JPP Journal of Pharmacy and Pharmacology

JPP 2004, 56: 19–25

Received May 06, 2003
Accepted September 23, 2003
DOI 10.1211/0022357022539
ISSN 0022-3573

Preformulation stability of Spantide II, a promising topical anti-inflammatory agent for the treatment of psoriasis and contact dermatitis

Loice Kikwai, R. J. Babu, Narayanasamy Kanikkannan and Mandip Singh

Abstract

Substance P is readily expressed in skin inflammatory disorders such as psoriasis and contact dermatitis. Spantide II is a peptide (MW 1668.76) that specifically binds to neurokinin-1 receptor (NKR-1) and blocks inflammation associated with substance P. The anti-inflammatory property of Spantide II makes it a suitable candidate to be studied as a topical formulation for the treatment of dermal inflammatory disorders. The objective of this study was to investigate the influence of pH, temperature, salt concentration and concentration on the aqueous stability of Spantide II. The stability of Spantide II was also assessed by circular dichroic (CD) spectroscopy and mass spectrometry (MS). The influence of various dermatological vehicles (ethanol, Transcutol, propylene glycol, *N*-methyl-2-pyrrolidone (NMP), ethyl oleate, isopropyl myristate and laurogylcol FCC (LFCC)) on the stability of Spantide II was investigated. A precise high-performance liquid chromatography (HPLC) assay was developed for analysis of Spantide II. At higher temperature (40 °C) the stability of Spantide II decreased with increase in pH ($P < 0.05$). Change in salt concentration did not appreciably affect the stability of Spantide II ($P > 0.05$). The concentration of Spantide II in the solution had no significant influence on its stability ($P > 0.05$). CD spectroscopy studies showed that Spantide II has a relatively stable α-helix structure in the liquid state. The stability of Spantide II was affected by the type of vehicle used in the study ($P < 0.01$) at different temperatures ($P < 0.05$). Spantide II at high temperature undergoes lysine–proline diketopiperazine degradation as evident in MS data. Spantide II was relatively more stable in ethyl oleate–ethanol, ethanol–water, ethanol and *N*-methyl-2-pyrrolidone. The results of this study indicate that ethyl oleate–ethanol (1:1) and ethanol–water (1:1) could be used as potential vehicles in the development of topical formulations of Spantide II.

Introduction

Cutaneous inflammation involves a series of reactions that set the stage for the repair of damaged tissue. It is mediated by immune and neurogenic mechanisms. A wide range of stimuli, including endogenous or exogenous chemicals, pathogens and physical trauma, can trigger inflammation. Long-term inflammation can lead to disorders such as psoriasis and contact dermatitis. Currently, these disorders are primarily treated with corticosteroids (Robertson & Maibach 1989), which target the immune system by lowering and inhibiting peripheral lymphocytes and macrophages. In addition, corticosteroids inhibit phospholipase A_2, which blocks the release of arachidonic acid, the precursor of prostaglandins and leukotriene, which are chemical mediators that are released in inflammatory processes (Mycek et al 1992). The main drawback of corticosteroids applied to the skin includes side effects such as stinging, itching, irritation, dryness, scaling, atrophy and hypopigmentation (Epstein et al 1963; Takeda et al 1988). Because the reduction of side effects is of importance in improving patient compliance, targeting another pathway of inflammation such as the neurogenic component can lead to promising anti-inflammatory therapies. With the exception of capsaicin, no therapies are available that specifically utilize aspects of neurogenic inflammation to treat inflammatory disorders.

There is increasing evidence that the release of neuropeptides from sensory nerves and their interaction with target skin cells play an important role in the pathogenesis of

Division of Pharmaceutics, College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, Florida, USA

Loice Kikwai, R. J. Babu, Narayanasamy Kanikkannan, Mandip Singh

Correspondence: M. Singh, Division of Pharmaceutics, College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, Florida, USA. E-mail: mandip.sachdeva@famu.edu

Funding: Support from NIAMS grant number AR47455-02 is gratefully acknowledged.

inflammatory skin disorders (Ansel et al 1996, 1997; Brain 1996). Substance P is a neurotransmitter of the central and peripheral nervous system, which has a diverse spectrum of inflammatory activity (Scholzen et al 1998). Substance P is a classical mediator of the triple response consisting of erythema, oedema and itching. Substance P is released by sensory neurons after a noxious stimulus provokes erythema and oedema via mast-cell-dependent and mast-cell-independent pathways. It is capable of up-regulating production of pro-inflammatory cytokines interleukin (IL)-1, IL-6, and IL-8 in murine and human keratinocytes (Brown et al 1990).

Spantide II is a peptide (MW 1668.76; NH_2-D-Lys (nicotinoyl)-Pro-β-(3-pyridyl)-Ala-Pro-3, 4-dichloro-D-Phe-Asn-D-Trp-Phe-D-Trp-Leu-Nle-NH_2) with an isoelectric point of 5.2 (Wang et al 1994). It specifically binds to neurokinin-1 receptor (NKR-1) and blocks pro-inflammatory activity associated with substance P. Spantide II has been shown to reverse the effects of substance P (Andoh et al 1998). Intradermal injection of 9 nmol Spantide II effectively blocked the plasma extravasation in the rat hind paw induced by 8 nmol intravenous substance P (Xu et al 1991). In an earlier study, we demonstrated the anti-inflammatory effect of Spantide II in an allergic contact dermatitis mouse model (Jaiani et al 2002). The long-term objective is to develop a topical formulation of Spantide II for the treatment of psoriasis and contact dermatitis.

To achieve therapeutic concentrations of Spantide II in the skin layers, it should be solubilized in a compatible topical vehicle that has optimal release and permeation characteristics. Peptides are vulnerable to both physical and chemical instability (Schmid 1970; Manning et al 1989). It is necessary to study their stability as a function of pH, temperature and salt concentration, which have been shown to affect the stability of peptides (Helm & Muller 1991; Brange et al 1992; Lee et al 1992). It has also been reported that the concentration of peptide in the solution has a significant influence on its stability (Wang 1999). The influence of pH, temperature, salt concentration and concentration of Spantide II on the stability of Spantide II was investigated. The stability of Spantide II was also assessed by circular dichroic (CD) spectroscopy and mass spectrometry (MS). In addition, the stability of Spantide II was determined in commonly used topical vehicles (ethanol, Transcutol, propylene glycol, *N*-methyl-2-pyrrolidone (NMP), ethyl oleate, isopropyl myristate and laurogylcol FCC (LFCC)) at different storage conditions.

Materials and Methods

Materials

Spantide II (assay > 95%) was custom-synthesized by Bio Peptide Co. LLC (San Diego, CA) and used without further purification. Ethanol, propylene glycol, ethyl oleate, isopropyl myristate, NMP, potassium chloride, mono and dibasic potassium phosphate, hydrochloric acid, potassium biphthalate, sodium hydroxide, trifluoroacetic acid (TFA) and boric acid were procured from Sigma Chemical Co. (St Louis, MO). Transcutol and LFCC were gifts from Gattefosse (Cedex, France). Ethanol USP (200 proof) was obtained from Florida Distillers Co. (Lake Alfred, FL). Water, acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (Atlanta, GA). All other chemicals were standard reagent grade.

High-performance liquid chromatography (HPLC) assay development

An HPLC system (Waters Corporation) along with a Vydac reverse phase C_{18} (300 Å pore size silica) analytical column (5 μm, 4.6 × 250 mm) was used for the analysis of Spantide II. This HPLC system consisted of an autosampler (model 717 plus), two pumps (model 515), and an ultraviolet (UV) detector (model 996 PDA). The mobile phases used were 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The mobile phase was filtered, degassed by sonication before use and analysis was run at a gradient of 32% to 68% (Solvent A:B, respectively), which was reversed to 68% to 32% (Solvent A:B, respectively) over 30 min, with a flow rate of 1 mL min^{-1}. The Spantide II content in the samples was analysed using a PDA-UV detector set at 230 nm. All injections were performed at room temperature, and the retention time of Spantide II was 22.6 min. The Millennium[32] software was used to control the HPLC system. To validate the HPLC method, the accuracy and precision of the HPLC assay was determined. Method validation was accomplished by performing intra- and inter-day assay of various concentrations of Spantide II. The precision of the method was assessed by determining the peak area of different injections in the range 0.1–10 μg of Spantide II. The study was repeated 3 times in one day to represent intra-day variation and 5 times on five consecutive days to represent inter-day variation of the analytical results. The coefficient of variation (%CV) of the peak areas was determined and represented the precision of the HPLC assay. Known concentrations of Spantide II (1.0, 5.0 and 10.0 μg) were assayed to determine the accuracy of the method. The study was repeated 3 times in one day to represent intra-day variation and 5 times on five consecutive days to represent inter-day variation of the assay of the compound. The coefficient of variation (%CV) of the concentration of Spantide II was determined to represent the accuracy of the HPLC assay.

Stability studies

Effect of pH and temperature

Spantide II was dissolved in ethanol at concentrations of 0.1 mg mL^{-1} and 1 mg mL^{-1} and these solutions were used for stability studies. Various buffer solutions (0.025 M) with pH 3 (acid phthalate buffer), pH 5 (potassium biphthalate buffer), pH 7 (phosphate buffer) and pH 9 (alkaline borate buffer) were prepared according to USP XIII procedures. Test samples were prepared by mixing a 1:1 ratio of each buffer with 0.1 mg mL^{-1} Spantide II stock solution to a final concentration of 0.05 mg mL^{-1}.

Four millilitres of each solution were placed in glass vials, tightly closed with polypropylene caps with silicone liners and stored at temperatures of 4, 25 and 40 °C. Samples were collected at 0, 4, 7, 14, 30, 60 and 90 days and the physical appearance and pH of the solutions were recorded. The Spantide II concentration of each solution was determined by HPLC.

Effect of salt concentration

Phosphate buffer solutions (0.025 M) with pH 7.4 and salt concentrations of 0.075, 0.1, 0.25, 0.5, 1 and 2 M were prepared. Salt concentrations were adjusted with potassium chloride. Test samples were prepared by mixing a 1:1 ratio of each buffer solution with 0.1 mg mL^{-1} Spantide II stock solution to a final concentration of 0.05 mg mL^{-1}. Four millilitres of each solution were placed in glass vials, tightly closed with polypropylene caps with silicone liners and stored at 4, 25 and 40 °C. Samples were collected at 0, 4, 7, 14, 30, 60 and 90 days and the physical appearance and pH of the solutions were noted. The Spantide II content of each solution was determined by HPLC.

Effect of Spantide II concentration

The stability of Spantide II as a function of concentration was determined using buffer solution (0.025 M) with pH 7.4 and salt concentration 0.075 M. Spantide II stock solutions (0.1 mg mL^{-1} and 1 mg mL^{-1}) were mixed with the buffer to a final concentration of 0.05 mg mL^{-1} and 0.5 mg mL^{-1}. Four millilitres of each solution were placed in glass vials, tightly closed with polypropylene caps with silicone liners and stored at 4, 25 and 40 °C. Samples were collected at 0, 4, 7, 14, 30, 60 and 90 days and the physical appearance and pH of the solutions were noted. The Spantide II content of each solution was determined by HPLC.

CD spectroscopy

Spantide II solution at a concentration of 0.01% w/v in 50% ethanol was prepared and stored at 25 °C and at 70 °C. At predetermined time intervals, samples were analysed by CD spectroscopy. CD spectra were obtained on a Jasco J-810 CD spectropolarimeter (Spectroscopic Co. Ltd, Tokyo, Japan) and recorded in the far UV region ($\lambda = 250-190$ nm) using a 2 mm quartz cuvette. The spectra were recorded at room temperature, with a scan speed of 10 nm min^{-1}, a bandwidth of 1 nm, and two accumulations.

Mass spectrometry

Spantide II solution was prepared at a concentration of 0.025% w/v in 50% ethanol and stored at 25 °C and at 70 °C. At predetermined time intervals, the sample was analysed by MS. Mass spectra were acquired on a JEOltheMSroute JMS.600H (JEOL Inc., Peabody, MA) mass spectrometer equipped with an electro spray inlet (ESI +) ionization probe. The spectra were obtained in the low-resolution mode with a voltage of 2.5 kV. Mass spectra were collected in the positive-ion mode, scanning from 100 to 1800 mass units every 5 s.

Effects of vehicles on stability

The stability of Spantide II in various vehicles was determined at 4, 25 and 40 °C over a time interval of 0, 4, 7, 30, 45 and 60 days. Samples were prepared by dissolving 0.5% w/v Spantide II in various vehicles or their binary mixtures with ethanol. Spantide II was readily soluble in ethanol, Transcutol, propylene glycol and NMP, but in the case of water, ethyl oleate, isopropyl myristate and LFCC, a 1:1 ratio of vehicle and ethanol was used to increase Spantide II solubility. The physical appearance of the solution was noted and Spantide II content of the solutions was determined by HPLC.

Statistical methods

The logarithmic concentration of Spantide II in the stability samples was plotted as a function of time. The degradation rate constant (K) was obtained from the slope of the logarithmic plot of Spantide II concentration versus time profiles by linear regression analysis using the GraphPad Prizm program. Statistical analysis of the data for effect of pH, temperature, salt concentration, Spantide II concentration and type of vehicle on the stability of Spantide II was performed using a two-way analysis of variance (GraphPad Prizm, San Diego, CA). In all cases, Tukey's test was used to identify individual differences within the variables. The differences were considered to be significant at $P < 0.05$.

Results and Discussion

HPLC assay

The HPLC method reported in this study was well validated to assess the stability of Spantide II. To our knowledge there is no reported HPLC assay method in the literature for Spantide II. The intra-day and inter-day precision and accuracy of the HPLC assay are summarized in Table 1. Precision is an expression of method repeatability and is represented by the coefficient of variation of the peak areas, which were < 6.47% (intra-day) and < 7.60% (inter-day) of the coefficient of variation. The accuracy of the HPLC method was represented as the coefficients of variation of the assay of Spantide II, which were < 5.3% (intra-day) and < 4.93% (inter-day). The detection limit of the HPLC assay for Spantide II was 0.1 μg mL^{-1}. Over the concentration range of 0.1–10 μg, the HPLC method demonstrated good linearity with a correlation coefficient of > 0.998. The data clearly demonstrates a reproducible and accurate HPLC assay for Spantide II.

Aqueous stability studies

The pH and physical appearance of the samples at various stability conditions showed no significant change during the entire duration of the study. The degradation rate constants (log K) were calculated from the ln concentration–time profiles and plotted as log K as a function of pH

Table 1 Validation of HPLC assay of Spantide II.

Qty (μg)	Intra day precision (n = 3)		Inter day precision (n = 5)	
	Peak area ($\bullet 10^5$)	%CV	Peak area ($\bullet 10^5$)	%CV
0.1	0.62 ± 0.02	3.50	0.60 ± 0.03	4.65
0.3	5.12 ± 0.13	2.55	5.19 ± 0.21	4.13
0.5	8.70 ± 0.15	1.77	10.74 ± 0.82	7.60
1.0	18.05 ± 0.76	4.19	18.97 ± 0.49	2.31
3.0	68.20 ± 2.45	3.60	68.54 ± 1.65	2.40
5.0	111.17 ± 3.93	3.53	113.55 ± 6.47	5.70
7.0	170.28 ± 11.01	6.47	175.94 ± 77.79	4.42
10.0	238.16 ± 7.16	3.00	245.70 ± 8.85	3.60
Qty (μg)	**Intra day accuracy (n = 3)**		**Inter day accuracy (n = 5)**	
	Concn	%CV	Concn	%CV
1.0	0.93 ± 0.04	4.75	0.94 ± 0.05	4.93
5.0	4.75 ± 0.25	5.29	4.92 ± 0.15	2.95
10.0	10.01 ± 0.03	0.33	10.03 ± 0.03	0.26

Peak areas and concentrations are expressed as means ± s.d.

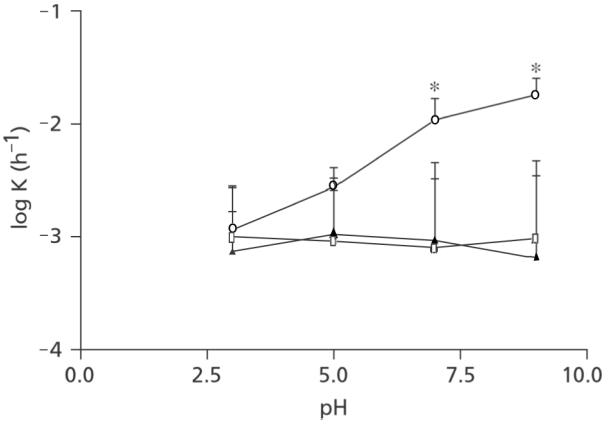

Figure 1 Effect pf pH on the degradation rate constant (log K) of Spantide II at different temperatures (□, 4 °C; ▲, 25 °C; ○, 40 °C). Data are means ± s.d., n = 3. *$P < 0.05$ vs pH 3.0.

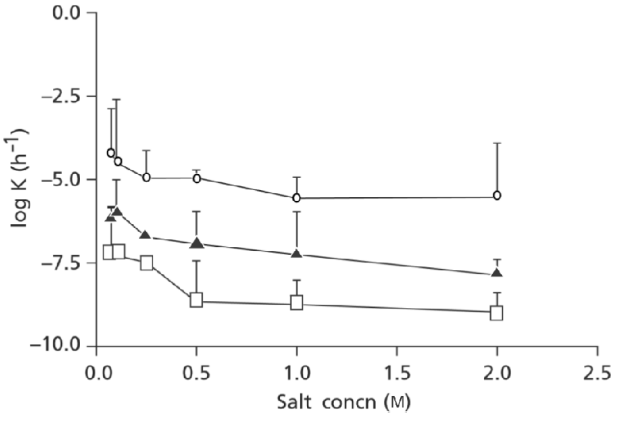

Figure 2 Effect of salt concentration on the degradation rate constant (log K) of Spantide II at different temperatures (□, 4 °C; ▲, 25 °C; ○, 40 °C). Data are means ± s.d., n = 3.

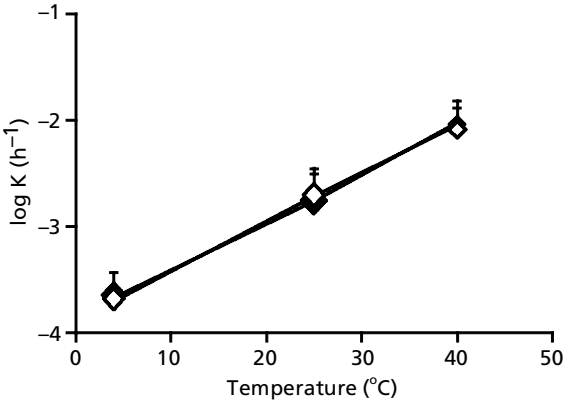

Figure 3 Effect of concentration on the degradation rate constant (log K) of Spantide II as a function of temperature (◇, 0.05 mg mL^{-1}; ◆, 0.5 mg mL^{-1}). Data are means ± s.d., n = 3.

(Figure 1). As shown in Figure 1, the log K did not increase as a function of pH at 4 °C and 25 °C. However, at 40 °C, there was a linear increase in the log K as a function of pH. The increase at pH 5.0 was not statistically significant compared with pH 3.0 ($P > 0.05$), whereas at pH 7.0 and 9.0 the increases were significantly higher than pH 3.0 ($P < 0.05$). It is also evident from Figure 1 that the degradation rate constant, at pH 3.0, did not increase as a function of temperature ($P > 0.05$). Thus, maximum stability was noted with pH 3.0 buffer at all temperatures. Peptides are typically more stable at a pH close to their isoelectric point (Wang 1999). Thus Spantide II, with an isoelectric point of 5.2, should be stable in the acidic range. The data in Figure 1 shows that Spantide II is susceptible to degradation at alkaline pH and is more stable at acidic pH.

Figure 2 shows the degradation rate constant of Spantide II as a function of salt concentration. It can be seen that with an increase in the salt concentration, the degradation of Spantide II was decreased. However, this decrease in the degradation of Spantide II (as shown by log K) was not statistically significant at 4, 25 and 40 °C ($P > 0.05$), indicating that there was no appreciable effect of salt concentration on the stability of Spantide II. At higher salt concentrations, the peptide stabilization possibly occurs by minimizing or preventing deamidation reactions (Bummer & Koppenol 2000). The effect of salt concentration on protein or peptide stability is complex because of ionic interactions on the surface or interior of protein or peptides (Kohn et al 1997). Protein or peptide aggregation is generally dependent on concentration, and this aggregation can lead to reduced stability. Hence, we studied the effect of concentration of Spantide II on its stability. Figure 3 shows the effect of Spantide II concentration on log K at different temperatures. The log K

profiles of 0.05 and 0.5 mg mL^{-1} Spantide II concentrations are similar ($P < 0.05$) at all temperatures, thus indicating that degradation of Spantide II was independent of its concentration.

Physical instability of peptides can be caused by various factors such as temperature, pH, denaturing agents, etc. Therefore, CD spectroscopy was used to demonstrate the physical stability of Spantide II as a function of temperature. CD spectroscopy in the far-UV region ($\lambda = 250$–190 nm) provides information on the secondary structure (α-helix, β-helix, β-turn and random coil) of the peptide. α-Helix in peptides is because of peptide folding by twisting in to a right-handed screw so that all the amino acids can hydrogen bond with each other. Negative peaks at 221 and 209 nm are characteristic of α-helix in peptides (Holzwarth & Dotz 1965). Figure 4 shows the CD spectra of Spantide II at 25 °C and 70 °C as a function of time. It is observed that Spantide II has negative peaks at 223 and 207 nm, which corresponds to some degree of α-helix secondary structure. At 25 °C, the CD spectrum of Spantide II did not show any change up to 60 days. Spantide II solution exposed to 70 °C for 10 days did not show any significant changes of the negative peaks, although at 14 days the intensity of the peak at 207 nm was slightly decreased. It was found that the peak intensity was dependent on the concentration of Spantide II (data not shown). This indicates that Spantide II has a relatively stable α-helix structure.

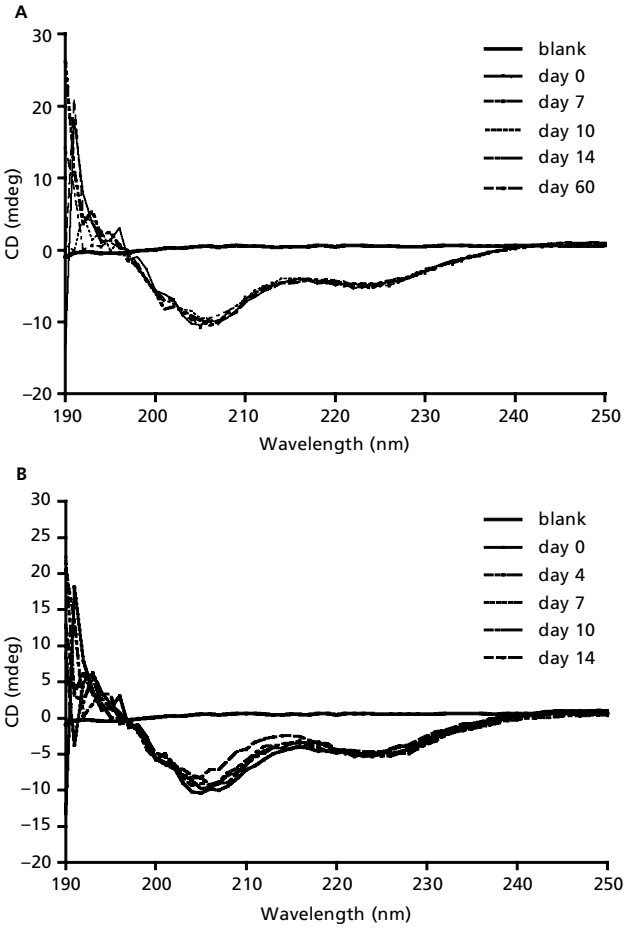

Figure 4 Circular dichroic spectra of Spantide II in aqueous ethanol at 25 °C (A) and 70 °C (B).

Figure 5 shows MS spectra of samples at day 0, day 60 at 25 °C and day 14 at 70 °C. Under the ESI condition, MS stability studies of Spantide II gave a number of ions having different charged states. As shown in Figure 5A, Spantide II yielded a singly charged ion $[M + H]^{1+}$ at m/z = 1669.8 and a more intense doubly charged ion $[M + 2H]^{2+}$ at m/z = 835.6 and, additionally, ions $[M + 3H]^{3+}$ at m/z = 557.4 and $[M + 4H]^{4+}$ at m/z = 418.4. The charged state at m/z = 279.2 is due to solvent contamination. No significant changes were observed in the MS spectrum of the sample stored for 60 days at 25 °C (Figure 5B). The sample exposed to 70 °C showed additional peaks at m/z = 1337.9, 670.5 and 331.3 in the MS spectrum corresponding to degradation products (Figure 5C). The peaks can be attributed to the Spantide II fragmentation due to diketopiperazine degradation reaction (Goolcharran & Borchardt 1998). The singly and doubly charged ions, $[M + H]^{1+}$ at m/z = 1337.9 and $[M + 2H]^{2+}$ at m/z = 670.5, correspond to the large peptide fragment (MW 1338.38). The singly charged ion, $[M + H]^{1+}$ at m/z = 331.3, corresponds to the small peptide fragment (MW 330.38). Based on the structure of Spantide II, the small peptide fragment is lysine–proline diketopiperazine (lys-pro DKP), and the remaining structure (-R_3) is the large peptide fragment as shown in Figure 5.

A preliminary accelerated stability study was designed to investigate the stability of Spantide II in various solvents, to screen the suitability of the various solvents as topical vehicles. No significant change in the physical appearance of samples was observed with any of the vehicles, except propylene glycol and Transcutol, where the solution turned pale yellow. The effects of various vehicles on the stability of Spantide II are summarized in Table 2. Two-way analysis of variance of log K values of various vehicles and temperatures showed that both significantly affected the stability of Spantide II ($P < 0.001$). At higher temperatures (25 and 40 °C), the log K values were significantly higher compared with 4 °C ($P < 0.001$). Spantide II was relatively more stable in ethyl oleate–ethanol, ethanol–H_2O and ethanol followed by NMP and isopropyl myristate–ethanol. Further studies with ethyl oleate–ethanol, ethanol–H_2O and NMP will be carried out with stabilizing agents and antioxidants to improve the stability of Spantide II in these vehicles.

Conclusion

The results of this pre-formulation study show that many factors can influence the stability of Spantide II. The use of higher pH resulted in significant degradation of Spantide II while salt concentration showed no appreciable effect on the stability of Spantide II. Spantide II concentration did not show any influence on its degradation pattern. Spantide II showed a stable α-helix structure as evident from CD

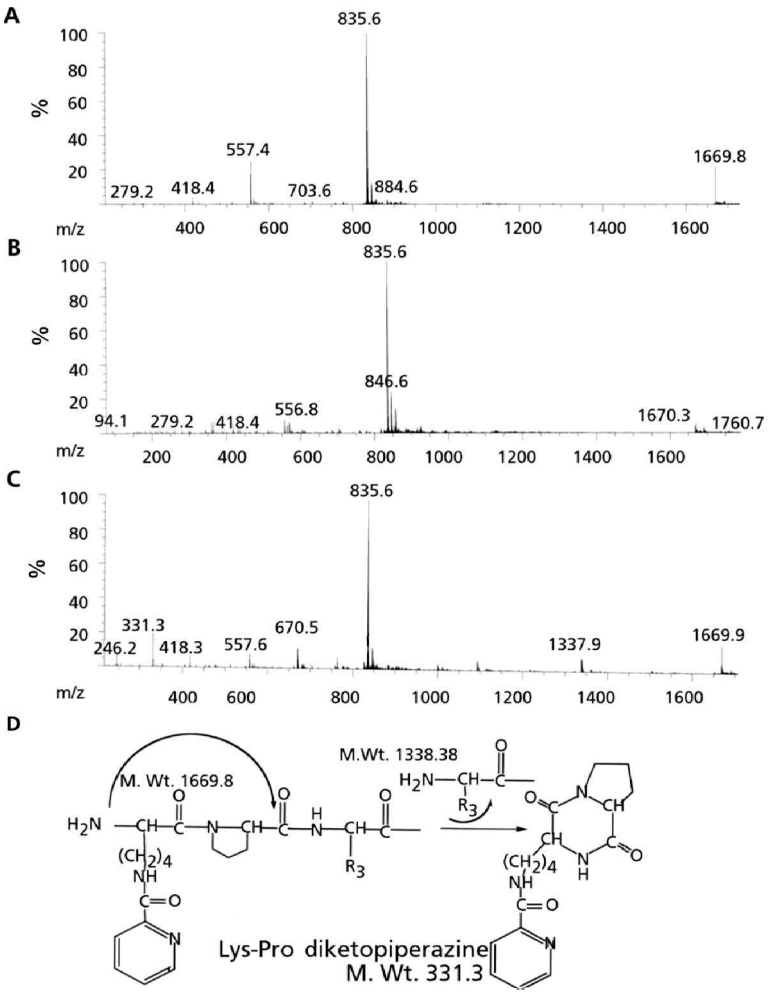

Figure 5 Mass spectra of Spantide II in aqueous ethanol at day 0 (A), day 60 at 25 °C (B) and day 14 at 70 °C (C). D. Spantide II undergoes rearrangement and cleavage to form a small peptide fragment (lys-pro DKP) and a large peptide fragment (R_3).

Table 2 Effect of vehicle on the stability of Spantide II.

Vehicle	log K		
	4 °C	25 °C	40 °C
Ethyl oleate–ethanol	−2.78 ± 0.0025	−2.89 ± 0.0022*	−2.30 ± 0.0036*
NMP	−2.75 ± 0.0009	−2.52 ± 0.0021*	−1.81 ± 0.0058*
LFCC–ethanol	−2.24 ± 0.0014	−2.31 ± 0.0009*	−2.20 ± 0.0055#
Propylene glycol	−1.85 ± 0.0015	−1.55 ± 0.0091*	−0.93 ± 0.0106*
Transcutol	−2.34 ± 0.0017	−1.80 ± 0.0047*	−0.99 ± 0.0138*
Ethanol	−2.75 ± 0.0011	−2.55 ± 0.0020*	−1.70 ± 0.0030*
Isopropyl myristate–ethanol	−2.87 ± 0.0021	−2.26 ± 0.0028*	−1.73 ± 0.0020*
Ethanol–water	−2.69 ± 0.0018	−2.26 ± 0.0021*	−1.94 ± 0.0016*

Values are means ± s.d., n = 3. *$P < 0.001$, #$P < 0.01$ versus respective vehicle at 4 °C.

spectroscopy. Spantide II degrades by the lys-pro DKP degradation pathway as is evident in MS data. Among non-aqueous solvents, ethyl oleate–ethanol (1:1), H_2O–ethanol (1:1) and NMP provided greater stability for Spantide II. Thus, these vehicles may be suitable for the development of topical formulations of Spantide II.

References

Andoh, T., Nagasawa, T., Satoh, M., Kuraishi, Y. (1998) Substance P induction of itch-associated response mediated by cutaneous NK1 tachykinin receptors in mice. *J. Pharmacol. Exp. Ther.* **286**: 1140–1145

Ansel, J. C., Kaynard, A. H., Armstrong, C. A., Olerud, J. E., Bunnett, N. W., Payan, D. G. (1996) Skin-nervous system interactions. *J. Invest. Dermatol.* **106**: 198–204

Ansel, J. C., Armstrong, C. A., Song, I., Quinlan, K. L., Olerud, J. E., Caughman, S. W., Bunnett, N. W. (1997) Interactions of the skin and nervous system. *J. Invest. Dermatol. Symp. Proc.* **2**: 23–26

Brain, S. D. (1996) Sensory neuropeptides in the skin. In: Geppeti, P., Holzer, P. (eds) *Neurogenic inflammation*. CRC Press, Boca Raton, pp 229–244

Brange. J., Langkjaer, L., Havelund, S., Volund, A. (1992) Chemical stability of insulin 1. Hydrolytic degradation during storage of pharmaceutical preparation. *Pharm. Res.* **9**: 715–726

Brown, J. R., Perry, P., Hefeneider, S., Ansel, A. C. (1990) Neuropeptide modulation of keratinocyte cytokine production. In: Oppenheim, P., Kluger, D., (eds) *Molecular and cellular biology of cytokines*. Wiley-Liss Inc., New York, pp 451–456

Bummer, P. M., Koppenol, S. (2000) Chemical and physical considerations in protein and peptide stability. In: McNally, E. J. (ed.) *Protein formulation and delivery*. Marcel Dekker Inc., New York, pp 5–70

Epstein, N. N., Epstein, W. L., Epstein, J. H. (1963) Atrophic striae in patients with inguinal intertrigo. *Arch. Dermatol.* **87**: 450–457

Goolcharran, C., Borchardt, R. T. (1998) Kinetics of diketopiperazine formation using model peptides. *J. Pharm. Sci.* **87**: 283–288

Helm, V. J., Muller, B. W. (1991) Stability of the synthetic pentapeptide thymopentin in aqueous solution: effect of pH and buffer on degradation. *Int. J. Pharm.* **70**: 29–34

Holzwarth, G., Dotz, P. (1965) The ultraviolet circular dichroism of polypeptides. *J. Am. Chem. Soc.* **178**: 350–355

Jaiani, L. T., Sachdeva, M., Jaychandra, B. R., Kanikkannan, N., Legat, F. J., Neparidze, N. G., Kikwai, L., Armstrong, C. A., Ansel, J. C. (2002) Inhibition of allergic contact dermatitis by a topically applied SP receptor antagonist. *J. Invest. Dermatol.* **119**: 655

Kohn, W. D., Kay, C. M., Hodges, R. S. (1997) Salt effects on protein stability: two-stranded alpha helical coiled-coils containing inter- or intrahelical ion pairs. *J. Mol. Biol.* **267**: 1039–1052

Lee, K. C., Lee, Y. J., Song, H. M., Chun, C. J., Deluca, P. P. (1992) Degradation of synthetic salmon calcitonin in aqueous solution. *Pharm. Res.* **9**: 1521–1523

Manning, M. C., Patel, K., Borchardt, R. T. (1989) Stability of protein pharmaceuticals. *Pharmacol. Res.* **6**: 903–918

Mycek, M. J., Harvey, R., Champe, P. (1992) Anti-inflammatory drugs. In: Harvey, R. A., Champe, P. C. (eds) *Lippincotts illustrated reveiws: pharmacology*. 2nd edn, Lippincott-Raven, Philadelphia, pp 401–418

Robertson, D. B., Maibach, H. I. (1989) In: Schleimer, R. P., Claman, H. N., Oronsky, A. (eds) *Anti-inflammatory steroid action*. Academic Press, San Diego, pp 494–524

Schimid, R. D. (1970) Stabilized soluble enzymes. *Adv. Biochem. Eng.* **12**: 41–118

Scholzen, T., Armstrong, C. A., Bunnett, N. W., Luger, T. A., Olerud, J. E., Ansel, J. C. (1998) Neuropeptides in the skin: interactions between the neuroendocrine and the skin immune systems. *Exp. Dermatol.* **7**: 81–96

Takeda, K., Arase, S., Takahashi, S. (1988) Side effects of topical corticoids and their prevention. *Drugs* **36** (Suppl. 5): 15–23

Wang, W. (1999) Instability, stabilization and formulation of liquid protein pharmaceuticals. *Int. J. Pharm.* **185**: 129–188

Wang, Z. Y., Feng, D. M., Wang, Y. L, Tung, S. R., Wong, K., Strichartz, G. R., Folkers, K., Hakanson, R. (1994) Pharmacological assessment of Spantide II analogues. *Eur. J. Pharmacol.* **260**: 121–128

Xu, X. J., Hao, J. X., Wiesenfeld-Hallin, Z., Håkanson, R., Folkers, K., Hökfelt, T. (1991) Spantide II, a novel tachykinin antagonist, and galanin inhibit plasma extravasation induced by antidromic C-fiber stimulation in rat hindpaw. *Neuroscience* **42**: 731–737