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# Isolation and identification of cyclic imide and deamidation products in heat stressed pramlintide injection drug product

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**Abstract**

This report summarizes the identification of six cyclic imide [Asu] and two deamidation products from a sample of pramlintide final drug product that had been stressed at 40°C for 45 days. The pramlintide degradation products were isolated by cation exchange high-performance liquid chromatography (HPLC) followed by reversed-phase HPLC. The isolated components were characterized by mass spectrometry (MS), tandem MS (MS/MS) and when necessary, by enzymatic (thermolysin) digestion followed by liquid chromatography/mass spectrometry (LC/MS) and sequence analysis. The isolated products were identified as [$Asu^{14}$]-pramlintide, [$Asu^{21}$]-pramlintide, [$Asu^{22}$]-pramlintide, [$Asu^{35}$]-pramlintide, [1–21]-succinimide-pramlintide, and [1–22]-succinimide-pramlintide. Also identified were [$Asp^{35}$]-pramlintide, the deamidation product of pramlintide at $Asn^{35}$, and [$Tyr^{37}$-OH]-pramlintide, the deamidation product of the pramlintide amidated C-terminal Tyr. Together these data support those presented earlier (C. Hekman et al., Isolation and identification of peptide degradation products of heat stressed pramlintide injection drug product. Pharm Res 1998;15:650–9) indicating that the primary mechanism of degradation for pramlintide in this pH 4.0 formulation is deamidation, with six of the eight possible deamidation sites observed to undergo deamidation. Gln-10 and Asn-31 are the only two residues subject to deamidation for which none is observed. The data indicate that the cyclic imide products account for $\approx 20\%$ of the total thermal degradation while the deamidation products account for $\approx 64\%$. The remaining degradation is due to peptide backbone hydrolysis. 

*Keywords:* Cyclic imide; Deamidation; Pramlintide; Degradation products; Heat stress; Peptide

## 1. Introduction

Human amylin, discovered at Oxford University and first reported 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

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glucose and other metabolic processes after meals [2]. In type I diabetes, 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 diabetes, however, both endogenous amylin and insulin are present at abnormally low levels [1].

Pramlintide is a synthetic analogue 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 contains 37 amino acids with the sequence:

K-C-N-T-A-T-C-A-T-Q-R-L-A-N-F-L-V-H-S-S-N-N-F-G-P-I-L-P-P-T-N-V-G-S-N-T-Y-$NH_2$

The peptide has 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 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. In a previous publication [6], we isolated and identified nine deamidation products and four products resulting from peptide backbone hydrolysis in a sample of pramlintide final drug product (FDP) which had been thermally stressed in pH 4.0 aqueous solution at 40°C for 45 days.

In this study, six degradation products containing cyclic imide residues derived from asparagine were isolated and identified in this same sample of heat stressed pramlintide injection drug product. These products are reaction intermediates that precede deamidation. The residues affected were identified by use of tandem mass spectrometry (MS/MS). The data confirm the existence of a cyclic imide intermediate for all except two deamidation sites identified previously in this peptide [6]. Two additional deamidation products were also isolated and the sites of modification identified by MS/MS and, when sufficient material was available, enzymatic digestion followed by liquid chromatography/mass spectrometry (LC/MS) and amino acid sequence analysis.

## 2. Materials and methods

### 2.1. Materials and chromatographic methods

These were as specified in Hekman et al. [6]. Briefly, samples of pramlintide injection final drug product, a preserved liquid formulation, containing 0.3 mg/ml pramlintide in a pH 4.0 isotonic formulation buffer, sterile filtered into 5 ml USP Type I glass vials with bromobutyl rubber closures, were thermally stressed at 40°C for 45 days. Subsequent to this forced degradation, the samples were subjected to solid phase extraction to remove excipients that may obscure the degradation products, and then isolated by semi-preparative strong-cation exchange (SCX) and reversed-phase high-performance liquid chromatography (HPLC). The strong cation exchange column employed was a PolySULFOETHYL Aspartamide column, 100 mm × 4.6 mm, 5 μm particle size, 300 Å pore size (Poly LC, Columbia, MD). Conditions were as previously specified [6]. Column loads were 50 μg for analytical analyses and 500 μg for preparative runs. The eluant from several preparative SCX runs was pooled prior to further analysis and/or isolation by reversed-phase HPLC. Reversed-phase separation was conducted as previously specified [6] except that a Waters Symmetry Prep $C_8$ column, 300 mm × 7.8 mm, 7 μm particle size, 100 Å pore size was used. Column loads were up to 200 μg for isolation of the degradation products.

### 2.2. Mass spectrometry and tandem mass spectrometry

Samples were analyzed on a Micromass (Beverly, MA) Quattro IIE triple quadrupole mass spectrometer operating in the positive ion electrospray mode. This instrument was configured with a Rheodyne manual injection valve (9125) fitted with a 50 μl sample loop which allowed the sample to be directly infused into the electrospray ionization source.

Each day, prior to use, the mass-scale calibration was performed in positive ion electrospray mode using sodium iodide as previously described [6]. Related substances were analyzed as received following HPLC desalt using a Supelcogel ODP-50 (4.6 mm × 2 cm) column (Supelco, Bellefonte, PA) with a water/acetonitrile gradient to elute the peak (final sample solvent was ≈40% acetonitrile, 60% water containing 0.1% acetic acid and 0.02% trifluoroacetic acid). Samples were introduced into the electrospray ionization source as a 50 μl plug in a flow stream composed of 49.75:49.75:0.50 water/acetonitrile/formic acid at a flow rate of 0.300 ml /h. The experimentally determined molecular weight of the pramlintide standard was typically 3949.7 ± 0.2 Da. During this sampling period, molecular weight determination was performed by single-stage mass spectrometry. Typical MS conditions included a scan range of 500–2000 Da/e with a 30 V sample cone voltage and a scan time of 5.0 s. If a sufficient quantity of sample was present, tandem mass spectrometry was performed on the $[M + 4H]^{4+}$ or $[M + 3H]^{3+}$ parent ions to elucidate the structure. MS/MS conditions employed a 30 V sample cone voltage, a scan range of 50–2000 Da/e, a scan time of 6.0 s, and $4.0 \times 10^{-3}$ mbar argon collision gas.

The mass spectrometer conditions for LC/MS of thermolysin-digested components were as specified previously [6] except that scan ranges were 300–1000 Da/e for the 30 V sample cone voltage and 70–1600 Da/e for the 60 V sample cone voltage. Scan times were 2.0 and 4.0 s for the sample cone voltages of 30 and 60 V, respectively.

### 2.3. Amino acid sequence analysis

Sequence analysis was conducted by Edman degradation using the ABI Procise 492 (Applied Biosystems, Foster City, CA).

### 2.4. Thermolysin digestion

This was conducted as described previously [6]. 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. 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).

## 3. Results

### 3.1. Isolation of related substances

The strong cation exchange HPLC analytical chromatogram of the heat stressed FDP sample (Fig. 1) revealed a number of peaks identified as degradation products by comparison to the initial unstressed pramlintide sample [6]. Nine of these peaks were isolated and identified previously [6]. These substances include pramlintide fragments [19–37] plus [20–37], [1–18], and [1–19] as well as the deamidation products [Iso-$Asp^{21}$], [Iso-$Asp^{3}$], [Asp/Iso-$Asp^{14}$], [Iso-$Asp^{22}$], [$Asp^{21}$] plus [$Asp^{22}$], and [Asp/Iso-$Asp^{35}$]. This report concentrates on five of the remaining peaks (Fig. 1) including two for which partial identification was reported previously [6].

Subsequent to isolation by strong cation exchange [6] the fractions corresponding to the peaks of interest were analyzed by reversed-phase HPLC to determine if the material collected was homogeneous or required further fractionation.

These data (not shown) indicated that all of the SCX fractions corresponding to peaks 1–5 in Fig. 1 appeared to contain multiple components. Further purification was achieved by removing the salt and acetonitrile by HPLC desalt followed by semi-preparative fractionation on the Symmetry Prep $C_8$ reversed-phase column as described previously [6]. The reversed-phase chromatogram of the unfractionated, heat stressed FDP (starting material) is shown in Fig. 2. The peak labels correspond to those in Fig. 1 and Table 1 and were determined based on the reversed-phase chromatograms of the individual SCX fractions. Material corresponding to peaks 1–5, 2B, 3B, and 5B (Fig. 2), was collected and frozen at −70°C. Thus, eight peaks were collected from the reversed-phase chromatography of these strong cation exchange fractions. They correspond to the original peaks 1–5 plus three additional peaks not separated by the cation exchange chromatography (Table 1).

### 3.2. Mass spectrometry of related substances

The eight components, isolated as described above, were desalted and subjected to analysis by MS. Mass spectra and tandem mass spectra were obtained for all samples. Single-stage mass spectra (Table 1) were composed of singly or multiply protonated molecular ions. These data were transformed into the mass domain by the MassLynx 2.2 NT software. The molecular weights listed in column 4 of Table 1 are the values obtained from the centroided transformed mass spectra. Tandem mass spectrometry provided fragment ions which facilitated structural elucidation as summarized in Table 1, columns 5–7. Structural assignments of peptide fragments ions followed the convention of Carr et al. [7]. 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

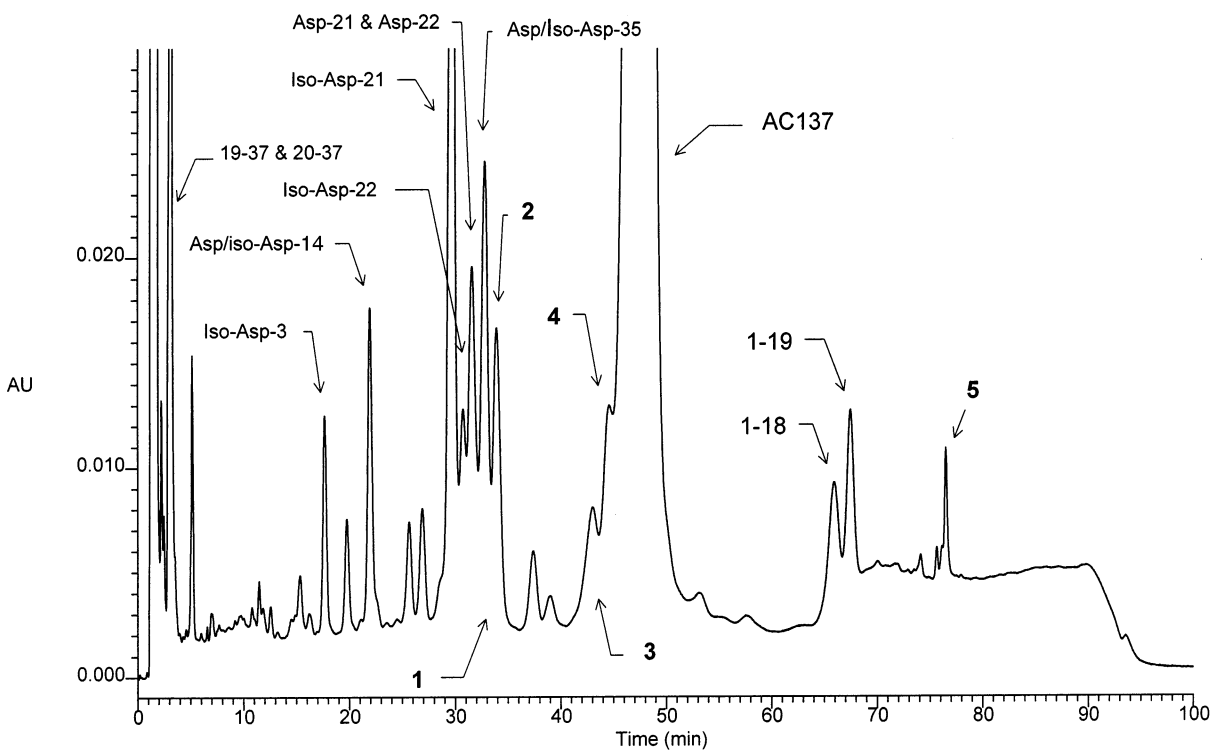


Fig. 1. Analysis of heat stressed (40°C, 45 days) Pramlintide (AC137) drug product by strong cation exchange HPLC. Column load was 50 μg. Peak numbers correspond to those in Fig. 2 and Table 1.

Table 1
MS and MS/MS data for heat stressed pramlintide FDP peaks

| Peak number | Area % by SCX | Ions (Da/e) | MW[a](Da) | Key fragment ions (Da/e)[b] | Proposed structure | Comments |
|---|---|---|---|---|---|---|
| Peak 1 | 0.39[c] | 984.2, 1311.8 | 3932.8 | $962.6 = b_{27}^{3+}$; $1049.0 = y''^{+}_{10}$; $1818.1 = b_{17}^{+}$; $589.4 = b_{11}^{2+}$ | Cyclic imide [Asn[14]]-pramlintide | The $b_{11}^{2+}$ and $b_{17}^{+}$ ions indicate that Asn[14] is cyclized |
| Peak 2 | ~0.69[d] | 988.7, 791.2, 1317.9 | 3950.8 | $968.3 = b_{27}^{3+}$; $1050.0 = y''^{+}_{10}$; $509.7 = y'_{10}b_{32}^{+}$; $768.7 = y'_{10}b_{35}^{+}$; $435.4 = y'_{10}b_{36}^{2+}$ | [Asp or iso-Asp[35]]-pramlintide | The mass-to-charge ratios of the $y'_{10}b_{32}^{+}$ and $y''^{+}_{10}$ indicate that the deamidated residue is in residues 33–37. The $y'_{10}b_{35}^{+}$ ion indicates that the deamidated residue is in the 33–35 region. Only possible site is at the Asn[35] |
| Peak 2B | ~0.35[d] | 988.7, 791.2, 1317.8. | 3950.7 | $968.2 = b_{27}^{3+}$; $1049.9 = y''^{+}_{10}$; $509.6 = y'_{10}b_{32}^{+}$; $767.8 = y'_{10}b_{35}^{+}$; $283.4 = y''^{+}_{2}$ | [Tyr[37]-OH]-pramlintide | The mass-to-charge ratios of the $y''^{+}_{2}$ and $y'_{10}b_{35}^{+}$ ions indicate that the deamidated residue is in residues 36–37. Only possible site is Tyr-amide-37. This is confirmed by the mass-to-charge ratio of the $y''_{2}$ ion and absence of the $y''_{1}$ ion |
| Peak 3 | 0.38[c] | 984.2, 1311.9, 787.7 | 3932.9 | $962.4 = b_{27}^{3+}$; $1049.0 = y''^{+}_{10}$; $1836.1 = b_{17}^{+}$; $1073.7 = b_{20}^{2+}$; $854.6 = b_{24}^{3+}$ | Cyclic imide [Asn [22]]-pramlintide | The b-type fragment ions observed indicate that the cyclized Asn imide is at Asn[22] |
| Peak 3B | 0.62[c] | 984.3, 787.7, 1311.8 | 3933.0 | $968.5 = b_{27}^{3+}$; $1032.1 = y''^{+}_{10}$; $509.9 = y'_{10}b_{32}^{+}$ | Cyclic imide [Asn[35]]- or [Tyr[37]]-pramlintide | Indicative ions to distinguish between the two possibilities were not observed |
| Peak 4 | 1.1 | 984.2, 1311.8, 787.7 | 3932.9 | $962.4 = b_{27}^{3+}$; $1049.1 = y''^{+}_{10}$; $1835.3 = b_{17}^{+}$; $1179.3 = b_{22}^{2+}$; $1131.2 = b_{21}^{2+}$, $786.7 = b_{22}^{3+}$ | Cyclic imide [Asn[21]]-pramlintide | The b-type fragment ions observed indicate that the cyclized Asn imide is at Asn[21] |
| Peak 5 | 0.06 | 566.0, 754.2, 1130.6 | 2259.8 | $918.2 = b_{17}^{2+}$; $426.4 = y''^{+}_{4}$; $202.3 = y''^{+}_{2}$; $1071.6 = b_{20}^{2+}$ | [1–21]-pramlintide-succinimide | The mass-to-charge ratio of the $y''_{2}$ indicates that residue 21 is a cyclized Asn succinimide |
| Peak 5B | 0.13 | 792.3 | 2374.1 | $917.8 = b_{17}^{2+}$; $540.7 = y''^{+}_{5}$; $229.0 = y''^{+}_{2}$ | [1–22]-pramlintide-succinimide | The mass-to-charge ratio of the $y''_{2}$ indicates that residue 22 is a cyclized Asn succinimide |

[a] The experimentally determined molecular weight of the pramlintide standard was 3949.7 + 0.2 Da.
[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] Taken from reversed-phase chromatography because peaks were better separated from the neighboring peaks by this method.
[d] Components migrate as a single peak by SCX with a total area percent of 1.04%. Analysis by reversed phase HPLC indicates the ratio of peak 2 to peak 2B is ≈ 2:1.

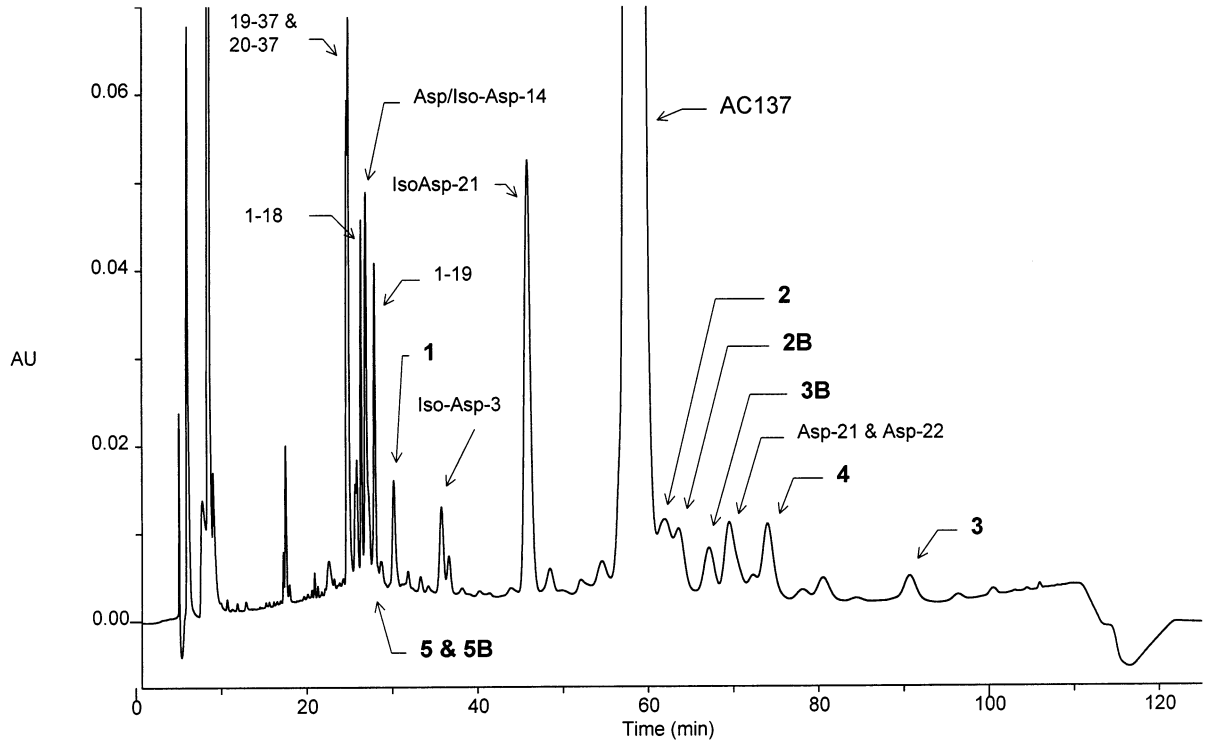


Fig. 2. Analysis of heat stressed (40°C, 45 days) Pramlintide (AC137) drug product by reversed-phase HPLC. Column load was 50 μg. Peak numbers correspond to those in Fig. 1 and Table 1. [Iso-Asp[22]]-pramlintide and [Iso-Asp[35]]-pramlintide are not resolved by this method.

product, while the 'y' fragment is generated by rearrangement.

### 3.3. Identification of pramlintide cyclic imides at $Asn^{14}$, $Asn^{21}$, $Asn^{35}$, and $Asn^{22}$

The pramlintide related substances corresponding to peaks 1, 3, 3B, and 4 all yielded molecular weights of 3933 Da (Table 1) which represents a loss of 17 Da from the pramlintide structure. This 17 Da loss appears to correspond to a loss of ammonia from the asparagine residues to form a cyclic imide. Cyclic imides have been reported [8,9] as intermediates in the formation of peptide deamidation products and, although these intermediates are generally considered unstable, a number of investigators have isolated and/or identified cyclic imides which are not only stable, but which account for 7–30% of the total degradation observed in their samples [9–11]. Thus, the presence of cyclic imides in this sample, which is known to contain nine deamidation products [6], would not be surprising.

Peak 1, which was identified previously [6] as pramlintide with loss of an ammonia or water molecule, was collected to obtain more complete characterization. The MS fragment ions $b_{11}^{2+}$ ($m/z$ 589.4) and $b_{17}^{+}$ ($m/z$ 1818.1) (Table 1) indicated that the $-17$ Da modification occurred between $Leu^{12}$ and $Val^{17}$ (inclusive). The only residue in this stretch of 6 amino acids able to lose an ammonia molecule is $Asn^{14}$. Thus these data support the assignment of a cyclic imide at $Asn^{14}$ for this component. An attempt was made to further confirm the site of cyclization by subjecting this material to thermolysin digestion followed by MS and sequence analysis. The MS results indicated, however, that the basic conditions of the ammonium bicarbonate buffer employed for the thermolysin digestion had converted this product

back into pramlintide (by reaction with the ammonia present in the medium). Thus, the site of cyclization could not be determined by this digestion method. This data did, however, serve to add support to the assignment of this product as a cyclic imide.

The MS/MS data for peaks 3 and 4, in particular the $y''^{+}_{10}$, $b^{3+}_{27}$, and $b^{+}_{17}$ fragment ions (Table 1), indicate that the minus 17 Da modification for both peaks 3 and 4 lies between residues 18 and 27 of pramlintide. The only possible sites for loss of ammonia within this 10 amino acid region are the Asn residues at positions 21 and 22. Formation of the $b^{3+}_{22}$ fragment ion ($m/z$ 786.7), which is present in the MS/MS spectra for peak 4 material but absent in the MS/MS spectra for peak 3, requires cleavage of the amide bond between $Asn^{22}$ and $Phe^{23}$. For the cyclic imide at position 21 this should be a facile cleavage. Thus, the $b^{3+}_{22}$ fragment ion is expected for the [$Asu^{21}$]-pramlintide product. However, for the cyclic imide at $Asn^{22}$ this cleavage would require more energy to cleave since two C–N bonds are required to cleave in order to form the $b^{3+}_{22}$ fragment ion. Consequently, the presence of the $b^{3+}_{22}$ ion is expected in much lower abundance for the [$Asu^{22}$] product. A review of the data indicates the presence of the $b^{3+}_{22}$ ion in the data for peak 4 but not for peak 3 suggesting that peak 4 corresponds to the [$Asu^{21}$]-pramlintide while peak 3 corresponds to the [$Asu^{22}$]-pramlintide.

Finally, the product ion spectrum of peak 3B (Table 1), in particular the $b^{3+}_{27}$, $y''^{+}_{10}$, and $y'_{10}b^{+}_{32}$ fragment ions indicate that the cyclized Asn in this material is between $Gly^{33}$ and $Tyr^{37}$. The only residues subject to loss of ammonia in this 5 amino acid region are $Asn^{35}$ and $Tyr^{37}$. Since a cyclic imide is unlikely at the C-terminal amidated $Tyr^{37}$, the data support the assignment of peak 3B as the pramlintide cyclic imide at $Asn^{35}$.

### 3.4. Identification of pramlintide fragments [1–21] and [1–22] with cyclized Asn succinimide

The MS/MS spectra for peaks 5 and 5B indicate that the material corresponding to peaks 5 and 5B are both modified pramlintide fragments. Peak 5 corresponds, by MW and fragmentation pattern, to [1–21]-pramlintide with a cyclic imide at $Asn^{21}$. Peak 5B is similar, except that the fragment is [1–22]-pramlintide and the cyclic imide is $Asu^{22}$.

### 3.5. Identification of pramlintide deamidation products

Peaks 2 and 2B were identified previously [6], as deamidation products of pramlintide with the deamidations occurring in the last ten residues of the peptide. These materials were collected a second time in an attempt to obtain a more complete characterization. The MS/MS spectra for these components are presented in Table 1. The data confirm the previous assignments and provide additional information as to the site of deamidation.

The $y'_{10}b^{+}_{32}$ and $y''^{+}_{10}$ fragment ions (Table 1) indicate that peak 2 is a deamidation product of pramlintide with the deamidation site between residues 33 and 37. The $y'_{10}b^{+}_{35}$ and $y'_{10}b^{2+}_{36}$ fragment ions further isolate the site of deamidation to $Asn^{35}$. Therefore, the data demonstrate that peak 2 is either [$Asp^{35}$]-pramlintide or [iso-$Asp^{35}$]-pramlintide.

The product ion data for peak 2B, in particular the $y'_{10}b^{+}_{35}$ and $y''^{+}_{2}$ fragment ions indicate that the deamidation site is either $Thr^{36}$ or the amidated C-terminal $Tyr^{37}$. Threonine is not an amino acid subject to deamidation. Therefore, the data indicate that peak 2B is the C-terminal deamidation product, [$Tyr^{37}$-OH]-pramlintide. This assignment is confirmed by the presence of $m/z$ 283, the $y''^{+}_{2}$ ion, and the absence of the $m/z$ 181, the $y''^{+}_{1}$ fragment ion for pramlintide. Furthermore, the strong cation exchange and reversed-phase relative retention time values of this component match those of authentic [$Tyr^{37}$-OH]-pramlintide.

### 3.6. Thermolysin digestion followed by liquid chromatography/mass spectrometry and sequence analysis

In an attempt to determine whether the peak 2 material corresponded to the [$Asp^{35}$] or [iso-$Asp^{35}$]-pramlintide product, the isolated material corresponding to peak 2 was subjected to enzymatic digestion with thermolysin as described

above. Subsequent to digestion, the thermolysin fragments were separated and analyzed by LC/MS. A flow splitter was employed to introduce 5% (by volume) to the electrospray ionization source, with the remaining 95% diverted to a fraction collector. The aberrant thermolysin fragments (relative to a pramlintide control) were collected and subjected to sequence analysis. The LC/MS data are summarized in Table 2.

The aberrant thermolysin fragments for peak 2 were [23–37]-pramlintide and [32–37]-pramlintide. The mass spectra for these fragments confirmed the site of deamidation as $Asn^{35}$ (Table 2) but were unable to distinguish whether the substance was the $Asp^{35}$ or iso-$Asp^{35}$ product. Subsequent amino acid sequence analysis of the 32-37 thermolysin fragment clearly indicated the presence of an Asp residue at position 35. The presence of an iso-aspartic acid, a beta amino acid that cannot be sequenced by Edman chemistry, would have resulted in sequence termination at residue 35. Thus these results confirm the identity of this component as [$Asp^{35}$]-pramlintide.

## 4. Discussion

This report summarizes the isolation and identification of eight related substances in pramlintide FDP which had been subjected to forced degradation at 40°C for 45 days. The components were present at levels ranging from $< 0.1\%$ to 1.1% of the total HPLC peak area. The total loss in pramlintide peak area (total degradation), relative to the control, was 12–14%. The eight related substances identified here fall into two categories: deamidation products and cyclic imides.

The two deamidation products identified as part of this study were [$Asp^{35}$]-pramlintide and [$Tyr^{37}$-OH]-pramlintide, the deamidation product of the C-terminal amidated $Tyr^{37}$. Both of these components were isolated as part of the study reported previously [6]. However, the exact location of the deamidated residues was not determined in that report. The work reported here employed MS/MS and chemical sequencing to allow the sites of deamidation to be determined conclusively.

Six of the components, peaks 1, 3, 3B, 4, 5, and 5B were identified as cyclic imides, The cyclized residues identified for the first four components were $Asn^{14}$, $Asn^{22}$, $Asn^{35}$, and $Asn^{21}$, respectively. Peaks 5 and 5B were identified as cyclic imides of pramlintide fragments [1–21] and [1–22], respectively. Although cyclic imides are frequently reported as mechanistic intermediates preceding deamidation [8,9], the lack of stability of these intermediates often precludes their isolation. Thus, the existence of these intermediates is often implied by the presence of both Asp and iso-Asp products rather than through direct isolation and identification. Nevertheless, several reports of the isolation of stable cyclic imide intermediates have been published [11–16]. The data presented here summarize the isolation and identification, through the use of MS/MS, of cyclic imide intermediates for four of the six known deamidation sites in this single sample of heat stressed pramlintide injection drug product. The two deamidation products for which no cyclic intermediates have been detected are [$Tyr^{37}$-OH]-pramlintide, the biologically inactive deamidation product of pramlintide at the C-terminal, for which no deamidation intermediate is expected, and [iso-$Asp^{3}$]-pramlin-

Table 2
MS data for thermolysin digested fragments of heat stressed FDP

| Peak number | Aberrant fragment | Digestion time (min) | Ions (Da/e) | Mass (Da) | Key fragment ions (Da/e) | Proposed structure |
|---|---|---|---|---|---|---|
| Peak 2 | 23–37 | 300 | 955.5 | 954.4 | None detected | FGPILPPTNVGSDTY[a] |
| | 32–37 | 300 | 640.4 | 639.4 | 460.3 = $b_4^+$<br>359.4 = $b_3^+$ | VGSDTY[a] |

[a] D represents Asp or Iso-Asp.

tide. The low levels of the [iso-Asp$^3$]-pramlintide product ($< 0.4\%$) coupled with the constrained environment around the Asn$^3$ residue make it likely that even if this cyclic imide is present, the levels would be lower than were pursued as part of this study (0.3%). The cyclic imides of pramlintide fragments [1–21] and [1–22] represent peptide backbone hydrolysis at the site of cyclic imide formation. This type of backbone hydrolysis has been reported as a competing side reaction during cyclic imide hydrolysis [17,18]. Thus, the presence of these truncated cyclic imides was not unexpected. Because of the low levels of these cyclic imide fragments, their hydrolysis products, [1–21]-pramlintide and [1–22]-pramlintide are not likely to be identified. The cyclic imide fragments were isolated because of their prominent elution profile by strong cation exchange HPLC. Together, these data add support to the work reported elsewhere [9] that these cyclic imide intermediates can, under some conditions, form as degradation products. Since the breakdown of these cyclic imides is base catalyzed, the low pH conditions employed in these studies may have stabilized these intermediates, allowing their isolation and characterization.

Together with the degradation products identified previously [6] the total number of related substances identified in this thermally stressed pramlintide FDP sample is 19. Four of the products are hydrolysis products, nine are deamidation products, and six are cyclic imides. The data indicate that the cyclic imide products account for approximately 20% of the total degradation products while the deamidation products identified previously [6] together with the two identified here account for approximately 64%. The remaining degradation is due to peptide backbone hydrolysis [6].

These data support those presented earlier [6] indicating that the primary mechanism of degradation for pramlintide in this pH 4.0 formulation is deamidation, with six of the eight possible deamidation sites observed to undergo deamidation. Gln-10 and Asn-31 are the only two residues subject to deamidation for which none is observed. The failure of these two residues to deamidate is not unexpected based on literature reports revealing that deamidation at Gln residues occurs at one-tenth the rate of deamidation at Asn residues [17]. The presence of the Arg residue, which has a bulky side chain, immediately adjacent to the Gln residue on the C-terminal side is expected to further slow the deamidation at the Gln residue [17,19]. The Asn$^{31}$ also has a bulky side chain on the adjacent C-terminal residue (Val) which is expected to hinder deamidation at this site [9,17,19].

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