Quantification of active infliximab in human serum with liquid chromatography–tandem mass spectrometry using a tumor necrosis factor alpha -based pre-analytical sample purification and a stable isotopic labeled infliximab bio-similar as internal standard: A target-based, sensitive and cost-effective method

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**ABSTRACT**

The therapeutic monoclonal antibody Infliximab (IFX) is a widely used drug for the treatment of several inflammatory autoimmune diseases. However, approximately 10% of patients develop anti-infliximab antibodies (ATIs) rendering the treatment ineffective. Early detection of underexposure to unbound IFX would result in a timely switch of therapy which could aid in the treatment of this disease. Streptavidin coated 96 well plates were used to capture biotinylated-tumor necrosis factor –alpha (b-TNF-α), which in turn was used to selectively extract the active form of IFX in human serum. After elution, IFX was digested using trypsin and one signature peptide was selected for subsequent analysis on liquid chromatography – tandem mass spectrometry (LC–MS/MS). The internal standard used was a stable isotopic labeled IFX bio-similar. The assay was successfully validated according to European Medicines Agency (EMA) guidelines and was found to be linear in a range of 0.5–20 μg/mL ($r^2 = 0.994$). Lower limit of quantification for the assay (<20% CV) was 0.5 μg/mL, requiring only 2 μL of sample. Cross-validation against enzyme-linked immunosorbent assay (ELISA) resulted in a high correlation between methods ($r^2 = 0.95$ with a $p_r = 0.83$) and the accuracy was in line with previously published results. In conclusion, a sensitive, robust and cost-effective method was developed for the bio-analysis of IFX with LC–MS/MS by means of a target-based pre-analytical sample purification. Moreover, low volume and costs of consumables per sample promote its feasibility in (pre)clinical studies and in therapeutic drug monitoring. This method should be considered as first choice due to its accuracy and multiple degree of selectivity.

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1. Introduction

Infliximab (IFX), an immunoglobulin G (IgG) based tumor necrosis factor alpha (TNF-α) blocking antibody, has been approved by the EMA and the FDA for the treatment of several autoimmune diseases, such as Crohn’s disease, ulcerative colitis, ankylosing spondylitis and rheumatoid arthritis to induce and retain remission [1,2]. One year after initiation of IFX therapy, 10% of patients lose response to the treatment [3]. It is speculated that this is largely due to induction of immunogenicity where patients develop anti-infliximab antibodies (ATIs) that bind to IFX rendering it ineffective. Therapeutic drug monitoring of the biological active fraction of IFX in patients’ serum has shown great promise to improve treatment outcomes in patients suffering from inflammatory autoimmune diseases [4–7].

The traditional way of analyzing monoclonal antibodies such as IFX is by ELISA. However, there are some noteworthy differences in sensitivity and selectivity attained with various IFX ELISA assays [8,9]. These differences are mainly attributed to the assay design for capturing and detecting the monoclonal antibody of interest. Furthermore, ELISA assays are vulnerable to cross-reactivity, this is when the detecting antibody lacks specificity and attaches itself to a soluble receptors or to endogenous IgG in serum instead of the therapeutic antibody of interest [8,10]. Moreover, ELISA methods lack the linear dynamic range and due to the higher risk of
cross reactivity requires an extensive validation [11]. It has been demonstrated that LC–MS/MS preceded by sample purification and protein digestion can provide the high sensitivity and specificity required for the quantification of monoclonal antibodies in serum [12]. Sample purification prior to MS analysis is necessary to remove serum proteins and salts that otherwise would interfere with the analysis. There have been various methods published dealing with sample purification for therapeutic antibodies in serum. Methods such as pellet digestion remove albumin from serum in the supernatant layer by using organic solvents or salts, here the pellet retains all IgGs including the therapeutic antibody [12–15]. Albumin accounts for approximately 50% of total serum proteins, thus the removal of albumin from serum enhances the sensitivity of the method [16]. Other approaches focus on capturing IgGs in serum by means of immuno-affinity purification using either protein A, protein G, or anti human Fc antibodies attached onto a solid support such as magnetic beads [17–23]. Thereafter, the therapeutic antibody is eluted and digested using trypsin and finally one ‘signature’ peptide (a non-endogenous peptide) is selected for quantification. However, these sample purification methods mentioned above are nonspecific for the active form of the therapeutic antibody. Furthermore, immunoglobulins (Ig’s) are amongst the most abundant proteins in serum [24,25]. These “naturally present” Ig’s are extracted together with the therapeutic antibody during these types of sample purifications and they pose a potential source of chromatographic interference and ionization suppression during MS analysis. In addition, high levels of Ig’s will compete with the therapeutic antibody for the binding sites during these types of immuno-affinity interaction, potentially harming recovery rates.

Indeed, a selective sample preparation would be able to eliminate or reduce the drawbacks found in the generic sample purification approaches described above. Therefore, in this manuscript we propose a selective purification of active IFX (infliximab with an unbound paratope) in human serum by means of its antigen TNF-α, followed by trypsin digestion and LC–MS/MS analysis. The proposed purification is based on immunoaffinity using biotinylated TNF-α (b-TNF-α) which has been fixed onto a streptavidin coated 96 well plate.

The benefit here are, cleaner extracts due to the use of selective interactions, which would lead to a reduction in the noise level and a lowering of the detection limit. Furthermore, due to the selective nature of the test, only the antibody of interest (IFX) is captured and eluted where this would require a minimal amount of the capturing antigen (b-TNF-α). Moreover, cleaner extracts would require a minimal amount of trypsin for the subsequent digestion. Also, due to the coupling of TNF-α onto a 96 well plate instead of beads, higher throughput, ease of use and faster sample preparation times are facilitated. These factors would reduce the cost of the test significantly and most importantly the use of a stable isotope labelled IFX bio-similar as internal standard (IS) would aid in obtaining accurate and precise results. This is due to the possibility of introducing the IS at the beginning of the sample preparation, thus providing ideal correction for losses occurred during sample pre-treatment, enzymatic digestion and LC–MS/MS analysis.

2. Materials and methods

2.1. Chemicals and reagents

Infliximab (Remicade™) was obtained from Janssen Biologics B.V. (Leiden, The Netherlands) as lyophilized powder and was reconstituted in distilled water to a final concentration of 10 μg/μL. 100 μL aliquots of this solution were pipetted in Eppendorf LoBind Microcentrifuge tubes and stored at −80 °C. Stable isotope Internal standard infliximab bio-similar was obtained from Promise advanced proteomics (Grenoble, France) as a 10 μg/10 μL solution. Biotinylated human recombinant TNF-α was obtained from ACRO biosystems (Newark, DE) and dissolved in Phosohate Buffered Saline (PBS), 0.1% Tween-20, 1% BSA to 50 μg/mL. Streptavidin high binding capacity coated 96 well plates were obtained from Thermo Fisher (Waltham, MA). MS grade modified trypsin was obtained from Promega (Madison, WI) and was dissolved to 1 μg/μL in 50 mM acetic acid and aliquoted in the Eppendorf LoBind Microcentrifuge tubes. Aliquots were stored at −80 °C. Drug-free human serum was obtained from BIO-RAD (Irvine, CA). All other reagents and LC–MS grade mobile phase solvents were obtained from Sigma (Saint Louis, MO).

2.2. Preparation of standards, internal standard and QCs

The working IFX solution (20 μg/mL) was prepared from the 100 μL aliquot (10 μg/μL) by adding 900 μL drug free human serum (DFHS) to obtain a concentration of 1 μg/μL. This solution was diluted further to 20 μg/mL in DFHS. Aliquots were stored in lobind eppendorf tubes at −80 °C. Standards at concentrations of 0.5, 1, 2.5, 5, 10, 20 μg/mL were prepared fresh from the working solution by dilution in DFHS. The 10 μg/mL IS solution was diluted to 25 μg/mL. Aliquots were stored at −80 °C. The working IS solution of (2.5 μg/mL) was prepared from the stock solution (25 μg/mL) by dilution in DFHS. Quality Control samples (QCs) at lower limit of quantification (LLOQ) (0.5 μg/mL), QC low (1.5 μg/mL), QC med (6 μg/mL) and QC high (15 μg/mL), were prepared in DFHS from a different stock solution to that used to make the standards. Aliquots were stored at −80 °C.

2.3. Instrumentation and chromatographic conditions

Sample purification was performed on a vibramax 100 plate shaker, Heidelberg Instruments (Schwabach, Germany). Sample drying was performed on a HETOVAR, VR-1 (Allerød, Denmark). All experiments were performed on an Ultimate 3000 UHPLC Dionex (Sunnyvale, CA) coupled to a TSQ Quantiva, Thermo Fisher (Waltham, MA). The analytical column was UPLC Acuity, BEH 2.1 × 50 mm, 1.7 μm particle size, Waters (Milford, MA) and was maintained at 50 °C. The mobile phases were: (a) 0.1% formic acid in water; (b) 0.1% formic acid in ACN. The LC gradients in minutes per percentage of mobile phase B were 0.0 (min)/10 % B, 3/35, 3.01/100, 4.25/100, 4.26/10 and 5.5/10. The flow rate was 0.5 mL/min and the run time was 5.5 min. The MS was operated in positive mode with spray voltage of 3.5 kV, Ion Transfer Tube Temperature 350 °C, vaporizer temperature 400 °C, aux gas pressure 15 Arb, sheath gas pressure 50 Arb, sweep gas pressure 0 Arb and collision gas pressure 2.5 mTorr. The precursor ions, product ions, collision energy and radio frequency (RF) lens settings are listed in Table 1 for IFX and for the internal standard.

2.4. Sample preparation for LC–MS/MS analysis

Sample preparation was based on immunoaffinity where active IFX was bound to b-TNF-α, which in turn was bounded to streptavidin coated 96 well plate (Fig. 1). b-TNF-α was coupled to a streptavidin coated 96 well plate by pipetting 5 μL b-TNF-α (50 μg/mL) and 195 μL PBS (0.1% Tween-20) in each well, followed by overnight mixing on a plate shaker (300 rpm) at room temperature. The plate was washed 3 times with 200 μL PBS (0.1% Tween-20). Then, in a lobind eppendorf tube, 200 μL PBS (0.1% Tween-20) was added, followed by 2 μL [standard, QC or serum sample] and 4 μL IS (2.5 μg/mL). This solution was mixed (gently) and transferred to the b-TNF-α pre-treated 96 well plate and allowed to bind for 1 h at room temperature on a plate shaker (300 rpm). Then, the wells were washed 3 times with 200 μL PBS.
Table 1

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Location</th>
<th>Precursor charge</th>
<th>Precursor [(m/z)]</th>
<th>Product [(m/z)]</th>
<th>Product Ion type</th>
<th>CE[\text{V}]</th>
<th>RF[\text{V}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DILLQPSAILSVPGER (Qualifier ion)</td>
<td>VL1</td>
<td>2+</td>
<td>948.53</td>
<td>545.25</td>
<td>y5</td>
<td>25</td>
<td>110</td>
</tr>
<tr>
<td>DILLQPSAILSVPGER (Qualifier ion)</td>
<td>VL1</td>
<td>2+</td>
<td>948.53</td>
<td>731.37</td>
<td>y7</td>
<td>25</td>
<td>110</td>
</tr>
<tr>
<td>DILLQPSAILSVPGER (Qualifier ion)</td>
<td>VL1</td>
<td>2+</td>
<td>948.53</td>
<td>1125.63</td>
<td>y11</td>
<td>25</td>
<td>110</td>
</tr>
<tr>
<td>DILLQPSAILSVPGER [13C,15N] (IS)</td>
<td>VL1</td>
<td>2+</td>
<td>953.53</td>
<td>555.25</td>
<td>y5</td>
<td>25</td>
<td>110</td>
</tr>
</tbody>
</table>

\[a\] CE: collision energy.
\[b\] RF: radio frequency lens.
\[c\] IS: internal standard.

Fig. 1. Principle of immunoaffinity purification of IFX using biotinylated TNF-\(\alpha\) and streptavidin-coated 96 well plate.

(0.1% Tween-20), followed by 5 times water wash to remove salts and tween. Bound IFX was extracted by adding 100 \(\mu\)L elution solution (48.5% MEOH: 48.5% H2O: 3% formic acid) and vortex mixed for 10 min at 900 rpm on a plate shaker. 100 \(\mu\)L extracts were transferred to a 2 mL glass vials and dried for 1 h at 45 °C on the HETOVA.
The samples, QC’s and standards were reconstituted in 150 \(\mu\)L Tris (50 mM, \(\text{pH}\) 9.5). Then, 5 \(\mu\)L DTT 100 mM was added, mixed for 5 min at 1500 RPM and allowed to react on a block heater set at 60 °C for 30 min. Samples were briefly spun down. Then, 5 \(\mu\)L trypsin (0.05 \(\mu\)g/\(\mu\)L) dissolved in acetic acid 2.5 mM was added and gently mixed. Then, the tubes were placed in an oven set at 37 °C for overnight digestion. Finally, trypsin activity was stopped through the addition of 30 \(\mu\)L formic acid 10% in methanol and 25 \(\mu\)L was injected and analyzed on LC–MS/MS.

2.5. ELISA method for IFX quantification

The ELISA reference method, used in this study to evaluate LC–MS/MS IFX results, was developed by Sanquin and validated according to ICH guidelines [26]. The steps involved are indicative of sandwich type ELISA. In brief, the procedure is based on capturing active IFX with TNF-\(\alpha\) which was bound to a monoclonal antibody on a polystyrene microtiter well plate. After washing the unbound serum components, biotinylated anti-IFX antibody was added. After washing, horseradish peroxidase (HRP) conjugated with streptavidin was added. Then, excess HRP was washed off followed by the addition of the substrate solution. HRP converts the substrate solution to a colored product in proportion to the amount of IFX present. Finally, a stop solution is added to terminate the reaction and the absorbance was measured in a microtiter plate reader.

2.6. Sample selection for comparative study

Routine Therapeutic Drug Monitoring samples were collected from patients who were treated with IFX. A portion of the sample was send for ELISA analysis at Sanquin (Amsterdam, The Netherlands) and the remainder was stored at −80 °C for LC–MS/MS analysis. The samples for the comparative study were selected based on their therapeutic range and on the presence of anti-IFX antibodies. The study was carried out on 24 samples from a concentration range of 0–20 \(\mu\)g/mL, of which 7 samples contained anti-IFX antibodies. All serum samples used in this study, were collected with patient’s consent.

2.7. Validation of infliximab LC–MS/MS method

A linear calibration curve was evaluated on three separate days and was established consisting of six standards ranging from 0.5 to 20 \(\mu\)g/mL. LLOQ was determined by calculating the ratio of LLOQ signal to the blank (DFHS) spiked with IS signal on three different days in 5 folds. Selectivity was tested using 6 blank human serum samples. Specificity was tested using spiked Adalimumab, Etanercept, Azathioprine and Cetuximab at 10 \(\mu\)g/mL in serum. Within-run and between-run accuracy and precision, which is a measure for repeatability and reproducibility of the analytical method, was validated by analyzing LLOQ, QC low, QC med and QC high in five folds during 3 days. The data obtained for each concentration level was evaluated with single factor ANOVA. Accuracy was expressed as percentage bias. Precision was expressed as percent coefficient of variation (% CV) and was calculated from the ANOVA derived mean squares (MS). Matrix effects of both IFX and IS were investigated by performing sample purifications on 6 randomly chosen blank human serum samples in duplicate, after washing and eluting, one set of extracts were spiked at QC low (1.5 \(\mu\)g/mL) and the other set were spiked at QC high (15 \(\mu\)g/mL) level. Clean extraction solutions were also spiked at QC low and QC high and were used as the matrix free reference. Then 4 \(\mu\)L IS (2.5 \(\mu\)g/mL) was added and samples were treated according to the procedure described above continuing on from drying step of 1 h at 45 °C. The IS-normalized matrix factor was calculated for each blank human serum sample by dividing the matrix factor (MF) of IFX (peak area

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IFX in the presence of matrix divided by peak area IFX in absence of matrix) by the MF of the IS. The matrix effect was expressed as% CV of the IS-normalized MF. Although lyophilized IFX can handle temperature extremes of up to 30 °C for a period of 12 months [27], the stability of IFX in serum is unknown. Furthermore, the effects of IFX denaturation due to sample handling (which could lead to IFX deactivation) could also reduce the storage period of the sample. Therefore, IFX stability was tested according to EMA guidelines at QC low and QC high level during 2 freeze and thaw cycles and the percentage bias was calculated.

3. Results and discussion

3.1. Method development

The 144 kDa molecular mass of infliximab exceeds the typical mass range of a triple quadrupole mass spectrometer. Therefore, protein digestion plays an integral part in LC–MS/MS analysis of biopharmaceuticals. We have opted for using trypsin to produce smaller peptides, because peptides liberated this way contain a terminal lysine or arginine, two basic amino acids that are easily ionized during electrospray ionization. During IFX digestion, a multitude of peptides were produced (Fig. 2). A signature peptide was selected based on four criteria: number (n) of amino acids in the peptide chain 6 < n < 20, the signal intensity, absence from the human genome (search performed on pBLAST® using the human database swissprot) and stability of the comprising amino acids. After careful consideration, D1ILITQSPAILSVSPGER peptide was selected as the signature peptide. This is a terminal peptide located in the variable light (VL1) portion of IFX which can be easily liberated. The peptides C44LEWVAEIR from the variable heavy chain (VHH4) and C12PSVFLAPSSK from the constant heavy chain (CH124) were monitored at 536.8 → 587.4 and at 593.8 → 699.4 m/z respectively solely to optimize the buffer type and strength for trypsic digestion. Stable isotopic labeled infliximab bio-similar, containing labeled arginine (13C6; 15N4) and lysine (13C6; 15N2) was chosen as the internal standard and was spiked at 5 μg/mL onto 2 μL samples, QC's and standards, thus requiring only 10 ng labeled IS. The SRM transitions of the precursors and the product ions were selected based on the ones that provided the lowest interference and the highest signal intensity. These transitions were found during MS optimization of collision energy settings (data not shown). Immunoaffinity sample purification was based on the bait and prey principal. Here 0.25 μg b-TNF-α was captured on top of streptavidin coated 96 well plate (Fig. 1) which in turn was used to capture biologically active IFX fraction in serum (IFX with at least one free paratope). 0.25 μg b-TNF-α corresponds to 9.6 pmol protein which falls within the manufacturer stated threshold of ~125 pmol biotinylated protein per streptavidin coated well. Furthermore, compared to the highest standard (0.04 μg or 0.27 pmol IFX), 0.25 μg b-TNF-α provides 18× molar excess to available IFX binding sites which is highly desirable for a fast extraction. The 96 well plates accommodated a high sample throughput, ease of use and a fast sample preparation time. The method was validated according to the latest EMA guidelines Rev. 1, with coming into effect date of 1 February 2012.

3.2. Streptavidin coated 96 well plate capacity test

Streptavidin has been reported to show a strong binding affinity towards biotin [28]. Furthermore, due to its near neutral isoelectric point, it possesses low non-specific binding. Based on these characteristics, b-TNF-α in conjunction streptavidin high binding capacity coated 96 well plates were chosen for the immunoaffinity purification. The capacity of these plates, which define the linear working range of the analytical method, was determined by the following test. The test was performed using the sample preparation procedure described above with some modifications, namely, the standard range was extended to 0.1–0.5–1–5–10–20–30–40–50–60–70–80–90 μg/mL and the internal standard was added after elution in this experiment, in order to correct solely for the losses observed after elution. As illustrated by Fig. 3, the calibration line starts to bend around 30 μg/mL IFX, which corresponds to 0.06 μg IFX per well. The therapeutic trough level range for patients treated with IFX lies between 3 and 7 μg/mL, therefore we have chosen to limit our calibration
range to the upper level of 20 \( \mu \text{g/mL} \) for therapeutic drug monitoring purposes. One hour binding time was selected to reduce the analysis time, however, an extended calibration range might be achieved by increasing the binding time.

### 3.3. Optimizing buffer type and strength for tryptic digestion

In this experiment we examined the buffer type and strength that yield the highest signals for three peptides (VL1, VH44 and CH124). The use of ammonium bicarbonate (ABC) and tris (hydroxymethyl) aminomethane (Tris) buffers both ranging from 50 to 200 mM buffer strength was explored. The buffers were adjusted with HCl to pH 8 for both buffer types at all buffer strengths. The test was performed in triplicate, 10 \( \mu \)L IFX working solution was diluted in multiple vials with 90 \( \mu \)L Tris or ABC at various buffer strengths, ranging from 50 to 200 mM with 50 mM increments. DTT was freshly made and added to the test solutions to obtain a final concentration of 2 mM. The vials were heated to 60°C for 60 min to reduce the disulfide bonds. After the vials were cooled to room temperature, 10 \( \mu \)L trypsin (0.01 \( \mu \)g/\( \mu \)L) was added and digested for 3 h at 37°C. Finally, 10 \( \mu \)L formic acid (10% in methanol) was added to stop the reaction and 25 \( \mu \)L was injected and analyzed on the LC–MS/MS.

Fig. 4 depicts the influence of buffer type and strength on the trypsin activity. Trypsin activity can be monitored through the amount of peptides formed, which in turn is linearly correlated to signal intensity. As shown, the signal intensity diminished with increasing buffer strength, indicating that trypsin is sensitive to high salt concentration. Furthermore, the rate of reduction is buffer type dependent. An increase in buffer strength in ABC resulted in a steeper decline in signal intensity of VH44 and CH124 compared to Tris. Because ABC is thermally labile and because it causes a steeper decline in signal intensity for VH44 and CH124, Tris at 50 mM buffer strength was selected for use as the digestion buffer.

### 3.4. Comparative study

24 samples were selected based on the concentration range (0–20 \( \mu \)g/mL) and the presence of ATls. A good correlation of \( R^2 = 0.95 \) was retrieved between ELISA assay and LC–MS/MS (Fig. 5) using Pearson’s linear regression. Furthermore, a Lin’s concordance correlation coefficient \( \rho_C = 0.83 \) was retrieved, indicating a substantial agreement between the two methods. Both methods reported no free IFX concentrations in the 7 patients that had developed ATls. However, LC–MS/MS IFX results were a factor 1.5× higher compared to ELISA assay, which is possibly due to the different interactions taking place during sample binding and analysis between the two assays. The ELISA assay requires two interactions, one for binding IFX to the plate and the other for IFX detection. The strength and/or efficiency of these two interactions might not be sufficient. In comparison, for the LC–MS/MS sample purification method, only one interaction is required to bind IFX to the plate. Moreover, the same between-assay differences were observed in a recent study of five IFX ELISA assays [9]. The results showed that the Sanquin and Dynacare ELISA assays provided approximately 1.5× lower concentrations compared to Janssen, KU Leuven and LabCorp in patients treated with IFX which is similar to what was found in this study.

### 3.5. Validation

A linear calibration curve was established with a mean correlation coefficient of \( R^2 = 0.994 \) using 6 standards, 0.5–1–2.5–5–10–20 \( \mu \)g/mL analyzed over three days. The mean regression line over the three days was \( Y = 0.275 \pm 0.00538 \) \( X \) – 0.0006 \( \pm 0.0174 \) with a residual sum of squares (RSS) of 0.041. The back calculated standard points are in concordance with EMA acceptance criteria of ±15% of the nominal value (Table 2). LLOQ was greater than the acceptance criteria of 5× the noise level (Fig. 6A). Selectivity and specificity were evaluated with 6 blank human serum samples and with spikes of 10 \( \mu \)g/mL of drugs commonly found in treated patients. Because cetuximab contains near identical light chain sequence to that of IFX it was also included in the specificity test. The signal ratios obtained from the samples mentioned above, were calculated in relation to LLOQ IFX signal and IS signal respectively and are displayed in Table 3.
Table 2
Statistics of the back calculated concentrations of the standard curve analyzed during three days.

<table>
<thead>
<tr>
<th>Nominal concentration (µg/ml)</th>
<th>0.5</th>
<th>1.0</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
<th>20.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.50</td>
<td>0.99</td>
<td>2.44</td>
<td>4.93</td>
<td>10.03</td>
<td>21.42</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.03</td>
<td>0.06</td>
<td>0.16</td>
<td>0.10</td>
<td>0.25</td>
<td>1.61</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>−0.07</td>
<td>1.00</td>
<td>−2.52</td>
<td>1.48</td>
<td>0.27</td>
<td>7.11</td>
</tr>
<tr>
<td>Imprecision (%)a</td>
<td>6.59</td>
<td>5.71</td>
<td>6.69</td>
<td>2.06</td>
<td>2.46</td>
<td>7.53</td>
</tr>
</tbody>
</table>

a Accuracy: Determined as (measured conc. - nominal conc.)/nominal conc.

b Imprecision: Expressed as co-efficient of variation (CV).

Table 4
Accuracy and precision validation data for QC’s at LLOQ, Low, Medium and High levels. Within-run data were based on 5 replicates and between-run data on 3 different days.

<table>
<thead>
<tr>
<th>QC</th>
<th>Precision (% CV)</th>
<th>Accuracy (% bias)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within-run</td>
<td>Between-run</td>
</tr>
<tr>
<td>LLOQ</td>
<td>10.4</td>
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</tr>
<tr>
<td>Low</td>
<td>5.3</td>
<td>7.5</td>
</tr>
<tr>
<td>Med</td>
<td>5.4</td>
<td>3.1</td>
</tr>
<tr>
<td>High</td>
<td>5.2</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Fig. 6. SRM of (A) IFX at the back (m/z 948.53 > 545.25) at LLOQ level overlaid with blank (DFHS) spiked with IS at the front. (B) IS at the back (m/z 953.53 > 555.25) overlaid with blank at the front.

can be seen all obtained signal ratios fall well within the tolerance of 20% of LLOQ and 5% of IS signal. Carry-over peak signals were also below 20% of LLOQ for IFX and below 5% of IS signal (Fig. 6). Within-run and between-run accuracy and precision all were within acceptance criteria of <15% of the nominal concentration and 15% CV, respectively (Table 4). The %CV of IS-normalized MF calculated over the 6 blank human serum samples for QC low and QC high were 7.2% and 6.6% respectively, indicating a slight ionization enhancement but falls within the acceptance criteria of 15%. Freeze and thaw stabilities for QC low and QC high over two days were within acceptance criteria (<15% nominal concentration).

4. Conclusion

A target-based pre-analytical sample purification for the quantification of active form of IFX in human serum was developed. Critical parameters such as the selection of signature peptide, buffer type and strength and the capacity of streptavidin coated 96 well plate were carefully evaluated and optimized. The method was validated according to the latest EMA guidelines and was found to be sensitive with LLOQ of 0.5 µg/mL while utilizing only 2 µL serum sample. The latter can mainly be attributed to the selective interactions of the proposed sample purification in combination with a highly sensitive mass spectrometer. Within-run and between-run accuracy and precision were all within acceptance criteria. The use of stable isotopic labeled IFX bio-similar as internal standard ensured corrections for loss during sample purification, digestion and LC–MS/MS analysis. The method is easy to perform, robust and can provide a high sample throughput due to the use of the 96 well plate format. Furthermore, due to the low sample volume required for the test, lower amounts of internal standard (10 ng) and biotinylated TNF-α (0.25 µg) were needed, reducing the cost of the analysis significantly. The method showed a strong correlation with the reference ELISA and the accuracy found to be in line with previously published ELISA results.

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