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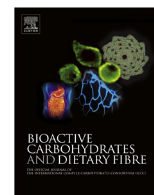

# Absorption and distribution of water-soluble hydroxypropyl chitosan in mice after oral administration

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ABSTRACT

Two kinds of water-soluble hydroxypropyl chitosan (HPCS) samples with different weight-average molecular weights ($M_w$) (HHPCS for higher $M_w$, $1.43 \times 10^5$ Da; LHPCS for lower $M_w$, $5.71 \times 10^3$ Da) were used to investigate their absorption and distribution in mice by oral administration after being labeled by fluorescein isothiocyanate. The result of *in vitro* degradation showed that HPCS degraded more easily in the presence of lysozyme than in pepsin. After oral administration of HPCS, HPCS would undergo a series of degradation and absorption under the action of tissue cells and enzymes in the body. The absorption and distribution of HPCS in organ tissues was significantly influenced by its $M_w$. With the decrease of $M_w$, the absorption rate and amount of HPCS increased. In fact, only a small amount of HPCS and its catabolite with low $M_w$ was absorbed by the tissues, and most HPCS was metabolized and excreted out of body rapidly. Additionally, there was some accumulation of HPCS in liver, and so HPCS content in liver and kidney was higher. The whole HPCS plasma concentration was lower instead, due to rapid plasma clearance. The absorbed HPCS molecules still maintained a relatively higher concentration even after 16 h in the tested tissues. The research results gave some valuable data for the application of water-soluble chitosan in food and biomedicine, especially as a potential candidate for drug delivery systems.
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## 1. Introduction

As a natural linear cationic polysaccharide, chitosan (CS) has attracted much scientific and industrial interest in the fields of biotechnology, pharmaceutics, food science, waste water treatment, agricultures and textiles (Elchinger et al., 2015; Mati-Baouche et al., 2014; Ravi Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Sandford & Stinnes, 1991; Shahidi, Arachchi, & Jeon, 1999), due to its unique bioactivity, including its excellent biodegradability, biocompatibility and nontoxicity (McAlindon, LaValley, Gulin, & Felson, 2000; Muzzarelli, 1993; Peng, Han, Liu, & Xu, 2005). In 1983, CS has been approved as feed additive by Food and Drug Administration (FDA). Especially for the pharmaceutical and biomedicine area, most studies indicated that CS has been considered as one of the most excellent candidates for drug delivery systems to enhance drug absorption, control drug release and target therapy as drug carriers (Agnihotri, Mallikarjuna, & Aminabhavi, 2004; Bansal, Sharma, Sharma, Pal, & Malviya, 2011; Bernkop-Schnürch & Dünnhaupt, 2012; Ravi Kumar et al., 2004; Sonia & Sharma, 2011; Wang, Tao, Zhang, Wei, & Ren, 2010).

Although many studies have been focused on the applications of CS in food engineering and biomedicine, its bioactive properties *in vivo* remained unclear, including bio-distribution, body absorption, biodegradation and toxicity, which were in close relation with drug action, medicine persistence and safety. Chae et al. investigated molecular-weight-dependent body absorption of CS *in vitro* and *in vivo*. CS oligosaccharides showed higher permeation and absorption profiles with negligible cytotoxic effect, which may be considered as a safe and potential candidate for pharmaceutical and biomedical applications (Chae, Jang, & Nah, 2005). Onishi et al. discovered that chitin with 50% deacetylate was highly biodegradable and was excreted quickly, and there was no accumulation in the mice after intraperitoneal administration (Onishi & Machida, 1999). As for CS micro-particles, Shimoda et al. analyzed its bioadhesive and absorptive characteristics (Shimoda, Onishi, & Machida, 2001; Takishima, Onishi, & Machida, 2002). Our previous study also found that the body absorption of CS was in close relation with its molecular weight and water-solubility (Zeng, Qin, Wang, Chi, & Li, 2008). CS with lower molecular weight had better

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solubility and was absorbed more easily by body tissues. For the absorption and distribution of chitosan, it was difficult for chitosan to be located *in vivo*, due to its colorless and no UV absorption, and so it was necessary for chitosan to be labeled using fluorescein. At present, isothiocyanate was used as fluorescein most commonly in most past studies (Chae et al., 2005; Onishi & Machida, 1999; Shimoda et al., 2001; Zeng et al., 2008).

In spite of these superior properties, plain CS has a major drawback: its solubility is poor above pH 6.0 (Qin et al., 2006). Now that most researches indicated that the bioactivity of CS was in close relation with its molecular weight and solubility, it is necessary to investigate the bioactivity of the water-soluble CS, such as carboxymethyl CS, and CS quaternary ammonium salt. Hydroxypropyl CS (HPCS) is another CS derivative with excellent water solubility, which was potentially suitable for drug carrier and food additive (Bansal et al., 2011; Sonia & Sharma, 2011; Wang et al., 2010). In the past few years, the structure, properties and use of HPCS were studied (Dong, Wu, Wang, & Wang, 2001; Peng et al., 2005), but little research about its metabolism *in vivo* was done. The safety evaluation of modified natural products is very important for their applications in food and drug carrier. In this paper, HPCS with different molecular weights was used to investigate its absorption and distribution in mice after oral administration by using fluorescein-labeling method.

## 2. Materials and methods

### 2.1. Materials

Initial CS ($M_w$, $1.86 \times 10^5$ Da; degree of *N*-deacetylation, DD, 90%) was supplied by Golden-shell Biochemical Co. LTD, China. Pepsin and lysozyme were purchased from Aladdin Co., whose activities were more than 2,500 units/mg dry weight and 5,000 units/mg dry weight, respectively. Fluorescein isothiocyanate (FITC) was purchased from Sigma Chemical Co. (USA). HPCS (degree of substitution, DS, 1.41) was obtained by etherification of CS with propylene oxide under alkaline conditions according to the previous study (Wang et al., 2014). Phosphate buffer solutions (PBS) were prepared using 0.2 mol/L sodium dihydrogen phosphate and 0.2 mol/L disodium hydrogen phosphate to form pH 5.5 and pH 6.86 solutions. All other reagents were of analytical grade and used directly without any further purification. Kunming female strain mice (4 weeks old) with a body weight ranging from 18 to 22 g were obtained from Hubei Experimental Animal Center (Wuhan, China).

### 2.2. In vitro degradation of HPCS

In order to understand the metabolism of HPCS in body, its enzymatic degradation was investigated imitating body metabolism using pepsin and lysozyme *in vitro*. Initially, HPCS (1.0 g) was dissolved in 500 mL phosphate buffer solution (pH 5.5), and then pepsin or lysozyme (10 mg) was added into the solution. The solution with and without enzymes was incubated at 37 °C, and the viscosity change was checked using the Ubbelohde viscosimeter at predetermined time.

### 2.3. Preparation of HHPCS and LHPCS samples

HPCS (10 g) was completely dissolved in 500 mL distilled water. UF-membrane of 10 kDa was used to separate the solution. The residue solution, which did not pass through the membrane, was precipitated by adding excessive acetone with 6 times volume. The solid was filtrated and washed with acetone 3 times to obtain HPCSsample with higher $M_w$ (HHPCS).

Lysozyme (0.1 g) was added into 500 mL 2 wt% HPCS aqueous solution. The solution was initiated under stirring at 37 °C for 8 h, and then the mixture was boiled for 10 min to deactivate the enzyme, and the enzyme residue was removed by filtration. The filtrate was separated by using UF-membrane. The fraction which passed through 10 kDa membrane but not 1 kDa membrane was precipitated in excessive acetone with 6 times volume, and the product was washed with acetone 3 times to get HPCS samples with lower $M_w$ (LHPCS). At last, the obtained HHPCS and LHPCS samples were dried by lyophilization.

The average molecular weight of the obtained HPCS samples was determined by gel permeation chromatography according to the previous paper (Qin, Du, Xiao, Li, & Gao, 2002). The $M_w$ of HHPCS and LHPCS were $1.43 \times 10^5$ Da and $5.71 \times 10^3$ Da, respectively. And their polydispersities were 1.87 and 1.16, respectively.

### 2.4. FITC labeling of HPCS (FITC-HPCS)

HPCS was labeled with FITC according to the previous study (Zeng et al., 2008). HPCS (0.6 g) was completely dissolved with double distilled water to form 2 wt% aqueous solution, and then methanol with 1.5 times volume was added under continuous stirring to form co-solvent system. Afterwards, a predetermined amount of FITC (HPCS/FITC, 60/1, w/w) dissolved in a small amount of acetone (about 2 mL) was added to the HPCS aqueous solution. The mixture was vigorously stirred for 24 h in dark at room temperature, and FITC-HPCS was obtained by precipitating the mixture in excess acetone with 6 times volume. The obtained FITC-HPCS was washed with acetone 6 times until there was no more visible absorption at 490 nm in filtrate with reference to FITC. Finally, the FITC-HPCS was obtained by lyophilization.

The absorbance strength of 5 mg/mL FITC-HPCS water/methanol (1/1.5, V/V) solution at the band of 490 nm was obtained using UV spectrophotometer. The content of FITC in FITC-HPCS (also known as labeling yield) was determined according to FITC standard curve using absorbance strength versus FITC concentration (Chae et al., 2005; Zeng et al., 2008). FITC contents of labeled HHPCS (FITC-HHPCS) and LHPCS (FITC-LHPCS) were 1.16% and 2.43%, respectively.

### 2.5. In vivo HPCS distribution test

Female mice (18–22 g body weight) were divided into groups with five mice each randomly. Before the administration of HPCS, the mice were fasted for 12 h but given water ad libitum, and then were administered 20 mg/mL FITC-HPCS aqueous solution through an oral gavage tube. The total volume of the administrated HPCS was 0.20 mL (at the dose of 200 mg/kg). The blood, liver, kidney, thymus, lung, heart and spleen were collected after the mice were sacrificed at the predetermined time. The blood samples from eyeballs were used directly after been suspended at room temperature overnight. The other various tissues were homogenized using glass homogenizer together with physiological saline (0.9 wt% NaCl solution), and then were centrifuged at 4,200 rpm for 20 min to separate the insoluble solids. Next, the 0.50 mL obtained sample solutions were added to 3.50 mL phosphorous buffer solution (pH 6.86). The fluorescence intensity of the resulting solutions was measured with an excitation (EX) wavelength at 490 nm using FLS 920 Fluorescence Spectrometer. The obtained fluorescence intensity at 520 nm of the emission (EM) wavelength was normalized with the standard FITC-HPCS solution, and the FITC-HPCS content of each tissue was determined from the corrected concentration and the tissue weight. The plotted data were the mean $\pm$ SD ($n=3$).

## 3. Results

### 3.1. Enzymatic degradation of HPCS

The degradation of HPCS with pepsin and lysozyme was achieved and the viscosity changes of the HPCS aqueous solution were shown in Fig. 1. In the case of the control without enzyme, the viscosity of HPCS solution was essentially unchanged even after the incubation at 37 °C for 24 h, which indicated that HPCS did not degrade in phosphate buffer solution with pH 5.5. After the incubation in the presence of pepsin or lysozyme, the viscosity of HPCS solution was found to decrease more or less. Chitosan could be decomposed under the chemical reagent and enzyme (Qin, Du, & Xiao, 2002). In the past, we used hemicellulase or lysozyme to degrade chitosan (Qin et al., 2002, 2003, 2006; Zeng et al., 2008). Onishi also proved that the in vitro biodegradability of Chitin was accelerated by incubation with lysozyme and murine plasma and urine (Onishi & Machida, 1999). This result also showed the enzyme-catalyzed degradation for HPCS. However, lysozyme appeared to cause a stronger catalysis than pepsin for the degradation of HPCS. HPCS was degraded slowly in the presence of pepsin, and the viscosity showed more than 75% initial value even after 24 h incubation. But for the presence of lysozyme, its viscosity was reduced rapidly to less than half its initial value within 1 h. And with the further prolongation of incubation time, the viscosity decreased very slowly. It is known that there are several enzymes in mammalian bodies, including pepsin and lysozyme. The results indicated that HPCS underwent extensive biodegradation by the enzymes after oral administration, which may be helpful for the absorption of HPCS by the tissues (Chae et al., 2005).

### 3.2. Absorption of HPCS in blood

It was reported that the content of CS in blood reflected the absorbed dose of CS by the small intestine (Chae et al., 2005). The concentration of different HPCS in plasma at different time was demonstrated in Fig. 2.

It could be seen from the graph that the absorption profiles of two kinds of HPCS were similar after oral administration. With the prolongation of time, the concentration of HPCS in blood increased at first, and then decreased continuously until approaching to zero. But for two kinds of HPCS with different $M_w$, the absorption amount and absorption rate were different. In the case of HHPCS, its content in blood reached the maximum at about 2 h while the time for LHPCS to reach the maximum in blood was at about 1 h, which indicated that the lower the $M_w$ was for HPCS, the faster the absorption rate was by the small intestine. And also, decreasing the HPCS molecular weight resulted in increasing its absorbed amount. The concentration of LHPCS in blood reached 2.20 μg/mL at 1 h, and instead the maximum content of HHPCS was only 0.84 μg/mL at 2 h because of poor absorption. All of these showed that HPCS with low $M_w$ was absorbed more easily by small intestine.

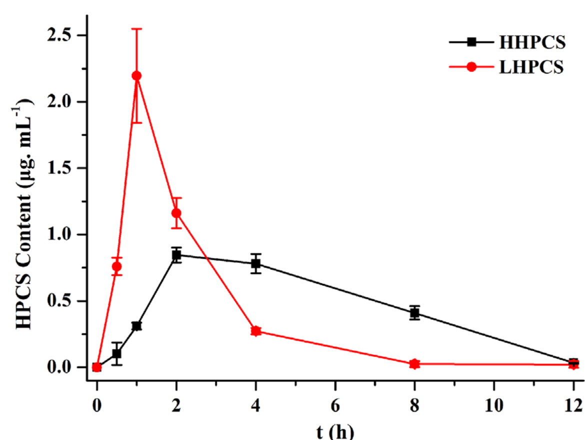


Fig. 2. The absorption of HPCS and their contents in plasma at different time after oral administration.

Although HPCS was absorbed rapidly by the small intestine, the absorbed HPCS in blood was also distributed to the other organ tissues rapidly so that both HPCS levels in blood decreased to zero after some time (12 h for HHPCS and 8 h for LHPCS). With the decrease of the HPCS $M_w$, it was dispersed more easily. As for LHPCS, its content in blood decreased rapidly from 1 h to 4 h. After 4 h, the rate of decrease slowed and it reached zero at 8 h. On the contrary, the blood concentration of HHPCS decreased gradually from 2 h to 12 h. Overall, the whole HPCS levels in blood were low, which may be related to two possibilities. One was that HPCS was absorbed rapidly by small intestine, and also the absorbed HPCS was transferred and cleared by blood to other organ tissues very quickly (Zeng et al., 2008). The other possibility could be that only little of the orally administered HPCS was absorbed by small intestine.

### 3.3. Distribution of HPCS in organs

Liver was the first barrier for exogenous compounds from blood after xenobiotics were absorbed by small intestine (Xiang, 2003). Therefore, the HPCS content in liver was an important index to reflect its absorption. From Fig. 3, the results indicated that the

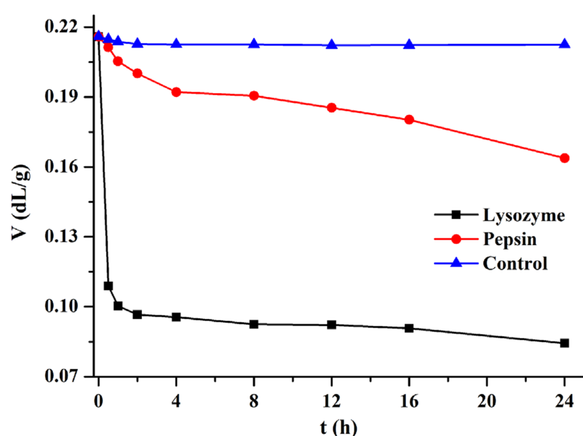


Fig. 1. The degradation and viscosity change of HPCS treated with pepsin and lysozyme.

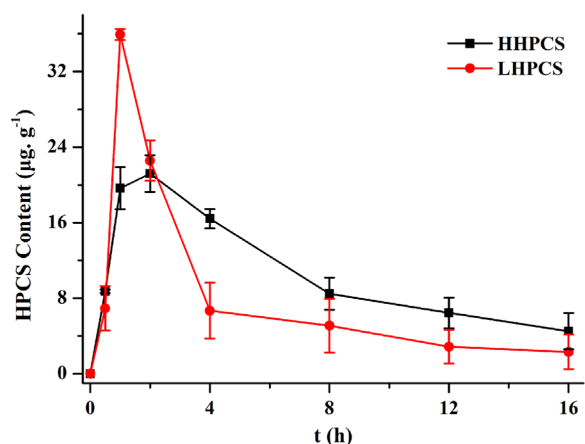


Fig. 3. The absorption of HPCS and their contents in liver at different time after oral administration.

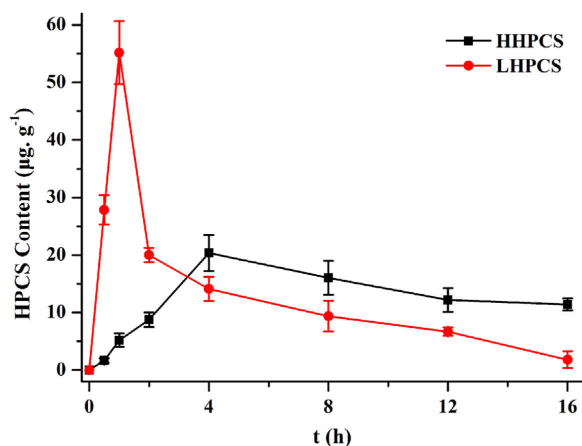


**Fig. 4.** The absorption of HPCS and their contents in kidney at different time after oral administration.

HPCS level in liver was much higher than that in blood, which suggested that there were some accumulation in liver for the absorbed HPCS in blood.

Comparing the concentration of two different HPCS, in the early stage, the LHPCS content in liver increased more rapidly than HHPCS content, and it also decreased rapidly. The LHPCS content increased to the maximum at 1 h, but it took about 2 h for HHPCS to reached a maximum. After 2 h, LHPCS had the lower concentration in liver than HHPCS. This result suggested that the absorbed CS smaller molecules from LHPCS were metabolized more easily and transferred to other tissues more rapidly than the absorbed larger molecules from HHPCS. And so there was higher liver accumulation for HHPCS although it had rapider blood clearance.

After metabolism by the various body organs, the metabolic products of absorbed HPCS were transferred into kidney at last. From the results of HPCS content in kidney in Fig. 4, it was found that HPCS reached rapidly into kidney at the beginning after administration. But LHPCS had the higher content than HHPCS before 2 h, which may be in relation to that LHPCS smaller molecules were absorbed more easily by the organ tissues and needed less time to be metabolized and transferred to kidney. Interestingly, it was observed that the total HPCS content in kidney was higher than that in liver at the early stage. Even LHPCS content in kidney (55 μg/g) was more than that in liver (36 μg/g) during the first 1 h. Two possible reasons came to mind, the first could be that HPCS was metabolized and excreted out of body rapidly. The second reason could be that little metabolism took place in body tissues and most HPCS was rapidly transferred to kidney.

Besides the liver and kidney, the distribution of HPCS in other body organs including thymus, lung, heart and spleen was also checked at predetermined time after administration. The resultant contents of HHPCS and LHPCS were showed in Fig. 5. From the graphs, it could be seen that the absorbed HPCS molecules almost were disseminated to the whole body, and only absorption amounts were different for different tested organs. Even HPCS molecules remained in body organs during the whole test period. It was also observed that with the decrease of HPCS $M_w$, there was a tendency that HPCS content in these various body organs increased. This suggested that HPCS molecules with low $M_w$ were absorbed more quickly by small intestine again and could penetrate the bio-membranes of every organs to reach other tissues more easily (Onishi & Machida,1999). All in all, the total concentration for two kinds of HPCS in these tested organs was generally lower in comparison with liver and kidney, which indicated that most HPCS was excreted out of body, and only small amount of HPCS was absorbed and circilated in the bloodstream.

## 4. Discussion

As a kind of natural polymer, most previous reports indicated that the physiological activity of CS was closely related to its absorption and body distribution *in vivo*. The molecular weight and water-solubility could be considered as two critical parameters for the absorption of CS *in vivo* (Chae et al., 2005; Zeng et al., 2008). Apparently, the intestinal absorption of CS *in vivo* was influenced by its water-solubility. The solid transport of CS was difficult while dissolved CS in fluid could be easily absorbed by small intestine. Our previous study also found that the body absorption of CS was in close relation with its molecular weight and water-solubility. The lower molecular CS with better solubility was found to be absorbed more easily (Zeng et al., 2008).

HPCS is the etherified product of CS with propylene epoxide. As a kind of water-soluble CS derivatives, its $M_w$ could be considered as a critical parameter for the absorption in mice after p.o. As the molecular weight of HPCS increased, the absorbing rate by the various organs decreased. It may be due to that the exterior cell tissues of body organs were just like osmotic membranes, which permitted HPCS with a certain size (less than 60 kDa) to pass through (Xiang, 2003). As the molecular weight increased, it became more difficult for HPCS to penetrate the intestinal membranes and be absorbed by body organs. After administration, most HPCS macromolecules were transferred directly from stomach to small intestine, and were excreted out of body at last due to their high molecular weights. Therefore, the absorbed amount of HPCS molecules was less than the administered amount.

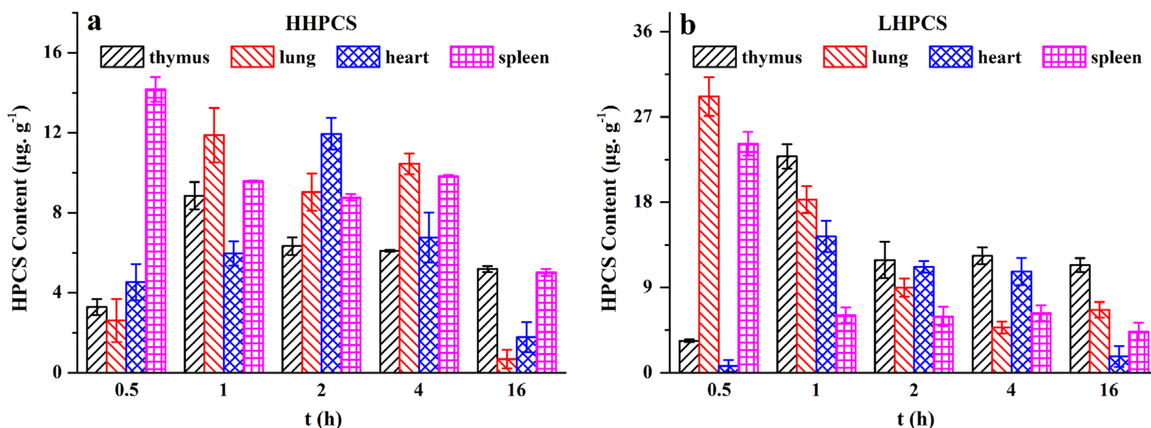


**Fig. 5.** The absorption of HPCS and their contents in other organs at different time after oral administration.

Naturally, there still left less HPCS which was absorbed by small intestine, and absorbed HPCS was distributed to all tested organs, such as blood, liver, kidney, thymus, lung, heart, and spleen. At the same time, some HPCS was decomposed by tissue enzymes, and the degraded products with smaller sizes were absorbed more easily through the bio-membranes of various organs.

The concentration of HPCS in blood was observed to be lower than that in liver over time. HPCS with high molecular weight was absorbed difficultly by small intestine. It was found the higher the molecular weight of HPCS was, the lesser the absorbed HPCS content was into bloodstream. On the other hand, it was associated with particularly rapid plasma clearance after absorption. The plasma clearance of HPCS became more difficult and it could keep in blood for a longer time with the increase of the HPCS $M_w$. It was interesting that two HPCS samples showed higher liver and kidney distribution after oral administration. The HPCS macromolecules from blood did not penetrate into liver cell tissues because liver excreted metabolite with molecular weight less than 5000 (Xiang, 2003). The result was that HPCS with higher $M_w$ was assimilated and transferred slowly by liver cells, and only was accumulated in liver. Thus HPCS with higher $M_w$ showed relatively higher liver distribution even after 16 h. As for kidney, it is the main excretion organ, and it just excretes xenobiotics with molecular weight lower than 500 (Xiang, 2003). The result indicated that HPCS was quickly eliminated from the body organs at the beginning. One reason was that HPCS was transferred directly to kidney after being absorbed with little metabolism, especially for HPCS with higher $M_w$. The other reason may be in relation with its extensive biodegradability by body tissues. After absorption, HPCS underwent the enzyme-catalyzed degradation in the body to become the products with uniformly small molecular sizes (Onishi & Machida, 1999). In addition, HPCS almost permeated into the other organ tissues, including thymus, lung, heart and spleen. With the increase of HPCS $M_w$, it was more difficult for HPCS molecules to permeate the bio-membrane and entered into other organs, and so the HHPCS content was lower than LHPCS content in these organ tissues.

## 5. Conclusion

In recent years, as a kind of natural biomacromolecule, chitosan was used widely in food engineering and biomedicine. And so, it is necessary to understand the interreaction of chitosan with biological tissues and its biological metabolism in *vivo*. In this study, the distribution and absorption of water-soluble HPCS derivatives with different $M_w$ was investigated *in vivo*. After oral administration, HPCS was absorbed and distributed to all over the body organs, such as liver, kidney, thymus, lung, heart and spleen, but most HPCS was excreted out of body directly without digestion at all. At the same time, the absorbed HPCS underwent a series of degradation and absorption under the action of tissue cell and enzyme *in vivo*. The absorption and distribution of HPCS in organ tissues was in relation to their molecular sizes closely. HPCS with lower molecular weight was more easily absorbed by organ tissues. Although HPCS was distributed the whole body, HPCS was concentrated mainly in liver and kidney while the other organs had less HPCS. Water-soluble HPCS could still maintain a relatively consistent and much high concentration for a long time, which suggested that HPCS could be a good candidate as drug carrier for a long-lasting targeted drug delivery system. The research results provided experimental basis for the further use of chitosan in food and biomedicine.

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