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RESEARCH PAPER

Development of a Lyophilized Parenteral Pharmaceutical Formulation of the Investigational Polypeptide Marine Anticancer Agent Kahalalide F

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ABSTRACT

Kahalalide F is a novel antitumor agent isolated from the marine mollusk Elysia rufescens; *it has shown highly selective in vitro activity against androgen-independent prostate tumors. The purpose of this study was to develop a stable parenteral formulation of kahalalide F to be used in early clinical trials. Solubility and stability of kahalalide F were studied as a function of polysorbate 80 (0.1%–0.5% w/v) and citric acid monohydrate (5–15 mM) concentrations using an experimental design approach. Stabilities of kahalalide F lyophilized products containing crystalline (mannitol) or amorphous (sucrose) bulking agents were studied at +5°C and +30°C ± 60% relative humidity (RH) in the dark. Lyophilized products were characterized by infrared (IR) spectroscopy and differential scanning calorimetry (DSC). Recovery studies after reconstitution of kahalalide F lyophilized product and further dilution in infusion fluid were carried out to select an optimal reconstitution vehicle. It was found that*

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a combination of polysorbate 80 and citric acid monohydrate is necessary to solubilize kahalalide F. Lyophilized products were considerably less stable with increasing polysorbate 80 and citric acid monohydrate concentrations, with polysorbate 80 being the major effector. A combination of 0.1% w/v polysorbate 80 and 5 mM citric acid monohydrate was selected for further investigation. Lyophilized products containing sucrose as a bulking agent were more stable compared to the products containing mannitol. The glass transition temperature of the sucrose-based product was determined to be +46°C. The amorphous state of the product was confirmed by IR analysis. A solution composed of Cremophor EL, ethanol, and water for injection (5%/5%/90% v/v/v CEW) kept kahalalide F in solution after reconstitution and further dilution with 0.9% w/v sodium chloride (normal saline) to 1.5 μg/m. A stable lyophilized formulation was presented containing 100 μg of kahalalide F, 100 mg sucrose, 2.1 mg citric acid monohydrate, and 2 mg polysorbate 80 to be reconstituted with a vehicle composed of 5%/5%/90% v/v/v CEW and to be diluted further using normal saline.

Key Words: *Citric acid monohydrate; Cremophor EL; Glass transition temperature; Kahalalide F; Marine antitumor agent; Parenteral lyophilized formulation; Polysorbate 80; Polypeptide; Sucrose*

INTRODUCTION

Kahalalide F is a cyclic depsipeptide derived from the marine mollusk *Elysia rufescens*, an organism living in the seas near Hawaii (Fig. 1) (1). Kahalalide F displays both in vitro and in vivo activity in various solid tumor models, including breast, colon, non-small-cell lung, and particularly prostate cancer (2). Preliminary data suggest the mechanism of action of the compound is on the lysosome level in the cell (3). On the basis of its selectivity, kahalalide F now is being further developed as a potential anticancer agent against androgen-independent prostate tumors (4,5).

The purpose of this study was to develop a stable parenteral dosage form to be used in early clinical studies. Based on the maximum tolerated dose (MTD) of 280 μg/kg body weight found in mouse toxicology studies, the starting dose for phase I clinical studies was set at 84 $\mu g/m^2$ body surface area (BSA), being 1/10 of the MTD_{mouse} equivalent (6). Therefore, a dosage unit content of 100 μg was considered as most appropriate to cover the expected phase I dosing range of approximately 150–1500 μg, assuming an average BSA of 1.7 m^2 and a maximum dosage level of 10 times the starting dose. In preformulation studies, it was found that kahalalide F displays limited solubility and stability in aqueous solution. Therefore, freeze-drying was selected as the formulation approach.

In this article, we present the investigations into the effects of various excipients on kahalalide F solubility and stability during formulation, freeze-drying, and storage. Furthermore, in view of the low infusion concentrations (down to 1.5 μg/ml) expected in the phase I clinical studies, the

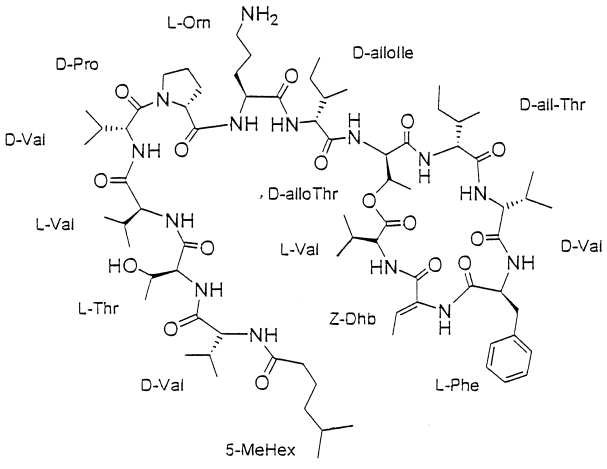

Figure 1. Chemical structure of kahalalide F ($C_{75}H_{124}N_{14}O_{16}$, MW 1477) 5-MeHex, 5-methyl-hexane; Val, valine; Thr, threonine; Pro, proline; Orn, ornithine; Ile, isoleucine; Phe, phenylalanine).

development of an appropriate reconstitution vehicle to avoid loss of drug substance as a consequence of sorption, for instance, is described.

EXPERIMENTAL

Materials

Kahalalide F was provided by Pharma Mar SA (Tres Cantos, Madrid, Spain). Mannitol, sucrose, polysorbate 80, ethanol absolute, and citric acid monohydrate, all European Pharmacopoeia III (Ph.Eur.) and/or U.S. Pharmacopoeia (USP) 24 grade, were purchased from Bufa (Uitgeest, The Netherlands). Water for injection (WfI) and 0.9% w/v sodium chloride for infusion (normal saline) were manufactured in house (Department of Pharmacy, Slotervaart Hospital/The Netherlands Cancer Institute). Cremophor EL and *tris*(hydroxymethyl)aminomethane (Tris) were obtained from Sigma (Zwijndrecht, The Netherlands). Acetonitrile and methanol were analytical grade and purchased from Biosolve (Amsterdam, The Netherlands). Trifluoroacetic acid was obtained from Merck (Amsterdam, The Netherlands).

Methods

Solubility Experiments

To screen for solubility, aqueous solutions at a target concentration of 50 μg/ml kahalalide F in WfI, 10 mM citric acid monohydrate, and 10 mM Tris with or without 0.1% w/v polysorbate 80 were prepared by dissolving 2.5 mg of the drug substance in 50.0 ml of the respective solution vehicles under continuous magnetic stirring in a hydrolytic class 1 glass container until complete visual dissolution. Additional solubility experiments were carried out in a similar way by dissolving 250 μg of kahalalide F in 5.0 ml of aqueous citric acid monohydrate (1, 5, and 10 mM) solutions containing 0.05% or 0.1% (w/v) polysorbate 80. Final kahalalide F concentrations and stability on dissolution were determined by high-performance liquid chromatographic (HPLC) analysis. All solubility experiments were conducted at room temperature (20C°–25°C).

Freeze-Drying

To obtain 100 μg/vial kahalalide F lyophilized product, formulation solutions at a concentration of 50 μg/ml kahalalide F were sterile filtered (Sartolab-P20 0.2 μm, Sartorius, Goettingen, Germany) and 2-ml aliquots were dispensed in hydrolytic class 1, type Fiolax 8-ml molded clear glass vials (Aluglas, Uithoorn, The Netherlands). Vials were subsequently semistoppered using type FM157/1 siliconized gray bromobutyl rubber stoppers (Helvoet Pharma, Alken, Belgium) and loaded into the freeze-dryer (Lyovac GT-4, Steris, Hürth, Germany) at room temperature.

Lyophilization of the formulation solutions containing mannitol as a bulking agent was performed by freezing the product to −40°C, followed by an annealing step (heating to −15°C and subsequent refreezing to −40°C), all at ambient pressure (1000 mbar). Primary drying was carried out at a chamber pressure of 0.15 mbar and a shelf temperature of −7°C, followed by secondary drying at +25°C and 0.15 mbar. Lyophilization of the formulations containing sucrose was performed by freezing the solutions to −45°C at ambient pressure. Primary drying was subsequently started by applying a chamber pressure of 0.1 mbar at −45°C, followed by additional drying steps at shelf temperatures of −25°C and −12°C and a chamber pressure of 0.1 mbar. Secondary drying was carried out at a shelf temperature of +25°C and a chamber pressure of 0.02 mbar. Completion of the drying process was determined by a pressure increase test. Vials were stoppered under vacuum.

Optimization of Formulation and Accelerated Stability Study

Stability of kahalalide F 100 μg/vial lyophilized product at an elevated storage temperature condition was examined as a function of polysorbate 80 and citric acid monohydrate concentration. A classical three-level factorial (2^3) experimental design was carried out using CARD software (Computer Aided Research and Design, version 5.0, S-Matrix, Eureka, CA). Polysorbate 80 formulation solution concentration levels were set at 0.1%, 0.3%, and 0.5% w/v (corresponding to normalized values of −1, 0, and 1, respectively); citric acid monohydrate levels were set at 5, 10, and 15 mM (normalized values of −1, 0, and 1, respectively). Mannitol at a formulation solution concentration of 50 mg/ml was used as a bulking agent. Kahalalide F was dissolved in the appropriate vehicle at a concentration of 50 μg/ml and subsequently lyophilized as described

Table 1

Levels of the Independent Variables (Polysorbate 80 and Citric Acid Monohydrate Concentrations) Evaluated in the Experimental Design (Replicate Formulations, 4,10; 6,11; 8,12)

Formulation	Polysorbate 80 Concentration (% w/v), X_1	Citric Acid Concentration (mM), X_2	$(X_1)^2$	$(X_2)^2$	X_1*X_2
1	0.3	15	0	1	0
2	0.3	5	0	1	0
3	0.1	5	1	1	1
4	0.1	15	1	1	−1
5	0.5	5	1	1	−1
6	0.1	10	1	0	0
7	0.5	10	1	0	0
8	0.3	10	0	0	0
9	0.5	15	1	1	1
10	0.1	15	1	1	−1
11	0.1	10	1	0	0
12	0.3	10	0	0	0

above. In total, 12 formulation compositions containing all possible polysorbate 80 and citric acid monohydrate levels were prepared, including three replicates (Table 1) and stored at +50°C in the dark. Also, blank formulations without kahalalide F were prepared and stored at +50°C. Kahalalide F content and chromatographic purity were determined on days 0, 1, 3, 7, 10, and 21 using HPLC analysis.

Characterization of Sucrose Vehicle

Aqueous solutions containing sucrose (50 mg/ml) with and without polysorbate 80 (0.1% w/v) and citric acid monohydrate (5 mM) were prepared and subsequently freeze-dried using the same procedure as for kahalalide F 100 μg/vial lyophilized product. Characterization of the physical state of the lyophilized products was performed by modulated differential scanning calorimetry (MDSC) and infrared (IR) analysis.

Long-Term Stability Study

Kahalalide F 100 μg/vial lyophilized product was prepared from formulation solutions containing 0.1% w/v polysorbate 80, 5 mM citric acid monohydrate, and 50 mg/ml mannitol or sucrose as bulking agents. Lyophilized products were stored at +5°C and +30°C ± 60% relative humidity (RH) in the dark and assayed for kahalalide F content and stability in time by HPLC analysis. Also, the physical state of the sucrose-based formulation in time was determined by MDSC and IR analysis.

Reconstitution Studies

Recovery after reconstitution and further dilution in normal saline of kahalalide F lyophilized product containing sucrose as bulking agent with WfI, 5%/5%/90%, 10%/10%/80%, and 15%/15%/70% v/v/v Cremophor EL/ethanol/WfI vehicles was studied. Lyophilized product was reconstituted with 2 ml of the respective vehicles and subsequently diluted with normal saline to theoretical concentrations of 15, 3, and 1.5 μg/ml in glass vials. All samples were analyzed for kahalalide F concentration by HPLC. Recovery after dilution was calculated with respect to the nominal concentration.

Analytical Methods

High-Performance Liquid Chromatography Analysis

Kahalalide F was assayed by a validated, stability-indicating reversed-phase HPLC method. The HPLC system consisted of a model SP8800 ternary pump (Thermo Separation Products [TSP], Fremont, CA), a model 996 photo-diode array detector (Waters, Milford, MA), and a model SP8880 autosampler (TSP). Analyses were carried

out with a Zorbax SB-C18 analytical column (4.6 mm ID × 150 mm, particle size 3.5 μm) (Rockland Technologies, Inc., Newport, DE) held at a constant temperature of +80°C using a model 7971 column heater (Jones Chromatography, Inc., Lakewood, CO). The mobile phase at a flow of 0.6 ml/min consisted of a linear gradient of acetonitrile containing 0.04% trifluoroacetic acid (35% to 90% in 25 min) and water containing 0.04% trifluoroacetic acid. An injection volume of 75 μl and a total run time of 30 min were employed. Ultraviolet (UV) detection was performed at 215 nm.

Under these conditions, the chromatogram of kahalalide F consisted of a single peak eluting at approximately 15 min. Kahalalide F formulation solutions and freeze-dried products (after reconstitution with 2 ml of the appropriate vehicle) were diluted fivefold with eluent (35%/65% v/v acetonitrile/water containing 0.04% trifluoroacetic acid) before injection into the HPLC system. Samples of kahalalide F infusion solutions (see Reconstitution Studies) were injected without further dilution.

Moisture Content

Residual water contents of the freeze-dried products were determined using a biamperometric Karl Fisher assay. The content of a vial was transferred quantitatively to the titration unit of a model 658KF titrino apparatus (Metrohm, Herisau, Switzerland) with previously dried methanol and subsequently titrated using Hydranal® titrant (Sigma, Zwijndrecht, The Netherlands). Analyses were carried out in triplicate.

Modulated Differential Scanning Calorimetry

Glass transition temperatures T_g of lyophilized products were determined using a DSC 2920 (TA Instruments, New Castle, DE) equipped with a liquid nitrogen cooling accessory (LNCA) for low temperatures. Samples of 2.5–5 mg were punched out of the freeze-dried cakes and subsequently transferred into aluminum pans, which were sealed immediately. An empty pan was used as a reference, and analyses were performed under a helium purge. The temperature scale and heat flux were calibrated with indium. Samples were heated to +150°C at a rate of 10°C/min. The T_g values were determined by taking the half-height between the baseline below and above the temperature range of the glass transition.

Infrared Spectroscopy

The IR spectra of the dried formulations and sucrose were recorded at +25°C using a model FTS 6000 infrared spectrometer (Bio-Rad, Veenendaal, The Netherlands). A total of 256 scans was recorded for each spectrum using a 4-cm^{-1} resolution. Sucrose samples and dried samples were prepared for IR analysis by grinding approximately 1 mg of material with approximately 200 mg of potassium bromide and annealing the powder into a disk using a hydraulic press. Spectra from 400 to 4000 cm^{-1} were obtained using Win-IR Pro software.

RESULTS AND DISCUSSION

Solubility Experiments

To screen for the solubility of kahalalide F over the pH range of 2–10, concentrations of kahalalide F in aqueous solutions at low (pH 2.6; 10 mM citric acid monohydrate), unadjusted (pH 7.6; WfI without buffer), and high pH (9.6; 10 mM Tris) levels with or without the addition of a nonionic surfactant (polysorbate 80, 0.1% w/v) were examined; a total of six formulation solutions were thus investigated. A target formulation solution concentration of 50 μg/ml kahalalide F was selected to obtain a freeze-dried product containing 100 μg of drug substance after 2-ml fillings.

Both citric acid monohydrate and Tris buffer were selected for their minimal pH change on freezing, which could influence kahalalide F stability during lyophilization (7,8). The nonionic surfactant polysorbate 80 is widely used in parenteral formulations as a solubilizing agent and in the prevention of adsorption of peptides to contact surfaces (8–10). Furthermore, polysorbate 80 at low concentrations is known to effectively reduce stress at the ice-water interface during freezing, which can be detrimental to polypeptide stability (11,12).

Figure 2A shows the results of the solubility screening of kahalalide F in the various formulation compositions. Rapid degradation of kahalalide F occurred on dissolution in the high pH Tris solutions, as concluded from the degradation peaks that appeared in the chromatograms immediately after

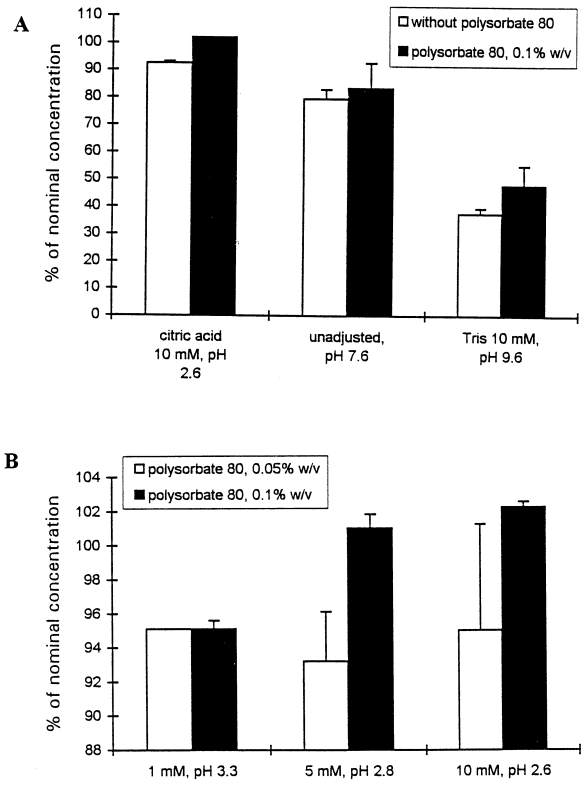

Figure 2. (A) Solubility of kahalalide F in aqueous solution at low, unadjusted, and high pH with and without polysorbate 80 at 0.1% w/v (% of nominal concentration ± SD); (B) solubility of kahalalide F in aqueous solution containing 1, 5, or 10 mM citric acid monohydrate and 0.05% or 0.1% w/v polysorbate 80 (% of nominal concentration ± SD).

dissolution. Kahalalide F visually dissolved completely in aqueous solutions containing no excipient, citric acid monohydrate, or polysorbate 80. However, less than 100% of the nominal drug concentration was recovered, as determined by HPLC analysis. As no degradation peaks were present in the chromatograms, this loss was most probably caused by sorption of drug substance to the container surface. The optimal formulation solution in terms of solubility contained both citric acid monohydrate (pH 2.6) and polysorbate 80, with a recovery of approximately 100% of the nominal concentration (Fig. 2A).

On the basis of these initial results, a formulation solution vehicle containing both polysorbate 80 and citric acid monohydrate was selected for further development. To determine the minimal levels necessary for complete dissolution, the solubility of kahalalide F was examined in solutions containing polysorbate 80 (0.05% and 0.1% w/v) and citric acid monohydrate (1, 5, 10 mM, corresponding to pH 3.3, 2.8, and 2.6, respectively). It was found that minimal concentrations of 0.1% w/v of polysorbate 80 and 5 mM of citric acid monohydrate are necessary for complete solubilization of kahalalide F (recovery of 101.0% ± 0.8% of the nominal kahalalide F concentration, $n = 3$) (Fig. 2B).

Stability Experiments

Based on the results of the solubility experiments, the stability of kahalalide F as a function of citric acid and polysorbate 80 concentrations was investigated after lyophilization and subsequent storage. As this is a multivariate problem, an experimental design approach was used to evaluate the influence of each independent variable (citric acid monohydrate, polysorbate 80) and interaction between the determinants (content and impurities level of lyophilized product) (13). A classical three-factorial (2^3) experimental design was used in which the minimal concentrations of citric acid monohydrate and polysorbate 80 necessary for complete dissolution of kahalalide F drug substance were taken as lower levels (0.1% w/v polysorbate 80 and 5 mM citric acid monohydrate, respectively), and 0.5% w/v polysorbate 80 and 15 mM citric acid monohydrate were used as upper levels (Table 1). To obtain data on the stability of kahalalide F lyophilized product within an acceptable time frame, an accelerated storage temperature condition of +50°C in the dark was applied.

Polypeptides and proteins are generally formulated with excipients such as sugars and polymers (e.g., sucrose, trehalose, dextran) to protect the substance during freezing and dehydration stresses encountered during lyophilization (7,14,15). These excipients remain amorphous during freezing, freeze-drying, and storage, thus increasing peptide drug stability by molecular interaction with the drug and/or acting as a "sink" for residual water (14,16).

An amorphous solid is characterized by its glass transition temperature T_g or collapse temperature. Near or above this temperature, the solid softens and mobility of the molecules increases significantly, resulting in accelerated chemical reaction rates (14). Due to interference of physical changes with

chemical stability, interpretation of results of accelerated stability testing of an amorphous solid at temperatures near or above T_g is difficult (17). For instance, the T_g of sucrose, widely used in lyophilized peptide formulations, is around +50°C (15,17). Therefore, to examine only the chemical stability of kahalalide F as a function of polysorbate 80 and citric acid monohydrate concentration, mannitol as a crystalline bulking agent was selected for this accelerated stability experiment. An annealing step was applied in the freeze-drying cycle to ensure complete crystallization of mannitol. Lyophilization resulted in excellent cakes with water contents below 1% w/w. From the initial content and purity determinations, it was concluded that lyophilization does not affect kahalalide F integrity.

Table 2 gives the zero-order rate constants for the decrease in kahalalide F content during storage as a function of polysorbate 80 and citric acid monohydrate concentration. No significant differences in degradation rates as a function of citric acid monohydrate and polysorbate 80 concentrations could be found, with a range of 64%–72% of the initial kahalalide F content remaining after 3 weeks of storage for all formulations. The chromatographic purity of the lyophilized product decreased rapidly for all formulations. The major degradation products appearing in the chromatographic profile of kahalalide F lyophilized product, however, also appeared in the chromatograms of blank, lyophilized vehicles stored under identical conditions as kahalalide F lyophilized product. Therefore, the increase in amount of degradation product originating from the pharmaceutical vehicle, as well as those originating from kahalalide F, was analyzed. In Table 2, normalized values for each formulation are given, with the highest impurity level taken as 100%. Highest levels of impurities, both vehicle related and originating from kahalalide F, were found for the formulation containing the highest concentrations of polysorbate 80 and citric acid monohydrate (0.5% w/v and 15 mM, respectively). Multivariate analysis resulted in the following second-order polynomial models for the (1) vehicle-related impurities level and (2) kahalalide F–related impurities level that fitted the observed data best, respectively:

$$Y = 116.1^{*}X_1 + 2.3^{*}X_2 - 8.1^{*}(X_1)^2 + 7.4^{*}(X_1X_2) + 6.7\ R^2 = 0.99 \quad (1)$$

$$Y = 115.6^{*}X_1 + 2.7^{*}X_2 - 12.0^{*}(X_1)^2 + 6.6^{*}(X_1X_2) + 7.1\ R^2 = 0.98 \quad (2)$$

where Y is the impurity level (normalized values %), and $X_{1,2}$ represent the polysorbate 80 and citric acid monohydrate levels (Table 2). As can be seen,

Table 2

Effect of Polysorbate 80 and Citric Acid Monohydrate Concentration on Stability of Kahalalide F 100 μg/Vial Lyophilized Product (Replicate Formulations 4,10; 6,11; 8,12)

Formulation	Content $k_{observed}$ (μg day^{-1}) $0.94 < R^2 < 0.99$	Impurities Level (Normalized Values %)			
		Vehicle Related		Kahalalide F Related	
		Observed	Predicted	Observed	Predicted
1	0.78	75.3	76.0	85.0	82.7
2	0.65	49.7	53.1	51.8	55.4
3	0.67	31.1	29.1	27.3	26.9
4	0.68	35.4	37.3	37.6	41.1
5	0.66	63.5	90.8	62.5	60.0
6	0.55	33.5	33.2	33.8	34.0
7	0.73	75.5	79.7	78.1	80.2
8	0.72	67.3	64.6	66.4	69.1
9	0.76	100	98.6	100	100.4
10	0.61	39.9	37.3	42.0	41.1
11	0.55	30.3	33.2	36.2	34.0
12	0.63	65.9	64.6	73.0	69.1

polysorbate 80 is the major effector on the impurity levels of the lyophilized product.

Table 2 shows that the predicted values for the impurity levels are in good agreement with the data observed. Furthermore, the normalized impurity levels as a function of polysorbate 80 and citric acid monohydrate concentrations have almost an identical profile (Figs. 3A, 3B). Lowest impurity levels for

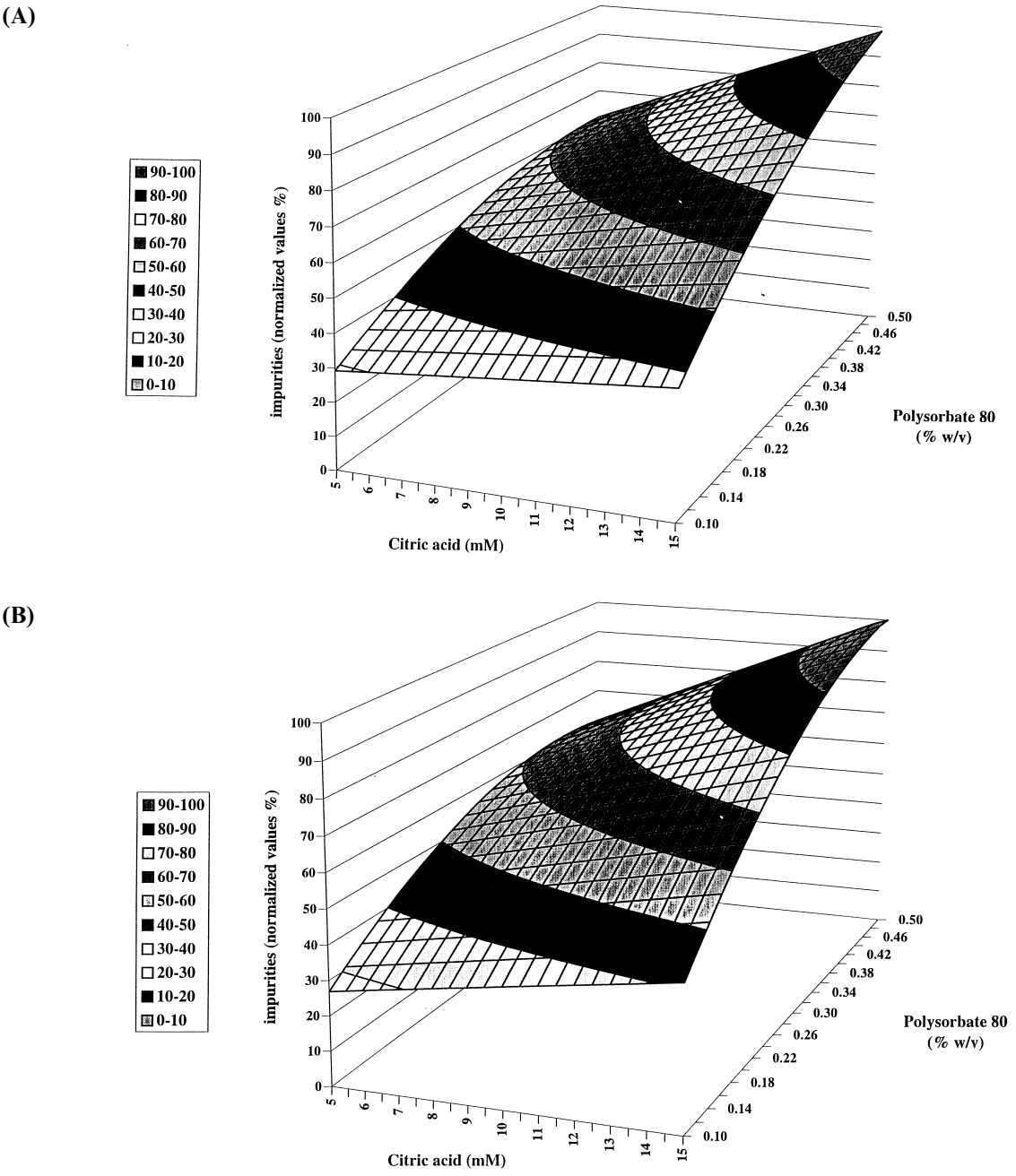

Figure 3. Three-dimensional surface plot illustrating the effect of citric acid monohydrate and polysorbate 80 on the stability of kahalalide F 100 μg/vial lyophilized product expressed as the normalized impurity levels (%): (A) vehicle-related impurities; (B) kahalalide F–related impurities.

both vehicle-related and kahalalide F–related levels were found for a formulation containing 0.1% (w/v) polysorbate 80 and 5 mM citric acid monohydrate, with predicted normalized impurities levels of 29.1% (lower and upper 95% confidence limits of 21.6% and 36.7%, respectively) and 26.9% (lower and upper 95% confidence limits of 19.4% and 34.3%, respectively), respectively. It can be concluded that both kahalalide F and the mannitol-based vehicle are less stable with increasing polysorbate 80 and citric acid monohydrate concentrations. Therefore, for further investigations, the combination of 0.1% (w/v) polysorbate 80 and 5 mM citric acid monohydrate was selected.

Table 3

Characteristic of Lyophilized Products

Lyophilized Product	T_g°C	Moisture Content (% w/w) ± SD ($n = 3$)
Sucrose	55	0.8 ± 0.03
Sucrose 5%, polysorbate 80 0.1%	58	0.7 ± 0.02
Sucrose 5%, polysorbate 80 0.1%, citric acid monohydrate 5 mM	53	0.6 ± 0.04
Kahalalide F 100 µg/vial, sucrose based	46	0.8 ± 0.07
Kahalalide F 100 µg/vial, mannitol based	not detected	0.8 ± 0.14

Selection of Bulking Agent

To examine the influence of the physical state of the bulking agent on the stability of kahalalide F lyophilized product, kahalalide F was freeze-dried using mannitol as a crystallizing excipient or sucrose as a noncrystallizing, amorphous excipient. Although crystalline bulking agents do not provide protection to peptides during lyophilization, some peptide drugs have been formulated successfully using mannitol as the bulking agent (16,18). Presumably, the amorphous fraction in these vehicles was sufficient to provide stability during long-term storage (8). In this experiment, a combination of 0.1% w/v polysorbate 80 and 5 mM citric acid monohydrate was used to solubilize kahalalide F since it was determined to be the vehicle with the highest stability. The mannitol formulation was freeze-dried, and an annealing step was included to ensure complete crystallization. Freeze-drying for both the mannitol- and sucrose-containing formulations resulted in excellent, white solid cakes.

Table 3 gives the characteristics of the lyophilized products. The T_g of +55°C found for the lyophilized blank sucrose product was comparable to data reported earlier (15,17). The T_g of +46°C determined for the sucrose-based lyophilized kahalalide F product seems adequate with respect to the physical stability at the intended long-term storage condition of +5°C and possible excursions to room temperature (e.g., during shipment). As expected, no T_g was observed for the freeze-dried mannitol formulations, indicating complete crystallization of the bulking agent.

Besides the applied annealing step, the addition of polysorbate 80 is reported to enhance mannitol crystallinity (12). The amorphous state of the sucrose-based formulations was confirmed by IR analysis. As can be seen in Fig. 4, the IR spectrum of sucrose raw substance (crystalline state) shows sharp, well-defined peaks that are absent in the spectra of the lyophilized products. This indicates the amorphous character of the freeze-dried products, which allows increased freedom of stretching and rotational states of the functional groups, resulting in bond broadening and smoothing. The additional peak at a wavelength of approximately 1700 cm^{-1} present in the lyophilized kahalalide F products can be attributed to the additional carbonyl functions present in the samples.

Table 4 shows the 3-month stability data for two batches of mannitol- and sucrose-based kahalalide F 100 µg/vial lyophilized product. Both formulations were stored at +5°C (intended long-term storage condition) and +30°C ± 60% RH in the dark and assayed for kahalalide F content and purity in time by HPLC analysis. The rapid decrease in chromatographic purity of the mannitol-based product versus no significant degradation of the drug formulated in the sucrose vehicle is illustrated in Fig. 5.

As can be seen, degradation peaks again were present in the blank formulations, indicating instability of the mannitol vehicle. While the moisture content of the mannitol-based lyophilized product stored at +5°C remained constant (0.8% ± 0.07% w/w), the moisture content of product kept at +30°C ± 60% RH dropped significantly (0.3% ± 0.06% w/w) after 3 months of storage.

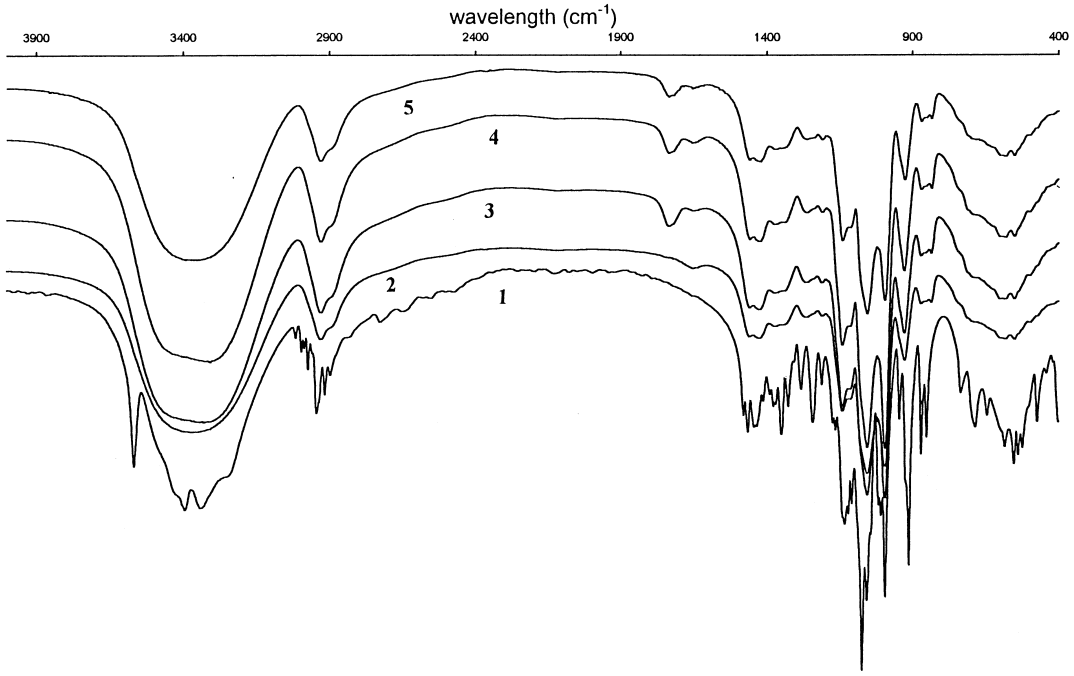

Figure 4. Infrared spectra (4000–400 cm^{-1}) of (1) crystalline sucrose from supplier's bottle; (2) blank lyophilized formulation composed of sucrose 100 mg, 0.1% w/v polysorbate 80, 5 mM citric acid monohydrate; (3) lyophilized kahalalide F (100 μg/vial) formulation; (4), (5) lyophilized kahalalide F (100 μg/vial) formulation after 3 months of storage at +5°C and +30°C ± 60% relative humidity, respectively.

Table 4

Stability of Kahalalide F 100 μg/Vial Lyophilized Product as a Function of Bulking Agent (Mannitol, Crystalline Versus Sucrose, Amorphous) During Storage at +5°C and +30°C ± 60% Relative Humidity (RH) in the Dark (2 Lots/Bulking Agent), Percentage of Initial Values

		1 Month		2 Months		3 Months	
Bulking Agent	Parameter (% ± SD)	+5°C	+30°C ± 60% RH	+5°C	+30°C ± 60% RH	+5°C	+30°C ± 60% RH
Mannitol	Content	102.2 ± 0.3	100.7 ± 0.3	101.9 ± 2.9	98.8 ± 1.6	102.3 ± 4.9	97.6 ± 5.2
	Purity	98.1 ± 0.9	93.5 ± 9.9	98.6 ± 3.3	82.9 ± 8.1	97.2 ± 1.2	85.9 ± 7.2
Sucrose	Content	102.5 ± 1.3	103.7 ± 1.4	99.3 ± 1.8	102.9 ± 3.7	99.3 ± 3.6	99.5 ± 0.6
	Purity	102.6 ± 3.6	101.8 ± 2.4	99.1 ± 0.1	99.9 ± 2.1	100.4 ± 5.3	99.5 ± 5.9

Sucrose-based lyophilized kahalalide F product stored at +5°C showed a slight increase in moisture content (1.1% ± 0.04% w/w), probably due to sorption of moisture from the lyophilization stopper. No significant difference with respect to the initial moisture content was found after 3 months of storage at +30°C ± 60% RH (0.6% ± 0.09% w/w). Fig. 4 shows that no crystallization of the sucrose-based kahalalide F lyophilized formulations occurred during the storage period at both +5°C and +30°C ± 60% RH (19).

In conclusion, kahalalide F formulated in the sucrose-based vehicle is stable for at least 3 months at +5°C and +30°C ± 60% RH in the dark. The

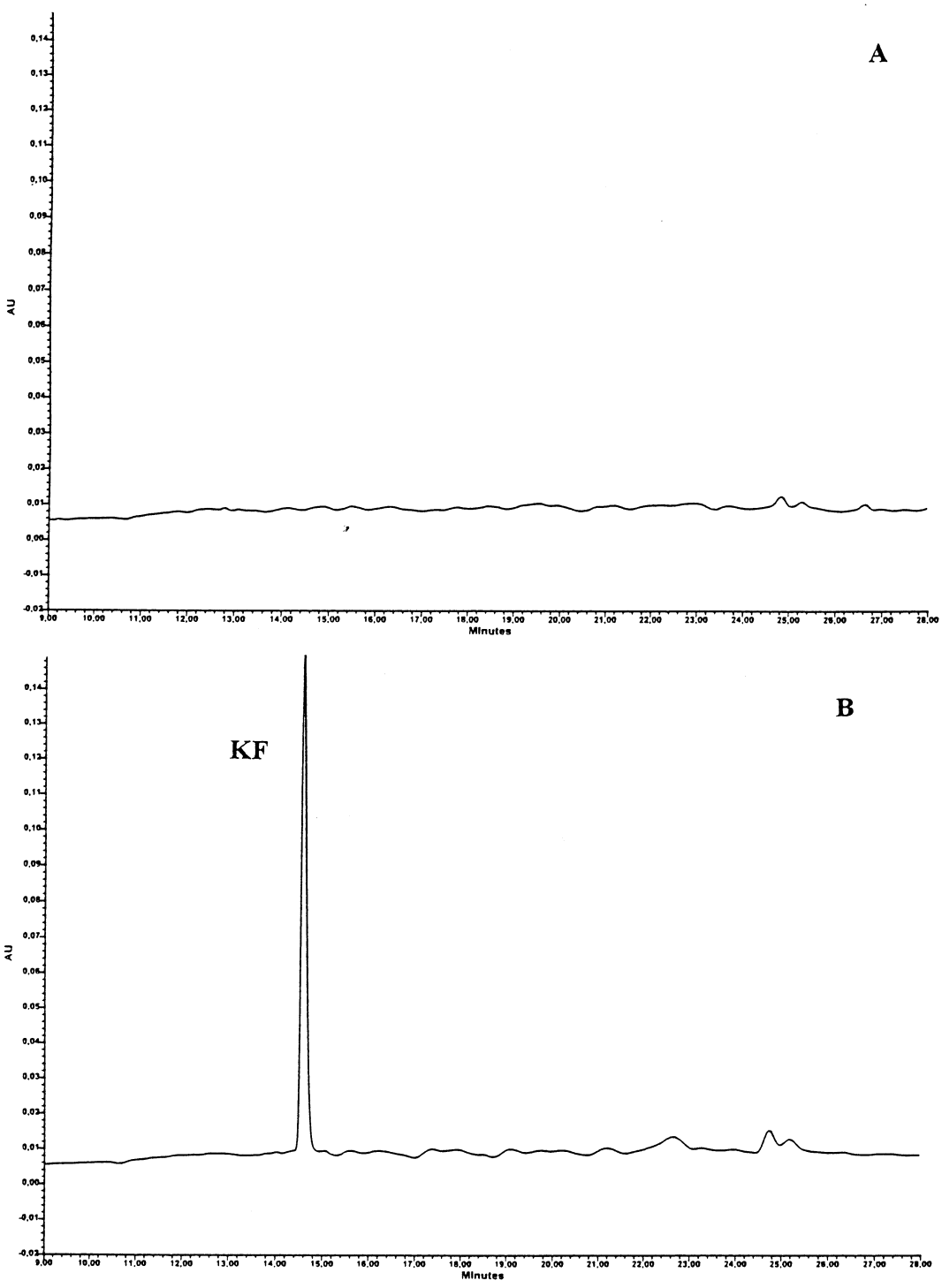

Figure 5. Chromatograms of lyophilized products after 3 months of storage at $+30^{\circ}C \pm 60\%$ relative humidity in the dark: (A) blank sucrose formulation; (B) kahalalide F (100 μg/vial) sucrose formulation (all lyophilized products contained 0.1% w/v polysorbate 80, 5 mM citric acid monohydrate, and 100 mg sucrose; KF is kahalalide F); (C) blank mannitol formulation; (D) kahalalide F (100 μg/vial) mannitol formulation (all lyophilized products contained 0.1% w/v polysorbate 80, 5 mM citric acid monohydrate, and 100 mg mannitol; KF is kahalalide F).

(continued)

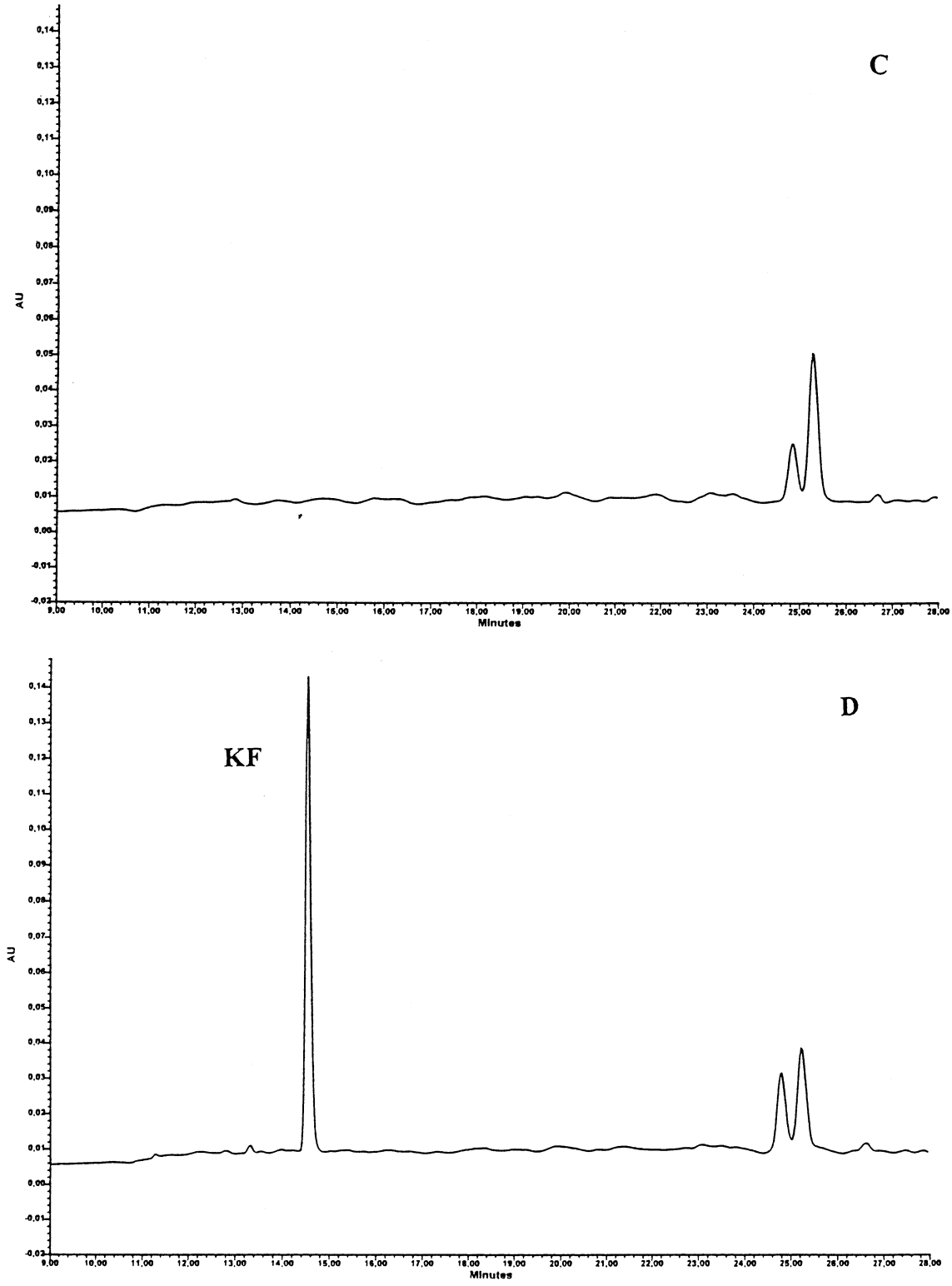

Figure 5. Continued

instability of the mannitol-based vehicle compared to the sucrose-based vehicle is probably related to their different physical states (amorphous versus crystalline).

Selection of Reconstitution Vehicle

Standard practice in the reconstitution of a lyophilized product is to add back a volume of pure water before direct intravenous administration or further dilution in infusion fluid. However, from initial solubility experiments, it was found that loss of kahalalide F occurs on dilution of formulation solution. Polypeptides are known for the occurrence of surface adsorption on reconstitution and subsequent dilution in infusion fluids (10,15). This can be prevented by the addition of a surfactant to the reconstitution vehicle.

We tested the suitability of a reconstitution vehicle composed of Cremophor EL, ethanol, and Water for Injection (CEW) for kahalalide F lyophilized product. The CEW vehicle has previously been used successfully in the solubilization of other drug substances (18). The nonionic surfactant Cremophor EL serves as a wetting agent during the dissolution of the lyophilized cake. Ethanol provides additional sterility assurance for the parenteral product, but above all, improves solubility. Normal saline was used as the diluent after reconstitution. Infusion concentrations of 3 and 1.5 μg/ml were selected on the basis of a starting dosage level of 150 μg and infusion volumes of 50 and 100 ml for a 24-h infusion duration. An infusion concentration of 15 μg/ml was selected as a hypothetical concentration at a maximum dosage level of 1500 μg and 100 ml infusion volume.

From the data presented in Table 5, it can be seen that reconstitution with WfI results in loss of kahalalide F on further dilution with normal saline. Apparently, the concentrations of both polysorbate 80 and citric acid monohydrate drop below a level to keep kahalalide F effectively in solution and prevent sorption. Complete recovery of kahalalide F after reconstitution and subsequent dilution, however, is found with all CEW vehicles tested. Parenteral administration of Cremophor EL is related to the occurrence of anaphylactic reactions (20).

On the basis of the expected maximum dose of 1500 μg in clinical phase I, a maximum dose of 1.5 ml of Cremophor EL will be administered intravenously to the patients using the 5%/5%/90% v/v/v CEW vehicle. In comparison, a 25-fold higher dose of Cremophor EL has been administered intravenously to patients treated with Taxol® (paclitaxel) (21). Based on experiences with similar formulations (22), no problems are expected with the intravenous administration of the 5%/5%/90% v/v/v CEW vehicle.

CONCLUSIONS

In conclusion, the development of a lyophilized parenteral formulation of the experimental marine anticancer agent kahalalide F was presented. A combination of 0.1% w/v polysorbate 80 and 5 mM citric acid monohydrate (pH 2.8) was required to obtain a stable 50 μg/ml aqueous solution of kahalalide F. Sucrose as an amorphous bulking agent, compared to mannitol as a crystalline excipient, resulted in a considerably more stable freeze-dried product. The T_g of the prototype formulation was found to be +46°C. Kahalalide F

Table 5

Recovery of Kahalalide F After Reconstitution and Further Dilution in Normal Saline (Percentage of Nominal Concentration ± SD, n = 3)

Cremophor EL/Ethanol/Water for Injection (WfI) Reconstitution Vehicle Composition (% v/v/v)	Infusion Solution Concentration (μg/ml)		
	15	3	1.5
WfI	93.8 ± 1.6	92.1 ± 2.1	85.8 ± 2.7
5/5/90	99.6 ± 0.5	100.8 ± 2.8	102.6 ± 1.7
10/10/80	100.5 ± 2.8	100.5 ± 4.0	102.9 ± 5.6
15/15/70	101.4 ± 2.2	102.4 ± 1.1	105.7 ± 2.7

100 μg/vial lyophilized product was stable for at least 3 months at +5°C in the dark. A reconstitution vehicle composed of 5%/5%/90% v/v/v Cremophor EL/ethanol/water for injection keeps kahalalide F in solution over the entire infusion concentration range anticipated in early clinical studies. Kahalalide F 100 μg/vial lyophilized product is scheduled for phase I clinical investigation in Europe and the United States by the end of 2001.

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