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Distribution of nobiletin chitosan-based microemulsions in brain following i.v. injection in mice

Jing Yao, Jian Ping Zhou *, Qi Neng Ping, Yun Lu, Liang Chen

Department of Pharmaceutics, China Pharmaceutical University, 24 Tongjiaxiang, Nanjing 210009, China

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Abstract

The purpose of this study was to characterize the in vitro properties of a number of chitosan-based microemulsions containing nobiletin and determine its distribution in mice brain following i.v. administration. The phase behavior and properties of chitosan-based microemulsions were investigated in a pseudo-ternary system composed of polyoxyethylene 35 castor oil/benzyl alcohol/medium-chain triglyceride/tea oil/water with the chitosan. The droplet sizes were found to be smaller than 25 nm by photo correlation spectrometer. The nobiletin-loaded hyaluronic acid chitosan-based microemulsion (HAC-ME) carried negative charge and nobiletin-loaded hydrochlorate chitosan-based microemulsion (HCC-ME) carried positive charge. The concentrations of nobiletin in tissues were determined by HPLC after i.v. administration of HAC-ME, nobiletin-loaded microemulsion (ME), HCC-ME and nobiletin solution. Based on AUC_{0-t}, MRT and C_{max}, HAC-ME delivered more nobiletin to the brain compared to nobiletin solution, ME and HCC-ME. The long-circulation effect might contribute to the higher AUC_{0-t} for HAC-ME in brain. On the other hand, the AUC_{0-t} in plasma and brain after i.v. administration of HCC-ME were not significantly increased relative to ME. These results indicate that HAC-ME may be presented as potential candidates for delivering more drugs into the brain.

Keywords: Chitosan-based microemulsions; Nobiletin; Hyaluronic acid chitosan; Hydrochlorate chitosan; Brain

1. Introduction

Nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone), a citrus polymethoxy flavonoid, is a candidate as an anti-inflammatory drug (Ishiwa et al., 2000; Lin et al., 2003). A number of studies have shown it to be effective in the treatment and precaution of the cancers such as gastric cancer (Delaney et al., 2001; Iwase et al., 2001; Yoshimizu et al., 2004). It may also be effective as a sunscreen reagent by protecting against photoinflammation and photoaging (Tanakaa et al., 2004). Nobiletin is a substrate for multi-drug resistance transporters (Ikegawa et al., 2000), leading to a low distribution to brain tissue and there by limiting its use for the treatment of brain tumors. For this purpose, identifying a means to improve the brain uptake and reduce the accumulation of nobiletin in other tissues would be helpful.

Microemulsions, which have a droplet size in the range of 10–100 nm, are a thermodynamically stable, isotropically clear products consisting of an oil phase, aqueous phase, surfactant/s and co-surfactant. Oil-in-water microemulsions are suitable for the incorporation of poorly water-soluble drugs and may increase the permeability for the oral (Kang et al., 2004; Ke et al., 2005), percutaneous (Sintov and Shapiro, 2004) and intranasal delivery (Zhang et al., 2004). Recently, the microemulsions have been shown to enhance of the uptake of drug by the brain (Tong et al., 2002; Yao et al., 2005), however, the conventional microemulsions exhibit toxicities due to the high concentration of surfactants and co-surfactants.

In addition to brain accumulation, microemulsions have been shown to accumulate in the reticuloendothelial system (RES). RES uptake can be altered by influencing the surface properties of the microparticles (Couvreur et al., 1995; Yang et al., 1999). Thus, it may also be possible to affect brain uptake by an analogous process. Specifically, microemulsions containing hyaluronic acid chitosan (HAC) increased the transport of the water-soluble Evans blue across the blood brain barrier (BBB) in a concentration dependent manner (Yao et al., 2006).

In the present work, a number of microemulsions were evaluated for their potential as brain selective delivery systems of

* Corresponding author. Tel.: +86 25 83271272; fax: +86 25 83301606.
E-mail address: zhoujpcpu@126.com (J.P. Zhou).

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nobiletin. The hydrochlorate chitosan (HCC) and HAC, which are the soluble chitosan derivates, were used to prepare the microemulsions along with polyoxyethylene 35 castor oil as the surfactant, benzyl alcohol as the co-surfactant and a 3:1 mixture of medium-chain triglyceride/tea oil as the oil phase. The effect of HAC and HCC on phase behavior and properties of the microemulsions and the in vivo distribution of nobiletin-loaded chitosan-based microemulsions were studied.

2. Materials and methods

2.1. Materials

Nobiletin was obtained from Chinabest Drugs Research Ltd. (Nanjing, China). Polyoxyethylene 35 castor oil USP24/NF19 (PEG-35 castor oil, Cremophor EL 35) was purchased from BASF (Ludwigshafen, Germany). Medium-chain triglyceride (Caprylic/capric triglyceride, Cradamol GTCC®) was a kind gift from CRODA (Singapore). Hydrochlorate chitosan (HCC, 88% *N*-deacetylated, viscosity 30 mPa s, Mw about 250 kDa) and hyaluronic acid chitosan (HAC, 90% *N*-deacetylated, inherent viscosity 510 g/ml, Mw about 500 kDa) were obtained from Golden-shell Biochemical Co. Ltd. (Yuhuan, China). Other chemicals were of HPLC or analytic grade.

2.2. Preparation of chitosan-based microemulsions

The preconcentrate of the conventional microemulsion was formed by mixing of surfactant, co-surfactant and oil in a fixed ratio of 4.5:1:1 (g/g). Nobiletin was then dissolved in the preconcentrate by stirring magnetically at 40 °C water bath. For HAC-ME (or HCC-ME), the resulting mixture was combined with the appropriate amount of 20% hyaluronic acid chitosan solution (or 2% hydrochlorate chitosan solution) by gentle stirring for 15 min. Excess distilled water was added to adjust the formulation to a final amount of water at 60% (g/g) and a final concentration of nobiletin at 7.5 mg/g.

2.3. Characterization of chitosan-based microemulsions

2.3.1. Particle size and zeta potential measurement

Photo correlation spectrometer (Zetasizer 3000HS, Malvern Instruments Corp., UK) was used to measure the particle sizes and zeta potential of the microemulsions following dilution with double-distilled water.

2.3.2. Electrical conductivity

The electrical conductivity was measured by a DDS-307 conductivity meter (Dapu Instruments Factory, Shanghai, China) equipped with a platinum conductance electrode, which was coated with platinum black. The temperature was thermostated at 25 ± 0.1 °C.

2.4. In vivo distribution study

Before administration, chitosan-based microemulsions were diluted with 5% glucose solution. The final concentration of HAC in HAC-ME was 0.2% and that of HCC in HCC-ME was 0.0625%.

The mice weighing 18–21 g (Center Animal Laboratory of China Pharmaceutical University, China) were randomly divided into four groups. Nobiletin solution with 10% ethanol (control group), ME, HAC-ME and HCC-ME were, respectively administered by tail vein injection to the mice at a dose of 4.5 mg/kg. At 5, 10, 15, 20, 30, 60, 90, 120, 180, 240 and 330 min after administration, the blood samples were collected from the ocular artery. The mice were then sacrificed, and the brain, liver, spleen, heart and kidney were carefully excised. Each tissue was quickly rinsed with saline and blotted with filter paper. After weighing, the tissue samples were homogenized with 2 ml saline. Blood samples were anticoagulated with heparin and centrifuged at 5000 rpm for 10 min to obtain plasma. The plasma and the tissues homogenates were stored in a freezer (−20 °C) until assayed. Measurements were made using five mice at each time point.

To 0.2 ml plasma or 1.5 ml liver tissues homogenates, 50 μl nimodipine in methanol (1.62 μg/ml, internal standard) and 0.5 ml acetonitrile were added and extracted with 1.5 ml acetoacetate for 5 min. For the other tissues homogenates were extracted with 3 ml acetoacetate/*n*-hexane (1:1) for 5 min. After centrifugation at 2800 rpm for 10 min, the organic phases were removed and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was reconstituted in 100 μl mobile phase as the sample solution for pre-emergency.

2.5. HPLC analysis of plasma and tissues samples

Aliquots of 20 μl were injected onto the HPLC system equipped with a Shim-pack CLC-ODS C18 column (5 μm, 150 mm × 4 mm, Kyoto, Japan). The HPLC system (LC-2010C, Shimadzu Corp., Kyoto, Japan) consisted of an autosampler and an UV detector set at 332 nm. The mobile phase was methanol–water (72:28, v/v), and the flow rate was 0.8 ml/min.

The standard curves of peak area ratio as a function of nobiletin concentration over a range of 37.2–1860.0 ng/ml were linear with regression coefficients (r) of 0.9998, 0.9966, 0.9972, 0.9982, 0.9991 and 0.9992 for plasma, brain, liver, kidney, spleen and heart samples, respectively. The extraction recoveries of nobiletin from plasma and tissues homogenates were greater than 90% and 85%, respectively. The within-day and between-day R.S.D.'s were less than 7% for the plasma and tissue samples. The lowest limit of quantitation (LLOQ) was 5 ng/ml.

2.6. Data and statistical analysis

Pharmacokinetic parameters in plasma and tissues were calculated using a statistical moment algorithm. The areas under the concentration–time curve (AUC) and the area under the first moment curve (AUMC) were calculated using the linear trapezoidal rule for infinite time. The mean residence time (MRT) was determined by dividing AUMC by AUC (Yang et al., 1999). The statistical differences among group means were assessed using the two-way unweighted means analysis of vari-

ance (ANOVA)-test and a value of $p < 0.05$ was considered statistically significant.

To evaluate the brain targeting of nobiletin-loaded chitosan-based microemulsion, two indexes were adopted (Iga et al., 1991; Chou and Donovan, 1997; Zhang et al., 2003):

(1) Relative uptake efficiency (Re):

$$\mathrm{Re}\,i = \frac{(\mathrm{AUC}_i)_\mathrm{m}}{(\mathrm{AUC}_i)_\mathrm{s}}$$

(2) Ratio of peak concentration (Ce):

$$\mathrm{Ce}\,i = \frac{(C_{\max\,i})_\mathrm{m}}{(C_{\max\,i})_\mathrm{s}}$$

where "m" and "s", respectively, represent tested group and reference group. AUC_i denotes the area under the concentration–time curve of the tissue i.

3. Results and discussion

3.1. Phase behavior

The pseudo-ternary phase diagrams at different concentration of chitosan are shown in Fig. 1. These are consistent with earlier reported pseudo-ternary phase diagram constructed by Djordjevic et al. (2005) and Ke et al. (2005). HAC and HCC did not change the maximum oil solubilization, but the oil-in-water microemulsion regions were somewhat increased. HAC and HCC are known to have weak surface activity, and their hydrocarbon chains may interact with the surfactant molecules and form a mixed micelle (Lv et al., 2005), which promotes the formation of the stable microemulsions. This in turn may enhance the stability of the microemulsion accounting for the increase in the area of oil-in-water microemulsion region observed at higher chitosan concentration.

3.2. Characterization of chitosan-based microemulsions

Fig. 2 demonstrates a typical conductivity behavior of a microemulsion as shown in the previous studies (Djordjevic et al., 2005; Lv et al., 2005). The conductivity of microemulsion and chitosan-based microemulsion systems depend on the water content and the type of chitosan. A steep rise in every curve was observed at a 65% weight fraction of water, which is the critical point where the mixture converts from a bicontinuous phase to an oil-in-water microemulsion (Lv et al., 2005). Probably because the increasing volume of water in the swelled droplets causes relatively stronger interaction between the interfacial membranes (Sintov and Shapiro, 2004). The addition of HAC and HCC does not influence the shape of the conductivity curve of the microemulsion. This implies that HAC and HCC do not change the phase transition of the microemulsions. However, at the same water content, the conductivity of microemulsion system was increased with the addition of HAC or HCC. The

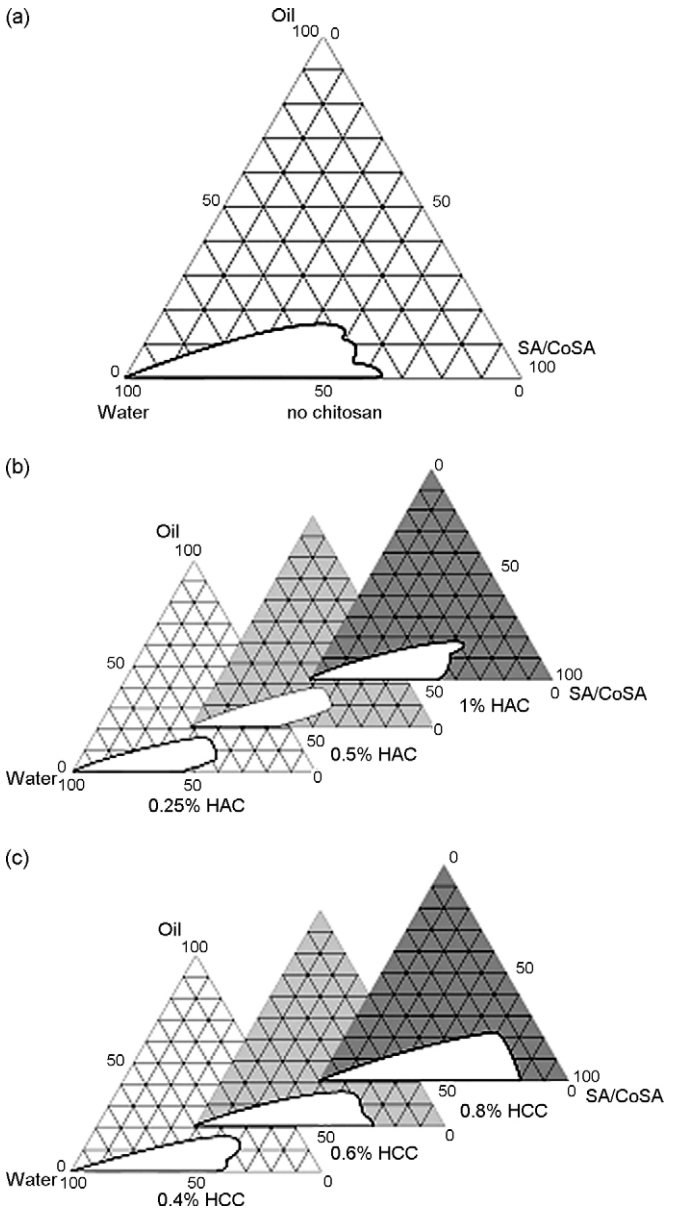

Fig. 1. Pseudo-ternary phase diagrams for (a) microemulsion with no chitosan, (b) HAC-based microemulsion system at various HAC concentration, and (c) HCC-based microemulsion system at various HCC concentration. The open spaces represent the o/w microemulsion regions.

addition of the positively charged HAC and HCC solutions along with the associated counterions appear to have affected the conductivity of systems. HCC may have a stronger electropositivity relative to HAC, therefore, the conductivity of HCC-ME was further increased.

In Fig. 3a, the effect of the chitosans on the droplet size of microemulsion is represented. The chitosans could influence droplet size by the factors such as the type and concentrations of chitosans. The droplet sizes of the chitosan-based microemulsion were the smallest when the concentration of HAC and HCC were 0.25% and 0.0125%, respectively. Their droplet sizes at this concentration were 10.6 and 12.2 nm, respectively. The effect of different concentration of HAC and HCC on the zeta potential is represented in Fig. 3b. It is shown that HAC-ME carried negative

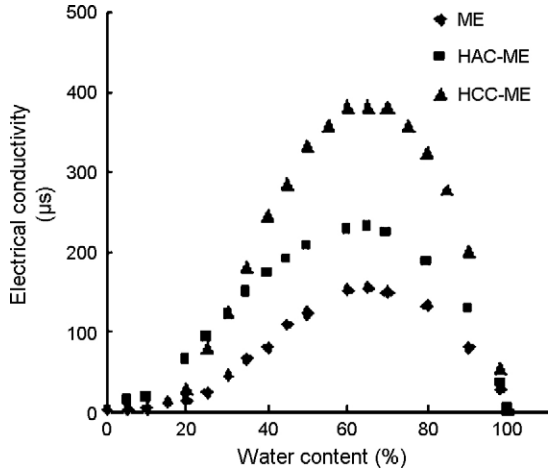

Fig. 2. Variation of electrical conductivity as a function of water content.

charge and HCC-ME carried positive charge, while the external surface of the chitosan-free microemulsion particles have a negative charge.

3.3. Distribution of nobiletin-loaded chitosan-based microemulsions

The mean plasma and brain tissues concentration–time profiles of nobiletin after i.v. administration are presented in Fig. 4, and the non-compartment pharmacokinetic parameters are given in Table 1. Plasma concentrations of nobiletin after i.v. administration of the three microemulsions were all increased relative to the control. HAC-ME yielded the highest level where the AUC_{0-t} value was 2.26 times (46,219 ng min/ml versus 20,436 ng min/ml) greater than the control group. This value was also considerably higher than that obtained after i.v. administration of chitosan-free microemulsions (*i.e.* ME group). Moreover, based on the AUC_{0-t} values, the brain uptake of nobiletin was far greater with the HAC-ME group in comparison to the other three groups, although the peak concentration (C_{max}) for HAC-ME and ME were similar. In comparison to ME group, the heart and kidney uptake of nobiletin was decreased. Thus, the presence of HAC in the microemulsions resulted in an increased concentration of nobiletin in the brain, but a decreased concentration in the heart and kidney. As to the ME group, the brain uptake of nobiletin was far greater than that of the control group, although it was less than that observed with the HAC-ME group. Overall, all of the microemulsions used in this study resulted in an increased distribution of nobiletin to the brain, which is consistent with previous studies of colloidal delivery systems (Kreuter, 2001; Yao et al., 2005). Moreover, the component in the formulation, Cremophor EL 35, has been shown to inhibit the multi-drug resistance (MDR) phenotype in cultured cells at concentrations likely to be achieved clinically (Yamazaki et al., 2000; Lo, 2003). Thus, the components found in the microemulsions or other colloidal delivery systems may enhance brain uptake of drug by inhibiting efflux drug transporters. Another possible mechanism may be related to the tight junction permeability, as suggested in the study by Sha et al. (2005), where the microemulsion containing labrasol increased the permeability of tight junctions in Caco-2 cells (Sha et al., 2005). Evans blue, which is water-

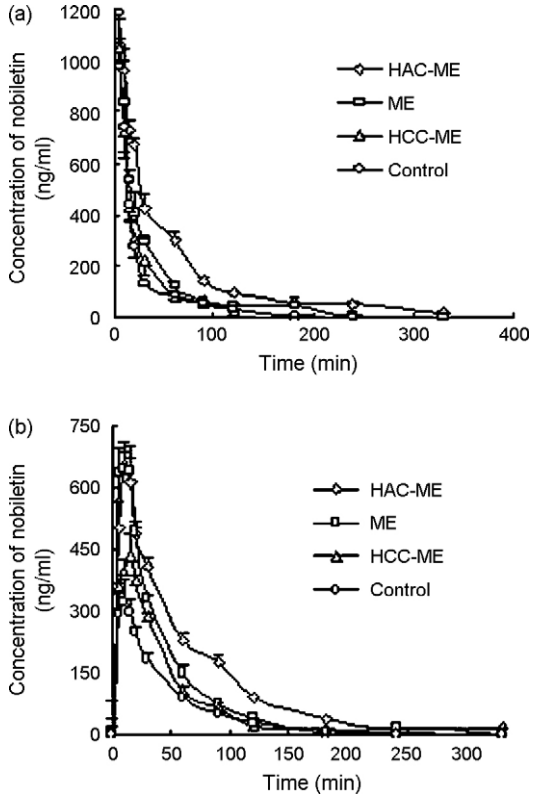

Fig. 4. Plasma (a) and brain (b) concentration–time curves of nobiletin after i.v. administration of control group (nobiletin solution), ME (chitosan-free microemulsion), HAC-ME (HAC-based microemulsion) and HCC-ME (HCC-based microemulsion). Results represent the mean ± S.D. of five mice.

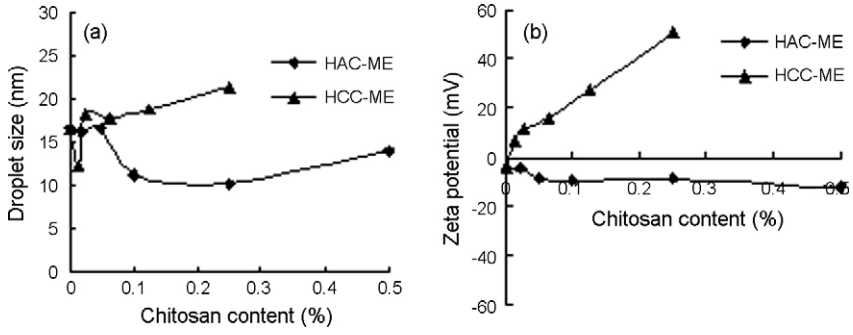

Fig. 3. Effect of chitosan content on droplet size (a) and zeta potential (b) of chitosan-based microemulsions.

Table 1
Pharmacokinetic parameters of nobiletin in various tissues following i.v. administration of control group, ME, HAC-ME and HCC-ME

Parameters	Tissues	Control	ME	HAC-ME	HCC-ME
AUC[a] (ng min/g)	Plasma[b]	20,436	27,412	46,219	20,953
	Brain	13,617	24,770	34,530	17,605
	Liver	20,382	25,965	32,105	21,908
	Kidney	22,546	41,102	33,637	23,850
	Spleen	17,180	13,907	18,483	24,884
	Heart	15,916	15,922	11,762	21,324
C_{max} (ng/g)	Brain	323 ± 50	646 ± 59**	651 ± 63**	397 ± 28ΔΔ
	Liver	1003 ± 83	1350 ± 53**	1122 ± 70ΔΔ	834 ± 36*,ΔΔ
	Kidney	1419 ± 83	1356 ± 154	1428 ± 242	1134 ± 34
	Spleen	1251 ± 67	840 ± 77**	503 ± 96**,ΔΔ	1789 ± 83**,ΔΔ
	Heart	896 ± 85	968 ± 61	595 ± 55**,ΔΔ	1264 ± 104**,ΔΔ
MRT (min)	Plasma	27.9	62.5	88.7	31.2
	Brain	50.3	48.4	62.6	44.4
	Liver	31.9	25.1	36.7	37.8
	Kidney	20.1	65.2	47.9	30.1
	Spleen	10.8	20.0	44.8	14.3
	Heart	13.8	12.6	19.7	16.0

Results of C_{max} are given as mean ± S.D., and the others are given as mean ($n = 5$). *$p > 0.05$ and **$p > 0.01$ *vs.* control group. Δ$p > 0.05$ and ΔΔ$p > 0.01$ *vs.* ME group.

[a] AUC values are calculated for 0–330 min.

[b] AUC value in plasma is given as ng min/ml.

soluble and can form a macromolecule compound because of the combination with plasma protein in the blood, was used as an indicator to study the effect of HAC-ME on the permeability of the BBB. The results of the in vivo distribution and the fluorescence microscopy imaging of brain showed that HAC-ME could increase the permeability of the BBB and it was concentration dependent (Yao et al., 2006).

Cationic delivery systems have been widely used for the delivery of drugs, proteins and genes to various targets including the liver, brain and kidney (Triguero et al., 1989; Opanasopit et al., 2002; Ma et al., 2006). However, in the present study, the AUC_{0-t} in plasma and brain after i.v. administration of HCC-ME was not significantly increased compared with the control group. Thus, not only were the beneficial effects not observed here, but the concentration of nobiletin in the spleen, liver and heart was also increased. The possible mechanism may be that the cationic HCC-ME systems showed a higher targeting efficiency in the liver and spleen, which is consistent with previous studies of cationic chitosan micelles (Huo et al., 2006) and cationic liposome complexes (Mahato et al., 1995). In these studies, the cationic chitosan micelles showed comparatively higher targeting to the spleen (Huo et al., 2006). The plasmid DNA/liposome complexes with a positive zeta potential were predominantly taken up by the liver within 5 min after intravenous administration (Mahato et al., 1995). Similar results were reported by Ma et al. (2005) for the cationized bovine serum albumin and by Nishida et al. (1991) for a cationic macromolecular conjugate of mitomycin C.

A prolonged residence in the blood was achieved with ME and HAC-ME. The MRT in the blood of ME and HAC-ME increased 2.24 and 3.18 times, respectively, when compared with the control group at same dose. The increase in the brain uptakes of nobiletin after i.v. administration of HAC-ME was directly related to the longer retention time of HAC-ME in the blood circulation, of which MRT (88.7 min) was markedly longer than those of ME group and control group.

Poor drug accumulation in the brain is known to be a result of rapid clearance of material from the blood and the extracellular fluid in the BBB (Huynh et al., 2006). So the prolonged drug retention time in the circulation and the inhibition of the drug excretion could help increase the drug accumulation in the brain (Calvo et al., 2001; Nakayama and Okano, 2005). The conventional nano-sized drug carriers are opsonized by plasma proteins in the bloodstream and trapped in the RES (Nakayama and Okano, 2005; Oku and Namba, 2005). Therefore, nanoparticles with reduced opsonization are expected to have prolonged circulation and to accumulate in non-RES tissues. To avoid RES trapping, two approaches have been considered. Nanoparticles may mimic cells circulating in the blood to escape host recognition as foreign substances, or they may be covered with a hydrophilic barrier to escape recognition. For the latter purpose, the hydrophilic polymers such as poly(ethylene glycol) (Dash et al., 2000; Oku and Namba, 2005), poly(*N*-vinyl-pyrrolidone) (Taeuchi et al., 2001; Park et al., 2003) and poloxamers (Muller et al., 1996) are widely used in the preparation of liposome (Dash et al., 2000; Taeuchi et al., 2001; Oku and Namba, 2005) and nanoparticles (Muller et al., 1996).

HAC is a hydrophilic polymer and easily soluble in water. The external surface of the microemulsion particles has a negative charge and are expected to be coated by the weakly positively charged HAC, which would result in the formation of a hydrophilic thin layer of chitosan molecule on the surface of the microemulsion. HAC may increase the hydrophilicity of microemulsion surface, which could inhibit the uptake of RES and prolong the retention time of nobiletin in the circulation. Moreover, the HAC layer outside the microemulsion particles

Table 2
Re[a] and Ce[a] of three microemulsions to control group in various tissues

Parameters	Tissues	Control	ME	HAC-ME	HCC-ME
Re	Brain	1	1.836	2.527	1.298
	Liver	1	1.274	1.575	1.075
	Kidney	1	1.823	1.625	1.058
	Spleen	1	0.809	1.076	1.448
	Heart	1	1.000	0.739	1.340
Ce	Brain	1	1.994	2.011	1.336
	Liver	1	1.346	1.119	0.831
	Kidney	1	0.956	1.006	0.799
	Spleen	1	0.671	0.402	1.430
	Heart	1	1.081	0.665	1.412

[a] $Re = (AUC_i)_m/(AUC_i)_{control}$; $Ce = (C_{max\,i})_m/(C_{max\,i})_{control}$ where "m", respectively, represent ME, HAC-ME and HCC-ME groups. "*i*" represent brain, liver, kidney, spleen and heart, respectively.

might retard the sedimentation of the drug in the system and improve the stability of drug-loaded microemulsion.

HAC shares similar properties with hyaluronic acid (HA). The latter consists of repeating units of D-glucuronic acid and *N*-acetyl-D-glucosamine, linked by β-1,4 and β-1,3 glycosidic bonds (Surendrakumar et al., 2003). Many studies have demonstrated that HA inhibits the phagocytosis of the macrophages at a dose and molecular weight dependent manner due to its mucoadhesive activity (Forrester and Balazs, 1980; Tamoto et al., 1994). This is another possible reason that HAC may prolong the existence time of HAC-ME in the blood and remain close to the main absorption site in the brain.

To elucidate and evaluate whether the microemulsions and chitosan-based microemulsions affect the distribution of nobiletin in the brain, relative uptake efficiency (Re) and ratio of peak concentration (Ce) were calculated and are given in Table 2. Compared with control group, Re of ME and HAC-ME in the brain were 1.836 and 2.527, respectively, and their Ce were 1.994 and 2.011 (all > 1). These are all higher than those of other tissues. It was suggested that ME and HAC-ME might increase the brain uptake capability of nobiletin. Similar results were reported by Tong et al. (2002) for nimodipine microemulsions and Yao et al. (2005) for cremophor-based microemulsions. In addition, comparing with ME, Re and Ce of HAC-ME in the brain exceeds 1 too (Table 3). It is concluded that HAC-ME were superior to the chitosan-free microemulsions for increasing the drug brain uptake.

Table 3
Re[a] and Ce[a] of chitosan-based microemulsions to chitosan-free microemulsion (ME) in various tissues

Parameter	Tissue	ME	HAC-ME	HCC-ME
Re	Brain	1	1.376	0.707
	Liver	1	1.236	0.844
	Kidney	1	0.818	0.580
	Spleen	1	1.329	1.789
	Heart	1	0.739	1.339
Ce	Brain	1	1.009	0.615
	Liver	1	0.831	0.618
	Kidney	1	1.053	0.836
	Spleen	1	0.599	2.131
	Heart	1	0.615	1.306

[a] $Re = (AUC_i)_m/(AUC_i)_{ME}$; $Ce = (C_{max\,i})_m/(C_{max\,i})_{ME}$ where "m", respectively, represent HAC-ME and HCC-ME groups. "*i*" represent brain, liver, kidney, spleen and heart, respectively.

Moreover, the addition of HAC might increase the solubilization of nobiletin in microemulsions and improve the stability of drug-loaded microemulsions (the data is not listed). And then, the inclusion of HAC allows for an about 20% reduction in the amount of surfactant required for the long-term stability of drug-loaded microemulsions in the same dose, which has benefit to decrease the potential side effects induced by the surfactants.

4. Conclusions

The use of HAC in the microemulsion formulations might significantly increase the brain uptake of nobiletin, and simultaneously reduce the concentration of drug delivered to the heart and kidney. These results indicate that HAC-ME may be advantageous for drug accumulation in the brain and may also help to reduce systemic toxicity. The prolongation of circulation time of nobiletin in HAC-ME may play an important role in the drug brain uptake. However, the AUC_{0-t} and MRT were not significantly increased by the addition of HCC. The reason for this is not clear. Further investigation may be necessary to elucidate the mechanism of drug delivery into the brain and improve the brain targeting efficiency.

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