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Short communication

Quantitative determination of insulin entrapment efficiency in triblock copolymeric nanoparticles by high-performance liquid chromatography

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Abstract

A rapid and effective isocratic chromatographic procedure was described in this paper for the determination of insulin entrapment efficiency (EE) in triblock copolymeric nanoparticles using reversed-phase high-performance liquid chromatography (RP-HPLC) with an ultraviolet/visible detector at low flow rate. The method has been developed on a Shimadzu Shim-pack VP-ODS column (150 mm × 4.6 mm, 5 μm, Chiyoda-Ku, Tokyo, Japan) using a mixture of 0.2 M sodium sulfate anhydrous solution adjusted to pH 2.3 with phosphoric acid and acetonitrile (73:27, v/v) as mobile phase at the flow rate of 0.8 ml min^{-1} and a 214 nm detection. The method was validated in terms of selectivity, linearity, precision, accuracy, solution stability, limit of detection (LOD) and limit of quantification (LOQ). The calibration curve was linear in the concentration range of 2.0–500.0 μg ml^{-1}, and the limits of detection and quantitation were 8 and 20 ng, respectively. The mean recovery of insulin from spiked samples, in a concentration range of 8–100 μg ml^{-1}, was 98.96% (R.S.D. = 2.51%, $n = 9$). The intra- and inter-assay coefficients of variation were less than 2.24%. The proposed method has the advantages of simple pretreatment, rapid isolation, high specificity and precision, which can be used for direct analysis of insulin in commercially available raw materials, formulations of nanoparticles, and drug release as well as stability studies.

Keywords: Insulin; Nanoparticles; Entrapment efficiency; High-performance liquid chromatography

1. Introduction

Protein drug delivery has become an important research area due to a large number of recombinant proteins that are now being investigated for therapeutic applications. However, proteins have very short in vivo half-lives and require multiple injections to achieve desirable therapeutic effects. One way to increase the therapeutic efficacy of these polypeptides is encapsulating them in a sustained dosage form that is capable of releasing the macromolecule continuously at a controllable rate [1,2]. Biodegradable polymers have been successfully used to deliver proteins and peptides with prolonged therapeutic effect. Biodegradable nanoparticles prepared from poly(lactic acid-*co*-glycolic acid) (PLGA) and its copolymers with poly(ethylene glycol)-monomethyl ether (mPEG), such as mPEG–PLGA, have been extensively used as protein delivery systems as well as injectable matrices for sustained drug release [3,4].

To achieve desirable therapeutic efficacy, proteins should be efficiently incorporated into nanoparticles, which mainly depends on several preparation-associated parameters. Among them, drug entrapment efficiency (EE) is of great significance to the screening of preparation method and to the quality control of the product [5,6]. An efficient quantitative measurement of drug EE within nanoparticles needs to be established. But three main issues should be taken into account. Firstly, different from ordinary preparations, nanoparticles, diffused in liquid medium, invariably require proper methods to separate unentrapped drug from drug-loaded nanoparticles for the determination of drug EE. A number of techniques, including gel-filtration, dialysis and ultracentrifugation are available for the study of drug EE. However, the separation was not an easy task. Secondly, in the process of preparation, storage and release of nanoparticles, drugs may degrade due to vigorous stirring, homogenization, sonication or other factors [7]. Thus, it is necessary to separate drug from its major degradation products for the determination of drug EE in nanoparticles. Moreover, after solidification of nanoparticles prepared by emulsion solvent extraction/evaporation technique, some organic solvents

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may remain in the nanoparticle suspensions because of incomplete evaporation or extraction. Commonly used organic solvents in emulsion technique for the nanoparticle formulations are dichloromethane, ethyl acetate, acetone and acetonitrile. Those remaining organic solvents may potentially cause interference with the determination of drug EE in nanoparticles, especially in the indirect method to determine the amount of drug entrapped by the difference between the total amounts of drug and the unentrapped drug presented in the aqueous medium after ultracentrifugation. Therefore, the potential interference from the above organic solvents should be considered in the determination of drug EE in nanoparticles.

In this study, insulin was chosen as the model drug and a rapid and effective isocratic chromatographic procedure for the determination of insulin EE in triblock copolymeric nanoparticles using RP-HPLC was developed. Among peptide molecules, insulin is of great interest for its extensive applications in the polytreatment of diabetes mellitus and is the most important regulatory hormones in the control of glucose homeostasis, consisting of 51 amino acid residues with a molecular weight of approximately 5800 Da. It is made up of two chains, an A chain of 21 amino acids and a B chain of 30 amino acids and two disulphide bridges link the two chains and an intrachain disulphide bridge is also found in the A chain [8]. Insulin is a water-soluble, unstable peptide for which the stability issues have been studied in details [8–11]. The preservation of insulin stability is essential for the maintenance of its biological activity not only in traditional parenteral formulations but also in novel drug delivery systems. All previous works showed that insulin degraded mainly through two mechanisms: deamidation and polymerization. Insulin is susceptible to chemical and physical degradation either in solution or in suspension. Deamidation at asparagine A21 is reported to be the main degradation product of insulin at low pH values, while in slightly acidic or alkaline medium, deamidation takes place at asparagine B3 [8]. Insulin can also form covalent dimers soluble in aqueous media through a transamidation reaction [9].

Separation of insulin and insulin-like peptides is a difficult task due to the slight differences in masses and charges. Available methods for the determination of insulin content in various formulations mainly include bioanalysis, UV–vis spectrometry, high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE), etc. [12–15]. Therefore, characteristic and sensitive analytical techniques such as HPLC and HPCE, which possess high separation effect, appear to be the method for the analysis of peptides with similar structure. HPLC has been widely employed to study insulin stability, which allows the simultaneous analysis of insulin and desamido insulin. Although many researches reported the determination of insulin content using HPLC method, most of the assays failed to achieve ideal sensitivity and resolution due to poor chromatographic conditions [11,12,16,17]. In the determination of insulin EE of nanoparticles using RP-HPLC, chromatographic procedure should effectively separate insulin from its principal degradation products, such as A21- and B3-desamido insulin, and eliminate the interference from the potential organic solvent presented in nanoparticle formulations or added in the preparation of sample solution for HPLC analysis, such as dichloromethane, ethyl acetate, acetone and acetonitrile, some of which usually have absorption at the wavelength of around 214 nm and thus probably interfere with the determination of insulin EE in nanoparticles under the same conditions. There are few reports about chromatographic procedure to separate insulin from its principal degradation products and eliminate the interference from the potential remaining organic solvent in the determination of insulin EE of nanoparticles or microparticles.

This paper aimed to develop a rapid and effective chromatographic procedure for the determination of insulin EE in triblock copolymeric nanoparticles using RP-HPLC. First, ultracentrifugation was selected to separate unentrapped insulin from insulin-loaded mPEG–PLGA–mPEG-nanoparticles (insulin-PELGE-NPs). Secondly, the chromatographic method proposed was assessed on selectivity, linearity, precision, accuracy, solution stability, LOD and LOQ. Additionally, the chromatographic method was applied to the determination of insulin EE in a direct method as well as the optimization of nanoparticle preparation.

2. Experimental

2.1. Materials and chemicals

The triblock copolymer, mPEG–PLGA–mPEG (PELGE), synthesized as described in literature [18,19] and confirmed by IR, NMR, Gel Permeation Chromatography (GPC), consists of a 2000 Da mPEG block connected to a 10624 Da PLGA block (LA/GA, 70/30) with M_n of 15 000 Da. Pure crystalline porcine insulin was purchased from Xuzhou Wanbang Bio-Chemical Co. (No. 0312A02, Jiangsu, China), with a nominal activity of 28 IU mg^{-1}, which can be used without further purification.

Acetonitrile of chromatographic grade was purchased from Tianjin Kermel chemical reagents development centre (Tianjin, China). Double distilled water was used for all solutions and dilution. Other chemicals were of analytical grade.

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a Waters 2690 separation module and a 996 Photodiode Array (PDA) detector; data were collected and processed using Millennium software version 2.1 (all equipment from Waters, Milford, MA, USA). Chromatographic separations were carried out on Shimadzu Shim-pack C_{18} reverse phase column, VP-ODS (150 mm × 4.6 mm, 5 μm) equipped with a Shimadzu Shim-pack G guard column (C_{18}, 10 mm × 4 mm, 5 μm) (Chiyoda-Ku, Tokyo, Japan).

The mobile phase consisted of a premixed isocratic mixture of 0.2 M sodium sulfate anhydrous solution adjusted to pH 2.3 with phosphoric acid and acetonitrile (73:27, v/v). It was newly prepared daily using double-distilled deionized water, filtered through a 0.22 μm membrane filter and degassed via an online degasser. The injection volume for samples and standards were 20 μl and eluted at a flow rate of 0.8 ml min^{-1} at 40 °C. The eluent was monitored at 214 nm (AUFS = 1).

2.3. Preparation of nanoparticles

Nanoparticles with a theoretical insulin loading of 5–10% (w/w) were prepared using a water-in-oil-in-water (w/o/w) emulsion solvent extraction/evaporation technique. In a typical procedure, 0.1 ml porcine insulin solution (5 mg ml^{-1}) in 0.01 M hydrochloric acid (HCl) was adjusted to pH 4.0 with 0.1 M sodium hydroxide (NaOH) and placed in 0.5 ml organic mixture of ethyl acetate and acetonitrile (9:1, v/v) containing PELGE (10 mg). A primary water-in-oil emulsion was prepared by sonication using a microtip probe sonicator at the energy output of 10 W (JY92-II ultrasonic processor, Ningbo Scientz Biotechnology Co., Ltd., China) for 50 s in ice bath. Thereafter, this primary emulsion was poured into 1 ml mixed aqueous solution of glycerin (0.5%, w/v) and Pluronic F-68 (0.5%, w/v) and probe sonicated at 40 W for 20 s in ice bath. The double emulsion was diluted in 5 ml Pluronic F-68 solution (0.5%, w/v) and the organic mixture was rapidly removed by evaporation under reduced pressure (Büchi, R-144 rotavaporator, Switzerland). The *z*-average diameters of insulin-loaded nanoparticles were in the ranges of 100 nm–160 nm with polydispersity index (PDI) value of about 0.15 calculated according to the International Standard on Photon Correlation Spectroscopy (PCS) by Zetasizer Nano ZS90 (Malvern Instruments Ltd., Malvern, UK).

2.4. Standard and standard solutions

The stock standard solutions of insulin were prepared in 0.01 M HCl at the concentration of 1 mg ml^{-1}. The standard curve solutions were prepared by diluting the stock standard solutions to: 2.0, 4.0, 8.0, 20.0, 40.0, 100.0, 200.0 and 500.0 μg ml^{-1} in 0.01 M HCl. All the solutions were kept at 4 °C in dark test-tubes.

2.5. Method validation

An important part of method validation is system suitability test, details of which are usually given in pharmacopoeias. System suitability parameters and validation parameters including method selectivity, linearity, precision, accuracy, stability, LOD and LOQ were set up.

Method selectivity was verified by comparing the chromatograms of nanoparticle samples, standard solutions and degradation products. The degradation products in acid and basic solution were obtained by dissolving insulin in 0.01 M HCl at 40 °C for 96 h [11,16,17] and in pH 7.4 phosphate buffered solution (50 mM) at 40 °C for two weeks [17], respectively. The solutions of degradation products were diluted with 0.01 M HCl to match the linear range of the calibration curve.

For the determination of linearity, standard calibration curve was used. The linearity was tested in the concentration range of 2–500 μg ml^{-1}. Twenty microlitres prepared sample solutions were injected directly to HPLC column in duplicate. A calibration curve was constructed using average peak areas versus known concentrations of insulin. The resulting regression equation was used to determine the concentration of the samples.

The measurements of intra- and inter-day variability were utilized to determine the precision of the developed method. The intra-day precision was evaluated by analyzing insulin repeatedly in the concentration range of 8–100 μg ml^{-1} ($n = 6$). The inter-day precision was evaluated by analyzing insulin in the concentration range of 8–100 μg ml^{-1} day by day over a period of six days ($n = 6$). Repeatability is determined by analyzing six parallel prepared sample solutions of the same batch of insulin-loaded nanoparticles as described above and evaluated by relative standard deviation (R.S.D.) of the nanoparticle EE calculated by calibration curve.

The accuracy of the method was evaluated by the recovery of insulin. Recovery experiments were performed by analyzing nine different spiked insulin samples at concentrations of 8, 40 and 100 μg ml^{-1}. All samples have been tested three times at each concentration ($n = 9$).

LOD and LOQ of insulin were calculated by means of signal-to-noise ratio method [11]. Also, short-term stability of standard solution was tested at 25 °C.

2.6. Preparation of sample solution

Definite quantity of freshly prepared insulin-PELGE-NP suspension (equal to 50 μg insulin) was ultracentrifuged for 1 h at 4 °C at 50 000 rpm (Optima MAX-E Ultracentrifuge, MLS-50 rotor, Beckman Coulter Inc., Fullerton, CA, USA) in order to isolate the entrapped insulin from the unentrapped insulin. The supernatant was removed and nanoparticle sediments were resuspended in water. The resuspended solution was ultracentrifuged twice under the same conditions. Two hundred microlitres acetonitrile was added into the sediments and the mixture was vortexed for 5 min. Then, 800 μl 0.01 M HCl was added and vortexed for 2 min. After centrifugation at 15 000 rpm (Allegra X-22R Centrifuge, F2402H rotor, Beckman Coulter Inc., Fullerton, CA, USA) at 4 °C for 20 min, supernatant was collected for further HPLC analysis.

3. Results and discussion

3.1. Methods development and optimization

In this work, attentions were mainly focused on the optimization of chromatographic conditions such as column selection, the composition and ratio of mobile phase, flow rate and column temperature. First, four analytical columns were tested in the development of this method including Shimadzu Shim-pack VP-ODS (150 mm × 4.6 mm, 5 μm), Shim-pack LC-ODS (150 mm × 6.0 mm, 5 μm, Chiyoda-Ku, Tokyo, Japan), Vydac Protein and Peptide C18 (218TP54, 5 μm, 300 Å pore diameter, Separations Group, Hesperia, CA, USA) and Intersil ODS-3 (150 mm × 4.6 mm, 5 μm, GL Sciences Co., Ltd., Japan). Parameters having been tested included theoretical plates, resolution (R) and symmetry factor.

A conventional stationary phase based on octadecylsilica sorbent Vydac Protein and Peptide C_{18} column was tested firstly. The result was not satisfactory in that the symmetry factor was around 2.0 at different mobile-phase ratios and flow rates; as for

other columns, the symmetry factors using LC-ODS and ODS-3 columns were in a range of 1.15–1.20 at different mobile-phase ratios and flow rates; however, the symmetry factor using Shimadzu Shim-pack VP-ODS column was no more than 1.08 with symmetrical peak shape and minimal peak tailing. Then, Shimadzu Shim-pack VP-ODS column was selected in this study.

Many RP-HPLC determinations of insulin used low pH solution of sodium sulfate anhydrous and acetonitrile as mobile phase in literatures [7,8,16,17] and in the United States Pharmacopeia [20]. The pH value of sodium sulfate anhydrous solution was usually adjusted to pH 2.3 with phosphoric acid, or with ethanolamine [20] and triethylamine [7,21]. Then, in this study three mobile phases adjusted to pH 2.3 with phosphoric acid, or with ethanolamine and triethylamine were tested, i.e. 0.2 M sodium sulfate anhydrous solution adjusted to pH 2.3 with phosphoric acid and acetonitrile; 0.2 M sodium sulfate anhydrous solution (0.27% phosphoric acid, v/v) adjusted to pH 2.3 with triethylamine and acetonitrile; 0.2 M sodium sulfate anhydrous solution (0.27% phosphoric acid, v/v) adjusted to pH 2.3 with ethanolamine and acetonitrile. The results showed some differences in theoretical plate, which was approximately 2900, 2750 and 2800 at a flow rate of 0.8 ml min^{-1} at 40 °C, respectively. Furthermore, chromatographic behaviors under various composition ratios (74:26, 73:27, 72:28, v/v) of mobile phase consisting of a premixed isocratic mixture of 0.2 M sodium sulfate anhydrous solution adjusted to pH 2.3 with phosphoric acid and acetonitrile, flow rates (0.8, 1.0, 1.2 ml min^{-1}) and column temperatures (35, 40 °C) were investigated. The results showed that retention time of insulin decreased with the increase of acetonitrile proportion in the mobile phase. When mobile phase was set at the ratio of 72:28 (v/v), a major problem was the interference of ethyl acetate, because the organic solvent, potentially remaining in the preparation of nanoparticles, had the retention time of about 7 min similar to insulin; however, at the ratio of 73:27 (v/v), retention time of ethyl acetate remained unchanged (still at about 7 min) and the interference to insulin was completely eliminated (data are not shown). When the mobile phase was set at the ratio of 74:26 (v/v), the insulin retention time was extended up to about 30 min which might increase the analysis time with insulin peak area decreasing slightly. Moreover, insulin peak area decreased significantly and peak tailing appeared with increasing flow rate; when column temperature increased from 35 to 40 °C, retention time of insulin increased from about 7 to 11 min and the theoretical plate was improved from about 2400 to 2900. But under the conditions of Section 2.2, baseline was straight and smooth, symmetry factor reached 1.01 and as shown in Fig. 1, the number of theoretical plates stayed around 3000, which was quite acceptable.

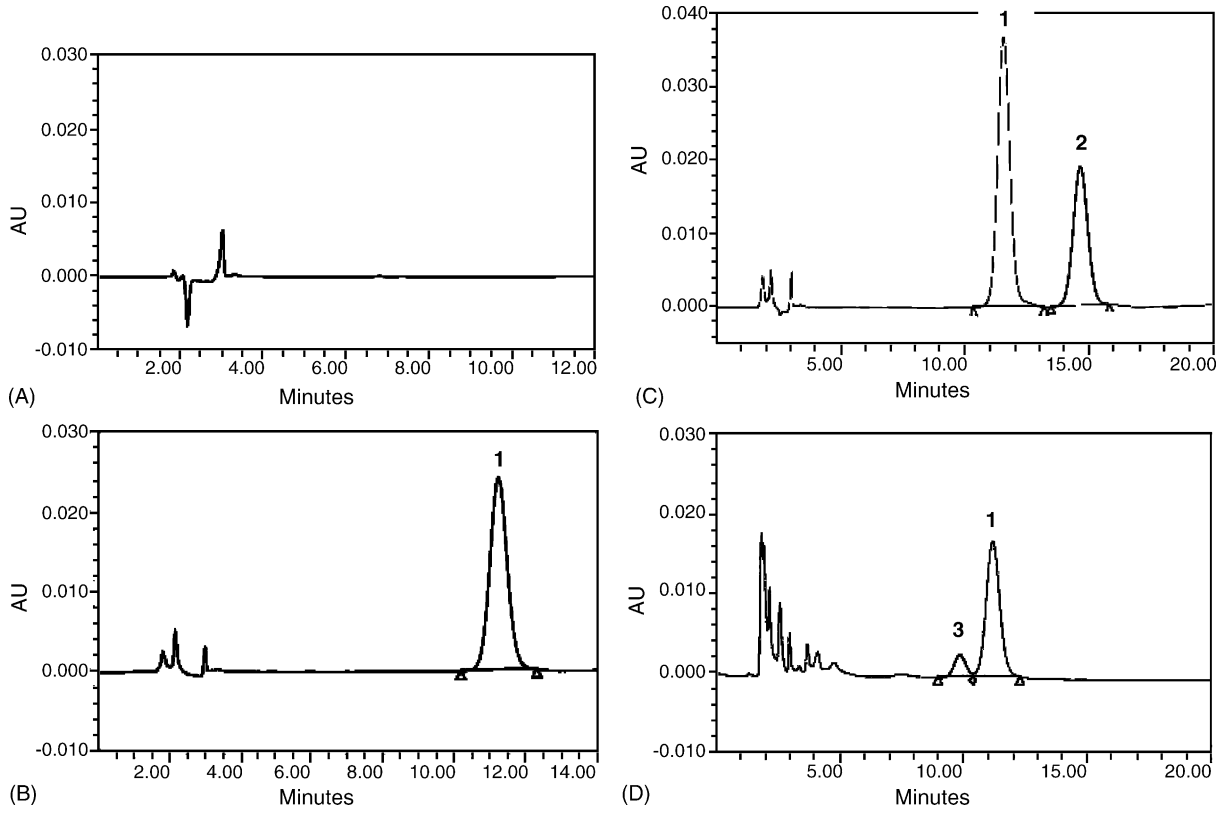

Fig. 1. Representative HPLC chromatograms using 0.01 M HCl as solvent (A), 40 μg ml^{-1} porcine insulin (B), insulin and its acid degradation product corresponding to a sample solution storing for 96 h at 40 °C in 0.01 M HCl (C), insulin and its basic degradation product corresponding to a sample solution storing for two weeks at 40 °C in phosphate buffered solution (50 mM, pH 7.4) (D). Peaks: 1, porcine insulin (11.19 min); 2, acid degradation product of insulin (14.59 min); 3, basic degradation product of insulin (9.85 min). Conditions: mobile phase, 0.2 M sodium sulfate anhydrous solution adjusted to pH 2.3 with phosphoric acid and acetonitrile (73:27, v/v); flow rate, 0.8 ml min^{-1}; detection wavelength, 214 nm; column temperature, 40 °C; injection volume, 20 μl.

3.2. Selectivity

In order to test the selectivity of the present method, insulin solutions were hydrolyzed by storing under different pH values at 40 °C. In literature, researchers developed the HPLC method to separate insulin and A21-desamido insulin usually under the conditions of acidic mobile phase [11,16]. Moslemi et al. [11] used a mixture of water and acetonitrile containing tetramethylammonium hydroxide as eluent for the analysis of insulin and its main degradation product, A21-desamido insulin. However, the RP-HPLC method had very narrow range of linearity, and resolution was no more than 1.8 ($R = 1.44$), with column efficiency less than 1000. Oliva et al. [16] developed the RP-HPLC method for the analysis of insulin and its degradation product (A21-desamido insulin) and validated in pharmaceutical preparations. Nevertheless, the baseline obtained was not smooth and its symmetry factor was more than 1.10, with column efficiency less than 1300. Kim and Peppas [22] developed a gradient elution to analyze the concentration and stability of insulin by RP-HPLC, which showed that symmetry factor of insulin was above 1.5, though the baseline was smooth. The chromatographic conditions developed by Hoyer et al. [23] and Kunkel et al. [12] failed to achieve agreeable resolution and symmetric peak shapes for insulin and its related substances. Most methods mentioned above were unable to separate insulin from B3-desamido insulin. Yomota et al. [17] developed a RP-HPLC method for separation of B-3 desamido insulin from human insulin under the conditions of alkaline mobile phase. Under acidic conditions, A21-desamido insulin was separately eluted, whereas B-3 desamido insulin was included in the human insulin peak. Thus, it is necessary to separate the insulin and derivative desamido insulin in a single analysis within a short time, combining a satisfactory resolution for separation.

In this study, the insulin degradation products in acid and basic solution was prepared according to the United States Pharmacoepia [20] and other reports [11,16,17,21], which were identified as A21- and B3-desamido insulin, respectively. We did not identify them due to difficulties to prepare the pure desamido insulin [24]. Fig. 1C and D shows the separation of insulin and its degradation products under the proposed chromatographic conditions in Section 2.2 in which HPLC coelution of the other two compounds would be A21- and B3-desamido insulin, respectively, comparing to the literatures above. The separation illustrated a good resolution of insulin and degradation product peaks with high selectivity, which was especially important for determining the purity and stability of insulin. The insulin degradation product in acid solution with concentration above 5% was prepared by storing in 0.01 M HCl at 40 °C for 48 h [11,20]. In this study, more than 20% the acid degradation product of insulin was obtained by increasing the storage time up to 96 h. Resolution was above 3.10, which demonstrated the baseline separation for the peaks in high concentration of the insulin degradation product. Fig. 1D showed fine resolutions and peak shapes of both insulin and the basic degradation product.

In addition, blank PELGE-NP solution was determined to further evaluate method selectivity. Results showed that there

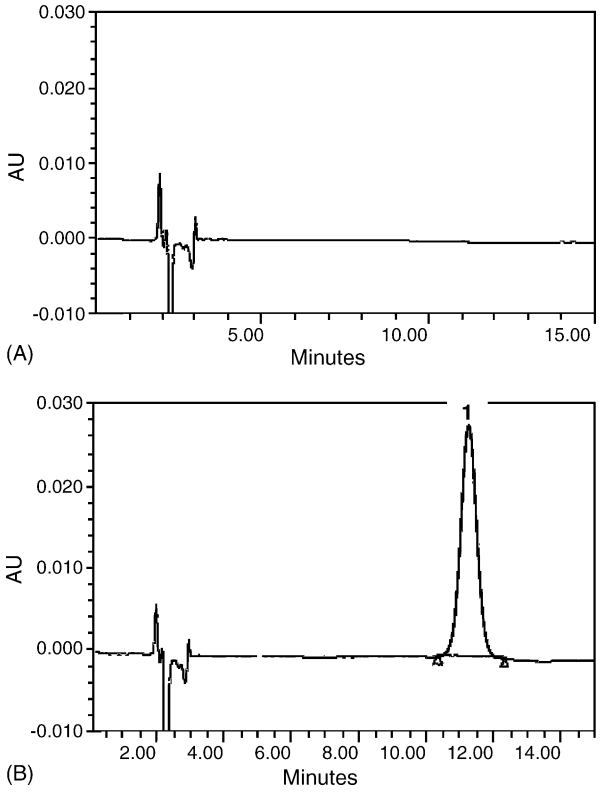

Fig. 2. Representative HPLC chromatograms for blank PELGE-NPs (A), porcine insulin-PELGE-NPs with the theoretical drug loading of 5% (B).

was no interference from nanoparticle matrices (Fig. 2A). Under proposed chromatographic conditions, no interference was observed from other components presented in the nanoparticle suspension or from chemical reagents (PELGE, glycerin and Pluronic F-68). For solvents commonly used in the preparation of nanoparticles, dichloromethane with retention time of 8.8 min and acetone of 3.3 min did not interfere with the determination of insulin.

From what has been discussed above, the developed HPLC method is highly selective for the determination of insulin and has achieved agreeable resolution, reasonable retention time, symmetric peak shapes of insulin and its related substances in PELGE-NPs.

3.3. Linearity

The linearity of the method was determined at eight concentration levels ranging from 2.0 to 500.0 μg ml^{-1} for insulin. The calibration curve was constructed by plotting mean area response (A) against concentration (C) of the drug. The results indicated that there existed an excellent correlation ($r = 0.99998$) between peak area and drug concentration within the concentration range indicated above.

3.4. Precision

The precision is determined by performing six replicate analyses of the same solution and evaluated by R.S.D. of the

Table 1
Recovery tests of insulin in insulin-PELGE-NPs ($n = 3$)

Actual concentration ($\mu g\,ml^{-1}$)	Calculated concentration ($\mu g\,ml^{-1}$)	Mean recovery (%)	R.S.D. (%)
8	7.72	96.52	1.55
40	40.56	101.40	0.32
100	99.75	99.75	0.11

peak area of insulin. Three concentrations of insulin (8, 40, 100 $\mu g\,ml^{-1}$) were prepared and analyzed in one day or six different days in order to evaluate intra-day or inter-day variations respectively. The R.S.D. of responses was calculated in each case. The method was found to be precise with the R.S.D. values of 0.64%, 0.83%, 1.24% for intra-day assays and 2.24%, 1.30%, 0.64% for inter-day assays, respectively.

3.5. Reproducibility

The insulin-PELGE-NPs suspension with theoretical insulin loading of 5% was processed according to the sample preparation method (see Section 2.6). Reproducibility of peak areas of six insulin-PELGE-NP injection sequences was shown as the R.S.D. for each peak. The result proved that the chromatographic conditions were of fine reproducibility with the R.S.D. value of 1.21% ($n = 6$).

3.6. Solution stability

In order to evaluate the stability of insulin solution during analysis, 40 $\mu g\,ml^{-1}$ insulin solution was analyzed over a period of 12 h at room temperature. Results showed that the retention time and peak area of insulin remained almost unchanged, the R.S.D. value of peak area was 1.93% ($n = 6$), and no degradation was observed within the indicated period. It is suggested that insulin solution be stable for at least 12 h, which is sufficient for the whole analytical procedure.

3.7. Accuracy

To study the reliability and suitability of the HPLC method, recovery experiments were carried out. The blank PELGE-NP suspension was processed according to sample preparation method (see Section 2.6). Sediments of blank PELGE-NPs, after dissolving in 200 μl acetonitrile, were spiked with 800 μl three different levels of insulin standard solution, concentrations ranging from 10, 50, to 125 $\mu g\,ml^{-1}$. Mixtures were processed (see Section 2.6) and analyzed. Accurate concentrations of insulin added and calculated concentrations following the sample analysis procedure were used to evaluate the percent recoveries of the method.

The mean values of the percent recoveries for each concentration of insulin are shown in Table 1. The mean recovery of insulin from spiked samples, in a concentration range of 8–100 $\mu g\,ml^{-1}$, was 98.96% (R.S.D. = 2.51%, $n = 9$). These results indicated a good accuracy of the proposed method for the determination of insulin in PELGE-NPs suspension.

3.8. Limit of quantitation and limit of detection

The LOQ and LOD of insulin were experimentally verified by six injections based on a detector signal-to-noise ratio of 10 and 3 (S/N = 10, 3), respectively [11]. The LOQ of insulin was found to be 20 ng and LOD was found to be 8 ng.

3.9. Entrapment efficiency

In literature, the EE of nanoparticles or microspheres was generally determined in an indirect way [25]. The amount of drug entrapped in nanoparticles or microspheres was determined by the difference between the total amounts of drug and the unentrapped drug after ultracentrifugation.

However, the aforementioned method would not be suitable to determine the amount of drug presented in the aqueous medium when the EE of nanoparticles was high. In such case, drug concentration in the aqueous medium was too low to be accurately determined, especially when supernatants were collected after several times of ultracentrifugation. In this study, the direct method was selected to determine the amount of drug entrapped in nanoparticle suspension. But reported direct methods for determining nanoparticle EE were always complex and time-consuming. Caliceti et al. [26] measured insulin content within nanoparticles by adding dichloromethane to nanoparticles and then mixing with water containing 0.05% trifluoroacetic acid (TFA). The two-phase mixture was stirred for 30 min and the organic solvent was left to evaporate. Finally, the samples were centrifuged and the solution volume was exactly determined.

In this study, EE was determined using acetonitrile extraction method to analyze the insulin content within the nanoparticles as previously described by Kawashima et al. [27], that is, the sediments of nanoparticle suspension after ultracentrifuge was dissolved in acetonitrile, and 0.01 M HCl was added to preferentially precipitate the polymer. In this study, sample solution had an identical retention time with that of the insulin standard (Compare Figs. 1B and 2B), this provided supportive evidence that insulin did not degrade to products of different chemical natures, such as the A21-desamido insulin (Fig. 2B). As for the acetonitrile extraction approach, the blank nanoparticles did not show any interference in the HPLC chromatograph, and insulin-spiked samples of blank nanoparticles had a mean recovery of 98.96% in the concentration range of 8–100 $\mu g\,ml^{-1}$. The satisfactory recovery contributed to further determination of insulin content in nanoparticles.

Nanoparticle suspensions with theoretical insulin loading of 5–10% were analyzed by proposed HPLC method. Under the

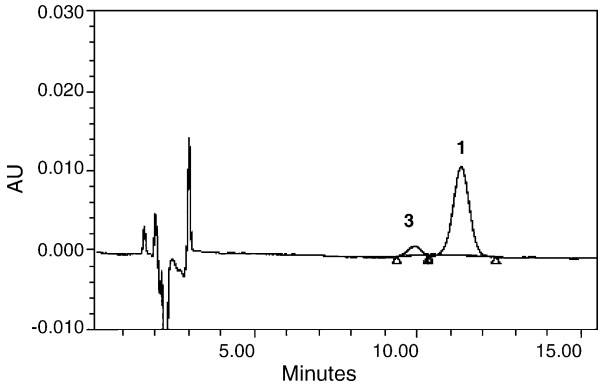

Fig. 3. Representative HPLC chromatograms for unentrapped insulin in which 0.01 M NaOH was used as the solvent for glycerin and Pluronic F-68.

developed chromatographic conditions for sample solutions, peak areas of sample solutions from insulin-loaded nanoparticles were measured and then insulin content in supernatant was calculated by calibration curve. Therefore, insulin EE could be calculated with the percent ratio of the actual amount of insulin incorporated within the nanoparticles to the total amount of insulin used in the preparation medium. Assay results for different theoretical amount of drug loadings from 5%, 8% to 10% were found to be 94.84% (R.S.D. = 1.34%), 89.02% (R.S.D. = 2.38%) and 57.81% (R.S.D. = 3.61%) ($n = 3$), respectively. The results demonstrated that the insulin EE could not lead to a corresponding increase with an increasing amount of drug used in the preparation of nanoparticles and implied that the fed drug possibly exceeds the incorporation capacity of the triblock copolymer to form nanoparticles, which was in agreement with the fact that drug incorporation efficiency into the nanoparticles is limited [28]. In this study, the actual drug loading contents calculated as the ratio between the amount of drug in nanoparticles and the amount of nanoparticle yield [29] were approximately 4.52%, 6.59% and 5.26%, respectively.

All the above results demonstrated that the validated LC method had successfully been applied to the assay of EE of insulin-loaded nanoparticles with three different levels of drug loading.

3.10. Method application

The analytical method for the separation of insulin and its two degradation products proved to be effective, fast and met the criteria for method validation. The method might be applied to direct determination of insulin in commercially available raw materials, optimization of the formulation of nanoparticles or microspheres, and drug release studies. The method might also conduce to the degradation process control in stability studies of pharmaceutical preparations, because the LOD and LOQ for degradation products were provided.

Fig. 3 represented an RP-HPLC chromatogram of the unentrapped insulin, in which 0.01 M NaOH was used as the solvent for the mixture of glycerin and Pluronic F-68 in the optimization of the formulation for insulin-PELGE-NPs. The result indicted that insulin would have degraded to B-3 desamido insulin in the alkaline medium solution, which was agreeable to literature [17].

4. Conclusions

A rapid and reliable isocratic RP-HPLC method for the determination of insulin EE in PELGE-NPs has been developed and validated. This assay has fulfilled the requirements to be considered as a reliable and feasible method, including selectivity, linearity, precision, accuracy, stability, LOD and LOQ. It is a highly specific and precise analytical procedure and its chromatographic run time of 15 min allows the analysis of a large number of samples in a short period of time. In short, this method is rapid and convenient in separating insulin from its principal degradation products and suitable for direct analysis of insulin in commercially available raw materials, formulations of nanoparticles, drug release studies and stability studies.

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References

[1] A. Vila, A. Sanchez, M. Tobío, P. Calvo, M.J. Alonso, J. Controlled Rel. 78 (2002) 15–24.
[2] K.S. Soppimath, T.M. Aminabhavi, A.R. Kulkarni, W.E. Rudzinski, J. Controlled Rel. 70 (2001) 1–20.
[3] Y.J. Kim, S. Choi, J.J. Koh, M. Lee, K.S. Ko, S.W. Kim, Pharm. Res. 18 (2001) 548–550.
[4] U. Bilati, E. Allémann, E. Doelker, J. Microencapsulation 22 (2005) 205–214.
[5] F.T. Meng, G.H. Ma, Y.D. Liu, W. Qiu, Z.G. Su, Colloids Surf. B 33 (2004) 177–183.
[6] T. Pitaksuteepong, N.M. Davies, I.G. Tucker, T. Rades, Eur. J. Pharm. Biopharm. 53 (2002) 335–342.
[7] P.G. Shao, L.C. Bailey, Pharm. Dev. Technol. 5 (2000) 1–9.
[8] J. Brange, L. Langkjaer, S. Havelund, A. Volund, Pharm. Res. 9 (1992) 715–726.
[9] J. Brange, S. Havelund, P. Hougaard, Pharm. Res. 9 (1992) 727–734.
[10] J. Brange, O. Hallund, E. Sorensen, Acta Pharm. Nord. 4 (1992) 223–232.
[11] P. Moslemi, A.R. Najafabadi, H. Tajerzadeh, J. Pharm. Biomed. Anal. 33 (2003) 45–51.
[12] A. Kunkel, S. Günter, C. Dette, H. Wätzig, J. Chromatogr. A 781 (1997) 445–455.
[13] N.V. Sergeev, N.S. Gloukhova, I.V. Nazimov, V.A. Gulyaev, S.V. Shvets, I.A. Donetsky, A.I. Miroshnikov, J. Chromatogr. A 907 (2001) 131–144.
[14] I. German, R.T. Kennedy, J. Chromatogr. B: Biomed. Sci. Appl. 742 (2000) 353–362.
[15] M. Lookabaugh, M. Biswas, I.S. Krull, J. Chromatogr. 549 (1991) 357–366.
[16] A. Oliva, J. Fariña, M. Llabrés, J. Chromatogr. B: Biomed. Sci. Appl. 749 (2000) 25–34.
[17] C. Yomota, Y. Yoshii, T. Takahata, S. Okada, J. Chromatogr. A 721 (1996) 89–96.
[18] Y.R. Duan, Z.R. Zhang, Y. Huang, C.Y. Wang, Key Eng. Mater. 254–256 (2004) 887–890.
[19] X. Sun, Y.R. Duan, Q. He, J. Lu, Z.R. Zhang, Chem. Pharm. Bull. 53 (2005) 599–603.

[20] Insulin/Official Monographs, in: United States Pharmacopeia 24/National Formulary 19, United States Pharmacopeial Convention, Inc., Rockville, MD, 1999, pp. 880–882.
[21] Z. Yu, T.L. Rogers, J. Hu, K.P. Johnston, R.O. Williams III, Eur. J. Pharm. Biopharm. 54 (2002) 221–228.
[22] B. Kim, N.A. Peppas, Int. J. Pharm. 266 (2003) 29–37.
[23] G.L. Hoyer, E.P. Nolan Jr., J.H. LeDoux, L.A. Moore, J. Chromatogr. A 699 (1995) 383–388.
[24] Y. Zhou, M. Ottens, E. Hansen, L.A.M. van der Wielen, J. Chromatogr. A 1061 (2004) 141–148.
[25] F. Cournarie, M. Chéron, M. Besnard, C. Vauthier, Eur. J. Pharm. Biopharm. 57 (2004) 171–179.
[26] P. Caliceti, S. Salmaso, N. Elvassore, A. Bertucco, J. Controlled Rel. 94 (2004) 195–205.
[27] Y. Kawashima, H. Yamamoto, H. Takeuchi, S. Fujioka, T. Hino, J. Controlled Rel. 62 (1999) 279–287.
[28] T. Govender, T. Riley, T. Ehtezazi, M.C. Garnett, S. Stolnik, L. Illum, S.S. Davis, Int. J. Pharm. 199 (2000) 95–110.
[29] F. Quaglia, G.D. Rosa, E. Granata, F. Ungaro, E. Fattal, M.I.L. Rotonda, J. Controlled Rel. 86 (2003) 267–278.