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Formulation and Evaluation of Epidermal Growth Factor in Pluronic F-127 Gel

M. D. DiBiase[1] and C. T. Rhodes[2]

[1]*Biogen, Inc., 14 Cambridge Center, Cambridge, Massachusetts 02142*
[2]*Department of Pharmaceutics, University of Rhode Island, Kingston, Rhode Island 02881*

ABSTRACT

Pluronic® F-127 gel was evaluated as a potential topical vehicle for epidermal growth factor delivery. The chemical stability of the polypeptide within the gel matrix was investigated using HPLC. Thermal stability studies were performed on the base gel formulation. Modifications to the formulation were made to improve physical characteristics and chemical stability. Humectants and antioxidants were investigated as potential formulation additives and the microbial status of the product was also evaluated. A formulation containing 10% propylene glycol as humectant showed both physical and chemical stability for 3 months when stored in a refrigerator. Kinetic studies were performed on thermal stability data obtained and apparent degradation rate constants were calculated. The degradation of the epidermal growth factor within the formulation appears to follow Arrhenius kinetic, with an apparent energy of activation of 93 kJ. per mole.

INTRODUCTION

Epidermal growth factor (EGF) is a single-chain polypeptide that stimulates the proliferation of many cell types, mainly those of epithelial and epidermal tissues. The mitogenic polypeptide has consequently been under investigation as a potential external adjunct to the wound-healing process. In the past 15 years numerous studies have demonstrated improved epithelial healing with application of EGF (1–4). However, the studies reported are generally implemented by incorporating EGF into a preexisting topical product immediately before application to the patient. Little information is available on formulating EGF and on the physical or chemical stability of the formulations once manufactured.

Pluronic® F-127 (Poloxamer 407) is a nonionic surfactant polyoxyethylene-polyoxypropylene block copolymer recently made available in pharmaceutical grade. The copolymer has been used in vehicles for ointments,

fluorinated dentifrices, eye applications, and contraceptive gels, and has been investigated as a potential artificial skin (5,6). In aqueous concentrations of 20–30%, Pluronic F-127 exhibits reverse thermal gelation (7). At 2°–8°C, 25% Pluronic F-127 in 0.05 M phosphate buffer pH 7.4 exists as a liquid, when warmed to room temperature or above the liquid gels, probably due to hydrogen bonding of the polymer ether oxygen atoms to water. The gel also possesses many favorable characteristics for use as a burn dressing. Not only does the gel provide a nontoxic, detergent covering to the wound, but specific studies suggest the Pluronic gel itself may have a beneficial action, accelerating wound healing over controls (6). It may also be possible to capitalize on the reverse thermal gelation property by producing a product stored in the refrigerator for increased stability. By then pouring the formulation onto the wound in its liquid form, the risk of cross contamination of the patient is greatly reduced and all contours and depths of the wound are covered before the formulation gels in situ. Previous studies at the University of Rhode Island (8) have shown Pluronic F-127 in 0.05 M phosphate buffer pH 7.4 to be a promising formulation base for EGF.

MATERIALS

EGF isolated from human urine was supplied by Serono Laboratories (Norwell, MA). Purity of the supplied material was stated as >98% biologically active EGF. Recombinant EGF produced from yeast was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Pluronic F-127 was a gift from BASF (Parsippany, NJ).

The isolated EGF was shown to exist of five fragmented and slightly modified forms of EGF: 1-52 EGF, 1-51 EGF, 1-50 EGF, and 1-51 and 1-52 EGF with oxidized methionine (methionine sulfoxide) at position 21 (Fig. 1) (13). The recombinant EGF was shown to exist as 1-52 EGF (Fig. 1) (9,13). Comparative studies during formulation showed no significant differences in the behavior of the two materials.

METHODS

Manufacture of Pluronic F-127 Gels

Pluronic F-127, 25% w/w, in 0.05 M phosphate buffer pH 7.4 containing 100 μg EGF/cm^3 was used as ini-

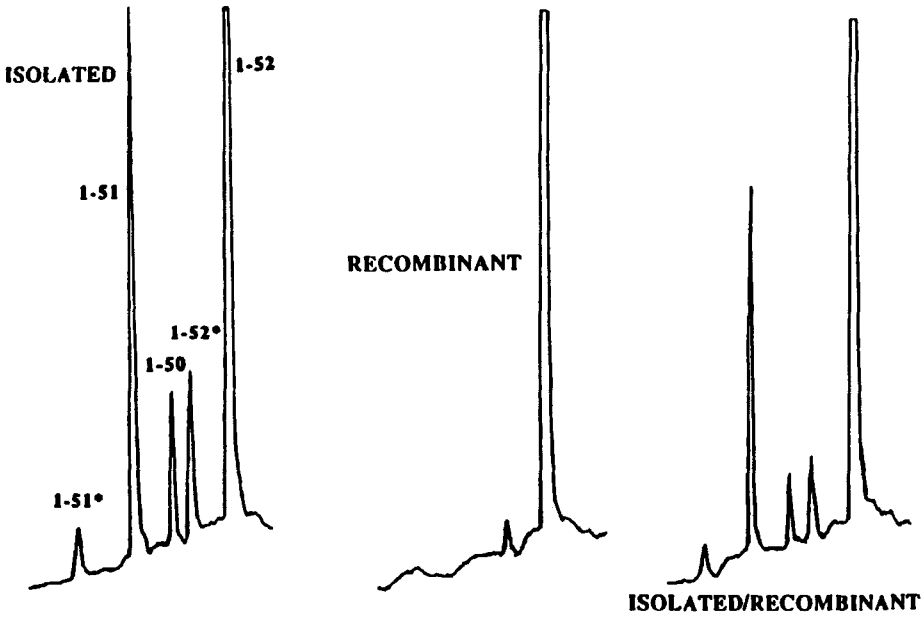

Figure 1. Comparison of urine isolated human EGF with yeast recombinant human EGF.

tial base formulation. The gels were prepared by the cold method (10) and any additional excipients investigated were added before overnight refrigeration of the mixture. Adding excipients to Pluronic F-127 gels can effect the temperature of gelation and the viscosity of the final formulation (11,12). Gelation temperature and viscosity were routinely monitored during manufacture. EGF in 0.05 M phosphate buffer was added after the Pluronic F-127 aqueous mixture had liquified and immediately before gelation. The concentration of EGF in phosphate buffer for addition to formulations was determined by ultraviolet spectroscopy at 276 nm with an experimentally determined extinction coefficient (E 1%, 1 cm) of 27.38.

Analytical Methods

Reverse-phase high-performance liquid chromatography (HPLC) was used to monitor the stability of the EGF directly in the formulation matrix. Slight adaptation of the previously used method (13) was necessary to evaluate the EGF directly in the formulation matrix. All samples of gel were diluted with 0.05 M phosphate buffer pH 7.4 to produce an analyte with a viscosity that could reproducibly be injected onto the column, and with a concentration high enough to be reliably detected. Repeated injection of the diluted gel resulted in decreasing EGF retention times due to polymer retention on the column. A standard washing procedure to remove the Pluronic, using acetonitrile followed by chloroform, permitted recovery of original retention times. The HPLC method repeatedly showed greater than 92% recovery of newly formulated EGF. The injection and washing procedure was validated as an acceptable method to obtain initial information on the growth factor within the gel. EGF quality controls and standard curves were repeatedly run on the column before, during, and after analysis of formulation samples. No significant changes in peak area or relevant retention time were seen for the EGF quality controls, and no significant differences were observed between the standard curves developed over the course of the study.

Standard curves developed for analysis of urine isolated EGF were obtained using the total area of all five peaks; samples were analyzed similarly. Independent standard curves were developed for analysis of gels containing yeast recombinant EGF.

Thermal Stability Studies

Individual 2-cm^3 samples of gel were stored under nitrogen at -20°, 4°, 25°, 37°, 43°, an 50°C in sealed polypropylene tubes. At appropriate intervals samples were removed and analyzed by HPLC. Urine isolated concentrations were determined using totaled areas of all five original peaks. Results were calculated on a percent-remaining basis. Following initial chemical stability analysis of the base formulation, additional excipients were formulated into the product and stability studies were repeated. Recombinant EGF concentrations were determined solely on 1-52 EGF content.

Viscosity Studies

Thermal viscosity studies on the gelation temperature and final viscosity of the formulation were performed using an RV Digital Brookfield Viscometer with a jacketed coaxial cylinder Small Sample Adaptor connected to a circulating bath with a temperature control of 4°C up to 50°C. Actual temperatures inside the cylinder were chart recorded by a thermocouple immersed in the formulation. Viscosity measurements, taken at 20 rpm using a cylindrical spindle with dimensions of 0.4 × 5.1 cm, were also chart recorded. Formulations at 4°C were introduced into the cylinder and the viscosity was recorded constantly as the temperature was raised at a rate of 5°C every min.

Microbial Analysis

Total bioburden microbial analysis was performed on a sample of nonsterile Pluronic F-127 gel made with sterile buffer and humectant. The analysis was performed in accordance with USP XXII and NASA Procedures for Microbial Examination of Space Hardware.

RESULTS

During the stability analysis of formulated EGF by HPLC, a number of chromatographic changes were noted. Some of the changes can be directly linked to specific EGF degradation mechanisms; others have yet to be identified. Two degradation mechanisms that have been identified are deamidation of the terminal asparagine and oxidation of the single methionine residue. Deamidated EGF is detected as a shoulder peak eluting

slightly later than the parent peak, and has been previously identified as a degradation product for EGF in solution at pH 7.4 (13). Oxidation of the methionine residue is detected by area changes from the 1-51 and 1-52 EGF peaks to the corresponding oxidized methionine peaks in the original chromatogram (Fig. 1). However, oxidation was only seen in samples stored at temperatures above 37°C and even then, it contributed only slightly to the overall degradation process. (Significant oxidation is detected in formulations stored at 25°C when aged samples of Pluronic F-127 are used.) Two other methods of degradation have yet to be identified: an HPLC chromatogram showing recovery of EGF from a Pluronic F-127 formulation matrix is shown in Fig. 2. The five original peaks can be identified easily, but there are also two new peaks eluting earlier in the chromatogram. These two peaks appear in the majority of EGF Pluronic gel samples analyzed, yet they do not increase in size any larger than shown in Fig. 2. A fourth degradation mechanism was noted by a general decrease in total chromatogram peak area as the stability studies progressed. The decrease in area occurred, to some extent, in all samples that exhibited degradation. At 50°C only 30% of the original total peak area was accounted for after 21 days; at 43°C, only 27% after 53 days. At lower temperatures the area loss was not so dramatic, and samples stored at 4° and -20°C showed no decrease in total peak area.

The results of the thermal stability analysis of EGF in 25% w/w Pluronic F-127 base formulation are shown in Fig. 3. Over a period of 100 days, no degradation was seen in samples stored at 4°C or -20°C. Samples stored at higher temperatures showed temperature-dependent degradation, with slight changes at 25°C and significant degradation at 50°C.

Addition of Humectants to Base Formulation

Results of viscosity studies on Pluronic F-127 gels containing various humectant excipients are shown in Fig. 4. Glycerin, propylene glycol, and two ethoxylated glucose derivatives were used as humectants, in concentrations of 10% w/w. All the humectants added to the formulation caused a decrease in gelation temperature and had an effect on the final viscosity of the formulation. Addition of Glucam E-20 produced a formulation with a lower final viscosity than the base Pluronic F-127 formulation. Previous studies suggesting that the release of EGF from Pluronic F-127 gel is related to formulation viscosity (8) indicate that higher viscosities are preferable. Studies were consequently continued only with the glycerin, propylene glycol, and Glucam E-10. The results of provisional stability studies of EGF in Pluronic F-127 25% gels containing 10% humectant are shown in Fig. 5. Stability analysis over a period of 45 days at 25°C showed significant degradation in the formulations containing Glucam E-10 and glycerin as humectants. The results of a detailed stability analysis of EGF in

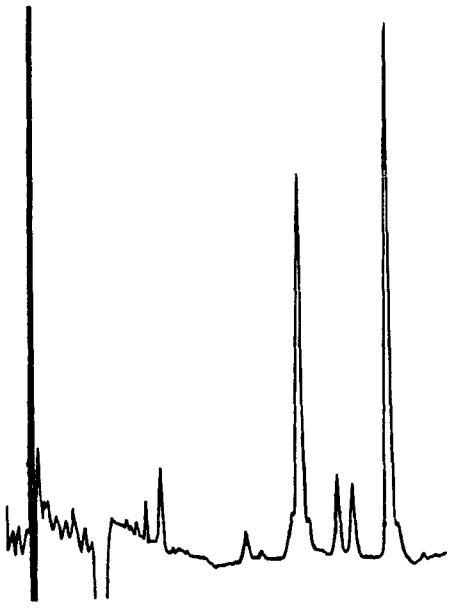

Figure 2. HPLC chromatogram showing the recovery of isolated EGF from the Pluronic F-127 formulation matrix. Two unidentified degradation peaks are shown early in the chromatogram.

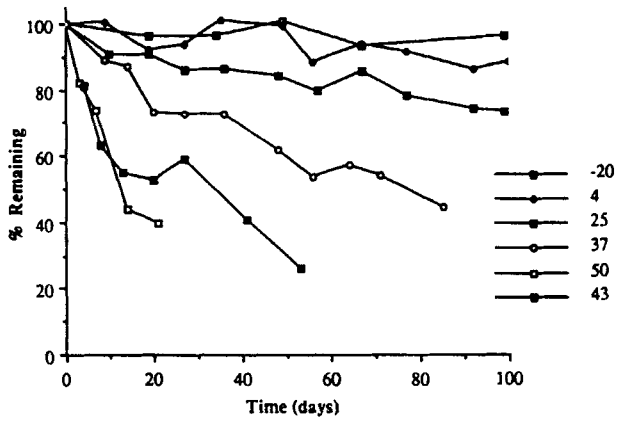

Figure 3. Stability data: EGF in Pluronic F-127 gel initial base formulation. Analyses performed on samples stored at -20°, 4°, 25°, 37°, 43° and 50°C.

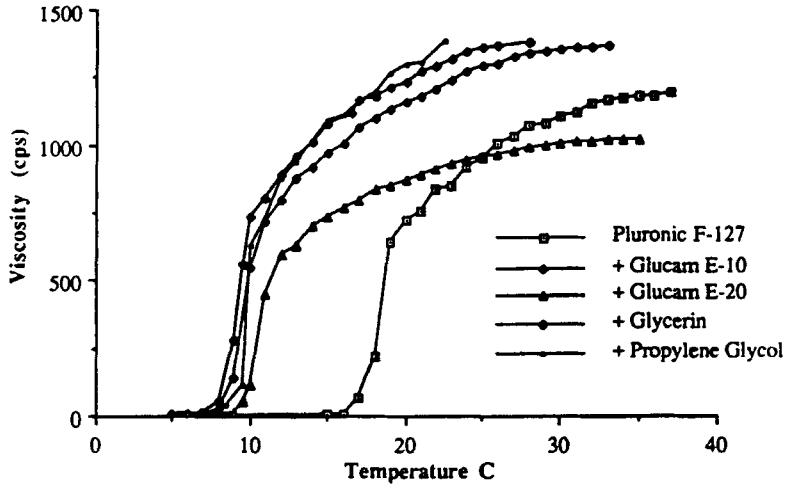

Figure 4. Effect of humectants on the gelation temperature and final viscosity of Pluronic F-127 25% w/w gel formulation.

Pluronic F-127 gel containing 10% propylene glycol are shown in Fig. 6.

Addition of Antioxidants to Base Formulation

Oxidation of the methionine residue of EGF can be induced within the formulation by using aged samples of Pluronic F-127. Aged samples of the polymer contain increased amounts of hydroperoxide formed by autoxidation of the poloxamer (Fig. 7) (14,15). Figure 8 shows the results of stability studies using various antioxidants incorporated into the gel to try to prevent or slow the oxidation process. EDTA was also investigated as a potential antioxidant or antioxidant synergist, but incorporation of the chelating agent retarded gelation of the base formulation to an unacceptable extent.

Formulation of Recombinant EGF

Figure 9 shows the results of a 60-day thermal stability study of yeast recombinant EGF in 25% Pluronic F-127 gel with 10% propylene glycol as humectant.

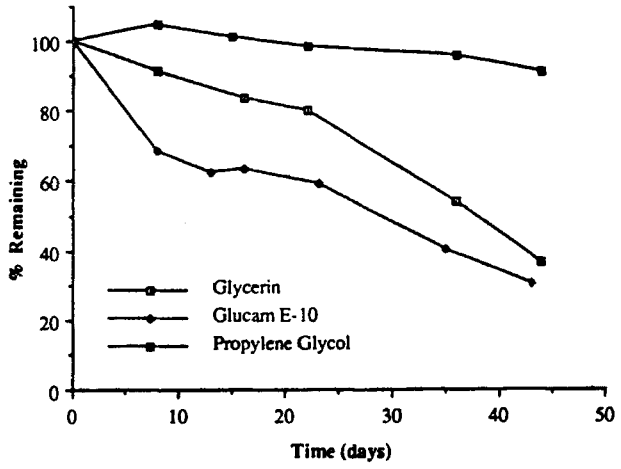

Figure 5. Stability data: Effect of humectants glycerin, Glucam E-10, and propylene glycol on the stability of EGF in Pluronic F-127 gel.

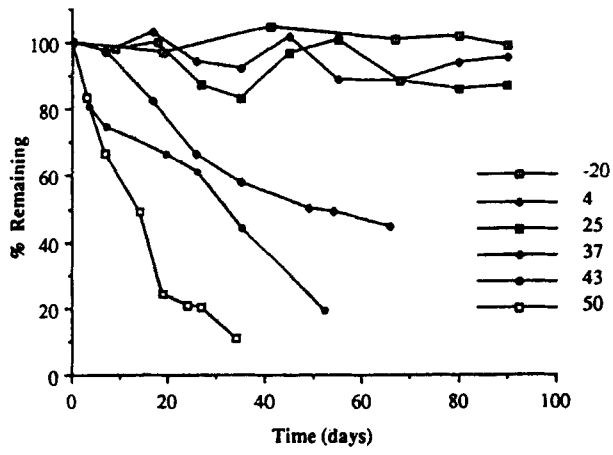

Figure 6. Stability data: EGF in Pluronic F-127 gel containing 10% propylene glycol as humectant. Analyses performed on samples stored at -20°, 4°, 25°, 37°, 43°, and 50°C.

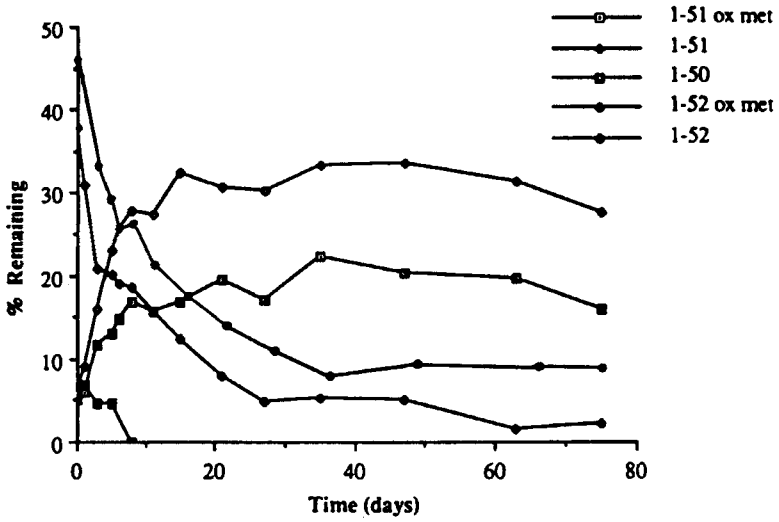

Figure 7. Oxidation of methionine residue of EGF in gel formulation by hydroperoxide present in aged Pluronic F-127. Oxidation is apparent by decreases in 1-51 and 1-51 EGF peak areas, and increases in 1-51 and 1-52 oxidized methionine peak areas.

Microbial Analysis

The microbial analysis showed no growth for the organisms screened which included aerobic bacterial, anaerobic bacteria, fungi, spore-forming aerobic bacteria, and spore-forming anaerobic bacteria. The average plate count for the formulation was therefore considered to be <5 per 50 ml of test article extract for the microbial groups listed.

Kinetic Analysis of Stability Results

Conventional kinetic analysis of the thermal stability data produced in Figs. 3, 6, and 9 gives the results shown in Table 1. Although the exact degradation mechanisms are unknown, good correlation can be obtained by applying first-order kinetics, and apparent rate constants for the degradation process can be calculated. Figure 10 shows application of Arrhenius kinetics to the

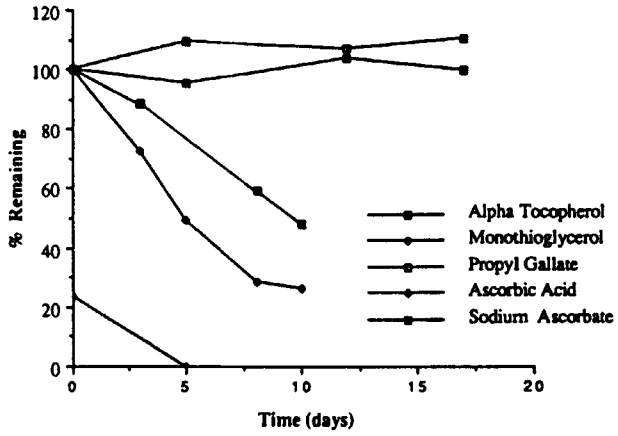

Figure 8. Stability data: Effect of antioxidants alpha-tocopherol, monothioglycerol, propyl gallate, ascorbic acid, and sodium ascorbate on induced EGF oxidation and the stability of EGF in Pluronic F-127 gel.

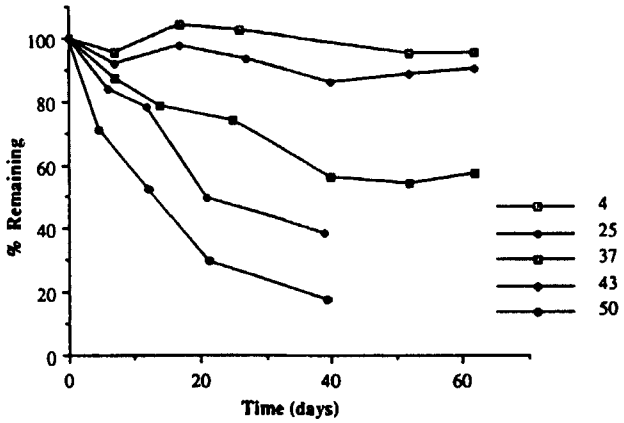

Figure 9. Stability data: Yeast recombinant human EGF in Pluronic F-127 gel containing 10% propylene glycol as humectant. Analyses performed on samples stored at 4°, 25°, 37°, 43°, and 50°C.

Table 1

EGF Degradation in Pluronic F-127 Formulations: Calculated Apparent First-Order Rate Constants

	Base Formulation			Base + 10% Humectant			Recombinant EGF		
Temp. °C	k (day^{-1})	r^2	n	k (day^{-1})	r^2	n	k (day^{-1})	r^2	n
25	0.0026	0.9435	11	—	—	—	—	—	—
37	0.0088	0.9780	11	0.0133	0.9845	8	0.0098		
	0.9037	7							
43	0.0208	0.9416	8	0.0262	0.9530	7	0.0253	0.9468	5
50	0.0459	0.9741	5	0.0652	0.09892	8	0.0424	0.9676	5

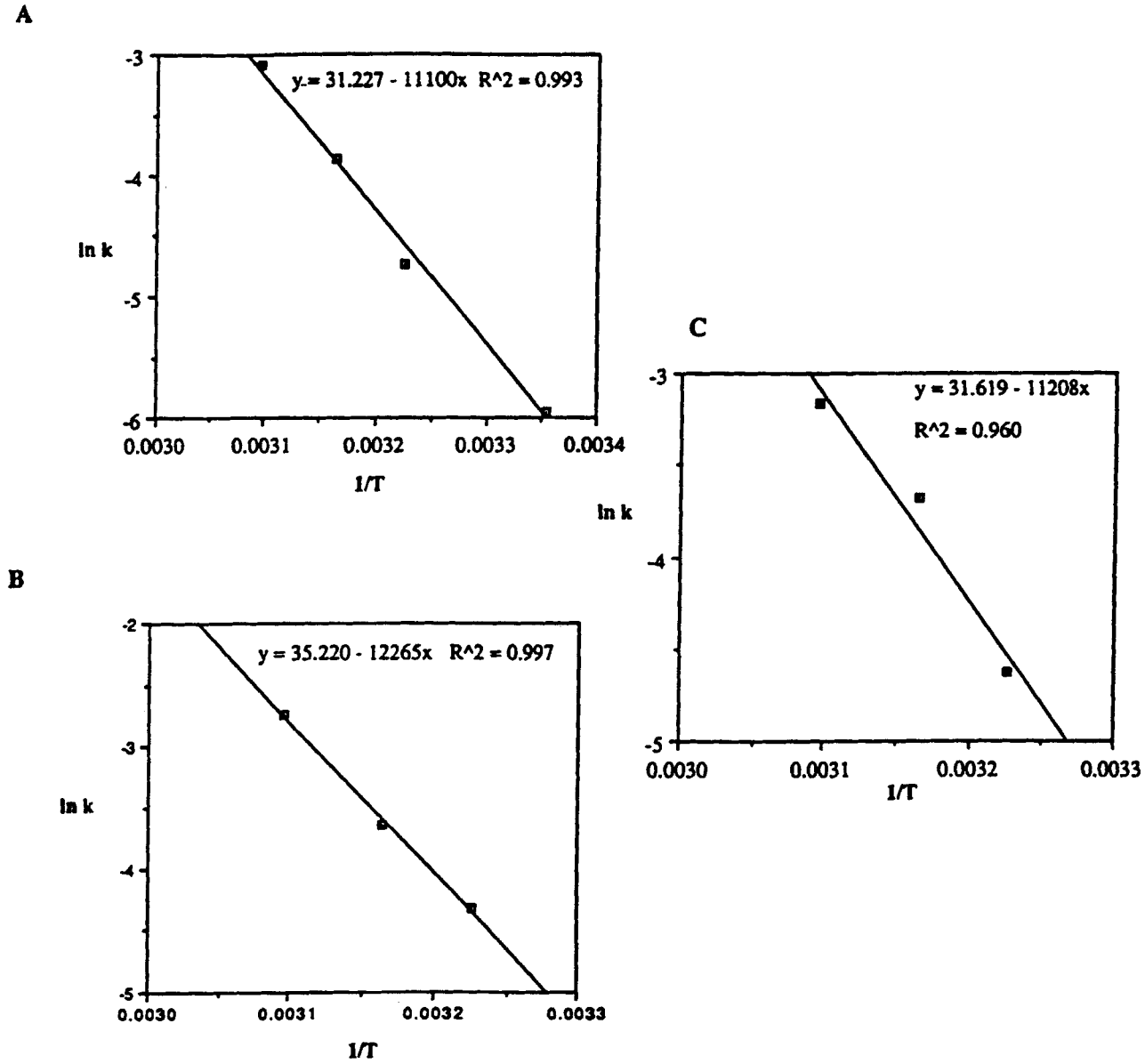

Figure 10. Application of Arrhenius kinetics to thermal stability data with calculated apparent energies of activation. A: Pluronic F-127 25% base formulation. Apparent E_a = 92.30 kJ/mol. B: Pluronic F-127 gel containing 10% propylene glycol as humectant. Apparent E_a = 101.96 kJ/mol. C: Recombinant EGF in Pluronic F-127 gel containing 10% propylene glycol as humectant. Apparent E_a = 93.18 kJ/mol.

apparent rate constant data and allows calculation of apparent energies of activation for the degradation.

DISCUSSION

Further formulation of pharmaceutical products after initial formulation evaluation is a common step in the development process. The initial base formulation of EGF in Pluronic F-127 gel showed excellent chemical stability for 3 months when stored at 2°-8°C, yet rapid dehydration of the formulation occurred when the product was left exposed to atmospheric conditions. Addition of humectants, at 10%, to the formulation retarded the dehydration process. Higher levels of humectant, while providing further improved hydration, increased the viscosity of the formulation, even at refrigerated temperatures, to an unacceptable level. Of the humectants studies, propylene glycol had the greatest potential for improving the formulation. Incorporation of propylene glycol significantly improved the physical characteristics of the formulation, while 3-month stability studies, when compared with those of the initial base formulation, showed no significant changes in degradation rates or mechanism.

It is believed that oxidation of the EGF at elevated temperatures is due to the presence of hydroperoxide impurities in the Pluronic F-127. The hydroperoxide is formed by autooxidation of the polymer (14,15). Although this reaction is inhibited by the addition of water (16), the elevated temperatures used in the study may induce the autoxidation. At the time the study was initiated, limited data were available on the modes of degradation of EGF. The use of aged Pluronic F-127 samples also suggests that oxidation played a significant role, even at room temperature, in EGF degradation. Investigations into preventing the oxidation with various antioxidants were therefore implemented. Later results using freshly obtained Pluronic F-127 indicated that oxidation at low temperatures was of little importance as a degradation mechanism, and that antioxidants were not essential for a product that was to be stored under refrigerated conditions. If, in the future, a product were to be developed that could be stored at room temperature, potential exposure to elevated temperatures may necessitate incorporation of an antioxidant. In this case, further studies would be required to find an antioxidant compatible with the EGF. The oil-soluble antioxidants alpha-tocopherol and propyl gallate did not adversely affect the formulation, but also did not prevent the oxidation. This is not immediately evident from Fig. 8 because results are calculated by summation of total peak area. Changes of area from one peak to another, as with oxidation, are not recorded but are evident from study of individual peaks in the chromatogram. The aqueous-soluble antioxidants ascorbic acid and sodium ascorbate did prevent oxidation but had adverse affects on the chemical stability of the EGF.

Studies using the yeast recombinant EGF in the formulation did not demonstrate any different degradation mechanisms or changes in degradation rate at elevated temperatures. Use of the genetically engineered material will be preferable for circumvention of human viral contamination and availability problems, and will be significantly more cost-effective in long-term use.

Microbial contamination of the product and infection of the wound are both important concerns that need to be addressed in formulation. The Pluronic F-127 gel can be steam sterilized (6) without losing its reverse thermal gelation properties. Concerns that the high temperature used in steam sterilization may induce autoxidation of the polymer have not been realized in studies where gels with humectant have been autoclaved and the EGF added aseptically after the sterilization process. HPLC studies of these formulations over a period of 14 days do not show signs of EGF oxidation. Even if further studies preclude the use of gel sterilization, microbial analysis of nonsterile Pluronic gel made with sterile buffer and humectant showed no evidence of microbial contamination. It is therefore hoped that the product could be manufactured as a unit dose without the addition of preservatives. Depending on the type of wound that the product will be applied to, incorporation of a compatible antibiotic may be a future possibility.

CONCLUSION

In conclusion, it appears feasible to develop a topical product of EGF in Pluronic F-127 gel with a shelf life of at least 3 months when stored in a refrigerator. Degradation of the EGF within this formulation at elevated temperature, as determined by HPLC, appears to follow Arrhenius kinetics, with good correlation, over a temperature range of 25° to 50°C.

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