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Monitoring of peptide acylation inside degrading PLGA microspheres by capillary electrophoresis and MALDI-TOF mass spectrometry

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Abstract

The purpose of this research was to assess the acylation reactions of peptides, salmon calcitonin (sCT), human parathyroid hormone 1–34 ($hPTH_{1-34}$) and leuprolide, in poly(lactic-co-glycolic acid) (PLGA) microspheres. Capillary electrophoresis (CE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) were used for determining and monitoring peptide acylation and quantitating acylation products in the degrading PLGA microspheres. In the degrading PLGA microspheres of sCT and $hPTH_{1-34}$, the acylation products were observed and determined to be adducts with glycolic acid units from degradable PLGA polymer by MALDI-TOF MS. In the microsphere of leuprolide, however, the acylation product was not observed even after 28 days of incubation at the release medium, which represents the different stabilities among peptides according to the primary structure. As the leuprolide contains tyrosine and serine having hydroxyl group of nucleophilic amino acids, the acylation reaction of peptide is shown to be mainly due to the primary amino groups of N-terminus or lysine residue. The complementary use of CE and MALDI-TOF MS will be useful for searching the counter measures as well as determining the peptide acylation in the manufactured formulations on the market.

Keywords: Microsphere; Peptide stability; Acylation; Capillary electrophoresis; Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

1. Introduction

Biodegradable poly(lactic-co-glycolic acid) (PLGA) microspheres have been widely used for long-term controlled delivery of various peptides and proteins [1–3]. Recently, sustained depot formulations of some of luteinizing hormone-releasing hormone (LHRH) and

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somatostatin analogues have become commercialized and several additional products are under clinical investigation. However, instability problems of peptides and proteins in the PLGA microspheres have been recognized as one of the major obstacles for the successful product development [4].

During the drug release period, the incorporated peptide or protein within the microspheres may experience the unfavorable microenvironment generated by the degradation of the PLGA polymers. The accumulation of PLGA degradation products lowers pH within degrading microspheres and local pH values between 1.5 and 4.7 have been recently reported [5–7]. The acidic microenvironment inside the microspheres can induce several chemical degradation reactions, e.g., deamidation and covalent dimerization of insulin [8], and has been regarded as the major factor of peptide and protein instability [9]. In addition, peptides and proteins with reactive amines may interact with the polymer resulting in the formation of undesired amide bond conjugates [10].

Recently, Lucke et al. [11] have suggested that the degradation of PLA and PLGA microspheres can even lead to covalent modifications of incorporated peptides by acylation with lactic and glycolic acid units. They found the evidence of peptide acylation in the microsphere by using LC-mass spectrometry (LC-MS). Similarly, Rothen-Weinhold et al. have also reported the formation of peptide impurities in polyester matrices during implant manufacturing and proposed the acylation mechanism between reactive amine groups with carbonyl functional group of lactic acid [12].

As the chemical modifications of peptide or protein may lead to a loss of activity or change of immunogenicity [13,14], it is essential to assess the possible interactions when developing controlled release systems for peptide and proteins. According to ICH guidelines, the amount of acylated peptides can be considered as impurity that should be investigated for toxicity and adverse pharmacological effects.

In this paper, the acylation reactions of the peptides in the PLGA microspheres have been monitored by capillary electrophoresis (CE) and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS). CE is a powerful analytical technique applicable to both small and macromolecular components of biological interest and appears to be highly promising with regard to separation efficiency, detection sensitivity, analysis speed and simplicity [15]. In addition, CE is a microanalysis technique, in which only a few nanoliters are injected. MALDI-TOF MS is a versatile and sensitive technique that is proving to be of great value in the molecular weight determination of proteins and peptides [16]. The technique has a mass range in excess of 300 kDa and is relatively insensitive to the presence of buffering agents, salts, and denaturants. These features make it the method of choice for characterization of peptides and proteins on the basis of molecular weight [17]. Both CE and MALDI-TOF MS provide the advantages of speed, high resolution, and small sample consumption for monitoring the peptide stability over conventional analytical methods such as HPLC.

As the nucleophilic groups, such as primary amines of N-terminus and lysine residue or the hydroxyl groups of tyrosine or serine, may be the major target for acylation reaction [11], two model groups were adopted: One group is salmon calcitonin (sCT) and human parathyroid hormone 1–34 ($hPTH_{1-34}$), which

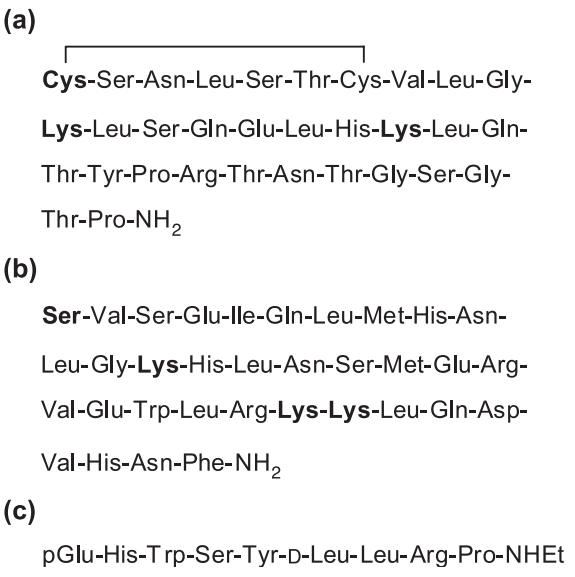

Fig. 1. Primary structures of peptides. (a) Salmon calcitonin (sCT); (b) human parathyroid hormone 1–34 ($hPTH_{1-34}$) and (c) leuprolide. The sCT and $hPTH_{1-34}$ have three and four primary amino groups of N-terminus and lysine residues, while leuprolide has blocked N-terminus.

have both primary amino groups in N-terminus and lysine residues and hydroxyl groups in tyrosine or serine. The other is leuprolide, which has only hydroxyl groups of tyrosine and serine without primary amino group because the N-terminus is pyroglutamyl residue (Fig. 1). In this study, PLGA microspheres of sCT, $hPTH_{1-34}$ and leuprolide were prepared and peptide stability against acylation reaction during in vitro release was investigated.

2. Materials and methods

2.1. Materials

PLGA (50:50) with free carboxyl end groups, Resomer RG503H, was obtained from Boehringer Ingelheim (Ingelheim, Germany). Leuprolide acetate ([Des-Gly^{10},D-Leu^{6},Pro-NHEt^{9}]-LHRH), salmon calcitonin and human parathyroid hormone (1–34) were obtained from Bachem (Torrence, CA, USA). Alpha-cyano-4-hydroxycinnamic acid (α-CHCA) and polyvinyl alcohol (m.w. 30 000–70 000) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methylene chloride (HPLC grade) were supplied by J.T. Baker (Philipsburg, NJ, USA). All other chemicals were of analytical grade and used as obtained commercially.

2.2. Peptide stability studies in the microspheres

PLGA microspheres were prepared by dispersing the homogeneous solution of RG503H polymer (PLGA 50:50, hydrophilic and non-end-capped

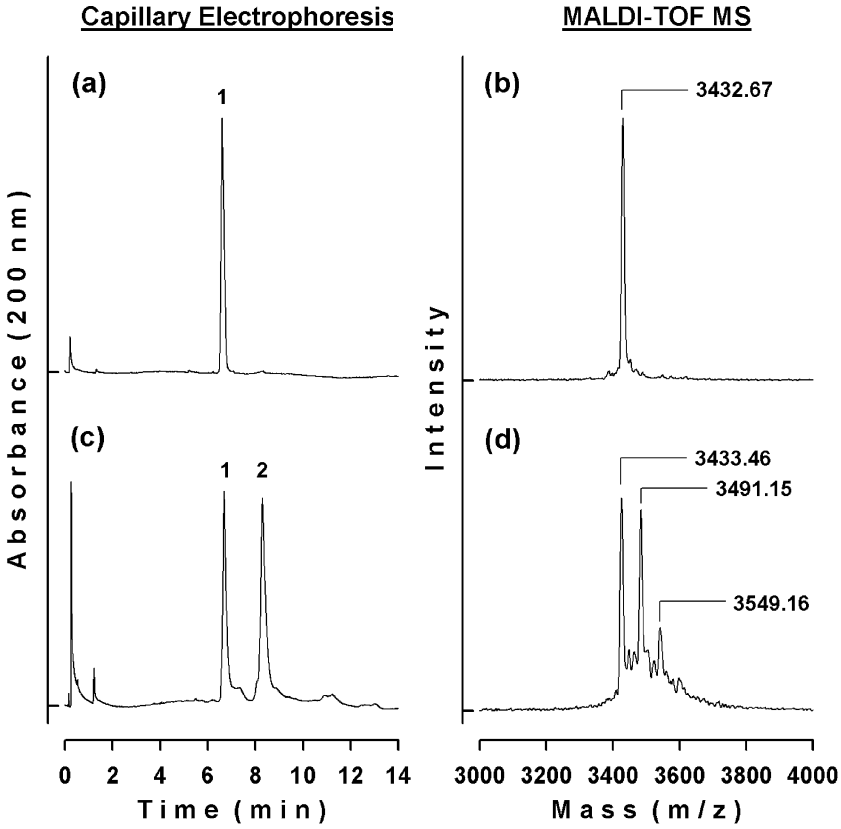

Fig. 2. CE and MALDI-TOF MS analysis of (a and b) sCT extracted from microsphere at 0 day; (c and d) sCT extracted from microsphere at 21 days of incubation in release medium at 37 °C. In the CE electropherogram, peak 1 is native sCT and 2 represents the acylated sCTs.

polymer) and peptide drug into a PVA solution followed by solvent extraction/evaporation [18,19]. The solidified microspheres were recovered by filtration and dried under vacuum at room temperature. The microspheres were stored at −20 °C until use.

For stability study of the peptides inside the microspheres, the microspheres were suspended in 10 mM phosphate buffer saline, pH 7.4 containing 0.02% w/w Tween 80 and 0.02% sodium azide for 28 days. The study was performed on a temperature-controlled shaker at 37 °C. Microsphere samples were removed periodically and centrifuged at 7000 rpm in a tabletop microcentrifuge for 2 min. The supernatant was discarded and the microspheres were dried by vacuum. The dried microspheres were extracted with 50% ACN by mechanical shaking for 30 min. At the end of extraction, the samples were centrifuged at 7000 rpm for 2 min and the supernatants were analyzed by CE and MALDI-TOF MS. The quantitation of native and acylated peptides was performed by CE method described below.

2.3. Capillary electrophoresis

CE was carried out using a Bio-Rad BioFocus 3000 CE System with a polyacrylamide-coated capillary, 50 μm I.D., 24 cm total length and 19.5 cm to the detector (Bio-Rad, Hercules, CA). Separation of each sample was performed in 100 mM phosphate buffer (pH 2.5) as the electrolyte for 40 min, and the UV absorption was measured at 200 nm. The capillary was rinsed with distilled water and buffer solution for 120 and 180 s, respectively, prior to each injection. Samples were loaded by applying a nitrogen pressure, and the voltage across the ends of the capillary was set at 10 kV. The temperature of the capillary and samples was maintained at 20 °C by a liquid cooling system.

The fraction collections were accomplished automatically by control of the Bio-Rad CE instrument. At designated times, the voltage was suspended, a collection vial positioned at the outlet, and the voltage reapplied to establish the separation field. To provide electrical contact between the outlet electrode and the fraction collection tube, 10 μl of running buffer was added to the tube.

2.4. MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry was performed using a Voyager Biospectrometry Workstation (PerSeptive Biosystem, MA). Samples were prepared by mixing 2 μL of aliquot with 2 μL of the matrix solution, a saturated solution of α-CHCA in 50% of water/acetonitrile with 0.3% trifluoroacetic acid. One microliter of the sample mixture was spotted into a well of the sample plate and dried by vacuum prior to mass spectrometry. Data for 2 ns pulses of the 337 nm nitrogen laser were averaged for each spectrum in a linear mode, and a positive ion TOF detection was performed using an accelerating voltage of 25 kV. Spectra were smoothened with a 19-point Savitzky-Golay filter and the external calibration

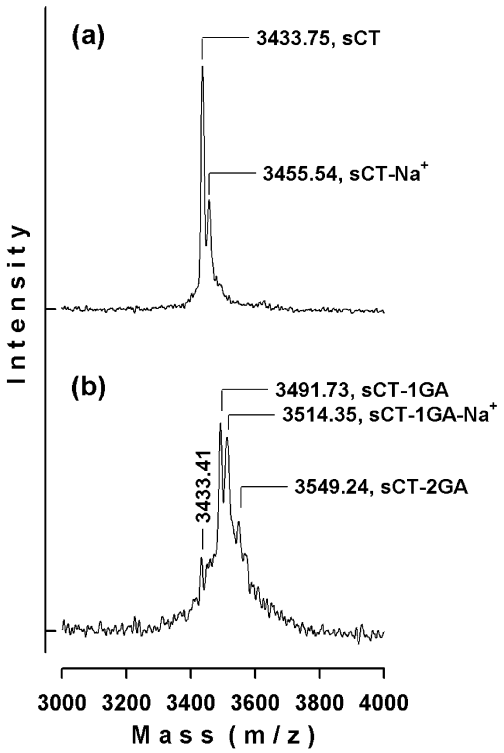

Fig. 3. Identification of two peaks separated in CE analysis of sCT extracted from microsphere at 21 days of incubation in release medium at 37 °C using by MALDI-TOF MS. (a) MALDI spectrum of sample collected from peak 1 of CE; (b) MALDI spectrum of sample collected from peak 2 of CE shown in Fig. 2c.

was performed using Mass Standard Kit 1 (PerSeptive Biosystems), a mixture of angiotensin I, ACTH (1–17), ACTH (18–39), ACTH (7–38) and bovine insulin.

3. Results and discussion

As the acylation reaction of incorporated peptide in the microsphere systems could have severe detrimental effects such as activity loss and toxicity, it is important to develop various analytical methods for assessing the possible interactions between peptides and polymer matrices. We here report useful micro-analytical methods to determine and monitor the acylation reaction of peptides inside the biodegradable microsphere systems.

3.1. Preparation and characterization of microspheres

The PLGA microspheres of sCT, $hPTH_{1-34}$ and leuprolide were prepared with RG503H polymer by solvent extraction/evaporation method. The average particle sizes of sCT, $hPTH_{1-34}$ and leuprolide microspheres were 20.51, 24.27 and 21.27 μm, respectively. All microspheres were spherical with a relatively non-porous surface when analyzed by scanning electron

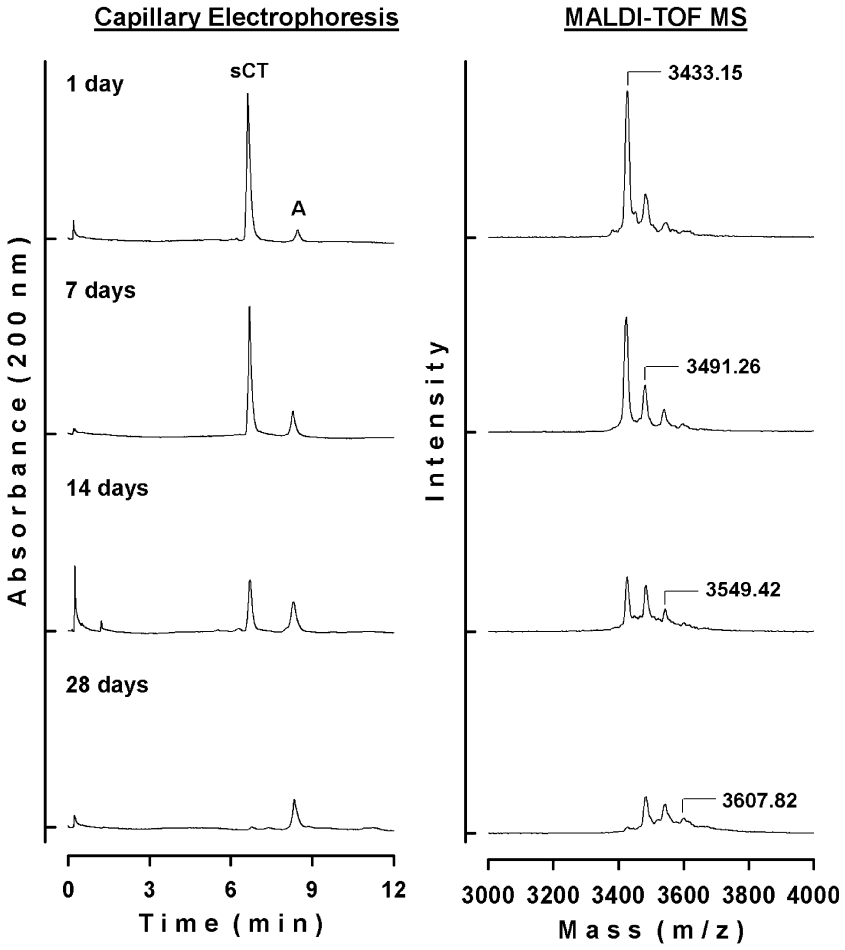

Fig. 4. Monitoring of acylation reaction of sCT inside degrading microspheres incubated for 1, 7, 14 and 28 days. In CE electropherogram, A represents the acylation product of sCT.

microscopy. The target load of the microspheres of sCT and $hPTH_{1-34}$ was 5%, and that of leuprolide microsphere was 18%. The actual peptide contents were determined by CE method using 0.1 M phosphate buffer (pH 2.5) as running buffer and 4.8% of sCT, 3.8% of $hPTH_{1-34}$ and 14.3% of leuprolide were incorporated into PLGA microspheres.

3.2. Acylation of sCT in degrading microspheres

To determine the structure of peptide non-released from the microsphere, sCT was extracted from microsphere samples during in vitro release study. The extracted samples were analyzed by CE and MALDI-TOF MS (Fig. 2). As shown in Fig. 2a

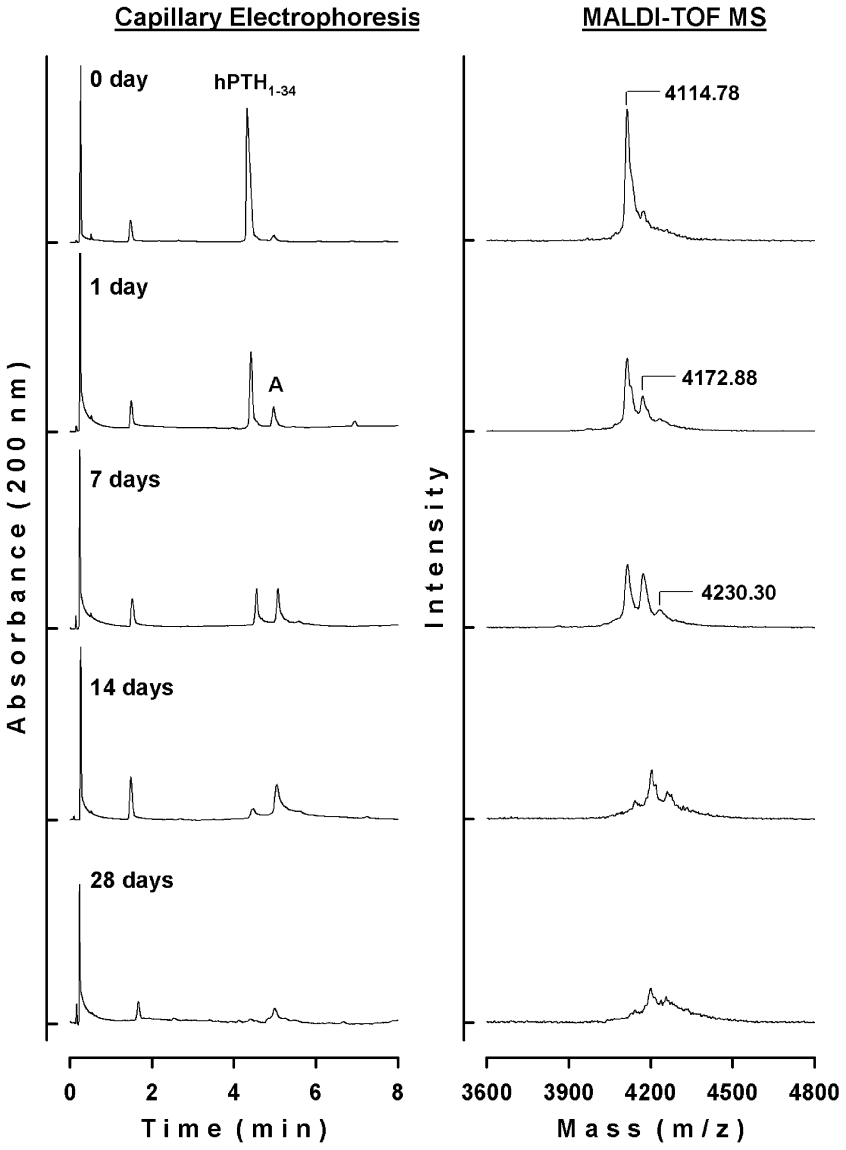

Fig. 5. Monitoring of acylation reaction of $hPTH_{1-34}$ inside degrading microspheres incubated for 0, 1, 7, 14 and 28 days. In CE electropherogram, A represents the acylation product of $hPTH_{1-34}$.

and b, only one single peak was detected in the electropherogram of CE and the mass was determined to be *m/z* 3432.67, indicating that the sCT remained unchanged in terms of its chemical integrity. However, after the release study of the sCT microspheres in phosphate buffer (pH 7.4) at 37 °C, the additional peak was observed in CE analysis (Fig. 2c) and the mass peaks of *m/z* 3491.15 and 3549.16 except the native sCT mass were presented in the MALDI-TOF mass spectrum (Fig. 2d). The difference of mass between intact sCT and the additional peaks was about 58 and 116 Da, which correspond to that modified with glycolic acid monomer and two monomer/dimer. To identify the two peaks of CE analysis, each peak fraction was collected and reanalyzed by MALDI-TOF MS (Fig. 3). The first peak fraction was determined to be intact sCT (*m/z* 3433.75) and the second peak fraction was the mixture of acylated sCTs (*m/z* 3491.73 and 3549.24). The adjacent mass peaks, *m/z* 3455.54 and 3514.35, are sodium salt adduct peak, which might be produced as the fraction contained phosphate buffer.

The acylation reactions of sCT inside the microsphere during the in vitro release study were monitored by CE and MALDI-TOF MS (Fig. 4). During the release study, the intact sCT peak rapidly decreased, whereas the acylated peaks predominantly appeared after 28 days of incubation. In the MALDI-TOF mass spectrum of 28 days of incubation, mass peak corresponding glycolic acid trimer complex (*m/z* 3572.82) was observed. As the MALDI-TOF MS is not quantitative, the residual content of sCT in the microsphere could only be determined by CE method, which showed linearity between 5 and 200 μg/ml with correlation coefficients of >0.99. However, as MALDI-TOF MS shows detailed information about the identities of peptide and degradation products, the complementary use with CE method is useful for monitoring peptide acylation in degrading microspheres.

3.3. Monitoring of $hPTH_{1-34}$ acylation in degrading microspheres

Similar to the sCT microspheres, $hPTH_{1-34}$ microspheres also showed the acylation reaction during the in vitro release test (Fig. 5). The mass of native $hPTH_{1-34}$ extracted from microsphere was determined to be *m/z* 4114.78 and the masses the acylation product were *m/z* 4172.88 and 4230.30. The mass difference between three peaks was about 58 Da, which indicates that the $hPTH_{1-34}$ was also modified by glycolic acid units from degrading PLGA. As $hPTH_{1-34}$ microspheres were exposed to the release medium buffer longer, the intact $hPTH_{1-34}$ peak was significantly decreased, while the acylation product peak was still observed after 28 days of the release test. Each peak of CE analysis was collected and reanalyzed by MALDI-TOF MS. The first peak fraction was determined to be intact $hPTH_{1-34}$ (*m/z* 4114.43) and the second peak fraction was the mixture of mono- (*m/z* 4172.46) and di-acylated $hPTH_{1-34}$ (*m/z* 4231.06). The residual contents of $hPTH_{1-34}$ and the acylation product in the microsphere were also determined by CE method (Fig. 6). The content of acylation products in the

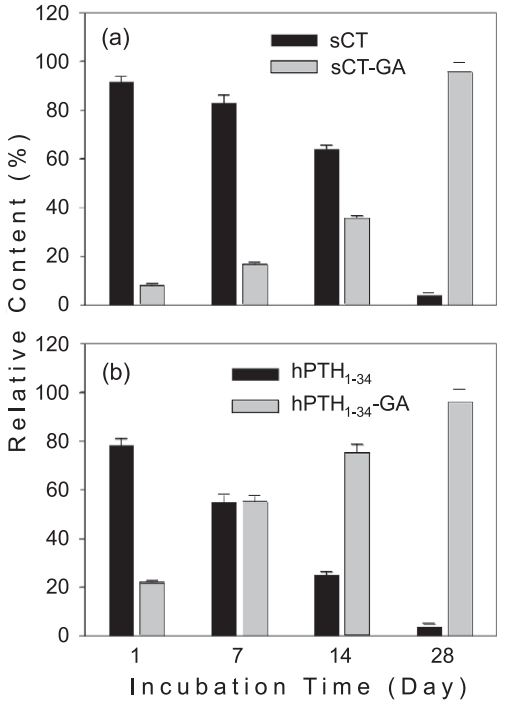

Fig. 6. Contents of (a) sCT and the acylation product (sCT-GA); (b) $hPTH_{1-34}$ and the acylation product ($hPTH_{1-34}$-GA) extracted from degrading PLGA microspheres after incubation for 1, 7, 14 and 28 days in the release medium. The contents of native and acylated peptides were determined as relative peak areas by CE method.

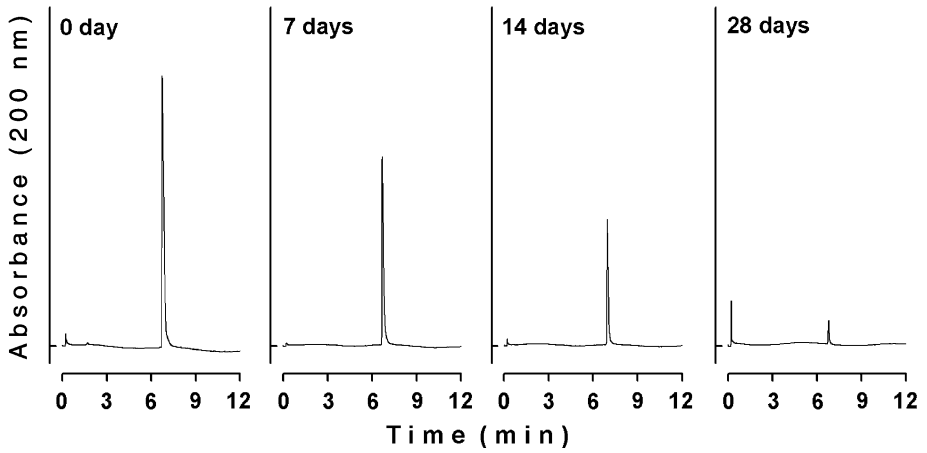

Fig. 7. Capillary electrophoretic monitoring of leuprolide inside degrading microspheres incubated for 0, 7, 14 and 28 days.

$hPTH_{1-34}$ microspheres increased faster than that in sCT microspheres. This may be due to greater number of amino groups in $hPTH_{1-34}$.

3.4. Stability of leuprolide in the microsphere during release test

Fig. 7 shows the CE electropherograms of leuprolide extracted from the PLGA microspheres over 28 days. Only a single peak was observed up to 28 days, indicating that leuprolide is very stable during in vitro release. In the MALDI-TOF MS of leuprolide extracted from the PLGA microsphere, the stability of leuprolide was also identified (Fig. 8). The molecular mass of leuprolide at 0 day sample was determined to be *m/z* 1210.79 and only one mass peak was apparent after 28 days. Therefore, leuprolide does not appear to undergo acylation in the PLGA microspheres. Lucke et al. [11] have proposed that, besides the primary amino group of N-terminus and lysine, the hydroxyl groups of serine or tyrosine can be also possible targets for acylation. As the N-terminus of leuprolide is blocked, it has only hydroxyl groups of tyrosine and serine as possible acylation targets. However, it did not show acylation reaction in the microspheres during the release test. Therefore, the

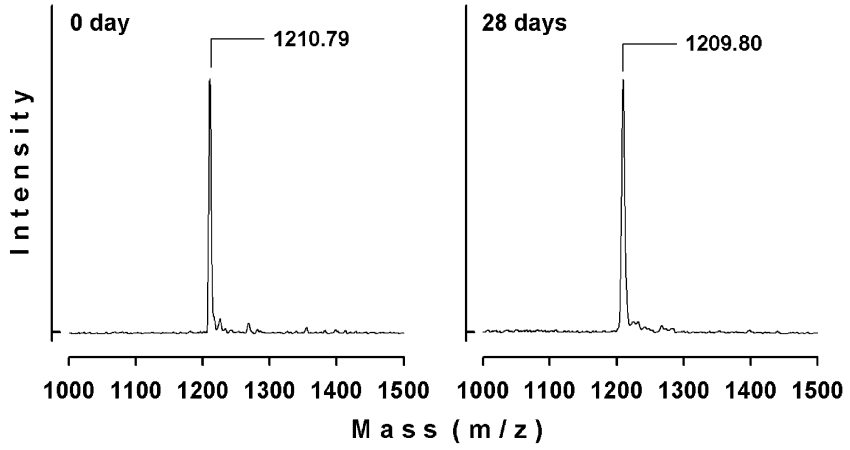

Fig. 8. MALDI-TOF mass spectra of leuprolide inside microsphere incubated for 0 and 28 days.

hydroxyl group may not react with degrading polymer. This suggests that the primary amino group of N-terminus or lysine residue is the major target for acylation reaction.

4. Conclusions

The CE and MALDI-TOF MS could be applied as a powerful tool for determining and monitoring the peptide acylation in the microsphere. They have merits of speed, high resolution and small sample consumption over other analytical tools. The sCT and $hPTH_{1-34}$ having the primary amino groups were acylated inside degrading microspheres, whereas leuprolide having only hydroxyl groups without primary amine showed no changes during the release test. This strongly suggests the primary amino group to be the major target for peptide acylation. As the acylation of peptides may pose a severe threat to the successful delivery of the bioactive molecules from controlled release systems, the development of analytical methods for monitoring acylation is important for both assessing stability and developing the counter measures to acylation.

Acknowledgements

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