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Development and validation of enzyme-linked immunosorbent assays for the measurement of infliximab and anti-drug antibody levels

Dong Xiang [a,1], Ninghong Li [a,b,1], Lu Liu [a], Hengyi Yu [a], Xiping Li [a], Tinghui Zhao [a], Dong Liu [a,**], Xuepeng Gong [a,*]

[a] Department of Pharmacy, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430030, China
[b] Department of Pharmacy, Nanchang First Hospital, Nanchang, 330008, China

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ABSTRACT

Infliximab and its *anti*-drug antibody (ADA) serum concentrations exhibit a strong correlation with clinical response and loss of response. The use of therapeutic drug monitoring to measure the concentration of infliximab and ADA can facilitate clinical decision-making, helping patients attain optimal therapeutic effects. However, there are still limitations to the existing infliximab and its ADA detection methods. Therefore, this study aimed to develop and validate enzyme-linked immunosorbent assay (ELISA)-based methods for measuring infliximab and its ADA levels in human plasma according to the general recommendations for immunoassays. Free infliximab is bound by recombinant TNF-α and detected using HRP-labeled *anti*-human antibody. The ADA is captured by on-plate-coated infliximab and recognized by biotin-labeled infliximab. Two bridging ELISA assays were developed and after assay optimization and validation, these assays have been applied in ten patients with inflammatory bowel disease (IBD). In infliximab detection assay, a standard curve ranging from 0.10 μg/mL to 8.0 μg/mL with great precision and accuracy has been established. Drug tolerance of the ADA assay was that 100 ng/mL ADA could tolerate at least 5.0 μg/mL infliximab in the plasma using a commercially available monoclonal *anti*-infliximab antibody as the positive control. The ADA screening and confirmatory assays achieved a sensitivity of 36.74 ng/mL and 37.15 ng/mL, respectively. All other assay characteristics met the requirements. The mean concentration of infliximab in eight patients with IBD was 7.88 (1.87–21.1) μg/mL, and the ADA levels were all negative. Moreover, the concentrations of infliximab in the remaining two patients were below the LLOQ and the ADAs were positive. Thus, accurate and sensitive ELISA methods have been developed and validated for the detection of infliximab and its ADA concentrations and have been successfully applied to clinical therapeutic drug monitoring.

* Corresponding author.
** Corresponding author.
E-mail addresses: liudong@tjh.tjmu.edu.cn (D. Liu), xue-penggong@tjh.tjmu.edu.cn (X. Gong).
[1] These authors have contributed equally to this work and share first authorship.

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Abbreviations

ADA	Anti-drug antibody
BSA	Bovine serum albumin
CCP	Confirmatory cut-point
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
HRP-*anti*-human IgG	Horseradish peroxidase-conjugated goat *anti*-human IgG (Fc specific) antibody
HMSA	Homogenous mobility shift assay
HQC	High quality control
HPC	High positive control
IBD	Inflammatory bowel disease
LLOQ	Lower limit of quantification
LPC	Low positive control
LQC	Low quality control
MPC	Middle positive control
MQC	Middle quality control
MRD	Minimal required dilution
OD	Optical density
PBS	Phosphate buffered saline
QC	Quality control
RT	Room temperature
SCP	Screening cut-point
SCPF	Screening cut-point factor
SERS	Surface enahanced raman spectroscopy
TNF-α	Tumor necrosis factor-α
ULOQ	Upper limit of quantification

1. Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn's disease, is characterized by chronic inflammation of the gastrointestinal system and is increasing in incidence and prevalence worldwide [1]. Tumor necrosis factor-α (TNF-α), a driver of inflammation, plays a critical role in the development and progression of IBD, and *anti*-TNF-α therapies have been the first-line treatment of IBD for more than two decades [2]. Infliximab, a chimeric IgG1 monoclonal antibody against TNF-α, is the first biotherapy drug approved by the U.S. Food and Drug Administration (FDA) in 1998 and has been widely used for the treatment of IBD as well as other inflammatory diseases [3].

Around 60 % of individuals with IBD initially exhibit a response to infliximab, which later diminishes [4]. The formation of *anti*-drug antibody (ADA) to infliximab, which bonds to and neutralizes infliximab, mainly results in the loss of response in IBD patients [5]. Additionally, high inter-individual clinical response variation has been observed [6], resulting in substantial challenges when treating IBD patients. Traditional management of patients receiving biological drugs is mainly based on empirical. Clinical symptoms are often employed to assess whether a dose adjustment is necessary. Drug switching is exclusively considered for patients exhibiting consistent non-responsiveness, potentially leading to a delay in disease treatment. With the in-depth knowledge and extensive application of therapeutic drug monitoring, the concentration-based strategy has become more personalized and effective [7].

The fundamental requirements for performing therapeutic drug monitoring are the availability of dose-response curves and methods for measuring drug concentrations. Several studies have demonstrated that the level of infliximab has a strong impact on IBD patients' response to treatment [8–10]. Low or undetectable trough concentrations of infliximab are associated with a reduction in drug effectiveness, while sufficient infliximab concentration is associated with sustained remission and treatment response [8,10]. Because low and undetectable trough concentrations are often a result of increased infliximab clearance caused by the formation of ADA, monitoring of ADA has clinical significance [11]. The combination of infliximab and its ADA concentration data in treatment can aid clinical decision-making.

Several methods could be used to measure the concentrations of infliximab and *anti*-infliximab antibodies, including enzyme-linked immunosorbent assay (ELISA) [12], homogeneous mobility shift assay (HMSA) [13], radioimmunoassay [14], and surface enahanced raman spectroscopy (SERS) [15]. The reagents for radioimmunoassay are radioactive and pose significant health risks to humans. HMSA combines ligand binding assay and size-exclusion chromatography and its reproducibility is low [16,17]. SERS is known to offer superior sensitivity and multiplexing ability, but unstable SERS substrates and poor reproducibility are drawbacks [18,19]. None of the above methods are suitable for clinical testing. ELISA has emerged as the most preferred method in therapeutic drug monitoring of biological agents owing to its swiftness, sensitivity, ease of use, and uncomplicated equipment [20].

In China, the number of IBD patients reached about two million by the end of 2017, and the incidence rate is still increasing every year [21]. However, no establishment methods or commercial kits are available to detect blood infliximab and its ADA concentrations

in clinics. Additionally, the published assays for infliximab and its ADA detection have limitations, including low sensitivity, high price, and poor drug resistance. In this study, we aim to develop reliable and sensitive ELISA-based methods for the determination of infliximab and its ADA concentrations in plasma samples, and apply these methods to plasma samples from Chinese patients with IBD.

2. Materials and methods

2.1. Reagents

Infliximab (Remicade®, 100 mg/vial) was purchased from Janssen Biotech, Inc. (Horsham, PA, US). Horseradish peroxidase-conjugated goat *anti*-human IgG (Fc specific) antibody (HRP-*anti*-human IgG) and Tween-20 were obtained from Sigma-Aldrich (Saint Louis, MO, USA). EZ-Link Sulfo–NHS–LC-Biotin was purchased from Thermo Fisher Scientific (Waltham, MA, USA). HRP-conjugated streptavidin was purchased from Jackson ImmunoResearch Inc. (West Grove, PA, USA) and 3, 3′, 5, 5′-Tetramethylbenzidine substrate (TMB) was obtained from SeraCare (Milford, MA, USA). Recombinant human TNF-α was purchased from Sino Biological (Beijing, China). *Anti*-infliximab monoclonal antibody (Isotype: IgG1; Code: HCA213) was purchased from Bio-Rad (Hercules, California, USA). Bovine serum albumin (BSA), phosphate buffered saline (PBS) and 1 mol/L Tris-HCl were obtained from Sangon Biotech Co., Ltd (Shanghai, China).

2.2. Reagent preparation

Pooled blank human plasma samples were obtained by mixing the blank plasma of 50 drug-naive individuals and used as the negative control. Infliximab stock solution was obtained by dissolving 10 mg infliximab lyophilized powder in sterile distilled water (10 mg/mL) and stored at −80 °C. Calibration standard and quality control (QC) samples were prepared by diluting the stock solution with pooled blank human plasma. Concentrations of calibrators were 0.050, 0.10, 0.25, 0.50, 1.0, 2.0, 4.0, and 8.0 μg/mL, and concentrations of QCs were 0.10, 0.30, 1.5, 6.0, and 8.0 μg/mL for the lower limit of quantification, low QC, medium QC, high QC, and upper limit of quantification (LLOQ, LQC, MQC, HQC, and ULOQ), respectively.

As to the ADA detection, positive control samples were prepared by spiking the *anti*-infliximab monoclonal antibody into pooled blank human plasma. The concentrations of low, medium and high positive control (LPC, MPC, and HPC) were 55.0, 500, and 1000 ng/mL, respectively. Moreover, the infliximab stock solution was incubated with a 20-fold molar ratio of Sulfo–NHS–LC-Biontin for 2 h on ice to obtain a 1.5 mg/mL biotinylated-infliximab solution.

2.3. Infliximab assay

The infliximab assay was carried out using a standard sandwich ELISA method, with a recombinant TNF-α protein serving as the capture protein. Briefly, the 96-well ELISA plates (Thermo Fisher, Waltham, MA, USA) were coated with 0.2 μg/mL recombinant human TNF-α and left for overnight incubation at 4 °C. After five washes with PBS-T (this method was used while cleaning the plates), non-specific binding was blocked for 2 h at room temperature (RT) by adding 200 μL/well blocking buffer (PBS with 1 % BSA and 0.05 % Tween-20). After washing, 100 μL of standards, QCs, and unknown samples were added with a 1:100 dilution in blocking buffer and shaken at 500 rpm for 2 h at RT. After washing the plates, HRP-*anti*-human IgG was added with a 1:18,000 dilution, and then incubated for 1 h at RT. After further washing, 100 μL/well TMB substrate was added and incubated for 20 min at RT. The color reaction was stopped by adding 100 μL 1 M H_2SO_4. The optical density (OD) value of all samples was read at 450 nm and 650 nm with a plate reader (Biotek, VT, USA).

2.4. ADA assay

A bridging ELISA was designed to detect *anti*-infliximab antibodies with the use of the drug for both capture and detection. Infliximab was diluted to 1 μg/mL in PBS and coated on 96-well plates overnight at 4 °C. After washing, the remaining protein binding sites were blocked for 2 h at RT by blocking buffer. Positive control, negative control, and unknown samples were diluted 1:20 in 300 mM acetic acid and incubated at RT for 15 min to dissociate immune complexes. The plate was once again washed and then 45 μL of Tris-BSA (1 mol/L Tris-HCl containing 0.5 % BSA) with or without containing 20.0 μg/mL infliximab was added to each well followed by adding 100 μL of the acid-dissociated plasma sample. After washing, 0.5 μg/mL biotinylated-infliximab solution was added and incubated for 1 h at RT, and then washed. HRP-conjugated streptavidin at a 1:40,000 dilution in 1 % (w/v) PBS-BSA was added to each well. After incubation for 30 min at RT and washing, TMB substrate was added and incubated for 20 min to visualize the enzymatic reaction. Finally, 1 M H_2SO_4 was used to stop the reaction and the OD values were measured at 450 nm.

2.5. Validation procedures

Infliximab and ADA assay validation were performed under the current FDA guidelines with minor modifications [22,23]. The following assay characteristics were tested to evaluate the infliximab assay performance and reliability of results: accuracy, precision, dilution linearity, prozone effect, selectivity, specificity, and stability of infliximab under different storage temperatures. As to the ADA assay, screening cut-point (SCP), confirmatory cut-point (CCP), establishment of LPC concentration, assay sensitivity, precision, selectivity, drug tolerance, and stability of positive control under different storage temperatures were tested and validated. The

calibration standard and QC samples were run in duplicate, and the mean was calculated. Results were discarded if the difference between the two values was greater than 20 %. Accuracy was accessed as the percentage difference (bias,%) between measured and nominal concentrations. Precision was evaluated as the coefficient of variation (CV, %).

2.6. Blood sample collection

These assays were applied to measure infliximab and its ADA concentrations in ten IBD patients treated with infliximab. All patients were on maintenance infliximab therapy at the recommended dose of 5 mg/kg every 8 weeks. Blood samples were collected on the 14th week after administration and before the next infusion. Patient samples were centrifuged (3500 *g*, 10 min, 4 °C) within 2 h after collection. The plasma was then transferred to Eppendorf tubes and stored at −20 °C until analysis. The committee on the Medical Ethics of Tongji Medical College affiliated with Tongji Hospital, Huazhong University of Science and Technology authorized this study (TJ-IRB20220302) and written informed consent was obtained from all patients.

3. Results

3.1. Infliximab assay optimization and validation

3.1.1. Infliximab assay optimization

During the development of the infliximab assay, the following parameters were evaluated through three QC levels: concentration of antigen for coating, time of coating, particular blocked solutions, time of blocking, incubation time, the concentration of HRP-conjugated IgG, and sample minimum required dilution (MRD). The signal/noise ratio between QC and negative control samples was calculated for assay optimization. Firstly, recombinant TNF-α coating concentrations at 0.050, 0.10, and 0.20 μg/mL were estimated under different HRP dilutions. Fig. 1A showed that the signal/noise ratio was optimal when the coating concentration at 0.20 μg/mL and HRP with a 1:180,000 dilution were selected. Secondly, QC and blank samples were diluted by 1:20, 1:50, and 1:100 in the blocking buffer for the optimization of sample MRD. As shown in Fig. 1B, the signal/noise ratio was the highest when the MRD was 1:100. Other conditions, such as particular blocked solutions, time of blocking, and incubation time were tested and had no significant effects on the signal/noise ratio.

3.1.2. Calibration curve

Six independent assessment runs were conducted for at least three different days. The ODs obtained from the calibration curves were fitted to a four-parameter logistic model to generate an *S*-shaped standard curve using Gen5 software (Biotek, VT, USA). The correlation coefficient (R^2) shows the goodness-of-fit of the curve. A typical curve graph was shown in Fig. 2. $R^2 \geq 0.996$ was obtained for each calibration curve. The accuracy biases for all the calibration curves were between −8.86 % and 11.23 %, with the highest CV of 14.89 % at all tested concentration levels.

3.1.3. Precision and accuracy

The five levels of LLOQ, LQC, MQC, HQC, and ULOQ (0.10, 0.30, 1.5, 6.0, and 8.0 μg/mL) were used to assess the precision and accuracy of the assay. Six independent analysis runs were evaluated on at least three different days by two analysts. Each plate contained three QC batches and one calibration curve. The precision and accuracy results were summarized in Table 1. The results of within-run and between-run precision and accuracy were less than 20.0 % and the method total error was below 30.0 % for all QC samples, which met the requirements.

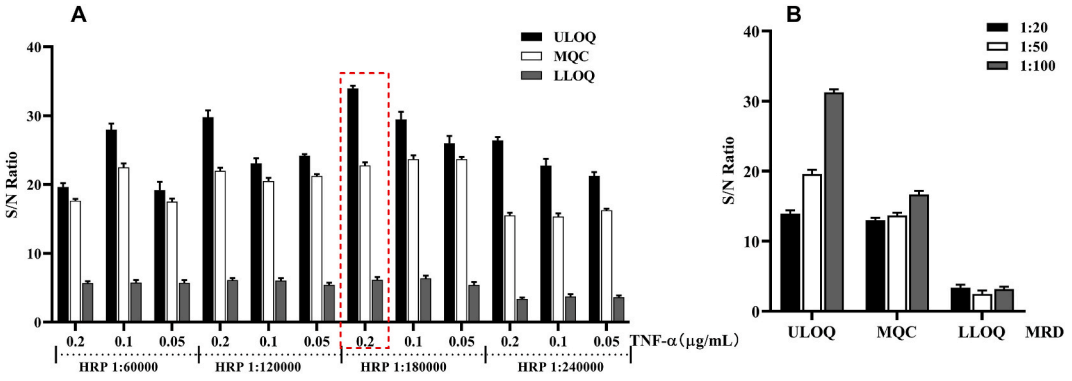

Fig. 1. Optimization of different recombinant TNF-α coating concentrations **(A)**, HRP dilutions **(A)**, and MRD **(B)**. The signal/noise (S/N) ratio between QC and negative control samples was calculated for assay optimization. Abbreviations: HRP, horseradish peroxidase-conjugated goat *anti*-human IgG antibody; ULOQ, the upper limit of quantification; MQC, medium quality control; LLOQ, the lower limit of quantification.

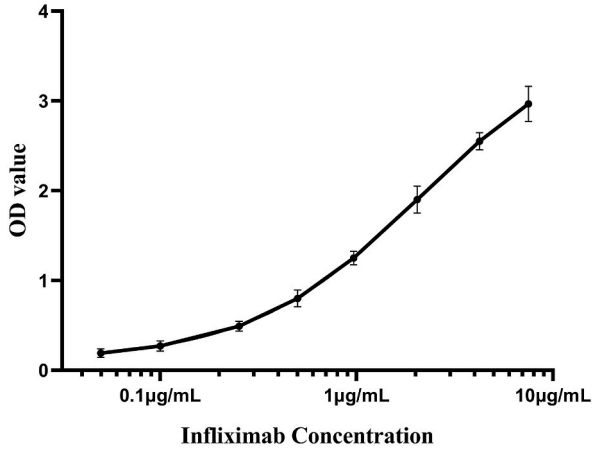

Fig. 2. Typical calibration curve for infliximab assay. A four-parameter logistic curve was produced using different infliximab concentrations ranging from 0.10 μg/mL to 8.0 μg/mL. The equation of this calibration curve was OD value = 3.221+(0.1016–3.221)/(1+[concentration/2144.077]$^{1.011}$), $R^2 = 0.996$.

Table 1
Precision and accuracy data for the infliximab assay.

	Measured concentration (μg/mL)		Precision (%CV)		Accuracy (Bias%)		Total error (%CV+%Bias)	
	within -run	between -run	within-run	between-run	within-run	between-run	within -run	between -run
ULOQ	8.26 ± 0.46	7.48 ± 0.88	4.49	11.83	3.29	−6.43	7.78	18.26
HQC	6.66 ± 0.09	6.49 ± 0.38	2.75	5.90	11.05	8.24	13.80	14.14
MQC	1.52 ± 0.03	1.59 ± 0.05	1.26	3.35	1.31	5.81	2.57	9.16
LQC	0.31 ± 0.004	0.32 ± 0.02	0.57	5.67	4.45	6.31	5.02	11.98
LLOQ	0.10 ± 0.006	0.11 ± 0.007	3.73	7.77	1.58	7.81	5.31	15.58

Abbreviations: LLOQ, the lower limit of quantification; LQC, low quality control; MQC, medium quality control; HQC, high quality control; ULOQ, the upper limit of quantification.

3.1.4. Dilution linearity and prozone effect

The dilution linearity test is essential for ensuring reliable and precise evaluation of samples with concentrations that exceed the linearity limit through dilution. Ultra-high QC samples (1000 μg/mL) were diluted in pooled blank human serum at ratios of 1:20, 1:50, 1:200, 1:333, 1:1,250, 1:2,500, 1:5,000, and 1:20,000. Three batches of dilution linearity samples were independently prepared. The bias of diluted samples at each concentration was within ±11.57 % and the precision across all dilutions ranged from 0.15 % to 5.15 %. Besides, the OD values of samples above ULOQ were all higher than ULOQ, thereby indicating that no prozone effect in our assay, even with the concentration of infliximab up to 50.0 μg/mL.

3.1.5. Selectivity and specificity

Selectivity and specificity were assessed by analyzing ten drug-naive plasma samples, five haemolysed plasma samples, and five lipaemic plasma samples. Each sample was spiked at the LLOQ and ULOQ concentrations. The results showed that the OD values of the blank were below that of LLOQ and the deviation from the nominal concentration at the LLOQ and ULOQ levels did not exceed ±25 % in at least 80 % of the samples evaluated.

3.1.6. Stability

To evaluate the stability of infliximab under different storage conditions, LQC (0.30 μg/mL) and HQC (6.0 μg/mL) samples were tested after 4 freeze-thaw cycles and storage at RT for 24 h or at 4 °C for 72 h. The long-term stability of infliximab was also measured after storing plasma samples at −20 °C and −80 °C for up to 3 months. Three batches of each control at storage conditions were

Table 2
Stability of infliximab under different storage conditions.

	RT for 24 h		4 °C for 72 h		4 freeze-thaw cycles		−20 °C for 3 months		−80 °C for 3 months	
	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV	%Bias	%CV
LQC	−0.11	1.96	−4.80	5.88	0.11	1.52	−2.56	2.04	1.39	1.35
HQC	8.66	5.71	8.60	5.46	4.88	3.88	−0.54	2.75	−0.19	2.66

Abbreviations: RT, room temperature; LQC, low quality control; HQC, high quality control.

prepared. The results showed that infliximab was stable at all examined conditions at LQC and HQC levels, with bias within ±8.66 % and CV less than 5.88 % (Table 2).

3.2. ADA assay optimization and validation

3.2.1. ADA assay optimization

Before beginning the validation of the ADA assay, the ideal assay conditions and key assay parameters were evaluated. Key reagents such as infliximab coating concentration, biotinylated-infliximab concentration, streptavidin-labeled HRP dilution, and sample MRD were mainly optimized with at least three different levels. The results showed that the best assay conditions could be obtained when the infliximab coating concentration was 1.0 μg/mL, the biotinylated-infliximab was 0.5 μg/mL, the dilution of streptavidin-labeled HRP was 1:40,000, and the sample MRD was 1:20 (Supplementary Fig. 1 and Supplementary Table 1). Other parameters such as blocking time and incubation time were tested and had little effect on the signal/noise ratio.

3.2.2. SCP and CCP

In the ADA screening assay, an unknown sample would be regarded as "putative positive" for the presence of ADA if its response is higher than the SCP. Screening positive samples need to be reconfirmed in a confirmatory assay to avoid false positive results. Putative positive samples can be affirmed as true positive if their signal inhibition rates are higher than the CCP [24].

A normalization factor, known as the screening cut-point factor (SCPF), was calculated to reduce variability between assays, plates, or analysts, and the floating SCP value of each plate was evaluated by the following equation: SCP=SCPF × mean response of negative control. The SCPF and CCP were evaluated by analyzing 54 drug-naive individual plasma samples in duplicates with or without the presence of infliximab according to the ADA assay steps. Screening and confirmatory tests were conducted on the same plate. A total of eighteen independent runs were completed by two analysts on at least three separate days, and each individual plasma sample yielded six data points. Therefore, a total of 324 data points were generated for the calculation of SCPF and CCP, respectively.

In the screening test, the signal/noise ratio was calculated by dividing the mean OD value of individual samples by the mean OD value of the negative control samples in each plate. The SCPF was obtained by calculating the 95 % percentile of the signal/noise ratios of all individual samples. In the confirmatory test, the percent inhibition was calculated based on the percent of signal reduction using the following equation: %inhibition=(1-sample mean OD with drug/sample mean OD without drug) × 100 %. The CCP was obtained by calculating the 99 % percentile of the percent inhibitions of all individual samples. The calculation formulas percentile (array, 0.95) and percentile (array, 0.99) in Microsoft Office Excel (version 2010) were used to calculate SCPF and CCP, respectively. As shown in Fig. 3A and **B**, the calculation results of SCPF and CCP were 1.16 and 13.48 %, respectively.

3.2.3. Assay sensitivity and establishment of LPC concentration

The assay sensitivity is the lowest concentration at which the *anti*-drug positive control consistently produces a positive result. To find the lowest concentration at which the signal reaches the cut points, *anti*-infliximab positive control was spiked into blank human plasma at 1000 ng/mL and diluted with pooled plasma to yield samples at concentrations ranging from 100 to 6.25 ng/mL. All diluted

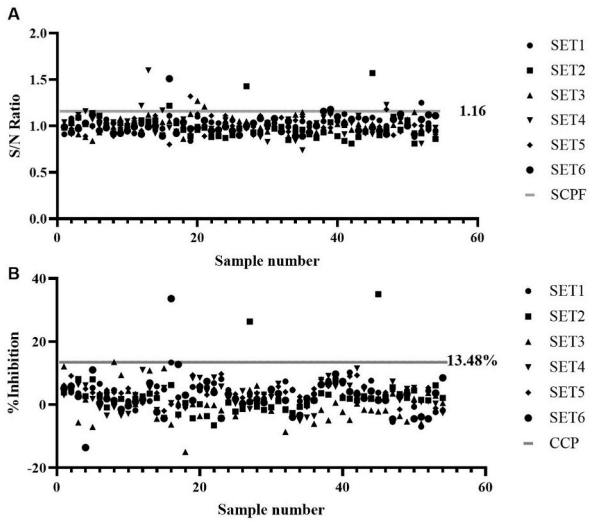

Fig. 3. Establishment of SCPF **(A)** and CCP **(B)** for ADA assay. A total of 54 drug-naive individual plasma samples were tested by two analysts in eighteen runs of screening assay and confirmatory assay performed on the same plates. Each individual plasma generated six data points. A total of 324 data points were generated to calculate SCPF and CCP, respectively. The calculation formulas percentile (array, 0.95) and percentile (array, 0.99) in Microsoft Office Excel (version 2010) were used to calculate SCPF and CCP, respectively. SCPF = 1.16, CCP = 13.84 %. Abbreviations: SCPF, screening cut-point factor; CCP, confirmatory cut-point.

samples were analyzed in both screening and confirmatory assays by two operators in four plates. Each plate contained three individual dilution curves for each of the two assays. The two-point linear regression model was used to fit these dilution curves. The concentrations of the positive control at SCP and CCP were determined to be 20.93 and 24.32 ng/mL, respectively. Hence, the assay sensitivity was calculated to be 36.74 ng/mL and 37.15 ng/mL with 95 % confidence for the screening and confirmatory assays, respectively.

The LPC was prepared by spiking *anti*-infliximab positive control into blank human plasma at a concentration that was statistically determined to give a 1 % assay rejection rate. The LPC was calculated by the following formula: LPC concentration = the mean concentration of positive control at the SCP + $t_{0.01}df \times$ SD of the mean concentration of positive control at the SCP. Where *df* is the estimated total degree of freedom, *SD* is the standard deviation of the sensitivity measurements, and *t* is the distribution. As a result, the LPC was assessed to be 55.0 ng/mL to obtain a 1 % assay rejection rate.

3.2.4. Assay precision and selectivity

The intra- and inter-assay precisions were evaluated. The negative control, LPC, MPC, and HPC (0, 55.0, 500, and 1000 ng/mL) were assessed with six plates and six independent batches of each plate. All plates were performed by two operators for at least three days. The intra-run and inter-run precisions of screening and confirmatory assays were shown in Table 3 and CVs at each positive control level were within 11.21 %.

Plasma samples from ten drug-naive human plasma samples, five haemolysed plasma samples, and five lipaemic plasma samples were chosen and spiked with the *anti*-infliximab antibody at the LPC and HPC levels. The spiked samples were analyzed along with their corresponding unspiked samples. No plasma samples were above SCP or CV $\geq$ 20.0 %.

3.2.5. Drug tolerance

The presence of infliximab in serum affects the sensitivity of the ADA assay. Specifically, ADA-drug complexes formed by ADA and infliximab prevent the detection of ADA in the test format. Evaluation of assay sensitivity in the presence of interfering infliximab, also known as assay drug tolerance, is crucial for understanding the applicability of the ADA detection method [25].

To assess drug tolerance, we deliberately added 100 ng/mL of *anti*-infliximab positive control to blank plasma in the absence or presence of increasing amounts of infliximab (0.16, 0.31, 0.63, 1.25, 2.5, 5.0, 25.0 μg/mL). Fig. 4 shows that even in the presence of 5.0 μg/mL infliximab, the OD value of the sample was still higher than SCP, suggesting that the presence of 5.0 μg/mL drug could be tolerated by our assay. It is sufficient to detect 100 ng/mL ADA in the majority of infliximab-treated individuals.

3.2.6. Stability

The stability of the *anti*-infliximab positive control was determined after 4 freeze-thaw cycles and storage at RT for 24 h, at 4 °C for 72 h and at −80 °C for 3 months. Three individual batches were prepared for LPC and HPC samples. The results showed that the *anti*-infliximab antibody can be stable under the above conditions, with no significant changes in OD values compared to newly generated samples (Table 4).

3.3. Clinical application

Infliximab and its ADA serum concentrations were measured by the established ELISA assays in 10 Chinese IBD patients (3 women and 7 men, average age 31.1 years) who receiving infliximab treatment. The serum concentrations of infliximab and ADA are shown in Table 5. Of the 10 IBD patients, the infliximab trough concentrations were below LLOQ in 2 patients. The median trough concentration of the other 8 patients was 7.88 (1.87–21.1) μg/mL. Significant variability in infliximab trough concentration was observed. When ADA was detected, 2 patients with infliximab concentrations below the LLOQ were positive for ADA, with ADA titers of 1:4 and 1:32, respectively. The other 8 patients with detectable drug levels were ADA-negative.

4. Discussion

Studies have shown that infliximab and its ADA serum concentrations have been linked with clinical response and loss of response [6,8–10]. Moreover, the clearance of infliximab is affected by many factors, including sex, body mass index, serum albumin concentration, and the use of concomitant immunosuppressive agents, especially ADA levels [6], resulting in large inter-individual variability in clinical effects. Therefore, several national guidelines and expert consensus recommend that infliximab

Table 3
Intra-run and inter-run precisions for the ADA screening and confirmatory assays.

	Screening assay		Confirmatory assay	
	Intra-run precision (%CV)	Inter-run precision (%CV)	Intra-run precision (%CV)	Inter-run precision (%CV)
NC	3.59	11.21	3.63	4.64
LPC	1.41	7.18	2.38	8.29
MPC	5.31	9.21	0.34	0.80
HPC	1.52	0.21	0.11	0.26

Abbreviations: NC, negative control; LPC, low positive control; MPC, medium positive control; HPC, high positive control.

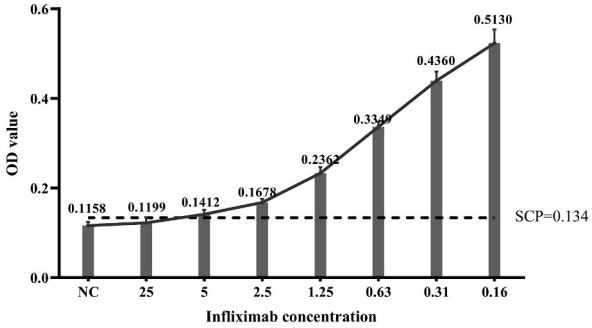

Fig. 4. Drug tolerance of the ADA assay. Different quantities (0.16–25.0 μg/mL) of infliximab were incubated with 100 ng/mL *anti*-infliximab positive control for 1 h at RT. After that, the samples were measured in an ADA screening assay.

Table 4
Stability of the *anti*-infliximab antibody at different storage conditions.

	RT for 24 h (%CV)	4 °C for 72 h (%CV)	4 freeze-thaw cycles (%CV)	−80 °C for 3 months (%CV)
HPC	0.67	0.02	0.16	1.22
	0.32	0.19	0.61	0.74
	0.88	0.10	0.82	0.97
LPC	9.09	0.23	0.39	0.74
	2.60	1.98	0.90	1.45
	0.56	1.17	0.73	1.07

Abbreviations: LPC, low positive control; HPC, high positive control; RT, room temperature.

Table 5
The serum levels of infliximab and ADA in 10 IBD patients.

Number	Gender	Age (years)	Infliximab concentration (μg/mL)	ADA titer
1	female	45	5.02	Negative
2	female	17	2.03	Negative
3	female	18	14.49	Negative
4	male	36	13.25	Negative
5	male	24	7.88	Negative
6	male	17	1.87	Negative
7	male	37	7.87	Negative
8	male	30	21.10	Negative
9	male	54	<0.1	Positive (1:4)
10	male	33	<0.1	Positive (1:32)

concentrations should be monitored to optimize patient treatment [26–29]. However, the main difficulty in therapeutic drug monitoring of biologics is the establishment of a reliable detection assay. Although several methods for the detection of infliximab and its ADA have been reported, there are still some shortcomings. In this study, we established sensitive, accurate, and specific ELISA methods to assess infliximab and its ADA levels in the human plasma.

ELISA methods can be improved by altering the assay parameters to increase the sensitivity or specificity, provide results faster, and boost the signal strength [30]. Typically, an optimization procedure is developed to improve the ratio between the signal of the positive and negative control samples, as well as the difference to the control conditions [31]. We optimized various parameters, such as reagent type and concentration, sample MRD, and incubation time in these two ELISA methods. The results showed that the concentration of coating antigen and HRP-conjugated IgG, the MRD of the sample in the infliximab detection assay, and the concentration of coating drug, biotinylated-drug, and streptavidin-labeled HRP and the MRD of the sample in the ADA detection assay were the key parameters with the greatest effect on the signal/noise signal. Generally, increasing reagent concentration enhances the signal/noise ratio, but also increases the background signal. Low sample dilutions result in high matrix interference, while high dilutions cause low response. Hence the appropriate reagent concentration and sample MRD need to be chosen.

In the infliximab detection assay, sufficient accuracy and precision within a wide calibration range of 0.10–8.0 μg/mL were obtained. Our method has a low LLOQ of 0.10 μg/mL, which is relatively low compared with other immunological methods with an LLOQ of 0.4–2 μg/mL [13,32,33]. In addition, the LLOQ of our method is much lower than the trough concentration of around 2–5 μg/mL reported in most studies in patients receiving the recommended infliximab regimen [34,35]. The prozone or hook effect is a common issue in immunological assays, where high levels of antibodies can cause saturation of antigen and form precipitation, resulting in a false negative [36]. However, in our assay, the prozone effect was absent, even at infliximab concentrations up to 50.0 μg/mL. The

matrix effect is often reported while analyzing the samples using an ELISA-based assay [37]. Various substances in the plasma matrix, such as free hemoglobin (hemolysis), and lipids (lipemia), will not interfere with the measurement of infliximab. Our method is a cheaper, simpler, and more practical option for clinics compared to previously reported methods, such as HMSA, SERS, and radioimmunoassay [16,18,19].

Evaluating ADA levels can be a challenge because of the potential drug interference and varying ADA characteristics, such as isotypes and affinities [38]. Drug interference can lead to undetectable or inaccurate quantification of ADA levels, making it difficult to assess its effect on pharmacokinetic parameters and clinical relevance [39]. The drug tolerance of an assay could be improved by using several sample pretreatment steps, such as acid dissociation. Several studies have shown the beneficial effects of acid dissociation on ADA assays, including enhanced drug tolerance and increased detection of ADA in patients [40]. In this study, our ADA assay was able to tolerate high concentrations of infliximab by acid dissociation using acetic acid, and 100 ng/mL ADA positive controls could be confirmed in the presence of at least 5.0 μg/mL infliximab. In contrast, some other reported methods were unable to detect ADA levels in the presence of infliximab [41,42].

The variability of ADA characteristics is another challenge for ADA detection. After exposure, several isotypes of drug antibodies such as IgM, IgE, and IgG would be produced, and different isotypes may have varying affinities for drugs, making it difficult to accurately quantify ADA levels [43]. Current assays for ADA detection are all semi-quantitative, and some present ADA as arbitrary units from an antibody calibrator [44,45], while others use titration values [14]. In this study, we use titer values to represent ADA levels in accordance with FDA guidelines [23]. Furthermore, biotinylated infliximab and streptavidin-labeled HRP were used in our ADA assay, which increased the sensitivity of the assay to 36.74 ng/mL, exceeding the typically acceptable ranges of 250–500 ng/mL in plasma for antibody assays in clinical studies [46].

Low or undetectable trough concentrations of infliximab are associated with the formation of ADA and poor clinical outcomes. Excessive infliximab concentrations, on the other hand, do not improve the treatment efficacy but will increase unnecessary drug costs [44]. By using therapeutic drug monitoring, the trough concentration of infliximab could be adjusted in time to control the drug levels within a reasonable range. The optimal trough concentration range of infliximab is generally considered to be 3–7 μg/mL [7,47]. Of the ten IBD patients included in our study, two patients had infliximab trough levels below the LLOQ with positive ADAs. As confirmed in our study, the presence of ADA is associated with low infliximab trough levels. The remaining eight patients had infliximab trough concentrations ranging from 1.87 to 21.1 μg/mL, and all were negative for ADAs. Our assay is reliable for the measurement of infliximab and ADA concentrations in human plasma samples.

The limitations of our method must be acknowledged. Firstly, it is important to note that the infliximab detection assay has a ULOQ of only 8.0 μg/mL, with some patients' trough concentrations already exceeding this level. Therefore, an improvement of the assay is required to increase the ULOQ level. Secondly, it is worth mentioning that the present method for ADA detection was a bridging format in which the labeled drug is used for both capture and detection. Therefore, the ADA method may have difficulty in detecting the IgG4 isotype as it is mainly monovalent and cannot be detected in bridging formats [43,48]. Thirdly, this study did not develop a neutralization assay, hence, distinguishing non-neutralizing and neutralizing ADA is not feasible. Lastly, the patient sample size in this study is limited, which restricts the analysis of dose-response relationships in Chinese patients and highlights the need for future expansion.

5. Conclusion

In conclusion, the present study has developed and validated two ELISA-based methods for the measurement of infliximab and its ADA concentrations, respectively, in human plasma. Both assays are accurate, precise, sensitive, and specific. A standard curve ranging from 0.10 μg/mL to 8.0 μg/mL was obtained with high precision and accuracy in the infliximab assay. The drug tolerance of the ADA assay was that 100 ng/mL ADA could tolerate at least 5.0 μg/mL infliximab in plasma. The sensitivity of the ADA screening and confirmatory assays was 36.74 ng/mL and 37.15 ng/mL, respectively. Other assay characteristics all met the requirements. These methods were successfully used for therapeutic drug monitoring of infliximab and *anti*-infliximab antibody levels in IBD patients.

Data availability statement

Data will be provided available on request.

CRediT authorship contribution statement

Xuepeng Gong, Dong Liu, and Dong Xiang: designated and supervised the study. Dong Xiang, Ninghong Li: performed the experiments; collected and analyzed data; wrote the manuscript. Lu Liu, Hengyi Yu, Xiping Li, and Tinghui Zhao: contributed to data collection and performed parts of the experiments. All authors reviewed the manuscript.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21858.

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