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Development of mucoadhesive microspheres of acyclovir with enhanced bioavailability

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ABSTRACT

Acyclovir-loaded mucoadhesive microspheres (ACV-ad-ms) using Ethylcellulose as matrix and Carbopol 974P NF as mucoadhesive polymer were prepared for the purpose of improving the oral bioavailability of acyclovir. The morphological properties of the microspheres were studied by optical microscopy and scanning electron microscopy (SEM). Drug loading and encapsulation efficiency was determined using HPLC method. In vitro and in vivo mucoadhesion of the microspheres was evaluated. Eggshell membrane was found to have a potential use for in vitro mucoadhesion measurement in place of stomach mucosa. In vitro drug release profiles and oral bioavailability of acyclovir in rats were also investigated. The release of the drug was influenced markedly by the medium pH and the proportion of Carbopol incorporated in the microspheres. The result of mucoadhesion study showed prolonged residence time of ACV-ad-ms in rats' gastrointestinal tract. In pharmacokinetics study, relatively steady plasma drug concentrations were observed within 8 h after oral administration of ACV-ad-ms to rats. The AUC_{0-t} and mean residence time (MRT) of ACV-ad-ms (6055.9 ng h/mL and 7.2 h) were significantly higher than that of ACV suspension (2335.6 ng h/mL and 3.7 h) ($P < 0.05$), which indicated that the bioavailability of acyclovir was greatly improved due to the prolonged retention of ACV-ad-ms in gastrointestinal tract.

1. Introduction

Acyclovir [9-(2-hydroxyethoxymethyl) guanine] (ACV), a synthetic purine nucleoside analog derived from guanine, is one of the most effective and selective antiviral drugs. ACV shows an antiviral effect on Herpes simplex virus HSV-1, HSV-2 and *Varicella Zoster* (VZV) virus through interfering with DNA synthesis and inhibiting viral replication (O'Brien and Camopli-Richards, 1989). Unfortunately, its absolute oral bioavailability is considerably poor (about 15–30%) because of its low water-solubility (about 0.2%, 25 °C) and short half-life (about 2.5 h) (Chiou and Barve, 1998). Therefore, Acyclovir must be taken in an oral dose of 200 mg five times daily, which cause compliance problems to patients. To overcome the oral absorption barrier, some prodrugs with enhanced solubility (such as valacyclovir) and different delivery systems containing ACV have been developed to improve its bioavailability, including α,β-poly (*N*-2-hydroxyethyl)-DL-aspartamide conjugate (Giammona et al., 1995), malonylchitosan microspheres (Stulzer et al., 2008), liposomes (Law et al., 2000), cyclodextrin complex (Bencini et al., 2008), etc.

It was also reported that the poor bioavailability of acyclovir is attributed to the short retention of its dosage forms at the absorption sites (in upper gastrointestinal tract to duodenum and jejunum) (Dhaliwal et al., 2008). Microspheres with bioadhesive polymer incorporated could contribute to improve absorption and enhance bioavailability of the drugs due to an intimate contact with the mucus layer, prolonged retention in the gastrointestinal tract, or specific targeting of drugs to the absorption site, etc. (Vasir et al., 2003). If the bioadhesive interactions occur primarily with the mucus layer of a mucous membrane, the phenomenon is referred to as "mucoadhesion" (Gu et al., 1988). Mucoadhesion can be obtained by either nonspecific or specific interactions with surface ligands at a mucosal surface. Over the last two decades, there has been considerable interest in bioadhesive/mucoadhesive drug delivery systems for its potential to optimize localized drug delivery, by retaining a dosage form at the site of action, or systemic delivery, by retaining a formulation in intimate contact with the absorption site (Bruschi and Freitas, 2005; Ahuja et al., 1997; Woodley, 2001). Despite the mucoadhesion, the advantage of using microspheres as oral mucoadhesive drug delivery system is that the small size microspheres can be trapped in the reductus of stomach and stay there longer. Besides, when the poor soluble drugs were loaded in the mucoadhesive microspheres, they were either adsorbed at the surface of the microspheres or highly dispersed in the inner part of the microspheres, which may help enhance the solubility of the drugs.

Mucoadhesion is a complex phenomenon. There were six kinds of theories that might explain the mechanism of mucoadhesion occurred between bioadhesive polymers and biological

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mucosal surface, including electronic, wetting, adsorption, diffusion, mechanical and fracture theories (Peppas and Sahlin, 1996). The occurrence of mucoadhesion has been said to experience two stages, the contact (wetting) stage followed by the consolidation stage (the establishment of adhesive interactions) (Smart, 2005). However, the in vivo mucoadhesion is influenced by many factors, such as various mucus turnover time, pH condition, and composition of different mucus. It is critical to employ an appropriate in vitro model to help evaluate the mucoadhesiveness and predict in vivo behavior of the formulation.

The purpose of this study was to prepare mucoadhesive microspheres of acyclovir in order to improve its oral bioavailability. The physicochemical characteristics, in vitro/in vivo mucoadhesive properties and pharmacokinetics in rats were investigated. Meanwhile, a new simple method using eggshell membrane as a substitute model for in vitro mucoadhesion study was developed and the correlations between different methods were also studied.

2. Materials and methods

2.1. Materials and animals

Acyclovir was purchased from Cheng Yi Pharmaceutical Co. (Zhe Jiang, China). Ethylcellulose was kindly provided by Colorcon Coating Technology (Shanghai, China). Carbopol 974P NF was kindly provided by Noveon, Inc. (USA). All other chemicals and solvent were of HPLC or reagent grade and used as received.

Wistar rats (220–250 g) and Kunming mice (25–30 g) were obtained from the Experimental Animal Center of Fudan University and maintained at $22 \pm 2\,^{\circ}C$ with free access to food and water. The animals used for the experiments were treated according to the protocols evaluated and approved by the Ethical Committee of Fudan University.

2.2. Microspheres preparation

The ACV loaded mucoadhesive microspheres (ACV-ad-ms) were prepared by emulsion solvent evaporation method and described as follows. 1.8 g of Ethylcellulose (Ec) and Carbopol 974P NF (Cb) with different Cb/Ec ratio (1/9, 1/7, 1/5, 1/3, w/w) were dissolved in 32 mL of ethanol; 0.6 g of ACV was added to the Ec–Cb solution under magnetic stirring. Then the suspension was quickly poured into 240 mL of light liquid paraffin containing 2.5% (w/v) of Span 80 and kept agitating at a rate of 10,000 rpm (FLUKO FA25, China) for 1 min to form a w/o emulsion. The emulsion was quickly shifted into a waterbath-jacketed beaker and was kept stirring at a rate of 600 rpm (Eurostar Power Control-visc, IKA-WERKE, Germany) for ethanol evaporation under reduced pressure. The system temperature was kept at 25 °C for 2 h and then gradually cooled to 15 °C until ethanol was thoroughly removed. The removal of residual oil was performed by washing the microspheres with petroleum ether for 3 times. The microspheres were dried under vacuum at room temperature for 12 h, and then strained twice: first through a 0.8-mm sieve and then through a 0.22-mm sieve. The microsphere fraction that passed through a 0.8-mm sieve and retained on the 0.22-mm sieve was collected and undergone further investigation.

Non-adhesive acyclovir loaded microspheres with only Ethylcellulose as matrix (ACV-ms) were prepared in a similar way.

The residual solvent content of petroleum ether in the product was detected using a GC method. The determination was achieved by a GC (Agilent6890, USA) with Diamonsil DM-624 column ($30\,m \times 0.53\,mm \times 3.0\,\mu m$). The experimental conditions were as following: a 2 μL volume of either a standard or sample solution was injected in the GC injection port, which was maintained at a temperature of 200 °C. The nitrogen carrier gas was set at a flow of 5.3 mL/min. The temperature of the flame ionization detector (FID) was set at 250 °C. The temperature of column started at 40 °C, stayed for 10 min, and then increased at a rate of 50 °C/min to reach a final temperature at 200 °C. And there was less than 0.01% of the residual petroleum ether found in the product.

2.3. Characterization of microspheres

2.3.1. Morphology of microspheres

The surface and inner part of the microspheres were observed respectively via Optical Microscopy (Nikon FA-35, Japan) and scanning electron microscopy (SEM, Hitachi S 502, Japan). For optical microscopy, the microspheres were directly observed under magnification (100×). Prior the SEM examination, samples of the microspheres were fixed on a brass stub using double-sided tape and then gold coated in vacuum by a sputter coater. And the pictures were taken at an excitation voltage of 20 kV.

2.3.2. Determination of drug loading and encapsulation efficiency

A certain amount of microspheres were ground to powder. About 100 mg of the powder was mixed with 2 mL of 0.4% NaOH solution and 90 mL of distilled water. The suspension was ultrasonicated for 2 h, and then diluted to 100 mL. After filtration through a 0.45 μm membrane filter, 1 mL of the filtrate was diluted to 10 mL. The acyclovir standard solution (20 μg/mL) was taken as reference. The samples were analyzed by HPLC (LC-10A, Shimadzu, Japan) equipped with a Diamond C18 column ($200\,mm \times 4.6\,mm$, 5 μm). The mobile phase of 8% methanol and 92% distilled water was used at a flow rate of 1 mL/min, and acyclovir was detected by UV detector at 254 nm.

2.4. In vitro drug release study

The in vitro release of acyclovir from mucoadhesive microspheres was measured using ChP XC basket type dissolution apparatus (Model ZRS-8, Tianjin University Precision Instrument Factory, China). About 60 mg of ACV-ad-ms were placed in the basket. The volume of dissolution medium was 900 mL and maintained at $37 \pm 0.5\,^{\circ}C$ at a rotation speed of 50 rpm. Different dissolution medium (pH 1.3 HCl solution, pH 3.6 phosphate buffered solution, and pH 7.4 phosphate buffered solution, respectively) was used for ACV release test. An aliquot of 4 mL of the solution was withdrawn at predetermined time intervals and replaced by 4 mL of fresh dissolution medium immediately. The samples were assayed via UV spectrophotometry (UV-240IPC, Shimadzu, Japan) at 252 nm after filtration through a 0.45 μm membrane filter. Another 10 mg of ground microspheres were dispersed in 150 mL of dissolution medium and were sonicated for 2 h. Then the suspension was filtrated through a 0.45 μm membrane filter. The absorbance of the filtrate was also measured at 252 nm for the total amount of acyclovir released. All dissolution tests were performed in triplicate.

The data obtained from the in vitro release studies were analyzed by various models such as the first order, Higuchi, and Korsmeyer–Peppas models. The equations were as follows:

$$\text{First order}: \quad Ln(Q_0 - Q_t) = k_1 t,$$

$$\text{Higuchi}: \quad Q_t = k_H t^{1/2},$$

$$\text{Korsmeyer–Peppas}: \quad Q_t = k_P t^n,$$

where Q_t is the amount of drug released in time t and Q_0 is the initial amount of drug in the microspheres, k_1, k_H, and k_P are release rate constants, n is the release exponent indicative of mechanism of release. In spherical matrices, if $n < 0.43$, a Fickian diffusion mediated drug release occurs; if $0.43 < n < 0.85$, non-Fickian transport

occurs; and erosion mediated release occurs if $n > 0.85$ (Ritger and Peppas, 1987).

2.5. Evaluation of mucoadhesive properties of microspheres

2.5.1. In vitro mucoadhesion test on mice stomach mucosa

The mucoadhesive properties of microspheres were evaluated by the method designed by Ranga Rao and Buri (1989) using stomach isolated from mice. First, Kunming mice were fasted for 24 h and the stomach was dissected immediately after the mice were sacrificed. The stomach mucosa were removed and rinsed with physiological saline. 100 particles of ACV-ad-ms or ACV-ms particles were scattered uniformly on the surface of the stomach mucosa. Then, the stomach mucosa with microspheres was placed in a chamber maintained at 93% relative humidity at room temperature. After 30 min, the tissues were taken out and fixed on a plate at an angle of 45°. The stomach mucosa was rinsed with simulated gastric fluid (pH 1.3, without enzymes) for 5 min at a rate of 22 mL/min. The microspheres remaining at the surface of stomach mucosa were counted, and the percentages of the remaining microspheres were calculated and the statistical significance of the differences between two groups was analyzed using the two-tailed *t*-test. A *p* value of less than 0.05 was termed significant.

2.5.2. In vitro mucoadhesion test using eggshell membrane as substitute mucosa

Eggshell membranes were employed as a substitute model for in vitro mucoadhesion evaluation. The eggshell membranes were obtained from fresh chicken eggs. After emptying the egg of its content, the external shell was removed, and the underlying membrane was isolated. Then similar procedure was carried out as mice mucosa to measure the in vitro mucoadhesion of the microspheres. The number of microspheres remaining on the surface of eggshell membrane was counted, and the adhering percent was calculated and statistically analyzed as above.

2.5.3. In vivo mucoadhesion

Wistar rats were fasted (water-fed) for 24 h and then divided into eleven groups randomly. Each group consisted of six rats. Five groups of rats were administered orally with 100 particles of ACV-ms or ACV-ad-ms with different Cb/Ec ratio (Cb/Ec = 1/9, 1/7, 1/5, 1/3, w/w), respectively. After administration, the rats were kept fasted until they were sacrificed by cervical dislocation after 2 h. The other six groups were administered with 100 particles ACV-ms or ACV-ad-ms (1/3), and sacrificed after 2 h, 4 h, and 6 h. The stomachs and small intestines were removed. The microspheres that remained in the gastrointestinal tract were counted, and the percentage of the remaining microspheres was calculated and statistically analyzed as above.

2.6. Pharmacokinetic study in rats

2.6.1. Animal experimental procedure

Wister rats were fasted for 24 h before the experiment and food was reoffered 4 h post-dosing. The rats were divided into two groups of five animals each. Each group was orally administered with ACV-ad-ms (Cb/Ec = 1/3, w/w) and Acyclovir suspension (ACV-sus), respectively, in 1 mL of saline, at the equivalent dose of 40 mg/kg body weight as acyclovir. Blood samples were collected from retro-orbital venous plexus of rats at predetermined time intervals (t = 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 24 h or 5 min, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 h, respectively), centrifuged to separate plasma for 10 min at 4000 rpm by using a Biofuge Primo R centrifuge (Heraeus Instruments, Germany), and stored at −20 °C until analysis.

2.6.2. Analytical procedure

For the analysis of plasma samples, Ganciclovir was used as internal standard. The procedure was as follows (Brown et al., 2002): 100 μL of plasma, 25 μL of internal standard (Ganciclovir, 10 μg/mL), and 25 μL of 20% perchloric acid were mixed by vortexing for 2 min and then centrifuged for 10 min at 15,000 rpm. 50 μL of supernatant was injected directly into the HPLC system.

The separation was achieved by a RP-HPLC (LC-10A, Shimadzu, Japan) (Palma-Aguirre et al., 2007) with YMC C18 column (150 mm × 4.6 mm, 5 μm) attached to a C18 guard column (7.5 mm × 4.6 mm, 5 μm) at 40 °C. The mobile phase consisted of methanol (1%) and H_2O (99%, adjusted to pH 2.0 with perchloric acid). Flow rate was set to 1 mL/min, and detection was carried out using a fluorescence detector at 260 and 375 nm of excitation and emission wavelength, respectively. The method was linear between 20 and 1500 ng/mL.

2.6.3. Pharmacokinetics and statistical analysis

The pharmacokinetic analysis was performed with WinNonlin 4.0.1 (Pharsight Corporation, CA, USA). The following pharmacokinetic parameters were derived from the non-compartmental analysis of the plasma concentration–time curve: the areas under the plasma concentration–time curve were calculated as $AUC_{0-\infty} = AUC_{0-t} + C_t/\lambda$, where t was the time of the last measurable plasma, C_t was the plasma concentration of this last sample. AUC_{0-t} was calculated by the trapezoidal rule with linear interpolation. The area under the first moment curve ($AUMC_{0-\infty}$) was also calculated by the trapezoidal and extrapolated to infinity. Mean residence time was calculated as MRT = AUMC/AUC, and peak plasma concentrations (C_{max}) was estimated directly from the experimental data.

To assess the statistical significance of the differences between two groups, the two-tailed *t*-test was used. A *p* value of less than 0.05 was termed significant.

3. Results

3.1. Characterization of microspheres

The optical and scanning electron micrographs of Acyclovir mucoadhesive microspheres (Cb/Ec = 1/3, w/w) are illustrated in Fig. 1. ACV-ad-ms were well-rounded spheres with uniform size distribution under optical microscope (Fig. 1(a)). Scanning electron microscopy (SEM) was utilized to observe the surface and inner part of the microspheres. It can be observed that the surface of the microsphere was uneven and there are some crystals and pores scattered on the surface (Fig. 1(b)). These pores are suspected to be formed during the solvent evaporation process, and might form passages to help the drug release from inner part of the microspheres. The crystals of acyclovir adsorbed on the surface of microspheres contribute to a burst release and help to achieve effective concentration quickly after oral administration. Fig. 1(c) shows the dense and porous inner part of the microspheres.

Data of drug loading and encapsulation efficiency of different formulations are shown in Table 1.

Table 1
Drug loading and encapsulation efficiencies for Avc-ad-ms with different weight ratio of Cb/Ec.

Cb/Ec (w/w)	Drug loading content (%)	Encapsulation efficiency (%)
1/9	16.08	64.63
1/7	18.17	72.33
1/5	18.38	73.58
1/3	18.53	73.65

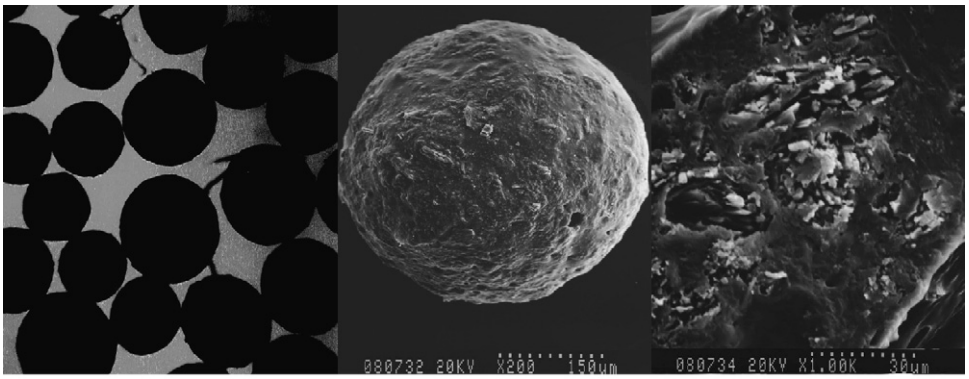

Fig. 1. Optical and scanning electron micrographs of ACV-ad-ms (Cb/Ec = 1/3, w/w): (a) microspheres under optical microscope (100×), (b) surface of the microsphere under SEM (200×), (c) inner part of the microsphere under SEM (1000×).

3.2. In vitro drug release

The in vitro release rate of ACV from mucoadhesive microspheres (ACV-ad-ms, Cb/Ec = 1/3) in different dissolution medium are shown in Fig. 2. The rate of drug release from microspheres was faster in pH 1.3 HCl and pH 7.4 phosphate buffers than in pH 3.6 phosphate buffers. Above 95% of acyclovir was released in 6 h at pH 1.3 and 7.4, while only 75% of acyclovir was released and gained a sustained-release in 12 h. This might be due to a result of the combination of gel forming ability of Carbopol and solubility of acyclovir at different pH. Carbopol is a highly crosslinked polymer which has the ability to swell up to 1000 times its original volume to form a gel when exposed to a pH environment above 4–6. Lots of carboxyl groups of Carbopol ionize at pH 3.6, resulting in repulsion between the anions and further increasing the swelling of the polymer, while the carboxyl groups will not ionize at pH 1.3 (Bonacucina et al., 2004). Therefore, the rate of drug release from the microspheres was significantly slower at pH 3.6 than that at pH 1.3. On the other hand, the solubility of acyclovir can be enhanced by increasing the medium pH, which might explain the relatively faster release of acyclovir from the microspheres at pH 7.4.

The influence of Carbopol to the drug release from mucoadhesive microspheres was also investigated in phosphate buffer with pH of 3.6. As shown in Fig. 3, the more Carbopol the microspheres loaded, the faster the drug was released. About 56.62%, 78.61%, 87.15%, and 95.6% of the ACV loaded in microspheres were released within 12 h from microspheres with weight ratios of 1/9, 1/7, 1/5 and 1/3 Cb/Ec, respectively. As Ethylcellulose (Ec) is widely regarded as a sustained-release matrix because of its properties of water-insoluble and low permeability, its effect on drug release could be influenced by Carbopol incorporated in the microspheres. Carbopol is a water-soluble polymer of acrylic acid, which can form hydrophilic passages inside the microsphere to help drug diffuse out. Therefore, the release of drug accelerated with the increase of Carbopol.

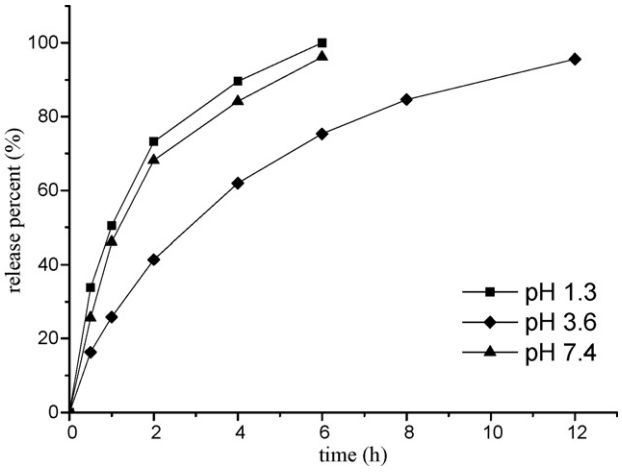

Fig. 2. Profiles of drug release from ACV-ad-ms (Cb/Ec = 1/3, w/w) in different medium.

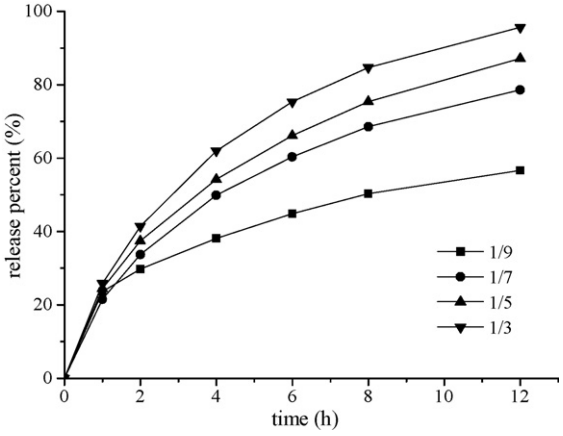

Fig. 3. Profiles of Acyclovir release from mucoadhesive-microspheres with different weight ratio of Cb/Ec.

Similar burst release of about 25% of ACV during the first hour in all formulations was observed because of the drug adsorbed on the surface of microspheres. This initial burst effect was beneficial to achieve the effective plasma concentration after administration of ACV-ad-ms.

Table 2 gives the correlation coefficients (R^2) obtained from different release models of Acv-ad-ms with different Cb/Ec ratios at pH 3.6. Considering the R^2 values, the Korsmeyer–Peppas model best fitted the formulation of Acv-ad-ms with Cb/Ec ratio of 1/9 and n is under 0.43, which indicates that Fickian diffusion dominates

Table 2
Release kinetics of Avc-ad-ms with different weight ratio of Cb/Ec.

Models	Formulation (Cb/Ec ratio)			
	1/9	1/7	1/5	1/3
First order R^2	0.9773	0.9915	0.9997	0.9916
Higuchi R^2	0.9954	0.987	0.9913	0.9792
Korsmeyer–Peppas R^2	0.9988	0.9894	0.9932	0.9838
n	0.3591	0.5266	0.5163	0.5342

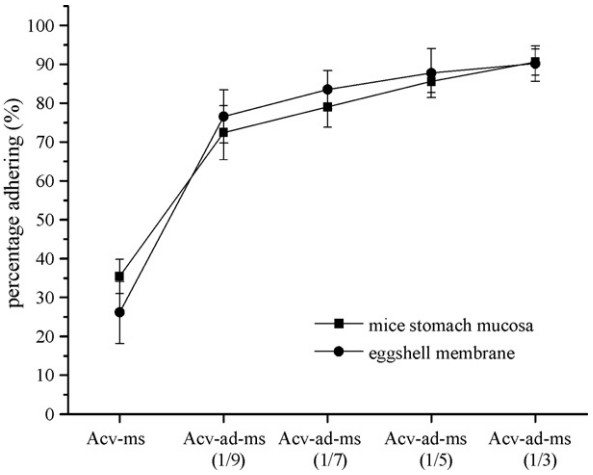

Fig. 4. In vitro mucoadhesiveness of microspheres to different mucosa ($n = 6$, mean ± S.D.).

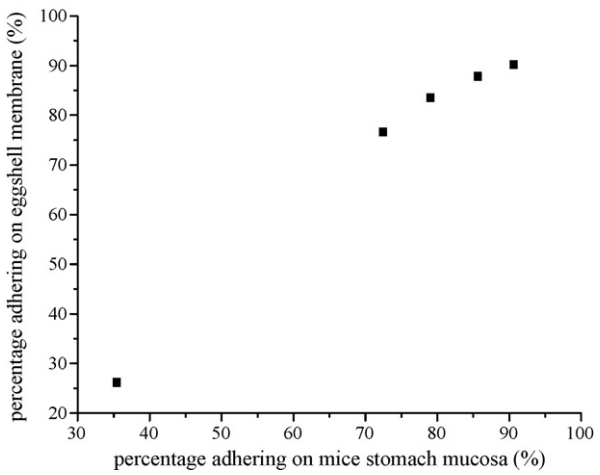

Fig. 5. Correlation between mice stomach mucosa and eggshell membrane in mucoadhesive evaluation in vitro ($R^2 > 0.97$).

the drug release through the swelling matrix and hydrophilic pores. However, the increase amount of Carbopol incorporated in the microsphers altered the release mechanism. The release profiles of Acv-ad-ms with Cb/Ec ratios above 1/9 were best fitted to the first order models, and gained an n value between 0.43 and 0.85 which indicated an anomalous (non-Fickian) mechanism. The increase of hydrophilic pores formed by Carbolpol facilitated the water penetrating into microspheres, accelerated the erosion of the swelling matrix and resulted in a combination of the diffusion and erosion mechanisms of drug release from microspheres.

3.3. Evaluation of mucoadhesion of microspheres

In vitro mucoadhesive properties of microspheres were evaluated according to the method Ranga Rao and Buri reported. As shown in Fig. 4, the remaining percentage was notably increased with Carbopol incorporated in the microspheres, which indicated that Carbopol has a strong ability to interact with mucus. The more Carbopol was incorporated, the better retention effect was observed. Significant differences were seen between ACV-ms and ACV-ad-ms ($P < 0.01$). And so were seen between ACV-ad-ms with Cb/Ec ratio of 1/3 and other ratios ($P < 0.01$).

Eggshell membranes were employed as a substitute to mimic animal mucosa in the evaluation of in vitro mucoadhesiveness in this study, since it is easily available and the experiment can be carried out in any laboratory. The results of microspheres remaining on eggshell membranes are also shown in Fig. 4, which were similar to the results obtained from mice stomach mucosa. The correlation of two methods was analyzed by calculating the correlation coefficient R^2, which measures the strength of a linear relation between two variables using Origin Program 8.0. As shown in Fig. 5, a good proportional correlation was recognized between mice stomach mucosa and eggshell membrane. The response was found to be linear with correlation coefficient exceeding 0.97, which indicated that eggshell membrane has a potential as a substitute for animal mucosa in mucoadhesion studies of mucoadhesive formulations.

In vivo mucoadhesive evaluation of microspheres was conducted in rats. The microspheres of different formulations remaining in the gastrointestinal tract were studied after being orally administered to rats. As shown in Fig. 6, the remaining percentage of ACV-ad-ms in stomach was significantly higher than that of ACV-ms 2 h after administration ($P < 0.05$), which was corresponding to the results of in vitro evaluation. While, the percentages of ACV-ms in small intestine were slightly higher than that of ACV-ad-ms due to their quick transition from stomach to small intestine.

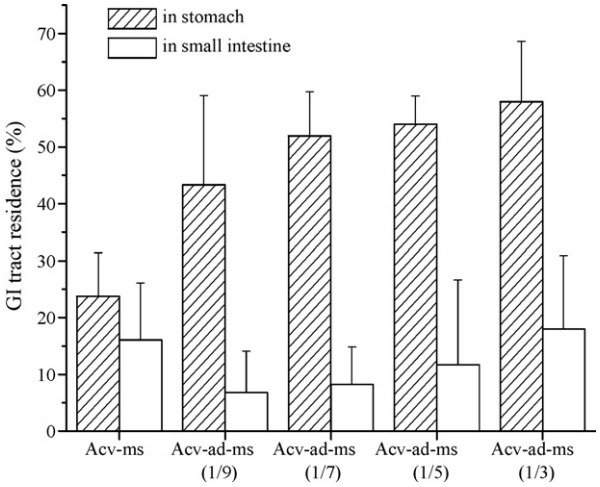

Fig. 6. Percentage of different microspheres retained in the stomachs and small intestines of rats in vivo in 2 h ($n = 6$, mean ± S.D.).

The correlation coefficient between in vitro and in vivo mucoadhesion studies was also investigated. As shown in Fig. 7, both methods showed good linear correlation. The coefficients were exceeding 0.97 or 0.93 between in vivo and in vitro studies, on stomach mucosa or eggshell membrane, which indicated that eggshell membrane could not only be a substitute of mucosa to measure in

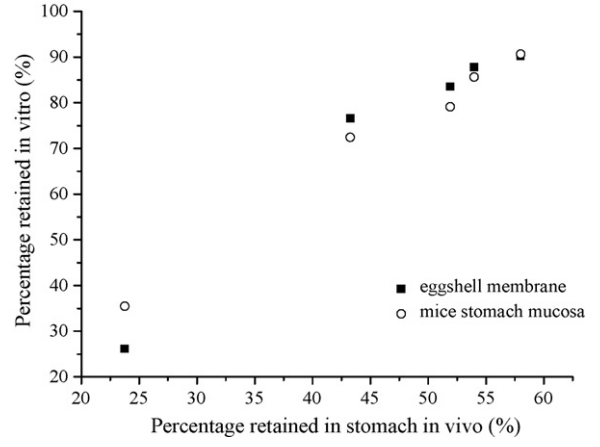

Fig. 7. Correlation between in vivo and in vitro mucoadhesive evaluation ($R^2 > 0.97$).

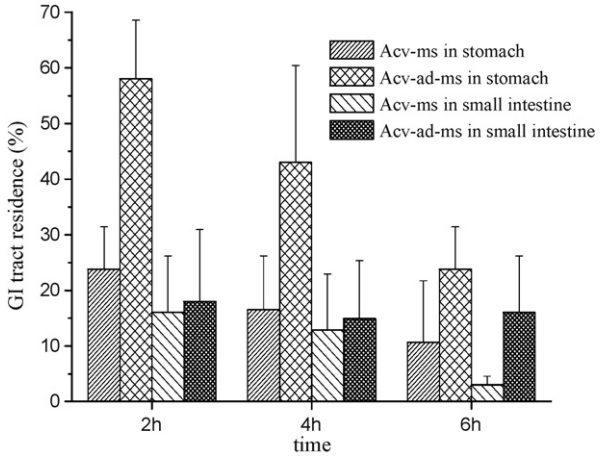

Fig. 8. Percentage of ACV-ms and ACV-ad-ms (Cb/Ec = 1/3, w/w) retained in the stomachs and small intestines of rats in vivo ($n = 6$, mean ± S.D.).

vitro mucoadhesion, but also could be used to predict the in vivo mucoadhesion of the microspheres.

The distribution of the microspheres in the gastrointestinal tract of rats was examined at 2, 4, and 6 h after oral administration as shown in Fig. 8. The remaining percentages of ACV-ad-ms (Cb/Ec = 1/3, w/w) in the stomach at each time interval were all significantly higher than that of ACV-ms ($P < 0.05$). The results indicated that Carbopol incorporated in the microspheres induced strong adhesion and deep penetration to the stomach mucous layer, which prolonged the residence time of the microspheres in the stomach. Most of ACV-ms had passed through the stomach in 2 h after administration. However, the small amount of ACV-ms remained in the stomach had a slow rate of gastric transmit, which could be attributed to the small particle size which caused the microspheres being trapped in the reductus of stomach. The remaining percentages of microspheres in the small intestine showed no difference between ACV-ms and ACV-ad-ms 2 h and 4 h after administration (16%, 13% and 18%, 15%). But after 6 h, the remaining percentages of ACV-ad-ms (16%) were notably higher than that of ACV-ms (3%) ($P < 0.05$).

3.4. Pharmacokinetics in rats

The mean plasma concentration of ACV-time profiles after oral administration of acyclovir loaded mucoadhesive microspheres and acyclovir suspension are illustrated in Fig. 9. After administration

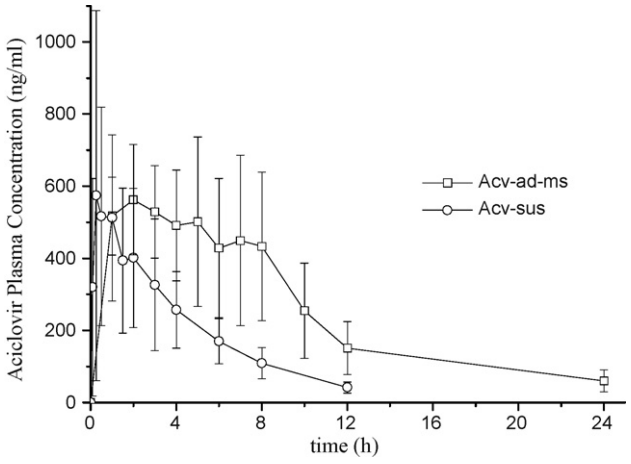

Fig. 9. Plasma concentration–time curves of two formulations of acyclovir after being orally administered to rats at a dose of 40 mg/kg ($n = 5$, mean ± S.D.).

Table 3
Pharmacokinetic parameters of ACV-ad-ms and ACV-sus after oral administration to rats at a dose of 40 mg/kg ($n = 5$, mean ± S.D.).

	C_{max} (ng/mL)	AUC_{0-t} (ng h/mL)	MRT (h)
ACV-ad-ms	627.2 ± 211.3	6055.9 ± 2587.6	7.2 ± 0.7
ACV-sus	750.5 ± 407.0	2335.6 ± 856.6	3.7 ± 0.4

of the drug suspensions, drug concentrations quickly reached their peaks within 1 h, and then decreased rapidly. On the other hand, the profiles for drug-loaded mucoadhesive microspheres were rather smooth. High plasma concentrations were observed in 1 h, which could be kept in a relatively steady state within 8 h, and slowly eliminated as the plasma concentration could still be detected after 24 h. However, for acyclovir suspension, the plasma concentration was below the limit of quantitation after 12 h.

The pharmacokinetic parameters are listed in Table 3. The C_{max} values for ACV-ad-ms and ACV-sus were 627.2 and 750.5 ng/mL, respectively, and the AUC_{0-t} (6055.9 ng h/mL) and mean residence time (MRT) (7.2 h) for ACV-ad-ms were significantly higher than that of ACV-sus (2335.6 ng h/mL and 3.7 h, respectively) ($P < 0.05$). The oral bioavailability of acyclovir was greatly improved as the relative bioavailability values were 259% for ACV-ad-ms compared with that of ACV-sus, which was attributed to the prolonged residence of microspheres in gastrointestinal tract and induced close contact of the drug at its absorption site to enhance the absorption (Kagan and Hoffman, 2008).

4. Discussion

Several studies have demonstrated that ACV is absorbed paracellularly by passive diffusion in the gastrointestinal tract and its poor solubility results in low oral bioavailability. Many in vitro everted sac and *in situ* single-pass perfusion experiments showed that absorption of ACV in the gastrointestinal tract primarily takes place at the upper and middle part of gastrointestinal tract (Meadows and Dressman, 1990; Lewis et al., 1986; Wilson et al., 1987). Hence it is an efficient way to enhance the bioavailability of ACV by introducing gastroretentive dosage form which can enable continuous drug delivery to the upper part of the gastrointestinal tract (Hou et al., 2003).

Mucoadhesive microsphere was one of the most promising gastroretentive formulations which could be retained in the stomach and keep continuous drug release. It has been reported that incorporation of mucoadhesive materials into microspheres significantly increase the gastrointestinal transit time of microspheres (Miyazaki et al., 2003; Takeuchi et al., 2003; Shimoda et al., 2001; Liu et al., 2005; Zheng et al., 2006). Carbopol is one of the currently most widely used mucoadhesive materials, thanks to its numerous carboxyl groups which facilitate the formation of hydrogen bonds with mucus. The mucoadhesion capability of Carbopol was easily affected by many factors such as pH and ionic strength (Singla et al., 2000). For example, at low pH (5.0 or less), the low ionization extent of carboxyl groups results in less swelling extent and thus stronger interaction with polysaccharides in mucus. On the other hand, the swelling ability of Carbopol greatly influenced the sustained-release properties of microspheres as shown in Part 3.2. We observed a negative correlation between mucoadhesion and drug release, demonstrating that special attention should be paid to select the optimal content of Carbopol to artfully balance mucoadhesion of microspheres and release of drug.

To achieve better absorption, sustained release of ACV is desirable because of the poor solubility and thus slow absorption rate of ACV. However, the release rate should also fit with the mean residence time of microspheres in stomach; otherwise the drugs could not be absorbed efficiently if microspheres were transported to the lower part of the gastrointestinal tract. In our study, almost all of

ACV was released from ACV-ad-ms (Cb/Ec = 1/3, w/w) at pH 1.3 by 6 h (Fig. 2) when about 23% of the microspheres still remained in the stomach of rats (Fig. 8), which indicated that ACV could be mostly absorbed in upper gastrointestinal tract.

As was shown by Fig. 9, mucoadhesive microspheres significantly enhanced the oral bioavailability of ACV, proving their potential to improve the gastrointestinal absorption of poor-soluble drugs with narrow absorption window. The mucoadhesive and sustained-release property of the studied microspheres assured a relative stable plasma drug concentration for 8 h. An obvious burst release (about 25%) of ACV was observed in vitro in the first hour (Fig. 2), which could be attributed to the adsorbed drug on surface of microspheres. This helped to explain the high plasma concentration of ACV observed in 1 h after oral administration of mucoadhesive microspheres (Fig. 9).

A proper method for measuring the mucoadhesive effect in vitro is critical to accurately predict in vivo behavior for mucoadhesive formulations. A lot of tests have been suggested to evaluate the in vitro mucoadhesion of microspheres, including the tensile stress measurement (Chichering and Mathiowitz, 1995), the rheological approach (Riley et al., 2001), the rinsing method, the everted sac technique (Santos et al., 1999), etc. Some of these tests need specific equipments and most of them employed animal mucosa, inevitably introducing practical difficulties like high cost and uncontrollable inter-individual diversity. A novel natural but non-animal tissue substrate, the eggshell membrane, was employed by Parodi et al. (1999) to measure the mucoadhesion of buccoadhesive tablets, which supplied more reliable values of mucoadhesion parameters compared with the synthetic membrane of polypropylene. In the present study, we used the eggshell membrane to substitute the animal stomach mucosa in the mucoadhesion evaluation of microspheres, based on the similarity between the eggshell membrane and the stomach mucus with respect to its composition and thickness. Eggshell membrane is composed of two individual membranes between egg albumin and eggshell, which comprised of proteins, lipids, minerals and carbohydrates (Sugino et al., 1997). The good correlation between the in vitro data from the eggshell membrane and the in vivo mucoadhesion studies (Fig. 5 and Fig. 7) demonstrated the potential of the eggshell membrane as substitute for the gastric mucosa. And the measurement of in vitro mucoadhesion of the microspheres might be greatly simplified by using eggshell membrane.

5. Conclusion

In the present study, mucoadhesive microspheres of acyclovir (ACV-ad-ms) were prepared by emulsion solvent evaporation method. Acv-ad-ms could remain in the stomach for an extended time and help ACV be better absorbed in the upper part of gastrointestinal tract. The preliminary pharmacokinetic study in rats showed the oral bioavailability of ACV in mucoadhesive microspheres was enhanced due to the prolonged residence time in stomach.

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