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Quantitation of free polyethylene glycol in PEGylated protein conjugate by size exclusion HPLC with refractive index (RI) detection

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ABSTRACT

In this study, size exclusion high performance liquid chromatography was evaluated for its application in separation and quantitation of free polyethylene glycol (PEG) and its PEGylated-protein-conjugate (PEG-conjugate). Although the large mass of the free PEG (2-fold greater than the protein) made separation difficult, chromatographic conditions were identified enabling resolution and quantitation of the free PEG, PEG-conjugate and non-PEGylated protein with Shodex Protein KW803 and KW804 columns in series and refractive index detection. The optimum resolution of 1.7 and 2.0 was achieved for the free PEG and PEG-conjugate as well as the free PEG and non-PEGylated protein using 20 mM HEPES buffer at pH 6.5. Under this condition, the plot of $\log_{10}$MW of all the pertinent analytes against retention time showed a linear relationship with a correlation coefficient of 1. Limited assay performance evaluation demonstrated that the method was linear in the concentration range of 10 to 250 μg/mL of free PEG with correlation coefficients of ≥0.99. When free PEG in this concentration range was spiked into PEG-conjugate samples at 1 mg/mL, the recovery was in the range of 78%–120%. Detection and quantitation limits were determined to be, respectively, 10 and 25 μg/mL for free PEG. The R.S.D. for intra- and inter-day precision was 0.09% or less for retention time measurements and 2.9% or less for area count measurements. Robustness testing was performed by deliberately deviating ±0.2 pH units away from the desired pH as well as by increasing the flow rate. These deviations resulted in no significant impact on area percent distribution of all species. However, separation was found to be sensitive to high ionic strength and buffer species.

1. Introduction

Since the first PEGylated protein drug (Adagen®) was approved for the treatment of severe combined immunodeficiency by the FDA in 1990 [1], at least five other PEGylated proteins and one PEGylated oligonucleotide are on the market for treatment of variety of conditions [2]. These include PEG-asparaginase (Oncaspar®) for treatment of acute lymphoblastic leukemia [3]; PEG-interferon α2a (Pegasys®) and PEG-interferon α2b (PEG-Intron®) for treatment of hepatitis C [4,5]; PEG-growth hormone receptor antagonist (Pegvisomant, Somavert®) for treatment of acromegaly [6]; PEG-G-CSF (PEGylated granulocyte colony stimulating factor, Neulasta®) for treatment of neutropenia during chemotherapy [7] and a branched PEG-anti-VEGF aptamer (MacugenTM), for treatment of age related macular degeneration [8]. PEGylated proteins have certainly increased the therapeutic value of the original proteins. The improvement may have resulted from any changes in the physical/biochemical/pharmacokinetic properties of the original properties, such as solubility, stability, duration of action, and/or toxicity/safety [2,9–14]. Such an improvement could also lead to more patient compliance.

However, addition of a PEG moiety to the original protein complicates the analytical assays for monitoring the quality and quantity of PEGylated protein products during the development, production and release. It is known that quantitative determination of free PEG in the presence of the PEGylated-protein-conjugate (PEG-conjugate) is challenging because free PEG has very limited UV resonance, preventing direct detection by UV or florescence, commonly used in HPLC systems [15]. Additionally, due to the large hydrodynamic radius of PEG in solution, the apparent molecular weight of free PEG could be 3–5 times greater than that of a protein of comparable molecular weight [2]. Thus, the size difference between free PEG and the PEG-conjugate is diminished, especially when the molecular weight of PEG is greater than the non-PEGylated protein. This results in difficulty resolving and quantifying the free PEG in the presence of the PEG-conjugate by size exclusion high performance liquid chromatography (SE-HPLC), the method commonly used for char-

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acterization and quantitation of molecules based on their size difference.

Traditionally, two analytical methodologies have been used for detection of free PEG [16–18]. Both are based on the formation of a barium iodide complex with PEG. The first involves separation of the free PEG by SDS-PAGE and PEGylated-protein followed by staining with a barium iodide reagent [16]; and the second utilizes UV–vis at 535 nm following reaction of the free PEG with barium chloride and iodine solution [17,18]. Both techniques have considerable limitations. SDS-PAGE analysis with iodine as a visualization agent is at best semi-quantitative and, as a common knowledge, SDS-PAGE analysis is extremely tedious and time consuming. This method, therefore, is not ideal for real-time, quantitative monitoring of the PEGylation process. Further, entry of PEG and PEG-conjugate into the gel could be limited by their large sizes. In comparison, the colorimetric method relying on the UV–vis absorbance at 535 nm only measures the total quantity of PEG and PEGylated protein, unless a separation is included [18], a challenging step as noted above. Additionally, low-sensitivity is associated with the colorimetric method, as background at 535 nm is high in the blank sample [15]. In searching for better tools to monitor the PEGylation process, attention has been turned to mass spectrometric techniques. Both MALDI and electrospray mass spectrometry have been reported as potential alternatives, but both techniques are more suitable for characterization than for quantitation [15,19,20].

Therefore, there is an immediate need for developing an easy, robust, and sensitive assay for monitoring free polyethylene glycol in PEG-conjugate samples during production process and stability studies. Such an assay would be an essential tool not only in maintaining product quality but also in satisfying regulatory requirements. In this paper, a quick and easy SE-HPLC method is reported for resolving and quantitating the free PEG in PEG-conjugate samples without any sample manipulation. This method uses Shodex Protein KW803 and KW804 columns in series with an UV and refractive index (RI) dual detector system and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) as a buffer in mobile phase. The free PEG, PEG-conjugate and non-PEGylated protein are successfully quantitated within 60 min.

2. Experimental

2.1. Materials

The model protein with a molecular weight of 22 kD was produced in house. Polyethylene glycol with a molecular weight of 43 kD was purchased from Shearwater Polymer Inc. The PEG-conjugate in phosphate buffered saline solution (PBS) was prepared in house. The concentration of PEG-conjugate was generally $\geq$5 mg/mL. It was diluted to 1 mg/mL using PBS before use. 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid and its sodium salt were purchased from Sigma–Aldrich with purity of 99.5% and 99%, respectively. Sodium phosphate monobasic, monohydrate was from J.T.Baker. Phosphate buffered saline (PBS) was purchased from Invitrogen Corporation. All reagents were used as received.

2.2. SE-HPLC method

Size exclusion high performance liquid chromatography was performed using Agilent 1100 series HPLC system (Agilent Technologies Inc., California, USA). Two tandem Shodex Protein KW803 (8.0 mm $\times$ 300 mm, 5 μm) and KW804 (8.0 mm $\times$ 300 mm, 7 μm) columns were used at ambient temperature. The mobile phase is an aqueous solution containing 20 mM HEPES buffer at pH 6.5 (prepared by dissolving 4.42 g of HEPES and 0.38 g of HEPES sodium salt in 1000 mL of water and filtered through a 0.45 μm filter before use) and the flow rate was 0.5 mL/min unless specified otherwise. Thirty microliters of sample was injected. The HPLC trace was monitored with a UV/vis detector at a wavelength of 214 nm and with a RI detector at an attenuation of 7.8×10^3. The chromatographic control system, data acquisition and analysis were performed using Turbochrome (PerkinElmer) software.

2.3. Sample preparation

Polyethylene glycol stock solution was prepared by dissolving 2.6 mg of lyophilized PEG powder in 2.6 mL PBS. Serial dilutions were made volumetrically using PBS to obtain 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, and 0.005 mg/mL of free PEG solution for the linearity study. Spike recovery samples were prepared by spiking a known amount of free PEG into 2 mg/mL PEG-conjugate solution at 1:1 ratio (V:V) so that the total protein concentration was 1 mg/mL.

3. Results and discussion

3.1. Resolving free PEG from PEG-conjugate

Because of the anticipated difficulty in revolving free PEG and PEG-conjugate, our initial efforts were focused on identification of conditions separating these two species. Several brands of SE-HPLC columns from a variety of vendors in combination with different mobile phase species, concentration, pH, ionic strength, and flow rate were evaluated to resolve free PEG from PEG-conjugate. Shodex Protein KW803 and KW804 in series were found to be able to separate free PEG from PEG-conjugate with a resolution of 1.7 when using 20 mM HEPES buffer at pH 6.5 with a flow rate of 0.5 mL/min as shown in Fig. 1A. After this, the non-PEGylated protein was included in the sample and it was resolved from free PEG with a resolution of 2.0. The elution order of these species was PEG-conjugate followed by free PEG and non-PEGylated protein. Multi-PEGylated species were also separated. These species were eluted before the desired product. Therefore, with one HPLC injection, the profile of all species present in the PEGylation process was obtained within an hour as shown in Fig. 1C.

3.2. Impact of ionic strength on resolution

It is a common practice to add sodium chloride into SE-HPLC mobile phase to eliminate non-specific interaction between column packing material and analytes so that the analysis is based solely on the size difference of the analytes. In this study, the potential impact of increasing ionic strength in the mobile phase on the resolution of free PEG and PEG-conjugate was examined. Addition of sodium chloride to HEPES buffer resulted in complete loss of resolution as demonstrated in Fig. 2C. Careful examination of the HPLC chromatogram revealed that the loss of resolution was due to a longer retention time of the PEG-conjugate on the column, whereas the retention time of free PEG remained unchanged. This resulted in the PEG-conjugate peak merging together with the free PEG peak. According to the vendor, Shodex Protein KW803 and KW804 columns are silica-based; these SE-HPLC columns are weakly anionic and slightly hydrophobic. Since proteins are amphoteric; they can be positively charged when mobile phase pH is below their pI and be negatively charged when mobile phase pH is above their pI. Proteins are also amphipathic, possessing both hydrophilic and hydrophobic properties. Therefore, non-specific interactions between column matrix and analytes can occur. Generally, these non-specific interactions can result in analytes being retained on a column longer than would be expected based on

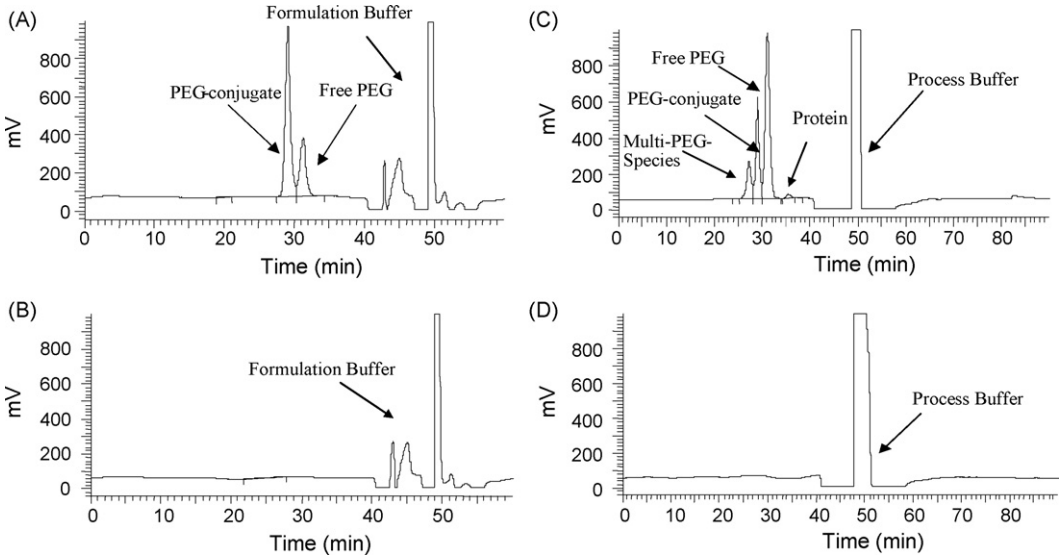

Fig. 1. Separation of free-PEG from PEG-conjugate and non-PEGylated protein: A: free PEG spiked into PEG-conjugate; B: formulation buffer; C: in-process sample; D: in-process buffer. Samples were analyzed using 20 mM HEPES pH 6.5 at flow rate of 0.5 mL/min. The injection volume was 30 μL. The peak was monitored by RI detector at attenuation of 7.8×10^3.

their size, due either to hydrophobic interactions with the column packing material or to an electrostatic attraction between the negatively charged column matrix and any positively charged character of the analytes. Alternatively, an analyte with a negative charge(s) may be eluted sooner than expected due to charge repulsion between column matrix and analytes. The optimized SE-HPLC mobile phase condition had pH of 6.5. At this running condition, PEG-conjugate and non-PEGylated protein should be negatively charged because they have a pI of about 5. Therefore, the interaction between PEG-conjugate and column matrix should be repulsive and result in shortened retention time. As discussed, the study showed the opposite trend. It is believed that this is likely due to increased hydrophobic interaction between PEG-conjugate and column packing material after addition of sodium chloride. Fig. 2C

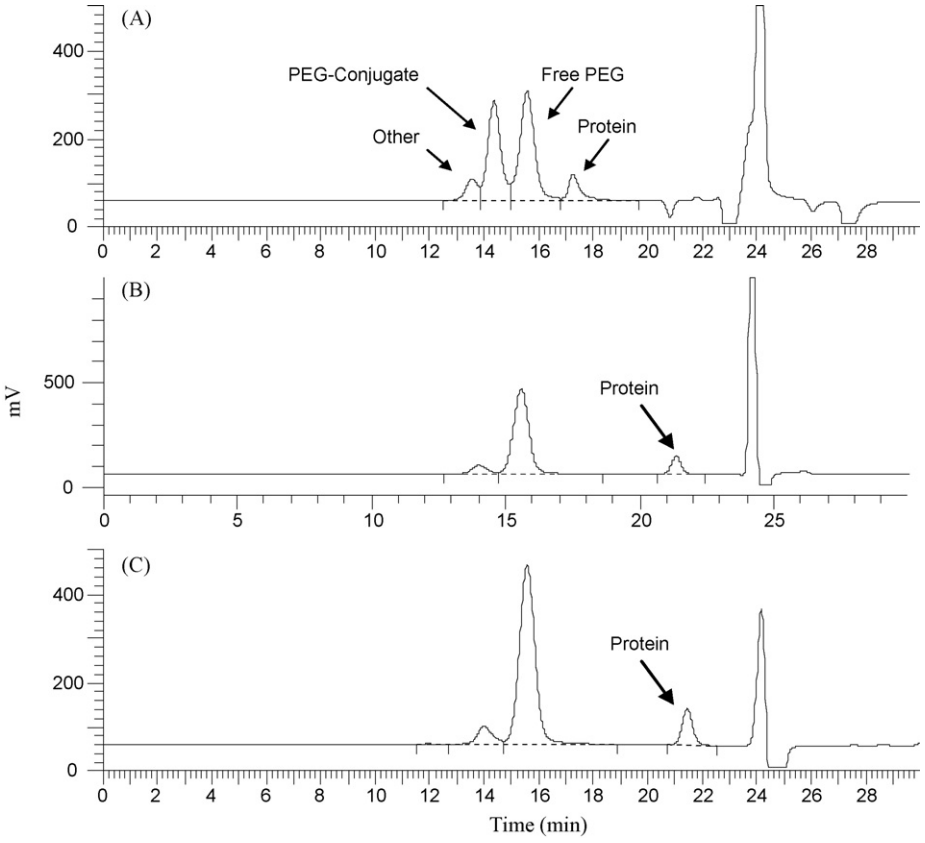

Fig. 2. Mobile phase impact on resolution: A: 20 mM HEPES buffer pH 6.5, free PEG and PEG-conjugate are resolved; B: 20 mM sodium phosphate buffer pH 6.5, free PEG and PEG-conjugate are not resolved; C: 20 mM HEPES pH 6.5 with 100 mM sodium chloride in it, free PEG and PEG-conjugate are not resolved. Samples were analyzed at flow rate of 1.0 mL/min. The injection volume was 30 μL. The peak was monitored by RI detector at attenuation of 62.5×10^3.

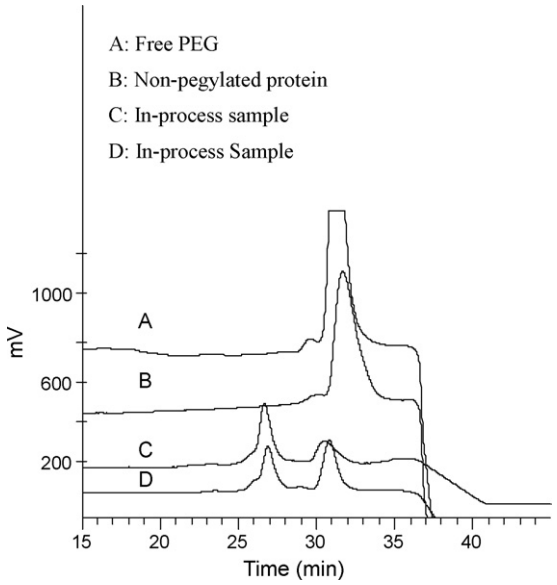

Fig. 3. Overlay of SE-HPLC chromatograms of free PEG (A), non-PEGylated protein (B) and in-process sample (C and D): A, B, and D were RI traces; C was UV 214 nm trace. Free PEG and non-PEGylated protein were overlapping with each other at this condition even though the resolution between free PEG and PEG-conjugate was better at this condition. Analysis was done using 10 mM HEPES buffer pH 6.5 at flow rate of 0.5 mL/min. The injection volume was 30 μL.

also demonstrated that retention time of non-PEGylated protein was lengthened as well. However, this impacts non-PEGylated protein much more than PEG-conjugate. This could be the result of PEG sheltering the protein from interaction with the column surface. The impact of sodium chloride on the interaction of the protein with the SE-HPLC column matrix observed in this study is consistent with that reported by Mant et al. for a series of non-PEGylated polypeptides [21]. In their studies, it was found that increasing sodium chloride concentration in SE-HPLC mobile phase increased hydrophobic interaction of polypeptides with column matrix; which resulted in an elution profile based on the hydrophobic character of the analytes not the size of the molecules [21].

3.3. Impact of buffer concentration on resolution

HEPES buffer was used in the mobile phase and its optimum concentration in mobile phase was found to be 20 mM. When HEPES concentration was increased from 20 to 50 mM, the resolution between free PEG and its PEG-conjugate was reduced from 1.7 to about 0.8. This reduction of resolution was caused by PEG-conjugate shifting to longer retention time whereas free PEG retention time remained unchanged. Decreasing HEPES concentration to 10 mM, the resolution between free PEG and PEG-conjugate increased to >2. However, the non-PEGylated protein started overlapping with free PEG as shown in Fig. 3. Interestingly, this overlap was caused by shortened retention time of non-PEGylated protein whereas that of the free PEG remained unchanged. Initially, it was thought that this apparent impact of buffer concentration on the resolution was likely caused by a decrease in ionic strength since the HEPES buffer at pH 6.5 was prepared with HEPES free acid and its sodium salt. The reduced ionic strength would reduce the hydrophobic interaction of PEG-conjugate and non-PEGylated protein with column matrix. However, by comparing to effect of adding 100 mM sodium chloride to HEPES mobile phase as shown in Fig. 2C, the reduction of sodium salt in this running condition is insignificant, but the impact on the retention time is significant. Therefore, the impact of mobile phase buffer concentration on retention time cannot be simply explained by the change in ionic strength. One possibility is that sodium chloride is small and can provide better shielding effect, which promotes greater hydrophobic interaction of the analytes with the column matrix.

3.4. Impact of buffer species on resolution

Buffer species in the mobile phase can also impact on the separation. Replacement of HEPES with phosphate buffer, a common buffer species used for size exclusion chromatography, resulted in a complete loss of resolution as demonstrated in Fig. 2B. Apparently, the protein related peaks, including those PEG-conjugate and non-PEGylated protein, shifted to a longer retention time; but the retention time of free PEG remained unchanged. As a result, the PEG-conjugate merged with free PEG resulting in a single peak. The peak shape/pattern is similar to that observed when 100 mM NaCl was added to the mobile phase containing 20 mM HEPES at pH 6.5 (Fig. 2C). Therefore, the peak shifting of protein related species might partially be the result of inorganic salt present in the mobile phase, which increases the hydrophobic interaction between the analytes and column matrix.

3.5. Other factors potentially affecting the assay

Other factors potentially affecting the resolution between free PEG and PEG-conjugate were also investigated, including mobile phase pH, flow rate, number and order of columns. Alteration of the mobile phase pH in excess of 0.3 pH units will result in a reduction in peak resolution between free PEG and PEG-conjugate (data not shown). Similarly, increasing the flow rate reduced the resolution and resolution R was 1.7, 1.5, and 1.3 at a flow rate of 0.5, 0.75, and 1.0 mL/min, respectively. The clear disadvantage associated with a lower flow rate is the increase in assay time and peak width. Increase in peak width would reduce the sensitivity of the assay. The potential impact of the number and order of columns were investigated as well. Experimental results showed that the column order had minimum impact on the resolution but the number of the columns did have significant impact. Use of a single column, either Shodex Protein KW803 or KW804 showed ~30% reduction in resolution. Increasing the number of columns to three in the order of KW803 → KW804 → KW804 did increase the resolution from 1.7 to 2.0. However, this resulted in a significant increase in analytical time and peak width, thus making the method less attractive and less sensitive as an in-process analytical tool. Since the analytical trace was monitored using a refractive index detector the detector attenuation setting was investigated. The sensitivity of the refractive index detector is adjustable by changing the detector attenuation. Decreasing the attenuation increases the sensitivity. However, the response and attenuation do not have a linear relationship and unlimited decreasing of the attenuation does not always increase the sensitivity due to baseline noise. In this study, an attenuation of 7.8×10^3 was found to be the optimum. Therefore, it was used throughout the study.

3.6. $Log_{10}MW$ vs. retention time

After all the above investigations, it was found that the optimum conditions for the assay include use of Shodex Protein KW803 and KW804 columns in series and a mobile phase containing 20 mM HEPES buffer at pH 6.5 and a flow rate of 0.5 mL/min. Under these conditions, the $\log_{10}$MW of PEG-conjugate, free PEG and non-PEGylated protein were plotted against their retention times. Linear regression of the curve let to the equation: $y = 0.075x + 6.91$ with correlation coefficient of 1; a perfect linear relationship was obtained (Fig. 4). Alteration in the buffer concentration, such as

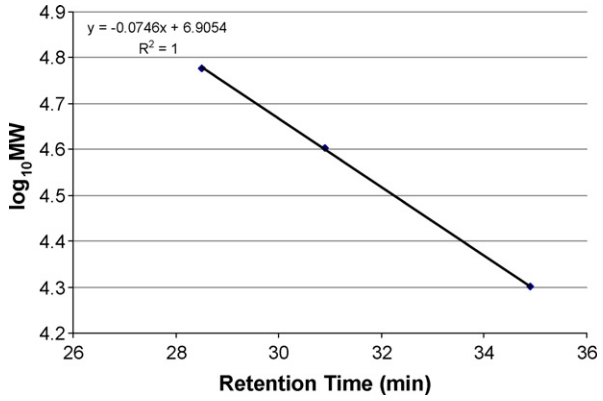

Fig. 4. Plot of $\log_{10}$MW vs. retention time of analytes: analysis was done using 20 mM HEPES pH 6.5 at flow rate of 0.5 mL/min.

10 mM HEPES or 50 mM HEPES, significant departure from the linear relationship was observed. This linear relationship suggests that any non-specific interactions between analytes and column matrix is likely to be minimal in the chosen mobile phase [21].

4. Preliminary evaluation of method performance

4.1. Linearity and accuracy

Linearity was assessed by varying the PEG concentration in sample matrix from 250 μg/mL down to 10 μg/mL with an injection volume of 30 μL. The absolute area count was plotted against PEG concentration. The equation of linear regression was $y = 193898604.3x - 1349706.2$ with a correlation coefficient of ≥0.99. When the free PEG in the concentration range from 250 μg/mL down to 10 μg/mL was spiked into PEG-conjugate samples (protein concentration at 1 mg/mL), the recoveries varied from 78% to 120%. The recovery obtained in this concentration range is considered acceptable when this assay is used as an in-process tool for monitoring PEGylation of proteins.

4.2. Limit of detection and quantitation

The limit of detection (LOD) was established as the minimum concentration at which the free PEG can be detected with signal-to-noise ratio of ≥3. The limit of quantitation (LOQ) was established as the minimum concentration at which the free PEG can be reliably measured with signal-to-noise ratio of ≥10 and with reasonable precision (±5% R.S.D.) and accuracy (≥70% and ≤130% spike recovery). With the above criteria, the LOD and LOQ for free PEG in the presence of PEG-conjugate were determined to be around 10 μg/mL and 25 μg/mL, respectively. At the LOD, the signal-to-noise ratio was 10. At the LOQ, the signal-to-noise ratio was 35 with a precision and accuracy of R.S.D. of 1.9% and recovery of 88%, respectively. The LOQ by this method is higher than the colorimetric method reported by others [18] due to poor baseline resolution and relatively broad peak width. However, this method improved sample throughput and simplified analysis procedure significantly compared to any of the quantitative analytical methods for free PEG in the published literature.

4.3. Repeatability

Repeatability was assessed using a single sample preparation and three replicate injections within the same day. For PEG retention time and PEG area count, the repeatability was demonstrated by R.S.D. of 0.03% and 1.6%, respectively. The repeatability by SE-HPLC analysis using RI detector was better than the colorimetric methods reported in the literature [18]. When compared to gel-based methods for quantitating free PEG, the SE-HPLC method offers much improved precision (gel is used as a qualitative tool [15]).

4.4. Intermediate precision and reproducibility

Intermediate precision was evaluated by two individual analysts with independent sample preparation and analysis using the same instrument. The data was pooled together for statistic analysis. Again, good intermediate precision for PEG retention time measurement and area count measurement was demonstrated with R.S.D. of 0.09% and 2.9%, respectively. This intermediate precision was better than the one measured using calorimetric method in the literature [18]. Reproducibility has not been performed due to early stage of the assay development and lack of a secondary laboratory to perform the analysis.

4.5. Specificity

Specificity is defined as the ability to discriminate between components of closely related structures as well as other species which are likely to be present in the sample, e.g. PEG-conjugate, non-PEGylated protein, chemical reagents for PEGylation and buffer species that may overlap with the peak of interest. Thus, it is necessary to demonstrate the absence of interferences. As shown in Fig. 1, specificity was demonstrated by near baseline resolution between free PEG and PEG-conjugate and baseline resolution between free PEG and non-PEGylated protein as well as the absence of any RI signals in the retention time of interested in when the process buffer matrix and placebo was analyzed.

4.6. Robustness

Robustness is an indication of analytical method reliability. It can be determined by deliberately varying key method parameters and assessing the impact on the method performance and final results. For this method, mobile phase preparation is one of the key parameters that could potentially introduce variation and affect performance. As discussed earlier, the method is sensitive to ionic strength and adjusting the mobile phase pH back and forth with sodium hydroxide and hydrochloric acid could increase the salt concentration in the mobile phase and lead to a reduction or even loss of resolution. The other factor is the flow rate. As a common knowledge, increasing flow rate increases the through-put. However, this will sacrifice the resolution and could potentially affect assay accuracy. To test the robustness of this method, the mobile phase pH was challenged, and the flow rate was also varied.

4.6.1. Mobile phase pH challenging study

In this study, an in-process sample without pre-treatment, containing free PEG, PEG-conjugate, high molecular weigh species (HMWS: multi-PEG-species), and non-PEGylated protein due to incomplete reaction, was used. While keeping all other method parameters constant, the mobile phase pH was intentionally altered by ±0.2 pH units (pH 6.3 and pH 6.7) to evaluate whether these changes would have any effect on the method performance. A minimum of three injections were performed under all pH conditions. The results showed that changing the pH did induce changes in retention times but the maximum variation of retention times were 1.92, 0.36, 0.08 and 0.50 min for HMWS, PEG-conjugate, free

Table 1
Mobile phase pH challenging studies.[a].

Assay parameters at different pHs	HMWS[b]	Conjugate[c]	PEG	Protein[d]
Retention time (min)				
Desired pH 6.5	28.99	28.59	30.93	34.54
Change at pH 6.3	−0.20	−0.36	−0.08	−0.50
Change at pH 6.7	−1.92	+0.11	−0.08	+0.21
Area distribution (%)				
Desired pH 6.5	7.99	34.98	45.84	11.19
Change at pH 6.3	+0.33	+0.92	−0.61	−0.63
Change at pH 6.7	+0.76	+0.13	−0.15	−0.73

[a] Single sample preparation, 3 injections per condition.
[b] High molecular weight species.
[c] PEG-conjugate.
[d] Non-PEGylated protein.

PEG, and non-PEGylated protein, respectively. The largest variation was seen at a pH of 6.7 (+0.2 units) where the retention time for HMWS was clearly decreased. However, this decrease in retention time was in the direction of improving the resolution between HMWS and PEG-conjugate. The variation of area percent distribution was 0.92% or less for all species. This demonstrated that the SE-HPLC method can be operated in a controllable mobile phase pH range from 6.3 to 6.7 without significantly impacting the accuracy of free PEG quantitation. A summary of the data is in Table 1.

4.6.2. Variation of flow rate

The same in-process sample used for pH challenging study was also used in this study. While keeping all other parameters unchanged the flow rate was increased from 0.5 to 1.0 mL/min. The variations in area percent distribution for HMWS, PEG-conjugate, free PEG, and non-PEGylated protein were 1.61%, 2.09%, 0.27%, and 0.76%, respectively. Among all the variations, the impact on free PEG quantitation was the least. This, again, suggests that the method can be operated under relatively broad range of flow rate without significantly affecting the quantitative output of the analyte.

5. System suitability and assay acceptance criteria

The system suitability test includes three replicate injections of in-process buffer/drug product diluent and reference standard, respectively. The reference standard used in the system suitability test is an artificially blended material containing known amount of PEG-conjugate, free PEG and non-PEGylated protein. The acceptance criteria for system suitability include: (1) the last injection of in-process buffer/drug product diluent must have no peaks within the retention time window for PEG-conjugate, free PEG and non-PEGylated protein (20–40 min). (2) The resolution between PEG-conjugate and free PEG as well as free PEG and non-PEGylated protein must be ≥1.3 and 1.6, respectively. (3) The R.S.D. of retention time for three replicate injections of PEG-conjugate, free PEG and non-PEGylated protein must be ≤5%. (4) The R.S.D. of peak area count for three replicate injections of PEG-conjugate, free PEG and non-PEGylated protein must be ≤5%, respectively.

The assay acceptance criteria include (1) system suitability must be met prior to sample injection. (2) All reference standard injections must meet resolution requirement. The resolution between PEG-conjugate and free PEG as well as free PEG and non-pegylated protein must be ≥1.3 and 1.6, respectively. (3) The R.S.D. of retention time in reference standard injections for PEG-conjugate, free PEG and non-PEGylated protein must be ≤5%. (4) The R.S.D. of peak area count in reference standard injections for PEG-conjugate, free PEG and non-PEGylated protein must be ≤5%, respectively.

6. Method comparison

The method reported here provides a simple and robust tool for monitoring free PEG in PEG-conjugate samples, thus offering advantages to the cumbersome gel and colorimetric methods traditionally used for PEG detection and quantitation. First, it does not need any sample pre-treatment whereas the colorimetric method mentioned earlier needs free PEG to be isolated from other PEG conjugated species before analysis can be performed, otherwise only the total amount of PEG-related species will be obtained [18]. Second, the SE-HPLC method reported here takes only 1 h to see the entire PEGylation profile whereas SDS-PAGE analysis takes at least 24 h. Finally, SE-HPLC analysis methods in general, have been proven to be a more reliable, robust, and accurate technique than the traditional colorimetric techniques and gel-based assays.

7. Conclusion

With tandem Shodex Protein KW803 and KW804 HPLC columns, free PEG was successfully separated not only from the PEGylated protein but also from the non-PEGylated protein. Extensive method development revealed that the HEPES buffer species, in addition to buffer pH and buffer concentration, is a key element resulting in the resolution of the free PEG and PEGylated protein. The optimum assay conditions were identified, including use of 20 mM HEPES buffer at pH 6.5 as the mobile phase, flow rate of 0.5 mL/min, and RI detector attenuation of 7.8×10^3. It is also shown that both addition of sodium chloride to the HEPES buffer or replacement of HEPES with phosphate buffer resulted in a complete loss of resolution between free PEG and PEG-conjugate.

Limited evaluation of assay performance demonstrates acceptable linear response, precision, accuracy, specificity, and robustness. Although the limit of detection and limit of quantitation seem to be slightly higher than those for the colorimetric method reported in the literature, this rapid SE-HPLC method (60 min) does not require additional sample manipulation as required by the colorimetric method, and quantitation proves to be much more accurate and reliable than the gel-based SDS-PAGE analysis. This SE-HPLC/RI technique has been successfully used as an in-process tool for monitoring the protein PEGylation reaction and would be an indispensable assay in the development of PEGylated protein products.

References

[1] J.M. Harris, R.B. Chess, Nat. Rev. 2 (2003) 214–221.
[2] F.M. Veronese, G. Pasut, Drug Discov. Today 10 (2005) 1451–1458.
[3] M.L. Graham, Adv. Drug Deliv. Rev. 55 (2003) 1293–1302.
[4] P. Bailon, A. Palleroni, C.A. Schaffer, C.L. Spence, W.J. Fung, J.E. Porter, G.K. Ehrlich, W. Pan, Z.X. Xu, M.W. Modi, A. Farid, W. Berthold, M. Graves, Bioconjug. Chem. 12 (2001) 195–202.
[5] Y.S. Wang, S. Youngster, M. Grace, J. Bausch, R. Bordens, D.F. Wyss, Adv. Drug Deliv. Rev. 54 (2002) 547–570.
[6] P.J. Trainer, W.M. Drake, L. Katznelson, P.U. Freda, V. Herman-Bonert, A.J. van der Lely, E.V. Dimaraki, P.M. Stewart, K.E. Friend, M.L. Vance, G.M. Besser, J.A. Scarlett, M.O. Thorner, C. Parkinson, A. Klibanski, J.S. Powell, A.L. Barkan, M.C. Sheppard, M. Malsonado, D.R. Rose, D.R. Clemmons, G. Johannsson, B.A. Bengtsson, S. Stavrou, D.L. Kleinberg, D.M. Cook, L.S. Phillips, M. Bidlingmaier, C.J. Strasburger, S. Hackett, K. Zib, W.F. Bennett, R.J. Davis, N. Engl. J. Med. 342 (2000) 1171–1177.
[7] O.B. Kinstler, D.N. Brems, S.L. Lauren, A.G. Paige, J.B. Hamburger, M.J. Treuheit, Pharm. Res. 13 (1996) 996–1002.
[8] Retina (Philadelphia, PA) 22 (2002) 143–152.
[9] C.J. Fee, J.M. Van Alstine, Chem. Eng. Sci. 61 (2006) 924–939.
[10] Y.S. Youn, J.Y. Jung, S.H. Oh, S.D. Yoo, K.C. Lee, J. Control Release 114 (2006) 334–342.
[11] P. Esposito, L. Barbero, P. Caccia, P. Caliceti, M. D'Antonio, G. Piquet, F.M. Veronese, Adv. Drug Deliv. Rev. 55 (2003) 1279–1291.
[12] P. Caliceti, F.M. Veronese, Adv. Drug Deliv. Rev. 55 (2003) 1261–1277.

[13] Y.S. Youn, M.J. Kwon, D.H. Na, S.Y. Chae, S. Lee, K.C. Lee, J. Control Release 125 (2008) 68–75.
[14] J.M. Boksoon Kwon, A.L. Zydney, J. Membr. Sci. 319 (2008) 206–213.
[15] F.M. Veronese, Biomaterials 22 (2001) 405–417.
[16] M.M. Kurfurst, Anal. Biochem. 200 (1992) 244–248.
[17] B. Skoog, Vox Sang. 37 (1979) 345–349.
[18] X.W. Gong, D.Z. Wei, M.L. He, Y.C. Xiong, Talanta 71 (2007) 381–384.
[19] Y.S. Youn, D.H. Na, S.D. Yoo, S.C. Song, K.C. Lee, J. Chromatogr. A 1061 (2004) 45–49.
[20] T. Yu, J.A. Traina, E. Pungor 2nd., M. McCaman, Anal. Biochem. 359 (2006) 54–62.
[21] C.T. Mant, J.M. Parker, R.S. Hodges, J. Chromatogr. 397 (1987) 99–112.