Immunoaffinity LC–MS/MS for quantitative determination of a free and total protein target as a target engagement biomarker

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Aim: IP-10 is a protein target for the treatment of Crohn's disease. Inhibition of IP-10 by anti-IP-10 mAbs neutralizes its various biological activities. The measurement of free IP-10 suppression as a target engagement biomarker is required for the assessment of drug effect on the target. **Results:** The development of highly sensitive immunoaffinity-LC–MS/MS assays for quantifying free and total IP-10 in cynomolgus monkey serum is reported for the first time. This paper details strategies for maximizing assay sensitivity by selecting digestion routes, and optimizing immunocapture to achieve full recovery and minimal matrix effect. For the free IP-10 assay, bioanalytical strategies have been established to minimize drug/ligand dissociation. **Conclusion:** The assays have been implemented for target engagement measurement, pharmacokinetic-pharmacodynamic correlation, and human dose projections.

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Measurement of target engagement biomarkers has been integrated into the drug discovery process to enable successful transition to development [1–7] and to improve drug discovery effectiveness and clinical success rate [8]. It has become an increasingly valuable tool for decision-making and prioritization of lead compounds. Target measurement provides crucial information to validate target and mechanism of action, elucidate disease pathophysiology, demonstrate target load and engagement, and determine dose-dependent pharmacodynamic (PD) effects. Target engagement is referred to as the ability of a drug to bind to a cellular target in living systems [9]. For a biotherapeutics directed against a soluble target (ligand), the drug binds to its target and as a result it inhibits the target's binding to its own receptor, leading to blockade of subsequent signal transduction and neutralizing the target's various biological activities. Following dosing with the anti-target antibody drug, the free target is reduced compared with the initial baseline levels. Therapeutic efficacy is driven by free target suppression, which is the magnitude and duration of free target decreasing *in vivo* following drug treatment [4].

IP-10 (CXCL10), which is an 8.7 kDa chemokine protein, regulates epithelial proliferation and basal TH1 cell trafficking during normal condition [10–13]. During inflammation, it recruits immune cells to the inflamed tissues of autoimmune diseases in response to inflammatory stimuli [10]. IP-10 has been shown to be upregulated during several chronic inflammatory conditions and autoimmune disorders [13], and is considered a viable target for the treatment of these diseases [11,12]. Anti-IP-10 monoclonal antibodies (mAbs) bind specifically to IP-10 and inhibit its binding to the receptor, C-X-C motif chemokine receptor 3 (CXCR3). The clinical activity of the anti-IP-10 mAbs has been observed in Crohn's disease [11]. The therapeutic efficacy of anti-IP-10 mAbs is driven by the magnitude and duration of free IP-10 suppression. The measurement of free IP-10 suppression as a target engagement biomarker had significant impact on the selection and development of anti-IP-10 mAbs in our drug discovery process. Given its similar affinity for IP-10 in cynomolgus monkeys and humans (Figure 1), the cynomolgus monkey model was used to predict duration and extent of free IP-10 suppression in human. The data provided the basis for differentiating anti-IP-10 mAb candidates and for selecting the most potent candidate for

Human	IP-10	1	VPLSRTVRCTCISISNQPVNPRSLEKLEIIIPASQFCPRVEIIATMKKKGEKRLNPESKA	60
			+PLSRTVRCTCISISNQPVNPRSLEKLEIIP SQFCP VEIIATMKKKGEKRLNPESKA	
Cyno	IP-10	1	IPLSRTVRCTCISISNQPVNPRSLEKLEIIPPSQFCPHVEIIATMKKKGEKRLNPESKA	60
Human	IP-10	61	IKNLLKAVSKERSKRSP	77
			IKNLLKAVSKERSKRSP	
Cyno	IP-10	61	IKNLLKAVSKERSKRSP	77

Figure 1. Sequence alignment between human and cyno IP-10, produced by Blast.

the clinical development. The data were also used for pharmacokinetic/pharmacodynamic (PK/PD) correlation assessment regarding safety and efficacy, and for human dose projection [14]. In this paper, we will focus on the development of immunoaffinity-LC-MS/MS (IA-LC-MS/MS) assays for quantifying free and total cynomolgus monkey IP-10 in serum.

IP-10 is present at low abundance, measured to be 30–60 pM in cynomolgus monkey serum. After dosing with anti-IP-10 mAbs, it was expected that free IP-10 levels would be significantly suppressed. Therefore, a highly sensitive method with a LLOQ of 1 pM was required to enable measurement of the magnitude and duration of free IP-10 suppression and to differentiate anti-IP-10 mAb drug candidates. In this work, IA-LC-MS/MS assays, which combine the advantages of both immunocapture and LC-MS/MS platforms for specifically isolating and detecting proteins of interest [15–23], have been developed. The strategies for maximizing sensitivity by selecting appropriate digestion routes and surrogate peptides, and optimizing the immunocapture procedure to achieve high recovery and minimal matrix effect will be detailed in this paper.

Surrogate matrix is commonly used for calibration standard preparation for biomarker assays as authentic matrix contains the endogenous biomarker [24]. It was recommended that when surrogate matrix was used, similar matrix effect and extraction recovery in both the surrogate and original matrices should be demonstrated [24,25]. In this paper, we highlight a procedure to generate IP-10-free blank cynomolgus monkey serum as the matrix for calibration standards.

Obtaining reliable measurements of both free and total ligand concentrations in the presence of high levels of drug is not straightforward, because the capture reagent might cause drug/ligand dissociation for free assays [14,26], and drugs might interfere the binding between the ligand and capture reagent for total assays. As a result, free ligand levels could be overestimated and total ligand levels could be underestimated. It is crucial to capture what is intended to be measured in order to generate meaningful data. Following dosing with a biotherapeutics, drug bound ligand levels are usually significantly elevated and can reach several orders of magnitude higher than free ligand levels. Free ligand is first separated from the complex by immunocapture prior to measurement using a specific inhibitory anti-ligand capture antibody. The inhibitory antibody is directed toward an epitope that is competitive to the antibody drug of interest. However, the anti-ligand capture reagent could shift the initial equilibrium and cause dissociation of drug–ligand complex. Even a small degree of dissociation from the high abundant drug–ligand complex can result in significant overestimate of free ligand. This issue becomes especially problematic when the drug has relatively low binding affinity and high off-rate. This is a common challenge for free ligand assays regardless of which bioanalytical platform is used, either ligand binding assay (LBA) or IA-LC-MS/MS. The key to a successful free assay is to minimize dissociation of drug–ligand complex [27]. There are very few published papers which show how to minimize drug–ligand complex [28]. Hansen *et al.* [28] examined the effect of preincubation and incubation time on the measurement of free ligand levels and demonstrated that longer incubation time led to overestimated free ligand levels. Lee *et al.* pointed out that it was challenging to develop and verify a free assay due to dynamic changes [14]. In this work, we have developed strategies to minimize dissociation of drug–ligand complex, which enabled the free IP-10 assays even in a challenging case, where the mAb has relatively low binding affinity and high off-rate.

Total IP-10 assays are also required to understand kinetics and equilibrium *in vivo* and perform PK/PD modeling. The typical total ligand assay format involves using a specific noninhibitory anti-ligand antibody as capture reagent.

It was challenging to identify a nonblocking antibody reagent for IP-10, since it is a relatively small protein (MW, 8.7 kDa), while both antibody reagent and antibody drug are large proteins (MW ~150 kDa). Even if both large antibodies bind to different epitopes on IP-10, capture antibody/IP-10 binding may be easily blocked or interfered by drug/IP-10 binding due to steric hindrance. The added challenge for developing an LBA assay is that it requires a third antibody as the detection reagent, to bind to different epitopes from the therapeutic mAb and from capture antibody without competing and interfering. Given these challenges, as far as we are aware, there has not been any total LBA assay available for human, cynomolgus monkey, and mouse IP-10. The details on screening and selecting a nonblocking antibody will be discussed. The total IP-10 assays were verified in the presence of two drug candidates: mAb-I as a high affinity drug and mAb-II as a low affinity drug, respectively.

Experimental

Materials & reagents

Recombinant cynomolgus monkey IP-10 was obtained from ProSpec. Biotherapeutics human IgG anti-IP-10 monoclonal antibodies mAb-I, mAb-II, mAb-III, and mAb-IV were developed in house as drug candidates. Anti-IP-10 mAb capture reagent, AHC2369 was purchased from Invitrogen Antibodies (MA, USA). Magnetic Dynabeads® protein G beads and magnetic streptavidin beads (Dynabeads MyOne Streptavidin T1) were purchased from Invitrogen Life Technologies (CA, USA). Sequencing grade modified trypsin was obtained from Promega Corporation (WI, USA). Sequencing grade Endoproteinase Glu-C was purchased from Roche Diagnostics (IN, USA). Phosphate-buffered saline (PBS) BioReagent and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (MO, USA). A stable isotope labeled (SIL) peptide, SIL-IIPPSQFC [CAM] PHVE, which was labeled with heavy valine (¹³C5 and ¹⁵N) with a mass shift of +6, was custom-synthesized at New England Peptide (MA, USA) and used for internal standard. Carbamidomethyl (CAM) corresponds to iodoacetamide alkylation product of the cysteine in the peptide. PBST buffer was prepared by adding 0.05% Tween-20 into PBS. Control cynomolgus monkey serum was from Bioreclamation, Inc. (NY, USA).

Instrumentation & LC–MS/MS conditions

An AB Sciex TripleTOF 5600™ mass spectrometer equipped with a DuoSpray ionization source was operated in positive ESI mode for peptide sequencing. A Q-TOF method consisted of a full scan TOF survey and information-dependent acquisition MS/MS product ion scans with a resolving power of greater than or equal to 30,000. Data were acquired using Analyst TF1.6 and processed using ProteinPilot™ 4.0 for peptide sequencing. The sequencing data were then imported into Skyline to build and optimize selected reaction monitoring (SRM) methods. An AB SCIEX Triple Quad 6500 system (ABI/MDS Sciex, ON, Canada) equipped with a TurboIonSpray™ source in positive SRM mode was employed for detection of surrogate peptide. Peptide IIPPSQFC[CAM]PHVE was selected as the surrogate peptide with SRM transition (*m/z*) of 712.4 > 599.3 (+2y10). A Shimadzu Nexera LC-30AD UHPLC system was coupled to the mass spectrometer for LC separation. A gradient was applied to a 2.1 × 50 mm, 1.8 μm ACQUITY™ UPLC™ HSS C18 column, which was set at 80°C, with a flow rate of 700 μl/min. The mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The gradient elution started at 2% B in 0.3 min, and increased linearly to 25% B in 3.7 min, followed by column washing and equilibrium. The run time was 5.8 min.

Standard, quality control & internal standard preparations

The IP-10 stock solution at 1 μM was prepared in a PBS buffer containing 0.1% BSA and stored at -80°C. The stock solution of SIL-IIPPSQFC[CAM]PHVE at 1 mM was prepared in water/acetonitrile (9:1) containing 0.1% formic acid and was stored at -80°C. The IP-10-free monkey serum, generated by using mAb-IV, was used as blank matrix for preparation of calibration standards. Calibration standards (1.00, 3.16, 10.0, 31.6, 100, 316, 1000 pM for the free assays, and 10.0, 31.6, 100, 316, 1000, 3160, 10,000 pM for the total assays) were prepared from the IP-10 stock solutions by serial dilutions in the IP-10-free blank monkey serum.

Preparations of mAb conjugated magnetic beads

Magnetic protein G beads and inhibitory anti-IP-10 capture antibodies, mAb-I or mAb-II, were used for the free assays. Magnetic streptavidin beads and noninhibitory anti-IP-10 capture antibody, AHC2369, were used for the total assays. A suspension of 500 μl of the magnetic beads was placed in a vial followed by separation and removal of supernatant with a magnet. The beads were washed three-times with 1 ml of PBST and resuspended in 500 μl

of PBST. For the free assays, 160 µg of anti-IP-10 capture antibody, mAb-I or mAb-II, was added to the protein G beads. For the total assays, 125 µg of anti-IP-10 capture antibody, AHC2369, was added to the streptavidin beads. The beads were incubated for 2 h at room temperature (RT) and washed four-times with 1 ml of PBST containing 1% BSA. After the final wash, the beads were resuspended in 500 µl of PBST buffer containing 1% BSA and stored at 4°C.

Immunoaffinity & digestion

For the free assays, mAb-I- or mAb-II-coupled magnetic protein G beads were diluted with PBST buffer with 1% BSA by tenfold and 20 µl of the diluted beads was added to 100 µl of serum samples in a 96-well plate (Eppendorf 1 ml Protein LowBind). The plate was gently incubated for 0.5 h at 7°C in a Thermomixer R (Eppendorf AG; Hamburg, Germany). For the total assays, 50 µl of serum samples was mixed with 200 µl of PBST buffer followed by the addition of 10 µl of AHC2369-coupled magnetic streptavidin beads. The plate was incubated for 1 h at RT. After incubation, the remaining steps were same for both free and total assays. The beads were washed three-times with PBST (300 µl each) and one time with 50 mM ammonium bicarbonate containing 0.05% zwittergent, followed by elution with 100 µl of 1% acetic acid. The samples were neutralized to an appropriate basic pH with ammonium bicarbonate in water, and then incubated at 99°C for 20 min for protein denaturation. Reduction was conducted by adding 10 µl of 20 mM dithiothreitol (DTT) in water and incubating at 60°C for 15 min. Alkylation was conducted by adding 10 µl of 100 mM iodoacetamide in water and incubating at RT for 20 min in dark. The samples were then digested for 22 h at 37°C using 0.5 µg of Glu-C. SIL internal standard was added followed by incubation for 20 min at 99°C to deactivate Glu-C digestion. The injection volumes for LC-MS/MS analysis were 50 µl and 10 µl for free and total assays, respectively.

Sample preparation for optimizing immunocapture procedure

Three samples, I, II and III were prepared in triplicates. Sample I and II were neat IP-10 samples at 1 nM in 50 mM ammonium bicarbonate with 0.1% BSA, and sample III was a IP-10-spiked serum sample at 1 nM. Sample I (100 µl) was incubated for 1 h at RT. Sample II and III (100 µl each) were mixed with 200 µl of PBST buffer and 10 µl of mAb-I coupled magnetic protein G beads, and incubated for 1 h at RT. The beads were washed using the same procedure as described in the 'Immunoaffinity & digestion' section, followed by the addition of 100 µl of 50 mM ammonium bicarbonate with 0.1% BSA. The remaining steps were same for sample I, II, and III. The samples were incubated at 90°C for 20 min for protein denaturation, followed by the same reduction, alkylation and digestion procedures as described in the 'Immunoaffinity & digestion' section.

Neat IP-10 samples (100 µl each) at a concentration of 1 nM in 50 mM ammonium bicarbonate containing 0.1% BSA were prepared in triplicates and processed by five different procedures respectively. Sample A: the samples were mixed with 10 µl of 50 mM ammonium bicarbonate containing 0.1% BSA (make-up buffer) and incubated for 1 h at RT. Sample B: the samples were mixed with 200 µl of PBST buffer and 10 µl of mAb-I-coupled magnetic protein G beads, and incubated for 1 h at RT. The beads were washed three-times with PBST and one time with 50 mM ammonium bicarbonate (300 µl each) followed by the addition of 110 µl of 50 mM ammonium bicarbonate with 0.1% BSA. Sample C: the samples were mixed with 10 µl mAb-I conjugated magnetic protein G beads and incubated for 1 h at RT. Sample D: the samples were mixed with 10 µl of mAb-I and incubated for 1 h at RT. Sample E: The samples were mixed with 10 µl of magnetic protein G beads. The remaining steps were same for all the samples. The samples were incubated at 90°C for 20 min for protein denaturation followed by the same reduction, alkylation, and digestion procedures as described in the 'Immunoaffinity & digestion' section.

Sample preparation for inhibition titration experiments

IP-10 was spiked at a concentration of 1 nM in the pooled monkey serum samples from three monkeys with increased drug levels. All samples were preincubated for 24 h to reach equilibrium and form IP-10/drug complex. The samples were then processed by the procedures for free and total IP-10 assays, respectively.

Depleted IP-10 blank monkey serum

A suspension of 30 µl of mAb-IV conjugated magnetic protein G beads, an in-house anti-IP-10 mAb antibody, was added to 12 ml of blank monkey serum in a 15 ml tube. The mixture was gently rotated for 30 min. The serum was separated from beads by a magnet, DynaMagTM-15. This depletion process was repeated two more times.

Results & discussion

Assay sensitivity improvement

An LLOQ of 1 pM was required to enable the assessment of the duration and extent of free IP-10 suppression. To achieve the required LLOQ, three aspects of optimization were carried out: selection of digestion route and surrogate peptides; optimization of immunocapture to achieve full recovery, minimal matrix effect and background noise; and generation of IP-10-depleted blank monkey serum for calibration standards.

Selection of digestion route & surrogate peptides

LC–MS/MS methods for protein analysis usually involves enzymatic digestion followed by measurement of surrogate peptides. Even with small proteins, such as IP-10 with a mass of 8.7 kDa, measurement of surrogate peptides is the method of choice to gain much better sensitivity than intact protein measurement. However, small proteins are much more challenging than large proteins in selecting of surrogate peptides due to very limited choices of peptides with the appropriate number of amino acids for good LC–MS/MS performance. These peptides might not provide sufficient signal response to achieve a desired LLOQ. Initially three digestion routes were proposed and tested. Trypsin is commonly used for digestion of proteins due to its high specificity and low cost. However, *in silico* trypsin digestion of IP-10 generated only two peptide candidates of appropriate size, CTCISISNQPVNPR and CLNPESK. LEIIPPSQFCPHVEIATMK is too long to offer a good LC–MS response. In the second digestion route, following trypsin digestion, chymotrypsin was added to further cleave LEIIPPSQFCPHVEIATMK into LEIIPPSQF and CPHVEIATMK. Third, Glu-C digestion was proposed as it generated more peptides of appropriate size. Since there are four cysteines and two disulfide bonds in the IP-10 molecule, digestion with and without reduction/alkylation was evaluated for all three digestion routes under their respective optimized digestion conditions. The peptides with the highest peak area under their respective digestion conditions are shown in Figure 2. The results showed that the peptide IIPPSQFC[CAM]PHVE from Glu-C digestion with reduction and alkylation, was sevenfold more sensitive than the peptide LEIIPPSQF, generated from trypsin/chymotrypsin digestion with reduction and alkylation. However, the peptide IIPPSQFC[CAM]PHVE posed a chromatographic challenge with a broad peak with peak width of 15 s at base under normal UHPLC conditions as shown in Figure 3, which would eventually compromise LLOQ. Proline–proline bond in peptides usually causes peak broadening, splitting, and even baseline separation due to the occurrence of a conformational equilibrium [29]. Gesquiere *et al.* reported that for the proline–proline bond containing peptide tested, almost baseline separation of two conformers was obtained at 0°C, and a single symmetrical peak was achieved at 50°C although the peak remained broad [29], suggesting that the column temperature had significant effect on peak shape. In this work, this issue was successfully resolved by increasing the column temperature from 50 to 80°C (Figure 3). As a result, a sharp peak was obtained and the baseline peak width was narrowed from 15 to 5 s. Finally, IIPPSQFC[CAM]PHVE from Glu-C digestion was selected as the surrogate peptide. Digestion temperature was maintained at 37°C as it was found that higher temperature led to lower digestion efficiency due to thermal instability of Glu-C.

Optimization of immunocapture to achieve full recovery, minimal matrix effect & background noise

An efficient immunocapture method with high recovery, low matrix effect, and low background noise is essential for a highly sensitive assay. During the initial method development, the recovery and matrix effect were evaluated and results are shown in Figure 4. The sample preparation procedures of samples I, II, and III were described in the Experimental section. Recovery was assessed by calculating peak area ratio of sample II or III/sample I. As shown in Figure 4, the immunocapture recovery was only 19% for the neat IP-10 sample. For the IP-10-spiked monkey serum sample, recovery was only 12%, which involved both recovery and matrix effect. To determine the cause of the low recovery, five neat IP-10 samples were processed with the procedures as described in the Experimental section, and the recoveries were summarized in Figure 5. Sample A was incubated only. Sample B was prepared by immunocapture with mAb-I coupled protein G beads followed by washing steps. Sample C was prepared by incubating with mAb-I coupled protein G beads only without washing. Sample D was incubated with mAb-I reagent only. Sample E was incubated with protein G beads only without mAb-I. The recoveries of samples B, C, and D, which involved mAb-I, were equally lower than the recoveries of samples A and E, which did not involve mAb-I. This result indicated that the formation of complex between the capture antibody (mAb-I) and the analyte (IP-10) was the cause of low recovery. The hypothesis is that, because of the formation of the complex, the cleavage sites of IP-10, which the capture antibody either bind to, or block due to steric hindrance, are protected and

Figure 2. LC-MS/MS chromatographic peak area of the peptides, which gave the highest peak area under their respective digestion conditions.

Sample: neat IP-10 digest.

not accessible to Glu-C. This issue was successfully resolved with full recovery (100%) achieved by using a harsh denaturation at 99°C for 20 min to 'inactivate' the capture antibody and release IP-10 for digestion. At 99°C, the capture antibody was permanently deactivated, and would not bind back to IP-10 when temperature returned to RT. The effect of denaturation temperature on peak area response is shown in Figure 6. Neat IP-10 samples at 1 nM were processed by four different procedures: denaturation at 90°C (a) or 99°C (b) for 20 min, followed by reduction, alkylation and digestion; immunocapture with mAb-I conjugated protein G beads at RT, denaturation at 90°C (c) or 99°C (d) for 20 min, followed by reduction, alkylation and digestion. The results showed that 100% recovery was obtained by increasing the denaturation temperature from 90 to 99°C.

After immunocapture, instead of direct on-bead digestion, elution of IP-10 from magnetic protein G beads with acid followed by neutralization to reach optimal pH for Glu-C digestion, was successfully incorporated into the assays to obtain cleaner samples. Figure 7 shows LC-MS/MS chromatograms with and without acid elution for the IP-10-spiked serum samples. With elution, the background peaks and noise levels were significantly reduced. The matrix effect was determined by comparing the peak area of SIL-IIPPSQFC [CAM] PHVE between a post-spiked sample and a neat SIL-IIPPSQFC [CAM] PHVE sample. The overall matrix effect is from 94 to 97%.

Even with the acid elution, the low recovery was still observed if 90°C denaturation was applied. Both IP-10 and antibody were eluted from protein G beads under acidic condition and quickly bound back when the samples were neutralized to basic pH prior to digestion. This observation further verifies that low recovery was caused by the formation of complex of the capture antibody and IP-10. To avoid the formation of the capture antibody/IP-10 complex, streptavidin beads conjugated with biotinylated mAb-I was also tried for the free IP-10 assay as the capture

Figure 3. Effect of column temperature on the peak shape. LC-MS/MS ion chromatograms of the surrogate peptide, IIPPSQFC[CAM]PHVE from IP-10 digest sample with the column temperature at 50°C (A) and 80°C (B).

Figure 4. Evaluation of the recoveries for the neat IP-10 and IP-10-spiked cyno serum samples. The digestion procedure: denaturation at 90°C, reduction, alkylation, and glu-C digestion.

antibody was not eluted from the beads during acid elution. However, the immunocapture recovery was only about 10%. For the same purpose, tosylactivated beads (Dynabeads™ M-280) cross-linked with mAb-I, were also tried but recovery was only 17%.

Figure 5. The IP-10 recoveries of the neat IP-10 samples with different sample pretreatments prior to digestion (denaturation at 90°C, reduction, alkylation, and glu-C digestion). Sample (A): incubating only. Sample (B): immunocapture with mAb-I coupled protein G beads followed by washing. Sample (C): incubating with mAb-I coupled protein G beads without washing. Sample (D): incubating with mAb-I reagent only. Sample (E): incubating with protein G beads only without mAb-1.

Generation of the IP-10-depleted blank monkey serum for calibration standards

The IP-10-depleted blank monkey serum was generated as the matrix for calibration standard preparation to minimize matrix difference between the calibration standards and study samples. IP-10 was removed by immunocapture using a specific anti-IP-10 mAb reagent. The LC-MS/MS ion chromatograms shown in Figure 8 demonstrated that endogenous IP-10 was removed. The peak in panel A represents the endogenous baseline level of IP-10 in the blank monkey serum. There was no corresponding peak in the IP-10-depleted blank monkey serum in panel B, while all other components in blank cynomolgus monkey serum were still maintained. The procedure for IP-10 depletion is simple with minimum cost and allows to handle around 12 × 4 ml serum in parallel.

With all of these optimizations, a highly sensitive IA-LC-MS/MS assay with a LLOQ of 1 pM has been developed. A representative chromatogram of the LLOQ is shown in Figure 8, panel C. As determined by this assay, baseline IP-10 levels in serum for normal monkeys were from 30 to 60 pM, indicating that the LLOQ of 1 pM was good enough to measure at least 97% of IP-10 suppression after drug treatment.

Development of the free IP-10 assays

For the free IP-10 assays, the inhibitory capture reagents only capture free IP-10 while drug-bound IP-10 is removed, as illustrated in Figure 9. The key to a successful free assay is to minimize dissociation of drug-ligand complex. We have examined the effects of the assay conditions on the measured free IP-10 recoveries and established strategies and optimal assay conditions for free assays. Under each assay condition, an inhibition titration test was conducted on the IP-10-spiked serum samples with increasing amount of drug, mAb-II, which was a challenging case for the free assay due to relatively low binding affinity. Table 1 shows the effect of the assay conditions on the free IP-10 recoveries (see the procedures in the 'Experimental' section). Column 3 in Table 1 shows the IP-10 recoveries obtained under the optimal conditions (see the free assay procedure in the 'Experimental' section) and other columns show the recoveries obtained with only one condition changed from column 3, including the capture antibody, incubation time, temperature and the amount of the capture antibody. The recovery of IP-10 was

Figure 6. The effect of denaturation temperature on IP-10 peak area. Sample: neat IP-10 in buffer. Sample (A): direct digestion without immunocapture. Denaturation temperature: 90°C. Sample (B): direct digestion without immunocapture. Denaturation temperature: 99°C. Sample (C): immunocapture with mAb-I coupled protein G beads. Denaturation temperature: 90°C. Sample (D): immunocapture with mAb-I coupled protein G beads. Denaturation temperature: 99°C.

Figure 7. LC-MS/MS ion chromatograms of the surrogate peptide IIPPSQFC [CAM] PHVE from the IP-10-spiked serum samples prepared by immunocapture with (red) and without (blue) elution of IP-10 from protein G beads. SRM: Selected reaction monitoring.

Figure 8. LC-MS/MS ion chromatograms of the surrogate peptide IIPPSQFC [CAM] PHVE from digestion of cyno serum samples. Sample (A): Blank cyno serum for baseline level of IP-10. Sample (B): The IP-10-depleted blank cyno serum used as blank matrix for calibration standards. Sample (C): IP-10 spiked at LLOQ of the method, 1 pM in the IP-10-depleted blank cyno serum. The samples were prepared by free IP-10 assay procedure. SRM: Selected reaction monitoring.

calculated by the peak area ratio of the samples with drugs and the sample without drugs. Therefore, the recovery for the sample without drugs was 100%. The measured IP-10 levels for this sample was around 1 nM, which matched the spiked concentration.

Figure 9. Schematics of LC-MS/MS based free and total ligand assays in the presence of anti-ligand mAb drug. **(A)** free IP-10 assay: only capture free IP-10 by using anti-IP-10 mAb drug as capture reagent. **(B)** total IP-10 assay: capture both free and drug bound IP-10 by using a non-inhibitory anti-IP-10 mAb as capture reagent.

Table 1. The effects of the assay conditions on free IP-10 recoveries.

mAb-II Con. (pg/ml)	Molar ratio of drug/IP-10	0.48 μ g mAb-II at 7°C for 30 min (%)	0.48 μ g mAb-III at 7°C for 30 min (%)	0.48 μ g mAb-II at 7°C for 60 min (%)	0.48 μ g mAb-II at 25°C for 30 min (%)	1.2 μ g mAb-II at 7°C for 30 min (%)
1.5E + 08	1000	1.1	9.5	10.4	8.3	2.6
1.5E + 07	100	11	32	48	46	16
1.5E + 06	10	45	61	90	69	49
1.5E + 05	1	89	96	94	94	83
1.5E + 04	0.1	100	96	97	89	96
1.5E + 03	0.01	101	97	92	89	100
1.5E + 02	0.001	98	98	105	95	101
1.5E + 01	0.0001	100	96	100	94	92
1.5E + 00	0.00001	102	100	90	100	107
0	–	100	100	100	100	100

- Selection of appropriate capture reagents: during the immunocapture process, both the drug and capture reagent compete for binding to the ligand. Therefore, the differences in binding affinity and on/off rates could cause a shift from the initial equilibrium. To reduce the dissociation of the drug–ligand complex, our strategy is to use the dosed therapeutic antibody drug as the capture reagent so that the drug and the capture reagent are identical and bind to the same epitope with the same binding affinity and on/off rate. The added benefit is that it saves significant time, effort, and cost in capture reagent screening. The data in columns 3 and 4 of Table 1 show the effect of capture antibody on the free IP-10 recoveries. Compared with mAb-II, mAb-III bind to the same epitope but has 25-fold higher binding affinity. As a result, the measured free IP-10 recoveries by using mAb-III as the capture reagent was much higher when the molar ratio of drug/IP-10 reached 10 and above.

Figure 10. Inhibition titration test for free IP-10 assay on IP-10 spiked serum samples with increasing amount of mAb-II.

- Incubation time: the incubation time was limited to 30 min because longer incubation time led to a shift from initial equilibrium. As shown in column 5, the free IP-10 recoveries were significantly increased with longer incubation time when the molar ratio of drug/IP-10 reached 10 and above.
- Incubation temperature: the incubation temperature was lowered to 7°C to reduce the binding exchange rate. The data in columns 3 and 6 in Table 1 show the effect of the incubation temperature on the free IP-10 recoveries. The free IP-10 recoveries were significantly increased with the molar ratio of drug/IP-10 at 10 and above due to the increased temperature.
- Amount of the capture antibody: to minimize dissociation, the amount of the capture antibody was optimized to a minimum. The data in columns 3 and 7 in Table 1 show more capture reagent led to higher free IP-10 recoveries when the molar ratio of drug/IP-10 reached 100 and above.

In addition, the dilution was avoided in the free IP-10 assay since dilution inevitably causes dissociation of ligand–drug complex [28]. With these strategies, we have developed the free IP-10 assays for both mAb-I as a high affinity drug and mAb-II as a low affinity drug, respectively. Figure 10 shows an inhibition titration graph for the IP-10-spiked monkey serum samples (1 nM) with increasing amount of drug (0 to 1500 µg/ml). The recovery decreased with the increased drug concentration, and the recoveries at 1.5E8 and 1.5E9 pg/ml were 1.1% and below quantitation limit (BQL), respectively. Titration of drug led to complete inhibition of the free IP-10 signal. The calculated IC₅₀ from the titration result was 8.3 nM, which is comparable to the K_d (~5 nM) from Biacore-based binding studies, further confirming that the assay is a free assay. The data generated from the free assay showed that the free IP-10 levels were fully suppressed following treatment with mAb-I in the monkey PK/PD studies.

Development of the total IP-10 assays

For the total IP-10 assays, the reagent and drug should bind to different epitopes of IP-10 without overlapping regions of recognition, and the binding between IP-10 and the capture reagent should not be affected by the binding between IP-10 and the drug. Therefore the capture reagent can capture both free and drug-bound IP-10, as illustrated in Figure 9. Since IP-10 is a small protein, two large antibodies (MW, 150 kDa) may easily block or interfere with each other by steric hindrance even if they bind to different epitopes on IP-10. A total of five capture reagents were screened by using inhibition titration test with drug levels up to 500 µg/ml. One capture antibody (AHC2369) was identified with good recoveries of IP-10 for the samples containing mAb-I and mAb-II, respectively. The verification of the total assay in the presence of mAb-II is shown in Figure 11. Although the LLOQ of 1 pM could be achieved for the total assays, the LLOQ of 10 pM is low enough as the total IP-10 levels were boosted following the drug treatment. A standard curve from 10 pM to 10 nM was defined for the total IP-10

Figure 11. Verification of total IP-10 assay in the presence of anti-IP-10 mAb-II showing consistent concentration obtained with drug levels up to 500 µg/ml. Spiked IP-10 in pooled cyno serum at 1000 pM.

assays to cover the concentration range for all the PK/PD samples. The data generated from the total assay showed that the total IP-10 levels were significantly increased following dosing with mAb-I in the monkey PK/PD studies.

Assay performance

Both free and total assays for the samples containing mAb-I were validated by evaluating accuracy, precision, recoveries, and selectivity. Three accuracy and precision runs with six replicates at each quality control (QC) level in each run were used to assess the accuracy and intra- and inter-assay precision. Accuracy was evaluated by calculating the percent deviation of the average measured concentrations at each QC level from the corresponding nominal concentrations. Good accuracy was obtained for all QC levels including LLOQ for both free and total IP-10 assays. The accuracy was within $\pm 7\%$ and $\pm 4\%$, for the free assay and total assay, respectively. The intra-assay and inter-assay precisions were 12 and 4%, respectively, for the free assay, and 8 and 10%, respectively, for the total assay. Specificity test was conducted by testing IP-10-depleted monkey serum blank matrix, blank matrix spiked with internal standard only, and blank matrix spiked with IP-10 at LLOQ level for six different lots. Specificity has been demonstrated for both free and total IP-10 assays as no interference peak was observed in the SRM channel of IP-10 for all of the six different lots of matrix tested. A linear least-squares regression with weighting factor of $(1/x^2)$ was used [30]. The representative calibration curves for both free and total assays are shown in Figure 12. LLOQs of 1 pM for the free assay (Figure 8, panel C) and 10 pM for the total assay were determined with sufficient S/N ratios and no interference peak was observed at the corresponding retention time. The assays have been employed to analyze thousands of the PK/PD study samples with demonstrated robustness and consistent data.

Conclusion

To the best of our knowledge, this is the first report of IA-LC–MS/MS assays for free and total monkey IP-10 measurement following anti-IP-10 mAbs treatment. An LLOQ of 1 pM has been achieved for the free IP-10 assay using only 100 µl serum. It was demonstrated that the strategies for maximizing assay sensitivity by selection of appropriate digestion route and surrogate peptide, and optimization of immunocapture procedure were very critical in the successful development of free and total IP-10 assays with low LLOQ. The suitable capture antibodies have been identified to capture what intend to be measured in the presence of high levels of anti-IP-10 mAb drugs for the free and total assays, respectively. The data were used to differentiate drug candidates, establish PK/PD relationship regarding safety and efficacy, and determine human dose.

Future perspective

The incorporation of target engagement biomarkers from drug discovery through development process has become increasingly important for decision-making and guidance of clinical trials. For biotherapeutics directed against

Figure 12. Representative standard curves of IP-10 for free and total assays in cyno serum.

soluble targets, efficacy is driven by free target suppression, which is a target engagement and efficacy biomarker. However, it can be highly challenging to measure decreasing levels of free target following biotherapeutic treatment since many of the soluble targets of therapeutic interests are of low abundance and rapid turnover [4]. Therefore, a highly sensitive method is required. As demonstrated in this work, IA-LC–MS/MS assays can fulfill sensitivity requirements for the accurate quantification of low abundance endogenous proteins and the reduced free target levels following drug administration. The strategies that we have established for sensitivity improvement have been not only used to gain the sensitivity for monkey IP-10 but also successfully applied to develop highly sensitive IA-LC/MS/MS assays for human IP-10 and other low abundance targets. The research demonstrated that IA-LC–MS/MS is a promising tool for measurement of proteins of low abundance.

Another significant challenge for free ligand measurement is that the drug/ligand dissociation under assay conditions can lead to overestimation of free ligand levels. This is a common challenge for free ligand assays regardless of the bioanalytical platform. The strategies that we have established in this work to minimize drug/IP-10 dissociation, have been successfully applied to the free assays for other protein ligands in our laboratory.

This research also demonstrated the advantages of IA-LC–MS/MS assay for total ligand (target) measurement in the presence of anti-target mAbs. Compared with LBA assay, this platform does not require a third antibody, in other words, detection reagent, to bind to IP-10 without interfering the bindings of drug/IP-10 and capture antibody/IP-10, respectively. It is challenging, costly, and time-consuming during the overall process to generate and identify capture-detection antibody pairs, especially for small proteins, such as IP-10.

Summary points

Aim

- The measurement of free IP-10 suppression as target engagement biomarker was required for the evaluation of drug effect on the target.
- A sensitive method with an LLOQ of 1 pM was required to enable evaluation of the duration and extend of free IP-10 suppression and to differentiate anti-IP-10 mAb drug candidates.
- Total IP-10 assays were also required to understand kinetics and equilibrium *in vivo*.
- Reliable determinations of both free and total ligand concentrations in the presence of the drugs are not straightforward because the capture antibodies might cause drug/ligand dissociation for the free assays, and the drugs might interfere the binding between the ligand and the capture antibodies for the total assays.

Results

- Developed the strategies to maximize sensitivity.
- Established strategies to develop free ligand assays in the presence of anti-ligand mAbs.
- Developed and verified free and total IP-10 assays.

Conclusion

- IA-LC–MS/MS assays for free and total IP-10 in monkey serum have been developed.
- LLOQ of 1 pM has been achieved for the free IP-10 assays by using only 100 µl serum.
- Serum target engagement was demonstrated. The data have been used to differentiate drug candidates, establish PK/PD relationship regarding safety and efficacy, and determine human dose.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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