Orthogonal HPLC Methods For Quantitating Related Substances and Degradation Products of Pramlintide

Wade Demond,[1] Richard A. Kenley,[2*] James L. Italien,[1] David Lokensgard,[3] G. Weilersbacher[2] and Keith Herman[4]

[1]Baxter Hyland, Glendale, CA
[2]Cabrillo Facility of Magellan Laboratories, Inc., 9250 Trade Place, San Diego, CA 92126
[3]Ligand Pharmaceuticals, Inc., 10275 Science Center Drive, San Diego, CA 92121
[4]Waters Corporation, Milford MA

ABSTRACT Pramlintide is a 37– amino acid peptide that is being evaluated as a drug candidate for treating people with type 1 and insulin-using type 2 diabetes. Two high-performance liquid chromatography (HPLC) methods were developed for quantitating related substance impurities in pramlintide drug substance as well as degradation products of pramlintide formulated for parenteral administration. The methods differ with respect to separation mode and therefore provide orthogonal information concerning related substances and degradation products. One method uses a reverse phase (RP) separation mode, and the other involves a strong cation exchange (SCX) separation. Method performance testing showed that the RP- and SCX-HPLC methods both afford a high degree of selectivity, accuracy, precision, and sensitivity. The limit of quantitation for determining spiked authentic samples of degradation products was shown to be approximately 0.1% (relative to intact pramlintide) for both methods. Relative retention times for known pramlintide degradation products were determined for both the RP- and SCX-HPLC methods, demonstrating the selectivities of the 2 methods as well as the orthogonality of the information. The methods were also shown to be diastereospecific with respect to separating pramlintide from authentic samples of D-isomers at Ala[5], Ala[8], Ala[5]-Ala[8], and Leu[12]. The methods did not resolve pramlintide, however, from diastereomers with D-isomers near the C- and N-termini, namely Lys[1],Cys[2], and Tyr[37].

KEYWORDS: Pramlintide, Peptide Analysis, RP-HPLC, SCX-HPLC, Orthogonal Separation, Diastereoselectivity.

INTRODUCTION

Amylin is a 37– amino acid peptide hormone that is produced in the pancreas and co-secreted with insulin in response to 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 drug synthesis and 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.

Figure 1 shows the pramlintide amino acid 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-*NH₂*

Notes:

1. Disulfide Bridge between Cys[2]-Cys[7]
2. Underscores show potential sites of deamidation at asparagine, glutamine, and C-terminal tyrosine
3. Boldface shows positions where three prolines in pramlintide replace alanine, serine, and serine in the human hormone, amylin
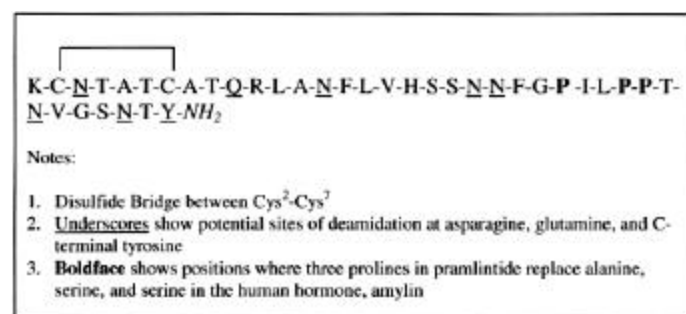
Figure 1. Amino acid sequence of pramlintide.

*)**Corresponding Author:** Richard A. Kenley; Cabrillo Facility of Magellan Laboratories, Inc., 9250 Trade Place, San Diego, CA 92126; telephone: 1-858-547-7810; facsimile: 1-858-578-0119; email: rkenley@cabrillolabs.com

The figure also shows the disulfide bridge between cysteines 2 and 7 and indicates the amino acid differences between pramlintide and amylin. Also, the pramlintide sequence contains no free carboxyl groups, even at the amidated C-terminus (tyrosine). All 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 salt with acetate as the counterion.

As with any drug substance, identifying and quantitating low levels of related substance impurities present in synthesis lots was a principal focus of the effort to develop a robust manufacturing process for pramlintide. Missed couplings, double-couplings, and L- to D-amino acid isomerizations are common errors that occur in peptide synthesis to yield, respectively, single-point amino acid deletion peptides, addition peptides, and diastereomers as related substances of the desired molecule. Specifically for pramlintide, the 37–amino acid length severely complicates detecting single amino acid modifications and requires highly selective analytical test methods for purity determinations.

An injectable, multidose liquid formulation was developed for pramlintide, consisting of a pH 4.0 aqueous solution of 0.3 to 1.0 mg/mL pramlintide with m-cresol added as an antimicrobial preservative. Degradation pathways for peptides in acidic aqueous solution frequently involve deamidation at asparagine and glutamine plus hydrolytic backbone cleavage [5-9]. The 37– amino acid length and the 8 potential deamidation sites of this formulation create a potential for many degradation products that differ from pramlintide by modifications at only a single amino acid.

Considering the foregoing, we undertook to develop high-performance liquid chromatography (HPLC) methods to determine the following: (1) the overall percentage purity of pramlintide (by area normalization) and (2) the percentage of individual related substance impurities and degradation products. The principal method design goals were threefold, namely to (1) develop 2 different methods involving orthogonal separation modes, (2) employ identical sample preparation steps for both HPLC methods, and

(3) apply both HPLC methods to drug substance and drug product analysis.

We developed a reverse-phase (RP) HPLC method and a strong cation exchange (SCX) HPLC method for analyzing pramlintide acetate drug substance and pramlintide injection drug product purity. This article describes the operating parameters and demonstrates method performance with respect to overall selectivity, orthogonal separation, and quantitation of related substances and degradation products at low levels in pramlintide acetate drug substance and pramlintide injection drug product.

MATERIALS AND METHODS

Test articles

Pramlintide was produced by either solid-phase or solution-phase synthesis, and isolated as a dry powder in the acetate salt form. Authentic samples of pramlintide degradation products and pramlintide D-isomers were similarly prepared. Sterile pramlintide drug product samples were prepared by aseptic processing at 0.3 to 1.0 mg/mL in pH 4.0 aqueous solution with m-cresol added as antimicrobial preservative. The samples were stored in 5-mL glass vials with bromobutyl rubber closures.

Abbreviations

For brevity, modified pramlintide structures use the naming convention [Xxx^{nn}], where Xxx is the 3-character abbreviation for the modified amino acid, and nn represents the amino acid sequence number in the pramlintide molecule. For example, the abbreviation [Asp^{21}] indicates a pramlintide modification in which the asparagine at position 21 has been converted to aspartic acid. Similarly, [D-Leu^{12}] indicates L- to D-isomerization of leucine at position 12. Backbone cleavage fragments of pramlintide use the naming convention [nn-mm], where nn represents the N-terminal amino acid of the pramlintide sequence in the fragment and mm represents the C-terminus of the fragment. For example, [1-18] represents the pramlintide fragment beginning with lysine[1] and ending with histidine[18].

Sample preparation

Working Reference Standard

Pramlintide working reference standard solutions were prepared (at 0.5 mg/mL in pH 4.0, 30- mM acetate buffer) from a master reference standard of pramlintide acetate whose peptide content was established by quantitative amino acid analysis.

Drug Substance Samples

For both the RP- and SCX-HPLC methods, pramlintide acetate samples were quantitatively weighed and diluted to 0.5 mg/mL in pH 4.0, 30-mM acetate buffer and directly injected (50-μg on column) for analysis.

Drug Product Samples

For both the RP- and SCX-HPLC methods, pramlintide injection samples were subjected to a solid-phase extraction step to remove mannitol and *m*-cresol. A 6-mL wide-bore C4 cartridge (Mallinckrodt Baker[Q: Please provide city and state of manufacturer]) was used. The procedure involved conditioning each cartridge with acetonitrile and then equilibrating each cartridge in water. A sample was then injected onto a cartridge, washed with water, and eluted with 40% acetonitrile in 0.1% trifluoroacetic acid (TFA). The collected sample was evaporated to dryness and dissolved in pH 4.0 30-mM acetate buffer at 2 mg/mL.

Force-Degraded Working Reference Standards

For analysis by RP-HPLC, a working reference standard solution was acidified to pH 2 with 0.1 N HCI and heated to 100 ° C for 30 minutes and rapidly cooled to room temperature to yield a solution with approximately 80% intact pramlintide remaining. For analysis by SCX-HPLC, a pramlintide solution made up in 4.5 mM aqueous sodium acetate pH 7.5 was heated to 100 ° C for 30 minutes and rapidly cooled to room temperature to yield a solution with approximately 80% intact pramlintide remaining. This solution was acidified with 15 μL acetic acid to give a pH 4.0 solution in 30 mM acetate buffer.

Spiked Samples for Determination of Quantitation Limits

A 0.5 mg/mL pramlintide working reference standard solution was prepared as described above. This working reference standard solution was then divided and half was spiked to contain 0.010 mg/mL (ie, 2% of the pramlintide concentration) of each of 6 degradation products, namely [*iso*-Asp^{21}], [Asp^{21}], [TyrCOOH^{37}], [1-18], [30-37], and pramlintide dimer. The spiked solution was then diluted with the unspiked working reference standard to give solutions containing 0.5 mg/mL pramlintide, and the 6 degradation products at 1.0%, 0.50%, 0.25%, 0.10%, and 0.050% of the pramlintide concentration.

Spiked Samples for Determination of Recovery After Solid-Phase Extraction

A 1.0 mg/mL pramlintide working reference standard solution was prepared as described above. This working reference standard solution was then spiked to contain approximately 0.010 mg/mL (ie, 1% of the pramlintide concentration) of each of 5 degradation products, namely [*iso*-Asp^{21}], [Asp^{21}], [TyrCOOH^{37}], [1-18], and pramlintide dimer. The spiked solution was treated by the solid-phase extraction step described above and analyzed by 3 different individuals. Spiked solution not treated by the solid-phase extraction step was analyzed as a control.

Force-Degraded Drug Product Samples

A 0.3-mg/mL pramlintide injection drug product formulation was maintained for 6 weeks at 40° C to yield a solution containing intact pramlintide at approximately 86% of initial purity (as determined by the RP- and SCX-HPLC methods). This force-degraded sample was maintained at 5° C until analyzed.

Test system

The 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, Model 62079 column oven, and either ExpertEase® or Millenium® 2020 integration software from Waters (Milford, MA).

Reagents

Buffers and solvents were HPLC grade or equivalent throughout. Chromatography reagents, sodium perchlorate, potassium phosphate monobasic, sodium phosphate, sodium hydroxide, potassium hydroxide, o-phosphoric acid, acetic acid, and acetonitrile were supplied by Fisher (Springfield, NJ). TFA was supplied by Pierce (Rockford, IL).

Method Operating Parameters

HPLC Operating Conditions

Table 1 shows the operating conditions for the RP- and SCX-HPLC methods.

Table 1. Operating Conditions for RP-HPLC and SCX-HPLC Methods

| Parameter | Value for Method: | |
	RP-HPLC	SCX-HPLC
Solvent Flow Rate	0.5 mL/min	0.8 mL/min
Detection Wavelength, Scale	220 nm, 1.0 AUFS	220 nm, 1.0 AUFS
Injection Volume	100 µL	100 µL
Column Temperature	55 ± 2 °C	40 ± 2 °C
Autosampler Temperature	6 ± 3 °C	6 ± 3 °C
Column Type	Waters Symmetry® C8	Poly LC PolySulfoEthyl A®
Column Dimensions	250 x 4.6 mm, 5 µm particle size, 100 °A pore size	100 x 4.6 mm, 5 µm particle size, 200 °A pore size

Mobile Phases

Table 2 shows mobile phase compositions for the RP- and SCX-HPLC methods. **Table 3** shows the gradient profile for the RP-HPLC method, and **Table 4** shows the gradient profile for the SCX-HPLC method.

.

Table 2. Mobile Phase Compositions for RP-HPLC and SCX-HPLC Methods

Method	Mobile Phase Buffer #	$[KH_2PO_4]$ mM	$[NaClO_4]$, mM	Acetonitrile ,%	pH[*]
RP-HPLC	1	85	0	6.05	3.0
RP-HPLC	2	85	0	22.5	3.0
RP-HPLC	3	85	0	26.9	3.0
SCX-HPLC	1	5	5	40	5.8
SCX-HPLC	2	5	260	40	5.8

[*] Apparent pH of mobile phase, adjusted after addition of acetonitrile.

Table 3. Mobile Phase Gradient Profile for RP-HPLC Method.

Gradient Time Minutes	Flow Rate mL/min	Buffer 1 %	Buffer 2 %	Buffer 3 %	Curve[a]
0.0	0.5	100	0	0	NA
1.0	0.5	100	0	0	Isocratic
16.0	0.5	0	100	0	Linear (6)
85.0	0.5	0	100	0	Isocratic
100.0	0.5	0	0	100	Linear (6)
110.0	0.5	0	0	100	Isocratic
110.5	0.5	100	0	0	Linear (6)
111.5	1.0	100	0	0	Isocratic
116.5	1.0	100	0	0	Isocratic
117.0	0.5	100	0	0	Isocratic
125.0	0.5	100	0	0	Isocratic

a. Waters Model 616 or 2690 controller.

Table 4. Mobile Phase Gradient Profile for SCX-HPLC Method.

Gradient Time Minutes	Flow Rate mL/min	Buffer 1 %	Buffer 2 %	Curve[a]
0.0	0.8	98	2	NA
6.0	0.8	85	15	Linear (6)
6.5	0.8	85	15	Isocratic
24.0	0.8	76	24	Linear (6)
61.0	0.8	76	24	Isocratic
81.0	0.8	12	88	Linear (6)
86.0	0.8	12	88	Isocratic
91.0	0.8	98	2	Linear (6)
115.0	0.8	98	2	Isocratic

a. Waters Model 616 or 2690 controller

RESULTS
Representative chromatograms

For the RP-HPLC method, **Figures 2**, **3**, and **4** show representative chromatograms, respectively, for blank, sample, and force-degraded working reference standard solution.

Drug product sample RP-HPLC chromatograms (not shown) are essentially identical to **Figure 3** Note the large number of degradation product species present in **Figure 4** and the excellent resolution of these species from the intact pramlintide peak. For the SCX-HPLC method, **Figures 5**, **6**, and **7** show representative chromatograms, respectively for blank, sample, and force-degraded working reference standard solution.

Figure 2.   RP-HPLC Method Chromatogram of Blank Injection.
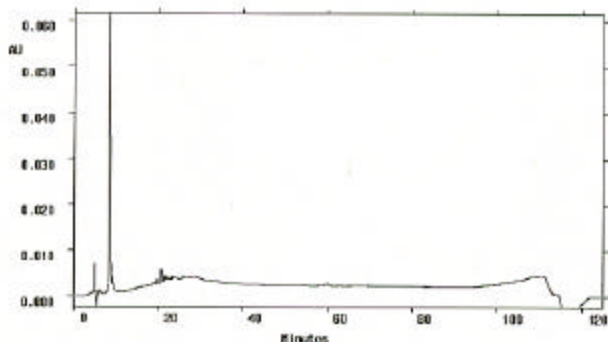
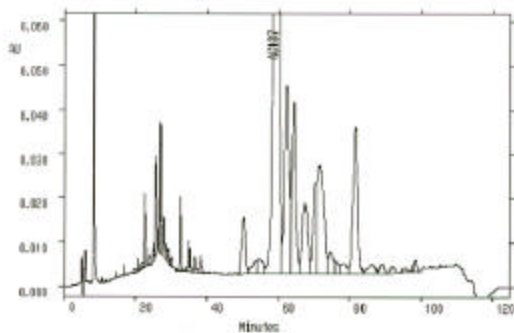Figure 3.   RP-HPLC Method Chromatogram of Pramlintide Acetate
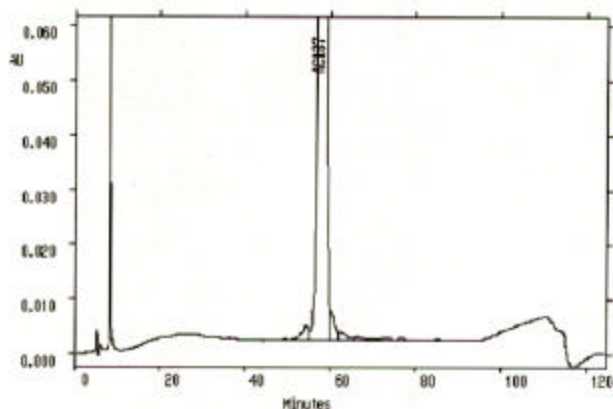
Figure 4.   RP-HPLC Chromatogram of Force-Degraded (15 min at 100 °C) Pramlintide Working Reference Standard Solution.

Figure 5.   SCX-HPLC Method Chromatogram of Blank Injection.
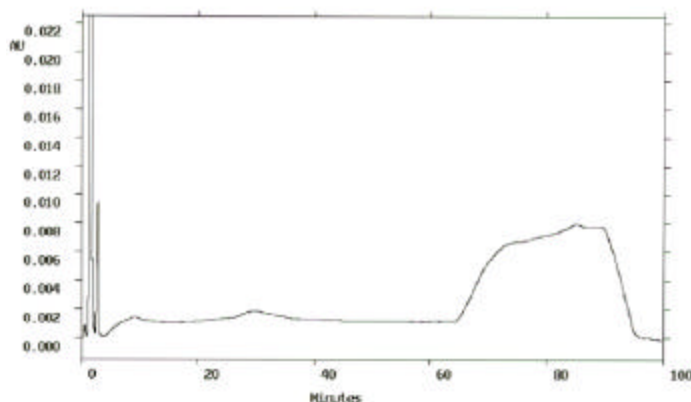

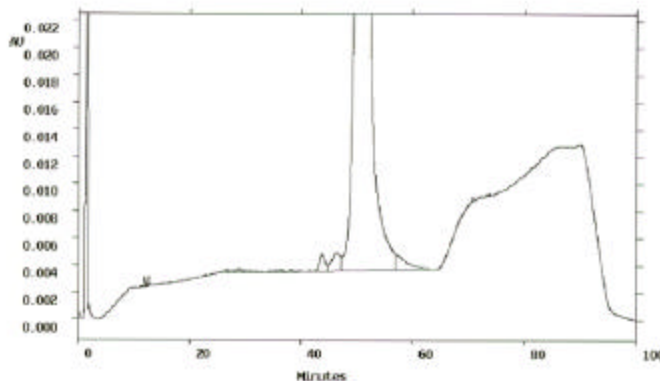Figure 6.   SCX-HPLC Method Chromatogram of Pramlintide Acetate
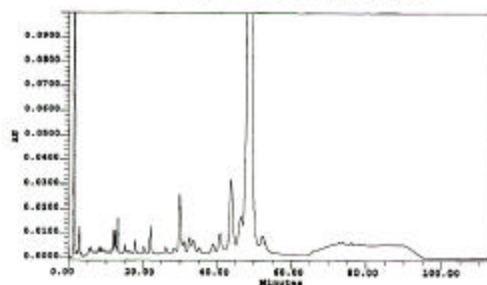

Figure 7.   SCX-HPLC Method Chromatogram of Force-Degraded (15 min at 100 °C) Pramlintide Working Reference Standard Solution.

Drug product sample SCX-HPLC chromatograms (not shown) are essentially identical to **Figure 6**. As for the RP-HPLC method chromatograms, many degradation product peaks are evident in the force-degraded working reference standard solution (**Figure 7**). Furthermore, comparing **Figure 4** with **Figure 7** reveals that the SCX-HPLC chromatogram "fingerprint" is distinctly different from the RP-HPLC method fingerprint, indicating significantly different selectivities for the 2 HPLC methods.

Both sets of chromatograms also demonstrate that relatively long isocratic mobile phase regimens and relatively long pramlintide elution times (approximately 60 minutes for RP-HPLC and approximately 50 minutes for SCX-HPLC) are needed to achieve the desired selectivities.

Quantitative method performance

Both the RP- and SCX-HPLC methods were tested to demonstrate method performance with respect to accuracy, linearity, recovery, reproducibility, repeatability, and limits of quantitation. To demonstrate linearity for pramlintide as a function of sample load, pramlintide working reference standard solutions at 0.40, 0.50, and 0.60 mg/mL were assayed and the HPLC area counts recorded. Least squares linear regression of area count versus concentration data demonstrated acceptable linearity for both the RP-HPLC and SCX-HPLC methods. Specifically, the squared correlation coefficient (R^2 value) for RP-HPLC determination of pramlintide concentration was 0.991 and the R^2 value for SCX-HPLC determination was 0.996.

To demonstrate linearity for pramlintide degradation products, a pramlintide standard solution was spiked with authentic samples of 6 known degradation products at 0.05% to 2% of the intact pramlintide and assayed (using area normalization) for degradation product content. **Table 5** shows the experimental results for the RP-HPLC method and **Table 6** shows the results for the SCX-HPLC method. In all cases, degradation product linearity was satisfactory ($R^2 > 0.998$).

Table 7 summarizes the results of RP-HPLC and SCX-HPLC method repeatability (1 analyst, 1 pramlintide lot, 6 replicate injections) and reproducibility (3 analysts, 4 pramlintide lots, 3 injections) testing for area percentage of intact pramlintide and selected impurities

In all cases, the precision of pramlintide (relative standard deviation [RSD]< 0.5%) and of low-level impurity (RSD < 11%) determinations was satisfactory.

To demonstrate the RP- and SCX-HPLC method limits of quantitation for pramlintide degradation products, the spiked solutions described above were assayed (using area normalization) for degradation product content. **Table 8** shows the experimental results for the RP-HPLC method and supports a quantitation limit of 0.1% for 5 of the 6 tested degradation products.

Table 5. RP-HPLC Method Linearity of Degradation Products Spiked Into Pramlintide Reference Standard Solution

Spike Level Nominal %[a]	Area Count For Spiked Degradation Product =					
	[30-37] RRT[b] =0.19	[1-18] RRT[b] =0.43	[*iso*-Asp[21]] RRT[b] =0.77	[TyrCO OH[37]] RRT[b] = 1.10	[Asp[21]] RRT[b] =1.2	Dimer RRT[b] =1.7
2.0	1226544	1243859	1252864	1198559	1375444	1159040
1.0	612277	632792	618184	588916	698420	552282
0.50	300220	312319	306324	284899	329671	235888
0.25	151069	157652	151255	155270	166770	103916
0.10	59745	65689	59559	67656	66506	33356
0.05	28327	32976	29029	28249	29708	12338
Equation[c]	y = 636047 (x) – 2602	y = 636112 (x) + 3299	y = 637367 (x) - 4940	y = 633592 (x) - 711	y = 640141 (x) - 5386	y = 651358 (x) - 36351
R^2	1.0000	0.9999	1.0000	0.9996	0.9998	0.9987

a. Expressed as a percentage of intact pramlintide peak.
b. RRT = retention time relative to intact pramlintide
c. Least Squares regression of area counts (y value) *versus* spike level (x value).

Table 6. SCX-HPLC Method Linearity of Degradation Products Spiked Into Pramlintide Reference Standard Solution

Spike Level Nominal %[a]	Area Count For Spiked Degradation Product =					
	[30-37] RRT[b] =0.14	[*iso*-Asp[21]] RRT[b]=0.60	[Asp[21]] RRT[b]=0.65	[TyrCO OH[37]] RRT[b] =0.72	[1-18] RRT[b] =1.3	Dimer RRT[b] =1.6
2.0	720393	822660	757480	830754	782990	500274
1.0	358923	419397	382381	423789	396734	264701
0.50	171796	197783	176021	190174	183953	134770
0.25	85089	101809	90836	96882	90367	68145
0.10	30792	42470	37735	38763	26963	25181
0.05	15335	24764	21588	21551	14680	10729
Equation[c]	y = 418475 (x) – 5493	y = 415981 (x) + 394	y = 416826 (x) - 2699	y = 418954(x) - 5265	y = 421786(x) - 8992	y = 416932(x) + 4141
R^2	0.9999	0.9997	0.9995	0.9993	0.9997	0.9988

a. Expressed as a percentage of intact pramlintide peak.
b. RRT = retention time relative to intact pramlintide
c. Least Squares regression of area counts (y value) *versus* spike level (x value).

Table 7. SCX-HPLC and RP-HPLC Method Repeatability and Reproducibility

Validation Parameter	Experimental Design	Test Method	Pramlintide Lot #	Analyte % Purity (Mean ± % Relative Standard Deviation) For		
				Pramlintide	Impurity at RRT = 1.09	Impurity at RRT = 0.86
Repeatability	One Person, One Lot, Six Injections/Lot	RP-HPLC	4	95.4±0.06	0.76±0.68	a
		SCX-HPLC	5	98.5±0.04	b	0.31±2.58
Reproducibility	3 Labs, Four Lots	RP-HPLC	1	97.3±0.24	0.36±9.3	a
	Three Injections/Lot	RP-HPLC	2	96.7±0.32	0.74±3.7	a
		RP-HPLC	3	97.8±0.23	c	a
		RP-HPLC	4	95.0±0.16	0.59±11	a
		SCX-HPLC	6	97.0±0.29	b	c
		SCX-HPLC	2	98.4±0.11	b	c
		SCX-HPLC	4	96.2±0.42	b	1.60±6.7
		SCX-HPLC	5	98.5 0.14	b	0.36±3.9

a. There is no detectable impurity peak at RRT = 0.86 by the RP-HPLC method
b. There is no detectabel impurity peak at RRT = 1.09 by the SCX-HPLC method.
c. There is no detectable impurity peak at the indicated retention time in this lot of pramlintide.

Table 8. RP-HPLC Method Recovery of Degradation Products Spiked Into Pramlintide Working Reference Standard Solution

Spike Level Nominal %[a]	%Recovery (± %RSD)[b] For:					
	[30-37] RRT[c]=0.19	[1-18] RRT[c]=0.43	[iso-Asp21] RRT=0.77	[TyrCOOH37] RRT[c]=0.1.10	[Asp21] RRT[c]=0.1.2	Dimer RRT[c]=1.7
1.0	100±0.06	102±0.2	99.2±0.7	98.8±0.8	102±0.6	95.8±4
0.50	99.3±0.1	102±0.2	99.2±0.5	96.5±1	97.1±2	82.5±1
0.25	98.9±0.5	102±0.2	97.0±0.2	104±0.4	97.4±3	72.1±2
0.10	98.3±0.6	106±0.0	96.1±1	114±4	97.2±5	58±8
0.05	93.8±3	107±1	93.9±2	95.3±39	87.4±6	43±10
Mean± %RSD (1 to 0.1% Spike)	99.2±0.9	103±2	97.9±2	103±7	98.5±3	77.1±21
Mean± %RSD (1 to 0.05% Spike)	98.1±3	104±2	97.1±2	102±7	96.2±6	70.3±29

a. Expressed as a percentage of intact pramlintide peak.
b. Mean and %RSD for five injections, except for 0.10% and 0.05% spike levels (three injections).
c. RRT = retention time relative to intact pramlintide.

The exception, pramlintide dimer, has a quantitation limit of approximately 0.25% by the RP-HPLC method. **Table 9** shows the results for the SCX-HPLC method and supports a quantitation limit of 0.1% for all 6 of the tested degradation products.

Table 9. SCX-HPLC Method Recovery of Degradation Products Spiked Into Pramlintide Working Reference Standard Solution

Spike Level Nominal %[a]	%Recovery (± %RSD)[b] For:					
	[30-37] RRT[c]=0.14	[iso-Asp21] RRT[c]=0.60	[Asp21] RRT[c]=0.65	[TyrCOOH37] RRT[c]=0.72	[1-18] RRT[c]=1.3	Dimer RRT[c]=1.6
1.0	99.6±0.7	102±0.6	101±1	102±1	101±2	106±2
0.50	95.1±0.6	96.0±0.4	92.7±0.3	91.3±3	93.8±0.7	108±3
0.25	93.9±0.8	98.3±0.2	95.3±0.5	92.8±1	91.8±2	108±4
0.10	85.1±1	103±3	99.8±2	93.2±3	69.2±3	101±7
0.05	86.1±0.0	119±3	112±3	103±8	75.2±10	85.6±13
Mean± %RSD (1 to 0.1% Spike)	93.4±7	99.9±3	97.2±4	94.8±5	89.1±16	106±3
Mean±%RSD (1 to 0.05% Spike)	92.0±7	104±9	100±8	96.5±6	86.3±16	102±9

a. Expressed as a percentage of intact pramlintide peak.
b. Mean and %RSD for five injections, except for 0.10% and 0.05% spike levels (three injections).
c. RRT = retention time relative to intact pramlintide.

To demonstrate recovery of degradation products after the solid-phase extraction procedure used for drug product samples, a pramlintide solution spiked to approximately 1.0% with 5 degradation products was analyzed by RP-HPLC and SCX-HPLC before and after application of the solid-phase extraction procedure. **Tables 10** and **11** summarize the data and demonstrate that,except for pramlintide dimer, which showed somewhat lower recoveries, the degradation product determinations were essentially unaffected by the solid-phase extraction step.

Table 10. RP-HPLC Determination of Recovery of Degradation Products Spiked Into Pramlintide Reference Standard Solution After Solid-Phase Extraction

Sample	Area % for Analyte =				
	[1-18] RRT[a]=0.43	[iso-Asp21] RRT[a]=0.77	[TyrCOOH37] RRT[a]=0.1.10	[Asp21] RRT[a]=0.1.2	Dimer RRT[a]=1.7
Control[b]	0.56	0.75	0.99	0.84	0.49
Analyst 1	0.56	0.75	1.00	0.84	0.29
Analyst 2	0.55	0.74	1.00	0.84	0.25
Analyst 3	0.53	0.74	0.95	0.82	0.21

a. RRT = retention time relative to intact pramlintide.
b. The control sample was not subjected to the solid-phase extraction step.

Table 11. SCX-HPLC Determination of Recovery of Degradation Products Spiked Into Pramlintide Reference Standard Solution After Solid-Phase Extraction

Sample	Area % For Analyte =				
	[1-18] RRT[a]=0.43	[iso-Asp[21]] RRT[a]=0.77	[TyrCOOH[37]] RRT[a]=0.1.10	[Asp[21]] RRT[a]=0.1.2	Dimer RRT[a]=1.7
Control[b]	0.61	0.66	0.95	0.68	0.26
Analyst 1	0.61	0.65	0.96	0.67	0.16
Analyst 2	0.62	0.65	0.95	0.67	0.13
Analyst 3	0.63	0.65	0.92	0.66	0.12

a. RRT = retention time relative to intact pramlintide.
b. The control sample was not subjected to the solid-phase extraction step.

Method Selectivity

Extensive studies with the RP- and SCX-HPLC methods demonstrated suitable resolution for both methods with respect to separating pramlintide from various related substance impurities in drug substance synthesis lots and degradation products from stress testing of drug substance. For brevity, these data are not shown. Rather, to demonstrate overall selectivity for the RP-HPLC method, **Figure 8** shows a representative chromatogram for a force-degraded pramlintide injection drug product sample (86% of initial purity remaining).

Figure 9 shows the corresponding SCX-HPLC method chromatogram for the same force-degraded drug product sample. Note the distinctly different "fingerprints" for the 2 methods and the large number of species that resolve from the intact pramlintide peak in both methods.

A comparison of **Figure 8** with **Figure 4** (the corresponding RP-HPLC method chromatogram for force-degraded working reference standard solution) shows comparable fingerprints and indicates that the solid-phase extraction sample preparation step used for drug product samples does not significantly perturb the hydrolytic degradation product profile.

A previous report [10] identified degradation products that result from 40° C stress testing of pramlintide injection drug product samples. **Table 12** lists the identified pramlintide injection degradation products and their retention times (relative to pramlintide) by the RP- and SCX-HPLC methods.

Figure 8. RP-HPLC Method Chromatogram of Force-Degraded (6 weeks at 40 °C) Pramlintide Drug Product.
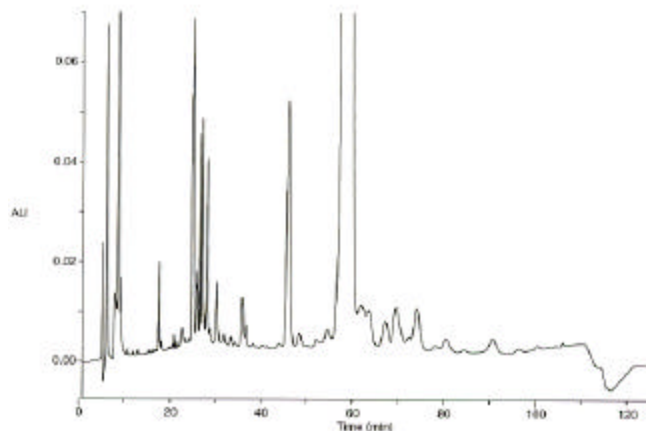
Table 12. RP-HPLC and SCX-HPLC Relative Retention Time Data for Pramlintide Drug Product Degradation Products

Identity[a]	Relative[b] Retention Time By Method:	
	RP-HPLC	SCX-HPLC
[iso-Asp[3]]	0.60	0.37
[Asp[3]]	1.4	0.58
[Asp[14]]+[iso-Asp[14]]	0.45	0.46
[iso-Asp[21]]	0.77	0.60
[Asp[21]]	1.21	0.65
[iso-Asp[22]]	0.98	0.62
[Asp[22]]	1.22	0.63
[iso-Asp[35]]	1.03	0.69
[Asp[35]]	1.08	0.73
[TyrCOOH[37]]	1.10	0.72
[1-18]	0.43	1.27
[1-19]	0.48	1.32
[19-37]	0.39	0.07
[20-37]	0.39	0.060

a. See Materials and Methods section for key to abbreviations used. Identification data from reference 9.
b. Retention time relative to pramlintide retention time.

Table 12 clearly demonstrates very different RP- versus SCX-HPLC selectivities toward the degradation products, thereby demonstrating the orthogonal nature of the two separation techniques. For example, [Asp[3]] elutes with relative retention time (RRT) = 1.4 by RP-HPLC and RRT = 0.58 by SCX-HPLC. Conversely, the [1-18] fragment elutes with RP-HPLC RRT = 0.43 and SCX-HPLC RRT = 1.27. Note also that several species do not resolve from pramlintide (RRT = 1.0) by one HPLC method but resolve well by the other. Thus, by applying only the 2 orthogonal HPLC methods it is

possible to be highly confident of detecting all the degradation product species.

Table 13 shows relative retention time data for authentic samples of 7 different pramlintide diastereomers. As also seen for the degradation product analyses summarized in **Table 10**, the data in **Table 13** demonstrate different RP- and SCX-HPLC selectivities for several of the D-isomers.

Table 13. RP-HPLC and SCX-HPLC Relative Retention Time Data for Pramlintide Diastereomers

Diastereomer[a]	Relative[b] Retention Time By Method:	
	RP-HPLC	SCX-HPLC
[D-Leu^{12}]	0.49	0.79
[D-Ala^5, D-Ala^8]	0.49	0.79
[D-Ala^8]	0.63	0.88
[D-Ala^5]	0.56	0.83
[D-Tyr^{37}]	1.00	1.00
[D-Lys^1]	0.97	0.92
[D-Cys^2]	0.94	1.00

a. See Materials and Methods section for key to abbreviations used
b. Retention time relative to pramlintide retention time.

DISCUSSION

The RP-HPLC method developed for pramlintide purity analysis uses conventional column material and mobile phase compositions. The long isocratic portion of the gradient program affords excellent separation between pramlintide and its degradation products and related substance impurities, albeit at the expense of a relatively long run time.

The SCX-HPLC method was chosen in anticipation [5-8] of deamidation as the primary degradation mode for the pramlintide drug product. Deamidation reactions at asparagine, glutamine, and C-terminal tryosine amide yield a unit charge decrease for the molecule at acidic pH, suggesting ion exchange chromatography as an appropriate separation mode for analysis. As with the RP-HPLC method, the SCX-HPLC method employs a lengthy isocratic section in the gradient program and features a relatively long run time.

Although the long run times are impractical, it was possible to simplify the overall analysis scheme and achieve significant operating efficiencies by developing two methods that share a common sample preparation and that apply both to drug substance and drug product samples.

Concerning RP- and SCX-HPLC method quantitative performance, **Tables 5 through 9** demonstrate that the methods perform satisfactorily with respect to accuracy, linearity, recovery, limit of quantitation, and precision (repeatability and reproducibility). Similarly, **Tables 8** and **9** demonstrate suitable recovery and precision for 6 different degradation products at levels as low as approximately 0.1% of intact pramlintide. A possible exception is the pramlintide dimer (the latest-eluting species by both the RP- and SCX-HPLC methods). **Tables 8** and **9** demonstrate reasonable recovery and precision for pramlintide dimer at levels as low as approximately 0.25%.

Figures 8 and **9** demonstrate the complexity of analyzing degraded pramlintide drug product samples and also give some evidence for differential selectivity between the RP- and SCX-HPLC methods based on the distinctly different chromatographic profiles for identical samples. **Table 12** clearly demonstrates the differential method selectivities insofar as most individual degradation product species exhibit significantly different relative retention times on the RP- versus the SCX-HPLC methods.

Similarly, the RP- and SCX-HPLC methods offer partial diastereospecificity with respect to 7 different pramlintide diastereomers. Thus [D-Leu^{12}], [D-Ala^5, D-Ala^8], [D-Ala^8], and [D-Ala^5] resolve well from pramlintide and show different relative retention times on RP- versus SCX-HPLC methods. The [D-Tyr^{37}], [D-Lys^1], and [D-Cys^2] species however, resolve poorly from pramlintide on both SCX-HPLC and RP-HPLC methods, indicating that isomerization near the N- or C-termini of the pramlintide molecule does not form a ready basis for diastereospecific separation.

In summary, the complexity of quantitating low levels of closely structurally related impurities and

degradation products in the 37– amino acid peptide, pramlintide, requires application of very highly selective HPLC techniques. Additionally, more complete characterization of pramlintide impurity and degradation product profiles is provided by multiple methods that provide some orthogonality with respect to separation mode. The RP- and SCX-HPLC methods described herein adequately satisfy these analytic requirements.

ACKNOWLEDGEMENTS

REFERENCES:

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

2.   Young AA. Amylin regulation of fuel metabolism. J Cellular Biochem. 1994;55 (Supp):12-18.

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

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

7.   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.

8.   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.

9.   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.

10.   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.