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A sensitive and high-throughput LC–MS/MS method for the quantification of pegylated-interferon-α_{2a} in human serum using monolithic C_{18} solid phase extraction for enrichment

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ABSTRACT

The analysis of pegylated-interferon-α_{2a} in patient serum samples is of high interest for clinic research trials, as this therapeutic protein has become an important antiviral treatment. In this study, an LC–MS/MS method for the absolute quantification of pegylated-interferon-α_{2a} in human serum was developed. The assay achieved a lower limit of quantification of 3.6 ng/mL (60 pM) with the use of a monolithic C_{18} solid phase extraction to enrich the target protein. The linear range of the assay was defined up to 54 ng/mL to measure the typical clinical pegylated-interferon-α_{2a} levels, and within this range, the precision and accuracy were found to be within ±20%. The method was applied to a clinical study and found suitable for high-throughput analysis of pegylated-interferon-α_{2a} in human serum. In addition, further investigations suggested the enrichment step may have general application to the sensitive analysis of other low molecular weight proteins.

1. Introduction

Pegylated-interferon-α_{2a} (PEG-IFN-α_{2a}) is interferon-α_{2a} (IFN-α_{2a}) covalently attached to 40 kD poly(ethylene-glycol) (PEG) at the lysine residues, and is a mixture of mono-pegylated positional isomers [1]. The pegylation results in sustained absorption, a longer half-life due to reduced renal clearance, and a strong antiviral response in the clinic [2]. PEG-IFN-α_{2a} has become a standard treatment for chronic hepatitis C virus (HCV) [3–6], and was recently approved for the treatment of hepatitis B virus (HBV) [7,8]. Therefore, monitoring clinical PEG-IFN-α_{2a} serum levels is important to evaluate therapeutic compliance and the antiviral response. It is also desirable to evaluate the pharmacokinetic properties of PEG-IFN-α_{2a} in patients following co-administration of other pharmaceutical agents to investigate possible drug–drug interactions.

The current assay for PEG-IFN-α_{2a} in human serum samples is an ELISA. Aside from limited availability of this assay, immunological approaches are in general costly and time-consuming to develop, and assay imprecision and matrix interferences are common issues. In contrast, LC–MS/MS is characterized by rapid assay development, good specificity (low interference), good assay precision and accuracy, and is increasingly being used to quantify macromolecules, such as therapeutic proteins and biomarkers [9–11]. However, the challenge for the LC–MS/MS approach is to achieve the required sensitivity to quantify proteins at low concentrations in matrices that contain large amounts of endogenous proteins, such as serum. Depletion of high abundance matrix proteins is one approach to reduce the complexity of the sample [12–14], but the current depletion technologies do not have the desired reproducibility and throughput [15,16]. Immunoaffinity methods, such as immunoprecipitation, can improve the assay specificity, and thereby markedly increase sensitivity to the subnanogram per millilitre level [17,18]. However, immunoprecipitation is time-consuming and requires a specific antibody, which may not always be available. Its application to some macromolecules may thus not be possible.

We have previously reported an LC–MS/MS approach for quantifying therapeutic proteins in serum or plasma, using a monoclonal antibody and human growth hormone as examples [19]. That method used two dimensional-solid phase extraction (2D-SPE) cleanup to achieve a detection limit of about 0.5 μg/mL in serum, which is still not adequate to detect PEG-IFN-α_{2a} at the expected levels in typical patients.

In the present work, a high-throughput methodology with an improved sensitivity was developed to support the analysis of PEG-IFN-α_{2a} in clinical samples. In this method, human serum samples were first denatured and reduced, then subjected to a

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monolithic C_{18} SPE for an enrichment of the target protein. The SPE-captured proteins containing PEG-IFN-α_{2a} were eluted, enzymatically digested and concentrated using a cation exchange SPE. The sample was then analyzed on the LC–MS/MS by monitoring a selected quantitative peptide derived from the target protein. The quantitation limit of this assay was 3.6 ng/mL (60 pM) with good precision and accuracy. The samples were processed in a high-throughput 96-well plate format. Furthermore, as this approach was not analyte-specific, it can be applied to other low molecular weight proteins.

2. Methods

2.1. Material

PEG-IFN-α_{2a}, at a concentration of 360 μg/mL, was purchased from Hoffman-La Roche, Inc (Nutley, NJ). IFN-α_{2a} was purchased from Sigma (St. Louis, MO). Somatropin (recombinant human growth hormone) reference standard was from the United States Pharmacopeia (Rockville, MD), while 96-well SPEC C_{18} plates (30 mg monolithic C_{18}) were purchased from Varian (Covina, CA). Sequence grade trypsin was purchased from Promega (Madison, WI).

2.2. Sample preparation

To a 200 μL aliquot of each human serum sample was added 400 μL 0.1 M ammonium bicarbonate buffer (pH 8) containing 8 M guanidine chloride, 5 mM dithiothreitol, and 10 ng of somatropin as internal standard (ISTD). Samples were then incubated at 70 °C for 1 h, and alkylated by an additional incubation with 0.02 M iodoacetamide for 2 h.

The samples were then acidified to pH 5, and loaded onto an equilibrated 96-well SPEC C_{18} (30 mg) plate. The plate was then filtered, washed with 500 μL water, and eluted with 2 mL of 50% acetonitrile in water. The eluate was dried with heated nitrogen and reconstituted in 500 μL of 0.1 M ammonium bicarbonate buffer (pH 8). The samples were further processed through Amicon 10 kD filter (Millipore, Billerica, MA) to remove possible *in vivo* proteolytic products of the target protein. And then trypsin (20 μg) was added to each sample for overnight digestion at 37 °C.

The digests of the serum samples were subjected to a cleanup using a 96-well Oasis MCX plate (30 μm) from Waters (Milford, MA). The eluate was evaporated to dryness with heated nitrogen and reconstituted in 50 μL of 20% acetonitrile in water prior to analysis by LC–MS/MS.

2.3. LC–MS/MS

The samples were analyzed on an LC–MS system comprised of a Shimadzu LC-20AD (Shimadzu Corporation, Columbia, MD) coupled to a TSQ Quantum ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA). A 10 μL sample was injected onto an ACE C8 column (5 μ, 50 × 2.1 mm) for separation with a flow rate of 250 μL/min. The gradient was started at 5% acetonitrile with 0.1% formic acid and linearly increased to 17% acetonitrile in 1 min, then to 27% acetonitrile in 4 min, and finally to 80% acetonitrile for 1.5 min. The resolved peptides were analyzed using a H-ESI probe (Thermo-Fisher). The vaporizer temperature of the probe was set at 300 °C and the spray voltage was 3.5 kV. Analysis was conducted using the selected reaction monitoring (SRM) mode with the collision energy set at 25 V. The MS/MS transition for the PEG-IFN-α_{2a} peptide "SFSLSTNLQESLR" was 741.5 (doubly charged) → 1047.5 (m/z), while 490.4 (doubly charged) → 719.0 (m/z) was monitored for somatropin peptide "LFDNAMLR". The ratio of the peak area of PEG-IFN-α_{2a} peptide to the peak area of somatropin peptide was used for quantification of PEG-IFN-α_{2a} in human serum samples.

2.4. Preparation of standards and quality control samples

PEG-IFN-α_{2a} solution at a concentration of 180 μg/0.5 mL was diluted 100-fold to 3.6 μg/mL in human serum, then further diluted with serum to 360 ng/mL (Stock A). Then 10 μL, 20 μL, 40 μL, 60 μL, 80 μL, 100 μL and 150 μL aliquots of Stock A were transferred to 1.5 mL tubes and human serum was added to a total volume of 1 mL. These samples were prepared as standards with concentrations of 3.6 ng/mL, 7.2 ng/mL, 14.4 ng/mL, 21.6 ng/mL, 28.8 ng/mL, 36 ng/mL and 54 ng/mL, respectively. Aliquots of 30 μL, 70 μL and 120 μL of Stock A were added to a volume of 1 mL with human serum and used as quality control samples with concentrations of 10.8 ng/mL, 25.2 ng/mL and 43.2 ng/mL, respectively.

2.5. Efficiency of monolithic C_{18} SPE enrichment

A human serum sample (200 μL) was denatured, reduced and alkylated using the procedure described above. The sample was loaded onto an equilibrated 96-well SPEC C_{18} (30 mg) plate. Vacuum was applied and then 500 μL of water was added to wash the plate. This filtered and washed solution was collected as the unbound fraction. The SPE-captured components were then eluted with 2 mL of 50% acetonitrile in water. The eluate was evaporated to dryness with heated nitrogen and reconstituted in 500 μL of 0.1 M ammonium bicarbonate buffer (pH 8) as the captured fraction. Un-processed human serum and the above unbound and captured fractions were finally assayed for protein content using the Bradford method (Biorad Laboratories, Hercules, CA).

2.6. Recovery

The recovery of PEG-IFN-α_{2a} through the sample process was tested at low, medium and high QC concentrations. One set of samples was spiked with PEG-IFN-α_{2a} before the sample process (as in the above procedure), and the other set spiked with PEG-IFN-α_{2a} digest after blank serum samples were processed. Both sets of samples were analyzed by LC–MS/MS. The recovery was calculated using the ratio of the peak area of PEG-IFN-α_{2a} in the first sample set to the peak area of PEG-IFN-α_{2a} in the second sample set. In parallel, the recovery of non-pegylated IFN-α_{2a} was tested using the same procedure. The recovery of ISTD was evaluated by spiking ISTD or ISTD digest before or after, respectively, the processing of the QC samples, and the LC–MS/MS peak areas of the ISTD were compared.

2.7. SDS-PAGE

To further investigate the monolithic C_{18} SPE enrichment procedure, un-processed human serum, and the two fractions (unbound and captured) from the extraction efficiency experiment were analyzed on SDS-PAGE. The protein samples (3 μg per well) were separated on a NuPAGE 4–12% bis–tris gel, 1.5 × 10 well (Invitrogen). The gel was stained using the SilverXpress™ silver staining kit (Invitrogen).

To test whether there was possible conversion of PEG-IFN-α_{2a} to IFN-α_{2a} during the sample processing prior to the SPE enrichment, PEG-IFN-α_{2a} standard was denatured, reduced and then alkylated using the procedure described previously. The sample was then analyzed on a NuPAGE 4–12% bis–tris gel, 1.5 × 10 well (Invitrogen) in parallel with an un-treated PEG-IFN-α_{2a} standard. The gel was stained with SilverSNAP Stain Kit II (Pierce, Rockford, IL).

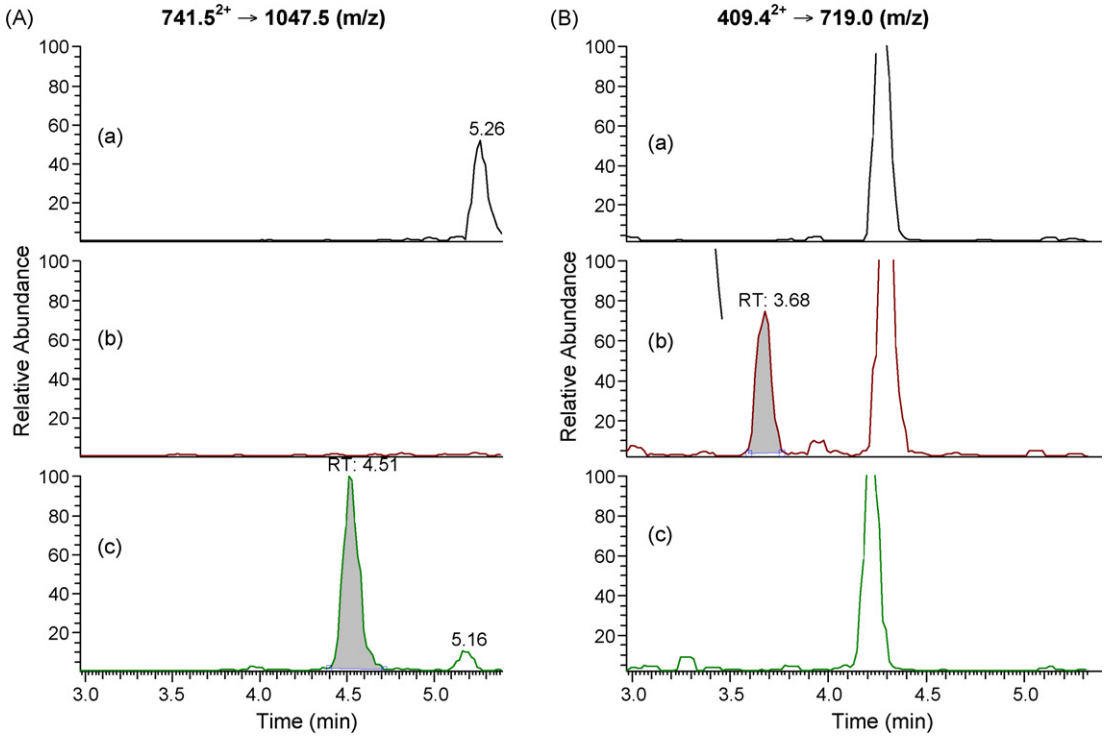

Fig. 1. Blank human serum (a), and human serum spiked with somatropin at 50 ng/mL (b) and human serum spiked with PEG-IFN-α_{2a} at 54 ng/mL (c) were analyzed using the procedure described. Column A shows the extracted ion chromatograms of 741.5^{2+} → 1047.5 (m/z). Column B shows the extracted ion chromatograms of 490.4 $^{2+}$ → 719.0 (m/z).

2.8. Clinical study of PEG-IFN-α_{2a}

Patients with hepatitis C virus (HCV) infection and compensated liver disease, who had not previously received antiviral therapy, were recruited for the clinical study. The patients were given 180 μg PEG-IFN-α_{2a} (PEGASYS®, Hoffman-La Roche, Inc.) by subcutaneous injection once a week, and oral administered daily doses of 1000–1200 mg (weight-based) ribavirin (Copegus®, Hoffman-La Roche, Inc.), an antiviral agent. Blood samples (5 mL) from seven of the patients were collected at 24 and 96 h post-dose during week 4 and 168 h post-dose during week 11. The blood was allowed to clot, then centrifuged at 1500 g and the serum was separated. The samples were stored at −80 °C until analysis.

3. Results

3.1. Selection of quantitative peptide for PEG-IFN α_{2a}

The basic principle for the LC–MS/MS analysis of PEG-IFN-α_{2a} is to quantify a selected peptide derived from the protein via enzymatic digestion. The selection of the quantitative peptide for PEG-IFN-α_{2a} was initiated using an *in silico* list of its tryptic peptides, with the exclusion of peptides having cysteine residues, lysine residues (which are potential pegylation sites), or other post-translational modifications. Three peptides, including one of the sequence "SFSLSTNLQESLR" were found to be unique from the human proteome (Blast search). The MS/MS transition of this peptide, 741.5^{2+} → 1047.5 (m/z) did not interfere with somatropin (internal standard) (Fig. 1A, b) or human serum digest constituents (Fig. 1A, a), and its LC–MS/MS peak had a better signal/noise (S/N) ratio than the other two potential peptides. Therefore, this peptide was selected as the quantitative peptide for PEG-IFN-α_{2a}. As shown in Fig. 2, a 3.6 ng/mL PEG-IFN-α_{2a} serum sample (the lower limit of detection) was processed and showed a peak with S/N about 40 (Fig. 2b), while no detectable signal of the same MS/MS transition was observed at the same retention time for a blank human serum sample (Fig. 2a). The specificity of the LC–MS/MS signal from the PEG-IFN-α_{2a} quantitative peptide was also confirmed in serum samples from six different human subjects. The interference of the endogenous IFN-α_{2a} was not detectable since its serum level is lower than 100 pg/mL [20].

Somatropin (human growth hormone) was used as the ISTD in this method, as its molecular weight is close to IFN-α_{2a}. It was

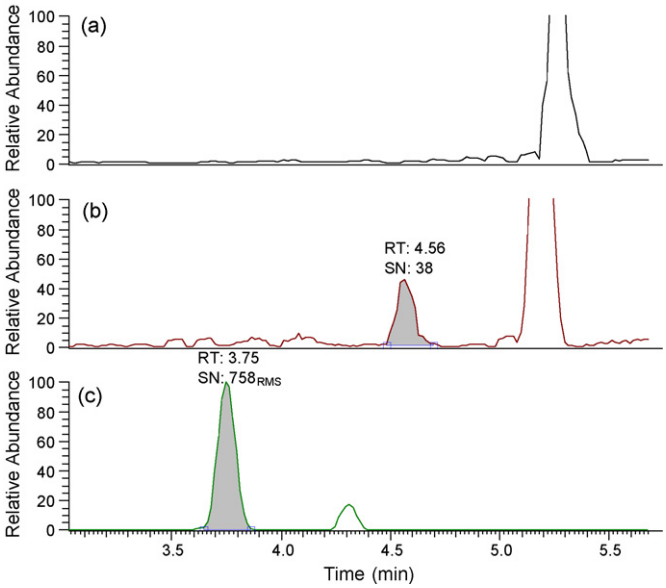

Fig. 2. Blank Human Serum (a), and human serum spiked with PEG-IFN-α_{2a} at 3.6 ng/mL and somatropin at 50 ng/mL (b and c) were analyzed using the procedure described in Fig. 1. Panel a and b are the extracted ion chromatograms of 741.5^{2+} → 1047.5 (m/z). Panel c is the extracted ion chromatogram of 490.4 $^{2+}$ → 719.0 (m/z).

spiked into serum samples at the beginning of the assay so that the recovery could be monitored during the entire sample preparation process. The quantitative peptide of somatropin was chosen as "LFDNAMLR" with an MS/MS transition of $490.4^{2+} \rightarrow 719.0$ (m/z). This peptide showed no interference from PEG-IFN-α_{2a} (Fig. 1B, c) or human serum digest components (Fig. 1B, a). As shown in Fig. 2c, the ISTD peptide eluted about 1 min earlier than the PEG-IFN-α_{2a} peptide. However, the LC gradient was minimal (17% to 27% increase in the organic phase in 4 min) at the elution time period of the two peptides, suggesting similar ionisation conditions at the mass spectrometer ion source for the two. Further, the matrix effect was substantially reduced after the two SPE cleanup steps [19]. As a result, the ISTD peptide was able to effectively monitor the LC–MS/MS analysis and contribute to the good validation results.

3.2. Efficiency of monolithic C_{18} SPE enrichment

Under standard PEG-IFN-α_{2a} clinical therapy (180 μg once a week), the serum concentrations of PEG-IFN-α_{2a} are in the range of about 16–32 ng/mL at steady state. The sample volume was established at 200 μL of serum to ensure adequate PEG-IFN-α_{2a} in the sample to meet the LC–MS/MS detection limit. However, direct digestion of 200 μL of human serum (about 18 mg of total proteins) is not feasible, and detecting this low concentration of PEG-IFN-α_{2a} in the presence of such a large amount of total serum protein is also difficult. In order to achieve the required assay sensitivity, a monolithic C_{18} SPE method was used to enrich PEG-IFN-α_{2a} versus the other serum proteins, which are 10^7 fold higher in abundance than the target analyte. In this approach, the denatured and reduced human serum samples were loaded onto a 96-well monolithic C_{18} SPE plate. After filtering and washing away the unbound serum components, the captured fraction was collected in a clean plate. The Bradford protein assay results showed that more than 80% of the total serum proteins were removed.

3.3. Recovery during sample processing

PEG-IFN-α_{2a} has been reported to be more resistant to enzyme digestion than IFN-α_{2a} [1,19]. In our approach, the proteins were first denatured, then reduced and alkylated. These treatments irreversibly break the protease resistance core of proteins to ensure the efficiency of trypsin digestion. After overnight reaction, the LC–MS/MS signal of the quantitative peptide was not further increased upon addition of more trypsin, suggesting the reaction was complete. The recoveries of PEG-IFN-α_{2a} and IFN-α_{2a} through the entire sample process were both found to be about 50% (Table 1). This result demonstrates the accuracy of the method, as it can also detect IFN-α_2, a possible *in vivo* conversion product. In addition, the ISTD protein had a similar and consistent recovery, even though it was neither a pegylated protein nor an isotope-labeled analog. Therefore, the chosen ISTD could effectively monitor the sample preparation process, including the crucial enrichment step.

3.4. Validation of LCMS bio-assay for PEG-IFN-α_{2a}

The described assay was developed to analyze human serum samples obtained from PEG-IFN-α_{2a} clinical studies. In the standard treatment regimen, the average trough concentration (C_{trough}) at steady state is about 16 ng/mL, and ranges from 2 ng/mL to 28 ng/mL (Physician Desk Reference, 2007), and the maximum concentration (C_{max}) is about twice as high. The range of the calibration curve was thus defined as 3.6 ng/mL to 54 ng/mL to cover the expected PEG-IFN-α_{2a} clinical concentrations. This calibration range showed a linear response with a regression coefficient of 0.99 (Fig. 3). In a 3-day validation, quality control samples (QCs) at concentrations of 10.8 ng/mL, 25.2 ng/mL and 43.2 ng/mL were prepared in six replicates and tested on each day. As shown in Table 2, accuracy within ±25% was achieved for 16 out of 18 (~90%) of samples at each QC level and on each day, and more than 70% were within ±20%. The precision (CV%) values of the 3-day validations were 17%, 19% and 15%, respectively, for the low, medium and high QCs. Overall, the

Table 1
Recovery of target proteins during sample processing.

Concentration [a] (ng/mL)	PEG-IFN-α_{2a}		IFN-α_{2a}		ISTD
	Recovery (%) [b]	RSD (%) [c]	Recovery (%)	RSD (%)	Recovery (%) [d]
10.8	54	2	49	14	44
25.2	62	7	43	15	56
43.2	37	10	63	10	66
Average	51		51		55

[a] The target concentrations were set at the low, medium and high QC concentrations.
[b] One set of the human serum samples was spiked with PEG-IFN-α_{2a} or IFN-α_{2a} before sample processing, and the other set of samples was spiked with the digests of PEG-IFN-α_{2a} or IFN-α_{2a} after blank human serum samples were processed. The recovery was calculated using the ratio of the peak area of PEG-IFN-α_{2a} or IFN-α_{2a} in the first set of samples over the peak area of PEG-IFN-α_{2a} or IFN-α_{2a} in the second set of samples.
[c] Relative standard deviation ($N = 3$).
[d] One set of QC samples of PEG-IFN-α_{2a} was spiked with ISTD before sample processing. The other set of QC samples was spiked with ISTD digest after blank serum sample processing. The recovery was calculated of by the ratio of the peak area of ISTD in the first set of samples over the peak area of ISTD in the second set of samples.

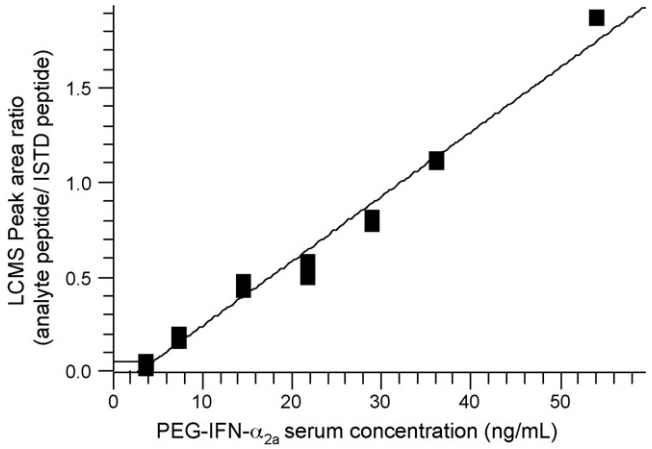

Fig. 3. The standards of PEG-IFN-α_{2a} in human serum were prepared at concentrations of 3.6 ng/mL, 7.2 ng/mL, 14.4 ng/mL, 21.6 ng/mL, 28.8 ng/mL, 36 ng/mL and 54 ng/mL. The standards were analyzed using the described LC–MS/MS method. The quantitative response shows a linear correlation with concentration (regression coefficient of 0.99).

Table 2
Accuracy and precision of quality control samples in a 3-day validation [a].

Accuracy [b]	Within ±15%	Within ±20%	Within ±25%	Over ±25%	Precision
Number of QCs in the category					
QC_{low} [c]	10	16	17	1	17%
QC_{medium}	13	14	16	2	19%
QC_{high}	11	14	16	2	15%
Day 1 [d]	8	16	16	2	
Day 2	13	15	16	2	
Day 3	13	16	17	1	

[a] Each QC level was tested with six replicates on each day.
[b] The difference of the back calculated concentration from the expected concentration.
[c] Concentrations of QC_{low} was 10.8 ng/mL, QC_{medium} was 25.2 ng/mL and QC_{high} was 43.2 ng/mL. A total of 18 QC samples at each level were tested during the 3-day validation.
[d] There were a total of 18 QC samples tested on each day.

Table 3
The concentrations of PEG-IFN-α_{2a} in clinical samples measured by the LC–MS/MS assay.

Patient	Week 4, 24 h Concentration[a] (ng/mL)	RSD (%)[b]	Week 4, 96 h Concentration (ng/mL)	RSD (%)	Week 11, 168 h Concentration (ng/mL)	RSD (%)
1	17.1	19	24.3	11	27.1	16
2	12.1	21	15.8	9	15.8	12
3	7.2	20	8.7	21	15.6	44
4	6.1	19	6.3	35	7.8	34
5	11.3	19	15.7	8	8.7	20
6	6.4	36	8.1	11	10.3	6
7	7.6	14	11	20	9.3	1
Average [c]	9.7	21	12.8	16	13.5	19
RSD (%)[d]	42		49		50	

[a] The average concentration from three separate LC–MS/MS runs.
[b] The relative standard deviation of values from three separate LC–MS/MS runs.
[c] Average concentrations at the same sampling time point from the seven patients.
[d] The relative standard deviation of values from seven patients.

accuracy and precision met the typical drug development criteria for a complex LC–MS/MS assay.

3.5. *Analysis of PEG-IFN-α_{2a} in clinical samples*

After proper validation, the LC–MS/MS assay was used to analyze human serum samples collected from a clinical study in which patients were given 180 μg PEG-IFN-α_{2a} by subcutaneous injection once a week. Steady-state serum levels of PEG-IFN-α_{2a} are normally reached within 5 to 8 weeks of once weekly dosing. Therefore, C_{trough} samples collected at 168 h after the 11th dose were selected for LC–MS/MS analysis. Administered subcutaneously, the C_{max} of PEG-IFN-α_{2a} is usually reached around day 3 or 4 post-dose. Serum samples taken at 24 h and 96 h after the fourth dose were also selected for analysis. Each of the samples was analyzed three times in separate runs. The mean ($N = 3$) concentrations are shown in Table 3. All sample concentrations were within the calibration range. The concentrations at 96 h (time point of C_{max}) after fourth dose were higher than at 24 h. The measured C_{trough} at steady state (week 11, 168 h) were comparable with the reported typical PEG-IFN-α_{2a} clinical concentrations. The relative standard deviation (RSD) of the sample concentrations measured from the three runs was primarily within ±20%, comparing well with the validation results. This incurred sample analysis demonstrated good assay reproducibility and indicated the developed methodology was suitable for clinical studies, where inter-individual variations were found to be above 40%.

4. Discussion

The conventional analytic method for proteins is immunoassay, which can be difficult to develop for pegylated proteins. Pegylation can occur at multiple lysine residues, resulting in positional isomers that do not necessarily have the same kinetics and thermodynamics of receptor-binding [21]. Their affinity to ligands immobilized to immunoassay plates are not necessarily equal. On the other hand, the LC–MS/MS approach has an advantage for this type of analyte, as it focuses on peptides unique to the matrix proteome, and avoids amino acid residues which can be potential modification sites. The assay is thus not affected by the biological activity of the protein, nor does it suffer from matrix interferences. In this study, the recovery of PEG-IFN-α_{2a} was the same as the non-pegylated IFN-α_{2a}, which suggested that the assay performance was not affected by the extent or position of pegylation of IFN-α_{2a}. Thus this LC–MS approach leads to simpler method development than typical immunoassays, which require specific agents in consideration of the complexity of pegylation.

A C_{18} SPE plate with a monolithic sorbent bed was chosen to enrich the analyte protein in this method. Unlike other polymeric SPE sorbents, which exclude most large molecules, the monolithic SPE has more active surface area, allowing retention of molecules of higher molecular weights. The SDS-PAGE analysis results (Fig. 4A) showed that many serum proteins (<40 kD) were enriched in the captured fraction of the monolithic C_{18} SPE. The target proteins in this study, IFN-α_{2a}, PEG-IFN-α_{2a}, and human growth hormone, all showed good and consistent recoveries through the monolithic SPE step. PEG-IFN-α_{2a} is a 60 kD molecule containing 40 kD PEG,

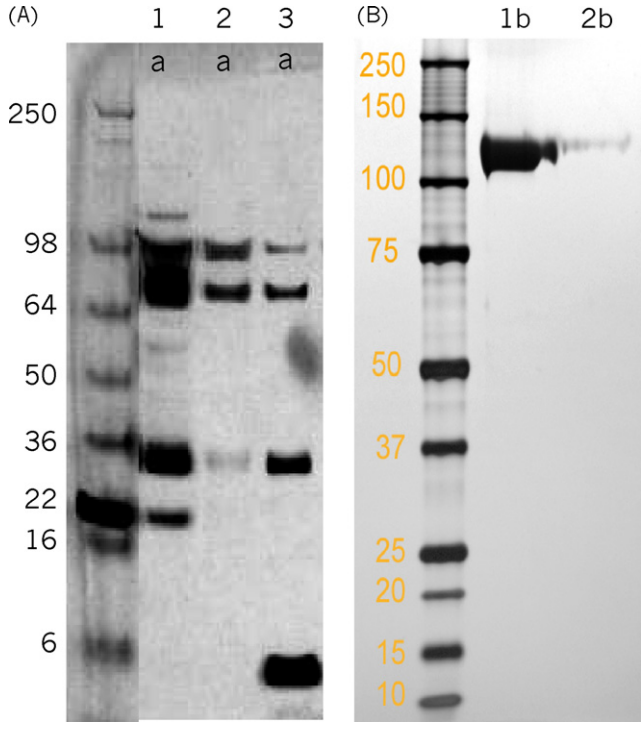

Fig. 4. (A) Human serum (200 μL) was denatured, reduced and alkylated, then extracted on a monolithic C_{18} solid phase extraction plate using the described procedures. The unbound fraction (lane 2a), the captured fraction (lane 3a) and an un-processed serum sample (lane 1a) were subjected to SDS-PAGE separation and silver staining. The same amount of protein was loaded onto each sample lane, (B) PEG-IFN-α_{2a} was denatured, reduced and then alkylated using the same procedure and subjected to SDS-PAGE separation and silver staining (lane 2b) in parallel with un-processed PEG-IFN-α_{2a} (lane 1b). The PEG-IFN-α_{2a} band is observed at a position higher than its molecular weight of 60 kD, which is due to the large hydrodynamic volume of poly(ethylene-glycol) (PEG), resulting in slower electrophoretic mobility, The denatured, reduced and alkylated samples (lanes 2a, 3a and 2b) show less silver staining due to the alkylation of sulfhydryl groups.

and the gel result (Fig. 4B) showed that the sample process did not remove PEG from the molecule before the SPE step. However, denaturing and reducing the protein disturbed the conformation of the molecule, and likely exposed the IFN-α_{2a} protein portion, allowing more interaction with the reverse phase C_{18} material. This enrichment removed more than 80% of the serum proteins and improved the assay sensitivity to achieve the desired quantitation limit. This high sensitivity is comparable to that of immunoaffinity enrichment, but does not require the use of an antibody. As this SPE enrichment method is not protein-specific, it may be a universal approach for the analysis of pegylated low molecular weight proteins and perhaps applied to other low molecular weight proteins, such as some therapeutic proteins and biomarkers.

This SPE enrichment step also effectively removes salts at the high concentrations typically used for protein denaturing and reduction. Desalting is a necessary step before trypsin digestion to ensure sufficient enzyme activity. Therefore this SPE step was found to offer effective enrichment of the analyte protein, as well as desalting to aid proper trypsin digestion, all in a 96-well high-throughput fashion.

5. Conclusion

A sensitive and high-throughput LC–MS/MS assay for PEG-IFN-α_{2a} using a monolithic C_{18} SPE enrichment has been developed. The assay achieved a quantification limit of 3.6 ng/mL (60 mol/mL) with acceptable accuracy and precision, and can be used to analyze serum samples from patients receiving standard PEG-IFN-α_{2a} therapy. As the enrichment approach is not protein-specific, it can be used as a universal method for the quantification of pegylated proteins which may present interference problems for immunological methods. The monolithic C_{18} SPE step enriches the low molecular weight serum proteins and showed consistently good recoveries for the target PEG-IFN-α_{2a}, as well as non-pegylated IFN-α_{2a} and human growth hormone (ISTD). This result suggests that the approach may have broad application to the analysis of low molecular weight proteins, such as other therapeutic proteins and biomarkers.

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References

[1] C. Dhalluin, A. Ross, L.A. Leuthold, S. Foser, B. Gsell, F. Muller, H. Senn, Bioconjugate Chem. 16 (2005) 504.
[2] R.K. Rajender, M.W. Modi, S. Pedder, Adv. Drug Deliv. Rev. 54 (2002) 571.
[3] S.J. Hadziyannis, G.V. Papatheodoridis, Expert Opin. Pharmacother. 4 (2003) 541.
[4] M.W. Modi, M.W. Lamb, M. Shiomi, Adv. Exp. Med. Biol. 519 (2003) 59.
[5] G.R. Foster, Expert Opin. Pharmacother. 4 (2003) 685.
[6] M.W. Fried, S.J. Hadziyannis, Semin. Liver Dis. 24 (Suppl 2) (2004) 47.
[7] W.G. Cooksley, Expert Opin. Pharmacother. 6 (2005) 1373.
[8] R.G. Gish, D.T. Lau, P. Schmid, R. Perrillo, Am. J. Gastroenterol. 102 (2007) 2718.
[9] O. Rinner, L.N. Mueller, M. Hubalek, M. Muller, M. Gstaiger, R. Aebersold, Nat. Biotechnol. 25 (2007) 345.
[10] S.P. Mirza, M. Olivier, Physiol. Genomics 33 (2008) 3.
[11] M. Mann, Nat. Biotechnol. 17 (1999) 954.
[12] G.S. Omenn, D.J. States, M. Adamski, T.W. Blackwell, R. Menon, H. Hermjakob, R. Apweiler, B.B. Haab, R.J. Simpson, J.S. Eddes, E.A. Kapp, R.L. Moritz, D.W. Chan, A.J. Rai, A. Admon, R. Aebersold, J. Eng, W.S. Hancock, S.A. Hefta, H. Meyer, Y.K. Paik, J.S. Yoo, P. Ping, J. Pounds, J. Adkins, X. Qian, R. Wang, V. Wasinger, C.Y. Wu, X. Zhao, R. Zeng, A. Archakov, A. Tsugita, I. Beer, A. Pandey, M. Pisano, P. Andrews, H. Tammen, D.W. Speicher, S.M. Hanash, Proteomics 5 (2005) 3226.
[13] M. Ramstrom, C. Hagman, J.K. Mitchell, P.J. Derrick, P. Hakansson, J. Bergquist, J. Proteome. Res. 4 (2005) 410.
[14] T. Plavina, E. Wakshull, W.S. Hancock, M. Hincapie, J. Proteome. Res. 6 (2007) 662.
[15] N. Seam, D.A. Gonzales, S.J. Kern, G.L. Hortin, G.T. Hoehn, A.F. Suffredini, Clin. Chem. 53 (2007) 1915.
[16] N. Zolotarjova, J. Martosella, G. Nicol, J. Bailey, B.E. Boyes, W.C. Barrett, Proteomics 5 (2005) 3304.
[17] B.L. Ackermann, M.J. Berna, Expert Rev. Proteomics 4 (2007) 175.
[18] M.J. Berna, Y. Zhen, D.E. Watson, J.E. Hale, B.L. Ackermann, Anal. Chem. 79 (2007) 4199.
[19] Z. Yang, M. Hayes, X. Fang, M.P. Daley, S. Ettenberg, F.L. Tse, Anal. Chem. 79 (2007) 9294.
[20] N.L. Anderson, N.G. Anderson, Mol. Cell Proteomics 1 (2002) 845.
[21] C. Dhalluin, A. Ross, W. Huber, P. Gerber, D. Brugger, B. Gsell, H. Senn, Bioconjug. Chem. 16 (2005) 518.