

Research Article

Acute Oral Toxicity and Kinetic Behaviors of Inorganic Layered Nanoparticles

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Received 25 June 2013; Revised 5 September 2013; Accepted 5 September 2013

Academic Editor: Xiaoming Li

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Layered double hydroxide (LDH) nanoparticles, also known as anionic clays, have attracted a great deal of interest for their potential as delivery carriers. Recent studies showed that LDH nanoparticles can efficiently deliver drugs or bioactive molecules into cells, which are highly related to their endocytic pathway. However, the efficient cell permeation capacity of LDH may also raise concern about their toxicity potential. In this study, the acute oral toxicity of LDH nanoparticles was assessed, and their kinetic behaviors, such as plasma concentration-time curve, tissue distribution, and excretion, were also evaluated in mice. No significant effects of oral LDH nanoparticles on behaviors, body weight gain, survival rate, and organosomatic index were observed up to the dose of 2000 mg/kg for 14 days. Serum biochemical parameters did not significantly increase, indicating that LDH nanoparticles did not cause acute liver or kidney injury. Plasma concentration of LDH nanoparticles rapidly decreased within 30 min depending on exposure doses, but they did not accumulate in any specific organ. Their excretion via urine and feces was observed within 24 h. These findings suggest that LDH nanoparticles do not exhibit acute oral toxicity and favorable kinetic behaviors in mice and, therefore, will be promising candidates for biological and pharmaceutical applications.

1. Introduction

In recent years, inorganic nanomaterials have been extensively developed for biomedical applications, such as diagnostic or imaging agents, tissue engineering, and drug delivery carriers [1–3]. Nanoparticles have large surface areas and high reactivity resulting from their small size. Moreover, their structure can be easily modified for target specific delivery [4]. This is fascinating to efficiently deliver target molecules to specific organs and consequently contributing to enhanced efficacy as well as reduced toxicity. Among them, layered double hydroxides (LDHs) nanoparticles, also known as anionic clays or hydrotalcite-like compounds, consist of positively charged metal hydroxide sheets and interlayers containing charge-balancing anions and water molecules to form lamellar structure [5, 6]. Thus, LDHs can intercalate anionic molecules, such as drugs, nucleotides, and biomolecules, into the interlayer spaces, release them in a controlled manner, and eventually protect them against harsh biological conditions, thereby attracting much attention as nonviral

delivery carriers [7, 8]. Recent studies have demonstrated that LDHs can efficiently deliver anticancer drugs or bioactive molecules into cells, leading to enhanced efficacy of intercalated molecules, which are closely related to their energy-dependent endocytic pathway, clathrin-mediated endocytosis [9–11]. However, the efficient cell permeation capacity of LDHs may cause undesirable harmful effects by interfering with biological cellular functions, which raises concern about their toxicity potential on human health.

Toxicological effects of LDH nanoparticles on human cells were recently investigated *in vitro*, demonstrating low cytotoxicity of LDHs compared to other inorganic nanoparticles such as iron oxide, silica, and carbon nanotubes [12]. Cytotoxicity of LDHs with respect to particle size [13], chemical composition [14], and structural stability [15] was also reported; ZnAl-LDH and 50 nm particles showed slightly higher toxicity than MgAl-LDH and 100 to 350 nm particles, respectively, and carbonate form of LDH with strong stability exhibited high toxicity compared to its chloride form possessing easy decomposition property in cell lines [16]. However,

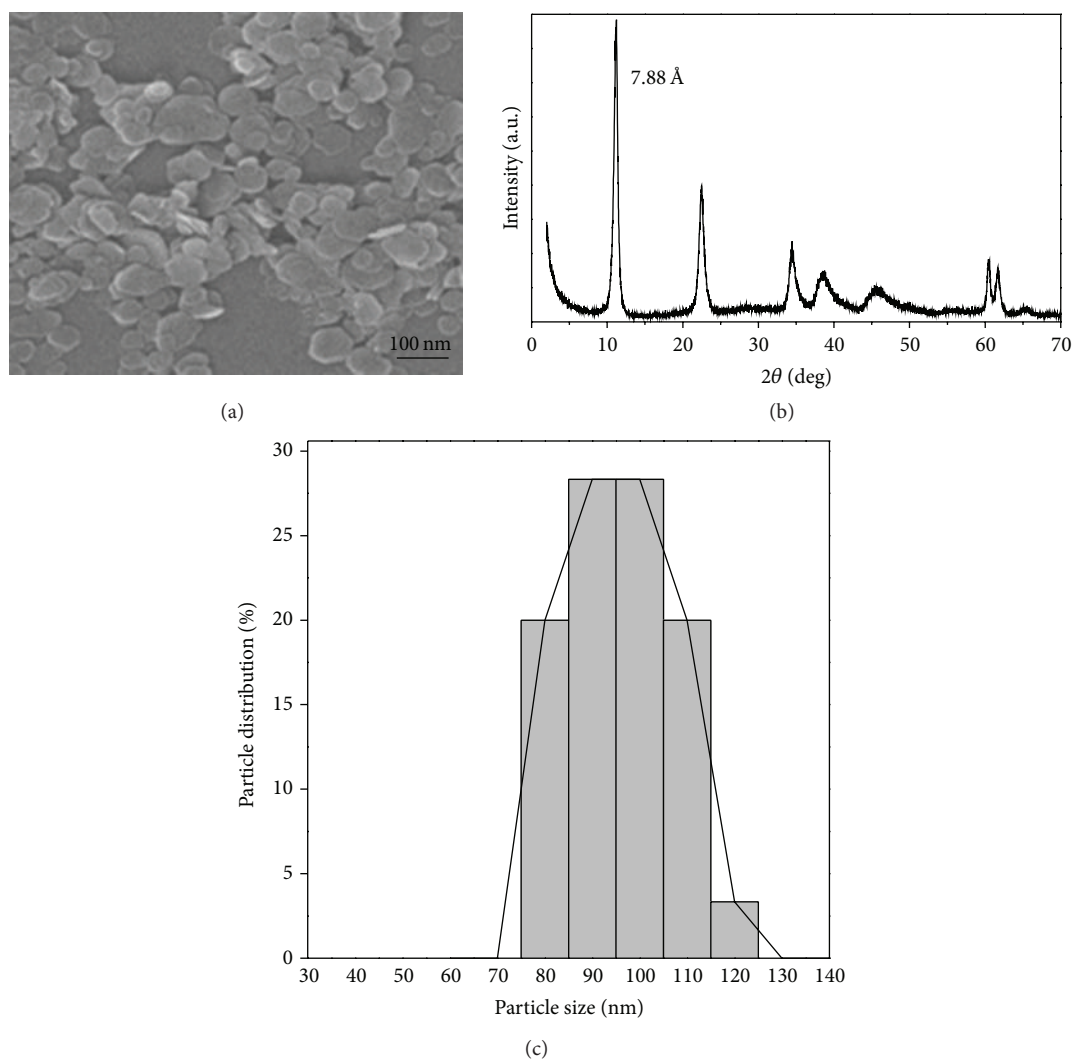


FIGURE 1: SEM image (a), PXRD pattern (b), and size distribution (c) of LDH nanoparticles. Particle size distribution was presented by randomly selecting 200 particles from SEM image and measuring each particle size.

research on toxicity evaluation of LDHs *in vivo* is extremely limited. Toxicity evaluation in whole animals provides more practical data, reflecting interaction of nanoparticles with immune systems, proteins, and dynamic body fluids at the systemic level. In particular, no information about acute oral toxicity of LDHs is available, although the gastrointestinal tract is one of the important routes by which nanoparticles get into the human body, for example, via oral intake of water and foods or drugs delivered by nanomaterials [17, 18]. Moreover, pharmacokinetics of LDHs have not been determined yet, which elucidate absorbed or accumulating amount as well as clearance kinetic profile *in vivo*.

In this study, the acute oral toxicity of LDH nanoparticles was evaluated in mice according to the OECD guideline. Pharmacokinetic, tissue distribution, and excretion studies were also performed after single-dose oral administration, aiming at understanding the relationships between toxicity potential of LDH nanoparticles and their kinetic behaviors in the body.

2. Materials and Methods

2.1. Preparation and Characterization of Nanoparticles. LDH nanoparticle, a chloride form of MgAl-LDH, was prepared by coprecipitation method; the salts $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, in a molar ratio $\text{Mg}/\text{Al} = 2$, were dissolved in decarbonated water and then precipitated by addition of NaOH/NaCl at a final pH of 10.5. The suspension was hydrothermally treated for 12 h at 100°C . The obtained white precipitation was centrifuged, washed with deionized water, and then freeze dried. Thus, prepared LDH nanoparticles were analyzed by powder X-ray diffraction (PXRD: Phillips PW3710 diffractometer with Ni-filtered $\text{CuK}\alpha$ radiation ($\lambda = 1.5418 \text{ \AA}$)) and by scanning electron microscopy (SEM: HITACHI S-4300).

2.2. Animals and Diets. Male Balb/c mice, aged 7 weeks and weighing 25–27 g, were purchased from G-Bio (Seoul, Republic of Korea). The animals were housed in plastic

laboratory animal cages in a ventilated room. The room was maintained at $20 \pm 2^\circ\text{C}$ and $60 \pm 10\%$ relative humidity on a 12 h light/dark cycle. Water and commercial laboratory complete food for mice were available *ad libitum*. They were acclimated to this environment for 7 days before receiving experimental treatment. All animal experiments were performed in compliance with the animal and ethics review committee of the Seoul Women's University.

2.3. Acute Toxicity Evaluation. The experiments to determine the LD_{50} (lethal dose 50%) values of LDH nanoparticles were designed in accordance with the method provided by the OECD guideline 423. Prior to dosing, food but not water was withheld for 4 h. LDH nanoparticles were homogeneously suspended in 0.9% saline by vortexing and ultrasonating the suspension for 5 min at 42 kHz (Vibra Cell Sonics & Materials Inc.). Four different concentrations of LDH nanoparticles (5, 50, 300, and 2000 mg/kg) were administered to each group of three mice via oral gavage in a stepwise procedure. Group of three mice, receiving identical volume of 0.9% saline, was served as a control group. Changes in body weight, behaviors, and symptoms were carefully recorded every day following the treatment. After 14 days, all organs were collected and organosomatic index was calculated by the following formula: $[\text{weight (g) of the organ}/\text{total body weight (g)}] \times 100$.

2.4. Blood Biochemical Analysis. Blood samples were also collected via tail vein, and the serum was obtained by centrifugation of the whole blood at 3000 rpm for 10 min. Liver function was evaluated based on the serum levels of alkaline phosphatase (ALP: ABD Bioquest), alanine aminotransferase (ALT: Bioo Scientific), and aspartate aminotransferase (AST: Bioo Scientific) according to the manufacturer's protocols. Nephrotoxicity was determined by measuring the concentrations of blood urea nitrogen (BUN: BioAssay Systems) and uric acid (UA: BioAssay Systems) in the serum according to the manufacturer's protocols.

2.5. Pharmacokinetics, Tissue Distribution, and Excretion Studies. For pharmacokinetic study, the blood samples (about 0.2 mL) were collected via tail vein at several time points (0, 5, 15, 30, 60, 120, and 240 min) after single-dose oral administration of LDH nanoparticles (5, 50, 300, and 2000 mg/kg). The blood sample at 0 h before oral administration was used to determine basal Mg level in the plasma. The blood samples were centrifuged at 3000 rpm for 15 min at 4°C to obtain the plasma and stored at -70°C before analysis. The following pharmacokinetic parameters were estimated using Kinetica program (version 4.4, Thermo Electron Corporation, USA): maximum concentration (C_{max}), time to maximum concentration (T_{max}), area under the plasma concentration-time curve (AUC), half-life ($T_{1/2}$), and mean residence time (MRT). To evaluate biodistribution of LDH nanoparticles, the tissue samples such as brain, heart, kidney, liver, lung, and spleen were collected at 0, 5, 15, 30, and 60 min following euthanasia by CO_2 . Urine and feces were also collected for 7 days.

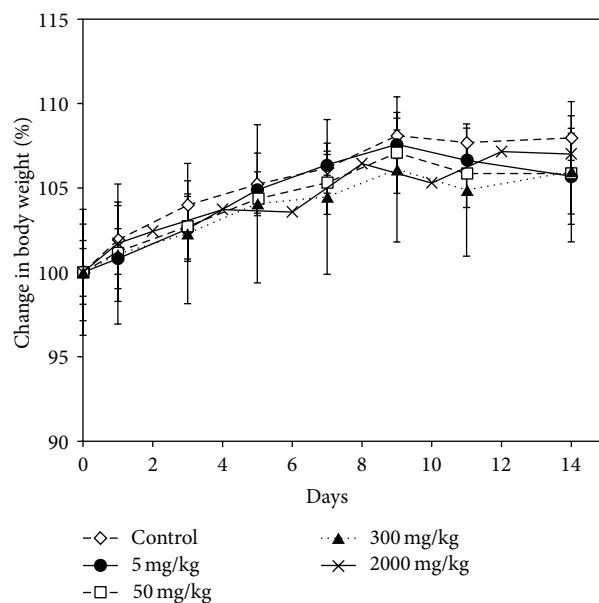


FIGURE 2: Changes in body weight in mice administered four different doses of LDH nanoparticles for 14 days.

2.6. ICP-AES Analysis. The plasma, tissue samples, urine, and feces were digested in 3 mL of ultrapure nitric acid. After adding 0.5 mL of H_2O_2 , each mixture was heated at about 160°C until the samples were completely digested. Then, the remaining nitric acid was removed by heating until the solutions were colorless and clear. The solutions were finally diluted to 10 mL with ultrapure water. Inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Jobin Yvon Hriba, JY 2000 Ultrace) was used to analyze Mg concentration, a main component of LDH, in the samples.

2.7. Statistical Analysis. Statistical analysis was performed using Student's *t* test for unpaired data, and *P* values of less than 0.05 were considered significant. All data were presented as mean \pm standard deviation (SD).

3. Results and Discussions

3.1. Characterization. The prepared LDH nanoparticles were analyzed by SEM (Figure 1(a)), showing homogenous size distribution with an average particle size of 91.8 ± 10 nm (Figure 1(c)). PXRD pattern showed typical characteristic peaks for LDH nanoparticles (Figure 1(b)), with the 003 reflection around $\sim 11^\circ$ (2θ) and *d*-spacing of 7.88 Å. From the SEM image and PXRD pattern, average aspect ratio was determined to be 3.5 as calculated by particle diameter/particle thickness, which is well consistent with the previous report [10]. LDH nanoparticles of about 100 nm in size were synthesized because it was found to be optimum for efficient cellular uptake [10].

3.2. Acute Toxicity. Figure 2 shows change in body weight after single-dose oral administration of LDH nanoparticles in mice. No significant difference in body weight gain was

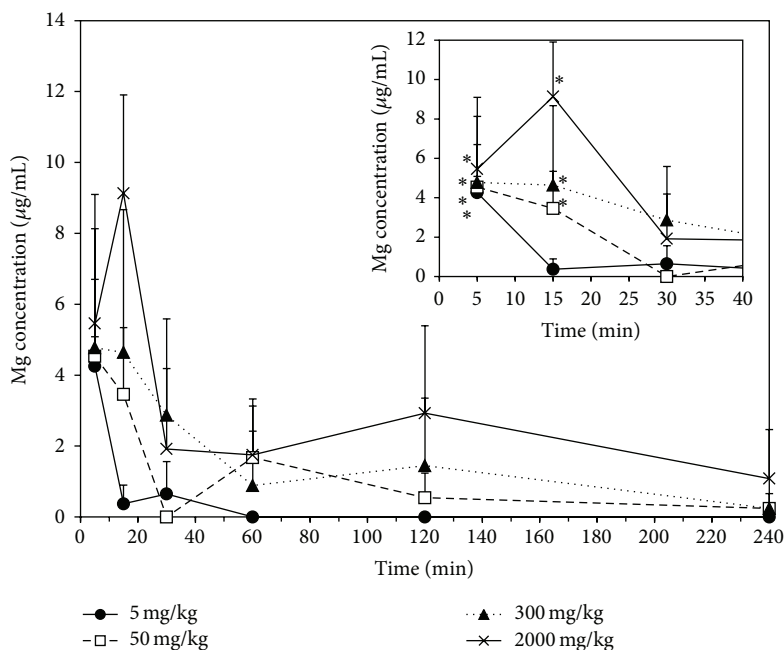


FIGURE 3: Plasma concentration-time curve of LDH nanoparticles after single-dose oral administration to mice. LDH concentrations were measured by analyzing a main component of LDH, Mg, using ICP-AES. The data were presented as increased Mg levels after subtraction of basal Mg level in untreated control group. Insert: higher magnification of plasma concentration-time curves for 30 min. *Significant difference from the control group ($P < 0.05$).

found between the control and the groups administered different doses of LDH nanoparticles ($P > 0.05$). Moreover, no mortality and abnormal behaviors or symptoms, such as decrease in food and water intake, diarrhea, loss of movement, and change in the size of eye's pupils and eye pigment, were observed in mice treated with even the highest dose for 14 days, indicating that the LD_{50} values were more than 2000 mg/kg. This result is an indication of low oral toxicity potential of LDH nanoparticles. Organosomatic indices of organs including brain, heart, intestine, kidney, liver, lung, spleen, and stomach were also assessed following oral administration of LDH nanoparticles (Table 1), showing no significant decrease or increase of organosomatic indices of all organs in mice administered up to 2000 mg/kg. This indicates that LDH nanoparticles did not affect any organ weight. All the results including changes in body weight, survival rate, and organosomatic index clearly suggest that orally administered LDH nanoparticles did not cause acute toxicity in mice.

3.3. Change in Blood Biochemical Parameters. Biochemical assay is generally and clinically used to evaluate target-specific tissue injury or disease. Elevated levels of ALP, ALT, or AST in the blood are an indication of the liver damage and have been associated with liver injury, since these metabolic enzymes are primarily expressed in the liver [18, 19]. On the other hand, urea, a major end product of protein catabolism in animals, is primarily produced in the liver and secreted by the kidney. A waste product generated from the degradation of purines, UA, is also filtered and removed by the kidney.

Thus, BUN or UA determination in the blood is very useful to assess kidney function [18, 20]. Table 2 showed that the levels of all biochemical parameters in the serum of mice treated with high doses, 300 and 2000 mg/kg of LDH nanoparticles, did not significantly increase in comparison with those in the control group after 14 days ($P > 0.05$). Biochemical parameters were analyzed after 14 days to evaluate possible delayed toxicity after reabsorption in the intestine as well as to examine the capacity of recovery of these organs after exposure to LDH nanoparticles. No effect of LDH nanoparticles on biochemical parameters was also found after 24 h (data not shown). This result clearly suggests that LDH nanoparticles did not cause acute liver or kidney injury by gastrointestinal ingestion.

3.4. Pharmacokinetics. Pharmacokinetic behaviors of oral LDH nanoparticles in mice are presented in Figure 3. The plasma concentration-time curve, measured by a main component of LDH, Mg, was dependent on the dose administered. However, significant increase in LDH levels was found only within 15 min in all the cases, and the peak concentrations rapidly decreased after 30 min even when 2000 mg/kg were administered. The pharmacokinetic parameters following oral administration were presented in Table 3. It is clear that all parameters increased as administered dose increased. Based on AUC values which elucidate the total amount of nanoparticles reaching the systemic circulation, absorptions (%) of 5, 50, 300, and 2000 mg/kg of LDH nanoparticles were determined to be about 2.85, 1.37, 0.40, and 0.14%, respectively. This result indicates that small amount of LDH

TABLE 1: Organosomatic indices of organs in different groups of mice at 14 days after oral administration.

Organ	Control	5 mg/kg	50 mg/kg	300 mg/kg	2000 mg/kg
Brain	0.88 ± 0.15	0.91 ± 0.11	0.79 ± 0.15	0.88 ± 0.09	0.86 ± 0.07
Heart	0.44 ± 0.12	0.34 ± 0.06	0.35 ± 0.02	0.24 ± 0.10	0.29 ± 0.04
Intestine	7.04 ± 0.49	6.03 ± 0.33	6.34 ± 0.36	5.59 ± 1.00	5.89 ± 0.90
Kidney	0.93 ± 0.11	0.88 ± 0.01	0.81 ± 0.02	0.78 ± 0.03	0.82 ± 0.03
Liver	3.81 ± 0.37	3.66 ± 0.28	3.33 ± 0.19	3.51 ± 0.13	3.35 ± 0.09
Lung	0.37 ± 0.02	0.41 ± 0.05	0.41 ± 0.05	0.41 ± 0.10	0.37 ± 0.02
Spleen	0.24 ± 0.09	0.23 ± 0.04	0.27 ± 0.03	0.21 ± 0.05	0.28 ± 0.02
Stomach	0.51 ± 0.17	0.23 ± 0.07	0.65 ± 0.24	0.58 ± 0.14	0.42 ± 0.26

All treated groups showed statistically no significant differences from the control group ($P > 0.05$).

TABLE 2: Changes in biochemical parameters in the serum of mice administered LDH nanoparticles.

Group	ALP (U/L)	ALT (U/L)	AST (U/L)	BUN (mmol/L)	UA (μ mol/L)
Control	120.30 ± 28.05	72.54 ± 22.65	166.52 ± 43.76	3.46 ± 1.01	156.81 ± 38.21
300 mg/kg	91.51 ± 33.77	80.13 ± 25.39	152.22 ± 39.92	3.62 ± 1.08	145.55 ± 29.52
2000 mg/kg	112.54 ± 17.51	85.76 ± 29.61	159.37 ± 39.97	6.62 ± 1.92	120.06 ± 28.54

All treated groups showed statistically no significant differences from the control group ($P > 0.05$).

ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; UA: uric acid.

TABLE 3: Pharmacokinetic parameters of different doses of LDH nanoparticles in mice after oral administration.

Dose	C_{\max} (mg/L)	T_{\max} (h)	AUC ($h \times mg/L$)	$T_{1/2}$ (h)	MRT (h)
5 mg/kg	4.15 ± 1.41	0.08 ± 0.00	0.79 ± 0.29	0.22 ± 0.20	0.35 ± 0.29
50 mg/kg	4.44 ± 0.32	0.14 ± 0.10	4.00 ± 2.36	0.73 ± 0.49	1.27 ± 0.82
300 mg/kg	7.24 ± 0.70	0.17 ± 0.10	8.74 ± 4.05	1.38 ± 0.67	2.15 ± 1.27
2000 mg/kg	9.36 ± 1.92	0.21 ± 0.08	11.96 ± 6.29	1.86 ± 0.10	3.14 ± 0.57

C_{\max} : maximum concentration; T_{\max} : time to maximum concentration; AUC: area under the plasma concentration-time curve; $T_{1/2}$: half-life; MRT: mean residence time.

nanoparticles could be absorbed into the blood stream via the gastrointestinal tract. About 15–30% of orally administered ZnO nanoparticles (2000 mg/kg), depending on surface charge, were determined to enter the systemic circulation [21], supporting low oral absorption of LDH nanoparticles compared to that of ZnO nanoparticles. It is worth noting that all MRT values were within 3.1 h even when 2000 mg/kg were administered, clearly indicating that the highest dose of LDH nanoparticles could be cleared from the body after 3.1 h. The low absorption and rapid clearance kinetic profile of LDH nanoparticles may explain their low toxicity as observed in Figure 2 and Tables 1 and 2, implying their low chronic toxicity potential in after long-term exposure.

3.5. Tissue Distribution. Figure 4 shows the tissue distribution profile of LDH nanoparticles following single-dose oral administration in mice. LDH levels did not significantly increase in the kidney, liver, lung, and spleen for 60 min after oral administration compared to basal Mg level in the tissues. Statistically increased Mg concentrations were not found in the other organs, such as brain and heart (data not shown). Statistical analysis also confirmed that significant difference was not found between the control and LDH-treated organs after 1 and 14 days (data not shown). These results suggest

that orally administered LDH nanoparticles did not significantly accumulate in any specific target tissue, which can be explained by their pharmacokinetic behaviors (Figure 3), such as rapid decrease in the plasma concentration-time curve, low absorption, and short residence time. On the other hand, tissue distribution of inorganic nanoparticles to target organs has been generally reported after oral administration; the kidney, liver, and lung were found to be target organs for accumulation of ZnO nanoparticles, regardless of particle size, surface charge, or gender [22], while silver nanoparticles were primarily distributed to the liver following single-dose oral administration to rats [23]. Hence, the fact that LDH nanoparticles were not found in any specific target tissue strongly supports their low toxicity potential.

3.6. Excretion Profile. Excretion patterns of LDH nanoparticles via urine and feces following oral administration are presented in Figure 5. Significantly increased Mg levels were found in urine and feces within 24 h but returned to normal levels after 24 h. A much higher Mg concentration was detected in feces than in urine, indicating that most LDH nanoparticles were eliminated via feces. This result also suggests that direct fecal clearance and biliary excretion play a major role in the elimination of LDH nanoparticles.

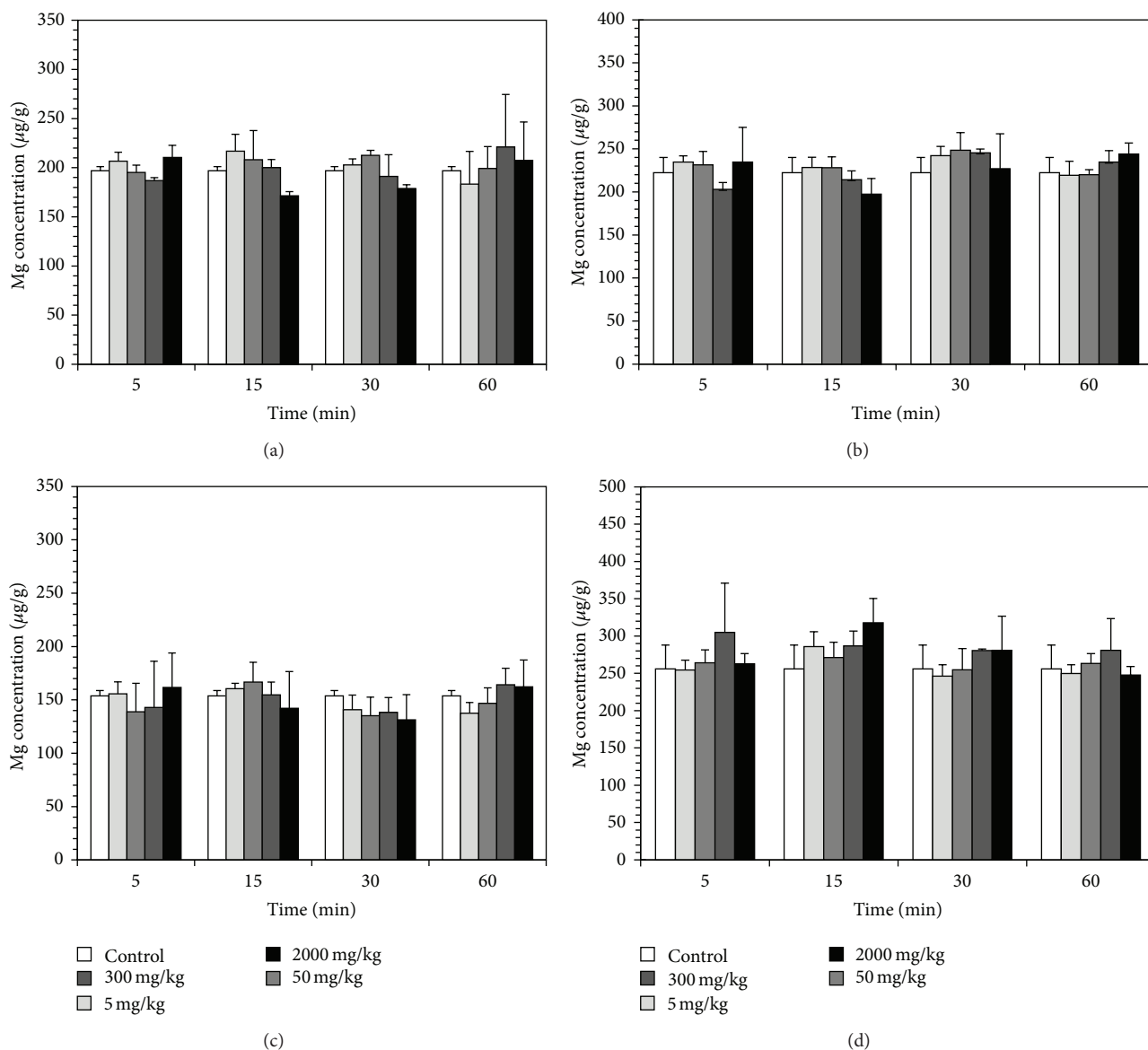


FIGURE 4: Tissue distribution of LDH nanoparticles after single-dose oral administration to mice. (a) Kidney, (b) liver, (c) lung, and (d) spleen. No significant significance was found between the control and LDH-treated groups.

4. Conclusion

The acute oral toxicity of LDH nanoparticles of about 100 nm was evaluated after administration of four different doses (5, 50, 300, and 2000 mg/kg) in mice. The results demonstrated that LDH nanoparticles did not cause any mortality, abnormal behaviors, symptoms, and body weight loss up to the highest dose administered, indicating that the LD_{50} values were more than 2000 mg/kg. Organosomatic indices of all organs in mice treated with LDH nanoparticles were not affected, thereby clearly supporting their low oral toxicity. Serum biochemical parameters did not significantly increase, suggesting that LDH nanoparticles did not induce liver or kidney injury. Pharmacokinetic and tissue distribution studies showed that LDH nanoparticles up to the dose of

2000 mg/kg were rapidly absorbed within 30 min and cleared from the body after 3.1 h, without significant accumulation in any specific target tissue. LDH nanoparticles were determined to be excreted via urine and feces within 24 h. Taken together, it is concluded that LDH nanoparticles do not cause acute oral toxicity in mice, which is strongly associated with their small absorbed amount by the gastrointestinal system and easy clearance kinetics at the systemic level.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

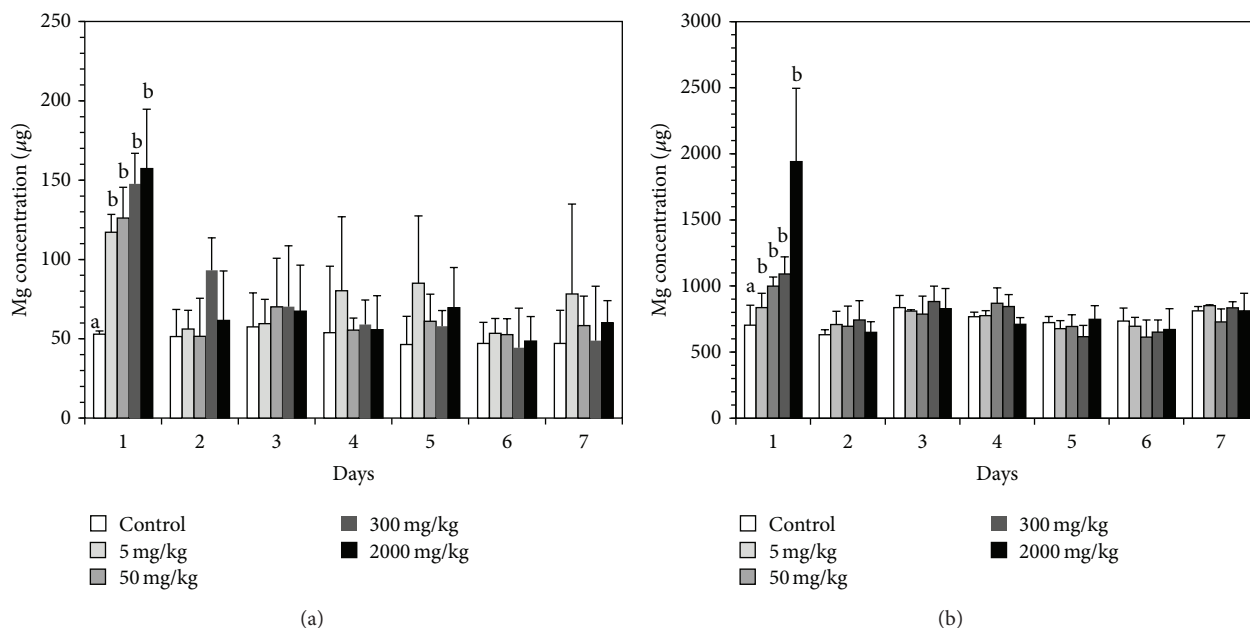


FIGURE 5: Excretion profiles of LDH nanoparticles via urine (a) and feces (b) after single-dose oral administration to mice.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0003570) and the National Research Foundation of Korea (NRF) Grant funded by the Korean government (MSIP) (2005-0049412) and partly by the National Research Foundation of Korea Grant funded by the Korean Government (KRF-2008-313-F00130).

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