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Synchronic release of two hormonal contraceptives for about one month from the PLGA microspheres: In vitro and in vivo studies

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ABSTRACT

A controlled drug release system based on the injectable PLGA microspheres loaded with gestodene and ethinyl estradiol was prepared and evaluated for the feasibility of monthly synchronic delivery of the two hormonal contraceptives. The scanning electron microscopy, light-scattering analyzer and gel permeation chromatography were used to study the morphology, particle size and molecular weight of the polymer microspheres, respectively. HPLC was utilized to determine the drug loading and the drug released, while a LC-MS-MS system was employed to analyze the plasma drug concentration. Result indicated that the PLGA particles obtained were spherical and appropriate in size. The formulation was stable during the test period. In vitro drug release from the microspheres for both drugs was sustained for about 30 days mostly by the diffusion mechanism. The plasma drug concentration-time profiles of the drug-loaded microspheres were relatively smooth after subcutaneous injection to rats for about 1-month, compared with that for drug suspension. In vitro and in vivo correlation was established. One of the most important facts is the synchronicity of the two contraceptives both in the release kinetics in vitro and the pharmacokinetic behaviors in vivo. Therefore, the synchronic delivery of two contraceptives is achieved for about 1 month by using the injectable PLGA-based microspheres.

1. Introduction

Hormonal contraceptives have played very important roles in family planning programs and have been used in clinical practice for more than 60 years. There has been a great increase in contraceptive use over the past 35 years in both developed and developing countries [1,2]. Ethinyl estradiol (EE) is a synthetic estrogen, while gestodene (GE) is a synthetic progestogen structurally related to levonorgestrel. They are commonly used as the estrogenic /progestogenic component in combination preparations [3,4].

The contraceptives can be formulated and administered by oral adminitration, injection, subdermal implants, transdermal patch or the vaginal ring [5]. However, oral route is the most commonly used since it is convenient and readily accepted by most patients. But oral contraceptives have high variations in blood concentration leading to blood-concentration-dependent side effects because of frequent administration [6–11]. And another drawback is that the reduction in efficacy due to the patient forgetting to take the medication, and resulting variation in drug levels in the blood descending to sub-therapeutic concentrations. Both side effects and forgetting to take the medication for the patient could be minimized by using a long-term controlled release system to ensure a longer period of steady blood drug concentration.

Currently, there are only a small number of commercially available products that utilize long-term controlled release technology. Norplant® is a silicone-based device for the delivery of levonorgestrel. However, the polymer used is not biodegradable, and the device has to be implanted and removed through surgical operation [12]. To overcome this problem, microspheres made of biodegradable polymer were developed for implantation under the skin without surgery [13].

Poly (D, L-lactide-*co*-glycolide) (PLGA), composed of poly (lactic acid) and poly (glycolic acid), is the most commonly used biodegradable copolymer. With the long history of safe human use in the form of surgical sutures, PLGA has been studied extensively as the injectable polymeric microspheres to prolong the release of therapeutic agents, such as antibiotics, anti-inflammatory drugs, anticancer drugs, steroids, peptides and proteins [14–21]. Some of the products are commercially available, for example, Lupron Depot®, with a controlled release of leuprolide acetate for 1 month [22–24] after subcutaneous administration.

There are only a few reports on the injectable PLGA microspheres containing contraceptives, and very few on the contraceptive combination. Recently, M. D. Dhanaraju et al. reported the preparation, morphology, drug loading, as well as the in vitro release of the PLGA microspheres loaded with levonorgestrel and EE [25].

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In literature, there is little information related to PLGA microspheres loaded with the drug combination. Hence many important issues remain to be explored and resolved, such as the exact control of drug release, synchronic release of different drugs, simultaneous determination of two or more components, precise determination of trace quantity of drugs (like hormonal contraceptives) in vivo, establishment of the correlation between in vitro and in vivo studies, and so on.

Accordingly, in the present study, a newly developed controlled release system based on PLGA microspheres loaded with both GE and EE was prepared and evaluated. Besides the evaluation of physicochemical characteristics, the in vitro degradation of the PLGA microspheres was also investigated. In vitro release studies were conducted to ensure the control release and specifically, synchronic release of two components for about 1 month. An unreported analytical method based on LC-MS technique was also developed to simultaneously determine two trace components in vivo samples for pharmaceutical analysis [26,27]. Finally, the correlation between in vitro and in vivo release was established.

2. Materials and methods

2.1. Materials

Poly (D, l-lactide-co-glycolide) (PLGA, 50:50, Av. Mw 12,000, Inherent visc. 0.17 dL/g) was purchased from Absorbable Polymers International (USA). GE and EE (microcrystalline powder, 99% purity) were obtained from Beijing Zizhu Pharmaceutical Co., Ltd (Beijing, China). Methylnorethindrone (99.1% purity) was supplied by Shenyang Pharmaceutical University (Shenyang, China). Dansyl chloride (99.0% purity) was from Sigma-Aldrich (USA). Poly (vinyl alcohol) (PVA 05-88, Mw 27,000–32,000) and sodium carboxymethycellulose were the products of Beijing Organism Chemical Industry Co. Ltd. Methanol, Acetonitrile (Merck Chemical Co.) and tetrahydrofuran (THF) were of HPLC grade. Other chemicals were of reagent grade. All solutions were prepared with distilled water.

2.2. Preparation of PLGA microspheres

An oil-in-water (o/w) emulsion solvent extraction/evaporation method was used for the preparation of GE and EE microspheres [28]. Briefly, 1250 mg of PLGA, 25 mg of GE and 10 mg of EE were dissolved in 10 ml of dichloromethane. This organic phase was slowly added to 300 ml of 1% PVA aqueous solution. The mixture was homogenized at 12,000 rpm for 10 min. The obtained emulsion was then added to 800 ml of 1% (w/v) PVA aqueous solution and stirred at 250 rpm, 25 °C for 6 h. The resulting microspheres were washed three times with distilled water and lyophilized for 24 h (ALPHA2-4, CHRIST, Germany).

2.3. Characteristics of microspheres

2.3.1. Observation by scanning electron microscopy (SEM)

The morphology of the microspheres was examined using a scanning electron microscope (SEM, JSM-5600LV, JEOL, Tokyo, Japan); images of microspheres were also taken after the in vitro drug release tests. Samples were prepared by placing microspheres onto an aluminium specimen stub, then dried overnight followed by sputter coated with gold prior to imaging (IB-3 Ion Coater, EIKO, Japan). Coating was performed at 2 mA for 3 min.

2.3.2. Particle size analysis

A light-scattering particle size analyzer (BT-9300, BETTER, China) with a circulation disperser (BT-600, BETTER, China) was used to determine the size distribution of the prepared microspheres. The lyophilized particles were suspended by a large mount of distilled water and analyzed under continuous stirring. Particle size was expressed as volume mean diameter in micrometers (SEM, $n=3$) of three batches.

2.3.3. Molecular weight of PLGA by gel permeation chromatography

Gel permeation chromatography (GPC) was performed to determine the mean molecular weight of PLGA, using a Waters ® system (Waters, USA) which included a Waters 515 HPLC pump, three Waters Styragel columns (HT2, HT3, HT4), a temperature control module with column heater, a Waters 2410 Differential Refractometer, and a Waters 2487 Dual λ Absorbance Detector. The pump flow rate was set at 1.0 ml/min and the columns were held at a constant temperature of 35 °C. Microspheres (10 mg) were dissolved in 10 ml of THF and filtered (0.22 μm). Only glassware (vials and syringes) was utilized to minimize possible contamination from plastic materials. The polymer solution injection volume was 50 μl. The data collection and analysis were performed using Waters Millennium[32] software. Weight average molecular masses were calculated based on polystyrene standards. All the measurements were conducted in triplicate and the mean values and standard deviations were reported.

2.3.4. High performance liquid chromatography

The concentrations of GE and EE in the in vitro studies were determined using HPLC system (HP1100, Angilent, USA), consisted of a pump and a UV-Vis detector set at 273 nm. The analytical column was a DIAMONSIL® C18 (4.6 mm × 250 mm) (Dikma technologies, Beijing, China) and the column temperature was set as 30 °C. The mobile phase consisted of methanol and water at a ratio of 65:35 (v/v) and the flow rate was 1 ml/min. Aliquot of 50 μl clear supernatant was injected into the HPLC system. The peak areas of GE and EE were recorded and the concentrations were calculated from a standard curve. The retention time of GE and EE was 18 min and 21 min, respectively.

2.3.5. Drug loading efficiency

To determine loading percentage of GE and EE in the microspheres, 10 mg of the freeze-dried microspheres were dissolved in 1 ml of dichloromethane. The resulting solution was then diluted to 10 ml with HPLC mobile phase and vortex-mixed (WH-861 Vortex Shaker, China). One milliliter of the dispersion was centrifuged for 10 min at 10,000 rpm (TGL-16G centrifuge, Shanghai, China). The supernatant was injected into HPLC system to determine the concentration of GE and EE. The drug loading efficiency (LE) was calculated as follows:

$$\mathrm{LE} = (\text{drug found in microspheres}/\ \text{drug added}) \times 100\%$$

All the measurements were conducted in triplicate and the mean values and standard deviations are reported.

2.3.6. In vitro drug release studies

Aliquot of 15 mg freeze-dried microspheres were suspended in 30 ml of 0.1 M PBS (pH 7.4) containing 0.05% Tween 80 and 0.02% sodium azide and stirred at 50 rpm in an air chamber thermostated at 37 ± 1 °C. At appropriate intervals, samples were collected and centrifuged at 3000 rpm for 5 min. Aliquot of 1 ml supernatant was taken and replaced with the equal value of fresh release medium. Then the supernatant was centrifuged at 10,000 rpm for 10 min. The amount of GE and EE in the collected supernatant was measured by the HPLC method (see Section 2.3.4). All the release tests were performed in triplicate.

2.3.7. In vitro degradation of the microspheres

Aliquot of 15 mg freeze-dried microspheres was dispersed in 30 ml of 0.1 M PBS (pH 7.4) containing 0.05% Tween 80 and 0.02% sodium azide. And the resulting suspension was dispersed in tubes and stirred at 50 rpm in an air chamber thermostated at 37 ± 1 °C. At the specific time point, the microspheres were collected by centrifugation (3000 rpm, 5 min), washed three times with double-distilled water and lyophilized. The samples were weighed and the weight reduction of the microspheres was evaluated using the following equation:

$$\text{Weight reduction} = M_2/M_1 \times 100\%$$

where, M_1 is the initial weight of microspheres and M_2 is the weight of the microspheres at certain time.

The degradation of microspheres was also determined by the GPC mentioned above. The surface morphology of the microspheres during the test was observed with SEM.

2.4. In vivo studies

2.4.1. Animals and experimental method

Sprague Dawley (SD) female rats (220±20 g, Peking University Animals Center) were used for the pharmacokinetic studies. All animal experiments complied with the requirements of the National Act on the use of experimental animals (PR China). All rats were fasted for 12 h before the experiments with free access to water.

The rats were randomly divided into two groups, namely the test group and control group. The animals in the test group were treated by subcutaneous injection of the suspension containing 10 mg of drug-loaded microspheres and 0.4 ml of dispersed solution (containing 0.5% CMC-Na and 0.05% Tween-80). The rats in control group were administered with the 10 mg of blank microspheres, with the same amount of GE and EE in ethanol, as well as the dispersed solution. All suspension used was vortex-mixed before administration. After administration, all the centrifuge tubes were collected and freeze-dried to determine the residue drug and calculate the exact amount of injected drug. For test group, the amount of GE and EE administrated was 127.14±3.27 μg and 51.33±4.13 μg, respectively; for control group, 137.63±1.56 μg and 53.83±2.08 μg, respectively.

At designed time point, 0.8 ml of blood samples was collected from the retroorbital plexus of the anesthetic rats, and centrifuged at 8000 rpm for 10 min within 2 h. Then 0.3–0.4 ml of plasma was obtained and stored at −20 °C before measurement.

2.4.2. Plasma concentration determination

2.4.2.1. Sample preparation. Aliquots of 0.2 ml of mixed plasma samples were transferred into 10 ml glass test tubes with glass stopper. Each sample was mixed with 20 μl of 5 μg/ml methylnorethindrone and 3 ml of redistilled acetoacetate by vortex, and then centrifuged at 3500 rpm for 10 min. The organic layer was evaporated to dryness under a stream of nitrogen at 45 °C.The residue was dissolved with 100 μl of sodium bicarbonate (0.1 M) and mixed with 100 μl of dansyl chloride in acetone (1.0 mg/ml), respectively. The tubes were kept in a water bath at 60 °C for 8 min to facilitate derivatization. After the reaction, the tubes were placed into water bath at room temperature. After the addition of 3 ml mixture of anhydrous diethyl ether and *N*-hexane (2:1, v/v), the tubes were vortex-mixed for 1 min and centrifuged at 3500 rpm for 10 min. Again, the organic layer was evaporated to dryness under the same condition mentioned above, and residue was dissolved with 100 μl of water-acetonitrile solution (20:80, v/v). Each sample was vortex-mixed for 1.5 min before analysis in a LC-MS/MS system.

2.4.2.2. Liquid chromatographic condition. An ACQUITY Ultra Performance LC™ liquid chromatograph integrated system, consisting of an autosampler, a multichannel mobile phase degasser, a column heater, two pumps (Waters, USA), and an ACQUITY UPLC™ BEH C18 column (2.1× 50 mm I.D, 1.7 μm, Waters, USA) was used for the chromatographic separation and analysis of GE, dansylated EE and internal standards. The mobile phases used were the mixture of water (A) and acetonitrile (B) in a gradient model. The ratio (A/B) of the two solvents in mobile phase was different in various periods: it was 70/30 between 0 and 0.5 min, 50/50 between 0.5 and 1.0 min, 30/70 between 1.0 and 1.5 min, 2/98 between 1.5 and 4.1 min and 70/30 after 4.1 min. The column was maintained at 40 °C and the flow rate was 0.35 ml/min. An aliquot of 10 μl sample each time was injected onto the system and all the column effluent was delivered to the mass spectrometer interface.

2.4.2.3. Mass spectrometric conditions. A Waters Quattro micro API triple quadrupole mass spectrometer (Waters, USA) with a turboionspray interface operated in the positive ionization mode and a Masslynx 4.1 data acquisition software were used in the multiple reaction monitoring (MRM) LC-MS/MS analyses. The optimized instrument conditions were as follows: TIS source temperature, 105 °C; TIS voltage, 3000 V; cone voltage, 30 V; curtain gas, N_2; gas temperature, 300 °C; gas flow rate, 500 L/h; cone gas flow rate, 50 L/h; collision air(Ar) pressure, $2.55e^{-3}$ mbar; collision energy, 30 eV for GE, 40 eV for dansylated EE and 25 eV for methylnorethindrone (inter standard).

The following precursors to product ion transitions were used for the multiple reaction monitoring: GE, m/z 311→109, m/z 311→91;

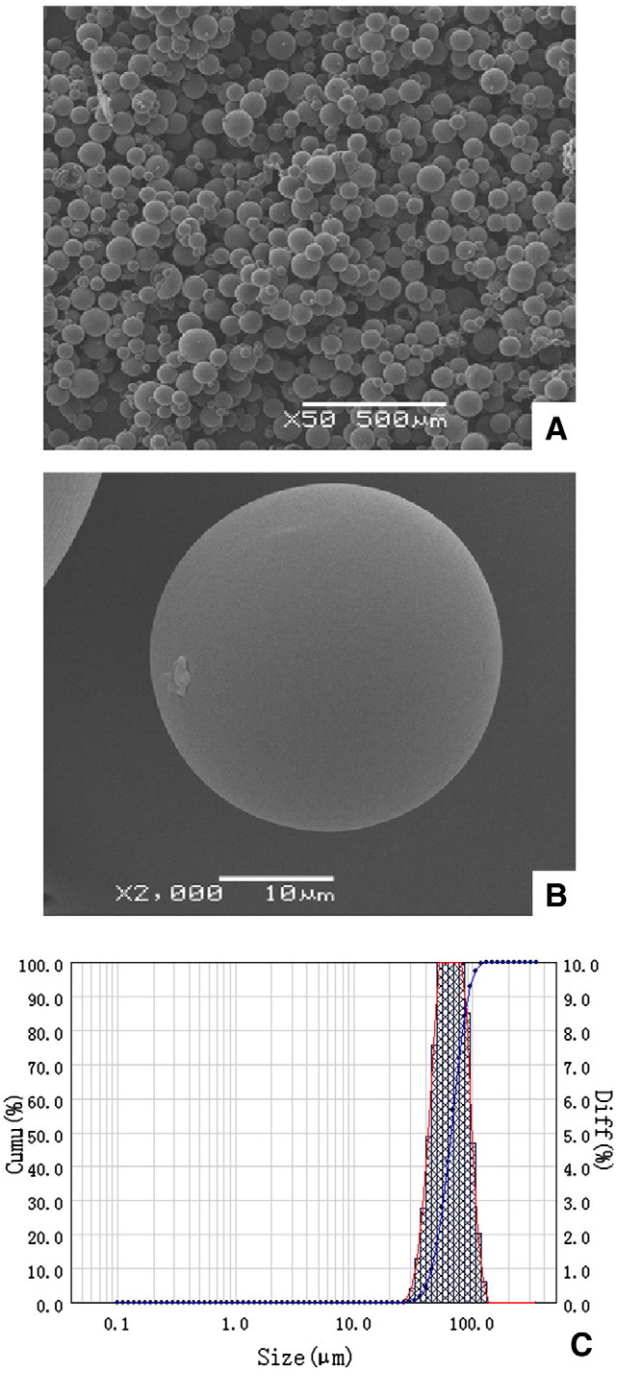

Fig. 1. Scanning electron microscopy photograph ((A) magnification of 50, and (B) of 2000 and particle size distribution (C) of GE and EE loaded PLGA microspheres.

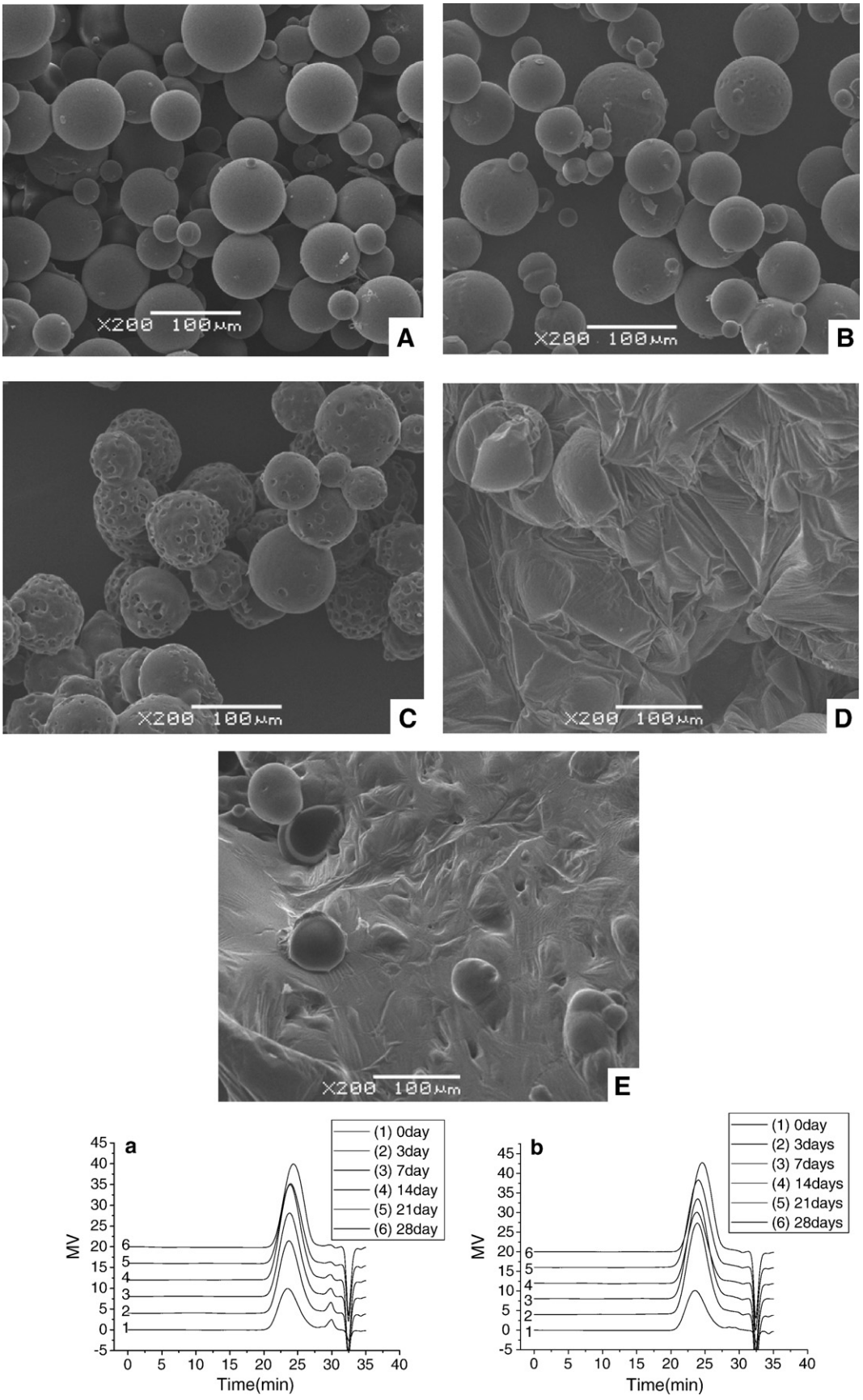

Fig. 2. Scanning electron micrographs (SEM (A: 0 day; B: 3 days; D: 14 days; E: 28 days) and gel permeation chromatography (GPC) chromatographs (a: GE and EE loaded MS; b: empty MS) of GE and EE loaded PLGA microspheres are different times of the degradation process in phosphate buffer pH 7.4 at 37 °C.

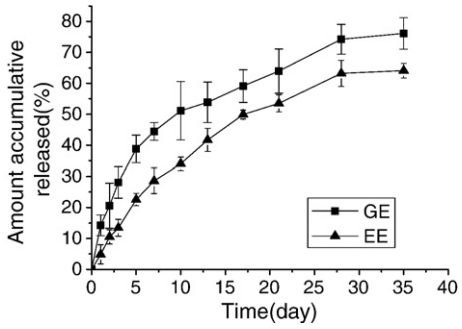

Fig. 3. In vitro cumulative drug release from GE and EE loaded PLGA microspheres in 0.1 MPBS (pH 7.4) at 37 C (n=3).

dansylated EE, m/z 530→171; methylnorethindrone, m/z 313→109, with dwell time of 50 ms.

2.4.3. Pharmacokinetics analysis

The plasma concentration-time data were analyzed with non-compartmental model by WinNonlin (Pharmasight, USA) to obtain the maximum plasma drug concentration (C_{max}), terminal elimination rate constant (k_e), terminal half-life ($t_{1/2}$), area under the plasma concentration-time curve from time 0 to last point (AUC_{0-t}) or infinity ($AUC_{0-\infty}$) and mean residence time up to last point (MRT_{0-t}). The correlation between the drug released (%) in vitro in PBS at 37 °C and the drug absorbed in vivo (F_a) was examined. The F_a was determined using the Wagner–Nelson method by the following equation: $F_a=(C_t/k_e+AUC_{0-t})/AUC_{0-\infty}$ [29]. The values of correlation coefficient (R^2), slope and intercept were calculated, respectively.

3. Results and discussion

3.1. Characteristics of PLGA microspheres

3.1.1. Morphology, particle size and GPC

The surface morphology of the microspheres was examined visually by scanning electron microscopy. Photomicrographs of PLGA microspheres loaded with GE and EE produced by solvent evaporation method were shown in Fig. 1A and B. It was observed that the drug-loaded microspheres were spherical in shape with a smooth surface. It shows that the microspheres were well sphere shaped, homogeneous, and no crystalline of drugs and fragment of polymer adhered. Generally, the injected microspheres should easily go through the syringe, so the particle size should be less than 250 μm, and it will be preferable if less than 125 μm.

As was seen from Fig. 1C, the particles of PLGA microsphers were mainly distributed around 60 μm, with the average value of 65.62± 4.56 μm. The results demonstrated that the particle size of the prepared system was uniform and appropriate for administration to rat via subcutaneous injection.

The GPC chromatogram of microspheres with or without drugs is illustrated in Fig. 2a (1) and b (1), respectively. The molecular weight of the microspheres is 6334 for the drug-loaded PLGA microspheres, and 6376 for the empty microspheres, indicating little impact resulted from the drug loading. The retention time for PLGA was 23 min, with no difference between the drug-loaded microspheres and the empty microspheres, showing that the encapsulation of drug is a physical process, and no chemical binding occurred. Complete elution of all components was within 35 min. There was another peak at about 30 min in the chromatogram, which was considered as the peak of drugs.

Table 1
Reduction in weight of microspheres with time as a result of polymer degradation (n=3)

Time (day)	Weight remained (%) (mean±SD)	
	GE and EE loaded MS	Empty MS
0	100	100
1	96.87±3.25	90.89±6.45
3	92.05±0.54	91.81±0.51
7	90.50±3.83	87.95±5.76
14	89.33±5.98	88.76±1.37
28	90.05±1.80	85.81±5.57

Table 2
Number-average molecular weight (Mn), weight-average molecular weight (Mw), polydispersity indices (PI) and evolution of the ratio of number-average molecular weight at different times of the degradation process (M_n) and the initial number-average molecular weight (M_n) of GE and EE loaded and empty PLGA microspheres after different time of incubation in phosphate buffer 0.1 M, pH 7.4 at 37 °C

	Time (days)	Mn	Mw	Polydispersity	M_n^1/M_n^0
GE and EE microspheres	0	4528	6334	1.399	1.00
	3	4391	6107	1.391	0.97
	7	4311	5965	1.384	0.95
	14	4247	5841	1.375	0.94
	21	4227	5679	1.343	0.93
	28	3795	4898	1.291	0.84
Empty microsphere	0	4787	6376	1.332	1.00
	3	4415	5914	1.339	0.92
	7	4343	5882	1.354	0.91
	14	4380	5953	1.359	0.91
	21	4172	5602	1.343	0.87
	28	3550	4565	1.286	0.74

3.1.2. Drug loading efficiency

The loading percentage of GE and EE determined from three batches was estimated to be 69.9±6.6% and 60.5±1.5%, respectively, with satisfactory reproducibility. The unencapsulated drugs were washed off in the preparation. The loading efficiency of the drugs in microspheres could be influenced by the molecular weight of PLGA, the preparation method and some other factors [30,31], including drug/polymer ratio, and incorporation of adjuvants in polymeric systems, solvent evaporation time, stirring speed and so on [32–34]. D. F. Chu reported that the encapsulation efficiency of huperzine A was influenced significantly by the molecular weight of PLGA [35].

3.1.3. In vitro drug release

Fig. 3 shows the in vitro release profiles of GE and EE from PLGA microspheres at the previously defined time. Relatively higher release rates were observed in the initial 5 days, which might be caused by the residual drugs on the surface of microspheres and possible minute cracks or porous channels in the microspheres formed during lyophilization. Then the release became slower after 10 days. From 10 to 35 days, the drug release rates were relatively constant, suggesting that

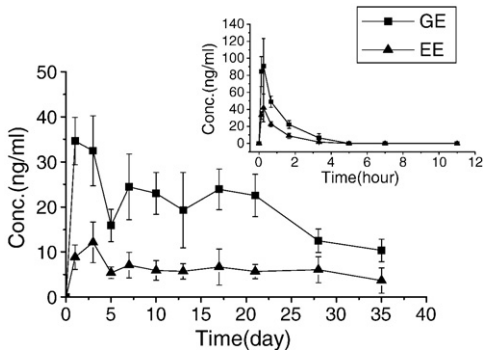

Fig. 4. Plasma concentrations vs. time (day) curves for GE and EE after s.c. administrations of the microspheres in rats. The insert shows the plasma concentrations vs. time (hour) curves for GE and EE after s.c. administration of the suspension in rats. Each point represents mean±S.D. (n=6).

Table 3
Pharmacokinetic parameters of GE and EE after s.c. administrations of the microspheres and the suspension in six rats (mean±S.D.)

Parameter	Administrations of microspheres		Administrations of suspension	
	GE	EE	GE	EE
C_{max}	34.74±5.40 ng/ml	12.20±5.01 ng/ml	95.70±30.03 ng/ml	42.86±15.59 ng/ml
k_e	0.05±0.01 d^{-1}	0.05±0.01 d^{-1}	0.85±0.24 h^{-1}	1.05±0.20 h^{-1}
$t_{1/2}$	18.15±4.56 d	15.50±3.56 d	0.88±0.27 h	0.68±0.15 h
AUC_{0-t}	689.72±114.20 ng·d/ml	216.91±87.83 ng·d/ml	100.77±16.91 ng·h/ml	44.40±10.44 ng·h/ml
$AUC_{0-\infty}$	956.18±198.75 ng·d/ml	303.31±162.67 ng·d/ml	111.96±22.08 ng·h/ml	46.08±10.80 ng·h/ml
MRT_{0-t}	28.03±3.88 d	25.78±6.21 d	1.21±0.33 h	0.98±0.16 h

the entrapped drugs began to release. About 76% of GE and 63% of EE released at the end of 35 days. Little drug release was noticed after 1 month. One of the important points is that the release rates of the two drugs from microspheres were similar, which was probably attributed to the similar physicochemical property of GE and EE, for instance, the similar hydrophobicity, MW (310.44 and 296.41 for GE and EE), melting point (196–202 °C and 181–185 °C for GE and EE) etc.

The in vitro release data were analyzed with Higuchi (GE: $Q=14.312t^{1/2}$ ($R^2=0.9491$); EE: $Q=11.209t^{1/2}$ ($R^2=0.9650$)), first-order (GE: $\log(Q_\infty-Q)=-0.0471t-0.1332$ ($R^2=0.9087$); EE: $\log(Q_\infty-Q)=-0.0572t-0.0566$ ($R^2=0.8758$)) or Weibull (GE: $\ln(\ln 1/(1-F(t))=0.6258\ln t-1.826$ ($R^2=0.9921$); EE: $\ln(\ln 1/(1-F(t))=0.8552\ln t-2.8501$ ($R^2=0.9914$)) equation, respectively. As a result, it was best fitted by the Weibull model because of the higher R^2 value (>0.99) of GE and EE.

The drug release from PLGA microspheres depended on various factors, including polymer composition, polymer molecular weight, percentage of drug loading, particle size, porosity the microstructure of microspheres [36–38], as well as the characteristics of the drug loaded. To a large extent, the prolonged GE and EE release from the prepared PLGA particles may be attributed to the hydrophobic property of the drugs and the polymer [25,39].

Two possible mechanisms may be involved: the dissolution/diffusion of drug from the matrices, and the matrix erosion resulting from degradation/dissolution of PLGA. Since the degradation of PLGA used takes quite long time, much longer than the release time (35 days) in this study [40,41], the polymer backbone may retain its integrity without significant degradation/dissolution, which has been proved in the following test. As a result, drug was supposed to release from the microspheres mainly by the diffusion mechanism.

As was shown in Fig. 3, the release was not completed at the end of 35 days. This situation could probably be improved in vivo since the polymer degradation might be accelerated and it might be the main cause for the drug release. As a matter of fact, the molecular weight of PLGA used in this study is rather small. It was reported by Dhanaraju et al. [25] that about 85% of levonorgestrel and EE released from the microspheres during a 105-day test. Compared between our observation and that reported by Dhanaraju et al., PLGA with lower MW (12,000) used in our test led to 35-day release, while PLGA with larger MW (70,000) resulted in 105-day release.

A month long in vitro release test is not acceptable for the subsequent quality control of the final products [42,43]. It is possible to reduce the in vitro release time by several strategies, such as addition of surfactant to the release medium, use of co-solvent, increase of the stirring speed, etc. But it should be noticed that the conditions might change the characteristics of the dosage forms [42], and result in a poor in vitro and in vivo correlation (IVIVC).

3.1.4. In vitro degradation of the microspheres

The weight loss of the microspheres in phosphate buffer (0.1 M, pH 7.4) at 37 °C for 28 days was listed in Table 1. As indicated in the table, there were some changes in weight loss for PLGA microspheres with or without drugs during the test period. Weight loss for empty particles was greater than that of drug-loaded particles, suggesting that the degradation might be related to the composition of the microspheres. But the weight loss at different time were also analyzed through *t* test, as a result, all the values of *p* were more than 0.3, so it was confirmed that there was no significant difference between these dosages.

The morphology of PLGA microspheres loaded with two drugs in phosphate buffer (pH 7.4, 37 °C) was observed with SEM at different times. As shown in Fig. 2, the drug-loaded microspheres were observed to be smooth in surface at day 0 (Fig. 2A) and became porous at the end of day 7 (Fig. 2C). Up to 4 weeks, most of microspheres could not keep their shapes (Fig. 2D, E), possibly due to the adherence and coalesce under the test conditions. According to the studies from the weight loss and the average molecular weight, the degradation may not be the main cause for the morphological changes of drug-loaded PLGA microspheres.

Fig. 2a and 2b shows the GPC chromatogram of drug-loaded and empty microspheres at different period in phosphate buffer (pH 7.4) at 37 °C. It was observed that the retention time had changed somehow, especially from 21 to 28 days, which indicated that the average molecular weight of the polymer had altered. The degradation behavior of the microspheres was also studied by determining the average molecular weight of the polymer at different period (Table 2). All the molecular weights were found to decrease with time. The average molecular weight of the drug-loaded microspheres prepared with PLGA 50/50 reduced by 16% and that of empty microspheres decreased by 26% during the 28 days. The ratio of polymer molecular weight at time *t* (Mn^t) and the initial (Mn^0) (Table 2) suggested polymer degradation. The result indicated that the capability of degradation of PLGA microspheres with or without drugs were relatively low, considering the designed dose regime of 1-month for this formulation. Of course, the degradation in vivo should be much faster.

3.2. In vivo studies

The plasma concentration of GE and EE-time profiles after subcutaneous injection of drug-loaded microspheres and drug suspension were presented in Fig. 4. After subcutaneous injection of the drug suspensions, drug concentrations reached their peaks within half hour, then decreased rapidly and got to bottom at about 5 h, suggested

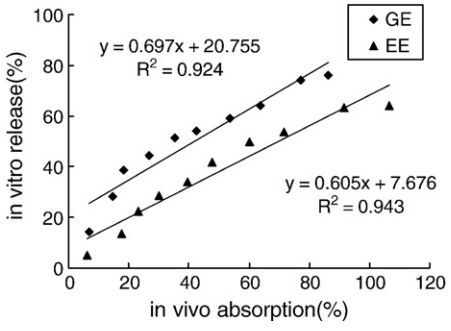

Fig. 5. Linear regression plots of cumulative absorption vs. percent dissolution of GE and EE from PLGA microspheres.

that the elimination of GE and EE were very fast. The peak value for GE and EE was about 90 ng/ml and 40 ng/ml, repectively.

On the contrary, the profiles for drug-loaded microspheres were rather smooth. The concentrations reached their maximum values within the first 5 days, specifically 34.62 ng/ml at day 1 for GE and 12.16 ng/ml at day 3 for EE, respectively. The relative steady state concentrations for both GE and EE reached from day 7 to day 35. It was also demonstrated that the tendency of concentrations change for both drugs was quite synchronic, although the drug levels for the two components were different due to the different doses. It indicated a synchronic release of both contraceptives in vivo.

The pharmacokinetic parameters are listed in Table 3. Obviously, much higher values in AUC, $t_{1/2}$ and MRT, as well as much lower values in C_{max} and k_e were obtained for the drug-loaded PLGA microspheres, compared with those for drug suspension.

This is the first time that a highly sensitive bioanalytical method used for the simultaneous determination of GE and EE in rat plasma was developed and validated using liquid–liquid extraction, derivatization and tandem mass spectrometric detection. The lowest limit of quantitation was 2.65 ng/ml for GE and 0.53 ng/ml for EE, using 0.20 ml of plasma sample. The use of chemical derivatization of EE with dansyl chloride significantly enhanced the detection sensitivity of the analyte in electrospray ionization. This validated method might be applied for the bioavailability in clinical pharmacokinetic and drug–drug interaction studies for these drugs. The results of our research indicated that GE and EE loaded PLGA microspheres were potentially effective in rats so far, briefly, compared with some reported research work [44], the C_{max} and $t_{1/2}$ were much higher or longer than that of GE implants administrated in rats. Of course, much more experiments and studies are required before this system could be proved to be effective in human.

3.3. The in vitro and in vivo correlation (IVIVC)

The linear regression plots of drug released (%) vs. F_a for the PLGA microspheres loaded with GE and EE were shown in Fig. 5 and the relative regression equations were also inserted. The good linear regression correlation was demonstrated between the percentage of drug released in PBS at 37 °C and the percentage of drug absorbed in rats of two drugs for the microspheres (GE: $R^2=0.924$, $p<0.01$; EE: $R^2=0.943$, $p<0.01$). Based on this result, it seems to be reasonable to predict the drug absorption in vivo through the release test in vitro.

The in vitro and in vivo correlation is always a crucial issue for the dosage forms in which the drug needs to be absorbed into circulation, since it is important for both formulation development and quality control. There are a large number of literatures related to the IVIVC of oral dosage forms [42,45,46], while only a few reports regarding that of s.c. injections [42,47]. It is unlikely to use the methods for oral formulation in the in vitro dissolution test of the controlled release system for s.c administration, due to the difference in milieu in vivo and the test duration. Therefore, it will be beneficial to develop an in vitro release process that will enable the prediction of the in vivo absorption of a subcutaneous injection. The data presented above suggested that the process with shake flask was acceptable in the IVIVC study for subcutaneous microsphere formulation.

4. Conclusion

Two kinds of hormonal contraceptives, GE and EE were successfully formulated in PLGA microspheres using an oil-in-water (o/w) solvent evaporation method in this study. It was proved that the preparation process was reproducible, while the PLGA particles obtained were spherical in morphology and appropriate in size. About 16% of degradation was determined for the drug-loaded microspheres according to the molecular weight during the test period of 28 days. A steady drug release from the microspheres for both drugs was observed in vitro, sustained for about 30 days and well described by Weibull model. Similarly, the relatively smooth profiles of the plasma drug concentration versus time after subcutaneous injection of drug-loaded microspheres to rats were obtained within the study period of around 1 month. Finally the good IVIVC was established. A very important fact indicated in this study is the synchronicity of the two components both in the in vitro release behavior and in vivo plasma level. Therefore, the present work has proved the feasibility of synchronic delivery of GE and EE for about 1 month by utilizing the injectable PLGA-based microspheres.

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