Characterization of a Solid State Reaction Product from a Lyophilized Formulation of a Cyclic Heptapeptide. A Novel Example of an Excipient-Induced Oxidation

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Purpose. To elucidate the structure of a degradation product arising from a lyophilized formulation of a cyclic heptapeptide, and to provide a mechanism to account for its formation.

Methods. Preparative HPLC was used to isolate the degradate in quantities sufficient for structural studies. A structure assignment was made on the basis of the compounds spectroscopic properties (UV, MS, NMR) and the results of amino acid analysis.

Results. The degradate was identified as a benzaldehyde derivative arising from the oxidative deamination of an aminomethyl phenylalanine moiety. The extent of formation of this product is influenced by the amount of mannitol used as an excipient in the formulation. A mechanism is proposed whereby reducing sugar impurities in mannitol act as an oxidizing agent via the intermediacy of Schiff base adducts which subsequently undergo tautomerization and hydrolysis.

Conclusions. Reducing sugar impurities in mannitol are responsible for the oxidative degradation of the peptide via a mechanism that involves Schiff base intermediates. This mechanism may be a potential route of degradation of other arylmethyl amines in mannitol-based formulations.

KEY WORDS: peptide stability; peptide formulation; oxidation; fibrinogen receptor antagonist; mannitol.

INTRODUCTION

L-367,073, acetylcysteine-asparagine-(5,5-dimethyl-4-thiazolidinecarbonyl)-4-aminomethyl-phenylalanine)-glycine-aspartic acid-cysteine cyclic disulfide, is a potent and specific fibrinogen receptor antagonist with potential utility in the treatment of a variety of cardiovascular conditions (1). Structurally, the compound is based on the known affinity of the GP IIb/IIIa receptor for the RGD (arginine-glycine-aspartic acid) sequence in fibrinogen (2). L-367,073 is a synthetic heptapeptide which is cyclized through a disulfide linkage (Figure 1). The compound lacks significant oral activity and must be administered by the intravenous route. Preformulation studies have demonstrated that the peptide does not possess sufficient solution stability to be formulated as a pre-made solution, and therefore a lyophilized formulation for reconstitution was developed for clinical use. The stability of the drug in the lyophilized formulation was monitored and found to be entirely satisfactory upon storage of the dosage form for up to 14 weeks at 5° and 30°C; however, longer term testing indicated that the drug exhibits instability at temperatures greater than 5°. In particular, storage of the lyophilized formulation for 1 year at 30°C results in the formation of a late eluting (by reverse phase HPLC) degradation product that had not been previously observed in stability studies using the drug as a neat solid or in buffered aqueous solution. In this report, we describe the isolation and identification of this degradate, and propose a mechanism for its formation which involves the pharmaceutical excipient (or more precisely, an impurity present in the excipient) acting as an oxidizing agent.

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MATERIALS AND METHODS

Preparation of Lyophilized Formulations

L-367,073 was obtained in >98% purity from the Department of Process Research, Merck Research Laboratories. Mannitol, USP was obtained from ICI Specialty Chemicals, and conformance to USP specifications was verified by internal testing prior to use. Lyophilized formulations were prepared using a Usifroid Model SMH 101 lyophilizer. For dosage form manufacture, a solution containing the peptide (5 mg/ml) and mannitol USP (20 mg/ml) was adjusted to pH 5.0 with sodium hydroxide and filled in 2.0 ml aliquots into 3 ml molded glass vials. The solutions were frozen at −38°C for 5 hrs, the chamber pressure was adjusted to 30 mTorr, and primary drying was conducted at −30°C for 5 hrs and 0°C for 10 hrs. The shelf temperature was then raised to 30°C at a rate of 6°C/hr, and secondary drying was conducted at 30°C for 10 hrs after which time the vials were stoppered under vacuum (<30 mTorr).

The determination of residual moisture levels in the lyophilized product was determined by Karl Fischer analysis using a Model 447 Coulomatic K-F Titrimeter (Fisher Scientific). Samples were taken up in methanol dried over molecular sieves for the analysis, and the results were corrected for the water content of the sieve-dried methanol as determined in a blank run.

Degradate Isolation

A pure sample of the unknown peptide degradation product was isolated from stressed (60°C/4 weeks) samples of the lyophilized dosage form using preparative scale HPLC. The preparative separation was accomplished on a Waters PrepLC 4000 instrument using a Vydac Prep 218TP column (250 × 22.5 mm) and a mobile phase consisting of 0.1% aqueous trifluoroacetic acid and acetonitrile. Using a linear gradient from 10% to 50% acetonitrile over 30 minutes at a flow rate of 20 ml/min, the intact peptide elutes at 6.5 min and the degradate elutes at 27 min. A sample loading of 25 mg (5 ml × 5 mg/ml) per injection was employed. The degradate fractions from 10 injections were combined, most of the acetonitrile and volatile trifluoroacetic acid was removed on a rotary evaporator, and the remaining solution was lyophilized to provide a white solid that was 97% pure by analytical HPLC.

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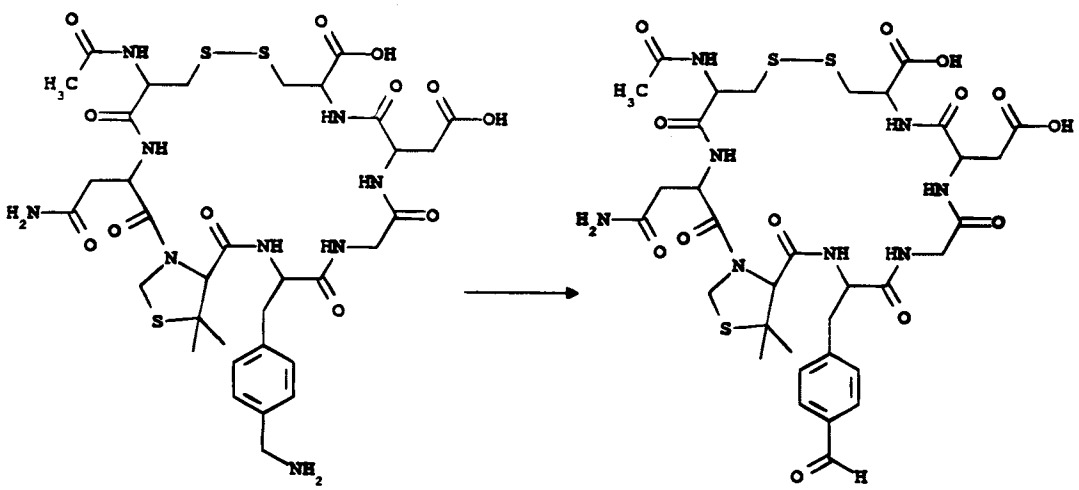

L-367,073

Fig. 1. Structure of L-367,073 and the oxidative degradation product.

Degradate Characterization

^{1}H NMR Spectroscopy

^{1}H NMR spectra were acquired on a Varian Unity-500 NMR spectrometer operating at 499.772 MHz. Samples for NMR measurements contained approximately 0.82 mg of the degradate dissolved in 0.65 ml DMSO-d_6. All spectra were acquired at 25°C and referenced internally to the residual proton of DMSO-d_5 at 2.49 ppm. Proton chemical shift assignments were made with the combined use of 2D TOCSY and NOESY experiments (3,4). All two-dimensional data sets were acquired in the hypercomplex mode (5) for phase-sensitive presentation. The TOCSY experiment was acquired with 1K complex points in t_2 and 512 points in t_1 consisting of 8 transients per increment. Spin-locking was performed with a MLEV-17 mixing sequence (6) for a duration of 50 ms. Data were zero-filled in t_1 to 1K points and multiplied by a Gaussian apodization function in both dimensions prior to Fourier transformation. Baseline correction was performed in F_2 by fitting the baseline to a second order polynomial. A NOESY spectrum was recorded with a mixing time of 500 ms. Solvent suppression was achieved by selective saturation of the residual water resonance during the 1.0 second recycle delay. Data sets consisted of 1K complex points in t_2 and 512 points in t_1 with 64 transients per increment using a relaxation delay of 1.0 seconds. Data sets were zero-filled to 1K points in t_1 and multiplied by a shifted Gaussian apodization function in both dimensions prior to Fourier transformation. ^{1}H chemical shift assignments are shown in Table 1.

Mass Spectrum

The fast atom bombardment mass spectrum of the degradate was obtained on a VG-70E magnetic sector mass spectrometer operating at an accelerating potential of 6 kVolts. The analyte (ca. 1 mg) was dissolved in 50 µl of methanol and 1–2 µl of the solution was mixed with an equal amount of dithiothreotol/dithioerythritol (magic bullet) matrix in methanol. Magnet scans were performed over the mass/charge range of 1200 to 140 at a scan rate of 6 second/decade.

Table I. ^{1}H NMR Chemical Shifts[a] for the Oxidation Product

Residue[b]	NH	H_α	H_β	Other
Cys 1	8.18	4.62	2.76, 2.91	
Asn	8.31	4.86	2.79	7.41, 7.91 (NH_2)
Dmt	—	3.96	—	4.74, 5.15 (H_δ)
				0.57, 1.30 (β-CH_3)
AmF	7.97	4.48	2.92, 3.36	7.48, 7.79 (Ar-H)
Gly	7.52	3.77		
Asp	8.05	4.61	2.57, 2.75	
Cys 2	8.50	4.63	2.88, 3.04	
CH_3CO				1.81
CHO				9.94

[a] Expressed in ppm referenced to internal DMSO-d_5 at 2.49 ppm.
[b] Abbreviations: Cys, cysteine; Asn, asparagine; Dmt, dimethylthioproline; AmF, aminomethyl phenylalanine; Gly, glycine.

UV Spectra

Ultraviolet spectra were recorded on a Perkin Elmer Lambda 6 spectrophotometer. All samples were dissolved in water at a concentration of ca. 15 µg/ml and spectra were recorded from 200 to 400 nm at ambient temperature.

Amino Acid Analysis

Samples of the intact drug or the degradate (ca. 0.5 mg each) were hydrolyzed for 20 hrs in 6 M hydrochloric acid at 110°C, then evaporated to dryness under vacuum. The residues were dissolved in commercial citrate buffer preparation (Beckman NaS diluent) and analyzed on a Beckman 6300 High Performance Amino Acid Analyzer. Results were calculated using response factors determined from authentic samples of the amino acids run just prior to the sample assay.

Stability Studies

Accelerated stability studies were conducted at 40° and 60°C. Solutions containing 5 mg/ml of peptide and either 5, 10, or 20 mg/ml of mannitol were adjusted to pH 5 with concen-

trated sodium hydroxide and filled and lyophilized as previously described. The freeze dried samples were stored in the dark in ovens maintained at the stated temperature to ±1°C. After 12 weeks of storage, the contents of the lyophilized samples were quantitatively transferred to a 10 mL volumetric flask and diluted to the mark with water. Analytical HPLC was performed on a Beckman Ultrasphere C18 column (250 × 2.4 mm, 5 μm) using a mobile phase of acetonitrile vs 0.01 M phosphate buffer (pH 3.5), and a gradient elution profile varying from 10% acetonitrile initially to 35% acetonitrile at 20 minutes was employed. The flow rate was set at 1.5 ml/min and detection was by UV at 260 nm.

Derivatizations with 2,4-Dinitrophenylhydrazine

2,4-Dinitrophenylhydrazine (DNPH) was purchased from Aldrich and was recrystallized from absolute ethanol prior to use. In a typical derivatization reaction, 4 ml of a stock DNPH solution (0.3 mg/ml in acetonitrile) was combined with 0.3 ml of 2 M aqueous hydrochloric acid and 3 ml of a stock solution of mannitol (15–30 mg/ml) in water. The mixture was incubated at ambient room temperature for 90 min, then brought up to a total volume of 10 ml with distilled water and analyzed by HPLC. The HPLC analyses used a Beckman Ultrasphere C18 column, with a mobile phase of 65% 0.01 M hydrochloric acid and 35% acetonitrile at a flow rate of 1 ml/min. Detection was by UV absorbance at 365 nm.

Two additional derivatization reactions were conducted. A blank run utilizing distilled water in place of the mannitol solution served as a control. In addition, a solution of mannose (Sigma Chemical Co., used as received) was derivatized and analyzed as described above. The mannose-DNPH adduct was used to estimate the levels of reducing sugar impurities in mannitol by assuming equal chromatographic response factors at the wavelength used for detection.

RESULTS AND DISCUSSION

Degradate Identification

Storage of the peptide in the lyophilized formulation for extended time periods at 30°C or for shorter time periods at higher temperatures results in the formation of a late-eluting degradation product. Significantly, this degradation product is not observed in stability studies with the peptide as a neat solid or in aqueous buffered solutions. It therefore seems likely that the excipient mannitol which is used in the formulation as a bulking agent is inducing the degradation of the active drug.

The degradation product was isolated by preparative HPLC in quantities sufficient for spectroscopic structure determination. The UV spectrum of the degradate exhibits an absorption maximum at 257 nm; in contrast, the drug has no significant absorbtivity above 230 nm. The shift to longer wavelength is indicative of additional conjugation in the molecule, and tends to rule out many of the commonly observed degradative mechanisms of peptide molecules such as amide bond cleavage/isomerization, disulfide bond reduction, or ester hydrolysis (7–9). These mechanisms are also rendered unlikely by mass spectral data which indicate a reduction in molecular mass of 1 for the degradation product relative to the intact peptide.

Amino acid analyses for aspartic acid (Asp), glycine (Gly), and p-aminomethyl phenylalanine (AmF) proved to be extremely useful in deducing a structure for the degradate. After 20 hr hydrolysis in 6 N hydrochloric acid, analysis of the intact drug gave Asp-2.06, Gly-1.05, AmF-1.02 mmol/mg (note that the asparagine residue appears as Asp in the assay). Under the same hydrolysis conditions, the Asp and Gly content of the degradate was not significantly different (Asp-1.93, Gly-0.94 mmol/mg), but the Amf content was reduced to 0.03 mmol/mg.

Consistent with the amino acid analysis, the ^{1}H-NMR data (Table 1) shows the dissappearance of the benzylic protons on the AmF residue. Of particular note in the spectrum is the appearance of a new resonance at 9.94 ppm which integrates for one proton. This region of the spectrum is generally diagnostic for acidic or aldehydic protons (10). The only structure that is consistent with the UV, mass spectral, and NMR results is the benzaldehyde derivative arising from oxidative deamination of the drug, as shown in Figure 1.

Mechanistic Aspects

The identification of the aldehyde degradate raises the question-how does the oxidation of the drug occur in the solid state in a lyophilized formulation sealed under vacuum? The oxidation occurs at a benzylic methylene carbon, and it is well established that benzylic hydrogens are susceptible to free radical oxidation (11). In such oxidations, however, the oxidizing species is molecular oxygen. Although we cannot completely rule out the possibility that small amounts of residual oxygen were present in the lyophilized samples, a free radical mechanism requiring a radical initiator and a stoichiometric amount of oxygen does not seem likely. Moreover, the oxidative degradate was not observed in stability studies with neat solid drug conducted under an air atmosphere. The implication is that the excipient mannitol is involved in the oxidation.

Of particular relevance to the mechanism of oxidation is the known oxidation of benzylamine to benzaldehyde in acidic aqueous formaldehyde solution (12). This reaction proceeds via an imine intermediate and does not require molecular oxygen. An analogous reaction involving reducing sugar impurities present in commercial mannitol provides a reasonable mechanism for the formation of an aldehyde from the aminomethylphenyl moiety of the drug. It is thus proposed that the oxidative degradate is formed via (1) Schiff base formation between the peptide primary amine and the aldehydic group of a reducing sugar impurity, (2) tautomerization to move the double bond into a more stable configuration in which it is conjugated with the phenyl group, and (3) hydrolytic cleavage of the new Schiff base to generate the observed degradation product. The sequence is illustrated in Figure 2.

The obvious requirement for this mechanism to be correct is the presence of reducing sugar impurities in the mannitol. The mannitol used in the lyophilized formulations conformed to pharmacopeal requirements; however, the USP test for reducing sugars gives only qualitative information (13). Therefore, the reducing sugar impurity level was determined chromatographically after conversion to the phenylhydrazone derivatives (14). The results show that phenylhydrazine reactive impurities (assumed to be sugar aldehyde groups) are present at approximately 0.1% w/w in the mannitol sample. This is a sufficient quantity to react with 2% of the amount of peptide present in the lyophilized formulation based on the relative amounts of peptide and mannitol and the molecular weights of 869 for the

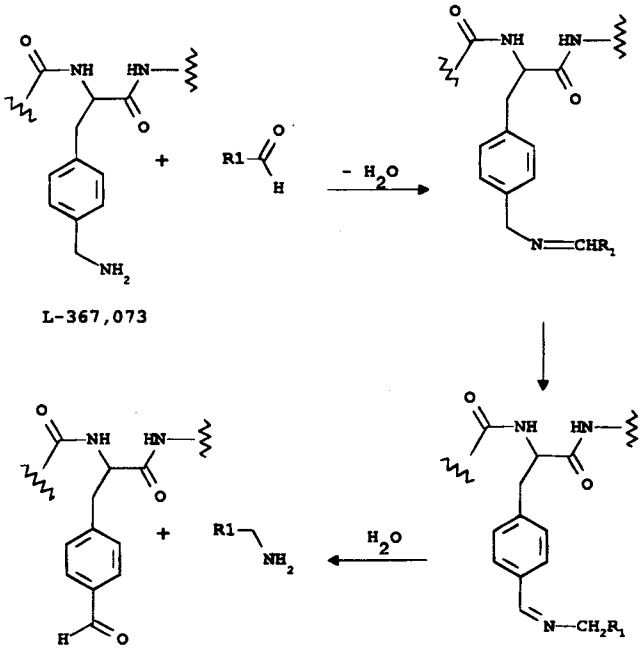

Fig. 2. Proposed route of formation of the oxidative degradation product.

peptide and 180 for a monosaccharide. Since we have not observed the degradate to form to an extent greater than this on a weight basis, we conclude that the mechanism cannot be ruled out based on mass balance.

Further support for the postulated mechanism is provided by the results of accelerated stability studies which were conducted during dosage form optimization. In these studies, the drug content of the formulation was held constant (10 mg per vial) and the amount of mannitol was varied between 10 and 40 mg/vial. Stability data were obtained after 12 weeks storage in constant temperature ovens at 40° and 60°C, and these results are shown in Table 2. The data show that the extent of oxidative degradation increases with increasing mannitol content at both temperatures studied. Moisture analyses on the freshly prepared lyophilized samples did not reveal any significant differences in water content (range 0.9 to 1.2% water), thereby eliminating varying water content as an explanation for the trends in the stability data.

The role of excipient impurities in promoting drug oxidation reactions has been described in the literature (15); however, previous studies have generally implicated free radical precursors as the initiators of oxidation. In contrast, the mechanism described herein does not rely on the involvement of radical or radical-precursor species, but does depend on the intermediacy of a Schiff base. In this respect, our mechanism is similar to that recently described by Stella et al. (16) in which the degradation of benzylguanine in aqueous polyethylene glycol solution was ascribed to the presence of formaldehyde in the organic solvent.

In summary, degradation of a cyclic peptide drug in a lyophilized formulation occurs to give an aldehyde as an oxidation product. The oxidative pathway does not require oxygen, but does depend on the presence of impurities in the pharmaceutical excipient used in the formulation. A mechanism involving Schiff base formation, double bond isomerization, and subsequent hydrolysis has been proposed to account for the formation of the degradate. It is remarkable that this series of reactions occurs within a lyophilized solid, and further studies will hopefully reveal additional details of the various reaction steps. Since mannitol finds widespread use in lyophilized pharmaceutical dosage forms, we mention that other compounds containing an arylmethylamine moiety may react in a fashion analogous to that described here.

Table II. Stability Data for Lyophilized L-367,073[a] (Expressed as Weight Percent of Oxidative Degradate) as a Function of Mannitol Level After 12 Weeks Storage

	Wt % Degradate	
mg Mannitol/vial	40°C	60°C
10	0.13	0.36
20	0.37	0.86
40	0.58	1.30

[a] Each vial contained 10 mg of peptide and was adjusted to pH 5 prior to lyophilization.

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