

# Simultaneous Analysis of Free Polyethylene Glycol (PEG) and PEGylated Proteins, and the Evaluation of Various PEG Reagents by HPLC and Charged Aerosol Detection



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## ABSTRACT

PEG reagents are used as surfactants throughout the pharmaceutical industry. They are also used to covalently modify peptides and proteins to improve physicochemical properties and bioavailability, and to reduce immunogenicity. PEG analysis can be difficult due to sample complexity; commercially available reagents differ not only in their nominal molecular weight (MW), but also with their polydispersity (the relative abundance of oligomers) and the lack a suitable chromophore limits the use of HPLC-UV detection.

Charged aerosol detection can measure any nonvolatile, and many semivolatiles compounds, typically with low nanogram sensitivity. Furthermore, as response is similar for all compounds and independent of chemical structure, it is ideal for measurement of PEG reagents. To show its great applicability, this work focuses on two areas: first, the use of isocratic gel permeation HPLC to measure 0.1% free PEG in the presence of PEGylated monoclonal antibodies (MAb); and second, the use of gradient reversed-phase HPLC to verify the composition of low MW PEG oligomers. In this latter study, methods were developed for the analysis of PEG reagents from both the upper and lower MW ranges. One objective of this work was to create both sensitive and efficient methods for the determination of nonionic surfactants, PEG 400 and 3000, with maximum resolution between the oligomers.

## INTRODUCTION

The functional role of excipients and surfactants in pharmaceutical preparations includes: modulating solubility and bioavailability of active pharmaceutical ingredients; increasing the stability of active ingredients in the dosage forms; helping active ingredients to maintain preferred polymorphic forms; maintaining the pH and/or osmolarity of liquid formulations; acting as antioxidants, emulsifying agents, aerosol propellants, tablet binders, and disintegrants; preventing aggregation or dissociation; and modulating immunogenic responses of active ingredients.<sup>1</sup>

PEG is being used more commonly in different stages of drug development. To fit the many applications where it can be used, PEG is available in a multitude of sizes and compositions. Smaller MW forms of free PEG, such as PEG 400 or PEG 3000, are widely used in drug manufacturing as excipients. Interestingly, PEG is also employed in the encapsulation of proteins (PEGylation) and the delivery of siRNA materials to facilitate their passage through the cell membrane. PEGylation is the process of covalent attachment of PEG polymer chains to another molecule, normally a biotherapeutic agent. The covalent attachment of PEG to another compound has several advantages:

- Masks the agent from the host's immune and metabolic systems;
- Increases the hydrodynamic size (size in solution) of the agent, which prolongs its circulatory time by reducing renal clearance; and
- Provides water solubility to hydrophobic molecules.<sup>1</sup>

Analysis of PEGylated biomolecules is important in drug development and QC. Excipient quality can influence the stability and bioavailability of the active drug component. The first part of this work, analysis of PEG 400 and PEG 3000, uses UHPLC with charged aerosol detection.

The analysis of PEG is difficult because it does not contain a chromophore (i.e., UV detection is limited) and it is difficult to ionize (i.e., mass spectrometer is not useable). UHPLC techniques can be used conveniently to separate and analyze the various oligomers of smaller PEG species using the Corona® Charged Aerosol Detector (CAD®). CAD is a sensitive, mass-based detector, especially well-suited for the determination of any nonvolatile analyte, independent of chemical characteristics. It has been used for many different types of assays, from low MW ions to the analysis of larger polymers such as PEG. The function and operation of the CAD is shown in Figure 1.

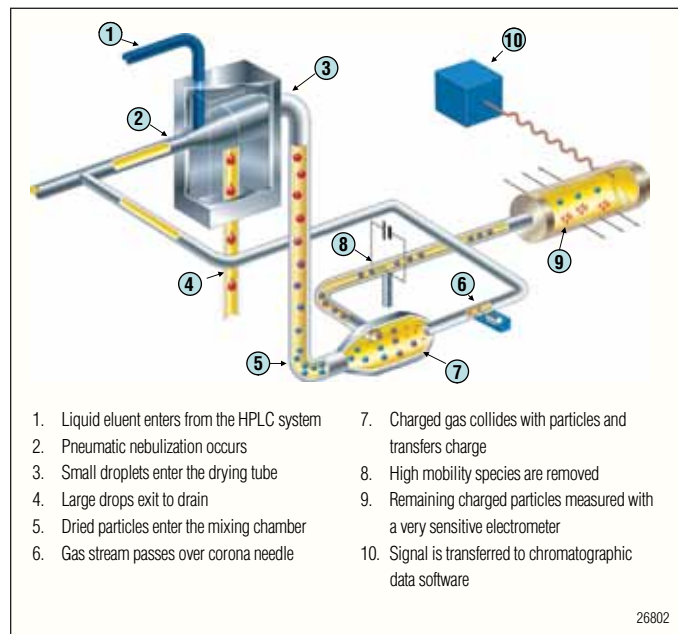


Figure 1. Corona CAD detector flow path schematic.

The analysis of free PEG and PEGylated proteins is complicated by the fact that the free PEG domain possesses no chromophore. Also the hydrated radius of the PEGylated protein and free PEG (30 kDa) are often similar, thereby making chromatographic separation challenging. Thus, analysis of free PEG from PEG-MAb by HPLC requires the use of different types of column materials, such as wide-pore reversed-phase, ion-exchange, or size-exclusion chromatography (SEC). The second part of this work extends the use of UHPLC-SEC techniques for the analysis of free PEG as an impurity in a PEGylated protein. The method also describes the use of SEC and the Corona CAD to resolve both PEG-MAb and free PEG.

## METHODS

### UHPLC Method Conditions 1: Reproducibility and Sensitivity of PEG 400 Method

HPLC System:	Dionex UltiMate® 3000 RSLC		
Column:	Acclaim® RSLC PolarAdvantage II (PA2), 2.2 µm, 2.1 × 50 mm		
Mobile Phase A:	DI water		
Mobile Phase B:	Acetonitrile		
Sample Preparation:	Sample was dissolved in DI water		
Injection Volume:	3 µL		
Detector:	Corona <i>ultra</i> ™		
Filter Setting:	Medium		
Gradient:	<i>Time (min)</i>	<i>Flow Rate (mL/min)</i>	<i>%B</i>
	0	0.75	1
	0.65	1	10
	2	1	20
	3	1	20
	3.4	1	1
	4.5	1	1

### UHPLC Method Conditions 2: Resolution of PEG 3000

Column:	ACQUITY® BEH C-18, 2.1 × 50 mm, 1.7 µm			
Mobile Phase A:	DI water			
Mobile Phase B:	Acetonitrile			
Sample Preparation:	Sample (24.5 mg/mL) was dissolved in DI water			
Detector:	Corona <i>ultra</i>			
Filter Setting:	High			
Gradient:	<i>Time (min)</i>	<i>%B</i>	<i>Time (min)</i>	<i>%B</i>
	0	6	32	29.5
	1	12	43	33
	3	17	47	50
	12	26	48	80
	17	27.5	49	80
	25	28.5	50	6

### SEC Method Conditions: Analysis of PEGylated Protein

Column:	Tosoh TSKgel®; G3000SWXL (SEC); 7.8 × 300 mm
Mobile Phase:	Water/acetonitrile (70:30)
Flow Rate:	1.0 mL/min
Column Temperature:	Ambient
Injection Volume:	10 µL
Detector:	Corona CAD
Filter Setting:	None
Sample Preparation:	5 mg/mL PEGylated MAb in PBS buffer
For Method Development:	1 mg/mL PEG (30 kDa) in water

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## RESULTS AND DISCUSSION

### Analysis of PEG 400

PEG 400 was analyzed using the UHPLC Method Conditions 1. The resolution of the different MWs is performed as shown in Figure 2.

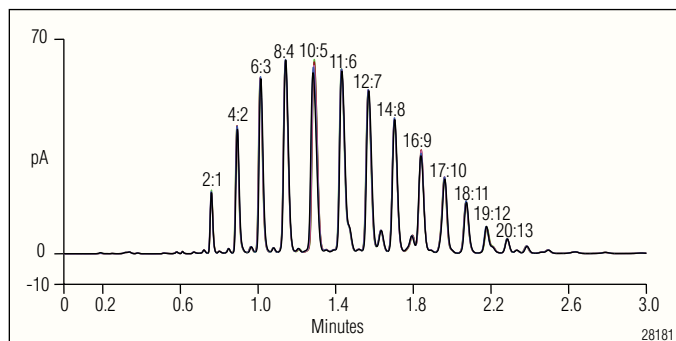


Figure 2. Overlay of five injections of ~53 µg on column of PEG 400.

Sensitivity of the CAD detector is demonstrated with the analysis of 7 ng on column of PEG 400 shown in Figure 3. The CAD detector offers excellent sensitivity and estimated mass on column of different subcomponents of PEG 400 ranged from 0.77 to 1.2 ng.

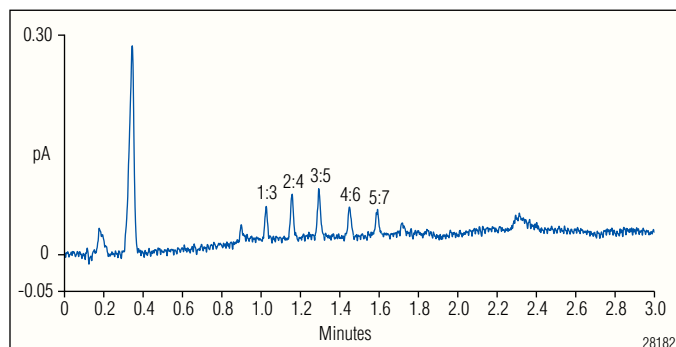


Figure 3. HPLC chromatogram of 7 ng (total) on column of PEG 400. Note the estimated masses on column of peaks 3, 4, 5, 6, 7 being 0.77, 1.1, 1.2, 1.1, and 0.93 ng, respectively.

A calibration curve for PEG 400 using injections of 13 different solution concentrations is presented in Figure 4. Data were fitted to an inverse second-order polynomial. All 13 oligomers had  $R^2$  values  $\geq 0.999$  from the high point to their LOQ.

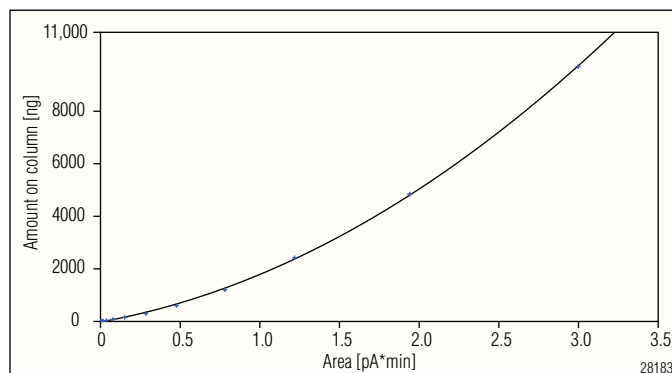


Figure 4. Calibration curve for PEG 400, 2–10,000 ng on column.

As a part of quality control specifications, lot-to-lot variation must be minimized, i.e., PEG characterization needs to be constant. Chromatograms of PEG 400 from two different suppliers are shown in Figure 5. Note the major components are similar both in retention time and relative amounts, although there are a significant number of minor, intermediate components that are resolved in one lot of PEG 400 (the upper chromatogram). The precision of the CAD was calculated and the variation in peak area for 10 of the 13 detected PEG 400 oligomers was less than 2% RSD.

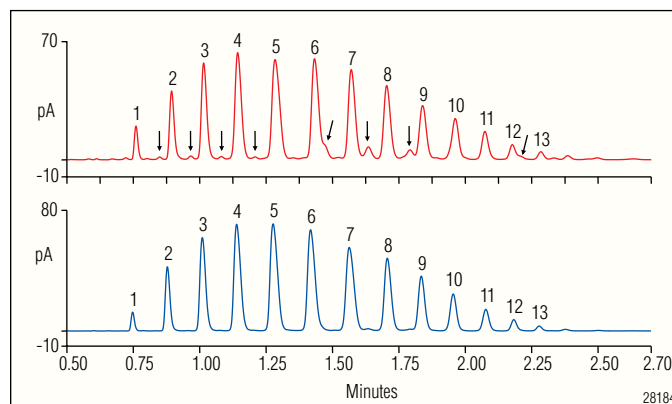


Figure 5. Two different lots of PEG 400 showing compositional differences (larger, minor peaks in top chromatogram).

## Analysis of PEG 3000

Using gradient elution with UHPLC Method Conditions 2, the components of the larger PEG 3000 are well resolved (Figure 6). Two injection amounts of 29 and 18  $\mu\text{g}$  on column were analyzed. A profile of the different PEG species present in the sample is clearly visible.

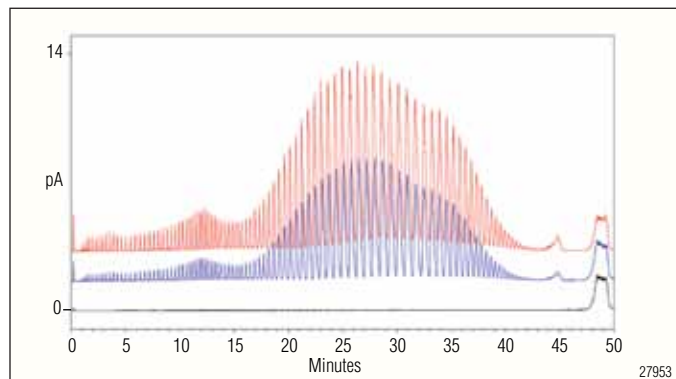


Figure 6. Overlay of 5  $\mu\text{L}$  (red) and 3  $\mu\text{L}$  (blue) injection of 6 mg/mL solution of PEG 3000 monodisperse solution (Fluka #81269) ~29  $\mu\text{g}$  and 18  $\mu\text{g}$  on column respectively. Black trace is 5  $\mu\text{L}$  injection of DI water.

## Analysis of Free PEG in the Presence of PEGylated Protein

The sensitive determination of free PEG in the presence of PEGylated proteins was accomplished using the CAD SEC method described above. Using these method conditions, free PEG is resolved from the PEGylated protein (Figure 7). The height of the PEG-MAB peak at a concentration of 5 mg/mL is within the detector's dynamic range, so it is not necessary to further dilute the sample.

SEC typically elutes the larger MW compounds first. But with this method, a small but clearly visible peak for free PEG is seen in Figure 7 with 30% acetonitrile as an optimal concentration for analyte response. The typical elution order expected for SEC is reversed due to hydrophilic interactions, causing the PEG-MAB to retain longer on this diol-based SEC column. When concentrations of organic content were increased, it caused the PEGylated protein to obscure the peak for free PEG. When 0.1% TFA is added to the mobile phase, typical size-exclusion mechanisms take precedence and the free PEG elutes after the PEGylated MAb, making quantification impossible (data not shown).

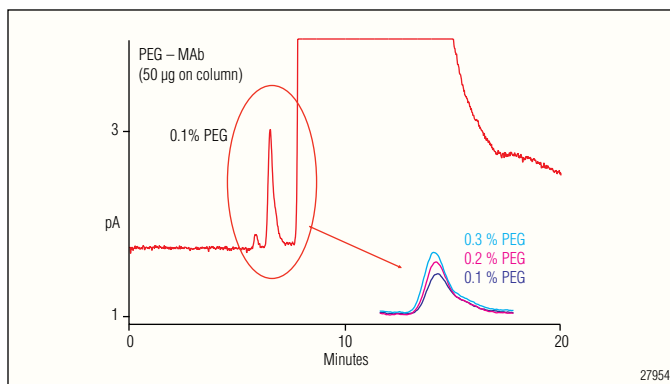


Figure 7. SEC-CAD chromatogram of free PEG at 0.1% in the presence of a PEGylated protein. Inset: Free PEG at 0.1, 0.2, and 0.3 w/w-%, relative to PEGylated protein.

Using these conditions, calibration curves for the free PEG were produced and illustrated good correlation ( $R^2 = 0.999$ ), as shown in Figure 8. The CAD has a dynamic range from 3 to 10,000 ng on column for free PEG. This is significantly larger than the range available using ELS detectors.

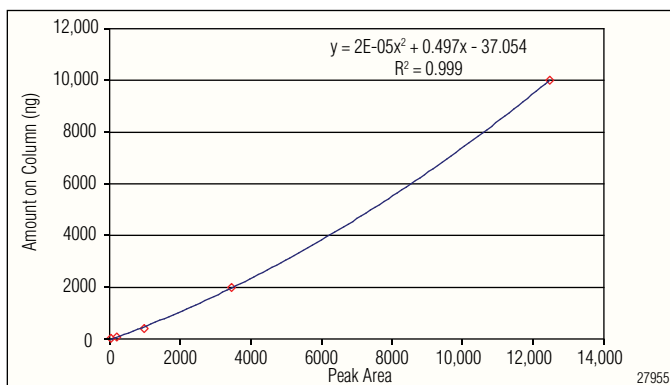


Figure 8. Calibration curve for the analysis of free PEG.

The sensitivity for this method was excellent, with a detection limit of 16 ng on column (1.6  $\mu\text{g}/\text{mL}$ ) of free PEG with a signal-to-noise (S/N) ratio of 5 (Figure 9). This represents a significant improvement over RI detection, where only 10  $\mu\text{g}/\text{mL}$  of free PEG could be detected.<sup>2</sup> Other researchers have also found that the CAD is more sensitive and reliable than RI or ELSD detectors for the analysis of free PEG.<sup>3,4</sup> The reproducibility of a 40  $\mu\text{g}/\text{mL}$  standard was evaluated under these conditions, and the %RSD was 2.6% ( $n = 5$ ).

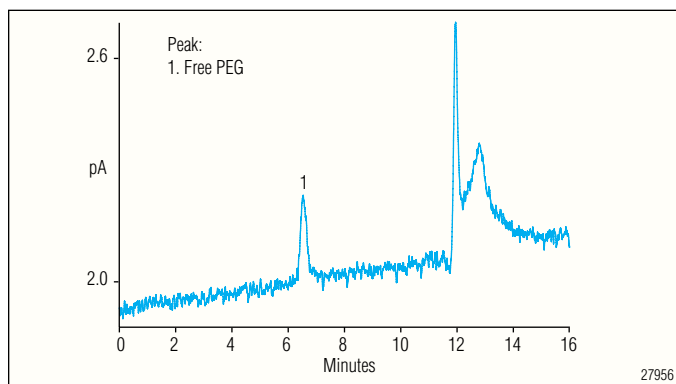


Figure 9. SEC-CAD chromatogram of free PEG (1.6 ppm; 16 ng on column; S/N ~5).

## CONCLUSION

Methods were developed for the determination of nonionic surfactants PEG 400 and 3000 with maximum resolution between the oligomers. The analysis time for PEG 400 was improved 4-fold when changing from a HPLC to a UHPLC method while still maintaining peak resolution (data not shown). UHPLC increases throughput while conserving solvent. The sensitivity for PEG 400 was in the low nanogram range. The CAD shows excellent precision for all of the different oligomers of PEG 400 (Figure 2).

The method using UHPLC with Corona *ultra* detection for the analysis of PEG 3000 shows the incredible resolution and sensitivity for measurement of analytes that only possess weak chromophores. The CAD has good sensitivity (low ng) to low level impurities so that trace amounts of the earlier eluting oligomers of PEG 3000 can be determined easily using the CAD. This provides a means to access excipient quality at early stages of product manufacturing, which can be an important factor in the stability and bio-availability of the active drug component.

A method for the determination of 0.1% free PEG impurities in a sample of PEGylated MAb was created using SEC-HPLC-CAD and a diol-based column. This column provides a separation based on molecular size and possibly another mechanism based on hydrophilicity of the analytes.

The CAD is suitable for the analysis of free PEG since it showed excellent sensitivity (LOD of 16 ng on column for PEG) and good reproducibility (2.6% RSD for 400 ng of PEG on column). This approach can readily detect 0.1% PEG in the presence of the PEGylated-MAb product.

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