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Chitosan-coated nano-liposomes for the oral delivery of berberine hydrochloride

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1. Preparation of nano-liposome-encapsulated berberine hydrochloride

Uncoated nano-liposomes composed of lecithin, cholesterol and dihexadecyl phosphate (in a 1:0.242:0.036 weight ratio) were prepared by thin film hydration followed by sonication.[1-3] The thin film hydration method originally reported by Bangham is the simplest, yet the most widely used method for liposome preparation. Briefly, 0.7296 g of lecithin, 0.1765 g of cholesterol and 0.0263 g of dihexadecyl phosphate were weighed in a 500 mL round bottom flask, 20 mL of ethanol was added, and the mixture was stirred at room temperature until the lipid is dissolved. A dried lipid film was prepared from this solution by rotary evaporation at 40 °C under reduced pressure. The BH solution (50 mL) was then loaded in a 50 mL flat-bottomed flask in a thermostated water bath at 60 °C before it was added to the flask with the dried lipid film and kept in the water bath at 60 °C for 30 min with stirring on a magnetic stirring plate. After hydration, the resulting multilamellar liposomal suspension was sonicated at 4 °C with a micro-tip probe sonicator, ultrasonic homogenizer (JY92-IIN, Ningbo Scientz Biotechnology Co., Ltd in China), to obtain small unilamellar liposomes. After sonication, the titanium particles released from the sonicator probe were removed by centrifugation at 1,500 rpm for 15 min.[4]

2. Release kinetics analysis of prepared liposomes

To examine the kinetics and mechanism of BH release from the liposomes, the data obtained from the in vitro release studies were analyzed by various models such as the first order, Higuchi, Hixson–Crowell and Korsmeyer–Peppas models.[5, 6] The equations used were as follows:

First order: $Ln(Q_0 - Q_t) = k_1 t$ (S1)

Higuchi: $Q_t = k_H t^{1/2}$ (S2)

Hixson–Crowell: $Q_o^{1/3} - Q_t^{1/3} = k_{HC} t$ (S3)

Korsmeyer–Peppas: $Q_t = k_{KP} t^n$ (S4)

where Q_t is the amount of drug released at time t and Q_0 is the initial amount of drug in the microspheres, k_1, k_H, k_{HC} and k_{KP} are the release rate constants, and n is the release exponent indicative of the mechanism of release.

The drug release data obtained for all the liposomes were fitted to different models including a first-order equation, the Higuchi model, the Hixson-Crowell model, and the Korsmeyer-Peppas model to analyze the mechanism of drug release and the diffusion kinetics. The fitting of each model was evaluated based on the correlation coefficient (R^2) values obtained. The R^2, k and n values obtained for each model fit are presented in Table S1.

Table S1 Correlation coefficient (R^2), k and n values from various drug release models for the liposomes (n = 3).

SGIF	Formulations	First-order		Higuchi		Hixson-Crowell		Korsmeyer-Peppas		
		R^2	K_1	R^2	k_H	R^2	k_{HC}	R^2	k_{KP}	n
SGF	**Uncoated nano-liposomes**	0.9120 ± 0.0029	0.1623 ± 0.0042	0.8316 ± 0.0051	20.98 ± 0.20	0.8528 ± 0.0033	0.0453 ± 0.0006	0.8963 ± 0.0034	28.07 ± 0.49	0.3790 ± 0.0036
	0.1% Chitosan-coated	0.6608 ± 0.0049	0.0900 ± 0.0026	0.8294 ± 0.0055	16.32 ± 0.22	0.4968 ± 0.0081	0.0250 ± 0.0010	0.8995± 0.0029	22.02 ± 0.55	0.3750 ± 0.0053
	0.3% Chitosan-coated	0.4924 ± 0.0148	0.0727 ± 0.0023	0.8040 ± 0.0105	14.81 ± 0.23	0.3061 ± 0.0219	0.0197 ± 0.0006	0.8963 ± 0.0035	20.65 ± 0.58	0.3613 ± 0.0059
SIF	**Uncoated nano-liposomes**	0.9365 ± 0.0022	0.2120 ± 0.0095	0.7856 ± 0.0096	23.13 ± 0.35	0.8634 ± 0.0037	0.0533 ± 0.0015	0.9070 ± 0.0030	33.29 ± 0.92	0.3477 ± 0.0055
	0.1% Chitosan-coated	0.6488 ± 0.0105	0.1373 ± 0.0065	0.7217 ± 0.0090	19.26 ± 0.36	0.4994 ± 0.0166	0.0397 ± 0.0015	0.9139 ± 0.0043	29.42 ± 0.82	0.3223 ± 0.0035
	0.3% Chitosan-coated	0.4649 ± 0.0079	0.1010 ± 0.0060	0.7376 ± 0.0153	17.14 ± 0.41	0.2450 ± 0.0103	0.0280 ± 0.0020	0.9252 ± 0.0030	26.07 ± 0.95	0.3240 ± 0.0056

SGIF: simulated gastrointestinal fluid; SGF: simulated gastric fluid; SIF: simulated intestinal fluid

The BH release rate constants (k_1, k_H, k_{HC}, k_{KP}) for uncoated liposomes were significantly higher than that of the chitosan-coated species ($p < 0.05$), indicating that chitosan coating provided a retention effect on the BH release. Moreover, all of the k values for the 0.3 % chitosan-coated samples were significantly lower than for the 0.1 % chitosan-coated samples. This is consistent with the aforementioned enhanced stability behavior and can also be explained by the coating layer building a dense shell on the surface of liposomes decreasing the fluidity of lipid bilayer, which leads to decreased membrane permeability.[7]

As shown in previous studies,[6, 8-10] the first-order equation represents a system where the release rate of the drug depends on the concentration of the drug in the system. The Higuchi model assumes that drug release is caused primarily by a diffusion mechanism. The Korsmeyer–Peppas model provides insight into the type of drug release mechanism taking place from swellable polymeric devices.

In SGF and SIF the uncoated liposomes obeyed the Korsmeyer-Peppas model and the first-order equation ($R^2 > 90$ %), whereas the chitosan-coated liposomes showed a good fit to the Higuchi and Korsmeyer-Peppas models ($R^2 > 80$ %) as compared to the other models. These results are in agreement with previous studies showing the applicability of the Korsemeyer-Peppas model for diffusion- and erosion-based drug release from liposomes.[6]

In spherical matrices, if $n < 0.43$, Fickian diffusion-mediated drug release occurs; if $0.43 < n < 0.85$, non-Fickian transport occurs; and erosion mediated release corresponds to $n > 0.85$.[11, 12] According to Table 3.2, the n value for the coated samples was smaller than that of uncoated samples in both SGF and SIF, moreover all the release exponents are < 0.5. For a spherical system, n has a limiting value of 0.50 in the case of Fickian diffusion.[11] The smaller n value obtained for the coated samples can be explained by increased steric hindrance caused by the polymer coating on the surface of liposomes.[13]

3. Validation of the analytical method

The HPLC method was validated for selectivity, linearity, limit of quantitation, precision, and accuracy according to current guidelines.[14]

3.1 Specificity and selectivity

The specificity of the method was investigated by comparing chromatograms for blank plasma with chromatograms obtained for the blank plasma spiked with authentic standards of BH and IS.

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. It was assessed by comparing chromatograms for different batches of blank rabbit plasma with the corresponding spiked rabbit plasma samples.

3.2 Calibration and linearity

Seven calibration standard solutions were prepared from the stock solutions at concentrations ranging from 0.01 to 5.0 μg/mL of BH, to encompass the expected concentration range in measured samples. The calibration curves were obtained by plotting the concentration of BH in plasma as a function of the peak area ratio for BH to IS.

The linearity of the calibration curve was evaluated by analysis of peak area ratios (BH/IS) for the BH concentrations spiked into blank plasma.

3.3 Limit of detection

The limit of detection (LOD), defined as the lowest concentration of analyte in a sample that can be detected, was determined by diluting the standard solutions of BH with methanol. The LOD, determined from the signal-to-noise (S/N) ratio for each compound in the analysis of a series of diluted solutions, corresponds to a S/N ratio of 3 for the analyte.

3.4 Precision and accuracy

The precision of an analytical method describes the closeness of individual measurements for an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of a biological matrix. The accuracy of an analytical method describes the closeness of the mean test results obtained to the true value (concentration) of the analyte. The accuracy is determined by replicate analysis of samples containing known amounts of the analyte.

The accuracy and precision were investigated by analyzing the QC samples at three different concentrations (0.01, 0.1, and 1.0 μg/mL) on 3 different validation days. The concentration of each sample was calculated using a calibration curve constructed on the same testing day.

Accuracy, defined as the relative error (*RE*) in the calculated value (*Cal*) of a standard from that of its true value (*T*), is expressed as a percentage (RE, %). It was calculated using Equation S5.

$$RE\ (\%) = \frac{(Cal - T)}{T} \times 100\ \% \quad (S5)$$

Precision was calculated as the relative standard deviation (*RSD*, %) following Equation S6.

$$RSD\ (\%) = \frac{Standard\ deviation}{Mean} \times 100\ \% \quad (S6)$$

The criteria used to assess the suitability of precision and accuracy were as follows: The *RSD* should not exceed 15 %, and the accuracy should be within 15 % of the actual value for the QC samples.

3.5 Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of analyte added to and extracted from the biological matrix, as compared to the detector response obtained for the true concentration of a pure authentic standard.

The extraction recovery (*ER*) of BH was analyzed at 0.05, 0.1, and 1.0 μg/mL. It was estimated by comparing the peak area of BH for pre-spiking samples (*pre-SS*) with those of post-spiking samples (*post-SS*). Pre-spiking samples refers to spiking BH and IS to yield the same concentrations as in the QC samples before sample preparation, while post-spiking sample means spiking neat solutions of BH and IS at the same concentrations as the QC samples into the extracted blank plasma. The extraction recovery of palmatine (IS) was evaluated in the same manner as for the QC

samples at 0.5 μg/mL. The extraction recovery was calculated by using Equation S7.

$$ER\ (\%) = \frac{pre - SS}{post - SS} \times 100\ \% \quad (S7)$$

Some result:

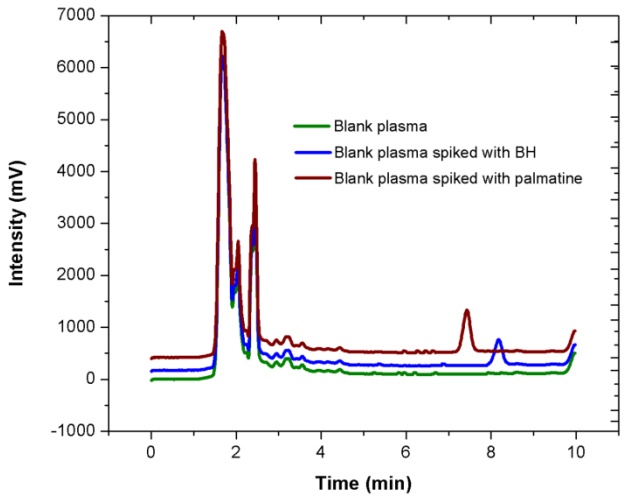

Fig. S1 The typical chromatograms for blank plasma, and plasma spiked with BH or palmatine.

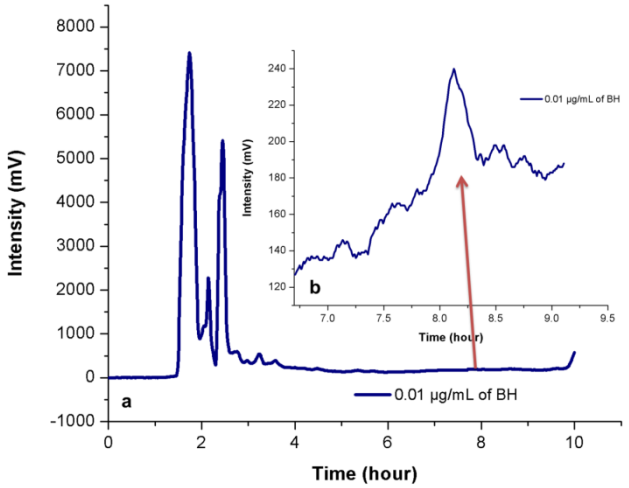

Fig. S2 Chromatograms for plasma spiked with 0.01 μg/mL of BH (a) and enlarged HPLC figure to show the BH limit of detection (b).

Table S2 Precision and accuracy of the determination of BH in rabbit plasma.

	Spiked concentration (μg/mL)	Measured concentration (μg/mL, Mean ± SD)	Precision (*RSD*, %)	Accuracy (*RE*, %)
Intra-day (n = 3)	0.05	0.044 ± 0.002	4.68	-12.28
	0.1	0.093 ± 0.003	3.19	-7.05
	1.0	0.916 ± 0.046	5.07	-8.43
Inter-day (n = 9)	0.05	0.046 ± 0.002	4.87	-8.99
	0.1	0.095 ± 0.003	3.19	-5.12
	1.0	0.940 ± 0.031	3.28	-6.03

Table S3 Percent extraction recovery measurement of BH in rabbit plasma (n = 3).

Added concentration (μg/mL)	Found concentration of pre-spiking samples (μg/mL, Mean ± SD)	Found concentration of post-spiking samples (μg/mL, Mean ± SD)	Recovery (%)
0.05	0.044 ± 0.002	0.048 ± 0.002	90.79 ± 6.95
0.1	0.093 ± 0.003	0.097 ± 0.002	96.06 ± 1.54
1.0	0.916 ± 0.046	0.971 ± 0.005	94.33 ± 4.75

Notes and references

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