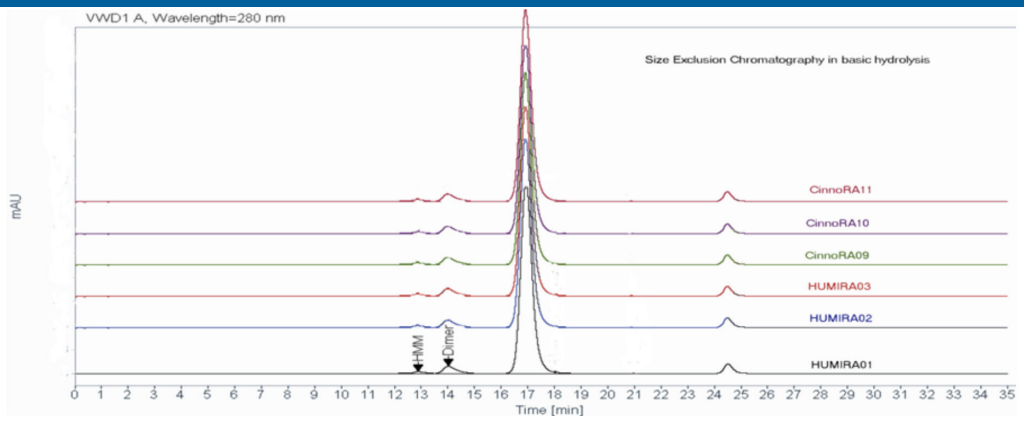


Figure 6: Interval plot of the basic hydrolysis of Cinnora® in comparison with Humira®

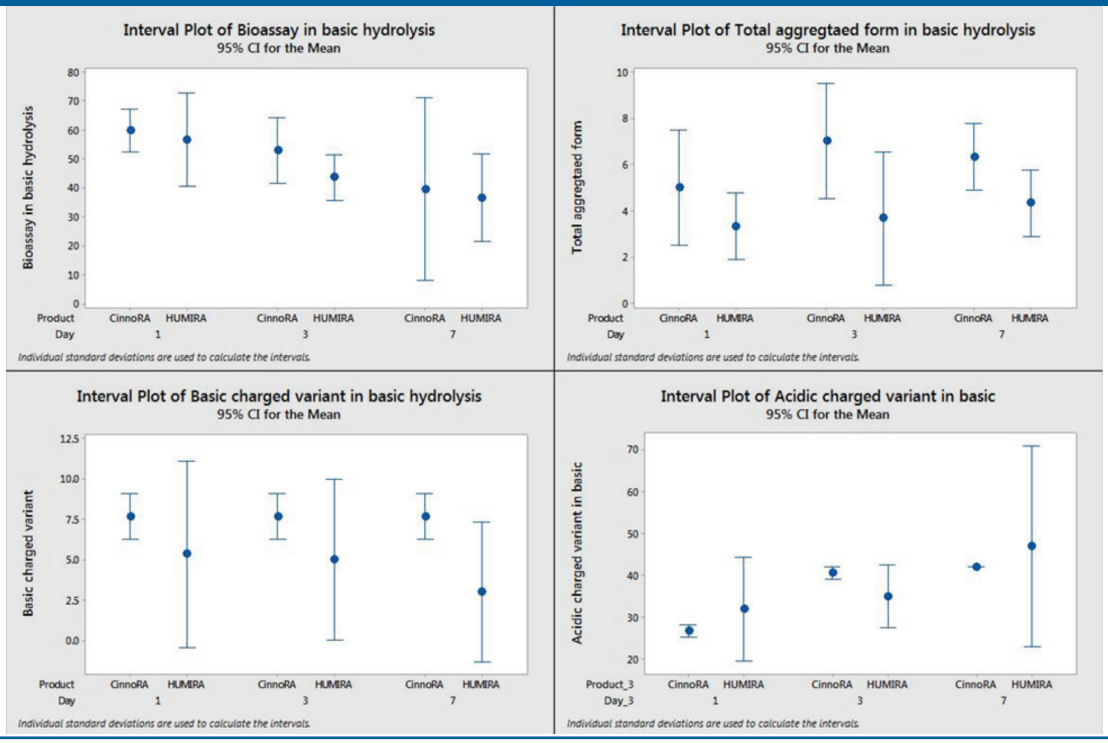
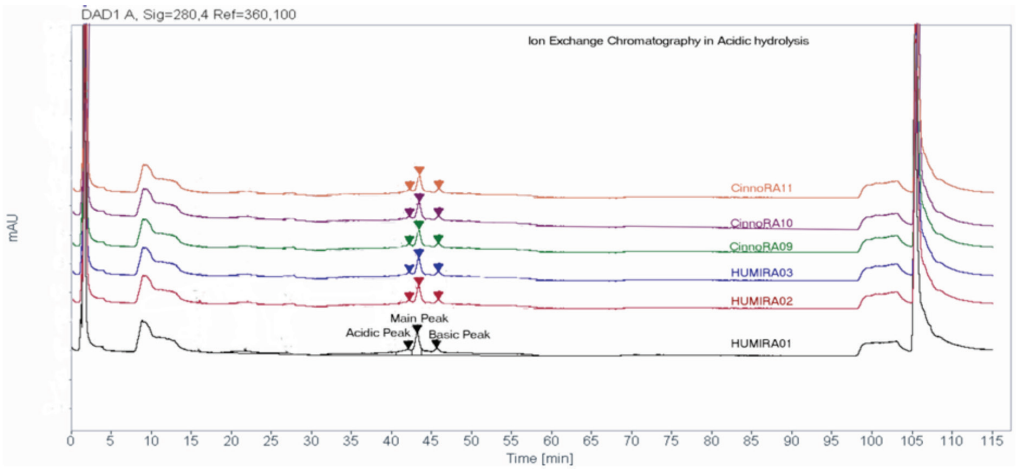


Figure 7: Comparison of acidic and basic charged variants by Ion exchange chromatography in acidic hydrolysis condition (day 1) between Humira® and Cinnora® is presented. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira® (01,02,03). This degradation pattern is followed for day 3 and 7.



Acidic hydrolysis:
Acidic hydrolysis was implemented by exposing the sample to HCL, and the degaradation is considrable in table 3. Besides, according to figure 7,8 and 9 all changes are comparable between Cinnora® and Humira®.

Oxidative stress:
As result of H_2O_2 exposure to adalimumab exerts its negative impact on the biological activity by increasing aggregate formation which is considerable in table 3. Significant difference between control and sample and similarity in degradation pattern of Cinnora® and Humira® was observed in figures 10,11 and 12.

Thermal stress:
Thermolytic degradation is implemented by 25±2ºC. The results of all batches of Cinnora® and Humira® are given in table 4 also figures 13,14 and 15 illustrate the comparability of all parameters in Cinnora® and Humira®.

Mechanical stress:
Slightly aggregates increasing was detected in mechanical stress, yet there is no significant difference between day 2 and day 4 (table 5). The result of Cinnora® and Humira® are completely similar and comparable (figure 16, 17 and 18).

Figure 8: Comparison of total aggregated forms by size exclusion chromatography in acidic hydrolysis condition (day 1) between Humira® and Cinnora® is demonstrated. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira (01,02,03). This degradation pattern is followed for day 3 and 7.

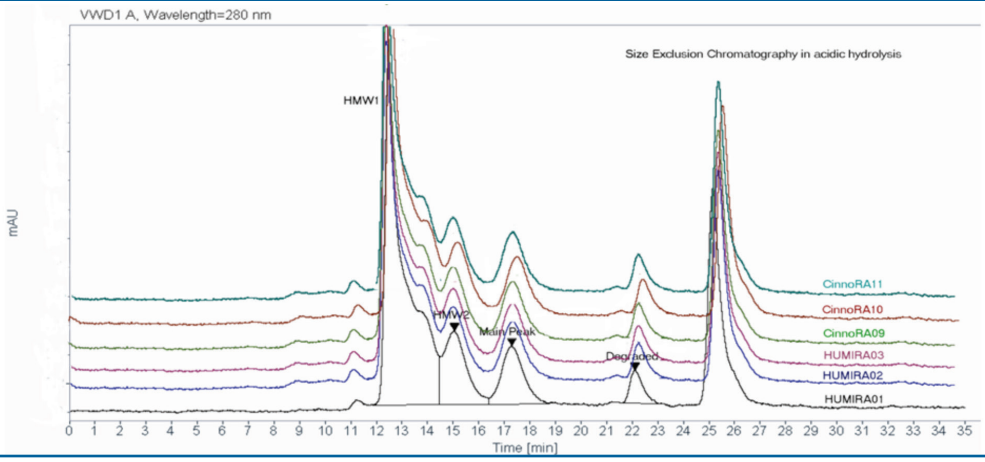


Figure 9: Interval plot of the acidic hydrolysis of Cinnora® in comparison with Humira®

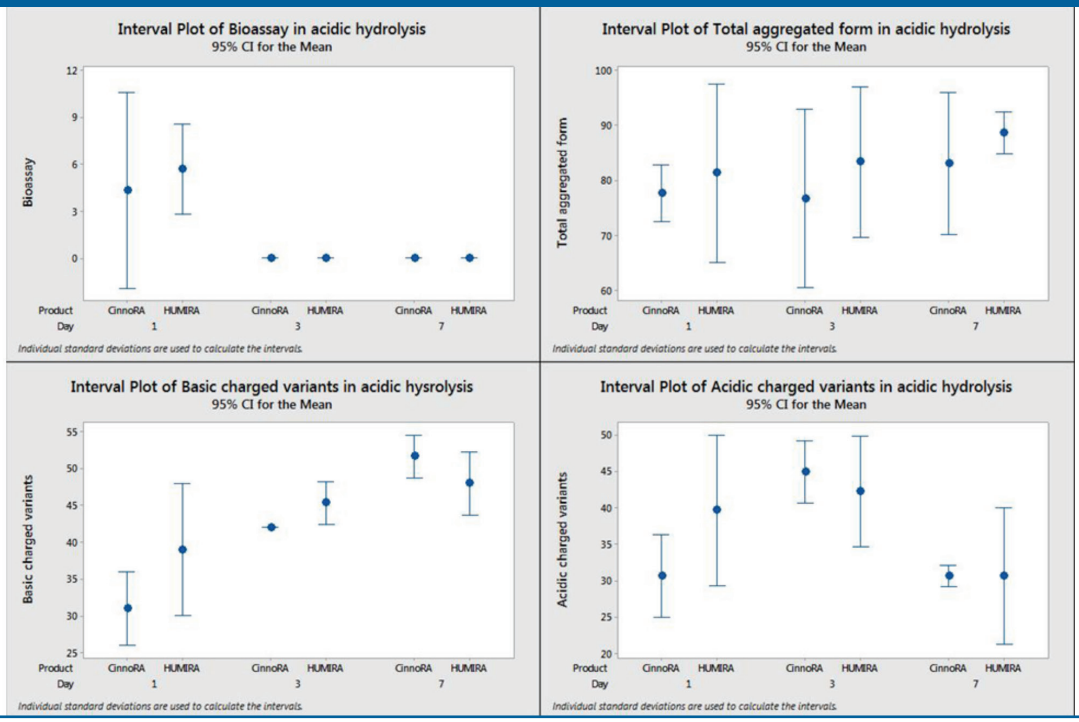


Figure 10: Comparison of acidic and basic charged variants by Ion exchange chromatography in oxidative stress condition (day 1) between Humira® and Cinnora® is given. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira® (01,02,03). This degradation pattern is followed for day 3 and 7.

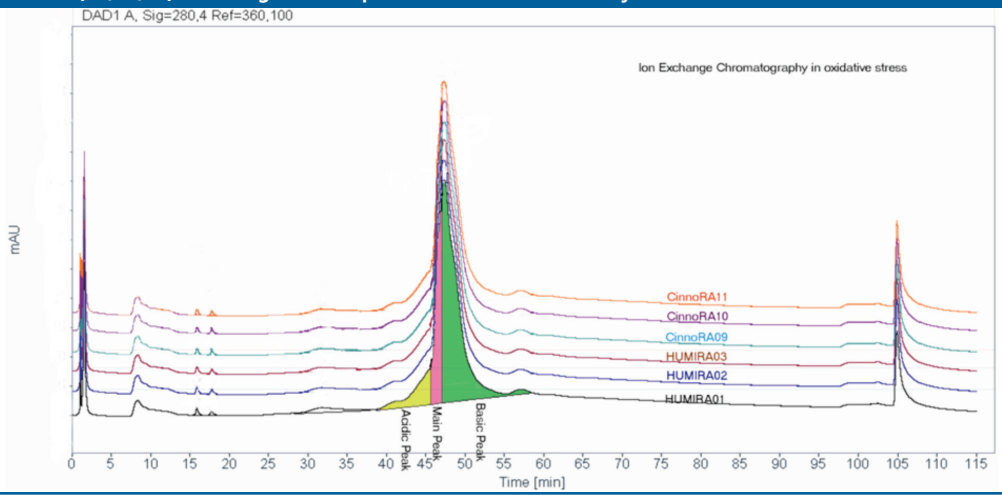


Figure 11: Comparison of total aggregated forms by size exclusion chromatography in oxidative stress condition (day 1) between Humira® and Cinnora® is demostarated. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira® (01,02,03). This degradation pattern is followed for day 3 and 7.

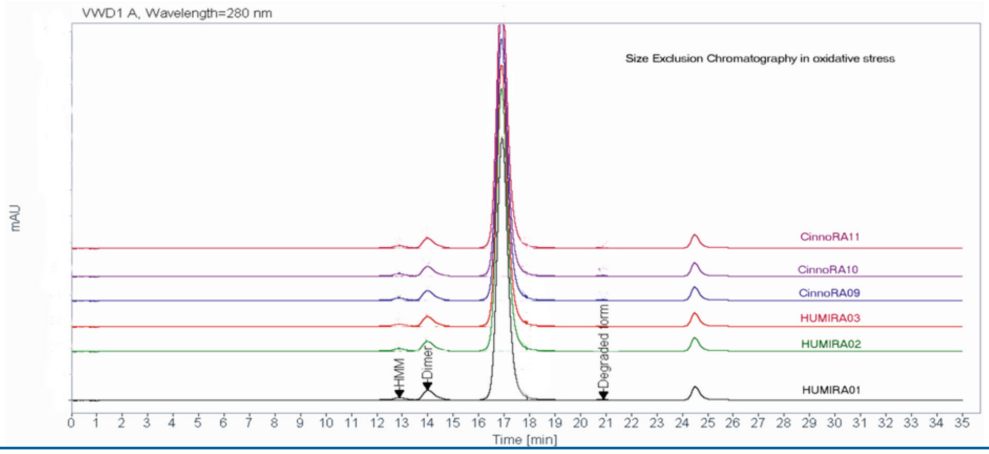


Figure 12: Interval plot of the oxidative stress of Cinnora® in comparison with Humira®

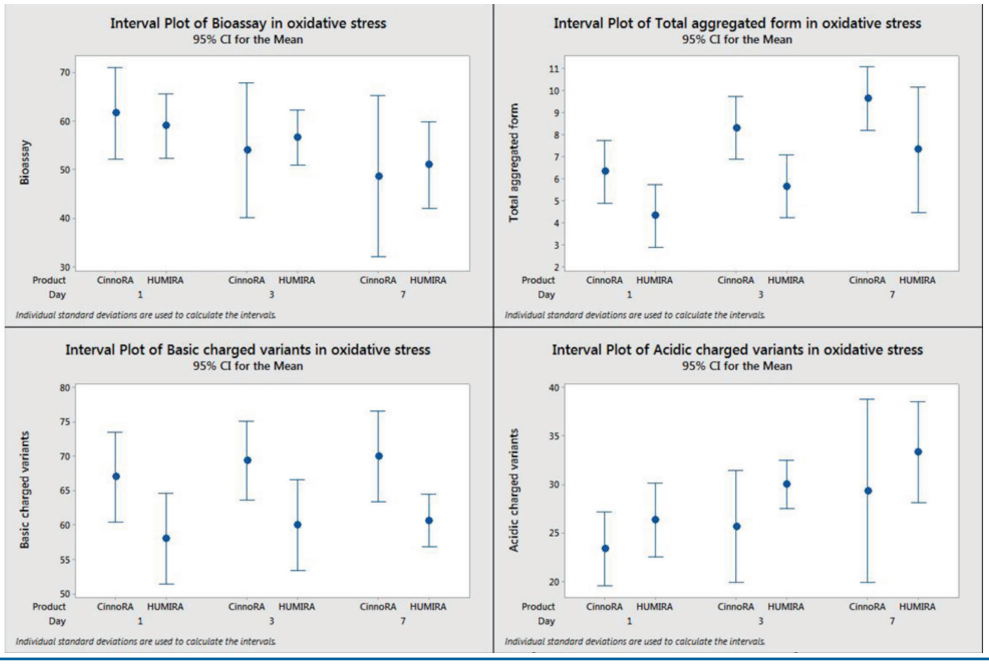


Table 4: The results of bioassay, total aggregated forms and acidic/basic charged variants of Cinnora® and Humira® in the thermal stress condition.

Conditions	Parameters	Samples	Day 1	Day 3	Day 7
Thermal	Bio assay	CinnoRA® 09	95	91	82
		CinnoRA® 10	91	92	81
		CinnoRA® 11	90	94	83
		HUMIRA® 01	97	91	84
		HUMIRA® 02	102	100	89
		HUMIRA® 03	96	93	85
	Total aggregated forms	CinnoRA® 09	4	5	5
		CinnoRA® 10	4	6	6
		CinnoRA® 11	5	5	5
		HUMIRA® 01	4	5	5
		HUMIRA® 02	3	3	4
		HUMIRA® 03	3	4	4
	Acidic charged variants	CinnoRA® 09	21	21	25
		CinnoRA® 10	21	23	26
		CinnoRA® 11	21	21	25
		HUMIRA® 01	20	21	24
		HUMIRA® 02	19	21	23
		HUMIRA® 03	20	22	23
	Basic charged variants	CinnoRA® 09	7	9	11
		CinnoRA® 10	9	10	11
		CinnoRA® 11	8	10	11
		HUMIRA® 01	5	6	8
		HUMIRA® 02	7	8	10
		HUMIRA® 03	6	8	11

Figure 13: Comparison of acidic and basic charged variants by Ion exchange chromatography in thermal stress condition (day 1) between Humira® and Cinnora® is demonstrated. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira® (01,02,03). This degradation pattern is followed for day 3 and 7.

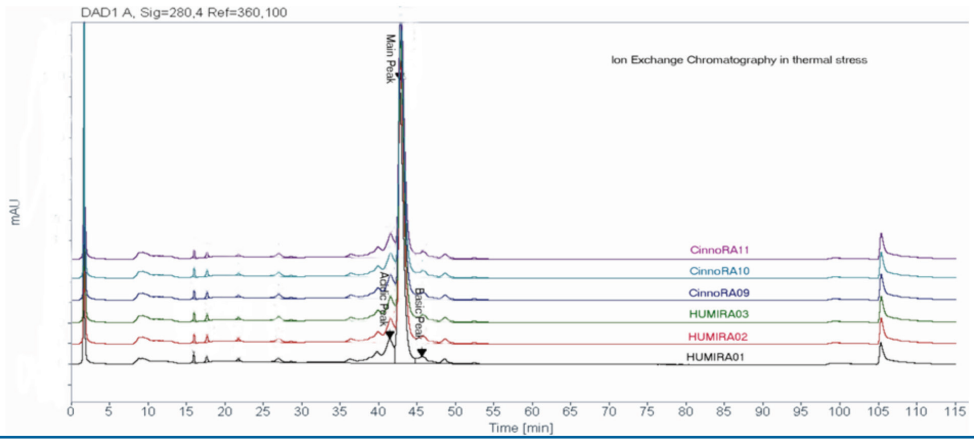


Figure 14: Comparison of total aggregated forms by size exclusion chromatography in thermal stress condition (day 1) between Humira® and Cinnora® is given. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira® (01,02,03). This degradation pattern is followed for day 3 and 7.

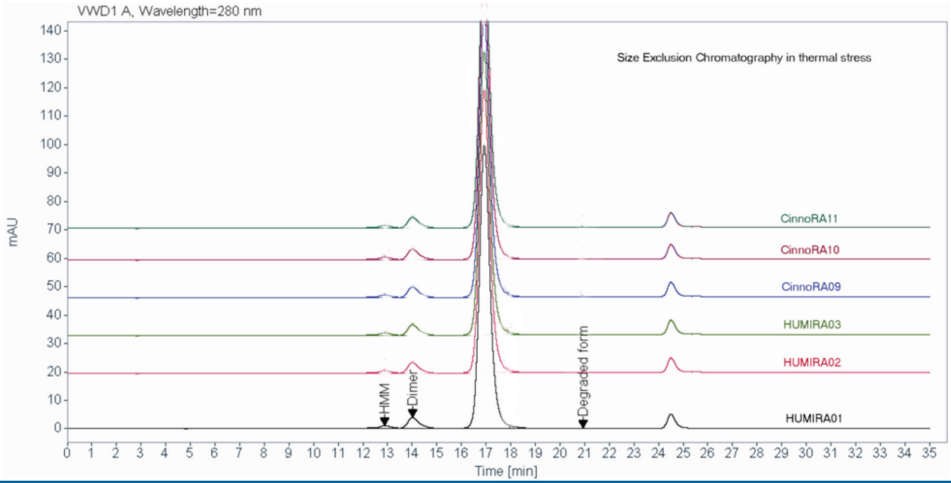


Figure 15: Interval plot of the thermal stress of Cinnora® in comparison with Humira®

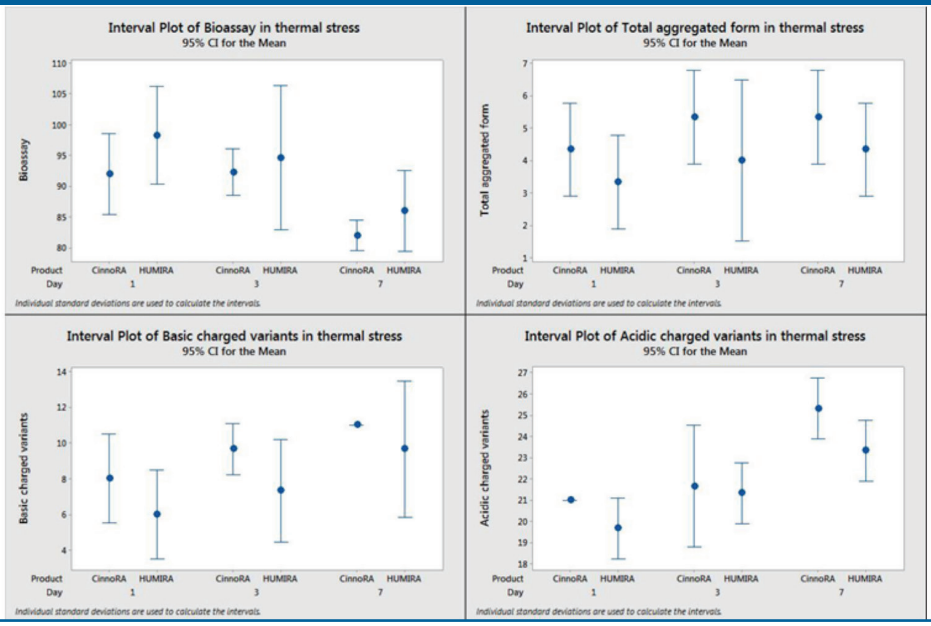


Table 5: The results of bioassay, total aggregated forms and acidic/basic charged variants of Cinnora® and Humira® in the mechanical stress condition

Conditions	Parameters	Samples	Day 2	Day 4
Mechanical	Bio assay	CinnoRA® 09	101	91
		CinnoRA® 10	95	89
		CinnoRA® 11	98	90
		HUMIRA® 01	99	94
		HUMIRA® 02	100	92
		HUMIRA® 03	95	90
	Total aggregated forms	CinnoRA® 09	5	5
		CinnoRA® 10	3	3
		CinnoRA® 11	5	6
		HUMIRA® 01	2	3
		HUMIRA® 02	4	5
		HUMIRA® 03	3	4
	Acidic charged variants	CinnoRA® 09	21	22
		CinnoRA® 10	20	22
		CinnoRA® 11	19	21
		HUMIRA® 01	18	19
		HUMIRA® 02	17	18
		HUMIRA® 03	18	20
	Basic charged variants	CinnoRA® 09	8	10
		CinnoRA® 10	9	9
		CinnoRA® 11	9	10
		HUMIRA® 01	14	14
		HUMIRA® 02	13	14
		HUMIRA® 03	10	12

Figure 16: Comparison of acidic and basic charged variants by Ion exchange chromatography in mechanical stress condition (day 1) between Humira® and Cinnora® is demonstrated. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira® (01,02,03). This degradation pattern is followed for day 3 and 7.

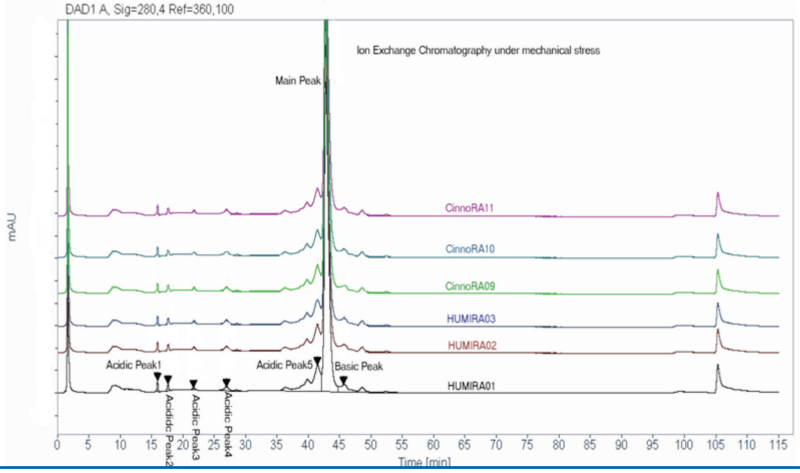


Figure 16: Comparison of acidic and basic charged variants by Ion exchange chromatography in mechanical stress condition (day 1) between Humira® and Cinnora® is demonstrated. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira® (01,02,03). This degradation pattern is followed for day 3 and 7.

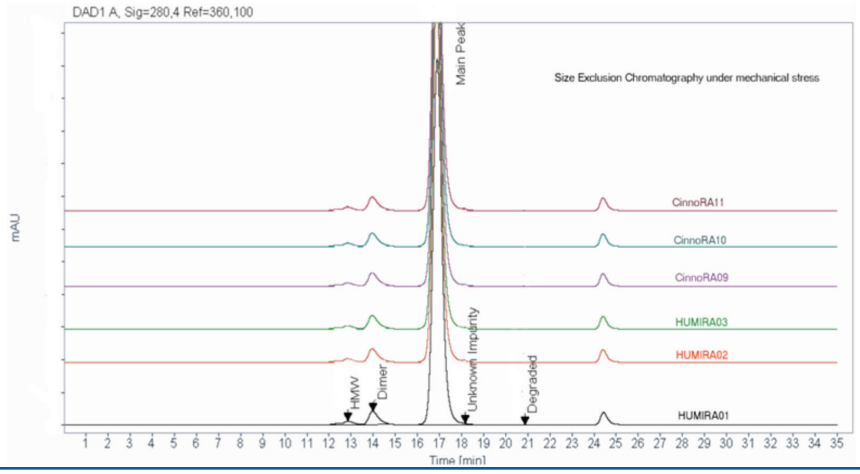
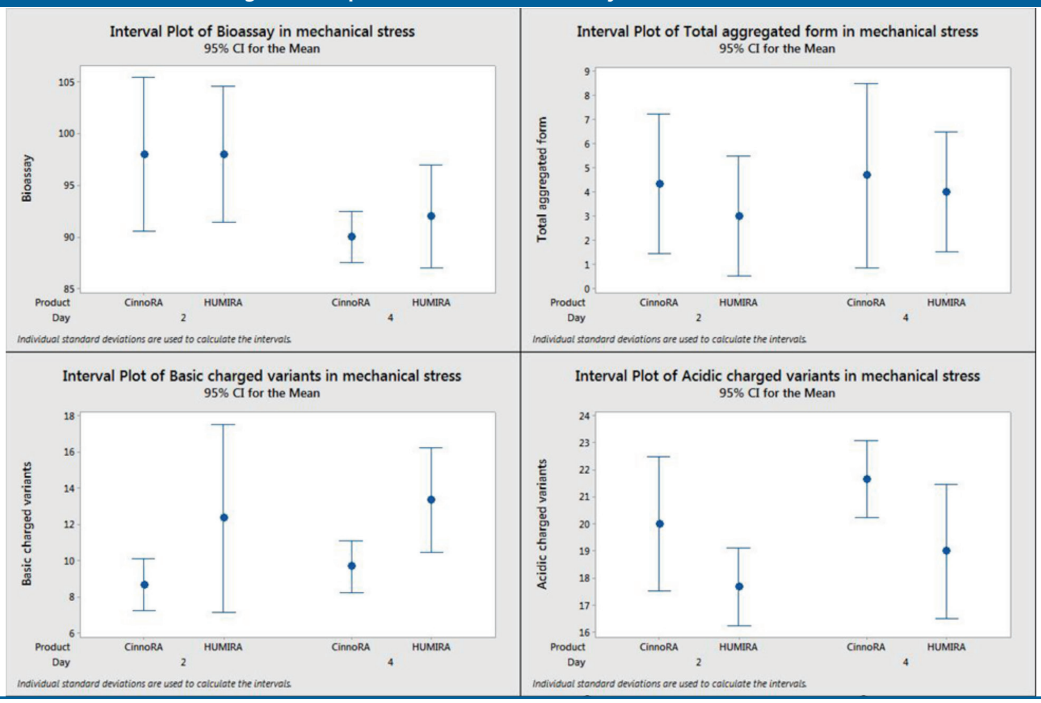


Figure 17: Comparison of total aggregated forms by size exclusion chromatography in mechanical stress condition (day 1) between Humira® and Cinnora® is given. Representative Ion exchange chromatography profile is presented for 3 batches of Cinnora® (09,10,11) and 3 batches of Humira® (01,02,03). This degradation pattern is followed for day 3 and 7.



Discussion

Photolysis:
Ion exchange and size exclusion chromatography and bioassay confirmed that acidic/basic charged variant, dimers and biological activity are identical and meet the acceptable range with similarity in all batches. Thus, the products are photo resistant in examined conditions.

Basic hydrolysis:
Changing pH significantly altered the mechanism of aggregate growth in adalimumab which leads to a decrease in antibody activity and could elicit an immunological response[43-45]; in addition, acidic charged variants increase in basic solution and protein get acidic properties. No significant differences in charged variants, high molecular weights and bioassays between Humira® and Cinnora® batches were observed (Figure 4, 5, 6).

Acidic hydrolysis:
Acidic hydrolysis caused the generation of a vast amount of aggregated forms which leads to precipitation and complete degradation of protein with no biological activity (table 3). Besides, basic charged variants increase in acidic condition. In all parameters, significant difference was observed between control and sample groups in table 3 which demonstrates the degratative effecs of HCL treatment on protein degradation.

Oxidation:
The oxidation of methionine to methionine sulfoxide presumably affects protein structure and stability by increasing basic charged variants reducing side chain hydrophobicity and increasing the capacity for hydrogen bonding [46]. As result of H_2O_2 exposure to adalimumab exerts its

negative impact on the biological activity by increasing aggregate formation which is considerable in table 3.

Thermal stress:
Thermolytic degradation is usually thought of as degradation caused by exposure to temperatures high enough to induce covalent bond breakage, that is, pyrolysis. Raising the temperature above accelerated condition (25±2°C) caused aggregated formation and biological activity decreases (table 4). Whereas, it should be considered the results in bioassay maintain still is an acceptable range as well as charged variants which demonstrate relative thermolytic stability in Cinnora® and Humira®.

Mechanical stress:
A slight increase in aggregated forms is noticeable other parameters meet the acceptable limits, so Cinnora® and Humira® are relatively resistant to mechanical stresses.

Totally the information provided by this study shows significant similarity in drug product characteristics, and based on their similarity in orthogonal characterization studies the comparability in degradation pattern can be considered. However, to get information of structural changes in order to develop a higher resistant medicine in exposure with different stress conditions some type of structural determinations, such as N-terminal amino acid analysis and glycosylation sites in drug product could be implemented.

Conclusions

A comprehensive force degradation strategy implemented to assess the similarity between biosimilar and innovator in protein degradation profile. Acidic and basic charged variants, aggregates, biological activities were evaluated and the results support the conclusion that Cinnora® is analytically similar to Humira® under different stress conditions.

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