Universal method for the determination of nonionic surfactant content in the presence of protein

A new analytical method has been developed for the quantitative determination of ethylene glycol-containing nonionic surfactants, such as polyethylene glycol 8000, polysorbate 80, and Pluronic F-68. These surfactants are commonly used in pharmaceutical protein preparations, thus, testing in the presence of protein is required. This method is based on the capillary gas chromatographic analysis of ethylene glycol diacetate formed by hydrolysis and acetylation of surfactants that contain ethylene glycol. Protein samples containing free surfactants were hydrolyzed and acetylated with acetic anhydride in the presence of p-toluenesulfonic acid. Acetylated ethylene glycol was extracted with dichloromethane and analyzed by gas chromatography using a flame ionization detector. The amount of nonionic surfactant in the sample was determined by comparing the released ethylene glycol diacette signal to that measured from calibration standards. The limits of quantitation of the method were 5.0 μg/mL for polyethylene glycol 8000 and Pluronic F-68, and 50 μg/mL for polysorbate 80. This method can be applied to determine thepolyethylene glycol content in PEGylated proteins or the final concentration of polysorbate 80 in a protein drug in a quality control environment.

Keywords: Ethylene glycol diacetate / Gas chromatography / Hydrolysis / Nonionic surfactants / Protein samples
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1 Introduction

Nonionic surfactants, such as PEG 8000, Pluronic F-68 or Pluronic L61, and polysorbate 80 (PS-80) are commonly used in biopharmaceutical manufacturing and formulation to increase protein stability and solubility. The use of nonionic surfactants in mammalian cell culture processes [1–3] and formulations of various proteins [4–6] presents unique challenges for quantitation.

Several difficulties arise in monitoring the concentration of nonionic surfactants in the presence of protein: (1) the analyte molecules are often complex and may contain dozens of oligomers; (2) electrochemical methods used in the detection of ionic surfactants [7] are not applicable; and (3) as these compounds do not possess characteristic chromophoric groups (Fig. 1), their detection by UV-visible absorption spectrometry is problematic. Nonionic detergents can be derivatized and quantitated by colorimetric detection [8–12] but these methods, albeit sensitive, are generally not specific and are labor intensive.

Multiple methods have been described for the analysis of these compounds, including CE [13–15], refractometry [16], SEC [17], and TLC [18]. The use of RP-HPLC has been reported for the analysis of poly(propylene glycol)s based on derivatization and fluorescence detection [19]. HPLC methods performed with evaporative light scattering [20–22], refractometric [23, 24], and fluorescence polarization detection [25] as well as MS [26, 27] are also useful tools for the determination of the molecular mass distribution or the composition of nonionic surfactants. In addition, a GC method for identification of the ester-type surfactants containing poloxoyethylene groups has been described [28]. Many of the methods listed above are afflicted by either cumbersome preparative steps or low sensitivity. Furthermore, quantitative analysis of PEG 8000, Pluronic F-68, or PS-80 in the presence of the protein component is usually complicated, due to adverse effects on method sensitivity and selectivity. There are clear advantages in an analytical method that allows the quantitative determination of surfactants containing ethylene glycol in protein samples without protein removal. Moreover, such a method could also be used for the assessment of the stoichiometry of conjugated PEG in PEGylated proteins.

The use of PEGylated proteins is becoming more common in the biopharmaceutical industry due to their role in modifying key properties of recombinant proteins, such as increasing circulatory half-life and reducing in vivo immunogenicity [29, 30]. Methods such as MS, SEC with multilangle light scattering detection and CE are useful...
for analyzing PEGylated proteins to monitor the degree of PEGylation, but they are not able to quantitatively determine the conjugated PEG levels [31–33]. Chemical agents, such as trinitrobenzene sulfonic acid, have been used in a titration analysis to determine the degree of protein PEGylation [34]. However, this colorimetric method measures modified lysine reactivity and quantitates the average degree of protein modification indirectly.

In the present manuscript, we describe and characterize the analytical performance of a general procedure for the determination of surfactants that contain ethylene glycol in protein samples as well as PEG conjugation levels in PEGylated proteins. The only required analyst intervention is sample dilution or salt removal steps using a low-molecular-weight dialysis cassette. The conditions for PEG acid hydrolysis have been adjusted to occur either within 90 min or overnight, resulting in low sample manipulation burden for the analyst. Acid hydrolysis conditions are compatible with GC detection and are less laborious than alkali-induced hydrolysis [35, 36].

2 Materials and methods

2.1 Chemicals and reagents

PEG 8000, Pluronic F-68, p-toluene sulfonic acid monohydrate, sodium bicarbonate, ethylene glycol diacetate (EGD), BSA, and dichloromethane (CH₂Cl₂) were obtained from Sigma–Aldrich (St. Louis, MO, USA); anhydrous acetic anhydride was obtained from Fluka (Buchs SG, Switzerland); HPLC-grade water, and PS-80 were purchased from J.T. Baker (Phillipsburg, NJ, USA); GL2 series PEG maleimide (MW 40 000) was obtained from NOF Corporation (Tokyo, Japan). The 3.5 kDa molecular weight cut-off Slide-A-Lyzer dialysis cassettes, used for desalting, were obtained from Pierce (Rockford, IL, USA) and Q Sepharose FF resin was from GE Healthcare (Little Chalfont, UK).

2.2 Instrumentation

An Agilent Technologies (Santa Clara, CA) 6890N/7890 GC system equipped with a split/splitless injector and a flame ionization detector was used. Data acquisition and processing were performed using Agilent ChemStation software.

A Perkin Elmer (Waltham, MA) Clarus 500 GC–MS equipped with a Perkin Elmer TurboMass Quadrupole mass spectrometer was employed for peak identification. Data were acquired in positive ion mode with a scan range of m/z 35–500 at a source temperature of 200°C. The NIST mass-spectroscopy library was used to identify the peaks present in a test sample. A Waters® (Columbia, MD) Micromass MALDI micro MX, MALDI-TOF MS was used to confirm PEG 40 000 content in PEGylated BSA.

2.3 Chromatographic conditions

A fused-silica DB-17 capillary GC column (30 m × 0.25 mm id, film thickness 0.25 μm) from J&W Scientific (Albany, NY, USA) was used. The initial oven temperature was 60°C. The temperature was increased at a rate of 2°C/min until 80°C was reached, then raised by 50°C/min until the final temperature of 200°C, which was held for 1 min. The helium carrier gas flow rate was 4 mL/min, the hydrogen flow rate was 30 mL/min, and the air flow rate was 400 mL/min. The front inlet temperature was 250°C, and the detector temperature was 280°C.

2.4 Sample preparation

2.4.1 Dialysis procedure

All protein samples containing more than 10 mM sodium chloride (NaCl) were dialyzed before hydrolysis using Pierce
0.5 mL capacity 3.5 K MWCO Slide-A-Lyzer dialysis cassettes. The cassettes were prewashed with phosphate-buffered saline (PBS) solution containing 50% ethanol, filled with 200 μL of sample and placed in a 1 L container with HPLC water for sample dialysis. The samples were dialyzed for 2 h and then evaporated to dryness using a Joan RC1010 vacuum centrifuge (Winchester, VA, USA).

### 2.4.2 Hydrolysis procedure

Activated acid was prepared by the slow addition of 6.7 mL acetic anhydride to 10.8 g of p-toluenesulfonic acid in a 100 mL glass bottle. The mixture was heated for 30 min at 125°C and cooled to room temperature before use.

Lyophilized protein samples were hydrolyzed with 400 μL of activated acid at 135°C for 1.5 h (for PS-80) or 18 h (for PEG 8000 and Pluronic F-68). Samples were cooled to room temperature, then 2 mL of saturated sodium bicarbonate was added; the released ethylene glycol diacetate was extracted with 3 mL of dichloromethane. The organic layer was separated and quantitatively analyzed by GC.

### 2.4.3 Preparation of calibration standards

A 500 μg/mL stock solution of PEG 8000, PS-80, or Pluronic F-68 was prepared in PBS and diluted with HPLC water to produce calibration standards at 5 μg/mL, 10 μg/mL, 15 μg/mL, 25 μg/mL, and 50 μg/mL. A 200 μL aliquot of each standard was lyophilized in a vacuum centrifuge for 3 h, treated with 400 μL of activated acid at room temperature, and incubated in an oven at 135°C for 1.5 h (for PS-80) or 18 h (for PEG 8000 and Pluronic F-68). After the standards were cooled to room temperature, 2 mL of saturated sodium bicarbonate were added and the released EGD was extracted with 3 mL of dichloromethane. The organic layer was separated and quantitatively analyzed by GC.

### 2.4.4 Preparation of PEGylated protein

Maleimide derivatization of PEG was used for the modification of the single-free sulhydryl group present in BSA. PEGylated BSA was produced by reacting BSA (3 mg/mL) in 25 mM sodium phosphate buffer, pH 9 with maleimide-activated branched PEG 40 000 at a PEG-to-protein ratio of 2.5:1.0. The reaction proceeded overnight at 2–8°C for 18 h. PEGylated BSA was purified from the reaction product by anion exchange chromatography. The reaction samples were adjusted to pH 7, loaded onto Q Sepharose FF resin that was equilibrated with 25 mM sodium phosphate, pH 7, and eluted by a linear salt gradient to 500 mM sodium chloride in 25 mM sodium phosphate, pH 7. Fractions containing protein with a SDS-PAGE profile consistent with PEGylated BSA were pooled and used for testing.

### 2.5 MALDI-TOF analysis

PEGylated BSA samples were diluted to 1 mg/mL with 0.1% TFA, mixed with desorption matrix (saturated sinapinic acid in 70% acetonitrile/30% of 0.1% TFA, v/v) and spotted in triplicate on a 96-well MALDI plate. Mass data (m/z) were collected on a Waters MALDI/MS spectrometer with BSA as the external mass calibrant and phosphorylase B as the system suitability standard.

### 3 Results and discussion

#### 3.1 Quantitation of free PEG in protein preparations

Free PEG in protein samples was measured using acid hydrolysis and simultaneous acetylation, followed by quantitative GC analysis of the resulting EGD. The GC method for the determination of free PEG was evaluated according to ICH Q2 [37] for the following: specificity, precision, accuracy, linearity, LOD, and LOQ.

#### 3.1.1 Specificity

The EGD peak at 8.27 min retention time (Fig. 2) was identified by GC–MS. The electron ionization mass spectrum was matched by comparison to the NIST MS database. Peak area was used for the assay quantitation results described below.

Potential interference from salts, buffer components, and the presence of protein in the quantitation of nonionic surfactants was evaluated. We found that the recovery of known PEG aliquots spiked into test samples was dependent on both the concentration of NaCl and the amount of protein present in the sample. Whereas PEG recovery was 86% in 10 mM NaCl, it decreased to only 28% in the presence of 200 mM NaCl (Fig. 3), clearly showing that increasing levels of NaCl impede poly(oxyethylene) hydrolysis and that, consequently, a desalting step is required before the addition of activated acid. We verified that various buffers spiked with 25 μg/mL of PEG 8000, dialyzed against water, and followed by hydrolysis and GC analysis did not show NaCl interference. Upon desalting, the recovery of PEG from spiked samples ranged from 99 to 102% (Table 1).

We evaluated the effect of protein concentration on PEG 8000 recovery in a similar fashion by spiking 25 μg/mL of the surfactant into samples containing protein concentrations typical of pharmaceutical formulations. The recovery of PEG 8000 was found to be inversely proportional to protein concentration with recovery decreasing from 91% in 10 mg/mL protein to 74% in 25 mg/mL and 59% in 50 mg/mL before leveling at higher protein concentrations (data not shown). Therefore, we recommend sample dilution to 10 mg/mL protein to achieve suitable analyte recovery.
Figure 2. Representative GC chromatogram of ethylene glycol diacetate (EGD) released after hydrolysis and acetylation of ethylene oxide polymer.

Figure 3. Effect of sodium chloride (NaCl) concentration on percent recovery of polyethylene glycol (PEG 8000) in a protein sample (10 mg/mL).

Table 1. Effect of buffer components on percent recovery of PEG 8000 (after desalting)

| Sample and buffer composition                      | Measured PEG 8000 (μg/mL) | % Recovery after desalting |
|---------------------------------------------------|---------------------------|----------------------------|
| 5 mM sodium phosphate, 100 mM NaCl, pH 8.0        | 25.6                      | 102                        |
| 50 mM Glycine, 30 mM NaCl, pH 7.8                  | 24.6                      | 99                         |
| 10 mM Tris, 25 mM NaCl, pH 7.8                     | 24.9                      | 100                        |

3.1.2 Precision

Method precision was assessed by analyzing six replicates of a 10 mg/mL protein sample spiked with 25 μg/mL of free PEG 8000. Before hydrolysis, samples were dialyzed using the procedure described in Section 2.4.2. Method repeatability, evaluated as % CV of the mean value of the six replicates, was 3.6%; and the percent recovery from a spiked protein sample was 82 ± 3% of the theoretical value (Table 2). The intermediate precision of the method was assessed by obtaining measurements from one analyst on two separate days, and from two different analysts (Table 3). The average % CV was determined as 4.2%. These results demonstrated that the method has excellent precision in determining free PEG content in protein samples.

3.1.3 Linearity and range

The linearity of the method was assessed from calibration curves generated using a range of PEG 8000 concentrations. PEG 8000 standards ranging from 5.0–50 μg/mL (0.005–0.05%) were prepared and analyzed as described in Section 2. A calibration curve was constructed by plotting the relative area of the released EGD peak for each PEG 8000 standard. The calibration curve was analyzed using a linear regression model; the correlation coefficient was 0.999 (Fig. 4A).

The LOQ, determined based on a S/N value greater than 10, corresponds to a PEG 8000 concentration of 5.0 μg/mL; this concentration matches the first point of the calibration curve. The LOD was determined by extrapolation to be 2.5 μg/mL, based on a S/N value of 3 (Table 4).
Table 2. Method Precision (repeatability) for PEG 8000, Pluronic F-68, and polysorbate 80 (PS-80)

| Analyte         | Measured concentration (μg/mL) | Average (μg/mL) | % CV | % Recovery |
|-----------------|-------------------------------|-----------------|------|------------|
| PEG8000         |                               |                 |      |            |
| a)              | 19.9                          | 20.6            | 3.6  | 82 ± 3     |
|                 | 21.0                          |                 |      |            |
|                 | 20.8                          |                 |      |            |
|                 | 19.5                          |                 |      |            |
|                 | 20.6                          |                 |      |            |
|                 | 21.5                          |                 |      |            |
| Pluronic F-68   |                               |                 |      |            |
| b)              | 10.1                          | 10.0            | 3.9  | 100 ± 4    |
|                 | 9.4                           |                 |      |            |
|                 | 10.3                          |                 |      |            |
|                 | 10.3                          |                 |      |            |
|                 | 9.6                           |                 |      |            |
|                 | 10.3                          |                 |      |            |
| PS-80           |                               |                 |      |            |
| c)              | 218.6                         | 197.3           | 6.0  | 99 ± 6     |
|                 | 198.0                         |                 |      |            |
|                 | 193.2                         |                 |      |            |
|                 | 198.2                         |                 |      |            |
|                 | 190.6                         |                 |      |            |
|                 | 184.3                         |                 |      |            |

a) A protein sample at 10 mg/mL was spiked individually with 25 μg/mL of PEG 8000.
b) A protein sample at 10 mg/mL was spiked individually with 10 μg/mL of Pluronic F68.
c) A protein sample at 10 mg/mL was spiked individually with 200 μg/mL of PS-80.

3.1.4 Accuracy

The accuracy of the method was determined from analysis of duplicate samples containing known amounts of PEG 8000. A protein sample was spiked with PEG 8000 at 5, 10, 25, and 50 μg/mL and analyzed as described. The average percent recovery was 85 ± 7% of the theoretical value and did not appear to be dependent on PEG concentration (Table 5). These results demonstrated that the method, used in conjunction with internal PEG standards, is accurate in determining free PEG content in proteins.

3.2 Applications to other surfactants and PEG-protein conjugate

Nonionic surfactants other than PEGs are often used as excipients in pharmaceutical formulations. Since PEG (PEG 8000), Pluronic F-68 or Pluronic L61, and PS-80 (among some of the most common solution additives) all contain oxyethylene subunits, we surmise that the analytical method described herein should be suitable for the quantitation of any of these polymeric compounds in protein samples (Fig. 1). We show in this section that this is indeed the case for these compounds.

3.2.1 Determination of Pluronic F-68 concentration

A series of Pluronic F-68 standards ranging from 5.0 to 50 μg/mL was hydrolyzed under the conditions described in Section 2.4.2. As expected, the calibration curve (Fig. 4B) shows linearity and sensitivity similar to that seen with PEG 8000 standards (Fig. 4A). Method precision was assessed by analyzing six replicates of a 10 mg/mL protein sample spiked with 10 μg/mL of free Pluronic F-68. Before hydrolysis, samples were dialyzed using the procedure described in Section 2.4.2. Method repeatability, evaluated as % CV of the mean value of the six replicates, was 3.9% and the percent recovery from a spiked protein sample was 100 ± 4% of the theoretical value (Table 2). The overall intra- and interday variations were 7.4% (Table 3). As shown in Table 5, the developed method demonstrated a good accuracy for analysis of Pluronic F-68 with the average recovery 108 ± 3%. The LOD and LOQ determined by the signal-to-noise approach were the same as for PEG 8000 (Table 4).

3.2.2 Determination of PS-80 concentration

Likewise, PS-80 was measured by EGD release after incubation with activated acid at 135°C for 1.5 h. The linearity of the method was assessed in the range of 30–500 μg/mL (0.005–0.05%) of PS-80 standard solutions, with standards and protein samples containing PS-80 prepared and analyzed as described in Section 2. The correlation coefficient of the calibration curve was 0.999 (Fig. 4C). The repeatability of the method was 6.0%, and the percent recovery from a spiked protein sample was 99 ± 6% (Table 2). The results indicate that the
method is particularly suitable for the quantitation of PS-80 in formulated protein samples, as the recovery was higher than that seen with PEG 8000 (82%). While we attribute the discrepancy in behavior to dissimilar preferential solvation of the protein by surfactants of different amphiphilic character, this hypothesis has not been examined in this work. The overall intra- and interday variations were 4.6% (Table 3). The average method accuracy for PS-80 quantitation was 102 ± 3%. The LOQ (S/N > 10) was determined as the PS-80 concentration that can be quantified in the test sample with acceptable precision under the stated operational conditions of the method and the analyte amount present in the bio-manufacturing process (Table 4). The quantitation limit evaluated in this manuscript is much lower than the amount of PS-80 in the analyzed sample matrices; therefore, the LOD was not evaluated.

3.2.3 Application to PEG-protein conjugates

Finally, the method described herein was tested with PEGylated BSA samples regarding the quantitation of PEG content as described in Section 2.4. It is known that BSA contains one free sulfhydryl group located on cysteine 34 that is surface exposed [34]. Maleimide-activated PEG enabled efficient PEGylation of the sulfhydryl group; the reaction resulted in formation of a stable, irreversible thioether bond in the conjugated protein. We used this well-characterized chemically modified system to assess the applicability of the GC method to the analysis of PEG conjugates.

The PEG-BSA conjugate sample was first dialyzed and subsequently hydrolyzed with activated acid. Released EGD was extracted with methylene chloride and analyzed by GC to determine the amount of PEG per mole of protein using a calibration curve generated from free PEG at concentrations of 5–50 μg/mL with a correlation coefficient of 0.999 (Fig. 5A). Analytical results were confirmed using MALDI-TOF-MS as an orthogonal method. GC and MS analyses reported identical derivatization levels of 1.0 mole of PEG per mole of protein, as expected.

4 Conclusion

A universal method using GC has been developed for the quantitation of ethylene glycol-containing polymers in protein samples. The content of PEG 8000, PS-80, and Pluronic F-68 was calculated based on the amount of EGD released from the test sample after hydrolysis. The temperature and duration of the hydrolysis reaction have been evaluated and optimized for each surfactant. In addition, it was shown that the presence of sodium chloride in a protein sample could impede poly(oxyethylene) hydrolysis, therefore necessitating a salt removal step before GC analysis. This method has sufficient specificity, precision, accuracy, and linearity to assess the content of free surfactants that contain ethylene glycol in protein samples.

This method has also been applied successfully to the determination of the degree of PEGylation in PEG-protein conjugates and is in agreement with results of MALDI-TOF-MS analysis used to confirm independently the content of PEG reported by the GC method.
Table 4. Determination of the LOD and the LOQ

| Analyte   | Spiked (µg/mL) | Measured concentration (µg/mL) | Average (µg/mL) | % Recovery | S/N | Mean S/N |
|-----------|----------------|--------------------------------|-----------------|------------|-----|----------|
| PEG8000   | 2.5            | 2.0                            | 2.1             | 82.9       | 4.9 | 4.0      |
|           | 5              | 3.8                            | 4.2             | 83.7       | 12.3| 19.8     |
| Pluronic F68 | 2.5            | 3.8                            | 3.1             | 124.0      | 8.8 | 8.0      |
|           | 5              | 4.6                            | 4.8             | 96.0       | 10.7| 10.7     |
| PS-80     | 50             | 49.4                           | 50.3            | 100.7      | 25.9| 21.8     |

S/N, A signal-to-noise ratio.

Only LOQ was ascertained for PS-80 assay as the method is not intended for use as a limit test.

Table 5. Method accuracy for PEG 8000, Pluronic F-68 and polysorbate 80 (PS-80)

| Analyte   | Spikeda (µg/mL) | Measured concentration (µg/mL) | Average (µg/mL) | % CV | % Recovery |
|-----------|-----------------|--------------------------------|-----------------|------|------------|
| PEG8000   | 5               | 3.8                            | 4.2             | 11.6 | 84 ± 10    |
|           | 10              | 9.6                            | 9.2             | 6.9  | 92 ± 6     |
|           | 25              | 19.1                           | 19.5            | 2.5  | 78 ± 2     |
|           | 50              | 42.5                           | 41.7            | 2.7  | 83 ± 2     |
| Pluronic F68 | 6               | 5.8                            | 6.7             | 11.8 | 112 ± 13   |
|           | 20              | 22.8                           | 21.5            | 5.7  | 107 ± 6    |
|           | 40              | 44.0                           | 42.2            | 5.0  | 106 ± 5    |
| PS-80     | 100             | 96.9                           | 97.9            | 1.0  | 98 ± 1     |
|           | 200             | 98.0                           | 98.8            | 3.7  | 102 ± 4    |
|           | 300             | 316.5                          | 314.6           | 1.3  | 105 ± 1    |

a) Spike amount was chosen based on amount of analyte typically present in the biomanufacturing process.

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