Removal of Peroxides in Polyethylene Glycols by Vacuum Drying: Implications in the Stability of Biotech and Pharmaceutical Formulations

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ABSTRACT

The purpose of this study was to investigate the utility of vacuum drying for removing peroxides from polyethylene glycols (PEGs). PEG solutions (PEG 1450 and PEG 20000) containing varying levels of peroxides were prepared by storing under different light and temperature conditions. PEGs containing low and high levels of peroxides were vacuum dried from dilute and concentrated solutions (2.5%, 7.5%, 15%, and 50% wt/vol of PEG 1450 and 2.5%, 7.5%, 15%, and 25% wt/vol of PEG 20000). Ferrous ion oxidation in presence of ferric ion indicator xylenol orange (FOX) colorimetric assay was used to determine the concentration of peroxides. Peroxide content in PEGs increased upon storage. The increase was more pronounced when PEGs were stored at higher temperatures and exposed to light. Vacuum drying at 0.1 mm Hg for 48 hours at 25°C resulted in greater than 90% decrease in the level of peroxides in all cases except when high peroxide containing 25% wt/vol solution of PEG 20000 or 50% wt/vol solution of PEG 1450 were dried. The reduction in the level of peroxides for PEGs dried from high peroxide containing 25% wt/vol solution of PEG 20000 and 50% wt/vol solution of PEG 1450 was found to be 88% and 52%, respectively. Oxidation of methionine in Met-Leu-Phe peptide was significantly reduced when vacuum-dried PEGs were used. Vacuum drying PEG solutions at low pressures is an effective method for the removal of the residual peroxides present in commercially available PEGs.

KEYWORDS: Freeze drying, peroxides, polyethylene glycol, proteins, vacuum drying.

INTRODUCTION

Polyethylene glycols (PEGs) are water-soluble synthetic polymers with a general formulae HO-$(CH_2CH_2O)_n$-H.[1] PEGs have found 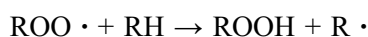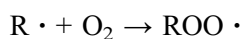various applications in the pharmaceutical and biotech industry and are widely used as cosolvents, as lubricants and stabilizers, as bases in topical products, as precipitants and crystallization agents for proteins, and as chemical agents for pegylation of proteins.[2-6] In addition, PEGs have been shown to stabilize proteins during freeze drying.[7] Most recently, stable formulations of dry protein powders have been developed by using PEG precipitation and vacuum drying.[8]

Despite their many uses, there is a concern with PEGs, because they contain low levels of residual peroxides, which accumulate on storage, and could potentially affect the stability of oxidation sensitive pharmaceuticals.[9-11] It is well documented in the literature that auto-oxidation can result in the formation of hydroperoxides and peroxide-free radicals in polyoxyethylene linkages containing substances such as polysorbate surfactants, poloxamers, and PEGs (see schematic below).[10-12] Auto-oxidation is initiated by metal- and/or light-induced decomposition of polyoxyethylene chains.[12] The process is propagated by the consumption of oxygen and formation of hydroperoxides and is terminated by the decomposition of hydroperoxides and/or collision among free radicals.[12] Several reports show that the formation and decomposition of peroxides occur simultaneously and are dependent on temperature, light, and the presence of oxygen.[10,13] Generation of peroxides and other oxidizing species such as peroxide and hydroxyl radicals, in formulations containing low levels of residual peroxides, can thus lead to fast degradation of the active drug resulting in significantly compromised therapeutic activity. The following is a schematic of the auto-oxidation process, where R for PEGs = $-(OCH_2CH)O-$.[12]
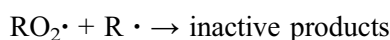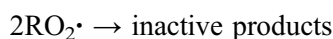
Initiation

RH + light or catalyst → R · + H ·

Propagation

R · + O_2 → ROO ·

ROO · + RH → ROOH + R ·

ROOH → ROO · + H ·

Termination

$2RO_2$· → inactive products

RO_2· + R · → inactive products

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The effect of peroxides in PEGs and of peroxides in poly-oxyethylenic nonionic surfactants on the oxidative stability of pharmaceuticals has been reported in the literature.[9,14-16] Peptides and proteins have amino acids such as cystine and methionine that are vulnerable to oxidative degradation.[17,18] Although the effect of peroxides in PEGs on protein stability has not been widely studied, there are some reports that describe the effect of peroxides in polysorbates on the oxidative stability of proteins in solution and in the lyophilized form.[13] Since proteins are often formulated in low concentrations, even trace amounts of peroxides can affect the stability of these biomolecules. Therefore, in order to increase the chemical stability of the active constituent, it is necessary that PEGs and polysorbates be purified before they are used in a formulation and adequate precautions should be taken to prevent the formation of peroxides during the storage of the formulation.

In a recent report, it was shown that complete removal of oxygen in the headspace of vials containing aqueous solutions of polysorbates (by storing the product either under nitrogen or in vacuum) resulted in a decrease in the concentration of peroxides.[13] Several authors have also shown that removal of oxygen from the headspace of ampoules or vials is an efficient way of preventing the degradation of the active drug.[13,14] However, maintaining vacuum and/or nitrogen environment in a certain type of vials (used in freeze drying) for a prolonged period of time is not an easy task. Alternatively, the levels of residual peroxides could also be controlled by the use of antioxidants. However, antioxidants can interact with the active constituent and lead to other instabilities such as the precipitation of protein.[19]

The removal of peroxides can be accomplished by using several methods reported in the literature.[20-22] An example of such a method is purification of PEGs by treating with sodium thiosulfate and passing through resin column.[21] Purification procedures reported in literature may be efficient but require chemical treatments, which are undesirable.

This report demonstrates that vacuum drying at low pressures is an efficient method for removing peroxides from PEGs. It is further shown that freeze drying also results in a decrease in the level of peroxides present in PEGs. The benefit of the vacuum-drying technique is demonstrated by a reduction in the rate of oxidation of methionine in a tripeptide in the presence of PEGs.

MATERIALS AND METHODS

Materials

Xylenol orange, hydrogen peroxide (30% wt/wt solution), iron (II) chloride (anhydrous beads), butylated hydroxytoluene, sulfuric acid (10N solution) and tripeptide, and Met-Leu-Phe (acetate salt) were purchased from Aldrich (St Louis,

MO). PEG 1450 (flakes) was purchased from Acros Organics (Geel, Belgium) and PEG 20000 (flakes) was purchased from Serva (Heidelberg, Germany). D-sorbitol was purchased from Fluka Chemicals (Buchs, Switzerland). Triple distilled water was used in the preparation of all solutions, and each solution was filtered through 0.22-μm millex filter (Millipore, Bedford, MA). All buffer reagents were of highest purity grade obtained from commercial sources and were used without further purification.

Stability Studies on PEG 1450 and PEG 20000

Fifty percent wt/vol and 25% wt/vol stock solutions were prepared for PEG 1450 and PEG 20000 in triple distilled water. The pH of the solutions was adjusted to 5.0 with 1N NaOH or 1N HCl as appropriate. Fresh solutions of PEGs were filled in 15-mL vials (12-mL fill with air in the headspace) and stored at 40°C under ambient light for 6 days. An incubator with glass covering was used to attain the desired temperature. The 6-day solutions were labeled as "type I high peroxide level solutions." Six-day samples were then kept for an additional 20 days in a dark oven at 25°C. The 26-day solutions were labeled as "type II high level peroxide solutions." For the preparation of the PEG solutions (50% wt/vol for PEG 1450 and 25% wt/vol for PEG 20000) under vacuum, the following procedure was used. The solutions (pH 5.0) in glass vials were placed in a freeze dryer and the shelf cooled down to −2°C (product temperature 0°C). After maintaining the samples for 15 minutes at −2°C, the pressure in the chamber was reduced to 0.1 mm Hg. The vials were then stoppered inside the freeze dryer (within the first 5 minutes of the vacuum reaching 0.1 mm Hg) and sealed manually with aluminum seals. High viscosity of the solutions prevented ejection of the samples (at 0.1 mm Hg) from the vials. Vials under vacuum were stored at 25°C for 48 hours. Peroxide level in fresh, type I, type II, and solutions under vacuum were measured by ferrous ion oxidation in presence of ferric ion indicator Xylenol orange (FOX) assay described in Determination of Peroxide Levels in PEGs.

Vacuum-drying Studies

The drying of the PEG solutions was done in a Durastop Freeze Dryer (FTS Systems, Stoneridge, NY) at a temperature of 25°C and a pressure of 0.1 mm Hg for 48 hours unless otherwise stated. The solutions were dried in 5-mL, 20-mm finish (1 mL fill) serum clear glass vials (Fisher Scientific, Millville, NJ) and stoppered with 20-mm, gray butyl, 3-prong lyophilization rubber stoppers (Fisher Scientific, Millville, NJ). All samples when dried were stoppered and sealed with tear-off 20-mm finish aluminum seals (Fisher Scientific, Millville, NJ). The pH of all the samples kept for drying was adjusted to 5.0 with 1N NaOH or 1N HCl as appropriate.

Freeze-drying Studies

Lyophilization of PEG solutions used the following steps: freezing to −40°C at 1°C/min, holding at −40°C for 1 hour, increasing the temperature to −10°C at 1°C/min, increasing the vacuum to 0.06 mm Hg, holding at −10°C for 16.66 hours, increasing the temperature to 25°C at 0.15°C/min, and holding at 25°C for 10 hours. Similar vials as those used in vacuum-drying studies were used and the fill volume was 1 mL. The pH of all the samples kept for drying was adjusted to 5.0 with 1N NaOH or 1N HCl as appropriate.

Determination of Peroxide Levels in PEGs

The FOX colorimetry assay was used to determine the peroxide levels of PEGs.[23] All dried PEGs were appropriately reconstituted in triple distilled water, and pH of all samples was adjusted to 5.0 prior to the assay with 1N NaOH or 1N HCl as appropriate. A brief description of the assay used in this study is as follows. Five microliters of 10% (wt/vol) butylated-hydroxy toluene (BHT) in ethanol was mixed with 50 μL of the sample and 950 μL of FOX reagent containing 250 μmol/L ferrous chloride and 100 mmol/L sorbitol in 25 mmol/L sulfuric acid. BHT is added to prevent generation of peroxides during the time of the assay. The solution was mixed well and incubated at room temperature for 20 minutes prior to reading the absorbance at 560 nm on a Cary 50 Bio, UV-visible (UV-vis) spectrophotometer (Varian, Walnut Creek, CA). Hydrogen peroxide was used to prepare standard curve (0-5 μmol/L). The intra-assay coefficient of variation of this method was 2.09% (n = 3), while the interassay coefficient of variation was 6.25% (n = 3) for a 2.5 μmol/L concentration solution of hydrogen peroxide. The limit of detection was 0.102 μmol/L. Since only 50 μL of the sample was used for the assay, the concentration (of PEGs) in the sample was adjusted such that the peroxide level was within the range of the H_2O_2 standard curve. Peroxide levels in PEGs were obtained as peroxide equivalent to H_2O_2 standards. The peroxide level was then calculated (by extrapolating) as micromolar equivalents (μEq) per gram of neat PEG (equivalent to H_2O_2 concentration in μmol/L). One micromolar equivalent of peroxides per gram PEGs (1 mEq/kg) reported

here is equivalent to a peroxide value of 2. Our assay and reporting procedure is similar to what has been used in a previously published study.[13]

Preparation of Met-Leu-Phe Stability Samples

A 1.6 mg/mL solution of the tripeptide was made in pH 5.0, 5 mmol/L acetate buffer. PEG 1450 and the tripeptide solution were mixed to obtain a 30% wt/vol concentration for PEG and 1.4 mg/mL concentration for the peptide. The samples were then incubated at 70°C for 46 hours.

Reverse-phase High-performance Liquid Chromatography of Met-Leu-Phe Samples

Reverse-phase High-performance liquid chromatography (RP-HPLC) was performed using Perkin-Elmer series 200 pump (Norwalk, CT) and Perkin-Elmer 785A UV-vis detector (Perkin-Elmer Corp, Norwalk, CT). The analysis of the oxidized and the native species of the tripeptide was performed on Peak Simple chromatography data system (SRI Instruments, Torrance, CA). A Vydac (Hesperia, CA) C-4 column (15 × 2.1 mm, 5 μm) was used. The mobile phase contained solvent A (0.1% trifluoroacetic acid [TFA] in water) and B (0.1% TFA and 90% acetonitrile in water). A gradient elution at a flow rate of 1 mL/min was used according to the following program: 95% A and 5% B for the first 5 minutes, from 95% A and 5% B at 5 minutes to 95% B and 5% A over the next 42 minutes, and finally 95% A and 5% B for the following 3 minutes. The tripeptide and its oxidized products were monitored at 214 nm. The oxidized species were identified by comparing the chromatograms of the samples to that obtained by the oxidation of the tripeptide by 100 mmol/L H_2O_2 in 5 minutes (Figure 1). The original tripeptide appeared as 2 closely eluting peaks with retention times of 19.0 and 19.5 minutes. The second peak indicated some impurity or degraded product in the peptide. However, both peaks oxidized and disappeared with time. It was not possible to quantify the extent of degradation of the 2 peaks individually since they were closely eluting. Hence, area under the curves of the original species (eluting at 19.0 and 19.5 minutes) and the oxidized species
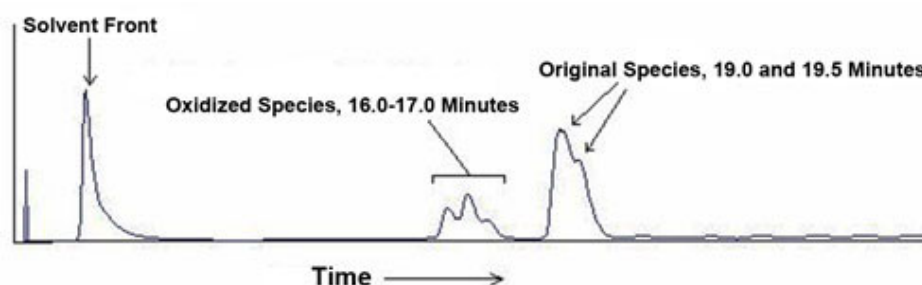

Figure 1. Separation and quantification of Met-Leu-Phe and its oxidized species by RP-HPLC.

(eluting between 16.0 and 17.0 minutes) were taken into account to calculate the percentage oxidation. The oxidized species appeared as a combination of closely eluting peaks that had retention times between 16.0 and 17.0 minutes.

RESULTS AND DISCUSSION

To study the effectiveness of vacuum drying for the removal of peroxides from PEGs, it was necessary to use solutions with relatively high levels of peroxides. It has been reported in literature that formation of peroxides in PEGs and polysorbates is accelerated by light, elevated temperature, and presence of oxygen.[9,12] It has also been shown that level of peroxides in cetomacrogol solutions increased even under ordinary storage conditions of room temperature and darkness.[12] Hence, PEG solutions were prepared by storing 50% wt/vol solution of PEG 1450 and 25% wt/vol solution of PEG 20000 under different conditions of light and temperature (see Methods section). Figure 2 shows the level of peroxides present in PEGs before and after storage. The level of peroxides increased from 42.85 ± 7.02 μEq to 731.56 ± 117.39 μEq (≈17.5-fold) in PEG 1450 and from 35.02 ± 12.0 μEq to 2400 ± 153.0 μEq (≈70-fold) in PEG 20000 after storing at 40°C for 6 days in ambient light. Peroxide levels in these samples further increased from 731.56 ± 117.39 μEq to 1366.436 ± 9.62 μEq in PEG 1450 and from 2400 ± 153.0 μEq to 4513.17 ± 235.0 μEq in PEG 20000 after storing for 20 days in the dark at 25 ºC. Several significant observations can be made from these results. First, the level of peroxides in both PEGs increased significantly even when solutions were stored in the dark at room temperature. Second, the level of

peroxides increased rapidly under light at elevated temperature. Third, the increase in the level of peroxides was higher for PEG 20000 than PEG 1450 under similar conditions of storage (70-fold for PEG 20000 and 17.5-fold for PEG 1450, during the first storage period). Since the fraction of water was higher in PEG 20000 solutions (25% wt/vol), the results suggest that water plays an important role in the formation of peroxides. Previous reports have also indicated that the rate of peroxide formation in several surfactant solutions (containing oxyethylene linkages) is related to the fraction of water present in the solution, and thus to the amount of the dissolved oxygen.[9,13] Our results are consistent with this observation.

Since removal of water results in the removal of the dissolved oxygen, the effect of vacuum drying on the level of peroxides present in PEGs was investigated. Therefore, PEG solutions with varying levels of peroxides (fresh and type I; see Methods section) were dried at 0.1 mm Hg and 25°C for 48 hours from dilute and concentrated solutions. PEG concentration was varied to affect the thickness (foam or compact mass) and the residual moisture of the dried product. Table 1 summarizes the results of the vacuum drying studies. Several significant observations can be made from this table. The level of peroxides fell below the detection limit (appears as zero) when fresh solutions of PEG 1450 and PEG 20000 were dried from dilute (2.5%, 7.5%, and 15.0% wt/vol solutions) as well as concentrated solutions (25% wt/vol for PEG 20000 and 50% wt/vol for PEG 1450). The level of peroxides for both PEGs reduced significantly when dilute solutions (2.5%, 7.5%, and 15% wt/vol solutions) with high levels of peroxides (type I) were dried. The reduction in the level of peroxides was also obtained when concentrated solutions (50% PEG 1450 and 25% wt/vol PEG 20000) containing high level of peroxides type I were dried. However, the reduction was smaller than the reduction obtained when dilute solutions were dried (from 731.56 ± 117.39 μEq to 381.0 ± 13.19 μEq when 50% wt/vol solution of type I PEG 1450 was dried and from 731.56 ± 117.39 μEq to 31.002 ± 4.70 μEq when 2.5% wt/vol solution of type I PEG 1450 was dried). Because the product was much more compact when PEGs were dried from concentrated solutions, it was anticipated that the compactness would affect the rate of drying, and hence the moisture content of the dried product. The measured residual moisture content of the dried PEG samples is listed in Table 1. Although the residual moisture content of all the dried products was less then 1%, the moisture content of PEGs that were dried from concentrated solutions was comparatively higher than those dried from dilute solutions. Hence, the smaller reduction in the level of peroxides when concentrated solutions were dried was resulted from the presence of higher residual oxygen entrapped within the thick mass.
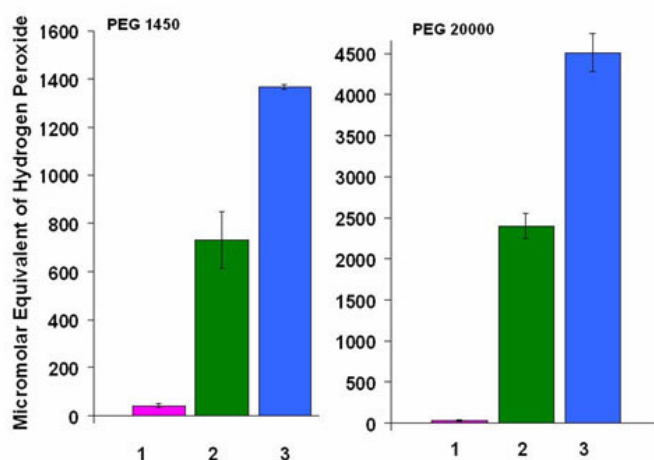

Figure 2. Effect of storage under different conditions on the level of peroxides (peroxide per gram of PEG) in PEG solutions. Key: 1, Fresh solution; 2, Fresh solution stored for 6 days under ambient light at 40°C; and 3, 6-Day solutions stored for additional 20 days in the dark at 25°C. Error bars are standard deviations (n = 3).

Table 1. Peroxide Level and Residual Moisture Content in Vacuum-Dried (48 hours at 25°C and 0.1 mm Hg) PEG Samples*

Solution Sample Dried	Peroxide Level† Before Drying	Peroxide Level† After Drying	% Moisture‡ in Dried PEG
2.5% wt/vol, fresh PEG 1450	42.85 ± 7.02	0	0.38 ± 0.065
7.5% wt/vol, fresh PEG 1450			0.33 ± 0.02
15% wt/vol, fresh PEG 1450			0.40 ± 0.085
50% wt/vol, fresh PEG 1450			0.86 ± 0.055
2.5% wt/vol, type I PEG 1450	731.56 ± 117.39	31.002 ± 4.7	0.45 ± 0.045
7.5% wt/vol, type I PEG 1450		30.0 ± 3.3	0.40 ± 0.08
15% wt/vol, type I PEG 1450		38.12 ± 5.9	0.46 ± 0.045
50% wt/vol, type I PEG 1450		381 ± 13.19	0.78 ± 0.025
2.5% wt/vol, fresh PEG 20000	35.01 ± 12.0	0	0.24 ± 0.04
7.5% wt/vol, fresh PEG 20000			0.24 ± 0.04
15% wt/vol, fresh PEG 20000			0.32 ± 0.035
25% wt/vol, fresh PEG 20000			0.39 ± 0.021
2.5% wt/vol, type I PEG 20000	2400 ± 153.0	30.37 ± 6.144	0.31 ± 0.07
7.5% wt/vol, type I PEG 20000		28.85 ± 3.43	0.31 ± 0.045
15% wt/vol, type I PEG 20000		53.64 ± 3.108	0.31 ± 0.005
25% wt/vol, type I PEG 20000		299.0 ± 42.0	0.51 ± 0.09

*PEG indicates polyethylene glycol.

†Peroxide levels are in micromolar equivalent of hydrogen peroxide present per gram of PEG. Errors bars are standard deviations (n = 3).

‡% moisture in dried sample. Errors represent deviations from average of 2 samples.

It has been reported that peroxide level decreases when polysorbate solutions are stored under vacuum.[13] Therefore, it was necessary to compare the rate of decrease of the peroxide levels in PEG solutions that were stored under vacuum and PEGs that were dried under vacuum. PEG solutions (50% wt/vol for PEG 1450 and 25% wt/vol for PEG 20000) were sealed under vacuum (see Methods section) and were stored for a time period that was equivalent to the time frame of the vacuum drying studies. The levels of peroxides did not decrease in any of the solutions stored under vacuum (Figure 3). In fact, the levels increased in almost all cases. Considering that peroxides were found to be nonvolatile and were not affected by low temperatures (data not shown), the above results clearly suggest that the decrease in the concentration of peroxides on drying is owing to the decomposition of the peroxides. Previous reports have shown that peroxide number in solutions of nonionic surfactants and PEGs stored in the presence of oxygen first increases owing to the induction and propagation of the radical reactions, reaches a plateau, and finally decreases
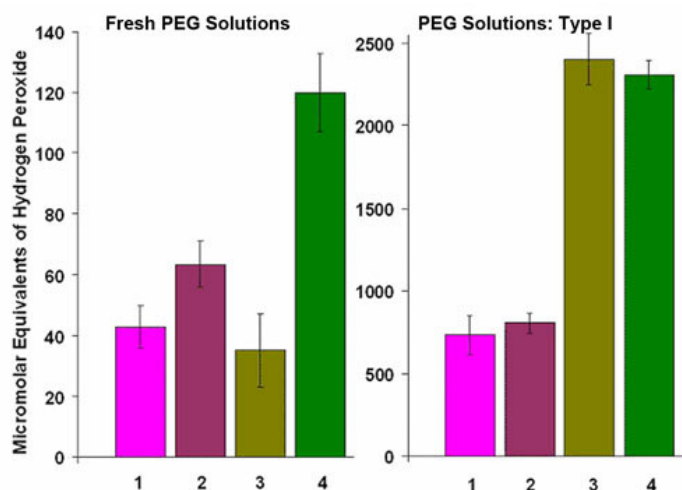

Figure 3. Effect of storage under vacuum at 25°C for 48 hours on the level of peroxides (peroxides per gram of PEG) in PEG solutions (25% wt/vol of PEG 20000 and 50% wt/vol of PEG 1450). Key: 1, PEG 1450 before storing; 2, PEG 1450 after storing; 3, PEG 20000 before storing; and 4, PEG 20000 after storing. Error bars are standard deviations (n = 3).
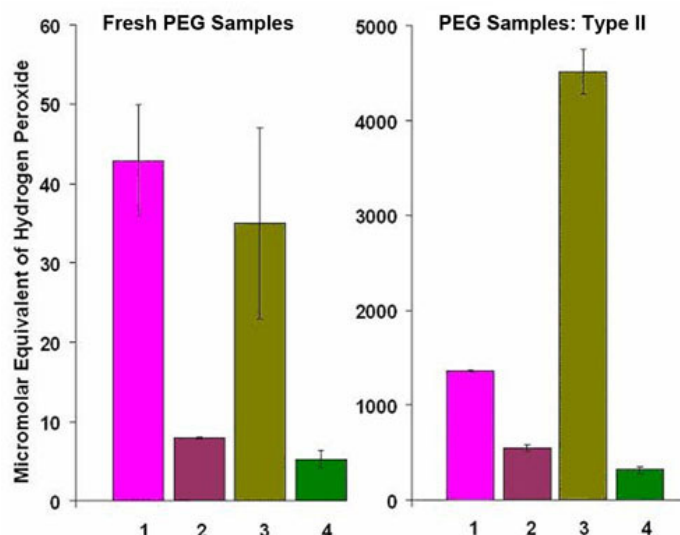

Figure 4. Levels of peroxides (peroxides per gram of PEG) in PEG samples before and after freeze drying (15% wt/vol solutions were freeze dried) in PEG 1450 and PEG 20000. Key: 1, PEG 1450 before drying; 2, PEG 1450 after drying; 3, PEG 20000 before drying; and 4, PEG 20000 after drying. Error bars are standard deviations (n = 3).
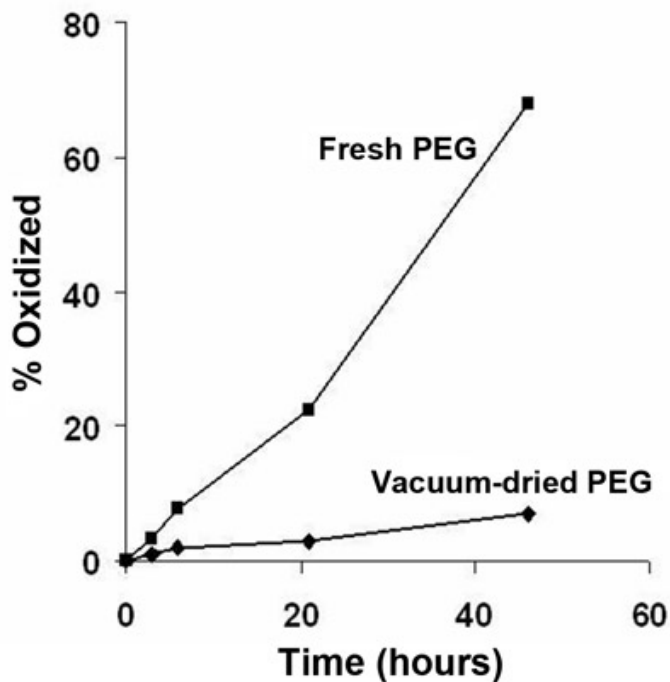
Figure 5. Oxidation of Met of Met-Leu-Phe in the presence of PEG 1450 at 70°C and pH 5.0.

due to the termination of the radical reactions.[10,13] Ha et al further showed that initial increase of the peroxide number in solutions of polysorbate 80 could be inhibited by storing the solutions under vacuum or nitrogen environment.[13] Storage of polysorbate 80 solutions under vacuum resulted in a decrease in the peroxide number because of reduction in available oxygen, which in turn reduced the formation of peroxides.[13] From the data reported in literature and results presented in this manuscript, it can be hypothesized that an increase in the concentration of components during drying step increases the rate of the termination reaction between peroxide radicals (to give inactive end products) along with a simultaneous and effective inhibition of the induction and propagation of the radical reactions (because of the absence of oxygen).

Since vacuum drying resulted in the decomposition of peroxides, the effect of lyophilization (freeze drying) on the level of peroxides present in PEGs was also investigated. For this purpose, 15% wt/vol solutions of PEG 1450 and PEG 20000 (fresh and high level type II solutions; see Methods section) were freeze dried. The residual moisture attained at the end of the freeze-drying cycle was found to be less than 1% in all the dried products. Evidently, freeze drying also resulted in a significant reduction in the levels of peroxides present in PEGs (Figure 4).

Finally, in order to test the effectiveness of the drying technique, rate of oxidation of methionine in a tripeptide (Met-Leu-Phe) in the presence of PEGs with low levels of peroxides, was studied. Two solutions of the tripeptide, one

prepared from fresh PEG 1450 and the other prepared from the vacuum-dried PEG 1450, were stored at 70°C. The samples were analyzed for the native and the oxidized species by RP-HPLC. Significant oxidative degradation of the tripeptide under these conditions was observed when fresh PEG 1450 was used (Figure 5), and effective inhibition of the oxidation was achieved when vacuum-dried PEG was used. The level of peroxides at the start of the study was 42.85 ± 7.02 μEq in solution containing fresh PEG and zero μEq in solution containing purified PEG, while at the end of the study (after 46 hours) the level of peroxides was 5421.4 in solution containing fresh PEG and 135.84 μEq in solution containing purified PEG. These results clearly illustrate the utility of the vacuum-drying technique for the removal of peroxides from PEGs.

CONCLUSIONS

Vacuum drying dilute PEG solutions at low pressures (0.1 mm Hg) is an effective method to purify PEGs. The method uses a simple approach and requires no chemical treatment. Removal of the residual peroxides present in the commercially available PEGs by this method just before the formulation can significantly improve the chemical stability of active ingredients.

ACKNOWLEDGMENTS

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