Utility of Nano-Sized, Water-in-Oil Emulsion as a Sustained Release Formulation of Glycyrrhizin

Kenjiro KOGA,*a Yuki NISHIMON,a Hisashi UETA,b Kenjiro MATUNO,b and Kanji TAKADA,c

a Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Hokuriku University; Ho-3, Kanagawa-machi, Kanazawa 920–1181, Japan; b Department of Anatomy (Macro), Dokkyo Medical University; 880 Kitakobayashi, Mibu, Tochigi 321–0293, Japan; and c Department of Pharmacokinetics, Kyoto Pharmaceutical University; Misasagi Nakauchi-cho 5, Yumashina-ku, Kyoto 607–8414, Japan. Received August 26, 2010; accepted November 26, 2010

The aim of this study was to determine the efficiency of nano-sized water-in-oil (w/o) emulsions that encapsulate glycyrrhizin (GZ) (Rp-I) as a sustained release formulation for subcutaneous administration. Four formulations were assessed in rats for 8—72 h: nano-sized water-in-oil (w/o) emulsion encapsulating GZ (Rp-I), GZ aqueous solution (Rp-II), oil-in-water (o/w) emulsion containing GZ (Rp-III), and w/o emulsion containing solid GZ (Rp-IV). All had a GZ concentration of 150 mg/ml. Over an 8-h period, GZ elimination in bile after subcutaneous administration of Rp-I, Rp-II, Rp-III, and Rp-IV (50 mg/kg GZ) was 10.8%, 97.0%, 81.0%, and 7.1%, respectively. The elimination of GZ into bile after the administration of Rp-IV was the lowest (30.5%) at the 72-h endpoint, dropping significantly from 48 to 72 h. On the other hand, the elimination rate of GZ after the administration of Rp-I was sustained at a constant level (1.8—2.1 mg/24 h) over 72 h. GZ concentration in liver at 72 h in Rp-I was highest (19.9 μg/g tissue) among the four formulations, suggesting that the release of GZ from the Rp-I formulation is constant, at least up to 72 h after administration. These results suggest that a nano-sized w/o emulsion is useful as a sustained release formulation for long-term therapy of chronic hepatitis.

Key words glycyrrhizin; nanoparticle; emulsion; sustained release; bile elimination; rat

Glycyrrhizin (GZ) is the main constituent of Glycyrrhiza glabra L. and chemically is the glycoside of glycyrrhetic acid. In addition to having anti-allergic, anti-inflammatory, and anti-hepatitis properties, GZ has interferon-inducing properties.1—3) Although oral (25 mg/tablet) and intravenous (2 mg/ml GZ solution containing glycine and L-cysteine) formulations are commercially used in Japan, the intravenous formulation is more effective for chronic hepatitis.4—6) This is because GZ is poorly absorbed from the intestinal tract,7) as GZ is hydrolyzed by β-glucuronidase in the large intestine.8) The intravenous formulation is administered 2 or 3 times per week over a prolonged period. Frequent hospital visits and pain from repeated intravenous injections are inconvenient, which direct results in reduced quality of life (QOL) for chronic hepatitis patients. Therefore, the development of a more patient-oriented formulation would be tremendously beneficial. Moreover, a patient-oriented formulation would likely increase compliance.

To address these issues and to reduce the unpleasant side effects of GZ, various type formulations were developed for oral,9) rectal,10) and nasal10) administrations. However, each of the bioavailability of GZ after the administration of non-vascular type formulations in dogs or rats was in the range of 2.4 to 20.2%.9,10) Namely, non-vascular route is inconvenient to improve the bioavailability of GZ. Therefore, we focused the subcutaneous administration to enhance the GZ bioavailability and to sustain the therapeutic effect of GZ. Previously, the preparation methods and phase behavior stability of a nano-sized water-in-oil (w/o) emulsion encapsulating GZ have been reported.11) Briefly, a 400-μg/ml GZ solution was dispersed into an oil phase (soybean oil and condensed ricinoleic acid tetraglycerin ester) using a Polytron. The solution was then evaporated until the water content in the emulsion reached approximately 9% (w/w).11) The dispersion properties of the nano-sized w/o emulsion were maintained at a constant level for 2 months. During this period, the average particle size was approximately 300 nm, and the size distribution was 135—421 nm (10 to 90% interval of particle size), guaranteeing pharmaceutical stability of the GZ emulsion. The next step was to investigate the release profile of GZ from the w/o emulsion, because the sustained release of GZ from the w/o emulsion is important for reducing the frequency of hospital visits, and thus elevating patient QOL.

Pharmacokinetic studies of GZ were investigated in detail in human,12,13) rodent (rat),14—16) and other species. The elimination half-life of GZ in chronic hepatitis patients after infusion of GZ (80 mg) is 4.4—4.7 h in serum.17) The elimination half-life of GZ in rats after the intravenous administration (20—50 mg/kg) is approximately 2—4 h in plasma.18,19) GZ is rapidly excreted into bile via multidrug resistance-associated protein 2 (MRP2) ATP-binding cassette transporter C2 (ABCC2) transporter.20) Considering the pharmacokinetics of GZ, the aim of this study was to clarify whether a nano-sized w/o emulsion (termed Rp-I) has sustained release compared to other formulations. Thus, GZ concentrations in bile, subcutaneous injection site, and liver were measured after the subcutaneous administration of GZ.

MATERIALS AND METHODS

Materials Glycyrrhizin monoammonium (GZ) was a gift from Kokey Co., Ltd. (Tokyo, Japan). L-Arginine and soybean oil were purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). Polyricinoleic acid tetraglycerol ester (CR-310) and polyethylene glycol-60 hydrogenated castor oil (HCO-60) were gifts from Sakamoto Yakuhin Kogyo Co., Ltd. (Osaka, Japan) and Nikko Chemicals Co., Ltd. (Tokyo, Japan), respectively. Egg yolk lecithin (PL-30S) was purchased from Kewpie Corp. (Tokyo, Japan). Indocyanine green (ICG) (product name: Diagnogreen for injection; 25
mg) was purchased from Daiichi Sankyo Company Ltd. (Tokyo, Japan). Other chemicals were of HPLC or reagent grade.

Animals The protocol of this study was approved by the Committee of Animal Use of Hokuriku University. All animal experiments were conducted in accordance with the Institutional Guidelines of Care and Use of Laboratory Animals. Male Sprague-Dawley rats (180—200 g) were housed for at least 10 d in a clean room. The rats were given free access to commercial chow and water and were maintained according to the Hokuriku University Animal Guidelines. For in vivo experiments in GZ formulations, the rats (250—280 g) were randomly divided into four treatment groups as four rats per group. For the experiments of ICG administration, two rats (250, 255 g) were used.

Preparation of GZ and ICG Formulations A GZ stock solution (400 mg/ml) was prepared at 60 °C in 100 mM phosphate-buffered solution, pH 7.4 (PBS), containing 8.0% (w/v) L-arginine, an inhibitor of gelation.21) The GZ stock solution was stored in a refrigerator. An aqueous formulation of GZ (150 mg/ml; Rp-II) was prepared by adding 100 mM PBS to the GZ stock solution. Preparation of an oil-in-water (o/w) emulsion of GZ was as follows: soybean oil (1.0 g), HCO-60 (0.12 g), and PL-308 (0.12 g) were blended uniformly by heating at 90 °C for 15 min on a block heater. The mixture was then cooled at room temperature. The o/w emulsion of GZ (150 mg/ml; Rp-III) was prepared by combining the soybean oil mixture (1.0 ml), GZ stock solution (1.16 ml), and 100 mM PBS (0.84 ml) and by using a Polytron (PT-MR 3100, Kinematica AG, Littau/Luzern, Switzerland) at 20000 rpm for 3 min for emulsification.

Preparation of an water-in-oil (w/o) emulsion of GZ was as follows: soybean oil (4.50 g) and CR-310 (0.50 g), a hydrophobic surfactant, were heated at 60 °C for 15 min. The mixture was then added to the lukewarm mixture, which was emulsified using a Polytron (PT-MR 3100) at 20000 rpm for 3 min. The w/o emulsion was placed into a 50-ml round-bottom flask, which was then set in a rotary evaporator (R-210, Buchi Labortechnik AG, Flawil, Switzerland) equipped with a vacuum controller (V-850, Buchi Laborteknik AG). The vacuum was initially set to 3100 mtorr at 40 °C; thereafter, the pressure was decreased at a rate of 10 hPa per min until the 72-h experiments were finished. The experimental endpoints were set to 72 h for Rp-I and Rp-IV, 8 h for Rp-II, and 30 h for Rp-III. Within two hours after the administration, in order to maintain continuous collection of bile, the rats were placed under restraint in a special cylindrical cage (a self-made plastic cage) limiting their movement. Water and commercial chow were set in the cage.

After the end of bile collection, the rats were anesthetized by inhalation of diethyl ether. Immediately, the subdermal site receiving the GZ formulation and liver were carefully removed and stored at 4 °C until the assay. The hair and epidermis of the subdermal site were removed, and then the subdermal tissue was homogenized with methanol (30 ml) containing 40 μg/ml chloramphenicol as an internal standard in order to extract the residual GZ in the subdermal space. The homogenized samples were centrifuged at 16000×g for 5 min. The supernatant (0.10 ml) and 100 mM PBS (9.90 ml) were mixed, filtered using a membrane filter (Dismic 13HP020, pore size 0.2 μm, Advantec Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and 10 μl of the filtrate was then injected into an HPLC system.

The excised liver (0.20 g wet weight), 100 mM PBS (0.8 ml), and stainless beads (two beads of 3.2 mm diameter and one bead of 5.4 mm diameter) were put into a 2-ml microtube. The liver was crushed by using a Beads Cells Disrupter/Micro-Homogenizing System (MS-100R, Tomy Seiko Co., Ltd., Tokyo, Japan) at 3000 rpm for 1 min at 4 °C. The liver homogenate (40 μl) and 10 μg/ml chloramphenicol (40 μl) and methanol (0.9 ml) were put into a 1.5-ml microtube, and were shaken for 20 min at 500 rpm using a Vortex Shaker (VR-36, TAITEC, Saitama, Japan). Chloramphenicol was used as an internal standard. The mixture was centrifuged at 16000×g for 5 min. The supernatant (0.8 ml) was transferred to a 10-ml glass tube and evaporated to dryness under a continuous stream of nitrogen gas and on a heat block set to 80 °C. After cooling the glass tube, 0.3 ml of acetonitrile/0.6% perchloric acid solution adjusted to pH 8.0 with 25% ammonia solution (2:8, v/v) was added to the glass tube, which was shaken using a touch mixer for 1 min. The solution (0.3 ml) was filtered using a membrane filter

set to calculate the particle size for Rp-I and Rp-IV.

In Vivo Experiments Rats were anesthetized with an intraperitoneal administration of sodium pentobarbital (50 mg/kg). After the hair of the abdomen and inner upper arm of the rats were carefully shaved, the rats were fixed in a supine position on an operation plate maintained at 32 °C. An abdominal incision was made, and the common bile duct was cannulated with polyethylene tubing (PE10, 20 cm in length; Becton Dickinson, Sparks, MD, U.S.A.). The tubing was guided through the left-side abdominal muscle and abdominal cavity to the bile duct using a pair of sharp-pointed tweezers. The tubing was fixed to the bile duct with surgical suture; the opposite end of the tubing was placed into a sampling glass tube. The opening in the abdomen was closed with surgical suture.

At 10:00 a.m., each formulation (Rp-I, Rp-II, Rp-III, or Rp-IV at 50 mg/kg) was administered subcutaneously into the rat’s back using a microsyringe. Bile was collected at the predetermined time (see Fig. 2). In animals receiving Rp-I and Rp-IV, bile samples were collected every hour for 10 h from 10:00 a.m. to 8:00 p.m. or from 8:00 p.m. to 10:00 a.m. until the 72-h experiments were finished. The experimental endpoints were set to 72 h for Rp-I and Rp-IV, 8 h for Rp-II, and 30 h for Rp-III. Within two hours after the administration, in order to maintain continuous collection of bile, the rats were placed under restraint in a special cylindrical cage (a self-made plastic cage) limiting their movement. Water and commercial chow were set in the cage.

After the end of bile collection, the rats were anesthetized by inhalation of diethyl ether. Immediately, the subdermal site receiving the GZ formulation and liver were carefully removed and stored at 4 °C until the assay. The hair and epidermis of the subdermal site were removed, and then the subdermal tissue was homogenized with methanol (30 ml) containing 40 μg/ml chloramphenicol as an internal standard in order to extract the residual GZ in the subdermal space. The homogenized samples were centrifuged at 16000×g for 5 min. The supernatant (0.10 ml) and 100 mM PBS (9.90 ml) were mixed, filtered using a membrane filter (Dismic 13HP020, pore size 0.2 μm, Advantec Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and 10 μl of the filtrate was then injected into an HPLC system.

The excised liver (0.20 g wet weight), 100 mM PBS (0.8 ml), and stainless beads (two beads of 3.2 mm diameter and one bead of 5.4 mm diameter) were put into a 2-ml microtube. The liver was crushed by using a Beads Cells Disrupter/Micro-Homogenizing System (MS-100R, Tomy Seiko Co., Ltd., Tokyo, Japan) at 3000 rpm for 1 min at 4 °C. The liver homogenate (40 μl) and 10 μg/ml chloramphenicol (40 μl) and methanol (0.9 ml) were put into a 1.5-ml microtube, and were shaken for 20 min at 500 rpm using a Vortex Shaker (VR-36, TAITEC, Saitama, Japan). Chloramphenicol was used as an internal standard. The mixture was centrifuged at 16000×g for 5 min. The supernatant (0.8 ml) was transferred to a 10-ml glass tube and evaporated to dryness under a continuous stream of nitrogen gas and on a heat block set to 80 °C. After cooling the glass tube, 0.3 ml of acetonitrile/0.6% perchloric acid solution adjusted to pH 8.0 with 25% ammonia solution (2:8, v/v) was added to the glass tube, which was shaken using a touch mixer for 1 min. The solution (0.3 ml) was filtered using a membrane filter
(Dismic 13HP020, pore size 0.2 µm), and 10 µl was injected into a HPLC system.

Since the exact volume of the bile collected could not be measured, the weight of the collected bile was measured, and then calculated the bile volume using its specific gravity (1.0 g/cm³). The bile sample (4 µl), 100 mM PBS (96 µl), 10 µg/ml chloramphenicol (100 µl), and acetonitrile/0.6% perchloric acid solution adjusted to pH 8.0 with 25% ammonia solution (2:8, v/v) (800 µl) were shaken for 30 s, followed by filtering with a membrane filter (Dismic 13HP020, pore size 0.2 µm). The filtered solution (10 µl) was injected into a HPLC system.

**GZ Assay by HPLC**  GZ concentrations in bile, subdermal tissue, and liver were determined using the same procedures as our previous report.22) The HPLC system was a semimicro type; it was equipped with a LC-10ADvp pump, SIL-20A autosampler, DGU-20As degassing apparatus, SPD-20A UV detector, CR7A-plus data processor (Shimadzu, Kyoto, Japan). Briefly, a Capcell Pak C18 column (1.5 mm inner diameter, 150 mm length) was used at 40 °C during separation. Detection was performed at a UV wavelength of 254 nm. Acetonitrile/0.6% perchloric acid solution adjusted to pH 8.0 with 25% ammonia solution (2:8, v/v) was used as the mobile phase; the flow rate was 0.08 ml/min. The detection limit was 0.2 µg/ml.

**Photo Dynamic Experiments**  To monitor over time the delayed drug distribution and the diffusion of hydrophobic formulation in the subdermal site, a w/o emulsion encapsulating GZ instead of GZ was prepared. ICG is a hydrophilic fluorescent dye and biocompatibility marker with excitation and emission spectra in the near-infrared wavelength range of 600 to 900 nm, and the maximum emission wavelength of ICG instead of GZ was prepared. ICG is a hydrophilic fluorescent dye and biocompatibility marker with excitation and emission spectra in the near-infrared wavelength range of 600 to 900 nm, and the maximum emission wavelength of ICG instead of GZ was observed during separation. Detection was performed at a UV wavelength of 254 nm. Acetonitrile/0.6% perchloric acid solution adjusted to pH 8.0 with 25% ammonia solution (2:8, v/v) was used as the mobile phase; the flow rate was 0.08 ml/min. Correlation coefficients of the calibration curves were 0.999 or better. The detection limit was 0.2 µg/ml.

**Photo Dynamic Experiments**  To monitor over time the delayed drug distribution and the diffusion of hydrophobic formulation in the subdermal site, a w/o emulsion encapsulating GZ instead of GZ was prepared. ICG is a hydrophilic fluorescent dye and biocompatibility marker with excitation and emission spectra in the near-infrared wavelength range of 600 to 900 nm, and the maximum emission wavelength of ICG instead of GZ was observed during separation. Detection was performed at a UV wavelength of 254 nm. Acetonitrile/0.6% perchloric acid solution adjusted to pH 8.0 with 25% ammonia solution (2:8, v/v) was used as the mobile phase; the flow rate was 0.08 ml/min. Correlation coefficients of the calibration curves were 0.999 or better. The detection limit was 0.2 µg/ml.

**Table 1. Characteristics of Prepared Four Type Formulations**

<table>
<thead>
<tr>
<th>Formulation Type</th>
<th>Water contents (%)</th>
<th>Average particle size and the size distribution range from 10 to 90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rp-I w/o emulsion</td>
<td>9.4</td>
<td>299—208—402</td>
</tr>
<tr>
<td>Rp-II aqueous solution</td>
<td>68</td>
<td>376—255—512</td>
</tr>
<tr>
<td>Rp-III w/o emulsion with solid GZ</td>
<td>1.5</td>
<td>312—5000 and 1000—2500 (two peaks)</td>
</tr>
</tbody>
</table>

*Water contents in formulations were analyzed using a Karl Fischer titration apparatus (870, KF Titroline Plus, Metrohm Shibata Co., Tokyo, Japan). The determination method was in detail described in ref. 11.*
slower than that in Rp-II, suggesting that the oil phase of the o/w emulsion inhibited the diffusion of the water phase containing GZ in subcutaneous regions, even though GZ was dissolved in the outer water phase of o/w emulsion. On the other hand, the cumulative elimination of GZ in Rp-IV was the lowest among the four formulations: The GZ elimination in bile at 8 h, 24 h, and 72 h was 7.1%, 14%, and 31%, respectively. As with the elimination kinetics of the Rp-I formulation, the elimination kinetics of GZ in Rp-IV showed that GZ was released in a sustained fashion for up to 72 h. Since Rp-IV contained solid GZ in w/o emulsions, it was speculated that the elimination rate of GZ in bile after the administration of Rp-IV would be slower than that of GZ in Rp-I. In fact, the eliminated amount of GZ in bile after the administration of Rp-IV was 0.64-fold compared to that of Rp-I. These results suggest that reduced water content in w/o emulsions delays hydration in the subcutaneous region and that much time is required to dissolve the dispersed solid GZ in Rp-IV.

To determine more precisely the characteristics of sustained GZ release from w/o emulsions, the rates of GZ elimination in bile were recalculated every 24 h after the administration of Rp-I and Rp-IV (Fig. 3). The elimination of GZ in Rp-I occurred at a constant rate for 72 h, i.e., the rate ranged from 1.80 to 2.12 mg/d. On the other hand, elimination of GZ in Rp-IV decreased from 1.40—1.41 mg/d at 48 h to 0.60 mg/d at 48—72 h. As the reason for the decrease in GZ elimination rate at 48—72 h in Rp-IV, it was predicted that the presence of solid dispersed GZ may be involved deeply the transfer rate of GZ from subcutaneous site to liver. Actually, dissolved-state GZ and solid-state GZ exist in Rp-IV. Although it was considered that the dissolved GZ in Rp-IV was transferred to liver as similar to GZ in Rp-I, solid GZ particles must be dissolved to some extent which can be passed vascular system such as vein and lymph capillary in order to transfer GZ from subcutaneous site to liver. Therefore, after 48 h, the proportion of solid GZ for residual GZ in the subcutaneous site will increase certainly. As a result, it was guessed that GZ elimination into bile decreased based on the decrease of transfer rate from subcutaneous site to liver.

These results indicate that Rp-I was a substantially superior formulation compared to Rp-IV in terms of sustained release in bile. It was hypothesized that the small and narrow-range polydispersity (208—402 nm in Rp-I) of the dispersed phase in w/o emulsions may be important for stabilizing the release rate of GZ from these emulsions.
GZ in the Subdermal Injection Site  Table 2 shows the residual GZ at the subdermal injection site following the administration of Rp-I, Rp-II, Rp-III, and Rp-IV. The residual GZ in Rp-II and Rp-III was 0% and 1.71%, respectively, of the administered GZ dose. On the other hand, the residual GZ in Rp-I and Rp-IV was 41.3% and 58.0%, respectively. These residual GZ values (%) were inversely proportional and correlated to the percentage of GZ eliminated in bile. These results suggest that the hydrophobic formulations of GZ (Rp-I and Rp-IV)—i.e., outer phase was oil—induced sustained release of GZ from the formulations. In general, the diffusion rate of the oil phase in the subdermal site was slow compared to that of the water phase after subcutaneous administration. This is because in the subdermis oil components are not susceptible to degradation. The distribution of a drug in blood or lymph relates to membrane permeability and physicochemical properties, such as surface tension, viscosity, etc.26,27) Therefore, the distribution of GZ from hydrophobic formulations (Rp-I and Rp-IV) is delayed compared to the distribution of GZ from hydrophilic formulations (Rp-II and Rp-III).

The purpose of the PDE experiments was to determine whether the diffusion rate of w/o emulsions at the subdermal site is remarkably slower than that of solutions like Rp-II. Figure 4 shows photographs of rats’ hind legs 15 min and 60 min after injecting ICG (right leg) and after injecting w/o emulsion encapsulating ICG (left leg). The ICG solution was rapidly absorbed into capillary blood vessels within 15 min. Furthermore, ICG also reached peripheral lymphatics. On the other hand, ICG in w/o emulsions remained in the vicinity of the administration site for 60 min. These results suggested that the outer oil phase inhibited the diffusion of w/o emulsion encapsulating ICG and/or the release of ICG from the w/o emulsion. The viscosity of lipophilic formulations is generally high. Therefore, one would expect the release of ICG from a w/o emulsion to be delayed compared to that from aqueous formulations of ICG. The expectation will be correspondent with the decrease of diffusion and/or release of GZ from Rp-I.

GZ Concentration in Liver  The GZ concentration in liver presumably reflects the therapeutic efficiency of GZ.6) Table 2 shows the concentrations of GZ accumulated in liver at each experimental endpoint after the administration of Rp-I, Rp-II, and Rp-IV. The concentration of GZ in the liver was highest in rats receiving Rp-I, suggesting that the influx velocity of GZ from the blood circulation to the liver reached a maximum at the experimental endpoint. These results indicate that the kinetics of GZ in Rp-I promotes the accumulation of GZ in liver at the endpoint. Indeed, the GZ concentration in liver after the administration of Rp-I was two-fold higher than that after the administration of Rp-IV. The difference in liver GZ concentrations after the administration of Rp-I and Rp-IV may depend on the rate of GZ release from the formulations, because GZ elimination into bile 48—72 h after Rp-IV administration was remarkably decreased compared to that after Rp-I administration (Fig. 3).

With regard to the efflux from liver to bile, GZ is a substrate of the efflux transporter MRP2, which is located on liver canalicular membranes.16) GZ is rapidly transported to bile via MRP2.20) It was reported previously that the relationship between GZ concentration in liver and the elimination rate of GZ into bile was linear under a steady-state infusion rate of <1 mg/h GZ.22) However, the elimination rates of Rp-I and Rp-IV at 48—72 h were 88.6 μg/h (2.13 ± 0.13 mg/24 h) and 25.0 μg/h (0.60 ± 0.94 mg/24 h), respectively, linear rela-

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Residual GZ in the subdermal site (%)</th>
<th>GZ concentration in liver (μg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rp-I</td>
<td>41.3 ± 5.4</td>
<td>19.9 ± 1.4</td>
</tr>
<tr>
<td>Rp-II</td>
<td>0</td>
<td>4.25 ± 0.21</td>
</tr>
<tr>
<td>Rp-III</td>
<td>1.71 ± 0.14</td>
<td>—</td>
</tr>
<tr>
<td>Rp-IV</td>
<td>58.0 ± 5.4</td>
<td>10.9 ± 6.4</td>
</tr>
</tbody>
</table>

The dose of GZ administered was 50 mg/kg. Hypodermal tissue and liver samples were sampled at the following times after GZ administration: 72 h for Rp-I and Rp-IV, 8 h for Rp-II, and 30 h for Rp-III. Data represent means±S.D. of four experiments.

Fig. 4. Photographs Taken at (A) 15 min and (B) 60 min after the Subcutaneous Injection of ICG Solution into the Right Leg and w/o Emulsion Encapsulating ICG into the Left Leg.

Injection volume of ICG was 50 μl in both formulations.
tionship between the GZ elimination rate and GZ concentration in liver was not observed. In fact, the ratios of average GZ elimination rate into bile (48—72 h) to GZ concentration in liver at 72 h in Rp-I and Rp-IV were 4.45 and 2.29, respectively. For the reason, it was considered the possibility that the influx of GZ to liver transiently decreased in Rp-I or increased in Rp-IV around the 72-h endpoint. The concentration of GZ in liver at the endpoint was clearly influenced by the blood circulation-to-liver influx velocity of GZ. In turn, the elimination of GZ in bile was influenced by the concentration of GZ in liver.

Furthermore, for an administered GZ dose of 50 mg/kg, the total percentages of GZ eliminated in bile and residual GZ in the subdermal injection site were >89%, suggesting that the amount of GZ distributed to blood, kidney, and other organs (excluding liver) was negligible and can be disregarded. Therefore, the proportions of GZ observed in bile, the subdermal site, and liver were consistent with the kinetics of GZ for its transport from the injection site to bile.

CONCLUSIONS

A nano-sized w/o emulsion of GZ (Rp-I) showed sustained elimination of GZ in bile at a relatively constant rate for 72 h. The sustained GZ elimination in bile was strongly affected by diffusion of the w/o emulsion and by the release of GZ from the emulsion to the perimeter of the subdermal site, based on the PDE observations with ICG. Moreover, Rp-I clearly supports sustained high GZ concentration in liver compared to other formulations in vivo experiments. Indeed, the average elimination rate of GZ in bile was 84.2 μg/h over 72 h and the GZ concentration in liver was 19.9 μg/g tissue at 72 h, when Rp-I (50 mg/kg as GZ) was administered subcutaneously. If GZ release from Rp-I will be maintained as zero-order elimination (84.2 μg/h), 6—7 d are needed until the GZ release finishes in the rats. Namely, GZ in Rp-I will slowly transfer from subcutaneous tissue to liver 20-fold periods as compared with GZ in Rp-II, the elimination of almost all GZ finished 8 h.

These results indicate that the nano-sized w/o emulsion encapsulating GZ, which can be subcutaneously administered, will be useful as a new sustained-release formulation for prolonged chronic hepatitis therapy.

Acknowledgements This work was supported in part by the Special Research Fund of Hokuriku University. We are grateful for the generous gifts of materials from Kokey Co., Ltd.; Sakamoto Yakuhin Kogyo Co., Ltd.; and the Nikko Chemicals Co., Ltd.

REFERENCES