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Studies on Acyclovir–Dextran Conjugate: Synthesis and Pharmacokinetics

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ABSTRACT

Acyclovir is an antivirus drug which has a good in vitro activity against hepatitis B virus. But because of the low solubility and low distribution in liver, the clinical application of acyclovir in hepatitis B was limited. To increase the solubility and the distribution in liver, acyclovir–dextran conjugate was synthesized by formation of Schiff's base. The solubility of obtained conjugate was 12 times greater than free acyclovir. Acyclovir will be slowly released from the obtained conjugate in pH 7.4 phosphate buffer solution (PBS) at 37°C with a rate constant of 0.0035 hr^{-1}. Pharmacokinetic studies of acyclovir and acyclovir–dextran conjugate were conducted in mice by i. v. administration. Acyclovir concentrations in plasma, liver and kidney were determined by HPLC method. Relatively higher distribution of acyclovir in liver was observed when i. v. acyclovir–dextran conjugate as compared with i.v. free acyclovir. The results of pharmacokinetic studies indicated that acyclovir–dextran conjugate will be a good candidate to treat hepatitis B.

Key Words: Acyclovir; Dextran; Acyclovir–dextran conjugate; Schiff's base.

INTRODUCTION

Acyclovir (Aciclovir) is a nucleoside antivirus drug with effective activity against members of the herpes group of DNA viruses, hepatitise B virus, etc. However, its clinical potential in the treatment of hepatitise B is limited by the low solubility of acyclovir, the severe renal toxicity and low distribution in liver caused by its poor water solubility.[1,2]

Recently, dextrans have been investigated as macromolecular carriers for delivery of drugs and proteins. It is demonstrated that dextrans may be of value in targeting therapeutic agents to the liver.[3] In this study, we aimed to increase liver distribution of acyclovir through conjugate with dextran. Acyclovir was coupled to dextran via an imine bond to the hydroxyl groups of dextran after periodate oxidation to form a Schiff's base. The pharmacokinetic

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characteristics of acyclovir–dextran conjugate in mice were investigated.

MATERIALS AND METHODS

Materials

Acyclovir was purchased from Xinxiang Pharmaceutical Co. Ltd, Henan Province, China; Dextran 40 (Mw 32,000∼42,000) was received from Huaiyin No.2 Pharmaceutical Co. Ltd, Jiangsu Province, China; Sodium periodate and Sephadex G-50 were supplied by Shanghai Chemical Co. Ltd, China; Sodium 1-Heptanesulfonate was obtained from Nippon Kayaku Co, Ltd, Japan; Dialysis tube with cut-off 1 kDa was obtained from sigma Chemical Co. Ltd, U. S. A.; Methanol is of HPLC grade. All other chemicals are of analytical grade.

Synthesis of Acyclovir–Dextran Conjugate

Oxidation of Dextran

0.4 g of dextran 40 was dissolved in 50 ml of water and mixed with 1.0 g of sodium periodate. The mixture was stirred for 4 h at room temperature in the dark. The degree of oxidation of dextran was determined by titrating of formic acid with a 0.01 N NaOH. At the end of the oxidation, 10 ml of ethylene glycol was added to remove excess periodate. To eliminate all the $NaIO_4$ and $NaIO_3$, obtained solution was dialyzed against DI water until the KI test was negative. The resulting solution was lyophilized under $-55^{\circ}C$ and 0.02 mbar for 24 hours using Christ Alphal-2 Freeze Dryer (Martin Christ Gefriertrocknungsantagen GmbH, Germany).

Conjugation of Acyclovir to the Activated Dextran

0.7 g of acyclovir was dissolved in 50 ml of 0.1 N NaOH. Then, 0.4 g of oxidized dextran (dissolved in 50 ml H_2O) was added, giving an approximate molar ratio of 2:1 (acyclovir : glycoside unit). The mixture was adjusted to pH 10 with the addition of 0.1 N NaOH and stirred for 6 h at room temperature in the dark. The resulting solution was concentrated to 2 ml by vacuum evaporation in a rotating evaporator (ZFQ85A Rotatory Evaporator, Shanghai Medical Instruments Company, China) under $20^{\circ}C$ and 0.1 MPa. The concentrated solution was applied to a gel filtration column (Sephadex G-50 column, 50 cm × 2.5 cm) with the elution with pH 7.4 sodium phosphate buffer (PBS). The flow rate of elution buffer was 1 ml/min and the effluent was monitored at 252 nm by 752C UV-spectrophotometer (Shanghai No. 3 Analytical Equipment Works). High-molecular weight fractions (acyclovir–dextran conjugate) were collected and lyophilized as the conjugate product. UV spectra and IR spectra of acyclovir, dextran and acyclovir–dextran conjugate were recorded.

Purity of the Conjugate Product, Bound Acyclovir Content in the Conjugate Product

Acyclovir content of the conjugate was determined by HPLC with a Waters 515 HPLC pump, Waters 2487 Dual Absorbance Detector and SR2000 HPLC Data Processing Workstation (Shanghai Sanrui Technology Co. Ltd). Chromatographic separations were achieved using Lichrospher RP18 (4.6 mm × 250 mm, 5 μm, Huaiyin Hanbang Sci-Tech Co., Ltd., China). The mobile phase was a mixture (4:96, v/v) of methanol and DI water. The flow rate of the mobile was maintained at 1.0 ml/min, and the effluent was monitored at an ultraviolet detection wavelength of 252 nm.

180 mg of conjugate were weighed and dissolved in 5 ml of DI water as the stock solution of conjugate. 200 μg of acyclovir were weighed and dissolved in 5 ml of DI water as the stock solution of acyclovir.

For determination of the purity of the conjugate product, 1 ml of the stock solutions were diluted to 2 ml with DI water and then detected by HPLC. Unbound acyclovir in the conjugate product was determined and the purity of the conjugate product was calculated as follows:

$$\text{Purity}\% = 100\% - (\text{A}/\text{C} \times 100\%) \quad (1)$$

where A is the amount (mg) of unbound acyclovir in a certain amount (C, mg) of the conjugate product.

Bound content of acyclovir in the conjugate was determined through elemental analysis. Because there is no nitrogen in dextran, bound content of acyclovir was calculated according the nitrogen content. As an alternate, hydrolysis method was used to determine the bound content of acyclovir in the conjugate product. One milliliter of the stock solutions were diluted to 2 ml with DI water and HCl to make final HCl concentration to 1.0 N, stored in room temperature for

12 hours. The resulting solutions were detected by HPLC. The bound content of acyclovir was calculated by:

$$\text{Bound content}\% = B/M \times 100\% \quad (2)$$

where B is the determined bound acyclovir (mol) after hydrolysis. M is the glucoside monomer amount (mol).

In Vitro Drug Release

Acyclovir–dextran conjugate (100 mg) was dissolved in 10 ml of phosphate buffers of pH 2.5, 7.4 and ammonia buffer of pH 10 (China Pharmacopeia 2000), respectively. Because the aqueous solubility of acyclovir is not high enough to prevent precipitation of acyclovir during in vitro experiments, especially in neutral condition, obtained solutions were dialyzed to separate the released acyclovir from the conjugate. The solution was put into dialysis sac (dialysis membrane of cut-off 1 kDa, sigma Chemical Co. Ltd, U. S. A.) and dialyzed against 60 ml of the same buffer solution as inside the dialysis sac in 37°C under shaking (100 rpm, THZ-C Incubation Vibrator, Taicang Experimental Equipment Company, Jiangsu Province, China). One milliliter of solution outside the sac were taken at predetermined time interval 2,8,12,24,48 hours and analyzed by HPLC. HPLC analysis was conducted as the condition as mentioned above.

The percentage of acyclovir released from the conjugate was calculated as the cumulative amount of acyclovir released divided by the total amount of acyclovir contained in the conjugate. The rate constants under different media were estimated by

$$\text{Log } C = a + k \times t$$

where C is the percentage of acyclovir released from the conjugate, k is the release rate constant.

In Vivo Disposition

Adult mice (20±2 g) (Experimental Animal Center of China Pharmaceutical University) were divided randomly into 22 groups. Each group consisted of six mice. 11 groups were acyclovir group, others were acyclovir–dextran conjugate group. 0.128 g of acyclovir was dissolved in 20 ml of physiological saline, giving an acyclovir concentration of 6.4 mg/ml. Several drops of 1 N NaOH were added to help the acyclovir dissolve completely. The pH of the solution was controlled at less than 9. 1.0 g of the conjugate was dissolved in 13.5 ml of physiological saline, giving an acyclovir concentration of 6.4 mg/ml. Acyclovir or acyclovir–dextran conjugate solution was injected through the tail vein of the mice at a dose of 65 mg/kg of acyclovir. At predetermined time interval of 0.25, 0.5, 0.75, 1, 1.33, 1.75, 2, 4, 6, 12, 24 hours after the administration, mice were exsanguinated and were sacrificed. The heart, liver, spleen, lung, kidney, brain, stomach, muscle were removed and weighed. Plasma was harvested by centrifugation at 4000 r/min for 15 min and analyzed by HPLC. Tissue samples were homogenized on a high-speed tissue comminuter (T25 high-speed tissue comminuter, IKA-Labortechnikstaufen, Japan). Proteins were precipitated by adding 1 ml 5% $HClO_4$. The samples were vortexed for 5 minutes, then centrifuged at 4000 r/min for 15 min. The supernatant was analyzed by HPLC.[4]

The chromatographic system used in determining the concentration of acyclovir in plasma and tissue supernatant was a Waters 515 HPLC pump, Waters 2487 Dual λ Absorbance Detector. Chromatographic separations were achieved using Lichrospher RP8 (4.6 mm × 250 mm, 5 μm, Huaiyin Hanbang Sci-Tech Co., Ltd., China) and a guard column (Waters, 4.0 mm × 4.0 mm). The mobile phase was a mixture (4:96, v/v) of methanol and aqueous 0.05 M sodium acetate solution containing 0.0025 M sodium 1-heptanesulfonate. The flow rate of the mobile was maintained at 1.0 ml/min, and the effluent was monitored at an ultraviolet detection wavelength of 252 nm. The column temperature was kept at 40°C.

Pharmacokinetic analysis was carried out with pharmacokinetic software, 3P97 (China Association of Pharmacology, Beijing, China). The plasma concentration—time profiles of acyclovir after i.v. acyclovir were fitted with two-compartment model, whereas those after i.v. the conjugate were fitted with two-compartment model with a release (absorption) phase. In the compartmental fitting, the weighting factor was 1/C (C is the experimental plasma concentration of acyclovir). The release (absorption) rate constant of acyclovir from the conjugate and the half-life of release were calculated. The areas under the concentration—time curve from time zero to time t (AUC_{0-t}) were determined by the trapezoidal rule. The areas from time t to infinity (AUC_{0-8}) were estimated according to the equation:

$$AUC_{0-\infty} = AUC_{0-t} + Ct/k$$

where Ct is the plasma concentration observed at time t, and k is terminal elimination rate constant of

acyclovir obtained from the slope of the linear portion of the curve by least square regression analysis.

RESULTS AND DISCUSSION

Synthesis of Acyclovir–Dextran Conjugate

Dextran conjugates can be synthesized by either direct conjugation or through a spacer molecule.[5] Drugs containing carboxylic acid group can be directly conjugated to dextran through formation of esters (e.g. the reaction between NSAIDs and dextrans). Conjugation of dextrans with most drugs lacking a carboxylic acid group requires activation of dextran molecule before the reaction can take place. For drugs containing amine group, drug can be conjugated with oxidized dextran and form Schiff's base. Because acyclovir contains amine group, acyclovir could be coupled with dextran via Schiff's base.

Acyclovir–dextran conjugate was synthesized by a two-step procedure (Fig. 1). Firstly, Dextran was oxidized by sodium periodate as the method reported by Kjell.[6] Then, Schiff's base between acyclovir and oxidized dextran were formed directly. Because Schiff's base is stable under basic condition, conjugation of acyclovir to oxidized dextran was conducted in 0.1 N NaOH solution.

To get rid of the free acyclovir from the conjugate, gel filtration separation was conducted. Absorbance of each fraction of the effluent was monitored by UV-spectrophotometer at 252 nm. Acyclovir–dextran conjugate and unconjugated acyclovir can be separated by gel-filtration on a Sephadex G-50 column. The conjugate portion was freeze-dried, giving a yellowish fluffy powder.

Acyclovir–dextran conjugate was identified by comparing UV and IR spectrum of acyclovir, dextran, oxidized dextran and acyclovir–dextran, respectively. No maximum absorption wavelength can be identified for dextran solution within the the range between 220 nm to 600 nm. The maximum absorption wavelengths (λ max) of and acyclovir–dextran in water were 222 nm and 266 nm, as compared with 226 nm for oxidized dextran and 252 nm for acyclovir. The

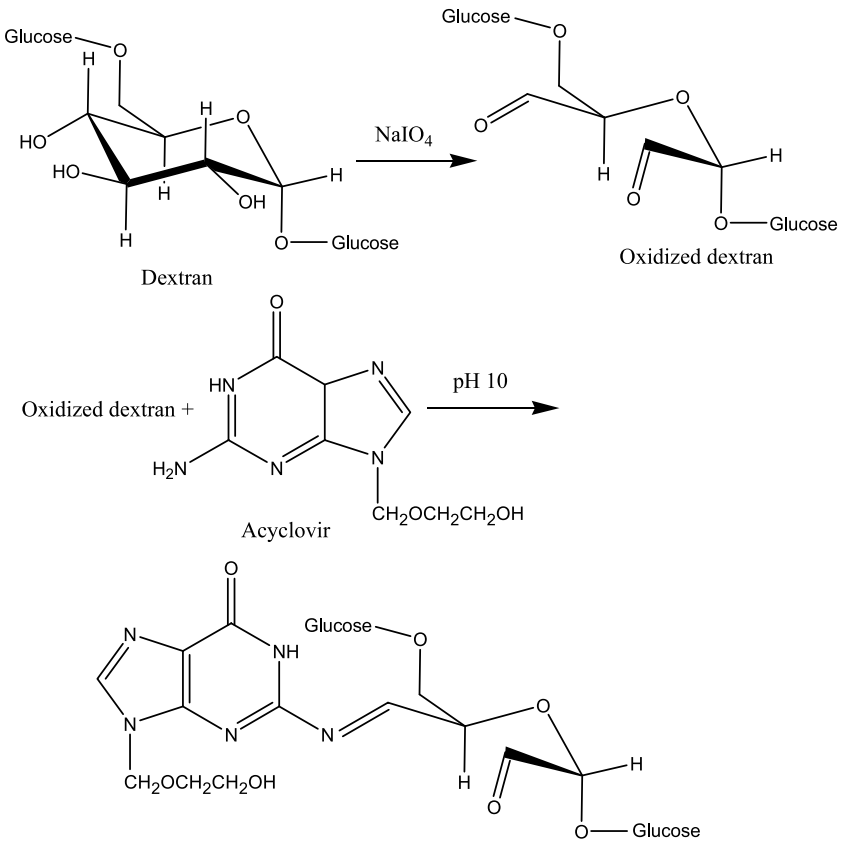

Figure 1. The schematic presentation of the conjugate reaction.

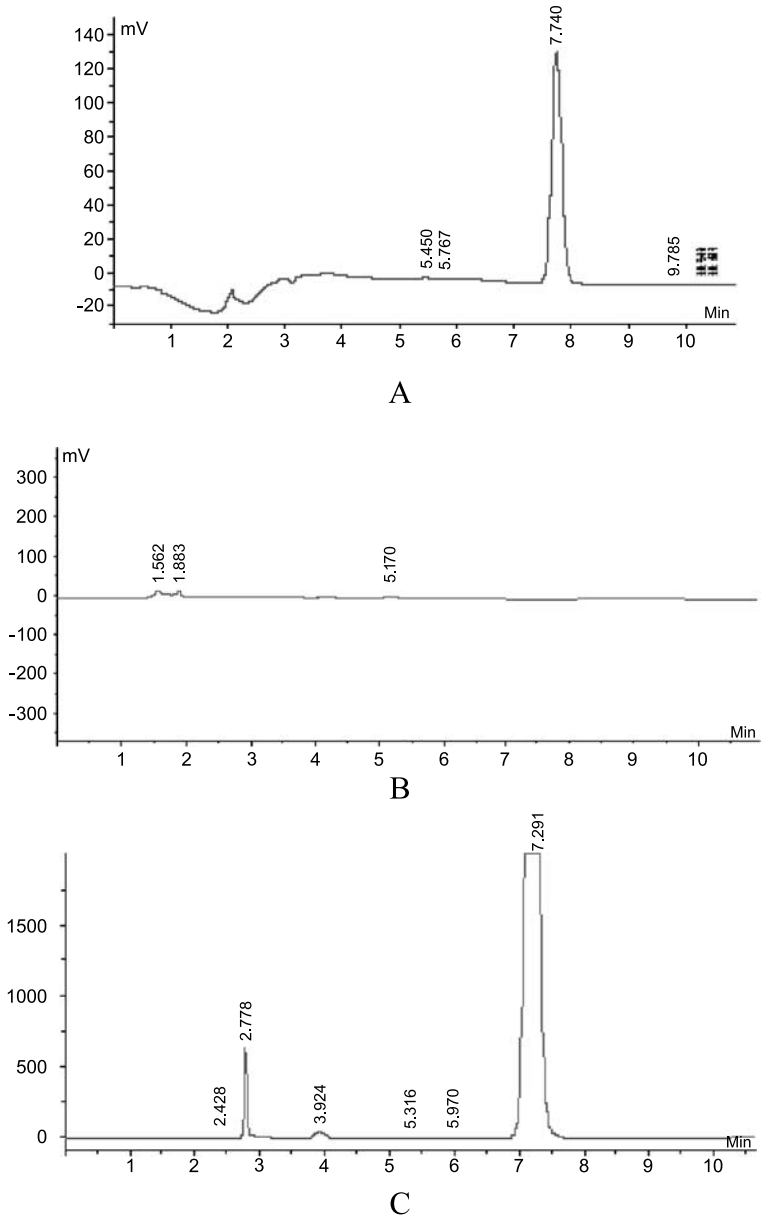

Figure 2. HPLC diagrams of acyclovir–dextran conjugate. A. Acyclovir; B. Acyclovir–dextran conjugate; C. Hydrolyzed products of B under pH 1.0 at room temperature for 12 hours.

change of ëmax of acyclovir and oxidized dextran might result from the formation of an imide bond, indicating that acyclovir–dextran conjugate had formed. In FT-IR spectrums of acyclovir, oxidized dextran, and the conjugate, shift of peaks of C=N and N–H were observed, which confirmed the formation of Schiff's base between acyclovir and oxidized dextran. Elemental analysis of conjugate indicated that N, C, H content in conjugate was determined as 2.12%, 33.83% and 5.20% respectively. According to elemental analysis, the conjugated content of acyclovir was calculated as 6.9%, that is bound acyclovir content was 6.9% (mol/mol).

Acyclovir–dextran conjugate before and after hydrolysis in 1 N HCl were analyzed by HPLC method. The HPLC diagram was shown in Fig. 2 in which conjugate showed no obvious peak (Fig. 2B) and the retention time of free acyclovir was 7.3 min (Fig. 2A). Acyclovir was released from the conjugate in pH 1.0 HCl solution (Fig. 2C). The purity of conjugate was determined as 98.3%. The results indicated

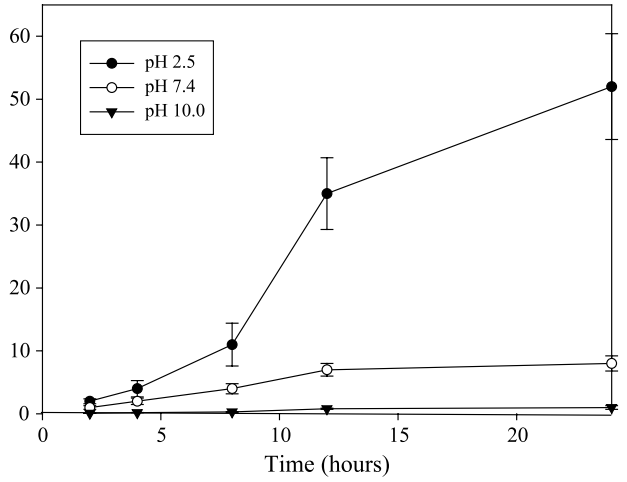

Figure 3. In vitro release profiles of acyclovir from the conjugate in different release media (n=6).

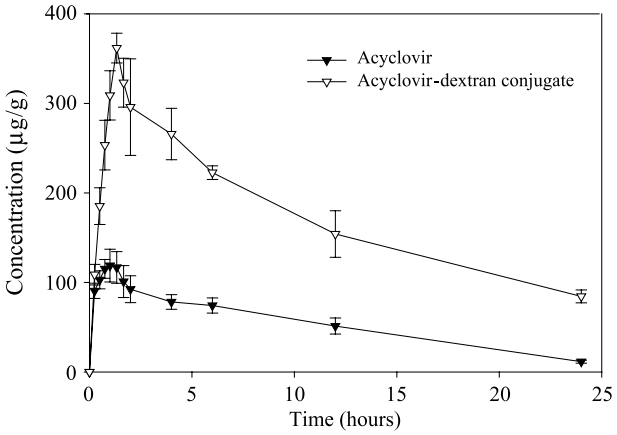

Figure 5. Concentration—time profile of acyclovir in liver after i.v. acyclovir and conjugate.

that gel filtration was an effective purification method for conjugate. The bound content of acyclovir in conjugate was determined as 7.4% (mol/mol) by hydrolysis method which is close to the result of element analysis, indicating acyclovir was completely released from the conjugate after hydrolysis in pH 1.0 HCl solution under room temperature for 12 hours.

In Vitro Drug Release

The release profile of acyclovir from conjugate was shown in Fig. 3. The release data was fitted well with first order kinetics. The rate constant was calculated as 0.0345 h^{-1} at pH 2.5 phosphate buffer solution (PBS), 0.0035 h^{-1} at pH 7.4 PBS, 0.0004 h^{-1} at pH 10.0 ammonia buffer solution, respectively. The release rate of acyclovir increased with decrease in pH. The results indicated the in vitro release of acyclovir from the conjugate was catalyzed by acid. Under pH 10.0, the percentage of acyclovir released from conjugate was not greater than 1% within 24 hours, indicating the conjugate is stable in basic condition. The half-life of the conjugate was estimated as 1505 hours at pH 10. The half-life of the conjugate was estimated as 200.6 and 20.1 hours at pH 7.4 and 2.5, respectively. Because of good stability of the conjugate under physiological pH, it is possible to deliver the conjugate to liver before acyclovir is released.

In Vivo Disposition

To elucidate the pharmacokinetic profiles of the conjugate, in vivo disposition of the conjugate was

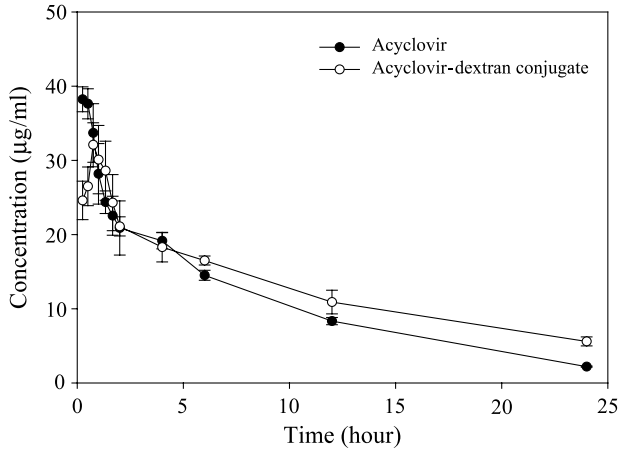

Figure 4. Plasma concentration—time profile of acyclovir after i.v. acyclovir and conjugate.

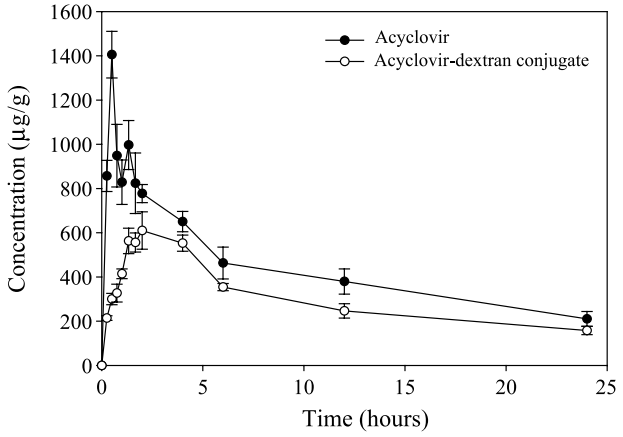

Figure 6. Concentration—time profile of acyclovir in kidney after i.v. acyclovir and conjugate.

investigated in mice. Figure 4 showed the acyclovir concentration—time profiles in plasma after i. v. administration of free acyclovir and acyclovir–dextran conjugate. The plasma concentration—time profiles of both acyclovir and the conjugate fit well with two-compartment model. Acyclovir plasma concentration after i.v. the conjugate showed a clear and continuous increase for more than 1 hour and then decreased, indicating that there is a release phase after i.v. the conjugate, which just like the absorption phase in oral administration. For conjugate, the half-life of release phase was calculated as 8.28 hours, and the terminal biological half-life was 14.47 hours, whereas the terminal biological half-life of acyclovir was 6.99 hours. The in vivo release half-life (8.28 hours) is much shorter than the in vitro release half-life (200 hours). This phenomenon might due to enzyme-catalyzed release of acyclovir in liver and will be beneficial to hepatic targeting.

The acyclovir concentration—time profiles in liver and kidney after i. v. administration of free acyclovir and acyclovir–dextran conjugate was illustrated in Figs. 5 and 6. Cmax of acyclovir in kidney is almost 14 times as the Cmax in liver after i.v. free acyclovir. The results confirmed the accumulation of acyclovir in kidney, which could be considered as the main reason of renal toxicity of acyclovir. However, Cmax of acyclovir in kidney is only 1.5 times as the Cmax in liver after i.v. the conjugate. The Cmax of acyclovir in kidney after i.v. conjugate is almost one third of that after i.v. free acyclovir, while the Cmax of acyclovir in liver after i.v. conjugate is almost as four times as that after i.v. free acyclovir. The areas under the concentration—time curve from time t to infinity ($AUC_{0-\infty}$s) of acyclovir in liver and kidney were determined by the trapezoidal rule developed by Bailer.[7] The differences of $AUC_{0-\infty}$s between free acyclovir and the conjugate in different tissues were statistically analyzed. The results (as shown in Table 1) indicated that the conjugate had significantly higher distribution in liver ($p<0.001$) and plasma ($p<0.01$) and lower distribution in kidney ($p<0.001$) as compared with free acyclovir. The results proved that conjugation with dextran could dramatically alter the disposition properties of acyclovir. The increased liver distribution and decreased kidney distribution of acyclovir would be beneficial to treat hepatitis B and reduce the renal toxicity of acyclovir.

Table 1. AUCs of acyclovir in plasma, liver, and kidney after i.v. acyclovir and conjugate.

	AUC (μg.h/g or μg.h/ml) $\bar{X} \pm SD$ (N=6)		
Organ	Free acyclovir	Conjugate	p
Plasma	279.5±55.6	384.6±22.1	$p<0.01$
Liver	1397.1±95.9	5494.0±252.2	$p<0.001$
Kidney	13728.4±946.2	9704.9±533.7	$p<0.001$

CONCLUSION

In present study, we synthesized a novel macromolecular prodrug of acyclovir, acyclovir–dextran conjugate, which had an extended circulating time in blood, less accumulation in kidney and to some extent liver targeting. It's the first report on liver targeting of acyclovir by macromolecular conjugation. Obtained conjugate showed good stability in basic solution. The acyclovir–dextran conjugate may have improved therapeutic potentials.

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