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APPLICATION NOTE

SHINING NEW LIGHT ON PHARMACOKINETIC ASSAYS WITH SIMPLE WESTERN

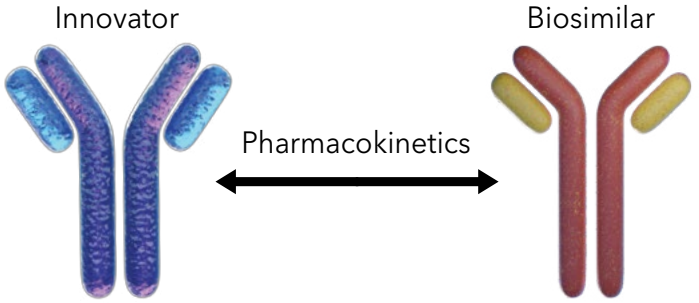
QUANTITATIVE PROTEIN CHARACTERIZATION IN COMPLEX SAMPLES

An important aspect of therapeutic drug characterization is pharmacokinetic (PK) studies, which are used to understand the fate of an administered drug. Typically, PK studies are conducted to look at drug absorption, bioavailability, distribution, metabolism, and excretion. These studies historically have utilized ELISA-based methods to quantify the remaining levels of drug in a patient post-administration.[1] However, given that biological drugs are quite complicated, and are known to change in response to stress, a one-dimensional test like ELISA may only provide limited information.

Instead, Simple Western™ from ProteinSimple, a Bio-Techne brand, is a capillary-based immunoassay that separates proteins based on Charge (i.e. isoelectric point) or Size (i.e. molecular weight) followed seamlessly by immunodetection directly in the capillary (think of an ELISA, but with protein separation by charge or size, see **FIGURE 1**). Because Simple Western assays rely on the specificity of antibodies recognizing separated proteins, followed by highly sensitive (low picogram) chemiluminescence detection, Simple Western is capable of protein analysis in highly complex samples like human serum. Further, Simple Western can process up to 96 samples in one overnight run, enabling relative quantitation of protein expression across samples or absolute quantitation of protein expression by incorporating standard curves alongside sample panels. This combination of highly specific measurements in complex samples and sample throughput make Simple Western an ideal tool for PK studies.

A NEW TOOL FOR BIOSIMILAR EVALUATION

The expiry of patent protection for many biological medicines presents an attractive opportunity for the development of biosimilars, which are amino-acid-copy drugs intended to offer comparable safety and efficacy to the off-patent innovator biologics. The approval of a biosimilar requires the demonstration of comparability with the innovator molecule through various analytical techniques, such as capillary isoelectric focusing (cIEF) and capillary electrophoresis sodium dodecyl sulfate (CE-SDS),[2] both of which can be performed on Maurice. Because these methods rely on direct detection of proteins by their UV or fluorescence spectra, they are relatively limited in their ability to analyze drugs in complex samples like serum, which in turn limits their use in PK assays. By contrast, Simple Western effectively combines cIEF/CE-SDS with highly specific immunodetection, enabling the detection of drugs in complex samples with unparalleled specificity (**FIGURE 1**). Therefore, Simple Western may provide new insights into the PK properties of innovator and biosimilar drugs to fast-track their development and approval.



	ELISA	Maurice CE-SDS	Maurice cIEF	**Simple Western**
Immunodetection	✓	✘	✘	✓
Charge separation	✘	✘	✓	✓
Size separation	✘	✓	✘	✓

FIGURE 1. A comparison of Simple Western and related assays used for PK and biosimilar evaluation of therapeutic monoclonal antibodies.

In this Application Note, we use Simple Western Charge and Size assays to evaluate the PK properties of adalimumab and two adalimumab biosimilars over the course of nine weeks in human serum. Adalimumab reduces inflammatory responses by inhibiting the binding of TNFα to its receptor. It was the bestselling biopharmaceutical until its patent expired in 2016, reaching $16 billion in global sales annually.[3] Several biosimilars have since been approved in the USA and Europe.[4]

While Simple Western Size detected small changes in drug stability over this time course, Simple Western Charge revealed a dramatic change in the charge heterogeneity of the drugs by the end of the study.

PK STUDY MATERIALS AND METHODS

EXPERIMENTAL SETUP

All experiments were performed on Peggy Sue™, a high throughput Simple Western system which is capable of both charge and size separation. Peggy Sue can automatically analyze up to 96 samples overnight without user intervention. This high throughput capacity of Simple Western is ideal for PK analysis across many time points and replicates. Adalimumab assay materials, Simple Western Charge materials and Simple Western Size materials are listed in TABLES 1, 2, and 3, respectively.

ITEM	VENDOR	PART NUMBER
Adalimumab Innovator	Provided by collaborator	N/A
Adalimumab Biosimilar 1	Provided by collaborator	N/A
Adalimumab Biosimilar 2	Provided by collaborator	N/A
Human Serum	Sigma	H3667
Anti-Adalimumab (Anti-Idiotype) Antibody	R&D Systems	MAB9616

TABLE 1. Adalimumab assay materials.

Adalimumab was spiked in human serum at a final concentration of 8.51 µg/mL and maintained on ice until time to aliquot. The adalimumab samples were aliquoted into a Random Access 96 well skirted PCR plate (Brooks Lifesciences, 4ti-0960/RA), and sealed with a Random Access Pierce Heat Seal Strong (Brooks Lifesciences, 4ti-05381/RA). The individually sealed wells were punched out and placed in a container with 3 packs of Boveda 2-way Humidity Control (62% humidity, 4 gram, B62-04-10P). The container was incubated at 37 °C. 10 tubes of adalimumab in human serum were separately snap-frozen on dry ice and stored at -80 °C to serve as frozen controls. With the container stored at 37 °C, a time course experiment was performed whereby samples were removed at the following time points: 0, 1, 4, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 336 (2 weeks), 504 (3 weeks), 672 (4 weeks), and 1512 (9 weeks) hours. Once removed, samples were immediately snap-frozen on dry ice and stored at -80 °C until the time course was complete. Then, the samples were analyzed by Simple Western Charge and Size on the same day on different Peggy Sue instruments. Samples were analyzed in technical triplicates for each time point.

ITEM	VENDOR	PART NUMBER
Charge capillaries	ProteinSimple	CBS700
Anolyte	ProteinSimple	040-337
Catholyte	ProteinSimple	040-338
Wash Buffer Concentrate	ProteinSimple	041-108
Ampholyte-free G2 Premix	ProteinSimple	040-967
pI 5-8 G2 Premix	ProteinSimple	040-973
pI 8-10.5 Ampholytes	Sigma	GE17-0455-01
pI Ladder 3	ProteinSimple	040-646
pI Standard 8.4	ProteinSimple	041-036
pI Standard 9.7	ProteinSimple	040-790
1 M L-Arginine	Sigma	A5006
Bicine/CHAPS Lysis Buffer and Sample Diluent	ProteinSimple	040-764
Antibody Diluent	ProteinSimple	040-309
Secondary Streptavidin-HRP	ProteinSimple	043-459-2
Peroxide	ProteinSimple	043-379
Luminol-S	ProteinSimple	043-311

TABLE 2. Simple Western Charge materials.

SIMPLE WESTERN CHARGE CONDITIONS

SAMPLE PREPARATION

Samples were thawed on ice, then diluted 1:7.5 in Bicine/Chaps Lysis Buffer and Sample Diluent followed by 1:4 in Master Mix for a final dilution of 1:30.

MASTER MIX PREPARATION

The Master Mix consisted of 90% pI 8-10.5 and 10% pI 5-8 with pI Ladder 3, pI Standards 8.4 and 9.7 and 40 mM L-Arginine. The pI 8-10.5 solution was prepared by mixing 8% pI 8-10.5 ampholytes in Ampholyte-free G2 Premix.

ANTIBODY PREPARATION

The primary antibody was prepared by diluting biotinylated anti-Adalimumab at 1:1000 in Antibody Diluent and incubated for 2 hours. The antibody was biotinylated in house with Biotin-XX-NHS. The detection antibody was Secondary Streptavidin-HRP (ready to use) and incubated for 30 minutes.

SEPARATION CONDITIONS

Default conditions were used except for the following: 45-minute separation, 180 seconds UV immobilization, and Size Luminol was used.

ITEM	VENDOR	PART NUMBER
12-230 kDa Peggy Sue or Sally Sue Separation Module	ProteinSimple	SM-S001
Bicine/CHAPS Lysis Buffer and Sample Diluent	ProteinSimple	040-764
Antibody Diluent 2	ProteinSimple	042-203
Secondary Streptavidin-HRP	ProteinSimple	043-459-2
Peroxide	ProteinSimple	043-379
Luminol-S	ProteinSimple	043-311

TABLE 3. Simple Western Size materials.

SIMPLE WESTERN SIZE CONDITIONS

SAMPLE PREPARATION

Samples were thawed on ice and diluted 1:50 in Bicine/Chaps Lysis Buffer and Sample Diluent, then mixed 1:1 with 2X Master Mix at a final dilution of 1:100. Samples were denatured under non-reducing conditions with 12.5 mM Iodoacetamide for 10 minutes at 70 °C.

ANTIBODY PREPARATION

The primary antibody was prepared by diluting biotinylated anti-Adalimumab at 1:1000 in Antibody Diluent 2 and incubated for 30 minutes. The antibody was biotinylated in house with Biotin-XX-NHS. The detection antibody was Secondary Streptavidin-HRP (ready to use) and incubated for 30 minutes.

SEPARATION CONDITIONS

Default conditions were used except for the following: 5 seconds UV immobilization.

PK STUDY RESULTS

SIMPLE WESTERN CHARGE

Simple Western Charge analysis of the adalimumab innovator, biosimilar 1, and biosimilar 2 revealed a main peak at a pI of approximately 8.6, with several smaller peaks in both the acidic and basic regions of the main peak (FIGURE 2A). This charge heterogeneity profile was consistent with previous results,[5] and a similar charge heterogeneity profile was obtained with purified adalimumab by cIEF analysis on Maurice (FIGURE 2B). After 1 hour incubation time, changes in the charge heterogeneity profile began to emerge. Most notable was the decrease in the basic peak area of all 3 molecules. By the end of the study, the charge heterogeneity had dramatically changed with the appearance of stress-associated peaks, indicating a severe compromise to the integrity of the antibody structure (FIGURE 3).

The Compass for Simple Western software automatically calculates the area under each peak to generate quantitative data. Thus, we examined the average peak area and average percentage of total peak area of the main, basic, acidic, and stress-associated acidic peaks from each time point. We observed a gradual decrease of main peak as the PK study progressed for all three drugs, ultimately resulting in approximately 70% decrease in signal compared to the frozen control by the end of the study (FIGURE 4).

We observed an increase in acidic peak area over time for all 3 drugs. The adalimumab innovator and biosimilar 1 increased approximately 300% in acidic peak area after 9 weeks compared to the frozen control, while biosimilar 2 increased approximately 50%. Interestingly, biosimilar 1 did not increase in % acidic peak area over time like the innovator and biosimilar 2 (FIGURE 5). The adalimumab innovator and biosimilar 2 basic peaks significantly decreased after 1 hour and were reduced to a shoulder after 4 hours, likely due to deamidation. Biosimilar 1 presented a basic shoulder throughout the study, resulting in a significant decrease at 9 weeks (FIGURE 6).

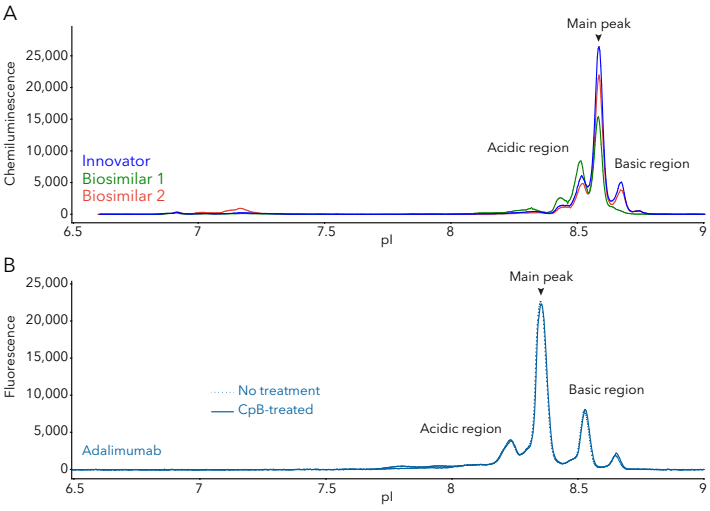


FIGURE 2. (A) Representative electropherograms resulting from Simple Western Charge analysis of the adalimumab innovator, biosimilar 1, and biosimilar 2 in human serum (frozen controls). (B) Maurice cIEF analysis of purified adalimumab with and without carboxypeptidase B (CpB) treatment.

All three drugs presented stress-associated acidic peaks which increased gradually over the study, resulting in a significant increase compared to the frozen control. These unexpected peaks might be due to an interaction between the drug and human serum (FIGURE 7).

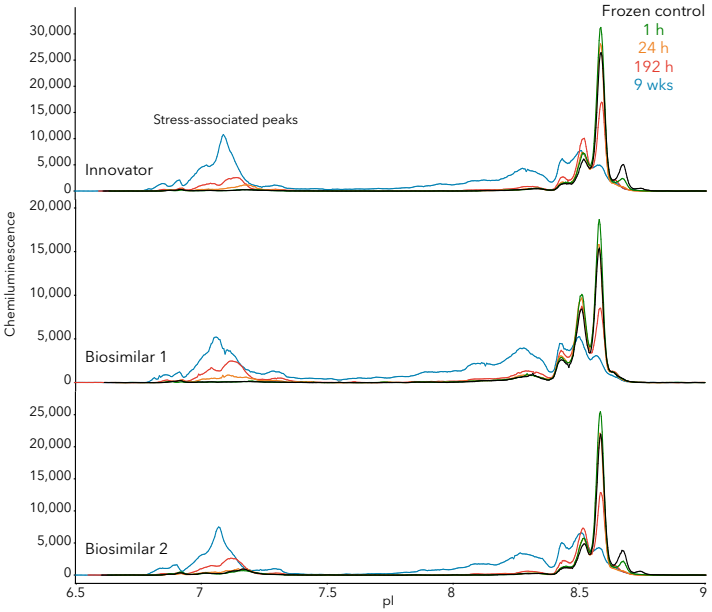


FIGURE 3. Representative electropherograms resulting from Simple Western Charge analysis of the adalimumab innovator (top), biosimilar 1 (middle), and biosimilar 2 (bottom) from the frozen controls and intermittent time points throughtout the study (1 hour, 24, hour, 192 hour, and 9 weeks).

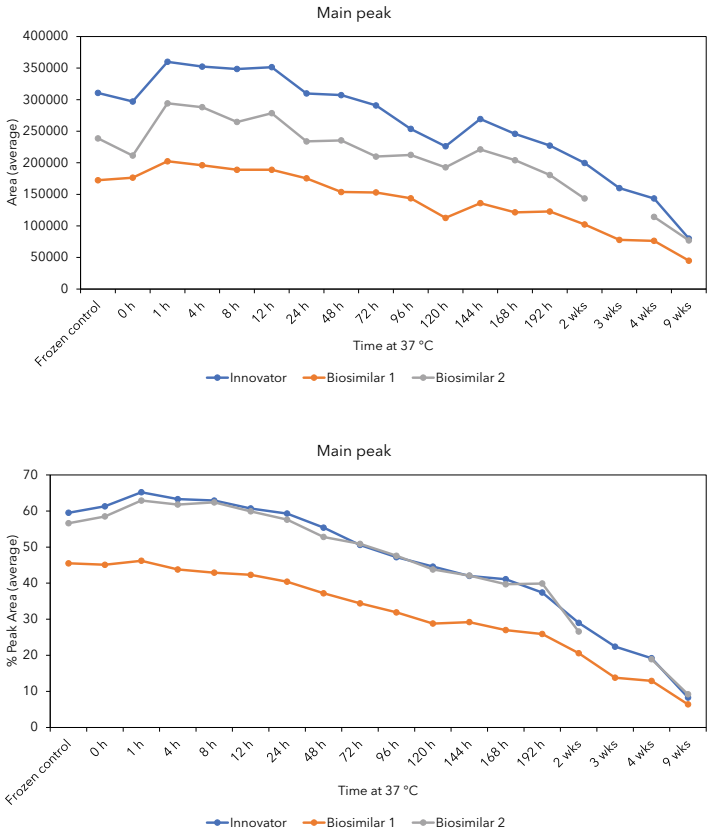


FIGURE 4. Average peak area and average % peak area resulting from Simple Western Charge analysis of the adalimumab innovator, biosimilar 1, and biosimilar 2 main peak over time.

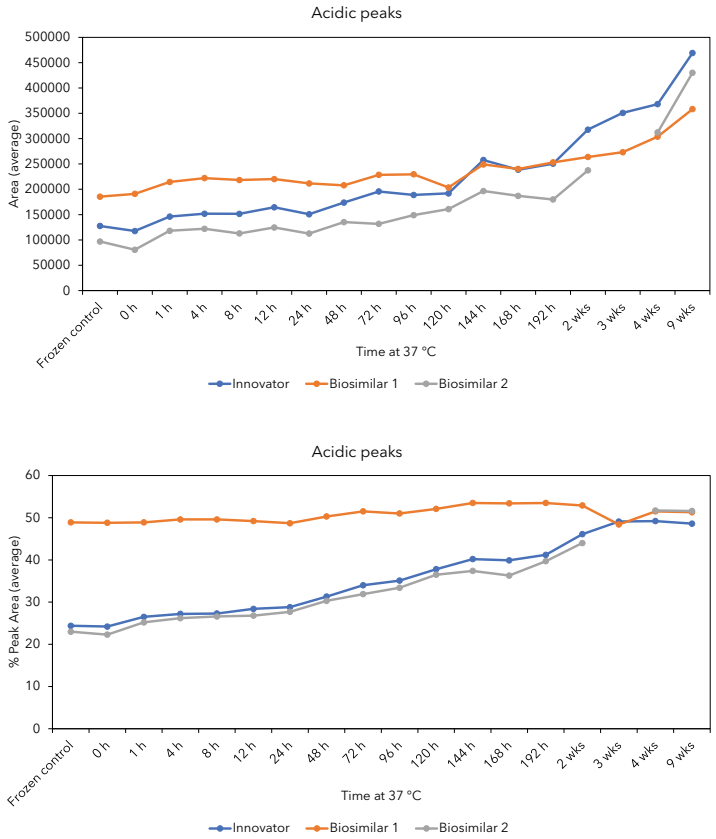


FIGURE 5. Average peak area and average % peak area resulting from Simple Western Charge analysis of the adalimumab innovator, biosimilar 1, and biosimilar 2 acidic peaks over time.

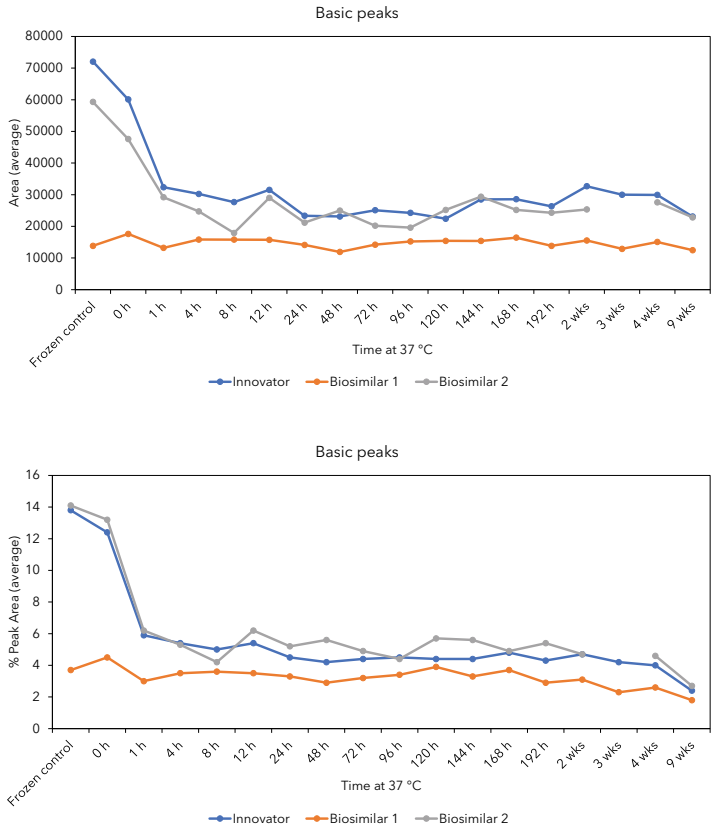


FIGURE 6. Average peak area and average % peak area resulting from Simple Western Charge analysis of the adalimumab innovator, biosimilar 1, and biosimilar 2 basic peaks over time.

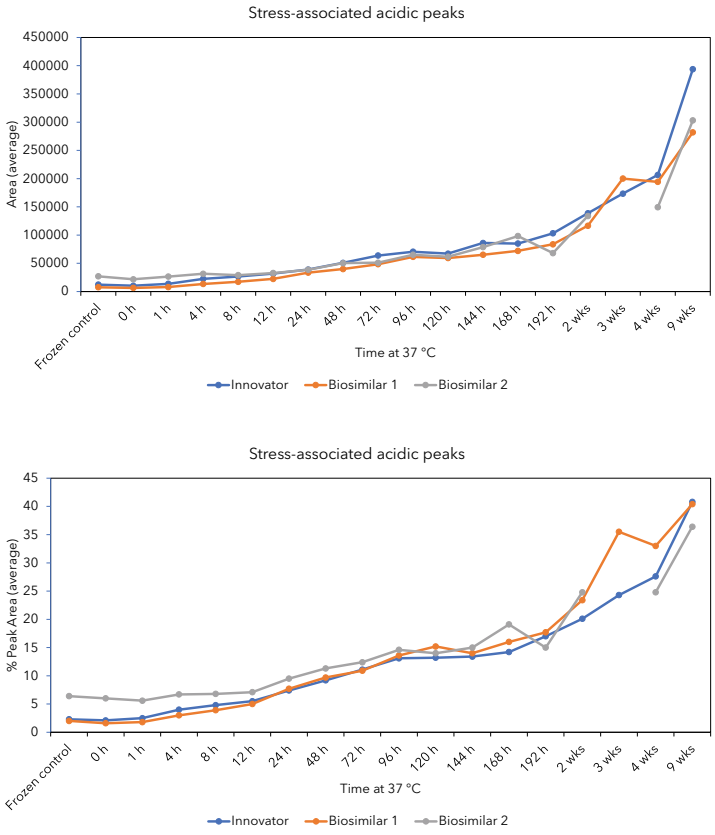


FIGURE 7. Average peak area and average % peak area resulting from Simple Western Charge analysis of the adalimumab innovator, biosimilar 1, and biosimilar 2 stress-associated peaks over time.

SIMPLE WESTERN SIZE

Simple Western Size analysis of the adalimumab innovator, biosimilar 1, and biosimilar 2 under non-reducing conditions revealed a well-defined peak at approximately 180 kDa that corresponded to intact IgG (**FIGURE 8**). This size separation profile was consistent with previous results.[5] Biosimilar 1 had a smaller peak at a higher molecular weight above 230 kDa. No differences were observed after 1 hour of incubation. After 9 weeks, the separation profile did not change dramatically, except for the appearance of lower molecular weight product that corresponded to IgG fragments (**FIGURE 9**).

We next examined the average peak area and average percentage of total peak area of the intact IgG peak, IgG fragments, and IgG aggregates for each time point using the signal quantification provided by Compass for Simple Western software. Some difference in intact IgG peak area was observed throughout for the three drugs; however, the % Peak area did not change much after 9 weeks, with only a ~10% decrease (**FIGURE 8**). IgG fragments remained steady for the first 2 weeks of the study, with a sizable jump at 9 weeks for the adalimumab innovator and biosimilar 2 (**FIGURE 10**). Likewise, IgG aggregates remained steady for the first 2 weeks of the study, with a sizable jump at 9 weeks for the adalimumab innovator and biosimilar 2. Biosimilar 1 did not change much since it had a sizeable aggregate peak from the beginning of the study (**FIGURE 11**).

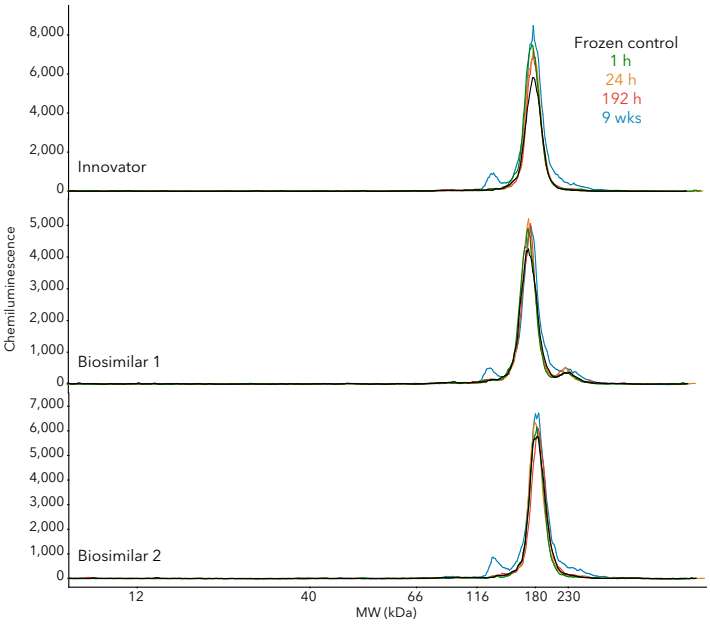


FIGURE 8. Representative electropherograms resulting from Simple Western Size analysis of the innovator (top), biosimilar 1 (middle), and biosimilar 2 (bottom) from the frozen controls and intermittent time points throughtout the study (1 hour, 24, hour, 192 hour, and 9 weeks).

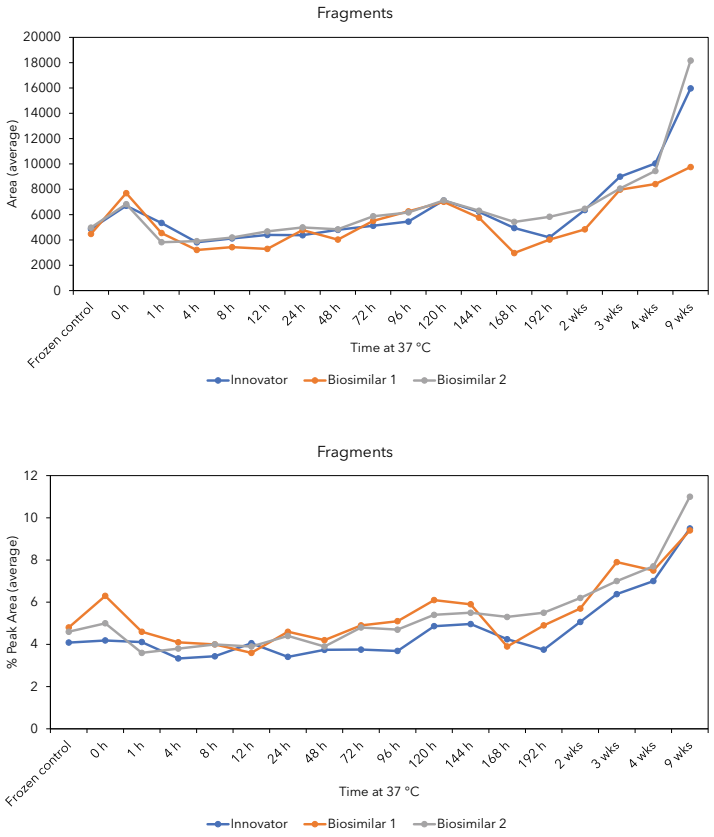


FIGURE 10. Average peak area and average % peak area resulting from Simple Western Size analysis of the adalimumab innovator, biosimilar 1, and biosimilar 2 IgG fragments over time.

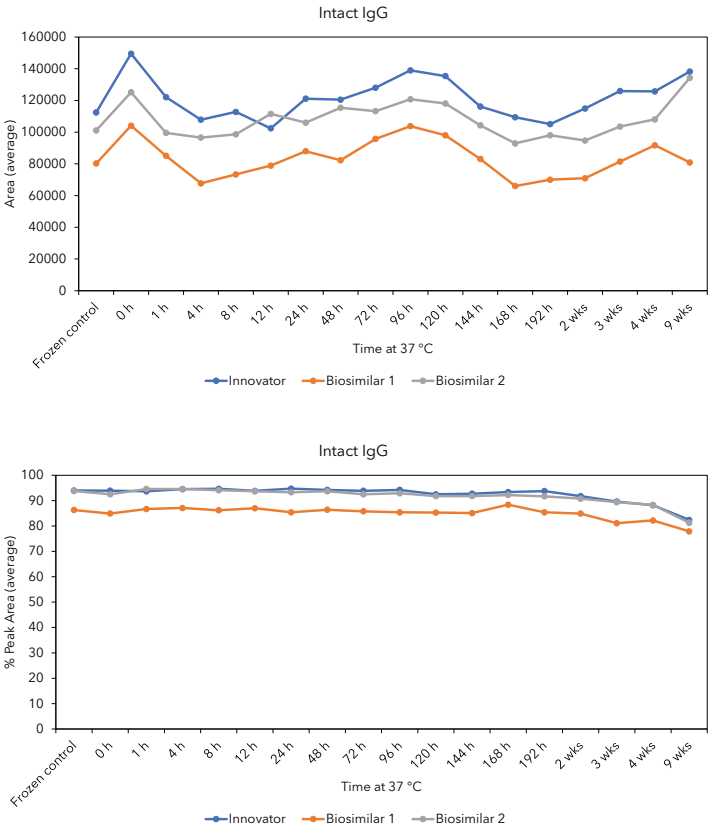


FIGURE 9. Average peak area and average % peak area resulting from Simple Western Size analysis of the adalimumab innovator, biosimilar 1, and biosimilar 2 intact IgG over time.

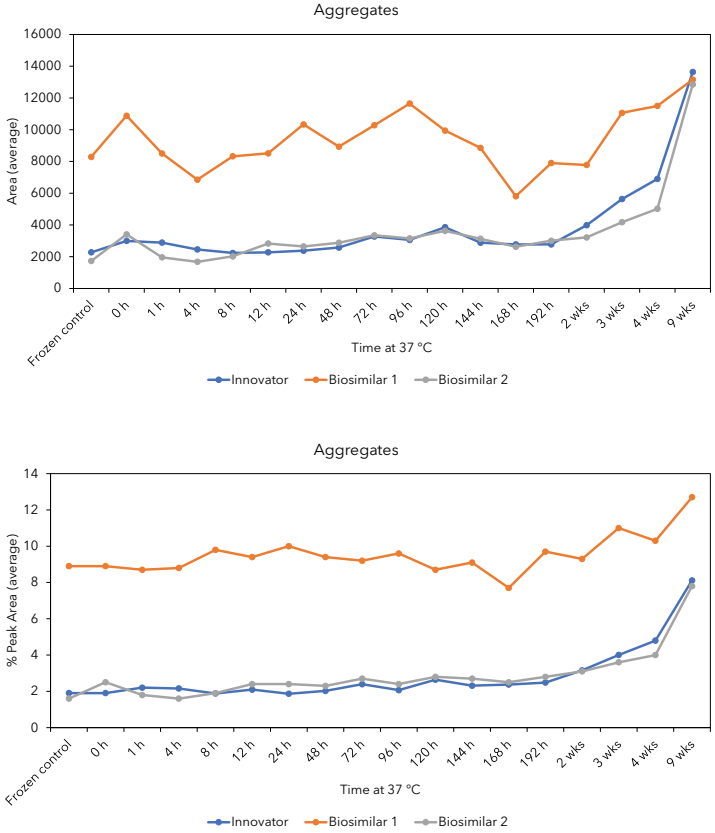


FIGURE 11. Average peak area and average % peak area resulting from Simple Western Size analysis of the adalimumab innovator, biosimilar 1, and biosimilar 2 IgG aggregates over time.

INFORMATION RICH PHARMACOKINETIC DATA WITH SIMPLE WESTERN

Simple Western combines the sensitive immunodetection of an ELISA with the separation power of cIEF and CE-SDS assays. This combination places Simple Western in a unique position to study the PK properties of therapeutic monoclonal antibodies and their biosimilars. Unlike ELISA, Simple Western provides high-resolution charge and size separation profiles of target molecules with minimal matrix effects that commonly plague ELISAs. And unlike cIEF and CE-SDS, Simple Western is an immunoassay that can detect even small amounts (low pg) of target protein in complex mixtures like human serum.

When utilizing Peggy Sue and Maurice cIEF as analytical techniques in tandem, users can obtain comparable charge separation profiles in samples from early stages of product development and to later stages of purified samples. Thus, easy method transferability between these platforms will enable protein monitoring that covers the entire protein therapeutic development pipeline, from target discovery to QC release and stability. For more information, refer to our Protocol on Method Transfer between Maurice and Peggy Sue.

In this study, Simple Western Charge and Size assays provided a complete picture of the PK activity of adalimumab in human serum. The Simple Western Charge assay provided the most wealth of information and was able to detect significant changes in the drug profiles over the course of the study, including the detection of unexpected stress-associated acidic peaks. The Simple Western Charge pharmacokinetic data suggest that the adalimumab innovator and biosimilar 2 are more similar than the adalimumab innovator and biosimilar 1. In summary, Simple Western is poised to be instrumental in understanding the PK properties of therapeutic monoclonal antibodies and potentially fast-tracking the regulatory approval of their biosimilars.

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