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Quantitative determination of cyclic polylactic acid oligomers in serum by direct injection liquid chromatography tandem mass spectrometry

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ABSTRACT

Polylactic acid (PLA) is a biodegradable polymer, currently used in pharmaceutical and surgical devices. There is a concern that cyclic polylactic acid (CPLA), which is a by-product of PLA synthesis, may be introduced into the human body as an undesirable contaminant. We carried out a quantitation investigation of the CPLA heptamer (CPLA-7) by liquid chromatography mass spectrometry (LC–MS). We found that CPLA-7 binds strongly with serum proteins and that only 62% of CPLA-7 was recovered after routine deproteination; therefore, we directly injected serum into the LC–MS/MS system after passage through a bovine serum albumin (BSA)-coated chromatographic column and found the recovery of CPLA-7 was improved to 84%, and that the detection (S/N = 3) and quantitation limit (S/N = 10 and below 15% relative standard deviation) were 1.5 and 2.5 ng/mL, respectively. We conclude that direct injection LC–MS/MS, using a BSA column, is a simple and effective quantitative analysis method for CPLA in serum.

1. Introduction

Biodegradable polymers have been widely used as industrial and medical materials, and their degradation products are present throughout the environment [1]. It is possible for biodegradable polymers or their products to enter the human body not only via the environment, but also as medical materials in drug delivery and surgical devices, and even as drugs themselves. It is thus important to develop an effective quantification method for biodegradable polymers or oligomers in a biological matrix rather than in environmental samples.

Numerous studies have examined the degradation of biodegradable polymers [2–4], among which, size exclusion chromatography (SEC) [5–7], NMR [8], X-ray diffraction [8], and laser diffractometry [9] have been used for the identification and quantitation of these polymers. Recently, mass spectrometry and X-ray photoelectron spectrometry (XPS) [10–13] have also been used for structural analysis of biodegradable polymers. Studies on decomposition products at the surface of poly(β-maleic acid) and polylactic acid (PLA) by secondary ion mass spectrometry (SIMS) and XPS have been reported [10–12]. Using TOF–SIMS, *in vitro* hydrolytic reactions of PLA and lactic-*co*-glycolic acid were studied [14]. Matrix-assisted laser desorption ionization mass spectrometry (MALDI–MS) is a soft ionization technique suitable for the analysis of thermally-labile or high molecular weight molecules and is particularly effective for structural analysis of synthetic polymers; several analyses of polystyrene, polyesters, or their copolymers have been reported [15–18]. Using the melt polycondensation method, Kéki et al. investigated the temperature dependence on by-product formation of PLA synthesis from D,L-lactic acid [19]. Tandem mass spectrometry is an effective technique for detailed structural analysis; for example, cyclic polylactic acid (CPLA) and linear polylactic acid (LPLA) can be differentiated by their different fragmentations [20].

PLA is one of the biodegradable polymers used in drug delivery and surgical devices, or as a material for postoperative adhesion prevention. PLA decomposes to lactic acid which is bio-resorbable and is harmlessly absorbed by human body. MALDI–MS analysis has revealed that chemical synthesis of PLA results in the formation LPLA as the principal product and CPLA as a by-product [19]. In addition, it was recently reported that CPLA can lower the activities of pyruvate kinase and lactic dehydrogenase, which may lead to the development of drugs suppressing FM3A ascites tumor cells [21]; therefore, it is important to develop an effective method of quantitatively analyzing CPLA in plasma or serum and to study its metabolism.

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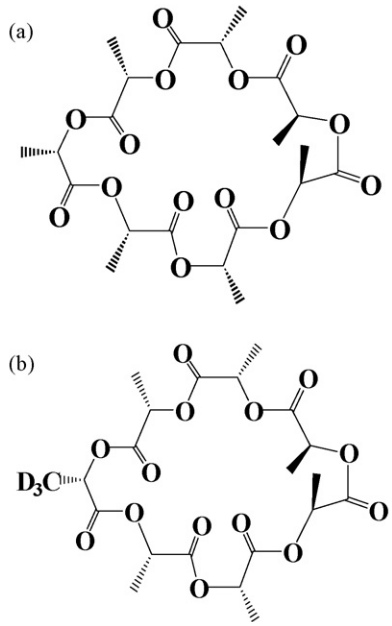

Fig. 1. Structures of (a) cyclic polylactic acid heptamer (CPLA-7) and (b) its deuterated isotope (CPLA-7-d_3).

Gas chromatography mass spectrometry (GC–MS) and liquid chromatography mass spectrometry (LC–MS) are commonly used for highly sensitive quantitation of organic compounds in plasma or serum. Quantitative analysis of plasma lactic acid using GC–MS [22] and ion-exclusion chromatography [23] has been reported, but our literature search yielded no reports on quantitative analysis of LPLA or CPLA oligomers.

In the present study, we used lactic acid, lactoyllactic acid, and L-lactate-3,3,3-d_3 to synthesize a heptamer of CPLA (CPLA-7) and its isotope CPLA-7-d_3. Due to strong binding between CPLA-7 and serum proteins, the LC–MS/MS analysis followed by regular deproteination resulted in low recovery of CPLA-7; therefore, we developed a method of directly injecting CPLA-7 contained in serum into LC–MS/MS, employing a bovine serum albumin (BSA)-coated column, and investigated the optimal conditions for this quantitation.

2. Experimental procedures

2.1. Synthesis of CPLA-7 and CPLA-7-d_3

The *O*-silylated trimers of lactic acid benzylester were obtained when *O-tert*-butyldimethylsilylated lactoyllactic acid was allowed to react with benzylated lactoyllactic acid or lactic acid via the Keck protocol. After selective deprotection of hydroxyl or carboxyl groups of the products obtained, the condensation reactions of these products using dicyclohexylcarbodiimide (DCC) catalyzed 4-(dimethylamino)pyridine (DMAP) were carried out to yield O-silylated oligo lactic acid benzylester. The protective groups were removed to produce a free heptamer of lactic acid. Finally, this heptamer was allowed to react with 2,4,6-trichlorobenzoyl chloride in the presence of amine, followed by DMAP-catalyzed cyclization under a dilute condition (Yamaguchi method). The mixture was purified by silica gel column chromatography to obtain monomeric CPLA-7 (Fig. 1a).

Benzyl L-lactate-3,3,3-d_3 was synthesized by reacting the sodium salt of L-lactic acid-d_3 (>98 atom%d, Isotec Inc.) with benzylbromide. The sililoxylactate hexamer was synthesized by condensation of linear lactate trimer benzylester and sililoxy lactate trimer benzylester. The sililoxylactate hexamer was then condensed with benzyl L-lactate-3,3,3-d_3 to form linear lactate heptamer-d_3, which was cyclized and purified as described in CPLA-7 synthesis to obtain monomeric CPLA-7-d_3 (Fig. 1b). The total yield of synthesis for CPLA-7-d_3 was ca. 50% just as for CPLA-7.

2.2. Reagents and standards

CPLA-7 and CPLA-7-d_3 were dissolved in acetonitrile/water (1:1, v/v) and diluted with water. A 100-ng/mL solution of CPLA-7-d_3 was used as the internal standard. Acetonitrile (LC–MS grade), phosphoric acid (HPLC grade) and ammonium acetate were purchased from WAKO Pure Chemicals (Osaka, Japan). Ultrapure water was obtained using a water distillation apparatus (RFD250NB, Advantec). Human serum from normal subjects was obtained from CHEMICON Int. Inc. (Temecula, CA, USA).

2.3. Sample pretreatment

For direct serum sample injection, sera containing 1% phosphoric acid, CPLA-7 solution and internal standard solution were mixed at a ratio of 2/1/1 (v/v/v). The solution was centrifuged (Centrifugeb 5417R, Eppendorf) for 3 min at 14,000 rpm, and the supernatant was injected directly into LC–MS/MS.

2.4. Apparatus

2.4.1. Liquid chromatography

HPLC was performed using an Agilent HP1100. A BSA-coated chromatographic column, the BSA-ODS-100V (2.0 mm × 50 mm, 5 μm particle size), supplied by Tosoh Co., Japan was used and was found to effectively remove proteins in the sample. The principle of separation for this column is as follows. Target compounds penetrate silica pores of column particles and are retained by the ODS interaction in these pores. At the same time, high molecular weight proteins do not penetrate the pores and are not retained. Furthermore, the surfaces of the particles are coated with polar BSA so that serum protein is readily eluted from the column, without retention or denaturalization. Binary mobile phases A (H_2O) and B (95% acetonitrile) were used with a flow rate of 0.3 mL/min and a gradient elution: programmed from 0% B (0–3.5 min) to 80% B (3.5–6.5 min), held at 80% B (6.5–8.5 min), returned to 0% B (8.5–9.0 min), and washed with 0% B (9–12 min). The injection volume was 5 μL. A cationizing solution (50 mM ammonium carbonate) with a flow rate of 0.06 mL/min was added at the post-column stage. Using a 6-way switch valve, flow between 7.5 and 9.5 min was introduced into the mass spectrometer and the flows before and after this time period were discarded as waste.

2.4.2. ESI–MS/MS

ESI mass spectra were obtained by a triple quadrupole mass spectrometer TSQ (ThermoQuest) in positive ion mode. The capillary temperature was 200 °C, spray voltage 4.5 kV, auxiliary gas 20 psi, and sheath gas 60 psi. The collision energy of the parent ion was set at 30 eV.

3. Results and discussion

3.1. Optimization of ESI–MS/MS

The ESI spectrum of CPLA-7 in Fig. 2 exhibits three molecular adduct ions at *m*/*z* 522, 527 and 543, corresponding to $[M+NH_4]^+$,

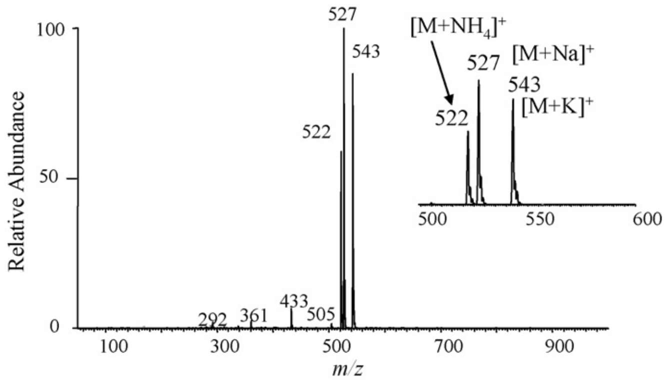

Fig. 2. ESI mass spectrum of CPLA-7.

$[M+Na]^+$ and $[M+K]^+$, respectively. M represents a CPLA-7 molecule. In order to determine the optimal ESI conditions, we first examined the relationship between capillary temperature and $[M+NH_4]^+$ intensity. The maximum $[M+NH_4]^+$ signal was obtained with a capillary temperature of 200 °C, i.e. 100 °C lower than usual. We attribute this to decomposition of $[M+NH_4]^+$ being suppressed at a lower temperature. The MS/MS spectrum of $[M+NH_4]^+$ *m*/*z* 522 (Fig. 3a) yields a series of A-type ions $[M\text{-}72n+H]^+$ which are derived from repeated elimination of lactate units (72 Da) and neutral NH_3. The elimination of NH_3 from ammonium adduct precursors in MS/MS spectra is well known. The fragment ions *m*/*z* 405, 333 and 261 are $[M-72n+H-28]^+$ (n = 1–3); these ions may be due to the elimination of either C_2H_4 or CO from the A-type ions $[M-72n+H]^+$ (n = 1–3) [20]. While the MS/MS spectra of $[M+NH_4]^+$ exhibited strong fragment signals, the MS/MS spectra of both $[M+Na]^+$ and $[M+K]^+$ provided only fragment ions with poor intensities.

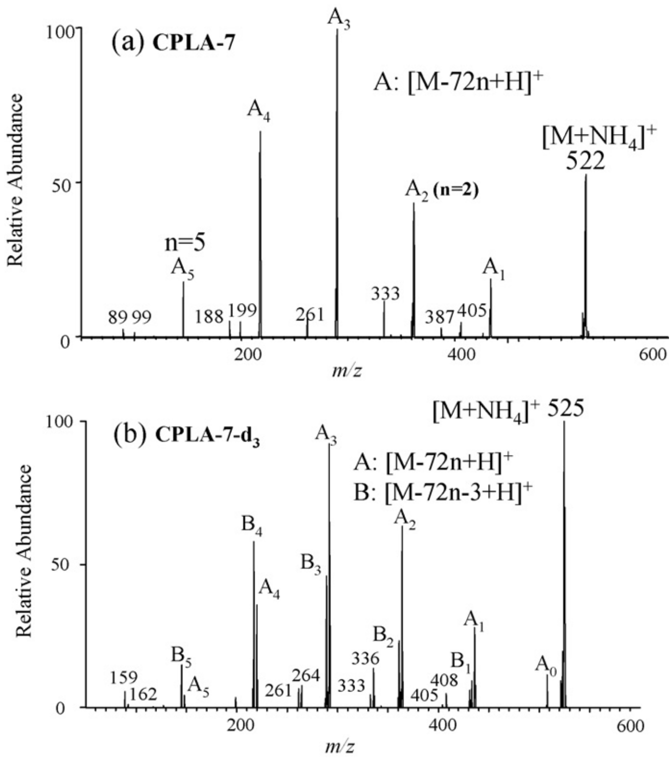

Fig. 3. ESI–MS/MS spectra of (a) CPLA-7 and (b) CPLA-7-d_3.

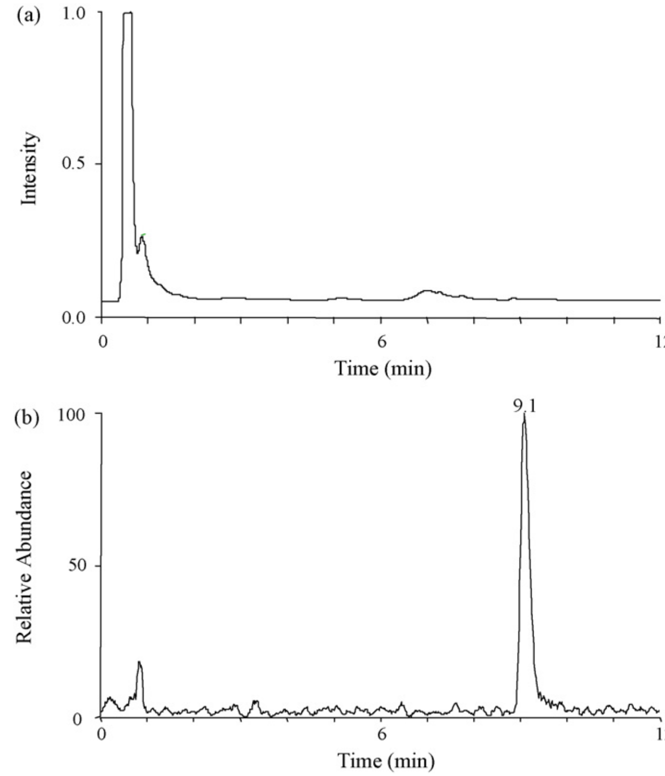

Fig. 4. (a) UV (285 nm) chromatograph and (b) SRM chromatograph, obtained by direct injection LC–MS/MS, for the serum sample (CPLA-7, 25 ng/mL).

From the above optimization study we chose 10 mM ammonium acetate as the cationizing solution for the subsequent selected reaction monitoring (SRM) experiments. The most significant SRM transition of *m*/*z* 522–289 (A_3 in Fig. 3a) was used for the quantitative determination of CPLA-7. Fig. 3b is the MS/MS spectrum of the ammonium adduct *m*/*z* 525 $[M+NH_4]^+$ of CPLA-7-d_3. A series of A-type ions $[M-72n+H]^+$ (n = 0–5) at *m*/*z* 508, 436, 364 and 292 are shown in Fig. 3b. Another series of ions, 3 Da less then the above ions, resulted from the elimination of a d_3-monomer (B-type ions in Fig. 3b). Each ion pair, *m*/*z* 408 and 405, 336 and 333, and 264 and 261, can be assigned to the 28-Da eliminations of either C_2H_4 or CO from the A- and B-type ions, respectively. We selected the MS/MS transition *m*/*z* 525 to 292 (A_3) as the internal standard of CPLA-7-d_3 for the SRM quantitative analysis.

3.2. LC–MS/MS analysis of CPLA-7 and CPLA-7-d_3

A tailing ion chromatogram was observed with the SRM analysis of CPLA-7, when the cationizing agent ammonium acetate was directly added to the mobile phase during the analysis. This phenomenon might result from an excessive interaction between $[M+NH_4]^+$ and silanol functions of the column. Therefore, we use the post-column addition of ammonium acetate solution and found tailing to be suppressed and the sensitivity of detection to be improved 1.5 fold. Fig. 4a shows the UV chromatogram (at 285 nm) obtained by direct injection of 25 ng/mL CPL-7 in serum. The large chromatographic peak eluted out around 1 min (Fig. 4a) was derived from proteins in serum. The proteins were eliminated as waste via the switching valve. Fig. 3b is the SRM ion chromatogram of the sample in which CPLA-7 was detected at 9.1 min.

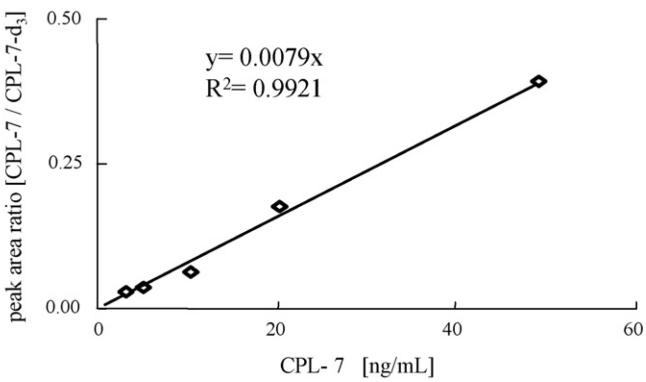

Fig. 5. Calibration curve of CPLA-7 by direct injection LC–MS/MS for serum sample.

3.3. Recovery of CPLA-7

3.3.1. Serum deproteination

As we found recovery of CPLA-7 to be particularly low when using the regular deproteination procedure, we examined CPLA-7 loss in the three deproteination steps: (a) protein precipitation by addition of 100 μL of acetonitrile in 100 μL of serum containing 0.5% phosphoric acid, (b) shaking the mixture at 0 °C to enhance precipitation, and (c) filtration by a PTFE membrane filter (Ultrafree-MC 0.2 μm, Millipore, Japan) to obtain the supernatant. We added CPLA-7 (25 ng/mL) to each of the above three deproteination steps to examine loss at each step. The recoveries in steps (a), (b) and (c) were 62% ($n = 3$ in 1 day), 70% and 94%, respectively. From these results, we calculated the loss of CPLA-7 due to co-precipitation with proteins to be 8%, that due to binding to proteins 24%, and that due to ionization suppression by impurities in serum 6%. In an attempt to release protein-bound CPLA-7 from proteins we introduced acetonitrile containing 1–5% DMSO or a 1:1 mixture of acetonitrile and isopropanol, but failed to improve CPLA-7 recovery. The attempt to use 1–5% of DMSO to recover CPLA-7 from protein precipitates in step (b) also failed. Therefore we conclude that serum deproteination is associated with significant losses of CPLA-7 causing misleading results, i.e. values much lower than the actual levels.

3.3.2. Direct injection method

The recovery of CPLA-7 by the method of direct serum sample injection was examined. When no phosphoric acid was added to the serum sample, the recovery average ($n = 3$) for CPLA-7 was close to 0%. This might be because CPLA-7 binds very strongly with serum proteins and is thus lost as waste, i.e. eliminated with the proteins. However, the recovery average ($n = 3$) of CPLA-7 increased to 84% when phosphoric acid was added to the serum sample. A portion of CPLA-7 might still bind to proteins, preventing complete recovery.

3.4. Quantitation of CPLA-7

A calibration curve was obtained for CPLA-7, by directly injecting the serum sample, as shown in Fig. 5. The within-day and between-day precision and accuracy for this method were determined by performing the experiments in triplicate. The detection limit (S/N = 3) and quantitation limit (S/N = 10 and below 15% relative standard deviation) were 1.5 and 2.5 ng/mL, respectively. The linearity of the curve was $R^2 = 0.9921$, the standard deviation and relative standard deviation at the quantitation limit were 0.006% and 12.2%, respectively. The relative standard deviation of serum samples containing CPLA-7 at 2.5 and 50 ng/mL were 13% and 7.1%, respectively. Thus, CPLA-7 quantitation was achieved in the 2.5–50 ng/mL concentration range.

4. Conclusion

For the ESI–MS measurements of CPLA-7, we detected three major molecular adducts of NH_4^+, Na^+ and K^+ ions. Among them the NH_4^+ adduct ion was the most sensitive for CPLA-7 quantitation. Our results show that the serum sample can be analyzed by direct injection after passage through a BSA-coated chromatographic column. Chromatographic peaks can be improved and the detection sensitivity increased by introducing the cationizing agent ammonium acetate using a post-column. Recovery of CPLA-7 in serum was 84% by the direct injection method, higher than by protein precipitation which was only 62%. This direct injection approach employing BSA column chromatography, developed in our laboratory, is a rapid, simple and effective quantification method for CPLA-7 in serum.

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