Chemical Modifications of Therapeutic Proteins Induced by Residual Ethylene Oxide

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ABSTRACT: Ethylene oxide (EtO) is widely used in sterilization of drug product primary containers and medical devices. The impact of residual EtO on protein therapeutics is of significant interest in the biopharmaceutical industry. The potential for EtO to modify individual amino acids in proteins has been previously reported. However, specific identification of EtO adducts in proteins and the effect of residual EtO on the stability of therapeutic proteins has not been reported to date. This paper describes studies of residual EtO with two therapeutic proteins, a PEGylated form of the recombinant human granulocyte colony-stimulating factor (Peg-GCSF) and recombinant human erythropoietin (EPO) formulated with human serum albumin (HSA). Peg-GCSF was filled in an EtO sterilized delivery device and incubated at accelerated stress conditions. Glu-C peptide mapping and LC–MS analyses revealed residual EtO reacted with Peg-GCSF and resulted in EtO modifications at two methionine residues (Met-127 and Met-138). In addition, tryptic peptide mapping and LC–MS analyses revealed residual EtO in plastic vials reacted with HSA in EPO formulation at Met-328 and Cys-34. This paper details the work conducted to understand the effects of residual EtO on the chemical stability of protein therapeutics. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

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INTRODUCTION

Common sterilization methods include moist heat (i.e., steam), dry heat, ionizing radiation (gamma or e-beam), hydrogen peroxide, and ethylene oxide (EtO). Each method has its advantages and limitations. Ionizing radiation, hydrogen peroxide, and EtO often leave residuals that are damaging to proteins and DNA.[1–4] EtO sterilization is particularly useful for sterilizing delicate medical devices and heat and/or moisture sensitive equipments or materials.[5,6] However, the difficulties caused by the potential hazards of EtO to patients, staff, and the environment as well as risk associated with handling a flammable gas make EtO sterilization a challenging task.[5] Because different materials adsorb and desorb EtO differently,[7] the allowable levels of residual EtO remaining in sterilized apparatus and materials need to be specified for individual products per regulations. Therefore, the sterilization process, including EtO concentration, sterilization time, temperature, and humidity as well as the aeration process conditions and time must be optimized for effectively eliminating residual EtO.[8]

Although EtO has been utilized for decades, protein degradation by EtO has not been widely reported in the literature. An initial model system found it forms adducts with methionine, cysteine, and histidine amino acid residues. The model experiments conducted by reaction of EtO with commercial proteins also reported a decrease in biological activity. The reduction in bioactivity appeared to be correlated with the electrophilic hydroxyethylation of an atom with one or more lone pairs of electrons, particularly nitrogen and sulfur.[1] Gas chromatography coupled with MS (GC–MS) and liquid chromatography coupled with MS (LC–MS) were applied in measuring small-molecule DNA and protein adducts released during EtO treatments, such as N-(2-hydroxyethyl) valine, N1-(2-hydroxyethyl) histidine, and S-(2-hydroxyethyl) cysteine.[4] The lack of information about tandem MS (MS/MS) analysis of EtO-modified peptides in proteins made site identification in protein sequence and quantitation of the modification analytically challenging. The challenges were because of the poor gas-phase fragmentation of peptides containing methionine residues modified by EtO. To date, identification of this modification is not widely described in the literature.

In this report, detection and identification of the protein degradation caused by residual EtO (low levels of EtO remaining in the different medical containers) using two therapeutic proteins, a PEGylated form of recombinant human granulocyte colony-stimulating factor (Peg-GCSF), and a recombinant human erythropoietin (EPO) formulated with human serum albumin (HSA), were studied. EtO sterilized delivery devices were filled with Peg-GCSF from a prefilled syringe (PFS) formulated at 10 mg/mL in 10 mM sodium acetate, 5% sorbitol, pH 4.0, 0.004% polysorbate 20 and incubated at an accelerated condition of 42 ± 1°C for 54 h. Samples were analyzed by several analytical assays to evaluate the impact of residual EtO on Peg-GCSF. Assay results showing the impact of EtO, including cation-exchange chromatography/HPLC (CEX–HPLC) and reversed-phase HPLC (RP-HPLC) are highlighted in this

Abbreviations used: ACN, acetonitrile; CEX, cation-exchange chromatography; CID, collision-induced dissociation; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPO, recombinant human erythropoietin; EtO, ethylene oxide; GC/FID, gas chromatography equipped with flame ionization detector; GC–MS, gas chromatography coupled with MS; Gdn-HCl, guanidine-HCl; HSA, human serum albumin; IAA, iodoacetic acid; LC–MS, liquid chromatography coupled with MS; MS/MS, fragmentation mass spectra; Peg-GCSF, pegylated recombinant human granulocyte colony-stimulating factor; PFS, prefilled syringe; PP1, post peak 1; PP2, post peak 2; PP3, post peak 3; RP-HPLC, reversed-phase HPLC; RT, retention time; TFA, trifluoroacetic acid.

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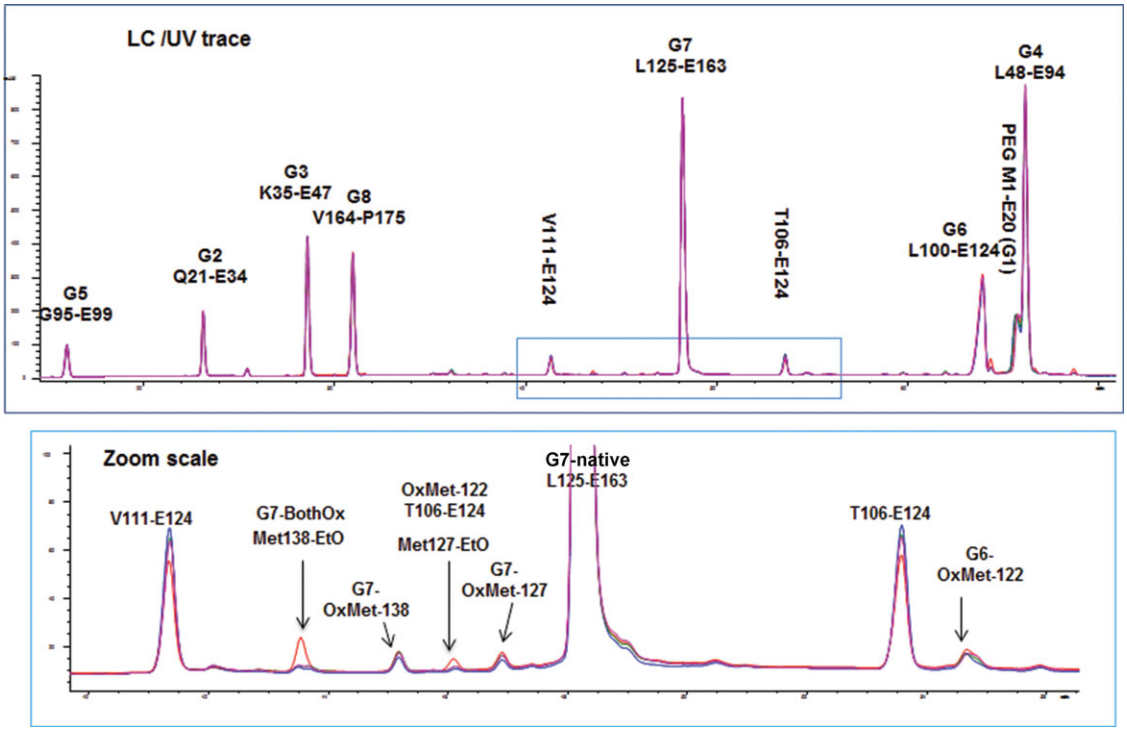

Figure 1. Identification of all minor species present in the retention time window of 40–56 min in Glu-C peptide map of Peg-GCSF. Blue trace, filled device control; red trace, stressed filled device; green trace, PFS control; pink trace, stressed PFS.

report. Endoproteinase Glu-C digested peptide maps analyzed by LC/MS were used for identification and characterization of any EtO modifications.

In a separate experiment, EPO was formulated at 0.0168 mg/mL in 20 mM sodium citrate buffer pH 6.9 with 100 mM sodium chloride and 2.5 mg/mL HSA. A 1.0 mL sample was filled in plastic vials which had been custom sterilized using an EtO process. Vials were incubated at 37 ± 1°C for up to 2 months after which they were analyzed by several assays to evaluate the impact of residual EtO on EPO with HSA.

EXPERIMENTAL PROCEDURES

Materials and Equipment

Chemicals and Materials

Sodium acetate, sodium chloride, glycerol, trifluoroacetic acid (TFA), tris(hydroxymethyl)aminomethane (Tris), Tris hydrochloride (Tris–HCl), urea, hydroxylamine, dithiothreitol (DTT), iodoacetic acid (IAA), ethylenediaminetetraacetic acid (EDTA), and guanidine-HCl (Gdn-HCl) were ACS grade (Sigma–Aldrich, St. Louis, Missouri). All organic solvents were of analytical or HPLC grade. Trypsin and Endoproteinase Glu-C was Roche sequencing grade (Roche Diagnostics Corporation, Indianapolis, Indinana). Vydac C-4 columns (250 × 2.1 mm^2, 5-μm particle size) were purchased from Grace Co. (Columbia, Maryland). PLRS-S columns (4.6 × 150 mm^2) and Varian Polaris C18 Ether columns (2.0 × 250 mm^2, 3-μm particle size) were purchased from Agilent Technologies (Santa Clara, California). Cation exchange columns (TSK gel SP-NPR, 4.6 × 35 mm^2, 2.5-μm nonporous particle) were from TosoHaas Bioscience Company (Phenomenex, part # CHO-8039; King of Prussia, Pennsylvania). Delivery devices were manufactured from Insulet Company (USA).

Instruments

A Thermo Scientific (Waltham, Massachusetts) Velos Ion Trap (LTQ) mass spectrometer was used for Glu-C peptide mapping analysis and CEX post peak characterization and an Agilent 1100 series HPLC was used for the RP-HPLC assay. A Dionex (Sunnyvale, California) Ultimate 3000 HPLC was used for the CEX–HPLC assay. Agilent headspace gas chromatography equipped with flame ionization detector (FID) was used for EtO analysis.

Protein Formulations

Peg-GCSF (pegfilgrastim) was formulated at 10 mg/mL in 10 mM sodium acetate, 5% sorbitol, pH 4.0, and 0.004% polysorbate 20. Epoetin alfa (recombinant human EPO) was formulated at 0.0168 mg/mL in 20 mM sodium citrate buffer (pH 6.9) containing 100 mM sodium chloride and 2.5 mg/mL HSA.

Methods

EtO-Degraded Peg-GCSF Sample Preparation

Peg-GCSF was loaded into an EtO sterilized delivery device from a glass PFS. The filled devices along with the initial PFS were subjected to storage at an accelerated condition of 42 ± 1°C for 54 h. The initial PFS was known to contain no residual EtO and served as a control at the stressed condition. Post incubation, the Peg-GCSF in the stressed containers and PFS control samples were each collected for testing. The PFS and filled containers at time zero (unstressed) were also included as controls. All samples were digested with endoproteinase Glu-C

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