Isolation and Identification of Peptide Degradation Products of Heat Stressed Pramlintide Injection Drug Product

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Purpose. This report summarizes the identification of nine deamidation and four hydrolysis products from a sample of pramlintide injection final drug product that was subjected to stress at 40°C for 45 days.
Methods. The pramlintide degradation products were isolated by strong cation exchange HPLC followed by reversed-phase HPLC. Subsequent to isolation, the molecular weight of each component was determined by liquid chromatography-mass spectrometry (LC/MS). Further characterization was accomplished by amino acid sequence analysis and/or enzymatic (thermolysin) digestion followed by LC/MS and sequence analysis.
Results. The isolated products were identified as [iso-Asp^{21}]-pramlintide, [iso-Asp^{3}]-pramlintide, and [iso-Asp^{22}]-pramlintide, the deamidation products of pramlintide with rearrangement at Asn^{21}, Asn^{3}, and Asn^{22}, respectively. Also found were [Asp/iso-Asp^{14}]-pramlintide, and [Asp/iso-Asp^{35}]-pramlintide, the deamidation products at Asn^{14}, and Asn^{35}, and [Asp^{21}]-pramlintide together with [Asp^{22}]-pramlintide. For the deamidations at the 14th and 35th residues, it could not be determined whether the substance corresponded to the Asp or the iso-Asp product. The [Asp^{21}] and [Asp^{22}] products could not be separated from each other chromatographically but were both identified in a single fraction. Two minor degradation products were also identified as deamidated species. However, the sites of deamidation remain unknown. Also identified were [1-18]-pramlintide, [1-19]-pramlintide, [19-37]-pramlintide, and [20-37]-pramlintide, the products of hydrolytic peptide backbone cleavage at amino acids His^{18}/Ser^{19} and Ser^{19}/Ser^{20}, respectively. One other product was isolated and tentatively identified as a cyclic imide intermediate preceeding deamidation.
Conclusions. The primary mode of thermally induced degradation for this peptide is deamidation. A second degradation mechanism is peptide backbone hydrolysis.

KEY WORDS: deamidation; hydrolysis; pramlintide; degradation products; heat stress; peptide.

INTRODUCTION

Human amylin, discovered in 1987, is co-secreted with human insulin from pancreatic β-cells in response to nutrients (1). It is now clear that amylin acts as a partner hormone to insulin in controlling blood glucose and other metabolic processes after meals (2). In type I diabetics, amylin and insulin appear to be absent. In insulin-resistant type II diabetes, high levels of insulin and amylin are found in the blood plasma (3). In late-stage type II diabetics, however, both endogenous amylin and insulin are present at abnormally low levels (1).

Pramlintide is a synthetic analog of human amylin in which residues 25, 28 and 29 of the human amylin are replaced with prolines. This peptide is currently under development (4) as an amylin hormone replacement therapy for treatment of diabetes. The pramlintide peptide (Fig. 1) contains 37 amino acids with a disulfide bridge between cysteines 2 and 7 and a C-terminal amidated tyrosine residue. Both the disulfide bridge and the C-terminal amidation are required for retaining full biological activity (5). Pramlintide also contains a significant number of labile residues, including six asparagines and one glutamine in positions 3, 14, 21, 22, 31, 35, and 10, respectively, as well as three serines and one histidine in positions 19, 20, 34, and 18, respectively. Thus, thermal degradation of this peptide in aqueous solution is anticipated to generate multiple products including several deamidation species. It is well known that deamidation is a major degradation pathway contributing to the instability of peptides (6,7), and that the diversity of deamidation products can be influenced by both endogenous (primary, secondary, and higher orders of intrinsic structures) (8–10) and exogenous factors (temperature, pH, buffer species, and excipients) (7, 11–14).

In this study, degradation of pramlintide was conducted at 40°C in pH 4.0 aqueous solution. Degradation products were isolated and characterized by mass spectrometry and, where sufficient material was available, amino acid sequence analysis. Nine deamidation products were found. Deamidation sites were identified for seven of these products. In addition to deamidation, a second degradation pathway involving peptide backbone hydrolysis at the His^{18}-Ser^{19} and Ser^{19}-Ser^{20} bonds was also observed. As the more common motifs for peptide hydrolysis do not generally involve serine residues, these hydrolysis products were unexpected.

MATERIALS AND METHODS

Materials

Sodium perchlorate, potassium phosphate, sodium hydroxide, o-phosphoric acid, acetic acid, sodium iodide, isopropanol, and acetonitrile were from Fisher (Springfield, NJ). Trifluoroacetic acid (TFA) was from Pierce (Rockford, IL). Ammonium bicarbonate was from Sigma (St. Louis, MO). EDTA was from Acros (distributed by Fisher). Thermolysin was from CalBiochem (San Diego, CA).

The HPLC equipment employed included a Hewlett Packard (Fullerton, CA) 1090 pump, injector, detector, and column

K-C-N-T-A-T-C/A-T-Q-R/L-A-N-F/L-V-H-S-
└—S–S—┘

S-N-N/F-G-P-I-L-P-P-T-N/V-G-S-N-T-Y-NH_2

Fig. 1. Primary Amino Acid Sequence of Pramlintide. Thermolysin cleavage sites are indicated with dashes. Bolded slashes indicate cleavages observed after 10 min. Lighter slashes indicate additional cleavages induced by allowing incubation to proceed for 300 min.

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oven controlled via Hewlett Packard's Chemstation, Version A.04.01. Also used were Waters (Milford, MA) 625 and 616 pumps and controllers, a Wisp 717 injector, and Model 486 detector.

Preparation of Heat Stressed FDP

Pramlintide injection final drug product (FDP) is a preserved liquid formulation. The samples used in this study contained 0.3 mg/mL pramlintide in a pH 4.0 formulation buffer, sterile filtered into 5-mL USP Type I glass vials with bromobutyl rubber closures. These samples were incubated at 40°C for 45 days and then stored at −70°C until processed.

Solid Phase Extraction (SPE)

Prior to sample analysis, excipients were removed from the samples by SPE to prevent them from obscuring any degradation products. SPE used 6-mL wide bore butyl (C_4) SPE cartridge columns (Mallinckrodt Baker, Phillipsburg, NJ). The cartridges were conditioned with acetonitrile and equilibrated in water. The sample was loaded onto the cartridge, washed with water, and eluted with 40% acetonitrile/0.1% TFA. The collected sample was evaporated to dryness in a SpeedVac® (Savant, Farmingdale, NY) and dissolved in 30 mM sodium acetate, pH 4.0, at approximately 2 mg/mL.

Strong Cation Exchange (SCX) HPLC

The SCX column employed was a PolySULFOETHYL Aspartamide column, 100 mm × 4.6 mm, 5 μm particle size, 300 Å pore size (Poly LC, Columbia, MD). The column temperature was 40°C with an autosampler temperature of 6°C. The solvent flow rate was 0.8 mL/min. The detection wavelength was 220 nm. The separation was achieved by gradient elution from 98% mobile phase 1 (5 mM KH_2PO_4, 5 mM $NaClO_4$, 40% CH_3CN, pH 5.8), 2% mobile phase 2 (5 mM KH_2PO_4, 260 mM $NaClO_4$, 40% CH_3CN, pH 5.8) to 24% mobile phase 2 over 24 minutes. This was followed by isocratic elution from 24–61 minutes with 24% mobile phase 2. A final gradient from 24% to 88% mobile phase 2 in 61–81 minutes was employed to elute the highly retained species. Column loads were 50 μg for analytical analyses and 500 μg for preparative runs. The eluate from several preparative SCX runs were pooled prior to further analysis and/or isolation by reversed-phase HPLC.

Reversed-phase HPLC

The reversed-phase column was a Waters Symmetry C_8, 250 mm × 4.6 mm, 5 μm particle size, 100 Å pore size. The column temperature was 55°C with an autosampler temperature of 6°C. The solvent flow rate was 0.5 mL/min. The detection wavelength was 220 nm. The separation was achieved by gradient elution from 100% mobile phase 1 (0.2M KH_2PO_4, 6.05% CH_3CN, pH 3.0) to 100% mobile phase 2 (0.2M KH_2PO_4, 22.45% CH_3CN, pH 3.0) over 16 minutes. This was followed by isocratic elution with 100% mobile phase 2 from 16-85 minutes. A gradient from 100% mobile phase 2 to 100% mobile phase 3 (0.2M KH_2PO_4, 26.90% CH_3CN, pH 3.0) was employed from 85–100 minutes followed by isocratic elution at 100% mobile phase 3 from 100–110 minutes to elute the highly retained material. Column loads were 50 μg for analytical runs and up to 200 μg for isolation of degradation products.

One fraction collected from SCX chromatography contained a mixture of two components that could not be resolved by the gradient listed above. Separation of these two components was achieved using a gradient from 50% mobile phase 1, 50% mobile phase 3 to 100% mobile phase 3 over a period of 30 minutes. Other operating conditions were unchanged.

Liquid Chromatography/Mass Spectrometry (LC/MS)

Samples were analyzed on a Micromass (Beverly, MA) Quattro IIE triple quadrupole mass spectrometer operating in positive ion electrospray mode. This instrument was configured with a Hewlett Packard 1050 HPLC, Rheodyne 7125 manual injection valve, a LC Packings 20:1 flow splitter to introduce 5% (by volume) to the electrospray ionization source, and a Gilson FC-203 fraction collector to collect the remainder of the sample. Prior to use, the mass scale calibration was performed in positive ion electrospray mode using sodium iodide. Mass spectra were recorded for scanning calibration, scan-speed compensation calibration and static calibration over an acquisition range of 20–2000 Da/e. The calibration range was from m/z 172 to m/z 1971.

Isolated degradation product samples were introduced into the mass spectrometer by reversed-phase gradient HPLC using a Zorbax (Mac-Mod, Chadds Ford, PA) SB-C8 column (4.0 × 12.5 mm, 5.0 μm particle size, or 4.6 × 150 mm, 3.5 μm particle size). The column was equilibrated and loaded in 1.0% acetic acid. Elution was achieved with acetonitrile containing 1.0% acetic acid or 0.05% TFA. The controlling software was MassLynx 2.2 NT. Detection employed an off-axis dynode and photomultiplier, set at 650V. Two scan functions were employed for each sample. The first employed a sample cone voltage of 30V (static) for molecular weight determination. The second utilized a voltage gradient of 18–75V to generate fragment ions. The scan ranges were 400–1500 Da/e, for the static condition, and 300–1500 Da/e for the gradient condition. Scan times were 2.96 s and 3.20 s for the static and gradient conditions, respectively.

The mass spectrometer conditions for LC/MS of thermolysin-digested components were as specified above except the second scan function employed a sample cone voltage of 60V to generate fragment ions. Scan ranges were 220–1500 Da/e for the 30V condition and 50–1500 Da/e for the 60V condition. Scan times were 2.0 and 3.95 s for the 30V and 60V conditions, respectively.

Amino Acid Sequence Analysis

Sequence analysis was conducted by Edman degradation using the ABI 470A and 477A (Applied Biosystems, Inc., Foster City, CA).

Thermolysin Digestion

The components to be digested, (0.5-2.0 μg) were dissolved in 9 μL of 100 mM ammonium bicarbonate, pH 8.5, and equilibrated for 10 min at 37°C. A 1.0-μL aliquot of a 0.1mg/mL thermolysin solution in 100 mM ammonium bicarbonate was added to the samples, after which they were incu-

bated at 37°C for 10 or 300 min. A 1.25-μL aliquot of a 50 mM EDTA solution was added to quench the reaction.

Under the conditions described, thermolysin proteolysis generates a time-dependent fragmentation pattern at hydrophobic amino acids such as leucine and valine. After quenching the reaction with sodium EDTA at 10 min, four peptide fragments, [1-11]-pramlintide, [12-15]-pramlintide, [16-22]-pramlintide, and [23-37]-pramlintide, are formed (see Figure 1). Continued reaction for 5 h yields secondary proteolysis fragments including [1-7]-pramlintide, [8-11]-pramlintide, [12-15]-pramlintide, [16-22]-pramlintide, [23-31]-pramlintide, and [32-37]-pramlintide.

Pramlintide degradation products containing altered amino acid sequences are expected to exhibit thermolysin peptide map(s) with a single peak or peaks of altered retention time. Alternatively, the altered sequence may inhibit thermolysin cleavage in the peptide. This inhibition of cleavage has been observed when the alteration is a deamidation of Asn to either Asp or iso-Asp. For example, the thermolysin cleavage at the Asn^{22}-Phe^{23} bond is inhibited in the pramlintide deamidation products [Asp^{21}]-pramlintide and [iso-Asp^{21}]-pramlintide (data not shown).

RESULTS

Isolation of Degradation Products by Strong Cation Exchange (SCX)

The SCX analytical chromatogram of the heat stressed FDP sample (Fig. 2) revealed the presence of multiple peaks corresponding to degradation products. The work reported here concentrated on the 11 peaks (Table 1 and Fig. 2) which were present in the highest concentration and were well separated from the pramlintide peak. The total extent of degradation after 45 days at 40°C was approximately 12–14%.

Previous work had demonstrated that no single chromatographic step is capable of adequately separating all of the pramlintide degradation products, indicating that multiple chromatographic steps would be required to purify all the components. For this reason and because of the high capacity of the SCX column, the SCX method was used for the initial semi-preparative HPLC isolation of pramlintide degradation products from the heat stressed FDP. Depending on the component(s) isolated, as many as five injections of 500 μg each of the heat stressed FDP (after SPE treatment to remove excipients) were chromatographed by SCX. Fractions were collected throughout the chromatography. The chromatograms of each run were compared and fractions from each run were pooled on the basis of the comparison. Subsequent to pooling, the fractions were analyzed by reversed-phase HPLC to determine if the material collected was homogeneous by reversed-phase chromatography or required further fractionation. The data (not shown) indicated that SCX fractions corresponding to peaks 3, 4, 6, 10, and 11 in Figure 2 contained a single peak. The remaining fractions, corresponding to the remaining SCX peaks, all contained more than one component. The reversed-phase chromatogram of the unfractionated, heat stressed FDP (starting material) is shown in Figure 3. The peak labels correspond to those in Figure 2 and Table 1 and were determined based on the reversed-phase chromatograms of the individual SCX fractions.

Isolation of Degradation Products by Reversed-phase Chromatography

A nitrogen jet was used to remove the acetonitrile from the SCX fractions containing peaks 5 and 7–9 (Figure 2). The remaining material was then loaded onto the reversed-phase column and fractionated as described above. Peaks 5, 7, 7B, 8, 9, and 9B (Fig. 3) were collected. The location of these peaks in the reversed-phase chromatogram can be ascertained from Figure 3. However, since Figure 3 represents an unfractionated sample, peaks 5 and 8 were not resolved from pramlintide in this chromatogram. When SCX fractions were chromatographed by this method all the pramlintide had already been removed by the SCX column so resolution from pramlintide was not an issue. As noted above, six peaks were collected from the reversed-phase chromatography of the four SCX peaks. They correspond to the original SCX peaks 5 and 7–9, plus two additional peaks not separated by the SCX chromatography. One of these peaks, peak 7B, eluted from the SCX column between peak 7 and peak 8. The other, peak 9B, co-migrated by SCX with peak 9. Peaks 9 and 9B were present in approximately a 2:1 ratio (based on reversed-phase chromatography). Thus, a total of thirteen components were collected from the original eleven SCX peaks (Table 1).

Analysis of components 1 and 2 from the SCX fractionation by reversed-phase chromatography revealed the presence of two degradation products that were poorly resolved from each other. Therefore, the reversed-phase gradient was modified, as specified above, to resolve these two peaks.

Mass Spectrometric Characterization of Degradation Products

The isolated components were analyzed using the LC/MS conditions described above. LC/MS was employed rather than MS because the purified components contained either phosphate or sodium perchlorate from the SCX and reversed-phase purification steps. Thus, the LC step was used both to desalt and further purify each component prior to MS analysis. Mass data obtained under low sample voltage conditions (Table 1) reveal the presence of singly- or multiply- protonated molecular ions that can be used to obtain the molecular weight of the molecule. These data were transformed into the mass domain by the MassLynx software. The molecular weights (column 4, Table 1) were obtained from the centroided transformed electrospray mass spectra (not shown). Under elevated voltage sample cone conditions, fragment ions were produced by collision induced dissociation (CID) which facilitated structural elucidation (columns 5–6, Table 1). Structural assignments of peptide fragment ions followed the convention of Carr, *et. al.* (15). The fragments identified with the "y" designation result from amide bond cleavage and contain the C terminus. The "b" fragments result from amide bond cleavage and contain the N terminus. The b fragment is a direct cleavage product while the y fragment is generated by rearrangement.

With the exception of peak 7B, all the components appeared to be either hydrolysis (components 1, 2, 10, 11) or deamidation products of pramlintide (components 3–9 and 9B). The deamidation assignments were based on the fact that the experimentally-determined molecular weight was 1 Da greater than the experimentally-determined molecular weight of pramlintide. Examining the sample cone CID mass spectra provided

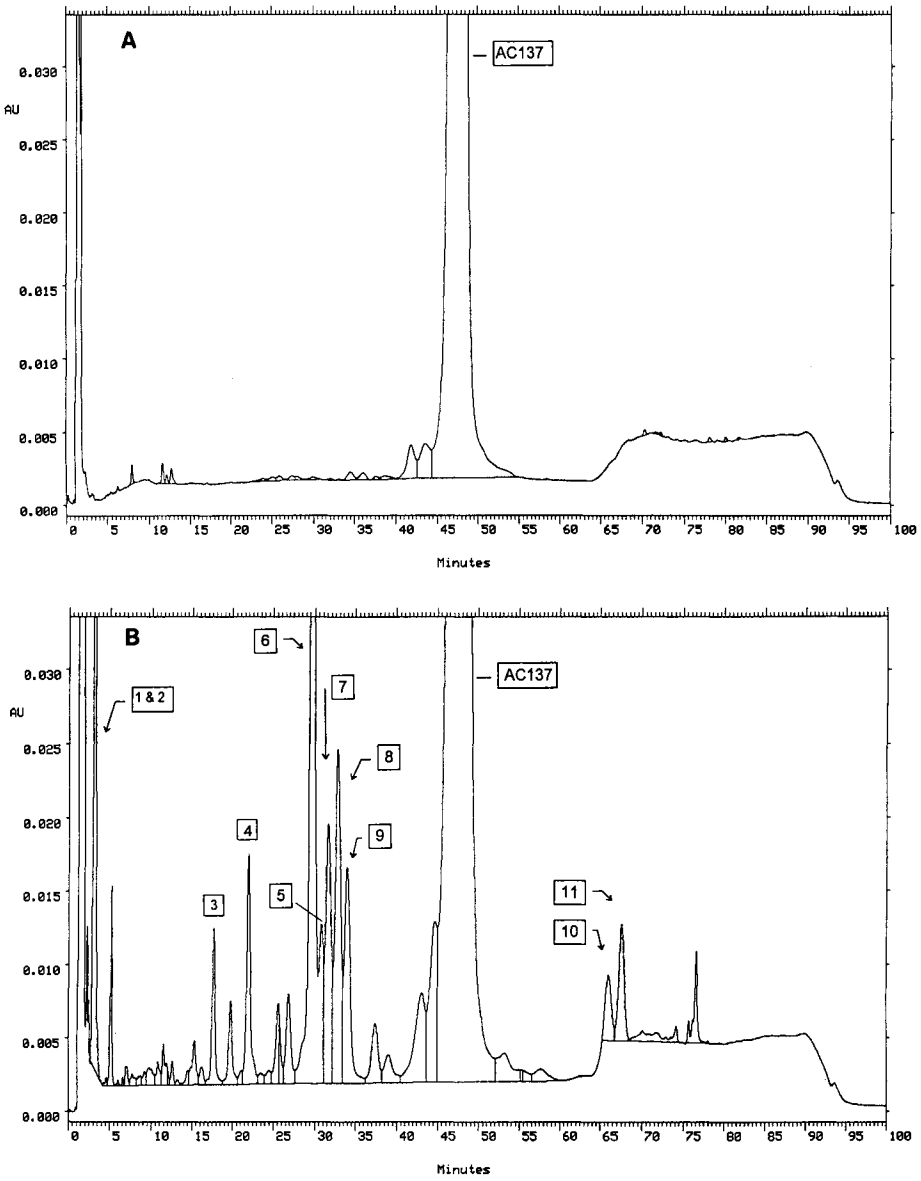

Fig. 2. Analysis of Pramlintide (AC137) Control (panel A) and Heat Stressed (40°C, 45 days) Pramlintide Injection (panel B) by Strong Cation Exchange HPLC. Column load was 50 μg. For peak index assignments, see Table 1.

information on the relative site of deamidation. Fragmentation of pramlintide produced a characteristic ion with the all-^{12}C isotopic peak at m/z 1048.7. This fragment is composed of the last ten residues (28–37) of the pramlintide molecule. When deamidation occurs at Asn^{31}, Asn^{35} or at the tyrosine-amide, the molecular weight of this C-terminal fragment increases by 1 Da and the all-^{12}C isotopic peak appears at m/z 1049.7. When deamidation occurs within the first 27 residues, the all-^{12}C isotopic peak of this C-terminal fragment appears at m/z 1048.7 (Fig. 4). Using this criterion, five of the components were deamidated in the first 27 residues of the molecule while the remaining three were deamidated in the last 10 residues (Table 1).

The molecular weight of component 1 (based on the $[M+2H]^{2+}$ (989.7) and $[M+3H]^{3+}$ (660.0) ions) was 1977.4 Da. The mass search, using BioLynx, listed two candidate sequences, [19-37]-pramlintide and [7-24]-pramlintide. The sample cone CID mass spectrum for this component yielded ions of y''_{10}, m/z 1048.4, and b_9, m/z 930.4, both generated from cleavage at the Leu^{27}-Pro^{28} in pramlintide. These ions would not be expected for [7-24]-pramlintide. Thus, the observed fragmentation pattern was consistent with a structure of [19-37]-pramlintide for component 1. When the mass spectrometric data were analyzed for component 2, both the molecular weight and fragmentation data were consistent with a structure of [20-37]-pramlintide.

The molecular weight of component 10, calculated from the $[M+2H]^{2+}$ (995.1), $[M+3H]^{3+}$ (663.9) and $[M+4H]^{4+}$ (498.3) ions, was determined to be 1989.2. The molecular mass search yielded one candidate sequence, [1-18]-pramlintide. Sample cone CID data revealed an extensive series of b-type fragment ions, including y''_8, y''_9, and y''_{10}. These fragment ions

Table 1. Pramlintide Degradation Products

Peak no.[a]	Area % by SCX	Ions (Da/e)	MW (Da)	Key fragments ions (Da/e)[b]	Proposed structure	Comments
1	<1.41	989.7, 660.0	1977.4	1048.4 = y''_{10}; 930.4 = b_9	[19-37]	
2	<1.41	946.2, 631.8	1890.4	1048.5 = y''_{10}; 843.2 = b_8	[20-37]	
3	0.38	988.5, 791.3, 1317.7	3950.4	968.3 = b_{27} (3+); 1048.3 = y''_{10} 1137.9 = b_{32} (3+)	deamidated pramlintide	Modification in 1-27 region
4	0.65	988.7, 791.0, 1317.7	3950.4	968.5 = b_{27} (3+); 1048.4 = y''_{10} 1137.6 = b_{32} (3+)	deamidated pramlintide	Modification in 1-27 region
5	<0.59	988.5, 1317.7, 791.2	3950.7	968.2 = b_{27} (3+); 1048.5 = y''_{10} 1137.6 = b_{32} (3+)	deamidated pramlintide	Modification in 1-27 region
6	3.21	988.4, 659.5, 790.9, 1317.6	3949.8	1048.4 = y''_{10}; 754.6 = b_{12} (+3) 792.4 = b_{22} (+3)	deamidated pramlintide	Modification in 1-27 region
7	<1.19	988.7, 791.0, 1317.6	3950.7	968.2 = b_{27} (3+); 1048.8 = y''_{10} 1137.7 = b_{32} (3+)	deamidated pramlintide	Modification in 1-27 region
7B	0.39[c]	984.1, 787.4	3932.6	962.5 = b_{27} (3+); 1048.7 = y''_{10} 1131.8 = b_{32} (3+)	pramlintide-NH_3 or pramlintide-H_20	Modification in 1-27 region
8	<1.52	988.5, 1317.9, 791.0	3950.6	967.9 = b_{27} (3+); 1049.5 = y''_{10} 1137.6 = b_{32} (3+)	deamidated pramlintide	Modification in 28-37 region
9	~0.69[d]	988.7, 1317.9, 791.2	3950.7	968.1 = b_{27} (3+); 1049.4 = y''_{10} 1137.6 = b_{32} (3+)	deamidated pramlintide	Modification in 28-37 region
9B	~0.35[d]	988.5 1317.4, 791.0	3950.5	968.1 = b_{27} (3+); 1049.4 = y''_{10} 1137.2 = b_{32} (3+)	deamidated pramlintide	Modification in 28-37 region
10	0.33	995.1, 663.9, 498.3	1989.2	600.1 = y''_{10} (+2)[e], 549.5 = y''_9 (+2) 931.0 = y''_{17} (+2)	[1-18]	
11	0.52	1038.6, 692.97, 520.11	2076.4	643.4 = y''_{11} (+2); 917.7 = b_{17} (+2) 974.4 = y''_{18}(+2)	[1-19]	

[a] See Figure 2.
[b] The fragments identified with the "y" designation result from amide bond cleavage and contain the C terminus. The "b" fragments result from amide bond cleavage and contain the N terminus. The b fragment is a direct cleavage product while the y fragment is generated by rearrangement.
[c] From reversed-phase chromatography rather than SCX because peak was separated from the neighboring peaks by reversed-phase.
[d] Components 9 and 9B migrate as a single peak on SCX with a total area percent of 1.04%. Analysis by reversed-phase indicates the ratio of 9 to 9B is approximately 2:1.
[e] Together with other b type fragment ions, the data yield a sequence [8-18] = ATQRLANFLVH.

provided sequence data for residues 8-18. Also, the presence of the N-terminal lysine was determined by fragmentation of the quadruply and triply protonated molecular ion to the y''_{17} ion, m/z 931.0. Thus, the molecular weight and fragmentation pattern are consistent with a structure of [1-18]-pramlintide for component 10.

When component 11 was analyzed in a similar manner, the search yielded two potential candidates, [1-19]-pramlintide and [7-25]-pramlintide. Because the signal for this component was weaker than that for component 10, sequence continuity could not be established. However, fragment ions such as m/z 974.4, representing the loss of lysine from the N-terminal residue, and m/z 917.7, representing the loss of His and Ser from the C-terminus, are consistent for a component with the sequence of [1-19]-pramlintide.

The 7B component had a molecular weight consistent with pramlintide which had lost either an ammonia or water molecule. This substance may represent a cyclic intermediate between pramlintide and deamidated pramlintide or, alternatively, a dehydrated serine residue. Unfortunately, the amount of this material collected was not sufficient for further identification.

Thermolysin Digestion and Sequence Analysis

Components 3, 4, 5, 7, and 8, all deamidation products, were enzymatically digested with thermolysin as described above. The digested fractions were separated and analyzed by LC/MS. The aberrant thermolysin fragments (relative to pramlintide) were collected during the analysis by LC/MS (Table 2) for sequence analysis (Table 3). All five thermolysin-digested deamidation products yielded 1 or 2 aberrant fragments when analyzed by LC/MS.

Identification of [iso-Asp^{21}]-Pramlintide

Component 6 was present in the FDP sample at approximately 3%. Because of the large quantity of material available, direct sequencing of this component was expected to yield at least the first 27 residues, without isolation of the affected thermolysin fragment. Sequencing of component 6 proceeded through the first 20 residues before terminating at position 21 (Table 3), indicating that the site of deamidation was not Asn^3, Gln^{10}, or Asn^{14}. Since this component was determined by mass spectrometry to be a deamidation product, with the modification in the first 27 residues of the pramlintide, the only possible sites were Asn^{21} and Asn^{22}. Sequence termination at position 21 was consistent with the presence of iso-aspartic acid, a beta amino acid that cannot be sequenced by Edman chemistry, at position 21. In addition, the relative retention time of an authentic standard of [iso-Asp^{21}]-pramlintide on both the SCX and reversed-phase methods (data not shown) were consistent with the assignment of component 6 as [iso-Asp^{21}]-pramlintide.

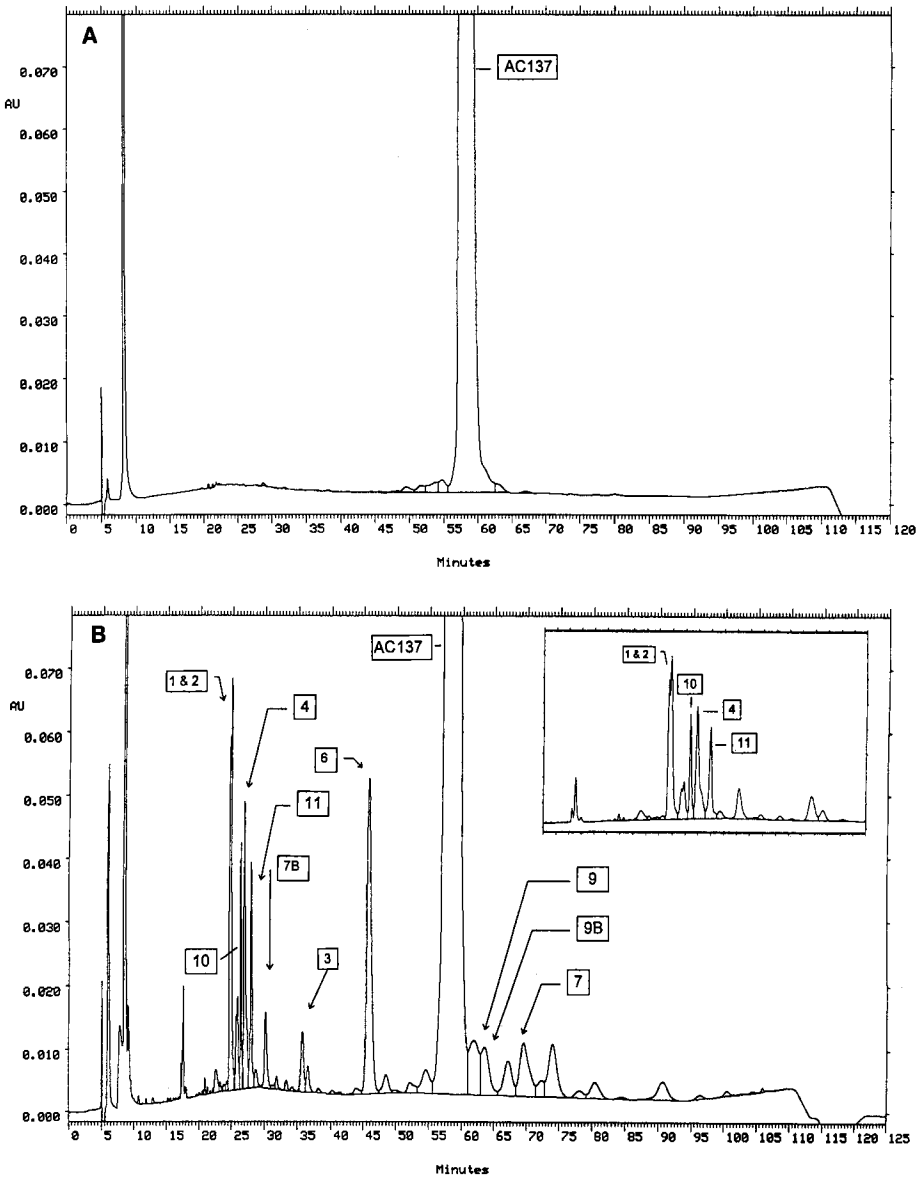

Fig. 3. Analysis of Pramlintide (AC137) Control (panel A) and Heat Stressed (40°C, 45 days) Pramlintide Injection (panel B) by Reversed-phase HPLC. Column load was 50 μg. Peaks 5 and 8 (see Table 1) are not resolved from pramlintide by this reverse-phase method. Panel B inset shows expanded view from 15 to 40 min.

Identification of Pramlintide Deamidation Products [iso-Asp[3]], [Asp/iso-Asp[14]], and [Asp/iso-Asp[35]]

There are two possible sites for deamidation in the thermolysin fragment 1-11, Asn^3 and Gln^{10}. The presence in the 1-11 thermolysin fragment of component 3 of a fragment ion at m/z 475, which contains Gln^{10} (Table 2), indicated that component 3 is the [Asp/iso-Asp^3] product of pramlintide rather than the [Glu^{10}] product. If the Gln^{10} was converted to Glu^{10} this fragment ion would have appeared at m/z 476. Sequence data obtained for component 3 thermolysin fragment 1-11 (Table 3) supported the assignment of this substance as [iso-Asp^3]-pramlintide.

The pramlintide thermolysin fragment 12–15 has only one possible sites for deamidation, Asn^{14}. Thus, the data indicate that component 4 corresponds to the [Asp/iso-Asp^{14}] product of pramlintide. The mass data added support to this assignment in that the full sequence of the thermolysin fragment was obtained from C-terminal fragment ions at m/z 352 (Ala-Asp-Phe), m/z 281 (Asp-Phe), and m/z 166 (Phe), and from N-terminal fragment ions m/z 300 (Leu-Ala-Asp), m/z 185 (Leu-Ala), and m/z 86 (Leu-CO_2). A similar fragmentation pattern was also observed from the 12-22 thermolysin fragment from component 4.

The pramlintide thermolysin fragment 32-37 has two potential deamidation sites, Asn^{35} and the C-terminal tyrosine. Thus, component 8 could be either the C-terminal deamidation product or the [Asp/iso-Asp^{35}]-pramlintide. The sample cone CID fragmentation pattern of the 32-37 thermolysin fragment

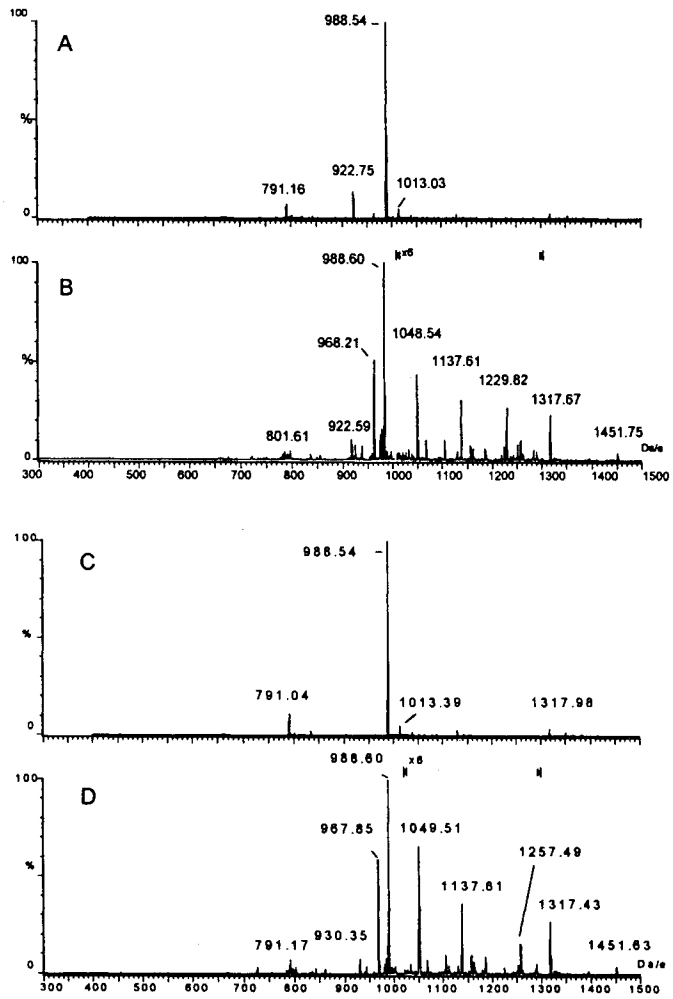

Fig. 4. Summed and background-subtracted mass spectra of components 5 (panels A and B) and 8 (panels C and D). Panel A and C were acquired under low sample cone voltage conditions and panels B and D were acquired under elevated sample cone voltage conditions.

from component 8 has fragment ions at m/z 460 and m/z 359 (Table 2), corresponding to the loss of tyrosine amide and the dipeptide threonine-tyrosine amide. If deamidation occurred at the tyrosine amide, these ions would appear at m/z 459 and m/z 358. Also, the 23-37 thermolysin fragment has fragment ions at m/z 1396 and 181 (data not shown), representing loss of tyrosine amide neutral and protonated tyrosine amide from this structure. Thus, the data indicate that component 8 is [Asp/iso-Asp^{35}]-pramlintide.

Insufficient material was collected from components 4 and 8 to obtain sequence data. Furthermore, low collision energy CID spectra do not provide the fragmentation information to distinguish between Asp and iso-Asp residues. Thus, we were not able to elucidate whether these products correspond to the Asp or the iso-Asp product.

Identification of [iso-Asp^{22}]-, [Asp^{21}]-, and [Asp^{22}]-pramlintide

The aberrant fragment identified for both components 5 and 7, thermolysin fragment 16-37, contains Asn residues at positions 21, 22, 31, and 35. The failure of thermolysin to cleave at the Asn^{22}-Phe^{23} bond, however, suggests that both deamidations occurred at either Asn^{21} or Asn^{22}. The mass spectrometric data (Table 1), indicating deamidation to be prior to position 27 for both components, adds support to this concept. Since sample cone CID does not produce fragmentation to distinguish between deamidation at position 21 and 22, with or without rearrangement, these sequences could not be determined by LC/MS. Sequence analysis of the 16-37 thermolysin fragment isolated from component 5 indicated that the Asn at position 21 was intact. However, the sequence signal was too low subsequent to the analysis of Asn^{21} to conclusively identify residue 22. These data suggest that component 5 was either [Asp^{22}]- or [iso-Asp^{22}]-pramlintide. A comparison of the relative retention times of component 5 on both SCX and reversed-phase chromatography with those for the authentic materials indicated a match to [iso-Asp^{22}]-pramlintide but not to [Asp^{22}]-pramlintide. Together the data show that component 5 is [iso-Asp^{22}]-pramlintide.

Table 2. Mass Spectrometric Data for Thermolysin Fragments of Pramlintide Degradation Products

Peak number[a]	Aberrant fragment	Digestion time (min)	Ions (Da/e)	Molecular weight (Da)	Key fragment ions (Da/e)	Proposed structure
3	[1-11]	10	599, 400	1195	475	Asp/iso-Asp^{3}
4	[12-15] & [12-22][b]	10	465, 609	464, 1217	352, 281, 166 1103, 1033, 918	Asp/iso-Asp^{14}
5	[16-37]	10	1165, 790	2326	641, 1048	Asp/iso-Asp$^{21 \text{ or } 22}$
7	[16-37]	10	1165, 790	2326	641, 1048	Asp/iso-Asp$^{21 \text{ or } 22}$
8	[32-37] & [23-37][c]	300	640	639	460, 359	Asp/iso-Asp^{35}

[a] See Table 1 and Figure 2.
[b] cmThese data are consistent with a deamidation at position 14 inhibiting the thermolysin digestion at the Phe^{15}/Lys^{16} bond.
[c] The 5 hour thermolysin digests do not result in complete conversion of the 23-37 fragment into the 23-31, and 32-37 fragments. Thus, the modification was evident in both fragments.

Table 3. Sequence Analysis Data for Thermolysin Digested Fragments of Pramlintide Degradation Products

Peak number[a]	Aberrant fragment	Amino acid sequence	Proposed structure
3	[1-11]	K[c]	iso-Asp^3
4	[12-15] & [12-22]	No Sequence Observed[d]	N/A
5	[16-37]	LVHSSNn[e]	Asp/iso-Asp^{22}
6	Full[b]	KXNTATXATQRLANFLVHSS	iso-Asp^{21}
7	[16-37]	N/D[f]	N/A
8	[32-37]	No Sequence Observed[d]	N/A
10	Full	KXNTATXATQRLANFLVH	[1-18]
11	Full	KXNTATXATQRLANFLVHS	[1-19]

[a] See Table 1 and Figure 2.
[b] No thermolysin digestion was performed. Entire isolated component was submitted for sequence analysis.
[c] Edman degradation sequence analysis cannot identify underivatized PTH-Cys (residue 2 of pramlintide). Therefore no signal was observed at residue position 2. PTH-Asn signal is expected at residue position 3 unless the amino acid at position 3 is an iso-amino acid or does not exist due to a peptide bond cleavage after Lys^1 or Cys^2. Thus these data, together with the mass spectrometric assignment of this substance as a deamidation product support the assignment of this substance as iso-Asp^3.
[d] Quantity submitted not sufficient for sequence.
[e] The observed signal of PTH-Asn at the theoretical Asn^{22} position was not sufficient to give a conclusive identification.
[f] N/D = Not done. Quantity collected was not sufficient for sequence analysis.

Sequence analysis of the 16-37 thermolysin fragment from component 7 could not be performed due to insufficient material. A comparison of relative retention times from reversed-phase and SCX chromatography of the sample with those for authentic materials revealed a match to both [Asp^{21}] and [Asp^{22}]-pramlintide. In a further attempt to distinguish which deamidation product corresponded to component 7, the original stressed FDP was spiked with authentic [Asp^{21}]-pramlintide and [Asp^{22}]-pramlintide. The spiked samples were analyzed by reversed-phase chromatography. These data (Figure 5) revealed that component 7 migrated with both the [Asp^{21}]- and [Asp^{22}]-pramlintide products. The [Asp^{21}] product aligned with the apex while the [Asp^{22}] product aligned more with the descending tail of component 7. In addition, a review of the reversed-phase analysis of the fractions collected from SCX revealed the presence of a shoulder running on the descending side of component 7 (data not shown) Since the mass spectrometric data did not reveal the presence of two substances in component 7, the mass spectral fragmentation patterns of both must be the same, as is the case for the [Asp^{21}]- and [Asp^{22}]-pramlintide products. Together these data suggest that component 7 contains

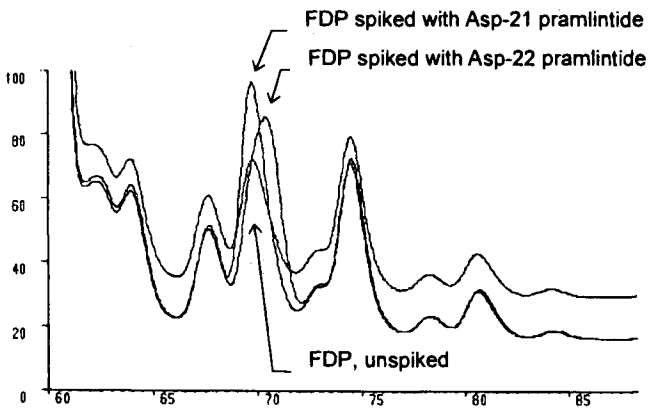

Fig. 5. Reversed-phase Analysis of Pramlintide (AC137) Injection Sample (40°C, 45 days) alone or spiked with [Asp^{21}]-pramlintide or [Asp^{22}]-pramlintide. Data are shown for analysis from 60–90 min only.

both the [Asp^{21}]- and [Asp^{22}]-pramlintide. Since both [iso-Asp^{21}]-pramlintide and [iso-Asp^{22}]-pramlintide have been identified in this sample, the identification of both the [Asp^{21}] and [Asp^{22}] products was not unexpected.

Deamidation in Residues 28-37

Components 9 and 9B were not collected in sufficient quantities to undertake sequence analysis. Molecular weight analysis of the components indicated (see above) that they corresponded to deamidation products in the 28-37 region of pramlintide. Thus, they were assigned as either [Asp/iso-Asp^{31}]-pramlintide, or the counterpart (Asp or iso-Asp) of the deamidation product at position 35 (component 8), or the C-terminal deamidation product of pramlintide.

Identification of [1-18] and [1-19]-Pramlintide

Components 10 and 11 were not subjected to thermolysin digestion because they appeared small enough to sequence in the absence of digestion. The sequence results (Table 3) indicated that component 10 consisted of the first 18 amino acid residues of pramlintide. Similarly, component 11 was identified as [1-19]-pramlintide. In addition, the relative retention times determined for these components by reversed-phase and SCX chromatographies corresponded to the relative retention times of authentic [1-18]- and [1-19]-pramlintide.

DISCUSSION

This report summarizes the isolation and identification of 13 components in a sample of pramlintide injection FDP that was stressed at 40°C for 45 days. Each component was present at greater than 0.3% of the total peak area. The total loss in pramlintide peak area (total degradation), relative to the control, was 12–14%.

The molecular weight of each of the 13 components was determined (Table 1). These data, together with MS fragmentation patterns, amino acid sequence analysis, and thermolysin digestion with sequence analysis, allowed the identification of

[iso-Asp^3]-pramlintide, [Asp/iso-Asp^{14}]-pramlintide, [iso-Asp^{22}]-pramlintide, [iso-Asp^{21}]-pramlintide, and [Asp/iso-Asp^{35}]-pramlintide. In addition, component 7 was identified as containing both [Asp^{21}]- and [Asp^{22}]-pramlintide. Also identified were the four hydrolysis products, [1-18]-pramlintide, [1-19]-pramlintide, [19-37]-pramlintide, and [20-37]-pramlintide. Three additional degradation products were characterized but not completely identified. These included two additional deamidation products (components 9 and 9B) and component 7B, a possible cyclic imide intermediate preceding deamidation.

A comparison of the peptide sequence of pramlintide with the sequence motifs predicted to be most prone to hydrolytic degradation (18) reveals the absence of the motifs noted for high reactivity: Asn-Gly, Asn-Ser, Asp-Gly, Gln-Gly, and Asp-Pro. Thus, pramlintide is predicted to be relatively stable to chemical degradation in a liquid formulation. In fact, pramlintide degrades quite slowly with only 12% degradation over 45 days at 40°C. Slow degradation at motifs other than "hot spots" does occur in other peptides and proteins, including Interleukin-1α, Interleukin-2, neocarzinostatin, parathyroid hormone and others (18).

The primary determinants of deamidation are backbone chain flexibility together with the presence of small hydrophilic residues surrounding the Asn residue to allow for cyclic imide formation (7,10,11,16,18). 2-D NMR of pramlintide in aqueous solution (data not shown) revealed a randomly coiled, flexible molecule, except for a small helical structure in the region of the disulfide bridge between Cys^2 and Cys^7. Hence, except for Asn^3 all other Asn residues are in flexible regions. The sequences surrounding the deamidated residues in pramlintide include: Cys-Asn-Thr (position 3), Ala-Asn-Phe (position 14), Ser-Asn-Asn (position 21), Asn-Asn-Phe (position 22), and Ser-Asn-Thr (position 35). The single asparagine residue not observed to deamidate, Asn^{31}, has a surrounding sequence of Thr-Asn-Val. The data indicate that the residues most subject to deamidation are Asn^{21} and Asn^{35}. The deamidation at the Asn^{21} motif (Ser-Asn-Asn) was the most prominent degradation peak observed, surpassing the [Asp/iso-Asp^{35}] by approximately 2-fold. It seems likely that the two hydrophilic residues, Ser on the N terminal side of Asn^{21} and Asn on the C-terminal side of Asn^{21}, facilitate deamidation at Asn^{21}. Deamidation at a similar motif, Ser-Asn-Asp, has been reported to be activating in the case of Interleukin-1β (18). Since Asn-Ser is a preferred deamidation sequence, possibly the similarity between Ser and Thr activates the Asn^{35} to deamidate, explaining the propensity to deamidate at position 35. Deamidation at a Ser-Asn-Thr motif was also observed in the Amylin Antagonist, a 24 amino acid peptide, and was explained as being due to the activation of the site by Thr (18).

The deamidation products at positions 22 and 14 are present in this stressed sample in considerably smaller amounts than observed for the Asn^{21} and Asn^{35} deamidation products. This is not unexpected since the C terminal adjacent amino acid in both cases is a Phe residue. Large bulky, hydrophobic groups are predicted to slow the rate of deamidation considerably (7,8,11,16,18).

The deamidation product at Asn^3, in the Cys-Asn-Thr motif was present in the smallest amount (0.38%). This Asn residue is in the most conformationally constrained part of the peptide due to the disulfide bond between Cys^2 and Cys^7. Disulfide bonds have been reported to render Asn residues much less reactive as a result of reduced flexibility (18). Deamidation in a Cys-Asn-Ser motif has been documented in tissue plasminogen activator (19).

In addition to sequence considerations, environmental variables, including buffer species, excipients, temperature, and pH may also play a significant role in determining the rate of peptide deamidation and the product distribution (7,11–14,20). Phosphate, in particular, has been shown to enhance deamidation rates (20). Comparison of Figures 3A and 3B indicates that deamidation does not result from the phosphate present in the mobile phase for the reversed-phase chromatography. Formulation excipients also showed little effect on the rate or nature of degradation (data not shown). As expected, temperature and pH had the greatest effect. In studies not shown here it was found that at pH 3 the iso-Asp products are absent, although the Asp products are still present. Furthermore, at pH 3 the hydrolysis products are considerably more prominent than at pH 4. This change in product distribution with pH is similar to that reported elsewhere (8,21). The absence of iso-Asp products at pH 3 suggests direct deamidation, rather than hydrolysis of a cyclic imide intermediate, as has been observed for other peptides/proteins (7,8,21). The presence, on the other hand, of both the Asp and rearranged iso-Asp products at pH 4 are suggestive of deamidation, at pH 4, through a cyclic imide intermediate. In the case of deamidation at Asn^{21} and Asn^{22} both the Asp and iso-Asp products were observed. Because peak 7 contains both the [Asp^{21}]-pramlintide and [Asp^{22}]-pramlintide products in an unknown ratio it is difficult to determine the ratio of iso-Asp/Asp product for these deamidations, although it is clear in the case of [iso-Asp^{21}]-pramlintide that the iso-Asp/Asp ratio is greater than 2.7. Unfortunately, we were unable to determine whether the deamidation products at positions 14 and 35 were the Asp or iso-Asp product. The deamidation at position 3 was clearly the rearranged product. The [Asp^3]-pramlintide product may also have been present. However, if it was present in significantly lower concentrations than the [iso-Asp^3]-pramlintide (0.38%), as is generally the case, it would not have been isolated. The iso-Asp/Asp ratio for deamidation through a cyclic imide intermediate is generally approximately 3:1(7,11).

A second degradation pathway involves peptide backbone hydrolysis at the His^{18}-Ser^{19} and Ser^{19}-Ser^{20} bonds. The more common motifs for peptide hydrolysis involve Asp-Y and X-Asp residues (12,17,20), though cleavages at Ser residues have been reported as minor degradation products in OKT4a (18) and other peptides (12). Such hydrolysis products in pramlintide were observed principally at lower pH's (data not shown) indicating the role of acid hydrolysis in the formation of these products.

Together these data suggest that the primary mode of thermally induced degradation for this peptide drug product is deamidation. The deamidations, although not occurring at sequence patterns noted for high reactivity, occurred in at least 5 different Asn residues. Two deamidation products, in the C-terminal half of the peptide, remain for which the site of deamidation is unknown. The mechanism of deamidation at pH 4 appears to proceed through formation of a cyclic imide intermediate which breaks down to give both the Asp and the rearranged, iso-Asp products. A second degradation mechanism is hydrolysis on the N-terminal side of both Ser^{19} and Ser^{20}.

The mechanism for this second degradation pathway is under investigation.

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