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Quantification of Alefacept, an immunosuppressive fusion protein in human plasma using a protein analogue internal standard, trypsin cleaved signature peptides and liquid chromatography tandem mass spectrometry

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ABSTRACT

Quantitative analysis of a therapeutic protein through use of surrogate proteotypic peptides was evaluated for the measurement of Amevive (Alefacept) in human plasma using liquid chromatography tandem mass spectrometry. Signature peptides were obtained through *in silico* and iterative tuning processes to represent Alefacept for quantification. Horse heart myoglobin was chosen as a protein analogue internal standard to compensate for errors associated with matrix effects and to track recovery throughout the entire sample pretreatment process. Samples were prepared for analysis by selective precipitation of the target proteins with pH controlled at 5.1 and heat denaturation at 45 °C followed by enzymatic digestion, dilution, and filtration. On-line extraction of the signature peptides was carried out using a Phenomenex Gemini C18 security guard column (4.0 mm × 2.0 mm) as a loading column and a Gemini C18 (100 mm × 2.1 I.D., particle size 5 μm) as the analytical (eluting) column. Tandem mass spectrometric detection was performed on a hybrid triple quadrupole linear ion trap equipped with electrospray ionization to positively ionize signature peptides for Alefacept and myoglobin. The method was linear for Alefacept (protein) concentrations between 250 and 10,000 ng/mL. Precision and accuracy for inter- and intra-assay for the lower limit of quantification was less than 20% (16.2 and 10.3, respectively). The method was validated according to current FDA guidelines for bioanalytical method validation.

1. Introduction

Autoimmune diseases rank third behind heart disease and cancer in the United States population and are the most common cause of diseases in the world [1]. Psoriasis is one of these immune mediated diseases, which affects approximately 7.5 million Americans [2]. It affects the skin and joints when the immune system conveys incorrect signals that accelerate the growth cycle of skin cells. Treatments include topical, phototherapy, traditional systemic and biological medications (biologics). Currently the biological medication of choice for treatment of psoriasis is injectable Alefacept, sold under the brand name Amevive.

Alefacept is a dimeric immunosuppressive fusion protein that blocks the activation of T white blood cells, and results in a reduction in skin inflammation. Alefacept consists of fused extracellular CD2-binding portion of the human leukocyte function antigen-3 (LFA-3) linked to the Fc (hinge, CH2 and CH3 domains) portion of human IgG1 [3]. It is approximately 91 kiloDaltons (kDa), with therapeutic levels between 500 and 6000 ng/mL [4].

Pharmacokinetic studies of biologics such as Alefacept are currently performed with immunoassays that can be costly and may require time-consuming method development. Immunoassays are also often associated with selectivity problems related to cross reactivity in biological fluids that may result in imprecise data and/or falsely elevated results [5]. It has been well established that liquid chromatography tandem mass spectrometry is the method of choice for small molecule clinical studies and has been gaining ground for large molecule quantification [6,7]. Use of signature peptides that act as surrogates for targeted protein quantification when coupled to liquid chromatography tandem mass spectrometry offers a potentially superior methodology for clinical studies and biomarker validation [8]. This approach requires proteolytic digestion to yield signature peptides that will ultimately be quantified using a triple quadrupole mass spectrometer with multiple reaction monitoring. Gerber et al. pioneered an absolute quantification (AQUA) strategy in 2003, which quantifies proteins using signature peptides and a synthetic, isotopically labeled peptide internal standard [9]. Quantification with synthetic peptide internal standards has been carried out successfully, however the lack of tracking the enzymatic digestion is a potential source of error. Addona et al. performed a multi-laboratory assessment of quantification using synthetic peptide internal standards for three experimental

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designs [10]. All laboratories yielded good precision and accuracy when synthetic internal standard peptides were incorporated into digested plasma. The imprecision proved significant however when synthetic peptide internal standards were added post-digestion for target proteins that were standardized in non-digested plasma. This indicates that a synthetic peptide internal standard may not sufficiently track digestion and therefore overall recovery. Isotopically labeled internal standards can be added before digestion however, most published works have done this after digestion. Protein internal standards may be more suitable because they can be digested with the target protein and therefore track digestion recovery.

Several approaches have been investigated for purification of target proteins. Immunodepletion of the highly abundant proteins (HAPs) in human plasma prior to enzymatic digestion is an effective method for reducing superfluous background in the matrix [11–15]. The drawbacks associated with this technique include expensive kits, possible imprecision, minimal volume applied, and minimal column life (<200 samples) [16]. Immunopurification of the target protein can also be accomplished and has also been shown to yield lower limits of detection [17–21]. However, the requirement for antibodies for immunopurification makes this technique less desirable. Stable Isotope Standards and Capture by Antipeptide Antibodies (SISCAPA) is an approach which provides an alternative through employing anti-peptide antibodies to capture the signature peptide and reduce matrix effects [6]. The SISCAPA approach has been shown to be effective in reaching low ng/mL levels in human plasma or serum [15,22–25], but production of antibodies for peptides is also time-consuming and costly. Other methods have employed two-dimensional solid phase extractions [26,27] for protein and/or signature peptide purification, which may require several optimizations. Yang et al. were able to reach low ng/mL concentrations (3.6 ng/mL LLOQ) for pegylated-interferon alpha (α_{2a}) using a monolithic C18 solid phase extraction for target protein enrichment and mixed mode cation exchange (Waters Oasis MCX) for digested sample clean-up prior to LC–MS/MS [27].

Control of digestion and tracking of signature peptides is critical to ensure precise and accurate results. Protein internal standards offer tracking of the digestion step due to introduction at the beginning of sample preparation. Under ideal conditions where all preparation steps are stoichiometric, an isotopically labeled protein internal standard may be appropriate as demonstrated in previous studies [28,29]; however, synthesis time and expense may still be limitations. Protein analogue internal standards are good candidates for this process since they may have similar intrinsic properties (pI, hydrophobicity) as the target protein. Protein analogue internal standards undergo the same preparation steps as the target protein, and signature peptides are generated in a similar fashion for quantification. The key to making this work is to match similar retention times for the signature peptides representing the target protein and analogue internal standard, respectively. Retention time similarities will facilitate correction of matrix effects. Yang et al. have successfully employed a protein analogue internal standard to yield precise and accurate results at ng/mL concentrations in biological fluids [26,27].

In the present work, our current method combines selective protein precipitation with use of a protein analogue internal standard (horse heart myoglobin) to quantify Alefacept in human plasma.

2. Experimental

2.1. Chemicals and reagents

Alefacept (Amevive) was purchased from Virginia Commonwealth University Medical Center Pharmacy. Horse heart myoglobin, sodium hydroxide, glacial acetic acid, hydrochloric acid, iodoacetamide, dithiothreitol, proteomics grade trypsin, and ammonium bicarbonate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Deionized water was obtained in-house using a Nanopure Diamond water system from Barnstead International (Dubuque, IA, USA). Methanol, isopropanol, acetonitrile were purchased from Burdick and Jackson (Muskegon, MI, USA). Acetic acid was procured from Curtin Matheson Scientific Inc. (Houston, TX, USA). Formic acid was obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA). K_2EDTA Human Plasma was obtained from BioChemed Services (Winchester, VA, USA).

2.2. Materials and equipment

Plasma samples were aliquoted into 1.5 mL microcentrifuge tubes purchased from VWR International (Westchester, PA, USA). Samples were filtered using nanosep MF 0.2 μm filters from Pall Life Sciences (Ann Arbor, MI, USA). All centrifugation was carried out using an Eppendorf 5804R centrifuge (Hamburg, Germany). All mixing was performed using a multi-tube vortex mixer from VWR International (Westchester, PA, USA). A syringe pump from Harvard Apparatus (Holliston, MA, USA) was used to infuse solutions for tuning optimization and post-column infusion.

2.3. Instruments and HPLC conditions

High performance liquid chromatography (HPLC) separations were performed using the following equipment: Shimadzu system controller SCL-10A VP, pumps LC-10AD VP, solvent degasser DGU14A (Shimadzu, Kyoto, Japan). An HTS PAL autosampler from CTC Analytics (Zwingen, Switzerland) and a CH-30 column heater from Eppendorf (Westbury, NY, USA) were used. Column trapping was performed using a Phenomenex Security Guard column (Gemini C18, 4× 2.0 mm) as the loading column and a Gemini C18 column (100 mm × 2.0 mm I.D., 5.0 μm) as the elution column, both from Phenomenex (Torrance, CA, USA). Three Shimadzu pumps were operated with one controller to apply the gradient conditions. Mobile phases from pumps A and C consisted of 0.1% formic acid and mobile phase B (0.1% formic acid in acetonitrile) was delivered from pump B. The initial loading conditions used 100% aqueous mobile phase (0.1% formic acid) from pump C. Following a short loading time (0.5 min), the diverter valve switched to position B and the gradient initiated. Mobile phase A and B were coupled together with a mixer and the elution conditions started with 5% mobile phase B (0.1% formic acid in acetonitrile). During loading of the sample, the mobile phases were delivered at 0.5 mL/min to provide sufficient flow through the trapping column along with continuous flow into the mass spectrometer. The flow was reduced to 0.25 mL/min during elution onto the analytical column and the column temperature was maintained at 45 °C. Gradient conditions are plotted in Fig. 1 for mobile phases A, B and C. A 10-port Cheminert switching valve and a microelectric actuator obtained from Valco Instruments Co. Inc. (Houston, TX, USA) was used to perform on-line column trapping. There were two autosampler rinses. The first rinse consisted of a cocktail of acetonitrile:methanol:isopropanol:water in the ratio of 40:20:20:20 and the second rinse was 1:1 mobile phases A and B. The mass spectrometer was an API 4000Qtrap hybrid triple quadrupole/linear ion trap from AB Sciex (Foster City, CA, USA) with Analyst 1.5 data acquisition software. The data analysis was performed using Quantitation Wizard processing software that accompanies Analyst. MRMPilot 2.0 (AB Sciex-Foster City, CA, USA) and was used to facilitate modeling of signature peptides. All nitrogen gas was generated from a Parker Hannifin (Haverhill, MA, USA) Tri-Gas Generator LC/MS 5000. Fig. 2 represents an overall schematic diagram of the instrument including the plumbing for column trapping.

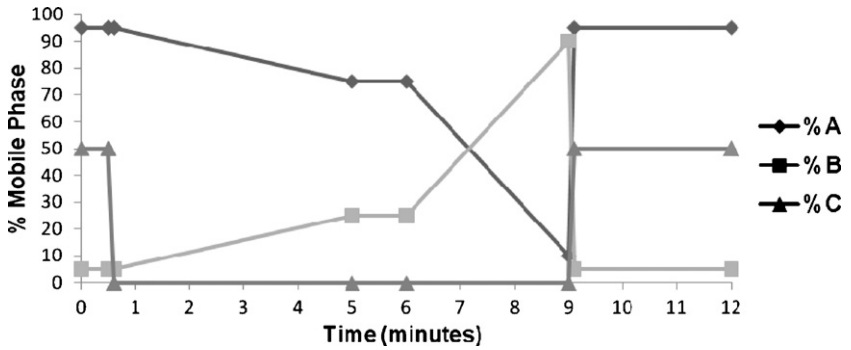

Fig. 1. Gradient conditions are plotted for mobile phases A, B, and C.

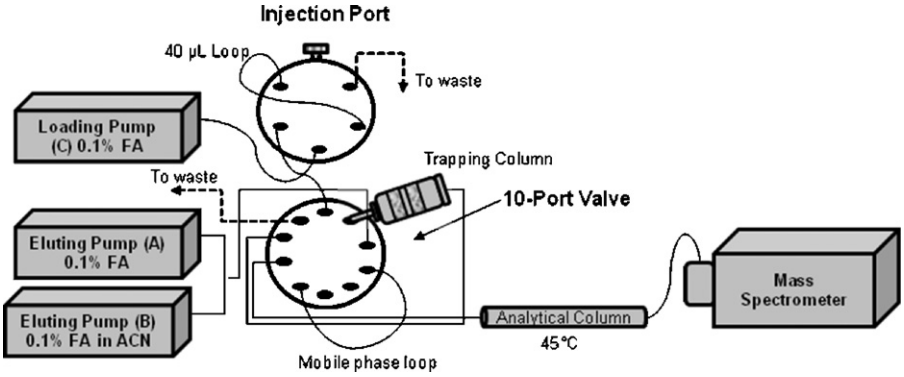

Fig. 2. Schematic of the overall instrument set-up including column trapping plumbing.

2.4. Mass spectrometer parameters

The mass spectrometer was operated in the positive electrospray ionization (ESI) mode with selected reaction monitoring (SRM) of signature peptides for Alefacept and horse heart myoglobin. Potential signature peptides were obtained through *in silico* digestions using MRMPilot 2.0 and sequence homology evaluations were performed using the basic local alignment search tool (BLAST-http://blast.ncbi.nlm.nih.gov/Blast.cgi). Signature peptides for Alefacept and myoglobin were optimized using multiple iterative processes of tuning, *in silico* predictions of cleaved peptides, SRM transitions, and collision energy voltages, and finally LC–MS/MS data collection. Tuning was performed two ways, direct infusion and LC–MS/MS injection by evaluation of the following scan types: Q1 full scan, precursor ion scan, product ion scan, and finally SRM to determine the final SRM(s) transitions that would represent Alefacept and myoglobin for signature peptide quantification. A 5 μg/mL solution of Alefacept and myoglobin prepared in 50 mM ammonium bicarbonate was digested with trypsin. This digested sample was used as a tuning solution for direct infusion at 10 μL/min tee'd with mobile phase at a flow rate of 0.20 mL/min. Mobile phases A and B composition was varied to evaluate intensity changes during tuning optimization. The same solution was also injected with similar gradient conditions described in Section 2.3 to determine the optimal conditions. The mass spectrometer parameters were tuned and optimized to achieve maximum sensitivity which resulted in the following parameters: source temperature 325 °C, ion spray voltage 5500 V, gas 1 (GS1) 38, gas 2 (GS2) 28, collision activated dissociation (CAD) was set on high, channel electron multiplier (CEM) 2200 V, declustering potential (DP) 109, entrance potential (EP) 15, and collision exit potential (CXP) set on 13. Alefacept and myoglobin signature peptides eluted at approximately 4.22 and 3.65 min, respectively. Table 1 SRM transitions, collision energies (CE), signature peptide information, and dwell times used during this study.

2.5. Stock solution and working solution preparation

The Amevive (Alefacept) vehicle was diluted with sterile water to yield a concentration of 7.5 mg/mL. This stock solution was subsequently diluted with water to prepare an intermediate working solution of 1 mg/mL. This working solution was then spiked in plasma to prepare the high standard (10,000 ng/mL) and high quality control (8000 ng/mL), which was then used to prepare the remaining calibration points and quality controls. Horse heart myoglobin was diluted in deionized water for the internal standard

Table 1
Optimized mass spectrometer conditions for signature peptides. Selected reaction monitoring (SRM) transitions and selected parameters.

Signature peptide	Q1	Q3	Signature peptide information	Dwell (ms)	Collision energy (V)
AQSP[a]	597.4	894.5	VAELENSEFR → LENSEFR (+2/y7)	200	31
AMSP1[b]	597.4	781.8	VAELENSEFR → ENSEFR (+2/y6)	200	31
AMSP2[c]	597.4	652.4	VAELENSEFR → NSEFR (+2/y5)	300	31
Myoglobin[d]	636.3	716.3	LFTGHPETLEK → ETLEK (+2/y6)	200	50

[a] Alefacept quantified signature peptide.
[b] Alefacept monitored signature peptide 1.
[c] Alefacept monitored signature peptide 2.
[d] Myoglobin signature peptide (analogue internal standard).

solution at a concentration of 1 mg/mL. Internal standard spiking solution was prepared fresh daily. Alefacept working solutions were prepared immediately prior to spiking into the plasma.

2.6. Preparation of calibration standards and quality control samples in human plasma

Pooled dipotassium EDTA human plasma from at least two donors was used to prepare the calibration standards and quality controls. A volume of 0.250 mL of the intermediate solution (1.0 mg/mL) of Alefacept was spiked into 25 mL plasma to obtain the high standard (10,000 ng/mL). The high standard was spiked into 10 mL volumetric flasks to prepare the remaining seven calibration standards (250, 500, 750, 1000, 2500, 5000, and 9000 ng/mL). A volume of 0.200 mL of the intermediate solution (1.0 mg/mL) of Alefacept was spiked into 25 mL plasma to obtain the highest quality control (HQC = 8000 ng/mL). The HQC standard was spiked into 25 mL volumetric flasks to prepare the remaining quality control samples representing the lower limit of quantitation (LLOQ) QC, low QC (LQC), medium QC (MQC) and high QC (HQC) quality controls, which were prepared at 250, 600, 2000, and 8000 ng/mL, respectively. A dilution control was prepared at 2.5 times the highest calibration standard (25,000 ng/mL), to evaluate dilution of samples within the calibration range. The dilution control was diluted five-fold with blank plasma to obtain a concentration within the calibration range (5000 ng/mL). Calibration standards (STDs) and quality control samples were freshly prepared for the first analytical run to establish a day zero nominal value for storage stability studies. The calibration standards and QC samples were prepared in a similar fashion to contain less than or equal to 5% (v/v) of the intermediate solution in order to simulate real matrix samples as much as possible. The STDs and QC samples were divided into aliquots of 0.750 mL each, and stored at −20 °C until analysis.

2.7. Sample preparation

The sample preparation procedure involved a selective precipitation followed by reduction, alkylation, trypsin digestion, dilution, and filtration prior to LC–MS/MS analysis. Each STD, QC, or plasma blank were aliquoted (0.350 mL) into 1.5 mL microcentrifuge tubes and diluted to 0.500 mL with 50 mM ammonium bicarbonate. Twenty microliters of myoglobin internal standard spiking solution (solution = 1 mg/mL or 58.9 μm) was added into the tube followed by brief vortex mixing. The pH was then adjusted to 5.1 with 1.0 M acetic acid and samples were incubated at 45 °C for approximately 10 min. The supernatant from the samples was then chemically reduced with 0.040 mL of dithiothreitol (100 mM of DTT) and incubated for 10 min at 45 °C. Following reduction, samples were alkylated with 0.080 mL 100 mM iodoacetamide and incubated for an additional 20 min in the dark. Samples were allowed to equilibrate to room temperature, and the pH was adjusted to 8.5 for optimal pH conditions for trypsin digestion with an approximate enzyme to substrate ratio of 1:20 (w/w). Samples were digested overnight for approximately 16 h at 37 °C. Digestion was stopped with 1 M acetic acid, followed by a dilution with 0.150 mL of 1:1 mobile phase A:mobile phase B. Samples were mixed thoroughly and filtered through 0.2 μm filters, pipette transferred to a 96-well 2 mL plate, and injected into the LC–MS/MS using a 50 μL injection.

2.8. Selective denaturation

Five different pH adjustments (pH 3.0, 4.7, 5.1, 7.6, 9.0) were applied to plasma samples incubated at five varying temperatures (22, 37, 45, 65, 95 °C) over the course of 10 min. The isoelectric points of two of the most abundant background proteins were investigated along with the average isoelectric points (pI) of Alefacept and myoglobin. Albumin (pI = 4.7), IgG (pI = 7.2), and the average pI of Alefacept (pI = 7.8) and myoglobin (pI = 7.2) were evaluated (average pI = 7.6). Adjustment of the pH to 5.1 was also evaluated based on previous experimental results with myoglobin. A recent study by Elena Saguer et al. indicated the secondary structure of serum albumin begins to unfold at pH 5, which leads to protein aggregation [30]. Employment of pH adjustments of 3.0 and 9.0 were evaluated to determine if pH adjustment well away from the pI is necessary to maintain the solubility of target proteins. Signature peptides were monitored for target and background proteins (albumin, IgG, transferrin) to evaluate the change in peak area response with pH adjustment and various temperature incubations. A sample was prepared in plasma at a concentration of 10 μg/mL and analyzed in triplicate for each condition. Background signature peptides were identified from *in silico* predictions, evaluations and literature sources [11], and were optimized accordingly. The background signature peptides monitored were: albumin (575 → 937), IgG (839–262), and transferrin (815–693).

2.9. Digestion time study

The trypsin incubation time was evaluated to determine if the digestion time could be reduced to improve throughput efficiency. The majority of the literature indicates overnight incubation times are needed; however, accelerated trypsin digestion by employment of microwave irradiation and convection has been successfully demonstrated by Lesur et al. [31], this was not available in our laboratory. A study was performed with 5 incubation time points (0.08, 1, 2, 4, and 20 h) to evaluate precision of quality control samples and the internal standard. Quality controls 1–3 were extracted in plasma as described above in triplicate with the different incubation periods for enzymatic digestion. Signature peptides were monitored for Alefacept and myoglobin. The +1 and +2 charge states of the myoglobin signature peptide were monitored, but the intensity for other myoglobin peptides was not sufficient for quantification or for any of the other experiments such as the digestion time study.

2.10. Matrix effects evaluations and recovery

Matrix effects were evaluated for potential ion suppression or enhancement of signature peptides along with monitoring of phospholipids as a surrogate for matrix effects [32]. A post-column infusion study was performed similarly to previous methods [33,34]. This experiment employed a sample that was extracted from 50 mM ammonium bicarbonate according to the sample preparation described previously as a clean matrix while injecting a blank plasma extracted sample as the matrix sample. The resulting profile was evaluated for any change in the ESI response of signature peptides for Alefacept and myoglobin. Phospholipids were monitored using the quasi-SRM transition 184–184 during this post-column infusion study as a likely candidate for suppression or enhancement of Alefacept and/or myoglobin signature peptides as suggested by previous phospholipid monitoring studies [32,35]. A post-extraction addition study to evaluate absolute matrix effects was also assessed using a similar method as suggested by Matuszewski et al. [36]. The experiment was performed by comparing the peak areas of processed blank plasma samples spiked with low, medium, and high concentrations of Alefacept (600, 2000 and 8000 ng/mL) in triplicate. Spiking was performed by diluting extracted blank plasma samples with a ratio of 1:1 with clean matrix extracted samples. A dilution factor of two was applied to the post-spike samples in order to compare the peak areas to the same non-diluted clean matrix extracted samples.

In addition to post-extraction addition investigations, a multiple donor source matrix evaluation was carried out as recommended

by Ismaiel et al. [37]. Six different sources were fortified with Alefacept at the LQC level (600 ng/mL) and analyzed in triplicate. Concentrations were calculated from the calibration curves analyzed in the same run.

A relative recovery experiment was performed for Alefacept in human plasma. Signature peptide peak areas of plasma extracted and solvent (50 mM ammonium bicarbonate) extracted quality control samples were compared. All samples were analyzed in triplicate at three different concentrations (LQC, MQC, and HQC). Absolute recovery was not performed due to the lack of standard peptides to represent the target and internal standard signature peptides.

2.11. Linearity

Eight calibration standards were prepared by serial dilution at concentrations of 250, 500, 750, 1000, 2500, 5000, 9000, and 10,000 ng/mL in dipotassium EDTA human plasma. All standards were analyzed in duplicate, calculating Alefacept signature peptide: myoglobin signature peptide internal standard peak area ratios for each concentration level. Standard curves were constructed using linear regression and a $1/x^2$ weighing factor was employed for the determination of Alefacept concentrations.

2.12. Precision and accuracy

Precision and accuracies were determined by analysis of QC samples at three different concentrations and were analyzed in three separate runs. QC concentrations were calculated from the calibration curves analyzed in the same run. Intra- and inter-assay precision and accuracy were determined by extracting LLOQ, low, medium, and high quality controls in six replicates for intra-assay performance. A dilution control was also evaluated for intra-assay performance with six replicates. Quality controls were extracted in triplicate in two additional runs for a total of three analytical runs for inter-assay performance.

2.13. Selectivity

Human plasma samples from six different sources were analyzed in duplicate to evaluate selectivity with regard to interferences. Each individual lot was extracted as described above (Section 2.7) with and without the addition of the myoglobin internal standard. Selectivity requirements were that the peak areas co-eluting with Alefacept must be less than 20% of the peak area of the average of LOQ samples of Alefacept for all six lots of blank plasma sources. Crosstalk interference was evaluated in pooled plasma requiring no more than 5% contribution from Alefacept to the myoglobin internal standard peak area. A high standard (10,000 ng/mL) was extracted absent of myoglobin in duplicate to evaluate the Alefacept contribution to the internal standard. The myoglobin internal standard contribution to Alefacept was evaluated in each run and in six different sources.

2.14. Stability and carryover

The stability evaluations of stock solutions were minimized due to daily preparation. Following the first validation run with freshly prepared calibration standards and quality controls, STDs and QCs were frozen at −20 °C and sub-aliquots were used each day for analysis. Long term storage stability was evaluated by using the day zero nominal value established by extraction of freshly prepared low and high controls. Post-preparative stability (PPS) was performed to evaluate extracted samples stored in the autosampler beyond 24 h at 5 °C in the event of an instrument malfunction requiring re-injection of samples. PPS was assessed from re-injection reproducibility after storage of quality control samples in the auto sampler for 48 h.

Carryover was assessed by injecting blank and/or extracted buffer samples immediately after each of the highest calibration standards (10,000 ng/mL) in an analytical run. The criterion for carryover in this experiment was the LQC must be accurate to within 15%.

3. Results and discussion

Signature peptides for target protein quantification have been evaluated various ways. Here, we present an alternative technology by coupling selective denaturation with a protein internal standard for the quantitative analysis of our target protein, Alefacept. Use of controlled pH and temperature allowed for a reduction in the major background proteins and maintained the target protein. The myoglobin internal standard was critical in order to obtain precise and accurate results. This investigation is a simple process and offers an alternative to isotope labeled proteins for internal standards and more complex purification processes such as immunoprecipitation.

3.1. LC–MS/MS

Signature peptides were identified using *in silico* predictions, direct infusion, and injection of solvent based samples to obtain the most intense signature peptides using positive electrospray ionization. Each precursor ion underwent extensive evaluation of gas pressures and voltages to obtain the most intense product ion peaks. Optimized collision energy voltages for product ions were comparable with the model generated recommendations made using MRMPilot. Optimization of the collision energies was critical in achieving maximum sensitivity. It was observed that changing collision energy ±10 units from decreased intensity significantly. Additionally, injection of clean digested samples to evaluate potential product ions yielded greater intensity than direct infusion experiments. Injecting a clean digested sample through an analytical column while performing a gradient elution may reduce the background interferences associated with the components in an enzymatic digestion even though the samples were prepared in a clean matrix. This digested sample is unlike a synthetic signature peptide due to the presence of components like DTT, iodoacetamide, and trypsin which are necessary in order to obtain a signature peptide for LC–MS/MS optimization. The final signature peptides are presented in Table 1 as mentioned in Section 2.2.

Chromatographic analysis of Alefacept and myoglobin signature peptides was investigated with various columns to resolve matrix peaks and ultimately produce similar retention times. An Ace C8, Aquasil C18, and a Phenomenex Gemini C18 were evaluated with a gradient of 0.1% formic acid and 0.1% formic acid in acetonitrile to achieve maximum sensitivity. The Phenomenex Gemini C18 provided the highest sensitivity, good peak shape, and closely related retention times for Alefacept and the internal standard (3.6 min versus 4.2 min). The first attempt at validation runoff the method resulted in complete loss of signal after 39 injections; therefore, to improve ruggedness of the method, on-line column-trapping was employed. Similar analytical columns that were initially evaluated for separation of Alefacept and myoglobin were evaluated again for trapping (loading) and analytical (elution) columns; however, high back pressure and longer retention times lead to the investigation of using a Phenomenex security guard column as a trapping column. The Gemini C18 guard column allowed for a short elution time onto the analytical column, which resulted in a 12 min run time. Loading time (elution time off the trapping column) was evaluated for intensity and peak shape, and resulted in an optimized loading time of 0.5 min before the valve switch onto the

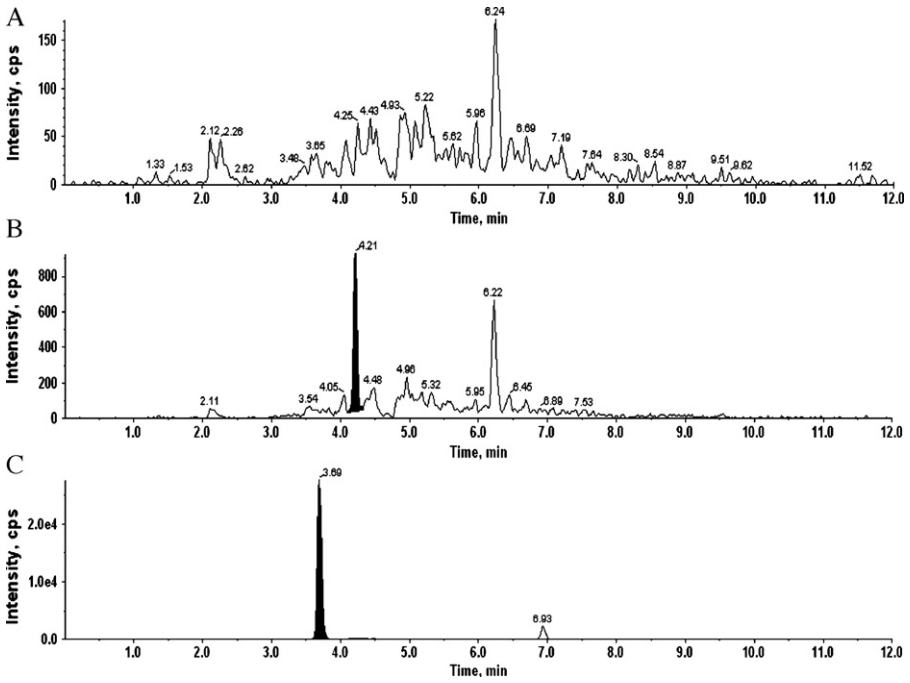

Fig. 3. (A) Representative blank human plasma chromatogram. (B) LLOQ chromatogram (250 ng/mL). (C) Representative myoglobin signature peptide internal standard.

analytical column. Introducing the on-line column trapping into the method improved the overall ruggedness. Following approximately 60 injections, the guard column was replaced; however, the same analytical column was used for the entire validation and has maintained performance for more than 400 injections. Examples of a blank chromatogram (A) and an LLOQ sample showing the Alefacept and myoglobin signature peptides (B and C), respectively are presented in Fig. 3.

3.2. Selective denaturation

Conditions were created where the target protein would undergo minimal denaturation, and representative background proteins would be more completely denatured and precipitated [38]. Using elevated temperature and pH adjustment sequentially, conditions were obtained away from the target protein (Alefacept and myoglobin) isoelectric points (pIs), in order to keep the target proteins soluble [38]. Charge repulsion between the charged random polypeptides in a protein keeps these peptides from aggregating, however, the closer the pH is to the pI of the target protein, the more likely aggregatation will occur [38]. The main goal was to use pH and temperature (which are generally not independent) to selectively denature background proteins. As temperature is increased, the hydrogen bonds of the protein are weakened, and adjustment of the pH would be expected to reduce the internal electrostatic forces of the protein and open it up to complete denaturation [38,39]. An initial study with myoglobin revealed potential reduction in major background proteins when the pH was adjusted to 5.1 at a denaturation temperature of 45 °C. This leads us to further investigate these conditions with our target protein since Alefacept has a relatively similar pI to myoglobin (7.2 versus 7.8). As mentioned in Section 2.8, five pH adjustments were evaluated with five different temperatures to attempt to denature background proteins and maintain target proteins as a purification step. Optimal conditions were determined by assessing the most intense peak areas obtained for the target proteins along with a reduction in major background proteins. Adjusting the pH to 3 under the five different temperatures yielded negligible differences in peak area response for both Alefacept and myoglobin signature peptides, and background protein signature peptides monitored showed negligible loss of response. Adjusting the pH to 7.6 or pH 9.0 resulted in loss of all signature peptide responses (including background signature peptides) below detection limits at temperatures of 22, 37, and 45 °C. Background protein and target signature peptide responses appeared again at 65 and 95 °C temperature conditions at both pHs. The increase in signature peptide peak area response under these temperatures may be due to a concentration effect associated with increased aggregation; these samples yielded supernatant volumes approximately three-fold less. The data was normalized to reflect the varying supernatant volumes in Fig. 4. At pH 4.7 (albumin pI) and 5.1, similar results in peak area responses for all temperature conditions evaluated was observed; however, background protein signature peptide peak are response was less using the pH adjusted to 5.1 at a particular temperature, 45 °C. Under these conditions, IgG background was reduced 25% as compared to 37 °C, and as much as a 10-fold reduction compared to 65 °C. However, denaturing at 65 °C with the pH adjusted to 5.1 did not result in acceptable target protein yields. The lower albumin signature peptide peak area response indicated that the presence of albumin was reduced at pH 5.1 and 45 °C. Using these results, it was decided to adjust pH to 5.1 and denature at 45 °C as an initial purification step. Transferrin background was a lesser concern under any conditions since the peak area response was consistently lower than the target protein signature peptide response. Fig. 4 represents a graphical representation of the temperature denaturation study with samples adjusted to pH 5.1, which shows the peak area responses for Alefacept, myoglobin, transferrin, IgG, and albumin signature peptides at the different temperatures.

3.3. Digestion time

Fig. 5(A) represents the results of the evaluation of trypsin digestion time at five different time points for triplicate analysis of controls (LQC, MQC, and HQC). The internal standard was also evaluated by averaging the peak area response obtained from the quality control samples for each incubation period. Analysis of variance was performed to determine if each time point was significantly different for quality controls and internal standard. This

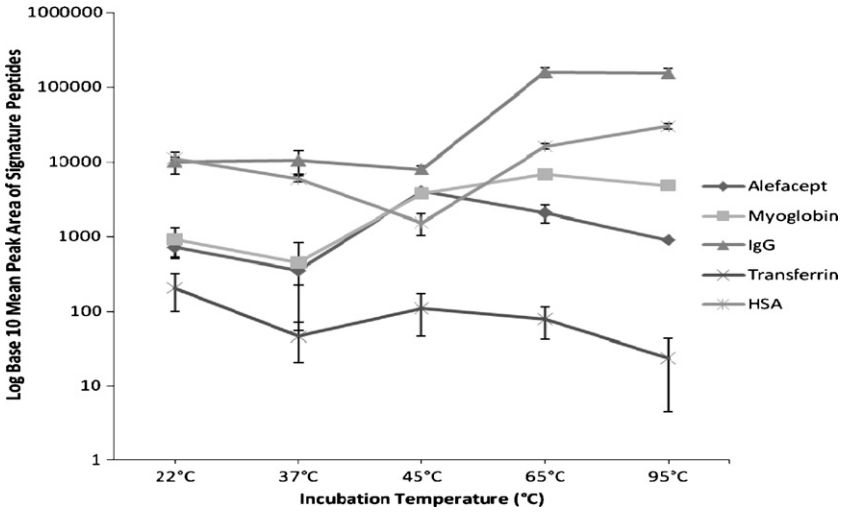

Fig. 4. Temperature denaturation study performed with pH adjusted to 5.1.

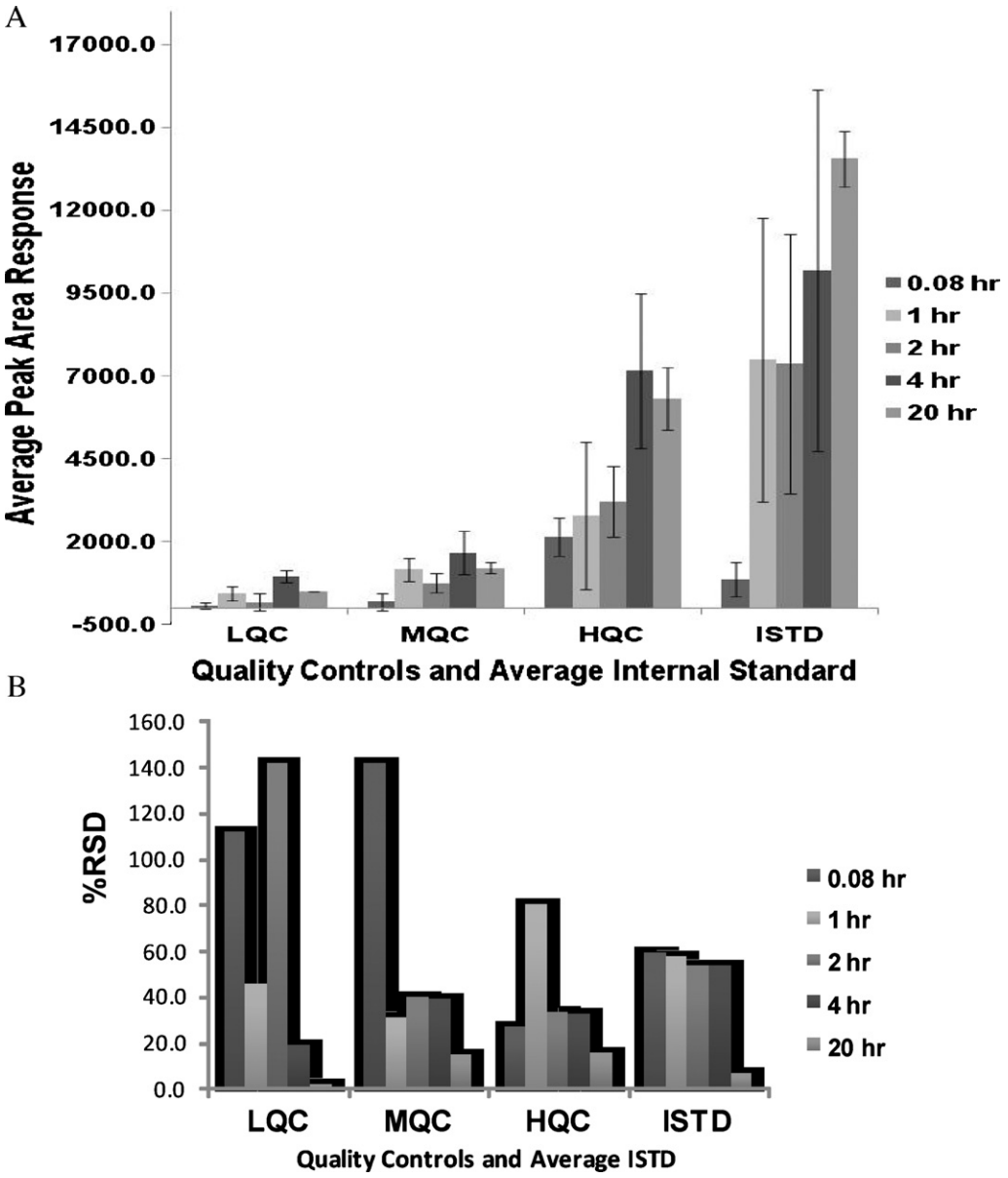

Fig. 5. (A) Incubation time for trypsin digestion results for LQC, MQC, HQC, and internal standard (ISTD) signature peptide peak area response with standard deviation error bars. (B) Comparison of relative standard deviation of 4 h incubation times for trypsin digestion.

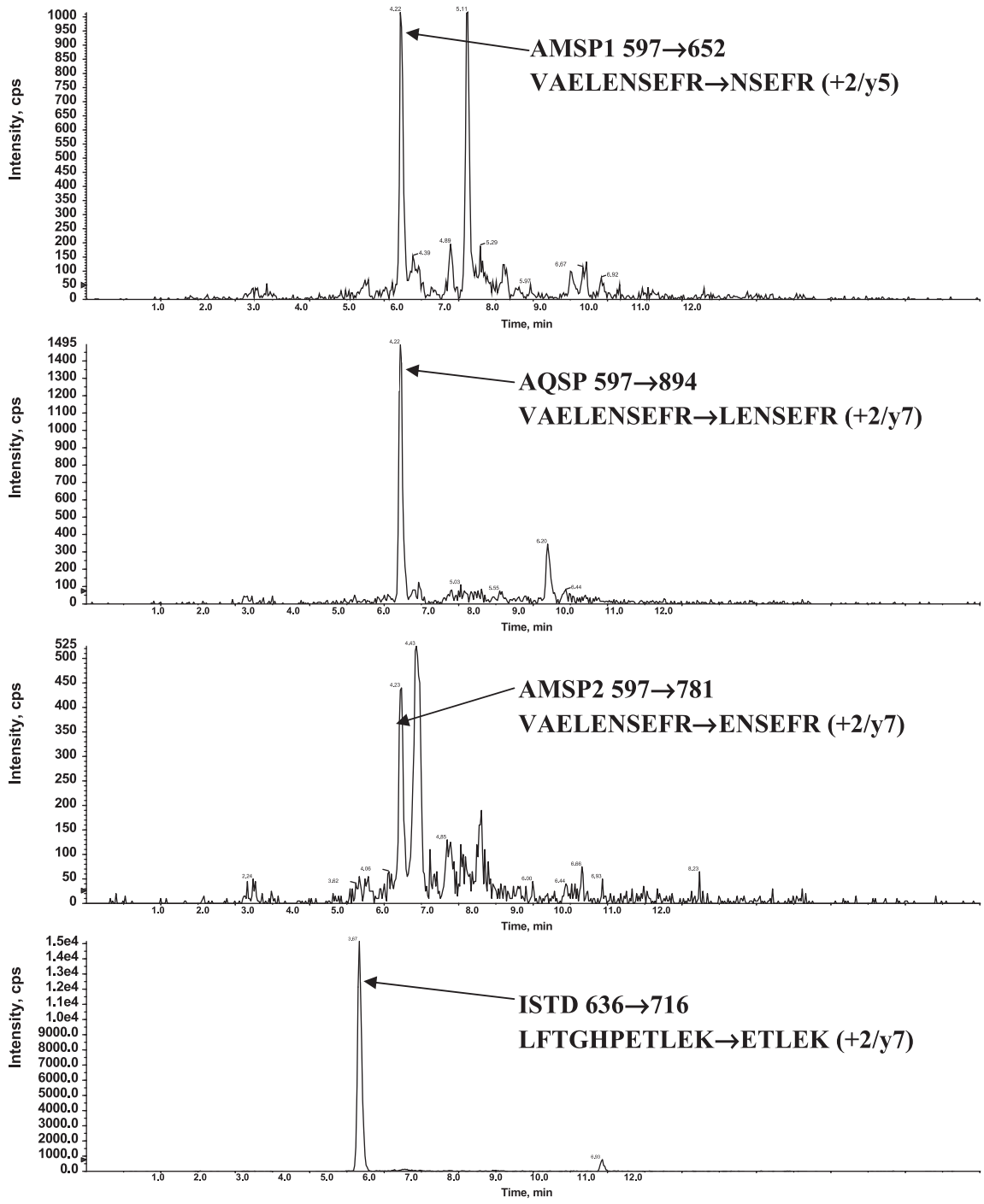

Fig. 6. (A) Alefacept monitored signature peptide 1 (AMSP1: 597 → 652). (B) Alefacept quantitative signature peptide (AQSP: 597 → 894). (C) Alefacept monitored signature peptide 2 (AMSP2: 597 → 781). (D) Myoglobin internal standard signature peptide (ISTD: 636 → 716).

revealed the quality controls under these conditions are not equal and less incubation time yielded high variability. In order to further explain the results, the relative standard deviations (%RSD) were plotted in Fig. 5(B). The comparison of %RSD revealed that the precision was less than 15% RSD employing 20 h incubation for all quality controls and less than 10% RSD for the internal standard response. Consequently, the 20 h incubation time for trypsin digestion was chosen to be more appropriate.

3.4. Matrix effects results

Ion profiles from the post-column infusion study revealed no clear suppression or enhancement at the retention times of myoglobin or Alefacept signature peptides. Phospholipids were monitored during the post-column infusion, which indicated their presence following extraction; however, all peaks were chromatographically resolved from Alefacept and myoglobin signature peptides. Even though phospholipids were present, ion profiles showed no suppression or enhancement as a result of phospholipids. In order to further evaluate matrix effects, a post-extraction addition study was performed as described in Section 2.11. The percent matrix effect was calculated as follows: Matrix effects = 100 × (post-spike peak area – solvent extracted peak area)/solvent extracted peak area. The studies revealed the presence matrix effects with the percent matrix effects resulting

Table 2
Multiple source matrix effect evaluation.

	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6
Average measured concentration (ng/mL) ± Std. Dev.	636.7 ± 55	652.7 ± 40	658.7 ± 49	687.0 ± 93	639.3 ± 26	650.7 ± 40
%RSD	8.7	6.3	7.6	13.6	4.2	6.2
%DFN	6.1	8.8	9.8	14.5	6.6	8.4

Table 3
Inter- and intra-assay precision and accuracy.[a]

	Average measured concentration (ng/mL) ± Std. Dev.	Inter-assay %bias	Intra-assay %bias	Intra-assay precision (%RSD)	Inter-assay precision (%RSD)
LLOQ	242.4 ± 39	−3.0	−10.0	10.3	16.2
LQC	665.3 ± 80	10.9	6.9	9.7	12.1
MQC	2086.7 ± 242	4.3	10.6	11.2	11.6
HQC	8169.2 ± 1033	2.1	1.9	9.0	12.6

[a] QCs analyzed $n = 6$ in 3 separate analytical runs.

in −50.8% for LQC, −73.3% for MQC, and −70.8% for HQC. A multiple source matrix effect study was also performed as discussed in Section 2.11. The results were acceptable (±15% for precision and accuracy) for all lots and are shown in Table 2.

3.5. Method validation

3.5.1. Linearity and limit of detection

The peak area ratio of Alefacept to myoglobin internal standard signature peptides in human plasma was linear as a function of concentration over the range 250–10,000 ng/mL. The calibration curves yielded acceptable reverse calculated residuals between −3% and 2.9%. Without use of the peak area ratios of analyte and internal standard signature peptides, calibration curves produced from absolute responses of Alefacept signature peptides versus actual concentration yielded reverse calculated residuals between −66% and 77%. Precision measured in terms of percent relative standard deviation ranged from 9.8% to 14.8%. The LLOQ for Alefacept was established at 250 ng/mL using the signature peptide approach.

Limit of detection (LOD) was determined by extracting three blank plasma samples and determining the peak to peak noise height at the elution time of Alefacept signature peptide. Standard deviation of these samples was calculated and LOD calculation was performed by: 3 times the standard deviation of the blank divided by the slope of the calibration curve. The LOD was 44 ng/mL and was assessed to determine if detectability could potentially be lower than the current immunoassay approach (80 ng/mL).

3.5.2. Selectivity

The selectivity of the method was evaluated in six different lots of blank human plasma. No endogenous peaks at the retention time of Alefacept or myoglobin signature peptide were observed for any of the lots. Fig. 3(A) shows a blank plasma representative chromatogram. Signature peptide selectivity was monitored throughout the entire study with three different product ions for the signature precursor ion. The MRM transitions monitored were 597 → 652, 597 → 894, and 597 → 781, which are depicted with the internal standard in Fig. 6. No apparent interferences were present with any of the transitions and 597 → 894 was chosen as the quantitative signature peptide because of its greater intensity. The 636 → 716 transition was chosen for the myoglobin signature peptide. The +1 charge state of the myoglobin precursor ion (1272) was monitored but was not used due to its lower intensity than the +2 charged precursor ion (636). Fig. 6 shows raw chromatograms which are labeled according to Table 2. Analyte interference studies were also acceptable with no peaks detectable at the retention time of Alefacept or myoglobin during these experiments.

3.5.3. Recovery and carryover

Relative recovery was performed to evaluate signature peptide recovery. Solvent extracted versus matrix extracted quality controls samples (LQC, MQC, and HQC) were analyzed in triplicate. Percent Recovery was obtained by dividing the matrix extracted samples by the solvent extracted samples and multiplying by 100. The results revealed 33.5, 24.9, 25.0% recovery for the LQC, MQC, and HQCs, respectively. Carryover was evaluated and no response (below detection limits) was present at the retention times for Alefacept or myoglobin signature peptides, and no biased versus LQC accuracy was observed.

3.5.4. Precision and accuracy

The precision and accuracy data are summarized in Table 3. Inter- and intra-day precision and accuracy were determined for the LLOQ QC, LQC, MQC and HQC samples. The intra-run accuracy (%bias) was within ±10.6% (maximum RSD of 11.2%) for all the concentrations including the LLOQ. Additionally, inter-run accuracy was within ±10.9% (maximum RSD of 16.2%) for all concentrations. The dilution quality control, precision and accuracy were less than 10%, 6.3% and 5.7%, respectively. The results indicate that the use of signature peptides for Alefacept with an analogue internal standard was both accurate and precise according to established acceptance criteria.

3.5.5. Stability

PPS was assessed from re-injection reproducibility after storage of quality control samples in the auto sampler for 48 h at 5 °C. Processed LQC, MQC, and HQC samples were stable and %bias was 10.1, 6.7, 11.3, respectively. Storage stability was evaluated in the final validation run by using the freshly prepared day zero nominal concentrations to quantify the frozen quality controls processed. Processed LQC and HQC samples were stable for 21 days at −20 °C and the %bias from day zero was 11.6 and 12.1, respectively.

4. Conclusions

Signature peptides employed as surrogates for target protein quantification is an attractive alternative to traditional immunoassays in biological fluids. Current methods for protein quantification by LC/MS have employed antibody-based purification through molecular recognition of target proteins and/or peptides in addition to immunodepletion of major background proteins [17,18,21]. Two-dimensional solid phase extractions have been used to reach low ng/mL levels and achieve precise and accurate results without the use of antibodies [27]. In this study, selective denaturation purification combined with the use of a carefully chosen protein

analogue internal standard and on-line extraction yielded precise and accurate results that meet FDA guidance acceptance criteria without the use of molecular recognition, immunodepletion, or off-line solid phase extraction. Internal standardization at the protein level was found to be necessary to compensate for matrix effects which hindered the ability to achieve precise and accurate data. Using Alefacept as a model therapeutic protein, the current method was able to achieve quantification limits necessary for evaluation of therapeutic levels (500–6000 ng/mL) without the need for multiple dilutions required in an ELISA assay that has a more limited range (80–900 ng/mL [3]). Employment of a more sensitive mass spectrometer may further lower quantification limits to be comparable to the enzyme-linked immunosorbent assay (ELISA) method (LLOQ = 80 ng/mL) [3].

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