Development of Acyclovir-loaded Bovine Serum Albumin Nanoparticles for Ocular Drug Delivery

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Abstract

The aim of the present study was to develop acyclovir (ACV) ocular drug delivery systems of bovine serum albumin (BSA) nanoparticles as well as to assess their in vivo transcorneal permeability. The ACV-loaded nanoparticles with 4 % and 8 % drug loading were prepared by desolvation method. Morphology of the nanoparticles under scanning electron microscopy was spherical in shape and uniform in size. The mean sizes and entrapment efficiencies of ACV-loaded BSA nanoparticles were in the range of 125 - 132 nm and 15 - 25 %, respectively. Increasing the amount of ACV added into the formulation led to significant reduction of entrapment efficiency of nanoparticles. The results from in vivo transcorneal permeation studies revealed that ACV-loaded BSA nanoparticles could readily permeate through the rabbits' cornea and bring about maximum ACV concentrations within 30 min and prolonged till 120 min.

Keywords: Nanoparticles, Acyclovir, Bovine serum albumin, Transcorneal permeability, Ocular drug delivery.

Introduction

The major problems encountered with the ophthalmic drug delivery are rapid and extensive precorneal loss caused by drainage and high tear fluid turnover [1]. Only 1 - 2 % of the instilled dose penetrates the cornea and reaches intracellular tissues, while a major fraction of the instilled dose is often absorbed systemically via the conjunctiva and nasolacrimal duct. Strategies to optimize the residence time of the preparations in the precorneal area by the use of viscous solutions, bioadhesive polymers, suspension, ointments and inserts possess several inherent disadvantages including blurred vision or difficulties in toleration or application into the eye. Hence, an “ideal” formulation would be easily dispensed, prolong residence time in the eye, and exhibit a controlled drug delivery over an extended period of time [2]. This goal might be achieved by using particulate carrier systems such as liposomes, microparticles and nanoparticles in ophthalmic drug delivery [3,4]. System of nanoparticles is one of ocular drug delivery systems which has been developed under the idea “prolonged residence time”. One of the rationalizations in supporting the therapeutic efficacy of nanoparticles is their mucoadhesive properties. The prolonged residence time can appreciably increase biological response and/or decrease the administration frequency [5,6]. In recent years, ophthalmic drug-loaded nanoparticles developed from poly-d,l-lactic acid, i.e., acyclovir [5], and bovine serum albumin, i.e., hydrocortisone [7] and pilocarpine [3,8] have been receiving appreciable interest since they are biodegradable and biocompatible. The pharmacokinetics of poly-d,l-lactic acid particles showed a controlled drug release, which could be elicited by mucoadhesion of the delivery system to the eye surface, thus allowing a long residence time and prolonged release compared to a drug suspension. The increase in drug bioavailability provided by PEG-coated poly-d,l-lactic acid particles could also be due to an enhancer effect [9]. Various in vivo studies in rabbits reported the prolonged effect of drug (pilocarpine, piroxicam) incorporated in albumin particles compared to commercial preparations or aqueous and viscous solutions [10]. Besides, it was reported that coadministration of pilocarpine-loaded albumin nanoparticles with various mucoadhesive polymers provided an effective means to

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improve the miotic response as well as the intraocular pressure-lowering response compared to the aqueous dispersion of the nanoparticles [3,8].

Acyclovir, an antiviral drug with a highly specific activity against herpes viruses, is widely used in the treatment of various ocular viral diseases. In particular, herpes simplex keratitis (in the most severe cases) is characterized by the spread of the virus into the deeper corneal layers, leading to damage of the stromal cells [11,12]. Therefore, treatment requires a suitable permeation of the antiviral drug through the epithelium in order to reduce the virus load. The topical application of acyclovir in ointments is limited by the low corneal penetration of the drug and by its poor water solubility (0.2 mg/ml). Many attempts have been made to improve the ocular bioavailability and the therapeutic efficacy of acyclovir by incorporating into colloidal systems such as liposomes [13,14] and nanoparticles [5]. In this study, acyclovir-loaded bovine serum albumin nanoparticles were developed after which their physicochemical properties and in vivo transcorneal permeabilities were assessed.

Materials and Methods

Materials

Acyclovir micronized (ACV) was obtained from Zhejiang Wuyi Pharmaceutical Factory, China. Bovine serum albumin (fraction V) (BSA) was purchased from Merck, Darmstadt, Germany. Sodium phosphate monobasic dihydrate, sodium phosphate dibasic dodecahydrate, sodium chloride, and hydrochloric acid 37 % were from Labscan, Ireland. Atropine sulfate (T.P. Drug Laboratories, Thailand), xylazine hydrochloride (Troy Laboratories, Australia), isofurane USP (Halocarbon, USA), and zinc sulfate heptahydrate (BDH, England)

Preparation of ACV-loaded BSA nanoparticles

ACV-loaded BSA nanoparticles were prepared by the following desolvation technique under aseptic condition. BSA 500 mg together with 20 or 40 mg of ACV were dissolved in 10 ml 1:1 - ethanol : sterile water for injection (SWI), into which 15 ml of ethanol was submersibly pumped (Model 505S, Watson Marlow, USA) through a 1.0-mm spinal needle at 2 or 4 ml/min with continuous stirring at 500 rpm. The nanoparticles obtained were subject to hardening process at 60 °C for 2 hr under constant shaking in water bath, and finally purified by ultracentrifugation (Model Coulter LE-80K, Beckman, USA) at 32,000 rpm for 1 hr at 4 °C and pellet washing with SWI. After 5 times washing, the nanoparticles were resuspended in 20 ml SWI and kept at 4 °C until use.

Physicochemical characterization of ACV-loaded BSA nanoparticles

Microscopic appearance and morphology

Scanning electron microscopy (SEM) (Model JSM-5410LV, Jeol, Tokyo, Japan) was employed to examine the morphology and surface structure of ACV-loaded BSA nanoparticles. The samples were air-dried and covered with osmium tetroxide vapor approximately 1 hr for border contrast, which were subsequently dehydrated with ethanol in series and finally coated with a thin layer of gold. The magnification used was 35,000x.

Particle size

The particle sizes of ACV-loaded BSA nanoparticles were determined by photon correlation spectroscopy (PCS) using a submicron particle analyzer (Model N4MD, Coulter Corporate Communication, Hialeah, Florida, USA). The samples were measured at 25 ºC with scattering angle of 90°.

Drug entrapment

The amount of ACV entrapped in nanoparticles was determined by subtracting the initial amount of drug added into the system by the amount of free drug in the supernatant determined spectrophotometrically (DU 650i, Backman, USA) at 255 nm during the purification step. The drug entrapment efficiency of ACV-loaded nanoparticles was calculated as follows:

\[
\% \text{ Entrapment efficiency} = \frac{\text{Amount of drug loaded}}{\text{Initial amount of drug added into the system}} \times 100 \tag{1}
\]

In addition, the drug loading capacity of ACV-loaded nanoparticles was calculated by the following equation:

\[
\% \text{ Loading capacity} = \frac{\text{Amount of drug loaded}}{\text{Amount of carrier BSA}} \times 100 \tag{2}
\]

Drug leakage

At predetermined time intervals (0, 2, 4, and 8 weeks), 2 ml of ACV-loaded BSA nanoparticles dispersions was accurately pipetted into an ultrafiltration tube (30,000 MWCO) and then centrifuged (Universal 30 RF, Hettich, Germany) at 5,000 rpm for 30 min. The amount of drug in filtrate was analyzed spectrophotometrically (DU 650i, Beckman, USA) at 255 nm. The percentage of drug leakage from the nanoparticles was estimated according to the following equation:

\[
\% \text{ Leakage} = \frac{\text{Amount of drug leaked}}{\text{Amount of drug loaded}} \times 100 \tag{3}
\]
In vivo transcorneal permeation study of ACV-loaded BSA nanoparticles

Animal care
Male New Zealand white rabbits (National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand) weighing from 2.0 - 3.0 kg, free of any sign of ocular inflammation or gross abnormality, were used. Animal procedures were ethically approved by the Institutional Animal Care and Use Committee, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The approved number was No. 2/2550. In this study, the Laboratory Animal Center, National Institute of Health (NIH), Department of Medical Science, Ministry of Public Health, Thailand, was the animal house. The rabbits were in conventional animal room with strictly hygienic conditions. The temperature and humidity of the animal room were 23 - 27 °C and 60 - 90 % relative humidity, respectively. Two rabbits were in the same stainless steel wire bottom cage with the size of about 17 x 14 x 25 in. The sterile shaved wood was bedded in the cage and changed every 2 days. Standard diet food and tap water were the routine feeding. The veterinarian routinely checked for health of rabbits every morning. Anterior chamber centesis was the technique to collect the aqueous humor for drug analysis. The rabbit was used only one time (not duplicate) in all studies.

Irritation test
According to Guideline for the Testing of Chemicals No. 405 (Acute Eye Irritation/Corrosion) of OECD (Organization for Economic Co-operation and Development) [15], 100 µl of aqueous dispersion of ACV-loaded nanoparticles was applied in a single dose to one of the rabbit eyes. The untreated eye served as the control. The degree of eye irritation/corrosion was evaluated by scoring lesions of conjunctiva, cornea, and iris, at specific intervals. The experiment would be terminated at any time when the animal showed continuing signs of severe pain or distress. Observations were made at regular time intervals of 1, 24, 48 and 72 hours, and scores were compiled for redness and swelling of the eye’s conjunctiva, response of the iris to light, corneal opacity, and presence of discharge. These scores were compared with a classification table to determine which test materials were considered to be eye irritants. To determine reversibility of effects, the animals should be observed normally for 21 days post administration of the test substance. If reversibility was seen before 21 days, the experiment should be terminated at that time.

Transcorneal permeation study
ACV levels in aqueous humor were monitored at 30, 60, 90, and 120 min after administrating 50 µl of aqueous dispersions of ACV-loaded nanoparticles into the conjunctival sac of rabbit eyes. Fifteen minutes before paracentesis, the rabbits were preanesthetized by intramuscular injection with atropine sulfate at 0.5 mg/kg dose and xylazine HCl at 5 mg/kg dose. Then five minutes before paracentesis, 4 % isofurane in oxygen was used as inhalation anesthetics for 2 min and maintained with 2 % of isofurane in oxygen during paracentesis. The paracentesis was closely supervised and checked by the veterinarian. Aqueous humor (150 µl) was withdrawn through the limbus by a syringe with 27G needle and stored at -20 °C. The samples were treated with a solution of 2 % w/v ZnSO₄·7H₂O at the ratio of 1:1, in order to deproteinize the aqueous humor, and then vortex-mixed and centrifuged (RC 5C Plus, Sorvall, USA) at 10,000 rpm for 1 hr at -10 °C.

The supernatant was analyzed for ACV by high performance liquid chromatography (HPLC) equipped with a high precision pump (LC-10AT VP, Shimadzu, Kyoto, Japan), UV-VIS detector (SPD-10A VP, Shimadzu, Kyoto, Japan) and system controller (SCL-10A VP, Shimadzu, Kyoto, Japan). A C₁₈ reverse-phase column (Mightysil RP-18 GP Aqua 4.6 x 250 mm, Kanto chemical, Tokyo, Japan) was used at ambient temperature. The mobile phase was 0.1 % v/v glacial acetic acid solution at a flow rate of 1.5 ml/min. The sample injection volume was 50 µl and the detection wavelength was 255 nm. ACV solutions in isotonic phosphate buffer pH 7.4 and aqueous humor ranging from 0.05 - 0.25 µg/ml were used for the calibration curves with the r² of 0.9990 and 0.9790, respectively. The retention times of ACV in both media were about 14.7 min so that the runtime was 33 min. The accuracy of HPLC analytical method in terms of mean values (n = 3) of % recovery of ACV at 0.05, 0.15, and 0.25 µg/ml were 92.97 ± 1.83, 92.69 ± 1.16, and 93.79 ± 1.05 %, respectively. The precision of the assay procedure expressed as % CV’s (n = 3) at 0.05, 0.15, and 0.25 µg/ml were 2.21, 1.90, and 1.13 % for within-run, and 2.75, 2.02, and 1.78 % for between-run, respectively.

Results and Discussion

Preparation of ACV-loaded BSA nanoparticles
The ACV-loaded BSA nanoparticles were prepared by desolvation technique. The basic principle of this technique is based on the desolvation and resolvation properties of proteins. Upon addition of desolating agent such as ethanol, desolvation along with a progressive modification of the protein tertiary structure is induced, leading to the formation of protein aggregates when a certain degree of desolvation is obtained. The initially clear solution gradually turns opalescent as the protein becomes less soluble in water and tends to form nanometric aggregates of the desolvated protein, leading to a milky suspension. The drug molecules previously bound to the macromolecules by protein binding are entrapped within the polymer network prior to hardening by heat. The particle size of such ACV-loaded BSA nanoparticles was around 130 nm.

Physicochemical characterization of ACV-loaded BSA nanoparticles
Microscopic appearance and morphology
The scanning electron photomicrograph (SEM) of ACV-loaded BSA nanoparticles is shown in Figure 1, which appeared to be spherical in shape and nearly uniform in size. The size of BSA nanoparticles indicated by SEM was about 100 - 200 nm, which was subsequently confirmed by PCS.

Particle size
The mean sizes of ACV-loaded BSA nanoparticles produced from 3 batches with different amounts of ACV added and solvent pump rates are shown in Table 1, which were in the range of 125-132 nm. The reproducibility among 3 batches could be indicated. The nanosize range of BSA nanoparticles would ensure an intimately and effectively diffusive mixing between the bulk aqueous solution containing drug and the organic solvent jet stream containing polymer submergingly pumped into it. It can be seen that increase in quantity of ACV added into the formulation or slower solvent pump rate generally resulted in significantly larger nanoparticles (P < 0.05, ANOVA, LSD).

Drug entrapment
The values of entrapment efficiency and loading capacity of ACV-loaded BSA nanoparticles shown in Table 1 were in the ranges of 12.15 - 24.75 % and 0.86 - 1.21 %, respectively. The reproducibility among 3 batches could also be observed. The entrapment efficiency was significantly increased with the reducing amount of ACV added into the formulation or more rapid solvent pump rate (P < 0.05, ANOVA, LSD). This may be due to mixing behavior between the bulk aqueous solution containing drug and the organic solvent jet stream containing polymer submergingly pumped into it. It is stated that the volume of liquid entrained at the nominal boundary of the jet per unit time, causing the mixing in action, is proportional to the volume of liquid leaving jet nozzle per unit time [16]. Therefore, the mixing rate became increasing as the solvent pump rate was increased, which in turn resulted in ACV being adsorbed or embedded at a higher extent into the precipitated BSA nanoparticles.

Drug leakage
The % drug leakages from all formulations of ACV-loaded BSA nanoparticles upon storage at 4 ºC for 2, 4, and 8 weeks were found to be less than 0.9 %, which was the minimal detectable limit. Neither alteration in physical appearance nor aggregation was observed for all formulations of nanoparticles upon storage up to 2 months.

In vivo transcorneal permeation study of ACV-loaded BSA nanoparticles
The ophthalmic colloidal formulations of ACV-loaded BSA nanoparticles chosen for further in vivo transcorneal permeation study in rabbits were those with 1.20 % loading capacities, the ACV concentration of which was 300 µg/ml.

Irritation test
Signs of irritation according to OECD guideline reported in terms of scores of irritation for ACV-loaded BSA nanoparticles are shown in Tables 2. It was found that all selected formulations of nanoparticles did not irritate the rabbit eyes so that further in vivo permeation study through rabbit cornea could be continued.

Transcorneal permeation study
The concentrations of ACV in rabbits’ aqueous humor at various time intervals after administrations of colloidal formulations of ACV-loaded BSA nanoparticles are shown in Figure 2. ACV-loaded BSA nanoparticles could readily permeate through the cornea into aqueous humor, whose maximum concentration about 18 ng/ml was reached within 30 min after administration. The drug concentrations could still be prolonged at this level up to 90 min, and then gradually declined till 120 min passed. The average values of in vivo “corneal permeability coefficient” (CPC) at 60 min after administrations of all ophthalmic formulations of ACV-loaded BSA nanoparticles studied was found to be 0.926 x 10⁻⁸ cm/sec.

Conclusions
ACV-loaded BSA nanoparticles could be reproducibly prepared by desolvation method. The nanoparticles obtained exhibited spherical shape and uniform nanosize about 100-200 nm with loading capacity of around 1-4 %. The in vivo transcorneal permeability of drug-loaded nanoparticles was shown to bring about adequate concentration in aqueous humor.

Authors’ contributions
PN prepared and characterized the physicochemical properties of ACV-loaded BSA nanoparticles. AP helped determine the entrapment of ACV-loaded BSA nanoparticles and draft the manuscript. WR conducted the in vivo transcorneal permeation study of ACV-loaded BSA nanoparticles. NS gathered and interpreted all the results as well as prepared the final manuscript.

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References


Figure 1. Scanning electron photomicrograph (SEM) of ACV-loaded BSA nanoparticles with 4 % drug loading. (35,000x magnification)

Figure 2. Concentration profiles of ACV in rabbits’ aqueous humor after administrations of colloidal formulations of ACV-loaded BSA nanoparticles with ACV concentration of 300 µg/ml. Each point and bar represents mean and standard deviation from 3 determinations, respectively.
Table 1. Particle size, entrapment efficiency, and loading capacity of ACV-loaded BSA nanoparticles with different drug loadings and solvent pump rates

<table>
<thead>
<tr>
<th>ACV loading</th>
<th>Solvent pump rate (ml/min)</th>
<th>Mean ± SD [^a]</th>
<th>Particle size (nm)</th>
<th>Entrapment efficiency (%)</th>
<th>Loading capacity (%)</th>
</tr>
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<tr>
<td>4 %</td>
<td>2</td>
<td>132 ± 8</td>
<td>21.46 ± 0.07</td>
<td>0.86 ± 0.00</td>
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<tr>
<td></td>
<td>4</td>
<td>126 ± 4</td>
<td>24.75 ± 0.11</td>
<td>0.99 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>8 %</td>
<td>2</td>
<td>125 ± 10</td>
<td>12.15 ± 0.17</td>
<td>0.97 ± 0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>131 ± 10</td>
<td>15.05 ± 0.10</td>
<td>1.20 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

\[^a\] Average from 3 determinations of 3 batches

Table 2. Score of irritation test for ACV-loaded BSA nanoparticles

<table>
<thead>
<tr>
<th>Time interval (hr)</th>
<th>Eye-side of administration</th>
<th>Score</th>
<th>Cornea</th>
<th>Iris</th>
<th>Conjunctivae</th>
<th>Chemosis</th>
</tr>
</thead>
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<td>1</td>
<td>L = No drug administration</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td>R = BSA nanoparticles</td>
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<td>0</td>
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<tr>
<td>24</td>
<td>L = No drug administration</td>
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<td>0</td>
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<tr>
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<tr>
<td>48</td>
<td>L = No drug administration</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>R = BSA nanoparticles</td>
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<td>72</td>
<td>L = No drug administration</td>
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</tr>
<tr>
<td></td>
<td>R = BSA nanoparticles</td>
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