doi.org/10.36721/PJPS.2024.37.1.REG.107-113.1

Release mechanism and pharmacodynamics of entecavir micro spheres

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Abstract: Entecavir, an effective anti-hepatitis B drug with low resistance rate, was designed as sustained-release micro spheres in our previous study. Here, we aimed to reveal the drug-release mechanism by observing the drug distribution and degradation behavior of poly (lactic-co-glycolic acid) and to investigate the pharmacodynamics of entecavir micro spheres. Raman spectroscopy was used to analyze the distribution of active pharmaceutical ingredients in the micro spheres. The results showed that there was little entecavir near the micro sphere surface. With increasing micro sphere depth, the drug distribution gradually increased and larger-size entecavir crystals were mainly distributed near the spherical center. The degradation behavior of poly (lactic-co-glycolic acid) was investigated using gel permeation chromatography. Changes in poly (lactic-co-glycolic acid) molecular weights during micro sphere degradation revealed that dissolution dominated the release process, which proved our previous research results. Pharmacodynamics studies on transgenic mice indicated that the anti-hepatitis B virus replication effect was maintained for 42 days after a single injection of entecavir micro spheres, similar to the effect of daily oral administration of entecavir tablets for 28 days. The entecavir micro spheres prepared in this study had a good anti-hepatitis B virus replication effect and it is expected to be used in anti hepatitis B virus treatment against hepatitis B virus.

Keywords: Entecavir, pharmacodynamics, micro sphere, release mechanism, drug delivery.

INTRODUCTION

With the widespread use of hepatitis B vaccines, the rate of infection has decreased across all age groups in various regions of the world (Hsu *et al.*, 2023). However, infections caused by hepatitis B virus (HBV) remain a serious global public health problem that impacts a large number of the population worldwide (Doan *et al*., 2023). To avoid excessive replication of HBV and recurrent attacks, patients must undergo standardized treatment for more than six months, which results in poor medication compliance (Jin *et al*., 2023; Gan *et al*., 2023).

Interferon and nucleoside analogues as anti-HBV drugs have been approved by the U.S. Food & Drug Administration (Ma *et al*., 2021). However, both interferon and nucleoside drugs have their own limitations (Zhang *et al*., 2011). Interferon has dual effects in regulating immunity and antiviral therapy, but it needs frequent intramuscular injection because of its short half-life, and it can cause many adverse reactions such as fever, headache, myalgia and mental disorders (Makokha *et al*., 2023). Moreover, it is unsuitable for those with a decompensated liver function. Nucleoside drugs can effectively inhibit HBV replication, but treatment takes at least 6 to 12 months and it can easily cause drug resistance; the virus is further prone to rebound after stopping drug treatment (Shu *et al*., 2019; Xiang *et al*., 2018). Therefore, pharmaceuticals with powerful anti-HBV effects and low drug resistance are urgently needed in clinical applications (Zhang *et al*., 2019).

In recent years, long-term oral antiviral preparations have been investigated by some studies. For example, injectable poly(lactic-co-glycolic acid) (PLGA) adefovir micro spheres for long-term therapy of hepatitis-B showed sustained release for 15 days after intramuscular injection in rats (Ayoub *et al*., 2018). A lipidic prodrug of entecavir was designed as a fatty acid ester micro suspension and pharmacokinetics in beagles indicated that the micro suspension sustained release for 28 days *in vivo* (Ho *et al*., 2018). PLGA micro particles of entecavir (MPs) were constructed using a spray-drying process for parenteral sustained delivery and the prepared MPs exhibited a prolonged release profile for more than 25 days *in vitro* (Kim *et al*., 2019). A sustained injectable *in-situ* implant and nanoparticles of entecavir were prepared using a PLGA polymer, which led to a long-term sustained release profile for up to 100 and 8 days, respectively (Ayoub *et al*., 2020). Although there have been some studies on the preparation of long-acting oral anti-hepatitis B drugs and their pharmacokinetics in animals, no pharmacodynamic research has been conducted to date.

Entecavir, a guanine nucleoside analogue, has a significant inhibitory effect on hepatitis B virus replication and is a nucleoside drug with a comparatively low resistance. In our previous research, extended-release entecavir-loaded PLGA micro spheres (ETV-MS) were

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formulated and the morphology, particle size, drug loading, *in-vitro* drug release and *in-vivo* pharmacokinetics in rats were evaluated in detail (Zhang *et al*., 2019). The results showed that the prepared entecavir micro spheres were circular in shape, with an average particle size of 86 µm; *in-vitro* and *in-vivo* studies indicated that dissolution dominated the release process.

Both the distribution of active pharmaceutical ingredients in micro spheres and hydrolytic degradation are important to the release behavior of a drug (Gasmi *et al*., 2015; Keles *et al*., 2015). Thus, the aim of this study was to further investigate the distribution of active pharmaceutical ingredients (APIs) in micro spheres and determine changes in the molecular weight of PLGA during degradation. We further investigated the drug release mechanism of the micro spheres. We, for the first time, performed pharmacodynamics studies of ETV-MS using commercial entecavir tablets as a reference to evaluate the inhibition on HBV DNA replication and provide a basis for clinical applications. This is the first pharmacodynamics study of entecavir long-acting formulations. The developed entecavir micro spheres could provide a new approach for the treatment of chronic hepatitis B.

MATERIALS AND METHODS

Materials

Entecavir with a purity of 99.2% was supplied by Shandong Boyuan Pharma Company (Jinan, China). Polyvinyl alcohol (PVA) was obtained from Aladdin (Shanghai, China). PLGA was obtained from Lakeshore Biomaterials (Alabama, USA). The diagnostic kit for quantitation of hepatitis B virus DNA (PCR-Fluorescence probing) was acquired from Sausure Biotech (Changsha, China). Dichloromethane (DCM) was purchased from Macklin Inc. (Shanghai, China). Tetrahydrofuran (THF) and acetonitrile were of HPLC grade and were obtained from EMD Millipore Corporation (Darmstadt, Germany). Unless noted otherwise, All chemicals and other solvents were of analytical grade and obtained commercially.

C57BL/6-HBV transgenic mice (females; 14.0±2.3g; ~5-6 weeks old) were obtained by injecting a fragment of HBV (type A, GenBank: AF305422.1) into the prokaryotic nucleus of C57BL/6NCrl mouse embryos, which were provided by Beijing Vitalstar Biotechnology Co., Ltd. The mice were fed in cages at 23±2°C under a standard 12-h light/dark cycle and provided with a standard diet and water. The research was carried out according to the guidelines of the Ethical Committee on Animal Experimentation of Jining Medical University (VST-SY-221116).

Preparation of entecavir micro spheres

The entecavir micro spheres were prepared by the solid-in-oil-in-water method as described previously (Zhang *et al*., 2019). Briefly, PLGA was dissolved in DCM, entecavir powder was crushed with an air flow grinder (SJM-50, Unique, China) to obtain drug particle sizes below 2 µm, and the pulverized entecavir powder was dispersed in the PLGA solution and sonicated for 20 min to form a homogeneous suspension (organic phase). PVA was dissolved in water at a temperature above 95°C. The organic phase was then slowly added to the PVA solution and homogenized to form an emulsion. The resultant emulsion was stirred to evaporate DCM and the micro spheres formed. The solidified microspheres were washed, lyophilized and stored in ampoules at -20°C before use.

The release mechanism of entecavir microsspheres

The drug distribution and degradation behavior of PLGA were detected using a Raman imaging spectrometer and a gel permeation chromatography (GPC) system, respectively. The pharmacodynamics parameters were analyzed by GraphPad Prism version 8.0 ($p<0.05$) through one-way analysis of variance (ANOVA).

Raman analysis of the APIs distribution in microspheres

The distribution of entecavir in micro spheres was observed using a laser micro-Raman imaging spectrometer (DXR2xi, Thermo Fisher, USA). A small number of micro spheres were laid on a clean glass slide, and the micro spheres were dispersed as well as possible. First, a 715- × 590-µm region and a 3-µm step size were selected for Raman imaging analysis. Then a small area was selected for fine scanning with a step size of 1.2 µm. A single microsphere with a diameter of approximately 50 µm was selected at last, the upper surface of the microsphere was chosen as the starting surface (defined as the depth of 0µm) and 3D Raman imaging was performed from the surface to the interior, every 2µm in the depth direction, to analyze the distribution of active pharmaceutical ingredients in the entecavir micro spheres. The excitation wavelength was 532 nm, and the laser power transferred to the sample was 10 mW (Lita *et al*., 2018).

In-vitro degradation

In-vitro degradation studies were carried out by dispersing microspheres into phosphate buffer (PBS, pH 7.4, 0.1 M) containing 0.02% Tween-80 in centrifuge tubes (Andhariya *et al*., 2019). The microsphere suspension was shaken at 45°C and 50 rpm. At 1h, 6h, 1, 2, 3, 4, 5, 6 and 7 days, aliquots of supernatant was withdrawn by centrifugation, the sediment consisting of microspheres was re-dispersed in equivalent fresh PBS. The micro spheres were washed and collected for gel permeation chromatography (GPC) analysis.

Determination of PLGA molecular weight

The molecular weight of PLGA was measured using an GPC system equipped with a Styragel HT3 column (7.8 × 300 mm, 10µm, Mw: 500-30 000) and a Styragel HT6E column (7.8 × 300 mm, 10µm, Mw: 5 000-10 000 000) in

series. The mobile phase was THF and the flow rate was 1.0 mL/min. The injection volume was 20μL. The column temperature and detector temperature were 30 and 35°C, respectively (Skidmore *et al*., 2019). A set of polystyrene standards was added with 1.5mL of THF to prepare the reference solution. Then, 1.5mL of THF was added to the lyophilized micro spheres, which were taken out from the release medium at predetermined time points, and the dissolved solution was used to test the molecular weight of PLGA.

Pharmacodynamics study

Study design

Eighteen transgenic mice were randomly divided into three groups (control group, microsphere group, tablet group); each group consisted of six mice. The mice in the micro sphere group received entecavir microspheres dispersed in menstruum, which was composed of saline containing 1% Tween 80 and 0.5% carboxymethylcellulose sodium. The mice in the tablet group received entecavir tablets, which were ground to a powder and dispersed in the same menstruum containing the micro spheres. The mental status, along with diet, urine, and excrement conditions and the activities of limbs were recorded every 24h until study completion. Body weights were recorded every 7d. Blood (0.1mL) was collected from the ophthalmic venous plexus respectively on 7, 14, 21, 28, 35 and 42d. All blood samples were centrifuged immediately at 1,500 × *g* for 5 min, and serum samples were collected and dissolved in PBS solution (Feng *et al*., 2021).

Uniformity of administration preparation

Suspensions were formed when micro spheres or crushed tablets were dispersed into the menstruum. Nine samples were taken from the upper, middle and lower layers of the suspension, i.e., three samples per layer, each having a volume of 0.1mL. The content of entecavir in each sample was determined using a HPLC (Agilent 1260) as described previously (Zhang *et al*., 2019), and the flow rate was 1.0mL/min. The injection volume was 100μL (Ashraf *et al*., 2017).

Determination of HBV DNA

The titer of HBV DNA was determined according to the instructions of the diagnostic kit for quantitation of hepatitis B virus DNA by PCR (ABI 7500, Real-Time PCR System, Thermo Fisher Scientific, USA). The reaction volume was 50μL and the PCR parameters were: 93°C for 2 min; 93°C for 45 s, 55°C for 60s in ten cycles; 93°C for 30 s, 55°C for 45 s in thirty cycles.

RESULTS

Preparation of entecavir micro spheres

The obtained entecavir microspheres were spherical in shape and had smooth surfaces, as shown previously (Zhang *et al*., 2019). The drug loading value was 13.0%. The particle analysis showed that the D(50) of the particles was 86.0 μm and the percentage below 250.0 μm was 100% with a span value of 1.146. The size of all micro spheres was less than 250.0 μm, which indicated that they met the requirements of needle permeability. Span is a parameter indicating the width of the sample particle size distribution. In our study, the value of the particle size span was close to 1, indicating that the particle size was close to a symmetrical distribution.

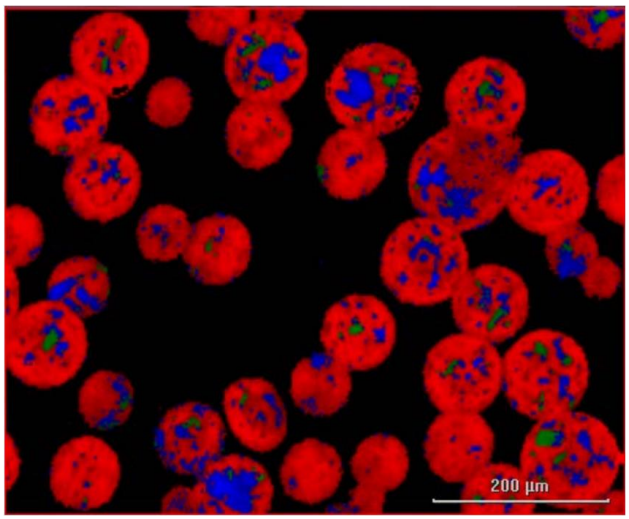

Fig. 1: Raman image of entecavir micro spheres with 3-μm step size

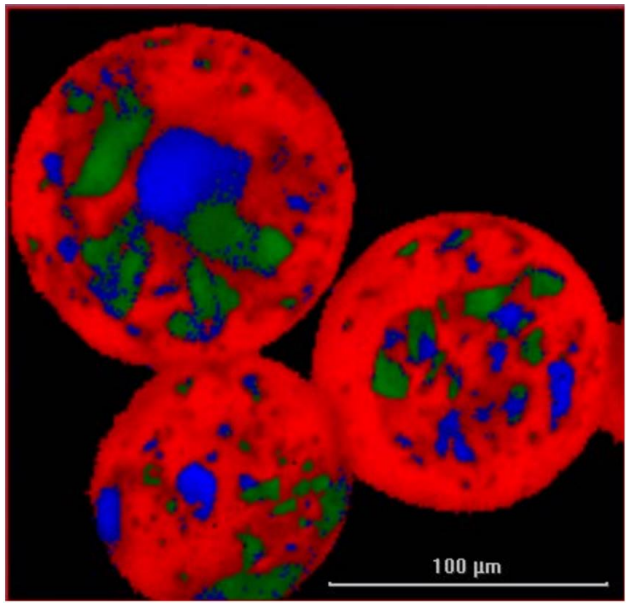

Fig. 2: Raman image of entecavir micro spheres with 1.2-μm step size.

Release mechanism of entecavir micro spheres

Distribution of APIs in micro spheres

A Dxr2xi micro imaging Raman spectrometer was used to analyze the distribution of entecavir in the micro spheres at different depths.

Table 1: Fitting equations for the release of entecavir from micro spheres

Mathematical model of drug release	Regression equation	R^2
Zero-order release model	y=3.129x+2.1203	0.9940
First-order release model	y=-0.1014x+0.3175	0.8046
Higuchi model	y=17.643x-11.585	0.9098
Korsmeyer–Peppas model	y=0.5212x+2.4603	0.8947

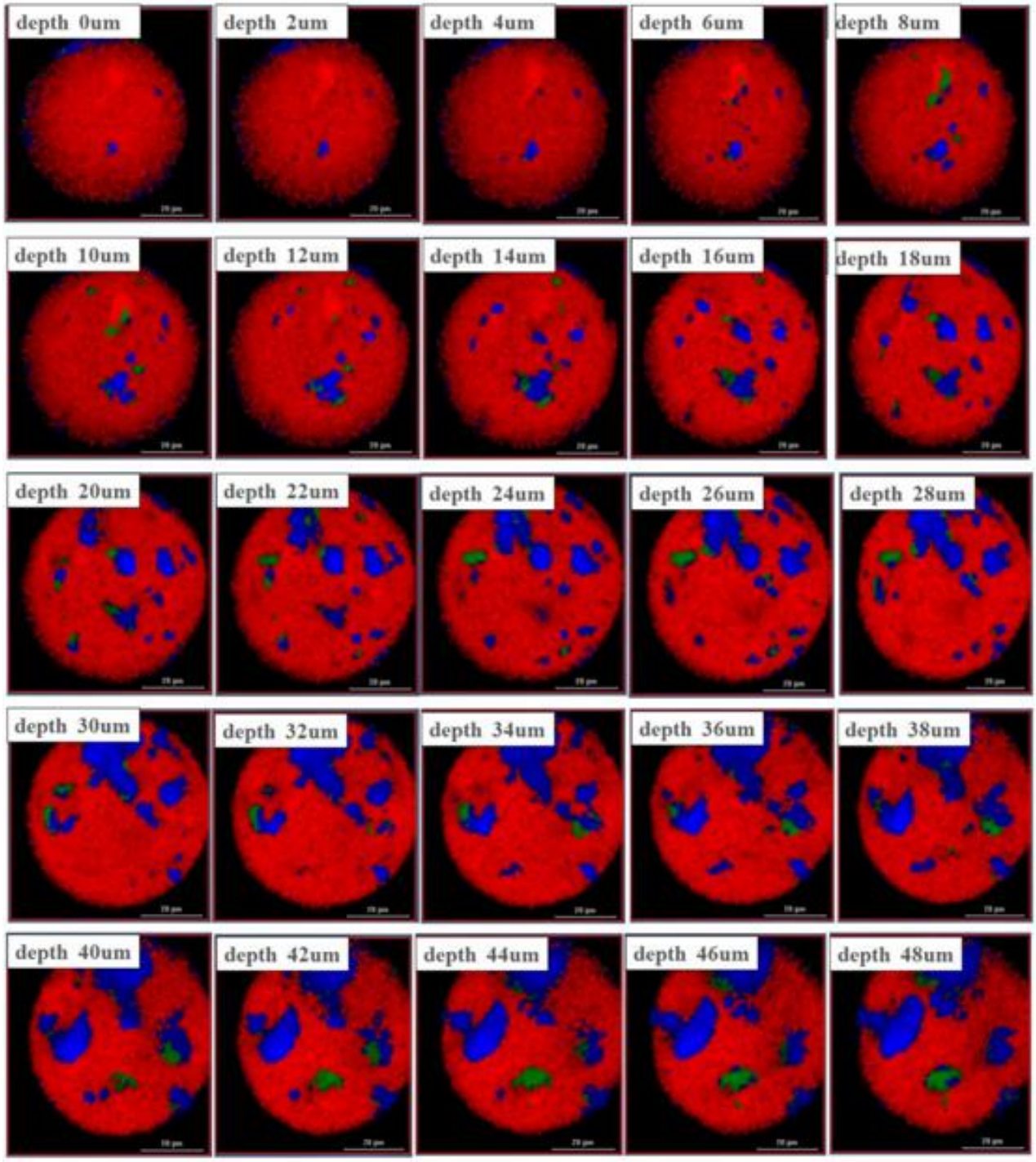

Fig. 3: Raman images of APIs at different depths of an entecavir micro sphere

First, a 715 × 590-µm region and a 3-µm step size were selected for Raman imaging analysis, and the results are shown in fig. 1. A small area was then selected for fine scanning with a step size of 1.2 µm, and the results are shown in fig. 2. Entecavir (blue and green) and APIs were uniformly dispersed in the PLGA matrix (red) micro spheres. A single micro sphere, approximately 50 µm in diameter, was selected and Raman imaging was performed with a 1-µm step size (fig. 3). Less APIs were distributed at the 0~18µm depth, more APIs were distributed at the 20~38µm depth and larger APIs were distributed at the 40~48µm depth. At the 46~48µm depth, the Raman signal weakened due to attenuation of the scattering signal. This indicated that the API distribution increased from the surface to the inside of the micro spheres.

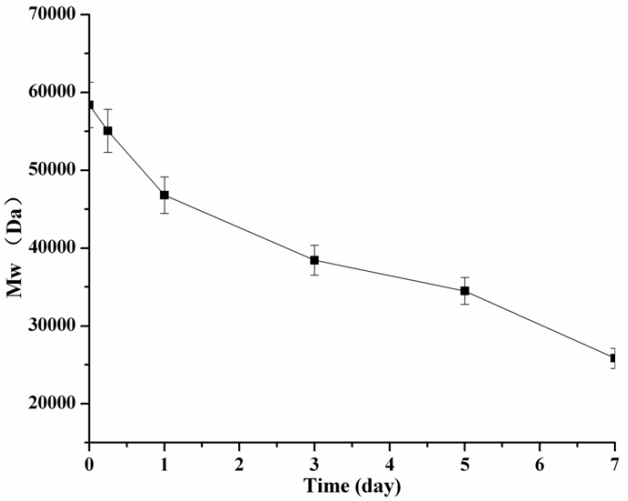

Fig. 4: Molecular weight-time profile of entecavir-loaded micro spheres

PLGA molecular weight during in-vitro degradation

The molecular weight of PLGA during degradation is presented in fig. 4. The molecular weight of PLGA changed nearly uniformly and this change was small in the initial stage of drug release. Along with water entering the micro spheres, PLGA began to degrade and its molecular weight gradually decreased. This was also confirmed by the equation fit to the drug release (table 1). The *in-vitro* release of entecavir microspheres was fit using regression analysis with Zero-order release model, first-order kinetics, the Higuchi equation, and the Korsmeyer Peppas equation, respectively (table 1). The results showed that the release rate of entecavir microspheres was almost constant *in vitro*.

Pharmacodynamics study

Uniformity of administration preparation

The relative standard deviation (RSD) of the concentration of the upper, middle and lower layers of entecavir tablets and micro sphere suspensions was 6.5 and 4.7%, respectively, which met the requirements of the regulations and ensured the accuracy and uniformity of the tablets and the micro sphere preparations.

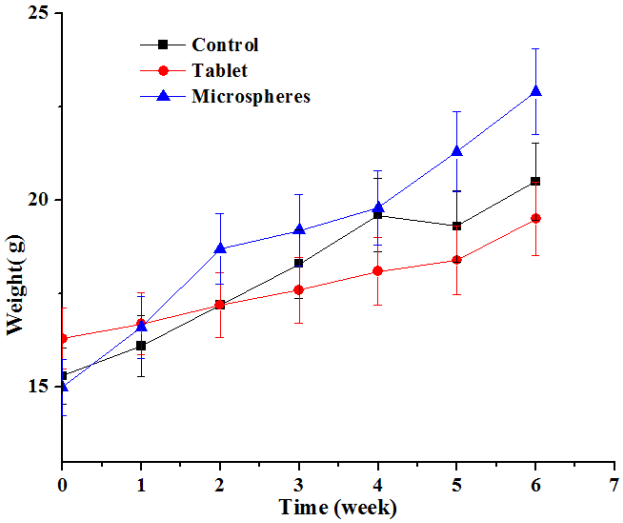

Fig. 5: Body weight-time profile of the control, tablet and micro sphere groups in mice

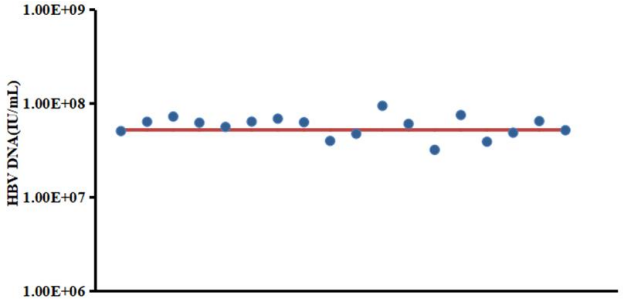

Fig. 6: HBV DNA copies in blank serum of model mice

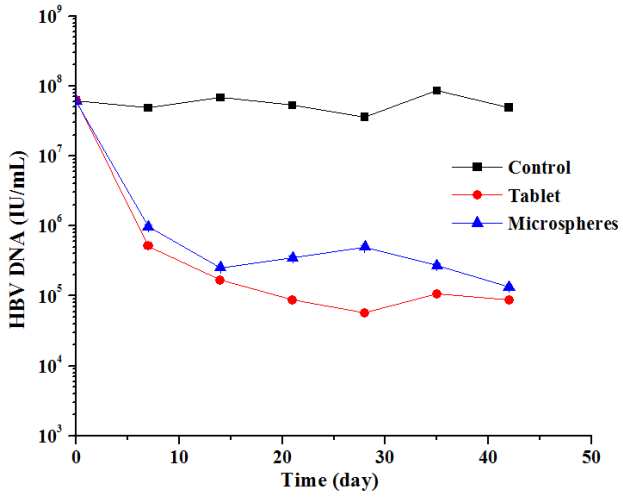

Fig. 7: HBV DNA-time profile of the control, tablet, and micro sphere groups in mice

Both the entecavir micro spheres and tablet powder are insoluble in water, so that suspensions were obtained via suspension in 0.9% sodium chloride injection. To increase the stability and uniformity of these suspensions, 1.0% Tween-80 and 0.5% CMC-Na were added into the sodium

chloride solution as dispersant and suspending agent, respectively, which increased the viscosity of injection and dispersed the drug powder (microsphere) in the injections more evenly (Yang *et al*., 2021).

Living states of C57BL/6-HBV transgenic mice

Food and drinking water of all C57BL/6-HBV transgenic mice were ingested normally, their appearance and hair were fine, their mental state and activities were normal, and no injury or animal death occurred during the whole experimental period. Statistics on weight changes are shown in fig. 5. The weight change in HBV transgenic mice among the control, tablet and micro spheres groups was not significant and the weight of all transgenic mice was between 15 and 23g.

Determination of HBV DNA

The HBV-DNA contents in blank serum samples were detected according to the operation instructions of the diagnostic kit for quantitation of hepatitis B virus DNA. The results of HBV-DNA copies in model mice (fig. 6) showed that the HBV-DNA content in the blank serum was above 1×10^7 IU/mL, suggesting that the HBV transgenic mice model was established successfully.

The results of HBV-DNA copies in the control, tablet and micro sphere groups are shown in fig. 7. Compared with the control group, HBV-DNA copies in the tablet and micro sphere groups decreased exponentially from above 1×10^7 IU/mL to 1×10^5 IU/mL, which met the standard for inhibiting HBV replication.

DISCUSSION

The pathogenesis of HBV infection is relatively complex. The first-line antiviral drugs for hepatitis B include entecavir, tenofovir and propofenofovir, which can effectively inhibit the replication of hepatitis B virus, delay the development of liver fibrosis and cirrhosis and reduce the incidence of liver cancer. However, to date, anti-HBV drugs can only inhibit HBV replication but not eliminate the virus, so that hepatitis B is a disease requiring lifelong treatment.

Although there have been some reports on the preparation of long-acting oral anti-hepatitis B drugs such as adefovir and entecavir (Ayoub *et al*., 2018; Kim *et al*., 2019), no pharmacodynamics research has been conducted so far. The decrease of HBV DNA copies is of great significance in the treatment of hepatitis B. Thus, the HBV DNA was detected to reveal the inhibitory effect of entecavir micro spheres on HBV in mice. The results showed that the inhibition rate and level by entecavir micro spheres and tablets with respect to HBV replication was almost the same. Single injection of entecavir micro spheres and intragastric administration of entecavir tablets resulted in an HBV-DNA decline that was almost at the same rate and level. The anti-HBV effect of the entecavir micro spheres lasted for 42 days and the entecavir micro spheres had a good anti-HBV replication effect in mice. The Studies on pharmacodynamics of entecavir micro spheres provide a reference for the subsequent clinical research.

The release of drugs from PLGA micro spheres is influenced by various factors such as PLGA hydrolysis and drug diffusion (Grizi *et al*., 2020). The morphological changes of entecavir micro spheres during degradation were observed using the scanning electron microscope in our previous study (Zhang *et al*., 2019). Combined with the scanning electron microscope results and molecular weight changes during accelerated release, it can be inferred that entecavir on the surface of the microspheres slowly dissolved into the release medium in the first phase of drug release because of the small solubility of entecavir in water. As water gradually penetrated into the micro spheres, entecavir close to the surface of the micro spheres diffused into the release medium. At this time, hydrolysis of PLGA was slow and the process of water entering the micro spheres was relatively long. Hydrolysis of micro spheres started from the surface (Wan *et al*., 2021). With the continuous dissolution and decomposition of the micro spheres, water gradually permeated into their interior and in the later release period, the molecular weight changed significantly. Therefore, the whole drug release process was controlled by dissolution and diffusion, as revealed by previous studies (Silva *et al*., 2015). Along with the layer-by-layer corrosion of micro spheres, the drug was released at a nearly constant speed.

CONCLUSION

The release mechanism and pharmacodynamics of entecavir micro spheres were successfully investigated. Entecavir crystals were mainly distributed near the spherical center of the micro spheres, and the release process was dominated by dissolution. The anti-HBV replication effect was maintained for 42 days in mice after a single injection of the micro spheres, which was similar to the effect of daily oral administration of entecavir tablets for 28 days. The entecavir micro spheres prepared in this study had a good anti-HBV replication effect and may have great potential for clinical use as an alternative treatment against HBV.

ACKNOWLEDGEMENT

This work was supported by Shandong Traditional Chinese Medicine Science and Technology Development Plan Project (2019-0452).

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