https://doi.org/10.48047/AFJBS.6.15.2024.4389-4417

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Development and Validation a Bioanalytical Bridge ELISA Method for Anti-Adalimumab Antibody Monitoring.

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Volume 6, Issue 15, Sep 2024

Received: 15 July 2024

Accepted: 25 Aug 2024

Published: 05 Sep 2024

doi: 10.48047/AFJBS.6.15.2024.4389-4417

Abstract

Adalimumab is a humanized IgG1 monoclonal antibody (Mab) used to treat rheumatoid arthritis, inflammatory bowel diseases, Crohn's disease, cancer and skin diseases. This drug specifically targets tumor necrosis factor alpha (TNF-α) by blocking its interaction with cell surface TNF-α receptors p55 and p75. Adalimumab diminishes inflammation, immunological reactions and thus minimizes tissue damage in autoimmune and inflammatory disorders. Of note, development of appropriate positive and negative controls is crucial for assay validation, interpretation and distinguishing antibody positive from antibody negative samples. Therefore, this study aimed to develop a bridge ELISA method using rabbit anti-adalimumab antibody as a positive control to screen anti-drug antibodies (ADAs). The positive control antibody was prepared by depleting human IgG specific antibodies and characterized for its specific reactivity with adalimumab, and without cross reactivity with human IgG. The method was developed and validated for the drug tolerant level by spiking the various concentrations of adalimumab with constant amount of rabbit anti-adalimumab antibody. The assay cut-off was determined by analyzing the mean concentration value of negative control + (2 x standard deviation). Remarkably, the precise identification and surveillance of ADAs are essential for enhancing biologic treatments and controlling immunogenicity. Moreover, the bridge ELISA has proven to be efficient among various approaches, due to its robustness, high sensitivity, and tolerance for variations in drug concentration. Of note, the newly designed bridge ELISA for anti-adalimumab antibodies exhibits superior performance, capable of detecting low ADA levels (0.0244 µg/mL) and conforming to rigorous EMA validation standards.

Keywords: Adalimumab, Arthritis, Inflammation, Bridge ELISA, Bioanalysis, Anti-drug antibodies, Therapeutic drug monitoring.

1. Introduction

Adalimumab, marketed as Humira from AbbVie, is a commonly used therapeutic monoclonal antibody that specifically targets and blocks tumor necrosis factor-alpha (TNF-α), an important cytokine implicated in inflammatory processes. Antibody- and protein-based biopharmaceuticals, together with related biological agents, have gained prevalence in recent years. The U.S. Food and Drug Administration (FDA) has recently approved the 100th monoclonal antibody (mAb) product. In 2020, the U.S. FDA approved 13 monoclonal antibodies for therapeutic application. These antibodies mostly contribute to antibody dependent cell-mediated cytotoxicity (ADCC), through complement-mediated cell lysis

(Quinteros et al. 2017). Adalimumab has a particular affinity for both soluble and membrane-bound TNF-alpha. The Fab portion of the antibody exhibits a strong affinity for TNF-alpha, effectively inhibiting its binding to cell surface receptors with great specificity. The primary mechanism of action of adalimumab is direct binding and blocking of TNF-α receptor-mediated activities (Caiazzo et al. 2018). Adalimumab blocks both TNFR1 and TNFR2 receptors by binding both soluble(s) and transmembrane TNF-α. Human anti-human antibodies, or HAHAs, are thought to be produced by adalimumab, which appears to induce immunization (Shealy and Visvanathan 2008). This antibody lowers levels of several indicators, including soluble ICAM-1, MMP, and IL-7 (Papamichael et al. 2019).The level of TNF-inhibitor in patient's serum and plasma varies greatly and is correlated with their symptoms. 20–30% of patients on adalimumab therapy experienced the development of idiotype antibodies against the drug. As a result of this, adalimumab concentration in patients may decline.

Due to adalimumab being a completely human IgG, the initial assumption was that the occurrence of anti-adalimumab antibodies (AAAs) would be less common compared to infliximab (Chimenti et al. 2016; Bonifati et al. 2016; Meade et al. 2024; Yanai and Hanauer 2011; Karmiris et al. 2009a; WEST et al. 2008; Chaparro et al. 2012). Recent studies have indicated that a significant percentage (ranging from 2.6% to 38%) of patients undergoing treatment for Crohn's disease or rheumatoid arthritis develop anti-adalimumab antibodies (Scheinfeld 2003; Mease 2007; Kivitz and Segurado 2007). These AAAs are found to be linked with low levels of adalimumab in serum and leads to decline in the effectiveness of the treatment (Gordon et al. 2006; Karmiris et al. 2009a; G. M Bartelds et al. 2007; Geertje M. Bartelds 2011).

Importantly, the identification and/or development of appropriate positive and negative controls is crucial as they are essential for assay validation. They are intimately associated with assay interpretation and for distinguishing antibody positive from antibody negative samples. Therefore, this study aimed to develop a bridge ELISA method using rabbit anti-adalimumab antibody as a positive control to screen anti-drug antibodies (ADAs).

2. Materials and methods

2.1. Materials

The Rabbit anti-adalimumab antibody (R-AAA) was raised in New Zealand white Rabbit, used as a positive control antibody. Adalimumab (Humira®) supplied as 100mg/ml was procured from AbbVie, Australia. Adalimumab HRP-conjugate was prepared in laboratory, Goat anti-

Rabbit HRP (Denovo Biolabs, India). All reagents used were of analytical grade and are supplied by Sigma-Aldrich, Himedia, Qualigens (India), Denovo Biolabs (India) were procured.

2.2. Rabbit Anti-Adalimumab Antibody Development (Positive Control)

The adalimumab drug was procured from a commercially available source. The whole drug was used as immunogen to raise antibody against adalimumab in New Zealand white Rabbit.

2.2.1 Host selection and immunization

The female New Zealand white rabbits of age 5-6 weeks were selected for pre-screening for presence of any cross-reacting (pre-existing) antibodies against adalimumab by ELISA.

An indirect ELISA was performed by coating the adalimumab on 96 well microtiter plate. Briefly, Coated the 96 wells microtiter plate with 100 µl of adalimumab at a concentration of 2 µg/ml using 1X Phosphate Buffered Saline (PBS), coating buffer pH 7.4. Incubated the ELISA plate overnight at 4ºC. The following day, allow the plate to reach room temperature and decant the contents of the plate and rinse with 1X PBS pH 7.4. The plate was blocked by adding 350 µl of blocking buffer (0.5% Gelatin, 1X PBS pH 7.4) and incubated at room temperature for 1 hour. Decanted the contents of the plate and rinse once with 1 X PBS buffer pH 7.4. The Rabbit serum dilutions from 1:125, 1:250 and 1:500 was prepared in antibody diluent buffer (0.5% Gelatin, 1X PBS pH 7.4 with 0.05% Tween-20). Added 100 µl/well of diluted rabbit serum and incubate the plate at room temperature for 1 hour. The antibody diluent buffer alone was added to ELISA plate as negative controls (NC). Decanted the contents of the plate and washed three times with 1X PBST (1 X PBS with 0.05% Tween 20 pH 7.4) followed by three washes with 1 X PBS pH 7.4 at three minutes interval each. Decanted off any residual buffer by inverting the plate and blotting it against clean paper towels. Prepared the working dilution Goat Anti Rabbit HRP conjugated antibody at 1:5000 in antibody diluent buffer. Added 100 µl of the detection antibody to the wells and covered with an adhesive strip sealer and incubate for 1 hour at room temperature. Decanted the contents of the plate and washed three times with 1X PBST followed by three washes with 1 X PBS pH 7.4 at three minutes interval each. Decanted off any residual buffer by inverting the plate and blotting it against clean paper towels. Added 100 µl of 1X substrate solution to each well. Incubate it for 15 minutes in dark. Added 50 µl of stop solution (2N H_2SO_4) to each well. Read the absorbance at 450 nm in a microplate reader after ensuring that there is no air bubble in any wells.

2.2.2 Immunization of rabbit

New Zealand white Rabbit was immunized with a dose of 500 µg of adalimumab with equal volume Freund's incomplete adjuvant (FIA) after preparing an emulsification after performing the pre-screening of rabbits to ensure the absence of any preexisting antibodies against adalimumab. The animals were maintained at In-vivo Biosciences animal house facility, Bangalore and the ethical approval for animal study was approved by the committee (approval no. INVOVO/002/2022). The antibody response was boosted with three booster doses in 15 days intervals with 250 µg of adalimumab with equal volume of Freud's complete adjuvant (FCA). The details of immunization are given in Table-1.

Table-1: Immunization Schedule for Rabbit Anti Adalimumab Antibody generation

Stage	**Immunization**	**1st Booster**	**2nd Booster**	**3rd Booster**
Antigen Dosage (µg)	1000	1000	1000	1000
Antigen: Adjuvant	1:1	1:1	1:1	1:1
Type of Adjuvant	FCA	FIA	FIA	FIA
Route of Administration	SC	SC	SC	SC
Total Volume Injected (µl)	2000	2000	2000	2000
Booster Sites	Multiple	Multiple	Multiple	Multiple

2.2.3 Determination of rabbit antisera titer

An antisera titer is a measurement of how much antibody has been produced by the experimental animal that recognizes a particular epitope, expressed as the inverse of the greatest dilution that still gives a positive result. The titer of rabbit anti-serum was determined by indirect ELISA (refer section 1.1 for indirect ELISA procedure). After second and third boosters, test bleed were performed by animal house personnels and the antiserum was received. The Rabbit serum dilution was prepared in antibody diluent buffer by serial double dilution (1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000 and 1:64000) for serum titre determination.

2.2.4 Antibody purification and depletion against human IgG

Adalimumab is a fully humanized antibody, which was used as immunogen to raise the anti-adalimumab antibody in rabbits. The polyclonal antiserum contains all for of antibody subtypes against the whole molecule. The protein-A affinity purified antibody from rabbit antisera has a potential risk of cross reacting against human IgG present in serum and may interfere with the assay. The antibody cross reactive was eliminated by purifying the antibody on protein-A affinity column, followed by loading the antibody elutes from protein-A affinity to over CN-

Br coupled Human IgG column. The antibody was depleted against Human IgG coupled column for several rounds and tested for its reactivity against adalimumab as well as against human IgG on indirect ELISA. The antibody purification was performed by using Rabbit anti-adalimumab serum (10 ml) on protein-A affinity column and followed by depletion on human IgG column.

2.2.5 SDS-PAGE analysis

A 12% resolving gel was cast using 30% acrylamide (Himedia, India), 1.5M Tris-HCl pH 8.8 (Qualigens, India), 10% SDS (Sigma-Aldrich, USA), 10% ammonium persulfate and TEMED, followed by 4% stacking gel using distilled water, 30% acrylamide, 1M Tris (pH 6.8), 10% SDS, 10% ammonium persulphate and TEMED. The 2 µg of purified anti-adalimumab antibody was mixed with 2X SDS-PAGE loading dye in a 1:1 ratio and loaded onto the 12% SDS-PAGE under reducing conditions. The electrophoresis was carried out at 100V till the tracking dye reached the end of the gel. The gel was stained using a 2X Coomassie stain solution and the protein bands were analyzed after de-staining the gel. The specific binding of anti-adalimumab antibody was tested by diluting the purified antibody (1.0 µg/ml to 0.001 µg/ml) in antibody diluent buffer against the adalimumab drug and the cross reactivity against Human IgG was evaluated by indirect ELISA.

2.3 Adalimumab immunogenicity bridge ELISA method development

A bridge ELISA, where the anti-adalimumab was bridged between the coated adalimumab and adalimumab drug conjugate, was developed utilizing the rabbit anti-adalimumab antibody as a positive control reagent.

2.3.1 Adalimumab HRP conjugate preparation

Adalimumab drug (Humira®) was conjugated to horse radish peroxidase (HRP) by Oxidation of polysaccharide residues in glycoprotein with sodium periodate provides a mild and efficient way of generating reactive aldehyde groups for subsequent conjugation with amine or hydrazide containing molecules via reductive amination. Cross-linking of sodium periodate activated HRP with amino group of antibody takes place under alkaline pH condition through the formation of Schiff base intermediates. HRP: Antibody 1:1 mass ratio (molar ratio 4:1).

HRP contains two moles of calcium per mole of the enzyme which are involved in maintaining the integrity of the active site *structure. Calcium* ions functions by maintaining the protein structure in heme environments as well as the spin state of the heme iron which promoted the

enzyme activity of HRP. The antibody conjugation was carried out as described below. Prepared adalimumab drug molecule (2 mg) at a concentration of 10 mg/ml by diluting the main stock of 50 mg/ml in 0.2M bicarbonate buffer pH 9.6 and dialyzed in 100 ml of 0.2M bicarbonate buffer volume for overnight. Next day, dissolved 2 mg HRP in 200 µl 50mM PBS pH 7.2 at a concentration of 10 mg/ml and added 50µL of freshly prepared Sodium meta periodate solution. The reaction was mixed to dissolve and protected from direct light by covering the tube with aluminum foil. Incubated in dark for 20 minutes at room temperature, in cyclomixer at medium rpm. A color change was observed from Brownish/gold to green as the reaction proceeded. Later, immediately purified the oxidized HRP using desalting column, which is equilibrated with 0.2M bicarbonate buffer pH 9.6. Eluted the HRP using the bicarbonate buffer. The collected fractions were measured using micro volume spectrophotometer, observe the absorbance at 403nm while measuring. Higher absorbance fractions were pooled together and concentrated using viva spin concentrator to around 10 mg/ml (200 µl). Mixed the antibody solution with the enzyme solution at a ratio of 1:1(V/V) and incubated for 2 hours at room temperature in a cyclomixer at a medium rpm. Added 40µL of 5M sodium cyanoborohydride to the reaction solution and incubated for 30 minutes at room temperature. The unreacted aldehyde site was blocked by addition of 20 µl of 1M ethanolamine, pH 9.6 to conjugation solution and incubated for 30min at room temperature. The conjugate was purified from excess reactants by passing through desalting column (PD10) which is equilibrated with 10mM PBS pH 7.2. fractions were eluted using 10 mM PBS pH 7.4. and quantified on microvolume spectrophotometer using IgG port. Selected the peak fractions and pooled to concentrate using viva spin concentrator. Filtered sterilize the HRP-conjugate by passing it through 0.22µ filter.

2.3.2 Adalimumab HRP conjugate working dilution determination

The working dilution of the adalimumab-HRP conjugate was determined by direct ELISA anti-adalimumab antibody coated 96 well microtiter ELISA plate. Briefly, Rabbit-anti-adalimumab antibody was prepared by diluting it to a concentration of 2 µg/ml in 1X PBS pH 7.4 and 0.1 ml/well was coated into a microtiter plate, incubated at 4°C overnight. Next day, the wells were blocked with 0.3 ml/well of 0.5% gelatin, 1X PBS, pH 7.4 blocking buffer, and incubated for 1 hour at room temperature. Adalimumab HRP conjugate was diluted in blocking buffer at a dilution of 1:250 to 1:20,48,000 in double dilutions and 0.1 ml/well was added in duplicates after discarding the contents of the wells. The plate was incubated for 45 minutes at room temperature. The plate was washed with 0.3 ml/well of 1X PBS, pH 7.4 wash buffer, for three

times and followed by three washes with 1X PBS with 0.05% Tween-20 wash buffer with three-minute incubation time between each wash. 20X TMB substrate was diluted 1:20 in ultra-pure water and added 0.1 ml/well. Incubated for 15 minutes at room temperature, in the dark. The reaction was stopped using 0.05 ml/well of 2N sulphuric acid and absorbance was measured at 450nm in an ELISA reader.

2.3.3 Bridge ELISA

Microtiter plate (Corning) was coated with 2 µg/ml of Adalimumab drug in 1X PBS pH 7.4, 0.1ml/well and incubated overnight at 2-8°C. Next day, the contents of the microtiter wells were discarded, and the wells were blocked with 0.3 ml/well of Blocking buffer containing 0.5% gelatin BSA (Sigma), 0.05% Tween 20 (Sigma) in 1X PBS pH 7.4 (Denovo Biolabs) and incubated for 1 hour at Room temperature (RT). After discarding the contents of the wells, the wells were washed once with 1X wash buffer containing 1X PBS, pH 7.4. Rabbit Anti-Adalimumab Antibody (ADA or Anti-Drug Antibody) was spiked into assay diluent or serum MRD 10 at final concentrations of standard calibrators (12.5 µg/ml to 0.0244 µg/ml, serial double dilutions) and mixed with 1:50,000 diluted adalimumab HRP conjugate in 1:1 ratio. Added 100 µl/well of the plate and incubated at RT for 45 minutes. The microtiter wells were washed three times with 1X wash buffer (1X PBS pH 7.4, 0.05% Tween-20) followed by three washes with 1X PBS pH 7.4 using the automated ELISA plate washer (Biobase), following 3 minutes soaking between each wash. Discarded the contents of the well and added 1:20 diluted TMB/H2O2 (Denovo Biolabs) 0.1 ml/well and incubated at RT for 15 minutes, The reaction was stopped by adding 2N H_2SO_4 0.05ml/well (0.05ml/well). Absorbance was read at 450nm using 620nm as a reference range in ELISA Microplate reader (Tecan F50, Germany). The absorbance (A_{450nm}), the mean (average) absorbance (A_{450nm}), standard deviation (SD), percent coefficient of variance (%CV) and percentage analytical recovery (%AR) for ADA spiked standard calibrator concentration (S) and assay negative control (NC) were determined.

2.3.4 Standard calibrators

Rabbit anti-adalimumab antibody (R-AAA) main stock (1.45 mg/ml) was diluted in assay matrix to prepare standard calibrators (100 µg/ml to 0.012 µg/ml) as detailed in Table-2. Each standard calibrator was analyzed in duplicate along with the negative control (no R-AAA spiked) by bridge ELISA described above, and the ELISA plate layout was designed as per Table-2. The linear range of the assay was determined by spike and recovery of standard calibrators. The %SD, %CV, %RE of each standard calibrator was determined using GraphPad

prism software, where a 4PL (parameter logistic) sigmoidal response with variable slope fitting was adapted.

Table-2: R-AAA standard calibrators (100 µg/ml to 0.012 µg/ml) preparation by double diluting the ADA main stock (1.45 mg/ml) and testing in bride ELISA.

STD	Final desired concentration µg/ml	Prepared concentration µg/ml	Required (µl)	Assay matrix at MRD 10 (µl)	Total volume (µl)	DF
S1	100.000	200.00	144.00	900.00	1044.00	7.25
S2	50.000	100.000	500.00	500.00	1000.00	2.00
S3	25.000	50.000	500.00	500.00	1000.00	2.00
S4	12.500	25.000	500.00	500.00	1000.00	2.00
S5	6.250	12.500	500.00	500.00	1000.00	2.00
S6	3.125	6.250	500.00	500.00	1000.00	2.00
S7	1.563	3.125	500.00	500.00	1000.00	2.00
S8	0.781	1.563	500.00	500.00	1000.00	2.00
S9	0.391	0.781	500.00	500.00	1000.00	2.00
S10	0.195	0.391	500.00	500.00	1000.00	2.00
S11	0.098	0.195	500.00	500.00	1000.00	2.00
S12	0.049	0.098	500.00	500.00	1000.00	2.00
S13	0.024	0.049	500.00	500.00	1000.00	2.00
S14	0.012	0.024	500.00	500.00	1000.00	2.00
NC	0.000	0.000	0.00	1000.00	1000.00	NA

2.3.5 Statistical analysis

Assay data variability was expressed in mean, standard deviation (SD), and coefficient of variation (CV). Curve fitting for standard calibrators and statistical analyses were performed GraphPad prism 10 version 10.3.1 using 4PL (parameter logistics) analysis. The standard calibrator linearity, accuracy, precision, sensitivity, selectivity, Inter/Intra assay variations, drug tolerance was calculated using MS Excel (Microsoft®) and with the formulas as below.

Concentrations were extrapolated from the A_{450nm} absorbance (OD) values and 4PL statistical calculations, obtained from the standard curve, using the formula- Recovered Concentration = 10^(LOG_{EC50} - (($LOG_{(((TOP-BOTTOM)/(ODA450nm-BOTTOM))}$-1))/HILLSLOPE)).

The %AR was calculated using the formula- %AR = (obtained concentration/actual concentration) x100.

The coefficient of variation (CV) of the slope was calculated using the formula- %CV = (standard deviation / mean of A_{450nm}) x100.

Relative error was calculated using the formula- RE=100 – obtained AR (or) %RE = 100% - %AR.

Note: RE can be depicted as an absolute value (ABS). If a negative value is obtained, the number is depicted as a positive number.

Total error was calculated using the formula- Total error = %CV + %RE.

2.4 Adalimumab ADA bridge ELISA validation

In compliance with the requirements and recommendations set by the FDA and the EMA method validation was carried out with particular regards to calibration Standard curve, matrix effect, selectivity, precision, accuracy, after optimizing the ELISA parameters, such as adalimumab drug coating, microtiter well blocking buffer, assay diluent, assay matrix, wash buffers, detection antibody, and Quality Control (QC) points namely Upper Limit of High QC (HQC), Low QC (LQC), Mid QC (MQC).

This method is intended to be used as a screening assay for adalimumab ADA (anti-drug antibody) testing, where Rabbit anti-adalimumab polyclonal antibody is used as a positive ADA control. This positive control ADA would mimic the ADA forms in the subjects undergoing the adalimumab therapy. The validation of ELISA method was undertaken on the following parameters.

2.4.1 Standard Calibrators

A standard calibration curve was established using eight nonzero concentrations (12.5 µg/ml to 0.098 µg/ml) of rabbit anti-adalimumab antibody (ADA) in assay matrix (MRD 10) by serial double dilution as mentioned in the Table-3. Each standard calibrator and negative control (NC) samples were assayed in duplicates on an ELISA plate, precoated with adalimumab drug. The ELISA procedure was carried out as described (refer 2.3.3) before and measured the absorbance at (A_{450nm}) and calculated percent analytical recovery (%AR), standard deviation (SD), percent coefficient of variations (%CV), percent relative error (%RE) each standard calibrator.

The 4PL analyzed sigmodal dose response with variable slope standard calibrator curve was acceptance for percent relative error (%RE). The percent relative error (%RE) of the back-calculated value for 75% standard calibrators should be within ≤20% of nominal concentration, except the ULOQ and LLOQ level where it should be within the ≤25% of nominal concentration.

Table-3: Preparation of standard calibrators (S1-S8; 12.5 µg/ml- 0.98 µg/ml) from ADA stock (1.45 mg/ml).

STD	Final desired concentration (µg/ml)	Prepared concentration (µg/ml)	Required (µl)	Assay Matrix (µl)	Total volume (µl)	DF
S1	12.500	25.00	18.00	1026.00	1044.00	58.00
S2	6.250	12.500	500.00	500.00	1000.00	2.00
S3	3.125	6.250	500.00	500.00	1000.00	2.00
S4	1.563	3.125	500.00	500.00	1000.00	2.00
S5	0.781	1.563	500.00	500.00	1000.00	2.00
S6	0.391	0.781	500.00	500.00	1000.00	2.00
S7	0.195	0.391	500.00	500.00	1000.00	2.00
S8	0.098	0.195	500.00	500.00	1000.00	2.00
NC	0.000	0.00	0.00	1000.00	1000.00	0

2.4.2 Precision and accuracy

Precision and accuracy of the assay was tested by Quality Controls (QCs) samples of 5 different concentrations such as upper limit of quantification (ULOQ), high-level (HQC), mid-level (MQC), low-level (LQC) and lower limit of quantification (LLOQ) within the calibration range were prepared independent of standard calibrators from ADA main stock 1.45 mg/ml by two analysts for total of six occasions on two different days. The prepared QCs were analysed on adalimumab drug coated 96-well ELISA plate in twelve (12) replicates for each of the QC point across all the accuracy and precision runs.

The repeatability was acceptable if the relative error (%RE) of the back calculated value for each level was ≤ 20% (≤ 25% at ULOQ and LLOQ), coefficient of variation (%CV) was ≤20% (≤ 25% at ULOQ and LLOQ), total error (sum of the %RE and %CV) was ≤ 30% (≤40% at ULOQ and LLOQ). Quality control samples used for evaluating accuracy and precision parameter were prepared by diluting the main stock of ADA in assay matrix at MRD-10 as per the Table-4 below.

Table-4: Preparation of quality control (ULOQ, HQC, MQC, LLOQ) of ADA by diluting the main stock of 1.45 mg/ml in assay matrix at serum MRD-10.

QC Samples	Final desired concentration µg/ml	Prepared concentration µg/ml	Required (µl)	Assay matrix at MRD 10 (µl)	Total volume (µl)	DF
ULOQ	12.500	25.00	34.48	1965.52	2000	58.00
HQC	6.250	12.5000	750.00	750.00	1500	116.00
MQC	0.550	1.1000	129.36	1340.64	1470	11.36
LQC	0.195	0.3906	532.64	967.36	1500	2.82

LLOQ	0.098	0.1953	500.00	500.00	1000	2.00

2.4.3 Drug tolerance

It is important to estimate the ADA against adalimumab therapeutic monoclonal antibody to ensure the drug bioavailability within the therapeutic window. Drug tolerance of an assay is defined as the ability of the assay to detect ADA in the presence of a defined concentration of adalimumab drug. This assay can estimate free anti-adalimumab antibody in the target sample matrix and can be utilized as a screening assay for free ADA detection. In this assay, a sample mix containing a constant amount of ADA, variable dilutions of adalimumab drug and constant dilution of adalimumab HRP conjugate were added on pre-coated adalimumab drug ELISA plate and incubated. Adalimumab drug was spiked at different concentration (20 µg/ml to 0.002 µg/ml) in a prediluted ADA at 10 µg/ml in assay matrix by serial double dilutions as per the table-28. A positive control was analyzed without adalimumab drug spike but with the ADA spiking at 10 µg/ml. The available free ADA reacts with coated adalimumab and produces a signal in proportion to the available free ADA.

Table-5: Drug tolerance sample preparation by diluting the adalimumab working stock (1000 µg/ml) in constant ADA spiked (10µg/ml) assay matrix at MRD-10. Each sample was diluted with adalimumab HRP conjugate (1:1) to achieve final adalimumab concentration.

S. N	Final adalimumab concentration µg/ml	Prepared concentration µg/ml	Required Volume (µl)	ADA spiked (10µg/ml) assay matrix at MRD 10 (µl)	Total volume (µl)	DF
DT1	20.000	40.00	8.35 from (WS)	1035.65	1044.00	125.00
DT2	10.000	20.000	500.00	500.00	1000.00	2.00
DT3	5.000	10.000	500.00	500.00	1000.00	2.00
DT4	2.500	5.000	500.00	500.00	1000.00	2.00
DT5	1.250	2.500	500.00	500.00	1000.00	2.00
DT6	0.625	1.250	500.00	500.00	1000.00	2.00
DT7	0.313	0.625	500.00	500.00	1000.00	2.00
DT8	0.156	0.313	500.00	500.00	1000.00	2.00
DT9	0.078	0.156	500.00	500.00	1000.00	2.00
DT10	0.039	0.078	500.00	500.00	1000.00	2.00
DT11	0.020	0.039	500.00	500.00	1000.00	2.00
DT12	0.010	0.020	500.00	500.00	1000.00	2.00
DT13	0.005	0.010	500.00	500.00	1000.00	2.00
DT14	0.002	0.005	500.00	500.00	1000.00	2.00
NC	0.000	0.000	0.00	1000.00	1000.00	0

All test samples with adalimumab drug, positive control (no adalimumab spiked) and negative control (no adalimumab and R-AAA spiked) were analyzed in two replicates. The measurements were performed by an analyst and recorded absorbance at 450 nm. Analysis of assay interference in the presence of adalimumab drug was achieved by comparing mean assay absorbance at each concentration of spiked adalimumab sample with a mean assay absorbance response of negative control serum at MRD-10. Multiple replicates of negative controls without adalimumab drug and R-AAA spiked were tested to establish a reliable cutoff of the assay.

2.4.4 Cut-off value determination

To determine a cut-off value for a bridge ELISA assay used to detect anti-drug antibodies (immunogenicity) of adalimumab requires to analyze positive and negative control analysis with a statistically significant number. At least twelve replicates were analyzed in a single ELISA plate run and the absorbance at 450 nm for negative controls (serum MRD 10) was recorded. This process ensures that the developed assay cut-off is reliable to distinguish between positive and negative samples. Rabbit anti-adalimumab antibody utilized as positive control at 10 µg/ml and normal serum at MRD-10 taken as negative control. The preparation of positive and negative controls as described in Table-3 below and the ELISA plate layout as mentioned in Table-6.

Table-6: Preparation of ADA positive and negative controls for assay cut-off determination

Sample ID	Conc of ADA (µg/ml)	Required Volume from Stock (µl)	Volume of Assay Matrix (MRD-10) (µl)	Total Volume (µl)	DF
Positive Control (PC)	10.00	17.24	25.00	2500.00	145.00
Negative Control (NC)	0.00	0.00	12.5000	2000.00	0.00

All ADA positive and negative samples were analyzed in duplicates by bride ELISA protocols. The cut-off value was calculated as per the formula: *Cut-off value= mean concentration value of negative control + (2 x SD)*

3. Results

3.1 Development of rabbit-anti adalimumab antibody

The rabbits (RD1 & RD2) were tested for any pre-existing antibody against adalimumab at serum dilution of 1:125, 1:250 and 1:500 by indirect ELISA. The absorbance value at 450 nm

for 1:250 and 1:500 dilution of rabbit sera was found within the acceptable range (OD ≤0.2), therefor RD1 animal was selected for adalimumab immunization.

Selected rabbit host (RD1) was immunized with adalimumab emulsified with FCA and followed by booster dose with adalimumab emulsified with FIA. The rabbit anti-sera titre was determined by indirect ELISA against coated adalimumab drug after second and third boosters. An antisera titer is a measurement of how much antibody has been produced by the experimental animal that recognizes a particular epitope, expressed as the inverse of the greatest dilution that still gives a positive result. The titre for rabbit antisera was determined as >1:64000 at OD ≥0.4 and OD ≥0.82 for second and third booster respectively

Rabbit antisera dilutions from second and third boosters were plotted against absorbance at 450 nm. The dilution effect was clearly overserved from lower to higher dilution of antisera (Figure-1).

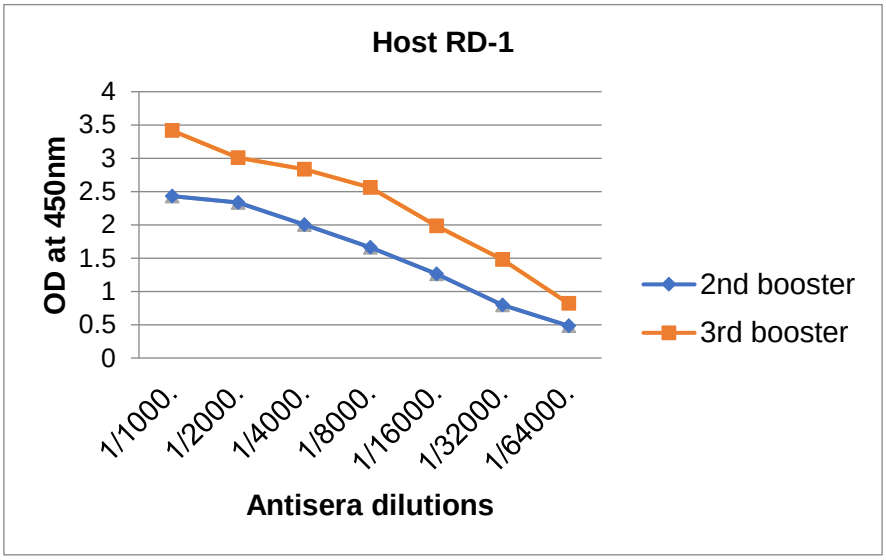

Figure-1: The ELISA data for second and third booster titer was plotted using serum dilution factor on X axis and absorbance at 450 nm on Y axis.

Further, the production bleed was performed after second and third boosters and collected 15 ml of antisera from each production bleed. The antisera were stored at -80°C for long-term storage. The rabbit antiserum from production bleed of third booster (10 ml) was purified on protein-A affinity chromatography followed by depletion on Human IgG coupled CN-Br column to eliminate the antibodies against human IgG and enrich the antibody pool specific to adalimumab CDR region. The purified antibody was quantified by using microvolume spectrophotometer and the purity of antibody was >95% by SDS-PAGE Coomassie staining (Figure-2).

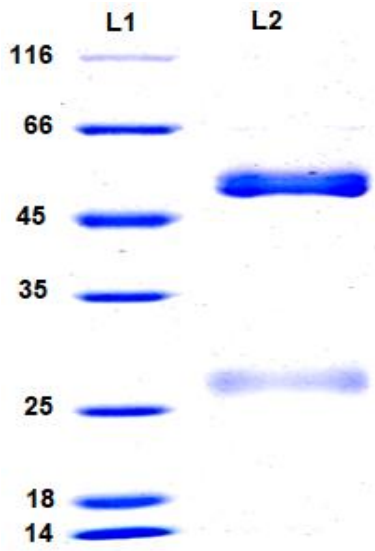

Figure-2: 12% SDS-PAGE analysis for Rabbit Anti Adalimumab Antibody ADA; L1 Protein molecular weight marker and L2 Purified Antibody (5 µg) after depletion against Human IgG coupled CN-Br column.

Next, rabbit anti-adalimumab antibody (R-AAA) reactivity was tested against adalimumab and Human IgG coated on ELISA. The ELISA result reveled that the Rabbit Anti-Adalimumab antibody was specifically reactive to adalimumab (Figure-3) at as low as 0.004 µg/ml whereas the ADA was non-reactive to human IgG. was purified on Protein-A affinity column from generated rabbit anti-adalimumab antisera. The anti-adalimumab antibody purified was further processed over Human IgG coupled column to deplete the anti-human IgG specific antibody. The ADA ELISA reactivity absorbance values for reactivity against adalimumab and human IgG are summarized in Table-7 and -8 respectively.

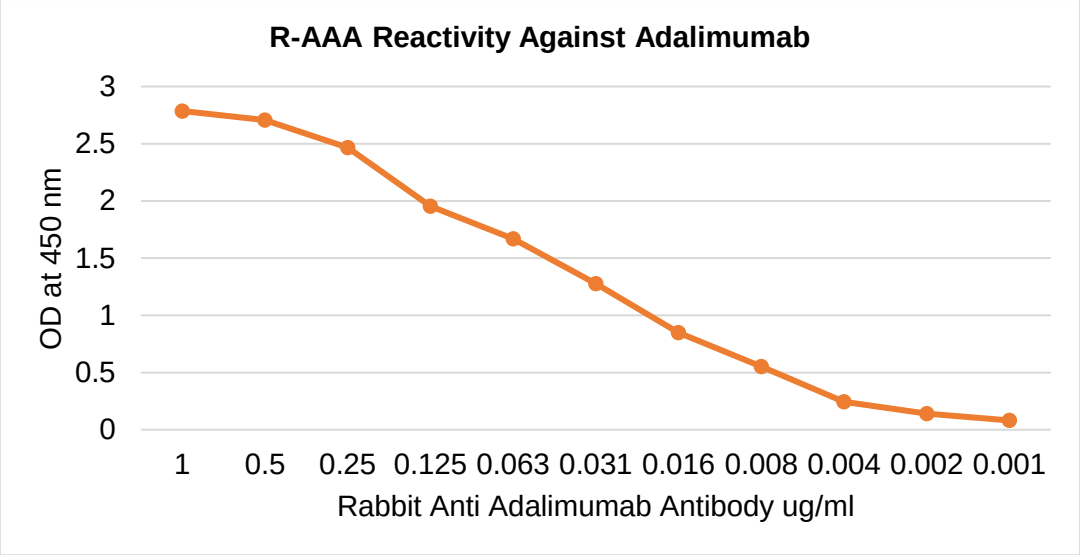

Figure-3: Purified Rabbit Anti-Adalimumab antibody concentration on X-axis and reactivity against Adalimumab absorbance at 450nm on Y-axis was plotted.

Table-7: Reactivity of purified Rabbit anti-Adalimumab antibody against adalimumab

Sl No.	Antibody Conc. (µg/ml)	OD at 450nm
1	1.000	2.7863
2	0.500	2.7083
3	0.250	2.467
4	0.125	1.9541
5	0.063	1.6688
6	0.031	1.277
7	0.016	0.8495
8	0.008	0.5527
9	0.004	0.2447
10	0.002	0.1406
11	0.001	0.0809
12	Negative Control	0.0113

Table-8: Cross reactivity of purified Rabbit anti-Adalimumab antibody against human IgG

Sl No.	Antibody Conc. (µg/ml)	OD at 450nm
1	1.000	0.0552
2	0.250	0.0676
3	0.063	0.0765
4	Negative Control	0.0789

The Affinity purified rabbit anti-adalimumab antibody is reactive to adalimumab and has no cross reactivity to Human IgG.

3.2 Bridge ELISA method development

3.2.1 Adalimumab HRP conjugate

Adalimumab HRP conjugate was prepared as per the method described in the section and working dilution was determined by direct ELISA. The data obtained from the direct ELISA showed the adalimumab HRP conjugate is highly sensitive and can be used at >1:32000 dilution (Table-9). Later, the dilution was further optimized for bridge ELISA at 1:50000 and the same was applied for all the method development and validation runs.

Table-9: Determination of working dilution for adalimumab HRP conjugate; the conjugate was serially double diluted and tested on anti-adalimumab antibody coated wells by direct ELISA.

Adalimumab HRP drug Conjugate dilution	OD1 at 450 nm	OD2 at 450 nm	Avg OD	Negative Control OD at 450 nm
1:250	3.261	3.386	3.324	0.005
1:500	3.188	3.351	3.270	0.006
1:1000	3.213	3.449	3.331	0.004
1:2000	3.176	3.405	3.291	0.003
1:4000	3.286	3.321	3.304	0.004
1:8000	3.180	3.204	3.192	0.004
1:16000	3.170	3.113	3.141	0.003
1:32000	2.915	2.984	2.950	0.004
1:64000	2.722	2.648	2.685	0.005
1:128000	2.084	2.322	2.203	0.005
1:256000	1.386	1.440	1.413	0.003
1:512000	0.794	0.840	0.817	0.005
1:1024000	0.499	0.463	0.481	0.003
1:2048000	0.233	0.250	0.242	0.004

3.2.2 Standard curve determination

A full assay range bride ELISA was performed as per the optimized parameters such coating of adalimumab, blocker or diluent buffer, assay matrix (MRD-10), adalimumab HRP conjugate etc., (Table-10) to determine the assay linear range. Anti-adalimumab antibody (ADA) can be reliably detected by Bridging ELISA. A signal response was shown over an ADA concentration range of 12.5 µg/ml to 0.098 µg/ml (Figure-4).

Table-10: Parameters optimized for bridge ELISA

Sl No	Optimization Parameter	Results
1	Coating concentration (Adalimumab)	2.0 µg/ml
2	ADA Standard Concentration points	12.5 µg/ml to 0.0244 µg/ml
3	Adalimumab-HRP dilution	1/50,000
4	Minimal Required Dilution (MRD)	1:10

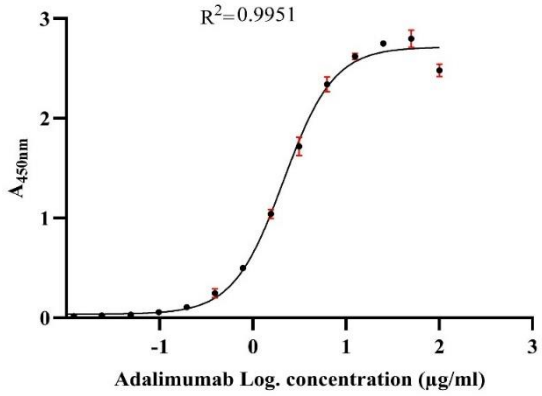

Figure-4: Standard calibrators linear range determination by spiking different concentration of ADA. 4PL analysis showed %RE ≤ 12% for each calibrator at 95% CI. The %CV and %RE were analysed for each standard calibrator and found within the acceptable range as per the EMA guidelines.

3.3 Bridge ELISA method validation

3.3.1 Calibration curve

The calibration curve generated using eight standard calibrators was fitted using GraphPad prism software. A 4PL sigmoidal dose response with variable slope model was adapted for the data analysis. The regression model was accepted as the %RE and %CV of the back-calculated value for at least 75% calibrators, within 20% of nominal concentration. The standard curve was within the acceptance criteria as %RE and %CV of ≤ 20% and total error (Inter and Intra assay) within 30% for all calibrators. The relative error (%RE) of the back-calculated value for 75% calibrator standards should be within ≤20% of nominal concentration, except at the ULOQ and LLOQ where the value should be ≤ 25%. The standard curve passed the acceptance criteria as %RE ≤20% for calibrators as shown in Figure-5.

The analyzed data for assay run within and between the analyst showed %CV of >20%, %RE of >20% and total error >30%, which qualifies the acceptance criteria described by the regulatory agencies.

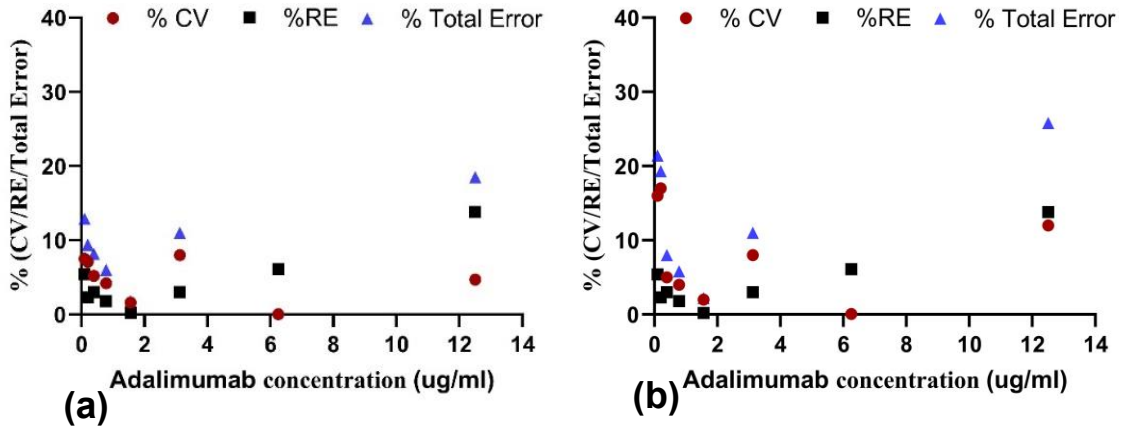

Figure-5: (a) Intra assay (b) Inter assay %CV, %RE and %total error for standard calibrators across all the six validation runs by two analysts over two or more days, analyzed using 4PL at 95% CI.

3.3.2 Precision and accuracy

Precision and accuracy were evaluated across all the six validation runs performed by two analysts. The data were analysed using GraphPad prism software, 4PL sigmoidal dose response with variable slope fitting. The data analysis graphical representation of %CV, %RE and % Total error in figure-6.

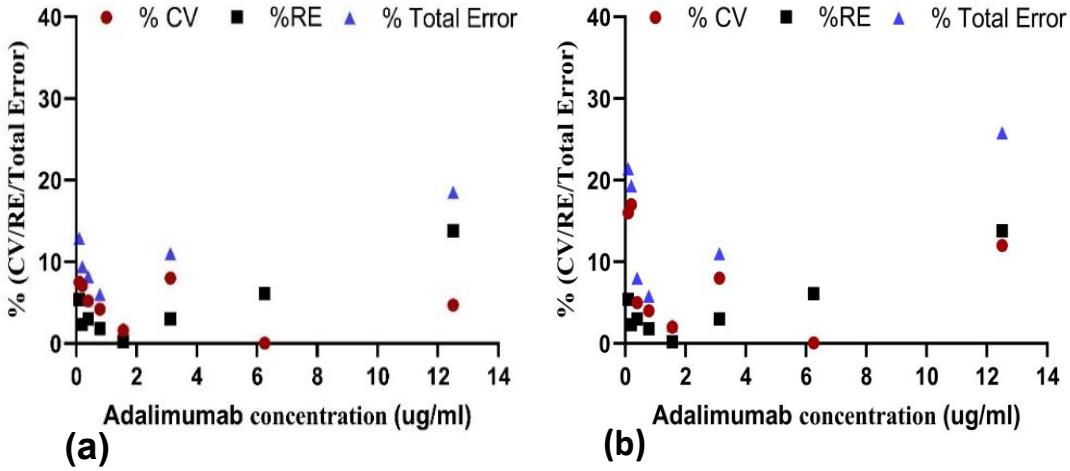

Figure-6: (a) Intra assay (b) Inter assay %CV, %RE and %total error for quality controls across all the six validation runs by two analysts over two or more days, analyzed using 4PL at 95% CI.

Accuracy (%RE) of all QC samples was within 20% across all the runs. Inter-assay and pooled intra-assay precision (%CV) of each QC sample was ≤ 20%. The total error was within 30%

(40% at ULOQ and LLOQ). The relative error (%RE) of all QC samples was within 20% across all batches. Inter-assay and cumulative intra-assay precision (%CV) of each QC sample was ≤ 20%. The total error was within 30% indicating the assay to be robust.

The inter-assay and intra-assay precision (%CV) of each QC sample were ≤20%. The total error was within 30% (≤40% at ULOQ and LLOQ). The obtained results showed that the developed immunogenicity bridge ELISA to screen ADA against rituximab was accurate and precise and passed the acceptance criteria set by the EMA guidelines.

3.3.3 Cut-off value

The assay cut-off was assessed using ADA negative controls. The mean value of negative control absorbance plus two standard deviations (Mean + 2SD). The value above and below the cut-off absorbance (0.0101 nm) is considered as a positive and negative for the ADA respectively (Table-11).

Table-11: cut-off value determination for ADA assay

Sample ID	OD 1 at 450 nm	OD 2 at 450 nm	AVG OD	SD	2SD	Cut-off Value
NC1	0.0067	0.0065	0.007	0.001	0.0027	0.0101
NC2	0.0091	0.0075				
NC3	0.0054	0.0057				
NC4	0.0092	0.0065				
NC5	0.0086	0.009				
NC6	0.007	0.008				
PC1 (10 ug/ml)	2.871	2.8542	2.8626	0.0267		
PC2 (10 ug/ml)	2.831	2.8942				

3.3.4 Determination of drug tolerance

The assay can tolerate the presence of 0.156 ng/ml adalimumab drug in presence of 10,000 ng/ml of ADA, and the ADA was reliably quantified by this assay (Table-12). The drug tolerance (cut-off) was calculated by; *Cut-off (Positive)= mean concentration value of negative control + (2 x SD)* when the test sample value was greater than the "cut-off" value, the sample was termed as "POSITIVE" for the presence of ADA and the test sample value lesser than the "cut-off" value, the sample was termed "NEGATIVE" for the presence of ADA at given concentration of free adalimumab. The calculated cut-off value in the experiment was analyzed to be 0.014. The test samples having an absorbance value greater than the cut-off value show a positive response for the presence of ADA and lower than the cut-off value indicating no ADA.

Table-12: Drug tolerance ELISA data analysis

Sample ID	Adalimumab Drug (µg/ml)	ADA (µg/ml)	OD at 450 nm	Result
DL1	20.000	10000.00	0.013	< cutoff
DL2	10.000	10000.00	0.013	< cutoff
DL3	5.000	10000.00	0.008	< cutoff
DL4	2.500	10000.00	0.007	< cutoff
DL5	1.250	10000.00	0.011	< cutoff
DL6	0.625	10000.00	0.011	< cutoff
DL7	0.313	10000.00	0.013	< cutoff
DL8	0.156	10000.00	0.413	> cutoff
DL9	0.078	10000.00	1.517	> cutoff
DL10	0.039	10000.00	2.223	> cutoff
DL11	0.020	10000.00	2.134	> cutoff
DL12	0.010	10000.00	2.343	> cutoff
DL13	0.005	10000.00	2.367	> cutoff
DL14	0.002	10000.00	2.342	> cutoff
PC (ADA)	0.00	10000.00	2.369	
NC	0.00	0.00	0.009	
NC (Mean)	0.009			
NC (SD)	0.002			
NC+2(SD)	0.014			
Cut-off	0.014			

4. Discussion

Antibody- and protein-based biopharmaceuticals, together with related biological agents, have gained prevalence in recent years. The U.S. Food and Drug Administration (FDA) has recently approved the 100th monoclonal antibody (mAb) product. In 2020, the U.S. FDA approved 13 monoclonal antibodies for therapeutic application. Therapeutic monoclonal antibodies exhibit a strong and consistent mechanism of action. By focusing on a specific structure, they demonstrate significant therapeutic efficacy and a favorable safety and tolerability profile, rendering them appropriate for various applications and a broad patient demographic (Pendley, Schantz, and Wagner 2003; Y. Chen et al. 2023; Baker 2004). Nonetheless, factors associated to drugs and patients influence immunogenic potential and may elicit an immune response to the biological agent, resulting in the production of anti-drug antibodies (ADA). Immunogenic reactions can modify drug dynamics and kinetics, resulting in modifications to safety and efficacy. Nonetheless, numerous obstacles exist with conventional methods for immunogenicity assessment (Guan et al. 2024; Meade et al. 2024).

Serum concentrations of adalimumab exhibit a good correlation with clinical remission and mucosal healing (Mazor et al. 2014; D.-Y. Chen et al. 2015; Radstake et al. 2009; Karmiris et al. 2009b). Presently, healthcare expenditures are predominantly influenced by the costs of medications, specifically anti-TNF biologics (Karmiris et al. 2009b; Bian, Ferrante, and Gils 2017). The immunological response to a biopharmaceutical, such as a monoclonal antibody (mAb) drug, can be identified through the assessment of anti-drug antibodies (ADAs) (van der Valk et al. 2014; Breedveld 2000; Weiner 2006; M.-S. Kim et al. 2007; Lázár-Molnár and Delgado 2016). The presence of anti-drug antibodies (ADAs) is a recognized contributor to the therapeutic failure of monoclonal antibodies (mAbs), such as adalimumab, which target and decrease tumor necrosis factor alpha (TNF-α). This can occur via the formation of drug-ADA complexes, leading to the neutralization of the drug's efficacy. These effects have led to a growing demand for sensitive, precise, and reliable ADA tests (Kessler, Goldsmith, and Schellekens 2006; Niels Vande Casteele et al. 2013; Krishna and Nadler 2016). A multitude of methodologies now exists for the detection, measurement, and characterization of ADAs (Suh, Kyei, and Hage 2022a).

Immunocapture using LC-MS offers several benefits, including the ability to determine antibody isotypes and analyze multiple classes of antibodies in a single assay (Huang et al. 2021; Suh, Kyei, and Hage 2022b; L.-Z. Chen, Roos, and Philip 2016; Duan et al. 2012). In recent years, several investigations have utilized immunocapture with LC-MS to identify anti-drug antibodies (ADAs) against monoclonal antibody (mAb) based treatments. An alternative method to achieve this is by indirectly measuring ADAs(Neubert et al. 2008; Jiang et al. 2014; Suh, Kyei, and Hage 2022a). One significant drawback of using an indirect immunocapture-LC-MS approach for ADA detection is the inability to determine ADA isotypes (Huang et al. 2021).

A bridging ELISA employs two binding agents, such as the target drug, to interact with distinct region on an ADA molecule (Wadhwa et al. 2015; N. Vande Casteele et al. 2012; Patton et al. 2005; Mi et al. 2016). One of these binding agents is captured to the ELISA plate and utilized to capture the ADA. Next, the second binding agent, which includes an enzyme label, is added into the mixture. It binds to the captured ADA and generates a signal that can be detected (Wadhwa et al. 2015). This approach exhibits greater tolerance towards variations in the concentration of the circulating drug compared to both the direct and indirect assay formats (N. Vande Casteele et al. 2012). The bridging ELISA is presently the predominant format used for detecting ADAs, owing to its numerous advantages (J. S. Kim et al. 2015; Brickelmaier et al. 1999; Bourdage et al. 2007; Feldman et al. 2003).

A bridge ELISA method for the detection of binding anti-adalimumab antibodies (ADA) in human serum was developed and validated according to published recommendations by EMA. The rabbit anti-adalimumab antibody was developed and characterized for its specific reactivity to adalimumab and no cross reactivity with human IgG. This was achieved by depleting the Rabbit anti-adalimumab against CN-Br coupled human Ig-G column, where antibodies specific to human IgG Fab and Fc were captured and eluted adalimumab specific antibodies. The purified rabbit anti-adalimumab antibody was utilized as a positive control antibody in bridge ELISA. The adalimumab HRP drug conjugation was prepared and tested for its suitability as detection reagent for this ELISA. The optimized ADA assay is robust and sensitive, as it can reliably detect the 0.0244 µg/mL of the anti-adalimumab drug antibody (ADA) in human serum at MRD-10, which exceeds generally accepted ranges of ADA in serum for antibody assays in clinical studies which is much lower detection level than the adalimumab available in the circulating blood in the body during adalimumab therapy. The immunogenicity assay is validated for its accuracy, precision, drug tolerance, sensitivity and assay cut-off value. The %CV, %RE and %Total error were estimated within the recommended acceptance criteria of ≤20%, ≤20% and ≤30% respectively at CI 95% and R^2 value >0.98. The calculated drug tolerance for the ADA in this assay of 0.156 µg/ml is far above the peak serum concentration of the drug reached after an injection of adalimumab i.v. in patients. Nonetheless, further studies may be warranted, as a drug tolerance assessment using the ADA can only partly mimic drug interference in true clinical samples since the individual ADA repertoire may vary.

Overall, this assay has appropriate performance characteristics, which should be further assessed for suitability in clinical trials and clinical routine testing. This assay can be used for testing for the presence of anti-Adalimumab antibodies in patient samples prospectively collected after start of therapy with Adalimumab and compared with the results from bioassays used for the detection of ADA. The assay will also help to address prediction of immunogenicity. Improvement in methods and a deeper understanding of immunogenicity may ultimately enable us to link ADA formation to a clinical loss of response, thus improving clinical care, and reducing treatment costs.

5. Conclusions

The precise identification and surveillance of ADAs are essential for enhancing biologic treatments and controlling immunogenicity. The bridging ELISA has been proven to be particularly efficient among the many approaches due to its robustness, high sensitivity, and tolerance for variations in drug concentration. The newly designed bridging ELISA for anti-

adalimumab antibodies exhibits outstanding performance, capable of detecting low ADA levels (0.0244 µg/mL) and conforming to rigorous EMA validation standards.

Funding

This work was not funded by any government or non-government agencies.

Ethics approval

The study did not require ethical approval.

Credit authorship contribution statement

Dinesh Kumar Saini developed the research idea; Dinesh Kumar Saini, Manjunath S. Devaramani, Hemalakshmi S and Syeda Zuhin Tabassum performed and analyzed experiments; Siddesha Jalahalli Mariswamy, Kiran Kumar Mudnakudu Nagaraju, Radhakrishna Shetty, Dinesh Kumar Saini, and Manjunath S. Devaramani, contributed to the design of the study, evaluation of the results, and writing of the manuscript. All authors have read and approved the manuscript.

Acknowledgments

The authors thank JSS Academy of Higher Education & Research (Deemed to be University), Mysuru, and Denovo Biolabs Pvt Ltd, Bengaluru for providing laboratory facilities and resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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