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International Journal of Pharmaceutics 191 (1999) 115–129

international journal of pharmaceutics

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Effect of peptide concentration and temperature on leuprolide stability in dimethyl sulfoxide

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Received 2 March 1999; received in revised form 17 June 1999; accepted 8 August 1999

Abstract

The effects of temperature and concentration on leuprolide degradation in dimethyl sulfoxide (DMSO) were explored. Leuprolide degradation products were analyzed by reverse phase high-performance liquid chromatography (RP–HPLC), size exclusion chromatography (SEC) and structurally characterized by mass spectrometry. Leuprolide solution stability in DMSO was characterized at 50, 100, 200, 400 mg/ml at 37–80°C for 2 months to 3 years. Leuprolide degradation products were identified by mass spectrometry and could generally be attributed to isomerization, hydrolysis, oxidation, or aggregation. The hydrolytic degradation products consisted primarily of backbone cleavage C-terminal to Trp^3, Ser^4, Tyr^5, Leu^6 and Leu^7, and oxidation of Trp^3 and β-elimination of Ser^4 were identified. Leuprolide degradation at 50°C, 65°C and 80°C proceeded in an exponential fashion ($E_a = 22.6 \pm 1.2$ kcal/mol); however, leuprolide degradation plateau'd after approximately 6 months at 37°C. Upon closer examination, degradation product peak areas were seen to vary with temperature. For example, aggregation products did not increase with time at 37°C, but aggregation peak intensities increased sharply with time at 80°C. Increasing the temperature also increased the proportion of leuprolide degrading via isomerization/hydrolytic pathways, and decreased the proportion degrading via oxidation. These variations suggested that solvent dielectric, free H^+ in an aprotic solvent, oxygen solubility, impurities and residual moisture may play a role. Leuprolide solubilized in DMSO yields adequate stabililty for a 1 year implantable osmotic delivery system, where use of a dry aprotic solvent results in conditions similar to solid state stability.

Keywords: Hydrolysis; Leuprolide acetate; Mass spectrometry; Oxidation; RP–HPLC

1. Introduction

Leuprolide (pGlu-His-Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt) is a potent luteinizing hormone releasing hormone (LHRH) agonist that stimulates the release of luteinizing hormone. Leuprolide has been used successfully for the palliative treatment of prostate cancer by saturating and downregulating pituitary receptors, resulting in the suppression of testosterone production (Nestor et al., 1982; Ogawa et al., 1988). Historically, LHRH agonists have been formulated in

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0378-5173/99/$ - see front matter
PII: S0378-5173(99)00289-6

controlled release microspheres administered as a depot injection lasting up to 3 months (Adjei and Hsu, 1993; Okada et al., 1994). Ideally, therapeutic effects could be maximized by long term continuous dosage of leuprolide with zero order release kinetics. With the advent of novel implantable osmotic delivery systems, continuous zero order release of leuprolide for 1 year is possible (Dionne et al., 1996).

The DUROS® implant is 4 mm in diameter by 45 mm in length and has a drug reservoir of ~150 μl, requiring formulation at high peptide concentrations in order to deliver a therapeutic dose for 1 year (Dionne et al., 1996). The peptide must also remain stable at physiological temperatures for the lifetime of the implant. Aqueous formulations of leuprolide did not provide the required chemical and physical stability (Hall et al., 1999). The incorporation of dimethyl sulfoxide (DMSO) into the formulation alleviated gelation and provided improved stability (Tan et al., 1996). Considering the extended 1 year duration of the delivery system, accelerated stability studies were crucial during formulation development. Temperature accelerated studies were utilized to estimate a 2 year shelf life plus a 1 year implant life; however estimations were not without difficulties. The objectives of this study were to provide acceptable stability at 37°C for the life of the implant, characterize the leuprolide degradation products and pathways in DMSO by mass spectrometry (MS), and propose a rationale for the change in degradation products with increasing temperature and leuprolide concentration.

2. Materials and methods

2.1. Stability studies

Solutions of leuprolide acetate (Mallinckrodt, St Louis, MO) were prepared in neat DMSO (Fisher, Pittsburgh, PA) at 50–400 mg/ml. Peptide solution formulation was dispensed into DUROS™ implant titanium alloy reservoirs (20–150 μl) and sealed with polymeric piston material. The sealed implants were placed into polyfoil bags and heat sealed. The polyfoil bags provided a moisture barrier, so that formulation water content was controlled and remained uniform over all leuprolide concentrations and storage conditions tested (1–3% water content by Karl Fischer). Sterile filling procedures were not utilized due to the bacteriocidal and bacteriostatic characteristics of DMSO and leuprolide. In addition, proteolytic products of leuprolide under aqueous conditions were characterized and not observed in these stability studies. Samples were stored at −80°C, 4°C, 37°C, 50°C, 65°C and 80°C for up to 3 years.

Samples were prepared for analysis by dissolution in water at approximately 600 μg/ml followed by reverse phase high-performance liquid chromatography (RP–HPLC) and size exclusion chromatography (SEC). The leuprolide area% from the resulting RP–HPLC chromatograms were normalized to −80°C controls. Evaluation of the relative growth of various degradation products was calculated from peak area% data.

2.2. Moisture content

Moisture content was monitored at varying intervals on stability, and was determined by Karl Fischer (KF) analysis using a Serradyne Aquatest 10 (Indianapolis, IN) moisture analyzer. Formulation was transferred from DUROS™ implants into pre-weighed dried vials and septum sealed. An aliquot of anhydrous methanol (~40 mg) was added to each vial. Samples were mixed, and aliquots were assayed for water content against an anhydrous methanol control. Moisture content results were routinely 1–3%.

2.3. Reverse phase HPLC

Leuprolide degradation products from stability samples were separated using a binary gradient HPLC system (Waters, Milford, MA) equipped with a HaiSil C-18 4.6 × 250 mm column (Higgins Analytical, Mountain View, CA) and UV photodiode array detection at 215 nm. Mobile phase A consisted of acetonitrile–100 mM sodium phosphate (5:95), pH 3.0, and mobile phase B was acetonitrile–water (95:5). The column was equilibrated at 10% B with a flow rate of 0.8 ml/min. A step gradient consisting of an isocratic hold at

10% B for 5 min, 10–21% B in 5 min, isocratic at 21% B for 20 min, 21–60% B in 15 min, 60–80% B in 1 min, and an isocratic hold at 80% B for 5 min.

Fractions were collected for mass spectrometry at 3 min intervals using the same methodology, with the exception that mobile phase A consisted of acetonitrile–20 mM sodium phosphate (5:95).

2.4. Mass spectrometry

Selected fractions were concentrated on a Speed Vac (Savant Instruments, Holbrook, NY) to a volume of 30 μl. Fractions (5–25 μl) were desalted on a C18 1.0 × 150 mm, 5 μm particle size, 300 Å column (Vydac, Hesperia, CA). Mobile phase was pumped (Hewlett Packard, Palo Alto, CA) at a flow rate of 50 μl/min with UV detection at 215 nm. Mobile phase A consisted of 2% acetonitrile–98% water–0.1% tetrahydrofuran (TFA) and mobile phase B was 90% acetonitrile–10% water–0.08% TFA. After sample injection, an isocratic hold at 0% B for 10 min was followed by a linear gradient from 0–63% B in 40 min. The outlet of the UV detector was connected to a 9:1 splitting tee, such that the flow rate introduced into the electrospray mass spectrometer (Perkin Elmer Sciex, API 300, Foster City, CA) was 5 μl/min. Typical operating voltages were: probe tip 4000 V, orifice plate 30 V and focusing ring 400 V. For molecular weight determinations, Q1 was scanned in 0.125 a.m.u. steps over the range m/z 70–1000 with a dwell time of 0.5 ms/step or m/z 400–1500 with a dwell time of 0.7 ms/step, resulting in scan times of 4.5 and 6.2 s, respectively. Resolution was set so that FWHM (full width at half maximum peak height) = 0.5 Da.

Collision induced dissociation (CID) spectra were generated using nitrogen as the collision gas, and the collision energy varied between 30 and 70 eV depending upon the precursor ion mass. Precursor ions were chosen from the most abundant of the singly or doubly charged state of a given peptide. Q1 and Q3 were set to unit resolution. In order to acquire the best quality CID spectra, Q3 scan rates for filtering fragment ions were varied according to the m/z value of the precursor ion. Unless otherwise stated, all quoted masses are measured monoisotopic values, including standard nomenclature for peptide fragment ions (Biemann, 1990).

2.5. Size exclusion chromatography

Leuprolide aggregation was assayed using a Pharmacia HR 10/30 10 × 100 mm column (Pharmacia Biotech, Piscataway, NJ). The mobile phase was 70% 100 mM ammonium phosphate, 200 mM sodium chloride, pH 2.0–30% acetonitrile. The flow rate was 0.5 ml/min, and leuprolide was detected at 215 nm. Peptide molecular weight standards (Sigma, St Louis, MO) were chosen for having pI values relatively close to leuprolide (pI 10.5), and included cytochrome c, growth releasing factor, angiotensin, Trp-Leu-Arg-Phe and bursin.

3. Results

3.1. Leuprolide degradation

Leuprolide saturation solubility in DMSO, at 25°C, is approximately 580 mg/ml free base, by RP–HPLC. Leuprolide does not precipitate out of solution at high concentrations, but forms a highly viscous solution into which no additional leuprolide powder can be incorporated. Leuprolide solubility was determined to be unaffected by decreasing temperature (-20°C to 50°C). Leuprolide solubility decrease slightly (10–15%) with increasing moisture (0–15% H_2O) and with increasing acetate (10–70 mg/ml ammonium acetate). However, these solubility effects were minor ($< 10\%$) when applied to leuprolide solutions of less than 400 mg/ml.

The effect of leuprolide concentration on stability was explored in order to afford a potent dose from an implantable system over a 1 year period of time. Leuprolide was solubilized at 50, 100, 200 and 400 mg/ml in neat DMSO and placed on accelerated stability for 2 months at 80°C (Fig. 1). Leuprolide demonstrated increased stability with increasing concentration, where 73% and 80% leuprolide remained at 50 and 400 mg/ml peptide, respectively. This indicated that leuprolide may be

self-stabilizing in DMSO and a good candidate for a miniature implantable osmotic delivery system.

The peptide concentration was fixed at 370 mg/ml leuprolide in neat DMSO. The formulation was placed on stability at −80°C, 37°C, 50°C, 65°C and 80°C for up to 3 years. Stability samples were characterized by RP–HPLC with good mass balance over the length of the study. For example, 100% of the peptide material could be accounted for after 3 years at 37°C. Mass balance was observed to decrease with increasing temperature, where only 99%, 88% and 91% could be accounted for after 2 years at 50°C, 2 years at 65°C and 6 months at 80°C, respectively. Given the identity of some of the degradation products, a decrease in the response factor was expected.

Peptide stability after 2 years was $94.6 \pm 0.2\%$, $85.4 \pm 1.2\%$ and $58.6 \pm 0.8\%$ at 37°C, 50°C and 65°C, respectively. Leuprolide stability at 37°C demonstrated $94.1 \pm 2.7\%$, $94.6 \pm 0.2\%$ and $93.7 \pm 0.6\%$ leuprolide remaining after 1, 2 and 3 years, respectively (Fig. 2). The RP–HPLC data were fit to pseudo first order kinetics, where the rate constants for overall loss of leuprolide were 1.10×10^{-3}, 6.35×10^{-3}, 2.18×10^{-2} and 1.08×10^{-1} $(\text{months})^{-1}$ for 37°C, 50°C, 65°C and 80°C, respectively (Fig. 3a). The initial rates of leuprolide degradation were then plotted versus the inverse of the absolute temperature (Fig. 3b). The Arrhenius plot appeared linear, where $E_a = 22.6 \pm 1.2$ kcal/mol with 95% confidence limits. Therefore, the estimated shelf stability (25°C) and physiologic stability (37°C) data appeared adequate for a 1 year implantable system.

Interestingly, the temperature dependence of leuprolide (370 mg/ml) degradation in DMSO did not always follow a smooth exponential decay. For example, leuprolide degradation at 50°C, 65°C and 80°C demonstrated accelerated exponential decay with time and temperature (Fig. 3a); however, the rate of degradation at 37°C appeared to plateau after the first 6 months (Fig. 2). This change in rate may be attributed to the consumption of residual water, other reactive substrates, the kinetics relating to the specific degradation pathway and changes in the major degradation pathway with temperature. Therefore, it should be stressed that the pseudo first order fit of the data is only an estimate.

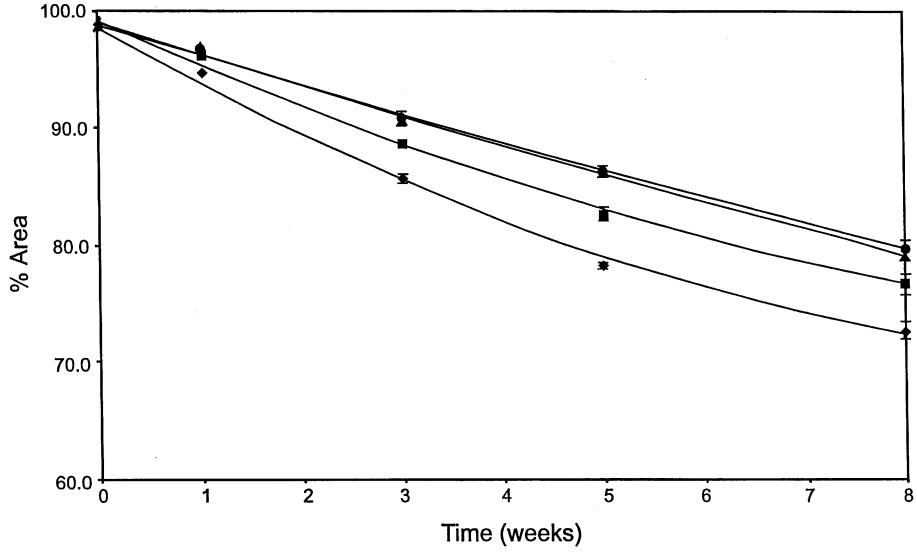

Fig. 1. Effect of concentration on leuprolide stability in DMSO, where 50 (◆),100 (■), 200 (▲) and 400 (●) mg/ml leuprolide at 80°C for 2 months ($n = 3$).

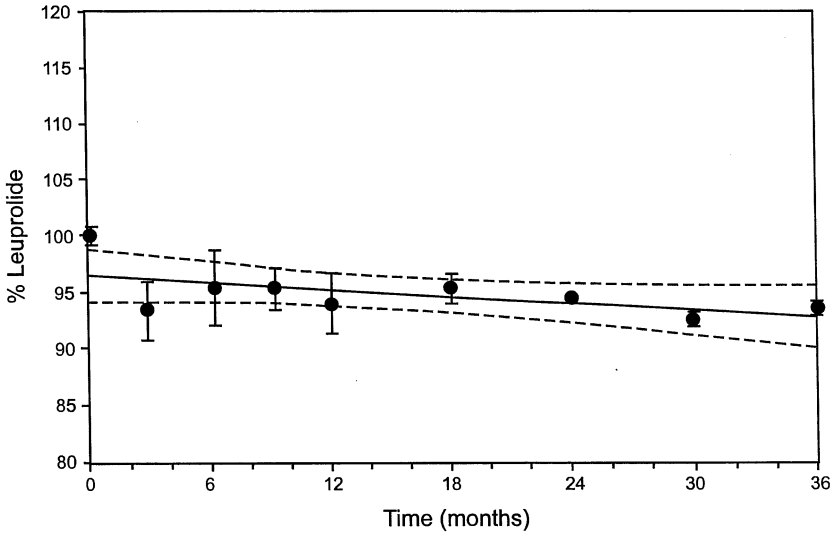

Fig. 2. Degradation of 370 mg/ml leuprolide in DMSO at 37°C for 3 years. The curved dotted lines denote 95% confidence intervals.

3.2. Degradation product identification

Leuprolide degradation products in DMSO were characterized by mass spectrometry and associated with their respective elution times for 370 mg/ml leuprolide in DMSO held at 80°C for 6 months (Fig. 4). The chromatogram represents a characteristic degradation profile of leuprolide in DMSO, where degradation products corresponding to hydrolysis, oxidation and isomerization were generally observed to elute prior to leuprolide (27.5 min). No additional peaks were observed at lower temperatures or at later time points (i.e. 2 years at 37°C).

Identification of the series of aggregates that eluted after leuprolide (33–40 min) was not pursued in these studies. However, the extent of irreversible aggregation was characterized by RP–HPLC and SEC. SEC indicated formation of irreversible aggregates two to three times larger than monomeric leuprolide; however, no larger aggregates were observed. No visible aggregation or precipitation was noted. Preliminary examination by mass spectrometry indicated that aggregates two times larger than leuprolide were not simply dimerized leuprolide. These aggregates could be partially resolved by RP–HPLC. Collection of the aggregate peaks from SEC and reinjec-

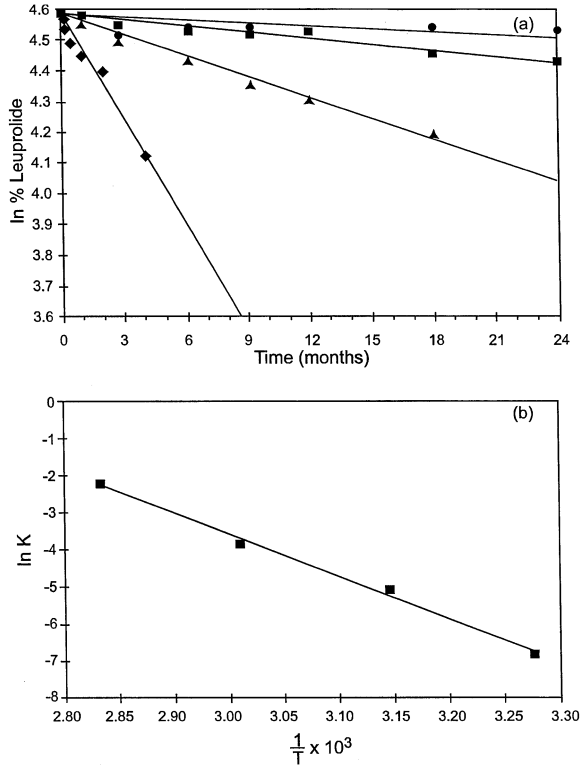

Fig. 3. Degradation of 370 mg/ml leuprolide in DMSO as a function of temperature illustrating: (a) pseudo first order fit, at 37°C (●), 50°C (■), 65°C (▲) and 80°C (◆); (b) resultant Arrhenius plot.

tion on RP–HPLC indicated that the retention times of the aggregates corresponded to the series of peaks eluting between 33 and 40 min. These multiple peak areas observed by RP–HPLC were summed and compared to the total peak area obtained for dimer and trimer, by SEC, with good agreement.

3.3. Isomerization

Two degradation products (22.5 and 24.0 min) were determined to have identical mass (MH^+ = 1209.8) and amino acid sequence (pGlu-His-Trp-Ser-Tyr-Leu-Leu-Arg-Pro-NHEt) and thus were considered to be isomers of leuprolide. The considerable peak widths produced by these two species suggested that several chromatographically unresolved leuprolide isomers were present (Fig. 4). Preliminary identification of racemized D and L amino acids comprising leuprolide was confirmed on a chiral HPLC column (data not shown). These isomers were only detected in stability samples and not in the leuprolide raw material. The identity of these isomers was not pursued; however, others have identified His^2, Trp^3 and Ser^4 as susceptible to isomerization (Motto et al., 1991; Okada et al., 1991; Oyler et al., 1991).

3.4. Hydrolysis

Several hydrolysis products were identified to be characteristic of peptide backbone cleavage C-terminal to Trp^3, Ser^4, Tyr^5, Leu^6 and Leu^7 residues resulting in Ser-Tyr-Leu-Leu-Arg-Pro-NHEt ($MH^+ = 775.4$), Tyr-Leu-Leu-Arg-Pro-NHEt ($MH^+ = 688.6$), Leu-Leu-Arg-Pro-NHEt ($MH^+ = 525.4$), and Leu-Arg-Pro-NHEt ($MH^+ = 412.4$) (Fig. 4). For example, a complete *y* ion series for Ser-Tyr-Leu-Leu-Arg-Pro-NHEt (*m*/*z* 143.2, 299.2, 412.4, 525.4, 775.4) produced by backbone cleavage of the amide bond confirmed the sequence of this peptide (Fig. 5a). Additional support was provided by the presence of the corresponding *b* ion series, excluding b_1.

The predominant hydrolytic cleavage occurred between Trp^3 and Ser^4, where pGlu-His-Trp-OH ($MH^+ = 453.2$) and pGlu-His-Trp-NH_2 (MH^+ = 452.2) produced the greatest peak heights relative to the other hydrolysis products. In addition, formation of pGlu-His-Trp-NH_2 was highly temperature dependent, where the peak height ratios of the amidated peptide formed at 80°C, to that formed at 65°C and 50°C, averaged 14:1.

Hydrolytic degradation products of LHRH analogs form readily in buffered aqueous solutions at accelerated temperatures and have been

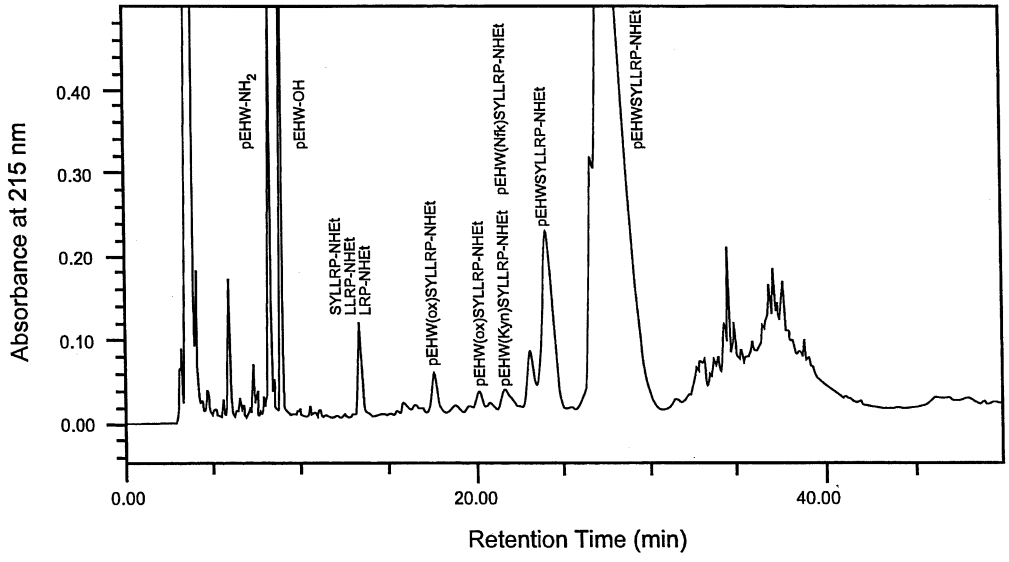

Fig. 4. Representative LC chromatogram of leuprolide degraded at 80°C for 6 months with identification of degradation products.

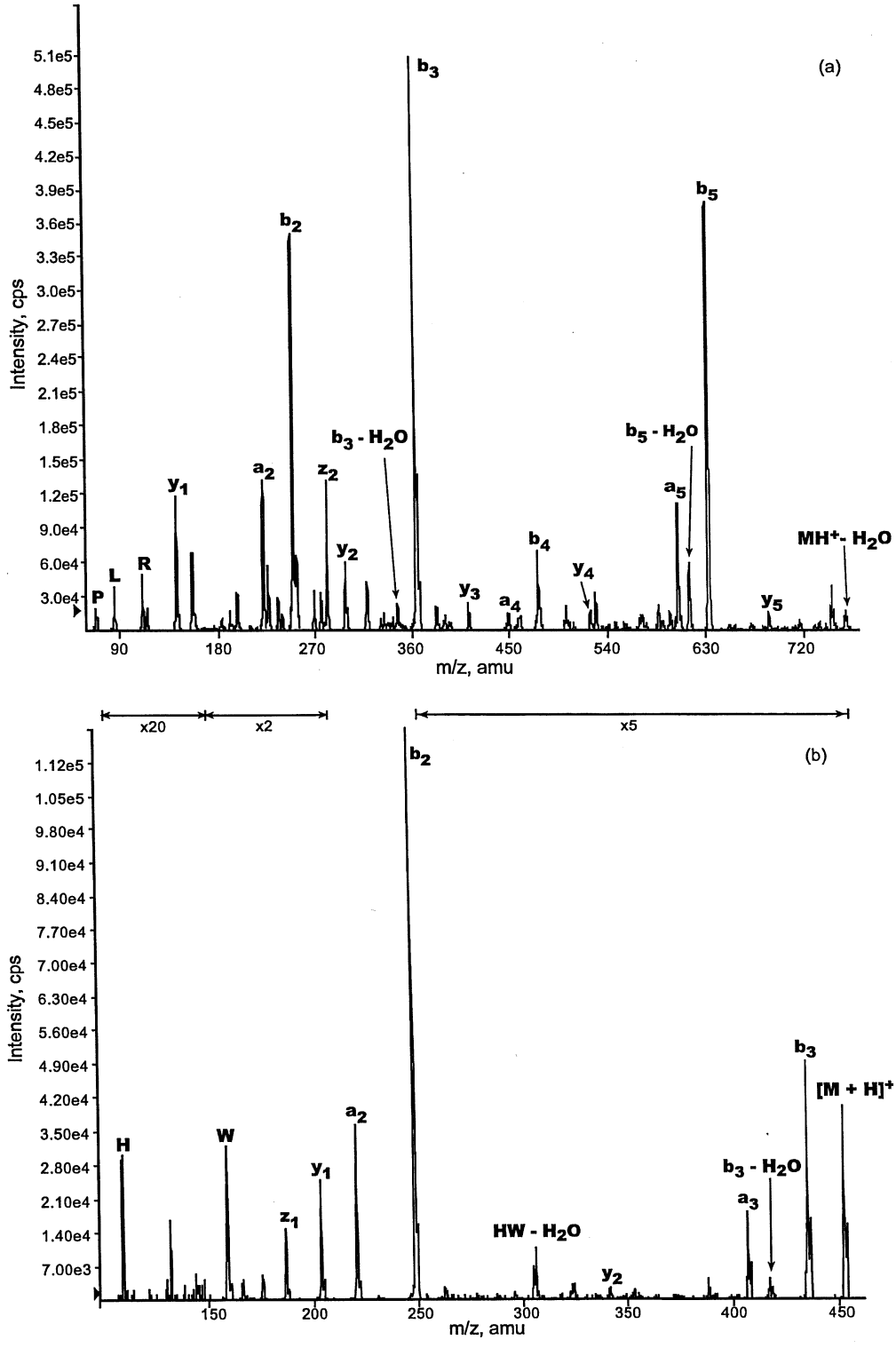

Fig. 5. MS/MS spectra of leuprolide hydrolytic products formed in DMSO: (a) Ser-Tyr-Leu-Leu-Arg-Pro-NHEt ($MH^+ = 775.4$); (b) pGlu-His-Trp-NH_2 ($MH^+ = 452.2$).

well documented (Johnson et al., 1986; Helm and Muller, 1990; Strickley et al., 1990; Motto et al., 1991; Okada et al., 1991; Oyler et al., 1991; Powell et al., 1991; Adjei and Hsu, 1993). Typical peptide backbone cleavage products characterized from aqueous formulations include: pGlu-His-Trp-OH, His-Trp-OH for fertirelin (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-NHEt) (Okada et al., 1991); His-Trp-Ser-Tyr-(D-N^{im}-bzl-His)-Leu-Arg-Pro-NHEt, Ser-Tyr-(D-N^{im}-bzl-His)-Leu-Arg-Pro-NHEt for histrelin (pGlu-His-Try-Ser-Tyr-(D-N^{im}-bzl-His)-Leu-Arg-Pro-NHEt) (Oyler et al., 1991); and His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, pGlu-His-Trp-OH, for LHRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) (Motto et al., 1991).

In addition to these direct cleavage products, researchers also reported identification of cyclo(His-Trp) ($MH^+ = 324.1$) (Motto et al., 1991; Okada et al., 1991; Oyler et al., 1991). However, cyclo(His-Trp), a diketopiperazine, was not detected in any of the leuprolide solutions formulated in DMSO. Evidence suggests that the decomposition of larger peptides, at elevated temperatures, occurs by N-terminal aminolysis, followed by reversible cyclization of dipeptides (Steinberg and Bada, 1981, 1983). Thus, it is conceivable that cyclo(His-Trp) could form by N-terminal hydrolysis of pGlu, from pGlu-His-Trp-OH, followed by cyclization of His-Trp-OH. Cyclo(His-Trp) was not detected, implying that further hydrolysis of primary degradation products was limited, probably due to the limited source of hydronium ions.

3.5. β-Elimination

Formation of pGlu-His-Trp-NH_2 ($MH^+ = 452.2$) (Fig. 5b) suggested a degradation route other than simple backbone cleavage. If cleavage of the amide bond had occurred, then a fragment containing dehydroalanine (Dha), Dha-Tyr-Leu-Leu-Arg-Pro-NHEt ($MH^+ = 757.5$), would have formed as the C-terminal fragment. A peptide having $m/z = 757.5$ was not observed in any of the degradation product fractions analyzed. Therefore, the amidated tripeptide was most likely formed from β-elimination of Ser, followed by hydrolytic cleavage between the α-carbon and amide nitrogen of the resulting dehydroalanine residue to yield the corresponding C-terminal fragment HO-Dha-Tyr-Leu-Leu-Arg-Pro-NHEt ($MH^+ = 758.5$) (Fig. 6). This mechanism was supported by the presence of a peptide with $m/z = 758.6$, eluting at approximately 33 min.

β-Elimination of Ser and Cys residues usually occurs under alkaline conditions and is accelerated at high temperatures (Nashef et al., 1977; Manning et al., 1989; Schrier et al., 1993). However, little data can be found regarding the occurrence of β-elimination in aprotic solvents.

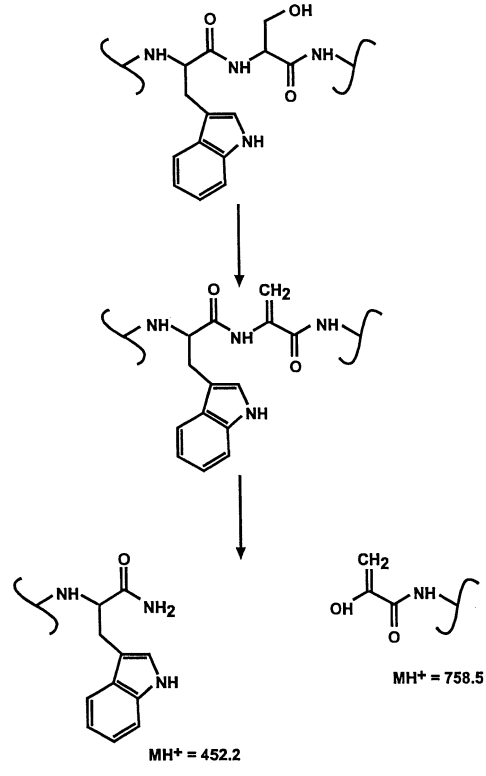

Fig. 6. β-Elimination of serine and backbone cleavage of leuprolide to yield an amidated tripeptide.

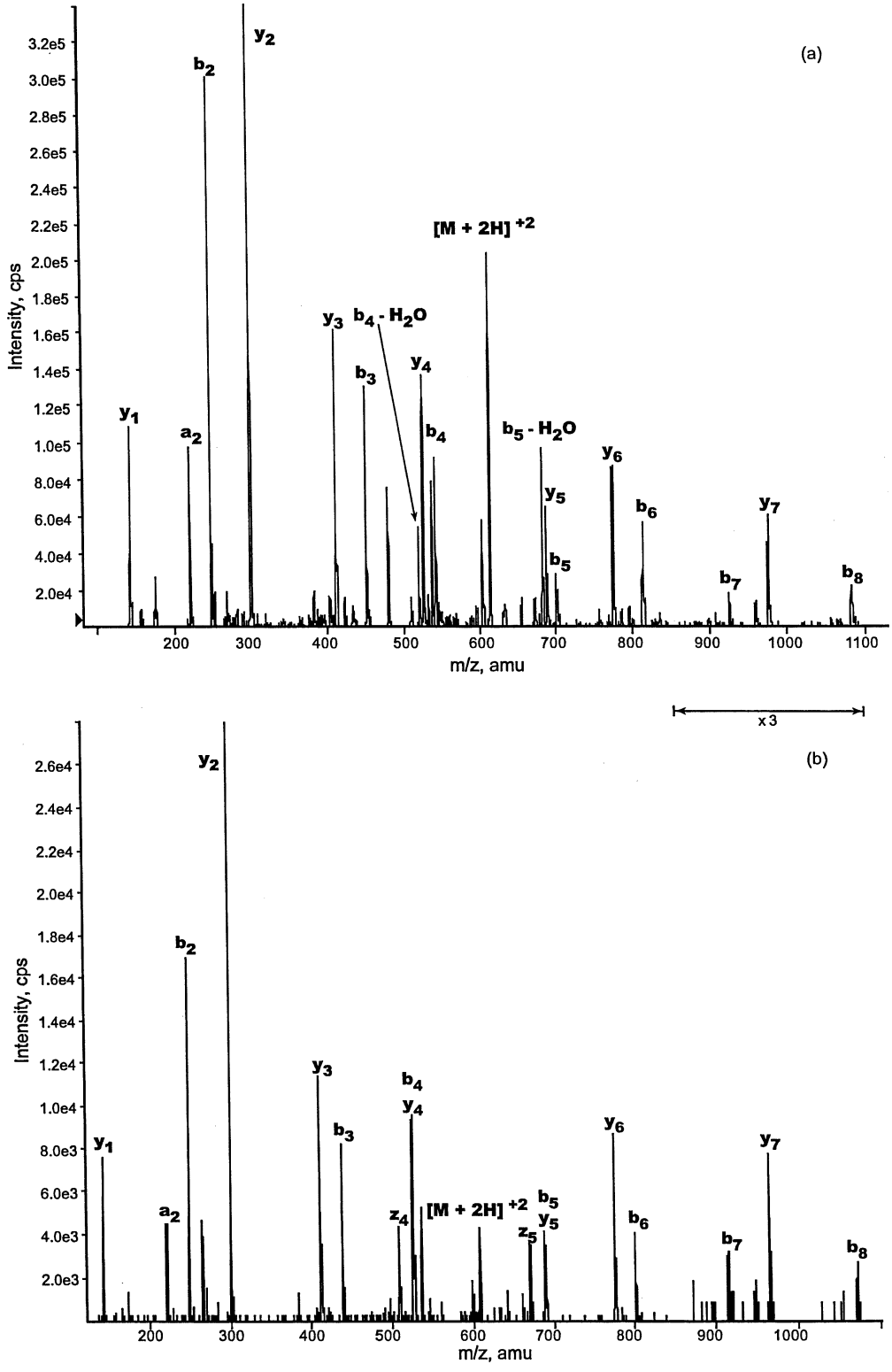

Fig. 7. MS/MS spectra of leuprolide oxidation products formed in DMSO, where tryptophan oxidation produced: (a) mono-oxidized leuprolide ($MH^+ = 1225.7$); (b) *N*-formylkynurenine ($MH^+ = 1213.8$).

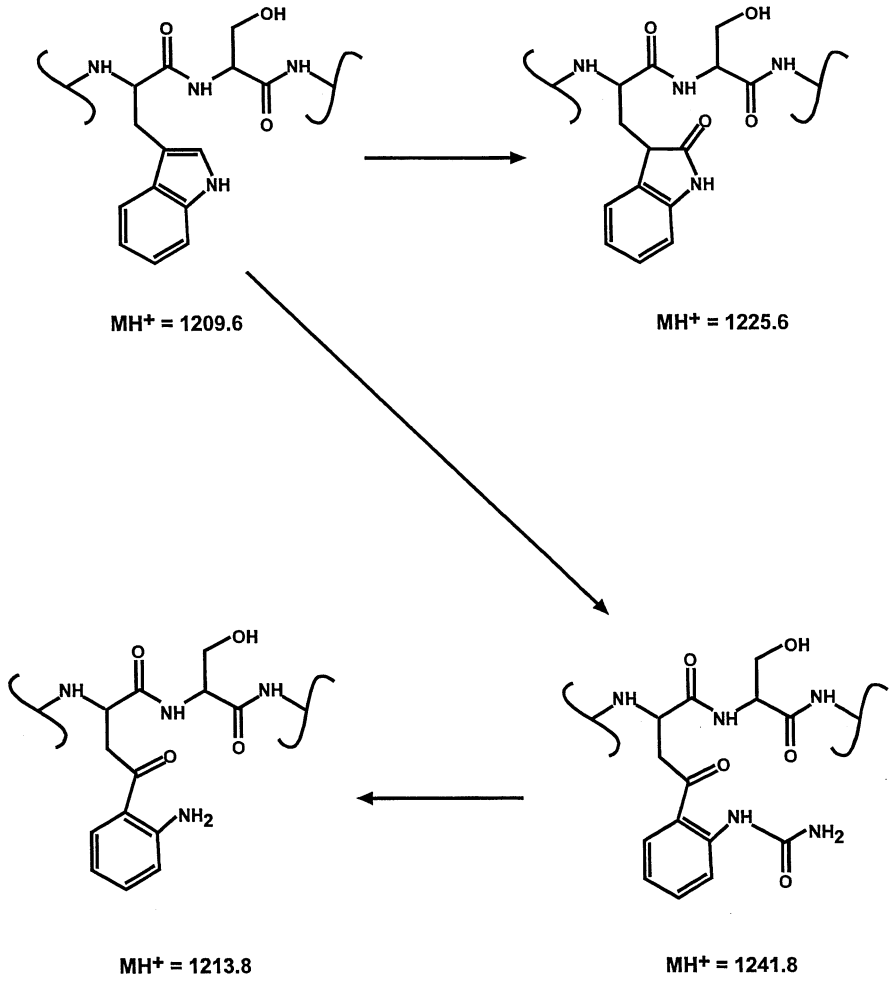

Fig. 8. Oxidation products of tryptophan in leuprolide.

3.6. Oxidation

Oxidative degradation of leuprolide formulated in DMSO yielded three products. One of these degradation products had m/z 1225.7, which was 16.1 Da greater than leuprolide ($MH^+ = 1209.6$) and eluted as chromatographically resolved isomers at retention times of 17.5 and 19.5 min. Interpretation of the collision induced dissociation (CID) spectra revealed the sequence to be mono-oxidized leuprolide, pGlu-His-Trp(ox)-Ser-Tyr-Leu-Leu-Arg-Pro-NHEt (Fig. 7a). Abundant y and b ions were observed in the CID spectrum due to the presence of His^2 and Arg^8 located near the N- and C-terminus, respectively. Covalent modification was unambiguously identified to be localized on Trp^3, since the mass difference between the y_7 and y_6 (977.5–775.7 = 201.8) C-terminal fragment ions corresponded to the residue mass of tryptophan (186.1), plus an oxygen atom (15.7). Concurrently, the same mass difference relationship existed between the corresponding b_2 and b_3 N-terminal fragment ions, providing further validation that the Trp side chain had been oxidized. A search of the literature on LHRH analogs and Trp oxidation did not reveal a peptide structure containing a mono-oxidized tryptophan. We propose that the oxygen atom was

added to the indole ring by a hydroxyl radical, possibly present as a contaminant in the DMSO (Fig. 8). However, further investigation is needed to confirm this hypothesis.

In a similar fashion, two additional tryptophan oxidation products were identified, with $m/z =$ 1241.8 and $m/z = 1213.8$. In both cases, oxidation of the indole ring by singlet oxygen, via a dioxetane intermediate, yielding *N*-formylkynurenine (Nfk) ($MH^+ = 1241.6$) and kynurenine (Kyn) ($MH^+ = 1213.7$) was postulated (Fig. 8) (Adam et al., 1993, 1994; Zhang and Foote, 1993; Zhang et al., 1994; Itakkura et al., 1994; Li et al., 1995). CID spectra from both oxidation products were compared with that of leuprolide and demonstrated that the tryptophan residue had been covalently modified. For example, comparison of the *b* ion series for pGlu-His-Kyn-Ser-Tyr-Leu-Leu-Arg-Pro-NHEt ($MH^+ = 1213.8$) with the *b* ion series from leuprolide depicted a 4 Da shift (Fig. 7b). The oxidized peptide produced a b_2 ion, $m/z = 249.2$, corresponding to pGlu-His-OH; however, the m/z values for b_3 through b_8 were all 4 Da greater than the corresponding series for leuprolide, indicating that the third residue from the N-terminus had been chemically modified. Furthermore, the mass differences between b_3 and b_2 ($439.4 - 249.2 = 190.2$) and between y_8 and y_7 ($965.6 - 775.5 = 190.1$) were equivalent to a tryptophan residue (186.1) plus 4.1 and 4.2 Da, respectively. Similarly, the seqence of pGlu-His-Nfk-Ser-Tyr-Leu-Leu-Arg-Pro-NHEt ($MH^+ = 1241.6$) was assigned. Additional structure elucidation of the modified tryptophan residues was not pursued.

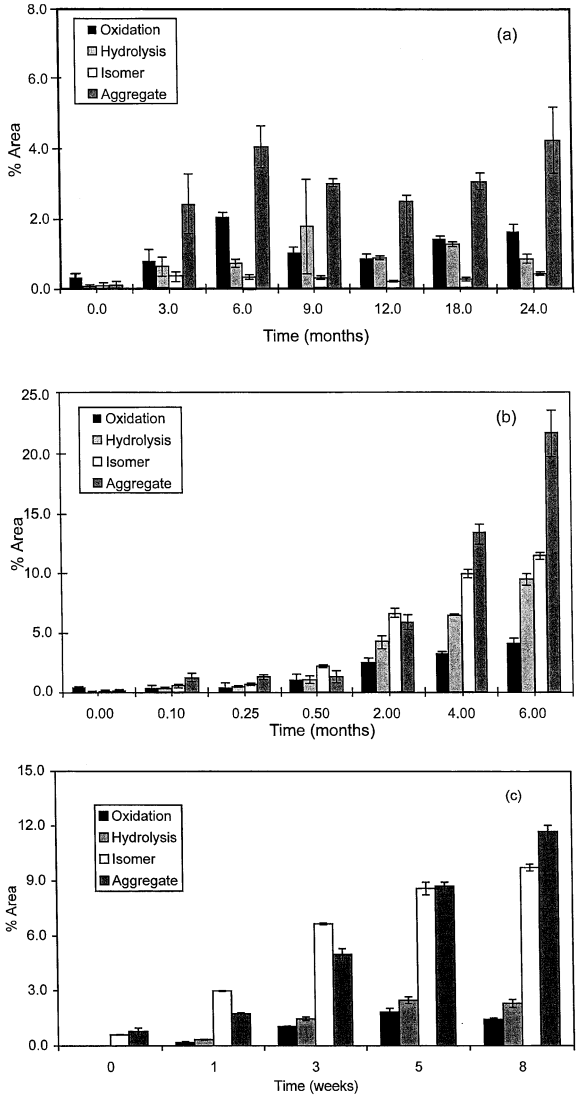

Fig. 9. Leuprolide degradation in DMSO at (a) 370 mg/ml 37°C, (b) 370 mg/ml 80°C and (c) 50 mg/ml 80°C ($n = 3$), illustrating the degradation product peak intensities.

4. Discussion

Leuprolide degradation products were grouped into four major pathways: hydrolysis/backbone cleavage, isomerization, oxidation and aggregation. Inspection of individual degradation product peak areas revealed variations over time with respect to temperature and leuprolide concentration. For example, leuprolide was observed to degrade more rapidly at lower peptide concentrations. Degradation products at varying leuprolide concentrations indicated that individual hydrolytic and isomerization degradation products decreased with increasing leuprolide concentration; however, this effect was minor. When summing individual degradation products into the four major groups, the overall proportion of degradation products was aggregation > isomerization > hydrolysis > oxidation for both low and high leuprolide concentrations at 80°C (Fig. 9).

Therefore, the variations of individual hydrolytic degradation products were not apparent when all of the hydrolytic products were summed and compared with other pathways. Overall, aggregation products were less sensitive than chemical degradation products over the concentration range tested, where approximately the same percent aggregate was produced from 50 to 370 mg/ml. In addition, isomerization initially appeared to be the major degradation pathway, and it is not until after several months, that aggregation predominated.

Characterization of the degradation product peaks over the temperature condition range demonstrated that their intensities changed with temperature (Fig. 9). At 37°C, there was little or no increase in the area percent of any degradation peaks from 6 to 24 months, suggesting the reaction had run out of effective substrates. At 80°C, the peak areas collectively continued to increase through the entire time period. Specifically, formation of pGlu-His-Trp-NH_2 was observed to be highly temperature dependent, while acceleration of oxidation from 37°C to 80°C was relatively small. Further, summation of peak area data for the degradation products at 37°C, 50°C, 65°C and 80°C demonstrated that their relative proportions changed with temperature. For example, at 37°C the proportion of degradation products was aggregation > oxidation > hydrolysis > isomerization, and at 80°C the proportions switched to aggregation > isomerization > hydrolysis > oxidation. Thus, under accelerated conditions, the relative importance of oxidation would be underestimated.

The linearity of the Arrhenius plot would be misleading when trying to predict the impact of a specific degradation pathway on overall stability. In addition, the pseudo first order fit should only be used as an estimate for stability, since several of the degradation pathways may be second order. Variations in degradation product peak intensity were expected considering that hydrolysis reactions generally have higher activation energies than oxidation reactions. However, the effect of temperature and leuprolide concentration may be further accentuated by other factors: the dielectric constant of the solvent, the source of free H^+ in an aprotic solvent, oxygen solubility as a function of temperature, stability of impurities at increased temperatures and residual moisture.

First, when comparing DMSO and water, as solvents, several characteristics become important: the aprotic nature of the solvent, the dipole moment ($\mu_{water} = 1.9$ D, $\mu_{DMSO} = 4.0$ D) and the dielectric constant ($\varepsilon_{water} = 80.0$, $\varepsilon_{DMSO} = 47.2$ at 20°C) (Lide, 1995). The aprotic nature limits the source of protons available for degradation, while the dipole moment allows solubilization of leuprolide. Interestingly, DMSO has a relatively high dipole moment, but, its dielectric constant is less than water. When comparing the degradation rate of leuprolide in water and in DMSO, leuprolide exhibits better stability in DMSO (Hall, et al., 1999). Therefore, as the dielectric constant decreases, the degradation rate decreases, consistent with studies on the rate of deamidation in protic organic cosolvents (Brennan and Clarke, 1993). Furthermore, inspection of the major degradation pathways at 37°C (water: hydrolysis > aggregation > isomerization > oxidation) (DMSO: aggregation > oxidation > hydrolysis > isomerization) indicated that decreasing dielectric constant was consistent with decreasing hydrolytic/isomerization processes (Hall et al., 1999).

Inspection of the shift in major degradation product pathways with temperature, in DMSO, yielded less obvious results. For example, as the temperature increases, the dielectric constant decreases ($\varepsilon_{DMSO} = 45.4$ at 37°C, $\varepsilon_{DMSO} = 39.2$ at 80°C) (Lide, 1995). However, the prevalence of hydrolysis/isomerization products was larger at higher temperatures than at low (37°C: aggregation > oxidation > hydrolysis > isomerization) (80°C: aggregation > isomerization > hydrolysis > oxidation), inconsistent with the assumption that increasing temperature and decreasing dielectric should result in decreased ratios of hydrolytic products (Brennan and Clarke, 1993). A clear relationship may be difficult to discriminate due to the relatively small change in dielectric constant with temperature, and may be further tempered by additional effects described later.

Second, as the temperature increases, the dissociation constant of residual water (1–3% residual moisture in formulation determined by Karl Fis-

cher) increases from 2.57×10^{-14} at 37°C to 23.4×10^{-14} at 80°C (Martin et al., 1983). The dielectric constant of DMSO will also affect the dissociation of residual water. Since the dielectic constant of DMSO is less than water, the residual water may be dissociated to a lesser extent in DMSO than in water. Furthermore, residual water may be more difficult to dissociate with increasing temperature as the dielectric constant continues to diminish. However, the effect of DMSO on the dissociation of residual water was assumed to be constant, since the ratio of water to DMSO did not change appreciably. Therefore, an increase in the dissociation of water to H^+ and OH^- with increasing temperature would accelerate hydrolytic degradation, consistent with the data. The source of free H^+ would also be consumed faster at higher temperatures than at lower temperatures. This effect would be primarily due to kinetics of the process and increased mobility of the reactants, resulting in an increased initial rate of degradation for hydrolytic and isomerization pathways.

Third, oxidation is usually less sensitive to temperature because oxygen solubility decreases with increasing temperature (Adam et al., 1993; Fransson et al., 1996). Therefore, reduction in the rate of oxidation with decreasing temperature is usually not as significant as other hydrolytic processes. As temperature decreases, solubilized oxygen increases, increasing the concentration of reactive species, indicating that leuprolide may be more susceptible to oxidation at lower temperatures.

Fourth, minimal oxidation at increased temperatures (80°C) could be accentuated by the degradation of other oxidizing agents to less reactive species, resulting in less leuprolide oxidation. If these reactive impurities were derived from the DMSO (trace metals, peroxides and free radical initiators), then as the leuprolide concentration increases, the ratio of impurities to leuprolide molecules decreases and may result in less leuprolide degradation. This may also suggest that at extremely high leuprolide concentrations, the dynamic flexibility of leuprolide is hindered and the mobility of the reactants is diminished. Residual water can also act as an antioxidant by facilitating recombination of free radicals (Hageman, 1988). At higher temperatures, dissociation of water is greater and can more effectively facilitate recombination. Minimization of water in an aprotic solvent may also result in an overall increase in oxidation (Hageman, 1988; Towns, 1995).

Fifth, in solid state stability, the effect of decreased water content on stability can result in decreased dynamic flexibility of the protein, decreased reactivity of water and decreased mobility of other reactive species through water (Hageman, 1988, 1992; Towns, 1995). This is especially prevalent below the required quantity of water to provide a hydrated monolayer (6–8%) (Hageman, 1988, 1992; Towns, 1995). However, 370 mg/ml leuprolide may have more flexibility in DMSO than in the solid state, but the remaining parameters apply.

The residual water in an aprotic solvent could remain preferentially bound to the charged and polar leuprolide side chains. Conversely, DMSO is a highly polar, hygroscopic solvent, where the water may be pulled away from the leuprolide and become equilibrated in the DMSO. Either way, the leuprolide molecules are effectively exposed to equivalent or less moisture than the lyophilized raw material. As leuprolide concentration in neat DMSO increases, the total water content increases in the formulation, but the environment of each leuprolide molecule may have equal or less associated water. Therefore, the increasing water content in the formulation with increasing leuprolide concentration did not result in increased degradation.

Lastly, less available residual water (i.e. activity), will result in less degradation, because water is a limited reactive species for hydrolytic processes. The extent of hydrolytic degradation in DMSO formulations appeared to be limited by the residual moisture present during the formulation process. Temperature accelerated studies indicated that increasing the formulation moisture content increased degradation. For example, increasing the water content to 15% resulted in a 4% decrease in leuprolide stability after 6 months at 50°C (Hall et al., 1999).

5. Conclusion

Leuprolide stability in DMSO was determined to be adequate for a 1 year implantable system. Leuprolide degradation profiles in an aprotic solvent, DMSO, differed from those observed in water. The degradation product peak intensities varied with temperature and leuprolide concentration. Specifically, the relative ratios of degradation products were observed to shift with temperature, indicating that temperature accelerated studies would not be indicative of the major chemical degradation pathways at 37°C. Increasing the temperature increased the proportion of leuprolide degrading via isomerization and hydrolysis reactions, and decreased the proportion degrading via oxidation. These data suggest that the rate of formation for individual degradants was dependent on activation energies, solvent dielectric, hydronium ion content, oxidation potential, impurities and water content in the formulation. Leuprolide solubilized in an aprotic solvent is an effective approach to limiting hydrolytic degradation pathways, where use of an aprotic solvent minimizes water content, similar to solid state stability.

Acknowledgements

We would like the thank Mandy Tan for analytical support. This product is being developed by ALZA Corporation on behalf of Crescendo Pharmaceuticals Corporation.

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