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Inductively coupled plasma mass spectrometry assay for quantification of free infliximab in serum

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ABSTRACT

TNF antagonists such as infliximab are effective for the treatment of several inflammatory and autoimmune diseases. Recent clinical studies have advocated the importance of measuring trough infliximab levels to guide treatment decisions. We have developed a novel assay for measuring serum free infliximab levels using inductively coupled plasma-mass spectrometry (ICP-MS). The method involves the incubation of patient serum in wells coated with recombinant TNF, followed by detection with lanthanide-labeled monoclonal anti-human IgG1 and ICP-MS analysis. Full method validation was performed and results for clinical samples tested with the new method were compared with those obtained from a capture ELISA and a cell-based assay. Validation of the ICP-MS assay revealed a lower limit of detection of 0.4 µg/mL in serum. The linear range of quantitation was 1–50 µg/mL. The within-run and between-run precision had a coefficient of variation (CV) of < 10%, and the accuracy of the assay had a CV of < 15%. In serum samples, the ICP-MS method was devoid of analytical interferences by high levels of hemoglobin, bilirubin and triglycerides. Serum sample results from 123 drug-naïve donors revealed a test cutoff at 0.5 µg/mL. Test results from clinical samples obtained by the ICP-MS method showed strong correlation with both the ELISA and cell-based assay. The ICP-MS methodology presented in this study is a robust method for measuring TNF antagonist serum levels, which makes it well suited for therapeutic drug monitoring in the clinical laboratory.

1. Introduction

Tumor necrosis factor (TNF) plays a central role in inflammation and the pathogenesis of autoimmune and chronic inflammatory diseases. The introduction of monoclonal antibodies targeting TNF into clinical practice has revolutionized the treatment of rheumatoid arthritis, ankylosing spondylitis, psoriasis, and inflammatory bowel disease (Smolen and Emery, 2011; Talley et al., 2011). Most patients respond favorably to TNF antagonists by showing dramatic improvements of the clinical symptoms, but many others fail to respond or develop clinical relapse after initial favorable response (Ordas et al., 2012). The mechanisms for the development of response failure can be attributed to many factors, including dosing and bioavailability issues, drug-related immunogenicity, and disease-specific differences in the pathogenic role of TNF (Bendtzen et al., 2009).

The benefits of laboratory testing for serum drug concentrations and detection of anti-drug antibodies (ADA) has been long recognized for TNF antagonists such as infliximab. Infliximab (IFX) is a chimeric mouse/human antibody against TNF, in which the variable region is of mouse origin, but the rest of the molecule is replaced by human sequences. Laboratory testing to measure IFX concentration and detect ADA allows for personalized treatment adjustment based on the most likely mechanism responsible for treatment failure. Randomized, controlled trials in Crohn's disease patients developing treatment failure to IFX have shown that laboratory test-guided strategy significantly reduces treatment costs per patient compared to the empirical approach, and enables precise and personalized medical management (Steenholdt et al., 2014). Although testing for serum concentrations of TNF antagonists and detecting ADA are mainly recommended for the diagnostic workup of treatment failure, a recent guideline from the American Gastroenterological Association has made recommendations on therapeutic drug monitoring in IBD (Feuerstein et al., 2017).

In response to clinical need, testing for serum concentration of IFX and other TNF antagonists has been adopted in many clinical laboratories. Most of the assays employed are binding-based methods including solid phase binding platforms such as ELISA (Baert et al., 2003),

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and liquid phase binding such as the HPLC-based homogenous mobility-shift assay (Wang et al., 2012). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Willrich et al., 2015) and a cell-based reporter gene assay (RGA) (Pavlov et al., 2016), are also used by clinical laboratories to measure IFX concentrations. Inductively coupled plasma-mass spectrometry (ICP-MS) is a very sensitive and precise method for absolute protein quantification (Beauchemin, 2006). ICP-MS is a technique that detects mass-to-charge differences in ions produced using high-temperature, inductively coupled plasma with final detection by mass spectrometry (Houk et al., 1980). ICP-MS for quantification offers advantages over other techniques due to several characteristics including high analytical throughput, excellent analytical sensitivity, wide linear analytical measurement range, and high tolerance to matrix effects (Liu et al., 2006). A recently developed technique for metal antibody labelling using polymer-based elemental tags offers an inexpensive alternative for indirect body fluid protein quantification by ICP-MS (Lou et al., 2007). The objective of this study was to develop an ICP-MS method to measure IFX in serum. This paper presents the analytical validation of this method for clinical use and comparison of the results to those obtained by ELISA and RGA.

2. Materials and methods

2.1. Patient samples

De-identified individual serum samples from patients treated with IFX were randomly selected from discarded samples after testing for IFX levels by RGA assay at ARUP Laboratories. The use of de-identified samples for assay validations by ARUP Laboratories was approved by the University of Utah Institutional Review Board.

2.2. Microplate preparation

Flat bottom MaxiSorp high-binding microplates (Thermo Fisher Scientific, Waltham, MA) were incubated overnight at 4 °C with 1 μg/mL of human recombinant TNF (R&D Systems, Minneapolis, MN) and 200 μL/well of 0.05 M phosphate buffer (pH 8.0) (Sigma Aldrich Corporation, St. Louis, MO). Next day the solution was removed, and the plates were dried under vacuum overnight and sealed with desiccant.

2.3. Tracer antibody labeling

Mouse monoclonal anti-human IgG1 (Thermo Fisher Scientific) was chelated with lanthanide Praseodymium-141 using the Maxpar® X8 Antibody Labeling Kit (Fluidigm, San Francisco, CA), following the manufacturer's procedure. Briefly, Maxpar® polymer was pre-loaded with lanthanide, and antibodies were partly reduced with Bond Breaker TCEP Solution (Thermo Fisher Scientific). Purified lanthanide-loaded polymer and purified partially reduced antibody were conjugated for up to 2 h at 37 °C and washed.

2.4. Preparation of assays standards and quality control samples

Assay standards and quality control samples were prepared by diluting commercially available infliximab (Remicade™, Janssen Biotech, Inc., Horsham, PA) in heat-inactivated normal human serum (BioIVT, Westbury, NY).

2.5. Principle of the ICP-MS assay

The principle of the IFX ICP-MS assay is illustrated in Fig. 1A. Briefly, incubation of serum containing IFX with TNF captured in microplate wells results in the formation of IFX-TNF immune complexes. To measure the concentration of IFX, an anti-human IgG1 monoclonal antibody labeled with a lanthanide is added to each well, incubated, and unbound labeled monoclonal antibodies washed out. TNF-IFX-anti-IgG1 immune complexes containing lanthanide tracer are released from microplate wells by acidic buffer, and applied to ICP-MS to measure lanthanide content (Fig. 1B). The concentration of infliximab in the serum is calculated using calibration curve with standards with known IFX concentration.

2.5.1. Assay protocol

Five microliters of each serum sample or IFX standards were mixed with 245 μL of PBST (0.01 M PBS and 0.05% Tween-20, pH 7.4, Sigma Aldrich Corporation), and 25 μL of each of these dilutions added to TNF-coated microplate wells containing 175 μL of sample diluent (ImmunoChemistry Technologies, Bloomington, MN). Microplates were incubated for 1 h at room temperature (RT), and washed on a microplate shaker for 3 cycles with 250 μL of PBST per well. Then, 200 μL of metal-labeled tracer antibody was added to each microplate well and incubated for another 1 h at RT, followed by 3 washing cycles with PBST. To extract lanthanide content, microplates were incubated for 1 min with 200 μL of 1% nitric acid containing iridium as an ICP-MS internal standard for normalization of praseodymium results (Sigma Aldrich Corporation). The content of each microplate well was analyzed by ICP-MS.

2.5.2. Inductively coupled plasma mass spectrometry (ICP-MS) measurement

For calculation of IFX levels, 150 μL of each assay sample was injected into an Agilent 7900 ICP-MS system (Agilent, Santa Clara, CA) using the MVX-7100 μL Workstation autosampler (Teledyne Cetac, Omaha, NE). Samples were injected at a flow rate of 550 μL per minute. Analysis was performed with MassHunter version 4.3, with the collision cell set to Standard mode; a single point peak pattern spectrum was collected. Three replicates, with 15 sweeps per replicate, were conducted for each analysis. Praseodymium, with a mass-to-charge ratio 141, had an integration time of 0.2 s, and the internal standard iridium, with a mass-to-charge ratio 193, had an integration time of 0.1 s.

2.6. Data analysis

Data obtained for standards was plotted, and IFX concentrations for each serum sample were calculated according to the linear approximation of the calibration curve. Confidence intervals for slope, intercept and R-square were calculated as central 90% of corresponding values obtained by resampling data 10,000 times with replacement. Data calculations were performed with R software (R Development Core Team, Vienna, Austria). Performance characteristics of the assay were calculated by EP Evaluator (Data Innovations, South Burlington, VT).

2.7. Free IFX assay performance validation

2.7.1. Analytical sensitivity

Limit of Blank (LOB) was determined by measuring replicates of standard curve blanks during multiple days. The LOB was calculated using the equation: LOB = Mean + 1.645 x SD of blank sample (normal human sera) (Armbruster and Pry, 2008). Limit of Detection (LOD) was determined using the equation: LOD = LOB + 1.645 x SD of low concentration samples (replicates of IFX-positive approaching the LOB) (Armbruster and Pry, 2008).

2.7.2. Assay precision

Three samples of normal human sera were spiked with different concentrations of IFX (high, medium, and low) and run in duplicates in 3 consecutive days, plus another day with 4 replicas each. Average results from each of the four days, for each of the 3 samples were used to calculate the coefficient of variance (CV) for between-run precision. Results from replicates of 4 tested on the same run were used to

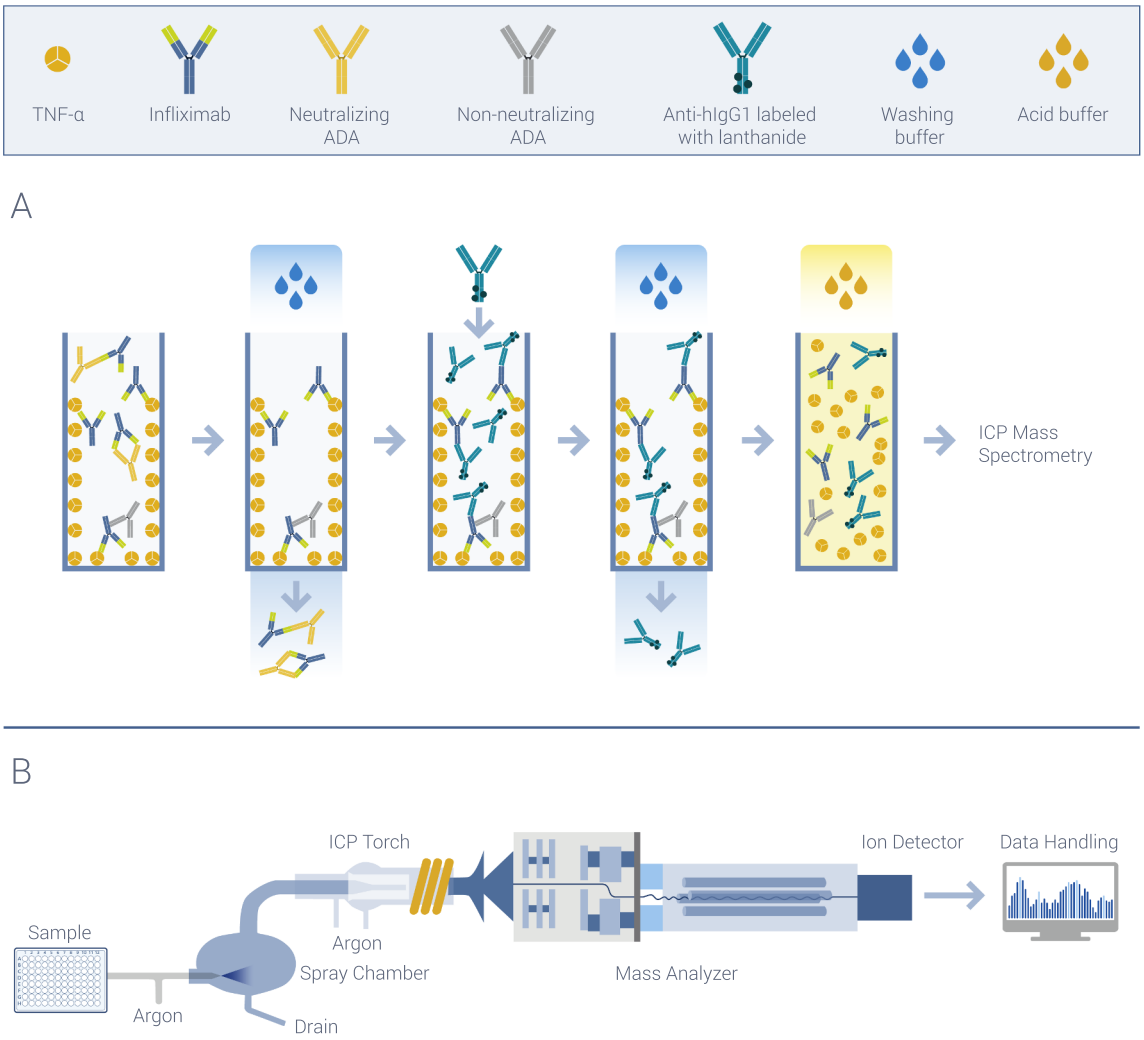

Fig. 1. Principle of ICP-MS method. A. Patient serum containing IFX is incubated in microplate wells with captured recombinant TNF. Unbound material (including IFX-neutralizing antibody complexes) is washed out, and monoclonal anti-human IgG1 antibodies labeled with lanthanide is added to the microplate. After second wash, complexes containing TNF, IFX and lanthanide-labeled monoclonal antibodies are removed from the microplate with acid buffer and applied to the ICP mass spectrometry. B. In ICP-MS, the sample is injected through a nebulizer into a spray chamber, generating a fine mist of aerosolized droplets. These droplets are then introduced into the torch of the instrument, containing argon flowing through the system. As the sample flows through, the ionized argon of the plasma passes energy to the more-easily ionized sample, breaking bonds and stripping the elements of electrons. This generates mostly singly charged, positive ions which are then focused into the mass analyzer. Finally, the ion beam goes through a quadrupole, where a combination of radio frequency and direct current voltages separates the ions by *m/z*. Those ions selected hit the detector and are proportional to the concentration in the sample. The concentration of IFX in the serum is calculated using calibration curve with standards of known IFX concentration.

calculate the CV for within-run precision. Average results from samples tested each day for each sample were also used for accuracy analysis (% error). The minimum acceptable criteria were < 20% for CV and < 25% for accuracy.

2.7.3. Assay linearity

Linearity of the IFX ICP-MS assay was evaluated using 5 normal human serum samples spiked with different concentrations of IFX (1, 13.25, 25.5, 37.75, 50 µg/mL), run in duplicates. The relationship between the expected and observed concentrations was evaluated by the R^2 and slope of the linear regression analysis. The minimum acceptable criteria were $R^2 \geq 0.80$ and slope = 1.00 ± 0.20.

2.7.4. Analytical specificity and interference studies

Three serum samples containing IFX and 2 IFX-negative control sera were spiked with following substances: bilirubin (7.25 mg/dL), hemoglobin (1800 mg/dL), and triglycerides (4700 mg/dL). Recovered concentrations of IFX were compared to corresponding values for the samples without interfering substances.

2.7.5. Sample stability evaluation

Two clinical samples were aliquoted and stored at RT for 24 h and 2 weeks, at 4 °C for 2 weeks, and − 70 °C for 2 weeks and 6 months. One set of samples was freeze-thawed 3 times. Samples were tested in duplicates at baseline, 24 h, 2 weeks and 6 months. Recovery was calculated as percent of the baseline. The minimum acceptable criteria was < 20% difference from baseline results.

2.7.6. Cutoff determination

De-identified residual waste serum samples from 123 drug-naïve donors (54 males, 69 females, age from 18 to 65 with median age of 29 years) were tested by the IFX ICP-MS assay. The cutoff for the IFX ICP-MS assay was determined by taking the mean value +2 x SD (95 percentile).

2.7.7. Comparison of ICP-MS with RGA and ELISA assays

Results of IFX levels obtained by ICP-MS were compared to those obtained by RGA and ELISA assays. RGA testing was performed at ARUP Laboratories, following a previously reported clinical laboratory protocol (Pavlov et al., 2016). ELISA testing was performed using the ImmunoGuide® product for measurement of free IFX using TNF-coated plates for capture, following the manufacturer's protocol (IBL America, Minneapolis, MN). Agreement between methods was calculated as percentage of number of samples which were concordant, either both positive or both negative in those tests over the total number of samples. The results for IFX quantitation using ICP-MS versus RGA and ELISA methods were compared using Deming regression analysis and Bland-Altman plots. Confidence intervals for the slopes, intercepts and R-squares were calculated as central 90% intervals of bootstrap data of 10,000 samplings with replacement.

3. Results

3.1. Validation of IFX ICP-MS assay

3.1.1. Analytical sensitivity

To establish the LOB, 156 blank samples were tested. The LOB was found to be equal to 0.3 µg/mL. The LOD was determined as LOB +1.645 x SD of 61 replicas of a sample containing 0.5 µg/mL of IFX. The LOD was found to be equal to 0.4 µg/mL.

3.1.2. Precision

Precision studies were performed using samples spiked with high, mid, and low concentrations of IFX, tested over different runs. The within-run and between-run CV results were < 8%, for low, mid, and high IFX-containing samples (Table 1). Accuracy of results ranged from 96% to 115%. The within-run and between-run CV's, as well as accuracy of results were within acceptable limits of the assay.

3.1.3. Linearity

Linearity assessment was performed on 5 human serum samples spiked with various amounts of IFX (1, 13.25, 25.5, 37.75, 50 µg/mL). The correlation value of the linear regression analysis for the assay (R^2 = 0.9976) demonstrated acceptable linearity.

3.1.4. Analytical specificity

Analytical interference experiments revealed that results of IFX samples spiked with hemoglobin, bilirubin and triglyceride concentrations up to 1800 mg/dL, 7.25 mg/dL, and 4700 mg/dL, respectively, were within the precision of the assay (± 8% of values for non-spiked samples).

3.1.5. Sample stability evaluation

Fig. 2 illustrates IFX results in two clinical samples tested at baseline, compared to results obtained from aliquots stored at RT, 4 °C, and − 70 °C or frozen at various times, with one set exposed to 3 freeze-thaw cycles. Recoveries of IFX results were found to be within 11% of baseline results, which are within acceptable range.

3.1.6. Cutoff for the IFX ICP-MS assay

The cutoff of the assay based on serum results from 123 drug naïve healthy individuals was set at 0.5 µg/mL.

3.2. Comparison of ICP-MS with RGA and ELISA assays

The performance of the ICP-MS assay was compared with two other methods used for measuring serum IFX levels. First, the performance of ICP-MS was compared to the clinically used RGA assay, by.

measuring 215 serum samples previously tested for IFX activity levels by the RGA method. Fig. 3 shows the correlation between IFX values reported by the two assays. A total of 206 samples were used in this analysis (9 samples exceeding reportable ranges for ICP-MS and RGA assay were excluded from the analysis). Very good correlation was observed between the RGA and ICP-MS method (slope 0.94 (90% CI: 0.87 to 1.01), intercept 0.39 (90% CI: -0.01 to 0.79); correlation coefficient R = 0.89). The average difference in infliximab concentrations determined by the two methods was 0.2 µg/mL. There were 2 out of 215 samples which were negative by the RGA (< 0.65 µg/mL) and positive by ICP-MS assay with values of 1.05 and 1.14 µg/mL infliximab. The agreement between the two methods was 99%. Importantly, the presence of ADA did not affect the correlation between the two methods, as even though 23 out of 215 serum samples used for comparison were positive for neutralizing ADA against infliximab by RGA, all 23 of them had IFX levels below the cutoff of drug positivity by both methods.

We also compared IFX results obtained with ICP-MS and a commercial ELISA assay using 86 patient sera. Fig. 4 shows the correlation between IFX values reported by these two assays. Eighteen out of 86 ELISA results exceeded the upper limit of this assay (20 µg/mL) and were excluded from the analysis. Acceptable correlation was observed between the ICP-MS and ELISA assays: slope 0.86 (90% CI: 0.78 to 0.95), intercept −0.52 (90% CI: -1.11 to 0.11), correlation coefficient R^2 = 0.85). The average difference in infliximab concentrations determined by the two methods was 1.8 µg/mL.

4. Discussion

Infliximab has revolutionized the therapy for chronic inflammatory and autoimmune diseases such as Crohn's Disease, ulcerative colitis, rheumatoid arthritis and others. However, a significant number of patients fail to respond from the beginning, or lose response to therapy over time after initial positive response. Secondary loss of response to therapy is most commonly due to the development of anti-drug antibodies, which will render the drug ineffective over time. Previous studies have demonstrated the importance of concurrent measurement of serum drug levels and ADA for accurate diagnosis of the causes of treatment failure to IFX (Velayos et al., 2013; Steenholdt et al., 2014; Steenholdt et al., 2015). More recent studies have advocated the importance of measuring trough IFX levels without the need for ADA testing. For instance, post induction trough IFX concentration was associated with durable sustained response in a multicenter, randomized, placebo-controlled study involving patients with Crohn's disease (Cornillie et al., 2014). A large randomized controlled study showed

Table 1
Assay precision and recovery of the ICP-MS assay.

	Intra-assay precision (n = 3)			Inter-assay precision (n = 4)		
	High	Mid	Low	High	Mid	Low
Expected (µg/mL)	31.0	16.0	8.0	31.0	16.0	8.0
Measured (mean, µg/mL)	32.1	17.0	9.2	31.7	15.4	8.5
SD (µg/mL)	2.6	1.4	0.4	1.4	1.2	0.5
CV (%)	8.1	8.2	4.3	4.4	7.8	5.9
Recovery (%)	104%	106%	115%	102%	96%	106%

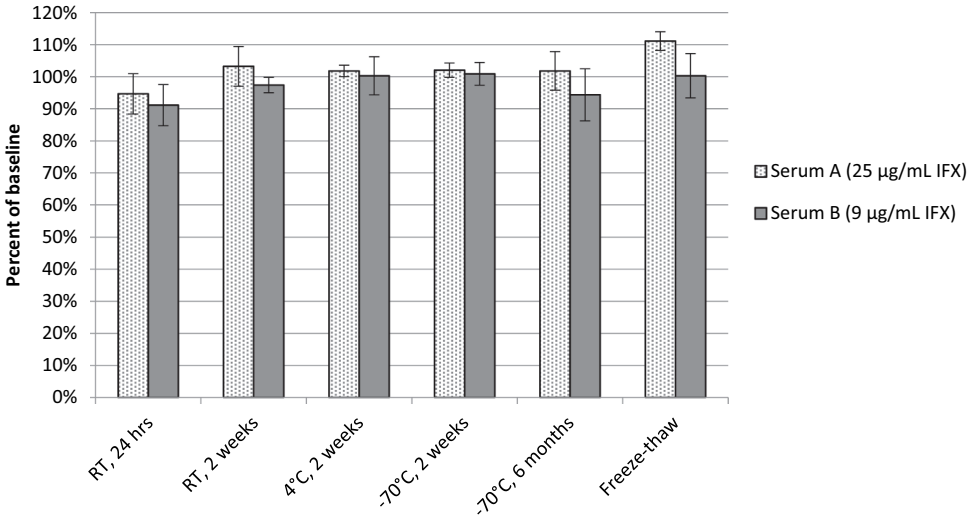

Fig. 2. Sample stability. Two clinical samples were aliquoted and stored at room temperature (RT) for 24 h and 2 weeks, at 4 °C for 2 weeks, and − 70 °C for 2 weeks and 6 months. One set of samples was tested after 3 freeze-thaw cycles. Recovery of IFX results was found within 11% of baseline results.

that targeting patient's IFX trough concentrations to 3–7 µg/mL resulted in a higher proportion of Crohn's disease patients in remission and overall more efficient use of the drug (Vande Casteele et al., 2015). Recently, the American Gastroenterological Association have recommended reactive therapeutic drug monitoring targeting trough concentrations of at least 5 µg/ml for IFX during maintenance therapy for patients with active inflammatory bowel disease (Feuerstein et al., 2017). These studies suggest the need for development of cost-efficient laboratory methods that can provide accurate and rapid results of trough drug levels to guide treatment decisions.

In this study, we report the development of a novel assay for measuring serum free IFX levels using ICP-MS. The methodology of this assay is fairly simple, requiring the incubation of IFX-containing patient serum in wells coated with recombinant TNF, followed by detection with lanthanide-labeled monoclonal anti-human IgG1 and ICP-MS analysis. Our results for the validation of the ICP-MS assay for measurement of IFX levels demonstrate that this assay is accurate, linear, precise and devoid of analytical interferences by high levels of hemoglobin, bilirubin and triglycerides.

Several methodologies are available for measuring IFX levels in the clinical laboratory, including ELISA (Baert et al., 2003), HMSA (Wang et al., 2012), LC-MS/MS (Willrich et al., 2015), and RGA (Pavlov et al., 2016). Methods such HMSA and LC-MS/MS measure total IFX, including free IFX and IFX bound to ADA, due to acid pre-treatment of the sample for HMSA, and trypsin digestion for LC-MS/MS, respectively. The ICP-MS assay described in this study is similar to capture ELISA methods which measure free IFX only, as neutralizing antibody-bound IFX is not available for binding to TNF in the assay. Levels of free IFX provide a better estimate of drug trough levels that can guide dose adjustments. Furthermore, due to the principle of the assay that it is based on drug capture by TNF, this method allows for measurement of any TNF antagonist by simply changing the drug standards and assay calibrators. Compared to ELISA, the ICP-MS assay described here has several advantages including a wider analytic dynamic range, better sensitivity due to very low background of rare earth elements in patient samples, lack of measurement challenges using spectrophotometry due to spectral interferences, and the ability to prepare and store plates for analysis without concern over signal degradation. ICP-MS also offers the potential for multiplexed testing of different TNF antagonists in the same reaction because of the availability of many lanthanides for antibody labeling. This would require the use of drug-specific antibodies against each TNF antagonist. Up to this date, there are several commercially available antibodies specifically against infliximab or adalimumab. Although ICP-MS instruments are relatively easy to operate, to date, these instruments are mainly available in national reference laboratories that measure a large number of trace and toxic elements. The use of ICP-MS has challenges for implementation in routine clinical testing including initial instrument costs, the need for a clean room environment and continuous argon gas supply, dedicated chemical disposal protocols, and specialized training for both operation and maintenance procedures, that are typically outside the level of expertise for the majority of clinical laboratory staff.

A good evaluation of a new method requires the comparison with others already in use. In our study, serum specimens were tested side-by-side using the ICP-MS, RGA, and ELISA methods. Good correlation of IFX levels was observed between the ICP-MS and the other two methods. The correlation between the ELISA method and ICP-MS is expected due to the fact that both methods measure free IFX in the sample. The correlation between the ICP-MS assay and RGA is remarkable considering that the ICP-MS assay and RGA use different methodologies and measure different parameters of IFX therapy (free drug concentration vs biological activity of drug, respectively). One explanation for this correlation is that the ICP-MS assay measures free IFX, which might correlate well with biological activity of free IFX in the RGA.

In summary, the ICP-MS methodology presented in this paper is a robust platform for measuring free serum drug levels in patients treated with TNF antagonists. The ICP-MS assay shows high precision, which makes it well suited for therapeutic drug monitoring in the clinical laboratory. The results of method comparison revealed that concentrations of IFX by ICP-MS disagreed slightly with those obtained by RGA and ELISA, suggesting that absolute drug concentrations cannot be directly compared between methods. This is likely due to inherent analytical sensitivity and specificity differences between the three assays. In the absence of a gold-standard assay available, it is unclear to determine if any method measures too high or too low IFX levels. For best clinical practice, it is recommended to use the same method for repeated measurements for the same patient. An international standard

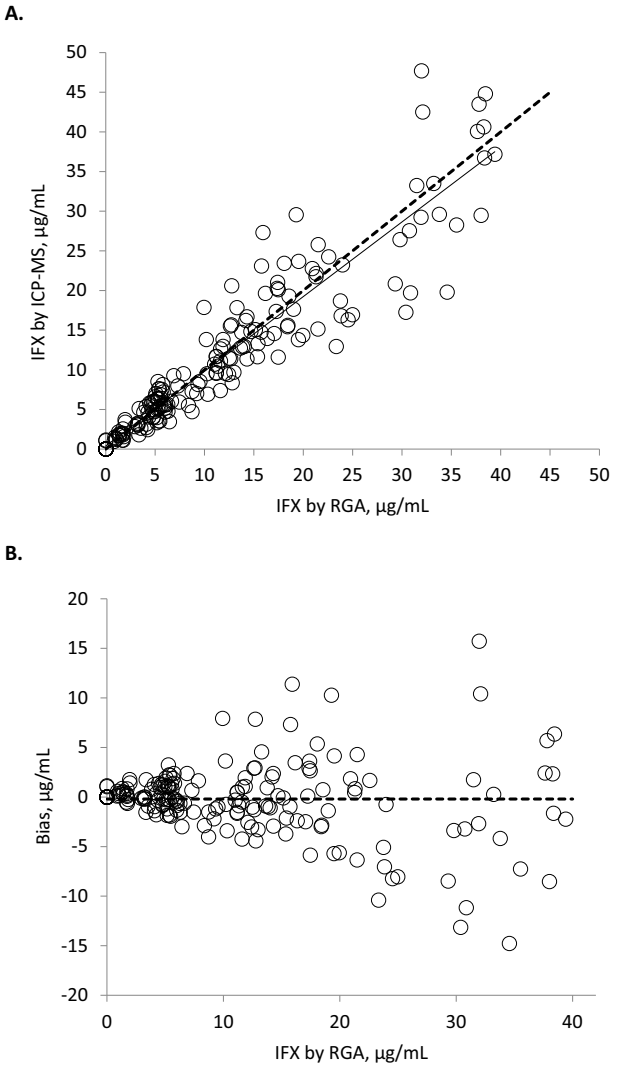

Fig. 3. Correlation between the ICP-MS assay and RGA on the measurement of IFX levels in 206 samples. A. Deming regression line is shown as a continuous line. The dashed line represents the 1:1 correlation. Correlation between the ICP-MS assay and RGA had a slope of 0.94 (90% CI: 0.87 to 1.01), intercept of 0.39 (90% CI: -0.01 to 0.79), and correlation coefficient $R = 0.89$. The dashed line represents the 1:1 correlation. B. Bias plot of infliximab concentrations (µg/ml) determined by the ICP-MS assay and RGA. The plot shows the difference between infliximab levels measured by ICP-MS minus the levels measured by RGA for individual samples, plotted against their RGA values. Mean difference between methods of infliximab concentrations was 0.2 µg/mL (dotted line).

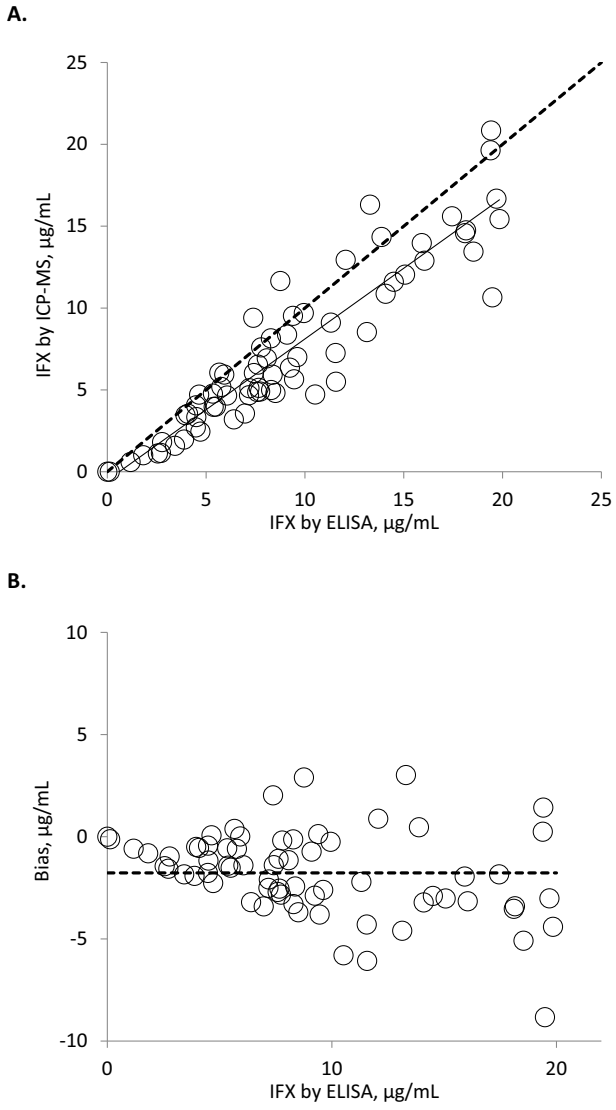

Fig. 4. Correlation between the ICP-MS assay and ELISA on the measurement of IFX levels in 68 samples. A. Deming regression line is shown as a continuous line. The dashed line represents the 1:1 correlation. Correlation between the ICP-MS assay and ELISA had a slope of 0.86 (90% CI: 0.78 to 0.95), intercept of 0.52 (90% CI: -1.11 to 0.11), and correlation coefficient $R = 0.85$ (90% CI: 0.79 to 0.90). B. Bias plot of infliximab concentrations (µg/ml) determined by the ICP-MS assay and ELISA. The plot shows the difference between infliximab levels measured by ICP-MS minus the levels measured by ELISA for individual samples, plotted against their ELISA values. Mean difference between methods of infliximab concentrations was 1.8 µg/ml (dotted line). slope 0.86 (90% CI: 0.78 to 0.95), intercept −0.52 (90% CI: -1.11 to 0.11), correlation coefficient $R = 0.85$.

for IFX has recently become available (https://www.nibsc.org/documents/ifu/16-170.pdf), which seems an important step towards future harmonization of current and new IFX assays. Future studies will be necessary to establish appropriate clinical cutoff levels for therapeutic drug monitoring using the ICP-MS assay.

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