Research paper

Development and validation of a homogeneous mobility shift assay for the measurement of infliximab and antibodies-to-infliximab levels in patient serum

Shui-Long Wang, Linda Ohrmund, Scott Hauenstein, Jared Salbato, Rukmini Reddy, Patrick Monk, Steven Lockton, Nicholas Ling, Sharat Singh *

Department of Research and Development, Prometheus Laboratories, Inc, 9410 Carroll Park Drive, San Diego, CA 92121, USA

ARTICLE INFO

ABSTRACT

Antibody-based drugs such as infliximab (IFX) are effective for the treatment of inflammatory bowel disease (IBD) and other immune-mediated disorders. The development of antibodies against these drugs may result in unfavorable consequences, including the loss of drug efficacy, hypersensitivity reactions, and other adverse events. Therefore, accurate monitoring of serum drug and anti-drug antibody levels should be an important part of therapy for patients being treated with an antibody-based drug. Current methods for the assessment of anti-drug antibodies and drug levels, involving various bridging ELISA and radioimmunoassay techniques, are limited by their sensitivity, interference, and/or complexity. To overcome these limitations, we have developed a non-radiolabeled homogeneous mobility shift assay (HMSA) to measure the antibodies-to-infliximab (ATI) and IFX levels in serum samples. Full method validation was performed on both the ATI- and IFX-HMSA, and the clinical sample test results were also compared with those obtained from a bridging ELISA method to evaluate the difference in performance between the two assays. Validation of the ATI-HMSA revealed a lower limit of quantitation of 0.012 μg/mL in serum. The linear range of quantitation was 0.029–0.54 μg/mL. The intra- and inter-assay precision was less than 20% of coefficient of variation (CV), and the accuracy (% error) of the assay was less than 20%. In serum samples, ATI as low as 0.036 μg/mL can be measured, even in the presence of 60 μg/mL of IFX in the serum. Sera from 100 healthy subjects were tested to determine the cut point of the assay. ATI-positive samples that had been previously analyzed by using a bridging ELISA from 100 patients were also measured by the new method. There was a high correlation between the two methods for ATI levels ($p < 0.001$). Significantly, the new method identified five false-positive samples from the bridging ELISA method. Validation of the mobility shift IFX assay also showed high assay sensitivity, precision and accuracy. The HMSA method may also be applied to other protein-based drugs to accurately detect serum drug and anti-drug antibody levels.

1. Introduction

Tumor necrosis factor-alpha (TNF-α) plays a pivotal role in the pathogenesis of inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and other autoimmune disorders (Suryaprasad and Prindiville, 2003; Kopylov et al., 2011; Sandborn et al., 2010). Protein-based drugs that block TNF-α

such as infliximab (a human-murine chimeric monoclonal IgG1κ) or adalimumab (a fully human monoclonal antibody) are effective in reducing disease activity of these inflammatory disorders (Tracey et al., 2008). However, over 30% of patients fail to respond to anti-TNF-α therapy, and many who initially respond later require higher or more frequent dosing due to a failure to maintain the initial response, especially in the IBD patient population (Hanauer et al., 2002; Gisbert and Panes, 2009; Regueiro et al., 2007). There is now compelling evidence that demonstrates that the loss of response in these patients is a result of a failure to achieve and maintain adequate therapeutic drug levels in blood and/ or from the formation of anti-drug antibodies (Miheller et al., 2012). Anti-drug antibodies could cause adverse events such as serum sickness and hypersensitivity reactions (Brennan et al., 2010; Emi et al., 2010), and it is hypothesized that their formation may also increase drug clearance and/or neutralize the drug effect, thereby potentially contributing to the loss of response. Moreover, recent data suggest that the standard dosing regimen for TNF-α-blocking drugs may be suboptimal in some IBD patients, and an individualized dosing regimen to achieve therapeutic drug levels may be important to maximize the initial drug response and to maintain remission (Colombel et al., 2012). Therefore, accurate monitoring of serum drug and anti-drug antibody levels should be an important part of therapy for patients being treated with protein-based drugs. While monitoring for serum drug levels and for the formation of anti-drug antibodies are routine components of early drug development and are mandatory during clinical trials (Shankar et al., 2006), these activities have generally not been adopted in clinical practice. This deficiency may be partially explained by technical issues of the available monitoring assays, which limit their utility as part of routine clinical practice.

Current methods for the assessment of anti-drug antibodies and drug levels in serum mainly utilize the bridging ELISA method (Baert et al., 2003) and, occasionally, the radioimmunoassay (RIA) method (Aarden et al., 2008). However, a major limitation of the bridging ELISA methods in measuring anti-drug antibody levels is the inability to accurately detect the antibodies in the presence of the drug in circulation due to cross-interference. Specifically, the circulating drug interferes with the capture of anti-drug antibodies by the same drug initially coated on the ELISA plate, thus limiting the ELISA's ability to detect anti-drug antibodies, resulting in a lower sensitivity for detection in the presence of IFX. Therefore, ELISA methods can only measure anti-drug antibodies accurately when there is no drug in circulation, which significantly limits its clinical utility. The disadvantages of the RIA method are associated with the complexity and safety concerns related to the handling of radioactive material as well as the prolonged incubation time needed to reach equilibrium for proper measurements. Therefore, there is a large unmet medical need to develop a simple and accurate assay that can overcome these limitations and provide clinicians with valuable quantitative measurements that they can then use to optimize the management of patients on biologic therapies. Here, we have developed and validated a novel homogenous mobility shift assay (HMSA) using size-exclusion high-performance liquid chromatography (SE-HPLC) to quantitatively measure

both induced antibodies-to-infliximab (ATI) levels and IFX levels in serum samples collected from IBD patients being treated with IFX.

2. Materials and methods

2.1. Materials

Individual serum samples from healthy controls were obtained from blood bank donors (Golden West Biologics, Temecula, CA). Sera from IBD patients treated with IFX were obtained from residual samples leftover after testing for ATI and IFX levels in our laboratories and the patient information was de-identified. Unless otherwise noted, all reagents and chemicals were obtained from either Thermo Fisher Scientific (Waltham, MA) or Sigma Aldrich Corporation (St. Louis, MO).

2.2. Conjugation of IFX and TNF-α

Commercially-available infliximab (Remicade™, Janssen Biotech, Inc., Horsham, PA) was buffer exchanged with phosphate buffered saline (PBS, pH 7.3) and labeled with AlexaFluor 488 (Life Technology, Carlsbad, CA) following the manufacturer's instructions. Briefly, a reaction mixture consisting of 10 mg of IFX, 154 μg of AlexaFluor 488 dye, and 1 mL 1× PBS (pH 8.0) was incubated in the dark at room temperature (RT) for 1 h with constant stirring. A desalting column was then used to remove free AlexaFluor 488, and the infliximab-AlexaFluor 488 conjugate (IFX-488) was collected. The protein concentration and labeling efficiency of the conjugate was measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The NanoDrop spectrophotometer measures the A_{280} value for the protein concentration and the A_{494} value for AlexaFluor 488 concentration. The approximate molar extinction coefficient of the AlexFluor 488 dye at 494 nm is $71,000 \text{ cm}^{-1}\text{ M}^{-1}$ and the labeling efficiency is calculated as follows:

$$moles\ dye\ per\ mole\ protein$$
$$= \frac{A_{494} \times dilution\ factor}{71,000 \times protein\ concentration(M)}$$

Only those conjugates containing 2 to 3 fluorescent dyes per antibody qualified for the ATI-HMSA.

The procedure for the labeling of recombinant TNF-α (RayBiotech, Inc, Norcross, GA) with AlexaFluor 488 was identical to that used for the labeling of IFX. The molar ratio of TNF-α to fluorescent dye in the reaction mixture was 1:6 and the resulting TNF-α-AlexaFluor 488 conjugate (TNF-488) contained 1–2 dye molecules per TNF-α.

2.3. Internal control (IC) for the HPLC analysis and preparation of IFX-488/IC and TNF-488/IC

Activated AlexaFluor 488 (1 mg) and 4 mL 1 M Tris buffer (pH 8.0) were mixed for 1 h on a magnetic stirrer at RT to block the active site on the dye. The resulting solution was buffer-exchanged with 1× PBS. The blocked AlexaFluor 488 was used as the IC and combined with either IFX-488 or TNF-488 at a molar ratio of 1:1. The resultant IFX-488/IC and TNF-488/IC were used to normalize the labeled IFX and TNF-α in

the reaction mixture used for HPLC analysis. The amount of IFX-488/IC and TNF-488/IC employed for the HPLC analysis was based on the IFX-488 and TNF-488 concentrations only.

2.4. Preparation of calibration standards, quality control samples and procedure for the ATI bridging ELISA

ATI-positive sera were prepared by pooling individual patient serum samples identified as containing high concentrations of ATI and negative for IFX as determined by ELISA method (Baert et al., 2003). In brief, the ATI bridging ELISA is a microplate based, double antigen formatted assay where IFX is coated on the solid phase 96-well plate to capture the ATI from the patient serum samples. The captured ATI is detected through binding to a biotinylated IFX. The amount of bound biotin on the microplate is determined with the addition of a neutravidin-HRP conjugate which transforms the substrate O-phenylenediamine to a chromogenic product that is measured in a microplate reader at 490 nm. In the bridging ELISA, an affinity purified polyclonal rabbit anti-mouse IgG $F(ab')_2$ (Thermo Fisher Scientific, Waltham, MA) is used to generate the standard curve for calculation of the relative amount of ATI in the patient serum sample. In HMSA, the relative amount of ATI in the pooled serum was estimated by comparing the fluorescent intensity of the ATI-IFX488 immune complex in SE-HPLC with a known concentration of IFX-488. The pooled ATI calibration serum was aliquoted and stored at $-70\ ^{\circ}C$. To generate a standard curve, one aliquot of the stock ATI calibration serum was thawed and diluted to 2% with normal human serum (NHS) in HPLC assay buffer ($1\times$ PBS, pH 7.3) to concentrations of 0.006, 0.011, 0.023, 0.045, 0.090, 0.180, 0.360, and 0.720 µg/mL. Three quality control (QC) samples were prepared by diluting the calibration serum in assay buffer with 0.1% BSA to yield the high (0.36 µg/mL), mid (0.18 µg/mL), and low (0.09 µg/mL) control concentrations. Similarly, IFX calibration standards were prepared by serially diluting a stock solution of 93.75 µg/mL in 100% NHS. After serial dilution, each standard was added to the assay plate and diluted with assay buffer containing 0.1% BSA to yield concentrations of 0.03, 0.06, 0.12, 0.23, 0.47, 0.94, 1.88 and 3.75 µg/mL of IFX and final NHS concentration of 4% in the reaction mixture. Three IFX QC samples were prepared by diluting the IFX calibration standard with assay buffer and 0.1% BSA to yield the high (0.63 µg/mL), mid (0.31 µg/mL) and low (0.16 µg/mL) control concentrations.

2.5. Assay procedures

2.5.1. ATI homogenous mobility shift assay (ATI-HMSA)
The assay was prepared in a 96-well plate format. In order to reduce interference from circulating drug, an acid dissociation step was employed. Briefly, a solution containing a 24 µL aliquot of serum sample, 5.5 µL 0.5 M citric acid (pH 3.0), and 10.9 µL HPLC grade water were added to each well and incubated for 1 h at RT to free the ATI in the patient serum samples from other bound proteins. Following the acid dissociation step, 6 µL of a 74 µg/mL IFX-488/IC solution was added and the reaction mixture was immediately neutralized with 27.6 µL of 10x PBS (pH 7.3). The plate was incubated for another hour at RT on an orbital shaker to complete the formation of the immune complexes. The incubated serum

samples were then diluted to a final serum concentration of 2% by pipetting 18.4 µL of each sample solution, 22.6 µL $10\times$ PBS (pH 7.3), and 259 µL HPLC grade water into the wells of a new 96-well plate. In this plate, the first four wells contained, respectively: 300 µL each of HPLC buffer as a blank, aqueous SEC1 column standard (Phenomenex, Torrance, CA) to monitor the resolution of the HPLC column, acid-dissociated 2% NHS, and acid-dissociated 2% NHS with 110 ng IFX-488/IC for calibrating the HPLC system. The next eight wells contained 300 µL each of the ATI calibration standards (0.006, 0.011, 0.023, 0.045, 0.090, 0.180, 0.360, and 0.720 µg/mL) with 110 ng IFX-488/IC for generating the standard curve. The next nine wells contained, respectively, 300 µL each of the three QC controls (high, mid and low) in triplicate with 110 ng IFX-488/IC to establish the precision and accuracy of the assay. The remaining wells were then filled with 300 µL of the prepared patient serum samples. After mixing on an orbital shaker for 1 min at RT, the samples were filtered through a MultiScreen-Mesh Filter plate equipped with a Durapore membrane (0.22 µm; EMD Millipore, Billerica, MA) into a 96-well receiver plate (Nunc, Thermo Fisher Scientific, Waltham, MA). The recovered solutions in the receiver plate were then transferred sequentially to the loading vials of an autosampler at 4 °C in an Agilent Technologies 1200 series HPLC system (Santa Clara, CA). A 100 µL aliquot from each vial was loaded onto a BioSep SEC-3000 column (Phenomenex, Torrance, CA) and the column effluent was monitored by a fluorescent detector at excitation and emission wavelengths of 494 nm and 519 nm, respectively. The chromatography was run at the flow-rate of 1 mL/min for a total of 20 min with $1\times$ PBS (pH 7.3) as the mobile phase. ChemStation Software (Agilent Technologies, Santa Clara, CA) was used to set up and collect data from the runs automatically and continuously. The time needed to process all the calibration standards, controls, and 35 patient serum samples was ~22 h for a single HPLC system.

2.5.2. IFX homogeneous mobility shift assay (IFX-HMSA)
The procedure for the IFX-HMSA was similar to the ATI-HMSA, except that the acid dissociation step was omitted in the preparation of the patient serum samples. IFX spiked in pooled NHS were used as calibration standards. The assays were performed by incubating the TNF-488/IC with serum samples or calibration standards to reach equilibrium. As in the ATI-HMSA method, the reaction mixtures were then filtered and analyzed by the SE-HPLC system.

2.6. Data analysis

Data analysis was performed with the use of a proprietary automated program run on R software (R Development Core Team, Vienna, Austria). Briefly, the R program opened the ChemStation files collected in the entire run's analyses and exported the raw spectra for an experiment of the user's choosing. The program then normalized the spectra, determined the area under each peak, and calculated the proportion of total peak areas shifted to the bound ATI/IFX-488 complexes over the total bound and free IFX-488 peak areas in the ATI-HMSA and in a similar manner for the IFX-HMSA. With these calculated data, a standard curve was generated by fitting a five-parameter logistic curve to the eight calibration samples using a non-linear least squares algorithm. The residual sum of

squares (RSS) was determined to judge the quality of the fit. Using this curve function, the five optimized parameters, and each sample's proportion of shifted area, concentrations for the unknown samples and the control samples (high, mid and low) were determined by interpolation. To obtain the actual ATI and IFX concentration in the serum, the interpolated results from the standard curve were multiplied by the dilution factor. In addition, the ATI values determined in our clinical laboratory are reported as ATI units/mL, where one ATI unit/mL is equivalent to 0.18 μg ATI protein/mL.

2.7. ATI-HMSA and IFX-HMSA assay performance validation

2.7.1. Characterization of the standard curves
Performance characteristics of the ATI-HMSA calibration standards in the concentration range of 0.006–0.720 μg/mL and the three QC samples (high, mid, and low) were monitored over 26 separate experiments, while the performance characteristics of the IFX-HMSA calibration standards in the concentration range of 0.03–3.75 μg/mL and the three QC samples were monitored over 38 separate experiments. Standard curve performance was evaluated by both the coefficient of variation (CV) for each data point as well as the recovery percentage of the high, mid, and low QC controls. Acceptance criteria were defined as CV <20% for each QC sample.

2.7.2. Assay limits determination
The limit of blank (LOB) was determined by measuring replicates of the standard curve blanks across multiple days. The LOB was calculated using the equation: $LOB = Mean + 1.645 \times SD$ (Armbruster and Pry, 2008). The limit of detection (LOD) was determined by utilizing the measured LOB and replicates of ATI or IFX-positive controls that contained a concentration of ATI or IFX that approached the LOB. The LOD was calculated using the equation: $LOD = LOB + 1.645 \times SD_{(low\ concentration\ sample)}$ (Armbruster and Pry, 2008). The lower and upper limits of quantitation (LLOQ and ULOQ, respectively) were the lowest and highest amounts of an analyte in a sample that could be quantitatively determined with suitable precision and accuracy. LLOQ and ULOQ were determined by analyzing interpolated concentrations of replicates of low concentration or high concentration serum samples containing spiked in IFX or ATI. The LLOQ and ULOQ were each defined as the concentration that resulted in a CV <30% and standard error <25%.

2.7.3. Assay precision
Nine replicates of ATI- or IFX-positive controls (high, mid, and low) were run during the same assay to measure intra-assay precision and accuracy. The minimum acceptable CV range was <20% and accuracy (% error) was <25%. Inter-assay precision was determined by running the assay standard and controls by the same analyst on different days and different instruments, followed by three analysts performing the same assay on different days with the same instrument. The minimum acceptable criteria were <20% for CV and <25% for accuracy.

2.7.4. Linearity of dilution
Linearity of the ATI-HMSA and the IFX-HMSA was determined by performing a two-fold serial dilution of an ATI- or an IFX-positive sample to graphically determine the relationship

between the observed and the expected concentrations. Both the R^2 value and the slope of each linear regression curve were calculated to evaluate the linearity of the assays.

2.8. Cut point determination

Serum samples from drug-naïve healthy donors ($n = 100$; Golden West Biologics. Temecula, CA) were analyzed to determine the screen cut point for the ATI-HMSA and IFX-HMSA. We set the cut point to have an upper negative limit of approximately 97.5%. It was calculated by using the mean value of individual samples interpolated from the standard curve plus 2.0 times the standard deviation (SD), where 2.0 was the 97.5th percentile of the normal distribution. Receiver operating characteristic analysis was also used to estimate the clinical specificity and sensitivity for the ATI-HMSA.

3. Results

3.1. HMSA principles

The principles of the ATI-HMSA and the IFX-HMSA are illustrated in Fig. 1A and B, respectively. The ATI-HMSA in Fig. 1A involved incubating an ATI-containing serum sample with IFX-488/IC at RT for 1 h to form IFX-488/ATI immune complexes. At the end of the incubation, the immune complexes and the remaining free IFX-488 were separated by SE-HPLC and the peak areas of the bound IFX-488 and the free IFX-488 were quantified by fluorescence detection. A pooled ATI-positive serum was used as the calibration standard. When serial dilutions of the ATI calibration standard were incubated with IFX-488, dose-dependent immune complexes were formed with concomitant reduction of the free IFX-488, all of which could be resolved by SE-HPLC analysis, as shown in Fig. 2A. Fig. 2B shows the standard curve generated by plotting the data from Fig. 2A. The lowest concentration of ATI in the standard curve was 0.006 μg/mL.

Fig. 1B illustrates the principle of the IFX-HMSA, which is similar to that of the ATI-HMSA. Incubation of the fluorescently labeled TNF-α (TNF-488) with the anti-TNF antibody IFX resulted in the formation of higher molecular weight immune complexes (TNF-488/IFX). The immune complexes and the remaining free TNF-488 were separated and quantified by SEC-HPLC. Purified IFX spiked in NHS at a concentration of 93.75 μg/mL was used as the IFX calibration standard. Using similar methodology to the ATI-HMSA, the immune complexes formed by combining the IFX calibration standards with TNF-488 were separated from the remaining free TNF-488 (Fig. 3A) and a standard curve was generated with the results (Fig. 3B).

3.2. Analytical validation of ATI- and IFX-HMSA

3.2.1. Validation of the standard curve and assay limits
To validate the standard curve, the performance characteristics of the ATI calibration standards within the concentration range of 0.006–0.720 μg/mL were monitored over 26 experiments by multiple analysts using different instruments over different days (Table 1). The mean RSS for the five-parameter fitted curve was <0.001 ($n = 26$) which was significantly better than our acceptability criterion of RSS = 0.01 (Fig. 2B). The error for the back-calculated values of the standards was
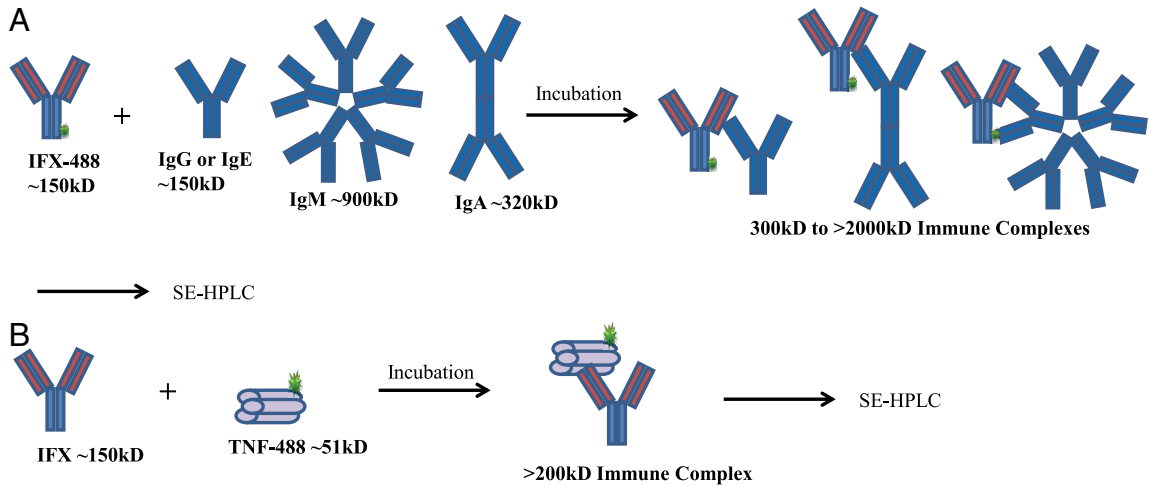
Fig. 1. Schematic illustration of the principles of the (A) ATI-HMSA and (B) IFX-HMSA. In (A), fluorescently labeled IFX (IFX-488; MW ~ 150 kD) is incubated with serum samples containing ATI (MW ~150–900 kD). The newly-formed immune complexes of ATI/IFX-488 have significantly higher MW than the free IFX-488, and can be separated and quantified by SE-HPLC with fluorescent detection. (B) Similarly, fluorescently labeled TNF-α (TNF-488, MW ~51 kD) binds to IFX in serum samples and the newly-formed immune complexes with increased MW of 51 kD to > 200 kD can then be separated and quantified.
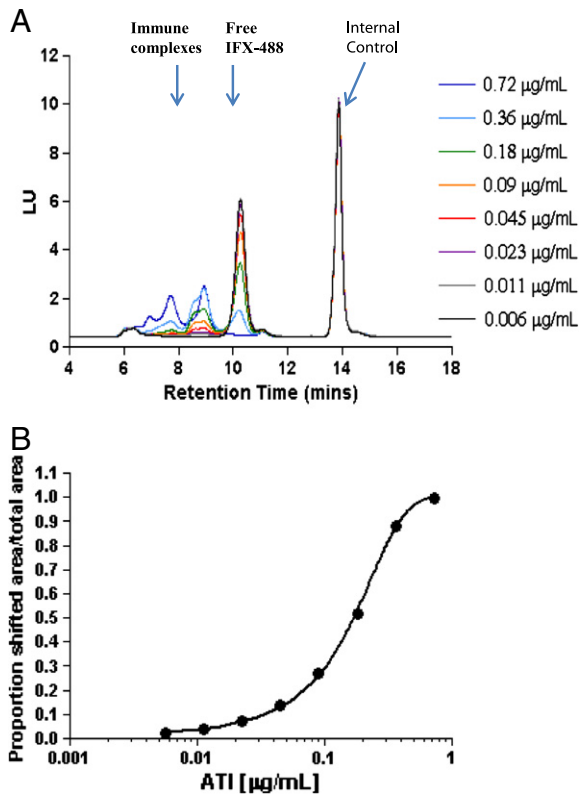

Fig. 2. Overlapping SE-HPLC chromatograms of (A) the ATI calibration standards and (B) the ATI-HMSA-generated standard curve. (A) The greater the amount of ATI calibration standard added to the IFX-488 reaction mixture, the greater the shift of free IFX488 (10–11.5 min) towards the formation of immune complexes (6.7–9.5 min); however there were no changes observed for the internal control (14 min). (B) The standard curve was generated by plotting the ratios of the proportion of the shifted area over total area vs. the concentration of ATI in the reaction mixture. LU = luminescent units.
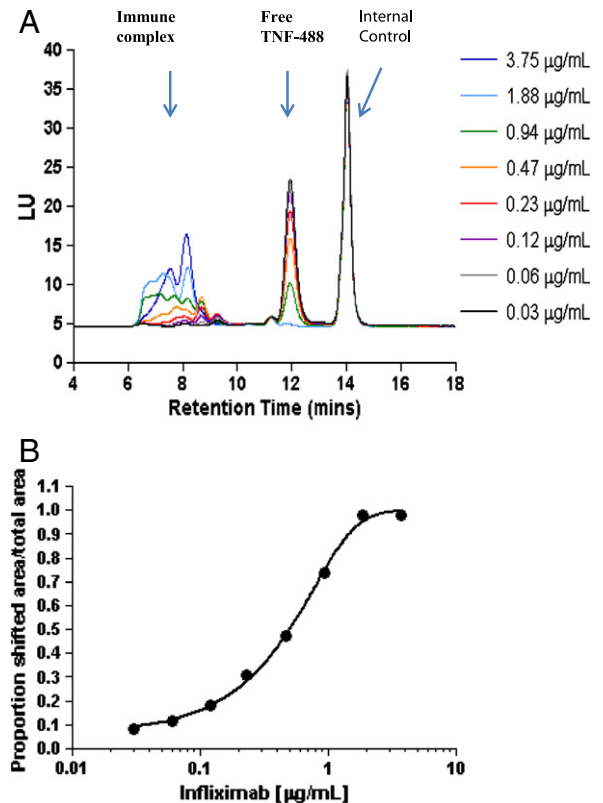
Fig. 3. Overlapping SE-HPLC chromatograms of (A) the IFX calibration standards and (B) the IFX-HMSA-generated standard curve. (A) The greater the amount of IFX calibration standard added to the TNF-488 reaction mixture, the greater the shift of free TNF-488 (11.5–12.5 min) towards the formation of immune complexes (6.7–9.5 min); however no changes were observed for the internal control (14 min). (B) The IFX-HMSA standard curve was generated using the same method as the ATI-HMSA. LU = luminescent units.

Table 1
Characteristics of the ATI-HMSA standard curves.

Standard (μg/mL)	Back calculated concentration (mean, μg/mL)	Error (%)	CV (%)	Normalized ratio (mean)	SD	CV (%)	n
0.720	0.707	1.89	1.06	0.992	0.007	0.74	26
0.360	0.373	3.64	1.33	0.771	0.037	4.83	26
0.180	0.172	4.7	1.54	0.438	0.033	7.53	26
0.090	0.091	0.97	1.58	0.235	0.02	8.54	26
0.045	0.050	11.25	3.2	0.127	0.012	9.51	26
0.023	0.026	16.27	6.2	0.07	0.009	12.77	26
0.011	0.012	2.71	28.71	0.043	0.011	26.77	26
0.006	0.000	92.12	169.83	0.029	0.01	33.01	26
0.000	0.006	N/A	N/A	0.011	0.003	22	60

within 30%, except for the lowest concentration (0.006 μg/mL). The CV was <10% for concentrations above 0.011 μg/mL and the dynamic range of the assay was two orders of magnitude. To establish the LOB, blank samples were tested (negative control, 0 μg/mL) along with the standard curve. The mean proportion value of the shifted area (immune complexes) over the total area determined from the blanks was 0.011 ± 0.003 ($n = 60$). The LOB was thus calculated to be 0.015 (mean $+ 1.645 \times$ SD) and the extrapolated ATI concentration from the standard curve was 0.006 μg/mL (Table 1). To determine the LOD, the extrapolated value of the lowest standard concentration (0.006 μg/mL) was obtained as 0.014 ± 0.003 μg/mL ($n = 26$). The LOD was calculated from the LOB and the SD from the lowest concentration in the standard curve with <30% error: $LOD = LOB + 1.645 \times SD_{(low\ concentration\ sample)}$ which was 0.012 μg/mL. The LLOQ for the ATI-HMSA assay was 0.011 μg/mL, which was determined by the interpolated concentrations of replicates of the low ATI concentration with CV <30%. The ULOQ for the ATI-HMSA assay was 0.54 μg/mL, which was similarly determined by the interpolated concentrations of replicates of the high ATI concentration with CV <20%. The effective serum concentrations corresponding to the LLOQ and the ULOQ for the ATI-HMSA were determined by multiplying the concentration with the dilution factor (50), which corresponded to 0.56 μg/mL and 27 μg/mL, respectively.

The performance characteristics of the IFX-HMSA standard curve in the concentration range of 0.03–3.75 μg/mL were similarly assessed over 38 experiments by multiple analysts using different instruments on different days (Table 2). The same methods were used to determine the LOB, LOD, LLOQ, and ULOQ as described for the ATI-HMSA. The LOB, LOD, LLOQ, and ULOQ for the IFX-HMSA were 0.0027, 0.0074, 0.039, and 1.36 μg/mL,

respectively. The effective IFX serum concentration for the LLOQ and ULOQ were 0.98 and 34 μg/mL (dilution factor = 25).

3.2.2. Assay precision and accuracy

To assess the precision and accuracy of the ATI-HMSA and the IFX-HMSA, two methods were used. First, we used the high, mid, and low QC samples in both assays to determine their recovery rate. As shown in Table 3, the ATI-HMSA intra-assay precision had a CV <4% and the accuracy rate was <12% error. The intra-assay precision and accuracy for the IFX-HMSA were <6% and <10% error, respectively (Table 4). Second, we tested the high, mid, and low control samples over different runs and instruments and by multiple analysts. The inter-assay precision of CV <15% and accuracy of <21% error were within the acceptable limits in both assays (Tables 3 and 4).

3.2.3. Linearity of dilution

To ensure accurate quantitative assessment, the positive samples of the assay must dilute linearly and in parallel with the standard curve. To determine this linearity of dilution, human serum samples containing a high-titer of ATI or a high concentration of IFX were used. The samples were diluted serially 2-fold and tested using the ATI-HMSA and the IFX-HMSA, respectively. The observed values of ATI or IFX were plotted with the expected levels of ATI or IFX in the serum. As shown in Fig. 4, both the R^2 values and the slopes of each linear regression curve for both assays show linearity.

3.2.4. Effects of potential interfering substances

We studied the effects of potential substance interference in both assays by spiking in common endogenous components of human serum and drugs methotrexate (MTX) and

Table 2
Characteristics of the IFX-HMSA standard curves.

Standard (μg/mL)	Back calculated concentration (mean, μg/mL)	Error (%)	CV (%)	Normalized ratio (mean)	SD	CV (%)	n
3.75	2.375	36.658	11.597	0.985	0.009	0.876	38
1.88	2.222	18.528	10.571	0.979	0.010	1.026	38
0.94	0.925	1.305	7.043	0.754	0.071	9.454	38
0.47	0.455	2.928	1.580	0.481	0.059	12.260	38
0.23	0.247	5.396	3.037	0.296	0.040	13.530	38
0.12	0.131	11.509	3.759	0.179	0.028	15.698	38
0.06	0.060	1.617	12.176	0.108	0.024	22.008	38
0.03	0.014	53.745	54.172	0.067	0.020	30.602	38

Table 3
Assay precision of the ATI-HMSA.

	Intra-assay precision ($n=3$)			Inter-assay precision								
				Run to run ($n=5$)			Analyst to analyst ($n=3$)			Instrument to instrument ($n=3$)		
	High	Mid	Low	High	Mid	Low	High	Mid	Low	High	Mid	Low
Expected (µg/mL)	0.36	0.18	0.09	0.36	0.18	0.09	0.36	0.18	0.09	0.36	0.18	0.09
Measured (mean, µg/mL)	0.40	0.18	0.09	0.38	0.18	0.09	0.39	0.18	0.09	0.35	0.17	0.07
SD	0.01	0.01	0.00	0.03	0.01	0.00	0.02	0.01	0.01	0.02	0.01	0.01
CV (%)	1.6	3.46	1.08	6.44	6.14	3.43	4.03	5.12	6.36	4.87	3.94	11.89
Accuracy (% error)	11.85	1.78	5.5	5.18	1.02	3.39	7.04	1.35	2.63	3.63	5.06	20.78

Azathioprine into the three QC samples (high, mid, and low) to determine their percent recovery. As shown in Table 5, no significant interference was observed in the physiological levels of serum substances and typical serum concentration of drugs in the ATI-HMSA and IFX-HMSA as assessed by the recovery of the mid QC samples in the presence of the potential interfering substances because of the recovery values were within ±10% of the mid QC control sample except for the lipemic serum sample at a concentration of 200 mg/mL in the IFX-HMSA and the TNF-α concentration at 250 ng/mL in the ATI-HMSA. TNF-α also had some interference in the IFX-HMSA when the concentrations were over 100 ng/mL because the recovery was greater than ±10% of the mid QC control sample value.

3.3. IFX drug tolerance of the ATI-HMSA

Substantial concentrations of IFX may be present in the serum from patients, even if the blood is drawn at the trough time point. As discussed previously, the presence of IFX in the patient serum significantly affected the quantitative measurement of ATI using the bridging ELISA assay. To address this issue with the HMSA-based assays, we evaluated the potential impact of IFX level in patient serum on ATI-HMSA results by adding increased amounts of IFX (6.6, 20, and 60 µg/mL) to each of the eight ATI calibration standards to assess the effects on the standard curve. As seen in Fig. 5, the ATI-HMSA could detect ATI levels as low as 0.036 µg/mL in the serum sample containing up to 60 µg/mL of IFX, which is much higher than the maximum therapeutic level reached after infusion of the patient with IFX.

3.4. Cut point determinations for the ATI-HMSA and IFX-HMSA

To establish the cut point for the ATI-HMSA and the IFX-HMSA, we screened 100 serum samples collected from IFX drug-naïve healthy subjects for the measurement of ATI and IFX levels. No shifting of the IFX-488 to the bound complex areas was found in most of the samples of the ATI-HMSA (Fig. 6A). The proportion of shifted area over the total area was near the LOB and the mean value of the extrapolated ATI from standard curve (multiplied by the dilution factor) was 0.73 ± 0.23 µg/mL as shown in Fig. 6B. The cut point for ATI was determined by taking the mean value $+2 \times$ SD, which yielded 1.19 µg/mL. Three samples contained ATI levels slightly higher than the cut point, which resulted in a clinical specificity of 97%. The same 100 serum samples were also used to establish the cut point for the IFX-HMSA (data not shown). The calculated cut point for IFX-HMSA was 0.98 µg/mL, yielding a clinical specificity of 95%.

3.5. Clinical validation of the ATI-HMSA

Currently one of the clinically validated methods for measuring ATI is by using bridging ELISA methodology (Baert et al., 2003), which over the last decade has been used to measure ATI in serum samples from IBD patients treated with IFX. To evaluate the performance of the HMSA to detect ATI in the presence of IFX compared to that of the bridging ELISA assay, we performed ATI-HMSA on 100 serum samples obtained from IBD patients that were previously tested to be positive for ATI by the bridging ELISA method. The proportion of shifted area over the total area and the interpolated ATI from the standard curve (multiplied by the dilution factor of 50)

Table 4
Assay precision of the IFX-HMSA.

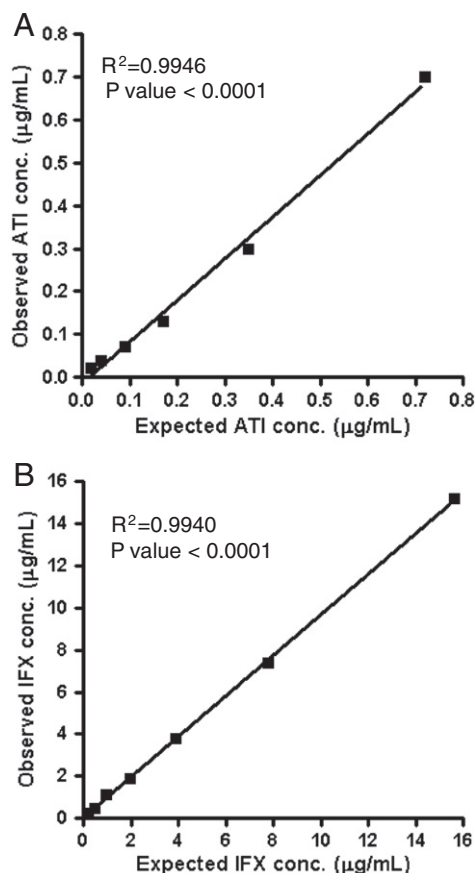|	Intra-assay precision ($n=3$)			Inter-assay precision								
				Run to run ($n=5$)			Analyst to analyst ($n=3$)			Instrument to instrument ($n=3$)		
	High	Mid	Low	High	Mid	Low	High	Mid	Low	High	Mid	Low
Expected (µg/mL)	0.63	0.31	0.16	0.63	0.31	0.16	0.63	0.31	0.16	0.63	0.31	0.16
Measured (mean, µg/mL)	0.63	0.34	0.17	0.64	0.34	0.18	0.62	0.33	0.16	0.64	0.34	0.19
SD	0.01	0.01	0.01	0.03	0.02	0.01	0.06	0.03	0.02	0.03	0.02	0.02
CV (%)	2.30	3.22	5.81	5.01	5.14	8.11	9.41	8.42	13.73	4.85	7.22	11.95
Accuracy (% error)	1.63	9.55	7.11	3.00	9.31	15.40	1.19	5.06	5.05	2.74	7.62	20.03

Fig. 4. Linearity of dilution for the (A) ATI-HMSA and (B) IFX-HMSA. Linearity of the ATI-HMSA and the IFX-HMSA were determined by a 2-fold serial dilution of a high-titer ATI-positive sample and a high concentration IFX-positive sample, respectively. The relationships between the observed and the expected concentrations were plotted. Panels A and B show that the R^2 value and the slope of each linear regression curve demonstrate good linearity.

the sensitivity was 95% (95% CI: 88.72%–98.36%), and the odds ratio was 47.50 when a 1.19 μg/mL cut point was used. Good correlation between the ATI values obtained from the ATI-HMSA and the bridging ELISA was also observed, with $p<0.0001$ and a Spearman r-value of 0.39 (95% CI: 0.2–0.55) as shown in Fig. 8. Upon re-testing the three samples from the healthy controls with the ATI concentration above the cut point (1.196, 1.201, and 1.219 μg/mL) using ATI-HMSA, the resulting ATI concentrations were all below the cut point. Thus we defined these results as false-positive. However, among the 100 ATI-positive IBD patient serum samples previously determined by the bridging ELISA, five of the samples were found to be ATI-negative (i.e., containing ATI concentrations below the cut point of 1.19 μg/mL). Repeatedly re-testing these samples showed no shift on the SE-HPLC chromatogram, thus we defined the five samples as true negative. The increased rate of false-positive ATI measurements with the bridging ELISA method may be attributed to an elevated level of nonspecific binding.

4. Discussion

Since the initial approval of the antibody drug IFX by the United States Food and Drug Administration for the treatment of Crohn's disease (CD) in 1998, the broad use of anti-TNF therapy in IBD has dramatically improved therapeutic outcome over the past decade (Targan et al., 1997; Colombel et al., 2010; Present et al., 1999; Rutgeerts et al., 1999; Hanauer et al., 2002). Nevertheless, there is a significant number of patients that either fail to respond (primary non-responders) or lose response (secondary non-responders) to anti-TNF treatments. There are many factors that may contribute to the loss of response to IFX in IBD patients, such as the development of a complication to the disease or uncontrolled disease activity (Miheller et al., 2012), in addition to the formation of ATI. ATI formation negatively affects drug efficacy by increasing the clearance of IFX and/or neutralizing its activity, therefore reducing the amount of active IFX in circulation (Baert et al., 2003; Hanauer et al., 2004; Farrell et al., 2003; Miele et al., 2004). In contrast, achieving an adequate serum IFX level is not only associated with improved treatment response but also appears to have a lower rate of ATI formation (Maser et al., 2006; Farrell et al., 2003). Thus there is an interdependent relationship between IFX levels and ATI, which underscores the importance of

are shown in Fig. 6C and D, respectively. The mean values of ATI in the patient serum samples were significantly higher than those in the drug-naïve healthy controls (mean ± SD = 9.57 ± 11.43 vs. 0.73 ± 0.29 μg/mL, $p<0.0001$) as shown in Fig. 7A. Receiver operating characteristic curve analysis of these samples (Fig. 7B) showed that the area under the curve was 0.986 ± 0.007 (95% CI: 0.973–0.999, $p<0.0001$),

Table 5
Effects of potential interfering substances in the ATI-HMSA and IFX-HMSA (All recovery values shown are from the mid QC controls of each assay).

| Substance	Typical range	Range tested	ATI-HMSA	IFX-HMSA
IgG	7–16 mg/mL	10 mg/mL	100.58 ± 3.51%	107.89 ± 0.65%
IgM	0.4–2.3 mg/mL	2.0 mg/mL	100.04 ± 0.93%	104.09 ± 0.32%
IgA	0.7–4.0 mg/mL	1.5 mg/mL	99.92 ± 3.34%	105.43 ± 2.09%
Rheumatoid factor	>30 IU/mL (RA patients)	Up to 774 IU/mL	96.22 ± 0.17% (774 IU/mL)	90.57 ± 0.62% (774 IU/mL)
Hemolyzed serum	<20 Hem. Index (HI)	100–300 HI	105.13 ± 3.66% (300 HI)	95.76 ± 0.78% (300 HI)
Lipemic serum	<150 mg/dL triglycerides	100–200 mg/dL	96.91 ± 2.49% (200 mg/dL)	113.70 ± 0.61% (200 mg/dL)
MTX	1.1 mM	2.0 mM	99.59 ± 0.18%	100.90 ± 2.71%
Azathioprine	3.6 μM	10.8 μM	96.27 ± 1.30%	100.49 ± 2.12%
TNF-α	6.2–6.6 pg/mL	0.01–250 ng/mL	110.46 ± 1.26% (250 ng/mL)	91.27 ± 0.90% (100 ng/mL)
TNF-β	100 pg/mL	0.01–250 ng/mL	94.58 ± 1.51% (250 ng/mL)	98.08 ± 0.25% (100 ng/mL)
sTNFR1	1.87–1.97 ng/mL	0.1–1000 ng/mL	99.76 ± 0.61% (1000 ng/ml)	94.32 ± 2.21% (1000 ng/mL)
sTNFR2	4.49–4.66 ng/mL	0.1–1000 ng/mL	96.34 ± 0.88% (1000 ng/mL)	99.20 ± 1.13% (1000 ng/mL)
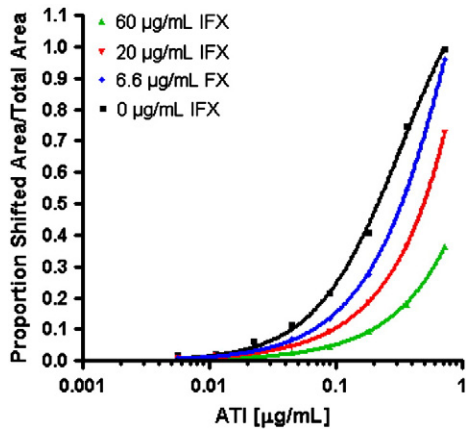
Fig. 5. ATI-HMSA drug tolerance. Interference by IFX in the ATI-HMSA was assessed by adding increasing doses of IFX (6.6, 20, and 60 μg/mL) in each of the eight ATI calibration standards to determine their effects on the generation of the standard curve. The results showed that the ATI-HMSA could detect an ATI level as low as 0.036 μg/mL in the presence of 60 μg/mL of IFX.

clinical study that correlated patient trough serum IFX concentration with response and remission (Maser et al., 2006). Recently, these findings were supported by a study of 115 UC patients where it was found that a detectable trough serum IFX level predicted clinical remission, endoscopic improvement, and a lower risk for colectomy, whereas, an undetectable trough serum IFX level was associated with less favorable outcomes (Seow et al., 2010). This proposed treatment strategy is in contrast to the most commonly used strategies of empirically increasing the dose, shortening the infusion frequency, or switching to another anti-TNF agent such as adalimumab or certolizumab pegol. A growing body of evidence suggests that serial monitoring of serum drug and ADA levels are important in the management and optimization of these therapies and thus may increase the overall response, the duration of response, and minimize adverse effects (Ordas et al., 2012).

Many clinicians have advocated the concurrent measurement of serum ATI and IFX levels in patients treated with IFX or other anti-TNF drugs and, indeed, monitoring of various anti-TNF drugs and their respective antibodies in IBD and RA patients has been studied in several clinical trials using a variety of methods (Miheller et al., 2012; Guerra et al., 2011). Different assay techniques were used to measure the ATI and IFX concentrations in the different trials, which may contribute to the inconsistent results obtained between studies. Many ELISA methods with different formats are available for commercial
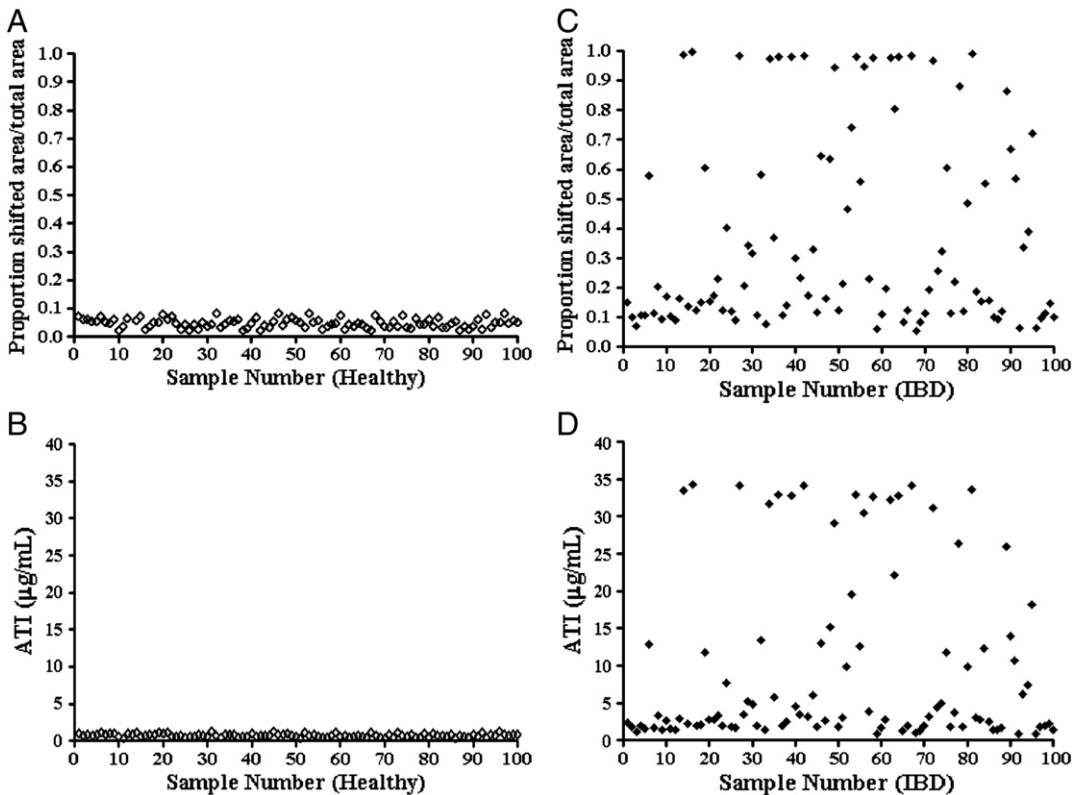
measuring and monitoring both IFX and ATI levels accurately. An evolving concept in the management of IBD patients with biologic therapy involves dose optimization using an individualized dosing regimen versus a standard "one-dose-fits-all" regimen to attain a personalized target therapeutic drug level (Ordas et al., 2012). This concept was demonstrated in a

Fig. 6. Clinical sample test and assay cut point determination for the ATI-HMSA. Serum samples from healthy donors and patients with IBD were analyzed by the ATI-HMSA (cut point value was calculated as the mean value plus 2.0 × SD). Panel A shows individual data from 100 healthy samples on the proportion of shifted area/total area obtained from the analysis. Panel B shows the interpolated ATI values. Panel C shows the individual data from 100 serum samples from patients with IBD on the proportion of shifted area/total area obtained from the analysis. Panel D shows the interpolated ATI values.
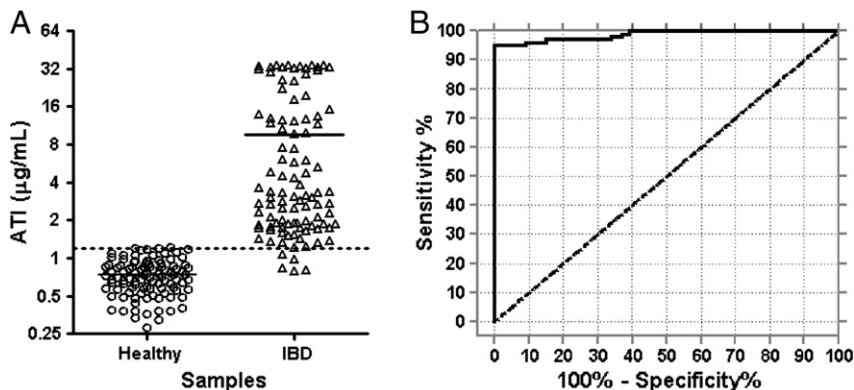
Fig. 7. (A) ATI concentrations in healthy control and IBD patient serum samples determined by the ATI-HMSA. The horizontal dotted line represents the cut point and the horizontal solid line represents the mean, the y-axis scale is Log 2. (B) Plot of the receiver operating characteristic (ROC) curve using data obtained from the ATI-HMSA analysis of healthy control and IBD patient serum samples.

use, but the reliability of these methods may be questionable because there is no standard available for comparison. The most common method for measuring serum ATI is the bridging ELISA as described by Baert et al. (2003). However other ELISA methods have also been described to detect IFX and ATI in serum samples from IBD and RA patients (Bendtzen et al., 2006, 2009; Ben-Horin et al., 2012; Imaeda et al., 2012). Some of these assays appear to be capable of detecting ATI in the presence of low concentrations of IFX, but the ATI-positive rates determined by these methods varied significantly (Kopylov et al., 2011; Imaeda et al., 2012). RIA has also been developed to measure serum ATI and IFX concentrations, and their clinical utility was compared to solid-phase ELISA methods (Wolbink et al., 2006; Bendtzen et al., 2006; Svenson et al., 2007). In general, RIA has some advantages over ELISA with fewer artifacts. However, RIA methodology is more complex compared to ELISA methodology and the use of radioactive materials is a major issue in many clinical labs. Nevertheless, despite the different ATI and IFX results obtained using the various methods, the clinical outcomes from most of the studies were similar, namely: 1) Detectable levels of ATI or high-titer ATI were correlated with low concentrations or undetectable trough levels of IFX, respectively, and 2) patients who were ATI-positive and

possessed low trough levels of IFX had a higher rate of loss of response to IFX treatment.

By taking advantage of homogenous fluid-phase methodology and avoiding the multiple washing steps of the ELISA format, we have developed an HMSA method with the ability to quantitatively measure IFX drug and ATI levels in IBD patient serum samples. This method was based on the incubation of IBD patient serum samples with fluorescent-labeled IFX to detect ATI levels or with fluorescent-labeled TNF-α to detect IFX levels. The immune complexes formed in the incubation mixture were separated from the free label by SE-HPLC and the amount of ATI or IFX in the samples was calculated from the resolved peak areas. A similar but more cumbersome method had been applied to measure the formation, distribution, and elimination of IFX and anti-IFX immune complexes in cynomolgus monkeys by using a radio-labeled monkey anti-IFX IgG to monitor the shifting of the immune complexes in the SE-HPLC (Rojas et al., 2005). The HMSA method overcomes many potential artifacts encountered in the solid-phase ELISA method because the antibody and antigen binding reactions takes place in a homogeneous liquid-phase condition. Also, the solid-phase ELISA method may only be able to detect high affinity antibodies because it involves many steps of washing and incubation that may potentially remove the antibodies bound with low affinity. Further advantages of the HMSA method include the potential detection of all immunoglobulin isotypes and all subclasses of IgG, including IgG_4. Analytical validation of the ATI- and IFX-HMSA showed that the assay performance was robust and not affected by potential interfering substances present in serum. Incorporation of an acid dissociation step during ATI-HMSA dramatically improved the drug tolerance of the assay and allowed for an accurate detection of ATI in the presence of high levels of IFX (up to 60 μg/mL) in serum. The use of fluorescent labeling and fluorescent monitoring of the SE-HPLC peaks significantly increased the analytical sensitivity for measuring ATI, which can reach a concentration of 0.011 μg/mL, compared with the suboptimal concentration of 200–500 ng/mL achieved by bridging ELISA. Re-analysis of clinical samples which had previously tested positive using a bridging ELISA method showed that 5% of them were negative using ATI-HMSA; otherwise, there was good correlation between the two
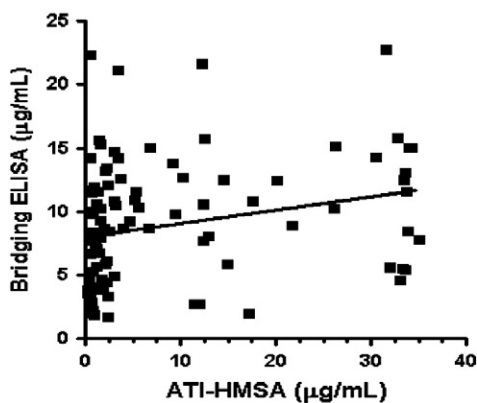

Fig. 8. Correlation of the ATI-HMSA and bridging ELISA on the measurement of ATI in IBD patient serum samples.

assays on the ATI-positive samples. The false-positive rate with the cut point of 1.19 μg/mL was 3%. However, this rate could be reduced by repeating the test if the result is within 10% of the cut point (i.e., 1.19–1.21 μg/mL). Additional patient samples are needed to verify the clinical utility of the ATI- and IFX-HMSA. Because a variety of anti-TNF drugs have been shown to induce antibody formation in clinical studies (Bartelds et al., 2011; Karmiris et al., 2009; Lichtenstein et al., 2010), the HMSA method may be applied to measure other antibody drug levels and anti-drug antibodies in patient serum samples.

In conclusion, the liquid-phase HMSA methodology presented in this paper for the purpose of measuring ATI and IFX in IBD patient serum samples overcomes many limitations encountered in the solid-phase ELISA and RIA methods. Validation of the ATI- and IFX-HMSA also showed higher sensitivity and drug tolerance compared to that achieved by the ELISA method. This liquid-phase HMSA format is a useful platform that can be broadly applied to detect anti-drug antibodies and drug levels for a variety of protein therapeutics during drug development and post-approval monitoring.

Disclosure Statement

Acknowledgments

References

Aarden, L., Ruuls, S.R., Wolbink, G., 2008. Immunogenicity of anti-tumor necrosis factor antibodies-toward improved methods of anti-antibody measurement. Curr. Opin. Immunol. 20, 431.

Armbruster, D.A., Pry, T., 2008. Limit of blank, limit of detection and limit of quantitation. Clin. Biochem. Rev. 29 (Suppl. 1), S49.

Baert, F., Noman, M., Vermeire, S., Van, A.G., D' Haens, G., Carbonez, A., Rutgeerts, P., 2003. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. N. Engl. J. Med. 348, 601.

Bartelds, G.M., Krieckaert, C.L., Nurmohamed, M.T., van Schouwenburg, P.A., Lems, W.F., Twisk, J.W., Dijkmans, B.A., Aarden, L., Wolbink, G.J., 2011. Development of antidrug antibodies against adalimumab and association with disease activity and treatment failure during long-term follow-up. JAMA 305, 1460.

Bendtzen, K., Geborek, P., Svenson, M., Larsson, L., Kapetanovic, M.C., Saxne, T., 2006. Individualized monitoring of drug bioavailability and immunogenicity in rheumatoid arthritis patients treated with the tumor necrosis factor alpha inhibitor infliximab. Arthritis Rheum. 54, 3782.

Bendtzen, K., Ainsworth, M., Steenholdt, C., Thomsen, O.O., Brynskov, J., 2009. Individual medicine in inflammatory bowel disease: monitoring bioavailability, pharmacokinetics and immunogenicity of anti-tumour necrosis factor-alpha antibodies. Scand. J. Gastroenterol. 44, 774.

Ben-Horin, S., Mazor, Y., Yanai, H., Ron, Y., Kopylov, U., Yavzori, M., Picard, O., Fudim, E., Maor, Y., Lahat, A., Coscas, D., Eliakim, R., Dotan, I., Chowers, Y., 2012. The decline of anti-drug antibody titres after discontinuation of anti-TNFs: implications for predicting re-induction outcome in IBD. Aliment. Pharmacol. Ther. 35, 714.

Brennan, F.R., Morton, L.D., Spindeldreher, S., Kiessling, A., Allenspach, R., Hey, A., Muller, P.Y., Frings, W., Sims, J., 2010. Safety and immunotoxicity assessment of immunomodulatory monoclonal antibodies. MAbs 2, 233.

Colombel, J.F., Sandborn, W.J., Reinisch, W., Mantzaris, G.J., Kornbluth, A., Rachmilewitz, D., Lichtiger, S., D'Haens, G., Diamond, R.H., Broussard, D.L., Tang, K.L., van der Woude, C.J., Rutgeerts, P., 2010. Infliximab, azathioprine, or combination therapy for Crohn's disease. N. Engl. J. Med. 362, 1383.

Colombel, J.F., Feagan, B.G., Sandborn, W.J., Van, A.G., Robinson, A.M., 2012. Therapeutic drug monitoring of biologics for inflammatory bowel disease. Inflamm. Bowel Dis. 18, 349.

Emi, A.N., de Carvalho, J.F., Artur Almeida, S.C., Bonfa, E., 2010. Immunogenicity of Anti-TNF-alpha agents in autoimmune diseases. Clin. Rev. Allergy Immunol. 38, 82.

Farrell, R.J., Alsahli, M., Jeen, Y.T., Falchuk, K.R., Peppercorn, M.A., Michetti, P., 2003. Intravenous hydrocortisone premedication reduces antibodies to infliximab in Crohn's disease: a randomized controlled trial. Gastroenterology 124, 917.

Gisbert, J.P., Panes, J., 2009. Loss of response and requirement of infliximab dose intensification in Crohn's disease: a review. Am. J. Gastroenterol. 104, 760.

Guerra, I., Chaparro, M., Bermejo, F., Gisbert, J.P., 2011. Utility of measuring serum concentrations of anti-TNF agents and anti-drug antibodies in inflammatory bowel disease. Curr. Drug Metab. 12, 594.

Hanauer, S.B., Feagan, B.G., Lichtenstein, G.R., Mayer, L.F., Schreiber, S., Colombel, J.F., Rachmilewitz, D., Wolf, D.C., Olson, A., Bao, W., Rutgeerts, P., 2002. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. Lancet 359, 1541.

Hanauer, S.B., Wagner, C.L., Bala, M., Mayer, L., Travers, S., Diamond, R.H., Olson, A., Bao, W., Rutgeerts, P., 2004. Incidence and importance of antibody responses to infliximab after maintenance or episodic treatment in Crohn's disease. Clin. Gastroenterol. Hepatol. 2, 542.

Imaeda, H., Andoh, A., Fujiyama, Y., 2012. Development of a new immunoassay for the accurate determination of anti-infliximab antibodies in inflammatory bowel disease. J. Gastroenterol. 47, 136.

Karmiris, K., Paintaud, G., Noman, M., Magdelaine-Beuzelin, C., Ferrante, M., Degenne, D., Claes, K., Coopman, T., Van, S.N., Van, A.G., Vermeire, S., Rutgeerts, P., 2009. Influence of trough serum levels and immunogenicity on long-term outcome of adalimumab therapy in Crohn's disease. Gastroenterology 137, 1628.

Kopylov, U., Mazor, Y., Yavzori, M., Fudim, E., Katz, L., Coscas, D., Picard, O., Chowers, Y., Eliakim, R., Ben-Horin, S., 2011. Clinical utility of antihuman lambda chain-based enzyme-linked immunosorbent assay (ELISA) versus double antigen ELISA for the detection of anti-infliximab antibodies. Inflamm. Bowel Dis. (Published on line 29 OCT 2011) http://dx.doi.org/10.1002/ibd.21919.

Lichtenstein, G.R., Thomsen, O.O., Schreiber, S., Lawrance, I.C., Hanauer, S.B., Bloomfield, R., Sandborn, W.J., 2010. Continuous therapy with certolizumab pegol maintains remission of patients with Crohn's disease for up to 18 months. Clin. Gastroenterol. Hepatol. 8, 600.

Maser, E.A., Villela, R., Silverberg, M.S., Greenberg, G.R., 2006. Association of trough serum infliximab to clinical outcome after scheduled maintenance treatment for Crohn's disease. Clin. Gastroenterol. Hepatol. 4, 1248.

Miele, E., Markowitz, J.E., Mamula, P., Baldassano, R.N., 2004. Human antichimeric antibody in children and young adults with inflammatory bowel disease receiving infliximab. J. Pediatr. Gastroenterol. Nutr. 38, 502.

Miheller, P., Kiss, L.S., Lorinczy, K., Lakatos, P.L., 2012. Anti-TNF trough levels and detection of antibodies to anti-TNF in inflammatory bowel disease: are they ready for everyday clinical use? Expert. Opin. Biol. Ther. 12, 179.

Ordas, I., Mould, D.R., Feagan, B., Sandborn, W.J., 2012. Anti-TNF monoclonal antibodies in inflammatory bowel disease: pharmacokinetics-based dosing paradigms. Clin. Pharmacol. Ther. 91, 635.

Present, D.H., Rutgeerts, P., Targan, S., Hanauer, S.B., Mayer, L., van Hogezand, R.A., Podolsky, D.K., Sands, B.E., Braakman, T., DeWoody, K.L., Schaible, T.F., van Deventer, S.J., 1999. Infliximab for the treatment of fistulas in patients with Crohn's disease. N. Engl. J. Med. 340, 1398.

Regueiro, M., Siemanowski, B., Kip, K.E., Plevy, S., 2007. Infliximab dose intensification in Crohn's disease. Inflamm. Bowel Dis. 13, 1093.

Rojas, J.R., Taylor, R.P., Cunningham, M.R., Rutkoski, T.J., Vennarini, J., Jang, H., Graham, M.A., Geboes, K., Rousselle, S.D., Wagner, C.L., 2005. Formation, distribution, and elimination of infliximab and anti-infliximab immune complexes in cynomolgus monkeys. J. Pharmacol. Exp. Ther. 313, 578.

Rutgeerts, P., D'Haens, G., Targan, S., Vasiliauskas, E., Hanauer, S.B., Present, D.H., Mayer, L., van Hogezand, R.A., Braakman, T., DeWoody, K.L., Schaible, T.F., van Deventer, S.J., 1999. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. Gastroenterology 117, 761.

Sandborn, W.J., Schreiber, S., Hanauer, S.B., Colombel, J.F., Bloomfield, R., Lichtenstein, G.R., 2010. Reinduction with certolizumab pegol in patients with relapsed Crohn's disease: results from the PRECiSE 4 Study. Clin. Gastroenterol. Hepatol. 8, 696.

Seow, C.H., Newman, A., Irwin, S.P., Steinhart, A.H., Silverberg, M.S., Greenberg, G.R., 2010. Trough serum infliximab: a predictive factor of clinical outcome for infliximab treatment in acute ulcerative colitis. Gut 59, 49.

Shankar, G., Shores, E., Wagner, C., Mire-Sluis, A., 2006. Scientific and regulatory considerations on the immunogenicity of biologics. Trends Biotechnol. 24, 274.

Suryaprasad, A.G., Prindiville, T., 2003. The biology of TNF blockade. Autoimmun. Rev. 2, 346.

Svenson, M., Geborek, P., Saxne, T., Bendtzen, K., 2007. Monitoring patients treated with anti-TNF-alpha biopharmaceuticals: assessing serum infliximab and anti-infliximab antibodies. Rheumatology (Oxford) 46, 1828.

Targan, S.R., Hanauer, S.B., van Deventer, S.J., Mayer, L., Present, D.H., Braakman, T., DeWoody, K.L., Schaible, T.F., Rutgeerts, P.J., 1997. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. N. Engl. J. Med. 337, 1029.

Tracey, D., Klareskog, L., Sasso, E.H., Salfeld, J.G., Tak, P.P., 2008. Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. Pharmacol. Ther. 117, 244.

Wolbink, G.J., Vis, M., Lems, W., Voskuyl, A.E., de, G.E., Nurmohamed, M.T., Stapel, S., Tak, P.P., Aarden, L., Dijkmans, B., 2006. Development of antiinfliximab antibodies and relationship to clinical response in patients with rheumatoid arthritis. Arthritis Rheum. 54, 711.