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Kinetics of Pramlintide Degradation in Aqueous Solution as a Function of Temperature and pH

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ABSTRACT The stability of the 37– amino acid peptide pramlintide, in aqueous solution, was studied as a function of pH and temperature. Samples of pramlintide formulated as a parenteral product were exposed to elevated temperatures and to realistic storage conditions for as long as 30 months. Pramlintide degradation was monitored by three high-performance liquid chromatography (HPLC) methods: a reversed-phase (RP-HPLC) and a strong-cation exchange (SCX-HPLC) method for percentage purity determination by area normalization, plus a second RP-HPLC method for potency determination *versus* external standards. The pH-rate profile for pramlintide shows increasing degradation rate constants with increasing pH over the range pH = 3.5 to 5.0. The Arrhenius expression for pramlintide degradation at pH = 4.0 over the temperature range 5°C to 50° C is $\ln(k_0) = 37.39 - 21.900/RT$, where k_0 is the zero-order rate constant (in %/mo) for pramlintide degradation. The pramlintide parenteral product formulated at pH = 4.0 is extremely stable, with percentage purity and percentage potency loss of only approximately 2% over 30 months at 5°C. The formulated pramlintide drug product has acceptable shelf life for long-term storage at 5°C and up to a 30-day patient use when stored at ambient temperature.

KEYWORDS: Pramlintide, Hydrolysis, pH, Arrhenius, RP-HPLC, SCX-HPLC, Orthogonal Separation

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INTRODUCTION

Amylin is a 37– amino acid peptide hormone that is produced in the pancreas and co-secreted with insulin in response to elevated serum glucose levels [1-3]. Pramlintide is a synthetic analog of amylin that retains the biological activity of the hormone while offering superior physical and chemical properties that facilitate development of a stable drug product for parenteral administration [4]. Pramlintide is being evaluated as a drug candidate for treating people with type 1 and insulin-using type 2 diabetes.[5-7]

Figure 1 shows the pramlintide amino acid sequence with the disulfide bridge between cysteines 2 and 7 and highlights the amino acid differences between pramlintide and amylin.

All of the carboxyl groups in pramlintide are amidated, rendering the molecule cationic (protonated lysine, histidine, and arginine) at acidic pH. Pramlintide may be isolated as a lyophilized salt with acetate as the counterion.

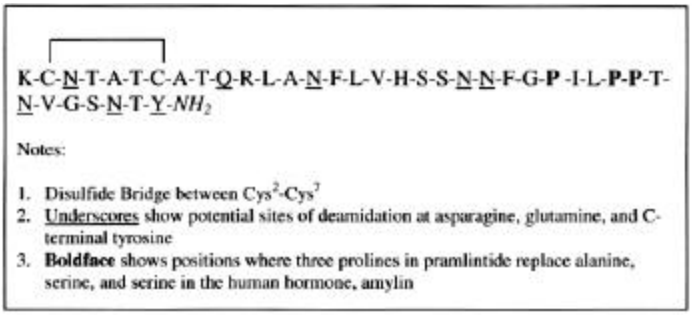


Figure 1. Amino acid sequence of pramlintide.

An injectable, multi-dose, liquid formulation for pramlintide drug product has been developed. The formulation uses pramlintide at 0.30 to 1.0 mg/mL concentrations and *m*-cresol as an antimicrobial preservative. Previous investigations have identified the significant pramlintide hydrolysis products [8] and demonstrated the performance of stability-indicating reversed-phase high-performance chromatography (RP-HPLC) and strong cation exchange (SCX-HPLC) analytical test methods [9].

This report details the kinetics of pramlintide degradation as a function of pH (range = 3.5 to 5.0) and temperature (range = 5 to 50°C). Also reported herein are results of potency and purity determinations of pramlintide injection drug product samples maintained as long as 30 months at 5° C.

MATERIALS AND METHODS

Test Articles

Sterile pramlintide injection drug product samples were prepared by aseptic processing at 0.3 or 0.6 mg/ml in pH 3.5 to pH 5.0 aqueous acetate buffer with *m*-cresol added as antimicrobial preservative. The samples were stored in 5-mL glass vials with bromobutyl rubber closures.

Working Reference Standard

Pramlintide working reference standard solutions were prepared at 0.5 mg/mL in pH 4.0, 30 mM acetate buffer. *m*-Cresol working reference standards were prepared at 0.225% (w/w) in pH 4.0, 30 mM acetate buffer.

Drug Product Samples

For both the RP- and SCX-HPLC percentage purity methods, pramlintide injection samples were subjected to a solid-phase extraction step to remove mannitol and *m*-cresol, as previously described [9]. For the RP-HPLC potency method, pramlintide injection samples were directly injected without additional workup.

Test System and Reagents

The analytical test system employed Waters (Milford, MA) equipment: Model 616 or 2690 solvent delivery, Model 717 autoinjector (with PEEK low dead-volume kit and refrigerated sample compartment), Model 486 detector, and Model 62079 column oven. Buffers and solvents were HPLC grade, or equivalent, throughout.

RP-HPLC Determinations of Pramlintide Potency and m-Cresol Concentration

Table 1 shows operating parameters for this test method, **Table 2** shows mobile phase compositions, and **Table 3** shows the gradient profile.

Table 1. Operating Conditions for RP-HPLC Potency Method

Parameter	Value For Method
Solvent Flow Rate	1.0 ml/min
Detection Wavelength, Scale	220 nm, 1.0 AUFS for pramlintide 240 nm, 1.0 AUFS for *m*-cresol
On Column Load	12 to 18 µg for pramlintide 45 to 67.5 µg for *m*-cresol
Column Temperature	50 ± 2 °C
Autosampler Temperature	6 ± 3 °C
Column Type	YMC ODS-AQ
Column Dimensions	50 x 4.0 mm, 3 µm particle size, 120 °A pore size

Table 2. Mobile Phase Compositions for RP-HPLC Potency Method

Mobile Phase Buffer #	[KH_2PO_4] M	Acetonitrile, %	pH*
1	0.22	9.5	2.0
2	0.22	50	2.0

- Apparent pH of mobile phase, adjusted after addition of acetonitrile.

Table 3. Mobile Phase Gradient Program for RP-HPLC Potency Method

Gradient Time Minutes	Flow Rate mL/min	Buffer 1 %	Buffer 2 %	Curve*
0.0	1.0	65	35	NA
10.0	1.0	40	60	Linear
10.1	2.0	0	100	Linear
10.3	2.0	0	100	Isocratic
10.4	2.0	65	35	Linear
12.9	2.0	65	35	Isocratic
13.0	1.0	65	35	Isocratic
18.0	1.0	65	35	Isocratic
Isocratic	0.1	65	35	Isocratic

* Waters Model 616 or 2690 controller.

Calculations

Because the extent of pramlintide percentage purity loss with time was low under all conditions studied (typically less than approximately 15% decrease from initial values), treating the decrease in percentage purity values by either zero-order or pseudo-first-order kinetic models will yield essentially identical comparisons. For simplicity, this report employs a zero-order model according to equation 1:

$$\%P = 100*(\%Purity_t / \%Purity_{t=0}) = I_0 + k_0*t \qquad (1)$$

where %P is the percentage purity at time t, relative to the initial percentage purity, %Purity is determined by area normalization using either the RP-HPLC %Purity method or the SCX-HPLC %Purity method, I_0 is the regression intercept, and k_0 is the zero-order degradation rate constant (regression slope) in %-mo^{-1}. Note that for the convention employed in equation 1, decreasing % of Initial Purity values with time, corresponds to a negative k_0 value.

Similarly, for loss in potency, this report employs a zero-order model according to equations 2 and 3:

$$LS_t = (\text{Observed RP-HPLC Potency}) / (\text{Label Strength, mg/ml}) \qquad (2)$$

$$\%LS = 100*(LS_t / LS_{t=0}) = I_0 + k_0*t \qquad (3)$$

where LS is the drug product potency relative to the label strength (label strength is either 0.3 mg/mL or 0.6 mg/mL), % LS is the label strength at time t, expressed as a percentage of initial LS, I_0 is the regression intercept, and k_0 is the zero-order degradation rate constant (regression slope) in %-mo^{-1}.

RESULTS

Control Experiments

Suitable control experiments demonstrated that formulated pramlintide injection pH and *m*-cresol were essentially invariant with storage time and temperatures under the conditions studied. There were no changes in visual appearance and no significant changes in the amount of visible or subvisible particulate matter. Furthermore, pramlintide degradation rate constants were independent of pramlintide over the concentration range 0.3 to 0.6 mg/mL.

Time and Temperature Dependence of %Purity and Potency Loss for Samples Formulated at pH = 4.0

Figure 2 shows the decrease in pramlintide %P values versus time for samples formulated at pH = 4.0 and maintained at 5, 15, and 25°C. The figure shows data for determinations by both the RP-HPLC and SCX-HPLC %Purity methods. Least-squares linear regression lines according to equation 1 are shown for the SCX-HPLC %Purity method data. The regression lines show a good fit to the zero-order kinetic model used for equation 1. Data points for both the RP-HPLC and SCX-HPLC %Purity methods are essentially identical, as would be expected if the two orthogonal methods are equally stability specific.

Figure 3 shows the decrease in pramlintide % Purity (by the RP-HPLC %Purity method) and the decrease in pramlintide potency (by the RP-HPLC potency method) versus time for samples formulated at pH = 4.0 and maintained at 5, 15, 25 and 40° C. Least-squares linear regression lines are shown for the RP-HPLC %Purity data according to equation 1. Here, the RP-HPLC %Purity data and the RP-HPLC potency data agree to within approximately 2%, but a trend toward slightly higher potency values compared with %Purity values may indicate that the RP-HPLC %Purity method is slightly more selective than the RP-HPLC potency method.

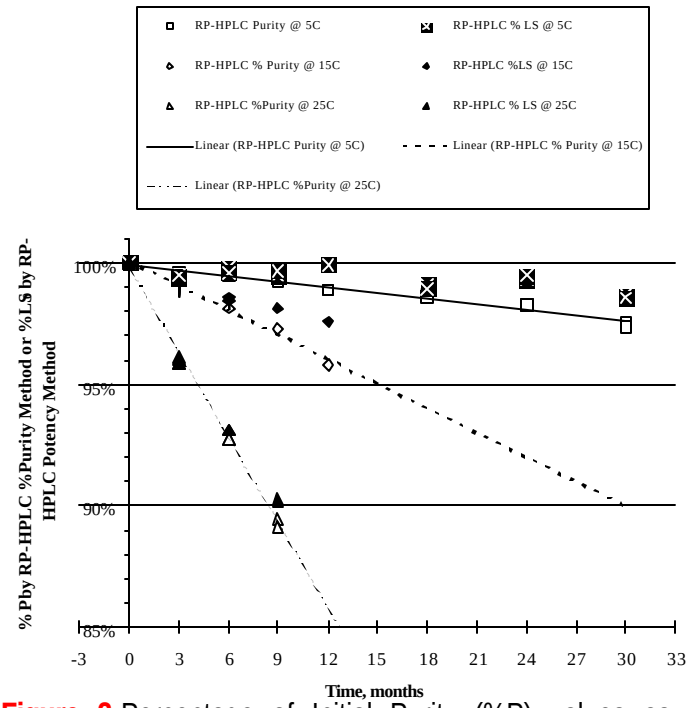
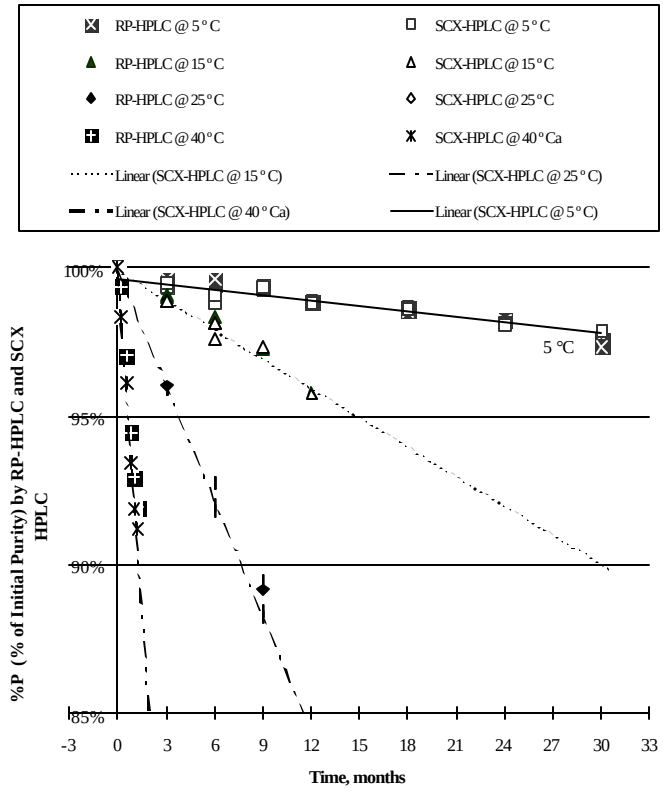


Figure 2. Percentage of Initial Purity (%P) values as determined by RP-HPLC and SCX-HPLC %Purity methods for pramlintide injection pH = 4.0 samples maintained at 5, 15, and 25°C.



Figures 2 and **3** demonstrate that pramlintide injection drug product samples maintained at 5° C are extremely stable, with less than approximately 2% loss in either %P or %LS over a 30-month period.

Rate Constants for %Purity Loss as a Function of Temperature for Samples Formulated at pH = 4.0

Table 4 shows zero-order rate constant (k_0) values for pramlintide degradation at 5, 15, 25, 30, 40, and 50° C.

Table 4. Zero-Order Rate Constant (k_0) Values for Pramlintide Degradation at pH = 4.0 as Determined* by SCX-HPLC %Purity Method

Lot	Temp	I_0	$-k_0$		R^2
number	°C	% Initial	%/mo	95 % CI	
3	50	99.6	23.1	0.997	0.996
1	40	99.9	7.68	0.94	0.992
4	40	100.1	8.34	1.17	0.994
3	30	100.6	2.99	0.47	0.952
1	25	99.5	1.29	0.26	0.988
2	25	99.8	1.29	0.060	0.998
1	15	99.8	0.402	0.20	0.934
2	15	99.9	0.333	0.076	0.962
2	5	99.7	0.000609	0.000125	0.895

*Calculated according to equation 1.

Figure 3. (left) Percentage of Initial Purity (%P) values as determined by RP-HPLC %Purity method and percentage of initial potency (%LS) values as determined by RP-HPLC potency method for pramlintide injection pH = 4.0 samples maintained at 5, 15, 25, and 40 °C.

Four different pramlintide injection drug product lots were used for these determinations, all made to pH = 4.0. The k_0 values were determined by least-squares linear regression analysis according to equation 1. Because the 3 analytical test methods employed showed essentially equivalent stability specificity (*vide supra*), **Table 4** shows data determined by the SCX-HPLC %Purity method only. **Table 4** also shows: regression intercept (I_0)

values, squared correlation coefficient (R^2) values for the regressions, and 95% confidence interval (CI) values for the k_0 values. All k_0 values in **Table 4** achieve statistical significance as indicated by k_0 values exceeding the 95% CI values

Figure 4 is a plot of $\ln(k_0)$ values versus reciprocal absolute temperature, according to equation (4):

$$\ln(k_0) = A - E_a/RT \qquad (4)$$

where A is the Arrhenius frequency factor, E_a is the activation energy (cal/mol), T is absolute temperature (° K), and R is the gas constant (1.98 cal/mol-°).

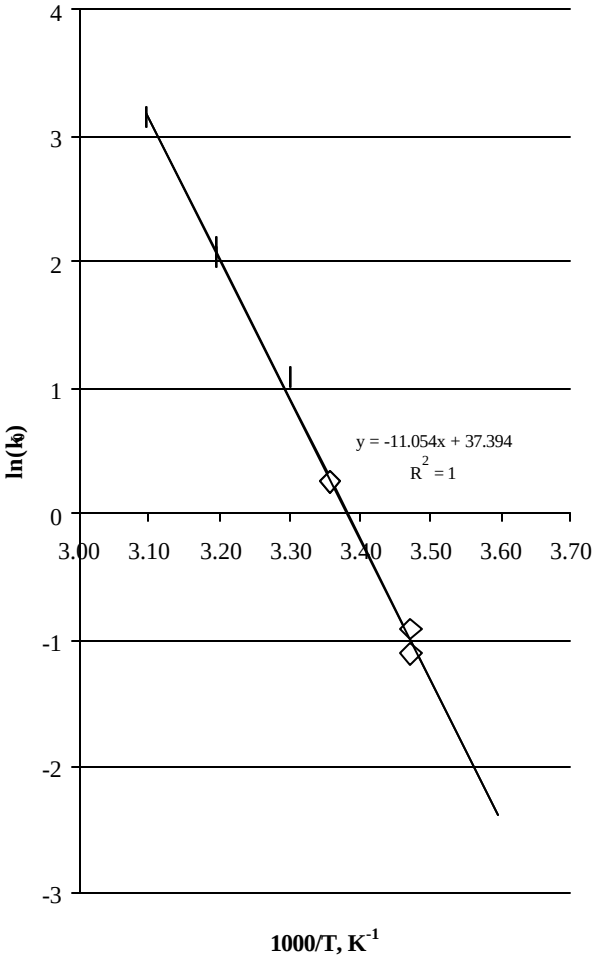


Figure 4. Natural logarithm of zero[th]-order degradation rate constant (k_0) values versus reciprocal absolute temperature as determined by SCX-HPLC %Purity method for pramlintide injection samples at pH = 4.0**.**

Figure 4 shows data for the 15, 25, 30, 40, and 50° C conditions, but excludes the k_0 value for the 5° C condition because the rate constant at 5° C is not statistically significantly different from zero. The figure shows that pramlintide degradation kinetics adhere well to equation (4) over the temperature range studied. From **Figure 4 ,** the Arrhenius frequency factor is 37.394 and the activation energy (slope*R) is 21 900 cal/mol.

pH Effect on Pramlintide Degradation Kinetics at 40° C

Table 5 shows k_0 values for pramlintide injection samples formulated at pH = 3.5, 4.0, 4.5, and 5.0 and maintained at 40° C. The k_0 values were determined by the SCX-HPLC %Purity method and calculated according to equation 1. **Table 5** shows that pramlintide degradation rate constant (k_0) values increase approximately 3-fold with increasing pH over the range studied.

Table 5. Zero-Order Rate Constant (k_0) Values for Pramlintide Degradation at 40 C as Determined * by SCX-HPLC % Purity Method

pH †	I_0 % Initial	$-k_0$ %/mo	95 % CI	R^2
3.5	99.6	7.18	1.1	0.993
4.0	100.1	8.34	1.2	0.996
4.5	100.1	13.4	0.45	0.994
5.0	100.0	22.9	1.8	0.998

*Calculated according to equation 1.
†Samples correspond to Lot #4 shown also in Table 4.

DISCUSSION

It has been reported that hydrolytic backbone cleavage and deamidation reactions predominate for peptide samples maintained at acidic pH [10-14]. We previously found that backbone cleavage and deamidation are the primary pathways for pramlintide degradation at pH = 4.0 [8].

This investigation focused on determining pramlintide hydrolysis kinetics over the pH range 3.5 to 5.0 and the temperature range 5°C to 50° C.

The analytical techniques used to monitor pramlintide hydrolysis included two methods for pramlintide %Purity that employ orthogonal separation modes, namely an RP-HPLC %Purity method and an SCX-HPLC %Purity method [9]. This investigation also utilized an RP-HPLC potency method that quantitated pramlintide versus external standards (rather than by internal area normalization). The RP-HPLC potency method provided a third axis of orthogonality for pramlintide degradation testing and is useful for detecting nonspecific physical losses (such as surface adsorption or aggregation) that might go undetected by the %Purity methods.

Figure 2 shows that the RP-HPLC and SCX-HPLC methods provide essentially identical %Purity information for samples formulated at pH = 4.0 and maintained at 5°C to 40 ° C. The equivalence in degradation rates is consistent with our previous observationsthat the RP-HPLC and SCX-HPLC %Purity methods are highly selective and resolve all major degradation products from intact pramlintide[9]. **Figure 2** also shows good adherence to the zero-order kinetic model chosen for this study.

Figure 3 shows agreement to within approximately 2% between the RP-HPLC %Purity method and the RP-HPLC potency method for samples. These results indicate that nonspecific physical losses are not significant for pramlintide in the pH = 4.0 formulation. The data show a possible trend toward slightly higher RP-HPLC potency values relative to the RP-HPLC %Purity values, which may indicate that the RP-HPLC %Purity method better resolves degradation products from intact pramlintide. It is worth emphasizing, however, that the possible bias between the two methods remains very small, even for extensively degraded samples.

The pH-rate dependence seen for pramlintide over the range pH = 3.5 to 5.0 shows that k_0 values increased approximately 3-fold over the range investigated.

Finally, **Figures 2** and **3** demonstrate that pramlintide injection samples formulated at pH = 4.0 are extremely stable under refrigerated storage, with %Purity and potency losses less than 2% over a 30-month period. Similarly, at 25° C (the anticipated patient use condition) the pH = 4.0 formulation offers excellent stability with %Purity and potency losses less than approximately 2% over a 1-month storage interval.

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REFERENCES

1. Young A, Pittner R, Gedulin B, VineW, Rink T. Amylin Regulation of carbohydrate metabolism. *Biochem Soc Trans.* 1995;23:325-331.

2. Scherbaum WA. The role of amylin in the physiology of glycemic control. *Experimental and Clinical Endocrinol and Diabetes. 1998;*106(2):97-102.

3. Amiel S. Amylin and diabetes. *Lancet.* 1993;341:1249-1250.

4. Janes S, Gaeta L, Beaumont K, Beeley K, Rink T. The selection of pramlintide for clinical evaluation. *Diabetes.* 1996;45(Suppl 2):235A.

5. Thompson RG, Gottlieb A, Organ K, Kolterman OG. Pramlintide, a human amylin analog reduced post-prandial plasma glucose, insulin and c-peptide concentrations in patients with type II diabetes. *Diabetic Medicine.* 1997;14(7):547-555.

6. Thompson RG, Peterson J, Gottlieb A, Mullane J. Effects of pramlintide, an analog of human amylin, on plasma glucose profiles in patients with IDDM: results of a multi-center trial. *Diabetes.* 1997;46(4):632-636.

7. Brower V. Amylin's pramlintide best of bad bunch of diabetes drugs. *Nature Biotechnol.* 1997;15(10):935.

8. Hekman C, Demond W, Dixit T, et al. Isolation and identification of peptide degradation products of heat stressed pramlintide injection drug product. *Pharm Res.* 1998;15:650—659.

9. Demond W, Lokensgard D, Kelley P, Herman K, Weilersbacher G, Kenley R. Orthogonal HPLC methods for quantitating related substances and degradation products of pramlintide, *AAPS PharmSciTech*,2000; 1 (1) article 6 (http://www.pharmscitech.com).

10. Ahern TJ, Manning MC, eds. *Stability of Protein Pharmaceuticals. Part A. Chemical and Physical Pathways of Protein Degradation.* New York, NY: Plenum Press; 1992.

11. Manning MC, Patel K, Borchardt RT. Stability of protein pharmaceuticals. *Pharm Res.* 1989;6:903-917.

12. Patel K, Borchardt RT. Chemical pathways of degradation III. Effect of primary sequence on the pathways of deamidation of asparaginyl residues in hexapeptides. *Pharm Res.* 1990;7:787-793.

13. Capasso S, Mazzarella L, Zagari A. Deamidation via cyclic imide of asparaginyl peptides: dependence on salts, buffers, and organic solvents. *Peptide Res.* 1991;4:234-241.

14. Robinson AB, Scotchler JW, McKerrow JM. Rates of nonenzymatic deamidation of glutaminyl and asparaginyl residues in pentapeptides. *J Am Chem Soc.* 1973;85:8156-8159.