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Identification of Chemically Modified Peptide from Poly(D,L-lactide-co-glycolide) Microspheres Under In Vitro Release Conditions

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ABSTRACT

The purpose of this research was to study the chemical reactivity of a somatostatin analogue, octreotide acetate, formulated in microspheres with polymers of varying molecular weight and co-monomer ratio under in vitro testing conditions. Poly(D,L-lactide-co-glycolide) (PLGA) and poly(D,L-lactide) (PLA) microspheres were prepared by a solvent extraction/evaporation method. The microspheres were characterized for drug load, impurity content, and particle size. Further, the microspheres were subjected to in vitro release testing in acetate buffer (pH 4.0) and phosphate buffered saline (PBS) (pH 7.2). In acetate buffer, 3 microsphere batches composed of low molecular weight PLGA 50:50, PLGA 85:15, and PLA polymers (≤10 kDa) showed 100% release with minimal impurity formation (<10%). The high molecular weight PLGA 50:50 microspheres (28 kDa) displayed only 70% cumulative release in acetate buffer with significant impurity formation (~24%). In PBS (pH 7.4), on the other hand, only 50% release was observed with the same low molecular weight batches (PLGA 50:50, PLGA 85:15, and PLA) with higher percentages of hydrophobic impurity formation (ie, 40%, 26%, and 10%, respectively). In addition, in PBS, the high molecular weight PLGA 50:50 microspheres showed only 20% drug release with ~66% mean impurity content. The chemically modified peptide impurities inside microspheres were structurally confirmed through Fourier transform-mass spectrometry (FT-MS) and liquid chromatography/mass spectrometry (LC-MS/MS) analyses after extraction procedures. The adduct compounds were identified as covalently modified conjugates of octreotide with lactic and glycolic acid monomers within polymeric microspheres. The data suggest that due to steric hindrance factors, polymers with greater lactide content were less amenable to the formation of adduct impurities compared with PLGA 50:50 copolymers.

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INTRODUCTION

Octreotide acetate, a synthetic octapeptide, has revolutionized the treatment of acromegaly, a condition in which excess growth hormone is produced from the anterior pituitary. The somatostatin analogue has been commercially formulated in both an injectable form (lactate buffer, pH 4.2) for subcutaneous administration 3 times daily and in a depot form (PLGA microspheres) for intramuscular administration on a monthly basis. Investigators found no significant difference in pharmacodynamic responses from chronic therapy with either the injection or the depot formulation.[1] The convenience for an acromegalic patient requiring chronic therapy over decades, however, justifies an optimal depot formulation with ideal release parameters and optimal drug stability within the dosage form.

Poly(D,L-lactide-co-glycolide) (PLGA) microspheres have been widely investigated for the delivery of bioactive peptides and proteins in humans and animals. In the United States and the European markets, several microsphere products exist for the delivery of compounds including luteinizing hormone releasing hormone (LHRH) analogues, somatostatin analogues, and recombinant growth hormone. The bioactive peptides appear most suitable for the dosage form due to high potency, low bioavailability, pharmacodynamic properties, and short biological half-lives. Much research has

focused on the formulation, in vitro release methodology, and in vivo PK/PD responses of microparticulate delivery systems.

Furthermore, attempts to characterize physical adsorption phenomena between peptide and polymer revealed a combination of hydrophobic and electrostatic forces that potentiate interactions.[2,3] These phenomena for peptide molecules, however, may not result in any potential loss of biological activity due to the lack of secondary and tertiary structures. For protein molecules, on the other hand, physical adsorption could represent a destabilization of secondary structures involving hydrogen bonding and tertiary structures involving folding of hydrophobic residues. Hence, for bioactive macromolecules, loss of drug activity requires major consideration during formulation and delivery.

For peptide molecules, the loss of biological activity, chemical potency, or chemical purity during manufacturing or at the time of delivery has not received much consideration until recently. Lucke et al described the acylation of salmon calcitonin (SCT) and atrial natriuretic peptide (ANP) in PLGA matrices (**Figure 1**).[4,5] The chemically modified peptides were discovered during the course of in vitro release testing in neutral phosphate buffer (pH 7.2). Furthermore, the authors determined that microenvironment pH and high local concentration of oligomers facilitated the formation of the peptide adducts. When analyzed by liquid chromatography/mass spectrometry (LC-MS), the adduct compounds were identified as 72 mass to charge ratio (m/z) units higher than the parent peptide, indicative of lactoyl substitution on the therapeutic molecule.

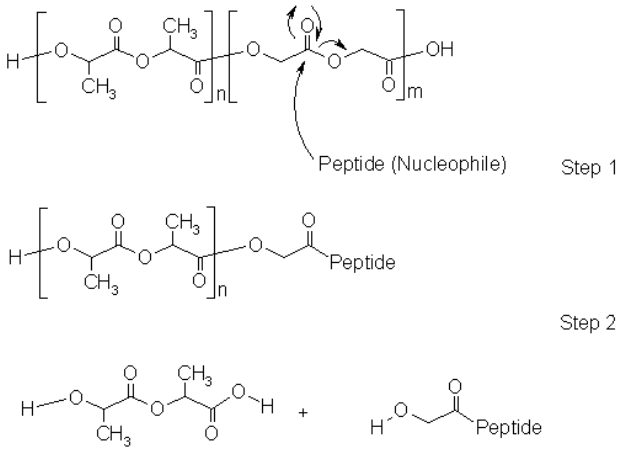

Figure 1. Proposed mechanism of reaction between peptide and polymer. Nucleophiles are -NH_2, -OH, and -SH (potential reactive centers on peptide).

Further evidence of peptide reactivity was provided when RC-160, a somatostatin analogue formulated into poly(D,L-lactide) (PLA) polymeric implants, was reported to undergo acylation with cyclic lactide dimers, which were present as polymer impurities from raw material synthesis.[6] The reaction occurred during the high temperature (120°C) melt extrusion procedure resulting in a lactoyl-lactyl adduct, which was identified with an m/z value 144 m/z units higher than the parent RC-160.

Preliminary investigation into octreotide acetate (H_2N-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol; MW = 1018.4) commercially formulated in PLGA microspheres, Sandostatin LAR depot (Novartis Pharma, Basel, Switzerland), revealed the presence of lactoyl (+72 m/z) and glycoyl (+58 m/z) adducts after in vitro incubation in PBS for 2 weeks. Hence, it may be postulated that octreotide acetate (**Figure 2**) could chemically interact with PLGA or PLA polymers either during the manufacturing process or during in vitro release testing. Furthermore, the extent of reaction occurring between peptide and polymer would vary as a function of the polymer-comonomer ratio. Based on steric factors, reactive nucleophiles present on amino acid side chains would react more readily with glycolic acid monomers as opposed to lactic acid monomers. The methyl group present on the alpha carbon adjacent to the ester carbonyl in lactic acid monomers would reduce reactivity in PLA-based microspheres as opposed to PLGA microspheres containing greater glycolide content in the comonomer ratio (**Figure 3**).

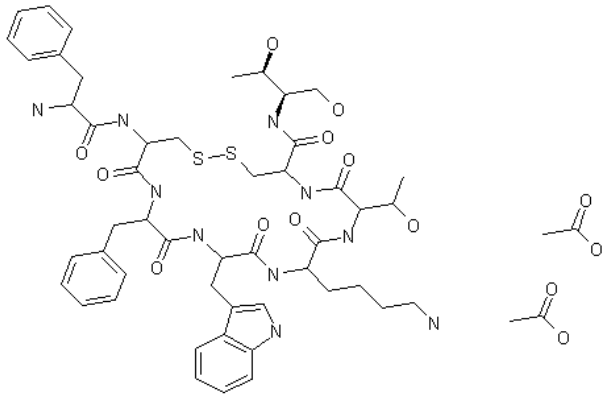

Figure 2. Structure of octreotide acetate.

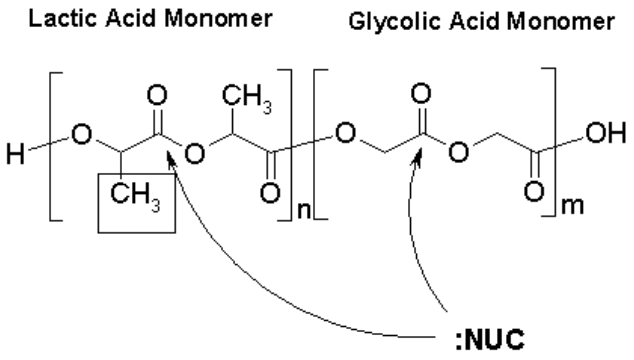

Figure 3. Proposed explanation of reduced reactivity of nucleophilic species with lactic acid monomers.

To test the above hypotheses, octreotide microspheres were prepared by a solvent extraction/evaporation method previously described.[7,8] Polymers utilized to manufacture microspheres included PLGA 50:50, PLGA 85:15, and PLA 100. The microspheres were subjected to in vitro release testing and in vitro incubation experiments for elucidation of the extent and the type of reactions taking place, respectively.

MATERIALS AND METHODS

Materials

Octreotide acetate (H_2N-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol; MW = 1018.4) was obtained from Bachem Inc (Torrance, CA). PLGA 50:50 copolymers were purchased as RG503H and RG502H from Boehringer Ingelheim (Ingelheim, Germany). PLGA 8515DL1AP copolymer and poly(l-lactide) 100DL1AP homopolymer were purchased from Alkermes Inc (Cincinnati, OH). All other chemicals used were of analytical reagent grade.

Microsphere Preparation

Drug loaded (octreotide acetate) PLGA and PLA microspheres were prepared by the solvent extraction/evaporation method, previously described.[7,8] The dispersed phase included methanol/methylene chloride solvent system and the continuous phase included 0.35% polyvinyl alcohol (PVA) aqueous solution. Octreotide acetate was dissolved in methanol and combined with the polymer solution dissolved in methylene chloride. This constituted the dispersed phase, which was slowly added via a syringe into the continuous phase (0.35% wt/vol solution of PVA at pH 7.2). The mixture was stirred at 5500 rpm with a Silverson L4R homogenizer (Silverson Machines, Waterside, UK). Continuous stirring at 40°C for 1 hour resulted in the extraction/evaporation of solvents from the formulation. Finally, hardened microspheres were recovered by vacuum filtration and washed with water. To evaporate any residual water content, microspheres were vacuum dried for 48 hours.

Microsphere Characterization

Microspheres were characterized by laser diffractometry using a Malvern 2600 laser sizer (Malvern Instruments, Malvern, UK). Particle sizes were expressed as volume median diameters in microns. Microspheres were also characterized for drug content and initial impurity content by an organic solvent extraction procedure using methylene chloride and buffer. The microspheres were dissolved in methylene chloride and the peptide was extracted with 0.1M acetate buffer (pH 4.0). Peptide concentration was determined by high-performance liquid chromatography (HPLC). Initial impurity content was determined by area percentage of the peaks eluting after the parent peptide peak on the chromatogram.

In Vitro Release Procedures

Octreotide microspheres prepared from polymers of varying comonomer ratio and molecular weight were subjected to in vitro testing in 0.02M PBS (pH 7.2) and in 0.1M acetate buffer (pH 4.0). Approximately 20 mg of microspheres were placed in a 15-mL round-bottom glass tube. After addition of 4 mL of buffer, each tube was continuously rotated 360 degrees using a wrist-shaker in a 37°C chamber. At specific time points, the supernatant of each tube was collected and analyzed by HPLC. To prevent the accumulation of peptide degradation products in solution, complete buffer replacements occurred at weekly intervals.

High Performance Liquid Chromatography Method for Peptide Measurements in Supernatant

Octreotide Acetate was analyzed by reversed phase (RP)-HPLC using an Alltech C18 start column, 250 mm × 4.60 mm (Alltech Associates, Deerfield, IL). A gradient elution method was utilized with mobile phase A (0.1% [vol/vol] trifluoroacetic acid in water) and mobile phase B (0.1% [vol/vol] trifluoroacetic acid in acetonitrile). The gradient was 80:20 (A:B) to 65:35

(A:B) over 18 minutes with a flow rate of 1.0 mL/min. UV absorbance was measured at 215 nm.

Incubation of Microspheres in Phosphate Buffered Saline

To identify the acylation products, peptide-loaded PLGA 50:50 microspheres were incubated in 0.1M PBS (pH 7.4) at 37°C. Collection of solid polymeric mass was performed at days 1, 4, 7, and 14 using vacuum filtration. The filtrates were subsequently extracted using methylene chloride and acetate buffer as mentioned above. For qualitative assessment, Fourier transform-mass spectrometry (FT-MS) analysis of buffer extracts was performed at all time points during the 2-week period. In addition, the buffer extract at the last time point (14 days) was analyzed separately by LC-MS/MS for chemical identification of impurities or related substances.

Fourier Transform Mass Spectrometry

Buffer extracts were placed under vacuum conditions for evaporation of water for subsequent incorporation into a α-cyanohydroxy cinnamic acid (α-CHCA) matrix. An Ion-Spec FT-MS was utilized for analysis of extracted peptide from microspheres.

LC-MS/MS

Liquid chromatographic separation was performed using an RP-HPLC with a Nucleosil, C18 column (250 × 4.6 mm) (Phenomenonex USA, Torrance, CA). The gradient elution utilized the same mobile phase A and mobile phase B as mentioned above with the gradient changing from 80:20 (A:B) to 40:60 (A:B) over 25 minutes. The UV detector at 220 nm was coupled to PE Sciex API-365 mass spectrometer (MDS Sciex, Ontario, Canada) with ionization mode using a turbo ion spray (positive detection). The scan range included 300 to 600 atomic mass units (amu). Since MS/MS information on octreotide and related substances was limited due to the low fragmentation of the molecule containing the disulfide bridge (**Figure 2**), the extracted material was treated with dithiothreitol (DTT) in phosphate buffer (pH 8.0) for 60 minutes at 40°C.

Statistical Evaluation of Data

Data are presented as means with SD. Paired *t* tests were performed to compare cumulative release of peptide with cumulative amount of impurity formation ($\alpha = 0.05$) using Microsoft Excel software. Further, summary results of extent of impurity formation were analyzed by 1-way analysis of variance (ANOVA) at α-level of 0.05 using Graph Pad Prism software (Graph Pad Software, Inc, San Diego, CA).

RESULTS AND DISCUSSION

Microsphere Characterization

The microspheres prepared by the solvent extraction evaporation method were characterized for drug/impurity content as well as particle size ranges. The microspheres were prepared with one of the following polymers: (1) low MW PLGA 50:50; (2) high MW PLGA 50:50; (3) PLGA 85:15; and (4) PLA 100. **Table 1** summarizes the relevant characteristics of each batch of microspheres. To test the effect of comonomer ratio on extent of acylation, 3 batches containing low MW polymer (≤10 kDa) were prepared. In addition, to test the effect of polymer MW on acylation, RG503H and RG502H 50:50 copolymers were utilized with values of 28 kDa and 10 kDa, respectively. The initial impurity content values were insignificant, thus indicating the present manufacturing process has little effect on the formation of detectable adduct compounds.

In Vitro Release in Acetate and Phosphate Buffer Systems

Previously, Bodmer et al reported the maximal solubility and stability of octreotide in acetate buffer (pH 4.0). Consequently, in vitro release testing of octreotide microspheres in acetate buffer resulted in 100% drug release with insignificant degradation products during testing.[9] In addition, Domb et al reported enhanced reactivity of amine compounds with polymer matrices at pH 7.4 as opposed to pH 4.0.[10] Therefore, the lower pH buffer system might be considered as a standard release media for quality control testing as in the case of microspheres containing orntide acetate, an LHRH antagonist decapeptide.[11] In the present study, however, the purpose was to investigate the formation of acylated peptide, hence a PBS media was also utilized to study the effect of physiological pH on the extent of reaction. In PBS buffer media alone, octreotide acetate does not display any significant instability for 21 days at 37(C with an apparent first order degradation constant of 4.37×10^{-3} $days^{-1}$ (unpublished laboratory data from University of Kentucky). Thus, any significant peptide instability could be a result of drug-polymer chemical interaction within the microsphere matrix.

Table 1. Microsphere Characteristics*

Microsphere Characteristic	High MW PLGA 50:50	Low MW PLGA 50:50	Low MW PLGA 85:15	Low MW PLA 100:0
Polymer	RG503H	RG502H	8515DL1AP	100DL1AP
Polymer manufacturer	Boehringer Ingelheim	Boehringer Ingel-heim	Alkermes	Alkermes
Polymer MW	28 kDa	10 kDa	10 kDa	8 kDa
Drug load, %	8.60%	8.55%	9.20%	8.47%
Initial impurity content (% of total drug load)	<1%	1.28%	<1%	<1%
Average particle size, μ	21.0	24.1	19.8	15.4

*MW indicates molecular weight; PLGA, poly(D,L-lactide-co-glycolide); and PLA, poly(D,L-lactide).

Figures 4 and **5** show the in vitro release profiles in 0.1M acetate buffer (pH 4.0). Specifically, **Figure 4** deals with the release profiles for batches of varying comonomer ratio and **Figure 5** shows curves from batches with varying molecular weights. In **Figure 4**, all batches release to approximately 100% with low MW 50:50 releasing the fastest (complete release achieved within 29 days). Both PLA and PLGA 85:15 microspheres, on the other hand, reach 100% release by day 43. From the data, the mechanisms of drug release can be empirically assessed, where PLGA 50:50 microspheres display a rapid initial burst with a subsequent erosion phase release. With PLA, the release is characterized by a reduced burst with a subsequent pseudo zero-order release phase. Further, PLGA 85:15 shows minimal burst release with a more rapid concentration independent release phase. In **Figure 5**, on the other hand, the high MW PLGA 50:50 batch demonstrates a sigmoidal type release profile, which typically is characterized by a lag time for release followed by a rapid erosive phase release. These observations on mechanisms are unique for the specific media surrounding the dosage form since mass loss, hydration, and drug release kinetics are drastically different in media of varying pH. This is predominantly due to the degree of ionization of free carboxylic end groups present on the polymer end groups and due to the solubility of active drug in media of varying pH, ionic strength, and buffer concentration.[11,12]

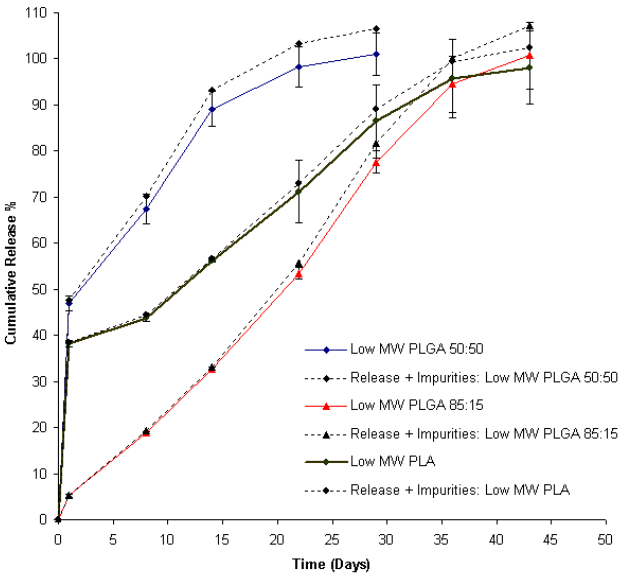

Figure 4. In vitro release in 0.1M acetate buffer (pH 4.0): microspheres with varying comonomer ratio.

Although the drug release profiles are of interest for characterization of the microspheres, the issue of chemical stability within the dosage form requires significant attention. In **Figures 4** and **5**, shadowing each release curve is a dashed line representing the release of parent octreotide plus impurity peaks present on each chromatogram. These peaks eluting after the parent compound were identified to be hydrophobic-related substances or octreotide adducts (see LC-MS/MS analysis from **Figure 11**). The hydrophobic peaks did not appear in any significant quantity in the initial drug content assay (<1%) as shown in **Table 1**. During the course of the release experiment, however, the presence of impurities increased with respect to the

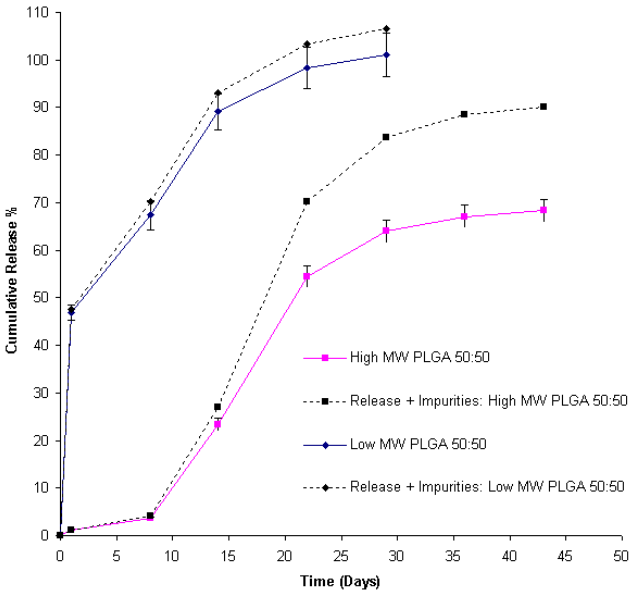

Figure 5. In vitro release in 0.1M acetate buffer (pH 4.0): microspheres with varying MW.

parent compound, octreotide. Assuming that the molar absorptivity coefficient under Beer's Law is the same for the parent compound as the adduct compounds, a proposed concentration was estimated at each time point in the release experiment based on area percentage of each chromatogram peak. The equation used is as follows:

$$\%TR = \%OR + \%OR * (API/APO) \quad (1)$$

where TR indicates total release (octreotide + impurities); OR, octreotide release; API, area percentage of impurities from chromatogram; and APO, area percentage of octreotide from chromatogram.

Although full release was observed for 3 out of 4 batches, additional release of impurities in addition to the parent compound exceeded the mass balance value of 100% for low MW PLGA 50:50, PLGA 85:15, and PLA 100 microspheres (**Figure 4**). The minor deviations could have been a result of acceptable analytical error (10%) or initial peptide-polymer acylation products undetectable in the zero time drug content assays (**Table 1**). **Figure 1** shows a reaction mechanism as proposed by Lucke et al where initial acylation of peptide occurs with the long chain polymer backbone (Step 1) with subsequent hydrolytic cleavage of modified peptide containing monomeric fragments of either lactic or glycolic acid subunits (Step 2).[4,5] To speculate on the present situation, peptide and long chain polymer conjugates (Step 1) could have formed during the manufacturing process and been detected as a result of cleavage of monomers from the polymer backbone during the time course of the in vitro release study (Step 2).

Table 2 shows the total cumulative release and the total impurity content release when curves approach the plateau region of release for high MW PLGA 50:50 microspheres (**Figure 5**). A statistical analysis was performed to compare the terminal result of the total octreotide release versus total release (parent + impurities). Hence, a paired *t* test was performed to assess the differences and to test statistical significance. From **Table 3**, the statistical calculation resulted in $P < .0005$, and if one were to assign $\alpha = 0.05$, then the 2 columns in **Table 2** would appear to be significantly different. Further, the paired *t* tests were also applied to all microsphere batches used in the study with results presented in **Table 3**. For all groups, the total release content appears to be statistically significant from total parent compound release with all *P* values < .05. One would argue as to the physical meaning and significance of comparing the theoretical curve (total release curve) with the observed data (octreotide release curve) for each batch. As a result, a 1-way ANOVA was performed to compare the total percentage impurity content at the termination of the release experiment for all batches.

The original hypothesis prior to the release testing included the premise that nucleophilic attack would be hindered with lactic acid monomers as compared with glycolic acid monomers. Hence, the percentage impurity content in microspheres batches of varying comonomer ratio should be assessed by a statistical test. From **Table 3**, performing the 1-way ANOVA to compare the mean percentage of impurities in each batch, the resultant *P* value was <.0001 with $\alpha = 0.05$. Further, a studentized rank method was performed to assess the order in which comonomer ratio affected the final impurity percentage. With 5% allowance, any mean with a difference greater than 1 percentage unit would be considered statistically significant. Hence, the following rank order was determined in the present study with respect to mean percentage of impurity content at the termination of release:

High MW PLGA 50:50 > Low MW PLGA 50:50, PLGA 85:15 > PLA 100

With the mean value of 5.140% for low MW PLGA 50:50 and the mean value of 5.874% for PLGA 85:15, statistical significance could not be found between the 2 batches of microspheres. Regardless, evidence exists for a potential effect of both varying comonomer ratio as well as polymer MW on the extent of impurity formation in 0.1M acetate buffer (pH 4.0). One

Table 2. Statistical Analysis of In Vitro Results for High MW PLGA 50:50 Microspheres in 0.1M Acetate Buffer (pH 4.0) at Termination*

Tube No.	% Octreotide Release	% Octreotide Release + Impurity†	Difference ($d = x1 - x2$)
1	66.67	88.83	22.16
2	67.29	88.40	21.11
3	71.07	93.25	22.18
Average (SD)	68.34 (2.38)	90.16 (2.69)	
			$\Sigma di = 65.45$
			$\Sigma di^2 = 1428.7$
			Mean d = 21.82
			n = 3

**d* indicates difference; MW, molecular weight; PLGA, poly(D,L-lactide-co-glycolide); Σdi, the sum of differences; and Σdi^2, the sum of the square of differences.
†Calculated based on area percentage of octreotide and impurities present at each time point during the course of the release experiment and the assumption that the molar absorptivity coefficient for each impurity is same as the parent compound.

Table 3. Summary of In Vitro Release in 0.1M Acetate Buffer (pH 4.0) (n = 3)*

Batch	% Octreotide Release (SD)	% Total Release (SD)†	Paired *T* Test ($\alpha = 0.05$)‡	Mean % Impurities (SD)§	1-way ANOVA ($\alpha = 0.05$)
PLGA 50:50 high MW	68.34 (2.38)	90.16 (2.69)	$P < .0005$	24.21 (0.641)	$P < .0001$
PLGA 50:50 low MW	101.8 (4.63)	106.6 (4.75)	$P < .001$	5.140 (0.201)	
PLGA 85:15 low MW	100.7 (7.25)	107.0 (7.31)	$P < .005$	5.874 (0.350)	
PLA 100:0 low MW	98.11 (7.96)	102.2 (8.37)	$P < .005$	4.034 (0.215)	

*ANOVA indicates analysis of variance; MW, molecular weight; and PLGA, poly(D,L-lactide-co-glycolide).
†Total percentage release (octreotide + impurities) calculated value based on the assumption that molar absorptivity coefficient for adduct compounds is same as parent compound.
‡Paired *t* test performed on each individual release tube within each treatment group.
§Calculation based on octreotide percentage release divided by total percentage release (octreotide + impurities) for each individual release tube, respectively.

would further argue, however, that in reality, a representative picture of chemical reactivity in vivo or at the site of injection (intramuscularly or subcutaneously) would be more accurate in a buffer system at physiological pH and ionic strength. Also, Domb et al previously reported a pH effect on the interaction between drug and polymer where accelerated reaction rates between alkyl amines and polyanhydrides occurred at pH 7.4 as opposed to pH 5.0.[10] As a result, the release experiment performed in acetate buffer (pH 4.0) was also performed in PBS (pH 7.2).

Figures 6 and **7** display the release profiles for the batches of microspheres of varying co-monomer ratio and molecular weight, respectively. In **Figure 6**, the microspheres display approximately 50% cumulative release by the termination of the experiment (day 50), unlike the release data obtained in acetate buffer (pH 4.0) where 100% release was observed for all batches by termination (day 43). For high MW PLGA 50:50, again in contrast to release observed in acetate buffer, the cumulative release in PBS was less than 20% by 50 days (**Figure 7**). The difference in release characteristics in the 2 buffer media can potentially be explained by the role of peptide solubility factors in surrounding media. In fact, solubility may provide a thermodynamic driving force for enhancing drug release from porous matrices. Similar observations were

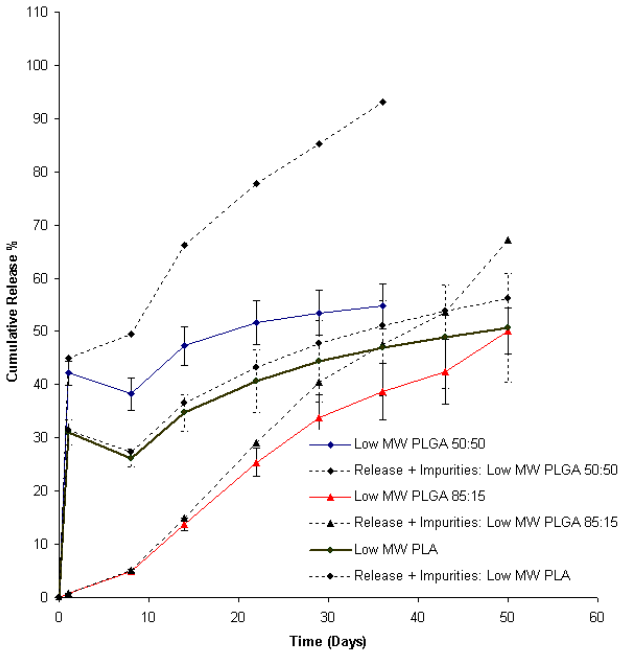

Figure 6. In vitro release in 0.02M PBS (pH 7.2): microspheres with varying comonomer ratio.

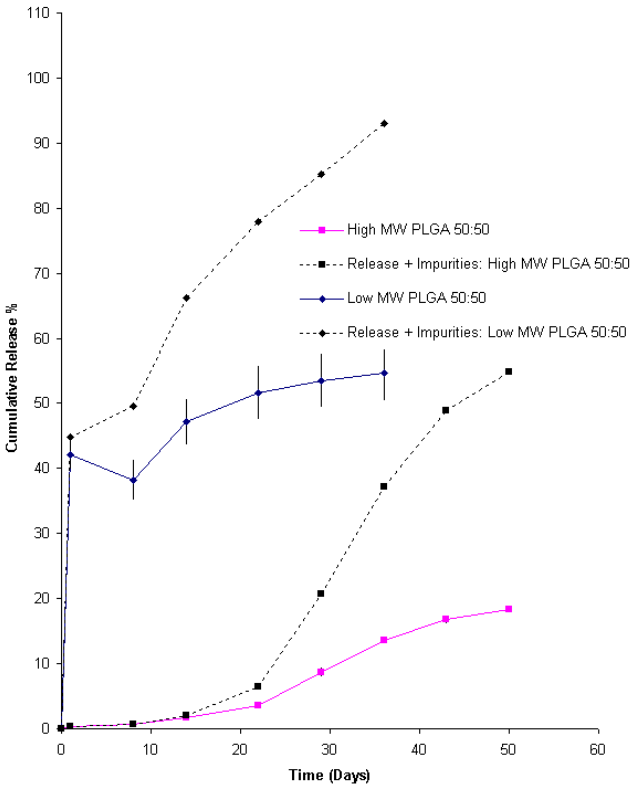

Figure 7. In vitro release in 0.02M PBS (pH 7.2): microspheres with varying MW.

reported when vapreotide (RC-160) microspheres were independently incubated in PBS (pH 7.2), 1% (wt/wt) BSA solution, and serum. The authors concluded that enhanced drug release from microspheres in BSA and serum as opposed to PBS was a result of enhanced solubilization of peptide in surrounding media.[12]

Although the release profiles obtained in PBS are of interest for characterization in media of physiological pH and ionic strength, the chemical reactivity in PBS is the issue that requires further analysis. By observing the total release profiles (octreotide + impurity) for each batch in **Figures 6** and **7**, one can conclude that the overall impurity formation is much greater in PBS as opposed to acetate buffer. **Table 4** shows the summary of in vitro release results for all batches including the cumulative percentage release of octreotide alone and with impurities (total percentage release) at termination. As with the data obtained in acetate buffer, a paired *t* test was also performed with a 1-way ANOVA for comparison of mean percentage of impurities. For the statistical comparison, the calculated *P* value was again <.0001. If one were to perform the studentized ranking procedure with 5% allowance, then one mean would be statistically different from another mean if the values were greater than 2.23 percentage units apart. Hence, in PBS, the mean percentages of impurities from all batches are statistically different from one other with rank order as follows:

High MW PLGA 50:50 > Low MW PLGA 50:50 > PLGA 85:15 > PLA 100

In summary of the results in different media, the extent of impurity formation or reactivity, detectable under HPLC conditions, was influenced by both the comonomer ratio and the MW of the polymer utilized in the microsphere preparation. The comonomer ratio phenomenon can be explained by the original hypothesis, in which nucleophilic reactivity toward lactide monomers is lower than glycolide monomers because of the methyl substituent on the alpha carbon (**Figure 3**). Thus, polymer with lower glycolide content would be less favorable for the formation of hydrophobic-related substances of octreotide. For the MW phenomenon, one could suggest that peptide residence time inside the microsphere dictates the extent of reactivity. Since the release curves for high MW 50:50 microspheres display lag times (PBS > acetate buffer), the exposure of peptide to high local concentration of polymeric or oligomeric materials of PLGA is greater as compared to low MW 50:50 microspheres, where burst release is significantly higher.

Table 4. Summary of In Vitro Release in 0.02M PBS (pH 7.2) (n=3)*

Batch	% Octreotide Release (SD)	% Total Release (SD)†	Paired *T* Test ($\alpha = 0.05$)‡	Mean % Impurities (SD)§	1-way ANOVA ($\alpha = 0.05$)
PLGA 50:50 high MW	18.18 (0.644)	54.20 (2.69)	$P < .001$	66.43 (0.678)	$P < .0001$
PLGA 50:50 low MW	54.70 (3.42)	92.88 (5.99)	$P < .001$	41.13 (1.17)	
PLGA 85:15 low MW	49.895 (4.63)	67.01 (5.77)	$P < .001$	25.55 (0.221)	
PLA 100:0 low MW	50.64 (10.3)	56.20 (12.3)	$P < .005$	9.68 (1.56)	

*ANOVA indicates analysis of variance; MW, molecular weight; PBS, phosphate buffered saline; and PLGA, poly(D,L-lactide-co-glycolide).
†Total percentage release (octreotide + impurities) calculated value based on the assumption that molar absorptivity coefficient for adduct compounds is same as parent compound.
‡Paired *t* test performed on each individual release tube within each treatment group.
§Calculation based on octreotide percentage release divided by total percentage release (octreotide + impurities) for each individual release tube, respectively.

Lucke et al proposed an explanation in which the high local concentration of polyesters inside the microsphere potentiates the physical mechanism of reaction due to proximity factors not present in dilute solutions of lactic acid.[5]

Incubation of Microspheres in PBS

Thus far, the appearance of peptide impurities has been observed in buffer media under in vitro release conditions. Further, the peptide reactivity has been assumed to be negligible in buffer media alone (PBS and acetate buffer) without the presence of polymeric microspheres or oligomers. In fact, weekly buffer replacements were performed to prevent accumulation of potential peptide degradation products in buffer media. Hence, the formation of hydrophobic-related substances was hypothesized to be a result of molecule residence inside the microsphere dosage form. In addition, the extent of reaction was a function of comonomer ratio and MW of the polymeric material.

Consequently, one would ascertain as to the chemical structure of the impurities or hydrophobic related substances. Further, the site of acylation on the peptide molecule requires further understanding in order to speculate on a mechanism of reaction. Lucke et al, identified reactive serine, lysine, and N-terminal groups as potential nucleophiles by LC-MS and tryptic digest analysis.[4] The researchers, however, only identified lactoyl conjugates (+72 m/z) indicative of reactions occurring in PLA polymers only. To further investigate the chemically modified peptides and the sites of acylation, incubation experiments were performed with high MW PLGA 50:50 microspheres in PBS (pH 7.4) at 37°C. Subsequently, independent tubes of PLGA microspheres were collected by vacuum filtration at days 1, 4, 7, and 14 for further extraction by organic solvents and acetate buffer. FT-MS analysis of the extracted material was performed at all time points and LC-MS analysis was performed at the terminal time point. The high MW 50:50 microspheres were chosen because of the highest extent of reaction and the long lag time observed from in vitro release. Hence, during the lag time (~14 days) for onset of drug release (**Figure 7**), the question remained on the type of reactions occurring inside the dosage form.

Figure 8 shows the mass spectrum for octreotide acetate prior to incorporation into PLGA microspheres. **Figure 9** demonstrates the FT-MS results through the time course of 14 days of incubation of microspheres in buffer media. In **Figure 8**, the mass spectrum of the raw material reveals no peaks with mass values greater than parent octreotide in the form of a noncovalent sodiated adduct (1018 m/z +23 m/z = 1041 m/z). In **Figure 9**, however, the presence of peaks with values greater than 1041 m/z is evident. For instance, at day 1 a peak is present at 1099.5 m/z, which is empirically indicative of a hydrophobic-related substance of octreotide. The covalently modified peptide is ~58 m/z units higher than the parent compound. At day 4, a minor peak in addition to 1099.5 m/z is present at 1113.5 m/z, which is 72 m/z units higher than the parent octreotide. Furthermore, at days 7 and 14, a peak appears at 1157.5 m/z (+116 m/z over octreotide) in addition to 1099.5 m/z and 1113.5

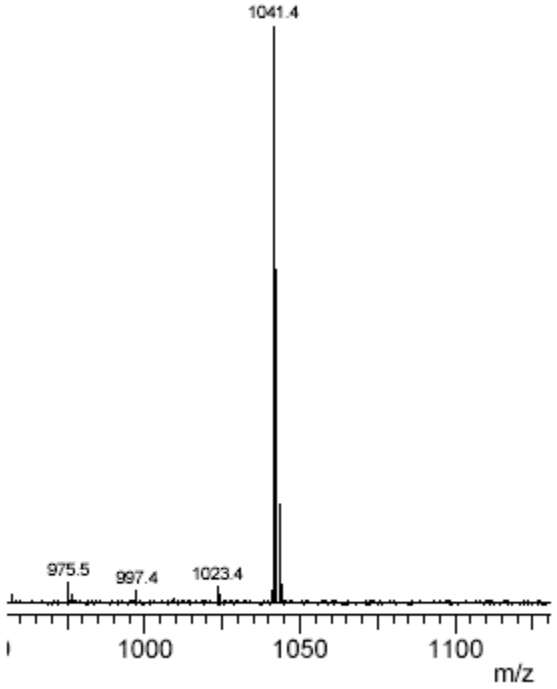

Figure 8. Mass spectrum of octreotide acetate dissolved in extraction media.

m/z. Judging by the proposed reaction mechanism shown in **Figure 1**, the m/z values are consistent with structures containing glycoyl adducts (+58 m/z), lactoyl adducts (+72 m/z), and glycoyl-glycyl adducts (+116 m/z).

Figure 10 presents hypothetical chemical structures for glycoyl adducts of octreotide, concurrent with the mass spectral results shown in **Figure 9**. In this representation, the pendant peptide molecule has either an n-terminus (left-sided figure extension) or an unmodified lysine residue (right-sided figure extension). Since the mass spectral results do not indicate the sites of acylation, the proposed monoglycoyl substitution in **Figure 10** could occur at either the N-terminus or the lysine residue. Furthermore, both a di-glycoyl substitution and a glycoyl-glycyl substitution can be deduced from the 1157.5 m/z (+116 m/z). The lactoyl substitution (not shown here), consistent with the 1113 m/z peak, again could either occur at the N-terminus or lysine residue. The lactoyl adduct is structurally similar to the glycoyl adduct with the addition of the methyl substituent on the alpha carbon.

LC MS/MS Analysis of Supernatant Extract

Although the mass spectra indicate the presence of hydrophobic-related substances, information on sites of acylation for each adduct requires further examination. The supernatant extract, therefore, obtained from the extract of incubated microspheres after 14 days in PBS was analyzed separately by LC-MS/MS. **Figure 11** shows a sample chromatogram of parent octreotide with hydrophobic adducts eluting after the main peak on a C18 reversed phase column. The chromatogram reveals at least 10 additional hydrophobic peaks eluting after octreotide at 10.83 minutes.

The peptide extract mixture was further treated with DTT for 1 hour to allow for molecular fragmentation by MS/MS. Subsequently, each of the 10 hydrophobic peaks, except peaks 5 and 10, were identified as adducts of octreotide containing either lacytoyl or glycoyl substitutions at 1 of the amine functional groups (N-terminus or lysine). **Table 5** details each of the adduct compounds from **Figure 11** where monosubstituted as well as di-substituted compounds were found. In addition, glycoyl-glycyl, glycoyl-di-glycyl, lactoyl-lactyl compounds were also identified. When correlating the information from **Table 5** with **Figure 11**, one can observe the change in retention time for the individual peaks due to disulfide bond reduction with DTT.

In addition, a pattern exists for the order of elution with respect to the site of substitution. For instance, prior to DTT treatment, lysine substituted compounds elute earlier from 11.30 minutes to 12.78 minutes, whereas N-terminal substituted compounds elute between 16.20 minutes and 18.87 minutes. As a result, the order of elution may provide an insight into the relative hydrophobicity of each of the compounds created from peptide-polymer interactions. Finally, the LC-MS/MS assay provides more detailed information on sites of substitution on the various compounds as opposed to FT-MS, where only 3 separate m/z peaks were qualitatively identified. The LC-MS/MS, in addition, allowed for identification of a greater number of adducts possibly because of the enhanced sensitivity of the instrument to miniscule concentrations.

Kinetics of Impurity Release from High MW PLGA Microspheres

With the identification of the major peaks in **Figure 11** through LC-MS/MS, an attempt was made to ascertain the relative percentage of each impurity present in the release data from **Figure 7**. The individual peaks from **Figure 11** were subsequently correlated with the peaks present from each chromatogram during the in vitro release experiment for high MW

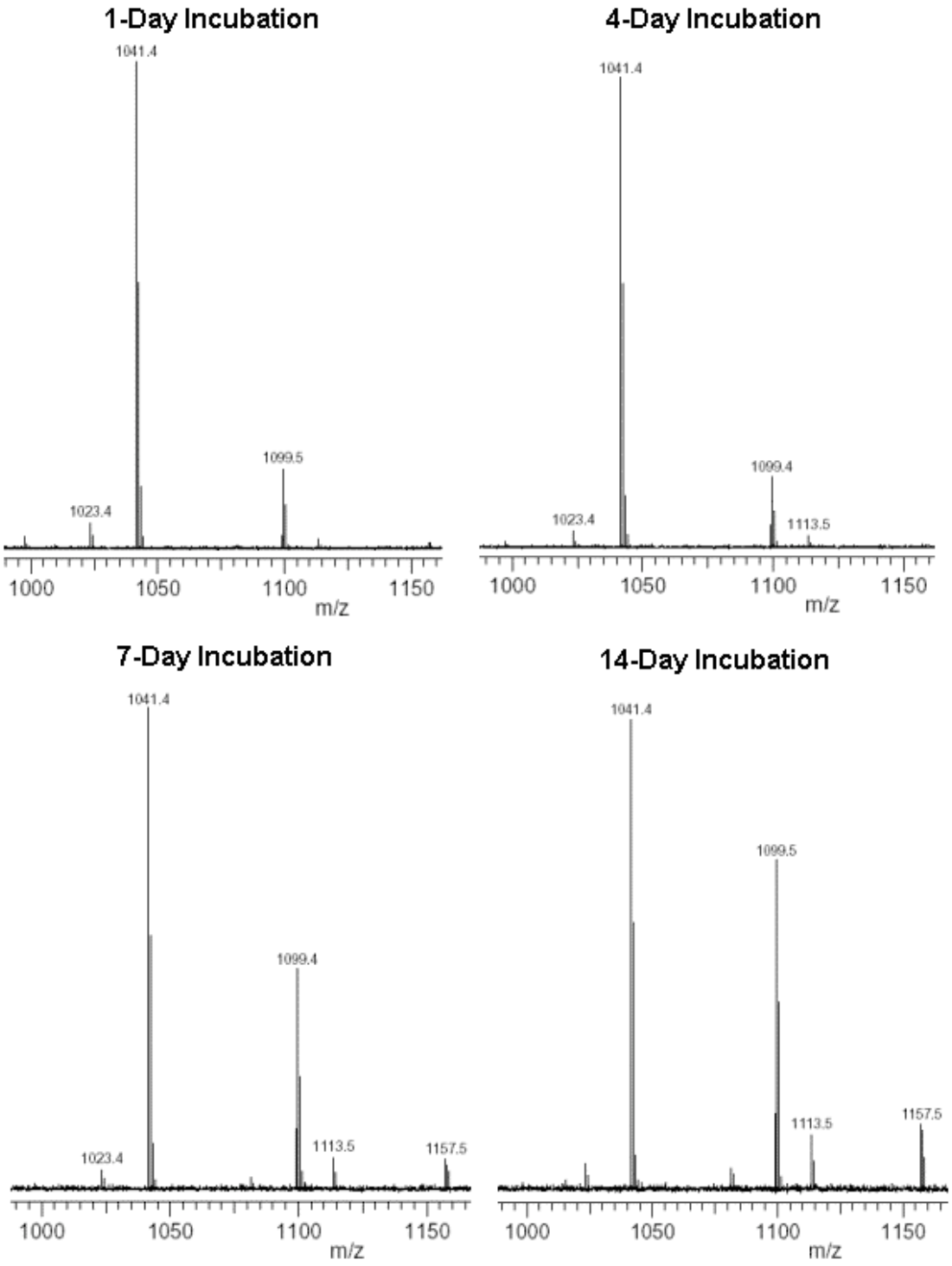

Figure 9. Mass spectra of extracts from high MW PLGA 50:50 microspheres after incubation in PBS (pH 7.4) for 1, 4, 7, and 14 days.

PLGA 50:50 microspheres. Assuming that the molar absorptivity coefficient is same for each impurity as octreotide, a theoretical concentration for each species was calculated as the chromophore regions (amide bonds) were not altered during acylation. **Figure 12**, therefore, shows total release (parent and all related peptides), octreotide release, and the release for selected acylated compounds in terms of concentration

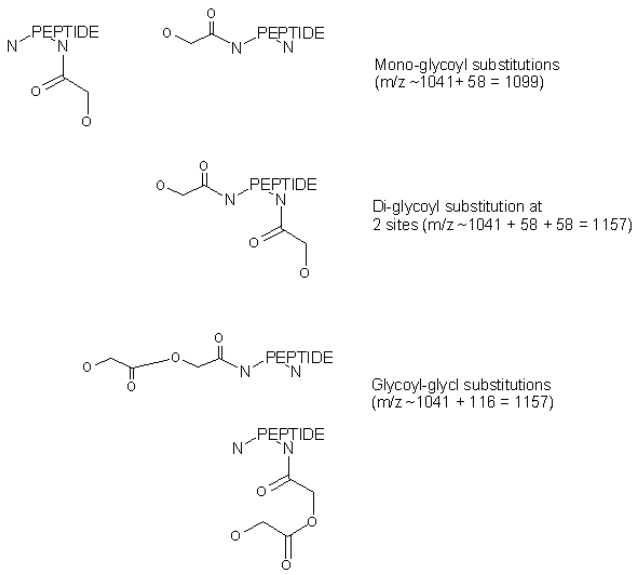

Figure 10. Proposed structures for glycoyl adduct compounds.

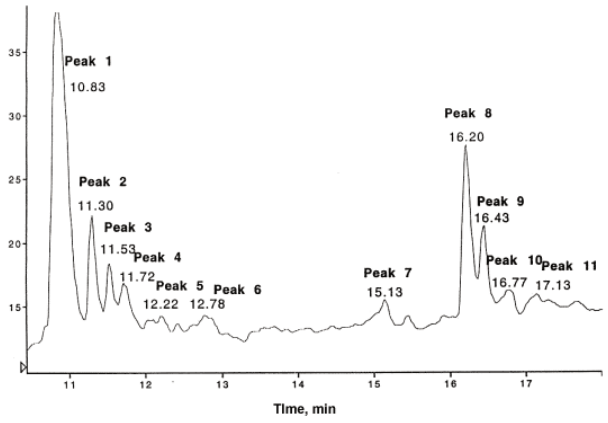

Figure 11. Chromatogram of octreotide and adducts (hydrophobic-related substances of parent peptide).

(μg/mL) versus time. The selected impurity compounds were chosen for analysis based on the relative order of elution and the ease of peak identification during the comparison of the LC chromatograms (from in vitro release) with the LC-MS/MS chromatogram in **Figure 11**.

In **Figure 12**, the profiles for the total release (parent + impurities) and the octreotide release through 43 days match the profile seen in **Figure 7** (cumulative percentage release versus time). That is, the profiles consist of a significant lag time prior to a linear release phase (matrix erosion controlled release) for all species. The most prevalent impurity species is the N-terminal glycoyl-substituted compound (~50 μg/mL cumulative release at 43 days). Next, the di-glycoyl-substituted compound (NH_2+g, Lys+g) and the lysine mono-glycoyl-substituted compound (Lys+g) appear similar in release (~30-35 μg/mL cumulative release at 43 days). Finally, the glycoyl-glycyl (Lys+g+g) and the glycoyl-di-glycyl (Lys+g+g+g) compounds show the lowest amount of release (8 and 4 μg/mL, respectively). From the results in **Figure 12**, one could also suggest that the greater nucleophilic activity of the N-terminus over the lysine residue is probably caused by pKa phenomenon. The N-terminal substituted compounds, in fact, show greater area percentage throughout the course of the release experiment and in the day-14 extraction of PBS-incubated microspheres. At physiological pH, a greater percentage of N-terminal amine groups could be un-ionized and in the more nucleophilic -NH_2 form as opposed to lysine groups in the ionized -NH_3^+ form.

CONCLUSION

During in vitro release testing of octreotide microspheres, hydrophobic-related substances or impurities were detected under HPLC conditions. Based on information in the literature and preliminary mass spectral data, the adducts were empirically presumed to be covalent modifications of the parent peptide at either the N-terminus or lysine residue. Under in vitro release conditions, microspheres manufactured from polymers of varying MW and comonomer ratio displayed significantly different amounts of impurity at the termination time point. In fact, the polymers of greater glycolide content in the comonomer ratio were demonstrating higher percentages of adduct compounds possibly because of the steric factors associated with lactic and glycolic acid monomers. FT-MS and LC-MS/MS analyses provided key evidence into the structural elucidation of the adduct compounds. From the results, if one were to choose a polymer for further development of a depot form of octreotide, low MW PLA appears to be viable because of lower impurity content. From the in vitro results in PBS, however, the PLA microspheres demonstrate a rapid burst release with minimal erosive phase drug release and hence do not exhibit an ideal zero-order release mechanism for a sustained release over time. Consequently, formulation processing parameters may require alterations in order to accommodate the use of PLA in a 1-month sustained-release dosage form.

Table 5. Peptide Sequence in Octreotide Peptide Mixture After DTT Treatment*

Peak No.	Retention Time, min	Peptide Sequence and Amino Acid Substitution	Product-Ion $[M+2H]^{2+}$ [m/z]
1	15.83	H-D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol	512
2	16.05	H-D-Phe-Cys-Phe-D-Trp-[Lys+g]-Thr-Cys-Thr-ol	541
3a	16.44	H-D-Phe-Cys-Phe-D-Trp-[Lys+g+g]-Thr-Cys-Thr-ol	570
3b	16.44	H-D-Phe-Cys-Phe-D-Trp-[Lys+l]-Thr-Cys-Thr-ol	548
4	16.87	H-D-Phe-Cys-Phe-D-Trp-[Lys+g+g+g]-Thr-Cys-Thr-ol	599
6	17.33	H-D-Phe-Cys-Phe-D-Trp-[Lys+l+g]-Thr-Cys-Thr-ol†	577
7	17.50	H-D-Phe-Cys-Phe-D-Trp-[Lys+l+l]-Thr-Cys-Thr-ol	584
8	18.08	H-[D-Phe+g]-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol	541
9	18.20	H-[D-Phe+g]-Cys-Phe-D-Trp-[Lys+g]-Thr-Cys-Thr-ol	570
11	18.87	H-[D-Phe+g]-Cys-Phe-D-Trp-[Lys+g+g]-Thr-Cys-Thr-ol	599

*DTT indicates dithiothreitol, g, glycoyl substitution; and l, lactoyl substitution.

†sequence of lactoyl-glycoyl substitution unknown.

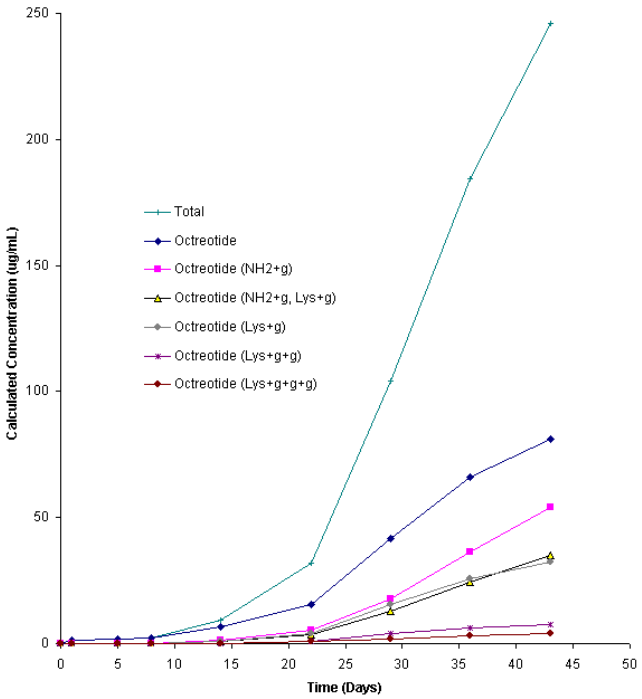

Figure 12. Release of peptide and related peptides from high MW PLGA 50:50 microspheres.

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