International Journal of Pharmaceutics 560 (2019) 27–34

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Entecavir-loaded poly (lactic-co-glycolic acid) microspheres for long-term therapy of chronic hepatitis-B: Preparation and *in vitro* and *in vivo* evaluation

Chunyan Zhang[a,b], Aiping Wang[a,c], Huiyun Wang[b], Maocai Yan[b], Rongcai Liang[a,c], Xiuting He[a], Fenghua Fu[a,c], Hongjie Mu[a], Kaoxiang Sun[a,*]

[a] School of Pharmacy, Collaborative Innovation Center of Advanced Drug Delivery System and Biotech Drugs in Universities of Shandong, Key Laboratory of Molecular Pharmacology and Drug Evaluation (Yantai University), Ministry of Education, Yantai University, Yantai, Shandong Province, People's Republic of China
[b] School of Pharmacy, Jining Medical University, Jining, Shandong Province, People's Republic of China
[c] State Key Laboratory of Long-Acting and Targeting Drug Delivery System, Shandong Luye Pharmaceutical Co., Ltd, Yantai, Shandong Province, People's Republic of China

ARTICLE INFO

Keywords:
Entecavir
PLGA
Biodegradable microsphere
Extended release
Chronic hepatitis-B

ABSTRACT

To avoid severe exacerbations in the load of hepatitis B virus (HBV) as a consequence of discontinuous use of anti-HBV drugs, entecavir (ETV), the first-line anti-HBV drug, was primally formulated as extended-release poly (lactic-co-glycolic acid) microspheres in the present study. Because ETV is slightly soluble in water and in some other organic solvents used for microsphere preparation, methods for solid-microencapsulation were employed to fabricate the ETV microspheres. The optimized microspheres were evaluated for their morphology, particle size, drug loading, *in vitro* drug release, and *in vivo* pharmacokinetics in rats. The optimized formulation was found to have a mean particle size of 86 μm and drug loading of 13%. Differential scanning calorimetry and powder X-ray diffraction indicated that ETV existed in crystal, amorphous, and molecular states in the microspheres. *In vitro* and *in vivo* release revealed that the dissolution of ETV dominated the release process. The morphology of the microspheres and changes in the morphology during *in vitro* release were assessed by scanning electron microscopy. The novel ETV-MS described in this study should have great potential for clinical use as an alternative treatment against HBV.

1. Introduction

Chronic hepatitis B virus (HBV) infection is estimated to affect 240 million people worldwide (Balogh et al., 2016). The number of new cases of HBV infection have plummeted since a vaccine against it has become available. However, some regions with a very high incidence of HBV still do exist (Pazgan et al., 2018). With the therapies in use presently, the viral load of HBV can be controlled at low levels but it is difficult to completely remove the virus from the body; thus, hepatitis B needs a prolonged treatment (Wang, 2014).

Entecavir (ETV) is one of the first-line anti-HBV drugs with a strong anti-HBV ability and a low drug tolerance (less than 1.2%, even after 5-years administration) compared to the existing drugs, such as adefovir and lamivudine, which show 18% and 66% increase in drug tolerance, respectively (Sacco, 2014). However, it is worth noting that anti-HBV drugs must be taken regularly for at least 2–3 years until requirements for the termination of treatment are met. Severe cases of acute exacerbation of hepatitis B have been reported in patients who had discontinued the anti-hepatitis B therapy, including treatment with ETV (Sarikaya et al., 2012; Yoon et al., 2013). Food intake has an obvious effect on the absorption of ETV from dispersible tablets (Zhang et al., 2010); taking ETV along with food results in a slight delay in drug absorption (from 0.75 h to 1–1.5 h), and a decrease in Cmax by 4.4%, as well as in the area under concentration–time curve (AUC) by 18% to 20%. To enhance the efficacy of ETV, it should be taken on an empty stomach every day; however, this aggravates adverse reactions, such as nausea, vomiting, abdominal pain, diarrhea, abdominal discomfort, and dyspepsia, resulting in poor patient compliance and poor therapeutic effect. Therefore, development of a sustained drug delivery system is necessary for the treatment of hepatitis B.

In recent years, there have been some reports on long-acting formulations of anti-HBV drugs. Adefovir, an important anti-HBV drug, has been microencapsulated into microspheres for long-term therapy of chronic hepatitis-B (Ayoub et al., 2018). Entecavir was designed as a novel lipidic prodrug to provide prolonged pharmacokinetic profile over 4 weeks, following parenteral administration (Ho et al., 2018). An injectable liquid crystal system for sustained delivery of ETV was also reported, which showed sustained release for 2 weeks in beagle dogs

* Corresponding author at: School of Pharmacy, Yantai University, No 30 Qingquan road, Yantai 264005, Shandong Province, People's Republic of China.
E-mail address: sunkx@ytu.edu.cn (K. Sun).

https://doi.org/10.1016/j.ijpharm.2019.01.052
Received 2 November 2018; Received in revised form 7 January 2019; Accepted 23 January 2019
Available online 31 January 2019
0378-5173/

(Lim et al., 2015).

The aim of this study was to develop ETV- poly(lactic-co-glycolic acid) (PLGA) microsphere for sustained release of ETV and long-term therapy of chronic hepatitis-B. Injectable PLGA microspheres have attracted a lot of attention and have found a wide range of applications owing to their biodegradability and biocompatibility, as well as because of their ability to deliver drugs in a controlled manner over periods of days to several months (Chu et al., 2018; Mao et al., 2012; Meng et al., 2018; Mitragotri et al., 2014). Herein, we prepared extended-release ETV-loaded PLGA microspheres to avoid severe acute exacerbation of hepatitis B, to reduce the chances of virus rebound and frequency of administration, and to improve the compliance of patients. Because ETV is slightly soluble in water and in some other organic solvents, solid-microencapsulation processes were employed to fabricate the ETV microspheres (ETV-MS). Factors affecting the release of microspheres, such as the process of preparation, the concentration of PLGA and emulsifier, and the ratio of ETV and PLGA, were carefully examined, and the optimized ETV-MS were evaluated for their morphology, particle size, drug loading, *in vitro* drug release, and *in vivo* pharmacokinetics in rats.

2. Materials and methods

2.1. Materials

Entecavir was kindly provided by Shandong Boyuan Pharma Company (Jinan, China). Poly(lactic-co-glycolic acid) was purchased from Lakeshore Biomaterials (Alabama, USA). Polyvinyl alcohol (PVA) (87%–89% hydrolyzed, MW 13–23 KDa) was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Dichloromethane (DCM) was purchased from Sinopharm Chemical Reagent (Hefei, China). Acetonitrile and methanol were of HPLC grade and were purchased from EMD Millipore Corporation (Darmstadt, Germany). All other reagents were of analytical grade.

Sprague Dawley rats (males; 200 ± 20 g; ~4–5 weeks-old) were provided by the Experimental Animal Center of Shandong Luye Pharmaceutical Co., Ltd. The rats were housed in steel cages in a room at 22 ± 1 °C under a standard 12 h light/dark cycle and were supplied with standard diet and water. All animal experiments were conducted in accordance with the guidelines of the Ethical Committee on Animal Experimentation of Yantai University (Yantai, China) and were in compliance with the EU Directive 2010/63/EU and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Preparation of ETV-PLGA microspheres

Three different solid encapsulation processes, namely *in situ* crystallization solid-in-oil-in-water (*in situ* S/O/W), traditional solid-in-oil-in-water emulsification solvent evaporation (S/O/W), and spray drying solid-in-oil (S/O) process, were used for the fabrication of ETV-MS.

2.2.1. The in-situ S/O/W method

The ETV-loaded PLGA microspheres were prepared using an *in situ* S/O/W method as described previously (Bao et al., 2006). Briefly, 2 g PLGA was dissolved in 10 mL DCM. Entecavir (300 mg) was dissolved in 2 mL dimethyl sulfoxide (DMSO). The ETV-DMSO solution was dispersed in the obtained PLGA solution of DCM to form fine particles because ETV was insoluble in DCM. The *in situ*-formed suspension was then added to 100 mL of a 1% (w/v) PVA solution and homogenized at 1000 rpm for 3 min. The resultant emulsion was stirred for 4 h at room temperature to evaporate DCM. The solidified microspheres were washed with de-ionized water and were collected by filtration. The prepared microspheres were freeze-dried in vacuum and stored at 4 °C for further use.

2.2.2. The S/O/W method

The ETV-loaded PLGA microspheres were prepared using the classical S/O/W solvent evaporation method (Gu and Burgess, 2015; Su et al., 2011; Wang et al., 2012). Briefly, 2 g PLGA was dissolved in 10 mL DCM. Thereafter, 300 mg ETV was dispersed in PLGA solution and sonicated in a bath sonicator for 20 min to form a homogeneous suspension. The suspension was further mixed using a homogenizer (T25 digital ULTRA-TURRAX, IKEA) at 10,000 rpm for 1 min. This organic phase was then slowly added to 100 mL of a 1% (w/v) PVA solution and homogenized at 1000 rpm for 3 min. The subsequent steps were same as described in Section 2.2.1.

2.2.3. The S/O method

Mini Spray Dryer (Buchi B-191, Switzerland) was used to prepare ETV-MS. Poly(lactic-co-glycolic acid) (2 g) was dissolved in 40 mL DCM, and 300 mg ETV was dispersed in DCM to form a uniform suspension by stirring with a magnetic stirrer at 600 rpm. The stirred suspension was pumped into the spray dryer at a flow rate of 6 mL/min. The spray conditions were as follows: an inlet temperature of 40 °C and an air-flow rate of 600 L/h.

2.3. Determination of particle size

The size of particles was measured using a laser diffraction particle size analyzer (Mastersizer 3000, Malvern, UK). About 200 mg ETV-MS was suspended in 0.1% Tween-20 solution at a stirring speed of 2100 rpm to prevent aggregation (Jiang et al., 2018). All measurements were made in triplicate and the results are reported as average diameter.

2.4. Scanning electron microscopy

The surface morphology and shape of microspheres were examined using a scanning electron microscope (SEM, EM-30PLUS, Coxem, Korea). Double-sided carbon tape was affixed on the aluminum stubs. Auto fine-coater was used to make a 2 nm thick layer above the sprinkled powder. These stubs were placed in the vacuum chamber of SEM and the microspheres were observed using a gaseous secondary electron detector (acceleration voltage: 20 kV) under different magnifications.

2.5. High performance liquid chromatography (HPLC)

The quantification of ETV was done using an HPLC system (Agilent 1260) equipped with a UV absorbance detector set at 254 nm. An ODS (4.6 mm × 25 cm, 5 µm) analytical column was used. The mobile phase consisted of methanol/water (20/80, v/v) and was used at a flow rate of 1 mL/min. The injection volume was kept at 100 µL. The retention time of ETV was found to be 5 min. The peak areas of ETV were recorded and the concentrations were calculated based on a standard calibration curve. The calibration curve showed good linearity over a concentration range of 1.04–104 µg/mL and the recovery was between 98.83% and 99.92%, with the relative standard deviation being less than 1.0%.

2.6. Drug loading and encapsulation efficiency

The amount of ETV encapsulated in the PLGA microspheres was determined by HPLC. Briefly, 10 mg of ETV-loaded PLGA microspheres were dissolved completely in 2 mL DCM. The mobile phase (30 mL) was added to extract the encapsulated ETV. This dispersion was centrifuged for 5 min at 1500 × *g* in a high-speed centrifuge (H1650, Xiangyi Laboratory Instruments, Changsha, China) and the supernatant was injected into the HPLC system to determine the concentration of ETV. The drug loading (DL) and encapsulation efficiency (EE) were determined as follows:

DL (%) = (amount of drug in microspheres/amount of

microspheres) × 100

EE (%) = (Actual drug loading/Theoretical drug loading) × 100

2.7. Differential scanning calorimetry (DSC)

The thermal characterization of ETV, PLGA, physical mixture, and freeze-dried ETV-MS was done using a differential scanning calorimeter (METTLER, DSC822^e) and thermograms were recorded. Briefly, about 2.5 mg of samples were weighed in an aluminum pan and sealed with an aluminum lid. The empty sealed aluminum pan was used as a reference. The DSC analysis was performed at 10 °C/min from 25 °C to 280 °C under an atmosphere of nitrogen (20 mL/min).

2.8. Powder X-ray diffraction (PXRD)

The powder X-ray diffraction (PXRD) analysis of ETV, PLGA, blank microspheres, and ETV-MS was performed using an X-ray diffractometer (D-MAX2500, Japan). The X-ray used was 40 kV/150 mA. The scanning range was 10–80° and the scanning rate was 2°/min.

2.9. Residual solvent analysis

The residual level of methylene chloride in the microspheres was determined by an Agilent 6890 N gas chromatograph (GC) equipped with a flame ionized detector (FID) and a DB-624 capillary column (30 mm × 0.32 mm, 1.8 µm). Dimethylsulfoxide was used as a solvent to dissolve the microspheres, and 1 µL of this solution was injected into the GC for analysis.

2.10. In vitro release of ETV

The *in vitro* release profile of ETV from PLGA microspheres was determined as follows: 30 mg of ETV-loaded PLGA microspheres were suspended in 30 mL of phosphate-buffered saline (PBS, pH 7.4, 0.1 M) containing 0.02% Tween-80 (Han et al., 2010). The microsphere suspension was shaken in a horizontal plane at 50 rpm in a shaking water bath maintained at 37 °C (HZS-HA, Harbin, China). At predetermined time intervals, the samples were centrifuged at 1500 × *g* for 10 min; 15 mL supernatant was withdrawn, and the sediment of microspheres was re-dispersed in an equal amount of fresh PBS. The supernatant was filtered through a 0.45 µm filter (MillexGV, Millipore, USA) and the ETV concentration in the filtrate was determined by HPLC as described above. All measurements were made in triplicate and the results were expressed as means ± SD.

2.11. In vivo pharmacokinetic study

Eighteen rats were randomly divided into three groups of six rats each. The rats in each group received ETV-physiological saline solution, ETV-MS (prepared with PLGA 6535 4A and 8515 5A, respectively) dispersed in 0.87% carboxymethylcellulose sodium at a single dose of 1.5 mg/kg by intramuscular injection. The blood samples were obtained via the orbital venous plexus and collected in heparinized tubes immediately prior to dosing and at 1 and 6 h, and 1, 2, 4, 6, 8, 10, 13, 16, 20, 23, 26, 29, 32, 35, 38, and 42 d for the ETV-MS groups after administration. All the blood samples were centrifuged immediately at 1500 × *g* for 5 min and the plasma samples were obtained and stored at −80 °C until the analysis.

The concentration of ETV in the plasma was determined by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS, AB Sciex API 4500 triple-quadrupole mass spectrometer, USA; SHIMADZU LC-30A High Performance Liquid Chromatograph, Japan). The separation of ETV was achieved using a Waters ACQUITY UPLC BEH Amide column (75 × 2.1 mm, 1.7 µm, Agilent, USA) with a mobile phase consisting of acetonitrile:0.2% formic acid (90:10, v/v). The flow rate was 300 µL/min. The mass spectrometer was operated using an electrospray ionization source with positive ion detection in multiple reaction monitoring mode. The ion spray voltage was 5500 V, ion transitions were *m/z* 278.1 → *m/z* 152.0 for ETV and *m/z* 266.1 → *m/z* 152.0 for the internal standard (lobucavir). The declustering potential was 77 and 77 V and collision energy was 27 and 28 V for ETV and the internal standard, respectively. Data were processed using a statistical software DAS v. 2.0 (Mathematical Pharmacology Professional Committee of China, China), using a two compartment model.

2.12. Analysis of the pharmacokinetic data

The plasma concentration–time data were analyzed with compartmental model with the DAS 2.1 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China) to obtain the pharmacokinetic parameters, including area under the plasma concentration–time curve (AUC), half-life ($T_{1/2}$), and mean residence time (MRT). The maximum plasma drug concentration (Cmax) and the time required to reach Cmax (t_{max}) were directly read from the plasma concentration–time data.

2.13. In vitro–in vivo correlation (IVIVC) analysis

The IVIVC analysis of the ETV-loaded PLGA microspheres was done by plotting the percent drug absorbed *in vivo* versus percent drug released *in vitro*. The percent drug released *in vitro* was obtained from the *in vitro* release data, and the trapezoidal method (Shen, 2015) was employed to calculate the fraction of drug absorbed *in vivo* from the plasma concentration data. A linear regression analysis was applied to fit the IVIVC plot and the coefficient of determination (R^2) was calculated to evaluate the IVIVC.

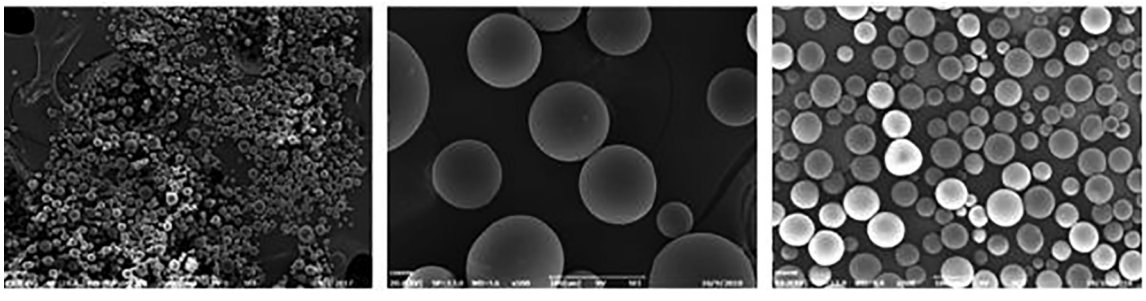

Fig. 1. Scanning electron microscope (SEM) microphotographs of ETV-MS prepared by spray drying S/O (a:2000x), in-situ S/O/W (b:500x), S/O/W (c:200x).

Table 1
Characteristics of ETV-MS.

Batch	Process	PLGA type[a]	Polymer inherent viscosity (dL/g)	ADL[b] (%)	EE[c] (%)	D (50) (μm)
1[d]	Spray drying S/O	5050 (A)	0.20	10.9	84.6	5.4
2	in-situ S/O/W	5050 (A)	0.20	3.0	24.0	74.5
3[d]	S/O/W	5050 (A)	0.20	8.0	62.9	88.0
4[d]	S/O/W	5050 (A)	0.40	10.2	77.8	84.0
5[d, e]	S/O/W	6535 (A)	0.40	11.8	90.7	83.0
6[d, e]	S/O/W	8515 (A)	0.50	13.0	99.5	86.0
7	S/O/W	5050 (E)	0.20	8.1	63.5	83.6
8	S/O/W	5050 (E)	0.60	11.3	86.8	85.0

[a] A: carboxylic group end-capped PLGA; E: ester end-capped PLGA.
[b] Actual drug loading.
[c] Encapsulation efficiency.
[d] This batch was chosen for the *in vitro* studies.
[e] This batch was chosen for the *in vivo* studies.

3. Results and discussion

3.1. Preparation of ETV-PLGA microspheres

The SEM images of ETV-MS obtained from three different methods are shown in Fig. 1. It is evident from the figures that ETV-MS were successfully fabricated and all of them were spherical and had smooth surfaces (Fig. 1a–c). The values of DL, EE, and particle size distribution of ETV-MS obtained using the different methods are shown in Table 1. It is clear from the data that DL, EE, and particle sizes were greatly influenced by the process of preparation.

Dimethylsulfoxide is a solvent with low toxicity potential and the solubility of ETV in DMSO at room temperature is 16.7 g/100 mL. We designed a novel precipitated solvent evaporation process—the in-situ S/O/W process—in which ETV was dissolved in DMSO and dispersed in DCM to form fine particles; the in-situ formed suspension was then added to the outer aqueous phase and was emulsified. According to Bao et al. (2006), the dissolution of low molecular weight proteins in DMSO and their dispersion in DCM can produce fine particles, and DL and EE as high as 15% and 90% can be obtained when the volume of DCM is great than or equal to that of DMSO. In our study, the ratio of the volumes of DCM and DMSO was 40:3, which is far greater than 1, but the values of DL and EE achieved were only 3% and 24%. The reason could be that, when the ETV suspension was added into the outer aqueous phase, ETV dissolved in DMSO partitioned into the outer phase with DMSO, because DMSO is miscible with water, leading to low DL and EE (Table 1).

Based on the low DL and EE obtained using the in-situ S/O/W method, to encapsulate solid particles of active pharmaceutical ingredients (API) directly, the spray drying S/O and S/O/W methods were used. For encapsulation of solids, diameter of the drug particle is very important in determining the DL and EE of the microspheres. For the S/O process, the prepared microspheres had a diameter of about 5 μm, and therefore, only small-sized APIs could be used. For the S/O/W process, the smaller was the particle size, the higher were the values of DL and EE that could be obtained (Fig. 2). Pneumatic jet milling was used to reduce the particle size of API; however, no change in the melting point was observed before and after pulverization of the API particles (Fig. 3e–f), which indicated that micronization did not change the crystal form of ETV.

Smaller microspheres with higher DL and EE were obtained by spray drying S/O. The results were in accordance with those obtained for celecoxib (a low solubility drug) microspheres prepared by electrohydrodynamic spraying (Bohr et al., 2011), which had a diameter in the 1–5 μm range. Owing to the smaller size of microspheres (Jiang et al., 2014), a burst rate of microspheres as high as 50% was obtained using the spray drying S/O method within 24 h (Fig. 5).

However, the microspheres prepared with the classical S/O/W process exhibited lower burst release, higher DL and EE, and longer release time. To enhance the DL as well as the EE, several formulations (shown in Table 1) as well as process parameters, such as the concentration of PLGA, ratio of ETV and PLGA, and emulsifier concentration, were carefully optimized (data not shown). It was found that 200 mg/mL of PLGA, an ETV to PLGA ratio of 1:7, and 1% w/v of PVA were suitable for the preparation of ETV-MS. Also, the uniform dispersion of ETV in DCM was also very important for the preparation of ETV-MS. In this study, bath sonicator and homogenizer were employed to break the ETV crystal into pieces so as to disperse it uniformly in DCM. The characteristics of ETV-MS prepared using the S/O/W method are detailed below.

3.2. Characteristics of ETV-MS

3.2.1. Drug loading and encapsulation efficiency

For the traditional S/O/W process, the diameter of API particles is very important for DL and EE of the microspheres. The comparison of EE values in different batches of microspheres produced by the particles before (particles with D(0.5) 20.10 μm) and after (particles with D(0.5) 2.18 μm) pulverization is presented in Fig. 2. The EE values in all the batches increased with the decrease in particle diameter. It can be concluded that smaller API particles would form microspheres with higher EE values. Therefore, pre-treatment is needed to reduce the particle size of the drug. In the present study, a spiral air pulverizer (Unique SJM-50, China) was employed for this purpose.

The comparison of the DL and EE values in batches 3, 4, 7, and 8 revealed that the EE value increased from 63% to 78% and 87% when the inherent viscosity of polymer changed from 0.20 to 0.40 and 0.60 dL/g, respectively (Table 1), i.e., it increased with the increase in inherent viscosity of the polymer. The probable reason for this could be as follows: higher viscosity leads to faster solidification of PLGA, resulting in a greater number of drug particles being encapsulated into the microspheres and a lesser number of particles flowing to the outer water phase.

3.2.2. Differential scanning calorimetry

The DSC thermograms of ETV, PLGA, mixture of ETV and PLGA, blank MS, and ETV-MS are presented in Fig. 3. Entecavir is a hydrate, that is, it contains a molecule of water bound to it, which is lost at 140 °C. The melting point of ETV is ~249–252 °C. These facts are consistent with the results represented graphically in Fig. 3. During the DSC experiment, PLGA melts with the increase in temperature; this changes the environment around the mixture, and may lead to the decrease in the melting point of ETV in the PLGA and ETV mixtures (Bozdağ et al., 2011; Bruni et al., 2010), as shown in Fig. 3b. The melting point of ETV (~235–240 °C) in the microspheres was significantly lower than that of its pure form. This suggests that the process for MS preparation could modify the crystalline characteristics of the drug. Furthermore, although ETV was added in the solid form to the

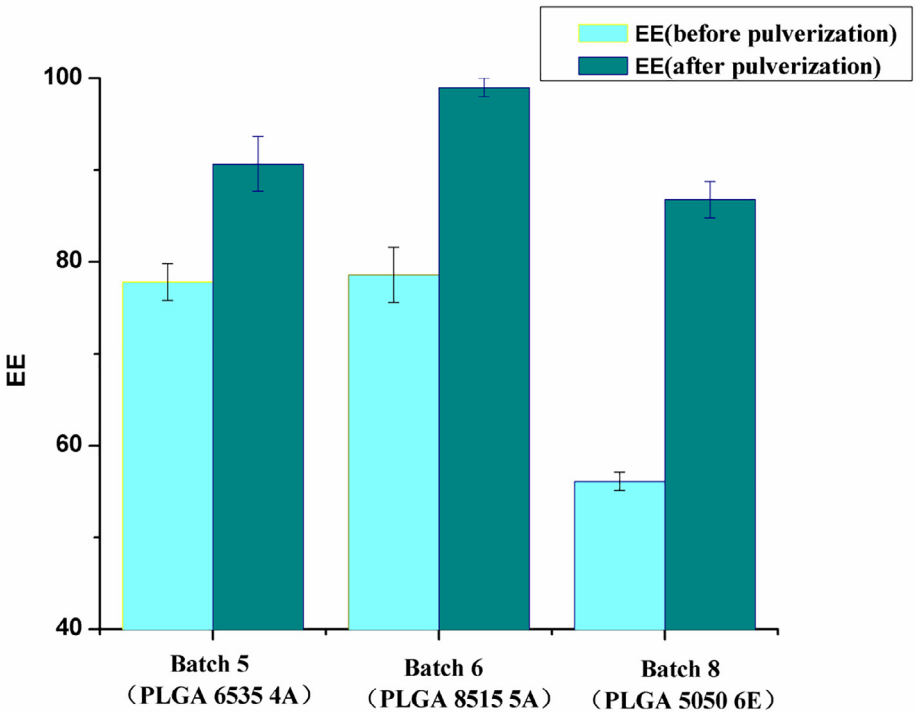

Fig. 2. Encapsulation efficiency comparison between microspheres prepared by particles before(D(0.5) 20.10 μm) and after (D(0.5) 2.18 μm) pulverization.

polymer solution (powder dispersed in DCM), it is to be expected that a fraction of ETV was dissolved in DCM and was, thus, incorporated into the MS matrix as a non-crystalline molecular dispersion during the maturation and consolidation of MS. This was also confirmed by PXRD (Fig. 4).

3.2.3. Powder X-ray diffraction

Based on the information derived from the PXRD spectrum, we can confirm whether the drug is amorphous or crystalline in the polymeric matrix. The PXRD patterns of ETV exhibited characteristic crystalline peaks at 2θ angles of 15.58 and 25.08, indicating its highly crystalline nature (Fig. 4). In contrast, the characteristic peaks for the drug were found to be significantly lowered in the microspheres, confirming that a part of ETV was either molecularly dispersed or was distributed in an amorphous form in the polymeric matrix.

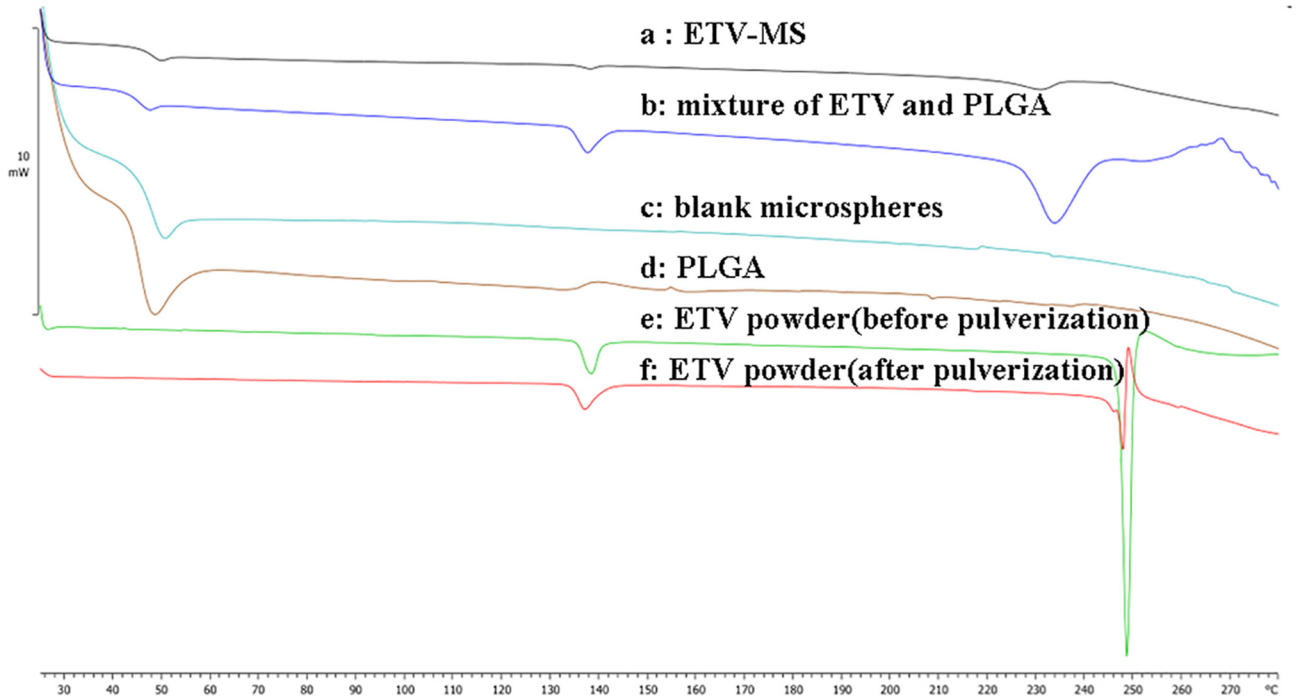

Fig. 3. Differential scanning calorimetry (DSC) microphotographs of (a) ETV-MS, (b) mixture of ETV and PLGA, (c) blank microspheres, (d) PLGA, (e) ETV powder (before pulverization), (f) ETV powder(after pulverization).

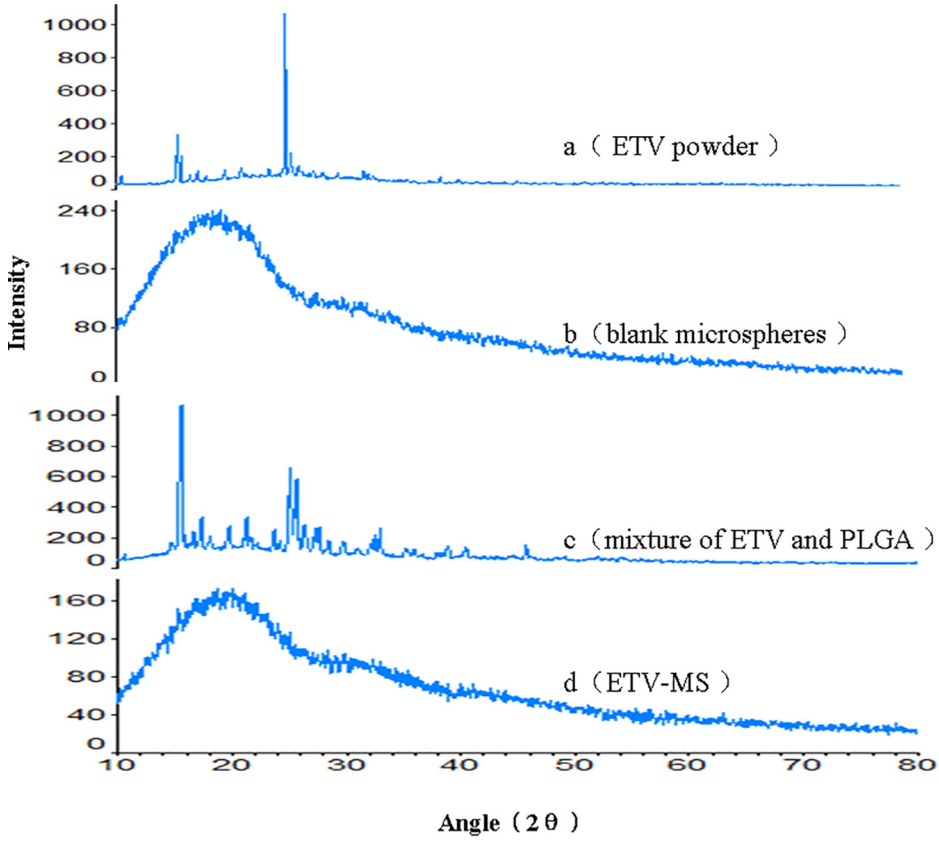

Fig. 4. Power X-ray diffraction (PXRD) spectrum of (a) ETV powder, (b) blank microspheres, (c) mixture of ETV and PLGA, (d) ETV-MS.

3.3. Determination of the content of residual solvent

Dichloromethane was used as an organic solvent for the preparation of ETV-MS. The determination of DCM in the final formulation is important because it falls under class 2 of residual solvent classification and the limit must be below 600 ppm. The residual content of solvent in the final product was found to be 33.8 ± 5.9 ppm, which confirmed the effectiveness of the stirring process in eliminating the organic

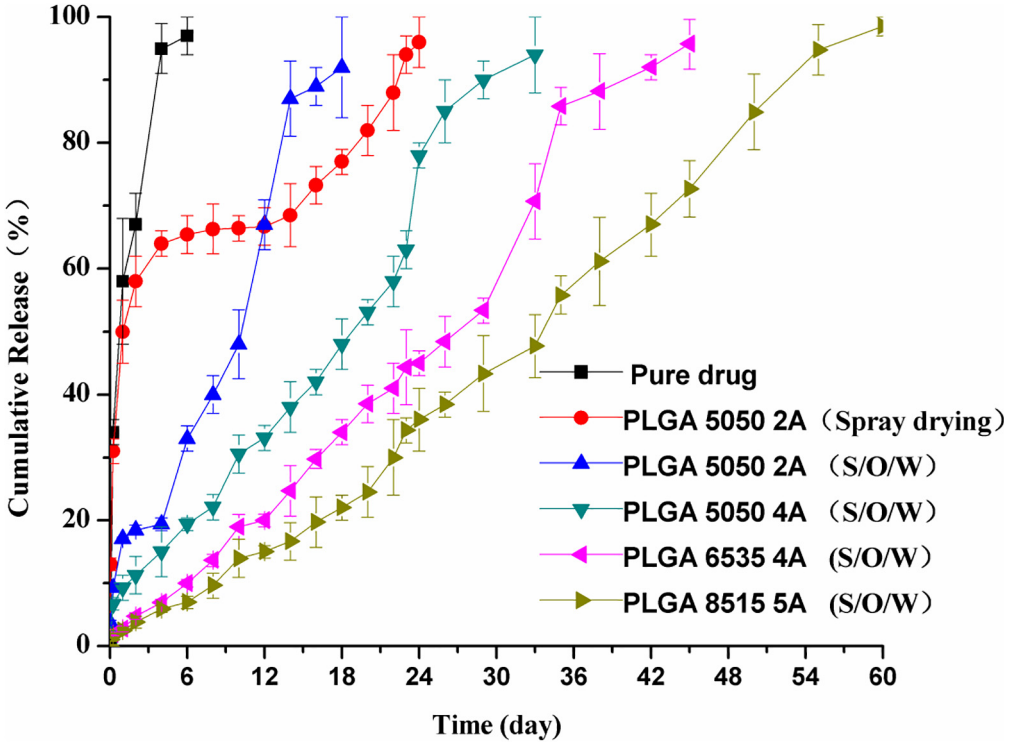

Fig. 5. *In vitro* cumulative release of pure entecavir and entecavir microspheres.

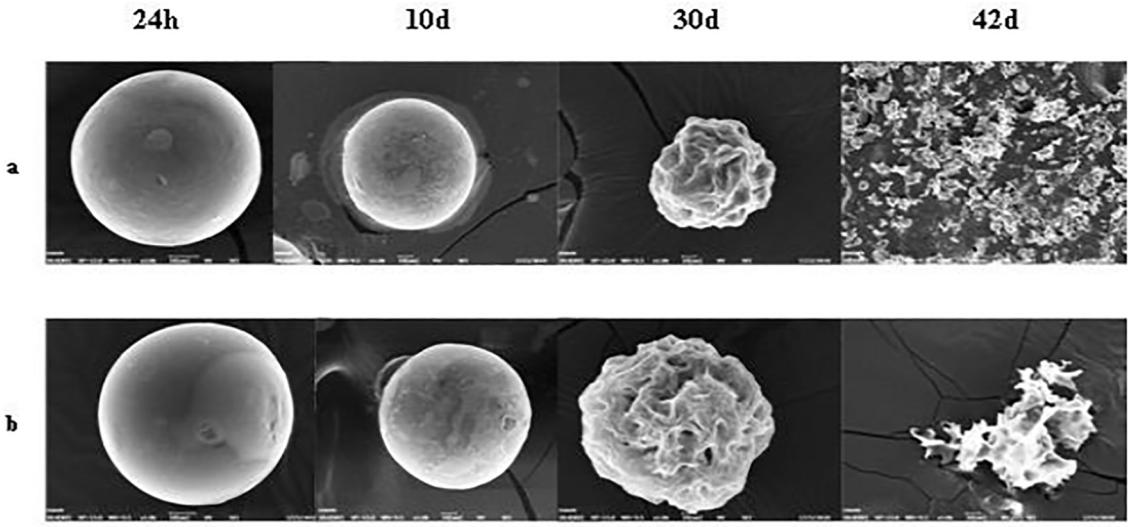

Fig. 6. SEM of entecavir microspheres prepared with different PLGA after exposing to release buffer for 24 h(2000x),10d(1000x), 30d(1000x)and 45d(1000x) (a) batch 5(PLGA 6535 4A), (b) batch 6(PLGA 8515 5A).

solvent from the microspheres.

3.4. Studies on the in vitro release of drug

The cumulative release profiles and morphological changes are shown in Figs. 5 and 6,. The release of free ETV occurred after 1 h and lasted for about 4 days owing to its slight solubility in water (Fig. 5). In contrast, a sustained release of ETV from the microspheres was observed from 16 to 60 days. Because of the low solubility of ETV, the API dissolution dominates the release behavior. The release of ETV is controlled by the dissolution and diffusion of API, and by the degradation of PLGA.

A comparison of the release curves obtained from batches 3 (PLGA 5050 2A) and 4 (PLGA 5050 4A) is shown in Fig. 5. The release from batch 4 microspheres lasted for 30 days whereas it lasted only for 16 days in the case of batch 3 microspheres. Therefore, the sustained release time was increased with the increase in the inherent viscosity of the polymer. The probable reason for this could be that with the increase in viscosity of the oil phase, the residual drug on the surface of microspheres is reduced, and the drug is more uniformly distributed in the microspheres, thereby, reducing the sudden release and extending the release time.

It is also clear from Fig. 5 that an increase in the ratio of lactide from 50 through 65 to 85 prolonged the time of the drug release as a result of the increase in hydrophobicity of PLGA. This is in accordance with the results of Zhang et al. (2014), Budhian et al. (2005) and Gabler et al. (2007) who reported that increasing the lactide content leads to slower diffusion of the drug from the polymer matrix.

To investigate the mechanism of ETV release from PLGA microspheres, the morphological changes after their exposure to the release buffer were analyzed (Fig. 6). It is obvious that the microspheres showed an almost smooth surface after exposure to the release buffer for 24 h. Only very little water was noticed to have diffused to the surface of the microspheres at 24 h. The microspheres were not deformed until day 10 and the volume of microspheres decreased while they were gradually degraded. The microspheres of batch 5 (PLGA 6535 4A) degraded completely on day 45 whereas drug fragments were still left in the microspheres of batch 6 (PLGA 8515 5A). The changes in the morphology during *in vitro* drug release were consistent with the release curve and confirmed the mechanism of *in vitro* release.

The *in vitro* release rate of microspheres prepared from small-sized APIs was slightly higher than that from large-sized APIs (data not shown), but the release rate *in vivo* was almost the same. This could be because of the fact that the *in vivo* release of ETV from the microspheres is mainly controlled by drug diffusion and polymeric erosion.

An ideal *in vitro* release method should first distinguish between different prescriptions. The *in vitro* release medium and pH value should simulate the tissue environment as much as possible. PBS buffer containing Tween 80 was usually used as the release medium. Considering that entecavir is slightly soluble in PBS, addition of Tween 80 can enhance the solubility and inhibit the aggregation of ETV microspheres The results showed that the release medium had a good discrimination, and there is a preliminary correlation between the *in vivo* and *in vitro* release.

3.5. Pharmacokinetic evaluation

Entecavir can be administered orally to patients at a dose of 0.5 mg per day; therefore, 15 mg would be needed for about a month (30 days) of treatment, which would provide 0.25 mg/kg body weight, considering the human body weight to be 60 kg. For rats, the dose was increased six times to 1.5 mg/kg after application of the human-equivalent-dose conversion factor.

The plasma concentration of ETV was monitored by LC-MS/MS to evaluate its *in vivo* pharmacokinetic behavior. The plasma concentration and pharmacokinetic parameters of ETV after intramuscular administration of a single dose of ETV-MS in rats are shown in Fig. 7 and Table 2. Interestingly, two similar concentration curves were obtained with low burst release in the first 24 h. The anti-virus activity of ETV is known to be maintained and is adequate when the concentration is over 1 ng/mL (Lim et al., 2015; Yan et al., 2013). Therefore, formulations maintaining a plasma drug concentration greater than 1 ng/mL at the last evaluated timepoint *in vivo* are considered to be optimal. Stable plasma drug levels in batches 5 (PLGA 6535 4A) and 6 (PLGA 8515 5A) were observed until day 42.

3.6. In vitro–in vivo correlation (IVIVC)

In this study, a point-to-point relationship between *in vitro* drug release and *in vivo* drug absorption was observed for ETV-loaded PLGA microspheres. A good linear relationship for the microspheres was observed between the fraction of ETV absorbed *in vivo* and the percent released *in vitro* ($y = 1.1229x - 1.113$; $R^2 = 0.9791$). Therefore, the IVIVC evaluation model could provide a good predictive value for further studies on ETV-MS.

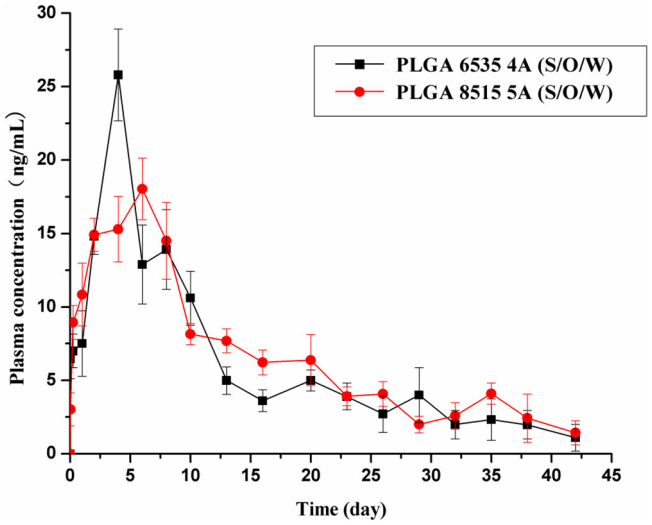

Fig. 7. Plasma concentration-time curve after a single injection of entecavir microspheres into Sprague Dawley rats (n = 6).

Table 2
Pharmacokinetic parameters after intramuscular injection of ETV-loaded PLGA microspheres(mean ± SD, n = 6).

Parameter	Batch 5	Batch 6
Tmax(h)	96.00 ± 0.00	120.00 ± 0.00
$T_{1/2}$	28.38 ± 8.66	58.04 ± 18.42
Cmax(μg/L)	25.79 ± 3.12	18.04 ± 2.11
AUC_{0-42}(μg/L * d)	382.95 ± 113.35	397.12 ± 127.68
$AUC_{0-\infty}$(μg/L * d)	417.37 ± 107.46	436.10 ± 112.07
MRT $_{0-\infty}$(d)	23.44 ± 4.16	31.86 ± 1.94

4. Conclusion

A novel ETV-loaded PLGA microsphere was primally built to provide prolonged pharmacokinetic profile, following parenteral administration. The influence of various formulations as well as of manufacturing parameters on drug loading and encapsulation efficiency, particle size, and *in vitro* and *in vivo* release were assessed. The optimized formulation was found to have a sustained release for about two months *in vitro* and the drug concentration in the plasma remained stable for 42 d in rats. This system was effective in prolonging the extension time of ETV *in vivo* and is expected to provide an optimized alternative for the treatment of HBV.

Acknowledgements

This work was supported by the State Key Laboratory of Long-Acting and Targeting Drug Delivery System and Shandong Luye Pharmaceutical Co., Ltd, China and the Supporting Fund for Teachers' research of Jining Medical University (JYFC2018KJ041), China.

Disclosure

The authors report no conflicts of interest in this work.

References

Ayoub, M.M., Elantouny, N.G., Elnahas, H.M., 2018. Injectable PLGA Adefovir microspheres; the way for long term therapy of chronic hepatitis-B. Eur. J. Pharm. Sci. 118, 24–31.
Balogh, J., Victor, D., Asham, E.H., et al., 2016. Hepatocellular carcinoma: a review. J. Hepatocell. Carcinoma 341–353.
Bao, W., Zhou, J., Luo, J., 2006. PLGA microspheres with high drug loading and high encapsulation efficiency prepared by a novel solvent evaporation technique. J. Microencapsul. 23, 471–479.
Bohr, A., Kristensen, J., Stride, E., 2011. Preparation of microspheres containing low solubility drug compound by electrohydrodynamic spraying. Int. J. Pharm. 412, 59–67.
Bozdağ-Pehlivan, S., Subaşi, B., Vural, I., et al., 2011. Evaluation of drug-excipient interaction in the formulation of celecoxib tablets. Acta Poloniae Pharmac. 68 (3), 423–433.
Bruni, G., Berbenni, V., Milanese, C., et al., 2010. Drug-excipient compatibility studies in binary and ternary mixtures by physico-chemical techniques. J. Therm. Anal. Calorim. 102 (1), 193–201.
Budhian, A., Siegel, S.J., Winey, K.I., 2005. Production of haloperidol-loaded PLGA nanoparticles for extended controlled drug release of haloperidol. J. Microencapsul. 22, 773–785.
Chu, L., Wang, A., Ni, L., 2018. Nose-to-brain delivery of temozolomide-loaded PLGA nanoparticles functionalized with anti-EPHA3 for glioblastoma targeting. Drug Deliv. 25, 1634–1641.
Gabler, F., Frauenschuh, S., Ringe, J., 2007. Emulsion-based synthesis of PLGA-microspheres for the in vitro expansion of porcine chondrocytes. Biomol. Eng. 24, 515–520.
Gu, B., Burgess, D.J., 2015. Prediction of dexamethasone release from PLGA microspheres prepared with polymer blends using a design of experiment approach. Int. J. Pharm. 495, 393–403.
Han, B., Gao, S., Zhang, X., 2010. Preparation of aclarubicin PLGA nanospheres and related in vitro/in vivo studies. Int. J. Pharm. 398, 130–136.
Ho, M.J., Lee, D.R., Im, S.H., 2018. Microsuspension of fatty acid esters of entecavir for parenteral sustained delivery. Int. J. Pharm. 543, 52–59.
Jiang, Y., Wang, F., Xu, H., Liu, H., Meng, Q., 2014. Development of andrographolide loaded PLGA microspheres: optimization, characterization and in vitro-in vivo correlation. Int. J. Pharm. 475, 475–484.
Jiang, Y., Zhang, X., Mu, H., et al., 2018. Preparation and evaluation of injectable Rasagiline mesylate dual-controlled drug delivery system for the treatment of Parkinson's disease. Drug Deliv. 25, 143–152.
Lim, J.L., Ki, M.H., Joo, M.K., 2015. An injectable liquid crystal system for sustained delivery of entecavir. Int. J. Pharm. 490, 265–272.
Mao, S., Guo, C., Shi, Y., Li, L.C., 2012. Recent advances in polymeric microspheres for parenteral drug delivery. Exp. Opin. Drug Deliv. 9, 1161–1169.
Meng, Q., Wang, A., Hua, H., 2018. Intranasal delivery of Huperzine A to the brain using lactoferrin-conjugated N-trimethylated chitosan surface-modified PLGA nanoparticles for treatment of Alzheimer's disease. Int. J. Nanomed. 13, 705–718.
Mitragotri, S., Burke, P.A., Langer, R., 2014. Overcoming the challenges in administering biopharmaceuticals:formulation and delivery strategies. Nat. Rev. Drug Discov. 13, 655–672.
Pazgan-Simon, M., Simon, K.A., Jarowicz, E., et al., 2018. Hepatitis B virus treatment in hepatocellular carcinoma patients prolongs survival and reduces the risk of cancer recurrence. Clin. Exp. Hepatol. 4 (3), 210–216.
Sacco, R., 2014. Use of entecavir for the treatment of complex forms of hepatitis B. Eur. Rev. Med. Pharmacol. Sci. 18, 1333–1343.
Sarikaya, M., Koçak, E., Köklü, S., 2012. Fatal fulminant hepatitis B after withdrawal of entecavir treatment in a patient with HBeAg seroconversion. Acta Gastro-Enterol. Belgica 75, 369–378.
Shen, J., Choi, S., Qu, W., Wang, Y., Burgess, D.J., 2015. In vitro-in vivo correlation of parenteral risperidone polymeric microspheres. J. Control Rel. 218, 2–12.
Su, Z., Shi, Y., Teng, L., Li, X., Wang, L., Meng, Q., 2011. Biodegradable poly(D, L-lactide-co-glycolide) (PLGA) microspheres for sustained release of risperidone: zero-order release formulation. Pharm. Dev. Technol. 16, 377–384.
Wang, A., Wang, L., Sun, K., Liu, W., Sha, C., Li, Y., 2012. Preparation of rotigotine-loaded microspheres and their combination use with L-DOPA to modify dyskinesias in 6-OHDA-lesioned rats. Pharm. Res. 29, 2367–2376.
Wang, J., 2014. Clinical utility of entecavir for chronic hepatitis B in Chinese patients. Drug Des. Develop. Ther. 8, 13–24.
Yoon, J., Park, D., Kim, C., 2013. A granulomatous drug eruption induced by entecavir. Ann. Dermatol. 25, 493–499.
Yan, J.H., Bifano, M., Olsen, S., 2013. Entecavir pharmacokinetics, safety, and tolerability after multiple ascending doses in healthy subjects. J. Clin. Pharmacol. 46, 1250–1258.
Zhang, Q.H., Yang, J., He, Y., 2010. Food effect on the pharmacokinetics of entecavir from dispersible tablets following oral administration in healthy Chinese volunteers. Arzneimittelforschung 60, 640–644.
Zhang, J.W., Wang, A.P., Li, P., 2014. Influences of PLGA/PLA and microsphere blends on the release of rotigotine from the microspheres. Chin. J. New Drugs 23 (4), 465–469.