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HPLC method validation studies on a specific assay for monomethoxypoly(ethylene glycol) succinimido carbonate (mPEG-SC)

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ABSTRACT

An UV-HPLC method for the determination of the potency of mPEG-SC, 5 kDa (**1**) has been developed and validated. Validation acceptance criteria that is typical of small molecule method validation was successfully applied for this method. The method relies on the HPLC separation of mPEG-OH, 5 kDa (**2**) and a derivatized form of mPEG-SC, 5 kDa (**1**). 1-Naphthylmethylamine reacts with mPEG-SC, 5 kDa (**1**) to form a stable, strongly UV absorbing carbamate **3**. When the eluent is analyzed by UV detection at 220 nm, the method was shown to be linear, precise and accurate. The limit of detection of the method was 0.1 μg.

1. Introduction

Polymeric drug substances represent a structurally diverse class of molecules such as peptides [1,2], carbohydrates [3,4], oligonucleotides [5–7] and pegylated small molecules [8–10] and the therapeutic importance of polymer drug conjugates has also been recently highlighted [11]. In contrast to small molecule HPLC method validation in which reports of method validation are routinely seen [12–16], there are few examples of HPLC method validation for polymeric substances. Validation of a HPLC method for polymeric excipients [17] and a SEC (size exclusion chromatography) method for the sunscreen, PEG-25 PABA [18] has appeared. As instrument-based separation methods continue to grow in sophistication [19], there will be increased pressure to validate HPLC methods for polymeric drug substances. For drug substances in which a polymer chain has been incorporated into the active pharmaceutical ingredient such as by the process of pegylation [20–22] or the formation of "plastic pills" [23,24], the polydispersity of the polymeric reagent will also influence the validation of such HPLC methods.

In a recent paper, we reported on a comparative study of three different methods, that were used to characterize the purity of monomethoxypoly(ethylene glycol) succinimido carbonate (mPEG-SC, 5 kDa (**1**)) [25]. For this work, a specific assay method was sought that could be used to assess differences in functional group activity and end group substitution of mPEG-SC, 5 kDa (**1**). As a result of the manufacture of mPEG-SC, 5 kDa (**1**), N,N′-disuccinimidyl carbonate (DSC) can be present in various lots of this material and differences would be expected as the reagent for the formation of the activated ester, DSC can assay in much the same way as mPEG-SC, 5 kDa (**1**) (Scheme 1). For this synthesis, mPEG-OH **2** reacts with DSC in the presence of a base to yield mPEG-SC **2** [26]. The importance of characterization of the degree of functionalization for maleimide-PEG as an approach towards control of PEG product variability has also recently been discussed [27].

Pegylation of small molecules imparts a variety of favorable pharmacokinetic and pharmacodynamic characteristics to drug substances [22] and many of these desirable characteristics such as increased blood circulation times are also observed when the PEG polymer is conjugated with a protein-based therapeutic [28]. For both of these applications, the mPEG polymer chain is incorporated into the final drug substance and the characteristics of the mPEG polymer will enhance the properties of the active pharmaceutical ingredient. Despite the commercial significance of mPEG-SC reagents [20] and their versatility for the preparation of novel pegylated polymers of pharmaceutical significance [29], a validated HPLC assay for these compounds has not yet been reported. Because of this situation and as a manufacturer of mPEG-SC (**1**) reagents, we decided to develop and validate a UV-HPLC testing method for mPEG-SC, 5 kDa (**1**).

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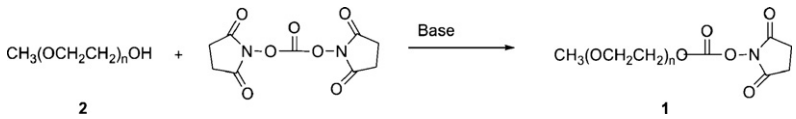


Scheme 1. Preparation of mPEG-SC, 5 kDa (**1**).

In our comparative study of three different methods used to assay mPEG-SC, 5 kDa (**1**), a titration assay emerged as the preferred method. The % R.S.D. (relative standard deviation) of an ELSD-HPLC (evaporative light scattering detection) method, which was based on the reaction of mPEG-SC (**1**) with benzylamine was shown to be too great to be considered useful for quantitative analysis and as a result, the less specific titration method emerged as the superior method. Because the benzylamine-based carbamate had a low extinction coefficient relative to the weight of the polymer, its low UV sensitivity precluded the use of UV detection for the analysis of the PEG carbamate. As a consequence, only detection by ELSD was evaluated and it was not possible to determine if the high % R.S.D. was due to the ELSD or was a characteristic of HPLC analysis of polymers [25]. Because of our need to develop and validate a HPLC method for mPEG-SC, 5 kDa (**1**), we decided to also compare detection by ELSD and UV in order to investigate if the precision of the method is a characteristics of the polymer sample or the detection method. We wish to report on our results from these validation studies.

2. Experimental

2.1. Chemicals

1-Naphthylmethylamine with a purity of at least 97% was used as received from Aldrich Chemical Company and was stored in a desiccator over potassium hydroxide flakes. HPLC grade solvents were obtained from Lab Express, Fairfield, New Jersey. Trifluoroacetic acid was purchased from Halocarbon. Distilled water was obtained from local vendors and was submicron filtered prior to use. Other chemicals were obtained from Aldrich Chemical Company. mPEG-SC, 5 kDa (**1**) was a product of internal manufacture and during storage, mPEG-SC, 5 kDa (**1**) samples were maintained under an argon atmosphere. Monomethoxypoly(ethylene glycol), mPEG-OH, 5 kDa (**2**) was obtained form Sunbio, Seoul, South Korea. The polydispersity of the mPEG-OH, 5 kDa (**2**) starting material was determined by the manufacturer to be 1.035.

2.2. Apparatus

The HPLC system consisted of a Thermo Separations degasser, P2000 gradient pump and an AS1000 autosampler. The eluent was analyzed by a Shimadzu SPD 10A UV detector connected in series to an Alltech ELSD 500 evaporative light scattering detector. Data collection and analysis was done by Chromeleon software by Dionex with a Dionex UCI-100 interface.

A Jupiter, 300 Å, C18, 5 μm column (Phenomenex) with dimensions of length of 250 mm and width of 4.6 mm operating at 35 °C was used. Gradient elution was employed with initial conditions of 65% trifluoroacetic acid (TFA) (0.1% (v/v)) and 35% acetonitrile at a flow rate of 0.8 mL/min. The initial conditions were maintained for 10 min and then changed to 40% TFA (0.1% (v/v)) and 60% acetonitrile over a 20 min period in a linear fashion. The injection volume was 10 μL and the sample compartment was maintained at ambient temperature. UV detection was done at 220 nm and the ELSD operated at a drift tube temperature of 115 °C and a nitrogen flow between 3.00 and 3.02 SLPM (standard liters per minute). The exhaust temperature was 56 °C.

2.3. Pre-method validation

Separate solutions of mPEG-OH, 5 kDa (**2**) and mPEG-naphthylcarbamate, 5 kDa (**3**) were prepared by combining 50 mg of the polymer and 5.0 mL of acetonitrile and diluting to volume in a 50 mL volumetric flask with water. The retention times were determined.

The derivatization procedure of mPEG-SC, 5 kDa (**1**) was as follows. 1-Naphthylmethylamine (100 mg) was diluted to volume with acetonitrile in a 100 mL volumetric flask. mPEG-SC, 5 kDa (**1**) (50 mg) was transferred to a 50 mL volumetric flask and 5.0 mL of the 1-naphthylmethylamine stock solution was added. The solution was held for 1 h at room temperature and diluted to volume with water. An aliquot of the sample was directly injected into the HPLC.

Two blank solutions were prepared. One consisted of 5.0 mL of acetonitrile diluted to volume in a 50 mL volumetric flask with water and for the other blank, 5.0 mL of the 1-naphthylmethylamine stock solution was diluted to volume in a 50 mL volumetric flask with water. Injections of these blank solutions showed no interference with mPEG-naphthylcarbamate, 5 kDa (**3**).

Five independent weighings of mPEG-SC, 5 kDa (**1**), which spanned a working concentration of 50–150% of analyte level were obtained. To each sample that was contained within a 50 mL volumetric flask, 5.0 mL of the 1-naphthylmethylamine stock solution was added and the derivatization procedure and sample preparation followed. Duplicate injections at each sample concentration were made. Program-based integration was found to be superior to manual integration of the peak. Values for peak start and peak end were used for the integration.

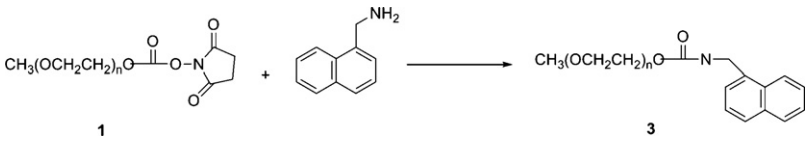
2.4. Method validation

For the linearity study of the method, a similar protocol as described in the pre-method validation work was followed except that mPEG-naphthylcarbamate, 5 kDa (**3**) was weighed and six independent weighings were done. The samples were dissolved in either 5.0 mL of acetonitrile or 1-naphthylmethylamine stock solution and diluted to volume in a 50 mL volumetric flask with water.

Triplicate preparations of mPEG-SC, 5 kDa (**1**) at concentrations of 80%, 100% and 120% of the analyte concentration were prepared. The samples were derivatized as described above and duplicate injections of each solution were obtained. The mean area counts from the sample were compared to the calculated area counts of the standard from the linearity curve. The accuracy was calculated.

Six independent weighings of mPEG-SC, 5 kDa (**1**) of 50 mg were obtained. The solutions were reacted under the standard protocol with 1-naphthylmethylamine and diluted as per the method. Duplicate injections were made and the average response in area counts per milligram of sample was calculated. The analysis precision was calculated from the % R.S.D. of these six samples. For the injection precision, six injections of a single preparation of mPEG-SC, 5 kDa (**1**) were obtained. The % R.S.D. was calculated.

For the determination of the limit of detection of the method, known concentrations of mPEG-naphthylcarbamate (**3**) were serially diluted. Six replicate injections of each dilution were made and the % R.S.D. was determined. The limit of detection was found when the % R.S.D. was greater than 5.0%, but less than 15.0%.



Scheme 2. Synthesis of mPEG-Naphthylcarbamate (**3**).

2.5. Preparation of N-(1-naphthylmethyl) O-monomethoxypoly(ethylene glycol)-carbamate (**3**)

Under a nitrogen atmosphere, 5.14 g (1.00 mmol) of mPEG-SC, 5 kDa (**1**) and 50 mL of anhydrous acetonitrile were combined at room temperature. To the solution, 1-naphthylmethylamine (0.44 mL (3.0 mmol) and 0.35 mL (2.0 mmol) of N,N-diisopropylethylamine (DIEA) were added. A precipitate of N-hydroxysuccinimide (NHS) formed almost immediately. The reaction was stirred for 4 h at room temperature. The precipitate was filtered and washed with acetonitrile. The solvent was evaporated under reduced vacuum at 40 °C on a rotary evaporator. To the residue, 200 mL of ethyl acetate was added and the mixture was heated until a solution was obtained. Cooling in an ice bath was followed by a filtration and washing with ethyl acetate. The solid was dried at 40–45 °C under vacuum. There was obtained 3.94 g of mPEG-naphthylcarbamate, 5 kDa (**3**) as a white solid in a 76% yield.

3. Results and discussion

Nimura and others have reported on the reaction of the aromatic amines with DSC to prepare activated carbamate reagents in which these reagents now contain a strongly absorbing chromophore. In this work, aniline, 4-bromoaniline and 1-naphthylamine were selected as the chromogenic component [30]. For the present study, 1-naphthylmethylamine was selected as the primary amine as it is an aliphatic amine and for the derivatization reaction, the reaction of this amine with mPEG-SC, 5 kDa (**1**) is likely to be instantaneous to form the stable mPEG-naphthylcarbamate, 5 kDa (**3**) (Scheme 2). In this derivatization reaction, 1-naphthylmethylamine reacts with the carbonate carbonyl of mPEG-SC, 5 kDa (**2**) to form a stable carbamate. As the Nimura conditions for the reaction of aliphatic amines with the aromatic activated carbamate esters involves reaction times of less than 10 min, our derivatization reaction times of 1 h and with our reported excess was considered more than adequate to ensure complete reaction of mPEG-SC, 5 kDa (**1**). Further, as a consequence of the development of a benzylamine titration assay, analytical reaction conditions were developed for the reaction of this primary aliphatic amine with mPEG-SC, 5 kDa (**1**) [25]. A two-fold molar excess and reaction times of 1 h were employed for the benzylamine titration assay whereas a three-fold molar excess and 1 h reaction time is used for the 1-naphthylmethylamine reaction.

Despite the expected similarities in reactivity between the primary amines, benzylamine and 1-naphthylmethylamine, 1-naphthylmethylamine was selected as the derivatization reagent as 1-naphthylmethylamine should impart desirable UV absorbing characteristics to the UV transparent PEG polymer. Naphthalene has an UV absorption at 221 nm with an extinction coefficient of 133,000 L/mol cm [31] whereas benzene has only a modest maximum extinction coefficient.

Our method was shown to be specific when a standard solution of mPEG-OH, 5 kDa (**2**) had a retention time of approximately 11.9 min and that of carbamate **3** had a retention time of 22.6 min (Figs. 1–3). Symmetrical ELSD peaks for mPEG-OH, 5 kDa (**2**) and carbamate **3** were observed with a peak asymmetry of 1.01 and 1.02 respectively. A more intense ELSD signal was obtained for carbamate **3** at the same concentration than for mPEG-OH **2** and this may be due to the fact that carbamate **3** elutes during the more organic-rich part of the gradient [32]. At the same sample concentration, the signal from the UV detection of carbamate **3** was less than for the ELSD.

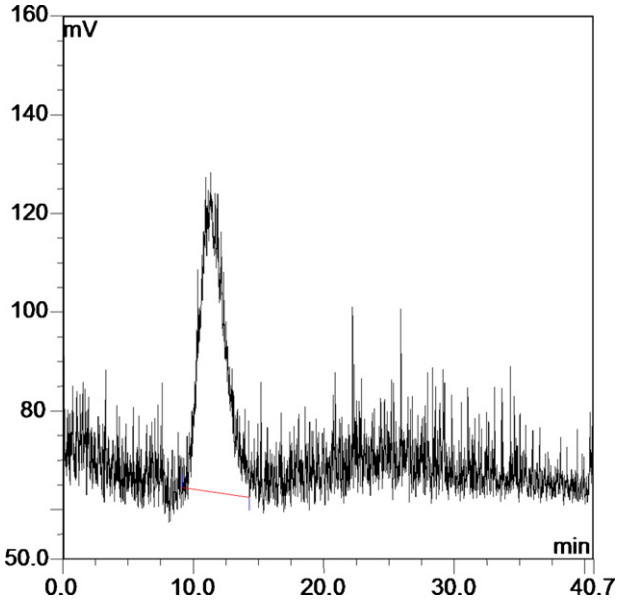


Fig. 1. ELSD-HPLC of mPEG-OH, 5 kDa (**2**) at a concentration of 1 mg/mL.

The peak asymmetry from the UV-HPLC was much higher than that from the ELSD for carbamate **3** and was 1.60. These differences are likely due to the polydispersity of the polymer and the fact that UV-HPLC is a measure of concentration whereas ELSD measures the mass of the analyte. Despite these differences, a reasonably shaped peak was obtained from the UV-HPLC.

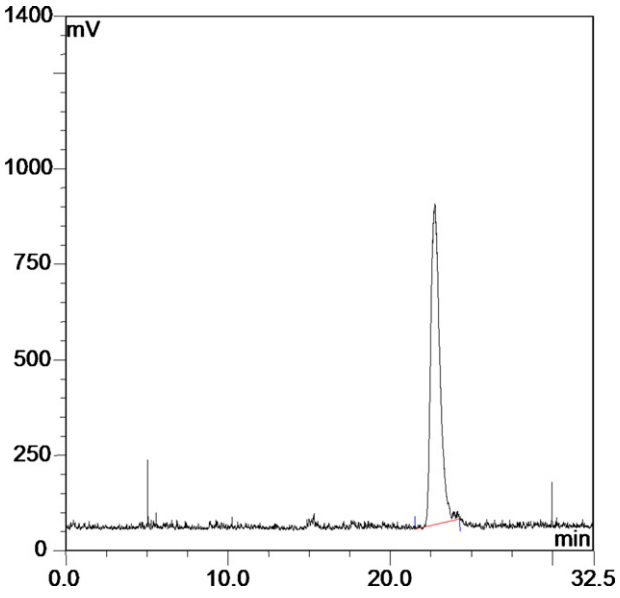


Fig. 2. ELSD-HPLC of mPEG-naphthylcarbamate (**3**) at a concentration of 1 mg/mL.

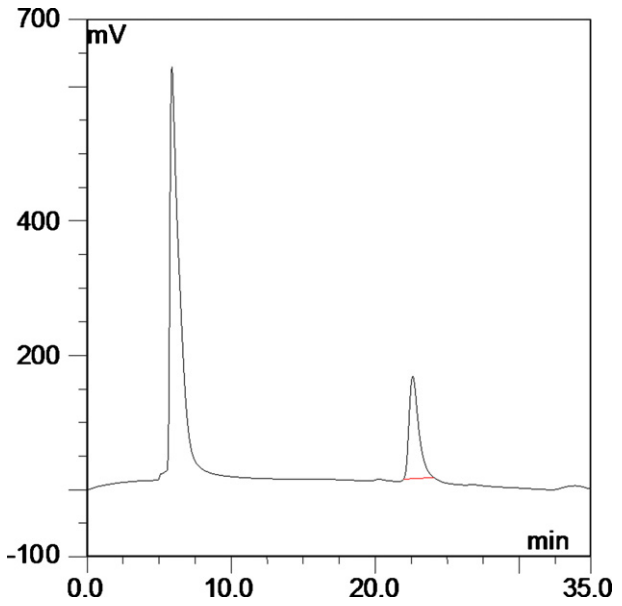


Fig. 3. UV-HPLC of mPEG-naphthylcarbamate (**3**) at a concentration of 1 mg/mL with the excess of the 1-naphthylmethylamine derivatization reagent.

1-Naphthylmethylamine elutes close to the void volume of the column with a retention time of 6 min as determined by UV-HPLC (Fig. 3). When DSC was reacted under similar conditions to the derivatization reaction, a broad peak, which was centered at about 14 min was detected. The component is likely the urethane **4** as a result of two moles of 1-naphthylmethylamine reacting with the difunctional reagent, DSC (Scheme 3). No interference was observed with the carbamate **3** peak. The by-product of the derivatization reaction, N-hydroxysuccinimide, a small molecule polar component is likely to be unretained under these chromatographic conditions [30]. Injections of the blank solutions also showed no interferences. As the conversion of activated ester **1** to carbamate **3** is complete under our analytical reaction conditions, the method is specific for mPEG-SC, 5 kDa (**1**) as assayed as carbamate **3** and the signal from a sample concentration of 1 mg/mL for the simultaneous detection by both UV and ELSD was adequate.

ELSD is fundamentally different from UV as the basis of the signal involves the scattering of light due to the presence of particles. The relationship between area response and analyte mass is [33]:

$$A = aM^b \quad (1)$$

where A is the area response, M is the mass of the analyte and a and b are values which are specific to the analyte and chromatographic conditions. Because of this relationship, evaporative light scattering detectors are often described as a mass detector [34]. Log–log plots should yield a linear curve [33]. UV detection is based on Beer's law and detection by this method is expected to be linear. Our initial linearity evaluation in the pre-method validation study should show whether HPLC analysis of polymers by either detection method is linear and if with our present method that a weight percent assay for mPEG-SC (**1**) might be possible.

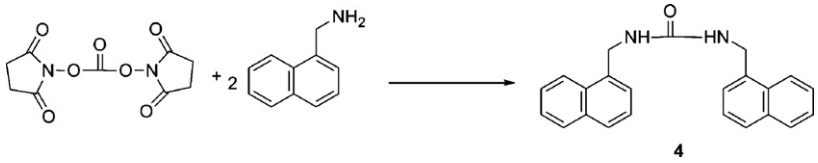
For two of the ten injections by analysis by ELSD of derivatized mPEG-SC, 5 kDa (**1**), the program-based integration failed to integrate the peak correctly. Interestingly, all ten injections by UV detection integrated the peak and little variation in the area was seen between duplicate injections. The failure of the integration software to consistently integrate the ELSD peak may be due to the fact that the shape of the peak is better when ELSD is used as the detection method. The faster rate of signal acquisition that is required for this ELSD signal may hamper its integration using conventional software. The use of peak start and peak finish as the means to integrate the carbamate **3** peak was shown further to be acceptable as the % R.S.D. of the retention times from the linearity curve, $n = 10$ was 0.38%.

With a calibration curve derived from a plot of area counts versus concentration (mg/mL), the UV-HPLC assay afforded a correlation coefficient for the linearity curve which is acceptable in small molecule method validation and was greater than 0.999 and was actually 0.9997. The slope was 137.6 and the y-intercept was −1.08, $n = 5$ with two replicate injections. For the UV-HPLC method, the y-intercept contributed less than 2.0% to the response at 100% of the analyte concentration.

A log–log plot improved the correlation coefficient of the ELSD calibration curve from 0.9891 to 0.9941. However, only two injections of each sample concentration were done and the known reduced precision of ELSD-HPLC methods [33] may not be overcome by only duplicate injections. Our proposed method would involve a three-point calibration curve and the analysis of two independent samples. With the constraints of typical working day and the time required for system equilibration, only duplicate injections are possible. Because we were satisfied with the UV-HPLC results, no optimization of the manufacturer suggested ELSD conditions to address the lack of precision was done. Despite the fact that an ELSD-HPLC method could detect and quantify UV-transparent polymers such as mPEG-OH, 5 kDa (**2**), a further evaluation of the ELSD-HPLC method was discontinued.

Having established that a linear response could be obtained by UV-HPLC for the analysis of polymer **1**, a standard of carbamate **3** was necessary. A standard of mPEG-naphthylcarbamate **3** was prepared by a procedure that parallels the derivatization reaction. The reaction was conducted in acetonitrile and DIEA was present to ensure a complete conversion of mPEG-SC, 5 kDa (**1**) to the carbamate **3**. When the standard mPEG-naphthylcarbamate **3** was used to determine a calibration curve, a linear curve again was found for the analyte. The correlation coefficient was 0.9991; the slope was 118.5 and the y-intercept was 0.968.

To minimize effects due to potential differences as a result of polydispersity between the analyte and reference sample, the mPEG-SC, 5 kDa (**1**) was prepared from the same lot of mPEG-OH, 5 kDa (**2**). By ^{13}C NMR, no extraneous resonances such as those due to mPEG-OH **2** or DSC were seen in the starting material for the reference carbamate **3** (Fig. 4) and the reference standard is of high quality. In d_6-DMSO and in addition to those resonances due to the PEG backbone and the methoxy resonance at 59 ppm, mPEG-SC, 5 kDa (**1**) had ^{13}C NMR resonances of 25 ppm for the methylene carbons of the succinimide ring; 68 ppm for the methylene group alpha to the oxygen atom of the carbonate group; 151 ppm for the carbonate carbonyl and 168 ppm for the succinimidyl carbonyls.



Scheme 3. Reaction of DSC with 1-naphthylmethylamine.

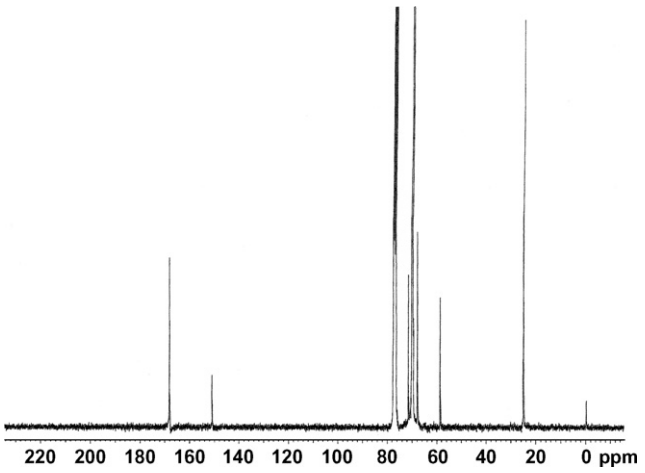


Fig. 4. ^{13}C NMR of mPEG-SC, 5 kDa (**1**) (lot A) in d_6-DMSO.

The alpha methylene protons for the starting mPEG-OH, 5 kDa (**2**) absorbs at 61 ppm. The ^{13}C NMR spectrum of DSC is characterized by a distinct resonance at 171 ppm. The titration assay for this sample of mPEG-SC, 5 kDa (**1**) (lot A) was 99.4%[25].

Similar but not identical regression equations were obtained when the standard was dissolved in the 1-naphthylmethylamine stock solution and a calibration curve was obtained for these solutions. For this situation, the correlation coefficient was 0.9993; the slope was 117.8 and the intercept was 2.38. Because this system most closely parallels the sample matrix, this calibration curve was used to determine the accuracy. The accuracy was evaluated at 80%, 100% and 120% of sample concentration (Table 1). Low potency mPEG-SC, 5 kDa (**1**) could be encountered as a result of an incomplete reaction to produce mPEG-SC, 5 kDa (**1**) or hydrolysis of mPEG-SC, 5 kDa (**1**) upon storage. An analyte of polydispersity different than the standard may influence the accuracy. Although an evaluation of the influence of polydispersity on the accuracy of the method was beyond the scope of the present work, the accuracy was evaluated at concentrations greater than and less than the standard. Accurate results were obtained with these concentrations. The benzylamine titration assay for this sample (lot B) of mPEG-SC, 5 kDa (**1**) was 102.9% [25]. As the HPLC wt% assay for this lot is within a reasonable specification of 98-102%, the method is accurate for mPEG-SC, 5 kDa (**1**). The average of the accuracy results gives a specific assay of 99.7% for this lot of mPEG-SC, 5 kDa (**1**). This value, which is close to 100% also supports the assertion that the derivatization reaction is complete.

The precision of the UV-HPLC method afforded an acceptable % R.S.D. for the analysis precision of 0.61%. The data set from a study of the injection precision of the method had a % R.S.D. of 0.75%. The close agreement between these values would suggest that the majority of the error is due to the chromatographic system and that variability due to such factors as handling milligram quantities of a moisture-sensitive compound, variability in the derivatization reaction or content uniformity of the sample is not significant. Both of these values are less than 2.0% and the method is considered precise.

Table 1
Accuracy results for mPEG-SC, 5 kDa (**1**), lot B.

% Target concentration	Recovery (%)		
80%	99.6%	99.0%	99.7%
100%	100.9%	98.8%	100.5%
120%	99.3%	98.8%	100.8%

Table 2
LOD determination for mPEG-naphthylcarbamate, 5 kDa (**3**).

Injection	Area counts			
	1[a] 10%[c]	0.5[a] 5%[c]	0.1[a] 1%[c]	0.05[a,b] 0.5%[c]
1	13.5714	6.6299	1.1640	0.2072
2	13.8381	6.9516	1.1571	0.3255
3	12.8396	6.7676	1.2146	0.3167
4	13.2477	6.5539	0.8529	0.1823
5	13.3935	6.7859	0.9417	0.2830
6	13.0714	6.4442	1.0845	0.3250
% R.S.D.	2.673	2.726	13.30	23.16

[a] Amount (μg).
[b] Manual integration.
[c] Area % of carbamate **3** at the present sample concentration.

Polyethylene glycols of molecular weight of 3000 have been derivatized to the corresponding bis-urethanes by reaction with 1-naphthylisocyanate. HPLC analysis was done on bare silica with fluorescence detection. Detection limits of about 0.1 ppm were reported [35]. Using our UV-HPLC method, which would not be as sensitive as a fluorescence detection method, the limit of detection (LOD) was higher and was 0.1 μg (Table 2). The % R.S.D. from six replicate injections was used to determine the LOD as this technique would parallel the method by which low-level impurities would be determined for samples of mPEG-SC, 5 kDa (**1**). Because mPEG-SC, 5 kDa (**1**) is a reagent of pharmaceutical interest and not a final drug product [36], this substance would not be subject to the same rigors as a final product. As such, a formal limit of quantification (LOQ) was not determined. The different methodologies that have been used to determine the limit of detection and limit of quantification in a drug substance have recently been discussed [37]. As can be gleaned from Table 2, the variability in replicate injections of a diluted sample relative to the injection precision of standard sample would suggest that the LOQ of the method is higher than 1 μg.

At concentrations less than 0.1 μg, the program-based integration did not integrate the peak and manual integration was necessary. An unacceptable % R.S.D. was observed under these circumstances. Using the standard deviation of the area counts from multiple injections of a blank [36], the LOD was calculated to be 5 μg. The LOD value of 0.1 μg would correspond to a detection limit for our current sample preparation of about 1% by area for impurities that are separated from mPEG-naphthylcarbamate, 5 kDa (**3**) and possess similar peak characteristics. An example of such an impurity would be the bis activated NHS ester of a PEG-diol as the PEG-diol can be an impurity in mPEG-OH **2** [38]. For this method, the analyte would be the corresponding bis-naphthyl carbamate.

4. Conclusion

Method validation acceptance criteria that is typically used in the validation of small molecule analytes was successfully applied to the validation of a HPLC method for a reactive polymeric mPEG-SC, 5 kDa (**1**) reagent. The method relies on the formation of a stable strongly UV absorbing carbamate **3** and its separation from the starting material of the synthesis and the hydrolysis product of mPEG-SC, 5 kDa (**1**), mPEG-OH, 5 kDa (**2**). The method is linear, precise and accurate and has a limit of detection of 0.1 μg. Because mPEG-SC **1** is used as the conjugation reagent in the preparation of the marketed product (PEG-INTRON), pegylated interferon [39] and succinimido carbonate chemistry is also employed to synthesize a branched activated ester [40] for the manufacture of PEGASYS, another form of pegylated interferon [41], this assay has commercial significance. In addition to its use for an assay for mPEG-SC,

5 kDa (**1**), the method and in particular the derivatization technique might be useful to optimize the amount of mPEG-SC **1** that is used in pegylation applications [42].

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