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Research Article

A Novel Oral Preparation of Hydroxysafflor Yellow A Base on a Chitosan Complex: A Strategy to Enhance the Oral Bioavailability

Guan-nan Ma,[1,2] Fang-lin Yu,[2] Shan Wang,[3] Zhi-ping Li,[2] Xiang-yang Xie,[2] and Xing-guo Mei[2,4]

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Abstract. Hydroxysafflor yellow A (HSYA), the main active pharmaceutical ingredient of the safflower plant (*Carthamus tinctorius* L.), is a hydrophilic drug with low oral bioavailability (BA). The objective of the present study was to improve the oral BA of HSYA by formulation design. The effect of several pharmaceutical excipients on enhancing BA, including Poloxamer 188 (P188), sodium caprate (SC), sodium deoxycholate, and β-cyclodextrin (β-CD), was investigated through animal models. Sodium caprate, with a relative BA of 284.2%, was able to improve the oral BA of HSYA. Furthermore, HSYA can bind with chitosan (CS) by Coulomb attraction and form a HSYA-CS complex. The preparation process was optimized, and the binding rate reached 99.4%. HSYA granules were prepared using a HSYA-CS complex and SC. The results of the pharmacokinetics showed that the relative BA of HSYA granules was 476%, much higher than HSYA/SC.

KEY WORDS: absorption enhancer; chitosan; hydroxysafflor yellow A (HSYA); oral bioavailability.

INTRODUCTION

Hydroxysafflor yellow A (HSYA) is the main active pharmaceutical ingredient of safflower, a traditional Chinese drug. HSYA has the effects of inhibiting coagulation and hypoxia, lowering blood pressure, and improving cardiovascular and cerebrovascular insufficiency; thus, it has been used clinically to treat cardiovascular and cerebrovascular diseases, such as myocardial ischemia, brain ischemia, coronary heart disease, and cerebral thrombosis (1).

HSYA is a type of chalcone and flavone. Its molecular formula (the molecular weight is 612.5) is shown in Fig. 1 (2). According to the biopharmaceutics classification system (BCS), HSYA is a class III drug with low membrane permeability and high water-solubility, which is most likely due to the hydroxy groups in its molecular structure. To date, only the injection of HSYA has been widely used in clinical therapy (3,4).

The main reason for the low oral bioavailability (BA) of HSYA is its low liposolubility, which can hinder its penetration across the epithelial monolayer. Some pharmaceutical excipients have been shown to enhance the permeability of BCS III drugs, such as sodium caprate (5), sodium deoxycholate (6), β-CD (7), and P188. Therefore, the above five excipients were selected to investigate their ability to enhance the oral BA of HSYA.

Furthermore, because chitosan is the only basic polysaccharide in nature that is positive charged, the properties of its bioadhesion and absorption enhancement make it a suitable carrier in mucosal drug delivery systems (8). These special properties could be utilized to prepare a HSYA-CS complex. Then, HSYA granules, which contain the HSYA-CS complex and a penetration enhancer, were prepared. Thus, many drug release cores will be formed and distributed on the surface of intestinal epithelium after these granules have been administered. A portion of the amino groups of each chitosan (CS) molecule bound with HSYA, and another portion of the amino groups, could adhere to the negatively charged intestinal mucosa by electrostatic interaction (9). Therefore, the action time and intensity between HSYA and the intestinal epithelia would be extended and strengthened. At the same time, the tight junctions of the intestinal cell monolayer would be opened or the permeability of intestinal cell would be increased due to the action of the absorption enhancer (10); thus, HSYA could permeate into the cell membrane, which could significantly enhance the absorption of HSYA. Based on the above idea, the preparation process of HSYA-CS was studied and optimized, and then a suitable dosage form was prepared, which would mainly include the HSYA-CS complex and the optimal absorption enhancer.

Guan-nan Ma and Fang-lin Yu contributed equally to this work.

[1] School of Chemical Engineering & Pharmacy, Wuhan Institute of Technology, Wuhan, People's Republic of China.
[2] Department of Pharmaceutics, Beijing Institute of Pharmacology and Toxicology, Haidian District, Beijing, People's Republic of China.
[3] Department of Pharmacy, Hubei Provincial Corps Hospital, Chinese People's Armed Police Forces, Wuhan, People's Republic of China.
[4] To whom correspondence should be addressed. (e-mail: ddsamms606@163.com)

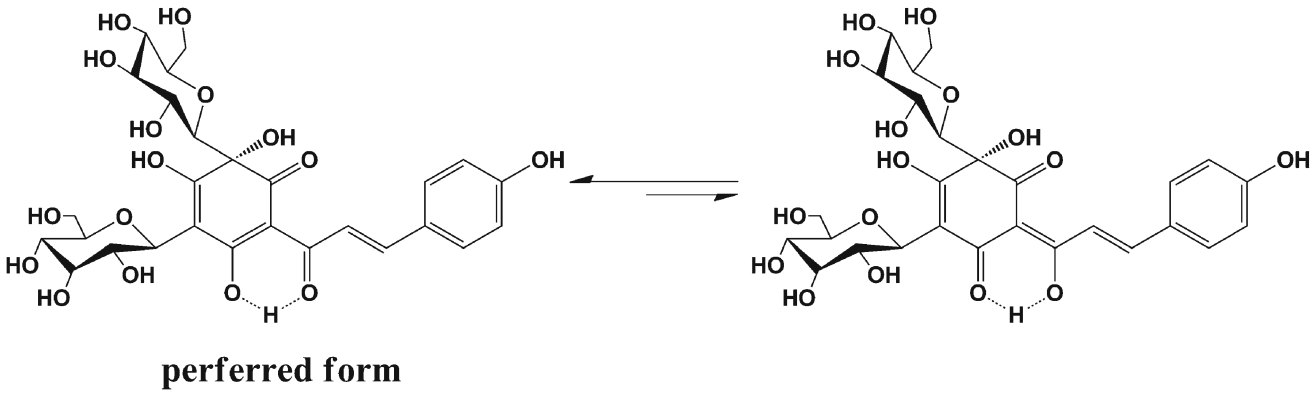

Fig. 1. Chemical structure of hydroxysafflor yellow A (HSYA) in solution

MATERIALS AND METHODS

Materials

Chemicals and Reagents

HSYA (85%) was supplied by Zhejiang Yongning Pharma (Yongning, Zhejiang, China). Sodium caprate (SC), trichloroacetic acid, and sodium polyphosphate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Four kinds of chitosan with molecular weights of 2, 4, 50, and 150 kDa were purchased from Nantong Xingcheng Biochemical Products Factory (Nantong, Jiangsu, China), and the deacetylation degree was more than 90% for all of them. Sodium deoxycholate was obtained from Acros Organics (New Jersey, USA). Poloxamer 188 and β-CD were purchased from Beijing Fengli Jingqiu Commerce Trade Co., Ltd. (Beijing, China). All other chemicals were of analytical grade and used as received.

Animals

Male Sprague-Dawley rats (180–220 g) were obtained from the Academy of Military Medical Sciences Laboratory Animal Center and housed under standard conditions. The rats were fed a standard laboratory diet and water. The procedures adhered to the recommendations provided by the Regulations for the Administration of Affairs Concerning Experimental Animals and were approved by the institutional animal care and use committee, National Beijing Center for Drug Safety Evaluation and Research (certification number: IACUC-2012-026, approval date: 2012-06-08). Before the experiments, all of the animals were fasted overnight (12 h) but had free access to water throughout the experimental period.

Methods

HPLC Analysis of Plasma Samples

Chromatographic conditions were performed according to the literature (11), with some modifications. The samples were detected by an Agilent 1200 HPLC system with Agilent Eclipse Plus C18 (4.6 mm×150 mm, 5 μm). The mobile phase consisted of a 0.76% potassium dihydrogen phosphate aqueous solution, methanol, and acetonitrile (77:5:18). The flow rate was 0.8 ml/min. The column oven temperature was 35°C and detection was performed at a wavelength of 403 nm.

Preparation of the CS-HSYA Complex and Characterization

HSYA and CS were accurately weighed at a mass ratio of 1:2, and then water was added to form a solution with the concentration of 100 mg/ml by HSYA. Then, the solution was stirred in a thermostatic water bath for an extended amount of time. HSYA and chitosan combined together and formed a complex under this condition. A purified complex was obtained after the process of freeze-drying and purification. Differential scanning calorimetry (DSC) was used to characterize the complex.

Influencing Factors to the Formation of HSYA-CS Complex

The yield of HSYA-CS complex was affected by several factors, including the molecular weight of CS, pH value, ratio of HSYA to CS, temperature, and reaction time. Therefore, some experiments were designed to inspect how these factors affected the binding rate of the HSYA-CS complex. The different levels for each factor were listed in Table I.

Determination of the Binding Rate

The process of purification was critical to determine the binding rate of HSYA to CS. HSYA easily dissolved in ethanol, but CS and the HSYA-CS complex did not. According to this principle, ethanol was added to the powder, which was obtained after freeze-drying, then vortex-mixed, and centrifuged. The free HSYA was dissolved in ethanol but the HSYA combined with CS was precipitated. The amount of free HSYA and HSYA combined with CS was detected by UV spectrophotometer at 403 nm, and the binding rate was calculated according to the formula below:

Table I. Levels for Each Factor of Binding Rate

Factors	Level
Molecular weight of CS	2, 4, 50, and 150 kDa
pH value	1.0, 3.0, 4.0, 5.0, 6.0, and 7.2
Ratio of HSYA to CS	1:1, 1:2, and 1:3 (mass ratio)
Concentration of HSYA	100, 200, 300, and 400 mg/ml
Temperature	30, 40, and 50°C
Reaction time	2, 4, 6, and 8 h

$$R(\%) = \frac{m_{bind}}{m_{free} + m_{bind}} \times 100\%$$

m_{bind} means the mass of HSYA which bind with CS; m_{free} means the mass of HSYA which is free in solution.

Preparation of Granules

The purified HSYA-CS complex was used to prepare HSYA granules. The formulation of HSYA granules consisted of the HSYA-CS complex, sodium caprate, HPMC K15M, and EC45. All excipients were mixed adequately by sieving through a 100-mesh screen (pore diameter: 150 μm) five times. A 50% ethanol solution was used to prepare soft material followed by granulation with an 18-mesh screen. HSYA granules were collected after drying for 4 h at 40°C.

Determination of HSYA in HSYA Granules

In total, 1 g of granules was weighed and adequately crushed in a mortar; 10 mg of the fine powder was accurately weighed and dissolved in water, then diluted to 100 ml. After stirring at 37°C for 30 min, the solution was filtered using a 0.45-μm filter membrane. The filtrate was collected and detected at a wavelength of 403 nm by UV spectrophotometer.

In Vitro Dissolution

Dissolution test for HSYA granules were performed using USP standard dissolution apparatus with a basket stirrer. The dissolution medium (900 ml of phosphate buffer solution, pH 6.8) was maintained at 37±0.5°C and stirred at 50 rpm. The samples were collected at 10, 20, 30, 45, 60, 90, 120, 180, and 240 min. At each sampling point, 10 ml of sample was withdrawn and an equal volume of fresh dissolution medium was used to maintain a constant volume. The samples were filtered and analyzed using a UV spectrophotometer (UV-1800, Shimadzu) at 403 nm.

Pharmacokinetic Study

Experimental Design

Thirty-six rats were randomly divided into 6 groups. All of the rats were fasted overnight (12 h) preceding the experiments. The first group was orally administered a HSYA solution as the control group. Four groups were orally administered HSYA/sodium caprate, HSYA/sodium deoxycholate, HSYA/β-CD, and HSYA/Poloxamer-188 solutions. This was intended to improve BA through enhancing the permeability of HSYA. The concentration of HSYA and the relevant excipients in the solutions were 6.25 and 0.1 mmol/ml, respectively. The concentration of the polymers, such as β-CD and P188, was calculated according to their structural units. According to the results of the excipients screening experiments, sodium caprate was selected as the absorption enhancer to prepare the HSYA granules. An aqueous suspension of HSYA granules was orally administered to the last group, which was intended to improve BA through extending the retention time of HSYA in the intestinal tract.

Each group was treated with different solutions with the HSYA dosage at 25 mg/kg. After oral administration, blood samples (0.2–0.3 ml) were obtained from the venous plexus of the eye socket at 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, and 480 min. Each sample was immediately transferred to a heparinized Eppendorf tube and then centrifuged at 4000 rpm for 8 min. Approximately, 100 μl was separated from the supernatant and stored at −20°C.

Preparation of Plasma Samples

The plasma samples were taken out of the refrigerator and thawed at 37°C. Then, 35 μl of 10% perchloric acid was added to 100 μl of the thawed plasma. Each sample was vortex-mixed for 3 min and freeze centrifuged for 20 min at 13,000 rpm. In total, 20 μl of the supernatant was injected into the chromatography system. The pharmacokinetic parameters were calculated by statistical moment theory.

Pharmacokinetic Parameter Calculation

The maximum plasma concentration of HSYA (c_{max}) and peak time (t_{max}) were determined directly from the mean plasma concentration-time curve (C-T curve). The area under the C-T curve (AUC_{0-8n}) and the area under the first-moment curve ($AUMC_{0-tn}$) were calculated using the linear trapezoidal rule. All of the values are expressed as the means ±standard deviations (SD).

The HSYA aqueous solution was taken as the control experiment to calculate the relative BA (F_{rel}). The relative BA values were calculated using the following formula:

$$F_{rel} = \frac{AUC_t \times D_r}{AUC_r \times D_t} \times 100\ \%$$

r represents reference formulation, t represents test formulation, and D means dosage.

Statistical Analysis

All values are expressed as the mean±standard deviation. One-way analysis of variance and Student's t test were used to examine the results, with $P<0.05$ accepted as the minimum level of significance.

RESULTS AND DISCUSSION

Determination of HSYA in Plasma Samples

Specificity

Blank plasma, HSYA mixed with blank plasma, and plasma samples after oral administration of HSYA to rats were detected under chromatographic conditions. The results indicate that the retention time of HSYA is 3.9 min, the drug peak is well-separated with an endogenous impurity peak (resolution>1.8), and the number of theoretical plates is more than 3000.

Standard Curve

Various quantities of HSYA were dissolved with methanol, and 20 μl of the solutions were transferred to Eppendorf tubes and vaporized in negative pressure. After 100 μl of blank plasma was transferred to those Eppendorf tubes, standard samples were prepared, and the concentrations of HSYA were 0.1, 0.2, 0.5, 1.0, 3.0, 5.0, and 10.0 μg ml^{-1}. The samples were treated according to the sample preparation method and detected by HPLC. The linear regression between the HSYA concentration and peak area was calculated and checked by the coefficient of determination. The equation was A=63.244C − 0.4072 with a determination coefficient of r=0.9998 (n=7). The linear relation of plasma samples was within the linear range (0.1–10.0 μg ml^{-1}). Several experiments were also performed, and the results confirmed that the precision, accuracy, and stability of the method met the requirements of bio-analysis.

Characterization of HSYA-CS Complex

Purified HSYA-CS complex was characterized by DSC; the result was displayed in Fig. 2. A decalescence peak appears in the DSC curve of HSYA-CS complex and the melting point is about 171.48°C, much different from HSYA, CS, and the mixture of them. It indicates that HSYA combined with CS through bonding interaction in HSYA-CS complex.

Influence of Different Factors on the Binding Rate of the HSYA-CS Complex

Molecular Weight of CS and pH Value

The pH value and molecular weight can significantly affect the dissociation and solubility of CS in solution, and they both affect the binding rate of the HSYA-CS complex. Therefore, the molecular weight and pH value of CS were investigated together in one experiment. Four kinds of CS, with molecular weights of 2, 4, 50, and 150 kDa, were investigated. The experiment was performed as follows: The experiment was divided into four groups according to the molecular weight of CS. For each group, CS and HSYA were accurately weighed at a mass ratio of 1:1, and then distilled water was added. The solutions were adjusted to pH 1.0, 3.0, 4.0, 5.0, 6.0, and 7.2 and stirred at 40°C for 6 h. Lastly, the solutions were adequately lyophilized for 36 h and a complex powder was obtained.

The binding rate of the HSYA-CS complex under different conditions was shown in Table II and Fig. 3. The pH value and molecular weight of CS could significantly affect the binding rate of HSYA to CS. The binding rate of HSYA to CS4k was much higher than other kinds of CS from pH 3.0 to pH 6.0, so CS4k was selected to perform the subsequent experiments. In addition, the binding rate could reach 98.65% if the pH of the HSYA and CS solution was not adjusted, so the pH was not adjusted during the preparation process in the following experiments.

Although CS4k is water-soluble, its solubility is much lower than HSYA, so the binding of HSYA to CS4k would decrease the solubility of HSYA to some extent, which leads to an improved BA for HSYA.

Ratio of HSYA to CS

The ratio of HSYA to CS was another important factor affecting the binding rate of the HSYA-CS complex. Based on the above results, CS4k was selected to prepare complex, and the mass ratios of 1:1, 1:2, and 1:3 for HSYA to CS4k (nearly equal to the molar ratio of 1:4, 1:8, and 1:12) were

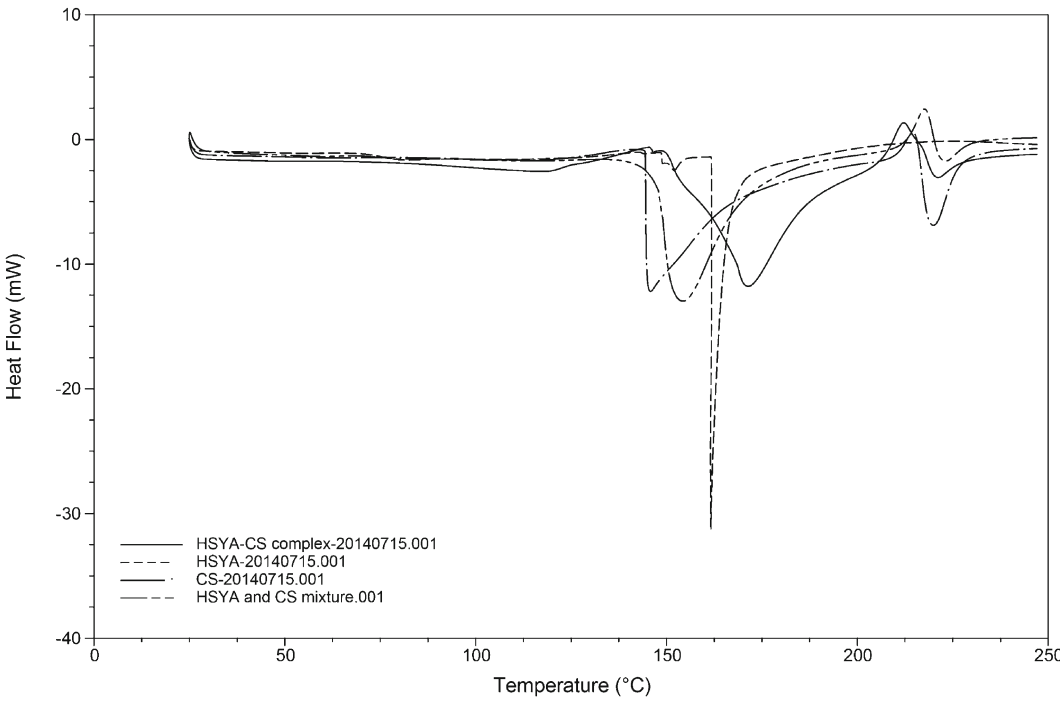

Fig. 2. The overlay DSC profile of HSYA, CS4k, HSYA-CS4k complex, and the mixture of HSYA and CS4k

Table II. The Influence of pH Value and Molecular Weight of CS on Binding Rate

pH	HSYA-CS150k	HSYA-CS50k	HSYA-CS4k	HSYA-CS2k
1.0	2.96%	3.75%	9.59%	14.27%
3.0	36.15%	39.24%	93.21%	94.29%
4.0	37.83%	41.17%	95.88%	92.36%
5.0	29.26%	26.13%	98.34%	84.43%
5.6	5.83%	6.1%	98.65%	73.84%
6.0	2.62%	3.81%	98.74%	69.97%
7.2	ND	ND	71.96%	62.46%

CS150k, CS50k, CS4k, and CS2k refer to chitosan with the molecular weight of 150, 50, 4, and 2 kDa
ND no data

investigated. The other process was according to descriptions in the "Methods" section.

The binding rates of HSYA and CS4k at the mass ratios of 1:1, 1:2, and 1:3 were 95, 98.9, and 99.3%, respectively. The results showed that the binding rate of HSYA to CS4k increased with an increased amount of CS4k. However, the increment was not significant when the ratio of HSYA to CS4k reached 1:3. Therefore, the mass ratio 1:2 was selected as the optimal ratio.

Concentration of HSYA

The binding rate of HSYA to CS4k was investigated when the concentration of HSYA was 100, 200, 300, and 400 mg/ml. The mass ratio of HSYA to CS4k was 1:2, and the action process was performed at 40°C for 6 h. A complex powder was obtained after freeze-drying. Then, the binding rate of HSYA to CS was determined.

The binding rates were 99.6, 99.7, 99.4, and 93% when the concentration of HSYA was 100, 200, 300, and 400 mg/ml, respectively. There was little change in the binding rates when the concentration of HSYA was between 100 and 300 mg/ml, but the rate decreased significantly if the concentration was more than 300 mg/ml. Furthermore, due to the improved concentration of HSYA, the freeze-drying time was shortened to 24 h. Therefore, 300 mg/ml of HSYA was selected as the optimal concentration.

The solubility of CS4k was much lower than HSYA, although the quantity of HSYA to CS4k was 1:2; the amount of CS4k dissolved in water was lower than the total amount while HSYA dissolved in water completely. Therefore, 400 mg/ml of HSYA resulted in a low binding rate of HSYA to CS4k.

Temperature

Temperatures of 30, 40, and 50°C were used to inspect the influence of temperature on the binding rate of the HSYA-CS complex. The other process was according to the results above and in the light of "Methods" section. Finally, the binding rate of HSYA to CS was determined.

The binding rates of HSYA and CS4k at 30, 40, and 50°C were 92.2, 99.4, and 99.6%, respectively. The results indicated that the binding rate increased with the increase in temperature, but it was reported that HSYA is not stable under high temperature (12), so 40°C was selected as the optimal temperature.

Action Time

To investigate the influence of action time on binding rate, 2, 4, 6, and 8 h were selected. The other process was according to the results above and in the light of "Methods" section.

The final binding rates were 96.3, 99.2, 99.4, and 99.4% for action times lasting 2, 4, 6, and 8 h, respectively. The results showed that the binding rate increased with the increase in action time. The binding rate increased with the increase of

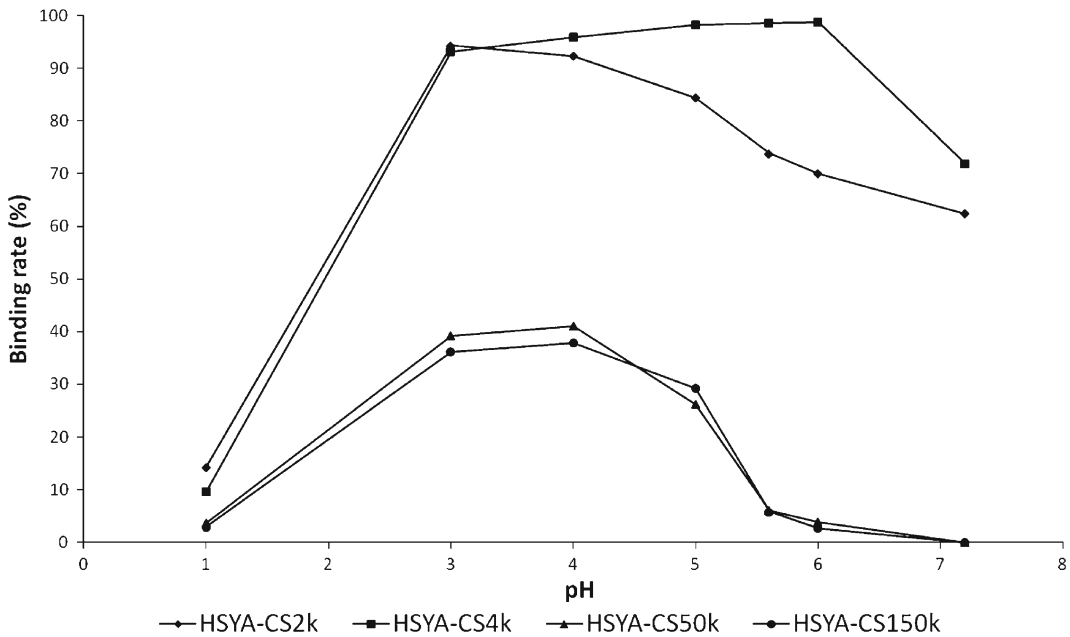

Fig. 3. The influence of pH value and molecular weight of CS on binding rate

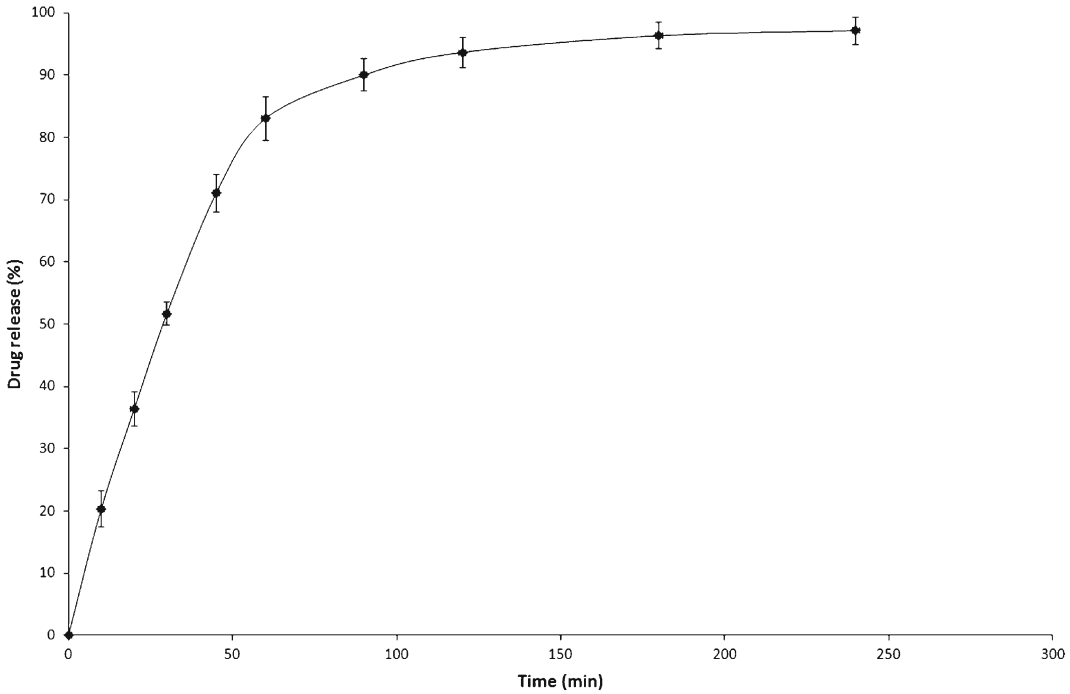

Fig. 4. *In vitro* dissolution curve of HSYA granules

action time. However, there was little change in the binding rates when the action lasted for 4, 6, and 8 h, so 4 h was selected as the optimal action time.

The Optimal Preparation Process of the HSYA-CS Complex

HSYA and CS4k were accurately weighed at a mass ratio of 1:2, then a solution was obtained after water was added, in which the concentration of HSYA was 300 mg/ml. The solution was stirred in a thermostatic water bath for 4 h to adequately facilitate action between HSYA and CS4k. A complex powder was obtained after freeze-drying for 24 h.

Content of HSYA in HSYA Granules

A series of standard HSYA solutions were accurately prepared. The drug concentrations were 2.5, 5, 7.5, 10, 15, and 20 μg/ml. Each solution was detected by ultraviolet-

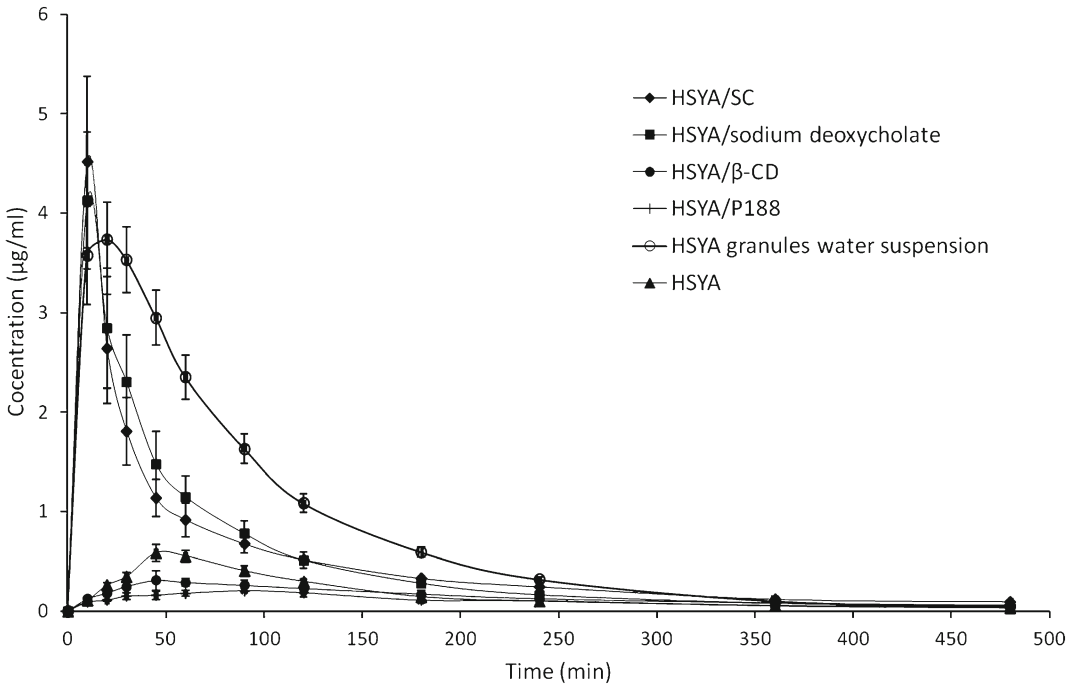

Fig. 5. Mean concentration-time profiles of HSYA after oral administration of HSYA, HSYA/SC, HSYA/sodium deoxycholate, HSYA/β-CD, and HSYA granules water suspension (n=6)

Table III. Main Pharmacokinetics Parameters of HSYA After Oral Administration of HSYA, HSYA/SC, HSYA/SD, HSYA/β-CD, and HSYA/P188 Aqueous Solution

Parameters	HSYA	HSYA/SC	HSYA/SD	HSYA/β-CD	HSYA/P188
C_{max} (μg/ml)	0.585±0.08	4.52±0.86	4.125±0.69	0.310±0.10	0.204±0.02
T_{max} (min)	45±10	10±5	10±5	60±11	90±18
MRT (min)	137.5±6.6	106.1±6	88.9±4	174.1±13	174.1±13
$AUC_{0\text{-}8h}$ (μg min/ml)	82.30±13.63	233.91±24.6	237.27±38.12	69.26±11.46	50.34±12.91
BA, F_{rel} (%)	100	284.2	288.3	84	61

The administration dosage of HSYA is 25 mg/ml, and the dosage of absorption enhancers are all 0.4 mmol/kg, among them β-CD and P188 are calculated according to their structural units

visible spectrophotometer at a wavelength of 403 nm. The linear regression between the HSYA concentration and absorbance was calculated, from which the linear equation and coefficient were determined: A=19.219C+0.0022, R^2=1 (n=6). The linear relation was within the linear range (2.5–20 μg/ml).

The HSYA granules were strictly treated according to determination method of HSYA in HSYA granules, and the content of HSYA was 19.5%.

In Vitro Dissolution

The mean dissolution profile of HSYA granules was displayed in Fig. 4. As it is seen from the figure, nearly 90% of HSYA is released from HSYA granules until 90 min. Apparently, HSYA granules has the effect of sustained release, and the sustained and synergistical release of HSYA and absorption enhancer is in favor of a higher bioavailability.

Pharmacokinetic Study

The Enhancing Effect of an Absorption Enhancer on the BA of HSYA

Figure 5 shows the mean plasma concentration-time of the HSYA, HSYA/sodium caprate, HSYA/sodium deoxycholate, HSYA/β-CD, and HSYA/P188 aqueous solutions after being orally administered to rats, and Table III shows the main pharmacokinetic parameters. The AUC values for HSYA after oral administration of HSYA/sodium caprate and HSYA/sodium deoxycholate to rats were significantly improved. The T_{max} of the HSYA/sodium caprate and HSYA/sodium deoxycholate groups were both 10 min, which clearly demonstrated that sodium caprate and sodium deoxycholate could greatly enhance the BA of HSYA early, but the effect of enhancement was not significant later. Medium-chain fatty acids and medium-chain glycerides can enhance the intestinal absorption of drugs (13–15). Sodium caprate, as a typical representative of a medium-chain fatty acid absorption enhancer, can act quickly *in vivo* but cannot sustain its effect. Therefore, the coordinated release of HSYA and sodium caprate should be designed during the development of the formulation. Figure 5 also shows that the AUC of HSYA declined after oral administration of HSYA/β-CD and HSYA/P188, which indicated that β-CD and P188 did not enhance the absorption of HSYA. The relative BA of HSYA after administration of HSYA/sodium caprate, HSYA/sodium deoxycholate, and HSYA/β-CD aqueous solutions was 284.2, 288.3, and 84.0%, respectively. Although the relative BA for HSYA/sodium deoxycholate is slightly higher than HSYA/sodium caprate, the toxicity of sodium caprate is much lower than sodium deoxycholate, so sodium caprate is a better absorption enhancer than sodium deoxycholate.

Table IV. Main Pharmacokinetic Parameters of HSYA After Oral Administration of HSYA Aqueous Solution and HSYA Granules Suspension

Parameters	HSYA	HSYA granules
C_{max} (μg/ml)	0.585±0.082	2.966±0.615
T_{max} (min)	45±10	20±5
MRT (min)	137.5±6.6	105.6±3.9
$AUC_{0\text{-}8h}$ (μg min/ml)	82.30±13.63	391.65±31.51
BA, F_{rel} (%)	100	476

The Enhancing Effect of HSYA Granules

The mean plasma concentration-time of an HSYA granule aqueous suspension after oral administration to rats and the main pharmacokinetic parameters were shown in Fig. 5 and Table IV, respectively. The AUC of HSYA significantly improved after oral administration of an HSYA granule water suspension to rats, and the relative BA was 476%. Compared with the C-T curve of HSYA/sodium caprate, the C_{max} of this curve was slightly lower, but the decline of plasma concentration with respect to time decreased, especially during the initial 30 min. Therefore, to some extent, HSYA granules extended the action time between the intestinal mucosa and HSYA. In addition, the plasma concentration was still much higher than other groups at 3 h. This was associated with the absorption enhancing effect of SC. Since the solubility of SC was much lower than HSYA and HSYA could be slowly released from HSYA granules, so it is not difficult to make a judgment that SC could be slowly and synergistically released with HSYA. Therefore, the effect time of SC was extended and contributed to the high BA of HSYA.

CONCLUSION

An HSYA-CS complex was prepared for the first time, and the preparation process was optimized. Furthermore, several pharmaceutical excipients were investigated to improve the oral BA of HSYA. Sodium caprate proved to be an excellent absorption enhancer. Therefore, based on the above results, HSYA granules were prepared in which the HSYA/CS complex and sodium caprate were the main ingredients.

Pharmacokinetic experiments were carried out for the HSYA granules, and the relative BA was 476%. The results showed that CS4k has a beneficial synergistic effect with sodium caprate, which can improve the absorption of HSYA remarkably.

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